WORLD HEALTH ORGANIZATION
INTERNATIONAL AGENCY FOR RESEARCH ON CANCER

IARC Monographs on the Evaluation of Carcinogenic Risks to Humans

VOLUME 94

Ingested Nitrate and Nitrite, and Cyanobacterial Peptide Toxins

LYON, FRANCE
2010

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This publication represents the views and expert opinions
of an IARC Working Group on the
Evaluation of Carcinogenic Risks to Humans,
which met in Lyon,

14–21 June 2006

2010

IARC MONOGRAPHS

In 1969, the International Agency for Research on Cancer (IARC) initiated a programme on the evaluation of the carcinogenic risk of chemicals to humans involving the production of critically evaluated monographs on individual chemicals. The programme was subsequently expanded to include evaluations of carcinogenic risks associated with exposures to complex mixtures, lifestyle factors and biological and physical agents, as well as those in specific occupations. The objective of the programme is to elaborate and publish in the form of monographs critical reviews of data on carcinogenicity for agents to which humans are known to be exposed and on specific exposure situations; to evaluate these data in terms of human risk with the help of international working groups of experts in chemical carcinogenesis and related fields; and to indicate where additional research efforts are needed. The lists of IARC evaluations are regularly updated and are available on the Internet at http://monographs.iarc.fr/.

This programme has been supported since 1982 by Cooperative Agreement U01 CA33193 with the United States National Cancer Institute, Department of Health and Human Services. Additional support has been provided since 1986 by the Health, Safety and Hygiene at Work Unit of the European Commission Directorate-General for Employment, Social Affairs and Equal Opportunities, and since 1992 by the United States National Institute of Environmental Health Sciences, Department of Health and Human Services. The contents of this volume are solely the responsibility of the Working Group and do not necessarily represent the official views of the U.S. National Cancer Institute, the U.S. National Institute of Environmental Health Sciences, the U.S. Department of Health and Human Services, or the European Commission Directorate-General for Employment, Social Affairs and Equal Opportunities.

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Intensive use of nitrogen fertilizers in crop culture and manure from animal feeding operations may be significant sources of nitrate in some regions like Brittany, France, leading to euthrophication and the excessive growth of green algae. The growth of peptide-toxin-producing cyanobacteria is favoured in waters where eutrophication occurs.

Ham, bacon, and some sausages are preserved with salt and sodium or potassium nitrite. Ascorbate is often added to inhibit the formation of *N*-nitrosamines before the cured meat is eaten. *N*-nitrosamines can also form in the stomach unless inhibited by vitamin C or other antioxidants. Kiwi and citrus fruits are rich sources of antioxidants.

Cover design: Roland Dray and Yann Grosse, IARC

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NOTE TO THE READER

The term 'carcinogenic risk' in the *IARC Monographs* series is taken to mean that an agent is capable of causing cancer under some circumstances. The *Monographs* evaluate cancer hazards, despite the historical presence of the word 'risks' in the title.

Inclusion of an agent in the *Monographs* does not imply that it is a carcinogen, only that the published data have been examined. Equally, the fact that an agent has not yet been evaluated in a *Monograph* does not mean that it is not carcinogenic.

The evaluations of carcinogenic risk are made by international working groups of independent scientists and are qualitative in nature. No recommendation is given for regulation or legislation.

Anyone who is aware of published data that may alter the evaluation of the carcinogenic risk of an agent to humans is encouraged to make this information available to the Section of IARC Monographs, International Agency for Research on Cancer, 150 cours Albert Thomas, 69372 Lyon Cedex 08, France, in order that the agent may be considered for re-evaluation by a future Working Group.

Although every effort is made to prepare the monographs as accurately as possible, mistakes may occur. Readers are requested to communicate any errors to the Section of IARC Monographs, so that corrections can be reported in future volumes.

IARC MONOGRAPHS ON THE EVALUATION OF CARCINOGENIC RISKS TO HUMANS

VOLUME 94
INGESTED NITRATE AND NITRITE AND CYANOBACTERIAL PEPTIDE TOXINS

Lyon, 14–21 June 2006

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[3] Receives research support from the Institute for Science and Health, which is funded by tobacco corporations.

[4] Holds a patent for congener-independent detection of microcystin and nodularin congeners.

[5] Writing on the exposure sections was funded by the US National Cancer Institute under a past contract to Technical Resources International, Inc., and a subcontract to the International Life Sciences Institute.

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PREAMBLE

IARC MONOGRAPHS ON THE EVALUATION OF CARCINOGENIC RISKS TO HUMANS

PREAMBLE

The Preamble to the *IARC Monographs* describes the objective and scope of the programme, the scientific principles and procedures used in developing a *Monograph*, the types of evidence considered and the scientific criteria that guide the evaluations. The Preamble should be consulted when reading a *Monograph* or list of evaluations.

A. GENERAL PRINCIPLES AND PROCEDURES

1. Background

Soon after IARC was established in 1965, it received frequent requests for advice on the carcinogenic risk of chemicals, including requests for lists of known and suspected human carcinogens. It was clear that it would not be a simple task to summarize adequately the complexity of the information that was available, and IARC began to consider means of obtaining international expert opinion on this topic. In 1970, the IARC Advisory Committee on Environmental Carcinogenesis recommended '...that a compendium on carcinogenic chemicals be prepared by experts. The biological activity and evaluation of practical importance to public health should be referenced and documented.' The IARC Governing Council adopted a resolution concerning the role of IARC in providing government authorities with expert, independent, scientific opinion on environmental carcinogenesis. As one means to that end, the Governing Council recommended that IARC should prepare monographs on the evaluation of carcinogenic risk of chemicals to man, which became the initial title of the series.

In the succeeding years, the scope of the programme broadened as *Monographs* were developed for groups of related chemicals, complex mixtures, occupational exposures, physical and biological agents and lifestyle factors. In 1988, the phrase 'of chemicals' was dropped from the title, which assumed its present form, *IARC Monographs on the Evaluation of Carcinogenic Risks to Humans*.

Through the *Monographs* programme, IARC seeks to identify the causes of human cancer. This is the first step in cancer prevention, which is needed as much today as when

IARC was established. The global burden of cancer is high and continues to increase: the annual number of new cases was estimated at 10.1 million in 2000 and is expected to reach 15 million by 2020 (Stewart & Kleihues, 2003). With current trends in demographics and exposure, the cancer burden has been shifting from high-resource countries to low- and medium-resource countries. As a result of *Monographs* evaluations, national health agencies have been able, on scientific grounds, to take measures to reduce human exposure to carcinogens in the workplace and in the environment.

The criteria established in 1971 to evaluate carcinogenic risks to humans were adopted by the Working Groups whose deliberations resulted in the first 16 volumes of the *Monographs* series. Those criteria were subsequently updated by further ad-hoc Advisory Groups (IARC, 1977, 1978, 1979, 1982, 1983, 1987, 1988, 1991; Vainio *et al.*, 1992; IARC, 2005, 2006).

The Preamble is primarily a statement of scientific principles, rather than a specification of working procedures. The procedures through which a Working Group implements these principles are not specified in detail. They usually involve operations that have been established as being effective during previous *Monograph* meetings but remain, predominantly, the prerogative of each individual Working Group.

2. Objective and scope

The objective of the programme is to prepare, with the help of international Working Groups of experts, and to publish in the form of *Monographs*, critical reviews and evaluations of evidence on the carcinogenicity of a wide range of human exposures. The *Monographs* represent the first step in carcinogen risk assessment, which involves examination of all relevant information in order to assess the strength of the available evidence that an agent could alter the age-specific incidence of cancer in humans. The *Monographs* may also indicate where additional research efforts are needed, specifically when data immediately relevant to an evaluation are not available.

In this Preamble, the term 'agent' refers to any entity or circumstance that is subject to evaluation in a *Monograph*. As the scope of the programme has broadened, categories of agents now include specific chemicals, groups of related chemicals, complex mixtures, occupational or environmental exposures, cultural or behavioural practices, biological organisms and physical agents. This list of categories may expand as causation of, and susceptibility to, malignant disease become more fully understood.

A cancer 'hazard' is an agent that is capable of causing cancer under some circumstances, while a cancer 'risk' is an estimate of the carcinogenic effects expected from exposure to a cancer hazard. The *Monographs* are an exercise in evaluating cancer hazards, despite the historical presence of the word 'risks' in the title. The distinction between hazard and risk is important, and the *Monographs* identify cancer hazards even when risks are very low at current exposure levels, because new uses or unforeseen exposures could engender risks that are significantly higher.

In the *Monographs*, an agent is termed 'carcinogenic' if it is capable of increasing the incidence of malignant neoplasms, reducing their latency, or increasing their severity or multiplicity. The induction of benign neoplasms may in some circumstances (see Part B, Section 3a) contribute to the judgement that the agent is carcinogenic. The terms 'neoplasm' and 'tumour' are used interchangeably.

The Preamble continues the previous usage of the phrase 'strength of evidence' as a matter of historical continuity, although it should be understood that *Monographs* evaluations consider studies that support a finding of a cancer hazard as well as studies that do not.

Some epidemiological and experimental studies indicate that different agents may act at different stages in the carcinogenic process, and several different mechanisms may be involved. The aim of the *Monographs* has been, from their inception, to evaluate evidence of carcinogenicity at any stage in the carcinogenesis process, independently of the underlying mechanisms. Information on mechanisms may, however, be used in making the overall evaluation (IARC, 1991; Vainio *et al.*, 1992; IARC, 2005, 2006; see also Part B, Sections 4 and 6). As mechanisms of carcinogenesis are elucidated, IARC convenes international scientific conferences to determine whether a broad-based consensus has emerged on how specific mechanistic data can be used in an evaluation of human carcinogenicity. The results of such conferences are reported in IARC Scientific Publications, which, as long as they still reflect the current state of scientific knowledge, may guide subsequent Working Groups.

Although the *Monographs* have emphasized hazard identification, important issues may also involve dose–response assessment. In many cases, the same epidemiological and experimental studies used to evaluate a cancer hazard can also be used to estimate a dose–response relationship. A *Monograph* may undertake to estimate dose–response relationships within the range of the available epidemiological data, or it may compare the dose–response information from experimental and epidemiological studies. In some cases, a subsequent publication may be prepared by a separate Working Group with expertise in quantitative dose–response assessment.

The *Monographs* are used by national and international authorities to make risk assessments, formulate decisions concerning preventive measures, provide effective cancer control programmes and decide among alternative options for public health decisions. The evaluations of IARC Working Groups are scientific, qualitative judgements on the evidence for or against carcinogenicity provided by the available data. These evaluations represent only one part of the body of information on which public health decisions may be based. Public health options vary from one situation to another and from country to country and relate to many factors, including different socioeconomic and national priorities. Therefore, no recommendation is given with regard to regulation or legislation, which are the responsibility of individual governments or other international organizations.

3. Selection of agents for review

Agents are selected for review on the basis of two main criteria: (a) there is evidence of human exposure and (b) there is some evidence or suspicion of carcinogenicity. Mixed exposures may occur in occupational and environmental settings and as a result of individual and cultural habits (such as tobacco smoking and dietary practices). Chemical analogues and compounds with biological or physical characteristics similar to those of suspected carcinogens may also be considered, even in the absence of data on a possible carcinogenic effect in humans or experimental animals.

The scientific literature is surveyed for published data relevant to an assessment of carcinogenicity. Ad-hoc Advisory Groups convened by IARC in 1984, 1989, 1991, 1993, 1998 and 2003 made recommendations as to which agents should be evaluated in the *Monographs* series. Recent recommendations are available on the *Monographs* programme website (http://monographs.iarc.fr). IARC may schedule other agents for review as it becomes aware of new scientific information or as national health agencies identify an urgent public health need related to cancer.

As significant new data become available on an agent for which a *Monograph* exists, a re-evaluation may be made at a subsequent meeting, and a new *Monograph* published. In some cases it may be appropriate to review only the data published since a prior evaluation. This can be useful for updating a database, reviewing new data to resolve a previously open question or identifying new tumour sites associated with a carcinogenic agent. Major changes in an evaluation (e.g. a new classification in Group 1 or a determination that a mechanism does not operate in humans, see Part B, Section 6) are more appropriately addressed by a full review.

4. Data for the *Monographs*

Each *Monograph* reviews all pertinent epidemiological studies and cancer bioassays in experimental animals. Those judged inadequate or irrelevant to the evaluation may be cited but not summarized. If a group of similar studies is not reviewed, the reasons are indicated.

Mechanistic and other relevant data are also reviewed. A *Monograph* does not necessarily cite all the mechanistic literature concerning the agent being evaluated (see Part B, Section 4). Only those data considered by the Working Group to be relevant to making the evaluation are included.

With regard to epidemiological studies, cancer bioassays, and mechanistic and other relevant data, only reports that have been published or accepted for publication in the openly available scientific literature are reviewed. The same publication requirement applies to studies originating from IARC, including meta-analyses or pooled analyses commissioned by IARC in advance of a meeting (see Part B, Section 2c). Data from government agency reports that are publicly available are also considered. Exceptionally,

doctoral theses and other material that are in their final form and publicly available may be reviewed.

Exposure data and other information on an agent under consideration are also reviewed. In the sections on chemical and physical properties, on analysis, on production and use and on occurrence, published and unpublished sources of information may be considered.

Inclusion of a study does not imply acceptance of the adequacy of the study design or of the analysis and interpretation of the results, and limitations are clearly outlined in square brackets at the end of each study description (see Part B). The reasons for not giving further consideration to an individual study also are indicated in the square brackets.

5. Meeting participants

Five categories of participant can be present at *Monograph* meetings.

(a) The Working Group is responsible for the critical reviews and evaluations that are developed during the meeting. The tasks of Working Group Members are: (i) to ascertain that all appropriate data have been collected; (ii) to select the data relevant for the evaluation on the basis of scientific merit; (iii) to prepare accurate summaries of the data to enable the reader to follow the reasoning of the Working Group; (iv) to evaluate the results of epidemiological and experimental studies on cancer; (v) to evaluate data relevant to the understanding of mechanisms of carcinogenesis; and (vi) to make an overall evaluation of the carcinogenicity of the exposure to humans. Working Group Members generally have published significant research related to the carcinogenicity of the agents being reviewed, and IARC uses literature searches to identify most experts. Working Group Members are selected on the basis of (a) knowledge and experience and (b) absence of real or apparent conflicts of interests. Consideration is also given to demographic diversity and balance of scientific findings and views.

(b) Invited Specialists are experts who also have critical knowledge and experience but have a real or apparent conflict of interests. These experts are invited when necessary to assist in the Working Group by contributing their unique knowledge and experience during subgroup and plenary discussions. They may also contribute text on non-influential issues in the section on exposure, such as a general description of data on production and use (see Part B, Section 1). Invited Specialists do not serve as meeting chair or subgroup chair, draft text that pertains to the description or interpretation of cancer data, or participate in the evaluations.

(c) Representatives of national and international health agencies often attend meetings because their agencies sponsor the programme or are interested in the subject of a meeting. Representatives do not serve as meeting chair or subgroup chair, draft any part of a *Monograph,* or participate in the evaluations.

(d) Observers with relevant scientific credentials may be admitted to a meeting by IARC in limited numbers. Attention will be given to achieving a balance of Observers

from constituencies with differing perspectives. They are invited to observe the meeting and should not attempt to influence it. Observers do not serve as meeting chair or subgroup chair, draft any part of a *Monograph*, or participate in the evaluations. At the meeting, the meeting chair and subgroup chairs may grant Observers an opportunity to speak, generally after they have observed a discussion. Observers agree to respect the Guidelines for Observers at *IARC Monographs* meetings (available at http://monographs.iarc.fr).

(e) The IARC Secretariat consists of scientists who are designated by IARC and who have relevant expertise. They serve as rapporteurs and participate in all discussions. When requested by the meeting chair or subgroup chair, they may also draft text or prepare tables and analyses.

Before an invitation is extended, each potential participant, including the IARC Secretariat, completes the WHO Declaration of Interests to report financial interests, employment and consulting, and individual and institutional research support related to the subject of the meeting. IARC assesses these interests to determine whether there is a conflict that warrants some limitation on participation. The declarations are updated and reviewed again at the opening of the meeting. Interests related to the subject of the meeting are disclosed to the meeting participants and in the published volume (Cogliano *et al.*, 2004).

The names and principal affiliations of participants are available on the *Monographs* programme website (http://monographs.iarc.fr) approximately two months before each meeting. It is not acceptable for Observers or third parties to contact other participants before a meeting or to lobby them at any time. Meeting participants are asked to report all such contacts to IARC (Cogliano *et al.*, 2005).

All participants are listed, with their principal affiliations, at the beginning of each volume. Each participant who is a Member of a Working Group serves as an individual scientist and not as a representative of any organization, government or industry.

6. Working procedures

A separate Working Group is responsible for developing each volume of *Monographs*. A volume contains one or more *Monographs*, which can cover either a single agent or several related agents. Approximately one year in advance of the meeting of a Working Group, the agents to be reviewed are announced on the *Monographs* programme website (http://monographs.iarc.fr) and participants are selected by IARC staff in consultation with other experts. Subsequently, relevant biological and epidemiological data are collected by IARC from recognized sources of information on carcinogenesis, including data storage and retrieval systems such as PubMed. Meeting participants who are asked to prepare preliminary working papers for specific sections are expected to supplement the IARC literature searches with their own searches.

For most chemicals and some complex mixtures, the major collection of data and the preparation of working papers for the sections on chemical and physical properties, on

analysis, on production and use, and on occurrence are carried out under a separate contract funded by the US National Cancer Institute. Industrial associations, labour unions and other knowledgeable organizations may be asked to provide input to the sections on production and use, although this involvement is not required as a general rule. Information on production and trade is obtained from governmental, trade and market research publications and, in some cases, by direct contact with industries. Separate production data on some agents may not be available for a variety of reasons (e.g. not collected or made public in all producing countries, production is small). Information on uses may be obtained from published sources but is often complemented by direct contact with manufacturers. Efforts are made to supplement this information with data from other national and international sources.

Six months before the meeting, the material obtained is sent to meeting participants to prepare preliminary working papers. The working papers are compiled by IARC staff and sent, prior to the meeting, to Working Group Members and Invited Specialists for review.

The Working Group meets at IARC for seven to eight days to discuss and finalize the texts and to formulate the evaluations. The objectives of the meeting are peer review and consensus. During the first few days, four subgroups (covering exposure data, cancer in humans, cancer in experimental animals, and mechanistic and other relevant data) review the working papers, develop a joint subgroup draft and write summaries. Care is taken to ensure that each study summary is written or reviewed by someone not associated with the study being considered. During the last few days, the Working Group meets in plenary session to review the subgroup drafts and develop the evaluations. As a result, the entire volume is the joint product of the Working Group, and there are no individually authored sections.

IARC Working Groups strive to achieve a consensus evaluation. Consensus reflects broad agreement among Working Group Members, but not necessarily unanimity. The chair may elect to poll Working Group Members to determine the diversity of scientific opinion on issues where consensus is not readily apparent.

After the meeting, the master copy is verified by consulting the original literature, edited and prepared for publication. The aim is to publish the volume within six months of the Working Group meeting. A summary of the outcome is available on the *Monographs* programme website soon after the meeting.

B. SCIENTIFIC REVIEW AND EVALUATION

The available studies are summarized by the Working Group, with particular regard to the qualitative aspects discussed below. In general, numerical findings are indicated as they appear in the original report; units are converted when necessary for easier comparison. The Working Group may conduct additional analyses of the published data and use them in their assessment of the evidence; the results of such supplementary

analyses are given in square brackets. When an important aspect of a study that directly impinges on its interpretation should be brought to the attention of the reader, a Working Group comment is given in square brackets.

The scope of the *IARC Monographs* programme has expanded beyond chemicals to include complex mixtures, occupational exposures, physical and biological agents, lifestyle factors and other potentially carcinogenic exposures. Over time, the structure of a *Monograph* has evolved to include the following sections:

1. Exposure data
2. Studies of cancer in humans
3. Studies of cancer in experimental animals
4. Mechanistic and other relevant data
5. Summary
6. Evaluation and rationale

In addition, a section of General Remarks at the front of the volume discusses the reasons the agents were scheduled for evaluation and some key issues the Working Group encountered during the meeting.

This part of the Preamble discusses the types of evidence considered and summarized in each section of a *Monograph*, followed by the scientific criteria that guide the evaluations.

1. Exposure data

Each *Monograph* includes general information on the agent: this information may vary substantially between agents and must be adapted accordingly. Also included is information on production and use (when appropriate), methods of analysis and detection, occurrence, and sources and routes of human occupational and environmental exposures. Depending on the agent, regulations and guidelines for use may be presented.

(*a*) *General information on the agent*

For chemical agents, sections on chemical and physical data are included: the Chemical Abstracts Service Registry Number, the latest primary name and the IUPAC systematic name are recorded; other synonyms are given, but the list is not necessarily comprehensive. Information on chemical and physical properties that are relevant to identification, occurrence and biological activity is included. A description of technical products of chemicals includes trade names, relevant specifications and available information on composition and impurities. Some of the trade names given may be those of mixtures in which the agent being evaluated is only one of the ingredients.

For biological agents, taxonomy, structure and biology are described, and the degree of variability is indicated. Mode of replication, life cycle, target cells, persistence, latency, host response and clinical disease other than cancer are also presented.

For physical agents that are forms of radiation, energy and range of the radiation are included. For foreign bodies, fibres and respirable particles, size range and relative dimensions are indicated.

For agents such as mixtures, drugs or lifestyle factors, a description of the agent, including its composition, is given.

Whenever appropriate, other information, such as historical perspectives or the description of an industry or habit, may be included.

(*b*) *Analysis and detection*

An overview of methods of analysis and detection of the agent is presented, including their sensitivity, specificity and reproducibility. Methods widely used for regulatory purposes are emphasized. Methods for monitoring human exposure are also given. No critical evaluation or recommendation of any method is meant or implied.

(*c*) *Production and use*

The dates of first synthesis and of first commercial production of a chemical, mixture or other agent are provided when available; for agents that do not occur naturally, this information may allow a reasonable estimate to be made of the date before which no human exposure to the agent could have occurred. The dates of first reported occurrence of an exposure are also provided when available. In addition, methods of synthesis used in past and present commercial production and different methods of production, which may give rise to different impurities, are described.

The countries where companies report production of the agent, and the number of companies in each country, are identified. Available data on production, international trade and uses are obtained for representative regions. It should not, however, be inferred that those areas or nations are necessarily the sole or major sources or users of the agent. Some identified uses may not be current or major applications, and the coverage is not necessarily comprehensive. In the case of drugs, mention of their therapeutic uses does not necessarily represent current practice nor does it imply judgement as to their therapeutic efficacy.

(*d*) *Occurrence and exposure*

Information on the occurrence of an agent in the environment is obtained from data derived from the monitoring and surveillance of levels in occupational environments, air, water, soil, plants, foods and animal and human tissues. When available, data on the generation, persistence and bioaccumulation of the agent are also included. Such data may be available from national databases.

Data that indicate the extent of past and present human exposure, the sources of exposure, the people most likely to be exposed and the factors that contribute to the exposure are reported. Information is presented on the range of human exposure, including occupational and environmental exposures. This includes relevant findings

from both developed and developing countries. Some of these data are not distributed widely and may be available from government reports and other sources. In the case of mixtures, industries, occupations or processes, information is given about all agents known to be present. For processes, industries and occupations, a historical description is also given, noting variations in chemical composition, physical properties and levels of occupational exposure with date and place. For biological agents, the epidemiology of infection is described.

(*e*) *Regulations and guidelines*

Statements concerning regulations and guidelines (e.g. occupational exposure limits, maximal levels permitted in foods and water, pesticide registrations) are included, but they may not reflect the most recent situation, since such limits are continuously reviewed and modified. The absence of information on regulatory status for a country should not be taken to imply that that country does not have regulations with regard to the exposure. For biological agents, legislation and control, including vaccination and therapy, are described.

2. Studies of cancer in humans

This section includes all pertinent epidemiological studies (see Part A, Section 4). Studies of biomarkers are included when they are relevant to an evaluation of carcinogenicity to humans.

(*a*) *Types of study considered*

Several types of epidemiological study contribute to the assessment of carcinogenicity in humans — cohort studies, case–control studies, correlation (or ecological) studies and intervention studies. Rarely, results from randomized trials may be available. Case reports and case series of cancer in humans may also be reviewed.

Cohort and case–control studies relate individual exposures under study to the occurrence of cancer in individuals and provide an estimate of effect (such as relative risk) as the main measure of association. Intervention studies may provide strong evidence for making causal inferences, as exemplified by cessation of smoking and the subsequent decrease in risk for lung cancer.

In correlation studies, the units of investigation are usually whole populations (e.g. in particular geographical areas or at particular times), and cancer frequency is related to a summary measure of the exposure of the population to the agent under study. In correlation studies, individual exposure is not documented, which renders this kind of study more prone to confounding. In some circumstances, however, correlation studies may be more informative than analytical study designs (see, for example, the *Monograph* on arsenic in drinking-water; IARC, 2004).

In some instances, case reports and case series have provided important information about the carcinogenicity of an agent. These types of study generally arise from a suspicion, based on clinical experience, that the concurrence of two events — that is, a particular exposure and occurrence of a cancer — has happened rather more frequently than would be expected by chance. Case reports and case series usually lack complete ascertainment of cases in any population, definition or enumeration of the population at risk and estimation of the expected number of cases in the absence of exposure.

The uncertainties that surround the interpretation of case reports, case series and correlation studies make them inadequate, except in rare instances, to form the sole basis for inferring a causal relationship. When taken together with case–control and cohort studies, however, these types of study may add materially to the judgement that a causal relationship exists.

Epidemiological studies of benign neoplasms, presumed preneoplastic lesions and other end-points thought to be relevant to cancer are also reviewed. They may, in some instances, strengthen inferences drawn from studies of cancer itself.

(*b*) *Quality of studies considered*

It is necessary to take into account the possible roles of bias, confounding and chance in the interpretation of epidemiological studies. Bias is the effect of factors in study design or execution that lead erroneously to a stronger or weaker association than in fact exists between an agent and disease. Confounding is a form of bias that occurs when the relationship with disease is made to appear stronger or weaker than it truly is as a result of an association between the apparent causal factor and another factor that is associated with either an increase or decrease in the incidence of the disease. The role of chance is related to biological variability and the influence of sample size on the precision of estimates of effect.

In evaluating the extent to which these factors have been minimized in an individual study, consideration is given to a number of aspects of design and analysis as described in the report of the study. For example, when suspicion of carcinogenicity arises largely from a single small study, careful consideration is given when interpreting subsequent studies that included these data in an enlarged population. Most of these considerations apply equally to case–control, cohort and correlation studies. Lack of clarity of any of these aspects in the reporting of a study can decrease its credibility and the weight given to it in the final evaluation of the exposure.

Firstly, the study population, disease (or diseases) and exposure should have been well defined by the authors. Cases of disease in the study population should have been identified in a way that was independent of the exposure of interest, and exposure should have been assessed in a way that was not related to disease status.

Secondly, the authors should have taken into account — in the study design and analysis — other variables that can influence the risk of disease and may have been related to the exposure of interest. Potential confounding by such variables should have been dealt with either in the design of the study, such as by matching, or in the analysis,

by statistical adjustment. In cohort studies, comparisons with local rates of disease may or may not be more appropriate than those with national rates. Internal comparisons of frequency of disease among individuals at different levels of exposure are also desirable in cohort studies, since they minimize the potential for confounding related to the difference in risk factors between an external reference group and the study population.

Thirdly, the authors should have reported the basic data on which the conclusions are founded, even if sophisticated statistical analyses were employed. At the very least, they should have given the numbers of exposed and unexposed cases and controls in a case–control study and the numbers of cases observed and expected in a cohort study. Further tabulations by time since exposure began and other temporal factors are also important. In a cohort study, data on all cancer sites and all causes of death should have been given, to reveal the possibility of reporting bias. In a case–control study, the effects of investigated factors other than the exposure of interest should have been reported.

Finally, the statistical methods used to obtain estimates of relative risk, absolute rates of cancer, confidence intervals and significance tests, and to adjust for confounding should have been clearly stated by the authors. These methods have been reviewed for case–control studies (Breslow & Day, 1980) and for cohort studies (Breslow & Day, 1987).

(*c*) *Meta-analyses and pooled analyses*

Independent epidemiological studies of the same agent may lead to results that are difficult to interpret. Combined analyses of data from multiple studies are a means of resolving this ambiguity, and well-conducted analyses can be considered. There are two types of combined analysis. The first involves combining summary statistics such as relative risks from individual studies (meta-analysis) and the second involves a pooled analysis of the raw data from the individual studies (pooled analysis) (Greenland, 1998).

The advantages of combined analyses are increased precision due to increased sample size and the opportunity to explore potential confounders, interactions and modifying effects that may explain heterogeneity among studies in more detail. A disadvantage of combined analyses is the possible lack of compatibility of data from various studies due to differences in subject recruitment, procedures of data collection, methods of measurement and effects of unmeasured co-variates that may differ among studies. Despite these limitations, well-conducted combined analyses may provide a firmer basis than individual studies for drawing conclusions about the potential carcinogenicity of agents.

IARC may commission a meta-analysis or pooled analysis that is pertinent to a particular *Monograph* (see Part A, Section 4). Additionally, as a means of gaining insight from the results of multiple individual studies, ad-hoc calculations that combine data from different studies may be conducted by the Working Group during the course of a *Monograph* meeting. The results of such original calculations, which would be specified in the text by presentation in square brackets, might involve updates of previously conducted analyses that incorporate the results of more recent studies or de-novo

analyses. Irrespective of the source of data for the meta-analyses and pooled analyses, it is important that the same criteria for data quality be applied as those that would be applied to individual studies and to ensure also that sources of heterogeneity between studies be taken into account.

(*d*) *Temporal effects*

Detailed analyses of both relative and absolute risks in relation to temporal variables, such as age at first exposure, time since first exposure, duration of exposure, cumulative exposure, peak exposure (when appropriate) and time since cessation of exposure, are reviewed and summarized when available. Analyses of temporal relationships may be useful in making causal inferences. In addition, such analyses may suggest whether a carcinogen acts early or late in the process of carcinogenesis, although, at best, they allow only indirect inferences about mechanisms of carcinogenesis.

(*e*) *Use of biomarkers in epidemiological studies*

Biomarkers indicate molecular, cellular or other biological changes and are increasingly used in epidemiological studies for various purposes (IARC, 1991; Vainio *et al.*, 1992; Toniolo *et al.*, 1997; Vineis *et al.*, 1999; Buffler *et al.*, 2004). These may include evidence of exposure, of early effects, of cellular, tissue or organism responses, of individual susceptibility or host responses, and inference of a mechanism (see Part B, Section 4b). This is a rapidly evolving field that encompasses developments in genomics, epigenomics and other emerging technologies.

Molecular epidemiological data that identify associations between genetic polymorphisms and interindividual differences in susceptibility to the agent(s) being evaluated may contribute to the identification of carcinogenic hazards to humans. If the polymorphism has been demonstrated experimentally to modify the functional activity of the gene product in a manner that is consistent with increased susceptibility, these data may be useful in making causal inferences. Similarly, molecular epidemiological studies that measure cell functions, enzymes or metabolites that are thought to be the basis of susceptibility may provide evidence that reinforces biological plausibility. It should be noted, however, that when data on genetic susceptibility originate from multiple comparisons that arise from subgroup analyses, this can generate false-positive results and inconsistencies across studies, and such data therefore require careful evaluation. If the known phenotype of a genetic polymorphism can explain the carcinogenic mechanism of the agent being evaluated, data on this phenotype may be useful in making causal inferences.

(*f*) *Criteria for causality*

After the quality of individual epidemiological studies of cancer has been summarized and assessed, a judgement is made concerning the strength of evidence that the agent in question is carcinogenic to humans. In making its judgement, the Working Group

considers several criteria for causality (Hill, 1965). A strong association (e.g. a large relative risk) is more likely to indicate causality than a weak association, although it is recognized that estimates of effect of small magnitude do not imply lack of causality and may be important if the disease or exposure is common. Associations that are replicated in several studies of the same design or that use different epidemiological approaches or under different circumstances of exposure are more likely to represent a causal relationship than isolated observations from single studies. If there are inconsistent results among investigations, possible reasons are sought (such as differences in exposure), and results of studies that are judged to be of high quality are given more weight than those of studies that are judged to be methodologically less sound.

If the risk increases with the exposure, this is considered to be a strong indication of causality, although the absence of a graded response is not necessarily evidence against a causal relationship. The demonstration of a decline in risk after cessation of or reduction in exposure in individuals or in whole populations also supports a causal interpretation of the findings.

A number of scenarios may increase confidence in a causal relationship. On the one hand, an agent may be specific in causing tumours at one site or of one morphological type. On the other, carcinogenicity may be evident through the causation of multiple tumour types. Temporality, precision of estimates of effect, biological plausibility and coherence of the overall database are considered. Data on biomarkers may be employed in an assessment of the biological plausibility of epidemiological observations.

Although rarely available, results from randomized trials that show different rates of cancer among exposed and unexposed individuals provide particularly strong evidence for causality.

When several epidemiological studies show little or no indication of an association between an exposure and cancer, a judgement may be made that, in the aggregate, they show evidence of lack of carcinogenicity. Such a judgement requires firstly that the studies meet, to a sufficient degree, the standards of design and analysis described above. Specifically, the possibility that bias, confounding or misclassification of exposure or outcome could explain the observed results should be considered and excluded with reasonable certainty. In addition, all studies that are judged to be methodologically sound should (a) be consistent with an estimate of effect of unity for any observed level of exposure, (b) when considered together, provide a pooled estimate of relative risk that is at or near to unity, and (c) have a narrow confidence interval, due to sufficient population size. Moreover, no individual study nor the pooled results of all the studies should show any consistent tendency that the relative risk of cancer increases with increasing level of exposure. It is important to note that evidence of lack of carcinogenicity obtained from several epidemiological studies can apply only to the type(s) of cancer studied, to the dose levels reported, and to the intervals between first exposure and disease onset observed in these studies. Experience with human cancer indicates that the period from first exposure to the development of clinical cancer is sometimes longer than 20 years; latent periods substantially shorter than 30 years cannot provide evidence for lack of carcinogenicity.

3. Studies of cancer in experimental animals

All known human carcinogens that have been studied adequately for carcinogenicity in experimental animals have produced positive results in one or more animal species (Wilbourn *et al.*, 1986; Tomatis *et al.*, 1989). For several agents (e.g. aflatoxins, diethylstilbestrol, solar radiation, vinyl chloride), carcinogenicity in experimental animals was established or highly suspected before epidemiological studies confirmed their carcinogenicity in humans (Vainio *et al.*, 1995). Although this association cannot establish that all agents that cause cancer in experimental animals also cause cancer in humans, it is biologically plausible that agents for which there is *sufficient evidence of carcinogenicity* in experimental animals (see Part B, Section 6b) also present a carcinogenic hazard to humans. Accordingly, in the absence of additional scientific information, these agents are considered to pose a carcinogenic hazard to humans. Examples of additional scientific information are data that demonstrate that a given agent causes cancer in animals through a species-specific mechanism that does not operate in humans or data that demonstrate that the mechanism in experimental animals also operates in humans (see Part B, Section 6).

Consideration is given to all available long-term studies of cancer in experimental animals with the agent under review (see Part A, Section 4). In all experimental settings, the nature and extent of impurities or contaminants present in the agent being evaluated are given when available. Animal species, strain (including genetic background where applicable), sex, numbers per group, age at start of treatment, route of exposure, dose levels, duration of exposure, survival and information on tumours (incidence, latency, severity or multiplicity of neoplasms or preneoplastic lesions) are reported. Those studies in experimental animals that are judged to be irrelevant to the evaluation or judged to be inadequate (e.g. too short a duration, too few animals, poor survival; see below) may be omitted. Guidelines for conducting long-term carcinogenicity experiments have been published (e.g. OECD, 2002).

Other studies considered may include: experiments in which the agent was administered in the presence of factors that modify carcinogenic effects (e.g. initiation–promotion studies, co-carcinogenicity studies and studies in genetically modified animals); studies in which the end-point was not cancer but a defined precancerous lesion; experiments on the carcinogenicity of known metabolites and derivatives; and studies of cancer in non-laboratory animals (e.g. livestock and companion animals) exposed to the agent.

For studies of mixtures, consideration is given to the possibility that changes in the physicochemical properties of the individual substances may occur during collection, storage, extraction, concentration and delivery. Another consideration is that chemical and toxicological interactions of components in a mixture may alter dose–response relationships. The relevance to human exposure of the test mixture administered in the animal experiment is also assessed. This may involve consideration of the following aspects of the mixture tested: (i) physical and chemical characteristics, (ii) identified

constituents that may indicate the presence of a class of substances and (iii) the results of genetic toxicity and related tests.

The relevance of results obtained with an agent that is analogous (e.g. similar in structure or of a similar virus genus) to that being evaluated is also considered. Such results may provide biological and mechanistic information that is relevant to the understanding of the process of carcinogenesis in humans and may strengthen the biological plausibility that the agent being evaluated is carcinogenic to humans (see Part B, Section 2f).

(*a*) *Qualitative aspects*

An assessment of carcinogenicity involves several considerations of qualitative importance, including (i) the experimental conditions under which the test was performed, including route, schedule and duration of exposure, species, strain (including genetic background where applicable), sex, age and duration of follow-up; (ii) the consistency of the results, for example, across species and target organ(s); (iii) the spectrum of neoplastic response, from preneoplastic lesions and benign tumours to malignant neoplasms; and (iv) the possible role of modifying factors.

Considerations of importance in the interpretation and evaluation of a particular study include: (i) how clearly the agent was defined and, in the case of mixtures, how adequately the sample characterization was reported; (ii) whether the dose was monitored adequately, particularly in inhalation experiments; (iii) whether the doses, duration of treatment and route of exposure were appropriate; (iv) whether the survival of treated animals was similar to that of controls; (v) whether there were adequate numbers of animals per group; (vi) whether both male and female animals were used; (vii) whether animals were allocated randomly to groups; (viii) whether the duration of observation was adequate; and (ix) whether the data were reported and analysed adequately.

When benign tumours (a) occur together with and originate from the same cell type as malignant tumours in an organ or tissue in a particular study and (b) appear to represent a stage in the progression to malignancy, they are usually combined in the assessment of tumour incidence (Huff *et al.*, 1989). The occurrence of lesions presumed to be preneoplastic may in certain instances aid in assessing the biological plausibility of any neoplastic response observed. If an agent induces only benign neoplasms that appear to be end-points that do not readily undergo transition to malignancy, the agent should nevertheless be suspected of being carcinogenic and requires further investigation.

(*b*) *Quantitative aspects*

The probability that tumours will occur may depend on the species, sex, strain, genetic background and age of the animal, and on the dose, route, timing and duration of the exposure. Evidence of an increased incidence of neoplasms with increasing levels of exposure strengthens the inference of a causal association between the exposure and the development of neoplasms.

The form of the dose–response relationship can vary widely, depending on the particular agent under study and the target organ. Mechanisms such as induction of DNA damage or inhibition of repair, altered cell division and cell death rates and changes in intercellular communication are important determinants of dose–response relationships for some carcinogens. Since many chemicals require metabolic activation before being converted to their reactive intermediates, both metabolic and toxicokinetic aspects are important in determining the dose–response pattern. Saturation of steps such as absorption, activation, inactivation and elimination may produce non-linearity in the dose–response relationship (Hoel *et al.*, 1983; Gart *et al.*, 1986), as could saturation of processes such as DNA repair. The dose–response relationship can also be affected by differences in survival among the treatment groups.

(*c*) *Statistical analyses*

Factors considered include the adequacy of the information given for each treatment group: (i) number of animals studied and number examined histologically, (ii) number of animals with a given tumour type and (iii) length of survival. The statistical methods used should be clearly stated and should be the generally accepted techniques refined for this purpose (Peto *et al.*, 1980; Gart *et al.*, 1986; Portier & Bailer, 1989; Bieler & Williams, 1993). The choice of the most appropriate statistical method requires consideration of whether or not there are differences in survival among the treatment groups; for example, reduced survival because of non-tumour-related mortality can preclude the occurrence of tumours later in life. When detailed information on survival is not available, comparisons of the proportions of tumour-bearing animals among the effective number of animals (alive at the time the first tumour was discovered) can be useful when significant differences in survival occur before tumours appear. The lethality of the tumour also requires consideration: for rapidly fatal tumours, the time of death provides an indication of the time of tumour onset and can be assessed using life-table methods; non-fatal or incidental tumours that do not affect survival can be assessed using methods such as the Mantel-Haenzel test for changes in tumour prevalence. Because tumour lethality is often difficult to determine, methods such as the Poly-K test that do not require such information can also be used. When results are available on the number and size of tumours seen in experimental animals (e.g. papillomas on mouse skin, liver tumours observed through nuclear magnetic resonance tomography), other more complicated statistical procedures may be needed (Sherman *et al.*, 1994; Dunson *et al.*, 2003).

Formal statistical methods have been developed to incorporate historical control data into the analysis of data from a given experiment. These methods assign an appropriate weight to historical and concurrent controls on the basis of the extent of between-study and within-study variability: less weight is given to historical controls when they show a high degree of variability, and greater weight when they show little variability. It is generally not appropriate to discount a tumour response that is significantly increased compared with concurrent controls by arguing that it falls within the range of historical controls, particularly when historical controls show high between-study variability and

are, thus, of little relevance to the current experiment. In analysing results for uncommon tumours, however, the analysis may be improved by considering historical control data, particularly when between-study variability is low. Historical controls should be selected to resemble the concurrent controls as closely as possible with respect to species, gender and strain, as well as other factors such as basal diet and general laboratory environment, which may affect tumour-response rates in control animals (Haseman *et al.*, 1984; Fung *et al.*, 1996; Greim *et al.*, 2003).

Although meta-analyses and combined analyses are conducted less frequently for animal experiments than for epidemiological studies due to differences in animal strains, they can be useful aids in interpreting animal data when the experimental protocols are sufficiently similar.

4. Mechanistic and other relevant data

Mechanistic and other relevant data may provide evidence of carcinogenicity and also help in assessing the relevance and importance of findings of cancer in animals and in humans. The nature of the mechanistic and other relevant data depends on the biological activity of the agent being considered. The Working Group considers representative studies to give a concise description of the relevant data and issues that they consider to be important; thus, not every available study is cited. Relevant topics may include toxicokinetics, mechanisms of carcinogenesis, susceptible individuals, populations and life-stages, other relevant data and other adverse effects. When data on biomarkers are informative about the mechanisms of carcinogenesis, they are included in this section.

These topics are not mutually exclusive; thus, the same studies may be discussed in more than one subsection. For example, a mutation in a gene that codes for an enzyme that metabolizes the agent under study could be discussed in the subsections on toxicokinetics, mechanisms and individual susceptibility if it also exists as an inherited polymorphism.

(*a*) *Toxicokinetic data*

Toxicokinetics refers to the absorption, distribution, metabolism and elimination of agents in humans, experimental animals and, where relevant, cellular systems. Examples of kinetic factors that may affect dose–response relationships include uptake, deposition, biopersistence and half-life in tissues, protein binding, metabolic activation and detoxification. Studies that indicate the metabolic fate of the agent in humans and in experimental animals are summarized briefly, and comparisons of data from humans and animals are made when possible. Comparative information on the relationship between exposure and the dose that reaches the target site may be important for the extrapolation of hazards between species and in clarifying the role of in-vitro findings.

(*b*) *Data on mechanisms of carcinogenesis*

To provide focus, the Working Group attempts to identify the possible mechanisms by which the agent may increase the risk of cancer. For each possible mechanism, a representative selection of key data from humans and experimental systems is summarized. Attention is given to gaps in the data and to data that suggests that more than one mechanism may be operating. The relevance of the mechanism to humans is discussed, in particular, when mechanistic data are derived from experimental model systems. Changes in the affected organs, tissues or cells can be divided into three non-exclusive levels as described below.

(i) *Changes in physiology*

Physiological changes refer to exposure-related modifications to the physiology and/or response of cells, tissues and organs. Examples of potentially adverse physiological changes include mitogenesis, compensatory cell division, escape from apoptosis and/or senescence, presence of inflammation, hyperplasia, metaplasia and/or preneoplasia, angiogenesis, alterations in cellular adhesion, changes in steroidal hormones and changes in immune surveillance.

(ii) *Functional changes at the cellular level*

Functional changes refer to exposure-related alterations in the signalling pathways used by cells to manage critical processes that are related to increased risk for cancer. Examples of functional changes include modified activities of enzymes involved in the metabolism of xenobiotics, alterations in the expression of key genes that regulate DNA repair, alterations in cyclin-dependent kinases that govern cell cycle progression, changes in the patterns of post-translational modifications of proteins, changes in regulatory factors that alter apoptotic rates, changes in the secretion of factors related to the stimulation of DNA replication and transcription and changes in gap–junction-mediated intercellular communication.

(iii) *Changes at the molecular level*

Molecular changes refer to exposure-related changes in key cellular structures at the molecular level, including, in particular, genotoxicity. Examples of molecular changes include formation of DNA adducts and DNA strand breaks, mutations in genes, chromosomal aberrations, aneuploidy and changes in DNA methylation patterns. Greater emphasis is given to irreversible effects.

The use of mechanistic data in the identification of a carcinogenic hazard is specific to the mechanism being addressed and is not readily described for every possible level and mechanism discussed above.

Genotoxicity data are discussed here to illustrate the key issues involved in the evaluation of mechanistic data.

Tests for genetic and related effects are described in view of the relevance of gene mutation and chromosomal aberration/aneuploidy to carcinogenesis (Vainio

et al., 1992; McGregor *et al*., 1999). The adequacy of the reporting of sample characterization is considered and, when necessary, commented upon; with regard to complex mixtures, such comments are similar to those described for animal carcinogenicity tests. The available data are interpreted critically according to the end-points detected, which may include DNA damage, gene mutation, sister chromatid exchange, micronucleus formation, chromosomal aberrations and aneuploidy. The concentrations employed are given, and mention is made of whether the use of an exogenous metabolic system *in vitro* affected the test result. These data are listed in tabular form by phylogenetic classification.

Positive results in tests using prokaryotes, lower eukaryotes, insects, plants and cultured mammalian cells suggest that genetic and related effects could occur in mammals. Results from such tests may also give information on the types of genetic effect produced and on the involvement of metabolic activation. Some end-points described are clearly genetic in nature (e.g. gene mutations), while others are associated with genetic effects (e.g. unscheduled DNA synthesis). In-vitro tests for tumour promotion, cell transformation and gap–junction intercellular communication may be sensitive to changes that are not necessarily the result of genetic alterations but that may have specific relevance to the process of carcinogenesis. Critical appraisals of these tests have been published (Montesano *et al*., 1986; McGregor *et al*., 1999).

Genetic or other activity manifest in humans and experimental mammals is regarded to be of greater relevance than that in other organisms. The demonstration that an agent can induce gene and chromosomal mutations in mammals *in vivo* indicates that it may have carcinogenic activity. Negative results in tests for mutagenicity in selected tissues from animals treated *in vivo* provide less weight, partly because they do not exclude the possibility of an effect in tissues other than those examined. Moreover, negative results in short-term tests with genetic end-points cannot be considered to provide evidence that rules out the carcinogenicity of agents that act through other mechanisms (e.g. receptor-mediated effects, cellular toxicity with regenerative cell division, peroxisome proliferation) (Vainio *et al*., 1992). Factors that may give misleading results in short-term tests have been discussed in detail elsewhere (Montesano *et al*., 1986; McGregor *et al*., 1999).

When there is evidence that an agent acts by a specific mechanism that does not involve genotoxicity (e.g. hormonal dysregulation, immune suppression, and formation of calculi and other deposits that cause chronic irritation), that evidence is presented and reviewed critically in the context of rigorous criteria for the operation of that mechanism in carcinogenesis (e.g. Capen *et al.*, 1999).

For biological agents such as viruses, bacteria and parasites, other data relevant to carcinogenicity may include descriptions of the pathology of infection, integration and expression of viruses, and genetic alterations seen in human tumours. Other observations

that might comprise cellular and tissue responses to infection, immune response and the presence of tumour markers are also considered.

For physical agents that are forms of radiation, other data relevant to carcinogenicity may include descriptions of damaging effects at the physiological, cellular and molecular level, as for chemical agents, and descriptions of how these effects occur. 'Physical agents' may also be considered to comprise foreign bodies, such as surgical implants of various kinds, and poorly soluble fibres, dusts and particles of various sizes, the pathogenic effects of which are a result of their physical presence in tissues or body cavities. Other relevant data for such materials may include characterization of cellular, tissue and physiological reactions to these materials and descriptions of pathological conditions other than neoplasia with which they may be associated.

(*c*) *Other data relevant to mechanisms*

A description is provided of any structure–activity relationships that may be relevant to an evaluation of the carcinogenicity of an agent, the toxicological implications of the physical and chemical properties, and any other data relevant to the evaluation that are not included elsewhere.

High-output data, such as those derived from gene expression microarrays, and high-throughput data, such as those that result from testing hundreds of agents for a single end-point, pose a unique problem for the use of mechanistic data in the evaluation of a carcinogenic hazard. In the case of high-output data, there is the possibility to overinterpret changes in individual end-points (e.g. changes in expression in one gene) without considering the consistency of that finding in the broader context of the other end-points (e.g. other genes with linked transcriptional control). High-output data can be used in assessing mechanisms, but all end-points measured in a single experiment need to be considered in the proper context. For high-throughput data, where the number of observations far exceeds the number of end-points measured, their utility for identifying common mechanisms across multiple agents is enhanced. These data can be used to identify mechanisms that not only seem plausible, but also have a consistent pattern of carcinogenic response across entire classes of related compounds.

(*d*) *Susceptibility data*

Individuals, populations and life-stages may have greater or lesser susceptibility to an agent, based on toxicokinetics, mechanisms of carcinogenesis and other factors. Examples of host and genetic factors that affect individual susceptibility include sex, genetic polymorphisms of genes involved in the metabolism of the agent under evaluation, differences in metabolic capacity due to life-stage or the presence of disease, differences in DNA repair capacity, competition for or alteration of metabolic capacity by medications or other chemical exposures, pre-existing hormonal imbalance that is exacerbated by a chemical exposure, a suppressed immune system, periods of higher-than-usual tissue growth or regeneration and genetic polymorphisms that lead to

differences in behaviour (e.g. addiction). Such data can substantially increase the strength of the evidence from epidemiological data and enhance the linkage of in-vivo and in-vitro laboratory studies to humans.

(*e*) *Data on other adverse effects*

Data on acute, subchronic and chronic adverse effects relevant to the cancer evaluation are summarized. Adverse effects that confirm distribution and biological effects at the sites of tumour development, or alterations in physiology that could lead to tumour development, are emphasized. Effects on reproduction, embryonic and fetal survival and development are summarized briefly. The adequacy of epidemiological studies of reproductive outcome and genetic and related effects in humans is judged by the same criteria as those applied to epidemiological studies of cancer, but fewer details are given.

5. Summary

This section is a summary of data presented in the preceding sections. Summaries can be found on the *Monographs* programme website (http://monographs.iarc.fr).

(*a*) *Exposure data*

Data are summarized, as appropriate, on the basis of elements such as production, use, occurrence and exposure levels in the workplace and environment and measurements in human tissues and body fluids. Quantitative data and time trends are given to compare exposures in different occupations and environmental settings. Exposure to biological agents is described in terms of transmission, prevalence and persistence of infection.

(*b*) *Cancer in humans*

Results of epidemiological studies pertinent to an assessment of human carcinogenicity are summarized. When relevant, case reports and correlation studies are also summarized. The target organ(s) or tissue(s) in which an increase in cancer was observed is identified. Dose–response and other quantitative data may be summarized when available.

(*c*) *Cancer in experimental animals*

Data relevant to an evaluation of carcinogenicity in animals are summarized. For each animal species, study design and route of administration, it is stated whether an increased incidence, reduced latency, or increased severity or multiplicity of neoplasms or preneoplastic lesions were observed, and the tumour sites are indicated. If the agent produced tumours after prenatal exposure or in single-dose experiments, this is also mentioned. Negative findings, inverse relationships, dose–response and other quantitative data are also summarized.

(*d*) *Mechanistic and other relevant data*

Data relevant to the toxicokinetics (absorption, distribution, metabolism, elimination) and the possible mechanism(s) of carcinogenesis (e.g. genetic toxicity, epigenetic effects) are summarized. In addition, information on susceptible individuals, populations and life-stages is summarized. This section also reports on other toxic effects, including reproductive and developmental effects, as well as additional relevant data that are considered to be important.

6. Evaluation and rationale

Evaluations of the strength of the evidence for carcinogenicity arising from human and experimental animal data are made, using standard terms. The strength of the mechanistic evidence is also characterized.

It is recognized that the criteria for these evaluations, described below, cannot encompass all of the factors that may be relevant to an evaluation of carcinogenicity. In considering all of the relevant scientific data, the Working Group may assign the agent to a higher or lower category than a strict interpretation of these criteria would indicate.

These categories refer only to the strength of the evidence that an exposure is carcinogenic and not to the extent of its carcinogenic activity (potency). A classification may change as new information becomes available.

An evaluation of the degree of evidence is limited to the materials tested, as defined physically, chemically or biologically. When the agents evaluated are considered by the Working Group to be sufficiently closely related, they may be grouped together for the purpose of a single evaluation of the degree of evidence.

(*a*) *Carcinogenicity in humans*

The evidence relevant to carcinogenicity from studies in humans is classified into one of the following categories:

***Sufficient evidence of carcinogenicity*:** The Working Group considers that a causal relationship has been established between exposure to the agent and human cancer. That is, a positive relationship has been observed between the exposure and cancer in studies in which chance, bias and confounding could be ruled out with reasonable confidence. A statement that there is *sufficient evidence* is followed by a separate sentence that identifies the target organ(s) or tissue(s) where an increased risk of cancer was observed in humans. Identification of a specific target organ or tissue does not preclude the possibility that the agent may cause cancer at other sites.

***Limited evidence of carcinogenicity*:** A positive association has been observed between exposure to the agent and cancer for which a causal interpretation is considered by the Working Group to be credible, but chance, bias or confounding could not be ruled out with reasonable confidence.

***Inadequate evidence of carcinogenicity*:** The available studies are of insufficient quality, consistency or statistical power to permit a conclusion regarding the presence or absence of a causal association between exposure and cancer, or no data on cancer in humans are available.

***Evidence suggesting lack of carcinogenicity*:** There are several adequate studies covering the full range of levels of exposure that humans are known to encounter, which are mutually consistent in not showing a positive association between exposure to the agent and any studied cancer at any observed level of exposure. The results from these studies alone or combined should have narrow confidence intervals with an upper limit close to the null value (e.g. a relative risk of 1.0). Bias and confounding should be ruled out with reasonable confidence, and the studies should have an adequate length of follow-up. A conclusion of *evidence suggesting lack of carcinogenicity* is inevitably limited to the cancer sites, conditions and levels of exposure, and length of observation covered by the available studies. In addition, the possibility of a very small risk at the levels of exposure studied can never be excluded.

In some instances, the above categories may be used to classify the degree of evidence related to carcinogenicity in specific organs or tissues.

When the available epidemiological studies pertain to a mixture, process, occupation or industry, the Working Group seeks to identify the specific agent considered most likely to be responsible for any excess risk. The evaluation is focused as narrowly as the available data on exposure and other aspects permit.

(*b*) *Carcinogenicity in experimental animals*

Carcinogenicity in experimental animals can be evaluated using conventional bioassays, bioassays that employ genetically modified animals, and other in-vivo bioassays that focus on one or more of the critical stages of carcinogenesis. In the absence of data from conventional long-term bioassays or from assays with neoplasia as the end-point, consistently positive results in several models that address several stages in the multistage process of carcinogenesis should be considered in evaluating the degree of evidence of carcinogenicity in experimental animals.

The evidence relevant to carcinogenicity in experimental animals is classified into one of the following categories:

***Sufficient evidence of carcinogenicity*:** The Working Group considers that a causal relationship has been established between the agent and an increased incidence of malignant neoplasms or of an appropriate combination of benign and malignant neoplasms in (a) two or more species of animals or (b) two or more independent studies in one species carried out at different times or in different laboratories or under different protocols. An increased incidence of tumours in both sexes of a single

species in a well-conducted study, ideally conducted under Good Laboratory Practices, can also provide *sufficient evidence*.

A single study in one species and sex might be considered to provide *sufficient evidence of carcinogenicity* when malignant neoplasms occur to an unusual degree with regard to incidence, site, type of tumour or age at onset, or when there are strong findings of tumours at multiple sites.

***Limited evidence of carcinogenicity*:** The data suggest a carcinogenic effect but are limited for making a definitive evaluation because, e.g. (a) the evidence of carcinogenicity is restricted to a single experiment; (b) there are unresolved questions regarding the adequacy of the design, conduct or interpretation of the studies; (c) the agent increases the incidence only of benign neoplasms or lesions of uncertain neoplastic potential; or (d) the evidence of carcinogenicity is restricted to studies that demonstrate only promoting activity in a narrow range of tissues or organs.

***Inadequate evidence of carcinogenicity*:** The studies cannot be interpreted as showing either the presence or absence of a carcinogenic effect because of major qualitative or quantitative limitations, or no data on cancer in experimental animals are available.

***Evidence suggesting lack of carcinogenicity*:** Adequate studies involving at least two species are available which show that, within the limits of the tests used, the agent is not carcinogenic. A conclusion of *evidence suggesting lack of carcinogenicity* is inevitably limited to the species, tumour sites, age at exposure, and conditions and levels of exposure studied.

(*c*) *Mechanistic and other relevant data*

Mechanistic and other evidence judged to be relevant to an evaluation of carcinogenicity and of sufficient importance to affect the overall evaluation is highlighted. This may include data on preneoplastic lesions, tumour pathology, genetic and related effects, structure–activity relationships, metabolism and toxicokinetics, physicochemical parameters and analogous biological agents.

The strength of the evidence that any carcinogenic effect observed is due to a particular mechanism is evaluated, using terms such as 'weak', 'moderate' or 'strong'. The Working Group then assesses whether that particular mechanism is likely to be operative in humans. The strongest indications that a particular mechanism operates in humans derive from data on humans or biological specimens obtained from exposed humans. The data may be considered to be especially relevant if they show that the agent in question has caused changes in exposed humans that are on the causal pathway to carcinogenesis. Such data may, however, never become available, because it is at least conceivable that certain compounds may be kept from human use solely on the basis of evidence of their toxicity and/or carcinogenicity in experimental systems.

The conclusion that a mechanism operates in experimental animals is strengthened by findings of consistent results in different experimental systems, by the demonstration of

biological plausibility and by coherence of the overall database. Strong support can be obtained from studies that challenge the hypothesized mechanism experimentally, by demonstrating that the suppression of key mechanistic processes leads to the suppression of tumour development. The Working Group considers whether multiple mechanisms might contribute to tumour development, whether different mechanisms might operate in different dose ranges, whether separate mechanisms might operate in humans and experimental animals and whether a unique mechanism might operate in a susceptible group. The possible contribution of alternative mechanisms must be considered before concluding that tumours observed in experimental animals are not relevant to humans. An uneven level of experimental support for different mechanisms may reflect that disproportionate resources have been focused on investigating a favoured mechanism.

For complex exposures, including occupational and industrial exposures, the chemical composition and the potential contribution of carcinogens known to be present are considered by the Working Group in its overall evaluation of human carcinogenicity. The Working Group also determines the extent to which the materials tested in experimental systems are related to those to which humans are exposed.

(*d*) *Overall evaluation*

Finally, the body of evidence is considered as a whole, in order to reach an overall evaluation of the carcinogenicity of the agent to humans.

An evaluation may be made for a group of agents that have been evaluated by the Working Group. In addition, when supporting data indicate that other related agents, for which there is no direct evidence of their capacity to induce cancer in humans or in animals, may also be carcinogenic, a statement describing the rationale for this conclusion is added to the evaluation narrative; an additional evaluation may be made for this broader group of agents if the strength of the evidence warrants it.

The agent is described according to the wording of one of the following categories, and the designated group is given. The categorization of an agent is a matter of scientific judgement that reflects the strength of the evidence derived from studies in humans and in experimental animals and from mechanistic and other relevant data.

Group 1: The agent is *carcinogenic to humans*.

This category is used when there is *sufficient evidence of carcinogenicity* in humans. Exceptionally, an agent may be placed in this category when evidence of carcinogenicity in humans is less than *sufficient* but there is *sufficient evidence of carcinogenicity* in experimental animals and strong evidence in exposed humans that the agent acts through a relevant mechanism of carcinogenicity.

Group 2.

This category includes agents for which, at one extreme, the degree of evidence of carcinogenicity in humans is almost *sufficient*, as well as those for which, at the other extreme, there are no human data but for which there is evidence of

carcinogenicity in experimental animals. Agents are assigned to either Group 2A (*probably carcinogenic to humans*) or Group 2B (*possibly carcinogenic to humans*) on the basis of epidemiological and experimental evidence of carcinogenicity and mechanistic and other relevant data. The terms *probably carcinogenic* and *possibly carcinogenic* have no quantitative significance and are used simply as descriptors of different levels of evidence of human carcinogenicity, with *probably carcinogenic* signifying a higher level of evidence than *possibly carcinogenic*.

Group 2A: The agent is *probably carcinogenic to humans*.

This category is used when there is *limited evidence of carcinogenicity* in humans and *sufficient evidence of carcinogenicity* in experimental animals. In some cases, an agent may be classified in this category when there is *inadequate evidence of carcinogenicity* in humans and *sufficient evidence of carcinogenicity* in experimental animals and strong evidence that the carcinogenesis is mediated by a mechanism that also operates in humans. Exceptionally, an agent may be classified in this category solely on the basis of *limited evidence of carcinogenicity* in humans. An agent may be assigned to this category if it clearly belongs, based on mechanistic considerations, to a class of agents for which one or more members have been classified in Group 1 or Group 2A.

Group 2B: The agent is *possibly carcinogenic to humans*.

This category is used for agents for which there is *limited evidence of carcinogenicity* in humans and less than *sufficient evidence of carcinogenicity* in experimental animals. It may also be used when there is *inadequate evidence of carcinogenicity* in humans but there is *sufficient evidence of carcinogenicity* in experimental animals. In some instances, an agent for which there is *inadequate evidence of carcinogenicity* in humans and less than *sufficient evidence of carcinogenicity* in experimental animals together with supporting evidence from mechanistic and other relevant data may be placed in this group. An agent may be classified in this category solely on the basis of strong evidence from mechanistic and other relevant data.

Group 3: The agent is *not classifiable as to its carcinogenicity to humans*.

This category is used most commonly for agents for which the evidence of carcinogenicity is *inadequate* in humans and *inadequate* or *limited* in experimental animals.

Exceptionally, agents for which the evidence of carcinogenicity is *inadequate* in humans but *sufficient* in experimental animals may be placed in this category when there is strong evidence that the mechanism of carcinogenicity in experimental animals does not operate in humans.

Agents that do not fall into any other group are also placed in this category.

An evaluation in Group 3 is not a determination of non-carcinogenicity or overall safety. It often means that further research is needed, especially when exposures are widespread or the cancer data are consistent with differing interpretations.

Group 4: The agent is *probably not carcinogenic to humans*.

This category is used for agents for which there is *evidence suggesting lack of carcinogenicity* in humans and in experimental animals. In some instances, agents for which there is *inadequate evidence of carcinogenicity* in humans but *evidence suggesting lack of carcinogenicity* in experimental animals, consistently and strongly supported by a broad range of mechanistic and other relevant data, may be classified in this group.

(*e*) *Rationale*

The reasoning that the Working Group used to reach its evaluation is presented and discussed. This section integrates the major findings from studies of cancer in humans, studies of cancer in experimental animals, and mechanistic and other relevant data. It includes concise statements of the principal line(s) of argument that emerged, the conclusions of the Working Group on the strength of the evidence for each group of studies, citations to indicate which studies were pivotal to these conclusions, and an explanation of the reasoning of the Working Group in weighing data and making evaluations. When there are significant differences of scientific interpretation among Working Group Members, a brief summary of the alternative interpretations is provided, together with their scientific rationale and an indication of the relative degree of support for each alternative.

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GENERAL REMARKS

This ninety-fourth volume of *IARC Monographs* contains evaluations of the carcinogenic hazard to humans of ingested nitrate and nitrite, microcystins and nodularin. This is the first *Monographs* review of these agents, which were nominated as high priorities for future evaluation by the most recent Advisory Group (IARC, 2003).

In view of the body's conversion of nitrate to nitrite and then to *N*-nitroso compounds, interpretation of the epidemiological studies on nitrate/nitrite ingestion was aided by mechanistic information on factors that accelerate or inhibit this conversion. Some epidemiological studies of high nitrate ingestion primarily from vegetables found reduced risks of gastric cancer, while other studies of nitrate/nitrite ingestion from nitrite-preserved meats found increased risks. Mechanistic studies show that the formation of *N*-nitroso compounds is accelerated by the presence of nitrosatable compounds (found in meat) and inhibited by vitamin C and other antioxidants (found often in vegetables). These inferences about *N*-nitrosamine formation are consistent with the epidemiological studies that examined interactions between nitrite and antioxidants and with the animal bioassays that investigated various combinations of nitrate/nitrite, nitrosatable compounds, and antioxidants. The cancer hazard from nitrate/nitrite ingestion cannot be determined without considering these other factors. Accordingly, the Working Group defined the agent not as "ingested nitrate or nitrite", but as "ingested nitrate or nitrite under conditions that result in endogenous nitrosation". This marks the first use of a mechanistic event (endogenous nitrosation) leading to carcinogenesis in the wording of an evaluation statement.

A summary of the findings of this volume appears in *The Lancet Oncology* (Grosse *et al*., 2006).

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THE MONOGRAPHS

INGESTED NITRATE AND NITRITE

INGESTED NITRATE AND NITRITE

This monograph focuses on ingested nitrate and nitrite ions, for which food and water are the major pathways of human exposure. The nitrate and nitrite salts described in Section 1.1 represent some of the potential anthropogenic sources of these compounds in food and water, and their use is presented in relation to exposure by ingestion; however, it is not the intention of this monograph to evaluate the carcinogenicity of any of the individual salts. The highly water-soluble nitrate and nitrite ions are of interest here and the patterns of occurrence and levels of human exposures to nitrate and nitrite ions are described in Sections 1.3 and 1.4.

1. Exposure Data

Nitrate and nitrite are naturally occurring ions that are part of the nitrogen cycle and are ubiquitous in the environment. Both are products of the oxidation of nitrogen (which comprises approximately 78% of the earth's atmosphere) by microorganisms in plants, soil or water. Nitrate is the more stable form of oxidized nitrogen but can be reduced by microbial action to nitrite, which is moderately chemically reactive. Chemical and biological processes can further reduce nitrite to various compounds or oxidize it to nitrate. Nitrate salts are used widely as inorganic fertilizers, and are also used in explosives, as oxidizing agents in the chemical industry and as food preservatives. Nitrite salts have been also used as food preservatives, especially to cure meats (Environmental Protection Agency, 1987; Health Canada, 1992; Pokorny *et al.*, 2006).

During the second half of the twentieth century, production of nitrogen compounds by humans increased dramatically—intentionally through the use of fertilizers or unintentionally as a by-product of the combustion of fossil fuels. In fact, it has been reported that, since the end of the twentieth century, humans have been adding more nitrogen compounds to the nitrogen cycle than all other sources combined (Fields, 2004).

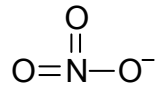
1.1 Chemical and physical data

1.1.1 *Nomenclature, molecular formulae, relative molecular masses and chemical and physical properties*

From Chapman and Hall/CRC (2006) and Toxnet (2006), unless otherwise indicated

Nitrate ion

Chem. Abstr. Serv. (CAS) Reg. No.: 14797-55-8
Deleted CAS Reg. Nos: 23746-18-1; 34236-35-6; 73394-83-9
Chem. Abstr. Name: Nitrate
IUPAC Systematic Name: Nitrate
Synonyms: Nitrate (NO_3^-); nitrate(1−); nitrate ion; nitrate ion (NO_3^-); nitrate ion(1−); nitrato; nitric acid, ion(1−)

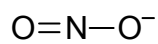


NO_3^- Relative molecular mass: 62.005

(*a*) *Acid*: Conjugated base of the strong acid HNO_3; pK_a = –1.3 (WHO, 2003a)
(b) Reactivity: Unreactive (WHO, 2003a)

Nitrite ion

Chem. Abstr. Serv. Reg. No.: 14797-65-0
Deleted CAS Reg. Nos: 12183-96-9; 114466-53-4
Chem. Abstr. Name: Nitrite
IUPAC Systematic Name: Nitrite
Synonyms: Nitrite (NO_2^-); nitrite(1−); nitrite anion; nitrite ion; nitrite ion (NO_2^-); nitrite ion(1−); nitrogen dioxide(1−); nitrogen dioxide ion(1−); nitrogen peroxide ion(1−); nitrous acid, ion(1−)



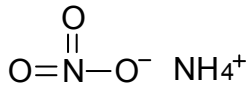
NO_2^- Relative molecular mass: 45.995

(*a*) *Acid*: Conjugated base of the weak acid HNO_2; pK_a = 3.4 (WHO, 2003a)
(*b*) *Reactivity*: Reactive; oxidizes antioxidants, the divalent iron (Fe^{2+}) of haemoglobin to trivalent iron (Fe^{3+}) and primary amines; nitrosates several amines and amides (WHO, 2003a).

Ammonium nitrate

Chem. Abstr. Serv. Reg. No.: 6484-52-2

Deleted CAS Reg. No.: 95255-40-6
Chem. Abstr. Name: Nitric acid, ammonium salt
IUPAC Systematic Name: Nitric acid, ammonium salt
Synonyms: Ammonium nitrate; ammonium nitrate (NH_4NO_3); Emulite; EXP 200; German saltpeter; Norge salpeter; Norway salpeter; Norwegian salpeter; Plenco 12203; Varioform I; ZhVK

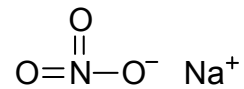


NH_4NO_3 Relative molecular mass: 80.043

(*a*) *Description*: White hygroscopic crystals; exists in five crystalline forms
(*b*) *Boiling-point*: Decomposes at about 210 °C, mostly into water and nitrous oxide (Merck & Co., 2005)
(*c*) *Melting-point*: 169.7 °C (Lide, 2005)
(*d*) *Density*: 1.725 g/cm^3
(*e*) *Solubility*: Very soluble in water (118.3 g/100 g at 0 °C; 213 g/100 g at 25 °C; 871 g/100 g at 100 °C) (Lide, 2005); soluble in acetone, ammonia, ethanol (3.8 g/100 g at 20 °C), isopropanol and methanol (17.1 g/100 g at 20 °C); insoluble in diethyl ether (Merck & Co., 2005)
(*f*) *pH*: 0.1 M solution in water is 5.43.

Sodium nitrate

Chem. Abstr. Serv. Reg. No.: 7631-99-4
Deleted CAS Reg. No.: 862599-22-2
Chem. Abstr. Name: Nitric acid, sodium salt
IUPAC Systematic Name: Nitric acid, sodium salt
Synonyms: Chile salpeter; niter; nitric acid sodium salt (1:1); salpeter; soda niter



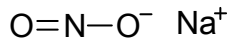
$NaNO_3$ Relative molecular mass: 84.995

(*a*) *Description*: Colourless trigonal (rhombohedral) crystals; undergoes transition to high-temperature polymorph at ~278 °C
(*b*) *Boiling-point*: 380 °C; decomposes on heating to form sodium nitrite (Weast, 1979).
(*c*) *Melting-point*: 306 °C
(*d*) *Density*: 2.26 g/cm^3 (Merck & Co., 2005)

(*e*) *Solubility*: Very soluble in water (92 g/100 g at 25 °C; 180 g/100 g at 100 °C); very soluble in ammonia; soluble in ethanol and methanol; very slightly soluble in acetone and glycerol; practically insoluble in dimethyl ether (Weast, 1979)
(*f*) *pH*: An aqueous solution is neutral (Merck & Co., 2005).

Sodium nitrite

Chem. Abstr. Serv. Reg. No.: 7632-00-0
Deleted CAS Reg. Nos: 32863-15-3; 56227-20-4; 82497-43-6; 82998-40-1
Chem. Abstr. Name: Nitrous acid, sodium salt
IUPAC Systematic Name: Nitrous acid, sodium salt
Synonyms: Nitrous acid soda; nitrous acid sodium salt (1:1)

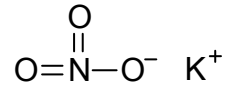


$NaNO_2$ Relative molecular mass: 68.985

(*a*) *Description*: White to pale yellow, hygroscopic, orthorhombic crystals (Lide, 2005)
(*b*) *Boiling-point*: > 320 °C (decomposes) (Lide, 2005); undergoes transition to high-temperature orthorhombic polymorph at 158 °C.
(*c*) *Melting-point*: 271 °C
(*d*) *Density*: 2.17 g/cm^3 (Merck & Co., 2005)
(*e*) *Solubility*: Very soluble in water (84.8 g/100 g at 25 °C); very soluble in ammonia; slightly soluble in ethanol (3 g/100 g at 20 °C) (Lide, 2005) and methanol (4.4 g/100 g at 20 °C); sparingly soluble in diethyl ether (0.3 g/100 g at 20 °C) (Weast, 1989)
(*f*) *pH*: An aqueous solution is alkaline (~9) (Merck & Co., 2005).

Potassium nitrate

Chem. Abstr. Serv. Reg. No.: 7757-79-1
Deleted CAS Reg. No.: 96193-83-8
Chem. Abstr. Name: Nitric acid, potassium salt
IUPAC Systematic Name: Nitric acid, potassium salt
Synonyms: Niter; nitre; nitric acid potassium salt (1:1); salpeter



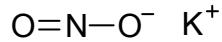
KNO_3 Relative molecular mass: 101.103

(*a*) *Description*: Colourless, orthorhombic crystals

(*b*) *Boiling-point*: 400 °C (decomposes) (Lide, 2005); undergoes orthorhombic to rhombohedral form transition at 128 °C; cooling the rhombohedral form gives a metastable rhombohedral polymorph that exists between 128 °C and 109 °C.
(*c*) *Melting-point*: 334 °C
(*d*) *Density*: 2.11 g/cm^3 (Merck & Co., 2005)
(*e*) *Solubility*: Very soluble in water (13 g/100 g at 0 °C; 38.3 g/100 g at 25 °C; 247 g/100 g at 100 °C); soluble in ammonia; insoluble in diethyl ether and ethanol (Weast, 1989; Lide, 2005)
(*f*) *pH*: ~7 (Merck & Co., 2005)

Potassium nitrite

Chem. Abstr. Serv. Reg. No.: 7758-09-0
Chem. Abstr. Name: Nitrous acid, potassium salt
IUPAC Systematic Name: Nitrous acid, potassium salt
Synonyms: Chile salpeter; niter; nitric acid sodium salt (1:1); salpeter; soda niter



KNO_2 Relative molecular mass: 85.093

(*a*) *Description*: Pale yellow, hexagonal crystals; white, hygroscopic crystals (Lide, 2005)
(*b*) *Boiling-point*: 537 °C (explodes) (Lide, 2005); undergoes transition from rhombohedral to cubic form at 70 °C; disproportionates on heating in the absence of air to form potassium nitrate (KNO_3), potassium oxide (K_2O) and evolving nitrogen.
(*c*) *Melting-point*: 440 °C
(*d*) *Density*: 1.915 g/cm^3 (Lide, 2005)
(*e*) *Solubility*: Very soluble in water (281 g/100 g at 0 °C; 312 g/100 g at 25 °C; 413 g/100 g at 100 °C); very soluble in ammonia; slightly soluble in ethanol (Weast, 1989; Lide, 2005)

1.1.2 *Physicochemical properties*

Concentrations of nitrate and nitrite can be expressed in different units, as either milligrams per litre (mg/L), milligrams of nitrate nitrogen per litre (mg/L nitrate-N) or milligrams of nitrite nitrogen per litre (mg/L nitrite-N). It can also be expressed in terms of moles (or millimoles); the latter simplifies the comparison of nitrate and nitrite with regard to exposure and their relative contributions to *N*-nitroso compounds. Conversion factors between the different units are given in Table 1.1 (Environmental Protection Agency, 1987; WHO, 2003a).

Table 1.1. Conversion factors between different units

	mM	mg/L NO_3^-	mg/L NO_3^--N	mg/L NO_2^-	mg/L NO_2^--N
mM	1	62	14	46	14
mg/L NO_3^-	0.016	1	0.226	–	–
mg/L NO_3^--N	0.0714	4.429	1	–	–
mg/L NO_2^-	0.0217	–	–	1	0.304
mg/L NO_2^--N	0.0714	–	–	3.29	1

From Environmental Protection Agency (1987); WHO (2003a)
NO_3^-, nitrate; NO_3^--N, nitrate nitrogen; NO_2^-, nitrite; NO_2^--N, nitrite nitrogen

1.2 Analysis

Spectrometric techniques are used for the determination of nitrate in water. Selected methods for the determination of nitrates and nitrites in various matrices are presented in Table 1.2. A molecular absorption spectrometric method is available for the determination of nitrite in drinking-water, raw water and wastewater (International Standards Organization, 1984b). A continuous flow spectrometric method for the determination of nitrite, nitrate or the sum of both in various types of water is suitable in undiluted samples (International Standards Organization, 1996). Nitrate and nitrite can also be determined in water by liquid chromatography (International Standards Organization, 1992).

WHO (2004a) has reported limits of detection for the following analytical methods: 0.1 mg/L (nitrate) and 0.05 mg/L (nitrite) by liquid chromatography; 0.01–1 mg/L (nitrate) by spectrometric techniques; 0.005–0.01 mg/L (nitrite) by a molecular absorption spectrometric method; and 22 µg/L (nitrate) and 35 µg/L (nitrite) by ion-exchange chromatography.

1.3 Production and use of major nitrate and nitrite salts

1.3.1 *Production and consumption volumes* (from Chemical Information Services (2006), unless otherwise specified)

(a) Ammonium nitrate

Ammonium nitrate was first described in 1659 by the German scientist Glauber, who prepared it by the reaction of ammonium carbonate and nitric acid. He called it ‘nitrium flammans’ because its yellow flame (from traces of sodium) was different from that of potassium nitrate. Historically, ammonium nitrate was manufactured by a double decomposition method using sodium nitrate and either ammonium sulfate or ammonium chloride. Modern commercial processes, however, rely almost exclusively on the neutralization

Table 1.2. Selected methods for the analysis of nitrate and nitrite in various matrices

Sample matrix	Sample preparation	Assay procedure	Limit of detection[a]	Limit of quantitation	Reference
Water (drinking-, rain-, ground-, surface)	React with potassium hydrogenphthalate or sodium carbonate/sodium hydrogencarbonate eluent; inject sample; compare peaks with standard solutions.	HPLC/CD-CE	0.1 mg/L (nitrate-N); 0.05 mg/L (nitrite-N)	Ranges of application: 0.1–50 mg/L (nitrate); 0.05–20 mg/L (nitrite)	International Standards Organisation (1992)
Water (drinking-, waste-, aqueous extracts)	Inject sample; compare with standards.	IEC/CD	0.002 mg/L (nitrate-N); 0.004 mg/L (nitrite-N)	NR	Environment Protection Agency (2000)
Water (drinking-, surface, saline; waste- (domestic and industrial))	React with brucine sulfate in sulfuric acid solution; measure absorbance at 410 nm.	Spectrophoto-metry	0.1 mg/L (nitrate-N)	Applicable range of concentrations: 0.1–2 mg/L nitrate-N	Environment Protection Agency (1997)
Water (waste-)	React with potassium hydrogenphthalate or other appropriate eluent; inject sample; compare peaks with standard solutions.	HPLC/CD-CE or UVD	0.1 mg/L (nitrate-N); 0.05 mg/L (nitrite-N)	Working ranges: 0.1–50 mg/L (nitrate); 0.05–20 mg/L (nitrite)	International Standards Organisation (1995)
Water (raw and potable)	React with sodium salicylate and sulfuric acid; treat with alkali; measure absorbance at 415 nm.	Spectrometry	0.003–0.013 mg/L (nitrate)	Range: up to 0.2 mg/L nitrate-N using max. test portion vol. of 25 mL	International Standards Organisation (1988)

Table 1.2 (contd)

Sample matrix	Sample preparation	Assay procedure	Limit of detection[a]	Limit of quantitation	Reference
Water (potable, raw, waste-)	React with 4-aminobenzene sulfonamide and orthophosphoric acid at pH 1.9; react diazonium salt formed reacts with *N*-(1-naphthyl)-1,2-diamino-ethane dihydrochloride; measure absorbance at 540 nm.	Spectrometry	0.001–0.002 mg/L (nitrite)	Range: up to 0.25 mg/L nitrite-N using max. test portion vol. of 40 mL	International Standard Organisation (1984a)
Water (ground-, drinking-, surface, waste-, sea-)	Reduce nitrate to nitrite with cadmium; react with sulfanilamide chloride and *N*-(1-naphthyl)ethylenediamine dihydrochloride; measure absorbance at 520-560 nm.	Spectrometry with FIA or CFA	NR (nitrate and nitrite)	Range of application: mass concentration, 0.01–1 mg/L nitrite-N; 0.2–20 mg/L nitrite-/nitrate-N (undiluted samples)	International Standards Organisation (1996)
Water (natural)	Inject sample.	IEC/SCD	0.05 mg/L (nitrate-N); 0.02 mg/L (nitrite-N)	Working ranges: 0.05–150 mg/L nitrate-N; 0.02–70 mg/L nitrite-N	Geological Survey (1987)
Water (low ionic strength)	Inject sample.	IEC/SCD	0.01 mg/L (nitrate-N)	Analytical range: 0.01–0.6 mg/L nitrate-N	Geological Survey (1985)
Water (low ionic strength)	React with sulfanilamide under acidic conditions; react couple diazo compound formed with *N*-(1-naphthyl)ethylenediamine dihydrochloride; measure absorbance at 540 nm.	Colorimetry	0.001 mg/L (nitrite-N)	Application range: 0.001–0.20 mg/L nitrite-N	Geological Survey (1989)

Table 1.2 (contd)

Sample matrix	Sample preparation	Assay procedure	Limit of detection[a]	Limit of quantitation	Reference
Water (surface, domestic, industrial, brine)	React with sulfanilamide under acidic conditions; react couple diazo compound formed with *N*-(1-naphthyl)ethylenediamine dihydrochloride; measure absorbance at 540 nm.	Colorimetry	0.01 mg/L (nitrite-N)	Application range: 0.01–1.0 mg/L nitrite-N	Geological Survey (1990)
Water (surface, ground-, waste-, drinking-)	Inject sample.	IEC/CSEC	0.003 mg/L (nitrate-N); 0.004 mg/L (nitrite-N)	NR	APHA/ AWWA/WEF (2005a)
Water (untreated and treated drinking-)	Inject sample.	IEC/DCD	0.017 mg/L (nitrate-N); 0.015 mg/L (nitrite-N)	NR	APHA/ AWWA/WEF (2005b)
Water	Dilute sample with reagent water; remove suspended solids by filtration; measure absorbance at 254 nm.	CIE/UVD	0.1 mg/L	NR	APHA/ AWWA/WEF (2005c)
Water (potable, uncontaminated natural)	Remove suspended solids by filtration; acidify with hydrochloric acid; measure absorbance at 220 nm (screening method).	UVS	NR (nitrate-N)	NR	APHA/ AWWA/WEF (2005d) Method B

Table 1.2 (contd)

Sample matrix	Sample preparation	Assay procedure	Limit of detection[a]	Limit of quantitation	Reference
Water (potable)	Remove suspended solids by filtration; acidify with sulfuric or hydrochloric acid; measure absorbance of second derivative at 230–210 nm.	UVS	0.05 mg/L (nitrate-N)	Applicable range: 0.05–2 mg/L nitrate-N	APHA/ AWWA/WEF (2005d) Method C
Water	Immerse electrode tips in sample; record potential reading when stable; compare with standards.	NIE	0.14 mg/L (nitrate-N)	Applicable range: 0.14–1400 mg/L nitrate-N	APHA/ AWWA/WEF (2005d) Method D
Water	Reduce nitrate to nitrite with cadmium–copper complex; react with sulfanilamide; react couple diazo compound formed with *N*-(1-naphthyl)ethylenediamine dihydrochloride; measure absorbance at 543 nm [cadmium reduction method].	Spectro-photometry	0.01 mg/L (nitrate-N and nitrite-N)	Applicable range: 0.01–1.0 mg/L nitrate-N	APHA/ AWWA/WEF (2005d) Method E

Table 1.2 (contd)

Sample matrix	Sample preparation	Assay procedure	Limit of detection[a]	Limit of quantitation	Reference
Water (potable, surface and saline; domestic and industrial waste-)	Reduce nitrate to nitrite with cadmium–copper complex; react with sulfanilamide; react couple diazo compound formed with *N*-(1-naphthyl)ethylenediamine dihydrochloride; measure absorbance [automated cadmium reduction method].	Colorimetry	0.5 mg/L (nitrate-N)	Applicable range: 0.1–10 mg/L nitrate-N	APHA/ AWWA/WEF (2005d) Method F
Water (potable and surface; domestic and industrial waste-)	Reduce nitrate to nitrite with hydrazine sulfate; react with sulfanilamide; react couple diazo compound formed with *N*-(1-naphthyl)ethylenediamine dihydrochloride; measure absorbance at 520 nm [automated hydrazine reduction method].	Colorimetry	0.01 mg/L (nitrate-N)	Applicable range: 0.1–10 mg/L nitrate-N	APHA/ AWWA/WEF (2005d) Method H
Water	Reduce nitrate to nitrite with cadmium–copper complex; react with sulfanilamide; react couple diazo compound formed with *N*-(1-naphthyl)ethylenediamine dihydrochloride; measure absorbance at 540 nm [cadmium reduction flow injection method].	Colorimetry with FIA	0.025 mg/L (nitrogen)	Applicable range: 0.00025–10 mg/L nitrate-N	APHA/ AWWA/WEF (2005d) Method I

Table 1.2 (contd)

Sample matrix	Sample preparation	Assay procedure	Limit of detection[a]	Limit of quantitation	Reference
Water	React with sulfanilamide under acidic conditions; react couple diazo compound formed with *N*-(1-naphthyl)ethylenediamine dihydrochloride; measure absorbance at 543 nm.	Spectro-photometry	0.005 mg/L (nitrite-N)	Applicable range: 10–1000 mg/L nitrite-N	APHA/ AWWA/WEF (2005e) Method B
Water (drinking- and waste-)	Inject sample.	IEC	0.5 mg/L (nitrate-N); 0.4 mg/L (nitrite-N)	Application concentration range: 0.3–100 µg/L (range differs for each analyte)	AOAC International (2000) Method 993.30
Water	Inject sample.	IEC/CSEC	0.42 mg/L (nitrate); 0.36 mg/L (nitrite)	Application concentration range: differs for each analyte	ASTM International (1997)
Water (high purity)	Inject sample; compare with standards.	IEC/CD-CE	0.0002 mg/L	Application concentration range: 0.02–100 µg/L	ASTM International (2000a)

Table 1.2 (contd)

Sample matrix	Sample preparation	Assay procedure	Limit of detection[a]	Limit of quantitation	Reference
Water (drinking-, waste-, other aqueous matrices)	Inject sample; measure absorbance at 254 nm.	CIE/UVD	0.08 mg/L (nitrate); 0.1 mg/L (nitrite)	Application concentration range: 0.1–50 mg/L	ASTM International (2000b)
Water (surface, saline, waste-, ground-)	Reduce nitrate to nitrite with cadmium–copper complex; react with sulfanilamide; react couple diazo compound formed with *N*-(1-naphthyl)ethylenediamine dihydrochloride; measure absorbance at 540 nm [automated and manual cadmium reduction method].	Spectro-photometry	0.05 mg/L (nitrate-N and/or nitrite-N)	Application range: 0.05–1.0 mg/L nitrogen	ASTM International (2004)
Water (surface and saline; domestic and industrial waste-)	React with brucine in sulfuric acid; measure absorbance at 410 nm.	Colorimetry	0.1 mg/L (nitrate-N)	Application range: 0.1–2 mg/L	AOAC International (2000) Method 973.50

Table 1.2 (contd)

Sample matrix	Sample preparation	Assay procedure	Limit of detection[a]	Limit of quantitation	Reference
Meat and meat products	Extract with hot water; precipitate proteins; filter; reduce extracted nitrates to nitrites with metallic cadmium; add sulfanilamide chloride and *N*-(1-naphthyl)ethylenediamine dihydrochloride; measure absorbance at 538 nm.	Photoelectric colorimetry or spectro-photometry	NR (nitrate)	NR	International Standards Organisation (1975)
Cured meat	Mix with water; add bromocresol green indicator; oxidize nitrites to nitrates; add sulfuric acid and phosphotungstic acid; add silver ammonium hydroxide; add sulfuric acid; add *meta*-xylenol; compare colour to standards.	Colorimetry	NR (nitrate and nitrite)	NR	AOAC International (2000) Method 935.48
Cured meat	React with sulfanilamide; react couple diazo compound formed with *N*-(1-naphthyl)ethylenediamine dihydrochloride; measure absorbance at 540 nm.	Colorimetry	NR (nitrites)	NR	AOAC International (2000) Method 973.31

Table 1.2 (contd)

Sample matrix	Sample preparation	Assay procedure	Limit of detection[a]	Limit of quantitation	Reference
Curing preparations	Mix potassium iodide and starch solution; bubble in carbon dioxide; add sulfuric acid; titrate with sodium thiosulfate.	Titration (nitrites)	NR (nitrites)	NR	AOAC International (2000) Method 964.13
Fruit, vegetables and derived products	Extract with hot water; precipitate proteins by addition of potassium hexacyanoferrate(II) and zinc acetate; filter precipitate; reduce nitrates to nitrites with metallic cadmium; add sulfanilamide chloride and *N*-(1-naphthyl)ethylenediamine dihydrochloride; measure absorbance at 538 nm.	Spectrometry	NR (nitrates and nitrites)	NR	International Standards Organisation (1984b)

Table 1.2 (contd)

Sample matrix	Sample preparation	Assay procedure	Limit of detection[a]	Limit of quantitation	Reference
Dried milk	Dissolve in water; precipitate fat and proteins; filter; reduce nitrate to nitrite with zinc powder and cadmium ion; add sulfanilamide chloride and *N*-(1-naphthyl)ethylenediamine dihydrochloride; measure absorbance at 538 nm.	Spectrometry	NR (nitrate)	NR	International Standards Organisation (1987)
Milk and milk products[b]	Dissolve in water; precipitate fat and proteins; filter; reduce nitrate to nitrite with copperized cadmium; react with sulfanilamide chloride and *N*-(1-naphthyl)ethylenediamine dihydrochloride; measure absorbance at 538 nm.	Spectrometry with FIA or SFA	NR (nitrate and nitrite)	NR	International Standards Organisation (2004a)
Milk and milk products[c]	Suspend sample in water; reduce nitrate to nitrite with cadmium; measure absorbance (nitrate determination); suspend sample in ammonium/sodium chloride solution (nitrite determination); measure absorbance at 550 nm.	Spectrometry with SFA (routine method)	NR (nitrate and nitrite)	NR	International Standards Organisation (2004b)

Table 1.2 (contd)

Sample matrix	Sample preparation	Assay procedure	Limit of detection[a]	Limit of quantitation	Reference
Milk and milk products[d]	Suspend sample in warm extraction buffer solution; separate fat by centrifuging and rapid cooling; reduce nitrate to nitrite with cadmium; react with sulfanilamide and *N*-(1-naphthyl)ethylenediamine dihydrochloride; measure absorbance at 540 nm.	Spectrometry with FIA	0.5 mg/kg (nitrate ions); 1.0 mg/kg (nitrite ions)	NR	International Standards Organisation (2004c)
Cheese (very hard, hard, semisoft, soft)	Mix cheese–water slurry; add zinc sulfate and sodium hydroxide; heat; cool; filter; reduce nitrate to nitrite; measure absorbance at 522 nm.	Spectro-photometry	≥1 µg/g nitrate	NR	AOAC International (2000) Method 976.14
Flour	Mix with water; react with sulfanilic acid; add α-naphthylamine hydrochloride solution; compare with standards.	Digestion	NR (nitrite-N)	NR	AOAC International (2000) Method 951.03
Fertilizer	Precipitate nitrate ions in acid solution as a complex with nitron reagent; filter precipitate; dry; weigh.	Nitron gravimetry	NR (nitrate)	NR	International Standards Organisation (1981)

Table 1.2 (contd)

Sample matrix	Sample preparation	Assay procedure	Limit of detection[a]	Limit of quantitation	Reference
Fertilizer	Mix with hot water; add sulfuric acid; add iron sulfate solution; assess colour change (purple, brown, reddish).	Colorimetry	NR (nitrates)	NR	AOAC International (2000) Method 920.01
Animal feed	Extract with cadmium and barium chloride solution; precipitate proteins in alkaline solution; reduce nitrates to nitrites with cadmium; measure absorbance at 540 nm.	Colorimetry	NR (nitrate-N and nitrite-N)	NR	AOAC International (2000) Method 968.07
Forages	Extract nitrate from sample into aluminum sulfate solution; compare with standards.	Potentiometric titration	0.50% potassium nitrate	NR	AOAC International (2000) Method 986.31
Infant food (containing meat)	Reduce nitrate to nitrite with spongy cadmium; measure absorbance at 530 nm	Spectro-photometry	100–300 mg/kg (as sodium nitrate)	NR	AOAC International (2000) Method 993.03

CD, conductivity detection; CFA, continuous flow analysis; CIE, capillary ion electrophoresis; CSEC, chemical suppression of eluant conductivity; DCD, direct conductivity detection; FIA, flow injection analysis; HPLC/CD-CE, high-performance liquid chromatography with conductivity detection and cation exchanger; IEC, ion-exchange chromatography; max., maximum; NIE, nitrate ion electrode; NR, not reported; SCD, specific conductivity detection; SFA, segmented flow analysis; UVD, ultraviolet detection; UVS, ultraviolet spectrophotometry; vol., volume

[a] The Working Group noted the limited validity of the methods that lack a limit of detection and/or quantitation.

[b] Includes whole and partly skimmed and skimmed dry milk, hard, semi-hard and soft cheeses, processed cheese, whey cheese, caseins and caseinates and dried whey.

[c] Includes milk, cheese, liquid and dried milk products and baby products.

[d] Hard, semi-hard and soft cheeses of various ages, processed cheese, whey powder, milk powder and milk-based infant food

of nitric acid, produced from ammonia through catalysed oxidation, with ammonia. The predominant commercial form has been the solid product that is used as a fertilizer. However, the use of ammonium nitrate as a component in urea–ammonium nitrate liquid fertilizer has grown to the extent that approximately half of the ammonium nitrate produced is actually marketed as a solution (Weston *et al.*, 2003).

World production of ammonium nitrate peaked in 1988 and then declined by 21% between 1986 and 2002 to 13.6 million tonnes (see Table 1.3). Eastern and western Europe recorded large declines while modest increases occurred in Africa and the USA. The former Soviet Union, the USA and Western Europe were the main producers. Together, these regions accounted for 72% of total world production in 2002; eastern Europe contributed approximately 7% and Africa contributed approximately 6%.

Table 1.3. World production and apparent consumption of ammonium nitrate in 1986 and 2002 (thousands of tonnes)

Geographical region	1986		2002	
	Production	Consumption	Production	Consumption
Former Soviet Union	4800	4684	3917	2185
Western Europe	5175	5295	3451	3674
Eastern Europe	2538	2337	1005	1124
Africa	621	747	800	811
Middle East	323	432	407	691
Eastern Asia[a]	715	708	405	754
Central and South America	250	264	314	620
North America				
Canada	403	270	392	257
Mexico	35	37	27	65
USA	2111	2256	2439	2727
Southwest Asia[b]	198	198	199	258
Oceania	85	94	158	167
Southeast Asia[c]	8	7	82	137
Japan	29	26	12	17
Total	17 291	17 355	13 608	13 487

From Suresh (2004a)

[a] Eastern Asia: Cambodia, China, Democratic People's Republic of Korea, Lao People's Democratic Republic, Mongolia, Viet Nam

[b] Southwest Asia: Afghanistan, Bangladesh, Bhutan, India, Nepal, Pakistan, Sri Lanka

[c] Southeast Asia: China (province of Taiwan), Indonesia, Malaysia, Myanmar, Philippines, Republic of Korea, Singapore, Thailand

In 2002, the USA and western Europe were the major consumers and accounted for 47% of world consumption. Eastern Europe and the former Soviet Union combined accounted for an additional 25% and Africa accounted for 5%. Apparent world consumption peaked at 17.7 million tonnes of nitrogen in 1988, and then declined by about 23% to 13.6 million tonnes in 2002. This decline in consumption is primarily attributed to the increased use of urea in the developing world and, more recently, because of safety concerns. It also results from a major reduction in consumption and production in eastern Europe and the former Soviet Union after 1988; consumption in this region decreased by 3.7 million tonnes of nitrogen between 1986 and 2002. In addition, consumption in western Europe fell because of a general decline in the use of nitrogen fertilizers following changes in the European Union agricultural subsidy policy (Suresh, 2004a).

In 2002, production of ammonium nitrate and calcium ammonium nitrate combined amounted to about 14.4 million tonnes of nitrogen. Western Europe is a major producer of calcium ammonium nitrate and accounted for approximately 55% of the combined production of ammonium nitrate and calcium ammonium nitrate (Suresh, 2004a).

Between 80 and 85% of ammonium nitrate is used worldwide as material for nitrogen fertilizers; explosives account for most of the remainder (see below) (Suresh, 2004a).

(b) Sodium nitrate

Sodium nitrate is produced worldwide primarily by leaching ores that contain nitrate with brine, followed by fractional crystallization. Alternatively, sodium nitrate can be obtained synthetically by the absorption of nitrous gases or by the neutralization of nitric acid.

The maximum world production of sodium nitrate of 3 million tonnes per year occurred around 1930, at which time the Chilean nitrate industry produced ~2.9 million tonnes. Production of synthetic sodium nitrate also peaked in the mid-1930s (730 000 tonnes per year). In 2004, total world production of sodium nitrate had declined to about 63 000 tonnes, of which 98% was produced in Chile from natural deposits. The remainder was manufactured in Germany and Mexico, generally as a by-product of the manufacture of nitric acid. In addition, China has been reported to manufacture unknown but significant volumes of sodium nitrate for domestic use (Pokorny *et al.*, 2006).

(c) Sodium nitrite

Sodium nitrite has been synthesized by several chemical reactions that involve the reduction of sodium nitrate. Industrial production of sodium nitrite is primarily by the absorption of nitrogen oxides into aqueous sodium carbonate or sodium hydroxide (Pokorny *et al.*, 2006).

(d) Potassium nitrate

Potassium nitrate is produced commercially by the reaction of potassium chloride and nitric acid and is the second largest source of non-chloride potassium fertilizer.

1.3.2 *Uses*

(a) Fertilizers

Ammonium nitrate is used primarily as a nitrogen fertilizer (Weston *et al.*, 2003). Its use as a fertilizer grew rapidly after the Second World War and accounted for about 90% of production in 1975, after which it was gradually replaced by urea (Weston *et al.*, 2003; Suresh, 2004a).

Sodium nitrate is also used as a fertilizer. From 1880 to 1910, it accounted for 60% of world production of nitrogen fertilizers. In the 1990s, it accounted for only 0.1%, although it was still preferred for some specific crops and soil conditions. This decline resulted from an enormous growth in the manufacture of other types of fertilizer and the use of less expensive nitrogen fertilizers that were produced from synthetic ammonia, such as urea, ammonium nitrate, ammonium phosphates, ammonium sulfate and ammonia itself (Pokorny *et al.*, 2006).

Potassium nitrate is more soluble than potassium sulfate and is used as a fully soluble fertilizer in applications such as fertigation [application of a soluble fertilizer via the irrigation system] and interior landscaping.

(b) Cured meat

Sodium nitrite has been used extensively in curing meat and meat products, particularly pork products such as ham, bacon and frankfurters; certain fish and poultry products are also cured with brines that contain sodium nitrite. The process may include dry curing, immersion curing, or direct addition or injection of the curing ingredients. Curing mixtures are typically composed of salt (sodium chloride), sodium or potassium salts of nitrite and nitrate and seasonings. Sodium nitrite acts as a colour fixative and inhibits the growth of bacteria, including *Clostridium botulinum*, which is the source of botulism toxin. Nitrite is a relatively strong reducing agent that has antibacterial properties; however, the preservation of foodstuffs can be attributed to a large degree to the high concentration of salts (including nitrate) that are employed during the curing process. In addition, nitrate can act as a reservoir from which nitrite may be formed by microbiological reduction (Pokorny *et al.*, 2006).

In addition to the preservation of meats, curing is a process that develops and maintains a stable red colour in cured and smoked meats, for which sodium or potassium nitrate or nitrite is responsible. When nitrate is used as the curing agent, the conversion (reduction) of nitrate to nitrite by bacteria in the meat or poultry is a necessary step in the development of the cured colour. The amount of nitrate that is reduced to nitrite is dependent upon the number of nitrate-reducing bacteria and several environmental

conditions such as temperature, moisture content, salt content and pH. Hence, the conversion rate and subsequent amount of nitrite that is formed is difficult to control. Similarly, the further reduction of nitrite to nitric oxide, which reacts with myoglobin (muscle pigment) to produce the cured colour, is affected by the same environmental conditions. When nitrite is used as the curing agent, there is no need for the nitrate reduction step, and the development of the cured colour is much more rapid. When the cured meat is heated, exposed to a more acid environment or left for a sufficient length of time under normal conditions, the nitric oxide–myoglobin is converted into a stable red pigment called nitrosohaemochrome. The time required for a cured colour to develop may be shortened with the use of cure accelerators, such as ascorbic acid and erythorbic acid or their derivatives, sodium ascorbate and sodium erythorbate. Cure accelerators speed up the chemical conversion of nitrous acid to nitric oxide, help to keep myoglobin in a reduced state and also serve as oxygen scavengers to prevent the fading of the colour of cured meat in the presence of sunlight and oxygen (Anon., 2003).

(c) Other uses

(i) *Sodium nitrate*

Sodium nitrate is used in several industrial processes, in most of which it acts primarily as an oxidizing agent. A major use is in the manufacture of medium- and high-quality glass, such as optical and artistic glass, television and computer screens and fibreglass. In the manufacture of explosives, sodium nitrate is used mainly in blasting agents. Another large application is as an ingredient in the production of charcoal briquettes. Sodium nitrate is also used in the manufacture of enamels and porcelain as an oxidizing and fluxing agent, in formulations of heat-transfer salts for heat-treatment baths for alloys and metals, in rubber vulcanization and in petrochemical industries. Other uses of sodium nitrate include the treatment of water, the melting of ice, in adhesives, cleaning compounds and pyrotechnics, in the nitration of organic compounds, in certain types of pharmaceutical production, in the refining of some alloys, for the recovery of lead, in the production of uranium and in the leaching of copper ore (Pokorny *et al.*, 2006). Nitrate is used for the treatment of angina pectoris (Parker & Parker, 1998).

(ii) *Potassium nitrate*

The major industrial use of potassium nitrate is as a component of specialty glasses, especially for cathode-ray tubes for television sets and computer monitors. Other important uses include fireworks, steel manufacture and in a eutectic mixture with sodium nitrite as a heat-transfer agent (Suresh, 2004b; Freilich & Petersen, 2005).

(iii) *Sodium and potassium nitrites*

Most of the industrial uses of sodium nitrite are based on its oxidizing properties or its liberation of nitrous acid in acidic solutions. Sodium nitrite is a convenient source of nitrous acid in the nitrosation and diazotation of aromatic amines and the production of azo dyes (Pokorny *et al.*, 2006).

Other applications of sodium nitrite include the syntheses of saccharin, synthetic caffeine, fluoroaromatics and other pharmaceutical products, pesticides and organic substances; as an inhibitor of polymerization; in the production of foam blowing agents; in the removal of hydrogen sulfide from natural gas; in textile dyeing; and as an analytical reagent (Pokorny *et al.*, 2006). Sodium and potassium nitrites are listed in the European and US Pharmacopeiae, which would indicate that they can be used in pharmaceutical preparations (Thomson Reuters, 2008). Sodium nitrite can be used as an antidote for cyanide poisoning and as a vasodilator (Lundberg & Weitzberg, 2005).

1.4 Occurrence

1.4.1 *Food*

Nitrate and nitrite are naturally occurring ions that are part of the nitrogen cycle and are ubiquitous in the environment. In addition, since the early 1900s, nitrate has been used extensively in agricultural activities, mainly as a fertilizer. The content of nitrate and nitrite in vegetables depends on the type of vegetable, the method of production, the use of fertilizer, the season and light. Nitrate and nitrite are also used as food additives in processed food as preservatives and colour fixatives in meat, poultry, fish and cheese (European Commission, 1995). Levels of nitrate and nitrite may be determined in raw commodities or in food as consumed. Also, two types of data are available: national surveys on food that is ready to eat and randomized samples that aim to control agricultural raw commodities. A national survey on nitrates and nitrites in food exists only for the United Kingdom and is based on the Total Diet Study method (Food Standards Agency, 1998a). Every year, 24 different towns throughout the United Kingdom are selected from which the food samples are bought. Every 2 weeks, instructions and equipment are dispatched to trained shoppers. Once bought, the foods are packed up and collected for delivery to the laboratory. At the laboratory, the food samples are prepared and, where necessary, cooked according to normal domestic practice. A portion of each food, relative to its contribution to the United Kingdom diet, is then combined with similar foods into 21 groups. A homogenous composite sample is then prepared for each group, and these are subdivided according to analytical requirements. The resulting samples are stored for 5 years and analysed for a range of contaminants such as heavy metals, dioxins, pesticides and nutrients as required, which allows the Food Standards Agency to estimate the average exposure of the population to these constituents. When available, these data are presented for each food category because they relate to average concentrations (pooled samples) of nitrate and nitrite in food as consumed. Such an approach provides a more realistic estimate of the concentrations that can be used to estimate long-term exposure.

(a) Nitrate

(i) *Vegetables and fruit*

Many data exist on the concentration of nitrate in vegetables but most of them are more than 20 years old. Reported values range from 30 to 6000 mg/kg (for reviews, see Walker, 1990; Gangolli *et al.*, 1994; European Commission, 1997). On the basis of published data, vegetables can be divided into three groups according to their nitrate content: low nitrate (< 100 mg/kg), medium nitrate (100–1000 mg/kg) and high nitrate (> 1000 mg/kg) (Chilvers *et al.*, 1984). Leafy vegetables such as lettuce, spinach, celery leaf and beetroot leaf have the highest nitrate concentrations (above 1000 mg/kg). In the Republic of Korea (Chung *et al.*, 2003), the concentrations of nitrate were found to be 4259 mg/kg in spinach while an average level of 1800 mg/kg was reported in radishes and Chinese cabbage. In Singapore (Dutt *et al.*, 1987), the concentrations of nitrate in salad, lettuce and spinach were 1360, 1470 and 4570 mg/kg, respectively. In Italy (De Martin & Restani, 2003), the concentration of nitrate in leafy vegetables was reported to range between 80 and 6250 mg/kg, with a higher mean concentration in those obtained by organic farming both for green salad (1680 versus 3009 mg/kg) and chicory (3232 versus 4629 mg/kg). In France (Malmauret *et al.*, 2002), the median concentration of nitrate in conventional and organic products was found to be 1591 and 1135 mg/kg, respectively, in spinach and 804 and 1221 mg/kg, respectively, in lettuce. In Poland, the concentration of nitrate in lettuce was found to be up to 3500 mg/kg (Nabrzyski & Gajewska, 1994). In the United Kingdom between 1996 and 1998 (Ysart *et al.*, 1999a*)*, the average concentration of nitrate in lettuce produced in glasshouses was 2382 and 3124 mg/kg in the summer and winter, respectively, whereas that in lettuce produced outdoors was 1085 mg/kg. In the same study, the concentration of nitrate in spinach was 1900 mg/kg. In a more recent survey in the same country (Food Standards Agency, 2004), the average concentration of nitrate in lettuce produced in glasshouses in summer was 2999 mg/kg (range, 676–4382 mg/kg; $n = 18$); in winter, the average was 3617 mg/kg (range, 1945–5720 mg/kg; $n = 33$). In lettuce produced outdoors, the concentrations were lower both in summer (average, 1140 mg/kg; range, 181–2656 mg/kg) and in winter (average, 1997 mg/kg; range, 810–3100 mg/kg); the average concentration of nitrate in spinach was 1815 mg/kg (range, 141–3909 mg/kg).

A second category of vegetables includes potatoes, cabbage and spring greens, which have concentrations that range between 100 and 1000 mg/kg. In Singapore (Dutt *et al.*, 1987), the concentrations of nitrate were 930, 340, 210, 150 and 140 mg/kg, respectively, in cabbage, green beans, aubergines, ladies finger [okra] and potatoes. In Poland, green beans contained up to 800 mg/kg (Nabrzyski & Gajewska, 1994). In France, concentrations of nitrate in carrots were 113 and 394 mg/kg, respectively, for conventional and organic products (Malmauret *et al.*, 2002). In the same study, French beans were found to contain 711 and 561 mg/kg nitrate, respectively, in conventional and organic products.

In a third category, vegetables such as asparagus or onions and fresh fruit, including tomatoes, had the lowest concentrations (less than 100 mg/kg). The average concentration of nitrate in fresh fruit in the United Kingdom (Food Standards Agency, 1998a) was found to be 27 mg/kg (range, 12–46 mg/kg). In Singapore (Dutt *et al.*, 1987), the concentrations of nitrate in tomatoes, asparagus, onions and mushrooms were found to be 60, 55, 35 and 15 mg/kg, respectively. In the Republic of Korea (Chung *et al.*, 2003), the concentrations of nitrate in onions, soya bean sprouts and green peppers were found to range between 23 and 76 mg/kg. In France (Malmauret *et al.*, 2002), the concentration of nitrate in tomatoes was found to be 19 and 1 mg/kg in conventional and organic products, respectively. In Poland (Nabrzyski & Gajewska, 1994), the concentration of nitrate in various fruit (currants, gooseberries, raspberries and cherries) ranged between 1.3 and 36 mg/kg, while a concentration of 58.7 mg/kg was found in strawberries.

(ii) *Cereal grains and their products*

The mean concentration of nitrate in bread and miscellaneous cereals obtained in total diet studies in the United Kingdom (Food Standards Agency, 1998a) was 7.2–11 mg/kg (range, undetected–20 mg/kg).

The mean nitrate contents reported by Walker (1990) and Gangolli *et al.* (1994) were between 0.5 and 16 mg/kg in cereals and cereal products worldwide. The variability of these results is easily explained by the various locations of production, seasons of sampling and type of products analysed. The concentration of nitrate increased after baking; darker breads or breads that contained rye had slightly higher levels. In bread crumbs and pasta, the concentrations were reported by the same authors to range between 15 and 24 mg/kg.

(iii) *Milk and dairy products*

In the Total Diet Study in the United Kingdom, the average concentration of nitrate in dairy products was 27 mg/kg and that in milk was 3.9–5.3 mg/kg (Food Standards Agency, 1998a).

The concentration of nitrates in cows' milk is generally below 5 mg/L (Walker, 1990; Gangolli *et al.*, 1994). In cheese without nitrate additives, the concentration of nitrates was found to be in the range of 1–8 mg/kg (Walker, 1990).

(iv) *Eggs*

The average concentration of nitrates in eggs was estimated in the United Kingdom Total Diet Study (Food Standards Agency, 1998a) to range between 4.4 and 5.4 mg/kg (range, undetected–12 mg/kg).

(v) *Cured meat*

A survey of nitrate and nitrite preservative samples obtained from supermarkets and other retail outlets in England and Wales has recently been completed. A total of 200 samples, 40 each from Wales, northern England, South East England, South West England and the Midlands, were purchased and analysed between December 1996 and

February 1997. The sampling programme was designed to reflect recent changes in shopping patterns towards freshly sliced produce available from supermarket delicatessen counters or local butchers' shops. Samples were transported to the laboratory and processed immediately (Food Standards Agency, 1998b).

Concentrations of nitrate in meat products in the United Kingdom Total Diet Study (Food Standards Agency, 1998a) were found to be 45 mg/kg (range, 14–101 mg/kg). In a more specific study on nitrate in 200 samples of cured meat (Food Standards Agency, 1998b), levels ranging from 1.4 to 445 mg/kg were found in bacon and ham and those ranging from 0.2 to 450 mg/kg were found in other cured meat products. The mean value of nitrate from all samples was found to be 62 mg/kg and was similar to that obtained in a previous survey. The difference between the two series of results can be explained by the inherent variability of nitrate content in processed meats when treated with additives and by the fact that the second study targeted meat products that were known to contain nitrates whereas the objective of the Total Diet Study was to sample various preserved meat available for consumers and analyse these for several chemicals.

(vi) *Fish*

In the United Kingdom Total Diet Study (Food Standards Agency, 1998a), the average concentration of nitrate in fish was estimated to be 11 mg/kg (range, 5–19 mg/kg).

The concentration of nitrate in fish was generally found to be below 2.5 mg/kg. It should be noted that, because nitrate and nitrite are authorized as food additives in certain countries, higher concentrations, ranging up to 380 mg/kg, were also reported (Walker, 1990).

(vii) *Beer*

In the United Kingdom, the Food Standards Agency (1998a) reported a mean concentration of nitrate in beers of 30 mg/kg (range, 10–100 mg/kg) in one survey. In a later survey, the mean concentration was found to be 16 mg/kg (range, 0.2–140 mg/kg) (Gangolli *et al.*, 1994).

(viii) *Breast milk*

The human mammary gland does not appear to concentrate nitrate, and, although human breast milk can contain nitrate, it has not been shown to be a significant source of exposure of infants (Greer *et al.*, 2005).

(b) Nitrite

(i) *Vegetables*

The average concentration of nitrite in vegetables is generally below 2 mg/kg. A mean concentration of 0.5 mg/kg was found in the United Kingdom Total Diet Study (Food Standards Agency, 1998a). In Korean vegetables (Chung *et al.*, 2003), an average concentration of 0.6 mg/kg was found.

(ii) *Cereal grains and cereal products*

The average concentration of nitrite in cereal products was reported to be 5 mg/kg in one study in the United Kingdom and 4 mg/kg in another study in the USA (Walker, 1990). In the 1997 Total Diet Study in the United Kingdom (Food Standards Agency, 1998a), the concentration of nitrite was found to range between undetected and 1.8 mg/kg.

(iii) *Fish*

The concentration of nitrite in fish was found to be below 0.6 mg/kg in several reports (Walker, 1990; Food Standards Agency, 1998a).

(iv) *Cured meat*

'Cured meat' generally means meat that has been preserved using curing ingredients consisting of food-grade salt and sodium or potassium nitrite, and includes ham, bacon and sausages; it may also include dry-cured meat or meat cured by other processes that do not use nitrite. From a practical point of view, analytical surveys, and in particular the Total Diet Study, are not designed to determine nitrite content only and investigate preserved meat in general. It is therefore difficult to rule out the fact that some samples of meat analysed may have been preserved or cured without the use of nitrites, which could lead to a 'dilution' of the reported average concentration. Nevertheless, such sampling is representative of the exposure of consumers to various preserved meats. In the European Prospective Investigation into Cancer and Nutrition (EPIC) cohort study, cured meat mostly meant nitrite-preserved meat, although some other meat preparations were included as cured meat (Linseisen *et al.*, 2002; Bingham & Riboli, 2004). In the USA and western Europe, but not in China, processed meat is a more abundant commodity than processed fish and is the main source of dietary nitrite. It is therefore essential that the effects of cured meat be distinguished from those of fresh meat.

In the United Kingdom Total Diet Study, the mean concentration of nitrite in cured meat was 4.1 mg/kg (range, 1.5–8.4 mg/kg), while, in a more specific study conducted in the United Kingdom, the average residue level of nitrite was found to be 24 mg/kg (range, 0.2–170 mg/kg) (Food Standards Agency, 1998a,b). [Such a result is probably related to the inclusion in the Total Diet Study of samples of meat products that did not contain nitrate or nitrite as additives.] A survey conducted in the USA (Cassens, 1997) of residual nitrite in 164 samples of cured meat in three trials showed average concentrations of 5–10 mg/kg (range, 0–48 mg/kg). Results reported in the 1970s showed average residual nitrite to be 52.5 mg/kg (range, 0–195 mg/kg).

In a survey from 1981, sausages (e.g. hot dogs) had a mean content of about 100 mg/kg nitrite and fried bacon and fried ham contained about 35 mg/kg nitrite (National Research Council, 1981). In a report that compiled 85 studies conducted between 1970 and 1991 in Canada and the USA of nitrite levels in cured meat, modelization of the results suggested some reduction in nitrite levels during the study period in most types of meat studied, except for frankfurters (Pogoda & Preston-Martin, 2001).

(c) Transformation of nitrate to nitrite and vice versa during storage

Transformation of nitrate to nitrite during storage, especially in home-prepared food, is known to occur. Evidence from studies on methaemoglobinaemia support the contention that the nitrate contained in vegetables is converted to nitrite before consumption (Bruning-Fann & Kaneene, 1993). Levels of nitrite up to 400 mg/kg have been reported in vegetables that have been damaged, poorly stored or stored for extended periods and in pickled or fermented vegetables (WHO, 1995, 2002a).

In contrast, levels of nitrite in cured meat appear to decrease during storage as it is converted into nitric oxide. The decrease is mainly due to its reactivity, and may average 70% (National Research Council, 1981).

(d) Formation of nitrosamines

The formation of nitrosamines from nitrite within preserved food is possible, and the formation of *N*-nitroso compounds in nitrite-preserved meat and fish has been reviewed (Tricker, 1997). The main sources of *N*-nitroso compounds in the diet are nitrite-preserved meat products (Tricker, 1997; Haorah *et al.*, 2001). Haorah *et al.* (2001) reported a mean concentration of 5.5 μmol/kg *N*-nitroso compounds in frankfurters, but only 0.5 μmol/kg *N*-nitroso compounds in fresh meat.

1.4.2 *Water*

The natural occurrence of nitrate and nitrite in soil results from microbial oxidation of ammonia which is derived from organic nitrogenous material such as plant proteins, animals and animal excreta (WHO, 1978). The background concentration of nitrate in sources of water in the USA is very low, and is generally less than 2 mg/L nitrate-N in groundwater and less than 0.6 mg/L in streams. Higher levels of nitrate are found in contaminated sources, and especially in groundwater (Mueller & Helsel, 1996).

Intensive use of nitrogen fertilizers in crop culture is usually the main source of water contamination, but manure and slurries from concentrated animal feeding operations may be a significant source in some areas. Other anthropogenic sources are disposal of municipal effluents by sludge spreading on fields and deposition of airborne nitrogen compounds emitted from the combustion of fossil fuels by industry and automobiles (Puckett, 1994; Mueller & Helsel, 1996). Point sources include septic tanks, old landfills and leaking sewage systems (WHO, 2003a).

As it is not bound to the soil and is very soluble, nitrate that is not taken up by plants leaches into groundwater or rivers. This occurs especially when the transit of water through soil is rapid, due to its geological composition (e.g. sandy soils). The main diffuse sources of contamination of surface water are agricultural run-off, drainage from roads and suburban lawns, and nitrogen transported by rain (Graffy *et al.*, 1996). Nitrite is a relatively unstable form of nitrogen that is rapidly converted to nitrate by bacteria; thus, the concentration of nitrite in environmental media such as water is usually very low, even when the concentration of nitrate is high (WHO, 1978; Health Canada, 1992).

Public water supplies are rarely heavily contaminated by nitrate because they are usually more closely protected and water authorities are obliged to comply with certain water quality standards. Private wells, the quality of which is not usually regulated, are at greater risk of contamination, in particular shallow wells that are located in agricultural areas. Nitrate is one of the major causes of eutrophication in surface water which leads to abundant growth of algae and aquatic plants. As a result, the concentration of nitrate in surface water is usually lower than that in groundwater (WHO, 1978; Health Canada, 1992).

The treatment of contaminated groundwater is very expensive and natural groundwater recharge can often take many years or decades. Despite a reduction in the use of commercial nitrogen fertilizers in Europe in the 1990s, no substantial reduction in the pollution of groundwater by nitrate has been observed in recent years (European Environment Agency, 2003).

(a) Nitrate

Many studies have been carried out on the occurrence of nitrate in sources of water as part of the characterization of water quality throughout the world; most of these have focused on special areas where waters were affected by specific sources of contamination, primarily agricultural activity. In most cases, the water studied was used for multiple purposes and the focus of the study was the impact of environmental sources of contamination.

(i) *Levels and sources of contamination of groundwater*

Groundwater is particularly vulnerable to contamination by nitrate. Data from several continents on the concentrations of nitrate in groundwater are summarized in Table 1.4.

Africa

Two studies were conducted in Morocco. Mourabit *et al.* (2002) evaluated 53 wells and springs located in the Loukkos area where agricultural activity was substantial. Wells were selected in arable areas where fertilizers were used extensively. Concentrations of nitrate-N ranged from 0 to 32.6 mg/L, and 23% of the samples (12/53) contained more than 11.3 mg/L. Shallow wells were more heavily contaminated than deep wells or springs, and extensive use of fertilizers was identified as the main cause of the contamination. Koukal *et al.* (2004) studied the quality of groundwater in the Fez area. Seven shallow wells that were located near rivers polluted by industrial discharges were sampled once; concentrations of nitrate-N ranged from undetectable to 9.4 mg/L.

Chippaux *et al.* (2002) studied representative samples of groundwater aquifers in Niamey, Niger. Seventeen shallow wells and 20 boreholes were monitored during 1998–99. The mean of concentrations in superficial aquifers (shallow wells) varied from 0.2 to 7.7 mg/L nitrate-N; those in borehole wells (depth, 50–70 m) were lower, and varied from 0.05 to 1.6 mg/L nitrate-N. The concentration of nitrate increased slightly during the dry

Table 1.4. Concentrations of nitrate[a] in groundwater of different regions of the world

Country, region	Type of water/identification	Population covered	Nitrate-N (mg/L)	Main activities or sources of pollution	Reference
Africa					
Morocco, Loukkos region	Shallow and deep wells	500 000	0–32.6	Mainly agricultural	Mourabit *et al*. (2002)
Morocco, Fez region	Shallow wells	850 000	<0.2–9.4	Extensive industrial development	Koukal *et al*. (2004)
Niger, Niamey	Shallow wells and boreholes	700 000	0.05–7.7	Large urbanized area	Chippaux *et al*. (2002)
Nigeria, Lagos and Ibadan	Shallow and deep boreholes	~8.2 million	1.4–13.4	Some wells near dumpsites	Sridhar (2000)
Senegal, Dakar	Dakar aquifer	2 million	9.7–81.1 (mean, 42.9)	Lack of sanitation, faulty latrines	Tandia *et al*. (2000)
Asia					
India–Pakistan	Superficial aquifer (Hudiara drain)	37 villages	2.2–14.6	Agriculture, discharge of untreated industrial and sewage waste	Afzal *et al*. (2000)
Japan, near Nagoya City	Kakamigahara aquifer	135 000	0.5–26.8 (mean, 9.1)	Agricultural, industrial and urbanized areas	Mohamed *et al*. (2003); Babiker *et al*. (2004)
Lebanon, Akkar region	Groundwater	NR	7.5–36.8	Agricultural area, salt-water intrusion	Halwani *et al*. (1999)
Philippines, Ilocoa Norte	Shallow wells		0–27	Intensively cropped (mostly rice–sweet pepper sequence)	Shrestha & Ladha (2002)
Turkey, Konya	Groundwater aquifers	850 000	Min., 0.4–3.0; max., 32–110; mean, 2.2–16	Rapidly growing urban area, multiple sources of contamination including agricultural and industrial activities	Nas & Berktay (2006)

Table 1.4 (contd)

Country, region	Type of water/identification	Population covered	Nitrate-N (mg/L)	Main activities or sources of pollution	Reference
Europe					
France, Brittany, Pyrenees and Allier regions	Wells and boreholes, 3 hydrological contexts		Averages, 6.8–24; range, <0.05–55.4	Animal manure, mineral fertilizers and wastewater discharges	Widory *et al*. (2005)
United Kingdom, England and Wales	Groundwater vulnerable to nitrate pollution (boreholes)		3.4–10.4	Agricultural areas	Lake *et al*. (2003)
United Kingdom, mid-Wales	Groundwater (boreholes)		0.0–1.3 (mean, 0.2)	Moorland and forested catchments	Neal *et al*. (2003)
North America					
USA, nationwide	Groundwater from 23 national water-quality assessment units (through 1992)		Median: forest land, 0.1; agricultural land, 3.4; urban land, 1.8	Forest, agricultural and urban land	Mueller *et al*. (1995)
USA, nationwide	Groundwater from major aquifers at 20 national water-quality units (1992–95)		Median: agricultural land use, 3.4; urban land use, 1.6; major aquifers, 0.5	Agricultural and urban land use	Nolan & Stoner (2000)
USA, California	Community wells operated by cities of Fresno and Clovis		2% >10.2; 29% 4.5–10.1	Sewage plants, septic tanks, agricultural use of fertilizer and wineries	Kloos (1997)
USA, Michigan	Groundwater (domestic wells)		0.0–48.6 (mean, 1.3)	Agricultural land use	Chowdhury *et al*. (2003)

Table 1.4 (contd)

Country, region	Type of water/identification	Population covered	Nitrate-N (mg/L)	Main activities or sources of pollution	Reference
USA, Ohio	Groundwater (subsurface flow)		2.0–25.5	Experimental watershed, surface run-off and subsurface flow; some agricultural activity	Owens & Bonta (2004)
USA, Oklahoma and Texas	Groundwater near Lake Texoma		Medians, 0.1–3.1; max., 12	Agriculture, oil production and residential areas	Kampbell *et al*. (2003) An *et al*. (2005)
USA, Texas	Groundwater from Gulf Coast aquifer	5 million	0.1–5.2 (median, 0.1)	Oil/gas production and agriculture; salt-water intrusion	Hudak & Wachal (2001)
USA, Texas	Bolson aquifers/El Paso		<0.1–11.7 (median, 0.4–1.7)	Farming, ranching and recreational land use	Hudak (2003)
USA, Texas	Nine counties, northeast (well water)	4.7 million	<0.07–23.0 (median, <0.07)	Urban and agriculture land use	Hudak & Sanmanee (2003)
USA, Washington State	Groundwater; shallow and deep wells (northwestern)		0–32.4	Excessive regional agricultural practices	Mitchell *et al*. (2003)
USA, West Virginia	Appalachian region (karst terrain)		0.1–3.5 (medians)	Agricultural activities	Boyer & Pasquarell (1995)
South America					
Venezuela, Maracaibo area	Groundwater	~2 million	0.2–9.5	Rural and urbanized areas	Morales *et al*. (2000)
Oceania					
New Zealand, Waikato region	Shallow aquifer		0.02–25 (average, 4.8)	Intensive grazing, gardening, pastoral land use and livestock	McLay *et al*. (2001)

max., maximum; min., minimum; NR, not reported
[a] All original values have been converted into mg/L nitrate-N.

season. Lack of sanitation and seepage from animal and human excreta into the superficial layers were postulated to be the source of pollution.

Sridhar (2000) studied 114 shallow and deep borehole wells in different major cities in Nigeria. Concentrations ranged from 1.4 to 13.4 mg/L nitrate-N. Lack of sanitation and proximity of waste dumpsites were identified as the main sources of contamination.

Tandia *et al.* (2000) studied the groundwater supply of Dakar, Senegal. Dug wells and piezometers were sampled in a vulnerable area [exact number of wells and samples not detailed]. The average concentration of nitrate-N was 42.9 mg/L with a range of 9.7–81.1 mg/L. A large increase in contamination was noted compared with previous samplings. The concentration of nitrate increased with proximity to latrines. Rapid demographic expansion and lack of adequate sanitation practices were described as the main causes of the contamination.

Asia and Oceania

In an industrial area of India and Pakistan, Afzal *et al.* (2000) studied the quality of water that received large quantities of untreated waste from factories. Twenty-one groundwater samples from superficial wells (depth, 1–2 m) were collected along the Hudiara drain; concentrations of nitrate-N ranged from 2.2 to 14.6 mg/L.

The quality of water was evaluated in Japan (Mohamed *et al.*, 2003; Babiker *et al.*, 2004) in the Kakamigahara groundwater basin that was previously known to be contaminated with nitrate due to agricultural activities. Fifty-seven samples were taken from domestic, farm, monitoring and public supply boreholes; 22% of the samples contained more than 9.9 mg/L N-nitrate and 90% contained more than 2.9 mg/L; the maximum level was 26.8 mg/L. A geographical information system analysis demonstrated an association between contamination with nitrate and intensive cultivation of vegetables. The concentration of nitrate was correlated with the depth of the groundwater and inversely correlated with the amount of precipitation. Some contamination was found in the urban area and was associated with nearby vegetable fields, with some urban sources or with sites of animal husbandry.

Halwani *et al.* (1999) studied 15 shallow wells in the northern region of Lebanon (Akkar plain) which was known to be susceptible to contamination by nitrate from agricultural activities. Concentrations of nitrate-N ranged from 7.5 to 36.8 mg/L, and all but one well had levels higher than 11.3 mg/L. Extensive fertilization and lack of vegetation during the winter were identified as the main causes of pollution of the groundwater by nitrate.

In the Philippines, Shrestha and Ladha (2002) monitored 21 water sources (eight from hand pumps on farms) that were located in a watershed where agriculture had been practiced for 1 year. Twelve (57%) had a concentration of nitrate-N above 10 mg/L with a maximum of 27 mg/L. Concentrations of nitrate were maximal in wells that were highest above sea level and increased slightly during the rainy season. The effect of high elevation might be due to a type of agriculture that needs greater amounts of fertilizer.

Nas and Berktay (2006) studied the contamination by nitrate of the groundwater source of the city of Konya in Turkey. Water samples were taken from 139 wells in 1998 and 156 wells in 2001. Average concentrations of nitrate-N increased from 2.2 mg/L in 1998 to 16.1 mg/L in 2001. The maximum concentration increased from 32 mg/L in 1998 to 110 mg/L in 2001. Several factors were postulated as possible causes of the contamination: run-off or seepage from fertilized agricultural land, municipal and industrial wastewater, refuse dumps, animal feedlots, septic tanks and private sewage disposal systems.

In New Zealand, McLay *et al.* (2001) analysed data from the Groundwater Nitrate Monitoring Project that was conducted in the Waikato region—the most intensely farmed area of this country. Water samples were taken twice a year (early autumn and spring) for 3 years (1995–98) from 88 borewells that covered diverse land uses. The concentrations of nitrate in groundwater, averaged over the 3 years, varied from 0.02 to nearly 25 mg/L nitrate-N. About 9% of the wells exceeded the 11.3-mg/L standard and 56% of the sites had a mean level higher than 3 mg/L. The concentration of nitrate was higher in areas where market garden crops were cultivated. High values were also detected in other farm areas.

Europe

In France, Widory *et al.* (2005) studied sources of contamination of groundwater by nitrate using an isotopic approach in wells and boreholes of three watersheds that had different hydrogeological contexts: fractured bedrock (Arguenon, Britanny), deep alluvial groundwater (Pia, Pyrenees) and subsurface alluvial groundwater (Ile du Chambon, Allier). In the first aquifer, nine sites were sampled five times over 1.5 years, and the mean concentration of nitrate-N was 24.0 mg/L (range, 0.7–55.4 mg/L). The main source of contamination, as suggested by analysis of ^{15}N and ^{11}B isotopes, was animal manure (especially pig farms). Wastewater sewage was also identified as a possible contributor. In the second watershed, eight sites were sampled twice at a 6-month interval, and the average concentration of nitrate-N was 11.5 mg/L (range, 2.3–31.4 mg/L). Mineral fertilizers were identified as the main contributor to contamination by nitrate, and wastewater discharge was also considered to be a possible source. In the third watershed, 16 sites were sampled twice at a 6-month interval, and the average concentration of nitrate-N was 6.8 mg/L (range, undetected–12.0 mg/L). Wastewater was identified as the main source of pollution of the groundwater.

Lake *et al.* (2003) monitored concentrations of nitrate in groundwater from agricultural areas of England and Wales using the results from more than 3700 sites. A geographical information system was used to identify areas that were susceptible to pollution by nitrate. Mean concentrations of nitrate-N ranged from 3.4 to 10.4 mg/L and declined with increasing depth, but class of vulnerability (based on surface leaching, soil characteristics, drift cover and type of aquifer) was a more important factor.

Neal *et al.* (2003) studied groundwater contamination by nitrates in mooreland and forested catchments in mid-Wales (United Kingdom). Twenty boreholes were studied,

most of which were monitored monthly over 1 year. Four boreholes were monitored every 2 weeks over 4 years after tree felling. The mean concentration of nitrate-N in boreholes before felling was 0.2 mg/L with a maximum of 0.5 mg/L. After tree felling, the concentration increased to a maximum of 1.3 mg/L. These increases lasted for at least 3 years after felling. Shallow depths contained higher concentrations of nitrate.

North America

Several studies have been conducted on the concentration of nitrate in groundwater in the USA. Major nationwide studies were carried out by the Geological Survey, and some states conducted their own studies.

Mueller *et al.* (1995) analysed data from 11 715 groundwater sites that were located in 23 study areas of the National Water-Quality Assessment Program (NAWQA) from 1970 to 1992. Water samples collected from wells (and a few springs) to monitor for compliance or to conduct research were selected after the elimination of those that were not considered to be representative. The median concentrations of nitrate-N were 0.2 mg/L in public water supplies, 1.3 mg/L in domestic wells and 2.4 mg/L in wells that were used for irrigation and stock water. The concentration in 1% of public water supplies exceeded the 10-mg/L standard compared with 9% of domestic supplies and 16% of samples from irrigation and stock water. Median levels of nitrate were higher in samples from agricultural land (3.4 mg/L) than those from urban land (1.8 mg/L) or forest land (0.1 mg/L). Highest levels were found in wells with a depth of less than or equal to 100 feet [30 m], in well drained soil and in areas where cropland was important. Among shallow wells, more than 20% exceeded the 10-mg/L guideline. Data on time trends were limited. Nevertheless, some areas showed a clear gradual increase in concentration of nitrate over the years (Georgia–Florida and Central Columbia Plateau). Nolan and Stoner (2000) analysed data from 2130 wells sampled in 20 study units of the NAWQA Program during 1992–95. Results were similar to those of Mueller *et al.* (1995).

In California, Kloos (1997) analysed data on concentrations of nitrate in 302 community wells in the Fresno-Clovis metropolitan area. Two annual test results were obtained in 1988–93. Nitrate was detected in samples from all wells. Six wells were closed because they had concentrations of nitrate-N that exceeded 10 mg/L and 86 (29%) had concentrations between 4.5 and 10 mg/L. Major sources of nitrate in groundwater in this area were sewage plant effluent, septic tank disposal systems and agricultural use of fertilizers.

Chowdhury *et al.* (2003) analysed data from 2435 domestic wells in Kalamazoo County, southwestern Michigan. The mean concentration of nitrate-N was 1.3 mg/L (range, 0–48.6 mg/L), and about 3% of the wells had concentrations above the 10-mg/L standard. The relative vulnerability of wells (based on the thickness of clay and partial clay layers and depth of the well) was associated with an increase in the concentration of nitrate. Agriculture was the largest land-use category of the county (about 40%).

Owens and Bonta (2004) conducted a study at the North Appalachian Experimental Watershed near Coshocton, Ohio. Concentrations of nitrate-N in groundwater were

assessed in four small watersheds over a 7-year period; they increased with degree of use of fertilizers (range, 13–25.5 mg/L) and decreased after 5 years of alternative management practice of grazing and growing hay with no use of fertilizers (range, 2.1–3.9 mg/L).

Kampbell *et al.* (2003) analysed data on water quality during drought conditions from 55 monitoring wells that surround Lake Texoma (border of Oklahoma and Texas). Median concentrations of nitrate-N were higher in samples from the agricultural zone (2.0 mg/L) and the area with septic tanks (1.4 mg/L), followed by those from land used for recreational purposes (0.6 mg/L) and oil production (0.1 mg/L). The maximal concentrations were 12.0 mg/L nitrate-N in the agricultural zone and 6.5 mg/L in the area with septic tanks. Concentrations of nitrate in groundwater were higher in sandy soils than in clay soils and increased during the drought season. The occurrence of nitrate in the agricultural area was explained by the application of fertilizers in vulnerable areas. An *et al.* (2005) extended this study over a 2-year period and found that residential areas had wider ranges of levels and slightly higher median levels of nitrate-N than agricultural areas (3.1 versus 2.5 mg/L).

Three studies were conducted in Texas. Hudak and Wachal (2001) evaluated concentrations of nitrate in 253 wells in the southeastern Gulf Coast aquifer and found that shallow wells and those located near cropland contained higher levels. Hudak (2003) evaluated groundwater supplies from 174 wells of five aquifers in the El Paso area. Median concentrations in aquifers ranged from 0.4 to 1.7 mg/L nitrate-N with maxima of 0.7–11.7 mg/L. The use of fertilizers was suspected to be the major source of nitrate in groundwater. Hudak and Sanmanee (2003) evaluated 110 wells in the Woodbine aquifer and found that the median concentration of nitrate-N was below the limit of detection (0.07 mg/L) with a maximum of 23 mg/L. The use of lawn fertilizers in urban areas and a combination of crop fertilizer and septic tank systems in agricultural areas were identified as possible sources of contamination.

Mitchell *et al.* (2003) studied the distribution of nitrate in the Abbotsford-Sumas aquifer in northwestern Washington State. A 22-month assessment of nitrate in groundwater was performed on a total of 504 samples from 26 domestic wells. The median concentration of nitrate-N ranged from 0 to 25.3 mg/L in shallow wells and from 0 to 13.5 mg/L in deep wells. The highest concentrations were linked to agricultural activity with a maximun of 32.4 mg/L. The impact of agricultural activity on a karst aquifer in southeastern West Viginia was assessed by Boyer and Pasquarell (1995). Two basins that had high levels of agricultural activity were compared with two others that were mostly forests. Water samples were taken weekly over several months. The median concentration of nitrate-N ranged from 2.8 to 3.5 mg/L in the agricultural areas and from 0.1 to 0.6 mg/L in the forest and areas with low levels of agriculture.

South America

Morales *et al.* (2000) studied the concentration of nitrate in groundwater in three water networks (one urban and two rural) in the Lake Maracaibo basin in Venezuela.

About 15 samples were taken at each site over several months. The mean concentrations of nitrate-N were 5.5 mg/L (range, 3.6–9.5 mg/L) in the urban network and 1.3 and 0.7 mg/L in the two rural areas. The sources of contamination of the urban network were not identified.

(ii) *Levels and sources of contamination of surface water*

The concentration of nitrate is usually lower in surface water than in groundwater, partly due to the uptake of nitrate by algae and other plants. Although the major sources of nitrate in surface waters are fertilizers and manure, in some areas, they are also contaminated by atmospheric sources (Graffy *et al.*, 1996). Several studies have recently been conducted worldwide and the results are presented in Table 1.5.

Africa

Ismail and Ramadan (1995) studied water samples from the Nile at several locations between Aswan City and Cairo. Levels of nitrate were very low (range, 0–0.2 mg/L nitrate-N). Koukal *et al.* (2004) analysed data on the quality of water from the Fez river in Morocco upstream from the Fez medina. The concentrations of nitrate-N ranged from undetectable to 2.5 mg/L.

Olajire and Imeokparia (2001) investigated the quality of water from six rivers in southwestern Nigeria (Osun State and Osogbo) in 1998. Mean concentrations of nitrate-N ranged from 4.6 to 8.9 mg/L. Intensive agricultural practices and inadequate disposal of domestic and municipal wastes were identified as the probable sources of contamination. Onyeike *et al.* (2002) studied levels of nitrate in streams located in four zones in Nigeria that had soils polluted from crude oil spillage and compared them with unpolluted waters. Concentrations of nitrate-N in the polluted areas ranged from 1.5 to 2.4 mg/L. Two control streams had concentrations of 0.6–0.8 mg/L.

Jonnalagadda and Mhere (2001) evaluated the quality of water from the Odzi River in the eastern highlands of Zimbabwe. River samples were collected at six sites over 9 months, and concentrations ranged from 0.4 to 1.0 mg/L nitrate-N.

Asia and Oceania

Cheung *et al.* (2003) studied the concentrations of nutrients in the Pearl River delta located in South China. Samples were collected at 16 sites along the estuary; concentrations of nitrate-N were typically inferior to 10 mg/L, except at one site where a concentration of 22 mg/L was measured. This high level was attributed to the influence of nearby cultivated areas.

Massoud *et al.* (2006) conducted an intense sampling programme to screen the water quality of water from the Abou Ali River in Lebanon. Three types of subcatchment were considered: mountainous forest, mountainous rural and urban. Samples were taken during three periods: the end of the dry season, and the peak and end of the wet season. Average concentrations of nitrate-N ranged from 1.8 to 3.4 mg/L in the forest area, from 1.8 to 4.7 mg/L in the rural area and from 2.9 to 4.4 mg/L in the urban area. There was little

Table 1.5. Levels of nitrate in surface water of different regions of the world

Country, region	Type of water	Population	Nitrate-N (mg/L)	Main activities or sources of pollution	Reference
Africa					
Egypt	River Nile between Aswan City and Cairo		0.0–0.2 (mean, 0.1)	Dam and industrial plants	Ismail & Ramadan (1995)
Morocco, region of Fez	River (main streams)	850 000	0–2.5	Extensive industrial development	Koukal *et al*. (2004)
Nigeria, southwestern	River Osum	557 000	4.6–8.9	Growing urbanization	Olajire & Imeokparia (2001)
Nigeria, Niger River delta	Streams		0.6–2.4	Heavily populated with surface oil pipelines	Onyeike *et al*. (2002)
Zimbabwe, eastern	River in plains and highlands		0.4–1.0	Some abandoned mines and mine dumps	Jonnalagadda & Mhere (2001)
Asia					
China, South	Pearl River delta	21.4 million	0–22	Industrial wastewater and domestic sewage	Cheung *et al*. (2003)
Lebanon, Tripoli area	Abou Ali River	350 000	1.8–4.7	Forest, rural and urban	Massoud *et al*. (2006)
Europe					
Germany, Saxony	Seven reservoirs and 22 tributaries	~2 million	0.28–6.0	Deforestation and reforestation	Ulrich *et al*. (2006)
Spain, South central (Castilla–La Mancha region)	Five rivers	~2 million	0.9–3.2 (pristine rivers); 2.7–8.7 (polluted rivers)	Mostly agricultural	Moreno *et al*. (2006)
United Kingdom, southern England	River Pang, tributary of the River Thames		5.0–8.4	Rural, sparsely populated	Neal *et al*. (2000a)

Table 1.5 (contd)

Country, region	Type of water	Population	Nitrate-N (mg/L)	Main activities or sources of pollution	Reference
United Kingdom, central England	Great Ouse River	1.6 million	5.4–15.7	Essentially rural, some important cities (Cambridge) and 500 sewage works	Neal *et al*. (2000b)
United Kingdom, southern central England	River Thames, upstream of London		4.2–12.9 (mean, 8.3)	Rural farming area	Neal *et al*. (2000c)
United Kingdom, Northeast England	River Wear		1.1–3.3 (mean, 2.1)	Arable farmland and former mining activity	Neal *et al*. (2000d)
United Kingdom, Aberdeenshire	Newmills burn		1.1–12.7 (mean, 6.1)	Pasture and farming activities	Petry *et al*. (2002)
North America					
USA, nationwide	20 national water-quality assessment units (different streams)		Agricultural land use, 1.1; agricultural/ urban land use, 0.8; urban land use, 0.8; forest and rangeland, <0.2	Forest, agricultural and urban land	Mueller e*t al*. (1995)
USA, Colorado, New Mexico	River Rio Grande		0.10–0.57 (median across 25 years)	Agricultural, residential and industrial uses	Passell *et al*. (2005)
Oceania					
Australia	River Tully		0.03–1.0 (mean, 0.1)	Cultivation of sugarcane and bananas	Mitchell *et al*. (2001)

difference in concentrations between the dry and wet seasons. The forested subcatchment was affected by nearby agricultural activity.

Mitchell *et al.* (2001) evaluated levels of nitrogen in the Tully River in North Queensland, Australia. Sugarcane and bananas were the main crops grown in the river catchments studied for the period 1989–2000. Concentrations of nitrate-N (plus nitrite) ranged from 0 to 1 mg/L (mean, 0.1 mg/L). Levels increased with agricultural activity, especially in the low-flow sectors.

Europe

Ulrich *et al.* (2006) conducted a study in seven acidified reservoirs that were affected by acid rain and 22 tributaries in the Erzgebirge region in southeastern Germany. The concentrations of nitrate-N in the reservoirs were monitored from 1993 to 2003 and ranged from 0.28 to 6.0 mg/L with a marked reduction in more recent years. When all study sites were considered, the authors estimated that the concentration of nitrate-N had decreased by an average of 41%. This decrease was found in 83% of the waters and was attributed to the possible effect of the regenerating vitality of forest vegetation and reforestation that could be associated with a reduction in deposition of atmospheric acidic sulfur.

Moreno *et al.* (2006) conducted a large study in Spain that compared relatively pristine and polluted rivers. The quality of water from five of the main rivers of Spain was assessed during 2001–03. Four main river ecotypes were studied: calcareous headwaters, siliceous rivers, plain rivers and large rivers. Polluted sites were selected in the Castilla–La Mancha River. In the unpolluted rivers, average concentrations of nitrate-N ranged from 0.9 to 3.2 mg/L. In comparison, polluted sites had average concentrations of 2.7–8.7 mg/L. The highest levels in relatively unpolluted rivers were found in calcareous headwaters. In polluted rivers, a strong relationship between the percentage of land used for agriculture and levels of nutrients was recorded.

Many studies have been conducted in the United Kingdom. Neal *et al.* (2000a,b,c,d) conducted a series of studies to evaluate the impact of agriculture on the quality of river water. The first (Neal *et al.*, 2000a) evaluated the River Pang, which is a tributary of the River Thames that drains a rural part of the Thames basin. Four sites were established along the River Pang and periodic sampling was carried out over a 2-year period. Concentrations of nitrate-N ranged from 5.0 to 8.4 mg/L and were higher during winter. This was attributed to increased surface run-off related to agricultural activities. The second study (Neal *et al.*, 2000b) was conducted over a 1-year period on the Great Ouse river that is located in central England. It flows through a highly agricultural area but also receives discharges from sewage works and industrial treatment plants. Concentrations of nitrate-N ranged from 5.4 to 15.7 mg/L. The higher concentration was associated with increased flow which is indicative of increased run-off from the agriculturally impacted soils. The third study (Neal *et al.*, 2000c) was carried out on the River Thames at a rural site downstream from Oxford, which has major agricultural activity. The data covered a 2-year period; concentrations of nitrate-N ranged from 4.2 to 12.9 mg/L with a mean of

8.3 mg/L and increased with higher flow that was related to run-off from agricultural land. The final study (Neal *et al.*, 2000d) was conducted over 1 year on the River Wear, which is located in northeastern England and has low agricultural input; the mean concentration of nitrate-N was 2.1 mg/L. Petry *et al.* (2002) studied the quality of water from the Newmills burn, Aberdeenshire, in agricultural parts of lowland Scotland. The mean concentrations of nitrate-N ranged from 4.28 to 7.08 mg/L with individual values in the range of 1.1–12.7 mg/L.

America

Mueller *et al.* (1995) analysed data on nutrients in more than 22 000 surface water samples in the USA. Data sets were compiled for each of the 20 NAWQA study units of the Geological Survey and were restricted to 1980–90. Samples were limited to one per month and several data sets were excluded because of potential bias (e.g streamflows of less than $1 ft^3/s$ [~$28 dm^3/s$]). Unit land use was assessed to determine the dominant type of use that affected each of the selected sites. Median concentrations of nitrate-N ranged from about 1 mg/L (for agricultural use of the land) to < 0.2 mg/L (for forest and rangeland). Large variations were observed within and between units. For instance, in the South Platte River Basin unit, median concentrations of nitrate-N were about 3 mg/L for agricultural land use. Generally, for non-point sources (such as agricultural activities), the concentration of nitrate increased with streamflow. The 10-mg/L drinking-water standard for nitrate-N was rarely exceeded in rivers and was less than 1% at agricultural sites. In forest/rangeland areas, the concentration of nitrate-N was generally less than 0.7 mg/L.

Passell *et al.* (2005) analysed 25-year temporal trends and spatial patterns for nutrients measured at six Geological Survey stations in the upper 600 km of the Rio Grande in Colorado and New Mexico. The monthly average concentration of nitrate-N (plus nitrite) ranged from 0.1 to 0.57 mg/L. The concentration of nitrate was stable in four sites and increased in two sites during this period. These two sites were characterized by predominant agriculture use of land.

(iii) *Levels of nitrate in drinking-water*

Few studies have been carried out that specifically evaluated the concentration of nitrates in drinking-water. Studies on the quality of water consumed by humans are presented in Table 1.6. Most studies were conducted in Europe and North America and some in Asian countries. Nitrite is usually integrated with the measurement of nitrate in these studies, and, therefore, most measurements refer to nitrate plus nitrite.

Few reports on public supplies were available. In China, a limited study reported that seven (64%) of 11 medium-sized cities (between 10 000 and 100 000 inhabitants) had water supplies with a concentration greater than 11.3 mg/L nitrate-N (Zhang *et al.*, 1996). Randomly selected public wells were also frequently found to be contaminated in an Indian region (28% above 11.3 mg/L; Manjappa *et al.*, 2003). In Saudi Arabia, a survey of representative samples of nationwide wells found that 13% had concentrations greater than 11.2 mg/L nitrate-N (Alaa el-Din *et al.*, 1994).

Table 1.6. Concentration of nitrate in drinking-water

Country, region	Location; type of water	Date; number of samples and source	Potentially exposed population	Nitrate-N (mg/L)	Reference
Asia					
China, Beijing, Hebei, Shanong, Tianjin	3 large cities, 11 towns and rural areas; mostly groundwater	1993–94; 69 locations, house wells and some waterworks	Millions	Major cities: 0–7.9; towns: 0–67.7 (7/11 >11.3); farmers' household wells: 0–67.7 (5/9 >23)	Zhang *et al*. (1996)
India, Karnataka State	61 public borewells selected at random	1999–2000; 3 samples per well	NR	0.02–69.6 (mean, 12.6; 28% >11.3; 16% >22.6)	Manjappa *et al*. (2003)
Saudi Arabia, nationwide	Groundwater, 1062 wells	1984–88; 4255 samples	NR	0–31.6 (13% >10.2; 1% >20.3)	Alaa el-Din *et al*. (1994)
Turkey, Ankara	Groundwater with potential use by military personnel	2002; 34 wells	NR	ND–14.7 (mean, 5.9)	Bakir *et al*. (2003)
Europe					
France	Cities with ≥5 000 inhabitants	1993–95; 2109 distribution units	73% of the French population	~6% >11.3 (max., 37.3)	Godet *et al*. (1998)
Greece, Thessaloniki province	Groundwater from 52 villages	2001; 52 samples of tap-water	NR	0.3–31.9 (mean, 5; 8% >11.3)	Fytianos & Christophoridis (2004)
Netherlands, Limburg region	Plateau of Schimmert; groundwater	1984–99; 16 natural springs	NR	9–45 (94% >11.3)	van Maanen *et al*. (2001)
Spain, Canary Islands	Island of Tenerife; 31 municipal areas	1999; 218 samples of tap-water	4.7 million tourists/ year	0.1–12.9 (means; 2% >11.3)	Caballero Mesa *et al*. (2003)
Spain, Galicia region	31 springs	2000–01; 185 samples	NR	0–12.2 (mean, 1.9; 6% >11.3)	Salgado *et al*. (2003)
United Kingdom, West Midlands	Private water supplies in rural areas	1995–96; 1297 samples	NR	21% >11.3, mostly <22.7	Harrison *et al*. (2000)

Table 1.6 (contd)

Country, region	Location; type of water	Date; number of samples and source	Potentially exposed population	Nitrate-N (mg/L)	Reference
North America					
Canada, New Brunswick	Carleton County (three rural areas); groundwater	1984–85; 300 rural wells	NR	10–17%, 10–20; 1.1–1.5% >20	Ecobichon *et al.* (1985)
Canada, Québec Province	Portneuf (near Québec City), rural area	1995; 71 private wells	52 500	20% >3; 6% >10	Levallois *et al.* (1998)
Canada, Québec Province	Ile d'Orléans (near Québec City), rural area	1995; 87 private wells	NR	41% >3; 5% >10 (max., 16)	Chartrand *et al.* (1999)
Canada, Saskatchewan	Rural area; groundwater	2000; 3425 private wells	500 000	14% >11.3; 6% >22.6 (max., 216)	Thompson (2001)
USA, nationwide	Rural area; groundwater	1990; 2213 domestic wells National Pesticides Survey National Alachlor Water Well Survey	NR	 2.4% >10 4.9 % >10	Spalding & Exner (1993)
USA, 18 states	Swine farms; groundwater	1991; 631 private wells	631 families	0.25–111.3 (mean, 4.0; 11.7% >10.2; 4.3% >22.6)	Bruning-Fann *et al.* (1994)
USA, Midwest	Nine states; domestic wells	1994; 5520 samples	5520 families	Mean, 8.4 (range, 0.01–266; 13.4% >10)	Center for Disease Control and Prevention/ National Center for Environ-mental Health (1998)
USA, Iowa	Statewide; groundwater	1988–89; 686 private rural wells	NR	<0.1–100 (mean, 6.2; 18.3% >10)	Kross *et al.* (1993)

Table 1.6 (contd)

Country, region	Location; type of water	Date; number of samples and source	Potentially exposed population	Nitrate-N (mg/L)	Reference
USA, New York State	Groundwater	1995–96; 419 private rural wells	NR	0–31.5 (16% >10)	Gelberg *et al*. (1999)
USA, Oregon	Umatilla and Morrow Counties; groundwater	1990–91; 80 private wells	219 people	25% >10 (median, 4.5)	Mitchell & Karding (1996)
USA, Pennsylvania	35 counties; groundwater	1989–92; 1583 private wells	NR	9.4% >10	Swistock *et al*. (1993)
USA, South Carolina	21 counties in Piedmont and Upper Coastal Plain; groundwater	2000–01; 70 private wells	NR	Average, 0.94 (29% >1)	Aelion & Conte (2004)

max., maximum; ND, not detected
NR, not reported

In France, a study of the quality of public water supplies reported that about 6% of distribution units that serve at least 5000 or more inhabitants had concentrations, at least once during 1993–95, greater than 11.3 mg/L nitrate-N (Godet *et al.*, 1998). A recent report of the European Environment Agency (2003) that used data from 28 countries concluded that pollution 'hot spots' occur in several European countries and that, in about seven of 17 countries, the guideline level of 5.65 mg/L nitrate-N is exceeded at more than a quarter of the sampling sites; the most affected area was the Republic of Moldovia where about 35% of sampling sites exceeded the 11.3-mg/L nitrate-N guideline.

Most of the studies in North America concerned quality of private well-water. Contamination of wells situated in agricultural areas was frequent: 5–10% or more of the wells had concentrations above 10 mg/L nitrate-N, occasionally with very high maxima. For instance, in Saskatchewan (Canada), a report on 3425 private wells found that 14% had a concentration of nitrate-N (plus nitrite) above 11.3 mg/L with a maximum of 216 mg/L. The sample was not taken at random. Since this province offers free measurements of nitrate to private well owners, it was considered to be a good example of the quality of the groundwater at least for some high-risk areas (Thompson, 2001). In another study of 631 private wells on swine farms in 18 states in the USA, about 12% of wells were found to have a concentration of nitrate-N greater than 10.2 mg/L, with a maximum of 111 mg/L (Bruning-Fann *et al.*, 1994).

(b) Nitrite

The content of nitrite in groundwater or surface water is generally negligible compared with that of nitrate. However, under anaerobic conditions, nitrate may be converted to nitrite through microbacterial contamination (Health Canada, 1992).

Few studies have specifically evaluated the presence of nitrite in sources of water. Table 1.7 presents results of recent available studies.

Concentrations of nitrite-N rarely exceeded 1 mg/L. The highest level reported (7.9 mg/L) was found in a private well located on a swine farm in the USA (Bruning-Fann *et al.*, 1994).

The use of chloramines as a drinking-water disinfectant has been responsible for the presence of nitrite in some distribution networks that used this technology (Health Canada, 1996). Ammonia, which is used in the formation of chloramines, can be oxidized to nitrite. This process can increase levels of nitrite in distribution systems by 0.2–1.5 mg/L (WHO, 2004a).

1.5 Exposure

Exposure to nitrate and nitrite may occur by inhalation during the spreading of fertilizer. This route of exposure is beyond the scope of this monograph and is not reviewed here.

1.5.1 *Food*

Nitrate is a potential hazard because it can be reduced to nitrite *in vivo* and in food; nitrites can react with amines or other nitrosatable substances present in food to produce *N*-nitroso compounds (Shephard & Lutz, 1989).

(a) Methodology

Dietary exposure can be estimated by several methodologies. One method uses food consumption surveys in combination with levels of the chemical of interest in the food items, and results in so-called modelled dietary exposure that can be based on several scenarios. This method provides deterministic or probabilistic assessments. Food consumption can be derived from individual data or from per-capita estimates; the latter are the only available and comparable data at international levels (FAO-WHO, 2005). Levels of nitrates and nitrites can be determined in raw commodities or in food as consumed, in 'total diet studies' (see Section 1.4). Levels in food as consumed are closer to real intake than those in raw commodities (WHO, 1999, 2002b, 2004b).

Another method is based on the analysis of the total content of a meal or of a diet during 1 or several days of a limited number of subjects, and is known as the 'duplicate diet method'. Results from such studies are available only for the Netherlands and the United Kingdom and are described below.

Table 1.7. Levels of nitrite in sources of water of different regions of the world

Country, region	Type of water/identification	Population	Nitrite-N (mg/L)	Main activities in the area or sources of pollution	Reference
Africa					
Morocco, Loukkos region	Groundwater	500 000	0–0.2	Mainly agricultural	Mourabit *et al*. (2002)
Niger, Niamey	Groundwater wells	700 000	0–0.03	Urban	Chippaux *et al*. (2002)
Senegal, Dakar	Dakar aquifer	2 million	0–1.43 (mean, 0.26)	Urban and wastes	Tandia *et al*. (2000)
Asia					
Saudi Arabia, nationwide	Groundwater wells; 4255 samples from 1062 wells		70%, not detected; 30%, 0.001–1.5	Septic tanks and agriculture	Alaa el-Din *et al*. (1994)
Europe					
Spain, Galicia region	Springs		0–0.27 (mean, 0.006)	Small towns and pilgrimage	Salgado *et al*. (2003)
Spain, Castilla–La Mancha region	Five rivers Pristine rivers Polluted rivers	~2 million	 0.006–0.014 0.02–0.4	Mostly agricultural	Moreno *et al*. (2006)
United Kingdom, central England	Great Ouse river	1.6 million	0–0.34	Essentially rural, some large cities (Cambridge) and 500 sewage works	Neal *et al*. (2000b)
United Kingdom, West Midlands	1297 private water supplies	5.3 million	0–1.5 (1% >0.03)	Mainly rural	Harrison *et al*. (2000)
North America					
USA, 18 states	Groundwater, 631 private wells		Detected in 1.2% of wells (max., 7.9)	Swine farms	Bruning-Fann *et al*. (1994)
South America					
Venezuela, Maracaibo area	Groundwater	~2 millions	<0.003	Rural and urbanized areas	Morales *et al*. (2000)

max., maximum

(b) Food consumption

International food consumption data can be extracted from the Global Environmental Measuring Survey food diets. These data are based on the availability of food for each country that is included (production plus imports minus exports) and are collected by the Food and Agricultural Organization (FAO). The data are then grouped on the basis of statistical analyses; this results in the formation of clusters of the countries included according to their dietary information and pattern. These data thus provide average per-capita consumption for given countries or regions. Additional information can be found at www.who.org/foodsafety.

(c) Dietary exposure assessment

(i) *Nitrate*

The average dietary ingestion of nitrate by adults in the United Kingdom using the 1997 Total Diet Study was estimated to be 57 mg/consumer per day, with a 97.5th percentile for consumers only for each type of food of 105 mg per person per day (Food Standards Agency, 1998a). Dietary exposure for adult consumers of vegetables commonly eaten in the United Kingdom were estimated at 93 mg per day and 140 mg per day for the mean and the 97.5th percentile, respectively (Ysart *et al.*, 1999b). In a vegetarian duplicate diet over 7 days, the average dietary exposure was estimated to be 53 mg per person per day and up to 84 mg per person per day when taking into account contributions from water and beer (Clarke *et al.*, 2003).

In the Netherlands, the mean dietary exposure of adults estimated by the 'duplicate diet method' in 1994 was 80 mg per person per day (range, 1–322 mg per day). These results are higher than those obtained in 1984–85 (52 mg per day) in the same country and with a similar methodology (Vaessen & Schothorst, 1999).

When the average concentration of nitrate in food from the United Kingdom total diet studies were combined with the corresponding consumption from the 13 clustered diets (FAO WHO, 2005), total dietary exposure ranged from 58 to 218 mg per day. Such a scenario is intended to cover the variability in average dietary patterns around the world. [This range does not take into account variability of nitrate concentration between countries or interindividual variation in the specific food consumed, as well as seasonal or other variations within countries.]

(ii) *Nitrite*

Almost all exogenous exposure to nitrite occurs through food. The average dietary exposure of adults in the United Kingdom using the 1997 Total Diet Study was estimated to range between 0.74 and 1.3 mg per person per day, with a 97.5th percentile for consumers only of 2.2 mg per person per day (Food Standards Agency, 1998a).

In the Netherlands, the mean dietary exposure of adults estimated by the 'duplicate diet method' in 1994 was below 0.2 mg per person per day in the autumn and 0.6 mg per

person per day in the spring (range, 0.1–16 mg per person per day) (Vaessen & Schothorst, 1999).

When the average concentration of nitrate in vegetables, cereals, meats and fish from the United Kingdom Total Diet Study was combined with the corresponding consumption from the 13 clustered diets (FAO WHO, 2005), total dietary exposure ranged from 0.7 to 1.6 mg per day.

The relative contribution of cured meats to total dietary intake of nitrite has decreased substantially over the past 30 years (National Research Council, 1981; Cassens, 1997).

1.5.2 *Drinking-water*

Groundwater is an important source of drinking-water in many countries, and is the main source of drinking-water in Europe (European Chemical Industry Ecology and Toxicology Centre, 1988; European Environment Agency, 2003). In the USA, about 15% of the population has a private water source, mainly from groundwater wells (Environmental Protection Agency, 2002); 40% of water drawn for public supply is groundwater and the drinking-water of more than half of the population is groundwater (Nolan & Ruddy, 1996). In Canada, 30% of the population (almost 9 million people) rely on groundwater for domestic use; two-thirds of them live in rural areas (Environment Canada, 2004/2006). Groundwater, and especially that taken from private wells located in agricultural areas, is the source that is most affected by contamination above the present guideline value of 50 mg/L nitrate (10 mg/L nitrate-N) (WHO, 2003a, 2004a).

Infants constitute a special case. Breast-fed infants are exposed to very little nitrate or nitrite, but bottle-fed infants can be exposed to nitrate from the water used to prepare their formula. In particular, breast-fed infants of mothers who ingest water with a high content of nitrate (up to 100 mg/L nitrate-N) are not considered to be at risk for over-exposure (Greer *et al.*, 2005). In contrast, bottle-fed infants are at risk for over-exposure in areas where the levels of nitrate in drinking-water exceed 50 mg/L (WHO, 2003a).

1.5.3 *Estimated daily intake of nitrate and nitrite from diet and water based on different scenarios of exposure*

Tables 1.8 and 1.9 present the relative contribution of food and water to the total nitrate and nitrite intake of adults according to different scenarios of exposure and consumption. The calculations are based on the average content of nitrate and nitrite in food in the United Kingdom and the average and 95th percentile of food consumption in the 13 clusters of the Global Environmental Measuring Survey studies. The different scenarios are: (i) average consumption of food and water, (ii) high consumption of vegetables (for nitrate) or food that is rich in nitrite (for nitrite), (iii) average consumption of water contaminated with 50 mg/L and (iv) high consumption of water contaminated at this level.

Table 1.8. Estimated daily intake of nitrate in various scenarios

	Average water intake (1.4 L/day)		Average food consumption	
	Average food consumption	High-vegetable diet	Average water intake (1.4 L/day)	High water intake (2.7 L/day)
	mg/day (%)[a]	mg/day (%)	mg/day (%)	mg/day (%)
Diet	52–80 (89–93)	140–220 (96–97)	52–80 (45–56)	52–80 (33–43)
Water	6 (7–11)	6 (3–4)	70 (47–57)	135 (63–72)
Total	58–86 (100)	146–226 (100)	122–150 (100)	187–215 (100)

[The values do not take into account variability of nitrate concentration between countries and location, or interindividual variation in the specific food consumed, or seasonal or other variations within countries. All values were calculated by the Working Group; these calculations were based on average and 97th percentile food consumption in the United Kingdom and average and 95th percentile water intake in the USA.]
[a] % of total daily intake

Table 1.9. Estimated daily intake of nitrite from food and water

	Average water intake (1.4 L/day)		Average food consumption	
	Average food consumption	High-nitrite food consumption	Average water intake (1.4 L/day)	High water intake (2.7 L/day)
	mg/day (%)[a]	mg/day (%)	mg/day (%)	mg/day (%)
Diet	0.74 (98)	2.2 (99)	0.74 (14)	0.74 (7.7)
Water	0.014 (2)	0.014 (1)	4.6 (86)	8.9 (92.3)
Total	0.754 (100)	2.214 (100)	5.34 (100)	9.65 (100)

[The values do not take into account variability of nitrite concentration between countries and location, or interindividual variation in the specific food consumed, or seasonal or other variations within countries. All values were calculated by the Working Group; these calculations were based on average and 97th percentile food consumption in the United Kingdom and average water intake in the USA. The high water intake is the 95th percentile of consumption in the USA.]
[a] % of total daily intake

1.5.4 *Studies of exposure that used biomarkers*

Due to the important fluctuation in nitrate concentration in both urine and saliva, 'spot samples' of either fluid give poor estimates of past exposure to nitrate. Therefore, 24-hour collection of urine is recommended to evaluate total recent exposure to nitrate (Packer *et al.*, 1989). Four studies evaluated the impact of water contamination on total exposure by

assessing urinary excretion of nitrate. In three of the studies, 24-hour collection of urine was used (Table 1.10).

In East Anglia (United Kingdom), Chilvers *et al.* (1984) studied 404 adult users of well-water who completed a diet diary over a 48-hour period and provided a 24-hour urine specimen and a sample of their drinking-water. Mean daily dietary intake of nitrate was estimated by multiplying the food content reported in the diary with the nitrate content of food estimated from the literature. Ingested nitrate from water was estimated by multiplying the mean of the consumption of tap-water on the 2 days of data collection by the concentration of nitrate (plus nitrite) in the water sample. When data were stratified by nitrate intake from food, there was a constant relationship between nitrate intake from water and urinary excretion of nitrate (plus nitrite): the slope of the regression line was 0.6–0.8. The relative contribution of water to total intake (food plus water) of exogenous nitrate increased constantly with increasing concentration of nitrate in the water.

Møller *et al.* (1989) studied 294 adults in Denmark who used underground water from waterworks. Dietary and liquid intake of nitrate (plus nitrite) was estimated by means of a duplicate portion technique for 1 day followed by collection of urine overnight. A sample of tap-water was also taken from the home at each visit. Nitrate concentration in food and liquid was determined using a standard protocol. The concentration of nitrate (plus nitrite) in the water and urinary excretion of nitrate (plus nitrite) were correlated. The relative contribution of water to total intake of exogenous nitrate was estimated to be around 60% when the concentration in water was about 11 mg/L nitrate-N, and around 70% when the concentration in water was 20 mg/L nitrate-N.

van Maanen *et al.* (1994) studied 60 adult women in the Netherlands who used groundwater from public water supplies and private wells. Participants were requested to answer a questionnaire about their food and water consumption and provide a 24-hour urine sample. A sample of water was taken from each private well. Nitrate intake from food was estimated using data from Dutch laboratory studies of nitrate content of food. There was a clear association between urinary nitrate and concentration of nitrate in the water, despite an apparent plateau for the most highly exposed groups. [The number of subjects was small in these two groups (six and ten, respectively).] The relative contribution of water to the total intake of exogenous nitrate was correlated with the concentration of nitrate in the water and was estimated to be 45% when this was about 30 mg/L nitrate-N.

Levallois *et al.* (2000) studied 187 adults in the Province of Québec (Canada) who used private wells as a source of drinking-water. Each participant collected a 24-hour urinary sample and answered a 24-hour dietary and liquid questionnaire. A sample of tap-water was taken in each home and was analysed for nitrate and nitrite. Using the same procedure as Chilvers *et al.* (1984), intake of nitrate (plus nitrite) from food and water was estimated. Two groups were assembled: one with a low concentration of nitrate in the water (< 3 mg/L nitrate-N; geometric mean, 0.18 mg/L) and the other with a moderate concentration of nitrate (geometric mean, 7.1 mg/L nitrate-N; range, 3–28 mg/L). The

Table 1.10. Summary of studies of nitrate intake from water that used urinary nitrate as a marker of internal exposure

Country	Area	Subjects and type of water consumed	Mean concentration[a] in water (mg/L)	Mean intake[a] from water (mg/day)	Mean intake[a] from food (mg/day)	% from water	% from food	Urinary nitrate-N (mg/day)	Reference
United Kingdom	East Anglia	404 adult well-water users	0 (n = 170) 6 (n = 106) 15 (n = 93) 35 (n = 35)	0 7.45 20.7 44.7	13.5 15.6 14.0 18.3	0 32 60 71	100 68 40 29	[19.4] [26.7] [31.6] [58.8]	Chilvers *et al*. (1984)
Denmark	West Himmerland (northern Jutland)	294 adults, aged 20–64 years, using 21 waterworks	0.07 (n = 115) 11 (n = 76) 19 (n = 103)	1.8** 13.8** 19.8**	8.7 9.2 12.6	17 60 61	83 40 39	8.2*** 12.4*** 16.4***	Møller *et al*. (1989)
Netherlands	Province of Limburg	60 adult women (44 with municipal supply and 16 using private wells)	0 (n = 21) 4.0 (n = 23) 5.0 (n = 6) 29.6 (n = 10)	0 5.7 7.9 24.7	36.6 37.5 27.1 31.4	0 13 23 45	100 87 77 55	10.4 14.5 24.2 26.2	van Maanen *et al*. (1994)
Canada	Province of Quebec	187 adults, aged 22–73 years, using well-water	0.18* (n = 100) 7.10* (n = 87)	0.25* 9.81*	23.0* 18.5*	1 35	99 65	16.86* 23.33*	Levallois *et al*. (2000)

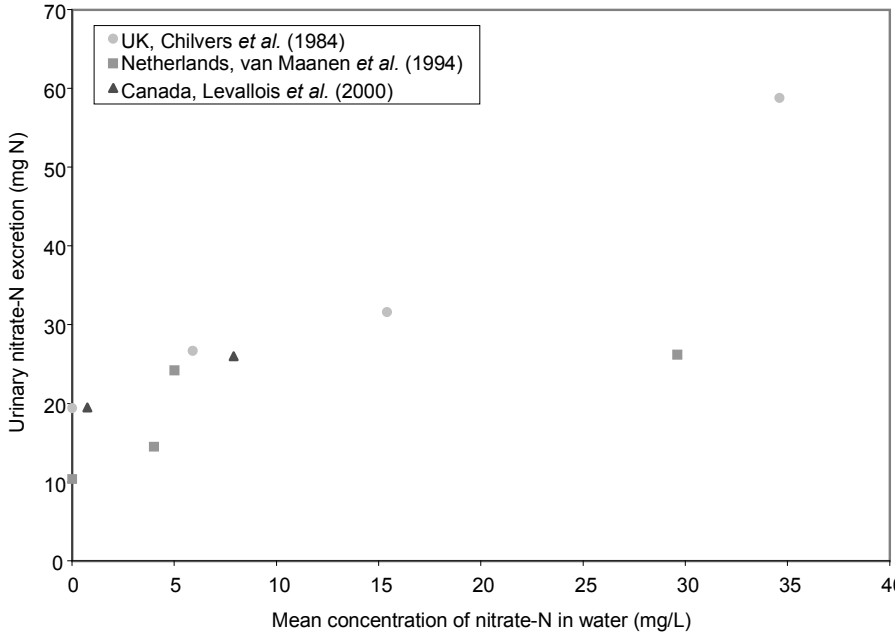
* Geometric means
** Ingested liquid, including tea and soups
*** Overnight sample only
[a] Values were converted to nitrate-N

impact of water on exposure to nitrate was clearly observed, with a 33% increase in urinary excretion in the most highly exposed group. The relative contribution of water to the total intake of exogenous nitrate in the moderately exposed group was estimated to be about 30%.

Because of differences in methodology between these studies and some limitations, especially with regard to the assessment of food intake, it is difficult to evaluate the relative importance of nitrate in water to total intake of nitrate. Nevertheless, it can be concluded that, when the concentration of nitrate is greater than the present standard (10 mg/L nitrate-N), nitrate in water becomes an important source of exposure to, and generally the principal source of intake of exogenous nitrate.

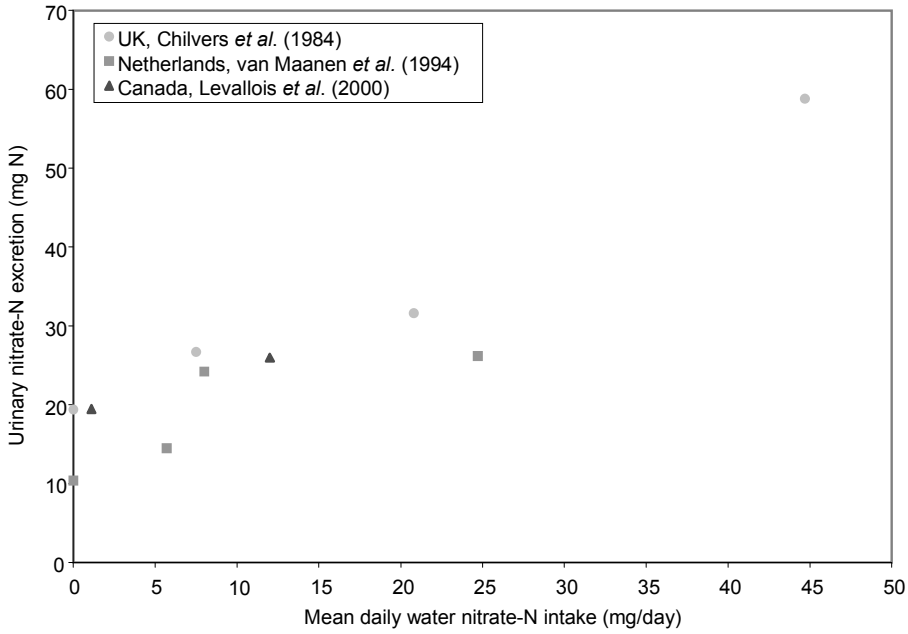
Figures 1.1 and 1.2 present the relationship found in these studies between 24-hour urinary excretion and concentration of nitrate in the water or daily intake of nitrate from water, respectively. From the available data, it can be concluded that urinary excretion increases steadily with increasing intake of nitrate from water.

Figure 1.1. Mean concentration of nitrate-N in water versus urinary nitrate-N excretion



Compiled by the Working Group

Figure 1.2. Mean daily water nitrate-N intake versus urinary nitrate-N excretion



Compiled by the Working Group

1.6 Regulations and guidelines

1.6.1 *Food*

(a) Acceptable daily intake

The Joint FAO/WHO Expert Committee on Food Additives (WHO, 2003b,c) has evaluated the health effects of nitrate and/or nitrite and confirmed the previous acceptable daily intake (ADI) of 0–3.7 mg/kg body weight (bw) per day for nitrate ion and has established an ADI of 0–0.06 mg/kg bw per day for nitrite ion (WHO, 1995). This ADI was also endorsed by the Scientific Committee on Food of the European Commission (1997). However, it was noted that these ADIs do not apply to infants under the age of 3 months. Bottle-fed infants under 3 months of age are most susceptible to methaemoglobinaemia following exposure to nitrate and/or nitrite in the drinking-water (WHO, 2003a).

(b) Permissible levels in food

At the international level, the *Codex alimentarius* elaborates guidelines for maximum permitted levels of residues in food, based on technological needs. In support of its work on food standards and codes of practice, the *Codex alimentarius* generates reputable texts

for the management of food safety and consumer protection based on the work of the best-informed individuals and organizations concerned with food and related fields. Countries have responded by introducing long-overdue food legislation and *Codex*-based standards and by establishing or strengthening food control agencies to monitor compliance with such regulations. The maximum permitted level for nitrate in the *Codex* draft General Standard for Food Additives is 37 mg/kg in cheese and cheese products, 3650 mg/kg in processed meat, poultry and game, 360 mg/kg in comminuted [powdered] meat poultry and game products and 220 mg/kg in semi-preserved fish and fish products. The maximum permitted level for nitrite is 17 mg/kg in cheese and cheese products, 420 mg/kg in processed meat, poultry and game (whole pieces and cuts) and 130 mg in comminuted processed meat and in processed fish (WHO, 2003c).

Permissible levels of nitrate (used as a source of nitrite) in meat and poultry products used to be in the 250–500 ppm [mg/kg] range, but in recent years the trend has been to reduce levels of nitrate (and combined nitrate and nitrite) to inhibit the formation of nitrosamines. In the European Union, it has been suggested that input levels of nitrate for heat-treated meat products could be reduced to 150 ppm and residual levels to 100 ppm. In Japan, permissible nitrate input for meat products is 70 ppm, measured as residual nitrite; in the European Union, up to 50 ppm sodium or potassium nitrate (as sodium nitrate) can be used in foie gras; and in the USA, nitrate cannot be used to cure bacon. The content of nitrate is limited to 200 mg/L in raw milk for cheese in Japan and to 50 ppm in cheese in the European Union (European Commission, 1995; Department of Agriculture, 1995; Ministry of Health and Welfare, 2000; Department of Agriculture, 2003; Food and Drug Administration, 2004; European Commission, 2005; Food Standards Australia New Zealand, 2006).

Permissible residual levels of nitrite (usually expressed as sodium nitrite) in meat and poultry products generally range from 50 to 200 ppm (Australia/New Zealand, European Union, Japan, USA). Permissible levels are typically 120–175 ppm for pork bacon (European Union, USA) and 70 ppm for whale bacon (Japan). Nitrite is also used to cure fish products; for example, in Japan, permissible residual levels are up to 50 ppm in fish ham and fish sausage and no more than 5 ppm in certain salted fish products; in the USA, levels up to 200 ppm are permitted in certain smoked fish (European Commission, 1995; Department of Agriculture, 1995; Ministry of Health and Welfare, 2000; Department of Agriculture, 2003; Food and Drug Administration, 2004; European Commission, 2005; Food Standards Australia New Zealand, 2006).

In some regions, additional limits are placed on baby foods. In the European Union, nitrate is limited to 200 ppm and, in the USA, no nitrate or nitrite can be added to food specifically produced for babies or young children (European Commission, 2004; Food and Drug Administration, 2004).

The addition of ascorbate as an antioxidant is also regulated in some countries. Regulations in the USA specify that, in addition to sodium nitrite or potassium nitrite, 550 ppm sodium ascorbate or sodium erythorbate should be added to prevent the formation of nitrosamines (Food Safety Inspection Service, 2006). The maximal levels

reported by the Directive 95/2/EC of the European Commission (1995) for most products, including pre-packed preparations of fresh minced meat, are 'quantum satis' [quantity required].

1.6.2 *Water*

The WHO (2004b) has established a drinking-water guideline value (short-term exposure) for nitrate and nitrite of 50 mg/L and 3 mg/L, respectively, to protect against methaemoglobinaemia in bottle-fed infants. For methaemoglobinaemia in infants (an acute effect), it was confirmed that the existing guideline value for nitrate of 50 mg/L is protective. For nitrite, human data reviewed by the Joint FAO/WHO Expert Committee on Food Additives (JECFA) support the current short-term guideline value of 3 mg/L, based on the induction of methaemoglobinaemia in infants. Toxic doses of nitrite that are responsible for methaemoglobinaemia range from 0.4 to more than 200 mg/kg bw. By applying the lowest level of the range (0.4 mg/kg bw), a body weight of 5 kg for an infant and a consumption of drinking-water of 0.75 L, the guideline value of 3 mg/L (rounded figure) was derived (WHO, 2003a, 2004b). A provisional guideline value (long-term exposure) for nitrite was set at 0.2 mg/L.

WHO has proposed this guideline value for nitrite associated with chronic exposure based on the analysis by JECFA of animal data that showed nitrite-induced morphological changes in the adrenal glands, heart and lungs. Using the JECFA ADI of 0.06 mg/kg bw per day, assuming a 60-kg adult who ingests 2 L of drinking-water per day and allocating 10% of the ADI for drinking-water, this guideline value of 0.2 mg/L nitrite (rounded figure) has been calculated. However, owing to the uncertainty surrounding the relevance to humans of the adverse health effects observed in animals and the susceptibility of humans compared with that of animals, this guideline value should be considered as provisional (WHO, 2003a, 2004b). The occurrence of nitrite in distribution as a consequence of the use of chloramine is intermittent, and average exposures over time should not exceed the provisional guideline value. Because of the possibility of the simultaneous occurrence of nitrite and nitrate in drinking-water, the sum of the ratios of the concentrations of each to its guideline value should not exceed 1.0 (WHO, 2003a, 2004b).

The Australian Government (2004) drinking-water guideline has adopted the WHO guidelines for infants under 3 months of age. However, the guideline is up to 100 mg/L nitrate for adults and children over 3 months of age.

The Canadian drinking-water (health-based) guideline for nitrate is 45 mg/L. Where nitrate and nitrite are determined separately, concentrations of nitrite should not exceed 3.2 mg/L (Health Canada, 1992, 2006).

In the USA, the Environmental Protection Agency maximum contaminant level for nitrates (measured as nitrogen) is 10 mg/L (45 mg/L nitrate); that for nitrites (measured as nitrogen) is 1 mg/L. The limit for nitrate in drinking-water was established as a safeguard

against infantile acquired methaemoglobinaemia. (Environmental Protection Agency, 2003).

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2. Studies of Cancer in Humans

2.1 Introduction

Ingestion of nitrate and nitrite can result in the endogenous formation of *N*-nitroso compounds in the presence of nitrosatable precursors that are contained in meats, fish and some common drugs (see Sections 1 and 4). Nitrate is ingested from dietary sources and drinking-water. Vegetables are usually the primary source when levels of nitrate in the drinking-water are below 50 mg/L, which is the regulatory limit in many countries. Many vegetables contain vitamin C and other compounds such as polyphenols that inhibit endogenous nitrosation. Whereas nitrate from both vegetables and drinking-water is reduced in the body to nitrite, sources from vegetables probably result in less endogenous formation of *N*-nitroso compounds because of the presence of inhibitors of nitrosation (see Section 1). For these reasons, the Working Group evaluated ingested nitrate from dietary sources and drinking-water separately.

Epidemiological studies that assessed the relationship between nitrate in the drinking-water and cancer have been primarily ecological in design and focused on stomach cancer. Fewer case–control and cohort studies were available for other cancer sites. Ecological studies can provide important information on causal inference when exposure circumstances contrast greatly (between regions or population subgroups) and migration of populations is limited, particularly if there is almost homogenous exposure within each region and there are no serious potential confounders (see Cantor, 1997; IARC, 2004).

However, inference from ecological studies of exposure to waterborne nitrate is more difficult because of the complexity of and intra-individual variation in endogenous nitrosation (see Section 4). Specific subgroups of a population that have higher exposure to nitrosation precursors (from nitrate in water and amines and amides in the diet) and lower exposure to inhibitors of nitrosation (e.g. dietary antioxidants) are probably at highest risk. Ecological studies of nitrate in drinking-water, therefore, are not liable to be highly informative unless levels of exposure are high and exposure to waterborne nitrate and other factors that affect nitrosation are homogeneous across the population groups. Therefore, the Working Group gave much greater weight to case–control and cohort studies in their evaluation.

Most case–control and cohort studies of exposure to nitrate in the drinking-water have been conducted in areas where levels of nitrate in drinking-water supplies are elevated due to the use of nitrogen fertilizers. Some of the highest levels of nitrate are found in shallow wells and surface water supplies which contain high levels in the spring due to run-off of excess nitrogen (see Section 1.4.2). Public water supplies are monitored routinely, and historical data on levels of nitrate are available; however, exposure to levels greater than the maximum contaminant limit in the USA of 10 mg/L (as nitrate-N) (Environmental Protection Agency, 2003) or the WHO drinking-water guideline of

50 mg/L as nitrate (WHO, 2004) are rare. Private wells tend to contain higher levels of nitrate than public supply wells because they are unregulated, are frequently shallower and more poorly constructed and are often located in close proximity to sources of contamination by nitrogen (crop fields, animal feed lots, septic tank systems). Since historical data from monitoring are available, most studies have focused on populations who use public water supplies and have excluded populations who use private wells with potentially higher exposures.

Occupational groups that may be at risk of ingesting nitrate were also considered. Workers in the manufacture of nitrate-based fertilizer can have high exposures to dusts that contain nitrate (> 10 mg/m^3) (see Section 1). One study (Al-Dabbagh *et al*., 1986) among men whose jobs entailed exposure to different levels of nitrates in dust (maximum, 5 mg/m^3) demonstrated that, at the end of a workday, salivary nitrate levels from highly exposed workers were approximately twice those of workers who had no exposure to nitrate in dust; however, dietary intake of nitrate was not controlled and the range of salivary levels of nitrate among highly exposed workers overlapped with those of unexposed men in several regions of England. Furthermore, a twofold variation in nitrate in saliva in non-occupationally exposed groups has been observed even under controlled conditions (Weisenberg *et al*., 1982; Bos *et al*., 1988). The Working Group considered that the evidence for exposure to nitrate via ingestion was lacking in these studies and could not be quantified; these studies were therefore not reviewed.

A few case–control studies have investigated exposure to nitrate from tap-water and have measured levels of nitrate and/or nitrite at the current residence or at the residence at the time of diagnosis of disease. Levels of nitrate and/or nitrite may change over time; therefore, considerable misclassification of exposure may occur if current levels of exposure are used to estimate past exposure. Variation in levels of nitrate across the distribution system of a public water supply could also introduce misclassification of exposure. In addition, nitrate and/or nitrite in water may be correlated with other environmental exposures such as agricultural pesticides that may be potentially relevant to some cancers.

Dietary intake of nitrite occurs primarily from the consumption of cured meats and fish, bakery goods and cereal products. Nitrite is also found as a contaminant of drinking-water but only in unusual circumstances (see Section 1.5). Several epidemiological studies evaluated the risk for specific cancers among subjects who had a higher intake of nitrite and a lower intake of vitamin C; this dietary pattern is liable to result in an increase in the endogenous formation of *N*-nitroso compounds. Studies of this design were considered by the Working Group to be particularly informative in the evaluation of human carcinogenicity.

Studies that estimated intake of nitrite from all dietary sources were reviewed, but these that only evaluated consumption of cured meat and risk for cancer were not reviewed specifically since they do not represent complete dietary nitrite intake. This is because many, but not all, cured meats contain nitrite and because other foods can also be

important sources of nitrite. The Working Group also noted the results of some studies that estimated intake of nitrite and preformed *N*-nitroso compounds.

Several ecological studies in high- and low-risk areas for stomach and oesophageal cancers evaluated the potential for endogenous formation of *N*-nitroso compounds using the *N*-nitrosoproline (NPRO) test developed by Ohshima and Bartsch (1981). Some studies also measured urinary excretion of nitrate, levels of nitrate and nitrite in the saliva and excretion of other specific *N*-nitroso amino acids. Excretion of NPRO was generally higher in high-risk areas; however, not all of the differences were statistically significant (Bartsch *et al*., 1992; van Maanen *et al*., 1996). Urinary and salivary levels of nitrate reflect exposures to nitrate from both dietary sources and drinking-water; therefore, these studies were evaluated as a separate group. However, the Working Group did not give substantial weight to these studies in their evaluation because of the ecological study design and because recent excretion of nitrate or levels of nitrate in saliva may not reflect past exposures.

2.2 Stomach and oesophageal cancer

2.2.1 *Ingested nitrate*

(a) Ecological studies (Table 2.1)

Several ecological studies have considered the relationship between exposure to nitrate and the risk for stomach or oesophageal cancer; most of these used mortality as the end-point. The concentration of nitrate in the drinking-water was often used as indicator of exposure, and some studies assessed its correlation with the occurrence of disease or investigated the differences in mortality or incidence of disease across exposure levels. Alternatively, the concentrations of nitrate were compared in populations who were classified according to different levels of risk. In many instances, urinary excretion of nitrate or its concentration in other body fluids in groups of individuals were used as indicators of exposure rather than levels in drinking-water and food. The main features and results of these studies are summarized in Table 2.1.

(i) *Nitrate in the drinking-water*

Asia

Two studies addressed the association between exposure to nitrate and the occurrence of tumours of the stomach and oesophagus in China. An early study (Wang *et al*., 1979) reported a positive significant correlation ($r = 0.23$) between concentrations of nitrate in the drinking-water (mean, 10.55 mg/L) and cancer of the oesophagus in Linxien, while a more recent study (Zhang *et al*., 2003) reported a concentration of 19.6 mg/L nitrate-N in well water in the county of Cixian, an area where mortality from oesophageal cancer was very high. These studies reported nitrate levels higher than the level (8 mg/L) in Chichen county, a low-risk area for oesophageal cancer.

Table 2.1. Ecological studies of ingested nitrate and nitrite and the risk for stomach and oesophageal cancer

Reference, location	Organ site (ICD code)	End-point	Exposure	Main results	Comments
Asia					
China					
Wang *et al.* (1979), 49 production brigades, Linxien County	Oesophagus (150)	Incidence, 1969–76	Nitrate in wells 1976–77, 4 seasons	Correlation (summer), $r = 0.233$; P-value < 0.05	Average concentration of nitrates, 10.56 mg/L
Zhang *et al.* (1984), 6 high-risk and 4 low-risk districts	Stomach (151)	Mortality ($\times 10^{-5}$): high risk (range), 31–113, low risk (range), 7–12	**Nitrate and nitrite in gastric juice; 809 (high risk) and 554 (low risk) subjects** Nitrate Nitrite	**High- versus low-risk areas (mg/mL)** 24.3 versus 30.9 ($P < 0.01$) 0.27 versus 0.10 ($P < 0.001$)	Concentrations are medians; subjects were patients with chronic stomach complaints.
Lu *et al.* (1986), Linxian (high-risk area) and Fanxian (low-risk area) counties	Oesophagus (150)	Mortality, 1978; rate $\times 10^{-5}$, men: Linxian, 151; Fanxian, 35	**Nitrate in 24 h-urine, 1982, healthy subjects (148 high risk, 96 low risk)** High-risk area Low-risk area	 94 mg 48 mg P-value = 0.001	Nitrate levels are median in mg/person/day.

Table 2.1 (contd)

Reference, location	Organ site (ICD code)	End-point	Exposure	Main results	Comments
Wu *et al.* (1993), 69 counties from 25 provinces	Stomach (151), oesophagus (150)	Mortality, 1975–75; cumulative rates, 0–64 years	**Nitrate in 12-h urine, 1982, healthy subjects (about 30 per xiang[a], 2 xiangs per county)**	**Correlation coefficient**	Nitrate was significantly associated (positive correlation) with tumours of nasopharynx and liver and leukaemia.
			Stomach	$r = -0.14$ (NS)	
			Oesophagus	$r = -0.10$ (NS)	
Lin *et al.* (2003), Lufeng County (low-risk area), Nan'ao County (high-risk area)	Oesophagus (150)	Mortality, age-adjusted rate ($\times 10^{-5}$); 10 (Lufeng); 110 (Nan'ao)	**Nitrate in 12-h urine (nmol/g creatinine), 120 healthy persons per county**		Daily intake of nitrate also higher in high-risk area; period to which mortality rates and urinary measurements refer not reported
			High-risk area	7.1	
			Low-risk area	3.1	
				P-value = 0.01	
Zhang *et al.* (2003), Cixian County (high-risk area), Chichen County (low-risk area)	Oesophagus (150)	Mortality, 1974–76 rate ($\times 10^{-5}$) men: Cixian, 147.7; Chichen, 8.3	**Nitrite and nitrate in well water, 1993–96 (33 in Cixian, 31 in Chichen; mg/L nitrogen), 1996**	**Cixian versus Chichen**	Rates adjusted for Chinese population; similar results for years 1993–95
			Nitrate	19.6 versus 8.0 ($P < 0.01$)	
			Nitrite	0.01 versus 0.002 ($P < 0.01$)	

Table 2.1 (contd)

Reference, location	Organ site (ICD code)	End-point	Exposure	Main results	Comments
Japan					
Kamiyama *et al.* (1987), Akita (high-risk area), Iwate (low-risk area) Prefectures	Stomach (151)	Mortality, 1969–78; rate $\times 10^{-5}$, men: Akita, 33, Iwate, 101	**Nitrate in 24-h urine, 104 healthy subjects, 1983** High-risk area Low-risk area	 116 mg 140 mg *P*-value = 0.07	Nitrate levels are median in mg/person/day.
Tsugane *et al.* (1992), Yokote, Saku (high-risk areas), Ninohe, Ishikawa (low-risk areas) cities	Stomach (151)	Mortality, 1985–87; rate, men ($\times 10^{-5}$): high-risk, 49, 43; low-risk, 30, 17	**Nitrate in 24-h urine, 134 adults, 40–49 years, 1989–90** High-risk area Low-risk area	*Mean nitrate (mg/day/person)* 120–166 167–203	Age-adjusted mortality (world population)

Table 2.1 (contd)

Reference, location	Organ site (ICD code)	End-point	Exposure	Main results	Comments
Europe					
Denmark					
Jensen (1982), towns of Aalborg and Aarhus and other towns in northern Jutland	Stomach (151), oesophagus (150)	Incidence (1968–72) from the Danish Cancer Registry	**Nitrate in drinking-water (mg/L) in 1976**	**Incidence ($\times 10^{-5}$)**	Incidence adjusted for age using European population; for stomach cancer, similar results for 5-year periods 1943–47 and 1963–67
			Stomach		
			Men		
			Aalborg: 27.1	41.5	
			Aarhus: 0.2	28.4	
			Women		
			Aalborg: 27.1	21.8	
			Aarhus: 0.2	13.2	
			P-value	> 0.0001	
			Oesophagus		
			Men		
			Aalborg: 27.1	5.6	
			Aarhus: 0.2	4.1	
			Women		
			Aalborg: 27.1	2.0	
			Aarhus: 0.2	2.4	
			P-value	NS	

Table 2.1 (contd)

Reference, location	Organ site (ICD code)	End-point	Exposure	Main results		Comments
Eastern Europe						
Zemla & Kolosza (1979), Zabrze, cities of Maciejov, Mikulczyce (low-nitrate and-nitrite districts) and Grzybowice, Pawlow (high-nitrate districts), Poland	Stomach (151)	Incidence, 1965–75	**Mean level in drinking-water (mg/L)** *Low (0.076–0.125)* Nitrate Nitrite *High (0.365–0.699)* Nitrate Nitrite	**Incidence rates (× 10^{-5})** *Men* *Women* 11.9 3.7 13.4 9.6 36.8 NR 52.6 4.8		Age-standardized rates, population of Zabrze; all rates but one significantly different from the city mean; no differences for women
Juhasz *et al.* (1980), 230 communities in Szabolcs-Szatmar county, Hungary	Stomach (151)	Incidence, 1975	**Nitrate in drinking-water** > 100 mg/L < 100 mg/L	**No. of communities ≥ 20 × 10^{-5}/ Total No. of communities** 127/205 12/25 Prevalence ratio, 1.29 (NS)		Prevalence ratio (proportion of communities with nitrate >100 mg/L divided by the proportion of communities with nitrate <100 mg/L); source of rates and period of nitrate measurement not stated
Zatonski *et al.* (1989), town of Opole (low risk), and Swierczow and Domaszowice rural areas (high risk), Poland	Stomach (151)	Mortality, 1980–84	**Urinary nitrate (24 h) from 50 (low-risk town) and 47 (high-risk areas) subjects** Low-risk areas High-risk areas	**Nitrate excretion (median, mg/day)** 76 106 $P = 0.0072$		Age-adjusted rates

Table 2.1 (contd)

Reference, location	Organ site (ICD code)	End-point	Exposure	Main results	Comments
Sandor *et al*. (2001), 192 communities of the county of Baranya, Hungary	Stomach (151)	Mortality, 1984–93; SMR	**Nitrate in drinking-water (mg/L), 1974–93** < 80 > 80	**SMR (95% CI)** 0.96 (0.79–1.13) 1.42 (1.25–1.58) Correlation: 0.59 (P = 0.072)	Expected deaths from national rates; correlation significant if nitrate log-transformed
Gulis *et al.* (2002), 60 villages in the district of Trnava, Slovakia	Stomach (151)	Incidence (1986–95) from the National Cancer Registry; SIR	**Nitrate in drinking–water (mg/L), 1975–95** 0–10 10–20 > 20	**SIR (95% CI)** 0.96 (0.71–1.30) 0.87 (0.69–1.10) 1.08 (0.87–1.35)	Expected cases calculated from the district rates
Italy					
Gilli *et al.* (1984), 1199 communities of the Piemonte region	Stomach (151)	Incidence (1976–79) from the Tumour Register of Piemonte and hospital releases; SIR	**Nitrate in drinking-water (recent years)** > 20 mg/L < 20 mg/L	**No. of communities with** SIR **significantly high/Total No. of communities** Relative Risk (95% CI) 10/155 1.0 (reference) 5/1059 13.7 (3.4–60.0)	Expected cases from regional rates (Piemonte)

Table 2.1 (contd)

Reference, location	Organ site (ICD code)	End-point	Exposure	Main results	Comments
Knight *et al.* (1990), Cagliari, Verona (low risk), Siena, Arezzo (high risk) cities	Stomach (151)	Mortality, 1976–80; age, 35–64 years	**Salivary nitrate and nitrite (nmol/mL) from hospital visitors (187 low-risk areas, 144 high-risk areas)** *Nitrate* Low-risk areas High-risk areas *Nitrite* Low-risk areas High-risk areas	 88.3 65.5 $P = 0.094$ 101.4 82.4 $P = 0.082$	Rates standardized to the world population; concentrations are geometric means.
Knight *et al.* (1992), urban areas of Florence (high-risk) and Cagliari (low-risk)	Stomach (151)	Mortality, 1980–92; age, 0–74 years	**Urinary nitrate from healthy subjects (39 in low-risk areas, 40 in high-risk areas)** *Nitrate (mg/12 h)* Cagliari (low-risk area) Florence (high-risk area) *Rate ($\times 10^{-5}$)* Men Cagliari Florence	 33.7 37.1 $P = 0.55$ 15.6 27.2	Rates standardized to the entire Italian population; concentrations are geometric means.

Table 2.1 (contd)

Reference, location	Organ site (ICD code)	End-point	Exposure	Main results	Comments
Spain					
Sanz Anquela *et al.* (1989), 9 areas (230 municipalities) of the province of Soria	Stomach (151)	Mortality and incidence, 1977–86	**Nitrate in drinking-water (mg/L) (1986)** Men Mortality Incidence	**Correlation coefficient** $r = 0.825$ ($P < 0.01$) $r = 0.701$ ($P < 0.05$)	Age- and sex-adjusted, overall province as standard population; incidence from pathology reports
Morales-Suárez-Varela *et al.* (1995), 258 municipalities of the province of Valencia	Stomach (151)	Mortality, 1975–80	**Nitrate in drinking-water (1968)** *Men* < 25 mg/L 25–50 mg/L > 50 mg/L *Women* < 25 mg/L 25–50 mg/L > 50 mg/L	**Rate ($\times 10^{-5}$)** 21.3 16.7 28.9 16.5 13.2 19.0	Significant differences for level >50 mg/L compared with <25 mg/L only for the age group 55–75 years (both men and women)

Table 2.1 (contd)

Reference, location	Organ site (ICD code)	End-point	Exposure	Main results	Comments
United Kingdom					
Hill *et al.* (1973), Worksop and 9 neighbouring 'control' towns	Stomach (151)	Mortality, 1963–71; SMR	Nitrate in drinking-water: Worksop, 93 mg/L; control towns, 15 mg/L	SMR, 1.27 ($P < 0.05$); all SMRs but one NS	Expected deaths calculated from national rates
Davies (1980), Nottinghamshire: 5 mining towns (including Worksop) and 4 non-mining towns	Stomach (151)	Mortality, 1958–75; SMR	*Men* Worksop Mining towns Non-mining towns *Women* Worksop Mining towns Non-mining towns	**SMR** 0.97 (*P*-value, NS) 0.92 (*P*-value, NS) 0.91 ($P < 0.5$) 1.23 (*P*-value, NS) 1.04 (*P*-value, NS) 0.86 ($P < 0.5$)	Expected deaths from national rates; populations in 1961 and 1971 plus adjustment for social class and mining

Table 2.1 (contd)

Reference, location	Organ site (ICD code)	End-point	Exposure	Main results	Comments
Fraser & Chilvers (1981), 32 rural districts of Anglia and Yorkshire	Stomach (151)	Mortality, 1969–73 and 1974–78; SMR	**Nitrate in drinking-water**	**SMR (1974–78)**	Expected deaths from national rates of population in 1971
			Men		
			Anglia		
			< 25 mg/L	0.90 ($P < 0.05$)	
			25–50 mg/L	1.07 ($P < 0.05$)	
			> 50 mg/L	1.29 ($P<0.05$)	
			Yorkshire		
			< 25 mg/L	0.99 ($P < 0.05$)	
			25–50 mg/L	1.05 ($P < 0.05$)	
			> 50 mg/L	NR	
			Women		
			Anglia		
			< 25 mg/L	1.00 ($P < 0.05$)	
			25–50 mg/L	0.98 ($P < 0.05$)	
			> 50 mg/L	1.05 ($P < 0.05$)	
			Yorkshire		
			< 25 mg/L	0.88 ($P < 0.05$)	
			25–50 mg/L	1.40 ($P < 0.05$)	
			> 50 mg/L	NR	
Beresford (1985), 229 urban areas of England, Scotland and Wales	Stomach (151)	Mortality, 1969–73; SMR	**Nitrate in drinking-water (1971) as mg/L nitrogen**	**Regression coefficient (SMR for a each mg/L)**	Expected deaths from national rates, population in 1971; results adjusted for social class.
			Men	–1.19 ($P < 0.05$)	
			Women	–2.02 ($P < 0.01$)	

Table 2.1 (contd)

Reference, location	Organ site (ICD code)	End-point	Exposure	Main results	Comments
Forman *et al.* (1985), Wales and Northeast (high risk), Oxford and Southeast (low risk)	Stomach (151)	Mortality, 1981; SMR	**Salivary nitrate and nitrite from selected subjects in each region (fasting samples)** *Nitrate (nmol/mL)* Low risk High risk *P*-value *Nitrite (nmol/mL)* Low risk High risk *P*-value	**SMR** Low risk, 0.59–0.91 High risk, 1.12–1.62 169 92.5 < 0.05 80.8 44.7 < 0.05	Fasting samples; similar results for both sexes and social class
Barrett *et al.* (1998), 148 water supply zones in Yorkshire	Stomach (151), oesophagus (150)	Incidence (1975–94) from the Yorkshire Cancer Registry	**Nitrate in drinking-water (average 1990–95)** *Stomach* Quartile 1, 2.4 mg/L Quartile 2, 5.0 mg/L Quartile 3, 13.7 mg/L Quartile 4, 29.8 mg/L *Oesophagus* Quartile 1, 2.4 mg/L Quartile 2, 5.0 mg/L Quartile 3, 13.7 mg/L Quartile 4, 29.8 mg/L	**Rate ratio (95% CI)** 1.00 1.02 (0.98–1.07) 0.86 (0.82–0.90) 0.91 (0.87–0.95) 1.00 1.01 (0.93–1.09) 1.01 (0.94–1.09) 1.06 (0.96–1.14)	Rates adjusted by age, sex, socioeconomic status and population density

Table 2.1 (contd)

Reference, location	Organ site (ICD code)	End-point	Exposure	Main results	Comments
North America					
Geleperin *et al.* (1976), Illinois, USA	Oesophagus (150)	Mortality, 1959–66; whites	**Nitrate in drinking-water** *Men* < 10 mg/L 40–45 mg/L, spring only > 40 mg/L, constant *Women* < 10 mg/L 40–45 mg/L, spring only > 40 mg/L, constant	**Rate ($\times 10^{-5}$)** 43.0 40.3 38.4 6.0 4.2 15.3	All comparisons NS
Van Leeuwen *et al.* (1999), 40 ecodistricts of Ontario, Canada	Stomach (151)	Incidence (1987–91) from the Ontario Cancer Registry	Nitrate in drinking-water (mg/L), 1987–91	Regression coefficient for ln(nitrate), b = –0.136 (–0.151, –0.122)	Age-standardized rates; results shown are for men.
South and Central America					
Cuello *et al.* (1976), several municipalities of the department of Nariño, Colombia	Stomach (151)	Incidence, 1968–72; 261 incident cases and 269 matched controls	**Nitrate in 173 water sources; nitrate and nitrite in urine and saliva of 282 subjects** Low-risk area High-risk area	**Nitrate (mg/L)** 1.7–14.6 12.5–39.0 (test not given) No differences in salivary nitrite	Level of risk by the case:control ratio in each area compared with the whole department

Table 2.1 (contd)

Reference, location	Organ site (ICD code)	End-point	Exposure	Main results	Comments
Zaldívar & Wetterstrand (1978), 25 provinces of Chile	Stomach (151)	Mortality (mean of 1960, 1962 and 1964)	**Nitrate in drinking-water (as mg/L nitrogen), 1953–75** Men Women	**Correlation coefficient** $r = 0.0335$ (NS) $r = 0.0486$ (NS)	Age-adjusted rates $\times 10^{-5}$
Armijo *et al.* (1981), Chillán, Linares (high risk), Antofagasta and Punta Arenas (low risk), Chile	Stomach (151)	Mortality	Nitrate in urine and saliva of 243 children aged 11–13 years (1977–80)	Antofagasta had significantly higher mean of urinary nitrate; no differences for nitrite in saliva	Actual values for concentration not provided, only graph and results of the test
Sierra *et al.* (1993), communities of Turrubares (high risk) and Hojancha (low risk), Costa Rica	Stomach (151)	Incidence (age-adjusted rate $\times 10^{-5}$): 66.1 (high risk), 26.8 (low risk)	**Nitrate in urine of children** Low-risk area ($n = 25$) High-risk area ($n = 26$)	**Nitrate (mmol/12 h)** 0.20 0.23 ($P = 0.75$)	Median: urinary nitrate excretion

Table 2.1 (contd)

Reference, location	Organ site (ICD code)	End-point	Exposure	Main results	Comments
International					
Hartman (1983), 12 countries (10 European, USA, Japan)	Stomach (151)	Mortality, 1974–75, adjusted rates ($\times 10^{-5}$)	Estimates of nitrate intake (mmol/day), 1970s	Correlation, $r = 0.88$	Correlation improved when nitrate-squared was used as a covariate.
Joossens *et al.* (1996), 24 countries	Stomach (151)	Mortality, (1984, 1986, 1987 or 1988), 45–74 years	**Nitrate in 24h urine 1986–87, 3303 adults aged 20–59 years** Men Women	**Correlation** $r = 0.63$ ($P = 0.001$) $r = 0.56$ ($P < 0.05$)	Age-adjusted rates, $\times 10^{-5}$, nitrate in mmol/day

CI, confidence interval; ICD, International Classification of Diseases; NR, not reported; NS, not significant; SIR, standardized incidence ratio; SMR, standardized mortality ratio

[a] Formerly called communes

Europe

Denmark

In Denmark, Jensen (1982) compared the incidence of cancer at several sites in the towns of Aalborg and Aarhus, which were known to have high and low levels of nitrate in the water (27.1 and 0.2 mg/L, respectively). Significantly higher rates of stomach cancer were reported for the high-nitrate area (Aalborg), but no differences were seen for oesophageal cancer.

Eastern Europe

In Poland (Zemła & Kolosza, 1979), significantly higher incidence rates of stomach cancer among men were observed in districts of the town of Zabrze that had higher levels of nitrate in the drinking-water.

In Hungary, a positive but non-significant association between the incidence of stomach cancer and the level of nitrate in the water was reported for the county of Szalbolcs-Szatmar (Juhasz *et al*., 1980), and a non-significant correlation between the level of nitrate in the water and mortality from stomach cancer was found in 192 communities of the county of Banya (Sandor *et al*., 2001); however, in the latter study, there was a significantly increased standardized mortality ratio (SMR) of 1.42 for communities that had concentrations of nitrate greater than 80 mg/L.

Gulis *et al*. (2002) analysed the incidence of stomach cancer in 60 villages in the district of Trnava, Slovakia. The number of observed cases from 1986 to 1995 was retrieved from the national cancer registry and expected cases were calculated for each village using the age-and sex-specific rates for the whole district. No association was observed between incidence rates of stomach cancer and levels of nitrate in the drinking-water. The villages were grouped into three categories according to levels of nitrate (0–10 mg/L, 10–20 mg/L and > 20 mg/L); standardized incidence ratios (SIRs) for the three groups were close to unity and none was significant.

Italy and Spain

A strong positive association was also observed between the incidence of stomach cancer and the level of nitrate in the drinking-water in the Piemonte area, Italy (Gilli *et al*., 1984).

The relationship between nitrate in the drinking-water and the risk for stomach cancer was also assessed in two studies in Spain. In the province of Soria (Sanz Anquela *et al*., 1989), a high-risk area for stomach cancer, a significant positive correlation was observed with both mortality ($r = 0.83$) and incidence ($r = 0.70$), while no clear pattern was observed in the analysis of 258 municipalities of Valencia (Morales-Suárez-Varela *et al*., 1995).

United Kingdom

Several studies compared indicators of the occurrence of stomach cancer with the concentration of nitrate in the drinking-water in defined geographical areas with a high degree of variability in exposure, ranging from 2.4 mg/L nitrate in some areas of Yorkshire to 93 mg/L in a town in Nottinghamshire. An early report showed a significant

increase in mortality from stomach cancer (standardized mortality ratio (SMR), 1.27; $P < 0.05$) in Worksop, a town that had high levels of nitrate (93 mg/L) in the drinking-water (Hill *et al*., 1973), but not in control towns where levels of nitrate were lower (15 mg/L). However, a re-analysis of mortality in the same area (Davies, 1980) calculated expected deaths using SMRs standardized by social class and occupational group at the national level and also using the proportion of subjects in each community by social class and occupation in mining. No increase in mortality was found when mining activity and social class were taken into account. A trend towards higher SMRs for stomach cancer with increasing level of nitrate in the drinking-water was observed in rural districts of Anglia and Yorkshire (Fraser & Chilvers, 1981) (although most estimates were not significant) while a significant inverse relationship was estimated in the analysis of 229 urban areas of England, Wales and Scotland (Beresford, 1985). Barrett *et al*. (1998) conducted a study in Yorkshire, where cancer incidence was available from a regional cancer registry. Ecological assignment was carried out by mean levels of nitrate in tap-water (148 water supply zones, average levels of monthly measurements in 1990–95) and some other variables (socioeconomic status, population density), while individual variables (age and sex) were available from the cancer registry (1975–94) for subjects who were diagnosed with tumours of the stomach (15 554), oesophagus (5399) and brain (3441). For each tumour site, the expected number of cases for each water supply zone, sex and age group was calculated and Poisson regression was employed to model the potential effects of levels of nitrate on incidence rates. For stomach cancer, a significant reduction in risk was found for the third (relative risk, 0.86; 95% confidence interval [CI], 0.82–0.90) and fourth quartile (relative risk, 0.91; 95% CI, 0.87–0.95), while no association was observed for cancer of the oesophagus. [Levels of nitrate in this region were modest: only 0.3% (31/9333) of samples contained > 50 mg/L, and 16% of water supply zones had average levels > 25 mg/L)].

North America

In the USA, Geleperin *et al*. (1976) did not find significant differences in mortality from cancer of the oesophagus with levels of nitrate in the drinking-water in Illinois.

Van Leeuwen *et al*. (1999) conducted an ecological study in 40 ecodistricts of Ontario, Canada. Levels of nitrate in groundwater were assessed for each district using regional data from the monitoring system for municipal water supplies, and sex- and age-standardized cancer rates for stomach and several other cancer sites were calculated from data from the population-based cancer registry (1987–91). Regression analysis weighted by population size was used to examine the correlation between levels of nitrate and incidence of cancer. A significant inverse association was reported between the incidence of stomach cancer and level of nitrate in the drinking-water among men.

South America

The risk for stomach cancer in relation to nitrate was investigated in Nariño, a department located in the South of Colombia (Cuello *et al*., 1976); two areas were defined

within the department based upon the differential distribution of subjects in a case–control study. Concentrations of nitrate in wells and artesian waters from the high-risk area (13–39 mg/L) were much higher than those in the low-risk area (1.7–15 mg/L), although no formal tests were provided.

Following a previous report that showed a strong positive relationship between mortality from stomach cancer and the use of nitrate fertilizers (Zaldivar, 1977), a correlation study was undertaken in Chile that compared mortality from stomach cancer and levels of nitrate in the drinking-water in 25 provinces (Zaldívar & Wetterstrand, 1978); no association was found; non-significant correlation coefficients were 0.03 and 0.05 for men and women, respectively.

(ii) *Biomarkers of nitrate*

Asia

Two Japanese studies compared excretion of nitrate in 24-h urine samples from subjects selected in areas of low and high risk for mortality from stomach cancer. Kamiyama *et al*. (1987) observed lower levels of urinary nitrate in the prefecture of Akita, an area where mortality from stomach cancer was high, compared with the low-risk area of Iwate: median levels of excretion of nitrate were 116 and 140 mg per day per person, respectively ($P = 0.07$). No association was observed for urinary nitrate in the high-mortality areas of Yakote and Saku cities compared with the low-risk areas of Ninohe and Ishikawa cities (Tsugane *et al*., 1992).

In China, Zhang et al. (1984) analysed levels of nitrate in the fasting gastric juice of subjects from six areas where mortality from stomach cancer was high and four where it was low and showed an inverse significant association. [However, this study was carried out among patients with chronic stomach complaints.] Three studies in China used excretion of nitrate in urine samples as an indicator of exposure. Wu et al. (1993) compared urinary nitrate in healthy subjects and mortality rates from 69 counties in 25 Chinese provinces and found inverse non-significant associations with mortality from both cancer of the stomach ($r = –0.14$) and cancer of the oesophagus ($r = –0.10$). Lu et al. (1986) had previously reported an excretion of 94 mg nitrate per person per day in Linxian County (an area that had the highest mortality from cancer of the oesophagus), which was significantly higher than the level of 48 mg measured in Fanxian county, an area that had much lower mortality. A very similar result was reported in a more recent study (Lin et al., 2003): the level of urinary nitrate was 7.1 nmol/g creatinine in subjects from Nan'ao county, a high-risk area, which was more than twice that measured in Lufeng county (3.1 nmol/g), where mortality from cancer of the oesophagus was much lower.

Europe

In Poland, Zatonski *et al*. (1989) reported higher levels of urinary nitrate in subjects from rural areas where mortality from stomach cancer was high compared with residents in the town of Opole that had lower mortality from stomach cancer.

In Italy, lower levels of nitrate in the saliva were found in subjects from regions with high mortality from stomach cancer (Siena, Arezzo) compared with those from Cagliari and Verona, where mortality rates were lower, although the differences did not reach statistical significance (Knight *et al*., 1990). In another Italian study, no differences were found in levels of urinary nitrate measured in children in Florence and Cagliari, areas with high and low mortality from stomach cancer, respectively (Knight *et al*., 1992).

Forman *et al*. (1985) compared concentrations of nitrate in the saliva of subjects from areas where mortality from stomach cancer was high (Northeast and Wales) with those from subjects in low-risk areas (Southeast and Oxford) and found an inverse relationship, with significantly higher levels of nitrate in areas that had lower mortality from stomach cancer.

South and Central America

Armijo *et al*. (1981) compared levels of nitrate in the urine and saliva of children aged 11–13 years from four cities in Chile, two with high (Chillán, Linares) and two with low mortality rates (Antofagasta, Punta Arenas) for stomach cancer. Children in the two areas with lower mortality had the highest and the lowest mean levels of urinary nitrate; however, children from only one of the low-risk areas (Antofagasta) had significantly higher urinary excretion of nitrate in pairwise comparisons with the other three areas.

In a study in Costa Rica (Sierra *et al*., 1993), children from two areas with high and low incidence rates of stomach cancer had similar levels of urinary excretion of nitrate.

International

In the multinational INTERSALT study (Joossens *et al*., 1996), the mortality rates for stomach cancer were compared with excretion of nitrate in 24-h urine samples from 24 countries; significant correlations of 0.63 and 0.56 were observed for men and women, respectively. Although the source of data to estimate average intake of nitrate at the country level was not specified, a review by Hartman (1983) reported a correlation of 0.88 between the mortality rates from stomach cancer and the average intake of nitrate in 12 countries.

(b) Cohort studies (Table 2.2)

The Netherlands Cohort Study is a prospective study of diet, other lifestyle characteristics and the risk for cancer that was started in September 1986 and initially included 58 279 men and 62 573 women aged 55–69 years at recruitment (van Loon *et al*., 1997, 1998). Usual diet was assessed by means of a 150-item semiquantitative food-frequency questionnaire. Intake of nitrates was estimated using data from the databank of contaminants in foods from the State Institute for Quality Control of Agricultural Products (RIKILT, Wageningen). Information on water intake was combined with data from all waterworks in the country to determine the concentration of nitrate in the drinking-water for each home address by postal code. A case–cohort approach was used for data analysis: a subcohort of 3500 subjects was randomly sampled and followed every

Table 2.2. Cohort studies of ingested nitrate and nitrite and the risk for stomach and oesophageal cancer

Reference, location, name of study	Organ site (ICD code)	Cohort description	Exposure assessment	Exposure categories	No. of cases/ deaths	Relative risk (95% CI)	Adjustment for potential confounders	Comments
van Loon *et al.* (1998), Netherlands, The Netherlands Cohort Study	Stomach (151)	Cohort of 58 279 men and 62 573 women, 55–69 years; prospective follow-up September 1986–December 1992 by regional cancer registries and national pathology register; case–cohort analysis of 282 incident cases (219 men, 63 women) and 3123 subcohort members (1525 men, 1598 women)	Self-administered 150-item food-frequency questionnaire; food composition and nitrate in drinking-water from databank on contaminants in food (nitrate) and a food composition database (nitrite)	**Total nitrate, quintile (mean, mg/day)**		**Rate ratio**	Age, sex, smoking, education. coffee, vitamin C, β-carotene, family history of stomach cancer, prevalence of stomach disorders, use of refrigerator or freezer	Similar results after exclusion of subjects with stomach disorders or those diagnosed during the first year
				I (59.8)	63	1.00 (reference)		
				II (84.7)	67	1.25 (0.84–1.86)		
				III (104.4)	42	0.74 (0.47–1.15)		
				IV (127.3)	54	0.92 (0.59–1.44)		
				V (179.8)	56	0.90 (0.53–1.55)		
						P trend = 0.30		
				Nitrate from drinking-water (mean, mg/day)				
				Q1 (0.02)	61	1.00 (reference)		
				Q2 (1.65)	54	0.93 (0.62–1.39)		
				Q3 (3.85)	53	0.87 (0.51–1.31)		
				Q4 (6.91)	57	0.83 (0.55–1.24)		
				Q5 (16.5)	57	0.88 (0.59–1.32)		
						P trend = 0.39		
				Nitrate from foods (mean, mg/day)				
				Q1 (55.8)	69	1.00 (reference)		
				Q2 (79.4)	61	1.02 (0.69–1.51)		
				Q3 (98.7)	45	0.71 (0.46–1.09)		
				Q4 (120.7)	49	0.80 (0.51–1.25)		
				Q5 (172.2)	58	0.80 (0.47–1.37)		
						P trend = 0.18		
				Total nitrite, quintile (mean, mg/day)				
				I (0.01)	47	1.00 (reference)		
				II (0.04)	51	1.20 (0.78–1.86)		
				III (0.09)	58	1.18 (0.77–1.82)		
				IV (0.16)	46	0.88 (0.56–1.37)		
				V (0.35)	80	1.44 (0.95–2.18)		
						P trend = 0.24		

Table 2.2. (contd)

Reference, location, name of study	Organ site (ICD code)	Cohort description	Exposure assessment	Exposure categories	No. of cases/ deaths	Relative risk (95% CI)	Adjustment for potential confounders	Comments
Knekt *et al.* (1999), Finland, several regions	Stomach (151)	9985 subjects, part of a multiphasic screening examination (the Mobile Health Clinic of the Social Insurance Institution); follow-up 1967–90 through nationwide Finish Cancer Registry; 68 incident cases of stomach cancer (43 men, 25 women)	Dietary history interview, referred to previous year	*Nitrate* Q1 Q2 Q3 Q4 *Nitrite* Q1 Q2 Q3 Q4	Not given	1.00 (reference) 1.01 (0.56–1.84) 0.52 (0.25–1.08) 0.56 (0.27–1.18) *P* trend = 0.09 1.00 (reference) 1.10 (0.58–2.11) 1.88 (1.01–3.49) 0.71 (0.28–1.78) *P* trend = 0.90	Age, sex, municipality, smoking, energy intake	No association with NDMA for stomach cancer; mean daily intake: 77 mg nitrate (92% from vegetables); 5.3 mg nitrite (94% from cured meats and sausages)

CI, confidence interval; ICD, International Classification of Diseases; NDMA, *N*-nitrosodimethylamine; Q, quartile

2 years for vital status and record linkage with all regional cancer registries. During a follow-up period of 6.3 years (up to December 1992), 347 cases of stomach cancer were identified. After exclusion of prevalent cases and those not microscopically confirmed or having incomplete dietary information, the analysis was carried out on 282 cases (219 men and 63 women) and 3123 subcohort members (1525 men and 1598 women). Total mean intake of nitrate in the subcohort was 111 mg per day (105 mg from food, 5.8 mg from drinking-water). No significant inverse associations were found for total nitrate intake, intake of nitrate from foods or intake of nitrate from water after adjustment for several factors including vitamin C intake. An analysis that excluded subjects with stomach disorders or those who were diagnosed during the first year did not modify these patterns substantially.

During 1966–72, a multiphasic screening examination was undertaken in several regions of Finland by the Mobile Health Clinic of the Social Insurance Institution. As part of the main study, data on food consumption were collected from 9985 individuals (5274 men and 4711 women) who had never had cancer (Knekt *et al*., 1999). Dietary information was collected by means of an interview, and several food composition databases were used to calculate intake of nitrate. During a 24-year period (1967–90), 68 cases of cancer of the stomach were ascertained through the Finnish Cancer Registry. A non-significant inverse association was observed for nitrate intake: compared with the first quartile, relative risks for the second, third and fourth quartiles of dietary nitrate were 1.01, 0.52 and 0.56, respectively, none of which was significant. All of these estimates were adjusted for age, sex, municipality, tobacco smoking and energy intake.

(c) Case–control studies (Table 2.3)

Several case–control studies have analysed the relationship between the occurrence of tumours of the stomach or oesophagus and intake of nitrate from foods, but very few provided separate estimates for the intake of nitrate from drinking-water. Most of these studies were carried out in populations who had relatively low levels of nitrate in the drinking-water and the majority of ingested nitrate was provided by diet.

China

One case–control study from Taiwan, China (Yang *et al*., 1998) was designed to assess the relationship between levels of nitrate in the drinking-water and mortality from stomach cancer. Information on the levels of calcium, magnesium and nitrate in the drinking-water were available from the Taiwan, China Water Supply Corporation for 252 of 361 municipalities. Cases were 6766 registered deaths from stomach cancer in residents from these municipalities from 1987 to 1991; age- and sex-matched controls were selected from among deaths from other causes excluding gastrointestinal disorders, cardiovascular diseases and some tumours. Levels of nitrate measured in 1970 in water in the municipality of residence was used as an indicator of exposure. No association was observed between mortality from stomach cancer and levels of nitrate in the drinking-water

Table 2.3. Case–control studies of ingested nitrate and nitrite and the risk for stomach and oesophageal cancers

Reference, study location, study period	Organ site (ICD code)	Characteristics of cases	Characteristics of controls	Exposure assessment	Exposure categories	No of exposed cases	Relative risk (95% CI)	Adjustment for potential confounders	Comments
China									
Yang *et al.* (1998), China (Province of Taiwan), 1987–91	Stomach (151)	6766 (4480 men, 2286 women) deaths from 1987 to 1991 obtained from the Bureau of Vital Status, residents in 252 (of 361) municipalities of the Province of Taiwan	6766 selected among deaths from other causes, excluding those associated with gastrointestinal problems, cardiovascular and cerebrovascular diseases and tumours of the bladder, prostate, lung, oesophagus, head and neck	Levels of nitrate in each municipality's treated drinking- water supply for the year 1990, from the Taiwan Water Supply Corporation	**Nitrate-N from drinking water, tertiles (mg/L) range (median)** < 0.22 (0.04) 0.23–0.44 (0.37) ≥ 0.45 (0.67) *P* for trend	 2109 2126 2531	 1.00 0.95 (0.87–1.03) 1.02 (0.93–1.11) 0.44	Age, sex, year of death	Significant inverse association with magnesium and calcium in drinking-water; relative risks for nitrate (tertiles) adjusted for calcium and magnesium: 1.10 (1.00–1.20) and 1.14 (1.04–1.25)

Table 2.3 (contd)

Reference, study location, study period	Organ site (ICD code)	Characteristics of cases	Characteristics of controls	Exposure assessment	Exposure categories	No of exposed cases	Relative risk (95% CI)	Adjustment for potential confounders	Comments
Europe									
Buiatti *et al.* (1990), 4 areas in northern and central Italy, 1985–87	Stomach (151)	1016 (640 men, 376 women) aged 75 years or under from all hospitals in the areas of study; 100% histologically confirmed; participation rate, 83%	1159 population controls from municipal lists of residents; stratified by age, sex according to distribution of cases; participation rate, 81%	Interviewer-administered structured questionnaire of 146 food items; Italian and British FCTs	**Nitrate, quintiles (mean, mg/day)** 1 (53) 2 (81) 3 (103) 4 (130) 5 (193) *P* for trend **Nitrite, quintiles (mean, mg/day)** 1 (2.1) 2 (2.8) 3 (3.4) 4 (4.1) 5 (5.9) *P* for trend	 NR NR 	 1.0 (reference) 0.9 (0.7–1.1) 0.9 (0.6–1.1) 0.7 (0.5–0.9) 0.9 (0.7–1.2) [0.68] 1.0 (reference) 1.0 (0.8–1.4) 1.2 (0.9–1.7) 1.4 (1.0–2.0) 1.9 (1.3–2.7 [0.005]	Age, sex, area, urban/rural residence, migration from south, socioeconomic status, familial history of stomach cancer, body mass index, total energy intake	Interaction between antioxidant nutrients (vitamin C plus α-tocopherol) and nitrosating/ nitrosable compounds (protein plus nitrite)

Table 2.3 (contd)

Reference, study location, study period	Organ site (ICD code)	Characteristics of cases	Characteristics of controls	Exposure assessment	Exposure categories	No of exposed cases	Relative risk (95% CI)	Adjustment for potential confounders	Comments
Boeing *et al.* (1991), Southeast Bavaria and Hesse, Germany, 1985–87	Stomach (151)	143 (50% men, 50% women) aged 32–80 years from five main hospitals in the two areas; 100% histologically confirmed; participation rate, 85%	579 in total; 328 hospital controls plus 251 visitor controls selected from the same hospitals as the cases; participation rate, 90% for hospital controls; unknown for visitors	Interviewer-administered questionnaire of 74 food items; German FCT	**Nitrate, quintiles (mg/day)** 1 (53) 2 (81) 3 (103) 4 (130) 5 (193) *P* for trend	NR	1.00 (reference) 0.93 (0.53–1.64) 0.61 (0.32–1.19) 0.61 (0.30–1.27) 1.26 (0.59–2.70) [1.0]	Age, sex, hospital, vitamin C, carotene, calcium	Both control groups were pooled in this analysis; having well-water supply (supposed high nitrate contents) had a relative risk of 2.26 (1.19–4.28) compared with having central water supply.
González *et al.* (1994), five provinces in northern and central Spain, 1988–89	Stomach (151)	354 (235 men, 119 women) aged 31–88 years from 15 public hospital, residents in the study areas; 100% histologically confirmed; participation rate, 100%	354 admitted to the same hospitals as the cases; individually matched by age (± 3 years), sex, area of residence	Interviewer administered diet history, structured by meals; English and Spanish FCTs	**Nitrate, quartiles (mg/day)** 1 (< 84) 2 (84–146) 3 (145–234) 4 (> 234) *P* for trend **Nitrite, quartiles (mg/day)** 1 (< 1.3) 2 (1.4–1.8) 3 (1.9–2. 4) 4 (> 2.4) *P* for trend	NR NR	1.00 0.80 (NR) 0.65 (NR) 0.45 (NR) 0.007 1.00 1.20 (NR) 1.09 (NR) 1.28 (NR) 0.38	Age, sex, area of residence, total caloric intake	CIs not provided; in this study a significant increased risk was observed with higher intake of NDMA; significant interaction between nitrosamines and vitamin C.

Table 2.3 (contd)

Reference, study location, study period	Organ site (ICD code)	Characteristics of cases	Characteristics of controls	Exposure assessment	Exposure categories	No of exposed cases	Relative risk (95% CI)	Adjustment for potential confounders	Comments
Hansson *et al.* (1994), five counties in central and northern Sweden, 1989–92	Stomach (151)	338 (sex distribution not given) aged 40–79 years resident in the study areas; 100% histologically confirmed; participation rate, 74%	679 randomly sampled from a population register in the areas of study; stratified by age, sex according to the distribution of the cases; participation rate, 77%	Interviewer administered FFQ of 45 items referred to adolescence and 20 years prior to interview; Swedish FCT	**Quartiles (Q; 20 years prior to the interview)** *Nitrate* Q4 versus Q1 *P* for trend *Nitrite* Q4 versus Q1	 NR NR 	 0.55 (0.38–0.80) 0.002 1.22 (0.82–1.81)	Age, sex	Mean intake of nitrate (controls), 42 mg/day
La Vecchia *et al.* (1994, 1997), Milan area, Italy, 1985–92	Stomach (151)	723 (443 men, 280 women) aged 19–74 years from four largest teaching and general hospitals; 100% histologically confirmed; participation rate, 95%	2024 (1189 men, 835 women) admitted to the same hospitals (excluding neoplastic and digestive wards); participation rate, 95%	Interviewer-administered FFQ of 29 items; Italian FCT	**Nitrate, quintiles (mg/day)** 1 (< 63) 2 (63–80) 3 (81–96) 4 (97–116) 5 (> 116) *P* for trend **Nitrite, quintiles (mg/day)** 1 (< 1.91) 2 (1.92–2.41) 3 (2.42–2.94) 4 (2.95–3.64) 5 (> 3.64) *P* for trend **Nitrite (mg/day)** < 2.7 ≥ 2.7	 228 156 117 117 105 123 128 126 153 193 NR 	 1.00 (reference) 0.64 (0.49–0.83) 0.50 (0.38–0.67) 0.52 (0.39–0.70) 0.43 (0.32–0.59) < 0.001 1.00 (reference) 0.98 (0.72–1.33) 0.99 (0.72–1.36) 1.15 (0.84–1.59) 1.35 (0.96–1.88) < 0.05 1.00 (reference) 1.44 (1.2–1.7)	Age, sex, education, family history of stomach cancer, body mass index, energy intake	Combined effect of nitrite with methonine high/high versus low/low, 2.45 (1.9–3.2)

Table 2.3 (contd)

Reference, study location, study period	Organ site (ICD code)	Characteristics of cases	Characteristics of controls	Exposure assessment	Exposure categories	No of exposed cases	Relative risk (95% CI)	Adjustment for potential confounders	Comments
Pobel *et al.* (1995), Marseilles, France, 1985–88	Stomach (151)	92 (59 men, 33 women; mean age, 67 years) from 8 major centres for gastric surgery in Marseilles; 100% histologically confirmed; participation rate, 100%	128 hospital (74 men, 54 women) controls from two specialized centres for re-education for trauma or injuries; stratified by age, sex according to the distribution of the cases; participation rate, 100%	Interviewer-administered diet history, structured by meals; French FCT	**Nitrate, tertiles** 1 2 3 *P* for trend **Nitrite, tertiles** 1 2 3 *P* for trend	 NR NR 	 1.00 0.49 (0.24–1.01) 0.76 (0.38–1.50) 0.96 1.0 0.83 (0.41–1.67) 0.88 (0.44–1.79) 0.49	Age, sex, occupation, total energy intake	Mean intake of nitrate (controls), 143 mg/day; no significant association with nitrate from different sources; a significant increased risk was observed with higher intake of NDMA.

Table 2.3 (contd)

Reference, study location, study period	Organ site (ICD code)	Characteristics of cases	Characteristics of controls	Exposure assessment	Exposure categories	No of exposed cases	Relative risk (95% CI)	Adjustment for potential confounders	Comments
Palli *et al.* (2001), Florence, 1985–87	Stomach (151)	382 (239 men, 143 women) aged 75 years or under from hospitals in the area of study; 100% histologically confirmed	561 population controls from municipal lists of residents; stratified by age, sex according to distribution of cases	Interviewer-administered structured questionnaire of 146 food items; Italian FCT	**Nitrate, tertiles (mean, mg/day)** 1 (62.6) 2 (93.2) 3 (107.4) *P* for trend **Nitrite, tertiles (mean, mg/day)** 1 (2.5) 2 (3.5) 3 (5.4) *P* for trend	NR NR 	1.0 (reference) 0.7 (0.5–1.0) 0.6 (0.4–0.9) 0.01 1.0 (reference) 1.4 (1.0–2.0) 1.4 (1.0–2.0) 0.04	Age, sex, urban/rural residence, social class, familial history of stomach cancer, body mass index, total energy intake	Estimates not adjusted for other dietary factors; a stepwise procedure retained only total protein, β-carotene and α-tocopherol intake as associated with risk for stomach cancer; no association with DMA or NDMA

Table 2.3 (contd)

Reference, study location, study period	Organ site (ICD code)	Characteristics of cases	Characteristics of controls	Exposure assessment	Exposure categories	No of exposed cases	Relative risk (95% CI)	Adjustment for potential confounders	Comments
North America									
Risch *et al.* (1985), Newfoundland, Manitoba and Toronto, Canada, 1979–82	Stomach (151)	246 (163 men, 83 women) aged 35–79 years from the province tumour registries (Newfound-land, Manitoba) or pathology reports of 21 hospitals in Toronto; 100% histologically confirmed; participation rate, 44%	246 population controls from provincial electoral lists or municipal lists of residents; individually matched to cases by age (within 4 years), sex, province of residence; participation rate, 58%	Interviewer-administered diet history; FCT from the USDA and other sources	Nitrate, per 100 mg/day Nitrite, per 1 mg/day	NR NR	0.66 (0.54–0.81) 1.71 (1.24–2.37)	Age, sex, province of residence (matched analysis), total food consumption, ethnicity	No association with NDMA
Rademacher *et al.* (1992), Wisconsin, USA, 1982–85	Stomach (151)	1268 (758 men, 510 women) deaths from stomach cancer in the Wisconsin Bureau of Health Statistics	1268 randomly selected from all other deaths (excluded other gastrointestinal problems); matched for sex, year of birth, year of death	Recorded levels of nitrate in municipal water sources (1970 edition) and those measured in private wells (data not provided)	**Level of nitrate-N (mg/L)** > 0.5 versus ≤ 0.5 > 2.5 versus ≤ 25 > 5.0 versus ≤ 5 > 10.0 versus < 10 Private wells versus public	 207 113 25 6	 0.92 (0.75–1.12) 0.97 (0.74–1.35) 0.86 (0.69–1.08) 1.50 (0.12–18.25) 1.09 (0.82–1.47)	Age, sex (matched analysis)	Nitrate-N in private wells was significantly higher than that in public supplies.

Table 2.3 (contd)

Reference, study location, study period	Organ site (ICD code)	Characteristics of cases	Characteristics of controls	Exposure assessment	Exposure categories	No of exposed cases	Relative risk (95% CI)	Adjustment for potential confounders	Comments
Rogers *et al.* (1995), three counties of Washington State, USA, 1983–87	Oesophagus (150)	645 upper aero-digestive tract cancers identified through the Cancer Surveillance System (SEER Program), of which 127 were oesophageal tumours (94 men, 33 women); participation rate, 67%	458 from random-digit dialling in the three counties; frequency-matched by sex, age (5-year interval)	Self-administered FFQ of 125 food items; food composition from report of the National Academy of Sciences	**Nitrate, tertiles (mg/day)** 1 (< 134) 2 (134–226) 3 (> 226) *P* for trend *High vitamin C* 1 (< 134) 2 (134–226) 3 (> 226) *Low vitamin C* 1 (< 134) 2 (134–226) 3 (> 226) **Nitrite, tertiles (mg/day)** 1 (< 1.06) 2 (1.06–1.60) 3 (> 1.60) *P* for trend *High vitamin C* 1 (< 1.06) 2 (1.06–1.60) 3 (> 1.60) *Low vitamin C* 1 (< 1.06) 2 (1.06–1.60) 3 (> 1.60)	 33 39 25 NR NR 26 28 43 NR NR 	 1.00 (reference) 0.71 (0.38–1.33) 0.44 (0.24–0.93) 0.078 1.00 (reference) 0.73 (NR) 0.44 (NR) 1.63 (NR) 1.44 (NR) 1.27 (NR) 1.00 (reference) 1.17 (0.57–2.38) 1.58 (0.73–3.44) 0.20 1.00 (reference) 2.24 (NR) 1.49 (NR) 2.93 (NR) 2.23 (NR) 5.07 ($P < 0.05$)	Age, sex, pack–years of cigarettes, drink–years of alcohol, energy intake, vitamin C, body mass index, level of education	Interaction between nitrite and vitamin C (NS); slightly increased risk (NS) for intake of NDMA; high NDMA with low vitamin C had a significantly increased risk versus low NDMA and high vitamin C.

Table 2.3 (contd)

Reference, study location, study period	Organ site (ICD code)	Characteristics of cases	Characteristics of controls	Exposure assessment	Exposure categories	No of exposed cases	Relative risk (95% CI)	Adjustment for potential confounders	Comments
Mayne *et al.* (2001); Engel *et al.* (2003), Connecticut, Washington state, New Jersey, USA, 1993–95	Stomach (151), cardia and non-cardia; oesophagus (150), SCC, ADC	Stomach: 255 cardia (217 men, 38 women), 352 non-cardia (244 men, 108 women); oesophagus: 282 ADC (235 men, 47 women), 206 SCC (166 men, 40 women) aged 30–79 years from three population-based tumour registries; 100% histologically confirmed; participation rate, 72% (overall)	687 from random-digit dialling and Health Care Finance Administration rosters: frequency-matched by age (5-year group), sex, geographic area; participation rate, 70%	Interviewer-administered FFQ of 104 items; FCT from the University of Minnesota and other databases	Nitrite, 75th percentile compared with 25th percentile	NR	*Oesophagus* SCC 1.13 (0.82–1.56) ADC 1.05 (0.80–1.38) *Stomach* Non-cardia 1.65 (1.26–2.15) Cardia 1.05 (0.79–1.40)	Age, sex, area (matching), race, proxy status, income, education, body mass index, cigarettes/day, years of consumption of beer, wine and liquor, sodium, energy intake	Interview with proxies for 30% of cases and 3.4% of controls

Table 2.3 (contd)

Reference, study location, study period	Organ site (ICD code)	Characteristics of cases	Characteristics of controls	Exposure assessment	Exposure categories	No of exposed cases	Relative risk (95% CI)	Adjustment for potential confounders	Comments
Mayne *et al.* (2001); Engel *et al.* (2003) (contd)		Stomach: 368 non-cardia	695 controls	Interviewer	**Nitrite, quartiles** 1 2 3 4 2–4 **High vitamin C** Low nitrite High nitrite **Low vitamin C** Low nitrite High nitrite	NR	 1.0 1.5 (1.0–2.4) 1.8 (1.1–3.0) 2.5 (1.4–4.3) 1.7 (1.1–2.6) 1.0 (reference) 2.10 (1.36–3.25) 2.25 (1.46–3.47) 2.95 (1.90–4.59)	Age, sex, area, race, respondent type, income, energy intake, smoking status, history of gastric ulcer	Levels (quartiles) of nitrite intake among controls by sex: men: 1.7–5.8, 5.9–7.5, 7.6–9.9, 10.0–39.2; women: 1.9–5.3, 5.4–6.9, 7.0–9.1, 9.2–31.2; increased risk of oesophageal ADC, SCC and stomach cardia cancer with low vitamin C intake (regardless of nitrite intake) compared with high vitamin C and low nitrite intakes

ADC, adenocarcinoma; CI, confidence interval; DMA, dimethylamine; FCT, food composition table; FFQ, food-frequency questionnaire; ICD, International Classification of Diseases; NDMA, *N*-nitrosodimethylamine; NR, not reported; NS, not significant; SCC, squamous-cell carcinoma; USDA, US Department of Agriculture

However, a weak but significant association was seen after adjustment for levels of calcium and magnesium in the water.

Europe

A large case–control study was carried out in Italy (Buiatti *et al*., 1990) based on 1016 cases and 1159 controls from two areas at high risk for stomach cancer (Florence–Siena and Forlí–Cremona–Imola) and two at low risk (Genoa and Cagliari). Dietary intake of nitrate (mg per/day) was derived from personal interviews using a structured questionnaire of 146 items and data on nitrate content in foods were obtained from Italian and British food composition tables. The association with risk for stomach cancer was assessed through the distribution of nitrate controls to define quintiles, taking the lowest as the reference category, and by means of a model that included age, sex, social class, familial history of stomach cancer and caloric intake. No association was found between dietary intake of nitrate and the risk for stomach cancer. Palli *et al*. (2001) updated the results of Buiatti *et al*. (1990) on the cases from the local cancer registry of Florence by expanding the series of controls and applying an updated Italian food composition table. Overall, this re-analysis included 382 cases and 561 controls. There was a significant reduction in risk for stomach cancer with increasing nitrate intake (P for trend = 0.01). [The authors stated that the effect of nitrate disappeared when adjustment was made for protein (associated with increased risk), and β-carotene and α-tocopherol (associated with protection against risk). However, adjusted estimates were not provided.]

Boeing *et al*. (1991) carried out a hospital-based case–control study in Southeastern Bavaria and Hesse (Germany). A total of 143 cases and 579 controls (patients and visitors) were interviewed using a standard questionnaire of 74 items coupled with a German food composition table to obtain dietary intake of nitrate. Information on the type of water supply for each residence was also collected. No association was observed between the risk for stomach cancer and quintiles of dietary intake of nitrate, adjusted for age, sex, hospital, vitamin C, carotene and calcium. Residence with a well-water supply was associated with an increased risk for stomach cancer (relative risk, 2.26; 95% CI, 1.19–4.28) compared with a central water supply. [The authors stated that private well-waters were supposed to have a higher content of nitrate, but information on levels of nitrate in water was not available for the areas under study.]

Another hospital-based case–control study that included 354 cases and 354 matched controls (González *et al*., 1994) was carried out in five provinces of Spain (Barcelona, La Coruña, Lugo, Soria and Zaragoza). A diet history method administered personally by a trained interviewer was used to assess habitual diet during a typical week of the year before the diagnosis (cases) or interview (controls); Spanish and British food composition tables were used to estimate the intake of nutrients. In the matched analysis (age, sex, area), an inverse association between risk for stomach cancer and increasing quartiles of nitrate intake was observed with a significant trend (P for trend = 0.007) after also adjusting for total caloric intake. The effect was more evident when the analysis was restricted to cases with diffuse-type histology.

Hansson *et al*. (1994) reported the results of a case–control study in Sweden. A food frequency questionnaire was administered by an interviewer to 338 cases of stomach cancer and 679 population controls who were identified through the population register; information on usual diet during adolescence and the 20 years before the interview was collected and nitrate intake was calculated from a Swedish food composition table. The highest quartile of nitrate intake, adjusted for sex and age, was significantly associated with decreased risk for cancer of the stomach (odds ratio, 0.55; 95% CI, 0.38–0.80; *P* for trend = 0.002). However, no association was observed when dietary intake of nitrate was further adjusted for vitamin C and α-tocopherol. [A plausible interpretation of such results could be that the decrease in risk is probably due to the inhibitory effect on nitrosation of vitamin C and vitamin E.]

A hospital-based case–control study carried out in Italy included 723 cases and 2024 controls selected from the four largest teaching hospitals from the Greater Milan area (La Vecchia *et al*., 1994, 1997). An interviewer administered a 29-item food-frequency questionnaire and an Italian food composition table was used to assess the usual dietary intake of nitrate. In a multivariate analysis that took into account potential dietary and non-dietary confounders, the highest quintile of nitrate consumption showed a significant reduction in the risk for stomach cancer (relative risk, 0.43; 95% CI, 0.32–0.59; based on 105 exposed cases; *P* for trend < 0.001) compared with the lowest quintile.

A hospital-based case–control study that included 92 cases and 128 controls was carried out in the area of Marseilles, France (Pobel *et al*., 1995). A questionnaire on diet history was administered by an interviewer at the hospital and a French food composition table was used to estimate the intake of nitrates. No association was observed between intake of nitrate and the risk for stomach cancer overall or when different dietary sources were considered separately, while a significant increase in risk was reported for the dietary intake of preformed nitrosamines. [Potential limitations of this study are the small sample size and the fact that controls were selected in two specialized medical centres of re-education for trauma or injuries, which may not provide a good representation of the dietary habits of the study base.]

North America

The first case–control study on risk for stomach cancer that reported results for dietary intake of nitrates was a Canadian study by Risch *et al*. (1985). Overall 246 cases of stomach cancer were identified from the cancer registries of two provinces of Canada and from the pathology reports of hospitals in Toronto; individually matched controls were selected from provincial or municipal lists of residents. Usual diet was assessed by the diet history method administered by an interviewer and nutrient intakes were calculated using a food composition table from the USA and other sources. The matched analysis with further adjustment for ethnicity showed a significant reduction in risk for stomach cancer for each 100 mg nitrate ingested per day (odds ratio, 0.66; 95% CI, 0.54–0.81). However, the effect of nitrate totally disappeared when adjustment was made for intake of vitamin C from vegetables.

Rademacher *et al*. (1992) reported a case–control study based upon death certificates to assess the association of stomach cancer with nitrate in the drinking-water. Cases were 1268 deaths from stomach cancer from 1982 to 1985 in Wisconsin (USA) and matched controls were selected from among deaths from causes other than gastrointestinal problems. Levels of nitrate recorded in 1970 for municipal sources or measured in private wells were used as indicators of exposure at the residence of the subject at the time of death. Using several cut-off points to define exposure, no significant associations between nitrate in drinking-water and mortality from stomach cancer were observed. [The authors noted that the levels of nitrate in private wells were measured at the time of the study, which was about 20 years later than those recorded for public sources.]

Only one study has evaluated the potential association between dietary intake of nitrate and oesophageal cancer. Rogers *et al*. (1995) conducted a population-based case–control study in Washington State (USA) of 645 tumours of the upper aerodigestive tract, that included 125 oesophageal cancers, which were identified through the cancer surveillance system and 458 frequency-matched controls. A self-administered, 125-item food-frequency questionnaire and a food composition table from the National Academy of Sciences were used to estimate the usual intake of nitrate. High consumption (> 226 mg per day) was significantly associated with a decrease in the risk for cancer of the oesophagus compared with lowest intake (< 134 mg per day) after adjustment for potential non-dietary and dietary confounders (including vitamin C intake).

2.2.2 *Ingested nitrite*

(a) Ecological studies

Most ecological studies that addressed tumours of the stomach or oesophagus focused mainly on the intake of nitrates whereas nitrite was rarely assessed. The main features and results of ecological studies on the relationship between intake of nitrite and tumours of the stomach or oesophagus are summarized in Table 2.1.

China

In China, Zhang *et al*. (1984) analysed levels of nitrite in the fasting gastric juice of subjects from six areas where mortality from stomach cancer was high and from four areas with low mortality rates and showed a significant positive association for nitrite.

The only ecological study that assessed the association between intake of nitrite and oesophageal tumours was carried out in China. Zhang *et al*. (2003) observed a significantly higher level of nitrite in well-water in the county of Cixian (0.01 mg/L), an area where mortality from oesophageal cancer is very high, compared with the low-risk county of Chichen (0.002 mg/L).

Europe

In a study in Italy (Knight *et al*., 1990), lower levels of nitrite in saliva were found in subjects from regions where mortality from stomach cancer was high (Siena, Arezzo)

compared with subjects from Cagliari and Verona, where rates were lower, although the differences did not reach statistical significance.

Only one study assessed measured levels of nitrite in the drinking-water in relation to stomach cancer. In Poland, Zemła and Kolosza (1979) reported a significantly higher incidence of stomach cancer among men in districts of the town of Zabrze that had higher levels of nitrite in the drinking-water.

In the United Kingdom, Forman *et al*. (1985) compared levels of nitrite in saliva in subjects from an area where mortality from stomach cancer was high (Northeast and Wales) with those from low-risk areas (Southeast and Oxford) and found an inverse relationship; levels were significantly higher in areas with lower mortality.

South America

The risk for stomach cancer in relation to ingested nitrite was investigated in the department of Nariño, Colombia (Cuello *et al*., 1976). No differences in levels of salivary nitrite were seen between two areas of high or low risk. Armijo *et al*. (1981) carried out a study in children aged 11–13 years from four provinces in Chile, two of which had high and two of which had low mortality rates for stomach cancer. No differences were observed in the concentrations of nitrite in saliva gathered from these children.

(b) Cohort studies

Two cohort studies (described in detail in Section 2.2.1(*b*)) analysed the association between ingested nitrite and the risk for cancers of the stomach or oesophagus; their main findings are summarized in Table 2.2.

In the Netherlands Cohort Study (van Loon *et al*., 1997, 1998), intake of nitrite was estimated using the database on food composition values from the Nutrition and Food Research Institute (TNO, Zeist). The association between stomach cancer and intake of nitrite, adjusted for age and sex, was not clear and did not show a clear dose–response relationship. The only significant estimate was for the fifth quintile of intake (age- and sex-adjusted relative risk, 1.49; 95% CI, 1.01–2.20), which became non-significant (relative risk, 1.44; 95% CI, 0.95–2.18) after simultaneous adjustment for vitamin C, β-carotene, level of education, tobacco smoking and use of a refrigerator or freezer. [The decreased risk after adjustment for vitamin C and β-carotene could reflect the potential inhibitory effect on nitrosation of such nutrients. The possible interaction between nitrite intake and vitamin C was not assessed; no data on dietary intake of nitrosamines were provided in this study.]

The Mobile Health Clinic of the Social Insurance Institution in Finland (Knekt *et al*., 1999) collected data on food consumption by means of a diet history interview, and several food composition databases were used to calculate intake of nitrite. After a 24-year follow-up (1967–90), 68 cases of cancer of the stomach were identified. Dietary intake of nitrite was not associated with risk for stomach cancer after adjustment for age, sex, municipality, tobacco smoking and energy intake.

(c) Case–control studies

The findings of case–control studies on the risk for stomach cancer and oesophageal cancer in relation to ingested nitrites are summarized in Table 2.3. Most studies that addressed nitrite intake also considered nitrate and have thus been described in full in Section 2.2.1(*c*). Only the few studies that addressed exclusively intake of nitrite are described in detail here.

Europe

A large case–control study that included 1016 cases of stomach cancer and 1159 controls was carried out in Italy (Buiatti *et al*., 1990). Nitrite intake (mg per day) was derived from personal interview using a structured questionnaire of 146 items and the application of food composition tables from Italian and English sources. The association with risk for stomach cancer was adjusted for age, sex, social class, familial history of stomach cancer, body mass index and caloric intake. There was a significant increase in risk with increasing levels (quintiles) of nitrite intake (P for trend = 0.005). Furthermore, an interaction was observed between antioxidants (vitamin C and α-tocopherol) and protein and nitrites: cases and controls were classified into three categories (low, medium, high) according to these two groups of compounds. Using the low/low category as the reference, the odds ratio for the high antioxidants/low protein and nitrite category was 0.4, while that for the low antioxidant/high protein and nitrite category was 2.1 (a fivefold difference in relative risk for the two extreme categories). Palli *et al*. (2001) updated the results of Buiatti *et al*. (1990) for the cases from Florence by expanding the series of controls and applying an updated Italian food composition table that included values for dimethylamine and *N*-nitrosodimethylamine (NDMA). Similarly to the previous study, a significant positive association with nitrite was observed (P for trend = 0.04); a positive association was also observed with estimated intakes of dimethylamine and NDMA.

Another Italian case–control study carried out in the four largest teaching hospitals of the Greater Milan area (La Vecchia *et al*., 1994, 1997) assessed nitrite intake through a 29-item food-frequency questionnaire and an Italian food composition table. A significant trend for an increased risk for stomach cancer was observed with higher intake of nitrite (categorized in quartiles), although none of estimates was statistically significant (P for trend < 0.05); however, when two levels of nitrite intake were considered in relation to the median (≥ 2.7 versus < 2.7 mg per day), a significant increased risk was observed (odds ratio, 1.44; 95% CI, 1.2–1.7). In this study, methionine was associated with an increased risk for stomach cancer and the combined effect of both compounds was assessed; the odds ratio for the category with high intake of nitrite and methionine was 2.45 (95% CI, 1.9–3.2) compared with the category with lowest consumption for both compounds.

A hospital-based case–control study of stomach cancer in four provinces of Spain (González *et al*., 1994) used a diet history method and Spanish and British food composition tables to estimate intake of nutrients. In the matched analysis of 354 pairs, a slightly increased risk for high intake of nitrite was observed, although the trend was not significant; the effect of nitrite was only evident for tumours of the intestinal type, while

no effect or inverse risk estimates were observed for diffuse-type tumours of the stomach. [In this study, there was a significant association with NDMA (results not provided) and an interaction with vitamin C: subjects who had a high intake of nitrosamines and a low intake of vitamin C had an odds ratio of 1.98 (95% CI, 1.3–3.1) compared with those who had a low vitamin C and low nitrosamine intake.]

Hansson *et al*. (1994) reported the results of a case–control study in Sweden of 338 cases of stomach cancer and 679 controls. Nitrite intake was calculated from a food-frequency questionnaire and the Swedish food composition table. The age- and sex-adjusted relative risk for the highest quartile of nitrite intake compared with the lowest quartile was 1.22 (95% CI, 0.82–1.81).

A hospital-based case–control study carried out in the area of Marseilles, France (Pobel *et al*., 1995) used a diet history questionnaire combined with a French food composition table to estimate the intake of nitrites. No association was observed in relation to risk for stomach cancer overall or when different dietary sources of nitrite (from vegetables or other foods) were considered separately. [Potential limitations of this study are described in Section 2.2.1(*c*).]

North America

Risch *et al*. (1985) reported the results from a population-based case–control study in two provinces of Canada and the area of Toronto. Usual diet was assessed by the diet history method which was administered by an interviewer and nutrient intakes were calculated using a food composition table from the USA and other sources. The matched analysis with adjustment for ethnicity and total caloric intake showed a significant increase in risk for stomach cancer for every milligram of nitrite consumed per day (odds ratio, 1.71; 95% CI, 1.24–2.37). [No adjustment was made for β-carotene, although this nutrient was also found to be associated with a decreased risk for stomach cancer. The interaction between intake of nitrite and antioxidant micronutrients was not assessed.]

Two case–control studies from the USA addressed the association of nitrite intake with the risk for oesophageal cancer alone or with tumours at other sites. Rogers *et al*. (1995) conducted a population-based study in Washington State and used a 125-item food-frequency questionnaire and a food composition table from the National Academy of Sciences to estimate the usual intake of nitrite. A positive but non-significant association was observed. Although no statistically significant interactions were found, the data suggested that individuals with both low intake of vitamin C and high nitrite intake had a higher risk for oesophageal cancer (odds ratio, 5.07; $P < 0.05$). [In this study, the pattern of estimates for NDMA was very similar to that reported for nitrites yielding a positive trend (P for trend = 0.06), although estimates for tertiles of intake were not statistically significant. There was a suggestion of an interaction of NDMA with vitamin C: those with higher tertiles of NDMA and low vitamin C intake had a three-fold significantly increased risk of oesophageal cancer ($P < 0.05$).]

A more recent population-based case–control study in the USA (Mayne *et al*., 2001) considered stomach and oesophageal tumours according to their localization or

histological type. Overall, 1095 cases were identified through population cancer registries; 458 frequency-matched controls were selected by random-digit dialling. Personal interviews were obtained using a 104-item food-frequency questionnaire and dietary data were linked to published databases on nitrite content. Separate results were provided for 352 non-cardia stomach cancers, 225 tumours located in the cardia, 206 squamous-cell type tumours of the oesophagus and 282 oesophageal adenocarcinomas. Adjusted for several potential confounders including total energy intake (but no nutrients), high intake of nitrite was significantly associated with an increased risk for non-cardia stomach cancer only. However, compared with persons with low nitrite and high vitamin C intakes, persons with low vitamin C intake had a significantly increased risk for cancers of the stomach (cardia and non-cardia) and oesophagus (adenocarcinomas and squamous-cell carcinomas). Intake of nitrite was further examined in cases of cardia stomach cancer from this study using categorization by quartiles according to the consumption of controls (Engel *et al*., 2003). A significant increase in risk was found for all levels of intake compared with the lowest.

2.2.3 *Preneoplastic lesions*

Superficial gastritis, chronic atrophic gastritis, intestinal metaplasia and dysplasia have been postulated to be part of the sequential process that leads to stomach carcinoma, and are therefore considered to be precursor lesions. The roles of nitrate, nitrite and nitrosation have been investigated in relation to this process, mainly in some areas in Colombia that are at high-risk for stomach cancer. In the Nariño area, a cohort of subjects was recruited between 1973 and 1983, all of whom had a gastroscopy and biopsy at baseline. Cross-sectional analysis of the first biopsy was carried out for 1670 subjects (Correa *et al*., 1990a). Taking normal mucosa as the reference category, positive versus negative level of nitrite in gastric juice (above or below the median) gave the following age-adjusted odds ratios: hyperplasia, 4.3; atrophy, 6.5; metaplasia, 8.3; and dysplasia, 16.8 (overall $P < 0.0001$); the corresponding values for nitrate (> 24 versus ≤ 24 mg/L) were 1.4, 1.8, 2.1 and 2.7 (P for trend < 0.0001). However, stomach nitrite failed to predict future changes in the same end-points (Correa *et al*., 1990b). Within this cohort, a further analysis was carried out for 263 subjects, including 117 cases of stomach cancer and 146 controls [not specified] (Chen *et al*., 1990). Odds ratios for the presence of nitrite in gastric juice and the risk for precursor lesions compared with subjects with normal mucosa, adjusted for age, sex, pH, sodium/creatinine ratio and addition of salt, were: superficial gastritis, 2.99 (95% CI, 0.88–10.17); chronic atrophic gastritis, 3.49 (95% CI, 0.84–14.40); intestinal metaplasia, 4.39 (95% CI, 1.36–14.16); and dysplasia, 24.72 (95% CI, 1.97–309.64). In the same region, Cuello *et al*. (1979) characterized the histopathology of stomach biopsies from 123 patients in a village of the department of Nariño, in the Andean region of Colombia. Measurable levels of nitrite in gastric juice were detected in patients who had chronic atrophic gastritis but not in those who had superficial gastritis or normal mucosa; the level was significantly elevated (4.2 mg/L;

$P < 0.01$) only among those who had intestinal metaplasia with dysplasia. There were no differences in the level of nitrate.

During 1989–90, a gastroscopic screening survey was carried out in a rural area of the province of Shandong (China), which has one of the highest rates of stomach cancer (You *et al*., 1996). Endoscopic biopsies, fasting gastric juice and overnight urine were collected; data were available for 583 persons (312 men and 271 women). The level of nitrite in gastric juice was above the detection level for 7% of subjects with superficial/chronic atrophic gastritis versus 17% of those with intestinal metaplasia ($P < 0.01$). Furthermore, among those with detectable values, the geometric mean gastric content of nitrite was 0.8 ng/mL for subjects with intestinal metaplasia compared with 0.4 ng/mL for those with superficial/chronic atrophic gastritis ($P = 0.06$). This pattern was similar for subjects whose gastric juice had a pH > 2.4, but not for the subgroup that had a lower pH. On the contrary, the level of nitrate in urine was higher among subjects who had superficial/chronic atrophic gastritis (281 ng/mL) than among those who had intestinal metaplasia (242 ng/mL), but the differences were non-significant.

2.3 Tumours of the brain

Epidemiological studies of brain cancer are challenging because of problems with the specificity of diagnosis and the difficulties of patients to recall past exposures while experiencing the effects of cerebral lesions, surgery or treatment. Numerous epidemiological studies have investigated the potential role of nitrate and nitrite in relation to the occurrence of central nervous system (mainly brain) tumours in adults and children. These were mostly case–control studies that estimated nitrate and nitrite consumption from either residential tap-water or other dietary sources and are summarized in Table 2.4.

In the studies that considered dietary exposure, participants, their mothers (childhood brain tumour studies) or proxy respondents (for deceased or disabled patients in some studies of adult brain tumours) were requested to recall consumption of selected foods; this information was then linked to data on the content of nitrate and nitrite in food. In each of the studies reviewed below, approximately 50 foods (range, 42–100) were included; in many, the authors stated that foods with a high content of nitrate, nitrite or *N*-nitroso compounds were intentionally well represented.

The studies that considered exposure from tap-water used past measures of nitrate in public utilities and/or measured nitrate and/or nitrite in newly collected water samples from the current home or the residence at the time of diagnosis. With the exception of one study (Ward *et al*., 2005), water testing relied on semiquantitative test strips.

As outlined in Section 2.1, studies were not reviewed if they only considered less direct potential indicators of exposure to nitrate and/or nitrite. However, the potential association between the consumption of cured meat and the occurrence of brain tumours has been studied extensively. Many studies suggested an association between brain tumours in children and consumption of cured meat by the mother during pregnancy and/or

Table 2.4. Case–control studies of nitrate and nitrite in the drinking-water and from dietary sources and the risk for adult and childhood central nervous system tumours

Reference, study location, period	Tumour type	Characteristics of cases and controls	Exposure assessment	Exposure categories	No. of exposed cases	Odds ratio (95% CI)	Adjustment for potential confounders	Comments
Adult								
Australia								
Giles *et al.* (1994), Melbourne and other cities of Victoria, Australia, 1987–91	Glioma primary	409 cases (243 men, 166 women) from registry/ 14 hospitals, aged 20–70 years; 100% histologically confirmed; response rate, 66%; 409 controls (243 men, 166 women) randomly selected from electoral roll; matched by sex, age; history of stroke, epilepsy, prior brain tumour excluded; response rate, 45%	Mailed questionnaire (59 foods), followed by in-person interview, linked to published tables of food content; proxy for 54.3% of male cases, 3.3% of male controls, 50.6% of female cases and no female controls	**Nitrate in the diet** *Men* T1 T2 T3 *Women* T1 T2 T3 **Nitrite in the diet** *Men* T1 T2 T3 *Women* T1 T2 T3	 74 95 74 73 48 45 60 90 93 61 47 58	 1.0 1.42 (0.89–2.26) 1.13 (0.68–1.86) 1.0 0.48 (0.25–0.91) 0.53 (0.28–0.96) 1.0 1.58 (0.96–2.61) 1.58 (0.96–2.58) 1.0 0.77 (0.43–1.37) 0.98 (0.55–1.72)	Alcohol consumption, tobacco smoking	Period for which diet was ascertained was not specified; among men, most food groups and nutrients were above null, and no adjustment was made for total energy intake [in this particular study, the Working Group believed this adjustment should have been made because of misclassification, not because energy intake is a potential risk factor].

Table 2.4. (contd)

Reference, study location, period	Tumour type	Characteristics of cases and controls	Exposure assessment	Exposure categories	No. of exposed cases	Odds ratio (95% CI)	Adjustment for potential confounders	Comments
Europe								
Boeing *et al.* (1993); Steindorf *et al.* (1994), Rhein–Neckar–Odenwald area, Germany, 1987–88	Brain, primary	226 cases (99 men, 127 women; 115 gliomas, 81 meningiomas, 30 acoustic neuromas) from 2 neurosurgery clinics, aged 25–75 years; 100% histologically confirmed; response rate, 97.8%; 418 controls (185 men, 233 women) from compulsory regional residential register; frequency-matched by sex, age; response rate, 72%	Diet: in-person questionnaire (42 foods, previous 5 years) linked to published tables of food content; proxy for 12% of cases, 3% of controls Water (test): semi-quantitative test strip estimation of tap-water at current home; available for 60.1% of cases and 90.4% of controls who had lived in the region > 1 year	**Nitrate in the diet** *Glioma* T1 T2 T3 *P* for trend **Nitrate in water (test)** Not detected 10 mg/L 25 mg/L 50–100 mg/L	 29 34 30 60 12 28 4	 1.0 (reference) 1.0 (0.6–1.8) 0.9 (0.5–1.5) 0.64 1.0 (reference) [0.5 (0.2–0.9)] [0.7 (0.4–1.2)] [0.3 (0.1–0.7)]	Age, sex, alcohol consumption, tobacco smoking Crude	Diet: odds ratios include 53 (23.5%) cases and no controls who did not live in the study region for at least 1 year since 1970 [approximately previous 17 years]. Water test: case water samples were sent to hospital, whereas control water samples were tested in home immediately after sample collection

Table 2.4. (contd)

Reference, study location, period	Tumour type	Characteristics of cases and controls	Exposure assessment	Exposure categories	No. of exposed cases	Odds ratio (95% CI)	Adjustment for potential confounders	Comments
Boeing *et al.* (1993); Steindorf *et al.* (1994) (contd)			Water (records): residential history linked to measures in 1971–87 from 69 water providers, plus other data for 2% with private well/spring; attempted for subjects living in region >1 year since 1970, 76.6% of cases and 100% of controls; data available for 168 cases and 406 controls (97% of each attempted)	**Nitrate in water (records)** *(mean nitrate mg/L)* Q1 (0–2.0) Q2 (> 2.0–11.3) Q3 (> 11.3–25.2) Q4 (> 25.2) **Nitrite in the diet** *Glioma* T1 T2 T3 *P* for trend	 45 38 44 41 26 31 36	 1.0 (reference) 0.99 (0.60–1.63) 1.12 (0.69–1.83) 1.00 (0.61–1.64) 1.0 (reference) 1.1 (0.6–1.9) 1.1 (0.6–2.0) 0.62	Age, sex (diet, tobacco smoking, bottled water considered) Age, sex, alcohol consumption, tobacco smoking	Water records: mean nitrate levels were very similar for cases and controls during each year (1970–87); consideration of tumour type or latency period did not alter conclusions.
Israel								
Kaplan *et al.* (1997), Tel Hashomer/ central Israel, 1987–91	Brain tumour, primary malignant or benign	139 cases (mostly glioma and meningioma; 63 men, 76 women) from one medical centre, aged 18–75 years; 100% histologically confirmed; response rate, 77%; 278 controls (126 men, 152 women); matched by age, sex, ethnic origin (one friend, one orthopaedic patient per case); response rate not specified	Questionnaire (100 foods) for the period 10 years before diagnosis, linked to published tables of food content; proxy for 30% of each group	Nitrate or nitrite in the diet	NR	No association [no odds ratios reported]	Total energy intake, matching variables	Analyses repeated by tumour type, and for the two control groups separately in subanalyses

Table 2.4. (contd)

Reference, study location, period	Tumour type	Characteristics of cases and controls	Exposure assessment	Exposure categories	No. of exposed cases	Odds ratio (95% CI)	Adjustment for potential confounders	Comments
USA								
Blowers *et al.* (1997), Los Angeles County, CA, USA, 1986–88	Glioma	94 women from tumour registry (county resident), alive and well enough to provide an interview, aged 25–74 years; 100% histologically confirmed; response rate, 67%; 94 female county residents; matched on neighbourhood of residence (at case diagnosis), age, race (black/white); response rate, 72%	In-person questionnaire (43 foods, 90% estimated ascertainment of nitrate and nitrite); no proxies used; complete nutrient data available for 91 (97%) case–control pairs	**Nitrate in the diet**	NR		Total food intake, body mass index	Only surviving female cases were included and may lead to survivor bias; ethnicity, religion, education, vitamin intake, diabetes and allergies, asthma and eczema considered but not included in the final models.
				Q1		1.0		
				Q2		1.3 (0.5–3.3)		
				Q3		1.1 (0.4–2.7)		
				Q4		0.7 (0.2–1.8)		
				P for trend		0.46		
				Nitrite in the diet	NR			
				Q1		1.0		
				Q2		0.6 (0.2–1.3)		
				Q3		0.4 (0.1–1.0)		
				Q4		1.4 (0.6–3.5)		
				P for trend		0.55		
				Nitrite from cured meat	NR			
				Q1		1.0		
				Q2		0.9 (0.3–2.4)		
				Q3		1.3 (0.6–3.0)		
				Q4		2.1 (1.0–4.6)		
				P for trend		0.07		

Table 2.4. (contd)

Reference, study location, period	Tumour type	Characteristics of cases and controls	Exposure assessment	Exposure categories	No. of exposed cases	Odds ratio (95% CI)	Adjustment for potential confounders	Comments
Lee *et al.* (1997), San Francisco Bay area, CA, USA, 1991–94	Glioma	494 cases (282 men, 212 women) from one cancer centre rapid-ascertainment system, aged > 20 years; 100% histologically confirmed; response rate, 82%; 462 controls (254 men, 208 women) from random-digit dialling; frequency-matched by age, sex, race/ethnicity (white, black, Asian, Hispanic, other)	Mailed questionnaire (79 foods, usual consumption in previous year) followed by in-person interview, linked to database of nutrients; 46% proxies for cases; available for 434 (94%) cases and 439 (99%) controls	**Nitrite with vitamin C**			Age, education, income	Analyses repeated to exclude proxy respondents: difference in nitrate intake became non-significant for women; increased risk in men with high nitrite and low vitamin C intake was not attenuated and remained significant; nitrite and vitamin C each dichotomized at the median.
				Men				
				Low nitrite, high vit C	36	1.0 (reference)		
				Low nitrite, low vit C	75	1.3 (0.7–2.3)		
				High nitrite, high vit C	58	1.0 (0.6–1.8)		
				High nitrite, low vit C	71	2.1 (1.1–3.8)		
				Women				
				Low nitrite, high vit C	24	1.0 (reference)		
				Low nitrite, low vit C	52	1.2 (0.6–2.4)		
				High nitrite, high vit C	69	1.6 (0.9–3.0)		
				High nitrite, low vit C	36	1.5 (0.7–3.1)		
						Mean weekly consumption (standard deviation)		
				Nitrate in the diet				
				Men				
				Cases		726.3 (26.8)		
				Controls		775.8 (26.3)		
				P-value		NS		
				Women				
				Cases		52.1 (22.8)		
				Controls		660.9 (41.2)		
				P-value		≤ 0.05		
				Nitrite in the diet				
				Men				
				Cases		6.5 (0.3)		
				Controls		6.0 (0.2)		
				P-value		NS		
				Women				
				Cases		4.9 (0.2)		
				Controls		4.6 (0.2)		
				P-value		NS		

Table 2.4. (contd)

Reference, study location, period	Tumour type	Characteristics of cases and controls	Exposure assessment	Exposure categories	No. of exposed cases	Odds ratio (95% CI)	Adjustment for potential confounders	Comments
Schwartzbaum *et al.* (1999), Ohio, USA, 1993–96	Glioma	40 cases (19 men, 21 women) from 1 hospital network at time of first surgery for possible brain tumour; median age, 52.5 years; all white; 100% histologically confirmed; response rate, 95%; 48 controls (22 men, 26 women) from same hospital network (orthopaedic or gynecological surgery patients with no malignant neoplasm, diabetes, pregnancy, obesity, gastro intestinal disease, coronary heart disease or stroke); matched by age, sex; all white; response rate, 92%	Nitrite: diet questionnaires (prior year) translated to nutrients via software and published tables; no proxies Vitamins C and E: pre-surgery serum analysed; available for 23 (57.5%) cases	**Nitrite from cured meat (mg/day)**			Protein, fat, total calories, cured meat, body mass index	Nitrite intake from cured meats: median, 0.6 mg/day in cases; 0.3 mg/day in controls; exclusion criteria for controls probably resulted in a group with a different diet than population controls; odds ratios for nitrite intake above the median from individual types of cured meats at null or below. *low = < median values, high = ≥ median values
				Q1 (< 0.16)	9	1.0 (reference)		
				Q2 (0.16–0.39)	7	2.0 (NR)		
				Q3 (0.40–0.91)	14	2.2 (NR)		
				Q4 (≥ 0.92)	10	2.1 (NR)		
				P for trend		0.36		
				Nitrite-serum vitamin C				
				Low nitrite* low vit C	8	1.0 (reference)		
				Low nitrite high vit C	1	0.1 (0.0–1.2)		
				High nitrite, high vit C	2	0.7 (0.1–9.4)		
				High nitrite, low vit C	12	1.6 (0.3–8.0)		

Table 2.4. (contd)

Reference, study location, period	Tumour type	Characteristics of cases and controls	Exposure assessment	Exposure categories	No. of exposed cases	Odds ratio (95% CI)	Adjustment for potential confounders	Comments
Chen *et al.* (2002); Ward *et al.* (2005), eastern Nebraska, USA, 1988–93	Glioma, primary	251 cases (139 men, 112 women) from cancer registry/11 hospitals in region, aged ≥ 21 years; all white; 100% histologically confirmed; response rate, 89% [among eligible]; 498 controls (283 men, 215 women) from a randomly selected control group (same base population) from a study of non-Hodgkin lymphoma (random-digit dialling, supplemented by Medicare records for age >65 years); frequency-matched on age, sex, vital status; all white; response rate, 71%	Diet: telephone questionnaire (48 food items), linked to published tables; available for 236 (94%) cases and 449 (90%) controls; proxies for 76% cases, 60% controls Public water: residence histories linked to nitrate water-quality monitoring data from 236 public water systems, 1947–85; available for 130 (52%) cases and 319 (64%) controls; ≥70% person–years after 1964 (when water data more complete); lag of 3–8 years between the exposure period and case diagnosis.	**Nitrate in the diet**			Energy, age, sex, farming, education, respondent, family history of central nervous system tumour	Subanalyses separate by respondent type, histological type (no meaningful differences); low statistical power to evaluate risk at public water system nitrate levels above 5–10 mg/L nitrate-N; only about 15% of cases and controls believed to be exposed to 10 mg/L nitrate-N in public water supplies >8 years. No interaction observed between vitamin C intake and dietary nitrite.
				Q1	59	1.0 (reference)		
				Q2	81	1.2 (0.7–1.9)		
				Q3	43	0.7 (0.4–1.2)		
				Q4	53	0.7 (0.4–1.2)		
				P for trend		0.1		
				Nitrate in water, public system, 1965–84 (Mean mg/L nitrate-N)			Age, sex, respondent, education, farming	
				Q1 (< 2.38)	24	1.0 (reference)		
				Q2 (2.38–2.57)	39	1.4 (0.7–2.7)		
				Q3 (2.58–4.32)	33	1.2 (0.6–2.3)		
				Q4 (> 4.32)	34	1.3 (0.7–2.6)		
				Nitrate with vitamin C				
				Q1/high vit C	8	1.0 (reference)	Birth year, sex, respondent, education, farming	
				Q2/high vit C	22	2.4 (0.8–6.9)		
				Q3/high vit C	13	2.0 (0.7–6.0)		
				Q4/high vit C	13	2.1 (0.7–6.4)		
				Q1/low vit C	15	2.3 (0.8–6.9)		
				Q2/low vit C	16	1.6 (0.5–4.8)		
				Q3/low vit C	17	1.7 (0.6–4.7)		
				Q4/low vit C	17	2.0 (0.7–5.8)		
				Years of nitrate ≥ 5 mg/L nitrate-N				
				0	22	1.0 (reference)	Birth year, sex, respondent, education, farming	
				1–4	62	1.3 (0.7–2.5)		
				5–9	20	1.8 (0.8–4.1)		
				≥ 10	26	1.1 (0.5–2.2)		

Table 2.4. (contd)

Reference, study location, period	Tumour type	Characteristics of cases and controls	Exposure assessment	Exposure categories	No. of exposed cases	Odds ratio (95% CI)	Adjustment for potential confounders	Comments
Chen *et al.* (2002); Ward *et al.* (2005) (contd)			Private water: tap-water samples from well used in 1985, laboratory analysed; available for 47 cases and 43 controls (75% and 60%, respectively, of those who had used a private well in 1985)	**Years of nitrate ≥ 10 mg/L nitrate-N**			Energy, age, sex, farming, education, respondent, family history of central nervous system tumour	
				0	85	1.0 (reference)		
				1–8	25	1.1 (0.6–2.1)		
				≥ 9	20	1.1 (0.6–2.0)		
				Nitrate in water, private well				
				< 10 mg/L nitrate-N	40	1.0 (reference)		
				≥ 10 mg/L nitrate-N	7	1.2 (0.4–4.1)		
				Nitrite in the diet			Birth year, sex, respondent, education, farming, β-carotene, fibre, calories	Chen *et al.* (2002)
				Q1	66	1.0 (reference)		
				Q2	66	1.0 (0.6–1.7)		
				Q3	57	0.9 (0.5–1.5)		
				Q4	47	0.8 (0.5–1.3)		
				P for trend		0.3		
				Animal sources (mg/day)			Birth year, sex, respondent, education, farming, β-carotene, fibre, calories	Ward *et al.* (2005)
				Q1 (< 0.29)	29	1.0 (reference)		
				Q2 (0.29–< 0.46)	25	0.8 (0.4–1.7)		
				Q3 (0.46–0.63)	31	1.3 (0.6–2.7)		
				Q4 (≥ 0.63)	36	1.3 (0.6–2.9)		
				Plant sources (mg/day)				
				Q1 (< 0.31)	34	1.0 (reference)		
				Q2 (0.31–< 0.43)	29	1.0 (0.5–2.1)		
				Q3 (0.43–0.59)	28	3.2 (1.2–8.3)		
				Q4 (≥ 0.59)	30	2.8 (1.0–8.2)		
				Preformed dietary intake + endogenous nitrite from water nitrate (mg/day)				
				< 1.4	21	1.0 (reference)		
				1.4–< 2.1	43	1.8 (0.9–3.6)		
				2.1–< 3.3	28	1.1 (0.5–2.3)		
				≥ 3.3	28	1.1 (0.5–2.5)		

Table 2.4. (contd)

Reference, study location, period	Tumour type	Characteristics of cases and controls	Exposure assessment	Exposure categories	No. of exposed cases	Odds ratio (95% CI)	Adjustment for potential confounders	Comments
Childhood								
Europe								
Cordier *et al.* (1994), Paris/Ile de France region, France, 1985–87	Intra-cranial tumour	75 cases (34 boys, 41 girls) from 13 hospitals in the region, aged 0–15 years, residents of Ile de France; 84% histologically confirmed; response rate, 69%; 113 controls (63 boys, 50 girls) from sample of residences in region, supplemented by telephone books; frequency-matched by birth year; response rate, 72%	Questionnaire (maternal diet during pregnancy) linked to French surveys and published tables of food content	**Nitrate in the diet** Q1 Q2 Q3 Q4 *P* for trend **Nitrite in the diet** Q1 Q2 Q3 Q4 *P* for trend	 NR NR NR NR NR NR 	 1.0 (reference) 1.5 (0.5–4.5) 0.5 (0.1–1.7) 1.5 (0.5–4.6) NS 1.0 (reference) 0.9 (0.3–2.7) 0.6 (0.2–2.3) 0.4 (0.1–0.4) NS	Age, sex, maternal age, maternal education	*N*-Nitroso compounds not considered; vitamin C non-significantly protective

Table 2.4. (contd)

Reference, study location, period	Tumour type	Characteristics of cases and controls	Exposure assessment	Exposure categories	No. of exposed cases	Odds ratio (95% CI)	Adjustment for potential confounders	Comments
Israel								
Lubin *et al.* (2000), Israel; 1984–93	Central nervous system tumour, primary	300 cases (149 boys, 151 girls) from all neurosurgical departments in Israel drawn from the Jewish population only, aged 0–18 years; histological confirmation not specified; response rate, 81.7%; 574 controls (293 boys, 281 girls) from population registry; matched by sex, birth year, birth hospital (Israel) or country (outside Israel); response rate, 78.2%	Questionnaire (100 foods) during pregnancy and childhood linked to published tables of food content	**Nitrate in the diet** *Pregnancy* T1 T2 T3 *P* for trend *Childhood* T1 T2 T3 *P* for trend **Nitrite in the diet** *Pregnancy* T1 T2 T3 *P* for trend *Childhood* T1 T2 T3 *P* for trend	 NR NR NR NR NR NR NR NR NR NR NR NR 	 1.0 0.86 [NR] 1.15 (0.8–1.7) 0.43 1.0 0.84 [NR] 1.17 (0.8–1.7) 0.46 1.0 1.04 [NR] 0.97 (0.6–1.7) 0.91 1.0 0.86 [NR] 0.86 (0.5–1.6) 0.64	Total energy intake, matching variables	Not specified whether controls were drawn from the Jewish population only as were cases.

Table 2.4. (contd)

Reference, study location, period	Tumour type	Characteristics of cases and controls	Exposure assessment	Exposure categories	No. of exposed cases	Odds ratio (95% CI)	Adjustment for potential confounders	Comments
North America								
Bunin *et al.* (1993, 1994), Canada/USA, 1986–89	Brain tumours, PNET (1993), astrocytic glioma (1994)	321 cases with no previous malignancy from 33 paediatric oncology groups, aged <6 years; 166 (100 boys, 66 girls) PNET; 89% histologically confirmed; response rate, [77%]; 155 (81 boys, 74 girls) astrocytic glioma; 78% histologically confirmed; response rate, [74%]; 321 controls from random-digit dialing; matched on telephone area code + next 5 digits, birthdate (1 year), race (black/other); 166 PNET controls (90 boys, 76 girls); response rate, [73%]; 155 astrocytic glioma controls (85 boys, 70 girls); response rate, [74%]	Structured telephone interview (53 foods during pregnancy, 60–75% estimated ascertainment of nitrate and nitrite), linked to databases of portion size and food content	**Nitrate in the diet** *PNET* Q1 Q2 Q3 Q4 *P* for trend *Astrocytic glioma* Q1 Q2 Q3 Q4 *P* for trend **Nitrite in the diet** *PNET* Q1 Q2 Q3 Q4 *P* for trend *Astrocytic glioma* Q1 Q2 Q3 Q4 *P* for trend	 NR NR NR NR 40 32 38 34 NR NR NR NR 40 31 31 42 	 1.0 1.18 (0.64–2.19) 0.61 (0.34–1.09) 0.44 (0.23–0.84) 0.002 1.0 0.7 (0.3–1.4) 0.9 (0.4–1.8) 0.7 (0.3–1.4) 0.43 1.0 1.58 (0.79–3.16) 1.04 (0.57–1.89) 1.11 (0.58–2.10) 0.80 1.0 0.8 (0.4–1.6) 0.8 (0.4–1.5) 1.3 (0.7–2.6) 0.54	None (child diet, family income, maternal tobacco smoking considered) Family income None (child diet, family income, maternal tobacco smoking considered) Family income	Inverse PNET–nitrate association attenuated and became non-significant (p for trend = 0.19) after adjustment for other food components (vitamins A, C and E, folate, nitrite and nitrosamine) and vitamin/mineral supplements [only crude risk estimates provided in full].

Table 2.4. (contd)

Reference, study location, period	Tumour type	Characteristics of cases and controls	Exposure assessment	Exposure categories	No. of exposed cases	Odds ratio (95% CI)	Adjustment for potential confounders	Comments
Preston-Martin *et al.* (1996); Pogoda & Preston-Martin (2001a,b), California (Los Angeles and San Francisco areas) and Washington (Seattle area) states, USA, 1984–91	Brain, cranial nerves, cranial meninges, primary	540 cases (298 boys, 242 girls) from 3 tumour registries, aged < 20 years, living in the study region at diagnosis in a home with a telephone; % histological confirmation not specified; response rate, [71%]; 801 controls (448 boys, 353 girls) from residents of the same counties and identified by random-digit dialling; frequency-matched by age, sex; response rate, [74%]	In-person questionnaire (47 foods, maternal intake during pregnancy), linked to published tables/software (1996 analyses) or year-specific nitrite levels from 26 food surveys of sodium nitrite levels (2001 analyses)	**Nitrite in the diet**			Age, sex, birth year, geographic area	No interaction with socioeconomic status (*p* for trend statistically significant for low and high socioeconomic groups); race and socioeconomic status considered but not included in final models.
				Q1	NR	1.0 (reference)		
				Q4	NR	1.1 (0.79–1.50)		
				[Other quartiles not reported]				
				Nitrite in vegetables only				
				Q1	NR	1.0 (reference)		
				Q4	NR	0.98 (0.71–1.3)		
				[Other quartiles not reported]				
				Nitrite in cured meat only (mg/day)				
				Q1 (<0.02)	122	1.0 (reference)		
				Q2 (0.02–0.29)	121	1.1 (0.78–1.5)		
				Q3 (0.30–1.28)	116	1.1 (0.78–1.5)		
				Q4 (> 1.28)	155	1.9 (1.3–2.6)		
				P for trend		0.003		
				Nitrite with prenatal vitamins supplements				
				Low nitrite/vit +	195	1.0 (reference)		Low nitrite = < 0.3 mg/day and high nitrite = ≥ 0.3 mg/day from cured meats
				High nitrite/vit +	219	1.3 (1.0–1.7)		
				P for trend		0.002		
				Low nitrite/vit –	46	1.5 (0.93–2.3)		
				High nitrite/vit –	47	2.2 (1.4–3.6)		
				P for trend		0.007		

Table 2.4. (contd)

Reference, study location, period	Tumour type	Characteristics of cases and controls	Exposure assessment	Exposure categories	No. of exposed cases	Odds ratio (95% CI)	Adjustment for potential confounders	Comments
Preston-Martin *et al.* (1996); Pogoda & Preston-Martin (2001a,b) (contd)				**Nitrite in cured meat only (mg/day)***				Re-analysis of Preston-Martin *et al.* (1986)
				None	102	1.0 (reference)		*Time-specific nitrite estimates from literature review
				0.01–0.49	293	1.1 (0.8–1.5)		
				0.50–0.99	68	1.9 (1.2–2.9)		
				1.00–1.99	28	1.3 (0.8–2.3)		
				2.00–2.99	12	1.8 (0.8–4.1)		
				≥ 3.00	11	3.0 (1.2–7.9)		
				Per mg/day		β = 0.22 (standard error, 0.08)		Interaction with vitamin use or intake not addressed
				P-value		0.008		

Table 2.4. (contd)

Reference, study location, period	Tumour type	Characteristics of cases and controls	Exposure assessment	Exposure categories	No. of exposed cases	Odds ratio (95% CI)	Adjustment for potential confounders	Comments
International								
Mueller *et al*. (2004), regions near Winnipeg, Canada; Paris, France; Valencia, Spain; and Los Angeles, San Francisco and Seattle, USA; and countries not collecting water (Australia, Israel, Italy), also included in full study; 1976–94	Brain, cranial nerves, cranial meninges, primary malignant	1218 cases in full study from cancer incidence registries in each area, aged < 20 years; > 90.2% histologically confirmed; response rate in each country, 71–88%; 2223 controls in full study from electoral roll, telephone directory, census, population register, health service rosters, or random-digit dialling; response rate in each country, 60–87%	Semi-quantitative test strip estimation of tap-water (collected in 1990s in Canada, France, Spain, USA) from the residence at the time of interview; available for 283 cases and 537 controls (28% of all subjects); including 185 cases and 341 controls, representing 86% subjects from the participating countries who still lived in pregnancy home; including 131 cases and 241 controls with no bottled water use in the home during pregnancy; test strip: minimum 10 mg/L nitrate, 1 mg/L nitrite	**Pregnancy home** *Nitrate in water (mg/L)* All None detected 10–< 25 25–< 50 ≥ 50 No bottled water None detected 10–< 25 25–< 50 ≥ 50 Astroglial tumours None detected 10–< 25 25–< 50 ≥ 50 PNET None detected ≥ 10 Other tumours None detected 10–< 25 25–< 50 ≥ 50	 128 31 12 14 NR NR NR NR NR NR NR NR NR NR NR NR	 1.0 (reference) 0.5 (0.3–0.9) 0.5 (0.2–1.1) 1.0 (0.4–2.2) 1.0 (reference) 0.9 (0.5–1.8) 0.7 (0.2–2.1) 1.5 (0.6–3.8) 1.0 (reference) 0.7 (0.3–1.3) 0.5 (0.1–1.5) 1.9 (0.7–5.7) 1.0 (reference) 0.2 (0.1–0.8) 1.0 (reference) 0.4 (0.2–0.99) 0.6 (0.2–1.6) 0.7 (0.2–2.1)	Study centre, age, sex, year of diagnosis	Among control pregnancy homes, nitrate and nitrite were very prevalent considering that most were on public water supplies at the time of conception: 39.6% any nitrate; 8.2% nitrate ≥ 50 mg/L; 10.0% any nitrite; 2.4% nitrite ≥ 5 mg/L. Results for pregnancy home only presented because these subjects were targeted (others who provided water samples may not be representative; among controls, participation of those not targeted varied according to the presence of nitrite).

Table 2.4. (contd)

Reference, study location, period	Tumour type	Characteristics of cases and controls	Exposure assessment	Exposure categories	No. of exposed cases	Odds ratio (95% CI)	Adjustment for potential confounders	Comments
Mueller *et al*. (2004) (contd)				**Nitrite in water (mg/L)**				Results for nitrate not alterered materially when individuals with nitrite detected excluded (nitrite interferes with nitrate measurement using these test strips)
				None detected	160	1.0 (reference)		
				1–< 5	19	1.7 (0.8–3.7)		
				≥ 5	6	2.1 (0.6–7.4)		
				No bottled water				
				None detected	NR	1.0 (reference)		
				1–< 5	NR	2.1 (0.8–6.0)		
				≥ 5	NR	5.2 (1.2–23.3)		
				Astroglial tumours			Center, age, sex, year of diagnosis	
				None detected	NR	1.0		
				1–< 5	NR	4.3 (1.4–12.6)		
				≥ 5	NR	5.7 (1.2–27.2)		
				PNET				
				None detected	NR	1.0		
				1–< 5	NR	0.9 (0.1–11.7)		
				≥ 5	NR	1.3 (0.1–30.9)		
				Other tumours				
				None detected	NR	1.0		
				1–< 5	NR	1.2 (0.3–4.2)		
				≥ 5	NR	0.7 (0.1–7.7)		

CI, confidence interval; NR, not reported; NS, not significant; PNET, primitive neuroectodermal tumours; Q, quartile; T, tertile; vit C, vitamin C; vit-, without vitamin supplement; vit+, with vitamin supplement

by the child (astrocytoma and astrocytic glioma: Preston-Martin *et al*., 1982; Kuijten *et al*., 1990; Bunin *et al*., 1994; primitive neuroectodermal tumours: Bunin *et al*., 1993; brain tumours: McCredie *et al*., 1994a,b; Sarasua & Savitz, 1994; Preston-Martin *et al*., 1996; Schymura *et al*., 1996; Pogoda & Preston-Martin, 2001a,b) but a few other studies did not confirm this (Howe *et al*., 1989; Cordier *et al*., 1994; Lubin *et al*., 2000; medulloblastoma: Bunin *et al*., 2005). Studies in adults have yielded mixed results. Some suggested an increased risk for brain tumours (meningiomas in women: Preston-Martin *et al*., 1982; astrocytoma versus population-based controls: Ahlbom *et al*., 1986; cohort study of glioma: Mills *et al*., 1989; meningioma in men: Preston-Martin *et al*., 1989; Preston-Martin & Mack, 1991; glioma and meningioma: Boeing *et al*., 1993; glioma: Giles *et al*., 1994; gliomas in women: Blowers *et al*., 1997; glioma: Lee *et al*., 1997; Schwartzbaum *et al*., 1999) whereas others did not (meningiomas in men: Preston-Martin *et al*., 1983; astrocytoma versus clinical controls: Ahlbom *et al*., 1986; hospital controls: Burch *et al*., 1987; cohort study of meningioma: Mills *et al*., 1989; glioma in men: Preston-Martin *et al*., 1989; Preston-Martin & Mack, 1991; glioblastoma: Hochberg *et al*., 1990; brain tumours: Kaplan *et al*., 1997; glioma: Chen *et al*., 2002). Meta-analyses of some of the studies cited above suggested an association between consumption of cured meat and the occurrence of brain tumours in children (Huncharek & Kupelnick, 2004) and adults (Huncharek *et al*., 2003), but Murphy *et al*. (1998) observed that trends in the incidence of brain tumour and consumption of cured meat in the two age groups do not support such an association [all three analyses, especially the latter, had limitations that limit inference]. Some studies observed a possible interaction between consumption of cured meat and intake of vitamins (e.g. vitamin C), fruit or vegetables (i.e. the greatest risk is for those who have high consumption of cured meat and a low intake of antioxidants) (Bunin, 1994; Preston-Martin *et al*., 1996; Blowers *et al*., 1997).

2.3.1 *Nitrate*

(a) Ecological studies

Barrett *et al*. (1998) conducted a study (described in detail in Section 2.2) in the Yorkshire region of England, where only 31 (0.3%) of all water samples in public supplies contained > 50 mg nitrate/L. The residence at diagnosis of 3441 (of 3812) cases of brain cancer was linked to a water supply zone ($n = 148$) and its corresponding measurements of nitrate. Relative to the quartile of the population that had the lowest mean concentration of nitrate in water, individuals in all other quartiles (higher levels of nitrate) had a very modest but statistically significant increase in risk for brain cancer (relative risk, 1.14; 95% CI, 1.04–1.26 for the second quartile; 1.13; 95% CI, 1.03–1.25 for the third quartile; and 1.18; 95% CI, 1.08–1.30 for the fourth quartile, adjusted for socioeconomic status and population density).

Van Leeuwen *et al*. (1999) conducted an ecological study (described in detail in Section 2.2) of 40 'ecodistricts' in Ontario, Canada, where levels of nitrate in water ranged from 0 to 91 mg/L nitrate-N. A model that included levels of atrazine as an a-

priori variable and all other potentially confounding factors that were selected according to *p*-value was used to estimate incidence ratios for brain cancer [it was not specified which confounders were retained nor whether any non-significant factors that affected the coefficient for nitrate were appropriately included.] The resulting incidence ratios were non-significantly increased in both men ($P = 0.072$) and women ($P = 0.101$) in relation to increasing levels of nitrate [coefficients not presented].

(b) Case–control studies in adults

Giles *et al*. (1994) conducted a case–control study of primary glioma and diet among adult residents in five major cities in Victoria, Australia, including Melbourne. Cases, aged 20–70 years, were identified at 14 hospitals that had neurological services and were checked against the cancer registry of the region. Controls with no history of stroke, epilepsy or brain tumour were randomly selected from the electoral roll of Victoria that covers 97% of the population. One control was matched to each case by sex and age and subjects with no dietary data were excluded, which resulted in 409 case–control pairs. Odds ratios (tertiles of intake), stratified by gender, were adjusted for alcohol consumption and tobacco smoking. In men, odds ratios for nitrate were modestly and non-significantly above null, while in women, nitrate was associated with a significantly decreased risk for glioma. No dose–response effects were observed in either sex. [Among men, risk estimates for most foods and nutrients, including vitamin C, were above null, which suggests that cases and/or their proxies possibly reported greater food intake in general than controls, although odds ratios were not adjusted for total energy intake. In this particular study, the Working Group believed that this adjustment should have been made because of misclassification, not because energy intake is a potential risk factor.]

Boeing *et al*. (1993) conducted a case–control study of primary incident brain tumours in adult residents of the Rhein–Neckar–Odenwald area of Germany. Cases aged 25–75 years at diagnosis were identified from two neurosurgery clinics that treated 95% of brain tumour patients in the region (115 gliomas, 80 meningiomas, 30 acoustic neuromas); 418 controls were identified by the residential register for the region and were frequency-matched by sex and age. Results were presented for glioma only; odds ratios for dietary intake of nitrate in tertiles were close to null after adjusting for age, sex, alcohol consumption and tobacco smoking. [Steindorf *et al*. (1994) indicated that all controls but only 76.6% of cases had lived in the study region for at least 1 year since 1970 (in the approximately 17 years preceding diagnosis).]

In the same study, Boeing *et al*. (1993) used semiquantitative nitrate test strips (10, 25, 50, 100, 250 and 500 mg/L nitrate) to estimate the levels of nitrate in drinking-water samples from the current residence for a subset of subjects: cases who sent a water sample to the hospital and controls for whom the interviewer conducted the testing during the in-home interview. Proportionally fewer cases than controls provided a water sample (an estimated 60% of cases and 90% of controls, based on Steindorf *et al*. (1994)). [The water-related results are difficult to interpret because only 76.6% of cases but all controls (Steindorf *et al*., 1994) had lived in the study region for at least 1 year since 1970 (in the

approximately 17 years preceding diagnosis); markedly fewer cases than controls provided water samples; a delay in water testing that applied only to cases raises the possibility that the estimates for cases may be systematically biased in relation to those of controls. It is unkown whether the 'current' residence was the residence at diagnosis.]

Steindorf *et al*. (1994), using data from the study by Boeing *et al*. (1993), focused on the 173 cases and 418 controls who had lived in the study region for at least 1 year since 1970. Historical information on residence was linked to data from water-quality monitoring that gave mean nitrate concentrations for each year in 1971–87 obtained from 69 public authorities and water-treatment plants to create indicators of exposure to nitrate in water for 168 (97.1%) cases and 406 (97.1%) controls. (Only 2% of subjects used private wells/springs and supplementary data were attempted for these individuals.) Nitrate values ranged from 0 to 97.8 mg/L and were very similar for cases (15.98 mg/L; standard deviation [SD], 15.61) and controls (16.16 mg/L; SD, 15.72), even when stratified by year of exposure. Odds ratios for estimated exposure to nitrate in quartiles yielded risk estimates for brain tumours at or very close to null, adjusted for age and sex (diet, use of bottled water and tobacco smoking were also considered). The authors indicated that the results were unchanged in subanalyses that considered latency and tumour type. The results of nitrate test strips obtained in Boeing *et al*. (1993) were presented for the subset of subjects (residents of the region) only; nitrate was associated with a decreased risk for brain tumours. [For the test strip results, most of the limitations noted for Boeing *et al*. (1993) apply. These do not apply to the results of water record linkage which suggested that these estimates are valid; when stratified by case status, there was good correlation ($r = 0.62$ in cases and $r = 0.59$ in controls) between the results of water record linkage and another non-gold-standard measure, the test strips. Although this also suggests that the test strips categorized subjects accurately according to nitrate levels within their respective case group (good precision), these results did not provide any information about possible non-differential or differential bias of the test strip measures.]

Kaplan *et al*. (1997) conducted a case–control study of primary adult brain tumours in central Israel. One hundred and thirty-nine cases, aged 18–75 years, were identified from one medical centre that served the region. Two controls (one friend and one orthopaedic patient from the region) of the same sex, age (within 5 years) and ethnic origin were located for each case. Analyses used nutrient densities (nutrient divided by total daily calories) or adjusted for total energy consumption, and were repeated by tumour type (glioma or meningioma) using only one control group at a time. The authors stated that total dietary intake of nitrate was not associated with the occurrence of brain tumours [the number of exposed cases and controls and the risk estimates were not provided].

Blowers *et al*. (1997) conducted a case–control study of gliomas in women and examined their diet. Cases, aged 25–74 years, were identified from a population-based tumour registry in Los Angeles County, CA, USA, and were restricted to 94 glioma patients who were alive and sufficiently well to undergo an interview. For each case, one female control who lived in the same neighbourhood as the case at the time of diagnosis

was selected and matched by age and race (black/white). There was no association between case status and dietary intake of nitrate in quartiles, adjusted for total food intake and body mass index (several other potential confounding factors were also considered). [Exclusion of deceased and very ill cases of glioma somewhat limits the interpretation of the results because surviving cases may not represent incident cases; therefore, selection bias may be possible and results cannot be generalized (such as for stage of tumour or treatment).]

Lee *et al*. (1997) conducted a case–control study of glioma in adults in the San Francisco Bay Area of California, USA. Four hundred and thirty-four cases were identified from a rapid case-ascertainment system at one cancer centre; 439 controls were selected using random-digit dialling and were frequency-matched by age, sex and race/ethnicity (white, black, Hispanic, Asian, other). All comparisons were stratified by sex and adjusted for age, education and family income. Mean weekly intake of nitrate was lower for cases than for controls in both men (non-significant) and women (significant). There was a statistically significant increased risk for glioma in men with high nitrate intake and low vitamin C intake (odds ratio, 2.1; 95% CI, 1.1–3.8; 71 exposed cases).

Chen *et al*. (2002) conducted a case–control study of diet and glioma in adults in eastern Nebraska, USA. Two hundred and fifty-one cases, aged ≥ 21 years, were identified through a cancer registry and 11 hospitals. Controls were mainly selected from among controls of a case–control study of non-Hodgkin lymphoma (that used random-digit dialling and Medicaid records in the same population) and were supplemented by others who were identified by random-digit dialling and death certificates; a total of 498 controls were included and were frequency-matched to cases by age, sex and vital status. Dietary nitrate calculated as nutrient intake in residual quartiles was adjusted for age, sex, respondent type, education, family history of central nervous system tumour and farming and was not significantly associated with the occurrence of glioma.

Ward *et al*. (2005) extended the study by Chen *et al*. (2002) of adult glioma and dietary nitrate by expanding intake of nitrate to drinking-water. This was largely assessed by linking residential histories to data from water quality monitoring for 236 public water sources from 1947 to 1984 (although most analyses presented included only records after 1964, when monitoring became more frequent); the study was restricted to the 130 (52%) cases and 319 (64%) controls who had > 70% person–years of use of public water after 1964 (and up to 1984, which resulted in a 3–8-year lag period before diagnosis). Mean levels assessed by this method ranged from 0 to 12 mg/L nitrate-N. Odds ratios for each quartile above the first quartile were slightly above null, but were non-significant and showed no dose–response pattern. Relative to low levels of nitrate in water and high vitamin C intake, all strata of exposure were above null but were non-significant. The number of years of use of a public water system that contained ≥ 10 mg/L nitrate-N was not associated with risk for glioma nor when 5 mg/L nitrate-N was used as the cut-off point, although the second to first quartile was non-significantly increased. In addition to these analyses, at the time of the interview, 75% of the cases and 60% of 72 controls who had used a private well in 1985 (47 cases, 43 controls) provided a tap-water sample that

was analysed for nitrate. Levels of nitrate ranged from < 0.5 to 67 mg/L nitrate-N. There was no association between this measure (< 10 mg/L nitrate-N versus ≥ 10 mg/L nitrate-N) and case status. The majority of participants relied largely on public water supplies, and only about 15% of cases and controls were believed to have been exposed to 10 mg/L nitrate-N from public water sources for more than 8 years.

(c) Case–control studies in children

Cordier *et al*. (1994) conducted a case–control study of brain tumours in children < 15 years of age in the Paris/Ile de France region of France. Seventy-five cases were identified from 13 hospitals and 113 controls were selected from children who lived in the region through the census agency and telephone books. Relative to the first quartile of nitrate intake during pregnancy, the risk for brain tumours in the offspring in the higher quartiles (adjusted for sex, the age of both the mother and child and maternal education in years) ranged from 0.5 to 1.5, with no dose–response pattern. [Cases and controls differed in several demographic characteristics, including the length of time that mothers had to recall diet during pregnancy, but adjustment was made for several factors as noted above. Unexpectedly, a greater proportion of cases were female than male, but case ascertainment appeared to be complete.]

Lubin *et al*. (2000) conducted a case–control study of children (aged 0–18 years) with brain tumours in relation to diet of both the children and the mothers (during the index pregnancy) in Israel. Three hundred cases, drawn from the Jewish population only, were identified from all neurosurgical departments in Israel and 574 controls were selected from the population registry and were individually matched to cases by sex, birth year and either hospital of birth (in Israel) or country of birth (outside Israel). Based on tertiles of intake, there was no association between the occurrence of brain tumours and intake of nitrate; odds ratios for the upper tertiles were close to null and non-significant relative to the first tertile of intake for both the mother during pregnancy and for the child. [It was not specified whether controls were also required to be Jewish, although intake of nitrate, such as that from pork products, may differ between Jewish and non-Jewish individuals. However, cases and controls were very similar with regard to the ethnic background of the mother as categorized as Asian/African, European/American and Israeli.]

A case–control study of selected types of brain tumour, specifically primitive neuroectodermal tumours (Bunin *et al*., 1993) and astrocytic gliomas (Bunin *et al*., 1994), in children aged < 6 years was conducted on cases identified from 33 paediatric oncology groups in the USA and Canada. One control per case (166 controls matched to primitive neuroectodermal tumours, 155 controls matched to astrocytic gliomas) was identified by random-digit dialling and matched to the case on telephone area code plus the first five digits, date of birth (within 1 year) and race (black/non-black). Increased intake of nitrate was associated in a dose–response fashion with a statistically significantly (P for trend = 0.002) reduced risk for primitive neuroectodermal tumours in the offspring (Table 2.4). However, the inverse association between primitive neuroectodermal tumours and nitrate was attenuated and became non-significant (P for trend = 0.19) after

adjustment for other food components (vitamins A, C and E, folate, nitrite and nitrosamines) and vitamin/mineral supplements. More than 20 other factors relating to the child, the mother and the child's diet were considered, including family income, duration of nausea during pregnancy that interfered with normal eating, duration of breast-feeding and maternal smoking; of these, it appeared that the child's diet of cured meat further attenuated the association reported (fourth versus first quartile of nitrate intake during pregnancy attenuated to 0.81). Dietary nitrate was not associated with risk for astrocyctic glioma in the offspring, and all odds ratios were at or below null after adjustment for family income (and consideration of other factors such as maternal smoking).

Mueller *et al*. (2004) conducted a case–control study of childhood brain tumours and intake of nitrate from drinking-water based on data from four countries: the USA (Mueller *et al*., 2001), Canada, France and Spain. These data were collected as part of a larger international case–control study that also included subjects from Australia, Israel and Italy, but water samples were not collected in these countries. In-person interviews were conducted with the biological mother and, if she was still residing where she had lived during the index pregnancy, a water sample was sought (and obtained from 86% of these women; 185 cases and 341 controls), although water samples were provided by 283 cases and 537 controls. [The Working Group believed that the risk estimates for residences during pregnancy were more valid in terms of exposure assessment because the estimates for post-pregnancy residences may be misclassified due to mothers potentially having changed residence after pregnancy.] Interviewers estimated nitrate using a semiquantitative Merckoquant test strip (10, 25, 50, 100, 250 or 500 mg/L). With adjustment for study region, age, sex and year of diagnosis, there was no increased risk for childhood brain tumours associated with nitrate in tap-water at the residence of pregnancy; however, there was a non-significantly increased risk associated with the highest category of exposure to nitrate after excluding women who used bottled water during pregnancy (odds ratio, 1.5; 95% CI, 0.6–3.8). [Mothers who drank any bottled water were excluded from subanalyses to test reliance on well-water.]

2.3.2 *Nitrite*

(a) Dietary intake of nitrite

(i) *Case–control studies in adults*

Giles *et al*. (1994) conducted a case–control study (described in detail in Section 2.3.1) of adult glioma that linked data on food intake from interviews to published tables of nitrite content. A modest borderline significant increase in risk for glioma was observed among men in relation to estimated dietary intake of nitrite, adjusted for alcohol consumption and tobacco smoking, but with no dose–response relationship. Corresponding odds ratios were close to null among women. [Interpretation of the possible association in men is limited, as noted in Section 2.3.1.]

Boeing *et al*. (1993) conducted a case–control study (described in detail in Section 2.3.1) of adult brain tumours in Germany. The risk for glioma associated with tertiles of dietary nitrite intake was close to null. Nitrite was rarely detected in drinking-water from the current homes of subjects using semiquantitative nitrite test strips. [However this does not reflect the nitrite burden from water in previous places of residence.] The risk estimate for glioma was close to null, while that for meningioma was markedly but non-significantly increased. [Study limitations are noted in Section 2.3.1.]

Kaplan *et al*. (1997) conducted a case–control study (described in detail in Section 2.3.1) of adult brain tumours in Israel. Consumption of approximately 100 foods in the period 10 years before diagnosis was assessed and linked to published tables. The authors stated that dietary intake of nitrite was not associated with the occurrence of brain tumours. [However, neither the number of exposed cases and controls nor risk estimates were provided.]

Blowers *et al*. (1997) conducted a case–control study (described in detail in Section 2.3.1) of glioma in women in southern California, USA. With regard to total nitrite intake and glioma, there was no pattern of association in the risk estimates (P for trend = 0.55), but glioma risk increased with increasing quartiles of nitrite intake from cured meats (P for trend = 0.07). [Study limitations are noted in Section 2.3.1.]

Lee *et al*. (1997) conducted a case–control study (described in detail in Section 2.3.1) of glioma in northern California, USA. All comparisons were stratified by sex and adjusted for age, education and family income. In both men and women, the mean weekly intake of nitrite of glioma cases was slightly [non-significantly] higher than that of controls. In men, the risk for glioma only increased among those with a nitrite intake above the median and a vitamin C intake below the median. This comparison was not significant [interaction p-value not provided.] [For other comments, see Section 2.3.1.] Also, non-participating controls who were asked about vitamin intake reported a higher vitamin intake than participating controls. [It is unknown whether participating and non-participating controls differed with respect to nitrite intake overall or differentially by category of vitamin C intake.]

Schwartzbaum *et al*. (1999) conducted a hospital-based case–control study of glioma and diet in Ohio, USA, among forty cases who underwent their first (exploratory) brain tumour surgery (all histologically confirmed) and 48 age- and sex-matched hospital controls who underwent orthopaedic or gynaecological surgery. Diet was ascertained through a questionnaire and was converted into nitrite intake from cured meats specifically from software and published tables. Just before surgery, 23 (53.5%) cases and 27 (56%) controls provided serum for which results (levels of three antioxidants) were available; these were used to examine the interaction between nitrite and vitamin C/α-tocopherol/γ-tocopherol. Cases had a higher dietary intake of nitrite from cured meat than controls; however, after adjustment for total energy intake, glioma was not associated with an intake of nitrite greater than the median from any of several specific types of cured meat (all odds ratios at or below null). Interaction odds ratios, adjusted for energy, suggested that the risk for glioma was highest among individuals who had an intake of

nitrite from cured meats above the median and levels of intake of vitamin C or α-tocopherol below the median; interaction *p*-values were not provided. [The exclusion criteria for controls that included several diseases related to diet (listed in Table 2.4) probably resulted in a control group that had a different diet (and prevalence of cigarette smoking) from population controls.]

Chen *et al*. (2002) conducted a case–control study (described in detail in Section 2.3.1) of adult glioma in relation to diet in Nebraska, USA. A questionnaire (48 foods) and published literature were used to assess total nitrite intake from the diet, which was not associated with the risk for glioma; there was no interaction between intake of nitrite and that of vitamin C.

Ward *et al*. (2005) (study described in detail in Section 2.3.1) extended the study by Chen *et al*. (2002) to consider the source of the dietary nitrite (animal versus plant) and to estimate the intake of endogenously formed nitrite from drinking-water (5% of the estimated nitrate intake from drinking-water). For most subjects, nitrate intake from water was estimated by linking histories of residence to data from public water-quality monitoring (mainly for 1965–85 for 130 (52%) cases and 319 (64%) controls). For a small subset of subjects who used private wells in 1985 and who provided a water sample, nitrite was estimated by laboratory analysis. As in the larger study, odds ratios for quartiles of total dietary intake of nitrite were close to null, and there was no interaction with intake of vitamin C (Chen *et al*., 2002). Odds ratios for nitrite from animal sources were also close to null and not statistically significant, but those for the upper two quartiles of nitrite from plant sources were markedly increased and statistically significant. When total nitrite intake from all dietary sources was combined with the estimated intake of nitrite from drinking-water, odds ratios were again close to null, with the exception of the second (versus the first) quartile (odds ratio, 1.8; 95% CI, 0.9–3.6). There was no interaction between this combined (diet and water) nitrite variable and vitamin C, nor between nitrite from water and either vitamin C or smoking status. [See comments in Section 2.3.1.]

(ii) *Case–control studies in children*

Cordier *et al*. (1994) conducted a case–control study (described in detail in Section 2.3.1) of childhood brain tumours in France. Maternal intake of selected foods during pregnancy was ascertained by structured interview and linked to a national survey and published tables to estimate total intake of nitrite. Odds ratios for brain tumours in the offspring were estimated by quartiles of nitrite intake, and a non-significant inverse dose–response relationship was observed [for possible limitations, see Section 2.3.1].

Lubin *et al*. (2000) conducted a case–control study (described in detail in Section 2.3.1) of childhood brain tumours and consumption of nitrite in childhood and during the index pregnancy in Israel. There was no association between brain tumours and intake of nitrite; odds ratios for the upper tertiles were close to null relative to the first tertile for intake by both the mother during pregnancy and the child. [See Section 2.3.1 for possible study limitations.]

Bunin *et al*. (1993, 1994) conducted a case–control study (described in detail in Section 2.3.1) of primitive neuroectodermal tumours and astrocytic gliomas in children < 6 years of age and their possible association with maternal dietary levels of nitrite in Canada and the USA. Intake of nitrite during pregnancy was not associated with the risk for either primitive neuroectodermal tumours or astrocytic gliomas in the offspring. Risk estimates for the latter were adjusted by family income, and numerous other potentially confounding factors were also considered (see Section 2.3.1).

Preston-Martin *et al*. (1996) conducted a case–control study of childhood brain tumours and diet in the states of California and Washington, USA. Five hundred and forty cases were identified from three tumour registries, and 801 controls, who were identified by random-digit dialling, were frequency-matched to cases by age and sex. A questionnaire (47 foods) was administered to biological mothers to identify the frequency and amounts of intake during the index pregnancy; these data were subsequently linked to published food tables and other software to determine levels of intake of nitrite. Odds ratios were adjusted for age, sex, birth year and geographical area; race and socioeconomic status were also considered but had no confounding effect. Risk for childhood brain tumour was not associated with total dietary nitrite or total intake of nitrite from vegetables. The highest quartile of nitrite intake from cured meats, however, was associated with a significantly increased risk for childhood brain tumours (odds ratio, 1.9; 95% CI, 1.3–2.6; 155 exposed cases), and yielded a significant *p* value for trend ($P = 0.003$); risk estimates for the second and third quartiles were close to null. The *p* value for trend was also significant among subjects with low and high socioeconomic status. When the authors considered the interaction between nitrite from cured meat and the use of prenatal vitamin supplements, no significant effect was observed, but the risk for brain tumours was increased in those with a high (above median) intake of nitrite from cured meats and no prenatal use of vitamins relative to those with an intake below the median plus prenatal use of vitamins (odds ratio, 2.2; 95% CI, 1.4–3.6; 47 exposed cases).

Pogoda and Preston-Martin (2001a) extended this work to refine the levels of nitrite linked to the earlier (Preston-Martin *et al*., 1996) data on diet using 26 food surveys with data on sodium nitrite to consider levels of nitrite in specific cured meats according to the year of consumption (index pregnancy). This updated study also allowed greater flexibility in the modelling (splines), with adjustment for the same confounding factors as before. Results were consistent with the previous study, and were more pronounced. The authors also estimated the increase in risk associated with 1 mg per day increase in nitrite consumption (from cured meat) during pregnancy, which was statistically significant ($P = 0.008$).

(b) Intake of nitrite from water

Mueller *et al*. (2004) conducted a case–control study (described in detail in Section 2.3.1) of childhood brain tumours and nitrite from drinking-water in Canada, France, Spain and the USA. Interviewers used semiquantitative Merckoquant test strips to estimate nitrite levels in tap-water samples from the current residence, which were sought

if this was the same residence as that of the index pregnancy. A sample was obtained from 86% of these women (185 cases and 341 controls), although water samples were provided by 283 cases and 537 controls. Among the latter, there was no increased risk for brain tumours in children in relation to the presence of nitrite in tap-water. [Subjects who no longer lived at the same residence as that at the time of pregnancy but who provided water samples may not be representative; among controls, participation of those who no longer lived at the pregnancy home varied according to the presence of nitrite.] When water samples were collected from homes in which the index pregnancy occurred, increasing levels of nitrite in water were associated with an increased risk for brain tumours in the offspring. This association was stronger (and statistically significant for the highest level of nitrite) when analyses were further restricted to women who did not drink any bottled water in the home during pregnancy. The increased risk was present only with respect to astroglial tumours; risk estimates for both primitive neuroectodermal tumours and other tumour types were close to null. [Among the pregnancy homes of controls, most of whom used public water supplies at the time of conception, nitrate and nitrite were very prevalent: 39.6% any nitrate, 8.2% nitrate ≥ 50 mg/L, 10.0% any nitrite and 2.4% nitrite ≥ 5 mg/L. Because levels of nitrite were usually low in public water supplies, it is unknown whether this reflects measurement error and, if so, whether it could differ by case status; however, it is unlikely to differ by histological tumour type, although the association was confined to astroglial tumours. Also, it should be noted that the levels of nitrite in tap-water may correlate with other substances in the water, such as pesticides and trichloromethanes.]

2.4 Cancer of the urinary tract

2.4.1 *Ingested nitrate*

(a) Ecological studies (Table 2.5)

Ecological studies in Europe have evaluated concentrations of nitrate in the drinking-water in relation to mortality from or incidence rates of cancer of the urinary tract (urinary bladder, lower urinary tract (comprised largely of bladder cancers) and kidney). Generally, these studies obtained concentrations of nitrate in water for a specific time period from public water supply databases and computed rate ratios, mortality rates or standardized incidence ratios (SIR) or compared incidence rates for regions grouped according to their average concentrations of nitrate.

In northern Jutland, Denmark, Jensen (1982) computed incidence rates (age standardized to the European standard population) of cancers of the urinary tract for Aalborg, a town that had high levels of nitrate in the water, and Aarhus, a town that had low levels. Levels of nitrate in municipal water averaged 27.1 mg/L in Aalborg in 1976 and had been detected since the 1930s, i.e. for at least 50 years; average levels in Aarhus were 0.2 mg/L in 1976 (described in detail in Section 2.2). No significant differences in incidence rates (per 100 000 persons) were observed between the two towns.

Table 2.5. Ecological studies of ingested nitrate and tumours of the urinary tract and genital tumours

Reference, location	Organ site (ICD code)	End-point	Exposure assessment	Exposure categories	No. of cases/ deaths	Main results		Adjustment for potential confounders	Comments
Jensen (1982), northern Jutland, Denmark	Urinary system, male genital organs, uterus, other female genital organs, breast	Incidence of urinary system cancers, 1968–72	Annual average concentrations of nitrate for the municipality of Aalborg (27.1 mg/L) and Aarhus (0.2 mg/L) in 1976	Mean nitrate (mg/L)		Incidence rate per 100 000		Rates age-standardized to the European standard population	
						Men	**Women**		
						Urinary tract			
				Aalborg		43.7	15.1		
				Aarhus		47.3	19.1		
						Male genital organs			
				Aalborg		49.9			
				Aarhus		47.8			
						Uterus			
				Aalborg			70.5		
				Aarhus			49.0		
						Other female genital organs			
				Aalborg			27.5		
				Aarhus			24.7		
						Breast			
				Aalborg		1.2	75.8		
				Aarhus		0.4	76.5		
Morales-Suárez-Varela *et al*. (1993, 1995), Valencia, Spain; 261 (1993) and 258 (1995) municipalities	Bladder (188), prostate (185)	Mortality, 1975–88	Concentrations of nitrate in public drinking-water, 1975–80 (1993) and 1968 (1995)	Mean nitrate (>50 mg/L) versus all other concentrations for municipalities		**Rate ratio (95% CI)** *Bladder* Men, 1.31 (0.64–2.71) Women, 2.1 (0.42–10.39) All, 1.38 (0.68–2.8)		Rate ratio based on mortality rates directly age-standardized to the 1981 Spanish population	Number of deaths not provided

Table 2.5 (contd)

Reference, location	Organ site (ICD code)	End-point	Exposure assessment	Exposure categories	No. of cases/ deaths	Main results	Adjustment for potential confounders	Comments
Morales-Suárez-Varela *et al*. (1993, 1995) (contd)				**Concentrations of nitrate in municipality (mg/L)**		**Mortality rates [per 100 000 persons/year]**	Age-adjusted mortality rates (direct method)	
					Men	*Bladder*		
				0–25	569	9.9		
				25–50	25	7.32		
				> 50	14	8.41		
					Women			
				0–25	112	2.03		
				25–50	0	–		
				> 50	0	–		
					Men	*Prostate*		
				0–25	925	16.9		
				25–50	58	16.9		
				> 50	40	24.04		
Van Leeuwen *et al*. (1999), Ontario, Canada	Bladder	Incidence of bladder cancer, Ontario Cancer Registry, 1987–91	Nitrate in drinking-water, 1987–91; 40 'ecodistricts' of Ontario	Mean concentrations of nitrate in the ecodistrict		No correlation was observed in women or men	Age-standardized	

Table 2.5 (contd)

Reference, location	Organ site (ICD code)	End-point	Exposure assessment	Exposure categories	No. of cases/ deaths	Main results	Adjustment for potential confounders	Comments
Gulis *et al.* (2002), Trnavas District, Slovakia	Bladder (188), kidney (189)	Cancer incidence from regional cancer registry, 1986–95	Concentrations of nitrate in 60 municipal supplies, 1975–95	**Average level of nitrate (mg/L)**		SIR (95% CI)	SIR based on age- (10-year), calendar year- and sex-specific incidence rates for the district	
						Bladder		
						Men		
				0–10	30	1.18 (0.82–1.68)		
				10.1–20	53	1.09 (0.83–1.43)		
				> 20	43	0.97 (0.71–1.30)		
						P for trend = 0.25		
						Women		
				0–10	13	1.32 (0.76–2.28)		
				10.1–20	15	0.81 (0.49–1.34)		
				> 20	14	0.82 (0.48–1.38)		
						P for trend = 0.13		
						Kidney		
						Men		
				0–10	14	0.99 (0.58–1.67)		
				10.1–20	29	1.06 (0.74–1.53)		
				> 20	26	1.11 (0.75–1.62)		
						P for trend = 0.53		
						Women		
				0–10	4	0.50 (0.19–1.32)		
				10.1–20	16	1.07 (0.66–1.75)		
				> 20	13	0.96 (0.55–1.64)		
						P for trend = 0.15		

Table 2.5 (contd)

Reference, location	Organ site (ICD code)	End-point	Exposure assessment	Exposure categories	No. of cases/ deaths	Main results		Adjustment for potential confounders	Comments
Volkmer *et al*. (2005), Bocholt, Germany	Urinary tract, testis, penis, prostate	Incidence of genitourinary tract cancers derived from medical facilities and practices, 1985–97	Concentrations of nitrate from two waterworks, 1957–85	Nitrate (60 mg/L versus 10 mg/L)	527 total	**SIR (95% CI)**		Age standardi-zation based on the German population in 1993	
						Transitional-cell carcinoma of the urinary tract			
					NR	Men	2.26 (1.34–3.79)		
					NR	Women	1.52 (0.78–2.96)		
					210	Total	1.98 (1.10–3.54)		
						Renal-cell carcinoma			
					NR	Men	0.61 (0.28–1.33)		
					NR	Women	2.96 (0.66–13.18)		
					57	Total	0.87 (0.34–2.22)		
					42	Testis	0.43 (0.21–0.90)		
					9	Penis	0.66 (0.14–2.88)		
					209	Prostate	1.06 (0.76–1.48)		

CI, confidence interval; ICD, International Classification of Diseases; NR, not reported; SIR, standardized incidence ratio

Morales-Suarez-Varela *et al*. (1993) studied cancer of the urinary bladder in Valencia, Spain, a region that has the highest reported levels of nitrate in the drinking-water in Europe. Mean concentrations of nitrate for 261 municipalities in the region were derived for the period 1975–80 from data obtained from the Department of Public Health, Hygiene and Environment. Mortality rates for urinary bladder cancer (International Classification of Diseases (ICD)-9 188) for the period 1975–88 were calculated from national death records and standardized directly for age and sex to the 1981 Spanish population. Compared to other regions, mortality rates in municipalities where water contained levels of > 50 mg/L nitrate were 38% higher overall, 31% higher among men and twice as high among women; however, these excesses were not statistically significant. [The Working Group noted that this study does not report the actual number of deaths from bladder cancer and the confidence intervals indicate imprecise rate ratios.] A subsequent report from Morales-Suárez-Varela *et al*. (1995) examined mortality rates for cancer of the urinary bladder (described in detail in Section 2.2). As in the previous study, deaths from cancer were obtained from national records (ICD-9 188 for bladder cancer) for 1975–88. Data from 268 municipalities were grouped into three categories of estimated concentrations of nitrate in the drinking-water (0–25, 25–50 and > 50 mg/L). Mortality rates for bladder cancer (directly age-standardized [standard population unspecified]) were similar across exposure categories for men: 9.9, 7.32 and 8.32 [per 100 000 person–years], respectively. In women, the rate was 2.03 [per 100 000 person–years] in the lowest exposure category and could not be computed for the other levels because there were no deaths.

An ecological study in Ontario, Canada (described in detail in Section 2.2) examined the incidence of urinary bladder cancer in relation to average concentrations of nitrate in 40 'ecodistricts' (Van Leeuwen *et al*., 1999). The authors indicated that no correlation was detected among men or women ($P > 0.25$), when levels of atrazine, alcohol consumption, tobacco smoking, level of education, income and occupational exposures derived from a provincial health survey were taken into account. [No correlations or risk estimates were provided.]

A study in the agricultural district of Trnava, Slovakia (Gulis *et al*., 2002), computed incidence rates for urinary bladder cancer in 60 villages that used municipal water systems (described in detail in Section 2.2). Villages were grouped into three categories based on the average concentrations of nitrate in their water supplies for the period 1975–95 (0–10, 10.1–20 and ≥ 20 mg/L nitrate). Using data from the regional cancer registry for the period 1986–95, sex-specific SIRs were computed for cancers of the urinary bladder (ICD-0 188) and kidney (ICD-0 189) with incidence rates for the entire district as the standard, stratified by age (10 years) and calendar year. SIRs did not increase with exposure category for either bladder or kidney cancer (SIRs for bladder cancer, 1.22, 1.18 and 1.32; SIRs for kidney cancer, 0.81, 1.05 and 1.05, respectively).

An ecological analysis was performed in a community of Bocholt, Germany, that was served by two waterworks over a period of 28 years (Volkmer *et al*., 2005). Concentrations of nitrate were distinct for the two waterworks: between 1957 and 1985,

one contained 60 mg/L nitrate and the other 10 mg/L, but both contained 10 mg/L nitrate from 1986 to 1997. Newly diagnosed cancers of the urinary tract among inhabitants of Bocholt were identified from medical departments (urology, pathology, oncology, radio-oncology) and outpatient urology practices between July 1985 and June 1997. Incidence rates in the two exposure groups were standardized to the German population for 1993. Rate ratios were higher in the area with higher levels of nitrate in 1957–85 for transitional carcinomas of the urinary tract (1.98; 95% CI, 1.10–3.54; overall rate ratio in men, 2.26; 95% CI, 1.34–3.79; overall rate ratio in women, 1.52; 95% CI, 0.78–2.96). For renal carcinomas, rate ratios were not increased overall among the high-nitrate group (0.87; 95% CI, 0.34–2.22); however, the rate ratio was above one among women (2.96; 95% CI, 0.66–13.18) and below one among men (0.61; 95% CI, 0.28–1.33), although, neither was statistically significant. [The investigators determined tobacco smoking histories from the medical records of the cases and these were not statistically significantly different between the two exposure groups; 15.9% in the high-exposure group and 20.6% in the low-exposure group had smoked > 20 pack–years; however, smoking could not be adjusted for in the analysis and the possibility of confounding remains. Further, the level of completion of case ascertainment is uncertain.]

(b) Cohort study (Table 2.6)

The US Iowa Women's Health Study (Weyer *et al*., 2001) evaluated nitrate in both the diet and drinking-water in relation to the incidence of cancers of the urinary bladder and kidney. This was a prospective study of 21 977 women who were 55–69 years of age in 1986 (response rate, 43%) and who used the same water supply for more than 10 years (87% used the same water supply for more than 20 years). The baseline postal questionnaire included basic demographic information, cigarette smoking history and a 126-item food-frequency questionnaire (the 1984 version of the Nurses' Health Study) which asked about usual diet in the previous year. A follow-up postal questionnaire in 1989 asked about the type of water system at the current residence and duration of use. Concentrations of nitrate in municipal water systems were analysed by the University of Iowa's Hygienic Laboratory in 1955–64, 1976–82 and 1983–88. Only communities that used a 'single source' (surface source or specific aquifer for 90% of their water) were included; users of private wells formed a separate stratum. Cancers diagnosed through to 31 December 1998 were determined by linkage to the Iowa State Cancer Registry. After adjustment for age and total energy intake, relative risks for kidney cancer were non-significantly elevated in the top three quartiles of dietary intake of nitrate (> 11.6 mg nitrate-N); however, there was no clear evidence of a trend in risk across quartiles. For urinary bladder cancer, relative risks were slightly and non-significantly elevated in the top three quartiles of dietary nitrate intake, but again there was no consistent increase with increasing exposure. Using average levels of nitrate in water (all years), relative risks were

Table 2.6. Cohort study of ingested nitrate and tumours of the urinary tract and genital tumours

Reference, location, name of study	Description of the cohort	Exposure assessment	Organ site (ICD code)	Exposure categories	No. of cases/ deaths	Relative risk (95% CI)	Adjustment for potential confounders	Comments
Weyer *et al.* (2001), Iowa, USA, Iowa Women's Study	Cohort of 21 977 Iowa women, 55–69 years of age in 1986, who had a valid driver's licence and no previous history of cancer and who had used the same water supply for > 10 years (87% > 20 years), linked to the Iowa Cancer Registry 1986–98; response rate, 43%	Mailed baseline questionnaire in 1986 included tobacco smoking and other factors and a 126-item food-frequency questionnaire (adapted from the 1984 version of the Nurses' Health Study); a follow-up questionnaire in 1989 included type of water system of current residence and duration of use.	Bladder, kidney, breast, ovary, uterine corpus	**Diet nitrate (mg nitrate–N)**			Age, total energy	Investigators did not examine interaction with vitamin C; for use of private wells, odds ratios were 1.31 (95% CI, 0.48–3.55) for bladder cancer and 1.07 (95% CI, 0.45–2.57) for kidney cancer.
						Bladder		
				< 11.6	9	1.00 (reference)		
				11.6–18.0	17	1.88 (0.84–4.24)		
				18.1–27.2	13	1.46 (0.62–3.47)		
				> 27.2	14	1.57 (0.66–3.75)		
						Kidney		
				< 11.6	12	1.00 (reference)		
				11.6–18.0	15	1.32 (0.62–2.83)		
				18.1–27.2	14	1.32 (0.60–2.89)		
				> 27.2	14	1.37 (0.61–3.06)		
						Breast		
				< 11.6	253	1.00 (reference)		
				11.6–18.0	252	0.98 (0.83–1.17)		
				18.1–27.2	265	1.04 (0.87–1.24)		
				> 27.2	254	0.99 (0.83–1.19)		
						Ovary		
				< 11.6	24	1.00 (reference)		
				11.6–18.0	28	1.12 (0.65–1.94)		
				18.1–27.2	28	1.10 (0.63–1.92)		
				> 27.2	22	0.85 (0.47–1.55)		
						Uterine corpus		
				< 11.6	71	1.00 (reference)		
				11.6–18.0	41	0.60 (0.41–0.88)		
				18.1–27.2	51	0.78 (0.54–1.12)		
				> 27.2	61	0.97 (0.68–1.39)		

Table 2.6 (contd)

Reference, location, name of study	Description of the cohort	Exposure assessment	Organ site (ICD code)	Exposure categories	No. of cases/ deaths	Relative risk (95% CI)	Adjustment for potential confounders	Comments
Weyer *et al.* (2001) (contd)		Nitrate concentrations were derived from municipal water supplies over the periods 1955–64, 1976–82, 1983–88; average nitrate level computed over the 33-year time frame for communities using a 'single source' for 90% of their water and average level for 1955–64 exclusively; users of private wells formed a separate stratum		**Mean nitrate in water (mg/L nitrate–N) 1955–88**			Age, education, tobacco smoking (never/former/current and pack–years), physical activity, body mass index, waist-to-hip ratio, total energy, intakes of vitamin C, vitamin E, and fruits and vegetables (for water nitrate), dietary nitrate, water source (ground/surface)	
						Bladder		
				< 0.36	7	1.00 (reference)		
				0.36–1.00	14	1.69 (0.66–4.30)		
				1.01–2.46	8	1.10 (0.38–3.20)		
				> 2.46	18	2.83 (1.11–7.19)		
						Kidney		
				< 0.36	9	1.00 (reference)		
				0.36–1.00	1	1.34 (0.55–3.25)		
				1.01–2.46	13	1.38 (0.56–3.41)		
				> 2.46	12	1.20 (0.46–3.16)		
						Breast		
				< 0.36	208	1.00 (reference)		
				0.36–1.00	209	1.03 (0.84–1.26)		
				1.01–2.46	185	0.97 (0.78–1.20)		
				> 2.46	208	1.03 (0.83–1.28)		
						Ovary		
				< 0.36	13	1.00 (reference)		
				0.36–1.00	19	1.52 (0.73–3.17)		
				1.01–2.46	24	1.81 (0.88–3.74)		
				> 2.46	26	1.84 (0.88–3.84)		
						Uterine corpus		
				< 0.36	44	1.00 (reference)		
				0.36–1.00	44	0.86 (0.55–1.35)		
				1.01–2.46	48	0.86 (0.55–1.36)		
				> 2.46	32	0.55 (0.33–0.92)		

CI, confidence interval

higher among those in the top quartile (> 2.46 mg/L nitrate-N) versus the lowest quartile (< 0.36 mg/L nitrate-N) for bladder cancer (relative risk, 2.83; 95% CI, 1.11–7.19); for kidney cancer, the relative risk was 1.20 (95% CI, 0.46–3.16) [the mean concentration for the top quartile was 5.59 mg/L nitrate-N]. Use of private wells was more weakly associated with bladder cancer (relative risk, 1.31; 95% CI, 0.48–3.55) and appeared to be unrelated to kidney cancer. Results were similar for an additional analysis that was restricted to 1955–64 and to women who had lived in the same residence for > 20 years. Relative risks for nitrate in water and use of private wells were adjusted for multiple potentially confounding factors such as age, education, tobacco smoking, pack–years of smoking, physical activity, body mass index, waist-to-hip ratio, total energy, vitamin C, vitamin E and fruit and vegetable intake, dietary intake of nitrate and type of water source (ground-versus surface). [Although the study did not ascertain sources of drinking-water outside the home, only 33% of women worked away from home; the study did not account for the amount of water consumed on an individual level. There was no assessment of potential effect modification, i.e. by vitamin C intake.]

(c) Case–control studies (Table 2.7)

One case–control study examined ingested nitrate in relation to cancer of the urinary bladder (Ward *et al*., 2003). Assessment of exposure to nitrate included evaluation of dietary intake and concentrations in the drinking-water. One small study of bladder cancer that analysed urinary concentrations of nitrate after diagnosis was not reviewed because the cancer potentially could have affected the levels of nitrate.

A large population-based case–control study of bladder cancer was conducted in Iowa, USA, and investigated intake of nitrate in both the diet and drinking-water (Ward *et al*., 2003). Cases included 1452 histologically confirmed bladder cancers that were newly diagnosed between 1986 and 1989, who were between the ages of 40 and 85 years and had no history of malignancy (except for basal-cell or squamous-cell carcinoma of the skin). These were identified by the Iowa State Cancer Registry and controls were selected from driver's licence records (for those aged < 65 years) and US Health Care Finance Administration records (for those aged 65 years and older). Reported response rates were 85% for cases and 82% for controls. Subjects were mailed a questionnaire that covered demographic information, history of cigarette smoking, occupation and lifetime residences and sources of drinking-water, together with a 55-item food-frequency questionnaire that asked about usual adult diet. Dietary levels of nitrate were based on the published literature and were analysed in quartiles. There was no evidence of an association between dietary intake of nitrate among men (odds ratio, 0.9; 95% CI, 0.7–1.1 for ≥ 119 versus < 59 mg nitrate per day) or women (odds ratio, 0.8; 95% CI, 0.5–1.3 for ≥ 127 versus < 62 mg nitrate per day). Estimates of exposure to nitrate from drinking-water were based on residential concentrations of nitrate. Questions included the amount of tap-water consumed in and outside the home, but the latter accounted for only 10% of consumption. Samples taken from the distribution system in a given year were averaged and linked to an individual by year and town. When no data were available for that year and

Table 2.7. Case–control studies of ingested nitrate and nitrite and tumours of the urinary tract and genital tumours

Reference, study location, period	Organ site	Characteristics of cases	Characteristics of controls	Exposure assessment	Exposure categories	No. of exposed cases	Relative risk (95% CI)	Adjustment for potential confounders	Comments
Barbone *et al*. (1993), Birmingham, AL, USA, 1985–88	Endo–metrial	168 women with endometrial cancer, 1985–1988, identified at the University of Alabama Hospital and a large private gynaecology–oncology practice in Birmingham, AL	334 women with an intact uterus attending the University optometry clinic; matched on age, race	Nurses' Health Study FFQ (116-item) administered in person with the assistance of the interviewer 103 cases and 236 controls	**Dietary nitrate (calorie adjusted mg)** T 1 (< 94.0) T 2 (94.0–<150.5) T 3 (≥ 150.5) *P* for trend	 48 29 26	 1.0 (reference) 0.7 (0.4–1.2) 0.4 (0.2–0.8) 0.0057	Age, race, years of schooling, total calories, use of unopposed estrogens, obesity, shape of obesity, smoking, age at menarche, number of pregnancies, diabetes, hypertension	

Table 2.7 (contd)

Reference, study location, period	Organ site	Characteristics of cases	Characteristics of controls	Exposure assessment	Exposure categories	No. of exposed cases	Relative risk (95% CI)	Adjustment for potential confounders	Comments
Wilkens *et al*. (1996), Oahu, Hawaii, USA, 1979–86	Lower urinary tract cancer (90% urinary bladder, 7% renal pelvis, 3% ureter)	261 cases (195 men, 66 women) of Caucasian or Japanese ancestry; 1977–86, hospitals covering 95.5% of eligible cases in the Hawaii Tumor Registry	522 population controls (390 men, 132 women); matched on sex, age (± 5 years), ethnic group; identified from Health Surveillance Program (annual survey of 2% of the population)	29-item dietary history of diet in a usual week one year prior to the reference date	**Dietary nitrites (µg/week)** *Men* Japanese T 1 T 2 T 3 *P* for trend Caucasian T 1 T 2 T 3 *P* for trend *Women* Japanese T 1 T 2 T 3 *P* for trend Caucasian T 1 T 2 T 3 *P* for trend	NR	 1.0 (reference) 1.2 (0.6–2.4) 2.0 (1.0–4.0) 0.05 1.0 (reference) 0.9 (0.5–1.6) 0.6 (0.3–1.2) 0.10 1.0 (reference) 0.5 (0.2–1.6) 0.9 (0.3–2.5) 0.84 1.0 (reference) 0.3 (0.1–1.1) 0.9 (0.3–2.4) 0.81	Age, cigarette smoking status, pack/years, employment in a high-risk occupation (both men and women), consumption of dark green vegetables (men), total vitamin C intake (women)	The mean intake of nitrate (µg/week) was reported as 1.95 in male cases, 1.84 in male controls, 1.14 in female cases and 1.32 in female controls [the Working Group questioned that the unit was more probably mg/week]. The odds ratios for nitrosamine in Japanese were 1.9 (95% CI, 0.9–4.3) and 3.0 (95% CI, 1.4–6.4) for tertiles 2 and 3 compared with tertile 1 in men and 1.0 (95% CI, 0.3–3.3) and 1.9 (95% CI, 0.6–5.8), respectively, in women.

Table 2.7 (contd)

Reference, study location, period	Organ site	Characteristics of cases	Characteristics of controls	Exposure assessment	Exposure categories	No. of exposed cases	Relative risk (95% CI)	Adjustment for potential confounders	Comments
Møller (1997), Denmark, 1986–88	Testicular cancer	514 men born between 1916 and 1970 identified by the Danish Cancer Registry; response rate, 88%	720 men selected randomly from the Danish population; matched on year of birth; response rate, 69%	Telephone interviews	**Born in high nitrate area**			Year of birth	
					Total	31	1.27 (0.99–1.64)		
					Non-seminoma	30	1.28 (0.91–1.80)		
					Seminoma	32	1.27 (0.93–1.74)		
					Lived in high nitrate area for the larger part of childhood				
					Total	32	1.40 (1.09–1.81)		
					Non-seminoma	33	1.49 (1.06–2.08)		
					Seminoma	33	1.36 (1.00–1.86)		
					High nitrate in childhood in the country				
					Total	18	1.10 (0.79–1.53)		
					Non-seminoma	17	1.04 (0.66–1.62)		
					Seminoma	19	1.14 (0.76–1.72)		
					High nitrate in childhood not in the country				
					Total	15	1.51 (1.03–2.20)		
					Non-seminoma	16	1.74 (1.07–2.82)		
					Seminoma	14	1.37 (0.86–2.20)		

Table 2.7 (contd)

Reference, study location, period	Organ site	Characteristics of cases	Characteristics of controls	Exposure assessment	Exposure categories	No. of exposed cases	Relative risk (95% CI)	Adjustment for potential confounders	Comments
Ward *et al*. (2003), Iowa, USA, 1986–89	Urinary bladder	1452 cases (1135 men, 317 women) newly diagnosed and histologically confirmed, aged 40–85 years, identified from the Iowa State Health Registry; response rate, 85%	2434 population-based controls (1601 men, 833 women) from driver's licence records or US Health Care Finance Administration; frequency-matched on age, sex at a different ratio in 1986–87 and 1988–89; response rate, 82%	Mailed questionnaire covering basic demographics smoking, occupation, residential and water source history, well depth and a 55-item FFQ on usual adult diet.	**Dietary nitrate (mg/day)** *Men* > 59 59–< 84 84–< 119 ≥ 119 *Women* < 62 62–< 90 90–< 127 ≥ 127 **Dietary nitrite (mg/day)** *Men* < 0.81 0.81–< 1.06 1.06–< 1.39 ≥ 1.39 *Women* < 0.58 0.58–< 0.75 0.75–> 0.98 > 0.98	NR	 1.0 (reference) 0.8 (0.7–1.1) 0.9 (0.7–1.2) 0.9 (0.7–1.1) 1.0 (reference) 1.2 (0.8–1.9) 0.9 (0.5–1.4) 0.8 (0.5–1.3) 1.0 (reference) 1.1 (0.9–1.4) 1.2 (0.9–1.5) 1.2 (0.9–1.6) 1.0 (reference) 1.0 (0.6–1.5) 0.8 (0.5–1.3) 1.0 (0.7–1.6)	Age, cigarette smoking, education, duration of chlorinated surface-water use, study period	Water analysis restricted to those who used Iowa public water systems with known nitrate level for ≥ 70% of their person–years starting in 1960. No evidence of a positive trend by years of use of a public water supply ≥ 10 mg/L nitrate-N. No positive association between use of private wells and risk.

Table 2.7 (contd)

Reference, study location, period	Organ site	Characteristics of cases	Characteristics of controls	Exposure assessment	Exposure categories	No. of exposed cases	Relative risk (95% CI)	Adjustment for potential confounders	Comments
Ward *et al*. (2003) (contd)				Residential concentrations of nitrate in water 1960 onwards; average concentrations and number of years of public water supply with ≥ 10 mg/L examined	**Nitrate in water (mg/L nitrate-N)**				
					Men				
					< 0.6	171	1.0 (reference)		
					0.6–< 1.40	171	0.9 (0.6–1.2)		
					1.40–< 3.09	164	0.8 (0.6–1.1)		
					≥ 3.09	116	0.5 (0.4–0.8)		
					Women				
					< 0.67	57	1.0 (reference)		
					0.67–< 1.18	44	0.7 (0.4–1.2)		
					1.18–< 2.48	38	0.6 (0.3–1.1)		
					≥ 2.48	47	0.8 (0.4–1.3)		
					High vitamin C	*(≥130 mg/day)*		Age, gender, smoking, duration, chlorinated surface water, education, study period	Nitrate (mg/L nitrate-N): low (< 1.25) high (≥ 1.25)
					Low nitrate	169	1.0 (reference)		
					High nitrate	134	0.7 (0.5–1.0)		
					Low vitamin C	*(<130 mg/day)*			
					Low nitrate	171	0.8 (0.6–1.1)		
					High nitrate	158	0.7 (0.5–0.9)		

CI, confidence interval; FFQ, food-frequency questionnaire; T, tertile

the sources did not vary more than 10%, a weighted average of annual averages in adjacent years was used (with greater weights closer to the year without data); missing values were assigned when no data were available within 10 years. The analyses were restricted to persons who used Iowa public water systems with known levels of nitrate for 70% or more of their person–years starting in 1960, and individual levels of nitrate were assigned based on the average levels after 1960. The risk for bladder cancer was unrelated to quartiles of nitrate-N (mg/L) among women and, if anything, lower among men. This risk was statistically significant for the highest exposure quartile (odds ratio, 0.5; 95% CI, 0.4–0.8 for ≥ 3.09 versus < 0.6 mg/L nitrate-N) and similar after accounting for number of years of use of water that contained levels of ≥ 10 mg/L nitrate-N after 1934. Estimates were adjusted for age, education, cigarette smoking, years of use of chlorinated surface water and study period. Further, there were no apparent interactions between exposure to nitrate from water and vitamin C intake, ever having had a bladder or kidney infection or smoking status. The authors also stated that duration of use of private wells and duration of use of wells less than 50 feet [15.2 m] deep were weakly inversely related to the risk for bladder cancer among men and unrelated to the risk among women [the point estimates were not provided].

2.4.2 *Ingested nitrite*

Two case–control studies of dietary intake of nitrite and cancer of the urinary bladder or lower urinary tract have been conducted, but no cohort studies. Case–control studies that used urinary concentrations of nitrite after diagnosis were excluded (for reasons provided in Section 2.4.1). The reviewed case–control studies of nitrite and urinary tract tumours are summarized in Table 2.7.

A population-based case–control study (Wilkens *et al*., 1996) from Oahu, Hawaii, USA, evaluated dietary intake of nitrites in relation to cancers of the lower urinary tract. The study included 195 men and 66 women of Caucasian or Japanese ancestry who were newly diagnosed with a lower urinary tract cancer between 1977 and 1986 at the seven largest civilian hospitals on the Island of Oahu (covering an estimated 95.5% of eligible cases based on the state cancer registry). Response rates were 89% for controls and 73% for living cases. Ninety per cent of the cases had tumours of the urinary bladder, 7% of the cancer of renal pelvis and 3% cancer of the ureter. Controls were selected from the Health Surveillance Program of the Hawaii State Department of Health (a survey of a 2% random sample of the population with participation rates of over 95%) and comprised 390 men and 132 women who had no history of a lower urinary tract tumour and who were matched to each case on sex, age (± 5 years) and ethnic group. A dietary history was obtained by personal interview that included a 29-item questionnaire that was designed to determine foods that contain nitrite and nitrosamines consumed in a usual week in the year before the reference date. Estimates of nitrite intake were obtained from published US Department of Agriculture tables. Among men of Japanese ancestry, there was a twofold risk for urinary tract cancers in the highest tertile of nitrite intake versus the

lowest tertile (odds ratio, 2.0; 95% CI, 1.0–4.0; *P* for trend = 0.05). The inverse was observed among Caucasian men (highest versus lowest tertile odds ratio, 0.6; 95% CI, 0.3–1.2), but not women. A statistically significant trend in risk (*P* = 0.01) was observed for dietary intake of nitrosamines among Japanese men (highest versus lowest tertile odds ratio, 3.0; 95% CI, 1.4–6.4). An elevated risk for nitrosamines was not statistically significant among Japanese women (highest versus lowest tertile odds ratio, 1.9; 95% CI, 0.6–5.8). Estimated intake of nitrosamines was unrelated to cancer of the urinary tract among Caucasian men or women. [The interaction between nitrite and vitamin C or other inhibitors of nitrosation was not evaluated.]

The population-based case–control study of bladder cancer from Iowa, USA (Ward *et al*., 2003; see Section 2.4.1 (*c*)), also estimated dietary intake of nitrite. When quartiles of intake were analysed, no significant association with bladder cancer was observed among men or women (odds ratio for men, 1.2; 95% CI, 0.9–1.6 for ≥ 1.39 versus < 0.81 mg nitrite per day; odds ratio for women, 1.0; 95% CI, 0.7–1.6 for > 0.98 versus < 0.58 mg nitrite per day).

2.5 Genital and breast cancer

2.5.1 *Ingested nitrate*

(a) Ecological studies

Ecological studies of exposure to nitrate in Europe have evaluated regional concentrations of nitrate in the drinking-water in relation to mortality from or incidence rates of testicular, penile, prostatic and breast cancer. These are described in greater detail in Section 2.4 and are summarized in Table 2.5.

In northern Jutland, Denmark, incidence rates (age-standardized to the European standard population) of cancers of the male genital organs, uterus and other female genital organs were computed for Aalborg, a town that had high levels of nitrate in the water, and Aarhus, a town that had low levels. Incidence rates (per 100 000 persons) for cancers of the uterus (cervix and corpus combined) were 44% higher in Aalborg, but no significant differences were observed in the rates of other cancers (Jensen, 1982).

A study in Valencia, Spain (Morales-Suárez-Varela *et al*., 1995), examined mortality rates (directly age-standardized [standard population unspecified]) for prostatic cancer (ICD-9 185) which were similar across exposure categories: 16.9, 16.9 and 24.04 [per 100 000 person–years].

(b) Cohort study

The US Iowa Women's Health Study (Weyer *et al*., 2001) examined nitrate in both the diet and drinking-water in relation to the incidence of breast, ovarian and corpus uterine cancers (summarized in Table 2.6). No association was apparent between cancers of the breast, uterine corpus or ovary and dietary levels of nitrate. When average levels of nitrate in water (all years) were used, relative risks were increased non-significantly for

ovarian cancer (relative risk, 1.84; 95% CI, 0.88–3.84), reduced for cancer of the uterine corpus (relative risk, 0.55; 95% CI, 0.33–0.92) and close to unity for breast cancer (relative risk, 1.03; 95% CI, 0.83–1.28). Use of private wells was more weakly related to ovarian cancer (relative risk, 1.55; 95% CI, 0.77–3.13) and appeared to be unrelated to cancers of the breast or uterine corpus. Relative risks for nitrate in water and use of private wells were adjusted for multiple potentially confounding factors. [There was no assessment of potential effect modification, i.e. by vitamin C intake.]

(c) Case–control studies

(i) *Endometrial cancer*

A hospital/clinic-based case–control study (Barbone *et al*., 1993; see Table 2.7) of endometrial cancer that was conducted in Birmingham, Alabama, USA, examined dietary intake of nitrate and included 103 women who were newly diagnosed with histologically confirmed endometrial cancer from June 1985 to December 1988 and were identified from the University of Alabama Hospital and a large private gynaecology–oncology practice in Birmingham. Controls were 236 women with an intact uterus matched on age and race who had attended the University optometry clinic. Information on reproductive history, medical history, anthropometric measurements and other factors was elicited through an in-person interview. Cases and controls also completed an interviewer-administered food-frequency questionnaire (the Nurses' Health Study 116-item questionnaire). Response rates were 93% for cases overall of whom 61% completed the nutritional assessment and 77% for controls overall of whom 71% completed the nutritional assessment. Dietary intake of nitrate was inversely related to the risk for endometrial cancer (odds ratio, 0.4; 95% CI, 0.2–0.8 in the highest versus lowest tertile of nitrate). These results were adjusted for age, race, years of schooling, total calories, use of unopposed estrogens, obesity, shape of obesity, tobacco smoking, age at menarche, age at menopause, number of pregnancies, diabetes and hypertension and changed only slightly with further adjustment for potassium (odds ratio, 0.5; 95% CI, 0.2–0.9).

(ii) *Testicular cancer*

Exposure to nitrate in relation to the incidence of testicular cancer was examined in a population-based case–control study in Denmark (Møller, 1997; see Table 2.7). Cases were 514 men (239 non-seminoma, 262 seminoma) who were born between 1916 and 1970 and were diagnosed with testicular cancer from 1986 to 1988. Controls were 720 men randomly sampled from the Danish population and frequency-matched on year of birth. Participants were interviewed by telephone. Of the cases approached, 88% participated, which represents 74% of the total incident cases in Denmark. Among controls, 69% of those approached took part in the study. The study assessed being born or living during childhood in areas known to have high groundwater levels of nitrate from manure and agricultural fertilizers. These regions included Aarhus, Viborg and Nordylland counties. In 1994, 22% of the waterworks in these areas contained more than 25 mg/L nitrate compared with 6% for the rest of the country. Odds ratios were slightly

increased among men born in the area that had high levels of nitrate (odds ratio, 1.27; 95% CI, 0.99–1.64); risk estimates were similar for both non-seminoma and seminoma tumours. The association was stronger for men who had lived in the area that had high levels of nitrate for the larger part of their childhood (odds ratio, 1.40; 95% CI, 1.09–1.81); again, the magnitude of the risk estimates were comparable for both histological types of tumour. The association appeared to be limited to men who had lived in the area that had high levels of nitrate during childhood but who did not live in the country (odds ratio for men who did not live in the country, 1.51; 95% CI, 1.03–2.20; odds ratio for men who lived in the country, 1.10; 95% CI, 0.79–1.53). [In light of the heterogeneity in nitrate in the exposure groups, and lack of consistency between the results for urban versus rural residence, the results argue against the association being related to nitrates.]

2.5.2 *Nitrite*

No cohort or case–control studies of have examined genital or breast cancers in relation to ingested nitrites.

2.6 Leukaemia and lymphoma

2.6.1 *Ingested nitrate*

(a) Ecological studies

Two ecological studies of levels of nitrate in the drinking-water and non-Hodgkin lymphoma were conducted in North America. Weisenburger *et al*. (1987) reported a twofold higher incidence of non-Hodgkin lymphoma among 25 counties in Nebraska where 20% or more of the private wells contained levels of nitrate-N greater than 10 mg/L compared with 25 counties where fewer than 10% of wells contained this level. A Canadian study (Van Leeuwen *et al*., 1999) (described in detail in Section 2.2) in 40 districts of Ontario evaluated the incidence of non-Hodgkin lymphoma in relation to levels of nitrate (weighted means of the levels in public and private wells). Incidence rates for non-Hodgkin lymphoma were not associated with the level of nitrate (range, 0.05–7.79 mg/L). The study also evaluated levels of atrazine, a crop herbicide that was weakly associated with higher rates of non-Hodgkin lymphoma among men but not among women.

Three ecological studies in Europe that evaluated levels of nitrate in drinking-water from public water supplies and the incidence of non-Hodgkin lymphoma had mixed results. A study in the agricultural district of Trnava, Slovakia (Gulis *et al*., 2002) (described in detail in Section 2.2) found a significant positive trend ($P = 0.021$) in incidence rates for non-Hodgkin lymphoma among men and women with increasing levels of nitrate in public water supplies (categories of nitrate: 0–< 2.3, 2.3–4.5, > 4.5 mg/L nitrate-N). Levels of nitrate were averaged over 20 years (1975–95) and incidence was calculated for the years 1986–95. An ecological study in the United

Kingdom (Law *et al*., 1999) evaluated levels of nitrate in 1990–95 (mean of monthly averages in water supply zones with homogeneous levels of nitrate and a population of < 50 000) in relation to the incidence of non-Hodgkin lymphoma in 1984–93 adjusted for population density. Overall, incidence was not related to levels of nitrate; however, incidence in 1984–88 (but not 1989–93) was positively associated with the average level of nitrate. [The authors interpreted the inconsistent association in the two time periods as not supportive of the hypothesis that levels of nitrate in the drinking-water are associated with non-Hodgkin lymphoma.] An ecological study in Sardinia, Italy (Cocco *et al*., 2003), compared incidence rates of non-Hodgkin lymphoma in 1974–93 with monitoring data on levels of nitrate in 1993 for 153 communes (median, 0.74 mg/L). There was no trend in the incidence of non-Hodgkin lymphoma among men or women by category of nitrate, although incidence rate ratios were significantly elevated for some categories of nitrate among men. Fifteen of the 153 communes had data on nitrate in 1971–84 when levels were higher (median, 1.83 mg/L); these higher levels of nitrate (≥ 5.7 versus < 1.81 mg/L) were not associated with incidence rates among men or women either.

One ecological study in Southwest England, United Kingdom (Foster *et al*., 1997), evaluated incidence (1984–88) of leukaemia subtypes and myelodysplasias in relation to levels of nitrate in public water supplies (46 water supply zones). The specific diagnostic groups that were evaluated included acute myeloblastic leukaemia, acute lymphoblastic leukaemia, chronic myeloid leukaemia, myelproliferative disorders and myleodysplasias. No significant differences in SIRs were observed for any of the leukaemias, myeloproliferative disorders or myleodysplasias in relation to levels (1984–88) of nitrate in public water supplies (range, 0–18.6 mg/L; weighted mean, < 10 mg/L; measurements in three of the 46 water supply zones exceeded 10 mg/L).

One study in 65 counties in China (Wu *et al*. 1993) (described in detail in Section 2.2) evaluated urinary levels of nitrate, 'nitrosation potential' (decrease in urinary NPRO after feeding ascorbic acid) measured by the NPRO test and excretion of *N*-nitroso amino acids among men aged 35–64 years in relation to mortality rates (ages, 0–64 years in 1973–75) for leukaemia. Mortality rates were significantly positively correlated with urinary excretion of nitrate, nitrosation potential and the sum of the *N*-nitroso amino acids.

(b) Cohort and case–control studies

A cohort study of women in Iowa (Weyer *et al*., 2001) (described in detail in Section 2.4.1) evaluated levels of nitrate in drinking-water from public supplies and dietary intake of nitrate. The water source at the current home was assessed in 1989 and cancer incidence was evaluated from the period of enrolment in 1986 through to 1998 (Table 2.8). The average level of nitrate was calculated from public monitoring data in 1955–88 for women who had used the same water supply for 10 or more years (396 towns/cities). Risk was also evaluated among women who used private wells in 1989, although no measurements of nitrate were available. In models adjusted for confounding factors that included intake of fruit and vegetables, intake of vitamins C and E, body mass index, education, physical activity and tobacco smoking, no significant

Table 2.8. Cohort study of nitrate in the drinking-water and nitrate and leukaemia and lymphoma

Reference, study location, study period	Organ site (ICD code)	Cohort description	Exposure assessment	Exposure categories	No. of cases	Relative risk (95% CI)	Adjustment for potential confounders	Comments
Weyer *et al*. (2001), Iowa, USA; 1986–98	Leukaemia	Cohort of 21 977 Iowa women, 55–69 years of age in 1986, who had a valid driver's licence and no previous history of cancer and who used the same water supply for > 10 years (87% > 20 years), 1986–98 linked to the Iowa Cancer Registry; response rate, 43%	Mailed baseline questionnaire in 1986 included tobacco smoking and other factors and a 126-item food-frequency questionnaire(adapted from the 1984 version of the Nurses' Health Study); a follow-up questionnaire in 1989 included type of water system of current residence and duration of use	**Average nitrate (mg/L nitrate-N) 1955–88**			Average nitrate: age, education, smoking, physical activity, body mass index, waist-to-hip ratio, total energy, vitamin C, E, diet nitrate, fruit and vegetables; nitrate in the diet: age, total energy	Nitrite in diet or interaction with vitamin C or smoking not evaluated
				< 0.36	31	1.0 (reference)		
				0.36–1.00	27	0.85 (0.49–1.47)		
				1.01–2.46	23	0.71 (0.39–1.29)		
				> 2.46	24	0.55 (0.29–1.04)		
				Nitrate in the diet (mg N/day)				
				< 11.6	37	1.0 (reference)		
				11.6–18.0	34	0.88 (0.55–1.40)		
				18.1–27.2	25	0.62 (0.37–1.04)		
				> 27.2	38	0.91 (0.56–1.46)		
	Non-Hodgkin lymphoma (200, 202)			**Average nitrate (mg/L nitrate-N) 1955–88**				
				< 0.36	27	1.0 (reference)		
				0.36–1.00	24	0.86 (0.48–1.55)		
				1.01–2.46	12	0.44 (0.20–0.95)		
				> 2.46	31	1.12 (0.61–2.06)		
				Nitrate in the diet (mg N/day)				
				< 11.6	22	1.0 (reference)		
				11.6–18.0	28	1.28 (0.73–2.24)		
				18.1–27.2	23	1.07 (0.59–1.94)		
				> 27.2	37	1.73 (1.00–3.00)		

CI, confidence interval; ICD, International Classification of Diseases

relationship was observed between average levels of nitrate in public supplies (median, 1.0 mg/L nitrate-N; interquartile range, 0.36–2.46 mg/L nitrate-N) and risk for leukaemia (94 cases) or non-Hodgkin lymphoma (105 cases). The relative risk for non-Hodgkin lymphoma decreased with increasing quartiles of average nitrate. The investigators did not evaluate the interaction with vitamin C or other factors that affect nitrosation. Use of private wells (> 10 years in 1989) was not associated with risk for either cancer. There was no trend in risk with increasing quartiles of dietary nitrate (highest quartile, > 27 mg per day), although the relative risk for leukaemia was marginally significant (1.73; 95% CI, 1.00–3.00).

The findings of case–control studies of nitrate in drinking-water and dietary intake of nitrate in relation to risk for non-Hodgkin lymphoma are summarized in Table 2.9. No case–control studies of other haematological malignancies have been conducted.

Three population-based case–control studies in the midwestern USA (Ward *et al*., 1996, 2006; Freedman *et al*., 2000) examined the relationship between average levels of nitrate in public water supplies over an average of 30 years and incident cases of non-Hodgkin lymphoma. The studies were similar in design, in that a history of source of residential water was collected and linked to data from public supply monitoring carried out by each town. The size of the population of the large majority of towns was < 50 000 and each study included over 100 utilites. Levels of nitrate were not monitored every year (the earliest year was 1935 for studies in Iowa and 1947 for the study in Nebraska) and values for missing years were imputed using weights based on the number of years since a measurement of nitrate. Data on measurements were most frequent by available after 1960. All studies evaluated potential confounding factors and analyses were limited to the populations who had spent 70–90% of their person–years in an area that had an estimate of nitrate during the exposure period to reduce misclassification by unknown (and probably higher) levels of nitrate from private wells. Two of the studies (Ward *et al*., 1996, 2006) also evaluated measurements of nitrate in private wells. These studies also assessed dietary intake of nitrate using a food-frequency questionnaire and a database developed from published values for nitrate in foods.

Ward *et al*. (1996) linked 156 cases of non-Hodgkin lymphoma and 527 controls to the average level of nitrate in public water supplies in 1947–84 in eastern Nebraska, USA. The population for these analyses was limited to people who had spent 90% or more of their person–years in the exposure period in an area that had an estimate of nitrate. The median exposure was 1.8 mg/L nitrate-N (interquartile range, 1.7–3.8 mg/L). Increasing quartiles of average nitrate were associated with an increasing risk for non-Hodgkin lymphoma and risk was elevated in the highest quartile for both men and women. Another measurement of exposure (years with a supply ≥ 10 mg/L nitrate-N versus no years at this level) was associated with a 50% increased risk (95% CI, 1.0–2.2). Those exposed to ≥ 10 mg/L nitrate-N were generally exposed for a short time period (first and third quartiles of duration were 2 years) and the odds ratio decreased to 1.1 after adjustment for occupational use of organophosphate insecticides and family history of cancer. Usual intake of tap-water was assessed and intake of nitrate from drinking-water was calculated

Table 2.9. Case–control studies of nitrate in the drinking-water or nitrate and nitrite in the diet and non-Hodgkin lymphoma (ICD code 200, 202)

Reference, study location, study period	Characteristics of cases and controls	Exposure assessment	Exposure categories	No of exposed cases	Relative risk (95% CI)	Adjustment for potential confounders	Comments
Ward *et al*. (1996), 66 counties of eastern Nebraska, USA, 1983–86	385 cases (white men and women) from Nebraska Lymphoma Study Group (population-based ascertainment of cases from hospitals and pathology laboratories across the state), aged 21 years and older; 40% of interviews with next-of-kin; 100% histologically confirmed; response rate, 90% 1432 population-based controls selected by random-digit-dialling for living controls aged < 65 years or from Health Care Finance Administration files and Nebraska mortality files for deceased controls; frequency-matched by race, age, sex, vital status; additionally matched by year of death	Interview-administered standardized FFQ designed to evaluate major sources of dietary nitrate, nitrite and vitamin C; lifetime history of water source; monitoring data on nitrate in 1947–84 from 138 public utilities in Nebraska; imputation of values for years without data on nitrate; analyses of nitrate in drinking-water: 156 cases, 527 controls with ≥ 90% person–years after 1947 with estimates of nitrate	**Average nitrate (mg/L nitrate-N) in public supplies 1947–79**				No interaction with vitamin C intake; average level of nitrate in 1965–79 (odds ratio, 1.12; 95% CI, 1.00–1.25) associated with risk but not 1947–64 (odds ratio, 0.99; 95% CI, 0.89–1.10); both metrics of nitrate in water showed similar results for men and women; self-reported and proxies.
			Q1, < 1.6	21	1.0 (reference)	Age, gender, family history of cancer	
			Q2, 1.6–< 2.0	67	1.4 (0.8–2.5)		
			Q3, 2.0–< 4.0	19	1.5 (0.7–3.0)		
			Q4, ≥ 4.0	47	2.0 (1.1–3.6)		
			Average nitrate/ vitamin C				
			Q1/High vitamin C	5	1.0 (reference)	Age, gender	
			Q2/High vitamin C	12	1.5 (0.5–4.6)		
			Q3/High vitamin C	11	1.4 (0.5–4.6)		
			Q4/High vitamin C	15	2.0 (0.7–6.1)		
			Q1/Low vitamin C	10	1.4 (0.4–4.5)		
			Q2/Low vitamin C	16	2.4 (0.8–7.4)		
			Q3/Low vitamin C	16	2.2 (0.7–6.7)		
			Q4/Low vitamin C	21	3.3 (1.1–9.9)		
			Dietary nitrate (mg N/day)			Age, gender, family history of cancer, vitamin C, carotenes	
			Q1, < 13	35	1.0 (reference)		
			Q2, 13–< 19	38	1.1 (0.6–2.0)		
			Q3, 19–26	20	0.8 (0.4–1.7)		
			Q4, > 26	11	0.7 (0.3–1.9)		
			Dietary nitrite (mg/day)			Results in text only for Q4 versus Q1, adjustment factors not stated	
			Q4 versus Q1		0.9 (0.5–1.7)		

Table 2.9 (contd)

Reference, study location, study period	Characteristics of cases and controls	Exposure assessment	Exposure categories	No of exposed cases	Relative risk (95% CI)	Adjustment for potential confounders	Comments
Freedman *et al.* (2000), Minnesota, USA, excluding residents of the four largest cities, 1980-82	329 cases (white men) ascertained from Minnesota hospital and pathology laboratory records, aged ≥ 30 years; 100% histologically confirmed; response rate, 89% (reported in Cantor *et al.*, 1992) 642 population-based controls (white men), and selected by random-digit dialling for ages < 65, a 1% random listing from Medicare files for living subjects aged 65 years and older and state death certificates for deceased cases; frequency-matched by age, vital status, state of residence	Interview-administered standardized questionnaire; lifetime history of water source; monitoring data on nitrate from 157 public utilities 1947–80; imputation of missing data (same method as Ward *et al*., 1996); analysis of nitrate: 73 cases, 147 controls with ≥ 90% person–years after 1947 with estimates of nitrate	**Average nitrate (mg/L nitrate-N) in public supplies 1947–75** ≤ 0.05 > 0.5–≤ 1.5 > 1.5	 41 19 3	 1.0 (reference) 1.4 (0.7–2.5) 0.3 (0.1–0.9)	Age	Exposure range, 0.1–7.2 mg/L nitrate-N; no differences for proxy/self-respondents

Table 2.9 (contd)

Reference, study location, study period	Characteristics of cases and controls	Exposure assessment	Exposure categories	No of exposed cases	Relative risk (95% CI)	Adjustment for potential confounders	Comments
Ward *et al.* (2006), Iowa, USA, 1998–2000	361 cases from Iowa Cancer Registry, aged 20–74 years; 100% histologically confirmed; response rate, 67% 276 population-based controls selected by random-digit dialling for ages < 65 and from Medicare eligibility files for ages 65–74 years; frequency matched by age, centre, race, gender	Interview-administered standardized questionnaire; complete history of water source; monitoring data on nitrate from public utilities and sample from private well at time of interview; analysis of nitrate: 181 cases, 142 controls with ≥ 70% person–years 1960 onwards with nitrate estimates; self-administered dietary questionnaire; modified version of Block FFQ with additional questions about high nitrate vegetables and processed meats	**Average nitrate (mg/L nitrate-N) in public supplies, 1960–2000** ≤ 0.63 0.63–1.36 1.37–2.89 ≥ 2.90 **Average nitrate in water (mg/day)/ vitamin C (mg/day)** High vitamin C/ < 3 mg nitrate Low vitamin C/ < 3 mg nitrate High vitamin C/ ≥ 3 mg nitrate Low vitamin C/ ≥ 3 mg nitrate	 40 49 46 46 31 38 13 13	 1.0 (reference) 1.3 (0.7–2.4) 1.0 (0.5–1.9) 1.2 (0.6–2.2) 1.0 (reference) 1.1 (0.5–2.3) 2.3 (0.7–7.0) 2.0 (0.6–5.9)	Age, sex, education	Intake of tap-water did not modify association; no significant interaction with vitamin C (<90 mg/day; ≥90 mg/day), red meat, cured meat, smoking status. Among subgroup with estimates of nitrate in water and diet, nitrate in water contributed a median of 13% (men) and 11% (women) towards total intake of nitrate.

Table 2.9 (contd)

Reference, study location, study period	Characteristics of cases and controls	Exposure assessment	Exposure categories	No of exposed cases	Relative risk (95% CI)	Adjustment for potential confounders	Comments
Ward *et al.* (2006) (contd)			**Nitrate in the diet (mg/day)**			Age, sex, education, study centre, race, dietary vitamin C, total energy	
			< 76	159	1.0 (reference)		
			76–113.9	116	0.75 (0.51–1.10)		
			114–169.9	111	0.71 (0.47–1.07)		
			≥ 170	80	0.54 (0.34–0.86)		
			Nitrite in the diet (mg/day)				
			< 0.71	82	1.0 (reference)		
			0.71–0.909	108	1.5 (1.0–2.3)		
			0.91–1.209	110	1.7 (1.1–2.7)		
			≥ 1.21	166	3.1 (1.7–5.5)		
			Nitrite in the diet from animal sources (mg/day)				
			< 0.16	101	1.0 (reference)		
			0.16–< 0.26	106	1.0 (0.7–1.5)		
			0.26–< 0.42	123	1.0 (0.7–1.6)		
			≥ 0.42	136	1.0 (0.6–1.7)		

CI, confidence interval; ICD, International Classification of Diseases; FFQ, food frequency questionnaire; Q, quartile

(in milligrams per day). Risks were higher among people who had a higher intake of nitrate from water (≥ 6.3 mg nitrate-N per day) and an intake of vitamin C below the median (< 130 mg per day) (odds ratio, 3.3; 95% CI, 1.1–9.9) compared with people whose intake of nitrate was < 2.5 mg nitrate-N per day and whose vitamin C intake was above the median; however, the statistical test for interaction was not significant. Increasing dietary intake of nitrate that came primarily from vegetables was associated with decreasing risks for non-Hodgkin lymphoma. Adjustment for vitamin C and carotenes attenuated the association and the inverse associations were not statistically significant.

Freedman *et al*. (2000) linked the average level of nitrate in Minnesota, USA, in 1947–75 (exposure was lagged 10 years) to 73 cases and 147 controls who lived in an area that had an estimate of nitrate for 90% of their person–years during the exposure period. Average levels of nitrate were low (highest exposure category, > 1.5 mg/L nitrate-N; median in this category, 2.4 mg/L nitrate-N) and were not associated with increasing risk; there was a significant inverse association for the highest category compared with ≤ 0.05 mg/L.

Ward *et al*. (2006) evaluated several indices of exposure to nitrate in Iowa, USA, including the average level of nitrate in 1960–2000 and the number of years that a public water supply with levels of nitrate ≥ 5 and ≥ 10 mg/L nitrate-N was used for 181 cases and 142 controls who lived in areas that had estimates of nitrate for 70% or more of their person–years during the exposure period. Increasing quartiles of the average level of nitrate were not associated with risk for non-Hodgkin lymphoma (median, 1.4; interquartile range, 0.6–2.9). Ten or more years of use of public supplies that had levels of > 5 mg/L nitrate-N was associated with a non-significant increased risk compared with no years of exposure at this level (odds ratio, 1.4; 95% CI, 0.7–2.9). One or more years of use of a supply that contained ≥ 10 mg/L nitrate-N was associated with a slight decrease in risk (odds ratio, 0.6; 95% CI, 0.2–1.5); exposure at or above this level was infrequent (11 cases, 10 controls). Levels of nitrate were measured in private wells at the time of the interview; 54 cases and 41 controls had used the well as their primary source of drinking-water for 5 or more years. Among this group, exposure levels were generally low; only eight cases and eight controls had levels of nitrate that were 5 mg/L nitrate-N or greater. Compared with those who had no detectable nitrate (< 0.2 mg/L nitrate-N), there was no association between levels of nitrate in private wells and risk for non-Hodgkin lymphoma (< 5 mg/L nitrate-N odds ratio, 1.7; 95% CI, 0.6–5.2; ≥ 5 mg/L nitrate-N odds ratio, 0.8; 95% CI, 0.2–2.5). For approximately half the study population, dietary intake was assessed using a 117-item food-frequency questionnaire that included questions about vegetables rich in nitrate and processed meats. Usual intake of nitrate from tap-water was also estimated. Among the subgroup that had estimates of nitrate from water and diet, nitrate from water contributed a median of 13% (men) and 11% (women) towards total intake of nitrate. Increasing quartiles of dietary intake were associated with an inverse risk for non-Hodgkin lymphoma after adjustment for dietary intake of vitamin C, education and study matching factors (age, sex, race and centre).

2.6.2 *Ingested nitrite*

Dietary nitrite was assessed in two case–control studies (Ward *et al*., 1996, 2006; Table 2.9). Quartiles of dietary nitrate were not associated with risk for non-Hodgkin lymphoma in the first study. Only the odds ratio for the highest versus the lowest quartiles were presented and no cut-off points were given (Ward *et al*., 1996). In the second study, quartiles of nitrite intake were significantly associated with an increasing risk for non-Hodgkin lymphoma; however, when animal and plant sources of nitrite were evaluated separately, the association was due only to plant and not to animal sources of nitrite (Ward *et al*., 2006). [The authors interpreted these findings as not supportive of a role for nitrite *per se* because animal sources of nitrite would be expected to show a stronger association with risk according to the nitrosation hypothesis.]

2.7 Oral, pharyngeal and laryngeal cancers

2.7.1 *Ingested nitrate*

A cohort study of head and neck cancers in Finland (Knekt *et al*., 1999) (described in detail in Section 2.2.1) estimated dietary intake of nitrate from a food-frequency questionnaire and a Finnish nutrient database (Table 2.10). Quartiles of dietary nitrate were not associated with an increased risk (48 cases).

Dietary intake of nitrate was evaluated in relation to oral and laryngeal cancers in one case–control study (Rogers *et al*., 1995) (described in detail in Section 2.2.1) which included 351 cases of oral cancer and 169 cases of laryngeal cancer in the Seattle metropolitan area in the USA (Table 2.11). Levels of nitrate were estimated from a 125-item food-frequency questionnaire and were linked to a database developed from the published literature. Tertiles of dietary intake of nitrate (highest tertile, > 226 mg per day [51 mg per day as nitrate-N]) were associated with significant inverse trends in the risk for oral ($P = 0.001$) and laryngeal ($P = 0.005$) cancers.

2.7.2 *Ingested nitrite*

The cohort study in Finland (Knekt *et al*., 1999) found no association between increasing quartiles of dietary nitrite and head and neck cancers, nor was NDMA intake associated with risk.

Tertiles of dietary intake of nitrite were not associated with the risk for oral or laryngeal cancers in the case–control study by Rogers *et al*. (1995); the odds ratios for the highest tertiles were below 1 (oral cancer, 0.66; 95% CI, 0.39–1.12; laryngeal cancer, 0.67; 95% CI, 0.34–1.34) (Table 2.11). The authors evaluated dietary intake of nitrate by level of vitamin C intake (> 195 versus < 195 mg per day) and by consumption of tea (less than once a week versus more than once a week), which are factors that inhibit endogenous nitrosation. Among persons who had a higher dietary intake of nitrate and lower consumption of either vitamin C or tea, no elevated risk was observed compared with

Table 2.10. Cohort studies of nitrate in the drinking-water and nitrate and nitrite in the diet and various cancers

Reference, study location, period	Organ site (ICD code)	Cohort description	Exposure assessment	Exposure categories	No. of cases	Relative risk (95% CI)	Adjustment for potential confounders	Comments
Knekt *et al.* (1999), several regions of Finland, 1966–72	Head and neck (140–148, 150, 161) (n = 48)	Cohort of 9985 persons with no cancer screened by the mobile health clinic of the Social Insurance Institution in several regions; cancer incidence follow-up through the Finnish Cancer Registry to 1990	FFQ; nitrate, nitrite, levels of NDMA estimated from a Finnish database (see Dich *et al.*, 1996 for more details of estimations of nitrate and nitrite); reproducibility estimated at intervals of 4–8 months with intraclass correlation coefficient: 0.48, nitrate; 0.73, nitrite; 0.53, NDMA; and 0.36, 0.25, 0.26, respectively for long-term reproducibility (4–7 years)	**Nitrate in the diet**			Age, sex, municipality, smoking, and energy intake	NDMA intake (Q2 versus Q1: odds ratio, 2.82; 95% CI, 1.11–7.11)
				Q1		1.0 (reference)		
				Q2		0.72 (0.32–1.60)		
				Q3		0.50 (0.21–1.20)		
				Q4		0.84 (0.39–1.81)		
				P for trend		0.95		
				Nitrite in the diet				
				Q1		1.0 (reference)		
				Q2		0.46 (0.19–1.12)		
				Q3		0.76 (0.35–1.66)		
				Q4		0.83 (0.36–1.88)		
				P for trend		0.77		
	Colorectum (153, 154) (n = 73)			**Nitrate in the diet**				NDMA intake (Q2 versus Q1: odds ratio, 2.12; 95% CI, 1.04–4.33)
				Q1		1.0 (reference)		
				Q2		1.01 (0.52–1.92)		
				Q3		0.98 (0.51–1.87)		
				Q4		1.04 (0.54–2.02)		
				P for trend		0.64		
				Nitrite in the diet				
				Q1		1.0 (reference)		
				Q2		0.82 (0.45–1.48)		
				Q3		0.94 (0.50–1.78)		
				Q4		0.74 (0.34–1.63)		
				P for trend		0.45		

Table 2.10 (contd)

Reference, study location, period	Organ site (ICD code)	Cohort description	Exposure assessment	Exposure categories	No. of cases	Relative risk (95% CI)	Adjustment for potential confounders	Comments
Weyer *et al*. (2001), Iowa, USA; 1986–98	Lung and bronchus	Cohort of 21 977 Iowa women, 55–69 years of age in 1986, who had a valid driver's licence and no previous history of cancer and who used the same water supply for > 10 years (87% > 20 years), 1986–98 linked to the Iowa Cancer Registry; response rate, 43%	Mailed baseline questionnaire in 1986 included tobacco smoking and other factors and a 126-item food-frequency questionnaire(adapted from the 1984 version of the Nurses' Health Study); a follow-up questionnaire in 1989 included type of water system of current residence and duration of use	**Average nitrate (mg/L nitrate-N) 1955–88**			Average nitrate: age, education, smoking, physical activity, body mass index, waist-to-hip ratio, total energy, vitamin C, E, diet nitrate, fruit and vegetables; nitrate in the diet: age, total energy	Nitrite in diet or interaction with vitamin C or smoking not evaluated
				< 0.36	56	1.0 (reference)		
				0.36–1.00	57	1.00 (0.67–1.47)		
				1.01–2.46	77	1.49 (1.02–2.17)		
				> 2.46	47	0.83 (0.53–1.30)		
				Nitrate in the diet (mg N/day)				
				< 11.6	76	1.0 (reference)		
				11.6–18.0	65	0.85 (0.61–1.19)		
				18.1–27.2	66	0.87 (0.62–1.22)		
				> 27.2	59	0.78 (0.55–1.11)		
	Colon			**Average nitrate (mg/L nitrate-N) 1955–88**				
				< 0.36	58	1.0 (reference)		
				0.36–1.00	86	1.53 (1.09–2.16)		
				1.01–2.46	92	1.54 (1.08–2.19)		
				> 2.46	64	0.98 (0.66–1.46)		
				Nitrate in the diet (mg N/day)				
				< 11.6	98	1.0 (reference)		
				11.6–18.0	78	0.79 (0.59–1.07)		
				18.1–27.2	90	0.93 (0.69–1.24)		
				> 27.2	97	1.00 (0.74–1.34)		

Table 2.10 (contd)

Reference, study location, period	Organ site (ICD code)	Cohort description	Exposure assessment	Exposure categories	No. of cases	Relative risk (95% CI)	Adjustment for potential confounders	Comments
Weyer *et al*. (2001) (contd)	Rectum			**Average nitrate (mg/L nitrate-N) 1955–88**				
				< 0.36	33	1.0 (reference)		
				0.36–1.00	25	0.72 (0.41–1.25)		
				1.01–2.46	32	0.95 (0.56–1.62)		
				> 2.46	16	0.47 (0.24–0.92)		
				Nitrate in the diet (mg N/day)				
				< 11.6	28	1.0 (reference)		
				11.6–18.0	39	1.42 (0.87–2.31)		
				18.1–27.2	27	1.01 (0.59–1.73)		
				> 27.2	28	1.06 (0.61–1.83)		
	Pancreas			**Average nitrate (mg/L nitrate-N) 1955–88**				
				< 0.36	17	1.0 (reference)		
				0.36–1.00	13	0.88 (0.42–1.84)		
				1.01–2.46	20	1.45 (0.73–2.88)		
				> 2.46	11	0.64 (0.27–1.56)		
				Nitrate in the diet (mg N/day)				
				< 11.6	19	1.0 (reference)		
				11.6–18.0	15	0.79 (0.40–1.56)		
				18.1–27.2	16	0.86 (0.44–1.69)		
				> 27.2	19	1.02 (0.52–1.99)		

Table 2.10 (contd)

Reference, study location, period	Organ site (ICD code)	Cohort description	Exposure assessment	Exposure categories	No. of cases	Relative risk (95% CI)	Adjustment for potential confounders	Comments
Weyer *et al*. (2001) (contd)	Oesophagus, stomach, small intestine, liver and bile ducts, gall-bladder, peritoneum/ retroperito-neum, other digestive			**Average nitrate (mg/L nitrate-N) 1955–88**				
				< 0.36	11	1.0 (reference)		
				0.36–1.00	12	1.39 (0.58–3.30)		
				1.01–2.46	16	1.83 (0.78–4.31)		
				> 2.46	16	2.09 (0.88–4.94)		
				Nitrate in the diet (mg N/day)				
				< 11.6	17	1.0 (reference)		
				11.6–18.0	19	1.18 (0.61–2.27)		
				18.1–27.2	20	1.32 (0.68–2.57)		
				> 27.2	15	1.03 (0.50–2.13)		

CI, confidence interval; FFQ, food-frequency questionnaire; ICD, International Classification of Diseases; NDMA, *N*-nitrosodimethylamine; Q, quartile

Table 2.11. Case–control studies of nitrite and nitrate in drinking-water and the diet and lung, nasopharyngeal, oral and laryngeal cancer

Reference, study location, period	Organ site	Characteristics of cases and controls	Exposure assessment	Exposure categories	No. of exposed cases	Relative risk (95% CI)	Adjustment for potential confounders	Comments
Goodman *et al.* (1992), Oahu, Hawaii, USA, 1983–85	Lung cancer	362 cases (226 men, 100 women), aged 30–84 years, residents of Oahu in five ethnic groups (Caucasian, Chinese, Japanese, Filipino, Hawaiian) identified by Hawaii Tumor Registry and diagnosed at seven major civilian hospitals; 100% histologically confirmed; response rates, 70% (men) and 63% (women) 865 population-based controls and selected by random-digit dialling (1983–84); random sample of participants of Oahu survey (refusals < 5%); supplemented controls > 65 with health care financing administration sample; matched by age, sex; response rates, 71% (men) and 67% (women)	In-person interview using standardized questionnaire; 130-item FFQ validated for this study population; data sources for nitrite, nitrate and NDMA not specified	**Nitrite in the diet (μg/week)** *Men* < 2000 2000–< 4000 4000–< 7000 > 7000 *P* for trend *Women* < 2000 2000–< 4000 4000–< 7000 > 7000 *P* for trend		 1.0 (reference) 1.2 (0.6–2.3) 1.7 (0.9–3.3) 2.0 (1.0–3.8) 0.02 1.0 (reference) 0.6 (0.2–1.2) 0.9 (0.4–2.1) 1.5 (0.5–4.1) 0.22	Age, ethnicity, smoking, β-carotene	Results consistent across ethnic groups; stronger associations (men) with cured meats among current versus past smokers; heavier smokers, squamous-cell versus adenocarcinoma; NDMA associated with stronger increased risks among men and women (*p* for trend: men, < 0.001; women, 0.04); dietary nitrate inversely associated with risk [data not shown]; 86% of intake from vegetables

Table 2.11 (contd)

Reference, study location, period	Organ site	Characteristics of cases and controls	Exposure assessment	Exposure categories	No. of exposed cases	Relative risk (95% CI)	Adjustment for potential confounders	Comments
De Stefani *et al.* (1996), Montevido, Uruguay, 1994–95	Lung cancer	320 cases (307 men, 13 women) from participating hospitals, all ages; average age, 63.1 years; histologically confirmed; response rate, 100% 320 hospital controls excluding diseases related to tobacco use; most frequent conditions: fractures (36%), eye disorders (22%), abdominal hernia (15%), trauma (9%), appendicitis (5%); frequency-matched by age, sex, hospital, residence (Montevideo, other counties); response rate, 100%	Interview-administered standardized questionnaire; 70-item FFQ focusing on main sources of nitrite, NDMA	**Dietary NDMA (μg/day)** ≤ 0.13 0.14-0.18 0.19-0.26 ≥ 0.27		 1.0 (reference) 0.88 (0.53–1.48) 1.77 (1.06–2.96) 3.14 (1.86–5.29)	Age, gender, residence, urban/rural status, family history of lung cancer, pack–years of tobacco smoking, total energy intake	Increasing risk with quartiles of NDMA for squamous-cell, small-cell and adeno-carcinoma; strongest association for squamous-cell and adenocarcinoma; dietary nitrite was estimated but results were not presented.

Table 2.11 (contd)

Reference, study location, period	Organ site	Characteristics of cases and controls	Exposure assessment	Exposure categories	No. of exposed cases	Relative risk (95% CI)	Adjustment for potential confounders	Comments
Ward *et al.* (2000), Taipei, Taiwan, China, 1991–94	Nasopharynx	375 cases identified from 2 referral hospitals in Taipei, aged < 75 years; 100% histologically confirmed; response rate, 99% of cases and 47% of mothers 327 population-based selected from national household registration system; matched by township/district, age, sex; response rate, 88% of controls and 63% of mothers	In-person interviews of cases and controls and their mothers; mothers asked about child's diet at age 10 and 3 years, and during weaning, and mother's diet during breast-feeding; cases and controls asked about diet as adult and age 10 years; nitrate, nitrite and nitrosamine levels (semi-quantitative) estimated from published values for Chinese foods and, when no values were available, from western foods	***Nitrite from soya bean products** Q1 (low) Q2 Q3 Q4 (high) ***Nitrite from other foods** Q1 (low) Q2 Q3 Q4 (high)	 NR NR NR NR NR NR NR NR	 1.0 (reference) 0.6 (0.2–1.3) 0.5 (0.2–1.1) 0.6 (0.3–1.4) 1.0 (ref) 1.7 (0.6–4.9) 3.5 (1.3–9.5) 2.0 (0.7–6.0)	Age, gender, ethnicity, vegetable intake	Results for mother's reports of intakes at age 3 years and weaning were similar (based on fewer foods);* intake at age 10 years as reported by mothers; nitrosamine intake from foods other than soya beans (as estimated by mothers) at ages 10 and 3 years, and weaning was positively associated with risk (weaning Q4 odds ratio, 3.9; 95% CI, 1.4–10.4)

Table 2.11 (contd)

Reference, study location, period	Organ site	Characteristics of cases and controls	Exposure assessment	Exposure categories	No. of exposed cases	Relative risk (95% CI)	Adjustment for potential confounders	Comments
Rogers *et al.* (1995), King, Snohomish and Pierce counties, Washington State, USA, 1983–87	Oral	351 cases from cancer surveillance system (part of NCI SEER); response rate, 73% (dietary component) 514 population-based controls selected by random-digit dialling; matched by age, gender; response rate, 76%	Personal interview; self-administered 125-item FFQ on foods high in nitrate, nitrite, NDMA; levels estimated from the published literature; nitrate in water estimated to be 1.3 mg/L	**Dietary nitrate (mg/day)** < 134 (T1) 134–226 (T2) > 226 (T3) *P* for trend **Dietary nitrite (mg/day)** < 1.06 (T1) 1.06–1.60 (T2) > 1.60 (T3) *P* for trend **Nitrite (mg)/ vitamin C** T1/high vitamin C T2/high vitamin C T3/high vitamin C T1/low vitamin C T2/low vitamin C T3/low vitamin C	 113 109 76 112 92 94 NR	 1.0 (reference) 0.66 (0.43–1.01) 0.46 (0.28–0.76) 0.001 1.0 (reference) 0.96 (0.61–1.51) 0.66 (0.39–1.12) 0.099 1.0 (reference) 1.62 1.01 2.40 1.64 1.30	All odds ratios adjusted for age, gender, education, pack–years of tobacco smoking, alcohol (drink–years), body mass index, total energy	No interaction of nitrite with vitamin C or tea consumption; NDMA tertiles associated with increasing risk (odds ratio highest versus lowest tertile, 1.82; 95% CI, 1.10–3.00) 95% CIs not given

Table 2.11 (contd)

Reference, study location, period	Organ site	Characteristics of cases and controls	Exposure assessment	Exposure categories	No. of exposed cases	Relative risk (95% CI)	Adjustment for potential confounders	Comments
Rogers *et al.* (1995) (contd)	Larynx	169 cases from cancer surveillance system (part of NCI SEER)		**Dietary nitrate (mg/day)**				
				< 134 (T1)	67	1.0 (reference)		No interaction of nitrite with vitamin C, tea consumption; NDMA tertiles associated with increasing risk (odds ratio highest versus lowest tertile, 1.70; 95% CI, 0.91–3.18)
				134–226 (T2)	49	0.50 (0.29–0.88)		
				> 226 (T3)	35	0.42 (0.22–0.80)		
				P for trend		0.005		
				Dietary nitrite (mg/day)				
				< 1.06 (T1)		1.0 (reference)		
				1.06–1.60 (T2)		0.98 (0.54–1.79)		
				> 1.60 (T3)		0.67 (0.34–1.34)		
				P for trend		0.107		
				Nitrite/vitamin C	NR		Age, gender, smoking, alcohol, body mass index, education, energy intake	95% CIs not given
				T1/high vitamin C		1.0 (reference)		
				T2/high vitamin C		1.04		
				T3/high vitamin C		0.65		
				T1/low vitamin C		1.65		
				T2/low vitamin C		1.22		
				T3/low vitamin C		0.95		

CI, confidence interval; FFQ, food-frequency questionnaire; NCI SEER, National Cancer Institue Survey of Epidemiology End Results; NDMA, *N*-nitrosodimethylamine; NR, not reported; Q, quartile; T, tertile

persons who had a low intake of nitrite and high intake of vitamin C or tea (95% CIs were not provided). The authors also estimated dietary intake of NDMA, which was associated with an 82% significant increased risk for oral cancer and 70% non-significant increased risk for laryngeal cancer.

2.7.3 *Nitrate and nitrite in saliva*

A small hospital-based study in Italy (Airoldi *et al*., 1997), in which oral, pharyngeal and laryngeal cancers were combined (36 men), found no differences between concentrations of salivary nitrate, nitrite or *N*-nitroso compounds in cases and controls. Selection of controls was not specified in detail and it was indicated that they were often relatives of the cases.

Another small hospital-based study of oral cancer in Egypt (44 cases, 40 control volunteers) measured levels of nitrate and nitrite in saliva (Badawi *et al*., 1998). Higher levels of salivary nitrite were associated with an increased risk for oral cancer (odds ratio, 4.3; 95% CI, 1.4–13.3 for > 40 μg/mL nitrite). [Levels in cases may have been affected by the disease; the controls were not population-based and did not appear to be matched to the cases by gender or age. Therefore, it was not possible to ascertain if their salivary levels are representative of the population.]

2.8 Nasopharyngeal cancer

No studies have evaluated nitrate ingested from drinking-water or diet and the risk for nasopharygeal carcinoma (NPC).

2.8.1 *Ingested nitrite*

(a) Ecological studies

Two ecological studies (Wu *et al*., 1993; Yi *et al*., 1993) in areas of China where mortality from NPC was both high and low (rates for other cancers varied) used the NPRO test to evaluate the hypothesis that higher endogenous nitrosation is associated with a higher risk for NPC. The study by Wu *et al*. (1993) (described in detail in Section 2.2) evaluated cancer mortality rates in 69 counties in relation to urinary nitrate, nitrosation potential and excretion of three *N*-nitroso amino acids in urine. Mortality rates (ages 0–64 years in 1973–75) for NPC were significantly positively correlated with urinary nitrate ($r = 0.28$, $P < 0.05$) and one *N*-nitroso amino acid ($r = 0.26$, $P < 0.05$) but not with nitrosation potential ($r = 0.06$, $P > 0.05$).

Yi *et al*. (1993) evaluated endogenous nitrosation potential among men and women in high- ($n = 37$) and low-risk ($n = 40$) districts in Zangwu County, Gaungxi region in 1990. Incidence rates of NPC varied 10-fold across the districts. The methods used were similar to those of Wu *et al*. (1993) except that a baseline 12-h urine sample was collected to measure background levels of nitrate and *N*-nitroso amino acids after a normal evening

meal. Background levels did not differ between the two areas. Proline intake increased excretion of NPRO of people in the high-risk but not of those in the low-risk district.

(b) Case–control studies

One case–control study of NPC in Taiwan, China (Ward *et al*., 2000), estimated dietary intake of nitrate during adulthood and at ages 3 and 10 years and maternal diet during pregnancy and breast-feeding (Table 2.11). Levels of nitrite were determined from the published literature using values for Chinese foods when available and from other published sources (mainly Japanese, USA and European data). Both the study subjects and their mothers were asked about the subject's diet at age 10 years. Intake of nitrite was assessed across all foods and results were presented separately for nitrite intake from soya bean products and from other foods (preserved meats, salted fish, preserved vegetables) because soya beans contain inhibitors of nitrosation. Based on self-reported adult dietary intakes and those at age 10 years, neither intake of nitrite from soya beans nor that from other foods was significantly associated with the risk for NPC. Based on maternal reports of the subject's diet at age 10 years, age 3 years and during weaning, intake of nitrite from soya bean products was inversely associated with risk, whereas, increasing intake of nitrite from other foods was positively associated with risk after adjustment for potential confounders, including vegetable intake. Dietary intake of nitrosamines was also estimated from the published literature and a semiquantitative level was assigned to each food. The results were similar to those for nitrite. Intake of nitrosamines from soya bean products was inversely associated with risk, whereas that from other foods was positively associated with the childhood diet based on maternal reports.

2.9 Colon and rectal cancers

2.9.1 *Ingested nitrate*

(a) Ecological studies

Gulis *et al*. (2002) (described in detail in Section 2.2) found a positive correlation between levels of nitrate in public water supplies and the incidence of colon but not of rectal cancer in Slovakia. Elevated levels of nitrate in public water supplies in 40 'ecodistricts' of Ontario, Canada, were positively correlated with age-standardized incidence ratios for colon cancer among women ($P = 0.048$) (described in detail in Section 2.2). In multiple variable regression analyses, age-standardized incidence ratios in women were no longer significantly associated with levels of nitrate in the drinking-water (Van Leeuwen *et al*., 1999). In Spain (Morales-Suárez-Varela *et al*., 1995) (described in detail in Section 2.2), no correlation was observed between levels of nitrate in 258 municipalities in 1968 (towns grouped by average nitrate level: 0–< 5.7, 5.7–11, > 11 mg/L) and mortality rates for colon cancer in 1975–80.

The study by Wu *et al*. (1993) in 69 counties of China (described in detail in Section 2.2) found no significant correlation between mortality rates (ages 0–64 years in 1973–75) for colorectal cancer and urinary concentrations of nitrate ($r = 0.16$).

(b) Cohort and case–control studies

A cohort study in Finland (Knekt *et al*. 1999) (described in detail in Section 2.2) enrolled men and women who were screened at mobile health clinics in 1966–72 and estimated dietary intake of nitrate from a food-frequency questionnaire and a Finnish database (Table 2.10). The incidence of colorectal cancer assessed through to 1990 (73 cases) was not associated with quartiles of dietary intake of nitrate.

A cohort study (described in detail in Section 2.4) of women in Iowa, USA (Weyer *et al*., 2001), found an inverse association with long-term average levels of nitrate (1955–88) in the public water supplies that served the current residence (minimum duration, 11 years) and incidence of rectal cancer (Table 2.10). The highest quartile of the average level of nitrate was not associated with an increased risk for colon cancer; however, relative risks were significantly elevated in the second and third quartiles. The authors did not evaluate the interaction with vitamin C intake. Increasing quartiles of dietary intake of nitrate were not associated with risk for colon or rectal cancers.

A population-based case–control study of colon and rectal cancer in Iowa, USA (De Roos *et al*., 2003), included 685 cases of colon cancer, 655 cases of rectal cancer and 2434 controls. The analyses of drinking-water were limited to those who had spent 70% or more of their person–years (1960–87) in an area with estimated levels of nitrate in public water supplies (376 colon cancers, 338 rectal cancers, 1244 controls). Overall, no relationship was observed between average levels of nitrate (1960–87) and years with a supply with > 5 or > 10 mg/L nitrate-N and risk for either colon or rectal cancer (Table 2.12). The authors evaluated potential interactions between exposure to nitrate in the drinking-water and factors that may affect endogenous nitrosation in the stomach or colon including vitamin C intake, meat intake and history of inflammatory bowel disease. For colon cancer, significant positive interactions were observed between exposures for 10 or more years to levels of above 5 mg/L nitrate-N and low vitamin C and high meat intake. Similar results were found for the metrics of nitrate in water. Quartiles of dietary intake of nitrate estimated from a food-frequency questionnaire and published levels in foods were inversely associated with risk for colon cancer and no association with rectal cancer was observed.

2.9.2 *Ingested nitrite*

A cohort study in Finland (Knekt *et al*., 1999) and a case–control study of colon and rectal cancer in Iowa, USA (De Roos *et al*., 2003), evaluated dietary intake of nitrite (Tables 2.10 and 2.12). Knekt *et al*. (1999) estimated dietary intake of both nitrite and NDMA. The incidence of colorectal cancer (73 cases) was not associated with increasing quartiles of dietary nitrite intake. However, the highest quartile of intake of NDMA was

Table 2.12. Case–control study of nitrate in drinking-water and nitrate and nitrite in the diet and colon and rectal cancers

Reference, study location, period	Organ site	Characteristics of cases	Characteristics of controls	Exposure assessment	Exposure categories	No of exposed cases	Relative risk (95% CI)	Adjustment for potential confounders	Comments
De Roos *et al*. (2003), Iowa, USA, 1988–89	Colon	685 cases identified from Iowa Cancer Registry, aged 40–85 years; white only; 86% deceased or too ill with proxy respondents; cases confirmed by histology (348) or positive cytology (28); response rate, 88%	2434 population-based selected from driver's licence records (aged <65 years) or from Health Care Finance Administration listing (aged > 65 years); matched by age, sex; response rates, 82% (aged < 65) and 80% (aged > 65)	Postal questionnaire; lifetime residential water source history; 55-item food-frequency questionnaire assessed nitrate, nitrite, vitamin C; nitrate monitoring data from 1960–87 from public utilities in Iowa; imputation of values for years without nitrate data; drinking-water analyses: 376 colon cases, 338 rectal cases, 1244 controls with ≥ 70% person–years 1960 onward with nitrate estimates	**Average nitrate (mg/L nitrate-N) in public supplies, 1960–89**			Age, gender	Significant interaction ($P < 0.10$) of water nitrate level with vitamin C, meat intake; odds ratio > 10 years nitrate at levels > 5 m/L nitrate-N and > median meat intake, 2.2 (1.4–3.6) versus 0 years > 5 mg/L nitrate-N and low meat intake; no significant for interaction for water nitrate with tobacco smoking, history of bowel inflammation, beverage consumption or high-fibre food intake
					≤ 1	172	1.0 (reference)		
					> 1–≤ 3	116	1.0 (0.8–1.3)		
					> 3–≤ 5	27	0.7 (0.4–1.1)		
					> 5	61	1.2 (0.8–1.7)		
					Years with nitrate >5 mg/L nitrate-N				
					0	240	1.0 (reference)		
					1–≤ 10	65	0.8 (0.6–1.1)		
					> 10	71	1.2 (0.9–1.6)		
					Vitamin C/years > 5 mg/L				
					High vit C/0 year	74	1.0 (reference)		
					Low vit C/0 years	97	1.4 (1.0–1.9)		
					High vit C/1–10 years	22	0.9 (0.5–1.5)		
					Low vit C/1–10 years	28	1.1 (0.7–1.9)		
					High vit C/> 10 years	24	1.1 (0.7–1.9)		
					Low vit C/> 10 years	35	2.0 (1.2–3.3)		

Table 2.12 (contd)

Reference, study location, period	Organ site	Characteristics of cases	Characteristics of controls	Exposure assessment	Exposure categories	No of exposed cases	Relative risk (95% CI)	Adjustment for potential confounders	Comments
De Roos *et al*. (2003) (contd)	Colon (contd)				**Dietary nitrate (mg/day)**				
					≤ 59.3	89	1.0 (reference)		
					59.4–86.86	68	0.8 (0.6–1.2)		
					86.7–122	68	0.8 (0.5–1.1)		
					> 22	55	0.7 (0.4–1.0)		
					Dietary nitrite (mg/day)				
					≤ 0.705	90	1.0 (reference)		
					0.706–0.94	73	1.1 (0.8–1.6)		
					0.94–1.26	48	0.9 (0.6–1.3)		
					> 1.26	69	1.5 (1.0–2.1)		
	Rectum	655 cases identified from Iowa Cancer Registry, aged 40–85 years; white only; 86% deceased or too ill with proxy respondents; cases confirmed by histology (348) or positive cytology (28); response rate, 88%			**Average nitrate (mg/L nitrate-N) in public supplies, 1960–89**			Age, gender, years with chlorinated surface water	No significant interaction with vitamin C, meat intake, tobacco smoking, history of bowel inflammation, beverage consumption or high-fibre food intake
					≤ 1	154	1.0 (reference)		
					> 1–≤ 3	98	0.8 (0.6–1.1)		
					> 3–≤ 5	30	0.7 (0.5–1.2)		
					> 5	56	1.2 (0.8–1.8)		
					Years with nitrate >5 mg/L nitrate-N				
					0	222	1.0 (reference)		
					1–≤ 10	57	0.8 (0.6–1.1)		
					> 10	59	1.1 (0.7–1.5)		

Table 2.12 (contd)

Reference, study location, period	Organ site	Characteristics of cases	Characteristics of controls	Exposure assessment	Exposure categories	No of exposed cases	Relative risk (95% CI)	Adjustment for potential confounders	Comments
De Roos *et al*. (2003) (contd)	Rectum (contd)				**Vitamin C/years**				
					> 5 mg/L				
					High vit C/0 year	87	1.0 (reference)		
					Low vit C/0 years	82	0.9 (0.7–1.3)		
					High vit C/1–10 years	15	0.5 (0.3–1.0)		
					Low vit C/1–10 years	24	0.8 (0.5–1.3)		
					High vit C/> 10 years	18	0.7 (0.4–1.3)		
					Low vit C/> 10 years	23	1.1 (0.6–1.8)		
					Dietary nitrate (mg/day)				
					≤ 59.3	56	1.0 (reference)		
					59.4–86.8	67	1.3 (0.9–1.9)		
					86.7–122	66	1.2 (0.8–1.8)		
					> 122	60	1.1 (0.8–1.7)		
					Dietary nitrite (mg/day)				
					≤ 0.705	74	1.0 (reference)		
					0.706–0.94	62	1.1 (0.7–1.6)		
					0.94–1.26	43	0.9 (0.6–1.4)		
					> 1.26	70	1.7 (1.1–2.5)		

CI, confidence interval; vit C, vitamin C

associated with a significant, 2.1-fold increase in risk. In the case–control study (De Roos *et al*., 2003), the highest quartile of dietary intake of nitrite was associated with an increased risk for colon cancer (odds ratio for the highest versus lowest quartile, 1.5; 95% CI, 1.0–2.1) and rectal cancer (odds ratio for the highest versus lowest quartile, 1.7; 95% CI, 1.1–2.5).

Meta-analyses of epidemiological studies that evaluated consumption of meat and the occurrence of colorectal cancer (Sandhu *et al*., 2001; Norat *et al*., 2002) reported significantly increased risks for the consumption of red meats and cured meats such as frankfurters, bacon, ham and luncheon meat. Although most cured meats are usually treated with nitrite, few studies estimated dietary intake of nitrite directly.

2.10 Pancreatic cancer

2.10.1 *Ingested nitrate*

One cohort study (Weyer *et al*., 2001) and one case–control study (Coss *et al*., 2004) evaluated nitrate in both the drinking-water and diet and risk for pancreatic cancer. Two additional case–control studies (Howe *et al*., 1990; Baghurst *et al*., 1991) evaluated dietary intake of nitrate alone.

A cohort study of women in Iowa, USA (Weyer *et al*., 2001; described in detail in Section 4.1), found no significant association with the long-term average levels of nitrate in public water supplies that served the current residence and the incidence of pancreatic cancer (Table 2.10); neither were quartiles of dietary intake of nitrate associated with risk.

A population-based case–control study in Canada of 249 cases and 505 controls (Howe *et al*., 1990) evaluated the risk for pancreatic cancer in relation to dietary intakes of nitrate using a food-frequency questionnaire and a database of nitrate values obtained from the published literature (Table 2.13). Modelled as a continuous variable and adjusting for age, sex, tobacco smoking and intake of fibre and calories, no significant association was observed between intake of nitrate and risk for pancreatic cancer.

Baghurst *et al*. (1991) conducted a population-based case–control study of pancreatic cancer in Australia among 104 cases and 253 controls (Table 2.13). Dietary intake of nitrate was estimated from a food-frequency questionnaire with nitrate values provided by Howe *et al*. (1990). Increasing quartiles of dietary intake of nitrate were associated with decreasing risk (odds ratio for highest versus lowest quartile, 0.45; 95% CI, 0.22–0.94) after adjustment for total energy, tobacco smoking and alcohol consumption.

A population-based case–control study (Coss *et al*., 2004) in Iowa, USA, included 376 cases of pancreatic cancer and 2034 controls. The analyses of drinking-water were limited to persons who had spent 70% of their person–years from 1960 onwards in an area with an estimate of levels of nitrate in the public water supply (189 cases, 1244 controls). The average level of nitrate in public water supplies (1960–87) and the number of years that the person used supplies with > 10 and > 7.5 mg/L nitrate-N were evaluated. No association was observed between any of the metrics of nitrate in the

Table 2.13. Case–control studies of nitrate in the drinking-water and nitrate and nitrite in the diet and pancreatic cancer

Reference, study location, period	Organ site	Characteristics of cases	Characteristics of controls	Exposure assessment	Exposure categories	No of exposed cases	Relative risk (95% CI)	Adjustment for potential confounders	Comments
Howe *et al.* (1990), metropolitan Toronto, Canada, 1983–86	Pancreas	249 diagnosed in 20 Toronto hospitals, aged 35–79 years, resident in greater metropolitan Toronto; 69% histologically confirmed, remainder clinically or radiologically; 194 interviews with proxies (62% spouse, 31% child); response rate, 46%	505 population-based selected from population rolls; matched by age, sex, residence in Toronto area; 194 interviews with proxies of selected controls (72% spouse; 19% child); response rate, 31%	Interview administered 200-item FFQ validated for the study population	Dietary nitrate, mean daily difference Q4 versus Q1: 35.1 mg *P* for trend Dietary nitrite, mean daily difference Q4 versus Q1: 1.6 mg *P* for trend		1.21 (0.72–2.02) 0.48 0.64 (0.34–1.22) 0.17	Age, sex, respondent status, total calories, fibre, cigarettes/day	Odds ratios estimated from continuous variable models; interaction with vitamin C not evaluated

Table 2.13 (contd)

Reference, study location, period	Organ site	Characteristics of cases	Characteristics of controls	Exposure assessment	Exposure categories	No of exposed cases	Relative risk (95% CI)	Adjustment for potential confounders	Comments
Baghurst *et al.* (1991), Adelaide, South Australia, 1984–87	Pancreas	104 (52 men, 52 women) from South Australian Cancer Registry; only 16 cases had next-of-kin respondents due to rapid enrolment; response rates for case series which included pancreas, gall-bladder and bile ducts was 62% for men and 63% for women	253 population-based from electoral rolls; matched on age, sex; response rates, 57% for men and 51% for women	Self-administered 179-item FFQ; validated for the study population; nitrate, nitrite, nitrosamine values from Howe *et al.* (1990; Canada) with local data for alcohol and cured meats from producers in Australia	**Dietary nitrate (mg/day)** Q1 Q2 Q3 Q4 *P* for trend **Dietary nitrite (mg/day)** Q1 Q2 Q3 Q4 *P* for trend		 1.0 (reference) 0.90 (0.46–1.77) 0.73 (0.36–1.48) 0.45 (0.22–0.94) 0.027 1.0 (reference) 1.01 (0.51–1.99) 0.65 (0.32–1.30) 0.92 (0.46–1.84) 0.49	Total energy, alcohol, tobacco	Interaction with vitamin C or animal sources separately not evaluated; nitrosamines associated with non-significantly increased risk (*P* for trend = 0.11); [histological confirmation not mentioned]

Table 2.13 (contd)

Reference, study location, period	Organ site	Characteristics of cases	Characteristics of controls	Exposure assessment	Exposure categories	No of exposed cases	Relative risk (95% CI)	Adjustment for potential confounders	Comments
Coss *et al.* (2004), Iowa, USA, 1985–87	Pancreas	376 (202 white men, 174 white women) identified from Iowa Cancer Registry, aged 40–85 years; 86% deceased or too ill with proxy respondents; cases confirmed by histology (348) or positive cytology (28); response rate, 88%	2034 population-based selected from driver's licence records (aged < 65) or from Health Care Finance Administration listing (aged > 65); matched by age, sex; response rates, 82% (aged < 65) and 80% (aged > 65)	Postal questionnaire; lifetime residential water source history; 55-item FFQ; monitoring data for 1960–87 from public utilities in Iowa; imputed values for years without data; drinking water nitrate analyses: 189 cases, 1244 controls with ≥ 70% person–years 1960 onward with nitrate estimates	**Average nitrate (mg/L nitrate-N) in public supplies, 1960–87**			Age, gender, cigarette use	No significant associations with years using a public supply with ≥ 7.5 or 10 mg/L nitrate-N; no interaction with smoking or vitamin C intake [data not shown]; association not significant after excluding proxies who were not spouses; no association for plant sources of nitrite among men or women [data not shown]
					< 0.6	50	1.0 (reference)		
					0.6–< 1.3	62	1.2 (0.79–1.8)		
					1.3–2.8	28	0.54 (0.33–0.89)		
					> 2.8	49	0.99 (0.64–1.5)		
					Dietary nitrate (mg/day)			Age, cigarette use, total calories	
					Men				
					< 58	26	1.0 (reference)		
					58–82	33	1.1 (0.63–1.9)		
					83–117	39	1.2 (0.70–2.0)		
					> 117	43	1.0 (0.60–1.8)		
					Women				
					< 63	39	1.0 (reference)		
					63–90	33	0.99 (0.58–1.7)		
					91–126	24	0.64 (0.36–1.1)		
					> 126	26	0.53 (0.29–0.97)		

Table 2.13 (contd)

Reference, study location, period	Organ site	Characteristics of cases	Characteristics of controls	Exposure assessment	Exposure categories	No of exposed cases	Relative risk (95% CI)	Adjustment for potential confounders	Comments
Coss *et al.* (2004) (contd)					**Dietary nitrite (mg/day)**				
					Men				
					< 0.75	15	1.0 (reference)		
					0.75–0.98	22	1.0 (0.52–2.0)		
					0.99–1.30	40	1.5 (0.81–2.9)		
					> 1.30	64	1.5 (0.79–3.0)		
					Women				
					< 0.56	18	1.0 (reference)		
					0.56–0.71	32	1.8 (0.94–3.4)		
					0.72–0.93	32	1.4 (0.72–2.6)		
					> 0.93	40	1.3 (0.65–2.5)		
					Dietary nitrite from animal sources (mg/day)				
					Men				
					< 0.22	9	1.0 (reference)		
					0.22–0.31	22	2.1 (0.95–4.8)		
					0.32–0.53	60	3.8 (1.8–8.0)		
					> 0.53	50	2.3 (1.1–5.1)		
					Women				
					< 0.13	13	1.0 (reference)		
					0.13–0.18	32	2.4 (1.3–4.7)		
					0.19–0.26	26	1.9 (0.94–4.0)		
					> 0.26	51	3.2 (1.6–6.4)		

CI, confidence interval, FFQ, food-frequency questionnaire; Q, quartile

drinking-water and risk for pancreatic cancer. No evidence of an interaction between the levels of nitrate in water and intake of vitamin C or tobacco smoking was found [results of analyses of interaction were not presented]. Increasing quartiles of dietary intake of nitrate were inversely associated with risk in women (odds ratio for the highest versus lowest quartile, 0.53; 95% CI, 0.29–0.97) but not in men (odds ratio, 1.0; 95% CI, 0.6–1.8). When analyses were limited to self-responding women and proxies who were husbands of the subjects, no significant association with risk was observed.

2.10.2 *Ingested nitrite*

The three case–control studies that evaluated dietary intake of nitrate also estimated dietary intake of nitrite using the same methods (Table 2.13).

Howe *et al*. (1990) modelled intake of nitrite as a continuous variable and found no association with the risk for pancreatic cancer. Baghurst *et al*. (1991) evaluated increasing quartiles of dietary intake of nitrite and found no association with the risk for pancreatic cancer; however, increasing quartiles of dietary intake of nitrosamines were associated with a non-significant positive trend in risk (P for trend = 0.11).

In the case–control study in Iowa (Coss *et al*., 2004), a slight elevated risk for pancreatic cancer was observed for the high quartile of consumption of dietary nitrite (odds ratio for men, 1.5; odds ratio for women, 1.3). When animal sources of nitrite were evaluated separately, risks were higher (odds ratio for highest versus lowest quartile: men, 2.3; women, 3.2) and were statistically significant.

2.11 Lung

2.11.1 *Ingested nitrate*

One cohort study (Weyer *et al*., 2001) (described in detail in Section 2.4) evaluated nitrate in the drinking-water and the diet and the risk for lung cancer among 252 cases. No significant relationship between average levels of nitrate (median, 1.0 mg/L nitrogen-N; interquartile range, 0.36–2.46 mg/L) in public water supplies and the risk for lung cancer was observed. Quartiles of dietary nitrate were not significantly associated with risk (odds ratio for highest versus lowest quartile, 0.78; 95% CI, 0.55–1.11).

One case–control study (Goodman *et al*., 1992) evaluated dietary intake of nitrate and the risk for lung cancer and reported an inverse association [results not presented in the paper; 86% of intake was reported to be from vegetables].

2.11.2 *Ingested nitrite*

A population-based case–control study in Oahu, Hawaii, USA, of 326 cases and 865 controls (Goodman *et al*., 1992) found elevated risks for lung cancer with increasing quartiles of dietary intake of nitrite among men and women (Table 2.11) with a significant positive trend among men (P for trend = 0.02). Risk estimates were adjusted

for ethnicity, tobacco smoking, and intake of β-carotene. Intake of nitrite was primarily from consumption of cured meat. The authors did not evaluate interactions with nitrite and vitamin C or other factors that affect endogenous nitrosation. The association with intake of individual cured meats was stronger among current smokers than among former smokers, and was somewhat stronger for squamous-cell carcinoma than for adenocarcinoma. Levels of NDMA were also estimated in this study and showed a stronger association with risk than nitrite among both men (P for trend < 0.001) and women (P for trend = 0.04).

2.12 Liver

No cohort or case–control studies have evaluated ingested nitrate and nitrite in relation to liver cancer.

Ecological studies

The study by Wu *et al*. (1993) in 69 counties of China (described in detail in Section 2.2.1) found a statistically significant positive correlation between mortality rates for liver cancer (ages 0–64 years in 1973–75) and urinary concentrations of nitrate ($r = 0.25$; $P < 0.05$). Mortality from these cancers was not significantly correlated with nitrosation potential, NPRO or specific *N*-nitroso amino acids.

An ecological study of cholangiocarcinoma in Thailand (Srianujata *et al*., 1984) evaluated salivary and urinary concentrations of nitrate and nitrite in men and women in low- (12 subjects) and high-risk (32 subjects) areas who consumed regular meals. Multiple samples were taken within 2 h after eating. Levels of nitrate and nitrite in saliva were significantly higher (two to five fold) among those living in high-risk areas. Urinary levels of nitrate were higher at some time points, but all levels of nitrite were similar. Mitacek *et al*. (1999) measured the levels of volatile nitrosamines, nitrate and nitrite in foods typically consumed in high- and low-risk areas for liver cancer in Thailand. Concentrations of nitrate and nitrite were not correlated with volatile nitrosamines. Specific volatile nitrosamines tended to be found at higher concentrations in foods that were consumed in the high-risk areas; however, no formal statistical analyses were conducted.

2.13 References

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3. Studies of Cancer in Experimental Animals

3.1 Nitrate

3.1.1 *Mouse*

Oral administration

In a study of the effects of concurrent administration of sodium nitrate and piperazine, one group of 40 male strain A mice, 7–9 weeks of age, was given 12.3 g/L sodium nitrate in the drinking-water for 25 weeks and was killed 13 weeks later. Controls were untreated. Other groups were fed piperazine (18.75 g/kg) alone in the diet or received piperazine and nitrate. The incidence of lung adenomas did not differ statistically between the sodium nitrate-treated mice (7/40), untreated controls (12/37), mice fed piperazine alone (7/33) or mice fed piperazine and treated with nitrate (15/37). The authors noted that less than 2.5% of the administered nitrate was reduced to nitrite *in vivo* in mice (Greenblatt & Mirvish, 1973).

Groups of 10 male and 10 female ICR mice, 8 weeks of age, were fed an experimental diet containing 0, 2.5 or 5.0% sodium nitrate for 2 years. No significant differences in food intake, body weight or tumour incidence were observed between untreated control and treated animals (Sugiyama *et al.*, 1979). [The Working Group noted the small number of animals per group.]

Groups of 100 female NMRI mice, weighing 27–31 g, were given drinking-water that contained 0, 100 or 1000 mg/L (0, 30 or 300 mg/kg body weight (bw)) calcium nitrate daily for 18 months. Survival of the high-dose group was lower than that of controls. No increase in tumour incidence was observed in the nitrate-treated groups (Mascher & Marth, 1993).

3.1.2 *Rat*

Oral administration

Groups of 15 male and 15 female MRC-derived rats, 8–10 weeks of age, were given 0 or 5 g/L sodium nitrate in the drinking-water for 84 weeks and served as controls in a study of nitrilotriacetic acid. The animals were killed 20 weeks later. Tumour incidences did not differ between nitrate-treated males and females and the untreated control groups (Lijinsky *et al.*, 1973a). [The Working Group noted the short duration of exposure.]

Groups of 10 male Sprague-Dawley rats, 2 months of age, received 0 or 4000 mg/L sodium nitrate in the drinking-water for 14 months. Body weights of the nitrate-treated group were lower than those of untreated controls. The nitrate-treated rats had a higher incidence of lung lesions compared with controls (4/4 versus 1/5). The nature of lung lesions was not described (Chow *et al.*, 1980). [The Working Group noted the small

number of animals, the short duration of exposure and the lack of histopathological examination.]

Groups of 50 male and 50 female Fischer 344 rats, 8 weeks of age, were fed a diet containing 0, 2.5 or 5% sodium nitrate for 2 years [equivalent to 0, 1250 or 2500 mg/kg bw sodium nitrate per day or 0, 910 or 1820 mg/kg bw per day expressed as nitrate ion]. Thereafter, tap-water and basal diet were given to all experimental groups and observation was continued until week 123. The survival rate of the treated animals was significantly higher ($P < 0.05$) than that of controls. Full histopathology was performed. The incidence of mononuclear-cell leukaemia was reduced ($P < 0.01$, χ^2 test) in the treated group compared with that in controls (males: control, 36%; low-dose, 4%; and high-dose, 2%; females: control, 28%; low-dose, 0%; and high-dose, 2%). No significant differences were observed in the incidence of any other types of tumour (Maekawa *et al.*, 1982). [The Working Group noted that Fischer 344 rats have a high incidence of spontaneous mononuclear-cell leukaemia].

Two groups of locally bred male white rats, weighing 120–140 g [age unspecified], received daily either a 0.05% solution of *N*-butyl-*N*-(4-hydroxybutyl)nitrosamine (BBNA) in the drinking-water for 30 days alone (18 effective animals) or, after treatment with BBNA was completed, 4 g/L sodium nitrate also in the drinking-water for 273 days (34 effective animals). A third group was treated with sodium nitrate alone at the same dose for 273 days (20 effective animals) and 20 effective animals in the fourth group served as untreated controls. All surviving rats were killed 10 months after the start of the experiment. Urinary bladders of all animals were examined histologically. BBNA alone induced urinary bladder carcinoma in one effective animal (5.6%), papillomas in four animals (22.2%) and papillary and nodular hyperplasia in eight animals (44.4%). Combined treatment with BBNA and sodium nitrate resulted in the development of carcinoma in six (17.6%; $P < 0.05$), papilloma in five (14.7%) and papillary and nodular hyperplasia of the urothelium in 22 rats (65%). No morphological changes of the urothelium were observed in males treated with sodium nitrate alone or in control animals. No spontaneous tumours of the urinary bladder were reported in historical controls of this strain of animal. The authors concluded that sodium nitrate significantly promoted bladder carcinogenesis induced by BBNA (Pliss & Frolov, 1991).

3.2 Nitrite

In most of the studies reviewed, mice or rats that were exposed to nitrite alone in the diet, by gavage or in the drinking-water did not have a higher incidence of tumours compared with untreated controls. It was noted that the negative findings may be due to the low doses of nitrite used, the short duration of exposure or the instability of nitrite. In some instances, when carcinogenic activity of nitrite was observed, the investigators noted the formation of *N*-nitroso compounds in the diet mix or in the stomach contents.

3.2.1 *Mouse*

(a) Oral administration in the diet

Groups of 20 male and 20 female C57BL/6 mice, 6 weeks of age, were fed 0.5% sodium nitrite in the diet (prepared freshly each week) for 1 year. Groups of 100 males and 100 females were untreated. The treated animals were observed for 120 days and did not exhibit a higher tumour incidence (males, 2/11; females, 2/12) than the untreated controls (males, 8/95; females, 17/92), however, when a third group of 50 males and 50 females was fed 0.58% butylurea and a fourth group of 50 males and 70 females was treated with 0.5% sodium nitrite and 0.58% butylurea, an increase in tumour incidence was observed in the latter groups (males, 35/39; females, 27/40) compared with animals treated with butylurea alone (males, 7/26; females, 4/24). The tumours reported in the latter groups were mainly malignant lymphomas (Krishna Murthy et al., 1979). [The Working Group noted the small number of nitrite-treated animals. The effective number of animals represents the number of animals evaluated histologically rather than the number necropsied.]

(b) Oral administration in the drinking-water

Six groups of male and female Swiss mice, 6–11 weeks of age, were administered secondary amines (6.25 g piperazine/kg of diet, 40 males and 40 females; 6.33 g morpholine/kg of diet, 20 males and 20 females; or 1.95 g *N*-methylaniline/kg of diet, 20 males and 20 females) in the feed (continuously) with or without sodium nitrite in the drinking-water (1.0 g/L, 5 days per week) for 28 weeks and were killed 12 weeks later. Forty male and 40 female mice received 1.0 g/L sodium nitrite in the drinking-water alone and served as nitrite controls and 80 male and 80 female mice were given tap-water and served as untreated controls. An increased incidence of lung adenomas was observed in male and female (combined) mice exposed to piperazine (48/75 versus 10/68), morpholine (20/35 versus 5/38) or *N*-methylaniline (23/38 versus 6/36) in the diet and sodium nitrite in the drinking-water compared with their respective controls. Sodium nitrite alone (14/74) or the secondary amines alone produced no increased incidence of lung adenomas compared with that in untreated controls (20/144). The authors concluded that the increased incidence of lung adenomas probably resulted from in-vivo nitrosation of secondary amines by nitrite (Greenblatt *et al.*, 1971).

In a study that was designed to evaluate the carcinogenicity of concurrent administration of amino acids (proline, hydroxyproline and arginine) and sodium nitrite, 30 male and 30 female Swiss mice, 5–7 weeks of age, received 1 g/L sodium nitrite in the drinking-water for 26 weeks and were killed at 38 weeks. Sixty untreated male and female controls were given tap-water. No increase in the incidence of lung adenomas was seen in nitrite-treated mice compared with untreated controls. No increase in the incidence of lung adenomas was seen in mice treated with a mixture of nitrite and proline, hydroxyproline or arginine in the drinking-water compared with their respective controls (Greenblatt & Lijinsky, 1972).

Swiss mice [number unspecified], 6–11 weeks of age, were given 1.0 g/L sodium nitrite in the drinking-water for 28 weeks while being fed methylurea or ethylurea. After an observation period of 12 weeks, they developed an increased incidence of lung adenomas (nitrite + methylurea, 105 adenomas, 16/26 mice [$P < 0.001$], 4.0±4.4 adenomas/mouse [$P < 0.001$]; nitrite + ethylurea, 134 adenomas, 25/31 mice [$P < 0.001$], 4.3±3.6 adenomas/mouse [$P < 0.001$]) compared with groups fed alkylureas alone. Two control groups were either given 1.0 g/L sodium nitrite in the drinking-water on 5 days per week for 28 weeks and were observed for another 12 weeks before they were killed or remained untreated. These were the same controls as those described in Greenblatt *et al.* (1971). No difference in the incidence of tumours was observed between the untreated and nitrite-treated groups (untreated, 26 adenomas, 20/144 mice, 0.2±0.4 adenomas/mouse; nitrite-treated, 16 adenomas, 14/74 mice, 0.2±0.5 adenomas/mouse) (Mirvish *et al.*, 1972).

Groups of 40 male strain A mice (locally bred), 7–9 weeks of age, were given 1.0 g/L sodium nitrite in the drinking-water on 5 days per week for 25 weeks and were killed 13 weeks later. In another study, groups of 40 male strain A mice were given 2.0 g/L sodium nitrite in the drinking-water on 5 days per week for 20 weeks and were killed 10 weeks later. Untreated controls were given tap-water. The incidence of lung adenomas in both groups of nitrite (1.0 or 2.0 g/L)-treated animals (11/37 or 7/39) was similar to that in the untreated controls (12/37 and 5/39). However, when the mice were exposed to piperazine at 6.25 g/kg in the diet and sodium nitrite in the drinking-water at levels of 0, 0.05, 0.25, 0.5, 1.0 or 2.0 g/L, the incidence of lung adenomas increased with increased intake of sodium nitrite in the drinking-water (11/39, 10/39, 20/40 ($P < 0.001$), 30/39 ($P < 0.001$), 37/37 ($P < 0.001$) and 39/40 ($P < 0.001$), respectively). Sodium nitrite may exert a dose-related carcinogenic effect in mice by nitrosating piperazine *in vivo* (Greenblatt & Mirvish, 1973).

In a study that was designed to evaluate the carcinogenicity of nitrilotriacetic acid, groups of 40 male and 40 female Swiss mice, 8 weeks of age, received 1 g/L sodium nitrite in the drinking-water on 5 days per week for 26 weeks or sodium nitrite in combination with 5 g/L nitrilotriacetic acid in the drinking-water and were killed at 37–38 weeks. Groups of 40 males and 40 females that were given tap-water served as untreated controls. No difference in weight, survival or tumour incidence was observed between the nitrite-treated, nitrite/nitrilotriacetic acid-treated and untreated control mice (Greenblatt & Lijinsky, 1974).

Groups of male and female Swiss mice were exposed transplacentally to sodium nitrite with or without the pesticide carbendazim. Pregnant mice were given 0 or 0.05% sodium nitrite in the drinking-water with or without intragastric intubation with five doses of 500 mg/kg bw carbendazim [frequency unspecified] throughout pregnancy. Offspring were killed at 252 days of age. A fourth group served as untreated controls. Of the offspring exposed to carbendazim and sodium nitrite, 7/10 (70%) developed lymphosarcomas. Only 1/256 (0.4%) untreated mice developed lymphosarcomas. No malignant lymphomas were found in the 82 sodium nitrite-exposed or 85 carbendazim-exposed offspring mice (Börzsönyi *et al.*, 1976).

Groups of pregnant Swiss mice, 4–6 weeks of age, received a single intragastric dose of 0 (control) or 10 mg/kg bw sodium nitrite in distilled water followed by 0 (control) or 0.1% sodium nitrite in the drinking-water on days 15–21 of pregnancy. The animals (parents and offspring) were killed 10 months after the beginning of the experiment. No increase in tumour frequency was observed in either parent animals (19 nitrite-treated, 18 control) or their offspring (62 male and 71 female nitrite-exposed, 70 male and 62 female control) (Börzsönyi *et al.*, 1978). [The Working Group noted the short duration of exposure.]

Groups of 50 male and 50 female ICR mice, 8 weeks of age, were exposed to 0.125, 0.25 or 0.5% sodium nitrite in the drinking-water for 109 weeks. A group of 20 males and 20 females served as untreated controls. No difference in tumour incidence, latency period or tumour type was observed between the nitrite-exposed and control mice (Inai *et al.*, 1979).

In a study that was designed to evaluate the carcinogenicity of cimetidine and nitrite combined, the effects of transplacental, transmammary and oral exposure to nitrite for life was studied in (C57BL/6 × BALB/c)F_1 mice (also called B6CF_1 mice). Female C57BL/6 and male BALB/c parent mice were exposed to 0.184 or 1.84 g/L nitrite in the drinking-water (starting 2 weeks before conception) and their offspring were also given nitrite in the drinking-water for life after delivery. Controls received nitrite-free water. Neither C57BL/6 dams (15–20/group) treated before and during pregnancy and until death nor female B6CF_1 mice treated from conception with the low or high dose of sodium nitrite developed more neoplasms than untreated controls. However, male B6CF_1 mice exposed to sodium nitrite from conception through gestation, lactation and life at a dose of 0.184 g/L had an increased incidence ($P < 0.029$) of lymphomas (16/52 versus 7/52 untreated controls) and an increased incidence ($P < 0.017$) of lung tumours [benign and malignant combined] (41/52 versus 30/52 untreated controls). The dose of 1.84 g/L had no effect on tumour incidence, survival or body weight (Anderson *et al.*, 1985). [The Working Group noted the absence of an increase in tumour incidence at the high dose.]

Male and female CBA and male and female C57Bl mice were subdivided into groups of 25, 30 or 40 male or female animals and were treated daily for the duration of the experiment (96 weeks) with sodium nitrite in the drinking-water (total dose consumed, ~0.02, 0.2, 1.6 or 2.0 g/mouse) alone or in combination with morpholine (total dose, 230 mg/mouse) added to the feed twice a week. One group of 30 male and 30 female C57Bl mice received a total dose of 230 mg morpholine alone in the feed. Two groups of 60 male and 60 female CBA mice and 30 male and 30 female C57Bl mice served as untreated controls. All tumours were examined histologically. Compared with untreated controls, statistically significant increases in the incidence of benign liver tumours (15/40 versus 9/53) in male CBA mice treated with 1.6 g sodium nitrite alone, of haemoblastosis (11/32 versus 3/53) in male CBA mice treated with 0.2 g sodium nitrite and morpholine and of malignant liver tumours (16/34 versus 11/57) in female CBA mice treated with 0.02 g sodium nitrate plus morpholine were observed (Iurchenko *et al.*, 1986). [The Working Group noted the limited reporting of the study.]

VM mice are susceptible to the spontaneous development of cerebral gliomas. A group of 100 male and female VM mice (equal sex ratios for all groups) were exposed to sodium nitrite *in utero* while suckling (pregnant mice were given 0.2% sodium nitrite in the drinking-water throughout pregnancy and suckling) and throughout their adult lives (0.2% sodium nitrite in the drinking-water). Another group of 200 male and female VM mice was administered 0.2% sodium nitrite in the drinking-water from the time of weaning. A third group of 200 male and female mice received distilled water from weaning and served as controls. The mice were maintained until their natural death. No excess incidence of nervous system tumours occurred in the nitrite-exposed groups (0/100 and 2/200) compared with controls (1/200). Survival and body weights were similar among the groups (Hawkes *et al.*, 1992).

Groups of 50 male and 50 female B6C3F_1 mice, approximately 6 weeks of age, received drinking-water that contained 0, 750, 1500 or 3000 mg/L sodium nitrite (equal to average daily doses of 0, 60, 120 or 220 mg/kg bw for males and 0, 45, 90 or 160 mg/kg bw for females) for 2 years. The survival rate of the treated groups was similar to that of the controls. The mean body weights of females at the highest dose were lower than those of controls throughout the study, and the treated groups generally consumed less water than controls. In female mice, the incidence of squamous-cell papilloma and carcinoma (combined) of the forestomach (1/50, 0/50, 1/50 and 5/50) showed a positive trend ($P = 0.011$). The incidence of hyperplasia of the glandular stomach epithelium was significantly greater in males at the highest dose than in controls (0/50, 0/50, 2/50, 10/50; $P \leq 0.01$, Poly-3 test) (National Toxicology Program, 2001).

(c) Gavage studies

A group of male (C57BL × C3H)F_1 mice, 15 days of age, was given a single intragastric dose of 50 μg/g bw sodium nitrite and animals were killed periodically up to 110 weeks. Controls were gavaged with distilled water (0.1 mL/g bw). Two groups that were given a dose of diethylamine hydrochloride (50 μg/g bw) or a dose of diethylamine hydrochoride (50 μg/g bw) followed by a dose of nitrite (50 μg/g bw) were also included in the study. There were from 30 to 31 mice per group. The incidence of hepatic tumours was: control, 2/17 (all cancers); nitrite, 2/11 (one haemangioma and one adenoma); diethylamine hydrochoride, 5/15 (two cancers and three adenomas); and diethylamine hydrochoride/nitrite, 14/23 [four cancers and 10 adenomas; $P < 0.05$ versus the nitrite-treated group; $P < 0.01$ versus the control group]. The authors suggested that carcinogens were formed *in vivo* by interaction of the two compounds (Rijhsinghani *et al.*, 1982).

Groups of 30 male and 30 female ICR mice, 5 weeks of age, were administered 10 weekly doses of ethylthiourea and sodium nitrite by gavage with the following combinations of treatment (ethylthiourea versus sodium nitrite, mg/kg bw per week): 0 versus 0 (untreated controls), 100 versus 0 (ethylthiourea-treated group), 0 versus 70 (nitrite-treated group), 25 versus 17.5 (low-dose ethylthiourea/nitrite-treated group), 50 versus 35 (mid-dose ethylthiourea/nitrite-treated group) and 100 versus 70 (high-dose ethylthiourea/nitrite-treated group). Mice were allowed to live without further treatment

up to 18 months after the first administration. No increase in tumour incidence was observed in the nitrite-treated or ethylthiourea-treated groups compared with untreated controls. An increased incidence of malignant lymphoma in the high-dose ethylthiourea/nitrite-treated group compared with the ethylthiourea-treated group in both males (13/30 versus 3/30; $P < 0.05$) and females (19/30 versus 7/30; $P < 0.05$), of lung adenoma and adenocarcinoma combined in the mid- and high-dose ethylthiourea/nitrite-treated group compared with the ethylthiourea-treated group in both males (22/30 and 25/30 versus 9/30; $P < 0.05$) and females (16/30 and 21/30 versus 4/30; $P < 0.05$), of lung adenocarcinoma in the high-dose ethylthiourea/nitrite-treated group compared with the ethylthiourea-treated group in both males and females (5/30 versus 0/30; $P < 0.05$), of forestomach squamous-cell carcinoma and squamous-cell papilloma/carcinoma combined in the high-dose ethylthiourea/nitrite-treated group compared with the ethylthiourea-treated group in both males (5/30 and 12/30 versus 0/30; $P < 0.05$) and females (5/30 and 8/30 versus 0/30; $P < 0.05$) and of uterine adenocarcinomas in the high-dose ethylthiourea/nitrite-treated group compared with the ethylthiourea-treated group (6/30 versus 0/30; $P < 0.05$) were observed (Yoshida *et al.*, 1993). [The Working Group noted the use of low doses.]

(d) Oral administration with known carcinogens

The effect of sodium nitrite administered in the drinking-water to CC57Br and AKR/J mice on the development of leukaemia and thymic lymphoma was investigated. Groups of 2-day-old CC57Br mice were infected with the Mazurenko leukaemia virus and were given 0 (control), 50 or 500 mg/L sodium nitrite in the drinking-water beginning 1 month after infection until death. The incidence of leukaemia and mean lifespean of leukaemic mice were, respectively: 19/24 and 229±28 days, 29/34 ($P < 0.05$) and 183±14 days ($P < 0.05$), and 26/34 and 194±6 days ($P < 0.05$). Groups of 2-month-old AKR/J mice were infected with the Gross leukaemia virus and given 0 (control) or 2000 mg/L sodium nitrite in the drinking-water until death. The incidence of thymic lymphoma and mean lifespan of mice with lymphoma were, respectively: 30/40 and 312.2±24 days and 36/40 and 246±38 days ($P < 0.05$). The authors concluded that sodium nitrite enhances the carcinogenic effect of leukaemia viruses *in vivo* (Ilnitsky & Kolpakova, 1997).

3.2.2 *Rat*

(a) Oral administration in the diet

(i) *Long-term carcinogenicity studies*

Groups of 30 male and 30 female weanling Wistar rats were fed a diet (prepared freshly each week) containing 40% canned meat and 0, 0.02 or 0.5% sodium nitrite (0, 200 or 5000 mg/kg of diet), with or without 1% glucono-δ-lactone (to lower the pH), for 2 years. Full histopathology was performed. No differences were observed in the

incidence of tumours or preneoplastic changes between the sodium nitrite-treated and control rats (van Logten *et al.*, 1972).

Groups of F_0 pregnant Sprague-Dawley rats were fed diets containing 0 or 1000 mg/kg sodium nitrite with or without 1000 mg/kg morpholine from mating onwards. The diets were prepared freshly each week, and the concentration of sodium nitrite decreased gradually; the weekly average concentration was 263 mg/kg. Randomly selected F_1 and F_2 offspring were maintained on the same diets and the experiment was terminated at week 125 of life of the F_2 generation. Results were presented for the F_1 and F_2 generations combined. No difference in survival was observed among the groups. Rats ($n = 96$) fed sodium nitrite in the diet developed a higher incidence (61%) of total tumours compared with 156 untreated controls (18%). The incidence of lymphoreticular tumours was also higher in sodium nitrite-treated (27%) than in untreated groups (6%). Rats ($n = 159$) fed sodium nitrite and morpholine combined developed a higher incidence (97%) of liver-cell carcinoma compared with 104 morpholine-treated controls (3%) (Shank & Newberne, 1976).

Four groups of male Wistar rats, 5 weeks of age, were fed a pelleted diet (CE2, CLEA) containing sodium nitrite (0.16%) and methylguanidine (0.16%) (Group 1; 20 rats), the pelleted diet containing 0.16% sodium nitrite (Group 2; five rats), the diet containing 0.16% methylguanidine (Group 3; five rats) or the pelleted diet alone (Group 4; 10 rats). The diets [storage conditions not specified] were used within 5 months after their preparation, and the study lasted 480 days. *N*-Nitroso compounds in the diet were determined with a thermal energy analyser. After 1 month, the basal diet contained only 5 ppb total *N*-nitroso compounds and less than 4 ppb NDMA, while *N*-nitrosopyrrolidine (NPYR) was not detectable. In the Group 2 diet, the concentrations of *N*-nitroso compounds were 2.52 ppm and 3.60 ppm at 1 and 5 months after preparation of the diet, respectively, those of NDMA were 1.86 and 3.36 ppm, respectively, and those of NPYR were 130 ppb and 110 ppb, respectively. No tumours were found in Group 4 rats. One rat in Group 2 died at 11 months with no detectable tumour. Among the remaining rats in Group 2, the incidence of liver tumours was 3/4 ($P < 0.02$); more precisely, the incidence of liver haemangioma was 2/4 and that of bile duct adenoma was 3/4. The authors concluded that the data demonstrated that *N*-nitroso compounds were formed in the feed during storage and possibly in the rat stomach (Matsukura *et al.*, 1977). [The Working Group noted the very small number of animals.]

In a follow-up report, three groups of 24 male Wistar rats, 8 weeks of age, were fed a pelleted diet mixed with 0 (control), 800 or 1600 mg/kg sodium nitrite for 646 days. The diets were prepared every 3 months and were kept at –10°C. The incidence of tumours was 0/19 in the control group, 1/22 (one hepatocellular adenoma) in the low-dose group and 5/19 ($P < 0.05$; two hepatocellular adenomas, one haemangioendothelioma, one hepatocellular carcinoma and one haemangioendothelial sarcoma) in the high-dose group. However, the nitrite diets were found to contain NDMA (1.26–2.01 ppm in the low-dose and 4.08–4.74 ppm in the high-dose diets) and NPYR (0.04–0.06 ppm in the low-dose and 0.13–0.11 ppm in the high-dose diets). *N*-Nitroso compounds were not detectable in

the control diet. The authors concluded that these preformed nitrosamines were probably the principal cause of the liver tumours (Aoyagi *et al.*, 1980).

Groups of male and female Fischer 344 rats, 6 weeks of age, were given 0 (50 males and 55 females) or 0.5% (20 males and 20 females) sodium nitrite in the feed for 1 year and were observed for a further 120 days. The incidence of neoplasms observed in controls was: males, 30/50 (60%); and females, 20/44 (45%); that in nitrite-treated groups was: males, 8/16 (50%); and females, 6/16 (37%). Although sodium nitrite in the feed did not cause a higher tumour incidence in male or female Fischer 344 rats, when 0.5% sodium nitrite and 0.58% butylurea were mixed together in the feed and given to 50 males and 50 females, a higher incidence of tumours (mainly Zymbal gland carcinoma, lung adenoma and squamous-cell papilloma and carcinoma of the forestomach) occurred (43/46 males and 44/45 females) compared with 50 male and 50 female rats fed 0.58% butylurea only (7/16 males and 5/16 females) (Krishna Murthy *et al.*, 1979). [Despite the short duration of exposure, a high incidence of treatment-related tumours was observed. The effective number of animals represents the number of animals histologically evaluated rather than the number necropsied.]

Feeding BD rats by gastric intubation from day 13 to day 23 of pregnancy with L-citrulline (100 mg/kg bw) and sodium nitrite (50 mg/kg per day) induced malignant tumours of the kidney (diagnosed as Wilms tumour) in 6/22 of the offspring. Neither of the controls given either compound alone developed tumours (Ivankovic, 1979). [The Working Group noted the limited reporting of the study.]

Groups of 68 male and 68 female Sprague-Dawley rat offspring [age unspecified] were administered sodium nitrite in the diet for up to 26 months. Groups 1–5 received 0, 250, 500, 1000 or 2000 ppm (mg/kg) sodium nitrite in an agar gel casein diet; Group 8 received urethane in agar diet; Groups 9–11 were fed commercial chow that contained 0, 1000 or 2000 ppm nitrite; Group 12 received urethane in chow; Groups 13–14 were fed an agar gel casein diet in a dry form that contained 0 or 1000 ppm nitrite. For Groups 1–14, the diets had been given to dams beginning 5 days before birth. Groups 15 and 16 were the respective dams of Groups 1 and 4 rat offspring that received 0 and 1000 ppm nitrite in agar diet. Groups 17–18 were only exposed to 0 or 1000 ppm nitrite in agar diet beginning at weaning. The experiment was terminated at 26 months. The incidence of malignant lymphomas was increased in nitrite-treated Groups 2–5 compared with control rats (Group 1) fed agar gel diet and increases were also observed in Groups 10, 11, 14, 16 and 18 compared with their respective control (i.e. Groups 9, 13, 15 and 17). The incidence of lymphoma in all control groups (combined) was 5.4% and that in all the treated groups (combined) was 10.2%. The increase was significant (Newberne, 1979). The findings were not confirmed following a review of the histology by a governmental interagency working group submitted to the US Food and Drug Administration. That working group arrived at different diagnoses and reported a smaller number of malignant lymphomas. Some of the lesions diagnosed as lymphomas by Newberne (1979) were classified as extramedullary haemopoiesis, plasmocytosis or histiocytic sarcomas. It was concluded that the incidence of lymphomas and other types of tumour was similar to that

arising spontaneously in Sprague-Dawley rats and no evidence existed that an increased incidence of tumours was induced by ingestion of sodium nitrite (Walker, 1990).

A group of 20 male and 20 female Fischer 344 rats, 8–9 weeks of age, was fed a mixture of 0.1% disulfiram with 0.2% sodium nitrite in a powdered diet for 78 weeks. The diet was mixed at first every 2 weeks and then weekly and was stored at 4°C until placed in the feeders every 2–3 days. Analysis of the food showed the presence of traces of *N*-nitrosodiethylamine (NDEA) after 7 days of storage and no more than 200 μg/kg after 17 days. Control groups of 20 male and 20 female rats were fed 0.2% sodium nitrite. A control group of 50 males and 50 females fed disulfiram only from a previous bioassay was also used (National Toxicology Program, 1979). The animals were observed until death. It was estimated that male rats consumed 30 mg disulfiram and 60 mg sodium nitrite per day and females 20 mg disulfiram and 40 mg sodium nitrite per day. Benign and malignant tumours were observed in the oesophagus (males, 7/20; females, 11/20), tongue (males, 0/20; females, 2/20), forestomach (males, 3/20, females, 0/20) and nasal cavity (olfactory adenocarcinomas; males, 2/20; females, 2/20). None of these tumours were observed in rats fed either disulfiram (National Toxicology Program, 1979) or sodium nitrite alone at similar doses. The authors hypothesized that these tumours were due to the reaction of disulfiram and nitrite in the stomach, which resulted in the formation of NDEA (Lijinsky & Reuber, 1980).

In a series of two experiments, the second of which began 3 months after the first, groups of 24 male and 24 female Fischer 344 rats, 7–8 weeks of age, were fed 0 or 2000 mg/kg (2000 ppm) sodium nitrite in the diet (the mixture was prepared weekly and no evidence of instability of sodium nitrite was found) for 104 weeks and observed until experimental week 127–130. The incidence of hepatocellular neoplasms in the two female groups (combined) exposed to sodium nitrite was significantly higher than that in the untreated controls (27/48 versus 8/48; $P < 0.05$); that in treated and untreated males was similar (9/48 versus 8/48). A decrease in the incidence of monocytic leukaemia was observed in treated males (6/48 versus 24/48) and females (4/48 versus 18/48). No treatment-related increases in other tumour types were observed (Lijinsky *et al.*, 1983). [This study lacked data for life parameters including growth curve and daily food consumption as well as intake of sodium nitrite. Thus, the effect of nutritional condition on the reduction in the incidence of leukaemia could not be measured.]

In a study of the effects of amines with sodium nitrite, groups of 20–24 male and 20–24 female Fischer 344 rats, 7–8 weeks of age, were fed 0 or 2000 ppm sodium nitrite in the diet for 106 weeks with or without 0.1% chlorpheniramine maleate, 0.2% diphenhydramine hydrochloride or 0.2% allantoin and were observed until death. Based on food intake, the males were estimated to have consumed 42 g and females 28 g sodium nitrite. Survival was similar in all groups. An increase in the incidence (13/24 versus 4/24; $P = 0.015$) of hepatocellular adenoma and carcinoma (combined) was reported in females but not in males (3/24 versus 5/24) treated with nitrite compared with untreated controls; the incidence of hepatocellular carcinoma was 3/24 versus 0/24 female controls. In male rats given diphenhydramine or chlorpheniramine maleate concurrently with sodium

nitrite, there was a significant increase in the incidence of hepatocellular adenoma and carcinoma combined (diphenhydramine + nitrite, 11/24 versus 4/24, $P < 0.05$; chlorpheniramine maleate + nitrite, 14/24 versus 3/24, $P < 0.05$) compared with their respective control groups given the amine alone; the incidence of hepatocellular carcinoma was 2/24 and 8/24 ($P = 0.0039$) versus 0/24 male controls, respectively (Lijinsky, 1984). [This study lacked data on life parameters including body weight and feed/water consumption during the experiment.]

In a two-generation study, male and female Wistar rats [age unspecified] were fed a diet of cured meat that had been treated with 0, 200, 1000 or 4000 mg/kg potassium nitrite (expressed as sodium nitrite) and were allowed to breed 10 weeks later. The diets were canned and stored for 1 month at room temperature and then at 4°C before use. The presence of volatile *N*-nitroso compounds was demonstrated only in meat to which 4000 mg/kg nitrite had been added: up to 30 µg/kg NDMA were found during the experiment. Reproduction outcomes were similar among the F_0 groups. After weaning, the F_1 generation rats (140, 120, 120 and 132 males and females) were fed the same diets as the F_0 generation and were killed at experimental week 132. There was no significant difference in survival or body weight among the groups. In males, the total incidence (all organ sites examined) of malignant tumours was increased with a significantly positive trend using the Armitage-Cochran test (males: 9/70, 10/60, 10/60 and 16/66; females: 15/70, 12/60, 11/60 and 15/66) [no *P* values provided]. The authors hypothesized that the increase in tumour incidence may be attributed to preformed nitrosamines present in the diet (Olsen *et al.*, 1984).

Two groups of 50 male Fischer 344 rats, 5–6 weeks of age, were fed 0.2 or 0.5% (w/w) sodium nitrite mixed in a reduced-protein diet for up to 115 weeks; the diets were prepared weekly. A control group of 20 male rats received the reduced-protein diet only. Body weight gain was decreased ($P < 0.05$) [~25%; read from figure] in the 0.5% nitrite-treated group. A dose-related decrease was noted in both the time of onset and the incidence of lymphomas (15/20, 14/50 ($P < 0.001$) and 10/49 ($P < 0.001$)), mononuclear-cell leukaemia (9/18, 7/39 and ($P < 0.05$) and 3/40 ($P < 0.001$)) and testicular interstitial-cell tumours (16/20, 31/48 ($P < 0.05$) and 7/50 ($P < 0.001$)) (Grant & Butler, 1989). [The Working Group noted the reduction in body weight.]

Three groups of 13 Sprague-Dawley rats [sex unspecified], 6 weeks of age, were fed Purina rat chow that contained wheat (10%), squid (10%, dehydrated at 60°C and then crushed to powder) or squid (10%) plus sodium nitrite (0.3%) for 10 months [no information on the frequency of preparation of the mixture or storage provided]. Levels of nitrosamines in the diets were not measured. Squid contains high levels of naturally occurring amines, such as trimethylamine-*N*-oxide and dimethylamine. The squid- and nitrite-treated rats had lower body weights compared with wheat-treated controls. The incidence of hepatocellular carcinoma was 0/13 (0%) in rats fed the diet that contained 10% wheat, 2/12, (16%) in rats fed the diet that contained 10% squid and 4/12 (33%) in rats fed the diet that contained 10% squid and 0.3% sodium nitrite (Lin & Ho, 1992). [The Working Group noted the small number of animals used.].

(ii) *Combined administration of nitrite and nitrosatable compound*

Seven groups of F_0 female Sprague-Dawley rats were fed from conception and for life a diet (prepared freshly each week) that contained nitrite or morpholine: Group 1, diet with no addition; Group 2, 1000 ppm nitrite and 1000 ppm morpholine; Group 3, 1000 ppm nitrite and 50 ppm morpholine; Group 4, 1000 ppm nitrite and 5 ppm morpholine; and Group 5, 50 ppm nitrite and 1000 ppm morpholine. After weaning, the F_1 generation rats were fed the same diet for life. The results for F_0 females and F_1 males and females were combined. Sodium nitrite at 1000 ppm combined with 50 or 1000 ppm morpholine (Groups 2 and 3) induced a dose-related increase in the incidence of hepatocellular carcinoma (24/122 and 156/159 versus 0/160), liver angiosarcoma (11/122 and 38/159 versus 0/160) and lung angiosarcoma (2/122 and 37/159 versus 0/169) compared with controls (Group 1). The incidence of metastatic hepatocellular carcinoma (10/122 and 109/159 versus 0/169) to the lung was also increased. The authors concluded that the data provided evidence of in-vivo nitrosation, presumably under acidic conditions in the rat stomach (Newberne & Shank, 1973). [The Working Group noted the absence of a group treated with morpholine only].

(b) Oral administration in the drinking-water

(i) *Long-term carcinogenicity studies*

Lijinsky *et al.* (1973a) reported negative results in a 2-year study when groups of 15 male and 15 female MRC rats, 8–10 weeks of age, were given 0 (untreated control) or 2000 ppm sodium nitrite in the drinking-water at a daily rate of 20 mL/rat with or without nitrilotriacetic acid for 84 weeks.

Groups of male and female MRC Wistar rats, 8 weeks of age, received 0 (control) or 3 g/L sodium nitrite in the drinking-water on 5 days per week for life (total dose, 63 g/kg). The incidence of forestomach squamous papillomas was significantly greater ($P < 0.002$ by the life-table method) in sodium nitrite-treated animals (18%; 5/22 males and 3/23 female) than in control animals (2%; 2/47 males and 0/44 female), which suggested that sodium nitrite was tumorigenic in this experiment (Mirvish *et al.*, 1980).

Three groups of 68 male and 68 female Sprague-Dawley rat offspring [age unspecified] were administered 0, 1000 or 2000 ppm sodium nitrite in the drinking-water for up to 26 months. Dams had been given 0, 1000 or 2000 ppm sodium nitrite in a semi-synthetic diet beginning 5 days before birth. The incidence of lymphomas was 5/136 (3.7%), 16/136 (11.8%), and 14/136 animals (10.3%) in the 0-, 1000- and 2000-ppm nitrite-treated groups, respectively. The incidence of other types of tumours was not increased (Newberne, 1979). A US governmental interagency working group, however, came to different conclusions following a review of the histology. That working group diagnosed only a small number of lesions as lymphomas and assessed an incidence of approximately 1% in both treated and control groups. This discrepancy concerned the differentiation between the lymphomas diagnosed by Newberne (1979) and the

extramedullary haemopoiesis, plasmacytosis or histiocytic sarcomas diagnosed by the interagency working group (Walker, 1990).

Groups of 50 male and 50 female Fischer 344 rats, 8 weeks of age, received 0, 0.125 or 0.25% sodium nitrite in the drinking-water for 2 years. Thereafter, tap-water and basal diet were given to all experimental groups and observation was continued until week 120. No carcinogenic effects were observed. The survival rate of the treated males was higher ($P < 0.05$) than that of controls. In females treated with 0.25%, mean body weight was decreased by more than 10% during the experiment relative to controls [statistics not provided]. There was a significant decrease in overall tumour incidence in high-dose females compared with controls: 73% (35/48) versus 92% (45/49; $P < 0.05$ by the χ^2 test). This reduced tumour incidence was due in part to a reduction in the incidence of mononuclear-cell leukaemias in treated animals and a high incidence in controls. [This neoplasm has a rather high spontaneous incidence in this rat strain.] Haematopoietic tumour incidence was 27% (13/49) in untreated females versus 8% (8/48) in 0.25% sodium nitrite-treated females ($P < 0.05$ by the χ^2 test). Treated males had a reduced incidence of haematopoietic tumours (most of which were mononuclear-cell leukaemias): 35% (16/46) in untreated males versus 10% (5/50) in 0.25% sodium nitrite-treated males and 10% (5/49) in the 0.125% sodium nitrite-treated males ($P < 0.05$ by the χ^2 test) (Maekawa *et al.*, 1982).

Groups of 24 male and 24 female Fischer 344 rats, 7–8 weeks of age, received 0 or 2000 ppm sodium nitrite in the drinking-water for 2 years. Survival of treated animals was slightly increased (average of time of death: 107 weeks for untreated males and 122 weeks for untreated females; 116 weeks for treated males and 130 weeks for treated females). The incidence of mononuclear-cell leukaemia was: control males, 12/24; control females, 11/24; treated males, 5/24; and treated females, 1/24 ($P = 0.0018$ versus untreated females by two-tailed Fischer test). No treatment-related differences were observed in other tumour types, while a slight, but a non-significant increase ($P = 0.060$) in the incidence of liver neoplasms (carcinomas and neoplastic nodules) was observed in females (control females, 0/24 carcinoma and 4/24 neoplastic nodules; treated females, 1/24 carcinoma and 10/24 neoplastic nodules) (Lijinsky *et al.*, 1983; Lijinsky, 1984). [This study lacked data on life parameters including growth curve, daily food consumption and intake of sodium nitrite. Thus, the effect of nutritional condition on the reduction in the incidence of leukaemia could not be measured.]

Groups of 50 male and 50 female Fischer 344/N rats, approximately 6 weeks of age, were exposed to 0, 750, 1500 or 3000 ppm sodium nitrite (equivalent to average daily doses of approximately 0, 35, 70, or 130 mg/kg bw for males and 0, 40, 80 or 150 mg/kg bw for females) in the drinking-water for 105 weeks. The survival rate of the treated groups was similar to that of controls. The mean body weights and water consumption of males and females exposed to 3000 ppm were less than those of controls throughout the study. The incidence of hyperplasia of the forestomach epithelium in both sexes at 3000 ppm was significantly greater than that in the control groups (males, 12/50 at 0 ppm versus 44/50 at 3000 ppm; females, 8/50 at 0 ppm versus 40/50 at 3000 ppm; $P < 0.01$ by

the Poly-3 test). In females, the incidence of fibroadenoma (multiple and single combined) of the mammary gland was increased only at 1500 ppm (31/50 versus 21/50; $P = 0.029$) and exceeded the historical range for National Toxicology Program (NTP) controls given NTP-2000 diet (all routes) (108/299; 36.1±6.2%). The incidences in the 750-ppm (54%) and 3000-ppm (50%) groups also exceeded the range for the NTP controls and the incidence in the control group (42%) equaled the highest incidence in the NTP-2000 diet historical control database. The incidence of multiple fibroadenoma was also increased at both 750 and 1500 ppm; however, these neoplasms occur with a high background incidence, and no increase was seen at 3000 ppm (multiple fibroadenomas: 7/50, 13/50, 13/50 and 5/50 at 0, 750, 1500 and 3000 ppm, respectively). The incidence of mononuclear-cell leukaemia was significantly decreased in both sexes at 1500 ($P \leq 0.01$) and 3000 ppm ($P \leq 0.001$) (incidence in males: 17/50, 12/50, 7/50 and 3/50 at 0, 750, 1500 and 3000 ppm, respectively; incidence in females: 15/50, 10/50, 1/50 and 1/50 at 0, 750, 1500 and 3000 ppm, respectively) and were less than the historical ranges for NTP controls (all routes) given NTP-2000 diet (130/299; 43.5±9.6%) (National Toxicology Program, 2001).

(ii) *Combined administration of nitrite and nitrosatable compounds*

In a study of electron spin resonance signal in the liver of rats treated with hepatocarcinogens, tumour induction was examined in male Holtzman rats [age unspecified, but it was stated that rats weighed about 140 g and were maintained on standard diet for 1–3 weeks before the experiment began] that were fed synthetic or chow diet with or without 0.067% 2-acetylaminofluorene (AAF) in the presence or absence of nitrite (40 mg/L nitrite-N) in the drinking-water for up to 8 months. A reduction in liver tumours was found in the group treated with nitrite and AAF–synthetic diet (24/38) compared with the group treated with AAF and maintained on distilled water (25/26). The authors concluded that nitrite inhibits the hepatocarcinogenicity of AAF (Commoner *et al.*, 1970) [statistical analysis was not performed] .

Induction of malignant tumours was examined in rats following administration of secondary or tertiary amines together with sodium nitrite in the drinking-water. Groups of 15 male and 15 female Sprague-Dawley rats [age unspecified] were given drinking-water on 5 days a week that contained 2000 ppm heptamethyleneimine hydrochloride + 2000 ppm sodium nitrite for 28 weeks, 1000 ppm aminopyrine + 1000 ppm sodium nitrite for 30 weeks or 250 ppm aminopyrine + 250 ppm sodium nitrite for 50 weeks, and were observed until death. Control groups of 15 male and 15 female rats were each given 2000 ppm heptamethyleneimine hydrochloride, 1000 ppm aminopyrine or 2000 ppm sodium nitrite. Treatment with aminopyrine + nitrite (1000 ppm) resulted in the death of all animals within 1 year and induced haemangiosarcomas of the liver in most cases (29/30). Of the 30 animals that received the lower dose of aminopyrine + nitrite, 14 died within the first year, 12 of which had liver haemangiosarcomas and two had other types of tumour. In the group treated with heptamethyleneimine hydrochloride + sodium nitrite, 23/30 animals died in the first year of treatment, and 20 had developed primary tumours

at one or more site. The most prevalent tumours were oesophageal papillomas (17 animals) and squamous-cell carcinoma of the lung (13 cases). Of the control animals, only one died with a large mammary tumour. The authors suggested that the interaction of secondary and tertiary amines with nitrite in the stomach may represent an important facet in the etiology of human cancer by generation of nitrosoamines in the stomach (Lijinsky *et al.*, 1973b).

Groups of 15 male and 15 female Sprague-Dawley rats, 8–10 weeks of age, were given approximately 20 mL of a drinking-water solution that contained either 0.2% heptamethyleneimine hydrochloride or this salt together with 0.2% sodium nitrite on 5 days a week for 28 weeks. Another group of 27 male and 30 female rats was given 0.2% sodium nitrite solution alone for 104 weeks. Most of the animals given heptamethyleneimine hydrochloride or sodium nitrite alone survived 2 years or more after the beginning of the treatment, and no tumours that could be attributed to the treatment were seen at death; tumours were those of endocrine origin that are found commonly in untreated controls. In the group that received the combined treatment, most females had died by 50 weeks and most males had died by 80 weeks, 27/30 of which had tumours that were not seen in either control group: a total of 16 had squamous-cell carcinomas of the lung; 25 had tumours (mostly papillomas) of the oropharynx, tongue, oesophagus and forestomach; eight had squamous-cell carcinomas of the nasal cavity; and four had metaplasias or papillomas of the larynx and trachea (Taylor & Lijinsky, 1975a). [Although tumour induction was evident following combined administration of heptamethyleneimine and sodium nitrite, statistical analysis for a comparison of the tumour incidence was not provided.]

Groups of 15 male and 15 female Sprague-Dawley rats, 8 weeks of age, were given combinations of 0.1% or 0.025% aminopyrine or 0.1% oxytetracycline with or without 0.1% or 0.025% sodium nitrite in the drinking-water. The experiment was terminated in experimental week 104 (aminopyrine-treated groups) or 130 (oxytetracycline-treated groups). Of 30 animals that received 0.1% aminopyrine and 0.1% sodium nitrite for 30 weeks, 29 developed haemangiosarcomas of the liver. The same tumour developed in 26/30 animals that received 0.025% aminopyrine and 0.025% sodium nitrite for 50 weeks. No animals in the control group that received 0.1% aminopyrine for 30 weeks developed this tumour. After treatment with 0.1% oxytetracycline and 0.1% sodium nitrite for 60 weeks, liver tumours were present in 4/30 rats (three hepatocellular tumours [tumour type not further specified] and one cholangioma). No liver tumours were observed in the controls treated with oxytetracycline for 60 weeks (Taylor & Lijinsky, 1975b).

The possible formation of *N*-nitroso compounds from ingested secondary or tertiary amines and nitrite was tested with 13 amino compounds that were administered to groups of 15 male and 15 female Sprague-Dawley rats, 8–10 weeks of age, on 5 days per week in the drinking-water as follows: 0.1% arginine with 0.2% sodium nitrite, 0.2% chlordiazepoxide with 0.2% sodium nitrite, 0.2% chlorpromazine alone or 0.1% chlorpromazine with 0.2% sodium nitrite, 0.1% cyclizine with 0.2% sodium nitrite, 0.18%

dimethyldodecylamine with 0.2% sodium nitrite, 0.1% dimethylphenylurea with or without 0.2% sodium nitrite, 0.1% hexamethylenetetramine with or without 0.2% sodium nitrite, 0.14% lucanthone with or without 0.2% sodium nitrite, 0.1% methapyrilene with 0.2% sodium nitrite, 0.1% methylguanidine with 0.1% sodium nitrite, 0.09% piperidine with or without 0.2% sodium nitrite, 0.1% tolazamide with or without 0.2% sodium nitrite or 0.08% trimethylamine oxide with or without 0.2% sodium nitrite. Duration of treatment was 50 weeks except for cyclizine and dimethyldodecylamine (80 weeks) and methapyrilene (90 weeks). After the end of treatment, the animals were kept until they died spontaneously or until they became moribund and were killed. Survival rates differed little between the various groups and only a few of the amines, either alone or in combination with nitrite, induced a significant incidence of malignant tumours. Chlordiazepoxide plus nitrite induced nervous system tumours in 4/30 effective animals (one spinal cord tumour in 15 effective females and one brain glioma, one malignant neurinoma of spinal nerves and one heart neurofibrosarcoma in 15 effective males); methapyrilene plus nitrite induced liver tumours in 9/29 effective animals (four cholangiocarcinomas, one hepatocellular carcinoma and one liver haemangiosarcoma in 14 females and one cholangiocarcinoma and two hepatocellular carcinomas in 15 males); and dimethyldodecylamine plus nitrite induced tumours of the urinary bladder in 3/24 effective animals (one papilloma in nine females and one transitional-cell carcinoma and one leiomyosarcoma in 15 males). Sodium nitrite-treated controls [described in Taylor & Lijinsky (1975a)] developed three thyroid adenomas, one thyroid adenocarcinoma, one multiple myeloma, one pancreatic islet-cell adenoma, one thymic lymphoma and one squamous-cell carcinoma of the ear in 30 females and two thyroid adenomas and two adenocarcinomas, one brain ependymoma, two pancreatic islet-cell adenomas and two carcinomas, one hepatocellular carcinoma, one parotid-gland squamous-cell carcinoma, one forestomach carcinoma and two gut adenocarcinomas in 26 males. The authors concluded that these results provide further evidence that ingestion of secondary and tertiary amines together with nitrite can lead to the formation of significant amounts of carcinogenic *N*-nitroso compounds in the stomach (Lijinsky & Taylor, 1977). [Statistical analysis was not provided and the control group of a previously published study in which a 0.2% sodium nitrite solution was given for 2 years to 26 male and 30 female rats was used as the concurrent control (Taylor & Lijinsky, 1975a).]

Two groups of 24 male and 24 female Fischer 344 rats, 7–8 weeks of age, were given drinking-water that contained 0.1% *N,N*-dimethyldodecylamine-*N*-oxide (DDAO) with or without 0.2% sodium nitrite on 5 days per week for 93 weeks. Two other groups of 24 male and 24 female control rats were given untreated drinking-water or 0.2% sodium nitrite in the drinking-water. At the end of the treatment period, the rats were observed until death. Any survivors were killed between week 127 and week 130 of the experiment. There was no marked life-shortening effect in any treatment group. Administration of DDAO alone did not induce an increased incidence of any tumour in comparison with the untreated control group. In male rats given DDAO concurrently with nitrite, a significant increase in the incidence of liver neoplasms (hepatocellular

carcinomas and neoplastic nodules, 10/24 animals versus 3/24 untreated controls; $P = 0.049$ by Fisher's exact probability test) was observed. The author suggested that the ingestion of DDAO under conditions where it could be nitrosated with nitrite in the stomach might present an increased carcinogenic risk (Lijinsky, 1984). [This study lacked data on life parameters including body weight and feed/water consumption during the experiment].

The carcinogenic activity of endogenously synthesized *N*-nitroso-bis(2-hydroxypropyl)amine (NDHPA) was investigated in rats administered 1% bis(2-hydroxypropyl)amine (DHPA) mixed into a powdered diet and 0.15% or 0.3% sodium nitrite dissolved in distilled water for 94 weeks (Yamamoto *et al.*, 1989). Groups of 20–28 male Wistar rats, 6 weeks of age, were divided into untreated controls (group 1) and groups treated with 0.15% sodium nitrite (group 2), 0.3% sodium nitrite (group 3), 1% DHPA (group 4), 1% DHPA + 0.15% sodium nitrite (group 5) or 1% DHPA + 0.3% sodium nitrite (group 6). The experiment was terminated at experimental week 94. Three to five animals from groups 1, 3, 4 and 6 were used to investigate urinary excretion of endogenously synthesized NDHPA at experimental weeks 24, 34 and 80. As a result, endogenous synthesis of NDHPA was clearly observed in rats given 1% DHPA and 0.3% sodium nitrite at any time-point (0.97–1.5 μmol/rat per dose), but not in the groups that received either of these precursors alone. Tumours of the nasal cavity, lung, oesophagus, liver (hepatocellular carcinoma) and urinary bladder (carcinoma) were found in rats treated with 1% DHPA and 0.15% or 0.3% sodium nitrite. In rats given 1% DHPA and 0.3% sodium nitrite, the incidence of tumours of the nasal cavity (papilloma and carcinoma, combined), lung (benign and malignant, combined) and oesophagus (papilloma) was 74% (14/19, $P < 0.01$ versus groups 1–5), 58% (11/19, $P < 0.01$ versus groups 1–4 and $P < 0.05$ versus group 5) and 11% (2/19), respectively. The tumour distribution was almost the same as that seen in rats given NDHPA (Konishi *et al.*, 1976; Mohr *et al.*, 1977; Pour *et al.*, 1979), indicating that endogenously synthesized NDHPA has similar carcinogenic activity to exogenously administered NDHPA in rats (Yamamoto *et al.*, 1989; Konishi *et al.*, 1990).

Induction of cancers and precancerous lesions of the oesophagus was examined in rats following the administration of precursors of *N*-nitrososarcosine ethyl ester. A total of 90 non-inbred male Wistar rats, 6 weeks of age, were given 2 g/kg bw sarcosine ethyl ester hydrochloride and concurrently 0.3 g/kg bw sodium nitrite, which are precursors of *N*-nitrososarcosine ethyl ester, in 2% sucrose as drinking-water. Group 1 (28 rats) received the precursors twice a week for 6 weeks followed by 8 weeks of observation and group 2 (34 rats) received the precursors once every 3 days for 7 weeks followed by 26 weeks of observation. Group 3 (28 rats) served as untreated controls. At the end of the treatment period, no tumour had developed in the oesophagus of three rats in group 1 that were killed. On subsequent observation, papillomas appeared in 1/3 rats in group 1 that were killed and carcinomas in 1/3 rats in group 2 that were killed within 4 weeks. The tumours induced in group 1 at the end of the experiment were mostly papillomas (all tumours, 12/20) and rarely carcinomas (3/20). When the observation was prolonged in

group 2, 100% (10/10) of the animals had carcinomas at observation week 20 (Xiang *et al.*, 1995). [No tumour incidences were reported for the untreated group.]

The effects of oral administration of ethylenethiourea (ETU) and sodium nitrite on the development of endometrial adenocarcinoma was investigated in female Donryu rats. A total of 119 females were divided into four groups. At 10 weeks of age, groups 1 and 3 received a single dose of polyethylene glycol (333 μL/kg bw) into the uterine cavity, while groups 2 and 4 were given *N*-ethyl-*N*-nitrosourea (ENU; 15 mg/kg bw), dissolved in polyethylene glycols in the same manner. ETU (80 mg/kg bw) and sodium nitrite (56 mg/kg bw) dissolved in distilled water were given orally to animals in groups 3 and 4 once a week by gavage from 11 to 51 weeks of age. Groups 1 and 2 received the vehicle alone. At termination (52 weeks of age), endometrial adenocarcinomas were observed in 29 (6/21), 13 (4/31) and 57% (21/37) of rats in groups 2, 3 and 4, respectively, but not in group 1 (0/21) (group 2 versus group 1, $P < 0.01$; group 4 versus group 1, $P < 0.001$; group 4 versus group 2, $P < 0.05$; and group 4 versus group 3, $P < 0.001$). Persistent estrus appeared earlier in groups 3 and 4 than in group 1. In groups 3 and 4, forestomach squamous-cell hyperplasias (16/31 and 12/37, respectively) and papillomas (14/31 and 19/37, respectively) were also observed, but with no difference between the two groups. All of the incidences were statistically increased ($P < 0.01$) versus group 1. The authors concluded that the results indicate that *N*-nitroso ETU formed in the stomach by concurrent oral administration of ETU and sodium nitrite induces endometrial adenocarcinomas by its mutagenic action, as well as promoting their development after initiation with ENU, presumably by influencing the hormonal balance as shown by the early appearance of persistent estrus (Nishiyama *et al.*, 1998). [Methods of statistical analysis were not shown.]

Six groups of 50 male and 50 female Fischer 344 rats, 6 weeks of age, were fed diets (basal diet) supplemented with 64%, 32% or 8% fish meal and were simultaneously given 0.12% sodium nitrite in the drinking-water (males, groups 1–3; and females, groups 7–9) or tap-water (males, groups 4–6; and females, groups 10–12). At experimental week 104, all surviving animals were killed and examined histopathologically. Treatment with fish meal dose-dependently increased the incidences and multiplicities of atypical tubules [tubular hyperplasia], renal-cell adenomas and renal-cell carcinomas in sodium nitrite-treated males. The incidence and multiplicity of each renal proliferative lesion in males were: atypical tubules: 73.4% ($P < 0.05$) and 1.95±1.82 ($P < 0.01$) in group 1, 42.2% ($P < 0.05$) and 0.61±0.84 ($P < 0.01$) in group 2 and 4.0% and 0.04±0.19 in group 3; adenoma: 67.3% ($P < 0.05$) and 1.40±1.33 in group 1, 25.5% ($P < 0.05$) and 0.38±0.73 in group 2 and 2.0% and 0.02±0.14 in group 3; and carcinoma: 57.1% ($P < 0.05$) and 1.61±1.98 ($P < 0.01$) in group 1 and 14.8% ($P < 0.05$) and 0.19±0.58 in group 2. Females were less susceptible than males to the induction of renal tumours: atypical tubules: 45.8% ($P < 0.05$) and 0.92±1.30 ($P < 0.01$) in group 7, 11.6% and 0.12±0.33 in group 8 and 6.3% and 0.07±0.26 in group 9; adenoma: 16.6% ($P < 0.05$) and 0.23±0.56 ($P < 0.01$) in group 7, 2.3% and 0.03±0.16 in group 8 and 2.1% and 0.02±0.14 in group 9; and carcinoma: 2.0% and 0.02±0.14 in group 7 and 0% and 0 in groups 8 and 9 (statistical

analysis by Fisher's exact probability test and one-way analysis of variance; comparison was made with the respective groups 4, 5, 6, 10, 11 or 12). The authors concluded that these results clearly indicate that concurrent administration of fish meal and sodium nitrite induces renal epithelial tumours (Furukawa *et al.*, 2000).

(c) Gastric intubation

Seven pregnant BD IX rats were treated on days 13–23 after conception with 100 mg/kg ethylurea and 50 mg/kg sodium nitrite daily by gavage. Ten of 13 newborn F_1 rats that died 77–211 days after delivery developed nine tumours of the nervous system (malignant neurinomas, mixed gliomas, medulloblastoma) and one myeloid leukaemia. Ethylurea only- and nitrite only-treated controls were available. No tumour development was observed in the treated mothers or in the ethylurea only- or nitrite only-exposed F_1 rats (Ivankovic & Preussmann, 1970). [This short communication does not provide any information on the number of animals in the control groups.]

A total 205 male and 195 female Wistar rats, 12 weeks of age, were divided into four groups and served as untreated controls (55 males and 45 females) or were treated by gastric intubation with aminopyrine alone (50 males and 50 females; 12.1 μmol; 2.8 mg/0.5 mL), with sodium nitrite alone (50 males and 50 females; 100 μmol; 6.9 mg/0.5 mL) or with aminopyine plus sodium nitrite (50 males and 50 females; doses identical to those of individual compounds) three times a week for 29 months. The males and females treated with aminopyrine plus nitrite had an increased incidence of various types of liver tumours (37/45 males and 27/42 females; $P < 0.05$ versus other groups by Fisher's exact probability test; untreated controls, 9/48 males and 3/41 females; aminopyrine alone, 3/44 males and 5/46 females; sodium nitrite alone, 13/44 males and 3/44 females). Unlike the other groups, more than half of the tumours in the aminopyrine plus nitrite-treated group were malignant (mainly hepatocellular carcinomas and cholangiocarcinomas). The incidence of tumours other than that of the liver did not differ between groups (Scheunig *et al.*, 1979).

Male Wistar rats, 50 days of age, were divided into two groups; treated animals ($n = 15$) were given extracts from homogenates of the mackerel pike (*Sanma hiraki* in Japanese) that had been incubated with sodium nitrite at pH 3.0 by stomach tube three times a week for 6 months. Control animals ($n = 10$) were given extracts from fish homogenates that had not been incubated with nitrite. The weekly amount of extract given was calculated to have mutagenic activity by the Ames test equal to that of a weeky oral dose of *N*-methyl-*N*′-nitro-*N*-nitrosoguanidine (MNNG), which is active in inducing adenocarcinomas of the glandular stomach in rats. The animals were held for an additional observation period of 18 months. Twelve to 18 months after administration, 8/12 treated animals developed tumours (two adenomas, two adenocarcinomas and one adenosquamous carcinoma of the glandular stomach, two squamous-cell carcinomas of the forestomach, two adenomas and one adenocarcinoma of the pancreas and one adenocarcinoma of the small intestine). Furthermore, preneoplastic lesions (including intestinal metaplasia and glandular hyperplasia of the glandular stomach as well as

squamous-cell hyperplasia of the forestomach) were noted in virtually all of the treated animals. No tumours were seen in eight control rats given the untreated fish extract. The authors concluded that extract of nitrite-treated fish was demonstrated to induce cancers of the glandular stomach in rats [although statistical analysis was not performed] (Weisburger *et al.*, 1980).

Transplacental carcinogenesis and chemical determination of 1-butyl-1-nitrosourea (BNU) in stomach content was assessed after simultaneous oral administration of 1-butylurea and sodium nitrite (Maekawa *et al.*, 1977). Solutions of either 100 mg/kg bw 1-butylurea and 50 mg/kg bw sodium nitrite (seven dams) or 1-butylurea alone (five dams) were administered daily by stomach tube to pregnant ACI/N rats (> 12 weeks of age at mating) from day 13 to day 21 of gestation. Neurogenic tumours were induced in their offspring. In the group treated with 1-butylurea plus sodium nitrite, the incidence of tumours and mean survival time of rats with nervous system tumours were 64% (23/36 male and female pups) and 309 (189–672) days, respectively. Thirty-nine neurogenic tumours developed in these 23 rats. Fourteen tumours of the peripheral nerves were all neurinomas. Among 25 tumours found in the central nervous system, gliomas were the most frequent (20/25). Localization and histological findings of the nervous system tumours were similar to those observed previously in rats whose dams had received BNU during pregnancy (Maekawa & Odashima, 1975). Neurogenic tumours did not develop in the offspring (0/23 male and female pups) of the dams that received 1-butylurea alone. In-vivo formation of BNU in the stomach content after intubation of 1-butylurea and sodium nitrite was determined. Four groups of 3–4 female ACI/N rats served as untreated controls (distilled water) or were treated with sodium nitrite alone, 1-butylurea alone or 1-butylurea plus sodium nitrite. In the group treated with 1-butylurea plus sodium nitrite, BNU was detected in the stomach content at levels of 25 ppm at 30 min and 23 ppm at 60 min after administration. The concentration of BNU in the stomach content corresponded to 48.3 and 29.2 μg/rat, respectively. BNU was not detected in the groups of untreated controls, or those treated with sodium nitrite alone or 1-butylurea alone (Maekawa *et al.*, 1977). [Statistical analysis was not performed for comparison of the data between groups.]

(d) Studies of interactions with carcinogens or antioxidants

In a lifetime study in which the effect of sodium ascorbate on tumour induction was examined in rats treated with morpholine and sodium nitrite or with *N*-nitrosomorpholine, a group of 40 male MRC Wistar rats, 8 weeks of age, was fed morpholine (10 g/kg of diet) on 5 days a week for 2 years and received 3 g/L sodium nitrite in the drinking-water (group 1). Another group of 40 rats was fed sodium ascorbate (22.7 g/kg of diet) in addition to this treatment (group 2). An untreated control group of 50 rats was also available. When ascorbate was ingested, the liver tumours induced by morpholine and nitrite showed a longer induction period (54 weeks in group 1 versus 93 weeks in group 2; $P < 0.001$), a slightly lower incidence (24/37 in group 1 versus 19/39 in group 2; statistically non-significant) and an absence of liver tumour metastases in the lungs

[statistical difference between groups 2 and 1 not provided], indicating, according to the authors, that ascorbate had inhibited the in-vivo formation of *N*-nitrosomorpholine. The incidence of forestomach tumours in group 2 was 21/39, including 7/39 squamous-cell carcinomas, whereas no forestomach tumours were found in group 1. The authors hypothesized that ascorbate may promote the action of *N*-nitrosomorpholine in this organ (Mirvish *et al.*, 1976). [The Working Group noted that statistical methods used in this study were unclear and the absence of sodium nitrite- or morpholine-treated control groups.]

Three groups of 40 male Sprague-Dawley rats, 4 weeks of age, were fed powdered basal diet and administered 0.1% aminopyrine and 0.1% sodium nitrite in the drinking-water (group A) on 5 days a week for 40 weeks, when the experiment was terminated. Animals in group B were similarly treated but the diet was supplemented with 0.7% ascorbic acid. Rats in group C were fed basal diet and tap-water *ad libitum*. With respect to survival, 12 animals died or were killed when moribund in group A, one animal accidentally died in group B and all group C animals survived in good condition. The tumour incidence was 31/37 and 14/39 in the groups A and B, respectively ($P < 0.001$ between the two groups) [method of statistical analysis not provided]. In group A, tumours were detected in the lungs, liver and kidneys of 25, 12 and 10 animals, respectively. In group B, tumours were found in the lung and kidneys of 13 and seven animals, respectively. Tumours were mainly lung adenocarcinomas, hepatocellular carcinomas and renal adenomas. These results suggested that ascorbic acid protected rats against the induction of liver tumours by aminopyrine and sodium nitrite, but that protection against the induction of lung and kidney tumours was not complete. The authors considered that the mechanism was in part due to the blockage of in-vivo nitrosation (Chan & Fong, 1977).

Groups of 40 male MRC-Wistar rats, 8 weeks of age, were fed 10 g/kg of diet morpholine and received 2 g/L sodium nitrite in the drinking-water for life without (group 1) or with (group 2) the addition of 22.7 g/kg of diet sodium ascorbate. Group 3 was untreated. No liver tumours were observed in rats in group 3. Rats in group 2 had a lower incidence of liver tumours (15/37) with a longer latency than group 1, with a survival period of 108±12 weeks in group 2 versus an incidence of 37/39 ($P < 0.05$ by the life-table method reviewed by Peto) and 55±12 weeks of survival in group 1, which made the authors consider that ascorbate inhibited the in-vivo formation of *N*-nitrosomorpholine (NMOR). Liver tumours were mainly hepatocellular carcinomas. In contrast, the incidence of forestomach papillomas was 1/30 in group 1, 14/37 in group 2 and 3/39 in group 3. The difference between groups 1 and 2 was not significant due to the shorter life-span of rats in group 1. Rats in group 1 and group 2 had a higher incidence of forestomach hyperplasia than those in group 3 ($P < 0.01$ for group 1 and $P < 0.05$ for group 2 versus group 3). The authors concluded that ascorbate may have enhanced induction of these lesions because of an action synergistic with that of NMOR. However, they found that it was more probable that the reduced dose of NMOR and concomitantly

increased survival produced by the ascorbate were solely responsible for the increased incidence of forestomach papillomas and other lesions in group 2 (Mirvish *et al.*, 1983).

Thioproline, which is readily nitrosated to form nitrosothioproline, is expected to act as a nitrite scavenger. The effect of thioproline as an inhibitor of the carcinogenesis induced by *N*-nitroso-*N*-benzylmethylamine precursors was examined. Two groups of male Fischer 344 rats, 6 weeks of age, were fed diets that contained 0.25% *N*-benzylmethylamine (group I, eight rats) or 0.25% *N*-benzylmethylamine plus thioproline (0.25% thioproline until week 17 and then 0.5%; group II, 10 rats). Both groups were given drinking-water that contained sodium nitrite (0.1% until week 17 and then 0.2%). The experiment was continued for 717 days. Squamous-cell carcinomas of the forestomach developed in 7/7 [6/7 in publication abstract] rats in group I and in significantly fewer (2/9 rats) in group II ($P < 0.01$ by the χ^2 test). The degree of tumour invasion was also lower in group II rats that were given thioproline than in group I rats (Tahira *et al.*, 1988). [The Working Group noted the lack of an *N*-benzylmethylamine-treated group.]

Groups of male non-inbred Swiss mice, 45 days of age, were given 1000 ppm dibutylamine (DBA) in combination with 2000 ppm sodium nitrite in the drinking-water, with or without either 30% soya bean in the diet or 5000 ppm ascorbic acid in the drinking-water, for a maximum of 12 months. An untreated control group was included. In each experimental group, some animals were killed after 7–9 or 10–12 months of treatment. Microscopical examination of the tumours observed in the urinary bladder and liver was performed. No tumours of the urinary bladder or liver were observed in the animals treated with DBA plus sodium nitrite plus soya bean for 7–9 months or 10–12 months (43 rats), in the animals treated with DBA plus sodium nitrite plus ascorbic acid for 7–9 months (20 rats) or in untreated controls (60 rats). Following treatment with nitrite plus DBA, the incidence of hepatomas was 3/20 (7–9 months of exposure) and 4/15 (10–12 months of exposure) and that of urinary bladder papillomas was 4/20 (7–9 months of exposure) and 6/20 (10–12 months of exposure). The authors concluded that a clear carcinogenic effect of the nitrosamine precursor (DBA) and sodium nitrite was demonstrated, and that soya bean and ascorbic acid had a protective effect (Mokhtar *et al.*, 1988)

Groups of 10 male Fischer 344 rats, 10 weeks of age, were treated with 0 or 0.3% sodium nitrite in the drinking-water and fed either 0.8% catechol or 2% 3-methoxycatechol in the diet for 24 weeks, when the experiment was terminated. Simultaneous administration of sodium nitrite with 3-methoxycatechol increased the formation of forestomach papillomas (5/10 versus 0/10, $P < 0.05$) compared with the groups treated with 3-methoxycatechol alone. In contrast, induction of adenomas in the glandular epithelium of the stomach was reduced (1/10 versus 10/10, $P < 0.01$; 2/10 versus 7/10 [$P < 0.05$]) compared with the respective catechol- and 3-methoxycatechol-treated groups (Hirose *et al.*, 1990).

Groups of 15 male Fischer 344 rats, 6 weeks of age, were given a single intraperitoneal injection of 100 mg/kg bw NDEA, four intraperitoneal injections of

20 mg/kg bw *N*-methylnitrosourea, four subcutaneous injections of 40 mg/kg bw dimethylhydrazine, 0.05% BBNA in the drinking-water for the first 2 weeks and 0.1% 2,2′-dihydroxy-di-*n*-propylnitrosamine in the drinking-water for the next 2 weeks of the initial 4-week initiation period. Starting 3 days after the completion of these treatments, animals were fed diets that contained 2% butylated hydroxyanisole, 0.8% catechol, 2% 3-methoxycatechol or basal diet either alone or in combination with 0.3% sodium nitrite until week 28, when a complete autopsy was performed. Sodium nitrite enhanced the development of forestomach lesions but inhibited that of glandular stomach lesions in rats that were given catechol or 3-methoxycatechol simultaneously with or without prior exposure to carcinogens. The incidence of carcinomas *in situ* (7/14 versus 1/15, $P < 0.05$ by Fisher's exact probability test) and squamous-cell carcinomas (14/14 versus 5/15, $P < 0.001$) of the forestomach was increased by sodium nitrite in addition to catechol and carcinogen treatment; the incidence of papillomas of the forestomach (5/10 versus 0/10, $P < 0.05$) was increased by sodium nitrite in addition to 3-methoxycatechol without carcinogen treatment. The incidence of submucosal hyperplasias and/or adenomas of the glandular stomach was decreased by sodium nitrite in addition to catechol or 3-methoxycatechol with or without carcinogen treatment. However, reductions in body weight, that probably reflected the decreased food consumption following co-administration of sodium nitrite, may have been a factor in the modification of the development of glandular stomach lesions. The authors concluded that sodium nitrite can stimulate cell proliferation and enhance carcinogenesis, particularly in the forestomach epithelium (Hirose *et al.*, 1993).

Groups of 15–20 male Fischer 344 rats, 5 weeks of age, were treated with an intragastric dose of 150 mg/kg bw MNNG and, starting 1 week later, were fed 2.0% butylated hydroxyanisole, 0.8% catechol or 2.0% 3-methoxycatechol in the diet or basal diet either alone or in combination with 0.2% sodium nitrite in the drinking-water until they were killed at week 52. All three antioxidants significantly enhanced forestomach carcinogenesis without any effect of additional treatment with sodium nitrite. The incidence of forestomach papillomas varied from 74 to 95% in the six treated groups: 75% (15/20) in the group treated with sodium nitrite and basal diet (not significant) compared with 50% (9/18) in the group fed basal diet only. The incidence of forestomach squamous-cell carcinoma varied from 85 to 100% in the two butylated hydroxyanisole- and two catechol-treated groups, was 42 and 50% in the 3-methoxycatechol- and the 3-methoxycatechol and sodium nitrite-treated groups, respectively, and was 25 and 33% in the groups given sodium nitrite and basal diet and basal diet alone, respectively. In the absence of MNNG pretreatment, there was no significant increase in the incidence of forestomach papillomas or squamous-cell carcinomas, except that a statistically non-significant increase in forestomach papillomas was observed in the group treated with 3-methoxycatechol and sodium nitrite compared with the 3-methoxycatechol-treated group (10/15 versus 4/15). A statistically non-significant increase in forestomach papillomas was also observed in the group treated with catechol and sodium nitrite compared with

the catechol-treated group (4/15 versus 0/15). Sodium nitrite had no effect on catechol- or 3-methoxycatechol-induced glandular stomach carcinogenesis (Kawabe *et al.*, 1994).

The effects on forestomach carcinogenesis of combined treatment with sodium ascorbate or ascorbic acid and sodium nitrite, with or without pretreatment with MNNG, were examined. Groups of 20 or 15 male Fischer 344 rats, 6 weeks of age, were given a single intragastric administration of 150 mg/kg bw MNNG in dimethyl sulfoxide:water (1:1) or vehicle alone, respectively. One week later, the animals received supplements of 1% sodium ascorbate or 1% ascorbic acid in the diet and 0.3% sodium nitrite in the drinking-water, alone or in combination, or basal diet until the end of week 52. In MNNG-treated animals, the incidence of forestomach papillomas and squamous-cell carcinomas was significantly enhanced in the group that received sodium nitrite alone (16/19 and 9/19, $P < 0.05$ and $P < 0.01$, respectively, by Fisher's exact probability test) compared with the group that received basal diet (6/20 and 2/20, respectively); a further significant increase in the incidence of carcinomas occurred following additional treatment with sodium ascorbate (15/19, $P < 0.01$ versus basal diet group, $P < 0.05$ versus sodium nitrite group by Fisher's exact probability test) or ascorbic acid (17/20, $P < 0.01$ versus basal diet group, $P < 0.05$ versus sodium nitrite group by Fisher's exact probability test). With no MNNG treatment, all animals that received sodium nitrite demonstrated mild hyperplasia, but no papillomas were observed. Additional administration of sodium ascorbate or ascorbic acid remarkably enhanced the grade of hyperplasia, and resulted in a 53% (8/15, $P < 0.01$ versus basal diet or sodium nitrite groups) and a 20% (3/15, not significant) incidence of papillomas, respectively. The authors concluded that these results demonstrate that sodium nitrite exerts a promoting effect on forestomach carcinogenesis and that sodium ascorbate and ascorbic acid act as co-promoters; also, combined treatment with sodium ascorbate or ascorbic acid and sodium nitrite may induce forestomach carcinomas (Yoshida *et al.*, 1994).

Groups of 15 male Fischer 344 rats, 6 weeks of age, were treated with an intragastric dose of 150 mg/kg bw MNNG and, starting 1 week later, were fed 0.5% *tert*-butylhydroquinone (TBHQ), 1% α-tocopherol or 1% propyl gallate in the diet or basal diet with or without 0.2% sodium nitrite in the drinking-water until they were killed at the end of week 36. In MNNG-treated animals, combined administration of α-tocopherol or propyl gallate with sodium nitrite significantly increased the areas ($P < 0.001$) and numbers ($P < 0.01$) of macroscopic forestomach nodules per animal (0.88±0.52 cm^2 and 8±5; 1.47±1.22 cm^2 and 7±3) compared with the respective values for animals treated with antioxidants alone (0.30±0.59 cm^2 and 3±2; 0.47±1.01 cm^2 and 3±3), and combined administration of α-tocopherol with sodium nitrite compared with administration of α-tocopherol alone significantly increased the multiplicity of forestomach papillomas (1.0±1.1 versus 0.2±0.4, $P < 0.05$) and tended to increase their incidence (9/15 versus 3/15, statistically non-significant). In rats not treated with MNNG, combined treatment with antioxidants and sodium nitrite significantly increased the incidence of mild or moderate hyperplasia of the forestomach (TBHQ, $P < 0.001$; α-tocopherol, $P < 0.05$; propyl gallate, $P < 0.01$). In the glandular stomach, atypical hyperplasias were observed

in all the MNNG-treated groups. Although the incidence showed a non-significant tendency to decrease with TBHQ treatment, additional administration of sodium nitrite caused a significant increase (60% compared with 13% in rats treated with TBHQ alone, $P < 0.05$). The authors concluded that co-administration of sodium nitrite with α-tocopherol, TBHQ or propyl gallate promotes forestomach carcinogenesis (Miyauchi *et al.*, 2002).

The effect of the antioxidants, 1-*O*-hexyl-2,3,5-trimethylhydroquinone (HTHQ) and ascorbic acid, on carcinogenesis induced by administration of aminopyrine and sodium nitrite was examined using a rat multiorgan carcinogenesis model. Groups of 20 male Fischer 344 rats, 6 weeks of age, were treated sequentially, during the first weeks, with an initiation regimen of NDEA (100 mg/kg bw, single intraperitoneal dose at commencement), *N*-methylnitrosourea (20 mg/kg bw, intraperitoneal injections on days 2, 5, 8 and 11), BBNA (0.05% in the drinking-water during weeks 1 and 2), *N*,*N*′-dimethylhydrazine (40 mg/kg bw, subcutaneous injections on days 14, 17, 20 and 23) and 2,2′-dihydroxy-di-*n*-propylnitrosamine (0.1% in the drinking-water during weeks 3 and 4). Initiation was followed by treatment with aminopyrine plus sodium nitrite, aminopyrine plus sodium nitrite plus HTHQ or ascorbic acid, sodium nitrite plus HTHQ or ascorbic acid, each of the individual chemicals alone or basal diet and tap-water as a control. Aminopyrine and sodium nitrite were given in drinking-water at a dose of 0.05% and HTHQ and ascorbic acid were given in the diet at a dose of 0.25%. All surviving animals were killed at experimental week 28, and major organs were examined histopathologically. In the group treated with aminopyrine and sodium nitrite, the incidence of hepatocellular adenomas and haemangiosarcomas was 19/20 and 7/20, respectively (both lesions, $P < 0.01$ versus untreated control by Fisher's exact probability test). When HTHQ, but not ascorbic acid, was administered with aminopyrine and sodium nitrite, the incidence decreased to 11/19 and 2/19 (adenomas, $P < 0.01$ versus aminopyrine plus sodium nitrite group by Fisher's exact probability test). In contrast, in the group treated with aminopyrine and sodium nitrite and the group treated with sodium nitrite alone, when HTHQ, but not ascorbic acid, was administered simultaneously, the incidence of forestomach squamous-cell carcinomas significantly increased in the aminopyrine plus sodium nitrite plus HTHQ-treated group (5/19, $P < 0.05$) and in the sodium nitrite plus HTHQ-treated group (8/20, $P < 0.01$), compared with 0/20 in the groups treated with aminopyrine plus sodium nitrite and sodium nitrite alone. The authors suggested that HTHQ can prevent [liver] tumour production induced by aminopyrine and sodium nitrite more effectively than ascorbic acid. In contrast, an enhancing or possibly carcinogenic effect of simultaneous administration of HTHQ and sodium nitrite only on the forestomach was suggested, while simultaneous treatment with the same dose of ascorbic acid and sodium nitrite may not be carcinogenic to the forestomach or other organs (Yada *et al.*, 2002).

Using a model of rat mammary carcinogenesis induced by 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine (PhIP) as an initiator, chemoprevention by synthetic and naturally occurring compounds of the carcinogenic effect was investigated in a series of

experiments. In one experiment, two groups of 6-week-old female Sprague-Dawley rats were given eight intragastric doses of 100 mg/kg bw PhIP during an initial 4-week period alone (20 rats) or simultaneously with 0.2% sodium nitrite in the drinking-water (10 rats). A third group of 10 rats received sodium nitrite alone for 4 weeks and a fourth group of 10 rats received basal diet alone. At the end of experimental week 48, all animals were killed and mammary tumours were examined histopathologically. No tumour was observed in the groups that received sodium nitrite or basal diet alone. The first tumour in the PhIP-treated with group appeared at week 13, whereas this was delayed until week 46 in the group treated with PhIP and sodium nitrite. Although the final incidence of mammary fibroadenomas and adenocarcinomas was not significantly different between the two groups (PhIP: adenocarcinomas, 11/20; fibroadenomas, 3/20; PhIP plus sodium nitrite: adenocarcinomas, 6/10; fibroadenomas, 2/10), the average volume of tumours was significantly reduced in the PhIP plus sodium nitrite-treated group compared to that in the group that received PhIP alone (0.3±0.4 versus 4.5±15.1 cm^3 [method of statistical analysis not provided]) (Hirose *et al.*, 2002).

3.2.3 *Hamster*

Groups of F_0 pregnant Syrian golden hamsters and their offspring [number and age unspecified] were fed various concentrations of nitrite (5, 50 or 1000 ppm) or morpholine (5, 50 or 1000 ppm) in an agar-gel diet from the time of conception. The F_1 generation was fed the same diet as the dams: a group of 30 hamsters was fed the diet containing sodium nitrite at 1000 ppm, a group of 22 hamsters was fed the diet containing morpholine at 1000 pm, another group of 23 hamsters was fed the untreated diet only and served as controls and groups of 16–40 animals were fed diets that contained combinations of the various concentrations of nitrite and morpholine. The survivors were killed when the group number was reduced to 20% of the initial population. No increase in tumour incidence was found between the groups, except that liver-cell carcinomas were found in 5/16 (31%) hamsters treated with 1000 ppm nitrite and 1000 ppm morpholine compared with 0/22 hamsters treated with 1000 ppm morpholine alone (Shank & Newberne, 1976). [The Working Group noted that hamsters appear to be less sensitive than rats to the carcinogenicity of nitrite.]

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4. Mechanistic and Other Relevant Data

4.1 Absorption, distribution, metabolism, and excretion

4.1.1 *Introduction*

The biochemistry and pharmacology of nitrate and nitrite have been under active investigation for hundreds of years (Gladwin *et al*., 2005), and several aspects, e.g. the endogenous synthesis of nitrate, have been discovered (Mitchell *et al*., 1916) and rediscovered (Green *et al*., 1981; Leaf *et al*., 1987). The recognition in the 1980s of the ubiquitous role of nitric oxide in mammalian biochemistry appeared to clarify the endogenous formation of nitrite and nitrate simply as potentially hazardous end-products of nitric oxide production. Indications of additional complexity often went unheeded. Doyle *et al*. (1981), for example, noted that nitrite reacted *in vitro* with deoxygenated haemoglobin to form nitric oxide, and Cortas and Wakid (1991), while investigating the oral biochemistry of nitrate and nitrite, noted an earlier suggestion by Wolff and Wasserman (1972) that production of nitrite by the oral flora may protect against the growth of clostridia. A growing body of research is now revealing that nitrate and nitrite participate in a metabolic cycle in which nitrite and nitric oxide can be interconverted depending on physiological needs and conditions.

The following sections cover absorption, distribution, metabolism and excretion of nitrate and nitrite. However, these processes are not independent since nitrate and nitrite, together with nitric oxide and other important compounds such as carbon dioxide, superoxide and hydrogen peroxide, participate in a complex set of regulatory pathways that have important consequences for both normal and abnormal physiology and biology. These pathways relate to—among others—neural signalling (Garthwaite 1995), regulation of blood pressure (Hunter *et al*., 2004; Crawford *et al*. 2006), inflammation (Stuehr & Marletta, 1985; Clancy *et al*., 1998) and ischaemia (Zweier *et al*., 1995; Dybdahl *et al*., 2005).

4.1.2 *Absorption*

Ingested nitrate enters the general circulation from the gastrointestinal system, which leads to maximum plasma concentrations within about 1 h, followed by clearance with a half-life of about 5 h (Wagner *et al*., 1983; Cortas & Wakid, 1991; McKnight *et al*., 1997). Above a certain level, about 25% of an oral dose of nitrate is re-circulated into the saliva, of which 20%–i.e. 5% of ingested nitrate–is converted into nitrite by oral bacteria (Spiegelhalder *et al*., 1976; National Research Council, 1981).

Exposure to nitrate/nitrite is primarily through ingestion and endogenous synthesis. Under normal conditions, endogenous synthesis of nitrate/nitrite occurs in many places in

the body, e.g. the liver, brain, blood vessels and sites of inflammation, by the conversion of arginine to nitric oxide which is mediated by a combination of constitutive and inducible nitric oxide synthases, followed by the reaction of nitric oxide with other cellular constituents, e.g. oxygen or peroxynitrite (Rodkey, 1976; Leaf *et al*., 1989a; Ignarro, 1990; Marletta *et al*., 1988, 1990; Moncada & Higgs 1991; Koppenol *et al*., 1992; Edwards & Plumb, 1994; Marletta, 1994; Pryor & Squadrito, 1995; Kelm, 1999). Nitrate/nitrite that is formed via this set of pathways is absorbed from the generating organ or tissue into the general circulation and constitutes a more or less steady-state baseline level in the body, which is then episodically supplemented by ingested nitrate/nitrite.

4.1.3 *Distribution*

Distribution of ingested nitrate and nitrite would be expected to reflect to some extent the levels of production in individual tissues and compartments. ^{13}N-Labelled nitrate administered to rats by gavage or intravenous injection was found to be distributed throughout the body, including the stomach (~20%), intestines (~20%), urinary bladder and kidney (~5%) and the remainder largely in the carcass within 1 h after dosing, which suggests fairly rapid distribution (Witter *et al*., 1979). When ^{13}N-labelled nitrite was given in the drinking-water to germ-free Sprague-Dawley rats for 48 h, detectable levels were found in the stomach, small intestine, caecum and large intestine but not in the urinary bladder (Witter & Balish, 1979). Parks *et al*. (1981) administered ^{13}N-labelled nitrate and nitrite intravenously or intratracheally to mice and rabbits and found that the label via both routes of administration was distributed uniformly throughout most of the body. In experiments with two human volunteers, most of an oral dose of [^{13}N]nitrate remained in the stomach for up to 1 hour (Witter *et al*., 1979).

While some earlier reports of nitrate plasma levels noted that nitrite was undetectable (Cortas & Wakid, 1991; Meulemans & Delsenne, 1994), recent advances in analytical techniques have allowed reasonably precise measurements of circulating plasma nitrite levels in mammals, which are generally in the range of 150–600 nM (Tsikas *et al*., 1998; Gladwin *et al*., 2000; Tsikas & Frölich, 2002; Kleinbongard *et al*., 2003).

In addition to saliva and plasma, nitrate and nitrite have been measured in the human brain, pancreas, lung, thyroid, kidney, liver, spleen and cerebrospinal fluid, generally at high nanomolar to mid-micromolar levels for nitrite and mid-to-high micromolar levels for nitrate (Friedberg *et al*., 1997; Goodman *et al*., 2002). Overall, based on analyses in animals and humans, nitrate and nitrite are widely distributed throughout the body—predominately as nitrate—although with large local variations.

4.1.4 *In-vivo formation of NPRO and other nitrosamino acids*

Ohshima and Bartsch (1981) introduced the use of excretion of NPRO in the urine as a measure of in-vivo nitrosation. They gave a volunteer 325 mg nitrate in beet juice and

500 mg L-proline, and determined NPRO in the urine collected over the next 24 h. They found 160 nmol (23 μg) NPRO in the 24-h urine. Ascorbic acid or α-tocopherol given simultaneously inhibited the formation of NPRO.

This was the first clear demonstration that humans can form *N*-nitroso compounds *in vivo*. NPRO is formed by gastric nitrosation of proline, absorbed into the blood and excreted quantitatively in the urine. The test is considered to be safe because NPRO is non-carcinogenic and is excreted quantitatively. Because of its carboxyl group, nitrosation of proline proceeds maximally at pH 1.5–2.7, rather than pH 3.0–3.3 which is usual for amines, but its rate of nitrosation is proportional to the square of the nitrite concentration, similarly to other amines, and its rate constant is intermediate compared with that of other amines (Mirvish, 1975). The NPRO test measures the nitrosating capacity of the stomach but not the gastric levels of amines and amides.

(a) Application of the NPRO test

Bartsch *et al*. (1990) summarized their research on the endogenous formation of *N*-nitroso compounds as measured by the NPRO test. The test (in which proline alone or proline plus vitamin C were given) has been applied to subjects from areas at high and low risk for cancer of the stomach in Colombia, Costa Rica, Japan and Poland, for squamous-cell cancer of the oesophagus in northern China and for nasopharyngeal cancer in southern China (Lu *et al*., 1986; Kamiyama *et al*., 1987; Zatonski *et al*., 1989; see also Mirvish 1994). In most cases, excretion of NPRO was greater in high-risk areas for these cancers than in low-risk areas. Patients infested with liver flukes in Thailand, who are at risk for liver cholangiocarcinoma, also showed increased excretion of NPRO (Bartsch *et al*., 1992). In most of these studies, vitamin C was found to reduce the formation of NPRO.

(b) Correlation of the NPRO test with gastric pH

In a study of patients who had gastric lesions, the yields of NPRO were highest when gastric pH was 1.5–2.0. The yield did not increase in cases who had advanced gastric lesions. The number of nitrate-reducing bacteria correlated positively with pH but not with urinary NPRO. These results indicate that NPRO was produced only in an acid-catalysed reaction (Hall *et al*., 1987).

In another study (Wagner *et al*., 1984a), excretion of NPRO by human volunteers was expressed after subtracting the amount of NPRO ingested in the diet. Mean basal excretion was 26±10 nmol per day, which was not reduced by ascorbic acid (2 g per day) or α-tocopherol (400 mg per day). After subjects ingested nitrate and proline, the mean excretion of NPRO was 100±81 nmol/day. When they were fed [^{15}N]nitrate, [^{15}N]NPRO was formed. Ascorbic acid inhibited [^{15}N]NPRO formation by 80%. Formation of another non-carcinogenic compound (*N*-nitrosothiazolidine-4-carboxylic acid (NTCA); see below) after ingestion of nitrate was completely inhibited by ascorbic acid. These results

indicated that a portion of urinary NPRO was produced by a mechanism that is not inhibited by ascorbic acid.

It has been suggested that there are two types of NPRO synthesis *in vivo* (Tannenbaum, 1987). One type incorporates ^{15}N from nitrite and is inhibited by ascorbic acid while the other type does not incorporate [^{15}N]nitrite, is not inhibited by ascorbic acid and does not, therefore, entail acid-catalysed nitrosation by nitrite (Wagner *et al*., 1985).

(c) NPRO formation in subjects with high levels of nitrate in their drinking-water

Urinary excretion of NPRO was increased in 44 men from a rural area in the USA, who drank well-water that contained levels of nitrate below or above 10 ppm nitrate-N. For 5 days, the subjects followed a diet low in NPRO and nitrate and (on days 4 and 5) ascorbate. The 24-h urine was collected on day 4 while normal activities were followed and on day 5 after an overnight fast and ingestion of 500 mg proline. Saliva samples were also collected. The concentration of nitrate in the drinking-water (< 10 or > 10 ppm nitrate-N) was significantly associated with urinary NPRO (< 1500 or > 1500 ng/day) on both days, with urinary nitrate, and with nitrite and nitrate in the saliva (Mirvish *et al*., 1992).

A similar study of 285 residents in rural Denmark also involved the ingestion of 500 mg proline and analysis of 12-h urine samples. The 24-h diet was recorded and analysed for nitrate. The concentration of nitrate in the drinking-water was weakly associated ($P = 0.08$) with an increase in the excretion of NPRO. Nitrate consumption in food was also measured. The risk for excreting excess NPRO increased significantly with total nitrate intake in food and water, and with tobacco smoking. Tea consumption reduced the excretion of NPRO, whereas the intake of vegetables did not have a clear effect. These findings show that a high intake of nitrate in water can increase the formation of NPRO (Møller *et al*., 1989).

In a study of subjects who consumed proline under controlled conditions, factors that affected excretion of NPRO were studied in healthy subjects after 5 days on a diet low in NPRO, nitrate, proline and (on days 4 and 5) ascorbate (Mirvish *et al*., 1995). In the standard test, subjects took 400 mg nitrate (as potassium nitrate) 1 h before a standard meal that contained 500 mg proline. Mean excretion of NPRO determined in 24-h urine was 26±2 (mean ± standard error (SE)) nmol compared with 5±1 nmol when subjects ingested proline alone. Similar results were observed in men and women. Doses of 200 and 100 mg nitrate produced mean NPRO excretory levels of 9±1 and 11±2 nmol/24 h, respectively. Nitrate produced the highest level of NPRO when given 1 h before the test meal. This time of 1 h reflects the 1–2 h required for salivary nitrite to peak after a dose of nitrate is taken (Spiegelhalder *et al*., 1976; Tannenbaum *et al*., 1976). When nitrate was given 15 min or 2 h before the meal, the NPRO yields were 17±2 and 19±4 nmol/24 h, respectively. Gavage of fasting subjects with nitrate and proline increased the formation of NPRO from three-to fourfold compared with when a meal was taken. Sodium ascorbate showed maximum inhibition of NRPO excretion from 2 h before to 1 h

after the test meal. When the dose of ascorbic acid was varied under the same test conditions as those described above, mean inhibitions compared with no ascorbic acid were 28, 62 and 60% for doses of 120, 240 and 480 mg ascorbic acid, respectively (Mirvish *et al*., 1998). As little as 100 mg ascorbic acid significantly reduced NPRO formation when taken with a meal containing proline. [The Working Group noted that these results strongly support the view that NPRO is produced by acid-catalysed nitrosation in the stomach.]

(d) Nitrosamino acids other than NPRO

In addition to NPRO, human urine contains NTCA (*N*-nitrosothioproline) and 2-methyl-NTCA. The precursor amines of these compounds are formed in the stomach by acid-catalysed condensation of formaldehyde and acetaldehyde, respectively, with cysteine (Tsuda *et al*., 1983). These nitrosamino acids are acid-labile and hence require appropriate analytical conditions. Excretion of NTCA and methyl-NTCA was increased in smokers (Tsuda *et al*., 1987). Thioproline is nitrosated about 1000 times faster than proline *in vitro*, and NTCA is probably not carcinogenic (Tsuda & Kurashima, 1991), but it seems less suitable than NPRO as a measure of gastric nitrosation because its formation depends on the levels of aldehydes and cysteine as well as nitrite.

(e) Other NPRO tests and related assays

Excretion of NPRO was reduced in patients who had elevated gastric pH and higher than normal bacterial counts in the stomach caused by premalignant gastric conditions or by treatment with cimetidine (Bartsch *et al*., 1989). This confirms that NPRO is produced in the stomach and suggests that, if products of gastric nitrosation initiate gastric cancer, they do so before premalignant conditions associated with achlorhydria develop.

Excretion of NPRO after ingestion of proline but not nitrate, was higher in groups of 25–50 healthy individuals from several areas with high cancer incidence than in similar groups from neighbouring areas with low incidence. This was shown for gastric cancer in Japan (Bartsch *et al*., 1989), Poland (Zatonski *et al*., 1989), Colombia (Stillwell *et al*., 1991) and Costa Rica (Sierra *et al*., 1993), for oesophageal cancer in northern China (Lu *et al*., 1986; Bartsch *et al*., 1989) and for nasopharyngeal cancer in southern China (Yi *et al*., 1993). In the NPRO tests conducted by Bartsch *et al*. (1989), 100 mg proline taken 1 h after the evening meal or after each of three daily meals and ascorbic acid (100–200 mg taken with proline) was shown to inhibit the formation of NPRO. Wu *et al*. (1993) examined pooled urines from 30 men from each of two villages in 69 counties in China with different cancer incidences. Significant associations were found between (*a*) NPRO yield and the decrease in NPRO yield after ingestion of ascorbic acid (the 'nitrosation potential') and (*b*) the incidences of cancer of the oesophagus and leukaemia, but not of gastric cancer or several other cancers. The urinary excretion of *N*-nitrosarcosine was strongly correlated with the incidence of oesophageal cancer ($P < 0.001$).

Excretion of NPRO was also elevated in patients with schistosomiasis in Egypt (Tricker *et al*., 1989) and those with liver fluke infestation in Thailand (Srivatanakul *et*

al., 1991; Ohshima & Bartsch, 1994). Presumably, these infestations promoted the synthesis of nitrogen oxide, which was converted to nitrate and hence increased nitrite levels and nitrosation in the stomach, as was demonstrated in Syrian hamsters infected with liver flukes (Ohshima *et al*., 1994).

Shapiro *et al*. (1991) examined the urinary excretion of NPRO and NTCA by subjects who had ingested sodium nitrate and proline, and compared these with results before and after use of an oral antiseptic (*Peridex*). This mouthwash solution reduced the number of nitrate-reducing bacteria in saliva by 94%, strongly reduced the reduction of nitrate to nitrite by saliva *in vitro* and inhibited the excretion of NPRO and NTCA by 62 and 74%, respectively. Individuals who had high levels of nitrate-reducing oral bacteria in their saliva showed significantly increased excretion of NPRO. These results suggest that gastric or oral nitrosamine formation is increased in individuals with poor oral hygiene.

Four studies showed that cigarette smoking increases the formation of NPRO, probably because of catalysis by thiocyanate or increased gastric acid secretion due to irritation by the smoke (Bartsch *et al*., 1989). Subjects who chewed quids of betel nuts and/or tobacco, but did not ingest proline or nitrate, showed increases in mean salivary and urinary NPRO, but the effects were not significant (Nair *et al*., 1985). When nitrate was given 1 h before a standard meal containing proline, chewing of gum and tobacco did not significantly affect the yield of NPRO after correcting for preformed NPRO derived from the tobacco. Chewing did not affect levels of nitrate but reduced levels of nitrite in the saliva, probably because of the increased flow of saliva (Mirvish *et al*., 1995).

Endogenous formation of *N*-nitrosamines – other than NPRO – following ingestion of nitrate in combination with an amine-rich diet was investigated by Vermeer et al. (1998, 1999).

A group of 25 female volunteers consumed during seven consecutive days a fish-meal rich in amines as nitrosatable precursors. The diet was supplemented with an aqueous solution of potassium nitrate (170 mg nitrate anion in 100 mL; the acceptable daily intake level). During one week before and one week after the test period a low-nitrate diet was consumed. The intake of nitrate caused a significant increase of mean nitrate concentrations in saliva and urine. *N*-nitrosodimethylamine (NDMA) and *N*-nitrosopiperidine (NPIP) were detected as volatile *N*-nitrosamines in the urine. While excretion of NPIP was not directly correlated with nitrate intake and composition of the diet, the excretion of NDMA was highly correlated with nitrate excretion and with salivary nitrate and nitrite concentrations. This study shows that nitrate intake in combination with a diet containing nitrosatable precursors increases the formation of carcinogenic *N*-nitrosamines (Vermeer et al., 1998).

In a further study with the same experimental design, the effects of ascorbic acid and green tea on NDMA excretion were investigated. Intake of 250 mg or 1 g of ascorbic acid per day resulted in a significant decrease in urinary NDMA excretion during days 4–7, but not during days 1–3 of the test week. Likewise, consumption of four cups of green tea per day (2 g) caused a significant decrease in excretion of NDMA during days 4–7, but not during days 1–3. In contrast, consumption of eight cups of green tea per day (4 g)

significantly increased the excretion of NDMA during days 4–7, again not during days 1–3. This may be a result of catalytic effects on nitrosation of the polyphenols in the tea. The results indicate that intake of ascorbic acid and moderate consumption of green tea can reduce endogenous formation of NDMA (Vermeer et al., 1999).

(f) Ascorbic acid and the NPRO test

Ascorbic acid inhibits the formation of *N*-nitroso compounds from nitrite and amines or amides, because it reduces nitrite to nitric oxide while it is itself oxidized to dehydroascorbic acid (Mirvish, 1975, 1994). Partly on the basis of these findings, all nitrite-preserved meat in the USA is currently prepared with 550 ppm sodium erythorbate (a non-vitamin isomer of ascorbate that also reduces nitrite to nitric oxide), and the level of nitrite in such foods has decreased accordingly (see Section 1).

Inhibition of NPRO formation by vegetable and fruit juices was shown to be due to both ascorbic acid and the phenolic fraction (Helser *et al*., 1992; Helser & Hotchkiss, 1994). Two inhibitory phenols in tomatoes were identified as *para*-coumaric and chlorogenic acids. The normal median level of ascorbic acid plus dehydroascorbic acid is 50 mg/L human gastric juice compared with 7 mg/L plasma, which indicates active secretion of 0.4 mg ascorbic acid per h into the stomach; however, this level is only 3.4 mg/L gastric juice in patients with chronic atrophic gastritis (O'Connor *et al*., 1989; Rathbone *et al*., 1989).

Gradually increasing doses of ascorbic acid were fed with a standard dose of proline on consecutive days to subjects on a low-ascorbic acid diet. Sodium nitrate (5.24 mmol) was given 2 h after the last meal; 30 min later, a 4.35-mmol dose of proline was given and the 24-h urine was analysed for nitrate, NPRO and total ascorbic acid. Nitrate balance was monitored using [^{15}N]nitrate. It was estimated from the dilution of urinary nitrate by [^{14}N]nitrate that nitrate synthesis was 1.28 mmol/day. NPRO excretion was reduced by 6 nmol/day for each increment of 0.05 mmol (9 mg) dietary ascorbic acid (Leaf *et al*., 1987).

4.1.5 *Metabolism (other than formation of N-nitroso compounds)*

The metabolism of nitrate and nitrite under normal physiological conditions has been investigated and reviewed extensively (Tannenbaum *et al*., 1978; Lundberg *et al*., 2004); the following is a brief summary.

Nitrate, either ingested or secreted into the mouth, is partly reduced by oral bacteria into nitrite (Spiegelhalder *et al*., 1976; Tannenbaum *et al*., 1976); the resulting mixture is swallowed and passes into the gastrointestinal system from which most is absorbed into the general circulation. Nitrite in plasma is converted by a rapid reaction with oxyhaemoglobin into nitrate (Rodkey, 1976) and this cycle then continues. Plasma nitrate and plasma nitrite levels are typically in the micromolar and nanomolar range, respectively (Kleinbongard *et al*., 2003). Thus, in the classic model, nitrate/nitrite exists

primarily as nitrate, which is the end-product of what has often been considered to be a detoxification and/or elimination mechanism for nitric oxide and nitrite.

The details of nitrite metabolism became a more complex when it was recognized that conversion of nitrite into nitric oxide can occur under certain physiological conditions such as hypoxia (Dejam *et al*., 2004; Gladwin *et al*., 2005; Kim-Shapiro *et al*., 2005). This represents a reversal of the well-known nitric oxide-to-nitrite/nitrate pathways. As noted earlier, experiments in the 1980s showed that the nitrite ion could react with deoxygenated haemoglobin to release nitric oxide (Doyle & Hoekstra, 1981; Doyle *et al*., 1981), but these experiments were carried out *in vitro* and their potential physiological relevance was not apparent. Zweier *et al*. (1995) reported that, in a perfused heart model for ischaemia, nitrite ion was converted *in vivo* directly into nitric oxide, which demonstrated that the earlier observations did in fact have biochemical implications. These studies have been followed by several related experiments indicating a renewed interest in the biochemistry of nitrite/nitrate. A model for nitrite/nitrate metabolism has emerged based on extensive and sometimes subtle interactions among ingested and endogenously synthesized nitrate, nitrite, nitric oxide and some related species, and the physiology of the organism. The details of these are beyond the scope of this monograph, but are discussed in several reviews (McKnight *et al*., 1997 Dejam *et al*., 2004; Lundberg *et al*., 2004; Gladwin *et al*., 2005; Kim-Shapiro *et al*., 2006).

4.1.6 *Excretion*

As detailed in previous sections, nitrate and nitrite are interconverted, primarily via bacterial reduction of nitrate in the mouth and the gut and oxidation of nitrite in the blood. The steady-state balance favours nitrate. Experiments with isotopically labelled nitrate indicate that about 60% of dietary nitrate appears in the urine within 48 h (Wagner *et al*., 1984b). Excretion, therefore, is mainly as urinary nitrate, typically in the approximate range of 0.4–3.5 mmol/day, with values towards the lower end for low-nitrate diets and towards the higher end for normal diets, although there can be large geographical variations (Lee *et al*., 1986; Hill *et al*., 1996; van Maanen *et al*. 1996). Patients with inflammatory bowel disease (Melichar *et al*., 1994), rheumatoid arthritis (Stichtenoth *et al*., 1995) and multiple sclerosis (Giovannoni *et al*., 1999), among other disorders, have been found to have elevated levels of urinary nitrate. Newborn infants had higher levels of urinary nitrate than adults when excretion was corrected for reduced kidney function and body size, which suggests that newborns produce more intravascular nitric oxide than adults (Honold *et al*., 2000). Urinary nitrite can be detected in the urine of people with bacterial infections, but this is due to bacterial reduction of nitrate to nitrite in the bladder (Tricker *et al*., 1991; Lundberg *et al*., 1997) and analysis of urinary nitrite has been suggested as a diagnostic aid for urinary tract infections (Liptak *et al*., 1993).

In summary, nitrate and nitrite participate in a dynamic interchange that involves ingestion, endogenous synthesis and excretion, mainly as nitrate in the urine. Dietary exposure is primarily as nitrate, approximately 5% of which is reduced in the mouth to

nitrite, which then passes into the stomach; some of the nitrate is absorbed into the circulation. Under acidic gastric conditions, the newly formed nitrite is converted partially to nitric oxide that participates in gastric functioning. Some of the gastric nitrite also passes into the general circulation where it is oxidized by haemoglobin into nitrate that then re-enters the cycle. Endogenous synthesis is mainly via the arginine-to-nitric oxide pathway followed by ultimate conversion of nitrogen oxide to nitrate/nitrate. Although this is the predominant relationship between nitrite and nitric oxide, it is now clear that, under some physiological conditions, e.g. acidic hypoxia, nitrite can be converted in the reverse direction to nitric oxide. There is evidence that nitrite may function partly as a storage system for nitric oxide, and that it may be involved in other important processes in the body.

4.1.7 *Endogenous formation of N-nitroso compounds*

Most of the evidence that nitrite or *N*-nitroso compounds may induce cancer at a particular site is based on the presence or formation of *N*-nitroso compounds at or near these sites, e.g. in the lumen of the stomach and colon, or on the excretion of *N*-nitroso compounds in urine or faeces. This section also discusses the formation of other potential carcinogens and of deaminated DNA bases from nitrite.

Nitrate is a potential hazard because it can be reduced to nitrite *in situ* in foods, as well as endogenously. This nitrite can react with amines or amides to form potentially carcinogenic *N*-nitroso compounds. In the human body, the oral cavity is the main site of reduction of nitrate to nitrite through high nitrate concentrations and high levels of nitrate-reducing bacteria. Thus, 25% of ingested nitrate is secreted into the saliva and 20% of the secreted nitrate (5% of ingested nitrate) is reduced to nitrite by oral bacteria (National Research Council, 1981). Although the majority of nitrate in the body arises from ingested nitrate (except during inflammation), there is—as noted elsewhere in this monograph—a substantial contribution from endogenous synthesis (Green *et al*., 1981). This is primarily via the arginine–nitric oxide pathway (Marletta *et al*., 1988; Rassaf *et al*., 2005), but some may arise via other precursors, e.g. ammonia, as has been found in rats (Saul & Archer, 1984).

(a) In-vivo nitrosation due to nitrite, dinitrogen tetroxide, bacterial action and inflammation

This subject has been reviewed (National Research Council, 1981). Tannenbaum (1987) listed three types of in-vivo nitrosation: acid-catalysed nitrosation in the stomach, the reaction of inhaled nitrogen dioxide (or, more correctly, dinitrogen tetroxide) in the lung and nitrosation mediated by macrophages and bacteria.

(i) *Acid-catalysed nitrosation in the stomach*

Gastric nitrosation is thought to be brought about by nitrite that arises from salivary reduction of nitrate. Since most saliva is swallowed, about 80% of gastric nitrite in the normal, acidic stomach is formed through reduction of ingested or endogenous nitrate. The remaining 20% of gastric nitrite arises from ingested nitrite in nitrite-preserved meat, fish and other foods. Gastric nitrosation may be catalysed by thiocyanate (Mirvish, 1983; National Research Council, 1981). In the achlorhydric stomach, bacteria proliferate and can reduce large amounts of nitrate to nitrite. Mean nitrite levels were 0.1–2.6 and 26–54 μM in fasting gastric juice of pH < 5 and > 5, respectively (Xu & Reed, 1993).

(ii) *Chemical acid-catalysed nitrosation*

The principal reactions involved in chemical acid-catalysed nitrosation have been reviewed (Mirvish, 1975; National Research Council, 1981), and are shown in the equations below. For nitrite to produce nitrosation, it must first be acidified to form nitrous acid (HNO_2; Equation [1]). This dimerizes with loss of water to form nitrous anhydride (N_2O_3; Equation [2]), which can react with amines to form nitrosamines (R_2N–NO; Equation [3]). For nitrosation of amines in the absence of catalysts (Equations [2] and [3]), the reaction rate is proportional to the square of the nitrite concentration (Equation [4]) and is maximal at pH 3.3 (the acid dissociation constant of nitrous acid). Nitrous acid can also be protonated to form the nitrous acidium ion ($H_2NO_2^+$; Equation [5]), which preferentially reacts with amides to form nitrosamides (RN(NO)COR′; Equation [6]). The rate of this reaction is proportional to the nitrite concentration and increases 10-fold for each one-unit drop in pH (Equation [7]). Because of the initial acidification step (Equation [1]), these reactions occur *in vivo* mainly in the acidic stomach (Mirvish, 1975; Leaf *et al*., 1989b). When catalysts such as iodide and thiocyanate are involved, the catalyst—taking thiocyanate (NCS^-) as an example—reacts with nitrous anhydride to form the active intermediate (ON–NCS; Equation [8]) that reacts with amines to form nitrosamines (Equation [9]). In this case, the nitrosation rate is proportional to the concentration of nitrite (Equation [10]).

In other reaction pathways, nitrous anhydride can be oxidized to give dinitrogen tetroxide (N_2O_4; Equation [11]). It can also reversibly decompose to yield nitrogen oxide and nitrogen dioxide (Equation 12). The nitrogen oxide generated in this way, or produced from arginine by nitrogen oxide synthase, can react with oxygen to form nitrogen dioxide (Equation [13]), which dimerizes to form dinitrogen tetroxide (N_2O_4; Equation [14]). Dinitrogen tetroxide reacts with secondary amines to produce nitrosamines (Equation [15]), with water to yield a mixture of nitrous and nitric acids (Equation [16]) or with amines to form a mixture of nitrosamines and nitramines (R_2N–NO_2), the latter of which are weak carcinogens (Equation [17]). Nitrous acid decomposes spontaneously in strong acid, especially at concentrations above 25 mM, to give nitrogen oxide and nitric acid (Turney & Wright, 1959; Equation [18]).

$NaNO_2 + H^+ \rightarrow HNO_2 + Na^+$ [1]

$2HNO_2 \leftrightarrows N_2O_3 + H_2O$ [2]

$N_2O_3 + R_2NH \rightarrow R_2N–NO$ (a nitrosamine) + HNO_2 [3]

$Rate = k \times [amine] \times [nitrite]^2$ [4]

$HNO_2 + H^+ \leftrightarrows H_2NO_2^+$ [5]

$H_2NO_2^+ + RNHCOR' \rightarrow RN(NO)COR'$ (a nitrosamide) + $H_2O + H^+$ [6]

$Rate = k \times [amide] \times [nitrite] \times [H^+]$ [7]

$N_2O_3 + NCS^- \rightarrow ON–NCS + NO_2^-$ [8]

$ON–NCS + R_2NH \rightarrow R_2N–NO + HNCS$ [9]

$Rate = k \times [amine] \times [nitrite] \times [halide\ or\ thiocyanate]$ [10]

$2N_2O_3 + O_2 \rightarrow 2N_2O_4$ [11]

$N_2O_3 \leftrightarrows NO + NO_2$ [12]

$2NO + O_2 \rightarrow 2NO_2$ [13]

$2NO_2 \leftrightarrows N_2O_4$ [14]

$N_2O_4 + R_2NH \rightarrow R_2N–NO + HNO_3$ [15]

$N_2O_4 + H_2O \rightarrow HNO_2 + HNO_3$ [16]

$N_2O_4 + 2R_2NH \rightarrow R_2N–NO + R_2N–NO_2$ (a nitramine) + H_2O [17]

$3HNO_2 \rightarrow 2NO + HNO_3 + H_2O$ (decomposition of nitrous acid) [18]

In the rate equations [4], [7] and [10], the square brackets refer to molar concentration, and the named compounds refer to total concentration irrespective of the state of ionization. Equations that give the particular ions involved, e.g. nitrite anion, nitrous acid, non-protonated amine and protonated amine, have been discussed in detail elsewhere (Mirvish, 1975; National Research Council, 1981). Equations [4] and [7] apply to secondary amines and Equation [10] pertains to *N*-alkylamides (Mirvish, 1975). The rate constants (k values) for at least Equations [1] and [2] may vary by factors of five to six orders of magnitude depending on the particular amine or amide that is nitrosated.

(iii) *Bacterial nitrosation in the stomach and intestine*

Bacterial nitrosation proceeds at neutral pH, is most likely to take place in the achlorhydric stomach and is mediated most efficiently by bacteria that can carry out denitrification (reduction of nitrate to nitrogen) (Leach *et al*., 1987; Calmels *et al*., 1991). In an in-vitro study, *Escherichia coli* reduced nitrite to nitric oxide and small amounts of

nitrogen dioxide under anaerobic conditions. When air was admitted, nitrosating agents were produced, which were presumably nitrous anhydride and dinitrogen trioxide that reacted with amines to form nitrosamines. The nitric oxide produced from nitrite was oxidized by dissolved oxygen to nitrogen dioxide, which dimerized to dinitrogen tetroxide or reacted with more nitric oxide to form nitrous anhydride. These reactions were inhibited by nitrate and azide, which indicates that the nitrite was reduced by nitrite reductase. The potential for nitrosamine formation was determined by the nitrosation of 2,3-diaminonaphthalene to form fluorescent naphthotriazole (Ji & Hollocher, 1988a,b; Misko *et al*., 1993).

In an in-vitro study on nitrosation by intestinal bacteria, more than 50 different strains were investigated. *E. coli* A10 catalysed the formation of nitrosamine from nitrite plus dimethylamine and other secondary amines. Other aerobic intestinal bacteria also catalysed these reactions, for which the optimum pH was 7.5. In this study, only one of 32 anaerobic species of bacteria catalysed nitrosamine formation (Suzuki & Mitsuoka, 1984).

Bacterial catalysis of nitrosation in the achlorhydric rat stomach was demonstrated when rats were treated with omeprazole to suppress gastric acid secretion and then gavaged with nitrosation-proficient *E. coli* A10 bacteria. Control rats did not receive omeprazole or *E. coli*. The rats were given nitrate and thiazolidine-4-carboxylic acid (the precursor of NTCA). Excretion of NTCA in the urine was 4.8±1.2 nmol/day in control rats, 2.8±07 nmol/day in rats treated with omeprazole and 19.2±3.5 nmol/day in rats that received omeprazole and *E. coli* A10 bacteria (Calmels *et al*., 1991).

In a study of the composition of gastric juice and urine in 45 patients on long-term omeprazole treatment—which would raise the level of gastric bacteria—and in 13 untreated controls, gastric pH was raised in the patient groups (60% of gastric juice samples had pH > 6) and gastric nitrite level was slightly elevated by the omeprazole treatment, but the concentrations of volatile nitrosamines in the stomach were not affected. Urinary excretion of NDMA was significantly higher in patients than in controls. Patients who tested positive for *Helicobacter pylori* showed somewhat higher gastric nitrite levels and urinary NDMA excretion than did uninfected patients (Vermeer *et al*., 2001).

(iv) *Nitrosamines in the blood*

Ellen *et al*. (1982) analysed the blood and urine of patients who took 2.5–9.0 g ammonium nitrate daily to prevent redevelopment of calcium phosphate kidney stones for the presence of volatile nitrosamines. NDMA and NDEA were not detected in the blood, whereas traces of NPYR of around 0.1 μg/L were found in six of 23 urine samples.

(v) *Nitrate and nitrite levels and nitrosation in the normal colon*

Chemical nitrosation in the human and rodent colon is not likely to occur because of the near-neutral pH and the low levels of nitrite. Normal faecal levels were 0–0.9 mg/kg

for nitrate and 0.3–0.9 mg/kg for nitrite (Saul *et al*., 1981). *N*-Nitroso compounds can be found endogenously within the colon because amines and amides produced mainly through bacterial decarboxylation of amino acids can be *N*-nitrosated in the presence of a nitrosating agent, mediated by anaerobic bacteria at an optimum of pH 7.5 (Mirvish, 1995; Calmels *et al*., 1988; Rowland *et al*., 1991; Bingham *et al*., 2002).

(vi) *Nitrite levels in the inflamed colon*

Ulcerative colitis was associated with elevated nitrite levels and decreased pH in colonic dialysates, which were collected by placing dialysis tubing filled with water or buffer in the colon of patients for 1 h (Roediger *et al*., 1986, 1990). Median values were 10.2 nmol nitrite/mL dialysate (mean pH, 7.1) in patients and 5.3 nmol/mL (mean pH, 7.5) in controls (this value in controls is not consistent with the very low levels in faeces reported by Saul *et al*., 1981). This nitrite probably derives from nitrogen oxide produced by inflammation in the mucosa and could yield *N*-nitroso compounds in the lumen. Nitrosating species derived from nitrogen oxide could also deaminate DNA bases directly. These reactions may help explain the risk for colon cancer in cases of ulcerative colitis (Gyde *et al*., 1988; Bernstein, 2000).

In a population-based case–control study, patients who had inflammatory bowel disease had a higher incidence of colon cancer if they drank water with high levels of nitrate (De Roos *et al*., 2003). In another study, levels of NDMA were measured in the faeces of patients with inflammatory bowel disease, who were given liquid nutrition at the hospital or had an unrestricted diet at home. Seventeen ulcerative colitis patients and 17 healthy controls were examined. NDMA was determined by extraction followed by gas chromatography–mass spectrometry. Mean levels of NDMA (as ng/g faeces) were: all controls, 1.4; all patients, 10.9; non-hospitalized patients, 14.3; and hospitalized patients, 2.4. Hence, diet can affect the level of NDMA in such cases. It was proposed that diet would modulate the risk for colon cancer in patients with inflammatory bowel disease, who should avoid foods that contain high levels of *N*-nitroso compound precursors, e.g. nitrite-processed meat (de Kok *et al*., 2005).

The amounts of faecal *N*-nitroso compounds increased 1.9-fold in mice that had acute colitis induced by dextran sulfate sodium compared with untreated mice (Mirvish *et al*., 2003).

4.2 Genetic and related effects

4.2.1 *Humans*

Kleinjans *et al*. (1991) studied chromosomal damage in the peripheral lymphocytes of human populations exposed to low (0.13 mg nitrate/L), medium (32.0 mg/L) and high (56.0–311.0 mg/L; mean 133.5±68.5 mg/L) levels of nitrate in the drinking-water in the Netherlands. The high nitrate levels were from private water wells. Nitrate contamination

of drinking-water caused dose-dependent increases in nitrate body-load as monitored by 24-h urinary nitrate excretion, but was not associated with an increase in the frequencies of sister chromatid exchange in peripheral lymphocytes.

van Maanen *et al*. (1996) compared the frequency of *HPRT* (hypoxanthine–guanine phosphoribosyltransferase) variants in the peripheral lymphocytes of human populations exposed to various levels of nitrate in the drinking-water (0.02 and 17.5 mg/L from public water supplies, 14 and 21 subjects, respectively; 25 and 135 mg/L from private water wells, six and nine subjects, respectively) in the Netherlands. Higher nitrate intake via drinking-water resulted in a dose-dependent increase in 24-h urinary nitrate excretion and in increased levels of salivary nitrate and nitrite. The mean log frequency of *HPRT* variants was significantly higher in the group with medium exposure from well-water (25 mg/L) than in the groups with low and medium exposure from tap-water (0.02 and 17.5 mg/L, respectively). Multiple regression analysis showed a significant correlation between frequency of *HPRT* variants and 24-h urinary nitrate excretion ($P = 0.02$) and salivary nitrite levels ($P = 0.03$) and between 24-h urinary excretion of NPYR (measured by gas chromatography-mass spectrometry) and 24-h urinary nitrate excretion ($P = 0.02$). [The Working Group noted that the volatile nitrosamines reported to be present in urine are generally metabolized and not excreted.] A small number of the well-water samples were mutagenic in the *Salmonella typhimurium* mutagenicity test after concentration over XAD-2 resin.

Tsezou *et al*. (1996) examined the frequency of sister chromatid exchange and chromatid/chromosomal aberrations in the peripheral blood lymphocytes of 70 children (12–15 years of age) who were exposed to elevated concentrations of nitrates (55.7–88.0 mg/L) in the drinking-water and 20 children from areas with low nitrate concentrations (0.7 mg/L) in Greece. The mean numbers of chromatid and chromosome breaks were significantly increased in children exposed to nitrate concentrations above 70.5 mg/L ($P < 0.01$), but there was no increase in the mean level of sister chromatid exchange. [The Working Group noted that other genotoxic agents present in the drinking-water could be responsible for the increase in chromosomal aberrations.]

4.2.2 *Experimental systems*

(a) Genotoxicity and mutagenicity

(i) *Nitrate* (see Table 4.1 for details and references)

Potassium nitrate and sodium nitrate were not mutagenic in the Ames test in several strains of *S. typhimurium*.

Sodium nitrate did not induce DNA single-strand breaks in Chinese hamster V79 cells *in vitro* as measured with an alkaline elution assay. Positive results were reported in an in-vitro test for chromosomal aberrations in Chinese hamster fibroblasts with

Table 4.1. Genetic and related effects of nitrate

Test system	Results[a]		Dose[b] (LED or HID)	Reference
	Without exogenous metabolic activation	With exogenous metabolic activation		
Salmonella typhimurium TA1535, TA100, reverse mutation	–	–	20.0 mg/plate[c]	Ishidate *et al.* (1984)
Salmonella typhimurium TA1535, TA100, reverse mutation	–	–	5 mg/plate	Ishidate *et al.* (1984)
DNA single-strand breaks, Chinese hamster V79 cells *in vitro*	–		1 mM, 2 h[d]	Görsdorf *et al.* (1990)
Chromosomal aberrations, Chinese hamster fibroblast cells *in vitro*	–	NT	1 mg/mL[c]	Ishidate *et al.* (1984)
Chromosomal aberrations, Chinese hamster fibroblast cells *in vitro*	+	NT	4 mg/mL, 24 h, 48 h	Ishidate *et al.* (1984)
Unscheduled DNA synthesis, mid-spermatids	–		600, 1200 ig, 3 days[e]	Alavantić *et al.* (1988)
Gene (8-azaguanine-resistance and ouabain-resistance C3HX101 mice, *in vivo*) mutation, hamster embryonic cells, *in utero*	–		500, ig × 1[d]	Inui *et al.* (1979)
Micronucleus formation, hamster embryonic cells *in utero*	–		500, ig × 1[d]	Inui *et al.* (1979)
Micronucleus formation, male Swiss mouse, polychromatic erythrocytes cells *in vivo*	±[f]		78.5, ig × 2	Luca *et al.* (1985)
Chromosomal aberrations, hamster embryonic cells *in utero*	–		500, ig × 1[d]	Inui *et al.* (1979)
Chromosomal aberrations, male Wistar rat bone-marrow cells *in vivo*	–[g]		2120, ig × 2	Luca *et al.* (1985)
Chromosomal aberrations, male Wistar rat bone-marrow cells *in vivo*	+[g]		78.5, ig × 2 weeks	Luca *et al.* (1985)
Chromosomal aberrations, male Swiss mouse bone-marrow cells *in vivo*	±[h]		706.6, ig × 2 weeks	Luca *et al.* (1985)
Chromosomal aberrations, male C57Bl mouse bone-marrow cells *in vivo*	+		50, 100, ip[e]	Rasheva *et al.* (1990)
Morphological transformation, hamster embryonic cells *in utero*	–		500, ig × 1[d]	Inui *et al.* (1979)
Sperm-head abnormality test	–[i]		600, 1200 ig, 3 days[e]	Alavantić *et al.* (1988)

[a] +, positive; –, negative; ±, positive at certain concentrations only; NT, not tested

[b] LED, lowest effective dose; HID, highest ineffective dose; ig, intragastric; ip, intraperitoneal; doses are converted to μg/mL for in-vitro experiments and mg/kg per day for in-vivo experiment unless otherwise specified.

[c] Potassium nitrate was used. All other experiments were carried out with sodium nitrate.

[d] Only one dose was examined.

[e] Only two doses were examined.

[f] 6 h after the last dose of nitrate; the higher doses (706.6 and 2120 mg/kg ip × 2) were negative.

[g] 24 h after the last dose of nitrate

[h] The higher dose (2120 mg/kg ig × 2 weeks) was negative.

[i] 11 and 17 days after the last dose of nitrate

sodium nitrate, whereas potassium nitrate gave negative results. As sodium chloride was also positive in the same assay, the chromosomal aberrations induced by sodium nitrate could be due to the high osmotic pressure and sodium ion concentration.

No micronucleus formation, chromosomal aberrations, morphological or malignant cell transformation or drug-resistant mutation were observed in embryonic cells obtained from Syrian golden hamsters that had been given oral doses sodium nitrate on days 11 and 12 of pregnancy.

Significant increases in the frequency of chromosomal aberrations were reported in the bone marrow of rats treated intragastrically with sodium nitrate for 2 weeks. The response was much weaker in mice. Similarly, increased cytogenetic changes were reported in the bone narrow of mice injected intraperitoneally with sodium nitrate.

When sodium nitrate was given to mice by gastric intubation, no increased in-vivo genotoxicity, as assayed by unscheduled DNA synthesis in early-to-mid-spermatids, and no sperm-head abnormality were observed.

(ii) *Nitrite* (see Table 4.2 for details and references)

Below pH 5.5, nitrite is converted to nitrous acid which can react with amino groups. Primary amines could thus easily be deaminated and secondary amines react with nitrous acid to form *N*-nitroso compounds. Early studies on the reaction of nitrous acid with RNA and DNA and on genetic effects of nitrous acid have been reviewed (Zimmermann, 1977). Nitrous acid can deaminate various nucleobases, including guanine, adenine and cytosine which are converted to xanthine, hypoxanthine and uracil, respectively. Nitrous acid can induce point mutations mainly by deamination of cytosine and adenine, but not of guanine because its deamination is rapidly lethal. More complex events may also be caused by nitrous acid-induced DNA–DNA and DNA–protein crosslinks (Zimmermann, 1977).

Sodium and potassium nitrite are mutagenic in *S. typhimurium* (strains TA100, TA1530, TA1535 and TA1950 with or without metabolic activation). Sodium nitrite gave weakly positive results in the *umu* test and negative results in the SOS chromotest.

Chronic feeding of 3-day-old larvae of *Drosophila melanogaster* with sodium nitrite at a concentration of 72.5 mM produced small single wing spots in the somatic mutation and recombination test.

Sodium nitrite did not induce DNA single-strand breaks in cultured mouse mammary carcinoma cells or in Chinese hamster V79 cells. However, it induced chromosomal aberrations in cultured mouse mammary carcinoma cells, hamster fibroblasts, Syrian hamster embryo cells, monkey fetal liver cells and HeLa cells *in vitro*. Sodium nitrite up to a concentration of 1 mM did not induce DNA single-strand breaks in Chinese hamster V79 cells *in vitro*, as measured with an alkaline elution assay. Sodium nitrite induced 6-thioguanine-resistant mutants in V79 hamster cells *in vitro,* and 8-azaguanine-resistant mutants in mouse mammary carcinoma cells *in vitro*. Incubation of newborn hamster cells *in vitro* with sodium nitrite resulted in aneuploidy, chromosomal aberrations and cell

Table 4.2. Genetic and related effects of nitrite

Test system	Results[a]		Dose[b] (LED or HID)	Reference
	Without exogenous metabolic activation	With exogenous metabolic activation		
SOS chromotest, *Escherichia coli* strain PQ37	–	–	6.9 mg/mL	Brams *et al.* (1987)
Salmonella typhimurium TA100, TA1535, reverse mutation	+	+	10 mg/plate	Ishidate *et al.* (1984)
Salmonella typhimurium TA100, reverse mutation	NT	+	1 mg/plate	Brams *et al.* (1987)
Salmonella typhimurium TA100, reverse mutation	NT	+	10 mg/plate[c]	Rubenchik *et al.* (1990)
Salmonella typhimurium TA100, TA1535, reverse mutation	+	+	1 mg/plate[d]	Prival *et al.* (1991)
Salmonella typhimurium TA100, reverse mutation	+	+	1 mg/plate	Zeiger *et al.* (1992); National Toxicology Program (2001)
Salmonella typhimurium TA100, TA1530, TA1535, reverse mutation	+	+	1 mg/plate	Balimandawa *et al.* (1994)
Salmonella typhimurium TA102, YG1024, DJ400, DJ460, reverse mutation	–	–	5 mg/plate	Balimandawa *et al.* (1994)
Salmonella typhimurium TA98, TA97, reverse mutation	NT	–	1 mg/plate	Brams *et al.* (1987)
Salmonella typhimurium TA98, reverse mutation	–	–	10 mg/plate	Zeiger *et al.* (1992)
Salmonella typhimurium TA1950, reverse mutation	NT	+	10 mg/plate[c]	Rubenchik *et al.* (1990)
umu test, *Salmonella typhimurium* TA1535/pSK1002, reverse mutation	(+)	NT	2.2 mg/mL, 2 h	Nakamura *et al.* (1987)
Drosophila melanogaster, somatic mutation, wing spot test	+		72.5 mM, 48 h[c]	Graf *et al.* (1989)
DNA single-strand breaks, C3H mouse mammary carcinoma cells *in vitro*	–	NT	100 mM, 24 h	Kodama *et al.* (1976)
DNA single-strand breaks, Chinese hamster V79 cells *in vitro*, alkaline elution	–		1 mM, 2 h[c]	Görsdorf *et al.* (1990)
Gene (8-azaguanine-resistant) mutation, C3H mouse mammary carcinoma cells *in vitro*	+	NT	1 mM, 48 h	Kodama *et al.* (1976)
Gene (6-thioguanine-resistant) mutation, Chinese hamster V79 cells *in vitro*	+	NT	0.05%, pH 4.95, 30 min	Budayová (1985)
Chromosomal aberrations, Chinese hamster fibroblasts *in vitro*	+	NT	0.25 mg/mL, 24 h	Ishidate *et al.* (1984)
Chromosomal aberrations, C3H mouse mammary carcinoma cells *in vitro*	+	NT	3.2 mM, 24, 48 h	Kodama *et al.* (1976)
Chromosomal aberrations, Syrian hamster embryo cells *in vitro*	+	NT	20 mM, 24 h	Tsuda & Kato (1977)
Chromosomal aberrations, BS-C-1 (African green monkey fetal liver cells) and HeLa cells *in vitro*	+	NT	0.265 mg/mL, 24 h[e]	Luca *et al.* (1987)
Cell transformation, Syrian hamster embryo cells	+		50, 100 mM, 24 h[e]	Tsuda *et al.* (1976)
Cell transformation, mouse BALB/c3T3 cells	+		10 mM, 72 h	Tsuda & Hasegawa (1990)
Morphological transformation, hamster embryonic cells *in utero*	+		125, ig × 1	Inui *et al.* (1979)
Aneuploidy, Syrian hamster embryo cells *in vitro*	+		50, 100 mM, 24 h[e]	Tsuda *et al.* (1976)

Table 4.2 (contd)

Test system	Results[a]		Dose[b] (LED or HID)	Reference
	Without exogenous metabolic activation	With exogenous metabolic activation		
Gene (8-azaguanine-resistance and ouabain-resistance) mutations, hamster embryonic cells *in utero*	+		125, ig × 1	Inui *et al.* (1979)
Micronucleous formation, hamster embryonic cells *in utero*	–		250, ig × 1	Inui *et al.* (1979)
Micronucleus formation, male ddY mouse polychromatic erythrocytes	–		200, ip or ig	Hayashi *et al.* (1988)
Micronucleus formation, male Swiss mouse polychromatic erythrocytes	±[f]		1.72, ig × 2	Luca *et al.* (1987)
Micronucleus formation, male Fischer 344/N rat polychromatic erythrocytes	–		100, ip × 3	National Toxicology Program (2001)
Micronucleus formation, male $B6C3F_1$ mouse, polychromatic erythrocytes	–		125, ip × 3	National Toxicology Program (2001)
Micronucleus formation, male and female $B6C3F_1$ mouse, polychromatic erythrocytes	-		5000 mg/L, dw, 14 weeks	National Toxicology Program (2001)
Host-mediated assay, *Salmonella typhimurium* G46 recovered from the peritoneal cavity of male ICR mice	–[g]		150, ig[c]	Couch & Friedman (1975)
Host-mediated assay, *Salmonella typhimurium* TA1530 recovered from the livers of female CD-1 mice	–[h]		120, pH 3.4, ig[c]	Edwards *et al.* (1979)
Host-mediated assay, *Salmonella typhimurium* G46 recovered from the livers of female CD-1 mice	–[h]		2320 µmol/kg, pH 3.4, ig	Whong *et al.* (1979)
Host-mediated assay, *Salmonella typhimurium* G46 recovered from the livers of female CD rat	–[h]		2170 µmol/kg, pH 3.4, ig	Whong *et al.* (1979)
Host-mediated assay, *Escherichia coli* K-12 *uvrB/recA* DNA repair recovered from blood, livers, lungs, kidneys and testicles of male NMRI mice	–[h]		70, 210, ig[c]	Hellmér & Bolcfoldi (1992)
DNA single-strand breaks, male Fischer 344 rat stomach mucosa *in vivo,* alkaline elution	–[h]		6.9 mg/rat, ig[c]	Ohshima *et al.* (1989)
Unscheduled DNA synthesis, early-to-mid spermatids *in vivo*	–[i]		60 & 120, 3 days[e], ig	Alavantić *et al.* (1988)
Unscheduled DNA synthesis, male Fischer 344 rat stomach mucosa *in vivo*	–[h]		6.9 mg/rat, ig[c]	Ohshima *et al.* (1989)
Chromosomal aberrations, male Wistar rat bone-marrow cells *in vivo*	+		1.72, ig × 2	Luca *et al.* (1987)
Chromosomal aberrations, male Swiss mouse bone-marrow cells *in vivo*	+		1.72, ig × 2	Luca *et al.* (1987)
Chromosomal aberrations, male Chinchilla rabbits bone-marrow cells, *in vivo*	±[f]		1.72 mg/kg dw, 3 months	Luca *et al.* (1987)

Table 4.2 (contd)

Test system	Results[a]		Dose[b] (LED or HID)	Reference
	Without exogenous metabolic activation	With exogenous metabolic activation		
Chromosomal aberrations, female albino rat, bone-marrow cells *in vivo*	+		1.25 g/L dw (210[j]), 13 days[c]	el Nahas *et al.* (1984)
Chromosomal aberrations, hamster embryonic cells *in utero*	–		500, ig × 1	Inui *et al.* (1979)
Chromosomal aberrations, liver cells from embryos after exposure of pregnant albino rats	+		1.25 g/L dw (210j), 13 days (days 5–18 of gestation)[c]	el Nahas *et al.* (1984)
Chromosomal aberrations, liver cells from embryos after *in utero* exposure	–		100 mg/mL dw (0.8 mg/mouse/day), 7–18 days of pregnancy	Shimada (1989)
Sperm-head abnormality test	+[k]		120, 3 days, ig	Alavantić *et al.* (1988)

[a] +, positive; (+), weakly positive; –, negative; ±, positive at certain concentrations only; NT, not tested
[b] LED, lowest effective dose; HID, highest ineffective dose; dw, drinking-water; ig, intragastric; ip, intraperitoneal; doses are converted to µg/mL for in-vitro experiments and mg/kg per day for in-vivo experiment unless otherwise specified.
[c] Only one dose was examined.
[d] Potassium nitrite was used. All other experiments were carried out with sodium nitrite.
[e] Only two doses were examined.
[f] The highest dose (46.66 mg/kg ig × 2) was negative.
[g] 3 h after nitrite dose
[h] 2 h after nitrite dose
[i] 17, 28 and 34 days after the last dose of nitrite
[j] estimated amount consumed per day
[k] 11 and 17 days after the last dose of nitrite

transformation. Similarly, incubation of mouse BALB/c3T3 cells with sodium nitrite resulted in the induction of transformed foci (type III foci) in a dose-dependent manner.

An in-vivo intrahepatic host-mediated mutagenicity assay in female CD-1 mice or female CD rats using *S. typhimurium* G46—introduced into the animals as the detecting organism—showed endogenous formation of NDMA from sodium nitrite and dimethylamine, but no increased mutagenicity was found when sodium nitrite was administered alone. Similarly, no increased mutagenicity was observed in the host-mediated assay using *S. typhimurium* G46 or TA1530 in male ICR mice or female CD-1 mice, respectively, given sodium nitrite. Sodium nitrite administered orally to NMRI mice also had no mutagenic effect in the *E. coli* K-12 *uvrB/recA* DNA repair host-mediated assay.

No increases in DNA single-strand breaks or unscheduled DNA synthesis were observed in the pyloric mucosa of rats given sodium nitrite orally. When sodium nitrite was given to mice, no increased unscheduled DNA synthesis in early-to-mid-spermatids was observed, but sperm-head abnormality was detected 11 and 17 days after treatment.

When sodium nitrite was given in the drinking-water to pregnant rats (days 5–18 of gestation) or non-pregnant rats, chromosomal aberrations were induced in the bone marrow of both pregnant and non-pregnant adults and in the embryonic liver. Sodium nitrite was given by gavage to Syrian hamsters on days 11 and 12 of pregnancy, and embryonic cells were obtained 24 h later. A dose-dependent increase in micronucleus formation and in 8-azaguanine- and ouabain-resistant mutations in embryonic fibroblasts was observed, as well as neoplastic transformation of the cells. However, sodium nitrite given to mice in the drinking-water on days 7–18 of gestation did not induce developmental toxicity or chromosomal aberrations in fetal liver cells. No increase in micronucleated polychromatic erythrocytes was found in bone-marrow cells when sodium nitrite was given intraperitoneally or by gavage to mice as single doses.

4.3 Nitrosamines and cancer

4.3.1 *Induction of cancer in rodents by nitrosamines*

In rodents, nitrosamines principally induce tumours of the liver, oesophagus, nasal and oral mucosa, kidney, pancreas, urinary bladder, lung and thyroid, whereas nitrosamides induce tumours of the lymphatic and nervous systems and (when given orally) of the glandular stomach and duodenum (see Section 3). The site of tumour induction depends on the *N*-nitroso compounds, the rodent species and other factors. The organ specificity probably stems from tissue-specific cytochrome P450 isozymes that activate the nitrosamines which alkylate DNA in the organ where they are activated.

Nitrosamides often induce tumours at the site of administration, e.g. in the glandular stomach when given orally, but can also act at distant sites. Some organ specificity of nitrosamides may arise because, unlike the liver, sensitive tissues such as the brain lack

the alkyltransferase (a DNA-repair enzyme) that regenerates guanine from O^6-alkylguanine (Rajewsky, 1983).

4.3.2 *Activation in organs other than the site of carcinogenesis*

Nitrosamines may become activated in one organ but alkylate DNA in another organ, where they cause cancer. DNA of blood leukocytes, including stem cells in which mutations could produce cancer, may be alkylated by nitrosamine metabolites produced in hepatocytes while blood circulates through the liver. After treatment with methylating carcinogens, including NDMA, the ratio of *N*7-methylguanine in leukocytes to that in hepatocytes was relatively constant at 0.01–0.03 (Bianchini & Wild, 1994).

Dibutylnitrosamine is a bladder carcinogen. It is metabolized in the liver to *N*-4-hydroxybutyl-*N*-butylnitrosamine, which is oxidized to *N*-3-carboxypropyl-*N*-butylnitrosamine (Okada & Ishidate, 1977). The latter is excreted in the urine. After absorption into the bladder mucosa, it could be activated by mitochondrial β-oxidation as was demonstrated in an in-vitro experiment (Janzowski *et al*., 1994).

4.3.3 *Studies related to the etiology of colon cancer*

The section on formation of *N*-nitroso compounds in the colon presents much of the evidence in support of the view that these compounds may contribute to colon cancer. Both fresh and processed red meat have been associated with colon cancer in epidemiological studies. Processed meat contains added nitrite and fresh and processed meat contain haeme, a component of myoglobin, which may promote the formation of *N*-nitroso compounds. Factors that affect excretion of *N*-nitroso compounds in the 24-h faeces of human subjects kept in a metabolic ward have been studied. Bingham *et al*. (1996) suggested that the association between consumption of red meat and the risk for colon cancer was based on the fact that red meat raises levels of *N*-nitroso compounds in the colon contents.

Faecal excretion of *N*-nitroso compounds in humans increased 3.7-fold when 420 g beef was consumed per day; it did not decline even after this diet was consumed for 40 days; it showed a dose–response relationship with the amount of beef consumed but was not affected when chicken or fish was eaten in place of red meat. Higher faecal levels of *N*-nitroso compounds were associated with longer transit times in the gut (Bingham *et al*., 1996; Bingham, 1999; Hughes *et al*., 2001; Bingham *et al*., 2002).

Faecal water derived from stools of individuals on different diets that contained various amounts of red meat were incubated with human adenocarcinoma cells (HT-29) and examined by the comet assay, which showed the presence of DNA strand breakage. The results were correlated with age, but not with the concentration of apparent *N*-nitroso compounds in the faecal water samples, with the amount of meat in the diet or with the concentration of apparent *N*-nitroso compounds in faecal homogenates (Cross *et al*., 2006). Possibly, the use of faecal water instead of faecal homogenates prevented

meaningful correlations from being established. Other studies have found that water extracts of faeces are directly mutagenic in the Ames test (Kuhnlein *et al*., 1981).

Colonic DNA of subjects from the United Kingdom (a high-incidence area for colon cancer) contains O^6-methylguanine. This supports the view that *N*-nitroso compounds in the colon alkylate colonic DNA (Povey *et al*., 2000). Levels of faecal *N*-nitroso compounds were higher in subjects who ate liver and blood sausages as sources of haeme, but not in subjects who consumed inorganic iron. This suggests that the haeme in myoglobin (the pigment in red meat) increases the formation of *N*-nitroso compounds in the colon and hence explains why red but not white meat is a risk factor for colon cancer (Cross *et al*., 2003).

In a recent study, humans were fed a red meat diet, exfoliated colonic mucosal cells were collected from the surface of faecal pellets and their DNA was analysed for O^6-carboxymethyl guanine by an immunohistochemical method. Levels of this adduct were higher after a red meat diet than after a control diet (Lewin *et al*., 2006). This adduct, as well as O^6-methylguanine, were reported earlier to be generated from the reaction of nitrosated glycine derivatives with guanine in DNA (Harrison *et al*., 1999).

4.3.4 *Studies in rats and mice on total apparent colonic and faecal N-nitroso compounds*

Despite all the research on faecal and colonic *N*-nitroso compounds, it has not been determined whether they are carcinogenic for the colon. The following evidence suggests that colonic *N*-nitroso compounds could cause colon cancer: (*a*) the high level of *N*-nitroso compounds after consumption of red meat and the low level after consumption of chicken or fish, as reported by Bingham (1999); (*b*) the finding that the colonic contents of subjects who eat red meat are genotoxic, as determined by the comet assay (Cross *et al*., 2006); and (*c*) the finding that *N*-nitroso compound precursors purified from hot dogs and then nitrosated are direct mutagens in the Ames test (Zhou *et al*., 2006).

In a study to assess the possible chemical transformation by reactive nitrogen–oxygen species of the heterocyclic amine 2-amino-3-methylimidazo[4,5-*f*]quinoline (IQ), which is formed during the cooking of red meat, various methods to generate these species were investigated in in-vitro systems. Conditions that produced nitric oxide, a nitrosating agent under oxidizing conditions, yielded a product that was identified by mass spectrometry and nuclear magnetic resonance as *N*-nitroso-IQ. The results demonstrate the chemical transformation and activation of IQ by reactive nitrogen–oxygen species and the further activation of its *N*-nitroso product to a DNA-reactive metabolite. The nitrosation of IQ was strongly potentiated by haemin, the ferric porphyrin component of haemoglobin (Lakshmi *et al*., 2002, 2005).

4.3.5 *Studies related to gastric cancer*

The incidence of gastric cancer is far higher in Japan and China than in the USA. This cancer was extremely common in the USA until the Second World War, but its rate has dropped dramatically since then. This decrease has been associated with the widespread introduction of home refrigerators after the war and the resultant decrease in the use of cured meat and an increase in the consumption of fresh vegetables and fruit. The hypothesis that *N*-nitroso compounds are a cause of gastric cancer was first suggested in 1971 (Mirvish, 1971) and has been reviewed several times (Preston-Martin & Correa, 1989; Boeing, 1991; Eichholzer & Gutzwiller, 1998; Jakszyn & Gonzalez, 2006).

Oral administration of certain nitrosoureas and related nitrosocarbamates, as well as the related compound MNNG, induced glandular stomach adenocarcinomas (similar to human gastric cancer) in rodents and other species (Druckrey *et al*., 1966; Ogiu *et al*., 1975; Maekawa *et al*., 1976; Sugimura & Kawachi, 1978).

Nitrosation of foods associated with gastric cancer produced direct mutagens in the Ames test (Marquardt *et al*., 1977). Treatment of extracts of mackerel fish with nitrite at pH 3 produced direct mutagens in *S. typhimurium*. When the extracts were administered thrice weekly for 6 months to Wistar rats, eight of the 12 rats developed gastrointestinal tumours 12–18 months later. Five of the rats developed adenomas or adenocarcinomas of the glandular stomach. Precancerous lesions were observed in nearly all treated animals. Feeding extracts that had not been nitrosated did not induce tumours (Weisburger *et al*., 1980).

(a) Role of Helicobacter pylori

Gastric infection with *H. pylori* contributes to the etiology of gastric cancer (Muñoz, 1994; Fischbach *et al*., 2005). Intestinal metaplasia of the stomach (a precursor lesion for gastric cancer) was associated with *H. pylori* infection, low levels of ascorbic acid and elevated bile acid levels, but not with elevated gastric nitrite or total *N*-nitroso compounds in gastric juice (Sobala *et al*., 1991). Eradication of *H. pylori* restored gastric ascorbic acid to normal levels (Sobala *et al*., 1993). In *H. pylori*-positive patients, low gastric levels of ascorbic acid were correlated with the extent of inflammation and with elevated gastric pH (Rood *et al*., 1994). Gastric adenocarcinomas that resembled human gastric cancer were induced in Mongolian gerbils given *N*-methylnitrosourea (MNU) in the drinking-water and inoculated with *H. pylori* before or after the carcinogen treatment, but not when they were treated with either agent alone (Sugiyama *et al*., 1998).

(b) O^6-Methylguanine in leukocyte DNA

Samples of peripheral blood were collected from randomly selected subjects from 17 populations with different incidences of gastric cancer. Leukocyte DNA was analysed for O^6-methylguanine by a competitive repair assay that can detect 0.05 fmol/μg DNA. Adducts were detected in 21 of 407 samples, including 16 of 102 samples from Portugal and Japan, where the incidence of gastric cancer is high. The presence of adducts was

significantly correlated with the incidence of gastric cancer and with a low (< 25 ng/mL) serum level of pepsinogen A, a marker of severe chronic atrophic gastritis (The EUROGAST Study Group, 1994). This suggests that the achlorhydric stomach produces methylating agents, which could be *N*-nitroso compounds or a compound formed by the nitrosation of methionine (Chen *et al*., 1996).

(c) Mutations in the P53 gene

Mutations in the *P53* gene were studied in 105 cases of gastric and colon cancer from Florence, Italy, to determine whether environmental factors contributed to the induction of gastric cancer in cases who had different types of *P53* mutation. The 33 cases who showed *P53* mutations ate a more traditional diet, including corn meal and meat soup, ate less fresh fruit and vegetables and had a higher nitrite intake than cases of gastric cancer who had no *P53* mutations. Of the 33 cases with *P53* mutations, 19 had G:C→A:T mutations at CpG sites. The 14 cases with mutations at non-CpG sites may have had higher exposure to dietary alkylating agents, whereas patients with *P53* mutations at CpG sites were more closely related to *H. pylori* infection (Palli *et al*., 1997).

4.4 Other relevant toxic effects

4.4.1 *Methaemoglobinaemia*

The most widely recognized effect of excess nitrate on health is methaemoglobinaemia, a condition whereby methaemoglobin is formed in the blood due to the interaction of haemoglobin with nitrite, the reduction product of nitrate. During this reaction, the iron atom is oxidized from the ferrous to the ferric state:

NO_2^- + oxyhaemoglobin (Fe^{2+}) → methaemoglobin (Fe^{3+}) + NO_3^-.

As a consequence, oxygen is less efficiently transported by the red blood cell.

The condition has been observed primarily in infants under 6 months of age, and particularly in those under 3 months of age (Comly, 1945; Bosch *et al*., 1950). Infantile methaemoglobinaemia is also commonly known as the blue baby syndrome. Infants are particularly susceptible because (*a*) fetal haemoglobin is more easily oxidized to methaemoglobin and a large proportion of fetal haemoglobin is still present in the infant's blood (60–80% at less than 3 months, 20–30% by 3 months of age) (National Research Council, 1981; WHO,1996a); (*b*) infants have a transient deficiency in nicotinamide adenine dinucleotide phosphate-dependent cytochrome b_5 methaemoglobin reductase, which is responsible for the reduction of methaemoglobin back to haemoglobin; (*c*) lower production of gastric acid (i.e. a higher gastric pH) in infants leads to greater reduction of nitrate to nitrite by gastric bacteria; (*d*) on a weight basis, infants have a higher intake of nitrate (in the diet and because of greater fluid intake relative to body weight); and (*e*) the occurrence of infantile gastroenteritis with acidosis and gastrointestinal infections increases the yield of nitrite and thus the formation of methaemoglobin.

In addition to methaemoglobin being incapable of binding oxygen, the oxidation of one or more of the haeme iron atoms in the haeme tetramer distorts the tetramer structure, so that the remaining non-oxidized haeme subunits bind oxygen avidly but release it less efficiently, which shifts the oxygen dissociation curve to the left. Therefore, methaemoglobinaemia becomes manifest as tissue hypoxia (Bradberry *et al*., 2001).

As the level of methaemoglobin increases, cyanosis (with a change in skin colour to bluish grey or brownish grey), anoxaemia and even death can result. Low concentrations of 0.5–3% methaemoglobin occur in normal people and concentrations up to 10% can occur without clinical signs (National Research Council, 1981). The signs and symptoms associated with increasing levels of methaemoglobin are: 10–20%, central cyanosis of the limbs and/or trunk; 20–45%, central nervous system depression (headache, dizziness, fatigue, lethargy) and dyspnoea; 45–55%, coma, arrhythmias, shock and convulsions; and > 60%, high risk of mortality (Fewtrell, 2004). Methylene blue is used as an antidote and ascorbic acid has been shown to have protective effects against nitrite-induced formation of methaemoglobin.

The current WHO (2003) drinking-water guideline (50 mg/L) and the Environmental Protection Agency (2003) drinking-water standard for the USA (45 mg/L or 10 mg/L nitrate-N) for nitrate were established to prevent infantile methaemoglobinemia (see Section 1). The level was based principally on the report of a worldwide survey in which no cases of methaemoglobinaemia were observed with concentrations of nitrate below 45 mg/L (Walton, 1951). Reported cases of methaemoglobinaemia have generally occurred in infants under 3 months of age who had consumed water that contained levels of nitrate higher than 22 mg/L nitrate-N (Craun *et al*., 1981). The absence of cases with levels of nitrate below 45 mg/L (< 10 mg/L nitrate-N) was confirmed in more recent reviews (Fan & Steinberg, 1996; WHO, 1996b, 2003). It was noted that gastrointestinal illness that results from exposure to bacterially contaminated water containing high levels of nitrate may also play an important role in methaemoglobinaemia (Craun *et al*., 1981).

Methaemoglobinaemia has been reported in children who were fed powdered milk or infant formula diluted with well-water that contained nitrate. Cases have also been reported in babies fed vegetables, particularly carrot juice and spinach, in which nitrate had been converted to nitrite through bacterial action during storage or transportation (Simon, 1966; Phillips, 1968; Keating *et al*., 1973).

Clinical cases of adult methaemoglobinaemia due to consumption of water contaminated by nitrate have not been reported, but cases in adults who consumed high doses of nitrate or nitrite by accident or as a medical treatment have been described (Cornblath & Hartmann, 1948).

In a recent literature survey for WHO, it was found that reports of nitrate concentrations in drinking-water higher than 50 mg/L were rarely paralleled by reports of methaemoglobinaemia (Hoering & Chapman, 2004). Another literature-based investigation for WHO (Fewtrell, 2004) did not identify exposure–response relationships that relate the level of nitrate in the drinking-water to methaemoglobinaemia.

Individuals may vary in susceptibility to the formation of methaemoglobin (WHO, 2002). The nitrate:nitrite conversion rate ranges from 5 to 7% for normal individuals to 20% for individuals with a high rate of conversion. Susceptible groups other than infants include people deficient in glucose-6-phosphate dehydrogenase (Foltz *et al*., 2006) or methaemoglobin reductase (Percy *et al*., 2005). In healthy individuals, low gastric pH suppresses the growth of nitrate-reducing bacteria and hence gastric levels of nitrite are low. Although the gastric pH in infants may be higher than that in adults, it is possible that gastrointestinal infections, inflammation and the ensuing overproduction of nitric oxide are major factors that contribute to infantile methaemoglobinaemia (WHO, 2002).

4.4.2 *Reproductive and developmental effects*

Some epidemiological studies have suggested an association between exposure to nitrates in the drinking-water and spontaneous abortions, intrauterine growth restrictions and various birth defects. These studies have uncertainties or limitations such as the lack of individual exposure assessment, the inability to rule out confounding factors or the small size of the study populations. Currently, there is insufficient evidence for an exposure–response or causal relationship between exposure to nitrate in the drinking-water and adverse reproductive or developmental effects (Manassaram *et al*., 2006), although some studies suggested an association.

Scragg *et al*. (1982) conducted a study among pregnant women in South Australia and reported an excess of malformations among newborns (mainly neural tube defects and defects that affected multiple systems) in association with drinking-water consumption from specific sources that differed in nitrate content. Women who used groundwater sources that contained levels of nitrate above 5 mg/L had a statistically significantly increased risk for delivering a child with congenital malformations than women who drank rainwater. The risk was higher for central nervous system defects.

In a follow-up study, Dorsch *et al*. (1984) found that women who consumed principally groundwater had a statistically significant increase in risk for giving birth to a malformed child compared with women who drank only rainwater during their pregnancy. Water from private bores mostly had nitrate levels in excess of 15 mg/L, and municipal water supplies derived from government bores had nitrate levels below or equal to 1 mg/L. The increase occurred specifically for malformations of the central nervous system and musculoskeletal system. There was a nearly threefold increase in risk for women who drank water that contained nitrate at 5–15 mg/L, and a fourfold increase for those who consumed > 15 mg/L nitrate. A seasonal gradient in risk was observed among groundwater consumers, ranging from 0.9 for babies who were conceived in winter, to 3.0 in autumn, to 7.0 in spring and to 6.3 in summer.

Ericson *et al*. (1988) examined all deliveries in Sweden in a case–control study of neural tube defects and did not report any difference in levels of nitrate.

In a Swedish study, a weak association was noted between nitrate in water at a level ≥ 2 mg/L and cardiac malformations (Cedergren *et al*., 2002).

Brender *et al*. (2004) examined exposure to nitrosatable drugs and pregnancies affected by neural tubal defects in relation to dietary nitrite and total nitrite intake (dietary nitrite plus 5% dietary nitrate) in a case–control study of Mexican-American women in Texas, USA. Interviews were conducted with 184 women who had had pregnancies affected by neural tubal defects and 225 women who had had normal livebirths. In addition, nitrate was measured in the usual source of drinking-water for 110 study participants. Nitrosatable drugs increased the probability (2.7-fold; 95% CI, 1.4–5.3) of a pregnancy affected by neural tubal defects. Women with nitrite intake of more than 10.5 mg per day had a 7.5-fold (95% CI, 1.8–45) probability of a pregnancy affected by neural tubal defects if they took nitrosatable drugs. A stronger association (14-fold) was also reported between exposure to nitrosatable drugs and neural tubal defects in women who used drinking-water containing ≥ 3.5 mg/L nitrate.

4.4.3 *Cardiovascular effects*

As discussed above, exposure to nitrate can result in methaemoglobinaemia after conversion to nitrite in the body. Nitrite, but not nitrate, is a smooth muscle relaxant and can produce hypotension especially in the vascular system at doses that overlap those that cause methaemoglobinaemia (National Research Council, 1995). High concentrations of methaemoglobin are associated with hypotension, rapid pulse and rapid breathing, as a result of the vasodilatory effects of nitrite.

A hospital-based study in Colorado, USA, reported on cardiovascular effects in nine communities in Weld County where community water supplies were evaluated as nitrate-free and nine areas where the drinking-water contained 19–125 mg/L nitrate. Among 487 cases of hypertension, there was no evidence of an increased risk for hypertension with increased nitrate consumption. Among residents in the nitrate-exposed communities, an earlier onset of hypertension was observed, which peaked at 50–59 years of age versus 70–79 years in the unexposed (Malberg *et al*., 1978).

4.4.4 *Effects on the thyroid*

(a) Nitrate

van Maanen *et al*. (1994) studied the effect of nitrate on the volume and function of the thyroid in humans exposed to nitrate in the drinking-water. The test populations consisted of healthy women volunteers who had no disease, did not use medication, were not pregnant and had no outdoor jobs. The study included two groups exposed to low (0.02 mg/L, $n = 24$) and medium (17.5 mg/L, $n = 27$) levels of nitrate in tap-water and two groups exposed to medium (≤ 50mg/L, $n = 19$) and high (> 50mg/L, $n = 12$) levels of nitrate in well-water. No iodine deficiency was observed in any of the groups. Concentrations of urinary and salivary nitrate were related in a dose-dependent manner to the consumption of water that contained nitrate. A dose-dependent difference in thyroid volume was observed between low and medium versus high exposure to nitrate, and this

thyroid hypertrophy was significant at nitrate levels exceeding 50 mg/L. Linear regression analysis showed a significant inverse relationship between thyroid volume and the concentration of thyroid-stimulating hormone in serum.

An epidemiological survey was conducted on the incidence of goiter among 181 children 6–14 years of age in the village of Karadzhalovo, a district of Plovdiv, Bulgaria, where the concentration of nitrates in drinking-water was constantly above the tolerable level (range: 78–112 mg/L during 1990–94). Population morbidity was assessed according to ICD-9 and recorded over 5 years (1990–94). The data were compared with those from 178 children of a control village in the same district, where nitrate levels in drinking-water were 28–48 mg/L during the same period. Non-parametrical analysis was used to make a comparative analysis of incidence of disease in both communities during the observation period. A statistically significant difference in the incidence of goiter in children in Karadzhalovo was found (40.9%) compared with that in children in the control village (Gatseva & Dimitrov, 1997; Gatseva *et al*., 1998).

Tajtáková *et al*. (2006) conducted an investigation to assess whether long-term intake of water that contained high levels of nitrate and meals from local agricultural products in eastern Slovakia influence the volume and function of the thyroid in school children. Thyroid volume, serum thyrotropin and anti-thyroperoxidase antibodies were measured in 324 children (10–13 years of age) from high-nitrate areas (51–264 mg/L) who received drinking-water from shallow wells and compared with those of 168 children of the same age from neighbouring low-nitrate areas (< 2 mg/L) and 596 children from the city of Kosice located in the vicinity of the low-nitrate area and also supplied by low-nitrate water. Thyroid volume in the children from high-nitrate areas was significantly larger compared with that in the two groups from the low-nitrate areas. The frequency of serum thyrotropin levels in the range of subclinical hypothyroidism (> 4.0 mU/L) in pooled age groups from the high-nitrate area was 13/324 (4.0%) and the frequency of anti-thyroperoxidase antibodies was 8/324 (2.5%), while the 109 children from the low-nitrate area did not show any of these changes. There were no differences in concentrations of total thyroxine or free triiodothyronine.

4.4.5 *Diabetes mellitus*

A prospective case–control study was conducted in Sweden on dietary factors and the risk for diabetes mellitus. The study population included 339 children aged 0–14 years who had recently developed type I diabetes mellitus and 528 control children matched for age, sex and county of residence. Foods were identified as rich in various nutrients and additives and 'nitrate or nitrite' but no quantitative data were given relating to the levels of nitrate or nitrite intake. A significant but non-linear trend was found for dietary intake of nitrates or nitrites in relation to type I diabetes mellitus. The trend was not affected when the results were standardized for possible confounders (Dahlquist *et al*., 1990).

In a nationwide Finnish case–control study on the epidemiology of type I diabetes, dietary intake of nitrite of children and mothers was positively associated with the risk for

type I diabetes independently from duration of mother's education, child's or mother's age, place of residence or mother's smoking status. The study population consisted of 684 case and 595 control children, 548 case–control pairs of fathers and 620 case–control pairs of mothers. Food consumption frequencies were obtained by structured questionnaire and data on nitrate and nitrite in household water were provided by the Finnish waterworks. The positive association between nitrite intake of the child and mother and risk for type I diabetes in children was seen in all age groups (0–4, 5–9 and 10–14 years) and in both boys and girls. The nitrate intake of the mother was associated with a decreased risk for type I diabetes in children (Virtanen *et al*., 1994).

The possible relationship between the incidence of type I diabetes and nitrate levels in drinking-water in the Netherlands was studied in an ecological study (van Maanen *et al*., 2000). Among a total of 2 829 020 children, 1104 cases of type I diabetes were diagnosed in children aged 0–14 years during 1993–95, and 1064 of these cases were included in the analysis. Two exposure categories were used: one was based on an equal number of children exposed to various levels of nitrate (0.25–2.08, 2.10–6.42 and 6.44–41.9 mg/L), and the other was based on cut-off values of 10 and 25 mg/L nitrate. An effect was observed with increasing age of the children on the incidence of type Idiabetes but no significant effects were found relating to sex or concentration of nitrate in the drinking-water. The 1.46-fold increase in the incidence rate ratio (95% CI, 0.88–2.43) at nitrate exposures > 25 mg/L was not significant, probably due to small numbers (15 observed, 10 expected).

Zhao *et al*. (2001) conducted a study in Cornwall and the former Plymouth Health Authority Regions in the United Kingdom to examine the relationships between nitrate, zinc and magnesium in the drinking-water and risk for childhood onset of type I diabetes mellitus. Children aged 0–15 years who had been diagnosed with type I diabetes mellitus between 1975 and 1996 (total, 517 subjects) were included. The mean concentration (± standard deviation) of nitrate was 6.62±5.02 mg/L (range, 0.49–31.9). The study found no association between nitrate in the drinking-water and risk for childhood onset of type I diabetes mellitus.

4.5 Mechanistic considerations

4.5.1 *Possible carcinogenic pathways involving nitrite*

Under acidic conditions, nitrite is converted to nitrous acid, which can then be converted to nitrosating agents such as nitrous anhydride, dinitrogen tetroxide and the nitrous acidium ion. Similar nitrosating agents can also be formed from nitrogen oxides in the presence of oxygen. Nitrogen oxide may be formed by synthases that are expressed in many cell types and by bacterial reduction of nitrite/nitrate. Once formed, nitrous anhydride or dinitrogen tetroxide can react with unprotonated amino groups at neutral pH, resulting in the formation of *N*-nitroso compounds.

Nitrosating agents react with secondary amines to form dialkyl or cyclic nitrosamines, which can cause a variety of types of damage to DNA as well as DNA mutations. Many of these compounds induce tumours experimentally at various sites of all species (more than 40, including primates) that have been tested to date (Searle, 1976; Lijinsky, 1979; Brown, 1999).

Nitrosamines can be oxidized *in vivo* by cytochrome P450 enzymes (Yang *et al*., 1990) to form α-hydroxynitrosamines that rapidly rearrange and decompose to form aldehydes and electrophilic diazohydroxides. The diazohydroxides react with nucleophilic sites in DNA to form premutagenic lesions (Park *et al*., 1977; Gold & Linder, 1979; Lown *et al*., 1984; Poulsen *et al*., 1987; Ukawa-Ishikawa *et al*., 1998). The mutagenicity of nitrosamines has been investigated extensively (Guttenplan, 1987). Nitrosation of *N*-alkylamides yields *N*-nitrosamides, which can form analogous alkylating species that do not require enzymatic oxidation, especially under basic conditions. For example, a group of over 30 nitrosamides with varying structures were all found to be direct-acting mutagens (Lee *et al*., 1977; Lijinsky & Andrews, 1979) and several of these have been shown to be direct-acting carcinogens in animals (Lijinsky, 1977; Sugimura & Kawachi, 1978; Yamashita *et al*., 1988; Wakabayashi *et al*., 1989). Hence, nitrosamines are probably activated mostly in the organs in which tumours develop.

Nitrosation of primary amines can lead directly to alkyldiazohydroxides or alkyldiazonium ions that, if formed in close proximity to DNA, can lead to alkylation of DNA bases. This reaction can also lead to deamination of DNA bases by nitrosating agents through the nitrosation of exocyclic amino groups. Examples include the deamination of 5-methylcytosine to thymine, of cytosine to uracil, of adenine to hypoxanthine and of guanine to xanthine. Nguyen *et al*. (1992) summarized the types of mutation induced by these deaminations. Deamination of 5-methylcytosine results in the formation of thymine which is a normal base and is not easily repaired. This base change leads to G:C→A:T transition mutations at CpG sites, which are frequently detected in many types of human cancer.

Early studies demonstrated that *N*-nitroso compounds form adducts at several sites in bases. The O^6-alkyl adducts of guanine are promutagenic and can induce G:C→A:T transitions. MNU-induced rat mammary tumours had a high incidence of c-H-*ras* oncogene mutations with G:C→A:T transitions at codon 12 (Zarbl *et al*., 1985; Inui *et al*., 1994). Similarly, NDMA-induced kidney tumours and MNU-induced oesophageal tumours in rats had a high incidence of mutated *p53* genes with G:C→A:T transition at codons 204 and 213 (Ohgaki *et al*., 1991). G:C→A:T transitions at both CpG and non-CpG sites are among the most common types of mutation found in *P53* genes from human tumours (Olivier *et al*., 2004).

Thus, either ingested or endogenously formed *N*-nitroso compounds are potentially carcinogenic through several related pathways. Examples of these in the context of the epidemiological findings presented earlier with regard to specific tumours are summarized below.

4.5.2 *Colon cancer*

The mechanism proposed for the association between consumption of nitrite-preserved meat and colon cancer is that *N*-nitroso compounds present in the meat pass down the gut to the colon and act there to induce cancer, or that *N*-nitroso precursors present in processed meat are converted to *N*-nitroso compounds in the stomach or intestines and that these *N*-nitroso compounds act as carcinogens in the colon. Evidence for this hypothesis is (*a*) that total *N*-nitroso compounds in the faeces increased nearly fourfold when volunteers consumed beef compared with those who did not (Hughes *et al*., 2001); (*b*) that total *N*-nitroso compound precursors from hot dogs, when partially purified and then nitrosated *in vitro*, were directly mutagenic in the *Salmonella* mutagenicity assay (Ames test); (*c*) that O^6-carboxymethylguanine was identified in the DNA of exfoliated colonic cells after human volunteers had consumed red meat (Lewin *et al*., 2006), which is the adduct expected from the reaction of nitrosated glycine derivatives with DNA (Harrison *et al*., 1999); and (*d*) that IQ, one of the heterocyclic amine carcinogens produced when meat is cooked at high temperatures (Sugimura *et al*., 1990; Nagao & Sugimura, 1993), reacts readily with nitrogen oxide in the presence of haemin and hydrogen peroxide to produce *N*-nitroso-IQ, which is a potent mutagen (Wolz *et al*., 1995).

4.5.3 *Oesophageal cancer*

The evidence that nitrosamines are a cause of oesophageal cancer can be summarized as follows (Mirvish, 1995): (*a*) in rats, oesophageal tumours are induced by many unsymmetrical and cyclic nitrosamines; (*b*) urinary excretion of NPRO was correlated in ecological studies with the incidence of oesophageal cancer (see Section 4.1.4); (*c*) O^6-methylguanine was detected in the DNA of normal oesophageal tissue of oesophageal cancer patients in China, which suggests the action of a methylating nitrosamine, such as NDMA (Umbenhauer *et al*., 1985); (*d*) tobacco-specific nitrosamines, especially N′-nitrosonornicotine and 4-(nitrosomethylamino)-1-(3-qyridyl)-1-butanone, induce oesophageal cancer in rats and are very probably the cause of human oesophageal cancer induced by tobacco smoking, which acts synergistically with alcohol.

4.5.4 *Brain tumours*

A large number of epidemiological studies have investigated a possible relationship between intake of nitrate/nitrite and tumours of the brain, and suggestive associations were apparent in several of these (see Section 2.3). However, the association is somewhat less straightforward than that for the colon and stomach, since the route from the digestive system to the site of action is less apparent. There is nevertheless reliable evidence that nitrosoureas can act at sites remote from the site of administration. For example, after an intraperitoneal injection of radiolabelled ENU into rats, Goth and Rajewsky (1974) found O^6-ethylguanine in the brain. The label was also found in non-target tissues (e.g. liver),

but the rate of clearance was lower in the brain, which led the authors to suggest that the longer persistence might allow more DNA replication in the brain and thus present a greater possibility of mutation induction and subsequent tumour formation.

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5. Summary of Data Reported

5.1 Exposure data

Nitrate and nitrite are naturally occurring ions that are part of the nitrogen cycle and are ubiquitous in the environment. Since the early 1900s, nitrate and other nitrogen compounds have been used extensively as fertilizers in agriculture. Moreover, nitrate and nitrite are added to meat and fish for preservation, as colour fixatives and as flavouring agents.

The natural background level of nitrate in groundwater is generally below 10 mg/L. Both groundwater and surface water can be contaminated by nitrate as a result of agricultural activities. Levels of contamination are usually significantly higher in groundwater, particularly in shallow wells. Concentrations that exceed the current WHO guideline of 50 mg/L, and exceptionally exceed 500 mg/L, have been reported in intensive agricultural regions. Nitrite is not frequently detected in drinking-water; when it is present, its concentrations rarely exceed 3 mg/L.

Human exposure to nitrate is principally from exogenous sources, i.e. through the ingestion of food and water, whereas exposure to nitrite is primarily endogenous (see Section 5.4 on the endogenous formation of nitrite).

The main potential contributors to ingested nitrate are vegetables, especially leafy vegetables. Other food sources include bakery goods, cereal products and cured meat. Water is generally a minor source of nitrate, but may become a major contributor to overall nitrate intake at concentrations above 50 mg/L. For an average adult consumer who lives in an area with low drinking-water contamination, total exposure to nitrate from food and water is estimated to be about 60–90 mg per person per day. For high consumers of vegetables, the intake of nitrate may reach 200 mg per person per day. Similar intakes could result from high consumption of water contaminated with more than 50 mg/L nitrate. The impact of contaminated water was confirmed by studies of total internal exposure to nitrate that was measured through 24-h urinary excretion.

The main sources of exogenous human exposure to nitrite are food (see Section 5.4 on the endogenous formation of nitrite). Over the past 30 years, the relative contribution of nitrite-cured meat to dietary exposure to nitrite for an average consumer has decreased substantially. Other sources of nitrite include cereal products and vegetables that are rich in nitrate. The total intake of exogenous nitrite is estimated to be about 0.75–2.2 mg per day for an adult with average food consumption and drinking patterns. Nitrite may react with amines and amides to form *N*-nitroso compounds during the storage of food.

5.2 Human carcinogenicity data

Ingested nitrate and nitrite have been studied in relation to the occurrence of many cancers; however, with the exception of stomach and brain cancers, few case–control or cohort studies are available for any given cancer site. Ingestion of nitrate and nitrite can result in the endogenous formation of *N*-nitroso compounds, particularly in the presence of nitrosatable precursors and in the absence of inhibitors of nitrosation such as vitamin C. Some of the epidemiological studies evaluated nitrite intake from cured meats or animal sources separately. Others evaluated risk among persons who had a higher nitrite and lower vitamin C intake, a dietary pattern that may result in increased endogenous formation of *N*-nitroso compounds. The Working Group gave these latter studies more weight in the evaluation of the literature.

Vegetables are the primary source of nitrate when levels of nitrate in the drinking-water are low. Because many vegetables contain vitamin C or other inhibitors of endogenous nitrosation, nitrate from these sources may result in less endogenous formation of *N*-nitroso compounds than nitrate in the drinking-water. The Working Group therefore considered the evaluation of nitrate ingested in the diet and in drinking-water separately.

5.2.1 *Gastric and oesophageal tumours*

(a) Ingested nitrate

The relationship between stomach cancer and nitrate in the drinking-water has been addressed in 15 ecological studies, two case–control studies and one cohort study. The two case–control studies (one in the USA and the other in Taiwan, China) used deceased cases and reported no association. A cohort study in the Netherlands addressed the contribution of nitrate from water as part of total nitrate intake separately from dietary sources; no association was observed. No clear evidence emerged from the ecological studies.

The association between the intake of nitrate from foods and stomach cancer was analysed in seven case–control and two cohort studies. Three case–control studies reported no association while four found a lower risk for stomach cancer with higher levels of dietary nitrate; no association between dietary nitrate and risk for stomach cancer was seen in the two cohort studies in the Netherlands and Finland. Most of these studies were carried out in populations who had relatively low levels of nitrate in the drinking-water (populations who used public water supplies); thus the majority of the ingested nitrate was from foods, of which vegetables were the main source. The inverse association found in some studies may be attributed to a high correlation between dietary levels of nitrate and high vegetable consumption.

Only one case–control study that was conducted in the USA assessed the relationship between oesophageal cancer and dietary intake of nitrate, for which an inverse association was found. In ecological studies of nitrate in the drinking-water, the pattern for

oesophageal tumours was similar to that described for stomach cancer, although the number of studies is substantially smaller. The few ecological studies provided no clear evidence for an association between nitrate in the drinking-water and oesophageal cancer.

(b) Ingested nitrite

The evidence for an association between dietary intake of nitrite and cancer of the stomach is based on seven well designed case–control studies and two cohort studies, all of which were carried out in Europe and North America.

Six of seven case–control studies found a positive association, which was significant in four. Two studies considered the potential interaction between dietary intake of nitrite and potential inhibitors of nitrosation: a large case–control study conducted in several areas of Italy reported a fivefold increase in risk for subjects who ingested high levels of nitrite and proteins and low levels of antioxidant micronutrients (vitamin C and α-tocopherol) compared with those who had a low intake of nitrite and proteins and a high intake of antioxidants. A multicentric study in the USA reported a significant increase in risk for stomach tumours located in both the cardia and non-cardia regions among subjects who had a high intake of nitrite and a low intake of vitamin C compared with those who had a low intake of nitrite but high vitamin C consumption. Four of the seven case–control studies also assessed the association between stomach cancer and preformed *N*-nitrosodimethylamine: one study in Canada and a study in Italy found no association, while one study in Spain and another in France found that a high intake of *N*-nitrosodimethylamine was positively associated with risk for stomach cancer. None of the studies reported effect estimates for nitrite adjusted for *N*-nitrosodimethylamine.

Two cohort studies were reviewed, one of which was conducted in the Netherlands and the other in Finland. In the Finnish study, no association was found between the risk for stomach cancer and dietary intake of nitrites or *N*-nitrosodimethylamine. The Dutch cohort reported a significant increase in risk for nitrite that was limited to the highest level of intake and became non-significant after adjustment for potential confounders, including vitamin C and β-carotene. However, effect modification of nitrite by vitamin C was not assessed in this study. Overall, the results were consistent for case–control studies, but none of the prospective studies found a clear positive association. Furthermore, none of the studies that were reviewed had taken into account potential confounding or effect modification by *Helicobacter pylori*, an important risk factor for stomach cancer, when assessing the effect of nitrite.

Only two case–control studies of oesophageal cancer, both of which were conducted in the USA, assessed the association with nitrite intake. Both were well designed and adjustment was made for the main risk factors for oesophageal cancer. Both studies reported a positive but non-significant association. When intake of vitamin C was taken into account, both studies observed a significant increase in risk for subjects who had a high intake of nitrite and a low intake of vitamin C compared with those who had low nitrite and high vitamin C consumption. The potential confounding effect of preformed *N*-nitroso compounds was not investigated.

5.2.2 *Brain tumours*

(a) Ingested nitrate

Overall, the Working Group evaluated 10 case–control studies of brain tumours, six of which were conducted in adults and four in children. In one of the four case–control studies of childhood brain tumours, current levels of nitrate in the tap-water in homes in which the pregnancies had occurred were estimated using non-validated measurements from semiquantitative water test strips. No significant association was observed, although the risk for astroglial tumours was non-significantly increased twofold for the highest category of exposure (≥ 50 mg/L as ion).

Two case–control studies of adult brain tumours estimated levels of nitrate in the drinking-water by linking information on residence to public water quality monitoring data from regions that had mainly low to moderate levels of nitrate contamination. One of them also measured nitrate levels in a current tap-water sample from users of private wells. Nitrate in drinking-water was not associated with risk for brain tumours in either of these studies.

None of the studies that focused on dietary sources of nitrate observed a positive association with brain tumours among adults or children. Some observed a decreased risk for brain tumours in relation to dietary nitrate. However, in studies that further adjusted for vitamin C intake, this association was attenuated or disappeared.

(b) Ingested nitrite

The Working Group evaluated 12 case–control studies that focused on nitrite in the diet or in drinking-water, five of which investigated brain tumours in children and four of which examined maternal diet during pregnancy as a possible risk factor for the development of brain tumours in the offspring. The largest case–control study that was conducted in western USA observed no association between estimated dietary intake of nitrite and the incidence of childhood brain tumours. However, when the source of dietary nitrite was considered, children born to mothers who had the highest category of intake of nitrite specifically from cured meat (> 1.28 mg per day) had an almost twofold increased risk for brain tumours; nitrite intake from vegetable sources was not associated with the occurrence of brain tumours. A re-analysis of these data using estimated nitrite levels in cured meats during the year of the pregnancy suggested a stronger association (a threefold increase in the highest category of intake of nitrite from cured meat; ≥ 3.0 mg per day). No increased risk in relation to overall dietary nitrite was observed in studies in Israel or France or in a study in North America that focused on children < under 6 years of age who had either astrocytic gliomas or primitive neuroectodermal tumours. These studies did not quantify nitrite intake from cured meat specifically.

Nitrite in the drinking-water was investigated in a study of the children who were included in the studies from western USA and France together with children from Spain. Current levels of nitrite in the tap-water in homes in which the pregnancies had occurred were estimated using non-validated measurements from semiquantitative water test strips.

This study reported a twofold increase in risk for brain tumours in the offspring for both categories of detectable nitrite in the drinking-water. This association was stronger among women who did not rely on bottled water and was confined to astroglial tumours.

Seven studies of dietary intake of nitrite and adult brain tumours were conducted; five were restricted to glioma and another study in Germany provided risk estimates for glioma only. No significant associations were reported for dietary nitrite intake overall. The largest study in California, USA, observed a twofold increase in risk among men who consumed levels of nitrite above the median and levels of vitamin C below the median; this pattern did not occur among women. Two small studies in the USA, one in Ohio and one among women in California, observed a positive association with intake of nitrite from cured meat; a larger case–control study in Nebraska, USA, observed no association with nitrite from animal sources but a threefold increase in risk for glioma among persons who had high consumption of nitrite from plant sources. The study from Nebraska observed no interaction between dietary intake of nitrite and vitamin C. No study reported risk estimates for meningioma or other tumour types.

5.2.3 *Tumours of the urinary tract*

(a) Ingested nitrate

Five ecological studies, one cohort and one case–control study of tumours of the urinary tract and nitrate intake in the diet and drinking-water were reviewed. These included investigations of cancers of the urinary bladder, kidney or all tumours of the lower urinary tract combined, of which bladder cancer predominated. Both analytical studies of intake of nitrate in the drinking-water were conducted in the USA and estimated exposure to nitrate among people who used public water systems; thus average levels were generally below 50 mg/L. In the cohort study of women from Iowa, risk for cancer of the urinary bladder was positively associated with average concentrations of nitrate in the water. No association was observed with renal cancer. This study did not evaluate any potential interactions between nitrate and inhibitors of nitrosation such as vitamin C. A largely non-overlapping case–control study of bladder cancer from Iowa examined nitrate intake from food. No association with estimates of nitrate intake from the diet or drinking-water was detected, and no interaction was observed between intake of vitamin C and ingestion of nitrate in water. Five ecological studies that were conducted in Europe were uninformative.

(b) Ingested nitrite

Two well-designed case–control studies of tumours of the urinary tract assessed dietary intake of nitrite; both used a food-frequency questionnaire to ascertain dietary history and both considered potentially confounding factors. A case–control study of cancers of the lower urinary tract from Oahu, Hawaii, USA, found an increased risk for cancer of the urinary bladder with greater dietary intake of nitrite among Japanese men. There was no association among Japanese women or among Caucasian men or women.

In a study of cancer of the urinary bladder from Iowa (largely Caucasian), dietary intake of nitrite was not associated with risk. Neither of the studies evaluated interactions with nitrosating agents or their inhibitors, such as vitamin C.

5.2.4 *Non-Hodgkin lymphoma*

(a) Ingested nitrate

One cohort study and two case–control studies, all of which were conducted in agricultural regions of the USA, evaluated ingestion of nitrate in the diet and drinking-water and risk for non-Hodgkin lymphoma. Another case–control study in the USA evaluated intake of nitrate in drinking-water only. The four studies that evaluated nitrate in drinking-water linked historical data on levels of nitrate in public water supplies to a residential history of water source. Increasing quartiles of the average nitrate level in public supplies were associated with an increased risk for non-Hodgkin lymphoma in one study in Nebraska where the highest average nitrate quartile was > 4.0 mg/L nitrate-N. The case–control study and cohort study in Iowa had slightly lower average exposures to nitrate (highest quartiles, > 2.5 and ≥ 2.9 mg/L nitrate-N); increasing quartiles of exposure were not associated with risk. The case–control study in Minnesota had the lowest exposure levels (highest quartile, > 1.5 mg/L nitrate-N) and observed an inverse association with risk for exposure at this level. The case–control studies in Nebraska and Iowa evaluated risk by comparing high and low strata of nitrate intake in water with strata of vitamin C intake. In the study in Nebraska, there was a statistically significant threefold increased risk among persons who had a higher nitrate intake from water and lower vitamin C intake compared with those who had a lower nitrate intake from water and high vitamin C intake. There was no evidence of such a pattern in risk in the case–control study in Iowa that used similar exposure categories. Five ecological studies evaluated nitrate levels in public water supplies and the risk for non-Hodgkin lymphoma. The Working Group considered that these studies provided little information for the evaluation because levels of nitrate were mainly below 10 mg/L and because of the limitations of the ecological study design.

Dietary intake of nitrate was associated with non-significant inverse risks in the Iowa cohort study and Nebraska case–control study; a significantly inverse association with higher intake of dietary nitrate was reported in the Iowa case–control study.

(b) Ingested nitrite

The relationship between ingested nitrite and non-Hodgkin lymphoma was evaluated in two case–control studies in the USA. Dietary nitrite was not associated with risk for non-Hodgkin lymphoma in one study but there was an increase in risk with increasing quartiles of nitrite intake in the second study. When plant and animal sources of dietary nitrite were evaluated separately, the positive association was observed only for plant sources.

5.2.5 *Colon and rectal tumours*

(a) Ingested nitrate

One case–control study and two cohort studies evaluated the intake of nitrate from drinking-water and dietary sources in relation to risk for cancers of the colon and rectum. The case–control study found no overall association between average levels of nitrate in drinking-water in public water supplies and risk for either type of cancer. However, for cancer of the colon, a significant twofold elevated risk was observed among persons who had a higher intake of nitrate from water and low vitamin C intake. A twofold significantly elevated risk was also observed with higher intake of nitrate from water and high meat intake. Average nitrate levels in public supplies were not associated with an increase in risk for cancer of the colon or rectum in the cohort study; however, elevated risks were observed in the middle exposure categories for colon cancer.

Dietary nitrate intake was not associated with the risk for colorectal cancer in the cohort studies. Higher dietary intake of nitrate was associated with a decreased risk for colon cancer in the Iowa case–control study, whereas there was no association with rectal cancer.

(b) Ingested nitrite

One case–control study in the USA and one cohort study in Finland evaluated dietary nitrite intake and risk for cancers of the colon and rectum. The case–control study found a 50% increased risk for colon cancer and a 70% increased risk for rectal cancer. Dietary intake of nitrite was not associated with risk in the cohort study.

5.2.6 *Other cancers*

(a) Ingested nitrate

Levels of nitrate in the drinking-water were investigated in analytical case–control studies of cancers of the pancreas, lung, ovary, corpus uteri, breast and testis.

Dietary intake of nitrate has been studied in case–control or cohort studies of pancreatic, oral, laryngeal, lung, ovarian, corpus uteri, endometrial and breast cancer. Three case–control studies of pancreatic cancer and two or fewer studies of cancer at other sites were available. There was no association or inverse association between cancers at these sites and dietary intake of nitrate.

(b) Ingested nitrite

Dietary nitrite intake was evaluated in case–control or cohort studies in relation to oral, laryngeal, nasopharyngeal, pancreatic and lung cancers. The number of studies of any given cancer site were few: three case–control studies of pancreatic cancer and two or fewer studies of cancers at other sites were available.

5.3 Animal carcinogenicity data

5.3.1 *Nitrate*

In three studies in mice, no evidence of carcinogenic activity of nitrate alone was observed whether it was administered in the drinking-water or in the diet at high concentrations. One study showed that less than 2.5% of the administered nitrate was reduced to nitrite in mice.

In four studies in rats, no increased incidence of tumours was observed when sodium nitrate alone was administered in the drinking-water or in the diet. One study showed that nitrate promoted urinary bladder carcinogenesis induced by *N*-butyl-*N*-(4-hydroxybutyl)-nitrosamine.

5.3.2 *Nitrite*

In most of the studies, mice or rats that were exposed to nitrite alone in the diet, by gavage or in the drinking-water did not have higher incidences of tumours compared with untreated controls. It was noted that the negative findings may be due to the low doses of nitrite used, the short duration of exposure or the instability of nitrite. In some instances, when a carcinogenic effect of nitrite was observed, the investigators noted the formation of *N*-nitroso compounds in the diet mix or in the stomach contents.

One study in female mice treated with nitrite in the drinking-water showed an increased trend in the incidence of forestomach papillomas and carcinomas combined. In one study in male mice exposed to nitrite in the drinking-water *in utero* (exposure of dams) and throughout life (until natural death), an increased incidence of lymphoma and lung tumours (benign and malignant combined) was observed.

In two studies, female rats that were fed nitrite in the diet had an increased incidence of hepatocellular carcinomas or adenomas and carcinomas combined. In one study in which rats were exposed to dietary nitrite *in utero* (exposure of dams) and throughout life, an increased incidence of lymphoreticular tumours was observed. In one study, rats that received nitrite in the drinking-water had an increased incidence of forestomach papillomas.

In many studies in mice that were exposed to nitrite by gavage, in the drinking-water or in the diet in combination with specific secondary or tertiary amines or amides (e.g. butylurea, *N*-methylaniline, piperazine, morpholine, methylurea, diethylamine hydrochloride, ethylurea, ethylthiourea, carbendazim or dibutylamine), the incidence of tumours, including benign and malignant lung tumours, malignant lymphomas, benign and malignant forestomach tumours, urinary bladder papillomas, benign and malignant liver tumours, or uterine adenocarcinomas was increased. In one of these studies, when piperazine was administered at a constant level in the diet with varying levels of sodium nitrite in the drinking-water, the increase in the incidence of lung adenomas was directly proportional to the levels of nitrite intake.

In many studies in rats, when sodium nitrite and specific secondary or tertiary amines or amides (e.g. morpholine, butylurea, disulfiram, aminopyrine, diphenhydramine, chlorpheniramine maleate, heptamethyleneimine hydrochloride, *N,N*-dimethyldodecylamine-*N*-oxide or bis(2-hydroxypropyl)amine) were mixed in the diet or given in the drinking-water or by gastric intubation, an increased incidence of tumours, including benign and malignant oesophageal tumours, haemangiosarcomas, hepatocellular adenomas and carcinomas, lung squamous-cell carcinomas or benign and malignant nasal cavity tumours was observed. In some of these studies, at a constant level of sodium nitrite, the tumour incidence induced was directly related to the levels of amine. When the level of amine was kept constant, tumour yield was also directly related to the level of sodium nitrite. When pregnant rats were given ethylurea and sodium nitrite in the drinking-water, neurogenic tumours developed in the offspring.

A dose-related increase in the incidence of renal-cell carcinoma was observed when rats were administered nitrite in the drinking-water in combination with varying amounts of fishmeal in the diet. Levels of *N*-nitrosodimethylamine in the stomach contents also showed a dose-related increase.

In one study, nitrite in the drinking-water marginally enhanced the carcinogenic effects of two different leukaemia viruses in infected mice.

Studies with antioxidants in rats showed that sodium ascorbate enhanced the incidence of forestomach tumours produced by concurrent administration of morpholine and sodium nitrite and that produced by initiation with *N*-methyl-*N*′-nitro-*N*-nitrosoguanidine and concurrent administration of sodium nitrite, while the concurrent administration of sodium ascorbate delayed the induction of liver tumours produced by morpholine and sodium nitrite. Ascorbic acid or soya bean prevented nitrite and dibutylamine from producing liver and urinary bladder tumours in mice and ascorbic acid protected against the induction of liver tumours by nitrite and aminopyrine in rats. Thioproline, a nitrate scavenger, decreased the incidence of forestomach carcinomas produced by nitrite and *N*-benzylmethylamine in rats. α-Tocopherol, *tert*-butylhydroquinone and propyl gallate enhanced the incidence of forestomach papillomas induced by concurrent administration with sodium nitrite after initiation with *N*-methyl-*N*′-nitro-*N*-nitrosoguanidine in rats. Concurrent administration of the antioxidant 1-*O*-hexyl-2,3,5-trimethylhydroquinone enhanced the incidence of forestomach tumours induced by aminopyrine and sodium nitrite in a rat multiorgan carcinogenesis model.

In rats, concurrent administration of sodium nitrite and 3-methoxycatechol produced forestomach papillomas, and catechol and 3-methoxycatechol increased the incidence of forestomach tumours produced by concurrent administration of sodium nitrite in a two-stage gastric carcinogenesis model.

In one study in hamsters, the incidence of liver-cell carcinomas was increased when sodium nitrite was fed together with morpholine.

5.4 Other relevant data

5.4.1 *Absorption, distribution, metabolism and excretion*

In humans, nitrate and nitrite participate in a dynamic interchange—the human nitrogen cycle—that involves ingestion, endogenous synthesis and ultimate excretion mainly as nitrate in the urine. Exposure by ingestion is primarily to nitrate, approximately 5% of which is reduced by oral bacteria to nitrite. The nitrate/nitrite mixture enters the gastrointestinal system in swallowed saliva. Nitrate and nitrite are absorbed from the upper intestine into the general circulation where the nitrite is oxidized by haemoglobin to nitrate that then re-enters the cycle. Endogenous synthesis is mainly via the arginine-to-nitric oxide pathway followed by ultimate conversion of nitric oxide to nitrate/nitrite. Nitrate and nitrite are widely distributed in the body, where nitrate predominates. Under some physiological conditions, e.g. hypoxia, nitrite can be converted in the reverse direction to nitric oxide; this pathway is suggestive of an important biological role for this anion.

5.4.2 *Endogenous nitrosation*

Nitrosation of proline to form the non-carcinogenic nitrosamine, *N*-nitrosoproline, has been used as a test of endogenous nitrosation, and a large number of studies have now demonstrated the nitrosation of proline in humans. In a typical experiment, proline and nitrate are administered and the nitrite that arises from oral reduction of the nitrate can then nitrosate proline in the stomach. Gastric nitrosation of proline in humans can be inhibited by vitamin C, as seen in animal and human experiments. Examples of endogenously formed carcinogenic nitrosamines are *N*-nitrosodimethylamine, *N*-nitrosopyrrolidine and tobacco-specific nitrosamines in humans. Nitrite-preserved meat contains substantial amounts of nitrosatable compounds (*N*-nitroso compound precursors). Other pathways for endogenous nitrosation include bacterially mediated and nitric oxide-related nitrosation.

5.4.3 *Genotoxic effects*

High intake of nitrate was associated with an increased frequency of hypoxanthine-guanine phosphoribosyl transferase gene mutants in peripheral blood lymphocytes in human populations who had been exposed to various levels of nitrate in their drinking-water. Another study showed an increase in the number of chromosomal aberrations, but not of sister chromatid exchange, in lymphocytes of children exposed to concentrations of nitrate in the drinking-water that exceeded 70.5 mg/mL.

Sodium nitrate gave negative results in the *Salmonella typhimurium* reverse mutation assay. Potassium nitrate caused chromosomal aberrations in Chinese hamster ovary cells *in vitro* in one study. In two in-vivo studies, sodium nitrate increased the frequency of chromosomal aberrations in rats and mice. Sodium nitrate did not cause chromosomal

aberrations, micronuclei, 8-azaguanine-resistant mutations, ouabain-resistant mutations or morphological transformation in the cells of hamster embryos after transplacental exposure. It was also negative in the mouse sperm-head abnormality test.

Sodium nitrite gave generally positive results in the *Salmonella* mutagenicity assay, but was negative in the SOS chromotest. It did not induce mutations in bacteria recovered in the host-mediated assay from rats or mice of various strains. In a single study, sodium nitrite induced somatic mutations in the wing-spot test in *Drosophila melanogaster*. It gave a positive response in several assays for chromosomal aberrations and micronucleus formation, both *in vitro* and *in vivo*. In several in-vitro studies, sodium nitrite was consistently positive in inducing aneuploidy, cell transformation, 8-azaguanine-resistant mutations, 6-thioguanine-resistant mutations and ouabain-resistant mutations. Similarly, sodium nitrite induced 8-azaguanine-resistant mutations, ouabain-resistant mutations and morphological transformation in cells of hamster embryos after transplacental exposure, but did not induce chromosomal aberrations or micronuclei in this assay. It was also positive in the mouse sperm-head abnormality test.

5.4.4 *Other toxic effects*

Nitrate, via reduction to nitrite as noted above, causes methaemoglobinaemia, especially in infants. High concentrations of methaemoglobin are associated with hypotension, as a result of the vasodilatory effects of nitrite. Epidemiological evidence suggests a possible association between nitrate in the drinking-water and spontaneous abortions, intrauterine growth restrictions, birth defects, childhood onset of diabetes mellitus, thyroid hypertrophy, hypertension and recurrent diseases (respiratory tract infection, diarrhoea, stomatitis) in children. No teratogenic effects were observed in tests with nitrate and nitrite.

5.4.5 *Mechanistic considerations*

Nitrosating agents—e.g. nitrous acid and nitrous anhydride—that arise from nitrite under acidic gastric conditions react with amines or amides to form nitrosamines or nitrosamides, and the induction of tumours in animals via endogenous synthesis of *N*-nitroso compounds has been demonstrated. Nitrosamines need to be activated metabolically by cytochrome P450 enzymes to electrophilic intermediates to exert a carcinogenic effect, while nitrosamides are direct-acting carcinogens. Nitrosation of primary amines produces electrophiles that can alkylate nucleophilic sites in DNA. Nitrosation of primary exocyclic amino groups on DNA, followed by deamination, may lead directly to mutations.

Ascorbic acid, a known inhibitor of nitrosation reactions, lowers the incidence of tumours in these experiments. The effect of ascorbic acid in the reduction of the risk for cancer that is associated with ingested nitrite has also been shown in epidemiological

studies (see Section 5.2). These observations support the role of endogenous nitrosation in tumorigenesis.

6. Evaluation and Rationale

There is *inadequate evidence* in humans for the carcinogenicity of nitrate in food.

There is *inadequate evidence* in humans for the carcinogenicity of nitrate in drinking-water.

There is *limited evidence* in humans for the carcinogenicity of nitrite in food. Nitrite in food is associated with an increased incidence of stomach cancer.

There is *inadequate evidence* in experimental animals for the carcinogenicity of nitrate.

There is *sufficient evidence* in experimental animals for the carcinogenicity of nitrite in combination with amines or amides.

There is *limited evidence* in experimental animals for the carcinogenicity of nitrite *per se*.

Overall evaluation

Ingested nitrate or nitrite under conditions that result in endogenous nitrosation is *probably carcinogenic to humans (Group 2A)*.

There is an active endogenous nitrogen cycle in humans that involves nitrate and nitrite, which are interconvertible *in vivo*. Nitrosating agents that arise from nitrite under acidic gastric conditions react readily with nitrosatable compounds, especially secondary amines and amides, to generate *N*-nitroso compounds. These nitrosating conditions are enhanced following ingestion of additional nitrate, nitrite or nitrosatable compounds. Some of the *N*-nitroso compounds that could be formed in humans under these conditions are known carcinogens.

CYANOBACTERIAL PEPTIDE TOXINS

CYANOBACTERIAL PEPTIDE TOXINS

1. Exposure data

1.1 Introduction

Cyanobacteria, also known as blue-green algae, are widely distributed in fresh, brackish and marine environments, in soil and on moist surfaces. They are an ancient group of prokaryotic organisms that are found all over the world in environments as diverse as Antarctic soils and volcanic hot springs, often where no other vegetation can exist (Knoll, 2008). Cyanobacteria are considered to be the organisms responsible for the early accumulation of oxygen in the earth's atmosphere (Knoll, 2008). The name 'blue-green' algae derives from the fact that these organisms contain a specific pigment, phycocyanin, which gives many species a slightly blue-green appearance.

Cyanobacterial metabolites can be lethally toxic to wildlife, domestic livestock and even humans. Cyanotoxins fall into three broad groups of chemical structure: cyclic peptides, alkaloids and lipopolysaccharides. Table 1.1 gives an overview of the specific toxic substances within these broad groups that are produced by different genera of cyanobacteria together, with their primary target organs in mammals. However, not all cyanobacterial blooms are toxic and neither are all strains within one species. Toxic and non-toxic strains show no predictable difference in appearance and, therefore, physicochemical, biochemical and biological methods are essential for the detection of cyanobacterial toxins.

The most frequently reported cyanobacterial toxins are cyclic heptapeptide toxins known as microcystins which can be isolated from several species of the freshwater genera *Microcystis*, *Planktothrix* (*Oscillatoria*), *Anabaena* and *Nostoc*. More than 70 structural variants of microcystins are known. A structurally very similar class of cyanobacterial toxins is nodularins (< 10 structural variants), which are cyclic pentapeptide hepatotoxins that are found in the brackish-water cyanobacterium *Nodularia*.

Table 1.1. General features of the cyanotoxins

Toxin group[a]	Primary target organ in mammals	Cyanobacterial genera[b]
Cyclic peptides		
Microcystins	Liver	*Microcystis, Anabaena, Planktothrix (Oscillatoria), Nostoc, Hapalosiphon, Anabaenopsis*
Nodularin	Liver	*Nodularia*
Alkaloids		
Anatoxin-a	Nerve synapse	*Anabaena, Planktothrix (Oscillatoria), Aphanizomenon*
Aplysiatoxins	Skin	*Lyngbya, Schizothrix, Planktothrix (Oscillatoria)*
Cylindrospermopsins	Liver[c]	*Cylindrospermopsis, Aphanizomenon, Umekazia*
Lyngbyatoxin-a	Skin, gastrointestinal tract	*Lyngbya*
Saxitoxins	Nerve axons	*Anabaena, Aphanizomenon, Lyngbya, Cylindrospermopsis*
Lipopolysaccharides	Potential irritant; affects any exposed tissue	All

From Sivonen & Jones 1999

[a] Many structural variants may be known for each toxin group.

[b] Not all species of the particular genus produce toxins.

[c] Whole cells of toxic species elicit widespread tissue damage, including damage to kidney and lymphoid tissue.

1.2 Chemical and physical properties

Cyclic peptides are comparatively large natural products that have a molecular weight in the range of 800–1100 but are relatively small compared with many other cell oligopeptides and polypeptides (proteins) (molecular weight, > 10 000). Nodularins and microcystins contain either five (nodularins) or seven (microcystins) amino acids; the two terminal amino acids of the linear peptide are condensed (joined) to form a cyclic compound.

The common structure of microcystins is cyclo(D-alanyl–L-*X*–D-erythro-β-methylaspartyl(iso-linkage)-L-*Z*-ADDA–D-glutamyl(iso-linkage)-*N*-methyldehydroalanyl) where ADDA stands for the β-amino acid, 3-amino-9-methoxy-2,6,8-trimethyl-10-phenyldeca-4(*E*),6(*E*)-dienoic acid, which is unique to microcystins and nodularins. The

main structural variation in microcystins is observed in the L-amino acid residues 2 (*X*) and 4 (*Z*), which are indicated by a two-letter suffix; for example, the common microcystin-LR contains leucine (L) in position 2 and arginine (R) in position 4 (Carmichael *et al*., 1988a; Falconer, 2005).

The ADDA side chain is a key structural element that is necessary for biological activity. Separation of the ADDA component from the cyclic peptide renders both components non-toxic (Carmichael, 1992). L Amino acids vary among toxins and a large number of combinations can be formed. To date, more than 70 microcystins have been discovered including non-toxic geometric isomers of microcystins-LR and -RR (Sivonen *et al*., 1992; Sivonen & Jones, 1999; Codd *et al*., 2005). Microcystins are stable at high temperatures for extended periods and are not denatured by boiling. They are non-volatile, resistant to changes in pH and are soluble in water, ethanol and acetone.

The common structure of nodularins is cyclo(D-methylaspartyl^{1}-L-arginine^{2}-ADDA^{3}-D-glutamate^{4}-Mdhb^{5}), in which Mdhb is 2-(methylamino)-2-dehydrobutyric acid. A few naturally occurring variations of nodularin have been found: two demethylated variants, one with D-aspartyl^{1} instead of D-methylaspartyl^{1} and the other with DMADDA^{3} instead of ADDA^{3}, and the non-toxic nodularin which has the 6(*Z*)-stereoisomer of ADDA^{3} (Namikoshi *et al*., 1994; Chorus & Bartram, 1999). The key difference between microcystins and nodularins is that the former usually occur as a mixture of several structural variants, whereas the variants of nodularins (i.e. demethylated or with modified ADDA) are rarely found.

1.2.1 *Nomenclature*

Microcystins

Nomenclature, Chemical Abstracts Service Registry (CAS) number and synonyms of the individual microcystins including microcystin-LR are listed in Table 1.2.

Nodularin

Chem. Abst. Serv. Reg. No.: 118399–22–7

CAS Name: Cyclo[(2*S*,3*S*,4*E*,6*E*,8*S*,9*S*)-3-amino-9-methoxy-2,6,8-trimethyl-10-phenyl-4,6-decadienoyl-D-γ-glutamyl-(2*Z*)-2-(methylamino)-2-butenoyl-(3*S*)-3-methyl-D-β-aspartyl-L-arginyl]

Synonyms: Cyclo[(*Z*)-2,3-didehydro-*N*-methyl-2-aminobutanoyl-erythro-3-methyl-D-β-aspartyl-L-arginyl-(2*S*,3*S*,4*E*,6*E*,8*S*,9*S*)-4,5,6,7-tetradehydro-9-methoxy-2,6,8-trimethyl-10-phenyl-3-aminodecanoyl-D-γ-glutamyl]; nodularin R; 1,4,8,11,15-pentaazacyclo-nonadecane, cyclic peptide derivative

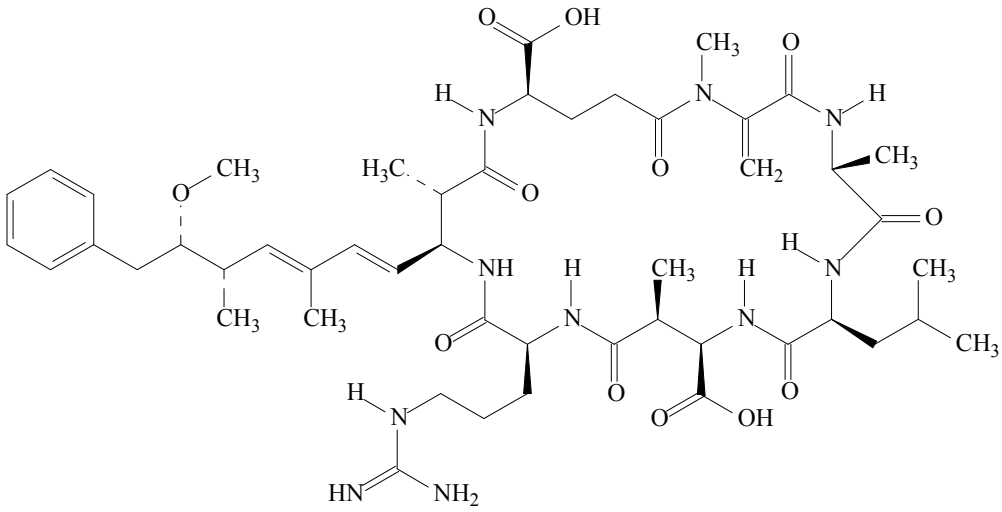
Table 1.2. Nomenclature of individual microcystins including microcystin-LR

CAS Name	CAS Registry number	Synonyms
Microcystin-LR	101043-37-2 Deleted CAS numbers: 847664-11-3; 128657-50-1	5-l-Arginine-microcystin LA; cyanoginosin-LA, 5-l-arginine; cyanoginosin LR; cyclo[2,3-didehydro-*N*-Me-ala-d-ala-l-leu-erythro-3-Me-d-β-asp-l-arg-(2*S*,3*S*,4*E*,6*E*,8*S*,9*S*)-4,5,6,7-tetradehydro-9-methoxy-2,6,8-trimethyl-10-phenyl-3-aminodecanoyl-d-γ-glu]; Toxin I (*Microcystis aeruginosa*); Toxin T17 (*Microcystis aeruginosa*)
Microcystin	77238-392	Cyanoginosin; Fast-Death Factor
Microcystin-LA	96180-79-9	Cyanoginosin-LA; Toxin BE 4
Microcystin-YM	101043-35-0	Cyanoginosin-LA, 3-l-tyr-5-l-met; cyclo(ala-tyr-Me-asp-met-ADDA-glu-MDHA); cyclo(ala-tyr-Me-asp-met-3-methoxy-2,6,8-trimethyl-10-phenyldeca-4,6-dienoic acid-glu-methyldehydrolanyl)
Microcystin-YR	101064-48-6	Cyanoginosin-LA, 3-l-tyrosine-5-l-arginine
Microcystin-RR	111755-37-4	Cyanoginosin-LA, 3-l-arginine-5-l-arginine
Microcystin-FR	111982-70-8	Cyanoginosin-LA, 3-l-phenylalanine-5-l-arginine
Toxin III (*Microcystis aeruginosa*)	118389-26-7	Cyanoginosin-LA, 3-l-arginine-4-d-β-aspartic acid-5-l-arginine; 3-desmethylmicrocystin RR; microcystin D
Toxin II (*Microcystis aeruginosa*)	120011-66-7	Cyanoginosin-LA, 4-d-β-aspartic acid-5-l-arginine; cyclo-ala-leu-isoasp-arg-ADDA-isoglu-*N*-MDHA; 3-desmethylmicrocystin LR; microcystin-A; toxin T16 (*Microcystis aeruginosa*)
Microcystin-LY	123304-10-9	Cyanoginosin-LA, 5-l-tyr
Microcystin-WR	138234-58-9	Cyanoginosin-LA, 3-l-tryptophan-5-l-arginine
Microcystin-AR	138258-91-0	Cyanoginosin-LA, 3-l-alanine-5-l-arginine
Microcystin-LL	154037-67-9	Cyanoginosin-LA, 5-l-leucine
Microcystin-LF	154037-70-4	Cyanoginosin-LA, 5-l-phenylalanine
Microcystin-LW	157622-02-1	Cyanoginosin-LA, 5-l-tryptophan

ADDA, β-amino acid, 3-amino-9-methoxy-2,6,8-trimethyl-10-phenyldeca-4(*E*),6(*E*)-dienoic acid; ala, alanyl; arg,arginyl; asp, aspartyl; CAS, Chemical Abstracts Services; glu, glutamyl; leu, leucyl; met, methionine; MDHA, methyldehydroalanyl ; Me, methyl; tyr, tyrosine

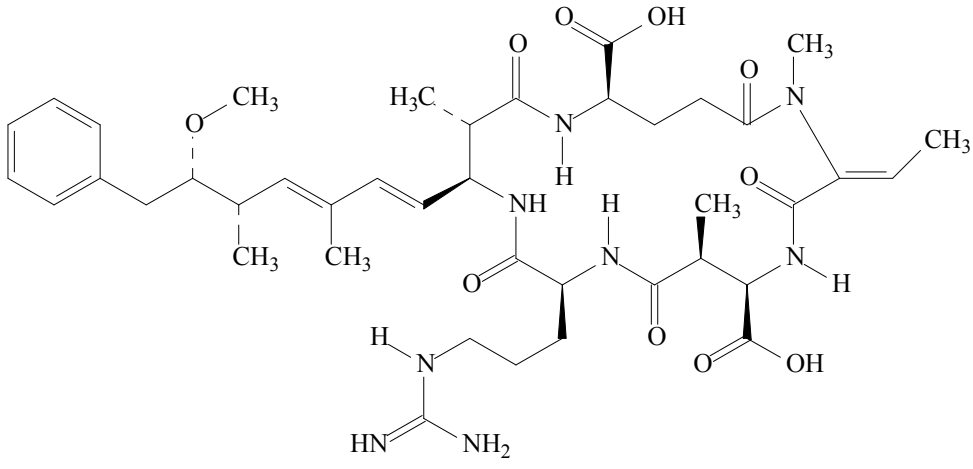
1.2.2 *Structural and molecular formulae*

Microcystin-LR



$C_{49}H_{74}N_{10}O_{12}$

Nodularin



$C_{41}H_{60}N_8O_{10}$

1.3 Analysis

A wide range of laboratory methods have been used to detect and identify cyanotoxins in water and solid matrices (including biomass); there is no single method that will provide adequate monitoring for all cyanotoxins.

Methods for determining microcystins and nodularins include: (a) physicochemical analysis by chromatographic separation (high-performance liquid chromatography [HPLC], gas chromatography, liquid chromatography) and detection either by ultraviolet absorbance (photodiode array detector) or mass spectrometry; (b) an immunoassay (enzyme-linked immunosorbent assay [ELISA]) for which several kits are commercially available; and (c) an enzyme assay that uses inhibition of protein phosphatase (Falconer, 2005).

An International Standards Organization (2005) method for the analysis of microcystin by HPLC is available, although currently no certified standard microcystins are available (McElhiney & Lawton, 2005). While chemical analysis differentiates between the structural variants of microcystin, immuno- and enzyme assays detect the sum of all microcystins in a sample. The systematic errors that are associated with the immuno- and enzyme assays are due to differences in reactivity between variants, but these assays are usually more rapid, require less elaborate equipment and may be cheaper when large numbers of samples are analysed (Falconer, 2005; McElhiney & Lawton, 2005).

ELISAs are widely used for the detection of microcystins and nodularins because of the ease of the procedure and the fact that they only require equipment that is readily available. Because of the many variants of microcystins and nodularins and the possible presence of metabolites and toxin covalently bound to protein phosphatases, antibodies give different results according to their respective affinities. This complicates the interpretation of quantitative data. ELISAs are therefore best used on simple well-defined samples. Many investigators have raised antibodies to microcystins and nodularins and have developed immunoassays that are more or less specific and vary largely in their reactivities to the different microcystin and nodularin variants or in their capabilities to detect non-cyclic degradation products (Chu *et al*., 1989; An & Carmichael, 1994; Bourne *et al*., 1996; Nagata *et al*., 1999; Baier *et al*., 2000; Fischer *et al*., 2001; Mikhailov *et al*., 2001; Zeck *et al*., 2001; McElhiney *et al*., 2002; Hilborn *et al*., 2005).

Inhibition of protein phosphatase measures free microcystins and nodularins and any metabolites that may still retain inhibitory activity. Conversely the method cannot detect or measure any microcystin that is covalently bound to cellular protein phosphatase or any metabolite that is not active. It is an assay that requires specific care, particularly in complex matrices such as cell or tissue extracts (Tencalla & Dietrich, 1997; Runnegar *et al*., 1999).

Other ways of determining toxicity are by bioassays in mice or other whole animals or in cells (Falconer, 2005).

HPLC or more advanced combinations of liquid chromatography and mass spectrometry may require complex steps of cleaning and concentrating but are best for the identification of microcystin and nodularin variants and the quantitation of toxins and metabolites. The disadvantage is that they require sophisticated equipment and expertise for reliable results (Azevedo *et al*., 2002; Hilborn *et al*., 2005).

1.4 Occurrence

Cyanobacteria are ubiquitous in water bodies with a great range of salinity and temperature, and occur in and on the soil as well as on rocks and in their fissures. In general, they are most abundant in nutrient-rich waters. Their growth is particularly favoured in lakes or water reservoirs where eutrophication occurs. Lake 'ageing' or eutrophication occurs primarily as a result of an increase in nutrients, in biological activity (productivity) and in sediments and organic matter from the watershed that fill the water basin. It is now accepted that human activities (e.g. domestic, industrial and agricultural wastes) play a significant role in the eutrophication or ageing process of the world's water bodies. In the seasonal cycle of freshwater phytoplankton that occur in temperate lakes, the appearance of cyanobacteria is probably due to the increased light and temperature at the end of spring. In water bodies that have a eutrophic to hypereutrophic nutrient status, the intensity and duration of cyanobacterial blooms are increased (Carmichael, 1996).

In addition to their wide range of social, economic and environmental impacts, cyanobacterial waterblooms which produce biotoxins are of particular concern for animal and human health; the more commonly occurring hepatotoxic biotoxins are microcystins and nodularins (Carmichael, 1996). In aquatic environments, these toxins usually remain contained within the cyanobacterial cells and are only released in substantial amounts during cell lysis or after cell death. This may occur naturally, although such events are short-lived, or through water treatment, e.g. by the application of copper sulfate to reservoirs to kill algae and cyanobacterial mass development. Release during some processes of drinking-water treatment may also be of concern (Falconer *et al*., 1983). Together with the high chemical stability and water solubility of microcystins, this containment has important implications for their environmental persistence in bodies of surface water and consequent human exposure (Sivonen & Jones, 1999).

1.4.1 *Concentrations of microcystin and nodularin in water bodies*

An increasing number of surveys worldwide have addressed the frequency of occurrence and the concentrations at which microcystins are found. Compilations of data (e.g. Sivonen & Jones, 1999; Fastner *et al*., 2001; Kardinaal & Visser, 2005) as well as overviews of case reports (e.g. Chorus, 2001) have been published, which cover a wide range of geographical regions and types of water body. More recently, survey results have also emerged from tropical settings (e.g. Morocco, Oudra *et al*., 2001; Kenya, Ballot *et al*., 2003; Bangladesh, Welker *et al*., 2005), most of which found that microcystins were

present in more than half of the samples tested, and that both the likelihood of the occurrence of microcystins and their concentrations increase with the abundance of cyanobacterial taxa.

Fewer data are available on the occurrence of nodularins. Results have focused on the Baltic Sea (e.g. Sivonen *et al*., 1989) and the Australian and New Zealand coastal areas (e.g. Carmichael *et al*., 1988b; Jones & Orr, 1994).

Table 1.3 (adapted from Sivonen & Jones, 1999) gives examples of concentrations of microcystin and nodularin reported in cyanobacterial bloom and water samples worldwide. Microcystin concentrations range from non-detectable to several milligrams per litre, and extremes of 10–25 mg microcystin-LR equivalents per litre have been reported in scum samples. Concentrations of micrograms per litre are often found, particularly when *Microcystis* spp. or *Planktothrix* spp. (syn. *Oscillatoria*) proliferate. Even in the absence of scum formation, these can cause levels of around 100 µg/L when both population density and toxin content per cell are high. For example, in a survey of Bangladeshi ponds in 2002 (Welker *et al*., 2005), microcystins were found in 39/79 ponds, 26 of which contained more than 10 µg/L and 18 more than 100 µg/L. Where cells accumulate, particularly in surface scums of *Microcystis* spp., concentrations in the range of milligrams per litre are not uncommon. With very few exceptions, these findings relate to cell-bound microcystin and, when the fraction dissolved in water was measured, it was usually very low (from < 1 to 5% of cell-bound microcystin; see Fastner *et al*., 2001 for a compilation of data from five studies).

Some structural microcystin variants are typically produced by certain genera or species, and the profile of microcystin variants can be quite typical for a given population of—for example—*P. agardhii* or *Microcystis* spp. (Fastner *et al*., 1999a). However, there is also overlap between taxa of microcystins; different strains of the same species show somewhat different microcystin profiles, and specific microcystin variants cannot be allocated unambiguously to certain cyanobacterial species.

In contrast, the production of nodularins appears to be species-specific. Laamanen *et al*. (2001) tested 345 single filaments from six different locations in the Baltic Sea using molecular methods for the allele to indicate nodularin production, and the results suggested that nearly all planktonic *Nodularia* (97%) in the Baltic sea produce this toxin. Interestingly, a close correlation was reported between the biomass concentration of *N. spumigena* and the concentration of nodularin which indicated a genetically very stable population of *Nodularia* that produces constant levels of nodularin (Chorus, 2001). In several Australian localities, Bolch *et al*. (1999) demonstrated that nodularin blooms within a water body tend to be clonal, which confirms the stability of toxin production.

Furthermore, the data of Laamanen *et al*. (2001) in the Baltic Sea suggest that *N. baltica* and *N. litorea* may both belong to the species *N. spumigena*, which may produce different morphotypes. If these results can be generalized and also hold true for other ecoregions, this would mean that nodularin is produced by only one species and that the large majority of strains of this species produce the toxin.

Table 1.3. Concentrations of toxin reported in cyanobacterial bloom or water samples worldwide

Location	Period of study	No. of positive samples (total no. of samples)	Toxins identified	Range of total concentrations (μg/g dry weight, unless otherwise indicated)
Microcystins				
Australia	1991	4	Microcystins, 24 unidentified	2100–4100[a]
Canada, Alberta	1990	37 (50)	Microcystin-LR	4–610
Canada, Alberta (3 lakes)	1990–93	168 (226)	Microcystin-LR	1–1550
China	1988	5 (10)	Microcystin-RR,-LR	200–7300
Czech and Slovak Republic	1995–96	(63)	Microcystin-LR	4–6835
Denmark	1992–94		Microcystin-RR,-LR	3–2800
Denmark	1993–95	198 (296)	Microcystins	5–1900
Finland	1994–95	17 (20)	Microcystin-LR	>10–800
France	1994	16 (22)	Microcystins	70–3970
France, Lake Grand-Lieu	1994	19 (30)	Microcystins	30–230
Germany	1992	8 (15)	Microcystin-LR	36–360
Germany	1993	17 (18)	Microcystins	0.15–36[a,b]
Germany	1995–96	385 (533)	Microcystins	1–5000
Germany	1997	34	Microcystins, several	1–25 000[b]
Japan	1990	12 (14)	Microcystin-RR, -YR, -LR	160–950
Japan	1988–92	11 (19)	Microcystin-RR, -YR, -LR	70–1610
Japan, Lake Suwa	1980–91	13	Microcystin-RR, -YR, -LR	30–2100
Japan	1986–88	4 (4)	Microcystin-RR, -YR, -LR	100–860
Japan	1992–95	18 (22)	Microcystin-RR, -YR, -LR	0.04–480[b]
Japan	1993–95	46 (57)	Microcystins	0.05–1300[a,b]
Japan	1993–94	12 (17)	Microcystins	0.06–94[a,b]
Japan	1989–94	10 (10)	Microcystins	300–15 600[a,b] 330–19 500[a,b]
Portugal	1989–92	12 (12)	Microcystin-LR plus six known and three unidentified microcystins	1000–7100
Portugal	1994–95	28 (29)	Microcystins	0.1–37[a,b]
South Africa	1985–86		Microcystin-FR, -LR, -YR, -LA, -YA	5–420
South Africa	1988–89	9 (9)	Microcystin-YR, -LR, -FR, -YA, -LA	40–630
United Kingdom	1992	3 (3)	Microcystins	17–131[a,b]
USA, Wisconsin	1993	9	Microcystins	1900–12 800[a]
Nodularins				
Australia, Tasmania, Orielton Lagoon	1992–93	7 (9)	Nodularin	2000–3500
Baltic Sea	1985–87	17 (23)	Nodularin	<100–2400
Baltic Sea	1990–91	6 (16)	Nodularin	300–18 000

Adapted from Sivonen & Jones (1999)
[a] Microcystin-LR
[b] Given as μg/L

Concentration per unit biomass of nodularin appears to be higher than that reported for microcystins: up to 18 mg/g dry weight of biomass were found in the Baltic Sea (Sivonen *et al*. 1989; Sivonen & Jones, 1999). While scum accumulations of *Nodularia* are likely to contain high concentrations of nodularin, concentrations in the open sea are rarely above a few micrograms per litre (e.g. Repka *et al*., 2004), merely because *Nodularia* filaments are less likely to accumulate.

In summary, microcystins are most liable to occur where cyanobacteria of the genera *Microcystis* or *Planktothrix* are found, and field populations of these genera that do not produce microcystins are rarely found. Nodularin is most liable to occur where *N. spumigena* is found. Microcystins are also found in populations of *Anabaena* spp., although less regularly (Fastner *et al*., 1999b), but have been reported less frequently in populations of other microcystin-producing taxa. However, it is currently unclear whether this is because such populations occur less frequently at sufficiently high levels to cause concern or whether they are less likely to contain microcystins. Some mat-forming cyanobacteria (e.g. *Phormidium* spp.) may contain microcystins, and, since such mats may become detached, exposure to microcystins may occur through this phenomenon (Mohamed *et al*., 2006). Microcystins and nodularins mainly occur as cell-bound entities. Extracellular concentrations greater than 1–5% of the intracellular concentrations have rarely been reported and are observed only under conditions that are detrimental for cell survival which trigger cell lysis and thus release microcystin. The consequences for risk assessment are that exposure is highest when cells are ingested or aspired.

Microcystins are very stable chemically (Harada *et al*., 1996). Although their photodegradation has been demonstrated (Tsuji *et al*., 1995; Welker & Steinberg, 1999), this process is usually of minor relevance, because water bodies that typically contain elevated concentrations of microcystin are usually quite eutrophic and consequently rather turbid. Microbial degradation of microcystins dissolved in water can be rapid (Jones *et al*., 1994). Lag phases are sometimes observed before degradation occurs, probably because bacteria that can degrade microcystins are not always present in sufficient numbers or need to adapt. However, once degradation begins, half-lives have been reported to be in the range of a few days and often only 1–2 days (Welker & Steinberg, 1999).

1.4.2 *Factors that influence concentrations of microcystin and nodularin*

The initial data obtained in molecular and physiological studies suggested that environmental factors as well as composition and dynamics of the cyanobacterial population are involved in the total concentration of the toxins in the water bodies. Clearly, however, more field experiments need to be conducted to assess fully the real impact of these two factors and to elaborate efficient water management (Dittmann & Börner, 2005).

(a) Population composition

Microcystins are produced by bloom-forming species of *Microcystis*, *Anabaena*, *Oscillatoria (Planktothrix)* and *Nostoc* (see Table 1.1), by a species of *Anabaenopsis* and by a soil isolate of *Haphalosiphon hibernicus*.

Nodularins have been found, with the exception of the marine sponge *Theonella*, only in *N. spumigena* (Table 1.1).

Cyanobacterial populations may be dominated by a single species or be composed of a variety of species, some of which may be not toxic. Even within a single-species bloom, there may be a mixture of toxic and non-toxic strains. Some strains are much more toxic than others, sometimes by more than three orders of magnitude. This means that one highly toxic strain, even when it occurs in small amounts among larger numbers of non-toxic strains, may render the bloom sample toxic (Sivonen & Jones, 1999; Janse *et al*., 2005).

Whether or not a strain produces the peptide toxins depends on its possession of the gene cluster that encodes for the multienzyme complex which is necessary for microcystin production (Kurmayer *et al*., 2003; Via-Ordorika *et al*., 2004). Strains both with and without these genes have been found for all potentially peptide-producing taxa known to date. Field populations of microcystins typically consist of a mixture of genotypes, i.e. with and without genes for microcystin production. The relative distribution of these genotypes, as well as the microcystin content of the respective clones, are major determinants of the concentrations of microcystin caused by a given cyanobacterial population. Gene probes are available to assess whether or not a given culture strain has the potential to produce microcystin (Tillett *et al*., 2000).

In contrast, for nodularins, populations of *N. spumigena* may contain only producer genotypes.

(b) Physiological responses

Numerous laboratory experiments with microcystin- or nodularin-producing strains of different cyanobacterial taxa have addressed the extent to which their net microcystin or nodularin production is affected by environmental conditions, i.e. availability of light, concentrations of nutrients and temperature (reviewed in Sivonen & Jones, 1999; Kardinaal & Visser, 2005). Contrary to earlier working hypotheses, levels of cyanobacterial toxins in field populations are not determined primarily by variations in environmental conditions that impact on the production rates of the cells but depend directly on the population sizes of cyanobacterial species and the relative distribution of genotypes with or without genes for microcystin production.

In some water bodies, the ratios of microcystins to biomass appear to vary rather rapidly, and there is some indication that levels of *Microcystis* spp. are higher at the beginning of the growing season (Kardinaal & Visser, 2005). In some water bodies, particularly those dominated by *P. agardhii*, the ratios remain stable throughout most of a growing season or even for several years (Janse *et al*., 2005). This may also be the case

for nodularin and *Nodularia*. Once this has been established for a given water body and particularly where cyanobacteria dominate the phytoplankton or form blooms, biomass estimates of the microcystin-producing cyanobacterial taxa may be useful site-specific surrogates for the approximation of concentrations of microcystin (Kardinaal & Visser, 2005).

1.5 Human exposure

Pathways of exposure to microcystins and nodularin in most settings are largely through water. In deriving its provisional guideline value for microcystin-LR, WHO (2003a) assumed an allocation factor of 80% to water as an exposure pathway. However, in specific settings, other pathways may gain major significance or be dominant. Therefore, to assess human health risks from microcystins, all potential exposure pathways should be considered. These include: recreational exposure, particularly to scums and in situations of high turbidity due to dispersed cyanobacterial cells, drinking-water, particularly in settings where particle removal is poor, haemodialysis during which surface water is used and treatment fails, occupational exposure to aerosols when surface water that contains cyanobacterial cells is used, e.g. in irrigation or for cooling water, 'health food' or dietary supplement tablets produced from cyanobacteria, and fish and mussels.

An important aspect of pathways of human exposure is that microcystins and nodularins do not appear to enter the human body through dermal exposure but chiefly through active transport mechanisms (see Section 4.1). Consequently, exposure requires ingestion or aspiration of water or food that contains cyanobacterial cells that have these peptides and/or dissolved cyanopeptides.

It is probable that the same human populations are exposed repeatedly to microcystins as a result of on-going contamination of freshwater sources, e.g. by *M. aeruginosa, P. agardhii* or *P. rubescens*, or brackish water sources that contain *N. spumigena.* Natural lakes and drinking-water reservoirs that are affected by these organisms regularly develop seasonal or perennial water blooms. Rural and less developed country populations that use surface water without treatment are also vulnerable to exposure. However, few studies on chronic exposure have been carried out either experimentally in animals or epidemiologically in human populations (WHO, 2003a).

1.5.1 *Recreational exposure*

Recreational exposure is the most probable pathway for ingestion of a high dose of microcystins or nodularins. Any water sport that involves immersion of the head invariably leads to some oral uptake or aspiration. Swimmers—if alerted to the hazard—might control their action to reduce ingestion and aspiration. Activities such as sailboarding, sailing in bad weather conditions or water skiing may lead to substantial uptake of water, and aerosol uptake through the spray generated by coastal wave action

may lead to exposure to nodularin. Children who play in shallow bays in which cyanobacterial scums tend to accumulate are particularly liable to swallow water.

Acutely lethal human intoxications through microcystins or nodularins appear to be improbable. [The provisional WHO tolerated daily intake (TDI) for microcystin-LR (0.04 µg/kg bw) may easily be exceeded through recreational exposure. This was illustrated by Chorus and Fastner (2001) using data from the Havel River in Berlin during a heavy, but moderately toxic bloom; half of the 28 samples taken on four occasions at 13 different sites contained more than 100 µg/L microcystins (as sum of all variants), four contained more than 1000 µg/L and two contained more than 10 000 µg/L. A re-calculation of their data for adult exposure (Table 1.4) shows that an adult would very probably ingest more than the TDI. If the cells contained five- to 10-fold more microcystin, swallowing only a few millilitres would already reach the TDI.] Similar concentration ranges of microcystins (mainly microcystin-LR) were detected in 25% of the 155 lakes in southwestern Germany that were monitored (Frank, 2002).

Table 1.4. Ingestion of scum material that would cause a dose above the WHO provisional tolerated daily intake for microcystin-LR (0.04 µg/kg bw): derivations from concentrations measured along the Havel River in July and August 1997

At 100 µg/L (0.1 µg/mL)		At 1000 µg/L (1 µg/mL)		At 25 000 µg/L (25 µg/mL)	
per kg	for a 100-kg adult	per kg	for a 100-kg adult	per kg	for a 100-kg adult
0.4 mL	40 mL	0.04 mL	4.0 mL	0.0016 mL	0.16 mL

From Chorus & Fastner (2001)

In many European cultures, permanently leased campsites or datchas are regularly used during holidays, on week-ends and, if sufficiently close to city flats, also on late afternoons and evenings. These are frequently located next to very eutrophic water bodies that harbour toxic cyanobacterial populations from July until late September or along the Baltic Sea coast; these water bodies are regularly used for swimming. Exposure thus occurs regularly over periods of several months. In subtropical and tropical settings, the cyanobacterial season and the period of recreational exposure may be substantially longer.

In summary, the estimation of recreational exposure requires a good understanding of the patterns of water use and occurrence of microcystins and nodularin. In view of the substantial, but hardly measurable, health benefits that populations often derive from the use of these settings, such exposure assessments should be carried out with care before interventions that curtail recreational use are implemented.

1.5.2 *Exposure through drinking-water and haemodialysis*

If the water is not treated to remove cyanobacterial cells, exposure scenarios for drinking-water can be similar to those outlined under Section 1.5.1 for recreational use. Disinfection in such situations probably does not degrade microcystins sufficiently, as chlorine is consumed by the high level of organic material. Such settings exist in many parts of the world, and a recent published example is ponds used in Bangladesh (Welker *et al*., 2005).

Where drinking-water is treated to remove particles, some break-through of microcystins may occur, although most of the microcystins are removed with the cells. When drinking-water is treated by initial oxidation (e.g. chlorine or ozone), microcystins are released from cells the process but may not be sufficient to oxidize all of the liberated microcystin (Hoeger *et al*., 2005). Examples of concentrations of cyanobacteria and toxins reported in drinking-water plants worldwide (Table 1.5) show that cyanobacterial toxin levels are usually well below 1 μg/L and rarely substantially above a few micrograms per litre.

Overall, exposure to microcystin through drinking-water can be assumed to be significant in settings where poorly treated surface water sources are used, whereas it is probably low or at least usually within the range of the provisional WHO guideline value in communities that are served by larger utilities that perform well-managed particle and organic contaminant maintenance, particularly when followed by an oxidation step.

Exposure through haemodialysis involves much larger amounts of water, i.e. approximately 120 L per treatment, which are effectively equivalent to an intravenous dose. This explains the severe impact of cyanotoxins on haemodialysis patients in Caruaru, Brazil, (Jochimsen *et al*., 1998), and highlights the importance of both the choice of the water source as well as excellent treatment of the water used in dialysis clinics.

1.5.3 *Occupational exposure*

Very few published data exist to demonstrate occupational exposure. However, scenarios can be estimated from the understanding of pathways of uptake and occurrence in surface waters. These would include any situation that leads to substantial ingestion or inhalation. Anecdotal evidence has been proposed from spray irrigation in agriculture and from aerosols produced by cooling the water used for mine drilling. Exposure would appear to be probable during large-scale and commercial harvesting and processing of cyanobacteria (e.g. for food supplements—see Section 1.5.4—and production of cosmetics). Estimates of uptake are hampered by the difficulty of estimating the volumes of water inhaled with such aerosols.

1.5.4 *Exposure through cyanobacterial dietary supplements*

Several regions in the world, e.g. Mexico, northern Africa and China, have a documented history of use of blue-green algae (*Spirulina* and *Nostoc* spp.) as a food source

Table 1.5. Examples of concentrations of cyanobacteria and/or cyanobacterial toxins in drinking-water before and after treatment in water plants worldwide

Location and source	Water treatment	Cyanobacteria	Raw water	Final water
Argentina, Bahía Blanca	NR	*Anabaena*/*Microcystis*	48×10^3–84×10^3 cells/ mL	276–2.5×10^3 cells/ mL
Australia, Queensland, reservoir	Flocculation/sedimentation, particulate activated carbon, slow filtration, chlorination	*Anabaena*, *Microcystis*	<2.200×10^3 cells/ mL <8 µg/L (microcystins)	<11×10^3 cells/ mL 0–0.5 µg/L (microcystins)
Bangladesh, lakes, ponds, reservoirs	NR	*Microcystis*	Samples positive for microcystins	samples positive for microcystins
Brazil, Itaparica Dam	Copper sulfate	*Anabaena*, *Microcystis*	NR	NR
Canada, Alberta, Camrose plant	Flocculation/sedimentation, slow filtration, chlorination, particulate activated carbon	NR	0.15–0.87 µg/L	0.09–0.18 µg/L
Canada, Alberta, Ferintosh plant	Flocculation/sedimentation, slow filtration, chlorination, granular activated carbon	NR	0.27–2.28 µg/L	0.05–0.12 µg/L
Czech Republic	NR	NR	≤8.7 µg/L	0–7.79 µg/L
China	NR	NR	0.28–35.3 µg/L	≤1.4 µg/L
Finland	Bank filtration Particulate activated carbon	*Planktothrix/Oscillatoria* NR	0.1–1.9 µg/L NR	0.01–0.1 µg/L ≤0.001 µg/L
France, Saint-Caprais reservoir	 Particulate activated carbon Particulate activated carbon	*Aphanizomenon* NR NR	63 µg/L NR NR	NR 33.2 ± 8.0 ng/L ≤0.001 µg/L
France, Lake Bourget	Ozonation, slow filtration	*Planktothrix/Oscillatoria*	<18×10^3 cells/ mL <5 µg/L	<6×10^3 cells/ mL <1 µg/L

Table 1.5 (contd)

Location and source	Water treatment	Cyanobacteria	Raw water	Final water
Germany, Dörtendorf, Weida Reservoir	Microsieve, flocculation/sedimentation, slow filtration	*Planktothrix/Oscillatoria*	7.5–10 µg/L	0–0.1 µg/L
Germany, Rostock, Warnow River	Ozonation, flocculation/sedimentation, slow filtration Ozonation, activated carbon filtration	*Microcystis* *Planktothrix/Oscillatoria*	10–28 µg/L 0.4–8.0 µg/L	0–0.2 µg/L 0.07–0.11 µg/L
Germany, Radeburg Reservoir	Bank filtration	*Aphanizomenon*/*Microcystis*	2–19 µg/L	≤0.06 µg/L
Israel, Lake Kinneret	Flocculation/sedimentation, chlorination	*Aphanizomenon*	$\leq 150 \times 10^3$ cells/ mL	NR
Italy, Lake Simbirizzi, Lake Flumendosa, Lake Mulargia	NR	*Planktothrix/Oscillatoria*	480 and 220 µg/g dry weight	NR
Korea, Republic of, Lakes and reservoirs	Mostly only rapid sand filtration	*Microcystis* (60%), *Anabaena* (30%), *Planktothrix/Oscillatoria* (10%)	0.6–171 µg/L	NR
Latvia, Baltezers, Lake Mazais	Slow filtration/bank filtration	*Aphanizomenon*, *Anabaena*, *Microcystis*	19–1229 µg/g dry weight; lake: ≤0.63 µg/L; infiltration basin: ≤0.25 µg/L	≤1.47 µg/L
Poland, Sulejów Reservoir	Flocculation/sedimentation, particulate activated carbon, rapid sand filtration, ozonation, chlorination	*Microcystis*	2.1–2.3 µg/L	0.5–0.8 µg/L

Table 1.5 (contd)

Location and source	Water treatment	Cyanobacteria	Raw water	Final water
Portugal, Crestuma-Lever reservoir	NR	*Aphanizomenon*, *Microcystis*	$\leq 12\times10^{3}$ cells/ mL (*Microcystis*) 4.7 μg/g dry weight (*Aphanizomenon*)	NR
Thailand	Partly without treatment	*Anabaena*, *Cylindrospermopsis*, *Microcystis*	NR	<1.0 μg/L
USA, Florida	NR	*Cylindrospermopsis*, *Microcystis*	NR	≤90 μg/L

Adapted from Hoeger *et al.* (2005); data come from field studies between 1980 and 2003
NR, not reported

(Carmichael *et al*., 2000; Jensen *et al*., 2001). In the twentieth century, blue-green algae supplements, which were primarily products that consisted entirely or partially of *Aphanizomenon flos-aquae* and *Spirulina* spp., represented an important economic activity (Carmichael *et al*., 2000), and were sold mainly in industrialized countries.

Blue-green algae supplements that consist of A. flos-aquae are specifically marketed and consumed for their putative beneficial health effects, e.g. increased alertness, increased energy, 'detoxification', elevated mood and weight loss (Jensen *et al*., 2001). More importantly, these supplements are marketed in some instances as a replacement for or alternative to the pharmacological therapy of 'attention deficit hyperactivity disorder' (Lindermann, 1995), and thus directly target the parents whose children present this disorder, providing a highly specific route of exposure to microcystins in small children.

Although producers and retailers of blue-green algae supplements maintain that batches that contain levels of microcystins above 1 μg/g dry weight are not marketed (Carmichael *et al*., 2000), independent investigations of microcystin contamination in these publicly available products have demonstrated toxin concentrations of up to 35 μg microcystin-LR equivalents/g dry weight. Although samples with toxin contamination greater than 10 μg microcystin-LR equivalents/g dry weight are the exception, several independent analyses detected more than 1 μg microcystin-LR equivalents/g dry weight in 50–100% of the blue-green algae products tested (Gilroy *et al*., 2000; Fischer *et al*., 2001; Lawrence *et al*., 2001; Dietrich & Hoeger, 2005; Bruno *et al*., 2006). Several studies (Lawrence *et al*., 2001; Bruno *et al*., 2006) have shown differences in detectable amounts of toxin when different detection methods were used. These differences appear primarily to stem from differences in the cross-reactivity of the microcystin congener of some of the ELISAs used but are also attributed to the lack of certified standards for five to 10 of the microcystin congeners that are commonly detected in blue-green algae supplements. Despite the latter findings, not all of these products contain high levels of microcystin (above 1 μg microcystin-LR equivalents/g dry weight). However, the levels of microcystin in a given brand can vary extensively from batch to batch (Gilroy *et al*., 2000) which does not allow for a proper assessment of human exposure, and specifically that of children, to microcystins.

Gilroy *et al*. (2000) calculated a TDI of 0.04 μg microcystin-LR equivalents/kg body weight (bw) per day based on a no-observed-adverse-effect level for microcystin-LR in mice of 40 μg/kg bw per day that was defined by Fawell *et al*. (1999); the application of a total 1000-fold uncertainty factor resulted in a provisional tolerable level for microcystins in blue-green algae supplements of 1 μg microcystin-LR equivalents/g dry weight. This level was adopted by the Oregon Health Division as a provisional regulatory standard for these products in 1997. This safe level translates into 2 μg microcystin-LR equivalents per adult per day. However, extrapolation of these daily doses to children (5–20 kg bw) shows that they would actually be exposed to three- to 12-fold higher daily doses than adults (Dietrich & Hoeger, 2005). Moreover, when assuming the worst case, i.e. blue-green algae supplements contaminated with 35 μg microcystin-LR equivalents/g dry

weight, the actual daily exposure of children could exceed the TDI by a factor 88–350, based on a maximum daily consumption of 2 g per day.

Contrary to the situation for food or water intake, in which a natural limitation of consumption can be assumed, daily consumption of blue-green algae supplements is largely dependent on the individual. Thus, overzealous parents may potentially severely increase the daily exposure of their child (Dietrich & Hoeger, 2005). The latter scenario is not improbable, as consumption of up to 20 g per day has been reported in the case of an adult (Schaeffer *et al*., 1999; Gilroy *et al*., 2000). Furthermore, contrary to water and food that are usually consumed together, e.g. during a meal, supplements are more probably treated as pharmaceuticals and are thus ingested on an empty stomach which may lead to higher potential uptake of microcystins from the gastrointestinal tract. Thus, the uptake from blue-green algae supplements should be treated entirely differently from the usual risk calculations for food and water.

1.5.5 *Exposure through food*

The order of importance of the individual food sources of exposure to microcystins varies between countries and largely depends on factors such as climatic conditions and irrigation practices, conditions for and traditions in agriculture and aquaculture (e.g. availability of cyanobacteria-free versus contaminated surface water), eating habits of the local population and, most of all, the affluence of the population in question. Indeed, a lack of regular income and the consequent discontinuity of sustenance forces poorer families to consume cyanobacteria-contaminated shellfish, crayfish or fish due to the inability to afford better quality food. Microcystins (and other cyanobacterial toxins, e.g. nodularin) accumulate in fish, crayfish and shellfish (Vasconcelos, 1999; Magalhães *et al*., 2001, 2003; Mohamed *et al*., 2003) at maximum concentrations of 300 µg/kg in the edible parts of fish, 2700 µg/kg in crayfish and 16 000 µg/kg in mussels (for discussions, see Falconer, 2005).

Microcystins have been reported to be taken up by commercially cultivated plants such as lettuce (*Lactuca sativa*) (Codd *et al*., 1999) and common beans *(Phaseolus vulgaris)* (Abe *et al*., 1996) when the toxins are present in the irrigation water or the growth media. The central leaves of lettuce were contaminated with 2.5 microcystin-LR equivalents/g dry weight that were not removable by washing, but no data were available on the actual level of contamination of beans with microcystins, as only inhibition of photosynthesis was determined following spray irrigation with microcystin-contaminated water. As some cyanobacteria fix nitrogen from the atmosphere and provide a valuable source of nitrogen to growing rice plants, cyanobacteria are welcome in rice fields (Rahman *et al*., 1996). However, although some of these cyanobacteria are presumably producers of microcystin, little is known about the mechanism(s) of uptake of microcystins into plants or the concentration of toxic cyanobacterial compounds in rice fields.

Few data are available on the accumulation of microcystins in livestock (e.g. cattle, swine, sheep), although these animals may frequently be exposed to microcystins and other cyanobacterial toxins through consumption of water contaminated with cyanobacteria (Beasley *et al*., 1983, 1989a,b). No carry-over of microcystins into milk (Orr *et al*., 2001) or meat (Orr *et al*., 2003) was observed in cows following administration of toxic *M. aeruginosa* in the drinking-water.

The actual exposure of humans (adults and children) to microcystins from food is difficult to estimate, especially since there is no general rule as to how much fish, shellfish, salad or rice is consumed daily per 'international adult or child'. It is possible that, in some regions of the world, children consume more than 0.1 kg fish or shellfish per day (Mohamed *et al*., 2003), especially when local populations are largely dependent on one type of food source (e.g. fish, shellfish or crustaceans, rice). Using the data from actual contamination of fish and shellfish reported by Vasconcelos (1999), Magalhães *et al*. (2001, 2003) and Mohamed *et al*. (2003) of up to 300 µg/kg edible fish, 2700 µg/kg crayfish and 16000 µg/kg mussels, there is a distinct possibility that exposure (subacute and chronic) of children occurs through microcystins in fish and shellfish (Dietrich & Hoeger, 2005). Daily consumption of 200 g fish contaminated with 300 µg microcystin-LR equivalents/kg edible fish by a 20-kg child would result in a total exposure of 60 µg microcystin-LR equivalents per day or 3 µg microcystin-LR equivalents/kg bw per day. Such an exposure would exceed the TDI of 0.04 µg microcystin-LR equivalents/kg bw per day proposed by the WHO by a factor of 75. In the worst case of highly contaminated mussels (16 000 µg microcystin-LR equivalents/kg mussel) and assuming the same daily consumption of 200 g, the TDI in a child would be exceeded by a factor 4000. Both of the latter calculations are, however, based on the assumption that all of the microcystin in the fish and shellfish is biologically and thus also systemically available to the exposed child.

1.6 Regulations, guidelines and preventive measures

1.6.1 *Drinking-water*

In 1997, WHO derived a provisional guideline value for microcystin-LR in drinking-water of 1 µg/L, based on a TDI of 0.04 µg/kg bw (WHO, 2004). The guideline is provisional because of the limitations of the database, particularly with regard to studies on long-term exposure and carcinogenicity. Moreover, it is limited to only one of several structural variants that occur as frequently in the same concentration range or even exclusively. In a supporting document to the *WHO Guidelines for Drinking-water Quality* (Chorus & Bartram, 1999), Falconer *et al*. (1999) recommended the use of concentration equivalents that include the other variants. An increasing number of countries are converting this WHO guideline into national regulations. Their approaches vary in dealing with the problem of the numerous microcystin variants that are typically found as mixtures in samples. While some (e.g. Canada) explicitly set the level to accommodate

for the presence of microcystins on the basis of general assumptions on their occurrence, others (e.g. Spain) simply refer to 'microcystin' without further specification.

In the third edition of the *WHO Guidelines for Drinking-water Quality* (WHO, 2004), the 1998 provisional guideline value for microcystin-LR was not changed, but is relevant to any hazard with emphasis on the need to consider national and regional conditions when converting any WHO guideline values into national standards and regulations. These may include issues of implementation, such as institutional capacity, and in particular the importance of a hazard for public health in relation to other prevalent hazards.

The publication of the WHO provisional guideline value for microcystin-LR in 1998 has led to national regulations on microcystins in drinking-water in several countries, and—in line with WHO's explicit emphasis on the need to use WHO guideline values in a nationally and locally adequate way to optimize the protection of public health—some countries have adapted their standard. Table 1.6 provides examples of national regulations and guidelines. In 2003, the European Union began discussions on whether microcystin-LR should be included in the forthcoming revision of its Drinking-water Directive.

Explicit guidelines for nodularin in drinking-water are known only for Australia. The Australian Government (2004) states that, due to the lack of adequate data, no guideline value is set for concentrations of nodularin. However, given the known toxicity of nodularin, the relevant health authority should be advised immediately if blooms of *N. spumigena* are detected in sources of drinking-water. Since there are some similarities between the toxicity of nodularin and microcystins, the guideline for microcystins could be used to derive cell numbers of *N. spumigena* that represent a preliminary indication of the potential hazard. It is recommended that notification and further assessment be made when cell numbers of *N. spumigena* exceed 40 000 cells/mL.

Table 1.6. Examples of regulatory approaches to cyanobacteria and microcystins in drinking-water

Country	Regulatory approach
Approaches motivated by compliance to a standard or guideline value	
Australia	Federal Drinking-water Guideline for total microcystins of 1.3 µg/L, expressed as microcystin-LR equivalents.
Brazil	Monthly monitoring of cyanobacteria in drinking-water resources; if cell counts exceed 10 000 cells/mL or biovolumes (determined from cell counts) exceed 1 mm^3 cell volume, weekly monitoring and analyses of toxins or toxicity testing are required; standard value for microcystins (variants not specified), 1 µg/L.
Canada	Maximum acceptable concentration for microcystin-LR in drinking-water, 1.5 µg/L; intended to be protective of human health against exposure to other microcystins that may also be present.

Table 1.6 (contd)

Country	Regulatory approach
Czech Republic	Mandatory monitoring of tap-water for microcystin-LR; limits, 1 µg/L; an update of the ordinance will include alternatives to analysis of microcystins such as quantification of cyanobacterial biomass in raw water or bioassays in conjunction with cell counts, and analyses of toxins only if thresholds for cyanobacterial biomass are exceeded.
France	Drinking-water Decree maximum limit is 1 µg/L microcystin-LR; analyses required if cyanobacteria proliferate in raw water.
Poland	Limit of 1 µg/L for microcystin-LR in drinking-water
Spain	Drinking-water Decree includes a limit for 'microcystin' (variants not specified) of 1 µg/L; sampling regimes specified in relation to size of population served; to be reviewed at 5-year intervals.
USA	In February 2005, the Environmental Protection Agency included cyanobacteria, other freshwater algae and their toxins on its 'contaminant candidate list' of unregulated contaminants, for which research is to be prioritized and data collected to determine whether regulation is necessary.
Indirect or implicit inclusion in drinking-water regulations	
Germany	National Drinking-water Ordinance stipulates drinking-water should contain no substances at concentrations that may be harmful to health, and the provisional WHO value for microcystin-LR defines such concentrations. The prerequisite for this approach is that drinking-water suppliers that use surface water usually monitor and acknowledge the phytoplankton in their resource and have effective treatment in place (as part of best practice and technical rules) and thus are aware of the cyanotoxin hazard.
Italy	No limit value has been implemented, but the national Drinking-water Decree considers 'algae' as an accessory parameter to be monitored when local authorities suspect a risk to human health; the provisional WHO Guideline of 1 µg/L for microcystin-LR is used as the basis for this assessment.
Hungary	The national Decree on Drinking-water Quality and the ordinance on monitoring include the number of cyanobacteria cells as a biological parameter to be monitored by microscopy, although no limit is given for cyanotoxins.
'Risk-based' approaches in regulations	
Australia	Fact sheets for each of the four cyanotoxins (microcystins, nodularin, saxitoxins and cylindrospermopsin) include the guideline value of 1.3 µg/L for the sum of all microcystin variants or a cell density of 6500 cells/mL for a highly toxic population of *Microcystis aeruginosa*. These values are not mandatory legally enforceable standards, but guidelines within a framework for analysing hazards and assessing risks for individual water supply systems and are being adopted by water authorities as agreed quality targets or as contract conditions for water supply, e.g. as targets and performance indicators for audits of process performance.

Table 1.6 (contd)

Country	Regulatory approach
New Zealand	Individual water safety plans are developed for each drinking-water supply system, following a comprehensive multi-barrier approach, in which process control is central. Hazard priorities are assigned by the Medical Officer of Health, with Priority 1 usually being assigned to pathogens and their indicators (i.e. *Escherichia coli*, *Giardia* and *Cryptosporidium*) and Priority 2 to cyanotoxins, when present at concentrations above 50% of the maximum acceptable value. Water safety plan development includes identification of barriers to contamination and eutrophicating nutrients in the catchment and in water treatment for the removal of cells and/or destruction of toxins. A 'Barriers to Contamination' guide assists suppliers to assess performance of barriers and to estimate risk of cyanotoxin occurrence. Plans include reporting and communication pathways, i.e. who receives which information and how often, and documentation.
Low regulatory level approaches including cyanotoxins in an understanding of good practice	
Denmark	Administrative units and research institutions collaborate to collate information, and the Danish National Environmental Research Institute posts a national overview of the occurrence of toxic cyanobacterial on its website.
Finland	Starting in the late 1980s, waterworks have been advised to monitor cyanobacteria microscopically, and, if cyanobacterial cells occur in raw or treated water, to analyse toxins. The Finnish Drinking-water Decree further stipulates that drinking-water should contain no substances harmful to health.

From Chorus (2005)

1.6.2 *Recreational water use*

Guidance on recreational water safety provided by WHO (Falconer *et al*., 1999; WHO, 2003b) is largely based on the occurrence of cyanobacteria as such, because it is at present unclear whether all important cyanotoxins have been identified, and the health outcomes observed after recreational exposure—particularly irritation of the skin and mucous membranes—are probably related to cyanobacterial substances other than well-known toxins. In addition, the WHO approach considers the particular hazard of liver damage by microcystins at high concentrations. This approach uses three levels of alert that are associated with incremental severity and probability of health effects.

The newly revised Bathing Water Directive (European Union, 2006) follows a risk-based approach and Article 8 of the Directive explicitly addresses toxic cyanobacteria. It stipulates that

(i) "When the bathing water profile indicates a potential for cyanobacterial proliferation, appropriate monitoring shall be carried out to enable timely identification of health risks" and

(ii) "When cyanobacterial proliferation occurs and a health risk has been identified or presumed, adequate management measures shall be taken immediately to prevent exposure, including information to the public".

The Bathing Water Profile describes the risk of pollution and explicitly includes an assessment of the potential for proliferation of cyanobacteria. Actions and frequency of monitoring should be related to the history and classification of the bathing water and to regional climatic conditions, and emphasis placed on bathing waters where risks may occur.

Several countries have regulations or guidelines that address cyanobacteria and/or cyanotoxins at recreational sites, and some include approaches to address the capacity of a water body to sustain large cyanobacterial populations. Examples of national regulations and guidance are included in Table 1.7.

Table 1.7. Examples of regulatory approaches to cyanobacteria and microcystins in water for recreational use

Country	Recreational sites
Australia	The monitoring of cell densities is often preferred to toxin limits because cell counting is widely available, cost-effective and is performed rapidly. The Federal Recreational Water Guideline provides values for three different parameters: 10 µg/L total microcystins or ≥ 50 000 cells/mL toxic *M. aeruginosa*; biovolume equivalent of ≥ 4 mm^3/L for the combined total of all cyanobacteria where a known toxin producer is dominant in the total biovolume; or 10 mm^3/L for total biovolume of all cyanobacterial material where known toxins are not present. A new approach is to assess the susceptibility for cyanobacterial growth from general monitoring data and historical information, including the scoring of water bodies as 'good, fair or poor'.
Denmark	Bathing Water Instruction requires when massive blooms occur that the material is investigated, the risk assessed and the authority alarm groups trigger posting of warning signs at the waterfront as well as dissemination of information particularly to local water body-user groups.
Finland	Health authorities were provided with guidelines in the late 1980s; a cost-effective monitoring network of nuisance algae occurrence is based on long-term data on occurrence collected since 1967, and now also includes the involvement of private citizens for visual monitoring.
France	Three levels of cyanobacterial cell density trigger management responses up to prohibition of water contact sports. Information on cell numbers is published on the internet within not more than 5 days of sampling.
Germany	Three-step guideline based on visual inspection and assessment of the nutrient capacity for blooms and assessment of cyanobacterial biomass, with thresholds for warning or closure. Sites may remain open if microcystin levels are low even when cyanobacterial levels are high.
Hungary	Addresses cyanobacterial blooms indirectly through a limit for chlorophyll-a.

Table 1.7 (contd)

Country	Recreational sites
Italy	Decree on Quality of Bathing Water addresses cyanobacteria indirectly: derogations above its limit for dissolved oxygen is granted only if not due to excessive proliferation of toxic algae. In 1998, the Ministry of Health provided a list of toxic algae and cyanobacteria of concern and analytical methodologies, and recommended a limit value of 5×10^6 cells/L for toxic algae species as a safe level for bathing activities.
Netherlands	Guideline of: 10 µg/L for issuing warnings; and 20 µg/L and scums for closure of bathing sites and continued monitoring

From Chorus (2005), Australian Government (2008)

1.6.3 *Measures to control human exposure*

The prevention of cyanobacterial proliferation in the water source is largely achieved through the reduction or prevention eutrophication, i.e. 'fertilization' of water bodies with plant nutrients, in most cases phosphorus and in some settings also nitrogen. It may require substantial reductions of concentrations within the water body and, where multiple and diffuse sources contribute to the total nutrient load of a water body, success may be slow. In such situations, other water-body management approaches that render growth conditions less favourable for cyanobacteria may be useful. These largely include physical measures, i.e. changes in the thermal mixing regime or flushing rate of the water body, and are possible only in some settings. When cyanobacterial proliferation cannot be prevented, other barriers against human exposure are necessary (see Chorus & Bartram, 1999, for a more detailed overview).

For drinking-water and dialysis units, control measures include offtake strategy and treatment: offtakes may be located away from surface scums or deeper horizons where cells may accumulate, or may occur through banks drilled close to the river, using the subsurface as a filter (bank filtration). Other commonly used particle removal techniques have often proven very successful and include flocculation combined with sedimentation and rapid filtration, dissolved air flotation, microfiltration and slow sand filtration (see Table 1.5). For some of these techniques, it is important that cells accumulated on filters be removed before they lyse and release their toxin content. When elevated concentrations of dissolved microcystins occur, these can be removed by oxidation (ozone or chlorination) and treatment with activated carbon. Comprehensive overviews of the state of the art of microcystin removal may be found in Falconer (2005).

For recreational exposure, no further barriers other than the prevention of cyanobacterial proliferation are available and keeping people out of the water under high-

risk conditions. Effective surveillance and public information strategies are key to achieving this (see Section 1.6.2).

For dietary supplements, tight monitoring of contamination with microcystins may be required, e.g. by the State of Oregon in the USA and the Food and Health Authorities of Switzerland. However, this cannot rule out the occurrence of other bioactive and potentially harmful cyanobacterial metabolites or other contaminants when cell material is harvested from scums on water bodies and pressed into tablets.

For occupational exposure through aerosols, either filtration of the water before use or application techniques to avoid generation of the aerosol may prevent exposure.

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2. Studies of Cancer in Humans

The incidence of hepatocellular carcinoma (HCC) in Southeast China is among the highest in the world, with annual rates in some counties reaching over 100/100 000 (Ferlay *et al.*, 2004). Risk factors in this region include infection with hepatitis B and C viruses and intake of aflatoxin B_1 from food items such as corn. Several epidemiological investigations have also suggested a role for cyanobacterial toxins, in particular microcystin, as a contributor to the overall risk for HCC. Three types of study have been conducted to evaluate the relationship between HCC and microcystin: comparisons of HCC mortality rates among groups who have different types of water source (ecological studies), cohort studies and case–control studies. In addition to studies of HCC, the incidence of colorectal cancer was investigated in an ecological study. Certain studies that the Working Group evaluated provided some data on levels of microcystins, and reported relative risks among persons who consumed water from sources that had elevated levels compared with those who consumed low levels. In other studies, the contrast used to calculate relative risk was between persons who had different types of water source, typically water from ponds, ditches or small rivers compared with well-water, that was sometimes characterized as being from deep or shallow wells. The Working Group did not have access to original publications of many studies of HCC, and only those studies for which summary information was available are reported here (see Section 5.2).

2.1 Hepatocellular carcinoma

2.1.1 *Ecological studies*

In a review, Yu (1995) presented a summary of findings from several studies. In Qidong County of Jiangsu Province, China, an endemic area for primary liver cancer, mortality rates of less than 20 deaths per 100 000 were observed in some districts compared with more than 60 deaths per 100 000 in adjacent locations. Mortality rates were higher in areas where drinking-water was drawn from ponds and ditches compared with rates in areas with deep wells. Six studies in Nantong City by different authors were cited. All showed that people who consumed water from ponds or ditches had higher rates of mortality from HCC (approximately 100 deaths/100 000) than people who drank well- or deep well-water (mortality rate, < 20 deaths/100 000). Yu (1995) also tabulated results from six evaluations of mortality from HCC, four from Qidong County for overlapping periods during 1972–83, one from Haimen County (1968–72) and one from Nanhui County (1981–84). Mortality rates for consumers of pond and ditch water were higher (range, approximately 60–140/100 000/year) than those for well-water users (range, approximately 0–15/100 000/year). In this Province, microcystin-producing cyanobacteria are abundant in surface waters, and significant amounts of microcystin were detected in

pond-ditch waters whereas no detectable levels were found in deep well-water; this provides supportive evidence that microcystins in drinking-water were partially responsible for the higher incidence of HCC (Chen *et al.*, 1996).

In a Chinese county that displayed high mortality from primary liver cancer, consumption of pond water with low microcystin concentrations (160 ng/L; $n = 27$) was correlated with a higher mortality rate than consumption of deep well-water with no detectable levels of microcystin (< 50 ng/L; $n = 25$) ($P < 0.01$ for deep well- in relation to pond or river water) (mortality rate, 115.05/100 000 for pond water versus 20.00/100 000 for deep well-water; $P < 0.01$) (Ling, 2000).

2.1.2 *Cohort studies*

In a cohort study of 77 682 persons in Nanhui County, who were followed from 1986 to 1991 (Yu *et al.*, 1995), a total of 202 deaths from HCC yielded relative risks of 1.16 (95% confidence interval [CI], 1.02–1.32) for consumption of pond and ditch water and 1.25 (95% CI, 1.09–1.43) for consumption of river water, using a case–cohort approach. The relative risk for history of hepatitis was 1.03 (95% CI, 1.02–1.04). The consumption of shallow and deep well-water (including tap-water) was protective and gave relative risks of 0.65 (95% CI, 0.59–0.73) and 0.20 (95% CI, 0.16–0.25), respectively. [The Working Group noted that the relative risk for surface water was approximately five times that for deep well-/tap-water].

A cohort study reported in the review by Yu *et al.* (1995) found rates of mortality from HCC of 121.96/100 000 for consumers of pond-ditch water (12 299 person–years), 77.81 for consumers of river water (5141 person–years) and 0 for consumers of well-water (1333 person–years) (Yu & Chen, 1994).

2.1.3 *Case–control studies*

Zhao *et al.* (1994) conducted a pooled analysis of 10 Chinese case–control studies of HCC, six from southern China and four from northern China, with a total of 920 cases and 920 controls. Water source (drinking pond-ditch water) was a risk factor in the pooled studies from southern China, but not in those from northern China. Additive and multiplicative models were used to evaluate risk and test for interaction of risk factors. In analyses of the data from southern China, the adjusted odds ratio was 1.60 (95% CI, 1.19–2.13) for consumption of pond water (multiplicative model), with similar findings from the additive model. Among users of non-pond water, the odds ratio for hepatitis antigen-positivity was 10.68 (95% CI, 7.94–14.37). The odds ratio for persons who used pond water and were positive for the hepatitis antigen was 17.04 (95% CI, 12.75–22.77). [A *P*-value for the interaction between HCC and pond water was not provided.]

A population-based case–control study of 99 incident cases of HCC diagnosed between October 1988 and October 1989 and 99 age- and sex-matched controls was undertaken in Fusui County, Guangxi Autonomous Region, China, using data from

interviews of study subjects (Zhang, 1993). Conditional logistic regression showed associations with drinking pond-ditch water (odds ratio, 3.70; 95% CI, 1.25–10.96) continuously for more than four decades relative to never-users of pond water. The findings were adjusted for ever having had a hepatitis B virus infection. [The authors did not present measurements or estimates of microcystins in the drinking-water.]

In the review by Yu *et al.* (1995), several case–control studies were mentioned. In Haimen County, the odds ratio for drinking pond-ditch water was 1.91 (95% CI, 1.01–4.74). Microcystin was found in several ponds and ditches of the high-endemic areas for HCC at levels of 0.061±0.086 μg/L; levels in well-water were 0.036±0.022 μg/L. [The Working Group noted that the levels of microcystin were relatively low.]

Yu *et al.* (2001) performed a meta-analysis of six case–control studies and calculated an odds ratio of 2.46 (95% CI, 1.69–2.59) for primary liver cancer following consumption of pond-ditch water, with a population-attributable risk of 30.39% (95% CI, 23.30–37.47%). In this study, much higher levels of microcystins were observed in pond-ditch water than in well-water.

2.2 Colorectal cancer

Four hundred and eight colon and rectal carcinomas diagnosed from 1977 to 1996 in eight townships were identified from the cancer registry of Haining City of Zhejiang Province, China. The type of drinking-water consumed by each patient was ascertained by interview with each case or a family member if the case was deceased. In addition, in June to September 1997, 640 samples were taken from the four types of water source in each of the eight townships and were analysed for levels of microcystins. The incidence of colorectal cancer was significantly higher among people who drank river water (relative risk, 7.94; 95% CI, 6.11–10.31) or pond water (relative risk, 7.70; 95% CI, 5.75–10.30) than among those who consumed well-or tap-water. The maximum levels of microcystins measured in well-, tap-, river and pond water were 9.13 ng/L, 11.34 ng/L, 1083.43 ng/L and 1937.94 ng/L, respectively ($P < 0.01$ for the contrast in levels of microcystins in river or pond water versus those in well-and tap-water). The incidence of colon cancer in each of the eight townships showed a significant positive correlation ($r = 0.881$; $P < 0.01$ Spearman rank) with the average concentration of microcystins in the township. The authors mentioned that findings for men and women were similar (Zhou *et al.*, 2000). [Cancer incidence was ascertained for the 20-year period of 1977–96 and the survey of concentrations of microcystins was conducted in 1997, after the period of diagnosis. Although this may lead to some misclassification of exposure, it was the opinion of the Working Group that levels of microcystins in 1997 were probably generally representative of levels over the previous 20-year period. The authors did not describe how they estimated denominators of population size for calculating the water source-specific estimates, and it was not stated whether incidence rates of colorectal cancer by township were sex- or age-adjusted.]

2.3 References

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3. Studies of Cancer in Experimental Animals

3.1 Pure microcystin-LR (see Table 3.1)

3.1.1 *Mouse*

A group of 13 male ICR mice, 5 weeks of age, received 100 intraperitoneal injections of 20 μg/kg bw microcystin-LR (five times a week) over 20 weeks and were killed after the end of the treatment (five mice) or after a 2-month withdrawal period (eight mice). Three non-treated mice were used as controls. Liver foci which were, according to the authors, probably benign tumours were induced in all 13 treated mice (Ito *et al.*, 1997). [The Working Group noted the small number of animals, the use of a single dose exposure regimen, inadequacies of statistical evaluations and the lack of results for the control animals.]

3.1.2 *Rat*

Groups of 10–16 male Fischer 344 rats, 7 weeks of age, were given a single intraperitoneal injection of 0 or 200 mg/kg bw *N*-nitrosodiethylamine (NDEA) in saline followed 2 weeks later by intraperitoneal injections of 0, 1 or 10 μg/kg bw pure microcystin-LR twice a week for 6 weeks and partial hepatectomy at the end of week 3. Phenobarbital (0.05%) was used as a positive control. In a second experiment, groups of 14–19 rats were initiated with NDEA followed 2 weeks later by two intraperitoneal injections of 0 or 10 μg/kg bw pure microcystin-LR, partial hepatectomy at the end of week 3 and intraperitoneal injections of 10, 25 of 50 μg/kg bw microcystin-LR twice a week for 5 weeks. The tumour-promoting activity was estimated by induction of glutathione *S*-transferase placental form-positive (GST-P) foci in rat liver. In the first experiment, rats treated with NDEA plus 10 μg/kg bw microcystin-LR had an increased incidence of GST-P foci per liver compared with NDEA-treated rats (26.0±8.1 versus 16.5±3.9/cm^2; $P < 0.005$). In the second experiment, the three groups of rats treated with NDEA plus 10 and 10 μg/kg bw microcystin-LR, NDEA plus 10 and 25 μg/kg bw microcystin-LR or NDEA plus 10 and 50 μg/kg bw microcystin-LR had an increased incidence of GST-P foci per liver compared with NDEA-treated rats (17.4±3.8 ($P < 0.01$), 32.7±11.1 ($P < 0.01$) and 44.4±10.3 ($P < 0.001$) versus 13.4±4.2/cm^2,respectively) (Nishiwaki-Matsushima *et al.*, 1992).

Groups of male Fischer 344 rats [initial number unspecified], 7 weeks of age, received a single intraperitoneal injection of 0 or 200 mg/kg bw NDEA in saline followed 2 weeks later by intraperitoneal injections of 0 or 25 μg/kg bw microcystin-LR. The tumour-promoting activity of microcystin-LR was evaluated on the basis of an increase in

Table 3.1. Summary of the liver tumour-promoting activity and/or carcinogenicity of intraperitoneal injection of microcystin-LR

Initiator	Microcystin-LR (μg/kg × times)	Species	Partial hepatectomy	Biomarker	Estimation	Reference
–	20 × 100 times	Male ICR mice	–	Neoplastic nodules	Weak carcinogen	Ito *et al.* (1997)
NDEA	10 × 12 times	Male Fischer 344 rats	+	GST-P foci	Tumour promoter	Nishiwaki-Matsushima *et al.* (1992)
NDEA	10–50 × 12 times	Male Fischer 344 rats	+	GST-P foci	Tumour promoter	Nishiwaki-Matsushima et al. (1992)
NDEA	25 × 20 times	Male Fischer 344 rats	–	GST-P foci	Tumour promoter	Ohta *et al.* (1994)
NDEA + aflatoxin B_1	10 × 12 times	Male Fischer 344 rats	+	GST-P foci	Tumour promoter	Sekijima *et al.* (1999)
Aflatoxin B_1	10 × 12 times	Male Fischer 344 rats	+	GST-P foci	Tumour promoter	Sekijima *et al.* (1999)

GST-P, glutathione *S*-transferase placental form-positive; NDEA, *N*-nitrosodiethylamine

three parameters: the number of GST-P-positive foci per liver (no./cm^2), the area of foci per liver (mm^2/cm^2) and the volume of foci per liver (v/v%) (see Table 3.2; Ohta *et al.*, 1994).

Groups of male Fischer 344 rats, 6 weeks of age, received an intraperitoneal injection of 0 or 200 mg/kg bw NDEA in saline 2 weeks before intraperitoneal injections of 0, 1 or 10 μg/kg bw microcystin-LR twice a week for 6 weeks. Other groups were also treated with aflatoxin B_1 (0.5 mg/kg bw) and NDEA, or aflatoxin B_1 alone, followed by partial hepatectomy (for dosing regimes and results, see Table 3.3; Sekijima *et al.*, 1999).

Table 3.2. Induction of liver GST-P foci by intraperitoneal administration of microcystin-LR or nodularin to rats initiated with NDEA

NDEA	Toxin (μg/kg) 20 times	Effective no. of rats	No. of foci/liver (no./cm^2)[a]	Area of foci/liver (mm^2/cm^2)[a]	Volume of foci/liver (v/v %)[a]
+	–	20	10.0±2.9	0.18±0.07	0.37±0.18
–	–	5	0	0	0
+	Microcystin-LR (25)	18	95.7±27.9*	4.74±2.23*	8.55±4.04*
–	Microcystin-LR (25)	17	1.6±1.4	0.02±0.02	0.04±0.03
+	Nodularin (25)	20	106.0±22.6*	39.87±10.51*	71.75±18.78*
–	Nodularin (25)	16	6.3±7.3	0.49±0.89	0.92±1.58

From Ohta *et al.* (1994)
GST-P, glutathione *S*-transferase placental form-positive; NDEA *N*-nitrosodiethylamine;
[a] Mean±standard deviation
* $P < 0.005$ versus NDEA control

Table 3.3. Induction of liver GST-P foci by microcystin-LR in rats initiated with NDEA and/or aflatoxin B_1

NDEA	Aflatoxin B_1	Microcystin-LR	No. of rats	No. of foci/liver[a]	Area foci/liver[a]
+	–	–	5	2.46±1.86	0.39±0.29
+	+	–	15	8.34 ± 3.60*	1.69±0.79*
+	+	1 μg/kg bw	13	10.72±6.74*	2.26±1.75*
+	+	10 μg/kg bw	13	9.16±4.70	1.96±1.03**
–	+	–	10	1.61±0.74	0.76±0.51
–	+	1 μg/kg bw	16	3.46±1.14***	2.24±1.35***
–	+	10 μg/kg bw	16	3.50±1.74	2.75±2.86

From Sekijima *et al.* (1999)
GST-P, glutathione *S*-transferase placental form-positive; NDEA, *N*-nitrosodiethylamine
[a] Mean±standard deviation
**P* <0.01 versus NDEA control
***P* <0.001 versus NDEA control
****P* <0.01 versus aflatoxin B_1 control

3.2 Microcystis extracts

Mouse

Groups of five male and five female Swiss mice, 3 weeks of age, were given extracts of *Microcystis aeruginosa* (1/4, 1/8 and 1/16 dilutions; equivalent to 28.3, 14.1 and 7 μg microcystin/mL) in the drinking-water or drinking-water alone (controls) for periods up to 1 year, during which mice were killed at various intervals. The incidence of tumours in mice was: 4/71 (one abdominal carcinoma, two lung carcinomas and one thoracic lymphosarcoma) in the high-dose exposure group, 0/150 for the mid- and low-dose exposure groups, and 2/73 (one uterine adenocarcinoma and one thoracic lymphosarcoma) in the control group (Falconer *et al.*, 1988). [The Working Group noted that details about examination of tumours was not reported.]

Groups of 20 female Swiss mice, 3 months of age, received a single dermal application of 0 or 500 μg 7,12-dimethylbenz[*a*]anthracene (DMBA) in acetone onto the shaved skin and, 1 week later, were given extracts of *Microcystis* (1/2 dilution; equivalent to 40 μg microcystin/mL) in the drinking-water or drinking-water alone. After 52 days of exposure, a significant increase ($P < 0.05$) in the weight of skin papillomas/mouse was reported [DMBA plus extract, 16 mg/mouse; DMBA, 2 mg/mouse; read from Figure]. No tumours were reported in the groups treated with the extract only or drinking-water alone [2.5 tumours/mouse and four tumours/mouse were reported in the groups treated with DMBA and DMBA plus extract, respectively; numbers of tumours were not significantly different] (Falconer & Buckley, 1989; Falconer, 1991). [The Working Group noted that the number of animals was not given.]

The possibility of promotion of growth of tumours initiated by two doses of 40 mg/kg bw *N*-methyl-*N*-nitrosourea at 1-week intervals was investigated in 115 C57 black mice [sex and age unspecified] exposed to 0, 10 or 40 μg/mL *Microcystis* toxins in the drinking-water (0, 29 or 89 μg/day/mouse). After 154 days, tumours of the duodenum, liver and lymphoid system were assessed. No effect of exposure to microcystin on the growth of these tumours was observed (Falconer & Humpage, 1996). [The Working Group noted the absence of a group treated with microcystins alone.]

To investigate the possibility that microcystin promotes colon tumours, a total of 176 male C57Bl/6 mice, 13 weeks of age, were given three weekly intraperitoneal injections of 0 ($n = 20$) or 5 mg/kg bw azoxymethane ($n = 156$) in saline. Three weeks later, azoxymethane-treated mice were exposed for 212 days to 0 ($n = 61$), 6 ($n = 53$) or 12 ($n = 42$) mg microcystin-LR equivalents/L (0, 382 or 693 μg/kg bw per day) in the drinking-water. Mice from each group were killed at 13, 22, 28 or 36 weeks after the first azoxymethane treatment. Examination of hypertrophic crypts in the colon showed a dose-dependent increase in the area of aberrant crypt foci after exposure to microcystin. Results were as follows: azoxymethane-only group (291 foci/57 mice), 25×10^3 μm^2; azoxymethane plus low-dose microcystin (280 foci/49 mice), 26×10^3 μm^2; azoxymethane plus high-dose microcystin (195 foci/38 mice), 29×10^3 μm^2 ($P < 0.05$). Only 1/16 untreated

mice had one foci and the rest had none (Humpage *et al.*, 2000). [The Working Group noted the absence of a group treated with microcystin alone.]

3.3 Nodularin

Rat

Groups of male Fischer 344 rats [initial number unspecified], 7 weeks of age, received an intraperitoneal injection of 0 or 200 mg/kg bw NDEA in saline followed 2 weeks later by intraperitoneal injections of 10 or 25 μg/kg bw nodularin twice a week for 10 weeks. Saline was injected into control animals. Nodularin showed strong tumour-promoting activity in rat liver on the basis of parameters such as the number of GST-P foci per liver (no./cm^2), the area of foci per liver (mm^2/cm^2) and the volume of foci per liver (v/v%). Treatment with NDEA followed by 25 μg/kg bw nodularin (20 rats) induced 106±22.6 foci per liver ($P < 0.005$), 39.87±10.51 mm^2/cm^2 foci per liver ($P < 0.005$) and 71.75±18.78% volume of foci per liver ($P < 0.005$) compared with 10.0±2.9 foci per liver, 0.18±0.07 mm^2/cm^2 foci per liver and 0.37±0.18% volume of foci per liver in NDEA alone-treated animals (20 rats). Treatment with NDEA followed by 10 μg/kg bw nodularin (19 rats) induced 0.25±0.09 mm^2/cm^2 foci per liver ($P < 0.025$). Treatment with 25 μg/kg bw nodularin alone (16 rats) induced GST-P foci at the same potency as NDEA alone (6.3±7.3 foci per liver, 0.49±0.89 mm^2/cm^2 foci per liver and 0.92±1.58% volume of foci per liver; statistically non-significant). The results suggest that nodularin is a stronger tumour promoter than microcystin-LR and has initiating activity equal to that of NDEA (Ohta *et al.*, 1994).

The tumour-promoting activity of nodularin was confirmed in groups of male Fischer 344 rats [initial number unspecified], 7 weeks of age, that received a single intraperitoneal injection of 0 or 200 mg/kg bw NDEA in saline and received intraperitoneal injections 2 weeks later of 0 or 25 μg/kg bw nodularin twice a week for 10 weeks. Animals were maintained up to experimental week 22 but were killed periodically. Results are presented in Table 3.4. GST-P foci decreased significantly after the cessation of intraperitoneal injections of nodularin. Following treatment with NDEA plus nodularin, GST-P foci displayed two types of hyperplastic nodules: homogenously stained dense nodules and heterogeneously stained pale nodules. The results suggest that nodularin is a promotor that induces hepatocyte proliferation (Song *et al.*, 1999).

Table 3.4. Induction of liver GST-P foci by nodularin in rats initiated with NDEA

Duration of the experiment	GST-P foci (no./cm^3)[a]			
	Nodularin only	NDEA only	NDEA + nodularin	
			Dense nodules	Pale nodules
Untreated, 8 weeks	0	0	0	0
8 weeks	6.4±2.0	11.4±2.0	71.0±43.0	0
10 weeks	6.8±2.0	10.0±2.1	87.8±16.2	0
12 weeks	8.5±2.4	9.5±2.0	124.5±38.3	0
15 weeks	7.5±1.8	6.9±0.9	83.7±6.8	14.5±2.9
18 weeks	5.2±1.0	6.4±1.8	60.0±11.4	13.2±4.5
22 weeks	7.2±3.6	7.6±2.2	59.4±17.8	18.0±4.5

From Song *et al.* (1999)
GST-P, glutathione *S*-transferase placental form-positive; NDEA, *N*-nitrosodiethylamine
[a] Mean±standard deviation

3.4 References

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4. Mechanistic and Other Relevant Data

A number of reviews that deal with various aspects of the toxicity of microcystins and nodularin have been published (Metcalf & Codd, 2004; Falconer & Humpage, 2005; Dietrich & Hoeger, 2005).

Over 70 variants of microcystins have been identified; most studies have used microcystin-LR, -RR, -YR and -YM, for which toxicity data are summarized in Section 4.4 (Gupta *et al.,* 2003).

Most work on nodularin has been carried out with the arginine pentapeptide, although the best structural work has been performed with motuporin (a more hydrophobic congener in which Arg is replaced by Val) (Bagu *et al*., 1995; Goldberg *et al*., 1995; Maynes *et al*., 2006).

4.1 Absorption, distribution, metabolism and excretion

4.1.1 *Humans*

There are only two reports in which the absorption of microcystin was documented by direct measurement of its association to tissues. Both reports referred to the same accident that occurred in Brazil, in patients who underwent haemodialysis in 1996 (see Section 4.4) (Pouria *et al.*, 1998; Carmichael *et al.*, 2001).

There are really no quantitative data on tissue distribution, metabolism or excretion in humans. A common finding in agreement with most animal studies is the accumulation and persistence of microcystins in the liver (Azevedo *et al*., 2002; Soares *et al.,* 2006). Although concentrated in the liver, microcystin was also detected in the sera of patients up to 2 months or longer after exposure (Hilborn *et al.*, 2005; Soares *et al.*, 2006).

Little is known about the metabolism of microcystins; indirectly, it probably occurs but nothing is known about any intermediates or products. The finding of unidentified peaks by mass spectrometry analysis could represent metabolic products of microcystins (Soares *et al.*, 2006).

The mode and extent of excretion in humans is not known either. It is unlikely to occur through the kidneys; many animal studies have shown that secretion in urine, when it occurs, is very limited.

There are no published records that directly and unequivocally link absorption of nodularin and its toxicity in humans. Many different animals are susceptible to the toxicity of nodularin (see Section 4.1.2); it is therefore most probably toxic in humans and this toxicity may be similar to that found in animals. In addition, nodularin shares some properties with the microcystins. Since microcystins have caused death in haemodialysis patients exposed to microcystin-contaminated water (Jochimsen *et al.*, 1998), nodularin is probably toxic to humans.

In a recent publication, Fischer *et al.* (2005) showed uptake of radiolabelled [^{3}H]dihydromicrocystin-LR in frog (*Xenopus laevis*) oocytes that express the human organic anion transporters polypeptides (OATPs) OATP1B1, OATP1B3 and OATP1A2, whereas no uptake was obtained in cells that express OATP2B1. All of these transporters are found in the liver and brain. [From calculations, the radioactivity taken up was very small] (Fischer *et al.* 2005). [If this pattern of differential species-specific uptake holds, then uptake of microcystin by the brain would be a property of this toxin that is peculiar to humans. This needs to be investigated in order to validate extrapolations between experimental models and humans.]

The uptake of nodularin has not been investigated (Fischer *et al.*, 2005).

4.1.2 *Experimental systems*

(a) Absorption and distribution

(i) *Whole animals*

Microcystins

Administration of microcystins to many species including mice, rats, cattle, sheep, swine and fish results in liver toxicity (Jackson *et al*., 1984; Falconer *et al*., 1986; Runnegar *et al*., 1986; Brooks & Codd, 1987; Galey *et al*., 1987; Robinson *et al.*, 1989, 1991; Williams *et al.,* 1995; Stotts *et al.*, 1997a,b; Tencalla & Dietrich, 1997). Most studies to investigate the uptake and tissue distribution of microcystins have used the intraperitoneal or intravenous routes, although a few have used oral administration.

Numerous studies used radiolabelled peptides but, depending on the radioactive labelling method used for microcystins or nodularin, the results may vary since the labelled products obtained may present different characteristics with both advantages and disadvantages (Table 4.1). A disadvantage of using different preparations with widely varying specific activities is that it is difficult to deduce whether or not variations between studies result in part from the choice of the labelled toxin. On the positive side, it reinforces any common findings that result from experiments that used these differently labelled tools.

Kinetic studies on the absorption and distribution of microcystin are described in Table 4.2.

In studies that used intravenous or intraperitoneal administration in different experimental models, with different protocols and variously labelled microcystins, the one common finding in almost all of them was the accumulation of microcystin in the liver and low concentrations in the kidney in animals as diverse as salmon, pigs and rodents. The intestinal concentration of microcystin was much more varied and probably reflects the different dosing and degrees of metabolism and secretion.

Not unexpectedly, the oral dose required to induce microcystin toxicity is higher than the intravenous or intraperitoneal dose. In most oral studies, microcystin is found in the liver. The pathology following oral administration of microcystin parallels broadly that

Table 4.1. Advantages and disadvantages of the different radioactive labelling methods for microcystins (MC) and nodularin (NOD)

Isotope	Toxin	Method	Advantages	Disadvantages	Specific activity (mCi/mmol)	Reference
^{14}C	MC-LR	Biosynthesis with $^{14}[C]NaHCO_3$	• Most stable labelled product • Structure closest to native peptide	• Time consuming labelling method • Low specific activity of the labelled product	2.6 1.38 > 0.9 0.08	Brooks & Codd (1987) Williams *et al.* (1997) Craig *et al.* (1996) Pflugmacher *et al.* (1998)
^{3}H	MC-LA MC-LR	Exchange with $^{3}H_2O$	• The two isomers obtained retain toxicity of the native peptide	• Dihydromicrocystins unable to form covalent bond to PP1 and PP2A • Potential differences of interaction, stability and metabolic properties with the native peptide	NR 194	Botes *et al.* (1984) Robinson *et al.* (1989)
^{3}H	MC-LR	Reduction with $^{3}[H]NaBH_4$	• The two isomers obtained retain toxicity of the native peptide	• Dihydromicrocystins unable to form covalent bond to PP1 and PP2A • Potential differences of interaction, stability and metabolic properties with the native peptide	170–310 22 700 32.6 1039 247	Meriluoto *et al.* (1990) Nishiwaki *et al.* (1994) Williams *et al.* (1995) Stotts *et al.* (1997a,b) Bury *et al.* (1998)

Table 4.1 (contd)

Isotope	Toxin	Method	Advantages	Disadvantages	Specific activity (mCi/mmol)	Reference
^{125}I	MC-YM MC-YM MC-YR	$Na^{125}I$ with lactoperoxidase/H_2O_2 $Na^{125}I$ with lactoperoxidase/H_2O_2 Iodogen	• Easy labelling procedure • High specific activity • Retain toxicity of the native peptide	• Limited to variants that contain tyrosine	NR NR NR	Falconer *et al.* (1986) Runnegar *et al.* (1986) Moorhead *et al.* (1994)
^{3}H	NOD	Reduction with $^{3}[H]NaBH_4$	• Retain toxicity of the native peptide		669–678	Spoof *et al.* (2003)

Adapted from Spoof *et al.* (2003)
H_2O_2, hydrogen peroxide; MC, microcystin; $NaBH_4$, sodium borohydride; $NaHCO_3$, sodium bicarbonate; NOD, nodularin; NR, not reported; PP1, protein phosphatase 1; PP2A, protein phosphatase 2A

Table 4.2. Absorption and distribution of microcystins and nodularin

Animal species	No. of animals	Analytical method(s)	Route of administration; dose	Time after dosing	Tissue	% of dose	Concentration of MC in tissue	Half-life	Reference
Microcystin *Mammals*									
							µg/kg tissue		Stotts *et al.*
Female Landrace cross specific pathogen-free swine (18–24 kg)	3 per dose	[^{3}H]Dihydro-MC-LR	iv; 25 µg/kg bw	4 h	Liver	64.6	633	3.7, 134 min[a]	(1997a,b)
					Kidneys	1.2	121		
					Lungs	1.75	62		
					Heart	0.22	17		
					Ileum	0.13	11		
					Spleen	0.04	9		
					Bile	4 (n = 1)			
			iv; 75 µg/kg bw	4 h	Liver	46.99	1110	3, 270 min[a]	
					Kidneys	2.19	654		
					Lungs	0.55	59		
					Heart	0.23	54		
					Ileum	0.20	57		
					Spleen	0.07	41		
					Bile	5.9 (n = 1)			
			Ileal loop; 75 µg/kg bw	5 h	Liver	49.6	1408		
					Kidneys	1.04	31		
					Lungs	0.65	69		
					Heart	0.81	19		
					Ileum	33.94	9165		
					Spleen	0.16	94		
					Bile	5.26 (n = 2)			

Table 4.2 (contd)

Animal species	No. of animals	Analytical method(s)	Route of administration; dose	Time after dosing	Tissue	% of dose	Concentration of MC in tissue	Half-life	Reference
Female albino rats (207–249 g)	10 (5 per time interval)	[^{125}I]MC-YM	iv; ~10 μg/kg bw	30 min	Liver Gut contents Kidney	21.7±1.1 7.0±0.3 5.6±0.2 0.9±0.5		2.1, 42 min[a]	Falconer *et al.* (1986)
				120 min	Urine (total) Liver Gut contents Kidney Urine (total)	19.2±0.3 9.4±1.1 5.3±0.4 1.9±0.2			
Male Swiss Webster mice (25–37 g)	25 (1–3 per dose)	[^{125}I]MC-YM mono- and di-iodinated peptides, results here for [^{125}I]MC-YM similar to [^{125}I]$_2$MC-YM	ip; ~80–240[b] μg/kg bw	At death (2–15 h) (lethal dose)	Liver Kidney	43–63 1–2			Runnegar *et al.* (1986)
				(24 h) (sublethal dose)	Liver Kidney	24–57 0.5–3			

Table 4.2 (contd)

Animal species	No. of animals	Analytical method(s)	Route of administration; dose	Time after dosing	Tissue	% of dose	Concentration of MC in tissue	Half-life	Reference
Male Han:NMRI mice (30±2 g)	4	[^{3}H]dihydroMC-LR epimers	iv; 15 µg/kg bw (0.5 µg/mouse)	45 min			**Relative concentration in counts (min × mass unit) plasma = 1**		Meriluoto *et al.* (1990)
					Liver		35		
					Intestine		7		
					Kidney		5		
					Spleen		1.5		
					Muscle		1		
					Brain		1		
					Plasma		1		
Male VAF/plus CD-1 mice (19–25 g)	5 per time interval	[^{3}H]MC-LR	ip; 45–101 µg/kg bw	At death or 6 h (surviving mice)	Liver	>50			Robinson *et al.* (1989)
					Intestine	~10			
					Carcass	~10			
					Kidney	~1			
					Heart, spleen, lung, skeletal muscle	All <1			
		[^{3}H]MC-LR	ip; 70 µg/kg bw	60 min	Liver	60		29 min	
					Kidney	1			
					Intestine	9			

Table 4.2 (contd)

Animal species	No. of animals	Analytical method(s)	Route of administration; dose	Time after dosing	Tissue	% of dose	Concentration of MC in tissue	Half-life	Reference
Male VAF/plus CD-1 mice (20–27 g)	6	[^{3}H]MC-LR	iv; 35 µg/kg bw	1 min	Liver	23±5			Robinson *et al.* (1991)
					Kidney	2.0±0.2			
					Intestine	5.2±0.9			
					Carcass	30±3			
					Plasma	25±4			
				60 min	Liver	67±4			
					Kidney	0.8±0.1			
					Intestine	8.6±0.7			
					Carcass	6±2			
					Plasma	Trace			
Female ICR mice	2–3 mice per time interval	[^{3}H]dihydroMC-LR	ip; ~80 mg/kg bw[c]	5 min	Liver	17±4.1			Nishiwaki *et al.* (1994)
				15 min		38.0±7.1			
				30 min		57.3±4.1			
				60 min		71.5±6.9			
				60 min	Small intestine	1.4			
					Large intestine	0.5			
					Kidney	0.5			
					Gall bladder	0.5			
					Lungs	0.4			
					Stomach	0.3			

Table 4.2 (contd)

Animal species	No. of animals	Analytical method(s)	Route of administration; dose	Time after dosing	Tissue	% of dose		Concentration of MC in tissue	Half-life	Reference
Female ICR mice	NR	[^{3}H]dihydroMC-LR	Oral; ~70 mg/kg bw[e]	6 h 6 days 6 h	Liver Liver Small intestine Large intestine Caecum Kidney Stomach Brain	0.68 0.41 0.2 0.2 0.15 0.05 0.05 0.01				Nishiwaki *et al.* (1994) (contd)
Male ICR mice (20–27 g)	2 per time interval	(1) [^{3}H]dihydroMC-LR and (2) ELISA with polyclonal anti-MC antibody	ip; 35 µg/kg bw			**[^{3}H]MC-LR %**	**ELISA %**			Lin & Chu (1994)
				15 min	Liver	16.3	16			
					Serum	5.3	5.7			
				12 h	Liver	89	89			
					Serum	23	23			
				24 h	Liver	71	71			
					Serum	15	21.5			

Table 4.2 (contd)

Animal species	No. of animals	Analytical method(s)	Route of administration; dose	Time after dosing	Tissue	% of dose	Concentration of MC in tissue	Half-life	Reference
Fish									
Atlantic salmon (80–160 g)	4 per time interval, only 1 at 46 h	[^{3}H]dihydroMC-LR	ip; 1 mg/kg bw				**µg/g tissue**		Williams *et al.* (1995)
				2 h	Liver	2.8±0.4	3.5		
				5 h		4.9±0.4	5.3		
				22 h		4.2±0.6	4.4		
				46 h		2.4	2.5		
				2 h	Muscle		0.1 or less		
				5 h	Muscle		0.1 or less		
				22 h	Muscle		0.3		
				46 h	Muscle		0.1 or less		
Atlantic salmon (100–130 g)	4 per time interval	[^{14}C]MC-LR	ip; 1 mg/kg bw				**µg/g tissue**		Williams *et al.* (1997)
				2 h	Liver	11.93±0.17	9.29±0.06		
				5 h		16.55±0.85	9.84±0.56		
				24 h		6.53±0.54	4.35±0.34		
				43.5 h		2.70±0.31	2.15±0.24		
				2 h	Muscle		0.15±0.02		
				5 h			0.26±0.04		
				24 h			NR		
				43.5 h			0.24±0.04		
				2 h	Carcass	40.7±3.5	0.43±0.04		
				5 h		39.3±3.0	0.42±0.03		
				24 h		13.2±1.5	0.14±0.02		
				43.5 h		6.1±0.7	0.07±0.01		

Table 4.2 (contd)

Animal species	No. of animals	Analytical method(s)	Route of administration; dose	Time after dosing	Tissue	% of dose	Concentration of MC in tissue	Half-life	Reference
Rainbow trout (60 g)	3 per time interval	(1) PP activity inhibition and (2) extractable MC-LR by PP activity inhibition	Gavage; freeze-dried cyanobacteria equivalent to 5700 µg/kg bw MC-LR*; dose toxic at 96 h			**PP inhibition (% of control)**	**Extractable MC-LR (ng/g liver, ng/mL plasma)**	3.3 h	Tencalla & Dietrich (1997); Fischer *et al*. (2000)
				1 h	Liver	60	144		
					Plasma		24		
				3 h	Liver	4	524		
					Plasma		520		
				12 h	Liver	20	226		
					Plasma		180		
				24 h	Liver	42	51		
					Plasma		62		
				48 h	Liver	41	40		
					Plasma		0		
				72 h	Liver	52	44		
					Plasma		0		

Table 4.2 (contd)

Animal species	No. of animals	Analytical method(s)	Route of administration; dose	Time after dosing	Tissue	% of dose	Concentration of MC in tissue	Half-life	Reference
Little skate (*Raja erinacea*) (0.6–1 kg)	2–3 skate per dose	(1) PP activity inhibition and (2) free MC-YM in plasma by PP activity inhibition				**% of dose/PP inhibition (% of control)[d]**			Runnegar *et al.* (1999)
			iv; 125 μg/kg bw	24 h	Liver	NR/93			
					Plasma	8.6/NR			
				48 h	Liver	NR/93			
				72 h	Liver	NR/93			
				7 days	Liver	NR/93			
			iv; 250 μg/kg bw	24 h	Liver	NR/93			
					Plasma	9.6/NR			
				48 h	Liver	NR/93			
				72 hr	Liver	NR/93			
				7 days	Liver	NR/95			
			iv; 500 μg/kg bw	24 h	Liver	NR/98			
					Plasma	10.2/NR			
				48 h	Liver	NR/94			
				72 h	Liver	NR/87			
				7 days	Liver	NR/99			
			iv; 32 μg/kg bw	24 h	Liver	NR/10			
			iv; 63 μg/kg bw		Plasma	2.45/NR			
				24 h	Liver	11/89			
					Plasma	4.2/NR			

Table 4.2 (contd)

Animal species	No. of animals	Analytical method(s)	Route of administration; dose	Time after dosing	Tissue	% of dose	Concentration of MC in tissue	Half-life	Reference
Nodularin									
BALB/c mice (20–25 g)	8	[^{3}H]dihydro-NOD	iv; 20 µg/kg bw	2 h			**cpm/mg**		Spoof *et al.* (2003)
					Liver	[1.6][e]	~15		
					Blood	[0.5]	~5		
					Intestine	[0.5]	~5		
					Kidney	[0.1]	~1		
					Spleen		trace		
					Bone		trace		
					Brain		trace		
					Muscle		trace		
					Lung		trace		

ELISA, enzyme-linked immunosorbent assay; ip, intraperitoneal; iv, intravenous; LD_{50},dose that is lethal to 50 % of animals; MC, microcystin; MC-LR*: corresponds to a conformational variant of microcystin-LR which is equally toxic; NOD, nodularin; NR, not reported; PP, protein phosphatase

[a] Biphasic disappearance curve of blood radioactivity

[b] Note: the LD_{50} of the peptide was 110 µg/kg for male mice in this study

[c] The doses given in the original article were: 2.4 µmol/mouse [~80 mg/ kg bw] and 2.1 µmol/mouse [~70 mg/ kg bw]. The Working Group noted that these reported doses are in great excess of the LD_{50} of [^{3}H]dihydromicrocystin-LR for mice (~110 µg/ kg bw). It is reasonable to deduce that the unit was misreported and that the real doses were 1000 times lower.

[d] > 90% inhibition of PP activity considered to be fully inhibited

[e] % of dose calculated by the Working Group

found after intravenous or intraperitoneal administration, although the amount of microcystin or microcystin-containing bloom that is needed for oral toxicity varies with species and on whether or not the animal is fasted. For fasted mice, the ratio between the oral and intraperitoneal dose required is ~30; for sheep, it is ~150; and for chickens, it is ~125 (Jackson *et al.*, 1984). In BALB/c mice, Yoshida *et al.* (1997) found that the intraperitoneal LD_{50} (dose that is lethal to 50% of animals) of microcystin-LR was 65.4 μg/kg bw and the oral LD_{50} was 10.9 mg/kg bw. In many of these studies, uptake of microcystin or microcystin-containing bloom was inferred by the resulting toxicity. The accumulation of microcystin in the liver can be very fast, within minutes after injection as was shown in mice (Robinson *et al.*, 1991; Nishiwaki *et al.*, 1994; Lin & Chu, 1994) and can last several hours and even several days as shown in salmon (Williams *et al.*, 1995, 1997).

Nodularin

A number of studies have demonstrated the toxicity of nodularin and, by inference, its absorption, both in field cases and in laboratory animals. In field cases, nodularin is taken up as cyanobacterial bloom that contains toxic strains of *Nodularia*. Laboratory animals are most frequently administered purified nodularin. Field cases attributed to nodularin poisoning include the deaths of a dog in South Africa (Harding *et al.*, 1995), sea birds in the Baltic Sea (Sipiä *et al*., 2004) and sheep in Australia (Main *et al.*, 1977). Laboratory animals tested include mice (Carmichael *et al.*, 1988; Eriksson *et al.*, 1988; Runnegar *et al.*, 1988), guinea-pigs and sheep (Main *et al.*, 1977). The primary target organ was the liver.

There is only one study in mammals of the distribution of nodularin (Spoof *et al.*, 2003; Table 4.2). Two hours after intravenous injection of a non-toxic dose of [^{3}H]dihydronodularin isomers (20 μg/kg bw) into mice, the toxin (and possibly its metabolites) was found concentrated in the liver. Intestine and blood also retained significant amounts of the labelled compound. [The Working Group noted that the experimental details were not sufficiently clear, the reported total activity was low and only a single time point was reported.]

Other studies have investigated the effect of nodularin in aquatic animals: from sea-ducks to zooplankton (Sipiä *et al.*, 2001a,b; Kankaanpää *et al.*, 2002; Sipiä *et al.*, 2002; Kankaanpää & Sipiä, 2003; Lehtonen *et al.*, 2003; Sipiä *et al.*, 2004; Kankaanpää *et al.*, 2005a,b). Adsorption and distribution in aquatic species are reported here (Table 4.3) because of the potential for bioaccumulation that would result in contaminated food sources. In fish from the Baltic Sea (such as flounders and cod), nodularin has also been reported to accumulate mainly in the liver. In flounders, the concentration of toxin was shown to increase strongly between May and August during the blooming period of *Nodularia*. However, when measured, none or only trace amounts of nodularin were detected in the muscle of fish even when the concentration in the liver was significant (Sipiä *et al.*, 2001a,b, 2002; Karlsson *et al.*, 2003).

Table 4.3. Detection and distribution of nodularin in aquatic animals

Species	Origin	Method of detection	Tissue	Quantitation	Reference
Flounders (*Platichthys flesus*)	Baltic Sea, 1995	LC–MS MALDI-TOF-MS	Liver	100–600 µg/kg wet wt	Karlsson *et al.* (2003)
Eiders (*Somateria mollissima*)	Baltic Sea, 2002	ELISA LC–MS	Liver	3–180 µg/kg dry wt	Sipiä *et al.* (2004)
Flounders (*Platichthys flesus*)	Baltic Sea, 1996–98	ELISA, HPLC, PP1 inhibition	Liver Muscle	25–140 µg/kg dry wt None detected	Sipiä *et al.* (2001a)
Cod (*Gadus morhua*)	Baltic Sea, 1996–98	ELISA, HPLC, PP1 inhibition	Liver Muscle	~55 µg/kg dry wt None detected	Sipiä *et al.* (2001a)
Flounders (*Platichthys flesus*)	Baltic Sea, 1999–2000	ELISA LC–MS MALDI-TOF-MS	Liver Muscle	Up to 400 µg/kg None detected	Sipiä *et al.* (2002)
Clams (*Macoma baltica*)	Baltic Sea, 2000	ELISA LC–MS MALDI-TOF-MS		100–130 µg/kg dry wt (one site, 1490 µg/kg)	Sipiä *et al.* (2002)
Mussels (*Mytilus edulis*)	Baltic Sea, 1999–2000	ELISA LC–MS MALDI-TOF-MS		40–130 µg/kg dry wt (one site, 1490 µg/kg)	Sipiä *et al.* (2002)

ELISA, enzyme-linked immunosorbent assay; HPLC, high-performance liquid chromatography; LC–MS, liquid chromatography–mass spectrometry; MALDI-TOF-MS, matrix-assisted laser desorption/ionization time-of-flight mass spectrometry; PP, protein phosphatase

Clams and mussels can also accumulate nodularin. Blue mussels collected from the northern Baltic Sea in 2000 contained about 40–130 µg/kg dry wt and, in the Gulf of Finland, toxin concentrations were up to 1490 µg/kg dry wt. Total hepatotoxin levels in mussels decreased from August to September, indicating at least partial detoxification/depuration of the toxin. However, in deeper-living wreck mussels, hepatotoxin levels continued to increase from August to September, indicating that portions of cyanobacterial hepatotoxins reach the sea floor (Sipiä *et al.*, 2002).

(ii) *In-vitro absorption*

Many studies have investigated the uptake of microcystins in isolated hepatocytes and its consequences. Most frequently, the source of hepatocytes is rats but cells from any other species would be expected to respond to microcystin in qualitatively the same way. It has been known for more than 20 years that uptake in hepatocytes is carrier-mediated, dose-dependent and saturable and that it can be inhibited (Runnegar *et al.*, 1981; Eriksson *et al.*, 1990a). Other cells (even non-parenchymal liver cells) are much less sensitive to the toxicity of microcystin and require much higher doses and longer incubation times for toxicity (Runnegar *et al.*, 1995a). [This finding of preferential uptake of microcystins in

hepatocytes may explain why the liver is the primary target of its accumulation and toxicity.]

(iii) *In-vitro and in-vivo inhibition of uptake*

A number of earlier studies on the toxicity of microcystin and/or *Microcystis* described the partial protection of the co-administration of a number of compounds. Runnegar *et al.* (1981) showed that at least some of these compounds protect rat hepatocytes *in vitro*: sodium cholate, deoxycholate, rifampicin (rifamycin SV) and bromosulfophthalein all inhibited the cytoskeletal changes (blebbing of the cells) induced by microcystin. With the introduction of the use of radiolabelled microcystins, this protection in rat hepatocytes was shown to result from inhibition of uptake (Runnegar *et al.*, 1991, 1995b). Runnegar *et al.* (1999) found a similar pattern of protection in skate hepatocytes which indicated the related nature of transporters in the uptake of microcystins in these very divergent species.

Fischer *et al.* (2005) recently expressed the human OATP family that catalyses the uptake of the cell-impermeable microcystins in *Xenopus laevis* oocytes (Section 4.1.1). The activity of these transporters is inhibited by bile acids and dyes which concords with the findings in hepatocytes by Runnegar *et al.* (1981). These transporters are present in the brain, as well as the liver, and may mediate uptake of microcystins across the blood–brain barrier, although this has not yet been demonstrated experimentally. However, some data suggest the possible transfer of microcystins across the blood–brain barrier in mice (Falconer *et al.*, 1988; Maidana *et al.*, 2006).

Hermansky *et al.* (1990) showed that 25 mg/kg bw rifampin given intraperitoneally before 100 µg/kg bw microcystin-LR protected mice from microcystin-LR toxicity. Runnegar *et al.* (1993) confirmed this in mice dosed intraperitoneally with 84 µg/kg bw microcystin-YM. This dose, when injected alone, inhibited protein phosphatase (PP) activity and [^{125}I]microcystin-YM accumulated in the liver of mice within 15 min of treatment. By 30 min, the liver had significantly increased in weight because of haemorrhage. Intraperitoneal injection of the antibiotic rifamycin (5 µmol/mouse) 5 min before microcystin-YM prevented the uptake of [^{125}I]microcystin-YM, the inhibition of PP activity and toxicity (increase in liver weight). This protection was still seen 24 h after treatment. Injection of rifampin (Hermansky *et al.*, 1990) or rifamycin (Runnegar *et al.*, 1993) after treatment with microcystins did not protect against toxicity.

No studies have reported factors that may inhibit uptake of nodularin.

(b) Metabolism

The proposed pathway for excretion of both microcystins and nodularin is conjugation to the thiol of glutathione (GSH), which may be excreted as such or processed to the γ-glutamyl cysteine conjugate and finally to the cysteine conjugate and then excreted.

(i) *Microcystins*

The metabolic reactions of microcystins have been shown to occur enzymatically *in vitro* with cell extracts from many sources (see Pflugmacher *et al.*, 1998). Formation of a GSH conjugate is the most probable pathway *in vivo* in view of the observed changes in GSH peroxidase and GST activities following treatment with microcystins in mice and *in vitro* (Pflugmacher *et al.*, 1998; Gehringer *et al.,* 2004).

Only one report identifies microcystin–GSH and –cysteine adducts *in vivo* following treatment with microcystin. Kondo *et al.* (1996) identified an HPLC peak that corresponded to a microcystin–GSH standard (chemically synthesized) as a microcystin-RR–GSH adduct. Nucleophilic reaction of the thiol group of GSH with the α-β unsaturated carbonyl of the methyl dehydroalanine moiety of microcystin results in the microcystin–GSH adduct. *In vivo*, the reaction would be catalysed by liver GSTs. Three hours after treatment, this HPLC metabolite constituted a small, unspecified percentage of the dose in mice injected intraperitoneally with microcystin-RR (20 μg/mouse, equivalent to about 600 μg/kg bw). Another peak was identified as the microcystin-LR–cysteine conjugate in the liver cytosol of rats 24 h after injection with 4 μg microcystin-LR/rat. Experimentally, the authors stated that results were at the limit of detection in a concomitant liquid chromatography–mass spectrometry analysis. A number of other unidentified metabolites that were more hydrophilic than microcystin-RR were also formed as reported previously for microcystin-LR (Robinson *et al.*, 1991).

The major mechanism that explains the high toxicity of microcystins is the ability of these molecules to bind covalently to and inhibit the Ser/Thr protein phosphatases 1 and 2A (see below). The bond between microcystins and protein phosphatases is very stable and influences metabolism (Runnegar *et al.*, 1995c).

The metabolism of microcystins would result from a balance between the two types of reactions described above.

Microcystin-LR, -YR and -RR–GSH adducts have been shown to retain some toxicity *in vivo*. Microcystin-LR–GSH retained 6% of the toxicity of the parent compound when injected intravenously into mice, while microcystin-LR–cysteine retained 14% of the toxicity (Kondo *et al.*, 1992). This indicates that GSH and cysteine adducts might themselves inhibit PP activity. [However, the possibility that the adducts convert back in part to the native microcystin *in vivo* and that this native toxin is the cause of toxicity was not considered. Whether the GST can catalyse the conjugation of GSH to microcystin when it is associated (non-covalently) with PPs has not been investigated either. The inhibitory binding between most PP inhibitors, including microcystins, is very tight and renders the interaction kinetically nearly irreversible (Takai *et al*., 1995).]

[In addition to GSH conjugation, many other factors may influence metabolism. The methyl dehydroalanine moiety could react with cytochrome P450 enzymes to form more soluble metabolites that retain, lack or have increased toxicity. Other possibilities include glucoronidation and compartmentation within the cell that could lead to the sequestration of some of the toxin. Decreased or increased activity or membrane localization of uptake and/or export of microcystin and its metabolites that may result from changes in

phosphorylation could also impact metabolism. Even catabolism of PP could lead to increases in active toxin within the cell. Metabolism of microcystin by endogenous enzymes and/or intestinal flora is a further possibility.]

(ii) *Nodularin*

Nodularin readily forms adducts with GSH. As for microcystins, it has not yet been fully demonstrated that the enzymatically formed GSH conjugate pathway applies to the metabolism and detoxication of nodularin. GSTs catalyse the conjugation of nodularin at the *N*-methyl dehydrobutyric residue to the thiol of GSH. These adducts can then be excreted as such or processed to the γ-glutamyl cysteine conjugate and finally to the cysteine conjugate. To date, the only evidence of this pathway of nodularin metabolism has been in aquatic animals.

In-vitro assays using the substrate 1-chloro-2,4-dichlorobenzene (Beattie *et al.*, 2003) showed that extracts from cysts, nauplii and adult brine shrimp (*Artemia salina*) contained significant amounts of GST. This GST activity was inhibited when tissue extracts from nodularin-treated *A. salina* were added to the assay. The partially purified GSTs catalysed the formation of nodularin–GSH efficiently. Feeding a culture of algae that contained nodularin to Australian black tiger prawns resulted in a change in the classes and distribution of GST enzymes (Pflugmacher *et al.*, 2005)

Few studies have reported the occurrence of nodularin–GSH adducts in tissue from animals exposed to nodularin. Sipiä *et al.* (2002) showed by mass spectrometry the presence of adduct in mussels. This is the only report that identified nodularin–GSH in animals exposed to nodularin. [Although no quantitation was given, by comparison of peaks in the measurements of the soft tissue of mussels, it is possible to estimate that they represent 20–30% of the native nodularin.] Other, unidentified nodularin-like compounds have been found (Lehtonen *et al.*, 2003) in clams (*Macoma baltica*).

[On balance, the metabolism of microcystin and nodularin is probably, at least partially, catalysed by hepatic GSTs. Because of the varied specificities and activities of GSTs between species and between individuals, the consistency in the response to microcystin and nodularin is remarkable, and similar toxicities are found across species as diverse as fish, pigs and mice.]

(c) Excretion

There is some evidence of biliary excretion of microcystin and its metabolites (Sahin *et al.*, 1996). In trout orally dosed with *M. aeruginosa* bloom containing microcystin-LR (equivalent to 4.6 mg microcystin-LR), free microcystin or its metabolites that retained the ability to inhibit PP1 were found in the bile by the PP inhibition assay. Cumulative excretion was not reported, but maximal inhibition was exerted by two samples 3 h after gavage, the concentration that inhibited PP was equivalent to 3.5 mg/mL of bile; another sample contained 475 ng/mL 48 h after gavage. Enterohepatic circulation could lead to the cycling of microcystin and its metabolites between the bile, intestine and liver.

It is not clear what role biliary excretion would play in acute microcystin intoxication. Studies in perfused rat liver (Pace *et al.*, 1991; Runnegar *et al.*,1995a) showed that bile formation decreased shortly after exposure of the liver to a toxic dose of microcystin. Bile flow was reduced by 50% within 15 min of perfusion; the amount of bile produced between 45 and 60 min was less than 5% of that in control livers treated in exactly the same way and, by 60 min, it had stopped completely (Runnegar *et al.*, 1995a). Pace *et al.* (1991) calculated that, after 60 min of perfusion, 1.7% of [^{3}H]microcystin-LR was found in the bile while liver-associated radiolabel was 16.8% of the total. The cessation of bile flow paralleled liver damage. With non-toxic doses of microcystin, excretion through the bile may play a significant role.

No radioactivity was detected in the urine of pigs that had been dosed intravenously or into the ileal loop with [^{3}H]dihydromicrocystin-LR and killed 4–5 h after injection (Stotts *et al.*, 1997b).

Mice treated intravenously with 35 μg/kg bw [^{3}H]microcystin-LR had excreted 9.2% of the dose within 6 days; most of the 9.2% was excreted within 24 h of treatment (only 6% at 6 h) (Robinson *et al.*, 1991). In the same study, radioactivity in the faeces of mice was maximal 6 h after injection and cumulative faecal excretion after 6 days was 14.5% of the dose. The parent toxin constituted about 60% of the 23.7% of the dose excreted in faeces and urine. A number of other more hydrophilic peaks constituted the remaining 35–40%.

From the above results, it can be concluded that microcystins are primarily excreted in the faeces as the parent compound or as yet unidentified hydrophilic metabolites.

Orr *et al.* (2001) gave drinking-water containing *Microcystis* cultures to four lactating Holstein-Friesian dairy cattle. The cattle consumed a total of 15 mg (1.21 μg/kg bw per day) microcystin over a period of 21 or 28 days. The milk produced was analysed by ELISA and HPLC and no microcystin was detected (detection limit, 2 ng/L of milk).

No data were available on the excretion of nodularin. The presence or absence of nodularin or its metabolites has not been determined in faeces or urine after dosing.

4.2 Genetic and related effects

4.2.1 *Genotoxicity* (see Table 4.4 for details and references)

The genotoxic properties of microcystin-LR have been studied extensively in a variety of test systems and the results have been contradictory.

Microcystin-LR was not mutagenic in *Salmonella typhimurium* or in *Bacillus subtilis* in the multigene sporulation test in the presence or absence of exogenous metabolic activation.

No cell transformation was observed in microcystin-LR-exposed Syrian hamster embryo cells and no chromosomal aberrations were observed in Chinese hamster ovary K1 cells exposed to microcystin-LR, although an increased frequency of polyploid cells was reported, which suggests that it is aneugenic.

Table 4.4. Genetic and related effects of microcystins and nodularins

Test system	Result[a]		Dose[b] (LED/HID)	Reference
	Without exogenous metabolic system	With exogenous metabolic system		
Microcystin-LR				
Salmonella typhimurium TA98, TA100, reverse mutation	–	NT	10000	Grabow *et al*. (1982)
Salmonella typhimurium TA98, TA100,TA102, reverse mutation	–	–	900	Repavich *et al*. (1990)
Bacillus subtilis, multigene sporulation test	–	–	900	Repavich *et al*. (1990)
Chromosomal aberrations, Chinese hamster ovary-K1 cells *in vitro*	–	–	100	Lankoff *et al*. (2003)
Cell transformation, Syrian hamster embryo cells *in vitro*	–	NT	10	Wang *et al.* (1998)
Polyploidy, Chinese hamster ovary-K1 cells *in vitro*	+	+	25	Lankoff *et al*. (2003)
Comet assay, human lymphocytes *in vitro*	+[c]	NT	1	Lankoff *et al*. (2004)
Chromosomal aberrations, human lymphocytes *in vitro*	–	NT	25	Lankoff *et al.* (2004)
Chromosomal aberrations, human lymphocytes *in vitro*	–	NT	0.5	Lankoff *et al*. (2006a)
Nodularin				
Micronucleus assay, human hepatoma HepG2 cells *in vitro*	+	–	2.5	Lankoff *et al.* (2006b)

[a] +, positive; –, negative; NT, not tested
[b] LED, lowest effective dose; HID, highest ineffective dose; in-vitro tests, μg/mL
[c] Complementary results revealed negative clastogenic properties

Microcystin-LR gave positive results in the comet assay in human cells, which suggests that it may be genotoxic. However, complementary experiments with a modified comet assay in human hepatoma HepG2 cells revealed an accumulation of unrepaired DNA strand breaks which indicates that DNA fragmentation reflected the intermediates of the cellular repair of oxidized purines and pyrimidines resulting from the action of reactive oxygen species. This suggests that microcystin-LR might induce formation of reactive oxygen species that cause DNA damage rather than have a direct clastogenic effect (Zegura *et al.*, 2003, 2004). [Due to inappropriate experimental design and statistical evaluation of the data, and because HepG2 cells do not, or very weakly, express the transporters necessary for microcystin uptake (Le Vee *et al.*, 2006), the Working Group raised serious doubt about the validity of the findings reported by Zegura *et al.* (2003, 2004).] However, a comparison of the positive results from the comet assay, the negative results from the chromosomal aberration assay and the positive results for apoptosis in human lymphocytes (Fladmark *et al.*, 1999) showed that the microcystin-LR-induced DNA damage observed in the comet assay might be related to apoptosis due to cytotoxicity rather than to genotoxicity (Lankoff *et al.*, 2004, 2006a).

[The Working Group raised doubts about the validity of positive results for the genotoxicity of microcystin-LR (Suzuki *et al.*, 1998; Ding *et al.*, 1999; Mankiewicz *et al.*, 2002; Maatouk *et al.*, 2004; Zhan *et al.*, 2004; Bouaïcha *et al.*, 2005) due to inappropriate experimental design, statistical evaluation and interpretation of the data.]

The aneugenic effect of nodularin was tested in a single study that reported a dose-dependent induction of centromere-positive micronuclei in HepG2 cells.

In conclusion, no evidence was provided for the mutagenic or clastogenic properties of microcystins or nodularins in non-mammalian or mammalian test systems. However, an increased frequency of polyploid cells as well as centromere-positive micronuclei were observed, which possibly suggests that both microcystins and nodularin are aneugenic.

4.3 Mechanisms associated with the tumour promotion of microcystin-LR and nodularin

4.3.1 *Relationship between structure and inhibition of protein phosphatases (PP)*

Although the structures of microcystin-LR and nodularin are not related to that of okadaic acid (Fujiki & Suganuma, 1993), Uemura and Hirata (1989) proposed that the okadaic acid molecule also has a cyclic structure with the formation of a hydrogen bond between the carbonyl group at C1 and the hydroxyl group at C24, which may provide evidence that microcystin-LR and nodularin act similarly to okadaic acid as inhibitors of PP1 and PP2A (Yoshizawa *et al*., 1990; see also MacKintosh *et al.*, 1990; Honkanen *et al*., 1991). Microcystin-LR and nodularin inhibited PP activity in a cytosolic fraction of mouse liver *in vitro*, with an IC_{50} (concentration that leads to 50% inhibition) of 1.6 nM for microcystin-LR and 0.7 nM for nodularin. In addition, both inhibited specific [^{3}H]okadaic acid binding to PP1 and PP2A in the cytosolic fraction of mouse liver, with

an IC_{50} of 1.3 nM for microcystin-LR and 2.3 nM for nodularin, and in the particulate fraction of mouse liver, with an IC_{50} of 11.0 nM for microcystin-LR and 8.0 nM for nodularin (Yoshizawa *et al*., 1990). Microcystin-LR and nodularin both show a planarity of peptide rings and similar relative spatial alignments of the ADDA and arginine side chains (Taylor *et al*., 1992). Furthermore, Quinn *et al*. (1993) used molecular modeling and identified common regions of microcystin-LR, okadaic acid and calyculin A. Moreover, the crystal structure of mammalian PP1 complexed with microcystin was determined by X-ray crystallography (Goldberg *et al.*, 1995; Maynes *et al.*, 2006). Microcystin was reported to bind to a PP1 catalytic subunit through the interaction with three distinct regions: the metal-binding site, the hydrophobic groove and the edge of the C-terminal groove near the active site. In contrast to microcystin-LR, Bagu *et al*. (1997) reported that nodularin does not bind covalently to PP1 or PP2A, and that microcystin-LR and motuporin (nodularin-V) are strikingly similar to okadaic acid and calyculin A. Maynes *et al.* (2006) recently elucidated that the crystal structures of dihydromicrocystin-LR or motuporin (nodularin-V) binds to human PP1c (γ isoform). Comparisons of the structures of the toxin:PP1 complexes explain why microcystins but not nodularins permanently modify PP by covalent binding to an active cysteine residue.

4.3.2 *Enzymatic inhibition of PP1 and PP2A*

Microcystin-LR strongly inhibited PP1 and PP2A with inhibition constant values below 0.1 nM, but inhibited PP2B 1000 times less potently (MacKintosh *et al*., 1990); microcystin-LR inhibits both PP1 and PP2A with similar potency, whereas nodularin and okadaic acid inhibit PP2A much more strongly than PP1 (Table 4.5; Honkanen *et al.*, 1991; Suganuma *et al*., 1992). Seven inhibitors of PP1, including microcystin-LL, -LV, -LM, -LF and -LZ (Z represents an unknown hydrophobic amino acid), were purified from blooms of *Microcystis aeruginosa* and inhibited PP1 with inhibitory concentration 50% (IC_{50}) values of 0.06–0.4 nM (Craig *et al*., 1993). However, the structure and functional relationships between 6*(E)*-ADDA microcystin-LR and -RR and 6*(Z)*-ADDA microcystin-LR and -RR were significantly different: the IC_{50} values of 6*(E)*-ADDA microcystin-LR and -RR for PP2A activity were 0.28 and 0.78 nM, respectively, whereas those of 6*(Z)*-ADDA microcystin-LR and -RR were 80 nM in both cases, indicating that 6*(Z)*-ADDA microcystins inhibited PP2A about 100 times more weakly than the parent microcystin-LR and -RR compounds (Nishiwaki-Matsushima *et al*., 1991). Runnegar *et al*. (1995c) raised antibodies specific for PP1 and PP2A and found that, in hepatocytes, microcystin forms secondary covalent bonds with the C-terminal of PP1 and PP2A catalytic subunits. This covalent binding of microcystin to PP1 and PP2A catalytic subunits was confirmed by Bagu *et al*. (1997). Microcystin-LR has been shown to inhibit nuclear protein phosphatases (Guzman *et al.*, 2003) and to be present in the nuclei (Guzman & Solter, 2002) of mouse hepatocytes following in-vivo administration.

Table 4.5. Inhibition of the protein phosphatases (PP) 1 and 2A compared with that of protein tyrosine phosphatase (PTP)

Agent	PP1 (IC_{50} nM)	PP2A (IC_{50} nM)	PTP (IC_{50} nM)	Reference
Microcystin-LR	0.1	0.10	>10 000	Suganuma *et al.* (1992)
Nodularin	1.8	0.027	NR	Honkanen *et al.* (1991)
Okadaic acid	3.4	0.07	>10 000	Suganuma *et al.* (1992)

NR, not reported; IC_{50}, concentrations that lead to 50% inhibition

4.3.3 *Cellular effects*

The biochemical consequence of the inhibition of PP1 and PP2A by microcystin-LR and nodularin is the accumulation of hyperphosphorylation of intracellular proteins in hepatocytes (Eriksson *et al.*, 1990b; Falconer & Yeung, 1992; Fujiki, 1992). These alterations in phosphorylation have been shown to result in major morphological changes in isolated human and rodent hepatocytes exposed to low (nM) concentrations of microcystins. The major consequence observed is the disaggregation of intermediate filaments composed of cytokeratins 8 and 18, which leads to the collapse of the cytoskeleton of the hepatocytes. This disaggregation secondarily results in detachment of the actin microfilament structure and contraction of the fibres. The characteristic blebbing of exposed hepatocytes is the result of this microfilament contraction in cells (Runnegar *et al.*, 1981; Falconer & Runnegar, 1987; Eriksson *et al.*, 1989; Yoshizawa *et al.*, 1990; Falconer & Yeung, 1992; Ohta *et al.*, 1994; Toivola *et al.*, 1997; Batista *et al.*, 2003).

At concentrations up to 9.6 μM, microcystin-YR did not induce any effects in human fibroblasts; it only induced morphological changes similar to those induced by okadaic acid following microinjection of concentrations of 670 μM (Matsushima *et al.*, 1990). This suggests that microcystin and nodularin do not easily penetrate into fibroblasts, and supports the finding that microcystin requires active uptake through OATPs (see Section 4.1). Similarly, the uptake of [^{3}H]dihydromicrocystin-LR was shown to be specific for freshly isolated rat hepatocytes, since its uptake in the human hepatocarcinoma HepG2 cell line, the human neuroblastoma SH-SY5Y cell line and the mouse fibroblast NIH/3T3 cell line was negligible (Eriksson *et al.*, 1990a).

4.3.4 *Apoptosis*

A number of studies have demonstrated that microcystins and nodularins cause apoptosis in cell cultures. At picomolar concentrations, microcystin-LR stimulated cytokinesis in primary mouse hepatocytes and reduced the rate of apoptosis, whereas

higher concentrations (nanomolar) inhibited cytokinesis and induced cell death (Humpage & Falconer, 1999).

Fladmark *et al.* (1999) reported that both microcystin-LR and nodularin induced caspase-3-dependent apoptosis in an ultra rapid manner in toxin-microinjected Swiss mice 3T3 fibroblasts, rat promyelotic (IPC-81) cells, normal rat kidney cells and human embryo kidney HEK 293 cells. It was also proposed that apoptosis induced by microcystins and nodularins correlates with PP inhibition and requires Ca^{2+}/calmodulin-dependent protein kinase II (Fladmark *et al.*, 2002).

The induction of apoptosis was also shown *in vitro* in microcystin-LR-treated rat hepatocytes and human lymphocytes (Mankiewicz *et al.*, 2001), Chinese hamster ovary K1 cells (Lankoff *et al.*, 2003) and colon carcinoma CaCo2 cells (Botha *et al.*, 2004) as well as in nodularin-treated primary rat hepatocytes (Herfindal *et al.*, 2005).

4.3.5 *Gene expression*

Sueoka *et al.* (1997) demonstrated that microcystin-LR and nodularin modulate the expression of oncogenes and tumour-suppressor genes and revealed a strong induction of tumour necrosis factor-α, and *c-jun*, *jun B, jun D*, *c-fos*, *fos B* and *fra-1* gene expression in primary cultured rat hepatocytes *in vitro*.

A two-stage carcinogenesis experiment that used initiation with NDEA and promotion with nodularin in Fischer 344 rats increased the incidence of liver preneoplastic foci. Increased transforming growth factor-β1 protein expression was seen to co-localize with GST-P expression in the preneoplastic foci (Lim *et al.*, 1999).

In summary, a number of studies have reported the involvement of microcystin-LR in epigenetic processes. These include stimulation of gene expression, cell survival/apoptosis and cell division. There is also evidence of the inhibitory effects of microcystin-LR on DNA repair (Lankoff *et al.*, 2004, 2006a). It was also found that cytokinesis was stimulated and the rate of cell division was increased by picomolar concentrations of microcystin-LR in primary mouse hepatocytes whereas nanomolar concentrations inhibited cytokinesis and induced apoptosis (Humpage & Falconer, 1999). These findings were supported by a combined transcriptomic and proteomic analysis with gene expression profiling in the liver of mice treated with microcystin-LR which demonstrated a modification of 61 of 96 apoptosis-related genes. At low concentrations, microcystin-LR increased the expression of the anti-apoptotic *Bcl-2* gene more that 4000-fold. At high concentrations of microcystin-LR, expression of the *Bcl-2* gene dropped markedly (Chen *et al.*, 2005).

4.4 Other relevant toxic effects

4.4.1 *Humans*

(a) Acute toxicity

The most relevant example of acute toxicity from cyanobacterial toxins was provided by exposure through renal dialysis. In Caruaru, Brazil, following renal dialysis during a single week in 1996, 116 of 131 dialysed patients developed disturbance of vision, vomiting, nausea, headache, muscle weakness and epigastric pain. Of these, 100 developed liver failure and 76 died. The deaths of 52 patients could be attributed directly to liver failure. Serum enzyme analysis showed an eightfold increase in aspartate transaminase (AST) and a fourfold increase in total bilirubin in patients with clinical symptoms (Jochimsen *et al*., 1998; Pouria *et al*., 1998; Carmichael *et al*., 2001). Examination of the cation filters in the dialysis unit showed the presence of microcystins-YR, -LR and -AR. The carbon filters also contained cylindrospermopsin; this compound is an alkaloid toxin from freshwater cyanobacteria, which is also responsible for human poisoning that results predominantly in liver damage, but through a different mechanism (Hawkins *et al*., 1985). Microcystins were present in the blood of patients at an average level of 2.2 ng/mL and in the liver at an average level of 223 ng/g. It was calculated that 19.5 μg/L microcystins were present in the dialysis water, and that the patients were probably exposed to 120–150 L (Carmichael *et al*., 2001).

Another report of human injury related to acute exposure to *M. aeruginosa* was among army recruits who carried out canoeing exercises in a lake that carried a heavy water-bloom of this cyanobacterium (Turner *et al*., 1990). The exercises involved swimming with a pack and also going underwater and coming back up while canoeing, both of which probably resulted in the inhalation and oral ingestion of water. Ten of the recruits reported symptoms of abdominal pain, nausea, vomiting, diarrhoea, sore throat, dry cough, blistering of the lips and mouth and headache. Two were hospitalized with pneumonia that was considered to be due to aspiration of bloom material. Serum enzymes indicative of liver damage, alanine transaminase (ALT) and aspartate transaminase (AST), were elevated in the most severe of the two cases investigated. Microcystin-LR was identified in the cyanobacterial bloom material from the lake (Turner *et al.*, 1990).

No data on nodularin were available to the Working Group.

(b) Subacute toxicity

Among the causes of human illness that have been associated with cyanotoxins in drinking-water sources, the following three show plausibility.

In Harare, Zimbabwe, a paediatrician noted that children whose homes were supplied with water from one reservoir developed seasonal acute gastroenteritis in the autumn, whereas children whose drinking-water came from other municipal reservoirs were unaffected. The reservoir that was the source of the water for the affected children had developed a summer water-bloom of *Microcystis*, which broke down in the early winter

at the time of the gastroenteritis. The paediatrician concluded that the lysed cyanobacteria in the water supply could be responsible for the illness (Zilberg, 1966).

A larger and more severe outbreak of gastroenteritis in children occurred in Brazil after a new hydroelectric dam was filled and was the source of drinking-water for several towns, including Paulo Alfonso that had a population of about 200 000 (Teixeira *et al*., 1993). Overall, approximately 2000 cases were recorded at the hospital, and 88 deaths resulted. Very high concentrations of cyanobacterial cells were detected in the water at the intakes of the drinking-water treatment plant, including *Microcystis.* The authors considered cyanobacterial toxins to be the probable cause of the outbreak, especially as patients who used only boiled water were among those affected.

A less damaging but more closely investigated occurrence of human injury from microcystins was reported in Australia in 1983 (Falconer *et al*., 1983). The laboratory in Armidale, New South Wales, that monitored reservoirs of drinking-water supplies for cyanobacterial blooms and regional cases of cyanobacterial poisoning of livestock observed a water-bloom of highly toxic *M. aeruginosa* growing in the city drinking-water reservoir. The water offtake for the treatment plant was located in a narrow bay that accumulated wind-drift of cyanobacterial scums. The drinking-water at Armidale was the subject of complaints of off-flavours and odours caused by *Microcystis*, to which the operating authority responded by applying copper sulfate to the reservoir by air. This caused rapid lysis of the cyanobacteria, and resulted in the liberation of free toxin into the water. A retrospective cohort study of serum indicators of liver damage was carried out. The data on liver enzymes from the regional pathology laboratory over a period of 18 weeks were sorted into analyses of samples taken during the 6 weeks of bloom development and termination by treatment with copper sulfate, the 6 weeks before that time and the 6 weeks after. Significantly increased levels of serum γ-glutamyl transferase were observed in the samples collected from the population who had drunk water from the affected reservoir only in the period when *Microcystis* was present and lysed. No increase was observed in the cohort population or during the periods before and after the water-bloom in either population. A smaller increase which was not statistically significant was seen in ALT, but no increase was seen in AST or alkaline phosphatase. While the average increase in γ-glutamyl transferase activity was approximately twofold, some samples showed a considerably larger increase. Serum enzyme activities had returned to normal by 6 weeks after the bloom. This evidence of liver damage was attributed to microcystins in the drinking-water supply. Unfortunately, analytical techniques that were sensitive enough to measure microcystins in drinking-water had not been developed at that time, so no actual exposure data were available to allow a dose–response determination.

(c) Immunotoxicity

No data were available to the Working Group.

4.4.2 *Experimental systems and natural exposure*

(a) Acute and subacute toxicity

Most of the structural variants of microcystins and nodularin are highly toxic in mice within a comparatively narrow dose range: LD_{50} following intraperitoneal injection were largely in the range of 50–300 μg/kg bw (Sivonen & Jones, 1999). Only a few non-toxic variants have been identified. In general, any structural modifications to the ADDA-glutamate region of the toxin molecule, such as a change in isomerization of the ADDA-diene (6*(E)* to 6*(Z)*) or acylation of the glutamate, renders microcystins and nodularin non-toxic. Linear microcystins and nodularin are more than 100 times less toxic than the equivalent cyclic compounds. Linear microcystins are thought to be microcystin precursors and/or bacterial breakdown products (Sivonen & Jones, 1999).

Microcystins and nodularin are primarily hepatotoxins. After acute or subacute exposure by intravenous or intraperitoneal injection of microcystins in mice or pigs, severe liver damage is characterized by a disruption of liver cell structure (due to damage to the cytoskeleton), a loss of sinusoidal structure, increases in liver weight due to intrahepatic haemorrhage, haemodynamic shock, heart failure and death. Other organs affected are the kidneys, lungs and intestines (for an extensive review of microcystin toxicity, see Sivonen & Jones, 1999).

(b) Chronic toxicity

Swiss albino mice were exposed to microcystin in the drinking-water for 1 year or longer in two studies (Falconer *et al*., 1988; Ueno *et al*., 1999). In one study, female BALB/c mice were exposed continuously to 20 μg/L microcystin-LR in the drinking-water for 18 months (mean cumulative toxin intake estimated at 35 μg/mouse for 18 months). No adverse effects of treatment were recorded, including no clinical changes, no observed liver histopathology or dysfunction and no liver tumours (Ueno *et al*., 1999).

In the other study, an extract of *M. aeruginosa* was administered in the drinking-water at six dose levels to male and female Swiss albino mice. Animals were killed at intervals up to 1 year. The highest dose administered was 56.6 μg/mL (approximately equivalent to 10 mg/kg bw per day), lower doses were 1/2, 1/4, 1/8 and 1/16 dilutions of this dose. Only the highest dose resulted in a reduced growth rate in both sexes of mice. Male mice were more adversely affected than females; at the 1/2 dilution, livers were significantly heavier in males, with elevated ALT levels, whereas female livers were heavier and serum contained significantly increased ALT only with the undiluted extract. Mortality clearly increased with dose throughout the range. Histopathological examination of the livers showed chronic active liver injury with hepatocyte necrosis, leukocyte infiltration and fibrosis in livers from mice receiving undiluted and 1/2 diluted extract. At lower concentrations of toxin, increased hepatic infiltration with neutrophils was seen. Histopathological examination of other tissues showed a significantly increased incidence of bronchopneumonia with age and dose, and a small number of cases of kidney damage at 1/4 and 1/8 dilutions from 31 weeks onwards (Falconer *et al.*, 1988).

In rats, daily intakes of lower doses of approximately 50 and 150 μg/kg bw pure microcystin-LR administered in the drinking-water for 28 days also led to increased liver weight, altered enzyme activities and histological injury to the liver (Heinze, 1999).

Intraperitoneal injections of microcystin-LR and -YR at a concentration of 10 μg/kg bw every other day for 8 months increased the numbers of TUNEL-positive cells in the cortex and medula of the kidney in rats (Milutinović *et al*., 2003).

It is apparent that microcystin-LR is predominantly hepatotoxic, whether administered acutely, subchronically or chronically, in all species investigated. However, toxic events due to consumption of or exposure to *M. aeruginosa* have been recorded in human and large animal populations with a wider range of clinical symptoms than hepatic injury alone, some of which are probably secondary to liver damage. It is also possible that poisoning by naturally occurring cyanobacterial water blooms leads to exposure to a range of toxic compounds, including several other microcystins and other cyanobacterial toxins with different toxic potentials.

No studies on the chronic toxicity of nodularin were available to the Working Group.

(c) Immunotoxicity

Only limited effects of microcystin or nodularin on the immune system of whole animals have been recorded. In rats administered microcystin-LR orally for 28 days, the number of leukocytes and lymphocytes in blood increased significantly (Heinze, 1999).

In a study of mice exposed to extracts of *M. aeruginosa* in the drinking-water for up to 1 year, the increased mortality could largely be attributed to pneumonia, not to liver dysfunction, which may reflect impaired immune function (Falconer *et al*., 1988).

In a more recent study, mice were injected intraperitoneally with a *M. aeruginosa* extract for 14 days and changes in the immune system were observed (Shen *et al*., 2003). At the highest dose (20 μg microcystin equivalents/kg bw), both spleen and thymus weights were decreased. Inhibition of lipopolysaccharide-induced B lymphocyte proliferation and a dose-dependent decrease in antibody-forming cells in mice that were immunized by sheep red blood cells were seen. [It should be noted that intraperitoneal injection of extracts may cause responses to other compounds, e.g. lipopolysaccharide endotoxins.] The proliferation of T cells following concanavalin A stimulation was not affected by treatment.

Nodularin administered intraperitoneally to mice decreased humoral immune responses to sheep red blood cells; the effect was heightened in animals given rifampicin to inhibit nodularin uptake into the liver (Yea *et al*., 2001).

Similar results were reported in an in-vitro study of mouse splenocytes, which demonstrated inhibition of lipopolysaccharide-stimulated lymphoproliferation in response to exposure to microcystin-LR and -YR and nodularin at concentrations of 10–50 μM. Concanavalin A-stimulated lymphoproliferation was suppressed by microcystin-YR and nodularin but not by microcystin-LR (Yea *et al*., 2001). [These concentrations of toxin in an in-vitro incubation are probably 10^3–10^4 times higher than those that occur in the blood during acute toxicity *in vivo*.]

A range of other in-vitro assessments of microcystin activity on immune cells have been undertaken, largely with the aim of clarifying the mechanism of toxicity. Macrophages incubated with microcystin-LR at approximately 100 nM (~0.1 μg/Ml) have been shown to respond by synthesis of tumour necrosis factor-α and interleukin-1β. These factors resulted in stimulation of intestinal secretory activity *in vitro* of rabbit ileum by supernatants from the incubation (Rocha *et al*., 2000).

Substrate adherence by polymorphonuclear leukocytes is an essential first step in the response to inflammation and phagocytosis of bacterial pathogens. Very low concentrations of microcystin-LR and nodularin (0.01–1.0 nM; ~0.01–1ng/mL) *in vitro* enhanced the early spontaneous adherence of polymorphonuclear leukocytes, but had no effect on late adherence or adherence of cells already stimulated by the peptide formyl-methionyl-leucyl-phenylalanine (Hernandez *et al*., 2000).

These limited results on immunomodulation by microcystin indicate that lower doses or concentrations may enhance immune response in some systems, but that higher doses are probably toxic, particularly to B lymphocytes.

4.5 References

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5. Summary of Data Reported

5.1 Exposure data

Microcystins and nodularin are cyclic peptide toxins that have a ring structure of seven and five amino acids, respectively, which comprise one unique phenyl deca-dienoic acid, four invariable D-amino acids and, in microcystins, two variable L-amino acids. There are approximately 70 variants of microcystin and several variants of nodularin. These peptides are produced naturally by cyanobacteria, an evolutionarily very ancient group of photosynthetic prokaryotic organisms. The bacteria occur in filamentous and coccoid forms (blooms) in free suspension in water or form layers (scums) on surfaces; they are distributed worldwide in water and soils, and on rock and plant surfaces.

Microcystin-LR (lysine-arginine) is the most extensively investigated cyanobacterial peptide toxin because it is frequently present in blooms in rivers and lakes. Nodularin occurs primarily in brackish waters. The peptide toxins are contained primarily within the cyanobacterial cells and are rarely released before the cells die. The concentration of toxin in water therefore depends on the cell content of microcystins or nodularin and the concentration of cells in the water. Due to widespread eutrophication in many settings, these toxins can occur at unnaturally high frequency and concentration. In natural water bodies and in water storage reservoirs, the concentrations of toxin vary widely from undetectable to several milligrams per litre in cyanobacterial scums.

A number of analytical techniques are used for the quantitation of cyanobacterial peptide toxins, and include high-performance liquid chromatography, liquid chromatography–mass spectrometry with a number of sophistications, enzyme inhibition assays and the enzyme-linked immunosorbent assay. Genetic probes are also available for the detection of the genes involved in the synthesis of these toxins.

Human exposure to these toxins occurs most frequently through the ingestion of water, i.e. through drinking or during recreational activities in which water is swallowed. Furthermore, cyanobacterial dietary supplements (blue-green algae supplements) are now on sale, and consumption of these toxins occurs from this source. Marine products such as fish, shellfish and crustaceans also accumulate nodularin, which remains stable and unchanged during cooking; this leads to their ingestion by humans.

WHO has adopted a provisonal guideline value for drinking-water supplies of 1 μg/L microcystin-LR, based on its subacute toxicity in mice. The toxicity of this microcystin variant is representative of that of other variants and of nodularin, and therefore provides a reasonable approximation of the toxicity of naturally occurring mixtures of these variants in water bodies. Many European countries and countries such as Australia, Brazil and Canada have adopted similar guideline values for microcystins, some of which are based on their total concentration in water samples.

Guidelines for recreational exposure have also been proposed by WHO; they are derived from the drinking-water guideline but are related to the concentrations of cyanobacterial cells found in water. They have also been implemented internationally, and include the emission of warnings at or the closure of recreational water sites when the specified levels of cyanobacterial contamination are reached.

5.2 Human carcinogenicity data

The Working Group reviewed several reports on ecological, cohort and case–control studies of the risk for hepatocellular carcinoma and source of drinking-water, some of which contained information on concentrations of microcystins in the water source. As access to the original publications was limited, the Working Group relied in many instances on summary information in review articles that lacked detailed descriptions of study methods and results. The studies of hepatocellular carcinoma included several ecological studies (12 from a review), two cohort studies (one in the review) and several case–control analyses (one meta-analysis, one pooled analysis and one additional case–control study). All studies conducted in the area of Southeast China that is endemic for hepatocellular carcinoma showed a positive association between the risk for hepatocellular carcinoma and water source; surface waters (pond, ditch or river waters) were associated with higher risks in contrast with either shallow or deep wells. Exposure assessment was limited in all studies to the use of these categorical measures. The few studies that reported concentrations of microcystins indicated that levels were much higher in surface waters than in well waters, but no study estimated the level of microcystins on an individual basis. In an analysis of pooled data from six case–control studies, the relative risk was 1.59; estimates of relative risk from other studies were generally in the range of 1.5–4, which raised the possibility that the observations were a consequence of confounding factors. Exposure to aflatoxin was not generally considered, and other contaminants or organisms in surface waters or factors related to water source were not evaluated. Some studies controlled for hepatitis B viral antigen or a history of hepatitis, which decreased the likelihood of confounding from this strong risk factor. An ecological study of colorectal cancer showed an association with concentration of microcystins, but confounding by other factors that are common to surface water sources in which the levels of microcystins were highest could not be ruled out. In summary, although many studies of hepatocellular carcinoma and one study of colorectal cancer found intriguing, positive associations with consumption of surface waters, in light of the quality of the published material available to the Working Group, it was not possible to associate the excess risk specifically with exposure to microcystin.

5.3 Animal carcinogenicity data

In one limited study in male mice, repeated intraperitoneal injections of microcystin-LR induced liver foci, which were probably benign tumours.

In three experiments in male rats that were initiated with *N*-nitrosodiethylamine and one experiment in male rats that were initiated with aflatoxin B_1, multiple intraperitoneal injections of microcystin-LR increased the incidence in the liver of glutathione *S*-transferase placental form-positive foci, which are considered to be preneoplastic lesions.

In one experiment in female mice that were initiated by skin application of 7,12-dimethylbenz[*a*]anthracene, *Microcystis* extracts given in the drinking-water increased the weight of skin papillomas per mouse.

One study in male mice that were initiated with *N*-methylnitrosourea and given *Microcystis* extracts in the drinking-water gave negative results.

In one experiment in male mice that were initiated by intraperitoneal injection of azoxymethane, exposure to *Mycrocystis* extracts in the drinking-water resulted in an increase in the area of aberrant crypt foci in the colon.

In two studies in male rats that were initiated with *N*-nitrosodiethylamine, multiple intraperitoneal injections of nodularin increased the incidence of glutathione *S*-transferase placental form-positive foci in the liver.

5.4 Other relevant data

Studies on the distribution of microcystins and nodularin have been carried out after intravenous and intraperitoneal administration to mice, rats and pigs of ^{125}I-, ^{14}C- or ^{3}H-labelled microcystins or nodularin. Kinetic studies showed rapid distribution into the liver and low accumulation in other tissues.

The cyanobacterial toxin microcystin does not permeate into cells. It is hepatotoxic because hepatocytes express transporters, which are organic anion-transporting polypeptides that permit the uptake of the toxin. A number of chemicals are known to compete for these transporters and inhibit the uptake of microcystins. In-vitro studies in hepatocytes have shown competitive inhibition of microcystin uptake by endogenous transporter substrates (e.g. bile acids) and xenobiotics (e.g. antibiotics).

The toxic action of microcystins is a consequence of their profound inhibition of cellular Ser/Thr protein phosphatases, which results in altered phosphorylation homeostasis. This impacts cell functions and structures that are controlled by changes in phosphorylation. The cellular metabolism of microcystins has not been elucidated but probably involves, at least in part, glutathione conjugation. Some data suggest that the conjugation of microcystins and possibly nodularins by glutathione occurs. It has been proposed, but not shown, that glutathione- and cysteine- adducts are excreted either in bile or back into the circulation together with native microcystin. No evidence was available of the involvement of cytochrome P450 in the detoxification of microcystins or nodularin.

Excretion of microcystins occurs primarily in faeces. A number of studies have shown that renal excretion, when it occurs, clears insignificant amounts of microcystins.

A common mechanism in the toxicity of microcystins and nodularin is the specific inhibition of Ser/Thr protein phosphatases 1 and 2A at picomolar concentrations in the cytoplasm and the nucleus *in vitro*. This inhibition results in hyperphosphorylation of

intracellular proteins, which is shown by the rapid disaggregation of intermediate filaments (cytokeratins) that form the cellular scaffold in human and rodent hepatocytes. Microfilaments become detached from the cytoplasmic membrane, which results in cell cytoskeletal deformation and bleb formation. Cell lysis and apoptosis follow, depending on the dose. Death results from dissolution of the liver structure and intrahepatic pooling of blood, which lead to overall haemorrhagic shock. Doses that are not immediately lethal can result in death from liver failure in large animals and humans several months after the initial exposure to microcystin.

The acute toxicity of microcystins in humans was shown unequivocally in the intoxication of haemodialysis patients in Caruaru, Brazil, who were exposed to microcystins in the dialysis water; this resulted in the death of more than 50 patients. Significant amounts of microcystin were detected in the livers and sera of these patients, and several incidents of hepatic disease and/or gastroenteritis have been reported after subacute intoxication with microcystins.

The toxicity of microcystins and nodularin has been described in rodents after intraperitoneal administration. The main injury was to the liver. Similar toxicity was also demonstrated in sheep and pigs in which hepatic damage was sustained for long periods after exposure.

Administration of cyanobacterial extracts that contain microcystins in the drinking-water to mice over a period of 1 year leads to liver damage. There are indications that chronic administration of microcystins results in immunotoxicity.

Nodularin has been studied less extensively than microcystin.

The mechanism(s) associated with the suspected carcinogenic activity of microcystins and nodularin is the enzymatic inhibition of protein phosphatases, which leads to downstream hyperphosphorylation of intracellular proteins.

There is no clear evidence that microcystins or nodularin are mutagenic or clastogenic in non-mammalian, mammalian or human cell systems. However, other mechanistic data indicate that both toxins are involved in epigenetic processes such as the modulation of oncogene and tumour-supressor gene expression, cell survival and/or apoptosis and the inhibition of DNA repair. In addition, an increased frequency of polyploid cells and centromere-positive micronuclei was observed, which suggests that both microcystins and nodularin are possibly aneugenic.

6. Evaluation and Rationale

There is *inadequate evidence* in humans for the carcinogenicity of microcystin-LR.

There is *inadequate evidence* in humans for the carcinogenicity of nodularin.

There is *inadequate evidence* in experimental animals for the carcinogenicity of microcystin-LR.

There is *inadequate evidence* in experimental animals for the carcinogenicity of *Microcystis* extracts.

There is *inadequate evidence* in experimental animals for the carcinogenicity of nodularins.

Overall evaluation

Microcystin-LR is *possibly carcinogenic to humans (Group 2B)*.

In three experiments in rats, Microcystin-LR promoted preneoplastic lesions of the liver. In a study in mice, microcystins promoted preneoplastic foci in the colon and a limited subchronic study with microcystin-LR resulted in persistent neoplastic nodules in mouse liver.

Strong evidence supports a plausible tumour promoter mechanism for these liver toxins. This mechanism is mediated by the inhibition of protein phosphatases 1 and 2A, an effect observed in rodents as well as in primary hepatocytes *in vitro*. The resulting hyperphosphorylation of intracellular protein leads to disruption of intermediate filaments that form the cellular scaffold in human and rodent hepatocytes. These toxins modulate the expression of oncogenes, early-response genes and of the cytokine, tumour necrosis factor α, and affect cell division, cell survival and apoptosis.

Microcystis extracts are *not classifiable as to their carcinogenicity to humans (Group 3)*.

Nodularins are *not classifiable as to their carcinogenicity to humans (Group 3)*.

LIST OF ABBREVIATIONS

(* in tables only)

AAF	acetylaminefluorene
ADC *	adenocarcinoma
ADDA	β-amino acid, 3-amino-9-methoxy-2,6,8-trimethyl-10-phenyldeca-4(*E*),6(*E*)-dienoic acid
ADI	accepted daily intake
ala *	alanyl
ALT	alanine transaminase
arg *	arginyl
asp *	aspartyl
AST	aspartate transaminase
BBNA	*N*-butyl-*N*-(4-hydroxybutyl)nitrosamine
BNU	1-butyl-1-nitrosourea
bw	body weight
CAS	Chemical Abstracts Service
CD *	conductivity detection
CE *	cation exchanger
CFA *	continuous flow analysis
CI	confidence interval
CIE *	capillary ion electrophoresis
CSEC *	chemical suppression of eluant conductivity
DBA	dibutylamine
DCD *	direct conductivity detection
DDAO	*N*,*N*-dimethyldodecylamine-*N*-oxide
DHPA	bis(2-hydroxypropyl)amine
DMA *	dimethylamine
DMBA	7,12-dimethylbenz[*a*]anthracene
dw *	drinking-water
ELISA	enzyme-linked immunosorbent assay
ENU	*N*-ethyl-*N*-nitrosourea
EPIC	European Prospective Investigation into Cancer and Nutrition
ETU	ethylenethiourea

FAO	Food and Agricultural Organization
FCT *	food composition table
FFQ *	food-frequency questionnaire
FIA *	flow injection analysis
glu *	glutamyl
GSH	glutathione
GST-P	glutathione *S*-transferase form-positive
H_2O_2 *	hydrogen peroxide
HCC	hepatocellular carcinoma
HID *	highest ineffective dose
HPLC	high-performance liquid chromatography
HPRT and *hprt*	hypoxanthine–guanine phosphoribosyltransferase gene
HTHQ	1-*O*-hexyl-2,3,5-trimethylhydroquinone
IC_{50}	concentration that leads to 50% inhibition
ICD	International Classification of Diseases
IEC *	ion-exchange chromatography
ig *	intragastric
ip *	intraperitoneal
IQ	2-amino-3-methylimidazo[4,5-*f*]quinoline
iv *	intravenous
k	rate constant
L	leucine
LC *	liquid chromatography
LD_{50}	dose that is lethal to 50% of animals
LED *	lowest effective dose
leu *	leucyl
MALDI-TOF*	matrix-assisted laser desorption/ionization time-of-flight
max. *	maximum
MC *	microcystin
Mdhb	2-(methylamino)-2-dehydrobutyric acid
Me *	methyl
met *	methionine
min. *	minimum
MNNG	*N*-methyl-*N*′-nitro-*N*-nitrosoguanidine
MNU	*N*-methylnitrosourea
MS *	mass spectrometry
$NaBH_4$ *	sodium borohydrate
$NaHCO_3$ *	sodium bicarbonate
NCI SEER *	National Cancer Institute Survey of Epidemiology End Results
ND *	not detected
NDEA	*N*-nitrosodiethylamine

NDMA	*N*-nitrosodimethylamine
NDPHA	*N*-nitroso-bis(2-hydroxypropyl)amine
NIE *	nitrate ion electrode
NMOR	*N*-nitrosomorpholine
NO_2^- *	nitrite ion
NO_2^--N *	nitrite nitrogen
NO_3^- *	nitrate ion
NO_3^--N *	nitrate nitrogen
NOD *	nodularin
NPC	nasopharyngeal carcinoma
NPRO	*N*-nitrosoproline
NPYR	*N*-nitrosopyrrolidine
NR *	not reported
NS *	not significant
NT *	not tested
NTCA	*N*-nitrosothiazolidine-4-carboxylic acid
NTP	National Toxicology Program
OATP	organic ion transporter polypeptide
PhIP	2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine
PP	protein phosphatase
PTP *	protein tyrosine phosphatase
Q *	quantile
R	argenine
SCC *	squamous-cell carcinoma
SCD *	specific conductivity detection
SE	standard error
SFA *	segmented flow analysis
SIR	standardized incidence ratio
SMR	standardized mortality ratio
T *	tertile
TBHQ	*tert*-butylhydroquinone
TDI	tolerated daily intake
tyr *	tyrosine
USDA *	US Department of Agriculture
UVD *	ultraviolet detection
UVS *	ultraviolet spectrophotometry
vit– *	without vitamin C supplement
vit C *	vitamin C
vit+ *	with vitamin C supplement
vol. *	volume

CUMULATIVE CROSS INDEX TO *IARC MONOGRAPHS ON THE EVALUATION OF CARCINOGENIC RISKS TO HUMANS*

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List of IARC Monographs on the Evaluation of Carcinogenic Risks to Humans

Volume 1
Some Inorganic Substances, Chlorinated Hydrocarbons, Aromatic Amines, *N*-Nitroso Compounds, and Natural Products
1972; 184 pages (out-of-print)

Volume 2
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1973; 181 pages (out-of-print)

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Certain Polycyclic Aromatic Hydrocarbons and Heterocyclic Compounds
1973; 271 pages (out-of-print)

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Some Organochlorine Pesticides
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