

Molecular approaches to diagnosing nutritional physiology in harmful algae: Implications for studying the effects of eutrophication

Sonya T. Dyhrman*

Biology Department MS#33, Woods Hole Oceanographic Institution, Woods Hole, MA 02543, United States

ARTICLE INFO

Article history:

Received 1 January 2007

Received in revised form 19 August 2007

Accepted 1 August 2008

Keywords:

Eutrophication

Genomics

Harmful algae

Nitrogen

Phosphorus

Proteomics

ABSTRACT

Every year harmful algal blooms (HABs) cause serious impacts to local economies, coastal ecosystems, and human health on a global scale. It is well known that nutrient availability can influence important aspects of harmful algae biology and ecology, such as growth, toxin production, and life cycle stage, as well as bloom initiation, persistence and decline. Increases in the rate of supply of organic matter to ecosystems (eutrophication) carries many possible ramifications to coastal systems, including the potential for nutrient enrichment and the potential for stimulation of harmful algal blooms. Traditional studies on algal nutrition typically use either cultured isolates or community level assays, to examine nutrient uptake, nutrient preference, elemental composition, and other metrics of a species' response to nutrients. In the last decade, technological advances have led to a great increase in the number of sequences available for critical harmful species. This, in turn, has led to new insights with regards to algal nutrition, and these advances highlight the promise of molecular technologies, and genomic approaches, to improving our understanding of algal nutrient acquisition and nutritional physiological ecology, in both cultures and field populations. With these developments increased monitoring of nutritional physiology in field populations of harmful algae will allow us to better discriminate how eutrophication impacts these groups.

© 2008 Elsevier B.V. All rights reserved.

1. Introduction

Eutrophication, or increases in the rate of supply of organic matter to an ecosystem (Nixon, 1995), carries many possible ramifications to coastal systems, including the potential for nutrient enrichment or the stimulation of harmful algal blooms (HABs). The relationship between HABs and coastal nutrient inputs, such as those resulting from eutrophication, is of particular concern. This relationship has been examined in numerous studies (Anderson et al., 2002, and references therein) but it is difficult to address, because the complexity of nutrient loading is matched by the complexity of nutritional strategies and capabilities within the phytoplankton community. In short, predicting what nutrients are fueling the growth of HABs, and how changes in nutrient type, concentration, and delivery, may influence HAB formation in different systems, remains a major ongoing source of study. There is no single straightforward way to ascertain the linkages between nutrient supply change and HABs, however, new advances in our molecular-level understanding of nutrient acquisition and meta-

bolism in key HAB species may help identify which pathways of nutrient acquisition are active, and where. These advances highlight the promise of molecular technologies and genomic approaches to examining the nutritional physiological ecology of HAB species and their responses to changes in nutrient composition and supply.

As highlighted above, the relationship between HABs and eutrophication is complex. HABs may be influenced by diverse nutrient assimilation strategies, nutrient supply, nutrient supply ratios, nutrient form, and community composition, among other variables (Anderson et al., 2002). For example, multiple forms of nitrogen (nitrate, ammonium, urea, amino acids, and humic substances) have all been shown to support the growth of toxic dinoflagellates or other HAB groups (Antia et al., 1991; Berg et al., 1997; Doblin et al., 1999; John and Flynn, 1999; Kudela and Cochlan, 2000; Dyhrman and Anderson, 2003). For dinoflagellates, nitrogen (N) source (e.g. nitrate versus urea) and N status (e.g. replete versus starved) can have important effects on toxin composition (reviewed in Poulton, 2001), and life history stage (reviewed in Pfister and Anderson, 1987). There are a number of proteins potentially involved in cellular N assimilation, such as urease, nitrate reductase, nitrite reductase, glutamine synthetase, amino acid oxidase, and nitrate transporters, among others. In

* Tel.: +1 508 289 3608.

E-mail address: sdyrman@whoi.edu.

many cases these proteins can be regulated by N source and N status (Antia et al., 1991; Hildebrand and Dahlin, 2000; Zehr and Ward, 2002). In other algae there are a number of N assimilatory proteins that have been identified and for which there is sequence information (e.g. glutamine synthetase; Robertson et al., 2001), but these proteins and pathways have not been sufficiently characterized in HAB model taxa.

Phosphorus (P) is another essential algal nutrient, and the concentration and composition of P may influence HAB dynamics such as growth, life-cycle stages and toxin production. P assimilation is critical to cellular function in algae, as P is incorporated into biomolecules such as phosphoproteins, nucleic acids, phospholipids and energy currency such as ATP (discussed in Cembella et al., 1984a,b; Palenik and Dyhrman, 1998). As is the case with other algae, most HAB taxa appear to be able to use both inorganic P and organic P (e.g. AMP and phytic acid) as sole P sources in axenic culture (discussed in Cembella et al., 1984a,b). There are a number of proteins potentially involved in algal P metabolism, including alkaline phosphatase, phosphate permease, polyphosphate synthetase, and C-P lyase. In many cases these proteins are regulated by P source or cellular P physiology (Schwarz and Forchhammer, 2005; Dyhrman et al., 2006b). Although much progress has been made on our molecular-level understanding of P assimilation in algae and other microorganisms (Dyhrman and Palenik, 1997, 2001; Scanlan and Wilson, 1999; Schwarz and Forchhammer, 2005), there is limited data on the genes involved with P assimilation in key HAB taxa.

Traditional studies on algal nutrition use either cultured isolates or community level assays to examine nutrient uptake (Kudela and Cochlan, 2000), nutrient ratios (Anderson et al., 2002 and references therein), and enzyme activity (Mulholland et al., 2003), among other approaches. These types of studies are very valuable, but with increasing sequencing resources and molecular technologies, molecular approaches to understanding algal nutrient acquisition and nutritional physiological ecology, in both cultures and field populations, are expanding our understanding of how algae respond to nutrients and eutrophication. In short, we are able to move beyond the outward responses of the cells to nutrients (e.g. changes in elemental composition or growth) to the underlying genetic and biochemical processes that dictate those responses in culture models. This level of molecular detail not only allows the promise of better predictive power with regards to a species' response to nutrients, but also provides the technological capability to specifically monitor that response in field populations.

The HAB field has arguably led the microbial oceanographic community in recognizing early on the value of molecular approaches, using them to identify and enumerate key harmful species within a mixed community. There is now considerable potential for using these same molecular and genomic level approaches to expand our understanding of how nutrients (supply, type, etc.) influence HABs. With the continued advance of sequencing technology and genomics, molecular approaches are also advancing, providing methodologies for addressing questions related to the complexity of algal nutrition, and how cells respond to changes in their geochemical environment. Ultimately, both a better understanding of HAB species nutrient physiology and nutrient dynamics are needed to identify the linkages between eutrophication processes and HABs.

The recent HARNNESS report (Harmful Algal Research and Response: A National Environmental Science Strategy 2005–2015) highlights the importance of enhancing our understanding of harmful species nutritional physiology. One mechanism by which to accomplish this is through the development of sequence information in key harmful groups. Increased sequence databases

are then rich sources for hypothesis building and testing in culture models and field populations. Ultimately, having these sequences may provide the targets and technology for tracking how harmful species respond to changing geochemical conditions. In the interest of brevity I focus herein on molecular-level studies with eukaryotic HAB taxa, but we emphasize that there is a rich literature available for nutrient metabolism in cyanobacteria (Schwarz and Forchhammer, 2005) and broader aspects of molecular microbial ecology and molecular biogeochemistry (Arrigo, 2005; DeLong and Karl, 2005; Caron, 2005; Zak et al., 2006).

2. Molecular studies of nutrient metabolism in culture models

Much of the molecular-level work focused on HAB nutrition has been with model organisms in culture (Erdner and Anderson, 2006). Current work in this area involves the expansion of our sequence information for harmful species, or strains, and efforts directed at the identification and regulation of pathways of nutrient assimilation. It is well understood that different species, and even different strains, can occupy a unique environmental niche with regard to nutrient metabolism. Increasing sequence databases, and detailed studies of the transcripts that are identified, may ultimately identify functional similarities between HAB taxa with regards to nutrient metabolism that will help to better predict HAB responses to eutrophication.

2.1. DNA and EST sequencing

Major advances in marine microbial genomics, such as the sequencing of *Thalassiosira pseudonana* (Armbrust et al., 2004), are providing insight into the genetic basis of algal nutrition for selected model species. For example, annotation of the *T. pseudonana* genome revealed novel genes for silica transport and cell wall formation, a complete urea cycle, and other genes implicated in the metabolism of a variety of exogenous nitrogen compounds (Armbrust et al., 2004). In recognition of the significant insights that can be provided with whole genome sequencing, the U.S. Department of Energy Joint Genome Institute (<http://www.jgi.doe.gov>) is now sequencing the *Aureococcus anophagefferens* genome (~56 Mb), and the *Pseudo-nitzschia multiseriens* genome (~250 Mb) (Table 1). The sequencing and annotation of these first full HAB genomes will allow researchers an unprecedented ability to assess physiological potential in these species, to probe the genome for clues as to how these model organisms use nutrients, and to examine what makes them successful in certain coastal systems.

Although whole genome sequences for HAB models are still rare, the sequence databases are constantly increasing. While ribosomal gene sequences dominated the databases for many years, there are now many thousands of functional gene sequences publicly available for HAB species from the groups including the dinoflagellates, diatoms, raphidophytes, prymnesiophytes and the pelagophyte *A. anophagefferens* (Table 1). This is particularly evident, and important for the toxic dinoflagellates (e.g. *Karenia brevis* and *Alexandrium tamarense*, Table 1). Estimates of dinoflagellate genome size vary, with estimates ranging from 3000 to 215,000 Mb, but are typically estimated to be larger than the human genome of roughly 3200 Mb (Erdner and Anderson, 2006 and references therein). As such, whole genome sequencing is not currently viable for dinoflagellates, but large-scale sequencing projects, such as the sequencing of expressed sequence tags (EST) (Tanikawa et al., 2004; Hackett et al., 2005; Lidie et al., 2005) are a practical alternative. Here EST sequencing can focus on the transcriptome, or the complete set of genes expressed under a

Table 1

Selected large-scale sequencing, and transcriptome profiling efforts, in model harmful algae, data from both available and upcoming resources is listed

Organism	Resource ^a	Reference ^b
<i>Alexandrium minutum</i>	EST	http://genome.imb-jena.de/ESTTAL/cgi-bin/Project.pl
<i>Alexandrium fundyense</i>	MPSS	Erdner and Anderson (2006)
<i>Alexandrium tamarense</i>	EST	Hackett et al. (2005)
<i>Aureococcus anophagefferens</i>	WGS	http://microbialgenome.org/announcement/seq2005.shtml
<i>Aureococcus anophagefferens</i>	SAGE Libraries	Dyhrman et al. (unpubl.)
<i>Fibrocapsa japonica</i>	EST	http://genome.imb-jena.de/ESTTAL/cgi-bin/Project.pl
<i>Heterocapsa triquetra</i>	EST	http://www.ncbi.nlm.nih.gov/dbEST/dbEST_summary.html (Waller et al., 2006)
<i>Karenia brevis</i>	EST	Lidie et al. (2005)
<i>Lingulodinium polyedrum</i>	EST	Tanikawa et al. (2004)
<i>Pfiesteria shumwayae</i>	SAGE Libraries	Coyne et al. (2004)
<i>Prymnesium parvum</i>	EST	http://genome.imb-jena.de/ESTTAL/cgi-bin/Project.pl http://www.ncbi.nlm.nih.gov/dbEST/dbEST_summary.html
<i>Pseudo-nitzschia multiseriis</i>	WGS	http://www.osl.gc.ca/conf/tox2007/abstracts.pdf (Armbrust et al., 2007)
<i>Pseudo-nitzschia multiseriis</i>	EST	Biossonneault (2004)
<i>Pseudo-nitzschia multistriata</i>	EST	http://genome.imb-jena.de/ESTTAL/cgi-bin/Project.pl

^a Abbreviations are as follows: EST, expressed sequence tag; WGS, whole genome sequence; SAGE Libraries, serial analysis of gene expression libraries; MPSS, massively parallel signature sequences.

^b Data from both available and upcoming projects is listed to highlight the diversity of sequencing projects, but this is not intended to be a comprehensive list.

given condition. EST projects for a variety of harmful alga taxa are now underway, with model species from several different groups including dinoflagellates, raphidophytes and diatoms (Table 1). A critical consideration is that majority of EST collections are being generated from a single strain in a single culture condition. Although very useful, single culture conditions inherently limit the diversity of possible expressed genes. Future work in this area will need to build EST collections from multiple strains grown under a variety of different conditions, so that nutrient responsive transcripts can be identified.

2.2. Sequence annotation

The annotation of available algal genomes and EST collections has allowed an unprecedented ability to assess physiological potential in these marine organisms. Genomic data has highlighted both the surprising presence of unique nutrient acquisition strategies, such as phosphonate metabolism (Dyhrman et al., 2006a), and the surprising absence of other nutrient acquisition strategies. For example, genomic data helped to determine that *Prochlorococcus* (MED4) lacks genes for nitrate reductase and nitrite reductase, and is only able to grow on ammonium. Conversely, *Prochlorococcus* (MIT9313) only lacks genes for nitrate reductase genes and is able to grow on both ammonium and nitrite (Moore et al., 2002). In short, whole genome sequences have played a critical role in identifying pathways of nutrient scavenging with the marine cyanobacteria, and annotation of the whole genome sequencing projects for the HAB taxa mentioned above may result in similar surprises.

The annotation of the growing collection of ESTs for dinoflagellates, and other HAB models (Table 1), will also expand our understanding of physiological potential. However, EST collections best yield information on the presence of expressed genes, not the absence, as may be more easily determined with a complete genome sequence. Whether the sequence information is from a whole genome sequence, or an EST collection, annotation is an ongoing challenge. Of the roughly 6000 unique ESTs sequenced in *A. tamarense*, only about 20% could be annotated using BLAST searches with GenBank (Hackett et al., 2005), and similarly only about 30% of the roughly 5000 *K. brevis* gene clusters could be annotated (Lidie et al., 2005). Ultimately, the annotation of large-scale sequencing projects will allow researchers to examine whether HAB species have common functional genes that are mechanistically linked to metabolism of particular nutrients through the proteins/enzymes that they encode. Alternatively, are HAB species missing common functional genes or the

metabolic pathways they encode? Basic insights such as these will help predict how different members of the phytoplankton community respond to eutrophication.

2.3. Transcript expression and regulation

The increasing number of sequences available for harmful algae is driving technology development, and as the cost of sequencing becomes more affordable, there are an increasing number of studies that examine transcriptional processes in key organisms (Lidie et al., 2005). Tools such as quantitative RT-PCR (qRT-PCR) and microarray chip technology are becoming tractable, and these approaches will allow scientists to assay (at the level of gene transcription) how different HAB species respond to changes in their exogenous nutrient environment. Methods for assessing gene expression on a genomic-scale in cultured HAB models include DNA microarrays (Lidie et al., 2005), serial analysis of gene expression (SAGE) (Coyne et al., 2004), and Massively Parallel Signature Sequencing (MPSS) (Erdner and Anderson, 2006), among others.

Microarrays use hybridization reactions to examine the expression of genes on a global scale. With the HAB models now slated for whole genome sequencing, and the growing collections of ESTs for key taxa, microarray-based studies are becoming more tractable. Given the large genome sizes of dinoflagellates, these approaches are constrained to EST derived arrays for dinoflagellates (Okamoto and Hastings, 2003; Lidie et al., 2005). The application of microarrays to expression studies in cultures of eukaryotic marine algae, and even more so in HAB taxa, is very much at its inception. However, the approach will become more common for studies of nutritional physiology as sequence databases continue to expand.

Another approach for genomic-scale expression studies is called SAGE (Velculescu et al., 1995), or Long-SAGE (Saha et al., 2002). In Long-SAGE a short 21 bp sequence tag from the most polyA proximal NlaIII restriction site of an mRNA molecule is used to uniquely identify the source gene from within the genome or sequence database. These short sequence tags are sampled from all NlaIII-positive transcripts in a mRNA sample and are linked together to form long concatenated molecules that are cloned and sequenced. Quantification of all tags provides a relative measure of gene expression (e.g. mRNA abundance). SAGE thus provides both the identity of expressed genes and levels of their expression. Detection level and sensitivity in SAGE are a function of sampling depth—the more tags sampled, the more likely detection of rare transcripts and the stronger statistical resolution of differential

abundance of transcripts among mRNA samples. SAGE differs from microarrays in that it can be applied in the absence of sequence information. For example, SAGE, and modifications of this approach, are useful tools for gene discovery (Coyne et al., 2004), even in the absence of a additional sequence information for gene identification, as was demonstrated by Coyne et al., 2004 with *Pfiesteria*. However, SAGE is typically applied in systems with enough sequence information (e.g. an EST collection) to map SAGE tags to genes for identification.

MPSS is a third example of a global transcriptional approach and it has been applied to examine nutrient-regulated genes in the dinoflagellate *Alexandrium fundyense* (Erdner and Anderson, 2006). This approach is similar to SAGE, in that it isolates and sequences short diagnostic sequence tags (signatures) that can be mapped back to sequence databases for gene identification. It differs from SAGE in that it uses proprietary technology to achieve a greater sampling depth, allowing the study of very rare transcripts (Erdner and Anderson, 2006). In the single MPSS study with a HAB model (*A. fundyense*), few signatures could be annotated, however the data revealed a dramatic, and complex transcriptional-level response to nutrient deprivation. For example, over 10,000 unique signatures had a statistically significant ($p < 0.05$) response with an expression ratio of >2 across the N-deficient and P-deficient libraries (Erdner and Anderson, 2006). Here again, increasing sequence databases will help improve annotation in this and other MPSS studies, offering another promising approach to examine how global expression patterns differ with changing exogenous nutrient type and supply.

2.4. Protein expression and regulation

Targeted protein level studies, and whole proteome (all the proteins expressed under a given condition) studies are complementary to the genomic approaches highlighted above, because protein work focuses on the products of the expressed genes. However, proteomics, or even targeted-protein work, has the advantage of directly examining the active agents in the cell, the proteins that are responsible for the observed biological activities. Merely knowing the genes expressed in a cell is not necessarily sufficient to elucidate overall function, particularly because of the potential for post-transcriptional regulation and RNA editing. This is especially true for dinoflagellates where, of the functional genes studied to date, many exhibit post-transcriptional regulation (Van Dolah et al., 1995; Fagan et al., 1999; Okamoto et al., 2001; Hackett et al., 2004). As a result, the proteome arguably reflects the external conditions encountered by the cell, and its internal response, better than the gene complement. Two-dimensional gel electrophoresis (2DGE), followed by mass spectrometry for protein identification, has now been used to compare the proteome of selected HAB species, under a range of conditions (Chan et al., 2004a,b, 2006). This approach is still limited by technical challenges, especially with protein identification, because of the dearth of sequence data available for many HAB species. As whole genome sequences become available, and sequence information increases, it will be easier to identify proteins of interest. A concerted focus on screening key taxa for proteins that are responsive to changes in exogenous nutrients, or nutrient supply, would be the next step towards identifying proteins that are linked to nutrient metabolism and predicting responses to eutrophication.

2.5. Protein function

A critical ongoing hurdle in algal molecular physiology is to identify the role of genes or proteins with no database homology.

However, it is equally important to concretely demonstrate the function of genes or proteins of interest that have a putative function. Although progress has been made in the area of transformation approaches for diatoms, there is no proven mechanism for gene silencing or knocking out a target gene in diatoms, dinoflagellates or other groups with harmful eukaryotic species (discussed in Walker et al., 2005). Despite the many challenges in this area, recent work by Bertomeu and Morse (2004) highlights an advance in regard to this last point, where they were able to positively identify the function of a dinoflagellate cyclin through functional complementation of a yeast cyclin mutant (Bertomeu and Morse, 2004). Additional work with complementation, and on the development of genetic systems for key species, is an important area of future work, especially as genes of interest and/or putative function are identified.

3. Molecular assessments of nutritional physiology in field populations

The application of molecular-level assays of nutritional physiology has been limited in field populations, mainly due to the challenges in identifying the physiology or nutrient acquisition strategy being employed in a single species, when it exists in a mixed community. Highlighted below are sections focused on methods for assaying gene or protein expression, and cellular activities in field populations.

3.1. Assaying gene expression in field populations

Assaying for the expression of genes encoding proteins related to nutrient metabolism using RT-PCR or qRT-PCR is a potentially powerful approach for tracking the nutritional physiology of key species in field populations. This approach is becoming increasingly common in marine cyanobacteria (Church et al., 2005; Dyhrman et al., 2006a), but the approach has been more limited thus far for marine eukaryotes. Ongoing work with model phytoplankton which have large sequence databases, such as the diatom *T. pseudonana*, highlight the promise of this type of approach and its future application to HAB models. For example, qRT-PCR protocols have been developed for the detection of the genes encoding nitrate reductase and glutamine synthetase II, which are both required for nitrate utilization in *T. pseudonana* (Schnitzler-Parker and Armbrust, 2005). The application of these assays to field populations of *T. pseudonana* may help to identify the sources of nitrogen being used by the population. Again, with the increasing sequence databases on functional genes for HAB species, the development of qRT-PCR assays and their application for functional genes in the field is not likely that far off.

Microarrays, which rely on hybridization reactions, may be difficult to apply to field populations, because of the challenges associated with isolating and enriching for the target species. Although there is a growing literature on the application of micro/microarrays for studies of functional gene diversity in marine systems, these arrays have been limited to detection of genes not their expression (Ward, 2005 and references therein). In the future, arrays may be able to detect mRNA and thus provide direct information about which genes in which groups are active in a sample. Technical challenges in quantification, detection limit, and obtaining enough high quality mRNA from samples currently limits this application (Ward, 2005 and references therein). However, in the case of HAB genera such as *Aureococcus* or *Karenia*, which can form high density, nearly monospecific blooms, the application of microarrays with field populations may be tractable. In summary, the challenge of the next few years is to develop qRT-PCR and other molecular technologies in a manner

that can be easily and affordably used to monitor the expression of nutrient-related functional genes in field populations of harmful algae.

3.2. Assaying protein expression in the field

Additional advances in protein detection and characterization will help to further define how HAB species respond to eutrophication in field populations. Species-specific screening of physiologically important proteins is possible for a number of different taxa, and protein targets (Peperzak and Dyhrman, 2005). For instance, we can now track the phosphorus physiology of *Prorocentrum minimum* using antibody probes for a phosphorus-regulated protein and whole-cell immunolabeling (Dyhrman and Palenik, 2001). The development and application of immunoprobes will be facilitated with the ongoing identification and annotation of genes and gene pathways related to nutrient metabolism.

Post-probing is a possible alternative to the direct application of a physiological immunoprobe in a target field population (e.g. no probe is available, or species identification and/or dual labeling is impractical). Given the number of species-specific immunoprobes (Peperzak and Dyhrman, 2005), this is a viable option for a number of HAB taxa. One example of post-probing is immunomagnetic bead separation (IMBS). This method involves coupling immunomagnetic beads to a target cell population through a species-specific immunoprobe, which can then be separated from a mixed species community via a magnet (Aguilera et al., 1996, 2002). By isolating or enriching the target field population, bulk measurements of cellular physiology such as cellular nutrient content, and cell quotas of nucleic acids and proteins may be applied (Aguilera et al., 2002). Researchers may also probe these enrichments with physiological immunoprobes that are not species-specific, thus broadening potential antibody choices.

As with the field application of microarrays discussed above, the application of global proteomic approaches to field populations may be challenging, except under high density, monospecific bloom scenarios. With targeted protein-level work in the field there also remain many challenges, including the development of target antibodies, and their application in a quantitative and specific manner. However, advances in HAB proteomics (Chan et al., 2006) and targeted-protein work (Dyhrman and Palenik, 2001) will likely continue to expand our understanding of pathways of nutrient metabolism in field populations.

3.3. Assaying species-specific activities in field populations

Many substrate analogs are available for assaying enzymes involved in the breakdown of organic matter and other processes related to nutrient metabolism in algae. However, application of these substrates is often limited to bulk, or whole community, applications. Significant progress in this regard has come in through the development of enzyme labeled fluorescence (ELF) substrates for the detection of enzyme activity in single cells. Although most enzyme substrates result in the production of soluble colored or fluorescent products, ELF substrates result in an insoluble fluorescent product (González-Gil et al., 1998 and references therein). ELF substrates for enzymes, such as alkaline phosphatase and *N*-acetylglucosaminidase, can specifically tag cells from target harmful species with these activities (Fig. 1). As alkaline phosphatase is commonly present in algae, and *N*-acetylglucosaminidase activity has been detected in HAB taxa (Fig. 1). These, and other ELF substrates, represent critical tools for assaying cell-specific enzyme activity in field populations.

IMBS also offers a potential approach for isolating the target population and then examining enzyme activity in a species-

specific manner. For example, urease activity was detected in field populations of *Alexandrium fundyense* that were enriched from Gulf of Maine plankton samples using IMBS (Dyhrman and Anderson, 2003). Given the potential significance of urea to algal nutrition (Anderson et al., 2002), molecular-level approaches for examining the presence, regulation and activity of the urease enzyme, or transcript, in field populations may be an important area of future work. Ongoing challenges in this area include the limited availability of enzyme substrates that can be applied for species-specific assays (e.g. ELF type substrates), and the validation work required for the development and application of both enzyme assays and post-probing approaches.

4. Molecular physiological ecology—a case study with alkaline phosphatase

The sections above highlight mechanisms by which the presence of a gene, its expression, and the activity that it encodes might be used to examine pathways of algal nutrient acquisition in field populations. In this section I illustrate this approach with a brief example, presenting a case study with alkaline phosphatase (Fig. 2).

Alkaline phosphatase is an enzyme commonly present in eukaryotic marine algae (Dyhrman and Ruttenberg, 2006 and references therein). It is typically surface associated and it will hydrolyze inorganic P from organic phosphomonoester sources for assimilation by the cell. Utilization of P from the organic phosphomonoester pool would allow cells to access a greater range of P forms than just the inorganic form. This is significant in that the concentration of dissolved organic P (DOP) can often meet or exceed the concentration of dissolved inorganic P, even in coastal systems (Dyhrman and Ruttenberg, 2006 and references therein). The presence of a gene (e.g. *phoA*) encoding alkaline phosphatase (Fig. 2) indicates a potential pathway for DOP metabolism. The *phoA* gene may be expressed when that pathway of DOP metabolism is active in the target population, which could be detected via qRT-PCR in field populations of harmful algae (Fig. 2). This type of expression analysis has been performed in cultures of a model coccolithophore (Xu et al., 2006), but not in field populations, or with harmful algal species. However, gene expression may not be entirely coincident with the expression, or presence, of the target protein. Assaying the target protein using an immunoprobe (Fig. 2) brings the investigator farther down this path (DNA, RNA, Protein, Activity), which may be advantageous in scenarios where there is post-translational regulation. Such a protein-based approach has been useful for identifying a putative alkaline phosphatase protein in cultures and field populations of the dinoflagellate *P. minimum* (Dyhrman and Palenik, 2001); data that highlight that DOP is a potential P source to *P. minimum* populations in Narragansett Bay, RI. The actual hydrolysis of the DOP is the end point of the biological cascade, which argues for the value of assaying the alkaline phosphatase activity itself (Fig. 2). Using ELF-based assays, cell-specific alkaline phosphatase activity has been examined in variety of systems to assess DOP hydrolysis by key phytoplankton groups or species (Dyhrman and Palenik, 1999; Ruttenberg and Dyhrman, 2005; Dyhrman and Ruttenberg, 2006).

In short, all of these molecular-level approaches may be used to examine P acquisition in a specific manner and track DOP hydrolysis in different species over time and with changes in community composition, nutrient form, and nutrient concentration. Yet in the end, it is perhaps most critical to understand the limitations and caveats of different assays, and target the point in the pathway (e.g. RNA, Protein, Activity) which best addresses the hypothesis.

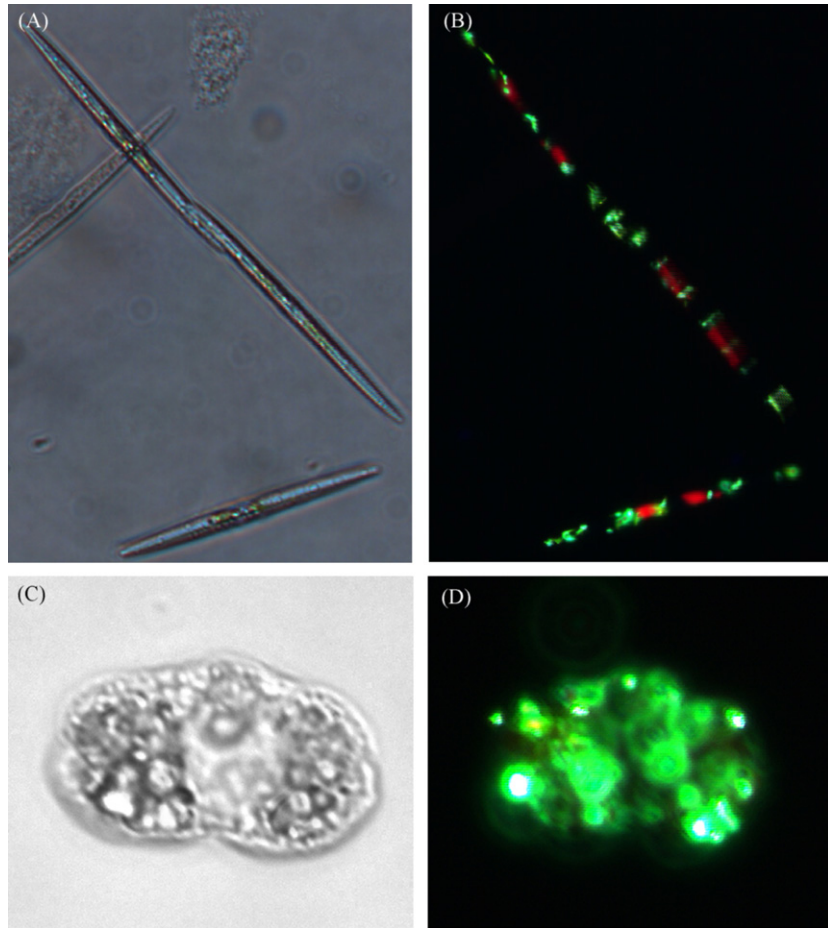


Fig. 1. Images of the HAB taxa *Pseudo-nitzschia* sp. (A) and *Karenia brevis* (C) assayed using enzyme labeled fluorescence (ELF) (B and D). ELF results in a fluorescent green precipitate at the site of enzyme activity. The green fluorescence indicates activity in cells ELF-assayed for *N*-acetylglucosaminidase activity (B) or ELF-assayed for alkaline phosphatase activity (D). Red indicates the chlorophyll autofluorescence (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article).

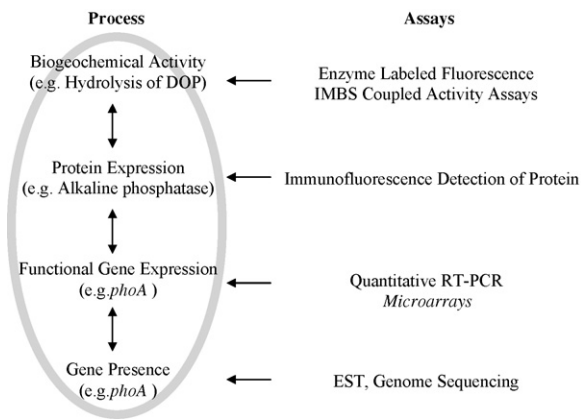


Fig. 2. A flow chart linking biogeochemical processes to functional genes. Conceptual linkages are listed under the Process heading and mechanisms for detecting different elements are listed to the right under the Assay heading. Examples are given for dissolved organic phosphorus hydrolysis. Acronyms are defined in the text.

5. Conclusions

Advances in sequencing have revolutionized our capacity to examine the genetic complexity of organisms at the whole genome level. With ever-growing sequence databases and whole genome

sequencing projects ongoing for HAB taxa, we are developing a new understanding of the basic pathways of nutrient metabolism in harmful algae. Ultimately, nutrient consumption is related to abundance, transcription, or activity of a particular functional gene, and the composition of the gene functional group (e.g. different algal species may regulate the same process differently). The HAB community is now poised to bring the full potential of these exciting molecular advances to our study of HAB nutritional physiology and how this may be influenced by eutrophication through the application of assays targeting nutrient acquisition pathways in field populations. Despite ongoing advances there remains a major need to link to phenotype and physiology to our understanding of ecological functioning and the influence of nutrients on strains/species within a complex community. Even with new, powerful, molecular-tools becoming available, it is critical to note that they should ideally be employed for long-term bloom monitoring so that they can be integrated with additional observations of abundance, nutrient uptake, nutrient concentrations, toxin production, grazing and other approaches currently used to examine bloom dynamics and how they relate to eutrophication processes.

Acknowledgements

I wish to thank Pat Glibert for the opportunity to contribute to this issue, and Sheean T. Haley for insightful comments on an

earlier version of this manuscript. I also thank two anonymous reviewers for their helpful comments. Production of this manuscript was in part supported by the Woods Hole Oceanographic Institution Ocean Life Institute through a fellowship to the author, National Oceanic Atmospheric Administration grant NA05NOS4781224 through the MERHAB Program, and the Environmental Protection Agency through the ECOHAB Program (R-83041501-0).[SS]

References

- Aguilera, A., González-Gil, S., Keafer, B.A., Anderson, D.M., 1996. Immunomagnetic separation of cells of the toxic dinoflagellate *Alexandrium fundyense* from natural plankton samples. *Mar. Ecol. Prog. Ser.* 143, 255–269.
- Aguilera, A., Keafer, B.A., Rau, G.H., Anderson, D.M., 2002. Immunomagnetic isolation of live and preserved *Alexandrium fundyense* cells: species-specific physiological, chemical and isotopic analyses. *Mar. Ecol. Prog. Ser.* 237, 65–78.
- Anderson, D.M., Glibert, P.M., Burkholder, J.M., 2002. Harmful algal blooms and eutrophication: nutrient sources, composition and consequences. *Estuaries* 25, 704–726.
- Antia, N.J., Harrison, P.J., Oliveira, L., 1991. The role of dissolved organic nitrogen in phytoplankton nutrition, cell biology and ecology. *Phycologia* 30, 1–89.
- Armbrust, E.V., Berges, J.A., Bowler, C., Green, B.R., Martinez, D., Putnam, N.H., Zhou, S., Allen, A.E., Apt, K.E., Bechner, M., Brzezinski, M.A., Chaal, B.K., Hadi, M.Z., Hallsten, U., Hildebrand, M., Jenkins, B.D., Jurka, J., Kapitonov, V.V., Kroger, N., Lau, W.W.Y., Lane, T.W., Larimer, F.W., Lippmeier, J.C., Lucas, S., Medina, M., Montsant, A., Obornik, M., Schnitzler-Parker, M., Palenik, B., Pazour, G.J., Richardson, P.M., Rynearson, T.A., Saito, M.A., Schwartz, D.C., Thamatrakoln, K., Valentin, K., Vardi, A., Wilkerson, F.P., Rokhsar, D.S., 2004. The genome of the diatom *Thalassiosira pseudonana*: ecology, evolution, and metabolism. *Science* 306, 79–86.
- Armbrust, E.V., Parker, M., Rocap, G., Holtermann, K., Marchetti, A., Jenkins, B.D., Bates, S.S., 2007. Whole genome sequencing of the toxic diatom *Pseudo-nitzschia multiseries*; an introduction to the study. In: 10th Canadian Workshop on Harmful Algae Book of Abstracts, p. 10.
- Arrigo, K.R., 2005. Marine microorganisms and global nutrient cycles. *Nature* 437, 349–355.
- Berg, G.M., Glibert, P.M., Lomas, M.L., Burford, M., 1997. Organic nitrogen uptake and growth by the Chrysophyte *Aureococcus anophagefferens* during a brown tide event. *Mar. Biol.* 129, 377–387.
- Bertomeu, T., Morse, D., 2004. Isolation of a dinoflagellate mitotic cyclin by functional complementation in yeast. *Biochem. Biophys. Res. Commun.* 323, 1172–1183.
- Biossoneault, K.R., 2004. Gene discovery and expression profiling in the toxin-producing marine diatom, *Pseudo-nitzschia multiseries* (Hasle) Hasle. Ph.D. Thesis. Massachusetts Institute of Technology, Cambridge, MA, USA.
- Caron, D.A., 2005. Marine microbial ecology in a molecular world: what does the future hold? *Sci. Mar.* 69, 97–110.
- Cembella, A.D., Antia, N.J., Harrison, P.J., 1984a. The utilization of inorganic and organic phosphorus compounds as nutrients by eukaryotic microalgae: a multidisciplinary perspective: Part 2. *CRC Crit. Rev. Microbiol.* 11, 13–81.
- Cembella, A.D., Antia, N.J., Harrison, P.J., 1984b. The utilization of inorganic and organic phosphorus compounds as nutrients by eukaryotic microalgae: a multidisciplinary perspective: Part 1. *CRC Crit. Rev. Microbiol.* 10, 317–391.
- Chan, L.L., Hodgkiss, I.J., Lu, S., Lo, S.C.L., 2004a. Use of two-dimensional gel electrophoresis proteome reference maps of dinoflagellates for species recognition of causative agents of harmful algal blooms. *Proteomics* 4, 180–192.
- Chan, L.L., Hodgkiss, I.J., Wan, J.M.F., Lum, J.H.K., Mak, A.S.C., Sit, W.H., Lo, S.C.L., 2004b. Proteomic study of a model causative agent of harmful algal blooms. *Prorocentrum triestinum* II: the use of differentially expressed protein profiles under different growth phases and growth conditions for bloom prediction. *Proteomics* 4, 3214–3226.
- Chan, L.L., Sit, W.H., Lam, P.K.S., Hsieh, D.P.H., Hodgkiss, I.J., Wan, J.M.F., Ho, A.Y.T., Choi, N.M.C., Wang, D.Z., Dungeon, D., 2006. Identification and characterization of a biomarker of toxicity from the proteome of the paralytic shellfish toxin-producing dinoflagellate *Alexandrium tamarense* (Dinophyceae). *Proteomics* 6, 654–666.
- Church, M.J., Short, C.M., Jenkins, B.D., Karl, D.M., Zehr, J.P., 2005. Temporal patterns of nitrogenase gene (*nifH*) expression in the oligotrophic north Pacific Ocean. *Appl. Environ. Microbiol.* 71, 5362–5370.
- Coyne, K.J., Burkholder, J.M., Feldman, R.A., Hutchins, D.A., Cary, S.C., 2004. Modified serial analysis of gene expression method for construction of gene expression profile of microbial eukaryotic species. *Appl. Environ. Microbiol.* 70, 5298–5304.
- DeLong, E.F., Karl, D.M., 2005. Genomic perspectives in microbial oceanography. *Nature* 437, 336–342.
- Doblin, M.A., Blackburn, S.I., Hallegraeff, G.M., 1999. Growth and biomass stimulation of the toxic dinoflagellate *Gymnodinium catenatum* (Graham) by dissolved organic substances. *J. Exp. Mar. Biol. Ecol.* 236, 33–47.
- Dyrhman, S.T., Anderson, D.M., 2003. Urease activity in cultures and field populations of the dinoflagellate *Alexandrium*. *Limnol. Oceanogr.* 48, 647–655.
- Dyrhman, S.T., Chappell, P.D., Haley, S.T., Moffett, J.W., Orchard, E.D., Waterbury, J.B., Webb, E.A., 2006a. Phosphonate utilization by the globally important marine diazotroph *Trichodesmium*. *Nature* 439, 68–71.
- Dyrhman, S.T., Haley, S.T., Birkeland, S.R., Wurch, L.L., Cipriano, M.J., McArthur, A.G., 2006b. Long serial analysis of gene expression for gene discovery and transcriptome profiling in the widespread marine coccolithophore *Emiliania huxleyi*. *Appl. Environ. Microbiol.* 72, 252–260.
- Dyrhman, S.T., Palenik, B.P., 1997. The identification and purification of a cell-surface alkaline phosphatase from the dinoflagellate *Prorocentrum minimum* (Dinophyceae). *J. Phycol.* 33, 602–612.
- Dyrhman, S.T., Palenik, B.P., 1999. Phosphate stress in cultures and field populations of the dinoflagellate *Prorocentrum minimum* detected using a single cell alkaline phosphatase activity assay. *Appl. Environ. Microbiol.* 65, 3205–3212.
- Dyrhman, S.T., Palenik, B.P., 2001. A single-cell immunoassay for phosphate stress in the dinoflagellate *Prorocentrum minimum* (Dinophyceae). *J. Phycol.* 37, 400–410.
- Dyrhman, S.T., Ruttenberg, K.C., 2006. Presence and regulation of alkaline phosphatase activity in eukaryotic phytoplankton from the coastal ocean: implications for dissolved organic phosphorus remineralization. *Limnol. Oceanogr.* 51, 1381–1390.
- Erdner, D.L., Anderson, D.M., 2006. Global transcriptional profiling of the toxic dinoflagellate *Alexandrium fundyense* using Massively Parallel Signature Sequencing. *BMC Genomics* 7, doi:10.1186/147-12164-7-88.
- Fagan, T., Morse, D., Hastings, J.W., 1999. Circadian synthesis of a nuclear-encoded chloroplast glyceraldehyde-3-phosphate dehydrogenase in the dinoflagellate *Gonyaulax polyedra* is translationally controlled. *Biochemistry* 38, 7689–7695.
- González-Gil, S., Keafer, B., Jovine, R.V.M., Anderson, D.M., 1998. Detection and quantification of alkaline phosphatase in single cells of phosphorus-limited marine phytoplankton. *Mar. Ecol. Prog. Ser.* 164, 21–35.
- Hackett, J.D., Anderson, D.M., Erdner, D.L., Bhattacharya, D., 2004. Dinoflagellates: a remarkable evolutionary experiment. *Am. J. Bot.* 9, 1523–1534.
- Hackett, J.D., Scheetz, T.E., Yoon, H.S., Soares, M.B., Bonaldo, M.F., Casavant, T.L., Bhattacharya, D., 2005. Insights into a dinoflagellate genome through expressed sequence tag analysis. *BMC Genomics* 6, 80, doi:10.1186/147-2164/6/80.
- Hildebrand, M., Dahlin, K., 2000. Nitrate transporter genes from the diatom *Cylindrotheca fusiformis* (Bacillariophyceae): mRNA levels controlled by nitrogen source and by the cell cycle. *J. Phycol.* 36, 702–713.
- John, E.H., Flynn, K.J., 1999. Amino acid uptake by the toxic dinoflagellate *Alexandrium fundyense*. *Mar. Biol.* 133, 11–19.
- Kudela, R.M., Cochlan, W.P., 2000. Nitrogen and carbon uptake kinetics and the influence of irradiance for a red tide bloom off southern California. *Aquat. Microb. Ecol.* 21, 31–47.
- Lidie, K.B., Ryan, J.C., Barbier, M., Van Dolah, F.M., 2005. Gene expression in Florida red tide dinoflagellate *Karenia brevis*: analysis of an expressed sequence tag library and development of a DNA microarray. *Mar. Biotech.* 7, 481–493.
- Moore, L.M., Post, A.F., Rocap, G., Chisholm, S.W., 2002. Utilization of different nitrogen sources by the marine cyanobacteria. *Prochlorococcus* and *Synechococcus*. *Limnol. Oceanogr.* 47, 989–996.
- Mulholland, M.R., Lee, C., Glibert, P.M., 2003. Extracellular enzyme activity and uptake of carbon and nitrogen along an estuarine salinity and nutrient gradients. *Mar. Ecol. Prog. Ser.* 258, 3–17.
- Nixon, S.W., 1995. Coastal marine eutrophication: a definition, social causes and future concerns. *Ophelia* 41, 199–219.
- Okamoto, O.K., Hastings, J.W., 2003. Genome-wide analysis of redox-regulated genes in a dinoflagellate. *Gene* 321, 73–81.
- Okamoto, O.K., Robertson, D.L., Fagan, T.F., Hastings, J.W., 2001. Different regulatory mechanisms modulate the expression of dinoflagellate iron-superoxide dismutase. *J. Biol. Chem.* 276, 19989–19993.
- Palenik, B., Dyrhman, S.T., 1998. Recent progress in understanding the regulation of marine primary production by phosphorus. In: Lynch, J.P., Deikman, J. (Eds.), *Phosphorus in Plant Biology: Regulating Roles in Molecular, Cellular, Organismic and Ecosystem Processes*. American Society of Plant Physiologists, Rockville, MD, pp. 26–38.
- Peperzak, L., Dyrhman, S.T., 2005. From microscope to magnet: probing phytoplankton population structure and physiology using mammalian antibodies. In: Subba Rao, D.V. (Ed.), *Algal Cultures, Analogues of Blooms and Applications*. Science Publishers Inc., Plymouth, UK, pp. 839–864.
- Pfiester, L.A., Anderson, D.M., 1987. Dinoflagellate reproduction. In: Taylor, F.J.R. (Ed.), *The Biology of Dinoflagellates*. Botanical Monographs. Blackwell Science Publications, Oxford, UK, pp. 611–648.
- Poulton, N., 2001. Physiological and behavioral diagnostics of nitrogen limitation for the toxic dinoflagellate *Alexandrium fundyense*. Ph.D. Thesis. Massachusetts Institute of Technology, Cambridge, MA, USA.
- Robertson, D.L., Smith, G.J., Alberte, R., 2001. Glutamine synthetase in marine algae: new surprises from an old enzyme. *J. Phycol.* 37, 793–795.
- Ruttenberg, K.C., Dyrhman, S.T., 2005. Temporal and spatial variability of dissolved organic and inorganic phosphorus, and metrics of phosphorus bioavailability in an upwelling-dominated coastal system. *J. Geophys. Res.* 110, C10S13, doi:10.1029/2004JC002873.
- Saha, S., Sparks, S., Rago, C., Akmaev, V., Wang, C.J., Vogelstein, B., Kinzler, K.W., Velculescu, V.E., 2002. Using the transcriptome to annotate the genome. *Nat. Biotech.* 20, 508–512.
- Scanlan, D.J., Wilson, W.H., 1999. Application of molecular techniques to addressing the role of P as a key effector in marine ecosystems. *Hydrobiologia* 401, 149–175.
- Schnitzler-Parker, M., Armbrust, E.V., 2005. Synergistic effects of light, temperature, and nitrogen source on transcription of genes for carbon and nitrogen

- metabolism in the centric diatom *Thalassiosira pseudonana* (Bacillariophyceae). *J. Phycol.* 41, 1142–1153.
- Schwarz, R., Forchhammer, K., 2005. Acclimation of unicellular cyanobacteria to macronutrient deficiency: emergence of a complex network of cellular responses. *Microbiology* 151, 2503–2514.
- Tanikawa, N., Akimoto, H., Ogoh, K., Chun, W., Ohmiya, Y., 2004. Expressed sequence tag analysis of the dinoflagellate *Lingulodinium polyedrum* during dark phase. *Photochem. Photobiol.* 80, 31–35.
- Van Dolah, F., Leighfield, T.A., Sandel, H.D., Hsu, C.K., 1995. Cell division in the dinoflagellate *Gambierdiscus toxicus* is phased to the diurnal cycle and accompanied by activation of the cell cycle regulatory protein cdc2 kinase. *J. Phycol.* 31, 395–400.
- Velculescu, V.E., Zhang, L., Vogelstein, B., Kinzler, K.W., 1995. Serial analysis of gene expression. *Science* 270, 484–487.
- Walker, T.L., Collet, C., Purton, S., 2005. Algal transgenics in the genomic era. *J. Phycol.* 41, 1077–1093.
- Waller, R.F., Patron, N.J., Keeling, P.J., 2006. Phylogenetic history of plastid-targeted proteins in the peridinin-containing dinoflagellate *Heterocapsa triquetra*. *Int. J. Syst. Evol. Microbiol.* 56, 1439–1447.
- Ward, B.B., 2005. Molecular approaches to marine microbial ecology and the marine nitrogen cycle. *Annu. Rev. Earth Planet. Sci.* 33, 301–333.
- Xu, Y., Wahlund, T.M., Feng, L., Shaked, Y., Morel, F.M.M., 2006. A novel alkaline phosphatase in the coccolithophore *Emiliania huxleyi* (Prymnesiophyceae) and its regulation by phosphorus. *J. Phycol.* 42, 835–844.
- Zak, D.R., Blackwood, C.B., Waldrop, M.P., 2006. A molecular dawn for biogeochemistry. *TRENDS Ecol. Evol.* 21, 288–295.
- Zehr, J.P., Ward, B.B., 2002. Nitrogen cycling in the ocean: new perspectives on processes and paradigms. *Appl. Environ. Microbiol.* 68, 1015–1024.