This Staff Report provides a summary of the *Development of Bioanalytical Techniques for Monitoring of Constituents/Chemicals of Emerging Concern (CECs)*[1] *in Recycled Water Applications for the State of California* Report*,* dated June 30, 2014. The bioanalytical techniques were developed and validated to examine the presence of unidentified CECs in recycled water used for groundwater recharge. Hereafter, the bioanalytical techniques are referred to as "bioassays."

Background

The Recycled Water Policy, adopted in 2009, directed the State Water Resources Control Board (State Water Board) to convene a "blue ribbon" advisory panel to guide future actions related to CECs. The Science Advisory Panel (Panel) was formed in the Spring of 2009, and composed of experts in the field of toxicology, biochemistry, epidemiology, and facility civil engineering to investigate the potential health risks posed due to the presence of CECs in recycled water. In 2010, the Panel submitted a report with several recommendations, including a monitoring strategy for CECs in recycled water. The Recycled Water Policy was amended, in early 2013, to include the Panel's recommendations, which established monitoring requirements for CECs in recycled water used for groundwater recharge. The Panel also encouraged the development of bioassays for CECs that are currently not identified (i.e. unknown chemicals), but may be potentially present in recycled water. In addition, an interpretive framework that includes appropriate trigger levels, and corresponding responses to CECs that pose a concern to human health is to be incorporated into the bioassay development. Below is a summary of development process for the bioassays and the interpretative framework used in the decision-making.

Cross-Coordination Development by the Research Team

An investigative team was assembled consisting of experts in the fields of chemistry, biochemistry, toxicology, and chemistry in conjunction with the expertise of the Southern California Coastal Research Project (SCCWRP). In addition, a Project Advisory Committee was assembled consisting of members from regulated, regulatory, research, and commercial services communities to provide feedback on the likelihood of the success and the applicability of the bioassays by the investigative team.

The investigative team was charged with several tasks including: (1) identifying the most promising bioassay endpoints relevant to recycled water and the protection of human health; (2) developing extraction protocols for recycled water samples; (3) comparing, evaluating and optimizing the performance of commercially available bioassay technology; (4) delivering written bioassay protocols for successfully optimizing endpoints; (5) providing interpretive guidance for

[1] Constituents of Emerging Concern (CECs) are defined as chemicals in personal care products, pharmaceuticals including antibiotics, antimicrobials; industrial, agricultural, and household chemicals; hormones; food additives; transformation products, inorganic constituents; and nanomaterials.

bioassay results; and (6) soliciting stakeholder feedback and guidance on the application of bioassays. This project was conducted in concert with a similar project, funded by the WateReuse Research Foundation, and led by an Australian consortium faced with similar water quality and quantity issues. Once developed, the bioassays were tested among 20 research laboratory worldwide. Hereafter, the combined teams (Australia and California investigative teams) will be referred to as the Methods Team.

Method Development Process

The Methods Team developed two method protocols; the sample extraction protocol and the bioassay protocol. The extraction protocol separates organic chemicals (in this case CECs) from the raw water sample. The bioassay protocol uses biological endpoints to identify the chemical and its corresponding concentration in the extracted sample.

Extraction Protocol

The water sample extraction protocol consists of passing a water sample (wastewater treatment sample) through two solid phase cartridges. The organic chemicals are absorbed onto the solid phase material. After the water sample completely passes through the solid phase material, solvent and pressure is applied to release the CEC from the solid phase material. The solvent and CECs mixture are to be used in the bioassay (concentrated sample extract).

Bioassay Protocol

Selection of the Biological Endpoint

Through an extensive literature search, the Methods Team identified eight endpoints that were sufficient in terms of performance potential and relevance for protecting of human health. These endpoints target mechanisms at the cellular level that initiates a biological response by the exposure to a foreign chemical (i.e., CEC). Each endpoint has a specific mode of action (MOA), and it only responds to those chemicals for the specific MOA.[2] The eight endpoints selected by the Methods Team included those associated with endocrine/reproductive disruption (e.g. estrogenicity, androgenicity), genetic modification and cancer (genotoxicity) and general cell health (cytotoxicity).

The U.S. Environment Protection Agency (US EPA) initially developed many bioassays to improve chemical screening and registration. These bioassays use engineered human, animal, or bacteria cell lines that are dosed with a small drop of sample concentrated extract into individual exposure wells (*in vitro*) on a plate. A plate contains many wells that allows for additional water sample testing, quality assurance and quality control (e.g., high throughput format). Chemicals in the sample extract are allowed to "activate" the array of genes associated with the engineered cells. After the activation period, each well is analyzed for amount of light

[2] Because foreign chemicals (CECs) can have similar characteristics to that of biological hormones, the chemicals are known to interfere with natural hormonal function in the body causing endocrine disruption.

that is emitted from CECs in the sample. The intensity and wavelength of the emitted light from each well is proportionate to the CEC concentration in the sample.

Fortunately, the selected bioassays targeting each of the endpoints were commercially available, and did not require further research and development. In addition, the most technologically advanced and relevant endpoints for water quality screening purposes were *in vitro* receptor-based transactivation bioassays. After the evaluation of commercially available bioassay kits from various vendors, the Methods Team selected the cell kits from Life Technology for further optimization. These kits were chosen due to its usability, performance, and in large part, the inclusion of culturable and cryo-preserved "frozen and use" (division arrested (DA)) formats. The kits include 96-well plate format, which allowed for intermittent application with little to no maintenance cost/effort and was capable with basic detection instruments. The Methods Team found that five of the eight endpoints were acceptable. To verify the usability of these methods as well as capability of producing consistent results, an inter-laboratory comparison study was conducted. Ten water extract samples representing different levels of treatment were sent to research laboratories. The study participants included all five members of the investigative team and represented 20 laboratories around the world. It was concluded that the most responsive and precise endpoint in the study include the estrogen receptor (ER) and the glucocorticoid receptor (GR) bioassays optimized by the team.

Table 1. Receptor-based transactivation cell bioassays recommended for future evaluation

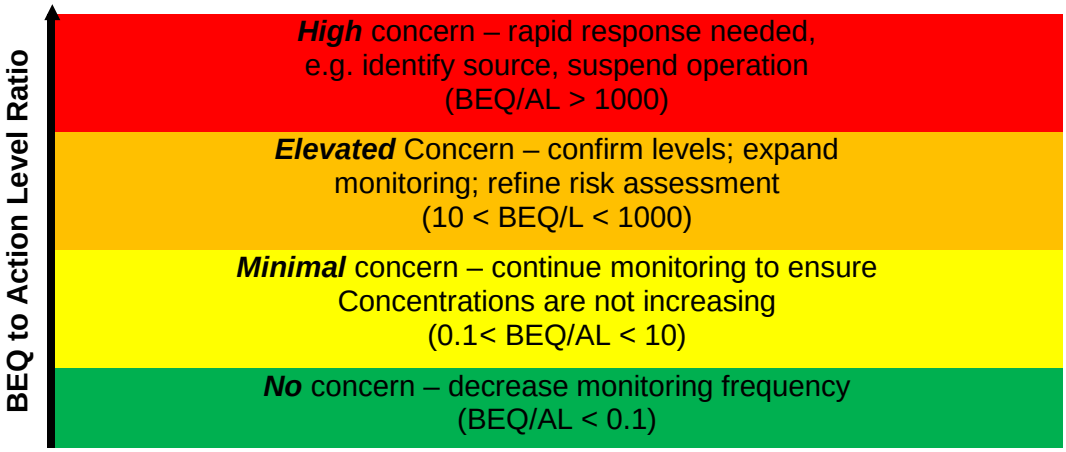
ENDPOINT	MODE OF ACTION	POTENTIAL ADVERSE OUTCOMES
estrogen receptor (ER)	Estrogen signaling (estrogenicity)	Impaired reproduction; feminization of males,
androgen receptor (AR)	Male sexual phenotype (androgenicity)	Impaired reproduction; masculinization of females
glucocorticoid receptor (GR)	Cortisol binding, gene transcription regulation	Development, immune function, diabetes
progesterone receptor (PR)	Embryonic development, cell differentiation	Cancer, diabetes, hormone resistance syndrome
cytotoxicity	General cell toxicity	Tissue damage, death

Interpretive Framework for Monitoring of Recycled Water Using In Vitro Bioassays

The Methods Team developed guidelines for data analysis and an interpretation process to be used in the decision making framework. The guidelines incorporate a three step process; data analysis, establishing thresholds and the decision making framework. Step 1 includes data analysis procedures along with tools to convert the bioassay results to concentrations that are equivalent to those of the reference toxicants, called "bioassay equivalent concentrations" (BEQs) e.g., 17ß-estradial for the estrogen-alpha (ERα) transactivation assay. Step 2 consists of applying the bioassay results considered health-based thresholds (e.g. no effect concentrations) as well as the difference in potency that CECs exhibits using *in vitro* bioassays

compared to effects that may occur *in vivo* (i.e., on individual test organisms, humans and human populations) to develop the appropriate bioassay-based action levels (ALs). Step 3 uses an interpretive framework that establishes ground rules for adaptive decision-making by managers based on the severity and persistence of AL exceedances.

Figure 1. Tiered decision making framework for monitoring of recycled water using *in vitro* bioassays



Note:
BEQ is the equivalent concentration of the CEC determined in Step 1
AL is the bioassay action level determined in Step 2

Next Steps

The next step to the bioassay endeavor is to coordinate a group of water utility and commercial laboratories and pilot the real world application of these methods. A pilot study would focus on technology transfer and real world simulation of how the bioassay could be used in a management context. The pilot study would include elements to address the following questions:

1. Could commercial service and water utility labs successfully preform the ER transactivation bioassay?

   Elements include: provide training on bioassay performance, data analysis and interpretations; and plan and conduct an inter-comparison exercise among commercial services and water industry laboratories.

2. Does the proposed bioassay interpretive framework provide managers with a robust decision making tool that better protects the beneficial uses of recycled water in California?

   Elements include: Collect and test recycled water samples across the State and analyze using both the ER transactivation bioassay and conventional chemical analysis; evaluate the bioassay results using the proposed interpretive framework and compare with decisions made based on chemical specific monitoring.

Further Research Recommendations

Several studies were identified to improve and/or better inform the application of bioassays for water quality screening purposes. These studies include the identification and optimization of a broader suite of endpoints (e.g., aryl hydrocarbon receptors, genotoxicity, oxidative stress, neurotoxicity, immunotoxicity), assessment of the efficacy of water extraction protocols for a diverse set of bioassay endpoints, and a cost-comparison between chemical-specific and bioassay-based screening level monitoring.