Evaluating Stressors in the San Francisco Estuary using Biomarkers

Report of an Independent Scientific Advisory Panel

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Executive Summary

An Independent Scientific Advisory Panel (Panel) was convened to assess ongoing research and advise on the development and application of biomarkers in the San Francisco Estuary (SFE). The overarching tasks put to the Panel were to:

- Assess the potential application of biomarkers for evaluating stressors and/or adverse effects on SFE fishes;

- Identify biomarkers that should be focused on in future SFE research; and

- Identify data gaps and develop a research framework to guide the role and application of biomarkers within the Bay-Delta ecosystem.

After reading background materials, the Panel convened on October 24-25, 2013, and listened to a series of presentations summarizing ongoing work in the SFE involving biomarkers, questioned the presenters, and had discussions among the Panel members. The Panel decided to build their recommendations off of an earlier 2007 committee report on a very similar subject, namely the use of biomarkers to investigate reasons for declines in populations of pelagic species in the SFE, known as POD species (POD being the acronym for pelagic organism decline).

In this report, we summarize the current state of biomarker development and application, and recap the progress we have observed to date in addressing the recommendations of the 2007 POD Committee (Anderson et al., 2007). Remaining data gaps are noted, and we make 12 specific recommendations for improving the utility of biomarker application in SFE fish species, in the short term. We then suggest two areas where future toxicogenomics research could be directed in a longer time frame.

Notable short-term needs include better understanding of the effects of both contaminant and non-contaminant stressors on early life history stages of SFE fish species, and a more thorough examination of a large dataset collected from field studies of Delta smelt. The confounding factor of abiotic parameters, such as salinity, on exposure of SFE species to toxicants, and effects on resulting health outcomes, is also noted as a research area needing attention, as well as more attention being paid to understanding effects of multiple stressors. Because the Panel was heavily weighted towards expertise in molecular biology, we also made several specific short-term recommendations concerning the development and application of molecular-level biomarkers in SFE fish species.

Future (longer term) directions for toxicogenomics research include developing algorithms to discriminate between gene expression profiles resulting from contaminant exposure and those arising from other environmental conditions (i.e., deriving ‘molecular signatures’), and investigations of possible epigenetic effects of contaminant exposures.

Biomarkers: definition and background.

While there is some debate regarding the precise definition of a *biomarker*, it is generally accepted that biomarkers represent quantitative sub-lethal indices measured at the molecular, biochemical, tissue and physiological levels that reflect either *exposure* to environmental contaminants, or resulting toxic *effects* (Hook et al., 2014). These quantitative biomarkers are of utility world-wide in biomonitoring and regulatory contexts, as they complement other toxicological tools such as toxicity testing and analytical chemistry analyses (Collier et al., 2013). Although not classically considered as biomarkers, *fish condition* indicators reflect functional aspects of individual health such as reproduction, growth, energetics, and histopathology, which can greatly complement biomarkers. It is widely recognized that fish condition indices can be part of biomarker monitoring programs and provide stronger linkages to population level effects. It was evident during the October 2013 biomarker workshop held at UC Davis that incorporation of fish condition indices is of particular interest in assessing the effects of nonchemical and chemical stressors in fish in the SFE.

Biomarker approaches in monitoring and aquatic toxicology are not new, with several biomarkers being in widespread use for decades (Stegeman et al., 1992). Despite their application in aquatic toxicology studies, there has not been extensive incorporation of biomarker approaches into a regulatory framework. Similarly, there has been little consistency among the scientific community with respect to agreement on criteria for the development and evaluation of biomarkers. Nonetheless, the following criteria seem to be consistent themes in biomarker development with respect to evaluating the suitability of a biomarker for use as a line of evidence:

a. Establishing a *dose-response relationship* for relevant contaminants of concern, and discriminating the toxic effects resulting from exposure to different classes of environmental chemicals (i.e., providing knowledge of chemical specificity). There is also a recognized need to apply chemical analytical and separative methods in tandem with biomarkers in order to identify specific chemical contaminant(s) responsible for the observed biomarker response

b. Evaluating the effect of *nonchemical stressors* (i.e., seasonality, temperature, sex differences, development, etc.) on the magnitude of biomarker responses. For example, several cytochrome P450 enzymes function in hormone metabolism, and these isoforms may be repressed in reproductively active animals. Accordingly, measurement of certain P450 biomarkers in reproductively active fish may be misleading. Similarly, hypoxic conditions may induce a down-regulation of CYP1A expression in some fish associated with alterations of nitric oxide and oxidant status (Rahman and Thomas, 2012).

c. Establishing *ecological relevance*, or at minimum, *linkages to higher-level biological or physiological effects* strengthens the utility of any biomarker. This is especially important in phenotypic anchoring of microarray data with physiological, behavioral, or morphological outcomes.

d. Establishing *temporal associations* among chemical exposure and the *onset of response,* as well as the *permanence of response* (i.e., how soon after exposure is the response detectable and how long after exposure does the response persist). Having this information helps the scientist evaluate how recently exposures may have taken place.

e. Evaluation of *methodological and logistical issues* such as sample handling, robustness of response, ease of measurement, cost of measurement, and technical reproducibility of results among laboratories.

While these criteria represent reasonable guidelines for biomarker development and validation, the lack of adequate scientific funding, as well as data gaps in the state of the science, reflect the reality that not all can be fully incorporated in biomarker studies. Given these criteria, very few, if any, *effects* or *exposure* biomarkers analyzed at the biochemical or physiological level have clearly established linkages at the individual, let alone population or ecosystem level effects. Similarly, impairment of behaviors that are critical to survival (i.e., swimming, feeding, predator-avoidance, prey selection) may ultimately lead to loss of fitness, but these important sublethal behavioral injuries have not yet generated clearly reliable sets of surrogate biomarkers.

An exhaustive review of biomarkers used in aquatic studies is outside the scope of this document and Panel report. However, it is important to note that several biomarkers have been widely incorporated in aquatic studies, and thus are largely accepted by the aquatic community. These particular biomarkers were a focus of the 2007 POD workshop (described below).

For example, commonly used biomarkers of *exposure* often measured in fish include the induction of *cytochrome P450* (i.e., CYP1A), at the messenger RNA, protein, or catalytic activity levels, for exposure to components to oil (Whyte et al. 2000; Lee and Anderson, 2005). The presence of certain types of fluorescent aromatic compounds (FACs) in the bile of fish indicates recent exposure to polycyclic aromatic hydrocarbons (PAHs) and is reviewed in Beyer et al., (2010), while induction of vitellogenin (VTG) in male or juvenile fish has been a commonly used biomarker of exposure to environmental estrogens (e.g. Denslow et al. 2004; Folmar et al. 1996; Jobling et al. 1998). Similarly, metallothionein (MT) mRNA levels in several fish species have been used to document exposures to certain metals (Chesman et al. 2007; Williams and Gallagher, 2013; Espinoza et al., 2012), and inhibition of acetylcholinesterase (AChE) activity has been widely used as a biomarker of exposure to, as well as effects of, carbamate and organophosphorus pesticides (reviewed in Fulton and Key 2001).

In 2007, a Committee reported recommendations for using biomarkers to help discern the role of chemical exposures on the decline of four pelagic fish species in the SFE. The technical document that reported the findings of the task force specifically focused on evaluation of biomarker techniques in future assessments of the POD problem (Anderson et al. 2007). The 2007 Committee reviewed biomarkers, fish life histories, considered chemicals of concern, and fish condition indicators. Conceptually, there was considerable overlap among the scientific issues associated with the pelagic organism decline (POD) in the SFE with the charge to the October 2013 Panel tasked

with evaluating and providing recommendations for biomarker incorporation on a larger scale within the SFE. The key questions examined in 2007 were:

1. How can biomarkers be used strategically to determine whether contaminants cause significant stress in the POD species?

2. How can biomarkers be integrated into a larger framework of investigation?

Some of the most important issues unique to the POD include geographic, seasonal, and temporal variability of the fish populations and contaminant inputs, difficulty in evaluating responses in such a complex water body, lack of a reference condition, and the need for a rapidly-progressing, problem-solving approach. Chemicals of concern (i.e., chemical *classes*, including pesticides, metals, polycyclic aromatic hydrocarbons, and others) were evaluated by the 2007 Committee to provide a framework for the biomarker discussions, because biomarkers are often selected based upon the presumed nature of chemical exposures (discussed more thoroughly in the next section). In contrast to the charge to the 2013 Panel, discussion and emphasis was placed on discriminating chemical versus non-chemical biotic factors (i.e. temperature, salinity, turbidity, etc.).

The 2007 Committee discussions led to the conclusion that, at that time, there was a poor understanding of the *spatial* and *temporal distribution of contaminants* and that there were *numerous compounds of potential significance* that had not been characterized, resulting in a high degree of uncertainty regarding selection of contaminant classes to focus upon. The 2007 Committee analyzed issues related to the feasibility of implementing specific biomarker studies, and this phase of the investigation focused upon logistics associated with various life stages for scientific investigations. Ultimately, a framework for strategic implementation of biomarkers in SFE studies was recommended based upon data gaps as well as the objectives of local regulatory agencies and scientists.

Integration of field and laboratory studies

In addition to the emphasis on field studies, the 2007 Committee recommended *laboratory studies* emphasizing the use of sensitive early life stages. This was primarily due to the fact that these animals are not easily obtained in the field. The Committee recognized the utility of emphasizing resident species toxicity tests using exposures to field-collected samples of water and other media coupled with toxicity identification evaluations (TIE), as well as limited exposures to mixtures. The rationale was that integrated laboratory studies could provide validation of biomarkers that could be applied in subsequent laboratory or field investigations. The laboratory-based studies also allow for rapid screening of environmental samples with a limited number of chemicals, and this may reduce uncertainty about what types of chemical exposures are of greatest concern, while also characterizing important effects in early life-stages. The Committee concluded that while TIE and field biomarker studies can provide insight related to effects from specific toxicants, quantitative responses characterized in the

laboratory could be compared to contaminant levels from field studies to help inform hazard assessments and direct additional investigations. Findings from the first tier of investigations would be used to devise a second tier that might include more extensive use of biomarkers. In particular, data that increase knowledge of potential contaminants of concern, the most significant life-stages for investigation, or of the most relevant types of effects, would be significant in defining future priorities. A hypothetical scenario was proposed in which histological analysis might reveal extensive damage to a particular tissue, which could be further investigated by implementing a targeted analysis of biomarkers that could clarify mechanisms underlying the tissue injury (i.e., modulation of immune function, DNA damage, or oxidative stress, etc.). Moreover, the additional targeted biomarker analysis would reduce the uncertainty associated with determining the effect of chemical exposures on fish health.

Overall, as far as integrating field and laboratory studies to advance the utility of biomarkers for assessing impacts of chemical contaminants on species of concern, two major data gaps were identified:

Laboratory investigations were recommended on embryos and early stage larvae using fish obtained from hatcheries. *In situ* deployments were also recommended for consideration. This priority was based upon assessments of the susceptibility of early life stages, as well as logistical issues associated with capture of embryos and larvae of some species in the field.

Implementation of biomarkers in one or more POD species using an *integrated program* that included both field and laboratory components was recommended. This recommendation was based on a review of the strengths and weaknesses of biomarker techniques at the time, and also evaluation of whether the biomarkers reflected primarily chemical exposures, or clear sublethal effects.

A framework was proposed that included incorporation of fish condition indicators related to reproduction, growth and energetics, and histopathology, along with molecular and physiological biomarkers, to provide an initial assessment of the POD species. Relative to biochemical and molecular indices, which require a relatively high level of scientific expertise for quantitation, several fish condition measurements are straightforward and have been proposed to link individual and population health. It was noted at the time that although alterations in fish condition indicators could support a link between contaminant exposures and effects, these indicators are not chemical-specific and thus could reflect contaminant exposures as well as nonchemical stressors. The issue of nonchemical stressors was a subject of considerable discussion in the 2013 workshop and initial conclusions by the 2013 Panel suggest that investigating the effects of both chemical and non-chemical stressors should be continued.

The 2007 Committee recommended that an iterative study design be incorporated into field studies, to include several indicators of *fish health*, and a small number of *exposure-linked* biomarkers (i.e., vitellogenin/choriogenin, CYP1A expression or activity,

and DNA adducts, although other exposure markers could be applied) measured in adult and juvenile fish sampled in the field. The 2007 Committee also recommended an *archiving program* so that additional fish tissues and environmental samples could be analyzed for biomarkers and contaminants, respectively, once preliminary data from the first set of analyses was obtained. Another benefit of tissue archiving would be to facilitate subsequent biomarker studies when chemicals of concern and locations of contamination are better characterized.

The 2007 Committee identified other data gaps. While recognizing that myriad potential biochemical and molecular biomarkers of exposure and effects had been generated, but not validated, in aquatic studies, the Committee advised a particular emphasis on three areas, including:

- Examination of neurological and neurobehavioral effects of pesticide exposure on POD species including acetylcholinesterase activity as a biomarker.

- Evaluation of multiple stressor responses focusing on the interaction between salinity stress and toxicant exposure using biomarkers of ion regulation and multixenobiotic resistance (MXR).

- A study or studies applying toxicogenomic techniques to one of the POD species.

The 2007 Committee also recommended that biomarker approaches be implemented within an integrated framework of investigations, and that the studies be adequate to discern significant contaminant-related effects within the confines of the best available techniques. Given logistical and potential funding constraints, the Committee further defined a minimum level of effort for implementation of the suggested framework. Specifically, they advised a minimum 3-4 year investigation focusing on two of the POD species to be selected based on policy concerns as well as the availability of hatchery stock. Studies would be conducted throughout the year, with intensive sampling in late winter and early spring due to the increased potential for contaminant exposures and because this is a period when early life stages of several species are present in the system.

Suggestions were also made regarding the need to develop a scientific consortium to provide adequate leadership for large projects, and also with need for strategies for *statistical* and *analytical chemistry analyses* as *essential elements* of all phases of the work. Also of priority was the need for detailed sampling and analysis plans, and ongoing consultations with fisheries modelers to facilitate sampling strategies. In summary, the 2007 Committee recognized biomarkers as useful tools that were underutilized in the context of the POD, but also that there were limitations in their use and application. Specifically, biomarkers should only be implemented within an integrated portfolio of other approaches.

Progress since 2007 in addressing recommendations.

In this section of the report we provide our assessments of the progress that has been made in addressing the research needs and data gaps that were highlighted in the 2007 POD biomarker report. We used information provided to us prior to and during the October 24-25, 2013 Advisory Panel meeting, as well as additional information assembled for us by Dr. Richard Connon following the Panel meeting. We also reviewed relevant literature found using the PUBMED search engine. This was a reasonable approach, given time constraints, but it is possible that some relevant studies were not identified through this process.

For the five (5) priority data gaps in the 2007 Committee report, (described above), we note the following progress:

1. Laboratory investigations and *in situ* field deployments, using early life stage embryos and larvae from hatchery-raised fish.

There have been some recent studies that strongly suggest the sensitivity of larval species to chemical contaminants (pesticides, PAHs) and other stressors in the Bay-Delta. One study in particular demonstrated maternal transfer of lipophilic organics in striped bass that were associated with adverse developmental outcomes in the offspring. Another study provided strong evidence of exposures to PAHs in juvenile striped bass from certain areas of the SFE. However we did not find information on successful field deployments of embryos or early stage larvae of native species. The development of stable, genetically diverse hatchery stocks of Delta smelt should serve as a foundation for these types of investigations.

Ostrach et al. (2008) reported maternal transfer of xenobiotics, primarily polychlorinated biphenyls and polybrominated diphenyl ethers, as well as some current use and legacy compounds, in egg samples from adult striped bass (*Morone saxatalis*) harvested from the Sacramento River. This study directly addressed a key data gap identified in the 2007 POD biomarker report. In addition to maternal transfer of xenobiotics to eggs, the authors demonstrated functional effects on larval development. As discussed below, striped bass have been a species of concern in relation to pelagic organism decline in this ecosystem. The developmental abnormalities reported in the 2008 study included abnormal yolk utilization, perturbed brain and liver development, and impaired growth of larvae of adult female striped bass that were associated with lipophilic organic chemicals. Relative to sampling presented during the 2013 workshop, sampling in Ostrach et al. (2008) was somewhat historical and occurred during 1999 to 2001. Although the results of this study demonstrated maternal transfer of complex mixtures of lipophilic organic pollutants that were strongly associated with developmental alterations in the offspring, addressing the effects of nonchemical stressors on development was not within the scope of the study.

Another study relevant to early life stage data gaps was conducted in juvenile striped bass, by Spearow et al. (2011). In contrast to the 2008 study, this study was directed toward analysis and evaluation of certain biochemical exposure biomarkers in juveniles collected from several sites in the SFE. To complement the field study, hatchery-reared bass were injected with extracts from semi-permeable membrane devices (SPMD) obtained from field deployments, followed by analysis of several exposure biomarkers (this is an example of *ex situ* exposure). The research group conducted comparisons to non-injected (negative) and also positive controls (i.e., chlorpyrifos for acetylcholinesterase inhibition and ß-napthoflavone for CYP1A induction). The use of positive chemical controls helped clarify the biomarker responses associated with the field sample and SPMD sampling. The fact that EROD and also another CYP-associated fluorescent substrate activity (benzyloxyresorufin-*O* deethylase, or BROD) was elevated in estuary fish relevant to hatchery controls, and was further associated with elevated PAHs from SPMD extracts, suggested PAH exposures to the fish and uptake into tissues. Although small increases in liver metallothionein levels (a relevant biomarker for metal exposures) were reported in juvenile bass injected with SPMD extracts from one site, the levels of MT induction were not strongly induced in comparison to fish exposed to metals in the field or laboratory. These data reflected either a likelihood of exposure of juvenile striped bass to metals, or possibly oxidative stress associated with exposure to multiple compounds that may have stimulated MT induction. In this work, biomarker responses were calibrated against positive control compounds for enzyme or biomarker induction, which was in line with recommendations by the 2007 POD Committee.

Other findings from this research group were presented in a final report to the California Department of Water Resources (Ostrach et al., 2009). In particular, the group reported additional evidence for sublethal contaminant exposures to juvenile striped bass occurring through the first 6 months of life. Of note was that exposures were related to sublethal stress and included potential immunosuppression associated with abnormal parasite loads and disease prevalence. Gross lesions that were observed in developing striped bass larvae in a 2006 analysis included abdominal edema, finfold edema, brain edema and necrosis of epithelial tissues in fish harvested from the Sacramento River. The authors further reported that the majority of these lesions were not seen in hatchery larvae and the prevalence of any lesions in hatchery larvae was relatively small. Similar lesions were also reported in a follow-up 2007 study in larvae from female striped bass harvested from the Sacramento River. This report strongly indicates that developing striped bass are sensitive to environmentally relevant contaminant exposures.

Another relevant laboratory investigation in an important POD species was reported by Connon et al. (2009), showing that larval Delta smelt, exposed to environmentally realistic concentrations of the pesticide esfenvalerate, exhibited altered swimming behavior, with younger animals being more affected compared to older (but still juvenile) animals. Moreover this study reported altered expression of several genes that offer promise for development of biomarkers that may help evaluate the effects of pesticides in early life history stages of native fish. This same laboratory also has reported that

larval Delta smelt exposed to copper at concentrations that were environmentally relevant also demonstrated altered swimming behavior, and similarly reported changes in gene expression that could be useful as biomarkers of exposure to this common toxic chemical in the SFE (Connon et al., 2011a). In a study of the effects of ammonia on larval Delta smelt these authors focused on gene expression, not behavior, and found an array of genes showing altered expression after exposure to this non-xenobiotic stressor (Connon et al., 2011b). Other investigators (Werner et al., 2010) have shown that while agricultural return water, contaminated with a variety of current use as well as legacy pesticides, was not acutely toxic to larval fathead minnows, it was toxic to their invertebrate prey base.

Panel Recommendations:

- *In situ and ex situ exposures of early life stages of fish. As examples of in situ exposures, recent publications have shown that wild herring embryos, either naturally spawned at, or transported to, sites impacted by oil spilled from the Cosco Busan in San Francisco Bay, developed lethal abnormalities, especially in the presence of sunlight (Incardona et al 2012a, b). Ex situ exposures could use SPMDs as reported in Spearow et al. (2011), or whole water or sediment samples collected from sites in the SFE. The lack of information concerning real world toxicity of ambient waters in the Delta to embryos and larvae of native fishes remains a critical data gap. Priority: high*

- *Added emphasis on exposure of embryos/larvae to PAHs and other components of fuels. In addition to the findings reported by Incardona et al., the study cited above using SPMDs (Spearow et al., 2011) indicated that PAHs were widespread in the waters of the lower Delta and Suisun Bay. Based on studies to date, it appears that developing embryos of fish are exquisitely sensitive to cardiotoxic effects of PAHs, especially tricyclic PAHs such as phenanthrene (Incardona et al., 2011). Understanding to what degree embryos of native fish in the Delta may be affected by PAH exposure is an important data gap. Priority: moderate.*

- *Effects of nonchemical stressors such as temperature and salinity on developmental stages of target species, including, but not limited to, salmonids, striped bass, and Delta smelt, or suitable surrogates where appropriate. Priority: moderate.*

2. Implementation of some biomarkers in POD species in integrated lab and field studies, including assessment of chemical contaminant exposure in field studies, to determine linkages.

There have been substantive field assessments conducted since 2007, and a number of useful laboratory studies, but we did not see the type of integrated lab and field studies that were called for in the 2007 report, nor have archived fish from field studies been systematically analyzed for contaminant residues.

The work summarized in Teh et al. (2012), and further presented during the Panel meeting, provides a considerable amount of information on biomarkers measured in

Delta smelt, collected from some parts of the Delta. Otoliths were used to assess fish growth and strontium isotope ratios in otoliths were measured in order to determine certain aspects of life history, including transitions into different salinity regimes. Morphometry was used to determine fish condition, HSI, and GSI, and nutritional status was determined by measuring RNA:DNA ratios and triacyl glycerides (TAG). While otolith analyses yielded useful information on fish growth rates and life history, nutritional and morphometric indices were highly variable. Catalytic activities of CYP1A, AChE, and Na/K-ATPase were all measured, fish tissues were examined by histology for disease conditions, and the presence of pathogens was determined. Reproductive status of the fish was also determined, and estradiol levels were measured. Frankly, the Panel was somewhat overwhelmed by the volume of data and the myriad correlations presented during the October 2013 meeting. There did not seem to be clear hypotheses being tested, nor had contaminant exposures been evaluated, at least with respect to the results presented at the meeting. Finally, there were a number of questions concerning methodology, and a lack of laboratory studies that could have demonstrated causality as well as magnitude of biomarker responses to different toxicant exposures. Spearow et al (2011) does represent a useful demonstration of an integrated approach to laboratory and field studies, as described above, but was conducted on fish obtained in 2005. The Panel is of the opinion that integrated laboratory and field investigations are still needed for POD species in the SFE.

Panel Recommendations:

- *The Teh et al (2012) study, and conclusions reached, would benefit from a more in-depth review focused specifically on that body of work. There appears to be much useful information there, but the Panel did not have the time or resources to thoroughly evaluate the data. Chemical analyses should be conducted on some of the archived samples from the large field investigation, to determine if the conclusions reached about contaminants affecting fish health are supported. Otolith studies were very informative and should be continued. Priority: high.*

- *Laboratory exposure studies should be conducted using hatchery reared Delta smelt, to determine the responsiveness of some of the biomarkers that are used as indicators of contaminant exposure in the field studies (e.g. CYP1A, as discussed during the panel meeting). The utility of measuring BROD activities, reported in one study, is questionable due to the lack of biochemical information on the specificity of this substrate for inducible CYP isoforms. Priority: moderate.*

A number of useful laboratory studies on resident species have been conducted, involving exposures to both chemical and non-chemical stressors, and are described in the next three sections as well as the section preceding this one.

3. Examination of neurological and neurobehavioral effects of pesticide exposure on POD species including acetylcholinesterase activity as a biomarker.

There are now an increasing number of laboratory studies which are establishing the

link between pesticide exposures in POD species and altered behaviors, such as reduced swimming speed, that are critical for survival in the real world. In addition to showing relationships between AChE activity and behavior, the approach used in POD species has also involved genomics, both for examining the effects of chemical contaminants as well as non-contaminant stressors on critical behavioral endpoints. As described below, the Panel believes that this line of research is useful and necessary, and should be continued.

Panel Recommendations:

- No further recommendations are made.

4. Evaluation of multiple stressor responses focusing on the interaction between salinity stress and toxicant exposure using biomarkers of ion regulation and multixenobiotic resistance.

Since 2007, there has been a modicum of SFE studies incorporating salinity as an arbitrator of exposure, in context of accompanying abiotic and biotic factors. To date, there is a paucity of research aimed at exposure-specific responses of multixenobiotic resistance (MXR) mechanisms.

Physical attributes of the SFE, with myriad freshwater-brackish water mixing zones, elevates changes in salinity to what might be considered a primary mediator of fish health, bioavailability and cumulative exposures. Estuarine salinity is likely to strongly influence the resident biome, including microbiologic assemblages, at select life stages, and is critical to phenotypic stabilization and biodiversity. Work in this area is described in studies by Connon and Hasenbein (Connon et al., 2011a; Connon et al., 2011b; Hasenbein et al., 2013) Among abiotic factors, investigators measured effects of turbidity, salinity, temperature, and ammonia on Delta smelt, and related those factors to higher order effects, including physiological stress and behavioral aspects such as feeding performance.

Green sturgeon (*Acipenser medirostris*), listed under the Endangered Species Act, were the subject of a recent study (Poletto et al., 2013) that suggests juveniles are not only capable of detecting salt water within the first year of life, but might also actively seek out saline environments, during continued development, as they navigate through watersheds. Another study (Durieux et al., 2011 suggested that salinity, in combination with other natural factors, exhibited no effect on acetylcholinesterase (AChE) activity in brains of striped bass. Acetylcholinesterase activity was positively correlated with water temperature and, to a minor extent, negatively with size of fish; no relationship between AChE and salinity was observed.

Panel Recommendations:

- *More investigation of ion regulatory mechanisms is needed due to the prevalence of non-chemical stressors in the SFE. While not constituting specific markers of*

exposure, changes in these measures may still provide important information relative to teleost health. Among the abiotic characteristics that modify toxicant bioavailability and resultant physiologic responses, salinity is arguably one of the most pervasive biologic pressures. Priority: low.

- *Investigate whether saline-tolerant phenotypes are appearing, because saltwater intrusion into estuarine systems is likely to become a more prominent issue with changing climate. Priority: low*

- *Experiments involving binary mixtures of different toxicant classes as well as non-chemical stressors (as discussed above) are needed, and constitute a reasonable approach to begin addressing the complexity of mixtures of stressors. A major challenge is that the number of mixtures to examine grows exponentially with the number of individual chemicals and exposure levels. In order to make this tractable, the initial focus should be on toxicants that are known to occur in the area, and are either thought to act on the same physiological pathways, or are known to affect detoxification mechanisms that act on the second chemical. Despite the inherent challenges, undertaking experiments involving multiple stressors should provide information to help support decisions concerning water operations in the region. Priority: moderate.*

5. Application of toxicogenomic techniques to one or more of the POD species.

Following the suggestions from the 2007 report, genomics approaches have been developed for three species found in the SFE: the Delta smelt (*Hypomesus transpacificus*), longfin smelt (*Spirinchus thaleichthys*) and silversides (*Menidia beryllina*) by the Connon and Brander groups. Currently there are oligonucleotide spotted arrays for Delta smelt and silversides using the Agilent systems, and an array for the longfin smelt is under development. While all of the studies in this realm are still in their infancy, results are beginning to be provided for both chemical and abiotic stressors in the Bay. It is important to continue to apply these methods both in the laboratory and the field across multiple life stages, different levels of contaminant exposures, and combinations of stressors. It is also critical to try to link changes in the most perturbed molecular pathways with corresponding changes in survival, growth and reproduction of individual fish, as well as population dynamics.

The Connon and Brander groups have collected excellent data on various chemical and non-chemical stressors in the Delta. They have used a combination of microarray and focused q-PCR for a set of target genes to characterize factors affecting Delta smelt. Among abiotic factors, they have measured the effects of turbidity, salinity, temperature and ammonia on Delta smelt and have related these to higher order effects including feeding performance and physiological stress (Hasenbein et al., 2013; Connon et al., 2011b). In addition, these groups examined the effects of anthropogenic contaminants on Delta smelt, including copper (Connon et al 2011a) and esfenvalerate (Connon et al., 2009). For copper, they found significant effects on neuromuscular, digestive and immune responses, and were able to link exposure to effects on swimming velocity. Esfenvalerate also affected swimming velocity, but apparently by a mechanism different than that of copper. Esfenvalerate also affected the expression of genes associated

with immune responses, apoptosis, redox, osmotic stress, detoxification, growth, and development (Connon et al., 2009). This group also identified gene transcription effects from exposures to sewage treatment effluents downstream from the Sacramento Regional Wastewater Treatment Plant (SRWTP) in addition to other locations of concern around the estuary (Hasenbein et al., 2014). What is still needed for these studies is quantitative assessment of exposures related to adverse outcome pathways (Ankley et al., 2010, discussed in more detail below).

Significant information about the natural life stages of Delta smelt has been provided by studies such as swimming performance (Swanson and Young, 1998) and observations of Delta smelt in captive populations (Fisch et al., 2013). Measurements of swimming velocity and endurance (Swanson and Young, 1998) need to be taken into account when analyzing contaminants that may affect swimming behavior. The captive program aims to maintain genetic diversity in the population and also determine appropriate management strategies for this species to ensure reliable production of successive generations (Fisch et al., 2013).

Because it is difficult to work with the Delta smelt in the laboratory, it is commendable that a more tractable surrogate species is being considered, the silversides (*Menidia beryllina*). Brander et al (2012) began to use this species as a new model organism for endocrine disruption, with the first exposures to estrogens in laboratory conditions, and have developed molecular biomarkers at both the mRNA and protein level for vitellogenin and choriogenin. It appears that silversides are much more sensitive to estrogens than other estuarine model species examined, for example, sheepshead minnow (*Cyprinodon variegatus*) (Folmar et al., 2000, 2002), and thus an excellent choice as a surrogate for the Delta smelt. Brander et al. (2013) have also tested silversides exposed to waters from different locations around San Francisco Bay. The authors reported the presence of both estrogens and androgens at some of the locations, and that the presence of both seemed to reduce the effects of exposures to estrogens alone (Brander et al., 2013). Higher order effects in this study included testicular necrosis, altered somatic growth and sex ratios skewed towards males.

This group has also exposed silversides to bifenthrin and permethrin, two insecticides thought to be present in the Bay, and found that *in vivo* exposures increase choriogenin, even though *in vitro* assays with the CALUX cell based assay showed that these chemicals are functional antagonists of the estrogen receptor. This suggested effects resulted either from metabolites (Brander et al., 2012b) or perhaps at higher points in the HPG axis as was demonstrated in steelhead *(Oncorhynchus mykiss*) under hypersaline conditions (Riar et al., 2013; Forsgren et al., 2013). Studies such as these will help identify molecular mechanisms by which contaminants affect adverse outcomes in fish. Continued work in this area is necessary to better connect the molecular initiating events from exposures to bifenthrin and permethrin to measured apical endpoints. It also serves as a cautionary tale about the limitations of our mechanistic understanding of physiology, and the dangers of overreliance on physiological models for predicting apical endpoints from early molecular events in the exposure-to-effect cascade.

Brander is now collaborating with Connon to develop a microarray for silversides that has potential to be an excellent tool to investigate transcriptional mechanisms by which contaminants are causing effects. Studies such as these, when linked with analysis of effects at higher levels of biological organization, may ultimately help with better understanding adverse outcomes and identification of stressors affecting fish in the SFE.

Another species of importance to the SFE is the green sturgeon (*Acipenser medirostris*), which is currently endangered. This fish migrates over long distances from estuary to estuary and up rivers to spawning sites. Huff et al. (2012) have modeled its migratory behavior based on the physical conditions in the environment. It appears to be sensitive to changes in temperature and salinity. A study by Sardella et al. (2008) measured ventilatory function and osmoregulatory control in this species with increasing temperature and salinity. Fangue's laboratory is studying the adaptability of green sturgeon to changes in salinity (Poletto et al., 2013). These studies provide relevant physiological information that can be used to anchor molecular studies. It is important to measure quantitative changes in molecular endpoints and to interpret these based on the life cycle and physiology of the species in question. One study performed proteomics studies to measure global changes in protein expression in fish that were exposed to selenium and high temperature. The main changes observed were for proteins involved in protein folding, protein synthesis, protein degradation, ATP supply and cellular structure (Silvestre et al., 2010). But it is not clear how these changes relate to higher order endpoints. To date there are no other 'omics studies with this species. Investigators should be encouraged to also use non-gel based proteomics measurements (such as those based on mass spectroscopy), because these are more cost effective in generating data that can be incorporated into pathway analysis paradigms.

Other fish species of interest in the SFE include striped bass (*Morone saxatilis*), threadfin shad (*Dorosoma petenense*), topsmelt (*Atherinops affinis*), Pacific herring (C*lupea pallasii*), splittail (*Pogonichthys macrolepidotus*) and the arrow goby (*Clevelandia ios*). These species have generally been studied to determine their physiological performance, behavior, or the presence of contaminants in fish tissues including mercury (Greenfield et al., 2013; Davis et al., 2012), PCBs (Greenfield and Allen, 2013), nonylphenol (Diehl et al., 2012), perfluoroalkyl compounds (Sedlak and Greig, 2012), oil spills (Incardona et al., 2012a) and contaminants that may target acetylcholinesterase activity (Durieux et al., 2011). No global molecular or apical endpoint studies have been conducted in these species to determine whether or not the tissue burdens of these contaminants are having adverse biological effects in the fish. Of these species, molecular tools are available only for striped bass (Reading et al., 2012) but could be developed quickly for other species, if deemed necessary.

<u>Incorporation of Adverse Outcome Pathways.</u> A recent trend in toxicology is the idea of progressing from molecular initiating events for a particular contaminant to higher-level individual or community effects using the Adverse Outcome Pathway (AOP) framework (Ankley et al., 2010). This framework is typically composed of a '*molecular initiating*

event' in which a chemical interacts with a biological target, a series of intermediate steps termed key events, and culminates in [an] adverse outcome. The 'prime mover', which is the molecular initiating event, apparently anchors the mechanistic process and offers credence to advance to population outcomes, defined as a sequential series of higher order effects to produce an adverse outcome with direct relevance to a specific risk assessment within a population (e.g., survival, development, reproduction, etc.). The AOP concept, originating in a framework developed by OECD (Organisation for Economic Co-operation and Development), tends to operate with reasonable limits when applied to human health. AOP models have recently been introduced in human risk assessment as pragmatic tools with multiple applications (Vinken et al., 2013).

It is not clear how the AOP framework would apply to ecotoxicology, where organisms are typically exposed to chemical mixtures, and with the inherent difficulty of separating out the effects of each chemical or natural stressor. Furthermore, the development and validation of an AOP requires a considerable amount of scientific data due to the fact that there needs to be a clear and unambiguous understanding of initiating molecular events, cellular and organism level responses, and also clear linkages to population effects. However, the framework does offer at least a guiding principle for interpreting molecular biomarkers in the context of the biochemical pathways in which they function.

Substantially more work needs to be accomplished in order to use molecular tools and biomarkers for assessment of conditions in the SFE. Specifically, linkage of gene expression with higher-level biological effects is needed. An ongoing concern (in the SFE context and elsewhere) is that gene expression and other exposure biomarker changes usually have not been quantitatively linked to individual, much less population, apical endpoints. As a result, it is difficult to distinguish between biomarker responses that may be consistent with population stability versus those that could be indicative of population decline. Biomarkers, as currently used, typically only provide qualitative indications about any ecological significance of environmental exposures. What is needed is to tie in molecular responses with adverse effects on growth, survival, and reproduction, and ultimately, to measures of population viability.

Evaluation and Interpretation of Multiple Molecular Biomarkers. In a system like the SFE, where multiple stressors (abiotic and biotic) co-occur, biomarker information has limited utility – just knowing stressors are present is unsurprising – what is needed is knowledge of which stressors are most important. It may be possible to rule some stressors out, but such conclusions are subject to significant caveats: perhaps measurements were not taken at the right time or place, or perhaps measurements are not sensitive enough to detect ecologically significant exposures. Thus, in this context, it is important to improve the interpretability of exposure biomarkers through more quantitative associations with indicators of individual or population condition.

Establishing binary quantitative 'cutoffs' of molecular measurements (corresponding to predictions of 'effect' or 'no effect' given a particular molecular response level) is one approach to establishing these relationships. Operationally, the binary cutoff could be identified using classification methods. Another approach is to develop continuous predictors, where the level of a measured molecular response is transformed into a

prediction of a particular level of impairment of growth, survival, reproduction, or population trajectory, using quantitative methods such as regression. Either of these two approaches are typically developed using controlled laboratory or mesocosm exposures, and then applied in environmental assessments by either making molecular measurements on wild-collected fish, on fish exposed in the lab to environmental samples (or fractionated material from environmental samples), or on fish that have been caged at sites of interest. Predictive relationships established using laboratory or mesocosm exposures should, however, be considered rough approximations to real-world relationships, due to inevitable differences in factors moderating dose responses that are hard to accurately reproduce during controlled exposures. Such factors include, but are not limited to, food availability, dietary composition, temperature fluctuation, predation, migratory stress, and co-occurring contaminants. In addition, application of relationships established using surrogate species to species of greater programmatic interest would be complicated by species differences in dose responsiveness that must also be accounted for. As a result, these cutoffs and relationships will typically not be exact or completely reliable, but may nevertheless provide very important information for consideration in a more comprehensive stressor characterization effort.

A third approach to studying causal connections between biomarkers and apical endpoints would be comparative evaluation of the strength of association between different classes of molecular responses and indicators of condition in wild fish. In this approach, one would collect fish from the wild, measure a range of molecular response levels, and evaluate the strength of association between different molecular responses and fish condition. The molecular responses with the highest levels of association to adverse fish condition would then suggest types of exposures with higher likelihood of causing impairment in fish condition. This sort of approach may reduce the need for expensive laboratory or mesocosm experiments, but would require consideration of the timescales over which molecular responses and condition indicators integrate stressor exposure information. In particular, most molecular responses reflect only the last few days of exposure, while many indicators of condition (such as fork length) reflect months of exposure history. This temporal mismatch complicates efforts to associate these measurements. Certain indicators of condition, such as otolith growth ring spacing, provide more detailed information on energy status over time, with resolution down to the day, and may therefore serve as more suitable condition indicators for association with molecular responses. Nevertheless, even otolith growth ring information is not straightforward to apply in this context, due to the dependency of growth ring deposition rate on the age and reproductive status of the fish. Accounting for these factors is possible, but requires care in experimental design and sophistication in the use of quantitative techniques. For this general approach to work, sample preservation techniques may have to be adjusted so that condition and molecular response measurements can be made in the same fish.

Predictive and associative approaches are in many ways complementary and are probably best implemented in parallel. The associative approach will in most cases be the least expensive to implement, and may be more directly interpretable due to the use of wild fish exposed under real-world conditions, and pairing with condition information

in the same fish. On the other hand, this approach may not be applicable to certain scenarios, such as estrogen exposure, which can dramatically affect reproduction without notably affecting most condition indicators. Another example would be stressors affecting predator avoidance, which could significantly reduce survival, again without major effects on the condition of surviving fish. Predictive approaches can provide useful information in cases such as these where the associative approach is likely to fail, since molecular responses are still possible to observe, and connections to reproduction and predator avoidance can be established under laboratory conditions. On the other hand, the predictive approaches depend on controlled experiments for their development, which require a great deal of time, money, as well as labor to carry out, and therefore can only be expected to be developed for a small handful of strongly suspected stressor classes. The associative approach, by contrast, can be carried out for a very large set of molecular responses, especially when methods such as microarray measurement are employed. Therefore a much broader 'net' can be cast using the associative approach, reducing the risk of missing potentially important stressor classes.

Once a suggestive relationship is identified between a particular molecular response and an apical endpoint, the responsible stressor still needs to be identified. Molecular responses to chemical contaminants are typically consistent with exposure to a variety of contaminants that can impinge on the same physiological pathways, and therefore substantial uncertainty about the identity of the responsible contaminant will persist. One approach to identifying the responsible contaminant is to look for 'usual suspects' in water samples (preferably collected in a way that they can be 'matched' to the molecular measurements) using chemical analysis. The 'usual suspect' list can sometimes be constructed based on local considerations (such as known inputs from mining, agriculture, or industrial activity). Other times, the molecular response will be fairly well characterized, with a well-known list of commonly occurring contaminants capable of eliciting the response, such as the up-regulation of vitellogenin, which is induced by a relatively small number of contaminants that are frequently found in environmental samples. For some other molecular responses, particularly in cases where a broad net has been cast using associative methods, a more open-ended approach may be more practical. In this latter case, environmental samples could be chemically fractionated and the biological activity of the fractions followed using an assay developed around the molecular response, until a single contaminant associated with the molecular response is identified. In any case, the connection between the suspect chemical and the apical endpoint should be corroborated by laboratory studies demonstrating a relationship between measured environmental levels of the contaminant and substantial effects on an apical endpoint predictive of impaired population trajectory.

<u>Panel Recommendations:</u>

- *As was pointed out in the 2007 POD report, it will be critical to integrate toxicogenomics, condition indicators (including, but not limited to histology), apical endpoint measurements, and chemical analysis in order to approach an understanding*

of stressors contributing most strongly to the POD. Toxicogenomics provides added value to this effort by allowing detection of effects on virtually all physiological pathways, with substantially improved stressor specificity, readout speed, sensitivity, and sample size requirements compared to traditional apical endpoint assays or condition indicators. Nevertheless, limitations of toxicogenomics with respect to interpretability in terms of growth, survival, reproduction, or population trajectory and limitations of specificity with regard to eliciting stressors dictates the need for applying these techniques in parallel with more traditional assays. It is also important to consider that toxicogenomics only provide a "snapshot " of the steady-state messenger RNA profiles in the tissue of interest, and at the time of sampling. It is also recommended that temporal experiments be conducted that may lead to a better understanding of changes that are reversible, or adaptive, versus those that lead to permanent tissue injury. Finally, because stage of development for early life stages or stage of reproduction for fish that are seasonal spawners influences the results from gene expression studies, it is also recommended that careful attention be paid to comparing similar life stages for reference fish and exposed fish. Priority: High

- *Continue to develop specific molecular toxicogenomics approaches (i.e., species-specific microarrays, RNA seq, genomic resources). Of particular importance is to use species that can also be exposed in the laboratory for verification studies. Species of interest include Menidia, top smelt, green sturgeon, and salmonids, among others. Priority: High*

- *Conduct pilot studies in the laboratory using model POD species together with toxicants known to occur in the area, to determine the potential added value over RNA abundance measures of employing other toxicogenomic methods for diagnosing fish condition and elucidating exposure history. Such methods include non-gel based global proteomics (such as mass-spectroscopy-based methods) and DNA methylation pattern determination. Priority: Medium*

- *Pair analytical chemistry with bioanalytical assays in the field to determine overall chemical equivalencies (TEQs) for endocrine related receptor transactivation. These could include assays currently in use by EPA or commercially available through several vendors. Priority: High*

Cross-walk of charge questions with Panel report

The charges laid to the Panel consisted of a three part overall purpose for convening the Panel, 7 "Questions", and 12 "Additional Questions". In our deliberations, the Panel felt that many of these purposes and questions were identical to the charges given to the 2007 POD biomarker Committee, and that there was considerable overlap between the 18 specific questions. We therefore focused our report on evaluating progress made in responding to the recommendations made in the 2007 report and provide our recommendations for addressing remaining data gaps, as well as recommendations for future directions in using biomarkers to characterize and help manage the SFE system. This approach was given at the end of the October 2013 workshop, and was generally

approved as a constructive way in which to frame our report. In this section of the report we do however provide our thoughts on whether, and how, we have addressed the original charges and questions given to us prior to the workshop.

The purposes given for the Panel to be convened were to:

1. Assess the potential application of biomarkers for evaluating stressors and/or adverse effects on SFE fishes;
2. Identify biomarkers that should be focused on in future SFE research; and,
3. Identify data gaps and develop a research framework to guide the role and application of biomarkers within the Bay-Delta ecosystem.

In this report we have provided specific recommendations for development and application of biomarkers to assessing stressors and effects in SFE fishes. While there is potential, we have in numerous places provided cautions concerning application. Given the complexity of the physical and ecological attributes of the SFE, at best we can say that biomarkers do have potential, but for most biomarkers, for the foreseeable future, it is not realistic to be able to conclude that "biomarker X measured in species Y tells you what the biological effect of stressor Z is". Biomarkers can only add to a thoughtful weight-of-evidence approach. In both our specific recommendations in response to the 2007 POD report, and suggested future directions, we have identified biomarkers, or classes of biomarkers, that we think should be focused on in SFE research. Finally, the framework proposed by the 2007 Committee was, and is, a reasonable framework to guide the development and application of biomarkers in the SFE system.

The seven "Questions" were:

1) How can biomarkers help us understand the relative health of organisms and the natural variability in these measured conditions?

2) What is the relative importance of biomarkers on individual organisms and population health?

3) How can current biomarker systems be used strategically to determine whether anthropogenic, physicochemical, and/or biological influences are causing significant stress in SFE species?

4) What is the relative importance of these stressors to individual and population-level impacts, and thus ecosystem functioning?

5) How can baselines, references or controls be established for field-based assessments?

6) How can spatio-temporal variability be incorporated into biomarker data analyses?

7) What are the relevant pros and cons that we need to be aware, or cautious, of?

Of these seven questions, the first four consist of two questions given to the 2007 Committee, and an updating of those same questions for this current Panel. Thus, we were actually being asked only questions 3 and 4 in the list of seven. For question 3, the Panel believes that we have given constructive recommendations on how to use biomarkers strategically to determine whether various factors are causing stress to the SFE ecosystem, especially in our responses concerning integration of field and laboratory investigations. Question 4, concerning the relative importance of different stressors, we believe to be beyond the ability of this Panel to determine with available information.

Questions 5, 6, and 7 were focused on how biomarkers can be used in field studies and in monitoring. The Panel has pointed out that there now exists a considerable amount of data, as well as archived tissues, that can potentially provide substantive baseline information, and we have given specific recommendations for evaluating that data (in depth review of the FLaSH study) as well as improving the knowledge base around those data (analytical chemistry on archived tissues). Regarding spatio-temporal variability and "pros and cons", there are numerous recommendations in this report that address many of those concerns, as there were in the 2007 POD report. Those issues essentially remain constant across field and monitoring studies, but they are amplified in a system like the SFE.

Additional Questions:

In general, many of the additional questions are addressed in the report, but often diffused among different sections. Below are brief assessments of how the additional questions were or were not considered.

- *What specific information on health condition should be obtained to support biomarker assessments and monitoring? Consider contaminant transport and fate, bioavailability, and bioaccumulation, in conjunction with physicochemical and biological influences.*

This question was not specifically addressed by the Panel, but in general we believe that there is value in measuring the following list of attributes in field and laboratory investigations: Fish size and condition, especially with respect to age; reproductive status, including fecundity of females, plasma levels of VTG and other hormones in both males and females; tissue structure, assessed by histopathology; immune function and disease status.

- *What are the current benefits and limitations of the use of single versus multi-biomarker approaches?*

While the report does not specifically address this issue, the Panel is of the opinion that reliance on any single biomarker, in a system as biologically and physically complex as the SFE, is not reasonable or recommended. Multiple biomarkers will be needed in

almost all field assessments, and these can be a combination of molecular, biochemical, and condition biomarkers. It is important to differentiate and incorporate biomarkers of *exposure* as well as biomarkers that discriminate toxicological *effects.*

• *What is the suitability of current biomarkers and/or novel approaches such as genomics, proteomics and metabolomics, to monitoring population health?*

There is a great deal of information in the report concerning the Panel's recommendations and cautions regarding linking current and "novel" biomarkers to higher levels of biological organization, including at organismal and population levels.

• *How can biomarker systems be used to assess effects of these stressors, and their interactions within 1) field populations? 2) laboratory studies? and 3) in-situ/ex-situ exposures?*

The report makes a number of specific recommendations for field, laboratory, and *in situ* applications of biomarkers. We did not specifically discuss *ex situ* exposures, but as long as care is taken to maintain the stability of the environmental matrices used for *ex situ* exposures, those types of exposures can be very useful.

• *How best can field and laboratory based studies be integrated, from a biomarker perspective?*

The report provides specific recommendations for improving recently conducted field studies, including conducting laboratory studies that would work towards such field/lab integrations.

• *How do non-lethal vs. lethal sampling limit the use of biomarker assessments?*

This was not specifically considered in detail by the Panel. There are practical limitations in sampling threatened species, and in general, a thoughtful experimental design should include power analysis with regards to sampling and biological endpoints, in order to use the fewest animals required. Alternatives would be studies using cultured or surrogate species wherever possible. In general, nonlethal sampling methods need to be carefully validated for analyte recovery and variability, which can confound data interpretation.

• *Should multiple species or a single species be selected as a model for biomarker investigations? Which species and why?*

The Panel is of the opinion that selecting a single species as a model for the SFE is too limited, and that as questions arise, appropriate species should be investigated. As well, in some cases the use of surrogate species, rather than native T&E species, will be required. There will be some situations where it is important to sample a species with high site fidelity, as opposed to a more migratory species, or both. This is largely driven by the question or hypothesis being tested.

• *How can AOPs or associations with higher levels of biological organization be integrated into the Delta monitoring approaches?*

The report provides the Panel's perspective on application of the AOP approach. In general we recommend caution, because a thoughtful and rigorous AOP necessitates a detailed understanding of initiating molecular events and subsequent outcomes at higher biochemical, tissue, and physiological levels. This requires a tremendous amount of data.

• *How best can we integrate life histories, and specific life stages into planned studies?*

The report provides specific recommendations on further work needed on early life stages, as this remains a critical need.

• *What additional information should be collected to aid interpretation of biomarker data?*

In addition to the health parameters given above, additional measures of animal fitness, such as behavior and swimming ability, can be very useful. However these are best measured in laboratory studies, as a complement to field studies. Information on abiotic parameters, such as salinity, temperature, and turbidity, should also be collected during field investigations, as described in this report. And, as discussed in the report, co-occurrence of chemical contaminants, either in biological tissues and fluids, or in environmental matrices at the time and site of sampling, is important to determine.

• *What analytical approaches would likely be most useful for interpreting biomarker data and understanding its environmental relevance?*

The TIE approach, as discussed in this report, is one way to help determine what chemicals might be contributing to biomarker responses. Although the Panel did not discuss bioanalytical assays that aggregate mode of action from contaminants that behave similarly, they can be useful tools. The use of bioanalytical assays was recommended in a report to the California Water Resources Control Board (Anderson et al., 2012), and a summary of those recommendations is added to this report as an appendix.

• *How do we extrapolate biomarker findings to fundamental fitness parameters such as survival, growth, and reproduction?*

Recommendations for linking biomarker data to fitness parameters (including behavioral endpoints) are found throughout the report.

Future directions for applications of toxicogenomics in the SFE

Development and Use of Molecular Signatures

The SFE is exceedingly complex because of ever-changing abiotic stressors, the overall geochemical condition of the entire area, thermal and hydrologic mixing, intrusion of salinity into fresh water systems, naturally occurring chemical compounds, and myriad chemicals discharged as a result of human activities. For toxicogenomics studies,

rather than trying to find specific gene responses for specific contaminant exposures, it is better to look for biochemical pathways of toxicity that correlate with higher order molecular indicators relating to adverse outcomes. It is recommended that investigators use a systems approach to interpreting complex toxicogenomic data and to develop algorithms to discriminate gene expression patterns and profiles that result from geophysical constituents from those that result from exposures to chemical toxicants. Gene expression libraries should be built based on exposures of sensitive species in scaled down mesocosms, that to the extent possible, replicate physiochemical conditions measured at regional sites throughout the SFE. Specific chemical compound exposures, and mixtures, should be employed on a background of natural abiotic factors. Generally, if the laboratory exposure conditions are too strictly controlled, it is possible to miss parameters that influence gene expression changes in the environment, as noted by Katagi (2010).

Training sets for learning gene expression contours and assembling unbiased expression patterns are detailed in many peer-reviewed references (Boscolo et al., 2008; de Ridder et al., 2013; Du et al., 2013; Foran et al., 2013; Harris and Ghaffari, 2008; Romualdi et al., 2003). This approach will necessarily require careful spatial calibration within aquatic systems, considering also diurnal factors, seasonality and the advent of changing climatic conditions.

The overall goal in developing molecular signatures should be to focus the reporting and use of molecular and cellular biomarkers in such a way that these tools can inform public policy and decisions made by resource managers. This could have application in many conservation efforts, including actions directed towards remediation and mitigation.

Epigenetics

SFE investigators could soon begin to study how contaminants interact with the epigenome of exposed animals, specifically DNA methylation and histone modification (Bossdorf et al., 2008; Crews and Gore, 2011; Crews and Gore, 2012; Kilvitis et al., 2014). Epigenetics is the intersection of genomes and the environment. As stated in one review (Beldade et al., 2011) external environmental cues can influence not only molecular mechanisms of adaptive developmental plasticity, but also trajectories that result in distinct phenotypes. Research accomplished over the last decade has indicated that germline-dependent epigenetic modifications (Crews and Gore, 2011) by chemicals such as endocrine disrupting chemicals (for example, vinclozolin) can be inherited by the offspring (Guererro-Bosagna et al., 2012; Anway and Skinner, 2008). Germline-independent epigenetic modifications that occur in adults are not inherited in a trans-generational manner.

Changes in DNA methylation, unlike DNA mutations, are potentially under constant environmental pressure. These changes are generally stable over the lifespan of individuals and have capacity to direct different scenarios of heritability (Angers et al., 2011). These characteristics make the epigenetic phenomena of DNA methylation a potentially important molecular process by which to monitor organismal change in context of changing environments.

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Appendix A. Summary of recommendations from 2012 report to the California Water Resources Control Board

Recommendations from SCCWRP Report

The California Water Resources Control Board assembled a Science Advisory Panel in October 2009 to make recommendations regarding chemicals of emerging concern that are entering California waters from discharge of treated municipal wastewaters or from stormwater. The Science Advisory Panel worked under the guidance of the Southern California Coastal Water Research Project and a final report was submitted in April 2012 (Anderson et al., 2012). The report developed a risk-based screening framework that is centered on knowing both the occurrence and toxicologic impact of individual CECs in various receiving waters in CA. It was clear that insufficient information exists on both occurrence and toxicology and the panel made specific recommendations for how to begin to fill the data gaps. In particular the panel thought that more effort should be devoted to (1) developing and using high throughput *in vitro* bioassays to screen for activity of CECs for receptors of interest; (2) establishing linkage between high throughput bioassays and higher order effects; and (3) developing strong adverse outcome pathways through the use of holistic molecular methods such as microarrays and firmly anchoring the gene changes to apical endpoints related to growth, reproduction, survival and susceptibility to disease. Along side these bioanalytical methods, there was a recommendation to get better information about source contribution, occurrence and toxicity of CECS through better fate and effects models.

Specific research recommendations:

1. Develop and validate high throughput bioanalytical assays to screen water and sediments with a focus on receptors of ecological relevance. These assays should measure the activity of chemicals by their mode of action.
2. Develop and validate adverse outcome pathways *in vivo* that are targeted by the contaminants. This would require whole animal exposures linked to effects in survival, reproduction, growth, and susceptibility to disease. “Omics” technologies are advantageous in pulling together complex higher order effects in a way that can be analyzed by systems approaches. It may be important to perform a set of whole organism experiments to start getting the linkages in place.
3. Develop a laboratory testable organism that can be used in the marine environment. It would be good to choose an organism for which general toxicity assays already exist.
4. Determine whether or not fish embryo assays would capture the full effects expected for other life stages.
5. Mixture experiments in the laboratory
6. Develop standard assays to measure antibiotic resistance in receiving waters
7. Develop standard protocols for extracting CECs from water, sediments and tissues.

8. Develop appropriate reference locations for monitoring
9. Use conceptual models to estimate occurrence, distribution
10. Improve and expand the application of conceptual models to estimate occurrence, distribution of CECs among different compartments (e.g. aqueous, particulate, sediment, organisms) to help with better monitoring.
11. Develop models to estimate predicted environmental concentrations
12. Broaden the analytical chemistry approach to identify CECs by mass spectrometry using a broad scanning method to identify unknowns.

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Appendix B. Discussion of salinity and other abiotic factors as they can affect the use and application of biomarkers in the SFE

SALINITY IN AQUATIC ECOSYSTEMS

Physical attributes of the Bay-Delta Estuary, with myriad freshwater-brackish water mixing zones, elevates changes in salinity to what might be considered a primary mediator of fish health, as well as bioavailability and cumulative exposures to contaminants. As a primary attribute, estuarine salinity is brought to bear on the resident biome at select life stages of the whole, and is critical to phenotypic stabilization and biodiversity. The factor is essential for developing and maintaining conservation and management strategies and has been elevated to critical status when considered in context of global climate change. Numerous research efforts - some in progress, using established assays indicative of altered salinity, have been undertaken pursuant to recommendations set forth in 2007, and there is acknowledgement that bioavailability of co-occurring chemical toxicants - and by extension, degree of respective exposures - might be mediated by normal organismal responses to shifts in salinity values. Noted also is the physiologic verity regarding life-stage specific capacity for ionic regulation. Measured activity of ion regulatory mechanisms do not constitute specific markers of exposure, but do provide essential companion data relative to teleost health. Among the multifactorial physiochemical abiotic characteristics that modify toxicant bioavailability and resultant physiologic responses, estuarine salinity contributes arguably one of the most pervasive and predictable biologic pressures.

Estuarine salinity varies with discharge; therefore, variance in precipitation coincident with changing climate might shift regions of low salinity and disease refuge. Considering the aforementioned, a study of oyster bottom habitat (Levinton et al., 2011) inferred negative impacts on reproduction and survival. The study also noted that temperature is an additional factor influencing survival of oysters and other aquatic species, and recent global temperature increases have amplified vulnerability to disease in regions of greater salinity. Salinity gradient and associated dynamics are among the primary physical characteristic of any estuarine ecosystem. Associated with salinity gradients of most estuarine ecosystems is the critical range of 5-8 PSU salinity, wherein key ***biotic*** and ***abiotic*** processes exhibit non-linear dynamics of rate changes. Acknowledged in a review of estuarine ecosystem processes (Telesh and Khlebovich, 2010), the above range of salinity serves two critical functions; the external ecological factor, and the homeostatic internal physiologic and cellular environment of aquatic organisms. It effectively partitions conditions for sustaining life of freshwater and marine faunas, delineates invertebrate communities with differential osmotic regulation, and establishes

distribution boundaries for remaining taxa. Taking into account spatiotemporal macrobenthic assemblages in the San Francisco Estuary and Sacramento-San Joaquin River Delta salinity gradients, Thompson et al (Thompson et al., 2013) identified five salinity gradient-specific benthic assemblages. Investigators noted that while most sites assemblages remained stable, a small number of sites exchanged designations of assemblage based on seasonal responses to salinity conversion owing to freshwater inflows. Sustainable food sources within estuarine systems are likewise subject to changing conditions of salinity. Along with salinity, nutrient enrichment and altered nutrient ratios are brought to bear across resident populations and communities. The continuum - estuarine to coastal - is subject to compound nutrient restrictions that occur among nitrogen, phosphorus, and silicon along the salinity gradient with seasonal influence. In one body of work (Rabalais, 2002), the author suggests that nitrogen is generally considered the primary limiting nutrient for phytoplankton biomass accumulation. When ecosystem thresholds are breached, catastrophic outcomes and disruption of ecosystem function might come to pass, including - but not limited to - toxic algal blooms, increased turbidity with associated loss of aquatic vegetation, paucity of dissolved oxygen, loss of habitat and biodiversity, shifts in food webs, and fishery crashes.

Data from a recent SFE-related study by Hasenbein et al. (2013) suggests that feeding behavior is influenced by conditions of turbidity, whereas salinity was found to be an important abiotic factor affecting cellular stress response in delta smelt. Investigators demonstrated greater abundances of delta smelt in low-salinity zones (0.5-6.0 ppt) within San Francisco Bay; a zone that is also presumed to exhibit most favorable turbidities. This body of work, relating biology of juvenile delta smelt to major abiotic factors, should help inform decisions made by resource managers in support of efforts to preserve this essential Bay-Delta teleost.

Several biomarkers have been considered in context of the Bay-Delta Estuary, with an eye on salinity as a modifying function of organismal response. Among them are the following; a recent investigation focused on effects of naturally occurring aquatic factors on activity of acetylcholinesterase (AChE) in striped bass (*Morone saxatilis*) (Durieux et al., 2011). This commonly measured biomarker was analyzed for spatiotemporal variability in brains of Young-Of-Year individuals collected monthly from August 2007 to January 2008, at 12 different sites in the San Francisco Estuary system. Authors indicated a positive correlation between enzyme activity and water temperature and, to a lesser extent, negatively with fish size while no relationship was detected with salinity. As with development and validation of any biomarker, irrespective of biologic hierarchy, knowledge of and function in context of system-specific abiotic factors must be an embedded convention. Another study (Werner, 2004) aimed at assessment of anthropogenic responses in SFE-dwelling exotic clam, *Potamocorbula amurensis*, in

view of salinous background, found that over a range of salinities, physiologic capacity to induce apposite levels of cellular *hsp*70 in response to heat-shock was considerably diminished. The author concludes that, in attempts to monitor aquatic organisms for health conditions and toxicant exposure, biomarkers applied in field studies should not be impinged upon by changes in naturally occurring ecological parameters such as salinity. This, according to the Principal Investigator, is especially important in estuarine environments, and for those indicators such as heat-shock protein, which are adequate companion markers, albeit comparatively nonspecific.

Saltwater intrusion into estuaries, likely to become an increasingly more prominent issue with changing climate profiles, creates stressful conditions for aquatic species that move about independent of hydrologic currents. In an attempt to form strong inference regarding gene flow as a function of changeable conditions of salinity within an estuary (Purcell et al., 2012), investigators evaluated the genetic structure of western mosquitofish (*Gambusia affinis*) populations that were previously shown to have developed adaptations for increased salinity tolerance. The study suggested advent of saline-tolerant phenotypes due to local adaptation. Overall there is conjecture that within limited genetic structure, juxtaposed with selection to saltwater incursions, species phenotype exhibited variance despite customary physical barriers to gene flow.

ABIOTIC FACTORS; PHYSIO- AND GEOCHEMICAL MODIFIERS OF BIOAVAILABILITY AND EXPOSURE

In numerous bodies of work engendered by SFE investigators and aimed at developing biomarkers for condition of aquatic organisms and exposures thereto, the byzantine attributes of abiotic conditions in aquatic ecosystems is widely acknowledged; however, challenges to development and validation of cellular biomarkers, in the face of such overwhelming complexities might be misjudged. Aquatic systems are reasonably delineated by physio- and geochemical factors which are influenced by regional geochemistry, sediment type(s), fate and transport of natural and manmade chemicals, local weather and air deposition, to name just a few, all of which can affect and modify the toxicity of pollutants by, among other mechanisms, altering stressor bioavailability and uptake. A vital manuscript, published in 1983 (Babich and Stotzky, 1983), was an appeal for the United States Environmental Protection Agency to consider, in all future assessments of aquatic systems, biologic effects modified by a host of abiotic factors. Features, highlighted in the above reference - although not an exhaustive inventory - likely to be present in most aquatic ecosystems are; **pH (acidity/alkalinity), Eh (oxidation-reduction potential), aeration status (aerobic, microaerobic, anaerobic), buffering capacity, inorganic anionic composition, inorganic cationic composition, water content, clay mineralogy, hydrous metal oxides, organic matter, cation exchange capacity, anion exchange capacity, temperature, solar radiation, hydrostatic pressure, and osmotic pressure**. Theoretically, minimal shifts

in values for individual, or any combinations of the above factors might significantly impact stressor bioavailability and toxicant uptake and, by extension, intercellular recruitment and primary gene products.

Describing the historical agricultural insecticide heptachlor, and terminal deposition of post-application residues (Fendick et al., 1990), investigators conclude that the lipophilic nature of this agrochemical results in the potential for significant bioaccumulation in all lipid-type compartments in the environment; however, the extent to which compartmentalization occurs is highly dependent on relative abiotic conditions. Many investigators presume overlap and commonality between causal factors of eutrophication (Total Nitrogen and Phosphorus [TNP]) and abiotic conditions. Investigators in one body of work, point out that models developed to simulate aquatic ecosystems (Koelmans et al., 2001) are generally categorized as standalone; distinct models devoted to eutrophication, contaminant fate, food web and food chain bioaccumulation. Because models tend to depict single issues, critical feedback interactions between food webs, nutrient, and toxicant cycles are inadvertently excluded from data sets; therefore, integration of critical measurements into a comprehensive modeling scheme is essential to evaluate the fate and risks of contaminants in systems wherein nutrient loading undergoes continuous change.

For a number of years, assorted groups of chemical compounds have been classified based on ability to cause substantial proliferation of peroxisome organelles in addition to hepatocarcinogenesis. In recent years it has been suggested that aquatic organisms living in coastal and estuarine areas, might be particularly susceptible to these compounds. Investigators issue a caveat (Cajaraville et al., 2003) that, when using peroxisome proliferation as a marker for exposure to this chemicals class, numerous biotic and abiotic factors have been shown to also initiate an increase in numbers of the organelles. Colloids and biofilm, also categorized as part of the abiotic sphere, apparently play a critical role in dispersion of metals into food webs. This is demonstrated by measured concentrations of As, Cu, Pb, and Zn partitioned between the two abiotic components. Authors suggest (Farag et al., 2007) that trophic transfer of Fe colloids is occurs by way of biologic constituents of which biofilm is composed, in addition to being an integral part of abiotic factors.

Biomarker responses, mediated by abiotic conditions, have been described in numerous bodies of work by means of diverse biologic organisms. Using estuarine bivalve mussels (*Mytilus edulis*) exposed to metals (Wepener et al., 2008), investigators indicate that sites yielding biomarker responses were clustered in a manner that reflected the influence of both internal exposure (uptake and bioaccumulation) and external exposure owing to physiochemical conditions. Researchers further noted that differences in biomarker responses clearly demonstrated influence of abiotic factors, distinct from metal pollution alone. In a separate study, investigators, using *Daphnia*

pulex (Scherer et al., 2013), assert that pollutant effects on aquatic species are unquestionably confounded by multiple abiotic and biotic stressors and further, results clearly illustrated that multiple stress factors can modify the response of an aquatic key species to pollutants.

Appendix B References

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Appendix C. Panel Members

Dr. Tracy Collier (Panel Chair) has over 40 years of experience in environmental toxicology. Dr. Collier currently serves as the Science Director for the State of Washington's Puget Sound Partnership, and is the chair of the State of California's Delta Independent Science Board. He worked for NOAA's Northwest Fisheries Science Center from 1972 until 2010, and since then has served as NOAA's Science Advisor to its Oceans and Human Health Initiative, technical advisor to NOAA for natural resource damage assessment for the Deepwater Horizon oil spill, and has consulted with the Haisla First Nation of British Columbia, Canada, concerning risks of diluted bitumen shipments through their territory. He received his PhD from the University of Washington in 1988, and has over 150 scientific publications. While Tracy enjoys thinking about how to develop and apply biomarkers, he thinks the term is slightly overused, and would prefer to be part of a panel on sustainability the next time.

Dr. Nancy Denslow is a professor in the Department of Physiological Sciences and in the Center for Environmental and Human Toxicology at the University of Florida. She has pioneered the use of molecular technologies for environmental toxicology especially focusing on endocrine disruption. She developed the first monoclonal antibodies for fish vitellogenins that were applied to quantify exposure of fish to estrogen-like contaminants in rivers in the US. She then developed estrogen receptor reporter assays utilizing fish estrogen receptors to study molecular initiating events for environmental xeno-estrogens. In addition, she has pioneered the use of microarray technology for non-model species, adapting technologies used for assessing toxicant effects on human health. She has served on two blue ribbon science advisory panels in California sponsored by the California Water Resources Control Board. She has served as an ad-hoc reviewer for EPA's FIFRA panel to review several EPA thrusts on endocrine disruption. She was awarded the University of Florida 2007 Pfizer Award for Research Excellence and was named the 2009-2011 University of Florida Research Professor. Nancy has over 150 peer-reviewed publications and is an inventor on four patents relating to protein factors, biomarkers for endocrine disruption and proteomics methodologies.

Dr. Evan Gallagher has been engaged in environmental toxicology research for 25 years, and joined the faculty of the University of Washington in 2004 as Sheldon D. Murphy Associate Professor of Toxicology. He has been using aquatic models to study the effects of cadmium, copper and chlorpyrifos in his University of Washington-Superfund Research Project since 2005. Dr Gallagher was formerly an Associate Professor at the University of Florida were he also served as Director of the Aquatic Toxicology Laboratory in the College of Veterinary Medicine. Dr Gallagher is the Director of the UW Superfund research program, and serves on the editorial boards of Toxicological Sciences and Environmental Research. He is also an active member of the UW Center for Ecogenetics and Environmental Heath (CEEH). Dr. Gallagher is a

member of the Society of Toxicology as well as the Society of Environmental Toxicology and Chemistry, and he maintains an active research and teaching program focused in the area of molecular and biochemical toxicology. His NIEHS Superfund project is directed towards understanding the mechanisms of pesticide- and metal-induced olfactory injury in salmon. Zebrafish are used to address epigenetic mechanisms of chemical olfactory injury. Other projects include funding from Washington Sea Grant to address the developmental toxicity of polybrominated diphenyl ethers (PBDEs) found in Pacific salmon, and also chemoprotection by omega-3 PUFAs against PBDE toxicity. He has continuing studies on the comparative biochemistry of glutathione transferases. Overall, his work involves environmental toxicological issues that cross ecosystem and human health boundaries.

Mitch Kostich is a research biologist with the USEPA's Ecological Exposure Research Division. He specializes in the application of machine learning algorithms to large biological and chemical datasets. He has served on emerging contaminant research planning work-groups for the White House Office of Science and Technology Policy and for the USEPA. For the last 10 years, the focus of his research has been the application of emerging technologies, including microarrays and high-throughput sequencing, to emerging contaminant research. His most recent research includes computational modeling and chemical analysis of pharmaceuticals as well as estrogenic contaminants in wastewater across the US. Other recent work involved development of molecular biomarkers indicative of fish pyrethroid exposures, and combined analysis using microarrays together with separative chemistry to identify contaminants responsible for toxicity of sediments from superfund sites.

Dr. David Lattier received his Ph.D. in 1989 from University of Cincinnati, College of Medicine and Cincinnati Children's Hospital Medical Center. Throughout Graduate studies, David was involved with sequencing the human gene for Adenosine Deaminase, during which he identified and isolated tissue-specific regulatory regions of the ADA gene that were incorporated into viral vectors used for initial trials of human gene transfer. In 1991, he was named NIH New Investigator in National Institute of Heart, Lung and Blood, Program of Excellence in Molecular Biology of Heart and Lung. In 1997, he joined the USEPA National Exposure Research Laboratory, Office of Research and Development. Dr. Lattier was appointed delegate to the USEPA *Genomics Task Force*, which was responsible for promulgating the *Interim Policy on Genomics* and shaping guidance regarding implications and potential applications of genomics research within USEPA, including research needs in areas of toxicogenomics and risk assessment. He also served as Chair of the Genomics Framework Performance-Based Quality Assurance Workgroup, a deliberative body appointed by USEPA Science Policy Council, which developed guidance for microarray-based data submission that outlines USEPA acceptance criteria for external genomics data in support of regulatory claims.