HHS Public Access

Author manuscript

Integr Environ Assess Manag. Author manuscript; available in PMC 2016 February 11.

Published in final edited form as:

Integr Environ Assess Manag. 2014 July ; 10(3): 327–341. doi:10.1002/ieam.1530.

The Role of Biomarkers in the Assessment of Aquatic Ecosystem Health

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Abstract

Ensuring the health of aquatic ecosystems and identifying species at risk from the detrimental effects of environmental contaminants can be facilitated by integrating analytical chemical analysis with carefully selected biological endpoints measured in tissues of species of concern. These biological endpoints include molecular, biochemical and physiological markers (i.e. biomarkers) that when integrated, can clarify issues of contaminant bioavailability, bioaccumulation and ecological effects while enabling a better understanding of the effects of non-chemical stressors. In the case of contaminant stressors, an understanding of chemical modes of toxicity can be incorporated with diagnostic markers of aquatic animal physiology to help understand the health status of aquatic organisms in the field. Furthermore, new approaches in functional genomics and bioinformatics can help discriminate individual chemicals, or groups of chemicals among complex mixtures that may contribute to adverse biological effects. While the use of biomarkers is not a new paradigm, such approaches have been underutilized in the context of ecological risk assessment and natural resource damage assessment. From a regulatory standpoint, these approaches can help better assess the complex effects from coastal development activities to assessing ecosystem integrity pre- and post-development or site remediation.

Keywords

fish; metals; oil; pesticides; assays; gene expression

INTRODUCTION

The input of anthropogenic contaminants to natural water systems has the potential to affect aquatic ecosystem health. Such inputs include controlled discharges, stormwater runoff and fugitive emissions, along with extreme events such as oil spills. When ecosystem degradation is identified, it is often difficult to determine whether effects are due to

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DISCLAIMER—The peer-review process for this article was managed by the Editorial Board without the involvement of G. Batley. The authors state that they have no conflicts of interest.

contaminants as opposed to non-chemical stressors such as turbidity, lack of oxygen, or lack of food due to changed in species composition resulting from habitat loss or over fishing.

Exposure to environmental contaminants can affect the survival of aquatic organisms via numerous mechanisms, including direct toxicity (both short- and long-term). More often the effects are more subtle, ultimately modulating organism fitness (Connon et al. 2009). Standardised laboratory measures of growth, reproduction and survival can be an unreliable estimation of the many indirect stressor effects in the field. More subtle changes in normal physiological function, such as appropriate reproductive behaviour, resilience to disease, and prey-capture abilities, may better indicate impacts on longer-term organism survival, reproductive output and ultimately, ecosystem health (Scholtz et al., 2012).

In order to better manage contaminants, it is desirable to be able to predict changes in ecosystem composition and function, as well as organism health, given a known set of environmental parameters and contaminant concentrations. It is also advantageous to have the ability to avoid losses in ecosystem structure and function before they occur, as opposed to conducting a retrospective analysis once an organism or system function has declined. However, this proposition is far more challenging than it seems, largely because of ecosystem complexity (for example, regular changes in the species composition and health of organisms in the field with tidal and seasonal cycles) and the presence of multiple stressors.

To further complicate environmental risk assessments, many contaminant exposure episodic in nature. Impacts such as oil spills, stormwater runoff following floods or heavy rains, release of antifoulants following ship groundings, release of material due to resuspension following dredging etc., are all likely to have a large influence on the health of organisms and their ability to recover (e.g. Scholz et al. 2011; Ward et al. 2013), even if they contribute minimally to overall contaminant concentrations over a larger spatial and temporal scale.

LIMITATIONS OF CURRENT METHODOLOGIES

The reductionist approach that is necessary to work with organisms in a laboratory setting may impair our ability to detect these sub-lethal, chronic impacts. Obviously, there are logistical constraints with the size and number of organisms that can be held in the laboratory. Complex behaviours, such as spawning aggregation or migration, cannot be reproduced in the laboratory. Long-term studies often pose logistical challenges as well. For example, determining the ultimate effects of chemical exposures that occur at larval phases on the reproductive output of a fish that takes years to reach sexual maturity are often outside the scope of a typical short-term research studies and also require extensive aquatic animal husbandry facilities to raise fish from larval to adult stages. In the field, organisms can move and potentially avoid contaminant hot-spots, whereas in the laboratory they are constrained to the test vessel (Ward et al. 2013). Furthermore, the ability to mimic natural stressors (e.g. hypoxia with tidal cycles, predator/ prey interaction, predator avoidance and food limitation) and complex natural exposure scenarios (e.g. stormwater-driven pulses, exposure to weathering oil following a spill, etc.) is extremely challenging, and often impossible. To further complicate laboratory studies, often only a small representative

component of the spectrum of environmental species can be successfully maintained under laboratory conditions, making it necessary to use surrogate species in laboratory tests (Simpson and Spadaro 2011; Kennedy et al. 2009). While surrogate species are often chosen because they are sensitive to toxicants in standardized laboratory tests, these surrogate species may have very different life histories and abilities to deal with chemical and non-biological stressors relative to the target aquatic species of concern.

Traditional ecotoxicity tests are designed to be rapidly and inexpensively conducted, to be easily replicated, and to have obvious ecological significance (Fisher and Hook 2002). These tests may typically involve an examination of acute toxicity, or more chronic effects on growth and reproduction (Simpson and Spadaro 2011; Kennedy et al. 2009). They are often designed for short-lived, fast-growing species, and are typically conducted over a few days for acute tests, with many chronic tests typically 10-28 days. While these tests certainly provide valuable information, they do not mimic the environmental scenarios discussed above. In particular, they may not be predictive where exposure is to either low-level contaminant concentrations or to episodic, very high concentrations, and effects are subtle in the way they effect physiology but nevertheless, decrease the organisms' fitness (Connon et al. 2009). To predict the subtle effects described above, it is necessary to move beyond the existing paradigm of standardized laboratory tests and beyond many of the traditional test endpoints. Here the use of molecular, biochemical or physiological biomarkers can provide unique insights into organism health.

BIOMARKER-BASED APPROACHES TO EVALUATING DEGRADED ECOSYSTEMS

Increasingly, environmental toxicologists are borrowing from the field of medicine to predict ecosystem health and function. Biomarkers are defined as detectable biochemical and tissue-level changes that indicate altered physiology (Smit et al. 2009). For example, a physician may analyse thyroid, kidney and liver function by combining a suite of biometric measurements and blood tests, as well as functional testing, and in some cases, imaging and pathology analysis. A similar multivariate biomarker approach involving multiple biological and physiological measurements, as well as analytical chemistry where appropriate, can also be used to assess the health of indicator species within ecosystems.

Biomarker approaches are not new, with several biomarkers being in use for decades (Stegeman 1978; Roesijadi 1980; Lee et al. 1981), although the acceptance of these approaches by regulatory agencies has been inconsistent. Water quality guidelines (e.g. ANZECC/ARMCANZ 2000) typically recommend that biomarker studies are best used to indicate exposure, as it is difficult to extrapolate between test results and ecosystem effects, even though they acknowledge that biomarker assays may be the best indicator of chronic stress (Hyne and Maher 2003). However, biomarkers, in general, have not yet been fully exploited in the context of an integrated monitoring system, and there are additional under-utilised advantages to incorporating a biomarker-based approach. Carefully chosen biomarkers may be the best approach to identify an early response to contaminants (Broeg et al. 2005) and are much more sensitive for identifying organism stress than whole animal responses (Smit et al. 2009), although it is not always clear the origin of the stress, and there

are often multiple stressors present. Biomarker responses can be measured in organisms collected from, or deployed in field sites, to integrate the effects of chemical and non-chemical stressors, reducing the need for complex laboratory exposure scenarios. To achieve these goals, however, it is important that a biomarker-based approach links mechanistic knowledge with a consideration of whole-animal physiology and population-level effects (reviewed in Connon et al. 2011).

In the following sections, the utility of different types of biomarkers that help to better understand environmental stressor effects on organism health is described.

CLASSES OF BIOMARKERS AND THEIR APPLICATION TO AQUATIC ECOSYSTEMS

Biomarkers have been classified by the extent that they reflect *exposure* to environmental stressors, or adverse health *effects* from contaminant exposures. Some biomarkers can also indicate *susceptibility* to adverse outcomes from environmental contaminants, although these have not been developed or incorporated in ecological assessment frameworks due to the fact that they largely rely on well-defined genetic databases and the incorporation of epidemiological studies, which are areas of future development in the field of aquatic toxicology. However, biomarkers that reflect both *exposure* and *biological effects* have been widely incorporated in field and laboratory studies, although the examples are few (Taylor and Maher 2010).

Exposure Biomarkers

Biomarkers of exposure to single or multiple contaminants with similar modes of action (Table 1) can show an early response to contaminants and are typically specific to a particular class of contaminants, e.g. biliary fluorescent aromatic compounds (FACs) for exposure to oil, or the induction of the egg-yolk precursor protein vitellogenin (vtg) for environmental estrogens (Broeg et al. 2005).

One of the most common biomarkers in this category is the measurement of the induction of a cytochrome P450 1A (i.e. CYP1A) at the messenger RNA, protein, or catalytic activity levels, as biomarkers of exposure to components of oil (Whyte et al. 2000; Lee and Anderson, 2005). Furthermore, the presence of some PAH metabolites excreted in the bile of fish (e.g. bile FACs) has also been used successfully to track the temporal and spatial effects of oil pollution (Aas et al. 2000). Oil biomarkers are particularly important as some components of oil can be rapidly metabolized in aquatic organisms (Whyte et al. 2000) or undergo environmental breakdown. However, elevations in oil exposure indices such as CYP1A activity have not been linked to higher level biological effects (Lee and Anderson 2005). Accordingly, measures of CYP1A as well as biliary FACs would have most predictive power in a regulatory context when used as part of a suite of biomarkers, including those discussed below that are more intricately linked to physiological and higher-level effects.

Induction of vtg in male or juvenile fish is a commonly used biomarker of exposure to environmental estrogens (e.g. Denslow et al. 2004; Folmar et al. 1996; Jobling et al. 1998).

This induction can be measured both at the protein (Folmar et al. 1996; Jobling et al. 1998, Johnson et al. 2008a) and at the mRNA level (Roy et al. 2003). Increased vtg levels have been linked to pharmaceuticals in sewage (Folmar et al. 1996; Jobling et al. 1998; Roy et al. 2003), to overall anthropogenic impact (Johnson et al. 2008a), as well as to phytoestrogens in pulp and paper mill effluents (Denslow et al. 2004).

Metallothionein (MT) has known roles in routine physiological processes involving essential metals and in detoxifying non-essential metals such as cadmium (Amiard et al. 2006). Different MT isoforms can also be responsive to oxidative stress (Hook et al., 2006). MT mRNA levels in fish have been shown to vary along a contamination gradient (Tom et al. 1998), and also have been shown to correlate with bioavailable metals in fish caged along contamination gradients (Chesman et al. 2007). MT mRNA levels were a highly sensitive indicator of laboratory cadmium exposures in Coho salmon (Williams and Gallagher, 2013). Interestingly, another study of Coho salmon indicated that measuring MT mRNA levels in the olfactory system of salmon was more sensitive than in the liver, suggesting tissue-specific differences in MT induction (Espinoza et al. 2012).

Acetylcholinesterase (AChE) inhibition has long been used as a biomarker for exposure to, as well as effects of, carbamate and organophophorus pesticides in both fish and invertebrates (reviewed in Fulton and Key 2001; Klumpp et al. 2002), although it is not always as effective a biomarker for molluscs due to avoidance behaviours (Cooper and Bidwell, 2006). AChE inhibition is a rare and in some ways ideal biomarker as it indicates both exposure and effects. Inhibition can be linked to both decreased swimming stamina and survival, although the exact relationships between these linkages tend to be species specific (Fulton and Key 2001). Exposure to pesticides and AChE inhibition are also thought to be contributing to the poor recovery of some Pacific salmon populations (Laetz et al. 2009).

Effects Biomarkers

Examples of effects biomarkers, i.e. indicators of physiological or biochemical changes as a consequence of exposure, are listed in Table 1. These indices may be direct measures (e.g. DNA damage, AChE inhibition), or indirect measures such as the impact on sub-cellular lysosomes. Theoretically, an organism with a quantitative change in an effects biomarker will undergo some loss of fitness. However, these associations can be difficult to demonstrate quantitatively.

Despite their advantages, a major disadvantage of effects biomarkers is that very few can be linked directly to exposures to specific classes of chemicals. However, there are some notable exceptions, including AChE activity, as well as the presence of PAH-DNA adducts (Table 1). However, as discussed later, effects biomarkers can be effectively incorporated with other diagnostic markers of fish health and also with analytical chemistry approaches to provide evidence for the contributions of chemical exposures.

Biomarkers of oxidative stress represent a unique sub-class of effects biomarkers that have been applied inconsistently in aquatic studies. Oxidative stress is part of the ageing process and occurs in all living organisms when reactive oxygen species (or their by-products) cause cell and tissue injury (Winston 1991; Kelly et al. 1998). It is well accepted that exposure to a

broad range of environmental chemicals, including pesticides, metals, and PAHs increase the level of cellular oxidative stress in aquatic organisms (Winston 1991; Kelly et al. 1998; Livingstone 2001; Pandey et al. 2003; Banni et al. 2005; Farombi et al. 2007; Taylor and Maher 2010). Aquatic organisms contain a full complement of antioxidant enzymes which comprise antioxidant defenses, including enzymatic components (e.g. superoxide dismutase (SOD), catalase and glutathione peroxidases) as well as small molecule antioxidants (e.g. glutathione). Since antioxidant defences can be quantitatively altered on exposure to contaminants, this has led to their exploitation as biomarkers in the field (Livingstone 2001; Pandey et al. 2003; Banni et al. 2005; Farombi et al. 2007). A breakdown in the antioxidant detoxification procedures with the potential for higher order effects has also been observed for metals in bivalves (Taylor and Maher 2010).

Elevated antioxidant enzyme parameters associated with increased lipid peroxidation have been observed in the fish, *Fundulus* sp. exposed to complex mixtures of organic chemicals (Bacanskas et al. 2004). Similar results have been reported for bivalves (Banni et al. 2005). Oxidative stress-associated parameters were particularly sensitive biomarkers of contaminants in receiving waters of a hydroelectric power plant (Sakuraguiet al. 2013).

Because the response of the organism may be both xenobiotic- and also tissue-specific, it is also important to examine several endpoints related to oxidative stress in different tissues. For example, Otto and Moon (1996) compared brown bullhead (*Ameriurus nebulosus*) collected from a system contaminated with PCBs to bullhead collected from a relatively non-contaminated site. Fish from the contaminated site had extremely high PCB concentrations in muscle compared to fish from the non-contaminated site, and SOD activity was increased in the kidney of fish from the contaminated site. However, the activity of another antioxidant enzyme, catalase, was lower in the kidney, and there were differences in the trends of expression of other enzymes. Studies such as these highlight the importance of tissue selection when evaluating oxidative stress in feral organisms.

Protective antioxidant enzymes and their non-enzymatic cofactors (e.g. GSH) can be overwhelmed during high levels of tissue oxidative stress, which can lead to the accumulation of metabolic by-products reflecting *oxidative damage*. These metabolic by-products include oxidized pigments such as lipofuscin, peroxidized cell membranes and their ensuing membrane breakdown products (i.e. lipid peroxides, malondialdehyde)as well as oxidative DNA and protein damage. These secondary products of oxidative damage can initially compromise the ability of the organism to maintain normal metabolic processes, and under chronic oxidative stress, to the disease states mentioned above. Lipid peroxidation, in particular, has been routinely measured in field studies to reflect chemical-induced oxidative damage.

It is important to note that there are quantitative differences in the normal levels of these antioxidants, as well as their induction capabilities among the various aquatic species (Kelly et al. 1998). Accordingly, oxidative stress biomarkers need to be carefully validated under controlled laboratory conditions Also, oxidative stress biomarkers and other enzyme based biomarkers are known to exhibit a “bell shaped dose response curve” with increasing toxicant dose or exposure time (e.g. Marigomez et al., 2013) This should be taken into

account when designing field experiments. Furthermore, these parameters are significantly affected by nutritional status, age, and other non-chemical factors.

Another commonly used effects biomarker is the measurement of the integrity and stability of lysosomal membranes detected by the uptake and retention of a cationic dye, neutral red. Dose-response relationships have been found for a range of aquatic contaminants, including both metals and organics (Moore et al. 2013). Lysosomal stability has been positively correlated with scope for growth, reproductive output, genotoxicity, total oxyradical scavenging capacity and protein synthesis and can be deemed as ecologically relevant (Ringwood et al., 2004). Edge et al. (2012), in a study of the Sydney rock oyster in contaminated estuaries containing both metals and PAHs, showed that lysosomal membrane stability was a more useful indicator of environmental stress than lipid peroxidation or concentrations of glutathione (GSH). Lysosomal stability correlated well with fertilisation, normal embryo development and estuary status.

Significant correlations were observed between lysosomal membrane stability and induction of the exposure marker MT in the digestive glands and gills and liposomal membrane stability, in mussels (*Mytilus galloprovincialis*) exposed to heavy meal contaminants (Domouhtsidou et al. 2004), but it was noted that other factors such as age, size and seasonal variation could contribute to MT induction. The aforementioned findings and those of other studies support the use of multiple biomarkers covering both exposure and effects, rather than the use of a single indicator.

Genotoxicity is another important effect that is commonly measured using biomarkers. For instance, DNA integrity (measured as strand breaks with the Comet Assay) was inversely correlated with total oxyradical scavenging capacity in mussels collected from a eutrophic lagoon in coastal Italy (Frenzilli et al., 2001). Both a spatial and temporal gradient was measured, with mussels having poor metrics in the areas with the worst water quality and in the summer, when temperatures were elevated. Both metrics were correlated with a loss of biodiversity in the area (Frenzilli et al., 2001). DNA damage (measured using the Comet Assay) was also found to correlate with concentrations of sediment contaminants in mussels deployed in San Diego Harbour, CA., USA (Steinert et al., 1998).

DNA damage can also be measured directly using DNA adducts. For instance, levels of DNA adducts were found to be elevated in fish captured near an aluminium smelter in western Norway (Aas et al., 2001). Adduct levels correlated to both EROD (indicating PAH exposure) as well as skin ulcers and fin erosion (Aas et al., 2001). \ DNA adducts were also used to measure the potential impacts of the Sea Empress oil spill (Harvey et al., 1999). DNA adducts were measured in a sponge, a mussel and several species of fish. Although elevated levels of adducts were not measured in either species of invertebrate, they were found in all three fish species. The return of these adduct to baseline levels after eighteen months indicated that fish populations were no longer under genotoxic threat (Harvey et al., 1999).

Transcriptomic Biomarkers that Integrate Exposure and Effects

Incorporation of transcriptomic, or genome-enabled biomarkers, is becoming increasingly recognized as a powerful approach that can yield information on both exposure and pathways of injury (i.e. biological effects), and thus provide somewhat of a bridge between exposure and effects. Genomic approaches are widely accepted in the context of biomarker generation (reviewed in Hook, 2010), however, these transcriptional approaches need to be validated with a phenotypic anchor, i.e. clearly defined and measurable effects at organ, tissue, or physiological levels. For example, whole tissue transcriptome profiling has been effectively used for screening adverse outcomes associated with drug and chemical toxicity in mammalian studies (Hu et al. 2000; Martin et al. 2006). Microarray approaches may be particularly useful in determining the contributions of various classes of environmental contaminants toward sub-lethal toxicity in mixture scenarios (Tilton et al. 2011; Osborn and Hook, 2013; Hook et al., 2014).

Increasingly, microarray-based gene-expression profiling, or other methodologies that measure overall gene expression, are being used as “discovery” tools to characterize response in both laboratory and field studies. These profiling techniques have the advantage of being able to be used when the stressors are unknown and being able to identify the causative agent of decline in the organism's health, since any field results can be compared to results from controlled laboratory studies. They are also inherently multivariate, in that transcript levels of biomarkers can be assessed simultaneously with the transcripts for gene products more closely associated with fitness. The disadvantage of these techniques is that interpreting the data can be challenging, especially given that the No Observable Transcriptional Effects Level (NOTEL) is often far below the concentrations at which toxic effects are seen (Poynton et al. 2007; Poynton and Vulpe 2009), and that the annotation of many genomes is incomplete. Van Straalen and Feder (2012) considered transcriptomics as a ‘super-biomarker’ because of the wealth of information generated but noted the current difficulty in linking the measured gene expressions to ecologically relevant effects.

CHALLENGES ASSOCIATED WITH ESTABLISHING LINKAGES BETWEEN EXPOSURE AND EFFECTS

The use of a comprehensive suite of markers within a single target tissue can sometimes yield results that are difficult to evaluate biologically. For example, McFarland et al. (1999) investigated site differences in liver antioxidant parameters in brown bullhead fish in a PAH-contaminated and a control site in Ohio, USA. In the aforementioned study, the levels of the enzymes glutathione reductase, glutathione *S*-transferase, and glutathione peroxidase were measured, but no site differences were present. In contrast, other antioxidant parameters such as SOD, catalase, and total tissue glutathione levels appeared to correlate with environmental exposure to PAHs. In another study, 11 biochemical markers of aquatic pollutants were evaluated in the livers of chub caught at several sampling sites of a river containing various contaminants (Machala et al. 2001). The results were tested against measured concentrations of organochlorine compounds, PAHs, and heavy metals. The biochemical markers of oxidative stress, including *in vivo* lipid peroxidation and *in vitro* production of ROS, did not correlate with the concentrations of the contaminants, while

glutathione-dependent enzymes formed a suitable battery of exposure biomarkers (Machala et al. 2001). These studies are among many that illustrate the complexities associated with drawing meaningful conclusions from differential antioxidant responses observed in the field. The section above summarizes the types of biomarkers that can be applied to the assessment of aquatic animal health. In the following sections, we provide some scenarios where these physiological markers have successfully been used in field scenarios.

DETERMINING THE SPATIAL AND TEMPORAL EXTENT OF CONTAMINATION

Biomarker-based approaches have been used to determine the spatial and temporal patterns of influence of a stressor in the field, i.e. extent of potential exposure. For instance, following a major oil spill, determining over what period of time the oil persists and whether organisms in the spill path were exposed is an important component of the ensuing risk assessment. Known oil exposure biomarkers, such as induction of CYP1A or EROD, are frequently used to assist in the assessment of hydrocarbon distribution and bioavailability.

Following a fuel oil spill in Micronesia, resource managers were uncertain as to whether the fuel oil partitioned into the water column, and if it did, if the effects of the fuel oil would be short-term or long-term. Downs et al. (2006) used protein-based biomarkers including heat-shock proteins, cytochrome p450s, heme oxygenase, SOD, catalase and DNA-repair enzymes that were indicative of exposure to fuel oil. The authors reported significantly different levels of expression between impacted and reference sites three months after the spill (Downs et al. 2006), indicating that there was sufficient fuel oil in the water column to have some impact on coral for at least three months.

Whether the oil released from the 1989 *Exxon Valdez* spill in Prince William Sound, Alaska, USA, caused long-term ecosystem damage, and if so, to what portions of the ecosystem, is still the subject of fierce debate (e.g. Incardona et al. 2012; Sellin Jefferies et al. 2013). Biomarker type responses were extensively used to assess these potential impacts. Aryl hydrocarbon hydroxylase levels were measured in Dolly Varden trout (*Salvelinus malma*) 120 and 460 days after the spill and were found to be elevated in fish collected from a heavily oiled site relative to those collected from a reference site (Collier et al. 1996). However, several years later, the oil response biomarkers were no longer elevated.

Hugget et al. (2003) collected fish from three operationally defined areas within Prince William Sound: (i) areas that were in the spill path and had oil deposited in the sediments at the time of the spill; (ii) areas in the spill path but had no oil deposited in the sediments at the time of the spill; and (iii) areas outside of the spill path. The researchers measured oil exposure biomarkers (EROD induction, CYP1A), immunohistochemistry, and bile FACs in near-shore and offshore fish species. These biomarkers were consistently found in the fish they collected, yet there were no significant difference in their levels between operational sites and other regions in the Gulf of Alaska. The data were used to argue that there was no ongoing exposure to Exxon-Valdez oil in Prince William Sound and that the exposure levels being measured in this study were due to other oil sources in the Gulf of Alaska, such as seeps and boat traffic.

Biomarkers levels were measured in the livers of demersal fish following the Prestige oil spill in Northern Spain (Martinez-Gomez et al. 2009) with the goal of determining the spatial and temporal extent of the impacts. EROD, GST, glutathione reductase and catalase activity were all measured, with EROD being the most sensitive. Patterns of biomarker induction matched the trajectory of spilled oil and patterns of oil deposition. In addition, levels of induction returned to baseline over a time period of two years at most sites (Martinez-Gomez et al. 2009). Two years after the spill, PAH concentrations were returning to background, but some sites still had a spill signature (Fernandez et al. 2010). To determine whether these concentrations of PAHs were having any biological effect, a variety of effects biomarkers were measured in mussels along a spatial gradient. At the spill sites, mussels had elevated oxidative stress markers, but no change in physiological markers that indicate changes in growth and reproductive capacity (Fernandez et al. 2010). Taken together, these data indicated that exposure to oil from the Prestige may have had an impact on fish health following the spill, but with a return to a normal physiological status within a few years.

Studies examining the spatial patterns of a stressor have not been confined only to oil. Microarray-based measures of gene expression have also been successfully used to distinguish contaminated field sites from relatively pristine ones in agricultural areas (Sellin Jeffries et al. 2012). Fathead minnows were caged in rivers having high concentrations of both agricultural pesticides and veterinary pharmaceuticals that have been shown to have anti-estrogenic properties. The patterns of hepatic gene expression were contrasted to minnows caged in rivers with low concentrations of these chemicals. The authors found that the patterns of gene expression could be used to differentiate high- and low-likelihood of impacts due to contaminant exposure. Microarray-based gene expression assays have also been used to differentiate between fish collected up- and downstream of a sewage treatment plant (Garcia–Reyero et al. 2008). These studies demonstrate the ability of transcriptomic biomarkers to differentiate between exposed and unexposed animals even when the exposures are to very low concentrations of complex mixtures. These gene expression patterns persisted despite the variability inherent in the transcriptome.

PREDICTING THE HEALTH OF ORGANISMS

Quantitative changes in biomarker levels have also been used to examine the health of organisms in contaminated waters. In these studies, the focus has been primarily but not exclusively on effects biomarkers. As a proof of concept, Broeg et al. (2005) used a multi-biomarker approach to differentiate amongst sites in the German Bight. They were able to distinguish both pristine and contaminated sites and to show a progression of disease from early to pathological stages in the demersal flounder *Platichthys flesus*. Their indices also correlated with other metrics of organism health such as immunological and lipid status. Dagnino et al. (2007) deployed caged mussels to a highly contaminated site near a refinery and used a series of effects-based biomarkers (oxidative stress markers, lipid accumulation and lysosomal stability, which has long been known to correlate with both exposure and effects of contaminants (see Ringwood et al. 1998)) to show a progression of deterioration in health with time. They also showed effects along a longitudinal gradient, using this series of biomarkers. This same series of biomarkers was recently used by Shaw et al. (2011) to

categorize the health of mussels from the Tamar estuary in the UK. In addition, they also categorized stress using microarray-based analyses of gene expression. Both lysosomal stability and oxidative-stress markers were correlated with PAH concentrations in the sediment, and the number of genes with altered patterns of transcription was highest at the most contaminated sites. However, no changes in neutral lipids with contamination level were observed (Shaw et al. 2011).

A similar study in Southern Portugal transplanted mussels from a metal-contaminated site to a PAH-contaminated one and vice versa (Serafim et al. 2011). Over four weeks, the metal-responsive biomarkers decreased in the former and increased in the latter, whereas the PAH-responsive biomarkers showed the opposite trend. These studies demonstrate the utility and specificity of biomarkers in identifying relevant stressors and showing gradients of pollution. Multiple biomarkers, including several measures of oxidative stress, genotoxicity, lysomal integrity, MT and a vitellogenin like protein, were also used to predict the health status of two sites on the Basque coast (Marigomez et al., 2013). Integrative biomarker indices that considered all of these metrics were predictive of environmental health status.

Steelhead trout (*Oncorhynchus mykiss*) numbers have been declining in the Columbia River basin (USA), and there has been concern that increased exposure to contaminants (associated with the dams used for hydroelectric power generation on the river) is associated with decreased pathogen resistance (Connon et al. 2012). Previous work (Fiest et al. 2005) with sturgeon (*Acipenser transmontanus)* collected from this system had shown good reproductive success and low contaminant body burdens in fish from areas of the river that were free-flowing, but poor reproductive success and high contaminant concentrations were observed in fish collected from areas behind dams. Gonad size, circulating hormone levels, and triglycerides were all negatively correlated to contaminant levels (Fiest et al. 2005) suggesting that exposure to dam-associated contaminants could be impacting fish health. To test the hypothesis that contaminant exposure is directly influencing the health of these fish, Connon et al. (2012) measured immune response, general stress and contaminant-exposure biomarkers in out-migrating salmon collected at various field sites along the Columbia River. They found that gene expression profiles correlated to pathogen presence and that changes in gene expression could be used to separate fish into good, fair and poor condition. They suggested that these transcriptomic assays could be used as a tool to monitor fish health and to inform resource management.

BEHAVIOUR-BASED BIOMARKERS

Some studies indicate that biomarker responses could be used to make field-based measurements of endpoints that are difficult to capture in laboratory-based studies. In laboratory-based studies, exposure to the pesticide esfenvalerate at ng/L concentrations altered swimming behaviour in the delta smelt (*Hypomesus transpacificus)* (Connon et al. 2009). These changes could be correlated to changes concentrations of genes involved in neuromuscular processes, immune responses, apoptosis and redox and metal binding. Some genes, such as aspartoacylase and creatine kinase, could be mechanistically linked to the disrupted physiological processes (Connon et al. 2009). Exposures to environmentally relevant concentrations of copper were also shown to decrease swimming speeds in the

endangered delta smelt (Connon et al. 2011). These behavioural changes also appeared to be related to changes in gene expression (Connon et al. 2011). The impact of low level exposures to certain contaminants, most notably pesticides and metals, has been linked to loss of olfactory ability in several fish species (Scholz et al. 2000; Tierney, Ross et al. 2007; Tierney et al. 2010; Baldwin et al. 2011).

Recently, Williams et al. (2013) linked molecular biomarker encoding olfactory receptors and signal transduction components to histological injury and loss of behavior in Coho salmon exposed to cadmium. This study is consistent with studies using the model organism zebrafish showing that olfactory antioxidant defense and signal transduction gene expression is quantitatively altered on exposure to the model trace metals copper and cadmium (Tilton et al. 2008; Wang et al. 2013). In the case of copper, the zebrafish study provided molecular insights into the ability of copper to impair sensory function in coho salmon, which has been linked with increased susceptibility to predation (McIntyre et al. 2008, 2012).

IDENTIFYING STRESSORS WITHIN COMPLEX SCENARIOS CONTRIBUTING TO ECOSYSTEM DECLINE

A combination of exposure and effects biomarkers can be used to identify the impacts of specific contaminant (e.g. Ernst and Peterson, 1994). A multiple biomarkers framework was used to determine whether specific contaminants were causing unknown species declines in waters of Puget Sound (USA), an urbanized estuary with limited circulation (Johnson et al. 2008b). The measures of total PAHs in sediments have been steadily increasing along with human population growth, and it was hypothesized that these increases are adversely impacting fish health. The multiple biomarker approach included determining levels of bile metabolites and CYP 1A activity to demonstrate exposure to PAHs, as well as showing that increased DNA adducts (which indicate the initiation of a cascade that can result in hepatic carcinogenesis) could all be correlated with increased PAHs in the sediments (Myers et al. 2003) and decreased fish health (reviewed in Johnson et al. 2008b). Over decades, this dataset has been successfully used to justify remediation and restoration efforts of several bays and waterways within Puget Sound (Johnson et al. 2008b) and the same biomarkers have been used to monitor the success of the remediation efforts. Analytical measures indicated that dredging sediments had removed PAH. Decreases in biomarker levels were indicative that bioavailable PAH concentrations decreased in a manner corresponding to the measured biomarkers (Myers et al. 2000, 2003). Since the measured PAH levels, biomarker levels and declines in fish reproductive status and increased tumour prevalence were all related, it was assumed that changes in biomarkers were predictive of PAH induced changes in fish health (reviewed in Johnson et al., 2008b).

Coral reefs are frequently subjected to multiple stressors, and are known to be in decline due to a variety of anthropogenic factors worldwide (Downs et al. 2012). Being able to predict which stressor was causing effects would enable remediation efforts to be prioritized. Multiple biomarkers were used to differentiate between chemical exposures and effects in corals from Guam (Downs et al. 2012). Coral tissue were collected from a series of sites separately influenced by herbicides and other pesticides, sedimentation, fuel and heat (at a shipyard), PCBs at a landfill site and antifoulants at a marina. Protein biomarker levels in

corals from these sites were compared to a reference site and profiles indicative of the purported stressors were distinguished (Downs et al. 2012). The biomarker changes were consistent with known modes of action of the contaminants. This approach raises the possibility of using biomarkers in the equivalent of a toxicity identification and evaluation (TIE) procedure (Osborn and Hook, 2013, Hook et al., in review).

CHALLENGES IN USING BIOMARKER-BASED APPROACHES

The interpretation of biomarker studies is not without its challenges. For instance, the relationships between tissue body burden and biomarker levels can be confounded by age, sex and reproductive status (Hylland et al. 2009) which may necessitate either sampling at a reproductively inert time of the year or sampling juveniles. Also, some putatively contaminant-specific biomarkers have been shown to be induced by non-contaminant stressors. For instance, metallthionein in oysters has been shown to respond to other non metal stressors, including handling stress, anoxia and antibacterial compounds (reviewed in Amiard et al., 2006). Humic substances common to surface waters have also been shown to induce CYP1A in some fishes (Matsuo et al., 2006).

Furthermore, a conceptual framework for the progression of toxicant injury to aquatic organisms from initial injury through to death is provided in Figure 1 (see also Ankley et al., 2010; Brain and Brooks, 2012). At all of these levels, the cell/organism is trying to maintain homeostasis, so that all of these changes (except death) may ultimately be reversible. This reversibility in itself has applications for the use of biomarkers in environmental assessment. For instance, the lack of biomarker induction in fish years after the *Valdez* spill (e.g. Huggett et al., 2003) or lack of transcriptomic changes in mummichogs following remediation of a chromium contaminated shipyard (Roling et al. 2007) has been used to show that particular stressors were no longer compromising health.

As shown in the framework depicted in Figure 1, organisms can undergo physiological changes to adjust to an external stressor, yet still maintain normal cellular function (termed ‘accommodation’, Nicholls et al. 2011). These changes are ’compensatory mechanisms’ that can mask toxicological impacts and complicate regulatory decisions. However, compensatory mechanisms have an associated energetic cost and can cause chronic stress and increase the organism's allostatic load (i.e. energetic demands or capacity to cope with stress). If the allostatic load is exceeded, the organism's health is negatively impacted (Nicholls et al. 2011).

Consequently, the reversibility of biomarker-like changes as a result of the maintenance of normal physiological function via compensatory mechanisms may compromise the overall fitness of the organism, even if the molecular and cellular changes return to a baseline state. An example of these costs is illustrated by a recent study by Kerabrum et al. (2102) in which juvenile fish were exposed to levels of crude oil relevant to levels encountered following an oil spill for a short time period (48-96 h), then allowed to depurate (recover) in clean water for a period of 4 weeks. At the end of this period, the condition of the fish was less than that of controls, even though the biomarker levels were decreasing to baseline levels. While in this case, elevations in exposure biomarkers were predictive of a long-term effect, a definite

relationship cannot be made (Lee and Anderson 2005). It is often unknown whether a particular change is reversible, and what cost the maintenance of normal physiological function has to the organism. It has been suggested that a future challenge for the field will be to collect sufficient data from experimentally exposed and field-captured organisms to better differentiate between protective molecular responses and those that are indicative of an impending disease (Shaw et al. 2011).

Since biomarker induction following toxicological insult can be a dynamic process between injury and repair, choosing the proper timing for sampling of biomarker responses is critical. One of the chief advantages of biomarker responses is that they are generally rapid (Kerabrum et al. 2012), e.g. transcriptomic changes can be measured over a period of hours for example (Hook et al. 2008, Hook et al. 2010), while protein changes typically are slower to induce but longer lasting (Nahrang et al. 2009). This can be an advantage in that effects are seen early, however, some knowledge of the kinetics of the response, either from literature data or experiment, is desirable before using biomarkers an indicator of effects.

USE OF MULTIPLE BIOMARKERS

In this review, we are advocating a thoughtful approach measuring multiple biomarkers, ideally across multiple levels (as depicted in Figure 1). Measuring across multiple levels of function will allow better estimation of the stressors that are causing changes in fitness (exposure biomarkers), as well as the likely changes in cellular function and fitness (effects and condition biomarkers). Redundancy in measures would also likely decrease the probability of a false positive due to a spurious result in a single measure. Balk et al. (2011) used a multiple biomarkers framework to evaluate the health of fish collected near offshore oil and gas platforms. The authors noted elevations in oil exposure biomarkers (biliary PAH metabolites and EROD), changes in oxidative stress markers, and changes in lipid ratios. When integrated, these data indicate that the haddock and cod collected in areas proximate to oil and gas platforms have decreased fitness due to exposure to petroleum hydrocarbons.

As acknowledged by Bartell (2006), effects at the cellular and sub-cellular level, using one or several biomarkers are perceived to be ecologically difficult to interpret and their ecological significance is often not known or readily estimated. For example, the extent to which endocrine disruption in fish and invertebrates affects reproductive processes (and imposex in gastropods) leading to adverse ecologically significant impacts remains unclear in many studies (Arcand-Hoy and Benson 1998; Depledge and Billinghurst 1999; Matthiesson 2000; Hecker and Hollett 2000). It is, however, becoming increasingly apparent that certain biomarkers (e.g. behaviour and reproductive changes) can directly affect fitness which can be linked to survival. One particular biomarker, inhibition of brain AChE, has been linked to population level impacts in Pacific salmon (Laetz et al. 2009). Despite the ongoing efforts to restore salmon habitats, salmon returns have not increased to historic levelsThe levels of AChE in the area would indicate sufficiently decreased survival to explain the lack of recovery (Laetz et al., 2009).

The work of Taylor and Maher (2010) examining the effects of metals on benthic organisms has highlighted the value of using multiple biomarkers in an exposure-dose-response model

to demonstrate the link between exposure and effect. The cascade of biomarkers shown in Figure 2 provides a link between molecular perturbations and higher order effects on the organism (e.g. Brain and Brooks, 2012).

USE OF BIOMARKERS IN AN ASSESSMENT FRAMEWORK

The latest approaches to contaminant impact assessment in both waters and sediments have evolved significantly from the measurement of contaminant concentrations and toxicity as recommended, for example, in the Australian and New Zealand water quality guidelines (WQGs) (ANZECC/ARMCANZ, 2000). These guidelines suggest the use of hierarchical assessment frameworks which initially consider chemistry (i.e. whether contaminant concentrations exceed guideline trigger values (TVs)). If guideline concentrations were exceeded, then toxicity testing was recommended. These tests often measure sub-lethal impacts on growth and reproduction in sensitive surrogate species in laboratory culture. While this approach is possibly adequate for many assessments, where TVs are not exceeded, it may not be adequately protective in scenarios where: (i) multiple contaminants exert non-additive effects (ii) toxicological effects are observed in species of concern although individual toxicant concentrations are below guidelines, or (iii) unknown (emerging) compounds are contributing to the observed toxicity. Here further studies, such as the application of TIE procedures are required.

The goal of ecological assessments is to determine whether an ecosystem is healthy. Not surprisingly, we can generally use a combination of traditional approaches such as diversity indices and analytical chemistry to identify either healthy or highly impacted ecosystems, but it is assessments in the transition zone between these two extremes, namely the moderately impacted sites, are more problematic. It was recognised that in such cases simply measuring chemistry and ecotoxicity was insufficient and additional lines of evidence were required to better assess the health of an ecosystem, in a weight-of-evidence approach (Chapman et al. 2002; Burton et al. 2002).

Traditionally, the recommended additional lines of evidence included bioaccumulation and an ecological assessment of species diversity and abundance. Both involved comparisons with reference sites (as do chemical concentration measurements in cases where background concentrations are above guidelines). Bioaccumulation at best indicates whether there are bioavailable contaminants that are accumulating above background concentrations. However, it is well known that bioaccumulation alone cannot provide a direct link to toxic effects (e.g. Wallace and Lopez 1997; Rainbow 2002; Rainbow and Luoma 2011). Biodiversity studies look at whole system health as distinct from organism health, and new genomic techniques are permitting more comprehensive assessments than simply assessing macroinvertebrate populations as was done in the past (e.g. Chariton et al., 2010). Biodiversity measurements, however, indicate a longer-term response than the short-term evidence from toxicity testing. It may take time before chronic impacts translate into the disappearance of particular species of organisms and they are replaced by more tolerant species and possibly a new ecosystem structure develops. In many cases, it is the short-term impacts that need to be assessed, to initiate a rapid management response, and it is here that biomarkers may have a role.

A number of international jurisdictions are quite prescriptive about the role of biomarkers. Biomarker responses are typically seen at concentrations below those for toxicity, and while they may be indicative of some type of physiological impairment as a consequence of exposure to one or more contaminants or to some other non-chemical stressors, the significance of this effect on the health of the organism and the extrapolation of this to longer-term effects on ecosystem health are frequently difficult to demonstrate. Considerable care must therefore be exercised before biomarker data can be used in the derivation of water quality guidelines that are traditionally based on chronic toxicity thresholds for growth, reproduction and survival. The Canadian water quality guidelines (CCME 2007), for example, admit non-traditional endpoints (e.g. behaviour, predator avoidance, swimming ability, swimming speed, etc.) and those reflecting physiological/ biochemical changes, including endocrine-disrupting ability, only if their ecological relevance can be demonstrated. This approach has also been proposed in the current revision of the Australian and New Zealand guidelines. Here the effects biomarkers in Table 1 need to be considered. To account for natural variability in organismal physiology, it would be ideal to compare any biomarker type responses to an established baseline of “normal” physiology. Optimizing baseline variables such as seasonality and sex differences increases the ability to discriminate nonchemical effects from those involving chemical stressors (Almeida et al., 2013).

Ecologically relevant effects have been defined as those that influence an organisms’ ecological competitiveness to an extent that they have a strong negative influence on survival, growth and reproductive ability (CCME 2007). As concluded by Lam (2009), for many biomarkers, links with ecosystem effects have not been adequately demonstrated. He concluded that the best use of biomarkers was to provide an early warning of impending environmental problems. Results for biomarkers that can be obtained rapidly (e.g. with 24-48 h of collection of organisms), makes them a potentially valuable component in an ecological assessment process. However, much of the literature (reviewed in McCarty and Munkittrick, 1996; Forbes et al., 2006) would indicate that in many cases decisions based on biomarker responses alone may result in incorrect decisions about health due to the high frequency of ‘false positive’ responses that cannot be explained and do not concur with any other lines of evidence (i.e. no toxicity, excellent abundance/diversity).

A scheme for evaluating multiple lines of evidence is shown in Figure 3. A reasonable approach is to use biomarkers as a supporting line of evidence to clarify issues associated with bioaccumulation or toxicity, although these responses are interrelated. The exposure biomarkers in Table 1 are used in a similar manner to bioaccumulation to provide evidence for exposure, while effects biomarkers need to be considered in relation to the ecological relevance of the response, both in the short term in relation to organism health and in the longer term with respect to ecosystem impacts, noting the difficulties in predicting the latter.

To determine the suitability of any biomarker for use as a line of evidence, the following basic criteria have been proposed by other investigators (Svendsen et al. 2004; Huggett et al. 1992):

(i) A clear dose-response relationship should be demonstrated for target contaminants, noting that in its application, the cause of response may include multiple stressors;

(ii) The sensitivity should ideally be greater than that for effects on growth and reproduction

(iii) Ecological relevance should be demonstrated;

(iv) The effects of confounding non-chemical factors such as season, temperature, size, etc. should be understood;

(v) Some knowledge of the chemical specificity is desirable to distinguish between non-specific biomarkers responding to a wide range of contaminants and those that are more specific to particular contaminants or contaminant classes;

(vi) The applicability of the biomarker for the chosen test species needs to have been demonstrated;

(vii) Time-response relationships for the biomarker need to be understood (how soon after exposure is the response seen and how long after exposure does the response persist);

(viii) Methodological concerns such as reproducibility, robustness, ease of use.

Although the above criteria are relevant to the development and incorporation of biomarkers, the state of the science dictates that most of these criteria cannot currently be fully realized by use of a single biomarker (Ankley et al., 2010; Brain and Brooks, 2012). We have discussed that there are very few effects or exposure biomarkers at the biochemical or physiological levels that have clear effects at the population, let alone the ecosystem, levels. Accordingly, most biomarkers would likely fail the ecological relevance criterion. However, as discussed, there exist several biomarkers, such as clearly impaired behaviours (i.e. swimming, feeding, predator-avoidance, prey selection), reproductive impairment, and AChE inhibition that have been associated with loss of organism fitness. Many can be seen as indications of decreased health of individuals, which would impact the health of populations and systems.

Similarly, quantifying disturbances at the ecological level are problematic due to natural ecosystem variation over time, as well as there are likely no truly undisturbed ecosystems due to atmospheric deposition of contaminants, global climate change, etc. What is reasonable, however, is for the biomarker under consideration (and, in particular, *effects* biomarkers) to have a reasonable linkage to adverse effects of the organism level.

While it is essential to clarify any biomarker with respect to time of onset and permanence of response, it is important to consider that a particular biomarker that responds rapidly to chemical exposure, but then rapidly returns to baseline with cessation of exposures, can be extremely valuable in reflecting *recent* exposures. For example, gene transcriptional responses (i.e. heme oxygenase, metallothionein, CYP1A) in many aquatic species can occur rapidly upon chemical exposure (i.e. within 12 hours), but may then decline. This phenomenon is also observed with *adaptive* responses associated with *oxidative stress*, such

as induction of tissue glutathione, whose tissue levels can rapidly return to homeostatic levels (Meister, 1974). Arguably, when used in conjunction with more long-lasting responses (i.e. liver DNA adducts, pre-neoclassic or neoplastic liver lesions) and also analytical chemistry, biomarkers in this category can provide the regulator with a more informed perspective of environmental degradation.

Non-chemical stressor effects are an extremely important consideration. In reality, however, even among the myriad of biomarkers that have been either used or proposed for use in aquatic toxicology, it can be argued that none have been fully characterized with respect to effects of non-chemical stressors such as salinity, temperature, seasonality, food deprivation, etc. While the use of single biomarkers may not provide a holistic measure of ecosystem health (e.g. Lee and Anderson 2005), this problem can be overcome by integrating multiple biomarkers or by integrating biomarkers with other chemical or toxicological effects data, will add strength to any inference. Integrating class-specific contaminant exposure biomarkers with effects biomarkers in a single assessment can predict overall organism health, and also identify causative agents. While these approaches would not address the reversibility of deleterious effects if the stressor is removed, or the capacity for organisms to avoid the stressors, they would still provide insight to a weight of evidence environmental risk assessment

A framework for using biomarker data as part of a line of evidence assessment of contaminant impacts is given in Figure 4.

CONCLUSIONS

Often, the use of analytical chemistry and the results of toxicity tests will provide assessors with the necessary tools to assess environmental risk from the effects of discharges, spills, dredging or other perturbations of water quality. For subtle toxicological effects, low level episodic exposures, combinations of natural and contaminant stressors, or situation with high societal scrutiny, these metrics alone may not be sufficient to predict longer term effects on ecosystem health. While evidence of bioaccumulation is indicative of contaminant bioavailability, measures of short-term impacts on organism fitness can provide an early warning of impending broader effects on ecosystem health that can trigger early management intervention. Biomarkers of both exposure and effects can provide evidence of physiological and biochemical impacts at the organism level, however, the demonstration that these impacts have broader ecological relevance is proving to be a challenge, although proving useful as a monitoring tool to assess the spatial distribution of contaminant impacts and the rate at which systems recover as management actions are implemented.

The strength of the inferences from biomarker studies can be enhanced by integrating the responses from multiple biomarkers that encompass specificity to different contaminant classes as well as reflecting combined impacts on organism fitness. Their greatest value is as a line of evidence to supplement the more conventional measures of chemistry, ecotoxicology and bioaccumulation in a weight of evidence prediction of ecological risk. Few biomarkers are at a stage of development that they could be considered for use in water quality guideline development on their own.

ACKNOWLEDGMENTS

The ideas for this paper were conceived while EPG was the recipient of a Distinguished Visiting Scholar Fellowship from CSIRO Land and Water. EPG was supported in part by the University of Washington Superfund Research Program, Grant #: NIEHS P42ES004696. This manuscript was improved by careful reading by Anthony Chariton and Stuart Simpson (CSIRO, Land and Water), as well as by two anonymous reviewers.

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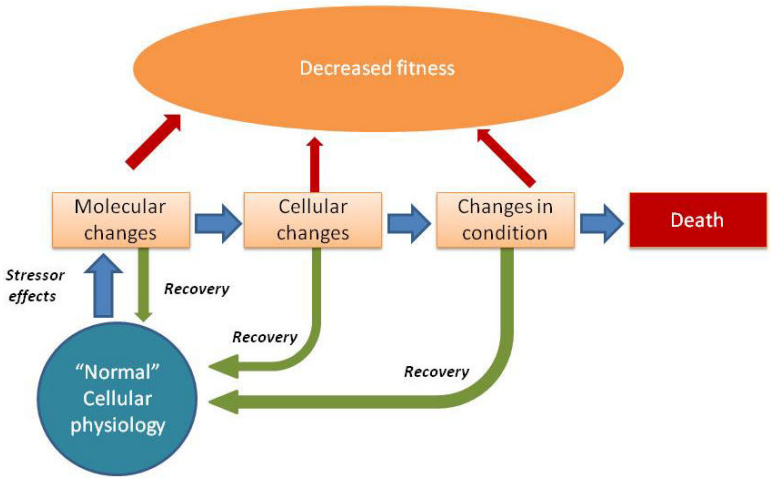

Figure 1.
Conceptual model of a cellular response to a stressor to be evaluated using biomarkers

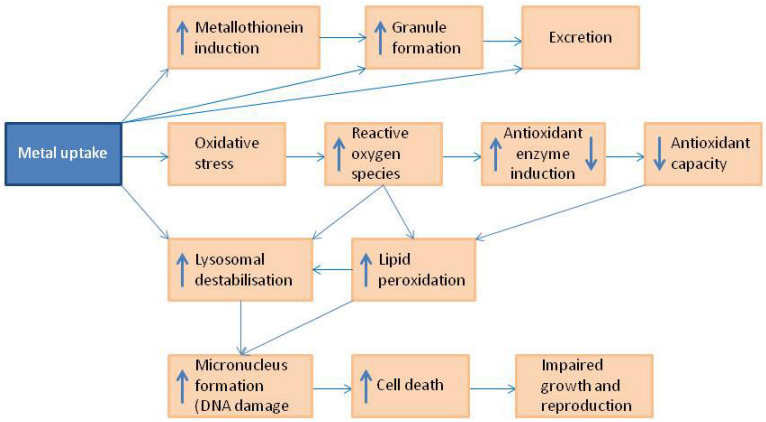

Figure 2.

Flow diagram showing a *cascade* of interlinked cellular reactions which can occur in response to metal exposure (from Taylor and Maher, 2010 with permission from Nova Science Publishers Inc.)

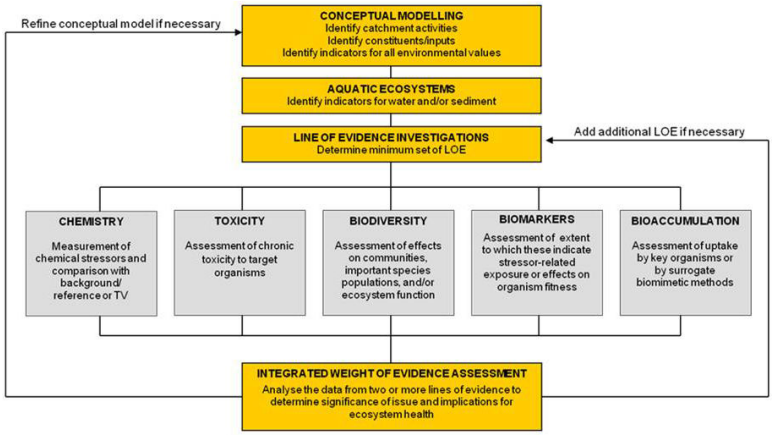

Figure 3.

A typical weight of evidence framework for water quality assessment (adapted from Humphrey et al. 2013)

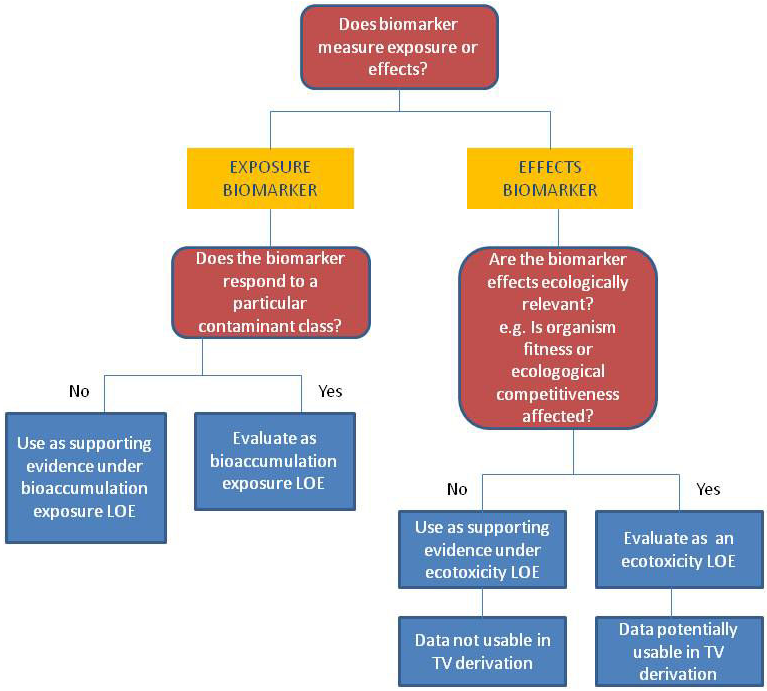

Figure 4.
Framework for evaluating biomarker results

Table 1

Examples of biomarkers that have been used to assess aquatic contaminants

Category	Measurement and Indication	References
Biomarkers of exposure		
Bile fluorescent aromatic compounds (FACs)	Metabolites of polycyclic aromatic hydrocarbons measured in the bile of fish, can reflect exposure to oil	Myers et al. 1991; Myers et al. 1994
Cytochrome P4501A mRNA or protein	An inducible isoform of the cytochrome p450 family that is measured in various tissues of fish and bivalves exposed to oil, and other chemicals that are Ah receptor agonists	Collier et al. 1996; Roberts et al. 2004
Ethoxyresorufin-o-deethylase (EROD), Aryl hydrocarbon hydroxylase	Catalytic activities of CYP1A enzyme described above	Huggett et al. 2003; Martinez-Gomez et al. 2009
Vitellogenin (vtg)	Egg yolk precursor protein induced on exposure to a broad range of estrogenic compounds	Folmar et al. 1996; Denslow et al. 2004
Metallothioneins	Metal-binding proteins that are induced on exposure to certain metals (Cd, Hg)	Roberts et al. 2005; Sakuragui et al. 2013; Williams and Gallagher 2013
Biomarkers of biological effects		
Heat-shock proteins (i.e. HSP 90)	Proteins that are induced in tissues of aquatic organisms as a generalized response to stress, including exposure to chemicals, hypoxia, and temperature	Downs et al.2006
Markers of oxidative stress (heme oxygenase, superoxide dismutase, glutathione, catalase, lipid peroxidation)	Enzymes, cofactors in metabolic products that can be quantitatively altered on exposure to pollutants. Oxidative stress is an adverse cellular and tissue level response to a variety of contaminants and non-chemical stressors	Ahmad et al. 2006; Nogueira et al. 2013; Patil and David 2013; Pereira et al. 2013; Williams and Gallagher 2013
Markers of lysosomal membrane stability	Sub-cellular organelles containing hydrolytic enzymes that are sensitive to toxicity leading to membrane rupture and leakage (metals)	Ringwood et al. 2004; Edge et al. 2012
Condition indices (hepatosomatic index, gonadal indices)	Decreases in organ weight relative to whole body weight can reflect organ toxicity or disease	Johnson et al. 2008a; Blazer et al. 2012
Circulating hormone levels	Levels of circulating hormones can be used to measure normal sexual and reproductive behaviour	Blazer et al. 2012
DNA damage measures (induction of DNA repair enzymes, presence of PAH-DNA adducts)	DNA damage is a reflection of exposure and genotoxic effects	Balk et al. 2011
Triglyceride levels, IGF1,	The levels of lipids (such as triglycerides) and of growth hormones (such as IGF1) can be used as a metric of the animals energy reserves	Balk et al. 2011
Biomarkers that integrate both chemical exposures and biological effects		
Acetylcholinesterase (AChE)	An enzyme that hydrolyzes neurotransmitters that is inhibited by exposure to carbamate and organophosphate pesticides	Laetz et al. 2009
Tissue transcriptome	Microarray and RNA sequencing of tissue transcriptome can identify cellular pathways perturbed by chemical and environmental stressors	Connon et al. 2012; Uren et al. 2013