Evaluation of Effects Based Methods for Regulating Metals in Aquatic Ecosystems

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Summary: The European Union is currently evaluating the use of Effects Based Methods (EBMs) as part of a more integrative strategy for regulating a number of substances of concern under the Water Framework Directive. This white paper evaluates the use of 13 EBMs that might be used in the regulation of metals. Each EBM was evaluated with respect to metal specificity and sensitivity, sensitivity to other classes of toxicants, and the strength of the relationship between effects measured by the EBM and effects observed at higher levels of biological organization such as the individual or population. The evaluation concluded that none of the EBMs evaluated met all three criteria of being sensitive to metals, insensitive to other classes of toxicants, and a strong indicator of effects at the whole organism or population level.

The current approach of regulating metals by using Biotic Ligand Model (BLM)-based Environmental Quality Standards (EQS) is sensitive only to metals and has strong links to effects at the individual and population level but does not consider the integrated effects of metal mixtures on aquatic systems. Given the lack of suitable EBMs for metals, it is recommended that continued development of mixture BLMs may be the most effective way to achieve the goal of a more holistic approach for regulating metals in aquatic ecosystems.

Introduction

The protection goal of the European Water Framework Directive (WFD) is to maintain waterbodies that have good chemical and ecological status and improve all of those that do not. Protection of water quality under the WFD is currently based on monitoring of water concentrations of individual substances and comparison to Environmental Quality Standards (EQS). However, the EU is considering incorporating Effects Based Methods (EBMs), a holistic approach to provide a more accurate assessment of risks and a more appropriate targeting of monitoring and measures under the WFD (with particular attention on of priority substances, specific pollutants and Watch List substances). This approach would group substances by mode of action (MoA) and then use MoA-specific effects assays to establish a trigger value for monitoring compliance, thus allowing for assessment of mixtures with the same MoA.

For some organics, the MoA is well-defined and there are, in some cases, EBMs developed that are MoA-specific. In contrast, for metals, there are a number of challenges related to MoA and EBM specificity. Further, in considering Adverse Outcome Pathways (Ankley et al. 2010) for these MoAs, the utility of EBMs for predicting ecological impacts at higher levels of biological organization is often limited or not well studied. These issues need careful consideration before such an approach could be successfully implemented for metals.

The objective of this white paper is to characterize and evaluate these issues. To accomplish this, a summary review of MoAs for metals is provided. This includes the relevant EBMs for each MoA and an evaluation of their specificity, sensitivity, and links to effects at higher levels of biological organization. A qualitative assessment of costs and ease of use for each EBM is also provided. Based on this evaluation, conclusions regarding the utility of EBMs for regulating metals in aquatic ecosystems and recommended paths forward are provided.

Effects Based Methods for Assessing for Metals in Aquatic Systems

The underlying MoAs for metals on aquatic organisms have been extensively investigated over the past 50 years. Metals exert toxicity on aquatic organisms by a variety of biological pathways, some of which are limited to specific metals or groups of metals. In general, our understanding of MoAs for metals is more developed for acute toxicity (relatively high metal concentrations in short-term exposures) compared to chronic toxicity (relatively low metal concentrations in exposures encompassing a significant fraction of an organism's life cycle). This is especially true with respect to linking MoAs to effects at higher levels of biological organization.

A comprehensive review of all metal MoAs is beyond the scope of this white paper. However, the following summarizes the more well-known MoAs. For each identified MoA, candidate EBMs are described, their specificity and sensitivity to metals assessed, and the strength of their linkage to effects at higher levels of biological organization evaluated. Table 1 summarizes this information.

Ion Homeostasis

Mechanisms of Action: Perhaps the most well characterized MoA for metals with direct links to acute and, in some cases, chronic metal toxicity is disruption of ion homeostasis. In freshwater, aquatic organisms diffusively lose major ions (particularly Na^+ and Ca^{2+}) to the environment and compensate for this loss by actively taking up ions against their concentration gradient via proteins involved in active transport. A range of divalent metals have similar charge and ionic radius such that they competitively or non-competitively inhibit proteins involved in active transport leading to a net loss of ions.

Effects Based Methods: Disruption of ion homeostasis can be measured directly using radio-isotopic techniques to measure unidirectional flux rates (Grosell and Wood 2002, Niyogi et al. 2015), and indirectly by measuring changes in net ion flux (Brix et al. 2012) or whole-body ion concentrations (Pane et al. 2003, Grosell and Brix 2009). Effects on unidirectional and net ion flux can manifest in a matter of hours, while effects on blood/hemolymph or whole-body ion concentrations typically requires days to weeks of exposure to detect. Use of radio-isotopic techniques, while sensitive, requires considerable expertise and is probably not practical as an EBM for routine monitoring. Measurement of blood/hemolymph ion concentrations is more sensitive than whole body ion measurements, particularly for Ca where high concentrations in bone and exoskeleton make it difficult to detect disruptions in ion homeostasis (Grosell et al. 2006).

Sensitivity and Specificity: Some metals can be generally classified as either Na-antagonists (Cu and Ag) or Ca-antagonists (Cd and Zn), while other metals are known to disrupt homeostasis of both ions (e.g., Pb) and Ni disrupts Mg and Ca homeostasis in some species (Hogstrand et al. 1994, Bury and Wood 1999, Grosell and Wood 2002, Rogers et al. 2003, Niyogi and Wood 2004, Pane et al. 2005, Niyogi et al. 2014). However, there are exceptions to these general classifications. Copper, for example, has been demonstrated to disrupt Ca homeostasis in snails (Brix et al. 2011). Disruption of ion homeostasis by metals has been demonstrated to occur at relatively low metal concentrations that are comparable to EQS in some cases (Grosell and Wood 2002, Brix et al. 2012).

Other toxicant classes are also known to disrupt ion homeostasis. Several pesticides, for example, have been shown to inhibit Na^+-K^+-ATPase activity in fish and invertebrates and/or reduce extracellular fluid ion concentrations (Moorthy et al. 1984, Suvetha et al. 2010). Similarly,

pharmaceuticals such as diclofenac have also been shown to reduce extracellular fluid concentrations of sodium and chloride in fish (Saravanan et al. 2011). Changes in ion concentrations and pH in the environment will also have significant effects on ion transport rates and whole-body ion concentrations.

Linkage to Higher Level Effects: Acute disruption of ionoregulation is directly linked to effects on organism survival. Acute loss of Na^+ leads to compensatory volume loss of extracellular fluid and ultimately cardiovascular collapse and death in aquatic organisms (Wood 2012). Acute loss of Ca^{2+} can interfere with intracellular signaling involved in a variety of physiological functions including nervous system function and muscle contraction (Wood 2012). Chronic loss of Ca^{2+} can affect growth processes involved in bone and shell formation, particularly in molluscs (Brix et al. 2011, Brix et al. 2012). There are no clear links between ionoregulatory disruption and other chronic endpoints such as reproduction, but they have not been extensively studied.

Oxidative Stress

Mechanisms of Action: Formation of reactive oxygen species (ROS) is a potential mechanism of metal toxicity in aquatic organisms. ROS can be generated by both endogenous and exogenous mechanisms and is regulated by a variety of non-enzymatic (e.g., glutathione) and enzymatic antioxidants (e.g., superoxide dismutase, catalase, glutathione peroxidase) (Regoli and Giuliani 2014).

One of the most important reactions for generation of ROS is the Fenton reaction, which leads to the formation of the hydroxyl radical ($^{\bullet}OH$):

$$metal^{n+} + H_2O_2 \rightarrow metal^{n+1} + {}^{\bullet}OH + OH^-$$

Once generated, ROS can directly cause DNA damage and also elicit changes in a variety of interacting signal transduction pathways that are related to the cell cycle and apoptosis. Disruption of these pathways (either stimulation or inhibition) leads to abnormal cell cycles and, potentially, to the formation of tumors (Valavanidis et al. 2006). ROS can also directly cause lipid peroxidation leading to cell membrane instability. Further, many of the products of lipid peroxidation are active mutagens that can cause DNA damage (Esterbauer et al. 1990).

Effects Based Methods: There are a number of endpoints that can be used to measure oxidative stress in aquatic organisms. Depletion of various antioxidants (superoxide dismutase, glutathione, catalase) is commonly used indicators of oxidative stress (Valavanidis et al. 2006). Patterns of relative depletions of different antioxidants can be complex and metrics such as Total Oxyradical Scavenging Capacity (TOSC) are used as integrative measures of oxidative stress (Nardi et al. 2018). The other common effects based EBM is characterization of lipid peroxidation via measurement of malondialdehyde (a secondary degradation product) using the thiobarbituric reactive substances test (Lushchak 2011). Finally, metallothioneins have also been used as a measure of oxidative stress induced by metals but the relationship between MT induction and oxidative stress is variable across species and exposure scenarios (Nardi et al. 2018).

Sensitivity and Specificity: Oxidative stress has been measured at concentrations approximating those associated with acute and chronic toxicity for some metals (Vieira et al. 2009, Zhang et al. 2010, Ransberry et al. 2015, Zheng et al. 2016) (Table 1). Oxidative stress is also induced by a number of other anthropogenic and natural stressors including PCBs, PAHs, pesticides, temperature, and salinity (Lushchak 2011).

Linkage to Higher Level Effects: Although oxidative stress can be detected at environmentally relevant metal concentrations, as far as we are aware, there have been no studies providing explicit links between metal-induced oxidative stress and individual or population level effects. This does not mean that such links do not exist, rather that they have not been characterized and quantified. As a result, it is not currently possible to relate an observed level of oxidative stress to effects at the individual or population level. Further, as described above, multiple metrics are often used to measure oxidative stress and these metrics often exhibit complex patterns on a species- and metal-specific basis further complicating data interpretation. What is clear, is that simply detecting a statistically significant difference in one, or even multiple, oxidative stress metrics does not necessarily equate to effects at the individual or population level.

Lysosomal Membrane Stability

Mechanisms of Action: Lysosomes are organelles that play a critical role in cell metabolism. They contain a variety of enzymes that digest proteins, nucleic acids, sugars, and lipids in a low pH environment. Lysosomes act by either fusing with autophagosomes or heterophagosomes, or by directly absorbing substances in the cellular environment. Both inorganic and organic contaminants have been shown to reduce lysosomal membrane stability which increases the probability of inappropriate release of lysosomal enzymes to the intracellular environment where they can cause damage.

Effects Based Methods: Lysosome membrane stability has primarily been studied in marine organisms, although there are a few examples in freshwater organisms as well (Monteiro et al. 2011). Lysosome membrane stability is commonly determined using the Neutral Red Retention assay. Reduced retention of the dye neutral red by lysosomes has been directly linked to membrane damage and impairment of the H^+-ATPase in the lysosomal membrane (Lowe et al. 1992). An alternative method to measure membrane stability is to measure the time required for a substrate of a lysosomal enzyme to penetrate the lysosome and react to form an enzyme product that can be visualized. The acid hydrolase β-N-acetylhexosaminidase is commonly used as the enzyme, though others have been used as well. Reduced time to maximum product formation is interpreted as degraded membrane stability (Moore 1988).

Sensitivity and Specificity: Many chemicals may trigger changes in lysosomal membrane stability, including metals, PAHs, PCBs, DDTs, and others (Nott et al. 1985, Grundy et al. 1996, Moore et al. 2006)**.** Copper has been shown to affect lysosomal membrane stability in mussels at concentrations at or slightly higher than EQS, while other metals require concentrations significantly higher than EQS to elicit effects (Regoli et al. 1998, Bolognesi et al. 1999, Brown et al. 2004) (Table 1).

Linkage to Higher Level Effects: Reductions in lysosomal membrane stability are strongly linked to increases in hepatic lesions in fish (Kohler 1991) and atrophy of the digestive gland in molluscs (Moore 1988). However, translation of these effects at the organ level to effects at the whole animal or population level has not been well characterized. As with some of the other MoAs for metals, the relationship between lysosomal membrane stability and effects at the individual or population level are likely to be metal- and species-specific. It should not be assumed that simply detecting a statistically significant effect on lysosomal membrane stability will result in a significant effect at the individual or population level of biological organization. To the best of our knowledge, no study has demonstrated this relationship.

DNA Damage

Mechanisms of Action: Mutagens are substances that induce damage to DNA structure causing effects on individual genes or groups of genes, while genotoxins are typically more broadly defined as substances that change the structure of number of genes via direct chemical interaction with DNA (De Lapuente et al. 2015). Metals have been shown to act as both mutagens and genotoxins.

Effects Based Methods: The Comet Assay, or Single Cell Gel Electrophoresis, is the most common EBM for assessing DNA damage by measuring breaks in DNA strands (Singh et al. 1988). The Comet Assay is relatively standardized and can be performed on a variety of cell types, although there are variations with respect to lysis buffer and conditions, denaturation conditions, and electrophoresis conditions (De Lapuente et al. 2015).

Sensitivity and Specificity: DNA damage has been observed at concentrations about an order of magnitude higher than EQS for Cd, Cr, and Cu (Bolognesi et al. 1999, Pellegri et al. 2014). In addition to metals, DNA damage can be caused by a wide range of toxicants including nanoparticles, pharmaceuticals, PAHs, and pesticides, as well as natural stressors such as UV radiation (De Lapuente et al. 2015).

Linkage to Higher Level Effects: DNA damage can occur in several ways. First DNA adducts can form in which a toxicant or other molecule is directly bound to DNA and alters expression of tumor suppression genes or oncogenes, potentially leading to the development of cancer. Alternatively, toxicant exposure can lead to DNA base oxidation or strand breaks. These latter two forms of DNA damage may be either repaired or mis-repaired (Martins and Costa 2017). When correctly repaired, effects at higher levels of biological organization on unlikely to be realized. When mis-repaired, a variety of effects including oncogenesis, teratogenesis, and cell apoptosis can occur. While DNA damage clearly provides a mechanism for effects at higher levels of biological organization, how a specific level of DNA damage translate to effects at higher levels of biological organization has been poorly studied and is likely to be both species- and metal-specific. As with many other metal MoAs, it should not be assumed that detecting a statistically significant increase in DNA damage will result in a detectable effect at the individual or population level of biological organization. To the best of our knowledge, no study has demonstrated this relationship.

Deformities (Chironomids/Diatoms/Amphibians)

Mechanisms of Action: Environmental monitoring of polluted sites and laboratory studies have shown associations between metal concentrations and the incidence of deformities in chironomid mouthparts, diatom frustules, and amphibian limb development. The specific mechanisms of action that causes these deformities vary by organism and metal and are not well understood in many cases.

Effects Based Methods: Chironomid mouthpart deformities have been used in a number of laboratory and field studies. However, deformity criteria are not standardized across the literature and assessment of morphological deformities is a subjective process. A recent comparative study of deformity assessment conducted by 25 experts revealed high variability in assessments (Salmelin et al. 2015). Diatom frustule deformities have also been used in number of laboratory and field studies and the methodology for assessment is well standardized (Lavoie et al. 2017, Pandey et al. 2017).

Sensitivity and Specificity: Deformities in chironomid mouthparts have been associated most commonly with exposure to Cd, Cu, Pb, Ni, and Zn. However, other contaminants have also

been shown to induce deformities including a variety of pesticides, nanoparticles, nonylphenol, phthalates, and steroidal estrogens (Gagliardi et al. 2016). Diatom frustule deformities have been linked to Cu, Ni, and Zn exposures, but are also induced by pesticides and excess nutrients (Lavoie et al. 2017). Metals such as Cr and Se are also associated with deformities in amphibians (Rowe et al. 1996), but other contaminants such as pesticides and natural stressors such as UV radiation and parasitic infection can also cause deformities (Bacon et al. 2013).

Linkage to Higher Level Effects: Deformities in these different aquatic organisms have been linked to individual, and in some cases, population level effects. For example, mouthpart deformities in chironomids have been correlated with effects on survival in laboratory studies (De Bisthoven et al. 2001). Similarly, diatom frustule deformities have been correlated with reductions in diatom abundance and changes in periphyton community structure (Pandey et al. 2014, Lavoie et al. 2017). Finally, amphibian deformities are associated with reduced grazing efficiency in tadpoles (Rowe et al. 1996) and impaired locomotion in adults (Bacon et al. 2013).

In Vivo Tests on Algae, Invertebrates and Fish

Mechanisms of Action: *In vivo* testing evaluates all MoAs for metals (and other toxicants), providing an integrative measure of their effects at the individual level of biological organization.

Effects Based Methods: Methods for conducting *in vivo* testing with algae, invertebrates and fish are well developed for a wide range of organisms (OECD 1996, OECD 2000, OECD 2006). Test methods for both acute and chronic exposures are available. In the United States, this type of approach has been widely used to monitor the impacts of point source discharges and receiving water body environmental quality for more than 30 years (USEPA 2002). A similar program was developed in Canada and has been in place for about 20 years (Environment Canada 2012).

Sensitivity and Specificity: *In vivo* testing of aquatic organisms is sensitive to metals as many of these species and test methods have been used to develop the species sensitivity distributions on which many EQS are based. The exceptions are metals such as Se where the primary mechanism of action is via dietary exposure (DeForest et al. 2017). For the same reasons, these methods are also sensitive to many other classes of toxicants (pesticides, PAHs, pharmaceuticals, etc.) at concentrations near their respective EQS. As a result, these test methods provide little ability to discriminate between classes of toxicants, much less specific metals. Additional test methods such as Toxicity Identification Evaluation procedures have been developed to identify specific causes of toxicity, but these methods require considerable expertise (USEPA 1992).

Linkage to Higher Level Effects: *In vivo* testing with aquatic organisms provides a direct measure of effects at the individual level. Mesocosm and field studies have demonstrated these effects are strongly related to effects at the population level for many metals (USEPA 1976, Clements et al. 1990, Versteeg et al. 1999, Roussel 2005).

Cytochrome P450

Mechanisms of Action: Cytochromes P450 (CYPs) are a family of hemoproteins involved in enzymatic detoxification pathways for a wide range of both biotic degradation productions and xenobiotic substances (Gagnon and Rawson 2017). There are a large number of isozymes involved in these degradation pathways with some isozymes being specific to certain substrates while others being more generic. Metals have generally been shown to reduce the activity of the CYP isozyme CYP1a1.

Effects Based Methods: Although CYP1a1 induction can be indirectly measured through changes in gene expression (Sorrentino et al. 2005), the most straightforward method to measure

induction is through measurement of EROD activity using a fluorometric technique (Pohl and Fouts 1980). EROD activity describes the CYP1a1 mediated deethylation of 7-ethoxyresorufin to resorufin and is directly related to the amount of CYP1a1 present (Gagnon and Rawson 2017).

Sensitivity and Specificity: A wide range of metals have been shown to inhibit CYP1a1 activity in aquatic organisms (Sen and Semiz 2007, Vieira et al. 2009) (Table 1). Inhibition has been observed at concentrations generally an order of magnitude higher than typical EQS with the exception of Pb where inhibition was observed near the EQS, but concentrations approximating EQS have not be routinely tested (Vieira et al. 2009, Zhang et al. 2010). In contrast to metals, toxicants such as PAHs, pesticides, and PCBs induce CYP1a1 activity (Gagnon and Rawson 2017). Studies to date suggest inhibition is a metal-specific effect while other toxicants lead to CYP induction. Consequently, when multiple classes of toxicants are present interpretation of CYP activity can be confounded (Sorrentino et al. 2005, Sen and Semiz 2007).

Linkage to Higher Level Effects: The induction of CYP1a is clearly linked to the metabolism of PAHs and subsequent formation of degradation products that are genotoxic as well as the potential upregulation of oncogenes and suppression of the immune system (Gagnon and Rawson 2017). Inhibition of CYP1a, as is generally the case with metal exposure, would presumably reduce the ability of organism to detoxify both biotic degradation products and xenobiotic compounds, but this has not been studied.

Acetylcholinesterase

Mechanisms of Action: Acetylcholinesterase (AChE) is an important enzyme that catalyzes the degradation of acetylcholine, a major neurotransmitter in the body. Inhibition of AChE leads to extended stimulation of the neuron by acetylcholine which can cause a variety of neural and muscular dysfunctions (Fulton and Key 2001, Moser and Padilla 2011).

Effects Based Methods: Acetylcholinesterase activity is readily measured using spectrophotometric techniques (Ellman et al. 1961, Sandahl and Jenkins 2002).

Sensitivity and Specificity: Metals such as As, Cu, Cd, Cr, Mo, and Pb have been demonstrated to inhibit AChE activity in a number of aquatic organisms (Forget et al. 1999, Diamantino et al. 2000, De Lima et al. 2013), while Cu can either stimulate (Tilton et al. 2011, De Lima et al. 2013) or inhibit AChE activity (Forget et al. 1999, Vieira et al. 2009). In many cases, AChE inhibition occurs at or below metal concentrations associated with acute survival effects, and near the EQS for Cu (Brown et al. 2004). Organophosphate and carbamate pesticides also potently inhibit AChE activity, as this is their primary mechanism of action (Fulton and Key 2001).

Linkage to Higher Level Effects: AChE inhibition has been directly linked to survival effects in both fish and invertebrates at metal concentrations associated with acute toxicity. Chronic sublethal effects of metal-induced AChE inhibition have not been demonstrated (Tilton et al. 2011).

Urease

Mechanisms of Action: Urease is a nickel metalloenzyme involved in the hydrolysis of urea into ammonia and bicarbonate. Urease can be found in most plants, bacteria, and in some aquatic invertebrates, but is absent in fish (Abolins-Krogis 1986, Muyssen et al. 2004). Inhibition of urease activity can lead to disruptions in nitrogen metabolism.

Effects Based Methods: Urease activity in metal contaminated soils is routinely measured to evaluate impacts on microbial soil nitrification (Kim et al. 2008). However, in water, urease activity is too low for this approach. Instead, the effects of toxicants on urease for aquatic samples

typically involves the isolation of urease from jack beans. The isolated enzyme is then exposed to water samples along with urea as a substrate for the enzyme. After a given reaction time (minutes) urease activity can be determined by directly measuring the amount of ammonia generated by the breakdown of urea (Jung et al. 1995). Alternatively, the assay is run in a phosphate buffered solution where the pH change caused by the formation of ammonium carbonate is measured (Brack et al. 2000). In some studies the assay is conducted at pH 5 (Brack et al. 2000), which will substantially increase metal bioavailability. In other variations, metal bioavailability is reduced, such as in the use of phosphate buffers (Olson and Christensen 1982).

Sensitivity and Specificity: There is considerable variability in the sensitivity of the assay to metals which may be due to differences in assay methods and effects on metal bioavailability. The assay is consistently sensitive to Cu at concentrations near the EQS, is variably sensitivity to Zn with effect concentrations near the EQS to more than two orders of magnitude higher (Olson and Christensen 1982, Jung et al. 1995, Brack et al. 2000). The assay is insensitive to Ni, which is not surprising given it is essential for enzyme function (Olson and Christensen 1982, Jung et al. 1995). The assay is relatively insensitive to Ag, Cd, Co, and Pb, though these results may be confounded by use of a phosphate buffer (Olson and Christensen 1982). Several studies indicate urease is insensitive to other classes of toxicants (e.g., pesticides) in water (Olson and Christensen 1982, Jung et al. 1995), although some classes of toxicants (e.g., PAHs) have not been studied.

Linkage to Higher Level Effects: Inhibition of urease activity has direct effects on nitrogen metabolism in microbes, algae, and aquatic plants and these are thought to lead to population level effects, at least in microbes and algae (Rai and Rai 1997).

Bacteria Reporter Assay

Mechanisms of Action: Genes involved in metal transport and detoxification often have metal responsive elements (i.e., metal binding sites) associated with the gene promoter that are triggered when metal enters the cell and binds to the metal responsive element (Van der Meer and Belkin 2010). The resulting transcriptional event results in either increased production of a specific protein, or a signaling cascade causing the production of multiple proteins.

Effects Based Methods: The bacteria reporter assay uses recombinant bacteria (e.g., *Escherichia coli*, *Staphylococcus aureus*) in which one or more responsive elements are linked to a reporter gene. Binding of the toxicant of interest (metal or organic compound depending on the specific responsive element) then activates the reporter gene. Different reporter genes have been used in this assay, but the most common are bioluminescent genes (e.g., LuxCDABE gene cassette) where the intensity of bioluminescence measured is related to the degree of transcriptional response (Van der Meer and Belkin 2010).

Specificity and Sensitivity: The sensitivity and specificity of this assay is dependent on the specific promoter(s) used in the assay. Typically, promoters with metal responsive elements are highly sensitive to specific metals at concentrations near the EQS and considerably less responsive to others (Tauriainen et al. 1998, Tauriainen et al. 2000, Riether et al. 2001, Ivask et al. 2009, Carvalho et al. 2014). Promoters with metal responsive elements are unlikely to be sensitive to other classes of toxicants, although no studies specifically testing this have been identified.

Linkage to Higher Level Effects: This assay uses recombinant bacteria to detect metals in the environment. The promoters involved in this detection system are often associated with genes involved in routine metal transport processes within a cell. Consequently, this assay provides a measurement of metal exposure with no direct links to effects.

ALAD

Mechanisms of Action: ALAD (δ-aminolevulinic acid dehydratase) is an enzyme that catalyzes the formation of porphobilinogen, a precursor of hemoglobin. ALAD requires a Zn co-factor for proper function, but Pb has a much higher affinity for the Zn binding site leading to ALAD disfunction and reductions in heme synthesis (Schmitt et al. 2002).

Effects Based Methods: ALAD activity can be directly measured in tissues using spectrophotometric techniques (Schmitt et al. 1993). Changes in gene expression can also be measured (Mager et al. 2008), although this will not directly characterize potential changes in enzyme activity.

Sensitivity and Specificity: ALAD activity is strongly correlated with blood Pb concentrations, making in a useful specific marker for Pb exposure (Hodson et al. 1977). Inhibition of ALAD activity has been demonstrated in a number of laboratory and field studies at environmentally relevant concentrations (Schmitt et al. 1993, Schmitt et al. 2002). Elevated blood Zn has been demonstrated to ameliorate the effect of Pb on ALAD activity (Schmitt et al. 1993).

Linkage to Higher Level Effects: Inhibition of ALAD reduces synthesis of the hemoglobin precursor. However, a number of studies demonstrate reduction in ALAD activity is inconsistent in causing corresponding reductions in hemoglobin concentration (Krajnovic-Ozretic and Ozretic 1980, Haux et al. 1986, Schmitt et al. 1993). Links to effects at higher levels of biological organization have not been demonstrated in aquatic studies.

Metallothioneins

Mechanisms of Action: Metallothioneins are a class of proteins that have sulfhydryl groups that actively bind metals. The concentration and particular forms of metallothioneins present controls the intracellular activity of essential and non-essential metals regulating their ability to interact with other proteins or generate ROS that might lead to oxidative stress. Numerous studies have shown MT induction in the presence of elevated metal concentrations in the environment (Amiard et al. 2006).

Effects Based Methods: Metallothioneins can be measured in multiple tissues using several different techniques including differential pulse polagraphy, spectrophotometrically, various immunoassays (e.g., ELISA), through characterization of gene expression, and several other methods (Ryvolova et al. 2011).

Sensitivity and Specificity: Metallothionein induction has been observed in the presence of multiple metals including Ag, Cd, Cu, Hg, Ni, Pb, V, and Zn, with Cd being the most potent and consistent inducer. In some cases, MT induction has occurred at concentrations approximating EQS, while in other cases MT induction has not occurred at metal concentrations more than 100-fold higher than EQS (Brown et al. 2004, Amiard et al. 2006). Other natural and anthropogenic stressors are also known to induce MTs including anoxia and freezing (English and Storey 2003), biochemical changes during the reproductive cycle (Baudrimont et al. 1997), pesticides (Erdogan et al. 2011), and gametogenesis (Oaten et al. 2017).

Linkage to Higher Level Effects: Metallothioneins are considered a biomarker of exposure and there are no known mechanistic links between MT induction and effects on individuals or populations.

eDNA Metabarcoding

Mechanisms of Action: Environmental DNA (eDNA) metabarcoding uses high throughput genetic sequencing of environmental samples to provide a characterization of the biological community present at a site. In characterizing the changes in the biological community, the approach integrates effects from all mechanisms of action.

Effects Based Methods: Environmental DNA can be obtained from water and/or sediment samples and extracted eDNA is amplified for specific marker genes (e.g., 18S) and then sequenced using a high throughput sequencing platform. Each unique sequence is then identified using existing taxonomic libraries and changes in community structure are assessed relative to a reference site (Baird and Hajibabaei 2012, Chariton et al. 2016). Using current techniques, sequence abundance is only weakly correlated with organism abundance, so data are normally analyzed only based on presence/absence although new techniques are being developed to allow assessment of organism abundance (Egge et al. 2013).

Sensitivity and Specificity: eDNA has much higher resolution than traditional bioassessment techniques, which substantially increases the ability to detect changes in community structure (Andujar et al. 2018, Yang et al. 2018). Changes in community structure reflect responses to both anthropogenic and natural stressors (Chariton et al. 2015). Consequently, similar to traditional bioassessment technique, eDNA metabarcoding currently lacks the ability to discriminate between metals and other stressors other than by qualitative methods (Chariton et al. 2016).

Linkage to Higher Level Effects: eDNA metabarcoding measures the presence/absence and potentially relative density of organisms at a site and consequently is a direct measure of population and community level effects.

Table 1. Summary of Sensitivity, Specificity, and Linkage to Individual/Population Effects for Metal EBMs

Mode of Action	*Metal Specificity*	*Other Toxicants*	*Metal Sensitivity*	*Link to Individual/Population Effects*	*Cost/Ease of Use*[1]
Ion Homeostasis	Na: Ag, Cu, Pb Ca: Co, Cd, Pb, Zn Mg: Ni	Pesticides, Pharmaceuticals, Salinity	Mixed: Effects detectable at concentrations near EQS in some cases but not all	Strong – Demonstrated links to survival and growth, but no links to reproduction demonstrated	Low/Simple
Oxidative Stress	As, Cd, Co, Cr, Cu, Fe, Hg, Ni, Ti, V, Zn	Pesticides, PAHs, PCBs, Dioxins, Temperature, Salinity	Mixed: Oxidative stress detected at environmentally relevant metal concentrations for some metals	Weak: No studies link to individual/population level effects	Moderate/Simple
Lysosomal Membrane Stability	Cd, Cu, Hg, Pb	PAHs, PCBs	Mixed: Effects near the EQS for Cu, but at higher concentrations for other metals	Moderate: Links to organ level effects, but individual and population level effects are not documented	Low/Simple
DNA Damage	Cd, Cr, Cu	Nanoparticles, Pesticides, Pharmaceuticals, UV Radiation	Moderate: DNA damage occurs at concentrations ~10-fold higher than EQS	Weak: Limited evidence of effect from DNA damage beyond the cellular level	Low/Moderate
Deformities	As, Cd, Cr, Cu, Hg, Ni, Pb, Se, Zn	Pesticides, Phthalates, Nanoparticles, Estrogens, Nutrients, Parasites, UV radiation	Mixed: Effects at concentrations near EQS for some metals but not others	Strong: Clear links between observed deformities and effects on individuals and populations	High/Moderate
In Vivo Testing	Non-specific	Many other toxicants	High: Effects demonstrated at concentrations near the EQS for most metals	Strong: Directly measures effects at the individual level with strong links to population level effects	High/Moderate
Cytochrome P450	Inhibition: Cd, Cu, Hg, Ni, Pb, Sb, Zn	Induction: PCBs, Pesticides, PAHs	Moderate: CYP1 inhibition occurs at concentration ~10-fold higher than EQS	Weak: Unclear how inhibition of CYP1a impacts organisms	Low/Simple

Acetylcholinesterase	As, Cr, Cu (inhibits and stimulates), Cd, Mo, Pb	Pesticides	Mixed: Effects have been demonstrated at concentrations causing acute toxicity and for Cu near the EQS	Strong: Strong correlation between AChE inhibition and acute effects on survival	Low/Simple
Urease	Ag, Cd, Co, Cr, Cu, Hg, Ni, Pb, Zn	Pesticides at high concentrations	Mixed: Effects for Cu near the EQS, variably for Zn, insensitive to Ag, Cd, Co, Ni, and Pb	Moderate: Effects on nitrogen metabolism and inferred effects at individual/population level	Low/Simple
Bacteria Reporter Assay	Ag, As, Cd, Co, Cr, Cu, Hg, Ni, Pb, Sb, Zn	Unlikely or only at very high concentrations	Detectable at concentrations near the EQS for most metals	Weak: No direct link to individual/population level effects	Moderate/Moderate
ALAD	Pb	Zinc ameliorates Pb effects	Mixed: Inhibition occurs near the EQS for Pb, but insensitive to other metals	Weak: Sporadic links to reduced haemoglobin concentrations	Low/Simple
Metallothionein	Ag, Cd, Cu, Hg, Ni, Pb, V, Zn	Anoxia, Freezing Gametogenesis, Pesticides	Mixed: Effects near the EQS in some cases but no effects observed in other cases where concentrations are 100-fold higher than EQS.	Weak: No studies linked to individual/population level effects	Moderate/Moderate
eDNA	Non-specific	All toxicants and natural stressors causing population level effects	High: Detects loss of metal sensitive taxa	Strong – Directly measures presence/absence of species	High/Difficult

[1] Cost/Ease of Use qualitatively was evaluated on a relative scale.

Discussion and Conclusions

Summary Assessment

The above summary describes the thirteen EBMs most prominent in the scientific literature that might be considered for use in regulating metals in aquatic systems under the WFD. Each EBM was evaluated with respect to metal specificity and sensitivity, as well as the links between EBM endpoints and effects at higher levels biological organization.

From this evaluation three key observations can be made:

1. ALAD and the bacteria reporter assay are the only EBMs that are specific to metals and ALAD only applies to a single metal, Pb. All other EBMs are also sensitive to at least one and often multiple other classes of toxicants and/or natural stressors;
2. Most EBMs have moderate sensitivity (effects ~5-10 fold higher than EQS) or mixed sensitivity (effects at EQS for some metals but not others). Only *in vivo* testing and eDNA metabarcoding are considered to have high sensitivity to metals generally, but they are also sensitive to many other classes of toxicants and natural stressors. It is possible the bacteria reporter assay could be developed to be sensitive to many metals.
3. One half of the evaluated EBMs (including ALAD and the bacteria reporter assay) are considered to have weak links to effects at the individual or population level. Ion homeostasis (except reproduction), deformities, *in vivo* testing, and eDNA metabarcoding are considered strong indicators of individual or population level effects.

These observations indicate that each of the currently proposed EBMs has at least one significant limitation that would make it poorly suited to monitoring compliance with multiple metal EQS in an integrative manner. Indeed, with perhaps the exception of ALAD and the bacteria reporter assay, none of the proposed EBMs is particularly well suited for monitoring compliance of even individual metals, much less metal mixtures.

In contrast, existing individual biotic ligand model (BLM)-based metal EQS are specific to the metals for which they were developed and clearly linked to the effects of metals at the individual and higher levels of biological organization. The primary limitation of existing BLM-based EQS is that, currently, they do not account for potential effects of metal mixtures.

Other Metal-Specific Considerations

There are two additional metal-specific considerations that need to be considered across the spectrum of proposed EBMs. The first is whether EBMs can account for metal bioavailability. EBMs explicitly do account for metal bioavailability as they will not respond unless a sufficient concentration of bioavailable metal is present in the system. However, as detailed above, some EBMs are not sensitive to some or many metals at concentrations near the EQS. As a result, some EBMs will not be particularly informative with respect to metal bioavailability. Further, EBMs may respond differently to factors that influence metal bioavailability. ALAD sensitivity to Pb, for example, is reduced by the presence of elevated Zn concentrations (Schmitt et al. 1993). However, in contrast to this antagonistic interaction for ALAD, Pb and Zn toxicity is strictly additive to the snail *Lymnaea stagnalis*, the most Pb-sensitive taxa tested to date (Cremazy et al. 2018). Hence ALAD is unlikely to predict the toxicity of Pb when elevated Zn is also present for the most Pb-sensitive aquatic organisms.

The other metal-specific issue is naturally elevated background metal concentrations in aquatic systems. In waterbodies where metal concentrations are naturally elevated, application of EQS which are generally based on toxicity studies in which the organisms are acclimated or adapted to relatively low metal concentrations can be problematic (Crommentuijn et al. 2000). It may be possible that sampling of local organisms adapted to naturally elevated background metal concentrations for EBMs may provide a useful tool for addressing this issue. For example, organism collected from such a location may not exhibit an oxidative stress response at naturally elevated background concentrations whereas a laboratory organism might. Alternatively, organisms may exhibit continual low-level responses to EBMs (e.g., elevated metallothionein concentrations) as part of their adaptation to this environment (Knapen et al. 2007). Exactly how specific EBMs will respond to naturally elevated background metal concentrations has not been studied in any detail and will need further evaluation.

Future Directions

Effect Based Methods are already used in some Member States as a weight of evidence approach (along with EQS) to demonstrate ecological impact. However, there is a clear need for development and implementation of tools that allow for a more holistic or integrative assessment of compliance with the objectives of the WFD, including consideration of unknown pollutants, substances which are difficult to detect, and mixtures. For some classes of organic toxicants, available EBMs may provide a viable option for achieving this goal. For metals, it is clear that currently available EBMs do not meet this need. Furthermore, since metals are easily and routinely measured, the added benefit of investing in development of EBMs for metals is unclear.

The ideal hypothetical EBM for metals would be sensitive to a suite of metals at or near the EQS for each metal. Further, the EBM would be insensitive to other classes of toxicants and either insensitive to, or account for, naturally occurring abiotic factors that influence metal bioavailability and toxicity. Even if an EBM could be developed that met all of these criteria, from a regulatory perspective it would still have limitations. While it would be useful for monitoring compliance of metal mixtures with respect to the WFD, if effects were detected at a site, it would not be able to inform the users of the particular components of the metal mixture that were driving non-compliance. This is obviously critical information for successful management of the non-compliance.

One tool currently under development that meets all of the above criteria and provides quantitative information on the relative contribution to non-compliance of individual metals in a mixture is the mixture BLM (mBLM). There has already been considerable progress in developing mBLMs in terms of both fundamental studies on how metals interact at metal binding sites (Komjarova and Blust 2009, Brix et al. 2016, Brix et al. 2017, Cremazy et al. 2019) and in the development of an appropriate modelling framework (Farley et al. 2015, Santore and Ryan 2015, Van Regenmortel et al. 2017, Nys et al. 2018). More study and model refinement is clearly needed including substantial field validation studies, but efforts to date indicate this approach is likely to lead to development of a practical tool for evaluating metal mixture toxicity in aquatic environments.

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