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Brief Communication

Evaluation of effects-based methods as monitoring tools for assessing ecological impacts of metals in aquatic ecosystems

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Abstract

Effects-based methods (EBMs) are considered part of a more integrative strategy for regulating substances of concern under the European Union Water Framework Directive. In general, EBMs have been demonstrated as useful indicators of effects on biota, although links to population and community-level effects are sometimes uncertain. When EBMs are sufficiently specific and sensitive, and links between measured endpoints and apical or higher level effects are established, they can be a useful tool in assessing effects from a specific toxicant or class of toxicants. This is particularly valuable for toxicants that are difficult to measure and for assessing the effects of toxicant mixtures. This paper evaluates 12 EBMs that have been proposed for potential use in the assessment 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 EBM endpoints and effects observed at the whole organism or population levels of biological organization. The evaluation concluded that none of the EBMs evaluated meet 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. Given the lack of suitable EBMs for metals, we recommended that the continued development of mixture biotic ligand models (mBLMs) may be the most effective way to achieve the goal of a more holistic approach to regulating metals in aquatic ecosystems. Given the need to further develop and validate mBLMs, we suggest an interim weight-of-evidence approach that includes mBLMs, macroinvertebrate community bioassessment, and measurement of metals in key macroinvertebrate species. This approach provides a near-term solution and simultaneously generates data needed for the refinement and validation of mBLMs. Once validated, it should be possible to rely primarily on mBLMs as an alternative to EBMs for metals. *Integr Environ Assess Manag* 2023;19:24–31. © 2022 The Authors. *Integrated Environmental Assessment and Management* published by Wiley Periodicals LLC on behalf of Society of Environmental Toxicology & Chemistry (SETAC).

KEYWORDS: Effects-based methods, metals in aquatic ecosystems, toxicant mixtures

INTRODUCTION

The objectives of the European Water Framework Directive (WFD; 2000/60/EC) include the aim to achieve and ensure "good status" of all water bodies throughout Europe through the updating and implementation of management plans at the river basin level. Under the WFD, the toxicity of chemical substances is currently considered using primarily a substance-by-substance approach. However, this single-chemical risk assessment approach for the management of chemical pollution has some limitations including the inability to analyze all substances that are present in the aquatic environment and to predict the effects of the mixture of substances present in the aquatic environment (Altenburger et al., 2015; Brack et al., 2017).

In response to the need for multistressor assessment tools, a technical report published in 2014 described the state-of-the-art for the use of effects-based methods (EBMs) in Europe, gave a series of recommendations for their use in the WFD, and included several fact sheets for different EBMs (Wernersson et al., 2015). In the technical report, it was concluded that the main use of EBM tools within the

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current WFD context would be as screening tools, to establish early warning systems and to take the effects from mixtures of pollutants or not routinely analyzed chemicals ("unknowns") into account. More recently, in 2016 the Water Directors endorsed the need for a more holistic approach, considering the presence of mixtures of chemicals acting together, to provide a more accurate assessment of risks and more appropriate targeting of monitoring and measures (Carere et al., 2021). This is also reflected in the new European Green Deal (European Union [EU], 2019), bringing further attention to the consideration and use of EBMs in a regulatory context.

The protection goal of the European WFD is to maintain water bodies that have good chemical and ecological status and improve all of those that do not (EU, 2000). Protection of water quality under the WFD is currently based on monitoring water concentrations of individual substances and comparing these concentrations to environmental quality standards (EQS). However, EBMs are considered a holistic approach to provide a more integrative assessment of risks and more appropriate targeting of monitoring and measures under the WFD. This approach would group substances by mode of action (MoA) and then use MoA-specific effects assays to establish trigger values for monitoring compliance, thus allowing for the assessment of mixtures with the same MoA (Brack et al., 2019).

For some organic substances, the MoA is well defined and there are, in some cases, EBMs developed that are MoA specific. Triazine herbicides, for example, are known to target photosystem II in plants while organophosphate pesticides are known to target acetylcholinesterase (Duke, 1990; Matsumura, 1975). In contrast, for metals, there are several challenges related to MoA and EBM specificity. More broadly, for EBMs in general, variability in nonchemical stressors and attributes (e.g., temperature and habitat) can confound the interpretation of EBM data. Further, linkages between the subcellular effects often measured by EBMs and effects on population, communities, and emergent properties of ecosystems are frequently obscured or unclear. In considering adverse outcome pathways (AOPs) (Ankley et al., 2010) for metal 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 the present paper was to evaluate a selection of EBMs with a specific focus on specificity and selectivity to metals, and their predictive potential for population or community effects. To accomplish this, we evaluated the 12 candidate EBMs being considered for use with metals (Carere et al., 2021). Three of these EBMs (deformity assessments for diatoms, chironomids, and amphibians; in vivo whole animal testing; and environmental DNA [eDNA] metabarcoding) target apical endpoints while the other nine EBMs target a specific MoA at a subcellular level of biological organization. Each EBM was evaluated using three criteria: specificity, sensitivity, and links to effects at higher levels of biological organization, such as individual, population, and community-level effects.

The remainder of this paper summarizes the evaluation of each EBM using these three criteria. The details of this evaluation for each EBM are provided in the Supporting Information. Based on our evaluation, conclusions regarding the utility of EBMs for regulating metals in aquatic ecosystems and recommended paths forward are provided.

METHODS

EBM specificity and sensitivity

A literature review was conducted to evaluate the specificity and sensitivity of each candidate EBM. The review was not intended to be comprehensive, but rather to identify key information regarding EBM specificity and sensitivity. For all proposed EBMs, previous literature reviews have been conducted summarizing the specificity and sensitivity of the EBM or the assay or analytical methods used in the measurement of the EBM. However, these reviews have typically been undertaken with a broader context of application and not focused on the applicability of the EBM for metals, which is the focus of this evaluation.

Specificity considers the range of metals, other chemical substances, and natural environmental variables (e.g., pH, salinity, and temperature) to which a particular EBM responds. Ideally, if the objective is to regulate metals in aquatic systems, an EBM would respond to multiple metals but not to other substances or environmental variables.

Sensitivity evaluates whether the EBM responds to metals at concentrations comparable to EQS. If EBMs are insensitive to metals at concentrations near the EQS, they will not provide adequate protection for aquatic systems. Conversely, if EBMs are sensitive at concentrations below EQS, they will infer negative effects at concentrations generally considered not to affect aquatic systems. The latter is particularly important for metals, where natural background concentrations of some metals can approach established EQS (Salminen et al., 2005).

Adverse outcome pathway analysis for EBMs

For EBMs that measure subcellular rather than apical (e.g., survival, growth, and reproduction) endpoints, it is important that there are documented direct links between the (sub)cellular endpoint measured and apical endpoints which, in turn, have strong links to effects on populations. Effects-based methods lacking this linkage are effectively biomarkers of exposure rather than effects, or at a minimum, their classification as a biomarker of effect is uncertain (Handy et al., 2003; Lam, 2009).

The underlying MoAs being targeted for the nine EBMs that do not measure apical endpoints were identified and an abbreviated AOP analysis was undertaken to evaluate the strength of linkages between endpoints measured by the EBM and effects at higher levels of biological organization (Altenburger et al., 2015; Ankley et al., 2010; Fay et al., 2017). Each of the nine EBMs measures a molecular initiating event (MIE) or key event (KE) within an AOP. We then evaluated available literature supporting the presence or absence of linkages from the MIE or KE measured by the EBM through to apical effects, such as survival, growth, and reproduction.

RESULTS

A more detailed description of each EBM evaluated along with supporting information on sensitivity, specificity, and linkages to effects at higher levels of biological organization are provided in the Supporting Information. Here, we summarize and synthesize our findings across EBMs to provide a broader view of the potential utility of EBMs for regulating metals in aquatic systems.

Metal specificity

Ideally, for an EBM to be useful as a monitoring tool for assessing the ecological impacts of metals on aquatic systems it should be responsive to multiple metals, but generally unresponsive to other substances and environmental variables. Our evaluation concludes that only one proposed EBM, the bacteria reporter assay, is likely specific to metals (Table 1). Promoters with metal-responsive elements are unlikely to be sensitive to other classes of toxicants, although no studies specifically testing this was identified. However, in general, bacterial assays with metal-responsive elements tend to be sensitive to only one or a few metals and we did not identify any assays that are sensitive to a broad suite of metals. All other EBMs are responsive to at least one other class of toxicants (e.g., pesticides and polycyclic aromatic hydrocarbons) and six of the EBMs were classified as being responsive to environmental variables, such as salinity, temperature, and UV radiation.

Metal sensitivity

Sensitivity to metals is another important characteristic to consider for candidate EBMs. If a candidate EBM is not sensitive to multiple metals at concentrations near existing EQS, it is unlikely to provide an adequate indicator of adverse effects from multiple metals in the environment. Candidate EBMs were grouped into three general categories with respect to sensitivity.

Two EBMs (in vivo toxicity testing and eDNA metabarcoding) were identified as having high sensitivity, meaning they were sensitive to a wide range of metals at concentrations approximating EQS (Table 1). This is not surprising given that EQS are derived from in vivo toxicity test data and eDNA metabarcoding can detect the loss of taxa sensitive to metals (Gillmore et al., 2021; Yang et al., 2018). Most of the other candidate EBMs were categorized as having mixed sensitivity, meaning they were sensitive to at least one metal but insensitive to other metals at concentrations near the EQS. Urease, for example, is sensitive to Cu at concentrations near the EQS, with some examples of similar sensitivity to Zn, but quite insensitive to a range of other metals (Ag, Cd, Co, Ni, and Pb) (Brack et al., 2000; Jung et al., 1995; Olson & Christensen, 1982). Two candidate EBMs, DNA damage and cytochrome P450, were characterized as moderately sensitive, meaning they are sensitive to multiple metals at concentrations ~10-fold higher than EQS, but insensitive to all metals at concentrations near the EQS (Table 1).

Linkage to higher levels of biological organization

Two of the candidate EBMs (in vivo toxicity testing and deformity assessments) directly measure effects on whole organisms, while eDNA metabarcoding can be used to estimate impacts at the population or community levels of biological organization. In contrast, the remaining nine candidate EBMs measure changes in response to a stressor at the (sub)cellular level of biological organization and typically directly measure either an MIE or KE in individual species (Ankley et al., 2010). Consequently, it is important to evaluate whether these responses are strongly linked to effects at higher levels of biological organization.

This evaluation was accomplished by undertaking a simplified AOP-type evaluation for each EBM, considering linkages from the MIE or KE to cellular, organ, and then whole organism responses. Details of this assessment can be found in the Supporting Information and are summarized graphically in Figure 1. Of the nine EBMs evaluated, only two (disrupted ion homeostasis and acetylcholinesterase inhibition) have well-documented links to effects at the whole organism level of organization. Three additional EBMs (reduced lysosomal membrane stability, reactive oxygen species production, DNA damage) have strong linkages to the organ level of organization, whereas links to the whole organism are postulated but not well documented.

The bacterial reporter assay and metallothionein induction have the weakest links to apical effects. In fact, both of these EBMs are actually components of the homeostatic control mechanisms involved in regulating concentrations of essential metals in organisms and by extension frequently respond to nonessential metals in a similar manner (Amiard et al., 2006; Tauriainen et al., 1998). The bacterial reporter assay uses recombinant bacteria to detect metals in the environment (Van der Meer & Belkin, 2010). 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. Metallothionein (MT) induction is a normal response of organisms to maintain homeostasis of some internally bioavailable metals and can also bind potentially toxic concentrations of metal taken up by an organism under scenarios of elevated metal exposure. In cases where MT induction is caused by exposure to elevated metals, it has long been postulated that protein synthesis of MT has a sufficient energetic cost to cause apical effects on the organism. However, to the best of our knowledge, this linkage has never been measured and so the linkage of MT induction to energetic effects at higher levels of biological organization is uncertain.

TABLE 1 Summary of sensitivity, specificity, and linkage to individual and/or population effects for metal effects-based methods

Effects-based method	Metal specificity	Other toxicants	Metal sensitivity	Link to individual/population effects
Ion homeostasis	Na: Ag, Cu, Pb Ca: Co, Cd, Pb, Zn Mg: Ni	Pesticides, pharmaceuticals, and salinity	Mixed: Effects detectable at concentrations near environmental quality standards (EQS) in some cases but not all	Strong—Demonstrated links to survival and growth, but no links to reproduction demonstrated
Oxidative stress	As, Cd, Co, Cr, Cu, Fe, Hg, Ni, Ti, V, Zn	Pesticides, polycyclic aromatic hydrocarbons (PAHs), polychlorinated biphenyl (PCBs), dioxins, temperature, and salinity	Mixed: Oxidative stress detected at environmentally relevant metal concentrations for some metals	Weak: No studies link to individual/ population-level effects
Lysosomal membrane stability	Cd, Cu, Hg, Pb	PAHs and 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
DNA damage	Cd, Cr, Cu	Nanoparticles, pesticides, pharmaceuticals, and 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
Deformities	As, Cd, Cr, Cu, Hg, Ni, Pb, Se, Zn	Pesticides, phthalates, nanoparticles, estrogens, nutrients, parasites, and 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
In vivo testing	Nonspecific	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
Cytochrome P450	Inhibition: Cd, Cu, Hg, Ni, Pb, Sb, Zn	Induction: PCBs, pesticides, and PAHs	Moderate: CYP1 inhibition occurs at a concentration ~10-fold higher than EQS	Weak: Unclear how inhibition of CYP1a impacts organisms
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
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
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
Metallothionein	Ag, Cd, Cu, Hg, Ni, Pb, V, Zn	Anoxia, freezing gametogenesis, and pesticides	Mixed: Sensitive near the EQS in some cases but insensitive in other cases where concentrations are 100-fold higher than EQS.	Weak: No studies linked to individual/ population-level effects
Environmental DNA	Nonspecific	All toxicants and natural stressors causing population-level effects	High: Detects loss of metal-sensitive taxa	Strong—Directly measures the presence/absence of species

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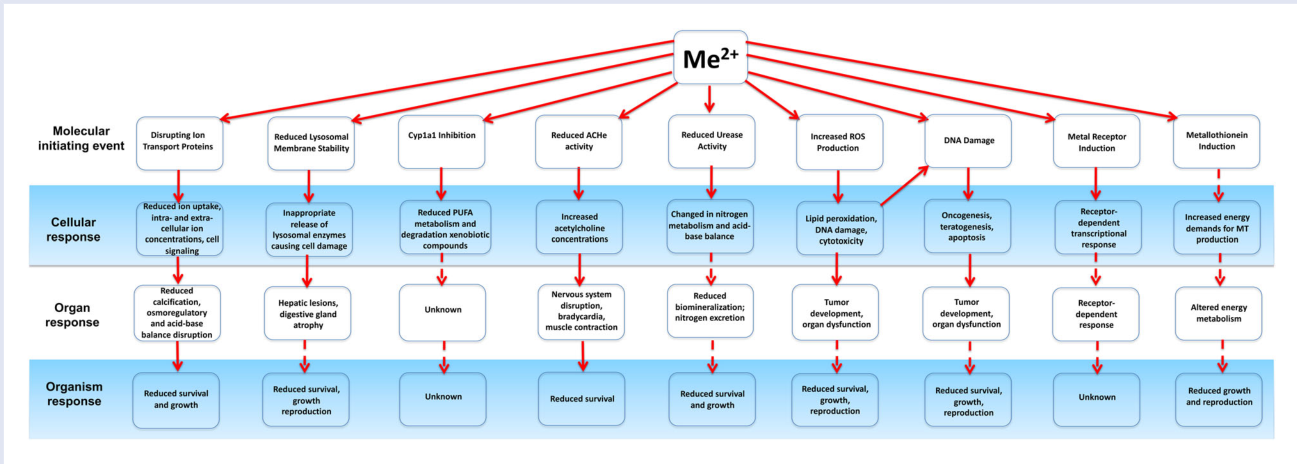

FIGURE 1 Adverse outcome pathway-type assessment of nine candidate effects-based methods. Solid arrows indicate strong support for linkage between two levels of the biological organization while dashed arrows indicate weak or no support for linkage. Note: No support may be due to data indicating that no linkage exists or there are no data to support the linkage. Details to support each evaluation are provided in the Supporting Information. AOP, adverse outcome pathway; MT, metallothionein; PUFA, polyunsaturated fatty acids; ROS, reactive oxygen species.

DISCUSSION AND CONCLUSIONS

Summary assessment

In this assessment, we evaluated the 12 EBMs most prominent in the scientific literature that might be considered tools for assessing the ecological impacts of metals on 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 of biological organization.

From this evaluation, three key observations can be made. First, the bacteria reporter assay is the only EBM with likely potential specificity to metals. All other EBMs are also sensitive to at least one and often multiple other classes of toxicants and/or natural stressors. Second, most EBMs have moderate sensitivity (effects at concentrations ~5- to 10-fold higher than EQS) or mixed sensitivity (effects at EQS for some metals but not others). In vivo testing and eDNA metabarcoding have high sensitivity to metals generally, but they are also sensitive to many other classes of toxicants and natural stressors. Only the bacteria reporter assay has the potential to be both sensitive and specific to multiple metals. Third, half of the evaluated EBMs (including the bacteria reporter assay) are considered to have weak links to effects at the individual or population level. Ion homeostasis, deformities, in vivo testing, and eDNA metabarcoding are considered strong indicators of individual, population, and community-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.

Other metal-specific considerations

There are two additional metal-specific issues that need to be considered across the spectrum of proposed EBMs. The first is whether EBMs can account for metal bioavailability, which can significantly change the toxicity threshold on a site-specific basis. Effects-based methods do explicitly account for metal bioavailability as they will not respond unless a sufficient concentration of bioavailable metal is present in an aquatic system. However, for in vitro EBMs, such as bacterial reporter assays, the effects of the required exposure system media may affect metal bioavailability and complicate the interpretation of metal bioavailability in water samples being assayed.

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 that 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). A sampling of local organisms adapted to naturally elevated background metal concentrations for EBMs may provide a useful tool for addressing this issue. For example, organisms 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

Effects-based methods are already used in some regulatory frameworks as part of a weight-of-evidence (WOE) approach to demonstrate ecological impact. However, there is a clear need for the 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 that are difficult to detect, and mixture effects. For some classes of organic toxicants, some available EBMs may provide a viable option in support of achieving this goal. For metals, it is clear that currently available EBMs do not meet this need.

The ideal EBM for metals would be sensitive to a suite of metals at or near the EQS for each metal and have clear linkages to effects at the individual, population, or community level of organization. Further, EBM would account for naturally occurring abiotic factors that influence metal bioavailability and toxicity. Even if an EBM could be developed that meets 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 is unlikely it would be able to inform the user of the particular metal(s) within the mixture that was driving noncompliance. This is obviously critical information for successful environmental management.

In the absence of at least one effective EBM for metals, an alternative tool currently under development that provides quantitative information on the relative contribution of individual metals to observed effects in a mixture is the mixture biotic ligand model (mBLM). There has been considerable progress in developing mBLMs in terms of both fundamental studies on how metals interact at metal-binding sites (Brix et al., 2016, 2017; Cremazy et al., 2019; Komjarova & Blust, 2009) and in the development of an appropriate modeling framework (Farley et al., 2015; Nys et al., 2018; Santore & Ryan, 2015; Van Regenmortel et al., 2017). While these developments are promising, there are still considerable uncertainties in existing models that limit their immediate application. More study and model refinement are clearly needed, including substantial field validation studies.

We suggest that in the interim, the most scientifically robust and practical path forward for effects-based monitoring of metal mixtures in aquatic systems is the development of a WOE approach that integrates the application of the mBLM and two additional tools for assessing metal effects on aquatic ecosystems: measurement of macroinvertebrate community abundance and composition (i.e., bioassessment) and metal bioaccumulation in benthic macroinvertebrates.

Direct measurement of changes in benthic invertebrate community abundance, composition, and structure has been used for decades to assess the impacts of metal mixtures on aquatic communities. Initial efforts to delineate the relative contributions of individual metals within a mixture to observed effects used relatively simple mixture models (Clements et al., 2000; Clements, 2004), while more recent efforts have used more sophisticated approaches with mBLMs (Balistrieri et al., 2015). An important limitation of the bioassessment approach is that it cannot explicitly discriminate the causes of observed effects, although approaches such as the river invertebrate prediction and classification system can account for effects of habitat and other natural factors that influence the expected biological community (Clarke et al., 2003). Consequently, within the context of assessing impacts from metal mixtures, data must be interpreted carefully when there is a potential for other stressor types (e.g., organic chemicals, nutrients, dissolved oxygen, and habitat) to impact the benthic invertebrate community.

Direct measurement of metal accumulation in benthic macroinvertebrates is also a useful tool in assessing the effects of individual metals and their mixtures on aquatic organisms. In particular, an approach in which metal accumulation in insensitive "accumulator" organisms is related to effects on more sensitive taxa has been demonstrated to be a potentially useful EBM for assessing effects on benthic macroinvertebrate communities. The conceptual details of this approach were developed in a SETAC Pellston Workshop (Adams et al., 2011) and several case studies indicate that at least for individual metals this approach is potentially useful (Bervoets et al., 2016; De Jonge et al., 2013; Luoma et al., 2010; Rainbow et al., 2012). We suggest it could be possible to also apply this approach in multimetal scenarios if it were used concurrently with mBLM approaches to facilitate data interpretation.

The WOE approach described above would serve two purposes. First, given the current uncertainties in existing mBLMs, the application of multiple tools in a WOE framework would provide a more scientifically robust assessment of potential impacts from metal mixtures. Second, the application of this approach would generate data sets that could be used to calibrate and ultimately validate the mBLM. Ultimately, with further optimization and sufficient validation, the goal would be to reduce the application of the mBLM as an alternative to EBMs for metals with periodic (every 3–5 years) confirmation of model performance using bioassessment.

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DATA AVAILABILITY STATEMENT

All information supporting this analysis is provided in the Supporting Information.

SUPPORTING INFORMATION

Supporting Information file provides a more detailed evaluation and references supporting Table 1 and Figure 1 of this brief communication.

REFERENCES

Adams, W. J., Blust, R., Borgmann, U., Brix, K. V., DeForest, D. K., Green, A. S., Meyer, J. S., McGeer, J. C., Paquin, P. R., Rainbow, P. S., & Wood, C. M. (2011). Utility of tissue residues for predicting effects of metals on aquatic organisms. *Integrated Environmental Assessment and Management*, *7*, 75–98.

Altenburger, R., Ait-Aissa, S., Antczak, P., Backhaus, T., Barcelo, D., Seiler, T. B., Brion, F., Busch, W., Chipman, K., de Alda, M. L., Umbuzeiro, G. A., Escher, B. I., Falciani, F., Faust, M., Focks, A., Hilscherova, K., Hollender, J., Hollert, H., Jager, F., … Brack, W. (2015). Future water quality monitoring—Adapting tools to deal with mixtures of pollutants in water resource management. *Science of the Total Environment*, *512–513*, 540–551.

Amiard, J. C., Amiard-Triquet, C., Barka, S., Pellerin, J., & Rainbow, P. S. (2006). Metallothioneins in aquatic invertebrates: Their role in metal detoxification and their use as biomarkers. *Aquatic Toxicology*, *76*, 160–202.

Ankley, G. T., Bennett, R. S., Erickson, R. J., Hoff, D. J., Hornung, M. W., Johnson, R. D., Mount, D. R., Nichols, J. W., Russom, C. L., Schmieder, P. K., Serrano, J. A., Tietge, J. E., & Villeneuve, D. L. (2010). Adverse outcome pathways: A conceptual framework to support ecotoxicology research and risk assessment. *Environmental Toxicology and Chemistry*, *29*, 730–741.

Balistrieri, L. S., Mebane, C. A., Schmidt, T. S., & Keller, W. (2015). Expanding metal mixture toxicity models to natural stream and lake invertebrate communities. *Environmental Toxicology and Chemistry*, *34*, 761–776.

Bervoets, L., De Jonge, M., & Blust, R. (2016). Identification of threshold body burdens of metals for the protection of the aquatic ecological status using two benthic invertebrates. *Environmental Pollution*, *210*, 76–84.

Brack, W., Aissa, S. A., Backhaus, T., Dulio, V., Escher, B. I., Faust, M., Hilscherova, K., Hollender, J., Hollert, H., Muller, C., Munthe, J., Posthuma, L., Seiler, T. B., Slobodnik, J., Teodorovic, I., Tindall, A. J., Umbuzeiro, G. A., Zhang, X., & Altenburger, R. (2019). Effect-based methods are key. The European Collaboratrive Project SOLUTIONS recommends integrating effect-based methods for diagnosis and monitoring of water quality. *Environmental Sciences Europe*, *31*, 10.

Brack, W., Dulio, V., Agerstrand, M., Allan, I., Altenburger, R., Brinkmann, M., Bunke, D., Burgess, R. M., Cousins, I., Escher, B. I., Hernandez, F. J., Hewitt, L. M., Hilscherova, K., Hollender, J., Hollert, H., Kase, R., Klauer, B., Lindim, C., Herraez, D. L., … Vrana, B. (2017). Towards the review of the European Union Water Framework Directive: Recommendations for more efficient assessment and management of chemical contamination in European surface water resources. *Science of the Total Environment*, *576*, 720–737.

Brack, W., Paschke, A., Segner, H., Wennrich,, R., & Schuurmann, G. (2000). Urease inhibition: A tool for toxicity identification in sediment elutriates. *Chemosphere*, *40*, 829–834.

Brix, K. V., Tellis, M. S., Cremazy, A., & Wood, C. M. (2016). Characterization of the effects of binary metal mixtures on short-term uptake of Ag, Cu, and Ni by rainbow trout (*Oncorhynchus mykiss*). *Aquatic Toxicology*, *180*, 236–246.

Brix, K. V., Tellis, M. S., Cremazy, A., & Wood, C. M. (2017). Characterization of the effects of binary metal mixtures on short-term uptake of Cd, Pb, and Zn by rainbow trout (*Oncorhynchus mykiss*). *Aquatic Toxicology*, *193*, 217–227.

Carere, M., Lettieri, T., Wernersson, A. S., Hanson, N., Buchinger, S., & Kase-Pasanen, R. (2021). *Technical proposal for effect-based monitoring and assessment under the Water Framework Directive*, 163 pp.

Clarke, R. T., Wright, J. F., & Furse, M. T. (2003). RIVPACS models for predicting the expected macroinvertebrate fauna and assessing the ecological quality of rivers. *Ecological Modelling*, *160*, 219–233.

Clements, W. H. (2004). Small-scale experiments support causal relationships between metal contamination and macroinvertebrate community responses. *Ecological Applications*, *14*, 954–967.

Clements, W. H., Carlisle, D. M., Lazorchak, J. M., & Johnson, P. C. (2000). Heavy metals structure benthic communities in Colorado mountain streams. *Ecological Applications*, *10*, 626–638.

Cremazy, A., Brix, K. V., & Wood, C. M. (2019). Using the biotic ligand model framework to investigate binary metal interactions on the uptake of Ag, Cd, Cu, Ni, Pb, and Zn in the freshwater snail *Lymnaea stagnalis*. *Science of the Total Environment*, *647*, 1611–1625.

Crommentuijn, T., Polder, M., Sijm, D. T. H. M., De Bruijn, J., & Van De Plassche, E. (2000). Evaluation of the Dutch environmental risk limits for metals by application of the added risk approach. *Environmental Toxicology and Chemistry*, *19*, 1692–1701.

Duke, S. O. (1990). Overview of herbicide mechanisms of action. *Environmental Health Perspectives*, *87*, 263–271.

European Union (EU). (2000). *EU Water Framework Directive 2000/60/EC.* "*Directive 2000/60/EC of the European Parliament and of the Council establishing a framework for the Community action in the field of water policy*."

European Union (EU). (2019). *Communication from the commission to the European Parliament, the European Council, the Council, the European Economic and Social Committee and the Committee of the Regions—The European Green Deal*, 24 pp.

Farley, K. J., Meyer, J. S., Balistrieri, L. S., De Schamphelaere, K. A. C., Iwasaki, Y., Janssen, C. R., Kamo, M., Lofts, S., Mebane, C. A., Naito, W., Ryan, A. C., Santore, R. C., & Tipping, E. (2015). Metal mixture modeling evaluation project: 2. Comparison of four modeling approaches. *Environmental Toxicology and Chemistry*, *34*, 741–753.

Fay, K. A., Villeneuve, D. L., LaLone, C. A., Song, Y., Tollefsen, K. E., & Ankley, G. T. (2017). Practical approaches to adverse outcome pathway development and weight-of-evidence evaluation as illustrated by ecotoxicological case studies. *Environmental Toxicology and Chemistry*, *36*, 1429–1449.

Gillmore, M. L., Golding, L. A., Chariton, A. A., Stauber, J. L., Stephenson, S., Gissi, F., Greenfield, P., Juillot, F., & Jolley, D. F. (2021). Metabarcoding reveals changes in benthic eukaryote and prokaryote community composition along a tropical marine sediment nickel gradient. *Environmental Toxicology and Chemistry*, *40*, 1892–1905.

Handy, R. D., Galloway, T. S., & Depledge, M. H. (2003). A proposal for the use of biomarkers for the assessment of chronic pollution and in regulatory toxicology. *Ecotoxicology*, *12*, 331–343.

De Jonge, M., Tipping, E., Lofts, S., Bervoets, L., & Blust, R. (2013). The use of invertebrate body burdens to predict ecological effects of metal mixtures in mining impacted waters. *Aquatic Toxicology*, *142–143*, 294–302.

Jung, K., Bitton, G., & Koopman, B. (1995). Assessment of urease inhibition assays for measuring toxicity of environmental samples. *Water Research*, *29*, 1929–1933.

Knapen, D., Reynders, H., VBervoets, L., Verheyen, E., & Blust, R. (2007). Metallothionein gene and protein expression as a biomarker for metal pollution in natural gudgeon populations. *Aquatic Toxicology*, *82*, 163–172.

Komjarova, I., & Blust, R. (2009). Multimetal interactions between Cd, Cu, Ni, Pb, and Zn uptake from water in the zebrafish *Danio rerio*. *Environmental Science & Technology*, *43*, 7225–7229.

Lam, P. K. S. (2009). Use of biomarkers in environmental monitoring. *Ocean & Coastal Management*, *52*, 348–354.

Luoma, S. N., Cain, D. J., & Rainbow, P. S. (2010). Calibrating biomonitors to ecological disturbance: A new technique for explaining metal effects in natural waters. *Integrated Environmental Assessment and Management*, *6*, 199–209.

Matsumura, F. (1975). *Toxicology of Insecticides*. Plenum Press.

Van der Meer, J. R., & Belkin, S. (2010). Where microbiology meets microengineering: Design and applications of reporter bacteria. *Nature Reviews*, *8*, 511–522.

Nys, C., van Regenmortel, T., Janssen, C. R., Oorts, K., Smolders, E., & De Schamphelaere, K. A. C. (2018). A framework for ecological rsk assessment of metal mixtures in aquatic systems. *Environmental Toxicology and Chemistry*, *37*, 623–642.

Olson, D. L., & Christensen, G. M. (1982). Effect of selected environmental pollutants and other chemicals on the activity of urease (*in vitro*). *Bulletin of Environmental Contamination and Toxicology*, *28*, 439–445.

Rainbow, P. S., Hildrew, A. G., Smith, B. D., Geatches, T., & Luoma, S. N. (2012). Caddisflies as biomonitors identifying thresholds of toxic metal bioavailability that affect the stream benthos. *Environmental Pollution*, *166*, 196–207.

Van Regenmortel, T., Nys, C., Janssen, C. R., Lofts, S., & de Schamphelaere, K. A. C. (2017). Comparison of four methods for bioavailability-based risk assessment of mixtures of Cu, Zn, and Ni in freshwater. *Environmental Toxicology and Chemistry*, *36*, 2123–2138.

Salminen, R., Batista, M. J., Bidovec, M., Demetriades, A., De Vivo, B., De Vos, W., Duris, M., Gilucis, A., Gregorauskiene, V., Halamic, J., Heitzmann, P., Lima, A., Jordan, G., Klaver, G., Klein, P., Lis, J., Locutura, J., Marsina, K., Mazreku, A., … Tarvainen, T. (2005). *Geochemical atlas of Europe. Part 1: Background information, methodology and maps* (p. 526). Geological Survey of Finland.

Santore, R. C., & Ryan, A. C. (2015). Development and application of a multimetal multibiotic ligand model for assessing aquatic toxicity of metal mixtures. *Environmental Toxicology and Chemistry*, *34*, 777–787.

Tauriainen, S., Karp, M., Chang, W., & Virta, M. (1998). Luminescent bacterial sensor for cadmium and lead. *Biosensors and Bioelectronics*, *13*, 931–938.

Wernersson, A. S., Carere, M., Maggi, C., Tusil, P., Soldan, P., James, A., Sanchez, W., Dulio, V., Broeg, K., Reifferscheid, G., Buchinger, S., Maas, H., Van Der Grinten, E., O'Toole, S., Ausili, A., Manfra, L., Marziali, L., Polesello, S., Lacchetti, I., … Kase, R. (2015). The European technical report on aquatic effect-based monitoring tools under the water framework directive. *Environmental Science Europe*, *27*, 7.

Yang, J., Xie, Y., Jeppe, K., Long, S., Pettigrove, V. J., & Zhang, X. (2018). Sensitive community responses of microbiota to copper in sediment toxicity test. *Environmental Toxicology and Chemistry*, *37*, 599–608.

