Chronic metal mixture toxicity: quantitative reappraisal and identifications of data gaps

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1. Introduction

Regulatory recognition that exposure to a cocktail of chemicals may cause chemical risks which are not captured by single chemical evaluations is growing. In the Chemicals Strategy for Sustainability, the EU Commission calls to systematically integrate the issue of "combined exposure" into chemical risk assessments. The metal mixture project within the MEED (Metal Environmental Exposure Data)-program aims to provide scientific evidence on metal mixture effects as input to the discussions on how to tackle the issue of exposure to unintentional mixtures for naturally occurring substances, like metals and inorganics. Recently, as a first step in characterising the European (mixture) risks due to inorganic substances, a set of 14 inorganic-priority contributing substances (I-PCS) were identified as substances contributing most to predicted mixture risks based on screening of European aquatic environmental monitoring data [1]. The identified I-PCS for freshwater are Ag, As, Ba, Cd, Co, Cr, Cu, Hg, Mn, Ni, Pb, Se, V, and Zn. The current research builds further on a recently published meta-analysis of mixture effects of Cd, Cu, Ni, Pb and Zn to aquatic organisms at the species-level, and expands the quantitative reappraisal to a broader set of metals, with a special focus on the I-PCS.

2. Materials and Methods

To identify relevant studies (published between 2007-2022), i.e. studies reporting on chronic metal mixture toxicity to aquatic organisms at the species-level, a literature study was performed building from a recent literature review [2] and a 'Web-of-Science' search focusing on mixtures of I-PCS.

Within the quantitative reappraisal, the following 3 questions [3] were answered. **Q1:** which mixture reference model, Concentration Addition (CA) or Independent Action (IA), is most accurate for predicting chronic metal mixture toxicity at the species level; **Q2:** How frequently do significant deviations from the mixture reference models, CA and IA, occur; **Q3:** How accurate is CA for predicting mixture effects at low effect concentrations? The quantitative reappraisal followed the methodology described by Nys et al. [3].

In addition, it was evaluated whether the conclusions of the literature study were dependent on the quality of the data and on the environmental relevance of the exposure concentrations. Experiments were considered 'high quality' if single metal exposures and mixture exposures were conducted simultaneously and if exposure concentrations were verified by analytical measurements. The environmental relevancy of the exposure concentrations was assessed based on a European environmental monitoring data (Waterbase).

3. Results and Discussion

Description of the dataset: In total, 30 chronic metal mixture studies were identified as useful for the quantitative reappraisal, covering 115 individual metal-mixture experiments (>1800 individual mixture treatments). The experiments combined 14 different metals in 33 different combinations, mostly binary (75) and ternary combinations (35), and a limited number of quaternary experiments (5). Metal-mixture toxicity data were identified for 24 species: 14 algae/plants, 4 vertebrates and 6 invertebrates. In total, 68 experiments were considered of high quality based on our criteria, of which 34 experiments tested mixture effects at environmentally relevant concentrations.

Q1: While IA clearly predicted mixture toxicity to invertebrates (orange squares; Figure 1.A) more accurately than CA, there was little difference between the models for algae (green triangles) when the entire dataset was considered. For vertebrates, there were too few and too uncertain data included in the analysis to draw firm conclusions. For some studies, a substantial over- or underestimation of mixture effects was observed. However, the most extreme over- and underestimations of mixture effects disappeared when only the high quality and environmentally relevant experiments were considered, suggesting that at environmentally relevant concentrations extreme under- or overpredictions of mixture toxicity are limited. CA resulted generally in the most conservative predictions, independent of thropic level or subset of the data considered.

Q2: Non-interactive and antagonstic mixture effects were clearly more frequently observed compared to significant synergistic effects, especially when evaluated relative to CA (41% non-interactive, 56%

antagonisms and 3% synergisms for the high quality and environmentally relevant experiments). For IA, the frequency of synergistic effects was slightly higher (18%), but were still less frequently observed compared to non-interactive (59%) and antagonistic (24%) effects.

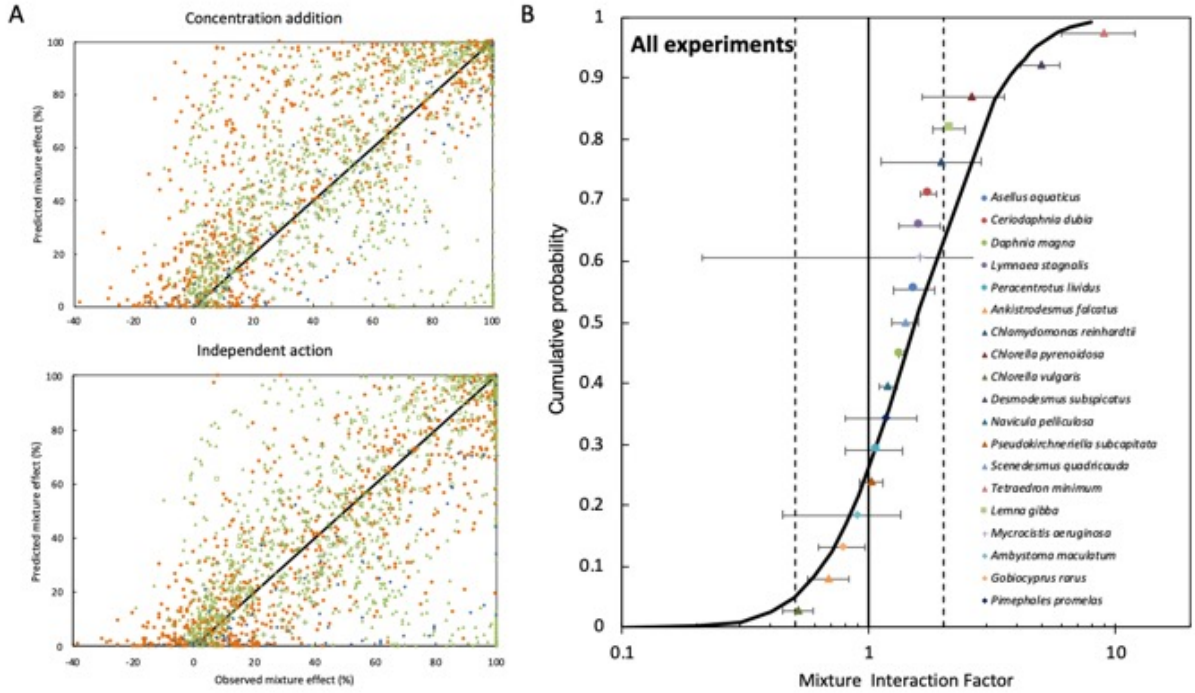

Figure 1: A) Predicted mixture effect (%) vs. observed mixture effect (%) using either CA (left panels) or IA (right panels) (all data shown). B) Species distribution of mixture interactions factors (MIFs) in chronic metal mixtures in all mixture experiments

Q3: The degree to which CA is protective at low effect concentrations has previously been expressed using the mixture interaction factor (MIF; [3]). The MIF is the mixture dose, expressed as ΣTU_{EC10}, that results in 10% mixture effect ($EC10_{\Sigma TUEC10}$) and is calculated for each experiment separately. The median MIF for the entire dataset is 1.3 (90th percentile range 0.5-4.0; Figure 1.B). The median MIF of the high quality and environmentally relevant experiments is also 1.3, but show a more narrow range (90th percentile range 0.7-2.6). Hence, overall, it can be concluded that CA overpredicts mixture toxicity at low effect levels by 1.3-fold on average.

Data gap analysis: The current study identified three main data gaps. First, no high-quality metal mixture toxicity datasets on vertebrates were identified. Second, the following I-PCS had no or few mixture data in the quantitative reappraisal: Ag, As, Ba, Co, Cr, Hg, Mn, Se and V. More specifically, there were no high-quality studies identified on mixtures of anionic I-PCS. Finally, only a limited number of studies combined 4 inorganics, while studies with 5 or more metals combined were not identified in the literature search.

4. Conclusions

In the present study, we evaluated metal mixture toxicity effects using a systematic analysis of mixture studies of I-PCS. Our study largely confirmed the observations from an earlier meta-analysis for a smaller subset of these metals [3]. The quantitative reappraisal showed that CA results generally in more conservative predictions compared to IA. However, metal mixture toxicity to invertebrates is most accurately predicted by IA, while there is less difference between both models for algae. Most experiments represented non-interactive effects or antagonistic effects, while significant synergisms were less frequently observed, certainly when evaluated relative to CA. Finally, CA overestimated mixture toxicity at low effect levels (i.e., 10% mixture effect) on average by 1.3-fold (median value). The results of the current analysis helped to identify important data gaps to prioritize for further testing. In addition, it will contribute to the discussion of how “combined exposure” to inorganic substances should be integrated in environmental risk assessment.

References - [1] ARCHE 2022. Report prepared for Eurométaux; [2] Martin et al. 2021. Environ. Internat.146: 106206; [3]. Nys et al. 2018. Environ. Toxicol. Chem. 37: 623-642

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Chronic metal mixture toxicity: From data gap analysis to the development of an environmentally and regulatory relevant experimental program

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In the Chemicals Strategy for Sustainability, the EU Commission calls to systematically integrate the issue of combined exposure into chemical risk assessments. The 'mixture effects of metals' project within the Metals Environmental Exposure Data Program (MEED) aims to provide scientific evidence on mixture effects as input to the discussions on how to tackle the issue of exposure to unintentional mixtures for naturally occurring substances. More specifically, it aims to focus on those metals and inorganics that are predicted to contribute most to the overall risks of unintentional mixtures in Europe, i.e. the so-called Inorganic-Priority Contributing Substances (I-PCS). In a previous prioritization exercise using European monitoring data, the following inorganics have been identified as I-PCS: Ag, As, Ba, Cd, Co, Cr, Cu, Hg, Mn, Ni, Pb, Se, V, and Zn. In addition, in a recent meta-analysis, it was observed that there is overall limited (high-quality) data on metal mixture toxicity to aquatic organisms of following I-PCS: Ag, As, Ba, Co, Cr, Hg, Mn, Se, and V. Moreover, there is only limited data for mixtures combining four or more metals.

Here, we present the development of a targeted testing program that aims to cover these data gaps. An experimental program was developed that considers metal mixture toxicity testing with algae *(Pseudokirchneriella subcapitata*) and invertebrates *(Daphnia magna)* at environmentally regulatory relevant concentrations and mixture combinations. The development of the experimental program followed a step-wise approach focusing on the identification of mixture risks of the I-PCS based on species sensitivity data (EC10) from the REACH registration dossiers. The most relevant mixture combinations were identified using a toxic unit-approach applied on the Waterbase, an European aquatic monitoring database. The followed approach ensured the environmental and regulatory relevancy of the experimental program by selecting the most relevant mixture size, metals, metal combinations and metal concentration (ratios) to be tested. The outcome of the experimental program, that is currently ongoing, will increase the scientific evidence on mixture toxicity of inorganics. As such, it will contribute to the discussions on the implementation of combined exposure into environmental risk assessment.

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Session: Towards a Better Understanding of Mechanisms of Metal Mixture and Their Risk Assessment

In the Chemicals Strategy for Sustainability, the EU Commission calls to systematically integrate the issue of combined exposure into chemical risk assessments. The 'mixture effects of metals' project within the Metals Environmental Exposure Data Program (MEED) aims to provide scientific evidence on mixture effects as input to the discussions on how to tackle the issue of exposure to unintentional mixtures for naturally occurring substances. More specifically, it aims to focus on those metals and inorganics that are predicted to contribute most to the overall risks of unintentional mixtures in Europe, i.e. the so-called Inorganic-Priority Contributing Substances (I-PCS). In a previous prioritization exercise using European monitoring data, the following inorganics have been identified as I-PCS: Ag, As, Ba, Cd, Co, Cr, Cu, Hg, Mn, Ni, Pb, Se, V, and Zn. In addition, in a recent meta-analysis, it was observed that there is overall limited (high-quality) data on metal mixture toxicity to aquatic organisms of following I-PCS in freshwaters: Ag, As, Ba, Co, Cr, Hg, Mn, Se, and V. Moreover, there is only limited data for mixtures combining four or more metals.

Here, we present the development of a targeted testing program that aims to cover these data gaps. An experimental program was developed that considers metal mixture toxicity testing with algae *(Pseudokirchneriella subcapitata*) and invertebrates *(Daphnia magna)* at environmentally regulatory relevant concentrations and mixture combinations. The development of the experimental program followed a step-wise approach focusing on the identification of mixture risks of the I-PCS based on species sensitivity data (EC10) from the REACH registration dossiers. The most relevant mixture combinations were identified using a toxic unit-approach applied on the Waterbase, an European aquatic monitoring database. The followed approach ensured the environmental and regulatory relevancy of the experimental program by selecting the most relevant mixture size, metals, metal combinations and metal concentration (ratios) to be tested. The outcome of the experimental program, that is currently ongoing, will increase the scientific evidence on mixture toxicity of inorganics. As such, it will contribute to the discussions on the implementation of combined exposure into environmental risk assessment.

Does the concentration addition model become a more conservative predictor of aquatic metal toxicity with increasing number of metals in the mixture?

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Session: Towards a Better Understanding of Mechanisms of Metal Mixture and Their Risk Assessment

Risk assessment of chemical mixtures is most conveniently regulated following the concentration addition (CA) model. The MAF (Mixture Assessment Factor) has been proposed as a tool to regulate individual chemicals while accounting for unintentional mixture effects predicted by CA. At low chronic effect concentrations, CA on average tends to overestimate mixture effects at the species level. A more accurate alternative for chronic metal mixture toxicity provides the independent action model (IA). A quantifier to assess the deviations of observed toxicity from toxicity predicted with CA is called the MIF (Mixture Interaction Factor), which indicates synergistic (MIF<1) or antagonistic (MIF>1) interactions, relative to CA, if present. However, most research has been conducted with well-studied metals, such as Cu, Zn, Ni, Cd, and Pb, in equitoxic test designs. Less attention has been paid to other metals and environmentally relevant concentration ratios. This study aims to fill this knowledge gap and will further test the hypothesis that IA is generally a more accurate model than CA for metal mixture toxicity. In addition, it will, based on theoretical and mathematical considerations, test the hypothesis that the MIF increases with an increasing number of metals in the mixture.

Ag, As, Ba, Cd, Cr, Cu, Mn, Ni, Pb and Zn have been identified previously as inorganic substances contributing most to predicted mixture risks. The experimental design consisted of testing single metals, and binary, ternary, quaternary and quinary mixtures combinations of those metals. To cover different trophic levels, the test organisms *Raphidocelis subcapitata* and *Daphnia magna* were chosen. For those species, we estimated that in a mixture experiment, five metals are usually sufficient to explain 90% of the toxicity of the mixture pressure (expressed as the sum of toxic units) of the whole mixture.

All tests followed a ray design at environmentally relevant concentrations and concentration ratios based on monitoring data from the European Environmental Agency (EEA) Waterbase. Additionally, an equitoxic ray design based on EC10 values was also tested. Each test was evaluated according to CA and IA, both models were compared for their ability to accurately predict observed toxicity, and the MIF was quantified. This study will contribute relevant experimental data for a better understanding of the joint toxicity of complex, but environmentally and regulatorily relevant metal mixtures.

Chronic metal-organic mixture toxicity: quantitative reappraisal and identifications of data gaps

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Session: Complex Mixtures in Chemical Risk Assessment: Challenges and Opportunities

In the Chemicals Strategy for Sustainability, the EU Commission calls to systematically integrate the issue of combined exposure into chemical risk assessments. Ecological risks of chemical mixtures have predominantly been studied within broad substance groups, (e.g. metals, pesticides), while chronic effects of metal-organic mixtures have rarely been assessed. The metal-organic mixture project within the Metals Environmental Exposure Data Program (MEED) aims to provide scientific evidence on effects of metal-organic mixtures. As a first step, a literature review and quantitative reappraisal of chronic mixture toxicity of metals and organic chemicals was conducted. The null hypothesis was that metals and organic chemicals act independently from each other, i.e. that the Independent Action (IA) model is an accurate predictor of mixture toxicity, and that it is on average more accurate than the concentration addition (CA) model.

Overall, focusing on the period 2007-early 2022, we identified only 4 chronic metal-organic mixture toxicity studies (13 individual experiments) that were of sufficient quality and relevance to be included into the systematic quantitative reappraisal. Those studies were dominated by tests with algae and with Cu, with limited other species and metals tested. Among these few studies, IA performed somewhat better than CA in predicting metal-organic mixture toxicity (lowest root mean square error), whereas CA was generally the most conservative (i.e. overestimates mixture effects more than IA). At low effect levels (10% mixture effect), CA performed relatively well, albeit some experiments suggested quite strong synergisms. However, these synergisms were unreliable and/or observed at unrealistic exposure concentrations (based on monitoring data reported in the Waterbase environmental monitoring database). In fact, most identified chronic metal-organic mixture studies were conducted outside of environmentally and/or regulatory relevant mixture concentrations, which means that it is not appropriate to draw any meaningful or general conclusions with respect to our central question whether IA is a better model to predict metal-organic mixture toxicity than CA, and how protective CA would be on average. Hence, there is a need to investigate metal-organic mixture toxicity at environmentally and regulatory relevant concentrations, with appropriately sensitive species and endpoints.

Which organic substances occur most frequently in hazardous and environmentally relevant binary mixtures with metals in European freshwater?

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Session: Complex Mixtures in Chemical Risk Assessment: Challenges and Opportunities

Ecological risks of chemical mixtures are predominantly assessed within a single compound group rather than simultaneously accounting for different groups of chemicals. Few studies are available describing the chronic mixture toxicity of metals and organic pollutants and most of these studies have been conducted at concentrations that have neither environmental nor regulatory relevance. This study aims to prioritize organic substances that potentially pose a aquatic mixture risk in binary pairs with metals. The analysis was based on monitoring data from the European Environmental Agency Waterbase. 16 metals considered in this prioritization had all been identified previously as inorganic substances potentially contributing to aquatic mixture risks. All organic substances with an individual CAS number, present in the Waterbase, were included in the analysis (598). 418 PNECs were derived from NORMAN's PNEC database, 29 from the Swiss Ecotox Center water quality parameters database, 84 PNECs were calculated using the Envirotoxdatabase.org database. 68 compounds were excluded because no PNEC could be found in any of the consulted databases, leaving 531 compounds for the analysis. 512 organics were measured together with at least one of the 16 metals. To priority-rank the organic substances, we calculated the percentage of mixtures at risk – based on the Hazard quotient (HQ = PEC/PNEC) – for each metal-organic pair. This percentage was calculated by dividing the number of samples shwowing risk (sum HQ > 1) where both the organic and metal contribute at least 10% to the risk by the number of samples where both compounds were measured together. The number of metals (out of 16) for which % mixture risk was >10% (priority) were counted and ranked in decreasing order. The (preliminary) top priority organic compounds belonged to various substance groups with different modes of action such as pesticides (imidacloprid), pharmaceuticals (diclofenac), Industrial chemicals (PAHs, Bisphenol-A). This prioritization identified realistic metal-organic mixtures driving the risk to aquatic communities in the field based on PNECs. It is possible, though, that this prioritisation may be somewhat biased to include more organic substances for which high assessment factors have been used in deriving their PNECs. Further laboratory test will be conducted to test the occurrence of mixture interactions between metals and organic substances in some of these identified priority mixtures.

REGIONAL EXPOSURE ASSESSMENT OF METALS IN THE AQUATIC FRESHWATER ENVIRONMENT.

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Environmental release rates of metals are expected to vary over time due to new uses and changes in used/manufacturer volumes, but also due to improvement of emission-reducing technologies and external conditions like climate change. As a consequence, previously collected regional ambient concentration levels that were used in a regulatory context (e.g. for exposure scenario development under REACH) may not be relevant anymore for current or future situation. An update of these regional metal concentration levels in the aquatic environment is part of Eurometaux's currently ongoing comprehensive "Metals environmental exposure gathering program" (MEED-program), covering today's and expected needs for tomorrow to comply with the Zero Pollution Ambition and biodiversity objectives.

Recent monitoring data (period: 2017-2021) of dissolved metal concentrations in EU surface freshwater systems were extracted from EEA's database on the status and quality of Europe's rivers, lakes and groundwater bodies (Waterbase). Data sets for 18 metals, covering up to 20 countries, were compiled and represented the basis for the derivation of country-specific reasonable worst-case (RWC) ambient PECs. The followed calculation methodology is stooled on the data treatment procedures that have been laid out in Guidance Documents for environmental risk assessment in the EU and includes an outlier analysis to minimize the impact of local point sources when predicting a representative ambient regional concentration.

Only quality-screened measured samples were considered. Data were categorized into sub-datasets (river basin, a specific waterbody, or an individual sampling location) that were sufficiently large to derive a meaningful 90^{th} percentile; the median of all 90P-values represents the ambient RWC-PEC.

Overall, the determined RWC-ambient PECs were within the same order of magnitude as those that were derived in the past (2000-2010)-period. Observed differences in country-specific RWC-ambient PECs for individual metals are likely caused by factors such as specific geological conditions, (over-)representation of sampling locations that represent major rivers affected by industrial activities (e.g., Rhine, Danube). For most metals, the calculated RWC-ambient PEC is typically situated between the 50^{th} and 90^{th} percentile of known baseline levels (FOREGS-dataset), making the latter a potential reference for estimating the RWC-ambient PEC for metals lacking monitoring data.