Metal mixture effects of Ni, Cu and Zn in a multi-species two-trophic level algal-daphnid microcosm can be predicted from single-trophic level effects: the role of indirect toxicity

Andreas Fettweis[*A] *and Simon Hansul*[B] *(co-first authors), Erik Smolders*[A]*, Karel De Schamphelaere*[B]

[A]Division of Soil and Water Management, KU Leuven, Kasteelpark Arenberg 20, 3001 Heverlee, Belgium

[B]Laboratory of Environmental Toxicology and Aquatic Ecology, Ghent University, Coupure Links 653 Building F - 2nd Floor, 9000 Ghent, Belgium

*Adress correspondence to andreas.fettweis@arche-consulting.com

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ABSTRACT

Effect assessments of metals are mostly based on single-metal, single-species tests, thereby ignoring metal-mixture effects and indirect effects through species interactions. Here, the combined effects of metal and species interactions were tested in two-trophic algal-daphnid microcosms. Metal-mixture effects on daphnid communities may propagate from effects on the generally more sensitive algal communities. Four different algal communities (three species each), with and without addition of the same daphnid community (three species) were exposed to single metals and one metal mixture (17:17:51 µg/L Ni:Cu:Zn). Daphnid densities were negatively affected by metals in the two-trophic test, the magnitude of which depended on the algal community composition. Algal densities were overall positively affected by the metals in the two-trophic test but negatively in the single-trophic test, illustrating an indirect positive effect in the two-trophic system due to a reduced grazing pressure. Metal effects on daphnid communities in the two-trophic test (day 21) were correlated with metal effects on the single trophic-level algal communities during exponential growth (R^2=0.55, p=0.0011). This suggests that metal effects propagate across trophic levels due to a reduced food quantity. However, the indirect positive effects on algal densities, resulting in abundant food quantity, suggests that metal effects can also propagate to daphnids due to a reduced food quality (not measured directly). Metal-mixture interactions on daphnid densities varied during exposure, but were additive or antagonistic relative to independent action when considering final daphnid densities (day 56). This suggests stronger indirect effects of the mixture than of the single metals. Overall, our study highlights the dynamic aspect of community-level effects, which empirical reference models such as independent action or concentration addition cannot predict.

INTRODUCTION

Effect assessment of metals is primarily based on single-species, single-metal data, while in the environment, metals are present as mixtures and species as a part of larger communities. Mixture effects are assumed to be non-interactive relative to reference models and species interactions are assumed not to affect community-level effects. This is in contrast to some experimental evidence and theoretical considerations. Species interactions could significantly modify community-level sensitivity to metals, compared to the underlying single-species sensitivities (De Laender et al., 2008) and mixture interactions might become more frequent at higher levels of organisation (Brook et al., 2008). Mesocosm experiments can be used to identify the effects of metals on the community and ecosystem level (Hommen et al., 2016), but are less suited to identify mechanisms. This is more easily done with more controlled microcosm experiments and theoretical models, which allow to move gradually across levels of organization and complexity.

Effects of single metals on single-trophic level communities

When moving from single-species systems to single-trophic level communities, a number of potential new driving forces for toxicity are introduced to the system. Most important are the consequences of nutrient limitation. In laboratory community tests, this can lead to compensatory dynamics, where less sensitive species can compensate for the loss of more sensitive species (if sensitivity is determined with respect to population-level endpoints). This results in a community structure in favour of the least sensitive species, also referred to as pollution-induced community tolerance (PICT, Yachi and Loreau 1999; McClellan et al. 2008; Baert et al. 2016) and is one of the major factors not taken into account by established community effect

assessment tools, such as species sensitivity distributions (SSDs). The potential for single-metal effects on community structure further depends on the relationship between species dominance without metal stress and their sensitivity to the metal. If the most dominant species are also the least sensitive, exposure to the metal can only increase their dominance. The maximum possible effects on relative species composition are then limited by the dominance without metal stress, but effects on species loss will be largest (Chesson, 2000). In contrast, if the most dominant species are most sensitive, metal stress will have more severe effects on the species composition of the community and less on species loss.

Data on single metal effects to aquatic single-trophic communities in controlled laboratory tests appears to be scarcer than corresponding data on multi-trophic communities. Only a few such studies in the peer-reviewed literature could be identified. Yu et al. (2007) studied the effects of copper on algal communities and found a lower sensitivity of the green algae *Microcystis aeruginosa* in the presence of other species, compared to the monoculture. A two-species community experiment involving *Daphnia magna* and *D. pule*x (Leblanc and Cochrane 1985) supported the idea that compensatory dynamics and release from competition are important factors in determining community-level effects. In contrast, Joonas et al. (2021) found no difference in the response of individual species in monocultures compared to communities, and therefore did not identify species interactions as a driving force for community-level effects. While the importance of indirect effects as a driving force for community-level metal effects appears theoretically plausible, some but not all experimental evidence supports this idea.

Effects of single metals on two-trophic communities

In two-trophic systems consisting of a producer (e.g. algae) and a primary consumer community (e.g. daphnids), additional mechanisms come into play. Indirect effects on the consumer community can be mediated by changes in food quantity and food quality. A first scenario is where the producer community is, overall, more sensitive than the consumer community. Here, food quantity to the consumer is expected to decrease as a function of metal stress, which in turn could change consumer community productivity and structure (Vanni, 1987). Furthermore, the nutrient status of producers can be affected by metal stress, such as an increased phosphorus (P) content of algal cells or a change in fatty acid composition (Fadhlaoui et al., 2020; Gao et al., 2016). At low effect levels, this may have a stimulating effect on consumers: if total producer biomass is insufficiently reduced to impact consumer ingestion rate, but nutritional value of producers is sufficiently increased to provide a net-increase in provision of a major limiting nutrient (R. Evens et al., 2012). Fettweis et al. (2018) found that daphnid reproduction decreases with changes in algal cell concentrations (i.e., food quantity) due to varying P-levels in a two-species microcosm, but that this effect was smaller at larger Zn concentrations due to an increase in algal P quotas (i.e., food quality). Hence, the effects of secondary stressors (e.g. Zn) are probably less pronounced when a primary limiting growth factor (P deficiency) prevails. Evens et al. (2012) also found that dietary Zn can increase the reproduction of daphnids due to an increased algal P content and that this increased nutritional benefit, due to a larger P uptake, might counteract direct Zn toxicity to *D. magna*. At higher effect levels, food quality can also have a negative effect on consumers due to dietary toxicity. Multiple studies have analysed the effect of dietary trace metal uptake in *Daphnia* and De Schamphelaere et al. (2004) found that dietary Zn possibly

accumulates in cells where vitellogenin synthesis or processing occurs and would, therefore, specifically target reproduction in *D. magna*. A second scenario is where the consumer community is more sensitive than the producer community. Here, grazing pressure on the producer community can decrease, which in turn can result in an increased productivity of the producers. In addition, if the consumers feed selectively on different producer species, the release from grazing pressure can be species-specific, introducing an additional mechanism of how indirect effects propagate across trophic levels. While the phenomenon of such trophic cascades have been observed (Brett & Goldman, 1996; Su et al., 2021), their role in determining effects of metals on two-trophic communities still deserves further investigation.

Effect of metal mixtures on two-trophic communities

When communities are exposed to metal mixtures, the relationships of the single-species sensitivities among the single metals across all algal species likely become important. A positive correlation of sensitivities among the single metals for all species in a community means that species in that community most sensitive to one metal (e.g. lowest EC50) are also those most sensitive to other metals, and similarly, that species most tolerant to one metal are also most tolerant to the other metals in that community. A negative correlation of sensitivities is the opposite, and means that the more sensitive species to one metal in a community are the more tolerant ones to another metal. Fettweis et al. (2021) demonstrated for freshwater phytoplankton species exposed to Cu, Ni and Zn that positive correlations are the rule and that the species most tolerant to all metals will preserve the community functioning under metal mixture toxicity, i.e. there is likely a large functional redundancy with respect to total biomass. In contrast, in the rare case of communities with negatively correlated sensitivities, this is not the case, and functional redundancy is expected to

be smaller under metal mixture toxicity. Therefore, we hypothesise that mixture effects on single-trophic level communities with negatively correlated sensitivities are stronger, i.e. a synergism relative to a reference model such as independent action (IA) and concentration addition, and can thus also potentially affect the other trophic levels to a stronger degree.

Effects of single metals on multi-trophic level communities have been investigated previously in a number of other microcosm and mesocosm experiments. For example, Van de Perre et al. (2016) studied the effects of a single metal (Zn) to multi-trophic level communities in combination with other environmental factors (temperature, phosphorus), and found statistic interactive effects to occur more frequently on community- than on species-level. Likewise, Van Regenmortel et al. (2018) experimentally studied effects of Cu-Ni-Zn mixtures on aquatic communities in microcosms and found consistent effects on community function and structure below the bioavailability-normalized HC5, but attributed this to a mismatch between the species present in the experiment and the species used to construct the SSD and derive the HC5. While researchers often acknowledged the importance of indirect toxic effects via trophic interactions (Gessner & Tlili, 2016; Schmitt-Jansen et al., 2008), relatively little is known about the role of trophic interactions in mediating effects of metals and metal mixtures on ecosystem functioning and structure. Current existing experimental evidence does not allow to draw clear conclusions about the relative importance of indirect effects via trophic interactions relative to direct effects. This can be mostly attributed to a lack of single-species information, including their sensitivity and competition behaviour, when studying community effects. Few studies describe metal mixture effects on aquatic communities, and those that do are mostly observational studies that do not allow to make predictions of the mixture effects using

a reference model, such as IA and CA (Clements et al., 2013; Stockdale et al., 2010) . Notable exceptions are Mebane et al. (2017) and Mebane et al. (2020). Mebane et al. (2017) tested the effects of Cd-Zn mixtures to aquatic insect communities in artificial streams and concluded that the IA model was generally accurate, with the exception of a few antagonisms that were found. Later, Mebane et al. (2020) also tested the effects of more complex metal mixtures (Cd-Cu-Ni-Zn), also in artificial streams on aquatic insect communities, and again found additive and antagonistic mixture interactions using the IA model. Those two studies are largely in support of the IA model being able to predict mixture effects at the community level. However, they are still case studies that do not allow to make generalisations under which biotic conditions such communities become more sensitive to metal and metal mixture exposure, and about how these effects cascade between trophic levels.

To investigate the role of indirect effects of metal mixtures across trophic levels, we conducted an eight-week laboratory microcosm experiment, using one three-species consumer community of daphnids and four different three-species producer communities of freshwater algae. In a point-design, all four combinations of daphnid and algal communities were exposed to a control, the single metals Cu, Ni and Zn, and their mixture. Communities were exposed at concentrations at which eight-week community dry mass of the daphnids was still unaffected in a previously conducted single-trophic experiment with the same daphnid community composition, but at which total algal dry mass was predicted to be reduced (Hansul et al., unpublished). We first hypothesised that effects on the algal communities (producers) would propagate to the daphnid communities (consumers) through changes in food quantity (reduction of algal dry mass), food quality (e.g., changes in algal P quota), or both, leading to either smaller or larger effects on the daphnid community, depending on the species

composition of the algal community and food preference of the daphnids. Secondly, assuming that propagation occurs mostly through effects on food quantity, we hypothesised that the extent of these effects can be predicted by the sensitivity of the algal community to the metal or the metal mixture. More specifically, for the metal mixture, we predict that the metal mixture sensitivities of the daphnid communities will be predictable from single metal sensitivities of the algal communities, by using the IA model to predict the metal mixture sensitivity of the algal communities. Any deviations from the IA model, i.e. synergisms or antagonism, will then follow from the correlation of single-species sensitivities among the single metals in each of the algal communities. Hence, we are predicting that the extent of mixture effects propagating from the algal communities to the daphnid communities via a change in food quantity can be explained using a combination of single metal sensitivities of the algal communities and an algal community parameter (i.e., the correlations of single-species sensitivities among the single metals) that is solely derived from data of single metals on single algal species. We also expect that the indirect effects on the daphnid community that cannot be explained by effects on food quantity are due to food quality. If propagation of metal toxicity occurs mostly through food quality, the extent of it may be explained by the nutritional quality of the algae or by shifts in the algal community composition resulting in changing food preferences for the daphnids.

MATERIALS AND METHODS

Four two-trophic level communities were selected, each consisting of three algal and three daphnid species. The communities differed only in the selection of algal species; all communities contained the same three daphnid species. The four algal communities were selected to have different correlation of sensitivities among

the single metals for all species in their respective community, and were also selected to have different single metal and metal mixture sensitivities. Single trophic-level algal communities were tested in parallel using the same algal communities as in the two-trophic test. Both the two-tropic test and the single trophic-level test were exposed to single metals at one concentration per metal (Ni, Cu and Zn), and one mixture treatment of those three metals at the same concentrations for each metal. A detailed comparison between the two-trophic test and the single-trophic level algal test are presented in Table 1.

Species and communities

Six unicellular freshwater algal species were studied to fill the role of primary producer in the two-trophic level communities. All species originate from the Culture Collection of Algae and Protozoa (CCAP) and comprise six Chlorophyta: *Ankistrodesmus falcatus* (CCAP 202/14B), *Chlorella vulgaris* (CCAP 211/11B), *Desmodesmus subspicatus* (CCAP 276/20), *Pseudokirchneriella subcapitata* (CCAP 278/4), *Scenedesmus quadricauda* (CCAP 276/16) and *Tetraëdron minimum* (CCAP 273/1). The algal species were successfully cultured for several months in regular COMBO medium in a temperature-controlled room at 20 ± 1 °C with a 16 h light/8 h dark-cycle and a light intensity of about 90 µmol photons m^{-2} s^{-1} (Kilham et al., 1998).

Three daphnid species were studied and fill the role of primary consumer in the two-trophic level communities. All species originate from Langerodevijver, Belgium (50°85′81″N, 4.63°99′60″E) and comprise *Daphnia magna*, *Daphnia pulex* and *Daphnia longispina*. Each daphnid species consists of a single genetic clone. Before onset of the experiments, the daphnid species had been cultured for two years in

modified COMBO medium in a temperature-controlled room at 20 ± 1 °C with a 16 h light/8 h dark-cycle and a light intensity of 80-100 µmol photons m^{-2} s^{-1}.

Test water

Modified COMBO medium with an initial pH of 7.5 was used as test water and prepared according to (Kilham et al., 1998) with some modifications to better represent environmental conditions. Water hardness of the medium was increased from 40 to 60 mg $CaCO_3$ L^{-1} compared to regular COMBO. Boric acid (H_3BO_3) was reduced from 388 µ*M* to 16 µ*M* B. Phosphorus (P) was added at an initial dose of 3 µM P and was supplement daily to all pots at a rate of 0.3 µ*M* P day^{-1} (as K_2HPO_4), resulting in a linearly increasing total concentration in solution and a final cumulative added concentration of 19.8 µ*M* P after 56 days. The daily P addition rate corresponds roughly to the amount of P that is added via algal biomass in standard daphnid experiments, that is 0.2 mg algal dry weight $daphnid^{-1}$ day^{-1} (OECD, 2012), with twelve initial daphnids per 0.5 L pot and assuming an algal P quota of 0.2%, which is typical for algal cultures at carrying capacity (Fettweis et al., 2021). The chosen P addition rate is environmentally realistic compared to typical sediment P release rates in rivers and lakes in Flanders (Van Dael et al., 2021). To replace ethylenediaminetetraacetic acid (EDTA) as complexing metal agent, 4 mg dissolved organic carbon (DOC) L^{-1} was added. The DOC originated from the Schwarzbach stream on the border of Belgium and Germany, about 10 meters before its confluence with the Roer stream (50°31'15.6"N 6°12'21.0"E) and was sampled using reverse osmosis, further processed and freeze-dried (Serkiz et al. 1990). This method does not significantly affect the protectiveness of DOC on metal toxicity to freshwater species (De Schamphelaere et al., 2005; Verheyen et al., 2014).

Two-trophic test

The four two-trophic level communities were exposed to one dose per single metal (17 µg Ni L^{-1}, 17 µg Cu L^{-1} and 51 µg Zn L^{-1}), one dose of a mixture of those three metals (17:17:51 µg Ni:Cu:Zn L^{-1}) and one control (no added metals). Single metal concentrations have been chosen at concentrations that would not directly adversely affect the total 8-week dry weight of daphnids under similar test conditions. In these single-species tests (Hansul et al., unpublished), the food source was a single algal species (*P. subcapitata*) and added at a constant daily rate of 2 mg dry weight L^{-1} in the form of frozen and defrosted suspension, so that algae were not able to grow. Otherwise, the test conditions were identical to the here described two-trophic test. Community structure, dry weight or total daphnid density at earlier time-points may be affected (Hansul et al., unpublished). The metal mixture is an environmentally realistic ratio for a range of contaminated streams in Flanders and the Netherlands (Van Regenmortel et al., 2017). Each treatment was tested using four replicates in food-safe polypropylene containers (Avamoplast, Belgium) with 500 mL modified COMBO medium. Initial daphnid density was four juveniles (<24h old) per pot per species, resulting in 24 total individuals L^{-1}. Initial nominal algal biomass density was 0.33 mg dry weight (DW) per pot per species, resulting in 2 mg total algal DW L^{-1}. Pots were randomly distributed over multiple plates and covered with a transparent plate. The test duration was 56 days and media were refreshed twice a week (Mondays and Thursdays, test starting on a Thursday) by replacing 25% of the old medium with fresh medium. Before each refreshment, all the daphnids were removed by filtering them over a 100 µm sieve. Care was taken to minimize the time that daphnids remained on the sieve, and it never exceeded 30 seconds. Algal cells pass through this sieve, with cell diameters ranging between 1 and 20 µm. Next the solutions were homogenised

296 and 25% was removed, thus also including 25% of the total algal biomass. New
297 medium of the corresponding treatment was added to have 500 mL total medium per
298 pot again. After the media were refreshed, the daphnids were gently transferred back
299 into their corresponding replicate.

300 *Single trophic-level algal test*

301 Simultaneously with the two-trophic level test, also the control, single metal and
302 metal mixture treatment were tested on only the single trophic-level algal communities.
303 The purpose of this additional test was to test how the metals affect the single trophic-
304 level algal communities and whether these metals effects correlate with those found
305 on the daphnid communities in the two-trophic test. Algal communities were tested in
306 24-well plates with 2.5 mL medium and four replicates. The test duration was 10 days
307 without any medium refreshments and was started at the same time as the two-trophic
308 level test. All other test parameters were identical to the two-trophic level test. As an
309 additional control treatment, the four single trophic-level algal communities (without
310 any daphnid species) were also setup in the same type of pots as those used for the
311 two-trophic test, with 500 mL medium and using four replicates (this is in addition to
312 the 24-well plates), and with identical initial algal densities and medium refreshments
313 as in the two-trophic test.

314 *Daphnia life-table test*

315 To understand the growth pattern and food preference of each daphnid, which
316 will support the analysis of the two-trophic data, we conducted 21-day life-table
317 experiments with the three daphnid species which were individually fed the six different
318 algal species. For each algal and daphnid species, 10 3rd brood daphnid juveniles
319 (<24h old) were reared individually in 50 mL polyethylene jars filled with 40 mL

modified COMBO medium. Algal species were harvested at carrying capacity, aliquoted and stored in -80°C for at least two days. Daphnids were fed daily with a defrosted algal suspension at a rate of 0.4 mg algal dry mass per daphnid per day. Media were renewed, reproduction and survival were recorded three times per week.

Biological monitoring

Algal cell concentrations were sampled twice a week by taking 200 µL of medium from the two-trophic level test right before each medium refreshment and after homogenising the solution. Cell concentrations were measured using a flow cytometer (BD Accuri C6) equipped with two lasers (488 and 640 nm). In the single-trophic level algal communities set up in 24-well plates, the cell concentrations were measured at day 3 and day 10. The flow cytometer measures six signals from each event (or algal cell): forward scatter signal (FCS), sideward scatter signal (SSC) and four fluorescence signals (FL1, FL2, FL3 and FL4). The signals of each event are characterised by an area (A), a height (H) value and a shared width (W) value, resulting in thirteen total parameters (6 signals x 2 + 1) measured for each event. Based on these parameters, algal species could be differentiated from one another using a clustering model (average validation accuracy of 90%) and algal dry weights could be estimated (cross-validation accuracy of 95%) as described in Fettweis et al., (2023).

The number of daphnids per species was inferred from image data using an image analysis script based on two Convolutional Neural Networks (CNNs), one trained to differentiate between daphnids and other objects (e.g., ephippia, algal cell agglomerates), the other trained to differentiate between the three daphnid species. Image data was acquired during medium renewals passing the entire test medium

through a 100 µm sieve, which was rinsed with a minimal amount of fresh test medium to transfer the community to a petri dish. As far as possible, dead animals were removed from the community with a transfer pipette. The petri dish was placed on a glass plate lit from below with a LED light. A digital single-lens reflex camera (EOS200D, Canon) was mounted above the petri dish and controlled by a TCL script, allowing to take 5 consecutive images at an interval of 5 seconds. The CNNs were used to infer the number of daphnids per species from each of the five images, representing five technical replicates. The mean number of daphnids per species was used for analysis. The CNNs had been trained with monoculture data generated under identical conditions as the multi-species controls to firstly differentiate between daphnids and other objects appearing in the images (cross-validation accuracy of 95%, n=610) and to differentiate between the three species. The CNN could differentiate well between *D. magna* (species-specific validation accuracy of 93%, n=163) and the other species, and with lower accuracy between *D. pulex* (validation accuracy=63%, n=163) and *D. longispina* (validation accuracy=70%, n=163). Classifications of the CNNs were screened manually for false detections of daphnids and were corrected where appropriate. The manual screening did not include checking for false species identifications. Image segmentation was done using Otsu's thresholding with the scikit-image package in Python and the CNN analysis was performed with Tenserflow (2.8.0).

The total dry mass of daphnid communities was measured at the end of the experiment. For this purpose, the communities were passed over a sieve, transferred to a Petri dish and living animals were isolated from remaining particles. The medium containing the cleaned-up daphnid community was vacuum-filtrated over a filter paper and rinsed repeatedly with tap water. Each community was transferred to a piece of

parafilm paper, dried for ≥48h at 60°C and the dry mass was determined on an analytical balance (Mettler Toledo, accuracy of ± 0.1 mg).

Physicochemical measurements

The pH was measured once every week right before and after medium refreshment in all pots. Temperature was recorded weekly in test vessels filled with deionized water. Dissolved (0.45 µm-filtered) metal concentrations were measured once every week right before and after medium refreshment in all pots using ICP-MS (Agilent 7700). Measured concentrations of Ni, Cu and Zn were then converted to free ion activities with the Windermere Humic Aqueous Model (WHAM VII, UK Centre for Ecology & Hydrology, Tipping et al. 2011). The added dissolved organic matter (DOM) is included in the model as colloidal fulvic acid (FA), assuming DOM contains 50% C and 65% of DOM are active fulvic acids, i.e. FA = 0.65 x 2 x DOC (Ritchie & Michael Perdue, 2003; Edward Tipping, 2002). The Fe(III) activity was assumed to be controlled by the solubility of the oxide using the default parameters in WHAM VII, log K_{sp} = -0.52 for $Fe(OH)_{2.49}$ or log K_{sp} = 3.09 for $Fe(OH)_3$. Major cations (Na, Ca, Mg and K) were added as measured dissolved concentrations and major anions (Cl, SO_4 and CO_3) as nominal concentrations. In addition, the default master database, which includes inorganic metal complexation constants for Ni, Cu and Zn, was changed to an updated version from the National Institute for Standards and Technology (Smith et al., 2004). Several recent metal mixture studies used similar assumptions for modelling FA-metal complexation (Nys et al., 2016, 2017; Edward Tipping & Lofts, 2015). Additionally, the ΔLK_2 value for Ni complexation on FA was changed from 1.57 to 2.35 corresponding to the adjusted best-fit parameters from Van Laer et al. (2006). The complete input file for WHAM VII can be found in supplemental information.

Statistical analysis

For the daphnids, the total daphnid density (total number of daphnids per litre), and the final daphnid biomass were used as endpoints. For the algae, only the total algal density (total estimated biomass concentration), was calculated. All endpoints were normalized by the control average per time-point and are denoted as relative response (*RR*). Mixture interactions were assessed using the independent action (IA) model. Predicted relative responses (*PRR)* were calculated with the IA model for each mixture treatment as in Jonker et al. (2005):

$$Independent\ Action\ (IA): PRR = \prod_{i=1}^{n} RR_i \qquad Eq.1$$

With *RR* the relative response of the *i*th single metal and *n* the number of components in the mixture (Ni, Cu and Zn).

The correlation of sensitivities among single metals for all species in an algal community (*r*), referred to as the correlation of sensitivities, is a parameter unique to each algal community and metal combination, and is calculated solely based on single metal test data on the single species. More specifically it is defined as the Pearson r correlation coefficient of relative responses at the tested single metal concentrations in the single trophic-level test across all species in an algal community for each combination of metals (Ni-Cu, Ni-Zn and Cu-Zn) at day 3 and at day 10. This parameter is further used to explain the mixture effects and mixture interactions (relative to IA) on community level.

RESULTS

Daphnia life-table test

The 21-day life-table test revealed that the three daphnid species had vastly different cumulative reproductions after 21 days depending on the specific algal species fed (Figure 1). Overall, the most ideal food sources for cumulative reproduction of all three daphnid species were *C. vulgaris*, *D. subspicatus* and *P. subcapitata*. *S. quadricauda* and *A. falcatus* were preferential food sources only for *D. pulex* and *D. longispina*, *D. magna* did not reproduce well when fed with only those species. All three daphnid species had a low cumulative reproduction when fed with *T. minimum*.

Performance of control treatments – two trophic

In the control, algal biomass initially increased up to 5 mg algal DW L^{-1}. Afterwards, algal biomass rapidly decreased and total daphnid densities started to increase (Figure 2). The dynamics of total daphnid density resembled delayed logistic growth and can be divided into an initial growth phase occurring within the first 21 days, followed by peak in daphnid densities (C4) and a stabilization (C1, C2, C4) or gradual decrease (C3) in daphnid densities towards day 56 depending on the algal community (C1, C2, C3 or C4) present. Final densities were between 209 and 378 daphnids L^{-1}, and were 39% higher in C1 than in the other communities. This coincided with two of the algal species present in C1 (*C. vulgaris* and *D. subspicatus*) that stimulated reproduction of all three daphnid species in the life-table test (21-day averages between 23 and 43 neonates), compared to other algal species (Figure 1). Algal densities in the two-trophic test remained low (<0.1 mg DW L^{-1}) for the remainder of the experiment, likely due to a continuous top-down control by daphnids. Based on

life-table tests conducted with the same *Daphnia* lineages at different food densities, reproduction and mortality are affected at food concentrations of at least 2 mg dry mass per daphnid per litre (Hansul et al., 2024), indicating that food limitation occurred throughout most of the experimental period after the daphnid population peak had been reached. While initial population growth rates were similar for *D. magna* and *D. pulex*, *D. magna* dominated the daphnid communities after the initial growth phase in all two-trophic communities, but the extent was community-dependent (Figure 3 and Figure SI 2). In community C2, the lower dominance of *D. magna* corresponds to the less ideal algal species (A. falcatus, S. quadricauda and T. minimum) as food source present in this community for *D. magna*, which was not the case for *D. duplex* and *D. longispina* (Figure 1). Overall, *D. longispina* was the least dominant species and their population densities remained low (<50 individuals L^{-1}) throughout the experiment, but there was a slight increase in relative community composition at the later stages. Final (day 56) daphnid community compositions were almost identical and independent of the algal community (Figure 3). Overall, the most dominant algal species were *D. subspicatus* and *A. falcatus*, followed by *P. subcapitata* and *S. quadricauda*, with the least dominant species being *T. minimum* and *C. vulgaris*. However, cells of *C. vulgaris* sticked to the polypropylene pots, even after thorough mixing, in the control and all metal treatments. This was indicated by a clear greening of the pots themselves and was also confirmed by microscopy to be *C. vulgaris* cells. Cell densities of *C. vulgaris* were, therefore, likely underestimated in all communities. Daphnids are filter feeders and generally cannot feed on cells sticking to the pots. However, these cells likely still took up dissolved P from solution. It is also possible that some of these cells slowly detach from the pots towards the solution over time when their biomass increases, thereby still being available as a food source to the daphnids.

Metal effects – two trophic

Relative responses of total daphnid densities (individuals L^{-1}) were correlated with relative responses of final (day 56) total daphnid dry weights (mg DW L^{-1}) in all communities, (R^2=0.90, slope=0.61, Figure SI 4), indicating that effects on total daphnid density could be used as a proxy for effects on productivity in the present test system.

In the two-trophic test, effects on total daphnid density and community structure to metal exposure depended on both the algal community and the exposure time (Figure 2 and 3). Data are presented for days 21 and days 56, the former indicating a time at which algal densities were large and daphnids were in their exponential growth phase. At day 56 algal densities were mostly depleted by grazing and daphnid densities approached a steady state. Effects on total daphnid densities on day 21 were overall more metal-dependent than community-dependent. In most communities, a delay was observed in peak total daphnid densities for Zn and an even longer delay was observed for the metal mixture, indicating a metal effect on daphnid growth rates independent of the specific algal community. Relative responses on daphnid densities at day 21 varied by a factor of 1.4 for Ni (*RR* between 0.83-1.13), factor 1.4 for Cu (*RR* between 0.90-1.26) and factor 1.3 for Zn (*RR* between 0.56-0.71) among the communities (Table 2). In contrast, in the mixture treatments, relative responses on daphnid densities varied by a factor of 3.5 (*RR* between 0.20-0.69) among the communities, i.e. more than those for the single-metal treatments (Table 2). All communities had notably lower daphnid densities during or part of the whole initial growth phase compared to the most toxic single metal (Zn). This more pronounced for communities C2 and C3. The effects of the mixture on daphnid densities on day 21 were synergistic ($p < 0.05$) relative to IA for communities C1, C2 and C3 and non-

488 interactive for community C4. The mixture responses on daphnid densities at day 21
489 in communities C2 (*RR* = 0.20) and C3 (*RR* = 0.37) were close to the most sensitive
490 endpoint of any of the single algal species in the community when tested in isolation
491 in the well-plates with daphnids (*RR* = 0.26 for *P. subcapitata* in C2 and 0.38 for *C.*
492 *vulgaris* in C3, Table SI 1). The algal density dynamics under Cu and Ni exposures
493 were similar as in the control. In contrast, a clear increase, delay and/or longer duration
494 of peak algal densities was observed in the Zn and mixture treatments. However, at
495 the same test concentrations, algal densities were adversely affected in the single-
496 trophic level algal tests (Table SI 1). Measured algal densities in community C3 were
497 notably low in all treatments, especially considering that the daphnid populations were
498 not largely affected compared to the other communities. This was probably related to
499 the cells of *C. vulgaris* sticking to the pots as described previously. The measured
500 algal biomass may, however, still be a good representation of the available fraction of
501 algal biomass for the daphnids.

502 Metal relative responses were overall smaller on day 56 than on day 21. Except
503 for community C1 (Ni, Cu and Zn) and C4 (Zn) where final daphnid densities were
504 negatively affected ($p < 0.05$) (Table 2). Mixture effects on daphnid densities at day
505 56 were non-interactive relative to IA for communities C1, C2 and C3, and antagonistic
506 ($p < 0.05$) for community C4. Overall, mixture effects during the growth phase (day 21)
507 were more synergistic than those observed on day 56, which were mostly non-
508 interactive (Table 2).

509 *Correlations with single-trophic level algal test*

510 Algal communities reached overall larger densities after three days in the
511 single-trophic level algal test than in the two-trophic test (Table 3). Final algal carrying

512 capacities (day 10) were clearly above the peak densities observed in the two-trophic
513 system, highlighting the top-down control in the latter. Relative responses of algal
514 densities to metal exposure in the single-trophic level algal test at day 3 (exponential
515 growth period) correlated linearly with relative responses of daphnid densities in the
516 two-trophic system at day 21 ($R^2 = 0.55$, $p < 0.001$, Figure 5). This suggests that metal
517 effects, including those of the single metals and metal mixtures, propagate from the
518 producers (algae) towards the consumers (daphnids). However, that correlation is
519 absent when relating these relative responses of algal densities to metal exposure at
520 day 3 (not shown) or day 10 to the daphnid densities at day 56 (Figure 5). To
521 understand this discrepancy between day 21 and day 56, and the lack of correlation
522 at day 56, it makes sense to further investigate the two influential data points at day
523 56. The first influential data point is the daphnid density in the mixture treatment of
524 community C3 which was unexpectedly large relative to the other communities (Table
525 2). This was also the community with the longest delay in reaching its peak density.
526 The second influential data point is the Zn treatment of community C4, where the
527 relative response on the total daphnid density was only 0.58, whereas it was 1.42 in
528 the corresponding single-trophic level algal test (Table SI 1). This is also reflected in
529 the relatively large algal densities of community C4 in the two-trophic test, which
530 overall suggests that the effect of Zn on the daphnid density was unlikely caused by
531 a decreased food quantity.

532 Synergistic mixture interactions relative to IA were observed in all the algal
533 communities in the single-trophic level algal test at day 3 (Table 3) and in three of the
534 daphnid communities in the two-trophic test at day 21 (Table 2). However, the extent
535 of these synergistic mixture interactions, which can simply be calculated as the
536 difference between the observed and predicted relative responses with IA, did not

correlate ($p > 0.05$) between both tests. When comparing the mixture interactions at day 10 in the algal communities in the single-trophic level algal test (two synergistic and two non-interactive, Table 3) and at 56 in the daphnid communities in the two-trophic test (one antagonistic and three non-interactive, table 2), the extent of these interactions again did not correlate ($p > 0.05$) between both tests. Overall, this suggests that the extent of these mixture interactions do not simply propagate from one trophic level to another. In addition, neither the mixture effects on the daphnid communities, nor the extent of the mixture interactions, could be explained ($p > 0.05$) by the mean correlation of single-metal sensitivities across all species in each of the algal communities, contrasting one of the starting hypotheses.

Water composition

Initial measured dissolved (<0.45 µm) metal concentrations in the control were 0.9 µg Ni L^{-1} (limit of quantification, LOQ=0.01 µg Ni L^{-1}), 4.0 µg Cu L^{-1} (LOQ=0.07 µg Cu L^{-1}) and 12 µg Zn L^{-1} (LOQ=0.06 µg Zn L^{-1}) for both the two-trophic and the single-trophic level algal test. Initial single metal and metal mixture concentrations corresponded largely with nominal concentrations, with measured dissolved (<0.45 µm) concentrations of 17 µg Ni L^{-1}, 17 µg Cu L^{-1}, 56 µg Zn L^{-1} and 20:16:52 µg Ni:Cu:Zn L^{-1} for the Ni, Cu, Zn and the mixture treatment, respectively. Metal concentrations decreased during the first 14 days and these concentrations regressed towards the nominal values by day 56. Final concentrations deviated at most by a factor of 2 from nominal ones. (Figure SI 1). The initial decrease in concentration is likely attributed to metal uptake in algae as biomass increased, with the subsequent rise beyond day 14 likely reflecting the periodic renewal and reduced algal biomass. Calculated initial free ion activities for the single metals were 8.4 (Ni^{2+}), 0.24 (Cu^{2+}) and 27 (Zn^{2+}) µg L^{-1}. For

561 the metal mixture, free ion activities were only slightly larger for each of the metals
562 with 9.5, 0.29 and 29 µg L^{-1} for Ni, Cu and Zn respectively.

563 The initial NPOC concentration was 1.9 mg L^{-1}, after which concentrations
564 increased until day 14 up to 20 mg L^{-1} (on average) and finally concentrations
565 decreased again down to 4.4 mg L^{-1} on day 56 (on average) (Figure SI 1). Initial
566 measured P concentrations were 2.8 µM P (on average) and generally depleted to
567 <0.1 µM P (LOQ) from day 7 onwards due to algal growth, illustrating that the daily P
568 additions still reflected P limited growth conditions except where metal toxicity affected
569 growth more (Figure SI 1).

DISCUSSION

Direct and indirect effects

Metals can affect the daphnid communities directly via waterborne toxicity and indirectly via the food chain. Direct effects are already captured in single species tests, such as the 21-day daphnid reproduction test, but indirect effects are mostly ignored in risk assessment as they are difficult to quantify, complex and are largely dependent on the specific testing conditions (Fleeger, 2020; Preston, 2002). Here, the two-trophic communities only differed in their algal community composition, and thus any variation in effect observed on the daphnid communities is a result of variation in indirect effects that propagated from the direct toxicity on the algal communities. This was especially observed for the mixture treatment, where relative responses of the daphnid communities differed more than a factor of 2 (0.2-0.7 on day 21, Figure 2 and Table 2). This variation was larger for the metal mixture than for the single metals(Fleeger et al., 2003), thus suggesting that indirect effects become more pronounced under mixture toxicity. However, it must also be noted that the free ion activities, and hence direct effects, of the metals in the mixture were slightly larger than those in the single metal treatments when using identical dissolved concentrations. This nuisance is partially inherent to more environmentally realistic two-trophic microcosm tests compared to single-species tests. In addition, daphnid densities during the initial growth phase were overall considerably affected by the mixture and effects were synergistic relative to IA for three of the communities on day 21 (Figure 2 and Table 2). Single metal and metal mixture effects clearly propagated between the algae and the daphnids when comparing endpoints during the exponential growth phase (Figure 5). This suggests that a reduction in algal densities due to metal stress indirectly affects the daphnids due to a reduced food quantity. However, this is contested by the

595 observation that algal densities were overall large in the mixture treatments at day 21
596 in the two-trophic test (Figure 2), and therefore, this suggests that food quantity is not
597 the only contributor to the indirect effects on the daphnids, but that likely also food
598 quality played a role. In addition, relative responses on the daphnid communities at
599 day 21 were consistently lower (i.e. higher toxicity) than on the algal communities
600 when tested in isolation during exponential growth (day 3, Table 2 and 3). All these
601 arguments hence suggest that indirect effects related to a combination of food quantity
602 and food quality can lead to synergisms relative to IA on the community-level. Although
603 indicators of food quality were not directly measured in this study, we inferred that it
604 likely plays a role because effects on food quantity could not completely explain the
605 effects on the daphnid community. It has to be considered however that direct effects
606 on the daphnid community may have occurred at concentrations where it was initially
607 not expected. The accuracy of IA-predicted relative responses of daphnid densities
608 varied as a function of the algal community composition, indicating community-specific
609 properties inducing deviations from IA.

610 The exact cause of a reduction in food quality can only be speculated, and could
611 be related to changes in algal community compositions that could lead to less
612 favourable algal species in the communities for the daphnids to consume (Figure 1,
613 most clear for community C2 and C4). Alternatively, foodborne metal toxicity, i.e.
614 dietary toxicity, due to metal accumulation by the algal species could also result in a
615 reduced food quality for the daphnids (De Schamphelaere et al., 2004; R. Evens et
616 al., 2012; Roel Evens et al., 2009). Changes in algal lipid composition or reduced P
617 quota can also affect the nutritional value for the daphnids (Fettweis et al., 2018;
618 Muller-Navarra, 1995; Ravet et al., 2003). Especially the large algal densities in the
619 Zn and mixture treatment likely resulted in reduced algal P quotas and increased Zn

620 quotas considering that Zn concentrations depleted up to a factor of 10 in solution at
621 the peak algal density (around day 14). It is overall clear that many indirect effects
622 related to food quality can negatively impact daphnids (Kilham et al., 1997). In addition,
623 the extent and the mechanisms behind each of the indirect effects related to food
624 quality are most likely metal-dependent. It would thus not be surprising that under
625 mixture toxicity multiple aspects related to food quality are affected simultaneously.

626 Stimulations of algal community biomass were also observed in the two-trophic
627 test that were not found in the single-trophic level algal communities, indicating a
628 positive indirect effect on the algal communities in the two-trophic test. These are
629 simpler to explain than the indirect effects on the daphnids, as the stimulations in algal
630 biomass were likely the result of a reduced grazing pressure by the daphnids. This
631 reduced grazing pressure either originated from a direct metal effect on the daphnids,
632 or an indirect metal effect related to food quality.

633 *Correlation of single metal sensitivities in communities affecting mixture effects*

634 The correlation of single-species sensitivities among the single metals for all
635 algal species in a community could not explain the mixture response of the daphnid
636 communities (Table 3), in contrasts with one of the starting hypotheses. While the
637 concept of correlation of sensitivities is intuitively appealing, the hypothesis could not
638 be tested as intended. That hypothesis was based on explaining the extent of mixture
639 effects on algal biomass (food quantity) propagating to the consumers via a change in
640 food quantity.

641 Our data did not provide clear evidence that effects on food quantity alone
642 explain indirect effects on the daphnid community, but rather that a combination of
643 food quantity with other factors – most likely related to food quality – has to be taken

644 into consideration to understand the propagation of metal mixture effects from algal
645 communities to daphnid communities.

646 *Growth dynamics*

647 Metal relative responses on daphnid densities were overall smaller during the
648 initial growth phase (day 21) than on final daphnid densities (day 56, Table 2).
649 Compensatory dynamics clearly resulted in an almost complete recovery of the
650 daphnid densities relative to the control, even for the metal mixture treatment (except
651 for community C1). Mixture effects on daphnid densities during the initial growth phase
652 (day 21) were mostly synergistic relative to IA, while final (day 56) daphnid densities
653 were non-interactive or antagonistic relative to IA (Figure 2). This probably originated
654 from the longer delay in peak daphnid densities, i.e. the overshoot phase, in the
655 mixture treatments relative to the single metal treatments where daphnid densities
656 were already in decline and started to reach their steady-state carrying capacities. If
657 the testing period would have been longer, long-term mixture effects might eventually
658 also have become more synergistic relative to IA. More importantly, these
659 observations indicate that part of the interactive mixture toxicity stems from daphnid
660 communities being in different phases of the same general growth pattern (i.e.
661 exponential growth, peak density or steady state) rather than a direct interactive effect
662 on the organism-level. It further highlights the dynamic aspect of community-level
663 effects, which is missing in empirical reference models such as IA.

664 In addition, effects observed during the growth phase might be at least equally
665 relevant as effects observed at carrying capacity, because these initial effects during
666 the growing period could propagate to higher trophic levels (e.g., to secondary
667 consumers) sooner in an ecosystem. Although effects on final daphnid densities can

still be significant, they can evidently only impact higher trophic levels when a steady state condition is being approached. In the environment, growth of algal and daphnid populations fluctuate largely and probably rarely reach their true carrying capacities (Scheffer et al., 1997). Hence, metal effects observed at a steady state may be less relevant than during growth.

Implications for risk assessment and ecological modelling

Testing single species separately and alongside the community is an essential part to understand the underlying mechanisms in a community experiment. Especially in the context of risk assessment, which is mostly designed around single-species tests, as this allows for a direct comparison between the metal effects on the single species and the metal effects on the community. Knowledge about the response of single species is therefore crucial to identify whether species interactions can result in stronger metal effects than what would be predicted based on the single species alone. However, simultaneously testing for single-species effects exacerbates the already high labour intensity of community experiments. In addition, these can be inherently difficult to control and generate data which is much more challenging to process (e.g., species-level taxonomic identification, inference of biomass from proxy measurements). These factors limit the practical feasibility and design of large-scale experimental testing of community-level effects, which would be needed for community-based ecological risk assessment. In the current study, testing the metals in a full concentration-response design was not possible and only a single concentration per metal with four replicates could be tested at most. Therefore, also mixture interactions could not be corrected for chemical interactions using free ion activities. To mitigate these problems, the further development of ecological models that simulate simplified multi-trophic systems can help in identifying and quantifying

693 the mechanisms resulting in both direct and indirect effects of metals and metal
694 mixtures (Forbes et al., 2011; Galic et al., 2010).

695 Our data suggest that most of the observed mixture interactions were the result
696 of shifts in the temporal dynamics of communities, as shown by fewer and less strong
697 deviations from IA as communities approached steady state in the mixture treatments.
698 For the ecotoxicological analysis of community data, the entire time-series data should
699 therefore first be taken into account, because an a priori choice of individual time-
700 points (as done in statistical analysis of standard toxicity data) might lead to varying
701 conclusions concerning the relative mixture effect from the same dataset, and
702 therefore unreliable information for use in risk assessment.

703 In addition, it is clear that effects on all trophic levels should be considered
704 alongside and relative to each other in risk assessment, as these can largely change
705 the dynamics of a multi-trophic system. Effects on one trophic level, can result in a
706 positive indirect effect on another trophic level. At the population and community level,
707 this can be seen as a stimulating effect. At the ecosystem level, however, such effects
708 might translate to undesired effects on ecosystem functions and services. For
709 example, stimulation of algal biomass can result in a larger frequency or extent of algal
710 blooms, which on its turn can lead to eutrophication.

CONCLUSION

We found effects of metal mixtures on two-trophic level communities to be driven to a considerable amount by indirect effects related to a combination of food quantity and food quality, including effects at concentrations at which the single species were still unaffected. The role of food quality in propagating metal effects across trophic levels was implicitly inferred because food quantity could not explain the indirect metal effects on its own. It is also clear that metal mixture effects can vary greatly from those predicted by IA, which is most likely explained by temporal shifts in community dynamics. Some of the observed mixture effects, e.g. an increase in algal biomass, could translate to adverse effects on ecosystem functions and services at concentrations and time-points where no adverse effects would be expected based on the single-species effects on their own. Consequently, further development of efficient experimental methods and mechanistic effect models are needed to improve the environmental relevance in the risk assessment of metals and metal mixtures.

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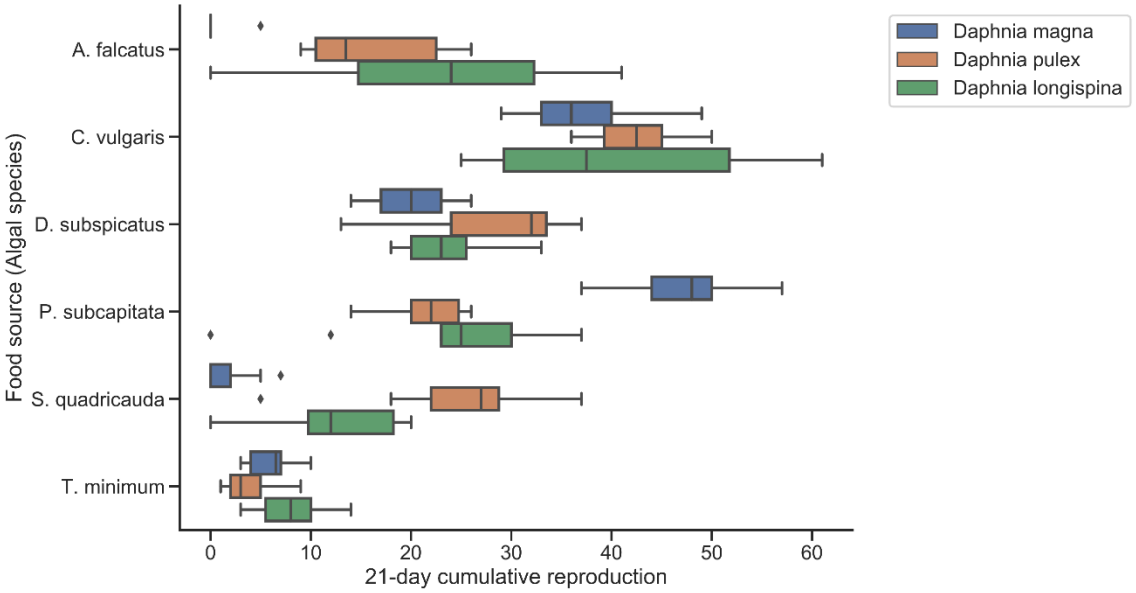


936

937 Figure 1: Cumulative reproduction of single daphnid species as affected by the food source,
938 i.e. the specific single algal species, in the 21-day life-table test. Boxplots show the median,
939 the interquartile range, 1.5-fold the interquartile range and outliers (diamonds), n = 10.

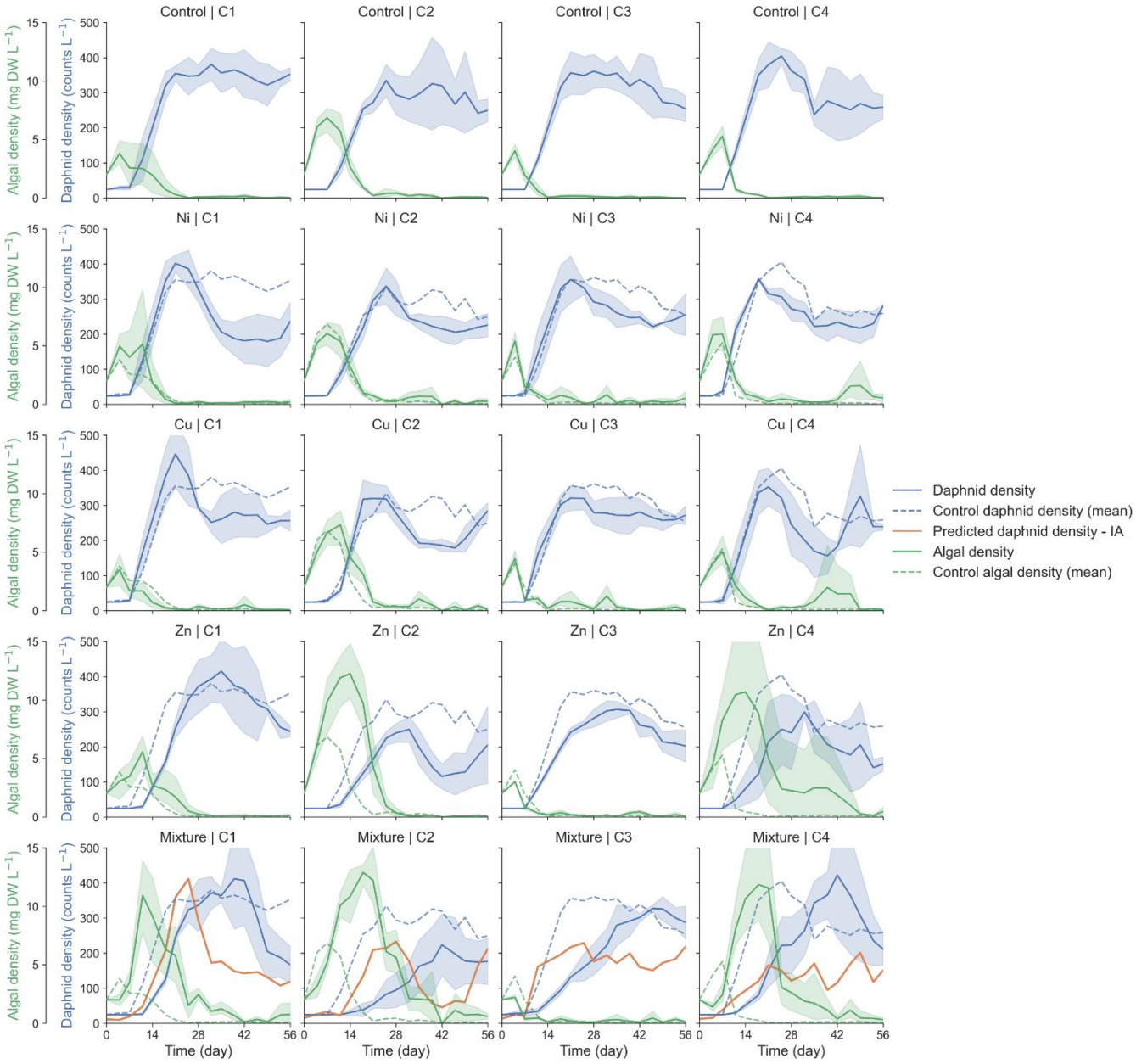
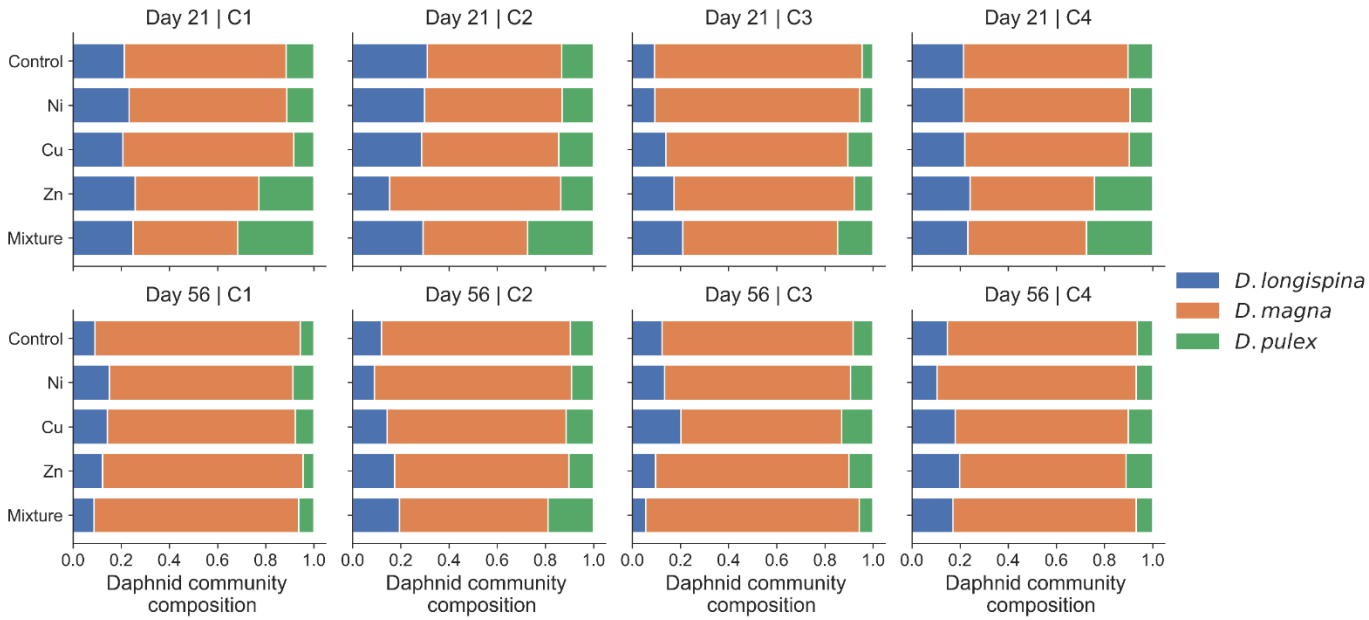


Figure 2: Dynamics of daphnid densities (daphnids L^{-1}) and algal densities (mg dry weight L^{-1}) for four two-trophic level communities (C1, C2, C3 and C4) in the control, the single metals (17 µg Ni L^{-1}, 17 µg Cu L^{-1} and 51 µg Zn L^{-1}) and the metal mixture (17:17:51 µg L^{-1} Ni:Cu:Zn). All two-trophic communities consist of the same daphnid community and only differ by the algal community composition. Predicted daphnid densities as calculated by the independent action (IA) model are shown in orange. Lines are means and shaded areas are 95% confidence intervals.



949

950 Figure 3: Average relative daphnid densities (daphnids L^{-1}) for each daphnid species in the
951 communities C1-C4 at day 21 and day 56 of the two-trophic experiment, n=4. The initial
952 relative densities were 0.33 for each species.

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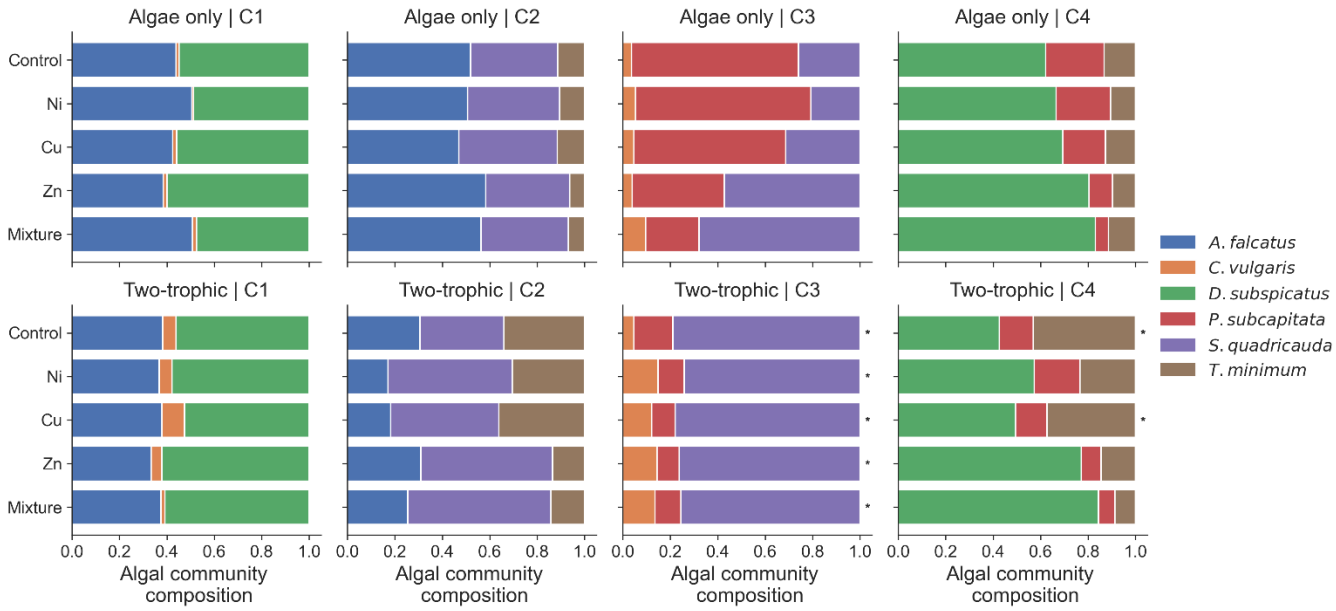

954

955 Figure 4: Average relative algal densities (mg DW L^{-1}) for each algal species in the algal
956 communities C1-C4 in the single-trophic level algal test at day 10 (top) and in the two-trophic
957 test at day 11 (bottom), n=4. The initial relative densities were 0.33 for each species and the
958 * symbol shows where the total algal density was smaller than 0.5 mg DW L^{-1}.

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960

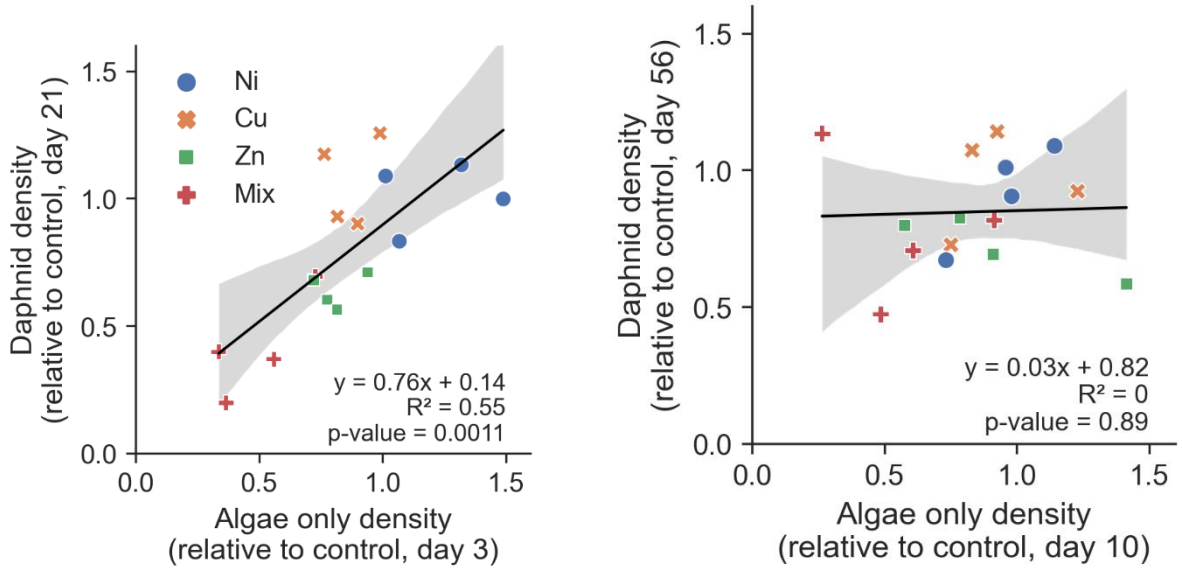

Figure 5: To test for indirect metal effects on the daphnids, the daphnid density (daphnids L^{-1}, relative to the control) in the two-trophic test is shown in relation to the algal density (mg dry weight L^{-1}, relative to the control) in the single-trophic level algal test for the single metals (17 µg Ni L^{-1}, 17 µg Cu L^{-1} and 51 µg Zn L^{-1}) and the metal mixture (Mix, 17:17:51 µg L^{-1} Ni:Cu:Zn). Each point per metal represents the mean of each of the different communities. Shaded areas are the 95% confidence region of the regression line, n=16.

970 Table 1: Comparison of the experimental setup between the two-trophic level algal-daphnid
971 test and the single-trophic level algal tests as described in the method section.

Two-trophic test	Single-trophic level algal test
Medium	
500 mL modified COMBO in 1L poly-propylene pots	2.5 mL modified COMBO in 24-well plates
25% (v/v) refreshed 2×week	Not refreshed
Treatment	
0.3 µ*M* P day^{-1}	
Control (no added metals)	
Single metals: 17 µg Ni L^{-1}, 17 µg Cu L^{-1} and 51 µg Zn L^{-}	
Mixture: 17:17:51 µg L^{-1} (mass ratios Ni:Cu:Zn)	
Algal Species	
Community 1 (C1): *A. falcatus, C. vulgaris* and *D. subspicatus*	
Community 2 (C2): *A. falcatus, S. quadricauda* and *T. minimum*	
Community 3 (C3): *C. vulgaris, P. subcapitata* and *S. quadricauda*	
Community 4 (C4): *D. subspicatus, P. subcapitata* and *T. minimum*	
(Initial density of 0.67 mg DW L^{-1} per species)	
Daphnid Species	
Community 1, 2, 3 and 4: *D. longispina*, *D. magna* and *D. pulex* Initial density of 8 daphnids L^{-1} per species	No daphnids
Test duration	
56 days	10 days

972

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974

Table 2: Daphnid densities (daphnids L^{-1}, relative to the control) in single metal (17 µg Ni L-1, 17 µg Cu L-1 and 51 µg Zn L-1) and the metal mixture (Mix, 17:17:51 µg L^{-1} Ni:Cu:Zn) treatment in the two-trophic test for two timepoints (day 21 and 56) with predicted responses in the mixture calculated as the product of the response to the single metals, i.e. following the independent action (IA) model. Values are means ± standard deviations, n=4. Standard deviations of IA predictions were calculated by propagation of the standard deviations of the single-metal responses. P-values were determined by two-sided t-test applied to the relative responses.

	Time	Daphnid density (relative to control)					S/A*
	(day)	Ni	Cu	Zn	Mix	IA	
C1	21	1.13 ± 0.07	1.26 ± 0.27	0.71 ± 0.08	0.69 ± 0.02	1.01 ± 0.25	Syn
	56	0.67 ± 0.15	0.73 ± 0.08	0.69 ± 0.04	0.47 ± 0.15	0.34 ± 0.09	n.s.
C2	21	1.09 ± 0.07	1.17 ± 0.16	0.60 ± 0.11	0.20 ± 0.04	0.77 ± 0.18	Syn
	56	0.90 ± 0.13	1.14 ± 0.09	0.82 ± 0.44	0.71 ± 0.26	0.85 ± 0.48	n.s.
C3	21	1.00 ± 0.19	0.90 ± 0.10	0.68 ± 0.04	0.37 ± 0.03	0.61 ± 0.14	Syn
	56	1.01 ± 0.23	1.07 ± 0.10	0.80 ± 0.18	1.13 ± 0.18	0.86 ± 0.29	n.s.
C4	21	0.83 ± 0.03	0.93 ± 0.14	0.56 ± 0.30	0.40 ± 0.13	0.44 ± 0.24	n.s.
	56	1.09 ± 0.01	0.92 ± 0.03	0.58 ± 0.08	0.82 ± 0.18	0.59 ± 0.08	Ant

* n.s. = not significant (p-value > 0.05), Syn = synergistic (p-value < 0.05), Ant = antagonistic (p-value < 0.05)

985 Table 3: Observed and predicted metal mixture effects on algal community densities (mg dry
986 weight DW L^{-1}, relative to the control) and the correlations of single metal sensitivities in the
987 single-trophic level algal tests. Predicted responses are calculated with independent action
988 (IA) from single metal effects (not shown, see Table SI 1). Values are means ± standard de-
989 viations, n=4.

	Time (day)	Algal density in the control (mg DW L^{-1})	Algal density in the mixture (relative to control)		S/A*	Correlation of single metal sensitivities**		
			Observed	Predicted (IA)		Ni-Cu	Ni-Zn	Cu-Zn
C1	3	10 ± 1	0.73 ± 0.05	1.23 ± 0.26	Syn	0.65	0.98	0.79
	10	44 ± 3	0.49 ± 0.05	0.50 ± 0.12	n.s.	0.79	0.89	0.98
C2	3	12 ± 1	0.37 ± 0.07	0.60 ± 0.06	Syn	0.58	0.20	-0.68
	10	50 ± 4	0.61 ± 0.10	0.71 ± 0.13	n.s.	0.73	0.96	0.52
C3	3	11 ± 1	0.56 ± 0.08	0.97 ± 0.12	Syn	0.95	0.79	0.94
	10	16 ± 1	0.26 ± 0.09	0.46 ± 0.13	Syn	0.99	0.50	0.60
C4	3	14 ± 1	0.34 ± 0.04	0.71 ± 0.05	Syn	0.96	0.50	0.24
	10	37 ± 5	0.92 ± 0.14	1.99 ± 0.61	Syn	0.15	0.81	-0.45

990 * n.s. = not significant (p-value > 0.05), Syn = synergistic (p-value < 0.05), Ant = antagonistic (p-value
991 < 0.05)
992 ** Pearson correlation coefficient of relative responses among the single metals for all algal species in
993 the community
994
995