FACULTY OF
BIOSCIENCE ENGINEERING

The effects of zooplankton community traits on interactive metal mixture toxicity:

Laboratory microcosm tests and pattern-oriented modelling using a Dynamic Energy Budget Individual-Based Model (DEB-IBM)

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Preface

First of all, I would like to thank professor Karel De Schamphelaere, promotor of this thesis, for the opportunity that I could participate in research that interests me and for the meetings we had to discuss the obtained results. Even more so I would like to thank Simon Hansul, my tutor, as he is by far the person I had the most contact with (mostly via video chat unfortunately) related to the thesis. Not only has he giving tons of really good advice, but he also was always prepared to answer all of my (stupid) questions which was very much appreciated. Thanks for everything!!!

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List of Abbreviations

ABM	Agent Based Model
CA	Concentration Addition
CNN	Convolutional Neural Network
CV	Coefficient of Variation
DEB	Dynamic Energy Budget
DOM	Dissolved Organic Matter
DOC	Dissolved Organic Carbon
EC	Effect Concentration
ERA	Ecological Risk Assessment
GUTS	General Unified Threshold model of Survival
HC	Hazardous Concentration
IA	Independent Action
IBM	Individual Based Model
ICP-OES	Inductive Coupled Plasma-Optical Emission Spectrometry
LC	Lethal Concentration
NOEC	No Observed Effect Concentration
PMoA	Physological Mode of Action
PNEC	Predicted No Effect Concentration
ROS	Reactive Oxygen Species
SD	Standard Deviation
SE	Standard Error
SSD	Species Sensitivity Distribution
TKTD	ToxicoKinetic-ToxicoDynamic
TOC	Total Organic Carbon
TU	Toxic Unit

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Abstract

English

In ecological risk assessment still a lot of single species exposed to single toxicant experiments are carried out. Since the goal is to protect species and communities in natural ecosystems this type of testing might be insufficient as a lot of the interactions species undergo in natural ecosystems are ignored. These have already been proven to affect the response to toxicity. Not only can interactions between species and the ecosystem affect the toxicological response, but toxicants often occur in mixtures. So, to include these interactions of both species and toxicants a microcosm setup with 3 binary *Daphnia* sp. communities exposed to metal mixtures of copper, nickel and zinc were performed with approximately equitoxic ratios on the community level. The outcome on the community endpoints was qualitatively compared to the outcome of a modelling study, in which the experimental setup was mimicked (binary communities exposed to a mixture of 3 toxicants in approximately equitoxic ratios) with a Dynamic Energy Budget-Individually Based Model (DEB-IBM). This to see if characterised community traits related to the toxicological response of the species in the modelling study could also be used on real-world data. It was found in this tested modelling setup that communities with a negatively correlated sensitivity analysis (in this thesis defined as a community with species that both are sensitive to at least one of the toxicants in the mixture compared to each other) were more likely to have a synergistic effect on community size at higher concentrations than positively correlated communities (one species is more sensitive than the other species for all of the toxicants) even with independent action assumed on the physiological level. Also, the sum of toxic units and 21-day survival probabilities of the species seemed to have a clear effect on the relative abundances of the species. The species which endures the least amount of stress based on these metrics were at high concentration levels often the most abundant species in the community. Linking the characterised community traits in the modelling study with the microcosm data was more challenging. The main problems were the relatively weak responses to the metal mixture which made it difficult to conclude if the same trends of the modelling study could be observed.

Nederlands

In ecologische risicobeoordeling wordt nog steeds vaak één soort blootgesteld aan één stressor. Aangezien het doel is om natuurlijke ecosystemen of specifieke soorten in hun natuurlijke habitat te beschermen, kan dit type testen onvoldoende zijn, omdat veel van de interacties die soorten ondergaan in natuurlijke ecosystemen worden genegeerd. Het is al bewezen dat deze de reactie op toxiciteit beïnvloeden. Niet alleen kunnen interacties tussen soorten en het ecosysteem de toxicologische respons beïnvloeden, maar stressoren komen ook vaak tezamen voor. Dus, om deze interacties beter te begrijpen tussen zowel de soorten als de stressoren, werd een experimentele opstelling met 3 binaire *Daphnia* sp. gemeenschappen blootgesteld aan een metaalmengsel van koper, nikkel en zink met ongeveer equitoxische verhoudingen op het gemeenschapsniveau beschouwd. De uitkomst op de gemeenschapseindpunten werd kwalitatief vergeleken met de uitkomst van een modelleerstudie, waarin de experimentele opstelling werd nagebootst (binaire gemeenschappen blootgesteld aan een mengsel van 3 stressoren in ongeveer equitoxische verhoudingen) met een Dynamic Energy Budget-Individually Based Model (DEB -IBM). Dit om te zien of gekarakteriseerde gemeenschapskenmerken die verband houden met de toxicologische respons van de soort in de modelleerstudie ook de respons verklaren in echte gemeenschappen. In de geteste modelleer opstelling werd gevonden dat gemeenschappen met een negatief gecorreleerde gevoeligheidsanalyse (in dit proefschrift gedefinieerd als een gemeenschap met soorten die beide gevoelig zijn voor ten minste één van de stressoren in het mengsel vergeleken ten opzichte van elkaar) meer kans hadden om een synergetisch effect te vertonen op de gemeenschapsgrootte bij hogere concentraties dan positief gecorreleerde gemeenschappen (de ene soort is gevoeliger dan de andere soort voor alle stressoren), zelfs als aangenomen wordt dat de stressoren niet interageren met elkaar op het fysiologische niveau. Ook de som van toxische eenheden en 21-dagen overlevingskansen van de soort leken een duidelijk effect te hebben op het relatieve voorkomen van de soort. De soort die op basis van deze statistieken de minste hoeveelheid stress ondergaat, was bij hoge concentratieniveaus vaak de meest voorkomende soort in de gemeenschap. Het koppelen van de gekarakteriseerde gemeenschapskenmerken in de modeleerstudie met de experimentele data bleek een uitdaging. De grootste problemen waren de relatief lage effecten die de stressoren met zich meebrachten, wat het moeilijk maakte om de link te leggen tussen de modelleerstudie en de geobserveerde data.

1 | Introduction

Ever since mankind started with the casting and manipulation of metals, the created materials and tools, mostly used for agriculture and war, were undoubtedly game-changing for the time (Lowe, 2017). This is also the era when the first trading routes were established, because seldom copper and tin ores were found in the same region to make bronze (Earle *et al.*, 2015). So, next to the technological improvements brought metal the first steps in trading and the society as known today. To this day it is still impossible to imagine a world without metals, they are virtually used everywhere around us.

As always there is also a flip side to this coin. The great advancements in metallurgy brought with them metal exposure to living beings and contamination of the environment which can potentially have negative effects. The first metal pollution dates back to the very first mining and metallurgy sites in the Bronze Age (Martínez Cortizas *et al.*, 2016). This is a problem that has persisted through time. In the Roman era increased levels of mercury and lead were found in human bones (López-Costas *et al.*, 2020). The first medical hypothesis about metal toxicity was formulated in the Renaissance (Riva *et al.*, 2012). It was not until 1980 that an increase in metal production did not lead to an increase in metal emissions (Nriagu, 1996). So, it took almost five centuries for science to mature, general awareness to grow and a feeling of urgency to develop within policymakers and industrials.

The first modern pieces of legislation that addressed these issues are "the Occupational Safety and Health Act" and the introduction of the environmental protection agency around 1970 in the USA with similar legislation being created in Europe, "the first Environmental Action Program" in 1973. Most of the environmental concerns initially came from a human health perspective like pollution of drinking water sources while in more recent legislation like "the Water Framework Directive" (European) from the year 2000 there was room for a "good status" of all water bodies which broadened the view on environmental protection (European Commission, sine anno).

Assessing the effects of metals/toxicants in a freshwater context is typically done by exposing different organisms like algae and fish to the chemicals/toxicants of interest. To gain insight in the toxicity, different endpoints like survival, growth, reproduction, etc. can be studied in function of the concentration to determine if these chemicals have an effect on the exposed organisms (European Commission, 2003). These experiments are often performed by exposing one species (or even one organism) to one toxicant. The tests are relatively easy to perform and gain insight in the toxicity, but they are simplified compared to real ecosystems (Jager, 2017; Van de Perre *et al.*, 2016). On top of that, this method is a black box (the mechanisms behind the toxicological response are not studied). When applying simplifications to study real-life interactions between toxicants and organisms, it is necessary to ensure that one gains useful information from the tests. In reality, organisms are often exposed to toxicant mixtures and organisms can interact with the environment and other organisms. By simplifying the ecotoxicological tests these interactions (between toxicants and organisms) are often ignored. Therefore, it is necessary that research is conducted where these interactions are not ignored, so that the importance can be determined and a more holistic approach to toxicity can be established.

2 | Literature study

2.1 Metal pollution

2.1.1 Metal pollution pathways

Metals are atoms, so they will not degrade like most organic pollutants. They can change their oxidation state and bind to different charged molecules or ions but will never disappear. Since all metals occur naturally on the planet even the most toxic ones like arsenic can occur in water streams without originating from anthropogenic pollution. For example, cobaltite and adamite are crystals that contain arsenic (Rice, sine anno). If these crystals were to weather and erode, the arsenic could end up in a water stream. Other natural causes could be forest fires and volcanic eruptions which are more incidental than the continuous rock weathering. Near urbanised areas the naturally present metal concentrations in waterbodies can be low compared to the pollution due to human activities (Tchounwou *et al.*, 2012).

Contamination can occur in different environmental compartments. In the atmosphere, metals can occur as a vapour (mostly mercury) or as small particles in the micrometer range (salts or pure metal) (National Research Council (US), 1997; Queensland governement, 2019). The metals could also be bound to airborne dust or soil particles. When a water layer starts to form on these particles the ionic metal species can act as catalysts and enhance radical formation in the air (Kleeman *et al.*, 2000; Deguillaume *et al.*, 2010). All airborne particles are under the effect of the atmospheric conditions like wind and rain which will affect the concentration and the spread. Since wind can carry particles and pollution for hundreds and sometimes even thousands of kilometres this is the pollution form with the biggest potential of spread (Steinnes *et al.*, 1989). The particles will not stay airborne forever and will deposit by two mechanisms: wet and dry deposition. Wet deposition is related to rain events while dry deposition is related to gravitational settling and diffusion and thus is a more constant process (Freedman, 1995). The main air pollution sources are transportation, fuel combustion (mostly coal), waste combustion, metal mining and melting. Depending on the region the contributions of each of the sectors may differ (Canadian governement, 2012; He *et al.*, 2013).

Anthropogenic metal emissions to soil can be mostly attributed to the deposition mechanisms and agricultural application of (organic) fertilizers and crop protection products (Zwolak *et al.*, 2019). For example, the application of manure as a fertilizer will increase the available copper and zinc in the soil. This can have negative effects on plant growth and can lead to groundwater contamination (Zhen *et al.*, 2020). Via groundwater metals can resurface in waterbodies, posing a potential threat as the metal pollution can become more widespread (Brunner *et al.*, 2017). The same threat exists when contaminated topsoil is swept up with rainwater, also known as runoff (He *et al.*, 2004). Depending on the metal, acidity and organic matter in the soil groundwater contamination can be a very slow process. In Li and Shuman (1996) lead and cadmium interacted in the studied soils with the organic matter causing the majority of the metal content to become insoluble, but zinc was more mobile and was thus detected deeper in the soil.

Lastly, direct emissions from human activities to waterbodies can also occur. Near urban areas, most of these direct emissions come from industry and households which may or may not be connected to a wastewater treatment plant (Scherer *et al.*, 2003). Although traditional activated sludge systems are not designed for metal removal, depending on operational conditions, metal concentration, metal type, etc. can play an important role in the removal. As metal removal is case specific there are some trends visible. For example copper is among the best-removed

Table 2.1: Thresholds for a selection of metals for rivers and lakes in Flanders which will not be used for drink water production with: * dependent on hardness of the water, most stringent provided and ** bioavailable concentration. All concentrations are in μg/l, except for b dissolved concentrations (VLAREM II - Bijlage 2.3.1 Basismilieukwaliteitsnormen voor oppervlaktewater Art. 3 §4)

	Co	U	As	Zn	B	V	Ba	Cd	Hg	Ni	Pb	Cu
Average environmental quality standard	0.5	1	3	20	700	4	60	0.08*		4**	1.2**	7
Maximum environmental quality standard								0.45*	0.07	34	14	

metals with removal efficiencies around 70% while nickel usually hovers around 20% (Stephenson and Lester, 1987; Yang *et al.*, 2015). In Flanders when the whole wastewater treatment plant is considered the removal efficiencies for nickel can climb up to 54% and arsenic seems to be removed the least at 40% (Vlaamse MilieuMaatschapij, sine anno).

Apart from the urban wastewater, metal production and mining facilities can have an enormous impact on metal concentrations in the neighbouring waters. The largest metal pollution comes from unmanaged waste rock. This is the rock/soil that remains after the extraction of the desired metal. If this waste rock was treated with acids the leaching of other non-target metals could be enhanced due to higher mobility of metal at low pH. This process also occurs naturally in sulphide-containing rocks. Sulphide exposed to air and water will induce bacterial growth of for example *Thiobacillus ferroxidans* which can oxidise sulphide to sulphuric acid creating low pH environments which will induce the dissolving of metals into the water fraction (Simate and Ndlovu, 2014). This acid and metal-rich leachate can runoff to rivers and lakes affecting the exposed ecosystems. This leaching of acidic, metal containing water is called acid mine drainage.

2.1.2 Status of metal pollution in surface waters

It is hard to get an idea of the overall surface water quality in the world as big differences can be observed between waterbodies. Although Zhou *et al.* (2020) gathered a lot of information on metal pollution of surface water from 1972 to 2010. Not only did the concentration of the individual metals increase throughout the years compared to 1970-1980 (relatively low pollution) but also the number of metals that exceeded the WHO and USEPA standards. This indicates that next to the metal pollution getting worse, a shift from single to multi-metal pollution has also happened. Zhou *et al.* (2020) found that the concentration and amount of metals above the WHO and USEPA standards is higher in developing continents compared to developed continents (North-America and Europe). Globally the main sources of pollution shifted from primary mining and manufacturing to secondary metal waste discharge. In 2010, when the mean concentration was calculated for all the sites, 10 out of 10 of the studied metals exceeded the WHO and USEPA standards: Cd, Pb, Cr, Hg, Zn, Cu, Al, Mn, Fe, As. The data could be biased because research on toxicity and pollution will be mainly focussed on polluted rather than unpolluted waters, but this data nonetheless indicates that metal pollution still is a problem.

When examining more recent data from Flanders in figure 2.1 the threshold exceedance percentage is shown for different metals in 2019. Cobalt seems to be the biggest problem together with uranium, arsenic and zinc. For none of the metals in the graph are there significant trends of increase or decrease over the last decade (Peeters, 2020). The industries lack of effort are not necessarily the origin. Some emissions from industry even decreased dramatically between 2010 and 2017, for example the copper emissions decreased by 23% and lead even by 86%. The

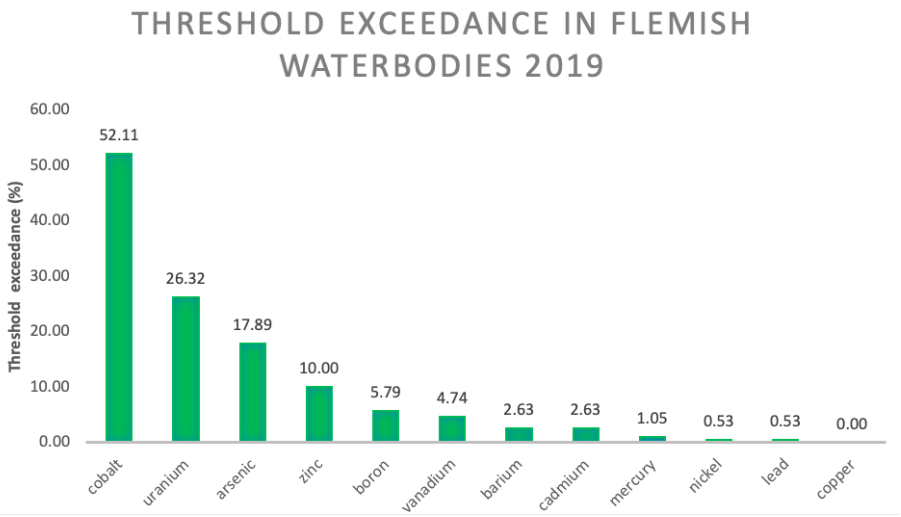


Figure 2.1: The percentage of waterbodies that exceed the threshold in all of the Flemish waterbodies for a selection of metals in 2019. The thresholds are mentioned in supplement two of VLAREM II. (data: Peeters (2020))

biggest pollution sources for arsenic, cadmium, chrome, mercury, nickel and lead are from deposition and rock erosion while copper pollution mostly comes from transport and zinc from the corrosion of buildings (Vlaamse MilieuMaatschapij, sine anno).

2.2 Ecological risk assessment (ERA)

2.2.1 Ecotoxicology

Ecotoxicology is the study of negative effects from chemicals on different levels of organisation ranging from (sub)individual to ecosystem level. The goal of ecotoxicity is to assess what the effects of certain pollutants will be (effects assessment). This can be done by research into: concentration-effect relationships, biotic and abiotic factors that determine toxicity and mode of action of the toxicant. Examples of studied effects (so-called endpoints) on the individual level are mortality, growth, fecundity, etc. and for populations, this can be monoculture yield, structure (*e.g.* ratio juveniles to adults), etc. It is possible to follow the relative effect in function of the toxicant concentration when the only variable in the test is the toxicant concentration (concentration-response curve). This way the effect concentration (EC_x) can be calculated. This is the concentration at which the observed endpoint effect is x% of the maximum effect for that specific endpoint. When mortality is the endpoint, lethal concentration or LC_x is used, which is the concentration that is lethal to x% of the organisms. Figure 2.2 shows an example for a concentration-response curve with mortality as an endpoint. It also shows visually the concept of the LC_{50} being the concentration at which 50% of the tested organisms die.

2.2.2 Risk assessment tools and assumptions

In ecotoxicology, the goal is to understand and determine negative effects in function of the toxicant exposure levels while in risk assessment these data will be used together with the environmental concentrations to assess failure risks of (components in) the ecosystem. Risk assessment can then be used in management to weigh the benefits of using a chemical against the harm it causes to the environment. Resulting in the determination of an "acceptable" risk level by policymakers.

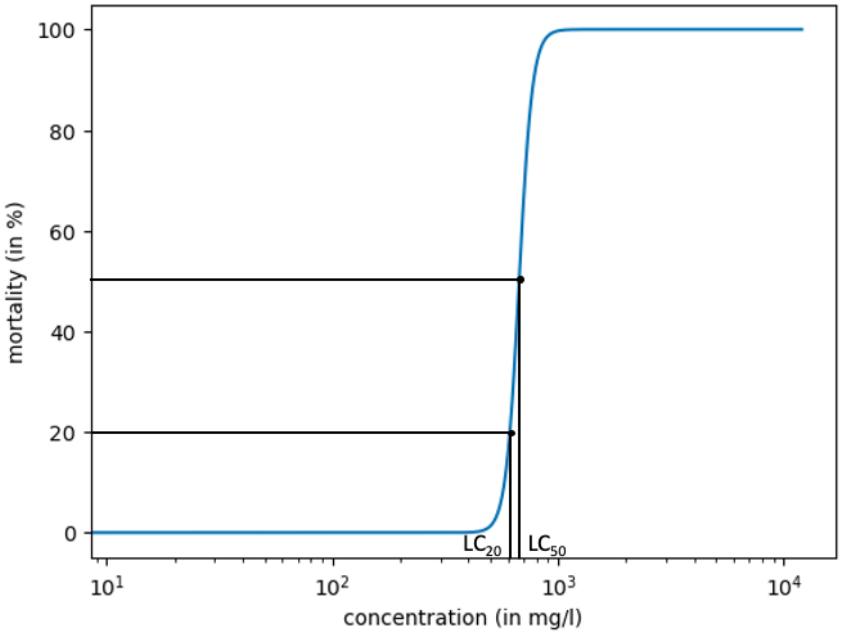


Figure 2.2: Example of a concentration-response curve with endpoint mortality

In Europe, for an effects assessment, the use of a PNEC or predicted no effect concentration is promoted (European Commission, 2003). The goal of a PNEC calculation is to determine a safe concentration for a pollutant at which the functioning of the ecosystem is guaranteed. There are two suggested ways of calculating the PNEC: via assessment factors or via statistical methods. Both of these methods aim to protect ecosystem functioning with data based on toxicity assessments from single species. This is understandable from a time/money perspective as these are simple and cheap, but are far from realistic and thus come with two important assumptions: the most sensitive species determines the ecosystem sensitivity and community functioning is guaranteed when protecting the ecosystem structure (European Commission, 2003). There are internationally accepted guidelines from organisations like OECD (Organisation for Economic Co-operation and Development) or ASTM (American Society for Testing and Materials) for these tests. The assessment factor method determines the PNEC based on the effect concentration of the most sensitive endpoint of the most sensitive species divided by an assessment factor which depends on the amount and diversity of the toxicological data gathered. These assessment factors are quite arbitrary and not scientific. For the statistical methods, an SSD (species sensitivity distribution) can be used preferably with chronic (long term) NOEC or no observed effect concentration data. A NOEC is the highest tested concentration in a bioassay where the organism did not show an effect on the studied endpoint. Fitting all the relevant toxicity data to a statistical distribution (SSD) will allow the calculation of the PNEC. This PNEC can be derived from this distribution as x% of the species in the ecosystem are affected by the chemical at a certain concentration. In Europe typically 5% is arbitrarily chosen, with the corresponding concentration called hazardous concentration (HC_5), to ensure the ecosystem will not be affected too much. This HC_5 can still be divided by an assessment factor ranging from 1 to 5 to account for uncertainties (for more info on PNEC calculation see European Commission (2003)).

Nonetheless, some community ecotoxicological studies have shown that simple toxicological data used in PNEC calculation like the HC_5 concentrations can be protective for the ecosystem (Maltby *et al.*, 2005; Van de Perre *et al.*, 2016). This will not always be the case as the use of an SSD comes with implicit assumptions that species tested are a representative subsample of the ecosystem and that functional traits are not related to species sensitivity. The last assumption

will not always hold up. Species affected by the pollutant can be compensated for by other species, so the ecosystem does not lose any of its functional traits (functional redundancy). In this case the HC_5 will be conservative when protecting functionality while in the opposite case an affected species can have a disproportional effect on ecosystem functioning (Baert *et al.*, 2017). This is for example the case when a keystone species (species with a high impact on the ecosystem as they are often crucial for the structure and functioning of the system) is among the most sensitive species in the ecosystem. Protection of ecosystem functioning and structure are goals that often pop-up in European regulations (Lilongwe, 1998).

A single species exposed to a single compound/pollutant is still a important way of collecting toxicity data until this day. This can be a problem because species interactions and mixture exposure often occur in real life. In rivers and lakes various sources of pollution often come together resulting in a mixture exposure. These species interactions are not always well represented by endpoints measured on the individual level like reproduction and mortality (Pereira *et al.*, 2019; Viaene *et al.*, 2015). A species may be tolerant for a certain stressor, but if its prey is very sensitive it will still greatly affect the tolerant species. Pereira *et al.* (2019) also found that observed reproduction effects on the individual level by cadmium only had minimal impact on population size likely due to reduced starvation, so ignoring the fact that an ecosystem is full of interactions can lead to wrong conclusions.

Mixture toxicity on the other hand can lead to increased stress for the exposed organisms or even have interactive effects. It is for example possible that a mixture is more toxic than one would expect based on the single toxicant exposures. This is called synergism and will be discussed in section 2.4.2. When organisms are exposed to multiple compounds at low levels, these effects can add up (Carvalho *et al.*, 2014). Furthermore natural stressors like temperature, heat, oxygen, biological (cyanobacteria), etc. are often neglected in risk assessment, while these most certainly also affect toxicity (Pereira *et al.*, 2019; De Coninck *et al.*, 2013). To get a comprehensive image of real environmental stress a lot of factors need to be taken into account.

To address the problem of ecological interactions, it has already been suggested to use population models as the ultimate goal of ERA is to protect populations and communities in real environments. These models can translate the individual level endpoints to population endpoints with no or minimal extra data. These model types have already been proven to be successful, but are only seldom used in risk assessment today despite existing general frameworks (David *et al.*, 2020; Pereira *et al.*, 2019; Vlaeminck *et al.*, 2021). Mostly due to limited guidance and often relatively complex models, some are only used by trained modellers (Raimondo *et al.*, 2018). This approach could increase the ecological realism in risk assessment, if the right setting can be created for the risk assessors and enough information and guidance is available.

2.3 Metal toxicity in freshwater

2.3.1 Bioavailability in aqueous environment

In a waterbody polluted with metals, it is important to realize that one metal can be present in different forms. This is called speciation and will greatly depend on physicochemical properties of the water (Gupta *et al.*, 2013). Speciation is of great importance when studying toxicity as not all forms have the same availability for uptake or toxicity (Allen *et al.*, 1980).

In figure 2.3 most of the metal interactions are depicted with their respective driving factors. Metals can interact with the sediment and suspended particles via adsorption, ion exchange (mostly clay) or by forming complexes (Bjerregaard *et al.*, 2014). The sediment can also con-

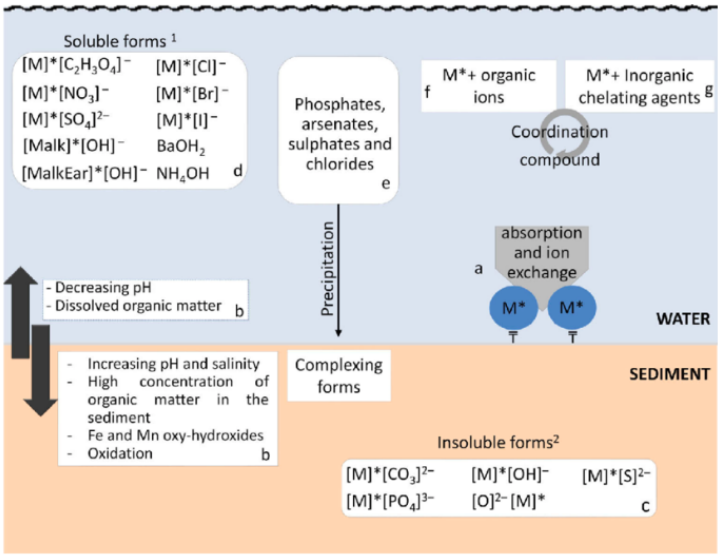


Figure 2.3: Metal (M*) distribution between water and sediment and interference factors in solubility. 1 and 2 are exceptions for some metals. Malk - Alkali metals. MalkEar - Alkaline earth metals (Magalhaes *et al.*, 2015)

tain organic matter which is overall negatively charged at neutral pH making it possible for positively charged metal ions to bind. In the water column, metal ions can interact with other dissolved organic and inorganic ions. The end product can stay in solution or precipitate like zinc phosphate. Organic molecules in the water column are generally referred to as dissolved organic matter (DOM) or dissolved organic carbon (DOC).

The amount of immobilisation greatly depends on the pH of the receiving water. The pH will affect hydrolysis, polymerization, aggregation, precipitation and proton competition for available ligands (Magalhaes *et al.*, 2015; Smith, 2009). Metals will be more abundantly present in the ionic form at low pH while at high pH they are more likely to be present as precipitates like oxides and hydroxides. Next to pH other positively charged ions can interact similarly with the ecosystem as toxic metals and thus are competing for the same target sites. The most important competitors are calcium and magnesium (measured as the hardness of the water). Lastly, the redox potential can influence the oxidation state of the metal and thus which interactions it can undergo.

DOC, pH, etc. have a large influence on the availability of the metals and subsequently the toxicity. Effect concentrations differences up to 20 fold for algae can be observed for the same total metal concentration, when the pH, hardness and DOC of the water were adjusted (De Schamphelaere *et al.*, 2005). A lower pH, lower hardness and lower DOC will lead, in the case of metals, to more soluble and free ion forms of the metals, which makes them more readily available for uptake by organisms. These water parameters can change throughout a year in a natural waterbody, so the same metal concentrations can become less or more toxic. Although it is difficult to generalize the free ion fraction because of physicochemical differences between waterbodies, Kalis *et al.* (2006) found that free ion fractions are generally low in natural waters. They determined the speciation of metals in rivers across Europe. The observed free ion fractions ranged from 0.015-0.63% for copper and 4.3-13% for zinc. Cadmium, nickel and lead were also analysed and were between 0.015 and 13%. Therefore, environmental relevant free ion concentrations are often in the nanomolar (nM) range.

2.3.2 Toxicokinetics and toxicodynamics

Every observed endpoint on every level of organisation will have its origin at the smallest of scales, the molecular level. For example, a chemical inhibits a certain protein which is important for the reproduction. When reproduction is altered this can lead to a slower population growth etc. Therefore understanding what happens at lower organisation levels might lead to insights on higher levels. On the scale of the individual are two important fields of study: toxicokinetics and toxicodynamics. The study of uptake, distribution, transformation and elimination of the toxicant (internal concentration) is called toxicokinetics, while toxicodynamics is linking the internal concentrations of the chemical to the effects on the different endpoints.

The metal pollution in the water column can thus only affect the exposed individuals when it can enter the organism. In section 2.3.1 bioavailability of metals was discussed and is key here. The most soluble/mobile forms in the water are the most toxic for organisms as these are more readily available for uptake. When accounting for bioavailability the free ion concentration is often considered as the most important fraction. This was shown by Erickson *et al.* (1996), they found that free ion activity of copper for different pH gives a more constant LC_{50} for fathead minnows, indicating that the toxicity of copper for this species is better predicted by the free ion activity than the total metal concentration. Ion activity is even more accurate than ion concentrations in this case because this theory includes the significant electrostatic interactions ions have.

The internal concentration in the target tissue (the place where the toxicant can interact with the body) will better predict the severity of toxic effects than the environmental concentration. This is not only because upconcentration can occur in certain tissues for certain chemicals, but there can also be a significant time effect (Jager and Ashauer, 2018). Before a chemical can have an effect, it has to be transported or reach the target site. When the concentration at the target site changes, the effect of the toxicant can also change over time. Changes in external concentration do not always directly raise internal concentrations. This delay can sometimes be important to include. That is why it can be useful to include toxicodynamics when studying toxicity. Nonetheless in experiments or large waterbodies, the external concentration is often quite stable and this can allow for an equilibrium between the environmental concentration and the internal concentration. The relationship between the external concentration and the effects of the toxicant can sometimes be a good approximation under these conditions.

The four major mechanisms that influence this internal concentration are aqueous uptake, dietary uptake, elimination, and growth (Tsui and Wang, 2007). Aqueous uptake consists of all toxicant uptake that happens via other structures than the gut like tissue adsorption. Next to bioavailability is hardness also important for aqueous uptake. Higher levels of hardness generally lead to lower toxicity of metal, as calcium and magnesium will compete for binding sites and uptake in the organism (Kozlova *et al.*, 2009; Tsui and Wang, 2007). Dietary uptake is the uptake via the guts, the metals are in or onto the food and suspended particles in the water column. Before take up the metals first need to go into solution by desorption from the particles. The effectiveness of desorption will determine if uptake via food is a major contributor to the toxicity.

Lastly, growth and elimination will be discussed as these processes will lower the internal concentration. Elimination is removal of the toxicant out of the body, with or without deactivating it first. Major elimination pathways are excretion and reproduction. Deactivation is possible via metallothioneins, which can act as detoxifying agents of the metals. These proteins can make the metals unavailable for the cellular receptors and can as a result no longer exhibit negative effects (Roesijadi, 1992; Amiard *et al.*, 2006). Significant amounts of maternal metal can be transferred to the eggs via reproduction. Growth will not lower the metal content inside the

organism but dilute it. The volume of the organism will increase while the metal mass will remain the same.

Once the metallic ions are in the body they can affect different pathways. Without going into detail the most important effect is ionoregulatory disruption, which might cause the creation of reactive oxygen species (ROS) generation. ROS generation can affect many cellular and physiological processes. Other effects may include: altering the essential metal uptake, toxic metals replacing essential metals in proteins, etc. (Davidson *et al.*, 2015). Most of the metals are only weakly mutagenic but can contribute to mutagenic properties of for example ultraviolet light as they can inhibit repair of DNA adducts (Davidson *et al.*, 2015; Hartwig, 1998). How these processes then affect the macroscopically observable endpoints (reproduction, growth...) is not always well understood, but it is clear that they undermine the normal processes in the organisms and cause unwanted effects. These effects are thus under the influence of bioavailability, dose, duration, exposure route and the organism. Intravariability of the species like age, gender, genetics, and nutritional status of the exposed individuals also affect the severity of the observed effects (Tchounwou *et al.*, 2012). Multi-generational exposure can alter the response to toxicants. Levinton *et al.* (2003) found that organisms inhabiting metal-contaminated environments developed some sort of tolerance which disappeared after the pollution was removed.

2.3.3 Mixture toxicity

When assessing mixtures it is important to have a reference situation. This can be based on the effects that the different toxicants have exposed separately to the organisms. The observed effect of the mixture can be more toxic (synergism) or less toxic (antagonism) than expected by assessing all the single toxicant effects. There are two well known and established reference models for determining synergisms and antagonisms:

1. Concentration addition (CA): The most important assumption in this method is that the toxicants act on the same target site and thus could be seen as dilutions of each other. In this reference model, the dose-response curves for all the toxicants need to be known. The experimental conditions need to be identical and the same endpoint needs to be studied, this is also the endpoint you will assess with the CA-method. The formula for a binary mixture is the following:
$$\frac{C_a}{EC_{x,a}} + \frac{C_b}{EC_{x,b}} = 1 \tag{2.1}$$
With C_i the concentration of compound i in the mixture and $EC_{x,i}$ the effect concentration of compound i that results in the same effect (x%) as the mixture. The need for the dose-response curves come from the fact that solving for x is required (Howard and Webster, 2009).

2. Independent action (IA): In this reference model the assumption is that the metals act on different receptors and thus act independently. The formula for a binary mixture would look like this:
$$R_a * R_b = R_{mix} \tag{2.2}$$
With R_i the chance of survival when an organism is exposed to a compound i (Cedergreen, 2014). The fact that the toxicants act independently also allows for relative effects to be multiplied. For example, if the biomass of an organism at a given timepoint is 4 mg in unstressed (control) conditions, single metal exposure to chemical a 2 mg and chemical b 3 mg then the IA predicted biomass is: $(\frac{2}{4} * \frac{3}{4}) * 4$ mg or 1.5 mg.

Both models have their respective assumptions, but in practice the interactions can be so complex that even when the chemicals are known to act on different sites that IA is not necessarily

better for predicting the mixture effect based on accuracy (Cedergreen *et al.*, 2008; Gao *et al.*, 2018). Nys *et al.* (2017) found that at environmental realistic concentrations CA often overpredicts the observed mixture effect for metals up to 3.6 times tested for *Daphnia*. When using the CA reference model in this case, antagonism could be concluded. IA and CA can also be applied on different levels of organization. For example, the population- or community-level, in this context the target site rationale does not hold any more. At these levels there are also other influence factors that determine the effect on the mixture.

Cedergreen (2014) used databases and reviewed scientific literature to collect toxicity data related to mixtures. Then she calculated IA model predictions as a reference and looked for antagonisms and synergisms for different categories of toxicants among other things metals. She only found limited cases that were synergistic or antagonistic for metals and these were mostly seen at very high concentrations, far above ecological relevant concentrations. Metal and organic compounds mixtures seemed to have a bigger tendency for synergism, but only a limited number of cases were studied which makes it hard to draw any conclusions.

Cedergreen (2014) also discussed that there are six important synergism pathways: bioavailability, uptake, internal transportation, metabolization, binding at the target site and excretion. Metabolization seemed to be an important pathway for all types of toxicants, while the most important pathways for metals are bioavailability and uptake. Due to competition for binding sites in the water column (DOC, sediment, inorganic ions etc.), a more toxic metal can become more available for uptake. Uptake of mixtures is influenced by competition at biological ligands or competitive inhibition of transport proteins. This shows once again that multi metal exposure can give deviating results from single metal exposures.

Certain toxicant mixtures are not always synergistic or antagonistic. The ratio between the compounds in the mixture and exposure level can also play a role, so-called dose ratio-dependent and dose level-dependent (Jonker *et al.*, 2005). Dose level-dependent deviation occurs when at low exposure levels of the mixture the deviation for the reference model is different than the mixture with high concentrations of the compounds. For example, at low concentrations of the components, the mixture might tend to be antagonistic but at high concentrations, synergism might occur. Dose ratio-dependent deviation takes place when the ratio of the mixture compounds determine the difference from a reference model, for example, high concentrations of compound 1 will lead to synergism while high concentrations of compound 2 lead to antagonism. Not only can the dose and the ratio change determine synergism, but the observed endpoint can also change the outcome (Cedergreen and Streibig, 2005).

2.4 Daphnids

2.4.1 Ecology

What are daphnids?

The taxonomic classification of *Daphnia magna* is portrayed in table 2.2. The genus *Daphnia* belongs to the phylum of the Arthropoda, so daphnids have an exoskeleton (which act as attachment place for muscles), a heterogeneously segmented body, paired jointed appendages and moult to grow. Furthermore are they planktonic species which means that daphnids float in the water column and their movement is under influence of the water current (Mauchline, 1998). Because daphnids are brachchiopoda they have flattened legs to create a water stream for the filtering apparatus which is important for food uptake. The fact that daphnids are cladocera means that their exoskeleton, which is called the carapace, is made out of chitin and they have two antennae for swimming. Cladocerans are often referred to as water fleas. The

size of cladocerans ranges from 0.5 mm to more than 6 mm. Males typically have smaller body size and a modified post-abdomen (Ebert, 2005). See figure 2.4 for detailed pictures of daphnids.

Daphnia spp. have two main pathways for reproduction (Ebert, 2005). First of all, daphnids reproduce via eggs that are grown in the brooding chamber under the carapace of female individuals. Once the eggs are hedged the newborns are released. There are 6 stages in their life before a female individual can produce eggs. Under unstressed conditions, this takes between 5 and 10 days. After that, every 3 or 4 days a new clutch of eggs can be produced via parthenogenesis (Ebert, 2005). This means that females produce clones of themselves (also females) by laying diploid eggs. These eggs can also become males under stressed conditions. The males become important when the organisms are starting to experience less favourable conditions (e.g. lower temperatures, shorter light periods, less food... (Baer and Owens, 1999)). Then they will start to reproduce sexually. Females will start to produce haploid eggs which need to be fertilised. These fertilised eggs will can form resting eggs that are encapsulated (Ebert, 2005). There are typically two eggs in the protective ephippium and the eggs can hedge when conditions start to become more favourable for the daphnids, but sexual reproduction does not necessarily to resting eggs. Resting eggs and sexual reproduction seem to be controlled by other environmental triggers (Hobæk and Larsson, 1990). This switch allows for a competitive advantage, parthenogenesis leads to rapid growth during favourable conditions while sexual reproduction increases the survival rate and the genetic diversity (Barton and Charlesworth, 1998).

Ecological niche and food web

Daphnia spp. can swim with their antennae, but it is not in their ability to withstand constant currents. This is why they are found mostly in freshwater lakes and ponds. *Daphnia* is also a very diverse and large genus with over 100 species which makes it possible to find daphnids all over the world. They have been found in very cold oligotrophic lakes as well as tropical ones. The different species can handle a broad range of fysico-chemical properties (Pietrzak *et al.*, 2013; Hebert, 1978). Hooper *et al.* (2008) determined the ecological niche for *Daphnia magna* based on the population growth rate for pH and calcium concentration. The definition of niche was fulfilled when the population growth rate was bigger then zero. The position of the laboratory niche boundary was determined at 0.5 mg Ca^{2+}/l as lowest concentration with a growth rate above zero and a pH between 5.75 and 9, though more Ca^{2+} is needed at lower pH values. These boundaries were accurate for natural systems although not a lot of the sites were colonized near these niche boundaries. This is probably due to other factors that could influence extinction when the population growth rate is low.

Table 2.2: Taxonomic classification *Daphnia magna* (ITIS, sine anno)

Hierarchy	
Kingdom	Animalia
Phylum	Arthropoda
Subfylum	Crustacea
Class	Branchiopoda
Order	Diplostraca
Suborder	Cladocera
Family	Daphniidae
Genus	*Daphnia*
Species	*magna*

Figure 2.4: Image of *Daphnia pulex* (left) and *Daphnia galeata* (right) at 40x magnification (Pearson, 2019)

When they occur they are often the most dominant plankton form in the water column making them very important in the food web (Ebert, 2005). Daphnids feed themself by creating a water current to their filter apparatus to catch single-celled algae, yeast and bacteria. To avoid predation themselves they stay relatively deep in the water column during the day to avoid being seen by predators and resurface every night to feed mostly on algae who live near the surface of the water for the best light conditions (Stich and Lampert, 1981). Typical predators are planktivorous fish, insects or salamanders.

Usually, before the growing season starts there are only limited or none females alive. When the growth season starts the resting eggs start to hatch and the females start to reproduce asexually and population numbers will start to boom. The population will reach a maximum size right after the drop in algae density due to high levels of predation by the daphnids. Afterwards, the *daphnia* population will drop due to starvation. Depending on the nutrient status of the lake one or multiple peaks in *daphnia* density can occur during one growing season. Predation on daphnids can also influence when and if these peaks occur (Ebert, 2005).

Why study daphinds?

When studying freshwater ecotoxicology in scientific literature or in risk assessment reports often species of the genus *Daphnia* are the test subject and there are good reasons why.

As discussed in the previous section daphnids can be a very dominant species compared to other planktonic species and are the primary consumers, so they link the autotrophic and heterotrophic communities. In some ecosystems, they are even considered keystone species since they can be the major food source for higher trophic levels. Chen *et al.* (2000) also proved that Zn and Hg concentrations in zooplankton can be an indicator for the internal concentrations of fish in lakes which might be consumed by humans. The metal concentration in the daphnids can than serve as an early warning tool. Apart from its relevance are daphnids easy to culture and they reproduce asexually under favourable conditions. This means that ones a clone has been separated from other individuals it will continue to create offspring genetically identical to the mother. This property makes it possible to perform different experiments on genetically the same organism. This trait can also be interesting for research around linking genetics with functional properties (Guan and Wang, 2006). Daphnids reproduce rapidly and have short lifespans. This makes it possible to do cost-effective multi-generational research which can lead to different insides than just single generation tests (Tsui and Wang, 2007). Lastly, if research were to be conducted in a real ecosystem these species are not commercially exploited, so this will not introduce a bias while studying fish populations can.

Intraspecific and interspecific interactions

Organisms will undergo interactions by sharing the environment with others. Such interactions, in theory, can happen with every species in the ecosystem. Some important interactions are for example predator-prey and sexual reproduction. There are two types that can be distinguished: population and community interactions. Population interactions refer to interactions between members of the same species at the same place and time while community interactions include all interactions between populations at the same place and time. In this section the definition of community will be more narrow: only interactions between different *Daphnia* species will be studied.

Daphnids are typically found in swarms in their natural habitat. This gives them an advantage as they can avoid predation, but at the cost of more competition for resources (Davies, 1985; Tessier, 1983). Higher densities do not only lead to higher competition, but also to higher con-

centrations of infochemicals (excreted by the daphnids themselfs) in the water which can affect their physiology and behaviour. Possible effects of crowding, without being related to food limitations, are reduced clutch size, reduced body size, reduced feeding rate, reduced growth rate etc. Burns (1995) also showed that some species under crowding conditions started to produce larger eggs, so the investment in reproduction (smaller clutch size, but bigger eggs) remained relatively constant, which is in line with other studies (Glazier, 1992). Apart from the above-mentioned effect can density also induce sexual reproduction (Barker and Hebert, 1990). These effects only occur at high levels of crowding, low levels seem to stimulate growth, body size and cluch size thus suggesting a curvilinear response (Burns, 1995).

In natural systems seldom only one *Daphnia* species is found in an ecosystem, most of the time multiple species coexist. Since the discussed crowding effects are not necessarily conspecific (species-specific), they can also affect other species (Matveev, 1993; Burns, 1995). The response to these infochemicals can differ between different species (and even clones) and there is likely a link with the body size of the *Daphnia* species (Burns, 2000). Large bodied organisms seem to react at higher levels of overcrowding than small-bodied organisms which could give them a competitive advantage when it is crowded, although this is not always observed (Frank, 1957). In Gliwicz and Lampert (1993) multiple *Daphnia* species of different size classes shared an aquarium. They found that in nutrient and food limiting conditions larger species dominated which the authors explained with the size-efficiency hypothesis. This hypothesis says that the larger zooplankton species filter apparatus can take up larger particles, so the exclusion would be due to food competition (Brooks and Dodson, 1965). Nonetheless, multiple species of different size can coexist in nature, so it is not just the size-efficiency hypothesis that plays a role. Possible explanations can be predation (planktivorous fish prefer larger organisms, while insect predators prefer smaller sized organisms), assimilation efficiency, abiotic factors etc. (Burns, 1969; Manca *et al.*, 2008; Houlahan *et al.*, 2007). This just shows how complex studying these interactions can be as even different clones of the same species can already differ in their responses (Burns, 2000).

2.4.2 Metal toxicity

Toxicokinetics

For *Daphnia*, aqueous uptake is possible via adsorption onto the exoskeleton. For zinc and cadmium, it was found that adsorption on tissue and exoskeleton were equally important (Yu and Wang, 2002). Uptake via the digestive tract originates mostly from food and suspended particles for filter feeders (Weltens *et al.*, 2000). The rate of desorption of the metal in the gut of daphnids will be determining for the dietary uptake. The assimilation efficiency of metals like zinc, cadmium and chrome range from 80% to 10% depending on the food density, while the assimilation efficiency for silver can be as low as 1% (Tsui and Wang, 2007; Komjarova, 2009). For most of the metals investigated in Tsui and Wang (2007), namely Hg, Cd, Ag, and Zn, aqueous uptake was the most important pathway for uptake whereas the important dietary metals are Se and MeHg. De Schamphelaere and Janssen (2004) showed that *Daphnia magna* only exposed to copper via dietary uptake did not seem to provoke any toxic effects, on the contrary, they saw it was beneficial for growth even for algae exposed to concentrations of 200 μg/l. Nonetheless, can foodborne toxicity enhance effects that come from waterborne toxicity.

Elimination of metals via reproduction can be significant for *Daphnia*. For example, up to 35 per cent of the maternal metal content can be transferred to the eggs (Tsui and Wang, 2007). For essential metals like copper elimination can also occur via special transport proteins which can actively excrete an oversupply (Burkhead *et al.*, 2009).

Metal mixtures uptake

When daphnids are exposed to different metals in a mixture some will have (partially) the same uptake path and will influence the uptake of each other. Komjarova (2009) examined the simultaneous metal uptake in green algae, zebrafish and *Daphnia magna* via stable isotopes of the metals: cadmium, copper, nickel, lead and zinc and the effects of sodium, calcium and pH. For *Daphnia magna*, cadmium and copper had the strongest suppressing effects on the other metals uptake rate except for lead. Next to suppressing effects were correlations found in uptake rate between nickel, zinc, cadmium (1) and copper, zinc (2). Because the slope of the uptake rates divided by the body weight were similar for *e.g.* Zn-Ni and Zn-Cd (in function of the external concentration) the author concluded that this could suggest that these pairs at least share a part of their uptake route. In other studies, it was already suggested that copper uptake was related to Na-metabolism, while zinc interferes with calcium uptake (De Schamphelaere *et al.*, 2007; Muyssen *et al.*, 2006). Although in Komjarova (2009) no effect of sodium was found on copper which could be due to the low copper concentration as she used environmental relevant concentrations. For zinc, a decrease in uptake was found with rising calcium concentration, but all uptake rates of all the metals were significantly affected by calcium (except for copper) probably due to competition for binding sites facilitating uptake.

Metal mixture toxicity

In Pérez and Hoang (2017) the mixture toxicity of cadmium and zinc was assessed for *Daphnia magna* in 21-day toxicity tests. The endpoints in this research were survival, reproduction, growth and metal accumulation. The cadmium concentration was constant across the experiments at 1.5 μg/l, while the zinc concentration varied between 10 and 200 μg/l. The used cadmium concentration already severely affected *Daphnia magna*. With increasing zinc concentrations, the negative effects first started to diminish at a concentration of 40 μg/l and from a zinc concentration of 160 μg/l it started to contribute to the toxicity. The less-than-additive effects can be explained by the lower cadmium concentrations in the body as the zinc concentrations got higher, clearly showing a dose-dependent mixture toxicity effect.

Nys *et al.* (2015) used 21-day reproduction tests to determine synergistic effects between zinc and nickel for *Daphnia magna*. They found that the mixture was non-interactive at low concentrations and synergistic at higher concentrations. More specifically, if both of the metals caused an effect over 20% on the reproduction, following the independent action model, synergism was observed. Concluding that synergism does occur in this mixture, but only at ecologically irrelevant concentrations.

Traudt *et al.* (2016) used 48-h survival tests to asses *Daphnia magna* toxicity to binary mixtures of cadmium, nickel, zinc and copper to assess synergism in these mixtures. Nickel caused (slightly) less-than-additive toxicity with cadmium and zinc, while in combination with copper greater-than-additive effects were registered. Once again showing that interactions can differ between the involved toxicants and that interactions can not be ignored studying mixtures. There are many more studies that discuss metal mixture toxicity for *Daphnia magna*, but this section was to illustrate that this type of research typically requires a case per case approach.

Effects of inter- and intraspecific interactions

Gust *et al.* (2016) tested if the population interactions described in section 2.4.1 have any effect on the toxicity response of *Daphnia magna* to lead and copper (no mixture). Not only did the experiments show that the 14-day LC_{50} was significantly lower in tests where populations were exposed compared to individual-level tests, but also a negative impact of lead on neonate (newborn) production was registered in the population tests. Lethal effects can thus be worse in

a population due to intra-specific interactions, but an effect on reproduction, for example, does not necessarily need to have a significant effect on population size (Pereira *et al.*, 2019; Vlaeminck *et al.*, 2021). This effect can be countered by reduced starvation in the toxicity tests. Next to these effects in chronic tests can crowding have an effect on recovery when exposed to pulses of toxicants, which is more relevant for pesticides (Liess and Foit, 2010). Much slower recovery of population structure and biomass was observed in high population density exposures to the pyrethroid Fenvalerate. Woo *et al.* (2020) did a similar observation that exposing organisms to toxicants at the peak phase of population growth (highest density, lowest food concentration) leads to slow recovery of the populations. Viaene *et al.* (2015) assessed next to intraspecific interactions also predation on *Daphnia magna* populations exposed to pyrene pulses. Predation seems to affect population size more then pyrene in this experiment. This was concluded from generalized linear models to test if the effects of pyrene and predation significantly explained size and structure of the population. They also observed that at high initial *Daphnia* concentrations combined with competition the effects of pyrene on *Daphnia* seemed to diminish. This observed effect is probably linked to the lower abundance of smaller life stages (juveniles) in the *Daphnia* population due to predation in the period before the first pulse. The juveniles are often more sensitive to toxicants and thus reduced deaths will be registered. This in combination with reduced predation because the *Chaoborus sp.* larvae (predator) might also be affected by pyrene.

2.5 Dynamic Energy Budget-Individual Based Model

2.5.1 Dynamic Energy Budget (DEB) theory

Dynamic energy budget aims to create a single framework which allows to be used to describe all living organisms. To achieve this it was important to base the theory on something all organisms have in common, metabolism: they take up resources out of their environments and use these to complete their life cycle (Jager, 2017). The model will thus consist of mass and energy balances on the individual level, different species will be modelled by using different parameters to alter the life history of the organism in the same or slightly altered model (for example autotrophs get energy from sunlight while heterotrophs will take up their food form their environment) (Martin *et al.*, 2012). The remaining part of this section will be based on Jager (2017), a book dedicated to modelling toxicant effects using DEB theory.

The major assumptions for this theory are: an organism consists of two compartments: structure and reserve, a relationship exists between volume and surface area of the organisms, metabolic processes like growth and assimilation of food are uncoupled, assimilated food is first becoming reserves before allocating to a metabolic process, strong homoeostasis (reserve and structure do

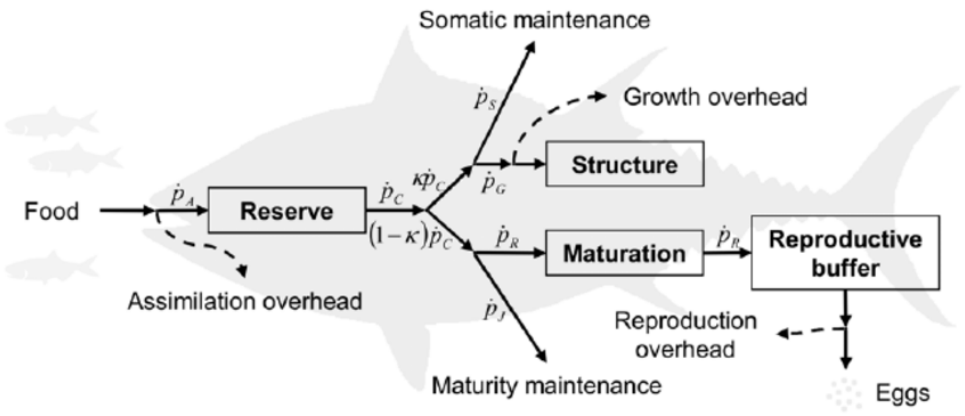


Figure 2.5: Schematic representation of a dynamic energy budget concept (Jusup *et al.*, 2011)

not change in composition) and weak homoeostasis (at a constant food concentration the ratio of the reserve to structure will come to an equilibrium).

Figure 2.5 shows the energy flows considered in DEB theory. The energy in the food cannot be fully used towards other metabolic processes thus only a certain fraction will be useful. The useful fraction will become reserves while the other fraction is lost. As discussed above the organism will be represented by two components: reserve and structure. Reserve is strictly defined as not maintenance costs. Structure does., while structure is everything else in the organism and requires energy for maintenance. This reserve will be mobilized towards two branches: structure and maturation. The allocation is in the standard model in fixed proportions called the kappa-rule. A fraction κ is allocated to structure and (1-κ) is allocated to maturation. Since the ratios are fixed there is no competition between structure and maturation for resources within the organism. Both of these energy fractions before being used for growth or reproduction are first allocated towards the maintenance of the structure and maturation component. Typical examples for maintenance of structure are muscle activity and creating concentration gradients across membranes. The remaining energy in the structure branch can be used for growth. What happens with the rest of the energy in the maturation branch depends on the life stage of the organism. In the standard DEB theory, three life stages are considered: embryo, juvenile and adult. In the embryo stage, the organism does not feed but uses the reserves of the mother, while in the juvenile stage it does feed but is not ready to reproduce itself. Juveniles need to invest a certain amount of energy in maturation before you can reproduce and thus become an adult. After turning into an adult, it is possible to use the maturation energy to store in a reproduction buffer which can be used to form offspring. For offspring production, it is important that when the organism reproduces that not half an egg/embryo is produced, only round numbers. Of course are modifications to the model (structure) possible if needed. For example some organisms lay only one egg at the time while others will lay eggclusters.

To use this framework in an ecotoxicological context, said energy flows, energy pools and mortality will be influenced by the toxicant. Effects on sublethal processes can explain part of the observed effect on mortality *e.g.* decrease in assimilation flux can lead to starvation and eventually death. Where and how the toxicant will exert an effect is called the PMoA (Physiological Mode of Action) of the toxicant.
Sublethal processes might not explain all of the observed effect on mortality, in which we need to model a part of the effect by a direct relationship between damage and survival probability. An example of a PMoA is somatic maintenance, a toxicant can lead to in increase of somatic maintenance which will lower the energy flow to structure. This will slow down growth. Other possible effects could be reduced food assimilation, increased energy cost for reproduction, change of energy allocation (κ) etc.
First of all, depending on the concentration, will the toxicant induce extra mortality. When the toxicant also has sublethal effects, it will act on a certain energy flows, which is called the PMoA (physological mode of action) of the toxicant. An example of a PMoA is somatic maintenance, a toxicant can lead to in increase of somatic maintenance which will lower the energy flow to structure. This will slow down growth and eventually lead to lower reproduction because ingestion rate scales with surface area. This will lead to a decrease in reserves compared to a non-exposed organism and thus slow down reproduction. Other possible effects could be reduced food assimilation, increased energy cost for reproduction, change of energy allocation (κ) etc.

2.5.2 Individual Based Modelling (IBM)

Individual-based models are alternatively known under the more generic name; Agent-Based models (ABM). In ABM's the modelled entities do not have to be individuals (like IBM) but could also be groups, organizations etc. The general idea of these models is that for every agent/individual in the studied area a set of state variables and characteristics are calculated and stored. This way every agent can have its own state and interactions with other agents and the environment. Since the existence of these type of models, they have been widely used in ecological modelling because of the need for implementation of local interactions (spatially explicit), adaptive behaviour (changes with for example life stages and environmental drivers), natural variability (size, ageing) and more to gain extra insights in ecosystems by making the models more complex and computationally heavy (Judson, 1994; Grimm, 2019).

As discussed, DEB tries to describe the use of resources in organisms by using mass and energy balances on the scale of one individual. It will translate environmental characteristics into individual performance (growth, reproduction...). This is where IBM can come in handy as it will allow using these individual traits to translate to population traits (Martin *et al.*, 2012). By modelling the individuals in the same environment competition will automatically occur like for a foodsource. Other population effects like density-dependent filtration rate (food uptake will change) or effects on other levels like predator-prey can be added fully in the function of the question that needs to be answered. This is also one of the major advantages of this combination, the fact that it is so versatile and has potential to by universally applicable (all species use same DEB-model only parameters change) (Martin *et al.*, 2012). Problems that can occur with IBM's are *e.g.* overparameterization, computation times, transparency, accessibility etc.

2.5.3 DEB-IBM in ecotoxicological research

In this last section, a couple of cases where a DEB-IBM has proven to be useful in ecotoxicology will be discussed. Pereira *et al.* (2019) is a beautiful example of this, in previous studies, they found that the reproduction of *Daphnia magna* is severely affected when exposed to nickel and 15°C. This would lead you to believe that the population of daphids would decrease when exposed to these conditions, but this was not the case. They found only minimal effects on the population level and tried to predict these results using DEB-IBM. The PMoA that best described the effects of nickel was growth costs. They found that the model predicted this outcome correctly and upon closer examination of the modelled data indications were found that this might be due to reduced starvation that compensates for the nickel mortality. This was checked by looking at the cause of death of the individuals predicted by the DEB-IBM. In Vlaeminck *et al.* (2021) more or less the same was tested as in Pereira *et al.* (2019), but now for a mixture of copper and zinc. They also used a DEB-IBM calibrated with individual level endpoint data to compare the results with a population level test. On the physiological level, they assumed that the metals would act independent of each other, thus the AI model was used. This allowed for the effects to be calculated independently and use of single substance toxicity data. It was found that the DEB-IBM could explain trends observed in the population experiment like the peak in the early stage of the experiment due to food abundance. Only the model used to model high zinc and copper concentration underpredicted the initial peak, but this did not seem to affect the model prediction a later timepoints. This showed that individual-level, single substance data can be used to predict mixture effects on a population level.

This framework has also been tested for other species and organic stressors like in David *et al.* (2020). In their research, they focussed on the effects of a pharmaceutical mixture in a mesocosm experiment. The five pharmaceuticals were: diclofenac, carbamazepine, irbesartan, acetaminophen and naproxen and the species of interest were three-spined stickleback. They

found that even at concentrations 100 times above environmental concentrations the population dynamics of the fish seemed to be minimally affected, while survival of female fish declined significantly. the other trophic levels did not see any negative effects. They were able to reproduce the observed decline in survival for female fish and also found a minimal effect on the population dynamics wit DEB-IBM. This indicates that there is some sort of compensation mechanism for the observed mortality in female fish which they think is related to a decrease in density-dependent mortality.

These examples clearly show that the use of mechanistic models can be beneficial to the understanding the influence of stressors as individual energy fluxes are modelled in comparison to statistical methods like dose-response curves (Vlaeminck *et al.*, 2021). Also, in mixtures, these type of models will allow assessing the effects of the individual chemicals although this would be influenced by the assumptions of the mode of action (IA and CA). Pereira *et al.* (2019) and Vlaeminck *et al.* (2021) showed that the traditional ecotoxicological data based on individual-level endpoints can be used to calibrate DEB-IBM models and after validation with population-level data it might be possible to use DEB-IBM as a prediction tool. This can lead to more ecological realism and better suits the aim of ecological risk assessment; protection of populations and communities, this with only a minimal need for extra data.

DEB-IBM's have proven that they can be useful in ecotoxicology, but some things require extra attention which can improve accuracy and prediction capabilities. So did Martin *et al.* (2013a) find that in their implementation the resource-dependent mortality of *Daphnia magna*, which is a function of the body size, does not accurately describe reality. In this case, it might be useful to estimate these effects form population experiments. By using this size-dependent submodel they were able to predict effects at other food levels and initial conditions.

Furthermore, to parametrise a DEB-IBM for ecotoxicological use, toxicological data is used. EC_x and LC_x values from a single point in time can be used, but PMoA effects on growth and reproduction, in reality, can change over time (EC_x is different in a 14- versus a 21-day test). A possible solution for a reproduction test could be to use all the measurement points in reproduction tests instead of only using the calculated EC_x. Another problem is that reproduction tests do not give an indication of which PMoA is affected by the toxicant as multiple PMoA's can affect reproduction output like a decreased feeding rate or higher embryonic mortality. This could be solved by measuring growth at the same time as reproduction, so it is possible to differentiate between the two effects (Martin *et al.*, 2013b). It is important as the effects of the chosen PMoA can have a profound effect on the outcome. (Martin *et al.*, 2014) did in silico experiments and found that two PMoA's that have the same effect on the individual level could lead to very different population responses from nearly no effect to complete extinction. It is possible to parametrise the DEB-IBM for the same reproductive output using different PMoA's for example. This indicates that only testing reproduction on the individual level is insufficient to use DEB-IBM's for extrapolation in risk assessment. Extras research into the PMoA's of toxicants might be useful to get the full potential out of DEB-IBM.

Lastly, due to different PMoA's and their effects on the population level is it possible that some endpoints do not seem to change under stress, for example, if a toxicant increases embryonic mortality (Martin *et al.*, 2014). In this case, the total biomass of the population might be relatively unaffected, but there will be a change in population structure (ratio adults over juveniles will increase). This can have significant effects on how to population responds to stress or predation and indicating that different endpoints are best considered in the assessment.

2.6 Goals of this thesis

The lack of real-life ecosystem complexity in traditional ecotoxicology for legal purposes requires assessing if the simplifications can be applied without losing valuable information on the systems. That is why in this thesis data of binary communities of *Daphnia* spp. exposed to metal mixtures will be analysed to assess the effects on the structure and size of the communities. By including toxicant and species interactions a more realistic view on toxicity will be assessed. On the other hand is the study not too complex like a small ecosystem with different species from different trophic levels, where a lot of interactions happen. This makes it often hard to prove which interactions are affected by the stressor and why certain species go extinct when exposed to toxicants (Van de Perre *et al.*, 2016). The metal mixture exposure experiments to *Daphnia* communities thus will have some degree of ecological complexity while still sufficiently simple.

Next to the microcosm study, similar experiments (binary communities exposed to toxicant mixtures) were repeated *in silico*. For the modelling, a Dynamic Energy Budget-Individual Based Model (DEB-IBM) was used. Because this is a relatively complex setting (multiple species, multiple toxicants) part of the goal for this thesis was to try and understand why the model behaves in certain ways. So far, there is no comprehensive study that demonstrates how the toxicity input parameters for a model like this relate to the output parameters. Secondly, A setting was created so that the modelled species were identical in their DEB-parameters and only differed in their sensitivity to toxicants. This way it was possible to isolate community traits related to the toxicity and analyse how they influenced the community level endpoints. This would not be possible in an experimental setup as changing species in the communities would not only change how the community is affected by the toxicants due to different tolerances for stress, but the species interactions would also change. So, this would confound one trait with other traits which makes it hard to conclude if the observed effects can be related to a specific trait. All of the community traits that will be defined in this thesis will use relatively simple ecotoxicological data like individual level LC_{50} values which is data that is more abundantly available than data on higher levels of organisation. In a legislative context most of the risk assessment is still being performed with acute and chronic single species testing. With the defined traits the goal is to try and explain some of the variability seen in the response to mixtures in a community context. The outcome of the community trait analysis for the modelling was coupled back in a qualitative way to the microcosm study to see if the same trends were observed.

3 | Materials & Methods

3.1 Pattern oriented modelling with DEB-IBM

All DEB-IBM modelling was done in Julia 1.5.2. The model implementation itself was not part of this thesis and the modifications described in section 3.1.1 were not self-implemented.

3.1.1 DEB-IBM model

The DEB-IBM model is mainly based on Pereira *et al.* (2019) of which some of the modifications compared to Jager (2017) and Martin *et al.* (2012) will be highlighted together with the modifications required to transform this model into a community model. In general, this model calculates the number of individuals per species that is present at a certain timestep in the community.

The first modification is related to the starvation response. When an animal is starving, the maintenance costs cannot be fulfilled anymore. In this model, changing the model equations for starvation was not considered. This will allow for the physical body size to decrease, together with the reproduction buffer for adults and the maturation buffer for juveniles. Death by starvation can only occur when the volume of the organism has shrunken to a certain percentage of the mamimum observed organism volume. When this condition is met, the individual has a fixed chance to die every timestep, given by equation 3.1, where the starvation constant is a fixed value.

$$P_{starvation} = 1 - (1 - starvation\ constant)^{\frac{1}{timestep}} \tag{3.1}$$

To make the model a better fit for zooplankton, a moulting state variable is included, which tracks the time since the last moult since this is how daphnids grow. The moulting is not used to model the discontinuous growth, but to incorporate the fact that produced eggs are typically carried in the brooding chamber and are released at moulting. In the model, it is assumed that the eggs hatch and become juveniles upon release. This is implemented by calculating the number of eggs that can be produced at moulting from the reproduction buffer and rounding up to obtain the amount of new juveniles in the current timestep.

The only form of inter- and intraspecific interactions included in the model is food competition. The order in which individuals are called is randomized at the beginning of every time step and thus determines the order in which they feed. The food density in the medium is updated immediately after an individual feeds. If the food concentrations are high enough the organisms can eat *ad libitum*. At lower food concentrations, the ingestion rate for the individuals will be lower.

The modelled organisms in this study can die due to three reasons: starvation (see first paragraph of this section), age (Jager, 2017) and due to the toxicant exposure. First of all, only lethal effects of the toxicant will be considered in this study to minimize complexity and to ease the relation of toxicity parameters to effect on a community level. This means that a higher concentration of a toxicant will directly lead to a higher chance of death, but will not have any other effect on the organism. The implementation of stress induced death is based on the reduced General Unified Threshold model of Survival (GUTS) described in Jager and Ashauer (2018). GUTS is an over-arching framework to which most of the ToxicoKinetic-ToxicoDynamic (TKTD) models belong. The reduced model can be represented as follows and incorporates lethal toxicity effects

on the physiological-level:

$$\frac{dD_{w,i}}{dt} = k_{d,i} * (C_{w,i} - D_{w,i}) \tag{3.2}$$

$$h_i = \frac{h_{max,i}}{1 + \left(\frac{D_{w,i}}{ED_{50,h,i}}\right)^{-\beta_{h,i}}} \tag{3.3}$$

$$P_{\text{survival stressor}} = \exp(-\int_0^t h_i(\tau)\, d\tau) \tag{3.4}$$

The external concentration $C_{w,i}$ (μg/l) is related to scaled damage ($D_{w,i}$ in μg/l) by the dominant rate constant $k_{d,i}$ (equation 3.2) with i standing for metal i. To calculate the hazard rate from scaled damage, the Hill equation (equation 3.3) is used in which three parameters are defined: $h_{max,i}$ (-), $ED_{50,h,i}$ (μg/l) and $\beta_{h,i}$ (-). $h_{max,i}$ is the maximum hazard rate, $ED_{50,h,i}$ the scaled damage that leads to a hazard rate which is 50% of the maximum hazard rate and $\beta_{h,i}$ is the slope of the log-logistic function. This equation deviates from the proposed framework by Jager and Ashauer (2018). From this hazard rate for a certain time interval, a survival probability can be calculated using equation 3.4. Death is thus handled as a stochastic process and these equations in a mixture exposure are independently constructed for all the toxicants. This way, independent action (IA) is assumed on the physiological level. In the model, the accumulation of toxicants starts when the juveniles are released from the brood chamber.

3.1.2 Modelling approach

The model was used to simulate 100 days with time steps of one hour. The sensitivity to a mixture of three toxicants was changed for the same binary community, by sampling different combinations for the three parameters in equation 3.3: h_{max}, $ED_{50,h}$ and β_h and k_d from equation 3.2 in combination with rearranging these parameters between the species in the community and changing the exposure concentrations.

The chosen DEB-parameters represent *Daphnia magna* and are based on the Add-my-pet database Kooijman and Gergs (2016) in combination with the estimated food-dependent parameters of Hansul *et al.* (unpublished) (food-dependent parameters in table 3.1). For both species in the binary community, the same DEB parameters were chosen. The DEB-parameters are not fully equal between two species in a community as a random factor will influence some of the parameters, like the maximum surface-area-specific ingestion rate, energy reserve at birth, maturation threshold, etc.

All four toxicity parameters are sampled from uniform distributions, with k_d between 1 and 8, h_{max} between 0.1 and 1, $ED_{50,h}$ between 1 and 100, and β_h between 3 and 15. The ranges of k_d, h_{max} and β_h are based on the distributions in the supporting information of Hansul *et al.* (unpublished) found for *Daphnia magna* exposed to copper, nickel and zinc (table 3.1). For the $ED_{50,h}$, a range of two orders of magnitude is chosen based on realistic differences between sensitivities for individual-level endpoints between different *Daphnia* species (Dalla Bona *et al.*, 2014; Mano *et al.*, 2020; Santos Medrano and Rico-Martínez, 2018). Note that the absolute values of the $ED_{50,h}$ and the external concentration do not matter in this simulation study, since only hypothetical scenarios are modelled. Only the difference between the $ED_{50,h}$ and external concentration will determine the severity of the toxicant effects. Therefore the $ED_{50,h}$ values were uniformly sampled in a range from 1 to 100. For the four TKTD-parameters six values needed to be sampled (two species and three toxicants). Once the parameters were assigned to a toxicant and a species, only the $ED_{50,h}$ was allowed to be exchanged between the

Table 3.1: Food dependent and TKTD parameters used in the DEB-IBM modelling. Based on Hansul *et al.* (unpublished).

Parameter	Unit	Value
Food dependent parameters		
p_{Am} (maximum specific assimilation rate)	$\frac{J}{cm^2 * day}$	260.4
F_m (filtration rate)	$\frac{J}{cm^2 * day}$	10.0
$\kappa_{E,X}$ (assimilation efficiency)	$\frac{J}{algal\ cell}$	3.7e-6
TKTD parameters		
k_d	$\frac{1}{day}$	Uniform(1-8)
h_{max}	-	Uniform(0.1-1)
$ED_{50,h}$	*e.g.* μg/l	Uniform(1-100)
β_h	-	Uniform(3-15)

species to create new exposure scenarios. The assigned parameters were used to create two types of sensitivity correlations on the physiological level. A positive correlation by exchanging the $ED_{50,h}$ values between the two species for the same toxicant, so that a certain species would consistently have the highest $ED_{50,h}$ for all the toxicants compared to the other species (figure 3.1). The negative correlation is constructed from the positive correlation by determining for which toxicant the difference of the natural logarithm between the $ED_{50,h}$ is the largest, which is similar to the biggest percentage difference. These $ED_{50,h}$ values were exchanged between the two species. This sampling and rearranging was done 100 times in fourfold replicates (for a total of 800 simulated communities).

From the TKTD-parameters the exposure concentrations were calculated. This was done using the same approach as the microcosm study (equation 3.9 and 3.10), but on the physiological level ($ED_{50,h}$ instead of LC_{50}). This approach would lead to equitoxicity, but on the physiological level. So, the community $ED_{50,h}$ is calculated for every toxicant by taking the geometric mean of the $ED_{50,h}$ values for the same toxicant between the species for example $ED_{50,h,species\ 1,toxicant\ 1}$ and $ED_{50,h,species\ 2,toxicant\ 1}$. Three concentrations were chosen based on these geometric means as follows: equal to the geometric mean, geometric mean divided by 2 and 4 for every toxicant. This means that for the three toxicant mixtures, the total sum of toxic units (TU) is equal to respectively 3 (1 TU per toxicant), 1.5 (0.5 TU per toxicant) and 0.75 (0.25 TU per toxicant).

The same approach was also used for the same set of parameters sampled before, but the concentrations were not based on the contributions calculated using the geometric mean of the $ED_{50,h}$ values, but using the 21-day LC_{50} values calculated using formulas 3.2 to 3.4.

In the microcosm study, the sensitivities expressed as LC_{50} values were often close to each other (see results table 4.2), so another simulation experiment was conducted with a uniform sampling of $ED_{50,h}$ values ranging from 1 to 2 (which implies only the double and not the 100-fold difference) and a smaller β ranging from from 3 to 8. k_d and h_{max} were sampled from the same distributions as previously described. This simulation was in all other ways identical to the previously discussed method except that not 100 parameter samples were chosen, but only 60. Concentrations were also calculated using the 21-day LC_{50} values. This is done because the calculated LC_{50} values for the three species were at maximum a 5-fold difference (and in most of the cases even 2-fold) when comparing the sensitivities between the species for the same toxicant (table 4.2).

The same toxicity parameters and concentrations were, next to the described mixture exposures,

also used to simulate the exposure to each of the single metals separately. From this data, it was possible to calculate the independent action predictions based on community endpoints. A control run (concentration levels for all toxicant equal to 0) was also performed to see how the community behaves in unstressed conditions.

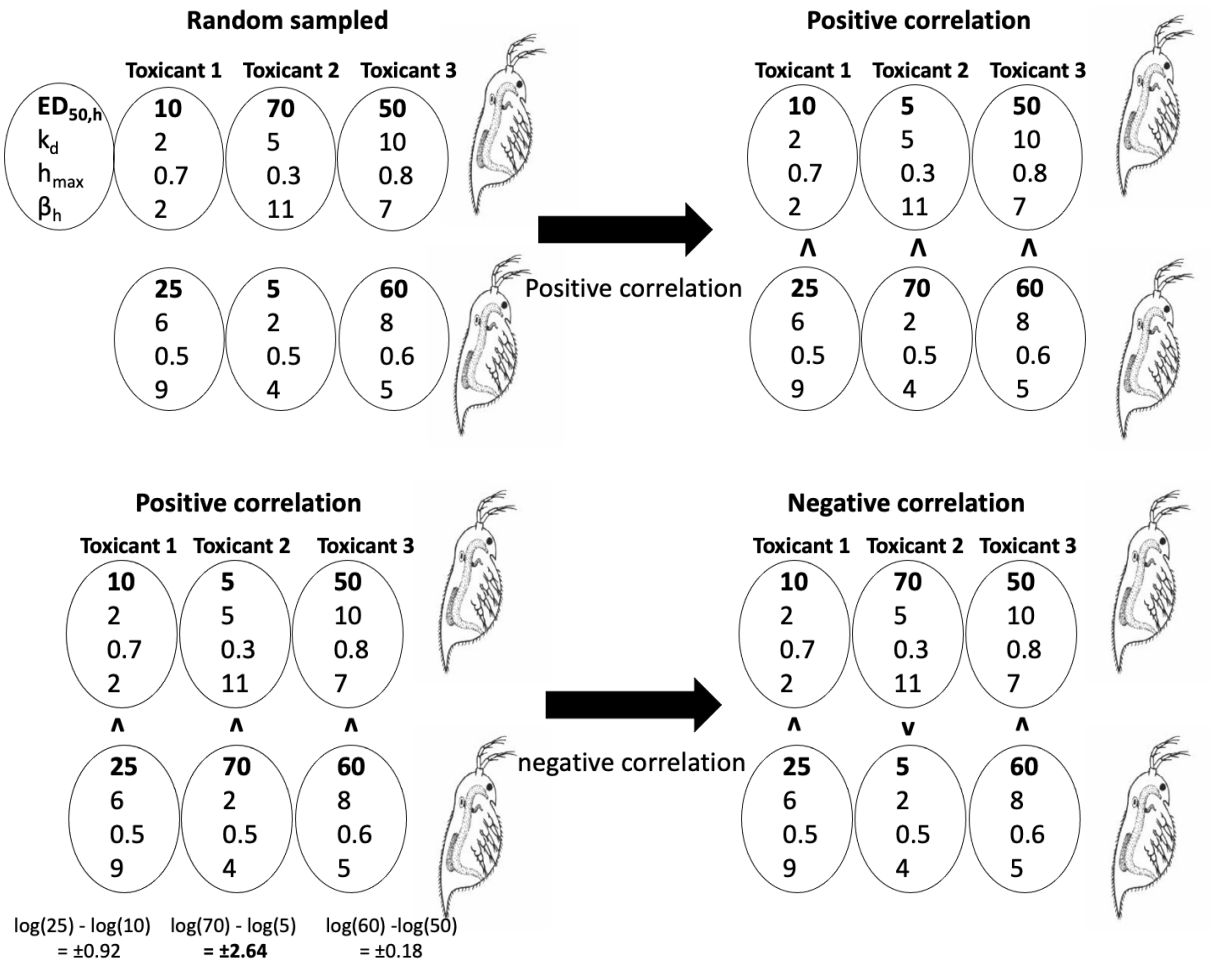


Figure 3.1: Visual representation of changing the sensitivity correlation with the circles containing the TKTD parameters for the different toxicants (*Daphnia* figure by Deken (2005))

3.1.3 Community endpoints analysis

Only every two days, the number of individuals per species was recorded as a trade-off between resolution and data storage. From this data, the community size and relative abundance for both species could be calculated for the control, the single metal exposures and the mixture exposure.

The main part of the analysis was focused on the situation at equilibrium and to create a more robust analysis the median of the last 14 days (7 data points) was calculated for the population sizes in the community and used as an equilibrium measure.

The relative response, compared to the control, of the community size exposed to the mixture was compared to the independent action prediction for the community size. The following ratio

was used:

$$\frac{\text{Relative response of the community size to the control}}{\text{IA predicited mixture effect on community size}} = \frac{\frac{\text{Community size mixture}}{\text{Community size control}}}{\prod_{i=1}^{3} \frac{\text{Community size toxicant i}}{\text{Community size control}}} \quad (3.5)$$

3.1.4 Evaluating equitoxicity

To assess if the concentration choices lead to equitoxicity on the community level, the equilibrium community sizes relative to the control were assessed. The coefficient of variation (mean divided by standard deviation) was calculated from the three relative community sizes, compared to the control of the single metal exposures. This coefficient indicates if the three relative community sizes are similar or not.

3.1.5 Community traits

Sensitivity correlation

The sensitivities for different toxicants can be plotted in a graph with the different species on the axis like figure 3.2 illustrates for two species. On the x- and y-axis the $ED_{x,h}$ values are represented for two species and a plotted point represents the $ED_{x,h}$ values for species x and y for the same toxicant. If the data point lies on the 1:1 line (x=y) both species have the same sensitivity for that toxicant, above the 1:1 line species x is more sensitive than species y and the other way around.

A positive sensitivity correlation is defined as a case where, based on the $ED_{50,h}$ in this thesis, a clear (relative to the other species) sensitive and insensitive species can be identified for the studied toxicants. For this simulation study if all three of the data points are above or below the 1:1 line the correlation was considered positive. A negative correlation was constructed by switching one pair (same toxicant, different species) of $ED_{50,h}$ parameters. In a negative correlation, the determination of a sensitive and insensitive species is more nuanced as both have, relative to each other, at least one toxicant that they are more sensitive to.

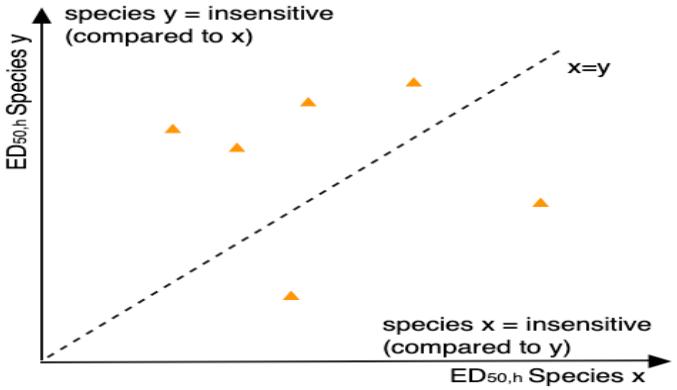


Figure 3.2: Relative response to IA of the community size at equilibrium with +: positive correlation and -: negative correlation

Toxic unit ratio

To calculate per species to what toxic unit amount they are exposed not the geometric mean is used (like in the concentration calculation), but just the LC_{50} of the species for the specific toxicant. In a mixture the toxic units can be summed to calculate to total stress of the mixture on the species following:

$$\frac{\sum_{n=1}^{3} TU_{i,species\ x}}{\sum_{n=1}^{3} TU_{i,species\ y}} \quad \text{with} \tag{3.6}$$

$$TU_{i,species\ x} = \frac{C_i}{LC_{50,i,species\ x}} \tag{3.7}$$

with $TU_{i,species\ x}$ the toxic unit (-) species x is exposed to for toxicant i, C_i the concentration (in μg/l)of toxicant i and $LC_{50,i,species\ x}$ the 21-day LC_{50} value for toxicant i exposed to species x (in μg/l). Species x is always the species exposed that has the lowest sum of toxic units so the ratio is always between 0 and 1.

Survival probability ratio

Independent action is assumed on the physiological level, so the toxicants independently affect the species mortality. This makes it possible to multiply the survival probabilities of the different single toxicant exposures to see how much mortality due to the toxicant is expected for a certain time horizon. The time horizon chosen was 21 days. The ratio was calculated as follows with i referring to toxicant i:

$$\frac{\prod_{n=1}^{3} P_{survival,i,species\ x}}{\prod_{n=1}^{3} P_{survival,i,species\ y}} \tag{3.8}$$

Species x is always the species with the lowest survival probability so the ratio is always between 0 and 1.

3.2 *Daphnia* microcosms

The experimental design, image processing and speciation modelling were not part of this thesis, but are described as I used some of the data in this thesis. I helped during the biological monitoring to gather the data. The experiment is part of a bigger project that includes full model calibration and validation, but the data will also be used in this thesis to see if community traits influence the response of metal mixture toxicity.

3.2.1 Test design

Three binary communities of *Daphnia* were exposed to single metals and mixtures of these metals to assess the effects of the metals on the community in a 56-day (8-week) experiment. A 56-day exposure time was chosen to ensure that at the end of the experiment the community structure and size would be close to equilibrium. The three communities were: *Daphnia magna* and *Daphnia pulex* (C1), *Daphnia magna* and *Daphnia longispina* (C2), *Daphnia pulex* and *Daphnia longispina* (C3) (table 3.3). The metals considered in this experiment were: zinc, nickel and copper in two concentrations for the single metal exposures and two for the mixture exposure. The mixture contained all three of the metals in the concentrations they were tested at in the single metal exposures, so 9 treatments were considered and the nominal concentrations are in table 3.2. These concentrations were chosen to create a set-up that is an approximation to equitoxicity on the community level. Equitoxicity is achieved when the contributions of the

Table 3.2: Nominal exposure concentrations for the different treatments in the microcosm study

	Copper (in μg/l)	Nickel (in μg/l)	Zinc (in μg/l)
Control	0	0	0
Copper 1	18	0	0
Copper 2	35	0	0
Nickel 1	0	53	0
Nickel 2	0	105	0
Zinc 1	0	0	88
Zinc 2	0	0	175
Zinc 1	18	53	88
Zinc 2	35	105	175

Table 3.3: Community composition in the microcosm experiments

	Species
Community 1 (C1)	*Daphnia magna* and *Daphnia pulex*
Community 2 (C2)	*Daphnia magna* and *Daphnia longispina*
Community 3 (C3)	*Daphnia pulex* and *Daphnia longispina*

different metals to the toxicity are equal to each other. This approximation was based on 21-day LC_{50} values. Contribution to toxicity is calculated as:

$$z_{i,x} = \frac{TU_{i,x}}{\sum_{i=1}^{N} TU_{i,x}} \quad \text{with} \tag{3.9}$$

$$TU_{i,x} = \frac{C_i}{LC_{i,x,geo\ mean}} \tag{3.10}$$

with $z_{i,x}$ the contribution of metal i on the x per cent effect level, TU the toxic units of metal i for the x per cent effect level, N the number of metals, C_i (μg/l) the exposure concentration and $LC_{i,x,geo\ mean}$ (μg/l) the geometric mean of the respective LC_x values from the species per metal i. To calculate the toxic units for the different metals, the geometric mean of the 21-day LC_{50} for the 3 species were used. The design is equitoxic when all the contributions ($z_{i,x}$) are equal for all the toxicants.

All treatments were tested for the 3 communities in fourfold replicates, so in total 108 communities were exposed (9 treatments, 3 communities, 4 replicates). The communities were in 1-litre food-safe polypropylene containers (Avamoplast, Lokeren, Belgium) filled with half a litre of medium. The used medium was a COMBO (Kilham *et al.*, 1998) medium, but modified by adding 55 mg $CaCl_2{\cdot}2H_2O$ l^{-1}, 55 mg $MgSO_4{\cdot}7H_2O$ $l^{-1}$and 1 mg H_3BO_3 l^{-1}. During the exposure, the beakers were covered with a transparent plastic plate to minimize evaporation and contamination. The different communities were randomly placed in the exposure room. Each community consisted of 6 neonates (0-24 h age) per species per experimental unit randomly selected on day 0. The single species cultures had been kept under the same conditions for more than a year and fed *ad libitum* with *Raphidocelis subcapitata* (also known as *Pseudokirchneriella subcapitata*). The algae were frozen at -80°C and defrosted on the day of feeding. For administering, the defrosted algae are diluted in the control medium. Freezing reduces algal growth in the microcosms, so it creates a better picture of how much food the daphnids have available. It will also avoid a decrease in pH as photosynthesis decreases the CO_2 content in the water which leads to an increase in pH and corresponding changes in metal bioavailability. The test media

and conditions were identical to the cultures except for the addition of natural DOC (4 mg l^{-1}) to test media instead of EDTA. Natural DOC had been sampled by reverse osmosis in November 2018 from the Schwarzbach stream (East-Belgium, 50.5210522N, 6.205860E). Metal-spiked test media were allowed to equilibrate for at least 48h before use. The communities were constantly exposed to a temperature of 20°C in a climate-controlled room and a light-dark cycle of 16:8 hours.

3.2.2 Biological monitoring

Feeding

Every day the communities were fed with the same algal mixture as the cultures, but not *ad libtum*. Approximately 2 mg C l^{-1} d^{-1} was added to the replicates. Here the algae were also first frozen before administering as previously described.

Image collection

Starting from day 0 every 4th and 7th day the *Daphnia* communities were removed from the medium by using a 150 μm sieve which is small enough to retain the smallest animals. The 1-litre beakers in which the communities are exposed are cleaned with a paper towel to remove all the sunken algae and debris on the bottom. Refilling the beakers is done with 75% of the old medium, that was in the beakers and 25% of the newly made medium. When the medium removal took place the communities were put in petri dishes with the new medium, to take images of the communities with a digital camera (Canon EOS200D, Canon, Tokyo, Japan) on a tripod on top of a lightbox. 5 photos were taken in a series with a 5 second time interval. The tripod was always on the lowest stand while the main zoom of the camera was at the maximum. So, the photos are always roughly on the same scale which is important for the image analysis. Afterwards, the communities were put in the beakers with the renewed medium (75% old medium/25% new medium).

Medium sampling and analysis

Every 7 days a sample was prepared per exposure treatment for the old (medium in which the communities were living) and the new (unused) medium to determine the major ion and metal concentrations. For the new medium, 10 ml was sampled for the control, all the single metals and mixture concentrations. For the old medium, a pooled sample of 10 ml was taken per exposure treatment (*e.g.* Zinc 1) for analysis. The samples were passed through a 0.45 μm filter (Acrodisc, Pall Life Sciences). This was also done every two weeks for TOC (Total Organic Carbon) measurements. The samples to determine the metal concentrations and major ions were acidified with concentrated nitric acid to a final concentration of 0.14 mol/l and analysed in an ICP-OES (Inductive Coupled Plasma-Optical Emission Spectroscopy). On the same occasions, pH was measured in old and new media. The TOC analysis was done using TOC-L (Shimadzu, Kyöto, Japan).

Day 56

On the 56^{th} day, the dry biomass of all communities was determined. This was done by first manually picking out the dead animals, debris and resting eggs from the communities to ensure almost all of the weight comes from the living daphnids. Afterwards, the living daphnids were vacuum filtrated and repeatedly rinsed with tap water. The rinsed communities were transferred to a small piece of parafilm paper and dried for at least 48h at 60°C. Dry mass was then determined with a fine scale.

3.2.3 Image processing

The species-specific individual counts were inferred with image analysis, using two convolutional neural networks (CNN), which had previously been trained to differentiate between daphnids and non-daphnid (debris, resting eggs, etc) and to differentiate between the three species. the validation accuracies of the two CNNs across all object sizes and species were 94% (differentiation daphnid vs non-daphnid) and 86% (differentiation between species).

By having a series of images from the same stationary camera position it was possible to determine if the individuals were immobilised or not. If an individual did not move it was not counted by the image analysis. The convolutional neural network to differentiate between the *Daphnia* species always classified all of the species (in this case 3), even though only 2 species are present in each of the communities. This misclassification was solved by splitting the individual count of the species that should not be present over the 2 species that are present. The ratio to allocate the individual to the other species is determined from the confusion matrix acquired during the testing phase of the model (appendix A.1). Next to the individual count is also the pixel area per species registered.

3.2.4 Speciation modelling

Speciation modelling was used to convert the measured metal concentrations with ICP-OES to free ion activities. For this WHAM VII was used (Tipping *et al.*, 2011). The input was the nominal total SO_4, CO_3, Cl and the measured DOC, pH, dissolved Na, Mg, K, Ca, Ni, Cu, and Zn. The DOC is entered in WHAM as fulvic acid.

3.2.5 Community endpoints analysis

For the analysis, three endpoints on the community level were considered: dry biomass (on 56th day), community size (based on individual count) and community structure (calculated as relative abundances based on the individual count). For the last two endpoints, the last week of the experiment was considered in the analysis (day 49, 53, 56).

The single metal exposed community data will also be used to calculate the independent action predicted effect of the mixture and because of this both single metal and mixture treatments needed to be tested at the same time (Cedergreen *et al.*, 2007; De Laender *et al.*, 2009). Simultaneous testing is needed because the test population used can vary in sensitivity to the toxicants over time which can be misinterpreted as an effect of the toxicants.

3.2.6 Community traits

Sensitivity correlation

Survival data of the single species in function of the concentration was used for *Daphnia magna*,*Daphnia longispina* and *Daphnia pulex* to fit concentration-response curves for the determination of the 21-day LC_{50} values for the different metals. Each individual was in a separate beaker and for every beaker and concentration level, there were 10 replicates. For copper, nickel and zinc were 5 concentration levels defined and the dissolved concentrations were measured. From the dissolved concentrations were the free ion activities calculated using speciation modelling. For every concentration was the mean and standard deviation determined. I was not involved in the gathering of these results.

The fitted concentration response curve is a log-logistic curve of the following from with i indicating the toxicant:

$$P_{mortality,i} = \frac{1}{1 + (\frac{C_i}{LC_{50,i}})^{\beta_i}} \tag{3.11}$$

To fit the concentration response curve a Monte-Carlo simulation was performed. So, for every concentration level, the concentration was randomly sampled from the respective normal distribution, but not allowed to be negative. From this fit, the LC_{50} and β were obtained. The sampling of the concentrations was repeated 1000 times for all the treatments. From the estimates of the LC_{50} values, the sensitivity correlation was determined as described in section 3.1.5. So, a positive correlation means that there is a species that is insensitive to all of the metals compared to the other species in the community and a negative correlation is a community where both species are sensitive and insensitive depending on the metal. LC_{50} values within one standard error (SE) of each other were not included in the determination of the correlations as they were classified as equal.

Sum of toxic units

To calculate the toxic unit contributions in the mixture treatments, the measured concentrations in the mixture at day 56 (table A.2) were divided by the respective calculated 21-day LC_{50} values per metal and species. Per species was the sum of the toxic units for the metals in the mixture calculated (equation 3.12). $C_{mixture,i}$ is the concentration of metal i in the mixture.

$$TU_{sum,mixture,species\ x} = \sum_{n=1}^{3} \frac{C_{mixture,i}}{LC_{50,i,species\ x}} \tag{3.12}$$

4 | Results

4.1 Pattern oriented modelling with DEB-IBM

4.1.1 Equilibrium

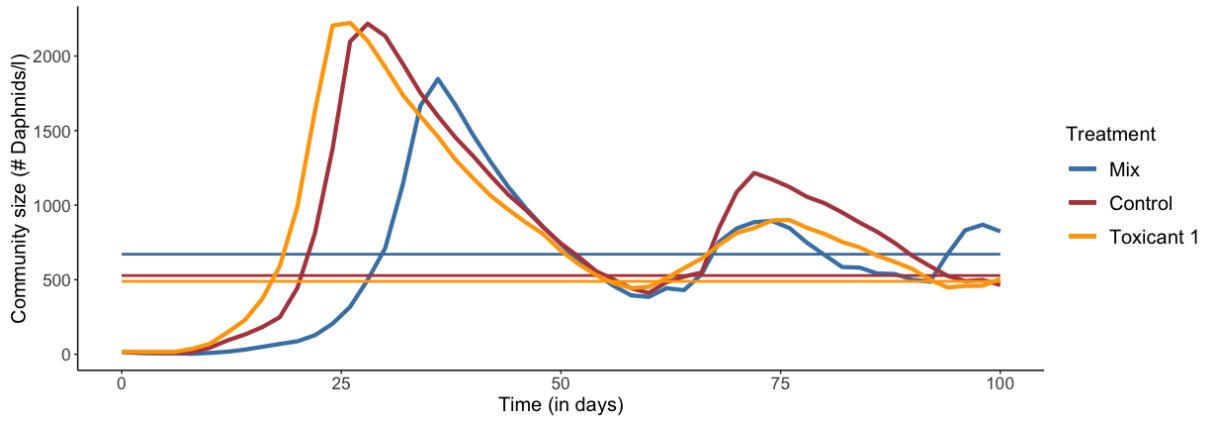


Figure 4.1: Simulated community size in function of time for a random community with the depiction of the last 14-day median as equilibrium measure

To evaluate at equilibrium what the response is to the different toxicants, it is important to determine a measure as the model will never reach a steady state. In figure 4.1 is the simulated community size depicted over time for the control (no toxicant), single toxicant exposure (only 1 of 3 depicted) and the mixture exposure (3 toxicants together) and shows that the different treatments can result in different community size responses over time. In this example, the control (no toxicants) and single toxicant 1 exposure initially have a very similar course, which starts to deviate after the initial community size peak, while the mixture exposure seems to follow a different path. When only considering day 100, the community size at equilibrium is higher for the mixture exposure than for the control and the single toxicant treatment. Thus, when only looking at day 100 the data suggest antagonism (less severe toxicity effects in the mixture than what would be expected based on the single metal responses, note that normally all the effects of single metals need to be assessed in the mixture before a real conclusion can be made on response compared to independent action), while at day 80 synergism would have been concluded (mixture community size lower than the single toxicant community size). These fluctuations over time, differences in height and timing of peaks make it difficult to conclude something from this data at a time point. To make this analysis less time-dependent not only the data from day 100 was used as equilibrium data but the median of the last 14 days as an attempt to find a more representable equilibrium community size under the different treatments. The horizontal lines depict the median of the last 14 days for this randomly selected modelled community.

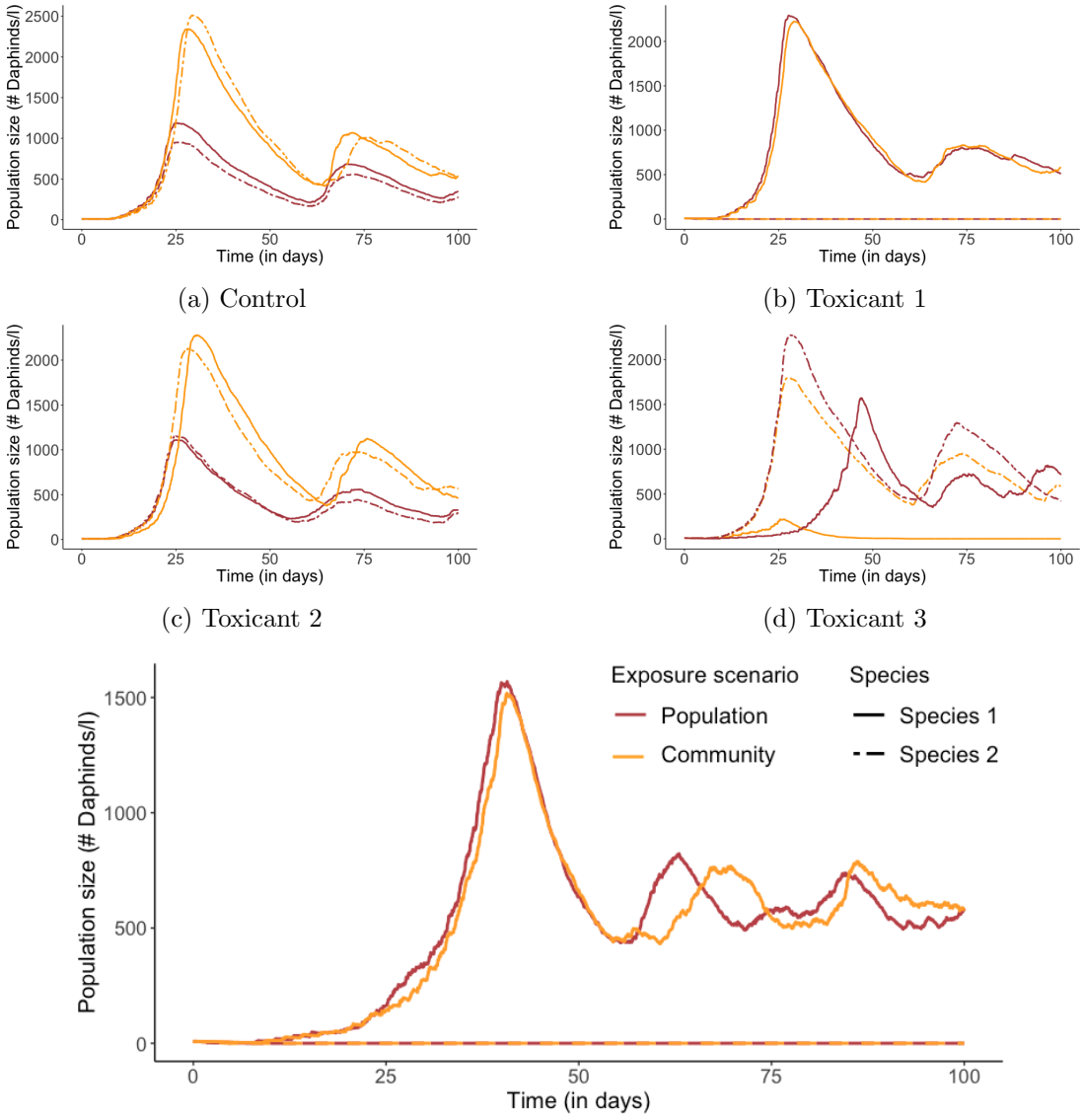
4.1.2 Competition

The only form of competition that exists between the species in the implemented DEB-IBM is food competition. In figure 4.2 is a simulated population size shown in function of the time for the 2 species exposed as a community and thus food competition will occur (yellow) and 2 species separately exposed (red) for one hypothetical community. In the community exposure, species 1 completely died-off exposed to toxicant 3 (figure 4.2 d) and species 2 exposed to toxicant 1 (figure 4.2 b). Both species died in one of the single toxicant treatments, but when exposed to a mixture of these treatments species 1 survived, while species 2 died off (figure 4.2 e). Looking at the population exposures (no competition) of both species in the mixture it becomes clear that species 2 cannot survive even without competition, while species 1 can. So, the full domination of one species in the mixture exposure can occur, while this species cannot survive in a community setting with less stress (single toxicant). This occurred in 7% of the cases (2400 simulated runs: 100 sampled TKTD parameters, 2 correlations, 3 concentrations and 4-fold replicates) in the dataset where the concentrations were based on the 21-day LC_{50} values. In which treatments this phenomenon occurred can be found in table 4.1. 90% of the time this occurred in the negatively correlated communities and 82% at the highest defined concentration level.

A figure containing extra information on the effects on reproduction and deaths related to the toxicant for the single toxicant 3 exposure as a community is depicted in figure 4.3. Here both species are again modelled in a community, so food competition can occur. At the first community size peak (around day 25), reproduction will come to a halt for both species. This while stress-induced deaths in both populations will keep on happening. Right before the reproduction start again (day 50), due to rising food availability, species 1 has completely died off in the community.

Table 4.1: Number of cases with no survival of both species in the single toxicant exposures, but one survives in the mixture

Concentration of the mixture			**Sensitivity correlation**	
3 TU	1.5 TU	0.75 TU	Positive	Negative
135	30	0	17	148



(a) Control

(b) Toxicant 1

(c) Toxicant 2

(d) Toxicant 3

(e) Mixture

Figure 4.2: Simulated evolution of population size over time for population and community exposures, the graphs are divided per treatment

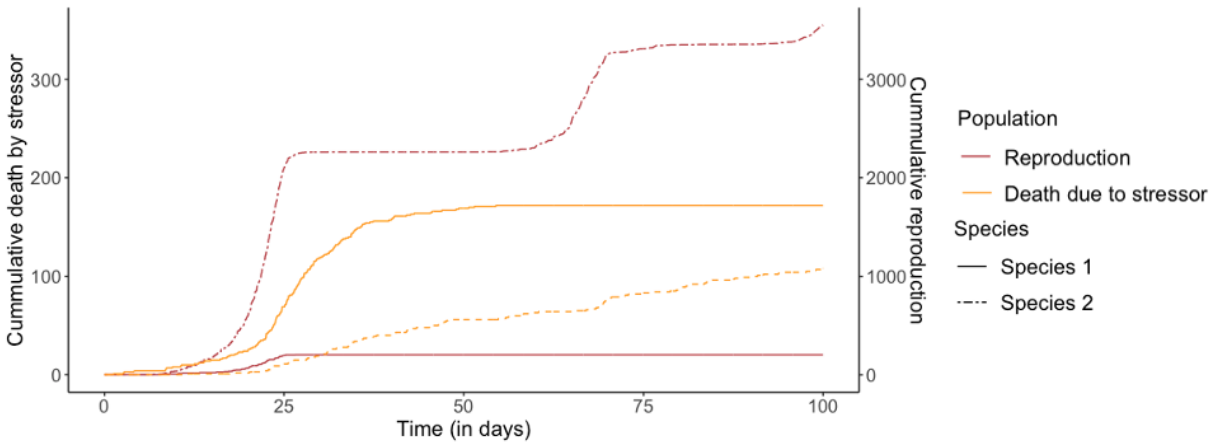


Figure 4.3: Simulated toxicant related deaths and reproduction in a community to toxicant 3 for the example as depicted in figure 4.2 d

4.1.3 Equitoxicity

The equitoxic design in the modelling study was initially based on the physiological level data and for a second run based on 21-day LC_{50} values calculated for the same set of sampled TKTD-parameters. In the microcosm setup, the observed 21-day LC_{50} values were used to make an approximation for equitoxicity on the community level. Equitoxicity would mean that the relative community sizes for the three single toxicant exposures are identical and thus the toxicants have the same effect compared to each other in the tested concentrations. This would mean that the coefficient of variation (CV) is low and equitoxicity on the community level was approached. In figure 4.4 the coefficient of variation for the simulated data is plotted per concentration level determined on the physiological level (concentrations based on the $ED_{50,h}$ values) for the top plot and the individual level (concentrations based on the LC_{50} values) for the bottom plot. The boxplots represent the different percentiles. The dots represent the 5% lowest values and 5% highest values of the data, within the box is 50% of the data contained (25% below and 25% above the median) with the thicker line in the box the median of the dataset, all boxplots in this thesis are represented in this way.

The percentile plots for the 2 lowest concentrations are very similar for both methods, but at the highest concentration were more cases registered with relatively high coefficients of variation. The CV-values recorded for the concentrations based on the physiological level were up to 1.73 for the highest concentration. In this case 2 of the 3 single toxicant exposures lead to no survival of the community, while the third toxicant did not affect the community size. In this instance, the toxicants will not contribute equally to the mixture due to the big differences in response in the single toxicant exposures. A case around the median for the lowest concentration for example is already a lot more equitoxic. A recorded case with a CV of 0.10 has relative community sizes of respectively 1.0, 0.95 and 1.15 for the three toxicants, which lie close to each other. There seems to be no relation between high CV values for both methods, so a high CV for concentrations based on the physiological level does not necessarily mean that these toxicity parameters give rise to a high CV when the concentrations are based on the calculated LC_{50} values (figure A.5).

The concentrations based on the individual level data were used in the further analysis due to the lower number of high CV values (maximum CV is 0.43 instead of 1.73). In the appendix A.2 can similar figures be found based on $ED_{50,h}$ equitoxicity and significant differences will be discussed in the main text. The simulation results related to the narrower $ED_{50,h}$ range (2-fold

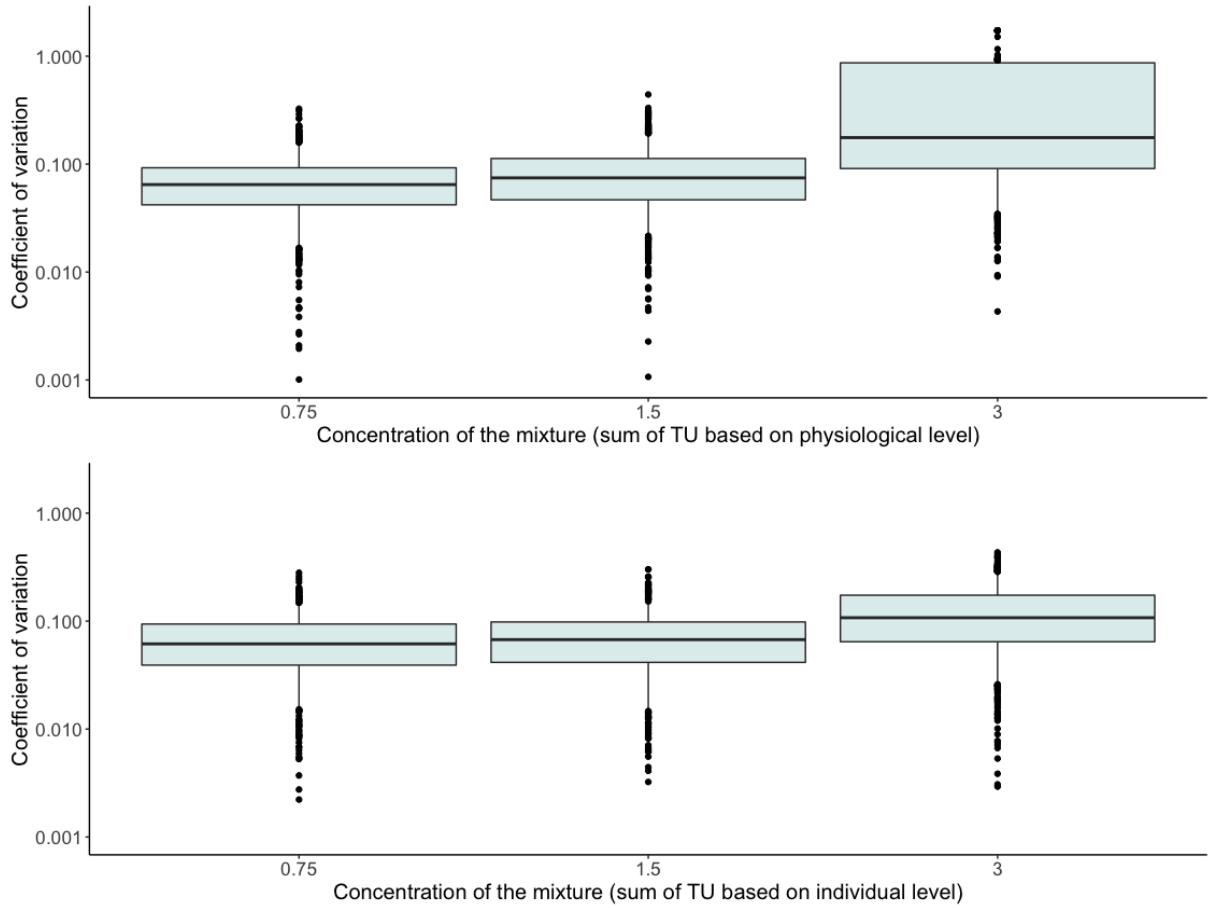


Figure 4.4: Achieved equitoxicity on the community level in the DEB modelling, top: concentrations based on $ED_{50,h}$, bottom: concentrations based on 21-day LC_{50} with the box containing the 25^{th} to 75^{th} percentile and the dots the 0^{th} to 5^{th} and 95^{th} to 100^{th} percentile

differences) are in appendix A.6. The results will not be discussed in the text as the finding in all cases are identical to the broader range (100-fold differences), but as expected were less pronounced.

4.1.4 Community traits

Sensitivity correlation

When assessing sensitivity different metrics can be used like EC_x, LC_x, $ED_{x,h}$ etc. For example, different LC_x values for a certain toxicant, if tested under the same conditions, can be compared to other species and this will give information on which species is more sensitive and will be affected more by the toxicant under the tested conditions. In a community, it can be particularly interesting to see how the different species have different sensitivities for the same toxicants. This could give a first idea of which species are expected to be overall more affected by the considered toxicants in a mixture. In this thesis, the correlation analysis is conducted on the physiological level ($ED_{50,h}$).

In figure 4.5 is the relative response shown for the simulated community sizes compared to the control (unstressed) community at equilibrium for all of the simulated communities. At the highest concentration are most of the relative community sizes for the negative correlation equal to 0 (median is 0), while for the positive correlation more than 75% of the cases the community

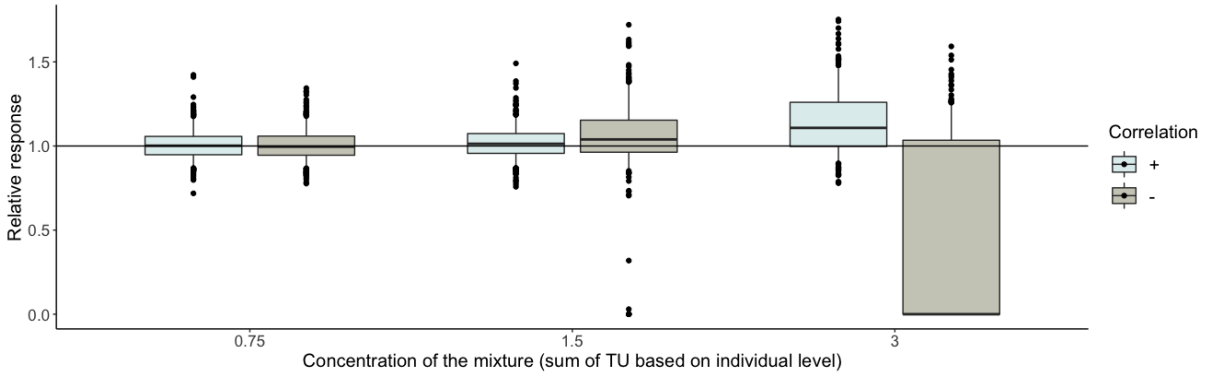


Figure 4.5: Simulated relative community size (compared to the control) at equilibrium for the mixture exposure with +: positive correlation and -: negative correlation and the box containing the 25^{th} to 75^{th} percentile and the dots the 0^{th} to 5^{th} and 95^{th} to 100^{th} percentile

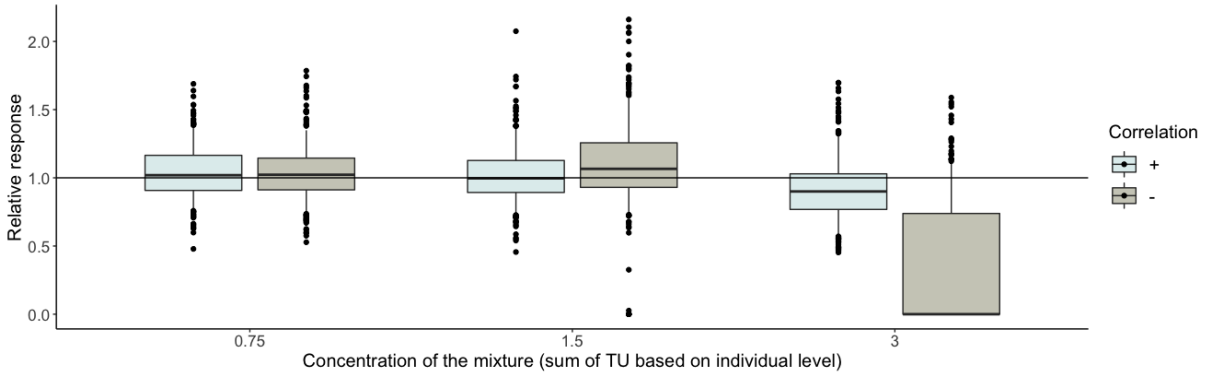


Figure 4.6: Simulated relative community size (compared to IA) at equilibrium for the mixture exposure with +: positive correlation and -: negative correlation and the box containing the 25^{th} to 75^{th} percentile and the dots the 0^{th} to 5^{th} and 95^{th} to 100^{th} percentile

size in the mixture exposure is bigger than the control. For the other exposure scenarios are the results very comparable with figure 4.6 which shows a similar distinction made with the concentrations and the correlations but this time not for the relative response compared to the control, but for the response compared to independent action. This ratio contains information on the expected effect that can be more or less severe than expected from the single metal toxicity data. A value of 1 means that the observed mixture effect and the IA prediction (in this case for the community size) are equal to each other. If the value is below 1 the observed effect is more severe than expected (synergism) and above 1 less severe (antagonism) than expected assuming non-interactivity of the metals.

When comparing the different correlations at the same concentration level, the biggest difference in response can be observed at the highest concentrations. Here both correlations tend to be more synergistic than the other concentrations and this goes especially for the negative correlation. Only for 46% (out of 400) was the community size not equal to 0 at day 100 for the negative correlation at the highest concentration level. For the other concentrations, the medians of the 2 correlations lie much closer to each other and to a relative response equal to 1 (additive effect), even the distributions of both correlations at the lowest concentration are quite similar. At the 1.5 TU concentration level is the distribution of the positive and negative correlation different. In the case of a negative correlation, in 10 cases the relative response was lower than 0.5. Synergistic mixture effects thus still occurred at the lower concentration level to a lesser extent. On the other hand, were also cases recorded that suggest antagonism

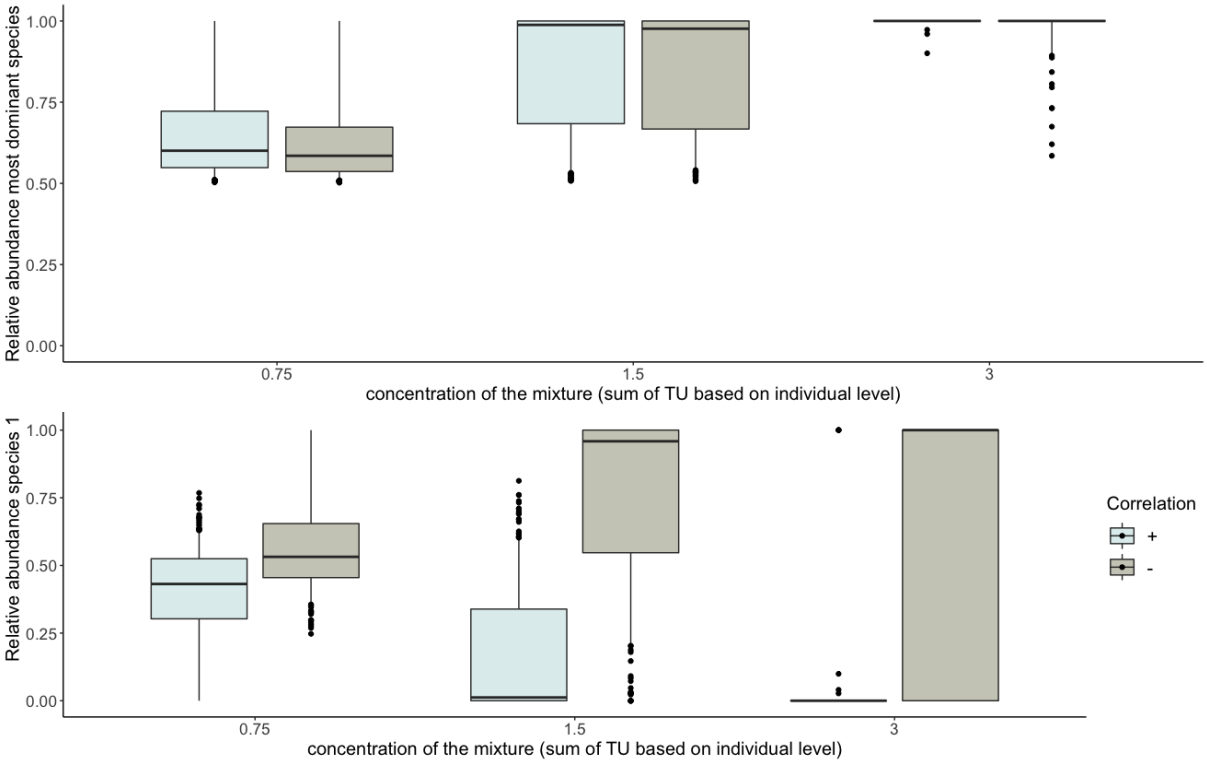


Figure 4.7: Simulated relative abundances of the species based on individual count at equilibrium for the mixture exposure, upper graph: Abundance of the most dominant species, bottom graph: Abundance of species 1 (sensitive to all of the single metals compared to species 2 in the positive correlation and to 2 out of 3 for the negative correlation defined on the physiological level) at equilibrium with +: positive correlation and -: negative correlation and the box containing the 25^{th} to 75^{th} percentile and the dots the 0^{th} to 5^{th} and 95^{th} to 100^{th} percentile

as for almost all concentrations and correlations except the negative correlation at the highest concentration there are cases with a relative response greater than 1.5.

The most obvious differences with the $ED_{50,h}$ based concentration plot (figure A.1) are the prediction of more synergistic effects even for the positive correlation at the highest concentration level.

In figure 4.7 are for the different concentrations and correlations the relative abundances of the species given exposed to the mixture at equilibrium. On the upper graph is the dominance (relative abundance of the most present species) plotted. The relative abundance is based on the individual count of the species. At the highest concentration level, it becomes clear that full domination of one species is more likely than at the other concentration levels. The median and distribution of the relative abundances of the most dominant species are very similar between the positive and the negative correlation. Although at the highest concentration a larger range of abundances was observed for the negative correlation than for the positive correlation.

Note that, when looking at the most abundant species, information is lost on which species dominate. That is information that is at the bottom of figure 4.7. Here the abundance of species 1 is plotted at equilibrium, this is the species that is sensitive to all of the toxicants in the positive correlation and the negative correlation still sensitive to 2 of the 3 toxicants. At the highest concentration in this plot, almost all of the communities consist of one species. In the positive correlation this is the insensitive species, while in the negatively correlated commu-

nities it is mainly the species that are still sensitive to two of the three toxicants. This trend remains visible in the lower concentrations when looking at the median of the treatments, but the distance between the median of the positive and the negative correlation becomes smaller at lower concentrations.

The most obvious difference with the community data generated with the concentrations based on the $ED_{50,h}$ (figure A.2) is at the highest concentration level. For the concentrations based on the $ED_{50,h}$ values it is not species 1 that is most dominant in the negative correlation, but species 2 (species that is only sensitive to 1 of the 3 toxicants).

Toxic unit ratio

The concentrations are defined based on the geometric mean of the 21-day LC_{50} values. Because the concentrations are based on the geometric mean does not mean that the individual species feel the calculated level of stress, since every species has its LC_{50} value which determines the stress level to which the individual is exposed. Compared to the sensitivity correlation does this approach also take the concentration of the toxicants into account. In figure 4.8 is the relative abundance of the most insensitive species, based on the sum of toxic units, plotted in function of the ratio of the sum of toxic units of the two species in the community for the different concentration levels at the different concentration levels of the considered mixture concentrations.

A high ratio would thus mean that both species experience a very similar level of stress based on the toxic units and a low value would indicate the opposite. The negatively correlated communities tend to have relatively higher toxic unit ratios (thus stress is more evenly distributed between the species) than in the positively correlated communities. Apart from that very similar behaviour is detected when it comes to the reaction in function of the TU-ratio. At higher values, both species can co-exist at the defined equilibrium, but if the TU-ratio becomes low enough, one species will completely dominate (relative abundance equal to 1) the community. This point seems to depend on the correlation and the concentration and seems to shift towards lower values at lower concentrations and is lower for positive than for negative correlations. For the two lowest concentration level this happens at lower TU-ratios for the positive correlation than for the negative correlation. In the negative correlation, even the most insensitive species (based on toxic units) can be dominated by the other species in the community (relative abundance equal to 0).

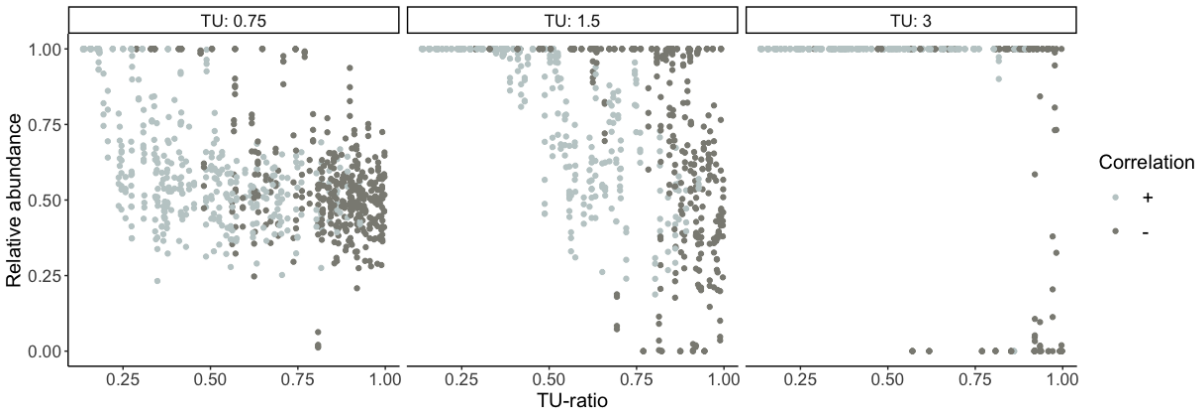


Figure 4.8: Simulated relative abundance, based on individual count, of the most insensitive species, based on the sum of toxic units, in function of toxic unit ratio at equilibrium with the concentration levels defined at the individual level for the mixture exposure with +: positive correlation and -: negative correlation

Note that at the highest concentration are 216 of the 800 tested communities not plotted as no survival of both species occurred at equilibrium.

21-day survival ratio

When the concentration response curve is known, in the case of mortality, the percentage of deaths can be determined at a certain concentration. This can be interesting in comparison to the toxic unit ratio since the toxic unit calculation only uses one point of the concentration response curve namely the EC_x or LC_x, while calculating the survival probability also takes into account that the concentration response can be very steep and thus the range of concentrations where a response is not 0 or 1 is small. The slope is parameter β in equation 3.11. In figure 4.9 is the simulated relative abundance of the most insensitive species, based on the product of the survival probabilities of the single toxicants, plotted in function of the defined 21-day survival ratio for the 3 different concentration levels.

In total 223 of the 2400 communities are not plotted. At the 2 lowest concentrations these were 7 communities that had no surviving individuals and at the highest concentration, this were 216 cases. For the 21-day survival ratio no longer a clear distinction between the positive and negative correlation can be made. Both react very similarly when considering the most insensitive species in function of the survival ratio. High ratios (meaning very similar survival probabilities in the mixture) lead to more balanced species distribution and co-existing species, while lower ratios seem to make it more difficult for the species to co-exist and the species that is insensitive will dominate in the community.

All of the cases with a relative abundance of the most insensitive species below 0.25 at the highest concentration level are no replicates of each other thus can be attributed to the randomness that is included in the model.

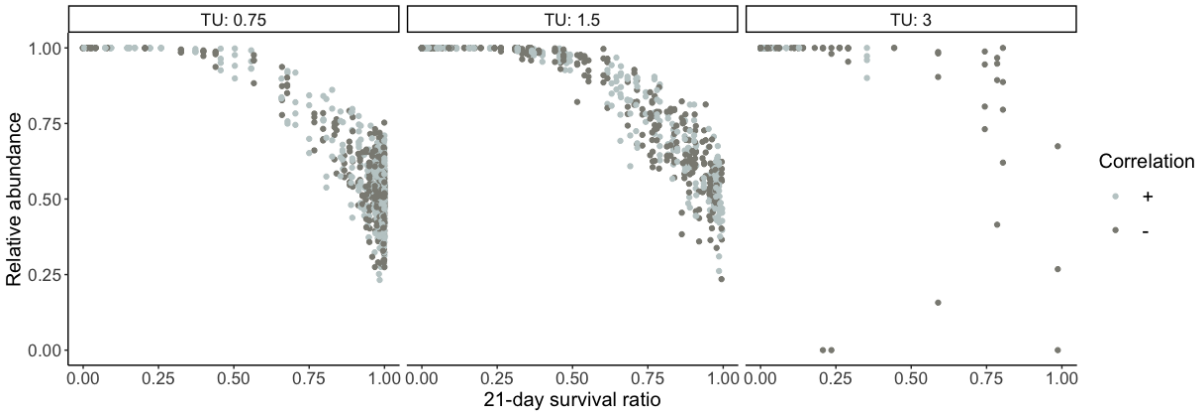


Figure 4.9: Simulated relative abundance, based on individual count, of the most insensitive species, based on the LC_{50} values, in function of 21-day survival ratio at equilibrium with the concentration levels defined at the individual level for the mixture exposure with +: positive correlation and -: negative correlation

4.2 Microcosm study

4.2.1 Community traits

Sensitivity correlation

From the calculated 21-day LC_{50} values can the sensitivity correlations be determined for the 3 communities. The LC_{50} and β values based on free ion activities and dissolved concentrations are in table 4.2. The calculated means were used for this analysis. In table 4.3 is depicted for the different communities which have a higher, lower or equal (within one standard error (SE) of each other) LC_{50} value for the same toxicant. Based on free ion concentrations are all of the communities positively correlated. Note that in community 3, 2 out of 3 LC_{50} were within one standard error, so only nickel was considered to determine the correlation. With *Daphnia magna* being the insensitive species in both community 1 and 2, while *Daphnia longispina* is the insensitive species in community 3. Based on the dissolved concentrations are communities 1 and 3 negatively correlated and community 2 positively with *Daphnia magna* the insensitive species of the 2.

sum of toxic units

The sum of the toxic units per mixture treatment is shown in table 4.4. If the calculated means for sum of toxic units are compared between the different species, per treatment, the data tends to suggest, based on the means, that *Daphnia longispina* and *Daphnia pulex* experienced very similar stress levels. This for both the calculations based on the free ion activity and the dissolved concentration, while *Daphnia magna* tends to have a lower sum of toxic units indicating that *Daphnia magna* would experience less stress when it is assumed that the metals act independently of each other.

Table 4.5 contains an overview of the different community traits for the 3 tested communities.

Table 4.2: 21-day LC_{50} and β estimation for concentration mortality curve with concentrations in free ion activity and dissolved concentrations

		Free ion activities		**Dissolved concentrations**	
Species	Metal	21 day-LC_{50} (in nM) (±SE)	β (±SE)	21-day LC_{50} (in μg/l) (±SE)	β (±SE)
D. longispina	Cu	1.68 (±1.05)	-5.21 (±11.28)	19.82 (±4.79)	-11.63 (±26.13)
D. longispina	Ni	901.54 (±59.24)	-7.75 (±5.15)	109.28 (±5.58)	-7.03 (±3.01)
D. longispina	Zn	1223.75 (±174.87)	-14.96 (±5.60)	1171.49 (±18.43)	-16.64 (±5.17)
D. magna	Cu	9.99 (±4.71)	-20.16 (±32.17)	47.01 (±8.93)	-41.37 (±114.73)
D. magna	Ni	1070.75 (±150.78)	-18.28 (±3.47)	106.70 (±9.93)	-19.85 (±2.75)
D. magna	Zn	1403.78 (±320.07)	-19.78 (±19.97)	170.11 (±15.61)	-18.14 (±4.16)
D. pulex	Cu	1.52 (±0.793)	-13.78 (±20.80)	24.36 (±6.77)	-53.41 (±302.89)
D. pulex	Ni	742.01 (±89.99)	-16.92 (±3.85)	97.24 (±8.19)	-19.22 (±2.33)
D. pulex	Zn	1354.81 (±106.70)	-7.29 (±3.15)	204.32 (±10.34)	-9.05 (±3.32)

Table 4.3: Sensitivity correlation for the communities in the microcosm based on free ion activities and dissolved concentrations with high and low referring to comparison of the value of the 21-day LC_{50} for the same toxicant between the species in the community and equal if the LC_{50} are within one standard error of each other

	Free ion activities			**Dissolved concentrations**		
LC_{50}	Copper	Nickel	Zinc	Copper	Nickel	Zinc
Community 1						
D. magna	high	high	equal	high	equal	low
D. pulex	low	low	equal	low	equal	high
Community 2						
D. magna	high	high	equal	high	equal	equal
D. longispina	low	low	equal	low	equal	equal
Community 3						
D. pulex	equal	low	equal	equal	low	high
D. longispina	equal	high	equal	equal	high	low

Table 4.4: Sum of toxic units based on 21-day LC_{50} values

Species	Mixture	sum of TU free ion activity (±SD)	sum of TU dissolved concentration (±SD)
D. longispina	Mix 1	0.55 (±0.31)	1.27 (±0.28)
D. longispina	Mix 2	1.50 (±0.95)	2.29 (±0.57)
D. magna	Mix 1	0.38 (±0.17)	0.96 (±0.18)
D. magna	Mix 2	0.96 (±0.41)	1.81 (±0.38)
D. pulex	Mix 1	0.60 (±0.33)	1.16 (±0.25)
D. pulex	Mix 2	1.63 (±1.01)	2.12 (±0.51)

Table 4.5: Overview community traits tested communities for both free ion activities and dissolved concentrations with +: positive correlated community and -: negatively correlated community and for the sum of toxic units is the rank of the means used with 1 = lowest sum of TU and 3 = highest sum of TU

		Sensitivity correlation		**Sum of toxic units**			
Species	Com	Free ion activity	dissolved	Free ion activity		dissolved	
				Mix 1	Mix 2	Mix 1	Mix 2
D. magna	1	+ (insensitive)	-	1	1	1	1
D. pulex	1	+ (sensitive)	-	3	3	2	2
D. longispina	2	+ (sensitive)	+ (sensitive)	2	2	3	3
D. magna	2	+ (insensitive)	+ (insensitive)				
D. pulex	3	+ (sensitive)	-				
D. longispina	3	+ (insensitive)	-				

4.2.2 Community endpoints

The community endpoint analysis was always performed on data gathered on the 56^{th} day and treated as equilibrium data. Figure A.6 shows the total community size for the last 7 days of the experiment and from this data it can be concluded that for all of the treatments the community size was relatively constant.

Dry biomass

On day 56 was the dry biomass determined for the communities (no distinction between the species) in all of the treatments. In figure 4.10 these results are plotted as concentration-response curves. The mean and standard deviation for the dry biomass in the control are in table 4.6. Community 1 (*Daphnia magna* and *Daphnia pulex*) and community 2 (*Daphnia magna* and *Daphnia longispina*) In the control had higher mean dry biomass content than community 3 (*Daphnia longispina* and *Daphnia pulex*). Community 3 had in all of the single metal and mixture 1 treatments biomass higher than in the control.

In the mixture 2 treatment, the standard deviation for community 1 and 2 is large as for both communities only 2 out of 4 replicates survived the 56-day exposure. In the case of community 3, all of the organisms in this treatment did not survive. None of the replicates of community 3 exposed to the highest mixture concentration made it past the first half of the 56 days, while the replicates of community 1 and 2 went extinct in the second half of the experiment. Community 3 consist of *Daphnia longispina* and *Daphnia pulex* and these species are also present in community 1 and 2 wherein some replicates they were able to survive.
In figure 4.11 is the mixture response shown compared to the Independent Action (IA) predicted dry biomass. Independent action was calculated by multiplying all the relative (to control) single metal biomasses. For example, multiplying relative biomass of copper 1; nickel 1 and zinc 1 treatment of community 1 to calculate the IA-predicted relative biomass for community 1 exposed to mixture 1. The IA-predicted biomass of community 3 is high because all of the single metal response compared to the control for community 3 were also high. In almost all of the cases (except community 1 exposed to mixture 1) is the mean independent action prediction higher than the real mixture effects. The data thus tends towards synergistic mixture reactions with the clearest case community 3. Community 3 has a mean IA-predicted effect on the biomass of around 4 (so based on the single metal data biomass 4 times higher than the control is expected), while all of the replicates went extinct in the real mixture exposure to the highest concentration.

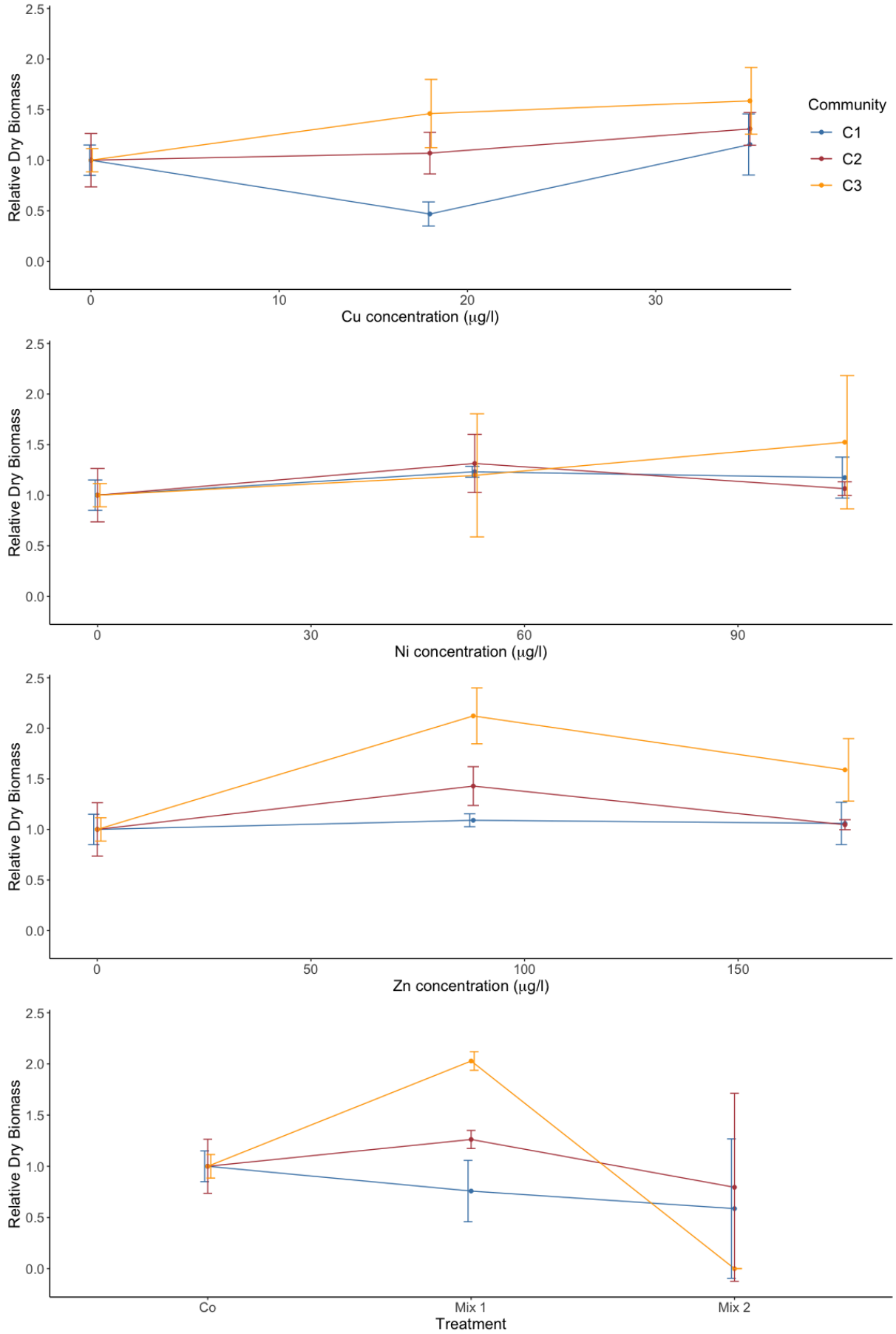


Figure 4.10: Observed relative biomass compared to the control on the 56th-day for the different treatments, from top to bottom: copper, nickel, zinc and mixture. On the x-axis are the nominal concentrations or the treatment and flags show interval mean ± SD

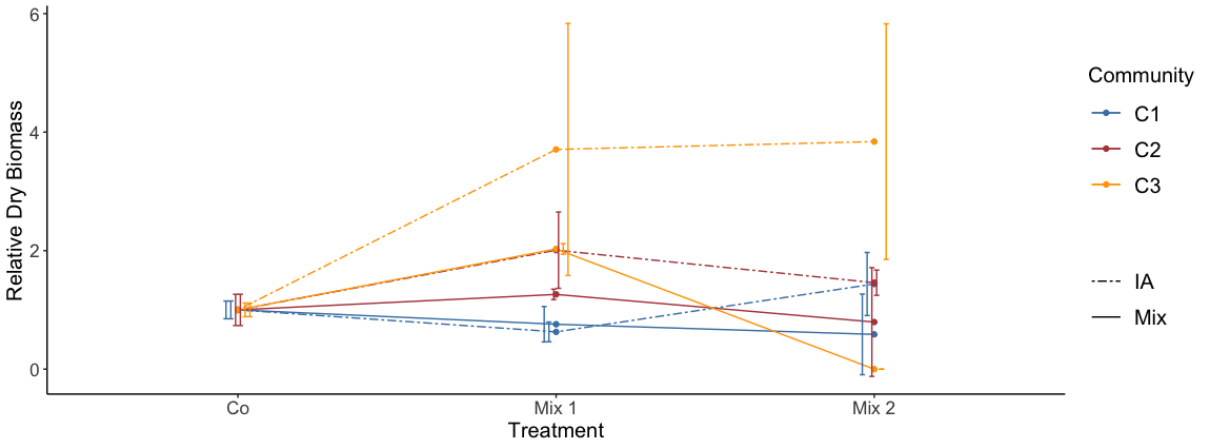


Figure 4.11: IA calculation compared to real mixture response for 56 day biomass. On the x-axis are the treatment and flags show interval mean ± sd

Table 4.6: Dry biomass at day 56 for the different control communities

	C1 (±SD)	C2 (±SD)	C3 (±SD)
Dry biomass control (in mg/l)	14.38 (±2.16)	12.36 (±3.26)	8.46 (±0.98)

Community size

On day 56, for all the different replicates, the individual count of the different species was determined using a convolutional neural network. In figure 4.12 these results are represented as concentration-response curves for the total community size (sum of the individual counts per replicate). On the y-axis is the relative community size compared to the mean of the control and on the x-axis is the nominal concentration for the single metal treatment graphs and the treatment for the mixture. The mean and standard deviation for the community size in the control are in table 4.7. The community size in the control of community 3 was almost twice as big as community 1 and 2. The high standard deviation in community 3 comes from the big differences between the control replicates as the lowest recorded community size was 259 Daphinds l^{-1} and the highest 524 Daphinds l^{-1}.

The largest mean deviation from the control is for community 3 for both the nickel treatments and the mixture 1 treatment. When looking at the IA-predictions (figure 4.13), the predictions are more severe than the observed mixture effects (based on the means) except for community 3 exposed to the highest mixture concentration. Here the mean IA-prediction is around 0.3, which indicates that a reduction in community size is expected based on the single metal exposures. Overall compared to the control for all of communities not a lot if deviation in community size is observed. For community 1 in mixture treatment 2 the IA-predicted response is worse than the observed indicating an antagonistic reaction.

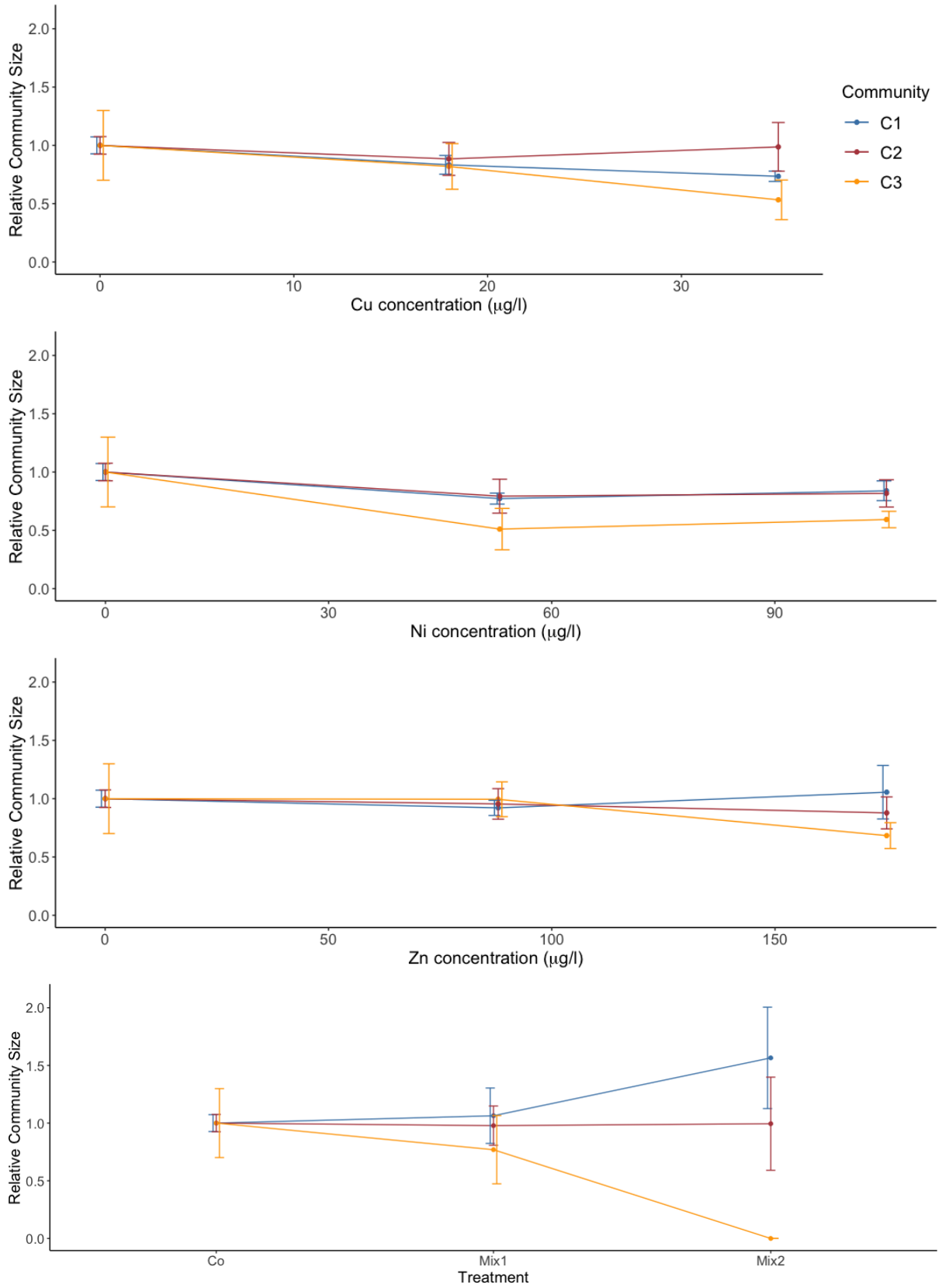


Figure 4.12: Observed relative community size compared to the control on the 56th-day for the different treatments, from top to bottom: copper, nickel, zinc and mixture. On the x-axis are the nominal concentrations or the treatment and flags show interval mean ± SD. Replicates without survival not included except for community 3 exposed to mixture 2

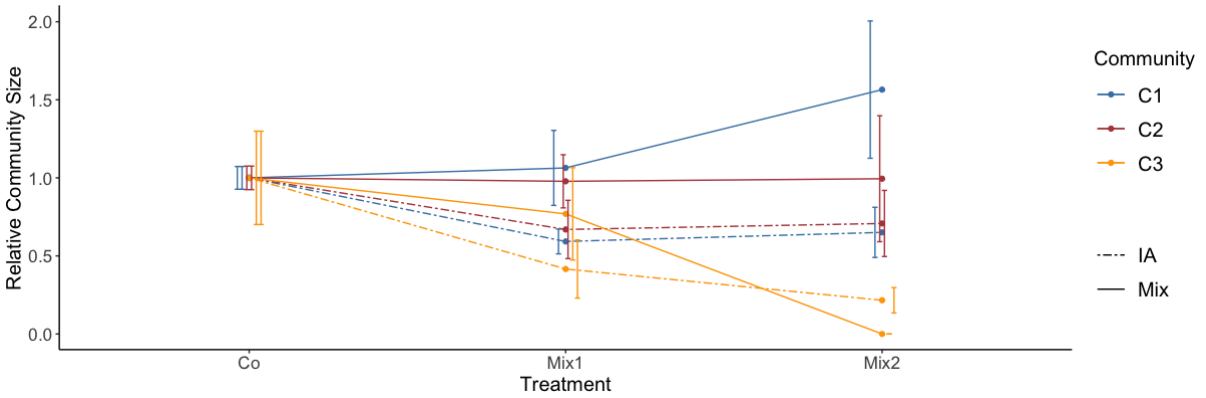


Figure 4.13: IA calculation compared to real mixture response for 56 day community size. On the x-axis are the treatment and flags show interval mean ± SD. Replicates without survival not included except for community 3 exposed to mixture 2

Table 4.7: Community size (in # Daphnids/l) at day 56 for the different control communities

	C1 (±SD)	C2 (±SD)	C3 (±SD)
Community size control (# Daphnids/l)	219.4 (±16.0)	222.2 (±16.7)	396.8 (±118.6)

Species abundance

On day 56 were for all the different replicates the individual counts of the different species determined using a convolutional neural network. In figure 4.14 these results are represented as concentration-response curves. On the y-axis is the relative abundance compared to the total community size per treatment and on the x-axis is the nominal concentration for the single metal treatment graphs and the treatment for the mixture. In the control was the relative abundance ratio of the species for the 3 different communities around 0.75/0.25. In community 1 and 2 *Daphnia Magna* dominated, while in community 3 *Daphnia Pulex* was the most present species.

In the copper treatments, based on the means, for community 3 it seems like the original domination of *Daphnia Pulex* becomes less strong as the concentration of copper increases. In the nickel and zinc treatments community 1 and 2 follow very similar responses to the toxicants. For both of the communities In the nickel exposures (clearest at the highest concentration) *Daphnia Magna* becomes less dominant compared to the other species in the community, while in the zinc treatments the opposite occurs. Here *Daphnia Magna* tends to have a higher relative abundance than in the control in both cases.

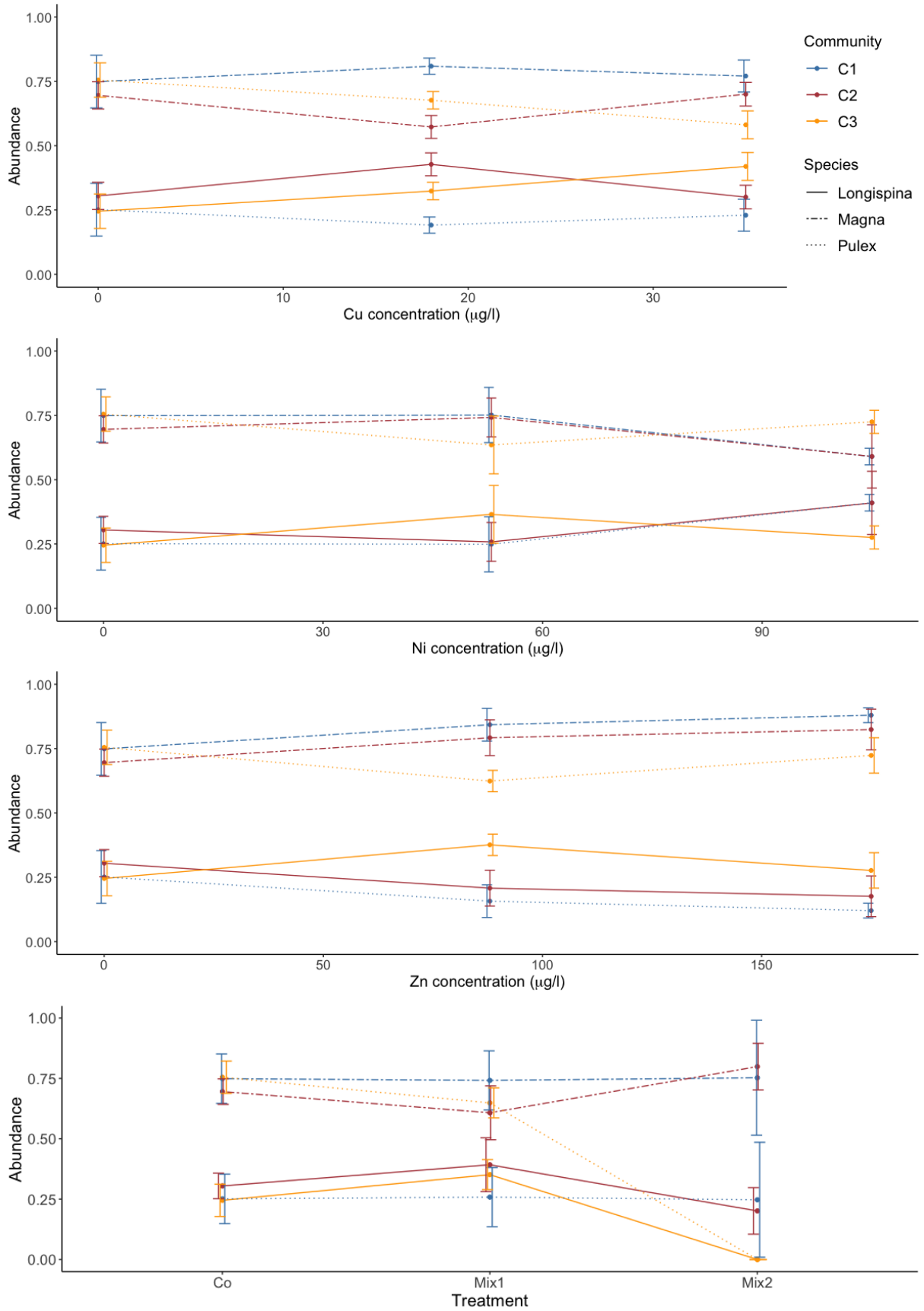


Figure 4.14: Observed relative abundance on the 56th-day for the different treatments, from top to bottom: copper, nickel, zinc and mixture. On the x-axis are the nominal concentrations or the treatment and flags show interval mean ± SD. Replicates without survival not included except for community 3 exposed to mixture 2

4.2.3 Linking the different endpoints

Community 1: *Daphnia magna* and *Daphnia pulex*

When comparing the different endpoints for community 1 in the mixture exposures the mean of the relative response is always relatively close to 1 (so situation in the control) and the relative abundances do not change a lot, except for the community size exposed to mixture 2, where an increase compared to the control was observed only considering the replicates with survival. The biomass response to mixture 2 shows a high standard deviation, but this is related to the fact that 2 replicates went extinct, the ones that survived had very similar biomass content to the control. A higher community size combined with no responses on biomass would indicate that smaller species or more juveniles are present in the community. To assess this looking at the relative abundances of the species can help. In the mixture 2 treatment, the abundances are 90% and 60% for *Daphnia magna* in the surviving replicates, so one higher and one lower response to the relative *Daphnia magna* abundance of the control was observed. The increase in community size in the replicate with only 60% *Daphnia magna* is solely due to an increase in population size of the smaller *Daphnia pulex* which could explain the higher observed community size mean with a relatively low impact on biomass as this is the smaller species of the 2.

Community 2: *Daphnia magna* and *Daphnia longispina*

Overall the response seems to be very similar to community 1 when assessing the effects of the different endpoints in the mixture treatments when considering the mean responses. Only considering the biomass in mixture 2, where 2 replicates died off, the biomass of the 2 surviving replicates is 1.5 times the biomass in the control. In mixture 2 did *Daphnia magna* seem to become more dominant based on pixel count (thus the amount of pixel in photo's that were identified as a certain species, appendix A.4) and relative abundance based on individual count. With the community size remaining relatively unchanged to the control in the mixture 2 treatment in combination with the increase in biomass would indicate that not more, but larger animals will be present. This was also observed as an increase in *Daphnia magna* population size, the larger species of the 2. Although an increase in biomass should relate to the pixel count as this is a measure for the surface area of the species and thus related to volume and biomass, but as mentioned did not go up. This could indicate that something went wrong in the biomass analysis like separation from the community from the debris etc.

Community 3: *Daphnia pulex* and *Daphnia longispina*

In all of the treatments (except for mixture 2) is the relative biomass compared to the control higher and in the case of mixture 1 treatment on average more than twice as high. This while the mean recorded community sizes were always lower than 1 compared to the control, the same trend is seen in the total pixel count. So, the data suggests more biomass without an increase in the size of the community and even without a positive effect on the pixel count (appendix A.4). The fact that no increase in pixel count was observed in any of the treatments, makes it difficult to explain why the biomass is higher compared to the control.

In all of the treatments (except for mixture 2), the mean abundances of *Daphnia longispina* go up, while *Daphnia pulex*, the most dominant in the control, seems to become less abundant.

4.2.4 Equitoxicity

To determine if the microcosm experiment approaches equitoxicity on the community level 6 one-way ANOVA models were constructed per community and concentration level for both dry biomass and community size at day 56. An example of such a model would contain for community 1 the single metal treatments data of copper 1, nickel 1 and zinc 1. For the dry biomass are the p-values and F-statistics given in table 4.8 and for the community size in table 4.9. Both homoscedasticity and normality of the residuals were visually checked. In both cases, 3 out of 6 models did seem to describe the data better with a model more complex than an intercept only model at the 5% significance level (p-value < 0.05) indicating that one of the single toxicants does have a significantly different response from the other toxicants. For concentration level 1, community 1 and concentration level 1, community 3 both dry biomass and community size had p-values registered under 0.05. This could indicate that the equitoxic approximation based on 21-day LC_{50} values was not equitoxic on the community level for these endpoints, but overall the deviation of equitoxicity on the community level will be acceptable.

Table 4.8: P-values and F-statistic for one-way ANOVA models based on for the dry biomass at day 56 with $*$: significant at 5% significance level

	Concentration level 1		Concentration level 2		
	F-statistic	p-value	F-statistic	p-value	
Community 1	93.71	9.44e-07 $*$	0.26	0.78	
Community 2	2.49	0.14	7.87	0.011 $*$	
Community 3	4.87	0.04 $*$	0.02	0.98	

Table 4.9: P-values and F-statistic for one-way ANOVA models based on the community size at day 56 with $*$: significant at 5% significance level

	Concentration level 1		Concentration level 2		
	F-statistic	p-value	F-statistic	p-value	
Community 1	5.23	0.031 $*$	5.21	0.031 $*$	
Community 2	1.36	0.305	1.17	0.353	
Community 3	7.84	0.011 $*$	1.50	0.27	

5 | Discussion

5.1 Pattern oriented modelling

5.1.1 Competition

When multiple species are exposed together as a community, under the tested simulation setting it was observed that being able to survive the induced stress in a single metal or mixture exposure is no longer enough to guarantee that the species can survive in a community exposure. Once the species can interact with each other and compete for the same resources other factors can start to play a role in determining the relative abundances of the species.

Communities where both species died in different single toxicant exposures, were in some instances able to survive the mixture treatment which induces more stress on the individuals. The death of the species in the single toxicant exposure cannot solely be attributed to the toxicant stress as in these instances at least one of the two species can survive the same stress level when it is not exposed as a community, but as a population (no food competition). Even one or both species can survive in the mixture what is even more evidence that the toxicants are not the only reason no survival was observed in the single toxicant exposures as a mixture even induces more stress on the individuals. This is where the food competition between the species comes into play. At the first population size peak, food limitations will start to occur. Food limitations will have an effect on the amount of energy the organisms have at their disposal and since energy investment in reproduction is subordinate to maintenance costs reproduction will come to a halt. This while stress-induced deaths will not stop as this is related to the external toxicant concentrations, which do not change. The death in the single toxicant exposure has to do with the extra induced mortality compared to the insensitive species for that toxicant. The extra mortality will reduce the growth rate, while the other species will relatively be less impacted creating these food limiting conditions while the sensitive species still has a relatively small population size. In the starvation period will the extra induced mortality due to the stressor compared to the insensitive species cause the sensitive species to die. Do note that the community size and relative abundance at the start of the simulation will not influence the outcome. This is in fact an example of competitive exclusion and by extension all of the cases were one species cannot survive, as two species are using the same resources only one can survive when their niches are exactly the same (Bøhn *et al.*, 2008). When the DEB-parameters are exactly the same, this will be the insensitive species.

This will only happen in negatively correlated settings as to die in the single toxicant exposure the species should be sensitive compared to the other species for that particular toxicant, but in the mixture overall insensitive to survive or even dominate the community. This was also observed in the modelling data as 90% of the cases were negatively correlated communities based on the $ED_{50,h}$ values (thus on the physiological level) and even 100% were negatively correlated based on the LC_{50} values (so using the same definitions of a positive and negative correlation but apply them on the calculated LC_{50} values). So, when trying to predict the outcome of mixture toxicity by analysing the species dynamics of the single toxicant exposures and dealing with a negatively correlated community it can be less straightforward to extrapolate the data as depicted under the simulation conditions.

5.1.2 Equitoxicity

The goal of the chosen concentrations in the modelling study was to approach equitoxicity on the community level. This was done in two ways: using the equitoxic ratio on the physiological level and using the equitoxic ratio on the individual level. The equitoxicity for the community size endpoint was relatively similar for both methods when only considering the lower concentrations, but at the highest defined concentration level, the equitoxic ratio based on the individual level toxicity data seemed to be a better approximation for equitoxicity on the community level. When using data of a higher level of organisation (physiological vs. individual level) more information on the toxicity of the stressors is already included. In the modelling study, only the $ED_{50,h}$ information is used to define the concentrations on the physiological level, while for the individual level the LC_{50} values were used, which do not only depend on $ED_{50,h}$ but also the other TKTD-parameters: k_d, h_{max} and β_h.

Defining equitoxicity in practice will mostly be done by using toxicity information on a lower organisation level to create an approximation of equitoxicity on a higher level of organisation. As equitoxicity can only be assessed after performing the experiment. From a time and money perspective, it is not ideal to do an identical test just to determine the equitoxic ratio. Note that the equitoxic ratio will be dependent on the endpoint and organisation level of interest.

The extrapolation of findings in an equitoxic setting has to be carefully analysed before applying them to pollution in natural ecosystems. The ratios of the toxicant concentrations in the environment can be very different from the equitoxic ratio (Koppel *et al.*, 2018; Su *et al.*, 2017) and thus the contributions of the different toxicants to the toxicity can be different from the equitoxic ratio.

5.1.3 Community traits

The idea behind looking at community traits was trying to identify certain characteristics of communities, related to their sensitivity to toxicants, to see if it is possible to find characteristics that leads to a certain response when exposed to toxicant mixtures.

Sensitivity correlation

The first trait considered was the sensitivity correlation. It was observed in the modelling study that synergistic responses were more common at high concentration levels and when communities have a negative sensitivity correlation. This is also something **??** found for *Daphnia magna* populations exposed to a mixture of nickel and zinc, but on the reproduction endpoint. So, the assumption of independent action of the toxicants on the physiological level does not mean that the response on higher organisation levels also follows the independent action model. The synergistic response can be explained by assessing the distribution of stress between the species. If the stress endured from the toxicants present is more evenly distributed between the species then both species will be affected by the mixture (under the form of extra mortality in this modelling setup). This while in a positive correlated binary community one species will be less affected by the toxicants in the mixture than the other species. This was also observed in the relative abundances in the positively correlated communities exposed to the mixture. The insensitive species was, and most clearly in the exposure to the highest concentrations, the most abundant species in the community.

This is also in a way what happens in the negatively correlated communities. Although there is no violation of equitoxicity on the community level, the effects of the toxicants on the individual level can be different from each other. To create the negative correlations the $ED_{50,h}$ values for

the toxicant with the biggest logarithmic difference were exchanged between the species. From the LC_{50} values was the concentration calculated using the geometric mean per toxicant was calculated. When the LC_{50} values are for example 10 and 90 μg/l the geometric mean will be 30. For the species with the LC_{50} of 10, this is triple the concentration at which 50% of the organisms died, while for the other species the concentration only has a minimal effect as the concentration is far below the 50% response. This shows that when the relative $LC_{50}/ED_{50,h}$ difference is large between the species for the same toxicant, it can play an important role in which species will be most abundant in the community. In the negative correlation the most abundant species was almost always the species which was insensitive to the toxicant with the biggest relative difference of the $ED_{50,h}$ values in almost indicating that the toxicant with the biggest relative difference can have a big influence on the species abundances in the community, but this is not always the case as for the two highest concentration levels the relative abundance of species 1 can take values between 0 and 100%. Upon closer examination of the LC_{50} values of both species, the reason is very similar to the finding related to the relative difference between the sensitivities for the same toxicant. In the cases where species 1 dominates, the LC_{50} values to which this species is sensitive to are relatively close to these of the insensitive species for these 2 toxicants. An example is given in table 5.1. This table contains the LC_{50} values for 2 cases with a negative sensitivity correlation where the abundance of species 1 differs greatly. In both cases species, 1 is insensitive to toxicant 2. As can be seen the differences between the LC_{50} values of the other toxicants are smaller in the cases of high abundances than low abundances of species 1. So, when just considering the concept of positive and negative sensitivity correlations it might explain some of the differences in response to the toxicant mixture, but the relative difference between the sensitivities for the same toxicant also play a role in the reaction to the mixture exposure and mainly which species will be most abundant in the mixture.

Note that the determination of the sensitivity correlation can be endpoint specific and only makes sense in approximately equitoxic setting on the organisation level of interest. If one toxicant is dominant in its effect on the community the other toxicants will on to a lesser extent influence the toxicity. In this thesis always the $ED_{50,h}$ values were used, but defining these correlations on for example on reproduction effects, population growth rate or even on the LC_{50} values is possible. the outcome of the analysis is likely to differ for the different endpoints.

Table 5.1: LC_{50} values for two cases with high and low abundance for species 1 for negatively correlated communities

	Low species 1 abundance (0%)			**High species 1 abundance (100%)**		
	$LC_{50,tox\ 1}$	$LC_{50,tox\ 2}$	$LC_{50,tox\ 3}$	$LC_{50,tox\ 1}$	$LC_{50,tox\ 2}$	$LC_{50,tox\ 3}$
Species 1	32.89	38.48	5.25	30.12	32.98	35.60
Species 2	74.96	1.85	50.39	51.72	3.23	66.06
difference	x2.3	x17.7	x9.6	x1.7	x10.2	x1.9

Toxic unit ratio

To integrate the relationship between the concentration and the $ED_{50,h}$ and/or LC_{50} values toxic units can be used. These can be calculated for all the species and toxicants separately and will give a better idea of how much stress both species in the community have to endure. The idea behind looking at the difference in stress-induced on the species is to try and find if these influence the relative abundances in the community.

In this modelling set-up, it was observed that at higher toxic unit ratios the response on relative abundance of the most sensitive species was relatively similar for both the negative and positive sensitivity correlation and leads to co-existing species in most of the cases. By definition of the ratio, this means that the sum of toxic units for both species is similar and thus the stress level will also be similar. Although the toxic unit ratio does seem to not explain everything, for certain values of the toxic unit ratio both the sensitive and the insensitive species can be dominant. This is mainly observed in negatively correlated communities. For such a case are the LC_{50} values and toxic units per species and toxicant in table 5.2. Based on the sum of toxic units it can be expected that species 2 will be more sensitive than species 1, but when the community dynamics are inspected it is species 2 that is more abundantly present in the community compared to species 1 at the mixture concentration of 3 TU. For this specific case is the sum of toxic units close to each other, but the main contributing factor to this behaviour is the fact that toxic units do not take the slope of the concentration response curves (β) into account, just the LC/EC_{50}. What is happening here is that the response of species 2 to toxicant 1 (TU = 2.19) does not have a steep slope. So, with concentrations around the LC_{50} value this will not be a problem, but once the concentrations start to deviate more form the LC_{50} value the slopes start to become more important. For species 2 exposed to toxicant 1 doubling the concentration compared to the LC_{50} will still allow for 15% survival of the species after 21-days in this case, while for other toxicants with higher β values this can be much closer to 0.

Table 5.2: 21-day LC_{50} values for a negatively correlated community in which the sensitive species is wrongfully identified by using toxic units at concentrqtion level in the mixture of 3 TU

	$LC_{50,tox\ 1}$	$LC_{50,tox\ 2}$	$LC_{50,tox\ 3}$	$TU_{tox\ 1}$	$TU_{tox\ 2}$	$TU_{tox\ 3}$	TU_{sum}
Species 1	77.73	30.60	9.41	0.46	1.40	1.50	3.36
Species 2	16.28	59.82	21.23	2.19	0.72	0.67	3.58

Survival probability ratio

The reason why the survival ratio describes the data better than the toxic unit ratio is that here information on the shape of the concentration response curve is taken into account while calculating the toxic unit only uses the EC_x/LC_x value and thus ignoring information on the slope of the fitted curve. Although this works very well in this modelling setup, it is important to realise that it is expected that this ratio describes the data well. This is because the stressors can only have an effect on mortality in this model and not on other parameters. Nonetheless, it still seems to be a good indicator of the stress distribution between the species and thus explains relatively well that high ratios (the survival probability exposed to the mixture is similar for both species) give rise to co-existing species, while at lower ratios the more insensitive species will start to dominate the community.

5.1.4 Modelling approach

The way the modelling design was set up was in most of the cases related to mimic choices that were made in the microcosm setup. These include for example that the concentration ratios of the different toxicants are based on the geometric mean of the 21-day LC_{50} values to approach equitoxicity on the community level and the extra simulation with the narrower ED_{50} range all in an attempt to recreate the microcosm setup tested better *in silico*.

In the analysis of the results, a binary approach to correlation was used (positive and negative) and the negative correlation was defined by taking the positive correlation but with the $ED_{50,h}$ couple with the biggest natural logarithm difference exchanged between the species. The fact that the negative correlations are always defined the same way will affect the outcome. Nonetheless, can the choice be justified, because when the positive correlation is fixed the created negative correlation is the most negative that can be created without starting to switch the $ED_{50,h}$ between the different toxicants. This was avoided as this will have a significant impact on the concentrations calculated and thus the concentration ratios between the positive and negative correlation will be different.

By defining the different correlations on the physiological level the correlations are not necessarily the same as on the individual or even higher levels of organisation and thus are endpoint specific. So, can combining an ED_{50} value with a low h_{max} lead to less mortality than combining it with a high h_{max}. This can cause correlation analysis to differentiate between different endpoints and different levels of organisation. So, did it happen in 1 of the 100 positively correlated communities that one species was sensitive for all of the considered toxicants on the physiological level, but insensitive to all on the individual level (based on the 21-day LC_{50} values).

It was opted to use the same DEB-parameters for both species in the modelled community, namely the ones from a *Daphnia magna*. If other DEB parameters would have been chosen it can be expected that the equilibrium densities change due to difference in assimilation efficiency, body size, maintenance costs etc. , but that the community trait findings will be relatively similar. Choosing the same DEB parameters for both species was done to isolate traits that are related to the sensitivity to the toxicants as no other factors that would otherwise need to be accounted for are at play in this setup. Because both species are identical the effect of their relative abundance will not influence the equilibrium community size they evolve to, since both species have similar energy usage, growth to the same size etc. This is also why the effects of the community traits on the community size were rather limited. Either community, with one or both species surviving, move towards the total community size equilibrium or both die and the community size at equilibrium is zero. Once two different species, with their respective DEB parameters, are chosen or more effects of the toxicants than just mortality are considered the relative abundance will influence the equilibrium community size.

5.2 *Daphnia* microcosms

5.2.1 Community traits

Out of the 3 tested species does *Daphnia magna* seem to be the most insensitive of the 3 species based on the correlation analysis and the stress level based on the sum of toxic units for the mixture. An important contributing factor to the differences in the sum of toxic units is the fact that the LC_{50} value for copper is around 5 times higher for the free ion activities and around 2 times higher based on the dissolved concentrations compared to the 2 other species, which are the biggest differences observed. Both zinc and nickel differences between the LC_{50} values are a lot smaller (under 2 times difference) between the species. Thus in the mixture exposures with *Daphnia magna* (community 1 and 2), it is expected, based on the defined traits in the modelling, that *Daphnia magna* will be less affected by the toxicants and thus has more chances to dominate the mixture exposure compared to *Daphnia pulex* and *Daphnia longispina*. This response could not be clearly observed. A contributing factor is that *Daphnia magna* already was the most abundant species in the control, but except for small deviations no fully die off of the other species was observed. It is possible that no complete die off of a species in any treatment is observed because resource competition can be more complex than implemented in the model. So, can particle size of the food create a niche as some species will be better at dealing with larger or smaller particles and the full exclusion of a species seen in the modelling study thus might be an extreme response (Brooks and Dodson, 1965).

Community 3 is negatively correlated based on the dissolved correlations and defined as a positive correlation on the free ion level, but for the free ion level, this was just based on one toxicant. On the free ion level, the data also suggest a negative correlation when including Zinc in the analysis, which is not included because the LC_{50} lie within one standard error of each other, but this is a pair of LC_{50} values that are very close to being included. In the modelling study, it was observed that negatively correlated communities would be more likely to be experiencing synergistic effects at high mixture concentrations. This is also observed when community 3 is exposed to the mixture 2 treatment as where complete die off of both species occurred. Although it could be questioned if this is related to the community trait. If these species are exposed in another community, but with the same stress level they can survive. If a species dies without or with minimal competition (as both of the species die in community 3) it is more likely that it would have died because of the stress-induced reasons than because of interaction effects between the species. If they die just from the exposure concentrations it is not expected that *Daphnia pulex* and *Daphnia longispina* can survive in the other mixture treatments which are not true. A possible hypothesis for the survival of *Daphnia pulex* and *Daphnia longispina* in the other communities could be the difference in physicochemistry of the water caused by the species. It could be that in the communities with *Daphnia magna* more excretion products were present and thus altering the amount of organic carbon in the water, which influences the distribution of the metals in different fractions (dissolved, free ion etc.) and thus possibly altering the bioavailability (Winch *et al.*, 2002). Since pooled samples were taken over the communities this could not be verified.

With the small differences between the LC_{50} values for the same toxicants and in some cases relatively high standard error were reported, stating that the communities are positively or negatively correlated can be difficult. It was also observed that the effects found in the modelling study with an $ED_{50,h}$ range that can differ up to 100-fold shows more extreme responses than in the narrower range modelling (which approaches the microcosm study better). This might be a contributing factor to the limited links that can be observed between the defined community traits and the community endpoint effects together with the overall limited response of the communities in the microcosm study.

5.2.2 Microcosm design

An analysis of the effects of community traits on the community response solely based on experimental data would take more than 3 communities as changing the species in the communities would not only lead to changes in sensitivity to the different toxicants but other confounding factors will be introduced as different species will lead to different interactions between the species which will also have an effect on the community dynamics and need to be accounted for to compare the results between the different communities. Even then due to the complexity of the systems it still might only be able to explain part of the observed effects.

In these types of experiments with limited concentration levels (2 apart from the control), it is important that the right concentration range is chosen as concentrations too low will not result in any effect, while concentrations too high would lead to complete die off of the species. In a mixture setup, this complicates even more as the toxicants can interact with each other (Nys *et al.*, 2015). This is also the reason why the single metal exposures do not show a lot of response to the toxicants since strong effects in the single metals would likely lead to complete die off in the mixture treatments which is not that interesting to study.

Concentrations needed to be specified In the design phase of the microcosm to which the communities would be exposed. Since the interest is in community effects for mixture exposures an equitoxic design on the community level would be ideal as equitoxicity will make sure that the mixture response is not dominated by one toxicant. Since no community response data on the toxicants were available the individual level LC_{50} values were used to approximate equitoxicity on the community level. Note that with a calibrated and validated DEB-IBM model the equitoxic ratio on the community level could be estimated instead of using experimental data which can be expensive and time consuming to gather.

In the modelling study clear community could be identified, but applying the finding on the microcosm data was not very successful. It could be that the less pronounced effects that were observed in the modelling study with the narrower $ED_{50,h}$ range really start to fade away when applying the concepts on microcosm data because of biological variability, the extrapolation from the concepts to a system where the species are not equal, changes in bioavailability of the metals over time, interactions of the species etc.

5.3 Measures for sensitivity correlation

In the analysis of correlation, a qualitative approach to correlation was used (defining to possible scenarios: a positive and a negative correlation). In a more generalized approach to toxicity, it might be better to try and quantify the correlation in a way. 2 possible methods will be discussed, but due to time limitations were they not tested on their effectiveness on describing community responses.

The first method will be the more qualitative approach to expressing correlation. The same visual representation of correlation will be used as in section 4.1.4. As discussed can this plot be divided into 2 zones of importance. Above the 1:1 line, where the $ED_{50,h}$ of species y is higher than species x and the other way around. In this approach, it is suggested to count the data points that are below and above the 1:1 line (figure 5.1, left). When the $ED_{50,h}$ values for a certain couple are the same, add 0.5 to each of the zone counts. It is also possible to establish a zone around the 1:1 line where the toxicants are considered equal. This zone can be based on a certain percentage of the average of the $ED_{50,h}$ couple. A percentage approach is recommended as fixing the interval could lead to wrong conclusions *e.g.* with a fixed interval of

[y-1,y+1] an $ED_{50,h}$ couple of 2 and 3 would be classified as equal, while 102 and 103 too and both lie on the outer boundary of the interval. Raising the concentration from 2 to 3 in the first case will be more impact-full on the stress level the species endure than raising it from 102 to 103.

Once the zone counts are calculated, it is possible to calculate how evenly distributed the data points are over the 2 zones using Pielou's evenness index (equation 5.1).

$$J' = \frac{H'}{H'_{max}} \quad \text{with} \tag{5.1}$$

$$H'_{max} = \ln(S) \quad \& \tag{5.2}$$

$$H' = -\sum_{i=1}^{S} p_i * \ln(p_i) \tag{5.3}$$

with H' the Shannon diversity index (equation 5.2) and H'_{max} (equation 5.3) the maximum possible value of the diversity index. S is the number of groups (here always 2) and p_i the proportion of each group. When the data points are evenly distributed over the 2 zones the index will be equal to 1 and when all of the data points are in one zone the index is equal to 0. So, if all the data points are in one zone this means that one species is consistently less sensitive to all of the toxicants than the other species (in this case on the physiological level). This measure gives an overall idea of which species overall has higher/lower $ED_{50,h}$ values compared to other species, but does not take into account how big the difference is between the $ED_{50,h}$ values per toxicant.

The second measure is displaced on the right side of figure 5.1 and does try to take the difference of the $ED_{50,h}$ per toxicant into account. The difference of the natural logarithm of the $ED_{50,h}$ values will make the differences easier to sum for the different toxicants as this is will take into account the relative difference rather than the absolute difference of the $ED_{50,h}$ values per toxicant (equation 5.4).

$$\text{Correlation metric} = \left| \frac{\sum_{i=1}^{N} (\ln(ED_{50,h,i,species\ y}) - \ln(ED_{50,h,i,species\ x}))}{\sum_{i=1}^{N} |(\ln(ED_{50,h,i,species\ y}) - \ln(ED_{50,h,i,species\ x}))|} \right| \tag{5.4}$$

with i the i-th toxicant. So, the sum of the differences is divided by the sum of the absolute values of the differences. By this division, the sign of differences (and thus if the datapoint lies above the 1:1 line or below) determines if this ratio deviates from 1. Afterwards, the absolute value of this division is used as the correlation metric to make the range between 0 and 1 and making the ratio independent on which species is on the x- and which species is on the y-axis. A correlation metric equal to 1 would mean that one species is more sensitive to all of the toxicants it is exposed to compared to the other species (here on the physiological level). When the metric gets closer to 0 it means that both species are sensitive and insensitive for certain toxicants compared to the other species.

Both metrics can be expanded for more species and more toxicants if needed. The downside to both of the methods is that in no way the exposure concentration is included in this analysis, which in the first place even determines if there is an effect of the toxicant on the species. In an equitoxic set-up will these metrics thus probably perform better.

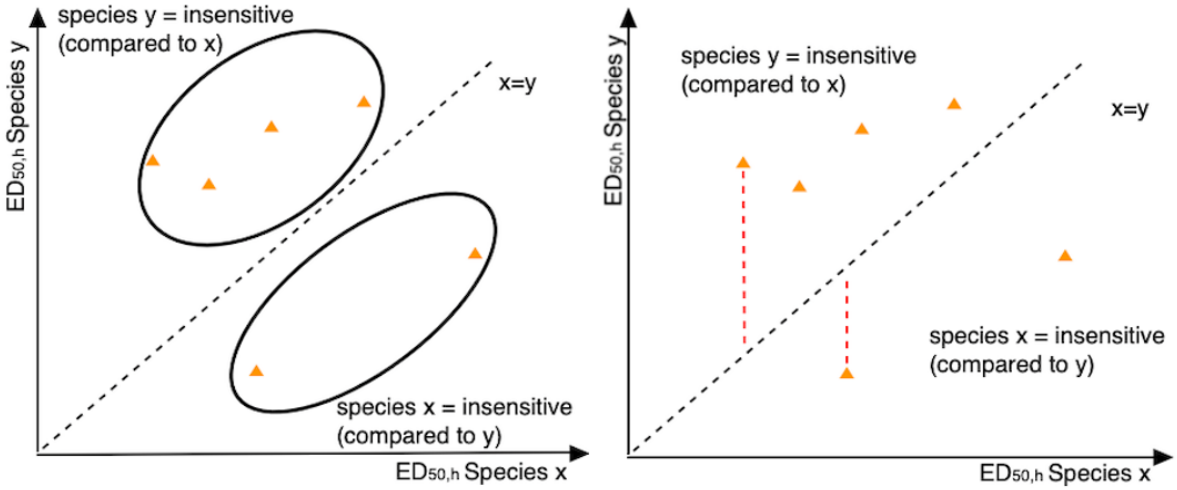


Figure 5.1: Visualisation of two approaches to quantify the sensitivity correlation, left: based on evenness, right: based on difference between the $ED_{50,h}$ per toxicant

6 | Conclusion

The goal of this thesis was to link experimentally observed data on mixture toxicity of zooplankton with the outcome of DEB-IBM simulations of similar systems to try and determine if traits related to the toxicological response of the species in the modelling study are also useful for describing experimental data. From the modelling study, it was possible to extract some relevant traits that (partially) explained the responses on the community level. Under the assumptions of the tested modelling setup, the communities with a negatively correlated sensitivity analysis were more likely to have a synergistic effect on community size at higher concentrations than positively correlated communities even with independent action assumed on the physiological level. Also, the sum of toxic units and 21-day survival probabilities of the species seemed to have a clear effect on the relative abundances of the species. The species which endures the least amount of stress based on these metrics were at high concentration levels often the most abundant species in the community.

Trying to project these finding on the outcome of the microcosm study was challenging and it became clear that even trying to mimic the experimental setup even more with a narrower $ED_{50,h}$ range the links between the microcosm study and modelling based on the defined community traits were minimal. The considered traits and findings in the modelling were proven wrong by comparing it to the microcosm study, but not a lot of effects of the toxicants were observed in the different treatments. This in combination with the possibility that other factors can influence the community-level response.

7 | Recommendations for further research

A first logical step as a follow-up of this thesis should be trying to determine if community traits can tell us something on the community response or if the concepts are just not inclusive enough or that other perhaps even more important factors play a role. To first assess if the traits concepts work it could be useful to select the testing organisms in the community setting based on the traits. To see if the findings of the modelling study explain anything at all it might be useful to try and maximize for example the differences between the LC_{50} values for the toxicants considered between the species. This will lead to stronger sensitivity correlations, lower toxic unit and lower survival probability ratios, which were in the modelling study the cases with the biggest effect on the community endpoints.

Another approach could be to see if the effects related to the traits that were observed in this simplified modelling setup are also observed when including for example more effects of the toxicants (like reduced assimilation, higher maintenance costs etc.) and using species that are not identical under the DEB framework. This way it can already be concluded if the finding from the simplified setup still is valid.

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A | Appendices

A.1 Confusion matrix of the species identification CNN

Table A.1: Confusion matrix for the species identification CNN, in the rows are the true labels and the columns are the predicted labels

	Daphnia longispina	*Daphnia magna*	*Daphnia pulex*
Daphnia longispina	0.815	0.037	0.138
Daphnia magna	0.042	0.930	0.029
Daphnia pulex	0.148	0.026	0.835

A.2 Modelling with concentrations based on $ED_{50,h}$

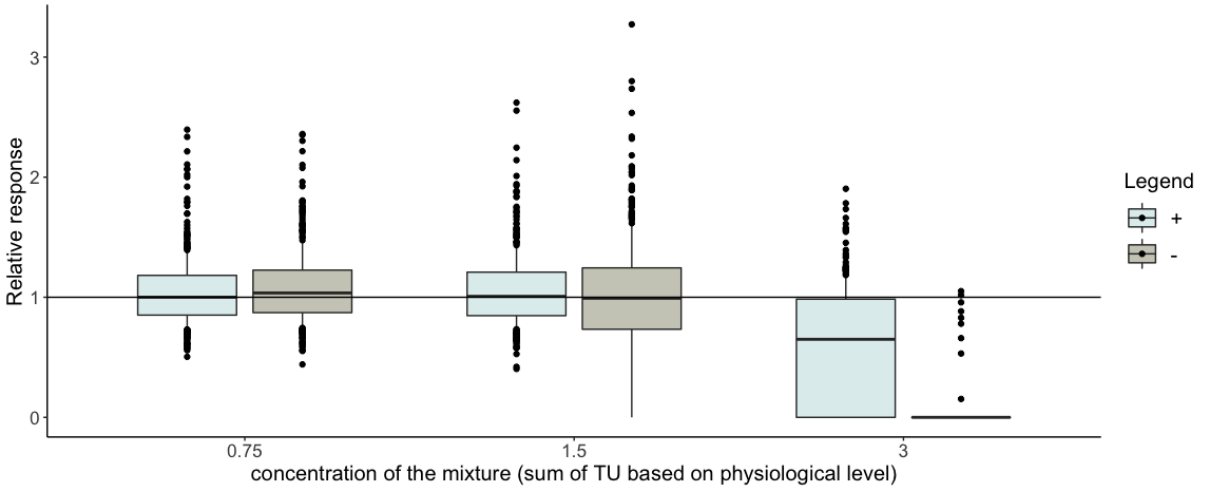

Figure A.1: Simulated relative community size, compared to IA, at equilibrium for the mixture exposure with concentrations based on the $ED_{50,h}$ values, +: positive correlation and -: negative correlation and the box containing the 25^{th} to 75^{th} percentile and the dots the 0^{th} to 5^{th} and 95^{th} to 100^{th} percentile

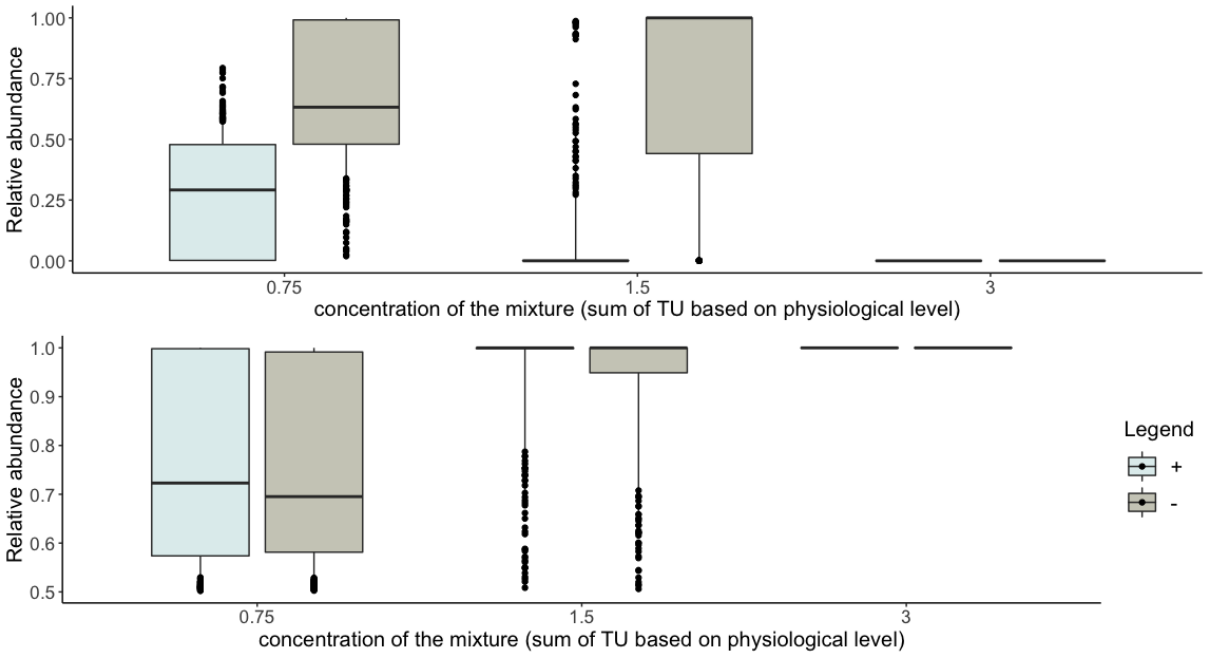

Figure A.2: Simulated relative abundance species, based on individual count, at equilibrium for the mixture exposure with concentrations based on the $ED_{50,h}$ values. Bottom graph: abundance of the dominant species, top graph: abundance of species 1, +: positive correlation and -: negative correlation and the box containing the 25^{th} to 75^{th} percentile and the dots the 0^{th} to 5^{th} and 95^{th} to 100^{th} percentile

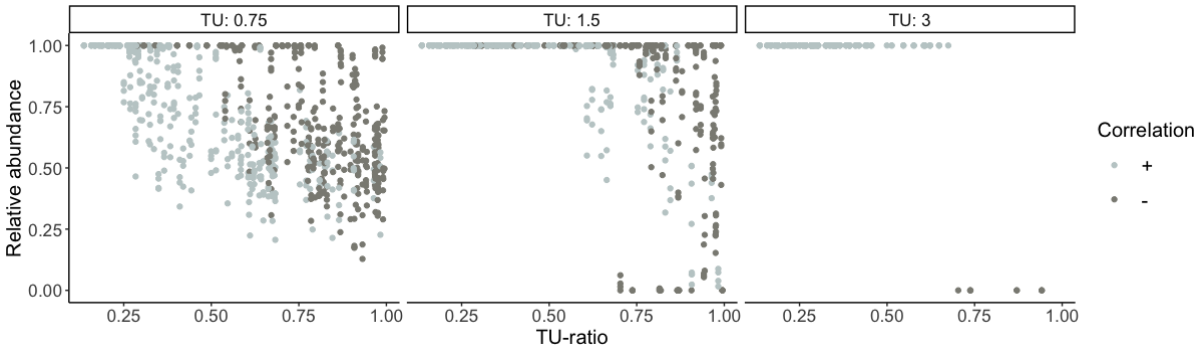

Figure A.3: Relative abundance species based on individual count in function of toxic unit ratio at equilibrium for the concentration levels defined at the physiological level with +: positive correlation and -: negative correlation

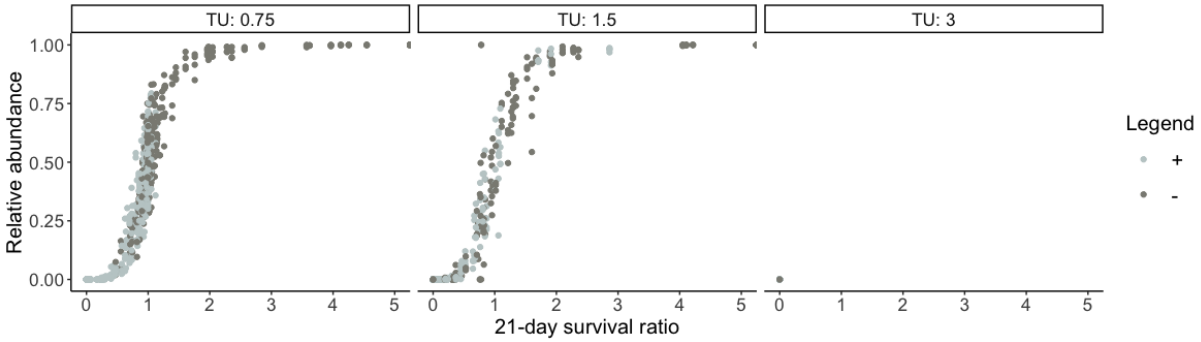

Figure A.4: Relative abundance species based on individual count in function of 21-day survival ratio at equilibrium or the concentration levels defined at the physiological level with +: positive correlation and -: negative correlation

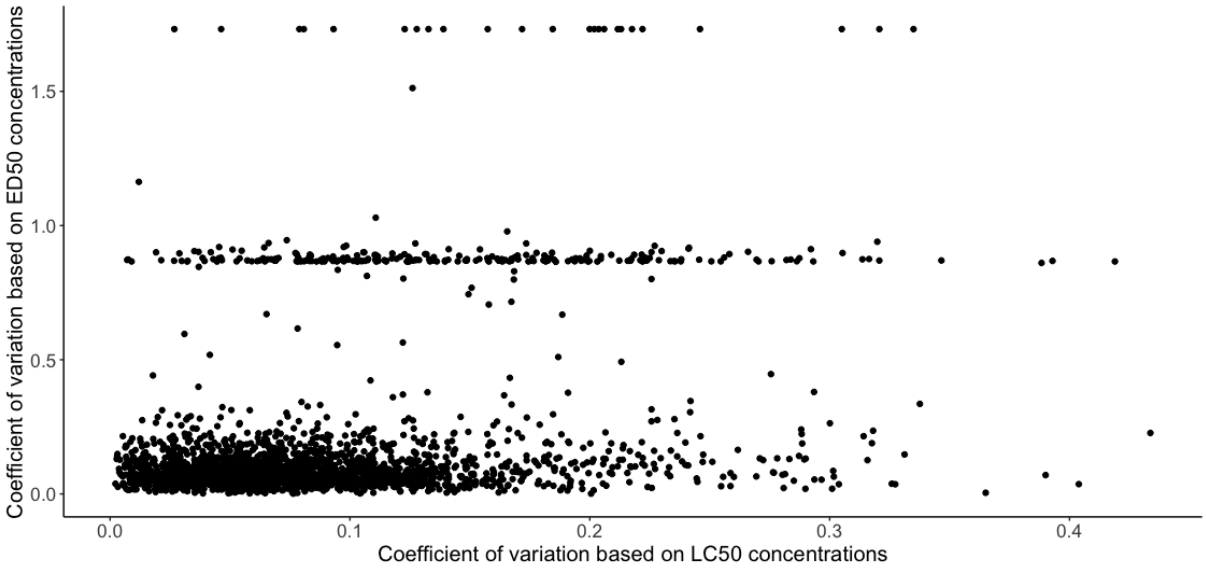

Figure A.5: Coefficient of variation based on LC_{50} plotted against coefficient of variation based on $ED_{50,h}$

A.3 Community size of the microcosms for the last week (day 49 to 56)

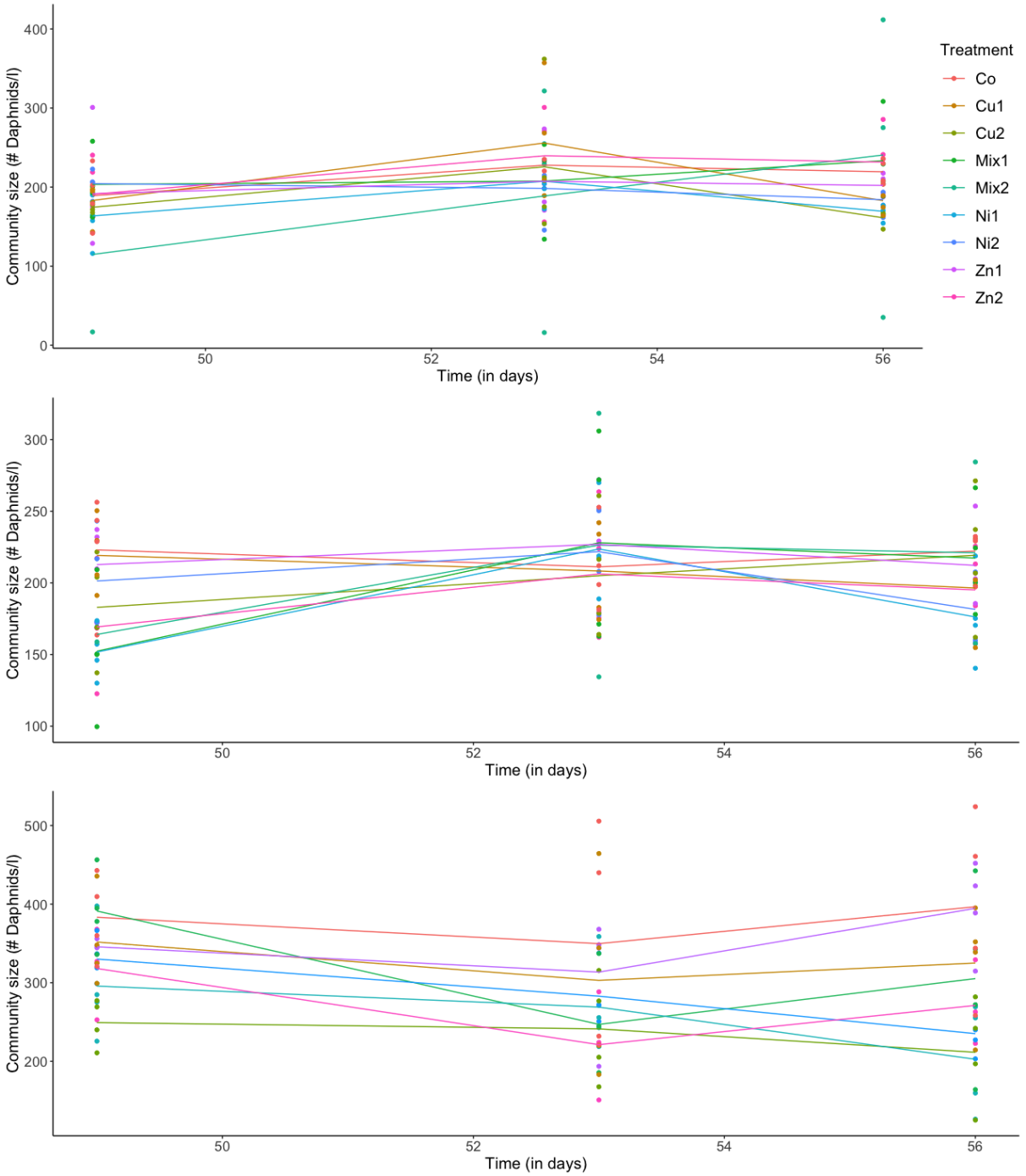

Figure A.6: Community sizes for the last week in the microcosm with the upper plot for community 1, middle plot for community 2 and lower plot for community 3

A.4 Pixel area of the communities on day 56

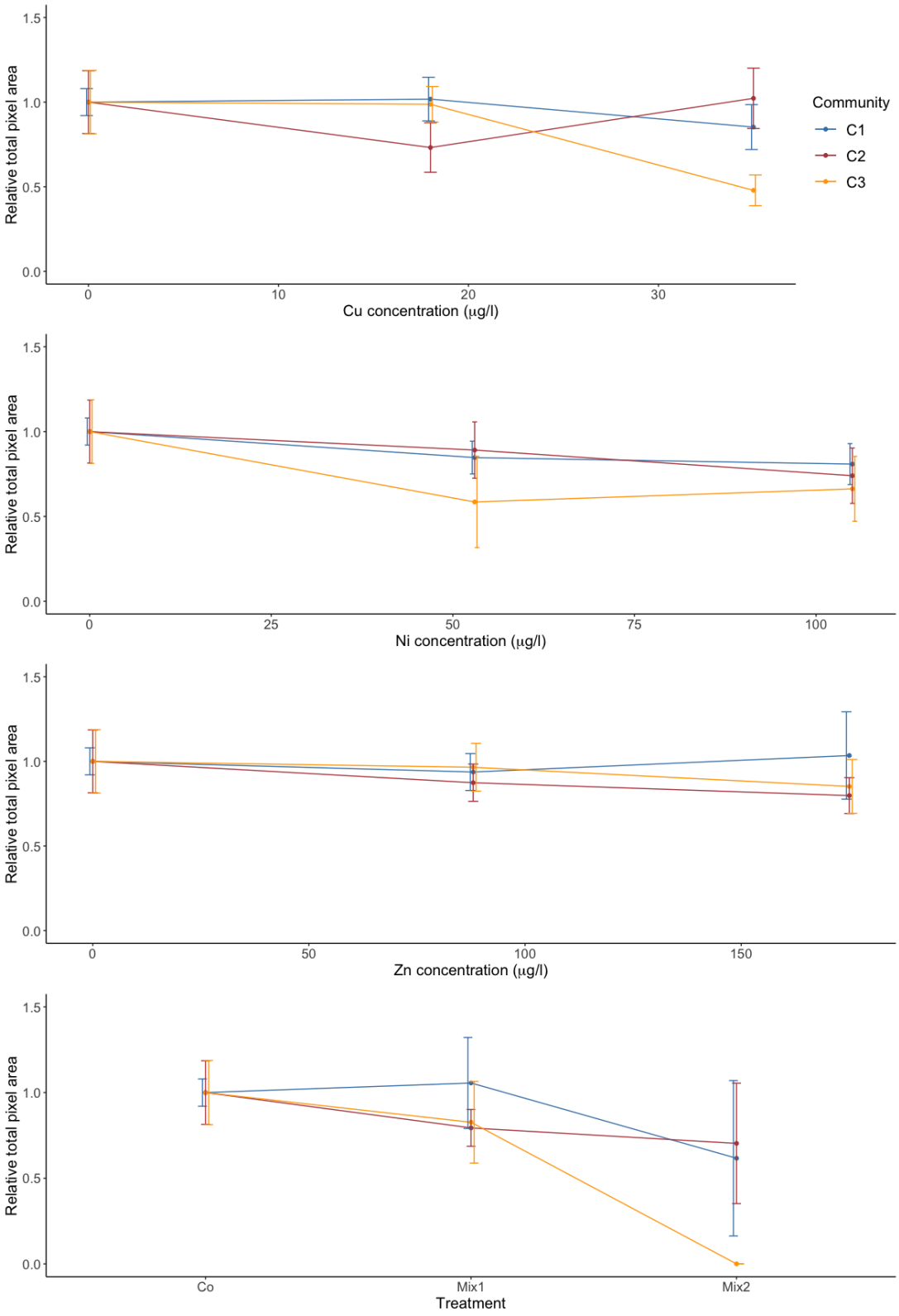

Figure A.7: Total pixel area of the communities on day 56 from top to bottom: copper, nickel, zinc and mixtures. Replicates without survival not included except for community 3 exposed to mixture 2

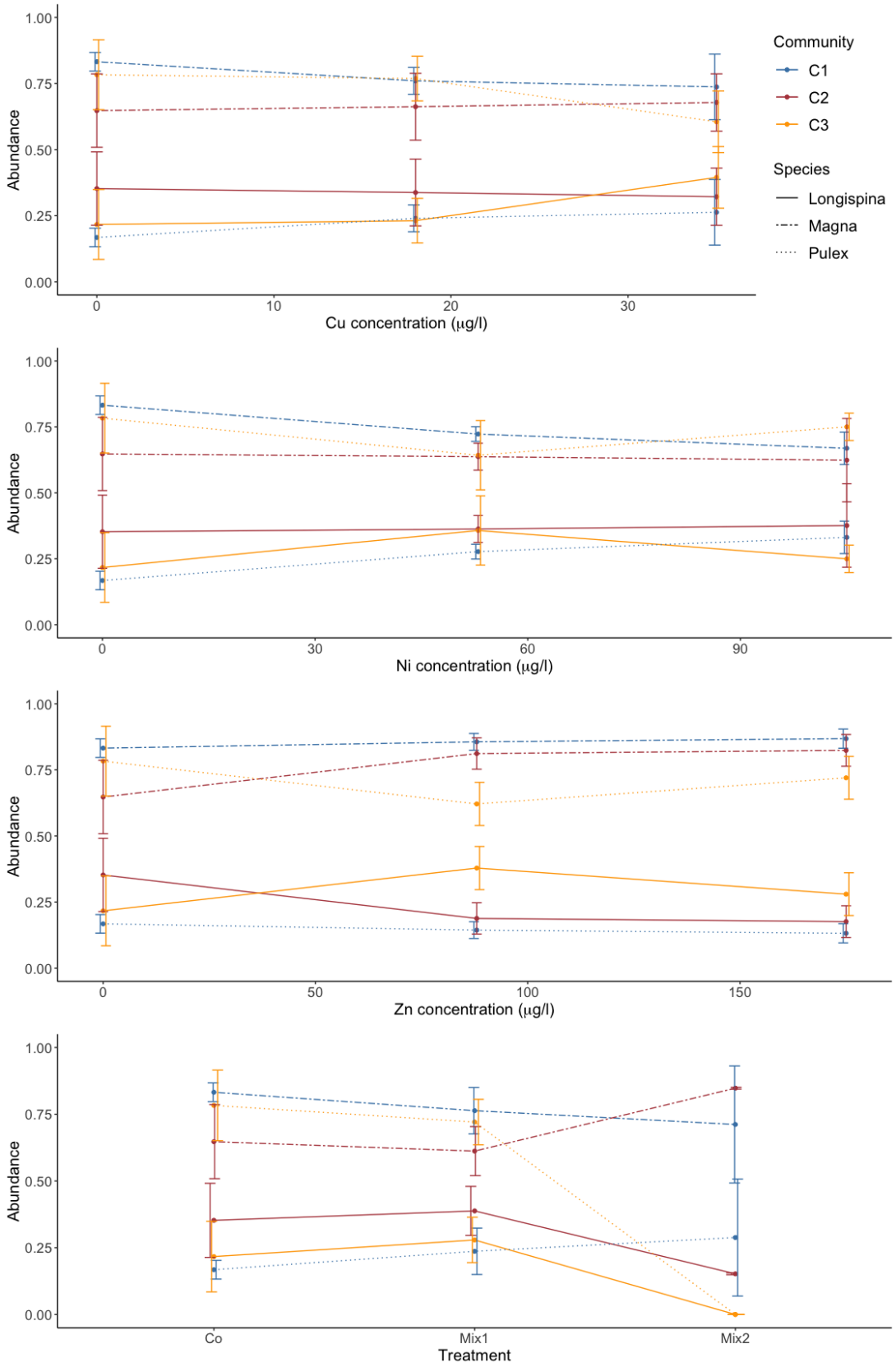

Figure A.8: Abundance of the species in the communities based on the pixel count on day 56 from top to bottom: copper, nickel, zinc and mixtures. Replicates without survival not included except for community 3 exposed to mixture 2

A.5 Measured metal concentrations in the microcosm at day 56

Table A.2: Modelled free ion activities (in nM) at day 56 for the different treatments

Treatment	Ni free	(±sd)	Cu free	(±sd)	Zn free	(±sd)
Co	1.19	0.56	0.00	0.00	6.87	4.77
Cu 1	2.82	1.44	0.07	0.09	5.32	4.36
Cu 2	4.21	2.45	0.37	0.60	4.74	3.64
Ni 1	146.04	61.35	0.01	0.02	5.89	4.96
Ni 2	383.93	126.78	0.02	0.03	5.44	3.95
Zn 1	3.44	1.13	0.01	0.01	189.75	67.09
Zn 2	3.99	2.10	0.01	0.02	456.25	202.14
Mix 1	201.63	66.12	0.23	0.23	237.94	94.23
Mix 2	517.56	130.24	0.79	0.94	559.44	195.63

Table A.3: Measured dissolved concentrations (in μg/l) at day 56 for the different treatments

Treatment	Ni dis	(±sd)	Cu dis	(±sd)	Zn dis	(±sd)
Co	2.32	1.02	1.18	0.83	3.44	2.42
Cu1	1.79	0.85	12.24	1.71	1.99	1.61
Cu2	1.59	0.59	24.20	3.11	1.55	1.18
Ni1	39.94	3.98	1.19	1.41	1.88	1.56
Ni2	80.65	6.00	1.04	1.27	1.56	1.17
Zn1	2.20	0.50	1.41	1.17	56.35	7.43
Zn2	1.78	0.64	1.07	1.12	114.14	16.77
Mix1	41.92	3.60	11.27	2.07	55.50	10.21
Mix2	83.68	7.71	17.27	5.58	112.71	20.80

Table A.4: Fractions of the total metal concentrations that is absorbed to Dissolved Organic Carbon (DOC) with proxy Fulvic Acid (FA) in speciation modelling

treatment	Ni to FA	sd	Cu to FA	sd	Zn to FA	sd
Co	0.94	0.01	0.86	0.36	0.67	0.67
Cu1	0.83	0.07	1.00	0.00	0.46	0.46
Cu2	0.72	0.12	0.99	0.01	0.48	0.48
Ni1	0.61	0.10	0.56	0.51	0.49	0.49
Ni2	0.49	0.10	0.66	0.48	0.53	0.53
Zn1	0.82	0.07	0.87	0.34	0.66	0.66
Zn2	0.75	0.12	0.75	0.45	0.61	0.61
Mix1	0.49	0.11	0.99	0.01	0.57	0.57
Mix2	0.34	0.08	0.98	0.01	0.50	0.50

A.6 DEB-IBM used with narrower $ED_{50,h}$-range

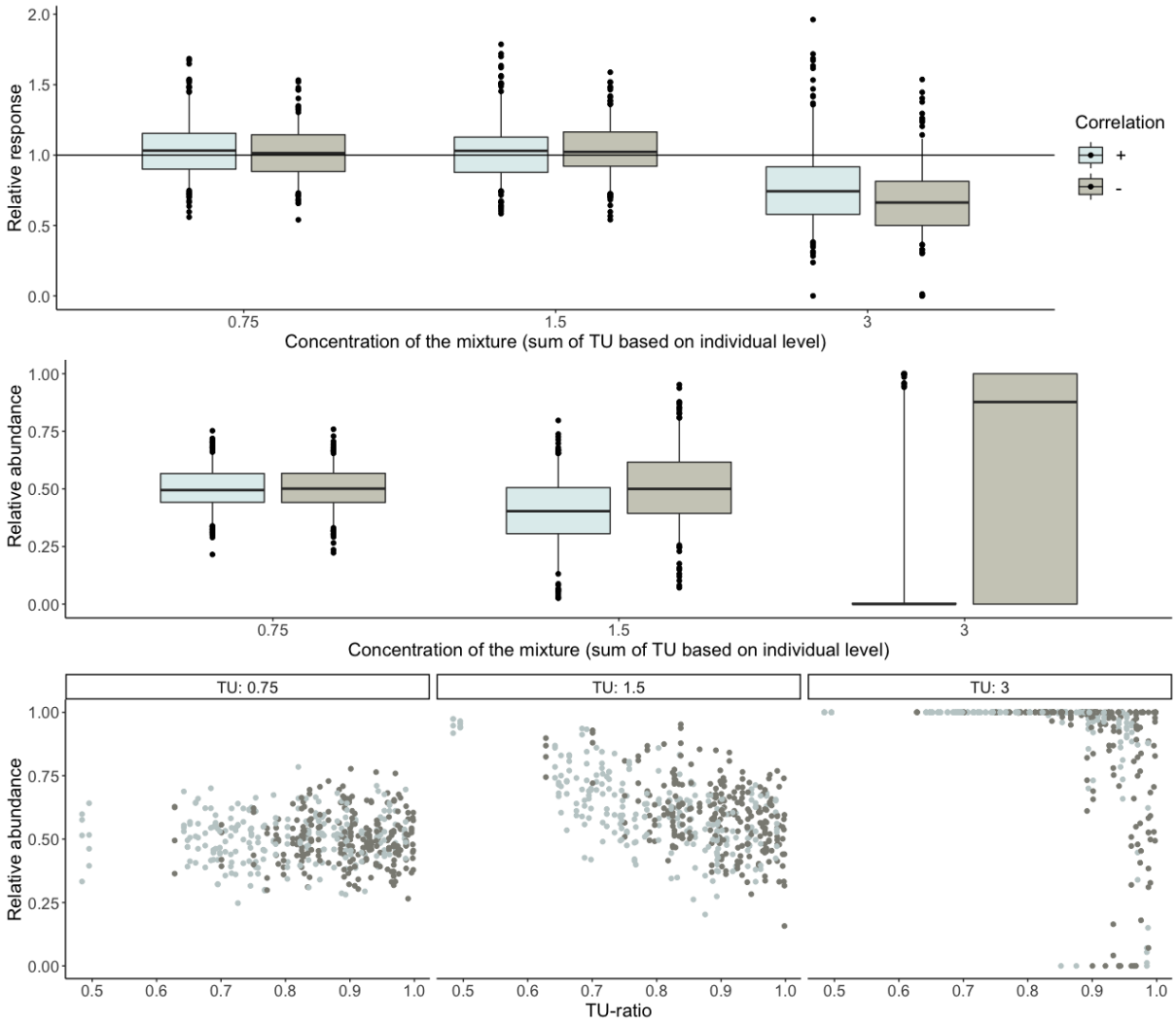

Figure A.9: Outcome of DEB-IBM modelling with narrower $ED_{50,h}$ range from top to bottom: relative response compared to IA, abundance of species 1 and abundance of the most sensitive species, based on toxic units