“Faith is taking the first step even when you don’t see the whole staircase”

(Martin Luther King Jr.)

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The combined and interactive effects of zinc, temperature and phosphorus on freshwater planktonic communities

Thesis submitted in fulfilment of the requirements for the degree of

Doctor (Phd) in Applied Biological Sciences

Dutch translation of the title:

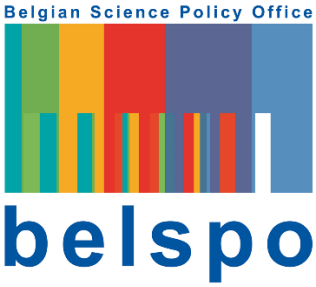
Het gecombineerde en interactieve effecten van Zn, temperatuur en fosfor op zoetwater gemeenschappen

Refer to this work as:

Van de Perre, D., 2018: The combined and interactive effects of Zn, temperature and phosphorous on freshwater communities. Thesis submitted in fulfilment of the requirements for the degree of Doctor (PhD) in Applied Biological Sciences, Ghent University, Ghent, Belgium.

ISBN-number: 978-9-4635707-7-0

The research conducted during this PhD dissertation was finically supported with funding from BELSPO IAP projects AquaStress (P7/31)



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List of abbreviations

ANOVA	Analysis of variance
BLM	Biotic ligand model
BOD5	5-day biochemical oxygen demand
DO	Dissolved oxygen
DIC	Dissolved inorganic carbon
DOC	Dissolved organic carbon
DOM	Dissolved organic matter
ECx	Effect concentration for x% of the organisms
ERA	Ecological risk assessment
HC5	5% hazardous concentration
HC50	50% hazardous concentration
IA	Independent action
LCx	Lethal concentration for x% of the organisms
LOEC	Lowest observed effect concentration
MATC	Maximum acceptable toxicant concentration
NOEC	No observed effect conversation
P	Phosphorus
PCA	Principal component analysis
PNEC	Predicted no effect concentration

PRC	Principal response curves
RDA	Redundancy analysis
SRP	Soluble reactive phosphorus
SSD	Species sensitivity distribution
T	Temperature
TOC	Total organic carbon
TP	Total phosphorus
WQC	Water quality criteria

Summary

The main goal of the ecological risk assessment of chemicals (ERA) is the protection of populations and communities and the correct effect assessment of chemicals on the structure and functioning of aquatic ecosystems. At present, ERA is mainly based on data obtained from standard ecotoxicity experiments. These experiments are typically conducted under standardized optimal conditions, at the species level and exposed at a single stressor at the time. However, these general ERA approaches are in sharp contrast with natural conditions. Natural populations and communities are often exposed to a mixture of multiple stressors that are biotic (e.g. food shortage, predation) and abiotic (e.g. eutrophication, non-optimal temperature or water chemistry, metals). Species interactions such as predation and competition for food are two major biotic factors that are able to significantly affect the responses of organisms to toxicants. Additionally, abiotic factors such as temperature (T) can also play an important role affecting the toxic effects of chemical pollutants (e.g. by influencing its bioavailability and toxicokinetics). Therefore, by ignoring ecological interactions and by not considering natural field conditions these single-species tests oversimplify the actual field situation and ERA may not be protective. **The aim of this PhD thesis was to investigate the combined effect of Zn with natural environmental stressors (temperature and/or phosphorous) at different organization levels (population vs. community) on freshwater organisms in order to increase the realism of current ERA**.

Do environmental factors such as temperature (T) and phosphorous (P) affect Zn toxicity to a freshwater plankton community? In Chapters 2 and 3 this was assessed by exposing a freshwater plankton community (zooplankton, phytoplankton and protozoa) to different levels of Zn (Reference, $HC5_{\text{-plankton}}$, $HC50_{\text{-plankton}}$), T (Reference

and a warmer regime) and P (Reference: low P addition and high P addition) in a full factorial design. During this experiment the abundances of plankton species, the community metabolism and the general properties of the water were monitored during 5 weeks.

Chapter 2 focused on the part of the freshwater microcosm study that was conducted to assess the direct and indirect effects of Zn on the community structure and function of a freshwater plankton community at a single level of T and P. At the highest Zn treatment ($HC50_{-plankton}$: predicted hazardous concentration for 50% of the organisms only based on the chronic toxicity data of plankton species that was first normalized to the target chemical properties of the water by using the Zn BLM's) a significant reduction in cladocerans increased the rotifers, ciliates and phytoplankton abundances. Additionally, the phytoplankton community shifted in dominance from grazing-resistant to edible species. In contrast to the SSD (Species sensitivity distribution) predictions, which identified phytoplankton as the most sensitive groups, only the total chlorophyll and two phytoplankton taxa were adversely affected at the highest Zn treatment. The HC5-plankton estimated from the bioavailability-normalized SSD was overall protective for the plankton community, however, the SSD was not able to correctly predict the species sensitivity ranking within their community context at the $HC50_{-plankton}$.

In chapter 3 it was assessed to what extent the toxicity of Zn is affected by temperature and phosphorus supply and how these T & P effects on Zn toxicity vary between the levels of organisation (population, functional group and community) and their endpoints in a community. Consistent interactions between Zn and T were only rarely found at the species level (4%), but were more frequently found at the functional group level (36%), for community structure (100%) and for community function (100%, such as Dissolved Organic Carbon concentrations and total chlorophyll). The majority of the Zn

× T interactions were only observed at HC50-plankton and generally indicated a smaller negative effect of Zn on these endpoints at higher T. The results thus suggest that higher T, e.g. related to global warming, decrease the toxic effects of the HC50-plankton Zn concentration on these endpoints. It is possible, however, that T itself was the main factor affecting the community (e.g. composition, species interactions) and this could obscure some of the expected Zn effects at the population and group level. Furthermore, P poorly affected the effects of Zn on the community at any level of organization. Interestingly however, 90% of all the Zn × T interactions observed at the species, group and community composition level were found at high P addition. However, a real explanation for this phenomenon could not be found within this PhD and needs further research. At the community level the different plankton groups (Zooplankton, phytoplankton and protozoa) were only consistently affected at the HC50-plankton under high P addition (cold and warm). However, under warm low P addition the phytoplankton community composition was consistently affected at the HC5-plankton and thus not protective for the plankton community. Collectively, our study with the model chemical Zn, suggests that temperature and phosphorus loading to freshwater systems should be accounted for in risk assessment, as these factors may modify the effects of chemicals on the structure and functioning of aquatic communities. Not doing so may underestimate risks in some and overestimate risks in other systems, depending on their temperature and phosphorous loading.

In Chapter 4 the combined effects of interspecies interaction (food competition), temperature and Zn was assessed by conducting a simple community experiment. Here, Daphnia longispina populations were exposed to different Zn, temperature and interspecific competition levels (No interspecific Brachionus competition= no Brachionus calyciflorus added; interspecific Brachionus competition= B. calyciflorus

added). Interspecific Brachionus competition and temperature by itself had a limited effect on the Daphnia abundances, but significantly interacted with the highest Zn concentration. Without Brachionus competition the highest Zn treatment had a stronger negative effect on the D. longispina population in the warm regime than in the cold regime. However, with Brachionus competition the highest Zn treatment had a reduced negative effect on the D. longispina juvenile abundances in the warm regime. This is probably due the fact that at the highest Zn treatment the B. calyciflorus were more numerous under cold conditions than under warmer conditions. Under cold condition the highest Zn treatment affected the juvenile abundance more negatively when Brachionus was present. Possibly the competition for food reduced the amount of energy that could be used for (1) reproduction (dynamic energy budget theory), resulting in fewer juveniles, or (2) to maintain enough energy to maintain normal body function when the metabolic costs increased due to Zn stress which could affect toxic effects observed at the population level. The present study illustrates that the influence of temperature and interspecific competition on the effect of Zn on the D. longispina abundance and should be considered when assessing ecological risks of chemicals.

In Chapter 5 the findings of this PhD thesis are combined, reviewed and summarized. Additionally, suggestions and possible directions for future research are provided in this chapter. Conventional ERA is generally based on the extrapolation of single-species ecotoxicity data to natural populations and communities (e.g. SSD method). Here, we tried to determine whether population level effects (single and interactions) of chemicals, observed in lower-tier experiments (as in Chapter 4) are similar to population level effects during high-tier experiments (as in Chapters 2 and 3). Stated otherwise: can lower-tier results be extrapolated to higher-tier experiments or to natural aquatic ecosystems? For this purpose, a D. longispina population experiment

(exposing D. longispina to different Zn and temperature) was conducted and was compared with the D. longispina population results from Chapters 2-4. At the highest Zn treatment the Zn effects observed in the lower-tier experiments are similar to population level effects during high-tier experiments. For D. longispina consistent Zn × T interactions were only observed in the lower-tier experiments at the highest Zn concentration. At the lowest Zn concentration the Zn effects goes from no effect (D. longispina population), to a positive effect ("D. longispina + small rotifers" community and "D. longispina + B. calyciflorus + small rotifers" community) to a negative effect (D. longispina in complex plankton community). The most likely explanations for this difference are biotic interactions. These biotic interactions can be very complex and can modify or even mask toxic effects of toxicants. In the complex community study (Chapter 2 and 3) for example, Mesostoma sp. predated selectively on the Daphnia populations, and it is unclear if the D. longispina population declines, in Chapters 2-3, were induced by Zn toxicity, Mesostoma sp. predation, a combination of both or by inter- and intra-specific interactions which could have had an effect on the species sensitivity to toxicants. The results from this PhD thesis indicate the importance of species interactions, T and P on Zn toxicity effects on aquatic organisms. By ignoring biotic interactions and environmental conditions ERA is in sharp contrast with natural conditions and the extrapolation of conventional ecotoxicological results from individuals to populations and ecosystems could be dubious. Therefore we believe that the combination of higher-tier experiments and ecological models are crucial for correctly predicting effects of chemicals on populations and communities.

Samenvatting

Het hoofddoel van de ecologische risico assessment (ERA) van chemicaliën is de bescherming van populaties en gemeenschappen en de correcte beoordeling van de effecten van chemicaliën op de structuur en functionering van aquatische gemeenschappen. Momenteel is de ERA vooral gebaseerd op data, verkregen uit standaard ecotoxicologische experimenten. Deze experimenten werden typisch uitgevoerd onder gestandaardiseerde, optimale condities, op soort niveau en slechts blootgesteld aan één stressor tegelijk. Echter staat deze algemene ERA benadering in sterk contrast met natuurlijke condities. Natuurlijke populaties en gemeenschappen worden vaak blootgesteld aan een verscheidenheid van multipele biotische (bv. voedselschaarste, predatie) en abiotische (bv. eutroficatie, non optimale temperatuur of water chemie, metalen) stressoren. Soortinteracties zoals predatie en competitie voor voedsel zijn twee belangrijke biotische factoren die een invloed hebben op hoe toxicanten organismen affecteren. Abiotische factoren zoals temperatuur (T) kunnen ook een belangrijke invloed hebben op de toxische effecten van toxicanten door het beïnvloeden van de bio beschikbaarheid en toxicokinetiek. Single-species testen zijn een over-simplificatie van natuurlijke omstandigheden doordat ze ecologische interacties negeren en geen rekening houden met de natuurlijke condities waardoor ERA mogelijk niet beschermend genoeg is. Het doel van deze PhD thesis was het onderzoeken van het gecombineerde effect van Zn met natuurlijke stressoren (fosfor en/of temperatuur) bij verschillende levels van organisatie (populatie vs. gemeenschap) op zoetwater organismen met als doel het verhogen van het realisme van ERA.

Kunnen omgevingsvariabelen zoals temperatuur (T) en fosfor (P) de Zn toxiciteit affecteren op een zoetwatergemeenschap? In hoofdstukken 2 en 3 werd onderzocht of temperatuur en fosfor een effect kunnen hebben op de Zn toxiciteit effecten op een

zoetwater gemeenschap. Dit was onderzocht door een zoetwater plankton gemeenschap (zoöplankton, fytoplankton en protozoa) bloot te stellen aan verschillende Zn (Referentie, HC5-plankton en HC50-plankton), T (Referentie en warm regime) en en P levels (Referentie en hoge P additie) in een full factorial design. Tijdens dit experiment de plankton abundantie en de generale waterkwaliteit parameters werden gemonitord gedurende vijf weken.

In Hoofdstuk 2 wordt de nadruk gelegd op het onderzoeken van de directe en indirecte effecten van Zn op de gemeenschapsstructuur en functie van een zoetwater planktonische gemeenschap. Bij de hoogste Zn concentratie (HC50-plankton) resulteerde de significante reductie van de cladoceren in de verhoging van de rotifeer, ciliaat en fytoplankton abundanties. Bovendien was er een verschuiving in de fytoplanktongemeenschap van een begrazings-resistente naar een begrazing gevoeligere gemeenschap. In contrast met de Single-Species Distributie (SSD) voorspellingen, die de fytoplanktongemeenschap als meest gevoelig voorspelde, werden enkel de totale chlorofyl en 2 fytoplankton taxa negatief beïnvloed door de hoogste Zn concentratie. Dus hoewel de HC5-plankton concentratie, die voorspeld werd door middel van de bio beschikbaarheid-genormaliseerde SSD, beschermend was voor de plankton gemeenschap, voorspelde de SSD niet de correcte soort gevoeligheid binnenin de gemeenschap.

In hoofdstuk 3 werd er onderzocht hoe de Zn toxiciteit werd beïnvloed door temperatuur en fosfor aanvoer en hoe deze T en P effecten op Zn toxiciteit variëren binnen de verschillende organisatorische niveaus (populatie, functionele groep en gemeenschap) en hun eindpunten binnenin een gemeenschap. Consistente interacties werden tijdens deze studie maar zelden waargenomen op soort niveau (4%), maar kwamen frequenter voor op groep (36%) niveau, gemeenschapsstructuur

(100%) en functie (100%, bv. totale chlorofyl). De meerderheid van de Zn × T interactions werden waargenomen bij HC50-plankton en verwezen naar een kleine Zn effect bij hogere T. Voorts werden er geen indicaties gevonden dat hoge P additie op zichzelf een effect had op de globale Zn toxiciteit. Belangstelling wekkend was het feit dat 90% van alle Zn × T interactions gevonden werden bij hoge P additie. Een explinatie voor dit phenomeen kan echter niet gevonden worden binnenin dit onderzoek. Op gemeenschapsniveau werden de verschillende plankton gemeenschappen bij hoge P additie enkel geaffecteerd bij HC50-plankton. Bij hoge T en lage P echter werd de fytoplanktongemeeschap reeds geaffecteerd bij de HC5-plankton en bleek dus niet beschermend voor de gemeenschap. De resultaten van hoofdstukken 2 en 3 illustreren dat T en P een effect kunnen hebben op Zn toxiciteitseffecten op een zoetwater planktonische gemeenschap. Hierdoor zouden zowel T als P als factoren in beschouwing moeten genomen worden voor risk assessment, omdat het niet incorporeren ervan kan leiden tot het onder-of over voorspellen van toxische effecten.

In hoofdstuk 4 werd het gecombineerde effect van interspecifieke interactie (voedselcompetitie) van temperatuur en Zn onderzocht door middel van het uitvoeren van een simpel gemeenschap experiment. Hiervoor werd een Daphnia longispina populatie experiment uitgevoerd, waarbij het werd blootgesteld aan verschillende Zn, T en interspecifieke competitie levels (Geen interspecifieke Brachionus competitie= geen Brachionus calyciflorus toegevoegd; interspecifieke Brachionus competitie= B. calyciflorus toegevoegd). Interspecifieke Brachionus competitie en temperatuur op zichzelf hadden een beperkt effect op de Daphnia abundanties, maar ze interageerden significant met de hoogste Zn concentratie. Zonder Brachionus competitie had de hoogste Zn concentratie een sterker negatief effect op de D. longispina populatie bij

warme condities. Wanneer er echter wel Brachionus competitie was, had de hoogste Zn concentratie een verminderd negatief effect op de D. longispina juveniele abundanties bij warme condities. Dit kan waarschijnlijk verklaard worden doordat de B. calyciflorus bij de hoogste Zn concentratie meer abundant waren onder koude omstandigheden dan onder warme. Onder koude omstandigheden had de hoogste Zn concentraties een groter negatief effect op de juveniele abundanties wanneer blootgesteld aan Brachionus competitie. Mogelijks resulterende de voedselcompetitie voor een verminderde hoeveelheid energie die gebruikt kon worden voor (1) voortplanting, wat resulteerde in minder juvenielen, of (2) voor het onderhouden van voldoende energie voor het onderhouden van de normale lichaamsfuncties wanneer de metabolisme kosten stijgen door Zn stress. Deze studie illustreert het effect van temperatuur en interspecifieke competitie hebben op de effecten van Zn op de D. longispina abundanties en zouden in rekening moeten gebracht worden bij ERA.

In hoofdstuk 5 werden de bevindingen van deze PhD thesis gecombineerd en samengevat. Aanvullend werden er in dit hoofdstuk ook suggesties gedaan naar toekomstig onderzoek. Conventioneel ERA is vooral gebaseerd op de extrapolatie van single-species ecotoxicologische data naar natuurlijke populaties en gemeenschappen (bijvoorbeeld doormiddel van de SSD methode). In hoofdstuk 5 werd getracht te bepalen of populatie level effecten (single en interacties) van chemicaliën, geobserveerd in lagere-tier experimenten (zoals in hoofdstuk 4) dezelfde zijn als effecten geobserveerd in hogere-tier experimenten (zoals in hoofdstukken 2 en 3). Anders gezegd: kunnen lagere-tier resultaten worden geëxtrapoleerd naar hogere-tier experimenten of naar aquatische ecosystemen? Om dit te onderzoeken werd een D. longispina populatie (D. longispina populatie blootstellen aan verschillende Zn en temperatuur) experiment uitgevoerd en vergeleken met de D. longispina populatie

resultaten van hoofdstukken 2-4. Bij de hoogste Zn concentratie werd geobserveerd dat de Zn effecten in de laagste-tier experimenten gelijkaardig zijn aan de populatie level effecten in de hoge-tier experimenten. Bij de laagste Zn concentratie kon vastgesteld worden dat de Zn effecten variëren van geen effect (D. longispina populatie), tot een positief effect ("D. longispina + kleine rotiferen" gemeenschap en "D. longispina + kleine rotiferen + B. calyciflorus" gemeenschap) en tot een negatief effect (D. longispina in een complexe plankton gemeenschap). Voor D. longispina werden consistente Zn × T interacties enkel geobserveerd in de lagere-tier experimenten bij de hoogste Zn concentratie. Bij de hoogste Zn concentratie werd geobserveerd dat de Zn effecten in de laagste-tier experimenten gelijkaardig zijn aan de populatie level effecten in de hoge-tier experimenten. De meest waarschijnlijke oorzaak voor dit verschil zijn biotische interacties. Deze biotische interacties kunnen heel complex zijn en deze kunnen de effecten van stressoren modificeren of zelfs maskeren. In de complexe gemeenschapsstudie (hoofdstuk 2-3) bijvoorbeeld, predateerde Mesostoma sp. zeer selectief op de Daphnia populaties. Hierdoor werd het onduidelijker of de geobserveerde populatie dalingen van de Daphnia het resultaat waren van Zn, Mesostoma sp. of door een combinatie van de 2. De resultaten van deze PhD thesis tonen de belangrijkheid aan van soorten interacties, T en P op de toxische effecten van Zn op aquatische organismen. Door biotische interacties en omgevingsfactoren te negeren staat ERA in sterk contrast met de natuurlijke condities en daarbij kan de extrapolatie van conventionele ecotoxicologische resultaten van individuen naar populaties en ecosystemen dubieus zijn. Hieruit werd afgeleid dat de combinatie van hogere-tier experimenten en ecologische modellen cruciaal zijn voor het correct voorspellen van effecten van chemicaliën op populaties en gemeenschappen.

1

General introduction and conceptual framework

1.1 ENVIRONMENTAL RISK ASSESSMENT

The main goals of the ecological risk assessment (ERA) of chemicals are the protection of populations or higher levels of organisation (communities) and the correct effect assessment of chemicals on the structure and functioning of aquatic ecosystem [1,2]. Currently, **conventional risk assessment** is mainly based on data obtained from standard ecotoxicity experiments, that are conducted under standardised optimal conditions (e.g. temperature, food, etc.), at the **individual** level (**single species** measuring e.g. survival or reproduction) and exposed to a single stressor at the time. Subsequently, these single-species ecotoxicity data are extrapolated to natural populations and communities. These extrapolations are generally based on statistical models such as species sensitivity distribution (SSD) [3,4]. In the SSD approach a probability distribution is fitted to a set of toxicity threshold data, derived from single-species toxicity tests (e.g. 10% effect concentration [EC10] or no observed-effect concentrations [NOEC]). These probability distributions are used to calculate the concentration that is protective to most of the single species [3,4]. Typically the HC_5 (the concentration at which 5% of the species is affected, i.e. 95% of the species is protected) is used as a safe environmental concentration. The SSD approach is commonly used and the major advantage of this technique is that it incorporates the data from different species [4]. In metal effect assessment studies the SSD approach is often combined with bioavailability models, which are used to normalize the chronic metal toxicity data (EC10 or NOEC) to the chemistry of the water body for which the assessment is performed (Figure 1.1) [5].

However, these general ERA approaches are in sharp contrast with natural conditions. **Natural populations** and **communities** are often exposed to a mixture of **multiple**

biotic (e.g. food shortage, predation) and abiotic (e.g. eutrophication, non-optimal temperature or water chemistry, metals) **stressors**.

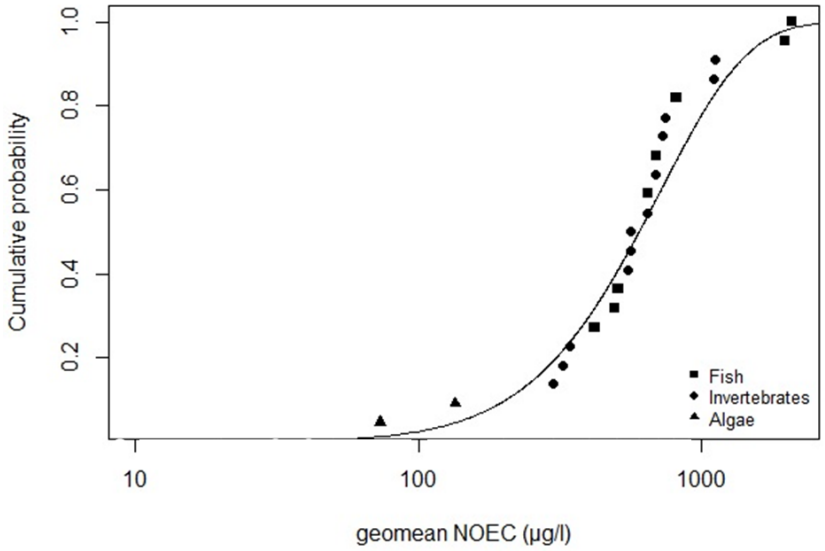


Figure 1.1: Cumulative probability plot of the NOECs, which are bioavailability normalized to the water characteristics of microcosms at the start of the test in chapter 2 and fitted Species Sensitivity Distribution curve. Normalizations were conducted using the Zn bioavailability models (BLM) for *Daphnia magna* [6], *Oncorynchus mykiss* [7,8] and *Pseudokirchneriella subcapitata* [7].

Species interactions such as predation and competition for food are two major biotic factors able to significantly affect the responses of organisms to toxicants [9]. Additionally, abiotic factors such as temperature can also be important drivers affecting the toxic effects of chemical pollutants by influencing their bioavailability and toxicokinetics [10,11]. By ignoring ecological interactions and by not considering natural field conditions these single-species tests oversimplify the actual field situation [9,12,13]. Hence, it might be possible that the effect of a pollutant is over or under protected via the current standardized ERA methods. More ecology and environmental realism is therefore needed in risk assessment [14,15]. As it is not possible to discuss

all possible biotic and abiotic factors affecting chemical pollutants, below we focus on metal toxicity (Zn), and the abiotic and biotic factors that are most relevant to this study.

1.2 Biotic and abiotic variables affecting metal ecotoxicity

Physicochemical properties of the water

Total or dissolved metal concentrations by themselves are no good predictors of toxic effects. This is because the physicochemical properties of the water have a major effect on metal bioavailability or, in other words, on metal toxicity [5,7,16]. Under certain physicochemical conditions the metal will be less bioavailable for the organisms and so less toxic (= higher NOEC) and vice versa. In order to adequately assess the potential impact of metal toxicity on the organism, bioavailability should be taken into account. For Zn the most critical variables for accurately predicting the chronic bioavailability and toxicity are pH, calcium (Ca^{2+}) and dissolved organic carbon (DOC) [5,7]. Another important factor affecting metal toxicity is temperature [10,11,17,18], but this will be discussed in detail later in this chapter (see 1.5).

pH

By influencing metal speciation and competition with the biotic ligand, pH has an important effect on metal toxicity. Speciation has been defined as “the distribution of an element amongst defined chemical species in a system” [19]. The speciation of the metal is of main importance since it determines whether it can be taken up by the organism (=bioavailable) or not. In non-dissolved form the metal can enter the organisms through dietary uptake but for most metals this is only a minor route for

metal uptake [20]. In dissolved form the metal can occur as free metal ions (Me^{2+}) or can be bound to an organic or inorganic ligand. Generally the Me^{2+} species is considered to be the most bioavailable and so the most toxic [16]. Lowering the pH generally increases the Me^{2+} concentration due to the competition between the Me^{2+} and the protons for the complexation with the organic ligands. Furthermore, the concentrations of inorganic ligands (e.g. OH^-, HCO_3^-, CO_3^{2-}) will determine the possibilities of complexation with Me^{2+} and so the metal toxicity (increased Me^{2+} inorganic ligand bindings = less toxic).

Hardness

Hardness of water is a term referring to the measured concentrations of Ca^{2+}, Mg^{2+} present in the water. These minerals can affect metal speciation and can affect metal toxicity by competitive interactions at the biotic ligand [16,21,22]. Heijerick et al. (2005) [6], for example, reported protective effects of the cations Ca^{2+}, Na^+, Mg^{2+} and H^+ on the chronic Zn toxicity to *Daphnia magna* and he explained the observed effect as competition effects with these cations at the site of action. Similar to Ca^{2+}, Na^+ and Mg^{2+} decrease the Zn toxicity but less effectively [22,23]. At present the protectiveness of cations such as Ca^{2+}, Na^+, Mg^{2+} and H^+ are generally accepted and are used to calculate the chronic bioavailability and toxicity of Zn [5,7].

DOC

Dissolved organic carbon (DOC) is the group of dissolved organic molecules with a wide varied composition and origin (e.g. decomposed dead organisms). Due to its thiol

groups (R-S-H) DOC can bind with metals (R-S-Me), making it bio-unavailable and reducing the accumulation and toxicity of the metal [6,24,25]. Waters with a low DOC concentration are generally more vulnerable to metal toxicity [24]. Therefore, accounting for DOC concentration is crucial for assessing metal toxicity [5].

Biotic variables

Species interactions

The competition for resources and predation are considered to be the most important species interactions. These interactions can occur between individuals of different species (interspecific interaction) or within the same species (intraspecific interaction). SSDs assume that species interactions have no effect on the sensitivity of the community to a chemical [15]. De Laender et al. (2008) [15] compared the traditional SSD approach with the SSD that took ecological interactions (eco-SSD) in account (by a mechanistic ecosystem model) and he found that for about 25% of the toxicants the traditional SSD was less strict (Higher PNEC). This implies the possibility that the traditional SSD derived HC5 are not protective for natural communities.

The competition for resources can refer to the competition for food, light, space or any other limited resource and can significantly modify the responses of organisms to toxicants [15,26–28]. For example, the toxic effects of a toxicant can decrease the abundances of the most sensitive species (direct effect) and this can result in an increased abundance of a more resistant species as a result of decreased competition (indirect effect) [9]. These indirect effects are frequently observed in microcosm and mesocosms studies [9,13,29,30]. Furthermore, the competition for food among species can reduce the amount of energy that can be used for reproduction (dynamic energy

budget theory [31]) which could enhance toxic effects observed at the population level [32–34] and recovery rates [35,36]. At the present, only few studies exist that investigate the combined effect of food competition and metal stress. One of these studies was conducted by Heugens et al. (2005) [17], who found that the adverse effects of cadmium (Cd) on a *D. magna* population were lower at higher food levels. Sarma et al. (2007) [37] investigated the combined effect of Zn and food concentrations on the competition between the rotifers *Anuraeopsis fissa* and *Brachionus rubens* and they illustrated the significant interactive effects between Zn and food concentration on the competitive outcomes.

Similar to competition for food, predation can play an important role on how organisms respond to toxicants. The chemical toxicant can affect the predator, prey or both. When the predator is negatively affected (direct effect) this can have a positive effect on the prey (indirect effect) [9]. For example, Van Wijngaarden et al. (2005) [29] observed that the phytoplankton species increased in abundance after an abundance reduction of the zooplankton grazers due to the application of the insecticide chlorpyrifos. Additionally, when exposed to a chemical stress, predator pressure can prevent density dependent compensation or recovery which can make the prey more sensitive to chemical stress. Gergs et al. (2013) [28] for example, found that the combination of predation and another stressor (p-353-nonylpheol) lead to the extinction of a *Daphnia* population, even though the effects of the single stressors were only small. Chemical stressors can also indirectly affect predator species by eliminating their food source [9]. Although the awareness of the influence of biotic interactions on toxicity effects of chemicals is growing [12,15,38] only a few studies exist investigating their combined effect, and those studies were mainly focused on pesticides [26,27,32,35]. To predict how biotic interactions influence the effects of metals, more research is needed.

1.3 Global change and its possible effect on chemical toxicity

The ecosystems panel defined global change as “the interactions between natural changes in the Earth’s physical and biological structure and the broader effects of human activity.” Global change includes natural and anthropogenic components and it is largely due to increased human population (increased resource consumption, technological advancement), which induces disturbances of natural systems. During the last century global change became a dire reality and its impact is expected to further increase in the future [39].

One of the main, and best known effects of global change is a global temperature increase. Climate models predict that climate change will induce a general temperature increase of 2°C to 4°C within the next century in temperate regions [40]. Extreme weather conditions such as storms (precipitation), heat waves and intense temperature fluctuations are predicted to occur more frequently. These increased rainfall intensities are predicted to induce additional eutrophication events due to increased phosphorous loading from land to lakes and streams [41]. Authors like Jeppesen et al. (2009) [41] and Moss et al. (2011) [42] predict that global climate change will not only induce additional eutrophication (increase nutrient influx due to increased rainfall intensities) but will also enhances its effects on aquatic ecosystems (Figure 1.2).

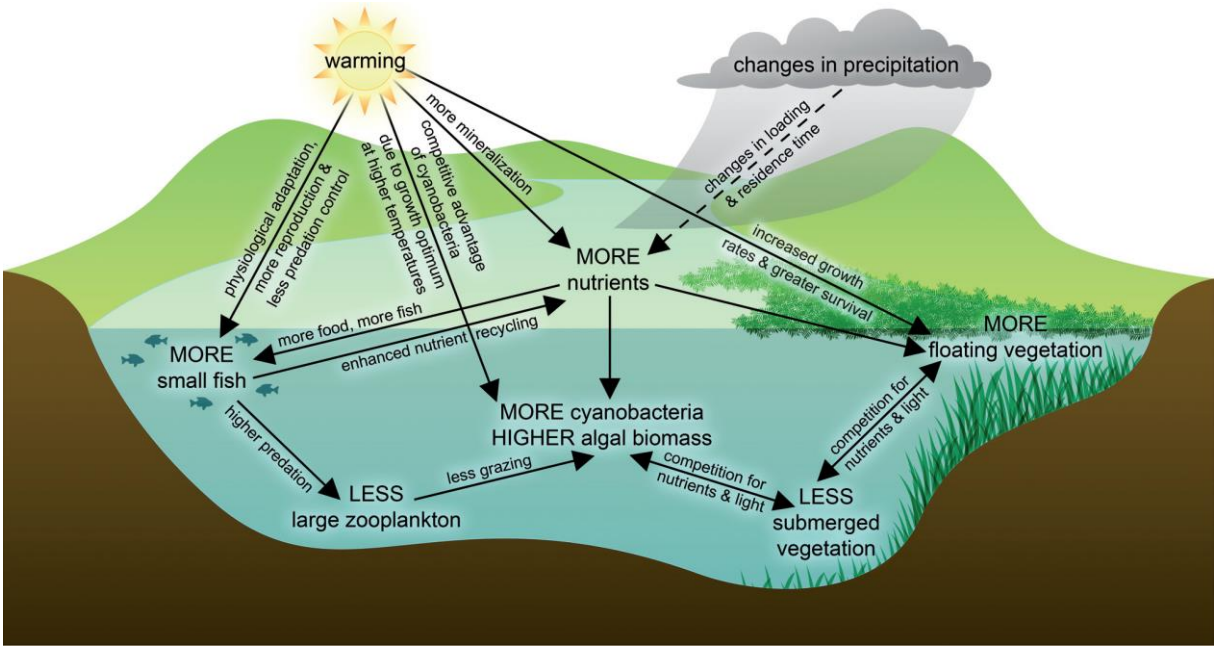


Figure 1.2: Example linking global climate change and eutrophication symptoms [42].

Recent studies have already revealed the importance of the effects global change can have on chemical pollution [10,39,43,44] and ecological risk assessment [45]. Moe et al. (2013) [43] for example, indicated that global climate change effects (e.g. temperature increase) can have a significant impact on species, species interactions and ecosystem processes and may affect the sensitivity of organisms to chemicals. In the light of the ongoing temperature increase and other global change effects it is crucial to understand and predict how these changes will affect chemical stressors effects on populations and natural communities. For this reason we focus in this study on the possible effects temperature and P can have on Zn toxicity effects on freshwater populations and communities. In the context of global change, and the predicted general temperature increase of up to 4°C within the next century, the two different water temperatures within this study will be based on the ambient water temperature

and the ambient +4°C. In section 1.5 the effects of temperature and P on metal toxicity are discussed in more detail.

1.4 Model systems: plankton community

In this study plankton communities were used as test systems. Plankton is an important part of aquatic ecosystems and consists of zooplankton, phytoplankton and protozoa.

Zooplankton

Zooplankton are heterotrophic organisms and are an important component of freshwater ecosystems. There are three important groups of zooplankton (Figure 1.3): rotifera, cladocerans and copepods. Small zooplankton, such as rotifers and copepod nauplii are also often called microzooplankton. Rotifers are the smallest (40 µm – 2mm) of the zooplankton and are filter feeders that mainly feed on algae and protozoa by using their corona (ciliated region). When conditions are favourable rotifers reproduce by parthenogenesis (asexually, clonal reproduction) [46]. However, under less favourable conditions (e.g. food shortage, high predation pressure, temperature, drought, high population density and chemical stress) the females will produce haploid eggs, which will develop into males and sexual reproduction and the formation of dormant eggs can occur.

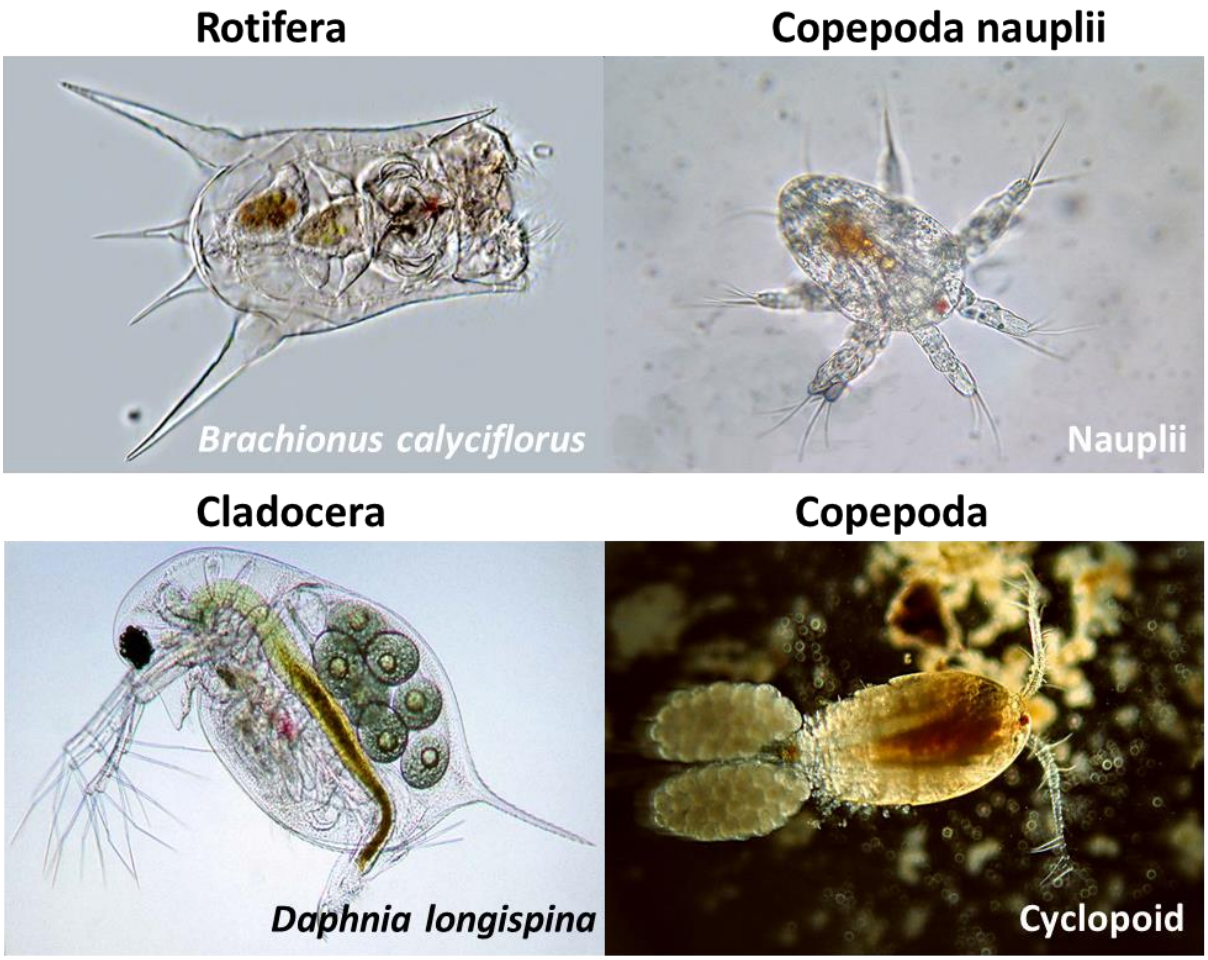


Figure 1.3: Examples of taxa of the major freshwater taxonomic zooplankton groups observed and used in this study.

Cladocerans and copepods are two important groups of the crustacean zooplankton. Most cladocerans are filter feeders (some are carnivorous) and mainly feed on algae, bacteria and protozoa. Cladocerans are commonly known as “water fleas” and their most well known genus is *Daphnia*. In many freshwater ecosystems *Daphnia* are the main grazers and have a huge impact on the phytoplankton community (biomass and species composition) [9,47,48]. This feature makes them a key stone species of many aquatic ecosystems. Cladocerans are cyclically parthenogenetic that will reproduce

asexually under favourable conditions and sexually under unfavourable conditions (resulting in dormant eggs: ephippia) [46,47].

Planktonic copepods are present in most freshwater ecosystems and are classified based on their antennae length: Calanoidea (antennae longer than body length) and Cyclopoidea (antennae shorter than body length). Generally copepods reproduce sexually, the eggs are fertilized and extruded in one (most Calanoidae) or two (Cyclopoidea) egg sacs [46]. After hatching, copepod nauplii will appear and they will undergo several larval stages before becoming an adult copepod. Therefore copepod life-time is often longer than those of cladocerans. Copepods will, under suboptimal conditions, produce resting eggs than can lay dormant in the sediment until better conditions.

Figure 1.4 illustrates a simplified traditional freshwater planktonic food web. Copepods and Cladocerans compete with rotifers for food and since they can ingest a wider range of algae cells they can outcompete the rotifers and prevent them to become abundant [46,48,49]. Additionally, rotifers can be damaged by being swept into the branchial chamber of the cladocera (: mechanical interference) [49]. On the other hand, epizoic rotifers (e.g. *Brachionus rubens*) can attach themselves onto the *Daphnia* carapax and this can have a negative effect on their host (associated with increased *Daphnia* mortality) [50]. Smaller rotifers require less food to reach maximum growth rates and are better adapted to live under low food environments [46,49]. Copepod and Cladoceran densities are greatly determined by predation intensities (e.g. planktivorous fish or invertebrates) [46,51,52]. Cladoceran predation by Turbellarians, such as *Mesostoma* sp. are known to have a great effect on the zooplankton community [30,52].

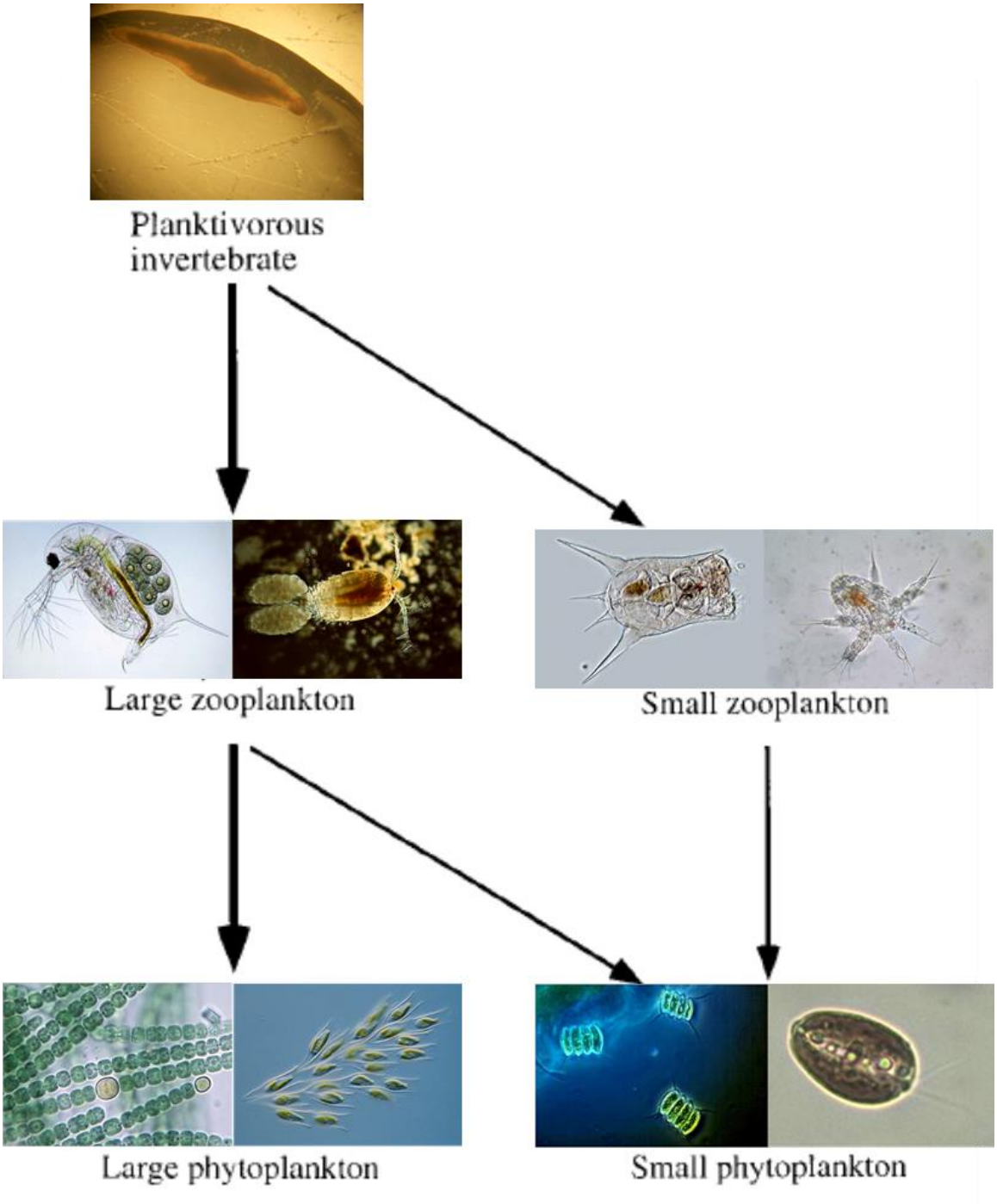


Figure 1.4: Simplified traditional freshwater pelagic food web (adapted from [46]).

Phytoplankton

The word phytoplankton can be broken down in phyto, which means plant and plankton, which means wandering. However, these “wandering plants” are not plants but algae that occur in the open water of lakes and large streams. The vast majority of the phytoplankton are photo autotrophic and are considered as primary producers. The algal cell wall is often made of cellulose and other polysaccharides and often contains silica, proteins and lipids. Since the chemical composition and the structure of the algae cell wall is species-specific it has been used for taxonomic classification. Diatoms for example have a very characteristic silicified cell wall (with sculpted ridges and grooves) which consists of two parts that fit over each other as a lid. Numerous phytoplankton species underwent several adaptations (e.g. spines, larger size, fast reproduction, mucus sheets) to withstand grazing pressure. The major planktonic freshwater taxonomic groups (Figure 1.5) are Bacillariophyta (diatoms), Cyanobacteria, Chrysophyta, Chlorophyta, Cryptophyta, Xanthophyta, Pyrrophyta (dinoflagellates) and Euglenophyta [46].

The plankton community composition is mainly correlated with environmental factors (e.g. nutrients, pollutants, light, temperature) and species interactions (e.g. interactions between other phytoplankton species or grazing pressure) [46]. Since phytoplankton communities are so well correlated with environmental factors they are often used as bio indicators [53–55].

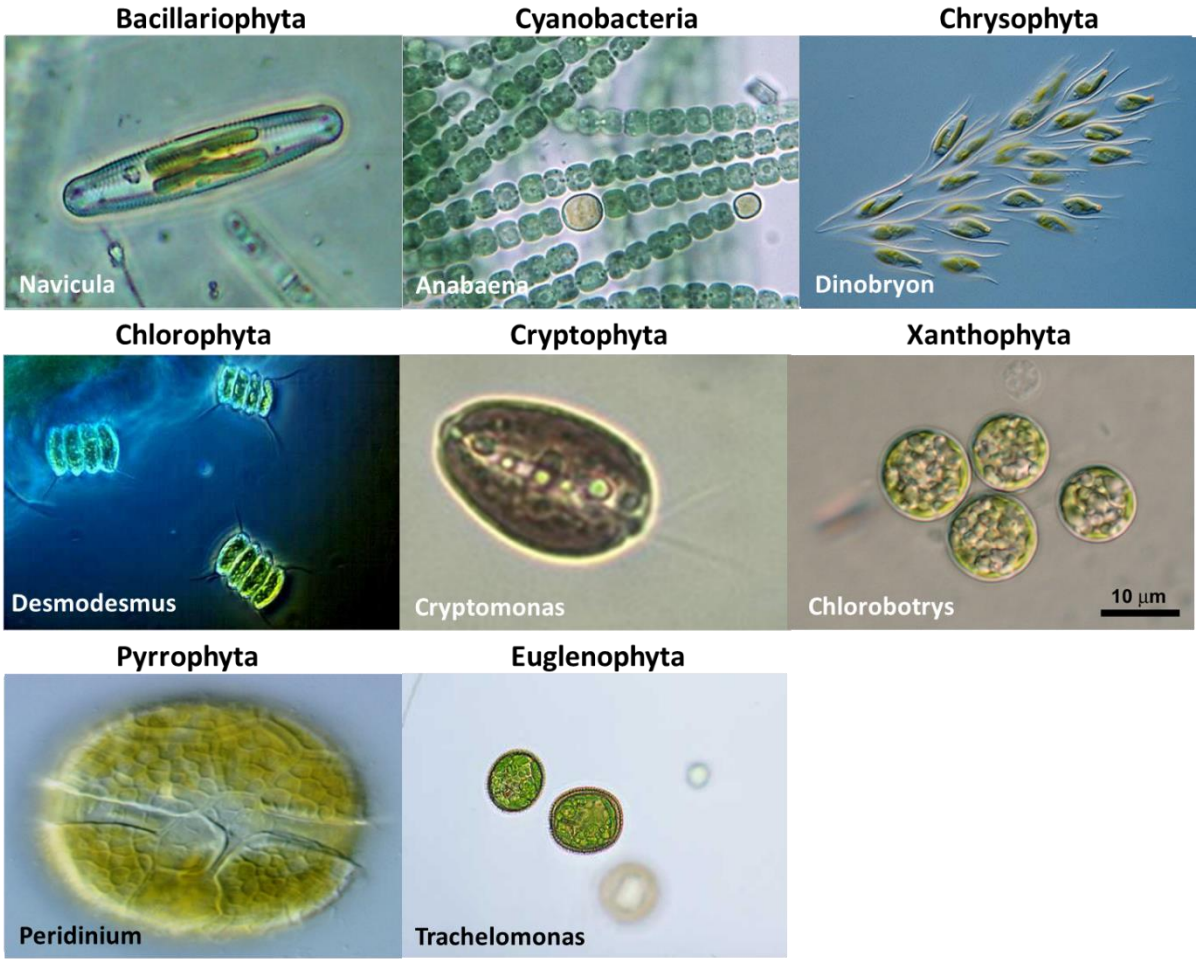


Figure 1.5: Examples of taxa of the major freshwater phytoplankton taxonomic groups observed in this study.

Protozoa

Protozoa or “first animals” are unicellular eukaryotic heterotrophic (or mixotrophic: heterotrophic but supplementing their feeding with photosynthesis) organisms that range from 1-300 µm. They are regarded as the most important bacterial consumers and have a short generation time (=high reproductive potential) [46,56]. This short generation time allows them to respond very fast to resources fluctuation. The classification of the different protozoa groups is based on their morphology and way of movement. Three groups are distinguished: heterotrophic flagellates, amoebae and

the ciliates (Figure 1.6). The amoebae use their pseudopodia (temporary “feet” expansions) for movement and feeding. The flagellates use flagella (whip-like structure) for locomotion and the ciliates cilia (short hairs).

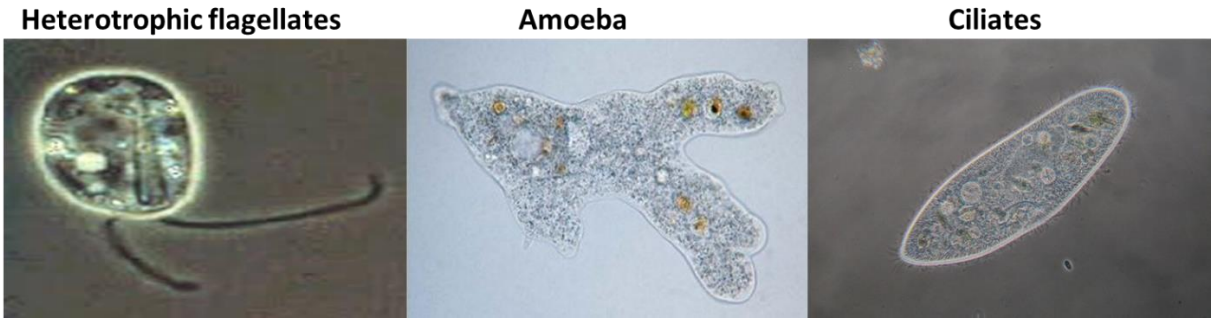


Figure 1.6: The different protozoa groups observed in this study.

Protozoa play a key role in the microbial loop (Figure 1.7) and its connection to the traditional food web [46]. Dissolved organic matter (DOM) excreted from organisms (e.g. phytoplankton and zooplankton) is consumed by bacteria. In turn, the bacteria are consumed by the protozoa and the zooplankton. Additionally, the zooplankton predate on the protozoa and have a major effect on their abundances [48,56,57].

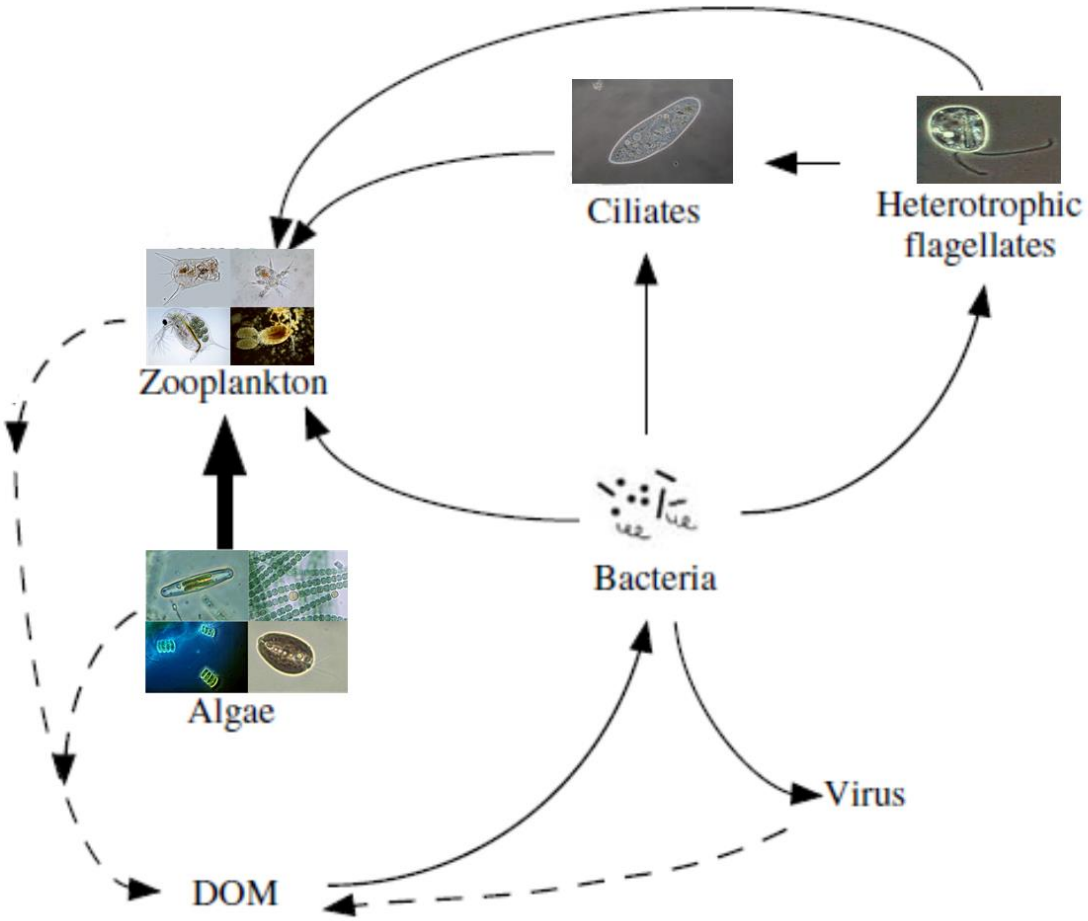


Figure 1.7: Carbon (Dissolved organic material: DOM) and nutrient transport into the microbial loop (dashed arrows) and the link with traditional food web (adapted from [46]).

1.5 Model stressors

Zinc

Zinc (Zn) is a natural component present in the soil, rock, coal, air and water [58]. It is an essential element, necessary for the growth and development of organisms (e.g. crucial component enzymes and proteins). A natural source of Zn addition to the environment is the weathering of Zn-containing bedrock. At present, Zn is mainly used in the galvanization industry and its use is still increasing, mainly in China and India.

The main anthropogenic sources for Zn to the environment are industrial activities such as steel processing, galvanisation and mining. The natural background dissolved Zn concentration in most European surface waters varies between 1 and 10 µg Zn/L (10^{th} – 90^{th} percentile) but can range up to 310 µg Zn/L in extreme cases (FOREGS). Although Zn is an essential element for many metabolic functions in most organisms, it can be harmful for aquatic ecosystems [5,13,59,60]. Based on *Daphnia* and fish Zn toxicity studies it has been shown that an important mode of action for Zn toxicity is the inhibition of Ca^{2+} uptake, which can lead to hypocalcaemia [61–63]. Muyssen et al. (2006) [63] investigated the chronic Zn effects on *D. magna* (e.g. growth, reproduction, mortality, respiration) and they found that *Daphnia* mortality mainly occurred during the first week. The internal Ca concentrations of the *Daphnia* suggested that their mortality was related with a decreased Ca concentration, which may occur due to the inhibition of Ca uptake [63]. An elevated Zn concentration in the water can also have a negative effect on the photosynthetic activities of the phytoplankton. This is due the fact that Zn can disturbed the electron transport within the PSII [64]. Additionaly, increasing Zn supply may induce P deficienty in algae [65,66]. Paulsson et al. (2002) [65] conducted an indoor flow-through microcosm study and she found that by increasing the Zn concentration from 2 µg Zn/L to 654 µg Zn/L the alkaline phosphatase activity (an indicator of P deficient) within the periphyton community increased by 2.5 fold. By influencing the P uptake and the bioavailibility of dietary P, Zn can affect the nutrient quality of organisms (e.g. C:P ration). Evens et al. (2012) [67] found that by increasing the Zn supply to 90 µg Zn/L, the C:P ratio of the algae *Pseudokirchneriella subcapitata* was reduced by half. He hypothesed that in his study, the reproductive outcome of *D. magna*, when exposed to Zn contaminated algae could more be related to Zn-induced, changes of the dietary P level than by direct dietary Zn toxicity [67]. Additionaly, it has

been well documented that algae with a lower P contect typicaly have a thicker cell wall which makes them more difficult to digest by zooplankton [46].

Temperature

Temperature is an important environmental factor that can have major effects on planktonic community composition and functioning [11,18,68–70], especially since most aquatic organisms are ectotherms. Ectothermic organisms are strongly influenced by temperature in terms of their metabolic rates, behaviour and physiological processes [11]. The mean water temperature in European waters are 15°C in late spring and 20°C in early summer [46]. However, the water temperature in rivers and ponds can range between 5°C up to 26°C and can fluctuate daily [46]. Generally at higher temperatures (within tolerance range) ectotherms such as *Daphnia*, mature faster and this can enhance population growth [17]. Extreme temperatures may be lethal for organisms. However, it is possible that organisms acclimate to temperatures outside their tolerance range (within genetic tolerance limits) [71].

Based on the Organisation for economic co-operation and development (OECD) guidelines most ecotoxicology studies with *Daphnia* are conducted at 20°C [72]. However, under natural conditions organisms are exposed to a wide range of fluctuating temperatures and as a result of global change the general water temperature is predicted to increase by 2°C to 4°C within the next century [40]. Temperature is known to be able to influence the toxic effects of pollutants by influencing their bioavailability and toxicokinetics [10,11,73]. A review study conducted by Noyes et al. (2009) [44] stated that a temperature increase generally enhances the toxicity of contaminants. To date most metal toxicity studies also indicated an

increased metal toxicity at higher temperatures [17,39,71,74–76]. One of these studies was conducted by Heugens et al. (2003) [74] who found that for a *Daphnia magna* population (monoclonal) the adverse cadmium (Cd) effects on growth were enhanced at higher temperature. She found that for *D. magna* the 24h-LC50 decreased nearly two-fold when increasing the temperature from 20°C to 23°C. This was for example also demonstrated for Zn by Bat et al. (2000) [76] for *Gammarus pulex* where the 96h-LC50 decreased from 12.1 mg/L at 15°C to 9.3 mg/L at 20°C and to 5.2 mg/L at 25°C. It is assumed that at higher temperatures the metabolic rates increase, which could increase metal uptake and accumulation [17,39,75]. However a recent study conducted by Pereira et al. (2016) [77] on *D. magna* reported that the chronical metal toxicity of Zn, Cu and Ni were generally higher at lower temperatures. For Zn, Cu and Ni she found that at 15°C the reproductive 21-day EC50 was 1.1, 1.4 and 1.3 times lower than at 20°C. Thus, to date, information concerning the effects of temperature on Zn (or metals in general) toxicity to freshwater organisms is very limited.

Phosphorus

Phosphorus (P) is an essential element for all life since it is a crucial component of cell metabolism (e.g. enzymes), DNA, RNA and the cell energy system (adenosine triphosphate). In water most of the P (usually 80 %) is included in the organic phosphorus fraction [46]. Phosphorus itself is taken up by the organisms as phosphate (PO_4^{3-}). In most freshwater ecosystems P is the limiting factor for primary producers growth. Sediment release, P influx from the catchment area and atmospheric deposition are the main influxes of P into the water. Lakes and rivers are often categorized based on their P concentrations. Aquatic ecosystems with a low P concentration (total P: 5-10 µg P/L) are categorized as oligotrophic and aquatic

ecosystems with a high P concentration (total P: 30-100 µg P/L) as eutrophic [46]. During the last decades, high anthropogenic P loading (e.g. agriculture and waste water) has caused many aquatic ecosystems to become eutrophic. One of the possible consequences of nutrient enrichments is the occurrence of cyanobacteria blooms which could be toxic, food web alternating and hypoxia generating [78]. Nutrient addition can directly affect the phytoplankton community (by altering biomass, size and nutritional quality), which can have and indirect effect on the zooplankton community [79–81]. However, extreme eutrophication is usually unfavourable for most zooplankton species, primarily due to the occurrence of unfavourable cyanobacterial booms [51].

Phosphorous can also affect metal toxicity to freshwater algae. At an elevated pH phosphates can precipitate with metal ions by complexation, making it bio-unavailable [82]. Additionaly, at luxury P supply phosphorus can be stored in algae cells in the form of polyphosphate [82]. These polyphosphates can sequester metal ions in the cell, decreasing the intracellular free metal ions and therefore reducing toxicity [83]. Serra et al. (2010) [84] and Twiss and Nalewajko (1992) [85] for example found that an increased P supply significantly decreased copper (Cu) toxicity on algae biomass (with up to a 3.6-fold increase in EC50). On the other hand, Gao et al. (2016) [86] found that at higher P supply, Zn was more toxic to *Pseudokirchneriella subcapitata* cell densities. At present too few studies have investigated the relationship between metal toxicity and P supply to draw consistent conclusions.

1.6 Problem formulation, objectives and conceptual framework

To protect the environment, a correct effect assessment of contaminants is crucial. However, from section 1.1 it is clear that conventional risk assessment is mainly focused on the effect assessment of a single stressor, conducted under optimal conditions and at the individual level. This is in contrast with natural conditions where aquatic communities are exposed to a mixture of constantly changing stressors, often under non-optimal conditions. As mentioned in section 1.1 and 1.5, species interactions and environmental conditions (e.g. temperature and P) can affect metal toxicity effects. Therefore, current ERA of metals may not be protective (or overprotective), by ignoring ecological interactions and by not considering natural environmental conditions. The objectives of this study can be divided in 3 main research questions:

1) Do environmental factors such as temperature and phosphorous affect Zn toxicity to a freshwater plankton community?
2) Do species interactions affect Zn toxicity effects on a freshwater plankton community?
3) Are the combined and interactive effects between Zn and temperature observed at the population level similar to the combined and interactive effects observed in more complex freshwater plankton communities?

The research conducted in this PhD thesis is described in 3 research chapters (chapters 2-4). The conceptual framework and outline of this PhD thesis is described in figure 1.8. In the final chapter 5, the conclusions and research perspectives are summarized.

In **Chapters 2** and **3** it is assessed whether **temperature** and **phosphorous** can affect **Zn** toxicity on a freshwater community. This was done by exposing a freshwater

plankton community (zooplankton, phytoplankton and protozoa) to different Zn, temperature and phosphorous levels in a full factorial design and monitoring the plankton abundances, community metabolism and general properties of the water during 5 weeks. Before we can understand how temperature and P interact with the Zn effects on a freshwater plankton community, we first need to understand the effect that Zn itself has on a freshwater plankton community. This is done in **Chapter 2** where the direct and indirect effects of **Zn** on the community structure and function of a freshwater community is assessed.

In **chapter 3** the combined and interactive effect of **Zn**, **temperature** and **P** on the structure and functioning of a freshwater community is assessed.

In the first two experimental chapters the whole planktonic community was used in the microcosm studies. However, to assess whether species interactions can affect Zn toxicity effects, using the whole planktonic community can make the assessment too complex. Therefore, in **Chapter 4** the combined effects of **interspecies** interaction (food competition), **temperature** and **Zn** was assessed by conducting a simple community experiment. Here, *Daphnia longispina* populations were exposed to different Zn, temperatures and two interspecific competition levels (No interspecific *Brachionus* competition= no *Brachionus calyciflorus* added; interspecific Brachionus competition= *B. calyciflorus* added).

In the final **Chapter 5**, the findings of this PhD thesis are combined, reviewed and summarized. Additionally, in **Chapter 5** we tried to determine whether population level effects (single and interactions) of chemicals, observed in lower-tier experiments (as in Chapter 4) are similar to population level effects during high-tier experiments (as in Chapters 2 and 3). Stated otherwise: can lower-tier results be extrapolated to higher-tier experiments or to natural aquatic ecosystems? For this purpose a small *D.*

longispina population experiment (exposing *D. longispina* to different Zn and temperature) was conducted and was compared with the *D. longispina* population results from **Chapters 2-4**. Additionally, suggestions and possible directions for future research are provided in this chapter.

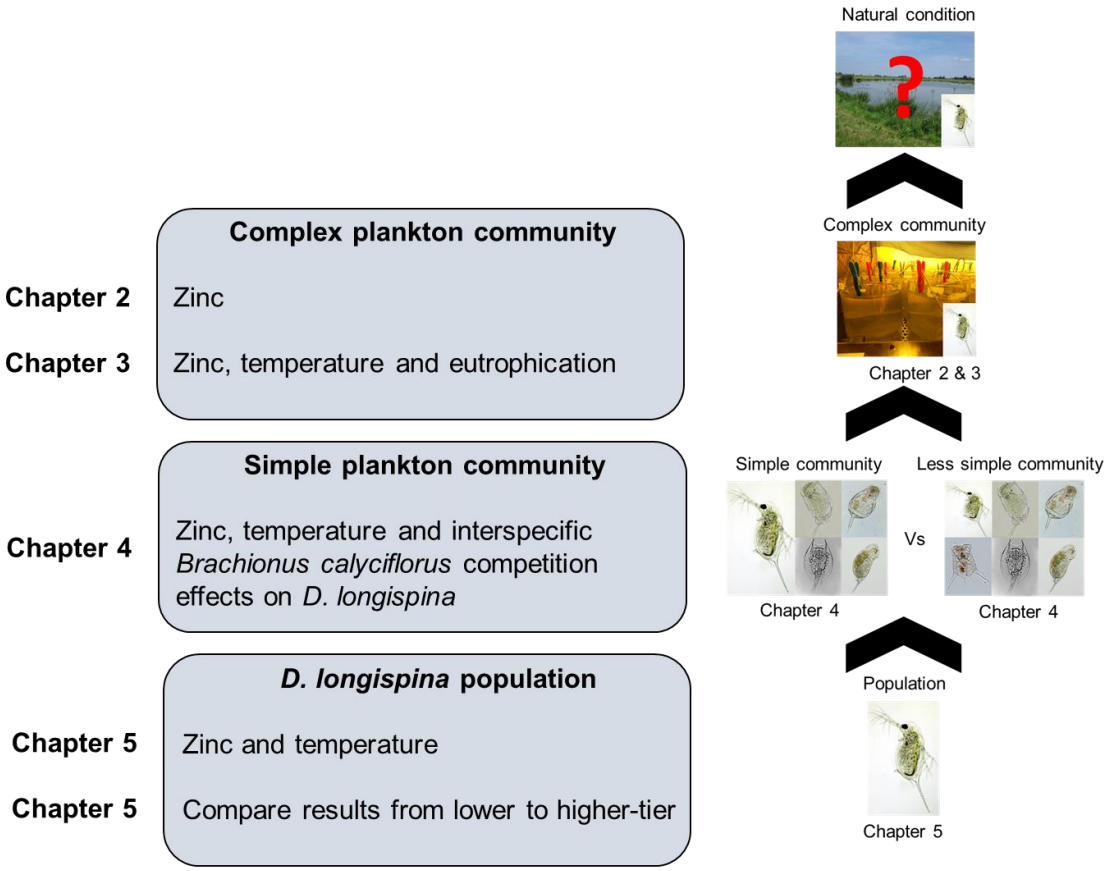


Figure 1.8: Conceptual framework and outline of the PhD thesis. Chapters 3 and 4 were used to answer the first question; Chapters 2 and 3 for the second question and chapters 2-5 for the third.

2

The effects of zinc on the structure and functioning of a freshwater community: a microcosm experiment

Redrafted after:

Van de Perre D, Roessink I, Janssen CR, Smolders E, Van Regenmortel T, Van Wichelen J, Vyverman W, Van den Brink PJ, de Schamphelaere K a. C. 2016. The effects of zinc on the structure and functioning of a freshwater community: a microcosm experiment. Environ. Toxicol. Chem. 29:730–741.

2.1 Introduction

Zinc (Zn) is a widely used heavy metal and the natural background concentration of Zn in European surface waters varies between 1 and 10 µg Zn/L (10^{th} – 90^{th} percentile) but can range up to 310 µg Zn/L in extreme cases [5]. Although Zn is an essential element for many metabolic functions in most organisms, it can be harmful for freshwater plankton communities [5,13,59,60,66]. Total or dissolved Zn concentrations by themselves are not good predictors of toxic effects, since Zn bioavailability varies under different conditions and needs to be taken into account to adequately assess the potential impact of Zn on aquatic ecosystems [5,16,87]. The Biotic Ligand Model (BLM) was developed to predict metal toxicity based on the local physico-chemistry of the water [16,87]. The most critical variables for accurately predicting the chronic bioavailability and toxicity of Zn are pH, Ca and dissolved organic carbon (DOC) [5,7]. Zn BLM's have been developed for *Daphnia magna* [6], *Oncorynchus mykiss* [7,8] and *Pseudokirchneriella subcapitata* [7]. To determine site specific HCx values (hazardous concentration affecting X % of the species within the community), chronic toxicity data obtained during chronic experiments (i.e. NOEC and EC10 values) are first normalized to the target water chemistry [6]. This normalization process takes into account both the speciation of Zn as well as the competition between Zn and other cations at the biotic ligand.

However, these BLM's are based on single species laboratory tests and the extrapolation of the results of laboratory single-species toxicity tests to natural populations and communities is one of the major challenges with the risk assessment of chemicals [13]. Laboratory experiments oversimplify the actual field situations by not considering natural field conditions and ecological interactions (intra-and interspecies) that play a major role in determining the community structure [9]. Micro-and

mesocosms are generally considered to be a realistic higher tier approach to investigate effects of chemicals on population and community level under relatively well controlled conditions [13,29,30,88,89]. In the past, microcosm studies for Cu [90] and Ni [91] have already confirmed the protectiveness of the HC5 for freshwater communities. For Zn, however, very few studies exist which have investigated the possible adverse effects Zn has on the whole planktonic community [60,92] and none of these studies measured all the necessary chemical variables (pH, DOC and Ca) needed to calculate the Zn bioavailability. Without bioavailability calculations there are many uncertainties to correctly assess the impact Zn has on the planktonic community [5]. Based on the Species Sensitivity Distribution (SSD) (Appendix A, Figure A1), constructed with the chronic tests of 22 freshwater species [5], it can be hypothesised that the phytoplankton taxa are the most sensitive to Zn stress in a freshwater plankton community and that as a consequence the zooplankton would be affected indirectly (by food web interactions). Although protozoa are an important part of the planktonic community [46], there are very few freshwater toxicology studies that have incorporated protozoa in their community analysis [59]. Assessing the effects of chemicals on the structure and function of the aquatic ecosystems is a major challenge in environmental toxicology [1,2].

The aim of the present study was to determine the direct and indirect effects of Zn on the community structure and function of a freshwater plankton community and to investigate if the normalized $HC5_{\text{-plankton}}$ (HC5 only based on the chronic toxicity data of plankton species that was first normalized to the target chemical properties of the water by using the Zn BLM's) for Zn is protective for the plankton community (populations and community). By only using plankton species for the HCx calculation it was possible to estimate the possible effect Zn has on the plankton community. For

this purpose, a microcosm study with a freshwater plankton dominated community, comprising of phytoplankton, zooplankton, protozoa and two macroinvertebrate species (*Mesostoma* sp. and *Lymnaea stagnalis*), was performed under semi-realistic conditions. The cosms were constantly exposed to three different Zn concentrations (background, $HC5_{\text{-plankton}}$ or $HC50_{\text{-plankton}}$), for five weeks. The responses of several biological and physico-chemical endpoints were studied during the five weeks of exposure. For every sampling date the No Observed Effects Concentrations (NOECs) were calculated for all population and community endpoints and compared with the calculated normalized HC5.

2.2 Materials and methods

2.2.1 Test systems

The experimental design consisted of three Zn treatments (background= control, $HC5_{\text{-plankton}}$ and $HC50_{\text{-plankton}}$) with three replicates for the Zn amended and four for the control, I.e. ten cosms in total. The experiment was carried out in indoor microcosms installed in a water bath (16 - 19 °C) for temperature regulation, in a climate controlled room (constant temperature 19 ± 2 °C; photoperiod 14 h at 70 ± 10 µmol) at Wageningen University (Wageningen, The Netherlands). Each microcosm consisted of a glass cylinder (diameter 0.25 m, height 0.35 m, volume 18 L), filled with a sediment layer of approximately 0.02 m and 14 L of water. The sediment and the water were collected in June 2013 from an uncontaminated mesotrophic ditch (Sinderhoeve Experimental Station, Renkum, The Netherlands; Appendix A Table A1). The microcosm set-up described by Van Wijngaarden et al. [29] has been used for this experiment to mimic plankton-dominated shallow freshwater systems. The

microcosms were randomly and evenly seeded with additional zooplankton from uncontaminated waterbodies from the Sinderhoeve experimental site. All macroinvertebrates were removed manually prior to the zooplankton seeding. Nutrients (NH_4NO_3:1 mg N/L; KH_2PO_4 and K_2SO_4: 1 mg K/L, 0.01 mg P/L) were added twice a week to stimulate the phytoplankton growth, starting 3 weeks before the actual start of the experiment (i.e. the pre-treatment period). During this period most of the water (removed to just above the sediment) from all the microcosms was taken out once a week and mixed in a central tank. After the mixing, the water was randomly returned to the cosms to ensure adequate mixing and similar start conditions in all test systems [89]. A light flow of compressed air was installed above the water surface to prevent the formation of a bacterial surface biofilm sealing off the cosm surface and to stimulate some gentle water movement. Water loss was replenished with demineralized water when needed. Two snails (*Lymnaea stagnalis*) were placed in every cosm to suppress periphyton growth.

2.2.2 Zinc application and analyses

After a pre-treatment period of three weeks, two sets of three microcosms received a first Zn dosing (=start of treatment, week 0). The four remaining were selected as controls. The two target Zn concentrations (75 µg/L: $HC5_{\text{-plankton}}$ and 300 µg/L: $HC50_{\text{-plankton}}$) were determined by fitting the SSD to BLM normalised chronic Zn toxicity data as explained in detail by Van Sprang et al. 2009 [5], based on the chemical properties of the water of the microcosms the day before the start of the treatments (Appendix A Table A1 and A2). For this, all invertebrate NOECs are normalized with the chronic D. magna BLM [6], all vertebrate NOEC values with the chronic O. mykiss BLM [9] and all algae NOEC values with the P. subcapitata BLM [11] using BLM software version

2.1.2 [5,93] (all parameter files used for this normalization with BLM modelling can be found in the Supportive information. Each parameter file corresponds to a toxicity data line (i.e. a NOEC or EC10 value) within the chronic zinc toxicity database published by Van Sprang et al. (2009) [6]).

Zinc was applied by dosing a Zn stock solution (in milli-Q water, dissolved $ZnCl_2$: 500 µM). The Zn stock solution was distributed evenly over the water surface of the microcosms by using a graduated cylinder or pipet and mixed by gentle stirring. Solution were sampled at frequent intervals to monitor Zn and Zn concentrations were adjusted by additional spiking to compensate for losses from the water column. For every sampling, two 10 ml water samples (one not filtered for measuring the total Zn concentration and one filtered through a 0.45 µm filter for measuring the dissolved Zn concentration, Acrodisc; Pall Life Sciences) were taken almost daily for Zn concentration measurement. The samples were taken after gently stirring using a syringe, approximately 15 cm under the water column. When Zn was dosed, samples were taken just before the Zn dosing and another at least 15 minutes after the Zn dosing. Samples for Zn measurement were acidified to 0.14 mol/L HNO_3. To prevent metal contamination, only acid (1% HNO_3) and sample washed (using 3 times 10 ml cosm water) syringes and filters were used to take samples. All Zn concentrations, except those of the controls, were measured using flame atomic absorption spectrophotometry (SpectrAA100; Mulgrave; Environment Canada: limit of quantification 20 µg Zn/L; method detection limit 6 µg Zn/L). The Zn concentrations of the controls were measured with inductively coupled plasma mass spectrometry (ICP-MS; Agilent 7700x, in the He mode using ^{72}Ge as internal standard: limit of quantification 3 µg Zn/L; method detection limit 1 µg Zn/L).

2.2.3 Zooplankton

Zooplankton was sampled weekly from each microcosm, starting 1 day before the start of the first Zn application. A total of 1 L of water was collected from several positions in the microcosms by using a perspex tube (length 0.4 m; volume 0.8 L) and was filtered through a plankton net (mesh width, 55 µm; Hydrobios, Kiel, Germany). The collected zooplankton was preserved with lugol. The filtered water was returned to the microcosms. Zooplankton was identified and counted using an inverted microscope. Cladocera and Rotifera were identified to the lowest practical taxonomic level. Copepoda were classified as Cyclopoida and Calanoida. All macro-zooplankton (i.e. Cladocera, adult and copepodite stadia of Copepoda and Ostracoda) individuals present in the sample were identified and counted. Abundances of micro-zooplankton (i.e. Rotifera, copepoda nauplii and *Chaetonotus* sp.) were determined and adjusted per litre, by counting a subsample of a known volume.

2.2.4 Phytoplankton and protozoa

The species composition of the phytoplankton and protozoa was identified to the lowest practical taxonomic level. One litre of water was collected in a similar way as for the zooplankton and was filtered through a plankton net (mesh width, 20 µm; Hydrobios, Kiel, Germany). A subsample of a known volume was settled overnight in sedimentation chambers and the concentrated organisms were counted along longitudinal transects according to Utermöhl et al. (1958) [94] with the use of an inverted microscope. At least 400 individual cells were counted and identified for every subsample and the abundances recalculated to numbers per litre. Colony-forming

algae were counted as a single individual. Bacillariophyceae were only identified as single cell diatoms or *Fragilaria* sp. colony chain.

The total chlorophyll concentration in every microcosm was measured on the same days as the other biological parameters. Two 10 ml water samples were taken 15 cm below the water column at the centre of every microcosm, during gentle stirring with a syringe and analysed by using a BBE Moldaenke GmbH Algae Lab Analyser.

2.2.5 Community metabolism and general chemical properties of the water

Dissolved oxygen (DO), temperature and pH were measured in the morning (start photoperiod) and evening (one hour before the end of the photoperiod) twice a week at mid water depth, starting one day before the start of the treatment. DO measurements were used to estimate net primary production ($DO_{evening\ day\ x}$ - $DO_{morning\ day\ x}$) and community respiration ($DO_{morning\ day\ x\ +1}$ - $DO_{evening\ day\ x}$) rates [95]. Measurements for DO, temperature and the pH were measured using a WTW 340i multi-meter. Conductivity was measured once a week by using a WTW LF 191 conductivity meter. Total and filtered (0.45 µm filter, Acrodisc; Pall Life Sciences) water samples were taken for nutrient analysis just before the biota samples were taken. Ammonium, total phosphorus (TP) and NO_2 + NO_3 were only measured 1 day before the start of the treatment and 2 and 5 weeks after the first treatment and were analysed using the ascorbic acid method (TP) and a Skalar 5100 auto analyser (NH3 and NO2 + NO3). The scalar 5100 auto analyser was also used to analyse the Soluble reactive phosphorus (SRP) concentration. Dissolved (in)organic carbon (DOC, DIC) were both measured with a total organic carbon analyser (TOC-5000; Shimadzu; limit of quantification 1.5 mg DOC/L; method detection limit 0.5 mg DOC/L). Total dissolved

phosphorus (TDP), Na, Mg, Al, Ca, Cr, Mn, Fe, Co, Ni, Cu, As, Mo, Cd and Pb were measured with ICP-MS (Agilent 7700x).

Additionally, a 5-day biochemical oxygen demand (BOD_5) test [96] was conducted every week for every microcosm with water that was filtered with a plankton net (mesh size, 55 µm; Hydrobios, Kiel, Germany).

2.2.6 Data analysis

Before univariate and multivariate analyses were performed, the zooplankton data were ln (2x+1) transformed and the phytoplankton/protozoa data were ln (1.67x+1) transformed where *x* is abundance value. This was done to down-weight high abundance values and to approximate a log-normal distribution of the data (see [97] for rationale). No Observed Effect Concentrations (NOECs) at the parameter or taxon level ($p \leq 0.05$) were calculated using the Williams test (analysis of variance) in the Community Analysis software [98,99]. This test assumes an increasing effect with increasing dose. NOECs were considered consistent when they showed statistically significant deviations in the same direction (adverse or beneficial) for at least 2 consecutive sampling dates.

The effects of the Zn treatment on the zooplankton, phytoplankton and protozoa communities were analysed by using the Principal Response Curves method (PRC). The PRC method is a multivariate technique which is based on the redundancy analysis ordination technique and was performed using the CANOCO 4.5 [100]. The PRC diagram shows the difference in species composition between the treatments and to the controls as they develop over time. This technique was specifically developed for analysing microcosm experiment data [101,102]. The results of the PRC analysis

can also be evaluated in terms of the fraction of the variance, that is explained by the factors' time and treatment. Only the fraction that is explained by treatment is shown in the PRC diagram. To assess the statistical significance of the effects of the treatments on the species compositions a Monte Carlo permutation test was performed together with the redundancy analysis. Monte Carlo permutation of the microcosms was used to test the significance of the PRC diagram in terms of displayed treatment variance, by using an F type test statistically based on the eigenvalue of the component [101]. For each sampling day a Monte Carlo permutation test was conducted, using the ln-transformed intended doses as the explanatory variables, to assess the significance of the treatment effects for each sample date. In case of a significant relationship between the treatment and the species composition, the treatment levels differ significantly from the controls were determined to derive NOECs at the community level ($NOEC_{community}$). The $NOEC_{community}$ were calculated by applying the Williams test [101,103] on the PCA sample scores resulting from a PCA analysis performed for each sampling date separately.

In order to evaluate the treatment effects, the observed effects were summarized into effect classes, which classifies effects based on reliability of the data and the magnitude of the effect, incorporating the Minimum Detectable Difference (MDD) as proposed by Brock et al. [89]. It is a practical guidance which is accepted by the European Food Safety Authority (EFSA) to analyse the statistical power of microcosm/mesocosms experiments and to demonstrate effects at population and community level. Hommen et al. 2015 [91] is an example of a microcosm, risk assessment study of nickel that used Brock et al. 2015 [16] as guideline.

Briefly, to determine the reliability (sufficient statistical power to demonstrate treatment related responses) of a taxon for analysis, the MDD categorizes the taxa in 3 different categories [16]:

- Category 1: The MDD of the taxon meets at least one of the following conditions during exposure period: < 100 % for at least 5 sampling moments; < 90 % for at least 4 sampling moments; < 70 % for at least 3 samplings or < 50 % for at least 2 samplings.
- Category 2: MDD of the taxon does not meet criterion for category 1 but a LOEC can be calculated for at least 1 sampling period.
- Category 3: MDD of the taxon did not meet the category 1 and 2 criteria.

An MDD of >100 % indicates that the statistical power of the test is too low to demonstrate treatment-related changes in abundance.

Category 1 and 2 taxa can be used for effect classification of the treatment-related effects. This results in 4 effect classes [89]:

- Effect class 1: No effect observed.
- Effect class 2: Slight effects. Effects only observed on individual samplings.
- Effect class 3: Clear short-term effects. Effects observed at, at least two subsequent sampling dates. Full recovery occurred after < 8 weeks.
- Effect class 4: Clear effect in short-term study. Study too short to demonstrate full recovery within study period (< 8 weeks).

All calculated MDD values of the different endpoints (e.g. taxa, algae classes, zooplankton groups and chlorophyll levels) were added in supporting information (Appendix A Table A3).

2.3 Results

2.3.1 Zinc concentrations

The average (± standard deviation) total dissolved Zn concentrations in the 75 (22.0 ± 4.9 µg/L) and 300 µg Zn/L (51.1 ± 13.87 µg/L) treatments were below their target concentrations during the first week (Figure 2.1). The target concentrations were achieved after one week. The average (± standard deviation) dissolved Zn concentrations between weeks 1-5 were within 2 % of the target concentration at the lowest dose (77.9 ± 17.8 µg/L) and within 4 % at the highest dose (287 ± 52.3 µg/L). In the controls, the mean dissolved Zn concentration were 5.1 ± 3.2 µg/L. For simplicity and because of the small deviations between average measured and nominal Zn, we will further refer to the nominal concentrations (75 and 300 µg Zn/L) throughout the paper.

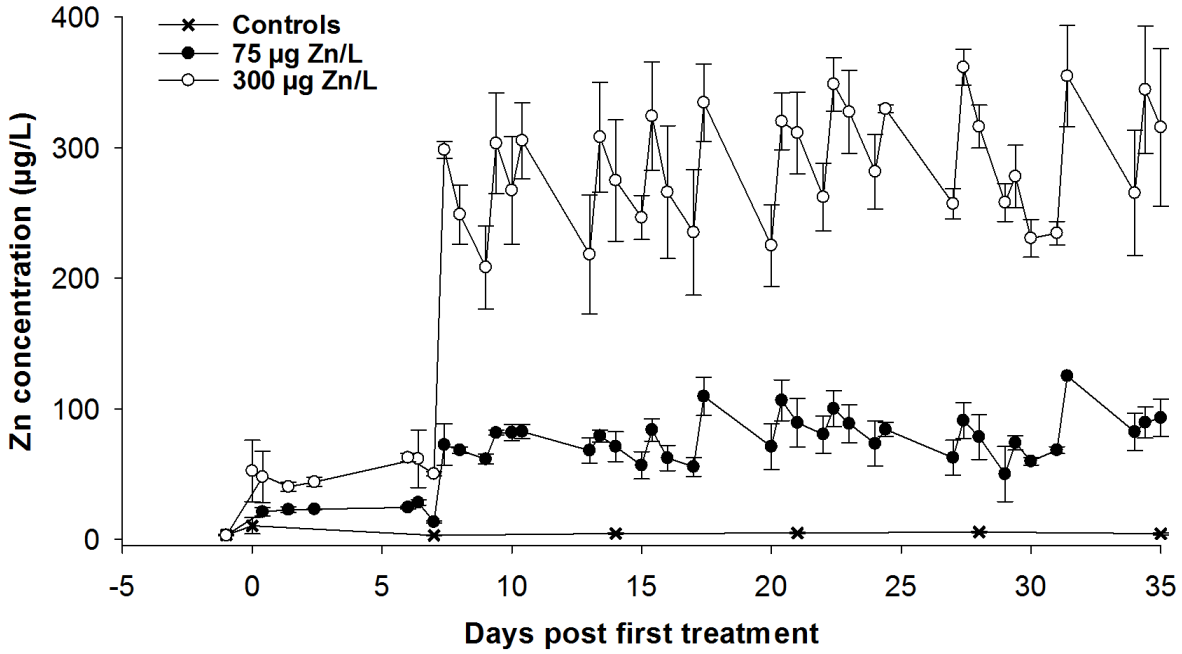


Figure 2.1: Measured dissolved Zn concentrations (µg/L) before and after spiking. Error bars are standard deviations.

2.3.2 Zooplankton

A total of 41 zooplankton taxa were identified. The majority of the taxa belonged to the micro-zooplankton (i.e. 32) followed by the macro-zooplankton (i.e. 9). Eight of these taxa fulfilled the MDD category 1 criterion for reliable statistical analysis (Appendix A Table A3). In the pre-treatment period (weeks –3 to 0), Cladocera abundances were low and all microcosms were mainly dominated by copepod nauplii and rotifers (*Auraeopsis fissa* and *Polyarthra remata*). During the exposure period (weeks 0-5), however, the control microcosms were dominated by, in decreasing order of abundance, rotifers (*A. fissa*, *Lecane* group *lunaris*, *Lecane* group *luna*), copepoda (nauplii, *Cyclopoida*) and Cladocera (*Chydorus sphaericus*, *Daphnia longispina* and *Simocephalus vetulus*). The species abundances of the different plankton groups (zooplankton, phytoplankton and protozoa) of the microcosms per sampling date are given in the appendix A (Table A7).

The PRC diagram of the zooplankton showed very little variation in community composition between treatment levels at the start of the experiment (Appendix A Figure A2 A). 14% of the variation in the zooplankton community composition between the different cosms, was explained by treatment, while 61% was explained by exposure time. The results from the Monte Carlo permutation tests and the $NOEC_{community}$ calculations (Figure 2.2 A) showed a significant difference ($p < 0.05$) in community structure between the controls and the highest Zn treatment throughout the experiment, starting from 2 weeks after exposure. The 75 µg Zn/L treatment community was only significantly affected at 2 weeks of exposure.

Cladoceran taxa like *D. longispina*, *C. sphaericus* and *S. vetulus* had a positive weight (species weight: b_k) within the PRC diagram, indicating that their abundances decreased in both treatment levels. Most rotifer taxa and Copepoda had a negative

affinity, implying an increase in abundance in the Zn treatments. Figure 2.3 shows the abundance values of some of the taxa for which a consistent significant treatment effect (Table 2.1) was found and a high affinity in the PRC diagram was noted.

At the population level, the highest Zn treatment had a significant negative effect on all Cladocera populations, especially on *D. longispina* which disappeared after 2 weeks of exposure and on *S. vetulus* (Figure 2.3 B and D). The lowest calculated consistent NOEC (< 75 µg Zn/L) was calculated for *S. vetulus*. The microcosms at the highest Zn treatment were dominated by copepods while cladocerans were the dominant macro-zooplankton group in the controls and low treatments (Figure 2.3 A). After three weeks, *D. longispina* and *S. vetulus* densities declined in all test systems, including the controls, possibly because of intensive predation by *Mesostoma* sp. (microtubellarian). After three weeks *Mesostoma* sp. became visible in all the microcosms (during the first 3 weeks *Mesostoma* sp. was only observed microscopically) and was not adversely affected by Zn treatments (Appendix A Figure A2). Between 2 and 4 weeks after the start of the treatment, rotifers in general (NOEC < 75 µg Zn/L) became significantly more abundant in the treated microcosms (Figures 2.3 C, E and F).

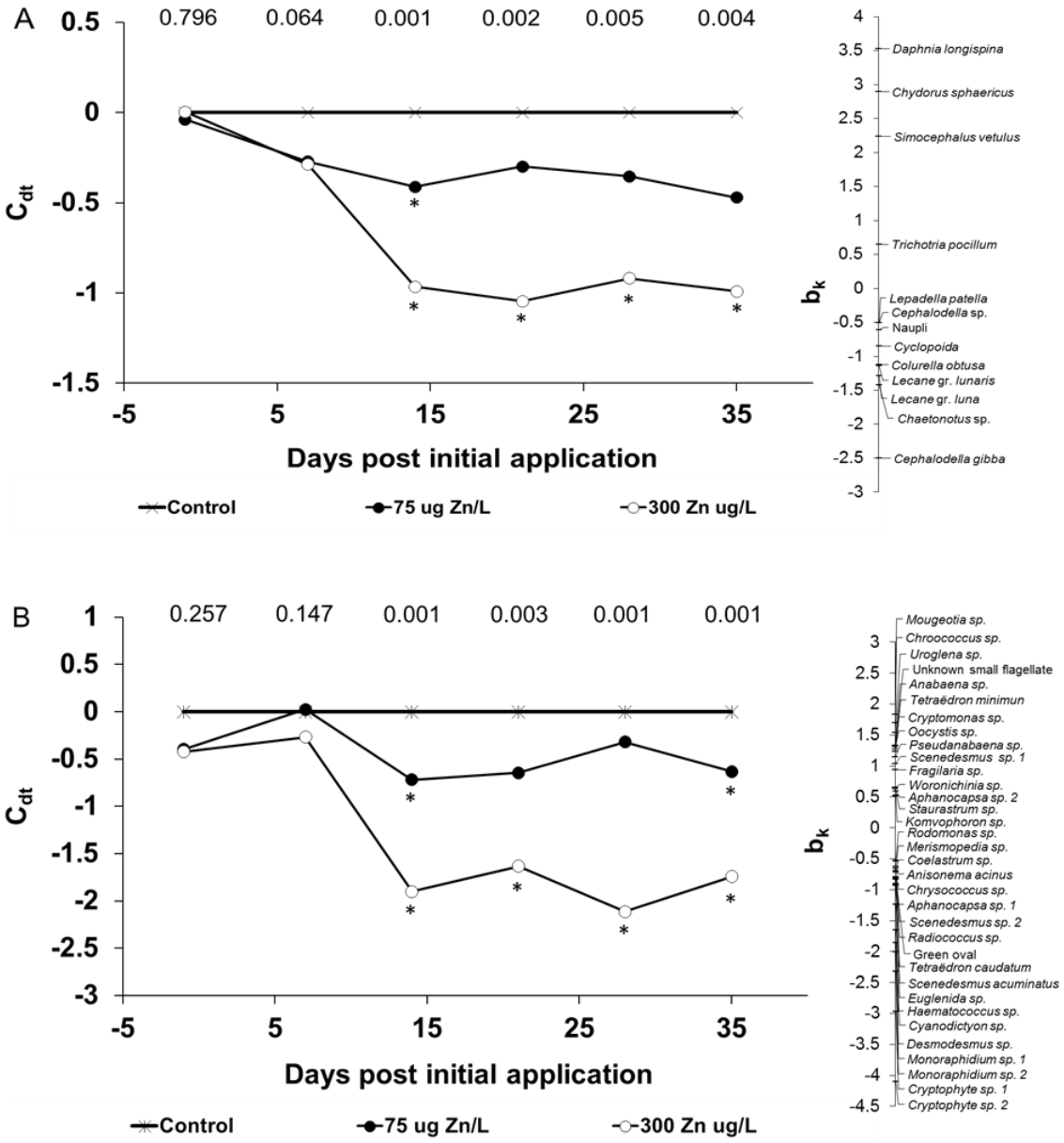


Figure 2.2: Principal response curve (PRC), resulting from the analysis of the zooplankton (A) and phytoplankton (B) data, indicating the effects of the different Zn treatments. The vertical axis represents the differences in community structure of the treatments compared to the controls expressed as regression coefficients (Cdt). The affinity of a taxon to the PRC is expressed as the species weight (bk). *: significant difference in community structure from the control ($p < 0.05$, Monte Carlo permutation test). Calculated Monte Carlo permutation test p values are plotted above the figures. See [101,102] for additional information.

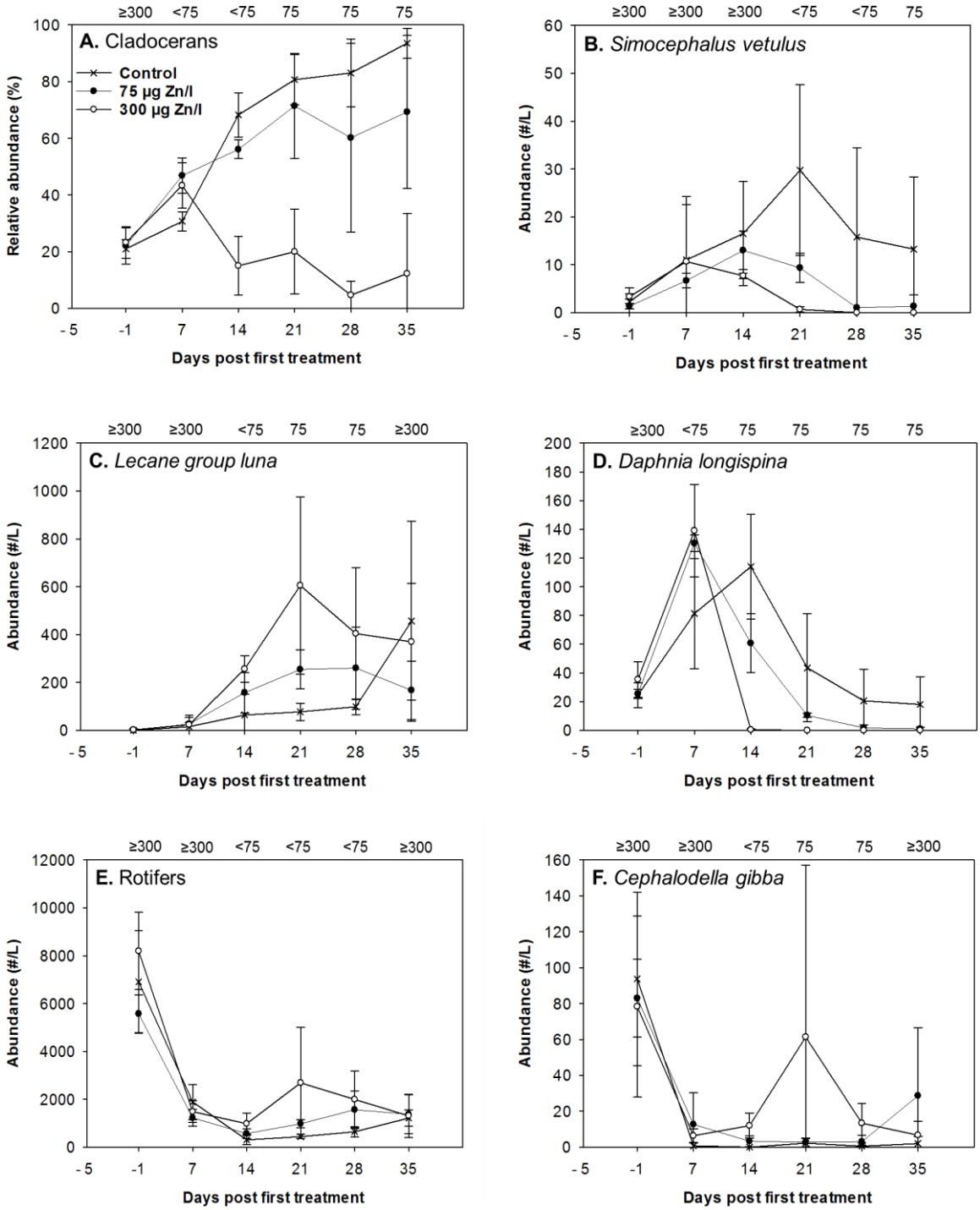


Figure 2.3: Population dynamics of some of the zooplankton taxa for which a consistent significant treatment effect and a high affinity in the PRC diagram was found. The geometric means (standard deviation as error bars) of the abundances (or relative abundances) per treatment concentration of Cladocerans in macro-zooplankton (A), *Simocephalus vetulus* (B), *Lecane* group *luna* (C), *Daphnia longispina* (D), Rotifers (E) and *Cephalodella gibba* (F) are shown. Calculated no-observed-effect concentrations are plotted above the figures.

Table 1: No-observed-effect concentrations (NOECs) (Williams test, $p < 0.05$) and observed effect classes per sampling date for the different plankton endpoints and species. The preselected effect classes refer to: 1= no effect; 2= slight effect; 3=clear short-term effects; 4: clear effect in short-term study; ↓= decrease; ↑= increase; ↑↓= increase and decrease on species and/or sampling date. [a] Effects observed only after 7 days of treatment.

		NOEC (µg/L)						Effect class	
Endpoint	Taxa	-1	7	14	21	28	35	75 µg/L	300 µg/L
Zooplankton									
PRC				< 75	75	75	75	2	4
Number of taxa						75↓	75↓	1	4↓
Cladocerans			< 75↑	< 75↓	75↓	75↓	75↓	2↓(↑[a])	4↓
	Daphnia longispina		< 75↑	75↓	75↓	75↓	75↓	2↑a	4↓
	Chydorus sphaericus			75↓	75↓	75↓	< 75↓	2↓	4↓
	Simocephalus vetulus				< 75↓	< 75↓	75↓	3↓	4↓
Copepods					75↑	75↑		1	3↑
	Nauplii				75↑	75↑		1	3↑
Rotifers				< 75↑	< 75↑	< 75↑		3↑	3↑
	Lecane gr. *lunaris*			< 75↑	75↑	< 75↑		2↑	3↑
	Lecane gr. *luna*			< 75↑	< 75↑	75↑		3↑	3↑
	Cephalodella gibba			< 75↑	75↑	75↑		2↑	3↑
Phytoplankton									
PRC				< 75	75	75	< 75	2	4
Number of taxa					75↓			1	2↓
Total chlorophyll			75↓			75↓	75↓	1	4↓
Cyanobacteria								1	1
	Anabaena sp.		75↓	< 75↓				2↓	3↓
	Aphanocapsa sp. 1			< 75↑	75↑			2↑	3↑
	Aphanothece sp.		75↓	75↑	75↑			1	3(↓[a])↑
Bacillariophyta				75↑				1	2↑
Chlorophyta				75↑	75↑	75↑	75↑	1	4↑
	Scenedesmus sp. 2					75↑	75↑	1	4↑
	Haematococcus sp.		< 75↓			75↑	< 75↑	2(↓[a])↑	4↑
	Desmodesmus sp.			75↑	< 75↑	75↑	75↑	2↑	4↑
	Monoraphidium sp. 2			< 75↑	75↑	75↑		2↑	3↑
Cryptophyta				75↑	75↑	75↑	75↑	1	4↑
	Cryptophyta sp. 2			< 75↑	75↑	75↑	75↑	2↑	4↑
	Cryptophyta sp. 1	75↑		75↑	< 75↑	75↑	< 75↑	2↑	4↑
Chrysophyta			75↓	75↑	75↑	75↑	75↑	1	4↑(↓[a])
	Uroglena sp.				75↓	< 75↓		2↓	3↓
	Chrysococcus sp.		75↓	75↑	75↑	75↑	75↑	1	4(↓[a])↑
Dinophyta								1	1
Euglenophyta				< 75↑	75↑	75↑		2↑	3↑
Protozoa									
PRC				75	75	75		1	3
Number of taxa								1	1
Ciliates			75↓	75↑	75↑	75↑	75↑	1	4↑(↓[a])
Bacterivorous ciliates				75↑	75↑	< 75↑	75↑	2↑	4↑
	R. brachykinetum			75↑		< 75↑	75↑	2↑	4↑
	Cyclidium sp.			75↑	75↑	< 75↑	75↑	2↑	4↑
Algivorous ciliates								1	1
Predaceous ciliates								1	1
Amoeba								1	1
Heterotrophic flagellates					75↑	75↑		1	3↑
	Codosiga botrytis				75↑	75↑		1	3↑

2.3.3 Phytoplankton

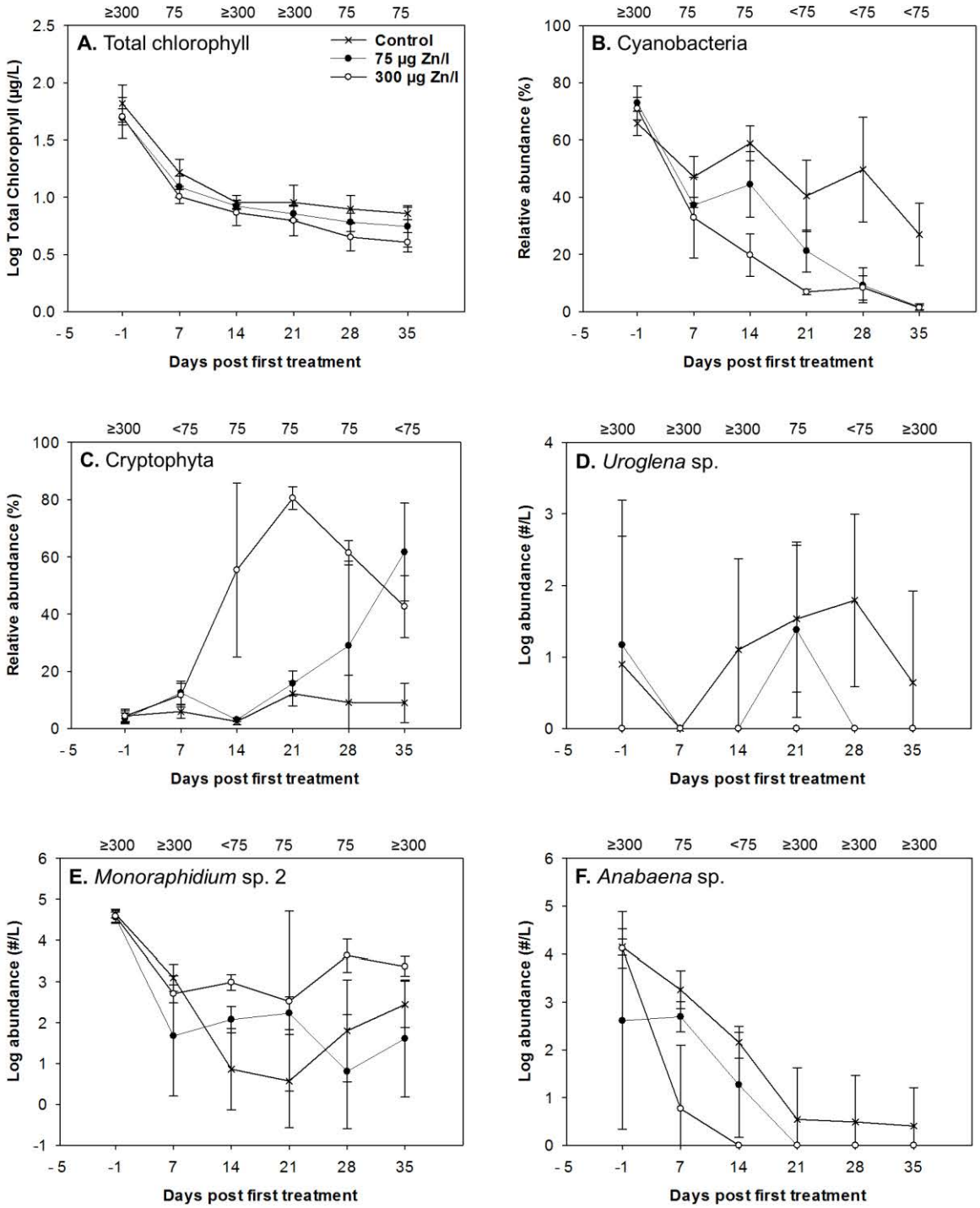
The phytoplankton community consisted of 83 different taxa, mostly belonging to the Chlorophyta (i.e. 38) and Cyanobacteria (i.e. 11). Six of the total number of taxa fulfilled the MDD category 1 criterion and 30 taxa were placed in category 2 (Appendix A Table A3). During the experimental period, the controls were characterized by Cyanobacteria (*Pseudanabaena* sp., *Aphanocapsa* sp. and *Anabaena* sp.), Chrysophyta (e.g. *Chrysococcus* sp.), Bacillariophyta and Chlorophyta (e.g., *Chlorococcus* sp., *Radiococcus* sp. and *Scenedesmus* sp.).

The PRC of the phytoplankton dataset (Figure 2.2 B) indicated that 20% of the total variance was explained by treatment and 37% by time. Significant treatment effects on the phytoplankton community were first observed 14 days after the first application. Monte Carlo permutation tests (Figure 2.2 B) showed that the community was affected by the highest Zn treatment throughout the experiment. The $NOfEC_{community}$ was below 75 µg Zn/L after 2 weeks and at the end of the experiment. In addition, a $NOEC_{community}$ of 75 µg Zn/L was observed on day 21 and 28. The species weights (b_k scores) indicated that most filamentous cyanobacteria (*Anabaena* sp., *Pseudanabaena* sp., *Woronchinia* sp.) and several non-filamentous cyanobacteria (*Chroococcus* sp., *Aphanocapsa* sp. 2) were adversely affected by the Zn treatments. In general it is observed that the abundance of most phytoplankton groups (Table 2.1) significantly increased in the highest treatment. No effects were observed for the Dinophyta abundances as a group and the Bacillariophyta abundances were only affected after 14 days of treatment. The Euglenophyta was the only phytoplankton group of which the abundance was (short term) affected by 75 µg Zn/L.

Table 2: No-observed-effect concentrations (NOECs) (Williams test, $p < 0.05$) and observed effect classes [89] per sampling date for the relative abundances of the different phytoplankton groups. Treatments resulted in significant increases (↑) or reductions (↓).Blank fields indicate that NOEC were equal to or higher than the highest tested concentration (300 µg/L). 1= no effect; 2= slight effect; 3=clear short-term effects; 4: clear effect in short-term study. [a] Effects observed only after 7 days of treatment.

	NOEC (µg/L)						Effect class	
	-1	7	14	21	28	35	75 µg/L	300 µg/L
Cyanobacteria		75↓	75↓	< 75↓	< 75↓	< 75↓	4↓	4↓
Bacillariophyta		< 75↑	75↓	75↓		75↓	2(↑)[a]	3↓
Chlorophyta				75↓			1	2↓
Cryptophyta		< 75↑	75↑	75↑	75↑	< 75↑	2↑	4↑
Chrysophyta				75↓			1	2↓
Dinophyta							1	1
Euglenophyta		< 75↑					2↓[a]	2↓[a]

The community structure (relative abundances) itself was significantly affected by Zn (Figure 2.4, Table 2.2). The relative abundance of the Cyanobacteria decreased significantly with increasing Zn (NOEC: < 75 µg Zn/L at 28 and 35 days). The relative abundance of the Cryptophyta, on the other hand, started to increase significantly after 14 days at the highest treatment and made up to 80 % of the phytoplankton population. Throughout the experiment the relative abundance of the Cryptophyta in the low treatment microcosms increased and became significantly different from the controls after 35 days. For the other phytoplankton groups no significant Zn effect was observed. The log abundances of six phytoplankton taxa, that could be used to calculate a consistent NOEC, are illustrated in Figure 2.4.


≥300 75 ≥300 ≥300 75 75
A. Total chlorophyll
Control
75 µg Zn/l
300 µg Zn/l
Log Total Chlorophyll (µg/L)
Days post first treatment
≥300 75 75 <75 <75 <75
B. Cyanobacteria
Relative abundance (%)
Days post first treatment
≥300 <75 75 75 75 <75
C. Cryptophyta
Relative abundance (%)
Days post first treatment
≥300 ≥300 ≥300 75 <75 ≥300
D. Uroglena sp.
Log abundance (#/L)
Days post first treatment
≥300 ≥300 <75 75 75 ≥300
E. Monoraphidium sp. 2
Log abundance (#/L)
Days post first treatment
≥300 75 <75 ≥300 ≥300 ≥300
F. Anabaena sp.
Log abundance (#/L)
Days post first treatment

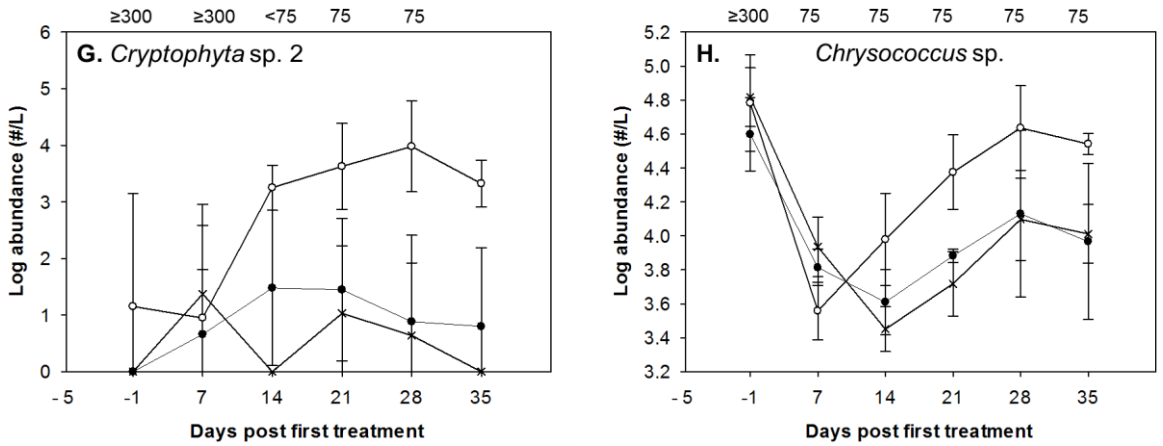


Figure 2.4: Total chlorophyll concentrations and population dynamics of some of the phytoplankton taxa for which a consistent significant treatment effect was found and a high affinity in the PRC diagram was found (standard deviation as error bars). The geometric means of the Total chlorophyll concentrations (A), the relative abundance values of the Cyanobacteria (B) and Cryptophyta (C) and the log abundances per treatment concentration of *Uroglena* sp. (D), *Monoraphidium* sp. 2 (E), *Anabaena* sp. (F), *Cryptophyta* sp. 2 (G) and the *Chrysococcus* sp. (H) are shown. Calculated no-observed-effect concentrations are plotted above the figures.

Anabaena sp. and *Uroglena* sp. (NOEC: 75 µg Zn/L) were the only taxa with a consistent NOEC that indicated a decrease in abundance in comparison with the controls, however these effects were only temporal. The lowest Zn treatment only had a positive, temporal effect on the abundances of *Monoraphidium* sp., *Desmodesmus* sp., *Haematococcus* sp., *Aphanocapsa* sp. 1, *Cryptohyte* sp. 1 and 2. A significant effect of the highest Zn treatment were observed for *Aphanothece* sp. and *Aphanocapsa* sp. 1 on day 7 and 14 after the start of the treatment. *Monoraphidium* sp. 2 was significantly affected by Zn, starting 14 days after the first application and this effect was significant throughout the experiment with exception of day 35. All other phytoplankton taxa displayed in table 1 showed effect class 4 effects [89]. The total chlorophyll concentration in the controls and the treatments declined after the start of

the experiment (Figure 2.4 A). Significantly lower total chlorophyll levels (NOEC: 75 µg Zn/L) were observed at 7, 28 and 35 days after the first treatment.

2.3.4 Protozoa

Twenty-three distinct protozoa taxa were identified. Most taxa were ciliates (i.e. 15), followed by heterotrophic flagellates (i.e. 5) and amoeba (i.e. 3). None of all these taxa could be placed in MDD category 1 but 8 fulfilled the MDD criterion 2 (Appendix A Table A3). The percentage of the total variance in the protozoa data, that is explained by treatment, was 20 % and 42 % was explained by time (Figure 2.5).

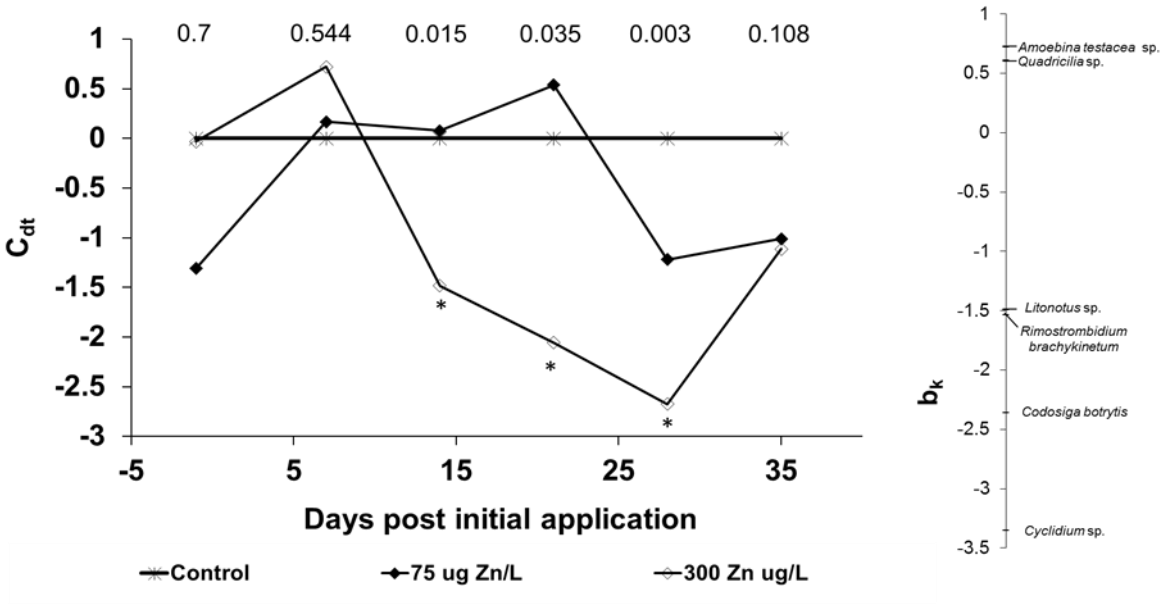


Figure 2.5: Principal response curve (PRC) resulting from the analysis of the protozoa data, indicating the effects of the different Zn treatments. The vertical axis represents the differences in community structure of the treatments compared to the controls expressed as regression coefficients (C_{dt}). The affinity of a taxon to the PRC is expressed as the species weight (b_k). *: significant difference in community structure from the control ($p < 0.05$, Monte Carlo permutation test). Calculated Monte Carlo permutation test p values are plotted above the figures. See [101,102] for additional information.

Monte Carlo permutation tests revealed significant differences in community compositions starting from 14 days up to 28 days after first application (Figure 2.5). For these sampling days a $NOEC_{community}$ of 75 µg Zn/L was calculated. Ciliates, in general, became significantly more abundant after 14 days till the end of the experiment in the highest treatment compared to the controls (Table 2.1). Bacterivorous ciliates (*Rimostrombidium brachykinetum* and *Cyclidium* sp.) in particularly became much more abundant (Figure 2.6 B and C, table 2.1). Heterotrophic flagellates (e.g. *Codosiga botrytis*) abundance was significantly higher (NOEC: 75 µg Zn/L) after 21 and 28 days only (Figure 6 A, table 2.1). A consistent NOEC could not be calculated for abundances of protozoa (Table 2.1).

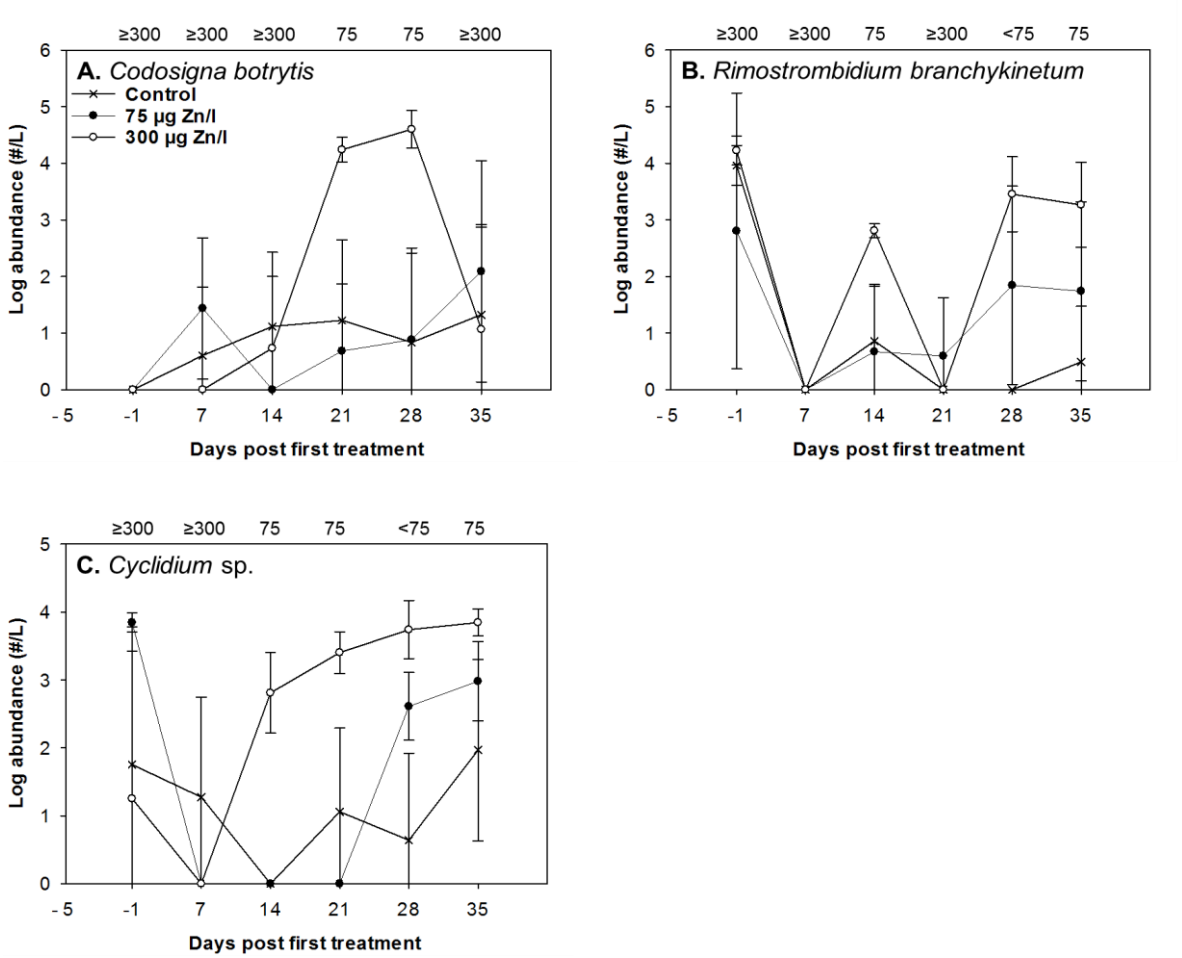


Figure 2.6: Population dynamics of some of the protozoa taxa for which a consistent significant treatment effect was found and a high affinity in the PRC diagram was found (standard deviation as error bars). The geometric means of the log abundances per treatment concentration of Codosigna botrytis (A), Rimostrombidium branchykinetum (B) and Cyclidium sp. (C) are shown. Calculated no-observed-effect concentrations are plotted above the figures.

2.3.5 Community metabolism and general chemical properties of the water

The physicochemical parameters: pH (morning, afternoon and mean), DO (morning, afternoon and mean), DOC and conductivity showed significant consistent treatment effects (Table 2.3, Figure 2.7). After 7 days of treatment the DO (afternoon and mean), primary production and the pH (morning and mean) were significantly lower for all the Zn treatments. Clear short term effects were observed for DO (with exception in the

morning and the mean value which had a consistent NOEC of 75 µg/L), productivity and respiration (Table 2.3) for both Zn treatments.

Table 2.3: No-observed-effect concentrations (NOECs) (Williams test, $p < 0.05$) and observed effect classes per sampling date for community metabolism and chemistry endpoints in microcosms. The numbers of the preselected effect classes [89] refer to: 1= no effect; 2= slight effect; 3=clear short-term effects; 4: clear effect in short-term study; ↓= decrease; ↑= increase; ↑↓= increase and decrease on species and/or sampling date. Blank fields indicate that NOEC were equal to or higher than the highest tested concentration (300 µg/L).

	NOEC (µg/L)											Effect class	
	-1	1	7	9	14	16	21	23	28	30	35	75 µg/L	300 µg/L
DO													
morning	—				75↓	75↓						1	3↓
afternoon			< 75↓		75↓	< 75↓	< 75↓		75↓			3↓	3↓
max-min	—		< 75↓		75↓	< 75↓	< 75↓		75↓			3↓	3↓
min-max	—		< 75↑		75↑	< 75↑	< 75↑		75↑			3↑	3↑
Mean			< 75↓		75↓	75↓	< 75↓					2↓	3↓
pH													
morning			< 75↓		75↓	75↓	< 75↓					2↓	3↓
afternoon			75↓		75↓	75↓	< 75↓		< 75↓			2↓	3↓
Mean			< 75↓		75↓	75↓	< 75↓					2↓	3↓
N													
NH_3		—	—	—		—	—	—	—	—		1	1
NO3 + NO2		—	—	—		—	—	—	—	—		1	1
P													
Total		—	—	—		—	—	—	—	—		1	1
SRP		—		—		—		—		—		1	1
DOC		—		—	75↓	—	< 75↓	—	< 75↓	—	< 75↓	4↓	4↓
Conductivity		—		—		—	75↑	—	75↑	—		1	3↑
BOD_5		—	< 75↑	—	75↑	—		—	75↓	—	75↓	2↑↓	4↓↑

The pH was slightly lower at 75 µg Zn/L but showed a clear short term effect at the highest treatment. Although the electrical conductivity was generally higher in the highest treatments compared to the controls, only significant differences were found

for day 21 and 28. After 14 days the DOC (Figure 2.7 A) became significantly lower in the highest treatments but throughout the experiment a consistent NOEC of < 75 µg Zn/L was calculated. The concentration levels of ammonia, NO_3 +NO_2, SRP and Total P did not show any significant treatment effects. The BOD_5 levels (Figure 2.7 C, Table 2.3) of the microcosms increased significantly with increasing Zn (NOEC: 75 µg Zn/L) after 7 and 14 days but the BOD_5 of the controls became significantly higher than the Zn treatments in the last 2 weeks of the experiment (NOEC: 75 µg Zn/L). The concentrations of calcium (Figure 2.7 D), chloride and potassium increased over the course of the experiment but did not show any significant treatment effects. The physico-chemical water parameters are given in the Appendix A (Table A5, 6).

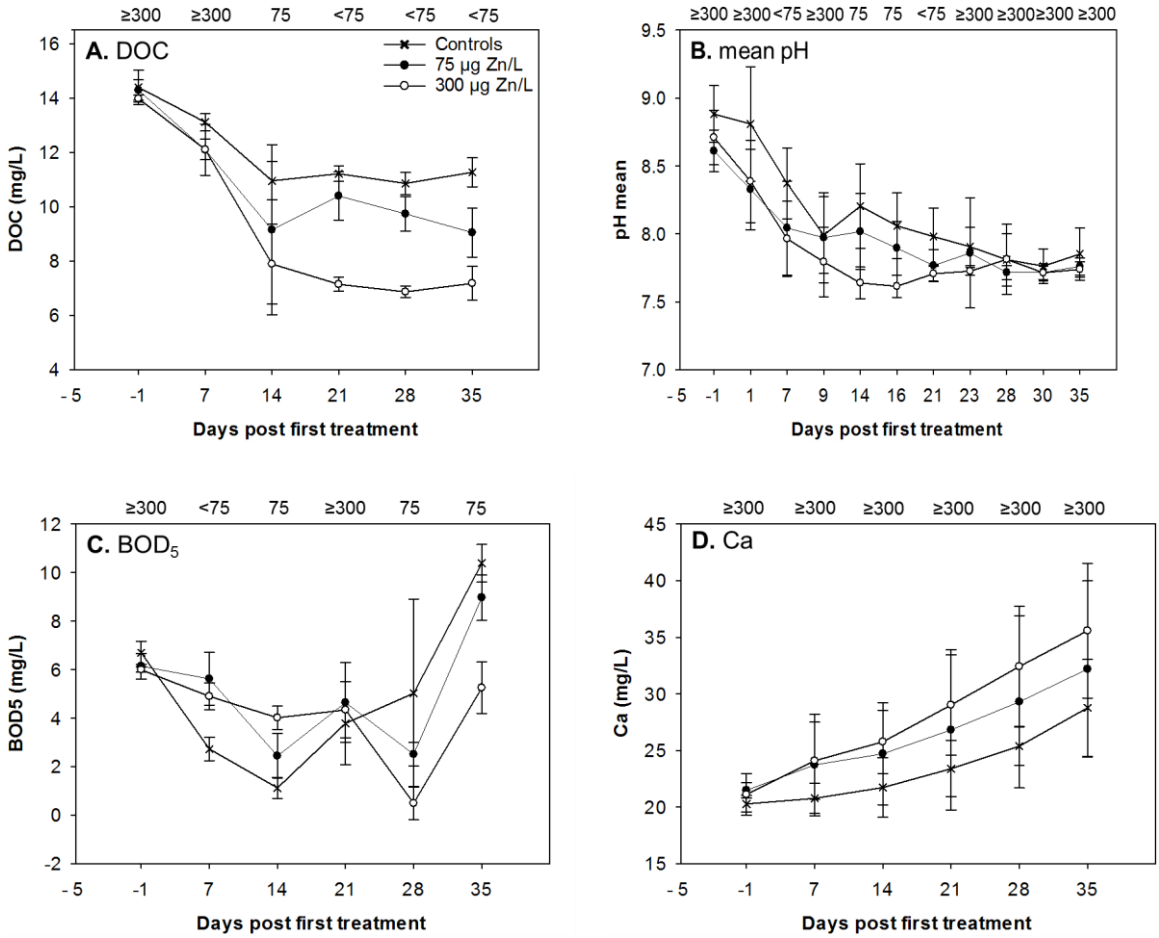


Figure 2.7: Geometric means of the dissolved organic carbon (DOC) concentrations (standard deviation as error bars) (A), the mean pH (B), the Biological Oxygen Demand after 5 days (BOD_5) (C) and the calcium concentrations (D) per treatment throughout the experiment. Calculated no-observed-effect concentrations are plotted above the figures.

2.4 Discussion

2.4.1 Zinc concentrations

During the first 7 days the Zn rapidly disappeared from the water column in all the treatments. Most likely due to losses by sorption and uptake by the biota and the sediment. After 7 days of treatment, we presume an equilibrium was reached between

the water column and the sediment and the target concentrations were met throughout the rest of the experiment. The low Zn concentrations during the first 7 days may explain the absence of clear Zn effects on the biota and the physico-chemical water characteristics before the 14th day of exposure.

2.4.2 Zn effects and community interactions

Clear Zn effects were mainly observed for the cladoceran populations at the highest treatment (Table 2.1 till 2.4 shows the classification of the treatment related responses by using the different effect classes described by Brock et al. [89]). These adverse effects probably resulted in an indirect, positive effect on rotifers and nauplii abundances, which appeared to be less sensitive to Zn stress (Figure 2.8 illustrates a schematic overview of the observed effects of the highest Zn treatment on the ecosystem structure of plankton-dominated microcosms). These indirect effects were likely the result of a reduced food competition [46], which is frequently observed in micro- and mesocosms studies [9,13,29,30]. In contrast to Marshall et al. [60], no adverse Zn effects were observed for the rotifers or for cyclopoide copepods. However, higher tolerance to Zn stress of Cyclopoida have also been suggested by Monteiro et al. [59] and Baudouin and Scoppa [104].

Despite the adverse Zn effect on the total chlorophyll levels and the primary production (community metabolites: pH, DO and DOC), only 2 algae taxa (*Anabaena* sp. and *Uroglena* sp.) showed a temporal adverse treatment effect (Table 2.1 and 2.3). Most phytoplankton groups and several taxa (e.g. *Cryptophyta* sp. 1, 2 and *Chrysococcus* sp.) in the present study showed a significant increase in abundance throughout the experiment at the highest Zn treatment. This is surprising, since phytoplankton is

considered to be the most sensitive group according to the Zn SSD (Appendix Figure A1; mean $HC5_{-phytoplankton}$: 46 µg Zn/L and mean $HC50_{-phytoplankton}$: 76 µg Zn/L; [5]) and Marshall et al. [60]. The most likely explanation for the increase in phytoplankton abundance is that Zn affects the top-down effects that the zooplankton has on the phytoplankton [9]. In our study, the reduced population size of the large cladoceran species like *D. longispina*, *S. vetulus* and *C. sphaericus*, likely reduced the grazing pressure on the phytoplankton population and probably altered the phytoplankton and protozoa community structure as mentioned by Arvola and Salonen (2001) [48]. Controls with high cladoceran grazing pressure were dominated by filamentous algae (e.g. *Anabaena* sp., *Pseudanabaena* sp., *Mougeotia* sp.) or colony forming (e.g. *Fragilaria* sp., *Scenedesmus* sp.1, *Oocystis* sp., *Chroococcus* sp., *Uroglena* sp.) taxa that were morphologically adapted to withstand grazing [46]. The Zn treatments reduced the Cyanobacteria dominance and increased the relative abundance of small (< 10 µm) *Cryptophyta* species (e.g. sp. 1 and 2, *Rodomonas* sp.) and other fast growing (r-strategy) and grazing sensitive species (e.g. *Chrysococcus* sp., *Monoraphidium* sp. and *Scenedesmus* sp. 2) [105]. The interaction (Linear regression: $p = 0.002$) between the *Daphnia* and the small *Cryptophyta* species in the control microcosms are shown in the Appendix A (Figure A3) to illustrate the effect the *Daphnia* has on them. Hypothetically these small species have a relatively low contribution to the total chlorophyll compared to the filamentous algae groups, which could explain the decline in total chlorophyll at the highest treatments. The ciliates (specifically bacterivorous ciliates) probably also benefitted from the disappearance of the large cladocerans, either due to less competition for food or due to a decreased predation pressure [48,106]. In addition to the planktonic food web interactions, the Zn

toxicity may also have changed the phytoplankton community structure, favoring the more Zn tolerant species.

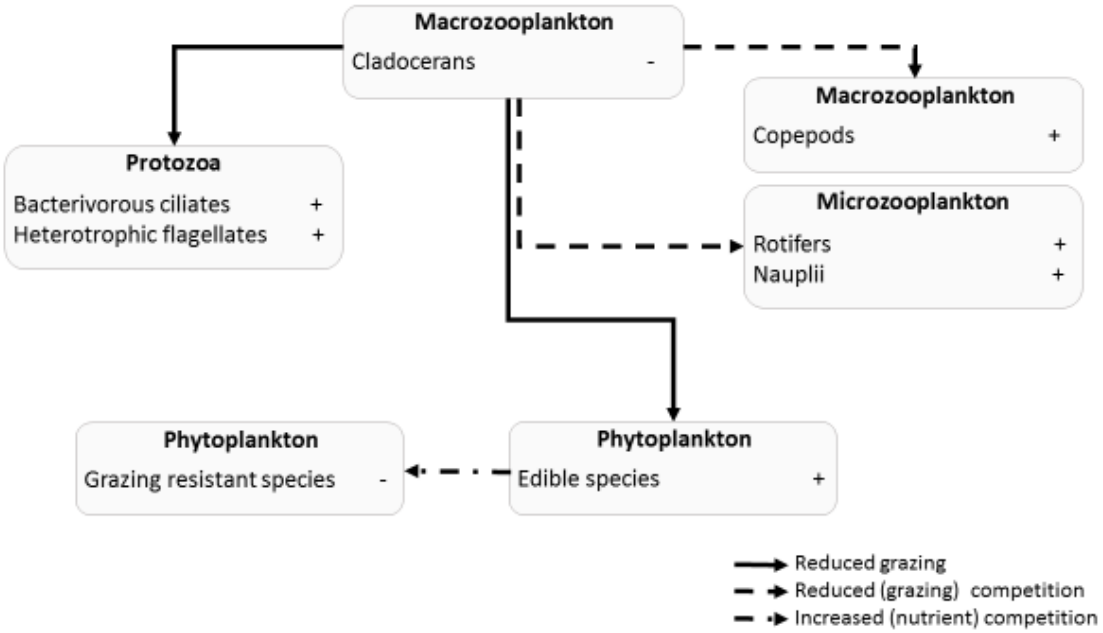


Figure 2.8: Schematic overview of the observed effects of the highest Zn treatment (300 µg/L) on the ecosystem structure of a plankton-dominated community (+: Increase; -: Decrease). At 300 µg Zn/L, a significant reduction in cladocerans resulted in an increase of rotifers, nauplii and copepod adults, which is probably the result from reduced grazing competition. Additionally, the reduced grazing pressure on the edible phytoplankton and protozoa population had a positive effect on their abundances and shifted the phytoplankton community dominance from grazing-resistant to edible species.

As grazers, large cladocerans, like *D. longispina*, have a large impact on the planktonic food web [47,106]. When these are removed or when their abundance declines this generally induces important planktonic community shifts [48]. In our study however, it is unclear if the *D.longispina* population declines were induced by Zn toxicity, *Mesostoma* sp., a combination of both or by inter- and intra-specific interactions which

could have an effect on the species sensitivity to toxicants [9,13,26]. *Mesostoma*'s are known to have a large effect on the zooplankton community [30,52] and so indirectly on the phytoplankton community. Although the *Mesostoma* predators possibly increased the stochasticity between the replicates, they are a natural part of the aquatic ecosystem. The zooplankton abundance plots (Figure 2.3 B - F) suggest that internal production more than kept up with sampling losses, thus avoiding concerns about population depletions over time due to sampling.

The normalized NOEC values (for reproduction) of *D. longispina* from the chronic Zn toxicity database (Appendix A Table A4) are higher than the observed NOECs of *D. longispina* in the microcosms. Because of the difference in chemical properties of the water, the *D. longispina* NOECs for the highest Zn treatments were a factor 1.6 times lower than those for the controls. Although after 14 days the BLM NOEC within the highest treatment cosms (NOEC: 279 µg Zn/L), was slightly lower than the target concentration, *D. longispina* went extinct. The *Daphnia* population sizes in the lowest treatments tended to be lower (significant for *S. vetulus*) than in the controls and the population even neared extinction after 28 day. Our data suggests that the observed Zn toxicity effects on *D. longispina* in a planktonic community are bigger than the predicted effects based on single species data. A possible explanation for this is the lack of genetic variation by using isoclonal population for ecotoxicity tests [5,107,108]. Other than *D. longispina*, *Anuraeopsis fissa* is the only zooplankton species included in both the Zn BLM [5] and our study. *A. fissa* is also considered the most sensitive rotifer taxa in the Zn SDD (mean normalized NOEC= 313 µg Zn/L table S4). Even when the normalized NOEC of *A. fissa* sharply declined after 14 days (normalized NOEC: 183 µg Zn/L and stayed around this concentration) in the highest treatments,

no adverse effect on the *A. fissa* abundance was observed throughout the experiment, which deviates from the predicted effects based on single species data.

2.4.3 Effects of Zn on chemical properties of the water and on HC5 or HC50

Due to the pre-treatment mixing, there was very little variation between all the different microcosms in chemical properties of the water (Appendix A Table A1) and biology (Figures 2.2 and 2.5) at the start of the experiment. These small variations in water properties were crucial to ensure that all microcosms started with the same Zn bioavailability [5]. A possible explanation for the decreasing pH and DO in the first week could be the increased grazing pressure (sharp decline in total chlorophyll: Figure 2.4 A; and phytoplankton densities: Figure 2.4 B - H) by the increased zooplankton abundances (Figure 2.3 A and B). In aquatic ecosystems calcium fluctuations are mainly explained by a combination of biotic (e.g. mollusk shell construction and bacteria), chemical (e.g. the pH-carbon dioxide-bicarbonate system) and physical (e.g. evaporation) processes [46]. In our study however, it is unlikely that evaporative water losses alone could explain the increase of the calcium concentration level throughout the experiment, and therefore biotic and chemical processes may also have contributed.

To our knowledge, very few microcosm community studies with metals are available that have also investigated the effect on the DOC concentration. Hommen et al. [91] did not found any significant effect of Ni on the DOC measured in the exposure system. Schaeffers [90] reported on a mesocosm experiment with copper and their results suggest a possible effect of Cu on the DOC (although the authors did not explicitly test the statistical significance of this effect). The consistent Zn effect (NOEC < 75 µg/L)

observed on DOC (Figure 2.7 A) throughout the experiment is an indication that the treatments had a significant effect on the microbial loop and on pelagic food web interactions [46,109]. When lowering both the pH and DOC, Zn becomes more bioavailable and therefore more toxic for the biota [5,7,16,87]. By changing the water properties, the Zn itself influenced its own toxicity. After the experiment was completed, new calculations of the $HC5_{\text{-plankton}}$ and $HC50_{\text{-plankton}}$ were performed to reflect the actual physico-chemistry that was recorded during the experiment in the various treatments and to more optimally reflect the plankton community (by including toxicity data for 3 rotifer species and not considering data from the epibenthic amphipod *Hyalella azteca*) (Appendix A Table A2 for details). The $HC5_{\text{-plankton}}$ and $HC50_{\text{-values}}$ values throughout the experiment are reported in Table 2.4. After 35 days of treatment the calculated $HC5_{\text{-plankton}}$ for controls were 1.3 times higher than for the 75 µg Zn/L treatments and 1.6 times higher than for the 300 µg Zn/L treatments. The same trend can be observed for the $HC50_{\text{-plankton}}$.

Table 2.4: Mean (± standard deviation) calculated $HC5_{\text{-plankton}}$ and $HC50_{\text{-plankton}}$ per sampling day and treatment, calculated as explained in [5], taking into account chronic Zn toxicity data for species mentioned in table A2

		-1	7	14	21	28	35
HC5 (µg Zn/L)	Control	85 ± 4	83 ± 4	69 ± 7	69 ± 4	64 ± 3	65 ± 3
	75	87 ± 2	74 ± 11	56 ± 21	61 ± 9	55 ± 6	51 ± 8
	300	84 ± 1	74 ± 4	46 ± 7	41 ± 3	38 ± 3	40 ± 5
HC50 (µg Zn/L)	Control	351 ± 31	271 ± 15	216 ± 32	201 ± 13	181 ± 8	189 ± 13
	75	316 ± 20	222 ± 40	174 ± 64	172 ± 17	158 ± 8	151 ± 12
	300	320 ± 21	214 ± 16	129 ± 18	122 ± 4	122 ± 6	124 ± 6

2.4.4 Zn risk assessment

The current study focused on the plankton communities and did not include fish or macroinvertebrates (except *Mestostoma* sp. and snails, which were not investigated), which are, according to literature, less sensitive to Zn than phytoplankton [5,7]. In our study, however, only two phytoplankton taxa showed a temporally consistent adverse Zn effect (NOEC: 75 µg Zn/L), which is almost a factor 2 higher than the lowest calculated normalized NOEC of *Pseudokirchneriella subcapitata* (range NOEC: 75 - 37 µg Zn/L), which is the most sensitive algae in the chronic Zn toxicity database (Table S2). Even at 300 µg Zn/L, most phytoplankton groups increased in abundance while the large cladocera were adversely affected. *S. vetulus* was the most sensitive taxon in our study (NOEC: < 75 µg Zn/L) and had a similar Zn sensitivity as the most sensitive cladocera in the chronic Zn toxicity database (*C. dubia*:table S2). This study thus demonstrates that community responses are not only dependent on the sensitivity of the organism alone [3,7] but also on the inter-and-intraspecific interactions [15,26], which can affect Zn risk assessment [5]. Therefore, it is recommended that additional multi-species Zn exposure experiments are carried out and these should preferably also include taxa belonging to higher trophic levels. This would enable a more complete understanding of how interspecific and intraspecific interactions can be taken into account in Zn risk assessment. Microcosm and mesocosm studies provide more realistic risk assessment than lower-tier single species tests and although these results are more difficult to interpret [29,89], they are essential and provide much extra information to Zn risk assessment studies.

According to the recommendations by Brock at al. [89] at least 8 populations of potentially sensitive taxa with an appropriate MDD (category 1) should be present in the test system to assure that the power of the statistical analyses conducted is high

enough to demonstrate possible treatment-related responses in terms of abundance. In our study, at least 14 different category 1 taxa, including cladocerans, rotifers, Chlorophyta, cyanobacteria, Cryptophyta and diatoms were observed, thus conforming the reliability of our study [89]. The multivariate analysis (PRC) conducted on the different plankton groups (zooplankton, phytoplankton and protozoa) revealed a consistent $NOEC_{community}$ of 75 µg Zn/L. At 75 µg Zn/L (Figure 2.2) the PRC curves for zooplankton and phytoplankton were only significantly different from the controls after 14 days and for the phytoplankton also at the end of the experiment. This is possibly due to community compensation (sensitive species replaced by less sensitive) or adaptation [9,66,110]. Protozoa have only been included in very few monitoring studies [59] and microcosm studies aiming to investigate the effects of toxicants on the community structure. Protozoa are an important link between the microbial loop and the pelagic food web [46] and can reveal important information about the effects the toxicants have on the aquatic community. In our study no consistent adverse effects were observed for any of the protozoa groups.

Another important thing that should be taken into account for Zn risk assessment is the indirect effect Zn has on the chemical properties of the water, with most importantly the effects on the DOC (see results and discussion). This resulted in a considerable difference in metal bioavailability in the microcosms between the different treatments and with time. For the treatment at 75 µg Zn/L, which is the consistent $NOEC_{community}$, the BLM-normalized $HC5_{-plankton}$ values ranged between 51 and 87 µg Zn/L throughout the experiment (Table 2.4). In this same treatment, the average measured Zn concentration during the last four weeks of exposure (77.9 µg Zn/L) was similar to or higher than the BLM-normalized $HC5_{-plankton}$ during the same period (i.e., between 51 and 74 µg Zn/L, mean: 59 µg Zn/L), suggesting that the BLM-normalized $HC5_{-plankton}$ is

equal to or lower than the consistent $NOEC_{community}$ of the different plankton groups and thus protective of the major structural and functional components of the plankton dominated community in the present study.

The latter suggestion of protectiveness of the $HC5_{-plankton}$ is based on an SSD-analysis in which only planktonic species were considered, which was done to match as closely as possible the pelagic community investigated in our microcosm experiment. However, current practice of SSD-analysis for risk assessment in regulatory context usually considers a wider range of species, including benthic and fish species to derive PNEC's (EU) or WQC (US), regardless of the type community or water body that is the target of protection. We therefore also investigated the 'protectiveness' of two HC5 values calculated according two 'regulatory' methodologies: (i) the $HC5_{EU\text{-}regulatory}$, calculated with the EU methodology as explained in [5] (and in fact identical to the method used to calculate $HC5_{-plankton}$, except that benthic and fish species were now also considered), and (ii) the $HC5_{US\text{-}regulatory}$, calculated with the US methodology as explained in [111]. HC5 values with these two methodologies for all treatments and time points in the microcosm experiment are reported in Supplemental data (Appendix Table A8).

Again focusing on the last four weeks of exposure (as above), we find that the mean $HC5_{EU\text{-}regulatory}$ for the 75 µg/L treatment is 73 µg/L. This value is only 24% higher than the $HC5_{-plankton}$ and still slightly lower (by 6%) than the measured exposure concentration of 77.9 µg/L in this 'no effect' treatment ($NOEC_{community}$). Thus, the $HC5_{EU\text{-}regulatory}$ can be considered protective for the investigated plankton-dominated community. The mean $HC5_{US\text{-}regulatory}$ for the 75 µg/L treatment is 159 µg/L, which is 2.2-fold higher than the $HC5_{EU\text{-}regulatory}$. A detailed comparison of the two methodologies is outside the scope of the present study, but important differences in methodology

include (i) species selection (no algae species considered in US), (ii) effect estimate selection (EC10 or NOEC in EU, EC20 or MATC in US), (iii) BLM application (three different BLMS for each trophic level in EU vs. a single BLM in US for all species), (iv) statistical estimation of HC5 (fitting SSD to all chronic toxicity data in EU vs. only four chronic toxicity data closest to the 5th percentile used for calculation). The $HC5_{US\text{-}regulatory}$ is also 2-fold above the $NOEC_{community}$. Thus, the present study provides no evidence of 'no community effects' at or above the $HC5_{US\text{-}regulatory}$. Yet, neither does it provide evidence of 'community effects' below the $HC5_{US\text{-}regulatory}$, as the mean $HC5_{US\text{-}regulatory}$ in the 300 µg/L treatment equals 133 µg Zn/L, which is 2.2-fold above the measured Zn concentration of 287 µg/L in this treatment (i.e. the $LOEC_{community}$). Altogether, the $HC5_{US\text{-}regulatory}$ is about half-way between the $NOEC_{community}$ and $LOEC_{community}$ by about 2-fold in each direction and thus the protectiveness (nor the non-protectiveness) of the $HC5_{\text{-}USregulatory}$ for the structure and function of the community in the present study cannot be determined. Microcosm testing at concentrations intermediate to those investigated here, and thus closer to this $HC5_{US\text{-}regulatory}$ would be required for a more definitive assessment.

2.5 Conclusion

The planktonic groups revealed a consistent NOECcommunity of 75 µg Zn/L, similar to or higher than the HC5-plankton, thus suggesting its protectiveness in this study. At 300 µg Zn/L a significant reduction in cladocerans resulted in an increase of rotifers, ciliates and phytoplankton abundances. Additionally, the phytoplankton community shifted in dominance from grazing-resistant to edible species. Contrary to the Species Sensitivity Distribution (SSD) prediction, which identified phytoplankton as the most

sensitive group, only the total chlorophyll and the abundance of 2 phytoplankton species were adversely affected at 300 µg Zn/L. Thus, although the $HC5\text{-}_{plankton}$ estimated from the bioavailability-normalized SSD was overall protective for the plankton community, the SSD was not able to correctly predict the species sensitivity ranking within their community context at the $HC50\text{-}_{plankton}$.

3

The combined and interactive effects of zinc, temperature and phosphorus on the structure and functioning of a freshwater community

Redrafted after:

Van de Perre D, Roessink I, Janssen CR, Smolders E, Van den Brink PJ, de Schamphelaere K a. C.. The combined and interactive effects of zinc, temperature and phosphorus on the structure and functioning of a freshwater community. Environ. Toxicol.. In preparation

3.1 Introduction

Metal pollution, together with eutrophication and climate change, can pose risks to aquatic ecosystems [40,43,84]. Understanding and being able to predict the effects of chemicals (e.g. metals) on the structure and function of the aquatic ecosystem under different environmental conditions (e.g. changing temperature and changing nutrient supply, e.g. phosphorus) is a major challenge in environmental toxicology [1,2,43,112]. This since climate models predict that climate change will induce a general temperature increase of 2°C to 4°C within the next century in temperate regions [40] and will also induce an increased phosphorus loading from land to lakes and streams due to increased rainfall intensity [41].

Conventional ecological risk assessment of chemicals is mainly based on single-species laboratory tests which evaluate the effects of a single stressor and which are conducted under optimal standard (e.g. temperature, food, pH) conditions. However, in reality aquatic communities are exposed to a mixture of stressors under different and often rapidly changing environmental conditions [113]. Many studies have already shown that environmental conditions can significantly alter the responses of organisms to toxicants [10,43,114–116]. This leads to interactive effects of environmental conditions and toxicants. The specific interaction type (synergistic: combined effect greater than expected; antagonism: combined effect smaller than expected; additive: combined effect as expected) itself can also vary by biologic response level (i.e. populations, community) and trophic level (i.e. autotrophs, heterotrophs) [115,116]. Jackson et al. (2016) [115] for example, conducted a meta-analysis on 88 freshwater studies and they found that the cumulative mean of the interactive effects was additive at the population level and antagonistic at the community level. However, antagonistic interactions were more common at the community level, while synergistic interaction

occurred more frequent at the population level [115]. This might be a result of the fact that at higher levels of biological organisation, compensatory species dynamics become more important and this may explain the difference in interaction type between the population and the community level (i.e. composition, function and diversity) [117].

Temperature (T) and phosphorous (P) are two important environmental factors that can have major effects on planktonic community composition and functioning [11,18,68–70,79–81,118,119] and both are also well known to influence the sensitivity of organisms to toxicants. Planktonic organisms are, like most aquatic organisms, ectotherms and they are strongly influenced by temperature on their metabolic rates, behaviour activity and physiological processes [11]. Temperature can also modify the toxic effects of pollutants by influencing their bioavailability and toxicokinetics [10,11]. A review study conducted by Noyes et al. (2009) [44] generally stated that an increase in temperature enhances the toxicity of contaminants. To date most metal toxicity studies also indicated an increased metal toxicity at higher temperatures [11,17,71,114]. As a limiting nutrient, variable P addition can directly affect the phytoplankton community by altering biomass, size and nutrient quality, which can have and indirect positive effect on the zooplankton community [79,81]. However, extreme eutrophication is unfavourable for most zooplankton species [51]. Phosphorus is known to both increase and decrease the metal toxicity to freshwater algae [84–86]. Serra et al. (2010) [84] and Twiss and Nalewajko (1992) [85] for example found that an increased P supply significantly decreased copper (Cu) toxicity on algae biomass. While Gao et al. (2016) [86] on the other hand found that Zn was more toxic to *Pseudokirchneriella subcapitata* cell densities at higher P supply. However, at the present few studies have investigated the relationship between metal toxicity and P supply to draw consistent conclusions.

Currently, very few 3-way interaction studies exist between nutrients/nutrition, temperature and a chemical factor, especially at the community level [6]. Heugens et al. (2003) [17] for example exposed a *Daphnia magna* population to different concentrations of cadmium (Cd), altered food supply and different temperatures and found significant 3-way interactions between these factors. These interactions indicated that the adverse Cd effects on *Daphnia* population growth were enhanced at higher temperature, whereas higher food concentrations protected the *Daphnia* population from Cd toxicity. Cd affected the *Daphnia magna* population more at low food levels and high temperatures [17]. Although, not a lot of data about three-way interactions is available, it is known that under eutrophic conditions the accumulation of polyphosphates by microalgae is stimulated by increasing temperature and this could counteract metal toxicity [82,84,85]. Phosphorus is also depleted faster at warmer temperatures, which hypothetically makes the primary producers more vulnerable to metal stress under phosphorus limitation at warm temperatures. If these interactions would propagate to the community-level, interactions at the community-level may be expected in either direction (antagonistic or synergistic). A major concern is that conventional risk assessment approaches, based on single species tests under optimized (low stress) environmental factors may underestimate the ecological impacts in case of a synergistic interaction between the toxicant and the environmental factor [114,120].

Against this background, this study was set up to identify to what extent the toxicity of Zn is affected by higher temperature (T) and by higher phosphorus (P) supply and how these T & P effects on Zn toxicity vary between the levels of organisation (population, functional group and community) in a freshwater community. Based on the limited evidence described above, it is speculated that Zn is more toxic at higher T. The

reduced Zn toxicity at higher P supply, if any, may be more important in warmer than in colder water. In addition, based on Jackson et al. (2016) [115], we speculate that interactive effects are more common at higher organization levels. To test this an indoor microcosm study was conducted with a freshwater plankton (zooplankton, phytoplankton and protozoa) dominated community. After a pre-treatment period of three weeks the microcosms were simultaneously exposed to three different Zn concentrations, two different T regimes (reference and a warmer regime) and two different P addition rates (Reference: low P addition and high P addition), for five weeks. During these five weeks the species (zooplankton, phytoplankton and protozoa) densities, community composition, biodiversity, total chlorophyll concentrations and physico-chemical endpoints were measured. The 2-way interactions between the Zn and each T or P factors were explored at the three levels of organisation and were tested with due attention to the direction, i.e. synergism where stress factors lead to more effects than purely additive and antagonism for the reverse.

3.2 Materials and methods

3.2.1 Test systems and experimental design

Forty indoor microcosms (diameter 0.25 m, height 0.35 m, volume 18 L) were installed in a water bath (16-19 °C) for temperature regulation, in a climate controlled room at Wageningen University (Wageningen, The Netherlands). Note that the water bath was divided in two compartments, in which the water temperature could be regulated seperately. To mimic a plankton-dominated shallow freshwater system, each microcosm was filled with a sediment layer of approximately 2 cm, 14 L of pond water and inoculated with a plankton dominated community that was collected in June 2013

from an uncontaminated mesotrophic ditch (Sinderhoeve Experimental Station, Renkum, The Netherlands; www.sinderhoeve.org). Two snails (*Lymnaea stagnalis*) were added to every cosm to suppress periphyton growth. Twice a week nutrients (NH_4NO_3:1 mg N/L; KH_2PO_4 and K_2SO_4: 1 mg K/L, 0.01 mg P/L) were added to microcosms to stimulate phytoplankton growth starting 3 weeks before the actual start of the experiment (i.e. the pre-treatment period). During the pre-treatment period most of the water from all the microcosms was taken out once a week and mixed in a central tank to ensure adequate mixing and similar start conditions in all test systems. Water loss was replenished with demineralized water when needed. A more detailed experimental set-up of the test systems has been described by Van de Perre et al. [121].

After the pre-treatment period the microcosms were exposed to three factors (P, T and Zn) simultaneously. The experimental design consisted of two temperature (cold: 16-19 °C and warm: 21-24 °C), two phosphorus (low P addition= 0.02 mg P/L a week and high P addition= 0.4 mg P/L a week) and three Zn treatments (background, 75 µg Zn/L and 300 µg Zn/L) in a full 2x2x3 factorial design, with three replicates for the Zn amended and four for the control (= no Zn added). The background Zn level is defined as the control. The two target Zn concentrations (75 µg/L: $HC5_{\text{-plankton}}$ and 300 µg/L: $HC50_{\text{-plankton}}$) were determined by using the Biotic Ligand Model (BLM) software version 2.1.2 [5,93] which fits the Species Sensitivity Distribution to BLM normalised chronic Zn toxicity data as explained in detail by Van Sprang et al. 2009 [5], based on the chemical properties of the water of the microcosms the day before the start of the treatments [121]. Only toxicity data of plankton species were used for the calculation of the $HC5_{\text{-plankton}}$ and 300 µg/L: $HC50_{\text{-plankton}}$.

The part of the experiment which focused on the Zn effects on the plankton community under cold low P addition conditions has already been published by Van de Perre et al. [121] and will not be addressed in detail in this manuscript but will be used to investigate possible interactions between Zn and the other factors.

3.2.2 Treatment applications and analyses

After the three weeks pre-treatment, the compartments of the water bath containing half of the microcosms was heated to 21-24 °C and the microcosms assigned to the Zn and P treatment received their first dosing (=start of treatment, week 0). Twice a week, together with the general nutrient dosing, additional phosphorous (KH_2PO_4: 0.19 mg P/L so a total of 0.2 mg P/L was dosed added every dosing) was added to the microcosms assigned to simulate a freshwater system that is exposed to a constant high P addition.

The Zn was dosed by distributing a Zn stock solution ($ZnCl_2$) evenly over the water surface and mixed by gentle stirring. At frequent intervals two water samples (one not filtered for measuring the total Zn concentration and one filtered through a 0.45 µm filter for measuring the dissolved Zn concentration, Acrodisc; Pall Life Sciences) were sampled from the microcosm to monitor the Zn concentration and to adjust the Zn concentration by additional spiking to compensate for losses from the water column. Additional samples were taken just before a Zn dosing and at least 15 minutes after the dosing. Inductively coupled plasma mass spectrometry (ICP-MS; Agilent 7700x, in the He mode using ^{72}Ge as internal standard: limit of quantification 3 µg Zn/L; method detection limit 1 µg Zn/L) was used to measure the Zn concentration in de control microcosms and all other Zn sampling were measured using flame atomic absorption

spectrophotometry (SpectrAA100; Mulgrave; Environment Canada: limit of quantification 20 µg Zn/L; method detection limit 6 µg Zn/L). Further details of the Zn treatment applications and analyses have been described by Van de Perre et al. [121].

3.2.3 Zooplankton, phytoplankton and protozoa

Plankton (zooplankton, phytoplankton and protozoa) samples were sampled from each microcosm every week, starting 1 day before the start of the treatments. For the plankton a total of 1 L of water was sampled from several random positions in the microcosms microcosms by using a Perspex tube (length 0.4 m; volume 0.8 L) and was filtered through a plankton net (zooplankton: mesh width, 55 µm; phytoplankton and protozoa: mesh width, 20 µm Hydrobios, Kiel, Germany). After filtering the remaining water was returned to the microcosm. Lugol was used to preserve the collected plankton samples. An inverted microscope was used to identify the plankton to the lowest practical taxonomic level.

All present macro-zooplankton (i.e. Cladocera, adult and copepodite stadia of Copepoda and Ostracoda) individuals were identified and counted. Copepoda were only classified as Cyclopoida, Calanoida and nauplii. Abundances of micro-zooplankton (i.e. Rotifera, nauplii and *Chaetonotus* sp.), phytoplankton and protozoa were determined by counting a subsample of a known volume and the abundances were adjusted per litre. Every subsample was settled overnight in sedimentation chambers and at least 400 individuals were counted and identified along longitudinal transects according to Utermöhl et al. [94]. Colonies of colony-forming algae were counted as a single individual. Diatoms (Bacillariophyceae) were only identified as single cell diatoms or *Fragilaria* sp. colony chains. The plankton species observed

during the experiment were classified in the following groups: zooplankton (rotifers, Cladoceran and Copepoda), phytoplankton (Bacillariophyceae, Chlorophyta, Chrysophyceae, Chytridiomycetes, Cryphtophyta, Cyanophyta, Dinophyta and Euglenophyta) and protozoa (Amoeba, heterotrophic flagellates and ciliates). The ciliates were additionally classified as: algivorous, bacterivorous, algivorous + bacterivorous, epiplanktonic, mixotrophic and predacious. Diversity calculations (Shannon index) were carried out using PRIMER 5.

Prior to every biological sampling, two 10 ml water samples were taken (15 cm below the water column at the centre of every microcosm after gentle stirring with a syring) from every microcosm for total chlorophyll analysis using a BBE Moldaenke GmbH Algae Lab Analyser.

3.2.4 Community metabolism and general chemical properties of the water

Measurements for dissolved oxygen (DO), temperature and pH were performed (WTW 340i multi-meter) in the morning (start photoperiod) and evening (one hour before the end of the photoperiod) twice a week at mid water depth, starting one day before the start of the treatments. The net primary production ($DO_{evening\ day\ x}$ - $DO_{morning\ day\ x}$) was estimated by using the DO measurements [95]. Once a week the conductivity was measured by using a WTW LF 191 conductivity meter.

Filtered (0.45 µm) and unfiltered water samples for nutrient analysis were taken before every biological sampling. A total organic carbon analyser (TOC-5000; Shimadzu; limit of quantification 1.5 mg DOC/L; method detection limit 0.5 mg DOC/L) was used for measuring the Dissolved (in)organic carbon (DOC, DIC). Measurements for total

dissolved P (TDP) and other elements, including Zn, were performed with ICP-MS (Agilent 7700x).

Soluble reactive phosphorus (SRP), Ammonium (NH_3) and NO_2 + NO_3 were analysed using a Skalar 5100 auto analyser. Total phosphorus (TP) was analysed by the ascorbic acid method.

Additionally, a standard 5-day biochemical oxygen demand (BOD_5) test [6] for every microcosms was conducted weekly with filtered (mesh size, 55 µm; Hydrobios, Kiel, Germany) microcosm water.

3.2.5 Data analysis

Interactive effects between Zn and T or P on the biological endpoints (plankton abundance) and on physico-chemical parameters for the different environmental conditions and sampling day were assessed. These endpoints were differentiated in different levels of organisation [Species level (1); group level, i.e. cladocera, chlorophyta (2); diversity, Shannon index (3); individual physico-chemical parameters (4); i.e. total chlorophyll, BOD_5, DO; community level (5)] and interactive effects were determined at the different levels or organisation. Before univariate and multivariate analyses were performed, the zooplankton data were Ln (2x+1) transformed and the phytoplankton and protozoa data Ln (1.67x+1) transformed where x is the abundance value. This was done to down-weight high abundance values and to approximate a log-normal distribution of the data (see [39] for rationale).

To identify how the toxicity of Zn is affected by increased T and increased phosphorus P the reference unstressed condition was defined as the Zn control under low P addition, cold conditions. To test whether the Zn interactions were dependent on the

Zn concentration, the data analyses were conducted for each Zn treatment separately (Zn low: 75 µg Zn/L and Zn high:300 µg Zn/L). First, a three-way ANOVA was performed to determine the significance ($p < 0.05$) of the three-way and two-way interaction terms for the first four types of endpoints. In case of a significant three-or two-way interaction, several more detailed two-way interactions were conducted to determine in which combinations of environmental conditions these interactions occurred. For example, when a significant Zn × T interaction was found, two additional two-way ANOVA analyses were conducted (by using the low P or high P data separately) to investigate under which environmental P conditions this significant Zn × T interaction occurred. Additionally, these two-way ANOVA analyses provide a formal statistical test of the Independent Action model [122]. Second, the observed combined effects (i.e. significant reduction or increase of the endpoint compared with the control) were compared with the predicted combined effects (independent action: sum of their single effects [122]) to determine whether the observed interaction was synergistic (observed Zn effect more than additive) or antagonistic (observed Zn effect less than additive). See De Coninck et al. (2013) [122] and Box 3.1 for further details on how the predicted effects were calculated and how the observed interactions were assessed. Interactions were only considered reliable when the ANOVA revealed the same type of interaction (synergistic or antagonistic) for at least two consecutive sampling dates In that case the interaction was defined as a ‘consistent interaction’.

Box 3.1 Determination of the interactive effects [122]

In this chapter we investigated whether the observed effects in the combined treatments, for the different treatment regimes (cold vs warm; low P addition vs high P addition), followed the independent action model. Bliss (1939) originally developed the independent action model which predicts the combined effects of binary stressors from observed effect in the individual stressor treatments [122,140]. In case of the Zn + T the following model was used (similar for Zn + P treatments):

$$E_{ZnT,predicted} = E_{Zn} + E_T - E_{Zn} \times E_T$$

Where

$$E_i = \frac{Ycontrol - Yi}{Ycontrol}$$

E_i is the observed fractional effect of the treatment i on endpoint Y relative to the control treatment. In our example i is either Zn, T or ZnT (combined Zn + T treatment). E_i can be positive (indicating a decrease in endpoint compared to the control) or negative (indicating an increase in endpoint compared to the control). When the two-way ANOVA found a significant Zn × T interaction we defined it as synergistic when the observed effect in the combined treatment was higher than the effect predicted with the independent action model [122,140]. This was the case when $E_{ZnT,\ observed} > E_{ZnT,\ predicted}$ in case $E_{ZnT,\ observed} > 0$ (The combined treatment causes a reduction of the endpoint compared to the control) or when $E_{ZnT,\ observed} < E_{ZnT,\ predicted}$ in case $E_{ZnT,\ observed} < 0$ (The combined treatment causes an increase of the endpoint compared to the control). The interaction was defined as antagonistic if the observed effect in the combined treatment was smaller than the effect predicted with the independent action model [122,140]. This occurs if $E_{ZnT,\ observed} > E_{ZnT,\ predicted}$ in case $E_{ZnT,\ observed} < 0$ or when $E_{ZnT,\ observed} < E_{ZnT,\ predicted}$ in case $E_{ZnT,\ observed} > 0$.

The Principal Response Curves method (PRC) [35] was used to analyse and illustrate the effects of Zn, T and P treatments on the plankton community composition and was performed by using CANOCO 5.0 [100]. The statistical significance of the single effects of the different treatments on the species compositions and their interactions were assessed by performing a Monte Carlo permutation test using the redundancy analysis (RDA). The Monte Carlo permutation test tested the significance of the PRC diagram in terms of displayed treatment variance, by using an F type test statistically based on the eigenvalue of the component [101]. This test was conducted for each sampling day, using the ln-transformed nominal treatment factor as the explanatory variables, to assess the significance of the treatment effects for each sample date. Interaction was tested by entering the interaction between Zn treatment and the environmental factor (P or T) as explanatory variables and the used factors and treatment as co-variables. For a more detailed description see Van Wijngaarden et al. (2006) [123].

Additionally, the Williams test (analysis of variance) as incorporated in the Community Analysis software [98,99] was used to calculate the No Observed Effect Concentrations (NOECs) for Zn at the parameter or taxon level ($p \leq 0.05$). NOECs at the community level ($NOEC_{community}$) were derived by applying the Williams test [101,103] on the Principal component analysis (PCA) sample scores resulting from a PCA analysis performed, separately for each sampling date. NOECs were only considered consistent when they showed statistically significant deviations in the same direction (adverse or beneficial) for at least two consecutive sampling dates.

3.3 Results and discussion

3.3.1 Zinc, phosphorous concentrations and temperature

The target temperature was generally achieved. During the experiment the mean temperature (± standard deviation) of the cold and the warm microcosms was 17.8 ± 0.7 °C and 22.3 ± 0.9 °C, respectively.

The target Zn concentrations were generally relatively well achieved, with the exception of the first week of the experiment. During the first week the measured dissolved Zn concentrations in the water column of the microcosms were below the target concentrations of 75 and 300 µg Zn/L (Table 3.1; Appendix B Figure B1 and Table B1). This was most likely the result of losses due to sorption by the sediment and biota. In the period thereafter (week 2-5), the target Zn concentrations in all microcosms were achieved to within 9% and 26% in the cold and warm microcosms, respectively (Table 3.1). The average dissolved Zn concentration in the warm microcosms was thus generally lower than in the cold microcosms. (Table 3.1). This may be due to higher sorption by sediment and biota at higher T. It is possible that the lower Zn concentrations during the first week and under warm condition concealed some of the Zn effects on the biota. Throughout this paper we will further refer to the nominal concentrations (75 and 300 µg Zn/L) for simplicity.

The TDP concentrations in the controls, low and high Zn treatments increased continuously throughout the experiment (Appendix B Figure B2). By the end of the experiment the mean TDP concentrations was 46 µg P/L under the low P addition and 840 µg P/L under high P addition. The phosphorus concentrations in the microcosms were not consistently affected by Zn or T (Appendix B Table B2 and B3). The measured TDP concentrations in the low P addition exceeded the 10 µg P/L that is defined as the

limit for oligotrophy. Thus the low P addition treatment should in fact be categorized as eutrophic (starting from 30-100 µg P/L) [46]. Similarly, the microcosms under high P addition can be categorized as hypereutrophic (>100 µg P/L)

Table 3.1: Average measured dissolved Zn concentrations (± standard deviation) in the 75 (low Zn) and 300 (high Zn) µg Zn/L treatments and the different multiple stressor treatment regimes during the first week of treatment ([a]) and between weeks 1-5 ([b]). * From chapter 2.

Week	Treatment	Cold		Warm	
		Low P*	High P	Low P	High P
1	Low Zn	22.0 ± 4.9	28.1 ± 13.8	25.5 ± 7.8	23.3 ± 7.9
1	High Zn	51.1 ± 13.9	54.8 ± 11.7	116.5 ± 81.6	164.4 ± 79.3
2-5	Low Zn	77.9 ± 17.8	82.0 ± 20.0	62.0 ± 15.0	58.1 ± 18.3
2-5	High Zn	287 ± 52.3	310.4 ± 55.9	222.5 ± 48.2	230.9 ± 55.9

3.3.2 Single stress treatments (effects of T and P addition)

The results of earlier studies that investigated the effects of experimental warming on zooplankton have been highly variable, ranging from negative [124] to small [79] or no clear effects [51]. The temperature increase during our study had a clear effect on the zooplankton community (Appendix B Table B4 and B11, Figure 3.1 A). Many of the zooplankton taxa experienced a clear short term positive (*A. nana* and *A. rectangular*), negative (Cyclopoida) or variable (from clear positive to clear negative: *Lecane* group *luna* and *lunaris* and *C. oblusa*) temperature effect. This may be explained by the fact that warming can affect competitive interactions and increase top-down regulation [11,79,118]. The species abundances of the plankton are given in the SI (Appendix B Table B5).

It has already been shown that nutrient addition can indirectly have a positive effect on the zooplankton community by altering biomass, size and nutritional quality of the phytoplankton [79–81]. However, in our study, high P addition by itself only slightly

affected the planktonic community (Appendix B Table B11, Figure 3.1 A-C). A possible explanation for this is the fact that throughout the experiment the phosphorus concentrations (TDP, TP and SRP) in the controls, low and high Zn treatments increased continuously (Appendix B Figure B2, Table B1). By the end of the experiment the measured TDP concentrations defined the microcosms that were under the low P addition as eutrophic and the microcosms that were under the high P addition conditions as hypereutrophic [46]. Therefore, it is unlikely that P was a limiting factor during this study.

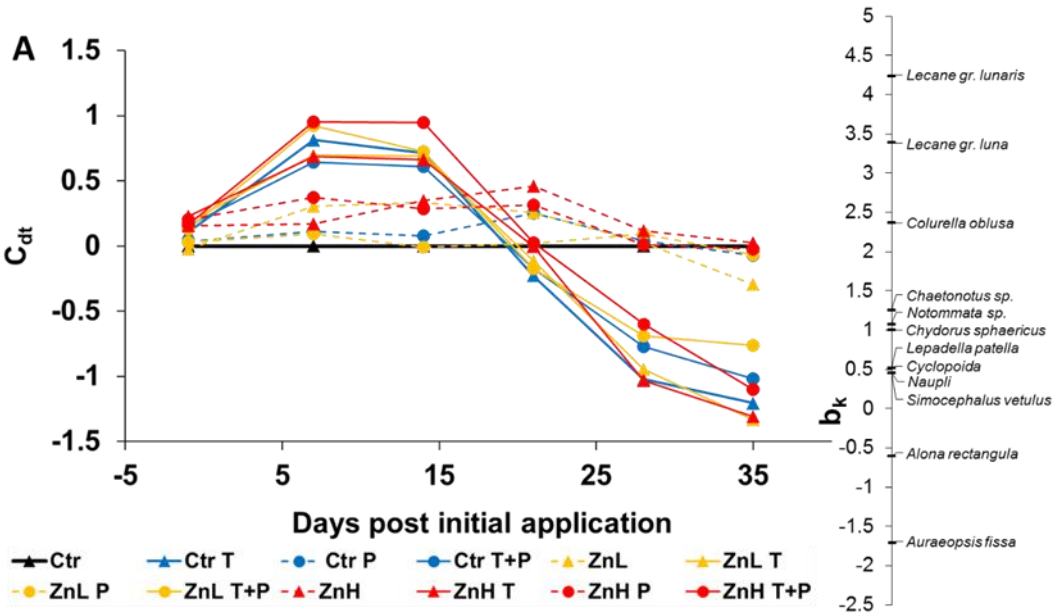


Figure 1 part 1: Principal response curve (PRC), resulting from the analysis of the global zooplankton (A), phytoplankton (B) and protozoa data (C), indicating the effects of the different Zn (Ctr: No Zn added; ZnL: 75 µ Zn/L; ZnH: 300 µg Zn/L), temperature (16-19 °C: Φ; 21-24 °C: T) and P treatments (Low P: Φ; High P: P) on the cold low P addition Zn control microcosms (Ctr). The vertical axis represents the differences in community structure of the treatments compared to the controls expressed as regression coefficients (C_{dt}). The affinity of a taxon to the PRC is expressed as the species weight (b_k). Species with a low b_k (between 0.5 and -0.5) are not shown. See [101,102] for additional information.

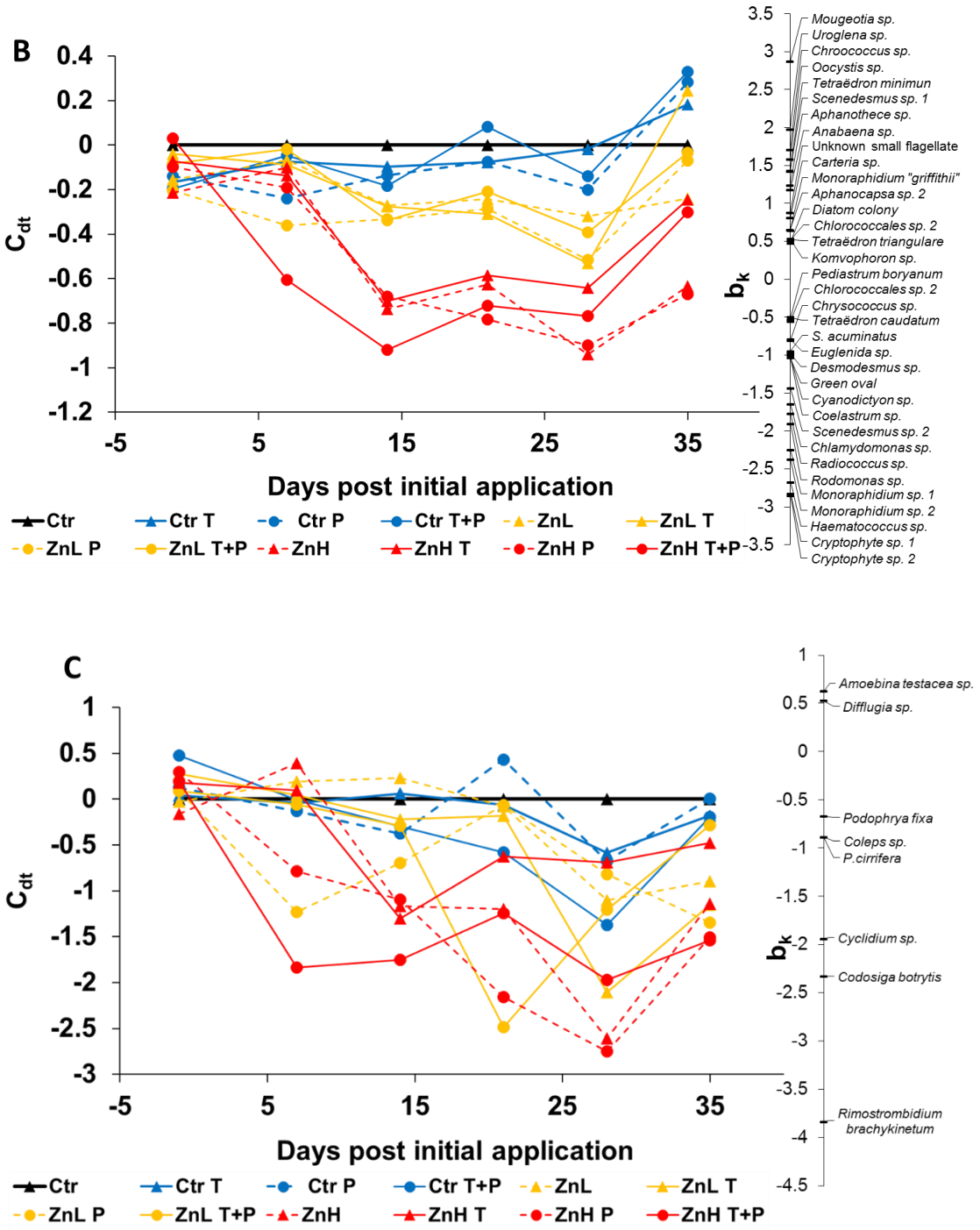


Figure 3.1 part 2: Principal response curve (PRC), resulting from the analysis of the global phytoplankton (B) and protozoa data (C), indicating the effects of the different Zn (Ctr: No Zn added; ZnL: 75 µ Zn/L; ZnH: 300 µg Zn/L), temperature (16-19 °C: Φ; 21-24 °C: T) and P treatments (Low P: Φ; High P: P) on the cold low P addition Zn control microcosms (Ctr). See [101,102] for additional information.

Generally the DO, DOC (Figure 3.2 A) and pH (Figure 3.2 B) of the Zn controls were consistently lower under warm than under cold conditions (Appendix B Table B1 and B15). The microcosms under warm conditions were also characterised by a significantly higher conductivity (Appendix B Table B1). At the end of the experiment the pH started to increase in the warm, high P addition microcosms (Figure 3.2 B). The high P addition induced a short term decrease of DO (morning and primary production), pH and NH3 (Appendix B Table B3). Under high P addition the DOC concentrations were consistently lower and the conductivity increased with increasing P throughout the experiment (Figure 3.2 A and Appendix B Table B3).

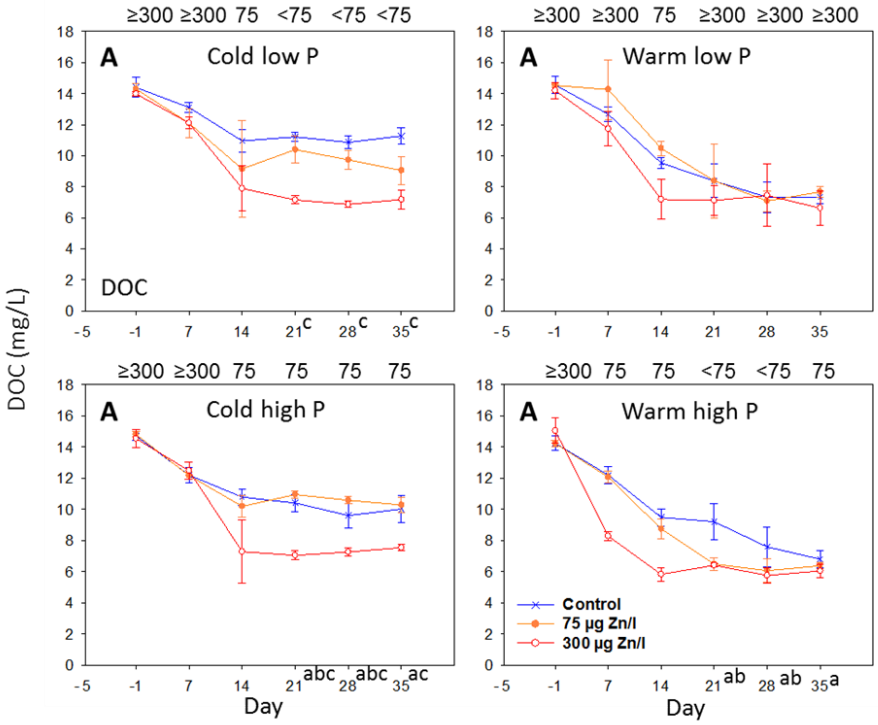


Figure 3.2 A: Dynamics of Dissolved organic carbon concentration (A) under cold low P addition, cold high P addition, warm low P and warm high P addition conditions. The means (standard deviation as error bars) of the different parameters for each Zn treatment and time point are shown. Calculated no-observed-effect concentrations are plotted above the figures.[a]: Consistent Zn (high) × T interaction; [b]: Consistent Zn (low) × T interaction; [C]: consistent Zn (low) × P interaction.

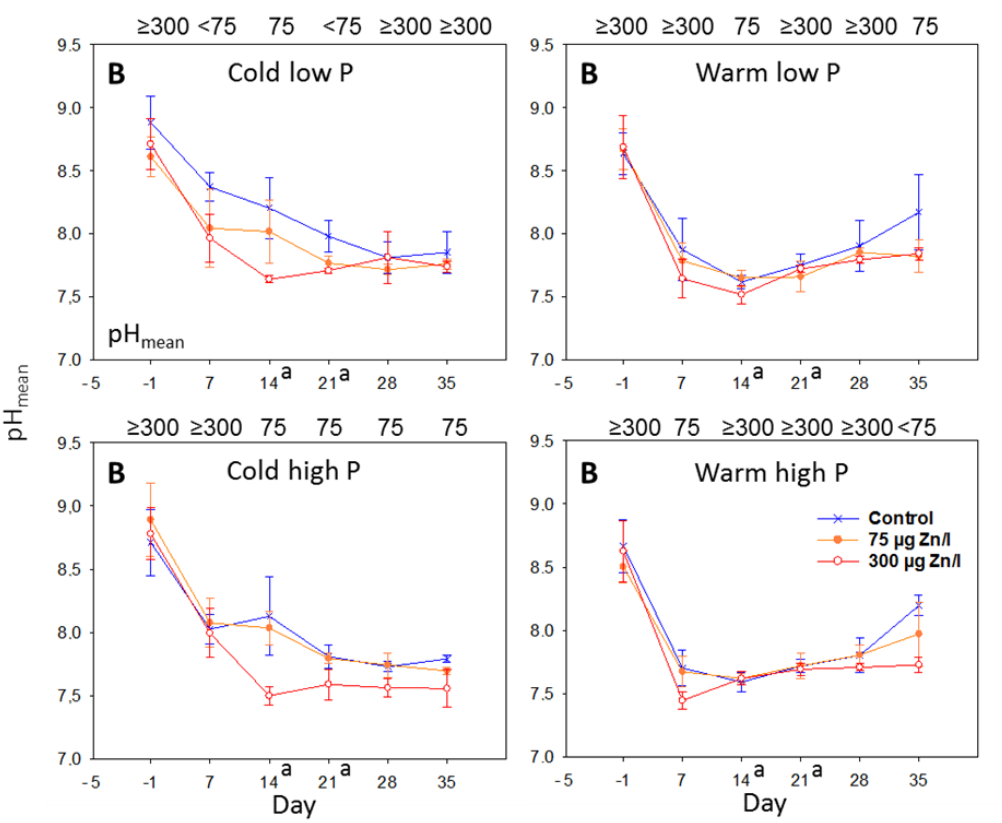


Figure 3.2 B: Dynamics of the pH_{mean} dynamics (B) under cold low P addition, cold high P addition, warm low P and warm high P addition conditions. The means (standard deviation as error bars) of the different parameters for each Zn treatment and time point are shown. Calculated no-observed-effect concentrations are plotted above the figures. [a]: Consistent Zn (high) × T interaction; [b]: Consistent Zn (low) × T interaction; [C]: consistent Zn (low) × P interaction.

3.3.3 Combined effects and interactions of T and P stress with Zn toxicity

3.3.3.1 Species and group level

During this study consistent interactions were only found for 3 out of 43 zooplankton species (*Cyclopoida* sp., nauplii and *Simocephalus vetulus*), 2 out of 86 phytoplankton species (Single cell diatoms and *Cryptophyte* sp. 1) and 1 out of 27 protozoa species (*Rimostrombidium brachykinetum*) (Table 3.2-4, Figure 3.3 and 3.5). None of these

consistent interactions were found under low P addition and with the exception of the *Cyclopoida* sp., all the consistent interactions were found between Zn and T at the highest Zn treatment.

Table 3.2: Statistical significance (p values two-way ANOVA) and calculation of the interactive effects (synergism: S; or antagonism: A) of Zn (Low Zn: L; High Zn: H) and the different factors of some of the different plankton species at different treatment regimes. The interaction type is based on comparing observed and predicted effects (independent action) using the methods explained in De Coninck et al.[122]. Only species for which a consistent interaction was found (with three-way ANOVA) are represented here. A consistent interaction was defined as an interaction of the same type (i.e. in the same direction) that was consecutively found for at least 2 consecutive sampling dates. See Appendix B table B7 and B8 for the statistical details on all species that showed consistent interactions.

Taxa	Day	Treatment regime	Interaction factors	Zn treatment	*p*	Interaction type
Zooplankton						
Cyclopoida sp.	14	High P	Zn × T	L	0.006	S
	21	High P	Zn × T	L	0.001	S
nauplii	28	High P	Zn × T	H	0.001	S
	35	High P	Zn × T	H	0.001	S
Simocephalus vetulus	21	High P	Zn × T	H	0.049	A
	28	High P	Zn × T	H	0.022	A
Phytoplankton						
Single cell diatoms	14	High P	Zn × T	H	0.049	A
	21	High P	Zn × T	H	0.032	A
Cryptophyte sp. 1	21	High P	Zn × T	H	0.005	A
	28	High P	Zn × T	H	0.012	A
Protozoa						
R. brachykinetum	21	High P	Zn × T	H	0.025	A
	28	High P	Zn × T	H	0.048	A

Among all plankton species identified, consistent Zn × T × P interactions were only found for 3 species (*Cyclopoida* sp., *Anisonema acinus* and *Nassula* sp.). However, further analyses for the *Anisonema acinus* and *Nassula* sp. revealed no consistent

two-way interactions effects for any of the factors (Appendix B Table B7 and B8). Further analysis on the *Cyclopoida* sp. abundance data revealed that under high P addition the Zn (low) × T interactions were consistently synergistic after 7 and 14 days of treatment, indicating a larger (up to 5-fold higher than predicted) negative Zn effect at higher T (Table 3.2 and Appendix B B7 and B8). At the highest Zn concentration consistent synergistic Zn × T interactions were calculated for the nauplii (Table 3.2 and 3.3 and Appendix B B9 and S B10, Figure 3.3 C). Under normal conditions Cyclopoida are considered to have a higher tolerance to Zn stress [59,104,121]. Under warm hypereutrophic conditions, however the nauplii were adversely affected at even the lowest Zn concentration (Figure 3.3 C). How the various plankton species were affected differently by the combination of Zn and the other stressors is explained in more detail in SI (Appendix B Figure B3 - 7 and Table B4, B6 and B12-15).

At the group level consistent interactions were only found at the highest Zn treatment and this for 2 out of 3 zooplankton groups (Copepoda and Cladocera), 3 out of 8 phytoplankton groups (Bacillariophyceae, Cryptophyta and Chlorophyta) and 1 out of 3 protozoa groups (Ciliate) (Table 3.2-4). The majority (6 out of 8) of the consistent interactions were found between Zn and T. The cladocerans (Figure 3.3 D) and the ciliates as a group showed an antagonistic Zn (high) × T interaction under high P addition, indicating a lower Zn effect at higher T (Table 3.3). As an example, figure 3.4 A illustrates the Zn and temperature effects and their interaction for the cladocerans after 35 days of exposure under the different P conditions. Here, it can clearly be observed that under high P addition the observed Zn + T effects on the cladoceran abundance were 1.5 times lower than predicted. The rotifers were the only zooplankton group that did not show any consistent interaction at the group level (Table 3.2 and 3.3). Furthermore, all consistent Zn × T interactions effects for phytoplankton taxa and

groups were antagonistic (Table 3.3). For the Bacillariophyceae these Zn × T interactions were only significantly antagonistic under low P addition while significantly antagonistic for the Cryptophyta under both low and high P addition (Table 3.2 and 3.3). Crain et al. (2008) indicated that the overall interactive effect across most studies were antagonistic for most autotrophs and he suggested that trophic level could be an important driver for interaction type since organisms with fundamentally different methods of energy acquisition may respond very differently to stressors [116].

Table 3.3: Statistical significance (p values two-way ANOVA) and calculation of the interactive effects (synergism: S; or antagonism: A) of Zn (Low Zn: L; High Zn: H) and the different factors of some of the different plankton species at different treatment regimes. The interaction type is based on comparing observed and predicted effects (independent action) using the methods explained in [122]. Only groups for which a consistent interaction (three-way ANOVA) was found are represented here. See appendix B Table B9 and B10 for the statistical details.

Taxa group	Day	Treatment regime	Interaction factors	Zn treatment	*p*	Interaction type
Zooplankton						
Copepoda	28	High P	Zn × T	H	0.001	S
	35	High P	Zn × T	H	0.001	S
Cladocera	28	High P	Zn × T	H	0.003	A
	35	High P	Zn × T	H	0.006	A
Phytoplankton						
Bacillariophyceae	14	High P	Zn × T	H	0.048	A
	21	High P	Zn × T	H	0.02	A
Cryptophyta	7	Warm	Zn × P	H	0.001	S
	14	Warm	Zn × P	H	0.04	S
	14	Low P	Zn × T	H	0.009	A
	21	Low P	Zn × T	H	0.032	A
	21	High P	Zn × T	H	0.001	A
	28	High P	Zn × T	H	0.007	A
Chlorophyta	7	Warm	Zn × P	H	0.029	S
	14	Warm	Zn × P	H	0.007	S
Protozoa						
Ciliate	21	High P	Zn × T	H	0.01	A
	28	High P	Zn × T	H	0.019	A

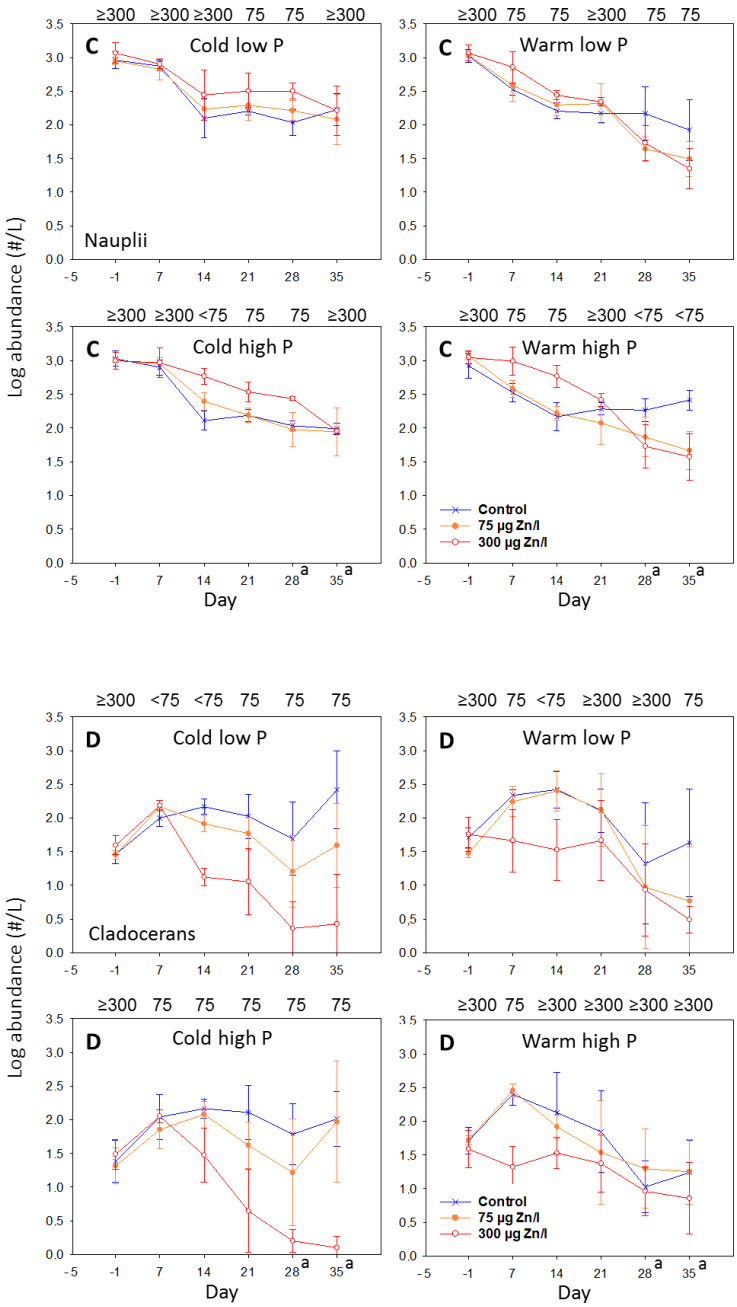


Figure 3.3 : Dynamics of Cladocerans (C) and copepod nauplii (D) under cold low P addition (Chapter 2), cold high P addition, warm low P and warm high P addition conditions. The means (standard deviation as error bars) of the different parameters for each Zn treatment and time point are shown. Calculated no-observed-effect concentrations are plotted above the figures. [a]: Consistent Zn (high) × T interaction; [b]: Consistent Zn (low) × T interaction; [C]: consistent Zn (low) × P interaction.

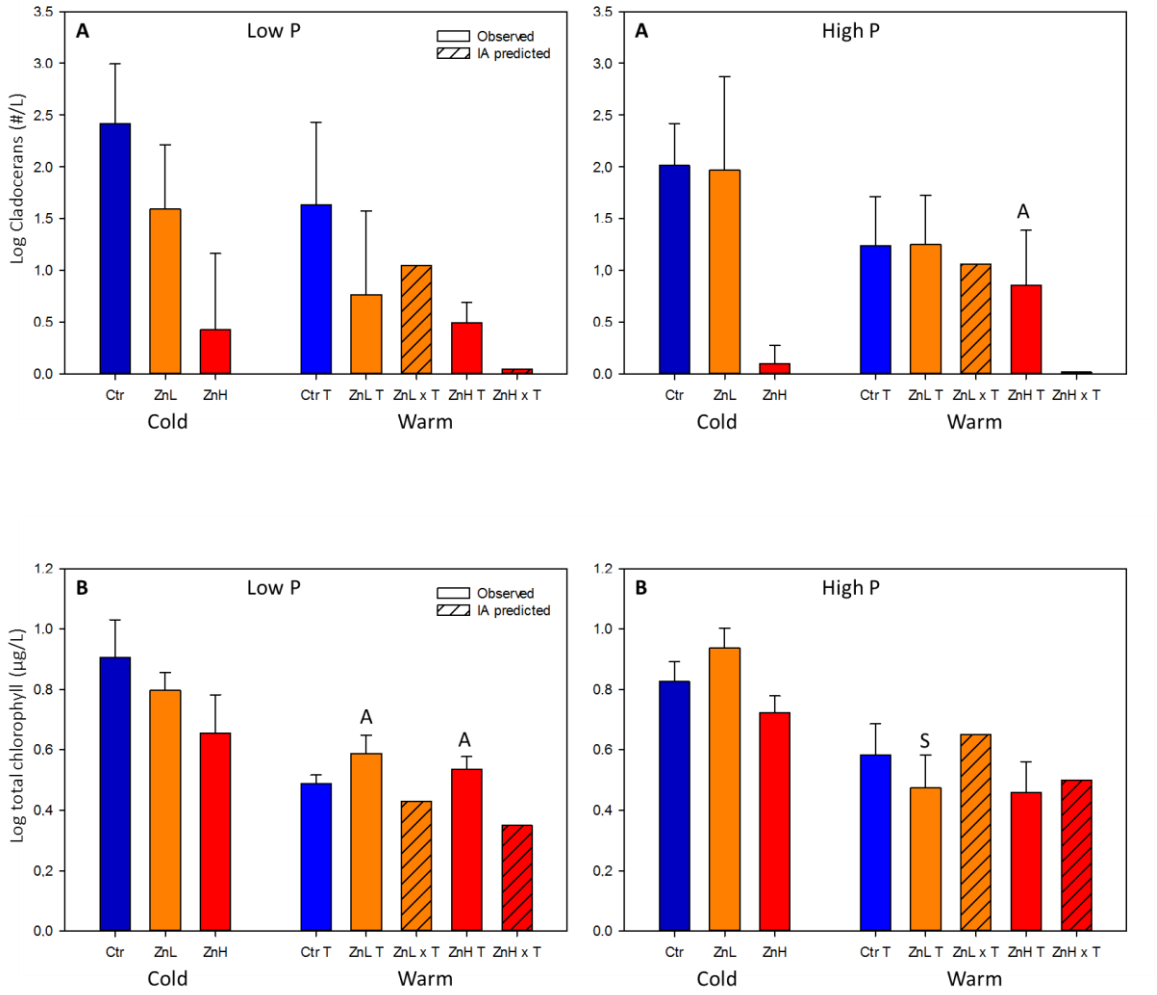


Figure 3.4: Log cladoceran abundance after 35 days of treatment (**A**) and total chlorophyll concentrations after 28 days of treatment (**B**) and predicted values using the Independent Action model (IA) under low P and high P addition conditions. Error bars indicate standard deviation. A: significant antagonistic Zn × T interaction; S: significant synergistic Zn × T interaction.

At the highest Zn treatment and under warm conditions consistent synergistic Zn × P interactions were found for the Chlorophyta and Cryptophyta as a group after 7 and 14 days of treatment. These results indicate that Zn had a larger positive effect (up to 30- and 6-fold higher than predicted) on the total abundances of the Chlorophyta and Cryptophyta at higher P (Table 3.3 and Appendix B B10). These interactions were not found under cold conditions. Therefore, it is possible that these Zn (high) × P interactions can be explained by the fact that, during the first week of the experiment, and only in the cold treatments, the measured Zn concentrations (300 µg Zn/L treatment) of the low P microcosms were significantly lower (by about 1.5-fold) than those of the high P microcosms (Table 3.1). Thus, high P addition by itself is concluded to have, at best, only slightly affected the overall Zn toxicity. Interestingly though, 90% of all the Zn × T interactions at the species and group level were found under high P addition, suggesting that a higher P loading, may enable a stronger influence of temperature on the effects of Zn on freshwater communities.

In summary, during this study 4% of the species (33% synergistic; 67% antagonistic) and 36% of the functional groups showed a consistent Zn × T interaction (20% synergistic; 80% antagonistic). This demonstrates that the Zn toxicity effects on the plankton community can be affected by temperature at the functional group level, but very limitedly at the species level (Table 2-4). Most authors observed an increased metal toxicity with increasing temperature due to an increased uptake and accumulation of the metal by the organism [11,18,74,114]. However, the majority of the consistent Zn × T interactions during this study were antagonistic, which suggest that Zn did not have a larger effect at higher T for most taxa and groups (Table 2 and 3). Our results are in line with Jackson et al. (2016) [115] who found that the interactive effects between stressors at the population level are usually additive and antagonistic

at the higher levels of organisation (functional group, community level). One of the possible explanations for the limited number of taxa that showed a consistent interactive effect is the fact that biotic interactions (indirect effects) within the community can mask or dampen possible interactive stressor effects that occur at the population level [116]. Indirect effects are frequently observed in microcosm studies [9,13,121] and can, for instance, be the result from a reduced food competition (or predation) [46]. Another possible explanation for the reason why interactions are more frequently found at the group level than at the species level, is the fact that species can sometimes completely disappear (or very low abundance) from the microcosms and reappear (e.g. through hatching of dormant eggs) in later samplings. On the other hand, the disappearance of this species can be compensated by the abundance increase of species with the same community function (functional redundancy), which could dampen the effects at higher levels of biological organisation [9]. Although these abundance fluctuations are a natural phenomenon it can limit the statistical power and the observation of consistent interactive effects. By combining the different species into functional groups we account for functional compensation and lower sampling errors and increased statistical power [89].

Table 3.4: Summary of the number of statistically significant interactive effects between Zn (Low: L and high: H) and the different factors (P and T) on the different organization levels of the different plankton species groups and ecosystem functions on at least one time point (following three-way ANOVA and Monte Carlo permutation test). The total number of assessed entities (N) at the different levels of organization is also reported. The number of consistent interactions are given between parentheses. The number of consistent interactions are given between parentheses. See Table 3.2,3.3, 3.6 and Appendix B B7, B9, B12 and B23 for details

			L	**H**	**L**	**H**	**L**	**H**
	level	**N**	**Zn × T**	**Zn × T**	**Zn × P**	**Zn × P**	**Zn × T × P**	**Zn × T × P**
Zooplankton								
	Species	43	5 (**1**)	14 (**2**)	5 (0)	6 (0)	5 (**1**)	4 (0)
	Group	3	1 (0)	3 (**2**)	1 (0)	1 (0)	0 (0)	1 (0)
	Community[a]	1	1 (0)	1 (**1**)	0 (0)	1 (0)	1 (0)	0 (0)
	Biodiversity[b]	1	1 (0)	1 (0)	0 (0)	0 (0)	1 (0)	1 (0)
Phytoplankton								
	Species	86	14 (0)	23 (**2**)	13 (0)	17 (0)	13 (0)	11 (0)
	Group	8	1 (0)	7 (**2**)	6 (0)	6 (**2**)	0 (0)	2 (0)
	Community[a]	1	0 (0)	1 (**1**)	1 (0)	1 (0)	0 (0)	0 (0)
	Biodiversity[b]	1	1 (0)	1 (0)	1 (0)	1 (0)	0 (0)	0 (0)
Protozoa								
	Species	27	1 (0)	5 (**1**)	4 (0)	6 (0)	2 (0)	6 (0)
	Ciliate group[c]	6	0 (0)	2 (0)	1 (0)	2 (0)	1 (0)	3 (0)
	Group	3	1 (0)	3 (**1**)	1 (0)	2 (0)	1 (0)	1 (0)
	Community[a]	1	0 (0)	1 (**1**)	0 (0)	1 (0)	0 (0)	0 (0)
	Biodiversity[b]	1	0 (0)	1 (0)	1 (0)	1 (0)	0 (0)	1 (0)
Function								
	BOD_5	1	0 (0)	1 (**1**)	1 (0)	1 (0)	1 (0)	1 (0)
	DOC	1	1 (0)	1 (**1**)	0 (0)	0 (0)	1 (**1**)	1 (0)
	pH_{mean}	1	0 (0)	1 (**1**)	0 (0)	0 (0)	0 (0)	0 (0)
	DO_{net}	1	0 (0)	1 (**1**)	1 (0)	0 (0)	1 (0)	1 (0)
	Total chlorophyll	1	1 (**1**)	1 (**1**)	0 (0)	1 (0)	1 (**1**)	1 (**1**)

[a]: Monte Carlo permutation test; [b]: Shannon index; [c]: Functional feeding group within the Ciliates

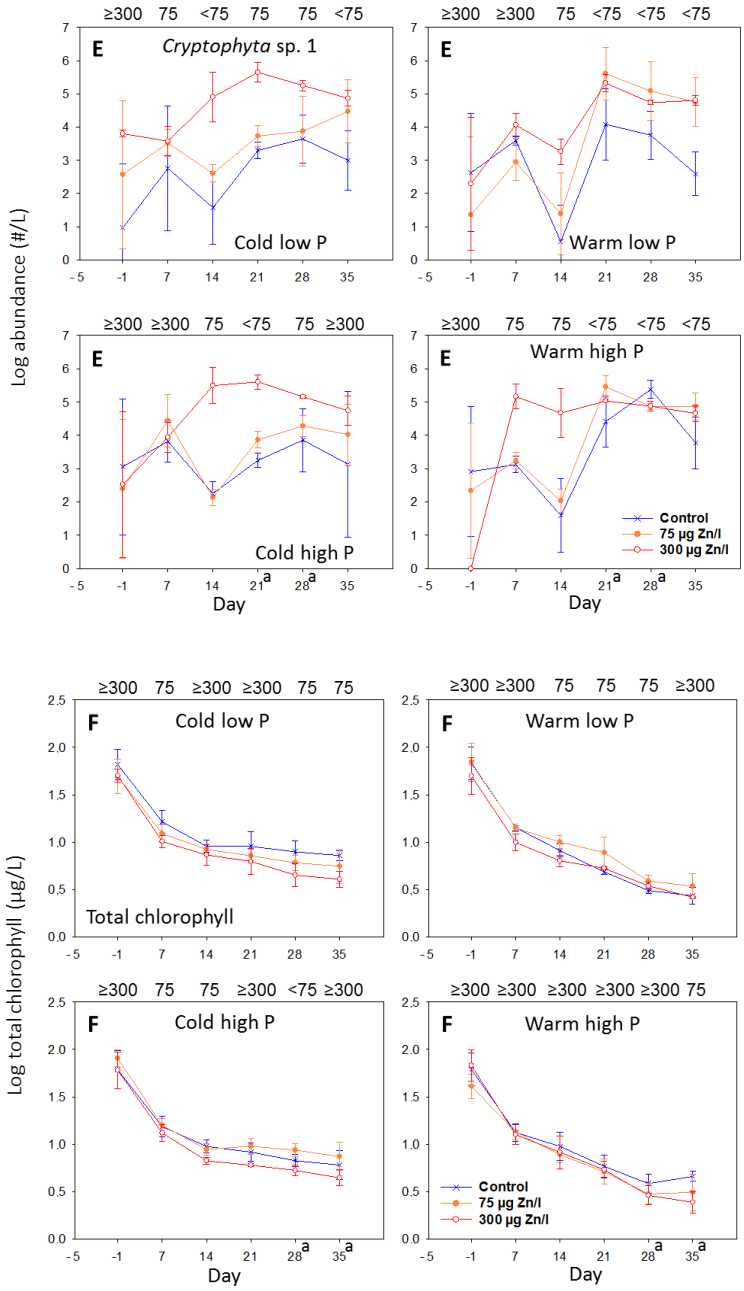


Figure 3.5: Dynamics of *Cryptophyta* sp. 1 (E) and total chlorophyll concentration (F) under cold low P addition (chapter 2), cold high P addition, warm low P addition and warm high P addition conditions. The means (standard deviation as error bars) of the different parameters for each Zn treatment and time point are shown. Calculated no-observed-effect concentrations are plotted above the figures. [a]: Consistent Zn (high) × T interaction; [b]: Consistent Zn (low) × T interaction; [C]: consistent Zn (low) × P interaction.

3.3.3.2 Biodiversity and community level

After 14 days of exposure and onwards significant Zn × T interactions (Monte Carlo permutation test) were observed at the zooplankton community composition level at the highest Zn treatment under high P addition (Table 3.5). The PRC diagram, visually revealed a clear temperature effect on the zooplankton community structure (Figure 3.1 A). The zooplankton community composition was consistently affected (3-way ANOVA on PCA-scores) by temperature and by the highest Zn treatment (Appendix B Table B11). Zn mainly adversely affected the cladocerans (Figure 3.2 A) like *Daphnia longispina*, *Simocephalus vetulus* and *Chydorus sphaericus* and this could differ among the different P and T treatment regimes. No consistent interactions between Zn, T & P were found for the Shannon biodiversity index for the zooplankton, phytoplankton or protozoa (Table 3.5).

At the phytoplankton community composition level significant Zn × T interactions were found after 7 and 14 days of treatment under high P addition at the highest Zn treatment (Table 3.5). A clear main Zn effect on the phytoplankton community structure was visually revealed after conducting a PRC analysis using the phytoplankton data from all the different treatments (Figure 3.1 B). The phytoplankton community composition was affected (3-way ANOVA on PCA-scores) by the highest Zn treatment after 14, 28 and 35 days of exposure (Appendix B Table B11). The species weights (b_k score) indicated that most filamentous cyanobacteria (*Pseudanabaena* sp., *Anabaena* sp. and *Woronchinia* sp.), several non-filamentous cyanobacteria (*Chroococcus* sp. and *Aphanothece* sp.) and colony or filament forming algae (*Scenedesmus* sp.1, *Mougeotia* sp., *Uroglena* sp., *Fragilaria* sp.) and were adversely affected by the Zn treatment. On the other hand most phytoplankton taxa with a negative species score belonged to the Chlorophyta (i.e. *Desmodesmus* sp., *Haematococcus* sp. and

Monoraphidium sp.1 and 2) and Cryptophyta (i.e. *Cryphtophyte* sp. 1 and *Rhodomonas* sp.) and indicated an increase in abundance due to the Zn treatments (Figure 3.1 B). The dominance shift from filamentous algae groups to small species (relative low contribution to the total chlorophyll) could possibly explain the decline in total chlorophyll at the highest Zn treatment (Figure 3.5 F and 3.6).

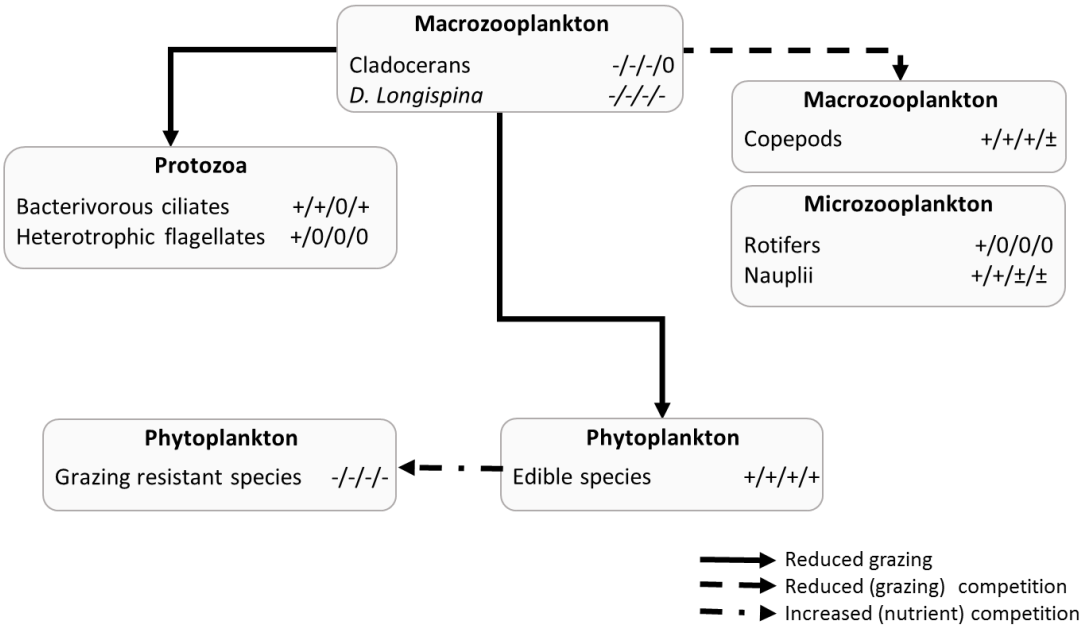


Figure 3.6: Schematic overview of the observed effects of the highest Zn treatment (300 µg/L) on the ecosystem structure of a plankton-dominated community (+: Consistent increase; -: Consistent decrease; ± Consistent increase during the first 2 weeks of exposure and a consistent decrease during the 2 last weeks, 0: No consistent effect) at the cold low P/ cold high P/ warm low P/ warm high P treatments. At 300 µg Zn/L, a significant reduction in cladocerans resulted in an increase of copepod adults and microzooplankton, which is probably the result from reduced grazing competition. Additionally, the reduced grazing pressure on the edible phytoplankton population had a positive effect on their abundances and shifted the phytoplankton community dominance from grazing-resistant to edible species.

One of the possible explanations for the dominance shift from filamentous algae groups to small species at the highest Zn treatments is the fact that biotic interactions (indirect effects) within the community can affect observed Zn effects [32]. Indirect effects are frequently observed in microcosm studies [9,13,121] and can be the result from a reduced food competition (or predation) [46]. One of the examples of this was at the highest Zn treatment, where Zn effects were mainly observed for the cladoceran populations in the cold (low and high P addition) and warm low P addition microcosms . Figure 3.6 illustrates a schematic overview of the observed effects of the highest Zn treatment on the ecosystem structure of plankton-dominated microcosms for the different treatment conditions. This decrease in abundance probably resulted in an indirect positive, top-down effect on the nauplii abundances and hypothetically on the rest of the plankton community [9,106]. By reducing the number of Cladocerans the Zn treatments indirectly increased the relative abundance of small (< 10µm) Cryptophyta species (e.g. *Rodomonas* sp., *Cryptophta* sp. 1 and 2) and other fast growing and zooplankton grazing sensitive species (e.g. *Monoraphidium* sp., *Scenedesmus* sp. 2 and *Haematococcus* sp.), at the expense of the filamentous algae (e.g. *Mougeotia* sp., *Anabaena* sp., *Pseudoanabaena* sp.) or colony forming (*Scenedesmus sp.1, Mougeotia sp., Uroglena sp., Fragilaria* sp.) algae (Figure 3.1 B). The observed phytoplankton patterns (PRC species scores) in figure 3.1 B are most likely the result of indirect effects (by a reduced grazing pressure caused by the direct negative effects of Zn on the cladocerans) and were observed for each of the different P and T conditions [9,48] (Figure 3.6 and Appendix B B6). However, for the zooplankton and protozoa community structure, the observed indirect effects of the reductions in cladocerans at the highest Zn treatment vary among the different treatment conditions. The rotifers, for example, only benefited from the disappearance of the large

cladocerans (=less food competition [9,48]) under cold, low P addition conditions while no rotifer abundance increase was observed under the other treatment conditions (Figure 3.6).

For the protozoa a consistent Zn × T interaction (between 21 and 28 days of treatment) was found at the community composition level under low P addition at the highest Zn treatment (Table 3.5). The PRC analysis, that was conducted with the protozoa data from all the different treatments, visually revealed no clear effect from any of the factors on the community structure (Figure 3.1 C). The protozoa community composition was affected (3-way ANOVA on PCA-scores) by the highest Zn treatment from 14 days onwards (Appendix B Table B11).

Table 3.5: Statistical significance (*p* values Monte Carlo permutation tests) of the interactive effects of Zn (L: 75 µg Zn/L; H: 300µg Zn/L) and the different factors (P and T) on the community structure of the different plankton groups at different treatment regimes. Significant ($p < 0.05$) are marked.

		low P		high P		Cold		Warm			
		L	H	L	H	L	H	L	H	L	H
Plankton group	Day	Zn × T	Zn × T	Zn × T	Zn × T	Zn × P	Zn × P	Zn × P	Zn × P	Zn × T × P	Zn × T × P
Zooplankton	-1	0.56	0.988	0.823	0.588	0.697	0.794	0.467	0.821	0.714	0.923
	7	0.186	0.068	0.028	0.084	0.583	0.836	0.064	0.253	0.035	0.377
	14	0.356	0.109	0.068	**0.031**	0.057	0.176	0.296	0.417	0.093	0.578
	21	0.831	0.437	0.412	**0.043**	0.123	0.501	0.512	0.834	0.58	0.678
	28	0.703	0.258	0.068	**0.011**	0.296	0.531	0.656	0.308	0.824	0.43
	35	0.562	0.205	0.4	**0.016**	0.31	0.818	0.348	0.045	0.366	0.083
Phytoplankton	-1	0.418	0.361	0.112	0.189	0.559	0.254	0.015	0.252	0.119	0.478
	7	0.217	0.657	0.344	**0.038**	0.038	0.244	0.561	0.063	0.056	0.164
	14	0.331	0.228	0.144	**0.028**	0.731	0.029	0.088	0.407	0.263	0.189
	21	0.29	0.72	0.445	0.174	0.749	0.477	0.41	0.677	0.764	0.567
	28	0.482	0.316	0.34	0.199	0.136	0.676	0.226	0.595	0.126	0.297
	35	0.592	0.12	0.224	0.341	0.118	0.066	0.242	0.124	0.391	0.484
Protozoa	-1	0.06	0.55	0.034	0.18	0.104	0.198	0.094	0.728	0.01	0.324
	7	0.494	0.398	0.311	0.24	0.363	0.321	0.063	0.082	0.238	0.561
	14	0.451	0.495	0.509	0.056	0.722	0.32	0.492	0.051	0.438	0.253
	21	0.198	**0.021**	0.158	0.098	0.263	0.003	0.108	0.853	0.209	0.089
	28	0.631	**0.016**	0.178	0.03	0.762	0.06	0.205	0.085	0.929	0.068
	35	0.234	0.362	0.301	0.14	0.479	0.874	0.409	0.261	0.288	0.86

3.3.4 Total chlorophyll, community metabolism and general chemical properties of the water

The physico-chemical water parameters data and how they were affected by the combination of Zn and the other stressors are given in the Appendix B (Table B1 and B3). Under high P addition the DOC was adversely affected by Zn in both the cold (at 300 µg Zn/L) and the warm (at 75 µg Zn/L) microcosms. However, in the warm microcosms with low P addition, no Zn effect on the DOC was observed (Figure 3.2 A). At the highest Zn treatment the DO was affected for a short term and was significantly lower than the control Zn under cold temperature conditions (Appendix B Table B3). The pH was consistently significantly lower at the highest Zn concentration and this for all the different regimes. The conductivity was not affected by Zn under high P addition and warm conditions and the NH_3 concentrations were significantly higher in the cold high P addition Zn treatments. In the high P addition microcosms the Biological oxygen demand after 5 days (BOD_5) significantly decreased with increasing Zn while in the low P addition microcosms the BOD_5 were only consistently affected at the highest Zn concentration (Appendix B Table B3). The consistent Zn effect on the DOC and BOD_5 can also be an indirect indication that the microbial loop and the pelagic food web were affect by Zn [46].

During this study, consistent interactions were found for all the main community metabolites (DOC, BOD_5, DO and pH) and for the total chlorophyll (Table 3.6). At the highest Zn treatment consistent antagonistic Zn × T interactions were found for the DOC, pH_{mean}, DO_{mean} and net primary production (DO_{net}). The Zn (high) × T interactions for the pH_{mean} were consistent under both P conditions while the Zn (high) × T interactions of the DOC, DO_{mean} and DO_{net} were only consistent under low P addition (Table 3.6). Under low P addition the observed effects (Zn high + T) on the DOC,

pH_{mean}, DO_{mean} and DO_{net} were on average 1.5, 1.8, 1.2 and 1.2 times less negative than predicted by the IA model (Appendix B Table B21). After 7 and 14 days of treatment consistent Zn (high) × T interaction were found for the Biological oxygen demand after 5 days BOD_5. These interactions were synergistic under high P addition while antagonistic under low P addition (Table 3.6). However, these BOD_5 results were only found during the first part of the experiment (= period below target Zn concentration) and should not be overemphasized.

At the lowest Zn concentration, the DOC was the only parameter for which consistent interactions were found: Consistent synergistic Zn (low) × T interactions under high P addition (on average 4 times more negatively affected than predicted) and consistent antagonistic Zn (low) × P interactions under cold conditions (on average 7 times less negatively affected than predicted) (Table 3.6, Appendix B Table B21).

Significant Zn × T × P interactions were observed for the total chlorophyll (Figure 3.5 F) concentration during the last 3 weeks of exposure at the lowest Zn and during the last 2 weeks at the highest Zn treatment (Appendix B Table B16). Under low P addition, the Zn × T interactions were antagonistic for the total chlorophyll at the lowest Zn treatment after 21 and 28 days of exposure and at the high Zn treatment after 28 and 35 days (Table 3.6). These Zn × T interactions indicated that under low P addition and high T the lowest and highest Zn treatment had a smaller adverse effect (3 and 1.4-fold, respectively) on the total chlorophyll concentration than predicted (Table S10). As an example, figure 3.4 B illustrates the effects and interactions between Zn and temperature on the total chlorophyll concentrations after 28 days of exposure under the different P conditions. No consistent Zn × T interactions were observed under high P addition. High P addition had no significant effect on the overall Zn effect (No Zn × P) on the total chlorophyll (Table 3.6). Therefore it can be concluded that, in contrast

to Gao et al. (2016) [86], no indications were found during this study that Zn was more toxic to the phytoplankton at higher P supply, neither at species or group level or at the level of community composition or total chlorophyll concentration in the system.

Table 3.6: Statistical significance (*p* values Three-and two-way ANOVA) and calculation of the interactive effects (synergism: S; or antagonism: A) of Zn (Low Zn: L; High Zn: H) and the different factors of the total chlorophyll, pH_{mean}, DOC, mean dissolved oxygen (DO_{mean}), net primary production (DO_{net}: $DO_{evening\ day\ x}$ - $DO_{morning\ day\ x}$) and BOD_5 at different treatment regimes. The interaction type is based on the observed and predicted effects using the methods explained in [122]. Only parameters for which a consistent interaction was found are represented here. See Appendix B B16 and B21 for the statistical details.

Parameter	Day	Treatment regime	Interaction factors	Zn treatment	p	Interaction type
BOD_5	7	Low P	Zn × T	H	**0,009**	A
	14	Low P	Zn × T	H	**0,005**	A
	7	High P	Zn × T	H	**<0,001**	S
	14	High P	Zn × T	H	**0,007**	S
DOC	21	Low P	Zn × T	H	**0,006**	A
	28	Low P	Zn × T	H	**0,005**	A
	35	Low P	Zn × T	H	**<0,001**	A
	21	High P	Zn × T	L	**0,002**	S
	28	High P	Zn × T	L	**0,025**	S
	21	cold	Zn × P	L	**0,041**	A
	28	cold	Zn × P	L	**0,006**	A
	35	cold	Zn × P	L	**0,010**	A
pH_{mean}	14	Low P	Zn × T	H	**0,011**	A
	21	Low P	Zn × T	H	**0,024**	A
	14	High P	Zn × T	H	**0,007**	A
	21	High P	Zn × T	H	**0,049**	A
DO_{mean}	14	Low P	Zn × T	H	**0,035**	A
	21	Low P	Zn × T	H	**0,019**	A
DO_{net}	14	Low P	Zn × T	H	0,026	A
	21	Low P	Zn × T	H	<0,001	A
Total chlorophyll	21	Low P	Zn × T	L	0.03	A
	28	Low P	Zn × T	L	0.034	A
	28	Low P	Zn × T	H	0.013	A
	35	Low P	Zn × T	H	0.013	A

3.3.5 Zn risk assessment

Microcosm and mesocosms studies are essential for Zn risk assessment because by including biotic interaction they provide a more realistic risk assessment than the lower-tier single species test [13]. Since microcosm studies can be difficult to interpret Brock et al. [89] worked out several guidelines and recommended that to ensure the statistical analysis, conducted to demonstrate a possible treatment related effect has enough power, at least 8 populations of potentially sensitive taxa with an appropriate MDD (category 1) are present in the study. In our study, at least 8 different category 1 taxa were observed under every treatment regime, including phytoplankton and zooplankton taxa but no protozoa, thus conforming the reliability of our study. Under high P addition (cold and warm) the multivariate analysis (PRC) conducted on the different plankton groups (Zooplankton, phytoplankton and protozoa) revealed a $NOEC_{community}$ of 75 µg Zn/L (Appendix B Figure B5 – 7). However, under warm low P addition the phytoplankton community composition was consistently affected at 75 µg Zn/L and a $NOEC_{community}$ of <75 µg Zn/L was calculated (Appendix B Figure B6 A). This is possibly due to the indirect effect the by Zn declined *D. longispina* (NOEC <75 µg Zn/L) populations had on the phytoplankton community (especially for *Cryptophyta* sp.1: Figure 3.5 E) (Appendix B Table B13).

Additionally, to the effect on the plankton community, Zn also indirectly affected the chemical properties of the water over time and hereby the metal bioavailability. During the experiment the pH and DOC levels were not only affected by temperature and eutrophication but also by the Zn treatments (with the exception of the warm low P addition microcosms and especially at high Zn). By lowering the DOC, Zn becomes more bioavailable and therefore more toxic [5,16].

3.4 Conclusions

During this study consistent interactions between Zn and the other factors (T and P) were rarely found at the species level, but they were frequently found at the group, community structure and functional level, thus largely confirming our hypothesis that stressor interactions occur more frequently at a higher level of organisation. Biodiversity was the exception, as no interactions were found for this community-level characteristic. We also found that 82% of all the consistent interactions at species or group level were observed at the highest Zn treatment (300 µg/L) and only 18% at the lowest (75 µg/L). In addition, the majority of the consistent interactions were found between Zn and T, indicating that Zn effects on plankton communities can be affected by temperature. Furthermore, these consistent Zn × T interactions were mainly antagonistic, which suggests that Zn did not have a larger, but rather a smaller effect at higher T for most taxa, groups and functions.

During this study, in contrast with our hypothesis that was based on single-species experiments, no clear indications were found that Zn was more toxic to the phytoplankton at a higher P supply, neither at the species or group level, nor at the level of community composition or total chlorophyll concentration in the system. Overall, high P addition by itself was concluded to have, at best, only slightly affected the overall Zn toxicity. Interestingly though, 90% of all the Zn × T interactions at the species and group levelwere found under high P addition. Additionally, with exception of the protozoa, all the observed consistent Zn × T interactions at the community composition level were only found under high P addition. Thus, high P addition clearly influenced the interactive effect between Zn and T. Collectively, our study suggest that temperature and phosphorus loading to freshwater systems should be accounted for in risk assessment of chemicals, as these factors may modify effects on aquatic

communities. Not doing so may underestimate risks in some and overestimate risks in other systems, depending on their temperature and phosphorous loading.

4

Combined effect of interspecies interaction (food competition), temperature and Zn on *Daphnia longispina* population dynamics

Redrafted after:

Van de Perre D, Janssen CR, de Schamphelaere K a. C.. Combined effect of interspecies interaction, temperature and zinc on *Daphnia longispina* population dynamics. Environ. Toxicol. Accepted February 2018.

4.1 Introduction

Currently the ecological risk assessment of chemicals (ERA) is generally based on results of single species laboratory tests conducted under standardised optimal conditions (e.g. temperature, food, etc.), which are then extrapolated to field conditions. Under natural conditions however, biological populations are constantly exposed to a variety of abiotic (e.g. temperature, nutrients) and biotic factors (e.g. competition, predation) which could interact with toxicant exposure effects [10,43]. The competition for resources between (interspecific) and within (intraspecific) species is one of the major biotic interactions and can significantly modify the responses of organisms to toxicants [15,26,27,125,126]. For example, a decrease of the most sensitive species due to toxic effects of the toxicant (direct effect) can result in an increase of a more resistant species (indirect effect) as a result of decreased competition or in a decrease of the consumer species due to starvation [9]. Furthermore, the competition for food among species can reduce the amount of energy that could be used for reproduction (dynamic energy budget theory [31]) or to withstand toxic stress [17] which could enhance toxic effects observed at the population level [34].

Although the awareness of the influence of interspecific interactions on toxicity effects of chemicals is growing [15,38], only a few studies have investigated combined interspecific competition and chemical stress, and those studies were mainly focused on pesticides [26,27,32,35,37]. In Chapter 2 [121] we conducted a freshwater microcosm plankton community experiment under the same cold conditions as in the present study, and they observed that Zn drove a *D. longispina* population to extinction at a concentration below its reported chronic NOEC derived from single-species tests. We hypothesised in Chapter 2 and 3 that interspecific competition had an important effect on the Zn toxicity effects.

Additionally, abiotic factors such as temperature (T) can also be important drivers affecting competition (e.g. affecting competitive interaction and top down regulation) [11,118] and the toxic effects of pollutants by influencing their bioavailability and toxicokinetics [10,11]. To date, most studies indicate that metal toxicity increases with increasing temperature [17,18,74,75,127]. In the context of global change, and the predicted general temperature increase of up to 4°C within the next century in temperate regions [40], it is crucial for ERA to understand the combined and interactive effects of chemical stressors and temperature.

At present, studies on how the combined effect of an abiotic factor and food competition affects the response of a population to a chemical stressor are even more underrepresented in the ecotoxicology literature. One of these studies was conducted by Heugens et al. (2006) [17] and they found that adverse cadmium (Cd) effects on *Daphnia* population growth were enhanced at higher temperature, whereas higher food concentrations protected the *Daphnia* population from Cd toxicity.

The aims of the present study were to determine the single effects of zinc, temperature and interspecific competition (comp) on a *Daphnia longispina* population and to determine whether temperature and/or interspecific competition can affect the zinc toxicity effects. To this end, a jar (0.5 L) study was conducted in which *D. longispina* populations (5 adults and 5 juveniles) were exposed to three different zinc (Zn) treatments (background, 29 µg Zn/L and 110 µg Zn/L), two different temperature regimes (“ambient“ or cold: 16-19 °C and “ambient +4°C” or warm: 21-24 °C) and two interspecific competition levels (No interspecific *Brachionus* competition= no *Brachionus calyciflorus* added; interspecific *Brachionus* competition= *B. calyciflorus* added) in a full 3x2x2 factorial design. Higher effects of the Zn treatment on the *D. longispina* population were expected under warm conditions [10,18,74], especially

when the populations were under an increased food competition (dynamic energy budget theory [31]).

4.2 Materials and methods

4.2.1 Test systems and experimental design

The experiment consisted of sixty 1 L glass jars filled with 0.5 L of filtered (0.20 µm) medium (originating from a mesotrophic ditch located at the Sinderhoeve Experimental Field Station, Wageningen, The Netherlands; general chemical properties of the water: Appendix C Table C1) and were installed in a water bath (ambient late spring water temperature mesotrophic ditch located at the Sinderhoeve Experimental Field Station: 16-19 °C) for temperature regulation and a 12h diurnal photoperiod (3500-4500 lux). One week before the applications of the different treatments (i.e. the pre-treatment period) forty-eight glass jars were inoculated with 5 *D. longispina* adults (carying eggs) and 5 juveniles. The classification of the *D. longispina* organism subclasses was based on their size (adults: ≥ 500 µm and juveniles: < 500 µm). The adult size class was determined by a pre-test in which the smallest (body length) egg carrying *D. longispina* female was determined and was used as cut-off value. The *D. longispina* organisms used during this experiment were obtained from a lab culture (16-19 °C) that originated from the same mesotrophic ditch (Sinderhoeve Experimental Field Station, Wageningen, The Netherlands). Twelve glass jars were designed to only contain *Brachionus calyciflorus* but these were only inoculated after the pre-treatment period.

After the pre-treatment period the jars were exposed simultaneously to three factors (Zn, T and *Brachionus calyciflorus* addition) for a period of 21 days. The applied Zn concentrations were based on the calculation of the hazardous concentration for x%

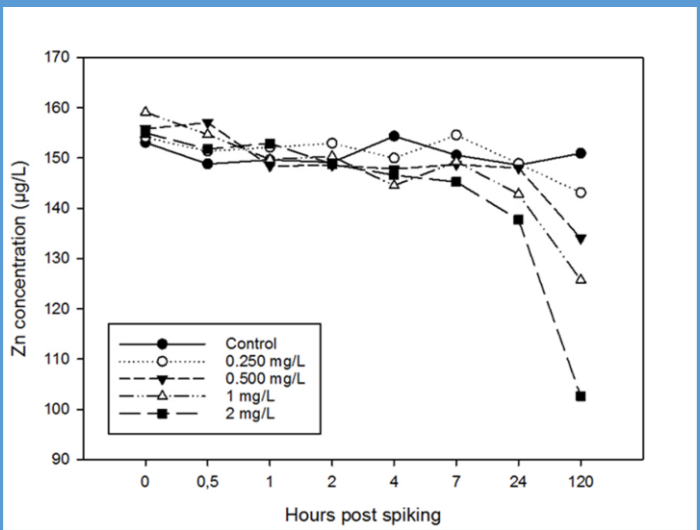
of the planktonic organisms, i.e. the $HCx_{plankton}$. This is the HCx that is based on the chronic Zn toxicity data of plankton species that were first normalized to the target properties of the exposure medium by using the Zn Biotic Ligand Model [121]). The two target water temperatures ("ambient" or cold: 16-19 °C and "ambient +4°C" or warm: 21-24 °C) were determined in context of global change, and the predicted general temperature increase of up to 4°C [40]. To simulate a more realistic community structure the *Daphnia*/Rotifer ratio (10:1450) used during this study was based on the starting communities of a microcosm study conducted by Van de Perre et al. (2016) [121]. The experimental design consisted of three Zn treatments (control = background Zn, i.e. no Zn added; 29 µg Zn/L= $HC5_{plankton}$ or Zn low and 110 µg Zn/L= $HC50_{plankton}$ or Zn high), two temperatures (cold: 16-19 °C and warm: 21-24 °C) and two interspecific competition levels (No interspecific *Brachionus* competition= no *B. calyciflorus* added; interspecific *Brachionus* competition= 1450 *B. calyciflorus* added) varied in a full 3x2x2 factorial design. Each treatment received four replicates.

The rotifer *B. calyciflorus* cysts were obtained from MicroBioTest (Mariakerke, Belgium) and a stock culture was started and maintained at 16-19 °C and 21-24 °C several weeks before the start of the experiment. The *B. calyciflorus* and the *D. longispina* in the stock cultures and during the experiment were fed daily with *Desmodesmus* sp. (1 g C/ml). The *Desmodesmus* sp. itself was frozen (for 1 week) to kill them and defrosted prior to feeding. This was done to prevent their growth in the jars, which made it possible to control the food supply, thus allowing food competition and limiting pH and Zn fluctuations (Box 4.1). To test whether the *B. calyciflorus* and the *D. longispina* populations would respond differently to dead or live *Desmodesmus* sp. feeding several pre-tests were conducted and none of these tests revealed a significant effect.

Twice a week the medium (acclimated to the right temperature) in the jars was changed completely to maintain the targeted Zn concentrations and water quality conditions.

Box 4.1 Investigating the relation between algae concentration and Zn losses

A small jar experiment (without sediment) was carried out to investigate whether the Zn loss during previous experiments could be explained by Zn algae uptake. Several concentrations (background, 0.25 mg/L, 0.50 mg/L, 1 mg /L and 2 mg/L) of a *Desmodesms* sp. solution were added to a 0.5 L jar. These jars were spiked one time with 150 µg Zn/L. Zn samples were taken after 0.5h, 1h, 2h, 4h, 7, 24 and 120h. Results strongly indicate that the higher the starting algae concentration, the more algae loss out of the water column. To contract this problem the *D. longispina* populations in the experiment in chapter 4 were fed with dead *Desmodesmus* sp. (by freezing and defrosting prior to use).



4.2.2 Zn treatment applications and general chemical properties of the water analysis

The media for the three target Zn concentrations were prepared by spiking $ZnCl_2$ into the water (target + 20% to compensate for Zn loss) and was acclimated to the target temperature at least one day before the medium change. Two water samples (one not filtered for measuring the total Zn concentration and one filtered through a 0.45 µm filter for measuring the dissolved Zn concentration, Acrodisc; Pall Life Sciences) were sampled from the jars before the medium was changed and from the new medium. The samples were taken after gently stirring using a syringe, approximately 10 cm under the water column and visually checked for *D.longispina* occurences (If *D. longispina* was present in the sample the sample was put back and retaken). The Zn samples were measured using an iCAP^{TM} 7000 Plus Serie ICP-OES (limit of quantification 2 µg Zn/L; method detection limit 0.5 µg Zn/L; Thermo Scientific, reference material TM28-4 and TMDA-70.2).

Water samples for nutrient analysis were taken simultaneously with the Zn samples. A total organic carbon analyser (TOC-5000; Shimadzu; limit of quantification 1.5 mg DOC/L; method detection limit 0.5 mg DOC/L) was used for measuring the Dissolved organic carbon (DOC) and dissolved inorganic carbon (DIC). Measurements for dissolved oxygen (DO), temperature and pH were performed (WTW 340i multi-meter) in the evening the day before changing the medium and in the morning, just before changing the medium.

4.2.3 Biological monitoring

By filtering the medium over 3 sieves (mesh size 500 µm, 200 µm and 15 µm) the *D. longispina* abundances of the adults and juveniles could be separated and counted. The sieve with mesh size 15 µm was used to retain the rotifers. The abundances of *D. longispina* and *B. calyciflorus* were monitored every time the medium was changed (-7, -3, 0, 4, 7, 11, 14, 18 and 21 days after the first treatment). The *B. calyciflorus* abundances were monitored by taking two 12.5 ml subsamples per jar and counted by using and inverted microscope (magnification 10x). After counting, the *Daphnia* and *Brachionus* were put back in the jars with fresh medium.

4.2.4 Data analysis

All statistical analyses were preformed using Sigmaplot 13. Prior to statistical analysis the abundances were log10-transformed to ensure the assumptions of normality (Shapiro–Wilkinson's W test) and homoscedasticity (Levene's test). In order to assess the main effects and interactions between Zn and temperature and interspecific competition on the *D. longispina* and *B. calyciflorus* abundances a series of ANOVA's (three and two-way) were used for every sampling day ($p < 0.05$). To test whether the Zn interactions were dependent on the Zn concentration, the data analyses were conducted for each Zn treatment (Zn low: 29 µg Zn/L and Zn high:110 µg Zn/L). First, three-way ANOVA's were performed to determine the significance ($p < 0.05$) of the three-way and two-way interaction terms for all endpoints. One way to interpret three-way interactions (Zn × T × comp) in our study is that it indicates that the Zn × T interaction is different with and without *Brachionus* (or competition with *Daphnia* in case of *Brachionus*) competition or that the Zn × comp interaction differs between

temperatures [122,128], which matches the questions we wanted to address in this work. In case of a significant three-or two-way interaction (Three-way ANOVA) several more detailed two-way ANOVA's (Zn × T and Zn × comp) were conducted (on a subset of the whole dataset) to get a better understanding about what these interactions implicated and to determine in which environmental conditions (cold and warm; no interspecific *Brachionus* competition and interspecific *Brachionus* competition) these interactions occurred. Additionally, these two-way ANOVA analysis provided a formal statistical test of the Independent Action model [122]. Secondly, interactive effects were evaluated relative to the Independent Action model and were classified as synergistic (combined effect greater than expected), antagonistic (combined effect smaller than expected) or additive (combined effect as than expected) [122]. In this chapter the cold microcosm jars, with no added Zn and no *Brachionus* added were defined as the reference condition (=control). See De Coninck et al. (2013) [122] and box 4.2 for further details on how the predicted effects were calculated and how the interactions were assessed. Additionally, pairwise comparison (Holm-Sidak method) was used to determine the single effect of Zn, temperature and interspecific competition. We defined statistically significant effects/interactions, that are significant for at least 2 consecutive sampling dates as "consistent" effects/interactions" [89].

Box 4.2 Determination of the interactive effects [122]

In this chapter we investigated whether the observed effects in the combined treatments, for the different treatment regimes (cold vs warm; No interspecific *Brachionus* competition vs interspecific *Brachionus* competition), followed the independent action model. Bliss (1939) originally developed the independent action model which predicts the combined effects of binary stressors from observed effect in the individual stressor treatments [122,140]. In case of the Zn + T the following model was used (similar for Zn + comp treatments):

$$E_{ZnT,predicted} = E_{Zn} + E_T - E_{Zn} \times E_T$$

Where

$$E_i = \frac{Ycontrol - Yi}{Ycontrol}$$

E_i is the observed fractional effect of the treatment i on endpoint Y relative to the control treatment. In our example i is either Zn, T or ZnT (combined Zn + T treatment). E_i can be positive (indicating a decrease in endpoint compared to the control) or negative (indicating an increase in endpoint compared to the control). When the two-way ANOVA found a significant Zn × T interaction we defined it as synergistic when the observed effect in the combined treatment was higher than the effect predicted with the independent action model [122,140]. This was the case when $E_{ZnT,\ observed} > E_{ZnT,\ predicted}$ in case $E_{ZnT,\ observed} > 0$ (The combined treatment causes a reduction of the endpoint compared to the control) or when $E_{ZnT,\ observed} < E_{ZnT,\ predicted}$ in case $E_{ZnT,\ observed} < 0$ (The combined treatment causes an increase of the endpoint compared to the control). The interaction was defined as antagonistic if the observed effect in the combined treatment was smaller than the effect predicted with the independent action model [122,140]. This occurs if $E_{ZnT,\ observed} > E_{ZnT,\ predicted}$ in case $E_{ZnT,\ observed} < 0$ or when $E_{ZnT,\ observed} < E_{ZnT,\ predicted}$ in case $E_{ZnT,\ observed} > 0$.

4.3 Results and discussion

4.3.1 Zinc concentrations and general water chemistry

During the experiment the average dissolved Zn concentrations (± standard deviation) of the controls were 2.9 ± 1.9 µg/L, 25.7 ± 13.3 µg/L in the lowest Zn treatment and 121 ± 24 µg/L in the highest Zn treatment (Table 4.1). The average DOC concentration was 5.0 mg/L ± 0.6 and TOC concentration 5.1 mg/L ± 0.9. During the experiment the average dissolved oxygen concentration was 9.0 mg/L ± 1.3 and the average pH 8.3 ± 0.3. On average the water temperature within the cold and warm treatments were 17.1 ± 0.4 and 21.5 ± 0.9.

Table 4.1: Mean (± standard deviation) of the average filterd Zn concentrations of the jar water before and after changing medium.

	New medium (µg Zn/L)	Before medium change (µg Zn/L)	Average mean (µg Zn/L)
Control	1.7 ± 1.1	3.0 ± 1.9	2.9 ± 1.9
Zn low	37 ± 7	18 ± 4	25.7 ± 13.3
Zn high	139 ± 9.8	100 ± 17	121.4 ± 24.4

4.3.2 Single effects of Zn, T and interspecific competition

4.3.2.1 *Daphnia longispina*

Under cold conditions and without *B. calyciflorus* competition the abundance of *D. longispina* adults was unaffected by Zn while *Daphnia* juvenile abundance was consistently affected at both Zn concentrations (Figure 4.1A and Table 4.2). From 11 days after the start of the experiment onward, the juvenile abundance was positively affected at the lowest Zn treatment (Figure 4.1E). As an essential element Zn itself can be a limiting factor and an addition of Zn could cause and increase in abundances [107,110,129,130]. Based on acclimation studies, Muyssen and Janssen (2005) [130] found that *Daphnia* exposed to a Zn concentration from 6 to 22 µg/L produce

significantly more offspring than *Daphnia* acclimated to lower or higher test concentrations. During our study (Chapter 4) the average Zn concentration (2.9 µg Zn/L) in the Zn controls was below the Zn optimum concentration for *Daphnia*, which could explain why the *D. longispina* juvenile abundances were consistently positively affected by the lowest Zn treatment (Table 4.2). Since the total *D. longispina* abundance largely consisted of juveniles it mainly reflected the population dynamics of the juveniles.

At the highest Zn treatment the *D. longispina* juvenile abundances were consistently negatively affected after 14 days of exposure onwards (Table 4.2 and Figure 4.1E). *Daphnia* juveniles are known to be more sensitive to toxicants than adults [26,131]. However, the recorded population variables in this study do not allow to determine whether the negative effect of the highest Zn treatment on the juvenile abundance resulted from direct mortality, reduced reproduction from the adults (e.g. reduced brood size, fraction of reproducing adults [132]) or from the combination of both. The NOEC (for reproduction) of *D. longispina* in the chronic Zn toxicity database [5] normalized to our medium characteristics is 245 µg Zn/L, which is more than double the highest Zn concentration used in this study, and thus no Zn effect on reproduction was expected.

Generally at higher temperatures *Daphnia* mature faster (i.e., lower the age at first reproduction) and this can enhance population growth. However, when comparing the cold control (No Zn nor *Brachionus* added) jars with the warm control jars no consistent temperature effect was observed for any of the different *D. longispina* subclasses throughout the experiment (adults: Figure 4.1A and 4.1C, juveniles: Figure 4.1E and 4.1G, total: Figure 4.1I and 4.1K; Table 4.2). This is possible due the fact the mean temperatures used in this study were within the OECD based temperature optimum for Daphna reproduction (18 – 22°C) [72].

In the present study interspecific *Brachionus* competition by itself had no significant consistent effect on any of the *D. longispina* subclasses (adults: Figure 4.1A and 4.1B, juveniles: Figure 4.1E and 4.1F, total: Figure 4.1I and 4.1J; Table 4.2). This was expected since rotifers are generally outcompeted by *Daphnia* and only have a limited effect on *Daphnia* abundance in food competition studies [26,27,133].

4.3.2.2 Rotifers

B. calyciflorus population densities declined sharply after 7 days of treatment and disappeared from most jars after 18 days (Figure 4.2). Their population densities were unaffected by the Zn and temperature treatments (Appendix C Table C3 and C4). Rotifers, like *B. calyciflorus* are also known to form dormant eggs (sexual reproduction) when under less favourable conditions (e.g. food shortage, chemical stress, temperature or high population density) [46]. Since these dormant eggs would only hatch after several weeks (after conditions are favourable again which could have been outside the duration of the experiment) it could partly explain why the *B. calyciflorus* abundance declined within the jars, even without the occurrence of *D. longispina*. However, this theory cannot be confirmed since the *B. calyciflorus* eggs were not counted during the experiment. Evaluating the effects of interspecific competition during our study became even more complex than anticipated due to the appearance of small rotifers (after 4 days) in the *Daphnia* jars (Figure 4.2). To avoid contamination the *D. longispina* individuals were transferred 3 times to clean medium just prior to the experiment. However, after 4 days of treatment and onward “small rotifer” species (*Lecane lunaris*, *Lepadella patella*, *Cephalodella* sp. and *Mytilina* sp.) detectably appeared in all the jars with *D. longispina* and their abundance further increased

throughout the experiment (Figure 4.2). With exception of the small rotifers, no other contaminations (e.g. algae or protozoa) were observed during this study. Most likely eggs of small rotifer species (or even the rotifers themselves) were attached to the carapax of the *Daphnia* and hatched later on [50]. Interspecific competition (by "*D. longispina* + small rotifers") had a consistent negative effect on the *B. calyciflorus* abundances (Figure 4.2A and 4.2B, Appendix C Table C3 and C4). When under interspecific competition (by "*D. longispina* + small rotifers") the *B. calyciflorus* abundance declined faster than when not under interspecific competition and even got extinct in some jars (Figure 4.2A and 4.2B). Cladocerans compete with rotifers for food and are generally known to outcompete (e.g. faster filtration rate, can ingest wider range of algae cells, bigger energy reserve) and suppress rotifer populations [26,27,133,134]. Additionally, cladocerans can damage the rotifers themselves (by being swept into the branchial chamber of the Cladocera: mechanical interference) and this can have a negative effect on the rotifer populations [134].

Interspecific competition for food also occurs between rotifer species [37] and it is possible that *B. calyciflorus* was suppressed by the small rotifers. The small rotifers were probably more biologically adept (e.g. reproduction potential, tolerance to starvation, mechanical interference and different food niche: e.g more bacterivorous) to co-exist with the *D. longispina.* Smaller rotifers require less food (energy) to reach maximum growth rates (or start reproduction) and are better adapted to live under low (limited) food environments [46,133]. The small rotifer abundance showed no consistent Zn effect (Figure 4.2F; Appendix C Table C3 and C4). The temperature increase had a consistent positive effect on the small rotifer abundance between 4 and 18 days of exposure (Figure 4.2F and 4.2H; Appendix C Table C3 and C4). The higher

small rotifer population density in the warm jars can potentially be explained by the positive effect temperature has on e.g. maturation rate and egg development rate [46].

Surprisingly, the small rotifer abundances were positively affected, i.e. increased abundance, by an increased interspecific (by "*D. longispina* + *B. calyciflorus"*) competition (Appendix C Table C3-4 and compare Figure 4.2F and 4.2I: "*D. longispina* competition" vs "*D. longispina* + *B. calyciflorus"* competition). This positive effect was found, starting after 11 days of exposure and in the period of the sharp *B. calyciflorus* decline. It can be hypothesed that the declining *Brachionus* and *Daphnia* abundances indirectly affected the small rotifer abundances by reduced food competition as observed in previous studies [9]. Although a continuous and solemnly *B. calyciflorus* competition pressure experiment could have led to different results, the occurrence of the small rotifers provided an unique opportunity to investigate a more complex system.

Table 4.2: Statistical significance (*p* values pairwise comparison control [cold, no Zn and no *Brachionus* added jars] vs treatment) Zn, temperature and interspecific competition effects at the different samplings. Significant ($p < 0.05$) values are flagged.

	Independent variable	Day after first treatment					
		4	7	11	14	18	21
D. longispina total	Zn low	0.764	0.491	0.012	0.003	0.085	0.119
	Zn high	0.71	0.51	0.842	0.008	0.001	0.001
	Competition	0.625	0.081	0.308	0.508	0.642	0.963
	Temperature	0.014	0.211	0.628	0.154	0.096	0.016
D. longispina adult	Zn low	0.395	0.796	0.879	0.84	0.741	0.601
	Zn high	0.58	0.644	0.662	0.786	0.134	0.077
	Competition	0.838	0.754	0.899	0.357	0.992	0.644
	Temperature	0.861	0.537	0.76	0.179	0.99	0.923
D.longispina juvenile	Zn low	0.832	0.495	0.003	0.006	0.117	0.032
	Zn high	0.821	0.568	0.707	0.009	0.001	0.001
	Competition	0.524	0.055	0.197	0.179	0.672	0.635
	Temperature	0.012	0.259	0.706	0.061	0.132	0.004

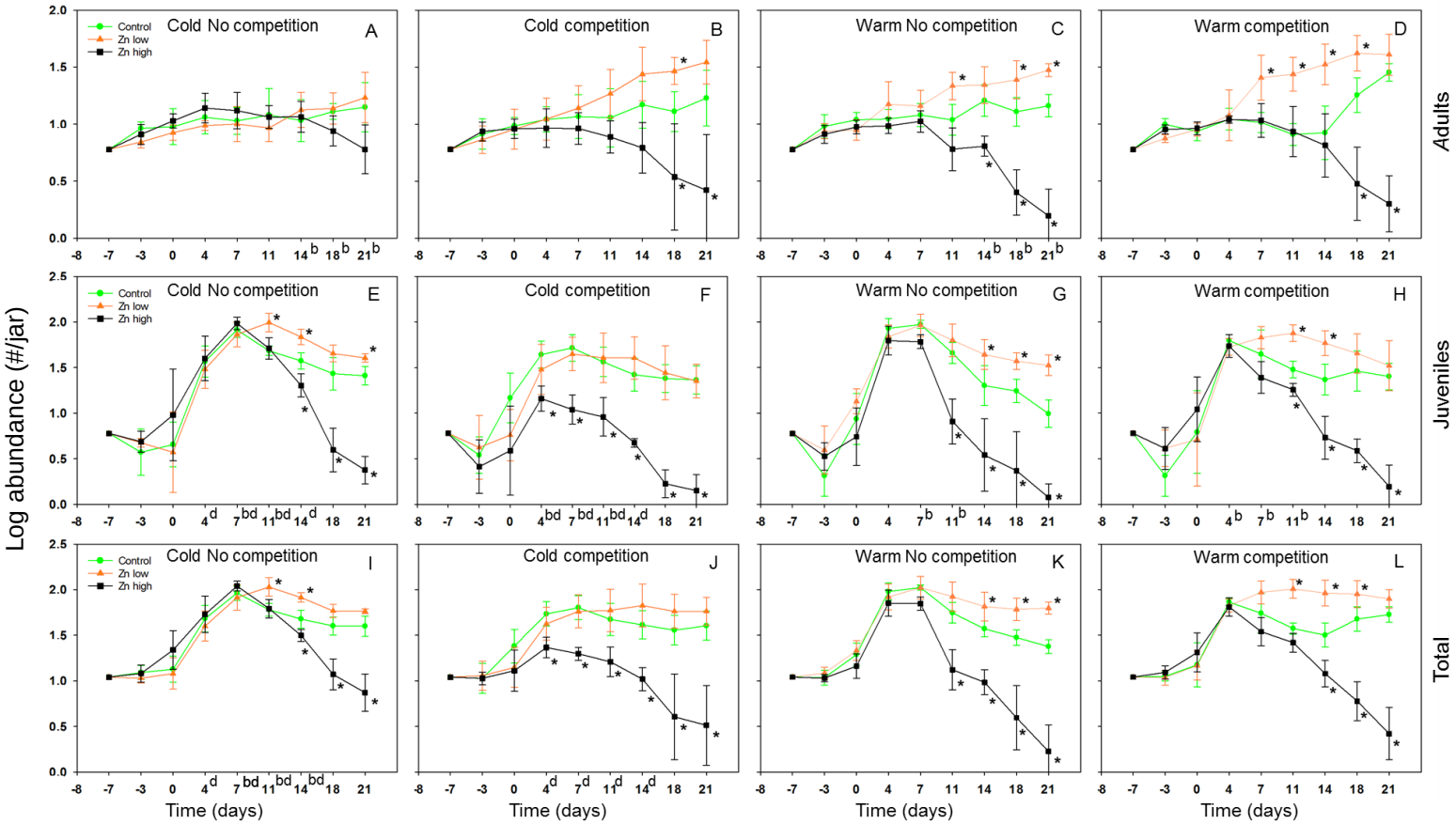


Figure 4.1: Population dynamics of *D. longispina* as shown by adult, juvenile and total abundance as a function of time for the different Zn treatments under the different temperature and *Brachionus* competition conditions. Error bars represent the standard deviation. *: significant Zn effect vs control ($p < 0.05$). [a]: consistent Zn (low) × T interactions; [b]: consistent Zn (high) × T interactions; [C]: consistent Zn (low) × *Brachionus* competition interactions; d: consistent Zn (high) × *Brachionus* competition interactions.

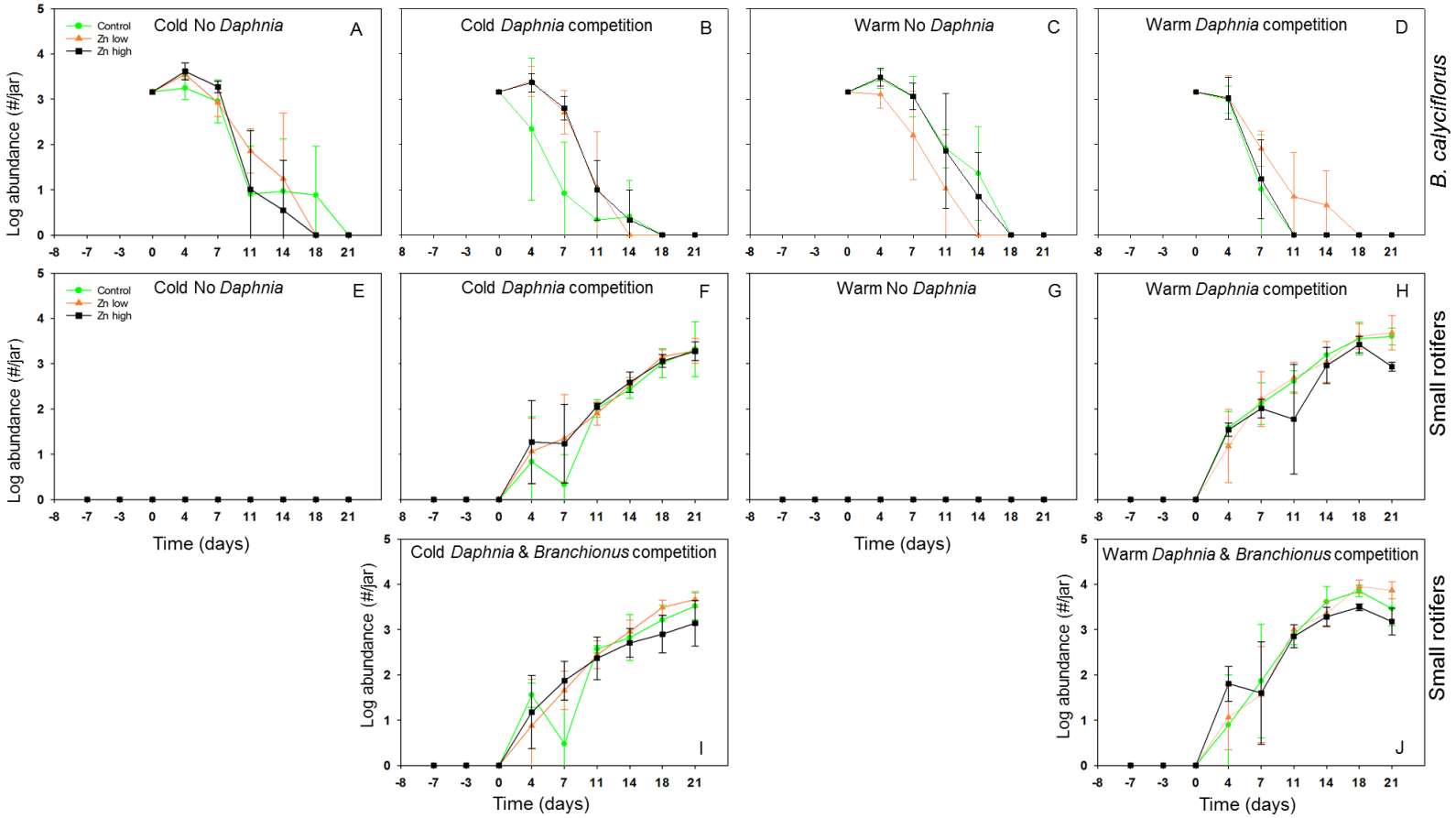


Figure 4.2: Population dynamics of *B. calyciflorus* and small rotifers as a function of time for the different Zn treatments under the different temperature and *Daphnia* competition conditions. The population dynamics of the small rotifers are further divided based on the occurrence *B. calyciflorus* (E-H: no *B. calyciflorus* present). Error bars represent the standard deviation. *: significant Zn effect vs control ($p < 0.05$).

4.3.3 Can interspecific competition affect Zn toxicity effects?

At the lowest Zn treatment the only consistent interaction (3-way ANOVA) that was observed was a Zn (low) × *Brachionus* competition for the *D. longispina* adults after 18 and 21 days of exposure (Table 4.3). However, when further analysing these interactions (2-way ANOVA), no consistent Zn (low) × *Brachionus* competition interactions were observed under any of the temperature conditions (Table 4.4). Additionally, it should be mentioned that in contrast to all other treatment regimes, no consistent positive Zn (low) effect was observed for the *D. longispina* juveniles (and total *D. longispina* population) when exposed to interspecific competition and cold conditions (Figure 4.1).

At the highest Zn treatment significant 3-way interactions between Zn, T and *Brachionus* competition were observed for the total *D. longispina* abundance between 4 and 14 days of exposure and for the juveniles between 4 and 11 days, but not for the adults (Table 4.5). When *B. calyciflorus* was present in the jars, it significantly affected the Zn effects on the *D. longispina* abundance (total and juvenile), as revealed by consistent Zn (high) × competition (Table 4.5 and 4.6). Under cold condition the highest Zn treatment affected the juvenile abundance sooner and more negatively (up to 9 fold) when simultaneously exposed to *Brachionus* competition (juveniles: Figure 4.1E and 4.1F, total: Figure 4.1I and 4.1J). For example, after 4 days of treatment the *D. longispina* juvenile abundance was unaffected by high Zn in the jars without *B. calyciflorus* (Figure 4.1E). However, in the jars with *B. calyciflorus* the *D. longispina* juvenile abundance, after 4 days of treatment, was 3.2 times lower in the high Zn treatment in comparison with the Zn control (Figure 4.1F). These Zn (high) × competition interactions were only observed under cold conditions and this from the start until the complete disappearance of *B. calyciflorus* (day 18) from the jars (Figure

4.2). Previous research has already confirmed that toxicity effects could increase under food stress [17,32,37,135], or otherwise stated, higher food levels could provide extra energy content to resist toxicity [17,34]. Especially the early life stages of *Daphnia* (juveniles) are more sensitive to starvation than adults and this may explain why the juveniles were more effected by the highest Zn treatment when *B. calyciflorus* was present than the adults in this study [136]. It is possible that, when under *Brachionus* competition, the food intake of the juveniles was insufficient to maintain enough energy to maintain normal body function when the metabolic costs increased due to Zn stress. Additionally, the competition for food could have reduced the amount of energy that could be used for reproduction (dynamic energy budget theory [31]) resulting in fewer juveniles which could affect toxic effects observed at the population level. For example, under cold conditions *B. calyciflorus* competition may have counteracted the positive effect the lowest Zn treatment addition had on the juvenile abundances, that were observed under the other treatment regimes (Figure 4.1). Warming itself is known to be able to modify competitive interaction [11,79,118] and this could explain why Zn × competition interactions in this study are only observed under cold conditions.

For the *B. calyciflorus* abundance No consistent significant 3-or 2-way interactions between Zn, T and competition (by "*D. longispina* + small rotifers") were found (Appendix C Table C3). Also no consistent significant 3-or 2-way interactions between Zn, T and competition (by "*D. longispina* + *B. calyciflorus*") were found for the small rotifer abundances (Appendix C Table C3 and C4).

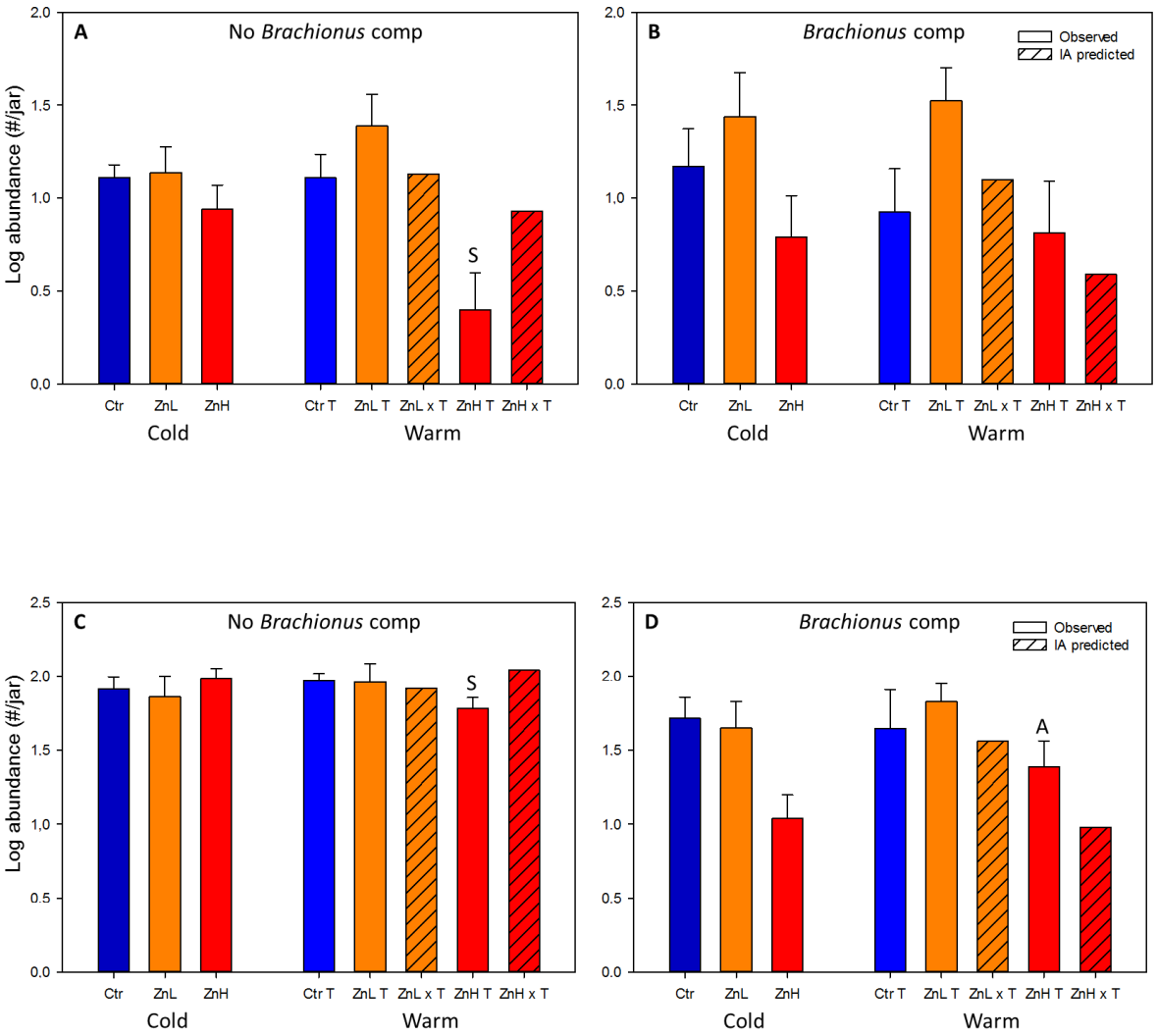


Figure 4.3: Log *D. longispina* adult abundance after 18 days of treatment (**A** and **B**) and juvenile abundance after 7 days of treatment (**C** and **D**) and predicted values using the Independent Action model (IA) with and without *Brachionus* competition. Error bars indicate standard deviation. A: significant antagonistic Zn × T interaction; S: significant synergistic Zn × T interaction.

Table 4.3: Statistical significance (*p* values Three-way ANOVA) of the main effects and interactions of the lowest Zn treatment and the different factors (competition and temperature) at the different samplings. Significant ($p < 0.05$) values are flagged and consistent interactions in bold.

	Independent variable	Day after first treatment					
		4	7	11	14	18	21
D. longispina total	Zn low (Zn L)	0.128	0.490	<0.001	<0.001	<0.001	<0.001
	Competition (comp)	0.473	0.003	0.031	0.710	0.098	0.005
	Temperature (T)	<0.001	0.122	0.983	0.368	0.286	0.644
	Zn L x Comp	0.988	0.241	0.593	0.344	0.977	0.101
	Zn L x T	0.626	0.117	0.189	0.211	0.261	0.081
	Zn L x Comp x T	0.798	0.272	0.048	0.240	0.681	0.115
D. longispina adult	Zn low (Zn L)	0.658	0.025	<0.001	<0.001	<0.001	0.002
	Competition (comp)	0.768	0.110	0.312	0.202	0.002	0.003
	Temperature (T)	0.331	0.062	0.168	0.390	0.011	0.037
	Zn L x Comp	0.927	0.068	**0.033**	**0.024**	**0.049**	0.767
	Zn L x T	0.274	0.063	0.007	0.176	0.198	0.775
	Zn L x Comp x T	0.420	0.342	0.706	0.294	0.242	0.128
D.longispina juvenile	Zn low (Zn L)	0.088	0.790	<0.001	<0.001	0.005	0.001
	Competition (comp)	0.456	<0.001	0.007	0.419	0.874	0.650
	Temperature (T)	<0.001	0.219	0.875	0.145	0.942	0.205
	Zn L x Comp	0.791	0.417	0.983	0.941	0.273	0.011
	Zn L x T	0.713	0.187	0.387	0.223	0.368	0.050
	Zn L x Comp x T	0.669	0.338	0.020	0.565	0.926	0.370

Table 4.4: Statistical significance (*p* values Two-way ANOVA) of the main effects and interactions of the lowest Zn treatment and competition for the different treatment regimes (cold and warm;) of the *Daphnia longispina* abundance at the different samplings. Significant ($p < 0.05$) values are flagged and consistent interactions in bold.

Experiment	Independent variable	Day after first treatment					
		4	7	11	14	18	21
Cold							
D. longispina adult	Zinc Low (Zn L)	0.606	0.782	0.655	0.090	0.013	0.095
	Competition (comp)	0.784	0.315	0.211	0.040	0.027	0.100
	Zn L x Comp	0.575	0.555	0.150	0.384	0.028	0.312
Warm							
D. longispina adult	Zinc Low (Zn L)	0.332	0.005	<0.001	0.002	0.001	0.001
	Competition (comp)	0.551	0.206	0.847	0.586	0.027	0.003
	Zn L x Comp	0.567	0.041	0.092	0.025	0.572	0.189

Table 4.5: Statistical significance (p values Three-way ANOVA) of the main effects and interactions of the highest Zn treatment and the different factors (competition and temperature) of the *Daphnia longispina* abundances at the different samplings. Significant ($p < 0.05$) values are flagged and consistent interactions in bold.

	Independent variable	Day after first treatment					
		4	7	11	14	18	21
D. longispina total	Zn high (Zn H)	0.011	<0.001	<0.001	<0.001	<0.001	<0.001
	Competition (comp)	0.017	<0.001	0.007	0.006	0.719	0.571
	Temperature (T)	<0.001	0.773	0.005	<0.001	0.382	0.019
	Zn H x Comp	0.075	0.001	0.959	0.158	0.217	0.134
	Zn H x T	0.447	0.753	0.102	0.188	0.399	0.069
	Zn H x Comp x T	**0.013**	**0.003**	**<0.001**	**0.002**	0.260	0.543
D. longispina adult	Zn high (Zn H)	0.715	0.791	0.113	0.005	<0.001	<0.001
	Competition (comp)	0.380	0.365	0.507	0.149	0.594	0.741
	Temperature (T)	0.566	0.909	0.109	0.278	0.186	0.210
	Zn H x Comp	0.538	0.534	0.616	0.669	0.170	0.097
	Zn H x T	0.682	0.904	0.866	0.552	**0.037**	**0.016**
	Zn H x Comp x T	0.190	0.172	0.102	0.018	0.330	0.494
D.longispina juvenile	Zn high (Zn H)	0.006	<0.001	<0.001	<0.001	<0.001	<0.001
	Competition (comp)	0.017	<0.001	0.002	0.087	0.962	0.275
	Temperature (T)	<0.001	0.502	0.007	0.002	0.937	0.010
	Zn H x Comp	0.051	<0.001	0.643	0.249	0.323	0.052
	Zn H x T	0.253	0.442	0.072	0.208	0.445	0.597
	Zn H x Comp x T	**0.011**	**0.003**	**<0.001**	0.052	0.324	0.634

Table 4.6: Statistical significance (*p* values Two-way ANOVA) of the main effects and interactions of the highest Zn treatment and the different factors (competition and temperature) for the different treamtent regimes (cold and warm; no competition and competition) of the *Daphnia longispina* abundance at the different samplings at . Significant ($p < 0.05$) values are flagged and consistent interactions in bold.

Experiment	Independent variable	Day after first treatment					
		4	7	11	14	18	21
Cold							
D. longispina total	Zinc High (Zn H)	0.055	<0.001	0.006	<0.001	<0.001	<0.001
	Competition (comp)	0.061	<0.001	<0.001	<0.001	0.079	0.199
	Zn H x Comp	**0.018**	**<0.001**	**0.004**	**0.004**	0.143	0.188
D.longispina juvenile	Zinc High (Zn H)	0,029	<0.001	0.002	<0.001	<0.001	<0.001
	Competition (comp)	0.066	<0.001	<0.001	<0.001	0.040	0.091
	Zn H x Comp	**0.014**	**<0.001**	**0.001**	**0.002**	0.108	0.250
Warm							
D. longispina total	Zinc High (Zn H)	0.101	0.019	<0.001	<0.001	<0.001	<0.001
	Competition (comp)	0.150	0.001	0.377	0.844	0.106	0.024
	Zn H x Comp	0.471	0.855	0.005	0.216	0.931	0.473
D.longispina juvenile	Zinc High (Zn H)	0,124	0.019	<0.001	<0.001	<0.001	<0.001
	Competition (comp)	0.131	<0.001	0.282	0.359	0.114	0.011
	Zn H x Comp	0.541	0.696	0.004	0.645	0.999	0.123
No Brachionus competition							
D. longispina total	Zinc High (Zn H)	0.597	0.131	<0.001	<0.001	<0.001	<0.001
	Temperature (T)	0.016	0.053	<0.001	<0.001	0.012	<0.001
	Zn H x T	0.268	**0.002**	**<0.001**	**0.002**	0.116	0.045
D. longispina adult	Zinc High (Zn H)	0.860	0.781	0.135	0.022	<0.001	<0.001
	Temperature (T)	0.153	0.731	0.082	0.570	0.002	0.014
	Zn H x T	0.235	0.253	0.188	**0.010**	**0.002**	**0.011**
D.longispina juvenile	Zinc High (Zn H)	0,583	0.108	<0.001	<0.001	<0.001	<0.001
	Temperature (T)	0.009	0.061	<0.001	<0.001	0.145	<0.001
	Zn H x T	0.358	**0.003**	**<0.001**	0.062	0.894	0.422
Brachionus competition							
D. longispina total	Zinc High (Zn H)	0.001	<0.001	<0.001	<0.001	<0.001	<0.001
	Temperature (T)	<0.001	0.260	0.425	0.714	0.312	0.914
	Zn H x T	0.010	0.070	0.038	0.236	0.858	0.452
D. longispina adult	Zinc High (Zn H)	0.497	0.571	0.458	0.061	<0.001	<0.001
	Temperature (T)	0.500	0.889	0.604	0.364	0.790	0.732
	Zn H x T	0.523	0.420	0.322	0.281	0.516	0.276
D.longispina juvenile	Zinc High (Zn H)	<0,001	<0.001	<0.001	<0.001	<0.001	<0.001
	Temperature (T)	<0.001	0.168	0.164	0.990	0.021	0.659
	Zn H x T	**0.005**	**0.049**	**0.022**	0.533	0.117	0.973

4.3.4 Can temperature affect Zn toxicity effects?

Our study indicated that the Zn effects on a *D. longispina* population can be affected by temperature and that the presence of *B. calyciflorus* can significantly affect the interactive effects between Zn and T. Consistent Zn (high) × T interactions were observed (two-way ANOVA) for the total *D. longispina* abundances and the different sub-classes (juveniles and adults) when there was no interspecific *B. calyciflorus* competition (Table 4.6). As an example figure 4.3 illustrates the Zn and temperature effects and their interactions on the *D. longispina* adult abundance after 18 days of treatment (Figure 4.3A and B) and juvenile abundance after 7 days of treatment (Figure 4.3C and D) with and without *B. calyciflorus* competition. Without *B. calyciflorus* present the *D. longispina* adult abundance under warm conditions after 14, 18 and 21 days of treatment was, on average 2.8, 7 and 21 times lower in the high Zn treatment in comparison with the Zn control (Figure 4.1A and 4.1C) while under cold conditions no significant Zn effect was observed. After 7 and 11 days of exposure the *D. longispina* juvenile abundance, under warm conditions and within the highest Zn treatments, was on average 1.5 and 5.5 times lower than in the Zn controls (Figure 4.1E and 4.1G). Without *B. calyciflorus* competition the observed Zn (high) × T interactions thus indicated that under warm conditions the highest Zn treatment had a more adverse effect on the juvenile and adult *D. longispina* abundances than predicted (=synergistic Zn (high) × T interaction) (Figure 4.3). This is in line with many other studies that observed an increased metal toxicity at higher temperature [11,12,18,74,75,127]. At higher temperatures it has been hypothesized that there is an increased metal toxicity due to a faster metabolism, resulting in an higher uptake and accumulation of the metal by the organism [11,18,74]. For *Daphnia*, however, no information is available about T effects on the elimination and detoxification rates. A

recent single clone study conducted by Pereira et al. [77] on *D. magna* (4 different single clones) reported that the chronic metal toxicity of Zn, Cu and Ni were generally higher at lower temperatures. This was the only study, however, that acclimated the test organisms to the different temperature treatments for 2 generations before applying the metal treatments. Furthermore her results for Ni were very clone dependent and she warned about extrapolating results about the effect of T on chemical toxcicity from single clone studies to the population level [77]. With *B. calyciflorus* competition present, consistent Zn (high) × T interactions were only found for the *D. longispina* juveniles (Table 4.6). However, the observed Zn (high) × T interactions for the juveniles abundances that were under *B. calyciflorus* competition showed a different interaction pattern than without *B. calyciflorus*. After 7 days of exposure, for example (similar for 4 and 11 days of exposure), these Zn (high) × T interactions indicated that under warm conditions and with *Brachionus* competition, the highest Zn treatment had a smaller adverse effect on the *D. longispina* juvenile abundance than predicted (=Antagonistic Zn (high) × T interaction) (Figure 4.3 D). Stated otherwise, with *B. calyciflorus* competition the highest Zn treatments was on average 2.2 times less toxic to the *D. longispina* juvenile abundances at higher temperatures. This is in contrast with our initial hypothesis that stated that higher Zn effects on the *D. longispina* abundances were expected under warm conditions [10,18,74], especially when the populations were under an increased food competition (dynamic energy budget theory [31]). However, this could potentially be explained by the fact that at the highest Zn treatment the *B. calyciflorus* were more numerous under cold conditions than under warmer conditions, indicating the importance of interspecific competition (Compare Figure 4.2B and 4.2D).

4.4 Conclusion

In the present study, interspecific *B. calyciflorus* competition by itself had a limited effect on the *Daphnia* abundances but it significantly interacted with Zn, as revealed by consistent Zn (high) × competition interactions. For example, under cold condition the highest Zn treatment affected the juvenile abundance more negatively when simultaneously exposed to interspecific *Brachionus* competition. Possibly the competition for food reduced the amount of energy that could be used for (1) reproduction (dynamic energy budget theory [31]), resulting in fewer juveniles, or (2) to maintain enough energy to maintain normal body function when the metabolic costs increased due to Zn stress which could affect toxic effects observed at the population level.

Additionally, our results show that the presence of *B. calyciflorus* can significantly affect the interactive effects between Zn and T. Without *B. calyciflorus* competition the highest Zn treatment had an increased negative effect on the *D. longispina* population (Total, adults and juveniles) at higher temperatures (= synergistic Zn (high) × T). With *B. calyciflorus* competition the highest Zn treatments had a reduced negative effect on the *D. longispina* juvenile abundances at higher temperatures (= antagonistic Zn (high) × T). However, this is probably due the fact that at the highest Zn treatment the *B. calyciflorus* were more numerous under cold conditions than under warmer conditions. No consistent interactive effects between Zn and T were observed for the *D .longispina* adults (or total abundance) when under *Brachionus* competition.

The present study clearly illustrated the influence of T and *Brachionus* competition on Zn toxicity and should be considered for ERA. Not doing so may under-or overestimate risks in aquatic ecosystems.

5

General conclusions and future research perspectives

5.1 Introduction

One of the objectives the European Union ERA is to “determine the maximum environmental concentrations of chemicals that do not cause adverse effects on the ecosystem” [38]. De Laender and Janssen [38] and Van den Brink [12] stated that to achieve this goal, the ERA should become more ecologically realistic. At present ERA is mainly based on data obtained from standard ecotoxicity experiments. These experiments are typically conducted under standardised optimal conditions (e.g. temperature, food, etc.), at the species level and exposed to a single stressor at the time. However, this contrasts with natural conditions, where natural populations and communities are most often exposed to mixtures of multiple biotic (e.g. food shortage, predation) and abiotic (e.g. eutrophication, non-optimal temperature or water chemistry, metals) stressors. Thus, by ignoring ecological interactions and by not considering natural field conditions, single-species tests oversimplify the actual field situation and ERA may be over or under-protective. Including ecological interactions and (non-chemical) environmental stressors in toxicity testing is only a logical step towards this objective.

The aim of this PhD thesis was to investigate the combined effect of Zn with natural environmental stressors (P and/or T) in aquatic systems at different organization levels (population vs. community) in order to increase the realism of current ERA. Based on the three original research questions, the key findings of this PhD thesis are summarized in this chapter and future research perspectives are suggested.

5.2 Do environmental factors such as temperature and phosphorus affect Zn toxicity to a freshwater plankton community?

5.2.1 Complex community (Zn, T and P)

In chapter 3 we assessed in a complex microcosm study, how the toxicity of Zn is affected by temperature (T) and phosphorus supply (P) and how these T & P effects on Zn toxicity vary between the levels of organisation (population, functional group and community) and their endpoints in a community. During this study consistent interactions between Zn and the other factors (T and P) were rarely found at the species level while frequently found at the group, community composition and functional level. The majority of the consistent interactions were found between Zn and T, demonstrating that the Zn toxicity effects on the plankton community can be affected by temperature. These consistent Zn × T interactions were mainly found at the highest Zn treatment ($HC50_{plankton}$) and were predominantly antagonistic, which suggests a less strong Zn effect at higher T for most taxa, groups and functions (e.g. total chlorophyll, DOC, DO_{net}).

During our study, no clear indications were found that high P addition by itself significantly affected the overall Zn toxicity. Thus, high P addition by itself is concluded to have, at best, only slightly affected the overall Zn toxicity. Interestingly though, 90% of all the Zn × T interactions at the species, group and community composition level were found under high P addition. This strongly suggests that high P addition influenced the interactive effect between Zn and T.

Additionally, in chapter 3, multivariate analyses (PRC) conducted on the different plankton group were used to calculate a $NOEC_{community}$ for each of the different environmental conditions. Under cold oligotrophic conditions and eutrophic conditions

(cold and warm) the plankton community composition was only significantly affected at the $HC50_{plankton}$. However, under warm oligotrophic conditions the phytoplankton community structure was already consistently affected at the $HC5_{plankton}$. This is possibly due to the negative effect of Zn on the *D. longispina* populations which indirectly affected the phytoplankton community. In addition to the effect on the plankton community, Zn, T and P also indirectly affected the chemical properties of the water over time and thereby the metal bioavailability. For instance, by lowering the DOC, Zn becomes more bioavailable and therefore more toxic (Box 5.1) [5,16].

Box 5.1 Indirect effect of Zn, T and P on $HCX_{-plankton}$ (Chapter 2 and 3)

During the experiment the pH and DOC levels were not only affected by temperature and the P addition rate, but also by the Zn treatments. For example, DOC was lower in Zn treatments and thus Zn becomes more bioavailable and therefore more toxic. This can be observed very clearly when comparing the calculated $HCx_{plankton}$ throughout the experiment in chapter 3 (Annex D table 5.1). Throughout the experiment the $HCx_{plankton}$ in the warm controls were clearly lower in comparison with the colder ones (up to 1.7-fold) and warming induced a much steeper HCx-plankton decline during the first 3 weeks. At the end of the experiment clear differences in $HC50_{plankton}$ were observed between the controls and the high Zn treatment (cold low P: 1.5 times lower; cold high P: 1.4 times lower; warm low P: 1.2 times lower; warm high P: 1.3 times lower). For the $HC5_{plankton}$ a clear decreasing trend can only be observed between the control and the low Zn treatment under cold eutrophic conditions. Most likely temperature itself had a greater effect on the DOC than the lowest Zn concentration, which can explain why at higher T the calculated $HC5_{plankton}$ are similar at the different Zn concentrations.

5.2.2 Simple community (Zn and T)

A simple community experiment (*D. longispina* + small rotifers; *D. longispina* + small rotifers + *B. calyciflorus*) was conducted in chapter 4 for assessing the combined effects of interspecific *Brachionus* competition (food competition), temperature and Zn. Without *Brachionus* competition the highest Zn treatment had an increased negative effect on the *D. longispina* population (adults, juveniles and total) at higher temperatures. With *Brachionus* competition no consistent interactive effects between Zn and T were observed for the *D .longispina* adults (or total abundance). However when under *Brachionus* competition, the highest Zn treatments had a reduced negative effect on the *D. longispina* juvenile abundances at higher temperatures. This is probably due the fact that at the highest Zn treatment the *B. calyciflorus* were more numerous under cold conditions than under warmer conditions. Our results show that T can have an effect on the Zn toxicity and that the presence of *B. calyciflorus* can significantly affect (most likely due to food competition) the interactive effects between Zn and T.

5.2.3 Population (Zn and T)

A simple population (or can be even defined as an extremely simple community) experiment (*D. longispina* + *Desmodesmus* sp.) was conducted in chapter 5 (see 5.4) for assessing the combined effects of Zn and T at lower biological organisation levels (See 5.4 for details). During this study consistent synergistic Zn (high) × T interactions were observed for the *D. longispina* population, indicating an increased negative Zn effect at higher temperature.

In conclusion: Collectively, our study suggests that temperature and phosphorus loading to freshwater systems should be accounted for in risk assessment of chemicals, as these factors may modify effects on aquatic communities. Not doing so may underestimate risks in some and overestimate risks in other systems, depending on their temperature and phosphorous loading. Additionally our results indicate that species interactions (food competition) can affect how temperature affects Zn toxicity.

Further research perspectives: To our knowledge very few studies have been conducted to investigate the adverse effects of Zn on the whole plankton community and none of these studies have investigated the combined effect of Zn (or any other metal) with temperature and/or P addition. With the upcoming global change, and the predicted occurrence of more climate extremes, it is crucial that more studies are conducted that investigate the combined and interactive effects of chemical stressors and environmental factors like temperature and nutrient addition. Although most of the Zn × T interactions in chapter 3 were observed under high P loading conditions, we did not conduct any of our additional experiments under these conditions. Therefore it would be interesting to rerun the simple community experiments (chapter 4 and 5) under high P (eutrophic) conditions.

Microcosm studies can be very time consuming (costly) and inherently complex to interpret. Furthermore, the huge amount of variables (biotic and abiotic) that could potentially influence how chemicals affect ecosystems makes it impossible to experimentally test each possible environmental scenario. Using models to a assess the effects of chemicals on communities under different scenarios could be a useful

tool for ERA. Some ecological models have already successfully been used to investigate and predict the effects of chemicals on communities [137] and ecosystems [112]. For example a recent theoretical, mechanistic fate and effect modelling study conducted by De Laender et al. (2015) [138] theoretically explored how direct and indirect chemical effects on an invertebrate pond community varied with changing ecological and exposure scenarios and he found that direct and indirect chemical effects are larger in mesotrophic systems than in oligotrophic systems. At present, correct model validation is one of the obstacles for creating realistic ecological models. This PhD dissertation provides an unique dataset that could be used to create new or validate existing ecological models (i.e. assess if the model accurately captures underlying processes).

5.3 Do species interactions affect Zn toxicity effects on a freshwater plankton community?

5.3.1 Complex community

In Chapter 2 the direct and indirect effects of Zn on the structure and function of a freshwater plankton community were assessed. Based on the Species Sensitivity Distribution, constructed with data from chronic toxicity tests with 22 freshwater species [5], it was hypothesised that the phytoplankton taxa would be the most sensitive to Zn stress in a freshwater plankton community and that, as a consequence the zooplankton would be affected indirectly (by reduced resource availability). However, our results strongly suggest that at the highest Zn concentration a significant reduction in cladocerans (e.g. *D. longispina*) resulted in increase of rotifers and ciliate abundance. These indirect effects were likely the result of a reduced food competition.

Contrary to the SSD predictions, which identified phytoplankton as the most sensitive groups, only the total chlorophyll and two phytoplankton taxa were adversely affected at the highest Zn treatment. Most of the phytoplankton groups showed a significant increase in abundance throughout the experiment at the highest Zn treatment. In addition, the phytoplankton community shifted in dominance from grazing-resistant to edible species. The results from Chapter 2 indicate that species interactions may have an important effect on the global Zn toxicity effect on a plankton community.

5.3.2 Simple community

A simple community experiment (*D. longispina* + small rotifers; *D. longispina* + small rotifers + *B. calyciflorus*) was conducted in chapter 4 for assessing the combined effects of interspecific *Brachionus* competition (food competition) and Zn. Interspecific *Brachionus* competition by itself had a limited effect on the *D. longispina* abundances but it significantly interacted with the highest Zn concentration. For example, the highest Zn treatment affected the juvenile *D. longispina* abundance more negatively when simultaneously exposed to *Brachionus* competition.

In conclusion: The results from chapter 2 and 4 strongly indicate that species interactions influence Zn effects on planktonic communities. This PhD thesis illustrates that a freshwater community is not just an aggregation of isolated entities but that species interact and that this can influence chemical effects. Thus, these interactiosn should be considered when assessing ecological risks of chemicals.

Further research perspectives: During the experiment conducted in chapter 4, the *B. calciflorus* abundance declined sharply and the population eventually went extinct. Additionally, the occurrence of small rotifers made it more difficult to assess how the strength of the *B. calciflorus* competition altered the effect of Zn on the *D. longispina* abundance. It would be interesting to rerun the experiment while avoiding small rotifers occurrence and by trying to keep the *B. calciflorus* abundance more constant (by adding additional *B. calciflorus*) within the interspecific competition treatments.

In chapter 4 we focused on the effects of interspecific food competition between *D. longispina* and *B. calcyflorus*. However, food competition is only one of the different species interactions that can affect chemical toxicity effects [9,26,27]. Predation for example is another major biotic interactions and can significantly modify the responses of organisms to toxicants [9,26,27]. At present very few studies have investigated the effects of species interaction on the toxic effects of chemicals and those studies were mainly focused on pesticides [26,27,32,35,37]. In order to increase the realism of current ERA of metals additional research is needed to investigate the effects of species interactions (especially predation) on metal effect on freshwater communities. Ecological models have been suggested as a one of the best options to improve effect assessment. Currently several initiatives have been taken to develop models for improving ERA by incorporating ecological interactions [15,26,138]. Although these models are available and can be used as a mechanistic basis to predict interactions between competition, predation and chemical toxicity, further steps (e.g.adding more complexity and testing) are needed before these models can be used for ERA to make accurate predictions [38]. The data generated in this PhD thesis could help validating existing ecological models.

5.4 Are the combined and interactive effects between Zn and temperature observed at the population level similar to the combined and interactive effects observed in more complex freshwater plankton communities?

Microcosm and mesocosm studies are considered to be a high-tier approach to assess the effects of chemicals at the population and community level [9,29,82,115]. Although microcosm and mesocosm studies (high-tier experiments) provide more realistic risk assessments than lower-tier single-species tests, they are more time consuming, costly and more difficult to interpret [29,89].

Here we try to determine whether population level effects (single and interactions) of chemicals, observed in lower-tier experiments are similar to population level effects during high-tier experiments. Stated otherwise: can lower-tier results be extrapolated to higher-tier experiments or to natural aquatic ecosystems? For this purpose, a simple population jar study was conducted in which *D. longispina* populations (24 jars with 5 adults and 5 juveniles) were exposed to three different zinc (Zn) treatments (background, $HC5_{\text{-plankton}}$ and $HC50_{\text{-plankton}}$) and two different temperature regimes (cold: 16-19 °C and warm: 21-24 °C) varied in a full 3x2 factorial design. Afterwards the results from this experiment (Zn effects, interactions) were compared with the *D. longispina* population (further denoted D) results from a simple community (*D. longispina* and small rotifers [*Lecane lunaris*, *Lepadella patella*, *Cephalodella* sp. and *Mytilina* sp.] further denoted D + R: chapter 4), a less simple community (*D. longispina*, small rotifers and *Brachionus calyciflorus* or D + R + B : chapter 4) and a complex community (zooplankton, phytoplankton and protozoa or COM: chapter 2 and 3)

(Figure 5.1). Since our main focus was on the Zn and T effects we only used the *D. longispina* abundance result of the low P treatments of chapter 3.

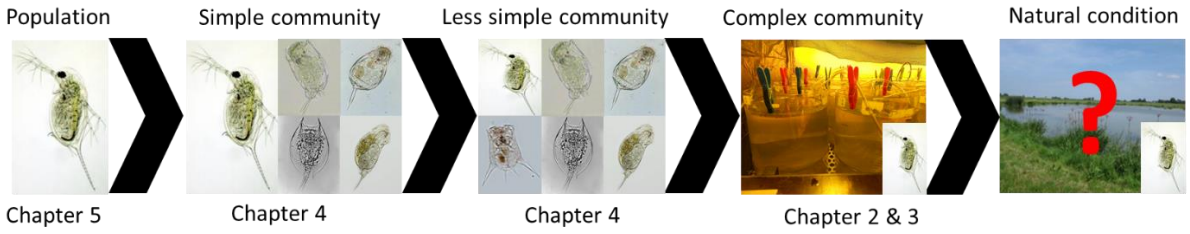


Figure 5.1: Schematic overview of 5.4. Are the Zn population effects observed at the high-tier similar to those at the lower-tier? Can the toxic effects of Zn observed at the lower-tier predict effects at the higher-tier?

To limit the genetic variation and stochasticity the *D. longispina* populations and the medium used in all our studies originated from the same mesotrophic ditch located at the Sinderhoeve Experimental Field Station (Wageningen, The Netherlands).

Table 5.1: Comparision of the experimental conditions used in chapters 2 till 5.

Experimental condition	Chapter 2 & 3	Chapter 4 A	Chapter 4B	Chapter 5
Biotic organisation	complex community	simple community	simple community	population
Volume	14L	0.5L	0.5L	0.5L
Initial density	mean 28 (adults + juveniles)	5 adults + 5 juveniles	5 adults + 5 juveniles	5 adults + 5 juveniles
D. longispina origin	Wageningen Sinderhoeve	Wageningen Sinderhoeve	Wageningen Sinderhoeve	Wageningen Sinderhoeve
D. longispina sampling season	Late spring	Autumn + begin winter (mix)	Autumn + begin winter (mix)	Autumn
Other organisms	zooplankton, fytoplankton, protozoa, Mesostoma sp.	small rotifers (*Lecane lunaris, Lepadella patella, Cephalodella sp. and Mytilina sp.*)	small rotifers + *Brachionus. calyciflorus*	/
Food source	phytoplankton	*Desmodesmus* sp. (dead)	*Desmodesmus* sp. (dead)	*Desmodesmus* sp. (alive)
Feeding	/	daily 1 g C/ml	daily 1 g C/ml	daily 1 g C/ml
Temperature	16-19 °C and 21-24 °C	16-19 °C and 21-24 °C	16-19 °C and 21-24 °C	16-19 °C and 21-24 °C
Zn treatments	Control, $HC5_{plankton}$, $HC50_{plankton}$	Control, $HC5_{plankton}$, $HC50_{plankton}$	Control, $HC5_{plankton}$, $HC50_{plankton}$	Control, $HC5_{plankton}$, $HC50_{plankton}$
Medium change	/	Medium change 2 times a week	Medium change 2 times a week	Medium change 2 times a week
Endpoints	1 time a week the abundances of adults + juveniles counted	2 times a week the abundances of the adults and juveniles were counted	2 times a week the abundances of the adults and juveniles were counted	2 times a week the abundances of the adults and juveniles were counted
Exposure duration	35 days	21 days	21 days	21 days

5.4.1 Zn toxicity from lower-tier to high-tier

Throughout all the studies in this thesis the *D. longispina* populations were exposed to the same Zn concentrations and two temperature (Table 5.1). Figure 5.2, table 5.2 and Appendix D (Table D2) shows the summary of the Zn effects and the Zn × T interactions of the *D. longispina* populations in the different tier experiments. At the highest Zn concentration the *D. longispina* populations were consistently adversely affected regardless of the complexity of the community. This was unexpected since the normalised NOEC (for reproduction) of *D. longispina* for the chronic Zn toxicity database [5], calculated for our different studies, were at least a factor 1.6 higher than the highest Zn concentration used in the different experiments (Chapter 3-5) and no effect of Zn was expected. Our data thus suggest that the used *D. longispina* populations in our studies were more sensitive to Zn than the isoclonal population (= lack of genetic variation) in the single-species ecotoxicity tests [5,107,108].

Another important observation is the fact that the magnitude of the adverse high Zn effect during the different studies differs (Figure 5.2). For example, after 14 days of treatment the whole *D. longispina* population got extinct at the highest Zn treatment in the population (D) and COM experiment (Chapter 3 and 5), while *D. longispina* population within the simple *D. longispina* and small rotifers community (Chapter 4) never got extinct (Figure 5.2). This can possibly be explained by the genetic difference between the different *Daphnia* populations (e.g. different sampling season and sampling size) used in our study [5,107,108]. *Daphnia* populations that are characterized with a higher genetic diversity, generally express a higher genotypic variability in tolerance to chemical stress [108]. For example, when a natural population is exposed to a chemical, genotypes that are more tolerant to this chemical will be favoured (i.e. maintain higher fitness) and this may allow the population to genetically

adapt this chemical toxicant [108]. For our study however, it is difficult to estimate whether eco-evolutionary dynamics played a role since we don't know the genotypic diversity of the inocula. To our knowledge, the *D. longispina* populations located at the Sinderhoeve Experimental Field Station ditch had never been exposed to high Zn stress prior to our experiments (natural, filtered Zn background concentration between 3 and 4 µg Zn/L), which makes it unlikely that the population was genetically adapted or acclimated to high Zn concentrations.

Table 5.2: Summary of the Zn effects and Zn ×T interactions on the different *D. longispina* populations: *D. longispina* population (D: chapter 5), *D. longispina* population in smaller rotifer community (D + R: Chapter 4), *D. longispina* population in smaller rotifer and *B. calyciflorus* community (D + R + B: chapter 4), *D. longispina* population in a natural, microcosm community (D + COM: chapter 2 and 3). Zn effects: no consistent effect (0), possitive effect (+), adverse effect (-).

	Population	Simple community		Complex community
	D	D + R	D + R + B	D + COM
Zn HC5 cold	0	+	+	0
Zn HC5 warm	0	+	+	-
Zn HC5 × T	No	No	No	No
Zn HC50 cold	-	-	-	-
Zn HC50 warm	-	-	-	-
Zn HC50 × T	Yes	Yes	No	No

At the lowest Zn concentration the Zn effects goes from no effect *(D. longispina* population), to a positive effect ("*D. longispina* + small rotifers" community and "*D. longispina* + *B. calyciflorus* + small rotifers" community) to a negative effect (*D. longispina* in complex plankton community) (Table 5.2 and figure 5.2). Although in the complex microcosm community experiment, the *D. longispina* populations were only significantly adversely affected at the lowest Zn concentration under warm conditions, their populations neared extinction at both temperatures (Chapter 2 and 3). One of the

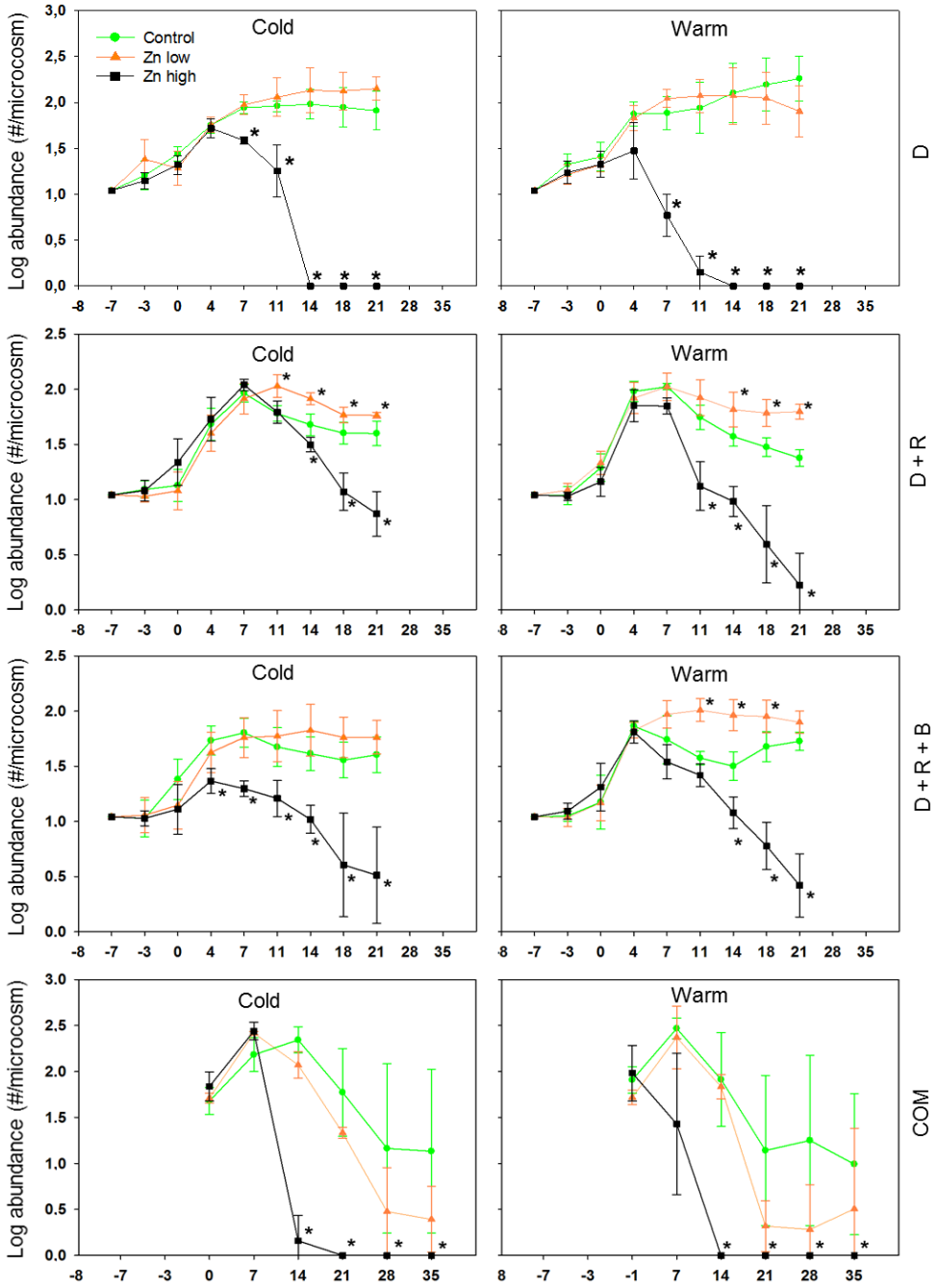


Figure 5.2: Population dynamics of the total *D. longispina* populations of the different Zn treatments under the different temperature of the different experiments: *D. longispina* population (D: Chapter 5), *D. longispina* population in the small rotifer community (D + R: Chapter 4), *D. longispina* population in the small rotifer and *B. calyciflorus* community (D + R + B: chapter 4), *D. longispina* population in a natural, microcosm community (COM: chapter 2 and 3). Error bars represent the standard deviation. *: significant Zn effect vs control ($p < 0.05$).

possible explanations for this are biotic interactions. Biotic interactions like competition for food and predation can have a major effect on the responses of organisms to toxicants [15,26,27] (see also 5.3). These biotic interactions can be very complex, can be affected by temperature and can modify or even mask toxic effects of toxicants. In the complex community study (Chapter 2 and 3). For example, *Mesostoma* sp. predated selectively on the *Daphnia* populations, which greatly affected the *D. longispina* population. Gergs et al. (2013) [28] found that the combination of predation and another stressor (p-353-nonylphenol) could lead to the extinction of a Daphnia population, although the effects of the single stressors were only small. It is unclear if the *D. longispina* population declines, in Chapters 2-3, were induced by Zn toxicity, *Mesostoma* sp. predation, a combination of both or by inter- and intra-specific interactions which could have had an effect on the species sensitivity to toxicants.

5.4.2 Zn × T: from lower-tier to high-tier

In our study, no consistent Zn (low) × T interactions were observed for the *D. longispina* population in any of the experiments. Consistent synergistic Zn (high) × T interactions were observed for the population experiment and the D + R community, indicating an increased negative Zn effect at higher temperature. No consistent Zn × T interactions were observed for the *D. longispina* populations of the more complex communities (Table 5.2). One of the possible explanations for this are biotic interactions. Biotic interactions can be very complex, are affected by temperature and can modify or even mask toxic effects of toxicants [15,26,27] (Chapter 3, 4). Another possible explanation for the fact that we don't observe consistent Zn × T interactions at the microcosm experiment is the difference in design (Table 5.1). In contrast to the other experiments, biology samples were only taken once a week during the complex community

experiment. By sampling only once a week temporally, short term (<1 week) interactions could not be observed.

In conclusion: The results from chapter 5 demonstrate that the results (especially for the lowest Zn treatments) observed in the lower-tier experiments can be different from population level effects during high-tier experiments. In other words, the extrapolation of lower-tier toxicology results to make predictions at higher-tier systems is complicated due to biotic interactions. Conventional ERA is generally based on the extrapolation of single-species ecotoxicity data to natural populations and communities (e.g. SDD method). The results from this PhD thesis indicate the importance of species interactions on Zn toxicity effects. By ignoring biotic interactions ERA is in sharp contrast with natural conditions and the extrapolation of conventional ecotoxicological results from individuals to populations and ecosystems could be dubious. Therefor we believe that the combination of higher-tier experiments and ecological models (e.g. [15,138]) are crucial for correctly predicting effects of chemicals on populations and communities.

5.5 Overall contribution of this PhD thesis to the future of ecological risk assessment

Currently, ERA is still mainly based on data obtained from standard ecotoxicity experiments, that are conducted under standardised optimal conditions, at the individual level and exposed to single stressors at the time. By ignoring biotic interactions and environmental conditions, ERA is in sharp contrast with natural conditions and the extrapolation of ecotoxicological results from individuals to populations and ecosystems could be dubious. This PhD thesis clearly illustrated that environmental factors, such as temperature and phosphorus, and biotic interactions can affect Zn toxicity effects on a freshwater community. Additionally, the results from this PhD thesis show that the results observed in the lower-tier experiments can be different from population level effects in high-tier experiments. A community is not just an aggregation of isolated entities but species interact and this can influence chemical exposure effects. In order to increase ecological realism, ERA should include temperature, phosphorus and biotic interactions in their assessment of chemicals, especially in the context of global change projections.

A

Supplemental material for Chapter 2

Tables

Table A1: Chemical properties of the water of the inoculation water from Sinderhoeve ditch and mean (± standard deviation) of the microcosm water at the start of the experiment.

Parameter	Sinderhoeve	Start experiment
Na (mg/l	4.15	4.57 ± 0.10
Mg (mg/l)	1.43	0.94 ± 0.05
Al (µg/l)	11.66	40 ± 6
P (µg/l)	10.95	12.2 ± 2.7
K (mg/l)	0.54	1.93 ± 0.10
Ca (mg/l)	20.12	20.89 ± 1.16
Cr (µg/l)	0.03	0.13 ± 0.18
Mn (µg/l)	34	5.6 ± 1.2
Fe (µg/l)	194	74.8 ± 2.2
Co (µg/l)	0.06	0.06 ± 0.03
Ni (µg/l)	0.26	0.46 ± 0.22
Cu (µg/l)	1.39	3.04 ± 0.89
Zn (µg/l)	3.04	3.05 ± 0.49
As (µg/l)	2.07	1.99 ± 0.11
Mo (µg/l)	0.14	0.95 ± 0.04
Cd (µg/l)	0.01	0.04 ± 0.01
Pb (µg/l)	0.11	0.038 ± 0.06
Mean pH	9.14	8.75 ± 0.21
DOC (mg/l)	13.6	14.2± 0.4
DIC (mg/l)	12.3	9.6 ± 0.7
Conductivity (µS/cm)	141	159 ± 6

Tabel A2: Species list used by the Biotic ligand model for the calculation of the $HC_{5\text{-plankton}}$ and $HC_{50\text{-plankton}}$ and the geomean of the normalized No-observed-effect concentrations per species at the start of the experiment.

Species	Phylum	Endpoints considered	Geomean NOEC normalized (µg Zn/L)
Algae			
Pseudokircheneriella supcapitata	Chlorophyta	Growth rate	73.07
Chlorella sp.	Chlorophyta	Growth rate	135.03
Invertebrate			
Ceriodaphnia dubia	Arthropoda	Reproduction	299.21
Daphnia magna	Arthropoda	Survival, reproduction	639.42
Daphnia longispina	Arthropoda	Reproduction	742.10
Hyalella azteca[a]	Amphipoda	Survival, reproduction	343.13
Anuraeopsis fissa[b]	Rotifera	Population growth rate	562.55
Branchionus rubens[b]	Rotifera	Population growth rate	562.55
Brachionus calyciflorus[b]	Rotifera	Population growth rate	725.39

[a] Taxon only used for HC_x calculations for experimental design (setting target Zn concentrations).

[b] Taxon only used for HC_x calculations for risk assesment.

Table A3: No-observed-effect concentrations and taxa classification of the class 1 and 2 taxa based on Minimum Detectable Difference values according to Brock et al [89]. Treatments resulted in significant increases (↑) or reductions (↓).

Taxa or endpoint	Cat.	-1	7	14	21	28	35
Single cell diatom	1	≥300(45)	≥300(47)	75↑(46)	≥300(60)	≥300(52)	≥300(61)
Scenedesmus sp. 2	1	≥300(98)	≥300(97)	≥300(98)	≥300(98)	75↑(95)	75↑(67)
Chrysococcus sp.	1	≥300(54)	75↓(41)	75↑(48)	75↑(44)	75↑(67)	75↑(60)
Cryptophyta sp. 1	1	75↑(120)	≥300(98)	75↑(99)	< 75↑(61)	75↑(92)	< 75↑(93)
Pseudanabaena sp.	1	≥300(35)	< 75↓(67)	≥300(71)	≥300(77)	≥300(86)	75↓(91)
Aphanocapsa sp. 1	1	≥300(37)	≥300(72)	< 75↑(41)	75↑(66)	≥300(92)	≥300(86)
# phytoplankton taxa	1	≥300(21)	≥300(16)	≥300(31)	75↓(14)	≥300(36)	≥300(18)
Bacillariophyceae	1	≥300(45)	≥300(46)	75↑(45)	≥300(59)	≥300(52)	≥300(57)
Chlorophyta	1	≥300(31)	≥300(61)	75↑(34)	75↑(42)	75↑(60)	75↑(70)
Chrysophyta	1	≥300(54)	75↓(41)	75↑(49)	75↑(43)	75↑(67)	75↑(59)
Cryptophyta	1	≥300(61)	≥300(61)	75↑(76)	75↑(59)	75↑(91)	< 75↑(90)
Cyanobacteria	1	≥300(33)	≥300(53)	≥300(58)	≥300(67)	≥300(79)	≥300(82)
Euglenophyta	1	≥300(65)	≥300(57)	< 75↑(55)	75↑(65)	75↑(96)	≥300(93)
Chydorus sphaericus	1	≥300(104)	≥300(86)	75↓(69)	75↓(75)	75↓(86)	< 75↓(88)
Cyclopoida	1	≥300(30)	≥300(26)	≥300(36)	≥300(61)	≥300(79)	≥300(87)
Simocephalus vetulus	1	≥300(110)	≥300(90)	≥300(65)	< 75↓(55)	< 75↓(80)	75↓(95)
Daphnia longispina	1	≥300(38)	< 75↑(36)	75↓(47)	75↓(66)	75↓(95)	75↓(94)
Colurella oblusa	1	≥300(114)	≥300(72)	≥300(96)	75↑(91)	≥300(81)	≥300(95)
Naupli	1	≥300(34)	≥300(31)	≥300(64)	75↑(47)	75↑(41)	≥300(68)
Lecane gr. *luna*	1	≥300(n.c.)	≥300(98)	< 75↑(72)	< 75↑(61)	75↑(65)	≥300(75)
Lecane gr. *lunaris*	1	≥300(173)	≥300(93)	< 75↑(65)	75↑(68)	< 75↑(53)	≥300(68)
# zooplankton taxa	1	≥300(31)	≥300(11)	≥300(21)	≥300(13)	75↓(26)	75↓(18)
cladocera	1	≥300(36)	< 75↑(27)	< 75↓(33)	75↓(71)	75↓(84)	75↓(90)
copepoda	1	≥300(32)	≥300(24)	≥300(55)	75↑(46)	75↑(39)	≥300(62)
rotifera	1	≥300(31)	≥300(58)	< 75↑(63)	< 75↑(64)	< 75↑(51)	≥300(62)
# protozoa taxa	1	≥300(21)	≥300(46)	≥300(44)	≥300(49)	≥300(55)	≥300(41)
Unknown small flagellate (7-10µm)	2	≥300(121)	≥300(n.c.)	≥300(n.c.)	75↓(35)	≥300(120)	< 75↓(91)
Tetraëdron minimun	2	≥300(105)	≥300(99)	75↓(81)	≥300(97)	≥300(153)	≥300(120)
Monoraphidium sp. 1	2	≥300(74)	≥300(90)	75↑(112)	≥300(118)	75↑(213)	≥300(103)
Monoraphidium sp. 2	2	≥300(36)	≥300(87)	< 75↑(113)	75↑(173)	75↑(99)	≥300(91)
Desmodesmus sp.	2	≥300(96)	≥300(92)	75↑(66)	< 75↑(110)	75↑(91)	75↑(96)
Radiococcus sp.	2	≥300(65)	≥300(103)	75↑(103)	≥300(121)	≥300(95)	≥300(92)
Green oval	2	≥300(96)	≥300(64)	≥300(100)	75↑(74)	≥300(90)	≥300(119)
Cosmarium sp.	2	≥300(137)	≥300(138)	75↓(90)	≥300(100)	≥300(212)	< 75↑(243)
Oocystis sp.	2	≥300(118)	≥300(103)	75↓(87)	≥300(97)	≥300(107)	≥300(105)
Haematococcus sp.	2	≥300()	< 75↓(59)	≥300(n.c.)	≥300(105)	75↑(93)	< 75↑(78)
Mougeotia sp.	2	≥300()	≥300(n.c.)	≥300(118)	≥300(107)	< 75↓(72)	≥300(107)
Uroglena sp.	2	≥300(125)	≥300()	≥300(106)	75↓(97)	< 75↓(88)	≥300(138)
Cryptomonas sp.	2	≥300(70)	≥300(106)	75↓(90)	≥300(118)	≥300(109)	≥300(102)

Taxa or endpoint	Cat.	-1	7	14	21	28	35
Rodomonas sp.	2	≥300(51)	75↑(n.c.)	≥300(112)	≥300(129)	≥300(175)	≥300(94)
Cryptophyta sp. 3	2	≥300()	≥300(100)	≥300()	75↑(72)	≥300(169)	≥300()
Cryptophyta sp.4	2	≥300()	75↓(39)	≥300(95)	≥300(94)	≥300(199)	≥300(108)
Anabaena sp.	2	≥300(97)	75↓(84)	< 75↓(77)	≥300(147)	≥300(153)	≥300(163)
Aphanocapsa sp.2	2	≥300()	≥300(87)	≥300()	≥300(161)	75↓(97)	≥300()
Chroococcus sp.	2	≥300()	≥300(92)	≥300(106)	≥300(101)	75↓(94)	≥300(94)
Peranema sp.	2	≥300()	≥300(145)	≥300()	75↓(96)	≥300(n.c.)	≥300(115)
Euglena sp.	2	75↑(119)	≥300(132)	75↑(92)	≥300(108)	≥300(104)	≥300(107)
Euglenida	2	≥300()	≥300(63)	≥300()	≥300(114)	75↑(97)	≥300(99)
unknown phytoplankton taxa	2	≥300(121)	≥300(n.c.)	≥300(165)	75↓(35)	≥300(120)	≥300(104)
Aphanothece sp.	2	≥300(95)	75↓(53)	75↑(79)	75↑(67)	≥300(95)	≥300(141)
Tetraëdron caudatum	2	≥300()	≥300()	≥300(n.c.)	≥300()	75↑(n.c.)	≥300(122)
Scenedesmus acuminatus	2	≥300()	≥300()	≥300()	≥300(n.c.)	≥300()	75↑(n.c.)
Cryptophyta sp. 2	2	≥300(n.c.)	≥300(110)	< 75↑(n.c.)	75↑(122)	75↑(152)	75↑(n.c.)
Merismopedia sp.	2	≥300(n.c.)	≥300(152)	75↑(n.c.)	≥300(n.c.)	≥300()	≥300(n.c.)
Cyanodictyon sp.	2	≥300()	≥300(n.c.)	≥300()	≥300(119)	75↑(135)	≥300()
Anisonema acinus	2	≥300()	≥300(n.c.)	≥300()	≥300(126)	≥300(206)	75↑(n.c.)
Chaetonotus sp.	2	≥300(n.c.)	≥300(n.c.)	≥300(n.c.)	≥300(152)	≥300(108)	75↑(157)
Cephalodella gibba	2	≥300(56)	≥300(208)	< 75↑(n.c.)	75↑(111)	75↑(175)	≥300(179)
Trichocerca bicristata	2	75↑(107)	≥300(59)	≥300(93)	≥300(103)	≥300(140)	≥300(97)
Lepadella patella	2	≥300(99)	≥300(60)	< 75↑(103)	≥300(98)	75↓(96)	≥300(161)
Amoebina testacea sp.	2	75↓(100)	≥300(126)	75↓(98)	≥300()	≥300(123)	≥300(103)
Amoeba sp.	2	≥300(116)	≥300(97)	≥300(100)	≥300(98)	≥300(69)	≥300(n.c.)
Difflugia sp.	2	≥300()	≥300(119)	≥300(143)	< 75↓(94)	≥300(n.c.)	≥300(148)
Cyclidium sp.	2	≥300(101)	≥300(102)	75↑(n.c.)	75↑(102)	< 75↑(119)	75↑(97)
Nassula sp.	2	≥300()	75↓(99)	≥300()	≥300(102)	≥300(127)	≥300(136)
Amoeba general	2	≥300(100)	≥300(73)	≥300(98)	≥300(98)	≥300(65)	≥300(107)
Ciliates general	2	≥300(46)	75↓(95)	75↑(95)	75↑(97)	< 75↑(102)	75↑(88)
Bacterivorous ciliates	2	≥300(52)	≥300(103)	75↑(99)	75↑(99)	< 75↑(115)	75↑(98)
Algivorous ciliates	2	≥300()	75↓(99)	≥300()	≥300(102)	≥300(127)	≥300(136)
Rimostrombidium brachykinetum	2	≥300(99)	≥300()	75↑(107)	≥300(n.c.)	< 75↑(n.c.)	75↑(135)
Litonotus sp.	2	≥300(n.c.)	≥300(119)	≥300(n.c.)	75↑(n.c.)	≥300(n.c.)	≥300(n.c.)
Codosiga botrytis	2	≥300()	≥300(124)	≥300(104)	75↑(103)	75↑(113)	≥300(103)
heterotrophic flagellates	2	≥300()	≥300(124)	≥300(105)	75↑(103)	75↑(103)	≥300(103)

Table A4: Biotic Ligand Model predicted No-observed-effect concentrations (NOECs) and observed community NOECS for *Daphnia longispina* and *Auraeopsis fissa* per sampling date.

Taxa	Day	NOEC BLM			NOEC community (µg/l)
		control	75 µg/L	300 µg/L	
Daphnia longispina	-1	805	689	711	≥300
	7	575	470	450	<75↑
	14	463	379	279	75↓
	21	425	366	272	75↓
	28	385	342	283	75↓
	35	407	334	286	75↓
Auraeopsis fissa	-1	610	523	539	≥300
	7	428	337	318	≥300
	14	336	266	183	≥300
	21	300	249	178	≥300
	28	265	230	186	≥300
	35	281	224	186	≥300

Table A5: Mean (± standard deviation) of the chemical properties of the microcosm water used for Biotic Ligand Model normalisation per sampling date.

Treatment	Day	pH	Ca (mg/l)	Mg (mg/l)	Na (mg/l)	K (mg/l)	SO4 (mg/l)	Cl (mg/l)	DIC mg/L	DOC (mg/l)
Control	-1	8.9(±0.2)	20.3(±0.7)	0.9(±0)	4.6(±0.1)	2(±0.2)	18.9(±0.5)	17.1(±0.4)	9.1(±0.6)	14.4(±0.6)
Control	7	8.4(±0.1)	20.8(±1.3)	0.9(±0.1)	4.6(±0.1)	2.4(±0.1)	19.2(±0.9)	17.4(±0.7)	10(±0.6)	13.1(±0.3)
Control	14	8.2(±0.2)	21.7(±2.6)	0.9(±0.1)	4.6(±0.2)	3(±0.1)	19.8(±1.7)	17.9(±1.3)	12(±1.1)	11(±0.7)
Control	21	8.2(±0.2)	21.7(±2.6)	0.9(±0.1)	4.6(±0.2)	3(±0.1)	19.8(±1.7)	17.9(±1.3)	12(±1.1)	11(±0.7)
Control	28	8(±0.1)	23.4(±2.5)	0.9(±0.1)	4.6(±0.1)	3.6(±0.1)	20.9(±1.6)	18.7(±1.2)	6.5(±0.7)	11.2(±0.3)
Control	35	7.9(±0.2)	28.8(±4.3)	1(±0.1)	4.6(±0.4)	4.6(±0.2)	24.1(±2.6)	21.1(±2)	12.9(±2.1)	11.3(±0.5)
75	-1	8.6(±0.2)	21.5(±0.7)	1(±0)	4.6(±0)	2(±0.1)	19.7(±0.4)	17.8(±0.3)	10(±0.6)	14.3(±0.4)
75	7	8(±0.3)	23.7(±4.5)	1(±0.2)	4.6(±0.2)	2.5(±0.3)	21(±2.8)	18.8(±2.1)	11.5(±2)	12.1(±0.9)
75	14	8(±0.3)	24.7(±4.5)	1(±0.1)	4.5(±0.1)	3.2(±0.3)	21.7(±2.8)	19.3(±2.1)	12.4(±0.4)	9.2(±3.1)
75	21	7.8(±0.1)	26.8(±7.1)	1(±0.2)	4.5(±0.1)	3.7(±0.1)	22.9(±4.2)	20.2(±3.2)	7(±1.3)	10.4(±0.9)
75	28	7.7(±0)	29.3(±7.6)	1.1(±0.2)	4.5(±0.1)	4.2(±0.2)	24.4(±4.4)	21.3(±3.3)	13.7(±2.6)	9.7(±0.6)
75	35	7.8(±0)	32.2(±7.8)	1.1(±0.2)	4.5(±0.3)	4.7(±0.2)	26.1(±4.4)	22.5(±3.2)	13.3(±2)	9.1(±0.9)
300	-1	8.7(±0.2)	21.1(±1.9)	0.9(±0.1)	4.5(±0.1)	1.9(±0)	19.4(±1.2)	17.6(±0.9)	9.8(±0.9)	14(±0.1)
300	7	8(±0.2)	24.1(±3.4)	1(±0.1)	4.7(±0.2)	2.4(±0.1)	21.3(±2.1)	19(±1.6)	11.5(±1.6)	12.1(±0.4)
300	14	7.6(±0)	25.8(±2.8)	1(±0.1)	4.5(±0)	3(±0.1)	22.3(±1.7)	19.8(±1.3)	13.8(±1.3)	7.9(±1.5)
300	21	7.7(±0)	29(±4.4)	1.1(±0.1)	4.4(±0.1)	3.6(±0.1)	24.3(±2.7)	21.2(±2)	8(±1)	7.1(±0.3)
300	28	7.8(±0.2)	32.4(±5.3)	1.2(±0.1)	4.4(±0)	4.2(±0)	26.3(±3.1)	22.7(±2.3)	15.7(±2)	6.9(±0.2)
300	35	7.7(±0.1)	35.6(±6)	1.2(±0.1)	4.3(±0.1)	4.6(±0.1)	28(±3.4)	24(±2.5)	12.3(±6.9)	7.2(±0.6)

Table A6: Mean (± standard deviation) of the chemical properties of the microcosm water per sampling date.

See excel sheet: Physico-chemical water parameters Van de Perre et al. 2016

Table A7: Species abundances per litre of the microcosms per sampling date.

See excel sheet: Species abundances Van de Perre et al. 2016

Table A8: Calculated HC_5 per sampling day and treatment, based on the EU [5] and US [111] methodology.

	Treatment	-1	7	14	21	28	35
HC_5 EU (µg Zn/L)	Control	156	126	97	97	89	82
	75	145	96	72	72	63	62
	300	146	96	49	46	47	46
HC_5 US (µg Zn/L)	Control	155	182	163	163	173	179
	75	182	186	144	162	153	150
	300	169	186	121	115	118	123

Figures

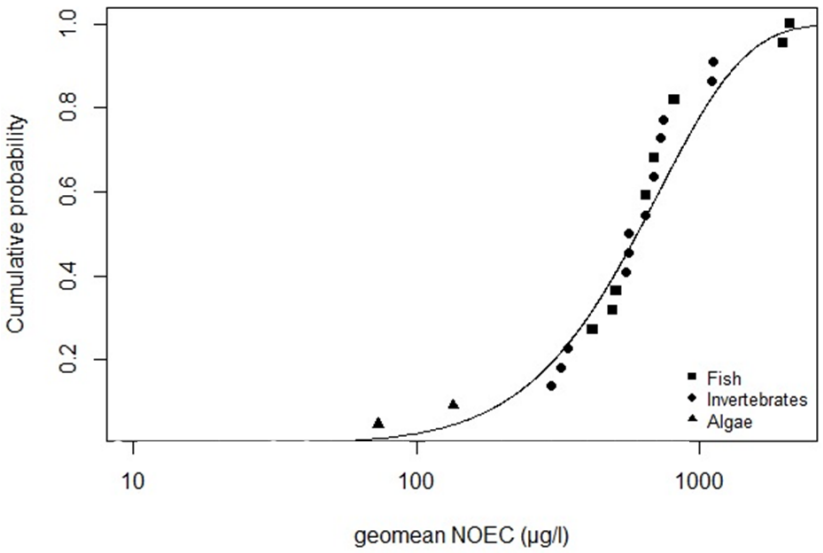


Figure A1: Cumulative probability plot of the normalized (to the water characteristics of microcosms at the start of the test) NOECs and fitted Species Sensitivity Distribution curve.

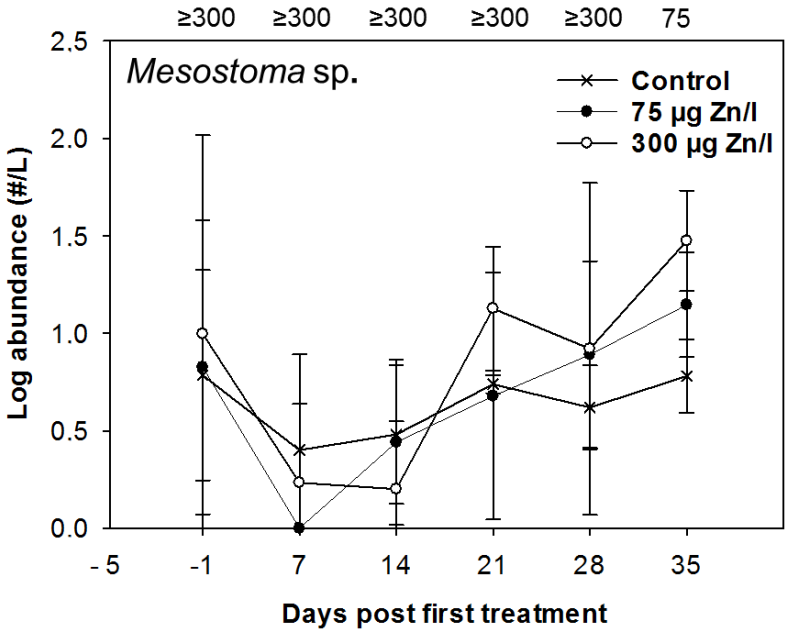


Figure A2: Population dynamics of the log abundances per treatment concentration of *Mesostoma* sp. (standard deviation as error bars). Calculated no-observed-effect concentrations are plotted above the figures.

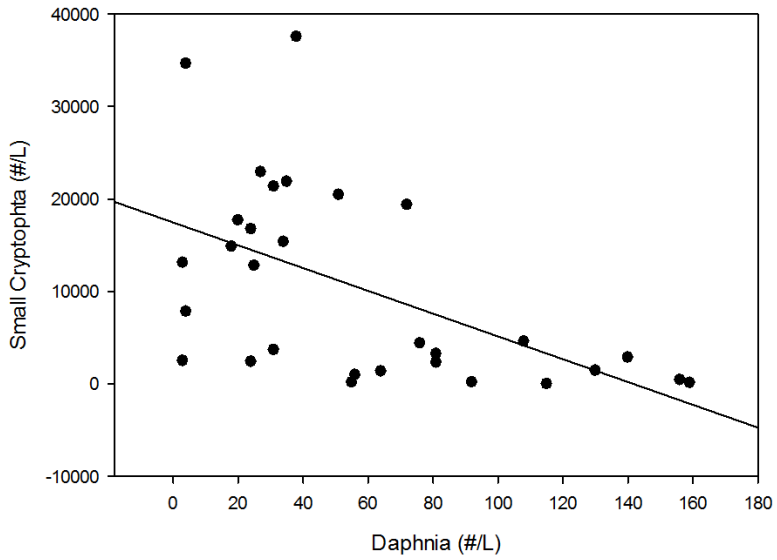


Figure A3: Interaction between the *Daphnia* and the small Cryptophyta species in the control microcosms.

B

Supplemental material for Chapter 3

Tables

Table B1: Mean (± standard deviation) of the chemical properties of the microcosm water per sampling date.

See excel sheet: Physico-chemical water parameters Van de Perre et al. 2017

Table B2: No-observed-effect concentrations (NOECs) (Williams test, $p < 0.05$) and observed effect classes [16] per sampling date for community metabolism and chemistry endpoints in the cold and warm low P addition Zn control microcosms. The numbers of the preselected effect classes [16] refer to: 1= no effect; 2= slight effect; 3=clear short-term effects; 4: clear effect in short-term study; ↓= decrease; ↑= increase; ↑↓= increase and decrease on species and/or sampling date. Blank fields indicate that NOEC were equal to or higher than the highest tested temperature.

	NOEC (µg/L)											Treatment levels
	-1	1	7	9	14	16	21	23	28	30	35	21-24°C
DO												
morning	—	< 22↓	< 22↓	< 22↓	< 22↓	< 22↓	< 22↓	< 22↓	< 22↓	< 22↓	< 22↓	4↓
afternoon		< 22↓	< 22↓	< 22↓	< 22↓	< 22↓	< 22↓	< 22↓	< 22↓	< 22↓		3↓
max-min	—			< 22↓	< 22↓	< 22↓	< 22↓				< 22↑	3↓(2↑)
mean		< 22↓	< 22↓	< 22↓	< 22↓	< 22↓	< 22↓	< 22↓	< 22↓	< 22↓		3↓
pH												
morning		< 22↓		< 22↓	< 22↓	< 22↓	< 22↓					3↓
afternoon			< 22↓	< 22↓	< 22↓	< 22↓	< 22↓				< 22↑	3↓(2↑)
mean			< 22↓	< 22↓	< 22↓	< 22↓	< 22↓					3↓
N												
NH3		—	—	—	< 22↑	—	—	—	—	—	< 22↓	3↓↑
NO3 + NO2		—	—	—	< 22↑	—	—	—	—	—	< 22↑	4↑
P												
Total		—	—	—		—	—	—	—	—		1
SRP		—	< 22↑	—		—	< 22↑	—		—		2↑
DOC		—		—	< 22↓	—	< 22↓	—	< 22↓	—	< 22↓	4↓
Conductivity		—	< 22↑	—	< 22↑	—	< 22↑	—	< 22↑	—	< 22↑	4↑
BOD5		—	< 22↑	—	< 22↑	—	< 22↑	—		—	< 22↓	3↑ (2↓)

Table B3: Observed effect classes (based on No-observed-effect concentrations per sampling date [89], see table B19, B20 and B21) for community metabolism and chemistry endpoints of the different treatment regimes. Treatments resulted in significant increases (↑) or reductions (↓).. 1= no effect; 2= slight effect; 3=clear short-term effects; 4: clear effect in short-term study

	Cold				Warm			
	¨Low P*		High P		Low P		High P	
	75 µg/L	300 µg/L	75 µg/L	300 µg/L	75 µg/L	300 µg/L	75 µg/L	300 µg/L
DO								
morning	1	3↓	1	3↓	2↑	2↑	1	2↑
afternoon	3↓	3↓	2↓	3↓	2↓	2↓	2↓	2↓
max-min	3↓	3↓	2↓	3↓	1	1	2↓	2↓
mean	2↓	3↓	2↓	3↓	2↓	2↓	2↓	2↓
pH								
morning	2↓	3↓	1	3↓	1	1	2↓	3↓
afternoon	2↓	3↓	2↓	4↓	2↓	3↓	2↓	3↓
mean	2↓	3↓	1	4↓	1	3↓	2↓	3↓
N								
NH3	1	1	4↑	4↑	2↑	2↑	1	2↑
NO3 + NO2	1	1	1	1	1	1	1	1
P								
Total	1	2↓	1	1	1	2↓	1	1
SRP	1	1	1	1	1	2↓	1	2↓
DOC	4↓	4↓	1	4↓	1	2↓	3↓	4↓
Conductivity	1	3↑	1	1	1	1	2↓	2↓
BOD5	2↑↓	4↓(3↑[b])	3↓ (2↑[a])	4↓	2↓(2↑[a])	3↓	4↓	4↓

[a] Effects observed only after 7 days of treatment; [b]: significant after 7 and 14 days of exposure
* From Chapter 2

Table B4: No-observed-effect concentrations (NOECs) and observed effect classes per sampling date for the different plankton endpoints and species in the **cold and warm low P addition Zn control** microcosms. Only species that could be used to calculate a consistent NOEC are represented here. The numbers of the preselected effect classes [89] refer to: 1= no effect; 2= slight effect; 3=clear short-term effects; 4: clear effect in short-term study; ↓= decrease; ↑= increase; ↑↓= increase and decrease on species and/or sampling date. Blank fields indicate that NOEC were equal to or higher than the highest tested temperature.

		NOEC (µg/L)						Effect class
Endpoint	Taxa	-1	7	14	21	28	35	21-24°C
Zooplankton								
PRC			< 22	< 22	< 22	< 22	< 22	4
Number of taxa							< 22↓	2↓
Cladocerans		< 22↑	< 22↑					2↑
	Alonella nana			< 22↑	< 22↑		< 22↓	3↑ (2↓)
	Alona rectangula				< 22↑	< 22↑		3↑
Copepods				< 22↓	< 22↓			3↓
	Cyclopoida			< 22↓	< 22↓			3↓
Rotifers				< 22↑		< 22↓	< 22↓	4↓
	Colurella oblusa		< 22↑	< 22↑	< 22↓	< 22↓	< 22↓	3↑↓
	Lecane gr. Luna		< 22↑	< 22↑		< 22↓	< 22↓	3↑↓
	Lecane gr. Lunaris		< 22↑	< 22↑	< 22↓	< 22↓	< 22↓	3↑↓
	Lepadella patella				< 22↓	< 22↓		3↓
Phytoplankton								
PRC			< 22	< 22	< 22	< 22		3
Number of taxa			< 22↑	< 22↑				3↑
Total chlorophyll					< 22↓	< 22↓	< 22↓	4↓
Cyanobacteria			< 22↓	< 22↓			< 22↓	3↓
	Pseudanabaena sp.		< 22↓	< 22↓	< 22↓	< 22↓	< 22↓	4↓
	Aphanocapsa sp. 1		< 22↓	< 22↓				3↓
Bacillariophyta			< 22↑		< 22↑			2↑
Chlorophyta			< 22↓	< 22↑	< 22↑			3↑(↓[a])
	Desmodesmus sp.			< 22↑	< 22↑			3↑
	Haematococcus sp.		< 22↓		< 22↓	< 22↓		3↓
Cryptophyta					< 22↑			2↑
	Cryptophyte sp. 4		< 22↓	< 22↓	< 22↑	< 22↑		3↓↑
Chrysophyta					< 22↓	< 22↓	< 22↓	4↓
	Chrysococcus sp.				< 22↓	< 22↓	< 22↓	4↓
Dinophyta								1
Euglenophyta					< 22↑			2↑
Protozoa								
PRC			< 22					2
Number of taxa			< 22↓					2↓
Ciliates			< 22↓					2↓
Bacterivorous ciliates			< 22↓		< 22↓			2↓
Algivorous ciliates					< 22↑			2↑
Predaceous ciliates					< 22↓			2↓
Amoeba			< 22↓					2↓
Heterotrophic flagellates								

Table B5: Species abundances per litre of the microcosms per sampling date.

See excel sheet: Physico-chemical water parameters Van de Perre et al. 2017

Table B6: No-observed-effect concentrations (NOECs) and observed effect classes per sampling date for the different plankton endpoints and species in the cold low P and high P addition Zn control microcosms. Only species that could be used to calculate a consistent NOEC are represented here. The numbers of the preselected effect classes [89] refer to: 1= no effect; 2= slight effect; 3=clear short-term effects; 4: clear effect in short-term study; ↓= decrease; ↑= increase; ↑↓= increase and decrease on species and/or sampling date. Blank fields indicate that NOEC were equal to or higher than the highest tested P addition.

	NOEC (µg/L)						Effect class
Endpoint Taxa	-1	7	14	21	28	35	High P
Zooplankton							
PRC							1
Number of taxa							1
Cladocerans							1
Copepods						< 200↓	2↓
Rotifers				< 200↑	< 200↑		3↑
Trichotria pocillum		< 200↓	< 200↓				3↓
Lecane gr. Luna				< 200↑	< 200↑		3↑
Phytoplankton							
PRC		< 200			< 200	< 200	3
Number of taxa				< 200↑			2↑
Total chlorophyll							1
Cyanobacteria					< 200↓		2↓
Bacillariophyta			< 200↑				2↑
Chlorophyta	< 200↓					< 200↑	2↑
Oocystis sp.					< 200↑	< 200↑	3↑
Cryptophyta							1
Chrysophyta			< 200↑				2↑
Dinophyta							
Euglenophyta			< 200↑				2↑
Protozoa							
PRC				< 200			2
Number of taxa			< 200↑			< 200↓	2↑↓
Ciliates			< 200↑	< 200↓			2↑↓
Bacterivorous ciliates			< 200↑	< 200↑			3↑
Algivorous ciliates		< 200↓		< 200↓			2↓
Predaceous ciliates				< 200↓			2↓
Amoeba						< 200↓	2↓
Heterotrophic flagellates							

Table B7: Statistical significance (*p* values Three-way ANOVA) of the interactive effects of Zn and the different factors (P and T) of the different species of the different plankton groups. Only species that could be used to calculate a consistent interaction are represented here.

				L	H	L	H	L	H
Plankton group	**Day**	**Species**	**N**	**Zn × T**	**Zn × T**	**Zn × P**	**Zn × P**	**Zn × T × P**	**Zn × T × P**
Zooplankton									
	-1	*Cephalodella gibba*		0.458	0.127	0.090	0.730	0.991	0.180
	7	*Cephalodella gibba*		0.029	0.162	0.937	0.813	0.516	0.080
	14	*Cephalodella gibba*		0.032	0.003	0.511	0.238	0.771	0.880
	21	*Cephalodella gibba*		0.484	0.104	0.580	0.369	0.863	0.183
	28	*Cephalodella gibba*		0.642	0.367	0.623	0.124	0.947	0.272
	35	*Cephalodella gibba*		0.580	0.313	0.176	0.017	0.860	0.099
	-1	*Lecane* gr. *luna*		NA	0.350	NA	0.761	NA	0.798
	7	*Lecane* gr. *luna*		0.502	0.082	0.758	0.225	0.061	0.844
	14	*Lecane* gr. *luna*		0.322	0.042	0.027	0.140	0.191	0.102
	21	*Lecane* gr. *luna*		0.662	0.215	0.001	0.033	0.536	0.022
	28	*Lecane* gr. *luna*		0.508	0.151	0.067	0.364	0.941	0.056
	35	*Lecane* gr. *luna*		0.413	0.868	0.552	0.404	0.819	0.553
	-1	*Cyclopoida* sp.		0.053	0.864	0.556	0.514	0.776	0.053
	7	*Cyclopoida* sp.		0.548	0.445	0.534	0.667	0.022	0.817
	14	*Cyclopoida* sp.		0.005	0.282	0.087	0.158	0.003	0.395
	21	*Cyclopoida* sp.		0.052	0.315	0.050	0.283	0.023	0.305
	28	*Cyclopoida* sp.		0.358	0.092	0.708	0.312	0.667	0.215
	35	*Cyclopoida* sp.		0.515	0.004	0.389	0.502	0.031	0.033
	-1	Naupli		0.268	0.658	0.470	0.779	0.381	0.291
	7	Naupli		0.563	0.005	0.631	0.450	0.614	0.682
	14	Naupli		0.325	0.601	0.683	0.045	0.498	0.875
	21	Naupli		0.577	0.094	0.117	0.956	0.321	0.656
	28	Naupli		0.008	<0.001	0.759	0.643	0.323	0.921
	35	Naupli		0.038	0.005	0.623	0.516	0.358	0.578
	-1	*Simocephalus vetulus*		0.570	0.061	0.120	0.772	0.217	0.506
	7	*Simocephalus vetulus*		0.176	0.112	0.186	0.819	0.044	0.515
	14	*Simocephalus vetulus*		0.842	0.775	0.187	0.461	0.074	0.785
	21	*Simocephalus vetulus*		0.961	0.026	0.891	0.906	0.441	0.944
	28	*Simocephalus vetulus*		0.109	0.012	0.043	0.232	0.858	0.445
	35	*Simocephalus vetulus*		0.736	0.470	0.265	0.536	0.780	0.261
Phytoplankton									
	-1	Single cell diatoms		0.980	0.657	0.330	0.755	0.184	0.592
	7	Single cell diatoms		0.175	0.016	0.015	0.006	0.237	0.133
	14	Single cell diatoms		0.234	0.003	0.440	0.212	0.947	0.451
	21	Single cell diatoms		0.083	0.008	0.567	0.470	0.672	0.277
	28	Single cell diatoms		0.731	0.075	0.516	0.566	0.599	0.305
	35	Single cell diatoms		0.138	0.012	0.049	0.427	0.258	0.587
	-1	Cryptophyte sp. 1		0.378	0.052	0.625	0.039	0.350	0.774

				L	H	L	H	L	H
Plankton group	**Day**	**Species**	**N**	**Zn × T**	**Zn × T**	**Zn × P**	**Zn × P**	**Zn × T × P**	**Zn × T × P**
	7	Cryptophyte sp. 1		0.155	0.243	0.654	0.516	0.482	0.087
	14	Cryptophyte sp. 1		0.768	0.617	0.262	0.896	0.588	0.760
	21	Cryptophyte sp. 1		0.116	0.003	0.756	0.470	0.479	0.459
	28	Cryptophyte sp. 1		0.879	0.009	0.144	0.047	0.075	0.178
	35	Cryptophyte sp. 1		0.627	0.796	0.362	0.338	0.773	0.497
	-1	Cryptophyte sp. 2		0.684	NA	0.091	NA	0.631	NA
	7	Cryptophyte sp. 2		0.592	0.003	0.735	0.865	0.903	0.158
	14	Cryptophyte sp. 2		0.110	0.012	0.384	0.723	0.431	0.806
	21	Cryptophyte sp. 2		0.361	0.203	0.675	0.560	0.245	0.143
	28	Cryptophyte sp. 2		0.777	0.074	0.423	0.339	0.326	0.178
	35	Cryptophyte sp. 2		0.858	0.011	0.919	0.329	0.105	0.140
	-1	Cryptophyte sp. 4		NA	NA	NA	NA	NA	NA
	7	Cryptophyte sp. 4		0.079	0.003	0.209	0.048	0.769	0.049
	14	Cryptophyte sp. 4		0.011	0.012	0.040	0.117	0.289	0.689
	21	Cryptophyte sp. 4		0.152	0.647	0.516	0.832	0.901	0.509
	28	Cryptophyte sp. 4		0.352	0.180	0.221	0.781	0.311	0.615
	35	Cryptophyte sp. 4		0.451	0.543	0.276	0.768	0.595	0.183
	-1	Unknown small flagellate		0.109	0.580	0.183	0.619	0.181	0.723
	7	Unknown small flagellate		0.175	0.752	0.014	0.292	0.105	0.971
	14	Unknown small flagellate		0.214	NA	0.032	NA	0.779	NA
	21	Unknown small flagellate		0.041	0.066	0.619	0.821	0.508	0.694
	28	Unknown small flagellate		0.201	0.789	0.089	0.986	0.874	0.532
	35	Unknown small flagellate		0.197	0.688	0.236	0.055	0.095	0.164
	-1	*Haematococcus* sp.		NA	NA	NA	NA	NA	NA
	7	*Haematococcus* sp.		0.252	0.007	0.356	0.352	0.958	0.605
	14	*Haematococcus* sp.		0.414	0.099	0.059	0.289	0.569	0.910
	21	*Haematococcus* sp.		0.376	0.860	0.903	0.581	0.092	0.395
	28	*Haematococcus* sp.		0.125	0.045	0.810	0.364	0.688	0.220
	35	*Haematococcus* sp.		0.025	0.042	0.030	<0.001	0.768	0.273
	-1	*Scenedesmus acuminatus*		NA	NA	NA	NA	NA	NA
	7	*Scenedesmus acuminatus*		NA	NA	NA	NA	NA	NA
	14	*Scenedesmus acuminatus*		NA	NA	NA	NA	NA	NA
	21	*Scenedesmus acuminatus*		NA	0.027	NA	0.175	NA	0.027
	28	*Scenedesmus acuminatus*		NA	0.001	NA	0.094	NA	0.946
	35	*Scenedesmus acuminatus*		NA	0.076	NA	0.021	NA	0.198
	-1	*Anisonema acinus*		NA	0.513	NA	0.309	NA	0.015
	7	*Anisonema acinus*		0.346	0.017	0.188	0.389	0.256	0.811
	14	*Anisonema acinus*		NA	NA	NA	NA	NA	NA
	21	*Anisonema acinus*		0.560	0.996	0.121	0.593	0.313	0.534
	28	*Anisonema acinus*		0.980	0.947	0.085	0.103	0.091	0.008
	35	*Anisonema acinus*		NA	0.161	NA	0.160	NA	0.017
Protozoa									
	-1	*Cyclidium* sp.		0.947	0.364	0.355	0.199	<0.001	0.314
	7	*Cyclidium* sp.		0.757	0.016	0.290	0.441	0.742	0.998

				L	H	L	H	L	H
Plankton group	**Day**	**Species**	**N**	**Zn × T**	**Zn × T**	**Zn × P**	**Zn × P**	**Zn × T × P**	**Zn × T × P**
	14	*Cyclidium* sp.		0.963	0.138	0.756	0.301	0.900	0.538
	21	*Cyclidium* sp.		0.073	0.028	0.032	0.459	0.296	0.625
	28	*Cyclidium* sp.		0.283	0.161	0.032	0.477	0.588	0.282
	35	*Cyclidium* sp.		0.607	0.402	0.371	0.703	0.885	0.958
	-1	*Nassula* sp.		NA	NA	NA	NA	NA	NA
	7	*Nassula* sp.		0.085	0.480	0.441	0.002	0.477	0.044
	14	*Nassula* sp.		0.353	0.077	0.225	0.289	0.084	0.013
	21	*Nassula* sp.		0.360	NA	0.381	NA	0.037	NA
	28	*Nassula* sp.		0.603	0.171	0.688	0.146	0.600	0.117
	35	*Nassula* sp.		NA	NA	NA	NA	NA	NA
	-1	*Rimostrombidium brachykinetum*		0.666	0.336	0.331	0.206	0.231	0.086
	7	*Rimostrombidium brachykinetum*		0.341	0.180	0.064	<0.001	0.840	0.032
	14	*Rimostrombidium brachykinetum*		0.755	0.594	0.828	0.263	0.240	0.576
	21	*Rimostrombidium brachykinetum*		0.462	0.016	0.462	0.007	0.101	0.016
	28	*Rimostrombidium brachykinetum*		0.880	<0.001	0.025	0.454	0.429	0.454
	35	*Rimostrombidium brachykinetum*		0.272	0.035	0.612	0.163	0.199	0.684

Table B8: Statistical significance (*p* values Three-and two-way ANOVA) and calculation of the interactive effects (Synergism: S; or antagonism: A) of Zn (Low Zn: L; High Zn: H) and the different factors (low P addition: O; high P addition: E; cold: C and warm: W) of the different plankton species at different treatment regimes. The interaction type is based on the observed and predicted effects based on De Coninck et al. (2013) [122]. Only species that could be used to calculate a consistent interaction are represented here.

Taxa	Day	Treatment regime	Interaction factors	Zn treatment	*p*	Observed effect	Predicted effect	Interaction type
Zooplankton								
Cyclopoida sp.	7	All	Zn × T × P	L	0.022			
	14	All	Zn × T × P	L	0.003			
	21	All	Zn × T × P	L	0.023			
	7	E	Zn × T	L	0.060			
	14	E	Zn × T	L	0.006	0.536	-0.138	S
	21	E	Zn × T	L	0.001	0.687	0.129	S
	7	W	Zn × P	L	0.100			
	14	W	Zn × P	L	0.018	-0.085	-0.097	A
	21	W	Zn × P	L	0.030	-0.453	-0.278	S
Naupli sp.	28	All	Zn × T	L	0.008			
	35	All	Zn × T	L	0.038			
	28	All	Zn × T	H	0.001			
	35	All	Zn × T	H	0.005			
	28	E	Zn × T	L	0.145			
	35	E	Zn × T	L	0.015	0.149	-0.153	S
	28	E	Zn × T	H	0.001	0.140	0.063	S
	35	E	Zn × T	H	0.001	0.192	-0.168	S
Simocephalus vetulus	21	All	Zn × T	H	0.026			
	28	All	Zn × T	H	0.012			
	21	E	Zn × T	H	0.049	0.936	0.997	A
	28	E	Zn × T	H	0.022	0.900	1.000	A
Cephalodella gibba	7	All	Zn × T	L	0.029			
	14	All	Zn × T	L	0.032			
	7	O	Zn × T	L	0.015	0.413	-10.3	S
	14	O	Zn × T	L	0.037	-2.89	-24.8	A
Lecane gr. *luna*	14	All	Zn × P	L	0.027			
	21	All	Zn × P	L	0.001			
	14	C	Zn × P	L	0.042	0.051	-0.482	S
	21	C	Zn × P	L	0.001	-0.109	-0.637	A
Phytoplankton								
Single cell diatoms	7	All	Zn × T	H	0.016			
	14	All	Zn × T	H	0.003			
	21	All	Zn × T	H	0.008			
	7	E	Zn × T	H	0.048	-0.118	0.015	S

Taxa	Day	Treatment regime	Interaction factors	Zn treatment	*p*	Observed effect	Predicted effect	Interaction type
	14	E	Zn × T	H	0.049	-0.094	-0.178	A
	21	E	Zn × T	H	0.032	-0.028	-0.207	A
Cryptophyte sp. 1	21	All	Zn × T	H	0.003			
	28	All	Zn × T	H	0.009			
	21	E	Zn × T	H	0.005	-0.529	-1.26	A
	28	E	Zn × T	H	0.012	-0.279	-0.905	A
Cryptophyte sp. 2	7	All	Zn × T	H	0.003			
	14	All	Zn × T	H	0.012			
	7	O	Zn × T	H	0.247			
	14	O	Zn × T	H	0.035	-8.11	-148	A
	7	E	Zn × T	H	0.002	-0.427	0.981	S
	14	E	Zn × T	H	0.145			
Cryptophyte sp. 4	7	All	Zn × T	H	0.003			
	14	All	Zn × T	H	0.012			
	7	E	Zn × T	H	0.011	-0.099	0.229	S
	14	E	Zn × T	H	0.054	0.637	0.962	A
Unknown small flagellate	7	All	Zn × P	L	0.014			
	14	All	Zn × P	L	0.037			
	7	C	Zn × P	L	0.006	0.000	-33.3	S
	14	C	Zn × P	L	0.088			
	7	W	Zn × P	L	0.531			
	14	W	Zn × P	L	0.195			
Haematococcus sp.	28	All	Zn × T	H	0.045			
	35	All	Zn × T	H	0.042			
	28	O	Zn × T	H	0.662			
	35	O	Zn × T	H	0.028	0.973	-0.193	S
	28	E	Zn × T	H	0.001	-0.449	0.936	S
	35	E	Zn × T	H	0.509			
Scenedesmus acuminatus	21	All	Zn × T	H	0.027			
	28	All	Zn × T	H	0.001			
	21	O	Zn × T	H	NA			
	28	O	Zn × T	H	0.001	-24.4	0.000	S
	21	E	Zn × T	H	0.038	-3.570	0.784	S
	28	E	Zn × T	H	0.099			
Anisonema acinus	28	All	Zn × T × P	H	0.008			
	35	All	Zn × T × P	H	0.017			
	28	W	Zn × P	H	0.007	0.235	0.867	A
	35	W	Zn × P	H	0.182			
	28	O	Zn × T	H	0.084			
	35	O	Zn × T	H	0.020	0.000	-37.7	S
	28	E	Zn × T	H	0.041	-3.17	0.791	S
	35	E	Zn × T	H	0.418			

Table S9: Statistical significance (*p* values Three-way ANOVA) of the interactive effects of Zn and the different factors (P and T) of the different functional groups of the different plankton groups. Only functional groups that could be used to calculate a consistent interaction are represented here.

			L	H	L	H	L	H
Plankton group	**Day**	**Group**	**Zn × T**	**Zn × T**	**Zn × P**	**Zn × P**	**Zn × T × P**	**Zn × T × P**
Zooplankton								
	-1	Copepoda	0.215	0.698	0.509	0.806	0.381	0.239
	7	Copepoda	0.842	0.006	0.758	0.522	0.053	0.862
	14	Copepoda	0.083	0.863	0.800	0.023	0.073	0.853
	21	Copepoda	0.511	0.066	0.092	0.827	0.223	0.592
	28	Copepoda	0.017	<0.001	0.771	0.260	0.377	0.474
	35	Copepoda	0.043	<0.001	0.788	0.313	0.176	0.226
	-1	Cladocera	0.638	0.359	0.548	0.557	0.295	0.678
	7	Cladocera	0.947	<0.001	0.492	0.123	0.101	0.518
	14	Cladocera	0.808	0.633	0.956	0.219	0.436	0.915
	21	Cladocera	0.542	0.043	0.439	0.434	0.893	0.479
	28	Cladocera	0.371	0.007	0.624	0.992	0.490	0.539
	35	Cladocera	0.970	0.007	0.105	0.369	0.882	0.420
Phytoplankton								
	-1	Bacillariophyceae	0.980	0.657	0.330	0.755	0.184	0.592
	7	Bacillariophyceae	0.173	0.016	0.015	0.006	0.246	0.127
	14	Bacillariophyceae	0.231	0.003	0.390	0.197	0.948	0.404
	21	Bacillariophyceae	0.073	0.005	0.597	0.492	0.643	0.220
	28	Bacillariophyceae	0.876	0.078	0.498	0.568	0.628	0.311
	35	Bacillariophyceae	0.114	0.012	0.036	0.381	0.289	0.593
	-1	Cryptophyta	0.531	0.934	0.538	0.638	0.051	0.470
	7	Cryptophyta	0.098	<0.001	0.039	0.009	0.658	0.021
	14	Cryptophyta	0.867	0.005	0.441	0.043	0.973	0.341
	21	Cryptophyta	0.089	<0.001	0.752	0.849	0.663	0.561
	28	Cryptophyta	0.902	0.004	0.154	0.043	0.112	0.190
	35	Cryptophyta	0.398	0.996	0.142	0.182	0.665	0.667
	-1	Chlorophyta	0.475	0.300	0.132	0.583	0.057	0.654
	7	Chlorophyta	0.540	<0.001	0.108	0.039	0.002	0.277
	14	Chlorophyta	0.336	0.540	0.270	<0.001	0.711	0.024
	21	Chlorophyta	0.677	0.009	0.649	0.853	0.427	0.841
	28	Chlorophyta	0.254	0.012	0.176	0.411	0.138	0.437
	35	Chlorophyta	0.619	0.263	<0.001	<0.001	0.138	0.490
	-1	Totale chlorofyl	0.557	0.907	0.798	0.290	0.102	0.818
	7	Totale chlorofyl	0.402	0.482	0.287	0.059	0.334	0.911
	14	Totale chlorofyl	0.636	0.655	0.184	0.927	0.193	0.495
	21	Totale chlorofyl	0.259	0.054	0.604	0.714	0.018	0.529
	28	Totale chlorofyl	0.926	0.053	0.928	0.864	0.003	0.027

			L	H	L	H	L	H
Plankton group	**Day**	**Group**	**Zn × T**	**Zn × T**	**Zn × P**	**Zn × P**	**Zn × T × P**	**Zn × T × P**
	35	Totale chlorofyl	0.842	0.513	0.767	0.309	0.026	0.015
Protozoa								
	-1	Ciliate	0.223	0.492	0.664	0.822	0.299	0.374
	7	Ciliate	0.413	0.005	0.077	0.037	0.633	0.946
	14	Ciliate	0.984	0.674	0.806	0.879	0.300	0.202
	21	Ciliate	0.284	<0.001	0.485	0.061	0.611	0.268
	28	Ciliate	0.751	0.031	0.019	0.315	0.740	0.851
	35	Ciliate	0.286	0.029	0.098	0.901	0.278	0.619
	-1	Bacterivorous ciliate	0.109	0.891	0.422	0.704	0.486	0.496
	7	Bacterivorous ciliate	0.756	0.028	0.035	0.450	0.930	0.373
	14	Bacterivorous ciliate	0.293	0.924	0.332	0.571	0.517	0.587
	21	Bacterivorous ciliate	0.458	0.019	0.212	0.202	0.389	0.607
	28	Bacterivorous ciliate	0.699	0.034	0.059	0.936	0.949	0.393
	35	Bacterivorous ciliate	0.278	0.216	0.833	0.527	0.850	0.816
	-1	Algivorous/bacterivorous ciliate	NA	NA	NA	NA	NA	NA
	7	Algivorous/bacterivorous ciliate	0.085	0.480	0.441	0.002	0.477	0.044
	14	Algivorous/bacterivorous ciliate	0.353	0.077	0.225	0.289	0.084	0.013
	21	Algivorous/bacterivorous ciliate	0.360	NA	0.381	NA	0.037	NA
	28	Algivorous/bacterivorous ciliate	0.603	0.171	0.688	0.146	0.600	0.117
	35	Algivorous/bacterivorous ciliate	NA	NA	NA	NA	NA	NA

Table B10: Statistical significance (*p* values Three-and two-way ANOVA) and calculation of the interactive effects (Synergism: S; or antagonism: A) of Zn (Low Zn: L; High Zn: H) and the different factors (Low P addition: O; High P addition: E; cold: C and warm: W) of the different plankton groups at different treatment regimes and the total chlorophyll. The interaction type is based on the observed and predicted effects based on De Coninck et al (2013) [122]. Only groups that could be used to calculate a consistent interaction are represented here

Taxa group	Day	Treatment regime	Interaction factors	Zn treatment	*p*	Observed effect	Predicted effect	Interaction type
Zooplankton								
Copepoda	28	All	Zn × T	L	0.017			
	35	All	Zn × T	L	0.043			
	28	All	Zn × T	H	0.001			
	35	All	Zn × T	H	0.001			
	28	E	Zn × T	L	0.234			
	35	E	Zn × T	L	0.008	0.102	-0.226	S
	28	E	Zn × T	H	0.001	0.147	-0.277	S
	35	E	Zn × T	H	0.001	0.185	-0.297	S
Cladocera	21	All	Zn × T	H	0.043			
	28	All	Zn × T	H	0.007			
	35	All	Zn × T	H	0.007			
	21	E	Zn × T	H	0.099			
	28	E	Zn × T	H	0.003	0.477	0.993	A
	35	E	Zn × T	H	0.006	0.640	1.000	A
Phytoplankton								
Bacillariophyceae	7	All	Zn × T	H	0.016			
	14	All	Zn × T	H	0.003			
	21	All	Zn × T	H	0.005			
	7	E	Zn × T	H	0.047	-0.116	0.014	S
	14	E	Zn × T	H	0.048	-0.097	-0.178	A
	21	E	Zn × T	H	0.020	-0.026	-0.211	A
Cryphtophyta	7	All	Zn × P	H	0.009			
	14	All	Zn × P	H	0.043			
	7	All	Zn × T	H	0.001			
	7	W	Zn × P	H	0.001	-0.365	-0.012	S
	14	W	Zn × P	H	0.040	-0.639	-0.297	S
	14	All	Zn × T	H	0.005			
	21	All	Zn × T	H	0.001			
	28	All	Zn × T	H	0.004			
	7	O	Zn × T	H	0.086			
	14	O	Zn × T	H	0.009	-0.231	-0.681	A
	21	O	Zn × T	H	0.032	-0.443	-0.904	A
	28	O	Zn × T	H	0.220			
	7	E	Zn × T	H	0.002	-0.116	0.015	S

Taxa group	Day	Treatment regime	Interaction factors	Zn treatment	*p*	Observed effect	Predicted effect	Interaction type
	14	E	Zn × T	H	0.194			
	21	E	Zn × T	H	0.001	-0.428	-1.058	A
	28	E	Zn × T	H	0.007	-0.249	-0.820	A
Chlorophyta	7	All	Zn × P	H	0.039			
	14	All	Zn × P	H	0.001			
	7	W	Zn × P	H	0.029	-0.263	-0.085	S
	14	W	Zn × P	H	0.007	-0.228	0.036	S
	21	All	Zn × T	H	0.009			
	28	All	Zn × T	H	0.012			
	21	E	Zn × T	H	0.074			
	28	E	Zn × T	H	0.014	-0.069	-0.308	A
Total chlorophyll	21	All	Zn × T × P	L	0.018			
	28	All	Zn × T × P	L	0.003			
	35	All	Zn × T × P	L	0.014			
	21	O	Zn × T	L	0.030	0.078	0.360	A
	28	O	Zn × T	L	0.034	0.350	0.520	A
	35	O	Zn × T	L	0.123			
	21	E	Zn × T	L	0.316			
	28	E	Zn × T	L	0.042	0.435	0.205	S
	35	E	Zn × T	L	0.117			
	28	All	Zn × T × P	H	0.027			
	35	All	Zn × T × P	H	0.015			
	28	O	Zn × T	H	0.013	0.406	0.611	A
	35	O	Zn × T	H	0.013	0.515	0.649	A
	28	W	Zn × P	H	0.062			
	35	W	Zn × P	H	0.010	0.117	-0.262	S
Protozoa								
Ciliate	21	All	Zn × T	H	0.001			
	28	All	Zn × T	H	0.031			
	35	All	Zn × T	H	0.029			
	21	E	Zn × T	H	0.010	-15.3	-392	A
	28	E	Zn × T	H	0.019	-0.397	-1.32	A
	35	E	Zn × T	H	0.089			
Bacterivorous ciliate	21	All	Zn × T	H	0.019			
	28	All	Zn × T	H	0.034			
	21	O	Zn × T	H	0.170			
	28	O	Zn × T	H	0.065			
	21	E	Zn × T	H	0.061			
	28	E	Zn × T	H	0.308			

Table B11: Statistical significance (*p* values ANOVA based on the PCA sample scores) of the main effects of Zn and the different factors (P and T) at the community level of the different plankton groups. Significant ($p < 0.05$) are marked.

		L	H	L	H	L	H
Day	**PCA score**	**Zn**	**Zn**	**T**	**T**	**P**	**P**
-1	Zooplankton	0.292	0.857	0.097	0.625	0.822	0.722
7	Zooplankton	0.159	0.265	<0.001	<0.001	0.655	0.904
14	Zooplankton	0.049	<0.001	<0.001	<0.001	0.453	0.243
21	Zooplankton	0.188	<0.001	0.003	<0.001	0.158	0.349
28	Zooplankton	0.378	0.028	<0.001	<0.001	0.086	0.066
35	Zooplankton	0.046	0.001	<0.001	<0.001	0.021	0.552
-1	Phytoplankton	0.561	0.799	0.115	0.324	0.760	0.655
7	Phytoplankton	0.963	0.580	<0.001	0.563	0.045	0.003
14	Phytoplankton	0.005	<0.001	0.429	0.102	0.174	0.106
21	Phytoplankton	0.092	0.934	<0.001	<0.001	0.597	0.525
28	Phytoplankton	0.052	<0.001	0.157	0.669	0.387	0.975
35	Phytoplankton	0.173	<0.001	0.348	0.160	0.914	0.270
-1	Protozoa	0.669	0.577	0.124	0.922	0.976	0.859
7	Protozoa	0.536	0.141	0.041	0.921	0.033	0.010
14	Protozoa	0.635	<0.001	0.376	0.292	0.014	0.062
21	Protozoa	0.017	<0.001	0.016	0.645	0.100	0.734
28	Protozoa	0.078	<0.001	0.081	0.291	0.387	0.010
35	Protozoa	0.081	<0.001	0.643	0.276	0.976	0.249

Table B12: Observed effect classes (based on No-observed-effect concentrations per sampling date [89], see table B14, B15 and B16) for the different plankton endpoints and species of the different treatment regimes. Only species that could be used to calculate a consistent NOEC are represented here. The numbers of the preselected effect classes [89] refer to: 1= no effect; 2= slight effect; 3=clear short-term effects; 4: clear effect in short-term study; ↓= decrease; ↑= increase; ↑↓= increase and decrease on species and/or sampling date

	Cold				Warm			
	Low P*		High P		Low P		High P	
Endpoint Taxa	75 µg/L	300 µg/L	75 µg/L	300 µg/L	75 µg/L	300 µg/L	75 µg/L	300 µg/L
Zooplankton								
PRC	2	4	2	4	2	3	1	3
Number of taxa	1	4↓	1	4↓	2↓	2↓	1	2↓
Cladocerans	2↓(↑[a])	4↓	1	4↓	1	3↓	1	2↓
Daphnia longispina	2↑a	4↓	1	4↓	3↓	3↓	4↓	4↓
Chydorus sphaericus	2↓	4↓	1	4↓	1	3↓	1	2↓
Simocephalus vetulus	3↓	4↓	1	4↓	1	2↓	1	1
Alonella nana	1	1	3↓	3↓	1	2↓	1	1
Copepods	1	3↑	2↑	3↑	1	3↑	2↓	4↓ (3↑[b])
Nauplii	1	3↑	2↑	3↑	1	3↓(3↑[b])	4↓	4↓ (3↑[b])
Rotifers	3↑	3↑	1	2↑	2↑	2↑	1	1
Lecane gr. *lunaris*	2↑	3↑	1	2↑	1	1	1	1
Lecane gr. *luna*	3↑	3↑	1	2↓	2↓	2↓	1	1
Cephalodella gibba	2↑	3↑	1	2↑	1	2↑	1	1
Lepadella patella	2↑	2↓↑	1	1	1	1	2↓	3↑(2↓)
Mytilina ventralis	1	1	1	1	1	1	1	3↑
Phytoplankton								
PRC	2	4	2	3	4	4	2	4
Number of taxa	1	2↓	2	3↓	1	2↓	1	2↑
Total chlorophyll	1	4↓	1	3↓	2↑b	3↓	1	2↓
Cyanobacteria	1	1	1	2↓↑	2↓	2↑	2↓	2↓↑
Anabaena sp.	2↓	3↓	1	3↓	2↓	2↓	3↓	3↓
Aphanocapsa sp. 1	2↑	3↑	2↑	3↑	1	3↑	1	2↑
Aphanothece sp.	1	3(↓[a])↑	1	1	1	1	1	2(↓[a])↑
Chroococcus sp.	2↓	3↓	2↓	3↓	1	2↓	2↑	2↑
Bacillariophyta	1	2↑	3↑	3↑	1	4↓	2↓	2↓↑
Single cell diatoms	1	2↑	3↑	3↑	1	2↓	2↓	2↑(↓[a])
Chlorophyta	1	4↑	2↑	3↑	1	2↓	4↓	4↓ (3↑[b])
Mougeotia sp.	2↓	2↓	3↓	3↓	3↓	3↓	4↓	4↓
Radiococcus sp.	1	2↑	2↑	3↑	1	1	1	1
Scenedesmus sp. 2	1	4↑	1	2↑	1	1	1	2↑
Scenedesmus acuminatus	1	2↑	1	1	1	4↑	1	2↑

Haematococcus sp.	2(↓[a])↑	4↑	2↑	4↑	2↑	3↑	1	4↑
Desmodesmus sp.	2↑	4↑	1	4↑	1	2↑	1	3↑
Monoraphidium sp. 1	2↓a	2↑(↓a)	1	4↑	1	2↓	1	3↑
Monoraphidium sp. 2	2↑	3↑	1	4↑	1	3↑	1	3↑
Chlamydomonas sp.	1	1	1	2↑	2↑	3↑	1	2↑
Tetraëdron minimun	1	2↓	1	3↓	1	2↓	2↓	2↓
Oocystis sp.	1	2↑(2↓)	1	2↓	1	2↑(2↓)	2↓(2↑)	3↓(2↑)
Cryptophyta	1	4↑	2↑	3↑	4↑	4↑	2↑(2↓)	3↑(2↓)
Rodomonas sp.	2↑	4↑	2↑	(2↓) 3↑	1	2↑	1	3↑
Cryptophyta sp. 1	2↑	4↑	2↑	3↑	4↑	4↑	2↑(2↓)	3↑(2↓)
Cryptophyta sp. 2	2↑	4↑	2↑	2↑	1	1	1	2↑
Chrysophyta	1	4↑(↓[a])	1	3↑	1	1	1	2↑
Uroglena sp.	2↓	3↓	2↓	4↓	3↓	3↓	2↓	2↓
Chrysococcus sp.	1	4(↓[a])↑	1	3↑	1	1	1	2↑
Dinophyta	1	1	1	2↓	1	1	2↓	2↓
Euglenophyta	2↑	3↑	2↑a	3↓	1	1	1	2↑
Euglenida sp.	1	2↑	1	3↓	1	1	1	2↑
Protozoa								
PRC	1	3	1	4	1	2	1	3
Number of taxa	1	1	2↑a	2↓	1	1	1	2↑
Ciliates	1	4↑(↓[a])	1	3↑	1	2↑	1	3↑
Bacterivorous ciliates	2↑	4↑	1	4↑	1	2↑	1	3↑
R. brachykinetum	2↑	4↑	2↑a	4↑	1	2↑	1	2↑
Cyclidium sp.	2↑	4↑	1	1	1	1	1	2↑
Algivorous ciliates	1	1	1	2↑	1	1	1	2↑
Predaceous ciliates	1	1	2↑	2↑	1	2↑	1	1
Amoeba	1	1	1	2↑	2↓(↑a)	2↓(↑a)	1	3↑
Difflugia sp.	1	2↓	1	2↓	2↓	2↓	3↓	3↓
Amoeba sp.	1	1	1	2↑	1	1	1	3↑
Heterotrophic flagellates	1	3↑	2↑	2↑	1	2↑	2↑	2↑
Codosiga botrytis	1	3↑	2↑	2↑	1	2↑	2↑	2↑

[a] Effects observed only after 7 days of treatment. [b] Effects observed only after 7 and 14 days of treatment. * From Chapter 2

Table B13: No-observed-effect concentrations (NOECs) (Williams test, $p < 0.05$) and observed effect classes per sampling date for the different plankton endpoints and species in the **warm low P addition microcosms**. Only species that could be used to calculate a consistent NOEC are represented here. The numbers of the preselected effect classes [89] refer to: 1= no effect; 2= slight effect; 3=clear short-term effects; 4: clear effect in short-term study; ↓= decrease; ↑= increase; ↑↓= increase and decrease on species and/or sampling date. Blank fields indicate that NOEC were equal to or higher than the highest tested concentration (300 µg/L)

	NOEC (µg/L)						Effect class	
Endpoint Taxa	-1	7	14	21	28	35	75 µg/L	300 µg/L
Zooplankton								
PRC		< 75	75			< 75	2	3
Number of taxa			< 75↓			75↓	2↓	2↓
Cladocerans		75↓	75↓			75↓	1	3↓
Daphnia longispina		75↓	75↓	< 75↓	< 75↓		3↓	3↓
Chydorus sphaericus		75↓	75↓			75↓	1	3↓
Copepods		75↑	75↑				1	3↑
Nauplii		75↑	75↑		75↓	75↓	1	3↓↑
Rotifers						< 75↑	2↑	2↑
Phytoplankton								
PRC			< 75		< 75	< 75	4	4
Number of taxa		75↓					1	2↓
Total chlorophyll		75↓	75↓		< 75↑		2↑	3↓
Cyanobacteria			75↑		< 75↓		2↓	2↑
Aphanocapsa sp. 1		75↑	75↑				1	3↑
Bacillariophyta					75↓	75↓	1	4↓
Chlorophyta	< 75↓		75↓				1	2↓
Mougeotia sp.			< 75↓	< 75↓	< 75↓		3↓	3↓
Scenedesmus acuminatus					75↑	75↑	1	4↑
Haematococcus sp.		75↑	< 75↑		75↑		2↑	3↑
Chlamydomonas sp.			75↑	< 75↑			2↑	3↑
Monoraphidium sp. 2		75↑	75↑		75↑		1	3↑
Cryptophyta			75↑		< 75↑	< 75↑	4↑	4↑
Cryptophyta sp. 1			75↑	< 75↑	< 75↑	< 75↑	4↑	4↑
Chrysophyta							1	1
Uroglena sp.				< 75↓	< 75↓		3↓	3↓
Dinophyta	75↓						1	1
Euglenophyta							1	1
Protozoa								
PRC			75				1	2
Number of taxa							1	1
Ciliates			75↑				1	2↑
Bacterivorous ciliates			75↑				1	2↑
Algivorous ciliates							1	1
Predaceous ciliates				75↑			1	2↑
Amoeba		< 75↑		< 75↓			2↓(↑[a])	2↓(↑[a])
Heterotrophic flagellates				75↑			1	2↑

[a] Effects observed only after 7 days of treatment.

Table B14: No-observed-effect concentrations (NOECs) (Williams test, $p < 0.05$) and observed effect classes per sampling date for the different plankton endpoints and species in the **cold high P microcosms**. Only species that could be used to calculate a consistent NOEC are represented here. The numbers of the preselected effect classes [89] refer to: 1= no effect; 2= slight effect; 3= clear short-term effects; 4: clear effect in short-term study; ↓= decrease; ↑= increase; ↑↓= increase and decrease on species and/or sampling date. Blank fields indicate that NOEC were equal to or higher than the highest tested concentration (300 µg/L)

		NOEC (µg/L)						Effect class	
Endpoint	Taxa	-1	7	14	21	28	35	75 µg/L	300 µg/L
Zooplankton									
PRC				< 75	75	75	75	2	4
Number of taxa						75↓	75↓	1	4↓
Cladocerans				75↓	75↓	75↓	75↓	1	4↓
	Daphnia longispina			75↓	75↓	75↓	75↓	1	4↓
	Chydorus sphaericus				75↓	75↓	75↓	1	4↓
	Simocephalus vetulus				75↓	75↓	75↓	1	4↓
	Alonella nana				< 75↓	< 75↓		3↓	3↓
Copepods				< 75↑	75↑	75↑		2↑	3↑
	Nauplii			< 75↑	75↑	75↑		2↑	3↑
Rotifers		75↓		75↑				1	2↑
Phytoplankton									
PRC				< 75	75		< 75	2	3
Number of taxa		75↓		75↓	< 75↓	75↓		2	3↓
Total chlorophyll				75↓	75↓	75↓		1	3↓
Cyanobacteria					75↑		75↓	1	2↓↑
	Anabaena sp.		75↓	75↓				1	3↓
	Aphanocapsa sp. 1			75↑	< 75↑			2↑	3↑
	Chroococcus sp.			75↓	75↓	< 75↓		2↓	3↓
Bacillariophyta		75↓	< 75↑	< 75↑	75↑			3↑	3↑
	Single cell diatoms	75↓	< 75↑	< 75↑	75↑			3↑	3↑
Chlorophyta				< 75↑	75↑	75↑		2↑	3↑
	Mougeotia sp.				< 75↓	< 75↓		3↓	3↓
	Radiococcus sp.			< 75↑	75↑	75↑		2↑	3↑
	Haematococcus sp.				< 75↑	75↑	< 75↑	2↑	4↑
	Desmodesmus sp.	75↓				75↑	75↑	1	4↑
	Monoraphidium sp. 1					75↑	75↑	1	4↑
	Monoraphidium sp. 2			75↑		75↑	75↑	1	4↑
	Tetraëdron minimun				75↓	75↓		1	3↓
Cryptophyta				75↑	< 75↑	75↑		2↑	3↑
	Cryptophyta sp. 1			75↑	< 75↑	75↑		2↑	3↑
	Rodomonas sp.			75↓		75↑	< 75↑	2↑	(2↓) 3↑
Chrysophyta				75↑	75↑	75↑		1	3↑
	Uroglena sp.				75↓	< 75↓	75↓	2↓	4↓
	Chrysococcus sp.			75↑	75↑	75↑		1	3↑
Dinophyta							75↓	1	2↓
Euglenophyta				75↓	75↓		< 75↑	2↑[a]	3↓
	Euglenida sp.				75↑	75↑		1	3↓
Protozoa									
PRC		75		75	75	75	75	1	4
Number of taxa				75↓			< 75↑	2↑[a]	2↓
Ciliates				75↑	75↑		75↑	1	3↑
Bacterivorous ciliates				75↑	75↑	75↑	75↑	1	4↑
	R. brachykinetum			75↑	75↑	75↑	< 75↑	2↑[a]	4↑
Algivorous ciliates		75↓				75↑		1	2↑
Predaceous ciliates					< 75↑			2↑	2↑
Amoeba					75↑			1	2↑
Heterotrophic flagellates			< 75↑		75↑			2↑	2↑

Table B15: No-observed-effect concentrations (NOECs) (Williams test, $p < 0.05$) and observed effect classes per sampling date for the different plankton endpoints and species in the **warm high P microcosms**. Only species that could be used to calculate a consistent NOEC are represented here. The numbers of the preselected effect classes [89] refer to: 1= no effect; 2= slight effect; 3=clear short-term effects; 4: clear effect in short-term study; ↓= decrease; ↑= increase; ↑↓= increase and decrease on species and/or sampling date. Blank fields indicate that NOEC were equal to or higher than the highest tested concentration (300 µg/L)

		NOEC (µg/L)						Effect class	
Endpoint	Taxa	-1	7	14	21	28	35	75 µg/L	300 µg/L
Zooplankton									
PRC			75	75				1	3
Number of taxa				75↓			75↓	1	2↓
Cladocerans			75↓					1	2↓
	Daphnia longispina		75↓	< 75↓	75↓	< 75↓	< 75↓	4↓	4↓
Copepods			75↑	75↑		75↓	< 75↓	2↓	4↓ (3↑[b])
	Nauplii		75↑	75↑		< 75↓	< 75↓	4↓	4↓ (3↑[b])
Rotifers								1	1
	Lepadella patella		75↑	75↑		< 75↓		2↓	3↑(2↓)
	Mytilina ventralis			75↑	75↑			1	3↑
Phytoplankton									
PRC			75	75	< 75	75	< 75	2	4
Number of taxa						75↑		1	2↑
Total chlorophyll							75↓	1	2↓
Cyanobacteria			75↑				< 75↓	2↓	2↓↑
	Anabaena sp.			< 75↓	< 75↓			3↓	3↓
Bacillariophyta			75↑				< 75↓	2↓	2↓↑
Chlorophyta			75↑	75↑		< 75↓	< 75↓	4↓	4↓ (3↑[b])
	Mougeotia sp.			75↓	< 75↓	< 75↓	< 75↓	4↓	4↓
	Desmodesmus sp.	75↑	75↑	75↑				1	3↑
	Oocystis sp.		75↓	75↓	< 75↓	< 75↑	< 75↓	2↓(2↑)	3↓(2↑)
	Haematococcus sp.		75↑	75↑		< 75↑	75↑	1	4↑
	Monoraphidium sp. 1			75↑	75↑			1	3↑
	Monoraphidium sp. 2		75↑	75↑	75↑			1	3↑
Cryptophyta			75↑	75↑	< 75↑	< 75↓	< 75↑	2↑(2↓)	3↑(2↓)
	Cryptophyte sp. 1		75↑	75↑	< 75↑	< 75↓	< 75↑	2↑(2↓)	3↑(2↓)
	Rodomonas sp.				75↑	75↑		1	3↑
Chrysophyta		< 75↓	75↑		75↑			1	2↑
Dinophyta							< 75↓	2↓	2↓
Euglenophyta			75↑					1	2↑
Protozoa									
PRC			75	75			75	1	3
Number of taxa						75↑		1	2↑
Ciliates			75↑	75↑				1	3↑
Bacterivorous ciliates			75↑	75↑				1	3↑
Algivorous ciliates				75↑				1	2↑
Predaceous ciliates		75↑						1	1
Amoeba						75↑	75↑	1	3↑
	Difflugia sp.			< 75↓	< 75↓			3↓	3↓
	Amoeba sp.					75↑	75↑	1	3↑
Heterotrophic flagellates					< 75↑		< 75↑	2↑	2↑

[b] Effects observed only after 7 and 14 days of treatment.

Table B16: Statistical significance (*p* values Three-way ANOVA) of the interactive effects of Zn (Low: L and high:H) and the different factors (P and T) of the total chlorophyll, pH_{mean}, Dissolved organic carbon (DOC), dissolved oxygen (DO), net primary production (DO_{net}: $DO_{evening\ day\ x}$ - $DO_{morning\ day\ x}$) and Biological oxygen demand after 5 days (BOD_5).

		L	H	L	H	L	H
Day	**Parameter**	**Zn × T**	**Zn × T**	**Zn × P**	**Zn × P**	**Zn × T × P**	**Zn × T × P**
-1	Total chlorophyll	0.557	0.076	0.798	**0.021**	0.102	0.198
7	Total chlorophyll	0.402	0.907	0.287	0.290	0.334	0.818
14	Total chlorophyll	0.636	0.482	0.184	0.059	0.193	0.911
21	Total chlorophyll	0.259	0.655	0.604	0.927	**0.018**	0.495
28	Total chlorophyll	0.926	0.054	0.928	0.714	**0.003**	0.529
35	Total chlorophyll	0.842	0.053	0.767	0.864	**0.026**	**0.027**
-1	BOD_5	0.877	0.457	0.212	0.701	**0.006**	0.355
7	BOD_5	0.260	**<0.001**	0.773	**<0.001**	0.910	**<0.001**
14	BOD_5	0.992	**<0.001**	0.762	0.055	0.146	0.443
21	BOD_5	0.810	0.130	**0.006**	**0.002**	0.174	0.051
28	BOD_5	0.360	0.994	0.992	0.969	0.426	0.768
35	BOD_5	0.428	0.789	0.775	0.285	**0.001**	0.862
-1	DOC	0.942	0.224	0.739	0.102	0.765	0.277
7	DOC	**0.047**	**<0.001**	0.545	0.058	**0.034**	**<0.001**
14	DOC	0.146	0.717	0.786	0.259	0.108	0.570
21	DOC	0.148	**0.007**	0.403	0.475	**0.020**	0.061
28	DOC	0.198	**0.006**	0.505	0.850	**0.012**	**0.025**
35	DOC	0.052	**<0.001**	0.076	0.131	**0.002**	0.104
-1	pH_{mean}	0.921	0.727	0.431	0.659	0.054	0.336
7	pH_{mean}	0.568	0.855	0.131	0.174	0.261	0.119
14	pH_{mean}	0.234	**<0.001**	0.741	0.796	0.730	0.443
21	pH_{mean}	0.322	**0.002**	**0.046**	0.651	0.467	0.726
28	pH_{mean}	0.850	0.842	0.371	0.437	0.760	0.364
35	pH_{mean}	0.138	0.058	0.641	0.257	0.620	0.923
-1	DO_{mean}	0.269	0.602	0.210	0.875	0.608	0.620
7	DO_{mean}	0.114	0.919	0.120	0.430	0.063	**0.033**
14	DO_{mean}	0.218	**<0.001**	0.384	0.651	0.640	0.237
21	DO_{mean}	0.770	**0.024**	**0.025**	0.889	0.462	0.553
28	DO_{mean}	0.590	0.116	0.052	0.786	0.751	0.443
35	DO_{mean}	0.250	0.768	0.524	0.547	0.660	0.787
-1	DO_{net}	0.489	0.158	0.860	0.685	0.552	0.165
7	DO_{net}	0.108	0.074	0.955	0.276	0.125	0.098
14	DO_{net}	0.053	**0.004**	0.913	0.702	0.650	0.855
21	DO_{net}	0.112	**<0.001**	**0.030**	0.190	**0.003**	**0.008**
28	DO_{net}	0.940	0.324	0.618	0.503	0.433	0.237
35	DO_{net}	0.464	**0.045**	0.782	0.847	0.396	0.800

Table B17: No-observed-effect concentrations (NOECs) and observed effect classes [89] per sampling date for community metabolism and chemistry endpoints cold low P addition and high P addition Zn control microcosms. The numbers of the preselected effect classes [89] refer to: 1= no effect; 2= slight effect; 3=clear short-term effects; 4: clear effect in short-term study; ↓= decrease; ↑= increase; ↑↓= increase and decrease on species and/or sampling date. Blank fields indicate that NOEC were equal to or higher than the highest tested P addition.

	NOEC (µg/L)											Treatment levels
	-1	1	7	9	14	16	21	23	28	30	35	High P addition
DO												
morning	—	<200↓	<200↓									3↓
afternoon			<200↓	<200↑			<200↓		<200↓			2↓(2↑)
max-min	—			<200↑		<200↓	<200↓		<200↓			3↓(2↑)
mean				<200↓					<200↓			2↓
pH												
morning		<200↓				<200↓	<200↓					3↓
afternoon			<200↓				<200↓	<200↓	<200↓			3↓
mean				<200↓				<200↓	<200↓			3↓
N												3↓
NH3		—	—	—	<200↓	—	—	—	—	—	<200↓	3↓
NO3 + NO2		—	—	—	<200↑	—	—	—	—	—	200↑	4↑
P												
Total		—	—	—	<200↑	—	—	—	—	—	<200↑	
SRP		—	<200↑	—	<200↑	—	<200↑	—	<200↑	—	<200↑	4↑
DOC		—	<200↓	—		—	<200↓	—	<200↓	—	<200↓	4↓

Table B18: No-observed-effect concentrations (NOECs) (Williams test, $p < 0.05$) and observed effect classes [89] per sampling date for community metabolism and chemistry endpoints in microcosms in the warm low P addition microcosms. The numbers of the preselected effect classes [89] refer to: 1= no effect; 2= slight effect; 3=clear short-term effects; 4: clear effect in short-term study; ↓= decrease; ↑= increase; ↑↓= increase and decrease on species and/or sampling date. Blank fields indicate that NOEC were equal to or higher than the highest tested concentration (300 µg/L)

	NOEC (µg/L)											Treatment levels	
	-1	1	7	9	14	16	21	23	28	30	35	75 µg/L	300 µg/L
DO													
morning	—	< 75↑								75↑		2↑	2↑
afternoon											< 75↓	2↓	2↓
max-min	—											1	1
mean											< 75↓	2↓	2↓
pH													
morning												1	1
afternoon					75↓	75↓					< 75↓	2↓	3↓
mean					75↓	75↓					75↓	1	3↓
N													
NH3		—	—	—		—	—	—	—	—	< 75↑	2↑	2↑
NO3 + NO2		—	—	—		—	—	—	—	—		1	1
P													
Total		—	—	—	75↓	—	—	—	—	—		1	2↓
SRP		—		—	75↓	—		—		—		1	2↓
DOC		—		—	75↓	—		—		—		1	2↓
Conductivity		—		—		—		—		—		1	1
BOD5		—	< 75↑	—		—	75↓	—	< 75↓	—		2↓(↑[a])	3↓

[a] Effects observed only after 7 days of treatment.

Table B19: No-observed-effect concentrations (NOECs) (Williams test, $p < 0.05$) and observed effect classes [89] per sampling date for community metabolism and chemistry endpoints in microcosms in the cold high P addition microcosms. The numbers of the preselected effect classes [89] refer to: 1= no effect; 2= slight effect; 3=clear short-term effects; 4: clear effect in short-term study; ↓= decrease; ↑= increase; ↑↓= increase and decrease on species and/or sampling date. Blank fields indicate that NOEC were equal to or higher than the highest tested concentration (300 µg/L)

	NOEC (µg/L)											Treatment levels	
	-1	1	7	9	14	16	21	23	28	30	35	75 µg/L	300 µg/L
DO													
morning	—				75↓	75↓		75↓			75↓	1	3↓
afternoon				75↓	75↓	75↓	75↓				< 75↓	2↓	3↓
max-min	—			< 75↓	75↓		75↓					2↓	3↓
mean					75↓	75↓		75↓			< 75↓	2↓	3↓
pH													
morning					75↓	75↓	75↓	75↓	75↓		75↓	1	3↓
afternoon					75↓	75↓	75↓		75↓	75↓	< 75↓	2↓	4↓
mean					75↓	75↓	75↓		75↓	75↓	75↓	1	4↓
N													
NH3		—	—	—	< 75↑	—	—	—	—	—	< 75↑	4↑	4↑
NO3 + NO2		—	—	—		—	—	—	—	—		1	1
P													
Total		—	—	—		—	—	—	—	—		1	1
SRP		—		—		—		—		—		1	1
DOC		—		—	75↓	—	75↓	—	75↓	—	75↓	1	4↓
Conductivity		—		—		—		—		—		1	1
BOD5		—	< 75↑	—		—	< 75↓	—	< 75↓	—	75↓	(2↑[a])3↓	4↓

[a] Effects observed only after 7 days of treatment.

Table B20: No-observed-effect concentrations (NOECs) (Williams test, $p < 0.05$) and observed effect classes [89] per sampling date for community metabolism and chemistry endpoints in microcosms in the warm high P microcosms. The numbers of the preselected effect classes [89] refer to: 1= no effect; 2= slight effect; 3=clear short-term effects; 4: clear effect in short-term study; ↓= decrease; ↑= increase; ↑↓= increase and decrease on species and/or sampling date. Blank fields indicate that NOEC were equal to or higher than the highest tested concentration (300 µg/L)

	NOEC (µg/L)											Treatment levels	
	-1	1	7	9	14	16	21	23	28	30	35	75 µg/L	300 µg/L
DO													
morning	—	75↑										1	2↑
afternoon											< 75↓	2↓	2↓
max-min	—	75↓		< 75↓					75↓			2↓	2↓
mean											< 75↓	2↓	2↓
pH													
morning			75↓							75↓	< 75↓	2↓	3↓
afternoon			75↓	< 75↓							< 75↓	2↓	3↓
mean			75↓	75↓							< 75↓	2↓	3↓
N													
NH3		—	—	—		—	—	—	—	—	75↑	1	2↑
NO3 + NO2		—	—	—		—	—	—	—	—		1	1
P													
Total		—	—	—		—	—	—	—	—		1	1
SRP		—	75↓	—		—		—		—		1	2↓
DOC		—	75↓	—	75↓	—	< 75↓	—	< 75↓	—	75↓	3↓	4↓
Conductivity		—		—		—		—	< 75↓	—		2↓	2↓
BOD5		—		—	75↓	—	< 75↓	—	< 75↓	—	< 75↓	4↓	4↓

Table B21: Statistical significance (*p* values Three-and two-way ANOVA) and calculation of the interactive effects (Synergism: S; or antagonism: A) of Zn (Low Zn: L; High Zn: H) and the different factors (low P: O; high P: E; cold: C and warm: W) of the community metabolism at different treatment regimes. The interaction type is based on the observed and predicted effects based on De Coninck et al (2013) Only groups for which at least one consistent interaction was found are represented here.

Parameter	Day	Treatment regime	Interaction factors	Zn treatment	*p*	observed effect	Predicted effect	Interaction type
BOD5	7	O	Zn × T	H	**0,009**	-0.57	-1.46	A
	14	O	Zn × T	H	**0,005**	-1.97	-14.50	A
	7	E	Zn × T	H	**<0,001**	0.18	-2.02	S
	14	E	Zn × T	H	**0,007**	0.50	-4.09	S
DOC	21	O	Zn × T	H	**0,006**	0.37	0.53	A
	28	O	Zn × T	H	**0,005**	0.33	0.58	A
	35	O	Zn × T	H	**<0,001**	0.42	0.59	A
	21	E	Zn × T	H	0,486			
	28	E	Zn × T	H	0,597			
	35	E	Zn × T	H	**0,025**	0.40	0.49	A
	21	O	Zn × T	L	0,566			
	28	O	Zn × T	L	0,285			
	35	O	Zn × T	L	**0,002**	0.32	0.48	A
	21	E	Zn × T	L	**0,002**	0.38	0.07	S
	28	E	Zn × T	L	**0,025**	0.37	0.13	S
	35	E	Zn × T	L	0,328			
	21	C	Zn × P	L	**0,041**	0.02	0.14	A
	28	C	Zn × P	L	**0,006**	0.02	0.21	A
	35	C	Zn × P	L	**0,010**	0.09	0.29	A
	21	W	Zn × P	L	0,099			
	28	W	Zn × P	L	0,259			
	35	W	Zn × P	L	0,116			
pH_{mean}	14	O	Zn × T	H	**0,011**	0.08	0.13	A
	21	O	Zn × T	H	**0,024**	0.03	0.06	A
	14	E	Zn × T	H	**0,007**	0.06	0.14	A
	21	E	Zn × T	H	**0,049**	0.02	0.04	A
Domean	14	O	Zn × T	H	**0,035**	0.27	0.34	A
	21	O	Zn × T	H	**0,019**	0.17	0.22	A
	14	E	Zn × T	H	**0,009**	0.23	0.38	A
	21	E	Zn × T	H	0,138			
DO_{net}	14	O	Zn × T	H	**0,026**	0.92	1.00	A
	21	O	Zn × T	H	**<0,001**	0.71	0.96	A
	14	E	Zn × T	H	0,062			
	21	E	Zn × T	H	0,072			

Figures

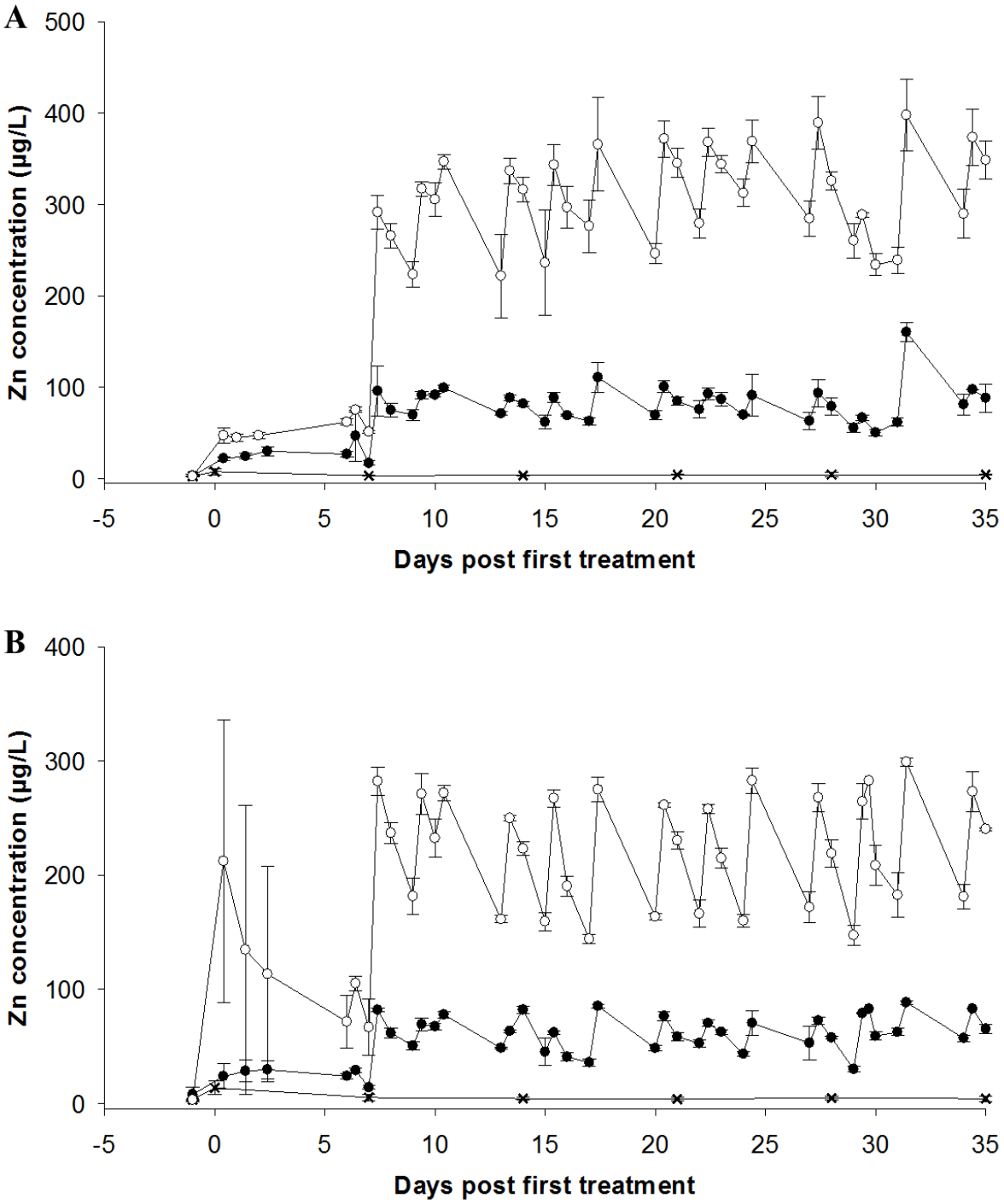


Figure B1: Measured dissolved Zn concentrations (µg/L) of the cold high P addition (A), warm low P addition (B) and warm high P addition (C) before and after spiking. Error bars are standard deviations.

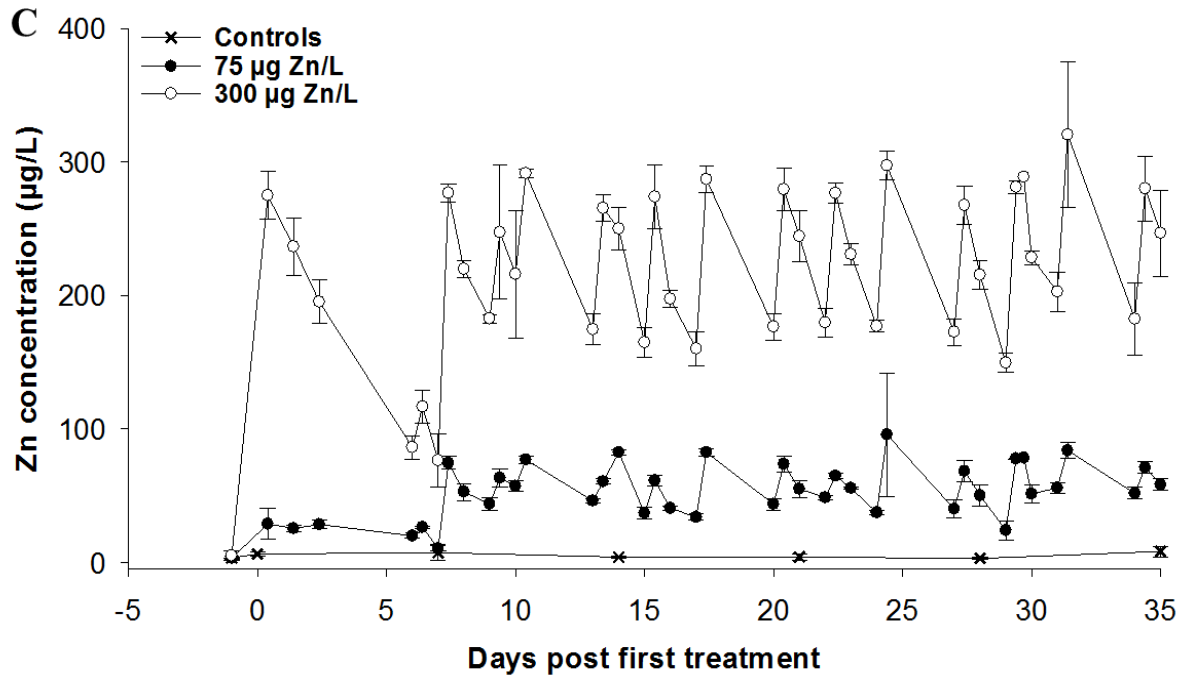


Figure B1: Measured dissolved Zn concentrations (µg/L) of the cold high P addition (A), warm low P addition (B) and warm high P addition (C) before and after spiking. Error bars are standard deviations.

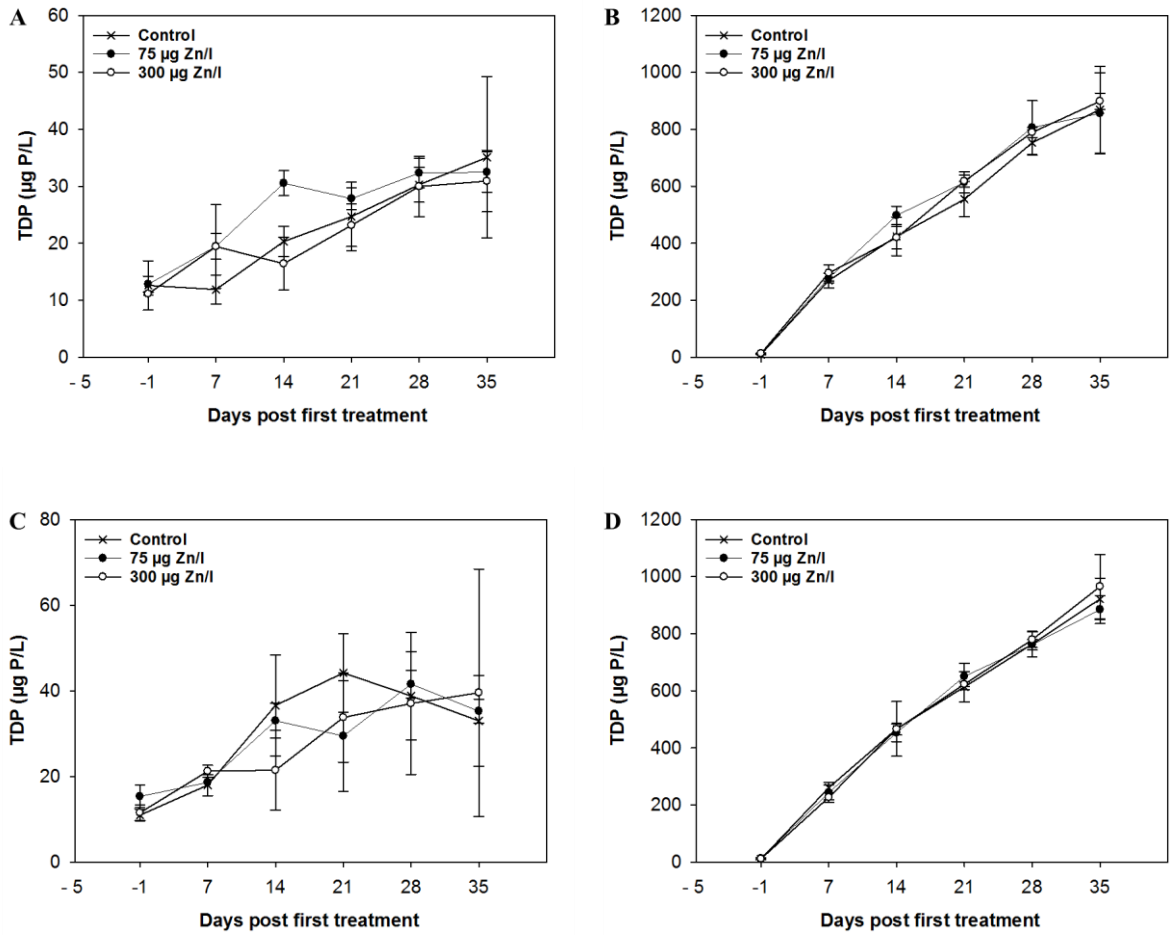


Figure B2: Measured total dissolved phosphorus (TDP) (µg P/L) of the cold low P addition (A), cold high P addition (B), warm low P addition (C) and warm high P addition (D) microcosm. Error bars are standard deviations.

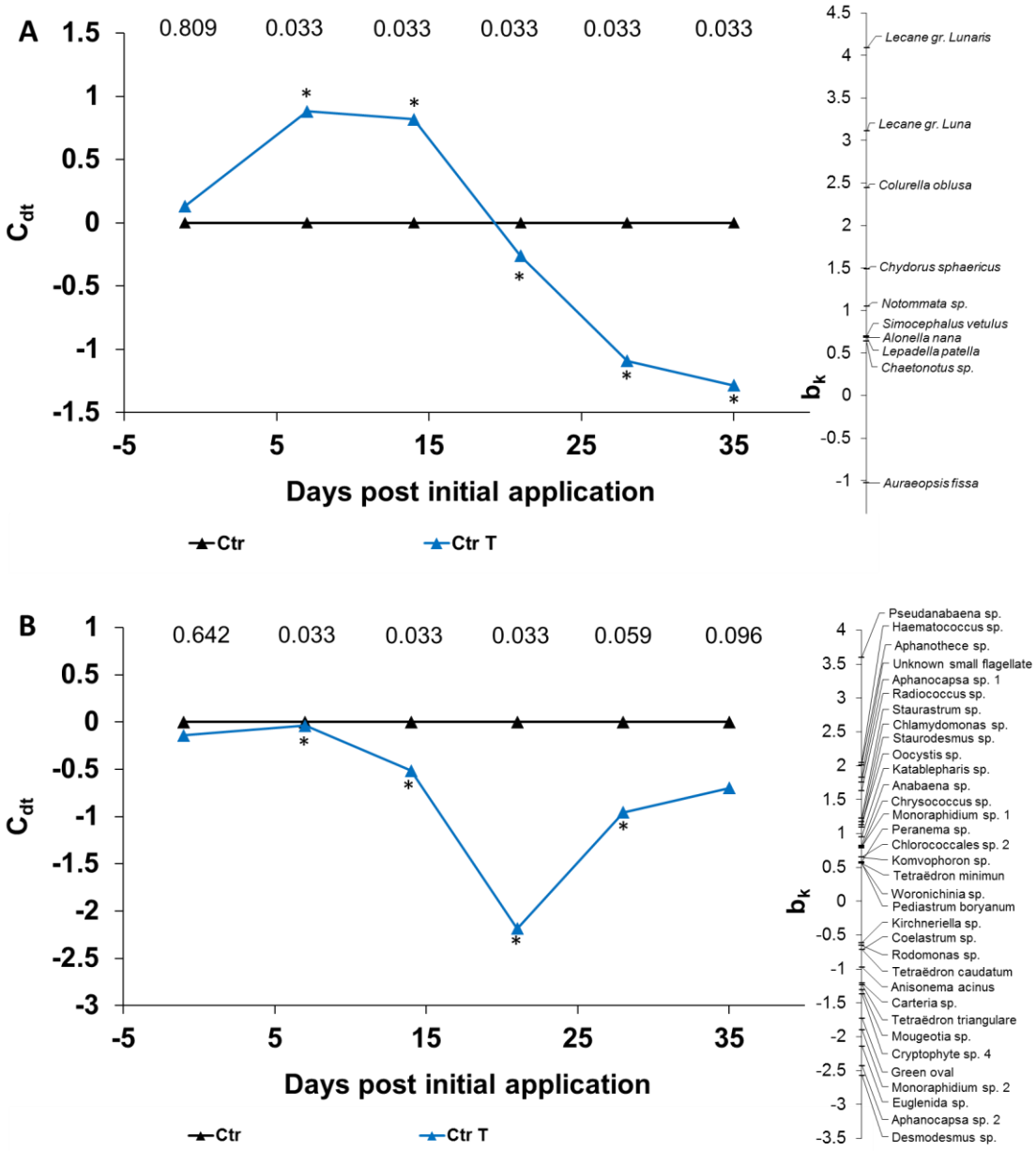


Figure B3: Principal response curve (PRC), resulting from the analysis of the zooplankton (A), phytoplankton (B) and protozoa data (C) indicating the effects of temperature in the low P addition microcosms without added Zn (16-19 °C: Ctr; 21-24 °C: Ctr T). The vertical axis represents the differences in community structure of the treatments compared to the controls expressed as regression coefficients (C_{dt}). The affinity of a taxon to the PRC is expressed as the species weight (b_k). Species with a low b_k (between 0.5 and -0.5) are not shown. *: significant difference in community structure from the control ($p < 0.05$, Monte Carlo permutation test). Calculated Monte Carlo permutation test p values are plotted above the figures.

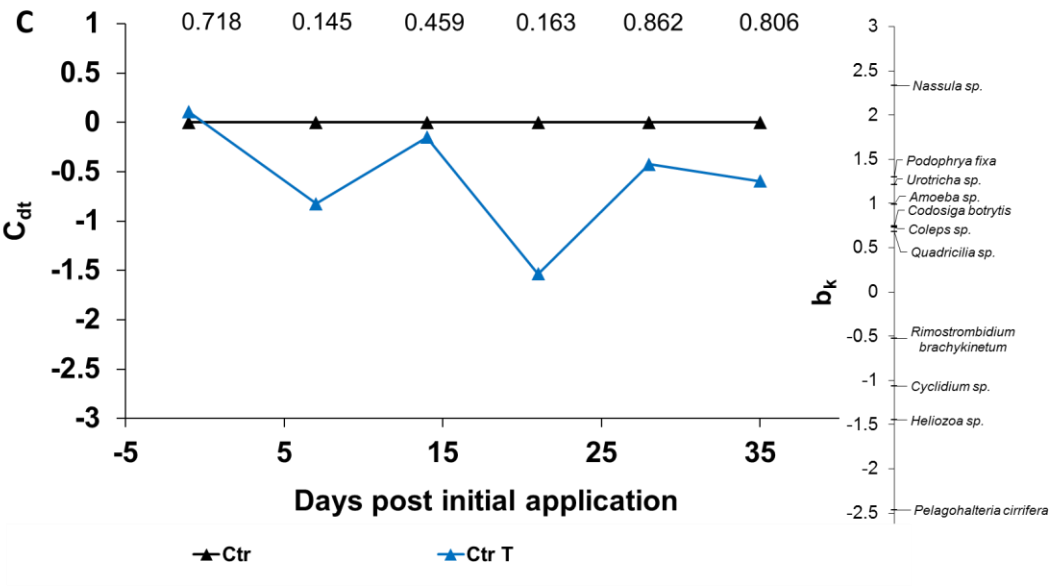


Figure B3: Principal response curve (PRC), resulting from the analysis of the zooplankton (A), phytoplankton (B) and protozoa data (C) indicating the effects of temperature in the low P addition microcosms without added Zn (16-19 °C: Ctr; 21-24 °C: Ctr T). The vertical axis represents the differences in community structure of the treatments compared to the controls expressed as regression coefficients (C_{dt}). The affinity of a taxon to the PRC is expressed as the species weight (b_k). Species with a low b_k (between 0.5 and -0.5) are not shown. *: significant difference in community structure from the control ($p < 0.05$, Monte Carlo permutation test). Calculated Monte Carlo permutation test p values are plotted above the figures.

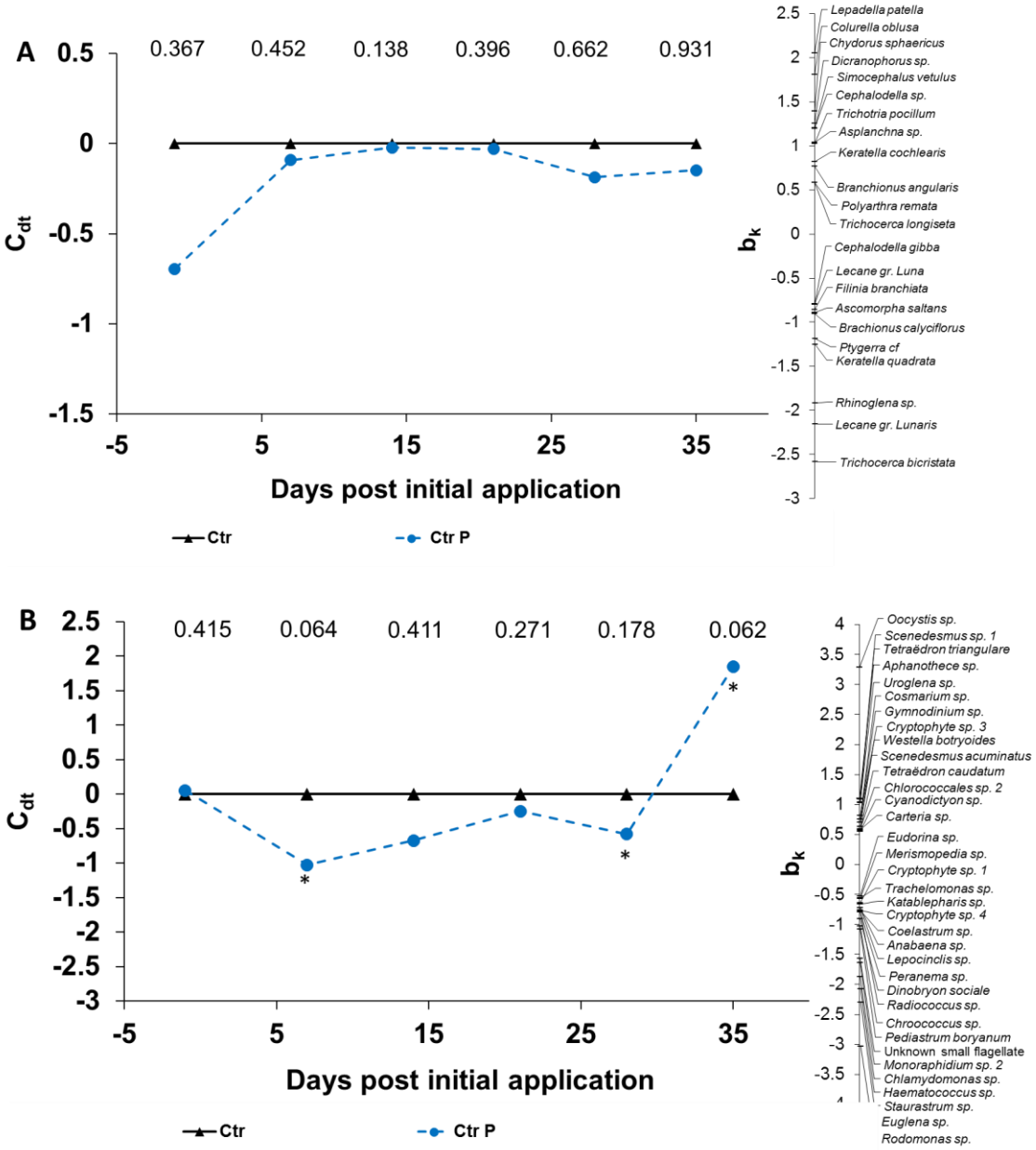


Figure B4: Principal response curve (PRC), resulting from the analysis of the zooplankton (A), phytoplankton (B) and protozoa data (C), indicating the effects of high P addition the cold microcosms without added Zn (Low P addition: Ctr; High P addition: Ctr P). The vertical axis represents the differences in community structure of the treatments compared to the controls expressed as regression coefficients (C_{dt}). The affinity of a taxon to the PRC is expressed as the species weight (b_k). Species with a low b_k (between 0.5 and -0.5) are not shown. *: significant difference in community structure from the control ($p < 0.05$, Monte Carlo permutation test). Calculated Monte Carlo permutation test *p* values are plotted above the figures.

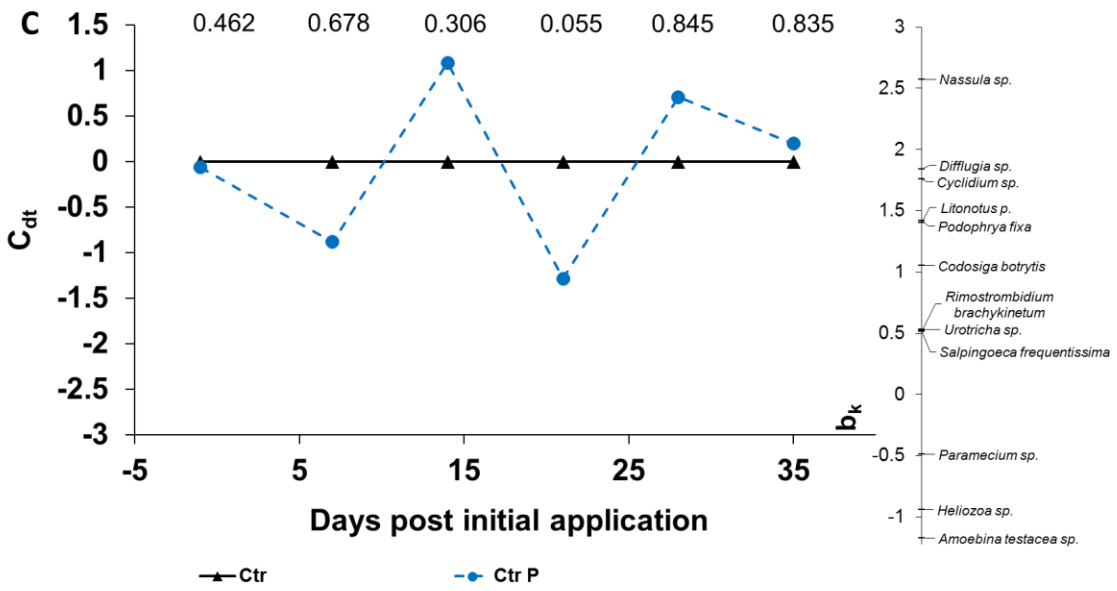


Figure B4: Principal response curve (PRC), resulting from the analysis of the zooplankton (A), phytoplankton (B) and protozoa data (C), indicating the effects of high P addition the cold microcosms without added Zn (Low P addition: Ctr; High P addition: Ctr P). The vertical axis represents the differences in community structure of the treatments compared to the controls expressed as regression coefficients (C_{dt}). The affinity of a taxon to the PRC is expressed as the species weight (b_k). Species with a low b_k (between 0.5 and -0.5) are not shown. *: significant difference in community structure from the control ($p < 0.05$, Monte Carlo permutation test). Calculated Monte Carlo permutation test p values are plotted above the figures.

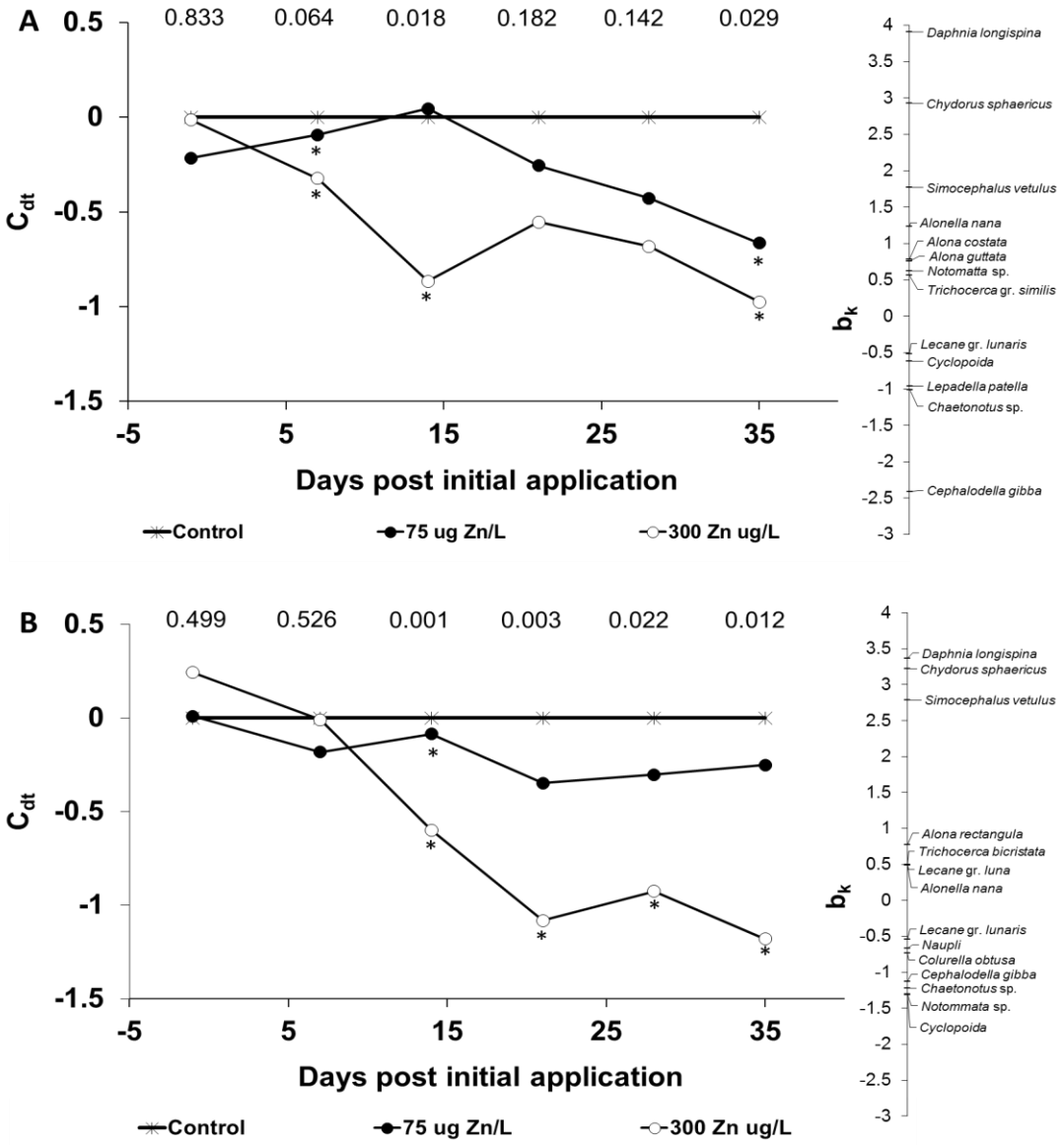


Figure B5: Principal response curve (PRC), resulting from the analysis of the **zooplankton** of the warm low P (A), cold high P addition (B) and warm high P (C) microcosms, indicating the effects of the different Zn treatments. The vertical axis represents the differences in community structure of the treatments compared to the controls expressed as regression coefficients (C_{dt}). The affinity of a taxon to the PRC is expressed as the species weight (b_k). *: significant difference in community structure from the control ($p < 0.05$, Monte Carlo permutation test). Calculated Monte Carlo permutation test *p* values are plotted above the figures.

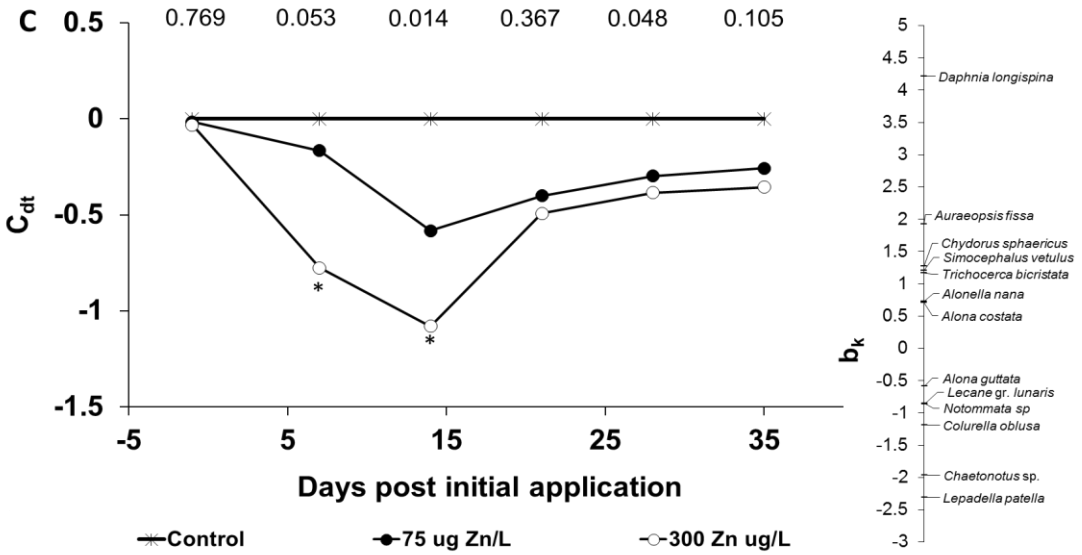


Figure B5: Principal response curve (PRC), resulting from the analysis of the **zooplankton** of the warm low P (A), cold high P (B) and warm high P (C) microcosms, indicating the effects of the different Zn treatments. The vertical axis represents the differences in community structure of the treatments compared to the controls expressed as regression coefficients (C_{dt}). The affinity of a taxon to the PRC is expressed as the species weight (b_k). *: significant difference in community structure from the control ($p < 0.05$, Monte Carlo permutation test). Calculated Monte Carlo permutation test *p* values are plotted above the figures.

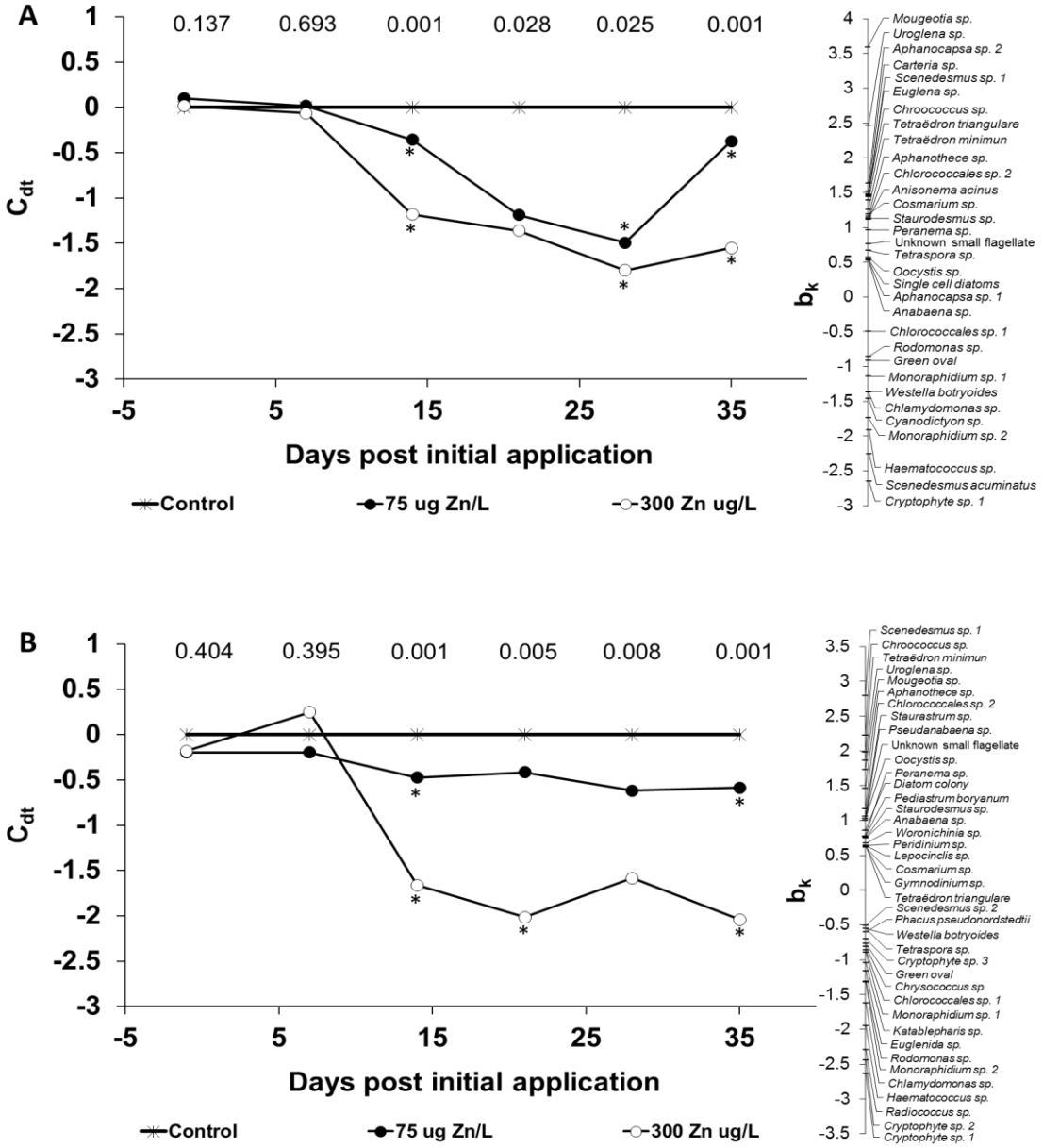


Figure B6: Principal response curve (PRC), resulting from the analysis of the **phytoplankton** of the warm low P (A), cold high P (B) and warm high P (C) microcosms, indicating the effects of the different Zn treatments. The vertical axis represents the differences in community structure of the treatments compared to the controls expressed as regression coefficients (C_{dt}). The affinity of a taxon to the PRC is expressed as the species weight (b_k). *: significant difference in community structure from the control ($p < 0.05$, Monte Carlo permutation test). Calculated Monte Carlo permutation test *p* values are plotted above the figures.

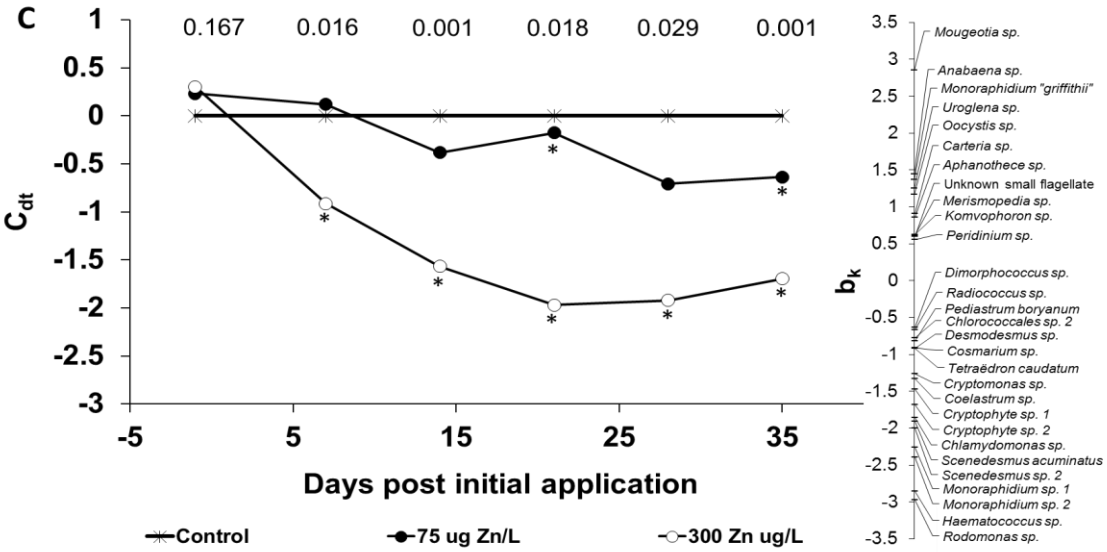


Figure B6: Principal response curve (PRC), resulting from the analysis of the **phytoplankton** of the warm low P (A), cold high P (B) and warm high P (C) microcosms, indicating the effects of the different Zn treatments. The vertical axis represents the differences in community structure of the treatments compared to the controls expressed as regression coefficients (C_{dt}). The affinity of a taxon to the PRC is expressed as the species weight (b_k). *: significant difference in community structure from the control ($p < 0.05$, Monte Carlo permutation test). Calculated Monte Carlo permutation test *p* values are plotted above the figures.

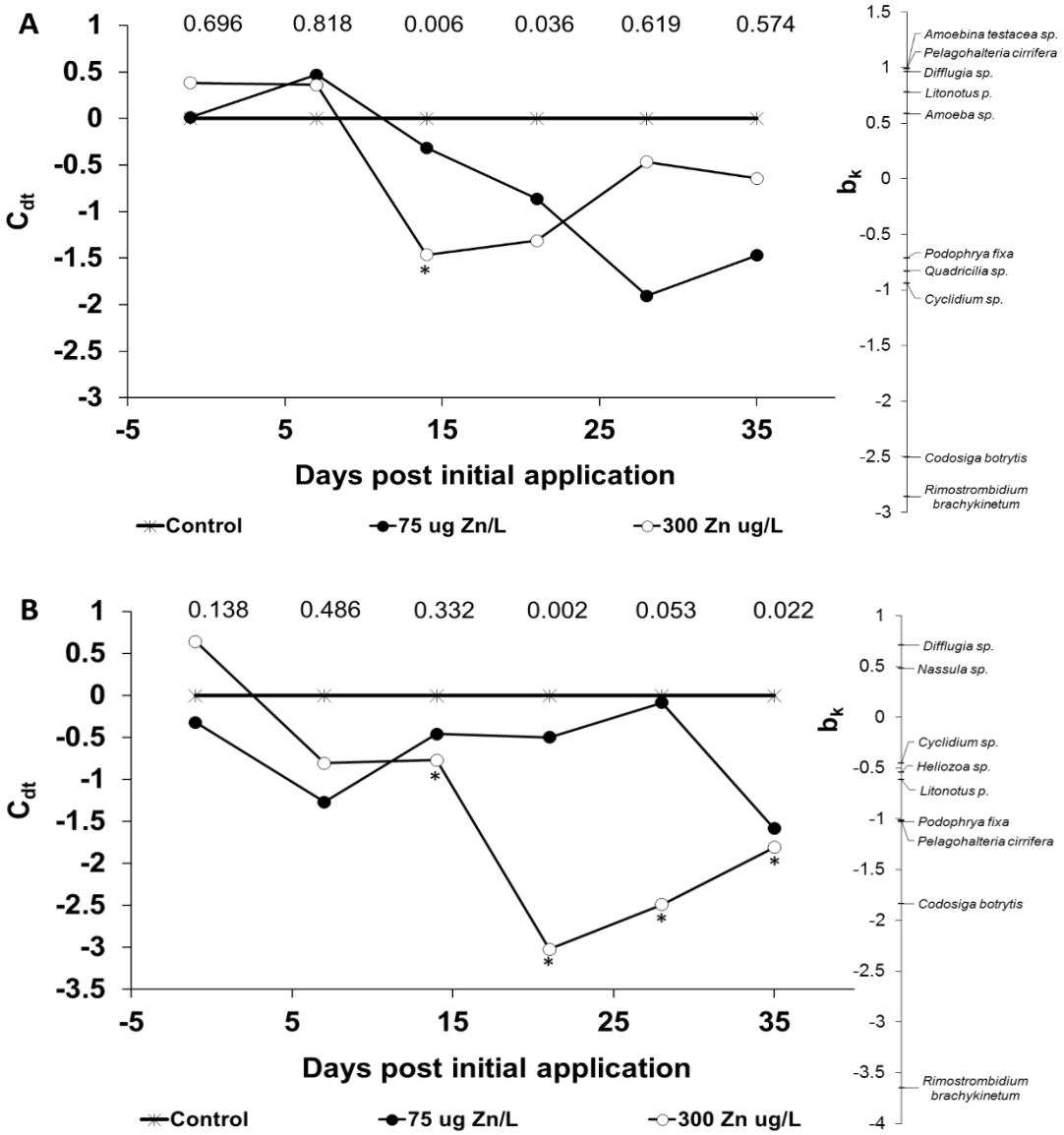


Figure B7: Principal response curve (PRC), resulting from the analysis of the **protozoa** of the warm low P (A), cold high P(B) and warm high P (C) microcosms, indicating the effects of the different Zn treatments. The vertical axis represents the differences in community structure of the treatments compared to the controls expressed as regression coefficients (C_{dt}). The affinity of a taxon to the PRC is expressed as the species weight (b_k). *: significant difference in community structure from the control ($p < 0.05$, Monte Carlo permutation test). Calculated Monte Carlo permutation test *p* values are plotted above the figures.

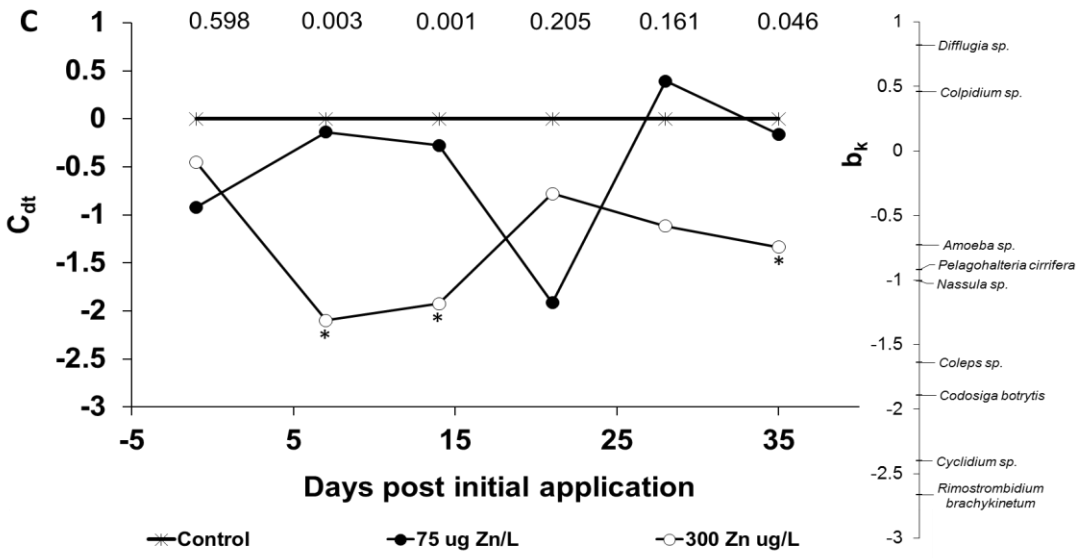


Figure B7: Principal response curve (PRC), resulting from the analysis of the **protozoa** of the warm low P (A), cold high P (B) and warm high P (C) microcosms, indicating the effects of the different Zn treatments. The vertical axis represents the differences in community structure of the treatments compared to the controls expressed as regression coefficients (C_{dt}). The affinity of a taxon to the PRC is expressed as the species weight (b_k). *: significant difference in community structure from the control ($p < 0.05$, Monte Carlo permutation test). Calculated Monte Carlo permutation test *p* values are plotted above the figures.

C

Supplemental material for Chapter 4

Tables

Table C1: Mean (± standard deviation) of the chemical properties of the jar water per sampling date.

Excel file Van de Perre et al. 2017

Table C2: Species abundances per litre of the jars per sampling date.

Excel file Van de Perre et al. 2017

Table C3: Statistical significance (*p* values Three-way ANOVA) of the main effects and interactions of the lowest Zn treatment and the different factors (competition and temperature) on the *Branchionus calyciflorus* and small rotifers abundance at the different samplings. Significant ($p < 0.05$) values are flagged and consistent interactions in bold.

	Independent variable	Day after first treatment					
		4	7	11	14	18	21
B. calyciflorus	Zn low (Zn L)	0.234	0.102	0.187	0.498	0.116	NA
	Competition (comp[a])	0.082	<0.001	0.009	0.046	0.116	NA
	Temperature (T)	0.967	0.225	0.762	0.622	0.116	NA
	Zn L x Comp[a]	0.205	0.003	0.224	0.274	0.116	NA
	Zn L x T	0.071	0.124	0.168	0.634	0.116	NA
	Zn L x Comp[a] x T	0.675	0.941	0.118	0.033	0.116	NA
Small rotifers	Zn low (Zn L)	0.557	0.107	0.894	0.711	0.128	0.216
	Competition (comp[b])	0.816	0.703	<0.001	0.002	0.003	0.199
	Temperature (T)	0.732	0.003	<0.001	<0.001	<0.001	0.096
	Zn L x Comp[b]	0.773	0.845	0.958	0.872	0.618	0.297
	Zn L x T	0.858	0.056	0.199	0.167	0.453	0.443
	Zn L x Comp[b] x T	0.205	0.646	0.871	0.761	0.813	0.767

[a]: "*Daphnia longispina* + small rotifers" competition; [b]: "*Daphnia longispina* + *B. calyciflorus*" competition

Table C4: Statistical significance (*p* values Three-way ANOVA) of the main effects and interactions of the highest Zn treatment and the different factors (competition and temperature) of the *Branchionus calyciflorus* and small rotifers at the different samplings. Significant ($p < 0.05$) values are flagged and consistent interactions in bold.

	Independent variable	Day after first treatment					
		4	7	11	14	18	21
B. calyciflorus	Zn high (Zn H)	0.100	0.024	0.549	0.409	0.116	NA
	Competition (comp)[a]	0.024	<0.001	0.001	0.019	0.116	NA
	Temperature (T)	0.669	0.130	0.657	0.971	0.116	NA
	Zn H x Comp[a]	0.452	0.091	0.605	0.480	0.116	NA
	Zn H x T	0.138	0.064	0.487	0.985	0.116	NA
	Zn H x Comp[a] x T	0.445	0.196	0.673	0.889	0.116	NA
Small rotifers	Zn high (Zn H)	0.365	0.111	0.148	0.254	0.057	0.012
	Competition (comp[b])	0.840	0.920	0.004	0.009	0.291	0.747
	Temperature (T)	0.337	0.004	0.119	<0.001	<0.001	0.881
	Zn H x Comp[b]	0.889	0.770	0.433	0.400	0.139	0.931
	Zn H x T	0.436	0.030	0.308	0.191	0.573	0.305
	Zn H x Comp[b] x T	0.093	0.587	0.140	0.687	0.734	0.171

[a]: *Daphnia longispina* + small rotifers competition; [b]: *Daphnia longispina* + *B. calyciflorus* competition

D

Supplemental material for Chapter 5

Tables

Table D1: Mean (± standard deviation) calculated $HC5_{\text{-plankton}}$ (µg Zn/L) and $HC50_{\text{-plankton}}$ (µg Zn/L) per sampling day and treatment, taking into account chronic Zn toxicity data for plankton species (Chapter 2 [5,121]).

		Cold (16-19°C)				Warm (21-24°C)			
		Low P*		High P		Low P		High P	
Treatment level	Day	HC5	HC50	HC5	HC50	HC5	HC50	HC5	HC50
Control	-1	85 ± 4	351 ± 31	89 ± 4	339 ± 36	92 ± 3	334 ± 30	90 ± 2	332 ± 37
	7	83 ± 4	271 ± 15	75 ± 4	221 ± 18	75 ± 2	215 ± 20	71 ± 4	193 ± 15
	14	69 ± 7	216 ± 32	65 ± 4	205 ± 29	52 ± 2	149 ± 6	52 ± 4	147 ± 10
	21	69 ± 4	201 ± 13	60 ± 5	174 ± 15	45 ± 5	141 ± 16	50 ± 6	150 ± 17
	28	64 ± 3	181 ± 8	54 ± 6	156 ± 14	39 ± 5	134 ± 13	41 ± 7	133 ± 20
	35	65 ± 3	189 ± 13	55 ± 5	166 ± 14	38 ± 3	147 ± 10	36 ± 3	139 ± 12
75	-1	87 ± 2	316 ± 20	88 ± 4	365 ± 43	91 ± 2	337 ± 23	91 ± 2	311 ± 15
	7	74 ± 11	222 ± 40	77 ± 3	227 ± 18	87 ± 14	245 ± 39	70 ± 5	189 ± 17
	14	56 ± 21	174 ± 64	64 ± 6	190 ± 22	57 ± 3	163 ± 6	48 ± 3	139 ± 9
	21	61 ± 9	172 ± 17	66 ± 2	182 ± 6	45 ± 12	137 ± 37	36 ± 2	114 ± 7
	28	55 ± 6	158 ± 8	61 ± 1	172 ± 8	38 ± 3	128 ± 9	34 ± 4	112 ± 12
	35	51 ± 8	151 ± 12	58 ± 3	164 ± 7	41 ± 2	136 ± 10	35 ± 1	123 ± 11
300	-1	84 ± 1	320 ± 21	89 ± 2	343 ± 37	90 ± 2	335 ± 42	94 ± 8	342 ± 24
	7	74 ± 4	214 ± 16	77 ± 4	224 ± 21	68 ± 8	183 ± 27	46 ± 1	124 ± 1
	14	46 ± 7	129 ± 18	42 ± 11	114 ± 24	40 ± 6	115 ± 18	34 ± 2	102 ± 6
	21	41 ± 3	122 ± 4	41 ± 1	115 ± 2	39 ± 4	122 ± 12	36 ± 1	112 ± 1
	28	38 ± 3	122 ± 6	41 ± 1	117 ± 5	40 ± 11	131 ± 28	32 ± 2	104 ± 7
	35	40 ± 5	124 ± 6	42 ± 1	120 ± 4	36 ± 5	121 ± 16	34 ± 2	109 ± 7

Table D2: Summary of the *p* values (ANOVA) of the Zn effects and Zn ×T interactions on the different *D. longispina* populations: *D. longispina* population (D: chapter 5), *D. longispina* population in smaller rotifer community (D + R: Chapter 4), *D. longispina* population in smaller rotifer and *B. calyciflorus* community (D + R + B: chapter 4), *D. longispina* population in a natural, microcosm community (D + COM: chapter 2 and 3). Significant ($p < 0.05$) values are flagged and consistent interactions in bold.

Experiment	Independent variable	Day after first treatment							
		4	7	11	14	18	21	28	35
Low Zn									
D	Zinc Low	0.703	0.144	0.274	0.669	0.905	0.621	NA	NA
	Temperature	0.110	0.953	0.972	0.820	0.515	0.654	NA	NA
	Interaction	0.661	0.320	0.865	0.507	0.218	0.020	NA	NA
D + R	Zinc Low	0.323	0.637	0.003	<0.001	<0.001	<0.001	NA	NA
	Temperature	<0.001	0.127	0.255	0.074	0.275	0.029	NA	NA
	Interaction	0.879	0.638	0.552	0.940	0.156	0.005	NA	NA
D + R + B	Zinc Low	0.823	0.020	0.002	0.002	<0.001	0.025	NA	NA
	Temperature	0.831	0.240	0.905	0.470	0.070	0.140	NA	NA
	Interaction	0.851	0.094	0.117	0.149	0.936	0.404	NA	NA
COM	Zinc Low	NA	0.524	NA	0.312	NA	0.04	0.077	0.171
	Temperature	NA	0.279	NA	0.069	NA	0.017	0.900	0.975
	Interaction	NA	0.140	NA	0.570	NA	0.520	0.743	0.765
High Zn									
D	Zinc High	0.033	<0.001	<0.001	<0.001	<0.001	<0.001	NA	NA
	Temperature	0.509	<0.001	<0.001	0.510	0.195	0.051	NA	NA
	Interaction	0.065	**<0.001**	**<0.001**	0.510	0.195	0.051	NA	NA
D + R	Zinc High	0.597	0.131	<0.001	<0.001	<0.001	<0.001	NA	NA
	Temperature	0.016	0.053	<0.001	<0.001	0.012	<0.001	NA	NA
	Interaction	0.268	**0.002**	**<0.001**	**0.002**	0.116	0.045	NA	NA
D + R +B	Zinc High	0.001	<0.001	<0.001	<0.001	<0.001	<0.001	NA	NA
	Temperature	<0.001	0.260	0.425	0.714	0.312	0.914	NA	NA
	Interaction	0.010	0.070	0.038	0.236	0.858	0.452	NA	NA
COM	Zinc High	NA	0.076	NA	<0.001	NA	<0.001	0.011	0.012
	Temperature	NA	0.097	NA	0.115	NA	0.285	0.912	0.843
	Interaction	NA	0.008	NA	0.445	NA	0.285	0.912	0.843

Table D3: Mean (± standard deviation) of the average filterd Zn concentrations of the microcosm water before and after changing medium.

	New medium (µg Zn/L)	Before medium change (µg Zn/L)	Average mean (µg Zn/L)
Zn low	53 ± 5	19 ± 13	37 ± 20
Zn high	169 ± 10	50 ± 22	109 ± 62

Throughout the experiment the dissolved Zn concentrations in the high and low Zn fluctuated greatly and Zn losses between medium changes could reach up to about 70% (Table 5.2). On average the concentrations of the lowest Zn treatment were 16% below target concentration and 23% lower from the highest Zn target concentration. The average dissolved Zn concentrations (± standard deviation) in the controls were 3.7 ± 2.8 µg/L. The average dissolved oxygen concentration throughout the experiment was 13.3 mg/L ± 1.6 and the average pH (9.1 ± 0.4), DOC (5.5 mg/L ± 0.5) and TOC (5.7 mg/L ± 0.7).

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Curriculum Vitae

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Personalia

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Day of birth:	19-03-1985
Place of birth:	Jette
Nationality:	Belgian
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Education

2008- 2010	**Master in Biology** (Environment: biodiversity & ecosystems): Vrije Universiteit Brussel Master Thesis: The determination and comparison of the ecological water quality of the Zenne basin by using diatoms and Macro-invertebrates Passed with great distinction
2003- 2008	Bachelor in Biology (Ecology): Vrije Universiteit Brussel Bachelor Thesis: Monitoring of the ecological water quality by using diatoms
1997- 2003	High school: Sint Donatusinstituut (Science & math)

Work experience

April 2013 - present Ghent University

Function: PhD student at Laboratory of Environmental Toxicology and aquatic Ecology

The combined and interactive effect of zinc, temperature and phosphorus on freshwater planktonic communities

July 2012 - November 2012 Belchim Crop Protection

Function: Trial Field assistant/ Lab manager

Tasks: Planning, managing, carrying out, follow up and reporting for all laboratory work and –tests. Laboratory manager of a three headed international team

December 2010 – September 2011 Aquafin NV

Function: Laborant II (Microscopy)

Tasks: Microscopy (biomonitoring active sludge: bacteria, protozoa,…), microscopic tests, stock management, process management water treatment plants, advising clients and directing technical staff

Publications

Van de Perre D, Roessink I, Janssen CR, Smolders E, Van den Brink PJ, de Schamphelaere K a. C.. The combined and interactive effects of zinc, temperature and phosphorus on the structure and functioning of a freshwater community. Environ. Toxicol.. Submitted (January 2018)

Van Regenmortel T, Van de Perre D, Janssen CR, De Schamphelaere K.A.C.. The effects of a Cu-Ni-Zn mixture on the structure, diversity and function of a freshwater planktonic community. Environ. Toxicol. submitted. (November 2017)

Van de Perre D, Janssen CR, de Schamphelaere K a. C.. Combined effect of interspecies interaction (food competition), temperature and Zn on Daphnia longispina population dynamics. Environ. Toxicol. Accepted. (February 2018)

Van de Perre D, Roessink I, Janssen CR, Smolders E, Van Regenmortel T, Van Wichelen J, Vyverman W, Van den Brink PJ, de Schamphelaere K a. C. 2016. The effects of zinc on the structure and functioning of a freshwater community: a microcosm experiment. Environ. Toxicol. Chem. 29:730–741.

Poster and platform presentations

Van de Perre D, de Laender F, Janssen CR and De Schamphelaere K. Combined effects of interspecies interaction, temperature & Zn on *Daphnia longispina* population dynamics. Poster presentation at SETAC Europe, 26th Annual meeting, Nantes, France (2016).

Van de Perre D, de Laender F, Roessink I, Van den Brink J, Smolders E, Janssen CR and De Schamphelaere K. The effects of zinc on the structure and functioning of a freshwater community: a microcosm experiment. Poster presentation at SETAC Europe, 26th Annual meeting, Nantes, France (2016).

Van de Perre D, de Laender F, Roessink I, Van den Brink J, Smolders E, Van Wichelen J, Janssen CR and De Schamphelaere K. Combined effects of temperature and zinc on a microcosm freshwater community. Poster corner presentation at SETAC Europe, 25th Annual meeting, Barcelona, Spain (2015).

Van de Perre D, de Laender F, Roessink I, Van den Brink J, Smolders E, Janssen CR and De Schamphelaere K. Combined and interactive effects of three stressors (phosphorus, temperature and zinc) in a freshwater community. Platform presentation at SETAC Europe, 24th Annual meeting, Basel, Switzerland (2014).

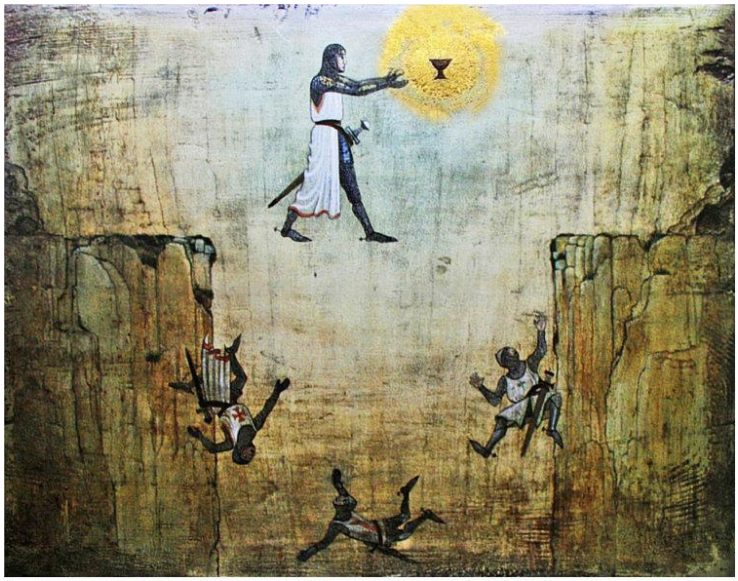
Attended conferences and Workshops

- SETAC Europe, 26th Annual meeting, Nantes, France
- SETAC Europe, 25th Annual meeting, Barcelona, Spain
- SETAC Europe, 24th Annual meeting, Basel, Switzerland
- RT-PCR training Applied Biosystems (2011)
- 1st taxonomic Workshop Nederlands-Vlaamse Kring van Diatomisten (*Achnanthidium minutissimum-pyrenaicum* complex), Aalst, Belgium (2010)
- 7th International Symposium "Use of Algae for Monitoring Rivers", Luxembourg (2009)

Dankwoord

“Faith is taking the first step even when you don’t see the whole staircase”

(Martin Luther King Jr.)



Sweet!!! Eindelijk!!!! Het zou gaan tijd worden!!! Deze woorden spookten door men hoofd na het schrijven van het laatste hoofdstuk van men doctoraat. Meer dan 4 jaar heb ik er uiteindelijk over gedaan om tot deze pagina’s te komen. Het zou niet mogelijk zijn geweest om op mijn eentje dit doctoraat te behalen. Daarom hier een korte hommage aan iedereen die er voor zorgde dat ik niet in de afgrond stortte.

Eerst en vooral zou ik graag mijn promotoren (Colin en Karel DS) bedanken. Zonder hun had ik nooit kunnen beginnen aan dit avontuur. Karel DS, ik zou jouw in het bijzonder willen bedanken voor de interessante discussies. Je hebt me echt omgevormd tot wetenschapper (leren kritisch nadenken, resultaten analyseren en neerschrijven). Je hebt ook je best gedaan om deze bioloog meer te laten denken als een bio-ingenieur.

Gedurende mijn doctoraat heb ik de kans gekregen om heel wat leuke mensen te leren kennen. Zonder jullie zou mijn doctoraat nooit zou leuk geweest zijn. Eerst en vooral de collega's natuurlijk. Ik genoot echt van de koffiemomentjes en onze zoektocht naar de beste burger (of de dichtstbijzijnde) van Gent. In het bijzonder zou ik graag mijn Ex plateau bureaugenoten Cecilia, Jan en Jonathan en verdieping genoten Maarten, Sasha en Jana (de valse rosse) willen bedanken. Ik ga onze 2de verdieping avonturen nooit vergeten. Zeker die keer niet dat Jan probeerde uit te leggen hoe een zwart gat werkt door middel van een zaklampje. Tijdens het doctoraat moest ik ook vaak naar het buitenland voor experimenten/stalen en SETAC congressen (What happend in Barcelona stays in Barcelona). Deze momenten hebben ook een speciaal plaatsje in mijn hart. Graag zou ik Anna, Ivo en Karel V willen bedanken voor de leuke momenten in Wageningen. Ons lijflied "Mama Luba" blijft tot heden het beste liedje om microcosm stalen te nemen ^^. Ivo, bedankt voor me zoveel te leren over microcosm experimenten en alles wat kan foutgaan. Jeroen, bedankt om mij het verschil te leren tussen kleine bolletjes en andere kleine bolletjes. De momenten aan de microscoop zouden veel minder leuk geweest zijn zonder uw gezelschap. Verder wil ik graag Tina, Emmy en Jolien (alias spek met rode bonen saus danser of doctoraat binder) bedanken voor de freestyle momenten op weg naar Wageningen. Het spijt me Emmy, Jolien en Nancy dat ik jullie soms overdonderd heb met waterstalen om te meten ^^. Zonder jullie hulp was dit doctoraat dan ook nooit gelukt. Marianne en Nancy wil ik ook in het bijzonder bedanken voor me te helpen met alle administratieve, bureaucratische rompeslomp en de leuke momenten. En natuurlijk niet te vergeten Marc om me te helpen bij het opstellen van de experimenten te Gent.

Aan mijn familie en vrienden, merci om er altijd voor mij te zijn en naar mijn gezaag te luisteren. A PhD can be dark and full of terrors. Dit weten mijn broer en Maikel maar al

te goed. Ze wisten dan ook dat een goed pak friet of een partijtje Magic de beste remedie was als het effe slecht ging. Mama en papa, dikke merci voor alles en voor altijd in mij te geloven. Ik heb mijn best gedaan om jullie in 5 jaar uit te leggen wat ik nu juist deed in Gent. Berta bedankt voor de leuke vrijdagen en voor mijn broer af en toe eens buiten te laten. Jentel, bedankt voor de fitness en bicky maison momenten. Ten slotte wil ik Sarah bedanken. Je was steeds mijn steun en toeverlaat en ik denk niet dat het zonder jouw gelukt zou zijn. Het lichtje in de duisternis.

Opwijk, December 2017