1 **Interactive effects between Zn, T and P on a microcosm**

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4 Corresponding author:

5 Dimitri Van de Perre

6 Laboratory of Environmental Toxicology and Aquatic Ecology, Environmental

7 Toxicology Unit (GhEnToxLab), Ghent University (UGent)

8 Jozef Plateaustaat 22, B-9000 Gent, Belgium

9 Phone +32 (0) 92643767

10 Fax +32 (0) 92643766

11 e-mail: Dimitri.VandePerre@Ugent.be

12 <http://www.ecotox.ugent.be/>

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

Dimitri Van de Perre,* † Ivo Roessink,‡ Colin R. Janssen, † Erik Smolders , §
Frederik De Laender, || Paul J. Van den Brink‡# and Karel A.C. De Schampelaere†

†Laboratory of Environmental Toxicology and Aquatic Ecology, Environmental
Toxicology Unit (GhEnToxLab), Ghent University (UGent), Jozef Plateaustraat 22, B-
9000 Gent, Belgium

‡Wageningen Environmental Research (Alterra), P.O. Box 47, 6700 AA Wageningen,
The Netherlands

§Division Soil and Water Management, Katholieke Universiteit Leuven, Arenbergpark
20, 3001-Heverlee, Belgium

||Research Unit of Environmental and Evolutionary Biology, University of Namur, Rue
de Bruxelles 61, 5000 Namur, Belgium.

Aquatic Ecology and Water Quality Management Group, Wageningen University,
P.O. Box 47, 6700 AA Wageningen, The Netherlands

Statement: * Dimitri.VandePerre@Ugent.be

ABSTRACT

Ecotoxicological studies mainly consists of single-species experiments evaluating the effects of a single stressor. However, under natural conditions aquatic communities are exposed to a mixture of stressors. This study was set up to identify how 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). Microcosm were exposed to three Zn concentrations (background, 75 µg Zn/L and 300 µg Zn/L), two temperatures (cold: 16-19 °C and warm: 21-24 °C) and 2 different phosphorous additions (oligotrophic: 0.02 mg P L⁻¹ week⁻¹ and eutrophic: 0.4 mg P L⁻¹ week⁻¹) for five weeks in full factorial design. Throughout the experiment 3% of the taxa showed a consistent Zn × T interaction (40% synergistic; 60% antagonistic). At the group level 36% of the functional groups showed a Zn × T interaction (20% synergistic; 80% antagonistic). Of all the interactions at the species and group level 90% were found under eutrophic conditions and 82% at the highest Zn treatment. Zn × P interaction (100% synergistic) were only found for the Chlorophyta and Cryptophyta under warm conditions. At the community level consistent Zn × T interactions were found at the highest Zn treatment for the zooplankton and phytoplankton under eutrophic conditions and for the protozoa under oligotrophic conditions.

Key words

Zinc, climate change, community level effects, multiple stressors, plankton

INTRODUCTION

Metal pollution, together with eutrophication and climate change, can pose risks to aquatic ecosystems [1–3]. 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. T and P) is a major challenge in environmental toxicology [3–5]. 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 [2] and will also induce an increased phosphorus loading from land to lakes and streams due to increased rainfall intensity [6].

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 [7]. Many studies have already shown that environmental conditions can significantly alter the responses of organisms to toxicants [3,8–11]. 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) [10,11]. Jackson et al. (2016) [10] for example, conducted a meta-analysis study based on freshwater studies and they found that the cumulative mean of the interactive effects, of all the investigated freshwater studies, were 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 [10]. At higher levels of biological organisation, compensatory species dynamics become more important [12] and this may explain the difference in interaction type a between the population and the community level (i.e. composition, function and diversity) [10].

Temperature (T) and phosphorous (P) are two important environmental factors that can have major effects on planktonic community composition and functioning [13–22] and both are also well known to influence the sensitivity of organisms to toxicants. Since most aquatic organisms are ectotherms, they are strongly influenced by temperature on their metabolic rates, behaviour activity and physiological processes [21]. Temperature can also modify the toxic effects of pollutants by influencing their bioavailability and toxicokinetics [9,21]. A review study conducted by Noyes et al. (2009) [23] generally stated that a temperature increase enhances the toxicity of contaminants. To date most metal toxicity studies also indicated an increased metal toxicity at higher temperatures [8,21,24,25]. As a limited factor phosphorus addition can have a directly affect the phytoplankton community (by altering biomass, size and nutrient quality), which can have an indirect positive effect on the zooplankton community [19,22]. However, extreme eutrophication is unfavourable for most zooplankton species [26]. Phosphorus is known to affect metal toxicity to freshwater algae [1,27,28]. Serra et al. (2010) [1] and Twiss and Nalewajko (1992) [28] for example found that an increased P supply significantly decreased copper (Cu) toxicity on algae biomass. Gao et al. (2016) [27] on the other hand found that Zn was more toxic to *Pseudokirchneriella subcapitata* cell densities at higher P supply. However, at the present too few studies have investigated the relationship between metal toxicity and P supply to draw consistent conclusions.

At the present very few 3-way interactions studies exist between nutrients/nutrition, temperature and a chemical factor, especially at the community level [6]. Heugens et al. (2003) [24] for example exposed a *Daphnia magna* population to different concentrations of Cd, altered food supply and different temperatures and found significant 3-way interactions between these factors. These interactions indicated that the adverse cadmium (Cd) effects on *Daphnia* population growth were enhanced at higher temperature, whereas higher food concentrations protected the *Daphnia* population from Cd toxicity. Although, not a lot of data about three-way interactions are 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 [1,28,29]. 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 community-level then interactions at the community-level may be expected in either direction (antagonistic or synergistic). A major concern is that conventional risk assessment approaches may underestimate the ecological impacts in case of a synergistic interaction between the toxicant and the environmental factor [8,30].

Against this background, this study was set up to identify how the toxicity of Zn is affected by temperature (T) and phosphorus (P) 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. Based on the limited evidence, it is speculated that Zn is more toxic at higher T and the reduced Zn toxicity at higher P supply, if any, may be more important in colder than in warmer water. In addition, we speculate that the synergistic effects of increased T and P supply on Zn toxicity are

less frequent at community than at population level. For this purpose a microcosm study was conducted with a freshwater plankton (zooplankton, phytoplankton and protozoa) dominated community, under semi-realistic conditions. After a pre-treatment period of three weeks the microcosms were simultaneously exposed to three different Zn treatments (background, HC5-plankton: 75 µg Zn/L and HC50-plankton: 300 µg Zn/L), two different T regimes (cold: 16-19 °C and warm: 21-24 °C) and two different P addition rates (oligotrophic P addition= 0.02 mg P L⁻¹ week⁻¹ and eutrophic P addition= 0.4 mg P L⁻¹ week⁻¹), for five weeks. During these five weeks the species densities, community composition, biodiversity, total chlorophyll concentrations and physico-chemical endpoints were measured.

MATERIALS AND METHODS

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). To mimic a plankton-dominated shallow freshwater system each microcosm was filled with a sediment layer of approximately 0.02 m, 14 L of 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 (NH₄NO₃: 1 mg N/L; KH₂PO₄ and K₂SO₄: 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. [31].

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 (oligotrophic P addition= 0.02 mg P/L a week and eutrophic P addition= 0.4 mg P/L a week) and three Zn treatments (background= control, HC5_{-plankton} [HC5 only based on the toxicity data of plankton species that was first normalized to the target properties of the water by using the Zn Biotic Ligand Model [32,33]] and HC50_{-plankton}) varied in a full 2x2x3 factorial design, with three replicates for the Zn amended and four for the control (= no Zn added).

The part of the experiment which focused on the Zn effects on the plankton community under cold oligotrophic conditions has already been published by Van de Perre et al. [31] and will not be addressed in detail in this manuscript but will be used to investigate possible interactions between Zn and the other factors.

Treatment applications and analyses

After a pre-treatment period of three weeks, the water bath containing half of the microcosms was heated up till 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 (KH₂PO₄: 0.19 mg P/L) was added to the microcosms assigned to simulate a freshwater system that is

exposed to a constant eutrophic P addition. For simplicity we will further refer to the microcosms who receive an additional phosphorous dose as eutrophic and those without as oligotrophic.

The two target Zn concentrations (75 µg/L: HC5-plankton and 300 µg/L: HC50-plankton) were determined by using the BLM software version 2.1.2 [34,35] which fits the SSD to BLM normalised chronic Zn toxicity data as explained in detail by Van Sprang et al. 2009 [35], based on the chemical properties of the water of the microcosms the day before the start of the treatments [31]. Zn was dosed by distributing a Zn stock solution (ZnCl₂) 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 correctly adjust the Zn concentration by additional spiking to compensate for losses from the water column. Additional Zn samples were taken just before a Zn dosing and at least 15 minutes after the dosing. An inductively coupled plasma mass spectrometry (ICP-MS; Agilent 7700x, in the He mode using ⁷²Ge 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 the 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. [31].

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 different plankton groups a total of 1 L of water was sampled from several random positions in the 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 different plankton groups 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. [36]. 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.

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 syringe) from every microcosm for total chlorophyll analysis using a BBE Moldaenke GmbH Algae Lab Analyser.

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 ($DO_{\text{evening day } x} - DO_{\text{morning day } x}$) were estimated by using the DO measurements [37]. Once a week the conductivity was measured by using a WTW LF 191 conductivity meter.

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

Total phosphorus (TP), Ammonium (NH_3) and $NO_2 + NO_3$ were sampled one day before the start of the treatments and two and five weeks after the first treatment and were analysed using a Skalar 5100 auto analyser.

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.

Data analysis

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 abundance value. This was done to down-weight high abundance values and to approximate a log-normal distribution of the data (see [38] for rationale).

Interactive effects between Zn and the different stressors on the biological endpoints (plankton abundance) and physico-chemical parameters for the different environmental conditions and sampling days were determined by comparing observed values with values predicted with the independent action model [39]. The different biological and physico-chemical endpoints were differentiated in different levels of organisation [Species level (1); group level (2): i.e. cladocera, chlorophyta; diversity (3): Shannon index; individual physico-chemical parameters (4): i.e. total chlorophyll, BOD₅, DO; community level (5)] and interactive effects were determined at the different levels or organisation. Firstly, three-way ANOVA were performed to determine the significance ($p < 0.05$) of the three-way and two-way interaction terms for all 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. Secondly, the observed combined effects were compared with the predicted combined effects (independent action: sum of their single effects [39]) to determine whether the observed interaction was synergistic (observed Zn effect more than additive) or antagonistic (observed Zn effect less than additive). Only consistent (statistically significant interactions for at least 2 consecutive sampling dates) ANOVA results that showed the same type of interaction (synergistic or antagonistic) were considered reliable.

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 [42]. 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 together with 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 [40]. This test was conducted for each sampling day, using the ln-transformed intended doses 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) [43].

Additionally the Williams test (analysis of variance) as incorporated in the Community Analysis software [44,45] 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 [40,46] on the 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 2 consecutive sampling dates.

RESULTS

Zinc and phosphorous concentrations and temperature

During the first week the dissolved Zn concentrations in the 75 and 300 µg Zn/L treatments were below their target concentration for the cold and warm microcosms (Table 1; **Figure S1 and table S1**). After one week the target Zn concentrations of the

cold and warm microcosms were achieved (Table 1). Between weeks 1-5 the average dissolved Zn concentrations of the cold eutrophic microcosms were within 9.3% of the target concentration at the lowest dose and within 3.4% at the highest dose. Under warm conditions the average dissolved Zn concentration between week 1 and 5 were generally 24% lower than those under cold conditions. The average dissolved Zn concentration between week 1-5 in the warm oligotrophic and warm eutrophic microcosm were within 17% and 23% of the target concentration at the lowest dose and within 26% and 23% at the highest dose, respectively. Throughout this paper we will further refer to the nominal concentrations (75 and 300 µg Zn/L) for simplicity.

Throughout the experiment the SRP concentrations ($\pm$ standard deviation) in the controls, low and high Zn treatments, under the o conditions, increased weekly (Figure S2). Under the oligotrophic P addition conditions the SRP concentrations ranged up till 46.5 µg P/L and the eutrophic P addition till 837.2 µg Zn/L. The SRP concentration in the microcosms did not show any consistent Zn or T effect (Table S2 and S3).

During the experiment the mean temperature ($\pm$ standard deviation) of the cold microcosms was 17.8 ± 0.7 °C and 22.3 ± 0.9 °C in the warm microcosm.

Combined effects and interactions between Zn and P, T

Zooplankton

At species level the only consistent Zn $\times$ T $\times$ P interactions at the lowest Zn treatment were calculated for *Cyclopoida* sp.. Further analysis revealed that under eutrophic conditions the Zn (low) $\times$ T interactions for the *Cyclopoida* sp. were consistent synergistic after 7 and 14 days of treatment (Table 2 and S7 and S8). At the highest Zn concentration consistent Zn $\times$ T interactions were calculated for copepod as a group

and more in particular the nauplii (Table 2 and 3 and S9 and S10, Figure 1 B). The cladocerans as a group (Figure 1 A), with *S. vetulus* in particular, revealed a consistent Zn $\times$ T interactions at the highest Zn treatment. At the highest Zn treatment and under eutrophic conditions these interactions were significantly synergistic for the Copepoda, nauplii and antagonistic for the Cladocerans as a group and *S. vetulus* (Table 2 and 3). As an example, figure 2 A illustrates the Zn and temperature effects and their interactions of the cladocerans after 35 days of exposure under the different P conditions. The rotifers were the only zooplankton group that didn't showed any consistent interaction at the species or group level (Table 2 and 3).

The PRC analysis, that was conducted with the zooplankton data from all the different treatments, visually revealed a clear temperature effect on the zooplankton community structure (Figure 3 A). The zooplankton community composition was consistently affected (3-way ANOVA on PCA-scores) by temperature and by the highest Zn treatment (Table S11). After 14 days of exposure and onwards significant Zn $\times$ T interactions (Monte Carlo permutation test) were observed at the zooplankton community composition level at the highest Zn treatment under eutrophic conditions (Table S12). The Shannon index for the zooplankton showed no consistent interactions.

A consistent NOEC_{community} of 75 μ g Zn/L was calculated for each of the treatment regimes, indicating a significant difference in zooplankton community structure between the controls and the highest Zn treatments. Zn mainly adversely affected the cladocerans (Figures 1 A) like *Daphnia longispina*, *Simocephalus vetulus* and *Chydorus sphaericus* and this could differ among treatment regimes. How the zooplankton community was affected differently by the combination of Zn and the other stressors is more explained in detail in SI (Figure S3, S4 and S5 and Table S4, S6 and

S13). The species abundances of the zooplankton and the other plankton groups are given in the SI (Table S5).

Phytoplankton

Consistent Zn $\times$ T $\times$ P interactions were only observed at the highest treatment for *Anisonema acinus*, however, further analysis revealed no consistent two-way interaction effects for any of the factors (Table S8). At the highest Zn treatment consistent Zn $\times$ T interactions were calculated for 2 different taxa and 2 phytoplankton groups (Table 4): the Bacillariophyceae (single cell diatoms) and Cryptophyta (*Cryptophyta* sp.1) (Table S7 and S9). For the Bacillariophyceae (group & single cell diatoms) and *Cryptophyta* sp.1 (Figure 1 C) these Zn $\times$ T interactions were only significantly antagonistic and under oligotrophic conditions while significantly antagonistic for the Cryptophyta as a group under all P conditions (Table 2 and 3).

At the highest Zn treatment and under warm conditions consistent synergistic Zn $\times$ P interactions were found for the Chlorophyta and Cryptophyta as a group (Table 3). These interactions were not found under cold conditions.

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 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 (Table S11). 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. *Cryptophyte* sp. 1 and *Rhodomonas* sp.) and indicated an increase in abundance due to the Zn treatments (Figure 5 B). The effects of Zn, in combination with T and, or P on the phytoplankton community are explained in more detail in SI (Figure S3, S4 and S6 and Table S4, S6 and S13). Under warm eutrophic conditions and under cold eutrophic conditions a NOEC_{community} of 75 µg Zn/L was calculated for the phytoplankton community (Table S13). However, under warm oligotrophic conditions the phytoplankton community composition was consistently affected at 75 µg Zn/L and a NOEC_{community} of <75 µg Zn/L was calculated.

At the community level significant Zn × T interactions were found after 7 and 14 days of treatment under eutrophic conditions at the highest Zn treatment (Table S12). No consistent interactions were calculated for the phytoplankton biodiversity (Shannon index) (Table S11). Significant Zn × T × P interactions were observed for the total chlorophyll (Figure 1 D) concentration during the last 3 weeks of exposure at the lowest Zn and during the last 2 weeks at the highest Zn treatment (Table S17). Under oligotrophic conditions, these 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). No consistent Zn × T interactions were observed under eutrophic conditions. As an example, figure 2 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.

Protozoa

At the species level consistent Zn $\times$ T $\times$ P interactions were calculated for the only algivorous/bacterivorous ciliate *Nassula* sp. at the highest Zn treatment (Table S5). However, further analysis revealed no consistent two-way interaction effects for any of the factors. None of the protozoa species showed a similar interactive effect (synergism or antagonism) for at least 2 consecutive sampling dates (Table 2 and 3). At the group level the ciliates as a group showed a consistent synergistic Zn $\times$ T interactions under eutrophic conditions at the highest Zn treatment (Table S7 and S9). For the protozoa no consistent interactions were found for biodiversity (Shannon index) but at the community composition level a consistent Zn $\times$ T (between 21 and 28 days of treatment) was found under oligotrophic conditions at the highest Zn treatment (Table S12). 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 3C). At the highest Zn treatment the protozoa community composition was consistently affected from 14 days onwards (Table S11). At the low Zn treatment eutrophication had a significant effect on the protozoa community after 7 and 14 days of treatment. How the protozoa community was affected by to the combination of Zn and the other stressor is more explained in detail in SI (Figure S3, S4 and S7 and Table S4, S6 and S13), but since this is not within the scope of this manuscript it will not be discussed any further. Under warm oligotrophic conditions the protozoa community composition was unaffected by the Zn treatments and a NOEC_{community} of >300 μ g Zn/L were found (Table S13). The protozoa communities under the other treatment regimes were only affected by the highest Zn treatments revealing a NOEC_{community} of 75 μ g Zn/L.

Community metabolism and general chemical properties of the water

427 Generally the DO levels, DOC (Figure 1 E) and pH (Figure 1 F) of the Zn controls were
428 consistently lower under warm conditions (Table S16). The microcosms under warm
429 conditions were characterised by a significantly higher conductivity (Table S1). At the
430 end of the experiment the pH started to increase in the warm eutrophic microcosms.
431 The eutrophic P addition had a short term lowering effect on the DO (morning and
432 primary production), pH and NH₃ (Table S3). Under eutrophic conditions the DOC
433 concentrations were consistently lower and the conductivity increased with increasing
434 P throughout the experiment (Table S3).

435 At the lowest Zn treatment consistent Zn × T × P interactions were found for the DOC
436 (Table 5). Further analysis revealed consistent Zn × T interactions under eutrophic
437 conditions and consistent Zn × P interactions under cold conditions (Table S22). At the
438 highest Zn treatments consistent Zn × T interactions were found for the DOC, DO_{mean},
439 net primary production (DO_{net}), pH_{mean} and BOD₅. The Zn × T interactions for the BOD₅
440 concentrations and the pH_{mean} were consistent under both P conditions. However, the
441 Zn × T interactions of the DOC, DO_{mean} and DO_{net} were only consistent under
442 oligotrophic conditions (Table S22).

443 Under eutrophic conditions the DOC was adversely affected by Zn in both the cold
444 (NOEC: 75µg Zn/L) and the warm (NOEC: <75µg Zn/L) microcosms. However, under
445 warm oligotrophic conditions, no Zn effect on the DOC was observed (Figure 1 E). At
446 the highest Zn treatment the DO was short term affected and significantly lower under
447 normal temperature conditions (Table S3). The pH was consistently significantly lower
448 at the highest Zn concentration and this for all the different regimes. The conductivity
449 levels were not affected by Zn under eutrophic and warm conditions and the NH₃
450 concentrations were significantly higher in the cold eutrophic Zn treatments. In the
451 eutrophic microcosms the BOD₅ levels significantly decreased with increasing Zn while

in the oligotrophic microcosms the BOD₅ were only consistently affected at the highest Zn concentration (Table S3).

All the physico-chemical water parameters data and how they were affected by the combination of Zn and the other stressor is more explained in detail in the SI (Table S1 and S3).

DISCUSSION

Zinc concentrations

During the first week the Zn rapidly disappeared from the water column in all the treatments. This is most likely the result of losses due to sorption by the sediment and biota. Starting after one week of exposure and onward, the target concentrations of the cold and warm microcosms were met, presumably because an equilibrium was reached between the water column and the sediment. Under warm conditions however the average dissolved Zn concentration was generally lower than those under colder conditions. This is probably due to the effect temperature has on sorption and uptake of Zn. 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.

Zn interactions at the population and community level

Throughout the experiment only 3% of the taxa (5 out of 156) showed a consistent interactive effect and all of them were Zn × T interactions (2 synergistic; 3 antagonistic). However at the group level 36% (5 out of 14) of the groups showed a Zn (high) × T interaction were 4 were antagonistic (lower Zn effect at higher T) and 1 synergistic (higher effect at higher T). Our results are in line with Jackson et al. (2016) [10] who

475 found that the mean interactive effects at the population level are additive and
476 antagonistic at the community level. One of the possible explanations for the limited
477 amount of taxa that showed a consistent interactive effect is the fact that biotic
478 interactions (indirect effects) within the community can mask or dampen possible
479 interactive effects at the population level [11]. Indirect effects are frequently observed
480 in microcosm studies [31,47,48] and can be the result from a reduced food competition
481 (or predation) [49]. One of the examples of this was observed during this study was at
482 the highest Zn treatment, where clear Zn effects were mainly observed for the
483 cladoceran populations in the cold (oligotrophic and eutrophic) and warm oligotrophic
484 microcosms (Figure 4 illustrates a schematic overview of the observed effects of the
485 highest Zn treatment on the ecosystem structure of plankton-dominated microcosms
486 for the different treatment conditions). These decrease in abundance probably resulted
487 in an indirect positive, top-down effect on the nauplii abundances and hypothetically
488 on the rest of the plankton community [48,50]. By reducing the number of Cladocerans
489 the Zn treatments indirectly increased the relative abundance of small (< 10µm)
490 Cryptophyta species (e.g. *Rodomonas* sp., *Cryptophyta* sp. 1 and 2) and other fast
491 growing and zooplankton grazing sensitive species (e.g. *Monoraphidium* sp.,
492 *Scenedesmus* sp. 2 and *Haematococcus* sp.), at the expense of the filamentous algae
493 (e.g. *Mougeotia* sp., *Anabaena* sp., *Pseudoanabaena* sp.) or colony forming
494 (*Scenedesmus* sp.1, *Mougeotia* sp., *Uroglana* sp., *Fragilaria* sp.) algae (Figure 3 B).
495 The observed phytoplankton patterns (PRC species scores) in figure 3 B are more
496 reflected by a reduced grazing pressure caused by the reduction of the Cladocerans
497 by the Zn treatments [48,51]. The dominance shift from filamentous algae groups to
498 small species (relative low contribution to the total chlorophyll) could possibly explain
499 the decline in total chlorophyll at the highest Zn treatment. Another possible

explanation for the reason why interactions are found more frequent 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. rest eggs) in later samplings. The disappearance of this species can be compensated by the abundance increase of a species with the same ecosystem function (functional compensation) [48]. Although these abundance fluctuations are a natural phenomenon it can limit your statistical power and the observation of consistent effects. By combining the different species into functional groups we partly included functional compensation and probably increased the statistical power.

Understanding the effects which each stressor affects the population or community responses, could help interpret the observed combined effects and interactions. The results of earlier studies that investigated the effects of experimental warming on zooplankton have been highly variable, going from negative [52] to little [19] or no clear effect [26]. The temperature increase during our study had a clear effect on zooplankton community (Table S4). 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. oblonga*) temperature effect. This can probably be explained due the fact that warming can affect competitive interactions and increase top-down regulation [16,19,21].

Most authors observed an increased metal toxicity with increasing temperature due to an increased uptake and accumulation of the metal by the organism [8,17,21,53]. Our study only partly confirmed this by revealing synergistic Zn $\times$ T interactions under eutrophic conditions for the copepoda. Under eutrophic conditions synergistic Zn $\times$ T interactions were observed for the *Cyclopoida* sp. at the low Zn concentration and at the high Zn concentration for the Copepoda as a group, with nauplii in particular. Under

normal conditions Cyclopoida are considered to have a higher tolerance to Zn stress [31,54,55]. Under warm hypereutrophic conditions, however the nauplii were adversely affected at even the lowest Zn concentration and the Copepoda as a group at the highest concentration. The cladocerans as a group and *S. vetulus* on the other hand showed an antagonistic Zn (high) $\times$ T interactions under eutrophic conditions, indicating a lower Zn effect at higher T. Furthermore, all the observed consistent Zn $\times$ T interactions effects for phytoplankton taxa and groups were antagonistic. 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 [11].

Although eutrophic P addition by itself only slightly affected the overall Zn toxicity, it clearly influenced its toxicity (Table 2 and 3). It has already been shown that nutrient addition can indirectly (by altering biomass, size and nutrient quality of phytoplankton) have a positive effect on the zooplankton community [18,19,22] but are toxic for most zooplankton species under extreme eutrophication [26]. Under warm conditions all the observed consistent Zn $\times$ P interactions in this study (Cryptophyta and Chlorophyta) were synergistic, indicating a higher Zn effect at higher P. Additionally it can be observed that the consistent Zn $\times$ T interactions for all the species, for most of the groups and for the zooplankton and phytoplankton community composition were only found under eutrophic conditions. The protozoa community was the only plankton community that showed a consistent Zn $\times$ T interactions under oligotrophic condition. Our results are in line with the results of a recent theoretical, mechanistic fate and effect modelling study conducted by De Laender et al. (2015) [56]. In this study Laender et al. 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 [56].

In our study significant Zn $\times$ T $\times$ P interactions were observed for a very limited amount of taxa and for total chlorophyll. After further analysis the total chlorophyll showed significant Zn (high) $\times$ T antagonistic interactions under oligotrophic conditions. Under eutrophic conditions no consistent interactions were observed for the total chlorophyll. The results indicate that the primary producers in this study were not more vulnerable to metal stress under phosphorus limitation at warmer temperatures. It is possible that in this study the phosphorus was not limiting enough to induce this effect.

Although protozoa are an important link between the microbial loop and the pelagic food web [49] very few community studies [31,54] have included them to investigate the effects of toxicants. In our study only the ciliates as a group showed an consistent antagonistic Zn $\times$ T interactions. Most likely the protozoa community was less affected by Zn, T or phosphorous addition and more by food web interactions [57].

Another important observation is the fact that most of the consistent interactions were observed at the group and community structure level and not at the species level nor for biodiversity. Understanding or predicting community responses are complicated due to redundancy (different species perform same ecosystem function, e.g. functional compensation), resilience (e.g. speed of recovery) and environmental stochasticity that can dampen possible interactive effects [11]. Understanding or predicting community responses to multiple stressors are complicated due to species interactions and should be investigated further.

Conclusions and implications for Zn risk assessment

To our knowledge very few studies have been conducted to investigate the adverse effects of Zn on the whole plankton community [31,58,59] and none of these studies have investigated the combined effect of Zn (or any other metal) with temperature and/or P addition. Aquatic ecosystems with shallow pond in particular are very vulnerable to warming and eutrophication [18,20,22]. Temperature stress can occur due to heated waste water discharges by factories, power plants or households or by climate extremes [21]. 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 [3,8,18].

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 [47]. Since microcosm studies can be difficult to interpret Brock et al. [60] 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 eutrophic conditions (cold and warm) the multivariate analysis (PRC) conducted on the different plankton group revealed a $\text{NOEC}_{\text{community}}$ of 75 $\mu\text{g Zn/L}$. At 75 $\mu\text{g Zn/L}$ the phytoplankton community structure was consistently affected under warm oligotrophic conditions and a $\text{NOEC}_{\text{community}}$ of <75 $\mu\text{g Zn/L}$ was calculated. This is possibly due to

598 the indirect effect the by Zn declined *D. longispina* (NOEC <75 µg Zn/L) populations
599 had on the phytoplankton community (especially for *Cryptophyta* sp.1).

600 Additionally to the effect on the plankton community, Zn also indirectly affected the
601 chemical properties of the water over time and hereby the metal bioavailability. During
602 the experiment the pH and DOC levels were not only affected by temperature and
603 eutrophication but also by the Zn treatments (with the exception of the warm
604 oligotrophic microcosms and especially at high Zn). The consistent Zn effect on the
605 DOC (cold eutrophic: NOEC 75 µg Zn/L; warm eutrophic: NOEC <75 µg Zn/L) and
606 BOD₅ (cold eutrophic: NOEC <75 µg Zn/L; warm eutrophic: NOEC <75 µg Zn/L) can
607 also be an indirect indication that the microbial loop and the pelagic food web were
608 affect by Zn [49]. By lowering the pH and the DOC, Zn becomes more bioavailable and
609 therefore more toxic [32,35].

610 During this study 3% of the species (40% synergistic; 60% antagonistic) and 36% of
611 the functional groups showed a consistent Zn × T interaction (20% synergistic; 80%
612 antagonistic) and thus demonstrating that the Zn toxicity effects on the plankton
613 community can be affected by temperature. This is confirmed by the observation of
614 consistent Zn × T interactions at the community structure for each of the different
615 plankton communities. Another important observation is the fact that 82% of the all the
616 consistent interactions at species or group level were observed at the highest Zn
617 treatment and not at the lowest. Eutrophic P addition by itself only slightly affected the
618 overall Zn toxicity, revealing only 2 consistent Zn × P interaction under warm conditions.
619 However, of all the interactions at the species and group level 90% were found under
620 eutrophic conditions. Additionally, with exception of the protozoa, all the observed
621 consistent Zn × T interactions at the community composition level were only found
622 under eutrophic conditions. It can thus be observed that eutrophic P addition clearly

influenced the combined effect between Zn and T. Therefore both temperature and P should be included in conventional risk assessment approaches, since at the present they don't include interaction between the toxicant and the environmental factor, which may under-or overestimate the toxic effects.

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Table 1: Average measured dissolved Zn concentrations ($\pm$ standard deviation) in the 75 (low Zn) and 300 (high Zn) $\mu\text{g Zn/L}$ treatments and the different multiple stressor treatment regimes during the first week of treatment (^a) and between weeks 1-5 (^b). * From Van de Perre et al. [31]

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

Table 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 the different plankton species at different treatment regimes. The interaction type is based on comparing observed and predicted effects (independent action) based on De Coninck et al. [32]. Only species for which a consistent (for at least 2 consecutive sampling dates) interaction (three-way ANOVA) was found and additionally, showed an consistent interaction effect are represented here. See supportive information table S7 and S8 for the statistical details on all species that showed consistent interactions.

Taxa	Day	Treatment regime	Interaction factors	Zn treatment	p	Interaction type
Zooplankton						
<i>Cyclopoida</i> sp.	14	Eutrophic	Zn $\times$ T	L	0.006	S
	21	Eutrophic	Zn $\times$ T	L	0.001	S
nauplii	28	Eutrophic	Zn $\times$ T	H	0.001	S
	35	Eutrophic	Zn $\times$ T	H	0.001	S
<i>Simocephalus vetulus</i>	21	Eutrophic	Zn $\times$ T	H	0.049	A
	28	Eutrophic	Zn $\times$ T	H	0.022	A
Phytoplankton						
Single cell diatoms	14	Eutrophic	Zn $\times$ T	H	0.049	A
	21	Eutrophic	Zn $\times$ T	H	0.032	A
Cryptophyte sp. 1	21	Eutrophic	Zn $\times$ T	H	0.005	A
	28	Eutrophic	Zn $\times$ T	H	0.012	A

Table 3: 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 and the different plankton groups at different treatment regimes. The interaction type is based on the observed and predicted effects based on De Coninck et al. [32]. Only groups for which a consistent (for at least 2 consecutive sampling dates) interaction (three-way ANOVA) was found and additionally, showed an consistent interaction effect are represented here. See supportive information table S9 and S10 for the statistical details on all species groups that showed consistent interactions.

Taxa group	Day	Treatment regime	Interaction factors	Zn treatment	<i>p</i>	Interaction type
Zooplankton						
Copepoda	28	Eutrophic	Zn × T	H	0.001	S
	35	Eutrophic	Zn × T	H	0.001	S
Cladocera	28	Eutrophic	Zn × T	H	0.003	A
	35	Eutrophic	Zn × T	H	0.006	A
Phytoplankton						
Bacillariophyceae	14	Eutrophic	Zn × T	H	0.048	A
	21	Eutrophic	Zn × T	H	0.02	A
Cryptophyta	7	Warm	Zn × P	H	0.001	S
	14	Warm	Zn × P	H	0.04	S
	14	Oligotrophic	Zn × T	H	0.009	A
	21	Oligotrophic	Zn × T	H	0.032	A
	21	Eutrophic	Zn × T	H	0.001	A
Chlorophyta	28	Eutrophic	Zn × T	H	0.007	A
	7	Warm	Zn × P	H	0.029	S
	14	Warm	Zn × P	H	0.007	S
Total chlorophyll	21	Oligotrophic	Zn × T	L	0.03	A
	28	Oligotrophic	Zn × T	L	0.034	A
	28	Oligotrophic	Zn × T	H	0.013	A
	35	Oligotrophic	Zn × T	H	0.013	A
Protozoa						
Ciliate	21	Eutrophic	Zn × T	H	0.01	A
	28	Eutrophic	Zn × T	H	0.019	A

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Table 4: Summary of the number of statistically significant (Three-way ANOVA and Monte Carlo permutation test) 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 on at least one time point. The N column indicated the total number of assessed entities at the different levels of organization. The number of consistent interactions are given between parentheses. See supportive information table 2,3 and S7, S9, S12 and S23 for details

			L	H	L	H	L	H
Plankton group	level	N	Zn x T	Zn x T	Zn x P	Zn x P	Zn x T x P	Zn x T x 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 (1)
	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 (0)	4 (0)	6 (0)	2 (0)	6 (1)
	Ciliate group ^c	6	0 (0)	2 (0)	1 (0)	2 (0)	1 (0)	3 (1)
	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)

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

Table 5: Summary of the number of statistically significant (Three-way ANOVA) interactive effects between Zn (Low: L and high: H) and the different factors (P and T) of the total chlorophyll pH_{mean} , Dissolved organic carbon (DOC), mean dissolved oxygen (DO_{mean}), net primary production (DO_{net} : $DO_{evening\ day\ x} - DO_{morning\ day\ x}$) and Biological oxygen demand after 5 days (BOD_5) on at least one time point. The number of consistent interactions are given between parentheses. See supportive information table S17 and S22 for details

	L	H	L	H	L	H
Paremeter	Zn x T	Zn x T	Zn x P	Zn x P	Zn x T x P	Zn x T x P
Total chlorophyll	0 (0)	0 (0)	0 (0)	0 (0)	1 (1)	1 (1)
BOD_5	0 (0)	1 (1)	1 (0)	1 (0)	1 (0)	1 (0)
DO_{mean}	0 (0)	1 (1)	1 (0)	0 (0)	0 (0)	1 (0)
DO_{net}	0 (0)	1 (1)	1 (0)	0 (0)	1 (0)	1 (0)
pH_{mean}	0 (0)	1 (1)	1 (0)	0 (0)	0 (0)	0 (0)
DOC	1 (0)	1 (1)	0 (0)	0 (0)	1 (1)	1 (0)

865 **Figures**

866 Figure 1: Dynamics of Cladocerans (A), copepod nauplii (B), *Cryptophyta* sp. 1 (C),
867 total chlorophyll concentration (D), Dissolved organic carbon concentration (E) and
868 pH_{mean} dynamics (F) under cold oligotrophic [From Van de Perre et al 2016], cold
869 eutrophic, warm oligotrophic and warm eutrophic conditions. The means (standard
870 deviation as error bars) of the different parameters for each Zn treatment and time
871 point are shown. Calculated no-observed-effect concentrations are plotted above the
872 figures. ^a: Consistent Zn (high) × T interactions; ^b: Consistent Zn (low) × T interactions;
873 ^c: consistent Zn (low) × P interaction.

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886 Figure 2: Log cladoceran abundance after 35 days of treatment (A) and total
 887 chlorophyll concentrations after 28 days of treatment (B) and predicted values using
 888 the Independent Action model (IA) under oligotrophic and eutrophic conditions. Error
 889 bars indicate standard deviation. A: significant antagonistic Zn $\times$ T interaction; S:
 890 significant synergistic Zn $\times$ T interaction.

Figure 3: 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 μ g Zn/L), temperature (16-19 °C: Φ ; 21-24 °C: T) and P treatments (Oligotrophic P: Φ ; Eutrophic P: P) on the cold oligotrophic 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.

Figure 4: 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 oligotrophic/ cold eutrophic/ warm oligotrophic/ warm eutrophic 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.