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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,[a,*] Ivo Roessink,[b] Colin R. Janssen,[a] Erik Smolders,[c] Frederik De Laender,[d] Paul J. Van den Brink,[b,e] and Karel A.C. De Schamphelaere[a]

[a]Laboratory of Environmental Toxicology and Aquatic Ecology, Environmental Toxicology Unit, Ghent University, Gent, Belgium
[b]Wageningen Environmental Research, Wageningen, The Netherlands
[c]Division of Soil and Water Management, Katholieke Universiteit Leuven, Heverlee, Belgium
[d]Research Unit of Environmental and Evolutionary Biology, University of Namur, Namur, Belgium
[e]Aquatic Ecology and Water Quality Management Group, Wageningen University, Wageningen, The Netherlands

Abstract: Ecotoxicological studies mainly consist of single-species experiments evaluating the effects of a single stressor. However, under natural conditions aquatic communities are exposed to a mixture of stressors. The present study aimed to identify how the toxicity of zinc (Zn) is affected by increased temperature and increased phosphorus (P) supply and how these interactions vary among species, functional groups, and community structure and function. Aquatic microcosms were subjected to 3 Zn concentrations (background, no Zn added, and 75 and 300 μg Zn/L), 2 temperatures (16–19 and 21–24 °C), and 2 different P additions (low, 0.02, and high, 0.4 mg P L^{-1} wk^{-1}) for 5 wk using a full factorial design. During the study, consistent interactions between Zn and temperature were only rarely found at the species level (4%), but were 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 × temperature interactions were observed at 300 μg Zn/L and generally indicated a smaller effect of Zn at higher temperature. Furthermore, no clear indication was found that high P addition by itself significantly affected the overall effects of Zn on the community at any level of organization. Interestingly, though, 90% of all the Zn × temperature interactions observed at the species, group, and community composition level were found under high P addition. Collectively, the results of our study with the model chemical Zn suggest that temperature and phosphorus loading to freshwater systems should be accounted for in risk assessment, because these factors may modify the effects of chemicals on the structure and functioning of aquatic communities, especially at higher levels of biological organization. *Environ Toxicol Chem* 2018;37:2413–2427. © 2018 SETAC

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INTRODUCTION

Metal pollution, together with eutrophication and climate change, can pose risks to aquatic ecosystems (Intergovernmental Panel on Climate Change 2007; Serra et al. 2010; Moe et al. 2013). 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) are major challenges in environmental toxicology (Sumpter 2009; De Laender et al. 2011, 2015; Moe et al. 2013). The importance of this line of research is supported by climate models, which predict that climate change will induce a general temperature increase of 2 to 4 °C within the next century in temperate regions (Intergovernmental Panel on Climate Change 2007) and will also induce an increased phosphorus (P) loading from land to lakes and streams due to increased rainfall intensity (Jeppesen et al. 2010).

Conventional ecological risk assessment of chemicals is mainly based on single-species laboratory tests that evaluate the effects of a single stressor and are conducted under optimal standard (e.g., temperature, pH) conditions. However, in reality aquatic communities are exposed to multiple stressors under different and often rapidly changing environmental conditions (Eggen et al. 2004). Many studies have already shown that

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* Address correspondence to Dimitri.VandePerre@Ugent.be
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environmental conditions can significantly alter the responses of organisms to toxicants (Crain et al. 2008; Holmstrup et al. 2010; Laskowski et al. 2010; Moe et al. 2013; Jackson et al. 2016). The specific interaction type (synergistic: combined effect greater than expected; antagonistic: combined effect smaller than expected; additive: combined effect as expected) can also vary by biologic response level (i.e., populations, community) and trophic level (i.e., autotrophs, heterotrophs; Crain et al. 2008; Jackson et al. 2016). Jackson et al. (2016), for example, conducted a meta-analysis on 88 freshwater studies and found that the cumulative mean of the interactive effects was additive at the population level and antagonistic at the community level. Synergistic interaction occurred more frequently at the population level compared with the community level (Jackson et al. 2016). This might be a result of the fact that at higher levels of biological organization, compensatory species dynamics become more important (i.e., are not present at species level), which may explain the difference in interaction type between the population and the community level (i.e., composition, function, and diversity; Baert et al. 2016).

Temperature and P are 2 important environmental factors that can have major effects on planktonic community composition and functioning (Donker et al. 1998; Heugens et al. 2001; Moss et al. 2003; Winder and Schindler 2004; Mooij et al. 2005; Graham and Vinebrooke 2009; O'Connor et al. 2009; Winder et al. 2009; Özen et al. 2013; Šorf et al. 2014), and both are also well known to influence the sensitivity of organisms to toxicants (Twiss and Nalewajko 1992; Donker et al. 1998; Heugens et al. 2001, 2006; Holmstrup et al. 2010; Serra et al. 2010; Gao et al. 2016). Like most aquatic organisms planktonic organisms are ectotherms, and temperature strongly influences their metabolic rates, behavior activity, and physiological processes (Heugens et al. 2001). Temperature can also modify the toxic effects of pollutants by influencing their bioavailability and toxicokinetics (Heugens et al. 2001; Laskowski et al. 2010). A review by Noyes et al. (2009) stated that in general an increase in temperature enhances the toxicity of contaminants. To date most metal toxicity studies have also indicated increased metal toxicity at higher temperatures (Donker et al. 1998; Heugens et al. 2001, 2006; Holmstrup et al. 2010). As a limiting nutrient, variable P addition can directly affect the phytoplankton community by altering biomass, algal cells size, and nutrient quality, which can have an indirect positive effect on the zooplankton community (Moss et al. 2003; Šorf et al. 2014). However, extreme eutrophication is unfavorable for most zooplankton species (McKee et al. 2002). Phosphorus is known to both increase and decrease the metal toxicity to freshwater algae (Twiss and Nalewajko 1992; Serra et al. 2010; Gao et al. 2016). Serra et al. (2010) and Twiss and Nalewajko (1992), for example, found that an increased P supply significantly decreased the toxicity of copper (Cu) to algae, as indicated by increases in algal biomass, whereas Gao et al. (2016) found that zinc (Zn) was more toxic to *Pseudokirchneriella subcapitata* with regard to cell densities at higher P supply. However, at present, only a few studies have investigated the relationship between metal toxicity and P, so it is not possible to draw consistent conclusions.

Currently, very few 3-way interaction studies have been done considering nutrients/nutrition, temperature, and a chemical factor, especially at the community level. Heugens et al. (2003), 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 among 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. Although few data about 3-way interactions are available, it is known that under eutrophic conditions the accumulation of polyphosphates by microalgae is stimulated by increasing temperature, which could counteract metal toxicity (Twiss and Nalewajko 1992; Powell et al. 2008; Serra et al. 2010). Phosphorus is also depleted more quickly at warmer temperatures, which hypothetically makes the primary producers more vulnerable to metal stress under phosphorus limitation at warm temperatures. If these interactions continue at the community level, they may be expected to occur 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 (Holmstrup et al. 2010; Van Den Brink et al. 2016).

Against this background, the present study was set up to identify to what extent the toxicity of Zn is affected by higher temperature and by higher P supply and how these temperature and P effects on Zn toxicity vary between the levels of biological organization (population, functional group, and community) of a freshwater ecosystem. Based on the limited evidence just described, it is speculated that Zn is more toxic at a higher temperature. The reduced Zn toxicity at higher P supply, if any, may be more important in warmer than in colder water. In addition, based on the work of Jackson et al. (2016), we speculate that interactive effects are more common at higher organizational levels. To test this hypothesis, an indoor microcosm study was conducted with a community dominated by freshwater plankton (zooplankton, phytoplankton, and protozoa). After a pretreatment period of 3 wk, the microcosms were simultaneously exposed to 3 different Zn concentrations, 2 different temperature regimes (reference and a warmer regime), and 2 different P addition rates (reference, low P addition, and high P addition), for 5 wk.

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). The water bath was divided into 2 compartments, in which the water temperature could be regulated separately. To mimic a plankton-dominated shallow freshwater system, each microcosm was filled

with a sediment layer of approximately 2 cm and 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). Two snails (*Lymnaea stagnalis*) were added to every microcosm 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 the microcosms to stimulate phytoplankton growth starting 3 wk before the actual start of the experiment (i.e., the pretreatment period). During the pretreatment 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 setup of the test systems has been described by Van de Perre et al. (2016).

After the pretreatment period, the microcosms were exposed to 3 factors (P, temperature, and Zn) simultaneously. The experimental design consisted of 2 temperature treatments (cold: 16–19 °C, and warm: 21–24 °C), 2 P treatments (low P addition = 0.02 mg P/L/wk and high P addition = 0.4 mg P/L/wk), and 3 Zn treatments (background, 75 μg Zn/L, and 300 μg Zn/L) in a full $2 \times 2 \times 3$ factorial design, with 3 replicates for the Zn-amended and 4 for the control (= no Zn added) treatments. The background Zn level is defined as the control. The 2 target Zn concentrations (75 μg/L: representing the hazard concentration, 5th percentile [$HC5_{\text{-plankton}}$]; and 300 μg/L: representing the $HC50_{\text{-plankton}}$) were determined by Van de Perre et al. (2016).

The part of the experiment that 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. (2016) 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 the pretreatment period, the compartments of the water bath containing half of the microcosms was heated to 21 to 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 P (KH_2PO_4: 0.19 mg P/L, so a total of 0.2 mg P/L was added at 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 almost daily (see the Supplemental Data for exact days of dosing) by distributing a Zn stock solution ($ZnCl_2$) evenly over the water surface and mixing by gentle stirring. At frequent intervals 2 water samples (1 not filtered for measuring the total Zn concentration and 1 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 min 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 the control microcosms, and all other Zn samples 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. (2016).

Zooplankton, phytoplankton, and protozoa

Plankton (zooplankton, phytoplankton, and protozoa) were sampled from each microcosm every week, starting 1 d 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 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). 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; then the abundances were adjusted per liter Every subsample was settled overnight in sedimentation chambers, and at least 400 individuals were counted and identified along longitudinal transects according to Utermöhl (1958). 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 in addition classified as: algivorous, bacterivorous, algivorous + bacterivorous, epiplanktonic, mixotrophic, and predacious.

Prior to every biological sampling, 2 10-mL water samples were taken (15 cm below the water column at the center of every microcosm after gentle stirring with a syringe) from every microcosm for total chlorophyll analysis using a BBE Moldaenke Algae Lab Analyser.

Community metabolism and general chemical properties of the water

Measurements for dissolved oxygen (DO), temperature, and pH were performed (WTW 340i multimeter) in the morning

(at the start of the photoperiod) and evening (1 h before the end of the photoperiod) twice a week at mid-water depth, starting 1 d before the start of the treatments, from which the net primary production ($DO_{evening\ day\ x} - DO_{morning\ day\ x}$) was estimated (Downing and Leibold 2002). Once a week the conductivity was measured by using a WTW LF 191 conductivity meter.

Filtered (0.45 μm) and unfiltered water samples were taken before every biological sampling for nutrient analysis. A total organic carbon analyzer (TOC-5000; Shimadzu; limit of quantification 1.5 mg dissolved organic carbon [DOC]/L; method detection limit 0.5 mg DOC/L) was used for measuring the DOC and dissolved inorganic carbon (DIC). Measurements for total dissolved P and other elements, including Zn, were performed with ICP-MS (Agilent 7700x). Soluble reactive phosphorus, ammonium (NH_3), and $NO_2 + NO_3$ were analyzed using a Skalar 5100 autoanalyzer. Total phosphorus was analyzed by the ascorbic acid method. In addition, a standard 5-d biochemical oxygen demand (BOD_5) test (International Organization for Standardization 1989) was conducted weekly for every microcosm, using microcosm water passed through a 55-μm filter (Hydrobios) microcosm water.

Data analysis

Interactive effects between Zn and temperature or P were assessed for the biological endpoints (plankton abundance) and for physicochemical parameters for the different sampling days. These endpoints were differentiated at different levels of organization (species level, 1: group level, e.g., Cladocera, Chlorophyta, 2; individual physicochemical parameters, e.g., total chlorophyll, BOD_5, dissolved oxygen, 3; community level, 4); then interactive effects were determined at the different levels. 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 downweight high abundance values and to approximate a log-normal distribution of the data (see Van den Brink et al. 2000 for the rationale).

To identify how the toxicity of Zn is affected by both increased temperature and 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; Zn high: 300 μg Zn/L). First, a 3-way analysis of variance (ANOVA) was performed to determine the significance ($p < 0.05$) of the 3- and 2-way interaction terms for the first 4 types of endpoints. In case of a significant 3- or 2-way interaction, several more detailed 2-way interactions were conducted to determine the combinations of environmental conditions in which these interactions occurred. For example, when a significant Zn × temperature interaction was found, 2 additional 2-way ANOVA analyses were conducted (by using the low P and high P data separately) to investigate under which environmental P conditions this significant Zn × temperature interaction occurred. In addition, these 2-way ANOVA analyses provide a formal statistical test of the independent action model (De Coninck et al. 2013). 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; De Coninck et al. 2013) 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) for further details on how the predicted effects were calculated and how the observed interactions were assessed. Interactions were only considered consistent when the ANOVA revealed the same type of interaction (synergistic or antagonistic) for at least 2 consecutive sampling dates In that case the interaction was defined as a "consistent interaction."

The principal response curves method (PRC; Van den Brink et al. 2009) was used to analyze and illustrate the effects of Zn, temperature, and P treatments on the plankton community composition and was performed by using CANOCO 5.0 (Ter Braak and Smilauer 2002). 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 redundancy analysis. The Monte Carlo permutation test computed 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 (Van den Brink and Braak 1999). This test was conducted for each sampling day, using the ln-transformed nominal treatment factor as the explanatory variable, to assess the significance of the treatment effects for each sample date. Interaction was tested by entering the interactions between Zn treatment and the environmental factor (P or temperature) as explanatory variables and the used factors and treatment as covariables. In addition, 3-way ANOVAs were performed on the principle component analysis (PCA) samples scores of the different plankton groups to analyze the effects of Zn, temperature, and P treatments at the community level. For a more detailed description, see Van Wijngaarden et al. (2006).

RESULTS AND DISCUSSION

Zn, P, and temperature

Measured Zn and P concentrations and temperature. The target Zn concentrations were generally 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 1 and Supplemental Data, Figure S1 and Table S1). This was most likely the result of losses due to sorption by the sediment and biota. In the period thereafter (weeks 2–5), the target Zn concentrations in all microcosms were achieved to within 9 and 26% of the target concentrations in the cold and warm microcosms, respectively (Table 1). During the first week of the experiment, the average dissolved Zn concentration at the highest Zn treatment was generally higher in the cold microcosms in comparison with the warm microcosm (Table 1). The average dissolved Zn

TABLE 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 and between weeks 2 and 5

		Cold		Warm	
Week	Treatment	Low P[a]	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
2–5	Low Zn	77.9 ± 17.8	82.0 ± 20.0	62.0 ± 15.0	58.1 ± 18.3
1	High Zn	51.1 ± 13.9	54.8 ± 11.7	116.5 ± 81.6	164.4 ± 79.3
2–5	High Zn	287 ± 52.3	310.4 ± 55.9	222.5 ± 48.2	230.9 ± 55.9

[a]From Van de Perre et al. (2016).

concentration in weeks 2 to 5 in the warm microcosms was also generally lower than in the cold microcosms. (Table 1). This finding may be due to higher sorption by sediment and biota at higher temperature. It is possible that the lower Zn concentrations during the first week (compared to weeks 2–5) and under the warm condition reduced some of the Zn effects on the biota. For simplicity, we will refer to the nominal concentrations (75 and 300 μg Zn/L) from this point on.

The total dissolved P concentrations in the controls and low and high Zn treatments increased continuously throughout the experiment (Supplemental Data, Figure S2). By the end of the experiment the mean total dissolved P concentrations was 46 μg P/L under the low P addition and 840 μg P/L under the high P addition. The P concentrations in the microcosms were not consistently affected by Zn or temperature (Supplemental Data, Tables S2 and S3). The measured total dissolved P 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; Brönmark and Hansson 2005). Similarly, the microcosms under high P addition can be categorized as hypereutrophic (>100 μg P/L).

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 and 22.3 ± 0.9 °C, respectively.

General patterns of the effects of Zn, temperature, and P on community metabolism and general physicochemical properties of the water. Generally the dissolved oxygen, DOC (Figure 1A), and pH (Figure 1B) levels of the Zn controls were consistently lower under warm than under cold conditions (Supplemental Data, Tables S1 and S15). The microcosms under warm conditions were also characterized by a significantly higher conductivity (Supplemental Data, Table S1). At the end of the experiment the pH started to increase in the warm, high P addition microcosms (Figure 1B). The high P addition induced a short-term decrease of dissolved oxygen (morning and primary production), pH, and NH_3 (Supplemental Data, Table S3). Under high P addition the DOC concentrations were consistently lower and the conductivity increased with increasing P throughout the experiment (Figure 1A and Supplemental Data, Table S3).

Under high P addition the DOC was adversely affected (concentration was reduced) 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 1A). At the highest Zn treatment the dissolved oxygen was affected for a short term and was significantly lower than the control Zn under cold temperature conditions (Supplemental Data, Table S3). The pH was consistently significantly lower at the highest Zn concentration and this was the case 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 BOD_5 significantly decreased with increasing Zn, whereas in the low P addition microcosms the BOD_5 values were only consistently affected at the highest Zn concentration (Supplemental Data, Table S3). 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 affected by Zn (Brönmark and Hansson 2005). The physicochemical water parameter data and how they were affected by the combination of Zn and the other stressors are given in the Supplemental Data (Tables S1 and S3).

General patterns of the effects of Zn, temperature, and P on the plankton community. The results of earlier studies that investigated the effects of experimental warming on zooplankton showed highly variable effects, ranging from negative (Strecker et al. 2004) to small (Šorf et al. 2014) or no clear effects (McKee et al. 2002). The temperature increase during our study had a clear effect on the zooplankton community (Figure 2A and Supplemental Data, Tables S4 and S11). The PRC diagram shows visually that temperature had the largest effect on the zooplankton community structure (Figure 2A). Many of the zooplankton taxa experienced a clear short-term positive (*Alonella nana* and *Alona rectangula*), negative (Cyclopoida), or variable (from clearly positive to clearly negative: *Lecane* group *luna* and *lunaris* and *Colurella oblusa*) temperature effect. This may be explained by the fact that warming can affect competitive interactions and increase top-down regulation (Heugens et al. 2001; Winder and Schindler 2004; Šorf et al. 2014). Detailed information on how the various plankton species were affected differently by the combination of Zn and the other stressors is provided in the Supplemental Data (Figures S3–S7 and Tables S4, S6, and S12–S15). In addition to temperature, the zooplankton community composition was

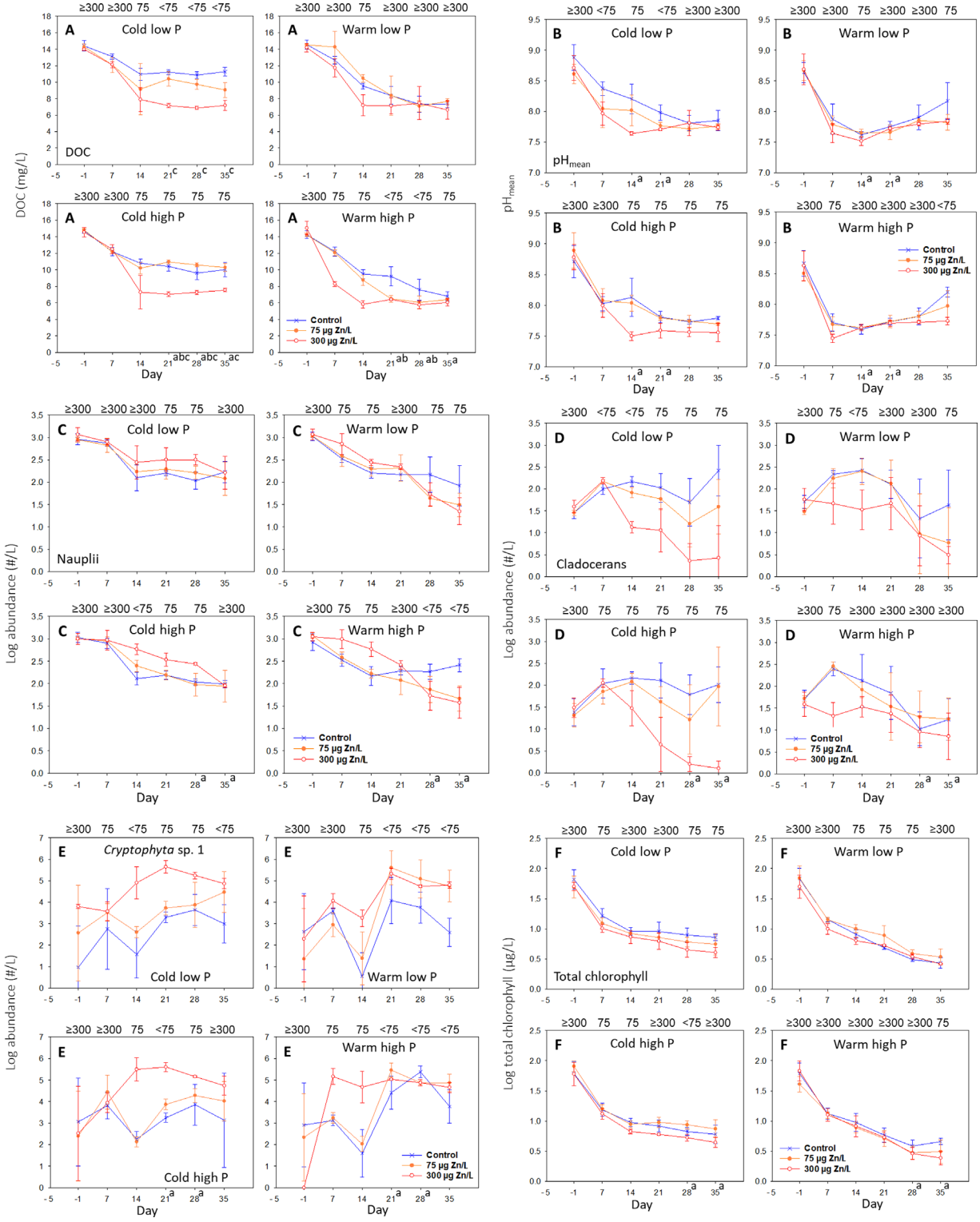

FIGURE 1: Dissolved organic carbon (DOC) concentration (**A**), pH_{mean} dynamics (**B**), dynamics of copepod nauplii (**C**), cladocerans (**D**), and *Cryptophyta* sp. 1 (**E**), and total chlorophyll concentration (**F**), and under cold low phosphorus (P) addition (from Van de Perre et al. 2016), 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) × temperature interactions; [b], consistent Zn (low) × temperature interactions; and [c], consistent Zn (low) × P interaction.

consistently affected (3-way ANOVA on PCA scores) by the highest Zn treatment (Supplemental Data, Table S11). Zinc mainly adversely affected the cladocerans (Figure 1D) like *Daphnia longispina*, *Simocephalus vetulus*, and *Chydorus sphaericus*, and this could differ among the different P and temperature treatment regimes (Supplemental Data, Figure S5).

A clear main Zn effect on the phytoplankton community structure was visually revealed after we conducted a PRC analysis

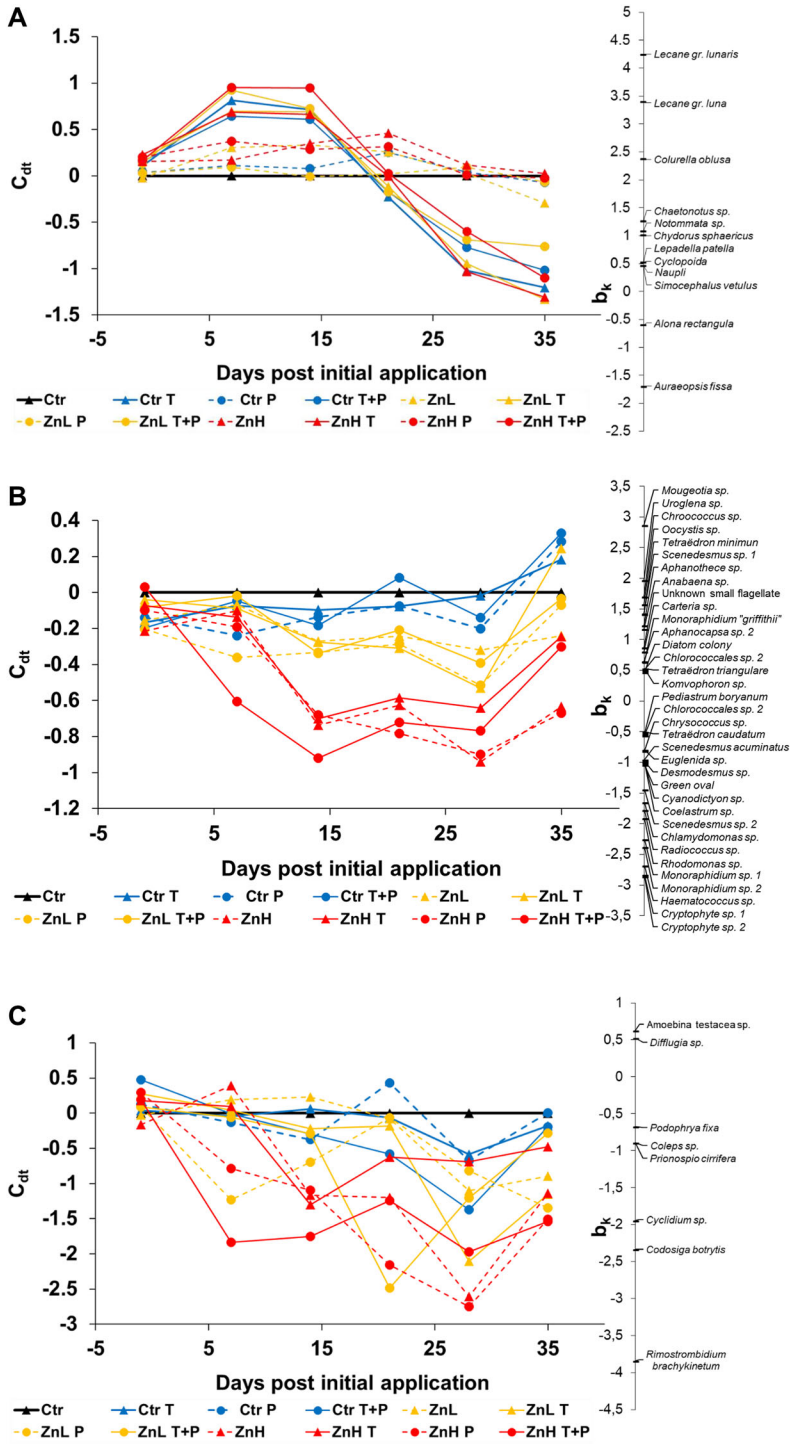

FIGURE 2: Principal response curve (PRC), resulting from analysis of the global zooplankton (**A**), phytoplankton (**B**), and protozoa data (**C**), indicating the effects of the different zinc (Zn: control [Ctr]: no Zn added; ZnL: 75 μg Zn/L; ZnH: 300 μg Zn/L), temperature (16–19 °C: Φ; 21–24 °C: temperature [T]) and phosphorus (P) treatments (low P: Φ; high P: P) on the cold low P addition Zn control microcosms. The vertical axis represents the differences in community structure of the treatments compared with 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.

using the phytoplankton data from all the different treatments (Figure 2B). The phytoplankton community composition was affected (3-way ANOVA on PCA scores) by the highest Zn treatment after 14, 28, and 35 d of exposure (Supplemental Data, Table S11). The species weights (b_k score) indicated that most filamentous cyanobacteria (*Pseudanabaena* sp., *Anabaena* sp., and *Woronichinia* sp.), several nonfilamentous cyanobacteria (*Chroococcus* sp. and *Aphanothece* sp.) and colony- or filament-forming algae (*Scenedesmus* sp.1, *Mougeotia* sp., *Uroglena* sp., and *Fragilaria* sp.) 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 2B). 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 1F; Van de Perre et al. 2016).

One of the possible explanations for the dominance shift from filamentous algae groups to small species at the highest Zn treatments is biotic interactions (indirect effects; Van de Perre et al. 2016). Indirect effects are frequently observed in microcosm studies (Jak et al. 1996; Fleeger et al. 2003; Van de Perre et al. 2016) and can result from reduced food competition (or predation; Brönmark and Hansson 2005). One of the examples of this phenomenon was seen 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 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. The decrease in abundance of the large cladocerans probably resulted in an indirect positive, top-down effect on the nauplii abundances and hypothetically on the rest of the plankton community (Jürgens 1994; Fleeger et al. 2003). By reducing the number of cladocerans, the Zn treatments indirectly increased the relative abundance of small (<10 μm) Cryptophyta species (e.g., *Rhodomonas* sp., *Cryptophyte* sp. 1 and 2) and other fast-growing and zooplankton-grazing sensitive species (e.g., *Monoraphidium* sp. 1 and 2, *Scenedesmus* sp. 2, and *Haematococcus* sp.), at the expense of the filamentous algae (e.g., *Mougeotia* sp., *Anabaena* sp.) or colony-forming algae (*Scenedesmus* sp.1, *Mougeotia* sp., *Uroglena* sp., *Fragilaria* sp.; Figure 2B). The observed phytoplankton patterns (PRC species scores) in Figure 2B 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 temperature conditions (Arvola and Salonen 2001; Fleeger et al. 2003; Figure 3 and Supplemental Data, S6).

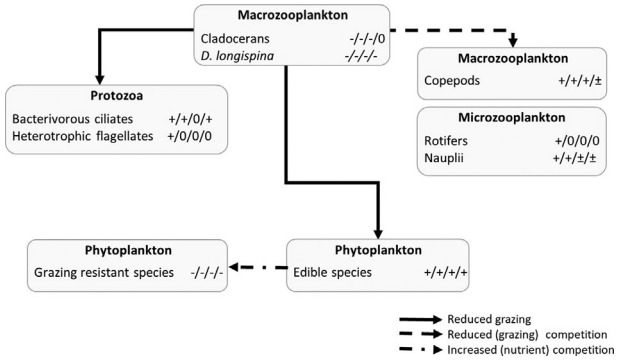

FIGURE 3: Schematic overview of the observed effects of the highest zinc (Zn) treatment (300 μg/L) on the ecosystem structure of a plankton-dominated community (+ = consistent increase; − = consistent decrease; ± = consistent increase during the first 2 wk of exposure and consistent decrease during the 2 last wk, 0 = no consistent effect) at the cold low phosphorus (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 in copepod adults and microzooplankton, which is probably the result of reduced grazing competition. In addition, 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. *D. longispina* = *Daphnia longispina*.

However, for the zooplankton and protozoa community structure, the observed indirect effects of the reductions in cladocerans at the highest Zn treatment varied among the different treatment conditions. The rotifers, for example, only benefited from the disappearance of the large cladocerans (= less food competition; Arvola and Salonen 2001; Fleeger et al. 2003) under cold, low P addition conditions, whereas no rotifer abundance increase was observed under the other treatment conditions (Figure 3).

The PRC analysis conducted with the protozoa data from all the different treatments visually revealed no clear effect on community structure from any of the factors (Figure 2C). The protozoa community composition was affected (3-way ANOVA on PCA scores) by high P addition after 7 and 14 d of treatment and by the highest Zn treatment from 14 d onward (Supplemental Data, Table S11).

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 (Moss et al. 2003; Özen et al. 2013; Šorf et al. 2014). However, in our study, high P addition by itself only slightly affected the planktonic community (Figure 2A–C and Supplemental Data, Table S11). A possible explanation is the fact that throughout the experiment the P concentrations (total dissolved P, total P, and soluble reactive P) in the controls and low and high Zn treatments increased continuously (Supplemental Data, Figure S2 and Table S1). By the end of the experiment the measured total dissolved P 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 (Brönmark and Hansson 2005). Therefore, it is unlikely that P was a limiting factor in any of the treatments.

Interactions of temperature and P stress with Zn toxicity

Species and group level. Consistent interactions (2- and 3-factor) were only found for 3 of 43 zooplankton species (Cyclopoida sp., nauplii, and *S. vetulus*), 2 of 86 phytoplankton species (single-cell diatoms and *Cryptophyte* sp. 1), and 1 of 27 protozoa species (*Rimostrombidium brachykinetum*; Tables 2–4 and Figure 1). 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 temperature at the highest Zn treatment. This shows that the interactions depended on the stressor intensity (i.e., the Zn levels), a finding also reported by Nys et al. (2015).

Among all plankton species identified, consistent Zn × temperature × P interactions were only found for 3 species (Cyclopoida sp., *Anisonema acinus*, and *Nassula* sp.). However,

TABLE 2: Statistical significance and calculation of the interactive effects (synergism or antagonism) of Zn and the different factors of some of the different plankton species under different treatment regimes[a]

Plankton group	Species	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							
	Rimostrombidium brachykinetum	21	High P	Zn × T	H	0.025	A
		28	High P	Zn × T	H	0.048	A

[a]The *p* values were calculated by 3- and 2-way analysis of variance (ANOVA). The interactive effects were either synergistic (S) or antagonistic (A), at low Zn (L) or high Zn (H). The interaction type is based on comparing observed and predicted effects (independent action) using the methods explained in De Coninck et al. (2013). Only species for which a consistent interaction was found (with 3-way ANOVA) are represented. 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 Supplemental Data, Tables S7 and S8, for the statistical details on all species that showed consistent interactions.
Zn = zinc; P = phosphorus; T = temperature.

further analyses revealed no consistent 2-factor interactions for *A. acinus* and *Nassula* sp. (Supplemental Data, Tables S7 and S8). Further analysis on the Cyclopoida sp. abundance data revealed that under high P addition the Zn (low) × temperature interactions were consistently synergistic after 7 and 14 d of treatment, indicating a larger (up to 5-fold higher than predicted) negative Zn effect at higher temperature (Table 2 and Supplemental Data, Tables S7 and S8). At the highest Zn concentration, consistent synergistic Zn × temperature interactions were calculated for nauplii (Tables 2 and 3 and Figure 1C, and Supplemental Data, Figures S9 and S10). Under normal conditions Cyclopoida are considered to have a high tolerance to Zn stress (Baudouin 1974; Monteiro et al. 1995; Van de Perre et al. 2016). Under warm hypereutrophic conditions, however, the nauplii were adversely affected at even the lowest Zn concentration (Figure 1C).

At the group level, consistent interactions (2- and 3-factor) were only found at the highest Zn treatment, and this was for 2 of 3 zooplankton groups (Copepoda and Cladocera), 3 of 8 phytoplankton groups (Bacillariophyceae, Cryptophyta, and

TABLE 3: Statistical significance and calculation of the interactive effects (synergism or antagonism) of Zn and the different factors of some of the different plankton groups under different treatment regimes[a]

Plankton group	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

[a]The *p* values were calculated by 2- and 3-way analysis of variance (ANOVA). The interactive effects were either synergistic (S) or antagonistic (A), at low Zn (L) or high Zn (H). The interaction type is based on the observed and predicted effects using the methods explained in De Coninck et al. (2013). Only groups for which a consistent interaction was found (with 3-way ANOVA) are represented. 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 Supplemental Data, Tables S9 and S10, for the statistical details on all species groups that showed consistent interactions.
Zn = zinc; P = phosphorus; T = temperature.

TABLE 4: Summary of the number of statistically significant interactive effects between Zn (low [L] and high [H]) and the different factors (phosphorus [P] and temperature [T]) on the different organization levels of the different plankton species groups and ecosystem functions on at least one time point (following 3-way analysis of variance and Monte Carlo permutation test)[a]

			L	H	L	H	L	H
Plankton group	Level	No.	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,b]	1	1 (0)	1 (1)	0 (0)	1 (0)	1 (0)	0 (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[b]	1	0 (0)	1 (1)	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[b]	1	0 (0)	1 (1)	0 (0)	1 (0)	0 (0)	0 (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]The total number of assessed entities (No.) at the different levels of organization is also reported. The numbers of consistent interactions are given between parentheses. See Supplemental Data, Table S2, S3, S5, S7, S9, and S21, for details.
[b]Monte Carlo permutation test.
[c]Functional feeding group within the ciliates.
BOD_5 = 5-d biochemical oxygen demand (test); DOC = dissolved organic carbon; DO_{net} = net dissolved oxygen.

Chlorophyta), and 1 of 3 protozoa groups (Ciliate; Tables 2–4). The majority (6 of 8) of the consistent interactions were found between Zn and temperature. The cladocerans (Figure 1D) and the ciliates as a group showed an antagonistic Zn (high) × temperature interaction under high P addition, indicating a lower Zn effect at higher temperature (Table 3). As an example, Figure 4A illustrates the Zn and temperature effects and their interaction for the cladocerans after 35 d of exposure under the different P conditions. It can clearly be observed that under high P addition the observed Zn + temperature 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 (Tables 2 and 3). Furthermore, all consistent Zn × temperature interactions effects for phytoplankton taxa and groups were antagonistic (Table 3). For the Bacillariophyceae, these Zn × temperature interactions were only significantly antagonistic under low P addition, whereas they were significantly antagonistic for the Cryptophyta under both low and high P addition (Tables 2 and 3). Crain et al. (2008) indicated that the overall interactive effects across most studies were antagonistic for most autotrophs, and they suggested that trophic level could be an important driver for interaction type because organisms with fundamentally different methods of energy acquisition may respond very differently to stressors (Crain et al. 2008).

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 d 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 and Supplemental Data, S10). These interactions were not found under cold conditions. 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 ~1.5-fold) than those of the high P microcosms (Table 1). Thus, the high P addition has, at best, only a slight effect on the overall Zn toxicity. Interestingly, though, 90% of all the Zn × temperature interactions at the species and group level were found under high P addition, suggesting that a higher P loading, may facilitate a stronger influence of temperature on the effects of Zn on freshwater communities. To our knowledge, the present study is the first to investigate metal × temperature × P interactions at the community level. Therefore, it is difficult to explain why 90% of all the Zn × temperature interactions were found under high P addition. We suggest 3 possible explanations. First, it has already been shown that extreme eutrophication can be unfavorable for most zooplankton species, primarily due to the occurrence of unfavorable cyanobacterial booms (McKee et al. 2002), and it is possible that these cyanobacterial booms (e.g., toxins or nutritional quality) affected Zn toxicity (which has, for example, already been shown for Cu; Hochmuth et al. 2015). Second, P can also affect certain aspects of the geochemistry of the water. For instance, at an elevated pH, phosphates can precipitate with Fe (forming $FePO_4$), making the Fe less bioavailable such that it can become a limiting factor for aquatic organisms (Powell et al. 2008). Finally, it is possible that the difference in P addition affected the bacterial community composition, which could have

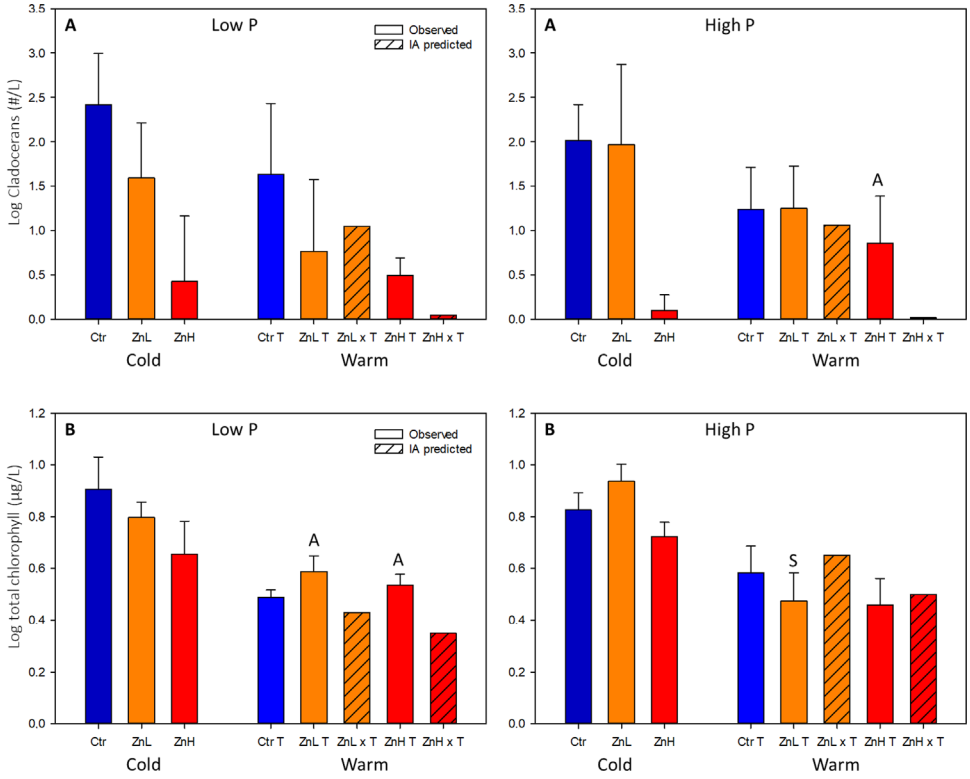

FIGURE 4: Log cladoceran abundance after 35 d of treatment (**A**) and total chlorophyll concentrations after 28 d of treatment (**B**) and predicted values using the independent action model (IA) under low and high phosphorus (P) addition conditions. Error bars indicate standard deviation. A = significant antagonistic zinc (Zn) × temperature interaction. T = temperature; S = significant synergistic Zn × temperature interaction; Ctr = control; ZnL = 75 µg Zn/L treatment; ZnH = 300 µg Zn/L treatment.

affected the overall plankton community dynamics (Brönmark and Hansson 2005). However, without the necessary variables measured, it is not possible to verify these hypotheses.

In summary, during the present study 4% of the species (33% synergistic; 67% antagonistic) and 36% of the functional groups showed a consistent Zn × temperature interaction (20% synergistic; 80% antagonistic). This finding 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 (Tables 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 (Donker et al. 1998; Heugens et al. 2001, 2003; Holmstrup et al. 2010). However, a recent chronic single-species study conducted by Pereira et al. (2017) on *D. magna* (4 different single clones) reported that the chronic metal toxicities of Zn, Cu, and Ni were generally higher at lower temperatures. The majority of the consistent Zn × temperature interactions during the present study were antagonistic, which indicates that Zn had a smaller effect at higher temperature for most taxa and groups (Tables 2 and 3).

Our results are in line with those of Jackson et al. (2016), who found that the interactive effects between stressors at the population level are usually additive and antagonistic at the higher levels of organization (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 may mask or dampen possible interactive stressor effects that occur at the population level (Crain et al. 2008). Indirect effects are frequently observed in microcosm studies (Jak et al. 1996; Fleeger et al. 2003; Van de Perre et al. 2016) and can, for instance, be the result of reduced food competition (or predation; Brönmark and Hansson 2005). Another possible reason why interactions are more frequently found at the group than at the species level is the fact that the variation in abundance is higher at the species than at the group level, due to the higher chance of detecting a group instead of detecting a species and the fact that the group abundance is a summary of the abundance of many species. On the other hand, the disappearance of one species can be compensated for by the abundance increase of species with the same community function (functional redundancy), which could dampen the effects at higher levels of biological organization (Fleeger et al. 2003). Although these abundance fluctuations are a natural phenomenon, they 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 (Brock et al. 2014).

Community level. Consistent Zn × temperature interactions were found at the community composition level for all the different plankton communities (zooplankton, phytoplankton,

and protozoa) and are in line with the main findings of our earlier analyses (species and group level). After 14 d of exposure and onward, significant Zn × temperature interactions (Monte Carlo permutation test) were observed at the zooplankton community composition level at the highest Zn treatment under high P addition (Table 5). At the phytoplankton community composition level, significant Zn × temperature interactions were found after 7 and 14 d of treatment under high P addition at the highest Zn treatment (Table 5). For the protozoa, a consistent Zn × temperature interaction (between 21 and 28 d of treatment) was found at the community composition level under low P addition at the highest Zn treatment (Table 5).

Total chlorophyll, community metabolism, and general chemical properties of the water. Consistent interactions were found for all the main community metabolites (DOC, BOD_5, dissolved oxygen, and pH) and for the total chlorophyll (Tables 5 and 6). At the highest Zn treatment, consistent antagonistic Zn × temperature interactions were found for the DOC, pH_{mean}, DO_{mean}, and net primary production (DO_{net}). The Zn (high) × temperature interactions for the pH_{mean} were consistent under both P conditions, whereas the Zn (high) × temperature interactions of the DOC, DO_{mean}, and DO_{net} were only consistent under low P addition (Table 6). Under low P addition, the observed effects (Zn high + temperature) 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 independent action model (Supplemental Data, Table S21). After 7 and 14 d of treatment, consistent Zn (high) × temperature interactions were found for the BOD_5. These interactions were synergistic under high P addition, whereas they were antagonistic under low P addition (Table 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, DOC was the only parameter for which consistent interactions were found: consistent synergistic Zn (low) × temperature 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 6 and Supplemental Data, Table S21).

Significant Zn × temperature × P interactions were observed for the total chlorophyll (Figure 1F) concentration during the last 3 wk of exposure at the lowest Zn and during the last 2 wk at the highest Zn treatment (Supplemental Data, Table S16). Under low P addition, the Zn × temperature interactions were antagonistic for the total chlorophyll at the lowest Zn treatment after 21 and 28 d of exposure and at the high Zn treatment after 28 and 35 d (Table 5). These Zn × temperature interactions indicated that under low P addition and high temperature, the lowest and highest Zn treatment had a smaller adverse effect (3- and 1.4-fold, respectively) on the total chlorophyll concentration than predicted (Supplemental Data, Table S10). As an example, Figure 4B illustrates the effects and interactions between Zn and temperature on the total chlorophyll concentrations after 28 d of exposure under the different P conditions. No consistent Zn × temperature 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 6). Therefore it can be concluded that, in contrast to Gao et al. (2016), no indications were found during the present study that Zn was

TABLE 5: Statistical significance (p values by Monte Carlo permutation tests) of the interactive effects of low and high zinc (Zn) treatments and the different factors (phosphorus [P] and temperature [T]) on the community structure of the different plankton groups under different treatment regimes[a]

		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

[a]The low Zn treatment was 75 μg Zn/L and the high was 300 μg Zn/L.
*Values significant at $p < 0.05$.

TABLE 6: Statistical significance and calculation of the interactive effects and the different factors 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 d (BOD_5) at different treatment regimes[a]

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

[a]The p values were calculated by 3- and 2-way analysis of variance (ANOVA). The interactive effects were either synergistic (S) or antagonistic (A), at low Zn (L) or high Zn (H). The interaction type is based on the observed and predicted effects using the methods explained in De Coninck et al. (2013). Only parameters for which a consistent (for at least 2 consecutive sampling dates that showed the same type of interaction effect) interaction (3-way ANOVA) was found are represented here. See Supplemental Data, Tables S21 and S16, for the statistical details on all species groups that showed consistent interactions.

more toxic to the phytoplankton at higher P supply, at either the species or group level or at the level of community composition or total chlorophyll concentration in the system. It is possible that in the present study the P was not limiting enough to induce this effect.

CONCLUSIONS

Consistent interactions between Zn and the other factors (temperature and P) were rarely found at the species level, but they were frequently found at the group, community structure, and functional levels, thus largely confirming our hypothesis that stressor interactions occur more frequently at a higher level of organization. We also found that 82% of all the consistent interactions at the 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 temperature, indicating that Zn effects on plankton communities can be affected by temperature. Furthermore, these consistent Zn × temperature interactions were mainly antagonistic, which suggests that Zn did not have a larger, but rather a smaller effect at higher temperature for most taxa, groups, and functions. Ecological risks of Zn could therefore be higher in cold than in warm aquatic systems.

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 × temperature interactions at the species and group level were found under high P addition. In addition, with the exception of the protozoa, all the observed consistent Zn × temperature 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 temperature. Collectively, our study results suggest that temperature and phosphorus loading to freshwater systems should be accounted for in risk assessment of chemicals, because these factors may modify the effects on aquatic communities. Not doing so may underestimate risks in some and overestimate risks in other systems, depending on their temperature and P loading.

Supplemental Data—The Supplemental Data are available on the Wiley Online Library at DOI: 10.1002/etc.4201.

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Data availability—Data and associated metadata are available in the Supplemental Data or on request from the authors (Dimitri.VandePerre@Ugent.be).

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