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The impact of phosphorus and temperature on the toxicity of zinc on benthic diatoms

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Abstract

The current index, IPS, developed for benthic diatoms and used for assigning the ecological status of streams in Sweden reflects on levels of nutrients and organic pollution. Metals are not considered in the index, which leads to the risk that metal contaminated streams can be assigned a high ecological status based on this index. Thus, an index that take into consideration the impact of metals in streams should be developed. In order to develop such an index, knowledge about if factors such as temperature and phosphorus affects metal toxicity to benthic diatoms could be useful. The aim of this study was to investigate if temperature and phosphorus affects the toxicity of Zn to benthic diatoms. Several laboratory experiments were performed with diatoms isolated from Swedish streams. The hypothesis was that temperatures above 20 °C would elevate toxic effects caused by Zn, which was partly confirmed. High temperatures (30-32 °C) caused a decrease in number of cells and in the maximum efficiency of the photosystem II in the highest concentration of Zn tested. The values of the approximate EC_{50} for each temperature and the amount of fluorescence per cells did however not show that temperature impact the toxicity of Zn. Thus, conclusively not all parameters investigated declared that temperature impacts the toxicity of Zn, but since some of them did, it cannot be said that temperature does not impact the toxicity of Zn. The hypothesis for the impact of phosphorus on Zn toxicity was that a higher concentration of phosphorus would increase tolerance to Zn and consequently lower concentrations of phosphorus was thought to increase the toxicity of Zn. The phosphorus effects were partly in-line with the hypothesis. The highest concentration of phosphorus tested caused a decrease in toxicity, detected as a higher median value of EC_{50} and as a higher amount of fluorescence in the highest concentration of Zn for most strains in one experiment. However, the lower amount of phosphorus only increased the toxicity, detected as a lower amount of fluorescence, in the highest concentration of Zn tested in two of the strains in one experiment. The results regarding the impact of phosphorus on Zn toxicity was not consistent in the experiments performed. However this study indicated that phosphorus and temperature could impact the toxicity of Zn. Therefore, in order to develop an index that reflect on the levels of metals more studies should be done to establish if temperature and phosphorus in fact impact the toxicity of Zn.

Contents

1. Introduction.....	2
2. Materials and Methods.....	4
2.1 Short-term experiment	4
2.1.1 Benthic diatoms	4
2.1.2 Media, volumes and concentrations.....	5
2.1.3 Cell count.....	5
2.1.4 Fluorescence measurement	6
2.1.5 Approximate EC ₅₀	6
2.2 Long-term experiment	6
2.2.1 Temperature	6
2.2.2 Volumes, concentrations and media	7
2.2.3 Strain	7
2.2.4 Fluorescence measurement and the use of fluorescence parameters	7
2.2.5 Approximate EC ₅₀	8
2.2.6 Cell count.....	8
2.3 Statistics	8
3. Results.....	10
3.1 Short-term experiment	10
3.1.1 Impact of Phosphorus on overall tolerance to zinc	10
3.1.2 Impact of Phosphorus on Zn tolerance in detail.....	12
3.2 Long-term experiment	15
3.2.1 The Zn and temperature effect on photosynthesis and level of stress.....	15
3.2.2 Number of cells.....	16
3.2.3 Approximate EC ₅₀	16
3.2.4 Fluorescence per cell.....	18
4. Discussion	20
4.1 Impact of phosphorus on the toxicity of Zn.....	20
4.2 The effect of temperature on Zn toxicity	22
5. Conclusion	24
Acknowledgements.....	25
References.....	26
Appendix.....	29

1. Introduction

Within the implementation of the Water Framework directive (WFD) in Sweden, lakes and streams are classified based on their ecological status according to a five graded scale (Naturvårdsverket, 2007a). The high status, top-ranking on the scale, reflects water bodies that are insignificantly affected by human activities (Naturvårdsverket, 2007a). The first step in the classification of the ecological status is to assess biological factors, which are indexes developed based on how an organism, than can reflect the status of the aquatic environment, responds to changes in the aquatic environment (Naturvårdsverket, 2007a). When assigning the ecological status of streams in Sweden, by the use of benthic diatoms as indicators, the current index, IPS, reflects levels of organic pollution and nutrients (Naturvårdsverket, 2007b). Furthermore, the additive parameters %PT and TDI, reflecting organic pollution and eutrophication, are also used to verify the classification (Naturvårdsverket 2007b). In addition there is an index developed for benthic diatoms that reflects the level of acidity in streams, but this index is not used to determine ecological status (Naturvårdsverket 2007b, Kahlert & Gottschalk, 2008).

In the present index, IPS, used for classifying the ecological status, the levels of metals are not considered, and metal contaminated streams have been assigned a high ecological status based on this index (Kahlert & Gottschalk, 2008). Thus, an index that take into consideration the impact of metals in streams should be developed (Kahlert & Gottschalk, 2008). In order to develop such an index, knowledge about what factors that can impact the toxicity of metals on benthic diatoms could be useful.

Benthic diatoms belong to the benthic algae and the periphytic community (Stevenson, 1996 pp. 4, 8), also called phytobenthos or biofilm (Sabater *et al.*, 2007). There are several reasons why benthic algae are a useful organism group for monitoring the aquatic environment (Lowe & Pan, 1996 pp 706). Firstly, they display an early response to changes in the environment. Benthic algae also constitute a part of the foundation of the food chain due to their role as primary producers, and thus changes in the benthic algae community could impact the aquatic ecosystem (Lowe & Pan, 1996 pp 706-707). Furthermore, due to their fixed location, the community of benthic algae is affected by spreading of pollutants in the aquatic environment (Lowe & Pan, 1996 p 707).

This study focuses on zinc (Zn), a metal with a dual nature. It is both essential for organisms but also potentially harmful at high levels (Maycock *et al.*, 2012). Thus, Zn has been shown to have negative short-term effect on the efficiency of the photosynthetic apparatus of biofilms (Corcoll *et al.*, 2012). In addition, short-term negative effects of Zn on the photosynthesis in an isolated culture of a benthic diatom have been reported (Ivorra *et al.*, 2002).

Abiotic factors such as temperature and phosphorus will not unlikely vary depending on location and season. Thus, if temperature and phosphorus affects the toxicity of metals on benthic diatoms, the response to metals could display geographical and seasonal variation. Therefore, knowledge about if

phosphorus and temperature affects the toxicity of metals to benthic diatoms could be needed when developing an index that also reflects the levels of metals.

In addition to Zn, the water temperature could affect the status of the benthic diatoms. It has been found that the number of benthic diatoms in the periphytic community decreases in temperatures above 20° C (DeNicola, 1996 p.174), suggesting that temperatures below 20° C are optimal for benthic diatoms. The impact of temperature on the toxicity of Zn to isolated Swedish benthic diatoms has to my knowledge not been addressed to date. Since the climate is changing (SMHI) knowledge about the impact of temperature the toxicity of Zn could also be useful, for predicting how benthic diatoms will respond to Zn in the future.

Phosphorus is a nutrient used by algae, and insufficient levels are believed to limit growth (Borchardt 1996 p. 197). Cellular polyphosphates in green algae has been shown to capture Zn ions (Wong, Wainright & Pimenta, 1994), which is believed to prevent intracellular toxic effects by Zn (Bates *et al.*, 1985). However, if polyphosphates are consumed, the bound Zn ions are thought to be released into the cell which is potentially harmful for the cell (Bates *et al.*, 1985). It has been suggested though that the release of Zn ions from polyphosphates in algae cells is inhibited by abundant amounts of phosphate available for growth since this would prevent the consumption of intracellular polyphosphates (Genter, 1996 p. 430).

The aim of this study was to investigate if temperature and phosphorus affects the toxicity of Zn to benthic diatoms, isolated from Swedish streams. My hypothesis regarding the impact of temperature on the toxicity of Zn was that the sub-optimal temperature above 20 °C would elevate toxic effects caused by Zn. The hypothesis about the impact of phosphorus was that higher concentrations of phosphorus would lead to increased tolerance to Zn in isolated benthic diatoms, and consequently that concentrations of phosphorus, lower than in a standard medium, would increase the toxicity of Zn.

2. Materials and Methods

The impact of phosphorus and temperature on the toxicity of Zn on benthic diatoms was investigated by performing two short-term experiment for the phosphorus impact and a long-term experiment for the effect of temperature. The strains of benthic diatoms used for the experiments had been isolated and cultivated at SLU (Swedish university of agriculture sciences). In both experiments Zn was added as ZnSO₄. At the end of both experiments all samples were fixed by the addition of Lugol's solution.

2.1 Short-term experiment

To investigate the impact of phosphorus on the toxicity of Zn to diatoms, a short-term experiment of 96 hours of incubation in 20 °C, with a light of 45 μmol·m⁻²·s⁻¹, was performed. The benthic diatoms were incubated in media with different phosphorus and Zn concentrations using a 12 well plate (3x4 wells) with 2 ml sample volume. On each plate a single strain (or species) was incubated in triplicates in the presence of different concentrations of phosphorus and Zn. The experiment was repeated twice. At the endpoint of the experiments cells were counted (second experiment) and fluorescence intensity (first and second experiment for most strains) measured.

2.1.1 Benthic diatoms

The benthic diatoms used were collected under circumstances given in table 1, and isolated at the Swedish University of Agriculture Sciences.

Table 1. The strains used for the short-term experiment were collected at different sites in Sweden. Data of pH and Tot P at site of collection were attained from the Swedish Diatom Isolate Database with the exception of data for pH and [Zn] for D4 and D14, which were obtained from the county administration board of Dalarna.

Benthic diatoms used for experiments					
Strain	Referred to as	Collection site	pH	Tot P $\mu\text{g/l}$	[Zn] $\mu\text{g/l}$
<i>Gomphonema parvulum</i> var. A3	GP A3	Börrumbäcken	6,87	111,4	
<i>Gomphonema parvulum</i> var. FLARK	GP FLARK	Flarkån	4,4	27	
<i>Brachysira neoexilis</i>	BNEO	Broströmmen	7,7	46,4	
<i>Fragillaria capucina</i>	FCAP	Smedmyrbäcken	4,3	32	
<i>Navicula</i> s.l.	D4	Plogens utlopp	7,2		260
<i>Navicula</i> s.l.	D14	Rullshyttebäcken	7,4		510

2.1.2 Media, volumes and concentrations

The total volume in each experimental unit (12-well plates) was 2 ml. ZnSO_4 was added at the final concentrations 0, 30, 300 and 1000 $\mu\text{g Zn l}^{-1}$ respectively. In the first experiment each strain was incubated in three, and in the second in four different concentrations of phosphorus. These were obtained by modifying the concentration of K_2HPO_4 in standard L16 (Lindström, 1991). In both experiments 161, 293 and 1613 $\mu\text{g P l}^{-1}$ were used. Due to a slight error, the phosphorus concentrations in the control of the first experiment were slightly higher (163, 309 and 1767 $\mu\text{g P l}^{-1}$) than in the Zn treatments. In the second experiment however, the concentrations of phosphorus in the controls (0 $\mu\text{g Zn l}^{-1}$) were equivalent to the concentrations used for the experimental concentrations of Zn (161, 293 and 1613 $\mu\text{g P l}^{-1}$). The additional fourth concentration of phosphorus in the second experiment was 148 $\mu\text{g P l}^{-1}$. Finally, cells in standard L16 were added to give a final concentration of 10 000 cells ml^{-1} . L16 was used because its TP concentration is relatively low in comparison to other standard media. The L16 medium was made according to the standard protocol (Lindström, 1991), with the exception of the EDTA concentration, which was 1/10 of the standard, leading to 70-80 % of the added Zn ions being bio-available (Kahlert, M. 2016-05-13, personal comment)

2.1.3 Cell count

In order to obtain the required concentration of cells ml^{-1} the cells were counted with the cell count chamber Neubauer Improved (Tiele 0,100 mm and 0, 0025 mm^2) in a Nikon Eclipse 80i microscope. Each strain was counted six times and the average result was used. To determine the number of cells that survived the second experiment the same equipment was used. For this each well on the 12-well plate was counted twice.

2.1.4 Fluorescence measurement

At the end of the experiment fluorescence intensity was measured for each plate and well, with a multifunctional micro plate reader Tecan Genios Pro 96/384, together with the Magellan data analysis software for fluorescence intensity with the excitation of 460 nm, emission of 685 nm and bandwidth of 20 nm. The emission wavelength (685 nm) corresponds to the wavelength of fluorescence originating from the chlorophyll a pigments (Govindjee, 1995). Measurements of such fluorescence can be used as a diatom biomass approximation (Larras, Montuelle & Bouchez, 2013). The maximum fluorescence possible to measure with this instrument is limited and thus when the fluorescence exceeded this level data could not be obtained. The fluorescence intensity was measured for all benthic diatoms in the first experiment and for GPAR A3, D4 and D14 in the second experiment.

2.1.5 Approximate EC₅₀

In order to define the Zn concentration causing a determined effect on the benthic diatom strains, an approximate EC₅₀ was used. An absolute EC₅₀ value refers to the concentration of a compound that causes a response equivalent to a half of the response in the absence of the compound (Sebaugh, 2010). The mathematical calculation of the EC₅₀ requires a regular response curve (Sebaugh, 2010). However due to a partly irregular response to Zn in my experiment, the following approach was used to determine the EC₅₀ value; the EC₅₀ was based on the linear equation for two or three points, in between which the corresponding value of half the control value in fluorescence or number of cells was found. The EC₅₀ was based on the average in fluorescence or number of cells, for this reason and for the fact that the response was somewhat irregular I chose to call it an approximate EC₅₀. Where half of the fluorescence or number of cells in the control (in the absence of Zn) did not correspond to a measured value, the approximate EC₅₀ was set to be above the highest concentration of Zn tested.

2.2 Long-term experiment

The long-term experiment was performed using an incubator with the possibility to incubate samples at 19 different temperatures, supposedly ranging from 4°C to 35 °C. Beneath the incubator was a light with 43 $\mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ directed towards the bottom of each vial in the incubator. For each temperature there was space enough for five vials giving the incubator capacity for 95 samples. In this experiment one benthic diatom strain was incubated at 15 temperatures, with five different concentrations of Zn.

2.2.1 Temperature

On the last day of the experiment the actual sample temperatures were measured and the actual temperatures ranged from 14° to 32°C (appendix, table 1).

2.2.2 Volumes, concentrations and media

The experiment was performed using glass vials with a total volume of 30 ml. The vials were autoclaved with WC medium made according to the standard protocol (see; Guillard & Lorenzen 1972) with the exception of the amount of added EDTA. This was added in 1/10 of the standard amount, as a result 70-80 % of the Zn ions added are available (Kahlert, 2016-05-13, personal comment). This media was then replaced with 10, 30, 100 or 300 μl of ZnSO_4 solution and 100 μl of cells cultivated in WC medium, in order to obtain a final concentration of 0, 10, 30, 100 or 300 $\mu\text{g Zn l}^{-1}$, respectively and the final concentration 100 cells ml^{-1} .

2.2.3 Strain

For the experiment the benthic diatom strain *Gomphonema parvulum* var. A3, referred to as GPAR A3 was used (Table 1).

2.2.4 Fluorescence measurement and the use of fluorescence parameters

Fluorescence can be an approximation of the function and efficiency of the photosynthesis of photosystem II because it's an alternative path for the conversion of absorbed light energy by the photosynthetic pigments, in photosystem II. The photosynthetic reaction starts when an electron in the chlorophyll pigment is excited by light energy photons, and transferred to an electron acceptor. This is called photochemical quenching (Maxwell & Johnson, 2000). Because the electron acceptor cannot accept another electron until it's been oxidized by the release of the electron to the following electron acceptor in the electron pathway, the course for the excited electrons at the chlorophyll pigments will either be to cause the re-emitting of a photon (fluorescence) or heat (Maxwell & Johnson, 2000, Consalvey *et al.*, 2005). Therefore, when all the primary electron acceptors are reduced, the more fluorescence occurs, or more energy is emitted as heat (Maxwell & Johnson, 2000, Consalvey *et al.*, 2005).

In order to determine the function of the photosynthetic apparatus, the level of stress and make an approximation of the algae biomass, the fluorescence parameters F_0 and F_m were measured with a PAM modulated fluorometer and the software Modflour, with the pulse and width set to 80 and 0,7 respectively. These parameters are the minimal (F_0) and maximum (F_m) levels of fluorescence in a dark-adapted sample. The minimal fluorescence occurs when the electron acceptor in the photosystem II has been oxidized, due to the lack of photons in the dark (Consalvey *et al.*, 2005). The instrument records this value by applying a measuring beam with an intensity level low enough to only measure the minimum fluorescence (F_0) (Schriber, Schilwa & Bilger, 1986). The instrument then sends out a beam of high intensity (called the saturating beam) that reduces the electron acceptors and causes the level of fluorescence to be at its maximum, as if no photochemical quenching would occur, and the F_m parameter is obtained (Maxwell & Johnson, 2000). Once these parameters have been measured the software automatically calculates the F_v/F_m parameter, which is the ratio between the difference in maximum

(F_m) and minimum (F_0) level of fluorescence and the maximum level of fluorescence (F_m) ($F_m - F_0 / F_m$) (Maxwell & Johnson 2000, Consalvey *et al.*, 2005, Schriber *et al.*, 1995). The F_v / F_m is equivalent to the potential of the photosystem II to harvest and utilize light for photochemistry (Maxwell & Johnson, Consalvey *et al.*, 2005). A decrease in the parameter F_v / F_m can be used as an indication of stress induced damage in vascular plants (Björkman & Demmig, 1987) and has been used for this purpose in algae (Honeywill, Paterson & Hogerthy, 2011). In the present study the F_v / F_m was used to determine if the toxicity of Zn was different in the temperatures tested, indicated as more stress and a lower potential in light utilization, by looking at an average relative difference to the control.

Since the F_0 parameter correlates with the concentration of chlorophyll a in algae and can be used to describe an approximation of the algae biomass (Honeywill, Paterson & Hogerthy, 2011), it was used as basis for the approximate EC_{50} .

The initial F_0 and F_m measurements were done 4 days after incubating the cells in the different temperatures, and the following second or third day during the rest of the experiment. The parameters were measured after a 15 min time period in darkness, suggested to be suitable according to Honeywill, Paterson & Hogerthy (2011).

2.2.5 Approximate EC_{50}

The approximate EC_{50} was calculated as in the short-term experiment, explained above, and was based on both F_0 and the average number of cells.

2.2.6 Cell count

In order to have a final cell concentration of 100 cells ml^{-1} , the cells were counted using the same equipment as in the short-term experiment with phosphorus. Once the experiment was finished the number of cells was also counted with this equipment and with a Herbert bacteria counter improved Neubauer (1/400 SQ.MM and 1/50 MM deep) with a Leitz Wetzlar M119 microscope. Each sample was counted two to four times.

2.3 Statistics

In order to determine if phosphorus had an effect on the toxicity of Zn, I tested if the approximate EC_{50} value in different concentrations of phosphorus within the same strain, and within the same concentration of phosphorus between strains, differed significantly. Furthermore, to determine the effect of phosphorus on the toxicity of Zn for each strain, I tested if the fluorescence intensity or numbers of cells significantly differed between phosphorus concentrations for the same concentration of Zn, where the standard deviation in average fluorescence intensity or number of cells did not overlap between phosphorus concentrations. For this I used the Minitab statistical software, with a two-sample t for the mean test, giving a CI interval and a p-value with an alpha value of $\leq 0,05$. An alpha value is

the level of risk to falsely reject the null hypothesis (Englund, Engstrand & Olsson, 2005 p. 143). With this level of alpha the CI interval covers an area in which the true difference can be found with a 95 % chance of being correct. Therefore, if the CI interval covers the value of zero the result cannot be said to be statistically significant on the 5 % level and there is no statistical difference at this level (Englund, Engstrand & Olsson, 2005 p. 167). In addition if the p-value exceeds 0.05 the result is not statistically significant of the 5 % level (Englund, Engstrand & Olsson, 2005 p. 141).

3. Results

3.1 Short-term experiment

3.1.1 Impact of Phosphorus on overall tolerance to zinc

When calculating the approximate EC_{50} it was apparent that the higher amount of phosphorus correlated with decreased toxicity of Zn, reflected by a higher EC_{50} value (table 1). The median value of approximate EC_{50} was $886 \mu\text{g Zn l}^{-1}$, based on average number of cells and fluorescence intensity from both experiments, for all the strains together, incubated in the highest phosphorus concentration (table 1a). For the cells incubated in lower phosphorus concentrations the median values of the approximate EC_{50} for all strains together was $295 \mu\text{g Zn l}^{-1}$ (table 1b) and $302 \mu\text{g Zn/l}$ (table 1c), respectively, based on average number of cells and fluorescence intensity from both experiments. Hence, cells in the lower concentrations of phosphorus showed approximately the same response to Zn.

That the approximate EC_{50} differed between diatom strains indicated that the impact of phosphorus varied between the diatoms (table 1). When comparing the approximate EC_{50} , based on fluorescence intensity, between strains (D14, GPAR A3 and D4) in each concentration of phosphorus, the EC_{50} for each strain did however not differ significantly. Neither was the difference between the approximate EC_{50} in different phosphorus concentrations for the same strain significant ($p > 0, 05$). The approximate EC_{50} of D4 in the highest phosphorus concentration could not be compared to other strains and concentrations of phosphorus due to the identical results in fluorescence intensity in both experiments ($>1000 \mu\text{g Zn/l}$).

Table 2. Result from the two replicated short term experiments shown as approximate EC₅₀ in µg Zn l⁻¹ for each strain incubated in different concentrations of phosphorus in L16 media, based on measured fluorescence intensity (both experiments) and average number of cells (second experiment). Fluorescence intensity was measured solely on the replicated experiment for the strains D14, GPAR A3 and D4.

1a)			
Strain	Approximate EC ₅₀ µg Zn/l in highest concentration of phosphorus (~1600 µg P l ⁻¹)		
	Fluorescence intensity first experiment	Fluorescence intensity second experiment	Average no. of cells/ml
D14	231	172	>1000
GPAR FLARK	160		>1000
GPAR A3	744	161	152
BNEO	>1000		886
D4	>1000	>1000	>1000
FCAP	>1000		650

1b)			
Strain	Approximate EC ₅₀ µg Zn/l in standard concentration of phosphorus (~300 µg P l ⁻¹)		
	Fluorescence intensity first experiment	Fluorescence intensity second experiment	Average no. of cells/ml
D14	228	170	314
GPAR FLARK	200		204
GPAR A3	460	143	254
BNEO	295		728
D4	584	677	732
FCAP	223		>1000

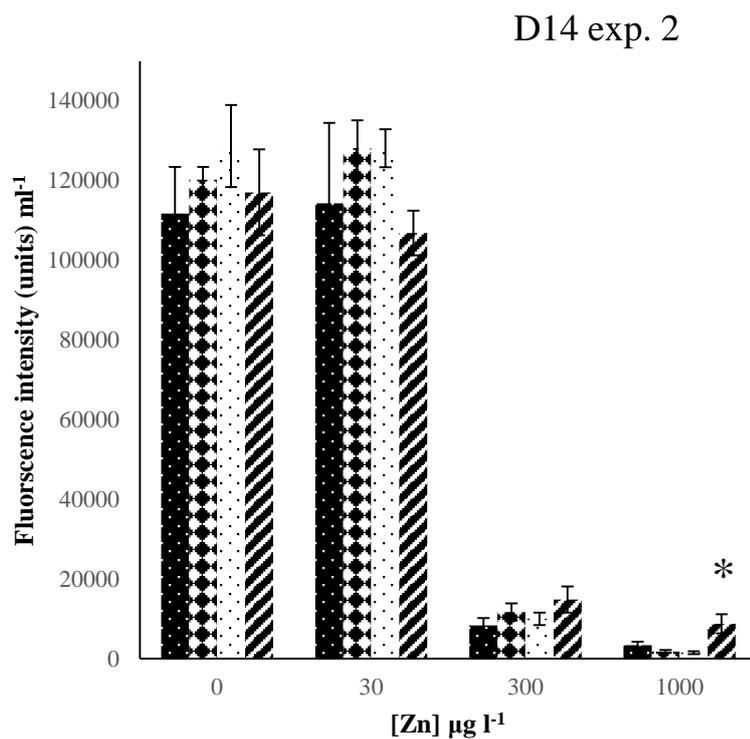
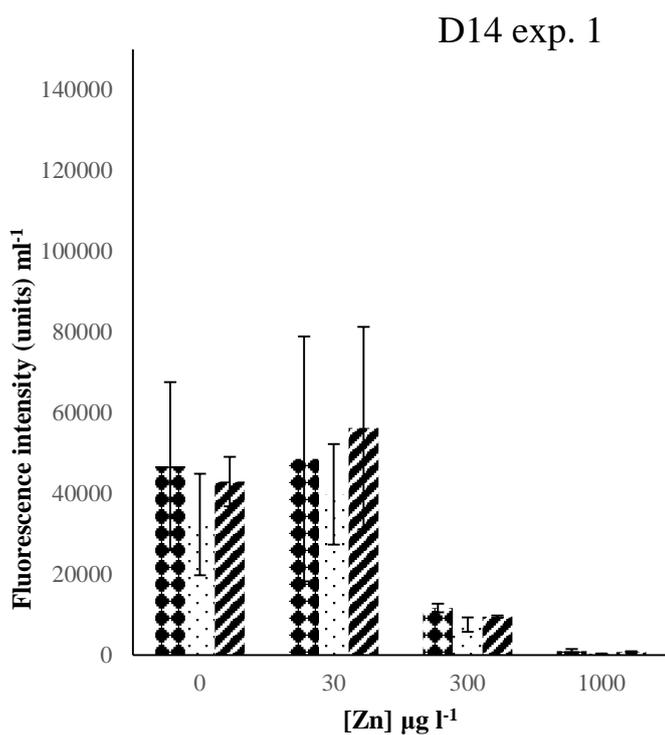
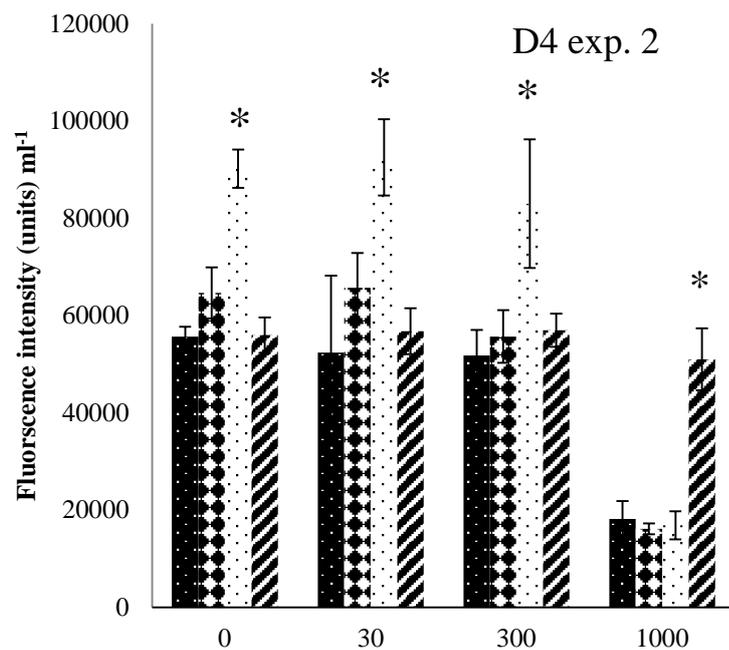
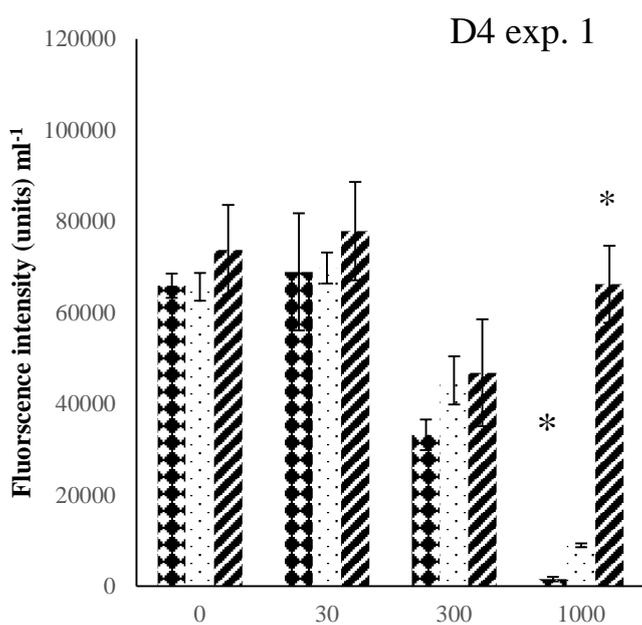
1 c)			
Strain	Approximate EC ₅₀ µg Zn/l in the lowest concentration of phosphorus (~160 µg P l ⁻¹)		
	Fluorescence intensity first experiment	Fluorescence intensity second experiment	Average no. of cells/ml
D14	215	188	247
GPAR FLARK	223		440
GPAR A3	315	156	216
BNEO	341		>1000
D4	302	702	578
FCAP	227		>1000

3.1.2 Impact of Phosphorus on Zn tolerance in detail

The impact of phosphorus on the toxicity of Zn differed between strains and experiments. When comparing the fluorescence intensity between phosphorus concentrations for the same concentration of Zn, most strains showed that the highest concentration of phosphorus decreased the toxicity of Zn in one experiment. The lower phosphorus concentration decreased the tolerance to Zn in two strains in one of the replicated experiments.

In the first experiment of D4, the fluorescence intensity in the highest concentration of phosphorus was significantly higher ($p < 0.05$) than the lower concentration of phosphorus, for the highest concentration of Zn (figure 1). In addition, increased toxicity of the highest Zn concentration was seen in the lowest concentration of phosphorus (figure 1), based on significantly ($p < 0.05$) lower fluorescence intensity than in the standard concentration of phosphorus (figure 1). In the second experiment of D4 the highest concentration of phosphorus decreased the toxicity of the highest concentration of Zn, indicated by the average fluorescence intensity and number of cells being significantly higher ($p < 0.05$) than the standard concentration of phosphorus (figure 1, appendix: figure 1). However, the lowest concentration of phosphorus did not increase the toxicity in comparison to the standard concentration of phosphorus, in the second experiment of D4 (figure 1).

D14 showed a similar trend in the impact of phosphorus to the toxicity of Zn in both experiments based on fluorescence intensity (figure 1) and average number of cells. No significant difference between phosphorus concentrations was detected for D14, neither in average number of cells (second experiment), nor in fluorescence intensity in the first experiment (appendix: figure 4, figure 1). However for D14 the fluorescence intensity was significantly higher ($p < 0.05$) in the highest concentration of phosphorus and Zn, than the lower concentration of phosphorus in the second experiment (figure 1), which indicates a decrease of Zn toxicity.



■ 161 µg P/l · 293 µg P/l ▨ 1613 µg P/l

■ 148 µg P/l ▨ 161 µg P/l · 293 µg P/l ▨ 1613 µg P/l

Figure 1. Average fluorescence intensity for D4 and D14 per ml of cell culture from the first and the second experiments as a function of the concentration of Zn. * denoting significant difference between phosphorus concentration in fluorescence intensity. Error bars illustrate standard deviation.

The impact of phosphorus on the toxicity of Zn differed between experiments for FCAP. In the first experiment of FCAP the highest concentration of phosphorus did indeed decrease the toxicity of Zn, since a significantly higher fluorescence intensity ($p < 0.05$) was detected, in comparison with the lower concentrations of phosphorus, for all concentrations with Zn (appendix, figure 2). Furthermore, the lower amount of phosphorus decreased the tolerance to Zn in the highest concentration of Zn and in the first experiment of FCAP, since the fluorescence intensity in the lowest concentration of phosphorus was significantly lower ($p < 0.05$) than in the standard concentration of phosphorus (appendix, figure 2). However, in the second experiment of FCAP, phosphorus did not impact the toxicity of Zn. Hence, there was no significant difference for the average number of cells between the different phosphorus concentrations for any Zn concentration (appendix, figure 3).

For BNEO the highest concentration of phosphorus decreased the toxicity of Zn in the first experiment. The highest concentration of phosphorus, in the highest concentration of Zn, caused a significantly higher fluorescence intensity ($p < 0.05$) than the lower concentration of phosphorus (appendix: figure 5). In the second experiment of BNEO, the highest concentration of phosphorus in the highest concentration of Zn showed a significantly higher average number of cells, in comparison to the standard concentration of phosphorus ($p < 0.05$). However based on the standard deviation there was no significant difference between the average number of cells in second lowest and the highest concentration of phosphorus in the highest concentration of Zn (appendix: figure 6). Thus the highest concentration of phosphorus cannot be said to have decreased the toxicity of Zn in this experiment.

The hypothesis that a higher amount of phosphorus would decrease the toxicity of Zn and that a lower amount of phosphorus would increase the toxicity of Zn was not detected in the first experiment of GPAR A3 (appendix: figure 7). However, in the second experiment of GPAR A3, the highest concentration of phosphorus decreased the toxicity of Zn, regarding fluorescence intensity, for both the second highest and the highest concentration of Zn (appendix: figure 8). The fluorescence intensity was significantly higher than in the lower concentrations of phosphorus in both the second highest ($p < 0.05$) and the highest concentration of Zn ($p < 0.05$) (appendix: figure 8). The lowest concentration of phosphorus did however not decrease the tolerance to Zn as believed, since the difference between the lower and the standard concentrations was not significantly different ($p > 0.05$) (appendix: figure 8). Furthermore, neither the lower nor the higher amount of phosphorus impact the average number of cells of GPAR A3 in the second experiment, since there was no significant difference, based on the standard deviation, between the phosphorus concentration for any Zn concentration (appendix; figure 9).

A decrease in Zn toxicity as a result of a higher phosphorus concentration was not seen in the two experiments of GPAR FLARK (appendix: figure 10, 11). Furthermore, in both experiments of GPAR FLARK, the lower amount of phosphorus did not increase the toxicity of Zn (appendix figure 10, 11). Hence, phosphorus did not impact the toxicity of Zn on GPAR FLARK.

3.2 Long-term experiment

3.2.1 The Zn and temperature effect on photosynthesis and level of stress

The impact of Zn and temperature on the maximum efficiency of the photosystem II and the level of stress was similar in the three lower concentrations of Zn (figure 2). However the highest concentration of Zn caused an average decrease in the potential of the photosystem II and an average increase of stress, for most temperatures (figure 2). The highest average decrease in the average of the maximum efficiency of the photosystem II was found in the highest temperatures and the highest concentrations of Zn (figure 2).

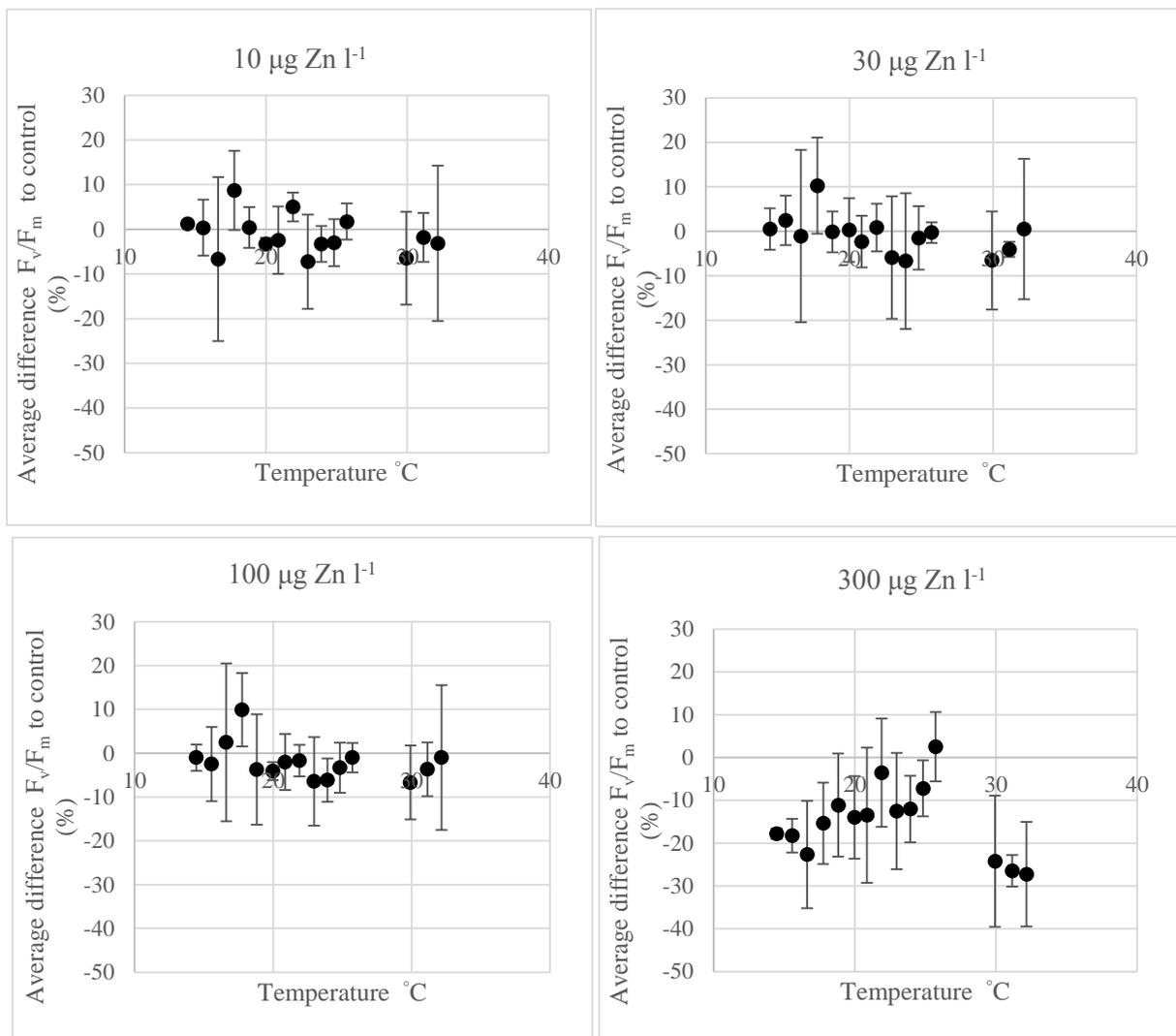


Figure 2. The relative average difference of F_v/F_m was based on an average from measurements on the 8th, 11th, 13th and 16th day of the experiment giving an $n=4$, represented as the black spots with standard deviation (error bars) and shown in different figures for the different Zn concentrations. The zero-line corresponds to the control making the spots below the line equivalent to a decrease in photosynthesis efficiency, and an indication of stress.

3.2.2 Number of cells

The number of survival cells was counted after the experiment as a measurement of the impact of temperature on the toxicity of Zn. The greatest average amount of counted cells (n= 2-4) was found approximately around the temperatures 20 – 26 °C. At the highest end of the temperature spectrum a decrease in the average amount of counted cells was observed in all the different Zn concentrations tested, even the control (figure 3). The decrease in average number of cells in the highest concentration of Zn was most apparent, and differed most from the control and the other Zn concentrations in 30-32 °C. In the highest temperature (32 °C) and the highest concentration of Zn, there were not any cells at the end of the experiment, indicating that they might have died (figure 3).

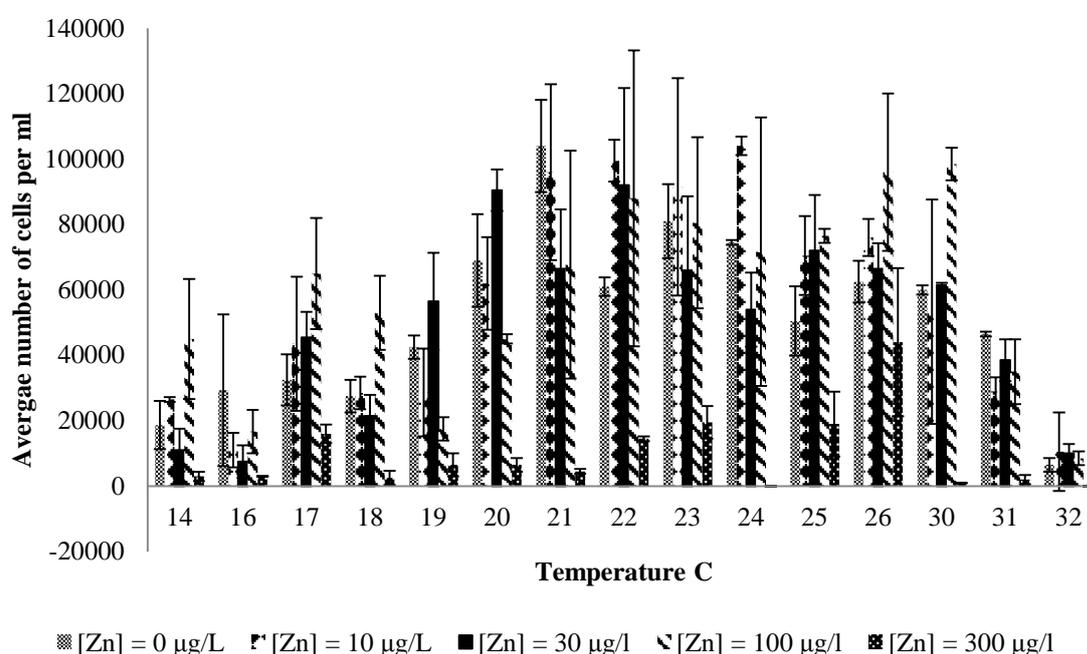


Figure 3. The average number of cells (n = 2-4) as a function of the average temperature in each line respectively where the temperature optimum for number of cells seemed to be in 21 °C for the control. Error bars illustrates the standard deviation. In temperature 24 °C the sample of the highest concentration of Zn was lost before cell count, therefore missing

3.2.3 Approximate EC₅₀

The approximate EC₅₀ based on both F₀ (for the 8th, 11th, 13th and 16th day of the experiment) and average number of cells per ml varied between 93 - >300 µg Zn l⁻¹ with no apparent trend for any temperature (Table 5).

Table 5. An approximate EC₅₀ for Zn (µg l⁻¹) was calculated based on F₀ per ml of cell culture from indicated days during the experiment, and the average cell number per ml of cell culture from the last day of the experiment. The approximate EC₅₀ shows a variation in which concentration of Zn that affect 50 % of the biomass in the control. Data for number of cells at 24 ° C, is missing, thus no approximate EC₅₀ based on average number of cells ml⁻¹ for this temperature.

Temperature ° C	Approximate EC ₅₀ µg Zn /l				
	Average number of cells ml ⁻¹ 16 th day	F ₀ 16 th day	F ₀ 13 th day	F ₀ 11 th day	F ₀ 8 th day
15	269,8	169,3	96,24	234,2	> 300,0
16	129,3	277,0	274,3	275,0	> 300,0
17	> 300,0	297,8	229,9	> 300,0	> 300,0
18	255,4	280,2	235,0	291,0	> 300,0
19	93,27	224,0	140,1	132,1	196,8
20	154,5	272,4	247,2	233,3	289,3
21	120,2	262,5	211,0	221,8	235,9
22	256,0	134,5	206,0	181,6	143,9
23	231,2	218,7	279,9	296,6	283,3
24	-	258,1	203,8	259,1	248,7
25	278,3	224,7	214,5	227,1	281,7
26	> 300,0	227,6	249,9	221,5	163,0
30	240,5	266,5	216,8	251,9	> 300,0
31	171,2	253,6	178,3	233,3	300,0
32	223,5	196,1	> 300,0	> 300,0	> 300,0

3.2.4 Fluorescence per cell

The result of the approximate EC_{50} based on F_0 and average number of cells for the last day varied, the ratio of F_0 and average number of cell, was therefore calculated in order to determine the following

- a) If the F_0 corresponds to the biomass in the experiment performed
- b) If Zn affects the function of the reaction centers, or the number of reaction centers in each cell
- c) If the temperature impacted the toxicity of Zn and if its effect caused an impact on such as the number of reaction centers on each cell or their functioning.

The average ratio between the F_0 and average cell number for each concentration of Zn and several temperatures, showed the average amount of minimal fluorescence per cell (Table 3). The ratio was calculated for three different temperature windows and for all temperatures (but 24° C and 32° C) in order to see if the temperature impacted the amount of fluorescence per cell in any of the Zn concentrations. The ratio was not consistent between concentrations in any of the temperature windows (Table 3), which indicate that F_0 did not correspond to the biomass. In order to analyse if the fluorescence per cell was significantly different between the different concentrations of Zn, in the long-term experiment, in one of the temperature windows, the same program and test was used as in the short-term experiment above. When comparing the ratios towards its corresponding control, respectively, a significant difference was solely found for the average ratio of highest concentration of Zn in the temperature window of all temperatures (except 24° C and 32° C). Therefore temperature did not significantly impact the amount of toxic effects, caused by Zn, on the photosystem II (c). Once looking at all temperatures, the F_0 did significantly deviate from an approximation of the biomass (a). Furthermore this means that Zn might affects the function or the number of reaction centers in the photosystem II on each cell (b). For the last day of the experiment the average cell count for the highest temperature (32° C) and concentration of Zn (300 µg Zn/l) was zero, hence the ratio became could not be calculated, thus the ratio between minimal fluorescence and average number of cells could not be incorporated (table 6).

Table 6. The average amount of fluorescence units per cell was obtained by the ratio between F_0 per ml (for each concentration of Zn) and average number of cells per ml. The ratio was different between the concentrations in all the temperature windows. Each ratio was compared to its corresponding control ($0 \mu\text{g Zn l}^{-1}$) and a significant difference between a Zn concentration towards the control was only found for all temperatures but 24°C and 32°C . * indicates significant difference between the sample and corresponding control (p-value <0.05).

[Zn] $\mu\text{g/l}$	Ratio F_0 and average number of cells			
	Temperature 14-23,25-31 $^\circ\text{C}$	Temperature 14-17 $^\circ\text{C}$	Temperature 20-23 $^\circ\text{C}$	Temperature 30-31 $^\circ\text{C}$
0	0.00016	0.00021	0.00016	0.00006
10	0.00016	0.00027	0.00011	0.00013
30	0.00020	0.00039	0.00007	0.00018
100	0.00022	0.00028	0.00021	0.00014
300	0.00007*	0.00008	0.00005	0.00015

4. Discussion

4.1 Impact of phosphorus on the toxicity of Zn

The aim of this study was to investigate the impact of phosphorus on the toxicity of Zn to isolated benthic diatoms strains, and the hypothesis was that a lower amount of phosphorus, in relation to a standard concentration, would increase the toxicity of Zn. This hypothesis can partly be confirmed by the decreased fluorescence intensity in some strains (D4 and BNEO) in one of the experiments. However there was only a minor difference in approximate EC_{50} between the standard and the lowest concentration of phosphorus. The hypothesis that a higher concentration of phosphorus would decrease the toxicity of Zn could be confirmed by the higher median value of the approximate EC_{50} . Furthermore it was partly confirmed by the higher fluorescence intensity and number of cells in most of the strains and in one or both (only for D4) of the experiments.

A possibility for the lack of difference between the phosphorus concentrations in one of the experiments for most strains, could be that metals can affect the function of the cellular membrane (Morin *et al.*, 2012). Guasch *et al.* (2004) reasoned that the formation of polyphosphates would not occur if uptake of phosphorus was prevented due to impermeabilization of the cell membrane by the presence of Cu. In addition, Kaneko, Shimada & Hirayma (2004) showed that the uptake of phosphorus from the medium of cultivated green algae (*S. capricornutum*) was profoundly decreased with increasing Zn concentrations (added as zinc nitrate). This decrease was apparent already at $10 \mu\text{g Zn l}^{-1}$, even after only 4 days, equal to the time for my experiment. Furthermore, except for growth, phosphorus is needed for the production of polyphosphates (Guasch *et al.*, 2004) and Bates *et al.* (1985) found that the concentration of polyphosphates increased at higher Zn concentrations. If Zn (added as ZnSO_4) can alter this reduction in uptake of phosphorus in benthic diatoms, polyphosphate cannot be synthesized and addition of extra phosphorous will not contribute to increased Zn tolerance, nor will a decrease in phosphorus levels cause an increased toxicity of Zn. This could perhaps also explain why there was no difference between the lower phosphorus concentrations regarding the approximate EC_{50} .

The hypothesis that a higher amount of phosphorus would decrease the toxicity of Zn was however verified by the median value of the approximate EC_{50} . However, the approximate EC_{50} result should be interpreted with caution, since it was based on an average in the fluorescence intensity or the number of cells, thereby not taking into account the variation in the data. In addition the approximate EC_{50} values were not significantly different between the replicates of fluorescence intensity between the phosphorus concentrations, or between strains in the same phosphorus concentrations.

One possible explanation for the fact that strains showed a higher tolerance to Zn in the highest concentration of phosphorus could be the presence of a tolerance mechanism that is enhanced by phosphorus. Different diatom taxa can have different tolerance mechanisms to metals (Morin *et al.*,

2012 and references therein). Perhaps one is related to preventing Zn from altering a reduction in the phosphorus uptake and thereby enabling the synthesis of polyphosphates. Such a tolerance mechanism could explain the result for FCAP in the first experiment in the highest concentration of phosphorus, which generated a higher fluorescence for all Zn concentrations. Furthermore, FCAP (*Fragilaria capucina*) has been reported as a metal-tolerant species (Morin *et al.*, 2012 and references therein). A tolerance mechanism that is enhanced by phosphorus could also explain why the fluorescence intensity in the second experiment of GPAR A3 was significantly higher in the higher concentration of phosphorus for the two highest concentrations of Zn.

The response to Zn as a function of the amount of available phosphorus was however not consistent between experiments. In the first experiment of GPAR A3 the highest concentrations of phosphorus did not decrease the toxicity of Zn, nor did the average number of cells in the second experiment of GPAR A3. Furthermore, the approximate EC₅₀ values between phosphorus concentrations for GPAR A3 was not significantly different, thus phosphorus did not seem to impact the toxicity of Zn to GPAR A3. In addition, the second experiment of FCAP did not show a decreased toxicity of Zn in the highest concentration of phosphorus.

The highest concentration of phosphorus only decreased the toxicity of Zn in the highest concentration of Zn for D4 (both experiments), D14 (second experiment in fluorescence intensity) and BNEO (first experiment). Perhaps this has to do with a tolerance mechanism that is enhanced by phosphorus but that requires the amount of phosphorus to be sufficient in relation to the amount of Zn. Thus the lower phosphorus concentrations were perhaps not sufficient enough in relation to a high concentration of Zn, but the highest concentration of phosphorus was. When inspecting the approximate EC₅₀ values for these strains both BNEO and D4 showed high EC₅₀ values for all phosphorus concentrations, in comparison to the other diatoms. However D14 showed low EC₅₀ values in comparison to the other diatoms, which indicates that this strain is not tolerant to Zn. Furthermore, the approximate EC₅₀ values for D14 did not significantly differ between phosphorus concentrations. In addition the highest concentration of phosphorus only caused a decrease in the toxicity of Zn in one experiment and this was not seen in the number of cells from the same experiment. Therefore D14 does not seem have a tolerance mechanism to Zn that is enhanced by phosphorus.

The higher approximate EC₅₀ values for D4 and the significant higher fluorescence intensity in the highest concentration of phosphorus and Zn might be a result of its natural environment. Benthic diatoms strains that have grown in an environment polluted by Zn have shown greater tolerance to amounts of Zn equivalent to the levels in their normal environment, in laboratory experiments (Ivorra *et al.*, 2002). At the collection site for D4 the concentration was 260 µg Zn/l. Due to its natural metal contaminated environment, perhaps this strain have developed tolerance mechanisms that is eased by phosphorus or that prevents damage to the functioning of the membrane so that phosphorus uptake can

still occur despite high levels of metals. This might apply to BNEO as well, which is a diatom that has been found in metal polluted streams (Kahlert & Gottschalk, 2008). Regarding BNEO it should be noted that the highest phosphorus concentration only caused a decrease in the toxicity of Zn in one experiment. In addition, at the collection site for D14 the levels of Zn was even higher than on that for D4, but this strain does not seem to be tolerant to Zn. The enhanced tolerance to Zn by addition of phosphorus, shown by D4, might therefore not be a result of adaption to high levels of Zn, since D14 did not show the same response. However, D4 showed the same response to the highest concentration of Zn in the highest concentration of phosphorus in both experiments. This could verify that D4 have a tolerance mechanism that is enhanced by phosphorus.

4.2 The effect of temperature on Zn toxicity

In this study the effect of temperature on the toxicity of Zn to a benthic diatom was investigated. The hypothesis was that temperatures above 20° C would increase the toxicity of Zn. It was found that the impact on the efficiency of the photosystem II for the lower concentrations of Zn was similar at different temperatures. However, the highest concentration of Zn did affect the efficiency, and the average of the potential of photosystem II was decreased for most temperatures. If observing the average decrease in the potential of the photosystem II, the hypothesis that higher temperatures did increase the toxicity of Zn can be confirmed. However, this was true solely for the highest concentration of Zn and first at 30 ° C. In addition, the average number of cells also indicated that the highest concentration of Zn at 30 ° C and above caused more toxic effects. It should not be left out that the lack of cells in the highest concentration of Zn and in 32 ° C could be a result of an error in the experimental performance. However, the cells in 30 ° C and 31 ° C and in the highest concentration of Zn did evidentially grow, since the number of cells were higher at the end of the experiments than the number added prior to the experiment. Due to the fact that these number of cells (in 30-31 ° C) were lower than in the other temperatures, the hypothesis is still confirmed, that temperatures above 20 ° C would increase the toxicity of Zn.

That the impact of the highest concentration of Zn was higher in the highest temperatures might have to do with the combination of a stress-related environment and a toxicant. If the impact of biological factors, such as nutrients, causes the organism to be in a better condition their tolerance to metals could be expected to increase (Wang 1987). Assuming that this reasoning also can be applied to the abiotic temperature factor, the result from this experiment would indicate that the degraded condition of the diatoms in the lower and the higher temperatures caused a stress that resulted in a lower tolerance to Zn. In addition, Cairns, Heath & Parker (1975) pointed out that the combined effect of two stresses could cause a greater stress together than separately, which in this case would be the combination of sub-optimal temperatures for the diatoms and Zn in the highest concentration tested. Furthermore, Wang (1987) concluded in a literature review, that in general the toxicity of metals to aquatic organisms

increases with elevated temperatures. Moreover, in a study of the impact of Hg to freshwater algae Val *et al.* (2016) found that an elevation of the temperature increased the toxicity of Hg, not significantly though.

The approximate EC_{50} showed a great variation over time and between F_0 and average number of cell. In addition, the ratio between F_0 and average number of cells revealed that the F_0 parameter did not correspond to the actual biomass, due to the lack of consistency in the ratio. For the temperatures 14-17 ° C , 19-22° C and 30-31° C there was however no significant difference between the ratios in the different Zn concentrations. The result for the average of all temperatures (with the exception of 24° C and 32° C) and the highest concentration of Zn was significantly lower than the control, with no additional Zn. This could indicate that Zn affects the number and or the functioning of reaction centers in photosystem II. A decreased number of reaction centers has been suggested as an explanation of lower F_0 values in a benthic diatom strain subjected to Zn (Ivorra *et al.*, 2002).

Effects on the photosynthetic apparatus on biofilms as a result of Zn exposure have also been shown by Corcoll *et al.* (2012). Thus Zn was shown to decrease the efficiency of the photosystem II, however the maximum efficiency, (the potential of the photosystem II) (F_v/F_m), as investigated here, was not as clearly reduced (Corcoll *et al.*, 2012). However, here I have found that Zn seems to influence the maximum efficiency of photosystem II, and perhaps the number and or functioning of the reaction centers in Photosystem II at 300 $\mu\text{g Zn l}^{-1}$.

The average related impact on the maximum efficiency of the photosystem II (F_v/F_m) and the number of cells caused by the highest concentration of Zn tested, could be related to higher temperatures. However, the F_0 and average cell number ratio and the approximate EC_{50} values did not shows any link between temperature and the toxicity of Zn. Thus, conclusively not all parameters investigated here declares that temperature impacts the toxicity of Zn, but since some of them do, it cannot be said that temperature does not impact the toxicity of Zn. Hence, the hypothesis that temperatures above 20 C would increase the toxicity of Zn is partly confirmed.

5. Conclusion

In this study phosphorus had an impact on the toxicity of Zn in some cases. This might indicate that phosphorus could cause a variation in the response to Zn, shown by the benthic diatoms in the field. If the levels of phosphorus are low in a Zn polluted stream the toxic effect of Zn might be greater than in a phosphorus rich stream with the same levels of Zn. However, due to the inconsistent results between experiments, more studies are required in order to fully determine if the level of phosphorus influences the toxicity of Zn. Furthermore, in order to determine if Zn affects the uptake of phosphorus and if some of the diatoms have tolerance mechanisms that prevent such an effect, the levels of phosphorus and Zn in media should be measured after the experiment. Since the added concentration of Zn and phosphorus is known, this would provide information about the damage caused by Zn or the prevented damage caused by phosphorus. Moreover, if Zn causes a decrease in the uptake of phosphorus it would be interesting to see if cells cultivated in different phosphorus levels prior to the experiment would react differently by the addition of metals. If so, this knowledge could be useful in order to determine how benthic diatoms in the field would respond to temporal outbreaks of metals and if this is dependent on the phosphorus levels in their environment.

This study indicates that temperature could impact the toxicity of Zn, even though not all parameters investigated pointed to a clear impact of temperature on the toxicity of Zn. Thus, more toxic effects as a decrease in number of cells or the maximum efficiency of the photosystem II, might be seen in the future if the climate changes causes temporal increase of water temperatures to 30 ° C or in a very hot period of the summer.

I conclude that more studies regarding the impact of phosphorus and temperature on the toxicity of Zn are needed since the result here is not fully consistent but at the same time indicates that phosphorus and temperature could impact the toxicity of Zn. Furthermore, these factors could therefore cause a geographical and seasonal variation in the response to Zn, shown by benthic diatoms in the field. Hence, when developing an index for benthic diatoms that reflects on the levels of metals in streams, it could be useful to further investigate the impact of phosphorus and temperature.

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Unpublished material:

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Appendix

Table 1. The average temperature was measured at the last day of the long-term experiment in each vial giving an $n=5$. \bar{x} shows the average and s the standard deviation.

Temperature °C	
\bar{x}	s
14,46	0,5128
15,56	0,4393
16,62	0,1789
17,76	0,2408
18,82	0,3834
19,98	0,3633
20,86	0,05477
21,90	0,3391
22,96	0,3130
23,92	0,0837
24,82	0,1304
25,72	0,2588
29,92	0,7050
31,14	0,2408
32,16	0,1673

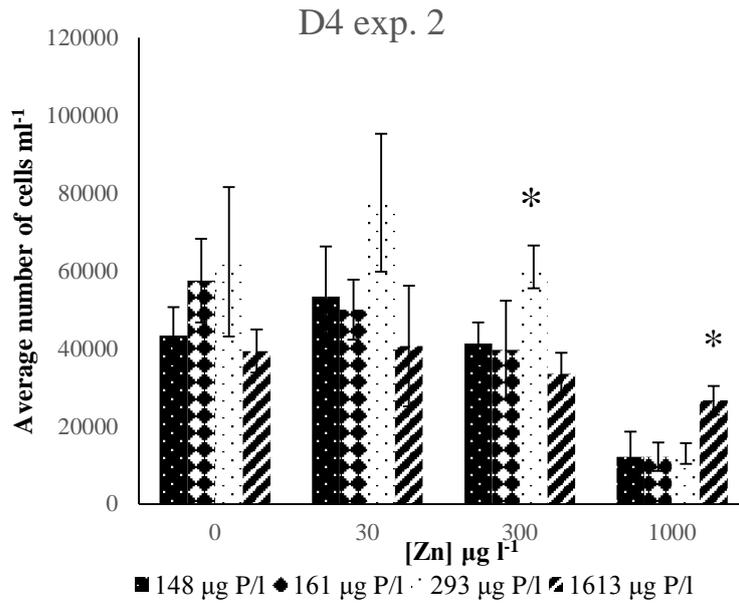


Figure 1. The average number of cells ($n=6$) as a function of the concentration of Zn for D4 and the second experiment, asterix * denoting significant difference between phosphorus strains.

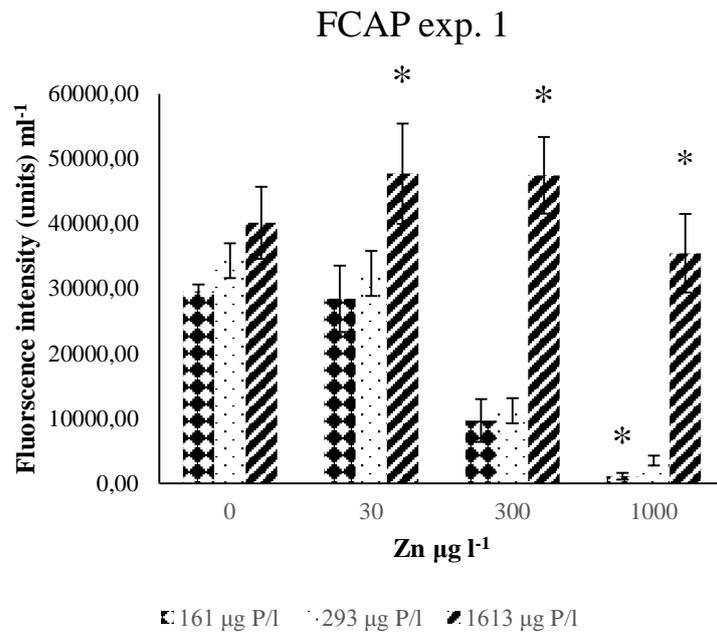


Figure 2. In the first experiment of FCAP the average fluorescence intensity ($n=3$) was measured and the result for the highest concentration was significantly different from the lower concentration in all Zn concentrations except the control (denoted as *). Furthermore, there was a significant difference between the lower concentrations of phosphorus in the highest concentration of Zn. In $300 \mu\text{g Zn l}^{-1}$ and $161 \mu\text{g P l}^{-1}$ there was an overflow of fluorescence that the machine could not measure, hence for this average fluorescence intensity $n=2$.

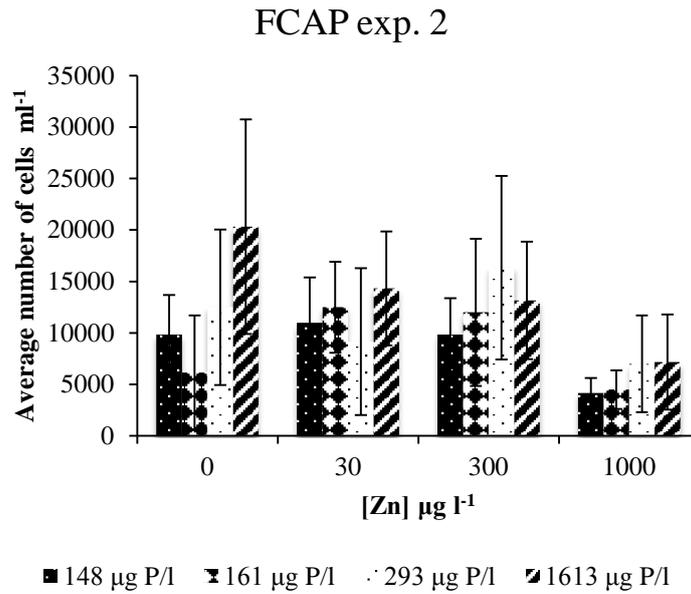


Figure 3. In the second experiment, the difference between phosphorus concentrations for FCAP was detected as the average number of cells ($n=6$). However there was no significant difference between concentrations for any Zn concentration, based on the standard deviation illustrated as error bars.

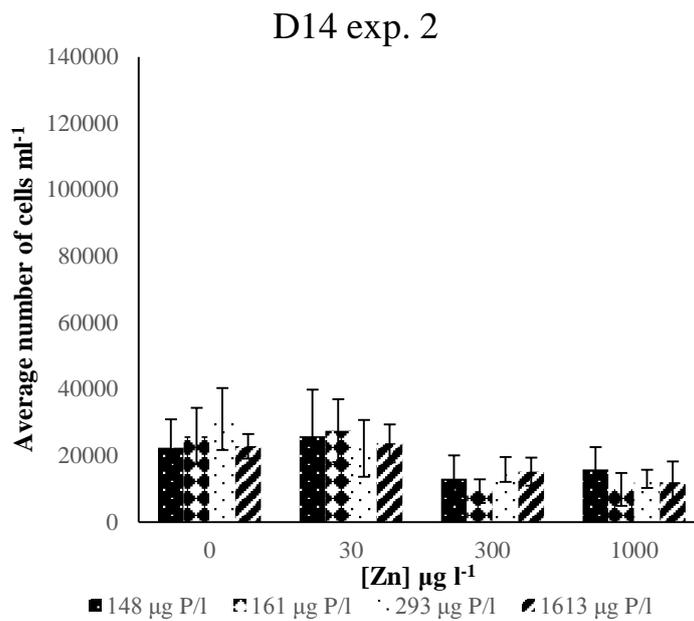


Figure 4. In the second experiment of D14 the result in average number of cells ($n=6$) was not significantly different between the phosphorus concentration and the Zn concentrations, based on the standard deviation illustrated as error bars.

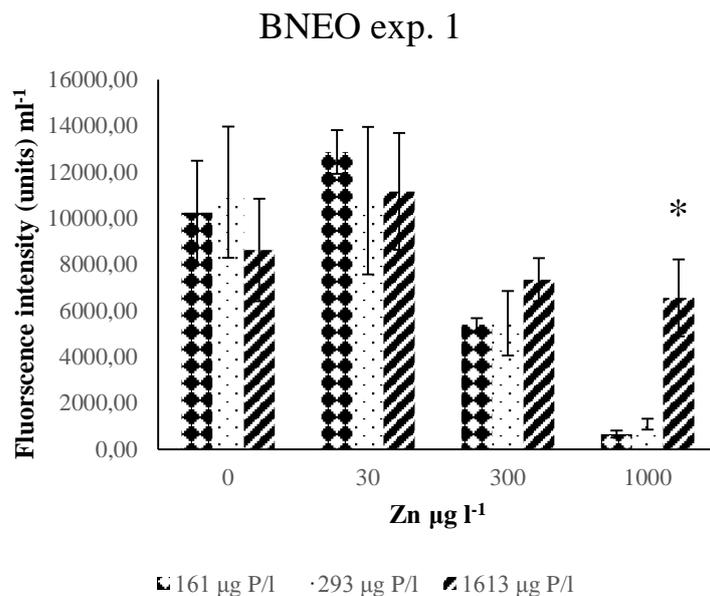


Figure 5. In the first experiment the average fluorescence intensity ($n=3$) was significantly higher in the highest phosphorus and Zn concentration, denoted as *. Error bars illustrates the standard deviation.

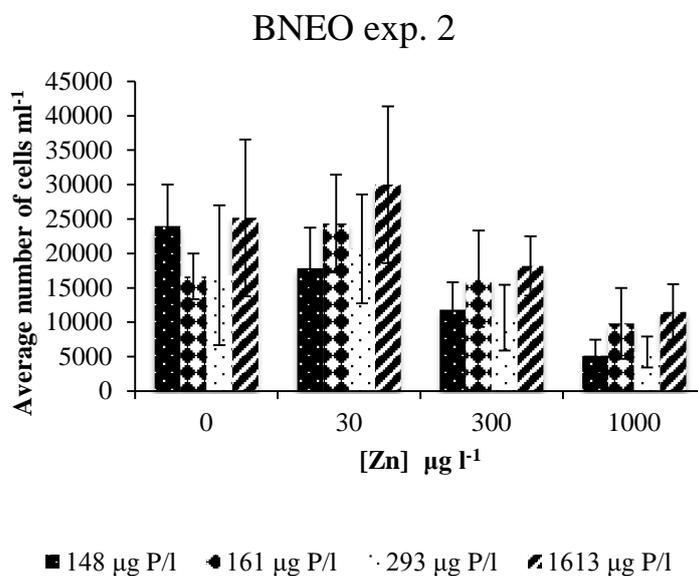


Figure 6. In the second experiment of BNEO the average number of cells ($n=6$) was not significantly different between the phosphorus concentrations for any Zn concentration, based on the standard deviation, illustrated as error bars. The highest concentration of phosphorus in the highest concentration of Zn showed a significantly higher fluorescence intensity in comparison to the standard concentration of phosphorus ($p < 0.05$). However based on the standard deviation there was no significant difference between the second lowest and the highest concentration of phosphorus in the highest concentration of Zn.

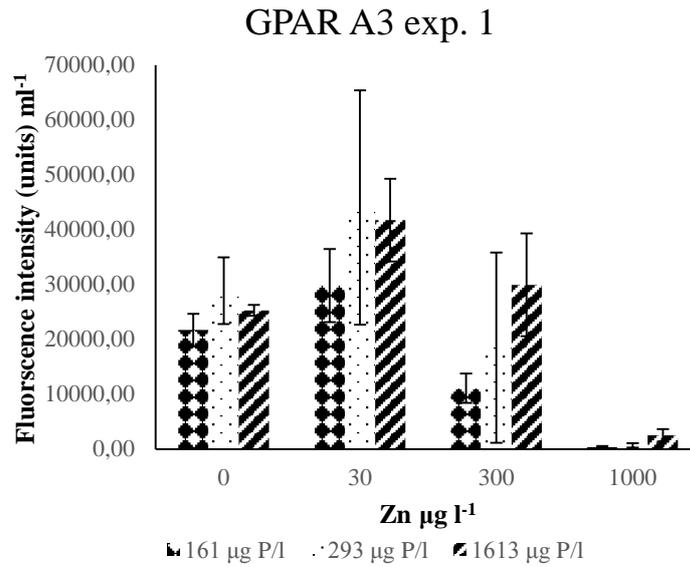


Figure 7. In the first experiment of GPAR A3 the average fluorescence intensity ($n=3$) was not significantly different between the phosphorus concentration in any Zn concentration. Error bars illustrates standard deviation.

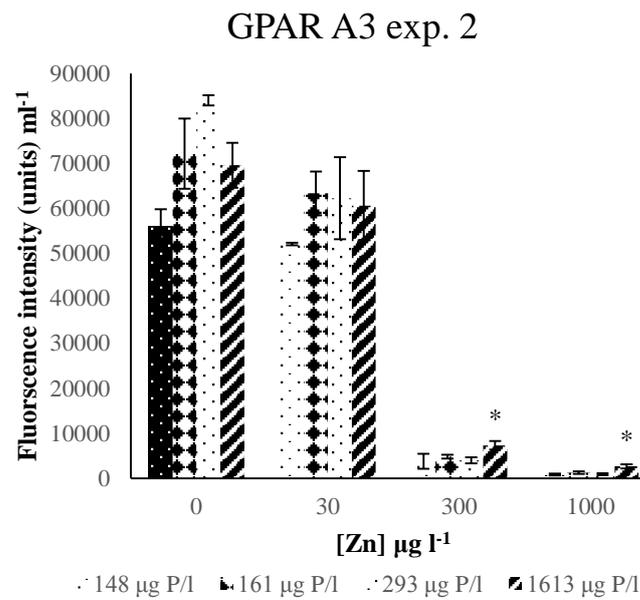


Figure 8. In the second experiment of GPAR A3 the fluorescence intensity was measured ($n=3$). The standard deviation is illustrated as error bars. In 300 µg Zn/l the highest concentration of phosphorus was significantly higher than the standard concentration of phosphorus and the lowest concentration of phosphorus. Furthermore, the highest concentration of phosphorus in the highest concentration of Zn was significantly higher than the standard concentration of phosphorus and the 160 µg P/l. The lower concentrations of phosphorus did not significantly differ in the any of the Zn concentrations.

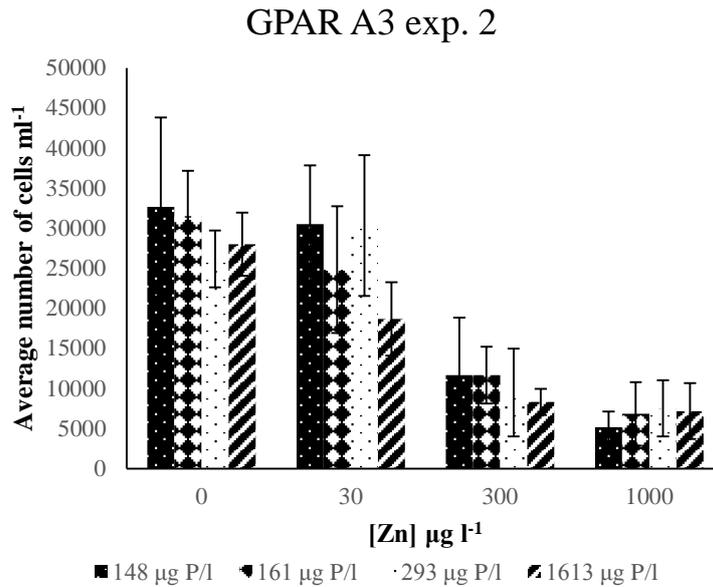


Figure 9. In the second experiment of GPAR A3, the number of cells was counted. The average number of cells ($n=6$) was not significantly different between the phosphorus concentrations in any Zn concentration, based on the standard deviation, illustrated as error bars.

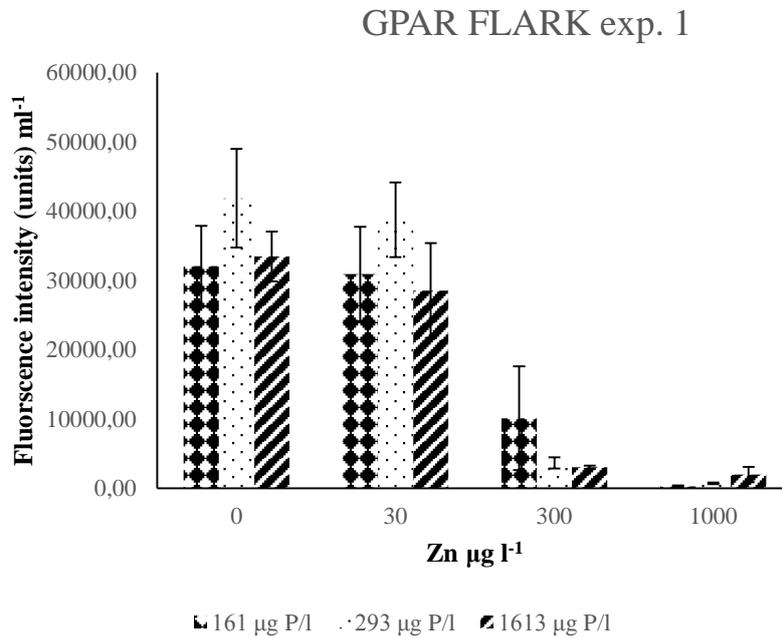


Figure 10. The average fluorescence intensity ($n=3$) was measured in the first experiment of GPAR FLARK. Standard deviation is illustrated as error bars. The lowest concentration of phosphorus showed a significantly lower fluorescence intensity than the standard concentration of phosphorus, in the highest concentration of Zn. However there was no significant difference between the lowest and the highest concentration of phosphorus, in the highest concentration of Zn. Thus the lowest concentration of phosphorus cannot be said to have increased the toxicity of Zn.

GPAR FLARK exp. 2

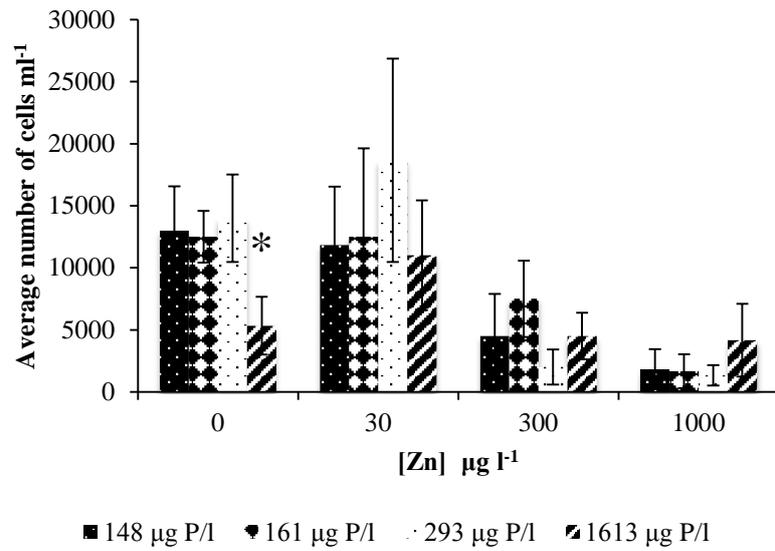


Figure 11. In the second experiment, the average number of cells ($n=6$) was significantly different in the control (no addition of Zn) between the highest and the lower concentrations of phosphorus. In the other concentrations of Zn however, there were no significant difference between phosphorus concentrations, based on the standard deviation illustrated as error bars.