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Faculty of Natural Resources and Agricultural Sciences

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Amanda Hedenborg

Department of Aquatic Sciences and Assessment
Degree project • 15 hec • First cycle, G2E

Uppsala 2016

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Supervisor: Maria Kahlert, SLU, Department of Aquatic Sciences and Assessment
Assistant supervisor: Sara Goncalves, SLU, Department of Sciences and Assessment
Examiner: Jens Fölster, SLU, Department of Aquatic Sciences and Assessment

Credits:15hec
Level: First cycle, G2E
Course title: Independent project in Environmental Science - bachelor project
Course code: EX0688
Programme/education: Bachelor programme in Environmental Science

Place of publication: Uppsala
Year of publication: 2016
Online publication: http://stud.epsilon.slu.se

Keywords: Freshwater diatoms, indicator for metal pollution, zinc pollution, pH, malformations

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Errata

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Page 3 Abstract
Old text:
…the approximated EC_{50} values of 0,91; 1,35 and 2,27 µg Zn l^{-1}
New text:
…the approximated EC_{50} values of 0,91; 1,35 and 2,27 mg Zn l^{-1}

Abstract

The aim with this study was to observe the zinc (Zn), tolerance for different strains isolated from sites with Zn pollution and to observe if lower pH decreases the Zn toxicity. Another aim was to see if malformations could be an indicator for Zn pollution.

Zn is found naturally in the bedrock, sediment and waterbodies. Mining is one activity which had led to high values of Zn > 60 µg Zn l^{-1} in some areas in Sweden. Today diatoms are used as indicators for organic pollution, the amount of nutrients and also for the pH condition. Due to problem with high values of metals, the diatom indicator has been developed further to also involve detection of metal pollution. Zn can affect diatoms growth rate, photosynthesis, cell division as well as their silica transport and thereby leading to malformed diatoms. In this study Zn toxicity was observed as decrease in number of cells, degree of malformations and as a decrease in the photosystem II efficiency.

The hypotheses for the study are the following: the tested strains isolated from polluted sites would have higher tolerance to Zn than diatoms isolated from non- polluted sites, Zn would be less toxic in pH 5 compared to pH 7 and malformations could indicate Zn pollution. The observed results confirmed the hypothesis regarding the tested strains having higher tolerance towards Zn than diatoms from non- polluted sites, by the approximated EC_{50} values of 0,91; 1,35 and 2,27 µg Zn l^{-1} respectively. However, the observed results for the hypothesis regarding the lower pH decreasing the Zn toxicity, were contradictory. The hypothesis was verified by higher EC_{50} values for the cell growth and fluorescence in pH 5 than in pH 7 for the short term experiments. Whereas the hypothesis was rejected by a higher degree of malformation in pH 5 than in pH 7 and by a higher cell growth in pH 7 than in pH 5 in the long term experiment. The different results regarding the pH effect on Zn toxicity might be explained by the different exposure times, probably the low pH is causing more stress to the cells pre-grown in pH 7 than it relieves the Zn toxicity. The long term experiment displayed significant more malformations in the higher Zn concentrations in comparison to the controls under both pH 5 and 7, and verified that malformations could detect high Zn concentrations and indicate Zn pollution.

Keywords: Freshwater diatoms, indicator for metal pollution, zinc pollution, pH, malformations

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1 Introduction

Zinc (Zn), is a heavy metal and toxic for many organisms in higher amount (Naturvårdsverket, 2010). Zn is used in the metal industry and there are emissions of Zn from oil combustion, wear of car tires and from use in the metal industry (Naturvårdsverket, 2010). Zn is also found naturally in the bedrock, sediment and in waterbodies (Naturvårdsverket, 2010). Therefore, mining plants can cause release of heavy metals such as Zn, hence old mining plants are usually polluted by heavy metals (Pandey & Bergey, 2005). The mine drainage also contains heavy metals and as the mine drainage is acid it increases the mobility of metal ions in the soil (Pandey & Bergey, 2015).

Diatoms are important in the aquatic food web as primary producers, they live in the ocean and in fresh water and are producing about 1/5 of all the oxygen (Saade & Bowler, 2009). Diatoms are photosynthetic organisms with chlorophyll a (chl a), and in addition they have carotenoid pigments, which give them their brown/golden colour (Debenest et al, 2013). Diatoms are unicellular organisms with characteristic cell walls, also called the frustule, made of silica, Si, (Debenest et al, 2013). The frustule is composed of two valves that are like a box and a lid (Debenest et al, 2013). There are two types of diatoms, pennate and centric, the pennate ones are only found in the benthic habitats (Debenest et al, 2013). The benthic diatoms live in a biofilm of mucilages that are composed of polysaccharides, the mucliages are important for the diatoms ability to attach to substrates (Debenest et al, 2013).

The present study was performed to observe if diatoms used to high Zn concentrations displays Zn tolerance and if the pH is affecting Zn toxicity. Furthermore, it was studied if Zn pollution could be detected by an increase of teratological cell forms.

1.1 Diatoms as an indicator for water quality

Sweden's national environmental quality objectives and EU: s water framework directive both have directions for the environmental monitoring (Berntsson, 2016). The environmental monitoring is used for observing if the environmental qualities are obtained and to notice new environmental changes (Berntsson, 2013). The Swedish agricultural university (SLU) is involved in the monitoring of freshwater in Sweden, where "Diatoms" is a sub-program in the environmental monitoring (Sonesten, 2015). The diatom sub-program gathers information about the environmental conditions, that can be used for classification of the ecological status (Kahlert, 2011). The program is also used for follow up of several of the Swedish national environmental quality objectives like, "Zero Eutrophication", "Flourishing

Lakes and Streams", "Natural Acidification Only" as well as "A Non-toxic Environment" (Berntsson, 2014). Freshwater diatoms are used as a biological indicator for water quality in streams in Sweden (Kahlert, 2016). Diatoms are good indicators because their responses to environmental changes are rather quick (a few days), and additionally it is possible to detect environmental changes happening up to several months ago, depending on the impact (Kahlert et al, 2016). Different diatom species have different preferences regarding pH and nutrients like phosphorous and nitrogen, therefore the species present in the water body can indicate the environmental conditions (Kahlert et al, 2016). There are several index for diatoms that are used to establish the water quality, IPS – shows the amount of organic pollution and the amount of nutrients, TDI- trophic diatom index displaying nutrients, %PT – percentage of frustules tolerant to organic pollution and ACID -that is used to determine the pH in the water body (Kahlert, 2016). To enable a further development of the diatom indicator to include detection of heavy metal pollution, a new method has been developed where the amount of malformation in the samples are analysed (Kahlert et al, 2016).

1.2 Zinc toxicity

Although Zn is a heavy metal, Zn is also an essential micronutrient for living organisms, due to its function as a building block for enzymes (Naturvårdsverket, 2010). Metals can affect diatoms in several ways. One is by metabolism dependent uptake, a process with a duration time of hours – days (Stevensson et al, 1996). Once Zn is inside the cell it can precipitate, bind to intracellular components or affect enzymes with proper binding sites (Stevensson et al, 1996). Intracellular Zn disturbs the silica transport to silica vesicles by poisoning the microtubules, resulting in malformed frustules (Falasco et al, 2009). The magnitude of Zn absorption is affected by the exposure time, rate of growth and the permeability for the membrane (Stevensson et al, 1996). The intracellular Zn increases with longer exposure time to high Zn pollution (Pandey et al. 2015). Zn is known to affect processes and decrease the diatoms functions in various ways. Zn has been observed to decrease the growth rate, due to being a stressor (Stevensson et al, 1996). Zn can also disturb photosynthesis by being a stressor that affect the CO_2 uptake or affecting the photosystem II function, causing chemical damage on pathways for the photosynthesis, or by indirectly affecting the photosynthesis by impairing enzyme activity and permeability for the membrane (Stevensson et al, 1996). Diatoms found in sites with high Zn pollution can show a decrease in size (Pandey and Bergey, 2015). The theory is that Zn disturbs the calcium, Ca, uptake and thereby affecting the function of Ca – ATPase negatively (Sbihi et al, 2012). Ca – ATPase is a function that is essential in cell division and can therefore affect the cell growth (Sbihi et al, 2012). Several

studies have observed changes in diatom communities due to Zn pollution, the amount of some Zn tolerant species increases whereas the amount for other species decreases (Ivorra et al, 2002).

Among the heavy metals Zn is considered one of the least poisonous, but it is also one of the most abundant in the environment in Sweden (Naturvårdsverket, 2010). Background values of Zn in Swedish water bodies are usually around 1-3 µg/L (Naturvårdsverket, 2007). In Sweden waterbodies are divided in the following groups in accordance with their Zn concentrations; 5 -20 µg low values; 20- 60 µg moderate values; 60- 300 µg high values and very high values > 300 µg (Naturvårdsverket, 2014).

1.3 Diatoms defence mechanisms to heavy metals

Diatoms have several defence mechanisms for handling stress from heavy metals, like producing phytochelatins, immobilize extracellular metals and incapacitate metal ions inside the cell (Branco et al, 2010). Phytochelatins are polypeptides that are rich in thiols and has a detoxifying mechanism for metals inside the cell (Lavoie et al, 2012). The phytochelatins task are to maintain the homeostasis for the essential metals inside the cell (Hirata et al, 2005). The homeostasis is maintained, by making complexes with metals inside the cell and transport them to the vacuole where they are incapacitated (Branco et al, 2010). Phytochelatins can be produced when diatoms are exposed to heavy metals, but there is no evident correlation between high concentration of intracellular Zn or high concentration of free Zn ions and the production of phytochelatins for freshwater diatoms (Lavoie et al, 2012). In contrary to Lavoie et al (2012) a study by Figueria et al (2014) observed that when the intracellular cadmium, Cd, increased a higher amount of intracellular Cd was bound to phytochelatins, which could indicate production of phytochelatins. Phycotchelatins affinity for Zn is lower than for other heavy metals like cadmium, probably due to the fact that Zn is an essential element to the cell (Hirata et al, 2005).

Diatoms also have defence mechanisms to stop heavy metals like Zn from entering the cell (Bahulikar & Kroth, 2008). Diatoms can produce extracellular polymeric substances, EPS, which consists of proteins and glycoproteins and carbohydrates (Bahulikar & Kroth, 2008). The EPS binds metals extracellular and protecting the inside of the cells from metals, making the diatoms more tolerant to heavy metals (Santos et al, 2012). Frustulins, a type of EPS, are a group of proteins found on diatoms frustules, where they enabling a protective coat against heavy metals (Santos et al, 2012). Frustulins are rich in acidic groups and cysteine groups, sulfhydryl is the functional group for cysteine, which enables frustulins high affinity for heavy metals (Santos et al, 2012). Frustulins are also considered to help the cell wall to maintain their shape and mechanisms (Santos et al, 2012). For diatoms exposed to

Cd the amount of frustulins have been observed to increase (Santos et al, 2012). It has also been observed that most of the Cd was found extracellular and that about 85 % of the Cd was binding to frustulins, indicating that frustulins are good at protecting the cells from metals (Santos et al, 2012).

Diatoms constantly exposed to high Zn values have been observed to have higher tolerance towards Zn, in comparison to diatoms not being exposed to high Zn values (Ivorra et al, 2002). It is considered to be a genetic adaption to high Zn that enables the tolerance (Ivorra et al, 2002). The photosynthetic function is disturbed at much lower Zn values for diatoms not used to high Zn than the diatoms exposed to high Zn concentrations (ivorra et al, 2002). The tolerance is thought to be obtained by a higher rate of phytochelatins and the vacuole sequestering the Zn from the cell, than the rate of uptake of Zn to the cell (Ivorra et al, 2002).

1.4 pH affecting zinc toxicity

For watercourses with pH > 6, Zn are in majority creating strong complexes with free hummus substances in the water, thereby decreasing the available Zn to affect organisms in the water (Berggren Kleja et al, 2006). If the pH decreases, more Zn is available as free Zn^{2+} in the water courses (Berggren Kleja et al, 2006). A decrease in the soil pH increases the solubility for Zn^{2+} (Berggren Kleja et al, 2006), therefore it is more available Zn ions that can affect diatoms in lower pH. Studies have also shown that cells cultured in acidic environments have to cope with the stress from regulating the cytosolic pH to neutral condition (Luis et al, 2014, Hevré et al). As the external pH decreased 2,1 units there was also a decrease in the internal pH with 0,94 units, which is corresponding to about 9 times higher H^+ concentration inside the cell (Hervé et al, 2012). The normal internal pH for the marine diatom *Thalassisoria weissflogi* has been measured to 7,35, which is an internal pH equivalent for other diatoms (Hervé et al, 2012).

Several studies have suggested that a low pH should have a shielding effect towards metals because of more hydrogen (H^+), ions that could compete with the metals ions for binding sites at the cell surface (Wilde et al, 2005). Wilde et al (2005) and Heijerick et al (2002) have observed that Zn toxicity is pH dependent, the IC 50 (the concentration needed to inhibit 50 % of a biological process) for Zn increased with decreasing pH, which indicates that more acidic pH is decreasing the Zn toxicity. Luis et al (2014) have also observed that lower pH could decrease metal toxicity. When Zn and copper (Cu), was added to cultures with diatom communities that already been exposed to high concentration of iron (Fe), and sulphate (SO_4^{2-}), the acidic condition decreased the toxic effect of Zn and Cu (Luis et al, 2014). In contrary to the studies observing a decreased toxicity for Zn with lower pH, some studies have reported that acidic pH does increase the Zn toxicity for diatoms that

have neutral or basic pH as their optimal pH conditions (Hevre et al, 2012; Hirst et al, 2002). When the external pH is altered from the optimal pH for the cell to a more acidic pH, the growth rate decreases (Hevré et al, 2012). Another study indicating that changed pH conditions affects diatoms are Hirst et al (2002) who found that within three days the dominant taxa had changed, when communities used to neutral pH were transplanted to an acidic condition. The contrary was found when diatom communities used to acidic condition were transplanted to neutral condition, where the species diversity increased and the dominant taxa had changed after 9 days (Hirst et al, 2002). When communities been transplanted, the dominant taxa differ in acidic and neutral condition, and 12 days after transplantation the original communities was no longer recognisable for both conditions (Hirst et al, 2002).

1.5 Malformations

Diatoms can have changes in morphology due to environmental factors causing stress. Pollutions from heavy metals like Zn are well known to cause malformations (Falasco et al, 2009). Morphological changes are also called teratological forms, the teratologcal forms involving the valve outline rises during the reproduction, which could lead to a population with teratologies (Falasco et al, 2009). All physiological mechanisms of why malformations occurs are not known (Cantonati et al, 2013). Nevertheless, Pandey et al (2015) observed a positive linear relationship ($r^2 = 0,87$) between the internal Zn concentration and the amount of malformations. The morphological changes are usually involving deformations in the frustules and patterns of striation (Falasco et al, 2009). Several studies have observed, valve outline malformations to be the most common type (Falasco et al, 2009; Pandey and Bergey, 2016); Cantonati et al, 2013). Pandey & Bergey (2016) have found that areas with high Zn pollution had a significant higher amount of malformed frustules (8,16 %) than sites with low Zn pollution (0,21 %). In Sweden a malformation degree of 4 % has been observed for sites with very high Zn concentrations ($Zn > 0,3$ mg l^{-1}) (Kahlert, 2012). The tendency to develop malformed frustules differs for species, *Fragilaria capucina* is a species that has been observed to have a high abundance of malformations in Zn polluted sites (Pandey et al, 2015; Pandey et Bergey, 2016). It is known that due to the excretion of waste products, a higher degree of malformation is commonly found in old culture media (Falasco et al, 2009). In the developed method using the degree of malformations as indication for toxicity, the preliminary classification system for malformed diatoms is the following: < 1% non or insignificant, 1-2 % low, 2-4 moderate, 4-8 high, > 8 % very high (Kahlert et al, 2016).

The aims for this study are to observe how pH conditions affect the Zn toxicity and if diatoms used to Zn pollution are more tolerant than diatoms not used to Zn.

Another purpose with the study is to test if malformations can indicate Zn pollution, as part of the development of the freshwater diatom indicator to include heavy metal pollution.

The first hypothesis for the study is that pH 5 will decrease the Zn toxicity compared to pH 7. The second hypothesis is that strains used to high Zn values from their collecting site will have a higher tolerance to Zn in comparison with diatoms collected from non polluted sites. The third hypothesis is that it is possible to detect Zn toxicity with malformations in diatoms.

2 Methods and materials

In this study three different Swedish diatom strains were used, the strains had been isolated earlier from different locations with different environmental conditions, see table 1. The long term experiment was only performed with one strain, whereas the short term experiments was performed with all three collected strains. All the strains have been cultured in WC medium with a pH around 7 (Guillard & Lorenzen, 1972). The cells have therefore probably adapted to the mediums pH even if they were isolated from sites with other pH conditions.

Table 1. Presenting the environmental conditions for pH and zinc concentration (mg/l), at the different locations were the diatoms in the study were collected.

Name:	Species:	Location:	pH:	Zinc concentration (mg/l)
D 16	*Fragilaria Gracilis*	Övre klingen	7,2	0,035
D 13	*Fragilaria Capucina*	Persbobäcken	7,3	0,36
FCAP	*Fragilaria Capucina*	Smedmyrbäcken	4,3	Unknown

2.1 Fluorescence measurements

Fluorescence is measured to observe the amount of biomass in samples. The fluorescence yield gives information about the amount of chlorophyll a, chl a, which is in relation to the amount of biomass (Honeywill et al, 2002). Fluorescence can be measured with a fluorimeter by applying light of a certain wavelength and measure the reemitted light (Maxwell and Johnsson, 2000). Light energy taken up by a chlorophyll molecule can either be used for photosynthesis, be reemitted as light (fluorescence) or dissipate as heat (Maxwell and Johnsson, 2000). There is a competition between these processes for the light absorbed by the chlorophyll molecule (Maxwell and Johnsson, 2000). In this study both the Fluorimeter Genios Pro 96/384 (Techan) and a Pulse Amplitude Modulated (PAM) fluorimeter (Fluorescence monitoring system, Hansatech) was used. Both instruments measure the fluorescence, the PAM fluorimeter will be described more below. When measuring the fluorescence with the Fluorimeter Genios Pro 96/384 the results were observed as the cells Chl a.

2.1.1 PAM fluorimeter

This type of fluorimeter has in contrary to a regular fluorimeter a modulated light source, that is switched on and off with high frequency (Maxwell and Johnsson, 2000). With this mechanism a PAM fluorimeter can measure fluorescence in light as well as in the dark (Maxwell and Johnsson, 2000). The photosystem II reaction centres are closed by a short flash of light that not affect the amount of energy that dissipates as heat or as fluorescence (Hanstech instruments Ltd). When the PSII reaction centres are closed there is no energy for photosynthesis and the maximum fluorescence, Fm, is obtained (Maxwell and Johnsson, 2000). The minimal fluorescence (F0), is corresponding to the chl a, thereby the F0 values indicates the amount of algae biomass (Honeywill et al, 2002). The variable fluorescence (Fv), is the change between F0 and Fm (Honeywill et al, 2002). If the efficiency for PSII changes negatively there is also a decrease in Fv/Fm, indicating that a stress factor is affecting the photosystem (Maxwell and Johnsson, 2000).

Because the detector is adapted to only detect fluorescence from the applied light, a PAM fluorimeter can also measure the fluorescence in light (Maxwell and Johnsson, 2000). When measuring in the light some of the PSII reaction centres are closed, and by applying the saturating pulse all of the PSII reaction centres closes and the maximum fluorescence yield, Fm´, is observed (Hansatech instruments Ltd). ePSII is measured in light and is a measurement of the amount of light in chlorophyll molecules related to PSII, that are used for photosynthesis (Maxwell and Johnsson, 2000). If the applied PAM light is similar in the experiments, one can assess the effectiveness of the photosynthesis under the experimental conditions.

2.2 Long term experiment

For the long term experiment diatoms from the strain D16 were cultured in WC medium. The experiment was carried out with a control with 0 Zn and the tested Zn concentrations were 0,03; 0,3 and 1 mg Zn l^{-1}. The control and the three Zn concentrations were tested at both pH 5 and pH 7, all treatments were made in triplicates. To reach the desired cell concentration of 100 cells ml^{-1} for the experiment the amount of cells was counted prior to the experiments, and the respective amount of cells was then added to the treatments. The cells were counted with the microscope Nikon Eclipse 80i and the counting chamber Neubauer improved, volume 1 µl.

2.2.1 Preparation of treatments

In the study WC medium was used that was prepared in accordance with the recipe, but the added EDTA was only 1/10 of the amount in the recipe (Guillard & Lorenzen, 1972). The medium was autoclaved before use. Twenty-four acid washed Erlenmeyer flasks were filled with 250 ml WC medium, the pH was adjusted to 5

respective to 7 for half of the Erlenmeyer flasks. The buffer MES and 0,1M HCl and 0,1 M NaOH were added to adjust the pH top 5 and 7 respectively. Zn stocks were made with $ZnSO_4$ and added to the Erlenmeyer flaks before the cells, the final volume was 250 ml.

2.2.2 Growth measurements with PAM

After the cells were added the Erlenmeyer flasks were put in a room with 21 ° C to grow. To be able to follow the growth of the diatoms, fluorescence was measured continuously whit a PAM Fluorimeter (Fluorescence monitoring system, Hansatech). The F0 value was measured to follow the growth of the cells and to be able to observe if Zn affected the photosystem II, the Fm was also measured. The fluorescence was measured for each replicate at day: 4, 6, 7, 10, 11, 12, 14 and 17 after the start of the experiment. The software program, instrument Modflur32 (Hansatech), was used to set the condition for the measurement and to register the results for the PAM measurements. The fluorescence was measured both in light and in the dark with an intensity of 80 % and 0,7 seconds duration for the saturation pulse. Before measuring Fv/Fm in the dark, the cells were adapted to the dark for 15 minutes. And before measuring esPII the cells were adapted to light for 15 minutes. In the light the actininc light source was set to intensity 1, and was switched to 0 when measuring in the dark. The Erlenmeyer flasks were placed above the PAM sensor when they were measured.

2.2.3 Counting the number of cells

The aim was to stop the experiment at the maximum cell growth level to be able to detect the effect of pH and Zn. After 18 days when most of the cells in respective condition seemed to have reach a more steady phase the experiment was stopped. Lugol was added to the Erlenmeyers to kill the cells and stop the experiment, they were then stored in an 8 ° C room. The number of cells in each replicate were counted six times with the microscope Leitz Wetzlar M119 with x 300 magnification and with the counting chamber improved Neubauer (volume 1 µl).

2.2.4 Counting the amount of malformations

To be able to see the malformations on the frustules, cells were washed several times and then concentrated in vails, oxidized with hydrogen peroxide and then cooked to obtain clean frustules. Permanent slides were made for each replicate. The frustules were analysed with the microscope Nikon Eclipse 80i, with x1000 oil immersion objective, and the counting chamber Neubauer improved (volume 1 µl). For each replicate the degree of malformations was analysed for 100 diatoms. In this experiment only the frustules, and not the fine structure of the cell surface, was analysed for malformations, because the fine structure is hardly visible in these algae. The

diatoms were divided in the groups normal frustules, frustules with slight malformation and frustules with high malformation. Pictures were used as a reference for normal frustules, frustules with slight malformation and frustules with high malformation (Kahlert, 2012).

According to the recently developed method the degree of malformation should be decided for at least 400 diatoms in the sample and the malformed diatoms are divided in the following groups: slightly malformed conformation, high malformed conformation, slightly malformed pattern and high malformed conformation (Kahlert et al, 2016). Note that in this experiment the degrees of malformations were only decided for 100 cells in each replicate.

2.3 Short term experiments

The short term experiments were a standard experiment, carried out for 96 hours with a cell concentration of 10 000 cells ml^{-1}. The number of cells were counted with the microscope Nikon Eclipse 80i and the counting chamber Neubauer improved with a volume of 1 µl. The strains in the study were D 13, D 16 and FCAP, which were cultured in WC medium. Each strain was made with a control and treatments with the following Zn concentrations 0,01; 0,03; 0,1; 0,3; 0,5; 1,0 and 1,5 mg Zn l^{-1}. The control and all Zn concentrations were tested in both pH 5 and pH 7. Three replicates were made for each strain with the specific conditions of Zn and pH.

Two short term experiments were performed and will be referred to as “Short term experiment 1” and “Short term experiment 2” in the report. Both short term experiments were prepared and performed as described above and in 2.3.1 Preparation of treatments. The results of the two short term experiments were analysed with two different methods.

2.3.1 Preparation of treatments

The short term experiment was performed with autoclaved WC medium. The medium was prepared in accordance with the recipe, but with only 1/10 of the EDTA in the recipe (Guillard & Lorenzen, 1972). The pH in the medium was adjusted to 7 and 5 respectively, with the buffer MES and 0,1 M HCl and 0,1 M NaOH. Zn stock for respective Zn concentration was made with $ZnSO_4$ and added in wells on plates. Later WC medium and cells were added to the wells with a final volume of 2 ml. The plates were placed in a 21 ° C room; every other day the plates were mixed to get an equal amount of light.

2.3.2 Short term experiment 1

After 96 hours the fluorescence were measured with the Fluorimeter Genios Pro 96/384 (Techan). The software used for the measurements was Magellan data analysis software, when measured the excitation and emission were set to 460 nm and 689 nm respectively with a band width of 20. The theory for fluorescence measurements is described above in 2.1 Fluorescence measurements.

2.3.3 Short term experiment 2

For the strain D 16, the fluorescence was measured after 96 hours, with the Fluorimeter Genios Pro 96/384 (Techan). And the software used for the measurements was Magellan data analysis software, with the same settings as describe in 2.3.3 Short term experiment 1.

For the strains D 13, D 16 and FCAP lugol was added to the wells to stop the experiment after 96 hours. The plates were stored in 8 ° C. The number of cells for each condition were calculated by counting the cells for each replicate two times, with the microscope Leitz Wetzlar M119 at x30 magnification and with the counting chamber improved Neubauer; volume 1 µl.

2.3.4 Calculating the EC_{50} values

For the first short term experiment, the number of cells were calculated from the calibrations curves for the fluorescence measurements. For both short term experiment the number of cells were plotted to the Zn concentrations. The number of cells for EC_{50} were calculated by:

$$= \frac{Number\ of\ cells_{\ Control}}{2}$$

Some of the curves did not reach their EC_{50} in the experiment, for those curves the Zn concentration corresponding to the number of cells for EC_{50} were calculated and added to the graph. For all cultures with different strains and pH conditions the best trend lines were found in Excel by adding a linear or polynomic line. The approximated EC_{50} values for Zn were calculated from the equations corresponding to the trend lines.

2.4 Statistics and data analysis

Excel 2016 were used to calculate the mean and standard deviation, and the graphs are made in Excel 2016. Statistical analysis was performed with MINITAB 17 and Excel 2016.

3 Results

3.1 Long term experiment

When the long term experiment was stopped after 18 days, the cells in the control; 0,03 and 0,3 mg Zn l^{-1} cultured in pH 7 most certainly had started dying, see figure 1. For the treatments cultured in pH 5 the cells were still in their growing phase when the experiment was stopped. The counting of the cells displayed no significant difference in number of cells between the control and any of the tested Zn concentrations in pH 7, see figure 1 (p-values > 0,05). For pH 5 there was a significant difference in number of cells between the control and the Zn concentration 0,03 mg l^{-1} (95 %; p-value < 0,05; n=6).

3.1.1 Cell growth

The means of F0 were compared at day 4 -the first measurement day and at day 11 - the last day where cells in all treatments were still increasing. The mean values for F0 are significantly higher at day 11 compared with day 4 for the controls and for all cultures both in pH 7 and in pH 5 (95 %; p-values < 0,05; n=3). When comparing the F0 values for day 11 for the same Zn treatments in pH 5 and 7, pH 5 had significantly lower values for 0,03 and 0,3 mg Zn $\mathbf{l^{-1}}$ (95 %; p-values < 0,05; n=3). First at the third measurement (day 8) the cells biomass were started to increase for all treatments. The treatments cultured in pH 7 with 1,0 mg Zn $\mathbf{l^{-1}}$ showed significantly lower biomass than the control for all measurements from day 7 to day 18 (95 %; p-values < 0,05; n=3). When comparing the different pH, the biomass for the control and cultures treated with 0,03 and 0,3 mg Zn $\mathbf{l^{-1}}$ in pH 7 were significantly higher than the biomass in pH 5 for several measurements, see figure 1 (95%; p-values < 0,05; n=3).

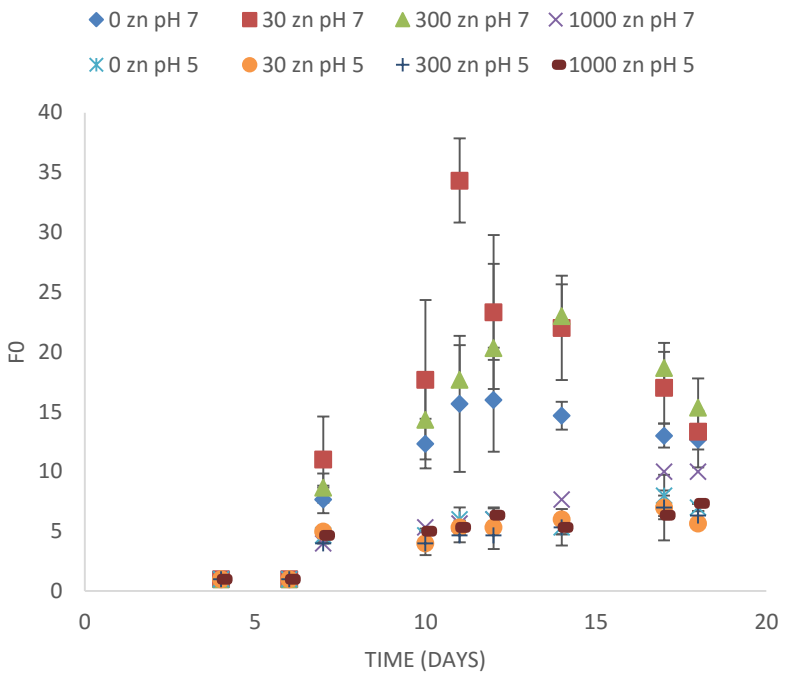


Figure 1. Mean values ± st.dev (95 %; n=3) of F0 for each treatment for all measurement days.

The number of cells in the long term experiment are presented in Figure 2, where Zn treatments in pH 7 have significantly more cells when compared to Zn treatments in pH 5 (95 %; p-values < 0,05; n=3), see figure 2. There was no observed difference in the number of cells for treatments with Zn compared to the controls in pH 7. This was also observed for treatments in pH 5, besides from the treatment with 0,03 mg Zn l^{-1}in pH 5 that displayed significantly less cells when compared to the control see figure 2 (95%; p-value < 0,05; n=3).

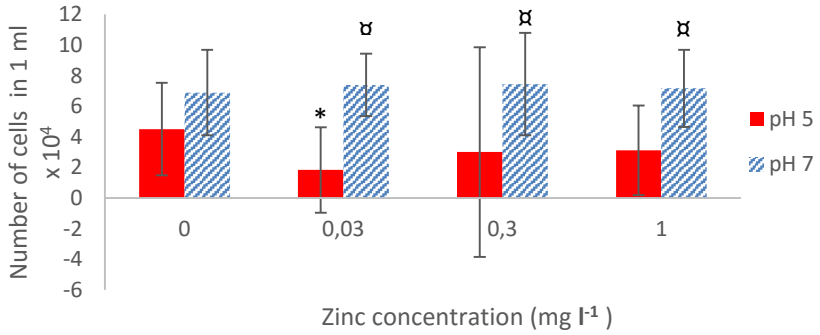


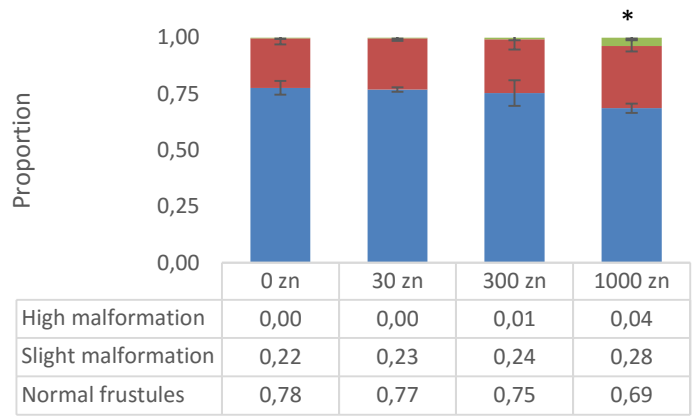
Figure 2. Displaying the results for the long term experiments presented as means ± st.Dev. * representing treatments with a significant (95%) lower amount of cells in comparison to the control, O representing treatments with a significant (95%) higher amount of cells in comparison to the control.

¤ representing treatments that have a significant (95%) higher number of cells, when comparing the same zinc treatments cultured in different pH.

3.1.2 Amount of malformations

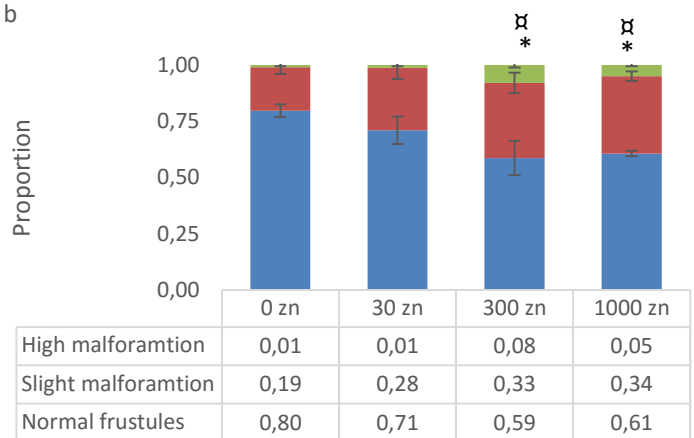
The analysis of malformations is presented in figure 3, there were significant differences in the amount of normal frustules between the control and the treatments with 0,3 and 1 mg Zn $\mathbf{l^{-1}}$ cultured in pH 5 (95%; p-values < 0,05; n=3). In pH 7 there was also a significant difference between the control and 1 mg Zn $\mathbf{l^{-1}}$ (95 %; p-value < 0,05). When comparison was made between pH 5 and pH 7, there was significant more malformations in pH 5 for the cultures that had been treated 0,3 and 1 mg Zn $\mathbf{l^{-1}}$ (95 %; p-values < 0,05; n=).

a



	0 zn	30 zn	300 zn	1000 zn
High malformation	0,00	0,00	0,01	0,04
Slight malformation	0,22	0,23	0,24	0,28
Normal frustules	0,78	0,77	0,75	0,69

b



	0 zn	30 zn	300 zn	1000 zn
High malforamtion	0,01	0,01	0,08	0,05
Slight malforamtion	0,19	0,28	0,33	0,34
Normal frustules	0,80	0,71	0,59	0,61

Figure 3. The proportion of malformations in the control and the treatments at pH 7 and pH 5 are displayed at a) and b) respectively. The results are presented as the mean ± 1 st.dev, for the proportion of normal frustules, frustules with slight malformation and frustules with high malformation. The frustules were analysed with the microscope Nikon Eclipse 80i and the counting chamber Neubauer improved. In the figure * represents treatments with a significantly lower amount of normal frustules than the control, ¤ represent treatments that have significantly more malformations than the same treatments in the other pH condition.

3.1.3 Zink affecting the photosystem

The Fv/Fm values from the PAM measurements are a good indicator for the efficiency of the cells photosystem II. For all treatments cultured in pH 5 there were no significant changes in Fv/Fm between the control and the treatments or between the same treatments for different days (95 %; n=3). There were also no observed differences in Fv/Fm values between the different pH treated with the same Zn concentrations. The treatment with 0,03 mg Zn $\mathbf{l^{-1}}$ cultured in pH 7, had a significant change in Fv/Fm between the first measurement day (day 4) and some of the measurements day at the end of the study (95%; p-values < 0,05; n=3).

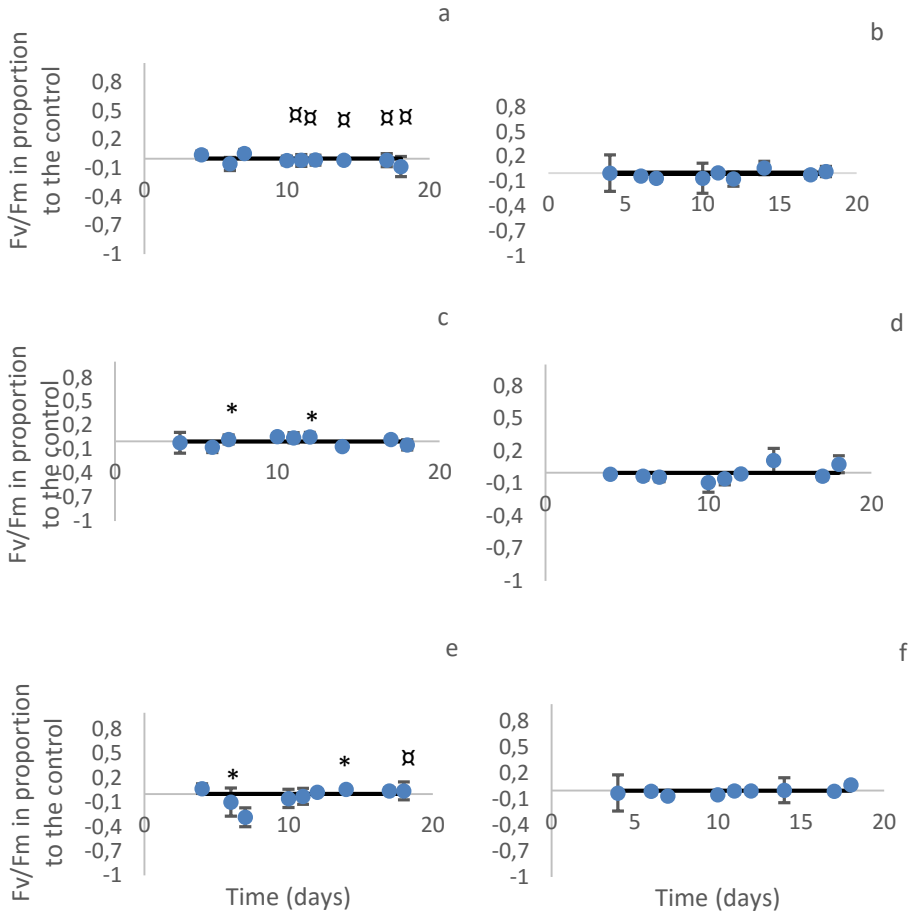


Figure 4. Results for the Fv/Fm in proportion to the control for the different treatments, presented as means ± St.Dev. a) 0,03 mg Zn l^{-1} pH 7, b) 0,03 mg Zn l^{-1} pH 5, c) 0,3 mg Zn l^{-1} pH 7, d) 0,3 mg Zn l^{-1} pH 5, d) 1,0 mg Zn l^{-1} pH 7 and e) 1,0 mg Zn l^{-1} pH 5. Students' t-test was performed for the Fv/Fm value (95 %; n=3). In the figure * represent Fv/Fm values that are significant different when compared to the control and ¤ represent Fv/Fm values that are significant different from the first measurement day.

3.2 Short term experiments

For both short term experiments cells from the strain FCAP were observed to be growing significantly better in pH 7 than in pH 5, for Zn concentrations 0 -0,3 mg Zn l^{-1}, see figure 5 (95 %; p-values <0,05; n=3). Cells from D 13 were growing better in pH 5 with the Zn concentrations 0,5 -1,5 mg Zn l^{-1} in the first experiment, in the second experiment there was no observed difference between the pH for any of the Zn concentrations, see figure 5 (95%; p-values < 0,05; n=3). Whereas D 16

are displaying a contradictory result. In the first short term experiment the number of cells was significantly higher for treatments with 0,3 -1,0 mg Zn l^{-1} cultured in pH 5 compared to pH 7, see figure 5 (95 %; p -values <0,05; n=3). In the second experiment the cells were, in contrary to the first experiment, observed to be growing significantly more in pH 7 with 1,0 and 1,5 mg Zn l^{-1} added than in pH 5, see figure 5 (95 %; p-values < 0,05; n=3). Even if the results are contradictory, the average result from both experiments is that the cells were growing better in pH 5 than in pH 7 with Zn concentrations ranging from 0,3 -1,5 mg Zn l^{-1}.

3.2.1 Approximated EC_{50} values

The EC_{50} values (the concentration corresponding to a 50 % reduction in the number of cells for the control) were calculated as describe under method and material. Since several of these strains did not display a linear or sigmoidal relationship for the number of cells and the Zn concentrations, the EC_{50} values for Zn presented in Table 2 and table 3, should be seen as approximations.

The EC_{50} values were calculated for the different strains cultured in both pH 5 and pH 7 for both short term experiments. In the first experiment the EC_{50} values are lower for cultures in pH 5 compare to cultures in pH 7 for all strains, see table 2. In the second experiment the EC_{50} values are more similar for the strains, but the EC_{50} value for D 16 cultured in pH 5 displays a lower EC_{50} value than in pH 7, see table 2.

Table 2. Approximated EC 50 values for the different strains cultured in different pH conditions for both short term experiments, presented as the average ± 1 st.dev (95%; n=3). The EC 50 values for the short term experiment 1 are based on the counted cell numbers in the treatments. The EC 50 values for the short term experiment 2 are based of fluorescence measurements that been calculated to the cell number in the treatments.

Approximated EC_{50} values for zinc (mg l^{-1}) after 96 h

	Experiment 1	Experiment 2	Average ± st.dev from both experiments
FCAP pH 7	1,53	1,89	1,71 ± 0,26
FCAP pH 5	3,54	2,12	2,83 ± 1,0
D 16 pH 7	0,52	1,02	0,76 ± 0,36
D 16 pH 5	1,77	0,35	1,06 ± 1,00
D 13 pH 7	1,13	1,01	1,07 ± 0,08
D 13 pH 5	2,18	1,07	1,63 ± 0,79

The average EC_{50} values were calculated from both experiments and both pH conditions for each strain, see table 3. The average EC_{50} values were also calculated from al strains for each pH condition, see table 3. Table 3 displays that regardless

pH condition the EC_{50} value are the highest for FCAP whereas D 16 had the lowest tolerance to Zn. It is also observed that pH 5 has a higher EC_{50} value than pH 7 and the average total EC_{50} value for the experiments is 1,51 ± 0,87 mg Zn l^{-1}.

Table 3. Approximated EC 50 values calculated as average from both experiments with both pH 5 and pH 7 to present an average EC 50 value for each strain, regardless pH conditions, and also for each pH condition, regardless the strain (95%; n=3).

Approximated EC_{50} values for zinc (mg l^{-1}) after 96 h	
	Average ± st.dev:
FCAP	2,27 ± 0,88
D 16	0,91 ± 0,64
D 13	1,35 ± 0,56
pH 5	1,84 ± 1,08
PH 7	1,18 ± 0,48
Total	1,51 ± 0,87

3.2.1 Cell growth - Short term experiment 1

The number of cells for the first short term experiment were calculated from a calibration curve for fluorescence measurements. When compared to the controls, all three strains were observed to be growing significantly less in treatments with 1,0 and 1,5 mg Zn l^{-1} when cultured in pH 7 (95 %; p-values < 0,05; n=3). Cells from D 16 and D 13 also grew significantly less in treatments with 0,5 mg Zn l^{-1} when cultured in pH 7 (95%; p-values < 0,05; n=3). The strains D 16 and D 13 cultured in pH 5 had a significantly decrease in number of cells for cultures treated with 1,0 and 1,5 mg Zn l^{-1} (95 %; p-values < 0,05; n=3). Whereas FCAP not displayed any difference in number of cells for cultures treated with Zn when compared to the control. As displayed in figure 5, several of the treatments with Zn cultured in pH 5 were growing better than the control (95%; p-values < 0,05; n=3). Regarding differences due to pH conditions, various results were observed for the strains, D 16 and D 13 had significantly more cells in cultures with pH 5 treated with 0,5; 1,0 and 1,5 mg Zn l^{-1}, whereas FCAP grew significantly better in pH 7 treated with low concentrations of Zn, se figure 4 (95%; p-values <0,05; n=3).

3.2.2 Cell growth - short term experiment 2

The cells from the strains D 16 and D 13 treated with 0,5; 1,0 and 1,5 mg Zn l^{-1} cultured in pH 5 and treatments with 1,0 and 1,5 mg Zn l^{-1} in pH 7 had significantly lower number of cells when compared with the controls (95%; p-values <0,05; n=3). FCAP had a significant more amount of cells for cultures with pH 7 in the control

and the three lowest zinc concentrations 0,01-0,1 mg Zn l^{-1}, whereas D 16 displayed significant more cells in treatments with 1,0 and 1,5 mg Zn l^{-1} cultured in pH 7 (95%; p-values <0,05; n=3).

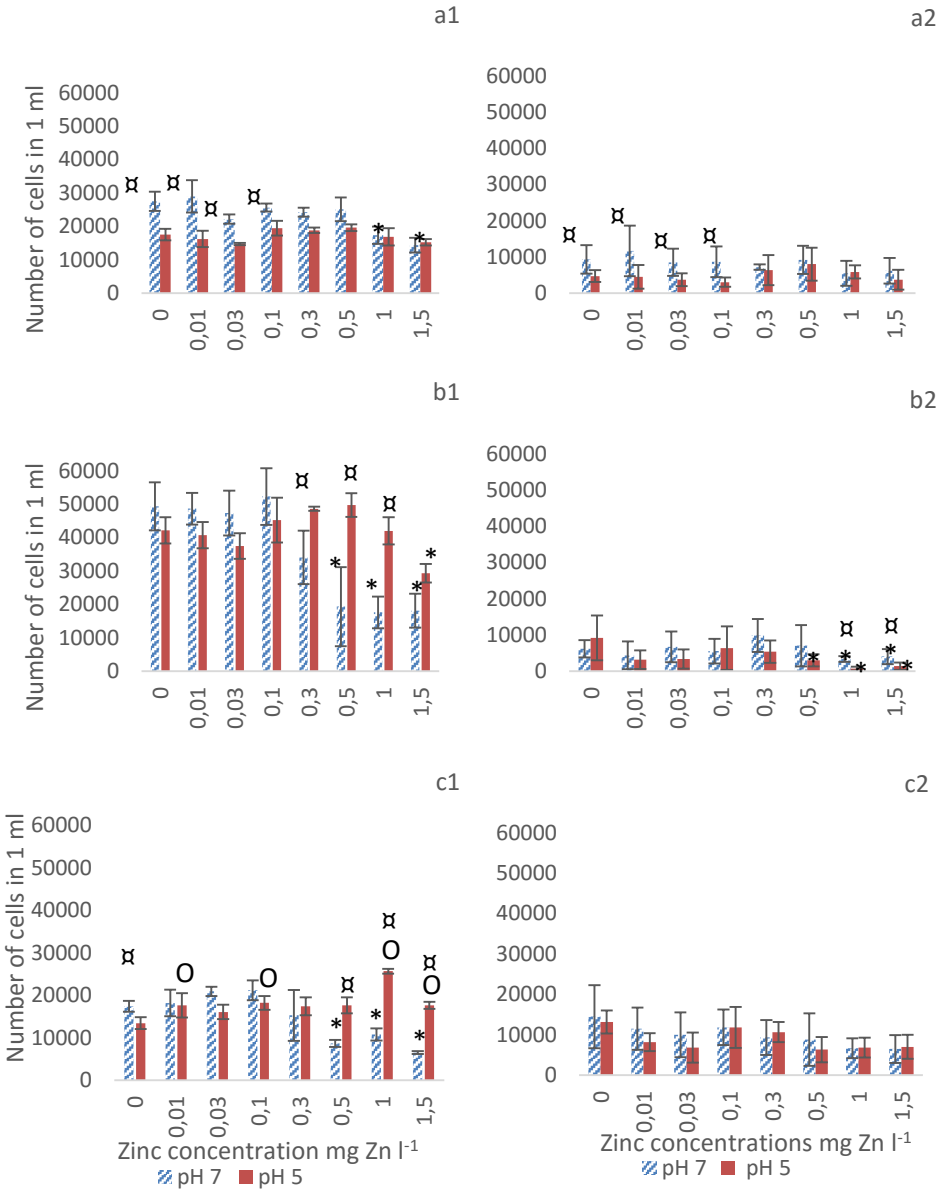


Figure 5. Displaying the results for the short term experiments presented as means ± st.Dev,. The results for short term experiment 1 are displayed in a1) FCAP, b1) D 16 and c1) D 13, and short term experiment 2 are displayed in a2) FCAP, b2) D 16 and c2) D 13. * representing treatments with a significant (95%) lower amount of cells in comparison to the control, O representing treatments with a significant (95%) higher amount of cells in comparison to the control. ¤ representing treatments that have a significant (95%) higher number of cells, when comparing the same zinc treatments cultured in different pH.

4 Discussion

The hypotheses for this study were that Zn would be less toxic in pH 5 than in pH 7, as well as the strains isolated from sites with high Zn concentrations should have higher tolerance to Zn than diatoms from non polluted sites. The last hypothesis was that Zn pollution could be detected by malformations in diatoms. The first and second hypotheses were verified by the results of EC_{50} values and the number of cells for the short term experiments. The last hypothesis about malformations indicating Zn pollution was proven by the long term experiment. In contrary the long term experiment rejects the first hypothesis about pH 5 decreasing the Zn toxicity.

4.1 pH conditions affecting the zinc toxicity

The short term experiments in the present study indicates that pH 5 could have a protective role for Zn, with the average EC_{50} values for all strains being higher for cultures with pH 5 than cultures with pH 7. The protective role of pH 5 for Zn is also proven by the observations, of a decreasing difference in the number of cells between the two pH conditions as well as the higher number of cells for cultures in pH 5 compared to pH 7, when the cells were treated with > 0,3 mg Zn l^{-1}. In accordance with my results, a lower pH having a decreasing effect on Zn toxicity have also been observed by Wilde et al (2005) and Heijerick et al (2002). However, the first hypothesis about pH 5 lowering the toxicity for Zn was not verified by the long term experiment. The first hypothesis is rejected by the high amount of cell biomass after 6 days for cultures with pH 7 and 0 -0,3 mg Zn l^{-1}, by the significantly higher amount of cells for Zn treatments cultured in pH 7 compared to pH 5, and finally by the significantly higher degree of malformations in treatments with 0,3 and 1,0 mg Zn l^{-1} in pH 5 compared to treatments in pH 7. In accordance with my observations of a lower pH affecting diatoms negatively, Hevré et al (2012), have observed a decrease in the internal pH when diatoms were exposed to lower pH than their optima for 3-6 days.

The results for the short term experiments could indicate that even though these strains are adapted to more neutral pH conditions, when the Zn concentrations are very high the acidic pH offers a protection for the cell by competing with Zn^{2+} to the cells binding sites. One possible explanation for why the long term experiment did not verify the first hypothesis is that the exposure time to Zn is 18 days, which is several days longer than the exposure time for the short term experiments (4 days). One idea is that the longer exposure time is affecting the cells more in pH 5, because the cells are both exposed to high Zn concentrations and they have to handle a lower

pH than they are used to. Thereby leading to lower numbers of cells and higher degrees of malformations in treatments with pH 5. My results could be explained by the fact that it is known that the amount of intracellular Zn are increasing with the exposure time (Stevensson et al, 1996), and that the intracellular Zn have been observed to have a positive relationship with the amount of malformations (Pandey et al, 2015). This theory could also be explained by the fact that the studies performed by Wilde et al (2005) and Heijerick et al (2002), where the Zn toxicity was observed to increase with increasing pH, were short term experiments with an exposure time of 48 h and 72 h respectively. Their observations could indicate, in agreement with my observations for the present study, that the lower pH is just offering a shielding effect towards Zn when the exposure time is a few days. This theory is also strengthened by the results from Hevré et al (2012), where diatoms transplanted to lower pH than their optimal grew less because of a decrease in the internal pH. The fact that there were no observations of an increase in the biomass the first 6 days for the long term experiment, indicates that 4 days was not enough time to observe how the cells were affected by the Zn and the pH conditions.

4.2 Zinc tolerance for diatoms

The second hypothesis about the strains showing tolerance to Zn is also verified. The approximated EC_{50} values differ for the strains, where FCAP seems to be the most tolerant. The strains D 13 and D 16 displayed lower tolerance to Zn than FCAP, but they still displayed a higher tolerance to Zn than diatoms from non polluted sites. The Zn value was unknown for FCAP isolation site, but since they have the highest observed EC_{50} value it is likely that their isolation site was highly polluted by Zn. D 13 was collected from a site with 0,36 mg Zn $\mathbf{l^{-1}}$ and D 16 from a site with 0,035 mg Zn $\mathbf{l^{-1}}$, D 16 is also displaying the lowest EC_{50} value of the tested strains. The EC_{50} value of Zn for diatoms depends on the specie and if they been exposed to Zn before. For different diatoms species collected from sites unaffected or with low Zn values the observed EC_{50} values have been ranging from 0,15-0,6 mg Zn $\mathbf{l^{-1}}$ after 72 - 96 h exposure (Sbihi et al, 2012). The observed EC_{50} values in this study are higher than 0,6 mg Zn $\mathbf{l^{-1}}$, indicating a tolerance to Zn. Ivorra et al (2002) concluded that the diatom species *Gomphonema parvulum* was able to inherit tolerance to Zn, even if the diatoms been cultured in medium without Zn for two years. This is probably also the reason why the strains in this study displayed a higher tolerance to Zn. The species *Fragilaria capucina*, is also a species that been observed to be tolerant in other studies and to be rather abundant in sites with high Zn values (Pandey & Bergey, 2015, Morin et al, 2012).

4.3 Malformation indicating zinc pollution

The hypothesis about malformations indicating Zn pollution is verified in the long term experiment. In the present study the observed malformation degrees increased with increasing Zn concentrations, and there were also observations of an increased degree of frustules with high malformations with higher Zn concentrations. The present study thereby indicates that Zn pollution could be detected by the amount of malformations.

The amount of malformations in this study is much higher than the ones observed in Swedish waters (4 %) by Kahlert (2012). A malformation degree > 8 % is classified as a very high frequency of malformations (Kahlert et al 2016). Hence, this study indicates a very high frequency of malformations for all Zn concentrations and the controls in the long term experiment. The high malformation degrees found in the treatments could be due to that diatoms excrete waste products which can result in malformations and that malformations can be passed through generations (Falasco et al, 2009; Smith et al, 2007). Thus the high malformation degree in this study could partly be due to excreted waste products, because the strains came from a culture, and inherent malformations. Still there is a significantly higher amount of malformations for high Zn concentrations compared to the controls in both pH 5 and pH. Therefore, I believe that the observed degree of malformations is related to the exposure of high Zn concentrations. These results indicate that malformations could be used as an indicator for Zn pollution.

Conclusions

The hypothesis about diatoms collected from Zn polluted areas being more tolerant to Zn was verified, the tested strains displayed a higher tolerance towards Zn in comparison with diatoms not collected from Zn polluted sites. The increased malformation degrees observed with increased Zn concentrations did also verify that malformations can indicate Zn pollution.

This study indicates that pH 5 could offer protection for the cell and have a decreasing effect of Zn toxicity in the initial exposure to Zn. But probably not when the diatoms are exposed to Zn for longer periods. It is likely that pH 5 protective effect decreases when cells are exposed to Zn in non-optimal pH condition for a longer time. Therefore, it would be interesting to do a similar experiment with cells from the same strains cultured both in neutral and acidic medium. Then it should be possible to observe if pH affects the Zn toxicity.

As observed in this study the different pH displayed different degrees of malformations, indicating that Zn pollution can be detected by different malformation de-

grees for acidic and neutral pH conditions. There were also high degrees of malformations in the controls, probably due to inherit teratological cell forms. It is therefore likely that diatoms living in watercourses that have recovered from Zn pollution, still could have a high degree of malformation even though the present Zn concentrations is decreased. The tested strains displayed different tolerance to Zn, and it is possible that also the tendency to develop malformations would have differed for the tested strains. More studies are needed to be able to determine an index for the detection of Zn pollution by malformations. The index, probably have to be adapted for different species of diatoms and also to the collections sites pH conditions.

Acknowledgement

I would like to thank my supervisor Maria Kahlert for the help and encouragement during my study. I would also like to thank my assistant supervisor Sara Goncalves for the help in the lab and for the invested time. At last I would like to thank my co -workers Josabeth Ström, Thibaut Imbert and Anders Lundqvist in the diatom project, for the help and support during this project.

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