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Swedish University of Agricultural Sciences

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Evaluating zinc content in aquatic ecosystem by estimating the responses of diatom biofilm to environmental and biological variables

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Abstract

Zinc is a common essential metal. At too high exposure it will become toxic.
Diatoms have a species-specific cell wall and they are easy to collect and to preserve. When exposed to environmental stress they could have teratological form, which is one of the reasons to have diatom as a bioindicator for metal contamination assessment. However, there are many variables could influence the bioavailability of zinc and other metals for diatoms. So in this review, we will evaluate the effects of different environmental and biological variables on zinc content in aquatic ecosystems. We also suggested here to have an interactive conceptual model to better assess what is known, what should be verified and what is needed in order to be better at evaluating the effects on diatom communities of zinc exposure.

Introduction

Zinc is widely used in the galvanization process, batteries and in the rubber industry (Blanck *et al.*, 2003). Thus, there is a lot of mining of zinc (Blanck *et al.*, 2003). Other sources include steel production, coal usage, waste and in traffic related emissions which could reach water bodies as atmospheric deposition (Blanck *et al.*, 2003; Gunawardena *et al.*, 2013). Zinc is of special concern since it is a relatively easily dissolved metal (Monteiro *et al.*, 2012; Corcoll, Bonet, Morin, *et al.*, 2012; Gunawardena *et al.*, 2013). In too high concentration, zinc could cause oxidative stress by breaking the oxidative balance inside a cell (Arunakumara & Zhang, 2007).

In accordance with EU directive Priority Substance Directive 2013/39/EU, a part of the Water Framework Directive, WFD, the Swedish Agency for Marin and Water Management regulates, in HVMFS 2013:19, the assessment of diatom status and zinc level water bodies in Sweden (Directive, 2012). The parameter IPS, which look at eutrophication and organic effect, and ACID, which show acidity, is used to classify diatoms in water bodies with the worst one determining status (Directive, 2012). For zinc in inland water bodies, a value of 5.5 $\mu g \cdot L^{-1}$ as a yearly average has been set as an upper limit (Directive, 2012). The value is regarding the amount of dissolved zinc which is bioavailable (Directive, 2012). The pH, water hardness and dissolved organic carbon can therefore be measured in to account for their effect on zinc bioavailability (Directive, 2012). The assessment of zinc could be done in several ways, one of which is that an expert judgment can replace the actual measurement if measurements are either lacking or the effect of substances on bioavailability is hard to conceptual model (Directive, 2012). Using diatom as a bioindicator of zinc has been suggested and studied intensively, and this is the main focus in this review and the effects of different variables will be discussed as well.

Diatoms are silicified microalgae with a large number of species (Sabater, 2009), and they are easy to collect and due to their cell wall they are easy to preserve. Quick growth of diatoms enables them to be used as an early warning system (Sabater, 2009). Also the species-specific silicon cell wall of a diatom is perforated, which allow gases and substances to be transported through, and transparent to allow light to reach its chloroplasts (Falasco et al., 2009a). The morphology of the cell wall can change after environmental stress producing teratological forms, which is defined as an abnormal manifestation due to environmental causes (Falasco et al., 2009a). What is more, Corcoll, Ricart, et al (2012) showed that benthic diatom, which is a member of a large community composed of several taxonomy groups such as green algae, cyanobacteria, bacteria, have a great potential for metal contamination assessment, since bioavailability is hard to conceptual model based on chemical analysis (Cornet & Corcoll Cornet, 2012; Guasch *et al.*, 2003; Ivorra *et al.*, 2000). Using diatom as bioindicator allows the bioavailability to be measured more directly, which will show how much of the zinc that is bioavailable and affect ecosystem (Cornet & Corcoll Cornet, 2012; Blanco & Bécares, 2010).

It is known that the bioavaliability is affected by variables such as pH, temperature (Wilde *et al.*, 2006; Monteiro *et al.*, 2012). The amount of zinc in food web is not only dependent on zinc concentration in water but on variables and biologic response. A lot of variables could

affect zinc bioavalibility, such as pH, UV, species, temperature, time, nutrients and other free cations. Here, in order to evaluate diatom as a bioindicator of zinc, a conceptual model will be introduced. In this conceptual model, all related studies were summarized, but they were separated into different sub-categories, and the details of their effect on zinc-diatom interactions were listed. What is more important is that it will also present the complex interactions of all those different variables, and their effect on diatom. For example, extracellular polysaccharide substances (EPS) are excreted by the biofilm cells, which can contribute to detoxification against metal exposure in biofilm. But, EPS itself could be affected by many factors, such as the age and composition of biofilms, which will give different evaluation or measurement of zinc effect on diatom. However, in this conceptual model, the individual effect of each variable is also discussed, so it will provide researchers a way of setting up an easy and clear lab experiment by controlling one or several factors and evaluate the influence of zinc on diatom in a controlled environment. But it will also give an opportunity to extend the laboratory results into more complex natural environments when many variables are present and affecting at the same time. This is quite important especially for decision makers, so that decisions could be made based on the true and complicated conditions. This conceptual model will be also beneficial to the studies on using diatom as bioindicator of zinc, since there are many studies that have been done on this subject, but showing very inconsistent results, which may be due to not knowing the complex interactions between environmental and biological variations.

In this following session, the effects of different environmental and biological variables on zinc-diatom interactions will be presented and discussed.

Variables

Each individual variable could fluctuate and this increases the difficulties in predicting and establishing the effect of zinc exposure on diatom.

pH

Biosorption of free zinc ions is pH dependent (Corcoll, Bonet, Morin, *et al.*, 2012) Biosorption consists of extracellular adsorption and intracellular uptake (Corcoll, Bonet, Morin, *et al.*, 2012). Extracellular adsorption is pH dependent (Wilde *et al.*, 2006). At a lower pH with more hydroxonium ions there are more free zinc cations avaliable in water that can adsorb with negatively charged active sites at the cell surface (Özer *et al.*, 2000; Fraile *et al.*, 2005; Ahuja *et al.*, 1999). At a higher pH there is less competition for the negatively charged cell surface from the hydroxonium ions which leads to increased biosorption (Fraile *et al.*, 2005). However, dissolved zinc is mostly occuring as free zinc ions at lower pH (Özer *et al.*, 2000; Fraile *et al.*, 2005; Ahuja *et al.*, 1999). At higher pH it precipitates and it becomes less bioavaliable to benthic algae (Gélabert *et al.*, 2006).

The most biosorption is reported as occuring at pH 5.0- 5.5 (Özer *et al.*, 2000; Fraile *et al.*, 2005; Crist *et al.*, 1994; Wilde *et al.*, 2006). Most studies have not been conducted at higher pH values due to zinc precipitation there (Özer *et al.*, 2000; Fraile *et al.*, 2005; Ahuja *et al.*, 1999). One that did reported even higher extracellular adsorption and with constant

intracellular uptake up to pH 8 despite a reduction in free zinc ions from 93 % to 78 % of total dissolved zinc (Wilde *et al.*, 2006). At very low pH, of around 3 or lower, there is negligable zinc adsorption (Fraile *et al.*, 2005; Rangsayatorn *et al.*, 2002).

Zinc intracellular concentration has been reported to be independent of pH (Wilde *et al.*, 2006). Another study reported an increase of zinc removal by freshwater diatoms when pH increased up until the complete removal of zinc at pH of around 8.5. At pH of 8.5 and over a decrease in free zinc ion concentration due to precipitation was noted but not under this (Gélabert *et al.*, 2006).

Light

When photons reach the photosynthetic processes of biofilms they go to chemicals where they are used to oxidize water to form hydrogen ions creating a hydrogen ion gradiant over the thylakoid membrane, in photochemical quenching (Corcoll, Bonet, Morin, *et al.*, 2012; Cornet & Corcoll Cornet, 2012). This also create reactive oxygen which can damage photosystem II (PSII) (Corcoll, Bonet, Morin, *et al.*, 2012; Cornet & Corcoll Cornet, 2012). When there are too much photons, non-photochemical quenching (NPQ) will be used instead of photosynthetic processes (Corcoll, Bonet, Morin, *et al.*, 2012; Cornet & Corcoll Cornet, 2012). Here de-epoxidation reaction (DR) and the xanthophyll cycle is used to dissipate the excess as heat in NPQ (Corcoll, Bonet, Morin, *et al.*, 2012; Cornet & Corcoll Cornet, 2012). The xanthophyll cycle can also remove the reactive oxygen (Corcoll, Bonet, Morin, *et al.*, 2012; Cornet & Corcoll Cornet, 2012).

A study reported that three biofilms with different light exposure were grown indoors under three different light intensities: low level light intensity (LL), medium level light intensity (ML) and high level light intensity (HL) for four weeks. Since there were several generations both phenotypic plasiticity and genetic adaptaiton were thought to play a role in the seen changes (Corcoll, Bonet, Leira, *et al.*, 2012).

The taxonomic groups green algae and diatoms differed significantly between LL on one hand and ML and HL on the other hand. The LL group had a higher abundance of diatoms, which are more efficient utilizing low level light exposure and more sensitive to zinc exposure, whereas the ML and HL had a higher relative abundance of green algae and had more biomass and more extracellular polymeric subtances (EPS). ML and HL also had more xanthophylls which minimize damage to photosynthetic processes from accumulation of reactive oxygen. Cyanobacteria had an even relative abundance in LL, ML and HL. The diatom species composition was significantly different between LL and the ML, HL. This suggest that the composition of the community has a significant effect on biofilm tolerance to zinc exposure. It also shows that xanthophyll may prevent zinc from blocking photosynthetic reactions and thereby causing an accumulation of reactive oxygen (Corcoll, Bonet, Leira, *et al.*, 2012).

A way for diatoms to remove excess light is through the xanthophyll cycle and the de-epoxidation reaction (DR). Both are important in non-photochemical quenching (NPQ). Without zinc exposure there is a strong correlation between NPQ and DR for varying light exposure. When exposed to zinc there has been a decoupling of NPQ from DR during

changing light intensites, suggesting damage to the xanthophyll cycle (Corcoll, Bonet, Leira, *et al.*, 2012).

Zinc exposure produce inhibition in photosynthetic processes (Corcoll, Bonet, Leira, *et al.*, 2012).

The light exposure history of the biofilm influence zinc toxicity. The LL biofilm was more sensitive to zinc which was shown when 1500 $\mu g \cdot L^{-1}$ zinc exposure for 6 hours inhibited 23% of photosynthetic processes. In ML and HL biofilms the level of inhibition when exposed to zinc was 10 and 6% respectivly. This indicate that structural and functional mechanisms of photoacclimation reduce sensitivity to zinc exposure (Corcoll, Bonet, Leira, *et al.*, 2012).

Photoacclimation to high level of light exposure have been reported to increase biomass and EPS which could possibly protect against zinc by giving mechanical protection and the EPS having negatively charged functional groups which would bind the free zinc ions (Corcoll, Bonet, Leira, *et al.*, 2012; Admiraal *et al.*, 1999; Duong *et al.*, 2010).

Zinc has been shown to increase the inhibitory effects of light exposure on photosynthetic activity. This suggest that both excessive light and zinc exposure target photosynthetic processes (Corcoll, Bonet, Leira, *et al.*, 2012).

The study also suggest that xanthophyll can prevent zinc from blocking photosynthetic processes and thereby keeping them from causing damage to them by the accumulation of reactive oxygen (Corcoll, Bonet, Leira, *et al.*, 2012).

Zinc exposure was also seen to cause a decoupling between of DR and NPQ during changes in light intensity showing a loss of phenotypic plasticity to handle those changes. This indicate that zinc exposure also target the xanthophyll cycle (Corcoll, Bonet, Leira, *et al.*, 2012).

Temperature

The effect of temperature on zinc biosorption is inconsistent among studies (Monteiro *et al.*, 2012). Some showed there is more metal biosorption with increasing temperature, while other showed that there is less or even no effect (Monteiro *et al.*, 2012). This may be due to different taxonomic groups, and even species as well as different heavy metals used in the studies. Typically studies show on an increase in metal uptake with increasing temperature (Monteiro *et al.*, 2012).

For a study on freshwater diatoms, zinc have been found to have an increased adsorption at higher temperatures (5 and 25°C). The seen difference was also dependent on pH with the most of the increase occuring between pH 5- 8 neutral pH values. At low pH values the total adsorption was low and at higher pH values there was a complete removal of zinc (Gélabert *et al.*, 2006).

A study on copper suggested that intracellular uptake of copper ions were affected by temperature. It increased from 6 °C to a maximum uptake at 25 °C. At higher temperature the intracellular uptake decreased. It was speculated that this was due to 25 °C being the optimum temperature range of metabolism. Extracellular adsorption was almost unchanged by

temperature, with a temperature increase from 6- 38 °C a small increase in adsorption towards a maximum at 38 °C was seen (Mehta *et al.*, 2002).

Time

Studies indicate that zinc exposure in a river affect biofilm differently depending on time and level of exposure (Corcoll, Bonet, Morin, *et al.*, 2012; Cornet & Corcoll Cornet, 2012). The difference in effect due to time has been attributed to that for a temporary exposure a high metal concentration is needed to affect the cells whereas a long-term exposure let the lower concentrations of metal penetrate the biofilm and reach the cells (Corcoll, Bonet, Morin, *et al.*, 2012; Cornet & Corcoll Cornet, 2012).

Early effects influence mainly funcion in the biofilm, such as an inhibition of photosynthetic processes or altering the photosynthetic pigment composition (Corcoll, Bonet, Morin, *et al.*, 2012; Cornet & Corcoll Cornet, 2012).

Long-term zinc exposure lead to structure changes (Corcoll, Bonet, Morin, *et al.*, 2012; Cornet & Corcoll Cornet, 2012). These include dominant taxa, diversity patterns and species composition within the biofilm (Corcoll, Bonet, Morin, *et al.*, 2012; Cornet & Corcoll Cornet, 2012). These strutural changes are expected to occur when the zinc has bioaccumulated (Corcoll, Bonet, Morin, *et al.*, 2012; Cornet & Corcoll Cornet, 2012).

Experiments have shown that biosorption of free zinc ions is fairly quick with the majority of adsorption occuring within 10- 100 minutes for dried freshwater diatoms (Gélabert *et al.*, 2006). This is echoed in studies for green algae and cyanobacteria and other heavy metals where the majority of biosorption occurred within one hour and the longest reported time until equilibrium was reached was two hours (Mehta *et al.*, 2002; Ahuja *et al.*, 1999; Sheng *et al.*, 2007; Chojnacka *et al.*, 2005; Özer *et al.*, 2000). Intracellular uptake rate have not been studied extensively. A study conducted on green algae and copper showed that extracellular adsorption occured within 10 minutes with intracellular uptake taking 30 minutes (Mehta *et al.*, 2002).

Nutrients

In a study, green algae cells exposed to 25 μM zinc for 48 hours had their intracellular protein content reduced by around 30% with a smaller reduction in carbohydrate content. After this the algae were transfered to a basal medium, without additional zinc. Even after 96 hours only 70% of the protein was restored (Tripathi & Gaur, 2006).
Interestingly, a more than 50% inhibition was detected in the uptake of nitrate ions when exposed to the same amount of zinc. The cells showed a about 60% recovery 96 hours after transfering them to the basal medium. This slow recovery was most likely due to some intracellular zinc remaining in the cells (Tripathi & Gaur, 2006).

Anion

The anions sulfate, chloride and nitrate have been seen to reduce Zn biosorption in cyanobateria .(Ahuja *et al.*, 1999) This is dependent on concentration as where inhibion of Zn biosorption increases with increasing anion concentration (Ahuja *et al.*, 1999).

The anions phosphate, carbonate and chloride have decreased free zinc ions as well as the reported zinc toxicity, as measured by inhibition in maximum luminescence, when introduced to cyanobacteria (Rodea-Palomares *et al.*, 2009).

Interestingly, low amounts of the anions phosphate and carbonate increase metal toxicity at low concentration (Rodea-Palomares *et al.*, 2009). These significant results were not attributed to speciation of the metals (Cu, Hg, Cd, Zn) (Rodea-Palomares *et al.*, 2009).

Cation

Different cations have different affinity for biosorption (Bradac *et al.*, 2010).

A study reported that the presence of free calcium ions and free magnesium ions inhibit free zinc ion biosorption through competition for binding sites on the cell surface of cyanobacteria *Oscillatoria anguistissima* (Ahuja *et al.*, 1999). Another study reported that Zn and Cd inhibit eachothers biosorption in the freshwater green algae *Scenedesmus vacuolatus* (Bradac *et al.*, 2010). It has also been reported that Mg inhibit Zn intracellular uptake in the cyanobacteria *Oscillatoria anguistissima* (Ahuja *et al.*, 2001). Thus, other cations may inhibit zinc toxicity.

It has been shown that free copper and zinc ions have competitive inhibition by binding to the same binding sites on the cell surface of the green algae *Chlorella vulgaris* (Mehta *et al.*, 2002).

Free copper, nickel and chrome ions showed noncompetitive and mixed inhibition suggesting that they bind to the same as well as different binding sites on the cell surface (Mehta *et al.*, 2002). Thus, the inhibition between free metal ions is dependent on the type of metal present.

It was reported that free zinc ion adsorption decrease when there is free sodium ions present and it decreases more with increasing amount of free sodium ions suggesting competition for binding sites on the cell surface. The effect varied between species *Thalassiosira weissflogii*, *Skeletonema costatum*, *Achnanthidium minutissimum* and Na*vicula minima* with a more pronounced, but varied, effect on the two freshwater species *Achnanthidium minutissimum* and *Navicula minima* (Gélabert *et al.*, 2006). Thus, the specific species affects the inhibition effect between different free metal ions. The adsorption to the carboxylate groups is reported as almost being the same between diatom species while total adsorption between them may vary, due to different cell wall thicknesses and composition (Gélabert *et al.*, 2006).

During sorption of Pb and Cd from solid hydroxides, Pb and Cd exchange places with the cations Ca, Mg, Na from the marine green algae *Rhizoclonium*'s cell surface (Crist *et al.*, 1994). It was stated that free zinc ions were exchanged for magnesium ions on the cell surface of cyanobacteria (Ahuja *et al.*, 1999). Simoultaneously with the ion exchange, an increase in pH have been seen in both instances (Crist *et al.*, 1994; Ahuja *et al.*, 1999). It was suggested that the sorption of Pb and Cd with the concurrent exchange of Ca, Mg and Na from the cell surface and OH from hydroxides at an original pH of 10 happened in stoichiometric amount (Crist *et al.*, 1994). Thus, the sorption of the solid Pb and Cd hydroxides can happen concurrently with the displacing Ca, Mg, Na ions from the cell surface of marine green algae

Rhizoclonium and at the same time raising the pH possibly by the release of hydroxide (Crist *et al.*, 1994).

A study noted that a tolerant strain of cyanobacteria, *Oscillatoria anguistissima*, that was accustomed to zink pollution also showed greater tolerance to other metals, Ni, Co, Cu, Cd, than a wild strain of the same species which was not used to zinc pollution (Ahuja *et al.*, 2001). Thus, tolerance to one metal may increase tolerance to other metals. The presence of Ca stimulated Zn uptake in the wild strain while Zn uptake was independent on Ca concentration in the tolerant strain (Ahuja *et al.*, 2001). Thus, this tolerance mechanism might alter the affect of other metals on the uptake of Zn and other cations may increase uptake of Zn (Ahuja *et al.*, 2001). The tolerant strain also exhibited an increased extracellular adsorption of zinc (Ahuja *et al.*, 2001).

Unstable conditions

Wide temperature and light ranges, along with drought and moisture can give rise to an abnormal cell wall structure (Falasco *et al.*, 2009a; Cornet & Corcoll Cornet, 2012). Hence, it is not ony the conditions in themselves but also the fluctuations that can function as a stress.

Biologic response

Biofilm

The biofilm consist of cells and an extracellular polysaccharide substances, EPS, excreted by the cells (Corcoll, Ricart, *et al.*, 2012; Duong *et al.*, 2010). The EPS is called a matrix and with the cells constitute a biofilm (Corcoll, Ricart, *et al.*, 2012; Laviale *et al.*, 2009; Guasch *et al.*, 2003; Duong *et al.*, 2010; García-meza *et al.*, 2005). The EPS consist mainly of polysaccharides (Crist *et al.*, 1994). Others have reported that the EPS also contain limited amounts of protein and fat (Bradac *et al.*, 2009; Loaëc *et al.*, 1997; Koukal *et al.*, 2007; Rangsayatorn *et al.*, 2002).

The EPS have many different functional functional groups, such as carboxyl, hydroxyl, phosphate, amino and sulfhydryl groups (Monteiro *et al.*, 2012). These have a net negative charge (Mehta & Gaur, 2005; Deng *et al.*, 2007; Rangsayatorn *et al.*, 2002; Volesky, 2007; Gupta & Rastogi, 2008; García-meza *et al.*, 2005). The functional groups are used to regulate adsorption of various particle, such as nutrients and metal ions (Corcoll, Ricart, *et al.*, 2012). The number of functional groups has been reported to be changed in response to the presence of metal exposure (Pérez-Rama *et al.*, 2002).
These functional groups have varying pKa values giving them different ranges of pH where they are deprotonated and mainly avaliable for metal binding (Monteiro *et al.*, 2012; Chojnacka *et al.*, 2005; Volesky, 2007).

This biofilm protect against metal exposure (Guasch *et al.*, 2004; Ahuja *et al.*, 1999; Corcoll, Ricart, *et al.*, 2012; Admiraal *et al.*, 1999; Duong *et al.*, 2010; Fraile *et al.*, 2005; Özer *et al.*, 2000; García-meza *et al.*, 2005; Bradac *et al.*, 2009). This could be due to adsorption on binding sites (Duong *et al.*, 2010; Guasch *et al.*, 2003; Corcoll, Ricart, *et al.*, 2012) or local conditions in the biofilm such as pH and hypoxia conditions (Serra & Guasch, 2009; Corcoll,

Ricart, *et al.*, 2012; Ivorra *et al.*, 2000; Guasch *et al.*, 2003). The high pH in the EPS facilitate metal precipitation (Ivorra *et al.*, 2000).

Biofilm protect against metal sorption deeper down in the biofilm due to reduced interaction between the cells and the environment (Corcoll, Ricart, *et al.*, 2012; Admiraal *et al.*, 1999; Duong *et al.*, 2010; Ivorra *et al.*, 2000). It has been suggested that this is a time dependent effect and, if there is a long-term metal exposure, there will be no difference between a thin and a thick biofilm (Guasch *et al.*, 2004).
An increasing biomass has been seen to decrease the specific zinc uptake in biofilms (Ahuja *et al.*, 1999). It has been suggested that this could be because of cell aggregation which would lessen to biosorption area (Ahuja *et al.*, 1999). It has also been suggested that aggregation may be the result of a non-symbiotic bacterial defence (Buhmann *et al.*, 2009). Another way in which a decrease in specific zinc uptake may occur is through electrostatic interactions (Fraile *et al.*, 2005; Özer *et al.*, 2000). As the cell density increases along with a decrease in the distance between cells this would masks active sites (Fraile *et al.*, 2005; Özer *et al.*, 2000). However, an increase in cell density also increase the number of active sites (Fraile *et al.*, 2005; Özer *et al.*, 2000). The overall effect is less of a specific zinc uptake for a cell (Fraile *et al.*, 2005; Özer *et al.*, 2000). For these reasons it may be better protection against metal eposure for smaller species (Cornet & Corcoll Cornet, 2012).

EPS production have been noted to increase when a biofilm is exposed to zinc (Corcoll, Ricart, *et al.*, 2012; Ahuja *et al.*, 2001).
One study reported an increase in EPS content of three times per unit of algal biomass (the algal biomass was decreased) by the benthic community when chronically exposed to copper compared with a control. The seen increase in EPS was attributed to a detoxification mechanism against metal exposure by binding the metal ions thus reducing bioavalibility and toxicity (Serra & Guasch, 2009).
Another study reported a 5.6 fold increase in EPS for a biofilm when exposed to 1000 μM zinc, the biofilm consisted mostly of green algae and cyanobacteria (García-meza *et al.*, 2005). Older biofilm generally have more biomass than young biofilm (Ivorra *et al.*, 2000).

It was also noted in a study that the composition of the EPS can be influenced when there is a Fe deficiency so that it has an increased adsorption of Fe (Koukal *et al.*, 2007). The adsorption increased from 1 to 11 nmol Fe (mg cell DW)$^{-1}$ s^{-1} (Koukal *et al.*, 2007).

Cell size and community composition

Diatoms have been seen to have a reduced cell size when exposed to zinc (Cornet & Corcoll Cornet, 2012). This has been coupled with a decreased metal uptake for smaller cells (Cornet & Corcoll Cornet, 2012).

At even higher levels of zinc exposure cells have been observed to swell after an initial cell division inhibition (Fisher *et al.*, 1981; Morin *et al.*, 2007). This was attributed to a decoupling photosynthetic process and cell division rate where photosynthetic activity continued without any cell division or the cell excreting the fixed carbon (Fisher *et al.*, 1981;

Morin *et al.*, 2007). It was suggested that possibly zinc adsorption to the cell surface caused diminished silicon uptake when the metal attached to the sulfhydryl groups, responsible for silicic acid uptake in diatoms (Fisher *et al.*, 1981). Silicon-limited cells swelled undistinguishable from copper polluted ones (Fisher *et al.*, 1981).

The diatoms are classified in three ecological guilds, the low profile, the motile and the high profile (Stenger-Kovács *et al.*, 2013; Berthon *et al.*, 2011). The low profile guild is found nearest the bottom of the biofilm while the high profile guild, often forming colonies, are nearer the top (Stenger-Kovács *et al.*, 2013; Berthon *et al.*, 2011). The motile guild consists of fastmoving diatom (Berthon *et al.*, 2011). The low guilds diversity have shown a significant correlation to low resources while the high guild show a significant positive correlation with high resources (Berthon *et al.*, 2011). Guilds are also known to affect the EPS composition (Ivorra *et al.*, 2000).

Small diatom species tightly attached to the substrate are more protected by the matrix than larger ones and are therefore more prolifirent in metal- polluted areas (Morin *et al.*, 2008; Berthon *et al.*, 2011; Corcoll, Ricart, *et al.*, 2012; Applications & Aug, 2012; Ivorra *et al.*, 2000). There is also the increase in abundance of smaller species when exposed to a metal pollutant which could function as a generall bioindicator (Morin *et al.*, 2012). The average cell size and the relative fraction between smaller and larger species (with a boundary of 500 μm^3 in cell size) have shown drastic differences when comparing the taxa from polluted and nonpolluted rivers (Morin *et al.*, 2007; Ivorra *et al.*, 2000). Between taxa there has been a seen selection towards more metal tolerant species which tend to be smaller (Morin *et al.*, 2007). However, another study noted that there was no significant decrease in average cell size for a community even though there was an increase in abundance of small- sized taxa (Corcoll, Ricart, *et al.*, 2012).

The metal uptake by individual cells is also dependent on their surface area with a smaller size giving a lowered uptake (Morin *et al.*, 2007). There have been noted a reduced cell size of diatoms within a taxa (Morin *et al.*, 2012, 2007). The diminished cell size within a taxa have been attributed to among other things inhibition of nutrient uptake and changes in photosynthetic activity due to metal saturation of the functional groups on the cell surface (Corcoll, Ricart, *et al.*, 2012). Small cell size in of itself have been seen to lead to teratological form, such as abnormalities in the valve of the diatom *Nitzschia palea* (Falasco *et al.*, 2009a).

Electrostatic interactions between the functional groups of cells may also play an important role in reducing metal uptake in smaller cells (Fraile *et al.*, 2005). As the cell density increases along with a decrease in the distance between cells this would masks active sites (Fraile *et al.*, 2005). However, an increase in cell density also increase the number of active sites (Fraile *et al.*, 2005). The overall effect is less of a specific zinc uptake (Fraile *et al.*, 2005).

However, cell size can also be influenced by the species present in the region (Corcoll, Ricart, *et al.*, 2012).

It has also been suggested that the decreased cell size during metal exposure is linked with an increase in the rate of vegetative reproduction which result in smaller diatoms, as compared to sexual reproduction that restores cell size to the original size (Morin *et al.*, 2012). Perhaps it is a combination of the aforementioned variables, including increased vegetative reproduction, together with the species-specific morphology and the functional components of the cell that dictates the benefit of certain cell sizes in certain amounts of metal exposure (Falasco *et al.*, 2009b).

Detoxification mechanism

Low amounts of phosphate and carbonate ions have been shown to increase zinc toxicity (Aslam *et al.*, 2012). Polyphosphate bodies, which are composed mainly of phosphate, act as a detoxification mechanism by making the intracellular zinc stable (Ahuja *et al.*, 2001). They also act as a nutrient storage (Rangsayatorn *et al.*, 2002; Morin *et al.*, 2012; Guasch *et al.*, 2004). The polyphosphate bodies are dependent on the presence of phosphorus (Guasch 2004). There was seen a direct positive correlation between increased copper exposure and an increase in the number of polyphosphate bodies (Guasch *et al.*, 2004; Rangsayatorn *et al.*, 2002). A negative connection between copper toxicity and phosphorus availability was also observed in biofilm (Guasch *et al.*, 2004).

Carbonate, in the form of bicarbonate, have been shown to be used as a carbon source for marine diatom, instead of carbondioxide, and could therefore be considered a nutrient (Tortell *et al.*, 1997).

In addition, biofilm have been shown to be more sensitive to copper when there is a low amount of phosphorus available (Guasch *et al.*, 2004).

It has been speculated that an indirect toxicity from zinc exposure come from the reduction in bioavailable phosphate, especially in environments with low phosphate concentration (Paulsson *et al.*, 2000). This is due to precipitation (Paulsson *et al.*, 2000). The resulting indirect zinc effect of a phosphate deficiency would be noticed as a decrease in biomass (Paulsson *et al.*, 2000). Note that photosynthetic activity and chlorophyll a content is a direct effect of zinc toxicity (Paulsson *et al.*, 2000).

These indirect effects would not be seen as a response to zinc exposure (Paulsson *et al.*, 2000). The direct zinc exposure was only toxic at 9.7 μM zinc and higher whereas the indirect nutrient deficiency effect was seen at 0.1- 0.4 μM of zinc (Paulsson *et al.*, 2000). Thus, the existing limits for zinc concentration, set at 5.5 $\mu g \cdot L^{-1}$ by the Swedish Agency for Marine and Water Management as a yearly average allows for the indirect toxic effect, which occurs at 0.1- 0.4 μM of zinc, to affect the biofilm (Directive, 2012; Paulsson *et al.*, 2000).

Effects from acidification and liming on zinc toxicity

In Sweden, liming is an often-used method to improve the pH value and theyreby the water quality (Naturvårdsverket, 2010). Hereby, using liming as an example for the conceptual model, I will discuss and present the effect of each variable and their interactive effect on zinc-diatom interactions. The water bodies in Sweden are often more acidic than their normal status due to anthropogenic causes (Naturvårdsverket, 2010). The effects of liming on zinc toxicity have not often been studied. I therefore used liming as an example to evaluate what effects it would have on zinc exposure for benthic algae.

Much of the zinc that reaches water bodies come from the catchment area, which it reaches often as either runoff or atmospheric deposition (Gunawardena *et al.*, 2013). Point sources, such as mining, do also occur (Ahuja *et al.*, 1999; Hill *et al.*, 2000; Sabater, 2000; Morin *et al.*, 2012). Liming with calcium carbonate would have the effect of raising the pH by binding hydronium ions as water molecules (Gunawardena *et al.*, 2013). Precipitation from zinc ions and hydroxide ions occur with increasing extent at higher pH (Ahuja *et al.*, 1999; Fraile *et al.*, 2005; Özer *et al.*, 2000; Gélabert *et al.*, 2006). Carbonate precipitation with zinc ions can occur, especially at pH of around 7 or higher (Patterson *et al.*, 1977). This suggests a decrease in available zinc ions at higher pH. However, there would also be less hydronium ions available for competition with zinc ions to the functional groups on cell surface which favors an increase in extracellular adsorption (Fraile *et al.*, 2005). The maximum biosorption have been reported at around pH 5.0-5.5 and at pH 8.0-8.5 respectively for various studies. The decrease in biosorption above pH 5.0-5.5 in the former studies were attributed to precipitation of the free zinc ions (Wilde *et al.*, 2006; Ahuja *et al.*, 1999; Fraile *et al.*, 2005; Özer *et al.*, 2000). In addition, the calcium ions from liming with calcium carbonate could compete with zinc ions when binding the cell surface (Ahuja *et al.*, 1999). If zinc ion concentrations are low when liming, for these reasons, it could lead to a zinc deficiency in diatom.

From this it would seem pretty clear that the different variables pH, biosorption, and influence from calcium and carbonate ions suggest there would be a decrease in biosorption of zinc when liming. However, things are not only related to chemical processes or as straightforward. There are many other variables that could lead to different results of liming. These variables are going to be discussed below, with an approxiamate overview figure detailing their interactions.

The conceptual model for zinc toxicity

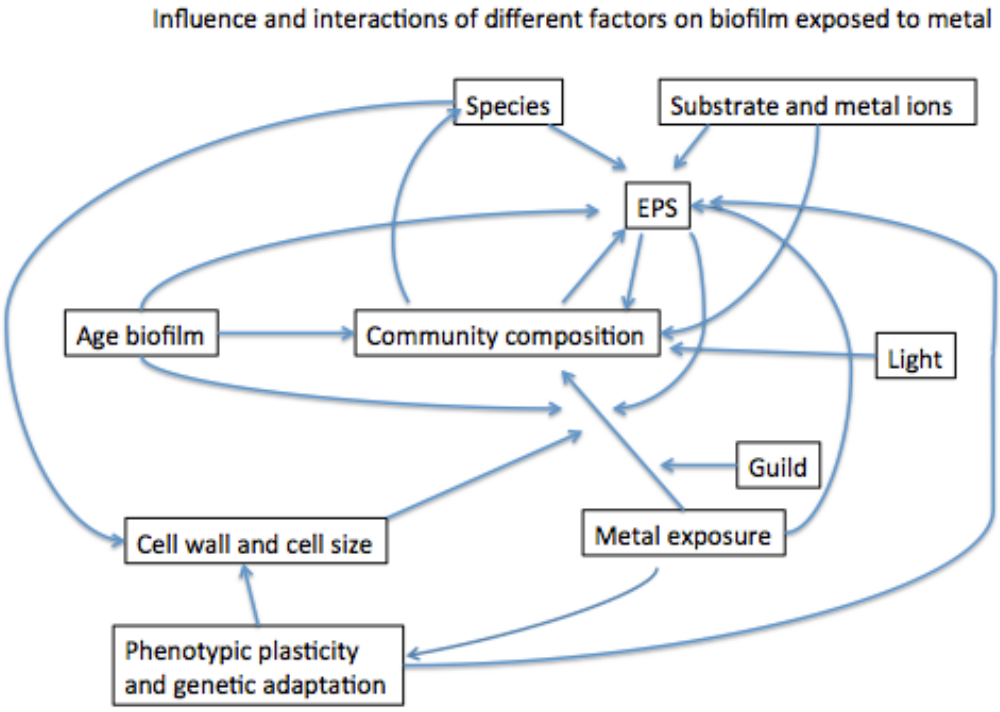

Figure 1. The main variables that influence zinc toxicity. Here is an overview of a simplistic conceptual model including the variables and biologic responses. Arrows show the main connections and the direction of influence.

Nutrients

The new bioavalibility of nutrients from liming will affect the EPS that in turn will affect metal exposure. When the nutrients are in low amounts it could lead to a reduced EPS production (Aslam *et al.*, 2012) (Figure 1).

Meanwhile, the composition of EPS can also vary with nutrient supply (Andersson *et al.*, 2011). This could affect the adsorption of nutrients that are deficient for biofilm, such as iron (Koukal *et al.*, 2007).
Different availability of nutrients will affect species differently leading to changes in the community composition (Sabater, 2009) (Figure 1).

It has also been noted that zinc ions could inhibit detoxification mechanisms in diatoms, by limiting the amount of phosphate and thereby on the intracellular polyphosphate bodies (Guasch *et al.*, 2004). A change in the amount of zinc ions could therefore affect the amount of bioavailable phosphate and therefore diatom detoxification mechanisms against zinc exposure (Guasch *et al.*, 2004; Ahuja *et al.*, 2001).

Carbonate, in the form of bicarbonate, which could be introduced from liming, have been shown to be used as a carbon source for marine diatom, instead of carbondioxide and could therefore be considered a nutrient (Tortell *et al.*, 1997).

Community composition

The community composition has an impact on the amount and composition of the EPS (Cornet & Corcoll Cornet, 2012; Falasco *et al.*, 2009b; Corcoll, Bonet, Leira, *et al.*, 2012). Tolerant species are known to become more common in the community when exposed to a stress, such as metal exposure, and they have different tolerance for the stress, which let the more sensitive species decrease in their abundance (Ahuja *et al.*, 2001; Morin *et al.*, 2012) (Figure 1).

Light

The effect of liming would be different based on light intensity. Biofilm is known to change community composition and amount and composition of EPS due to different light intensities (Corcoll, Bonet, Leira, *et al.*, 2012), see Figure 1. This was shown to affect the biofilms sensitivity to zinc exposure (Corcoll, Bonet, Leira, *et al.*, 2012) (Figure 1).

Species

Depending on the biofilm species present the biofilm will react differently to the effects of liming, such as the effect on nutrients. Biofilm is mainly composed of cyanobacteria, green algae, diatom and bacteria (Cornet & Corcoll Cornet, 2012; Guasch *et al.*, 2003; Ivorra *et al.*, 2000). Biofilm will behave differently depending on which bacteria are present. Different microbial strains produce different compositional EPS (Ivorra *et al.*, 2000) (Figure 1). Some microbial strains even have been shown to produce several different types of EPS (Andersson *et al.*, 2011). Microbial strains could switch type of EPS being produced due to presence of EPS produced by other strains, changes of local conditions and nutrient deficiencies (Andersson *et al.*, 2011). Bacteria could feed on the EPS excreted by benthic algae, and they are suggested to be able to communitcate with diatom. Moreover, bacteria could be attracted and stimulate the production of diatom EPS (Buhmann *et al.*, 2009).

EPS

The amount and composition of EPS can be adjusted to changes in environmental variables, for instance, liming. These changes in EPS could influence the community composition by affecting metal exposure and nutrient avalibility which could be preferred by different species (Ivorra *et al.*, 2000; Andersson *et al.*, 2011; Zeng *et al.*, 2016) (Figure 1).

Guild

There is a higher abundance of the low profile guild in metal polluted water bodies (Morin *et al.*, 2008; Berthon *et al.*, 2011; Corcoll, Ricart, *et al.*, 2012; Applications & Aug, 2012; Ivorra *et al.*, 2000). Metal exposure is probably reduced after liming, and the abundance of the low profile guild could be rivaled by the emerging presence of a high profile guild which is more

sensitive to metal exposure (Morin *et al.*, 2008; Berthon *et al.*, 2011; Corcoll, Ricart, *et al.*, 2012; Applications & Aug, 2012; Ivorra *et al.*, 2000) (Figure 1).

Age biofilm

Biofilm of different age will have different response to the changes when liming. The age of the biofilm could affect the EPS and the community composition (Figure 1). An older biofilm have a different composition and a different, typically larger, amount of EPS and are less sensitive to metal exposure (Ivorra *et al.*, 2000) (Figure 1).
Older thick biofilms have been shown to have a reduced nutrient demand and they instead rely more on internal reuse of nutrients and a decreased uptake of contaminents (Ivorra *et al.*, 2000).

Metal exposure

Metal exposure has also been seen to lead to changes in the form of phenotypic plasticity and genetic adaptation in the cells (Corcoll, Bonet, Morin, *et al.*, 2012; Cornet & Corcoll Cornet, 2012; Klerks & Weis, 1987; Posthuma & Vanstraalen, 1993; Ivorra *et al.*, 2002; Admiraal *et al.*, 1999; Ivorra *et al.*, 2000; Corcoll, Bonet, Leira, *et al.*, 2012; Ahuja *et al.*, 2001) (Figure 1).
Metal exposure can influence the amount of EPS excreted by the cells. In general, with more metal exposure comes an increase in EPS production (Ahuja *et al.*, 2001; Serra & Guasch, 2009; García-meza *et al.*, 2005; Morin *et al.*, 2012). Community composition has been known to adapt to metal exposure by increasing the abundance of tolerant species (Guasch *et al.*, 2004).

Phenotypic plasticity and genetic adaptation

The metal exposure affect phenotypic plasticity and genetic adaptations processes such as cell size and cell wall, community composition and EPS production. When liming, the new metal exposure could affect these processes and the biofilm (Figure 1).

The cells in biofilm can also respond to metal exposure with phenotypic plasticity and genetic adaptation (Corcoll, Bonet, Morin, *et al.*, 2012; Cornet & Corcoll Cornet, 2012; Klerks & Weis, 1987; Posthuma & Vanstraalen, 1993; Ivorra *et al.*, 2002; Admiraal *et al.*, 1999; Ivorra *et al.*, 2000; Corcoll, Bonet, Leira, *et al.*, 2012; Ahuja *et al.*, 2001). This is shown in altering the number of binding sites on the cell wall, an ability to increase photoprotective mechanisms and through adapting a smaller cell size (Corcoll, Bonet, Leira, *et al.*, 2012; Santos *et al.*, 2013; Morin *et al.*, 2012), see Figure 1.

One phenotypic plasticity response is that the number of binding sites on the cell wall have been shown to dramatically increase during metal exposure leading to more extracellular adsorption and another response is improved intracellular detoxification mechanisms, such as polyphosphate bodies (Koukal *et al.*, 2007; Ahuja *et al.*, 2001; Falasco *et al.*, 2009b; Stauber & Florence, 1990; Rangsayatorn *et al.*, 2002; Serra & Guasch, 2009; Morin *et al.*, 2012).

The reported smaller cell size during metal exposure would give more mechanical protection inside the biofilm as well as a reduced cell surface area (Ivorra N, Hettelaar J, Tubbing GMJ, Kraak MHS, 1999; Khoshmanesh *et al.*, 1997; Tlili *et al.*, 2011; Mehta & Gaur, 2005; Morin *et al.*, 2012)**.**

Cell wall and cell size

Small cell size in diatoms is often seen in the presence of metal exposure (Ivorra N, Hettelaar J, Tubbing GMJ, Kraak MHS, 1999; Tlili *et al.*, 2011) (Figure 1).

This could be due to a larger relative cell surface area with more unoccupied binding sites, less biosorption for a small cell giving a reduced specific cell uptake of metals, better mechanical protection within the matrix, and an increased ability to aggregate with other cells. This increase in aggregation would also increase electrostatic interactions, since the smaller cells can come closer to eachother, so that a masking effect occurs between the binding sites. An alternative suggestion is that cells have increased vegetative reproduction during metal stress. Vegetative reproduction in diatom leads to decreased cell size whereas sexual reproduction restores it.

Different species also have different cell wall structure and composition that affect adsorption of a metal, even by the same functional group (García-Ríos *et al.*, 2007; Gélabert *et al.*, 2006). These have been seen as different adsorption by different species (García-Ríos *et al.*, 2007; Gélabert *et al.*, 2006).

It is difficult to say how liming would affect zinc exposure for benthic algae since we do not know how it would interact with all other variables and how diatom would respond. By setting up a conceptual model it gives information about what is known, what to verify and what variables are missing. Then they should correlate the estimated outcome with the seen result.

As seen from the liming example we can see that this is very complex. It is not easy to use diatom since so many factors can influence it.

Final discussion

A conceptual model is here seen as an interactive list of all variables and biological responses whereas a normal experimental design may only be concerned with a few variables. Bench experiments could still be used. It is necessary to think of them in order to attribute seen changes and effects, to study and have an overview to the many interacting components of the conceptual model.

In this conceptual model, the variables are structured and how they could influence others at different concentrations (just deficiency, optimum and too much) and presence so they can be accounted for. By just looking at individual variables it will be rather general, and even possibly leads to the wrong direction of the future studies, if we just make a simple

correlation with zinc and diatom. Instead, a conceptual model shows the quite complex networks of the whole interactions of zinc and diatoms.

For researchers could start with using a part of it, and then extend it with adding more factors, for example to do rather simple bench experiments, which shows both the general/individual influence of each variable, but also give a interacted network of all the mentioned variables. This could be used for structuring the studies, experiments and find out what is the things affecting the organisms.

As an example, the often seen increased toxic effect on diatom during zinc exposure and low carbonate or phosphate ion concentrations could more easily be seen when using a conceptual model view which incorporate the interactions of variables. There are a number of ways in which zinc exposure could affect diatom. A direct zinc exposure could lead to teratological form. There is a detoxification mechanism, polyphosphate bodies, in diatom which is dependant on supply of phosphate (Ahuja *et al.*, 2001). If zinc ions reduce the bioavalibility, through precipitation, of phosphate this would leave the diatom more susceptible to the zinc effect by decreasing the ability of the detoxification mechanism (Guasch 2004; Paulsson *et al.*, 2000). An indirect effect in which zinc exposure could affect diatom is by reducing the phosphate, or carbonate, bioavalibility until a deficiency of these nutrients occur for the diatom (Paulsson *et al.*, 2000). This effect should be more pronounced in oligotroph, nutrient-poor, water bodies since there is only a limited amount of nutrients, such as carbonate and phosphate, to begin with (Paulsson *et al.*, 2000). This indirect effect is especially pronounced in oligotrophic water bodies, due to a dependence on other variables. This should perhaps lead to different upper levels of zinc exposure for low and high-nutrient water bodies.

This intricate connection between variables and biologic responses is favored by a conceptual model approach that sees an overview of the system.

Conclusion

There are many variables that affect the impact of zinc exposure. It is therefore difficult to set a standard value. The interactions of these variables should be included in the assessment of the acceptance of a certain metal exposure.

There may be a new method to use diatoms at bioindicators, there has been noted an increase in polyphosphate bodies and a decrease in motility of cells when there is metal exposure. These changes could be measured using equipment, as opposed to expertise in diatom anatomy. It would be required to use live cells, and not just a cell wall, to assess metal exposure (Pandey & Bergey, 2016).

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