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Valve teratologies and Chl *c* in the freshwater diatom *Tabellaria flocculosa* as biomarkers for metal contamination

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Abstract

Freshwater benthic diatoms, routinely used for bioassessment, are potential metal indicators because of their known capacity to reflect changes in water chemistry. Nevertheless, the effect of metals on benthic freshwater diatoms is still poorly understood. Metals, such as Zn and Cu, are not only essential as micronutrients, but also one of the main anthropogenic pressures in aquatic ecosystems. However, metal stress cannot be detected with the current European standard methods using bioindicators for environmental assessment, so new tools need to be developed. We studied the effects of Zn and Cu on the freshwater diatom *Tabellaria flocculosa.* This diatom isolated from a Zn and Cu contaminated stream, was exposed to different concentrations of Zn and Cu, and the metal effects were assessed by measuring physiological ((growth, fluorescence, pigments' content (Chl *a*, *c*, carotenoids)) and morphological parameters (teratological forms). We found that at environmental concentrations occurring in European streams, Zn and Cu showed toxic effects on *T. flocculosa*. Toxic effects induced by Zn were only observed at high concentrations (500 and 1000 μg/L), resulting in fluorescence changes, an increase of Chl *c* cellular content and an increase of teratological forms (with an increase of more heavily deformed valves with increasing metal concentrations). Increasing Cu exposure induced several changes, such as increasing the cellular content of Chl *a* and *c*, while carotenoids' cellular content decreased. Cu also increased the number of *T. flocculosa* frustule deformations. Cu did not affect the cellular fluorescence. Our results thus show that the frequency of diatom teratologies could potentially be used to assess metal stress for the studied metals Zn and Cu. Additionally, the analysis of algal pigments could potentially separate the effects of different metals.

Keywords: Zinc; Copper; Physiological indicators; Pigments; Valve teratologies

1 Introduction

Anthropogenic activities such as mining, industry and agriculture release metals into the environment (Corcoll et al., 2012b; Millennium Ecosystem Assessment, 2005). Although metals are one of the most notable stress factors in aquatic ecosystems, little is yet known about their effects on the metabolism of aquatic organisms such as algae (Jamers et al., 2009; Kalff, 2002). Algae are furthermore a promising group for the development of biological indicators and cellular markers that will allow the detection of metal contamination of aquatic ecosystems and their recovery after restoration (Pandey and Bergey, 2018).

Zn and Cu are examples of metals known to be toxic but which are also essential micronutrients playing important roles for cell functioning. Zn is a cofactor of several enzymes, such as carbonic anhydrase and rubisco (Hassler et al., 2005), carboxypeptidases, aminopeptidases and phospholipase C and in proteins with Zn finger domains (Godinho et al., 2014; da Silva and Williams, 2001). Cu functions as a cofactor of metalloenzymes, such as the Cu/Zn Superoxide dismutase (SOD) and as a component of photosynthesis and electron carrier, plastocyanin (Festa and Thiele, 2011; Jamers et al., 2013a; Larkum, 2016; Peers and Price, 2006).

However, when these metals exceed physiological concentrations they may become toxic to algae (Chen et al., 2013; Corcoll et al., 2012b), being associated with photosynthesis inhibition for example. The level of toxicity is species dependent, dependent on metal bioavailability, and on the cellular targets of each metal (Corcoll, 2012; Mallick and Mohn, 2000). Zn and Cu are considered growth inhibitors because they may cause structural damage to the photosynthetic apparatus such as the ultra-structure of the thylakoid membrane, change in pigments' composition (Corcoll et al., 2011) and inhibition of cell division (Filippis et al., 1981; Jamers et al., 2013b; Pawlik-Skowrońska, 2003, 2001; Stauber et al., 1990).

It is not clear at what concentrations these metals stop being beneficial and become toxic. Metals occur naturally in freshwater in low concentrations. Background concentrations of 0.6 µg Zn/L and 0.23 µg Cu/L are common for European streams (Salminen et al., 2015) and can reach up to 5 µg Zn/L and up to 0.5 µg Cu/L in Swedish streams and lakes (Swedish Environmental Protection Agency, ~~1999~~2000). However, at highly polluted sites such as mining areas, metal concentrations can be much higher: levels of up to 1.2–9.3 mg Zn/L and 0.84–3.7 mg Cu/L have been measured at contaminated sites in Sweden (Larson, 2010). Based on the Water Framework Directive (Directive 2000/60/EC) and the daughter directive on Priority Substances (Directive 2008/105/EC, 2013/39/EC) (EP, 2013), each EU member state implemented a national strategy against water pollution. The national legal document that classifies water quality in Sweden (HVMFS, 2016), defines concentrations above 12 µg Cu/L and 20 µg Zn/L as toxic for freshwater habitats, resulting in a classification of *"less than good ecological status"* (HVMFS, 2016). In Portugal other threshold values for Cu and Zn concentration are defined (7.8 µg/L Cu and Zn) (APA, 2016).

We studied the effects of different concentrations of Zn and Cu on the freshwater diatom *Tabellaria flocculosa* (Roth) Kützing. Freshwater benthic diatoms are excellent water quality indicators (Hering et al., 2006) because of their short life cycle and consequently fast response to environmental changes, their sensitivity to different stress factors, and the siliceous frustules' preservation which allows for a relatively easy and direct identification of taxa (Chen et al., 2013; Morin et al., 2012; Rimet, 2012). Additionally, diatoms often represent the major autotrophic proportion of biofilms (Corcoll et al., 2012b). Based on this diverse and widespread group of algae and its species richness, taxonomic composition and shifts, several diatom indices based on autecology have been developed. These are commonly used within the WFD, and other countries outside of Europe, mainly to assess eutrophication and high concentrations of organic matter (Cemagref, 1982; Corcoll et al., 2012b; Kelly et al., 2009, 2012; Smol and Stoermer, 2010).

Diatoms have been shown to develop morphological alterations of the siliceous cell wall under metal stress (Cerisier et al., 2018; Corcoll et al., 2012b; Ferreira da Silva et al., 2009; Lavoie et al., 2017, 2012; Luís et al., 2011; Luís et al., 2016; Morin et al., 2012; Pandey et al., 2018; Pandey and Bergey, 2018; Riato et al., 2018). These morphological alterations of the siliceous cell wall are called teratologies. Several studies have already suggested the use of diatom teratologies to detect environmental stress (Cattaneo et al., 2004; Rimet, 2012; Smol and Stoermer, 2010). Riato et al. (2018) have already developed a regional diatom-based index for the assessment of mining impacts in wetlands in the Mpumalanga Highveld region in Africa. Other studies have developed diatom indices to monitor the impact acid mine drainage (AMD) (Fernández et al., 2017; Ferreira da Silva et al., 2009). For example, Fernández et al., 2017 developed and validated the ICM (*Índice de Contaminación por Metales* or Metal Pollution Index) for Guadiamar River (SW Spain). However, these bioindices cannot be used universally, as they are restricted to a specific bioregion and its environmental cofactors, a problem already highlighted by Rimet (2012). Another problem is that many other stress factors aside from metals can potentially cause diatom teratologies (Arini et al., 2012; Falasco et al., 2009a,b; Pandey et al., 2018) or alterations in pigment content (Juneau et al., 2007). Furthermore, little is known about which metals and which concentrations cause a given frequency of teratological forms, and how variable teratology formation is under different environmental conditions (Cantonati et al., 2014). Teratological forms, and cell size reduction, were associated in particular with high concentrations of Cu and Zn (Cantonati et al., 2014; Cattaneo et al., 2004; Falasco et al., 2009a; Ferreira da Silva et al., 2009; Lavoie et al., 2012; Morin et al., 2008a,b).

The effects of Zn and Cu on algae including diatoms have been extensively evaluated in marine species (Admiraal et al., 1999; Contreras et al., 2005; Debelius et al., 2009; Florence et al., 1983; Hassler et al., 2005; Mallick, 2004; Nielsen and Wium-Andersen, 1970; Paulsson et al., 2002; Rijstenbil et al., 1994; Ritter et al., 2008, 2010; Stauber and Florence, 1987; Torres et al., 2008). Fewer studies on freshwater biofilms or single species have been published (Jamers et al., 2013b; Knauert and Knauer, 2008; Luis et al., 2014; Nielsen and Wium-Andersen, 1970; Pandey and Bergey, 2016; Tripathi et al., 2006). These freshwater studies focused mainly on measuring some physiological and morphological parameters such as growth, chlorophyll-*a* fluorescence, pigment content and, when including diatoms, frustule deformations. However, those previous freshwater studies were mainly focused on the diatom community level and did not combine the analysis of physiological and morphological parameters with frustule deformations on the species level (Cantonati et al., 2014; Luis et al., 2013).

Bringing together the use of deformities for metal contamination monitoring combined with physiological parameters (growth, fluorescence and pigments content) could help us to better understand diatoms' response to metal contamination and their tolerance mechanisms (Gonçalves et al., 2018a, b) and to find specific markers that could be used as early warning tools (Arini et al., 2012; Morin et al., 2008a,b). This knowledge can help limnologists to better understand diatoms' and biofilms' response to metal contamination at an early stage. Thus, these studies can help create and improve environmental guides, not only to prevent impairment but also to follow ecosystem recovery after restoration.

This work aimed to study the effects of Zn and Cu exposure and to find possible markers of it on the freshwater benthic diatom *Tabellaria flocculosa* (Roth) Kützing 1844 by evaluating physiological parameters such as growth, fluorescence capacity, pigments' content and valve morphology. Experiments were carried out at environmentally relevant Zn and Cu concentrations. Contrary to earlier assumptions that characterise *T. flocculosa* as a very sensitive species to pollutants (Hofmann et al., 2013; Morin et al., 2012; Olenina et al., 2006), our strain was isolated from a permanently metal contaminated stream, as we aimed to better understand the response developed by a species which

has established tolerance to metals (Gonçalves et al., 2018a, b). Our hypotheses were i) Zn and Cu exposure induce stress in *T. flocculosa* cells, negatively affecting physiological parameters such as growth and photosynthetic capacity and increasing the frequency of valve teratologies, ii) this cellular response differs for the different metal (Zn or Cu) exposure, and iii) for different concentrations of the metal used.

2 Material & methods

2.1 Region characterization, diatom isolation and culture

The Dalarna region in Sweden is known for high metal pollution (Larson, 2010). The region contains old (approx. 1000 years) and new mines and a history of mine effluents draining to the surrounding waters (Larson, 2010). Metals have been found to reach concentrations above 580 μg Cu/L and 1000 μg Zn/L, with the highest measured value of 1800 μg Cu/L and 9300 μg Zn/L (Larson, 2010).

The species used for this study, *Tabellaria flocculosa,* was isolated from a stream in this region with 500 μg Zn/L, 6.2 μg Cu/L and pH ~ 5 (water parameters summarized in supplementary Table 1S) (Larson, 2010). The isolation of a single cell was made by micropipette technique to agar plates (Andersen, 2005; Round et al., 1990) followed by transference to WC (Wright's cryptophyte) liquid medium (Guillard and Lorenzen, 1972). *T. flocculosa* culture is now part of Swedish University of Agricultural sciences diatom culture collection.

2.2 Zn and Cu laboratory experiments

2.2.1 Experimental setup

The strain was pre-cultured in WC medium at pH 5 until exponential growth was reached. Cells at exponential growth were used for Zn or Cu experiments by transferring approximately 250 000 cells to new sterile flasks containing 250 ml of WC modified medium (1000 cells/ml), supplemented with different Zn or Cu concentrations, using 3 to 5 replicates for each condition depending on the total number of cells from the stock culture. The experiments were performed at 20 ± 2 °C with a 12 h/12 h light/dark cycle at 75 μmol/m^2/s in WC medium during 96 h. These experiments where repeated twelve times.

2.2.2 Experimental medium and metal concentrations

The WC medium was modified by using just 1/10 of the EDTA/trace metal solution to ensure that the added Zn or Cu would be present in solution (calculations done by Visual MINTEQ ver. 3.0). Zn concentration in WC medium alone (control) was 2.2 μg/L.

Cu concentration in WC medium alone (control) was 1 μg/L.

We used nominal metal concentrations of 0, 30, 500 and 1000 μg/L for Zn exposure (added as $ZnSO_4$). For Cu experiments, nominal concentrations used (added as $CuSO_4$) were 0, 0.3, 6 and 10 μg/L. The chosen experimental metal concentrations are all environmentally relevant for Swedish streams and reflected i) natural background metal concentrations in Swedish reference streams, ii) a concentration which has been considered as threshold for toxic impact of metals, and iii) high metal concentrations expected to represent a high metal toxicity. Metal concentrations in Swedish reference sites are on average below 10 μg Zn/L and 0.5 μg Cu/L, values up to 3 μg Cu/L in non-impacted streams are not uncommon (HVMFS, 2016), which fits quite well with our nominal control concentration of Zn and Cu in the WC medium (2.2 μg Zn/L; 1 μg Cu/L). A Zn concentration of 30 μg/L is considered a risk level for toxic effects for freshwater organisms (European Union, 2010; HVMFS, 2016), and is therefore considered to represent the boundary between good and moderate ecological water quality of Swedish surface waters (Swedish Environmental Protection Agency, 2000~~Naturvårdsverket, 1999~~). According to Swedish Environmental Protection Agency (~~1999~~2000), 500 μg Zn/L is considered a very high concentration for Swedish waters. However, 12% of all sites studied from Dalarna region have concentrations above 500 μg Zn/L. The selection of 1000 μg Zn/L as an extremely high concentration was chosen but still allowing growth of *Tabellaria flocculosa*. Furthermore, 1000 μg Zn/L is also the threshold concentration where all tested organisms experienced toxic effects under Zn exposure (European Union, 2010). Cu concentration of 0.3 μg/L was chosen as median concentration occurring in European streams, and 6 and 10 μg Cu/L as concentrations commonly noted in metal contaminated streams (Salminen, 2005). The selection of 10 μg Cu/L and not higher concentrations ensured sufficient biomass for analyses, and not a total growth inhibition of *T. flocculosa*.

2.3 Growth determination

At the end of the exposure time, 96 h for physiological parameters and 10 days for teratologies, 1 ml of each replicate was preserved with Lugol's solution for cell counting. Cell density was measured by direct counting in a Neubauer chamber using Nikon Eclipse 80i microscope with 100x magnification Nikon objective Plan Apo (numerical aperture 1.40).

2.4 Fluorescence

Fluorescence was measured using a Tecan micro-plate reader (Tecan Infinite® M1000 Microplate Reader, Tecan Trading AG, Switzerland) set with a 430 nm excitation and a 680 nm emission filter (Esteves et al., 2017). The

fluorescence measured corresponds to photosystem II (PSII) chlorophyll-a emission (~683 nm) after blue light excitation (Luis et al., 2013; Simis et al., 2012). Based on light microscope direct counting in Neubauer chambers (in Section 2.3), calibration per number of cells was made and results are presented as an estimation of fluorescence per cell.

2.5 Pigment content

T. flocculosa cells were harvested by centrifugation (5000 rpm, 15 min), cell pellets were resuspended in deionized water, vortexed and centrifuged again. The washing procedure was repeated two more times to ensure the removal of all the Zn or Cu from the culture medium. Pigments were extracted with 1 ml of 90% acetone from the pellet. The extract was protected from light and maintained in the cold. To break the cells, samples on ice were sonicated for 20 s. The extract centrifuged at 4000×g for 10 min at 4 °C. Chlorophylls a and c and carotenoids were determined spectrophotometrically, and the concentration calculated following the procedure of Jeffrey and Humphrey (1975). Results are presented in μg pigment per million cells (μg/M cells).

2.6 Diatom teratological forms – treatment and identification

T. flocculosa cells were harvested and washed as described in Section 2.3. For this parameter, cells were grown 10 days instead of 96 h in order enable the formation of teratologies (Falasco et al., 2009a; Lavoie et al., 2017; Morin et al., 2012). The resulting pellet was treated by addition of H_2O_2 30% at room temperature for 24 h followed by oven heating at 80–90 °C overnight, in order to eliminate the organic matter and to obtain clean diatom suspensions. Cooled samples were centrifuged (3500 rpm, 15 min) and washed tree times in MQ-water. Permanent slides were mounted using the high refractive index (1.74) medium Naphrax ®. A total of 400 valves were counted for each sample discriminating normal cells from deformed ones by using a Nikon Eclipse 80i light microscope with Plan Apo objective (numerical aperture 1.40, 1000× magnification).

Teratological forms type 1 (Falasco et al., 2009a), valve outline deformation, were divided in three levels of severity: Deformed – D; Highly Deformed – HD; and Extremely Deformed – ED according to Kahlert, 2012. Examples of these levels of severity are presented in Fig. 4.

2.7 Statistical analysis

To test the null hypothesis that an increase of Zn or Cu concentrations does not cause a change in any of the measured parameters, we used Permutation Multivariate Analysis of Variance (PERMANOVA) (Anderson, 2005). All data analysis was performed with the software PRIMERv6 (Clarke and Gorley, 2006) with the add-on PERMANOVA+ (Anderson et al., 2008). To run the PERMANOVA tests we considered 9999 Monte Carlo permutations and pairwise comparisons between Zn or Cu conditions. This null hypothesis was tested for Growth, fluorescence, pigments content and valve deformations described in Section 2. Values of p ≤ 0.05 revealed that the groups differed significantly, indicated in figures by different lowercase letters.

3 Results
3.1 Growth

The growth of *T. flocculosa* after 96 h of metal exposure was inhibited by 20% under 500 μg/L, and significantly inhibited by 50% by the highest Zn concentration of 1000 μg Zn/L compared to the control (Fig. 1A).
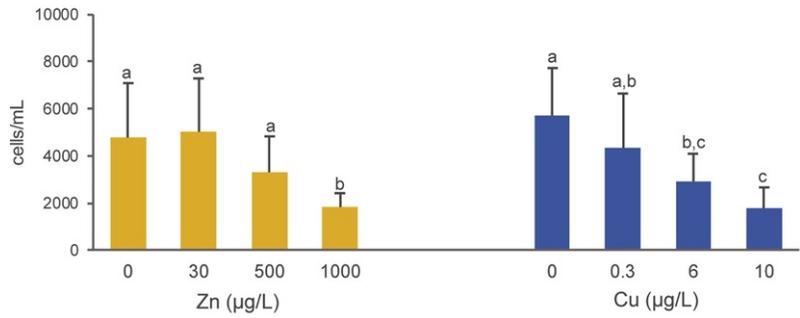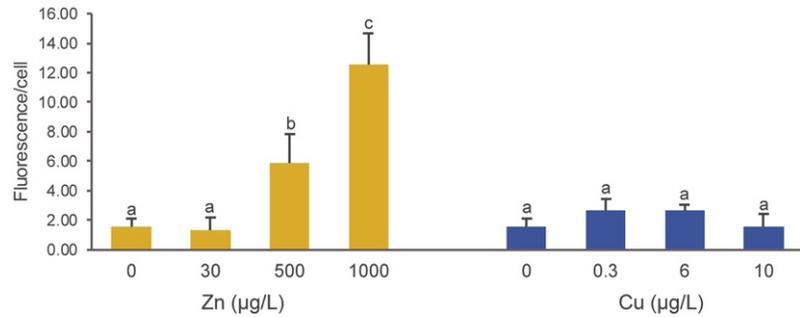
Fig. 1 A) Growth of *Tabellaria flocculosa* (cells/mL) exposed for 96 h to different Zn and Cu concentrations. Values are means (+ standard deviation) of 12 independent experiments. B) Fluorescence per cell from *T. flocculosa* exposed for 96 h to different Zn and Cu concentrations. Lowercase letters indicate significant differences between Zn or Cu exposures $p < 0.05$.

Also, exposure to the highest Cu concentrations (6 and 10 µg/L) inhibited growth significantly, with approximately 50% and 70% of growth inhibition compared to the control. Growth was already decreased by 25% at 0.3 µg/L (Fig. 1A).

3.2 Fluorescence per cell

The fluorescence per cell did not show significant differences between Zn control and 30 µg/L, however increases 3 to 7-fold were observed for cells exposed to higher concentrations (500 and 1000 Zn µg/L) (Fig. 1B). Cu exposure did not induce differences in cell fluorescence (Fig. 1B).

3.3 Pigments

Cellular content of chlorophyll *a* did not show significant differences in Zn exposures compared to the control, we noted however an increase at 1000 µg/L (Fig. 2A). Chlorophyll *c* content also increased per million cells (M cells) (2-fold) significantly at 1000 µg/L (Fig. 2B). An opposite trend was verified for carotenoids' content per M cells. Although not significant, a decrease was seen with the increase of Zn exposure (Fig. 2C).
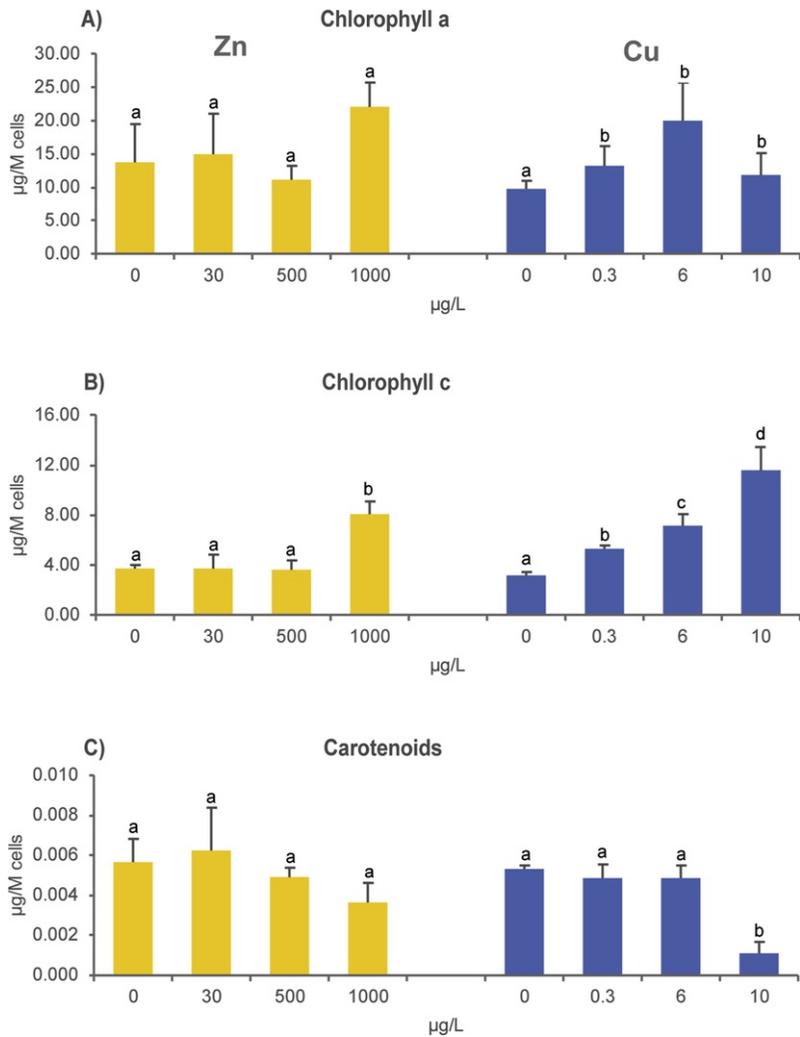
Fig. 2 Pigments content A) Chlorophyll *a*, B) Chlorophyll *c*, C) carotenoids (µg/M cells) in *T. flocculosa* exposed for 96 h to different Zn and Cu concentrations. Values are means (+ standard deviation) of independent experiments. Lowercase letters indicate significant differences between Zn or Cu exposures $p < 0.05$.

The exposure to Cu induced more expressive differences in *T. flocculosa* pigments' content. Chlorophyll *a* content per M cells increased in all Cu concentrations (0.3, 6 and 10 µg/L) compared to the control, the highest value was measured at 6 µg/L (Fig. 2A). A steady increase of chlorophyll *c* per M cells with the increase of Cu concentrations was shown with a 3-fold increase at 10 µg/L compared to the control (Fig. 2B). Carotenoids content per M cells presented no significant differences at 0.3 and 6 µg/L compared to the control, but a significant decrease at 10 µg/L compared to the remaining conditions was shown (Fig. 2C).

3.4 Teratological ~~percentages~~ forms ~~and additional observations on living cells~~

For both Zn and Cu, teratological forms were observed in the control condition (Fig. 3 A, C). The total percentage of teratological forms increased significantly at 500 and 1000 µg Zn/L, compared to the control, with the special contribution of significantly higher extremely deformed shapes (Fig. 3B, Fig. 4). Cu concentrations (0.3, 6, 10 µg/L) all caused a significant increase of teratological forms compared to the control (Fig. 3C). The concentration of 6 µg Cu/L showed the highest frequency of teratologies. In Cu exposure the proportion of valve deformities 1D:1HD:1ED was maintained for all treatments (Fig. 3D). The type of *T. flocculosa* teratological forms in response do Zn and Cu is mainly
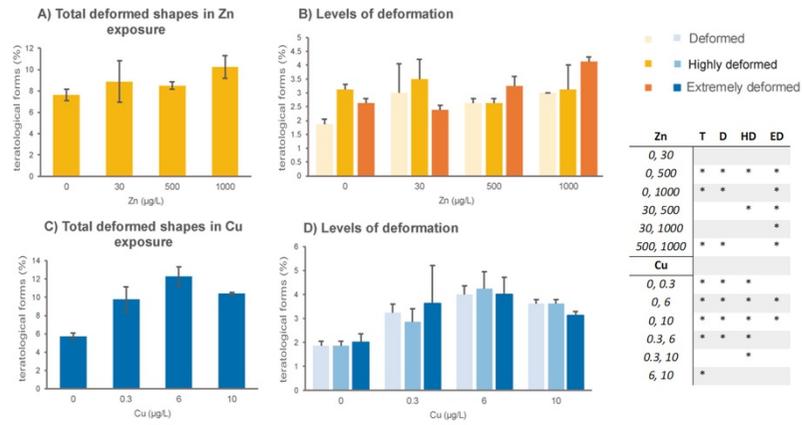
the loss of double symmetry (Fig. 4, Fig. 5).

Fig. 3 Teratological forms percentage of *T. flocculosa* exposed for 96 h to different Zn concentrations: A) total deformed shapes, T; B) levels of deformation: Deformed D, Highly deformed HD and Extremely deformed ED; and Cu concentrations: C) total deformed shapes, T; D) levels of deformation: Deformed, D, Highly deformed HD and Extremely deformed ED. Values are means (+ standard deviation) of independent experiments. Table of significance on the right, * indicates significant differences between metal exposures, $p < 0.05$.
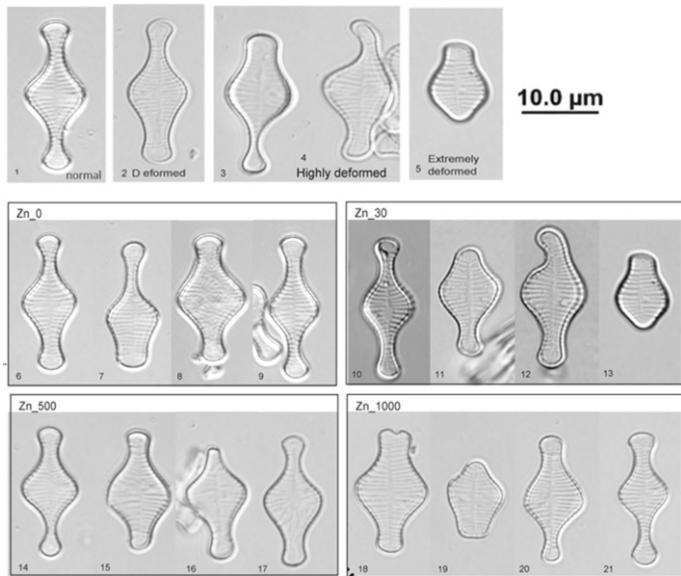

Fig. 4 *Tabellaria flocculosa* deformed valves resulting from Zn exposure. Examples of levels of deformation: 1 – normal shape; 2-Deformed (D); 3, 4 – Highly deformed (HD) and 5 – Extremely deformed (ED). 6 to 9 – Control. 10 to 13 – 30 µg Zn/L; 14 to 17 – 500 µg Zn/L; 18 to 21 – 1000 µg Zn/L.
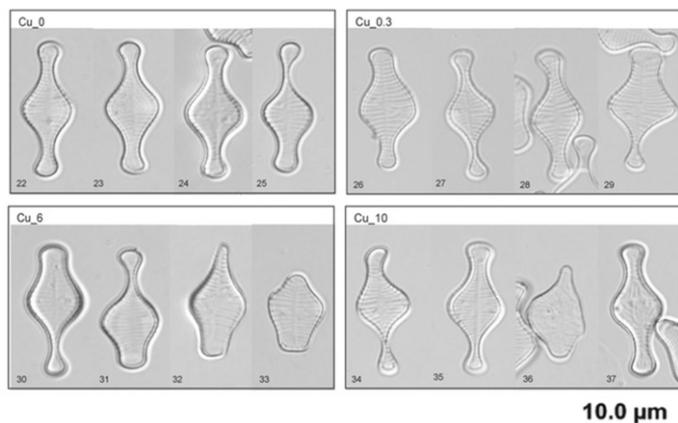
Fig. 5 *Tabellaria flocculosa* deformed valves resulting from Cu exposure. 22 to 25 – Control. 26 to 29 – 0.3 µg Cu/L; 30 to 33 – 6 µg Cu/L; 34 to 37 – 10 µg Cu/L.

4 Discussion

We studied the response of the freshwater benthic diatom *T. flocculosa* (isolated from a metal contaminated stream) to Zn or Cu exposure by measuring physiological and morphological parameters (Gonçalves et al., 2018a, b).

We showed that Zn and Cu at concentrations equal to those found in European streams have negative effects on *T. flocculosa,* confirming our first hypothesis (i)*.* The second hypothesis (ii) was also confirmed since the negative effects on *T. flocculosa* were more notable at high concentrations of Zn (500 and 1000 µg/L) and for all the increasing Cu exposures. Finally, we have also found differences in *T. flocculosa's* response to Zn and Cu (iii). Exposure to high concentrations of Zn resulted in fluorescence changes, an increase in Chl *c,* and an increase in teratological forms with increases in metal concentration. Exposure to Cu did not show changes in fluorescence. However, increases in cellular contents of Chl *a* and *c,* an increase in teratological forms and a decrease in carotenoids' content were observed with increasing Cu concentrations compared to control.

At low concentrations of Zn (30 µg/L) *T. flocculosa* did not show growth alterations or changes in any other parameters evaluated in this study compared to the control, which was somewhat unexpected as we had previously shown effects at this Zn concentration (Goncalves et al., 2018b). The reason might be that effects on pigments' content are not only dependent on metal concentration but also on exposure time. Short-term exposure, as used in the present study, and low concentrations might not be able to induce measurable alterations of these physiological parameters (Corcoll et al., 2011). Also, Stauber et al. (1990) reported that at Zn concentrations below 500 µg/L, only slight effects were noticed in cell division rate, respiration and photosynthesis of the marine diatom *Nitzschia closterium*. However, cellular analysis (biochemical and metabolomics) showed cellular alterations at this concentration (Gonçalves et al., 2018b), indicating that physiological parameters might not be sufficient as markers of stress at low concentration in a short-term bioassay, and that a physiological approach might need additional tests, for example, stress enzymes activity. Contrarily to the observations in other studies where Zn had induced differences (decreases) in Chl *a* fluorescence (e.g. Corcoll et al., 2012a, 2011), in our study Zn seemed to increase the fluorescent emission without, however, affecting the photosynthetic processes of *T. flocculosa* since no alteration was shown in pigments content with the exception of Chl *c* at 1000 µg Zn/L. The lack of alteration of pigments content might be explained by an adaptation process of *T. flocculosa* to high Zn concentration as this strain was isolated from a highly polluted mining site.

Fluorescence (Chl *a* fluorescent emission on PSII) per cell increased at 500 and 1000 µg Zn/L. These results might have several explanations. First, the increased fluorescence could be an attempt by *T. flocculosa* to improve the PS II efficiency for energy production needed for cellular defence mechanisms towards Zn, explored in Gonçalves et al. (2018b). Energy production would be increased by activation of the chlorophyll cycle (increase in Chl *a* and Chl *c* at 1000 µg Zn/L) also supported by the increase of phytol (chlorophyll component), as found in Gonçalves et al. (2018b).

Second, Rashid et al. (1994) described Zn toxicity related to its interference with Ca^{2+} and Mn^{2+} binding on the oxidizing side of PSII and with the electron transport rate. This interference of Zn on PSII might also explain variance on fluorescence found in *T. flocculosa.*

Ultimately, fluorescence was measured in living cells after 96 h exposure, however, some of those living cells when observed under the light microscope were not very "healthy", since the protoplast was not occupying the whole cell compartment. When measuring fluorescence, these cells possibly had a disturbed fluorescence emission.

Chl *c* increased in both metalś exposure and we proposed studying it as a possible biomarker of metal stress, particularly of Cu exposure. However, Chl *c* is not usually used to study metal monitoring contamination. Chl *c* are porphyrins with an acrylic acid side chain not esterified with phytol (Dougherty et al., 1970; Helfrich et al., 2003) which have a role in transporting and ensuring electron transportation to Chl *a* reactions (Büchel, 2003; Hoffman et al.,

2011; Kuczynska et al., 2015). This supports the idea that, at 1000 μg Zn/L and at Cu exposure, cells were trying to increase the source of electrons for Chl *a* for energy production. Additionally, Chl *c* was capable of showing a biomarker-like response for Cu, with linear response. Thus, we suggest Chl *c* evaluation in future studies of metal assessing in diatoms, especially for Cu exposure.

Carotenoids are very important for diatom cells, not only as accessory pigments but also because of their antioxidant properties, preventing oxidative stress damages, being correlated with Chl *a* degradation state in diatom cells (Arini et al., 2012; Bonet et al., 2012). Carotenoids should therefore also be taken in account in metal exposure studies. In the present study the carotenoids' cellular content was not sensitive enough to distinguish different metal concentrations. However, a decrease in carotenoids was observed at 10 μg Cu/L, distinguishing moderated to high stress. For example, the xanthophyll cycle, functions as a light-harvesting complex. It uses the excess of energy produced by stress exposure (e.g. high radiation light or contaminants) to avoid photoinhibition (blockage of light-dependent photosynthesis reactions), and so prevents the photosynthetic process from stopping (Corcoll et al., 2012a). Corcoll et al. (2012a) concluded that the use of pigments such as xanthophylls/carotenoids could detect short-term effects of low to moderate metal pollution.

The increase of teratological valves of 1–2% in comparison to the control marked the effects of high concentrations of Zn (500 and 1000 μg Zn/L) while for all tested Cu concentrations the increase of teratologies was about 5% in relation to the control. The observed total frequencies of teratologies in our study are seemingly very high when compared to values found in the natural environment (Arini et al., 2012). We acknowledge that our experiments were performed with a cultured strain, and that high teratological frequencies often are observed in diatom cultures. Nevertheless, we expect that our results showing a significant increase of teratologies under metal exposure are relevant for natural environmental conditions as well, since earlier studies found similar results (Cantonati et al., 2014; Cattaneo et al., 2004; Falasco et al., 2009a; Morin et al., 2012; Pandey and Bergey, 2016). The observed changes were mostly in the valve outline with loss of double symmetry for both metals in accordance with Falasco et al. (2009a,b). Despite the small but significant increases, teratologies were able to indicate metal exposure in this study, supporting the use of these morphological aberrations for biomonitoring (reviewed in Lavoie et al., 2017; Morin et al., 2012). Teratological forms can also be related to other factors, such as disturbances on silicate uptake which can be caused by metal exposure (Cattaneo et al., 2004). Fisher et al. (1981) proposed that Cu could bind to sulphydryl groups on the diatom cell membrane reducing silica uptake and thus create conditions of silica limitation. It is known that metals in general interact with sulphydryl groups and thus with cell components such as proteins, which can suffer damage. In diatom frustule silicification process, a protein template is needed for silicic acid polymerization to opaline silica, therefore a disruption of these proteins could also be an explanation for the abnormal frustules production (Debenest et al., 2008; Fisher et al., 1981).

Simultaneous observations of teratologies along with cell size reduction have been observed at metal contaminated sites including those with high levels of Zn and Cu (Cattaneo et al., 2004; Luís et al., 2011). According to Cattaneo et al. (2004), cell size reduction appears to be a general response to elevated metal concentrations, whereas valve distortions are associated in particular to Cu. Nevertheless, in other studies from mining sites with a mixture of metals (not only Cu), diatom teratologies were also registered (e.g.Arini et al., 2012; Leguay et al., 2016; Luís et al., 2016, 2011; Morin and Coste, 2006). In our study teratological forms were higher with Cu exposure, but also appeared when exposed to Zn.

Morphological changes of diatom frustules reflect a consequence of metabolomic changes even in diatom species from metal polluted sites, which are obviously tolerant to metal exposure (Cattaneo et al., 2004, Gonçalves et al., 2018a, b). In order to better understand these cell aberrations in different pollution scenarios there is a need for more studies which not only evaluate teratological forms, but which also investigate the cellular process behind teratologies.

5 Conclusions

Our study showed that Zn, at high concentrations, and Cu are toxic for the freshwater benthic diatom *T. flocculosa*. Different markers were observed for both metals.

No effects were observed in *T. flocculosa* when exposed to 30 μg Zn/L. However, exposure to higher Zn concentrations was marked by increases in fluorescence, in teratologies (500 and 1000 μg Zn/L), and in Chl *c* concentration (1000 μg Zn/L).

For Cu exposure, most of the physiological parameters showed a response at low concentration (0.3 μg Cu/L), including increases in Chl *a,* Chl *c,* teratological forms and for high Cu concentrations also a decrease in carotenoids (10 μg Cu/L).

This study combined commonly used physiological parameters with the evaluation of morphological changes, contributing to a better understanding of the changes diatom undergo under Zn or Cu stress.

We suggest that parameters such as Chl *a*, Chl *c* combined with diatom valve teratologies should be tested as biomarkers for assessing Zn and Cu impacts in diatoms. Further research should test and validate these results, designing new studies using higher environmental complexity levels, such as at biofilm level.

Acknowledgements

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ecolind.2019.01.032.

References (Please add the following 3 references to the reference list:
-Swedish Environmental Protection Agency, 2000. Lakes and watercourses. Environmental quality criteria. Report 5050. Stockholm. 105 p.
-Luís, A.T., Bonet, B., Corcoll, N., Almeida, S.F.P., Da Silva, E.F., Figueira, E., Guasch, H., 2014. Experimental evaluation of the contribution of acidic pH and Fe concentration to the structure, function and tolerance to metals (Cu and Zn) exposure in fluvial biofilms. Ecotoxicology 23, 1270–1282. doi:10.1007/s10646-014-1270-2
- Rashid, A., Carom, E.L., Ekramoddoullah, A.K.M., 1994. Molecular mechanism of action of Pb 2+ and Zn 2+ on water oxidizing complex of photosystem II, FEBS Letters.)

Admiraal W., Blanck H., Buckert-De Jong M., Guasch H., Ivorra N., Lehmann V., Nystrom B.A.H., Paulsson M. and Sabater S., Short-term toxicity of zinc to microbenthic algae and bacteria in a metal polluted stream, *Water Res.* **33**, 1999, 1989–1996, https://doi.org/10.1016/S0043-1354(98)00426-6.

Andersen R.A., Algal Culturing Techniques, 2005, Elsevier Academic Press.

Anderson M., Gorley R. and Clarke R., Permanova+ for Primer: Guide to Software and, 2008, Statisticl Methods.

Anderson, M.J., 2005. PERMANOVA: a FORTRAN computer program for permutational multivariate analysis of variance.

Arini A., Feurtet-Mazel A., Morin S., Maury-Brachet R., Coste M. and Delmas F., Remediation of a watershed contaminated by heavy metals: a 2-year field biomonitoring of periphytic biofilms, *Sci. Total Environ.* **425**, 2012, 242–253, https://doi.org/10.1016/j.scitotenv.2012.02.067.

Bonet B., Corcoll N. and Guasch H., Antioxidant enzyme activities as biomarkers of Zn pollution in fluvial biofilms, *Ecotoxicol. Environ. Saf.* **80**, 2012, 172–178, https://doi.org/10.1016/j.ecoenv.2012.02.024.

Büchel C., Fucoxanthin-chlorophyll proteins in diatoms: 18 and 19 kda subunits assemble into different oligomeric states, *Biochemistry* **2003** (42), 2003, 13027–13034, https://doi.org/10.1021/bi0349468.

Cantonati M., Angeli N., Virtanen L., Wojtal A.Z., Gabrieli J., Falasco E., Lavoie I., Morin S., Marchetto A., Fortin C. and Smirnova S., Achnanthidium minutissimum (Bacillariophyta) valve deformities as indicators of metal enrichment in diverse widely-distributed freshwater habitats, *Sci. Total Environ.* **475**, 2014, 201–215, https://doi.org/10.1016/j.scitotenv.2013.10.018.

Cattaneo A., Couillard Y., Wunsam S. and Courcelles M., Diatom taxonomic and morphological changes as indicators of metal pollution and recovery in Lac Dufault (Québec, Canada), *J. Paleolimnol.* **32**, 2004, 163–175, https://doi.org/10.1023/B:JOPL.0000029430.78278.a5.

Cemagref, 1982. Etude des méthodes biologiques d'appréciation quantitative de la qualité des eaux. Lyon.

Cerisier A., Vedrenne J., Lavoie I. and Morin S., Assessing the severity of diatom deformities using geometric morphometry, *Bot. Lett.* **1-9**, 2018, https://doi.org/10.1080/23818107.2018.1474800.

Chen X., Mao X., Cao Y. and Yang X., Use of siliceous algae as biological monitors of heavy metal pollution in three lakes in a mining city, southeast China, *Oceanol. Hydrobiol. Stud.* **42**, 2013, 233–242, https://doi.org/10.2478/s13545-013-0079-6.

Clarke, K., Gorley, R., 2006. PRIMER V6: user manual-tutorial.

Contreras L., Moenne A. and Correa J.A., Antioxidant responses in Scytosiphon lomentaria (Phaeophyceae) inhabiting copper-enriched coastal environments, *J. Phycol.* **41**, 2005, 1184–1195, https://doi.org/10.1111/j.1529-8817.2005.00151.x.

Corcoll N., The use of pulse amplitude modulated fluorescence techniques for metal toxicity assessment in fluvial biofilms, 2012, University of Girona.

Corcoll N., Bonet B., Leira M. and Guasch H., Chl-a fluorescence parameters as biomarkers of metal toxicity in fluvial biofilms: an experimental study, *Hydrobiologia* **673**, 2011, 119–136, https://doi.org/10.1007/s10750-011 0763-8.

Corcoll N., Bonet B., Leira M., Montuelle B., Tlili A. and Guasch H., Light history influences the response of fluvial biofilms to Zn exposure, *J. Phycol.* **48**, 2012a, 1411–1423, https://doi.org/10.1111/j.1529-8817.2012.01223.x.

Corcoll N., Bonet B., Morin S., Tlili A., Leira M. and Guasch H., The effect of metals on photosynthesis processes and diatom metrics of biofilm from a metal-contaminated river: a translocation experiment, *Ecol. Indic.* **18**, 2012b, 620–631, https://doi.org/10.1016/j.ecolind.2012.01.026.

Debelius B., Forja J.M., DelValls Á. and Lubián L.M., Toxicity and bioaccumulation of copper and lead in five marine microalgae, *Ecotoxicol. Environ. Saf.* **72**, 2009, 1503–1513, https://doi.org/10.1016/j.ecoenv.2009.04.006.

Debenest T., Silvestre J., Coste M., Delmas F. and Pinelli E., Herbicide effects on freshwater benthic diatoms: Induction of nucleus alterations and silica cell wall abnormalities, *Aquat. Toxicol.* **88**, 2008, 88–94, https://doi.org/10.1016/j.aquatox.2008.03.011.

Dougherty R.C., Strain H.H., Svec W.A., Uphaus R.A. and Katz J.J., The structure, properties, and distribution of chlorophyll c, *J. Am. Chem. Soc.* **92**, 1970, 2826–2833, https://doi.org/10.1021/ja00712a037.

EP, 2013. Directive 2008/105/EC,2013/39/EC, Official Journal of the European Union.

Esteves S.M., Keck F., Almeida S.F.P., Figueira E., Bouchez A. and Rimet F., Can we predict diatoms herbicide sensitivities with phylogeny? influence of intraspecific and interspecific variability, *Ecotoxicology* **1-13**, 2017, https://doi.org/10.1007/s10646-017-1834-z.

European Union, 2010. European Union Risk Assessment Report, Environment. doi: 10.2788/40041.

Falasco E., Bona F., Badino G., Hoffmann L. and Ector L., Diatom teratological forms and environmental alterations: a review, *Hydrobiologia* **623**, 2009a, 1–35, https://doi.org/10.1007/s10750-008-9687-3.

Falasco E., Bona F., Ginepro M., Hlúbiková D., Hoffmann L. and Ector L., Morphological abnormalities of diatom silica walls in relation to heavy metal contamination and artificial growth conditions, *Water SA* **35**, 2009b, 595–606, https://doi.org/10.4314/wsa.v35i5.49185.

Fernández M.R., Martín G., Corzo J., de la Linde A., García E., López M. and Sousa M., Design and testing of a new diatom-based index for heavy metal pollution, *Arch. Environ. Contam. Toxicol.* **1-23**, 2017, https://doi.org/10.1007/s00244-017-0409-6.

Ferreira da Silva E., Almeida S.F.P., Nunes M.L., Luís A.T., Borg F., Hedlund M., Sá C.M., Patinha C. and Teixeira P., Heavy metal pollution downstream the abandoned Coval da Mó mine (Portugal) and associated effects on epilithic diatom communities, *Sci. Total Environ.* **407**, 2009, 5620–5636, https://doi.org/10.1016/j.scitotenv.2009.06.047.

Festa R.A. and Thiele D.J., Copper: an essential metal in biology, *Curr. Biol.* **21**, 2011, R877–R883, https://doi.org/10.1016/j.cub.2011.09.040.

Filippis L.F., Hampp R. and Ziegler H., The effects of sublethal concentrations of zinc, cadmium and mercury on Euglena, *Arch. Microbiol.* **128**, 1981, 407–411, https://doi.org/10.1007/BF00405922.

Fisher N.S., Jones G.J. and Nelson D.M., Effects of copper and zinc on growth, morphology, and metabolism of Asterionella japonica (Cleve) 1, *J. Exp. Mar. Bio. Ecol.* **51**, 1981, 37–56, https://doi.org/10.1016/0022-0981(81)90153-2.

Florence T.M., Lumdens B.G. and Fardy J.J., Evaluation of some physico-chemical techniques for the determination of the fraction of dissolved copper toxic to the marine diatom Nitzschia Closterium, *Anal. Chim. Acta* **151**, 1983, 281–295.

Godinho R.M., Cabrita M.T., Alves L.C. and Pinheiro T., Imaging of intracellular metal partitioning in marine diatoms exposed to metal pollution: consequences to cellular toxicity and metal fate in the environment, *Metallomics* **6**, 2014, 1626, https://doi.org/10.1039/C4MT00105B.

Gonçalves S., Kahlert M., Almeida S.F.P. and Figueira E., Assessing Cu impacts on freshwater diatoms: biochemical and metabolomic responses of Tabellaria flocculosa (Roth) Kützing, *Sci. Total Environ.* **625**, 2018a, 1234–1246, https://doi.org/10.1016/j.scitotenv.2017.12.320.

Gonçalves S., Kahlert M., Almeida S.F.P. and Figueiraa E., A freshwater diatom challenged by Zn pollution: biochemical, physiological and metabolomic responses of Tabellaria flocculosa (Roth) Kützing, *Environ. Pollut.* 2018b.

Guillard R.R.L. and Lorenzen C.J., Yellow-green algae with chlorophyllide C, *J. Phycol.* **8**, 1972, 10–14, https://doi.org/10.1111/j.1529-8817.1972.tb03995.x.

Hassler C.S., Behra R. and Wilkinson K.J., Impact of zinc acclimation on bioaccumulation and homeostasis in Chlorella kesslerii, *Aquat. Toxicol.* **74**, 2005, 139–149, https://doi.org/10.1016/j.aquatox.2005.02.008.

Helfrich M., Bommer B., Oster U., Klement H., Mayer K., Larkum A.W.D. and Rüdiger W., Chlorophylls of the c family: absolute configuration and inhibition of NADPH:protochlorophyllide oxidoreductase, *Biochim. Biophys. Acta Bioenerg.* **1605**, 2003, 97–103, https://doi.org/10.1016/S0005-2728(03)00081-1.

Hering D., Johnson R.K., Kramm S., Schmutz S., Szoszkiewicz K. and Verdonschot P.F.M., Assessment of European streams with diatoms, macrophytes, macroinvertebrates and fish: a comparative metric-based analysis of organism response to stress, *Freshw. Biol.* **51**, 2006, 1757–1785, https://doi.org/10.1111/j.1365-2427.2006.01610.x.

Hoffman G.E., Puerta M.V.S. and Delwiche C.F., Evolution of light-harvesting complex proteins from Chl c-containing algae, *BMC Evol. Biol.* **11**, 2011, 101, https://doi.org/10.1186/1471-2148-11-101.

Hofmann G., Werum M. and Lange-Bertalot H., Diatomeen im Süßwasser-Benthos von Mitteleuropa, 2013, Koeltz Scientific Books, Koeltz, Königstein; Lauterbornia Onlinestore.

HVMFS, 2016. Miljögifter i vatten – klassificering av ytvattenstatus Vägledning för tillämpning av HVMFS 2013:19. Göteborg.

Jamers A., Blust R. and De Coen W., Omics in algae: paving the way for a systems biological understanding of algal stress phenomena?, *Aquat. Toxicol.* **92**, 2009, 114–121, https://doi.org/10.1016/j.aquatox.2009.02.012.

Jamers A., Blust R., De Coen W., Griffin J.L. and Jones O.A.H., An omics based assessment of cadmium toxicity in the green alga Chlamydomonas reinhardtii, *Aquat. Toxicol.* **126**, 2013a, 355–364, https://doi.org/10.1016/j.aquatox.2012.09.007.

Jamers A., Blust R., De Coen W., Griffin J.L. and Jones O.A.H., Copper toxicity in the microalga Chlamydomonas reinhardtii: an integrated approach, *BioMetals* **26**, 2013b, 731–740, https://doi.org/10.1007/s10534-013-9648-9

Juneau P., Qiu B. and Deblois C.P., Use of chlorophyll fluorescence as a tool for determination of herbicide toxic effect: review, *Toxicol. Environ. Chem.* **89**, 2007, 609–625, https://doi.org/10.1080/02772240701561569.

Kahlert, M., 2012. Utveckling av en miljögiftsindikator – kiselalger i rinnande vatten. Report 2012/12, Länsstyrelsen Blekinge län, Karlskrona, 40p.

Kalff J., Limnology: Inland Water Ecosystems, 2002, Prentice Hall.

Kelly M., Bennett C., Coste M., Delgado C., Delmas F., Denys L., Ector L., Fauville C., Ferréol M., Golub M., Jarlman A., Kahlert M., Lucey J., Ní Chatháin B., Pardo I., Pfister P., Picinska-Faltynowicz J., Rosebery J., Schranz C., Schaumburg J., van Dam H. and Vilbaste S., A comparison of national approaches to setting ecological status boundaries in phytobenthos assessment for the European Water Framework Directive: results of an intercalibration exercise, *Hydrobiologia* **621**, 2009, 169–182, https://doi.org/10.1007/s10750-008-9641-4.

Kelly M.G., Gómez-Rodríguez C., Kahlert M., Almeida S.F.P., Bennett C., Bottin M., Delmas F., Descy J.-P., Dörflinger G., Kennedy B., Marvan P., Opatrilova L., Pardo I., Pfister P., Rosebery J., Schneider S. and Vilbaste S., Establishing expectations for pan-European diatom based ecological status assessments, *Ecol. Indic.* **20**, 2012, 177–186, https://doi.org/10.1016/j.ecolind.2012.02.020.

Knauert S. and Knauer K., The role of reactive oxygen species in copper toxicity to two freshwater green algae, *J. Phycol.* **44**, 2008, 311–319, https://doi.org/10.1111/j.1529-8817.2008.00471.x.

Kuczynska P., Jemiola-Rzeminska M. and Strzalka K., Photosynthetic pigments in diatoms, *Mar. Drugs* **13**, 2015, 5847–5881, https://doi.org/10.3390/md13095847.

Larkum A.W., Photosynthesis and light harvesting in algae, In: *The Physiology of Microalgae,* 2016, Springer International Publishing; Cham, 67–87, doi: 10.1007/978-3-319-24945-2_3.

Larson D., Metallpåverkade sjöar och vattendrag i Dalarna. Konsekvenser av en tusenårig gruvhistoria. Rapporter från Länsstyrelsen i Dalarnas län, 2010, Miljöenheten Länsstyrelsen Dalarna; Falun.

Lavoie I., Hamilton P.B., Morin S., Kim Tiam S., Kahlert M., Gonçalves S., Falasco E., Fortin C., Gontero B., Heudre D., Kojadinovic-Sirinelli M., Manoylov K., Pandey L.K. and Taylor J.C., Diatom teratologies as biomarkers of contamination: are all deformities ecologically meaningful?, *Ecol. Indic.* **82**, 2017, 539–550, https://doi.org/10.1016/j.ecolind.2017.06.048.

Lavoie I., Lavoie M. and Fortin C., A mine of information: Benthic algal communities as biomonitors of metal contamination from abandoned tailings, *Sci. Total Environ.* **425**, 2012, 231–241,

https://doi.org/10.1016/j.scitotenv.2012.02.057.

Leguay S., Lavoie I., Levy J.L. and Fortin C., Using biofilms for monitoring metal contamination in lotic ecosystems: the protective effects of hardness and pH on metal bioaccumulation, *Environ. Toxicol. Chem.* **35**, 2016, 1489–1501, https://doi.org/10.1002/etc.3292.

Luis A.T., Coelho H., Almeida S.F.P., da Silva E.F. and Serodio J., Photosynthetic activity and ecology of benthic diatom communities from streams affected by Acid Mine Drainage (AMD) in pyritic mines, *Fundam. Appl. Limno* **182**, 2013, 47–59, https://doi.org/10.1127/1863-9135/2013/0370.

Luís A.T., Durães N., de Almeida S.F.P. and da Silva E.F., Integrating geochemical (surface waters, stream sediments) and biological (diatoms) approaches to assess AMD environmental impact in a pyritic mining area: Aljustrel (Alentejo, Portugal), *J. Environ. Sci. (China)* **42**, 2016, 215–226, https://doi.org/10.1016/j.jes.2015.07.008.

Luís A.T., Teixeira P., Almeida S.F.P., Matos J.X. and Da Silva E.F., Environmental impact of mining activities in the Lousal area (Portugal): chemical and diatom characterization of metal-contaminated stream sediments and surface water of Corona stream, *Sci. Total Environ.* **409**, 2011, 4312–4325, https://doi.org/10.1016/j.scitotenv.2011.06.052.

Mallick N., Copper-induced oxidative stress in the chlorophycean microalga Chlorella vulgaris: response of the antioxidant system, *J. Plant Physiol.* **161**, 2004, 591–597, https://doi.org/10.1078/0176-1617-01230.

Mallick N. and Mohn F.H., Reactive oxygen species: response of algal cells, *J. Plant Physiol.* **157**, 2000, 183–193, https://doi.org/10.1016/S0176-1617(00)80189-3.

Millennium Ecosystem Assessment, Ecosystems and Human Well-being: Synthes, 2005, Island Press; Washington, DC.

Morin S., Cordonier A., Lavoie I., Arini A., Blanco S., Bonet B., Duong T.T., Torne E., Becares E., Coste M., Faggiano L., Laviale M., Pe F., Fortin C., Guasch H. and Sabater S., Consistency in diatom response to metal contaminated environments, *Emerg. Priority Pollutants Rivers* 2012, 117–146, https://doi.org/10.1007/978-3-642-25722-3.

Morin, S., Coste, M., 2006. Metal-induced shifts in the morphology of diatoms from the Riou Mort and Riou Viou streams (South West France). Use algae Monit. rivers VI 91–106.

Morin S., Coste M. and Hamilton P.B., Scanning electron microscopy observations of deformities in small pennate diatoms exposed to high cadmium concentrations, *J. Phycol.* **44**, 2008a, 1512–1518, https://doi.org/10.1111/j.1529-8817.2008.00587.x.

Morin S., Duong T.T., Dabrin A., Coynel A., Herlory O., Baudrimont M., Delmas F., Durrieu G., Schäfer J., Winterton P., Blanc G. and Coste M., Long-term survey of heavy-metal pollution, biofilm contamination and diatom community structure in the Riou Mort watershed, South-West France, *Environ. Pollut.* **151**, 2008b, 532–542, https://doi.org/10.1016/j.envpol.2007.04.023.

Naturvårdsverket, 1999. Bedomninsgrunder for miljokvalitet. Stockholm. (Please replace by Swedish Environmental Protection Agency, 2000. Lakes and watercourses. Environmental quality criteria. Report 5050. Stockholm. 105 p. This reference (which is the English translation of Naturvårdsverket 1999) was also corrected in the text.)

Nielsen E.S. and Wium-Andersen S., Copper ions as poison in the sea and in freshwater, *Mar. Biol.* **6**, 1970, 93–97, https://doi.org/10.1007/BF00347237.

Olenina, I., Andersson, A., Wasmund, N., Busch, S., 2006. Biovolumes and size-classes of phytoplankton in the Baltic Sea. Helsinki Commission.

Pandey L.K. and Bergey E.A., Metal toxicity and recovery response of riverine periphytic algae, *Sci. Total Environ.* **642**, 2018, 1020–1031, https://doi.org/10.1016/J.SCITOTENV.2018.06.069.

Pandey L.K. and Bergey E.A., Exploring the status of motility, lipid bodies, deformities and size reduction in periphytic diatom community from chronically metal (Cu, Zn) polluted waterbodies as a biomonitoring tool, *Sci. Total Environ.* **550**, 2016, 372–381, https://doi.org/10.1016/j.scitotenv.2015.11.151.

Pandey L.K., Lavoie I., Morin S., Park J., Lyu J., Choi S., Lee H. and Han T., River water quality assessment based on a multi-descriptor approach including chemistry, diatom assemblage structure, and non-taxonomical diatom metrics, *Ecol. Indic.* **84**, 2018, 140–151, https://doi.org/10.1016/J.ECOLIND.2017.07.043.

Paulsson M., Månsson V. and Blanck H., Effects of zinc on the phosphorus availability to periphyton communities from the river Göta Älv, *Aquat. Toxicol.* **56**, 2002, 103–113, https://doi.org/10.1016/S0166-445X(01)00189-8.

Pawlik-Skowrońska B., When adapted to high zinc concentrations the periphytic green alga Stigeoclonium tenue produces high amounts of novel phytochelatin-related peptides, *Aquat. Toxicol.* **62**, 2003, 155–163, https://doi.org/10.1016/S0166-445X(02)00080-2.

Pawlik-Skowrońska B., Phytochelatin production in freshwater algae Stigeoclonium in response to heavy metals contained in mining water; effects of some environmental factors, *Aquat. Toxicol.* **52**, 2001, 241–249, https://doi.org/10.1016/S0166-445X(00)00144-2.

Peers G. and Price N.M., Copper-containing plastocyanin used for electron transport by an oceanic diatom, *Nature* **441**, 2006, 341–344, https://doi.org/10.1038/nature04630.

Riato L., Leira M., Della Bella V. and Oberholster P.J., Development of a diatom-based multimetric index for acid mine drainage impacted depressional wetlands, *Sci. Total Environ.* **612**, 2018, 214–222, https://doi.org/10.1016/j.scitotenv.2017.08.181.

Rijstenbil J.W., Derksen J.W.M., Gerringa L.J.A., Poortvliet T.C.W., Sandee A., Van den Berg M., Van Drie J. and Wijnholds J.A., Oxidative stress induced by copper: defense and damage in the marine planktonic diatom Ditylum brightwellii, grown in continuous cultures with high and low zinc levels, *Mar. Biol.* **119**, 1994, 583–590.

Rimet F., Recent views on river pollution and diatoms, *Hydrobiologia* **683**, 2012, 1–24, https://doi.org/10.1007/s10750-011-0949-0.

Ritter A., Goulitquer S., Salaün J.P., Tonon T., Correa J.A. and Potin P., Copper stress induces biosynthesis of octadecanoid and eicosanoid oxygenated derivatives in the brown algal kelp Laminaria digitata, *New Phytol.* **180**, 2008, 809–821, https://doi.org/10.1111/j.1469-8137.2008.02626.x.

Ritter A., Ubertini M., Romac S., Gaillard F., Delage L., Mann A., Cock J.M., Tonon T., Correa J.A. and Potin P., Copper stress proteomics highlights local adaptation of two strains of the model brown alga Ectocarpus siliculosus, *Proteomics* **10**, 2010, 2074–2088, https://doi.org/10.1002/pmic.200900004.

Round F.E., Crawford R.M. and Mann D.G., Diatoms: biology and morphology of the genera, In: Round F.E., Crawford R.M. and Mann D.G., (Eds.), *Google Livros,* second ed., 1990, Cambridge University Press.

Salminen R., (Ed), *Geochemical Atlas of Europe. Part 1: Background Information, Methodology and Maps,* 2005, Geological Survey of Finland.

da Silva J.J.R.F. and Williams R.J.P., The Biological Chemistry of the Elements: The Inorganic Chemistry of Life, 2001, Oxford University Press.

Simis S.G.H., Huot Y., Babin M., Seppälä J. and Metsamaa L., Optimization of variable fluorescence measurements of phytoplankton communities with cyanobacteria, *Photosynth. Res.* **112**, 2012, 13–30, https://doi.org/10.1007/s11120-012-9729-6.

Smol J.P. and Stoermer E.F., The Diatoms: Applications for the Environmental and Earth Sciences – Google Livros, second. ed., 2010, Cambridge University Press.

Stauber J., Florence T. and Humphrey F., Mechanism of toxicity of zinc to the marine diatom Nitzschia closterium, *Mar. Biol.* **105**, 1990, 519–524.

Stauber J.L. and Florence T.M., Mechanism of toxicity of ionic copper and copper complexes to algae, *Mar. Biol.* **94**, 1987, 511–519, https://doi.org/10.1007/BF00431397.

Torres M.A., Barros M.P., Campos S.C.G., Pinto E., Rajamani S., Sayre R.T. and Colepicolo P., Biochemical biomarkers in algae and marine pollution: a review, *Ecotoxicol. Environ. Saf.* **71**, 2008, 1–15, https://doi.org/10.1016/j.ecoenv.2008.05.009.

Tripathi B.N., Mehta S.K., Amar A. and Gaur J.P., Oxidative stress in Scenedesmus sp. during short- and long-term exposure to Cu2+ and Zn2+, *Chemosphere* **62**, 2006, 538–544, https://doi.org/10.1016/j.chemosphere.2005.06.031.

Appendix A. Supplementary data

The following are the Supplementary data to this article:

Multimedia Component 1

Supplementary data 1

Graphical abstract

Highlights

- Zn and Cu were toxic to *T. flocculosa* at relevant environmental concentrations.

- Teratological forms were good markers for high concentrations of Zn.

- Increases in cellular Chl *c* content and teratological forms distinguished different Cu exposures.

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Missing references:

-Swedish Environmental Protection Agency, 2000. Lakes and watercourses. Environmental quality criteria. Report 5050. Stockholm. 105 p.

-Luís, A.T., Bonet, B., Corcoll, N., Almeida, S.F.P., Da Silva, E.F., Figueira, E., Guasch, H., 2014. Experimental evaluation of the contribution of acidic pH and Fe concentration to the structure, function and tolerance to metals (Cu and Zn) exposure in fluvial biofilms. Ecotoxicology 23, 1270–1282. doi:10.1007/s10646-014-1270-2

- Rashid, A., Carom, E.L., Ekramoddoullah, A.K.M., 1994. Molecular mechanism of action of Pb 2+ and Zn 2+ on water oxidizing complex of photosystem II, FEBS Letters.

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