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Assessment of five live-cell characteristics in periphytic diatoms as a measure of copper stress

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Journal of Hazardous Materials

Title: Assessment of five live-cell characteristics in periphytic diatoms as a measure of copper stress

Running Head: Periphytic diatoms as potential bioindicators of copper stress

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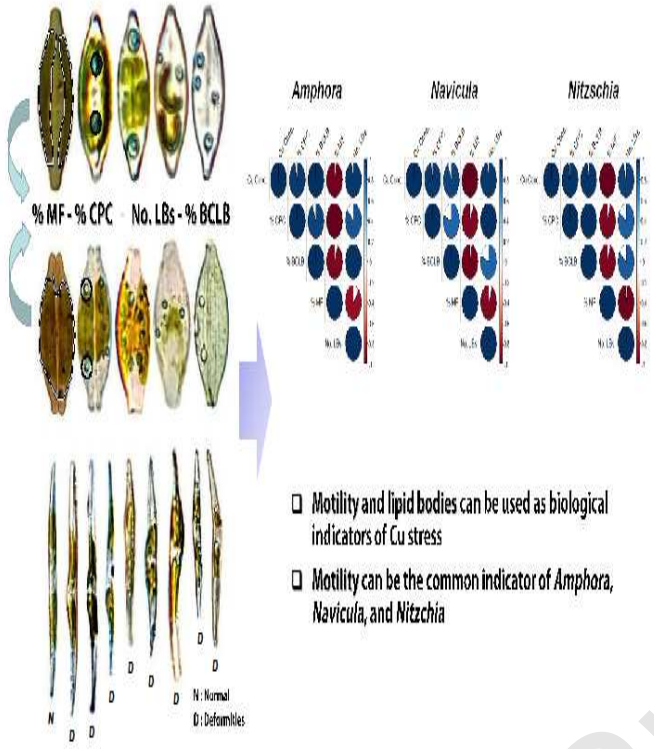
Graphical abstract



Highlights

- Cu stress alters the motility and morphological parameters of diatoms.
- These parameters include protoplasmic content and lipid bodies.
- Motility and lipid bodies can be used as biological indicators of Cu stress.
- Motility can be the common indicator of *Amphora*, *Navicula* and *Nitzchia*.

Abstract

Metal pollution of fluvial systems remains a major problem and biomonitoring can be a useful tool for assessing the metal contamination. To assess their potential as new bioindicators of copper stress, we treated a field-collected live periphytic diatom community (dominated by *Amphora*, *Navicula*, and *Nitzschia*) with dissolved Cu under optimal growth conditions. We studied the effects of Cu on five live-cell attributes: motility, protoplasmic content, lipid body number and biovolume, and frustule morphology. In all three genera, motility and protoplasmic content decreased, whereas the LB number, biovolume and deformity increased when Cu and exposure time increased. The sensitivity to Cu was highest for % MF, % CPC and % BCLB in *Navicula* and the LB number and deformity in *Nitzschia*. *Amphora* appeared to be more tolerant to Cu in comparison with other genera. The five cell attributes were inter-related. A heatmap showed that a recommended indicator for rapid screening of Cu toxicity was % BCLB for *Amphora* and % MF for *Navicula* and *Nitzchia*. % MF might be the most common representative indicator that can be applied to all three genera to evaluate the lethal effects of Cu stress if only one of the five cell attributes must be selected.

Keywords

Deformities; lipid bodies; motility; periphytic diatoms; protoplasmic content

1. Introduction

Periphytic diatoms are an attached, autotrophic, and perennially growing biological assemblage inhabiting various aquatic systems. Diatoms are major contributors to primary productivity and are the basis of the trophic chain in aquatic system. Many diatom species are

cosmopolitan, and because they divide frequently, their populations can respond quickly to stressful conditions (Pandey et al., 2017).

Periphytic diatoms are also of special interest for water quality bioassessment because they have a short generation time, are not costly to collect, and are relatively easy to identify and enumerate (Kim Tiam et al., 2019). For these reasons, diatom-based biological monitoring tools for fluvial ecosystem have been developed and adopted in many countries (Coste et al., 2009; Kelly et al., 1995; Lavoie et al., 2014). However, traditional bioassessment of toxicity using diatoms has several methodological drawbacks, in particular the use of cleared and mounted diatom frustules (the hard, porous silicaceous exterior of diatoms), a time-consuming process requiring expertise. In addition, permanent fixation of diatom frustules can distort crucial features of live diatoms (organic material, motility, protoplast content, and lipid bodies), thereby hindering ecotoxicological studies based on those features. Permanent fixation of diatom frustules may also interfere with the examination of deformed frustules, as only one view of the fixed frustule out of four (two valve and two girdle views) can be prepared.

As a supplement or alternative to traditional cell counting, therefore, live diatom features (e.g., motility, integrity of the protoplasmic content, size and number of lipid bodies, and cell wall deformities) are being explored as biomonitoring tools (Pandey et al., 2017). These newer metrics are quick, easy, require less human expertise, have good reproducibility, have standard protocols and, most importantly, can be adopted world-wide. These parameters are alteration in the cell membrane and cytoplasmic content and change in lipid body formation and frustule deformities.

Diatoms are well known for their transparent silica frustules and, as a result, it is very easy to observe the intracellular changes under light microscopy. Morin et al. (2016) found that under

eutrophication and high amounts of organic matter and heavy metals, high percentage of cytological abnormalities in diatoms for the detection of changes of the water quality associated with toxic pollution.

Diatoms are unique in storing lipids, which occur in intracellular lipid bodies (LBs), as a reserve food material. In diatoms, LBs become more prominent (in terms of number as well as in size) under various types of environmental and anthropogenic disturbances (Gillett et al., 2011; Gillett et al., 2009). The effects of heavy metal stress on LBs are relatively unexplored (Pandey et al., 2015).

Diatoms are well known for their robust, species-specific, ornamented silica frustules, which are replicated from generation to generation (Falasco et al., 2009a). However, these silica frustules are occasionally prone to alteration under various types of environmental (Svensson et al., 2014; Winder et al., 2009) and anthropogenic stress. These alterations include the production of various morphological forms (phenotypic plasticity), size reduction and even frustule deformation (Cox et al., 2012; Kociolek and Stoermer, 2010; Morin et al., 2012). Morphological abnormalities caused by metal pollution have been reported in different classes of algae, but these are especially apparent in diatoms (Falasco et al., 2009a).

Raphid diatoms are known for their motility and show characteristic movements closely related to the shape of their raphe system (Round et al., 2007). Anthropogenic perturbations alter the movement rate and patter of live diatoms. The possible use of diatom motility as a tool for measuring the health of water bodies has been little studied. (Gupta and Agrawal, 2007) reported an inhibitory effect of several metals on the motility rate of two lab cultured diatoms, *Navicula grimmei* and *Nitzschia palea*.

However, the use of these metrics for ecotoxicological and bioassessment testing needs further study and demonstration in order to better determine concentration-dependent and/or time-dependent responses and to gain wider acceptance.

Copper (Cu) is an essential component of enzyme cofactors in photosynthesis, nitrogen fixation, and other processes that result in marine phytoplankton growth, but dissolved Cu concentrations exceeding tolerance levels have toxic effects on phytoplankton (Morel and Price, 2003; Sunda, 1975, 2012). In this study, we assessed the effects of Cu toxicity on a periphytic diatom community under laboratory conditions at multiple cellular levels: i.e., the morphological (deformities), behavioral (motility), and physiological (lipid bodies and alteration in protoplast content) levels. Particular efforts were made to assess the effect of Cu toxicity on the protoplast content of live diatom cells, which has recently been investigated under pesticide treatments (Wood et al., 2014; Wood et al., 2016). We also investigated the relationship between these behavioral and physiological parameters under Cu stress, an aspect that may clarify the pathway of Cu toxicity inside diatom cells.

2. Materials and Methods

2.1. Sample collections

Periphytic diatom communities were collected from wastewater treatment and reclamation plants in Incheon (37°21′56" N, 126°38′23" E), South Korea. Random sampling was performed, and dominant genera were later selected and identified based on studies, conducted by Cox (1996) and Taylor et al. (2007), and the ANSP algal image database. Periphyton samples collected by scraping colonized cobbles with a blade and brush were placed in 50-ml plastic centrifuge tubes. In the laboratory, samples were mixed with 30 mL unfiltered water from the

collection site, shaken, and centrifuged at 3,500 rpm with 3,000 g force for 10 min (Sigma 3-30KS, USA). The supernatant was decanted, and the process was repeated at least three times. Next, the pellet was maintained in 20 mL of unfiltered water from the collection. The experiment started approximately 30 min after the preparation of the pellet.

2.2. Preparation of copper solution

Copper solutions with three different concentrations (1.0, 2.0, and 3.0 mg Cu L^{-1}; pH 7.0–6.8) were prepared by the serial dilution of a standard Cu (1,000 mg L^{-1}) solution (Showa, Japan) with unfiltered water from the collection site. This concentration range was chosen based on the literature relating to Cu contamination in fluvial ecosystems (WHO, 2004). For each investigation, three replicates (500 frustules each) were used for the control and Cu treatments. The current experiments were performed in the laboratory, and the effects of three different concentrations of copper on three major diatom genera were studied for 5 days under optimal environmental conditions for growth (photon flux density 80±3 µmol m^{-2} s^{-1}, light period 12:12 LD, pH 7.8 and temperature 17±1°C) except for copper treatments. Our focus was to determine the effect of copper, not other environmental factors, on the live features of the three diatom groups (*Amphora*, *Navicula*, and *Nitzschia*).

2.3. Parameters

At the start and end of the experiment, community analyses and measurements of diatom characteristics were performed using slides of both living and acid-cleaned diatoms at 400× and 1000× magnification with an Axiostar Plus microscope (Carl Zeiss, Germany). The percentage of motile diatom frustules was determined by counting the number of moving cells among 500 diatoms examined at 400× magnification. Photographs of live diatom frustules were taken at 1000× magnification before and after exposure to Cu solutions. These were used to measure the

contraction of the protoplasmic content relative to the full protoplasmic size (Fig. S1) and determine the changes in the number and biovolume of lipid bodies represented as percentage of cell biovolume (Fig. S2).

Lipid bodies (LBs) were investigated in live diatom cells following the protocols described by Pandey et al. (2015). The size and number of LBs in individual species were measured using a camera and computer attached to a calibrated microscope (Nikon 450) using 400× and 1000× magnifications. The biovolume of each LB was calculated, assuming that the LBs were more or less spherical, so that the mathematical formula for a sphere ($V = 4/3\pi r^3$, where V is the volume and r is the measured radius of an LB) could be applied. The biovolumes of individual diatom species were calculated using geometrical formulae given by Hillebrand et al. (1999). The percent biovolume of all LBs inside individual diatom cells was calculated by summing the volume of each LB (total contribution of all lipid bodies) divided by the cell biovolume.

Numbers of lipid bodies were manually counted in 500 live frustules. The percentage contraction of protoplasmic content (CPC) and percent biovolume contribution of LBs (BCLB) were calculated by measuring the changes in the perimeter of the protoplasmic content (PPC) and lipid bodies (PLB) in the control and metal-treatment groups. Motic software (Motic Microscopes, Hong Kong) was used to measure the sizes of cells and cellular components. The percent contraction of protoplasmic content (% CPC) was calculated using the following equation:

$$\% \text{ CPC} = 100 \times \left(\frac{PPC \text{ in metal treatment}}{PPC \text{ in control}}\right)$$

Similarly, the percent biovolume contribution of lipid bodies (% BCLB) per cell was calculated as follows:

$$\% \text{ BCLB} = 100 \times \left(\frac{PLB\ in\ metal\ treatment}{PLB\ in\ control}\right)$$

Deformities in diatom frustules were investigated in both live and acid-cleaned samples. In live samples, deformities were examined at a 1000× magnification using an oil immersion. To acid-clean diatoms, samples were treated with aqua regia (1:3 (v/v) HNO_3/HCl for 24 h, followed by washing with distilled water until the pH of the decanted water reached 7 (Pandey et al., 2014). Acid-cleaned frustules were examined for deformities using a scanning electron microscope (SEM) (JSM-7001F, USA). Deformed diatomic frustules were divided into four types: (1) deformed valve circumference; (2) distorted striations, non-uniform distribution, uneven and forked striae; (3) raphe modifications; (4) mixed deformations (more than one type of deformation in the same frustule). This categorization is based primarily on reports (Falasco et al., 2009a; Pandey et al., 2015; Pandey et al., 2014). Type 3 deformation (modifications in the central and longitudinal regions), defined by Falasco et al. (2009a, b), was categorized as Type 2 because these modifications were primarily caused by abnormalities in striations.

2.4. Statistical analysis

Data were statistically analyzed by one-way analysis of variance (ANOVA) followed by Tukey's HSD test to compare various means. Using R software (R Development Core Team, 2019), the data on % MF, % CPC, % BLBs, Number of LBs, and Cu concentrations were subjected to Pearson correlation analysis to determine the dependent variables that correlated directly with the proposed treatments. The "ggplot2" package was accessed to create the heat map using the "cor" functions to generate the coefficient matrices. To facilitate the visualization of correlations, correlation coefficients were inserted into heatmap cells. Pearson's correlation analysis were performed by the XLSTAT software (Microsoft Corporation, USA).

3. Results and Discussion

3.1. Frustule motility

For all three diatom genera, the percentages of motile frustules decreased when the concentrations of Cu and exposure time increased (Fig. 1). Among the three genera, *Navicula* sp. was the most sensitive and *Amphora* sp. the least sensitive to Cu toxicity.

Motility is a unique feature of raphid diatoms, which show characteristic motility patterns that depend upon the shape of the raphe (straight, curved, or sigmoid) in each species (Round et al., 2007). Altered motility in diatoms has been reported under stresses associated with nutrient levels, temperature, water availability, light intensity, UV, pH, and organic substances (Coquillé et al., 2015; Gupta and Agrawal, 2007; Svensson et al., 2014), but only a few studies have addressed the effects of heavy metal stress. Gupta and Agrawal (2007) found that a Cu concentration range from 1–200 mg L^{-1} produced an inhibitory response in terms of the percentage of gliding cells and cell gliding period in *Navicula grimmei* and *Nitzschia palea*. Swaying (turning at either end of the cell) in *Nitzschia palea* stopped completely (compared to 2–3 turns per min in untreated cells). This reduced total motility is probably due to alterations of the normal motility patterns (straight for *Navicula* sp., curved for *Nitzschia* sp., and sigmoid for *Gyrosigma* sp.). For example, Pandey and Bergey (2016) reported slow, jerky motility in diatom species sampled from severely impacted (by Cu and Zn) sites than in those from less-impacted sites in Rajasthan, India. The exact mechanism of motility reduction in diatoms under Cu exposure is not known, but it may be due to disruption of the cytoplasmic homeostasis and cytoskeletal system (actin and myosin filaments) of diatom cells, which ultimately leads to slow and altered motility patterns (Pinto et al., 2003).

3.2. Protoplasmic content

All three diatom genera showed a higher percentage contraction of protoplasmic content (% CPC) under Cu treatments when compared to the respective controls over the course of the experiment (Fig. 2). The effect was concentration- and time-dependent, with a greater reduction in the protoplasmic content at higher Cu concentrations and after longer exposures. *Amphora* sp. and *Nitzschia* sp. were the most and least sensitive to Cu toxicity, respectively.

Wood et al. (2014, 2016) developed an effective method for testing the toxicity of the pesticide atrazine by measuring changes in the condition of the protoplasm in the diatom genera *Navicula*, *Gomphonema*, *Ulnaria*, *Achnanthidium*, *Cymbella*, *Amphora*, and *Eunotia* after 48 h of pesticide treatment. A healthy condition was represented by an intact protoplasm, whereas empty silica frustules indicated an extremely unhealthy condition. This methodology is fast and requires no taxonomic expertise to relate diatom health to the toxicological status of the waters they inhabit.

The exact mechanism of alteration of the diatom protoplasmic contents is uncertain, but Cu is well known to be a redox-active metal, and inside algal cells it causes membrane destruction through generation of reactive oxygen species (Tripathi and Gaur, 2004). Pinto et al. (2003) reported that heavy metals also cause cytoplasmic acidification and membrane depolarization, and thereby disturb the cytoplasmic homeostasis of cells. This may be the cause of the deterioration of the protoplasmic content in diatom cells under Cu exposure. The percentage contraction in protoplasmic content (% CPC) represents a new and important index of the physiological condition of live diatoms and the health status of a diatom community, and may be a useful tool for ecotoxicological studies of these algae.

3.3. Number and biovolume of lipid bodies

It was notable that number of LBs were significantly higher in *Nitzschia* exposed to Cu of higher than 2 mg L^{-1} for only 3 days, whereas a similar toxicity was recorded for the other two genera after exposure to 3 mg L^{-1} (Fig. 3). The NLBs were the highest (4) in *Amphora* sp. (4) although the percent increase in NLBs after the Cu exposure was the highest in *Nitzschia* sp. (%).

In all three genera, after 4 days, the percentage biovolume contribution of lipid bodies (% BCLB) increased when Cu concentrations and exposure time increased (Fig. 4). Diatoms characteristically store lipid bodies as a reserve food material, and these bodies may be more prominent under various types of stress (Ramachandra et al., 2009), particularly nitrogen starvation (Jiang et al., 2012). In our experiment, lipid body induction was significantly greater (in both number and biovolume) in the genera *Navicula* and *Nitzschia* under Cu stress, a result consistent with previous studies (Pandey and Bergey, 2016; Pandey et al., 2015). A higher induction of lipid bodies in *Amphora* under Cu stress is reported here for the first time. Gautam et al. (2017) also reported increased lipid body induction in *Gomphonema pseudoaugur* collected from bodies of water contaminated with heavy metals (Cu, Zn, Ni, Cd, and Se) in Haryana, India. In a laboratory study, lipid content in the diatom *Haslea ostrearia* was higher under Cu stress than in the control treatment, but the difference was not statistically significant (Joux-Arab et al., 2000). Lelong et al. (2013) found increased lipid content in Cu-starved cells of *Pseudo-nitzschia* but slightly decreased lipid content under combined Cu and Fe limitation. Stress does not always induce lipid body formation: organic Hg and Cd stress resulted in lower fatty acid and sterol content in the marine diatom *Asterionella glacialis* (Jones et al., 1987). In general, the induction of lipid bodies in diatom frustules under heavy metal stress is not well understood, but it may be related to evolutionary events (endosymbiosis) through which diatoms have acquired unique sets of metabolic and regulatory genes that have facilitated their colonization of aquatic

habitats with wide ranges of temperature, pH, and nutrient availability (Vinayak et al., 2015). This colonization ability reflects a very flexible metabolism that allows diatoms to adapt to environmental and anthropogenic constraints (Masmoudi et al., 2013; Roháček et al., 2014). Such adaptation often involves metabolic shifts resulting in the production of secondary metabolites such as carotenoids and/or lipids (Bertrand, 2010; Darko et al., 2014; Ramachandra et al., 2009).

Lipid body induction is a newly identified and important parameter reflecting the physiological condition of live diatoms. It provides information on the health status of a diatom community, and has potential application to ecotoxicological studies of these algae.

3.4. Deformities

We examined the diatoms of the three genera in the control and Cu-treated samples for deformities after 5 days of incubation (Figs. S3 and S4). *Nitzschia* sp. showed the highest frequency of deformed frustules. The effect was concentration dependent, with significantly higher percentages of deformed frustules in the medium (2%) and high (8%) Cu concentrations than in the control (0.5%). For *Navicula* sp. and *Amphora* sp., the frequency of deformed frustules in the Cu treatment groups did not differ significantly from those of the control groups (Fig. S5). We also examined the relative proportions of various deformity types in the three tested genera (Table 1). In the controls, only Type 1 (valve outline) deformity was found in all three genera. Additional deformity types were found in the Cu treatment groups, namely, Type 2 (striation pattern) in *Navicula* sp. and Types 3 (deformed raphe) and 4 (mixed deformity) in *Nitzschia* sp. Deformities commonly do not occur evenly among diatom taxa, and here the percentage of deformed frustules was increased by Cu stress only in the genus *Nitzschia*.

Deformities can be classified by type, and whereas deformities of the frustule outline (Type 1) were the most common in controls, additional deformity types (i.e., deformity in striae = Type 2 and in the raphe = Type 3, and mixed deformities = Type 4) were found under Cu stress. Similarly, Pandey et al. (2014, 2015) and Pandey and Bergey (2016) reported multiple types of deformities in Cu-stressed *Nitzschia* in both laboratory and field studies. Although the exact mechanism of induction of abnormalities in diatom cells is not clearly understood, it may be related to the impairment of silica uptake and its subsequent deposition into the cell wall. Inhibition of silica uptake due to binding of metal ions to sulphydryl groups of the cell membrane has been suggested by Fisher et al. (1981). It has also been proposed that metal ions may harm the microtubular system required for the movement of silica toward silica deposition vesicles (Falasco et al., 2009a). Notwithstanding the underlying causes, the present study makes a strong case for the use of morphological abnormalities in diatoms in biomonitoring of metal pollution in fluvial ecosystems.

The low percentage of deformed frustules even under stress conditions is a major concern in assessing the use of deformities as a biomonitoring tool. The incorrect identification of diatom species and the lack of proper training to distinguish the deformed frustules from normal ones are the main reasons for lower counts of deformity in diatom assemblages. In addition, permanent slide preparation results in an inability to examine all sides of individual diatoms for deformities. Examining all four views (two valve and two girdle views) of diatom frustules would almost certainly increase the percentage of deformed cells counted in samples with deformities.

3.5. Relationships among parameter endpoints

We compared the values of four live periphytic diatom parameters (percentage motile frustules, percent contraction of protoplasmic content, number of lipid bodies, and percent biovolume contribution of lipid bodies) in the three genera exposed to three different concentrations of Cu for 5 days to determine their inter-relationships (Fig. 5). Under Cu stress, the alteration of the protoplasmic content and lipid bodies (number and biovolume) increased, whereas the frustule motility decreased gradually over the experimental period. The cellular mechanisms involved in the morphological and behavioral changes reported in this study are still poorly understood. These responses are likely to be interrelated and connected to changes in the diatom cytoskeleton (microtubules, actin filaments, and microfilaments). Poulsen et al. (1999) found that motile diatoms (*Craticula* sp., *Nitzschia* sp., *Pinnularia* sp., and *Craspedostauros australis*) treated with latrunculin (an anti-actin drug) and butanedione monoxide (an anti-myosin drug) lost their motility, suggesting that actin-myosin proteins are essential for the movement of diatoms. In the same study, at high concentrations of nocodazole (which affects the polymerization of microtubules), a significant proportion of treated cells showed contracted chloroplasts and vesiculation of the protoplasm, thereby indicating unhealthy conditions or even cell death (Poulsen et al., 1999). Desiccation and the resulting ionic imbalance cause contraction of protoplasts, which may eventually disintegrate (Schmid, 1979). Treatment with microtubule inhibitors can also produce abnormal diatom frustules (Duke and Reimann, 1977; Lee and Li, 1992). Alternatively, Rijstenbil et al. (1994), suggested that Cu may produce lipid peroxidation effects on membranes, which could induce frustule deformities.

In general, heavy metals in algal cells appear to alter membrane polarity, causing cytoplasmic acidification, which ultimately leads to the disruption of cytoplasmic homeostasis (Pinto et al., 2003). In diatoms, cellular imbalance due to Cu stress results in cytoskeleton poisoning, affecting

microfilaments and microtubules. Consequently, a cascade of events inside the cell is progressively manifested as alterations in motility, protoplasma, lipid bodies, and diatom morphology. Pearson's correlation analysis (Fig. 6) showed that the percent contraction of protoplasmic content ($r^2 > 0.92$), lipid body number ($r^2 > 0.88$), and lipid body biovolume ($r^2 > 0.88$) were positively correlated ($r^2 > 0.88$). Conversely, frustule motility was negatively correlated ($r^2 > –0.891$) with Cu concentrations. It was also notable that a similar strength of correlation was displayed among various indicators after exposure for longer than 4 days.

4. Conclusion

Our study offers new insight into the use of live periphytic diatoms as tools to assess the biological effects of Cu stress using the motility, number, and biovolume of lipid bodies and frustule deformities. The sensitivity to Cu was highest for % MF, % CPC, and % BCLB in *Navicula* sp. and the number of lipid bodies and deformity in *Nitzschia* sp. Furthermore, *Amphora* sp. appeared to be more tolerant to Cu when compared with other genera. However, there are some controversies regarding the segregation of metal-tolerant and sensitive species in diatoms. Duong et al. (2008) reported different sensitivities in *Ulnariaulna*, depending on seasonal variability. *Achnanthidium minutissimum*, which is frequently dominant in lotic environments subjected to toxic events, is generally considered an indicator of metal pollution (Cattaneo et al., 2004; Stevenson and Bahls, 1999; Takamura et al., 1990); however, it also indicates good general water quality (e.g., Coste et al., 2009; Lavoie et al., 2006).

The common rapid response to Cu exposure and close inter-relationships among the five cell attributes indicate that they can be used as integrated bioindicators. These new parameters are relatively easy, quick, and inexpensive to measure, and can be applied globally, with no need for

expertise in diatom taxonomy. Therefore, these new non-taxonomical metrics reported in the present study can effectively deal with the shortcomings of traditional metrics while ensuring compliance to the globally accepted protocols. Furthermore, non-taxonomic metrics allow for comparisons across sites and even geographical locations (countries and continents) because they are independent of taxonomic similarity or differences. Thus, the refinement in current protocols, particularly through building an integrated index to evaluate Cu toxicity, can improve the feasibility of the current methods and widen their applicability across locations globally as well as improve bioassessment and ecotoxicological assessment studies of diatoms and contribute to the development of efficient and effective biomonitoring strategies. Furthermore, % BCLB for *Amphora* and % MF for *Navicula* and *Nitzschia* can be the recommended indicators for the rapid screening of Cu toxicity. Because % MF is also the second recommended indicator for *Amphora*, it can be used as the common indicator of all three genera to evaluate the lethal effects of Cu stress. The exposure time for the testing based on the proposed cell attributes was at least 4 days. This testing method will be interesting in that slow diatoms undergo fast environmental screening.

Author statement

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Declaration of interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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References

Bertrand, M., 2010. Carotenoid biosynthesis in diatoms. Photosynth. Res. 106, 89-102.

Cattaneo, A., Couillard, Y., Wunsam, S., Courcelles, M., 2004. Diatom taxonomic and morphological changes as indicators of metal pollution and recovery in Lac Dufault (Québec, Canada). J. Paleolimnol. 32, 163-175.

Coquillé, N., Jan, G., Moreira, A., Morin, S., 2015. Use of diatom motility features as endpoints of metolachlor toxicity. Aquat. Toxicol. 158, 202-210.

Coste, M., Boutry, S., Tison-Rosebery, J., Delmas, F., 2009. Improvements of the Biological Diatom Index (BDI): Description and efficiency of the new version (BDI-2006). Ecol. Indic. 9, 621-650.

Cox, E.J., 1996. Identification of Freshwater Diatoms From Live Material: Chapman & Hall London. Weinheim, New York, Tokyo, Melboum, Madras.

Cox, E.J., Willis, L., Bentley, K., 2012. Integrated simulation with experimentation is a powerful tool for understanding diatom valve morphogenesis. Biosystems 109, 450-459.

Darko, E., Heydarizadeh, P., Schoefs, B., Sabzalian, M.R., 2014. Photosynthesis under artificial light: the shift in primary and secondary metabolism. Philos. Trans.R. Soc. B Biol. Sci. 369, 20130243.

Duke, E.L., Reimann, B.E.F., 1977. The ultrastructure of the diatom cell, in: Werner, D. (Ed.), The biology of diatoms. Blackwell Scientific Publications, Oxford, UK, pp. 13-45.

Duong, T.T., Morin, S., Herlory, O., Feurtet-Mazel, A., Coste, M., Boudou, A., 2008. Seasonal effects of cadmium accumulation in periphytic diatom communities of freshwater biofilms. Aquat. Toxicol. 90, 19-28.

Falasco, E., Bona, F., Badino, G., Hoffmann, L., Ector, L., 2009a. Diatom teratological forms and environmental alterations: a review. Hydrobiologia 623, 1-35.

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

Fisher, N.S., Jones, G.J., Nelson, D.M., 1981. Effects of copper and zinc on growth, morphology, and metabolism of *Asterionella japonica* (Cleve). J. Exp. Mar. Biol. Ecol. 51, 37-56.

Gautam, S., Pandey, L.K., Vinayak, V., Arya, A., 2017. Morphological and physiological alterations in the diatom *Gomphonema pseudoaugur* due to heavy metal stress. Ecol. Indic. 72, 67-76.

Gillett, N.D., Pan, Y., Manoylov, K.M., Stevenson, R.J., 2011. The role of live diatoms in bioassessment: a large-scale study of Western US streams. Hydrobiologia 665, 79-92.

Gillett, N.D., Pan, Y., Parker, C., 2009. Should only live diatoms be used in the bioassessment of small mountain streams? Hydrobiologia 620, 135-147.

Gupta, S., Agrawal, S., 2007. Survival and motility of diatoms *Navicula grimmei* and *Nitzschia palea* affected by some physical and chemical factors. Folia Microbiol. 52, 127-134.

Hillebrand, H., Dürselen, C.D., Kirschtel, D., Pollingher, U., Zohary, T., 1999. Biovolume calculation for pelagic and benthic microalgae. J. Phycol. 35, 403-424.

Jiang, Y., Yoshida, T., Quigg, A., 2012. Photosynthetic performance, lipid production and biomass composition in response to nitrogen limitation in marine microalgae. Plant Physiol. Biochem. 54, 70-77.

Jones, G.J., Nichols, P.D., Johns, R., Smith, J.D., 1987. The effect of mercury and cadmium on the fatty acid and sterol composition of the marine diatom *Asterionella glacialis*. Phytochemistry 26, 1343-1348.

Joux-Arab, L., Berthet, B., Robert, J.-M., 2000. Do toxicity and accumulation of copper change during size reduction in the marine pennate diatom Haslea ostrearia? Mar. Biol. 136, 323-330.

Kelly, M., Penny, C., Whitton, B., 1995. Comparative performance of benthic diatom indices used to assess river water quality. Hydrobiologia 302, 179-188.

Kim Tiam, S., Lavoie, I., Liu, F., Hamilton, P.B., Fortin, C., 2019. Diatom Deformities and Tolerance to Cadmium Contamination in Four Species. Environments 6, 102.

Kociolek, J., Stoermer, E., 2010. Variation and polymorphism in diatoms: the triple helix of development, genetics and environment. A review of the literature. Vie Milieu 60, 75-87.

Lavoie, I., Campeau, S., Grenier, M., Dillon, P.J., 2006. A diatom-based index for the biological assessment of eastern Canadian rivers: an application of correspondence analysis (CA). Can. J. Fish. Aquat. Sci. 63, 1793-1811.

Lavoie, I., Campeau, S., Zugic-Drakulic, N., Winter, J.G., Fortin, C., 2014. Using diatoms to monitor stream biological integrity in Eastern Canada: an overview of 10 years of index development and ongoing challenges. Sci. Total Environ. 475, 187-200.

Lee, M., Li, C.-W., 1992. The origin of the silica deposition vesicle of diatoms. Bot. Bull. Acad. Sinica 33, 317-325.

Lelong, A., Bucciarelli, E., Hégaret, H., Soudant, P., 2013. Iron and copper limitations differently affect growth rates and photosynthetic and physiological parameters of the marine diatom Pseudo□nitzschia delicatissima. Limnol. Oceanogr. 58, 613-623.

Masmoudi, S., Nguyen-Deroche, N., Caruso, A., Ayadi, H., Morant-Manceau, A., Tremblin, G., 2013. Cadmium, copper, sodium and zinc effects on diatoms: from heaven to hell—a review. Cryptogamie, Algologie 34, 185-225.

Morel, F.M., Price, N., 2003. The biogeochemical cycles of trace metals in the oceans. Science 300, 944-947.

Morin, S., Cordonier, A., Lavoie, I., Arini, A., Blanco, S., Duong, T.T., Tornés, E., Bonet, B., Corcoll, N., Faggiano, L., 2012. Consistency in diatom response to metal-contaminated environments, Emerging and priority pollutants in rivers. Springer, pp. 117-146.

Morin, S., Gómez, N., Tornés, E., Licursi, M., Rosebery, J., 2016. Benthic diatom monitoring and assessment of freshwater environments: standard methods and future challenges. Aquatic Biofilms, 111.

Pandey, L.K., Bergey, E.A., 2016. 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, 372-381.

Pandey, L.K., Bergey, E.A., Lyu, J., Park, J., Choi, S., Lee, H., Depuydt, S., Oh, Y.-T., Lee, S.-M., Han, T., 2017. The use of diatoms in ecotoxicology and bioassessment: Insights, advances and challenges. Water Res. 118, 39-58.

Pandey, L.K., Han, T., Gaur, J., 2015. Response of a phytoplanktonic assemblage to copper and zinc enrichment in microcosm. Ecotoxicology 24, 573-582.

Pandey, L.K., Kumar, D., Yadav, A., Rai, J., Gaur, J., 2014. Morphological abnormalities in periphytic diatoms as a tool for biomonitoring of heavy metal pollution in a river. Ecol. Indic. 36, 272-279.

Pinto, E., Sigaud□kutner, T.C., Leitao, M.A., Okamoto, O.K., Morse, D., Colepicolo, P., 2003. Heavy metal–induced oxidative stress in algae. J. Phycol. 39, 1008-1018.

Poulsen, N.C., Spector, I., Spurck, T.P., Schultz, T.F., Wetherbee, R., 1999. Diatom gliding is the result of an actin□myosin motility system. Cell Motil. Cytoskel. 44, 23-33.

Ramachandra, T., Mahapatra, D.M., Gordon, R., 2009. Milking diatoms for sustainable energy: biochemical engineering versus gasoline-secreting diatom solar panels. Ind. Eng. Chem. Res. 48, 8769-8788.

R Development Core Team, 2019. R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. http://www.r-project.org/index.html.

Rijstenbil, J., Derksen, J., Gerringa, L., Poortvliet, T., Sandee, A., Van den Berg, M., Van Drie, J., Wijnholds, J., 1994. 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, 583-590.

Roháček, K., Bertrand, M., Moreau, B., Jacquette, B., Caplat, C., Morant-Manceau, A., Schoefs, B., 2014. Relaxation of the non-photochemical chlorophyll fluorescence quenching in diatoms: kinetics, components and mechanisms. Philos. Trans. R. Soc. Lond. B Biol. Sci. 369, 20130241.

Round, F.E., Crawford, R.M., Mann, D.G., 2007. Diatoms: biology and morphology of the genera. Cambridge university press.

Schmid, A.-M.M., 1979. Influence of environmental factors on the development of the valve in diatoms. Protoplasma 99, 99-115.

Stevenson, R.J., Bahls, L.L., 1999. Periphyton protocols. Rapid bioassessment protocols for use in wadeable streams and rivers: periphyton, benthic macroinvertebrates, and fish. EPA.

Sunda, W.G., 1975. The relationship between cupric ion activity and the toxicity of copper to phytoplankton. Massachusetts Institute of Technology.

Sunda, W.G., 2012. Feedback interactions between trace metal nutrients and phytoplankton in the ocean. Front. Microbiol. 3, 204.

Svensson, F., Norberg, J., Snoeijs, P., 2014. Diatom cell size, coloniality and motility: trade-offs between temperature, salinity and nutrient supply with climate change. PLoS One 9.

Takamura, N., Hatakeyama, S., Sugaya, Y., 1990. Seasonal changes in species composition and production of periphyton in an urban river running through an abandoned copper mining region. Jap. J. Limnol. 51, 225-235.

Taylor, J., van Vuuren, M.J., Pieterse, A., 2007. The application and testing of diatom-based indices in the Vaal and Wilge Rivers, South Africa. Water SA 33, 51-60.

Tripathi, B.N., Gaur, J., 2004. Relationship between copper-and zinc-induced oxidative stress and proline accumulation in *Scenedesmus* sp. Planta 219, 397-404.

Vinayak, V., Manoylov, K.M., Gateau, H., Blanckaert, V., Hérault, J., Pencréac'h, G., Marchand, J., Gordon, R., Schoefs, B., 2015. Diatom milking: a review and new approaches. Mar. Drugs 13, 2629-2665.

WHO, 2004. Copper in drinking-water: background document for development of WHO guidelines for drinking-water quality [Internet]. World Health Organization, WHO/SDE/WSH/03.04/88.

Winder, M., Reuter, J.E., Schladow, S.G., 2009. Lake warming favours small-sized planktonic diatom species. Proc. Biol. Sci. 276, 427-435.

Wood, R.J., Mitrovic, S.M., Kefford, B.J., 2014. Determining the relative sensitivity of benthic diatoms to atrazine using rapid toxicity testing: a novel method. Sci. Total Environ. 485, 421-427.

Wood, R.J., Mitrovic, S.M., Lim, R.P., Kefford, B.J., 2016. How benthic diatoms within natural communities respond to eight common herbicides with different modes of action. Sci. Total Environ. 557, 636-643.

Figure legends

Figure 1. % MF in the genera *Navicula*, *Nitzschia*, and *Amphora* exposed to low (1.0 mg Cu L^{-1}), medium (2.0 mg Cu L^{-1}), or high (3.0 mg Cu L^{-1}) concentrations of Cu for 5 days. Control, 0.5 mg Cu L^{-1}. Bars bearing different lower-case letters are significantly different from each other ($P < 0.05$, Tukey's HSD test).

Figure 2. % CPC in the genera *Navicula*, *Nitzschia*, and *Amphora* exposed to low (1.0 mg Cu L^{-1}), medium (2.0 mg Cu L^{-1}), or high (3.0 mg Cu L^{-1}) concentrations of Cu for 5 days. Control, 0.5 mg Cu L^{-1}. Bars bearing different lower-case letters are significantly different from each other ($P < 0.05$, Tukey's HSD test).

Figure 3. No. LBs in the genera *Navicula*, *Nitzschia*, and *Amphora* exposed to low (1.0 mg Cu L^{-1}), medium (2.0 mg Cu L^{-1}), or high (3.0 mg Cu L^{-1}) concentrations of Cu for 5 days. Control, 0.5 mg Cu L^{-1}. Bars bearing different lower-case letters are significantly different from each other ($P < 0.05$, Tukey's HSD test).

Figure 4. % BCLBs in the genera *Navicula*, *Nitzschia*, and *Amphora* exposed to low (1.0 mg Cu L^{-1}), medium (2.0 mg Cu L^{-1}), or high (3.0 mg Cu L^{-1}) concentrations of Cu for 5 days. Control, 0.5 mg Cu L^{-1}. Bars bearing different lower-case letters are significantly different from each other ($P < 0.05$, Tukey's HSD test).

Figure 5. Relationships between percent of motile frustules, percent decrease in protoplasmic content, number of lipid bodies, and percent biovolume contribution of lipid bodies in the genera *Navicula*, *Nitzschia*, and *Amphora* at the highest Cu concentration (3.0 mg L^{-1}) used in the experiments.

Figure 6. Heatmap of the Pearson correlation coefficients obtained from motile frustules, percent decrease in protoplasmic content, number of lipid bodies, and percent biovolume contribution of lipid bodies. The numbers in the heatmap cells indicate the correlation coefficients.

Figure 1.

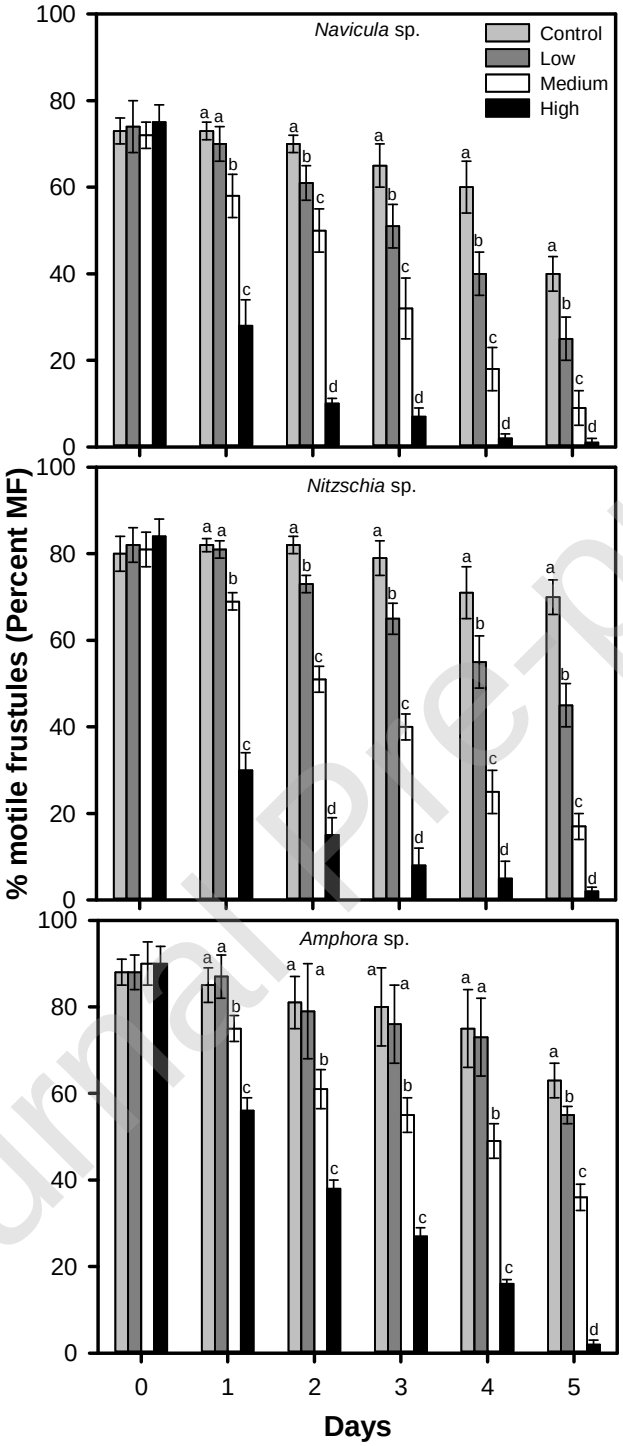

Figure 2.

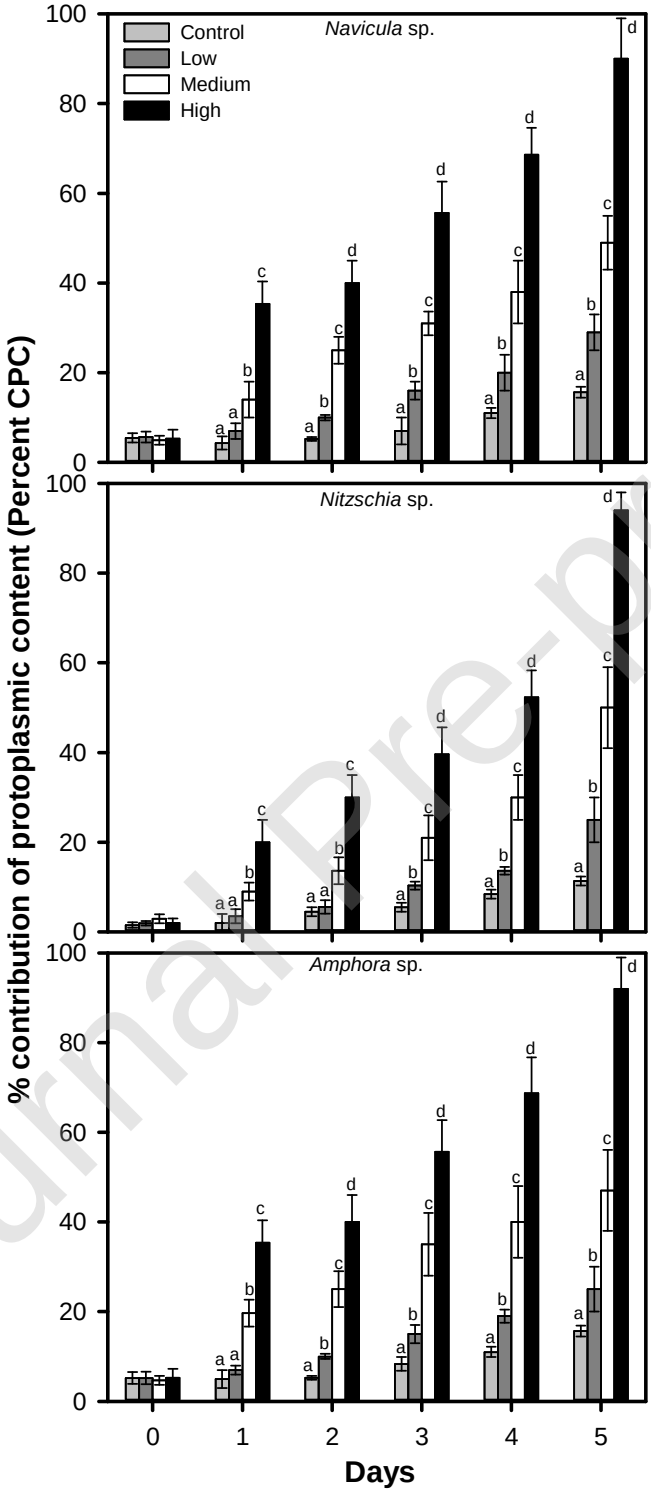

Figure 3.

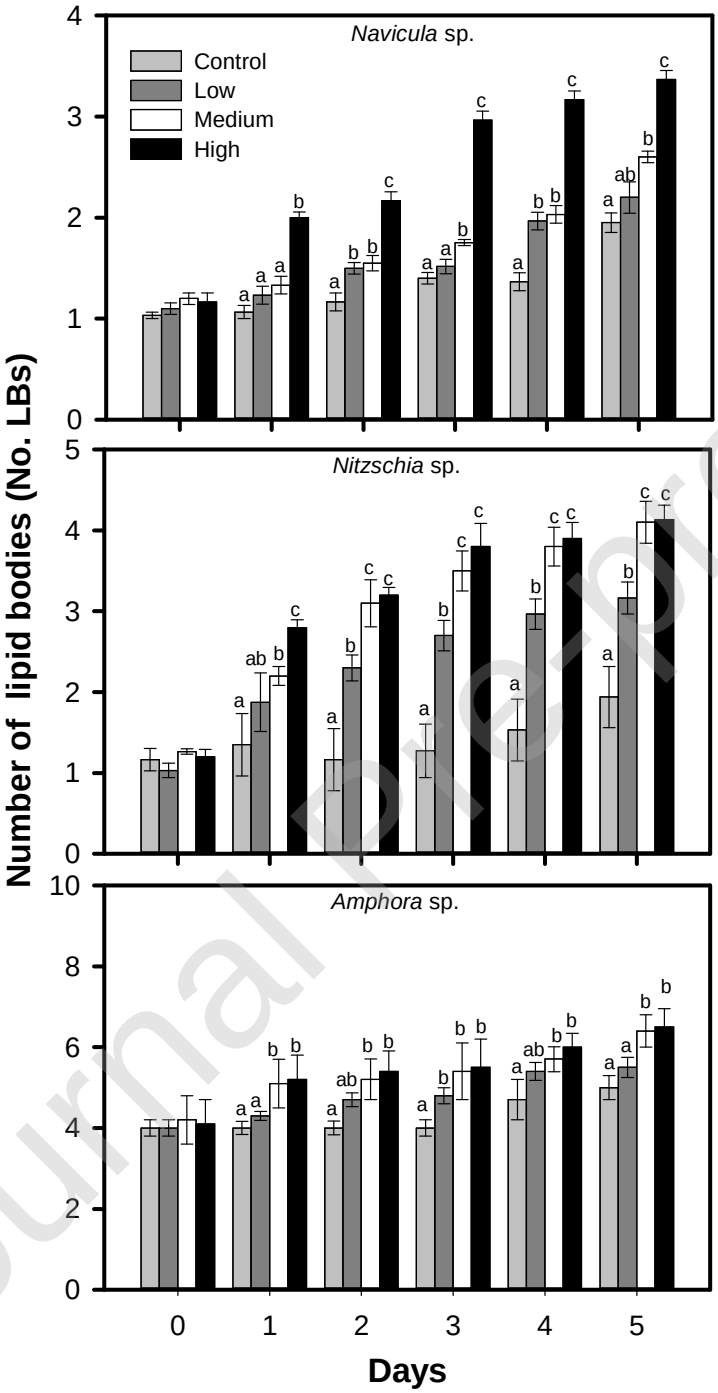

Figure 4.

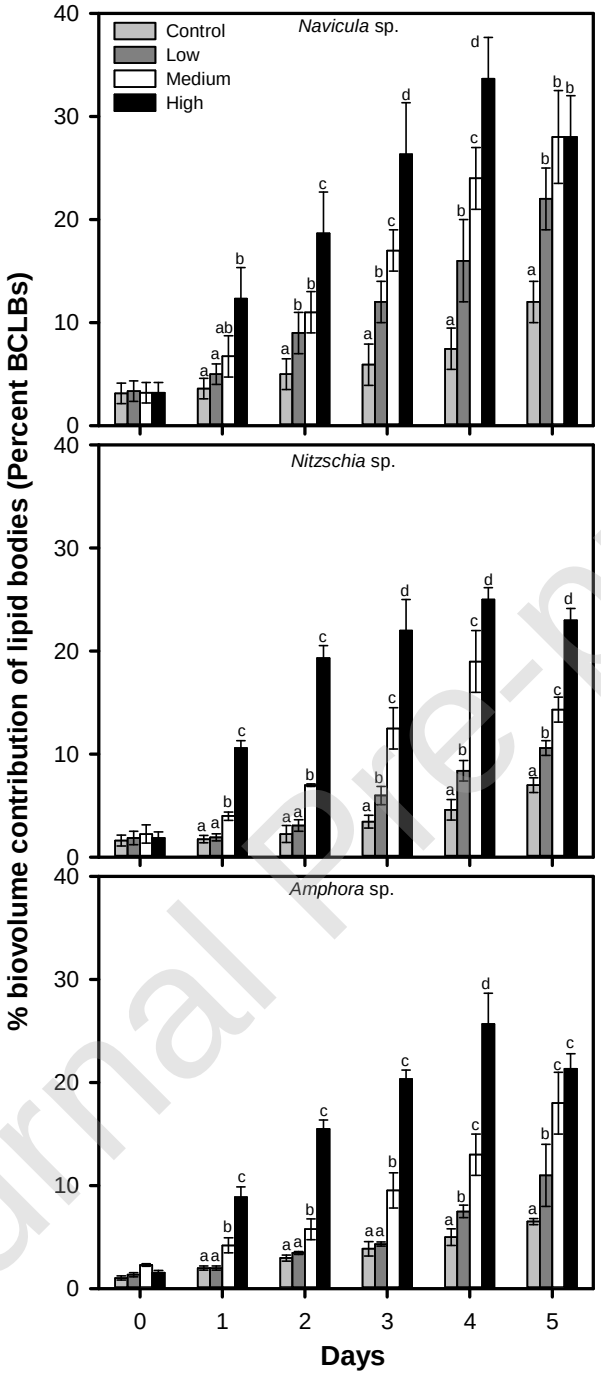

Figure 5.

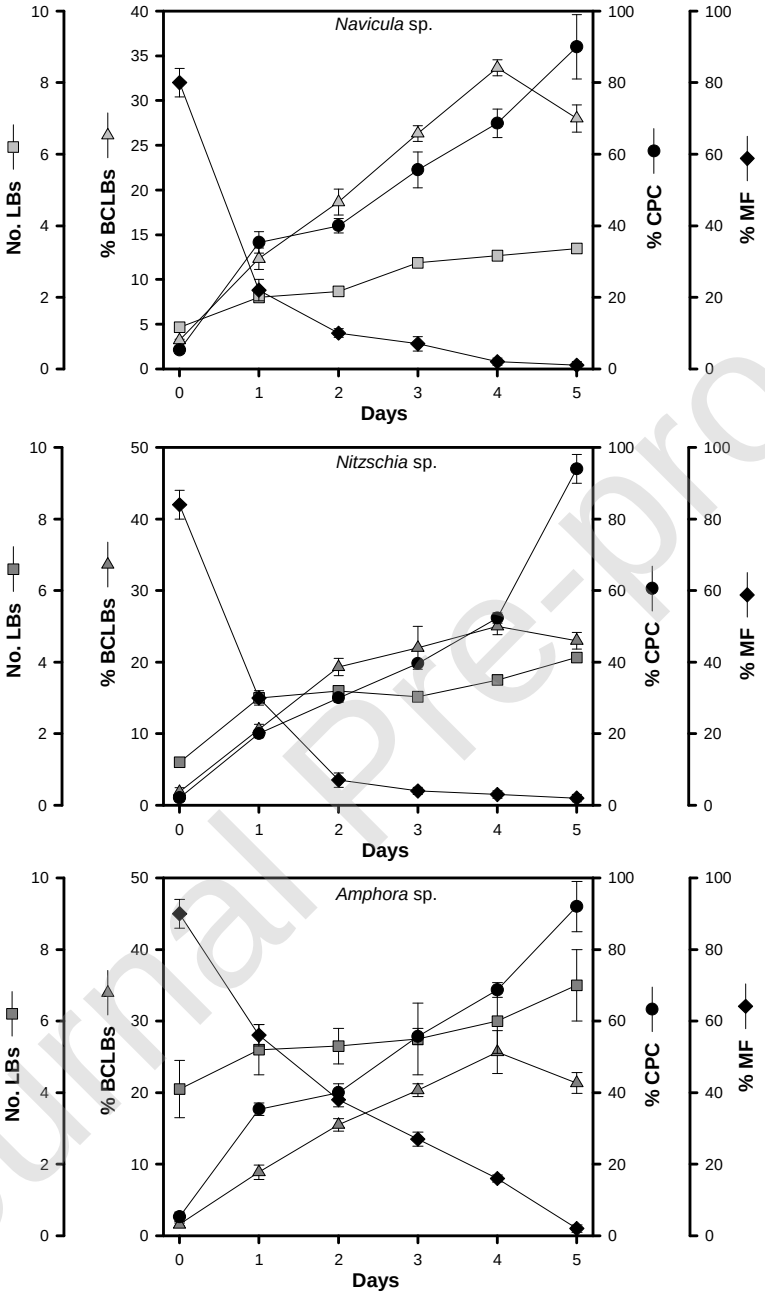

Figure 6.

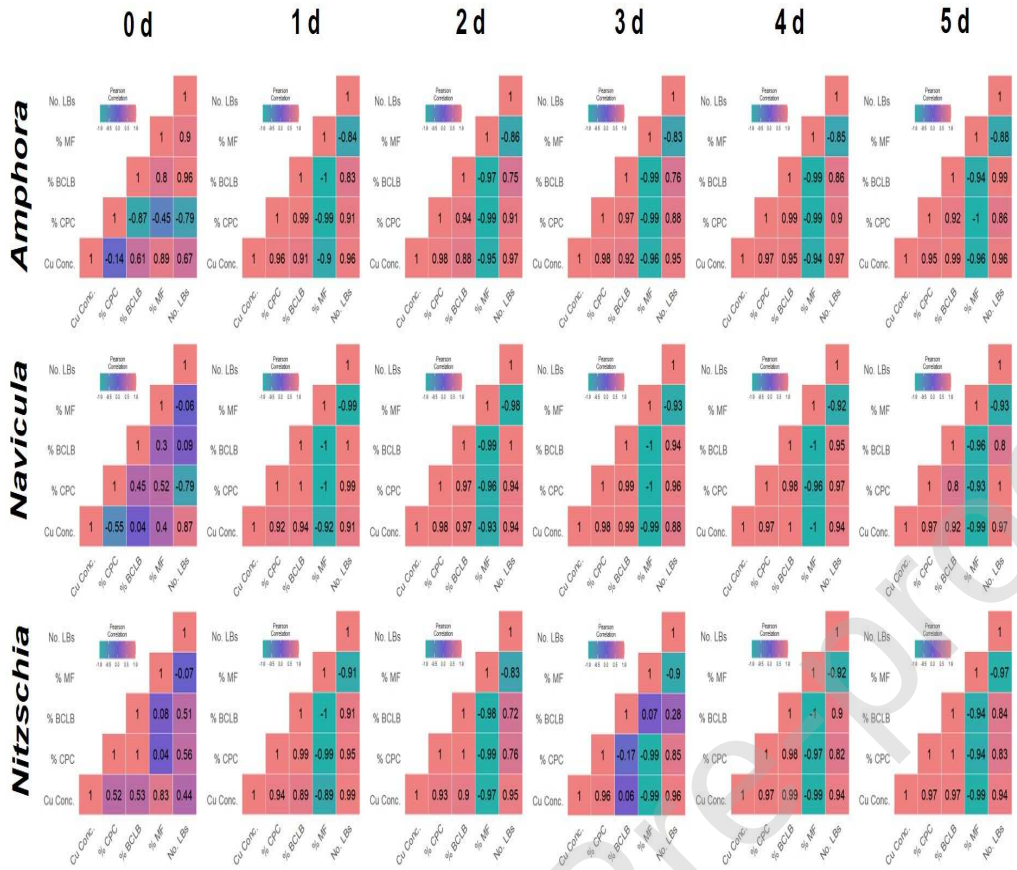

Table 1. Frequencies of the four deformity types in the three diatom genera after incubation with Cu.

		% deformity types			
		Deformed valve (Type 1)	**Deformed striations pattern (Type 2)**	**Deformed raphe (Type 3)**	**Mixed (Type 4)**
Navicula sp.	Control (0.89)	100 (0.89)	0	0	0
	3 ppm (1.20)	50 (0.80)	50 (0.40)	0	0
Nitzschia sp.	Control (0.69)	100 (0.69)	0	0	0
	3 ppm (8.00)	50 (5.00)	0	25 (1.50)	25 (1.50)
Amphora sp.	Control (0.70)	100 (0.70)	0	0	0
	3 ppm (0.70)	100 (0.70)	0	0	0

Shown are the percentage occurrence of each deformity type in the three genera after 5 days of incubation in 1.0 mg Cu L^{-1}, and in the control treatment. Numbers in parentheses show crude values of total deformed frustules (%) counted in the permanent slides.