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Effects of zinc on *in vivo* fluorescence, chlorophyll *a* and growth of the diatom *Conticribra weissflogii* (Thalassiosirales, Thalassiosiraceae)

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Abstract: Diatoms are vital primary producers in marine ecosystems, and their growth may be affected by harmful concentrations of zinc in the environment. However, limited information exists on the response of some important physiological parameters to zinc as contaminant. In the present study, we evaluated the effect of zinc exposure on growth, chlorophyll *a* and *in vivo* fluorescence of the diatom *Conticribra weissflogii* under laboratory conditions. Cell numbers were significantly reduced after 48 h exposure to 450 and 900 µg Zn L^{-1}. Growth rates estimated after 96 h exposure (in div day^{-1}) were 0.81, 0.85, 0.69 and 0.59 for cells exposed to control, 90, 450 and 900 µg Zn L^{-1}, respectively, and it was significantly reduced at 900 µg Zn L^{-1}. There was a tendency of increase in chlorophyll *a* per cell after 3 h exposure to zinc. However, the ratio of *in vivo* fluorescence to chlorophyll *a* strongly decreased after 3 h exposure to zinc, suggesting that the mechanism of toxicity would involve a zinc-induced damage to the photosystem II. Possible metabolic routes for this damage and its environmental implications are discussed.

Key words: *Conticribra weissflogii;* ecotoxicology; metal; microalgae

Resumo: **Efeitos do zinco sobre fluorescência *in vivo*, clorofila *a* e crescimento da diatomácea *Conticribra weissflogii* (Thalassiosirales, Thalassiosiraceae).** Diatomáceas são de importância vital para a produção primária em ecossistemas marinhos, e seu crescimento pode ser afetado pela poluição por zinco. Entretanto, existe limitada informação sobre a resposta de importantes parâmetros fisiológicos após a exposição a este contaminante. Assim, o presente estudo avaliou o efeito da exposição ao zinco no crescimento, clorofila *a* e fluorescência *in vivo* da diatomácea *Conticribra weissflogii* sob condições de cultivo em laboratório. Incremento na densidade celular (i. e. crescimento) foi significativamente reduzido após 48 h de exposição a 450 e 900 µg Zn L^{-1}. Taxas de crescimento após 96 h de exposição, estimadas em divisões por dia, foram 0,81, 0,85, 0,69, e 0,59, para os tratamentos controle, 90, 450 e 900 µg Zn L^{-1}, respectivamente, sendo significativamente menor que o controle na maior concentração testada. Células expostas ao zinco apresentaram tendência aumentar o conteúdo de clorofila após 3 h. No entanto, a razão de fluorescência *in vivo* por clorofila *a* diminuiu significativamente no

mesmo período, o que sugere um mecanismo de toxicidade envolvendo danos ao fotossistema II. Possíveis rotas metabólicas e as implicações ambientais são discutidas.

Palavras-chave: *Conticribra weissflogii;* ecotoxicologia; metal; microalga

Introduction

Phytoplankton plays a major role in primary production in aquatic environments, being responsible for almost the total primary production in oceanic environments (Grant Gross 1987). Phytoplankton is a group of single celled organisms (although many can aggregate in chains or colonies) that may be continuously and directly exposed to environmental contaminants, which can affect their physiology and growth rates. Indeed, growth rate is an ecologically relevant parameter commonly affected by environmental stressors. Growth rate is also an index of net production and can be determined by following increases in cell number (cell mL^{-1}) with time (Kinne 1984). Another parameter indicating photosynthetic health and consequently the potential for primary production is the fluorescence of chlorophyll *a*. In intact algae cells, it provides information on the absorption, distribution and utilization of energy in photosynthesis (Cid *et al*. 1995). Additionally, reduction in phytoplankton growth rate and photosynthesis may cause rearrangements in the marine food web (Yap *et al*. 2004). Therefore, understanding aspects of the interaction physiology-contaminant involved in phytoplankton growth is of ecotoxicological concern.

Diatoms are the most representative group in marine nano- and microphytoplankton and one of the most important in terms of carbon fixation (Martin-Jézéquel *et al*. 2000, Gonçalves-Araujo *et al*. 2012). The marine diatom species *Conticribra weissflogii* (Grunow) (Stachura-Suchoples & Williams (Guiry 2014) (previously *Thalassiosira weissflogii* (Grunow), Fryxell & Hasle 1977) is ubiquitous and frequently dominant in phytoplankton biomass in estuarine and coastal areas worldwide. This species is also important in aquaculture activities, being pointed as a significant source of live food for several cultured organisms (Lavens & Sorgeloos 1996). Despite the ecological and economical importance of the diatom *C. weissflogii*, the effect of chemical pollutants on growth rate and physiology of this species is not completely understood. Growth of diatoms, in general, is limited by the environmental availability of nutrients and light (Grant Gross 1987, Gonçalves-Araujo *et al*. 2012). However, chemical pollutants can potentially disturb primary production and growth rates. In fact, a more thorough understanding of the responses of phytoplankton growth rate and toxic mechanisms associated with anthropogenic chemical contaminants is required before accurate predictions of the effects of environmental pollution on oceanic primary productivity could be made (Cloern 2001).

In this context, zinc is one of the most common contaminants in aquatic systems and potentially toxic to organisms at high waterborne concentrations (Bozcaarmutlu & & Arinç 2007). Natural total and dissolved zinc concentrations in coastal and estuarine waters are in the $\mu g\ L^{-1}$ range (Prazeres *et al*. 2012), usually around 5-15 $\mu g\ L^{-1}$ (Berner & Berner 1996). The level considered to be safe for biota protection according to Brazilian water criteria is 90 $\mu g\ L^{-1}$ (CONAMA 2005). However, anthropogenic contamination has increased zinc concentrations in the environment, which in some impacted areas can be as high as 1 mg Zn L^{-1} (Azetsu-Scott *et al*. 2007). Growth impairment and cytotoxicity are among the many effects of zinc on phytoplankton (Yap *et al*. 2004). However, there is lack of information on physiological mechanisms involved in short- and long-term toxic effects of zinc in marine diatoms.

In light of the above, the present study aimed to evaluate the toxic effects of zinc on cell growth rate, *in vivo* fluorescence and chlorophyll *a* content. Differently from several studies concerning zinc mainly as nutrient (at very low limiting environmental concentration) (Morel *et al.* 1994, Varela *et al.* 2011), the present work evaluates potential growth impairments related to zinc contamination. The experimental concentrations are comparable to ranges from natural levels (Berner & Berner 1996, Kinne 1984) to contaminated waters (Azetsu-Scott *et al*. 2007), where some toxicity to aquatic organisms are expected, contributing to understanding of the potential sub-lethal toxicity of zinc to the microalgae *C. weissflogii*.

Materials and Methods

C. weissflogii was obtained from the Universidade Federal do Rio Grande culture collection (isolated in November 2003, registered at algae bank as THAL WEIS 01). The algae were cultivated in Guillard f/2 medium (Guillard & Ryther 1962) prepared in artificial seawater (salinity 30 ± 2) made with commercial marine salts

(Coralife®, Franklin, WI, USA) dissolved in nanopure water. Temperature (20 ± 1ºC) and white fluorescent light (100 µmol photons m^{-2} s^{-1}) were set according to average values recommended (Lavens & Sorgeloos 1996). Before zinc exposure, microalgae were cultured until the stationary growth phase, using a batch culture method in the control f/2 media. The acclimation period lasted 10 days, according to what is indicated for volumes up to 2 L (Lavens & Sorgeloos 1996). At the starting of the experiment, microalgae were transferred to a fresh f/2 control media or exposed to additional 90, 450 and 900 µg Zn L^{-1} (nominal concentrations). It is worth to mention that control Guillard f/2 media presented 14,5 ± 3 µg total Zn L^{-1} and 12,2 ± 3 µg dissolved Zn L^{-1}. Final zinc concentrations in the water were verified by atomic absorption spectrometry (Avanta Plus, GBC, Australia), according to procedures described in Prazeres *et al*. (2012). The nominal 90 µg L^{-1} was measured as 98.2 ± 1 and 85.5 ± 7 µg L^{-1} of total and dissolved Zn, respectively. The nominal 450 µg L^{-1} as 460.0 ± 3 and 430.2 ± 2 µg L^{-1} of total and dissolved Zn, respectively. Finally, the treatment with nominal 900 µg L^{-1} presented 956.5 ± 1 and 919.5 ± 3 µg L^{-1} of total and dissolved Zn, respectively. Control and zinc-treated Guillard f/2 media were prepared 24 h before introduction of diatoms into test chambers to allow chemical equilibration of zinc with seawater. For practical purposes we refer hereafter to the nominal concentrations.

The experiment was started by inoculating 50 mL of the stationary culture in 500 mL of fresh experimental media (control or with additional zinc), triggering an exponential growth phase during zinc exposure. Approximately the same number of cells was inoculated in each treatment (Fig. 1). Temperature and light levels were the same as in the acclimation period. Tests were run in triplicates for 96 h. However, different sampling times were used for different biological responses. The endpoints were measured in the shortest time possible to report their first interaction with the contaminant in the exposure media (Table I). Cell growth was analyzed every 24 h up to 96 h. Aliquots (50 mL) from all experimental flasks were collected and cells were counted using a hemocytometer (Neubauer chamber) under optic microscope. *In vivo* fluorescence intensity was measured every 24 h using a fluorometer (Turner Designs TD-700, Sunnyvale, CA, USA). Growth rate (div.d^{-1}) was calculated from increments in cell number over the experimental period, using increments in $\log_2$ (cell concentration) (Kinne 1984). Special attention was given to the first doubling in population size (in terms of cell numbers), which was the time when major effects of Zn were observed (3 h and 48 h). Results of cell density (Cell. mL^{-1}) over 96 h period were used to estimate growth rate. As mentioned, the 96-h specific growth rate was obtained from a linear regression of cells concentration (*log*-transformed) on exposure time.

In order to assess the effect of Zn on the cell chlorophyll content, the 72-h period exposure was selected. This was the shortest exposure time to statistically assure that more than 95% of microalgae cells performed cellular division within all experimental media, i. e. cell chlorophyll content was assessed mostly in cells exposed since division. Thus, after 3 and 72 h exposure, microalgae cells were collected and chlorophyll *a* was extracted for 24 h in 90% acetone. Chlorophyll *a* content was measured using a fluorometer (Turner Designs TD-700, Sunnyvale, CA, USA) following the non-acidification fluorometric method (Welschmeyer 1994). Another fluorometer of the same model (but assembled with appropriate lamps and optical filters) was used to measure *in vivo* chlorophyll fluorescence over the experimental period. The chlorophyll *a* cell content (*f*g chlorophyll *a.* $cell^{-1}$) was calculated dividing the total extracted chlorophyll *a* by the number of microalgae cells after 3 and 72 h exposure. Likewise, the *in vivo* fluorescence per unit chlorophyll *a* was obtained by dividing the *in vivo* fluorescence by total chlorophyll *a* measured at each experimental condition after 3 and 72 h exposure. Results of zinc-exposed microalgae were expressed as a percentage of the control.

Data presented here are expressed as mean ± 1 standard error of mean. Significant differences were accessed by Kruskal-Wallis analysis of variance followed by Dunn's test. The Spearman coefficient was used to assess linear correlations. Whenever linear correlations were detected, a linear regression was performed. In all analysis $a = 0.05$.

Results

Based on cell counting, no significant effect of zinc on the diatom cell density was observed after 3 h exposure. However, cell density of microalgae under control conditions (50 ± 10.10^5 cell mL^{-1}) was significant higher than those of microalgae under 48 h exposure to 90 µg Zn L^{-1} (28 ± 3.10^5 cell mL^{-1}), 450 µg Zn L^{-1} (25 ± 2.10^5 cell mL^{-1}) and 900 µg Zn.L^{-1} (23 ± 4.10^5 cell mL^{-1}) (Fig. 1). After 48 h exposure fluorescence of zinc-exposed cells was significant higher (Fig. 1).

Table I: Endpoints studied and zinc exposure times used in the present study for the diatom *Conticribra weissflogii*.

Endpoint	Exposure time				
	3h	24h	48h	72h	96h
Short-term effect on cell doubling	X		X		
Long-term effect on cell density and growth rate	X	X	X	X	X
In vivo fluorescence	X	X	X	X	X
Total chlorophyll *a* content	X			X	
Cell chlorophyll *a* content	X			X	
Fluorescence per chlorophyll *a* contents	X			X	

After 96 h exposure, the effect of zinc was smaller, but growth rate reduction was also observed (Fig. 2). The growth curve parameters are presented in Table II, in which "*a"* represents the rate of cell doubling per day. Similar response was observed after 96 h exposure when growth rate was estimated based on the *in vivo* fluorescence (not shown). However, no significant difference was observed among treatments when the growth regression coefficients from *in vivo* fluorescence are considered, which suggests some disagreement of *in vivo* fluorescence and cell density results.

Total chlorophyll *a* (Fig. 3A) and average chlorophyll *a* cellular content (Fig. 3B) were not significantly different among treatments after 3 or 72 h exposure. However, chlorophyll *a* per cell trended to increase at high zinc concentration (N=12, r^2=0.96, p<0.001) after 3 h exposure (solid bars on figure 3B). Furthermore, the *in vivo* fluorescence intensity per unit of chlorophyll *a* was markedly reduced after 3 h exposure to zinc. Thus, fluorescence was not equally correlated to total chlorophyll *a* or to chlorophyll *a* cell content in exposed and control cells. In fact, a significant and negative relationship between this parameter and zinc concentration was observed after 3 h exposure to zinc (N=12; r^2 = 0.99; p<0.005) (Fig. 3C and D).

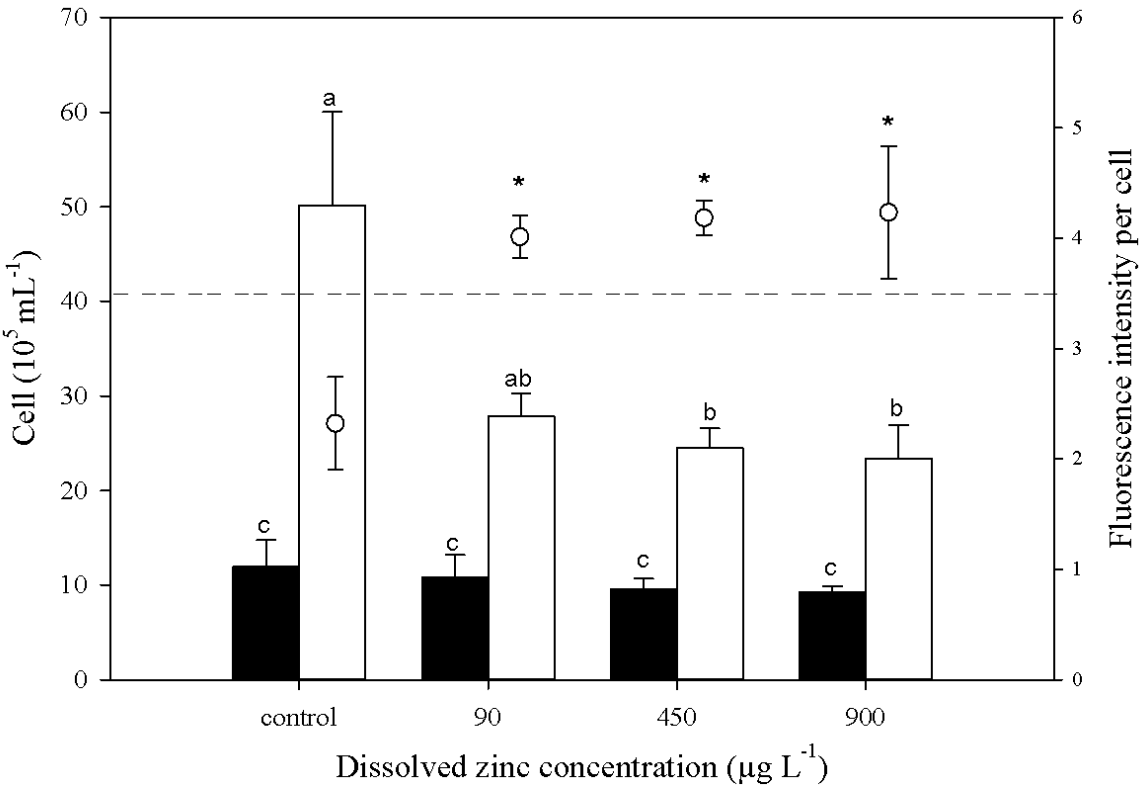

Figure 1. Short-term effects of zinc on cell density and fluorescence of the diatom *Conticribra weissflogii.* Cell density (10^5 mL^{-1}) is shown at beginning of the experiment (3 h, solid bars) and after 48 h (open bars) exposure. Fluorescence intensity per cell is shown after 48 h exposure (open circles). Data are expressed as mean ± 1 standard error of mean. The average level of fluorescence in the control culture is shown as dashed line (no difference was detected in fluorescence per cell among treatments at 3 h). Different letters indicate significantly different mean values of cell density, while the star indicates significantly different fluorescence per cell among treatments (p<0.05). Approximately the same number of cells (4 ± 1 10^5 cell mL^{-1}) were inoculated at each experimental treatment.

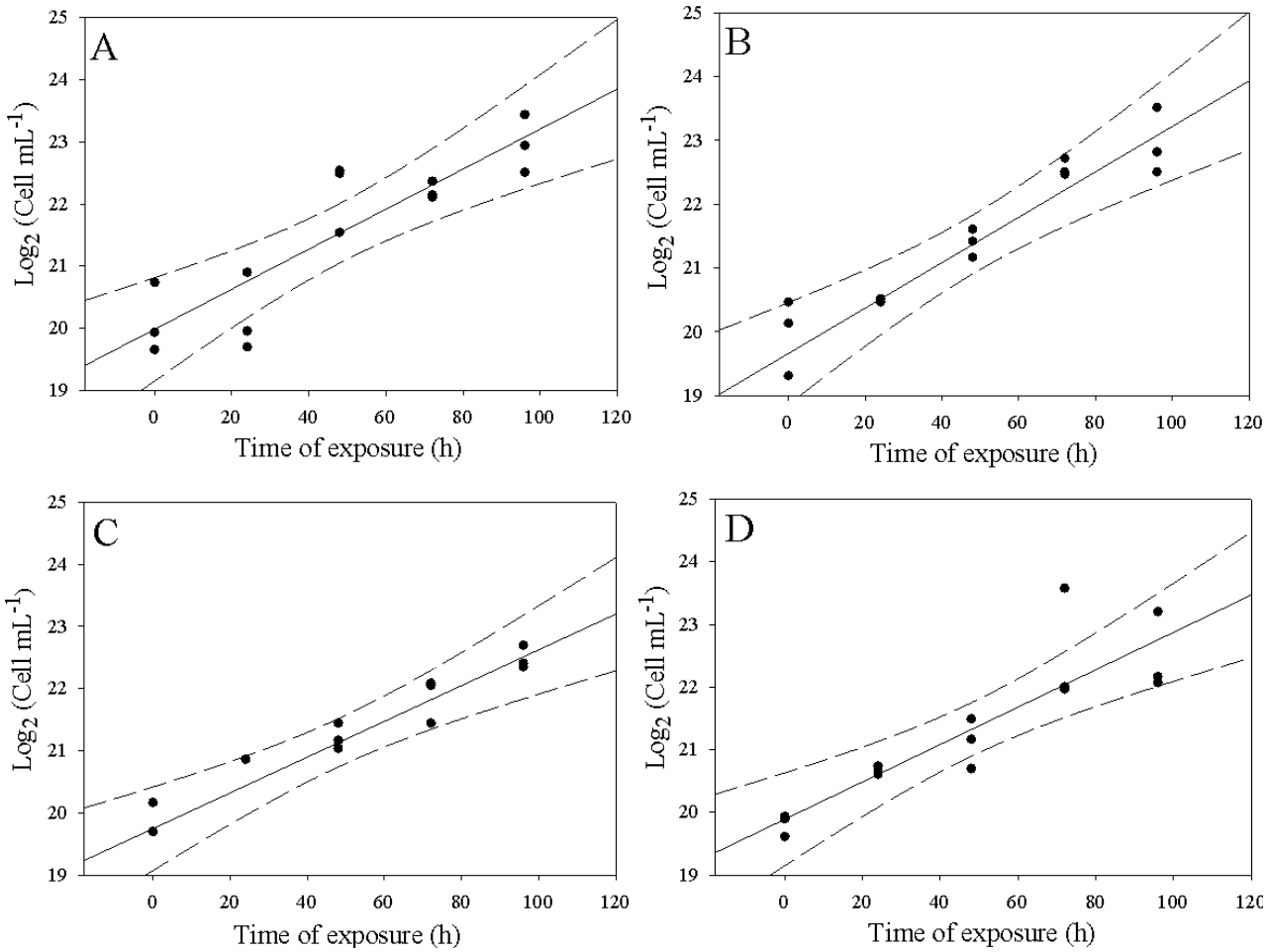

Figure 2. Cell density of the diatom *Conticribra weissflogii* as a function of the exposure time to different concentrations of zinc [control (A); 90 µg Zn L^{-1} (B); 450 µg Zn L^{-1} (C); 900 µg Zn L^{-1} (D)]. Regression line and 99% confidence interval limits are represented by the solid and dashed lines, respectively.

Table II. Cell density and growth rate (*a*: cell div.day^{-1}) for the diatom *Conticribra weissflogii* after 96 h of zinc exposure. Different superscript lowercase letters indicate mean values significantly different among treatments.

Zn concentration (µg L^{-1})	Cell density* (10^5 cell mL^{-1})	Logarithm growth function parameters		
		*a***	r^2	p
Control	85 ± 16[a]	0.81 [a]	0.83	<0.01
90	85 ± 18 [a]	0.85 [a]	0.93	<0.01
450	59 ± 5 [b]	0.69 [ab]	0.99	<0.001
900	46 ± 3 [b]	0.59 [b]	0.96	<0.001

*At the beginning of experiment it was inoculated 4 ± 1 .10^5 cell mL^{-1} in each experimental treatment.

** *a* represents the number of cell doubling per day and it was estimated trough the slope of a linear correlation between cell density (log-transformed) and time.

Discussion

Diatom sensitivity to zinc

Short-term environmental exposure of organisms to metals may occur in the case of point source contamination such as dredging, which normally enhances metals levels in the water for several hours (Van den Berg *et al*. 2001). In the present work, the growth rate of the diatom *C. weissflogii* was found significantly affected after 48 h exposure to zinc at concentrations above 450 µg L^{-1}. Although 90 µg Zn L^{-1} did not alter *C. weissflogii* growth rate, considerable reduction in the diatom biomass (in terms of cell numbers) was still observed under this treatment within that period. It is

worth noting that 90 µg Zn L^{-1} is considered the upper limit within the Brazilian water criteria for aquatic biota protection (CONAMA 2005). In fact, cells 48 h-exposed had their concentration reduced by 44, 52 and 54% at 90, 450 and 900 µg Zn L^{-1}, respectively. These results agree with those reported by Fisher *et al*. (1981) for the pennate freshwater diatom *Asterionella japonica* and also for other diatom species in the literature (Brœk *et al*. 1980) (Fig. 4). According to Fisher *et al*. (1981) growth inhibition induced by metal exposure was related to uncoupling between photosynthesis and cell division, corresponding to equivalent reduction in biomass production and carbon fixation.

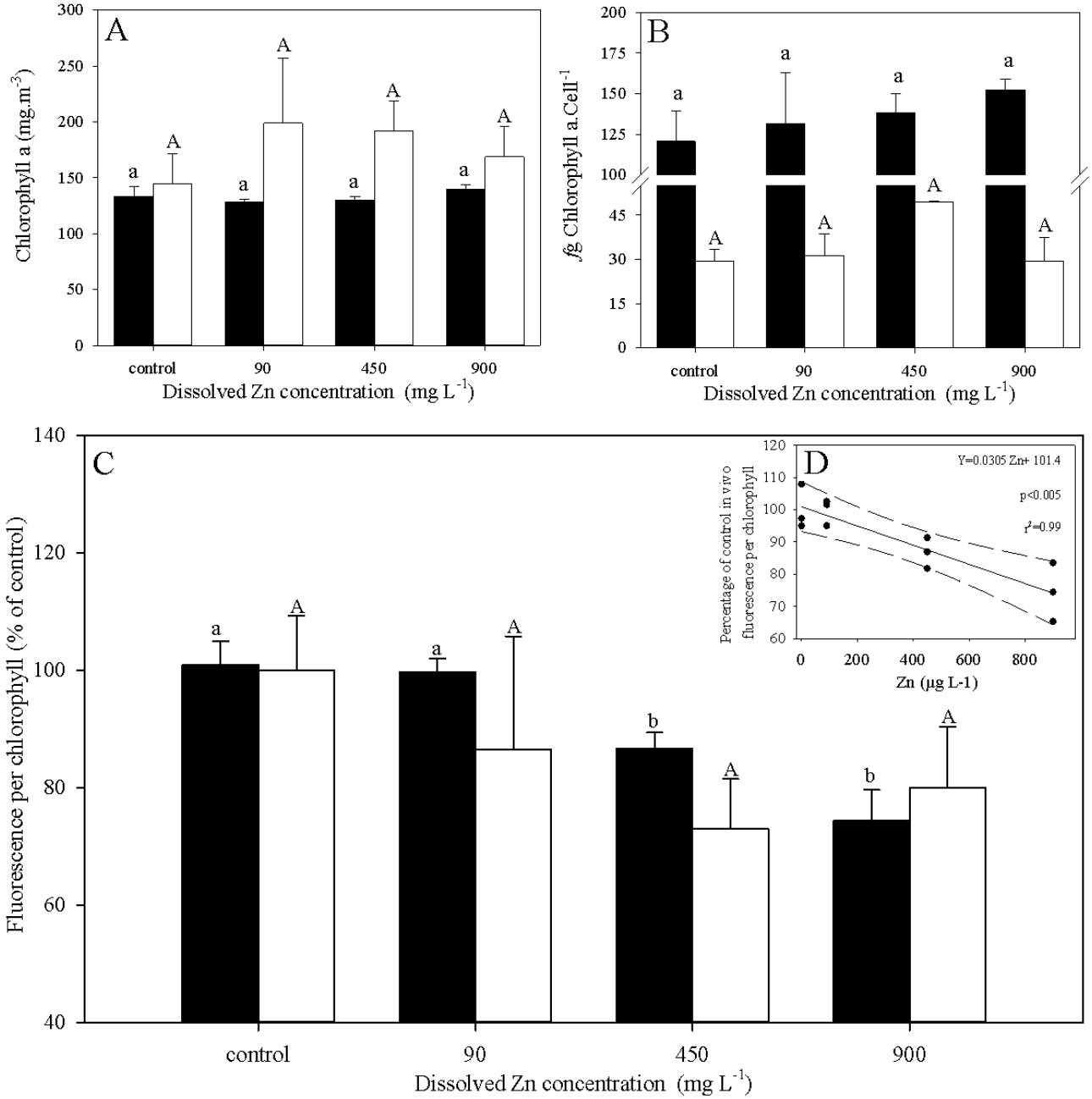

Figure 3: Total chlorophyll *a* (A), chlorophyll *a* cell content (B) and *in vivo* fluorescence intensity per total chlorophyll *a* (C) of *Conticribra weissflogii* after 3 h (solid bars) and 72 h (open bars) exposure to zinc. Insert in panel C, plot D represents the relationship between the *in vivo* fluorescence intensity per total chlorophyll *a* (as a percentage of control) after 3 h exposure and zinc concentration in the test medium. Data are expressed as mean ± 1 standard error of mean. Different lowercase and uppercase letters indicate significantly different mean values among treatments after 3 and 72 h exposure, respectively ($p<0.05$).

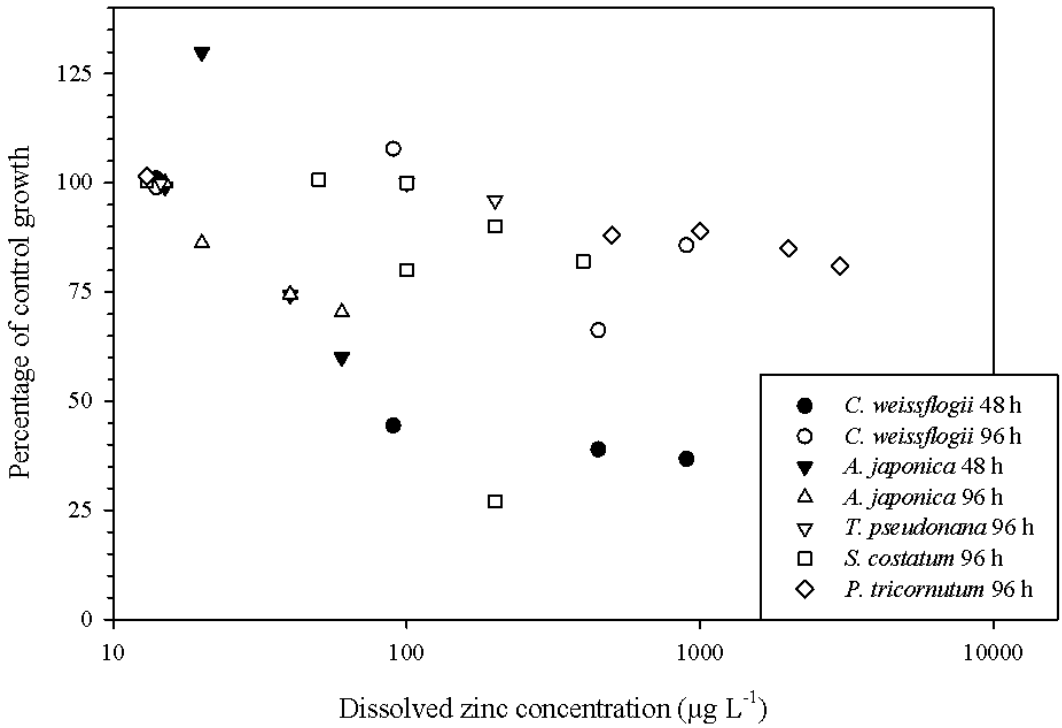

Figure 4: Effect of zinc on diatoms growth after 48 h (black filled symbols) or 96 h (white filled symbols) exposure for *Conticribra weissflogii* (present study), *Asterionella japonica* (Fischer *et al*. 1981), and *Thalassiosira pseudonana, Skeletonema costatum,* and *Phaeodactylum tricornutum* (Brœk *et al*. 1980).

Cell density of *C. weissflogii* was comparatively less affected by zinc after 96 h than after 48 h exposure (Fig. 4). This could be explained considering a higher bioavailability of this metal in the first exposure hours, thus causing more toxicity. In fact, *T. pseudonana* and other diatoms assimilate zinc within the first 10 h of exposure (Brœk *et al*. 1980). Yap *et al*. (2004) found bioconcentration approximately ~120-fold waterborne in microalgae cells 48 h-exposed to zinc. Additionally, microalgae are able to produce phytochelatin, a metal-chelating compound, after 48 h exposure to metals (Gekeler *et al*. 1988). Phytochelatins act chelating either intracellular or extracellular metal as a way of metal regulation (Lee *at a*l. 1996). In fact, after 48 h exposure toxic heavy metals may bind to phytochelatins and any sulfhydryl-rich compounds, which would contribute to decrease their toxicity over time. In diatoms, detoxification mechanisms also include frustule formation (Fisher *et al*. 1981). Sulfhydryl metabolism is important for synthesis of siliceous exoskeleton (Lewin 1954) and for effective metal detoxification in organisms (Machado *et al*. 2013, Machado *et al.* 2014). Moreover, the frustule formation itself is a way to eliminate intracellular zinc, which is passively or actively incorporated into the frustules (Jaccard *et al*. 2009). Therefore, the lower effect of zinc observed in *C. weissflogii* at longer exposure times could be explained by the decrease in bio-available toxic zinc. Several generations of cells grew over the experimental period; and most added zinc may have become unavailable after incorporation into the organism's frustules or binding with excreted metabolites. However, effects of zinc on growth rate were still observed even after 96 h exposure, reflecting significant differences in daily doubling rate (*a*) (Table II). The highest *a* value was estimated as 0.85 div day^{-1} at 90 µg Zn L^{-1}, which was close to the value observed for the control diatoms (*a* = 0.81 div day^{-1}). At 450 and 900 µg Zn L^{-1}, *a* values were 0.69 and 0.59 cell div day^{-1}, respectively, indicating a reduction in growth rate ranging from 15 to 27 % respective to the growth of control diatoms.

Mechanism of zinc toxicity

Fluorescence per unit chlorophyll *a* markedly decreased as a function of zinc concentration (Fig. 3) together with a trend to increase in chlorophyll *a* cell content after zinc exposure. Although photosystem II activity was not directly measured, let us discuss as those responses together suggest that there was damage to this photosystem.

The use of *in-vivo* chlorophyll fluorescence has been recognized as a valuable tool to probe

photosynthetic function because of its nondestructive measurements and high sensitivity (Eberhard *et al*. 2008). It is based on the principle that a portion of light absorbed by chlorophyll is re-emitted as fluorescence. In healthily growing cells, fluorescence levels are expected to follow the general increasing trend in biomass and chlorophyll *a*. However, this was not the case in zinc-exposed cells in the present work, in which reductions in growth rate around 30- 50 % caused by zinc exposure were associated with increases in fluorescence per cell (Fig. 1). In addition, zinc-exposed cells showed decreased fluorescence per unit chlorophyll *a* at higher Zn concentrations (Fig 3C). Effects decreasing fluorescence of chlorophyll normally indicates a negative action of stressors on photosynthesis, such as nutrient limitation or the effect of toxic compounds. In this context, more common is that phytoplankton toxicants like copper, which induce restraining in nutrient absorption, acid-base unbalance, and reduction in photosynthesis or growth rate, are reported to increase cell chlorophyll and cell fluorescence (Cid *et al*. 1995). After 72 h exposure, although the algae presented significantly lower cell numbers, zinc-exposed *C. weissflogii* showed the same levels of bulk chlorophyll *a* as the control. Therefore, a trend of increase in chlorophyll content per cell with increasing zinc concentration was observed. In fact this trend was significant even after a short period of exposure (3 h). After 72 h exposure, the increasing trend in chlorophyll per cell with zinc exposure was less marked, but still significant, suggesting that zinc exposure induced less functional chlorophyll, which stimulated cells to produce more of this pigment, culminating in higher chlorophyll *a* content in zinc-exposed cells.

As previously mentioned, aquatic toxicants normally increase *in vivo* fluorescence intensity per unit chlorophyll. However, decreases in this parameter are particularly well reported as related to dysfunction in photosystem II. In fact, the environmental stressor reducing fluorescence intensity per chlorophyll is excessive light ultra-violet radiation (Heraud & Beardall 2000) due to damage (or nonphotochemical quenching) caused to the photosystem II (Waring *et al*. 2010). Photosystem II is the major structure responsible for *in vivo* fluorescence response, and when damaged it can cause decreases in fluorescence yield of the photosystem centers (Eberhard *et al*. 2008), which is consistent with the less functional chlorophyll discussed above. Moreover, damage to the photosystem II induced by over-exposure to light causes shifts in cellular chlorophyll content (Waring *et al*. 2010) also observed in the present study. Given the similarity of effects, the present data suggests that the mechanisms of toxicity of zinc to *C. weissflogii* might be analogous to light stress and associated with damage to the photosystem II. Under light-stress, photosystem II damage is submitted to phosphorylation of some specific polypeptides, acid-base unbalance and deep oxidation of pigments (Eberhard *et al*. 2008), conditions likely to occur after zinc exposure. Besides the oxidative stress, it has also been shown that zinc can affect uptake of essential ions such as manganese and silicon (Lewin 1954, Fisher *et al*. 1981, Sunda & Huntsman 1996). Manganese plays an important role in water oxidation at the photosystem II. Sunda & Huntsman (1996) demonstrated that zinc exposure could decrease manganese uptake by the diatom *T. pseudonana*. In fact, manganese deficiency would additionally lead to decrease in manganese-containing water-oxidizing centers in the photosystem II, resulting in lower functionality. This would affect electron donor side of the photosystem II, implying a reduction in photosystem II electron transport, which is consistent with the observed decrease in fluorescence. Furthermore, damage to photosystem II is recoverable when cells are under more favorable conditions (Heraud & Beardall 2000). Recovery related to decreased zinc in experimental media could explain the reduced effect of zinc on fluorescence per chlorophyll after 96 h exposure, when zinc was less bioavailable. Taken altogether, our findings on zinc effects on cell chlorophyll content and *in vivo* fluorescence in *C. weissflogii* provide evidence of zinc damage to diatoms photosystem II.

Conclusions

Results reported in the present study show that the physiology and growth rate of the diatom *C. weissflogii* are affected by zinc at concentrations higher than 90 μg L^{-1} within the first 48 h exposure. This implies that *C. weissflogii* in environments with concentrations slightly above Brazilian saltwater quality criteria might experience pollution-derived impairments in primary production. Reductions of 54 % in cell density compared to control were observed at 900 μg Zn L^{-1}. Similarly, reductions of 28 % in growth rate are expected at this Zn level after 96 h exposure. The zinc effect of decreasing fluorescence per chlorophyll *a* and increasing fluorescence and chlorophyll *a* per cell provides indication that sub-lethal toxicity mechanism are likely associated with damage to the photosystem II. Finally, further studies are needed for scaling the effects and better elucidate the mechanism of

zinc-photosystem II interaction in this marine microalga.

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