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PROLIFERATION OF THE DIATOM *CYCLOTELLA MENEGHINIANA* AFFECTED BY EXPOSURE TO LEAD

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Abstract: Elevated levels of pollutants, including heavy metals, resulting from human activities, have raised concerns regarding their adverse impacts on natural ecosystems. Among these, aquatic organisms, particularly non-target microalgae, bear substantial consequences from such contaminants. This study endeavored to evaluate the impact of the heavy metal lead (Pb) at concentrations of 2.5, 25, and 250 μg L^{-1} on the growth and lipid content of the diatom *Cyclotella meneghiniana* in a 14-day toxicity test conducted under controlled laboratory conditions. The findings indicate that lower Pb concentrations (2.5 and 25 μg L^{-1}) fostered algal proliferation, whereas exposure to 250 μg L^{-1} resulted in complete inhibition in two distinct endpoints. Additionally, no significant fluctuations in algal lipid content were discernible between exposure to 2.5 and 25 μg Pb L^{-1} and the control group. This study stands as a pioneering effort in unveiling the toxicity of Pb on *C. meneghiniana*, shedding light on the species' susceptibility to this heavy metal and underscoring the necessity of incorporating this species into ongoing ecological risk assessments of Pb.

Keywords: heavy metals, stimulus effect, lipid content, algal density, ecological risk assessment

1. INTRODUCTION

The world is presently witnessing an unparalleled surge in economic growth driven by a multitude of human activities. This surge has led to the rapid escalation of both natural and anthropogenic pollutants (Nguyen et al., 2020, 2021, 2022, 2023a; Pham et al., 2023). Notably among these pollutants, heavy metals stand out as a significant factor of water contamination, posing severe threats to a wide array of aquatic organisms, particularly microalgae (Atici et al., 2008, 2012; Ali et al., 2019; Ahmed et al., 2020). Consequently, this disruption has far-reaching consequences for the functioning of aquatic ecosystems due to the critical and multifaceted roles played by microalgae (Gheorghe et al., 2017; Qu et al., 2018; Aziz et al., 2023).

Lead (Pb) contamination in water commonly originates from additives employed in paint, gasoline, and industrial processes involving elevated temperatures, such as coal combustion, smelting, cement production, battery manufacturing, glass production, thermometer crafting, and electronic switch fabrication (Lattanzio & Clark, 2020; Singh et al., 2022). It is widely recognized as one of the most hazardous elements impacting global health due to its resistance to biodegradation and persistent presence in the environment, leading to its accumulation in living organisms and subsequent development of disorders and diseases (Malik et al., 2012; Dung et al., 2013; Srinivasan, 2013; Ayangbenro & Babalola, 2017; Ali et al., 2019).

In aquatic environments, the levels of residual Pb can vary significantly depending on specific geographical areas. Concentrations of Pb in water samples collected from Malaysian rivers ranged from 0.74 to 2.56 μg L^{-1} (Ahmed et al., 2020), while those detected along the Ghana coast ranged from 4.08 to

70.73 µg L^{-1} (Appiah-Opong et al., 2021). The Pb concentrations in water collected from the secondary irrigation channels in Indonesia varied from 370 to 1,690 µg L^{-1} (Tahir et al., 2021). Nevertheless, in certain cases, Pb concentrations in wastewater have been detected at remarkably elevated levels. For instance, concentrations as high as 551 mg L^{-1} were identified in Italian storage battery facilities, while Indian radiator manufacturing industries exhibited even higher levels at 709 mg L^{-1} (Dao & Beardall, 2016).

Diatoms, classified under the Bacillariophyceae class, are minuscule photosynthetic unicellular algae that wield a pivotal influence in aquatic ecosystems, both biologically and chemically (Vidoudez & Pohnert, 2012; Richardson-Coy & Teed, 2013; Giri et al., 2022). These ubiquitous microalgae hold promise as potential bioindicators in water quality assessment due to their responsiveness to various physicochemical parameters of aquatic environments (Morin et al., 2012). Numerous toxicity studies have indicated that diatoms, as metal toxicity indicators (Jamali et al., 2012), manifest heightened sensitivity to heavy metals compared to other aquatic species (Hörnström, 1990; Hirst et al., 2002; De Jonge et al., 2008; Pandey et al., 2018). Among them, the genus *Cyclotella* was found to be sensitive to heavy metals (Morin et al., 2007; Duong et al., 2008; Morin et al., 2012; Li et al., 2021); therefore, this genus is potential for heavy metal-related toxicity testing (Afriana et al., 2022). Notably, within the *Cyclotella* genus, *Cyclotella meneghiniana* has received relatively little attention in toxicity testing, despite indications that it may be frequently exposed to stressors. This centric diatom species features valves measuring 8.0–30.3 µm in diameter and is found in various habitats, ranging from fresh to brackish water, with a well-documented and widespread ecological distribution (Sang et al., 2013). *C. meneghiniana* resides as planktonic cells in various water bodies, including reservoirs, estuaries, rivers, and lakes (Finlay et al., 2002; Bharati et al., 2019). It is considered one of the most dominant diatom species, comprising approximately 30% of the total algal abundance and 60% of the total biomass in Goczałkowice Reservoir, Poland (Timm et al., 2009).

Ecotoxicological research has delved into the impact of lead (Pb) on diatoms, revealing notably low values of the effective concentration for 50% inhibition in 96-h toxicity testing (96-h EC_{50}) for endpoints related to proliferation. These 96-h EC_{50} values are instrumental in assessing the ecological risks associated with Pb exposure. For instance, a 96-h EC_{50} of 1,400 µg Pb L^{-1} was reported for the growth of the diatom *Chaetoceros calcitrans* (Jensen et al., 2000), while values of 350 µg Pb L^{-1} and 200 µg Pb L^{-1} were observed for the growth of diatoms *Odontella mobiliensis* and *Coscinodiscus centralis*, respectively (Karthikeyan et al., 2021). Despite these findings, there remains a critical gap in research regarding the evaluation of Pb toxicity in *C. meneghiniana*. This gap is particularly noteworthy given the crucial ecological roles this species plays in aquatic environments, as previously mentioned. Therefore, there is an urgent need for an initial study to investigate the sensitivity of *C. meneghiniana* to Pb exposure.

This study aimed to examine the development of the diatom *C. meneghiniana* under controlled laboratory conditions while exposing it to a range of Pb concentrations spanning from 2.5 to 250 Pb L^{-1}. We consider this study as a vital preliminary toxicity test, which will help us assess the sensitivity of *C. meneghiniana* to Pb exposure. The results from this study will serve as a valuable reference point for incorporating this species into the existing ecological risk assessment framework for Pb.

2. MATERIALS AND METHODS

2.1. Microalgal isolation and chemicals for study

The algal sample was collected from the Nhieu Loc-Thi Nghe Canal, Phu Nhuan District, Hochiminh City, using a phytoplankton net with a mesh size of 20 µm (Sournia, 1978). The diatom *C. meneghiniana* (Kutzing, 1844) was morphologically identified following the systematic taxonomy described by Krammer et al., (2004) and isolated under a microscope using the pipetting and washing method (Belcher & Swale, 1982) (Fig. 1). After isolation, the alga was cultured in Z8 medium supplemented with silicate at a salinity of 2‰ (Kotai, 1972) under laboratory conditions with a temperature of 27 ± 1°C, a light intensity of approximately 2,500 lx, and a light-dark cycle of 12 h:12 h (APHA, 2017).

The metal solution $Pb(NO_3)_2$ at a concentration of 1,000 mg L^{-1} (grade for the ICP/MS equipment) was obtained from Merck (Germany) and used as a stock solution for the experiments. All the test media including Z8 and Z8 containing Pb (pH around 7.2) were filtered through a sterilized 0.22 µm filter (Millipore Corporation) prior to the start of the experiments. For the culture media, the hardness and the alkalinity (determined by titration) (APHA, 2017) ranged from 30–40 mg $CaCO_3$ L^{-1}, and from 42–50 mg $CaCO_3$ L^{-1}, respectively.

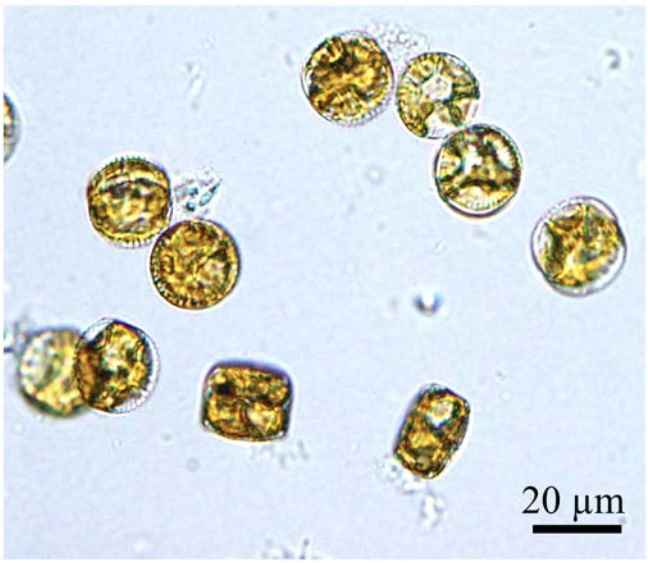

Figure 1. Light microscope view of the morphology of the diatom *Cyclotella meneghiniana*

2.2. Experimental setup, algal enumeration, and lipid analysis

The exposures of *C. meneghiniana* to Pb were conducted according to Muhaemin, (2004) with minor modifications. Briefly, the alga was incubated in 250 mL flasks containing 150 mL of the medium at three different Pb concentrations of 2.5, 25, and 250 µg L^{-1}. The test concentrations were chosen based on the Vietnam Technical Regulation for surface water safety (QCVN 08-MT:2015/BTNMT), and the Pb concentrations found in nature (Ning et al., 2011). Besides, the control was prepared in parallel with the Pb exposures by culturing the alga in the medium without metal addition. There were three replicates (n = 3) with a similar initial density of the alga in each test concentration. The pH values in each treatment including the control were measured (Metrohm 744) at the beginning and end of the test and did not alter significantly, ranging from 6.6 and 8.0. The experiment on the growth of the alga lasted 14 days in the laboratory conditions as mentioned above. At the start and every two days of the experiment, 2 mL sub-samples from each culturing flask were collected, fixed with Lugol solution, and the alga was counted with a Sedgewick Rafter counting chamber (Graticules Optics, England) under the microscope (Optika B150, Italy) (Sournia, 1978). Every time of algal enumeration, at least 400 cells of *C. meneghiniana* were counted to get a reliable algal density as guided by Sournia, (1978). At the start of the experiment, the mean densities of *C. meneghiniana* in the flasks of the control, 2.5 µg Pb L^{-1}, 25 µg Pb L^{-1}, and 250 µg Pb L^{-1} were 3022, 2933, 3244, and 2756 cells mL^{-1}, respectively.

Based on Mishra et al., (2014), Park et al., (2016), and Khan et al., (2021), the sulpho-phospho-vanillin (SPV) colorimetric method for lipid quantification of microalgae was used to estimate the lipid contents of control *C. meneghiniana* and *C. meneghiniana* treated with 2.5 and 25 µg Pb L^{-1} at day 14. The lipid content in the *C. meneghiniana* treated with 250 µg Pb L^{-1} was not measured because no alga survived on the 6th day of incubation before the experimental termination. For the SPV reaction, 2 mL of concentrated sulfuric acid was added to a 100 µL algal sample before the mixture was heated for 10 minutes at 100°C, and then cooled for 5 minutes in the ice bath. Next, 5 mL of phospho-vanillin reagent was added, and the sample was kept in the incubator shaker for 15 minutes at 37°C at 200 rpm for pink color development. Absorbance was measured at 530 nm using a spectrophotometer to quantify the lipid content within the sample. To prepare the phospho-vanillin reagent, 0.06 g vanillin was dissolved in 1 mL absolute ethanol, 9 mL deionized water, and mixed well. Subsequently, 40 mL of concentrated phosphoric acid was added to the mixture, and the complete reagent was stored in a dark bottle until use. The standard lipid stocks were prepared using commercial canola oil (20 mg in 10 mL chloroform, final concentration 2 mg mL^{-1}). The standard curve (R^2 = 0.9919) of lipid content was built consisting of 9 points (from 0 to 40 µg mL^{-1}), and formulating y = 116.7x – 0.0339, where y is absorbance and x is lipid content (µg mL^{-1}). Teflon-covered glass vials were used throughout all experiments.

2.3. Data analysis

Data analysis was conducted using R software (version 4.2.2) within the Rstudio environment

(version 2023.06.0). To model the non-linear relationship between density and exposure time, the "drc" package was employed, utilizing a four-parameter logistic regression model: $\text{Density} = a + \frac{b-a}{1+e^{-c(\text{exposure time}-d)}}$. Within this equation, parameters *a* and *b* represent the lower and upper curve limits respectively, *c* signifies the slope, and *d* corresponds to the inflection point.

Our primary focus was directed towards comparing the slope of each Pb exposure to the control group, indicating variations in the speed of density increase. This was achieved by examining the non-overlapping nature of the 95% confidence intervals associated with this parameter.

For evaluating the diatom density and lipid content at day 14 between the Pb-exposed organisms and the control group, a one-way analysis of variance (ANOVA) was applied. If statistical significance was observed, the Dunnett test was subsequently conducted (Nguyen et al., 2023b). A significance level of $p \leq 0.05$ (two-tailed) was employed as the threshold for rejecting the null hypothesis.

3. RESULTS

3.1. Development of *Cyclotella meneghiniana* exposed to lead

The results demonstrated a progressive increase in the density of *C. meneghiniana* over the course of the 14-day experiment, both in the control group and in groups exposed to 2.5 and 25 µg Pb L^{-1}. This trend was depicted by an S-shaped curve modeled using the four-parameter logistic regression (Figure 2). However, for the organism exposed to 250 µg Pb L^{-1}, the density of *C. meneghiniana* reached zero by day 6. The prediction curve indicates that exposure to 2.5 and 25 µg Pb L^{-1} led to an enhanced density of *C. meneghiniana* starting from day 6, in comparison to the control group. This difference was supported by the steeper slopes and the non-overlapping 95% confidence intervals of the regression model (parameter *c*) in both Pb exposure groups, as compared to the control group (Table 1). The density increment due to Pb exposure continued throughout the experiment, as confirmed by the ANOVA analysis on day 14, which revealed a significant effect of Pb exposure on this endpoint (*p*-value < 0.001, Table 2). This effect was further emphasized by Dunnett's post hoc test (*p*-value < 0.001, Figure 2).

4. DISCUSSION

4.1. Effect of lead: Stimulation of diatom density, with negligible effect on lipid content, at 2.5 and 25 µg L^{-1}

It is important to note that Pb does not have an essential role in the growth of organisms due to its absence of physiological function (Marella et al., 2020). Nevertheless, exposure of organisms to Pb concentrations within their tolerance limits often triggers various responses across different test subjects. These subjects encompass a range of organisms, including green algae (e.g. *Dunaliella tertiolecta* and *Scenedesmus quadricauda*), cyanobacteria (e.g. *Microcystis aeruginosa*), and even higher-level organisms like the water flea (Berglind et al., 1985; Starodub et al., 1987; Saçan et al., 2007; Bi et al., 2013). The emergence of these responses is likely linked to the increased content of polysaccharides within cell walls, subsequently boosting photochemical efficiency and facilitating cellular division (Ouyang et al., 2012). This aligns with the heightened production of NADPH (Nicotinamide Adenine Dinucleotide Phosphate) in

Table 1. Comparison of daily density increase (day^{-1}) between lead-exposed diatoms and control group. Data are presented as model estimations and their 95% confidence intervals (in brackets), derived from the four-parameter logistic function. The value highlighted in bold signifies a lack of statistical significance in the estimation

0 µg L^{-1}	2.5 µg L^{-1}	25 µg L^{-1}	250 µg L^{-1}
0.125	0.252	0.295	**−11.819**
(0.0448, 0.205)	(0.2100, 0.295)	(0.2401, 0.350)	**(−65.992, 42.354)**

Table 2. ANOVA results for the impact of lead on diatom density and lipid content at day 14

	df	Sum of squares	Mean squared	*F* value	Pr(> *F*)
Density					
Treatment	3	556.813×10^6	185.604×10^6	8640	2.230×10^{-14}
Residuals	8	171.852×10^3	21.481×10^3		
Lipid content					
Treatment	2	7.658	3.829	0.852	0.473
Residuals	6	26.974	26.974		

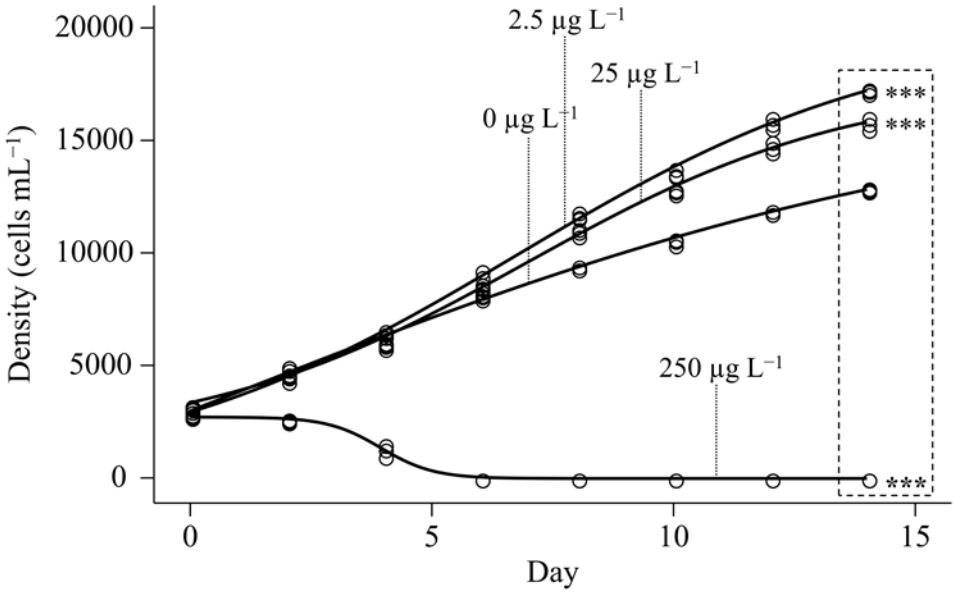

Figure 2. Diatom density increases over exposure time. The lines represent the regression curve modeled using a four-parameter logistic function. "***" indicates a significant difference in density at day 14 (framed data) for exposed organisms compared to the control, with a two-tailed significance level of $p \leq 0.001$

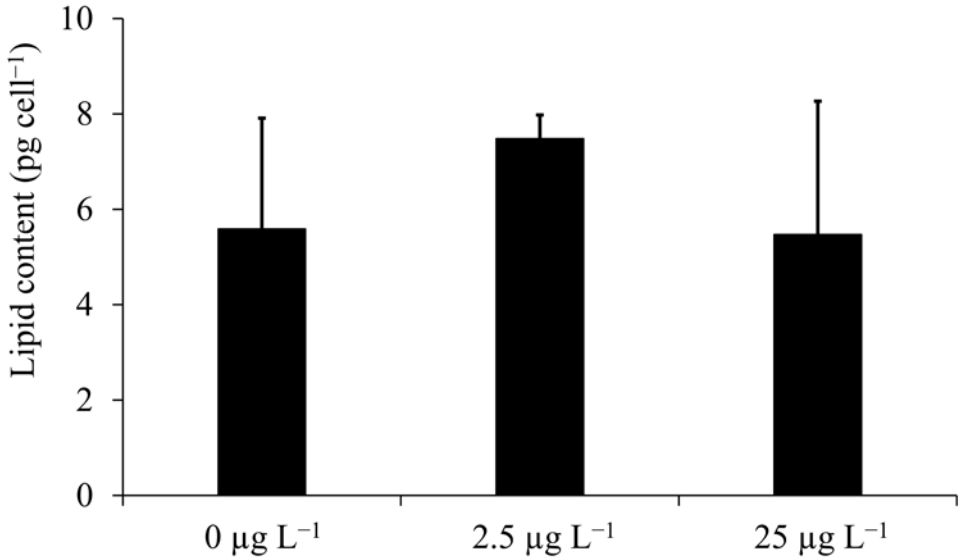

Figure 3. Diatom lipid content at day 14. Data is presented as mean ± standard deviation (n = 3). Data for the exposure to 250 µg lead L^{-1} are not available due to the absence of viable diatom cells at day 14

challenging conditions due to its necessity in generating cell lipids (Shi et al., 2020). In line with this, Bi et al., (2013) documented a rise in polysaccharide production, revealing a positive correlation with the formation of *M. aeruginosa* colonies at concentrations ≥ 5 mg Pb L^{-1}. The present study stands as a pioneering effort in deciphering the long-term effects of Pb on a freshwater diatom, shedding light on the intricate connection between Pb exposure and the observed physiological responses. However, to fully comprehend whether these enhancements are beneficial, detrimental, or neutral in terms of Pb exposure, further investigations into other endpoints, such as cell volume and other cell components, are necessary.

The lipid content in diatom cells is known to be influenced by various stress conditions, including changes in light intensity, salinity (Zhu et al., 2016), temperature fluctuations, and exposure to heavy metals (Hedayatkhah et al., 2018). Under favorable environmental conditions, diatoms tend to maintain low lipid levels, but they accumulate higher lipid content in stressful conditions (Yi et al., 2017). This serves as a self-defense mechanism, enabling them to

synthesize lipids as a reservoir of energy-rich carbon to ensure normal physiological functions (Hellier et al., 2015; Shi et al., 2020). For example, *Scenedesmus* sp. exhibited increased lipid content at Pb concentrations of 0.5 and 1 mg Pb L^{-1}. These discussions underscore the significance of maintaining normal or elevated lipid content to withstand challenging conditions. While a definitive conclusion regarding the stimulation of lipid content is not reached due to high variability, our observations in this study could provide support for the link between Pb exposure and the preservation of lipid content. Additionally, the effects of Pb on lipid synthesis and storage might be less direct compared to its interference with specific biological activities related to cell division. Consequently, the disruptions caused by Pb might not always lead to immediate changes in lipid content. As an example, *Scenedesmus* sp. lipid content remained unaffected at a low concentration of 50 µg Pb L^{-1} but increased when exposed to higher concentrations of 500–1,000 µg Pb L^{-1}, and then decreased at an extreme concentration of 10,000 µg Pb L^{-1} (Pham et al., 2020). These results suggest that algal lipid content could serve as a potential and straightforward endpoint for evaluating the impact of environmental stressors on the development of microalgae, with careful consideration.

4.2. Effect of lead: Complete inhibition of diatom at 250 µg L^{-1}

The excessive production of reactive oxygen species (ROS) stands out as a pivotal mechanism triggering cell death. This phenomenon has been elucidated in the context of *Scenedesmus acutus* and *Chlorella* sp. (Dao & Beardall, 2016). Additionally, Pb, given its inherent properties, has the capacity to interact with both lipid bilayers and proteins within cell membranes. This interaction results in the release of cellular contents, compromising cell viability and functionality (Piotrowska-Niczyporuk et al., 2015). Moreover, it is worth noting that the impairment of cell membranes can extend to perturbations in cellular homeostasis, a cascade that inevitably leads to cell death (Pinto et al., 2003). It is important to consider that Pb's toxicity mechanism might not exclude the inhibition of photosynthesis. In fact, Pb has the capacity to disrupt the structure and functioning of chloroplasts, where photosynthesis takes place, resulting in diminished light absorption and subsequent inhibition of cell growth (Carfagna et al., 2013).

The complete inhibition of *C. meneghiniana* at a concentration of 250 µg Pb L^{-1} by day 6 highlights the high susceptibility of this species to Pb exposure. In a related study, Pinto et al., (2003) observed partial inhibition of Pb up to 103.6 mg L^{-1} in the green alga *Acutodesmus obliquus* over a 7-day test period. Additionally, *C. meneghiniana* demonstrated higher sensitivity compared to the green alga *Cladophora crispata*, which was not completely inhibited by Pb even at a concentration of 20 mg L^{-1} by day 20 (Jabbar, 2010). Similarly, the densely populated cyanobacterium *Spirulina platensis* exhibited growth at 0.5 mg Pb L^{-1} but was inhibited at higher concentration of 1.0 mg Pb L^{-1} during a 22-day test (Mohy El.Din, 2017). Furthermore, Pham et al., (2020) and Nanda et al., (2021) found that the 96-h EC_{50} for *Scenedesmus sp.* was 4,760 µg Pb L^{-1}, whereas it was as high as 565,000 µg Pb L^{-1} for *Chlorella sorokiniana*. Notably, *C. meneghiniana* exhibited similar or even higher sensitivity compared to other diatoms, such as *Chaetoceros calcitrans*, *Odontella mobiliensis*, and *Coscinodiscus centralis*, as indicated by a 96-h EC_{50} of ≥ 200 µg Pb L^{-1} for proliferation (Jensen et al., 2000; Karthikeyan et al., 2021). These analyses collectively highlight the high sensitivity of *C. meneghiniana* to Pb exposure and emphasize the importance of including this species in updating current ecological risk assessments for Pb.

5. CONCLUSIONS

Our study represents the first description of *C. meneghiniana*'s responses to environmentally detected concentrations of Pb. It underscores the species' high sensitivity compared to other previously tested algae, particularly in terms of inhibition concentration. Our findings suggest that Pb exposure at sublethal doses is unlikely to significantly affect cell lipid content, possibly indicating that lipid synthesis and storage are not primary targets of Pb or that lipid content serves as a self-defense mechanism. These results provide valuable insights into the toxicity of Pb to *C. meneghiniana*, serving as a critical scientific resource for the current ecological risk assessment processes related to Pb.

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