Molecular Characterization of Microalgae *Thalassiosira* sp. Based on Genetic Marker tufA and Potential Test for Bioremediation of Heavy Metal Lead (Pb)

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Abstract

Heavy metal lead (Pb) pollution in aquatic environments poses a serious threat to aquatic ecosystems and human health. The microalga Thalassiosira sp. has potential as a bioremediation agent for heavy metals; however, further studies are needed to maximize its capability. This study aims to molecularly characterize Thalassiosira sp. using the tufA gene, evaluate the effects of Pb on growth and cell morphology, and assess its Pb uptake efficiency. Methods included DNA isolation, phylogenetic analysis, growth measurement using a hemocytometer, Pb uptake testing via ICP-OES, and cell morphology analysis. Results showed that the tufA gene could molecularly characterize Thalassiosira sp., with a phylogenetic similarity of 99.53% to Conticriba weissflogii (MH571875.1). Pb significantly inhibited growth at concentrations of 0.03 ppm and 0.05 ppm and caused cell aggregation and pigment degradation at higher concentrations (2 ppm). This microalga exhibited the highest Pb uptake efficiency at lower concentrations (0.03–0.05 ppm), reaching 100%, while at 2 ppm, the efficiency decreased to 40%. This study highlights the potential of Thalassiosira sp. in remediating Pb, providing a foundation for the development of microalgae-based biotechnological applications.

Keywords: *Lead; Bioremediation; Thalassiosira sp.*

INTRODUCTION

Increasing carbon emissions and heavy metal pollution in waters pose serious threats to aquatic ecosystems and human health (Shahid *et al*., 2020). Heavy metals such as Pb often accumulate and are difficult to degrade naturally (Edelstein & Ben-Hur, 2018; Vardhan *et al*., 2019). With its toxic properties, ability to accumulate biologically, and potential to cause mutagenesis and cancer at low concentrations (Priya *et al*., 2022; Sharma *et al*., 2021), Pb is one of the most dangerous heavy metals in the aquatic environment (Collin *et al*., 2022).

Cases of Pb pollution in Indonesia are increasing every year. At Surabaya Beach, Pb levels reached 0.04–0.22 mg/L, while at Branta Pesisir Beach, Pamekasan, they reached 0.12–0.33 mg/L, which has exceeded the threshold of 0.05 mg/L based on the Decree of the Minister of Environment Number 51 of 2004 (Suastuti *et al*., 2021; Fadlilah *et al*., 2023). Similar pollution was also found in the Pekalongan River, Central Java, with Pb levels of 1,854–2,318 mg/L due to batik industry waste (Maharani *et al*., 2023). In the coastal area of Prigi Bay, lead concentrations reach 0.22-0.60 mg/kg in sea air and 0.40-0.57 mg/kg in sediment (Yona *et al.,* 2020). This contamination comes from human activities which include the disposal of industrial, agricultural and household waste (Azdem *et al.,* 2024).

As an environmentally friendly technology, bioremediation is an efficient solution to reduce heavy metal pollution in various environments (Crecca *et al*., 2023). Microalgae, including the genus

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Thalassiosira , have unique abilities in bioabsorption and bioaccumulation of heavy metals through the formation of organometallic complexes that neutralize toxic effects (Leong & Chang, 2020; Chakravorty *et al*., 2023). Several species of *Thalassiosira* have been reported to be able to remediate Cu, Zn, and Cd (Halder, 2014; Saini & Dhania, 2019; Mishra *et al*., 2020; Chasapis *et al*., 2022). However, to date, no studies have tested the potential of *Thalassiosira* sp. to remediate Pb.

Microalgae species are known to have significant potential in remediating heavy metals, including Pb. *Nannochloropsis oculata, Chorella* and *Porphyridium* effectively absorb Pb, Cd and Cu (Waluyyo *et al*., 2020; Soeprobowati & Hariyati, 2012). Exposure to Pb can have a negative effect on the growth of microalgae, but in some species, it can increase acclimatization and production of secondary metabolites as a stress response (Dahmen-Ben *et al.,* 2022). The effectiveness of *Thalassiosira* sp. as a Pb biorediator, it is a significant solution for reducing heavy metal pollution in waters. This study aims to characterize the molecular properties of *Thalassiosira* sp. using the tufA gene, as well as to assess its potential in remediating Pb heavy metals. The results of this study are expected to be the basis for the development of microalgae-based bioremediation technology to deal with Pb pollution in waters.

MATERIAL AND METHODS

The microalgae used in this study were *Thalassiosira* microalgae isolates from Brackish Water Aquaculture (BBPBAP) in Jepara, Indonesia.

Microalgae Cultivation

Thalassiosira sp. microalgae were obtained from BBPBAP, Jepara, and cultured using a batch culture system. Culture was carried out by preparing the media using a ratio of 1:4, namely 50 mL of microalgae culture in 200 mL of sterile fresh water added with 30 g of NaCl to achieve a salinity of 30 ppt (Kusumaningrum & Zainuri, 2016; Lasmarito *et al*., 2022). Nutrients in the form of Walne fertilizer were added as much as 0.1 mL for every 10 mL of saline water (Prihardianto *et al*., 2021). Culture was carried out at room temperature around 21°C with LED lights with an intensity of 1,000–10,000 lux as a light source. The aeration system was used as an oxygen supply during the cultivation process (Gildantia *et al*., 2022). Cell density measurements were carried out by creating a standard curve based on cell density calculation data using a haemacytometer (Kusumaningrum *et al*., 2019).

DNA Isolation

Thalassiosira sp. DNA isolation was carried out using the CTAB method from Doyle & Doyle (1987). Microalgae cells were separated from the culture medium by repeated centrifugation at 8000 rpm to obtain pellets. The pellets were then incubated with CTAB buffer at 65°C, homogenized, and added with Chloroform Isoamyl Alcohol (CIA) before being centrifuged again. The supernatant was discarded and precipitated with isopropanol at -20°C overnight. After centrifugation, the pellets were washed with 70% alcohol, dried, and dissolved in TE buffer.

Qualitative and Quantitative Analysis of DNA

Thalassiosira sp. DNA was measured using a Nanodrop spectrophotometer at wavelengths of 260 nm, 280 nm, and 230 nm. Qualitative and quantitative tests serve to measure the concentration and purity of DNA.

Amplification of Microalgae tufA Gene

TufA gene amplification was carried out using a PCR thermalcycler machine with a DNA template of *Thalassiosira* sp. isolates with a concentration of more than 100 ng/mL and a purity level between 1.8–2.0 (Vieira *et al*., 2016; Adhikary & Kumar, 2022).

The amplification process used the tufA forward primer (5'-GGNGCNGCNCAAATGGAYGG-3') and tufA reverse primer (5'-CCTTCNCGAATMGCRAAWCGC-3'), as well as a MyTaq Master Mix mixture consisting of 25 µL mastermix, 1 µL forward primer, 1 µL reverse primer, 7.5 µL ddH_2O, and 3 µL template DNA. The PCR reaction included predenaturation at 95°C for 1 minute, followed by denaturation at 95°C for 15 seconds, annealing at 55°C–56°C for 15 seconds, extension at 72°C for 10 seconds, and postextension at 72°C for 5 minutes. The annealing temperature was optimized using a PCR gradient machine.

The amplification results were analyzed using 1% agarose gel electrophoresis. A total of 3 µL of DNA sample was mixed with 3 µL of marker consisting of 1 µL of loading dye and 2 µL of DNA ladder, then inserted into the gel well. Electrophoresis was run at 100 volts for 25 minutes, and the results were visualized using a UV transilluminator to observe the DNA bands.

Sequencing and Phylogenetic Analysis

The DNA sequencing process of *Thalassiosira* sp. was carried out by PT. Genetika Science Indonesia. The sequencing data were edited using BioEdit software to perform alignment and consensus creation, then converted into FASTA format. Nucleotide homology analysis was performed via BLAST at NCBI (http://www.ncbi.nlm.nih.gov), which compares the similarity of base sequences with the GenBank database using the parameters Max Score, Query Coverage, E-value, and percentage of nucleotide identity (Boratyn *et al*., 2019; Torkian *et al*., 2020; Samal *et al*., 2021). The phylogenetic tree was constructed using MEGA 11 software with the Neighbor-Joining method, supported by a Bootstrap value of 500 to produce an accurate topology based on the nearest branch length and the efficiency of the analysis process (Moniz *et al*., 2014).

Experimental

Experiments to test the potential of *Thalassiosira* sp. as a bioremediation agent for lead (Pb) were designed using a Completely Randomized Design (CRD) (Chen *et al*.. 2024). Pb stock solution (1000 ppm) was added to the culture until it reached a final concentration of 0, 0.03, 0.05, and 2 ppm, with each concentration carried out in three replications. Observations were made on the level of Pb absorbed by *Thalassiosira* sp. from day 0 to day 10, which includes the exponential phase of microalgae growth. The independent variable in this study was the concentration of heavy metal Pb. Control variables included temperature 21°C, aeration using a rotary shaker at a speed of 50–100 rpm, light intensity 3000 lux (equivalent to a 40 watt lamp), and the use of Walne fertilizer as a nutrient source. The dependent variables consisted of Pb heavy metal absorption efficiency, microalgae growth rate, and changes in microalgae cell morphology.

Analysis of the Effect of Lead (Pb) Concentration on Growth, Morphology, and Absorption Efficiency of *Thalassiosira* sp.

Analysis of the effect of lead (Pb) concentration on the growth and density of *Thalassiosira* sp. cells was carried out using an Optilab microscope and hemacytometer, starting from day 0 to reaching the exponential phase. The data obtained were analyzed using ANOVA with a confidence level of 0.05, followed by a post hoc test. Changes in cell morphology due to Cd treatment were observed using an Olympus CX23 light microscope with a magnification of 400x to 1000x. The efficiency of Pb absorption by *Thalassiosira* sp. was measured on days 0 and 11 using ICP-OES and calculated using the following formula:

$$Eff = \frac{t0 - tn}{t0} \times 100\%$$

Adsorption efficiency (Eff) refers to the percentage of metal removed from the environment during the study. It is calculated by comparing the initial metal concentration (t0) at the beginning of the experiment with the final metal concentration (tn) at the end of the experiment. This provides

a measure of how effectively the material or organism, in this case *Thalassiosira* sp., is able to reduce the concentration of contaminants, expressed in parts per million (ppm). The results of the efficiency test of the ability of *Thalassiosira* sp. in absorbing heavy metal Pb with different concentrations are presented in percentage form (Halima *et al*., 2019).

RESULTS AND DISCUSSION

Thalassiosira sp. cultivation was carried out for 21 days in a batch culture system on a laboratory scale. The growth curve of *Thalassiosira* sp. for 21 days showed growth phases including the lag phase, exponential phase, stationary phase, and death phase (Figure 1). The lag phase occurred until the 2nd day due to the adaptation process to the new environment (Vermeersch *et al*., 2019). The exponential phase occurred on the 3rd to 10th day which was indicated by a significant increase in cell density because the microalgae utilized optimal conditions to carry out rapid cell division (Himeoka & Kaneko, 2017). The stationary phase was characterized by stable cell density which was indicated on the 11th to 14th day because it entered a period of balance between growth and cell death (Jaishankar & Srivastava, 2017). The death phase occurred on the 15th to 21st day due to decreased metabolic activity and decreased viability, causing the rate of cell death to exceed the rate of cell division (Schiraldi, 2023).

Culture color intensification occurred on day 0 to day 10 . On day 0, the culture color was light brown, then changed to a darker brown on day 10. Color intensification in *Thalassiosira* sp. occurs due to increased cell density and biomass (Brzychczyk *et al*., 2020); de Almeida Moreira *et al*., 2021), so that the concentration of fucoxanthin pigment in the culture also increases (Goss *et al*., 2022).

Molecular Characterization of the tufA Gene

DNA isolation from each sample was analyzed at wavelengths of 260 nm, 280 nm, and 230 nm to evaluate the purity and concentration of DNA (Fialova *et al*., 2020; Antane *et al*., 2024). The results in table 1, the purity of the microalgae DNA *Thalassiosira* sp. is in the range of 1.8-2.0, which indicates that the isolated DNA has a high level of purity with minimal contamination. Purity values below 1.8 indicate contamination by protein or organic matter, while values above 2.0 indicate contamination by phenol or RNA (Zulkarnain *et al*., 2023). The absorption ratio at 260 nm/230 nm is used to assess the quality of nucleic acids, while the absorption ratio at 260 nm/280 nm is used to assess the level of contamination by protein (Ghaheri *et al*., 2016). According to Ermavitalini *et al*. 2021, DNA purity plays an important role in the success of PCR amplification, where the purer the DNA, the clearer the DNA bands are visible on visualization under UV light after electrophoresis.

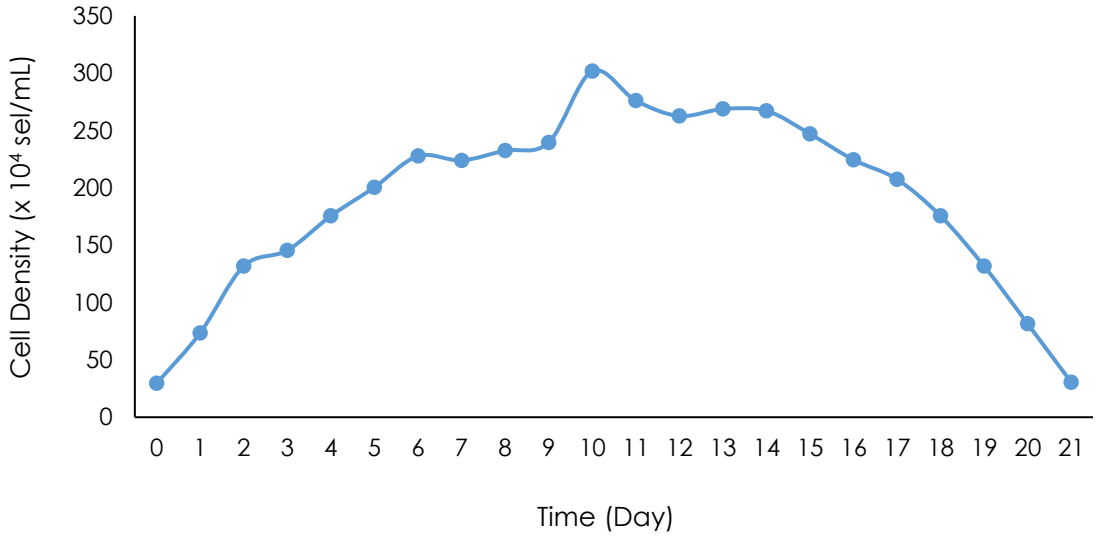

Figure 1. Growth Curve of *Thalassiosira* sp.

Table 1. Nanodrop DNA Spectrophotometer Results of microalgae *Thalassiosira* sp.

Sample Name	Concentration (ng/µL)	A_{260}	A_{280}	Ratio A_{260}/A_{280}	Ratio A_{260}/A_{230}
Thalassiosira sp.	572.6	11.452	5.572	2.06	1.97

Table 2. The results of the sequence homology of *Thalassiosira* sp.

BLAST Sequence	Accession	Query Cover	E-Value (%)	Percent Identity (%)
Conticriba weissflogii	MH571875.1	95%	0.0	99.53%

The results of amplifying Thalassiosira sp. DNA isolates using the tufA gene are shown in Figure 3, it can be seen that the results of the two *Thalassiosira* DNA bands with the best amplification were at an annealing temperature of 56°C, where the DNA fragment bands were clearly visible and thick. A clear and thick DNA fragment band indicates successful amplification of the tufA gene target (Levin *et al*., 2018). The clarity and thickness of the band at an annealing temperature of 56°C indicate that this temperature provides optimal conditions for the primer to bind the DNA target specifically, minimizing the formation of by-products such as primer dimers or non-specific amplification.

In the study of Joseph *et al*. (2018), it was shown that *Thalassiosira* weissflogii obtained a size of 845 bp for the partial chloroplast tufA gene region, which is recorded in the NCBI GenBank with accession number MH571875. This is also supported by the study of Vieiria *et al*. (2016) that the tufA gene has a range of 758-901 bp, but Sauvage *et al*. (2020) found the latest range that the length of the tufA base pair can reach up to 1230 bp.

The sequencing results showed that the *Thalassiosira* sp. isolate has similarities with several species found in the GenBank database. Based on this database, the highest similarity of the *Thalassiosira* sp. isolate with the tufA gene is the *Conticriba weissflogii* sequence ID MH571875.1 (Table 2).

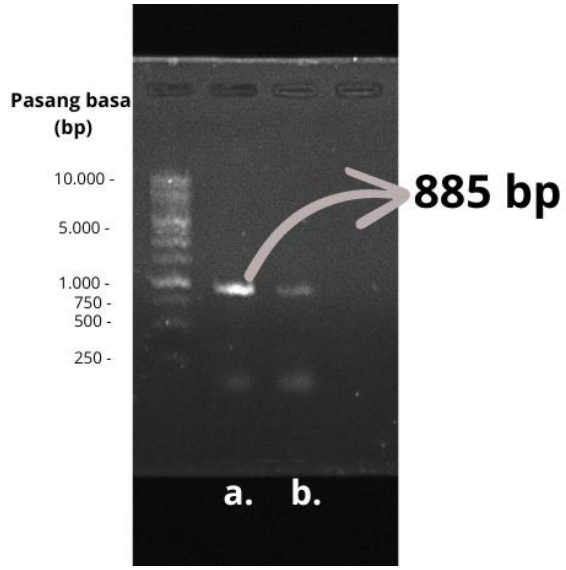

Figure 2. Visualization of DNA fragments of *Thalassiosira* sp. tufA gene. Description: (a.) *Thalassiosira* sp. annealing temperature 56°C; (b.) *Thalassiosira* sp. annealing temperature 58°C

Based on the results of the sequence analysis of the *Thalassiosira* sp. sample with the *C. weissflogii* sequence ID MH571875.1 has a high percent identity value and a low e value. Boratyn *et al*. (2019) added that a high percent identity, namely above 90%, provides greater confidence that the search results are truly relevant, indicate a very close relationship, and represent homologous sequences. Research by Bussard *et al*. (2017) also showed that an e value approaching 0 has significant similarities between the sample sequence and the comparison sequence, thus reflecting real homology. The results of the phylogenetic analysis confirmed that the *Thalassiosira* sp. sequence. in this study was in the same clade as *C. weissflogii* ID MH571875.1, with a very high bootstrap value of 100% (Figure 4).

The Effect of Different Concentrations of Pb Metal on the Growth of *Thalassiosira* sp.

Thalassiosira sp. microalgae cells were harvested for Pb metal concentration testing in the exponential phase, precisely on the 10th day. This phase is characterized by the most active cell growth rate, where cells divide rapidly and their numbers increase exponentially. In the exponential phase, cell metabolism is at its peak, so that cells actively absorb nutrients and reproduce. This high metabolic activity also allows cells to absorb and accumulate substances from their environment more effectively, including heavy metals such as Pb.

Based on the growth curve of *Thalassiosira* sp. in Figure 5, it shows that the control treatment has the highest average cell density at the peak of the exponential phase and the highest average total growth, namely 301.23 x 10^4 cells/mL, compared to the average cell density at the peak of the exponential phase and the average total growth in cultures that had been given various Pb heavy metal treatments. Interference of Pb with metabolic nutrients has been reported by Tripathi & Poluri (2021) as one of the main factors causing microalgae to experience a significant decrease in growth rate. Zamani-Ahmadmahmoodi *et al*. (2020) also showed how lead (Pb) toxicity affects the growth rate of microalgae. The growth inhibition of *Thalassiosira sp.* is influenced by the concentration of Pb applied. Treatments with Pb concentrations of 0, 0.03, 0.05, and 2 ppm revealed variations in cell density, where lower Cd concentrations resulted in higher cell densities. This occurs because higher Pb concentrations induce stronger toxic effects on the cells of *Thalassiosira sp.* This reduced growth in microalgae causes a shift in metabolism towards the synthesis and accumulation of triglycerol (Thompson, 1996). Apart from this, there is also a decrease in protein in microalgae which occurs due to the formation of Reactive Oxygen Species (ROS) in algal cells (Palma *et al*., 2002). High Pb concentrations also cause a decrease in chlorophyll a, b and carotenoid contents which affect the

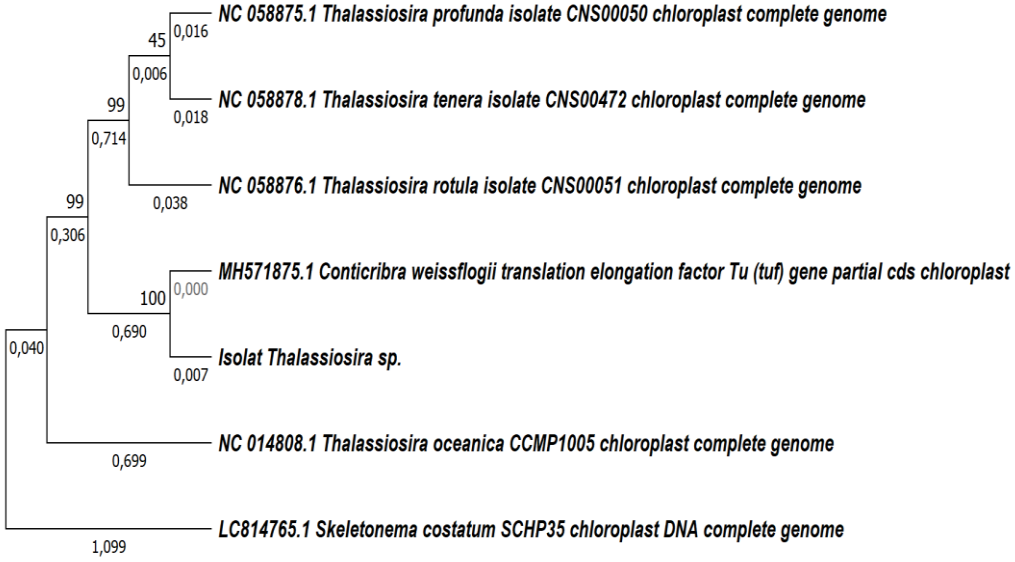

Figure 3. Phylogenetic tree of *Thalassiosira* sp. isolate

photosystem II process which depends on these compounds (Dao and Beardall, 2016; Nanda *et al.*, 2021). This causes a decrease in microalgae growth in growth media contaminated with high levels of Pb.

One Way Anova test (95% confidence level) showed that the concentration of heavy metal lead (Pb) had a significant effect on the growth of *Thalassiosira* sp. with a p value (Sig.) of 0.000, which is much smaller than 0.05. The results of the follow-up test (post hoc LSD) showed that all p values in the treatments were less than 0.05. This shows that the difference in the growth rate of the microalgae *Thalassiosira* sp. among the Pb treatment groups (control, 0.03 ppm, 0.05 ppm, and 2 ppm) is quite large and consistent, indicating that the concentration of Pb significantly affects the growth rate of *Thalassiosira* sp. at all treatment levels.

The Effect of Differences in Lead (Pb) Concentration on the Morphology of Microalgae *Thalassiosira* sp. Cells.

This research examined the effectt of the heavy metal Pb on the morphology of *Thalassiosira* sp.. through microscopic observation. Figure 6 shows that most treatments experienced cell aggregation and some experienced pigment degradation. In the control, normal *Thalassiosira* sp. cells have the characteristics of rectangular cell shape and the cell color looks yellowish green, according to research by Park *et al*. (2017). However, at Pb concentrations of 0.03, 0.05, and 2 ppm, the cells became slightly swollen (not rectangular) and showed erosion of photosynthetic pigments.

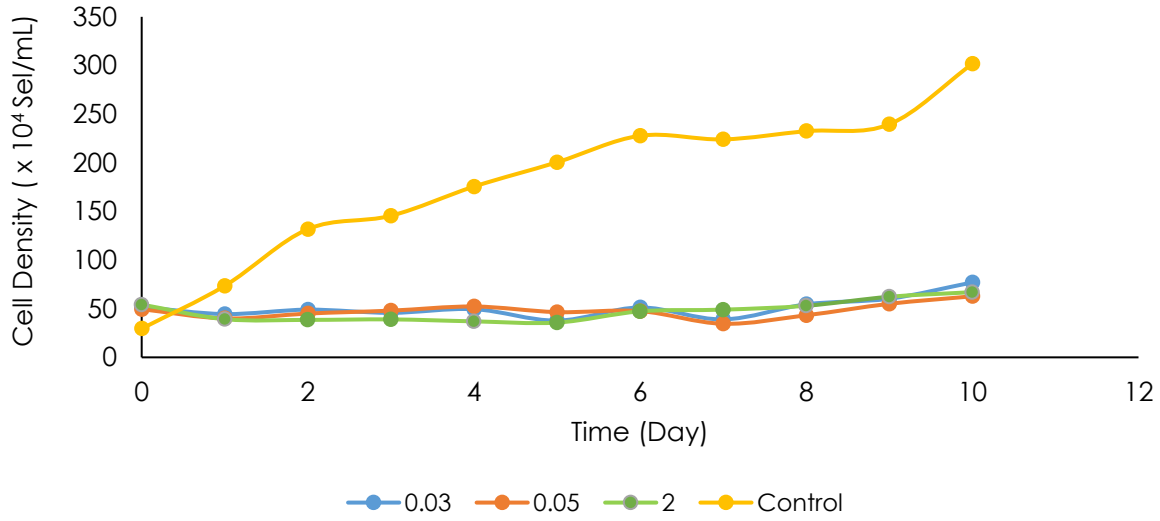

Figure 4. Growth curve of *Thalassiosira* sp. with Pb Heavy Metal Treatment

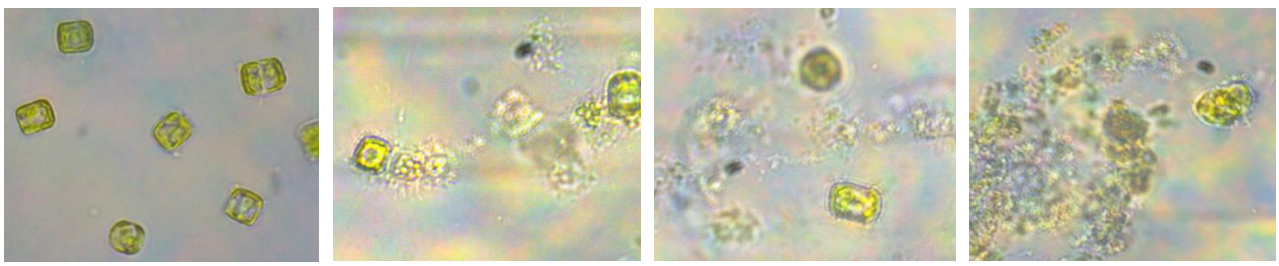

Figure 5. Morphology of *Thalassiosira* sp. Cells at Differences in Lead (Pb) Concentration with 1000x Magnification. (a.) 0 ppm, (b) 0.03 ppm, (c) 0.05 ppm, (d) 2 ppm.

The higher the concentration of Pb as shown in Figure 6, shows that the changes in *Thalassiosira* sp. cells are increasingly visible. At 0.03 ppm, cells lose their rectangular shape, and aggregation begins. At 0.05 ppm, cells swell, and pigment degradation occurs. At 2 ppm, cell aggregation becomes pronounced, accompanied by significant morphological changes. Solomonova *et al*. (2023) in their research showed that microalgae experience cell aggregation when exposed to contaminants as a form of adaptive response in cellular defense mechanisms. Chen *et al*. (2024) added that pigment degradation, such as chlorophyll loss, reduces photosynthetic efficiency and indicates cellular damage.

In several studies conducted by Bauenova *et al*. (2021), exposure to heavy metals causes changes in the structure of microalgae cell organelles. Concentrations of Cr (VI) as much as 50 mg/L and Cd (II) as much as 0.5 mg/L in the Parachlorella kessleri growth medium caused the disintegration of thylakoids in the stroma. Changes in cell organelles that function for photosynthesis will reduce the ability of microalgae to produce food. In addition, vacuole enlargement occurs due to structural changes in the cytoplasmic membrane and excessive starch accumulation.

Potential of *Thalassiosira* sp. in absorbing Heavy Metal Pb with various concentrations

Data collection of heavy metal Pb concentration on the 10th day using ICP-OES showed the ammount of absorption by *Thalassiosira* sp.. Based on Khan *et al*. (2022), ICP-OES analysis was performed simultaneously, enabling the direct quantification of metal concentrations by utilizing the compatibility of each metal's emission spectrum with a specific optical system. In this research, the Pb analysis was performed at a wavelength of 220.35 nm, and the Pb absorption efficiency of *Thalassiosira* sp. is summarized in Table 3.

Based on table 3, the average result of Pb heavy metal absorption from treatments other than control was 80% with the highest percentage being 100% in the 0.03 ppm and 0.05 ppm concentration treatments and the lowest percentage at 2 ppm was 40%. The microalgae species Scenedesmus obliquus can absorb up to 85.5% Pb through surface biosorption and intracellular bioaccumulation mechanisms (Danouche *et al*., 2022). The average result of Pb heavy metal absorption was 80%, indicating that the *Thalassiosira* sp. species is very effective in remediating Pb heavy metals. According to Raji *et al*. (2023), heavy metal absorption efficiency is categorized as very effective (>70%), effective (50–70%), fairly effective (30–50%), and less effective (<30%), depending on the sorbent type and application conditions.

Microalgae *Thalassiosira* sp. have a complex adaptive mechanism to survive Pb heavy metal stress, starting with the adsorption, where negatively charged exopolysaccharides (EPS) in the cell wall bind Pb^{2+} to reduce toxicity (Zhou *et al*., 2024). In the absorption phase, Pb^{2+} enters the cytoplasm and forms stable, non-toxic complexes with chelators like phytochelatin and metallothionein (Ferrari *et al*., 2024). During the accumulation phase, these complexes are stored in vacuoles or vesicles to prevent metabolic disruptions. In the transformation phase, Pb^{2+} is converted into a more stable and

Table 3. Pb Data Absorption by *Thalassiosira* sp.

Treatment	Pb Concentration (ppm)			Percentage of Pb Absorption Efficiency	Average Value
	Initial	Final	Total Reduction		
0 ppm (Control)	0	0	0	-	
0.03 ppm	0.03	0	0	100%	80%
0.05 ppm	0.05	0	0	100%	
2 ppm	2	1.2	0.8	40%	

non-reactive form through chelation with sulfhydryl groups (-SH) on the chelators. These stabilized complexes are either permanently stored in vacuoles or released into the environment via exocytosis. Additionally, antioxidant enzymes such as superoxide dismutase (SOD) and glutathione peroxidase are produced to mitigate oxidative stress, ensuring cells maintain their normal functions (Cavalletti *et al*., 2022; Pradhan *et al*., 2022).

The study samples indicated as *Contricriba weissflogii* provide new insights into its potential as a bioremediation agent for Pb, surpassing previous genus-level studies on other heavy metals by Saini & Dhania (2019), Mishra *et al*. (2020), and Chasapis *et al*. (2022). The study samples showed efficient adsorption, sorption, and adaptive responses to, supported by 99.53% homology of the tufA gene with Conticriba weissflogii (MH571875.1). Unlike C. weissflogii studied by Joseph *et al*. (2018), whose Pb bioremediation mechanism is still unexplored, this study reveals initial insights into Pb bioremediation through a mechanism involving adsorption, sequestration, accumulation, transformation, chelation, and formation of antioxidant enzymes, similar to its mechanism for remediating Cd (Chen *et al*., 2024). These findings confirm the compatibility of the species for Pb remediation.

CONCLUSION

This study concluded that the tufA genetic marker in *Thalassiosira* sp. isolates showed a homology level of 99.53% with Conticriba weissflogii (partial tufA gene, chloroplast, MH571875.1), making it an effective tool for molecular characterization. Exposure to Pb at concentrations of 0.03 ppm, 0.05 ppm, and 2 ppm caused significant inhibition of the growth of *Thalassiosira* sp., with Pb treatment causing cell aggregation, pigment degradation, and lysis in response to heavy metal stress. Nevertheless, *Thalassiosira* sp. showed great potential in absorbing heavy metal Pb with an average absorption efficiency reaching 80%. The highest absorption of 100% occurred at concentrations of 0.03 ppm and 0.05 ppm, while the lowest absorption of 40% occurred at a concentration of 2 ppm. These results show that *Thalassiosira sp.* could serve as an effective bioremediation agent for mitigating Pb pollution in aquatic ecosystems.

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