Molecular Characterization of *Nannochloropsis* sp. Based on *tufA* Genetic Marker and Potential Test of *Nannochloropsis* sp. as a Cadmium (Cd) Heavy Metal Bioremediation Agent

Sulis Setyowati[1], Hermin Pancasakti Kusumaningrum[2*], Muhammad Zainuri[3], Agung Suprihadi[2], Heradita Kaniaazzahra Doktorasaintifika[4], Azalia Puspa Herida[3], Candra Wahyuningsih[3], Siti Nur Jannah[2], Wijanarka[2]

[1]Biology Study Program, Biology Department, Faculty of Science and Mathematics, Diponegoro University
[2]Biotechnology Study Program, Biology Department, Faculty of Science and Mathematics, Diponegoro University
[3]Oceanography Department, Faculty of Fisheries and Marine Science, Diponegoro University
Jl. Prof Jacub Rais, Diponegoro University, Tembalang, Semarang. 50275, Indonesia
[4]Senior High School 1 Semarang
Jl. Taman Menteri Supeno No.1, Mugassari, Kota Semarang, Jawa Tengah 50249
Corresponding author: herminpk@live.undip.ac.id

Abstract

Cadmium (Cd) heavy metal pollution not only affects aquatic ecosystems but also has toxic effects on human health. Bioremediation using microalgae *Nannochloropsis* sp. is considered more economical and sustainable for overcoming heavy metal pollution. This study aims to molecular characterization of *Nannochloropsis* sp. with *tufA* gene markers, determine the effect of different Cd concentrations on the growth and morphology of *Nannochloropsis* sp. and the ability of *Nannochloropsis* sp. to absorb Cd concentrations. The methods used include DNA isolation, quantitative and qualitative DNA analysis, amplification of *tufA* gene, sequencing result analysis, phylogenetic tree analysis, and bioremediation test. The results of molecular characterization showed that *Nannochloropsis* sp. has similarities with *N. oceanica* strain BR2 plastid (CP044614.1) with 60% bootstrap value. *Nannochloropsis* sp. grew at different Cd concentration (0, 2, 4, and 6 ppm) but optimal at 0 ppm Cd concentration. Morphology of *Nannochloropsis* sp. showed morphological differences in the cell structure. The cell was broken and the color turned increasingly clear. Different concentrations of Cd metal on *Nannochloropsis* sp. growth significantly affected the decline in *Nannochloropsis* sp. growth. *Nannochloropsis* sp. has the potential to absorb heavy metal Cd with an efficiency that increases as the concentration reaching 62.6% at 6 ppm. It can be concluded that molecular characterization using the *tufA* marker was effective in showing that *Nannochloropsis* sp. had closed relation with *N. oceanica* strain BR2 plastids. Cadmium (Cd) exposure significantly reduced growth and caused cell damage, while *Nannochloropsis* sp. effectively adsorbed Cd and lowering Cd levels by 3.76 ppm.

Keywords: Bioremediation, Cadmium, ICP-OES, *Nannochloropsis* sp., *tufA*

INTRODUCTION

Heavy metals are generally found in low concentrations in the environment. Heavy metals are one of the most worrisome particulate components in the atmosphere because they can become persistent pollutants after being deposited in the environment (Lv *et al.,* 2023). Heavy metal contamination on earth has reached more than 25%, and water contaminated with heavy metals has a detrimental impact on humans due to its potential toxicity and carcinogenicity (Briffa *et al.,* 2020). One of the heavy metal elements that are generally considered is cadmium (Cd). Cd as haeavy metals has wide distribution in nature. This metal is unnecessary and too toxic for most living organisms. Its toxicity is 2 to 20 times higher than most other heavy metals (Nahvi *et al.,* 2017).

Cd heavy metal pollution has been found in several parts of Indonesia. In Saguling Reservoir, Bandung, West Java has been exposed to Cd reaching 0.31 mg/L, where the determination of the Cd threshold refers to PP No. 82 of 2001 concerning Water Quality Management and Water Pollution Control for water designation with class

*Corresponding author
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II criteria, namely the maximum level of Cd of 0.01 mg/L (Jais *et al.,* 2020). In addition, several rivers in Central Java (Kaligarang, Juwana, Bengawan Solo, Kali Agung, Kali Banger) show that the five rivers have been polluted with heavy metal cadmium (Cd) quite high because it has exceeded the threshold limit set by the World Health Organization (WHO) and the Ministry of Environment (KLH) (Susanti *et al.,* 2014).

The presence of Cd in high concentrations will pose a threat to health and the environment because Cd is a toxic inorganic metal more than the heavy metal lead (Pb) (Purnamawati *et al.,* 2015). The impact of Cd heavy metal pollution is quite dangerous, it is necessary to prevent or overcome it by designing remediation strategies. Modern biological techniques are considered more economical and can be a sustainable approach to removing heavy metals. The utilization of microalgae as bioremediation has been widely used to overcome pollution in waters, because its large availability in waters, relatively fast reproduction, wide microalgae toxicity range, many wastes that can be remediated, and is non-pathogenic (Purnamawati *et al.,* 2015).

The microalga *Nannochloropsis* sp. has potential in extreme environmental tolerance, can be used as a bioassorbent of heavy metals such as Zn^{2+} and Cd^{2+} for bioremediation purpose and easy to cultivate (Hala *et al.,* 2014; Rizal *et al.,* 2020). In this study, *Nannochloropsis* sp. used were obtained from BBPBAP Jepara and molecular characterized using the *tufA* gene. It is important in responding to oxidative stress due to heavy metal Cd by increasing the synthesis of protective proteins such as HSP70 (Viera *et al*., 2016; Khan *et al*., 2015; Mishra *et al*., 2018).

This study aims to reveal the potential of *Nannochloropsis* sp. as a bioremediation agent of heavy metal cadmium (Cd) and identify based on molecular character using genetic marker *tufA*. The potential was measured based on the efficiency of Cd absorption, the effect of Cd concentration on growth, differences in cell morphology, and cadmium metal absorption activity at various concentrations.

METHODS AND MATERIALS

The microalgae used in this study was isolates of the microalgae *Nannochloropsis* from Brackish Water Aquaculture Fisheries (BBPBAP) on Jepara, Indonesia.

Microalgal culture

Cultivation of *Nannochloropsis* sp. microalgae was carried out using Walne fertilizer at a dose of 0.1 mL per 10 mL of saline water, mixed into 200 mL of isolate and 800 mL of saline water with a salinity of 30 ppt. *Nannochloropsis* sp culture was carried out at room temperature ranging from 25°C with a white lamp (20 watt) as a source of illumination and aeration system as a source of oxygen supply for 21 days cultivation. The container was closed to avoid contamination and labeled (Kusumaningrum *et al.,* 2016). Cell density measurements were made by making a standard curve based on cell density calculation data using a haemacytometer (Muhaemin, 2016).

DNA isolation

DNA isolation of *Nannochloropsis* sp. was performed using the CTAB method of Doyle & Doyle (1987). Microalgal cells were separated from the culture medium through repeated centrifugation at 8000 rpm To obtain the pellet. The pellet ware then incubated with CTAB buffer at 65°C, homogenized, and added Chloroform Isoamyl Alcohol (CIA) before recentrifugation. The supernatant ware removed and precipitated with isopropanol at -20°C overnight. After centrifugation, the pellet ware washed with 70% alcohol, dried, and dissolved in TE buffer.

Analiysis of quality and quantity of DNA

The DNA ware measured using NanoDrop Software at wavelength 260 nm, 280 nm. The qualitative and quantitative test serves to measure the concentration and purity of DNA

Amplification of gene *tufA* Microalgae

Amplification of the *tufA* gene was performed using a thermalcycler PCR machine. Nannochloropsis sp. isolate DNA with a concentration of more than 100 ng/mL and purity of 1.8-2.0 ware used as template (Fama *et al.,* 2000; Vieira *et al.,* 2016). Amplification was performed with tufA forward (5'-GGNGCNGCNCAAATGGAYGG-3') and reverse (5'-CCTTCNCGAATMGCRAAWCGC-3') primers, using MyTaq Master Mix: 25 µL mastermix, 1 µL of each primer, 7.5 µL ddH_2O, and 3 µL template DNA. The PCR process consisted of pre-denaturation at 95°C for 1 minute, denaturation at 95°C for 15 seconds, annealing at 55°C-56°C for 15 seconds, extension at 72°C for 10 seconds, and post-extension at 72°C for 5 minutes. The PCR

mixture ware then inserted into a PCR gradient machine for annealing temperature optimization. The amplification results were analyzed using 1% agarose gel electrophoresis. A total of 3 µL of DNA sample and 3 µL of marker (1 µL loading dye and 2 µL DNA Ladder) were put into the gel wells. Electrophoresis was run at 100 volts for 25 minutes. After that, the gel was placed on a UV transluminator to visualize the DNA bands.

Sequencing and Phylogenetic Analysis

DNA sequencing of Nannochloropsis sp. was carried out by PT Genetika Science Indonesia. The sequences were edited using Bioedit software for alignment and consensus, then converted to FASTA format. Base homology analysis was performed with BLAST at NCBI (http://www.ncbi.nlm.nih.gov). This analysis compares nucleotide similarity with the Genbank database, based on Max Score, Query Coverage, E-value, and nucleotide identity (Tindi *et al*., 2017). and phylogenetic tree construction using MEGA 11 with the Neighbor-Joining tree method and a Bootstrap value of 1000 was used for phylogenetic tree construction, because this method is considered fast and produces accurate topology based on the length of the nearest branch (Moniz *et al.,* 2014).

Experimental

The experimental design for testing the potential of *Nannochloropsis* sp. as a cadmium (Cd) bioremediation agent used a completely randomized design (CRD) (Zamani-Ahmadmahmoodi *et al.,* 2020). Cd stock solution was added to the culture to achieve concentrations of 0, 2, 4, and 6 ppm, with three replicates for each concentration and a control. The experiment observed the level of Cd absorbed by *Nannochloropsis* sp. from day 0 to day 11 (exponential phase). The independent variable was Cd concentration, the control variables were temperature (25°C), light intensity (20 watts), and aeration, while the dependent variable was total Cd content.

Analysis of the Effect of Cadmium (Cd) Concentration on Growth, Morphology, and Absorption Efficiency of *Nannochloropsis* sp

Analysis of the effect of cadmium (Cd) concentration on the growth and cell density of *Nannochloropsis* sp. was conducted with an optilab microscope and hemacytometer from day 0 to the exponential phase, then analyzed with ANOVA at a confidence level of 0.05 and further tests (Selvika *et al*., 2016). Differences in cell morphology after Cd treatment were observed using a Zeiss Primostar light microscope with a magnification of 400x to 1000x. The efficiency of Cd uptake by *Nannochloropsis* sp. was measured on days 0 and 7 using ICP-OES, and calculated with the following formula:

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

Absorption 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. This provides a measure of how effectively the material or organism, in this case *Nannochloropsis* sp., was able to reduce the concentration of the contaminant, expressed in parts per million (ppm). The results of the efficiency test of the ability of *Nannochlopsis* sp. in absorbing heavy metal Cd with different concentrations are presented as a percentage (Wiyarsi and Priyambodo, 2013 in Halima *et al.,* 2019)

RESULTS AND DISCUSSION

Cell Growth of *Nannochloropsis* sp.

Nannochloropsis sp. cultivation was carried out for 21 days using a batch culture system. During the 21-day cultivation, *Nannochloropsis* sp. underwent adaptation, exponential, stationary, and death phases (Figure 1). The lag phase occurred until day 4 with an insignificant increase in density, as a process of cell adaptation to the new environment (Arfah *et al.,* 2019; Sarifah *et al.,* 2019). The exponential phase occurs on day 4 to day 11 with cell density increasing more than twice (Mukhlis *et al.,* 2017). The stationary phase occurs on days 12 to 16, and the death phase begins on days 17 to 21, characterized by a decrease in cell density (Arfah *et al.,* 2019).

On day 0, the culture colour was yellow and started to turn green on day 9, then intense green on day 11, influenced by light intensity and nutrients (Figure 2.). Excess light can cause stress in *N. oceanica*, decreasing chlorophyll a and carotenoids, but increasing zeaxanthin (Ye *et al.,* 2024). The yellow-orange color on day 0 indicates

the initial stress of the isolate due to adaptation to the new environment (Wang & Jia, 2020). The increase in zeaxanthin indicates the effort of *N. oceanica* IMET1 to stabilize the thylakoid membrane to protect against stress damage. Damage to the photosynthetic antenna system and photosynthetic reaction center II (PS II) occurs after excessive light exposure, causing pigment bleaching. The intense green color indicates the exponential to stationary phase, with a peak in cell density on day 11, it is important for determining harvest time.

Molecular Characterization of *tufA* Gene

DNA isolation of each sample was analyzed at wavelengths of 260 nm and 280 nm used to evaluate DNA purity and concentration (Onyemata *et al.,* 2021). The results in Table 1. showed that the DNA purity values of microalgae *Nannochloropsis* sp. were between 1.8 - 2.0, indicating a pure isolation with low levels of contamination. Purity values below 1.8 indicate protein or organic matter contamination, while values above 2.0 indicate contamination by phenol and RNA (Zulkarnain *et al.,* 2023). The quality of nucleic acids is indicated by the absorption ratio at 260 nm/230 nm and the degree of contamination by proteins is indicated by the absorption ratio at 260 nm/280 nm (Iqhani and Khakvar, 2020). According to Ermavitalini *et al.,* (2021) stated that DNA purity affects the success of amplification. The purer the DNA, the clearer the DNA bands formed are seen in visualization under UV light after electrophoresis.

The amplification results of *Nannochloropsis* sp. DNA isolates using the *tufA* gene are shown in Figure 3. showed the results of the amplification of the *tufA* gene in *Nannochloropsis* sp. there are one bands where the bottom band looks smear called the dimer primer. Dimer primers are formed when two or more primers that stick together and bind instead of binding the desired DNA target (Xie *et al.,* 2022). Primers are short nucleotides measuring 12-20 bases that are used as attachment points for DNA polymerase enzymes during the DNA elongation process of a specific gene through polymerization chain reaction (PCR), so dimer primers also have a short size which is generally below 100 bp. The existence of dimer primers is due to inappropriate or insufficiently high annealing temperatures.

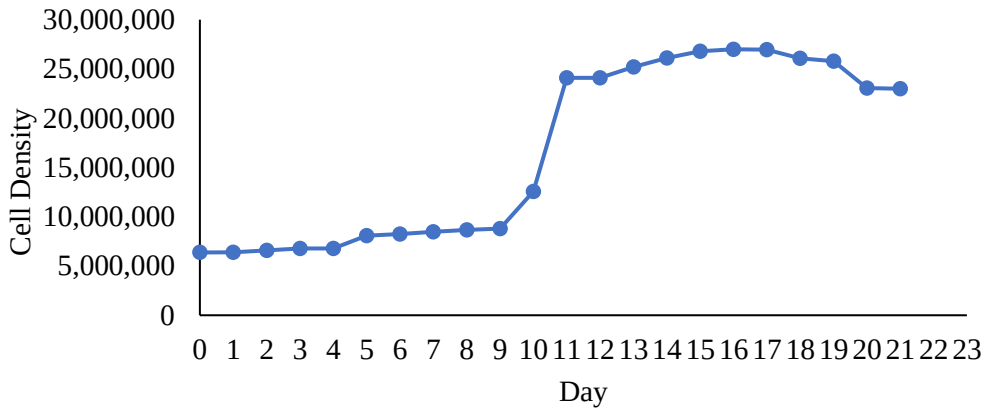


Figure 1. *Nannochloropsis* sp. growth curve

(a) (b) (c)

Figure 2. Culture of *Nannochloropsis* sp. (a) Day 0, (b) Day 9, (c) Day 11

Of the three visible bands, the clear and thick band was taken because it showed the best amplification results at 56ºC annealing temperature. In the research of Khosravinia *et al.,* (2023) stated that *Nannochloropsis* oceanic obtained a base pair size with the *tufA* gene of 769 bp, this has been recorded in GenBank with the accession number MN721843. This is also supported by Fama *et al.,* (2002) in his research with the *tufA* gene which obtained base pair sizes in several types of Chlorophyta which ranged from 758-901 bp. Viera *et al.,* (2016) stated that DNA amplification in Chlorophyta using the *tufA* gene as a genetic marker obtained a base length of about 826 bp.

The sequencing results showed that the isolates of *Nannochloropsis* sp. there are similarities with several species contained in the GenBank database. Based on the database, the highest similarity of *Nannochloropsis* sp. isolates with the *tufA* gene was *N. oceanica* strain BR2 plastid sequence ID CP044614.1 (Table 2.). Based on the results of sequence analysis of *Nannochloropsis* sp. samples with *N. oceanica* strain BR2 plastid ID sequence CP044614.1 has a fairly low percent identity value and a fairly high Max score value.

Tomar *et al.,* (2014) stated that in BLAST selection, the higher the score and the smaller the e-value, the better the result. Pearson (2013) added that percent identity above 30% indicates conservative similarity, although values below 20% can still show homology if supported by relevant e-values. An e-value of 0.0 indicates similarity, and score bits of more than 50 indicate reliable results. Phylogenetic analysis showed that the *Nannochloropsis* sp. sequences in this study are not in the same clade as *N. oceanica* strain BR2, with a weak bootstrap value of 60% (Figure 4.), it is still considered better than morphological analysis.

Effect of Different Concentrations of Cd Metal on the Growth of *Nannochloropsis* sp.

Cell harvesting for testing the concentration of Cd metal on the growth of *Nannochloropsis* sp. was carried out during the exponent phase precisely on day 11. The exponent phase was

Table 1. NannoDrop Spectrophotometer Results of DNA of microalgae *Nannochloropsis* sp.

Sample	Concentration (ng/µL)	Ratio A_{260}/A_{280}	Ratio A_{260}/A_{230}
Nannochloropsis sp.	256.8	1.82	1.61

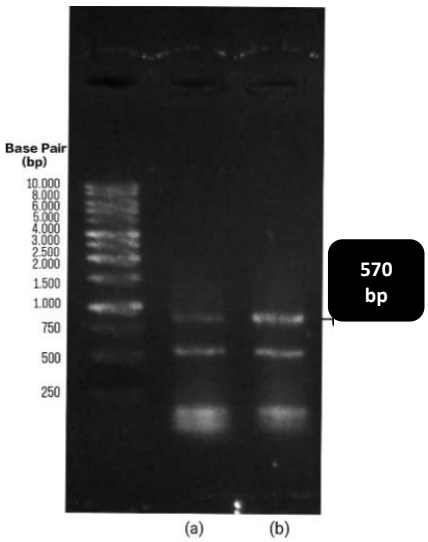


Figure 3. Visualization of *tufA* fragments of *Nannochloropsis* sp.
Note: (a) 55°C annealing; (b) 56°C annealing.

characterized by the most active cell growth rate so that the cells divide rapidly and the number increases exponentially. In addition, cell metabolic activity is very active in absorbing nutrients and proliferating. High metabolism causes cells to more actively absorb and accumulate substances from their environment, including heavy metals.

Table 2. *Nannochloropsis* sp. sequence homology results

Sample	Results BLAST	Accession	E-value	Query Cover (%)	Per Ident (%)	Max Score
Nannochloropsis sp.	*Nannochloropsis oceanica*	CP044614.1	2e-15	61	65.34	80.6

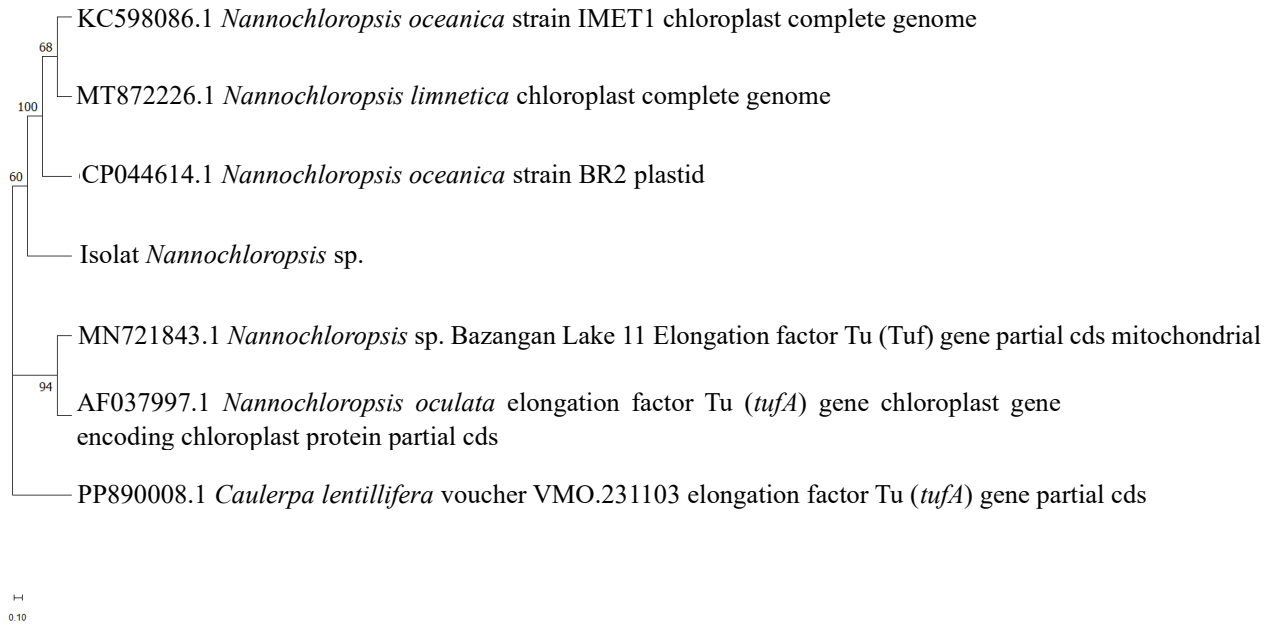


Figure 4. Phylogenetic tree of isolates of *Nannochloropsis* sp.

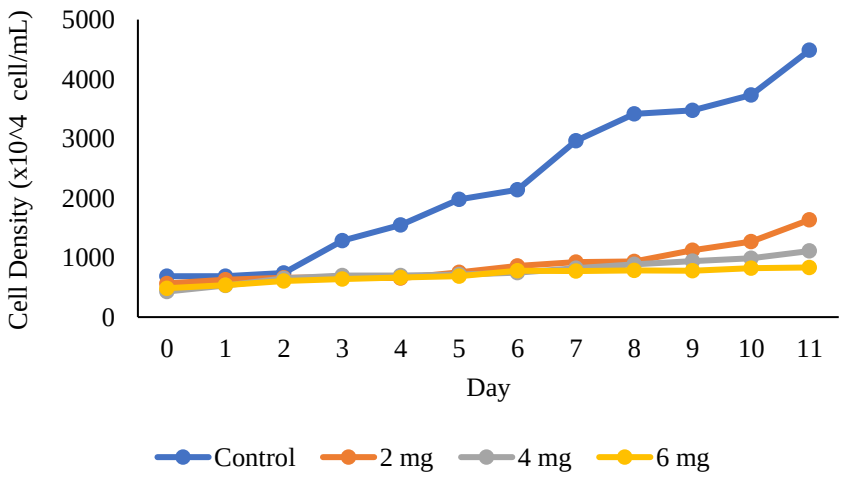


Figure 5. Growth curve of *Nannochloropsis* sp. with the treatment of Cd concentration

Based on the growth curve graph in Figure 5., the control treatment showed the highest *Nannochloropsis* sp. cell density in the exponential phase, it was 4485.5 x 10^4 cells/mL, compared to cultures treated with heavy metal Cd at various concentrations (0, 2, 4, and 6 ppm). Permana *et al.,* (2022) exposure to cadmium has been shown to affect the physiology and morphology of phytoplankton by inhibiting cell growth, damaging chloroplasts and photosynthetic pigments such as chlorophyll (chlorosis).

The inhibitory effect of Cd on the growth of *Nannochloropsis* sp. depends on the concentration given. In the treatment of Cd concentrations of 0, 2, 4 and 6 ppm showed a difference in the amount of cell density where the lower the concentration of Cd given, the higher the cell density. This is because the higher the concentration of Cd given, the greater the toxic effect experienced by *Nannochloropsis* sp. cells.

One Way Anova test showed that the concentration of heavy metal cadmium (Cd) has a significant effect on the growth of *Nannochloropsis* sp., with F count of 288.61 greater than F table 4.07 at a significant level of 0.05. BNT (LSD) 0.05 further test showed that the concentration of 6 ppm was significantly different from 0, 2, and 4 ppm, and 0 ppm was significantly different from 2, 4, and 6 ppm, but the concentrations of 2 and 4 ppm showed no significant difference.

Effect of Cd Metal with several different concentrations on *Nannochloropsis* sp. cell

This study observed the effect of heavy metal Cd on the morphology of *Nannochloropsis* sp. using a microscope. Figure 6. showed that the higher the concentration of Cd, the more cell damage, such as changes in shape, color, and cell wall damage. In the control, *Nannochloropsis* sp. cells was normal with a yellowish green color and round shape, in accordance with the characteristics of cells according to Santri *et al.* (2021). However, at Cd concentrations of 2, 4, and 6 ppm, the cells changed to pale, not round, and showed damage to the cell walls that were broken or cracked.

The higher the Cd concentration as shown on Figure 6. exhibited that, the more severe the cell damage of *Nannochloropsis* sp. At 2 ppm, cell wall lysis occurred at 4 ppm the cell color changed and wall damage became more obvious. While at 6 ppm, the cells became pale to transparent, the cell wall was severely lysed, and some cells changed morphology. Carfagna *et al.* (2013) stated that Cd inhibits PS II by damaging thylakoids and reaction centers, visible from ultrastructural changes and damage to photosynthetic organelles in cells. Kusuma and Zulaika (2014) added that in *Chlorella* sp., Cd treatment causes cell death, pale color changes, and cell wall rupture.

Potential of *Nannochloropsis* sp. in absorbing Heavy Metal Cd with various concentrations

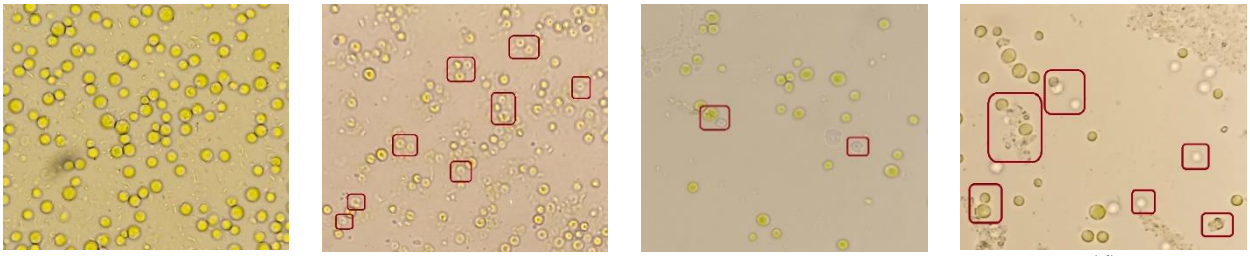
Data collection of heavy metal Cd concentration on day 11 using ICP-OES showed the amount of absorption by *Nannochloropsis* sp. According to Rinawati *et al.* (2008), ICP-OES analysis was carried out simultaneously, allowing direct measurement of metal levels due to the suitability of the emission spectrum of each metal with a particular optical system. In this study, the Cd assay was conducted with a wavelength of 214.43 nm, and the efficiency of Cd absorption by *Nannochloropsis* sp. is presented in Table 3.

Based on Table 3. *Nannochloropsis* sp. showed the ability to absorb heavy metal Cd, with the highest decrease of 62.6% at a concentration of 6 ppm, and an average absorption of 24.2%. Cd absorption increases with higher concentrations, in accordance with the statement of Widiyani and Dewi (2014) on *Chlorella vulgaris* and Hala *et al.,* (2013) on *N. salina* which reached 92.92% at 10 ppm Cd. Absorption efficiency is influenced by cell size, surface area, and cell wall functional group content. Other studies have also shown *Nannochloropsis* sp. to be effective in remediating other metals such as Cu^{2+} (Martínez-Macias *et al.,* 2019).

The absorption of heavy metal Cd by *Nannochloropsis* sp. occurs in two phases: adsorption (passive phase) and absorption (active phase). The passive phase involves absorption on the algal cell wall through carboxyl, hydroxyl, and sulfate phosphate groups (Kaplan, 1988; Naja & Volesky, 2011). High adsorption on *Nannochloropsis* sp. occurs due to the presence of functional groups such as amines and carboxylates that allow strong interactions with metal ions (Vishnu, 2006; Nisak *et al.,* 2013). In the active phase, Cd is absorbed through the cell membrane, bound by proteins such as metallothionein (MT) and phytochelatins (PCs), which help reduce metal toxicity through chelation (Balzano *et al.,* 2020; Yan *et al.,* 2023). After binding, heavy metals enter the vacuole, where ions and metabolites are stored until the cell reaches saturation and dies.

Table 3. Data of Cd absorption by *Nannochloropsis* sp.

Treatment	Cd (Concentration ppm)			Percentage of Cd absorption efficiency*	Mean
	Initial	Final	Initial -Final		
0 ppm	0	0	-	-	24,2%
2 ppm	2	1.42	0.58	29%	
4 ppm	4	1.82	2.18	54.5%	
6 ppm	6	2.24	3.76	62.6%	



(a) (b) (c) (d)

Figure 6. Morphology of *Nannochloropsis* sp. after treatment with different concentrations of Cd microscopically at 1000x magnification Description: red box indicates that *Nannochloropsis* sp. cells have been damaged and morphological changes from what it should be. 0 ppm (a), 2 ppm (b), 4 ppm (c), 6 ppm (d).

Organic compounds in the cell, including chlorophyll, help bind heavy metals such as Cd through reactive groups, forming complex compounds that stabilize and accumulate metals in the cell, as well as increase the concentration of H^+ ions to maintain internal pH balance (Purnamawati *et al.,* 2015). This allows *Nannochloropsis* sp. to adapt and survive in polluted conditions, minimizing Cd poisoning. Research by Hassan *et al.* (2023) showed that *N. oculata* can improve kidney function and reduce oxidative stress in sheep exposed to Cd. It is highly efficient in reducing Cd (Table 3) and adapting in polluted environments.

CONCLUSION

This study concluded that the genetic marker *tufA* in *Nannochloropsis* sp. isolates showed similarity to *N. oceanica* strain BR2 plastids, making it an effective tool for molecular characterization. Cadmium (Cd) application significantly impacted growth reduction, causing cell structure damage and discoloration due to chloroplast damage. In addition, *Nannochloropsis* sp. showed high potential in adsorbing Cd, with the highest absorption efficiency of 62.6% at a concentration of 6 ppm, successfully reducing Cd levels in the contaminated environment by 3.76 ppm.

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