Journal of Tropical Biodiversity and Biotechnology
Volume 08, Issue 03 (2023): jtbb88143
DOI: 10.22146/jtbb.88143

Research Article

Bioremediation of Mercury- Polluted Water in Free Water Surface-Constructed Wetland System by *Euglena* sp. and *Echinodorus palifolius* (Nees & Mart.) J.F. Macbr.

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Keywords:
Anatomy
Euglena
Echinodorus palifolius
FWS-CW
growth

Submitted:
19 August 2023

Accepted:
30 November 2023

Published:
13 December 2023

Editor:
Furzani Binti Pa' ee

ABSTRACT

Mercury accumulation in the aquatic environment can be highly harmful. The body takes mercury vapor through the lungs, then absorbs mercury metal through the digestive system, and then the blood carries the metal to the brain. Bioremediation is the process of breaking down or converting harmful compounds into non-toxic forms, which can be accomplished through phytoremediation or phycoremediation. The goal of this study was to examine the growth and anatomy of *Euglena* sp. after being cultured in the mercury-containing FWS-CW waste treatment system. The ability of *Euglena* sp. and *Echinodorus palifolius* to bioremediate mercury at different concentration as well as association and non-association treatments. This study was carried out in a bioreactor known as FSW-CW (Free Water Surface-Constructed Wetlands). Plant growth (plant height and number of leaves), chlorophyll content, diameter of root and petiole, metaxylem diameter of root, petiole, and leaves, cortical thickness of root and leaves, and petiole anatomy were all measured. Water temperature, pH, salinity, and light intensity were all measured as environmental parameters. Mercury treatment reduced *Euglena* density (183.5 cells. $mL^{-1}10^3$ in control and 12.6 cells. $mL^{-1}10^3$ in 100 ppm mercury treatment) and number of *E. palifolius* leaves, but not plant height and chlorophyll. Root and petiole diameters were affected by the mercury treatment, petiole diameter decreased unless the concentration was 100 ppm, whereas root diameter actually increased. The diameter of the root metaxylem increased, but the petioles and leaves, as well as the thickness of the root cortex, did not provide a significant response. The growth of *E. palifolius* was still optimal in the presence of *Euglena* in mercury-containing medium.

INTRODUCTION

Heavy metals and metalloids including mercury are known to be extremely poisonous and carcinogenic, which puts human health and ecological variety at serious risk (Leong & Chang 2020; Tripathi & Poluri 2021) Hg metal will accumulate in the environment and can precipitate and form complex compounds with organic and inorganic materials. Mercury can accumulate in the environment and contribute to global mercury pollution (Al-Sulaiti et al. 2022). Humans are exposed to mercu-

ry both directly and indirectly. Direct exposure occurs when mercury vapor (HgO) oxidises in the atmosphere or when mercury metal ($MeHg^{+}$) forms. Inorganic mercury ions (Hg^{3+}) are another kind of mercury exposure. The body takes mercury vapor through the lungs, then it absorbs mercury metal through the digestive system and then the blood, which carries the metal to the brain. One effort to overcome mercury pollution is phytoremediation (Ekawanti & Krisnayanti 2015; Abad et al 2016; Krisnayanti & Probiyantono 2020). Heavy metals will be degraded in plant tissue (Dixit et al. 2015) or evaporates through the transpiration process into the atmosphere (Kumar et al. 2023). Phytoremediation using the constructed wetlands method which utilises media and plants as a way to reduce pollutant levels is an alternative for dealing with environmental pollution (Metcalf 2003; Bilgaiyan 2023). Prasetya et al. (2020) showed a decrease in mercury in the water media given by *E. palifolius* and zeolite in the SSF-CW (Subsurface-Constructed Wetlands) waste treatment system. *E. palifolius* or water jasmine is an ornamental plant that can live in various seasons and is able to act as a pollutant reducer by expanding the area of microorganisms on the roots and forming an oxygen-rich rhizosphere zone (Sari et al. 2018). *E. palifolius* can reduce the phosphate content by 93.81% in the Free Water Surface-Constructed Wetlands (FWS-CW) system (Prasetya et al. 2020). FWS-CWsystem utilises surface water flow and interactions between vegetation and biofilm bonds in the water phase through microbial degradation, filtration, and sedimentation. The Subsurface Flow System drains wastewater horizontally through granular media and passes through contact with aerobic, anoxic, and anaerobic zones on the surface (Knight et al. 2000; Vymazal 2013; Stefanakis et al. 2014).

As of now, thermal treatment and capping and dredging have been used to reduce mercury levels in the environment, which is the best method for minimising mercury contamination in aquatic systems (Wang et al. 2004; Kumar et al. 2023). However, these methods are quite expensive. Solitary algae and *Cryrtomium macrophyllum*, which have high resistance to mercury are still being used in efforts to develop mercury clean up agents. No species that can behave as hyperaccumulators have been discovered, despite efforts to improve Hg solubility by adding chelators like potassium iodide, sodium thiosulfate, and ammonium thiocyanate (Xun et al. 2017). Due to low biomass, delayed phytoremediation processes, and slow plant development, heavy metal hyperaccumulators are hard to detect (Singh et al. 2021).

Algae can regenerate quickly and high biomass (Devars et al. 2000; Majid et al. 2014). *Euglena* can convert Hg^{2+} to Hg^{0} with mercury reductase activity. Estimates of the biological evaporation rate of Hg^{2+} range from 0.7 to 4 nmol (10^{6} cells/hour) (Rodriguez-Zavala et al. 2007). *E. gracilis* wild type (Z-strain) and *E. gracilis* var. saccharophila (B-strain) tolerates and accumulates beat metals up to 1000 ppm Pb, 600 ppm Cd, and 80 ppm Hg. The mechanism of *E. gracilis* to tolerate and to absorb heavy metals involves the adsorption of metal ions to the cell wall or intracellular binding to thiol compounds and finally accumulating in chloroplasts, mitochondria, and cytoplasm for detoxification (Moreno-Sanches et al. 2017). Algae have a significant contribution in removing heavy metals from solution (Danouche et al. 2021). Microalgae can also be used as a potential reservoir for removing toxic heavy metals such as lead (Pb), mercury (Hg), cadmium (Cd) and arsenic (As) (Kumar et al. 2018). Bioremediation using mercury (Hg)-volatilising and immobilising bacteria is an eco-friendly and cost-effective strategy for Hg-polluted farmland (Chang et al. 2022)

Plants can reduce metal uptake by exuding organic acids such as citric, malic and oxalic exudate which chelate metal ions in the soil. Water-soluble mercury (Hg^{+}, Hg^{2+}) is often retained by cell wall components. In root aplastic transport, Hg^{2+} can bind to oxygen-containing molecules, such as organic acids or sulphur-rich proteins in the cell wall, such as extension and expansions (Shah et al. 2021). This study is innovative in the way it employs groups of higher plants (*E. palifolius*) and algae (*Euglena* sp.) to remove mercury from the Free Water Surface-Constructed Wetlands (FWS-CW) waste treatment system. The strain of *Euglena* utilised is IDN 28. *Euglena* sp. solitary has been proven to remediate mercury, as has *E. palifolius*. These two types of living creatures are associated with reducing the concentration level of mercury in water. *E. palifolius* can chelate toxicants, while algae can absorb metals (Ubando et al. 2021). So, the association of the two is expected to have a better impact on heavy metal remediation. The purpose of this study was to analyse the growth of *Euglena* sp. and anatomy of leaves, petiole and roots of *E. palifolius* after being grown in the mercury-containing FWS-CW waste management system and mercury content in chloroplasts of *Euglena* sp.

MATERIALS AND METHODS

Materials

Materials used in this research were *Euglena* culture, *E. palifolius*, *Euglena* sp. strain IDN 28 mono-culture obtained from Nogotirto Algae Park (microalgae cultivation at Yogyakarta, Indonesia) while *E. palifolius* was obtained from rice fields in Bantul, Yogyakarta, Indonesia which had been acclimatised before treatment.

Methods

Medium preparation and mercury treatments

Euglena sp. was cultivated in regular mass cultivation medium with 5 salinity treatments for 18 days. The mercury level used in the study was 0, 25, 50, 75 and 100 ppm, respectively. The justification of mercury was carried out by Hg_2Cl addition. Before cultivating *Euglena* sp., CM medium was made into 1 litter of distilled water. Composition of CM medium was : $(NH_4)2SO_4$, KH_2PO_4, $MgSO_4$ $7H_2O$, $CaCl_2$ $2H_2O$, $Fe_2(SO_4)_3$ $7H_2O$, $MnCl_2$ $4H_2O$, $CaSO_4$ $7H_2O$, $ZnSO_4$ $7H_2O$, $CuSO_4$ $7H_2O$, Na_2MoO_4 $2H_2O$, Vitamin B1, Vitamin B12, H_2SO_4. *Euglena* sp. grown in CM medium under light intensity conditions of 2000 lux (Khatiwada et al. 2020). The culture was placed at a temperature of 25ºC, and aerator circulation was carried out at a speed of 150 rpm.

The treatment used in the study were E1 (*Euglena* sp. with 0 ppm mercury), E2 (*Euglena* sp. with 25 ppm mercury), E3 (*Euglena* sp. with 50 ppm mercury), E4 (*Euglena* sp. with 75 ppm mercury), E5 (*Euglena* sp. with 100 ppm mercury), P1 (*Euglena* sp. and *E. palifolius* with 0 ppm mercury), P2 (*Euglena* sp. and *E. palifolius* with 25 ppm mercury), P3 (*Euglena* sp. and *E. palifolius* with 50 ppm mercury), P4 (*Euglena* sp. and *E. palifolius* with 75 ppm mercury), and P5 (*Euglena* sp. and *E. palifolius* with 100 ppm mercury) based on previous study.

Growth measurement

The number of *Euglena* sp. was counted using a manual cell counting method with a modified haemocytometer (Suyono et al. 2015). *Euglena* sp. cell culture was homogenised and then taken as much as 900 microliters. The sample was then put into a 2 mL microtube and added with 100 microliters of 70% alcohol. The sample was then placed on a 1mm

Neubauer haemocytometer and observed under a light microscope, then the image in the microscope was observed with Optilab. After the image from the optilab was saved, the number of cells in the sample was counted. The cells counted were in the five medium boxes in the large box in the middle of the haemocytometer section. The parts of the medium box that were measured are the top right and left corners, the bottom right and left corners, and the middle. After obtaining the calculation results, they were then entered into the formula. Measurements were taken on day 9th. The calculation of specific growth rate of *Euglena* sp. was using specific growth rate formula:

Number of cells (cells/mL) = the measurement result x 5 x 10^4 sel/mL (Suyono et al. 2015). During treatment, measurements of the plant's height of *E. palifolius*, number of leaves were taken on day 18th.

Anatomy Measurements

Anatomical preparations were made using the paraffin method using Johansen's protocol (1940). Samples were sliced with a rotary microtome with a thickness of 6-12µm. The preparations were dried on a hot plate at 45°C until Canada balsam was dry. Observations made with the aid of an optilab-equipped light microscope. Measurements were made with the Image Ruster application. The parameters measured were root, petiole and leaf metaxylem diameters, root and petiole diameters and root and leaf cortex thickness.

ANOVA was used to assess growth data for *Euglena* sp. and *E. palaefolius* with a 95% confidence level, followed by the DMRT test (for quantitative data) with software IBM SPSS 2.0 and Graphpad Prims 10.

RESULTS AND DISCUSSION

The Environment Parameter

Environmental parameters measured include temperature, salinity, pH and light intensity. The average daily temperature is 32.4°C (Table 1 and 2) The average intensity of sunlight is 3,568 lux taken at 10.00 am. All treatment has not significantly different temperature and intensity of light.

The salinity of treatment E1 (control treatment *Euglena* sp.) was quite high and differ from E2, E3, E4 and E5. ($p < 0.05$) (Table 2) Meanwhile, the association *Euglena* sp. and *E. palifolius* for mercury in P1, P2 and P3 treatments showed almost the same salinity but not in P4 and P5. The pH of the water in all treatments showed acidic conditions or low pH, both remediation with *Euglena* alone and the association of *Euglena* with *E. palefolius*. All treatment has not significantly different pH. This means that the optimum pH for the life of *Euglena* sp. fulfilled. In con-

Table 1. The environment parameter in FWS-CW reactor with *Euglena* sp.

Concentrations of $HgCl_2$ treatment	E1	E2	E3	E4	E5
Water temperature (°C)	$31.7^a \pm 2$	$32.4^a \pm 1.5$	$32.7^a \pm 1.5$	$32.6^a \pm 1.5$	$32.5^a \pm 1.5$
Salinity (ppm)	$1149^b \pm 325$	$660^a \pm 299$	$438^a \pm 228$	$601^a \pm 43$	$650^a \pm 77$
pH	$1.5^a \pm 0.5$	$1.8^a \pm 0.2$	$1.9^a \pm 0.2$	$1.9^a \pm 0.2$	$1.8^a \pm 0.5$
Light intensity (lux)	$3355^a \pm 1{,}958$	$2646^a \pm 756$	$2750^a \pm 964$	$3064^a \pm 1{,}060$	$3468^a \pm 1{,}457$

Note: E1, E2, E3, E4, and E5 please see Method section, medium preparation and mercury treatments. Different superscript letters above number indicate significant ($p<0.05$) difference.

Table 2. The environment parameter in FWS-CW reactor with *E. palifolius* and *Euglena* sp.

Concentrations of $HgCl_2$ treatment	P1	P2	P3	P4	P5
Water temperature (°C)	32.2[a]±1.7	32.6[a]±1.6	32.6[a]±1.6	32.4[a]±1.6	32.8[a]±1.8
Salinity (ppm)	652[a]±85	599[a]±172	650[a]±275	1025[b] ±68	1128[b]±273
pH	1.9[a]±0.7	2[a]±0.8	2.3[a]±1.05	1.6[a]±0.4	1.5[a]±0.04
Light intensity (lux)	3884[a]±2728	2967[a] ±1,516	3449[a]± 1,560	3752[a] ±1658	3752[a] ± 2767

Note: P1, P2, P3, P4, and P5 please see Method section, medium preparation and mercury treatments. Different superscript letters above number indicate significant ($p<0.05$) difference.

trast to the other pH settings, *Euglena* sp. exhibited the maximum growth rate at pH 3.5 (Nurafifah et al. 2023).

The growth rate of *Euglena*

The growth pattern of *Euglena* sp. in the mercury treatments of 0 ppm, 25 ppm, 50 ppm, 75 ppm and100 ppm is presented in Figure 1.

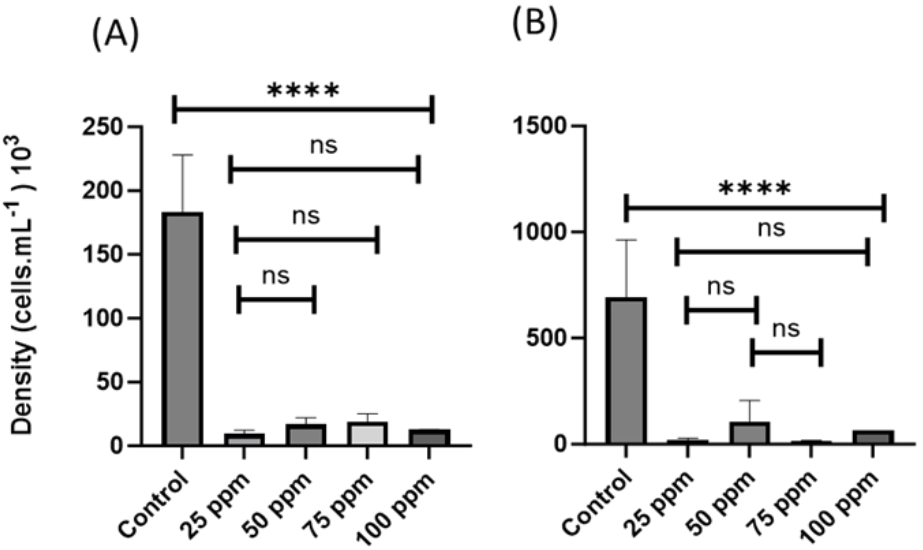

Figure 1. Density of *Euglena* sp. (A) and density of *Euglena* sp. in association with *E. palifolius* (B) in FWS-CW reactor on day 9th. Note: ****= significant ($p<0.05$) difference. Ns= no significant difference.

Euglena's density was highest in control *Euglena* and *Euglena* association with *E. palifolius*. However, the density of *Euglena* in the P4 treatment (75% mercury treatment in the *Euglena* and *E. palifolius* association) was higher than the other mercury treatments).

Based on Figure 1B. *Euglena*'s density was highest in controls (P1) and P3 showed the highest density among other mercury treatments. This shows that *Euglena* is able to survive in high concentrations of mercury with the association of *E. palifolius*. This is possible because *Euglena* is able to convert Hg^{2+} to Hg^0 with mercury reductase activity (Khatiwada et al. 2020). The estimation of the biological evaporation rate of Hg^{2+}, range from 0.7 to 4 nmol (10^6 cells/hour) (Rodriguez-Zavala et al. 2007). *E. gracilis* wild type (Z-strain) and *E. gracilis* var. saccharophila (B-strain) tolerates and accumulates beat metals up to 1,000 ppm Pb, 600 ppm Cd and 80 ppm Hg. The mechanism of *E. gracilis* to tolerate and absorb heavy metals involves adsorption of metal ions to the cell wall or intracellular binding to thiol compounds and finally accumu-

lation in chloroplasts, mitochondria and cytoplasm for detoxification. (Moreno-Sanches et al. 2017; Hader & Hemmersbach 2022). Algae have a significant contribution in removing heavy metals from solution (Danouche et al. 2021). Carboxyl, hydroxyl, sulphate and amino groups containing elements O, N, S, P, play a direct role in the binding of heavy metal ions (Rangabhashiyam & Balasubra-Manian 2019; Danouche et al. 2021). The ability to photosynthesise, motility abilities, and stress-sensitive *Euglena* pigments (heavy metals, salinity and toxins) are the reasons for the ease of bioassay measurements on algae (Ahmed & Hader 2010). The IAA hormone synthesised from the roots of *E. palifolius* can influence the growth of Euglena (Hakim et al. 2023).

The addition of mercury treatment aims to determine whether the microalgae can be resistant to the stress in their surroundings. Heavy metals such as mercury are one of the heavy metals that are produced by human activities today. Microalgae are very sensitive to this substance, and their growth rates and biological macromolecules will undergo physiological and biochemical changes due to the influence of heavy metals. Based on the hormesis phenomenon, low mercury concentrations can stimulate the growth and metabolism of microalgae. That way even though there is exposure to heavy metals, microalgae can still grow but with a note that heavy metals are used on a small scale (Abdelfattah et al. 2023). The ability of algae to grow and develop under stressful conditions has also been reported by Rangkuti et al. (2023) that under salinity stress conditions of 10 ppm, *Spirulina* grew after the 10th day.

The Growth of *E. palifolius*

The number of leaves (Figure 2A) of *E. palifolius* control remained the highest. Meanwhile, all mercury treatments showed no significant difference in the number of leaves. This indicates a substantial effect of mercury stress on the number of *E. palifolius* leaves within 18 days of treatment. The plant height of *E. palifolius* (Figure 2B) at 25 and 50 ppm mercury treatment was not significantly different with control but not with the 75 and 100 ppm treatments. This shows that the high growth of *E. palifolius* is still tolerant to mercury at concentrations of 25 and 50 ppm. This is possible because *E. palifolius* can neutralise mercury from water. One of the biological mechanisms of metal hyperaccumulation is through the interaction of the rhizosphere, namely the process of interaction of plant roots with the planting medium (soil and water). Hyperaccumulator plants can dissolve metal elements in the rhizosphere and absorb metals, so that the absorption of metals by hyperaccumulator plants exceeds that of normal plants (McGrath et al. 1997; Fu et al. 2021; Shah et al. 2021).

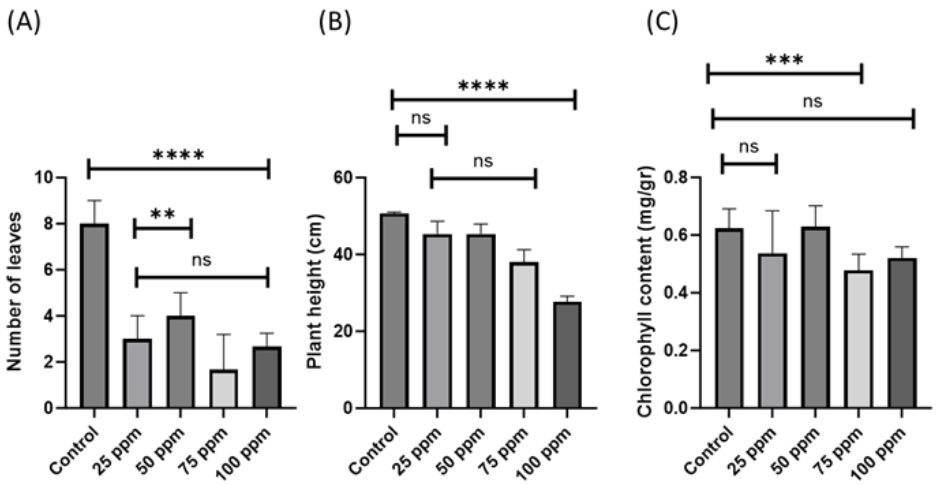

Figure 2. Number of leaves (A), plant height (B), and chlorophyll content of *E. palifolius* in mercury treatments on day 18th (C). Note: ****= significant ($p<0.05$) difference. Ns= no significant difference.

There is no significant difference in the total chlorophyll content in *E. palifolius* (Figure 2C) in treatment and control. Evidence that the chlorophyll content was not significantly different between the control and the treatment showed no effect of mercury treatment on the chlorophyll content of *E. palifolius* leaves. The heavy metal stress can substitute Mg ions inside the chlorophyll structure. (Yan & Hao 2018). Unlike carotenoids, the emphasis on the excess antioxidant capacity of carotenoids can cause a decrease in carotenoids. It can cause the carotenoid structure to be damaged by toxic ion pressure (Zamani-Ahmadmahmoodi et al. 2020; Indahsari et al. 2022). However, in contrast to *Spirulina*, carotenoids increased at a salinity of 10‰-30‰ (Rangkuti et al. 2023). The contents of carotenoid were higher than total chlorophyll (Gojkovic et al. 2022).

The diameter of petiole (Figure 3A) of *E. palifolius* in the 100 ppm mercury treatment actually had the largest diameter (0.15 µm), while control had 0.08 µm and treatment 25 ppm mercury had 0.1 µm diameter of petiole. The diameter of the petiole in the 100 ppm treatment was not significantly different from the control but significantly different from the other treatments. This shows that the 100 ppm treatment encouraged *E palifolius* plants to spread their petioles. Figure 4 clearly shows the petiole aerenchyma treated at 100 ppm broader. This aerenchyma is used by plants in addition to being able to stay afloat and provide air supply for respiration; it is also used to store mercury that plants have absorbed.

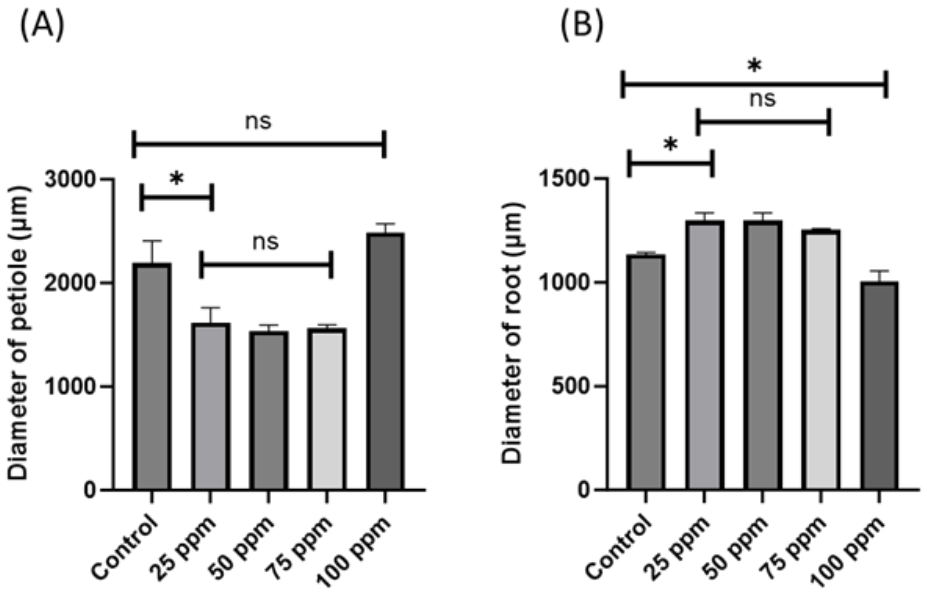

Figure 3. Diameter of petiole (A) and diameter of root of *E. palifolius* in mercury treatments on day 18th (B). Note: *= significant ($p<0.05$) difference. Ns= no significant difference.

The diameter of root (Figure 3B) in the 25, 50, and 75 ppm of mercury treatments was larger than the control (*Euglena* and *E. palifolius* association). There is a kind of root mechanism to counteract the pollutant in the form of mercury, so that at high concentrations the roots grow stretched rapidly as happened with transgenic tobacco (Hussein et al. 2007) and stem of *E. palifolius* (Wardana et al. 2023).

Anatomy of the rhizome of *Echinodorus* sp. those grown in aquatic systems showed vascular cylinders containing xylem and phloem cells. In contrast, those produced in terrestrial systems showed cortex with air gaps and amyloplasts with parenchyma cells in the cortex and the centre cylinder. Anatomy of the trunk of *Echinodorus* sp. those that grow on the ground are very similar to *Echinodorus* sp. those that grow in water. The rhizome has a cortex formed by aerenchyma with little space between cells. The central cylinder is of the atactostele type, transport bundles distributed randomly on the stele (Claro et al. 2009).

The petiole anatomy of *E. palifolius* in media containing 25, 50, 75, and 100 ppm of mercury appeared to have an anomaly, there is an accumulation of mercury inside the epidermis and around the metaxylem (Figure 4). It can be seen that the cells that make up the transport bundles in the control treatment are much larger and rounder than the other treatments, whereas in the P5 treatment, the cells that make up the transport bundles are smaller.

The root diameter (Figure 3A) corresponds to the root metaxylem diameter, indicating that metaxylem growth promotes root diameter enlargement. The diameter of the roots of *E. palifolius* in the treatment of mercury 25.50 and 75 decreased but increased again at a high concentration of 100 ppm. The high mercury concentration encourages metaxylem to increase in diameter to absorb mercury and store it in cell organelles (Marrugo-Negrete et al. 2015).

The petiole diameter (Figure 3A) decreased at concentrations of 25, 50, and 75 and increased at a concentration of 100 ppm, which was inconsistent with the metaxylem petiole diameter because the petiole diameter was supported by the growth of aerenchyma used as a mercury reservoir (Figure 5). Plants that are submerged and in anoxic environments will change in terms of morphology and anatomy. They will, specifically, have a lot of aerenchyma (Yuan et al. 2022).

Under stress conditions on heavy metals, aquatic plants such as *E. palifolius* will experience anatomical changes, mainly in the roots. This condition is due to the role of the heart of the first gate of defence against pollutants. This anatomical difference can be seen in the diameter of the metaxylem and the thickness of the cortex. The cortical cells of stressed aquatic plants will show an increase. The aerenchyma of aquatic plant roots experienced a significant increase in heavy metal stress and metaxylem. This condition is an attempt by plants to accumulate heavy metals, so they do not enter further and become toxic to plants (Batool et al. 2014; Napaldet et al. 2019; Li et al. 2023).

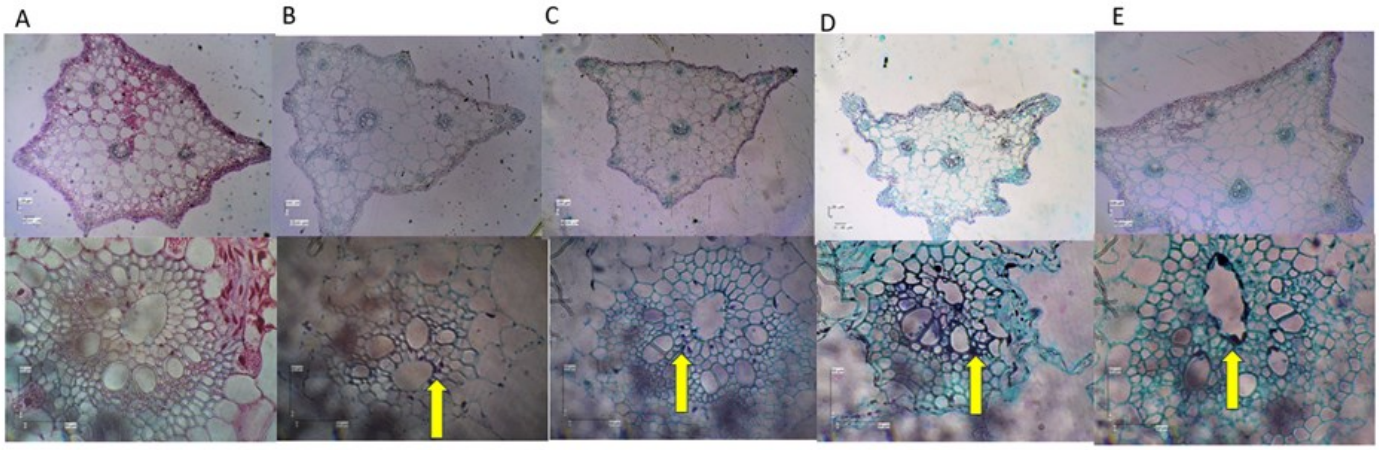

Figure 4. Anatomy of petiole (upper) and petiole metaxylem (lower) of *E. palifolius* after being exposed to mercury s for 18 days Notes: (A)=control, (B) = 25 ppm, (C) = 50 ppm, (D) = 75 ppm, (E) 100 ppm (100 times magnification).

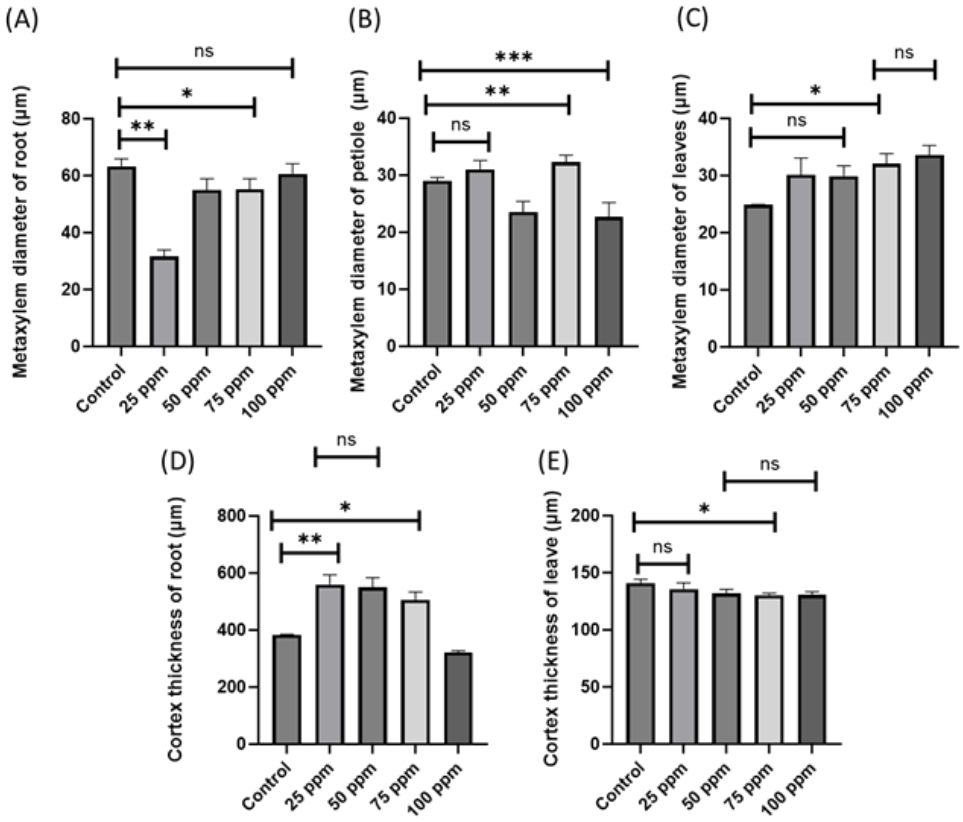

Figure 5. Metaxylem diameter of root (A), petiole (B), leaves (C), cortex thickness of root (D) and cortex thickness of leave (E) *E. palifolius* in mercury treatments on day 18th. Note: *= significant ($p<0.05$) difference. Ns= no significant difference.

CONCLUSION

Mercury treatment reduced *Euglena* sp. density and number of *E. palifolius* leaves, but not plant height and chlorophyll. Root and petiole diameters were affected by the mercury treatment, petiole diameter decreased unless the concentration was 100 ppm, whereas root diameter actually increased. The diameter of the root metaxylem increased, but the petioles and leaves, as well as the thickness of the root cortex, did not provide a significant response. Growth of *E. palifolius* was still able to grow optimally on condition of association with *Euglena* sp. in media containing mercury.

AUTHORS CONTRIBUTION

The contribution of each author in this research is D.U.S. prepared, created and wrote the initial draft; B.S.D developed and designed the methodology and reviewed the manuscript; H.T.B.M.P verified the research output and reviewed the manuscript, E.A.S designed the research and coordinated the responsibility for the research activity planning.

ACKNOWLEDGMENTS

This study was a part of a first author dissertation. The Ministry of Research and Technology/National Research and Innovation Agency of the Republic of Indonesia has funded this research with PDD (*Penelitian Disertasi Doktor*) grant scheme (033/E5/PG.02.00/2022).

CONFLICT OF INTEREST

There is no conflict of interest in this study.

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