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Morpho-physiological analysis of aquatic plants for phytoremediation of wastewater from gold mine wastewater treatment installation (IPAL)

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Abstract. Several types of aquatic plants have a potential role in reducing pollutants from contaminated water through phytoremediation processes. The purpose of this study was to analyze the capacity of aquatic plants in phytoremediation process of cyanide and heavy metals from gold mine liquid waste, based on the growth and heavy metal deposition in the root and shoot. The study was conducted using a completely randomized design with two factors, i.e., the concentration of gold-mine wastewater (0%, 50%, and 100%) and the combination of two aquatic plants from 4 species (*Eichhornia crassipes*, *Neomarica longifolia*, *Hydrilla verticillata*, and *Pistia stratiotes*). The results showed that gold mine wastewater generally reduced the growth rate of all the plants, especially at 100% wastewater concentration. The treatment with gold mine wastewater caused an increase in lipid peroxidation specified by the increase of root and leaf MDA content, while it decreases leaf chlorophyll content significantly. The best combination of aquatic plants for gold mine phytoremediation was shown by *N. longifolia* – *P. Stratiotes* based on the smallest dry weight reduction and the greatest reduction of cyanide, Pb and Hg from the media.

1. Introduction

The development of gold mining companies in Indonesia is currently managed by several parties, including state-owned companies, foreign companies as well as artisanal miners, which causes environmental problem. Gold extraction utilizes technology using cyanide and other chemical compounds to obtain gold from rocky materials by leaching technology [1, 2]. Consequently, the waste produced from gold mining contains some amount of cyanide and heavy metals which if the wastes are dumped directly into water bodies or soil it causes an environmental problem and even causes the death of living things [3, 4, 5]. To reduce detrimental effects, gold mining waste needs to be neutralized using some methods such as physical, chemical, and biological remediation. Biological remediation technique (phytoremediation) has been considered to be easier, effective, efficient, and environmentally friendly than other methods [6, 7, 8].

Phytoremediation is a process utilizing the plants to reduce contaminant from the environment [4]. Plants that are able to accumulate metals in high concentrations are called hyperaccumulators. For the aquatic environment, utilization of some aquatic weeds such as *Hydrilla*, *Ceratophyllum*, *Lemna Minor*, and *Wolffia* to reduce heavy metal contaminant had been effectively proven [4, 9]. The implementation of those plant can be done by combining various aquatic plants to reduce various heavy [5, 10]. The



ability of aquatic plants to accumulate heavy metals and cyanide in gold mine waste has not been widely studied, although types of aquatic plants have been known to have the ability to accumulate metals. Some aquatic plants have a fast life cycle and have the ability to clean the water ecosystem so that they can be used as phytoremediator [11]. However, combining more than one plant to reduce contaminants in the aquatic environment may have positive or negative result due to differences in physiological response among the species. Therefore, the investigation of those combination to reduce contaminant is required. This study aimed to analyze the response of aquatic plant combination to the reduction of cyanide and heavy metals from gold mine wastewater collected from water treatment installation (IPAL) of gold mines based on morphological, anatomical and physiological changes and reduction of cyanide and heavy metal.

2. Materials

The materials used in this study were four aquatic plants namely *Eichornia crassipes*, *Neomarica latifolia*, *Pistia stratiotes* (collected from the Department of Biology IPB), and *Hydrilla verticillata* (collected from Taman Buah Mekar Sari, Cileungsi, Bogor, Indonesia). As treatment material, the gold mining wastewater was taken from the Effluent tank of wastewater treatment installation (IPAL) of PT.ANTAM Pongkor, Bogor West Java, Indonesia.

Methods

2.1. Plant preparation and wastewater treatment

Four species were prepared in establishment media for several weeks using Hoagland solutions (10%) based on the previous research conducted by Srivastava et al. [5]. At the same times, gold mine wastewater from IPAL was prepared before the treatment was applied. After three weeks, the combination of 2 aquatic plants was prepared by weighing 25 g of each plant and then were grown in the treatment media. The experiment was carried out using a Completely Randomized Design with two factors. The first factor was plant combination which had six levels, i.e. *E. crassipes*- *N. longifolia* (T1), *E. crassipes*- *H. verticillata* (T2), *E. crassipes*- *P. stratiotes* (T3), *N. longifolia*- *H. verticillata* (T4), *N. longifolia*-*P. stratiotes* (T5), *H. verticillata*- *P. stratiotes* (T6). The second factor was gold mine wastewater treatment, which contained three levels, i.e. 0%, 50% and 100% of gold mine wastewater. The combination of plants was grown in the treatment media for 30 days.

2.2. Observation of Plant Growth

After 30 days of treatment, plant growth was measured to observe: the increase of root length, shoot dry weight, root dry weight, and the dead-root dry weight. The leaf area was measured using the scanner (HP 1050) and was calculated using the imageJ application [12]. Shoot and root dry weight were measured after the specimen was dried using the oven at a temperature of 70°C during 4-7 days until the dry weights were constant. Dead roots which were collected from the bottom of the containers were placed in the sieve (USA Standard No. 8) and then was dried in the oven for 4-7 days until constant weight at the temperature of 70°C.

2.3. Lipid Peroxidation Analysis

Lipid peroxidation was analyzed based on the Thiobarbituric acid (TBA) test by malondialdehyde (MDA). The 0.5g of leaves were crushed and added with 5 ml of Trichloroacetic acid (TCA) 0.5%, and then the solution was centrifuged with the rotation speed of 3000 rpm, at 4°C for 25 minutes. After that, 2ml of the leaves extract was taken and then added with 3 ml of TCA 0.5% in TBA 0.1%. The solution obtained was heated in a water bath at 80°C for 30 minutes. After cooling, the solution then was centrifuged with the speed rotation of 3000 rpm at 40°C for 30 minutes. The supernatant obtained then was measured for its absorbance at wavelengths of 450, 532 nm, and 600 nm [13].

2.4. Chlorophyll and carotenoids

Analysis of photosynthetic pigments includes chlorophyll a, chlorophyll b and carotenoids was performed based on the method of Quinet *et al.* [14]. Pigment content was calculated based on equation [15].

2.5. Analysis of Cyanide and heavy metals in the waste water

Analysis content of Pb and Hg was done before and after the treatment using methods of APHA ed. 22nd 3111 B 2012. The cyanide content was analyzed using the methods of APHA 4500 CN 22nd ed.-E, 2012.

2.6. Histochemical analysis of metals in leaves and roots

The histochemical observation was carried out by making transverse incision leaves and roots using a sliding microtome (G.S.L. I Bismensdorf WSL, Switzerland). The incision was soaked for a view second to metal detector dye contained 0.5 mg/ml of Dithizone [16]. The thickness of the incision leaves *E. crassipes*, and *N. latifolia* were 5 µm, *H. verticillata* leaves was 10 µm, while the leaf of *P. stratiotes* was 12 µm. The thickness incision of the root *E. crassipes* and *N. latifolia* were 7 µm, while *H. verticillata* and *P. stratiotes* were 10 µm.

3. Result and discussion

3.1. Pb, Hg and cyanide content in the wastewater

Analysis of the initial content of liquid waste from gold mine IPAL is necessary to know the levels of heavy metals (Pb, Hg) and cyanide content of the wastewater that was used for the treatment. The purpose of this analysis was to find out the content of Pb, Hg, and cyanide in the wastewater. The table 1 shows that the content of Pb, Hg, and cyanide waste liquid IPAL gold mine exceeded the threshold that is allowed to be released into the environment. Pb concentration was more than twice, and Hg was eight times higher than the threshold concentration, while cyanide (CN) was only slightly higher than the threshold concentration (Table 1). It means that it was able to pollute the environment and living things around the waters when it was released directly without treatment.

Table 1. The concentration of Pb, Hg, and cyanide in the liquid waste from gold mine IPAL that was used in the treatment.

Metals/ Cyanide (CN)	Methods	Unit	Threshold concentration	Result Analysis
Pb	APHA ed. 22nd 3111 B 2012	mg/L	0.030	0.070
Hg	APHA ed. 22nd 3111 B 2012	mg/L	0.002	0.016
CN	APHA ed.22nd 4500 CN-E, 2012	mg/L	0.020	0.0243

3.2. Growth parameters in response to wastewater

The response of plant growth to gold mine IPAL wastewater treatment showed varies among the plant combination. The growth can be represented by some parameters such as the increase of root length, leaves the area, shoot dry weights, root dry weight, and the dead roots. For plant growth, this experiment showed that the interaction of plant combination and concentration of IPAL wastewater significantly ($P < 0.05$) influenced these parameters (Figures 1 and 2).

In general, IPAL wastewater treatment caused the decrease of root length, leaf area, shoot dry weight, and root dry weight, but it caused the increase of dead roots significantly ($P < 0.05$). Meanwhile, there was variation among plant combinations. Figure 1a showed that the combination of T4 had the greatest root length decrease (55.90%), while T4 had the smallest one (9.75%) when the plants exposed to 100% IPAL wastewater. For leaf area, the combination of T4 has also exhibited the most reduction (35.93)

when it was subjected to 100% of IPAL wastewater, while the combination of T2 (17.40%) was the smallest (Figure 1b). The presence of cyanide and heavy metals in the media caused the plant underwent roots and leaves stunted. This is in line with the experiment carried out by Asati et al. [17] which found that the growth of roots and shoot was hampered by the presence of heavy metals because these elements are toxic even in small quantities.

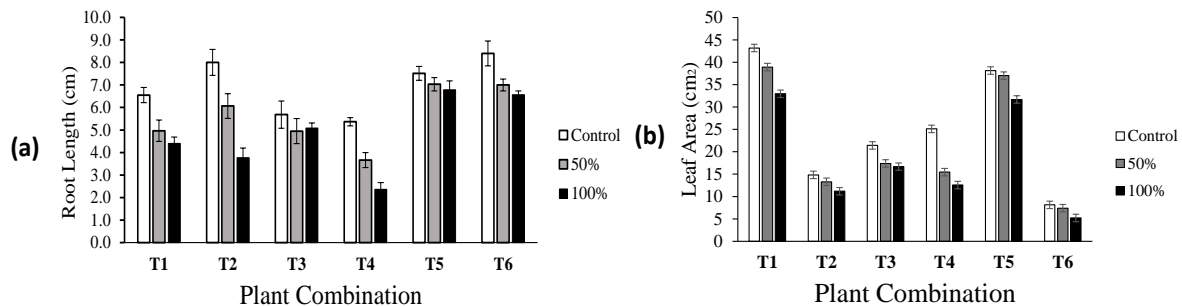


Figure 1. The growth of plants with waste treatment IPAL gold mine. Increase the length of the root (a), (b), leaves from the 6 combinations of water plants, *E. crassipes*- *N. longifolia* (T1), *E. crassipes*- *H. verticillata* (T2), *E. crassipes*- *P. stratiotes* (T3), *N. longifolia*- *H. verticillata* (T4), *N. longifolia*-*P. stratiotes* (T5), *H. verticillata*- *P. stratiotes* (T6). The line bars indicate the standard of error at 5% of α .

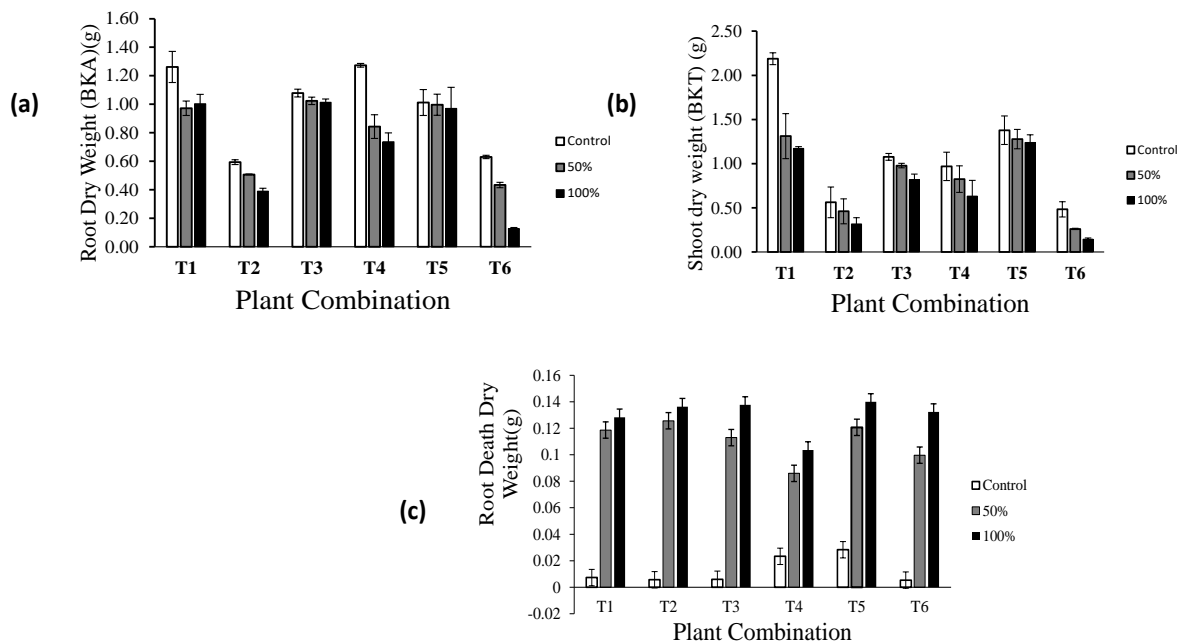


Figure 2. Root dry weight (a), shoot dry weight (b), and the weight of dead roots (c), of the 6 combinations of aquatic plants in response to gold mine IPAL wastewater at 0, 50 and 100%; *E. crassipes*- *N. longifolia* (T1), *E. crassipes*- *H. verticillata* (T2), *E. crassipes*- *P. stratiotes* (T3), *N. longifolia*- *H. verticillata* (T4), *N. longifolia*-*P. stratiotes* (T5), *H. verticillata*- *P. stratiotes* (T6). The line bars indicate the standard of error at 5% of α .

Not only root length and leave the area, shoot and root dry weight also significantly influenced ($P < 0.05$) by the interaction between plant combination and concentration of IPAL liquid waste. The largest root dry weight loss was experienced by the combination of the T6 (79.37%), while the smallest decline experienced by T5 which only reduced 4.12% in response to 100% IPAL liquid waste (Figure 2a). At the contrary, 100% treatment dramatically increased dead root with the maximum was found in the combination of T3 while the least was found in T4 (Figure 2c).

Growth is generally a manifestation of physiological responses of plants to their environment, and the stress due to the presence of for example heavy metal causes disruption of cell metabolism which resulted in the changes of plant growth as a whole [18]. Some other researcher also presented that the treatment with gold mine industrial liquid waste caused a significant decrease in shoot and dry root weight of terrestrial plant *Reutealis trisperma* [19]. From Table 1, we also found that the levels of Pb exceeded the threshold that is allowed, and this may at least cause the decrease in plant growth. Sharma and Dubey [20] stated that lead was very influential substance to the growth of roots and shoots, which normally caused by a decrease in cell division, photosynthesis, and protein synthesis. According to Ivanov and Seregin [21], stunted growth is a primary symptom if the plants are grown on media containing heavy metals, either hyperaccumulator or plants that are sensitive to heavy metal. Plant response towards heavy metal may undergo inhibition of growth or have the ability to produce enzymes and certain compounds as part of a defense mechanism which determines plant adaptability [22].

3.3. The content of MDA roots and Leaves

Malondialdehyde is the product of lipid peroxidation in the cell when the plant undergoes stress such as drought, salt, as well as heavy metal stress [19]. In this experiment, the measurement of MDA content in the leaves and roots was carried out after 30 days of the treatment. In general, the levels of MDA in the leaves and the roots was significantly increased ($P < 0.05$) due to the treatment of gold mine IPAL liquid waste. The treatment with 100% liquid waste caused a dramatic increase of MDA content in the root with the greatest number in plant combinations of T6 (122%), while the smallest increase was shown by a combination of T5 (8%). Meanwhile, a rise in the level of leaf MDA content was largest in a combination of T1 plants (98.74%). And the smallest was experienced by the combination of T5 (22.85%). The differences may have an indication of plant capability to tolerate contaminant in the treatment. In line with this finding, Hilmi et al. [23] also found that in addition to inhibition of growth, gold mines wastewater also caused the increase of malondialdehyde in *R. trisperma*.

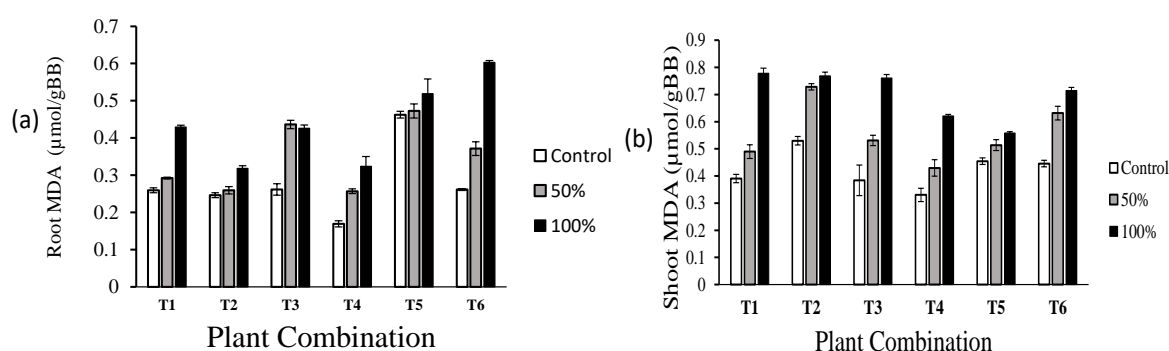


Figure 3. MDA content of roots(a) and Shoot (b), of the 6 combinations of aquatic plants in response to gold mine IPAL wastewater of 0, 50 and 100%; *E. crassipes*- *N. longifolia* (T1), *E. crassipes*- *H. verticillata* (T2), *E. crassipes*- *P. stratiotes* (T3), *N. longifolia*- *H. verticillata* (T4), *N. longifolia*-*P. stratiotes* (T5), *H. verticillata*- *P. stratiotes* (T6). The line bars indicate the standard of error at 5% of α .

Increased levels of MDA showed that the plants underwent stress and therefore, the plant may make a defense effort against stress. An antioxidative mechanism such as peroxidase activity is among the enzymes that have been investigated to have an important role in the plants that are tolerant of heavy metals such as in *Melastoma malabathricum* [24]. Peroxidase is a key enzyme that maintains the cell metabolisms against oxidative stress, and therefore, it will be produced more in the plant organs [13, 25, 26]. The combination of plant T5 had the lowest levels of MDA, even under 100% of IPAL wastewater (Figure 3). This shows that T5 combination is the most tolerant to gold mine IPAL wastewater. This date is also associated with the growth data, which showed that T5 combination had the least reduction of growth in response to gold mine IPAL wastewater (Figures 1 and 2). Ferdhiani et al. [27] suggested that plant that was resistant to heavy metal stress had a significant increase of peroxidase enzyme activity which was able to reduce lipid peroxidation, and consequently, the plants did not experience growth declining too much under heavy metal stress.

3.4. Chlorophyll and Carotenoid Content

The value of pigment content including chlorophyll-a, chlorophyll-b, total chlorophyll, and carotenoids in all the combination plants generally declined in response to gold mine IPAL wastewater, especially at 100% of wastewater (Figure 4). On the average, a combination of T3 had the lowest all pigment content, while T4 had the highest. For total chlorophyll, T2 combination underwent the highest chlorophyll reduction in response to gold mine IPAL wastewater at 100%, while T4 combination was the smallest (Figure 4). The carotene content also had an almost similar pattern, where T2 combination had the biggest reduction while T3 combination had the smallest.

Photosynthetic pigment reduction is obvious when the plant undergoes environmental stress such as drought and heavy metals. Because this factor can cause chlorophyll biosynthesis hampered due to the inhibition of the enzyme work [28, 29]. Inhibition of chlorophyll biosynthesis may occur because heavy metal such as lead can reduce the uptake of Fe and Mg, which caused alterations in biosynthesis of chlorophyll [30, 31]. In addition, heavy metals may reduce plant photosynthesis, particularly through the inhibition of the enzymes that work in chlorophyll biosynthesis, and in many cases also cause chloroplast membrane damage [32].

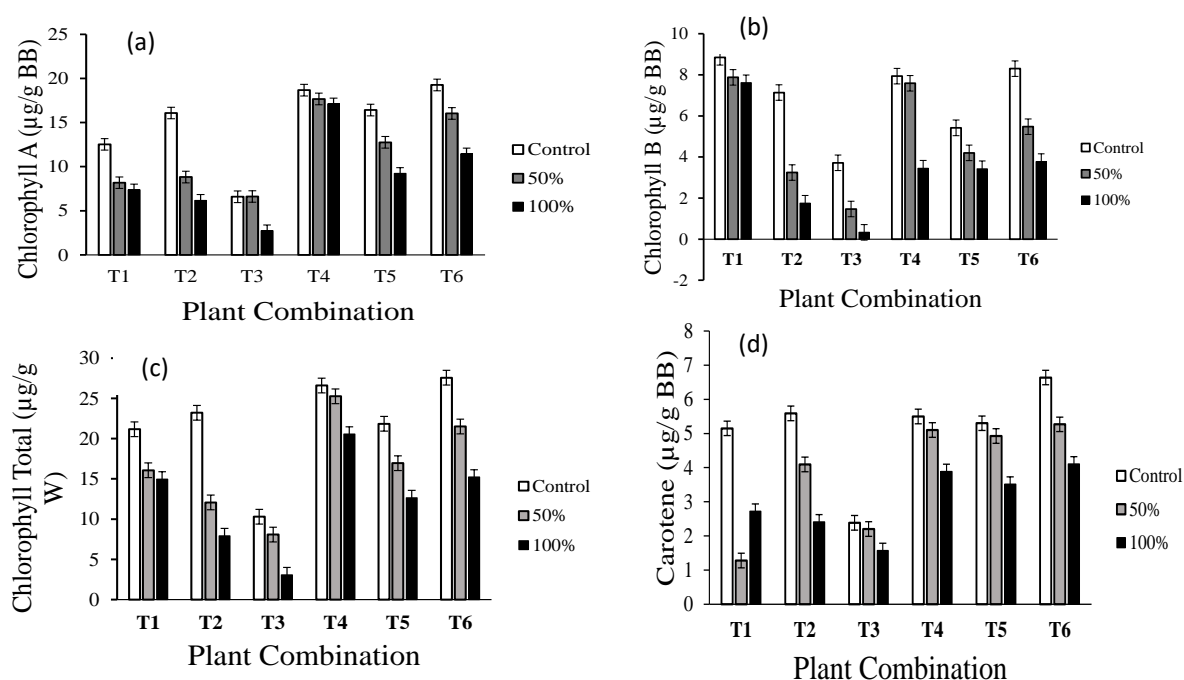


Figure 4 The content of chlorophyll a (a), Chlorophyll b (b), (c) Total Chlorophyll, and Carotenoids (d) of the 6 combinations of aquatic plants in response to gold mine IPAL wastewater at 0, 50 and 100% , *E. crassipes*- *N. longifolia* (T1), *E. crassipes*- *H. verticillata* (T2), *E. crassipes*- *P. stratiotes* (T3), *N. longifolia*- *H. verticillata* (T4), *N. longifolia*-*P. stratiotes* (T5), *H. verticillata*- *P. stratiotes* (T6). The line bars indicate the standard of error at 5% of α .

3.5. Histochemical analysis of root and leaf tissues

The histochemical analysis was carried out to observe Pb and Hg accumulation inside the roots and leaves tissues after treated by gold mine IPAL wastewater (Figure 5). The use of dithizone to detect the presence of metal accumulation inside plant tissues is indicated by red color for metals Pb accumulation and brown color for Hg accumulation. Figure 5 showed that metal was accumulated inside the leaves of all the species (1b, 2b, 3b, and 4b). The accumulation of metals Pb and Hg was higher in the epidermis tissues. Plants have a special transporter protein to transport metals into the cytoplasmic and vacuole, and this depends on the capacity of defense mechanisms of each species. Mercury (Hg) ions is a mobile ion group, so it is easier for the plant to transfer this ion to the shoot of the plants. Hg²⁺ ion can also be stored in the vacuole and epidermal cells of leaves. It would be at the higher amount on a shoot of hyperaccumulator plants [33].

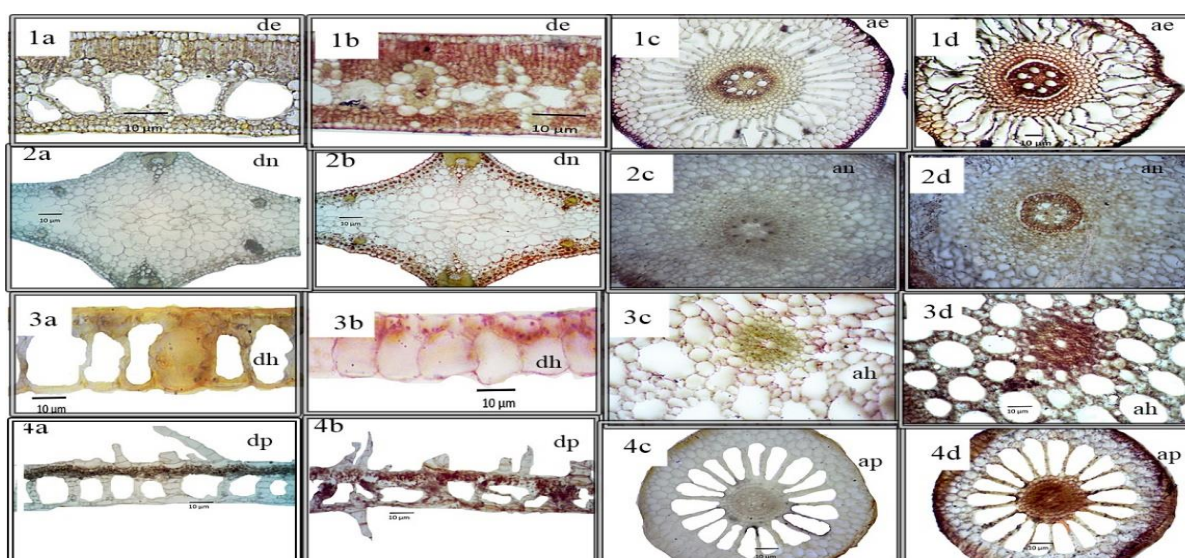


Figure 5 Transverse cross section of the roots and the leaves of the plants after the treatment using gold mine IPAL wastewater at 0 and 100%. Red and brown colors indicate the accumulation of Pb and Hg which shown on the xylem and epidermis. Note: the leaves *E. crassipes* (de), the roots of *E. crassipes* (ae), the leaves of *N. longifolia* (an), the root of *N. longifolia* (an), leaf *H. verticillata* (dh), the roots of *H. verticillata* (ah), the leaves of *P. stratiotes* (dp), the roots of *P. stratiotes* (ap). The plant was given treatment stress (1a) de 0%, (1b) de 100%, (1 c) ae 0%, (1 d) ae 100%, (2a) dn 0%, (2b) and 100% (2 c), 0%, (2d) 100%, (3a) dh 0%, (3b) dh 100%, (3 c) ah 0%, (3d) ah 100%, (4a) 0%, dp (4b) dp 100% (4 c) ap 0%, (4 d) ap 100%.

The root of the transverse incision is shown in Figure 5 (1-4, c and d). Metal accumulation inside the roots was evidence that Pb and Hg were transported inside the plant. There is special transporter at the root membrane so that the metal would be easily absorbed. Immobilization may occur during the process and storage of heavy metals, especially in the vacuoles of the root. Furthermore, the metal will be transported towards the leaves through xylem [34]. On the hyperaccumulator plant, metal would be transferred to the leaves through xylem tissues follows the path of transpiration and then would be accumulated inside the vacuole. Liu et al.[35] and Ratheesh et al. [36] also suggested that the

translocation of metals occur through the xylem so that the accumulation of metals happen through transport chain from the roots to the shoot [37, 38].

3.6. Analysis of the content of cyanide and heavy metals

The concentration of heavy metals and cyanide inside the medium before and 30 days after treatment was observed to calculate the capacity of plants to reduce contaminant from the media. The decrease of cyanide, Pb and Hg content in the media had similar pattern among all the combinations (Table 2), suggesting that all the plants had the capacity to reduce cyanide, Pb and Hg contaminant. Even though not significantly different among the plant combinations, the greatest cyanide, Pb and Hg reduction were shown by the combination of T5 which reduced from 2.4×10^{-2} to 0.08×10^{-2} , 7×10^{-2} to 1×10^{-2} , and 1.63×10^{-2} to 0.95×10^{-2} respectively (Table 2). This data shows that the combination T5 is the best combination to reduce cyanide, Pb, and Hg from contaminated media. This result is in accordance with the observation recorded from histochemical analysis (Figure 5). The combination of T5, which consisted of *N. longifolia* and *P. stratiotes* showed clear staining which indicated of Pb and Hg accumulation compared to other two plants (Figure 5 [2a-d] and [4a-d]). The same result of histochemical analysis had also been presented by other authors for Pb accumulation in the terrestrial plant such as *R. trisperma* [23].

Table 2. Concentrations of cyanide and heavy metals (Pb and Hg) in the media before (control) and after 31 days application with 0, 50 and 100% of gold mine wastewater with 6 combination plants, *E. crassipes*- *N. longifolia* (T1), *E. crassipes*- *H. verticillata* (T2), *E. crassipes*- *P. stratiotes* (T3), *N. longifolia*- *H. verticillata* (T4), *N. longifolia*-*P. stratiotes* (T5), *H. verticillata*-*P. stratiotes* (T6).

Waste	Content of waste water	Plants Combinations					
		T1	T2	T3	T4	T5	T6
Cyanide (mg/L)	Control	$2.4 \times 10^{-2}a$	$2.4 \times 10^{-2}a$	$2.4 \times 10^{-2}a$	$2.4 \times 10^{-2}a$	$2.4 \times 10^{-2}a$	$2.4 \times 10^{-2}a$
	50 %	$0.09 \times 10^{-2}b$	$0.13 \times 10^{-2}b$	$0.13 \times 10^{-2}b$	$0.36 \times 10^{-2}b$	$0.08 \times 10^{-2}b$	$0.13 \times 10^{-2}b$
	100%	$0.09 \times 10^{-2}b$	$0.10 \times 10^{-2}b$	$0.13 \times 10^{-2}b$	$0.08 \times 10^{-2}b$	$0.08 \times 10^{-2}b$	$0.09 \times 10^{-2}b$
Pb (mg/L)	Control	$7 \times 10^{-2}a$	$7 \times 10^{-2}a$	$7 \times 10^{-2}a$	$7 \times 10^{-2}a$	$7 \times 10^{-2}a$	$7 \times 10^{-2}a$
	50 %	$2 \times 10^{-2}bc$	$3 \times 10^{-2}bc$	$3 \times 10^{-2}bc$	$3 \times 10^{-2}bc$	$2 \times 10^{-2}d$	$2 \times 10^{-2}d$
	100%	$3 \times 10^{-2}b$	$3 \times 10^{-2}b$	$2 \times 10^{-2}bc$	$3 \times 10^{-2}bc$	$1 \times 10^{-2}e$	$3 \times 10^{-2}b$
Hg (mg/L)	Control	$1.63 \times 10^{-2}a$	$1.63 \times 10^{-2}a$	$1.63 \times 10^{-2}a$	$1.63 \times 10^{-2}a$	$1.63 \times 10^{-2}a$	$1.63 \times 10^{-2}a$
	50 %	$0.95 \times 10^{-2}bc$	$0.95 \times 10^{-2}bc$	$0.95 \times 10^{-2}bc$	$0.95 \times 10^{-2}bc$	$0.91 \times 10^{-2}c$	$0.95 \times 10^{-2}bc$
	100%	$0.97 \times 10^{-2}b$	$0.97 \times 10^{-2}b$	$0.97 \times 10^{-2}b$	$0.97 \times 10^{-2}b$	$0.95 \times 10^{-2}bc$	$0.97 \times 10^{-2}b$

Note: The numbers followed by similar letter on the same variables is not significantly different based on DMRT test at $\alpha = 0.05$.

4. Conclusion

The treatment using gold mine IPAL wastewater at 50 and 100% caused growth inhibition of all combined plants with T5 combination exhibited the list affected than others. The treatment induces the increase of malondialdehyde content while decrease chlorophyll and carotenoids content of all combinations of plants. Metal (Pb and Hg) absorption by plants was detected inside roots and leaves tissues of all the plant indicated by histochemical analysis. The combination number T5, which consisted of *Neomarica longifolia* and *Pistia stratiotes* was the best among all combination based on growth sustainability and the lower MDA content. This combination also demonstrated the highest capacity to reduce cyanide, Pb, and Hg from the media compared to other combination.

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