Comparative phytotoxicity of Azolla pinnata and Lemna minor in Treated Palm Oil Mill Effluent

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Abstract

The phytotoxicity of Azolla pinnata and Lemna minor was assessed when exposed to Treated Palm Oil Mill Effluent (POME) for their survival and growth tolerance in a 5-day exposure conducted in an open laboratory at Universiti Kebangsaan Malaysia. A total of 34 100 mL-containers were used, with three replicates for each concentration, including plant less control and plant control. 50 mL of treated POME in different concentrations (100%, 75%, 50% and 25%) was filled in each container and exposed to 3 g Azolla pinnata or Lemna minor with observation conducted daily. After two days of exposure, 40% of A. pinnata died in 100% concentration and almost 100% died after 5 days of exposure for all concentrations. On L. minor, only 5% mortality was observed for 100% concentration on the fourth day and remained healthy until the end of five days. At the end of 5-day phytotoxicity exposure, the highest removals were 63.0%, 70.5%, 51.0%, 65.4% and 53.8% respectively for COD, BOD, Ammonia, Phosphate And Nitrates by A. pinnata, while the maximum removals recorded by L. minor were 61.0%, 54.0%, 9.8%, 61.4% and 31.6% respectively, giving evidence that A. pinnata is more effective in removing pollutants than L. minor although its survival in the treated POME is lesser than L. minor.

Keywords: Azolla Pinnata; Lemna Minor; Phytotoxicity; Palm Oil Mill Effluent (POME).

1. Introduction

Malaysia is one of the major producers of crude palm oil, besides Indonesia, in the world which accounted for 31.9% in 2016 [1]. Total revenue of the world's palm oil is expected to rise again in 2017 over the previous years due to a recovery in the palms after the effects of El Nino, which occurred in 2016 [2]. The highest production statistics of selected food crops in Malaysia is also on the production of palm oil compared to rice, natural rubber, cocoa and kenaf, to an increased production of palm oil by 2.4% in 2015 [3]. The increase of oil palm products in Malaysia will also increasing generate wastewater from palm oil mills each year. Palm oil production process requires large volume of water with approximately 5-7.5 tons of water is required to produce 1 ton of palm oil and 50% of that water will contribute to the generation of wastewater [4] which is also commonly known as palm oil mill effluent (POME). Basically, palm oil processing will produce wastewater during sterilization, separation of sludge and also during cyclone process. Typically, oil palm wastewater is treated in several stages involving few processes of cooling pond, anaerobic, aerobic and final discharge. Many POME treatment schemes currently used by the Malaysian palm oil industry including membrane bioreactor and biological method were discussed in details by [5-7]. However, the application of these technologies is often still difficult to comply with the stringent environmental regulation in Malaysia. Phytoremediation, a fairly new technology in tropical country like Malaysia, is proposed to further polish treated POME which is still rich with nutrients and most of the time hardly meets the stringent environmental regulation, using native ferns that are later to be used as livestock feeds. Phytoremediation is one of the green technology processes utilizing plants to reduce the concentration of pollutants in contaminated soil, water or air using plants or herbs that attempt to control, degrade, or remove metals, pesticides, solvents, cracking materials, crude oil, and various contamination materials [8]. It is low cost, easy to handle and environmental friendly that make use of plants together with microorganism to remove or detoxify contaminations in wastewater [9-12]. More than 500 species of plants are listed as a potential plant for phytoremediation [13], most of them are temperate plants. One of this research output is to identify tropical and native plants in Malaysia that can be used to further polish palm oil mill effluent as well as later be used as livestock feeds. Phytoremediation technology has been widely used to treat various industrial wastes but not much for POME. There are studies utilizing floating plants or duckweeds to treat POME, due to the fact that the properties of the floating plants themselves are easy to multiply and efficient to treat pollutants [14]. In addition, Eichhornia crassipes can also reduce COD up to 33-45% in POME after 6 days of study [15], [16] had applied emergent plants of Vetiver and found that Vetiver was able to reduce COD and BOD up to 70.60% and 93.33% in pure POME. Other plants such as Pistia stratiotes [17], Lysia oryzoides [18] and Ludwigia peploide [19] are also able to reduce COD in POME up to 39.1-59.66%, 27% and 20%, respectively. Therefore, the uses of other types of duckweeds and, emergent and submerged plants are still further explored to be used for POME treatment by considering their capabilities in pollutant removal, survival and their tolerance in the effluent. In this study, two types of duckweed, Azolla pinnata and Lemna minor were selected to polish treated POME so that at the

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end they can be converted to be livestock feed for fish, chickens and ducks. Both are native aquatic and floating ferns that can be used to treat domestic [20] and industrial wastewater [21-23]. Both of these ferns are considered as duckweed, with the duckweed dispersal worldwide is due to the adaptation of plants that lead to different species [24]. Duckweed is a plant with fast reproduction and can absorb a large amount of nutrients such as Nitrogen (N) and Phosphorus (P) from agricultural and municipal [25-29]. L. minor consists of small-sized monocotyleden plants floating on a calm surface of the pond [30-31]. Its plant structure is relatively simple and only consists of leaves and roots [24]. Among different plant-based systems, L. minor has been applied with success in different countries for the removal of nutrients and heavy metals, combining efficient wastewater treatment and important biomass production [11-32]. A. pinnata is also a floating-free aquatic plant belonging to the Azollaceae family. It is widely distributed in Asia and along the tropical African coast [33]. It has one cavity in the center of the leaf, which hosts symbiotic cyanobacteria, Anabaena azollae [34]. This symbiotic absorbs N₂ from the atmosphere and produces high nitrogen levels in the A. pinnata plant tissue, creating the plant to be useful as a green manure where it has been used for several centuries [35-37], [22]. Some advantages for Azolla is that it can grow rapidly and double its biomass in every three days. It produces more than 4 to 5 times the protein compared with hybrid A. pinnata, A. japonica, A. mexicana, A. microphylla, A. nilotica, A. pinnata and A. rubra. Species of A. pinnata and A. microphylla are usually found in Malaysia. They can grow naturally on calm waterways like abandoned rivers, canals and ponds [39]. Both of these plants are also able to absorb heavy metals found in wastewater [40-41]. This study aims to determine the tolerance and survival of these two plants (A. pinnata and L. minor) in treated POME as to select the right concentrations of POME to be used in the next stage of plant uptake in larger scale (pilot scale) by these plants in treated POME.

2. Materials and method

2.1. Plant collection and propagation

The two healthy and fresh plants, A. pinnata and L. minor were obtained from a greenhouse at Universiti Kebangsaan Malaysia. The plant was cultured in Houglund medium as suited by [42-43] to ensure plant stock is sufficient for future research activities. Some plants were taken and placed in a beaker filled with tap water and left for 10 min to remove the remnants attached to the plant roots. Then, the plants were washed with tap water, filtered and dried using tissue paper to reduce the percentage of water at the plant [44]. Both plants’ growth was observed physically in terms of their colour change. Figure 1 compares an image of healthy A. pinnata and L. minor with the dead ones. Healthy A. pinnata is a dark green, while L. minor is light green (Figure 1). A. pinnata will change its color to dark brown while L. minor will change its green color to white when dies.

![Azolla pinnata](image1)

![Lemna minor](image2)

**Fig. 1**: Azolla Pinnata and Lemna Minor Discoloration when Healthy and after Death.

2.2. Experimental design of phytotoxicity test

The purpose of this study is to illustrate how duckweeds, which are small, simply constructed, floating aquatic plants, and suitable for dealing with toxicity [45]. The duckweed is able to grow rapidly in a solution containing high nutrients and potentially to reduce toxicity in waste solution. Under controlled conditions, the duckweeds test is excellent for determining the toxicity of water pollutants, and duckweeds are essential as a model of aquatic plants in Eco toxicity assessment [46]. [45] and [47] also discussed duckweed as a mechanism that can remove toxicity with the aid of additional nitrogen in which duckweed cultivation on nutrient-rich sewage has described the diversity and the potential of this plant for water recovery and for the remediate use of growth. To achieve this toxicity study, a total of 34 100 mL-containers were prepared for the phytotoxicity test. Sixteen containers were used for each plant (A. pinnata and L. minor). Each container contained 50 mL of treated POME obtained from a crude palm oil mill in Dengkil, Selangor with different POME concentration of 100%, 75%, 50% and 25% [(Vtreated POME/Vtotal) x 100%]. For example, 75% v/v of treated POME was prepared by taking 37.5 mL of treated POME and adding 2.5 mL tap water to obtain 50 mL of solution. Table 1 lists down the characteristics of the treated POME obtained from the palm oil mill. Each dilution was triplicated and control solution was also provided for each dilution without the plants. Another container acting as plant control containing plants with 50 mL tap water. Each container was loaded with 3 g of L. minor or A. pinnata as illustrated in Figure 2.

![Experimental Set-Up for Phytotoxicity Test](image3)

**Table 1**: Characteristics of Treated POME

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>COD</td>
<td>836 ± 21 mg/L</td>
</tr>
<tr>
<td>BOD₅</td>
<td>400 ± 17 mg/L</td>
</tr>
<tr>
<td>NH₃-N</td>
<td>15.2 ± 0.4 mg/L</td>
</tr>
<tr>
<td>PO₄³⁻</td>
<td>13.7 ± 0.5 mg/L</td>
</tr>
<tr>
<td>NO₃⁻</td>
<td>28.0 ± 12 mg/L</td>
</tr>
<tr>
<td>pH</td>
<td>9.56 ± 0.3</td>
</tr>
</tbody>
</table>

![Figure 2: Experimental Set-Up for Phytotoxicity Test.](image4)
2.3. Physical observation of plant

Observations were made every day starting from day 0 to day 5 and quantified with the percentage of plants that can survive for each concentration of treated POME. Table 2 shows the physical indicator of *A. pinnata* and *L. minor* observed for healthy and dead ones. *A. pinnata* will turn brown from the center of the fronds and eventually become black when it dies. For *L. minor*, it turned white when died, as shown in Table 2. The tolerance and survival of both plants are quantified based on the area of the healthy ones compare to the total area covered by the whole fern on the water surface and also on the colour change.

<table>
<thead>
<tr>
<th>Plant</th>
<th>Fresh / Healthy</th>
<th>Dead</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Azolla pinnata</em></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Lemna minor</em></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table 2: Indicator for Healthy and Dead Plants for Azolla Pinnata and Lemna Minor

2.4. Analysis of wet biomass and water quality parameters

Physicochemical parameters of the treated POME, including total biomass, Chemical oxygen demand (COD), biological oxygen demand (BOD₅), pH, ammonia nitrogen (NH₃-N), nitrate nitrogen (NO₃⁻), and dissolved orthophosphate (PO₄³⁻), were observed throughout the 5-day exposure. Experiments were initiated by analyzing POME for each dilution of 100, 75, 50 and 25% and on each day of sampling, water samples were taken from each container for the analysis of water quality parameters. The plants were harvested each day 1, 3 and 5 for their growth characteristic namely wet biomass. The total biomass was analyzed by filtering plants using a 1 mm sieve [48] to instantly reduce the water content of the plant and weighed to obtain the wet weight of each sample [49]. The pH was measured with an IQ 150 Multiprobe (IQ Scientific Instruments, Spectrum Technologies, Plainfield, U.S.A and the rest of parameters were analyzed using the HACH test kits [50]. The pH was measured with an IQ 150 Multiprobe (IQ Scientific Instruments, Spectrum Technologies, Plainfield, U.S.A and the rest of parameters were analyzed using the HACH test kits [50]. All these water quality parameters were determined according to the Standard Methods [30].

3. Results and discussion

3.1. Effect of treated pome on plant growth

The ability of *A. pinnata* to survive in treated POME of different dilutions was observed throughout 5 day-exposure as shown in Table 3. No colour changes were observed on the first day of exposure. Unlike on the second day, only 60% of the plants survived in 100% treated POME (no dilution), but in 75%, 50% and 25% dilution, most plants in the containers were still alive and healthy. On the third day, only 30% of the plants were still green at 100% POME, while on the other dilutions it had shown a significant decrease. Physically, it can be seen on the fourth and fifth day that all the plants turned black and died indicating that *A. pinnata* failed to resist the toxicity of the treated POME solution. Moreover, the longer the exposure, the darker the colorization of the plant leading to invariably death of plants. [48] also showed *Azolla* sp. plants could not withstand toxicity on remaining wastewater but [21] found that it is capable of treating heavy metals (Hg and Cd) in wastewater.

![Table 3: Physical Observation of the Toxicity of Treated POME on Azolla Pinnata](Image)

Similar amount (3 g) of *L. minor* was also exposed to the treated POME with different dilution for 5 days. For *L. minor*, it was observed that healthy light green color of the plants remained unchanged until the third day of exposure (Table 4). The plants were still fresh in all dilutions of treated POME (25% up to 100%). On day 4, it was found that only 5% of the plants turned white indicating death signs in the 100% treated POME and remained the same until the end of 5-day exposure. This is proven that *L. minor* is highly resistant to toxicity even in 100% treated POME. [50] had also demonstrated the suitability of *L. minor* to treat final POME and, [51] also indicated the capability of this plant to treat laboratory wastewater.

![Table 4: Physical Observation of the Toxicity of Treated POME on Lemna Minor](Image)

Figure 3 illustrates the wet biomass of both plants compared to the control plant throughout the 5-day exposure. The growth of *A. pinnata* increased on the first day and decreased with time until the fifth day. The wet biomass of *A. pinnata* in all the concentrations of treated POME decreased significantly compared to that in the plant control. Similar trend was observed on the physical growth of *A. pinnata*, as explained previously, in which the plants turned black, a sign of dead plants, starting the first day of exposure as shown in Table 3. This is because the plants cannot tolerate high nutrient content in POME solution. For *L. minor*, the
growth of plants in all concentrations of treated POME did not show any significant effect compared to the control plants. The wet biomass of all plants increased on the first day and then gradually decreased until day 5, following similar trend with the control plant. This result is in line with the physical growth as captured in Table 4. A study conducted by [52] also show similarity with the decrease of biomass volume for the study using synthetic hydrocarbon wastewater. In contrast to the study conducted by [50], the results showed an increase in the amount of biomass on the L. minor carried out at pilot scale.

![Fig. 3: Wet Biomass of Azolla Pinnata and Lemna Minor throughout the 5-Day Phytotoxicity Test to Treated POME.](image)

3.2. Comparative tolerance of Azolla pinnata and Lemna minor

The physical observation for the plant tolerance and survival was quantified in terms of the number of plants that still remained fresh and green. Figure 4 clearly shows that L. minor has higher resistance (95%) to the toxicity of treated POME with only 5% became white and died compared with A. pinnata. Up to day 5, L. minor was still able to survive while A. pinnata started to die starting day 3 and mostly died on day 5.

![Fig. 4: Comparison on the Phytotoxicity of Treated POME on Azolla Pinnata and Lemna Minor.](image)

3.3. Characteristics of wastewater throughout the 5-day exposure

The results of water quality parameters (NO₃⁻, PO₄³⁻, NH₄-N, pH, BOD₅ and COD) for POME were depicted in Figure 5. On nitrate content, it is found that the maximum nitrate removal of 53.8% is observed in the 100% treated POME for A. pinnata. While L. minor also showed a decline with a loss of nitrates to 13 mg/L which is 31.6% removal at 75% treated POME solution over a 5-day trial. As well as, in 50% and 25% solutions, both plants showed a decrease to 5 mg/L for A. pinnata and 4 mg/L for L. minor. Overall, it shows that A. pinnata is a potential phytoremediator in all distributions. The reduction of nitrate-nitrogen in different wastewater was in agreement with previously reported study by [53] that A. pinnata can reduce the nitrate content up to 88% in the Hoagland solution, and achieve 100% nitrate reduction from the study solution using well water and sewage water. [51] reported that L. minor can remove nitrate from municipal wastewater, sewage water and seafood processing plant wastewater with 66 to 99% removal. Ammonia is a measure of water health in rivers, lakes, and groundwater.

![Fig. 5: Characteristic of Water Quality Throughout the 5-Day Phytotoxicity Test.](image)
the trial for 100% treated POME due to the absorption and adsorption or direct taken by plants [58], compared with the phosphate concentration in the control which was only 10.7 mg/L. There is a slight drop in phosphate levels in the treated POME in the early stages when the duckweeds grew with the abundance of more phosphate yields [57]. The decrease of phosphate in the 75% treated POME was less for both plants due to lack of phosphate in the diluted solution. A more significant decrease can be seen in A. pinnata compared to L. minor in all POME concentrations.

The pH in treated POME for both plants on day zero was slightly alkaline conditions at pH 9.56 and decreased gradually towards neutral conditions due to the release of hydrogen ions when ammonia was converted to nitrates [60]. The pH in 25% POME concentration for L. minor decreased 17.3% to pH 7.8 in 5 days compared to the value of pH 9.43 for the control plant. The highest pH drop for A. pinnata occurred in the 25% treated POME of 0.67 which is 7.1% drop compared to 100% treated POME which is only 0.20 drop on day 5. [51], and, [60] reported the reduction in alkalinity values from and pH 8.9 to pH 7.7 for L. minor. [53] stated that reduction of pH at all points can be attributed to the fact that ammonia and nitrate can be reduced by this study. This is because the absorption of ammonia and nitrogen compounds usually promotes biological responses that produce hydrogen ions contributing to lower pH [61]. pH reduction is also the reason why ammonia, phosphate and nitrate can also be reduced [54].

In this study, COD and BOD contents were also investigated to identify the ability of A. pinnata and L. minor as phytoremediation plants to improve the quality of treated POME. POME contains high COD and BOD concentrations of organic pollutants that must be removed before POME can be released into the environment. The results of this study show that both L. minor and A. pinnata can reduce organic pollutants from treated POME for all POME concentrations within the 5-day exposure. The maximum removals of 63.0% and 61.0% for COD were observed in the 25% treated POME with final COD concentrations were 364 mg/L and 352 mg/L for A. pinnata and L. minor respectively. In addition, the removal of BOD was 70.5% and 54.0% with final readings were 34 mg/L and 53 mg/L for A. pinnata and L. minor respectively, compared to the control of 115 mg/L. While, an increase in COD and BOD readings can be observed for both plants in 100% and 75% POME concentrations. This is because on the fifth day, the crops had died entirely and, in this case, the photosynthesis process had stopped and no oxygen can be supplied to the solution [56]. In the early stages when plants began to grow and spread, POME treated with plants showed a greater amount of reduction in BOD [28]. A study by [62] also showed a reduction of COD and BOD for treating wastewater from chicken farm using A. pinnata. Another study conducted by [63], the reduction of COD and BOD was very significant using A. filiculas with 94.6% and 74% removal, respectively, in treating wastewater from the textile factory. Other study by [48] has also shown that L. minor can reduce COD and BOD in synthetic wastewater showing a significant reduction of 92.2% and 94.7%. [64] reported that COD removal was in the range of 70-80% in discharge duckweed system.

4. Conclusion

Among the four different dilutions of treated POME, L. minor displayed higher tolerance and survival level compared to A. pinnata, 95% of L. minor remained fresh and green even in the 100% treated POME. However, A. pinnata mostly died at the end of 5-day exposure even in the lowest dilution (25%) of treated POME. Through this physical observation, it could be concluded that L. minor is a fern that is able to grow in treated POME containing high nutrient. This study was preceded to analyze the efficiency of L. minor and A. pinnata to remove nutrients and organic content from treated POME. A. pinnata had removed 53.8%, 51.0%, 65.4%, 70.5% and 63.0% respectively for COD, BOD, ammonia, phosphate and nitrates with the final effluent concentrations reached 12 mg/L, 7 mg/L, 3.70 mg/L, 212 mg/L and 34 mg/L in 25% treatment POME concentrations for. Compared to A. pinnata, L. minor could only remove in average 31.6 % NO₃⁻, 9.8% NH₄-N, 61.4% PO₄³⁻, 17.3% pH, 54.0% BOD and 61.0% COD in all dilutions of treated POME with the respective final effluents as 13 mg/L, 12.8 mg/L, 4.13 mg/L, 224 mg/L, 53 mg/L, respectively. On overall, it can be concluded that A. pinnata works well in the treatment of treated POME compared to L. minor in terms of water quality although L. minor has a strong resistance in the high content of nutrients and organic carbon. Further study will be carried out to balance out the potential of phytoremediation and also the survival and tolerance of the plant species for the sustainability of the animal feed.

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