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Research paper

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,*

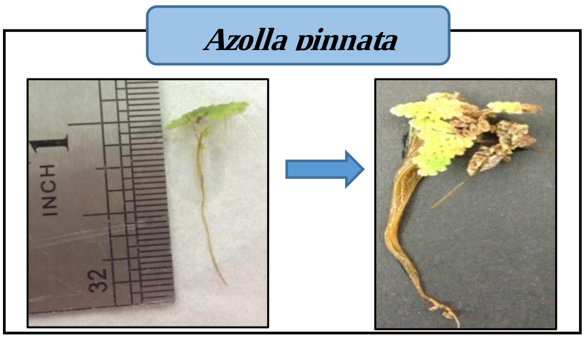
1. Introduction

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 increasingly 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 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 monocotyledon 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 symbiont absorbs N_2 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 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 *Napier* and *Lucern* [38]. There are at least eight-azolla species worldwide which is *A. caroliniana*, *A. circinata, 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 Hoagland 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.



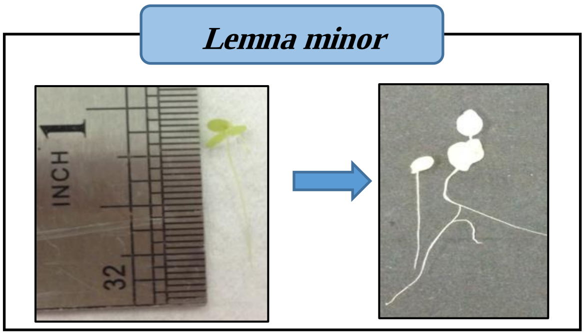


Fig. 1: *Azolla Pinnata* and *Lemna Minor* Discoloration when Healthy and after Death.

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 remediative 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% [($V_{treated\ POME}/V_{total}$) 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.

Table 1: Characteristics of Treated Pome

Parameter	Value
COD	836 ± 21 mg/L
BOD_5	400 ± 17 mg/L
NH_4-N	15.2 ± 0.4mg/L
PO_4^{3-}	13.7 ± 0.5 mg/L
NO_3^-	28.0 ± 12mg/L
pH	9.56 ± 0.3

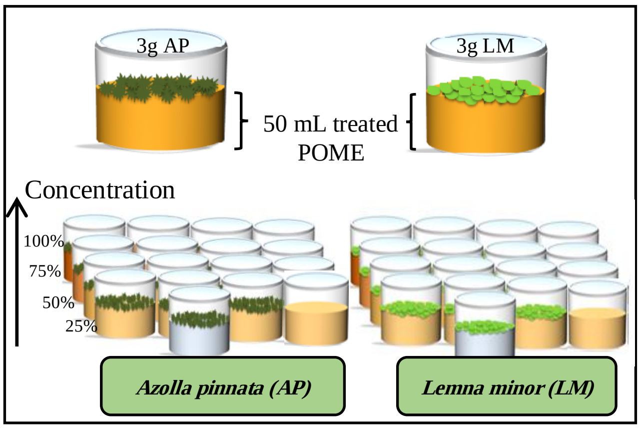


Fig. 2: Experimental Set-Up for Phytotoxicity Test.

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

Plant	Fresh /Healthty	Dead
Azolla pinnata		
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 (BOD_5), pH, ammonia nitrogen (NH_4-N), nitrate nitrogen (NO_3^-), and dissolved orthophosphate (PO_4^{3-}), 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 with an UV spectrophotometer (DR 6000, Hach Company, Loveland, CO, USA). 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*

POME Concentration (%)	Exposure duration (days): 1, 2, 3, 4, 5
100	
75	
50	
25	
0 (Control)	

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 the 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*

POME Concentration (%)	Exposure duration (days): 1, 2, 3, 4, 5
100	
75	
50	
25	
0 (Control)	

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.

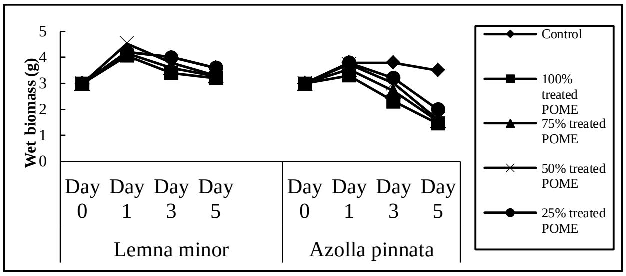


Fig. 3: Wet Biomass of *Azzola Pinnata* and *Lemna Minor* throughout the 5-Day Phytotoxicity Test to Treated POME.

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.

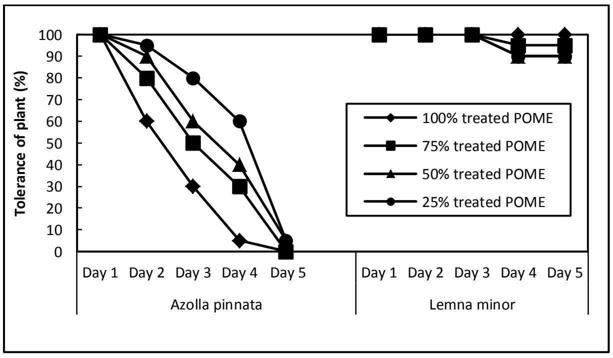


Fig. 4: Comparison on the Phytoxicity of Treated POME on *Azolla Pinnata* and *Lemna Minor*.

3.3. Characteristics of wastewater throughout the 5-day exposure

The results of water quality parameters (NO_3^-, PO_4^{3-}, NH_4-N, pH, BOD_5 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.

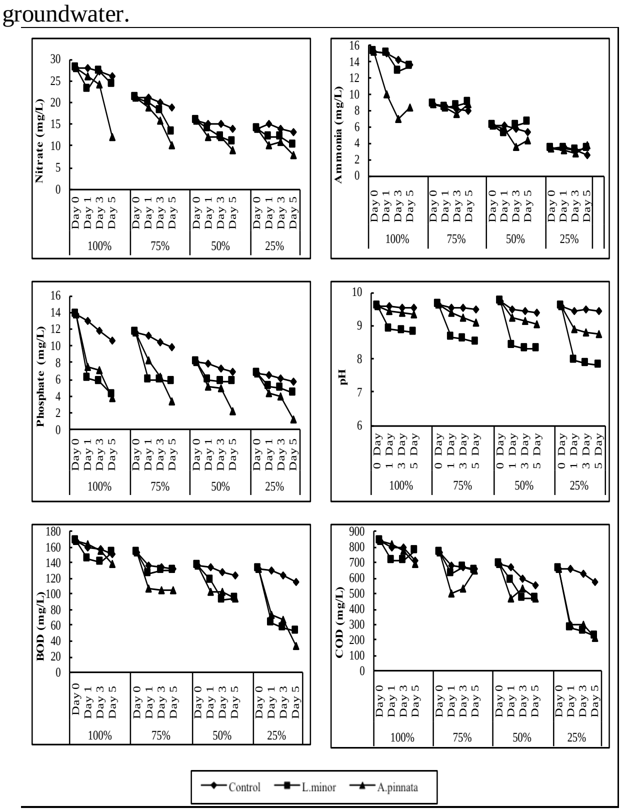


Fig. 5: Characteristic of Water Quality Throughout the 5-Day Phytotoxicity Test.

It is a toxic substance commonly found in POME. Ammonium intake is very important for the treatment of POME, in which ammonium is the primary form of nitrogen. The results of this study show that ammonia can be partially removed from treated POME using both species. *A. pinnata* was capable of removing ammonia from 14.3 to 7 mg/L which is 51.0% reduction compared with the control within 3 days in 100% treated POME as shown in Figure 5. The extraction rate of NH_4-N after 5 days in all dilution solutions by *L. minor* was less favorable, only 9.8% removal in 100% treated POME. The ammonia concentration showed increment after 3 day of cultivation for 75%, 50% and 25% treated POME. The NH_4-N concentrations at 50% and 25% treated POME increased significantly and reached 3.4 and 3.8 mg/L at the end of 5 days, respectively for *A. pinnata*. This was mainly attributed to the breakdown of duckweed tissue, which was degraded by bacteria and exoenzymes, and eventually released NH_4^+ [42]. Duckweed deliberately absorbs ammonia from nitrites due to the nitrogen content in ammonium forms that is directly altered to plant proteins [54]. Due to the huge potential for wastewater treatment, duckweed is given the opportunity to increase the future through genetic engineering. The simple protocol for transferring gene to minor *L. minor* has been shown to produce more specific nutrients or other pollutants [55]. If the ammonia content in the water supply is too high, it can be very toxic to humans. [29] studied the nitrogen extraction by *L. minor* and found that these species growing in a 1:1 mixture of NH_4^+ and NO_3^- preferably took ammonia against nitrate particularly in low nitrogen availability. Excessive ammonium speeds up eutrophication in the open pool and produces nitrate formation when released into underground water. Therefore, it is vital to remove it from wastewater.

[56] reported that floating plants such as *Lemna sp.* requires high concentration of phosphorus to grow in water. [57] also found that *Lemna* consistently released the largest amount of ammonia and phosphorus from storm water within 8 weeks. During the 5-day exposure, the phosphate from the treated POME solution was used by *A. pinnata* and *L. minor* for growth and its concentration was reduced 65.4% to 3.7 mg/L and 61.4% to 4.13 mg/L at the end of

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 crop 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 % NO_3^-, 9.8% NH_4-N, 61.4% PO_4^{3-}, 17.3% pH, 54.0% BOD_5 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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