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PHYTOREMEDIATION OF PALM OIL MILL FINAL DISCHARGE WASTEWATER USING SELECTED AQUATIC MACROPHYTES

MOHD FAIZAL BIN HAMZAH

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ABSTRACT

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This study assesses the treatment of palm oil mill (POM) final discharge wastewater using a cost effective technology approach of phytoremediation. POM final discharge wastewater obtained from final distribution pond at KL Kepong Berhad Palm Oil Mill, Tanjong Malim was treated with three species of local macrophytes, *Leersia oryoides*, *Pistia stratiotes* and *Ludwigia peploides*. The phytoremediation performance was evaluated by monitoring the changes and removal efficiency (%) of BOD_5, COD, $N\text{-}NH_3$, TSS and pH level. The characteristics of nutrient uptake were determined by measuring the percentage of C, H and N elements, bioconcentration factor (BCF) and translocation factor (TF) of Ca, Mg, K, Na, Fe and Zn in leaf, stem and root organs of the macrophytes. The microbial population in the rhizosphere was identified using partial 16S rRNA molecular technique. It was found that BOD_5, COD, $N\text{-}NH_3$ removal efficiencies of 93%, 30%, and 82% were achieved for *Pistia stratiotes*, 90%, 27% and 80% for *Leersia oryzoides* and 93%, 20% and 80% for *Ludwigia peploides*, respectively after 15 days of treatment. The N percentages were increased in all studied macrophytes leaves. The BCF values for Fe and Zn were found highest in *Ludwigia peploides* than other elements, 1.47×10^4 L/kg and 1.18×10^4 L/kg, respectively. Low TF values (<1.0) obtained for Fe and Zn indicated that most of them were retained in root for phytostabilization. In this study, *Bacillus megaterium*, *Pseudomonas aeroginosa* and *Bacillus cereus* that usually involved in denitrification process was identified in *Pistia stratiotes*, *Ludwigia pepoides* and *Leersia oryzoides* roots, respectively which confirmed the macrophytes-microorganisms interaction in the phytoremediation. These results suggest that phytoremediation of POM final discharge wastewater was feasible with *Ludwigia peploides* as the most tolerance macrophytes. The finding is of prominence important for improvement of the existing palm oil mill effluent (POME) treatment system.

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FITOREMEDIASI AIR BUANGAN AKHIR KILANG KELAPA SAWIT MENGGUNAKAN MAKROFITA AKUATIK TERPILIH

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ABSTRAK

Kajian ini menilai kaedah rawatan air buangan akhir kilang kelapa sawit (POM) dengan menggunakan teknologi kos efektif iaitu fitoremediasi. Air buangan akhir kilang kelapa sawit yang diperolehi dari kolam pengagihan akhir kilang kelapa sawit KL Kepong Berhad Tanjong Malim dirawat dengan tiga spesis makrofita tempatan iaitu *Leersia oryzoides*, *Pistia stratiotes* dan *Ludwigia peploides*. Prestasi fitoremediasi dinilai melalui pemerhatian terhadap perubahan dan kecekapan penyingkiran (%) BOD_5, COD, $N\text{-}NH_3$, TSS dan pH. Ciri-ciri pengambilan nutrien ditentukan dengan mengukur peratusan elemen C, H dan N, faktor biokonsentrasi (BCF) dan faktor translokasi (TF) bagi Ca, Mg, K, Na, Fe dan Zn di dalam organ daun, batang dan akar makrofita. Populasi mikroorganisma di rizosfera dikenalpasti menggunakan teknik molekular 16S rRNA. Dapat diperhatikan bahawa kecekapan penyingkiran BOD_5, COD, $N\text{-}NH_3$ ialah masing-masing 93%, 30%, 82% bagi *Pistia stratiotes*, 90%, 27%, 80% bagi *Leersia oryzoides* dan 93%, 20%, 80% bagi *Ludwigia peploides* selepas 15 hari rawatan. Peratusan N meningkat di dalam semua daun makrofita yang dikaji. Nilai BCF bagi Fe dan Zn didapati paling tinggi di dalam *Ludwigia peploides* berbanding dengan elemen-elemen lain iaitu masing-masing 1.47×10^4 L/kg dan 1.18×10^4 L/kg. Nilai TF yang rendah (<1.0) yang diperolehi bagi Fe dan Zn menunjukkan bahawa kebanyakan elemen-elemen ini ditahan di akar bagi proses fitostabilisasi. Kajian mendapati *Bacillus megaterium*, *Pseudomonas aeroginosa* dan *Bacillus cereus* yang biasanya terlibat dalam proses denitrifikasi telah dikenalpasti pada akar *Pistia stratiotes*, *Ludwigia peploides* dan *Leersia oryzoides* yang mana mengesahkan interaksi makrofita-mikroorganisma di dalam fitoremediasi. Dapatan ini mencadangkan fitoremediasi air buangan akhir POM boleh dilakukan dengan *Ludwigia peploides* sebagai makrofita paling toleran. Dapatan kajian ini adalah sangat penting untuk sistem rawatan efluen kilang kelapa sawit (POME) sedia ada.

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LIST OF ABBREVIATIONS

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BCF – Bioconcentration Factor

BLAST – Basic Local Alignment Search Tool

BOD_3 – 3-day Biochemical Oxygen Demand

BOD_5 - 5-day Biochemical Oxygen Demand

COD - Chemical Oxygen Demand

DO - Dissolved Oxygen

HPC – Heterotrophic Plate Count

HRT – Hydraulic Retention Time

POM – Palm Oil Mill

POME – Palm Oil Mill Effluent

POM FD – Palm Oil Mill Final Discharge

RDP II – Ribosomal Database Project II

TF – Translocation Factor

TS – Total Solid

TSS – Total Suspended Solid

VFA- Volatile Fatty Acids

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CHAPTER 1

INTRODUCTION

1.1 Background of Study

Phytoremediation is a method that use plants or plant organs to remove, degrade, or render harmless hazardous materials present in soil and water. The emerging technology may offer a cost effective, non-intrusive, and safe alternative to conventional soil clean up techniques by using the ability of certain tree, shrub, and grass species to remove, degrade or immobilize harmful chemical from soil or water (Vishnoi & Srivasti, 2008). For many years macrophytes have been known to play an important role in removing pollutants as tertiary treatment (Curia, Koppe, Costa, Feris & Gerber, 2011). The application of phytoremediation for various kind of wastewater such as landfill leachate, palm oil mill effluent (POME) and sewage might be an intriguing approach. In Malaysia, palm oil industry is among the largest agricultural

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sector that contributes to the production of POME (Tang, Huang, Scholz & Li, 2009). POME is a liquid waste produced during palm oil processing in palm oil mill. About 0.67 tonne of POME is generated for every fresh fruit bunch (FFB) processed (Ma, 2009).

In year 1970s, the subject of POME and its impact on the environment had become an issue of much concern to the government and the public. The government has taken action in enacting the Environment Quality Act in 1974 and specific regulations for POME in 1977. The regulations of governing discharges into water courses and lands came into force on 1st July 1978. It is mandatory for all palm oil mills to treat their wastewaters on site to an acceptable level before it is allowed to be discharged into watercourses (Table 1.1) (Environmental Quality Act 1974).

In response to these issues, most of the palm oil mills initiate POME treatment through physical and biological methods using open ponding system. Various designs and configurations of ponding systems are used in Malaysia by 421 palm oil mills in Malaysia (Ma, 2009; MPOB, 2010). Figure 1.1 shows a typical system of POME treatment with several ponds, each for different function as indicated. Usually the open ponding system is used as its involved low cost of maintenance and capital operating system. The treated POME also known as palm oil mill (POM) final discharge wastewater is produced after the biological and physical treatment and usually still contain several types of pollutants particularly BOD, COD N-NH3 and TSS that requires tertiary treatment before being discharge into the watercourses.

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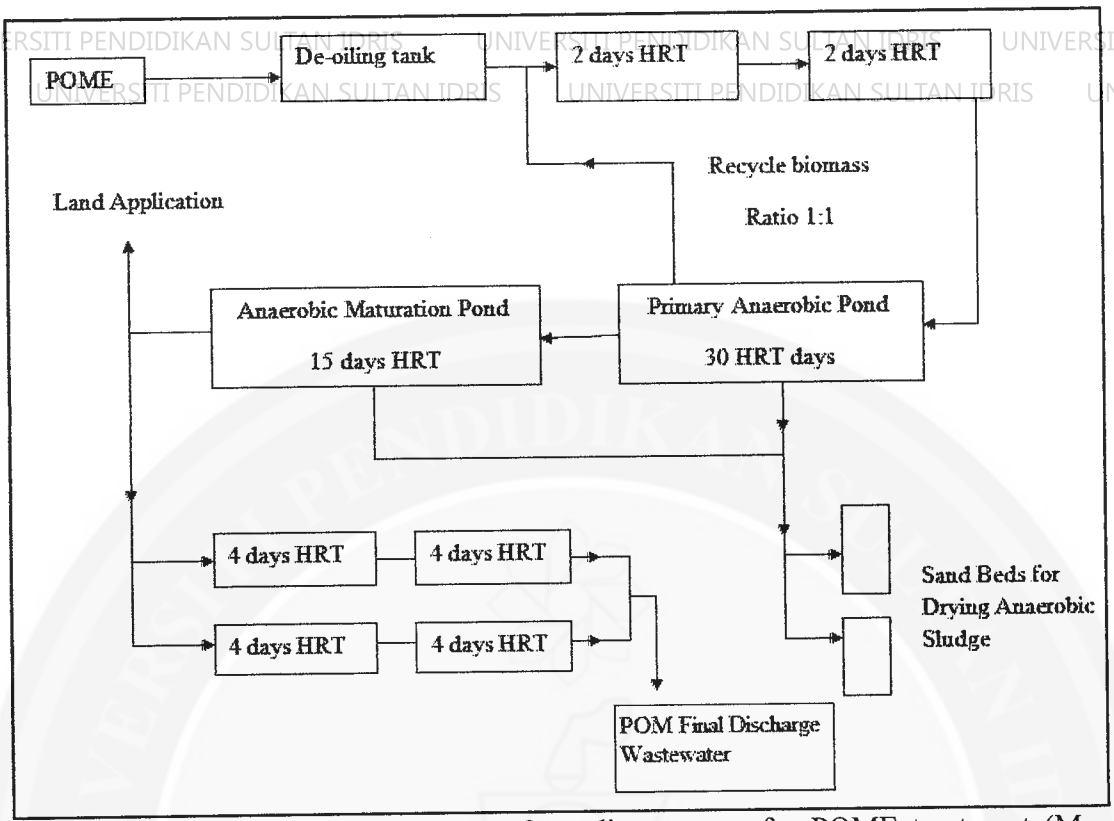


Figure 1.1. Schematic flow diagram of ponding system for POME treatment (Ma, 2009)

Phytoremediation is among the cost effective technology potentially used for bioremediation. This process depends on the plants to draw contaminated soil water to the rhizosphere (root zone) and in which plant uptake of contaminants or microbial activities provide desired removal of contaminants (Crites, Middlebrooks & Reed, 2006). Microbial community thrive up during phytoremediation because macrophytes provide large surface area for microbial biofilms and supply oxygen to microbial community through their roots (Greenway, 2007; Vymazal & Kropfelova, 2008). Furthermore, microbial communities also play important role in decomposition of organic matter (Cronk & Fennessy, 2001).

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There is several factors influence phytoremediation efficiency using aquatic macrophytes. Among them are the rate of nutrient uptake, bioavailability of nutrient, age of macrophytes, types of macrophytes roots and phytotoxicity effect (Cronk & Fennessy, 2001; O'Sullivan, Moran, & Otte, 2004; Wu et al., 2011; Cheng et al., 2009; Pierce, Moore, Larsen & Pezeshki, 2010). The characteristics of wastewater such as recalcitrant properties also affect the performance of phytoremediation due to the limited biodegradation of organic matter (Calheiros et al., 2009). High strength of wastewater with large amount of chemical oxygen demand (COD) could inhibit the growth of macrophytes (Sooknah & Wilkie, 2004). For this reason, macrophytes are used to remediate palm oil mill (POM) final discharge wastewater which has lower strength of pollutants than the raw POME (Table 1.1). This research discussed phytoremediation of POM final discharge wastewater efficiency using selected aquatic macrophytes *Leersia oryzoides, Pistia stratoites* and *Ludwigia peploides*.

Previous studies have shown that aquatic macrophytes have great ability in removing pollutants during phytoremediation of various wastewaters. The ability of *Leersia oryzoides* in phytoremediation had been studied to remediate permethrin in mesoscosm experiment (Moore, Kroger, Cooper & Smith, 2009) and different macronutrient allocations in its organs were observed (Pierce et al., 2010). *Pistia stratoites* was known could improve water quality by removing nitrogen and phosphorous from eutrophic stormwaters (Lu, He, Graetz, Stoffela & Yang, 2010).

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Table 1.1

Characteristics of POME and POM final discharge wastewater

Parameter (mg/L)[a]	Raw POME[c]	POM final discharge wastewater[d]		Standard limits (mg/L)[e]
	Range	Range	Average	
BOD_3[b]	25,000	490-612	549	100
Chemical Oxygen Demand (COD)	30,000-50,400	1025-1073	1049	-
Ammoniacal nitrogen (NH_3-N)	-	38-42	40.5	100
Total Suspended Solid (TSS)	18,000	660-750	710	400
Total Solid (TS)	40,500	5150-5300	5233	-
pH	4.7	8.44-8.5	8.48	5.0-9.0
Total Alkalinity (as $CaCO_3$)	-	13401-13511	13457	-

Note. [a] mg/L except for pH,
[b] BOD_5 express for POM final discharge wastewater and BOD_3 for raw POME at three days at 30°C
[c] Adapted from Bhatia, Othman & Ahmad (2006)
[d] This study
[e] Environmental Quality Act (1974)

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1.2 Significance of Study

1.2.1 Phytoremediation as an Environmental and Cost Effective Technology for Tertiary Treatment of POM Final Discharge Wastewater

As one of the leading agricultural industries in Malaysia, the palm oil industry has a yearly production of more than 13 million tons of crude palm oil and the plantations area covers 11 % of Malaysian land. However, the production of such amounts of crude palm oil had resulted in even larger amounts of POME produced, which estimated at nearly three times the quantity of crude palm oil (Wu, Mohammad, Jahim & Anuar, 2009).

POME can cause land and water pollution when discharged untreated due to the presence of high organic load and their phytotoxic properties (Nwoko, Ogunyemi, Nhwocha & Nnorom, 2010). Therefore it is imperative to find an economical and environmental friendly solution for this problem. Phytoremediation, being more cost-effective and fewer side effects than physical and chemical approaches, has gained increasing popularity in industry and research studies (Lone, He, Stoffela & Yang, 2008). Furthermore, Malaysia has a wide range of plant diversity, which provides high opportunity in finding the potential macrophytes to treat the environmental problem caused by POME discharges.

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1.2.2 Compliance to Malaysian Environmental Regulation

Most of Malaysian palm oil mills are located near to the rivers for easy access to water supply. As the location of mill is close to the river, mills conveniently discharge POME into the river. To some extent, this benefits the ecosystem as some nutrients can fertilize the river and enhance the growth of microplanktons which are food to aquatic life. However, POME in excess, as it degrades will deplete oxygen in water and suffocates the aquatic life (Ma, 2000). Uncontrolled discharge of POME can easily pollute water resources (Lim et. al, 2009).

Watercourses are most vulnerable to pollution by unregulated discharge of POME (Lim et al., 2009). Because of that, Malaysia had enacted Environmental Quality Act (1978) to ensure palm oil mills comply with parameter limits for POME discharges, which is 100 mg/L of BOD_5 for watercourse and 5000 mg/L for land application (Ma, 2009). Nevertheless, treated POME discharged from ponding systems sometimes could not comply with these parameter limits (Mohammad, Alam, Kabbashi & Ahsan, 2012).

In Malaysia, pond system is widely used for a secondary treatment of POME (Ma, 2009). This secondary treatment is based on biological approach namely anaerobic and aerobic lagoons. However, it is not efficient enough to treat POME (Ahmad, Ismail & Bhatia, 2005). This is due to the low organic loading rate applied, which is about 0.2 to 0.35 kg/m^3/day of BOD (Ahmad et al., 2005). In addition, the large size and configuration of the ponds made the biological process difficult to be controled and monitored. Usually, the mixing process at the facultative and aerobic

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zones could be only done by the biogass bubbling hence difficult to reduce BOD to the standard limit imposed by the government (Ma, 2009; Poh & Chong, 2009). Therefore, an appropriate tertiary treatment is necessary to reduce strength of POM final discharge wastewater. In comparison with other tertiary treatments such as dissolved air floatation (DAF) and reverse osmosis (RO), phytoremediation approach is more cost effective, less chemical usage and energy saving. Table 1.2 shows several aspects of comparison between phytoremediation with other tertiary treatment such as DAF and reverse osmosis.

In response to these issues, POM final discharge wastewater using macrophytes is suggested. The feasibility study of phytoremediation using three macrophytes species was performed. Throughout the study, the performance of the phytoremediation was monitored and evaluated. The characteristics of nutrient uptake during phytoremediation were determined to obtain a better understanding of the mechanism of phytoremediation process. The microbial population isolated from macrophytes root was enumerated and identified using 16S rRNA molecular technique. This information is useful in understanding the contribution of microorganisms in the phytoremediation process. To date, very limited data on the phytoremediation of POM final discharge wastewater can be obtained for Malaysia, hence, it might be valuable to improve the existing POME treatment system.

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Table 1.2

Comparison of three tertiary treatment of wastewater; dissolved air floatation (DAF), reverse osmosis (RO) and phytoremediation

Aspect of Comparison	Dissolved air flotation (DAF)	Reverse osmosis (RO)	Phytoremediation
Cost	Involve high cost for construction (civil work and electromechanical), chemicals, sludge treatment and disposal (El-Gohary, Tawfik & Mahmoud, 2010)	High cost compared to phytoremediation for instalment, maintainance, and specialized personnel (Zhang, Ji & Xi, 2006)	Low cost equipment and not involve highly specialized personnel (Erakhrumen, 2007)
Chemical usage	Use of chemical such as ferric chloride for coagulation and flocculation the suspended matter (Azbar & Yonar, 2004)	Use of NaCl rejection mounted with RO membrane (Zhang et al., 2006)	No chemical are used
Energy	Use of electrical energy (El-Gohary et al., 2010)	Use of electrical energy (Zhang et al., 2006)	Require human labour for site preparation (EPA, 2000)

1.3 Objectives

The objectives of this study are:

1. To evaluate the phytoremediation performance of POM final discharge wastewater using three different macrophytes species.
2. To determine the characteristics of nutrient uptake using phytoremediation of POM final discharge wastewater.
3. To identify microbial population involved in phytoremediation of POM final discharge wastewater.

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CHAPTER 2

LITERATURE REVIEW

2.1 Macrophytes for Phytoremediation

2.1.1 Classification of Macrophytes

A macrophyte is a plant that grows permanently on submerged land (Lim, 2002). Macrophytes can be classified into three types, emergent, submerged and floating (Greenway, 2007; Crites et al., 2006; Srivastava, Gupta & Chandra, 2008) based on their morphological and growth forms (Figure 2.1). Greenway (2007) classified macrophytes into two main divisions based on their functions: the rooted macrophytes and non-rooted macrophytes. Types and description of macrophytes are summarized in Tables 2.1 and 2.2. The main difference between rooted and non-rooted

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macrophytes is rooted macrophytes rely on sediment for anchorage while non-rooted macrophytes are free-floating or suspended in water column (Greenway, 2007).

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Table 2.1
Rooted macrophytes classification according to Greenway (2007)

Type of macrophyte	Descriptions
Emergent	This type of macrophyte has roots/rhizomes anchored in the sediment, and its stems and leaves emerge on water surface (e.g. reeds, bulrush, sedges). These macrophytes are usually found in shallow water column with water depth below 1 m. The root and rhizome systems procured oxygen from aerial organ because they live in permanently anaerobic condition
Floating-leaved	Maximum depth for these macrophytes can live is 1.5 m. It has leaves floating on water surface while its roots/rhizomes anchored onto sediment (e.g. water lilies).
Submerged	All of macrophytes organs are submerged and their lives and existence depend on light and oxygen (e.g. *Potamogeton, Triglochin, Vallisneria*).
Creepers or vines	Plants with anchoring in shallow sediment, floating stems and leaves, with adventitious roots in the water (e.g. *Bacopa monniera, Ipomopa aquatica, Ludwigia peploides, Persicaria strigosum*)
Trees and shrubs	Woody plants that dominate seasonally inundated swamp forests, riparian zones and floodplains (e.g. *Melaleuca, Salix, Alnus*)

Table 2.2
Non-rooted macrophytes classification (Greenway, 2007)

Type of macrophyte	Description
Floating plants	Surface leaves and stems with roots which hang down into the water (e.g. water hyacinth (*Eichornia*), duckweed (*Lemna*), *Azolla*).
Submerged plants	Stems and leaves below the water surface; adventitious roots may be present but non-attached (*Ceratophyllum, Hydrilla*).
Creepers or vines	Floating stems and leaves with adventitious roots in the water (e.g. *Ipomea aquatica, Paspalum distichum*)

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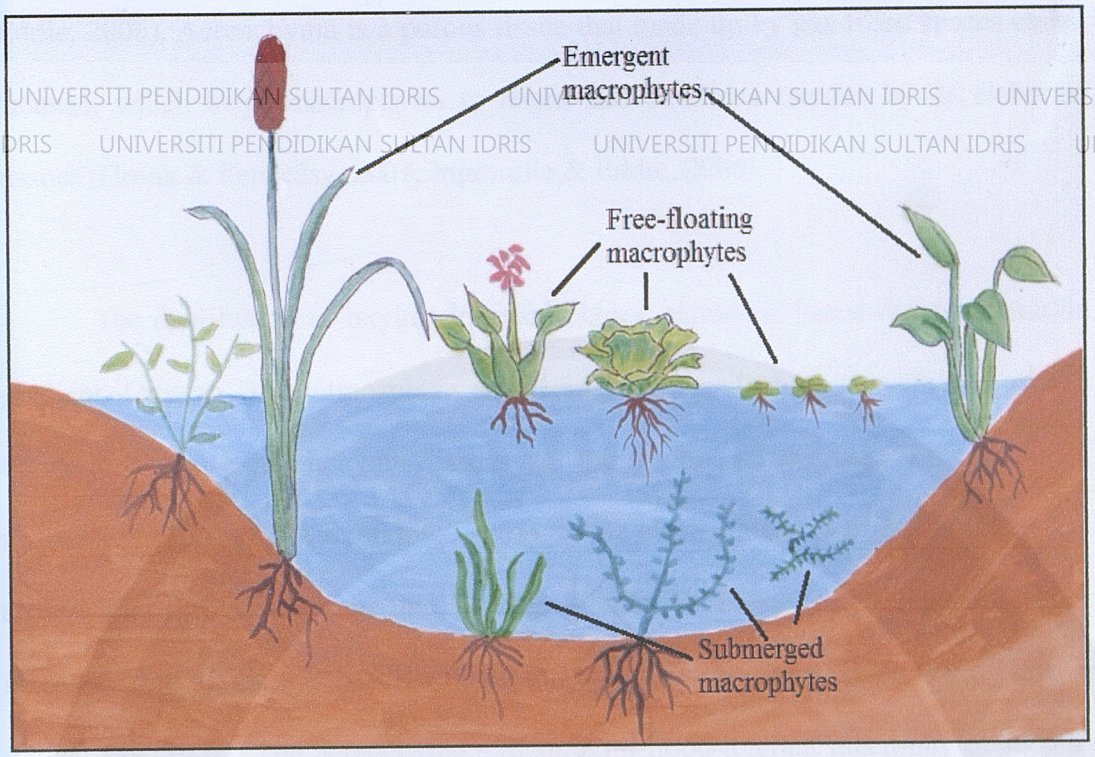


Figure 2.1. Three main types of macrophytes (Adapted from Greenway, 2007)

2.1.2 Adaptation of Macrophytes

Aquatic macrophytes have several adaptations to live in severe environment that less of carbon dioxide and oxygen. With their adaptations, aquatic macrophytes are able to absorb water and exchange gases over all surfaces of submerged tissues. The permanently submerged species usually have the most modified forms of gaseous exchange. In the majority of genera, the aquatic leaves lack stomata either on the upper or on the lower leaf structure, whereas in partly submerged ones, stomata are present only on the upper surface, thus increasing surface area for gases exchange. Numerous flotation devices have evolved independently across many lineages of aquatic plants and often seem to be derived from the aerenchyma system (Ingrouille &

Eddie, 2006). Aerenchyma is a porous tissue that made up by gas-filled spaces called lacunae, which helps macrophytes to retain and distribute oxygen by its elaborate tissues (Cronk & Fennessy, 2001; Ingrouille & Eddie, 2006).

The distribution of oxygen in macrophytes' tissues is based on concentration gradient. Oxygen from atmosphere and from by-product of photosynthesis will be transported along aerenchyma tissues to the roots and difusses into water at rhizosphere (Vymazal & Krofelova, 2008). In the harsh waterlogged environment that always has limited oxygen, this process is vital to create aerobic condition for microbial population that involve in various microbial processes such as nitrification (Greenway, 2007). The aerenchyma structures in flood-tolerant macrophytes such as *Leersia hexandra* roots are tightened with lignin and suberin while secondary walls in the outer layer of cortex are densely and thickly packed (Deng, Ye & Wong, 2009). The structure is comparable to the actinorhizal plants (dicots) that live in waterlogged area which root nodules surrounded by a periderm (cork and cork cambium) making it less impermeable to gas (Pawlowski, 2011). The floating macrophytes such as water hyacinth, *Eichornia crassipes* (pontederiaceae), and *Pistia*, have petioles that are inflated as floats (Ingrouille & Eddie, 2006).

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In *Ludwigia* (Onagraceae) the vertical shoots have aerenchyma in the basal regions and in the roots. In addition, there are spongy pneumatophores that arise from the rhizome and these probably increase aeration in this macrophyte. Some of the free floating aquatic aroids have root systems that hang in the water (e.g. *Lemna*) but others (e.g. *Wolffia*) are rootless. Aquatic plants also have active transpiration, a necessary means of transport of materials around the plant. Since submerged plants

cannot lose the water of transpiration through evaporation, they do so by guttation. This takes place via specialised 'water-pores' or hydathodes at nerve endings, and on the undersides of floating leaves, and is regulated in a layer of tissue known as the epithem (Ingrouille & Eddie, 2006). As submerged macrophyte lives in rich oxygen water, it makes this type of macrophyte not suitable to treat wastewater with high BOD (Dhote & Dixit, 2007).

2.1.3 Use of Macrophytes to Treat Water and Wastewater

In natural ecosystems, aquatic macrophytes play several vital roles to sustain the ecosystems. Excessive nutrient load from the water can be removed by macrophytes, thus preventing eutrophication of the water body (O'Hare et al., 2010). Also nutrient and mineral ions can be absorbed from water column by aquatic macrophytes. The slowing water current by aquatic macrophytes can favour sedimentation of suspended particles, and trap particulate matter and this indirectly influence metal retention (Greenway 2007; Lim, Tay & Kho, 2002; Idris, Abdullah, Hung & Wang, 2010). Wind protection from aquatic macrophytes also can reduce sediment resuspension. Researches about using aquatic macrophytes as a means for treatment of wastewater to mitigate variety of pollution level are abound (Srivastava et al., 2008). The characteristics of three macrophytes species; *Leersia oryzoides*, *Ludwigia peploides* and *Pistia stratiotes* is discussed in the following section.

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2.1.3.1 *Leersia oryzoides*

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Leersia oryzoides (Figure 2.2) is a grass type macrophyte with rhizomes that can be found abundantly in wildlife habitat such as wetlands, ditches and streams and reported to have high tolerance to acidity as low as pH 3.0 (USDA, 2008). It was also reported that *Leersia oryzoides* has great ability in uptake of iron (Fe) and this ability accelerates when there is an increase in water level which provides more available Fe for uptake (Pierce et al., 2010). When grown in enriched environment with nitrogen and phosphorous, *Leersia oryzoides* demonstrates luxury uptakes and assimilates more of both nutrients compared to non-enriched treatments (Kroger, Holland, Moore & Cooper, 2007).



Figure 2.2. Leersia oryzoides

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2.1.3.2 *Ludwigia peploides*

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Ludwigia peploides (Figure 2.3) is also known as water primrose in English and Inai Pasir in Malay language (Lim, 2002). *Ludwigia peploides* is an invasive macrophyte species in which its invasion of many types of fresh water ecosystem can lead to serious problems of management of this macrophyte (Lambert, Dutartre, Coudreuse & Haury, 2010). However, *Ludwigia peploides* posses several characteristics that are suitable for phytoremediation such as rapid growth, high nitrogen accumulation and can allocate most of its biomass and nitrogen into its above ground tissues. In addition, *Ludwigia peploides* have rapid decomposition when died (Hussner, 2010), in which decomposition can release nutrients into water regime. Greenway (2003) found that *Ludwigia peploides* are able to grow and spread successfully in secondary treated sewage effluent with NH_4-N concentrations 7.7 – 18.6 mg/L. Therefore, by discerning their potential and ability in phytoremediation, this macrophyte will be useful instead of only become an invasive and pest species of macrophyte.



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Figure 2.3. Ludwigia peploides

2.1.3.3 *Pistia stratiotes*

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Pistia stratiotes (water lettuce) (Figure 2.4) is a spongy macrophyte and consists of a shell-like rosette of light yellow-green leaves. A tuft of long unbranched fibrous roots extends from a central extension of the underwater stolon. Reproduction is predominantly by budding either from the main plant or from stolons. Water lettuce appears to thrive best in still water or areas of nominal flow. It grows mainly as free-floating plant, but can survive as a semi-rooted plant for prolonged periods (Neuenschwander, Julien, Center & Hill, 2009). As free-floating macrophyte, it buoyant and rigid leaves are built by cells turgor and extensive lacunate mesophyll tissue which contain more than 70% gas by volume (Wetzel, 2001). *Pistia stratiotes* is very useful for phytoremediation because it can grow rapidly and have high capacity to assimilate nutrient (Greenway, 2007). *Pistia stratiotes* is also reported to have great ability in removing heavy metals such as zinc (Zn), iron (Fe), cadmium (Cd), copper (Cu) and chromium (Cr) from wastewater (Mishra & Tripathi, 2008).



Figure 2.4. Pistia stratiotes

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2.1.4 Role of Macrophyte Roots in Phytoremediation

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Plant uptake of contaminants by its root and microbial activity plays important role in phytoremediation (Crites et al., 2006). Greenway (2007) and Lim et al. (2002) stated that macrophyte roots are supporting microbial biofilm growth by becoming substrate for microbial growth. Iasur-Kruh, Hadar, Milstein, Gasith and Minz (2009) also found that there is a highly complex bacterial diversity in macrophyte root biofilms. The relationship between macrophyte root and microbial biofilm allows macrophytes to assist microbial biofilm growing by supplying oxygen through its root, in which oxygen play a pivotal role in the activity and metabolism performed by microorganism in the macrophyte rhizosphere (Stottmeister et al., 2003).

Macrophytes have the capability to transport oxygen into the root zone and this can oxidizes microenvironment and stimulates carbon and nitrogen transformations (Greenway, 2007; Vymazal & Kropfelova, 2008). However, macrophytes that live in anoxic condition develop special aerenchyma structure in root cells, tightened with lignin and suberin to steer oxygen transport to the apex. This will prevent radial oxygen loss (ROL) to rhizosphere and enhance tolerance of macrophytes to high metals concentration (Deng, Ye & Wong, 2009). Metals that are well studied and proved can be removed by macrophyte are iron (Fe), zinc (Zn) and alkali earth metals such as calcium (Ca), magnesium (Mg), potassium (K) and sodium (Na) (Deng et al., 2009; O'Sullivan et al., 2004; Morari & Giardini, 2009).

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2.2 Mechanisms of Phytoremediation

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Phytoremediation is an increasingly popular green technology approach based on plant ability to accumulate, translocate, remove, destroy or sequester harzardous contaminants from the environment (Glick, 2003; Rahman & Hasegawa, 2011). In previous studies, various contaminants have been successfully treated by phytoremediation technique such as petroleum hydrocarbons, chlorinated solvents, metals, radionuclides, and nutrient such as nitrogen and phosphorous (Crites et al., 2006). There are several mechanisms involved in phytoremediation and can be categorized as phytoextraction, phytofiltration, phytostabilization, phytovolatization and phytodegradation (Garbisu & Alkorta 2001).

2.2.1 Phytoextraction

Phytoextraction is the removal of a contaminant from polluted soil, ground water or surface water by plants. The contaminants are uptake by plants roots and translocate within the plants (EPA, 2000). Plants store the pollutants in their organs and pollutants are removed by harvesting the plants (Singh, Dhania, Sharma & Jaiwal, 2007). Among the factors that determine the efficiency of phytoextraction are plant biomass productions and site arability, availability and solubility of metals and nutrients, the ability of plant to accumulate nutrients and metals in its tissues and regulation of phytoextraction process (Simeonova & Simeonov, 2006).

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2.2.2 Phytoaccumulation

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Phytoaccumulation is a process that involved accumulation of metals by plants and the ability of plant to accumulate metals is expressed by biocencentration factor (BCF) and bioaccumulation factor (BAF) (Fairbrother, Wenstel, Sappington & Wood, 2007; Zou, Li, Zhang, Yu & Huang, 2012). Plants that accumulate high amount of metals are called hyperaccumulator (Lu, Tian, Zhang, Zhang, Yang & Jiang, 2010). The hyperaccumulators that have been most extensively studied by scientific community include *Thlaspi sp.*, *Arabidopsis sp.*, *Sedum alfredii sp.* (both genera belong to the family of Brassicaceae and Alyssum). *Thlaspi sp.* are known to hyperaccumulate more than one metal, i.e., *T. caerulescens* for cadmium (Cd), nickel (Ni), lead (Pb) and zinc (Zn), *T. goesingense* for Ni and Zn, *T. ochroleucum* for Ni and Zn, and *T. rotundifolium* for Ni, Pb and Zn (Prasad & Freitas, 2003). The metal ion accumulated in the aerial parts that can be removed to dispose or burnt to recover metals (Garbisu & Alkorta, 2001). There are several factors that cause accumulation rate of pollutants during phytoaccumulation. As described by Trapp, Ucisik, Romano & Larsen (2007), pollutant removal by plants depends on three factors; mass of plant, velocity of uptake of pollutants and enzymatic reaction rate.

2.2.3 Phytostabilization

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In phytostabilization, the plant roots absorb the pollutants from the soil and keep them in the rhizosphere, rendering them harmless by preventing them from leaching (Garbisu & Alkorta, 2001). This process takes advantage of plant root ability to alter

soil environment conditions, such as pH and soil moisture content. Many root exudates cause metals to precipitate. Thus diminish bioavailability of metals (Susarla, Medina & McCutcheon, 2002). Phytostabilization is used in the treatment of soil, sediments and sludges. Phytostabilisation has several advantages such as low cost, ecosystem restoration enhanced by revegetation, and disposal of hazardous materials and biomass and soil removal are not required. However, when plants are not removed, the contaminants are remained in place, so the vegetation and soil required long-term maintenance to hamper re-release of the contaminants and impending leaching (EPA, 2000).

2.2.4 Phytodegradation

Phytodegradation is also known as phytotransformation in which contaminants are taken up by plants and metabolic processes within the plants will breakdown the contaminants or contaminants will be brokedown externally by enzymes produced by plant and enzyme co-factors (EPA, 2000; Susarla et al., 2002). Uptake process is dependent on hydrophobicity, solubility, polarity and acidity of contaminants. Moderately hydrophobic organic compounds are most readily taken up by and translocated within plants. By-products of degradation process will be used in lignification of cell walls, mineralisation or complete degradation or organic compounds which convert them into water and carbon dioxide (Barbafieri & Tassi, 2011). Contaminants degradation due to enzymes produced by a plant can occur in an environment free of microorganisms (for example, an environment in which the microorganisms have been killed by high contaminant levels). Plants are able to grow

in sterile soil and also in soil that has concentration levels which are toxic to microorganisms. Thus, phytodegradation potentially could occur in soils where biodegradation cannot (EPA, 2000). The example of phytodegradation is degradation of polychlorinated biphenyls (PCB) with up to three chlorine atoms by *Phragmites australis* (Chu, Wong & Zhang, 2006).

2.2.5 Rhizodegradation

Rhizodegradation is the breakdown of an organic contaminant in soil through microbial activity that is enhanced by the presence of the root zone. Rhizodegradation is also known as plant-assisted degradation, plant-assisted bioremediation, plant-aided *in situ* biodegradation, and enhanced rhizosphere biodegradation (EPA, 2000). Rhizodegradation of pollutants occur in rhizophere because plants and microorganisms often have symbiotic relationships making the root zone or rhizophere an area of very active microbial activity (Wenzel, 2009). Certain plant roots release substances that become nutrients for microorganisms like bacteria and fungi. This results in increased biological activity of the microbes immediately in the area surrounding the root zone (rhizosphere). By encouraging a microbiologically active rhizosphere, the plants facilitate accelerated digestion (biodegradation) of wide variety of organic contaminants (Sinha, Valani, Sinha, Singh & Herat, 2009).

Many organic compounds are degraded by microorganisms located in the rhizospheres (on the roots) of plants. The enhanced rhizosphere biodegradation results from the ability of certain plants to provide favourable habitats for soil microbes to act

(Hrynkiewicz & Baum, 2011). Macek et al. (2008) reported that rhizosphere play a dominant role in degradation of PCBs (Polychlorinated Biphenyls). The water hyacinths (*Eichhornia cressipes*) works on the same biological principle. It harbours several microbes in its root zone which perform the task of biodegradation of heavy metals in polluted water and also helps in absorption and adsorption of chemical impurities. Filtration, sedimentation and attachment of microbes in the plant root surfaces is thought to be one of the processes of microbial reduction in wetlands. It is plausible that the microbial attachment to the root surface reduces the settling of bacteria and viruses in constructed wetlands (Karim, Manshadi, Karpiscak & Gerba, 2004)

2.2.6 Rhizofiltration

Rhizofiltration is the adsorption or precipitation onto plant roots, or absorption of contaminants into the roots that are in solution surrounding the root zone, due to biotic or abiotic processes. Plant uptake, concentration, and translocation might occur, depending on the contaminant. Exudates from the plant roots might cause precipitation of some metals. Rhizofiltration first results in contaminant containment, in which the contaminants are immobilized or accumulated on or within the plant. Contaminants are then removed by physically removing the plant. Rhizofiltration is efficient when contaminants are in low concentration and the condition is high-water-content. Rhizofiltration is not efficient in soil medium, sediments or sludge because the contaminants need to be in solution in order to be absorbed into plant system (EPA, 2000).

Examples of rhizofiltration systems are natural and constructed wetlands which have been successfully utilised for the removal of a wide range of pollutants, including plant macronutrients, metals, organic compounds and biological contaminants, such as coliform bacteria and parasites, and they also improve pH, Biological Oxygen Demand (BOD) and Chemical Oxygen Demand (COD) (Otte & Jacob, 2006). Plant selected for rhizofiltration treatment must be vigour, with ample fibrous root system to make absorption of contaminant efficient (Dushenkov & Kapulnik, 2000). An example of rhizofiltration experiment was conducted by Garnett, Murch, Krishnaraj, Dixon and Saxena (2002) show that scented geraniums plant are able to tolerate excess salt up to 100 mM, but beyond that, scented geraniums shows phytotoxicity with stunted growth. Another study carried out by Abhilash et al. (2009) showed that *Limnocharis flava* is a suitable candidate for the phytofiltration (>93%) of Cd from low-level Cd-contaminated water.

2.2.7 Phytovolatilization

There is a specific phytoremediation process in removal of volatile organic contaminants which is called phytovolatilization. Phytovolatilization involves three stages which commence with uptake of volatile organic contaminants by plant roots. Second stage is transport of contaminants to leaves and third stage involve releasing the volatile contaminants into the air in the process of volatilization which occur at the stomata of plant leaves (Singh et al, 2007). During phytovolatilization, phytodegradation also takes place when contaminants are modified to enable for release during plant metabolism and plant transpiration (EPA, 2000; Crites et al,

2006). Phytovolatilization is relevant for all compounds that are quickly translocated and have a high vapour pressure or a high Henry's Law constant and mainly occur during sun shining (Trapp & Karlson, 2001)

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2.3 Phytoremediation Performance of Wastewater

Phytoremediation of various type of wastewater using macrophytes was aboundly reported. The performance of these phytoremediation is summerised in Table 2.3:

Table 2.3
Phytoremediation of various kind of wastewater using macrophytes

Macrophyte species	Type of wastewater	Findings	References
Schoenoplectus tabernaemontani	Agricultural wastewater	• Accumulation of Ca, Mg, Na, K, Mn occurred mainly in above ground tissue and Fe, Zn, Cu and B in below-ground tissue.	Tanner (2001)
Eichornia crassipes	Diluted lemon industry effluent	• Water hyacinth reduced diluted effluents of BOD and COD to 70 and 61%, respectively. • Reduction in the effluent colour due to the metabolic action of the plants on pigments.	Navarro et al. (2012)
Phragmites australis	Oil refinery wastewater	• Removal efficiency above 90% was obtained.	Moreno et al. (2002)
Reed (*Scirpus sp.*) and Cattail (*Typha sp.*)	Metal-mechanical industry effluents	• Removal of NH_4-N (56%), TKN (61%) and phosphorus (73%) • Vertical flow promotes a	Curia et al. (2011)

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		higher aeration of effluent, facilitating the nitrifying bacterial action and reduced NH_4-N level.	
Typha angustifolia, Desmostachya bipinnata, and *Sacharum bengalense*	Acid mine drainage	• *Typha angustifolia* increased pH from 2.9 to 7.1 after 48 hours of retention. • *Typha angustifolia, Desmostachya bipinnata,* and *Sacharum bengalense* survived and thrived in acidic conditions.	Sheoran (2006)
Eichornia crassipes, Azolla pinnata and *Lemna minor*	Dairy effluent	• Nitrogen removal by *Eichornia crassipes, Azolla pinnata* and *Lemna minor* were 71.8, 62.5 and 60.13%, respectively. • Combination of *E. crassipes* and *L. minor* was found to be the best for removal of nitrogen and phosphorus from the dairy wastewater with 78.8 % N and 69.4% P were removed, respectively.	Tripathi and Upadhyay (2003)

2.3.1 Biodegradable Organic Matter Removal

Microbial degradation plays a dominant role in the removal of soluble biodegradable organic matter (BOD/COD) in wastewater (Lim, 2002). The BOD removal mechanism in the attached biofilms is similar to the trickling filters. Bacteria form biofilms on macrophytes roots help in enhancing phytoremediation process by degrading organic materials (Greenway, 2007). Biodegradation occurs when dissolved

organics is carried into biofilms on the submerged plant stems, the roots system and surrounding soil or media by diffusion process. Macrophyte roots have two main roles in BOD removal, the first is to provide a support medium for microbial degradation to take place and second is to convey oxygen to the rhizosphere and microbial community for aerobic degradation to occur (Idris et al., 2010; Lim, 2002).

Pevious researches proved that macrophytes play an important role in removing BOD_5 during phytoremediation of domestic wastewater (Hench et al., 2003; Bashal, 2010). BOD removal could be reduced when macrophyte litters decayed however, this eventually will increase BOD_5 level back (Karathanasis, Potter & Coyne, 2003).

2.3.2 Chemical Oxygen Demand (COD) Removal

Chemical oxygen demand (COD) is a measurement of organic strength based on oxygen required for the oxidation of organic coumpound and widely used in study of domestic and industrial waste (Sawyer, McCarty & Parkin, 2003). Many researches have been done to examine the feasibility of macrophytes for reducing COD in wastewater. However, these research show variable amount of COD that could be removed by macrophytes. For example, Bashal (2010) found that *Pistia stratiotes* could remove 43.8 mg/L COD from domestic wastewater. Mixture of macrophytes in constructed wetlands removed 231 mg/L of COD from industrial wastewater (Hadad, Maine & Bonetto, 2006). Tang et al. (2009) noted that macrophyte only play a small role in removing COD from wastewater during phytoremediation. It was also reported

that high COD level could affect macrophyte growth. Sooknah and Wilkie (2004) found dairy manure wastewater with 2010 mg/L COD halted *Pistia stratiotes* growth.

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High COD in wastewater indicates the presence of high organic carbon (Sawyer et al., 2003). During phytoremediation decayed macrophytes used to treat the wastewater release large amount of organic matter which contributes to high COD in its environment (Cronk & Fennessy, 2001). Organic matter usually contains approximately 45-50% carbon (C), could be used by denitrifiers as a source of energy for denitrification process (Idris et al., 2010). Furthermore, prolong of retention time during phytoremediation promotes hydrolysis of organic matter that trapped in macrophytes roots to proceed smoothly (Song et al., 2006).

2.3.3 Ammoniacal Nitrogen Removal

Both free living and symbiotic microorganisms are capable of fixing atmospheric N_2, while nitrogen in the form of ammonium ion (NH_4^+) and nitrate (NO_3^-) could be directly taken up by plants (Maathuis, 2009; Vymazal & Kropfelova, 2008). Previous studies show that various macrophytes have high ability in removing ammonia from wastewater, such as *Phragmites australis*, *Phragmites communis*, *Zizania latifolia* and *Typha angustifolia* (Moreno et al., 2002; Kim, Park, Lee & Kang, 2011; Bashal, 2010). Excess in ammonia in submerged macrophyte tissues can cause oxidative stress which is quelled by antioxidant defence system. However, antioxidant defence system can contribute to the production of reactive oxygen species which can cause deterioration of macrophytes (Nimptsch & Pflugmacher, 2007).

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Wetland system provide better picture on how nitrogen transformation in nature are takes place (Lim et al., 2002). In wetland system, for instance, free-water surface occurs when two zones develop; the upper layer of media is oxidised (aerobic zone) and lower layer is reduced (anaerobic zone). The organic N is mineralized to NH_4^+-N in both layers during the ammonification process (Lim et al, 2002; Idris et al, 2010), however oxidized layer (aerobic) favours ammonification more than the reduced zone (Saeed & Sun, 2012). The oxidized layer and the submerged portions of macrophytes are important sites for nitrification in which ammoniacal nitrogen (NH_3-N) are converted to nitrites NO_2^--N by *Nitrosomonas* bacteria and eventually converted to nitrates NO_3^--N by the *Nitrobacter* bacteria (Idris et al, 2010). The product of nitrification, NO_3^- is either taken up by the macrophytes or diffuses into the reduced zone (anaerobic) where it is converted to N_2 and N_2O by the denitrification process (Idris et al., 2010).

At a higher pH of 10, some NH_3-N will be lost to the atmosphere by volatilization process (Lim et al, 2002; Idris et al, 2010). NO_3^- in the reduced zone is removed through denitrification, leaching and some by plant uptake. However, reduced zone is replenished by NO_3^- from oxized zone through diffusion process. Atmospheric oxygen diffuses into rhizosphere through the leaves, stems, rhizomes, and roots of macrophytes thus creating an aerobic layer that is similar to that oxidized layer.

It was reported that *Pistia stratiotes* can remove more than 50% of NH_4^+ compared to unvegetated plot treating eutrophic stromwater (Lu, He, Graetz, Stoffella & Yang, 2010). Recent finding shows that ammonia is not only removed by ammonia-

oxidizing bacteria but also by ammonia-oxidizing archaea which can be found in various habitats, including wastewater treatment system (You, Das, Dolan & Hu, 2009).

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2.3.4 Solids Removal

In a wetland, solids that are able to settle usually are removed by physical sedimentation which involves gravitational settling of solids and constituent pollutants. The macrophyte roots give secondary effect to settleable solid through filtration and adsorption. Then, solids that trapped in roots system are degraded by bacteria (Lim et al., 2002; Vaillant, Monnet, Sallanon, Coudret & Hitmi, 2003). However, this biological process plays only a minor role in solid removal (Song et al., 2006). The decaying macrophyte litters can increase TSS in phytoremediation treatment and hence re-poluted the wastewater (Karathanasis, Potter & Coyne, 2003).

2.3.5 pH Changes

Some macrophytes are sensitive to low pH level for optimum growth and the availability of HCO_3^- in water can affect their growth (Brandrud, 2002). Uptake of carbon dioxide (CO_2) by macrophyte roots can reduce carbonic acid in water and hence will increase water column pH (Cronk & Fennessy, 2001). Equation 2.1 shows how CO_2 diffuses into water to form carbonic acid (H_2CO_3) and dissociates to form hydrogen ions and bicarbonate (Equation 2.2). At pH 8.3 and above, bicarbonate

dissociates to form carbonate (CO_3^{2-}) and hydrogen ion (Equation 2.3) (Sawyer et al., 2003; Cronk and Fennessy, 2001). If waters contain appreciable amount of calcium ions ($Ca^{2+}$and CO_3^{2-}), calcium carbonate ($CaCO_3$) will precipitates (Equation 2.4) and this will increase pH level (Zhang et al., 2010; Sawyer et al., 2003). However, $CaCO_3$ precipitation only occurs at pH lower than 10 (Sawyer et al., 2003).

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$$CO_2 + H_2O \longleftrightarrow H_2CO_3 \qquad \text{(Equation2.1)}$$

$$H_2CO_3 \longleftrightarrow HCO_3^- + H^+ \qquad \text{(Equation2.2)}$$

$$HCO_3^- \longleftrightarrow CO_3^{2-} + H^+ \qquad \text{(Equation 2.3)}$$

$$Ca^{2+} + CO_3^{2-} \longleftrightarrow CaCO_3 \qquad \text{(Equation 2.4)}$$

A balance equilibrium of pH system developed by macrophytes is to ensure the uptake of alkali and alkaline earth metals such as Ca, Na, Mg, and K from surrounding water into roots tissue will release H^+ (Cronk & Fennessy, 2001). This argument was supported by Zhang et al. (2010) which showed that macrophytes could stabilize the alkalinity of wastewater in constructed wetland. However, when macrophytes died and decayed, these alkaline nutrients would be released again into water and caused an increase in pH level (Azeez, Prusty & Jagadesh, 2009).

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2.4 Nutrients Uptake and their Functions in Phytoremediation

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2.4.1 Mechanism of Nutrient Uptake and Allocation

The rooted macrophytes take up nutrients that are vital for their growth and reproduction primarily through their root systems while some uptake are via immerse leaves and stems from surrounding water (Brix, 2003). Rooted macrophytes remove these nutrients from the sediment, whereas submerged and floating macrophytes and algae remove the nutrients directly from the water column. These inorganic nutrients are assimilated and converted into organic matter (biomass) and became relatively unavailable until death and decay (Greenway, 2007).

Figure 2.5 depicts mechanism of metal uptake, commence by pumping out H^+ and secretion of chelators in which will mobilized metal towards root plasma membrane. To create a balance system between, inside and outside of roots, uptake of positively charged ions such as Mg^{3+}, Ca^{2+}, Na^+, K^+ and Fe^{2+} will release hydrogen ion (H^+) into the environment (Cronk & Fennessy, 2001). These positively charge ions will enter into root cells via plasma membrane through various types of pumping systems and precipitated in vacuole (Figure 2.5 (b)) (Vymazal & Kropfelova, 2008). However, if macrophytes live in excess of iron ions, iron will precipitate outside of root cell wall and gives brownish colour to roots (Siqueira-Silva, da Silva, Azevedo & Oliva, 2012; Deng et al., 2009). From the roots, metals are transported to the xylem through root symplast. Then, metals are translocated through xylem to reach leaves via plasmodesmata and some metals stored in trichomes (Figure 2.5 (c) and (d)). In the xylem, metals present in the form of hydrated ions or metal-chelate complexes are

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unloaded to shoots. After metals enter cell wall, they are distributed into organells by the process called 'trafficking' (Figure 2.5 (e)) which is mediated by specific metallochaperones and transportation of metals is localized to inner of membranes in each cell (Vymazal & Kropfelova, 2008).

2.4.2 Factors Influence Nutrient Uptake

2.4.2.1 Growth Rate

Plants with high growth rate will reduce time needed to reach a large biomass. In phytoremediation processes that involve plant roots, such as rhizodegradation, rhizofiltration and phytostabilization, fast growth of roots are desirable to enhance pollutants removal. However, in phytoextraction, high growth rate of above ground tissue are necessary for maximum pollutants removal (EPA, 2000). High growth rate accelerates plant nutrient uptake. (Jampeetong, Brix & Kantawanichkul, 2012). Macrophytes tend to take up more nutrients that are needed for their growth when increase in nutrient supplement and this leads to higher nutrient content (Cronk & Fennessy, 2001). Increase nutrient supply load also can increase floating-leave macrophyte biomass such as *Trapamaximowiczii* (Li, Liu & Guan, 2010) and hence raising floating macrophyte tolerance with metal contamination. Floating macrophytes show this characteristic are *Lemna minor* and *Spirodelapolyrhiza*, attribute to a very useful wastewater treatment (Leblebici & Aksoy, 2011).

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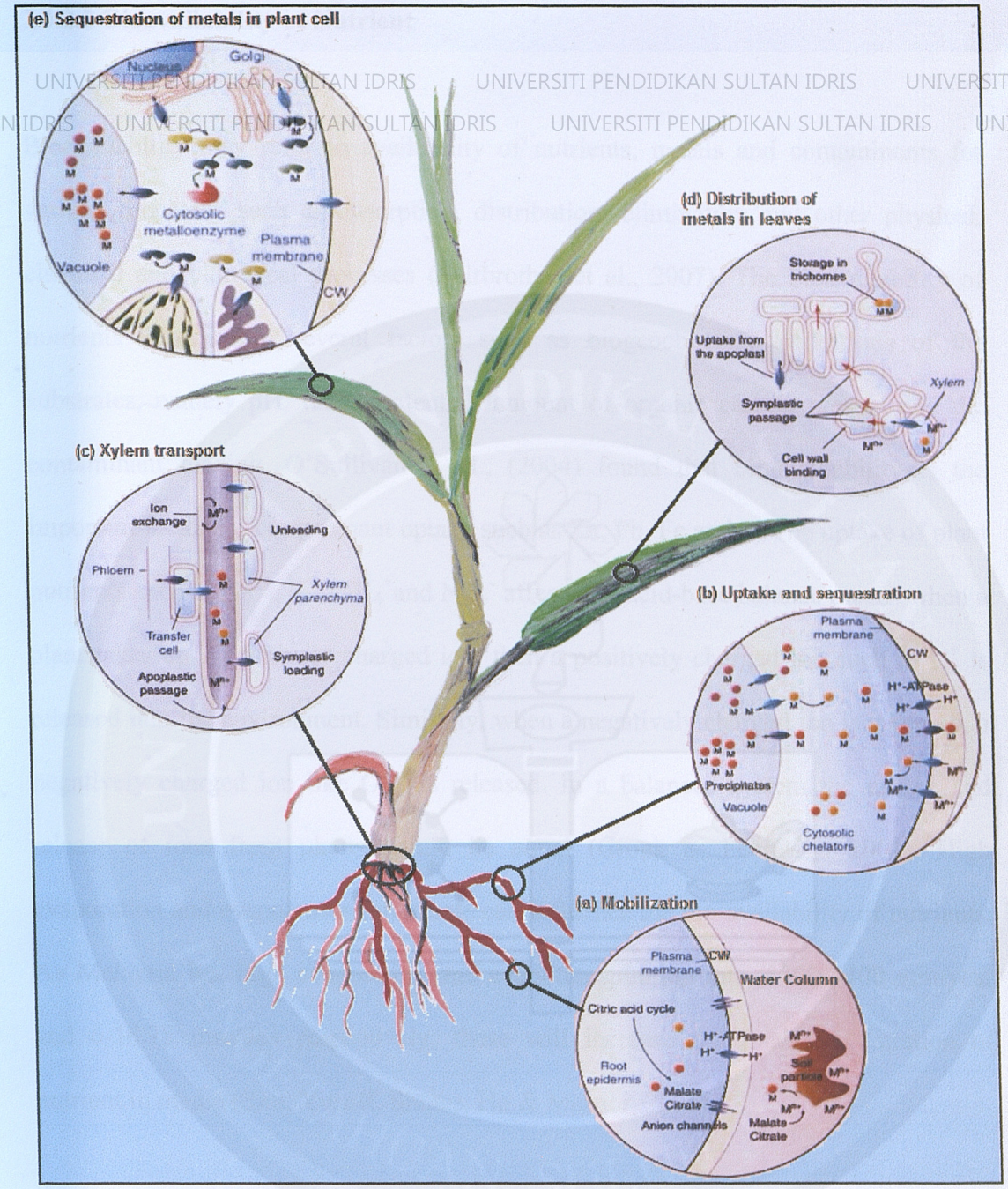


Figure 2.5. Molecular mechanisms proposed to be involved in transition metal accumulation by plants. (a) Metal ions are mobilized by secretion of chelators and by acidification of the rhizosphere. (b) Uptake of metals via various pumping systems in plasma membrane. (c) Transportation of metals from roots to xylem. (d) Distribution of metals into leaves. (e) Sequestration of metals in plant cell. CW = cell wall, M = metal, filled circles = chelators, filled ovals = transporters, bean-shaped structures = metallochaperones. Adapted from Vymazal and Kropfelova (2008).

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2.4.2.2 Bioavailability of Nutrient

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Bioavailability may refer to availability of nutrients, metals and contaminants for various processes such as absorption, distribution, elimination and other physical, chemical and biological processes (Fairbrother et al., 2007). The bioavailability of nutrients depends on several factors such as biogeochemical conditions of the substrates, namely pH, redox potential, amount of organic carbon present and the contaminant loading. O'Sullivan et al., (2004) found that bio-availability is the important factor for contaminant uptake such as Zn, Pb, Fe and S. The uptake of plant nutrients such as Ca^{2+}, K^{+}, $NH_4^{+}$and NO_3^{-} affects the acid-base balance means when a plant takes up a positively charged ion, then a positively charged ion such as H^{+} is released into the environment. Similarly, when a negatively charged ion is taken up, a negatively charged ion like OH^{-} is released. In a balanced system the uptake and release of ions from plants would be equal (Cronk & Fennessy, 2001). High evaporation and evapotranspiration rate can influence the bio-availability of nutrients. As Malaysia has high evaporation and evapotranspiration rate, 1300-1400 mm/year and 6-16.78 mm/day respectively, these will increase the final concentration of nutrient in media (Sim, Yusoff, Shutes, Ho & Mansor, 2008).

2.4.2.3 Bioconcentration Factor (BCF) and Translocation Factor (TF)

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Bioconcentration factor (BCF) is an important identification for efficiency of phytoremediation experiments because it shows the ability of a plant to accumulate metals (Marchiol, Sacco, Assolari & Zerbi., 2004). BCF is the ratio of metal

concentration in plant organs (mg/kg) to the metal concentration in media (mg/L) hence BCF values are noted by L/kg (Soda et al., 2012; Khellaf & Zerdaoui, 2009). Translocation factor (TF) shows the ability of plant to translocate metals from roots to above ground tissues and a high TF value indicates that the plant has high capability in transporting metals (Soda et al., 2012). Aquatic macrophytes have higher BCF and TF values compare to terrestrial plants and algae (Ahmad, Ghufran & Zularisam, 2011) indicating the macrophytes' efficiency in accumulating and translocating metals in their tissues. Low TF values shows that macrophytes are only transporting small amount of metals absorbed in roots to the above ground tissue (Sundberg-Jones & Hassan, 2007). However this type of plants are good in phytostabilization process which only involve stabilization of media by plant roots from metals contaminants (Zou et al., 2012).

2.4.2.4 Age of Macrophytes and Decay Rate

Nutrient uptake tends to be high in the early stage of growing and diminishing as plant matured and senesced (Vymazal & Kropfelova, 2008; Wu et al. 2011). Nutrient uptake during phytoremediation becomes less efficient when macrophytes died and decay, and plant mass may even diminish because of wilting (Trapp et al, 2007; Cronk & Fennessy, 2001). Decomposition of macrophytes may also release large amounts of nutrients in water and thus re-polluted water (Cronk & Fennessy, 2001; Shilla, Asaeda, Fujino & Sanderson, 2006; Hu et al., 2010).

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When macrophytes decompose, nutrients that are more labile such as sodium and potassium are easily leaching into surrounding water (Azeez et al., 2009). Compare to other type of macrophytes, grasses have slow decomposition rate and may show better nutrient uptake (Karathanasis et al., 2003). Nevertheless, the decayed plant roots and falling residues could act as food sources for microbial populations (Karathanasis et al., 2003). Detritus from decayed macrophytes also can supply carbon for denitrification process, thus enhance denitrification (Lim, 2002).

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2.4.2.5 Types of Macrophyte Roots

Macrophytes that have fibrous roots are found more efficient in nutrient uptake compared to rhizomatic type macrophytes. This is due to large surface area in fibrous roots and great number of roots for nutrient uptake. Macrophytes with fibrous root exhibit more efficient uptake of nitrate and ammonia but there is no different in COD removal between fibrous and rhizomatic macrophytes (Cheng et al., 2009). Previous study shows that rice plants grown in stagnant condition can adsorb more iron (Fe) because the plants develop more adventitious roots which provide more surface area for adsorption compared to the plants grown in aerated condition (Deng, Wu, Wu, Deng & Wong, 2010).

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2.4.2.6 Effect of Phytotoxicity

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Essential elements for plants can become toxic and contaminant to plants if exceed certain range of concentration (Hodson & Bryant, 2012). Phytotoxicity happens when plants cannot tolerate to high amount of contaminants (Arienzo, Christen & Quayle, 2009). Toxicity of wastewater also gives effect to phytoremediation because excess in nutrient uptake beyond luxury uptake point can lead to nutrient toxicity in macrophytes (Figure 2.7) (Reddy & De Busk, 1987).

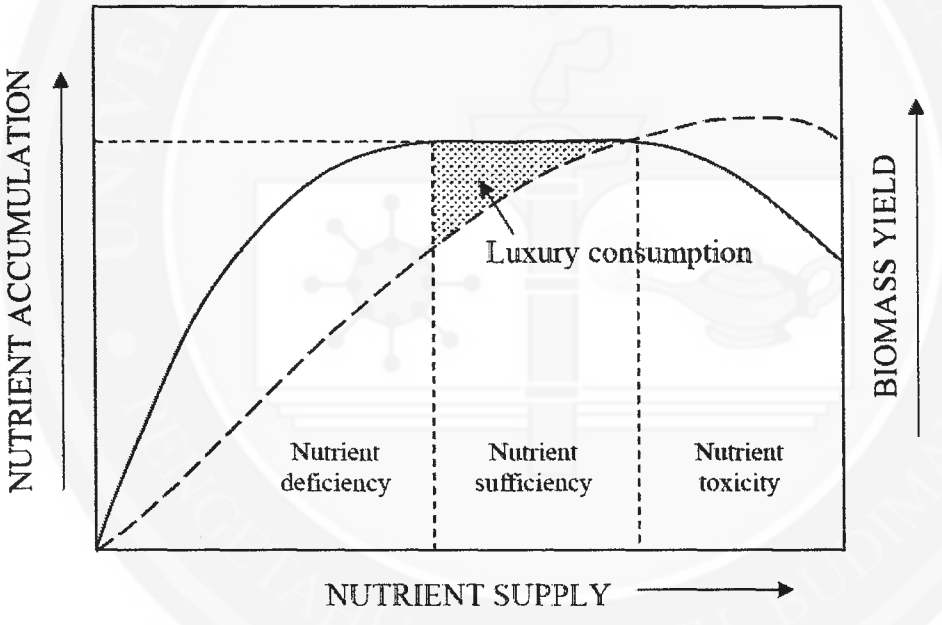


Figure 2.7. Relationship between nutrient supply and nutrient accumulation (----) and biomass yield (—) (Reddy & De Busk 1987)

Other factors that can lead to phytoxicity include low pH, high COD and high salinity which prevent macrophyte growth (Arienzo et al., 2009). Phytotoxicity can also be exemplified by plants that live in reduced soils (anoxic) which suffer from metabolic stress results in decrease of nutrient uptake and translocation of nutrients (Pierce et al., 2010). Bodini, Cicalini and Santori (2011) found the length of exposure

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time affect the phytotoxicity among shrubs in olive mill wastewater varied greatly. They found that *Salix sp.* and *Laurus nobilis* and, later, *Pinus mugo* suffered phytotoxic effect of olive mill wastewater after six months treatment but *Cupressus sempervirens* and *Quercus ilex* proved tolerant to the length of exposure time. Sooknah and Wilkie (2004) noted that high COD from undiluted wastewater and high salinity can stop the growth of macrophytes. High ammonia level can cause submerged macrophyte, *Vallisneria natans* diminished in total chlorophyll (a and b) and prolonged exposure of 2.8 mM NH_4CL for four days can cause water-soaked symptoms and weak chlorosis (Wang et al., 2008). According to Cronk and Fennessy (2001), excess of iron can also lead to phytotoxicity and the concentration is varied among the macrophyte species. *Pistiastratiotes* was reported could grow healthy without phytotoxicity effect at concentration of 5 mg/L and was able to remove 90% of iron at this concentration (Mishra & Tripathi, 2008). Macrophytes that suffered toxicity of iron may show symptoms of discoloured leaves, reduced photosynthesis activity and decreased root respiration (Mishra & Tripathi, 2008).

2.4.3 Allocation of Nutrients in Macrophytes Organs

Previous research that compare nutrient uptake between macrophyte organs shows intriguing results. It was found that different macrophyte organs differed in their nutrient uptake associated with types of nutrients. For example, Tanner (2001) found accumulation of Ca, Mg, Na, K, Mn occurred mainly in the above ground tissues while Fe, Zn, Cu and B mainly accumulate in below-ground tissues especially in soft-stem bulrush. While research on Italian ryegrass reveals the roots assimilated more

nutrients than shoots (Xian, Hu, Chen, Chang & Zou, 2010). This result coincides with Pierce et al. (2010) finding, in which Fe uptake was higher in root compared to the aboveground organs. They also found that Fe uptake by *Leersia oryzoides* was associated with water regime volume, in which, increase in water regime by flooding can increase solubility of Fe, hence will increase available Fe in water thus enhance Fe uptake by *Leersia oryzoides* root. However, Lu et al. (2010) found minimal differences between root and shoot of *Pistia stratoites* in N removal due to different nutrient uptake (Jampeetong et al., 2012). The free-floating macrophytes were observed to have higher nitrogen uptake rate than the emergent macrophytes (Jampeetong et al., 2012).

2.4.4 Transformation of Nutrients in Macrophytes

Nutrients play a vital role in plants. From 92 chemical elements in surface layer of earth, only 16 elements are considered essential for all plants as described in Table 2.4

Table 2.4
Sixteen essential plant nutrients (Glass & Parker, 2009)

Classification	Nutrients
Non-mineral	Carbon (C), Hydrogen (H), Oxygen (O)
Primary	Nitrogen (N), Phosphorus (P), Potassium (K)
Secondary	Calcium (Ca), Magnesium (Mg), Sulfur (S)
Micronutrients	Boron (B), Chlorine (Cl), Copper (Cu), Iron (Fe), Manganese (Mn), Molybdenum (Mo), Zinc (Zn)

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2.4.4.1 Carbon (C)

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Under aerobic conditions, macrophytes carried out photosynthesis process as source of glucose (Equation 2.5) subsequently converted to energy in respiration process (Equation 2.6):

$$6CO_2 \rightarrow 12H_2O + \text{light} \longrightarrow C_6H_{12}O_6 + 6O_2 + 6H_2O \quad \text{(Equation 2.5)}$$

$$C_6H_{12}O_6 + 6O_2 \longrightarrow 6CO_2 + 6H_2O + 2e^- + \text{energy} \quad \text{(Equation 2.6)}$$

(Vymazal & Kropfelova, 2008)

However, natural wetlands are lack of oxygen, thus macrophytes are more likely to undergo anaerobic processes initially with fermentation process and subsequently by methanogenesis process to produce energy (Ingrouille & Eddie, 2006; Vymazal & Kropfelova, 2008). Carbon constitutes a large percentage in plant as structural component and organic compounds such as starch and carbohydrate which are important for energy production (Hopkins & Hunner, 2004). In phytoremediation study, Malecki-Brown, White and Brix (2010) reported that emergent macrophytes have higher total carbon than submerged macrophytes after phytoremediation of municipal wastewater. Macrophyte that suffer ammonia toxicity from water column could diminish in carbohydrate level, because a lot of energy will be used to transport NH_4^+ out ofmacrophytes, hence could also decrease overall carbon level (Cao, Xie, Ni, Zhang & Xu, 2009).

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2.4.4.2 Nitrogen (N)

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Eighty percent of our atmosphere consists of nitrogen. However, the extremely stable form of atomic N (N_2) is not available to plants (Maathuis, 2009). In wastewater, nitrogen can exist in various form, namely ammoniacal nitrogen (NH_3-N) which includes unionized form (NH_3) and ionized (NH_4^+), organic nitrogen, and oxidized nitrogen; nitrite (NO_2^-) and nitrate (NO_3^-) (Lim, 2002; Idris et al., 2010) but most of the inorganic nitrogen present in wastewater treatment wetlands is in the form of ammonia (NH_3) and ammonium (NH_4^+) (Cronk & Fennessy, 2001). The removal of nitrogen from wastewater is achieved through three main mechanisms: nitrification/denitrification, volatilization of ammonia (NH_3), and uptake by macrophytes (Vymazal & Kropfelova, 2008; Idris et al., 2010). Nitrate (NO_3^-) from nitrification process and ammonium ion (NH_4^+) could be taken up directly by macrophyte (Lim, 2002).

Denitrification is more likely to occur in anoxic condition and involves several bacteria genus namely *Alcaligenes*, *Bacillus* and *Pseudomonas* that can utilise nitrate as alternative electron acceptor (Eusebio et al., 2007; Metcalf & Eddy, 2003). This process require carbon as energy source which can be obtained from wastewater BOD and macrophyte biomass decomposition (Kadlec & Wallace, 2009; Leverenz et al., 2010). Macrophytes can provide reduced zone in their roots which favour denitrification process for NO_3^-removal (Caselles-Osorio, Villafane, Caballero & Manzano, 2011). Decayed macrophytes not only supply carbon for denitrification process but also supply organic nitrogen for ammonification process which convert amino acids to mineralized form of ammonia, the ammonium ion (NH_4^+) (Vymazal &

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Kropfelova, 2008). The two forms of nitrogen used for assimilation are NH_4^+ and nitrate anions (NO_3^-) and almost all green plants, fungi and bacteria are able to reduce them in biosynthesis of amino acids and protein. The biochemical pathway is:

$$NO_3^- \rightarrow NO_2 \rightarrow NH_2OH \rightarrow NH_4^+ \rightarrow R - NH_2 \qquad \text{(Equation 2.7)}$$

(Lim, 2002)

NO_3^- is reduced either in the roots or transported to the leaves, where it is further reduced in the presence of light (Miller & Cramer, 2004). However, it has been reported that nitrate reduction is predominant in macrophyte roots because of high nitrate reductase content in roots compared to shoots (Cedergreen & Madsen, 2003). Oxygen shortage (anaerobic) may induce nitrate reductase activity (De la Haba, Aguera, Benitez & Maldonado, 2001).

The atmospheric nitrogen or organic nitrogen is not readily absorbed by green plants without the help of symbiotic prokaryotes (Sinha, 2004). The foremost function of N is to provide amino groups in amino acids. N is also prolific in nucleotides, where it occurs incorporated in the ring structure of purine and pyrimidine bases. Nucleotides form the constituents of nucleic acids and also have many other important functions such as in energy homeostasis, signalling and protein regulation. In addition, N is essential in the biochemistry of many non-protein compounds such as co-enzymes, photosynthetic pigments, secondary metabolites and polyamines. In ample supply, NO_3^- is deposited in vacuoles where it significantly contributes to generate cell turgidity (Maathuis, 2009). Much of the assimilated nitrogen must be transported to the shoot through the vascular tissues. Whereas certain bacteria reduce and fix

nitrogen, others oxidize it, which adversely affects the plant metabolism. When organisms die and decay, their nitrogenous compounds become available to the roots of living plants. However, because these compounds are highly reduced, they can be used as an energy source if oxygen is available (Mauseth, 2009).

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2.4.4.3 Calcium (Ca)

Plants in general have high amount of calcium (Ca) in their tissues and universally known protective agent in plants against adverse soil factors (Plieth, 2005). Ca ions are involved in many mechanisms in biochemical and physiological activities such as stabilization of cell wall structures, acidic growth, ion exchange, and control of the activities of wall enzymes. All these properties originate from the tight binding of Ca ions to the pectins present in the cell walls (Carpin et al., 2001). In plant cells, the cytosolic concentration of free Ca^{2+} is normally lower than about 10^{-7} mol/L. These very low Ca^{2+} concentrations are maintain by ATP-dependent pumps (Ca^{2+}-P-ATPases) which accumulate Ca^{2+} in vacuole or transport Ca^{2+} via the plasma membrane to the extracellular compartment (Heldt, 2005).

Addition of mineral elements such as Ca into soils to improve plant growth is a standard agricultural practice (Hirschi, 2004). Increase of Ca concentration in nutrient solution has positive effects on the contents and yields of effective components in medicinal plants (Supanjani, Tawaha, Yang & Lee, 2005). Study by Xiao et al. (2011) showed that macrophyte *Cyperus malaccensis* has higher Ca concentrations in shoots than compared to roots. *Cyperus malaccensis* shoots have Ca BCF range from 1.20

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L/kg to 2.28 L/kg and TF value range from 1.43 to 2.24 which indicates high accumulation and transportation rate of Ca in this macrophyte organ (Xiao et al., 2011). These results however are contrary with Morari and Giardini (2009) which asserted that Ca mainly accumulated in roots of macrophytes.

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2.4.4.4 Magnesium (Mg)

Magnesium enters plant through transporters in a passive way. Mg^{2+} is probably best known for its central position in the chlorophyll molecule where it coordinates covalently with four nitrogen atoms from the porphyrin ring, and Mg^{2+} has further crucial roles in photosynthesis, particularly in its capacity of promoting the light reactions in the stroma (Maathuis, 2009). During carbon fixation in photosynthesis process, Mg acts as an activator for vital enzymes, ribulose bisphosphate carboxylase and phosphoenol pyruvate carboxylase (Hopkins & Hunner, 2004). Most of cellular Mg^{2+} also plays an important role as enzyme cofactor in stabilization of nucleotides (Maathuis, 2009). In phytoremediation study, it was reported that concentration level of Mg varied between different species of macrophytes after phytoremediation treatment of municipal wastewater (Malecki-Brown et al., 2010). It has also been reported that Mg mainly accumulated in macrophyte roots, however the accumulation become not significant when macrophyte roots decomposed and easily release it back to wastewater (Morari & Giardini, 2009; Shilla et al., 2006).

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2.4.4.5 Potassium (K)

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In general, during phytoremediation of wastewater, K is absorbed by roots and significantly translocated to macrophyte leaves (Morari & Giardini, 2009). The presence of K in wastewater can influence uptake of N, in which increase of K in wastewater can increase uptake of N by macrophytes (Tanner, 2001). However, Rossmann, Teixeira de Matos, Abreu, Fonseca e Silva & Borges, (2012) asserted that increase of K around the roots would decrease transpiration rate and consequently diminished the uptake of other nutrients, which caused by great osmotic potential difference. K is an essential nutrient and the most abundant cation in plant cells (Szczerba, Britto & Kronzucker, 2009).

Another role of K is in the osmo-regulation of stomata apertures (Hopkins & Hunner, 2004). A study by Cochrane & Cochrane (2009) confirmed the role of K in the modulation of plant stomata apertures. If K is deficient for a plant, it probably activates a signaling mechanism which leads to the translocation of mobile K^+ ions from old to new leaves to support stomata aperture osmo-modulation. This causes the breakdown of stomata in the old leaves and subsequent necrosis with the consequent loss of considerable leaf surfaces for the transpiration, gas exchange and photosynthesis.

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2.4.4.6 Sodium (Na)

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Previous study by Morari and Giardini (2009) shows that Na mostly accumulated in macrophyte stems during phytoremediation. Rhizofiltration test shows that excess of Na cause plants to suffer from phytotoxicity with stunted growth, and diminished some amount of chlorophyll *a* (Garnett et al., 2002). Physiologically, Na enters plant through non-selective voltage-independent cation channels in which Ca^{2+} and K^{+} may inhibit sodium uptake but not Na efflux in pepper plants (Rubio, Flores, Navarro & Martinez, 2003).

However if K^{+} is deficient, Na can replace it and act as a cofactor to certain enzymes, such as regulating stomata opening in osmoregulation and cell elongation. Na is also important for photosynthesis process in C4 and CAM plants, in which Na is required to regenerate phosphoenolpyruvate (PEP) from pyruvate (Pilon-Smits et al., 2009). In aquatic halophytes *Zosteramanina* L., nitrate (NO_3^-) uptake is enhanced by Na in which uptake of nitrate can also occur in high-affinity Na-symport system (Garcia-Sánchez, Jaime, Ramos, Sanders & Fernandez, 2000).

2.4.4.7 Iron (Fe)

Because of redox-active nature of Fe, it plays a pivotal role in plant metabolisms such in photosynthesis, mitochondrial respiration, nitrogen assimilation, nitrogen assimilation, hormone biosynthesis (ethylene, gibberellic acid, jasmonic acid), production and scavenging of reactive oxygen species, osmoprotection and pathogen

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defence (Hänsch & Mendel, 2009). The uptake of Fe by macrophytes is related to bioavailabilty of Fe, in which Pierce et al. (2010) found increase of wastewater level can increase solubitiy of Fe and enhance Fe uptake. It was also reported that Fe concentration level below than 5 mg/L can be 90% removed by *Pistiastratiotes* (Mishra &Tripathi, 2008).

Furthermore, in wider ecosystem, Lesley, Daniel and Paul (2008) found that oxidation of iron to form iron hydroxide precipitates was the dominant removal process in proximal wetland system while in distal parts where iron concentrations are much lower biotic removal processes appeared to become more important. Excess of Fe can lead to Fe precipitation at macrophyte roots (Siquera-Silva et al., 2012). The precipitation of Fe (oxy-) hydroxides in the rhizosphere is due to radial oxygen loss (ROL) and action of microorganisms (Jacob & Otte, 2003). Deng, Ye and Wong (2009) found that lignin and suberin deposition in macrophyte roots such as in *Leersia hexandra* form a tight barrier for ROL lead to a high tolerance of macrophytes to Fe and Zn. This was supported by Xiao et al. (2011) which showed that BCF values tend to be higher in roots of macrophytes (*Cyperusmalaccensis*) compared to shoots with the range from 0.07 L/kg to 0.18 L/kg. Macrophytes that have low Fe TF ratios indicate low translocation capacity of Fe (Xiao et al., 2011).

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2.4.4.8 Zinc (Zn)

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Zinc (Zn) is microelements that essential for macrophytes with concentration of 100 mg/kg of tissue dry weight (Sasmaz, Obek & Hasar, 2008). Zn acts as an activator for various enzymes, for instance alcohol dehydrogenase, carbonic anhydrase and superoxide dismutase (Hopkins & Hunner, 2004). Macrophytes such as *Hydrilla verticillata* and *Ipomea aquatica* were reported as good zinc accumulators, however most of zinc accumulated were stored in root and not transported to leaves, indicated by low translocation factor (TF) (Sasmaz et al., 2008). Similar phenomenon has also occurred in grass macrophytes such as *Phragmites australis*, which roots have higher Zn bioconcentration ability than above ground organs (Lesage et al., 2007).

2.5 Microorganisms involvement in Phytoremediation

2.5.1 Role of Microorganisms in Phytoremediation

Several studies on relationship between microorganism and plant used for biological wastewater treatment are well documented (Osemet al., 2007). The predominant microorganisms involved are gram-negative rhizosphere bacteria. This is due to their ability to utilize growth substrates available in the rhizosphere and tolerate with toxic environments (Chaudhry, Blom-Zandstra, Gupta & Joner, 2005). More ecological stable system produced with interaction between the plant root system and biological regime of the substrate, collectively known as rhizosphere (Weber, Gehder & Legge, 2008). This rhizosphere acts as substrate for microbial growth and microorganism that

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forms a biofilm in roots (Lim et al., 2002; Greenway 2007). This biofilm community are probably can be attributed to the environmental factors such as availability of organic matter and nutrients, dissolved oxygen level, pH and salinity (Osem et al., 2007).

Macrophytes and photosynthetic micro-organisms also improve overall water quality by producing oxygen during photosynthesis which diffuses into the water column. Emergent macrophytes transport oxygen down their stems into the roots where it diffuses into the sediment to produce an aerobic microenvironment around the root zone (rhizosphere) in which aerobic microorganism will utilize this oxygen. The interaction between macrophytes and microbes is essential for nitrogen removal. There are three processes in which microorganism involved in removing and transforming nitrogen which are ammonification, nitrification and denitrification (Greenway, 2007).

Microorganisms also involve in degrading organic matter into simple end products in aerobic biological oxidation process. In this process, microorganisms oxidized the dissolved and particulate carbonaceous organic matter into simple end products and additional biomass, as represented by the following equation:

$$v_1 \text{ (organic material)} + v_2O_2 + v_3NH_3 + v_4PO_4^{3-} \xrightarrow{\text{microorganisms}} v_5 \text{ (new cells)} + v_6CO_2 + v_7H_2O \qquad \text{(Equation 2.8)}$$

where v_i = the stoichiometric coefficient

In Equation 2.8, oxygen (O_2), ammonia (NH_3) and phosphate (PO_4^{3-}) are used to represent the nutrients needed for the conversion of the organic matter to simple end products (i.e., carbon dioxide (CO_2) and water. The term shown over the directional arrow is used to denote the fact that microorganisms are needed to carry out the oxidation process. The term 'new cells' is used to represent the biomass produced as a result of the oxidation of organic matter. Microorganisms also remove nitrogen and phosphorous (Tchobanoglous, Burton & Stensel, 2003). Several studies have been done to determine relationship between plants and microorganism in constructed wetlands. Studies show that microbial processes within the rhizosphere of constructed wetlands are crucial to wastewater treatment (Greenway, 2007). Planted wetlands usually had a higher total microbial activity than unplanted wetlands (Zhao, Liu, Zhang, Hu & An, 2010). There are many factors contributed to the establishment of diverse assemblages of microbial population such as interaction between macrophyte roots, microorganisms and substrate (Calheiros et al., 2009). Sun et al. (2010) suggested possible competition between microbes and plant roots for nutrients in low organic matter soil. However, Liu, Xia and Park (2007) found that, nitrogen is primarily removed by macrophytes and nitrification by microbes only plays a minor role in nitrogen removal mechanism.

2.5.2 Microbial Population and Identification in Phytoremediation

The most important and popular method to identify bacteria and their relationship is by using 16s rRNA gene (Sacchi et al., 2002). Sequencing of microbial biofilm community by using 16s rRNA gene fragment showed the high complexity of

bacteria. The diversity of bacterial population is influenced by the depth of microcosm tank which is related to nitrification potential (Iasur-Kruh et al., 2010). Many researchers found that macrophytes give less effect on diversity of bacteria community exist during phytoremediation (Osem et al., 2007; Baptista, Davenport, Donnelly & Curtis, 2008; Iasur-Kruh et al., 2010). In fact, random processes become a vital part to microbial community assembly in natural and constructed wetland in which archaea, bacteria and sulphate-reducing bacteria seems to be no more similar than if they had been randomly assembled from the same source of population (Baptista et al., 2008).

However, Vacca, Wand, Nikolausz, Kuschk and Kästner (2005) found that macrophytes can stimulate specific bacterial community that are different from the bulk soil. They found some bands of PCR-single strand conformation polymorphism that only appear in rhizosphere and not in bulk soil. Macrophytes can give benefit to microbial community. Exudates from macrophyte *Phragmites australis* did not suppress new bacteria community introduced into rhizosphere, and bacteria community in rhizosphere indeed were doubled by magnitude (Vacca et al., 2005). Types of macrophytes alone are not just a factor that affects microbial community in rhizosphere because another factors such as pH, availability of nutrients, salinity, dissolved oxygen (DO) and other environmental factors also contribute their growth (Osem et al., 2007). However, there are two major factors which are responsible for vast diversity and abundances of microbial community which are oxygen supply and labile organic carbon from the macrophyte roots system (Kofoed, Stief, Hauzmayer, Schramm & Hermann, 2012). Toxicity of heavy metals can contribute to abundance of bacteria from *Proteobacteria* phylum because of decrease in radial oxygen loss and

lower redox potential which is favouring *Proteobacteria* populations (Somenahally, Hollister, Loeppert, Yan & Gentry, 2011).

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There are wide varieties of bacteria that capable of denitrification process including *Pseudomonas*, *Alcaligenes*, *Bacillus* and *Agrobacterium* (Vymazal & Kropfelova, 2008). At high COD (>200 mg/L) these denitrifiers will prevail over anammox bacteria population because of abundance of organic carbon for denitrification process (He et al., 2012). Iasur-Kruh et al, (2010) found that *Acidobacteria*, *Proteobacteria* and *Bacteroidetes* are dominant population in wetland microcosm biofilm while bacteria strain that have 98% similarity to *Nitrosomonas sp.* and *Nitrospira sp.* found in the upper layer of tank. This is due to oxidized layer supports the nitrification process (Lim et al., 2002). Recently, more research focuses on removal of faecal coliform, especially in phytoremediation of domestic wastewater which found *Salmonella*, *Shigella*, *Yersinia*, and coliphage the predominant microbial species (Hench et al., 2003).

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CHAPTER 3

PHYTOREMEDIATION PERFORMANCE OF POM FINAL DISCHARGE WASTEWATER USING *Leersia oryzoides*, *Ludwigia peploides* AND *Pistia stratiotes*

3.1 Introduction

In phytoremediation technique, plants are used to remove, degrade or render harmless hazardous materials present in soil and water. The emerging technology may offer a cost effective, non-intrusive, and safe alternative as compared to conventional soil clean up techniques. Phytoremediation performances of wastewater using macrophytes are well documented (Osem et al., 2007; Tanner et al., 2001). Among the wastewater treated include dairy effluent, oil refinery wastewater, lemon industry effluent and olive mill wastewater (Tripathi and Upadhyay, 2003; Moreno et al., 2002; Navarro et al., 2012; Bodini et al., 2011). To date very limited information on phytoremediation of POM final discharge wastewater can be obtained. This

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information is important and useful to determine the feasibility of phytoremediation for POM final discharge wastewater. This chapter discussed the selection of macrophytes for phytoremediation of POM final discharge wastewater and the performance of phytoremediation process.

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3.2 Methodology

3.2.1 Selection of Tolerant Macrophytes to POM Final Discharge Wastewater

Seven macrophytes were selected for tolerance test of POM final discharge wastewater. The selected species were *Jussiaea repens*., *Salvinia spp.*, *Limnocharis flava*, *Elodia spp.*, *Eichhornia crassipes*, *Leersia oryzoides*, and *Ludwigia peploides*. These species were selected for tolerant test because of their well reported role as phytoremediators and its abundance in waterlogged area in Malaysia. The aquatic macrophytes were obtained from several identified areas, including the abandoned tin mine lakes, open deserted land and paddy fields at Kalumpang, Hulu Selangor, Kuala Selangor and Tanjung Malim, Perak, Malaysia. Macrophytes were grown in one litre POM final discharge wastewater. The tolerance test was conducted for four weeks. Observation was conducted on wilting and deterioration sign over the period of screening. Three macrophytes that have highest tolerance were selected for the phytoremediation study.

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3.2.2 Macrophytes Species

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The three selected species, *Leersia oryzoides*, *Pistia stratoites* and *Ludwigia peploides* were cultured in dechlorinated water for acclimatization process for about two to four weeks. New adventative emergent shoots or growing rosettes with almost similar initial weight (25-35) g obtained from macrophytes were used in the phytoremediation study. The description of the selected macrophytes is described below:

3.2.2.1 *Leersia oryzoides*

Scientific name: *Leersia oryzoides* (L.) Sw.

Local and common names: Padi angin, Sickle grass, Rice cutgrass (USDA Plant Fact Sheet, 2008)

Family: *Poaceae*

Description: Figure 3.1 shows *Leersia oryzoides*. The plant spreads by scaly, underground rhizomes and often forms dense colonies. Its stalks (stems) are upright to sprawling, velvety at the nodes (joints), and 90 to 125 (150) cm tall. Leaf blades are 7 to 30 cm long, 6 to 15 (20) mm wide, and yellow-green in colour. They have a coarse or rough upper surface and are lined with tiny teeth along the margins. The flowerhead (panicle) is open, often drooping, and 10 to 20 cm long. Some panicles remain enclosed within the lower leaf sheaths and release seeds upon decay of the stem. The seed hull is covered with minute bristles that readily cling to clothing or fur for dispersal (USDA Plant Fact Sheet, 2008).

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Habitat: *Leersia oryzoides* can be found in a variety of wet, sunny, and partially shaded sites. However, it is most common near streams, ponds, or beaver areas, and in ditches, canals, or freshwater marshes. This species thrives best in nutrient rich mud and slow moving or stagnant water.



Figure 3.1. Leersia oryzoides

3.2.2.2 *Pistia stratiotes*

Scientific name: *Pistia stratiotes* (L.)

Local and common name: Kiambang (Malay), Water lettuce

Family: *Araceae* (*Arum*)

Description: Figure 3.2 shows *Pistia stratiotes*. It is a floating herb in rosettes of gray green leaves, rosettes occurring singly or connected to others by short stolons. Its roots are numerous and feathery. The leaves are often spongy near base, densely soft

pubescent with obvious parallel veins, slightly broader than long, widest at apex, to 15 cm (6 in) long. Flowers are inconspicuous, clustered on small, fleshy stalk nearly hidden in leaf axils, with single female flower below and whorl of male flowers above. Fruit arising from female flower as a many-seeded green berry (Langeland & Cherry, 2008).

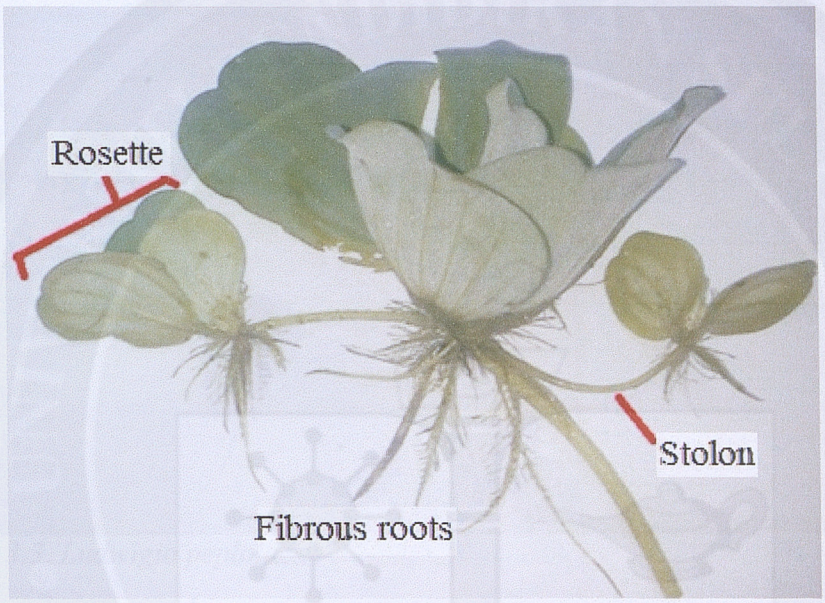


Figure 3.2. Pistia stratiotes

3.2.2.3 *Ludwigia peploides*

Scientific name: *Ludwigia peploides* (Kunth) P. H. Raven

Local and common name: Inai pasir (Malay), Floating primrose-willow, creeping water primerose

Family: *Onagraceae*

Description: Figure 3.3 shows *Ludwigia peploides*. It is a perennial, matted, floating and creeping plant. Its leaf is less than 10 cm long, arranged in alternate and the blade

shape is oblong to round. Its flower has 5 to 6 sepal and fruit is reflexed in which seeds are embedded in the inner fruit wall. This plant can be found in ditches, stream banks and lake shores and naturally distributed in Eurasia, Australia and United States (Oregon, California and Southern States) (University of California, 2012).

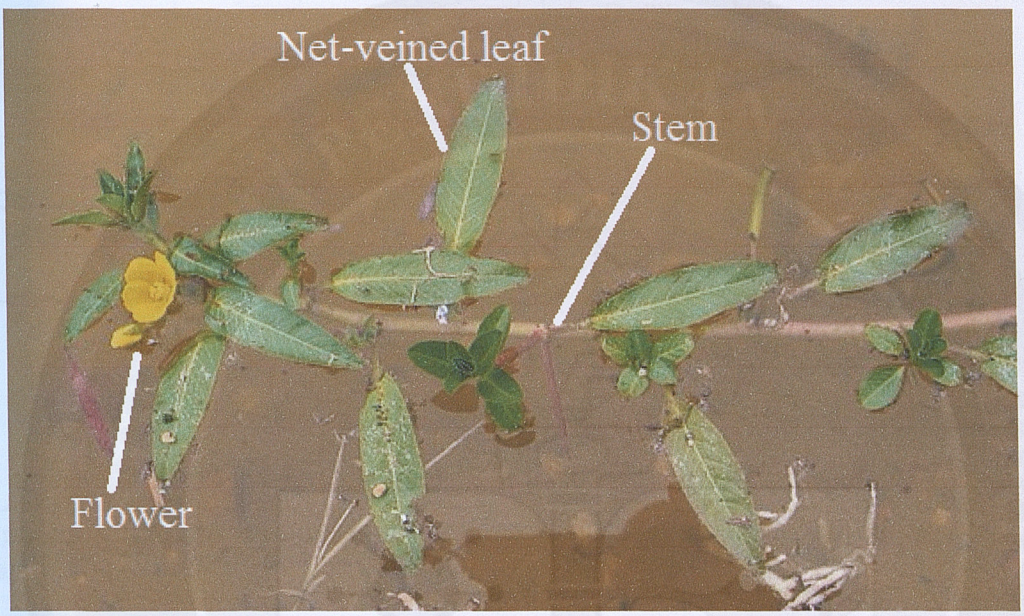


Figure 3.3. Ludwigia peploides

3.2.3 POM Final Discharge Wastewater

POM final discharge wastewater was collected from the final distribution ponds (Figure 3.4) at KL Kepong Palm Oil Mill, Changkat Asa, Tanjong Malim, Perak. The characteristics of POM final discharge wastewater were analysed in laboratory and the details were summarized in Table 3.1. The process flow of POME treatment at KL Kepong Palm Oil Mill using ponding system that involves anaerobic and aerobic lagoons is shown in Figure 3.4.

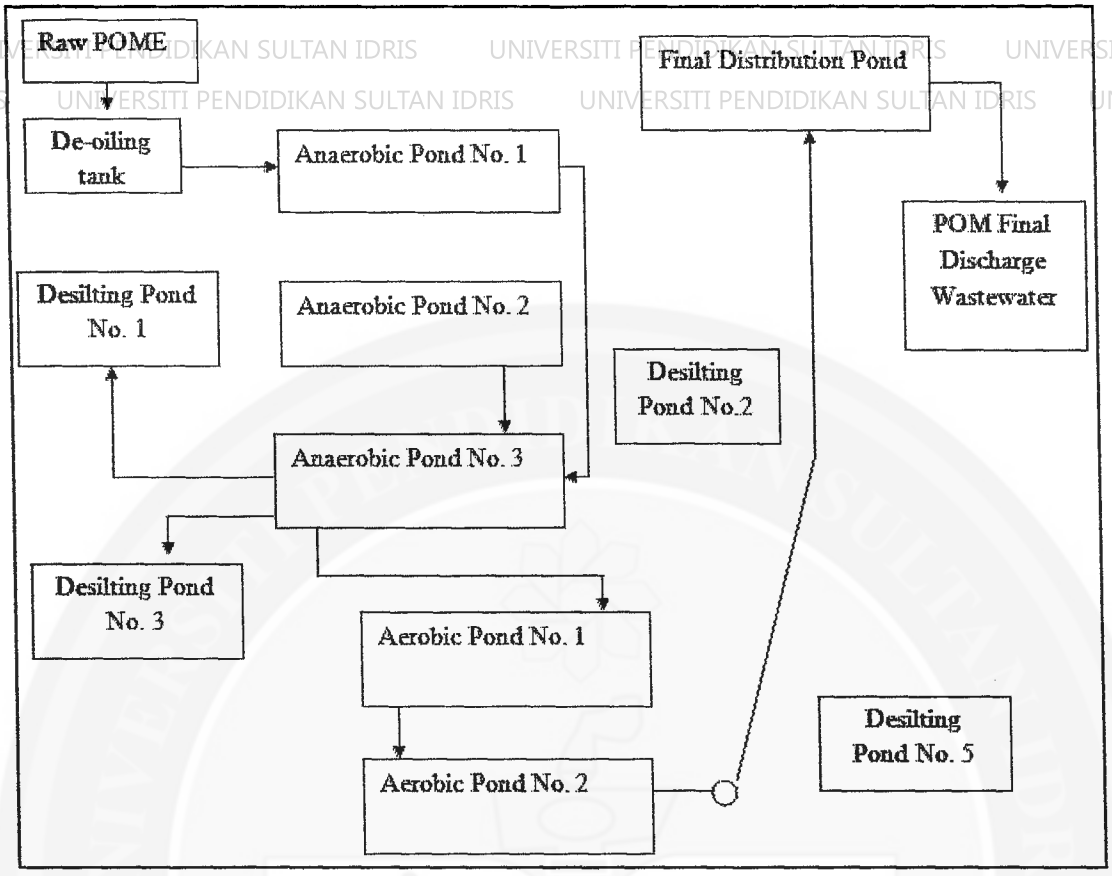


Figure 3.4. Treatment process of POME at KL Kepong Palm Oil Mill Changkat Asa, Tanjung Malim, Perak.

3.2.4 Experimental Set Up

The experiments were conducted at laboratory scale using 33 cm x 20 cm x 10 cm open containers with 6.5 L working volume. Six litres of POM final discharge wastewater was used in every treatment with four replicates. All treatments used macrophytes; *Leersia oryzoides*, *Pistia stratiotes*, and *Ludwigia peploides*, that weighed ranging from 25 g to 35 g. Containers without vegetation were also set up as controls. The containers were placed in shady area near the laboratory to control light intensity factor.

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Table 3.1

Characteristics of POM final discharge wastewater used in this study

Parameter (mg/L)*	Range (mg/L)	Mean ± SD mg/L
BOD_5	490-612	549.3±61.1
COD	1025-1073	1049±33.9
Ammonia NH_3	43-45	44 ±1
Total Solid	4860-5351	5150±212.3
Total Suspended Solid	3506-3713	3600±150.4
Total Volatile Solid	1200-1480	1333.3±140.5
Total Alkalinity (as $CaCO_3$)	13401-13511	13457 ± 50.7
pH	8.44-8.5	8.48±0.03
Mg	219.3-221.7	220.17±1.33
Ca	11.20-11.22	11.21±0.01
K	172.55-207.8	194.15±18.92
Fe	0.65-1.56	1.03±0.47
Na	5.68-5.73	5.70±0.02

Note. *unit in mg/L except for pH

3.2.5 Phytoremediation Process

The BOD_5, COD, NH_3-N, pH and Total Suspended Solid (TSS) were monitored throughout the phytoremediation process. Measurements for NH_3-N were conducted daily, BOD_5 COD, TSS and pH for every three days, and metals content before and

after the treatment. Plant weight was monitored weekly. The phytoremediation experiment was repeated for three times.

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3.2.5.1 Chemical Analyses

All chemical analyses were determined according to the Standard Method (APHA, 2005). BOD_5 was measured based on five day period of oxygen consumption at 20°C and COD was determined by closed reflux method using HACH COD reactor and HACH DR 4000 spectrophotometer. Ammoniacal Nitrogen (NH_3-N) was analysed using Nessler method. TSS was measured according to Standard Method APHA 2540 D. Sample was filtered through a glass-fibre filter and the residue retained on the filter was dried to a constant weight at 103 to 105 °C. Calculation of TSS was conducted using the following formula:

Total Suspended Solid (mg/L) = ((A-B) × 1000/ sample volume, mL)

where:

A= weight of filter + dried residue, mg

B= weight of filter, mg

pH was measured by pH meter (Orion Star LogR, USA) and calibration was done properly before each measurement.

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3.2.5.2 Plant Weight

The biomass of each macrophytes species was monitored weekly by measuring the wet weight of macrophytes gravimetrically. The macrophytes morphological changes such as physical appearance of the leaves for wilting signs were also observed as described by Soltan and Rashed (2003).

3.2.6 Data Analysis

The removal efficiencies (r, %) of pollutants was calculated as described in the formula below (Juang & Chen 2007) :

$$r = ((C_0 - C_e) / C_0) \times 100$$

where,

r : removal efficiency

C_0: initial pollutant concentration

C_e: final pollution concentration

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3.2.7 Statistical Analysis

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In order to examine the significant different among the treatments, analysis of variance (ANOVA) was performed using SPSS 17.0 software. A probability of 0.05 or lower was considered to be significant.

3.3 Results and Discussions

3.3.1 Selection of Macrophytes

Table 3.2 shows the screening result of macrophytes in the selection of species that tolerant to POM final discharge wastewater. Species selection for phytoremediation is important to ensure maximum pollutant removal (Brisson & Chazarenc, 2009). Plants selected for phytoremediation process should also survive and have appropriate growth in the harsh environment treated (EPA, 2000). From Table 3.2, four macrophytes species *Ludwigia peploides*, *Elodia spp.*, *Pistia stratiotes* and *Leersia oryzoides* were observed healthy during the first week of experiment. *Elodia spp.* however suffered complete wilting in the third week than other macrophytes that survived in the first week which only suffered partial wilting (*Ludwigia peploides*, *Leersia oryzoides*) and *Pistia stratiotes* showed the least wilting. Hence these three macrophytes were selected for further phytoremediation treatment of POM final discharge wastewater.

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Table 3.2
Selection of macrophytes that tolerant to POM final discharge wastewater

Macrophyte	Observation of wilted leaves of macrophytes			
	Week 1	Week 2	Week 3	Week 4
Ludwigia peploides	Healthy	¼ Wilting	Partial wilting	Complete wilting
Salvinia spp.	Partial wilting	¾ Wilting	Complete wilting	Complete deteriorated
Limnocharis flava	⅓ Wilting	¾ Wilting	Complete wilting	Complete deteriorated
Elodia spp.	Healthy	Partial wilting	Complete wilting	Complete deteriorated
Eichhornia crassipes	¼ Wilting	¾ Wilting	Complete wilting	Complete deteriorated
Pistia stratoites	Healthy	¼ Wilting	¾ Wilting	Complete deteriorated
Leersia oryzoides	Healthy	¼ Wilting	Partial wilting	Complete wilting

3.3.2 Phytoremediation Performance

Table 3.3 summarized the characteristics of POM final discharge after the phytoremediation treatment. BOD_5 and NH_3-N of POM final discharge wastewater after treatment were found below parameter limits in the range of 12-58 mg/L and 63-158 mg/L, respectively. However, TSS concentration were found exceed the parameter limit in the range of 1280-7760 mg/L.

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Table 3.3
Characteristics of POM final discharge wastewater after phytoremediation treatment

Parameters[a]	Phytoremediation tanks	Range (mg/L)	Mean±SD (mg/L)	Discharge limit[b]
BOD_5	*Leersia oryzoides*	40-58	52±10.4	100
	Ludwigia peploides	28-50	36.5±9.9	
	Pistia stratiotes	12-52	36.5±18.1	
COD	*Leersia oryzoides*	717-820	756.3±55.6	-
	Ludwigia peploides	799-941	840.3±67.6	
	Pistia stratiotes	734-842	770±48.8	
NH_3-N	*Leersia oryzoides*	6.25-7.75	7.1±0.8	100
	Ludwigia peploides	11.75-12.75	12.3±0.4	
	Pistia stratiotes	12.25-15.75	13.4±1.4	
TSS	*Leersia oryzoides*	4500-6850	5650±1175	400
	Ludwigia peploides	1960-7760	3862.5±2813.8	
	Pistia stratiotes	1280-2240	1760±417	
pH	*Leersia oryzoides*	9.10-9.13	9.11±0.02	5-9
	Ludwigia peploides	8.86-9.02	8.94±0.07	
	Pistia stratiotes	8.87-8.91	8.89±0.02	

Note. [a] mg/L except for pH
[b] Environmental Quality Act (1978)

3.3.2.1 BOD_5 Removal

Figures 3.5 to 3.9 represent phytoremediation performances of POM final discharge wastewater. The removal efficiency of organic pollutant was determined by measuring BOD_5 and COD concentration throughout the experiments. Figures 3.5 (a) and (b) show that macrophyte species used were able to remove biodegradable organic compounds in POM final discharge wastewater. It was found that type of macrophyte species significantly affected the removal of BOD_5 ($p=0.001$). Throughout the

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experiment, BOD_5 was in the wane. As seen in Figure 3.5-(a) BOD_5 level was lowest in *Leersia oryzoides* tanks on Day 9. However on Day 15, BOD_5 levels were lower in *Pistia stratiotes* and *Ludwigia peploides* tank. The BOD_5 removal efficiency for *Leersia oryzoides, Pistia stratoites* and *Ludwigia peploides* were 90.6%, 93.3% and 93.3%, respectively with mean value of final concentration of 52 mg/L, 36.5 mg/L and 36.5 mg/L. These results correspond to previous research (Bashal, 2010), which demonstrated that *Pistia stratiotes* had the efficiency to remove 50.11% of BOD_5 in pilot scale constructed wetlands. A reduction of BOD_5 concentration was also observed in control containers and this might be due to the presence of natural microorganisms in POM final discharge used. The findings demonstrate *Leersia oryzoides* as the most efficient species in removing BOD_5 compared to the other two species (Figure 3.5 (a)). Greenway (2007) and Lim et al. (2002) reported that, BOD was principally removed by microbiological activity associated with plants and litter by providing surfaces for biofilm growth in wetland system.

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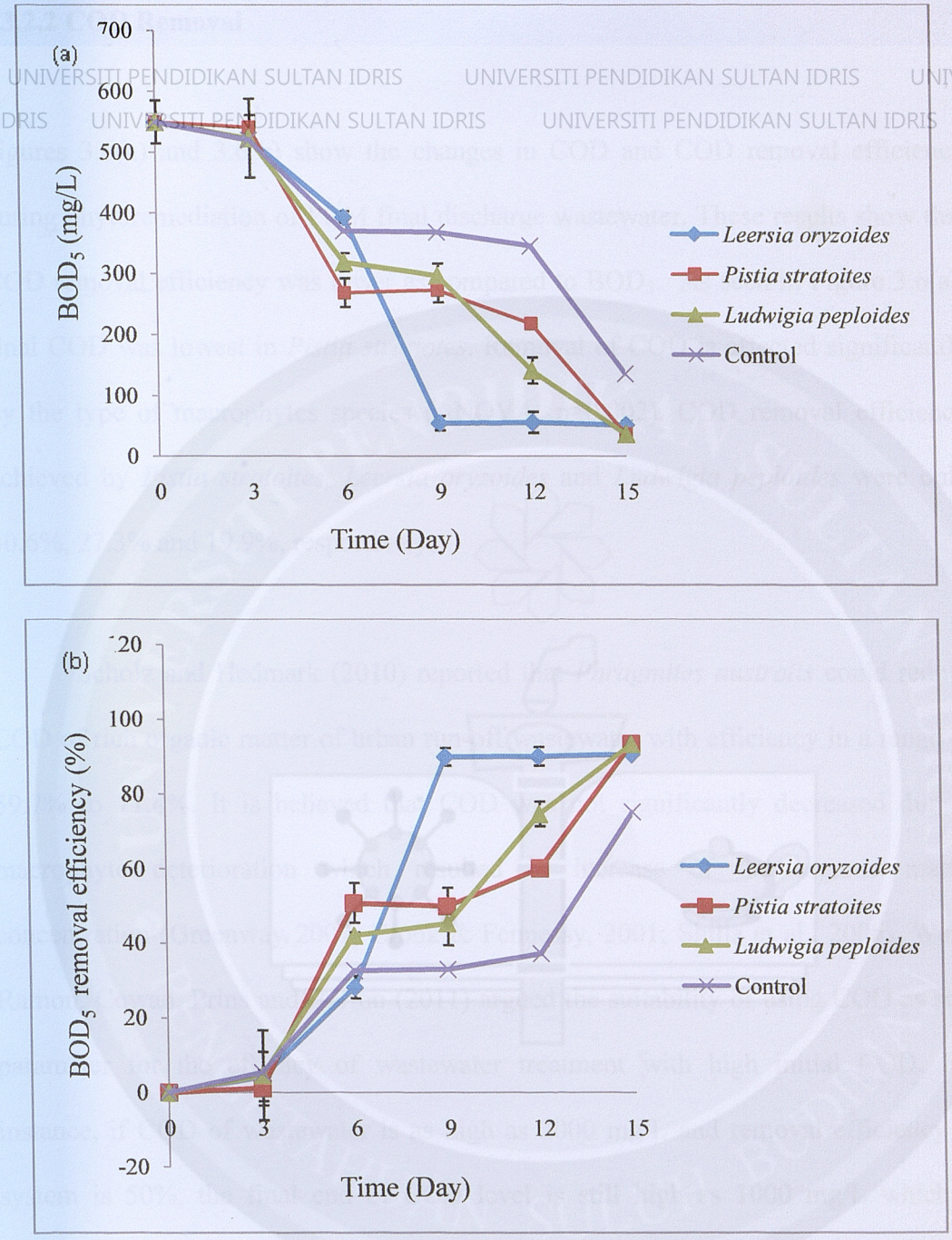


Figure 3.5. Changes in BOD_5 (a) and BOD_5 removal efficiency (b) of POM final discharge wastewater treated with three macrophytes species.

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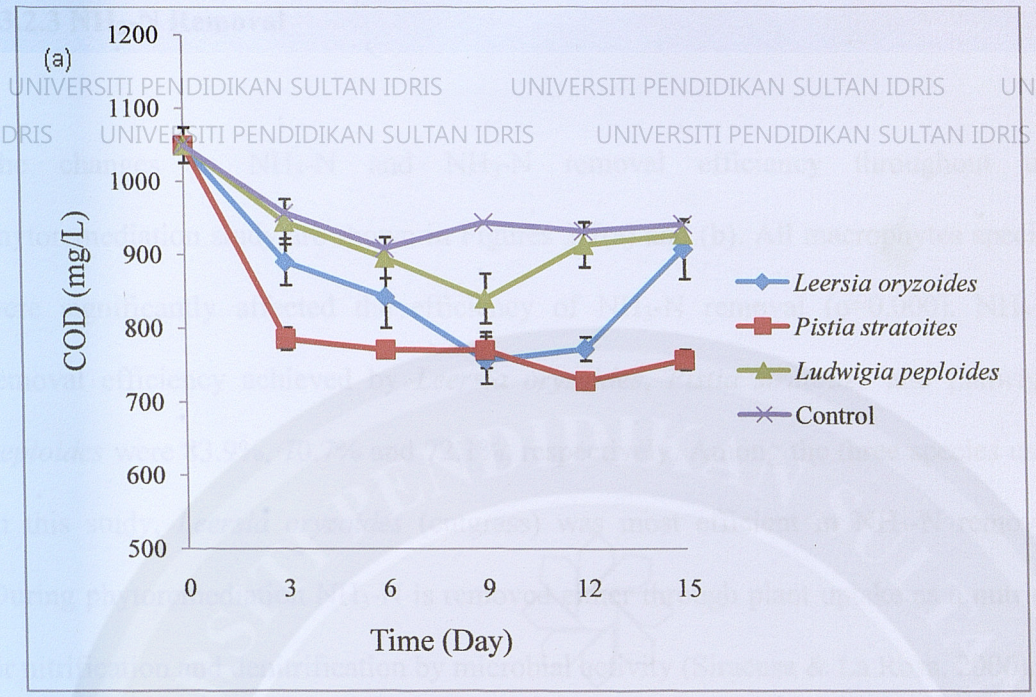
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3.3.2.2 COD Removal

Figures 3.6(a) and 3.6(b) show the changes in COD and COD removal efficiency during phytoremediation of POM final discharge wastewater. These results show that COD removal efficiency was lower as compared to BOD_5. As seen in Figure 3.6(a), final COD was lowest in *Pistia stratiotes*. Removal of COD is affected significantly by the type of macrophytes species (ANOVA, p=0.002). COD removal efficiency achieved by *Pistia stratoites, Leersia oryzoides* and *Ludwigia peploides* were only 30.6%, 27.3% and 19.9%, respectively.

Scholz and Hedmark (2010) reported that *Phragmites australis* could reduce COD of rich organic matter of urban run-off wastewater with efficiency in a range of 59.7% to 71.4%. It is believed that COD was not significantly decreased due to macrophyte deterioration which resulted in increase of the organic matter concentration (Greenway 2007; Cronk & Fennessy, 2001; Shilla et al., 2006). Welz, Ramon, Cowan, Prins and Burton (2011) argued the suitability of using COD as sole parameter for the efficacy of wastewater treatment with high initial COD. For instance, if COD of wastewater is as high as 2000 mg/L and removal efficiency of system is 50%, the final end of COD level is still high as 1000 mg/L which is unsuitable to be released into environment. Hence other parameter should also be considered such as ammoniacal nitrogen. Small removal of COD in previous study confirmed that macrophytes only play a minor role in removal of COD (Tang et al., 2009).

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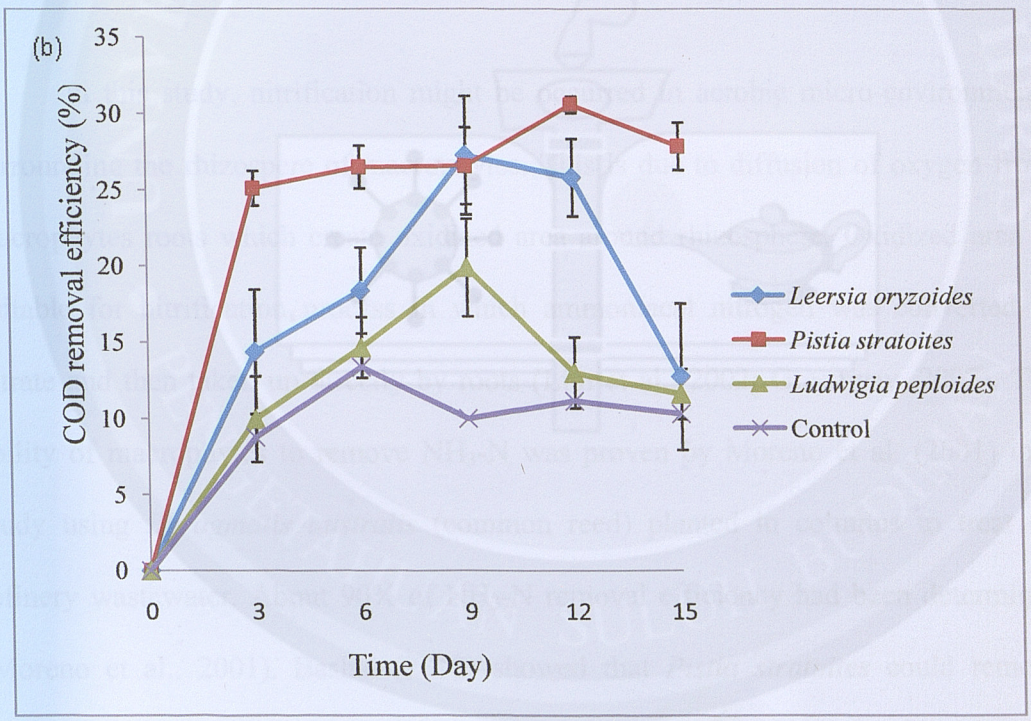


Figure 3.6. Changes in COD (a) and COD removal efficiency (b) of POM final discharge wastewater treated with three macrophytes species

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3.3.2.3 NH_3-N Removal

The changes in NH_3-N and NH_3-N removal efficiency throughout the phytoremediation study are shown in Figures 3.7(a) and (b). All macrophytes species were significantly affected the efficiency of NH_3-N removal ($p=0.000$). NH_3-N removal efficiency achieved by *Leersia oryzoides*, *Pistia stratoites* and *Ludwigia peploides* were 83.9%, 70.7% and 72.1%, respectively. Among the three species used in this study, *Leersia oryzoides* (cutgrass) was most efficient in NH_3-N removal. During phytoremediation NH_3-N is removed either through plant uptake as a nutrient or nitrification and denitrification by microbial activity (Siracusa & La Rosa, 2006).

In this study, nitrification might be occurred in aerobic micro-environments surrounding the rhizospere of macrophytes. This is due to diffusion of oxygen from macrophytes roots which create oxidized area around rhizosphere. Oxidized area is suitable for nitrification process in which ammoniacal nitrogen was converted to nitrate and then taken up directly by roots (Lim et al., 2002; Greenway, 2007). The ability of macrophytes to remove NH_3-N was proven by Moreno et al. (2001) in a study using *Phragmatis australis* (common reed) planted in columns to treat oil refinery wastewater. About 90% of NH_3-N removal efficiency had been determined (Moreno et al., 2001). Bashal (2010) showed that *Pistia stratoites* could remove 86.44% of total nitrogen (from 20.7 mg/L to 2.8 mg/L) in constructed wetland while NH_3-N removal was about 86.66% (from 10.8 mg/L to 1.44 mg/L). These findings show that macrophytes play an important role to facilitate NH_3-N removal during phytoremediation. Lim et al. (2002) noted that some NH_3-N which exists in the form of NH_3-N is lost to the atmosphere by the volatilization process at higher pH (>10).

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However, volatilization process might not be occurred in this study as POM final discharge wastewater is lower than pH 10 (pH 8.44-9.11). Figure 3.7(a) shows that at the end of phytoremediation, NH_3-N slightly increased. It is believed that the increase in NH_3-N might be caused by ammonification of decaying macrophytes (Vymazal & Kropfelova, 2008).

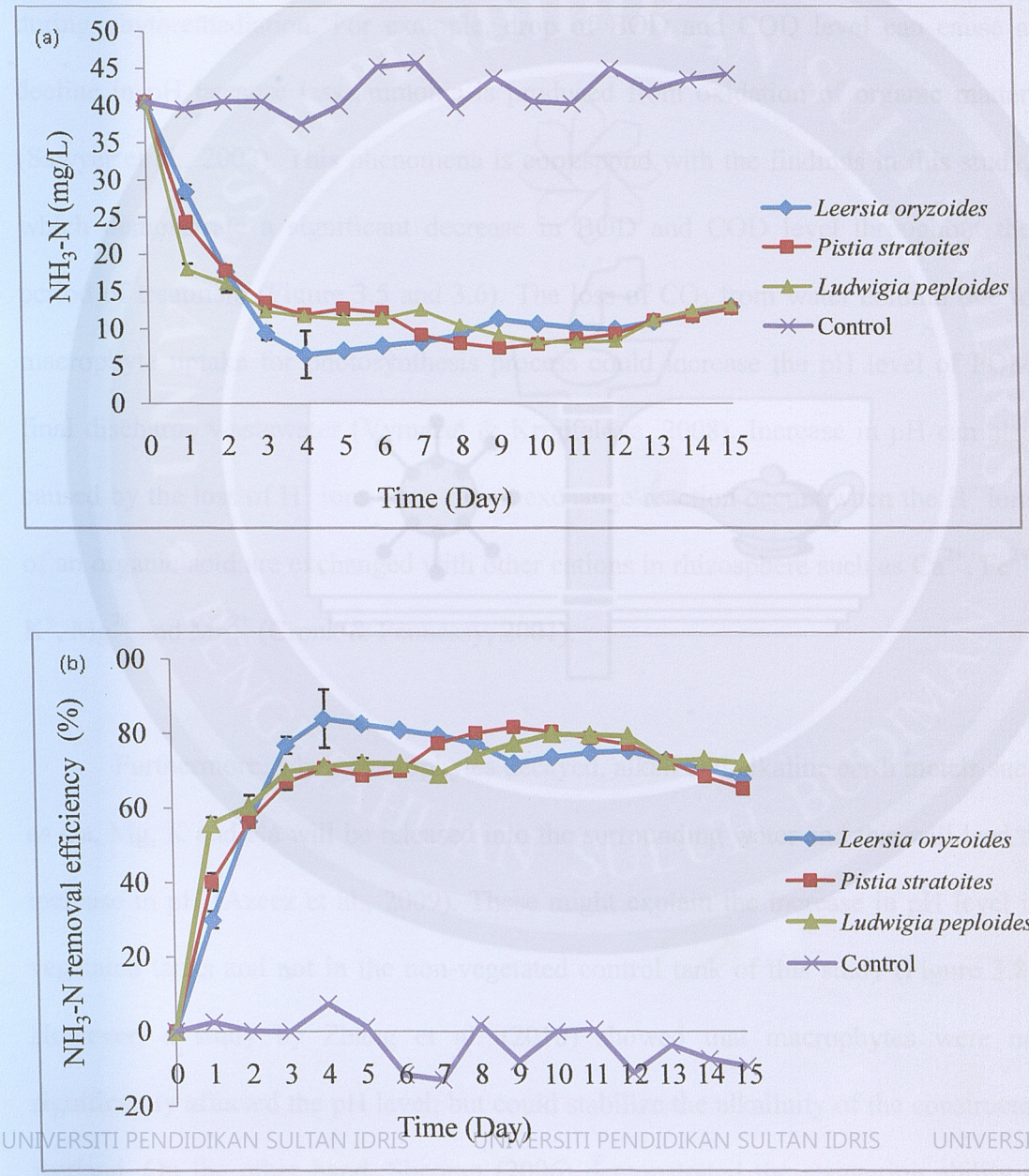


Figure 3.7. Changes in NH_3-N (a) and NH_3-N removal efficiency (b) of POM final discharge wastewater treated with three different macrophytes species

3.3.2.4 Changes in pH level

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Figure 3.8 shows pH level of POM final discharge wastewater throughout the experiment. Type of plant species was found significantly affected the pH level ($p=0.000$). The pH level of all macrophytes increased gradually during the treatment, but decreased in the control tank. There are many factors that affect the pH level during phytoremediation. For example, drop of BOD and COD level can cause a decline in pH because less ammonia is produced from oxidation of organic matter (Sawyer et al., 2003). This phenomena is correspond with the findings in this study, which demonstrate a significant decrease in BOD and COD level throughout the period of treatment (Figure 3.5 and 3.6). The loss of CO_2 from water column due to macrophyte uptake for photosynthesis process could increase the pH level of POM final discharge wastewater (Vymazal & Kropfelova, 2008). Increase in pH can also caused by the loss of H^+ ions from cation exchange reaction occurs when the H^+ ions of an organic acid are exchanged with other cations in rhizosphere such as Ca^{2+}, Fe^{2+}, K^+, Mg^{2+} and Mn^{2+} (Cronk & Fennessy, 2001).

Furthermore, when macrophytes decayed, alkali and alkaline earth metals such as Ca, Mg, K and Na will be released into the surrounding water and this will lead to increase in pH (Azeez et al., 2009). These might explain the increase in pH level in vegetated tanks and not in the non-vegetated control tank of this study (Figure 3.8). However, a study by Zhang et al. (2010) showed that macrophytes were not significantly affected the pH level, but could stabilize the alkalinity of the constructed wetland. On the other hand, Sheoran (2006) demonstrated the staggering ability of

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grasses macrophytes, *Typha angustifolia* to increase pH of acid mine drainage from 2.9 to 7.8 only in two days of retention time.

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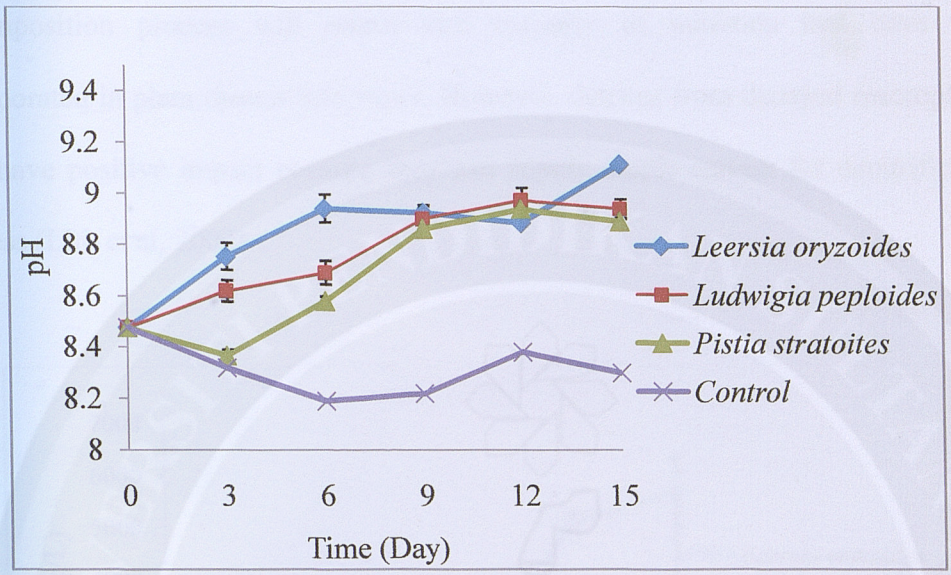


Figure 3.8. Changes in pH level of POM final discharge wastewater treated with three macrophyte species

3.3.2.5 Changes in TSS

TSS level in POM final discharge wastewater treated with three macrophytes species and control tank is shown in Figure 3.9. A gradual increase of TSS was observed throughout the treatment period in all vegetated tanks and non-vegetated (control). However, the type of macrophyte species did not significantly affected the increase of TSS, (ANOVA, $p = 0.63$). Among the three macrophyte species, the highest mean of TSS was achieved by *Leersia oryzoides* (5650 mg/L) on the final day of experiment (Day 15th) followed by *Ludwigia peploides* (3862 mg/L). This occurred most probably because of the higher decay rate of *Leersia oryzoides* long rhizomatic roots than *Pistia stratoites*, which has shorter fibrous roots. Several studies have shown the

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accumulation of decaying biomass increased TSS and harvesting macrophytes at the end of growing season could improve water quality in constructed wetlands (Karathanasis et al., 2003; Nahlik & Mitsch, 2006). Crites et al. (2006) noted that decomposition process will return vast majority of nutrients that have been incorporated in plant tissues into water. However, detritus from decayed macrophytes also have positive impact because they can supply ample carbon for denitrification process (Lim et al, 2002).

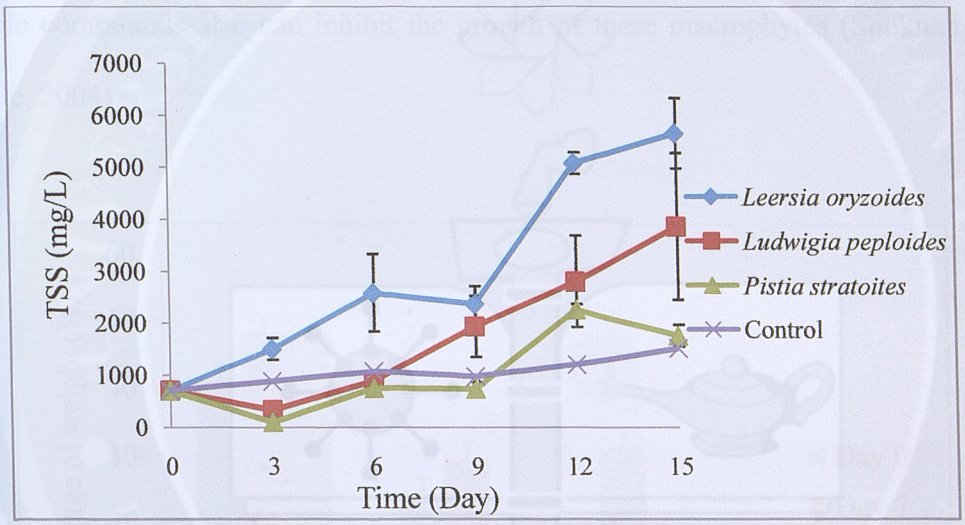


Figure 3.9. Changes in total suspended solid (TSS) of POM final discharge wastewater treated with three macrophytes species

3.3.3 Plant Weight

Figure 3.10 shows the changes in plant weight percentage throughout the phytoremediation treatment. Plant weight increment was observed in *Leersia oryzoides* and *Ludwigia peploides*. However, a decrease of weight occurred in *Ludwigia peploides* on day 14 that might due to its maximum uptake limit of nutrients

(Reddy & DeBusk, 1987). In this study, an increase of plant weight was corresponded to the NH_3-N removal (Figure 3.7) as ammonium ion is assimilated into protein (Vymazal & Kropfelova, 2008). Rapid decrease of *Pistia stratoites* weight was observed and this might be caused by more porous structure of *Pistia stratoites* compared to the other two macrophytes which led to their vulnerability to pollutant stress. In a phytoremediation study carried out by Sooknah and Wilkie (2004), a high COD of anaerobically digested flushed dairy manure wastewater could stop the growth of *Pistia stratiotes* and *Hydrocotyle umbellata*. Salinity and uncharacterized soluble compounds also can inhibit the growth of these macrophytes (Sooknah and Wilkie, 2004).

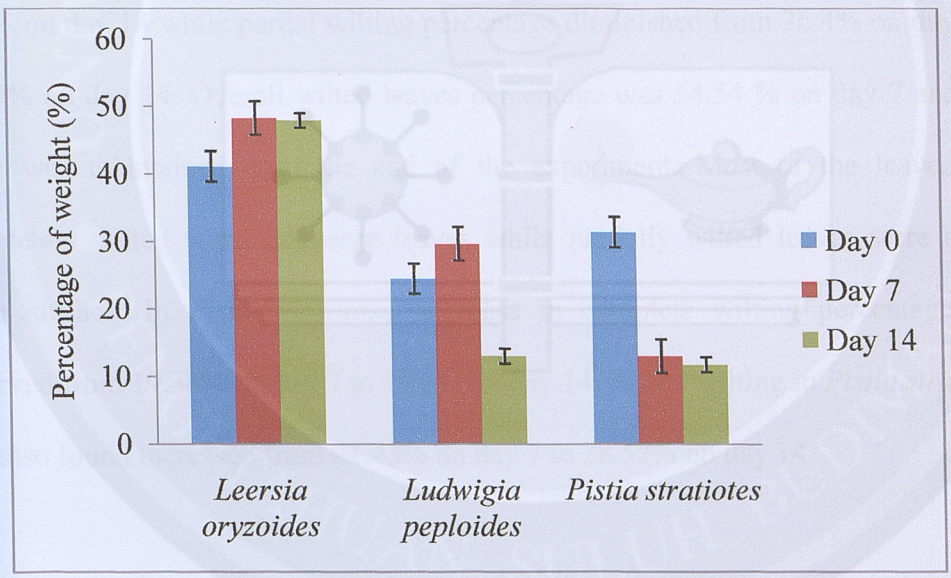


Figure 3.10. Changes in plant weight during phytoremediation of POM final discharge wastewater

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3.3.4 Morphological Changes of Macrophytes

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Figures 3.11 and 3.12 show the wilting percentages and morphological changes of macrophytes throughout the phytoremediation study. Wilting was not observed in all macrophytes during the initial day of experiment (day 0). However, throughout the experiment, the leaves of all macrophytes were gradually wilted. In *Leersia oryzoides*, complete wilting increased from 14.26% on day 7 to 47.62% on day 14 and partial wilting occured from 28.57% on day 7 to 38.1% on day 14, respectively. On day 7, the lowest complete wilting percentage (14.26%) was found in *Leersia oryzoides* than two other macrophytes. In *Ludwigia peploides*, most of the leaves that wilted were submerge leaves. Complete wilting percentage was increased from 18.2% on day 7 to 36.4% on day 14 while partial wilting percentage diminished from 36.4% on day 7 to 15.15% on day 14. Overall wilted leaves percentage was 54.54 % on day 7 and this value was maintained until the end of the experiment. Most of the leaves that completely wilted were submerge leaves while partially wilted leaves were above water surface. In *Pistia stratoites*, increase in complete wilting percentage was observed from 19.38% on day 7 to 39.8% on day 14. Partial wilting in *Pistia stratiotes* was also found increased from 21.43% on day 7 to 28.57% on day 14.

It was observed that wilting exceeded 50% in all macrophytes species with *Leersia oryzoides* suffered severe wilting which was about 85.72%. According to Sooknah and Wilkie (2004), a high COD level of from wastewater can inhibit macrophytes growth. In this study, only 19.9% to 30.6% of COD were able to be removed from POM final discharge wastewater. High Fe strength in wastewater also cause phytotoxicity as Cronk and Fennessy (2001) reported that this toxicity varies

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among the macrophytes and some even suffer phytoxicity at as low as 1 mg/L level of Fe. Wilting sign may cause by a decrease in chlorophylls; a physiological change that happens when macrophytes accumulate more metals (Tewari, Singh, Singh & Rai, 2008).

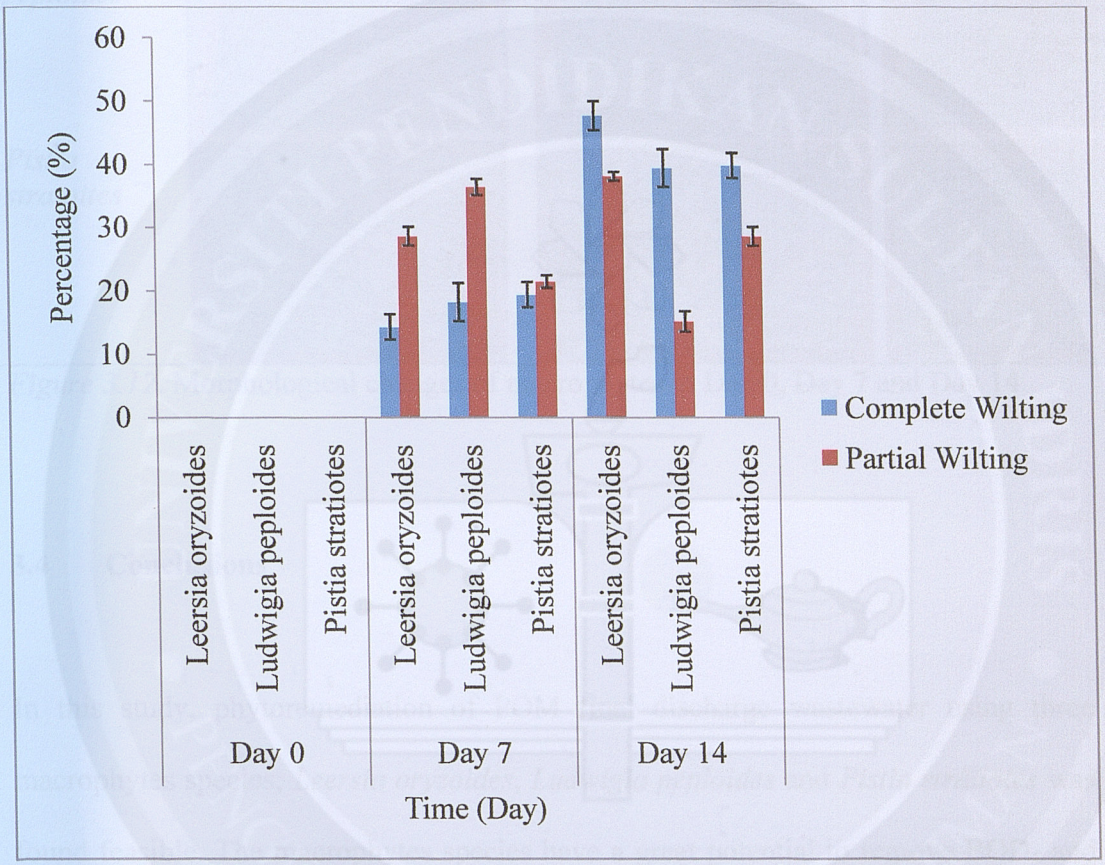


Figure 3.11. Wilting of macrophyte's leaves throughout the phytoremediation study

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Macrophyte Species	Time		
	Day 0	Day 7	Day 14
Leersia oryzoides			
Ludwigia peploides			
Pistia stratoites			

Figure 3.12. Morphological changes of macrophytes in Day 0, Day 7 and Day 14

3.4 Conclusions

In this study, phytoremediation of POM final discharge wastewater using three macrophytes species; *Leersia oryzoides*, *Ludwigia peploides* and *Pistia stratiotes* was found feasible. The macrophytes species have a great potential to remove BOD_5 and NH_3-N from POM final discharge wastewater. *Leersia oryzoides* has been identified as the most efficient macrophyte for NH_3-N and COD removal while *Ludwigia peploides* and *Pistia stratiotes* were excellent in removing BOD_5 (93.3 %). However the macrophytes studied have also suffered phytotoxity which resulted in the sign of wilting. Among the three species, *Ludwigia peploides* was found resistant to pollutant found in POM final discharge wastewater with lowest wilting leaves. The ability of this species to tolerate with the pollutant strength might be useful for POM final

discharge wastewater treatment at larger scale. Besides, the characteristics of POM final discharge wastewater after phytoremediation using this species is comply with the discharge limit imposed by DOE, particularly for BOD_5, NH_3-N and pH.

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CHAPTER 4

CHARACTERIZATION OF NUTRIENT UPTAKE IN MACROPHYTES DURING PHYTOREMEDIATION

4.1 Introduction

POME is rich with nutrients and its discharge into river water can lead to eutrophication and depletion of oxygen in water (Ma, 2009). Phytoremediation is an important mechanism in removing nutrients and pollutants, by which there is a possibility to reduce water pollution. Uptake of nutrients during phytoremediation involves plants that grow in or near water bodies. To accelerate nutrient removal, contact between active zones of nutrient absorption and the wastewater should be maximized to optimize nutrient removal and incorporation into plant biomass (Greenway, 2007). Several studies on nutrients allocation in macrophytes organ during phytoremediation have been reported. Among the studies was done by Cronk and Fennessy (2001), who found that nitrogen percentage was higher in macrophytes

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leaves than other organs such as stem and root. The feasibility of phytoremediation study depends on biocencentration factor (BCF) that determine how efficient a plant could absorb metals, transport them into its organs and store them (Marchiol et al., 2004). Translocation factor (TF) determines the ability of plants to transport absorbed metals from roots to leaves (Ahmad et al., 2011). However, macrophytes that have low translocation factor (TF) value can reduce the amount of metals accumulate in leaves (Soda et al., 2012).

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This chapter discussed the characterization of the nutrients uptake during the phytoremediation study by measuring the C, H and N elements percentages in leaf, stem and root organs of the macrophytes species. The BCF and TF values of metals for each studied organs were also calculated to understand the ability of the macrophytes to absorb and transport the nutrients.

4.2 Methodology

4.2.1 Preparation of Macrophytes Sample for CHNO-S and Atomic Absorption Spectrometry (AAS) Analysis

Macrophytes samples prepared for CHNO-S and AAS analysis were taken before and after phytoremediation treatment. The macrophytes leaves, stems (except for *Pistia stratoites* which has no stem) and roots were harvested from each treatment tank. The samples were washed initially with tap water followed by distilled water. Then the macrophytes samples were dried in an oven at 105°C and ground with mortar and

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pestle. Sample in the form of powder were used for CHNO-S analysis. For AAS analysis, about 0.5-3.0 g of the ground plant samples were placed into 250 ml conical flask. 50 ml of 2M hydrochloric acid (HCl) was added into the flask for macrophyte digestion. The sample mixture was then shaken overnight with orbital shaker and centrifuged for 20 minutes and filtered using 0.45 μm cellulose acetate membrane. The filtrate was then analysed for metals.

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4.2.2 Atomic Absorption Spectrometry (AAS)

Prepared macrophytes samples were analysed with AAS model Analyst 220, according to the Standard Methods APHA. For each metal, three optimum standard solutions within the range and blank reagents were prepared to obtain calibration curves. About 1 to 2 ml of each sample was analysed using AAS. Results from analysis of each samples displayed three readings, means and standard deviations.

4.2.3 CHNS-O Elemental analysis

Prepared macrophytes samples in the form of powder were weighed to about 3 mg. Sample was then put in a tin vessel and placed in an auto sampler for analysis. Helium gas was purged for combustion at temperature of 1700-1800 °C. The oxidation of sample was aided by Tungstenoxide (WoO_3) catalyst. The combustion produces CO_2, H_2O, N_2 and SO_2 and these gasses were measured with thermal conductivity detection

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(TCD) where peak height was converted to the exact percentage using acetanilide as standard.

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4.2.4 Bioconcentration Factor (BCF)

In general, efficiency of phytoremediation can be determined from BCF value. In this study, BCF for Ca, Mg, K, Na, Fe and Zn were calculated using Equation 4.1 (Khellaf and Zerdaoui, 2009; Soda et al., 2012) :

$$BCF = C_p/C_w \qquad \text{(Equation 4.1)}$$

Where C_p is metal concentration in macrophytes organs (mg/kg) and C_w is metal concentration in POM final discharge wastewater (mg/L). Macrophytes with high BCF value indicate high capability to accumulate metals (Soda et al., 2012).

4.2.5 Translocation Factor (TF)

Translocation factor (TF) was determined to investigate translocation of metals in macrophytes from roots to leaves. Translocation factor was calculated using Equation 4.2 (Soda et al., 2012):

$$TF = C_i/C_r \qquad \text{(Equation 4.2)}$$

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Where C_i is metal concentration in leaves and C_r is metal concentration in macrophytes roots. Higher TF value indicates that macrophytes have high capability to translocate metals.

4.3 Results and Discussions

4.3.1 Nitrogen (N) Percentage in Macrophytes Organs

N level in macrophyte organs before and after phytoremediation treatment is shown in Table 4.1 and Figure 4.1. Before treatment, leaf of *Leersia oryzoides* and *Pistia stratoites* had the highest amount of N than their roots and stems. This result corresponds to Greenway (2007) in her study on phytoremediation of municipal wastewater, that N amount was higher in macrophytes' leaves (*Phragmites australis*). In this study, the highest level of N after phytoremediation treatment was in leaves of *Leersia oryzoides* with 3.02 %. The percentage of N in all studied organs of *Leersia oryzoides* was highest than *Ludwigia peploides* and *Pistia stratoites.* In general, N was found high in all studied macrophyte organs except in stems and roots of *Ludwigia peploides*. This might be due to translocation of assimilated ammonium and nitrate from roots to leaves and stems which then transported to the other organs by the vascular tissues (Vymazal & Kropfelova, 2008). The percentage of N for all macrophytes was increased after the phytoremediation, showed the contribution of senescence organs in nutrient translocation from stems and roots to the new growing shoots or storage organs (Greenway, 2007).

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Table 4.1
Nitrogen (N) percentage in different macrophytes organs before and after phytoremediation treatment

Species	Organ	Before (%)	After (%)	Percentage difference (%)
Leersia oryzoides	Leaf	0.77	3.02	2.24
	Stem	bdl[a]	2.57	2.57
	Root	0.26	1.44	1.18
Ludwigia peploides	Leaf	0.90	2.09	1.19
	Stem	1.74	0.34	-1,41
	Root	1.41	0.77	-0.64
Pistia stratoites	Leaf	1.61	1.84	0.23
	Root	1.48	2.03	0.55

Note. [a]bdl: below detection limit

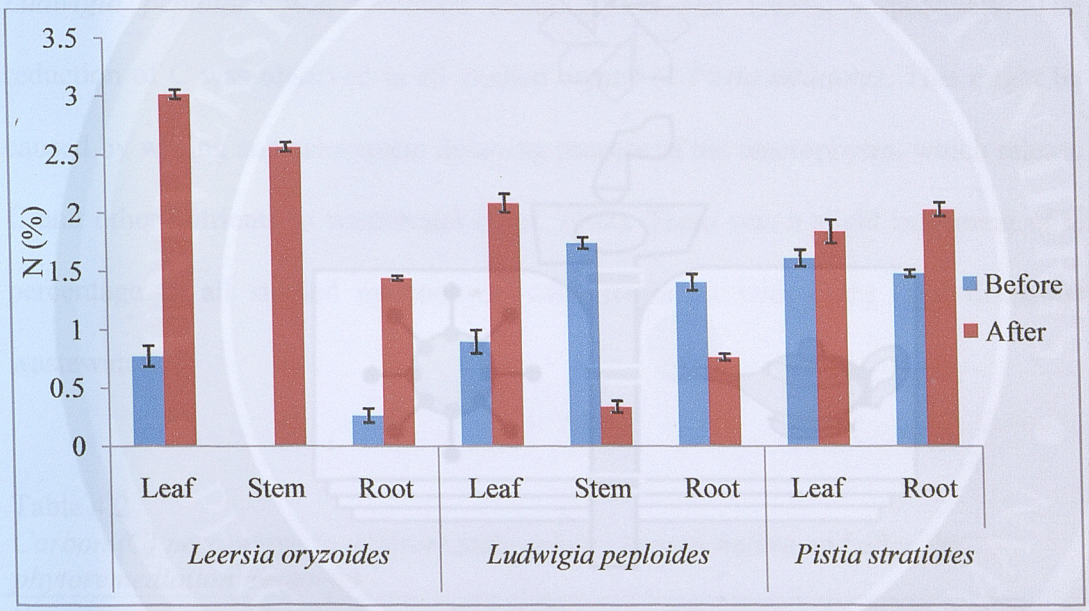


Figure 4.1. Nitrogen (N) percentage in different macrophytes organs before and after phytoremediation treatment

4.3.2 Carbon (C) Percentage in Macrophytes Organs

Table 4.2 and Figure 4.2 show the carbon (C) level in all macrophytes organs before and after phytoremediation study. In general, C percentages were high in all studied organs of all macrophytes species in the range of 24.76% to 42.06%. This might be

due to an ample amount of C in plants in the form of carbohydrate, lipid, protein and nucleic acid molecules (Berg, 2008). The major processes of C transformation to organic molecules under aerobic conditions are photosynthesis and respiration which dominate aerobic horizons (aerial and aerobic water) with H_2O as the major electron donor in photosynthesis and oxygen as the terminal electron acceptor in respiration.

The C percentage in *Leersia oryzoides* leaves was increased subtly from 39.90% to 39.99% than in stem and root. The C percentage in leaves and stems of *Ludwigia peploides* was increased about 2.95% and 1.93%, respectively. The reduction of C was observed in all studied organs of *Pistia stratiotes*. This might be caused by wilting and subsequent decaying process of the macrophytes, which release C and other nutrients to wastewater (Lim, 2002). There was a slight increment of C percentage in all studied macrophytes after treatment with POM final discharge wastewater.

Table 4.2
Carbon (C) percentage in different macrophytes organs before and after the phytoremediation treatment

Species	Organ	Before (%)	After (%)	Percentage difference (%)
Leersia oryzoides	Leaf	39.90	39.99	0.09
	Stem	37.69	36.83	-0.86
	Root	38.63	31.61	-7.02
Ludwigia peploides	Leaf	39.11	42.06	2.95
	Stem	36.77	38.70	1.93
	Root	40.34	32.31	-8.02
Pistia stratoites	Leaf	39.60	31.53	-8.07
	Root	30.80	24.76	-6.04

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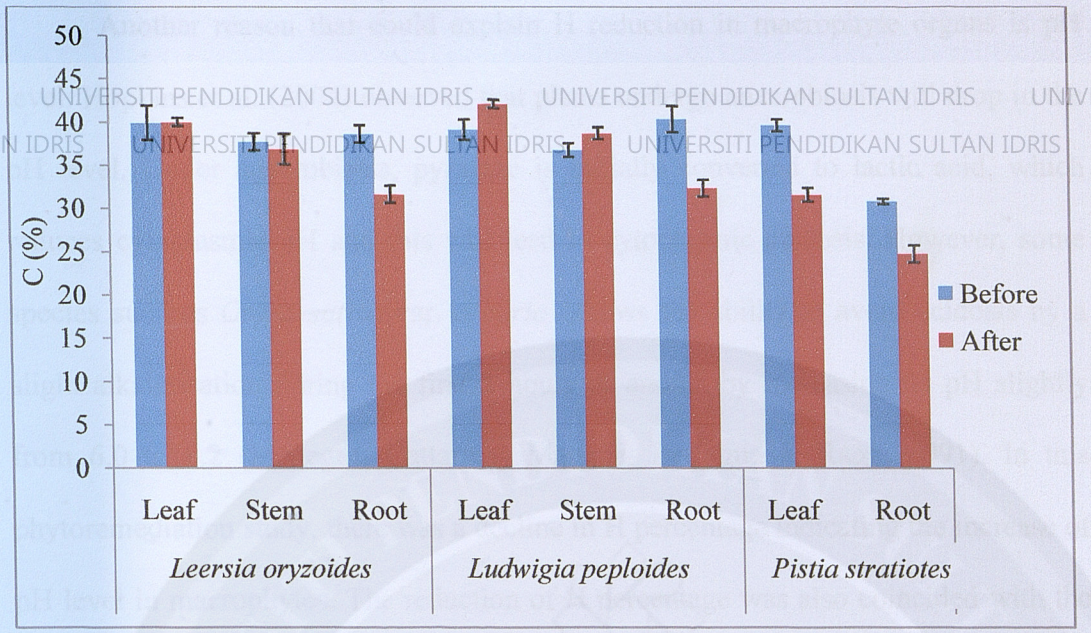


Figure 4.2. Carbon (C) percentages in different macrophyte organ before and after the phytoremediation treatment

4.3.3 Hydrogen (H) Percentage in Macrophytes Organs

The hydrogen (H) level and its concentration during the phytoremediation treatment are shown in Table 4.3 and Figure 4.3. In general, a reduction in hydrogen level was observed in all studied macrophyte organs, except in *Leersia oryzoides*' stem with 1.95% increment (Figure 4.3). Hydrogen percentage in other macrophytes showed significant decrease particularly in *Pistia statoites*' root. The reduction in H can be explained by H ion exchange with positively charged ions such as Mg^{+}, Ca^{2+} and K^{+} via it roots. When positively charged ions are taken up by macrophytes, H^{+} are released to rhizosphere creating an equally balanced ion concentrations (Cronk & Fennessy, 2001). This is supported by the increase of Ca, Mg and K in macrophyte organs, which are discussed in later subtopics of this chapter.

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Another reason that could explain H reduction in macrophyte organs is pH level. Hopkins et al. (2004) observed that plants undergo anaerobiosis will drop in the pH level. Under anaerobiosis, pyruvate is initially converted to lactic acid, which reduces cytoplasmic pH and this will lead to cytoplasmic acidosis. However, some species such as *Oryza sativa* var. *arborio* shows the ability to avoid acidosis by a slight alkalinisation during the first 8 hours of anoxia by increasing its pH slightly from 6.0 to 6.2 (Menegus, Cattaruza, Mattana, Beffagna & Ragg, 1991). In this phytoremediation study, there was a decline in H percentage indicating the increase of pH level in macrophytes. The reduction of H percentage was also coincided with the reduction of N and C as both elements usually hold H to form a molecule.

Table 4.3
Hydrogen (H) percentage in different macrophyte organ before and after the phytoremediation treatment

Species	Organ	Before (%)	After (%)	Percentage difference (%)
Leersia oryzoides	Leaf	5.95	5.44	-0.52
	Stem	3.04	4.98	1.95
	Root	5.44	4.35	-1.09
Ludwigia peploides	Leaf	5.27	5.15	-0.12
	Stem	5.03	4.89	-0.15
	Root	5.04	4.3	-0.74
Pistia stratoites	Leaf	5.31	4.52	-0.78
	Root	4.49	3.34	-1.15

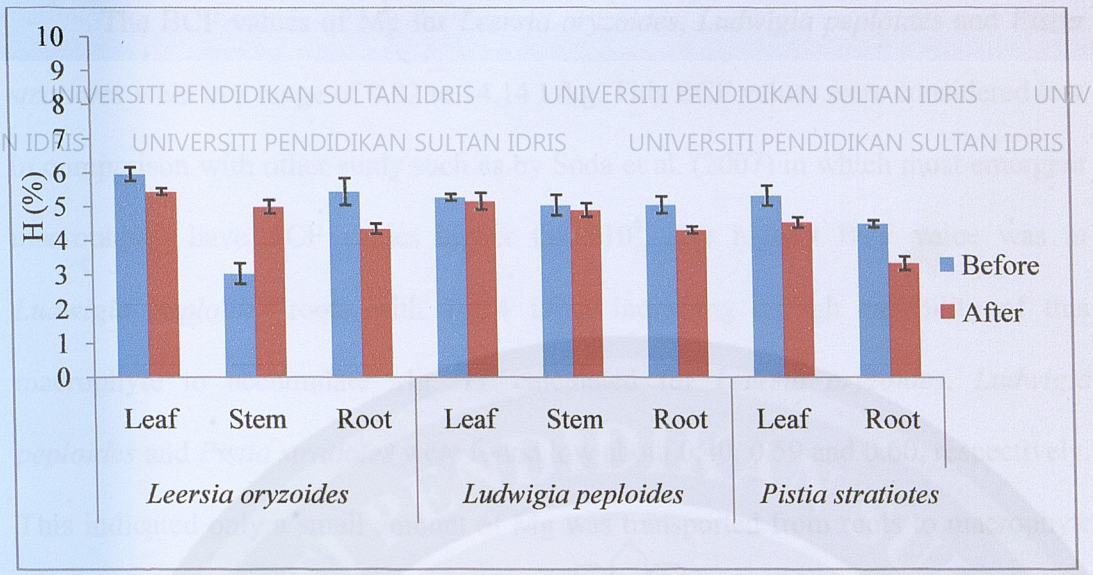


Figure 4.3. Hydrogen (H) percentage in different macrophyte organs before and after phytoremediation treatment

4.3.4 Magnesium (Mg)

Table 4.4 and Figure 4.4 show Mg concentration in three macrophytes organs and in POM final discharge wastewater, before and after treatment and its BCF and TF values. Increase in Mg concentrations was observed in all macrophytes' organs. In leaves, Mg is found as a structural component of chlorophyll which important for photosynthesis (Maathuis, 2009). POM final discharge wastewater had sufficient of Mg concentration to provide adequate nutrients to macrophytes. When comparing Mg level (Table 4.4) in all studied organs of macrophytes, the highest Mg concentration was observed in roots, about 25.08 mg/L, 65.66 mg/L and 101.09 mg/L in *Leersia oryzoides*, *Ludwigia peploides* and *Pistia stratiotes*, respectively. The increase in Mg probably because of the active ion exchange process by macrophytes roots (Cronk & Fennessy, 2001).

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The BCF values of Mg for *Leersia oryzoides*, *Ludwigia peploides* and *Pistia stratiotes* were in a range of 0.82 to 14.14 L/kg. This BCF values were considered low in comparison with other study such as by Soda et al. (2007) in which most emergent macrophytes have BCF values higher than 10^4. The highest BCF value was in *Ludwigia peploides* roots with 14.14 L/kg, indicating a high capability of this macrophyte to accumulate Mg. TF calculated for *Leersia oryzoides*, *Ludwigia peploides* and *Pistia stratiotes* were found low about 0.40, 0.59 and 0.60, respectively. This indicated only a small amount of Mg was transported from roots to macrophyte leaves.

The Mg levels in POM final discharge wastewater after phytoremediation treatment increased in *Leersia oryzoides* and *Pistia stratiotes* by 95.1 mg/L and 32.65 mg/L, respectively. Only Mg concentration in *Ludwigia peplodes* was reduced about 132.15 mg/L (Table 4.4). Increase in Mg level in POM final discharge wastewater in *Leersia oryzoides* and *Pistia stratiotes* might be related to a higher level of wilting and decaying processes observed in both macrophytes than *Ludwigia peploides* (Chapter 3). The previous chapter (Chapter 3) discussed that *Leersia oryzoides* and *Pistia stratiotes* suffered total wilting on day 14^{th} of treatment (85.72% and 68.37%, respectively) while *Ludwigia peploides* showed only about 54.54% of wilting leaves. This finding was in accordance to the low percentage level of Mg found in POM final discharge wastewater after treated with *Ludwigia peploides*. Plant decomposition released large amounts of nutrients in water and re-polluted the water (Cronk & Fennessy, 2001; Shilla et al., 2006; Hu et al., 2010). As *Ludwigia peploides* has the least wilting leaves, therefore less Mg was released into POM final discharge wastewater.

Table 4.4
BCF and TF of Mg in different macrophytes organs

Species	Organ	Initial (mg/L)	Final (mg/L)	Concentration difference (mg/L)	BCF (L/kg)	TF
Leersia oryzoides	Leaf	0.11	15.46	15.35	0.82	0.40
	Stem	7.712	16.99	9.28	6.56	
	Root	13.12	38.2	25.08	2.02	
	POM FD	220.2	315.3	95.1		
Ludwigia peploides	Leaf	0.392	44.78	44.39	8.48	0.59
	Stem	11.43	29.54	18.11	5.59	
	Root	9.04	74.7	65.66	14.14	
	POM FD	220.2	88.05	-132.15		
Pistia stratoites	Leaf	29.0	87.2	58.2	5.75	0.60
	Root	43.4	145.3	101.9	9.58	
	POM FD	220.2	252.85	32.65		

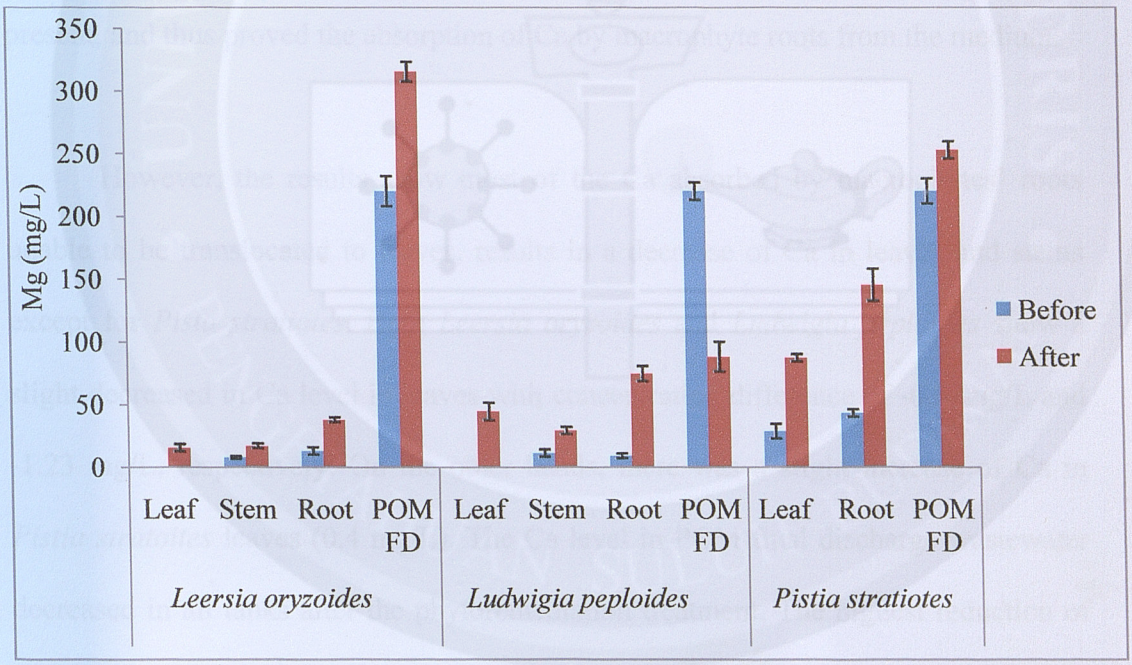


Figure 4.4. Mg concentration in different macrophytes organs before and after the phytoremediation treatment

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4.3.5 Calcium (Ca)

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The Ca content was increased in the roots of the three studied macrophytes with the highest level of Ca observed in *Pistia stratiotes* root (12.59 mg/L), followed by *Leersia oryzoides* roots (12.56 mg/L), (Table 5.5 and Figure 5.5). *Leersia oryzoides*'s roots have the highest concentration difference of Ca throughout the phytoremediation study, about 10.31 mg/L. The concentration of H^+ ions and other metals could affect the uptake of Ca by competing for binding sites on roots and low pH value in water indicates the presence of high concentrations of H^+ ions which inhibit Ca uptake (Cronk & Fennessy, 2001; Batty & Younger, 2004). In this study, the POM final discharge wastewater was basic in pH (Chapter 3, page 77) that show less H^+ ions present, and thus proved the absorption of Ca by macrophyte roots from the medium.

However, the results show most of the Ca absorbed by macrophytes' roots unable to be translocated to leaves, results in a decrease of Ca in leaves and stems except for *Pista stratiotes*. Both *Leersia oryzoides* and *Ludwigia peploides* show a slight decreased in Ca level in leaves with concentration difference of -0.62mg/L and -1.23 mg/L, respectively. On the other hands, there was a slight increase of Ca in *Pistia stratoites* leaves (0.4 mg/L). The Ca level in POM final discharge wastewater decreased in all tanks after the phytoremediation treatment. The highest reduction of Ca from POM final discharge wastewater was in *Pistia stratiotes* (7.5 mg/L) indicated active Ca uptake by this plant. The precipitation of Ca ions to calcium carbonate might also occurred as POM final discharge wastewater pH was below 10, a pH condition that favoured the process (Sawyer, et al. 2003).

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BCF values of Ca in all macrophytes organs were higher than BCF values of Mg in the range of 3.60 to 56.57 L/kg. *Pistia stratiotes* has the highest BCF value, 56.57 L/kg and 56.17 L/kg for leaves and roots, respectively. This result was in accordance with reduction of Ca in POM final discharge wastewater, which shows that *Pistia stratiotes* has the highest capability in absorbing Ca from POM final discharge wastewater as compared to the other macrophytes studied. However, the TF value obtained in this study show a slight Ca translocation from roots to leaves of *Pistia stratiotes*.

Ca is generally immobile and plant that suffers Ca deficiency will initially shows symptoms of necrosis in shoots (Hopkins and Hunner, 2004). Ca ions are also important in a number of physiological roles in plants such as altering membrane permeability, cell signalling and cation-anion balance in vacuoles (Berg, 2008; White & Broadley, 2003). Previous study showed Ca content in macrophytes was related to K uptake (Azeez et al., 2009). This is because both nutrients are important for macrophytes growth. The concentration of Ca for adequate growth in plants is normally around 0.5% of shoot dry matter. The developing tissues of macrophytes are relying on the immediate supply of Ca as it cannot be mobilised from older tissues (White & Broadley, 2003).

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Table 4.5
BCF and TF of Ca in different macrophytes organs

Species	Organ	Initial (mg/L)	Final (mg/L)	Concentration difference (mg/L)	BCF (L/kg)	TF
Leersia oryzoides	Leaf	6.67	6.05	-0.62	15.41	0.48
	Stem	2.08	1.41	-0.67	3.60	
	Root	2.25	12.56	10.31	31.98	
	POM FD	11.21	6.55	-4.66		
Ludwigia peploides	Leaf	12.01	10.78	-1.23	35.87	0.86
	Stem	8.68	5.78	-2.90	19.22	
	Root	11.47	12.53	1.06	41.69	
	POM FD	11.21	5.01	-6.2		
Pistia stratiotes	Leaf	12.19	12.59	0.4	56.57	1.01
	Root	8.51	12.50	3.99	56.17	
	POM FD	11.21	3.71	-7.5		

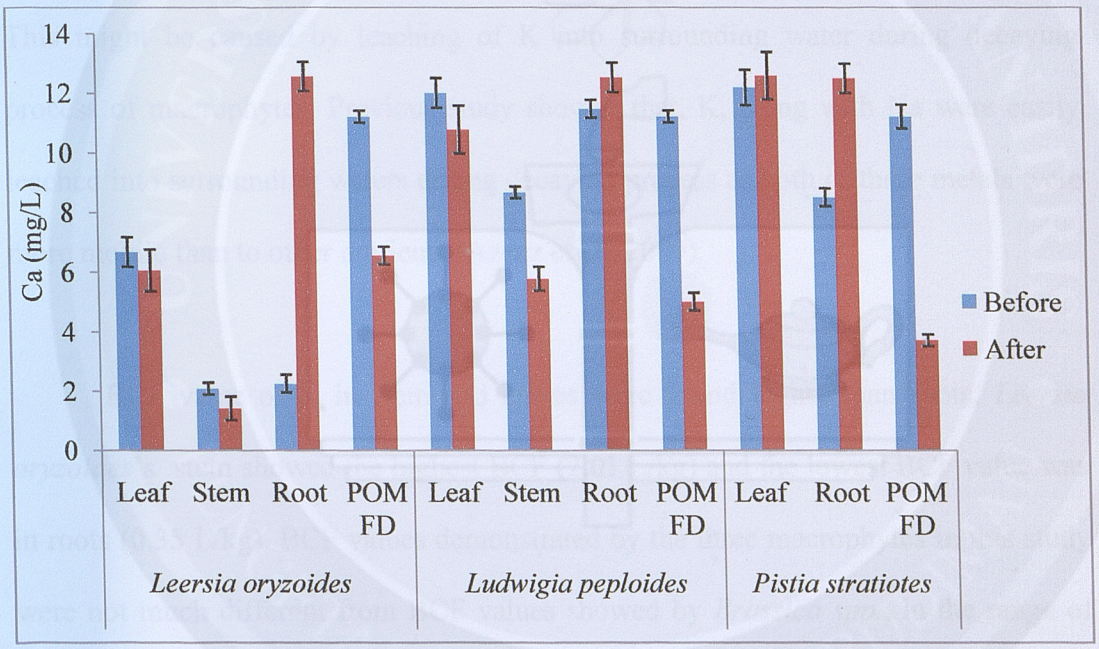


Figure 4.5. Ca concentration in different macrophytes organs before and after the phytoremediation treatment

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4.3.6 Potassium (K)

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The K level in all macrophytes' organs and in POM final discharge wastewater was increased except in *Leersia oryzoides*'s roots. The highest K level after the treatment was in *Pistia stratiotes*'s leaves, with an increase about 8.9 mg/L. K is the most abundant cation in higher plants, which takes part in many essential processes including enzyme activation, protein synthesis, photosynthesis, phloem transport, osmoregulation, stomatal regulation and tropism (Cochrane & Cochrane, 2009). The K concentration in POM final discharge wastewater also increased after the treatment. This might be caused by leaching of K into surrounding water during decaying process of macrophytes. Previous study showed that, K, along with Na were easily leached into surrounding waters during decaying process as both of these metals were more mobile than to other nutrients (Azeez et al., 2009).

BCF value of K in stem and leaves were found higher than roots. *Leersia oryzoides*'s stem showed the highest BCF (2.01 L/kg) and the lowest BCF value was in roots (0.35 L/kg). BCF values demonstrated by the three macrophytes in this study were not much different from BCF values showed by *Brassica spp.*, in the range of 0.45 to 1.91 L/kg (Marchiol et al., 2004). The highest TF value for K was observed in *Leersia oryzoides* species (4.95) suggesting that *Leersia oryzoides* has the highest capability in absorbing and translocating K from POM final discharge wastewater to its organs.

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Table 4.6
BCF and TF of K in different studied macrophytes organs

Species	Organ	Initial (mg/L)	Final (mg/L)	Concentration difference (mg/L)	BCF (L/kg)	TF
Leersia oryzoides	Leaf	17.8	20.8	3	1.72	4.95
	Stem	18.4	24.3	5.9	2.01	
	Root	12.25	4.2	-8.05	0.35	
	POM FD	194.15	201	6.85		
Ludwigia peploides	Leaf	7.0	13.6	6.6	1.12	1.9
	Stem	12.25	20.9	8.65	1.72	
	Root	4.6	7.15	2.55	0.59	
	POM FD	194.15	202.5	8.35		
Pistia stratiotes	Leaf	15.55	24.45	8.9	2.00	1.18
	Root	11.85	20.65	8.8	1.69	
	POM FD	194.15	204	9.85		

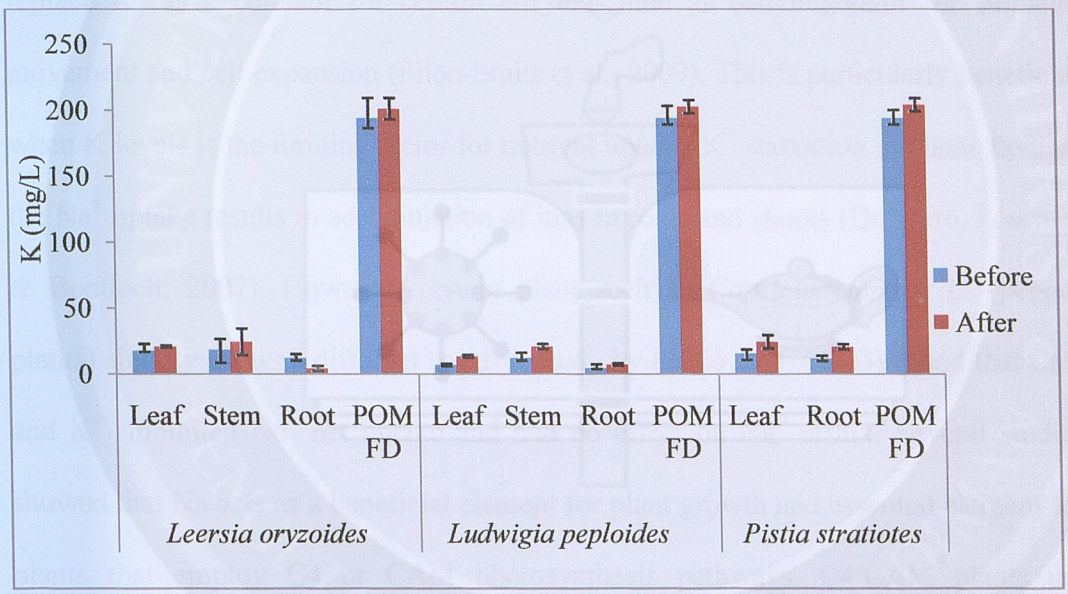


Figure 4.6. K concentration in different macrophytes organs before and after the phytoremediation treatment

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4.3.7 Sodium (Na)

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Increase in Na level was observed in all macrophyte leaves after the phytoremediation treatment. The highest Na level was found in *Pistia stratiotes*'s leaves followed by *Ludwigia peploides* and *Leersia oryzoides* with concentration of 2.2 mg/L, 1.15 mg/L and 0.6 mg/L, respectively. It was suggested that Na^+ can enters the plants mainly via non-selective cation transporters, as it is chemically similar to K^+ (Davenport & Tester, 2000). In this process Na^+ transporters are transporting Na out of the root into the vacuole or into the shoot phloem. Owing to the similarity of Na^+ to K^+, Na^+ can replace K^+ as a cofactor for certain enzymes, and as osmoregulator for stomatal movement and cell expansion (Pilon-Smits et al., 2009). This is particularly beneficial when K levels is the limiting factor for nutrient uptake. K^+ starvation in plants favours the Na^+ uptake results in accumulation of ions in roots and shoots (Quintero, Fournier & Benlloch, 2007). However, certain plant such as *Capsicum annum* L. (pepper plants) slightly shows a different trend. A study by Rubio et al. (2003) found that Ca^{2+} and K^+ inhibited Na^+ net uptake and had no effect on Na^+ efflux. Several studies showed that Na acts as a beneficial element for plant growth and essential element for plants that employ C4 or CAM photosynthesis pathways. C4/CAM plants use phosphoenolpyruvate (PEP) to fix atmospheric carbon for photosynthesis and Na is needed for the regeneration of PEP from pyruvate (Pilon-Smits et al., 2009).

Table 4.7 shows that *Pistia stratiotes* has the highest BCF value in leaves and roots with 11.95 L/kg and 11.41 L/kg, respectively. The lowest BCF was found in *Leersia oryzoides* roots (2.42 L/kg). The BCF value of Na was higher than K indicating that Na is more mobile to be accumulated in all studied macrophyte organs

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than K. However, the TF value was not much different between these three species with a TF range of 0.88 to 1.09. This result indicated that the translocation of Na was not affected by the type of macrophytes species.

The Na level in POM final discharge wastewater was also increased after the experiment with concentration difference of 3.08 mg/L, 2.7 mg/L and 2.88 mg/L in *Leersia oryzoides*, *Ludwigia peploides* and *Pistia stratoites*, respectively. Decaying process of macrophytes results in the release of Na into surrounding water (Azeez et al., 2009). Similar to the Mg, the highest Na was found in POM final discharge wastewater treated with *Leersia oryzoides*, followed by *Pistia stratiotes* and *Ludwigia peploides*. *Ludwigia peploides* suffered less wilted leaves than other two macrophytes species thus minimised the Na release into the wastewater.

Table 4.7
BCF and TF of Na in different macrophytes organs

Species	Organ	Initial (mg/L)	Final (mg/L)	Concentration difference (mg/L)	BCF (L/kg)	TF
Leersia oryzoides	Leaf	0.8	1.4	0.6	2.66	1.09
	Stem	1.5	5.525	4.03	10.49	
	Root	1.55	1.275	-0.28	2.42	
	POM FD	5.7	8.775	3.08		
Ludwigia peploides	Leaf	0.3	1.45	1.15	2.88	0.88
	Stem	2.15	2.475	0.33	4.91	
	Root	5.625	1.65	-3.98	3.27	
	POM FD	5.7	8.4	2.7		
Pistia stratoites	Leaf	3.95	6.15	2.2	11.95	1.04
	Root	10.2	5.875	-4.33	11.41	
	POM FD	5.7	8.575	2.88		

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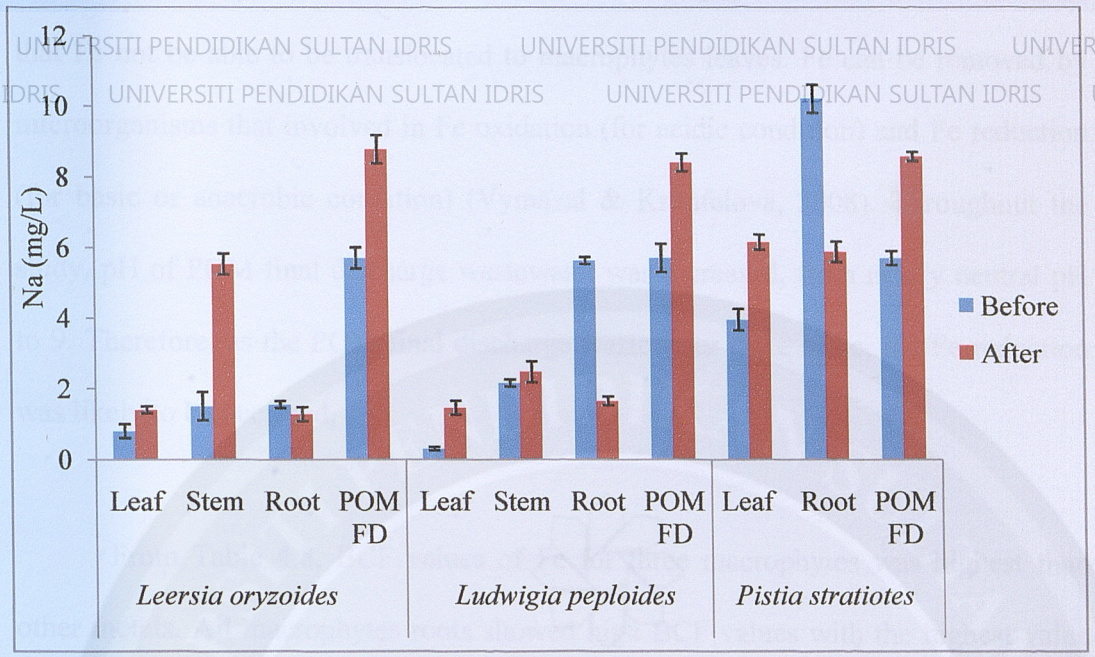


Figure 4.7. Na concentration in different macrophytes organs before and after the phytoremediation treatment

4.3.8 Iron (Fe)

From Table 4.8 and Figure 4.8, show an increase of Fe level in all *Leersia oryzoides* studied organs and the highest accumulation was observed in root. Results obtained from this study was in accordance to Tanner (2001), Pierce et al. (2010) and Xiao et al. (2011) which found Fe mainly accumulated in organ below ground. *Leersia oryzoides* were able to absorb and retain Fe in roots than in leaves and stems. The Fe concentration in *Leersia oryzoides* roots was increased from 30.33 mg/L to 55.88 mg/L. Fe concentration in *Ludwigia peploides* and *Pistia stratiotes* were decreased to 17.67 mg/L and 13.30 mg/L, respectively. This indicated that *Leersia oryzoides* roots able to absorb Fe from surrounding water regime than the other two types of macrophytes. The Fe content in POM final discharge wastewater reduced in all tanks

reflected that it might have been absorbed by the macrophytes roots. It was also found that Fe not be able to be translocated to macrophytes leaves. Fe can be removed by microorganisms that involved in Fe oxidation (for acidic condition) and Fe reduction (for basic or anaerobic condition) (Vymazal & Kropfelova, 2008). Throughout the study, pH of POM final discharge wastewater was increased, from nearly neutral pH to 9. Therefore, as the POM final discharge wastewater quite basic, the Fe reduction was likely to be occurred.

From Table 4.8, BCF values of Fe for three macrophytes was highest than other metals. All macrophytes roots showed high BCF values with the highest value found in *Ludwigia peploides*, about 1.47×10^4 L/kg. The BCF values of three macrophytes roots obtained in this study was in accordance to previous researches on heavy metals accumulation (Soda et al., 2012; Sundberg-Jones & Hassan, 2007). However, the TF values for Fe was low than other nutrients such as Mg, Ca, Na and K because most Fe absorbed were retained in the roots and only small amount were transported to the macrophytes leaves (Xiao et al., 2011). Due to immobility of heavy metals, plant with high BCF and low TF values for root is useful for phyostabilization process (Zou et al., 2012).

Fe is important metal for plant life. As a redox-active metal, Fe involved in photosynthesis, mitochondrial respiration, nitrogen assimilation, hormone biosynthesis (ethylene, gibberellic acid, jasmonic acid), production and scavenging of reactive oxygen species, osmoprotection, and pathogen defence (Hänsch & Mendel, 2009). However, only certain types of plants can accumulate Fe. For example, *Leersia oryzoides* leaves accumulate about 0.14 mg/L of Fe. Fe^{2+} is a positively

charged ion that plays an important role in osmoregulation or creating ionic balance system between inside plant tissue and surrounding water environment. In this study, only *Leersia oryzoides* roots were able absorbing great amount of Fe from POM final discharge wastewater.

Table 4.8
BCF and TF of Fe in different macrophytes organs

Species	Organ	Before	After	Concentration difference (mg/L)	BCF (L/kg)	TF
Leersia oryzoides	Leaf	0.486	0.630	0.14	0.78×10^{2}	1.13×10^{-2}
	Stem	0.666	0.942	0.28	1.17×10^{2}	
	Root	25.55	55.88	30.33	6.85×10^{3}	
	POMFD	0.493	0.134	-0.36		
Ludwigia peploides	Leaf	1.714	0.635	-1.08	5.29×10^{2}	3.59×10^{-2}
	Stem	1.668	0.448	-1.22	3.73×10^{2}	
	Root	55.58	17.67	-37.91	1.47×10^{4}	
	POMFD	0.493	0	-0.49		
Pistia stratiotes	Leaf	1.761	0.990	-0.77	0.98×10^{2}	7.44×10^{-2}
	Root	78.51	13.30	-65.21	1.32×10^{3}	
	POMFD	0.493	0.168	-0.33		

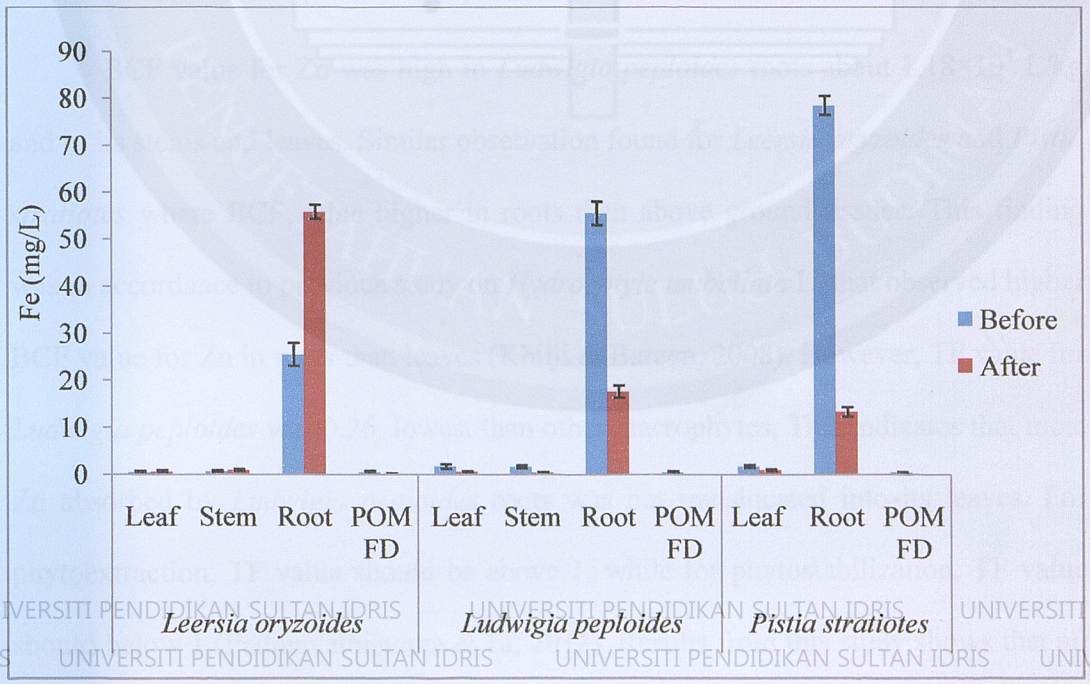


Figure 4.8. Fe concentration in different macrophytes organs before and after the phytoremediation treatment

4.3.9 Zinc (Zn)

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Table 4.9 shows Zn concentration in macrophyte organs was low than other metals. Zn is microelements required by plant in small amount, therefore shows not much difference between deficiency and toxicity level (Hodson & Bryant, 2012). After the phytoremediation experiment, Zn concentrations were increased in *Ludwigia peploides* (leaves and roots) and *Pistia stratiotes* (leaves). The BCF value of Zn was highest in *Ludwigia peploides* root with concentration difference of 2.33 mg/L. In general, Zn concentration in all macrophytes roots before and after phytoremediation were higher than above ground organs. This results was in accordance to previous research where the accumulation of Zn in roots might be due to the immobility of Zn as heavy metals (Lesage et al., 2007; Vymazal & Kropfelova, 2008). Although Zn is required in small amount by plants, it is important as enzymes activator (Hopkins & Hunner, 2004).

BCF value for Zn was high in *Ludwigia peploides* roots about 1.18×10^4 L/kg and in its stems and leaves. Similar observation found for *Leersia oryzoides* and *Pistia stratiotes* where BCF value higher in roots than above ground tissues. This finding was in accordance to previous study on *Hydrocotyle umbellata* L. that observed higher BCF value for Zn in roots than leaves (Khilji & Bareen, 2008). However, TF value for *Ludwigia peploides* was 0.26, lowest than other macrophytes. This indicates that most Zn absorbed by *Ludwigia peploides* roots was not translocated into its leaves. For phytoextraction, TF value should be above 1, while for phytostabilization, TF value should below 1 (Padmavathiamma & Li, 2012). Results from this study shows that all

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macrophytes have TF value below 1 which might explain the phytostabilization mechanism during the phytoremediation study of POM final discharge wastewater.

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Table 4.9
BCF and TF of Zn in different macrophytes organs

Species	Organ	Before (mg/L)	After (mg/L)	Concentration difference (mg/L)	BCF (L/kg)	TF
Leersia oryzoides	Leaf	0.66	0.59	-0.06	4.32×10^2	0.54
	Stem	0.90	0.76	-0.15	5.47×10^2	
	Root	3.09	1.11	-1.98	8.01×10^2	
	POMFD	0.01	0.023	-0.013		
Ludwigia peploides	Leaf	1.25	1.66	0.41	3.07×10^3	0.26
	Stem	1.7	1.18	-0.52	2.18×10^3	
	Root	4.07	6.4	2.33	1.18×10^4	
	POMFD	0.01	0.009	0.008		
Pistia stratoites	Leaf	0.51	0.64	0.131	3.45×10^2	0.46
	Root	14.38	1.39	-12.99	7.47×10^2	
	POMFD	0.001	0.03	0.03		

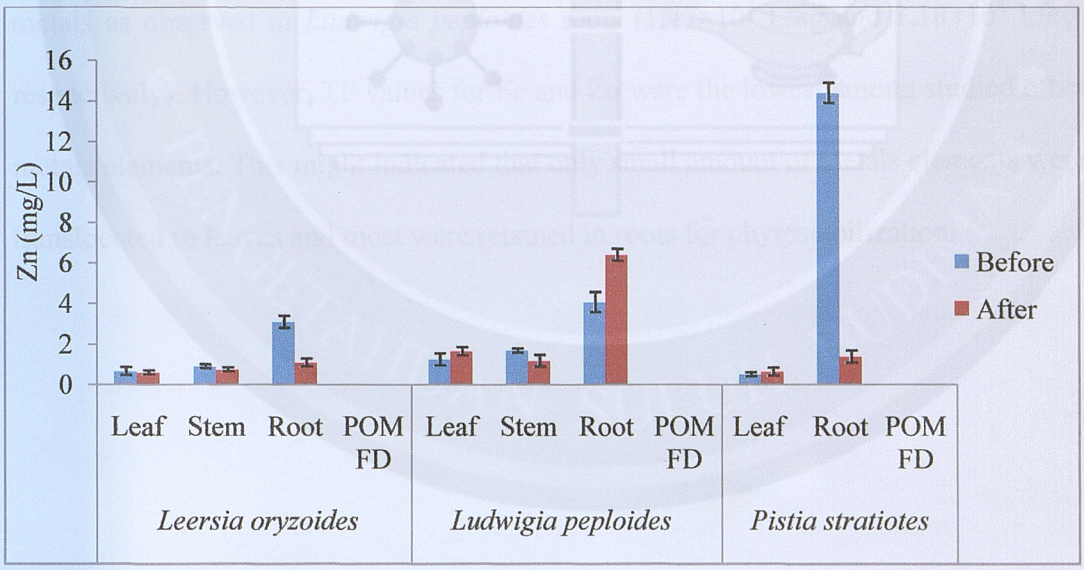


Figure 4.9. Zn concentration in different macrophytes organs before and after the phytoremediation treatment

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4.4 Conclusions

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N percentages were increased in all studied macrophytes leaves. This study also found that C was high in all organs before phytoremediation treatment and remain high after the phytoremediation with subtle decreased. It was also observed that H percentages were reduced, indicated the increased of pH in macrophytes. It was found that BCF values of Mg were increased in all studied macrophytes roots with 14.14 L/kg in *Ludwigia peploides* roots. This study also showed that Mg and Ca were absorbed mainly in macrophytes roots through phytoaccumulation. Phytoaccumulation also observed in *Leersia oryzoides* leaves as TF value of K was highest among studied metals with value of 4.95. For Na, the highest BCF values were varied among macrophytes organ with the highest value measured in *Pistia stratiotes* leaves (11.95 L/kg). BCF values for heavy metals (Fe and Zn) were the highest among studied metals as observed in *Ludwigia peploides* roots (1.47×10^4 L/kg and 1.18×10^4 L/kg, respectively). However, TF values for Fe and Zn were the lowest among studied other metals elements. This might indicated that only small amount of metals elements were translocated to leaves and most were retained in roots for phytostabilization.

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CHAPTER 5

MICROBIAL POPULATION IDENTIFICATION IN PHYTOREMEDIATION OF POM FINAL DISCHARGE WASTEWATER

5.1 Introduction

Among the important aspect during phytoremediation is the role of microorganisms in this process and its interaction with macrophytes. This interaction has been well documented in previous studies (Osem et al., 2007). For example, interaction between macrophytes and microbes during phytoremediation is essential for nitrogen removal. Macrophytes provide oxygen through its roots to microbial colony at rhizosphere which involve in nitrification process (Greenway, 2007). Previous research shows that some bacteria such as from genus *Pseudomonas* and *Bacillus* are playing pivotal role for denitrification in activated sludge (Srinandan, Shah, Patel & Nerurkar, 2011). During phytoremediation, macrophytes roots supply substrate and oxygen for microbial biofilm growth (Kurniadie, 2011). Sequence of the 16S rRNA gene has

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been widely used as a molecular clock to estimate relationships among bacteria (phylogeny). Recently it has also become important as a molecular tool to identify unknown bacteria to the genus or species level (Sacchi et al. 2002). In this chapter, the determination of heterotrophic microbial populations before and after phytoremediation and bacterial identification is discussed in relation to the effectiveness of phytoremediation of POM final discharge wastewater.

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5.2 Methodology

5.2.1 Scanning Electron Microscopy (SEM)

The morphology of bacteria at three macrophytes roots was observed with Hitachi UHR FESEM field emission scanning electron microscope. The samples were pre-fixed with 4% of glutaraldehyde solution and subsequently washed, post-fixed with 1% of osmium tetraoxide and dehydrated in a graded series of acetone solution. The samples were dried by the critical point method and sputter coated with gold (Yusof et al., 2010).

5.2.2 Microbial Enumeration

Rhizosphere heterotrophic bacterial numbers were measured after phytoremediation study in all tank including the controls. Microbial parameters were measured according to heterotrophic plate count (HPC) from APHA Standard Methods (2005).

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Enumeration of bacteria in the rhizosphere was performed by placing 1 g of whole young roots in a 100 ml Erlenmyer flask containing 30 ml of sterile saline (0.85% NaCl). After vigorous shaking to homogenize the solution (200 strokes per min) at 30°C for 30 min, the solution was serially diluted, plated on solid nutrient agar (Difco), incubated at 25 ± 2°C for 72 h, and counted. Dilution plate of every plant species that showed 30-300 bacterial colonies were counted for their total population.

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5.2.3 Isolation of Pure Colony

Each colony from HPC that showed different morphology was further inoculated onto nutrient agar and incubated for 24 hours at 25°C which is optimum temperature for microorganism from plant roots (Bashan, 1986). Pure isolates were stored on nutrient agar at 4°C until further characterization.

5.2.4 Molecular Identification of Isolates

Pure isolates were stored on trypticase soy (TS) agar at 4°C for further characterization. All isolates were subjected to PCR analysis, comprising DNA extraction, amplification of 16S rDNA, purification of PCR products and DNA sequencing.

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5.2.5 DNA Extraction

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DNA was extracted using Ultraclean™ soil DNA kit (MO Bio Laboratories, Inc., Carlsbad, CA, US) according to manufacturer's instructions. About 0.25-1.00 g of microbial isolates were placed into 2 mL bead solution tube and then gently vortexed to mix. About 60 µl of Solution 1 contains SDS and other disruption agents required for complete cell lysis was added and vortexed horizontally on flat-bed vortex pad with tape for 10 minutes at maximum speed. Bead solution tubes were then centrifuged at 10,000 × *g* for 30 seconds. 400-450 µL of supertanants were transferred into the 2 mL collection tubes and 250 µl of Solution 2 contains Inhibitor Removal Technology (IRT) was added, a reagent to precipitate non-DNA organic and inorganic material including humic substances, cell debris, and proteins. The sample was vortexed for 5 seconds, incubated at 4°C for 5 minutes and centrifuged for 1 min at 10,000 × *g*.

A 450 µL of sample was transferred to a clean 2 ml collection tube by discarding the pellet. A 900 µl of solution 3 (patented Inhibitor Removal Technology® (IRT), a second reagent to precipitate additional non-DNA organic and inorganic material including humic acid, cell debris, and proteins), was then added and vortexed for 5 seconds. It is important to remove contaminating organic and inorganic matter that may reduce DNA purity and inhibit downstream DNA applications. Approximately 700 µl of supertanant was loaded onto spin a spin filter and centrifuged at 10,000 ×*g*. About 200 µl of solution 4 that contained high concentration of salt solution to bind DNA was added for washing. The flow through was discarded and centrifuged again for 1 min. Spin filters were carefully placed in a

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5.2.5 DNA Extraction

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new 2 ml collection tube by avoiding splashing any solution 4 onto it. A 50 µl of solution 5 (ethanol based wash solution used to further clean of DNA that is bound to silica filter membrane in the spin filter) was added to the centre of the white filter membrane and centrifuged at 10,000 × *g* for 30 seconds. Spin filter discarded and DNA extract was ready. The DNA was viewed by electrophoresis.

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5.2.6 Polymerase Chain Reaction (PCR) Amplification

Partial 16 S rDNA fragments of the entire bacterial community were amplified from the extracted genomic DNA by PCR using the general bacteria primers, 27F (AGA GTT TGA TCC TGG CTC AG) and 1492R (ACG GTT ACC TTG TTA CGA CTT). The PCR ingredient (total volume, 25 µL, Fermentas ExTaq DNA polymerase) consisted of 2.5 µL 10× PCR buffer, 0.2 µL ExTaq DNA polymerase, 0.5 µL 5 mM dNTP, 2.5 µL $MgCl_2$ (25 mM), 0.5 µL of each primer, and 1.0 µL of the above template and an adequate amount of sterile distilled water was added to reach the desired volume. The PCR program consisted of an initial 4 min denaturation step at 95°C followed by 35 cycles of repeated denaturation at 95°C for 45 sec, annealing at 56°C for 45 sec, and extension at 70°C for 1 min followed by a final extension at 72°C for 3 min. The samples were amplified using a PCR Thermal Cycler Dice, Takara, Japan. The amplified and purified products were first separated by horizontal electrophoresis through 0.8% (w/v) agarose gel solution. The gel was stained with ethidium bromide and visualized under UV light box to visualize the DNA separated pattern under UV light.

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5.2.7 PCR Purification and DNA Sequencing

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PCR purification was performed using Agencourt Ampure Xp, Beckman Coulter Genomics. A 40 µL of PCR product was added with agencourt onto SPRI Plate 96R for 10 minutes. The supernatant was then washed with 70% absolute ethanol before dried at room temperature. The purified PCR product was viewed by electrophoresis. Samples were then sent to the First BASE Laboratories Sdn. Bhd. (Selangor, Malaysia) for DNA sequencing. Primer 750R for soil and environmental sample was used in sequencing. The sequences were compared to the NCBI (National Centre for Biotechnology Information) Genbank using the BLAST search tool which is available at: http://www.ncbi.nlm.nih.gov/ and Ribosomal Database Project II (RDP) through the website (http://rdp.cme.msu.edu/seqmatch/seqmatch_intro.jsp) to identify the nearest bacterial species relatives of the partially sequenced 16S rRNA genes. The BioEdit version 7.1.3.0 program was used to edit, assemble and align all of 16S rRNA gene sequences.

5.3 Results and Discussions

5.3.1 Bacterial Morphology

Figures 5.1-5.3 show the morphology of bacteria colonizing the roots of the studied macrophytes after phytoremediation. All macrophyte roots were dominated by straight rod shaped bacterial cells with size of about 2.33 µm × 0.7 µm. These bacteria were confirmed to be from genus *Bacillus* according to its morphological feature. *Bacillus*

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sp. are well known bacteria involved in treatment tank of domestic wastewater (Ajithkumar et al., 2001). Bacteria in the form of biofilm could involve in various pollutants removal such as BOD_5 and NH_3-N (Lim 2002; Greenway, 2007). This finding also in accordance to Osem et al. (2007) which found that macrophyte roots (*T. domingensis*) were colonized by large population of bacteria after treatment of domestic kitchen wastewater using hydroponic system.



Figure 5.1. Bacterial population observed in *Leersia oryzoides* root (4000 X magnification)

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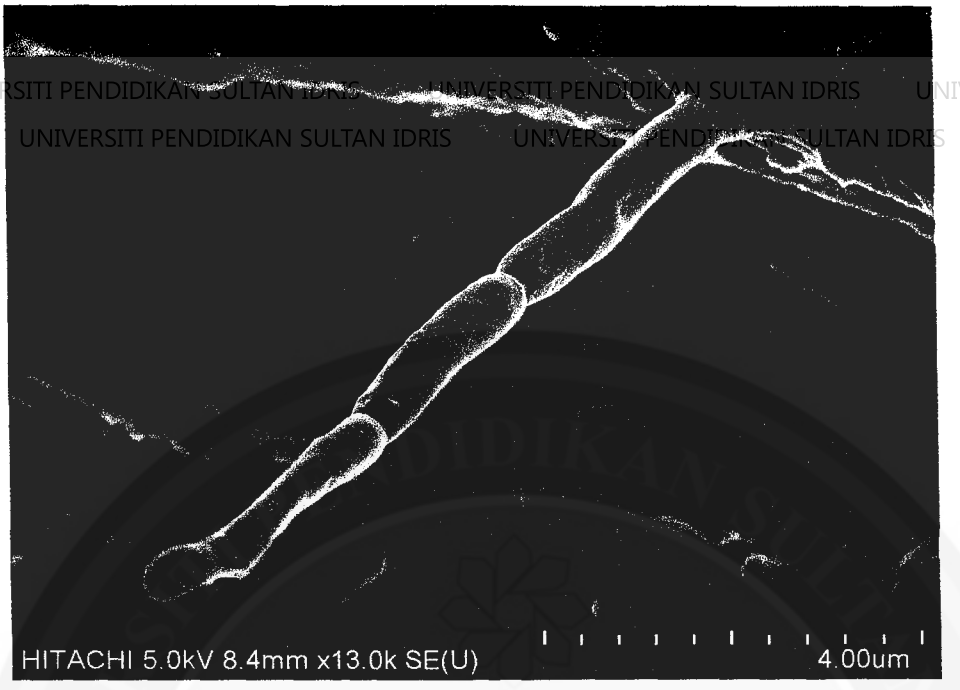


Figure 5.2. Bacterial population observed in *Ludwigia peploides* root (13000 X magnification)

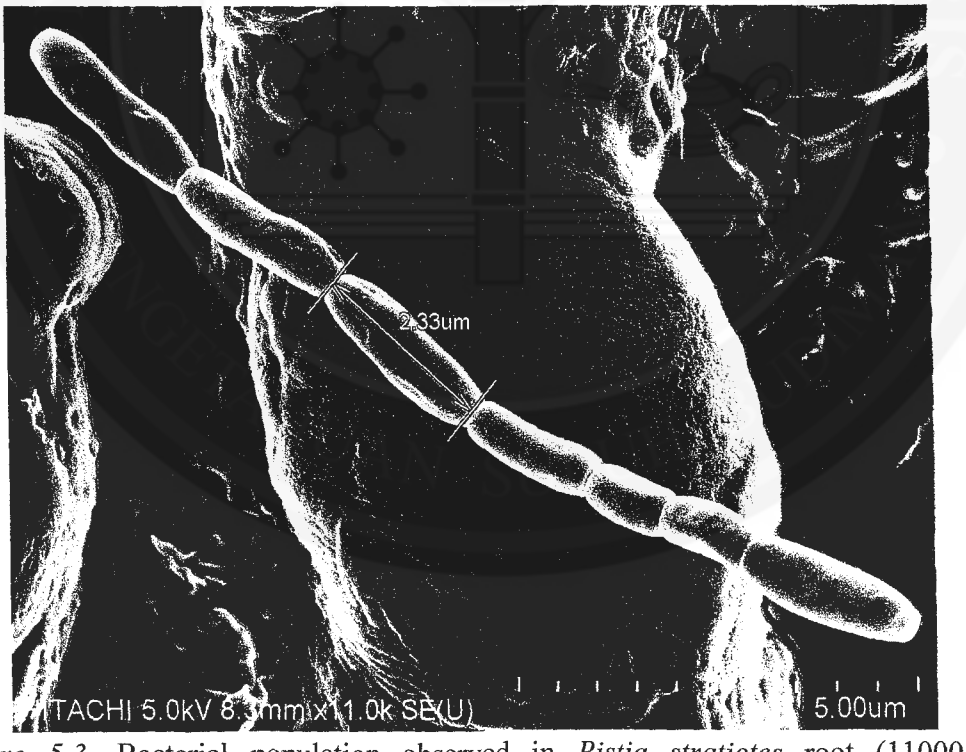


Figure 5.3. Bacterial population observed in *Pistia stratiotes* root (11000 X magnification)

5.3.2 Microbial Populations

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Table 5.1 shows the increment of microbial population in the roots of the studied macrophytes during phytoremediation. The initial heterotrophic bacterial number in rhizosphere of *Leersia oryzoides* rhizosphere was 5.71×10^4 CFU/ml, 1.24×10^4 CFU/ml for *Ludwigia peploides* and 5.71×10^3 CFU/ml for *Pistia stratiotes*. On day 15th the bacteria population in roots increased with the highest population was in *Leersia oryzoides* followed by *Ludwigia peploides* and *Pistia stratiotes*, 1.2×10^5 CFU/ml, 2.5×10^4 CFU/ml and 1.5×10^4 CFU/ml, respectively. In contrast, the number of heterotrophic bacteria was reduced in POM final discharge wastewater after the experiment. The bacterial population in POM final discharge wastewater was reduced from 1.16×10^4 CFU/ml to 4.5×10^2 CFU/ml, 4.43×10^2 CFU/ml and 2.26×10^2 CFU/ml for *Leersia oryzoides*, *Ludwigia peploides*, and *Pistia stratiotes*, respectively after the treatment period.

The increase in microbial population in the roots of the studied macrophytes and the reduction of bacterial population in POM wastewater indicates the favourable condition for microbial growth. POM final discharge has provided ample nutrition sources for the growth of microbes. This result supports previous findings by Greenway (2007) and Iasur-Kruh (2010) that macrophyte roots play an important role as substrate for colonization and growth of microorganisms in which they are responsible for various pollutants degradation. The macrophyte roots also release oxygen and create oxidized conditions at rhizosphere that stimulate both aerobic decomposition of organic matter and growth of nitrifying bacteria (Lim, 2002; Herrmann, Saunders & Schramm, 2009). Furthermore, Vacca et al. (2005) found that

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macrophytes (*Phragmites australis*) were able to stimulate development of specific bacterial communities in which PCR-single strand conformation polymorphism analysis result shows some bands appeared in the rhizosphere that were not present in the bulk soil.

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5.3.3 DNA Extraction and Polymerase Chain Reaction (PCR)

DNA extractions were carried out to further analysis the bacterial colonies. Figures 5.4 and 5.5 show band of DNA extracts after extraction and after PCR amplification. Six DNA extracts from macrophytes roots were obtained using PowerSoil DNA Kit. Agarose gel electrophoresis indicated that DNA samples were all in the range of 10-20 kbp. PCR amplification was performed by universal primer 16S rRNA genes for bacteria (12F and 1492R). Figure 5.5 shows that 16S rRNA genes were successfully amplified and have the size of nearly 1.5 kbp. Strong bands were detected from band 5 (*Leersia oryzoides*) and band 6 (*Leersia oryzoides*) after amplification, indicating this hypervariable region were well amplified for bacterial identification (Pereira et al., 2010).

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Table 5.1

Heterotrophic bacterial count for the studied macrophytes root and POM final discharge wastewater before and after the phytoremediation study.

	Before phytoremediation		After phytoremediation		
Sample	Mean of heterotrophic bacteria (CFU/ml)	Population range (CFU/ml)		Mean microbial population (CFU/ml)	Population range (CFU/ml)
Leersia oryzoides	$5.7 \times 10^4 \pm 2.4 \times 10^4$	3.0×10^4-7.3×10^4		$1.2 \times 10^5 \pm 2.4 \times 10^3$	1.1×10^5-1.2×10^5
Ludwigia peploides	$1.2 \times 10^4 \pm 3.5 \times 10^3$	8.57×10^3-1.5×10^4		$2.6 \times 10^4 \pm 7.1 \times 10^3$	2.0×10^4-3.3×10^4
Pistia stratoites	$5.71 \times 10^3 \pm 2.5 \times 10^3$	1.3×10^4-1.9×10^4		$1.5 \times 10^4 \pm 1.2 \times 10^4$	4.71×10^3-2.9×10^4
POM final discharge wastewater	$1.6 \times 10^4 \pm 4.2 \times 10^3$	1.3×10^4-1.9×10^4	*a*	$4.5 \times 10^2 \pm 6.4 \times 10^2$	0-9×10^2
			b	$4.43 \times 10^2 \pm 2.0 \times 10^2$	2.14×10^2-5.57×10^2
			c	$2.26 \times 10^2 \pm 3.3 \times 10^2$	0.26×10^2-6.08×10^2

Note. *a* POM final discharge wastewater treated with *Leersia oryzoides*
b POM final discharge wastewater treated with *Ludwigia peploides*
c POM final discharge wastewater treated with *Pistia stratiotes*

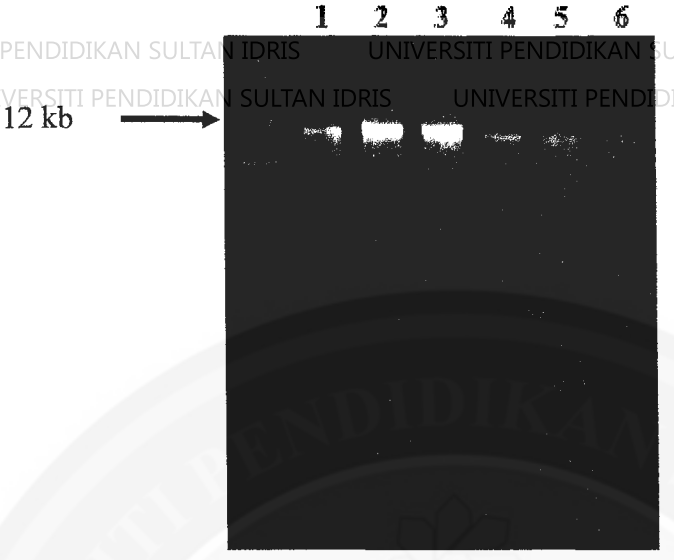


Figure 5.4. Gel gradient electrophoresis of DNA extracts from six microorganisms isolated from macrophytes roots; (1-2) bacteria from *Pistia stratiotes* root, (3-4) bacteria from *Ludwigia peploides* root, (5-6) bacteria from *Leersia oryzoides* roots

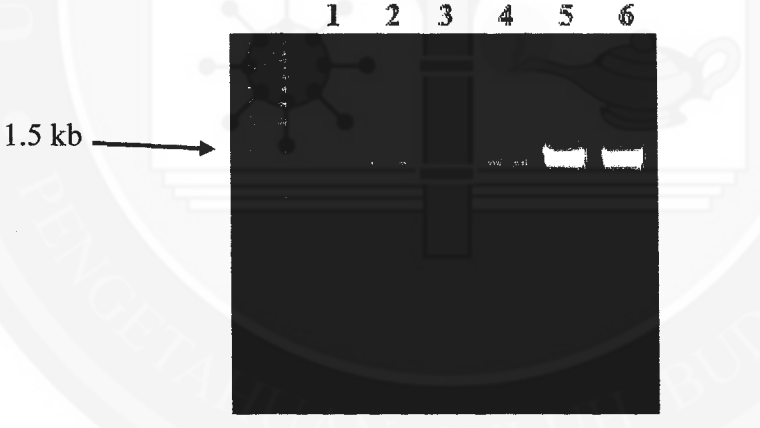


Figure 5.5. Gel gradient electrophoresis of PCR product after PCR amplification. Strong bands observed from band 5 and 6

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5.3.4 Molecular Identification of Bacteria Isolated from Macrophytes Root

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5.3.4.1 Selected Bacteria Sequence from *Pistia stratiotes* roots

Table 5.2 describes the similarity identification of all samples from pure culture of bacteria that colonize the roots of macrophytes based on BLAST database, whilst Table 5.3 describes the bacterial identification using RDP II database. The results show the bacteria from sample 1 were closely related to *Bacillus megaterium* (NCBI and RDP II) with similarity of 100% and 95%, respectively.

Table 5.2
Bacterial similarity identification from BLAST database for selected bacterial DNA samples

Sample	Source of bacteria	Accession	Description	Maximum identification
1	*Pistia stratiotes* root	HE981752.1	*Bacillus megaterium* partial 16S rRNA gene, isolate A8-1	100%
2	*Pistia stratiotes* root	AB680017.1	*Brevundimonas diminuta* gene for 16S rRNA, partial sequence, strain: NBRC 3140	99%
3	*Ludwigia peploides* root	EU931560.1	*Pseudomonas aeruginosa* strain ZFJ-5 16S ribosomal RNA gene, partia	100%
4	*Ludwigia peploides* root	FJ544319.1	*Pseudomonas mendocina* strain 81006 16S ribosomal RNA gene, partial sequence	100%
5	*Leersia oryzoides* root	AY512827.1	*Acidovorax avenae* 16S ribosomal RNA gene, partial sequence	89%
6	*Leersia oryzoides* root	JX077093.1	*Bacillus cereus* strain - Y111 16S ribosomal RNA gene, partial sequence	99%

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Table 5.3

Bacteria similarity identification from RDP II database for selected bacterial DNA samples

Sample label	Source of bacteria	Accession	Bacteria similarity	S_ab score
1	*Pistia stratiotes* root	S000330853	*Bacillus megaterium*; MO31; AY553118	1.000
2	*Pistia stratiotes* root	S000413819	*Brevundimonas diminuta*; IFO 3140; D49422	0.963
3	*Ludwigia peploides* root	S001169573	*Pseudomonas aeruginosa*; ZFJ-5; EU931560	0.991
4	*Ludwigia peploides* root	S000702052	*Pseudomonas mendocina*; DQ641475	0.967
5	*Leersia oryzoides* root	S000001712	*Acidovorax sp.* KSP2; AB076843	0.499
6	*Leersia oryzoides* root	S000006757	*Bacillus cereus*; AH 527; AF290555	0.993

Bacillus is a well known gram positive bacteria used in industries for production of various extracellular enzymes such as amylases (Yang et al., 2006). Previous research showed that bacteria from *Bacillus* genus including *B. cereus* and *B. thurigiensis* have high similarity percentage, more than 99% of 16S rRNA partial sequence which indicate their close phylogenetic relationship (Helgason et al., 2000). *Bacillus* species were abundant in marsh of *Phragmites australis* (Borsodi, 2007). Among eubacteria found in soil, *Bacillus megaterium* was reported abundance (Dong & Reddy, 2010). *Bacillus megaterium* showed play a vital role in bioremediation of olive oil wastewater in bioreactor (Eusebio et al., 2007). In his study, COD removal and phenolic compounds were 87% and 80%, respectively indicating this bacterium

were well adapted by stress conditions imposed by special type of jet-loop bioreactor.

Bacillus and *Pseudomonas* are heterotrophic denitrifying bacteria, as its use nitrate as an alternative electron acceptor in partially anoxic conditions that favouring denitrification (Metcalf & Eddy, 2003).

Results from BLAST and RDP II show that Sample 2 was closely related to *Brevundimonas diminuta*. *Brevundimonas diminuta* is a gram negative pathogen bacterium used in the pharmaceutical and drinking water treatment industries (Donofrio, Bestervelt, Saha & Bagley, 2010). *Brevundimonas diminuta* also reported can accumulate nitrite during denitrification (Srinandan et al., 2011). *Brevundimonas spp* was found to adsorb more light metals than heavy metals during bioremediation (Fosso-Kankeu, Mulaba-Bafubiandi, Mamba, & Barnard, 2011). *Brevundimonas sp.* ZF12 strain isolated from hot spring water with high ^{226}Ra content able to remove 50% of cadmium at a concentration level of 250 ppm (Masoudzadeh et al., 2011).

5.3.4.2 Selected Bacteria Sequence from *Ludwigia peploides* roots

The bacteria isolated from *Ludwigia peploides* root (Sample 3) was closely related to *Pseudomonas aeruginosa* with 100% similarity using BLAST. It is a gram negative bacterium that produces several proteolytic enzymes (Jellouli et al., 2008). *Pseudomonas aeruginosa* is also biosurfactant-producing bacteria as found in oil-containing wastewater (Yin et al., 2009). *Pseudomonas fluorescens* showed efficient denitrification in activated sludge (Srinandan et al., 2011) and able to degrade benzene, toluene, ethylbenzene and xylenes in fibrous bed bioreactor (Shim, Shin &

Yang, 2002; Srinandan et al., 2011). The genus of *Pseudomonas*, *Aeromonas* and *Vibrio* are important denitrifiers in aquatic environment while *Bacillus*, *Micrococus* and *Peudomonas* are important denitrifiers in soil (Vymazal, 2007).

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Bacterial identification from Sample 4 (*Ludwigia peploides*) shows that it was related to *Pseudomonas mendocina* with 100% similarity (BLAST) and 0.967 (RDP II). Previous study showed that K uptake by *L. Sativa* increased significantly if *Pseudomonas mendocina* was inoculated at root (Alguacil, Kohler, Caravaca & Roldan, 2009). A study conducted by Srinandan et al. (2011) found that *Pseudomonas mendocina* and *Pseudomonas pseudoalcaligenes* were among the denitrifying bacteria in which *Pseudomonas mendocina* could reduce up to 96.46% of nitrate.

5.3.4.3 Selected Bacteria Sequence from *Leersia oryzoides* roots

Sample 5 obtained from *Leersia oryzoides* was found closely related to *Acidovorax sp.* with similarity of 89% (BLAST) and 0.499 (RDP II). The genera of *Acidovorax* are members of the β-proteobacteria which primarily cause leaf spot and streak disease in a variety of host plants. The most significant pathogens within the genus *Acidovorax* belong to the species *A. Avenae* (Fegan, 2006). *Acidovorax* is also abundantly found in wastewater treatment system. For example 10.4% of the bacteria isolates from petrochemical activated sludge is aerobic *Acidovorax* genus (Shokrollahzadeh, Azizmohseni, Golmohammad, Shokouhi & Khademhaghighat, 2008). *Acidovorax avenae* subsp. *avenae* LMG 17238 could be successfully used for denitrification by

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using various carbon sources (Nalcaci, Boke & Ovez, 2011). *Acidovorax avenae* also could degrade chlorobenzenes (Monferran, Echenique & Wunderlin, 2005).

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Bacteria isolated from *Pistia stratiotes* root were identical to *Bacillus cereus* (Tables 5.2 and 5.3). Previous research reported that fermentation of raw POME with *Bacillus thurigiensis* under several conditions could produce bioinsecticide (Wu et al, 2010). *Bacillus cereus* is an ubiquitous facultative anaerobic bacterium and used oxygen as the terminal electron receptor during its respiration (Wang, Chong & Jiang, 2012). Pure cultures of *Bacillus cereus* MTCC 9817 strain AKG1 and *B. cereus* MTCC 9818 strain AKG2 were reported capable to degrade phenol at concentration up to as 2000 mg/L (Banerjee & Goshal, 2010). *Bacillus cereus* and *Bacillus megaterium* are alkali tolerant bacteria that can be found in marsh of *Phragmites australis* (Borsodi et al, 2007). These bacteria are able to survive at rhizosphere as the high pH in phytoremediation tank can favour their growth.

5.4 Conclusions

During the phytoremediation, macrophytes play important role in supplying substrate and oxygen for bacterial growth and metabolism resulted in increase of bacterial population. Bacterial DNA sequences from rhizosphere showed that the bacterial involved in phytoremediation of POM final discharge wastewater were closely related to *Bacillus megaterium* and *Brevundimonas diminuta* for *Pistia stratiotes*, *Pseudomonas aeruginosa* and *Pseudomonas mendocina* for *Ludwigia peploides*, *Acidovorax avenae* and *Bacillus cereus* for *Leersia oryzoides*

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CHAPTER 6

GENERAL DISCUSSION

6.1 Phytoremediation Performance

The phytoremediation performance of POM final discharge wastewater was evaluated in this study. It was found that BOD_5 was removed greatly by three species of macrophytes from POM final discharge wastewater in which BOD_5 after the treatment were complied with discharge limit of Environmental Quality Act (1978). This removal was assisted by rhizosphere microorganism in which macrophytes contribute indirectly by supplying substrate and oxygen through their roots for microorganism growth. This was supported by microbial enumeration results which were increased in vegetated tank. However, only 19.9 % to 30.6% of COD was removed, and this removal was lower than BOD_5. It was comprehensible since COD was a measure of total oxygen required for oxidation of total organic strength while BOD_5 measures total oxygen required by bacteria for stabilizing biodegradable organic matter. The

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recalcitrant property of POM final discharge wastewater should be considered similar to pulp mill wastewater which contain about 12% of fibre from the total nutrient in POME (Idris & Al-Mamun, 2003). During the extraction of fresh fruit bunch, huge amount of fibre were produced and discharged in POME and subsequently to POM final discharge wastewater which cannot be decomposed by microorganism and thus contributed to recalcitrant property of COD. However, decomposable organic matter that also constituted to COD were also used as energy source in denitrification process in which several denitrifiers were identified.

Result from this study showed that NH_3-N was nearly completely removed. The uptake and subsequent assimilation of ammonium by macrophytes were contributed in this removal as results showed that nitrogen percentages were increase in all macrophytes leaves after phytoremediation treatment. The ammonium ions and nitrate were used as nutrients in macrophytes. Nitrate is a product of nitrification process in which NH_3-N oxidised by *Nitrosomonas* to produce nitrite that further converted to nitrate by *Nitrobacter* bacteria. The nitrate was reduced in anaerobic zone during phytoremediation of POM final discharge wastewater by denitrifiers to produce N_2 and N_2O which released back to atmosphere (Lim 2002; Idris et al., 2010). The present of denitrifiers in this phytoremediation treatment were confirmed by molecular identification in Chapter 5. This study confirms the role of both bacteria and macrophytes in removal of NH_3-N.

Wilting is a sign of phytoxicity in which plant suffered this condition will decrease in their chlorophylls (Tewari et al., 2008). In this phytoremediation treatment, high recalcitrant COD was believed the main cause of wilting and

subsequent decay of macrophytes. Increase of wilting percentages of macrophytes also cause the increment of TSS throughout this treatment that released back nutrient to POM final discharge wastewater. These nutrients also contain alkaline earth metals such as Ca, Mg, K, and Na which contributed to increase of pH in this study. Therefore, the nutrient uptake was also studied to determine the characterization and its interrelationship with phytoremediation efficiency.

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6.2 Characterization of Nutrient Uptake

Nutrient uptake in phytoremediation treatment is important for macrophytes growth and to reduce excess of nutrient in wastewater. In this phytoremediation treatment, N percentages in leaves were increased after phytoremediation treatment which indicates that NH_3-N taken up by macrophytes from POM final discharge wastewater were assimilated by macrophytes in their leaves for various function. Among non-mineral element studied in this treatment, C and H show the highest percentages in macrophytes organs as it become the major element in macrophytes structures. However C and H percentages were slightly reduced in all macrophytes organs after the treatment. Wilting and subsequent decaying process reduced both C and H in macrophytes. Atmospheric C in the form of CO_2 becomes source of C for macrophytes growth. In this study, macrophytes weight decreased at day 14 (Chapter 3, page 76) which shows that growth of macrophytes were reduced and in accordance with reduction of C in macrophytes organs. However for N, it was increased because of senescence and wilting symptom led to N transport to leaves for storage. As macophytes died and decomposed, C and N were released into POM final discharge

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wastewater in the form of organic matter and nitrogenous compounds. The nitrogenous compounds could be assimilated by macrophytes while organic matter used as energy source for microorganism population growth. This describes the increment of N in macrophytes leaves and decrease of both C and H in its organ.

Positively charged ions such as Ca^{2+}, Mg^{2+}, Na^{+}, Fe^{2+} and Zn^{+} may compete with H^{+} at binding site of macrophytes roots. This was in accordance with pH value of POM final discharge wastewater which was slightly increased (Chapter 3, page 74). This indicates that H^{+} was loss from POM final discharge wastewater as it were taken up by macrophytes along with other positively charged ions.

The BCF values for Ca, Mg, Na, Fe and Zn were low than previous studies by Soda et al. (2012). The low of BCF value might be affected by other pollutants in wastewater as suggested by Galal and Shehata (2014). In this study, metals such as Ca, Mg, Na, Fe and Zn were phytostabilised in macrophytes roots as the TF value obtained below 1. The metals elements absorbed were retained in macrophytes roots and only small amount were transported to the leaves. Only K ions were phytoaccumulated in macrophytes leaves with BCF value above 1. Fe and Zn show the lowest TF values among metal elements studied, indicated that these two elements were highly phytostabilised in macrophytes roots than Ca, Mg and Na. Phytostabilisation of Fe and Zn by macrophytes roots shows the tolerance of macrophyte to these elements in which Deng, Ye and Wong (2009) suggested caused by prevention of radial oxygen loss produced in anoxic condition to destruct macrophyte cells by deposition of lignin and suberin. However radial oxygen loss also results in precipitation of Fe at macrophyte roots (Jacob & Otte, 2003).

6.3 Microbial Identification in Phytoremediation

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Morphology of bacteria found in this phytoremediation treatment was in the form of rod-shaped *Bacillus*. This finding was in accordance with 16S rRNA molecular identification results, in which two *Bacillus* species were identified such as *Bacillus megaterium* and *Bacillus cereus*. Microbial population had increased in macrophytes roots after phytoremediation treatment. This increment was assisted by both macrophytes and POM final discharge wastewater as macrophytes supply substrate and oxygen through it roots while POM final discharge supply nutrients for microorganism growth. This creates macrophytes-microorganisms interaction in which these microorganisms involved in BOD_5 and NH_3-N removal through ammonification, nitrification and denitrification (Greanway, 2007). Rhizodegradation process is a type of phytoremediation mechanism that involved symbiotic relationship between plant rhizosphere and microorganisms (Wenzel, 2009).

In the process of oxidation of organic matter, microorganisms consume O_2, NH_3-N and PO_4^{3-} as nutrient to produce simple end product such as CO_2 and water, and biomass (new cell) (Tchobanoglous et al., 2003). Previous study by Nwoko (2010) showed that fermented POME could increase microbiological activities in which microbial biomass had increased. However, without substrates supplied by macrophytes roots, microorganism could not increase their growth resulted in decrease of phytoremediation efficiency. The DNA of bacteria isolated from macrophytes rhizosphere was successfully extracted and amplified using PCR technique. The amplification of 16S rRNA region was successful with a strong band

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in gel electrophoresis in which this region are well known for their hypervaribility but still bearing species specific sign for identification of bacteria (Pereira et al., 2010).

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Similarity identication by both BLAST and RDP II database showed percentages between 99% to 100%. This high similarity percentage indicates their close phylogenetic relationship (Helgason et al., 2000). The present of Bacillus species in macrophytes rhizosphere were re-confirmed with molecular identification, in which two species identified as *Bacillus megaterium* and *Bacillus cereus* from *Pistia stratiotes* and *Leersia oryzoides* roots, respectively. According to previous studies, all bacteria identified in this phytoremediation treatment were denitrifiers in which these bacteria became the final electron acceptor in denitrification process. Denitrification process occurred by using carbon as energy source from biodegradable organic matter of POM final discharge wastewater and macrophytes biomass decomposition (Kadlec & Wallace, 2009; Leverenz et al., 2010). After phytoremediation treatment, BOD_5 and NH_3-N greatly removed (Chapter 3 page 68 and 72) which indicated that denitrifiers identified in this phytoremediation treatment used biodegradable organic matter as energy source in denitrification.

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CHAPTER 7

CONCLUSIONS AND RECOMMENDATIONS

7.1 Conclusions

Phytoremediation of POM final discharge wastewater was feasible using *Pistia stratoites, Lersia oryzoides* and *Ludwigia peploides*. The phytoremediation performance shows that BOD_5, COD and N-NH_3 removal efficiencies of 93%, 30% and 82%, were achieved for *Pistia stratoites*; 90%, 27% and 80%, for *Lersia oryzoides* and 93% , 20% and 80%, for *Ludwigia peploides,* respectively. *Ludwigia peploides* shown higher phytoremediation performance than *Leersia oryzoides*. In the phytoremediation study, NH_3-N and BOD_5 were removed greatly from POM final discharge wastewater by all studied macrophytes. However, only about 30% of COD can be removed by the three studied macrophytes. *Ludwigia peploides* was found as

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the most tolerance macrophytes to POM final discharge wastewater, than *Pistia stratiotes* and *Leersia oryzoides*.

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C, N and H elements accumulation in leaves were varied after the phytoremediation study with increased of N percentage and reduced in H percentage. The C percentages were high in all studied macrophytes organs and slightly reduced after the phytoremediation study. It was found that bioconcentration factor (BCF) and translocation factor (TF) of metals were varied among the studied macrophytes. The BCF values for Mg, Fe and Zn were highest in *Ludwigia peploides* roots, about 14.14 L/kg, 1.47×10^4 L/kg and 1.18×10^4 L/kg, respectively. Meanwhile, the BCF values for Ca and Na were highest in *Pistia stratiotes* than other macrophytes, about 56.57 L/kg and 11.95 L/kg, respectively. BCF value for K was highest in *Leersia oryzoides* stem, about 2.01 L/kg and also have highest TF value among studied metals with 4.95. The high value of BCF in certain organs showed the phytoaccumulation ability of studied macrophytes. The TF values of K, Na and Z were highest in *Leersia oryzoides*. *Pistia stratiotes* shown highest TF values than other studied macrophytes for Mg, Ca and Fe. It was found that metals elements such as Fe and Zn in the three studied macrophytes has low TF values than other metals due to its immobilization in roots and less translocation to leaves and stems which indicated that these metals were phytostabilised in macrophytes roots.

Microbial population in macrophytes rhizospheres increased after the phytoremediation. The morphology of the bacteria observed was *Bacilli*. Molecular identification of bacteria isolated from macrophytes' root was found closely related to *Bacillus megaterium* and *Brevundimonas diminuta* for *Pistia statiotes*, *Psedomonas*

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aeruginosa and *Pseudomonas mendocina* for *Ludwigia peploides* and *Acidovorax avenae* and *Bacillus cereus* for *Leersia oryzoides*.

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7.2 Recommendations

It is recommended that phytoremediation could be used as a tertiary treatment for POM final discharge wastewater in order to comply with standard limits imposed by Malaysian government. In addition, aeration and water circulation could be included in a future study to increase COD removal. Harvesting of wilted and decayed macrophytes might also reduce the amount of nutrient leached to POM final discharged wastewater. A study on phytotoxicity effect of POM final discharge wastewater on macrophytes at cellular and molecular level could be carried out to enhance the understanding of phytoremediation.

With these intriguing findings and performance at lab scale treatment, it is recommended to scale up phytoremediation of POM final discharge wastewater. The macrophytes can be grown in ditches and cascade flat bed in land application to avoid leaching of nutrients to river stream. Valuable findings from this research can also be used for large scale phytoremediation such as constructed wetland. This constructed wetland acts as tertiary treatment before POM final discharge wastewater entering the water stream. Kinetic uptake of metals could be further studied by using radioactive fluorescent label of metals which can determine the final destination of metals in macrophytes organs after uptake and translocation. Complete bacteria profiling existed in the scale up phytoremediation could be established using various molecular

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methods such as denaturing gradient gel electrophoresis (DGGE) for community analysis of bacteria at species level. Bacteria identified from this study also can be inoculated to macrophytes roots for other phytoremediation treatments to enhance degradation and removal of pollutants. These bacteria also can be subjected to research on bioconcentration of metal; whether the present of certain species bacteria can enhance or inhibit certain metals uptake by macrophytes. Phylogenetic tree of all bacteria involved in phytoremediation also could be established to understand the genetic relationship.

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APPENDICES

Appendix 1: Statistical Data (One Way Anova)

Descriptives

Reduction of BOD from average inital BOD

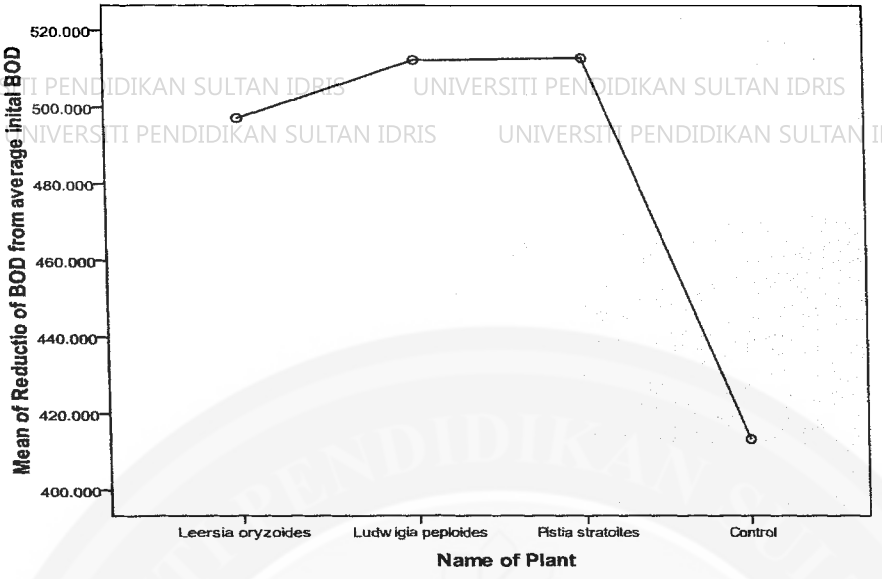
	N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum
					Lower Bound	Upper Bound		
Leersia oryzoides	3	497.33300	10.392305	6.000000	471.51708	523.14892	491.333	509.333
Ludwigia peploides	4	512.33300	9.451631	4.725816	497.29335	527.37265	499.333	519.333
Pistia stratoites	4	512.83300	18.064699	9.032349	484.08803	541.57797	497.333	537.333
Control	1	413.33300	.	.	.	.	413.333	413.333
Total	12	500.49967	30.540088	8.816164	481.09542	519.90391	413.333	537.333

ANOVA

Reductio of BOD from average inital BOD

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	8796.667	3	2932.222	16.034	.001
Within Groups	1463.000	8	182.875		
Total	10259.667	11			

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Descriptives

Reduction of Ammoniacal nitrogen in 5 days

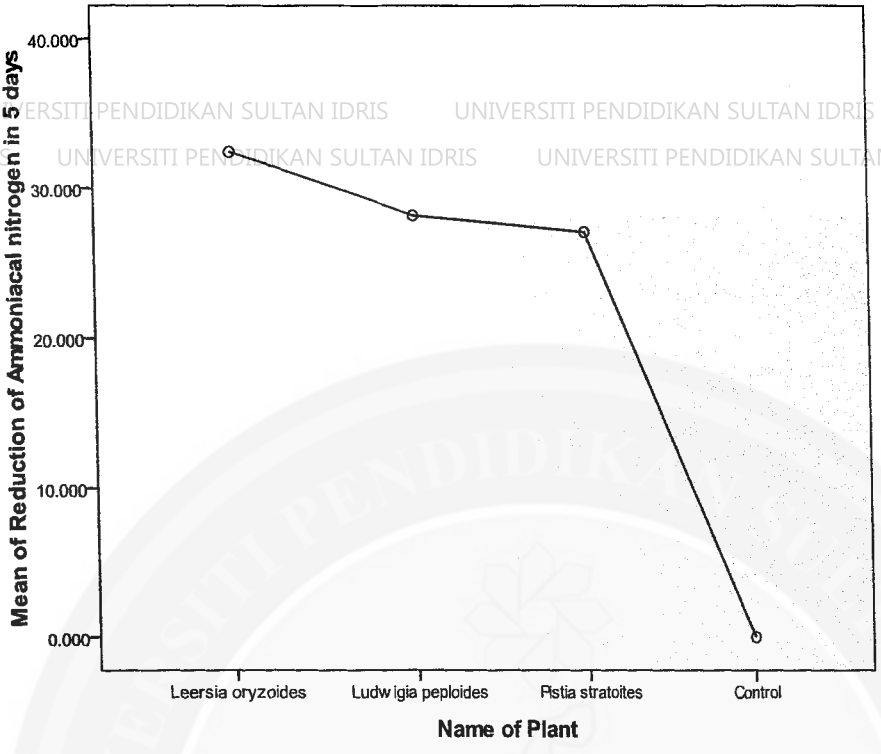
	N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum
					Lower Bound	Upper Bound		
Leersia oryzoides	3	32.41667	.144338	.083333	32.05811	32.77522	32.250	32.500
Ludwigia peploides	4	28.18750	.426956	.213478	27.50812	28.86688	27.750	28.750
Pistia stratoites	4	27.06250	1.572882	.786441	24.55969	29.56531	24.750	28.250
Control	1	.00000	.	.	.	.	.000	.000
Total	12	26.52083	8.675631	2.504439	21.00860	32.03307	.000	32.500

ANOVA

Reduction of Ammoniacal nitrogen in 5 days

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	819.922	3	273.307	272.952	.000
Within Groups	8.010	8	1.001		
Total	827.932	11			

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Descriptives

Reduction of COD

	N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum
					Lower Bound	Upper Bound		
Leersia oryzoides	3	141.66667	70.209211	40.535307	-32.74268	316.07602	61.000	189.000
Ludwigia peploides	4	121.75000	34.528973	17.264487	66.80670	176.69330	88.000	161.000
Pistia stratoites	4	292.25000	27.427176	13.713588	248.60724	335.89276	266.000	324.000
Control	1	108.00000	.	.	.	.	108.000	108.000
Total	12	182.41667	90.043887	25.993431	125.20551	239.62782	61.000	324.000

ANOVA

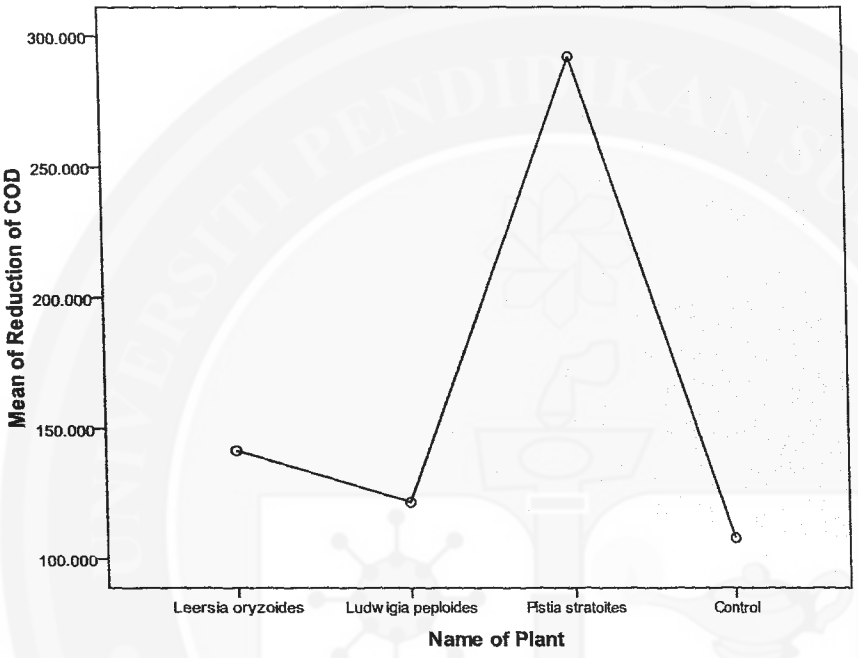
Reduction of COD

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	73494.750	3	24498.250	12.489	.002
Within Groups	15692.167	8	1961.521		

ANOVA

Reduction of COD

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	73494.750	3	24498.250	12.489	.002
Within Groups	15692.167	8	1961.521		
Total	89186.917	11			



Descriptives

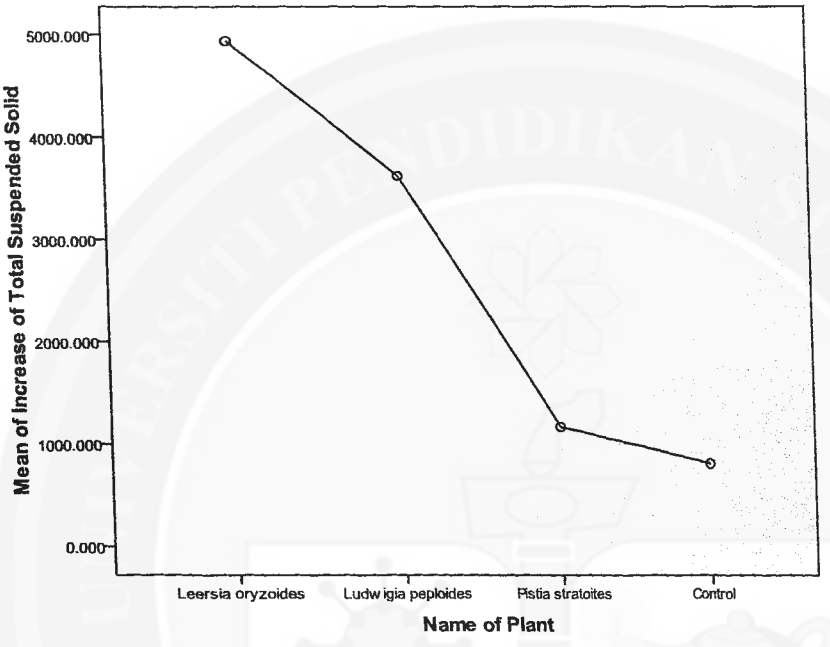
Increase of Total Suspended Solid

	N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum
					Lower Bound	Upper Bound		
Leersia oryzoides	3	4940.00000	1175.797602	678.847062	2019.15684	7860.84316	3790.000	6140.000
Ludwigia peploides	4	3620.50000	2532.766801	1266.383400	-409.69717	7650.69717	940.000	7050.000
Pistia stratoites	4	1170.00000	416.973221	208.486610	506.50256	1833.49744	570.000	1530.000
Control	1	810.00000	.	.	.	.	810.000	810.000
Total	12	2899.33333	2203.969532	636.231201	1498.99790	4299.66877	570.000	7050.000

ANOVA

Increase of Total Suspended Solid

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	3.090E7	3	1.030E7	3.657	.063
Within Groups	2.253E7	8	2816415.375		
Total	5.343E7	11			



Descriptives

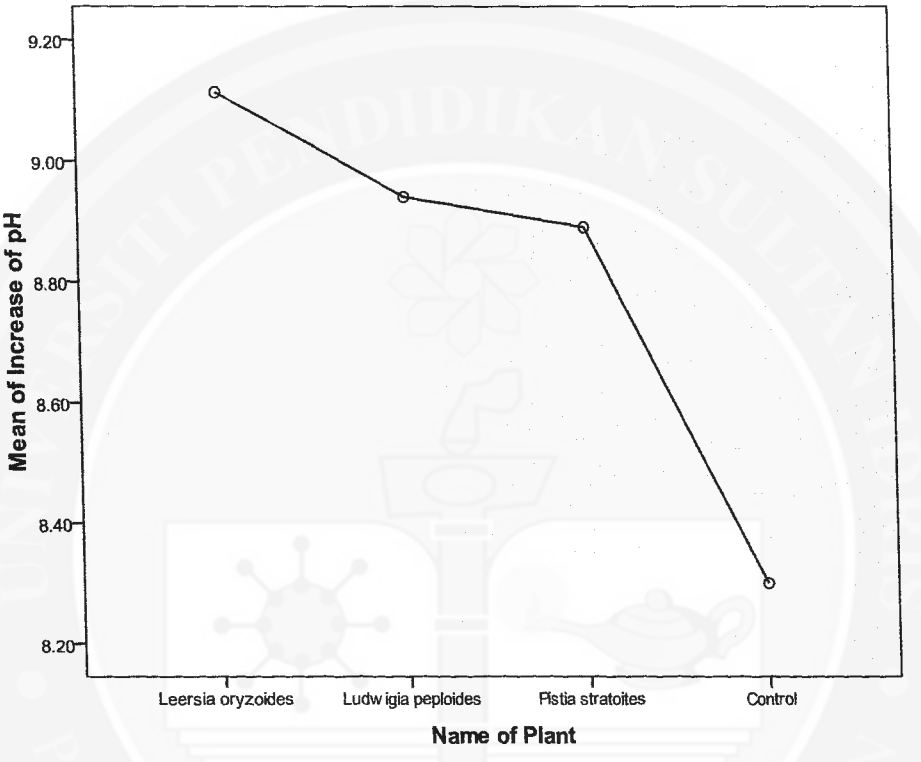
Increase of pH

	N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum
					Lower Bound	Upper Bound		
Leersia oryzoides	3	9.1133	.01528	.00882	9.0754	9.1513	9.10	9.13
Ludwigia peploides	4	8.9400	.07303	.03651	8.8238	9.0562	8.86	9.02
Pistia stratoites	4	8.8900	.01633	.00816	8.8640	8.9160	8.87	8.91
Control	1	8.3000	.	.	.	.	8.30	8.30
Total	12	8.9133	.21710	.06267	8.7754	9.0513	8.30	9.13

ANOVA

Increase of pH

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	.501	3	.167	77.405	.000
Within Groups	.017	8	.002		
Total	.518	11			



Appendix 2 : Atomic Absorption Spectroscopy (AAS) Calibration Curves

AAS Calibration Curve, Mg

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Sequence No.: 40
Sample ID: Calib Std 3
Analyst:

Autosampler Location:
Date Collected: 11/11/2011 11:10:37 AM
Data Type: Original

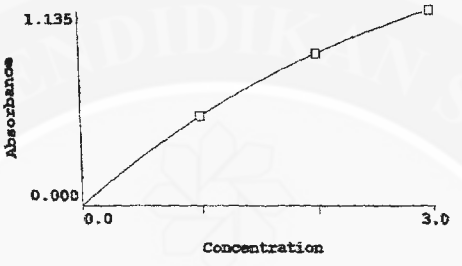
Replicate Data: Calib Std 3

Repl #	SampleConc mg/L	StndConc mg/L	BlnkCorr Signal	Time	Signal Stored
1		[3]	1.135	11:10:37	No
2		[3]	1.139	11:10:42	No
3		[3]	1.131	11:10:47	No

Method: Plant Mg -Master Page 2 Date: 11/11/2011 11:11:29

Mean:	[3]	1.135
SD:	0	0.0038
%RSD:	0	0.34

Standard number 3 applied. [3]
Correlation Coef.: 1.000000 Slope: 0.61020 Intercept: 0.00000



Calibration data for Mg 285.21 Equation: Nonlinear Through Zero

ID	Mean Signal (Abs)	Entered Conc. mg/L	Calculated Conc. mg/L	Standard Deviation	%RSD
Calib Blank 1	0.0000	0	0.000	0.00	0.3
Calib Std 1	0.5195	1.0	1.000	0.01	1.1
Calib Std 2	0.8831	2.0	2.000	0.01	0.6
Calib Std 3	1.1348	3.0	3.000	0.00	0.3

Correlation Coef.: 1.000000 Slope: 0.61020 Intercept: 0.00000

AAS Calibration Curve, Ca

Sequence No.: 96
Sample ID: Calib Std 3
Analyst:

Autosampler Location:
Date Collected: 11/11/2011 3:19:56 PM
Data Type: Original

Replicate Data: Calib Std 3

Repl #	SampleConc mg/L	StndConc mg/L	BlnkCorr Signal	Time	Signal Stored
1		[3]	0.294	15:20:00	No
2		[3]	0.294	15:20:05	No
3		[3]	0.294	15:20:10	No

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AAS Calibration Curve, Na

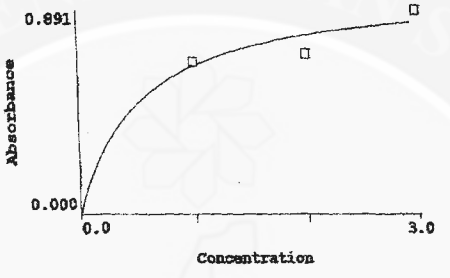
Sequence No.: 120 Autosampler Location:
Sample ID: Calib Std 3 Date Collected: 11/11/2011 3:53:14 PM
Analyst: Data Type: Original

Replicate Data: Calib Std 3

Repl #	SampleConc mg/L	StndConc mg/L	BlnkCorr Signal	Time	Signal Stored
1		[3]	0.887	15:53:18	No
2		[3]	0.889	15:53:23	No
3		[3]	0.896	15:53:28	No

Mean:	[3]	0.891	
SD:	0	0.0050	
%RSD:	0	0.56	

Standard number 3 applied. [3]
Correlation Coef.: Slope: 1.82274 Intercept: 0.00000
S-shaped calibration curve detected. Two-coefficient equation used.



Calibration data for Na 589.00 Equation: Nonlinear Through Zero

ID	Mean Signal (Abs)	Entered Conc. mg/L	Calculated Conc. mg/L	Standard Deviation	%RSD
Calib Blank 1	0.0000	0	0.000	0.00	0.1
Calib Std 1	0.6598	1.0	1.083	0.01	0.9
Calib Std 2	0.6978	2.0	1.293	0.00	0.6
Calib Std 3	0.8907	3.0	4.818	0.00	0.6

Correlation Coef.: Slope: 1.82274 Intercept: 0.00000

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AAS Calibration Curve, Fe

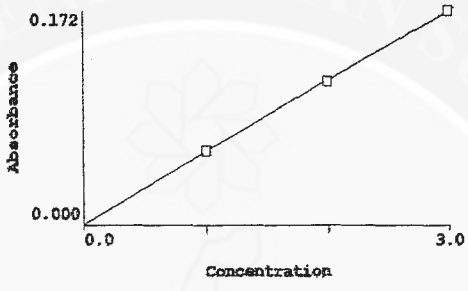
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Sequence No.: 5 Autosampler Location:
Sample ID: Calib Std 3 Date Collected: 11/11/2011 9:48:29 AM
Analyst: Data Type: Original

Replicate Data: Calib Std 3

Repl #	SampleConc mg/L	StndConc mg/L	BlnkCorr Signal	Time	Signal Stored
1		[3]	0.173	09:48:36	No
2		[3]	0.175	09:48:41	No
3		[3]	0.168	09:48:46	No
Mean:		[3]	0.172		
SD:		0	0.0034		
%RSD:		0	1.98		

Standard number 3 applied. [3]
Correlation Coef.: 0.999819 Slope: 0.05973 Intercept: 0.00000
S-shaped calibration curve detected. Two-coefficient equation used.



Calibration data for Fe 248.33 Equation: Nonlinear Through Zero

ID	Mean Signal (Abs)	Entered Conc. mg/L	Calculated Conc. mg/L	Standard Deviation	%RSD
Calib Blank 1	0.0000	0	0.000	0.00	658.7
Calib Std 1	0.0591	1.0	1.005	0.01	9.7
Calib Std 2	0.1145	2.0	1.979	0.00	1.7
Calib Std 3	0.1717	3.0	3.016	0.00	2.0

Correlation Coef.: 0.999819 Slope: 0.05973 Intercept: 0.00000

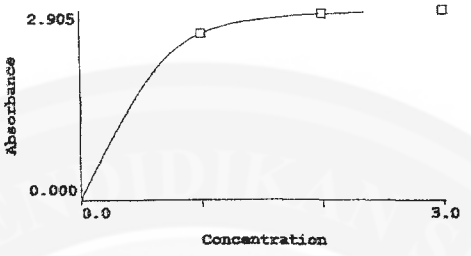
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AAS Calibration Curve, Zn

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Mean:	[3]	2.905
SD:	0	0.0423
%RSD:	0	1.46

Standard number 3 applied. [3]
Correlation Coef.: 1.000000 Slope: 3.67220 Intercept: 0.00000



Calibration data for Zn 213.86 Equation: Nonlinear Through Zero

ID	Mean Signal (Abs)	Entered Conc. mg/L	Calculated Conc. mg/L	Standard Deviation	%RSD
Calib Blank 1	0.0000	0	0.000	0.01	0.9
Calib Std 1	2.5517	1.0	1.000	0.01	0.3
Calib Std 2	2.8485	2.0	2.000	0.01	0.4
Calib Std 3	2.9051	3.0	3.000	0.04	1.5

Correlation Coef.: 1.000000 Slope: 3.67220 Intercept: 0.00000

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Appendix 3: Biocentration Factor (BCF) and Translocation Factor (TF) calculations

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Concentration of plant extract = 3g/50 ml
= 3000 mg/0.05L
=60,000 mg/L

Concentration Mg = 15.35 mg/L

Concentration in mg/kg = 15.35 mg/L / 60 000 mg/L
=15.35 mg/0.06 kg
=257.67 mg/kg

BCF value
= Mg concentration in plant organ (mg/kg) / Mg concentration in POM final discharge wastewater (mg/L)

= 257.67/15.46
= 0.82 L/kg

TF value
= Mg in plant (mg/kg) / Mg in POM final discharge wastewater (mg/kg)
= 257.67 mg/kg / 5255 mg/kg
= 0.40

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Appendix 4: Bacterial isolation results

Source of bacteria/label	Morphological Colour
Leersia oryzoides 1	Yellowish-red
Leersia oryzoides 2	Yellowish-red
Leersia oryzoides 3	White
Leersia oryzoides 4	White
Leersia oryzoides 5	White
Leersia oryzoides 6	White
Leersia oryzoides 7	White
Leersia oryzoides 8	Yellowish-red
Leersia oryzoides 9	White
Leersia oryzoides 10	White
Leersia oryzoides 11	White
Leersia oryzoides 12	White
Ludwigia peploides 1	White
Ludwigia peploides 2	White
Ludwigia peploides 3	Yellowish-red
Ludwigia peploides 4	White
Ludwigia peploides 5	Yellowish-red
Ludwigia peploides 6	White
Ludwigia peploides 7	White
Ludwigia peploides 8	White
Ludwigia peploides 9	Yellowish-red
Ludwigia peploides 10	Yellowish-red

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Ludwigia peploides 11	White
Ludwigia peploides 12	White
Ludwigia peploides 13	White
Ludwigia peploides 14	Yellowish-red
Ludwigia peploides 15	White
Ludwigia peploides 16	Yellowish-red
Pistia stratiotes 1	Yellowish-red
Pistia stratiotes 2	White
Pistia stratiotes 3	White
Pistia stratiotes 4	White
Pistia stratiotes 5	Yellowish-red
Pistia stratiotes 6	White
Pistia stratiotes 7	Yellowish-red
Pistia stratiotes 8	Yellowish-red
Pistia stratiotes 9	White
Pistia stratiotes 10	White
Pistia stratiotes 11	White
Pistia stratiotes 12	Yellowish-red
Pistia stratiotes 13	Yellowish-red

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Appendix 5: Bacterial similarity identification based on BLAST and RDP II database

Table 1: Bacterial similarity identification from BLAST of sample 1

Accession	Description	Maximum identification
HE981752.1	*Bacillus megaterium* partial 16S rRNA gene, isolate A8-1	100%
JX402436.1	*Bacillus sp.* SG21 16S ribosomal RNA gene, partial sequence	100%
JX393073.1	*Bacillus megaterium* strain TDB-2 16S ribosomal RNA gene, partial sequence	100%
JX315318.1	*Bacillus aryabhattai* strain SCSGAB0134 16S ribosomal RNA gene, partial sequence	100%
JX315306.1	*Bacillus aryabhattai* strain SCSGAB0090 16S ribosomal RNA gene, partial sequence	100%
JX315300.1	*Bacillus megaterium* strain SCSGAB0051 16S ribosomal RNA gene, partial sequence	100%
JX312585.1	*Bacillus megaterium* strain IARI-BC-13 16S ribosomal RNA gene, partial sequence	100%
JX312581.1	*Bacillus megaterium* strain IARI-BC-9 16S ribosomal RNA gene, partial sequence	100%
JX312579.1	*Bacillus aryabhattai* strain IARI-BC-6 16S ribosomal RNA gene, partial sequence	100%
JX293332.1	*Bacillus megaterium* strain S63 16S ribosomal RNA gene, partial sequence	100%

Table 2: Bacteria that show above 95% RDP II similarity score when compared with partial sequence from sample 1

Accession	Bacteria similarity	S_ab score
S000330853	*Bacillus megaterium*; MO31; AY553118	1.000
S000366978	*Bacillus sp.* R-16769; AJ748259	1.000
S000466502	*Bacillus sp.* XL-2004; AY788910	1.000
S000478929	*Bacillus sp.* EC2; AY864632	1.000
S000544166	*Bacillus megaterium*; AC46b1; AJ717381	1.000
S000546351	*Bacillus sp.* Fa29; AY131222	1.000
S000621767	*Bacillus megaterium*; 2-37-4-1; DQ267829	1.000

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S000626362	*Bacillus sp.* Y-h; DQ302416	1.000
S000626900	*Bacillus sp.* M9H; DQ323079	1.000
S000635309	*Bacillus megaterium*; B188; DQ289998	1.000

Table 3: Bacterial similarity identification from BLAST of sample 2

Accession	Description	Max Ident
AY972871.1	Alpha proteobacterium BAL284 16S ribosomal RNA gene, partial sequence	100%
HM448991.1	*Brevundimonas sp.* DB-4 16S ribosomal RNA gene, partial sequence >gb|JN592618.1| Brevundimonas sp. IARI-T-26 16S ribosomal RNA gene, partial sequence	99%
GQ250440.2	*Brevundimonas olei* strain MJ15 16S ribosomal RNA gene, partial sequence	99%
AB680017.1	*Brevundimonas diminuta* gene for 16S rRNA, partial sequence, strain: NBRC 3140	99%
FN663626.2	*Brevundimonas sp.* OU-6 16S rRNA gene, strain OU6	99%
EU730914.1	*Brevundimonas diminuta* strain 207 16S ribosomal RNA gene, partial sequence	99%
EU434468.1	*Brevundimonas diminuta* strain a242 16S ribosomal RNA gene, partial sequence	99%
AB167229.1	*Brevundimonas diminuta* gene for 16S rRNA, partial sequence, strain:c79	99%
HM217129.1	*Brevundimonas diminuta* strain DSS1 16S ribosomal RNA gene, partial sequence	99%
JQ994366.1	*Brevundimonas sp.* AD67 16S ribosomal RNA gene, partial sequence	99%

Table 4: Bacteria that show above 95% RDP II similarity score when compared with partial sequence from sample 2

Accession	Description	S_ab score
S000413819	*Brevundimonas diminuta*; IFO 3140; D49422	0.963
S000469687	*Brevundimonas diminuta*; c66; AB167225	0.965

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S000469691	*Brevundimonas diminuta*; c79; AB167229	0.980
S000516381	alpha proteobacterium BAL284; AY972871	1.000
S000981215	*Brevundimonas diminuta*; NK 2.18-3; EU352761	0.967
S001015470	*Brevundimonas diminuta*; a242; EU434468	0.977
S001046201	*Brevundimonas diminuta*; 207; EU730914	0.976
S001240608	*Brevundimonas diminuta*; XW2a; EU545397	0.967
S001264396	*Brevundimonas sp.* S-SL-1; FJ529023	0.967
S001264853	*Brevundimonas naejangsanensis* (T); BIO-TAS2-2; FJ544245	0.967

Table 5: Bacterial similarity identification from BLAST of sample 3

Accession	Description	Max Ident
AM992186.2	*Pseudomonas pseudoalcaligenes* partial 16S rRNA gene, isolate TS IW	100%
FJ159437.1	*Pseudomonas sp.* Gc-5-a 16S ribosomal RNA gene, partial sequence	100%
EU931560.1	*Pseudomonas aeruginosa* strain ZFJ-5 16S ribosomal RNA gene, partia	100%
JX122108.1	*Pseudomonas sp.* MOB13 16S ribosomal RNA gene, partial sequence	100%
JX122106.1	*Pseudomonas sp.* MOA1-19 16S ribosomal RNA gene, partial sequence	100%
JX122097.1	*Pseudomonas sp.* CDA1-15 16S ribosomal RNA gene, partial sequence	100%
AB638853.1	*Pseudomonas sp.* 38B gene for 16S rRNA, partial sequence	100%
JN679850.1	*Pseudomonas fluorescens* strain 1573 16S ribosomal RNA gene, partial sequence	100%
FJ587079.1	*Pseudomonas pseudoalcaligenes* strain MOB13 16S ribosomal RNA gene, partial sequence	100%
FJ159438.1	*Pseudomonas sp.* Gc-6-b 16S ribosomal RNA gene, partial sequence	100%
JF513155.1	*Pseudomonas nitroreducens* strain ES-18 16S ribosomal RNA gene, partial	99%
GQ280106.1	*Pseudomonas sp.* JS-36 16S ribosomal RNA gene, partial sequence	99%

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Table 6: Bacteria similarity identification from RDPII of sample 3 which shows >95% similarity

Accession	Description	S_ab score
S000702052	*Pseudomonas mendocina*; DQ641475	0.955
S000722495	*Pseudomonas pseudoalcaligenes*; B50; DQ837704	0.955
S000893437	*Pseudomonas sp.* RM12W; EF675623	0.958
S001169573	*Pseudomonas aeruginosa*; ZFJ-5; EU931560	0.991
S001170679	*Pseudomonas sp.* Gc-5-a; FJ159437	0.991
S001243326	*Pseudomonas pseudoalcaligenes*; JM2; FJ472854	0.952
S001243327	*Pseudomonas mendocina*; JM3; FJ472855	0.952
S001243329	*Pseudomonas pseudoalcaligenes*; JM5; FJ472857	0.952
S001292430	*Pseudomonas pseudoalcaligenes*; TS IW 01; AM992186	0.981
S002409408	*Pseudomonas pseudoalcaligenes*; 2-3; HQ285878	0.955

Table 7: Bacterial similarity identification of sample 4 partial sequence using BLAST

Accession	Description	Max Ident
FJ544320.1	*Pseudomonas pseudoalcaligenes* strain 81007 16S ribosomal RNA gene, partial sequence	100%
FJ544319.1	*Pseudomonas mendocina* strain 81006 16S ribosomal RNA gene, partial sequence	100%
FJ544382.1	*Pseudomonas pseudoalcaligenes* strain sw04 16S ribosomal RNA gene, partial sequence	99%

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JQ034362.1	*Pseudomonas sp.* L-6(2012) 16S ribosomal RNA gene, partial sequence	99%
JN544146.1	*Pseudomonas mendocina* strain PRM6 16S ribosomal RNA gene, partial sequence	99%
JX025167.1	*Pseudomonas mendocina* strain TSM4 16S ribosomal RNA gene, partial sequence	99%
JQ652571.1	*Pseudomonas mendocina* strain PMLR-1 16S ribosomal RNA gene, partial sequence	99%
AB681730.1	*Pseudomonas nitroreducens* subsp. *thermotolerans* gene for 16S rRNA, partial cds, strain: NBRC 102205	99%
JN836325.1	*Pseudomonas mendocina* strain CY003 16S ribosomal RNA gene, partial sequence	99%
JF911369.1	*Pseudomonas nitroreducens* strain EAPn13 16S ribosomal RNA gene, partial sequence	99%

Table 8: Bacterial similarity identification of sample 7 partial sequence from RDP- II

Accession	Description	S_ab score
S000702052	*Pseudomonas mendocina*; DQ641475	0.967
S000722495	*Pseudomonas pseudoalcaligenes*; B50; DQ837704	0.966
S000965734	*Pseudomonas sp.* SBR-camb; EF151241	0.968
S001243326	*Pseudomonas pseudoalcaligenes*; JM2; FJ472854	0.966
S001243327	*Pseudomonas mendocina*; JM3; FJ472855	0.966
S001243328	*Pseudomonas pseudoalcaligenes*; JM4; FJ472856	0.966
S001243329	*Pseudomonas pseudoalcaligenes*; JM5; FJ472857	0.966
S001243332	*Pseudomonas pseudoalcaligenes*; JM8; FJ472860	0.966
S001264935	*Pseudomonas pseudoalcaligenes*; sw04; FJ544382	0.981
S001264936	*Pseudomonas pseudoalcaligenes*; sw05; FJ544383	0.975

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Table 9: Bacteria similarity identification from BLAST of sample 5

Accession	Description	Max Ident
AY651926.1	*Comamonadaceae* bacterium MPsc 16S ribosomal RNA gene, partial sequence	90%
AY512827.1	*Acidovorax avenae* 16S ribosomal RNA gene, partial sequence	89%
AB547156.1	*Acidovorax sp.* KBT017 gene for 16S rRNA, partial sequence	89%
JN700196.1	*Acidovorax oryzae* strain YNA110 16S ribosomal RNA gene, complete sequence	89%
JF505947.1	*Acidovorax oryzae* strain KNUC9013 16S ribosomal RNA gene, partial sequence	89%
HQ834240.1	*Pseudacidovorax sp.* NH-1 16S ribosomal RNA gene, partial sequence	89%
FJ581042.3	*Pseudacidovorax sp.* ptl-2 16S ribosomal RNA gene, partial sequence	89%
NR_044241.1	*Pseudacidovorax intermedius* strain CC-21 16S ribosomal RNA, partial sequence >gb|EF469609.1| Pseudacidovorax intermedius strain CC-21 16S ribosomal RNA gene, partial sequence	89%
AF214644.1	Nitrogen-fixing bacterium MI753 16S ribosomal RNA gene, partial sequence	89%
AB547157.1	*Acidovorax sp.* KBT209 gene for 16S rRNA, partial sequence	89%

Table 10: Bacteria similarity identification from RDP II of sample 8

Accession	Description	S_ab score
S000001712	*Acidovorax sp.* KSP2; AB076843	0.499
S000735291	*Acidovorax sp.* S20; AY741158	0.495
S000776648	*Acidovorax sp.* 3Re21; EF182718	0.500
S001265111	*Acidovorax sp.* SD2; FJ560467	0.500
S001265113	*Acidovorax sp.* SD3; FJ560469	0.500
S001745186	*Acidovorax sp.* N13; GU086421	0.495
S001783358	*Acidovorax sp.* enrichment culture clone ECC3-16; GU056303	0.495

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S002304145	*Acidovorax sp.* KBT005; AB547155	0.500
S002304146	*Acidovorax sp.* KBT017; AB547156	0.500
S002304147	*Acidovorax sp.* KBT209; AB547157	0.500

Table 11: Bacterial identification result from BLAST for sample 6

Accession	Description	Max ident
JX077093.1	*Bacillus cereus* strain -Y111 16S ribosomal RNA gene, partial sequenc	99%
JX077092.1	*Bacillus cereus* strain -Y19 16S ribosomal RNA gene, partial sequence	99%
JX077088.1	*Bacillus anthracis* strain -Y11 16S ribosomal RNA gene, partial sequence	99%
JQ433949.1	*Bacillus sp.* KV1 16S ribosomal RNA gene, partial sequence	99%
JQ433946.1	*Bacillus sp.* TN3 16S ribosomal RNA gene, partial sequence	99%
JQ433945.1	*Bacillus sp.* SI1 16S ribosomal RNA gene, partial sequence	99%
JQ433944.1	*Bacillus sp.* ZRI 16S ribosomal RNA gene, partial sequence	99%
JQ958837.1	*Bacillus sp.* 7(2012) 16S ribosomal RNA gene, partial sequence	99%
JX047445.1	*Bacillus sp.* SDT14 16S ribosomal RNA gene, partial sequence	99%
JX035961.1	*Bacillus thuringiensis* strain JDM138 16S ribosomal RNA gene, partial sequence	99%

Table 12: Bacteria similarity identification from RDP II of sample 9

Accession	Description	S_ab score
S000000601	*Bacillus thuringiensis*; WS 261; Z84584	0.993
S000004484	*Bacillus sp.* AH 540; AF290557	0.993
S000006577	*Bacillus thuringiensis*; WS 2626; Z84588	0.993
S000006757	*Bacillus cereus*; AH 527; AF290555	0.993
S000007475	*Bacillus thuringiensis*; ATCC33679;	0.993

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	AF290549	
S000007738	*Bacillus thuringiensis*; WS 2614; Z84593	0.993
S000013815	*Bacillus thuringiensis*; AF160221	0.993
S000014039	*Bacillus cereus*; ATCC43881; AF290550	0.993
S000014773	*Bacillus sp.* AH 533; AF290556	0.993
S000015508	*Bacillus cereus*; ATCC14579; AF290547	0.993

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