Effects of Ectophytic Bacteria in Phytoremediation

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ABSTRACT

Treating wastewater has become one of the challenges faced by people living in an urban area. Due to anthropogenic activities, the water quality has been decreasing drastically, and we felt the need to address the issue at the earliest. Therefore, this study was conducted to analyze the effectiveness of symbiotic bacteria in phytoremediation using two aquatic plants, *Echinodorus cordifolius* (L.) Griseb. and *Lepironia articulata* (Retz.) Domin. Aquatic plants were treated with sodium hypochlorite to sterilize plant roots, and cultured in domestic wastewater for five days. The water samples were analyzed for the levels of phosphate (PO_4^{3-}), ammonium (NH_4^+), nitrate (NO_3^-) and nitrite (NO_2^-). In addition, roots were collected for 16S rRNA gene-based metagenomics analysis of bacterial composition. The results showed that the removal percentage of PO_4^{3-}, NH_4^+, NO_3^-, and NO_2^- of unsterilized plants were higher than sterilized plants. Moreover, the 16S rRNA gene-based metagenomics analysis revealed that the dominant bacterium in control *E. cordifolius* was *Calothrix* sp. The level of *Calothrix* was lower in sterilized *E. cordifolius* (38.88%) when compared with control (46.19%) and unsterilized *E. cordifolius* (49.69%). In control of *L. articulata*, *Clostridium* was a dominant bacterium. The proportion of *Clostridium* was lower in sterilized *L. articulata* (1.31%) when compared with control (13.72%) and unsterilized (49.02%) *L. articulata*. In conclusion, the results suggested that selected aquatic plants were effective in the removal of phosphate and nitrogen. Also, bacterial compositions presented in these plants were changed after sodium hypochlorite treatment. This study suggested that symbiotic bacteria might affect the removal of phosphorus and nitrogen from domestic wastewater.

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CONTENTS

ABSTRACT…… v

ACKNOWLEDGEMENTS…….…….. vi

CONTENTS…… viii

LIST OF TABLES…….. xi

LIST OF FIGURES…… xii

LIST OF ABBREVIATIONS…… xiv

LIST OF PAPER…… xvi

CHAPTER 1: INTRODUCTION……. 1

1.1 General introduction…….……... 1

1.2 Research questions……..... 4

1.3 Hypothesis ……..……... 4

1.4 Objectives……... 4

1.5 Conceptual framework of the experiment……... 4

CHAPTER 2: LITERATURE REVIEW……. 5

2.1 Sources of sludge and contaminants in wastewater……..... 5

2.2 Effect of untreated wastewater on physiology and the health

of animals and human ……..……..……..... 6

2.3 Methods of wastewater treatment..... 7

2.4 Importance of phytoremediation and bioremediation……..... 7

2.5 Interaction between plants and microbes in phytoremediation ……. 8

2.6 Nitrogen cycle…… 11

2.7 Phosphorus cycle……... 12

2.8 Molecular technique on microbial community……. 13

2.9 Plants morphology of selected plants……... 14

2.9.1 The Mud King (*Echinodorus cordifolius* (L.) Gpiseb.)….. 14

2.9.2 The Grey Sedge (*Lepironia articulata* (Retz.) Domin)..... 15

CHAPTER 3: MATERIALS AND METHODS…….…...……... 16

3.1 Material collection……..…….……..……... 16

3.2 Acclimation of plants/preparation of mud-clay soil and wastewater….16

3.3 Experimental condition……... 16

3.4 Water analysis……..……... 17

3.4.1 Determination of Phosphate……..…….…….. 17

3.4.2 Determination of ammonium…….……..……..... 18

3.4.3 Determination of nitrite…….…….…….. 20

3.4.4 Determination of nitrate…….…….. 21

3.4.5 Determination of dissolve oxygen (DO)…….…… 23

3.5 Microbial community analysis…….....……... 25

3.6 Statistic analysis…….. 26

CHAPTER 4: RESULTS…….…… 27

4.1 Temperature, pH and DO……..…….……. 27

4.2 Initial and final fresh weight of the plants…….…….. 28

4.3 Phosphate (PO_4^{3-}) removal……..……..…….. 29

4.4 Removal percentage of Nitrogen (NH_4^+, NO_3^-, NO_2^-)…….…… 30

4.4.1 Ammonium (NH_4^+) removal…….…… 30

4.4.2 Nitrite (NO_2^-) removal…….…… 31

4.4.3 Nitrate (NO_3^-) removal……... 32

4.5 Microbial community composition..... 33

CHAPTER 5: DISCUSSION…..……..……..….. 37

CHAPTER 6: CONCLUSIONS……..….....…… 43

REFERENCES……..…..……..…….…….. 44

APPENDIX…….…….…… 57

Appendix 1: Acclimatizing the *Echinodorus cordifolius* and *Lepironia articulata* in the greenhouse for 14 days ……...…..…… 57

Appendix 2: Experimental set up for selected aquatic plants*;* *Echinodorus cordifolius* and *Lepironia articulata* with five replicates with control.…….…… 57

VITAE……..…..... 58

LIST OF TABLES

Table		Page
1	Characteristics of domestic wastewater before the experiment (day 0)	27
2	Temperature, pH, and dissolved oxygen (DO) of domestic wastewater after five days of the experiment	28
3	Fresh weight (g) of plants before and after an experiment	29
4	Richness (genus-based), diversity index and evenness of the bacterial community in control *Echinodorus cordifolius* (before treatment), sterilized *E. cordifolius* (after treatment), unsterilized *E. cordifolius*, control *Lepironia articulata*, sterilized *L. articulata*, and unsterilized *L. articulata*.	34

LIST OF FIGURES

Figure		Page
1	Conceptual framework of the two major experiments	4
2	Pictorial representations of microbial assemblages in biofilm	9
3	Nitrogen cycle	11
4	Phosphate cycle	13
5	Mud king (*Echinodorus cordifolius* (L.) Griseb)	14
6	Grey Sedge (*Lepironia articulata* (Retz.) Domin	15
7	Absorbance values of standard at 880 nm versus phosphate concentration	18
8	Absorbance values of standard 630 nm versus ammonium concentration	20
9	Absorbance values of standard at 540 nm versus nitrite concentration	21
10	Absorbance values of standard 540 nm versus nitrate concentration	22
11	Removal percentage of phosphate (PO_4^{3-}) in sterilized *Echinodorus cordifolius*, unsterilized *E. cordifolius*, sterilized *Lepironia. articulata*, and unsterilized *L. articulata*	30
12	Removal percentage of ammonium (NH_4^+) in sterilized *Echinodorus cordifolius*, unsterilized *E. cordifolius*, sterilized *Lepironia articulata*, and unsterilized *L. articulata*	31
13	Removal percentage of nitrite (NO_2^-) in sterilized *Echinodorus cordifolius*, unsterilized *E. cordifolius*, sterilized *Lepironia articulata*, and unsterilized *L. articulata*	32

14	Removal percentage of nitrate (NO_3^-) in sterilized *Echinodorus cordifolius*, unsterilized *E. cordifolius*, sterilized *Lepironia articulata,* and unsterilized *L. articulata*	33
15	Bacterial community composition in control *Echinodorus cordifolius*, sterilized *E. cordifolius* and unsterilized *E. cordifolius*, Control *Lepironia articulata*, sterilized *L. articulata* and unsterilized *L. articulata* at the phylum, class and genus levels	36
A-1	Acclimatizing the Echinodorus cordifolius and Lepironia articulata in greenhouse for 14 days.	57
A-2	*Experimental set up for selected aquatic plants:* Echinodorus cordifolius *and* Lepironia articulata *with five replicates with controls.*	57

LIST OF ABBREVIATIONS

Abbreviations

APHA	American Public Health Association
DNA	Deoxyribonucleic acid
DO	Dissolve oxygen
EC	*Echinodorus cordifolius*
g	Gram
h^{-1}	Per hour
KNO_3	Potassium nitrate
KIO_3	Potassium iodate
K_2HPO_4	Di-potassium hydrogen phosphate
kg	Kilogram
LA	*Lepironia articulata*
L	Litre
min	Minute
mg L^{-1}	Milligram per liter
NH_4Cl	Ammonium chloride
NH_4^+	Ammonium
$NaNO_2$	Sodium nitrite
NO_3^-	Nitrate
NO_2^-	Nitrite

PO_4^{3-}	Phosphate
PRC	Polymerase chain reaction
SD	Standard deviation
s	Second

LIST OF PAPER

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CHAPTER 1

INTRODUCTION

General introduction

As the population increase, the rapid development of living standards has been improved in the world, and mostly settled in the cities and towns which generate large amounts of different contaminants causing significant issues to the municipal worker (Zhuang et al., 2015). Water is vulnerable to pollution because water is a universal solvent that can dissolve more substances than other liquids in the world (Zhang et al., 2019). Therefore, water is easily polluted by chemicals or toxic substances from industries, factories, towns, and farms, which are readily dissolved and mixed into water and cause water pollution (Sun et al., 2017). According to the U.S. Environmental Protection Agency, nearly half of rivers, streams and lakes are contaminated and prohibited for swimming, fishing, and drinking (US Environmental Protection Agency, 2016).

Moreover, untreated domestic wastewater is identified as a leading cause of eutrophication (Chen et al., 2011). Eutrophication is caused by excess releasing of nitrogen and phosphorus in water and categorized as one of problems that threaten the water quality worldwide. Excess of nitrogen and phosphorus can cause algal blooms containing a toxic from blue-green algae that can be harmful to human and animals (Pan et al., 2019). Contaminated water also contains pathogens, such as disease-causing bacteria and viruses from human and animal wastes, which are the causes of illness from drinking contaminated water. Diseases are spreaded by consuming of contaiminated water including cholera, giardia, and typhoid (Pan et al., 2019; Niederberger and Glanville-Wallis, 2019)

Various methods, such as solidification, filtration, oxidation, reverse osmosis, lagoon treatment, and electrochemical treatment have been used to approach environmental pollutants (Aleya et al., 2019; Rasheed et al., 2018; Khan et al., 2004).

However, the vast cost for construction, and chemical reagents, and the production of secondary pollutants lead to implementation challenges (Dasgupta et al., 2015)and not more than 60% of the total operating cost is spending on buying equipment, which might be unaffordable for developing countries (Baghapour et al., 2011).

Phytoremediation is a biological wastewater treatment using plants. It is a concept of treating wastewater which eliminates the contaminants from wastewater by symbiotic bacteria or microbes existing in the root of the aquatic plants (Roongtankiat et al., 2007). Phytoremediation is considering as the most effective eco-friendly method which is done with different types of mechanisms, such as, biofilm interaction, sedimentation, filtration, and chemical precipitation (Gupta et al., 2012; Hammer et al., 1989). The phytoremediation system using aquatic plants is the favored treatment needed in the developing countries because it is quite cheap to construct, fewer skill required for operation with low maintenance cost (Kirkpatrick, 2005; Mahmood et al., 2005). Besides water quality improvement, phytoremediation has other benefits to the ecosystem, such as promoting biodiversity, providing habitat for aquatic organisms and wildlife (Dixon et al., 2003).

Nowaday, more than 400 aquatic plants are known and used for treating wastewater (Gupta et al., 2012). Many researchers have been reported the impressive removal rates of inorganic nitrogen (nitrate (NO_3^-), ammonium ($NH_4^{+)}$, and phosphate (PO_4^{3-}) by using aquatic plants in nutrient-rich wastewater (Lu, 2009). For example, some studies reported that aquatic plants *Nelumbo nucifera* and *Cyperus alternifolius*, and green alga *Scenedesmus obliquus* were able to remove contaminated phosphate in domestic wastewater within five days (Thongtha et al. 2014; Martinez et al., 2000). Similarly, a study showed that aquatic plants *Canna generalis* and *Echinodorus cordifolius* could remove ammonium, nitrite, and nitrate from contaminated water within six days (Nakphet et al., 2017). In recent years, many researchers have studied and improved the methods of contaminant removal by using different aquatic plants and associated microbes for improvement of polluted environments (air, soil, and water).

In addition to phytoremediation, bioremediation has also been considered as an efficient and eco-friendly method for removing aquatic pollutants (Liu et al., 2019). In bioremediation, microbial communities exist in the form of detrital bacterial mat, biofilm, planktonic microbes and bacterial assemblages in plant rhizosphere (Battin et al., 2003). The presence of microbes or symbiotic bacteria in the roots of aquatic plants plays an essential role in removing inorganic pollutants. These microbial communities are related to nitrification, denitrification and metal ion reduction to improve water quality (Battin et al., 2003; Hahn, 2006). Various aquatic plant species have been tested for their efficiency in phytoremediation and bioremediation (Maggioni et al., 2009). *Echhornia crassipes* and *Pistia stratiotes* exhibits high potential in pollutant removal (Lu et al., 2010). Lu and colleagues (2017) showed that water hyacinth together with its nitrifying and denitrifying microbial associates removed 99.0% of ammonium (NH_4^+). Similarly, water lettuce, together with its microbial associates, removed 93.6% of phosphorus in wastewater (Lu et al., 2017). Furthermore, *Pseudomonas*, *Nitrosomonas*, *Dechloromonas*, and *Geobacter* have been reported as the nitrogen-removal microbes in the biofilm (Gao et al., 2016; Sotres et al., 2016). A cyanobacterium, *Anabaena azollae*, symbiosed with aquatic plants also involves in nitrogen fixation and absorb nitrogenous compounds from wastewater (Forni et al., 2001). Therefore, the integrated approach of using aquatic plants and their microbial associates might be an alternative solution for nitrogen and phosphorus removal.

Hence, this study aims to examine the ability of two aquatic plants, *Echinodorus cordifolius* and *Lepironia articulata*, to remove phosphate and nitrogen from the wastewater, and clarify the functions of symbiotic microbes presented in their roots in term of removal of phosphate and nitrogen from wastewater. The experiments were divided to 2 experiments. First, two aquatic plants, *Echinodorus cordifolius* and *Lepironia articulata*, were examined the ability of plants and their microbial associates to remove phosphate and nitrogen in wastewater. Second, 16S amplicon sequencing was performed to identify the plants' microbial communities involving in nitrogen and phosphate removal for understanding the function of microbial community in phytoremediation.

1.2 Research questions

- Do the selected aquatic plants effectively remove phosphate and nitrogen in wastewater?
- How do symbiotic bacteria in selected aquatic plants help in the removal of phosphate and nitrogen?
- What kind of symbiotic bacteria are found in effective aquatic plants?

1.3 Research hypothesis

- Symbiotic bacteria found in plant roots might be related to the elimination of nitrogen and phosphorous in wastewater.

1.4 Objectives

- To determine the efficiency of phosphorus and nitrogen removals by selected aquatic plants and their associated symbiotic bacteria
- To clarify the roles of symbiotic bacteria in phytoremediation
- To identify the bacterial community in the selected aquatic plant roots

1.5 Conceptual framework of the experiments

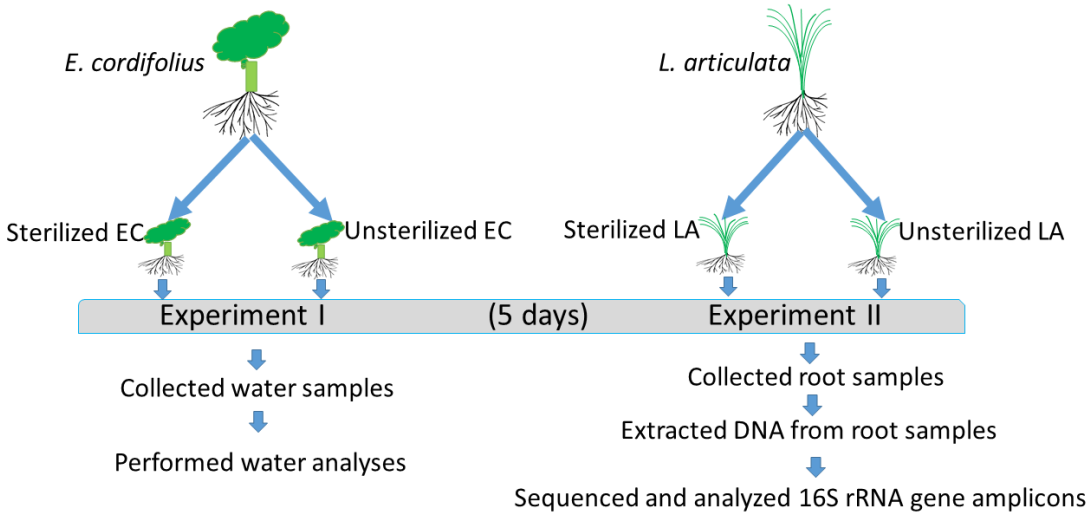

Figure 1 Conceptual framework of the two major experiments

CHAPTER 2

LITERATURE REVIEW

Anthropogenic activities around the world cause contamination in water bodies so, restoring is the big deal for human to solve this problem. However, the best remedy for wastewater treatment is phytoremediation, which is considered as the best eco-friendly and less expensive methods. The previous studies and their findings related to phytoremediation using plants and microbes are shown in this chapter

2.1 Sources of sludge and contaminants in wastewater

Wastewater is contaminated water that have been released by anthropogenic activities, and ultimately affected water quality of natural aquatic ecosystem (Gosh and Singh, 2005). The primary sources of wastewater are discharged by domestic residences, commercial properties, industries, schools, monasteries, offices, and hotelier (Hussain et al., 2018). The contaminants in wastewater consist of organic and inorganic compounds. Organic contaminant is a type of hydrocarbon pollutants, such as organic matter (liquid manure, sewage treatment sludge, etc.), organochlorides (DDT) and polychlorinated biphenyls (PCB). The inorganic contaminant is naturally found in environment, however, human activities have altered to drastically increase the amount of inorganic contaminant in the environment. The contaminations of organic and inorganic compounds mostly are acidity wastewater released by industries, contaminated ammonium from waste of food processing, chemical waste from industrial by-products, and oil leakage from shipwrecks (Burton and Pitt, 2001).

Furthermore, fertilizers containing high contents of nitrate and phosphate are run off into water bodies (Burton and Pitt, 2001). Industries, laboratories, and the hospital also produce a large amount of nitrogen and phosphate, which almost 50% are discharged as waste (Burton and Pitt, 2001).

2.2 Effects of untreated wastewater on physiology and the health of animals and human

The leading causes of pollution are due to anthropogenic activities, which changes the pH of water into acidity and causes eutrophication (Gooddy et al., 2016). This leads to increase algal blooming and cause hypoxic water (less oxygen in water) because algae consume oxygen in high level. Moreover, algae also emit the carbon dioxide from physiological respiration leading to decrease the pH of water bodies (increase acidity). Eutrophication in the water bodies increases the concentration of soluble organic pollutants depending on the degree of eutrophication (Cenk et al., 2017). This phenomenon affects the water quality of natural water resources leading to impact on aquatic organisms. For example, algal blooming occurs on the surface of the water, which blocks the sunlight and disrupts the growth of other aquatic plants. Moreover, these algae consume a lot of oxygen for respiration during nighttime, then oxygen is not enough for physiological activities of fish and other aquatic animals. This may lead to the death of aquatic animals (Gooddy et al., 2016; Cenk et al., 2017).

Nitrogen naturally exists in many forms, and most of the common nitrogen compounds are ammonium, nitrite, and nitrate (Larsdotter, 2006). Nitrite contamination in drinking water becomes a problem when nitrite concentration elevates to high level (Michael, 2015). The high content of ammonium in wastewater causes the nitrification and hypoxia, consequently causes the death of aquatic organisms, decreases the water quality, and harms to the aquatic ecosystem (Juan et al., 1998). Ammonia-nitrogen is a neutral molecule, which diffuses through the epithelial membrane of aquatic organisms and blocks oxygen transfer in the fish's gills. Those fishes suffering from ammonia-nitrogen show sluggish and come to the water surface to gasp for air (Udeh, 2004). The higher concentration of phosphate also causes eutrophication in ponds, rivers, and sea (Wang and Wang, 2009). It also causes the water quality problems, such as water spoilage and algal toxins (Thongtha et al., 2014).

Pathogens are the microorganisms that cause waterborne diseases to human or animals. (Harrison, 2001). Some of microorganisms are popularly found in wastewaters that have caused diseases, such as *Burkholderia pseudomallei*, *Cryptosporidium*

parvum, Giardia lamblia, Salmonella spp. and *Norovirus* spp. (Schueler, 2000). The contamination of these pathogens leads to cause many diseases: typhoid, paratyphoid, bacillary dysentery, gastroenteritis, and cholera.

2.3 Methods of wastewater treatments

There are many kinds of wastewater treatments, for example, solidification, filtration, reverse osmosis, lagoon treatment and electrochemical treatment. These treatments have been widely, but these methods need the municipal expertise to treat wastewater. The advantages of using these treatments are: 1) easy to get rid of solidified pollutants, 2) tranformation of polluted wastewater into clean water, 3) elimination of pungent smell, and 4) high efficiency in organic degradation (Dasgupta et al., 2015 Aleya et al., 2019). However, developing countries can not afford to buy the equipments for construction of the treatment plants. Moreover, lots of chemical reagents are required in the processes, and massive secondary pollutants are produced as by-products. The electricity is needed to run the treatment system for 24 hours, which is expensive for operation (Dasgupta et al., 2015; Aleya et al., 2019). Therfore, using of biological methods is an alternative way to solve these problems.

2.4 Importances of phytoremediation and bioremediation

Phytoremediation (phyto (Greek) refers to plant, and remediation (Latin) refers to restoring balance) is restoring the balance of contaminated environment to remove or degrade the pollutants from the contaminanted soils, sledge, sediments, surface water, and groundwater by using plants (Reichenauer et al., 2008). Phytoremediation is considered as a cost-effective approach of remediation, which plants uptake and metabolize all sorts of various pollutants in the various form presented in the environment (Gupta et al., 2012). Plants can bioaccumulate, degrade, or reduce harmful contaminants from the soil, water, and air. Heavy metals and organic pollutants are considered as primary targets for phytoremediation (Barchanska et al., 2019).

Numerous aquatic plants have been used for the wastewater treatment. *Pistia stratiotes, Phragmites australis, Typha latifolia, Nuphar luteum, Eichhornia crassipes, Myriophyllum spicatum, Lemna obscura, L. minor, L. majus,* and *L. gibba* have been

widely studied for the uptakes of phosphate and nitrogen from wastewater (DeBusk et al., 1995; Kasselmann, 1995; Srivastava et al., 2008).

Bioremediation is a process to treat polluted media including soil and water by stimulating the growth of microorganisms to degrade the target pollutants. This method is also considered as less expensive and more suitable for remediation (Hussain et al., 2018). Most of bioremediation processes involve oxidation and reduction by using microbes as a mediator to change the toxic compounds to less or non toxic compounds. An electron acceptor (commonly an oxygen) is used to stimulate the oxidation of reduced pollutants (e.g., hydrocarbons), while an electron donor (commonly an organic compound) involves in reduction of oxidized pollutants (e.g., nitrate, perchlorate, oxidized metals, chlorinated solvents, explosives, and propellants) (US Environmental Protection Agency, 2013). Cyanobacteria, *Calothrix*, involves in the removal of nitrogen by fixing and using nitrogen for their metabolisms (Nieto et al., 1989; Reuter et al., 1986). *Clostridium* is also related to the nitrogen cycle and able to utilize the nitrogen. Moreover, Alphaproteobacteria also helps in ammonium oxidation and denitrification (Wani et al., 2011; Ye et al., 2016). Bioremediation related technologies are phytoremediation, mycoremediation, bioventing, bioleaching, landfarming, bioreactor, composting, bioaugmentation, rhizofiltration, and biostimulation (Hussain et al., 2018).

2.5 Interaction between plants and microbes in phytoremediation

The aquatic macrophytes are divided into four different clusters: 1) emergent macrophyte (e.g. *Phragmites australis*), 2) floating-leaved macrophyte (e.g. *Nelumbo*), 3) free-floating macrophyte (e.g. *Pistia stratiotes*), and 4) submerged macrophyte (e.g. *Chara, Hydrilla*) (Srivastava et al., 2008). The spreading of aquatic plants and microbial classes mostly depends on the nutrient status of water bodies in the following order: oligotrophic, mesotrophic, eutrophic, and hypertrophic (Buosi et al., 2011). The microbial assemblages mostly are observed as a biofilm covering the leaves and roots of macrophytes, and planktonic micro algal-bacterial assemblages that help in removal of contaminants by nitrification, denitrification, sulfate and metal ion reduction (Battin et al., 2003; Cotner and Biddanda, 2002; Paerl and Pinckney, 1996). These cycles

greatly influence the flow of aquatic ecosystems, improving of water quality and reducing of the pollutants (Battin et al., 2003; Hahn, 2006).

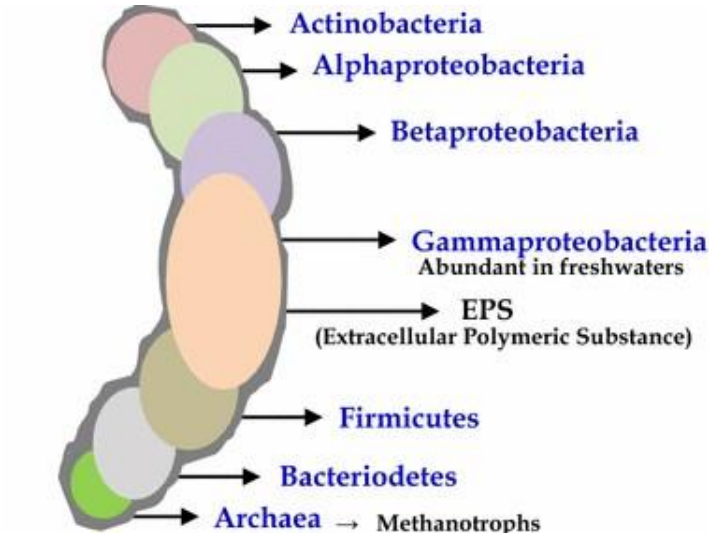

Figure 2 Illustration of microbial assembly in a biofilm (Srivastava et al., 2017)

A microbial community in the biofilm mostly found on the leaves, rhizosphere and the solid places of debris (Figure 2). The rhizosphere is part of root that is the most active zone contacted with water or soil presented with many kinds of microbial communities (Munch et al., 2007). Microbes in the water body interact with plants to receive organic nutrients and oxygen, while aquatic plants receive protective immunity and mineral interchange (Srivastava et al., 2017). The roots of marine plants also give more surface for a benthic bacterial community to rest, and act as a modified niche to supply of nutrients, organic carbon and oxygen (Stottmeister et al., 2003). Therefore, the formation of the microbial community as a biofilm on the surface of the aquatic plant is specific to plant species. Stout (2006) found the effects of plant-microbe interaction on *Lemna minor* whereby microbial association in the plant roots do not allow to uptake the Cd ion to evade the pass of this contaminated metal into the plants. The plant-microbe interaction in aquatic bodies be determined by numerous factors, such as, pH, dissolve oxygen, dissolved organic contaminants, toxic organic

contaminants, redox conditions, and the accessibility of nutrients (Buosi et al., 2011; Gray et al., 2004; Schauer et al., 2005).

In general, microbes have two types of symbiotic relationship with aquatic plants: 1) endophytic microbes which is the colonization of bacteria in the internal tissues of plants (such as nitrogen fixing diazotrophs, other nutrient assimilators, and arbuscular mycorrhizal fungi), and 2) ectophytic microbes which means microorganisms remaining outside of the plant (for examples, ammonia-oxidizing bacteria, and methanotrophic bacteria) (Nielsen et al., 2001; Sorrell et al., 2002; Wei et al., 2011; Weyens et al., 2009). Ectophytic interaction is an essential plant-microbe interaction because numerous biological reactions occur at the interactive surface of plant leaves and roots, which influences the elemental cycles in an aquatic ecosystem (Laanbroek, 2010). Biofilm in a marine ecosystem depends on the availability of different nutrients, such as mineral elements, phosphorus, and nitrogen, for their proper growth. The higher amounts of nutrients cause to eutrophication of water body followed by toxic production (Giaramida et al., 2013). Aquatic plants uptake excessive nutrients from the wastewater and prevent the growth of algae. Floating macrophytes, such as *Eichhornia crassipes* and *Ipomoea* sp., are important to decrease the levels of inorganic compounds (such as ammonium and nitrate). A study has shown that aerobic chemoautotrophic bacteria *Nitrosomonas* and *Nitrobacter* oxidize and transform ammonium to nitrate (Wetzel, 2001). The presence of ammonia-oxidizing microorganisms and archaea play an important role in nitrification and denitrification on the rhizoplane with the presence of *amoA* gene (Herfor et al., 2007; Wang et al., 2009; Wei et al., 2011). The environmental consequence of the plant-microorganism interactions has been broadly studied in constructed marshes and found that plant-microbe interaction in a water body and their ability to eradicate the pollution depends on types of interactions (Stottmeister et al., 2003; Tara et al., 2005; Münch et al., 2007; Nahlik and Mitsch, 2006; Vymazal et al., 2001; Vymazal, 2007). Therfore, aquatic plants–microbe relationship not only benefits to each other, but also greatly improve the water quality by removing of contaminants from the contaminated water at rhizosphere. However, some environmental conditions, such as eutrophication with

high abundance of toxic elements in the wastewater can cause damage to biofilm (Calheiros et al., 2009; Giaramida et al., 2013).

2.6 Nitrogen cycle

Though nitrogen is covered 78% in the atmosphere, it is very less availability in the soil for physiological processes in plants and microbes. So, the nitrogen cycle is required to convert the atmospheric nitrogen into different formed of nitrogen, such as ammonium, nitrite and nitrate (Figure 3). The nitrogen cycle take place both biological and physical processes, such as fixation, ammonification, nitrification, and denitrification (Simon et al., 2013).

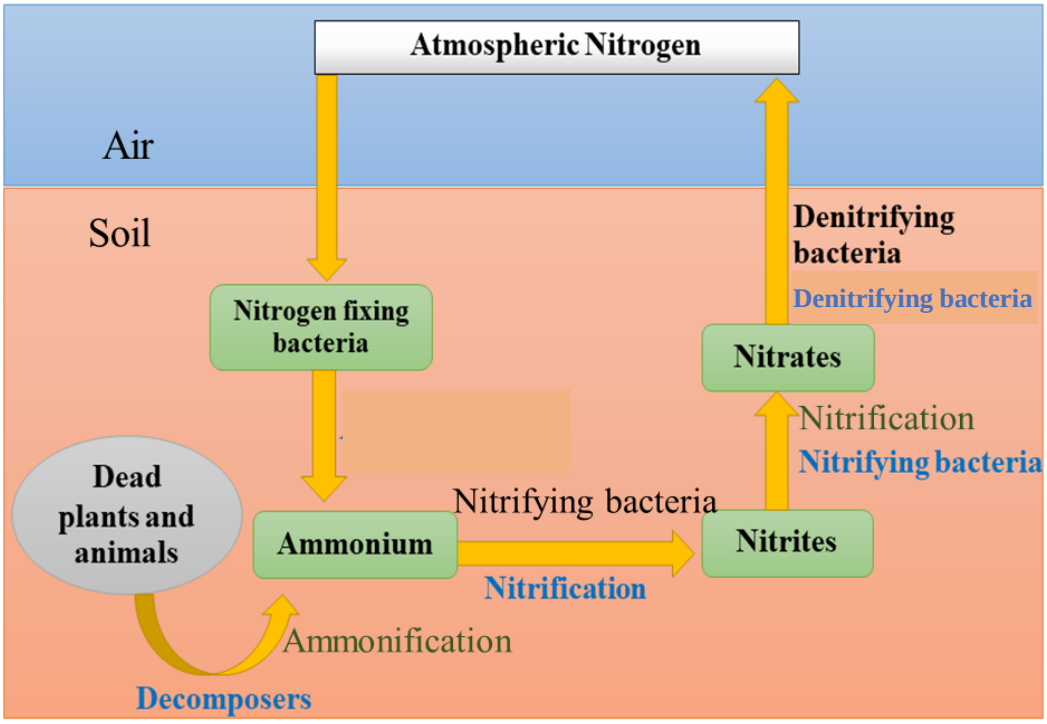

Figure 3 Schematic diagram of nitrogen cycle. The importance of bacteria in the cycle is immediately recognized as being a key element in the cycle that involve in transformation of nitrogen compounds (Simon et al., 2013)

The atmospheric nitrogen is converted into ammonium in the soil by lightning strikes, and mostly fixed through nitrogen fixation by symbiotic bacteria presenting in the plant roots. Nitrogen element in ammonium form is a usable form for plants uptake (Simon et al., 2013). Some of the microbes involving in fixation of nitrogen are diazotrophs, archaea, *Azotobacter*, and *Rhizobium*, which usually live in the roots of leguminous plants, such as pea, locust tree and alfafa (Moir, 2011).

Some of the important conversion involved in the nitrogen cycle is ammonification, where living organism dies or excretion of animal waste contain nitrogen in the form of organic, but slowly with the help of bacteria or fungi converted into ammonium (NH_4^+) called ammonification (Sparacino-Watkins et al., 2013). The process of changing from ammonium to nitrite or nitrate is called nitrification. Some of the bacteria involved in this process are *Nitrosomonas*, which converts ammonium to nitrite, and *Nitrobacter* converts nitrite into nitrate (Simon et al., 2013). The final process for nitrogen cycle is denitrification, which *Pseudomonas* and *Paracoccus* converts the nitrate back to nitrogenous gas under anaerobic conditions (Sparacino-Watkins et al., 2013; Simon et al., 2013).

2.7 Phosphorus cycle

In phosphorus cycle, atmosphere can not provide phosphate, but it is generated or produce from the primary and secondary minerals, and other organic sources like domestic wastewater from both human and animals (Silva et al., 2000). Phosphorus is deposited in the soil ranging from 0.001-1 mg L^{-1} (Brady and Weil, 2002). Plants uptake the phosphorus into the form of orthophosphate and also uptake certain forms of organic phosphate (Figure 4). Diffusion plays vital role in moving phosphorus to the root surface. However, the presence of plant-associated microbes, also uptake the phosphorus for their metabolism and makes less availability to the plants (Silva et al., 2000).

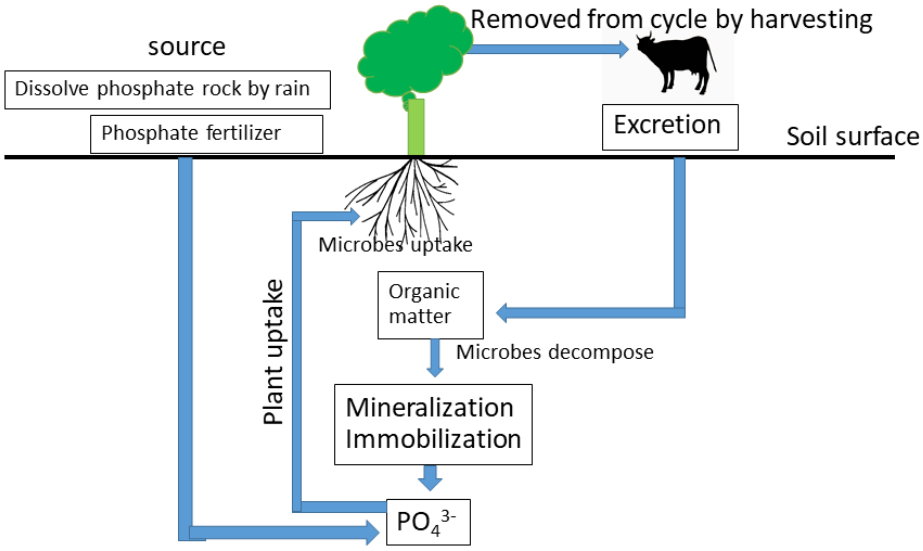

Figure 4 Schematic diagram of phosphorus cycle (Silva et al., 2000)

2.8 Molecular techniques on microbial community

Microorganisms are important for the phytoremediation. They help to degrade the contaminants from the polluted areas, and promote the plant growth. (Gerhardt et al., 2009). Thongtha and colleagues (2014) proved that not only plants involved in the removal of phosphorus, but microorganism also involved in the removal of phosphorus. They also found that phosphorus absorption was not only done by soil, but also absorbed by plants and microorganiams in wastewater.

The latest technology provides information on overall patterns of a bacterial composition by using bacterial DNA. Truu (2009) showed his finding using molecular methods, such as denaturing gradient gel electrophoresis (DGGE) and terminal restriction fragment length polymorphism (TRFLP) fingerprints of amplified 16S rDNA fragments, which easily give information about the overall pattern of the microbial community of biofilm (Truu et al., 2009).

2.9 Plant Morphology of selected plants species.

2.9.1 Mud King (Echinodorus cordifolius (L.) Griseb.)

Mud king (*Echinodorus cordifolius*) is a species of aquatic plants belonging to Family Alismataceae (Figure 5). It is a native plant of Mexico, the West Indies, Central America, Paraguay and Texas in United States. It is a perennial herb with rhizomes and upright leaves. It is inflorescences aquatic species with 3 - 15 flowers. It is easily cultivated in neutral to soft water in tropical to sub-tropical temperatures with high intensity of light. The rhizomes become thick as it has grown to adult stage with a dense mass of roots, which absorb lots of nutrients from the wastewater (Brown,2007; Torit et al., 2012).

Figure 5 Mud King (*Echinodorus cordifolius* (L.) Griseb.)

2.9.2 Grey Sedge (Lepironia articulata (Retz.) Domin)

Lepironia articulata or **Grey Sedge** belongs to Family Cyperaceae (Figure 6), which is found in Madagascar, India, Sri Lanka, Southern China, Southeast Asia, New Guinea, various islands of the Western Pacific, and northern and eastern Australia (Govaerts and Simpson, 2007). Grey sedge has creeping rhizomes covered by brown ovate scales with 3 or 4 leaf sheaths at the base (Flora of China, 2012). This plant is effective for the removal of effluents from household greywater (Wurochekke et al., 2014).

Figure 6 Grey Sedge *(Lepironia articulata* (Retz.) Domin)

CHAPTER 3

MATERIALS AND METHODS

3.1 Plant, wastewater and mud-clay soil collection

Two aquatic plants, *Echinodorus cordifolius* and *Lepironia articulata* were used in this study. We collected *E. cordifolius* from Prince of Songkla University, Hat Yai campus (7° 0′ 17.84 ″ N, 100° 29′ 39.24 ″ E), and *L. articulata* from a nearby mosque in HatYai, Songkhla, Thailand (7° 4′ 36.84″ N, 100° 29′ 33.72″ E). Wastewater for this study was collected from the sewage treatment plant II located near Songklanagarind Hospital, Hat Yai, Songkhla, Thailand (7° 0′ 17.28″ N, 100° 29′ 36.24″ E). The mud-clay soil was obtained for plant culture from the local shop.

3.2 Acclimatization of aquatic plants

The *Echinodorus cordifolius* and *Lepironia articulata* were cleaned using tap water to remove soil particles attached on the roots. Then, each of *E. cordifolius* and *L. articulata* was planted with 5 kg of autoclaved mud-clay soil in a plastic pot and submerged in autoclaved 10 L of tap water.

3.3 Experimental condition

The experiment was set into five groups with three replicates.

1) control (autoclaved wastewater + autoclaved mud-clay soil)
2) sterilized *E. cordifolius* + autoclaved mud-clay soil + autoclaved wastewater
3) unsterilized *E. cordifolius* + autoclaved mud-clay soil + autoclaved wastewater
4) sterilized *L. articulata* + autoclaved mud-clay soil + autoclaved wastewater
5) unsterilized *L. articulata* + autoclaved mud-clay soil + autoclaved wastewater

After acclimatizing both plants for 14 days, we selected plants with 36-38 g fresh weight and transferred to 4 L plastic pot. Each plastic pot contained 2 L of autoclaved wastewater and 1 kg of autoclaved clay soil. To prepared the sterilized plants, the roots of *E. cordifolius* and *L. articulata* were sterilized by soaking in 1% sodium hypochlorite for 30 minutes, and washed twice with MilliQ for 3 minutes. Then, the prepared sterilized and unsterilized plants were cultured for five days in the following described conditions. The wastewater volume in each pot was maintained using autoclaved MilliQ water daily.

3.4. Wastewater analysis (APHA, 1992)

3.4.1 Phosphate (PO_4^{3-})

To prepared 4.5 M sulfuric acid (H_2SO_4) (reagent 1), 25 ml of conc. H_2SO_4 was slowly added to approximately 70 ml distilled water under cool condition, added distilled water to 100 ml, and kept in a polyethene bottle. To prepared 20% ascorbic acid solution (reagent 2), 2 g of ascorbic acid was dissolved in 10 ml of distilled water, then added 10 ml of reagent 1. The 10% molybdate solution (reagent 3) was prepared by dissolving 1.25g of ammonium heptamolybdate tetrahydrate ($(NH_4)_6Mo_7O_{24}.4\ H_2O$) in 12.5 ml of distilled water. The antimony tartrate solution (reagent 4) was prepared by dissolving 0.05 g of potassium antimony tartrate ($K(SbO)C_4H_4O_6$) in 2 ml of distilled water. The mixed reagent (reagent 5) was prepared by mixing 12.5 ml of reagent 3 with 35 ml of reagent 1, then, added 2 ml of reagent 4, while stirring.

The standard phosphorus (PO_4^{3-}) stock solution (10 µM) was prepared by drying potassium dihydrogen phosphate (KH_2PO_4) at 105˚C and cooling in a desiccator. Then, 0.1361 g of KH_2PO_4 was dissolved in distilled water and added up to 100 ml by using a volumetric flask. The working phosphate (PO_4^{3-}) standard series was prepared by dilution with distilled water.

To prepare the standard curve, 0.5 ml of reagent 2 was added into working standard, and mixed, then added 0.5 ml of reagent 5, mixed and standed for 10 min. Colour of the solution changed into blue. The absorbance at 880 nm was measured with a spectrophotometer (DRAWELL DV-8200, China) and prepared standard curve by

plotting the absorbance values of standards versus the phosphate concentration, as shown in Figure 7.

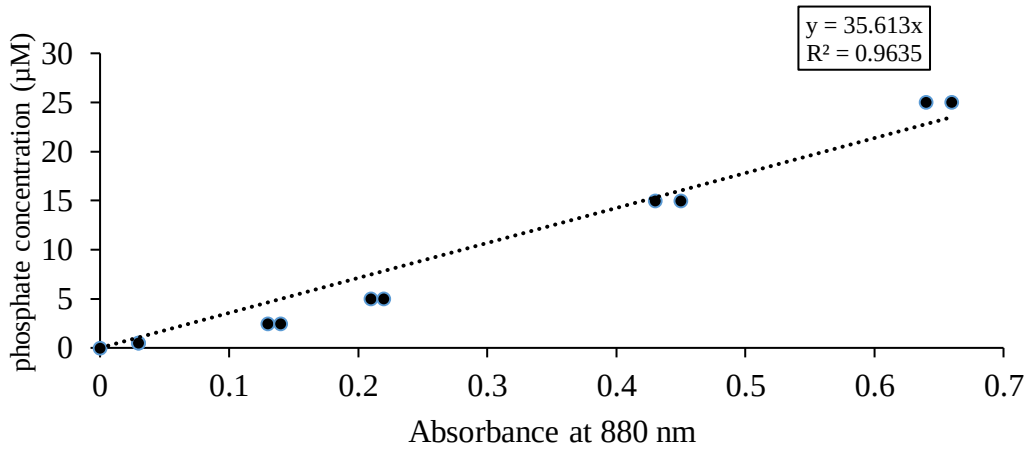

Figure 7 Absorbance values of standards versus phosphate concentration

For wastewater samples, the wastewater was collected and filtered using 0.45 µm filter to removed solid particles, and phytoplankton. Then, 25 ml of filtered wastewater was added in a conical flask (prepared two replicates), and the blank was prepared by adding distilled water instead of wastewater (prepared two replicates). Then, 0.5 ml of reagent 2 was added and mixed to 25 ml of wastewater or blank. Subsequently, 0.5 ml of reagent 5 was added, mixed, and standed for 10 min. The colour of the solution changed into blue. The absorbance at 880 nm was measured with a spectrophotometer (DRAWELL DV-8200, China) and determined the phosphate concentration from the standard curve.

$$\text{Concentration of phosphate } (\mu M) = \frac{\text{Absorbance at 880 nm}}{\text{Slope of standard calibration curve}}$$

3.4.2 Ammonium (NH_4^+)

Ammonium-free water was prepared by adding 2-3 pellets of NaOH in 1 L of distilled water, then boiled for 5 min and cooled it down. Ammonium-free water were used for chemical preparation and washed glassware in this experiment. To prepared 1 M sodium hydroxide, 4 g of NaOH was added in 75 ml distilled water, then adjusted the volume to be 100 ml in a volumetric flask. The phenol reagent (A) was prepared by dissolving 16 g phenol in 60 ml of ethyl alcohol, and added 120 ml of ammonium-free water. To prepared solution B, 0.12 g of di-sodium nitroprusside dehydrate was dissolved in 20 ml of ammonium-free water. After that, solution A and solution B were mixed, and kept in the brown bottle at 8˚C. The citrate solution was prepared by dissolving 48 g of trisodium citrate dihydrate and 4 g of Na-EDTA in 120 ml of distilled water, then added 2 ml of 1M NaOH and boiled until the water become lower than 100 ml. Then, ammonium-free water was added into100 ml in a volumetric flask for adjusted solution. To prepare 6% sodium hypochlorite, the stock standard ammonium (10 µM) was prepared by drying NH_4Cl at 100˚C for 1 hour and dissolving 0.0535 g of NH_4Cl in ammonium-free water.

To prepared standard curve (Figure 8), 1 µM, 5 µM, 10 µM, 25 µM, and 50 µM of stock standard ammonium were used, and added 1 ml of phenol, 0.5 ml of citrate solution, and again added 1 ml of sodium hypochlorite, mixed it properly and standed for 30 min. The solution changed into blue colour. Finally, absorbance at 630 nm was measured, then plot absorbance versus ammonium concentration.

For the sample, 25 ml of filtered wastewater was used, and added 1 ml of phenol, 0.5 ml of citrate solution, and 1 ml of sodium hytochlorite, mixed and standed for 30 min. Solution changed into blue colour, and finally measured absorbance at 630 nm in spectrophotometer (DRAWELL DV-8200, China).

$$\text{Concentration of ammonium } (\mu M) = \frac{\text{Absorbance at 630 nm}}{\text{Slope of standard calibration curve}}$$

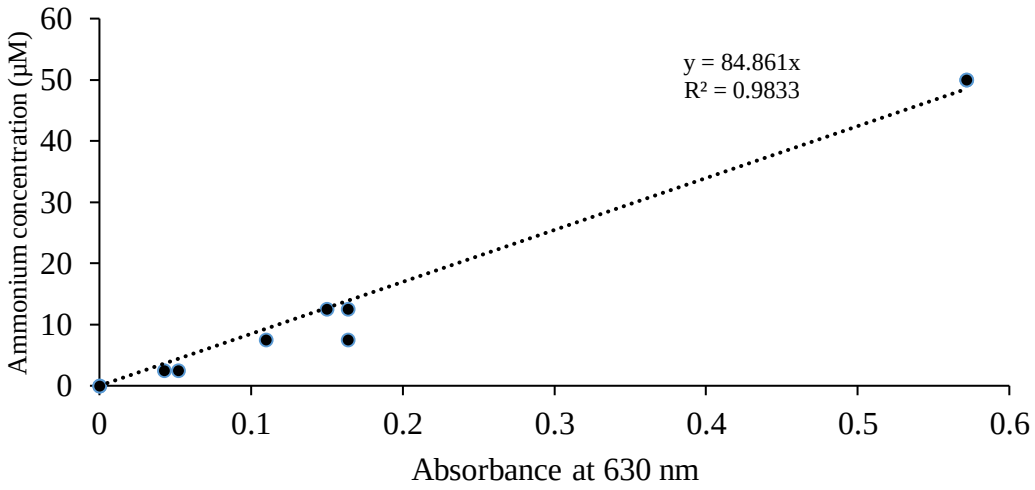

Figure 8 Absorbance values of standards at 630 nm versus ammonium concentration

3.4.3 Nitrite (NO_2^-)

Sulfanilamide solution was prepared by adding 1 g of sulfanilamide in a mixture of 10 ml conc. HCl and 50 ml distilled water, then added distilled water up to 100 ml. To prepare NED solution, 0.1 g of N-(naphthyl)-ethylenediamine dihydrochloride (NED) was dissolved in 100 ml distilled water. I prepared standard nitrite (NO_2^-) stock solution (10 µM) by drying anhydrous $NaNO_2$ at 105˚C, cooling in a desiccator, and dissolving 0.0690 g of $NaNO_2$ in 100 ml distilled water using a volumetric flask.

To prepare standard curve, 1 µM, 5 µM, 10 µM, 25 µM, and 50 µM of stock solution were used, and added 1 ml of sulfanilamide reagent, 1 ml of NED reagent and mixed properly, standed for 15 min which the solution turned into red colour. Finally, the absorbance at 540 nm was measured using a spectrophotometer (DRAWELL DV-8200, China) andthe concentration of nitrite against absorbance at 540 nm was plotted as shown in Figure 9.

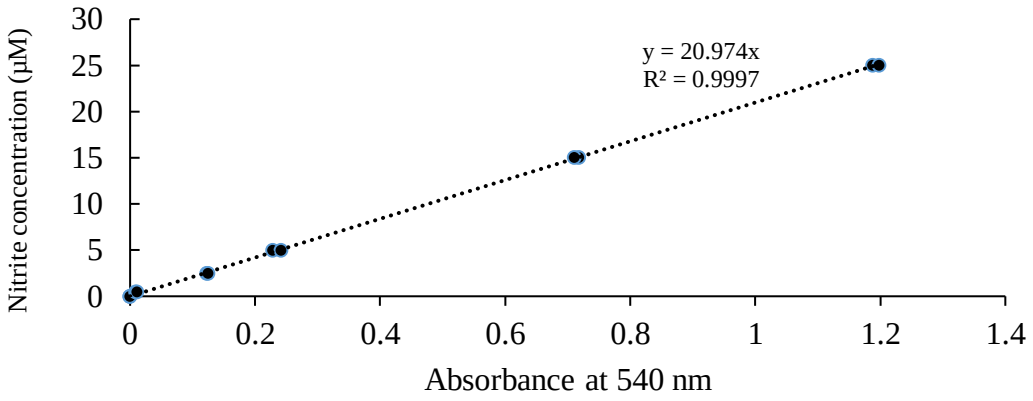

Figure 9 Absorbance values of standards at 540 nm versus nitrite concentration.

The sample wastewater was collected and filtered to removed solid particles and phytoplankton, and autoclaved it to remove the presence of bacteriaTwenty-five milliliters of filtered samples were pipetted and added into a flask (2 replicates), then added 1 ml of sulfanilamide reagent and 1 ml of NED reagent, mixed properly and standed for 15 min. Then, the solution turned to red. Finally, absorbance at 540 nm was measured following the equation by using a spectrophotometer (DRAWELL DV-8200, China) and the concentration of nitrite in µM unit was calculated

$$\text{Concentration of nitrite } (\mu M) = \frac{\text{Absorbance at 540 nm}}{\text{Slope of standard calibration curve}}$$

3.4.4 Nitrate (NO_3^-)

To prepare sulfanilamide solution, 1 g of sulfanilamide and 10 ml of conc. HCl was added to 50 ml of distilled water. Then, the volume was adjusted to 100 ml by adding distilled water. The NED solution was prepared by dissolving 0.1 g of N-(naphthyl)-ethylenediamine dihydrochloride (NED) in 100 ml distilled water. For the standard nitrate (NO_3^-) stock solution (10 µM), anhydrous KNO_3 (AR grade) was dried at 105˚C, and cooled in a desiccator. After that, 0.1011 g of KNO_3 was dissolved in 100 ml distilled water using a volumetric flask.

To prepare standard curve, 1 µM, 5 µM, 10 µM, 25 µM, and 50 µM of standard nitrate stock solution, were used andadded 1 ml of sulfanilamide reagent and 1 ml of NED reagent, mixed properly and standed for 15 min. The solution turned into red. The absorbance of nitrate was measured at 540 nm using a spectrophotometer (DRAWELL DV-8200, China), and finally plotted the standard curve as showned in Figure 10.

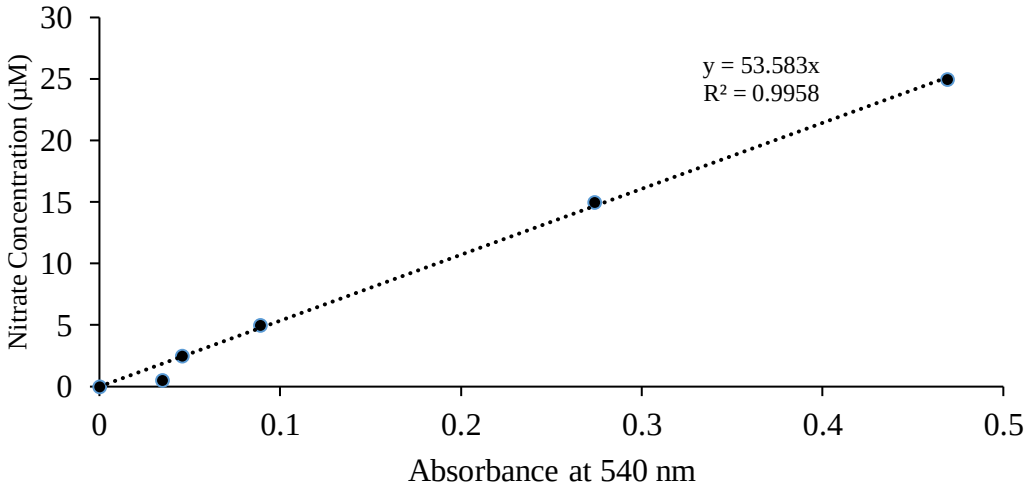

Figure 10 Absorbance values of standards at 540 nm versus nitrate concentration

To prepare ammonium chloride buffer, 10 g of ammonium chloride (NH_4Cl) was dissolved in 1 L of distilled water, then adjusted pH to 8.5. Copper sulfate solution was prepared by dissolving 1 g of copper sulfate ($CuSO_4$) in 100 ml distilled water. Then, cadmium granules were washed in HCl for 5 min, and washed again with distilled water for 2-3 times. After that, cadmium granules were put in 2% copper sulfate, and stirred them until blue colour of copper sulphate disappeared, and brown colloids appeared. Then, cadmium granules were washed with distilled water until colloids disappeared for 8-10 times and soaked cadmium granules in ammonium chloride buffer (pH 8.5).

To prepare the column, 50 ml ammonium chloride buffer was poured into the column, then cadmium granules was slowly added to make 20 cm height. Again, 50 ml of ammonium chloride buffer was added, then adjusted the flow rate as 10 ml per 1 min. After flow rate was adjusted, 60 ml of mixture solution between wastewater and

ammonium buffer (1:1) was added. Then, 35 ml of solution was discarded, and only 25 ml of solution was collected. After that, 25 ml of ammonium chloride buffer was added to washed the left over samples, and continue with other samples as mentioned. For the blank, distilled water was used instead of wastewater sample. Then, the 25 ml of collected sample or blank was added with 1 ml of sulfanilamide reagent, and 1 ml of NED reagent, mixed and standed for 15 min. The solution turned to red. Finally, the absorbance at 540 nm was measured using a spectrophotometer (DRAWELL DV-8200, China).

$$\text{Concentration of nitrite/nitrate } (\mu M) = \frac{\text{Absorbance at 540 nm}}{\text{Slope of standard calibration curve}}$$

$$\text{Concentration of nitrate } (\mu M) = \text{Concentration of nitrite/nitrate} - \text{Concentration of nitrite}$$

3.4.5 Dissolve oxygen (DO)

To prepare the manganous sulfate reagent (reagent 1), 36.5 g of $MnSO_4 \cdot H_2O$ was dissolved in 100 mL distilled water until the solution becomes pink. Then, the alkaline potassium iodide solution (reagent 2) was prepared by dissolving 30 g of KOH in 50 ml distilled water. Then, 60 g potassium iodide (KI) was added into a mixture of reagent 1 and reagent 2, then solution became transparent. To prepare 0.01 N standard thiosulfate solution (reagent 3), 2.9 g $Na_2S_2O3 \cdot 5H_2O$, and 0.05 g sodium carbonate (Na_2CO_3) was dissolved in 1 L of distilled water. And, prepararion of starch indicator solution (reagent 4), 1.0 g of soluble starch was dissolved in 100 ml distilled water, and boiled until completely dissolved. In preparation of 0.01 N standard iodide solution (reagent 5), potassium iodate (KIO_3) was dried at 105˚C for 1 hour, and cooled it. After drying, 0.3567 g KIO_3 was dissolved in 1 L of distilled water. And final reagent (6) was conc. sulfuric acid (H_2SO_4).

To collect the wastewater, BOD bottle was rinsed twice with wastewater, and filled the bottle slowly to avoid air bubbles occur in the BOD bottle. Then, 1 ml of

reagent 1 and 1 ml of reagent 2 were added to form a precipitated manganese (III) hydroxide. Then, the bottle was closed with gound-glass stopper to avoid oxygen in the air, the bottle was inverted upside-down, and precipitate was mixed. Finally, the BOD bottles were labeld, and taken to laboratory.

DO in wastewater was evaluated by adding 1 ml of conc. H_2SO_4 into BOD bottle. Wastewater wasinverted upside-down until the precipitates were completely dissolved. After that, 50 ml of the clear solution was pipetted into the conical flask using a volumetric pipette and titrated with 0.01 N sodium thiosulphate until the colour of solution becoming to pale yellow. After that, starch solution was added until the colour changed into blue. Titration was repeated with 0.01 N sodium thiosulphate until blue colour disappeared. Each sample was done in duplication. Finally, the DO with a unit of mg L^{-1} was calculated as the following equation.

$$\text{DO (mg L}^{-1} = \frac{88.8 \times \text{f} \times \text{B}}{\text{A}}$$

A = volume of 0.01 N sodium thiosulphate used in titration of 0.01 N KIO_3

B = volume of 0.01 N sodium thiosulphate used in titration of water sample

To prepare the blank, 25 ml of distilled water was added with 1 ml of conc. H_2SO_4, 1 ml of manganous sulphate solution, and 1 ml of alkaline iodide solution. The solution was inverted upside-down to mix well.

For standardization of sodium thiosulphate, 50 ml of blank was pipetted, then 5 ml of 0.01 N KIO_3 was added by using a volumetric pipette, mixed and standed for 2 min. The solution became clear and brown, then titrated with 0.01 N sodium thiosulphate until colour of solution becoming pale yellow. Starch solution was added until the colour changed to blue. After that, 0.01 N sodium thiosulphate was titrated until the blue colour disappeared. Finally, the volume of 0.01 N sodium thiosulphate was used for calculation of DO.

3.5 Microbial community analysis

To investigate the bacterial composition, samples were collected at the end of the experiment. Plant roots were washed two times in autoclaved milliQ water to remove clay from plant roots and collected in a plastic tube for DNA extraction. One hundred milligrams of plant roots were homogenized in 200 µl Buffer A solution (100 mM Tris-HCl, 100 mM ethylenediaminetetraacetic acid (EDTA), 100 mM NaCl, and 0.5% sodium dodecyl sulfate (SDS), pH 7.5) using pestle, and incubated at 65°C for 30 min. Then, 400 µl of mixture between 5 M potassium acetate and 6 M lithium chloride (1:2.5) was added, mixed, incubated on ice for 10 min, and centrifuged for 15 min at 15000 rpm. Five hundred microliters of the supernatant were transferred to a new microcentrifuge tube and mixed with 300 µl of isopropanol, then centrifuged for 15 min at 15,000 rpm. Finally, the supernatant was discarded, and the precipitate was cleaned with 70% ethanol, dried until ethanol completely evaporated, and re-suspended in 50 µl of MilliQ. All DNA samples were kept at -20˚C until use in for amplification of 16S rRNA gene.

The V3-V4 hypervariable region of bacterial 16S rRNA gene was amplified using 2x KAPA HiFiHotStart Ready mix DNA polymerase (Kapa Biosystems Ltd., London, UK) with primers with overhang adapter sequences (forward primer: 5'–TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCCTACGGGNGGCWGCAG–3', and reverse primer: 5'-GTCTCG TGGGCTCGGAGATGTGTATAAGAGACAGGACTACHVGGGTATCTAATCC-3') (Johnston, et al. 2017). Cycle condition was 3 min at 95°C followed by 25 three-temperature cycles (30 s at 95°C, 30 s at 55°C, and 3 min at 72°C), then a final extension of 72°C for 5 min. Libraries were purified using AMPure XP beads (LABPLAN, Naas, Ireland) following protocol for preparation of the Illumina 16S metagenomics sequencing library. Dual Indices from the Illumina Nextera XT index kit (Illumina, San Diego, USA) were added to the amplicons of partial 16S rRNA gene using 2x KAPA HiFiHotStart Ready mix DNA polymerase (Kapa Biosystems Ltd, London, UK) under the following condition: 3 min at 95°C followed by 9 cycles of 30 s at 95°C, 30 s at 55°C and 3 min at 72°C, then a final

extension of 72°C for 5 min. Libraries were purified using AMPure XP beads (LABPLAN, Naas, Ireland).

The barcoded amplicon libraries were pooled, diluted and denaturated following a protocol of the Illumina Miseq library preparation. The sequencing was run on the Illumina Miseq using the 600 cycle Miseq reagent kit (version 3) with paired 301bp reads. All sequence data produced in this study have been deposited to NCBI SRA repository and are available via series accession number PRJNA542551.

Paired-end read sequences generated from Illumina Miseq were processed using Illumina 16S Metagenomics (version 1.0.1) workflow in BaseSpace-Illumina (https://basespace.illumina.com/). Each read was blasted against the Illumina-curated version of the Greengenes database (greengenes.secondgenome.com/downloads/database/13_5) to determine the operational taxonomic units (OTUs) which corresponded to the 16S rRNA gene sequence. Taxon which did not inform enough to further classification such as "Unclassified at Kingdom level" and "Viruses" were excluded from subsequent diversity analysis. OTU richness, Shannon diversity index and Evenness based on the genus of bacteria were calculated.

3.6 Statistical analysis

The results of wastewater parameters were shown in mean ± SE. The normality and the homogeneity of variance were tesed using the Bartlett test and Shapiro-Wilk test. Then, multiple comparisons among the treatments were performed using one-way ANOVA and Tukey HSD test in software R, version 3.5.2. (R Development Core Team, 2018). The initial and final fresh weight were compared using Welch's t-test. The significant difference between treatments was set at $p < 0.05$.

CHAPTER 4

RESULTS

In this study, the ability of aquatic plants *Echinodorus cordifolius* and *Lepironia articulata*, and their microbes to improve the quality of domestic wastewater were investigated. The parameters of phosphate, ammonium, nitrate, nitrite, DO, pH, temperature and fresh weight of the plants on the initial day and compared with the values of those parameters in the last day of the experiment were measured. The results suggested that unsterilized *E. cordifolius* and *L. articulata* significantly exhibited higher ability to remove phosphate and nitrogen from wastewaterwhen compared to controls ($p < 0.05$).

4.1 Temperature, pH and DO

Temperature, pH and DO of wastewater at the beginning and the end of the experiment were measured. The results showed that the temperature, pH and DO of the initial wastewater were 31.45 ± 0.27°C, 6.55 ± 0.00 mg L^{-1} and 7.89 ± 0.20 mg L^{-1}, respectively (Table 1).

Table 1 Characteristics of domestic wastewater before the experiment (day 0). Data are shown as mean ± SD.

Parameter	Initial wastewater (day 0)
Temperature (^oC)	31.45 ± 0.27
pH	6.55 ± 0.00
DO (mg L^{-1})	7.89 ± 0.20
PO_4^{3-} (µM)	46.04 ± 0.04
NH_4^+ (µM)	57.35 ± 4.05
NO_2^- (µM)	68.62 ± 1.81
NO_3^- (µM)	556.49 ± 6.96

After culturing aquatic plants for five days, there was significant differences between water temperature of control treatment and the other treatments. However, pH and DO were not different among the treatments (Table 2).

Table 2 Temperature, pH, and dissolved oxygen (DO) of domestic wastewater after five days of the experiment. Data are shown as mean ± SD. Values marked by different letters indicate significant differences between treatments (Tukey HSD test, $p < 0.05$). (EC and LA means *E. cordifolius* and *L. articulata*, respectively.)

Parameters	After experiment (day 5)				
	Control	Sterilized EC	Unsterilized EC	Sterilized LA	Unsterilized LA
Temp. (°C)	35.1 ± 0.2[a]	33.2 ± 0.1[b]	33.4 ± 0.3[b]	33.4 ± 0.3[b]	33.6 ± 0.2[b]
pH	4.48 ± 0.45	4.53± 0.23	4.01 ± 0.05	4.26 ± 0.27	4.01 ± 0.05
DO (mg L^{-1})	5.95 ± 0.51	6.55 ± 1.31	6.31 ± 0.36	5.98± 0.62	5.86 ± 0.3

4.2 Fresh weight of aquatic plants

This study used the same initial fresh weight of *E. cordifolius* and *L. articulata* and cultured these plants in the domestic wastewater. After 5 days, fresh weight of unsterilized *E. cordifolius* was significantly different from sterilized and unsterilized *L.*

articulata (Tukey HSD test, $p < 0.05$), however, no significant difference between sterilized and unsterilized *E. cordifolius* (Tukey HSD test, $p > 0.05$). Final fresh weight of both sterilized and unsterilized *L. articulata* slightly decreased from the beginning, but the difference between initial and final fresh weight was not found (Welch's t-test, $p > 0.05$) (Table 3).

Table 3 Fresh weight of plants before and after an experiment. Data are shown as mean ± SD. Values marked by different letters indicate significant differences between treatments (Tukey HSD test, $p < 0.05$).

Fresh weight of the plants	Initial Fresh weight (g)	Final Fresh weight (g)
Sterilized *E. cordifolius*	37.10 ± 0.44	37.90 ± 2.59^{ab}
Unsterilized *E. cordifolius*	38.03 ± 0.67	43.06 ± 4.80^{b}
Sterilized *L. articulata*	36.87 ± 0.06	31.35 ± 2.89^{a}
Unsterilized *L. articulata*	36.76 ± 0.65	35.63 ± 1.06^{a}

4.3 Phosphate (PO_4^{3-}) removal

Unsterilized plants used in this study significantly exhibited a higher degree of phosphate removal when compared to that of sterilized plants (Tukey HSD test, $p < 0.05$). After five days, sterilized *E. cordifolius* removed 93.64 ± 0.51 %, unsterilized *E. cordifolius* removed 97.50 ± 0.53 %, sterilized *L. articulata* removed 92.20 ± 0.39 %, and unsterilized *L. articulata* removed 96.35 ± 0.79 % of

phosphate (Figure 11).

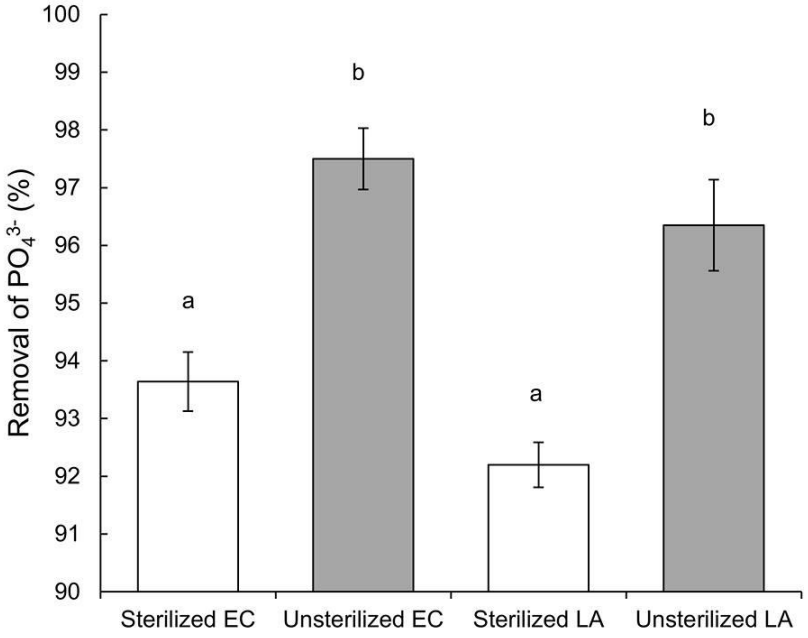

Figure 11 Removal percentage of phosphate (PO_4^{3-}) in sterilized *E.cordifolius*, unsterilized *E. cordifolius*, sterilized *L. articulata*, and unsterilized *L. articulata*. (n = 3 replicates). Data are shown as mean ± SD. Different letters indicate significant differences between treatments. (Tukey HSD test, $p < 0.05$). EC and LA means *E. cordifolius* and *L. articulata*, respectively.

4.4 Nitrogen (NH_4+, NO_3^-, NO_2^-) Removal

4.4.1 Ammonium (NH_4^+) removal

The results showed that unsterilized plants used in this study significantly higher removed ammonium when compared to that of unsterilized plants (Tukey HSD test, $p < 0.05$). Sterilized *E.s cordifolius* removed 58.44 ± 1.60 %, unsterilized *E. cordifolius* removed 69.92 ± 5.10 %, sterilized *L. articulata* removed 58.33 ± 5.71 %, and unsterilized *L. articulata* removed 70.69 ± 3.49 % of ammonium (Figure 12).

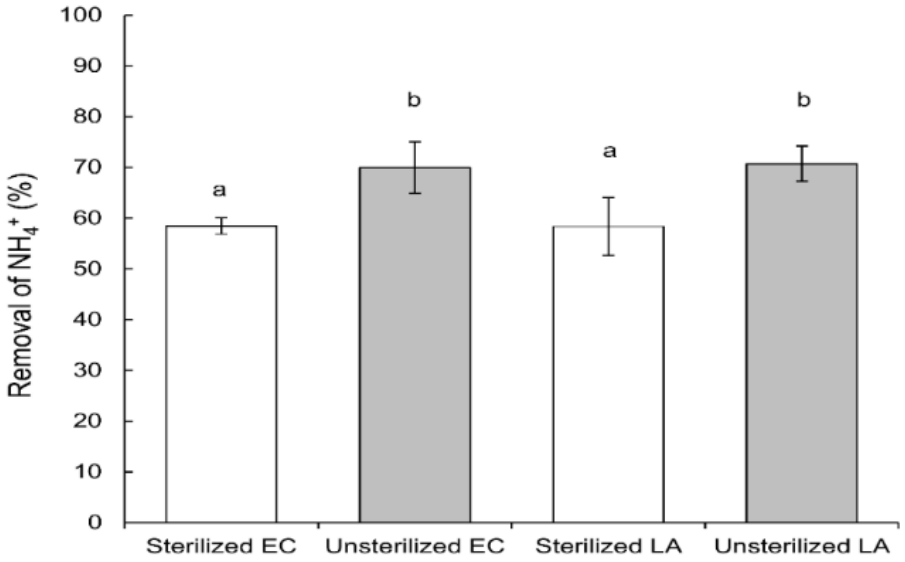

Figure 12 Removal percentage of ammonium (NH_4^+) in sterilized *E. cordifolius*, unsterilized *E. cordifolius*, sterilized *L. articulata*, and unsterilized *L. articulata*. (n = 3 replicates). Data are shown as mean ± SD. Different letters indicate significant differences between treatments. (Tukey HSD test, $p < 0.05$). EC and LA means *E. cordifolius* and *L. articulata*, respectively.

4.4.2 Nitrite (NO_2^-) removal

Unsterilized and sterilized plant significantly exhibited a difference of nitrite removal (Tukey HSD test, $p < 0.05$). Sterilized *E. cordifolius* removed 59.11 ± 3.68 %, unsterilized *E. cordifolius* removed 80.45 ± 1.48 %, sterilized *L. articulata* removed 54.46 ± 5.02 %, and unsterilized *L. articulata* removed 82.06 ± 2.47 % of nitrite (Figure 13).

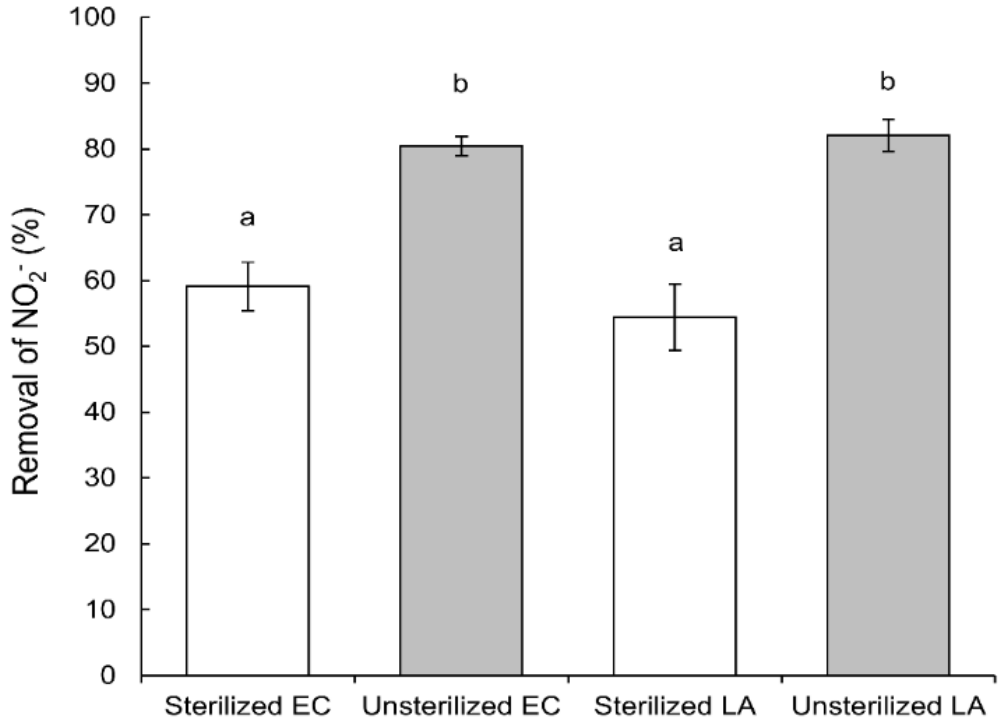

Figure 13 Removal percentage of nitrite (NO_2^-) in sterilized *E. cordifolius*, unsterilized *E. cordifolius*, sterilized *L. articulata*, and unsterilized *L. articulata*. (n = 3 replicates). Data are shown as mean ± SD. Different letters indicate significant differences between treatments. (Tukey HSD test, $p < 0.05$). EC and LA means *E. cordifolius* and *L. articulata*, respectively.

4.4.3 Nitrate (NO_3^-) removal

Sterilized *E. cordifolius* removed 12.24 ± 1.09 %, unsterilized *E. cordifolius* removed 51.90 ± 5.78 %, sterilized *L. articulata* removed 5.37 ± 1.58 %, and unsterilized *L. articulata* removed 53.57 ± 2.77 % of nitrate (Figure 14). There was a significant difference in nitrate removal between unsterilized and sterilized plants (Tukey HSD test, $p < 0.05$).

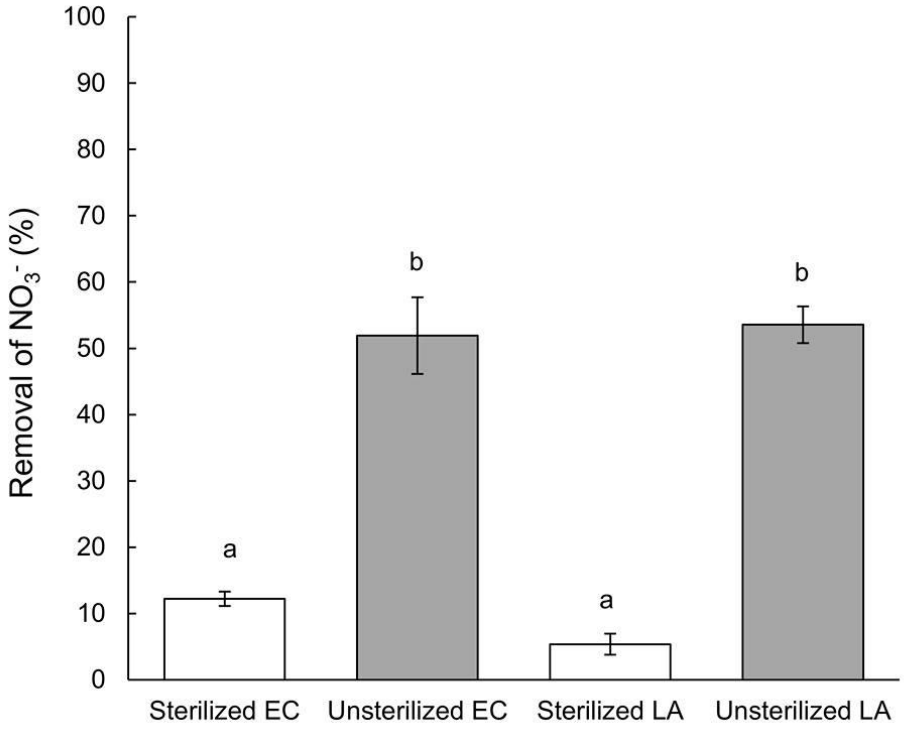

Figure 14 Removal percentage of nitrate (NO_3^-) in sterilized *E. cordifolius*, unsterilized *E. cordifolius*, sterilized *L. articulata,* and unsterilized *L. articulata*. (n = 3 replicates). Data are shown as mean ± SD. Different letters indicate significant differences between treatments. (Tukey HSD test, $p < 0.05$). EC and LA means *E. cordifolius* and *L. articulata*, respectively.

4.5 Bacterial community composition

Plants roots of sterilized and unsterilized plants on the initial day and after five days of the experiment were collected, and performed 16S amplicon sequencing using Illumina Miseq to investigate the bacterial community of plant roots. The results showed that each experimental set displayed different level of bacterial diversity (genus-based); sterilized *E. cordifolius* (2.68), unsterilized *E. cordifolius* (2.16), sterilized *L. articulata* (4.48), and unsterilized *L. articulata* (2.37) (Table 4).

Table 4 Richness (R), Diversity (H) and Evenness (J) of the bacterial community in six different samples - control *E. cordifolius* (before treatment), sterilized *E. Cordifolius* (after treatment), unsterilized *E. cordifolius* (after treatment), control *L. articulata*

(before treatment), sterilized *L. articulata* (after treatment), and unsterilized *L. articulata* (after treatment).

Sample ID	Richness (R)	Shannon Diversity Index (H)	Evenness (J)
Control *E. cordifolius*	518	2.39	0.0046
Sterilized *E. cordifolius*	559	2.68	0.0048
Unsterilized *E. cordifolius*	534	2.16	0.0040
Control *L. articulata*	448	3.47	0.0077
Sterilized *L. articulata*	644	4.86	0.0075
Unsterilized *L. articulata*	598	2.37	0.0039

Cyanobacteria (40.38%), Proteobacteria (37.33%), Firmicutes (8.37%), Actinobacteria (6.76%), and Bacteroidetes (0.87%) were the five most predominant phyla in sterilized *E. cordifolius*. Cyanobacteria (51.88%), Proteobacteria (34.52%), Firmicutes (7.53%), Actinobacteria (2.22%), and Bacteroidetes (1.01%) were the five most predominant phyla in unsterilized *E. cordifolius* (Figure 15A).

Proteobacteria (55.98%), Firmicutes (9.95%), Actinobacteria (8.42%), Bacteroidetes (3.39%), and Verrucomicrobia (3.18%) were the five most predominant phyla in sterilized *L. articulata*. Firmicutes (59.24%), Proteobacteria (24.21%), Bacteroidetes (12.08%), Actinobacteria (1.02%), and Cyanobacteria (0.36%) were the five most predominant phyla in unsterilized *L. articulata* (Figure 15B).

Nostocophycideae (39.77%), Alphaproteobacteria (18.63%), Betaproteo - bacteria (5.23%), Clostridia (6.31%), and Gammaproteobacteria (1.39%) were the five most predominant classes in sterilized *E. cordifolius*. Nostocophycideae (50.75%), Alphaproteobacteria (18.86%), Betaproteobacteria (8.59%), Clostridia (6.59%), and Gammaproteobacteria (4.71%) were the five most predominant classes in unsterilized *E. cordifolius* (Figure 15C).

Deltaproteobacteria (18.65%), Betaproteobacteria (13.37%), Alphaproteo-bacteria (13.40%), Actinobacteria (7.99%), and Gammaproteobacteria (7.45%) were

the five most predominant classes in sterilized *L. articulata*. Clostridia (51.28%), Bacteroidia (11.47%), Betaproteobacteria (8.45%), Bacilli (8.12%), and Alphaproteobacteria were the five most predominant classes in unsterilized *L. articulata* (Figure 15D).

The 559 and 534 bacterial genera were identified in sterilized and unsterilized *E. cordifolius*, respectively (Table 4). *Calothrix* (39.01%), *Hyphomicrobium* (9.03%), *Cystobacter* (5.54%), *Heliorestis* (4.61%), and *Candidatus Liberibacteria* (3.65%) were the five most predominant genera in sterilized *E. cordifolius*. *Calothrix* (49.88%), *Rickettsia* (10.55%) *Heliorestis* (5.78%), *Methylotenera* (2.89%), and *Phaeobacter* (2.35%) were the five most predominant genera in unsterilized *E. cordifolius* (Figure 15E).

The 644 and 598 bacterial genera were identified in sterilized and unsterilized *L. articulata*, respectively (Table 4). *Cystobacter* (8.95%), *Rubrivivax* (2.56%), *Methyloversatilis* (1.82%), *Haliangium* (1.69%), and *Clostridium* (1.32%) were five most predominant genera in sterilized *L. articulata*. *Clostridium* (49.06%), *Prevotella* (10.33%), *Bacillus* (4.05%), *Thiomonas* (3.86%), *Rickettsia* (2.87%), and *Ammoniphilus* (2.26%) were the five most predominant genera in unsterilized *L. articulata* (Figure 15F).

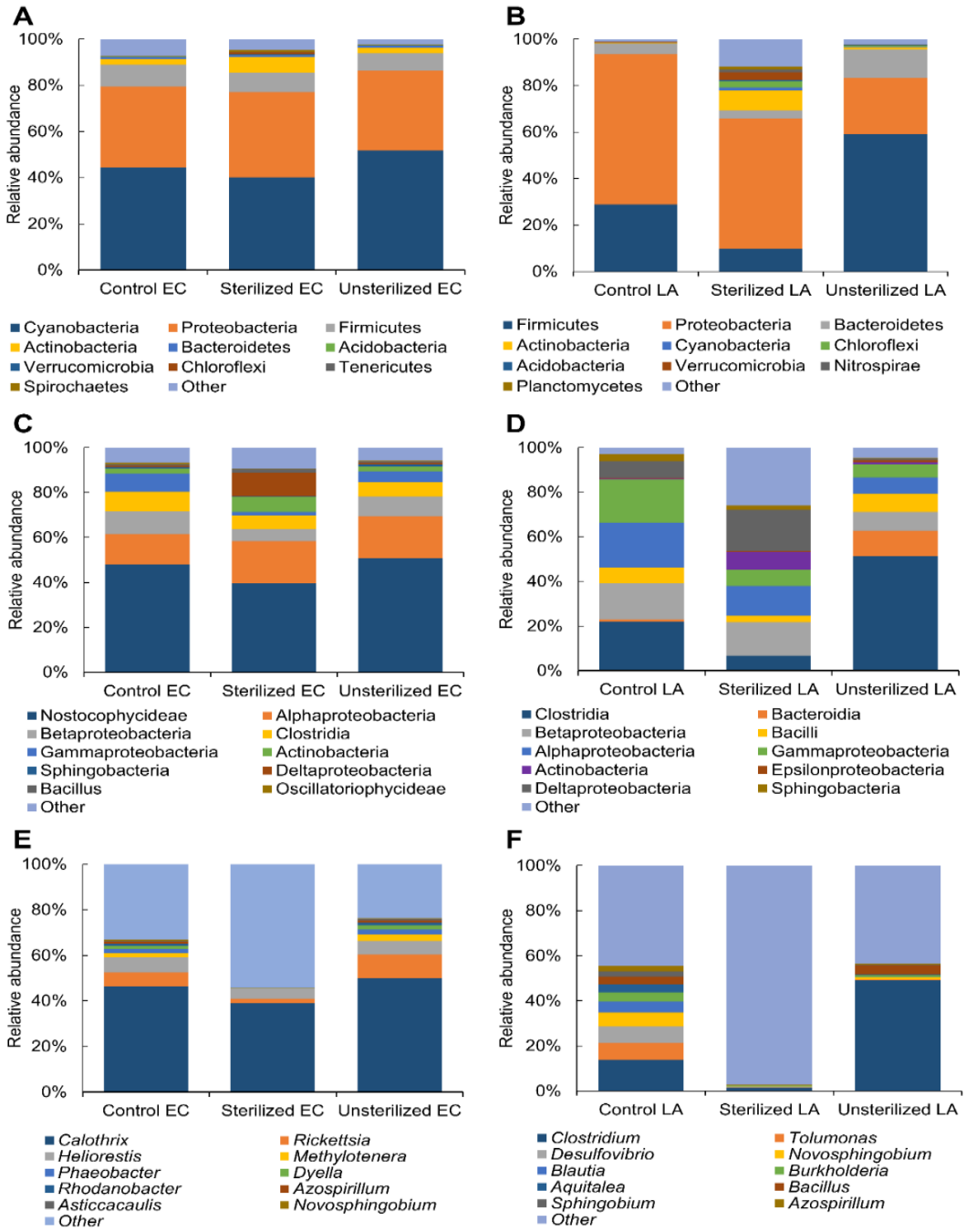

Figure 15 Bacterial community composition in control *E. cordifolius* (before treatment), sterilized *E. cordifolius* (after treatment) and unsterilized *E. cordifolius* (after treatment), Control *L. articulata* (before treatment), sterilized *L. articulata* (after treatment) and unsterilized *L. articulata* (after treatment) at the phylum (A and B), class (C and D), and genus (E and F) level (16S Metagenomics analysis). EC and LA means *E. cordifolius* and *L. articulata*, respectively.

CHAPTER 5

DISCUSSION

The ability of aquatic plants *E. cordifolius* and *L. articulata* with and without their associated microbes to remove phosphate and nitrogen from the sampled wastewaterin this study. The concentration of wastewater, phosphorus, and nitrogen before and after treating with sterilized and unsterilized *E. Cordifolius* and *L. articulata* were measured and the change of bacterial taxa presented in both sterilized and unsterilized plants using 16S rRNA gene amplicon analyses were investigated.

After five days of the experiment, higher degree of phosphate removal was observed in unsterilized *E. cordifolius* (97.50%) when compared to that of unsterilized *L. articulata* (96.35%). There were significant differences in the removal of phosphate in unsterilized plants when compared to sterilized groups The ability of these aquatic plants to remove phosphate from wastewater was similar to results from previous studies (Martinez et al., 2000; Yadavalli and Heggers, 2013). Interestingly, the removal efficiency of phosphorus found in this study was higher than those in previous studies. For example, hyacinth removes 18.76% and water lettuce removes 15.25% of phosphorus after 30 days of treatments (Gupta et al., 2012; Shah et al., 2014). Therefore, unsterilized *E. cordifolius* and *L. articulata* have a high efficiency for removal phosphorus. Moreover, the relative abundance of bacterial composition was also higher in unsterilized plants compared to sterilized plant. For instance, Cyanobacteria in unsterilized *E. cordifolius* (51.88 %) was higher when compared to sterilized *E. cordifolius* (40.38 %). Similarly, the relative abundance of Firmicutes in unsterilized *L. articulata* was increased to 59.24 % when compared to sterilized *L. articulata* (9.95 %). These indicated that Cyanobacteria and Firmicutes preferred to colonize in the unsterilized plant.

Higher degree of phosphorus removal was observed in sterilized *E. cordifolius* (93.64%) when compared to that of sterilized *L. articulata* (92.20%). However, there

was no significant difference in the removal of phosphorus in sterilized plants. Treatment of 1% sodium hypochlorite (NaClO) might remove the symbiotic bacteria present on the root surface in this study, which affects no difference in removal of phosphorus in sterilized plants. Previous studies also showed that 97-100% of different bacteria were removed from parrotfeather (*Myriophyllum aquaticum*), wheat (*Triticum aestivum*) and spinach (*Spinacia oleracea*) when they were exposed to 0.525% NaClO solution for 15 s and rinsed with deionized water (Medina et al., 2000). Furthermore, studies found that sterilized treatment of cobalt-60 irradiation and autoclaving eliminated microorganisms and found a lack of microbial growth on potato-glucose agar, plate count agar, and nutrient broth (Wolf et al., 1989). Interestingly, Xia and Ma (2005) proved that a microorganism removed 12% of phosphorus, and 69% was uptake by *Eichhornia crassipes*. These showed that removal of phosphorus in sterilized plants was mostly done by plants, though bacterial composition was present in sterilized plants. As per the 16S amplicon analyses, the relative abundance of Cyanobacteria (40.38%) and Proteobacteria (55.98%) in sterilized *E. cordifolius* and *L. articulata* was reduced than those in control *E. cordifolius* (49.39%) and *L. articulata* (64.66%), respectively. These indicated that growth of Cyanobacteria and Proteobacteria decreased as compared to the control. Studies had been reported that both the phylum are essential for carbon and nitrogen cycling (Nieto et al., 1989; Santos et al., 2019).

The removal efficiency of phosphorus was significantly higher in unsterilized plants than sterilized plants. For example, in this study, unsterilized *E. cordifolius* removed the higher amount of phosphorus (97.5%) than sterilized *E. cordifolius* did (93.63%). Furthermore, unsterilized *L. articulata* removed the higher amount of phosphorus (96.35%) than sterilized *L. articulata*did (92.20%). A similar result was demonstrated in a previous study where unsterilized *E. crassipes* removed 0.01659 h^{-1} of ethion (organophosphate pesticides), which was higher than that of sterilized *Eichhornia crassipes* (0.00930 h^{-1}) (Xia and Ma, 2005). Moreover, the relative abundance of bacterial composition was higher in unsterilized plants than that of sterilized plants. For instance, the relative abundance of *Clostridia* was 51.28% higher in unsterilized *L. articulata* than that of sterilized *L. articulata* (6.62%). Furthermore, the relative abundance of Nostocophycideae was 50.75% higher in unsterilized *E.*

cordifolius compared to that of sterilized *E. cordifolius* (39.77%). In addition, one of the previous studies also illustrated that the relative abundance of Proteobacteria (63.55%) was higher in unsterilized soil than sterilized soil (35.80%) (Hou et al., 2017). These results suggested that the relative abundance of bacteria was higher in unsterilized plants than that of sterilized plants.

Higher nitrogen (ammonium, nitrate, and nitrite) removal was observed in unsterilized *L. articulata* (70.69% of ammonium, 61.92% of nitrate, and 82.06% of nitrite) when compared to that of unsterilized *E. cordifolius* (69.92 % of ammonium, 61.49% of nitrate, and 80.45% of nitrite). However, we did not observed the significant difference of nitrogen removal between these two unsterilized plants. In previous studies, higher removal of nitrogen was reported by Chen and colleagues (2017). They found that removal of nitrogen was 92.8% by *Oryza sativa* higher than that of *Lactuca sativa* (90.7%) in 13 days. The nitrogen removal depends on different factors and number of the days of the experiment conducted.

The relative abundance of *Calothrix* (49.88%) was higher in unsterilized *E. cordifolius* than that of control *E. cordifolius* (46.38%). Similarly, the relative abundance of *Clostridium* (49.06%) in unsterilized *L. articulata* was higher than that of control *L. articulata* (49.06%). This result suggests that both phytoremediation and bioremediation might have involved in the removal of nitrogen.

Higher nitrogen (ammonium, nitrate, and nitrite) removal was observed in sterilized *E. cordifolius* (58.44% of ammonium, and 59.11% of nitrite) when compared to that of sterilized *L. articulata* (58.33% of ammonium and 54.46% of nitrite). In contrast, a different fashion of nitrate removal was investigated in this study. Sterilized *L. articulata* removed 24.15% of nitrate which was higher than that of sterilized *E. cordifolius* (13.17%). No significance was found between sterilized *E. cordifolius* and sterilized *L. articulata* in the removal of ammonium, but a significant difference was found between sterilized *E. cordifolius* and sterilized *L. articulata* in the removal of nitrite and nitrate.

The relative abundance of Betaproteobacteria in sterilized *L. articulata* (15.12%) was lower than that of control *L. articulata* (16.29%). Similarly, the relative abundance of Betaproteobacteria in sterilized *E. cordifolius* (5.23%) was lower when

compared to that of control *E. cordifolius* (9.96%). This is congruent with the results from Santos et al., (2019), where they also found a decrease in Betaproteobacteria when it is exposed in the substrates.

Higher nitrogen (ammonium, nitrate, and nitrite) removal was observed in unsterilized *E. cordifolius* (69.92 % of ammonium, 61.49% of nitrate, and 80.45% of nitrite) when compared to that of sterilized *E. cordifolius* (58.44% of ammonium, 59.11% of nitrite, and 13.17% of nitrate). Similarly, significantly higher nitrogen (ammonium, nitrate, and nitrite) removal was investigated in unsterilized *L. articulata* (70.69% of ammonium, 61.92% of nitrate, and 82.06% of nitrite) when compared to that of sterilized *L. articulata* (58.33 % of ammonium, 54.46% of nitrite, and 24.15% of nitrate). Similar results were also reported by Wang et al., (2008) that the removal of pyrene by *Typha Orientalis* in unsterilized soil was 77% higher than that of sterilized soil (59%).

Our 16S amplicon analyses revealed that the relative abundance of top two dominant bacteria groups was higher in unsterilized plants compared to that of sterilized plants. For instance, *Calothrix* (49.88%) in unsterilized *E. cordifolius*, and *Clostridium* (49.06%) in unsterilized *L. articulata* were higher, when compared to that of sterilized *E. cordifolius* (39.01%), and *L. articulata* (1.32%), respectively. *Calothrix* and *Clostridium* were the predominant bacteria and most relative abundance in this study. The presence of bacteria in both sterilized and unsterilized plants suggested that the methods used for sterilizing plants in this study removed higher ectophytic bacteria than that of endophytic bacteria. The higher removal of these ectophytic bacteria might have resulted in bacterial abundance difference between the sterilized and unsterilized groups.

Presence of higher abundance and diversity of bacteria in unsterilized plants might be a factor that resulted in removing more nitrogen in the unsterilized plants *Calothrix* and *Clostridium* were the predominant bacteria found in this study, which are belonging to Cyanobacteria and Firmicutes, respectively. The presence of a high abundance of these taxa might be incongruent with the high nitrogen reduction in the unsterilized *E. cordifolius* and *L. articulata*. Cyanobacteria used nitrogen for their metabolism, and *Calothrix* exhibited N-fixation activity (Nieto et al., 1989; Reuter et

al., 1986) Furthermore, *Clostridium* is related to nitrogen cycle and utilize the nitrogen. Alphaproteobacteria also helps in ammonium oxidation and denitrification (Wani et al., 2011; Ye et al., 2016).

Many studies have proved that symbiotic nitrogen-fixing bacteria freely lived in the rhizosphere where the roots of the plants released amino acids, sugar and organic acids which served as food for bacteria (Shrivastava et al., 2104). The growth of the bacteria is enhanced in rhizosphere due to roots exudates, and most of the microbes lives at root parts such as epidermis, root hair, cortex, and or in the rhizosphere. In return, symbiotic bacteria supply some essential nutrients, vitamins, and antibiotics, which are required for plant growth. These activities make rhizosphere to become the most important environment for exchanging nutrients between plants and microbes (Hartmann et al., 2008; Shrivastava et al., 2014). Furthermore, the relative abundance of bacteria showed that Cyanobacteria and Firmicutes grown more in unsterilized *E. cordifolius* and *L. articulata*, this results suggests that tissues of roots are specific to different kinds of bacteria to develop symbiotic association within aquatic plants (Gewin, 2010).

Endophytic colonization associated with plants-microbes has been studies by several researchers (Reinhold and Hurek, 2011). Bacteria living in the rhizosphere are able to enter into the inner parts of the root and survive as endophytic bacteria. For example, Proteobacteria and Actinobacteria were colonized in the roots of *Arabidopsis thaliana*, and enhance mutual benefits for *A. thaliana* (Schulze Lefert, 2012). Some of the studies also proved that endophytic bacteria can infiltrate the root cortex and survive as endophytic bacteria till they are released back into the soil when root senescence occurs (Bulgarelli et al., 2012). Therefore, the presence of microbiomes in the rhizosphere is effective for mutual benefits, moreover, most of the contaminated organic and inorganic has been removed through plant-microbe interaction (Gewin, 2012). Moreover, plant-growth-promoting rhizobacteria (PGPR), such as *Serratia*, *Bacillus*, *Pseudomonas*, and *Azospirillum*, which live in rhizosphere and helps in plant growth, and prevent the growth of pathogens (Kloepper, 1991; Montesinos et al., 2002). Therfore, ectophytic and endophytic bacteria helps in plants growth and development

with implication of phytoremediation enhancement (Hardoim et al., 2008; Weyens et al., 2008).

Presence of oxygen in the rhizosphere is also important for the pollutant degradation. Oxygen enters the soil by suction effects of water when it flows, moreover, oxygen is also inputted by the aquatic plants (Stottmeister et al., 2003). Most of the aquatic plants cannot survive without supply of oxygen (Crawford and Braendle, 1996). Plants roots system has ability to get oxygen from the atmosphere pass through the special tissues formed in rhizome, called aerenchyma (gas chamber), then enter to the roots (Stottmeister et al., 2003). The aerenchyma distributed almost 60 % of the total tissue volume (Grosse and Schro'der, 1986). Flow of gas through the tissue is taken by diffusion under both high and low pressure (Allen, 1997). For instance, gas transport in the plant has been studies in *Typha latifolia*, and found that different solubility of the oxygen depends on the level of pressure (Bendix et al., 1994; Allen, 1997). The microbes survive in the rhizosphere due to available oxygen, which can be used for their metabolism, and as well as for the respiration (Grosse et al., 1996). In this study, dominant bacteria in *E. cordifolius* and *L. articulata* are different. This might be because the tissue structures, especially aerenchymal tissues in *E. cordifolius* and *L. articulata* are found in different levels leading to involve in the level of oxygen transfer into the plant roots and rhizosphere. This might affect to the bacterial composition in plant roots. However, anatomy of plant tissues was not observed in this study, so, this should be investigated in the further experiments. Moreover, all the taxa of symbiotic bacteria in this study were identified using a software (Illumina Basespace), and we do not experimentally culture the above bacteria for the treatment of wastewater. Therefore, further research is needed to carry out for the improvement of wastewater treatment.

CHAPTER 6

CONCLUSIONS

The use of aquatic plants *E. cordifolius* and *L. articulata* together with their associated bacteria efficiently helps in removal of phosphorus (PO_4^{3-}) and nitrogen (NH_4^+, NO_3^-, and NO_2^-) from domestic wastewater. Unsterilized plants are better in the removal of phosphorus and nitrogen than that of sterilized plants. However, symbiotic bacteria presented in their roots also involves in the degradation of phosphate and nitrogen along with the uptake of selected plants. The dominant bacterium found in *E. cordifolius* is cyanobacteria *Calothrix*. And, the dominant bacterium found in *L. articulata* is firmicutes *Clostridium.* These bacteria in unsterilized plants has higher relative abundance than sterilized palnts (Table 5). These predominant bacteria presented in this study might be related in phytoremediation to eliminate phosphate and nitrogen from wastewater. In conclusion, *E. cordifolius* and *L. articulata* are efficiently useful for removals of excessive phosphate and nitrogen, and their associated bacteria also helps in phytoremediation.

Table 5 Phosphate and nitrogen removal, and number of bacterial 16S rRNA gene sequences in *E. cordifolius* and *L. articulata* plants

	PO_4^{3-}	NH_4^+	NO_3^-	NO_2^-	Read % phylum	Read % class	Read % genus
Sterilized EC	93.64	58.44	59.11	12.24	40.38 Cyanobacteria	39.77 Nostocophycideae	39.01 Calothrix
Unsterilized EC	97.50	69.92	80.45	51.90	51.88 Cyanobacteria	50.75 Nostocophycideae	49.88 Calothrix
Sterilized LA	92.20	58.33	54.46	5.37	9.95 Firmicutes	6.59 Clostridia	1.32 Clostridium
Unsterilized LA	96.35	70.69	82.06	53.57	59.24 Firmicutes	51.28 Clostridia	49.06 Clostridium

For further application, construction of treatment ponds with shallow reservoirs to mimic the wetland might be better to grow these two aquatic plants for the wastewater treatment to remove excess of nitrogen and phosphate. The cross-talk between the bacteria and plants are needed to study and deeply understand the mechanisms and functions of symbiotic bacteria in phytoremediation. However, the methods to control the amount of these plants in the treatment ponds should be considered, for example, the use of these plants as an energy source instead of coal.

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APPENDIX

Figure A-1 Acclimatizing the *Echinodorus cordifolius* and *Lepironia articulata* in greenhouse for 14 days.

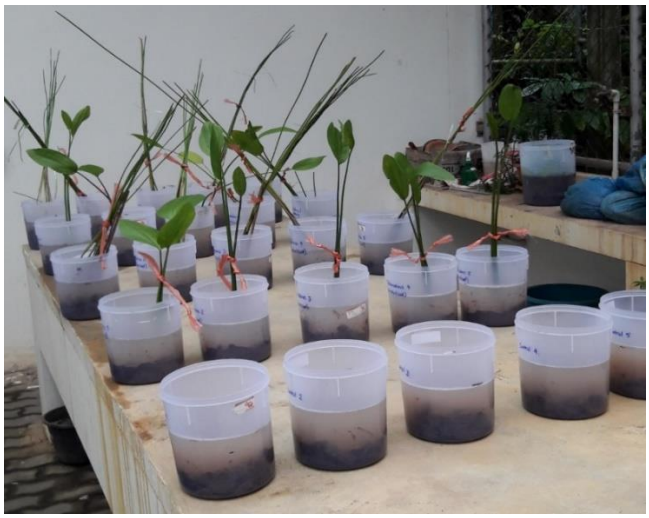

Figure A-2 Experimental set up for selected aquatic plants: *Echinodorus cordifolius* and *Lepironia articulata* with five replicates with controls.

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