STUDY THE PERFORMANCE OF DUCKWEED AND EFFECTIVE MICROBES IN REMOVING ARSENIC FROM THE SOIL OF PADDY FIELD AND REDUCING ARSENIC IN PADDY

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A project report submitted in partial fulfillment of the requirements for the award of Bachelor of Engineering (Hons) Environmental Engineering

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DECLARATION

I hereby declare that this project report is based on my original work except for citations and quotations which have been duly acknowledged. I also declare that it has not previously and concurrently submitted for any other degree or aware at UTAR or other institutions.

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APPROVAL FOR SUBMISSION

I certify that this project report entitled "**STUDY THE PERFORMANCE OF DUCKWEED AND MICROBE IN REMOVING ARSENIC FROM THE SOIL OF PADDY FIELD AND REDUCING ARSENIC IN PADDY"** was prepared by CHIN SZE JING has met the required standard for submission in partial fulfillment of the requirements for the award of Bachelor of Engineering (Hons) Environmental Engineering at Universiti Tunku Abdul Rahman.

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Specially dedicated to my beloved grandmother, mother and father

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STUDY THE PERFORMANCE OF DUCKWEED AND EFFECTIVE MICROBES IN REMOVING ARSENIC FROM THE SOIL OF PADDY FIELD AND REDUCING ARESNIC IN PADDY

ABSTRACT

Paddy is a source of carbohydrate for human consumption and statistically, more than 90% of Asian consumes rice. The more the intake of rice, more arsenic that are present in the soil are absorbed by the paddy. Arsenic is a metal that is harmful to the human health which is why phytoremediation plant (duckweed) and effective microbes were used in this research to reduce to the arsenic concentration in paddy soil and paddy grain.

Duckweed is able to absorb arsenic from the contaminated soil. The results show that the concentration of arsenic in soil will affect the concentration of arsenic in duckweed and also the reproduction rate of duckweed. Besides that, duckweed and effective microbes have the ability to decrease the arsenic concentration in paddy plant, but the method which is using the mixture of effective microbes and duckweed in reducing the arsenic in paddy had the least potential in reducing the arsenic concentration in paddy.

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LIST OF SYMBOLS/ ABBREVIATIONS

^{o}C	Degrees Celsius
^{o}F	Degrees Fahrenheit
%	Percentage
±	Both plus and minus operations
cm	Centimetre
mg/ha.d	Milligram per hectare per day
mg/kg	Milligram per kilogram
mg/L	milligram per litre
mL	millilitre
nM	Nano molar
Ppb	Parts-per-billion
rcf	Relative centrifugal force
µg/L	Microgram per litre
µL	Microliter
µM	Micro molar
µm	Micrometre
As	Arsenic
As^{III}	Arsenite
As^{V}	Arsenate
H_2O_2	Hydrogen Peroxide
AMF	Arbascular mycorrhizal fungi
BGA	Blue green algae
DMA	Dimethylarsinic acid
EM	Effective microorganisms

EPS	Extracellular polysaccharide
HPLC	High-performance liquid chromatography
Hsp70s	Heat shock protein 70s
ICPMS	Inductivity Coupled Plasma Mass Spectrometer
PD	Panicle differentiation
PGPRs	Plant growth-promoting rhizobacteria
PI	Panicle initiation
WHO	World of Health Organisation

CHAPTER 1

INTRODUCTION BRIEFING

1.1 Introduction

Rice is the most widely consumed staple food for a huge part of the world's human population. Though not all the place that has the suitable soil condition and environmental factors to support the growth of the healthy paddy. Paddy, is an annual grass with hollow, round, jointed culms; flat, narrow, sessile leaf blades joined to the leaf sheaths with collars; well-defined, sickle-shaped, hairy auricles; small acute to acuminate or two cleft ligules; and terminal panicles. With its ever increasing consumption throughout the world and this lead to the rise in plantation of paddy. Thus, natural arsenic that is present could be absorbed by the paddy and into the rice that will be consumed later.

Arsenic is a semi-metallic element that can be found in the environment such as:

i. Erosion of arsenic-containing rocks
ii. Volcanic eruptions
iii. Contamination from mining and smelting ores
iv. Previous or current use of arsenic-containing pesticides

There are two types of arsenic which are organic and inorganic. Inorganic arsenic (As) is identified as a non-threshold, class 1 human carcinogen, because it will cause damage to the health such as bladder and skin cancers (Chien et al, 2014). Besides that, according to Environmental Protection Agency (2007), exposure of chronic arsenic will cause:

i. Kidney damage and failure
ii. Anemia
iii. Low blood pressure and shock
iv. Central nervous system symptoms such as headaches, weakness and delirium
v. Increased risk of diabetes
vi. Adverse live and respiratory effects, including irritation of mucous membranes

Therefore, The World Health Organisation (WHO) has set concentration limits of arsenic for drinking water at 10 μg /L and for foodstuffs at 2 mg/ L (Mkandawire and Dudel, 2005).

Soil microorganisms play important roles in soil function, particularly in nutrient cycling, thus contributing a lot to sustainable crop production. The contribution of synthetic nitrogen fertilizers to crop production is unequivocal in agricultural systems. In general, the highest amount of nitrogen uptake by a crop is derived from the mineralization of soil organic matter. Soil microbes are vital for this mineralization process, and soil with higher nitrogen-supplying capacity is considered to have better soil fertility (Tojo and Hirasawa, 2014). Microorganisms have the potential to decrease the soil pollutants (Azizur etal, 2007).

Phytoremediation is a plant-based green technology used to remove environmental pollution. It is inexpensive and environmental friendly, thus it is a common method for water and soil remediation (Azizur et al, 2007). Some plants such as water hyacinth, azolla and duckweed have the ability to accumulate toxic metal at high concentration. In this experiment, the uptake and accumulation of arsenic by great duckweed *(Spirodela polyrhiza)* will be studied.

1.2 Problem Statements

It is hard to test the arsenic concentration in soil even the soil sample is taken from the same location due to the arsenic mobilization. Duckweed is a small floating macrophyte that can be transported with flowing water (raining) (Iqbal, 1999). Therefore, the water level must be maintained to prevent it from being flushed away. Few researches use duckweed as a companion with paddy. Besides that, the reproduction rate of duckweed is hard to investigate due to the very small size and rapid growth rate of duckweed. (Lemon, Posluszny and Husband, 2001). Lastly, the effective microbes provided need to be stored in a temperature less than 4^{o}C.

1.3 Study Objectives

i. To study the performance of duckweed in removing arsenic from paddy soil at different time frame.
ii. To investigate the reproduction rate of duckweed under shade in room temperature.
iii. To study the performance of effective microbes in removing arsenic from paddy soil.

CHAPTER 2

LITERATURE REVIEWS

2.1 Paddy

Paddy is an annual grass with round, hollow, jointed culms; narrow, flat, sessile leaf blades joined to the leaf sheaths with collars; well-defined, sickle-shaped, hairy auricles; small acute to acuminate or two cleft ligules; and terminal panicles (Karen Moldenhauer st al, 2015)

2.1.1 Types and Characteristics of Paddy

There are two species of paddy which are Asian rice (*Oryza Sativa*) and African rice (*Orayza glaberrima*) (DH. Beighley, 2012). However, the two major types of rice grown nowadays belong to the species of Asian rice which are *Oryza sativa indica* and *Oryza sativa japonica*. They can be differentiated by the area where they grow. *Japonica* types have either medium or short kernels and sticky when cooked, while *Indica* types have long kernels and mostly not sticky (DH. Beighley, 2012). **Table 2.1** shows the habit and distribution of different species of rice.

Table 2.1: Habit and Distribution of Different Species of Rice (Farmer's Portal, 2015)

Species	Habit	Distribution
Oryza sativa	Annual, cultivated	South and South-east Asia
O. nivara	Annual, wild	South and South-east Asia
O. rufipogon	Perennial, wild	Tropical Asia, Australia
O. glaberrima	Annual, cultivated	Tropical west Africa
O. barthii	Annual, wild	Sub-Saharan Africa
O. longistaminata	Perennial, wild	Tropical west Africa
O. glumaepatula	Parennial, wild	Tropical west Africa
O. meridionalis	Wild	Tropical Australia
O. officinalis	Perennial, wild	South and South-east Asia
O. minuta	Perennial , wild	Philippines
O. rhizomatis	Wild	Sri Lanka
O. eichingeri	Wild	Sri Lanka, Tropical Africa
O. punctata	Wild	Tropical Africa
O. latifolia	Wild	Central and South America
O. alta	Wild	Central and South America
O. grandiglumis	Wild	South America
O. australiensis	Wild	Tropical Australia
O. granulata	Wild	Tropical Asia
O. meyeriana	Wild	South-east Asia
O. longiglumis	Wild	Indonesia, Papua New Guinea
O. ridleyi	Wild	South-east Asia, Papua New Guinea
O. schlechteri	Wild	Papua New Guinea
O. brachyantha	Wild	Tropical Africa
P. coarctata	Perennial, wild	South Asia

2.1.2 Paddy Growth Stages

Figure 2.1 shows the growth stages for paddy in Arkansas. The life cycle of rice mostly take about 110 to 150 days in Arkansas. There are three stages of development in paddy growth:

i. Vegetation
ii. Reproduction
iii. grain filling and ripening/ maturation

In vegetation stage, the leave will start to emerge and increase their height. There are 6 steps in the vegetative stage:

i. Seed Germination
ii. Seedling Emergence
iii. Pre-Tillering
iv. Tillering
v. Maximum Tillering
vi. Vegetative Lag Phase

The seed requires two days for germination. At this step, the seed coat will become soft and elastic when the seed is soaked with enough amount of water. The temperature at this stage is very important, the optimum temperature is 87 ^{o}F. If the temperature is not in the range of 50 ^{o}F to 107 ^{o}F, the germination requires more time. At seedling emergence step, the first internode (mesocotyl) has elongated and pushed the tip of the rice through the soil surface. Then the seed needs to be transplanted to soil that cover not more than 0.75 inch because the mesocotyl at this step is still short. Seeding germination and emergence mostly take about 5 to 28 days, depending on the environment while pre-tillering takes 15 to 20 days for the development of the first fourth leaves. Tillering starts when the fifth leaf is visible and this step will continue until the maximum tiller number is reached, which is the step of maximum tillering. Vegetative lag phase is the step between end of tillering and beginning of reproductive stage, the height and stem diameter will increase in a slow rate. From tellering to vegetative lag phase takes about 24 to 67 days.

The reproductive stage takes about 30 days. 6 steps occur during this stage:

i. Panicle Initiation (PI)
ii. Internode Elongation
iii. Panicle Differentiation (PD)
iv. Booting
v. Heading
vi. Anthesis

Panicle Initiation (PI) occurs in the uppermost node of the culm which the panicle premordia starts to produce a panicle. Internode elongation and panicle differentiation (PD) are connected which the internode starts to elongate and the branching of panicle is visible. These steps will continue until the paddy reaches its full height. In the booting step, the flag leaf sheath will swell due to the increasing of the panicle size as it grows up the leaf shealth. Heading will take more than 10 to 14 days. In this step, the panicle will start to push up from the boot. Anthesis, also known as flowering, only lasts for 1 to 2 1/2 hours.

Ripening stage takes 30-45 days. During ripening stage, the light intensity is very important as more than 60 % of the carbohydrates need photosynthesis for grain filling which the grain will increase its size and weight by trans-locating of the sugars and starch from culms. At the end of this stage, the grain will change from green colour to gold colour. There are 4 steps in this stage:

i. Milk Stage Soft
ii. Dough Stage
iii. Hard Dough Stage
iv. Maturity

At milk stage, the kernel is filled with white milky stage starch grain. In soft dough stage, the grain is still soft but firmer than the milk stage. In hard dough stage, the grain is firm but the moisture content is still higher than 22 %. In maturity stage, the grain is hard and the moisture content is about 20 to 22 %. In this stage, the grain is ready for harvest (Karen Moldenhauer et al, 2015).

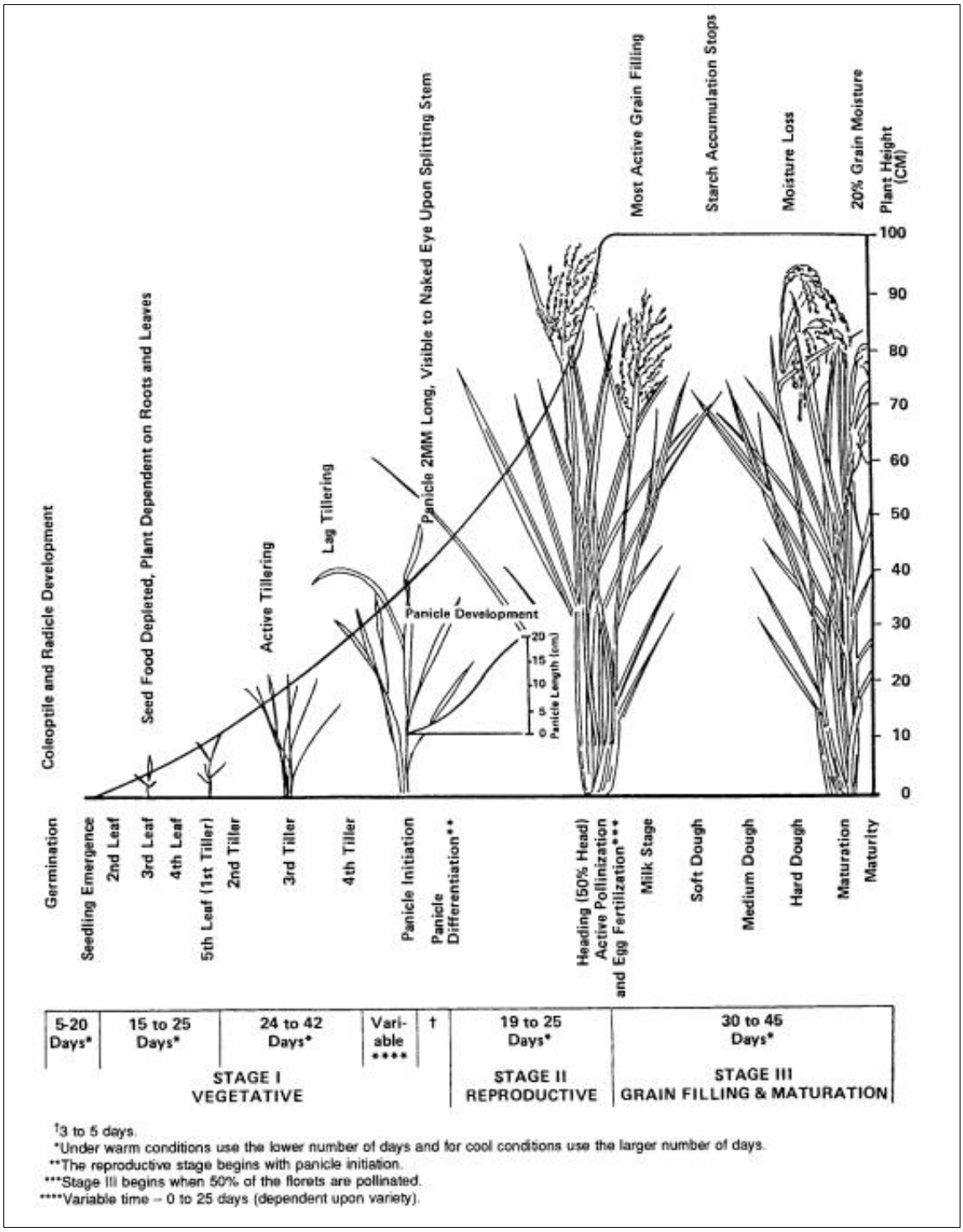

Figure 2.1: Development Stages of Paddy in Arkansas (Karen Moldenhauer et al, 2015)

2.2 Arsenic

Arsenic has an atomic number of 33 and 74.92 relative atomic mass. Arsenic is known as metalloid or semi-metal because its chemical and physical properties are between a metal and a non-metal. Besides that, it is under the Group VA of Periodic Table and has 4 oxidation states which are –3, 0, +3, and +5 but the major oxidation states of arsenic are arsenite, As^{III}, and arsenate, As^{V} (IARC, 2012). There are three major groups of arsenic compounds:

i. -inorganic arsenic compounds
ii. -organic arsenic compounds
iii. -arsine gas

According to International Agency for Research on Cancer (2012), the inorganic arsenic is carcinogenic. It has been found that arsenite is more toxic than arsenate due to its solubility in water is 4 to 10 times higher than arsenate, but arsenate is the major contaminant in ground water as it is thermodynamically more stable (Tangahu et al., 2011). The mean concentrations of arsenic in soil and sediment are 5mg/kg, but overall it ranges from 1mg/kg to 40mg/kg (IARC, 2012). From the research of Azizur Rahman et al, (2008), arsenic concentration in rice grain was 0.57 ± 0.02 mg/ kg and the highest concentration is in the grains grown on soil treated with 40 mg As/ kg soil. Azizur also stated that the acidity of soil will affect the arsenic uptake by plant. When the pH of the soil is below 5, the uptake of arsenic by paddy may also be increased.

2.2.1 Arsenic in Paddy

Rice is a main source of inorganic arsenic, because rice is a staple food in Asia and other countries (Naito et al, 2015). The major species of arsenic in rice are As^{III}, As^{V}, and dimethylarsinic acid (DMA) (Naito et al, 2015). The uptake of arsenic by paddy is through the root, with accompany of phosphate. The mobilisation of some absorbed arsenic from sediments and the paddy root area is caused by reducing of ferric iron to ferrous iron (Mkandawaire and Dudel, 2005).

From the research of H.K Das, (2002), the highest accumulation of arsenic is in the roots of paddy (2.4 mg/kg), following by the stem (0.73 mg/kg) and the lowest is in the rice grain (0.14 mg/kg). Besides that, the concentration of inorganic arsenic is the highest in bran, following by brown rice and the lowest is white rice. According to the research of Naito et al. (2015), the total arsenic concentrations in brans were 3 times higher than brown rice and 4 to 8 times higher than white rice. The concentration of arsenic in paddy plant at different stages will have different concentration. At panicle initiation stage, the concentration of arsenic and straw were 20.67 mg/kg while at maturity stage, the concentration was 20.67 mg/kg in straw and 1.67 mg/kg in husk, when the rice plant was grown in 60 mg of As/ kg soil (Azizur Rahman.et al, 2008).

2.3 Phytoremediation Plant

Phytoremediation is a technological solution used to remove pollutants from contaminated soil. It is affordable as it is environmental friendly and has low cost. **Figure 2.2** shows the uptake of organic and inorganic contaminant through the phytoremediation technology. For organic contaminants, the mechanisms are phytovolatilization, phytodegradation, rhizofiltration, rhizodegradation and phytostabilization while for inorganic contaminants, the mechanisms are phytovolatilization, phytoaccumulation, phytoextraction, rhizofiltration and phytostabilization. The definition of the uptake mechanisms on phytoremediation technology is shown in **Table 2.2** (Tangahu et al., 2011).

Table 2.2: Definition of Uptake Mechanisms on Phytoremediation Technology (Tangahu et al., 2011)

Mechanism on Phytoremediation technology	Definition
Phytovolatilisation	The uptake of a contaminant by a plant from the soil or water. The contaminant is passing through the plant and released out to the atmosphere.
Phytoextraction	The uptake of a contaminant by plant and translocation of the contaminant into the plant shoots.
Phytodegradation	The contaminants were taken up by plants and breakdown through metabolic processes or the breakdown of contaminants by the enzymes produced by plants.
Phytoaccumulation	The uptake of a contaminant and accumulated in plant shoots and leaves.
Rhizodegradation	The breakdown of contaminants in the soil through microbial activity that is enhanced by the presence of the root zone
Phytostabilisation	Some of the species of plant have the ability to immobilize the contaminants.
Rhizofiltration	Adsorption or precipitation onto plant roots or absorption into and sequesterization in the roots of contaminants that are in solution surrounding the root zone.

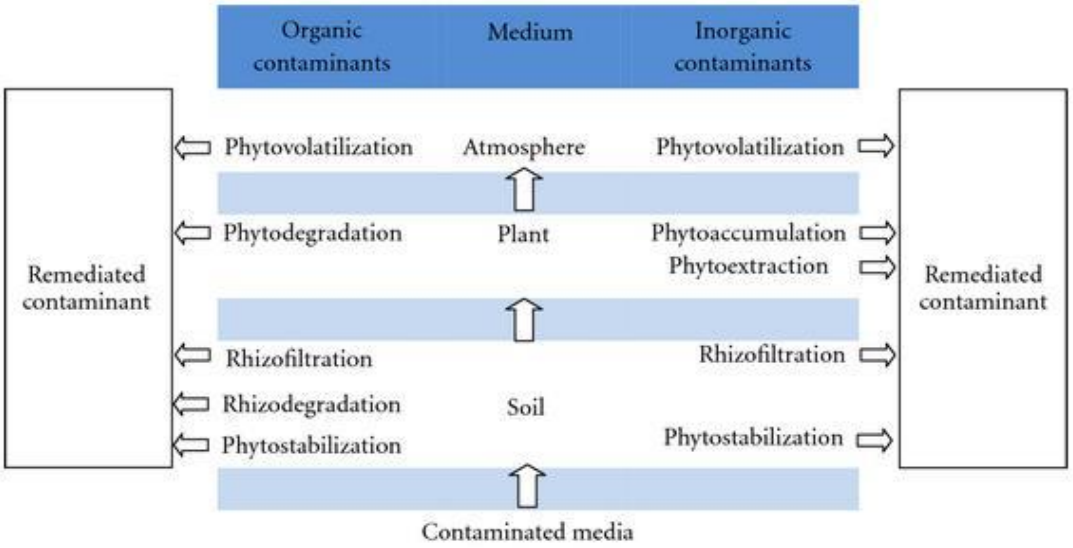

Figure 2.2: Uptake Mechanisms on Phytoremediation Technology (Tangahu et al., 2011)

The phytoremediation technology is effective in reducing contaminants, low cost, acceptable by public and environmental friendly, but it is a time consuming process, and will be affected by climate, level of contaminant, root depth and soil chemistry (Tangahu et al., 2011).

2.3.1 Azolla

Azolla is a fern frond that growing on the water surface. It is triangular/ polygonal shape. Different species have different range of diameter; the diameter of small species is from 1 to 2.5 cm while the diameter of *A. nilotica* and. *A. filiculoides* is more than 15 cm. Azolla is made up of alternate leaves and adventitious roots which connected to a main stem while some leaves have secondary stem develop at the axil (Ferentinos, Smith and Valenzuela,2002).

The water temperature and pH will affect the growth rate of azolla. The optimum temperature is approximately 30 ^{o}C. When the temperature is more than 35 ^{o}C, the growth rate will decrease and azolla will die when the temperature is above

45 ^{o}C. (Watanabe and Berja, 1983; Ashton and Waimsley, 1976). In a water temperature of 25 ^{o}C, azolla is able to live at a pH range of 3.5 to 10, but the optimum growth rate is at a pH range of 5 to 7 (Ashton, 1976; Cary and Weerts, 1992). Azolla can only live in humidity above 60 %, else it will become dry and fragile and the optimum humidity is at a range of 83 % to 90 % (Watanabe, 1983).

The concentration level of nitrogen in water will affect the growth rate of Azolla. At a 5nM concentration of nitrate, azolla achieves optimum growth rate. However, at the same concentration of ammonium- nitrogen (5 nM), the growth rate of azolla decreases (Singh et al, 1992).

2.3.2 Duckweed

Duckweed belongs to the family of azolla. However, it does not have stems or leaves; it only has a slightly round frond and a small rootlet that hangs down from underneath (Fcps.edu, n.d.). There are 14 species of *Lemnaceae*, which are:

i. *L. aequinoctialis*
ii. *L. disperma,*
iii. *L. ecuadoriensis,*
iv. *L. gibba,*
v. *L. japonica,*
vi. *L. minor,*
vii. *L. minuta,*
viii. *L. obscura,*
ix. *L perpusilla,*
x. *L. tenera,*
xi. *L. trisulca,*
xii. *L. turionifera,*
xiii. *L. valdiviana*
xiv. *L. yungensis,*

Besides that, *Spirodela*, *Wolffia* and *Wolffiella* belong to the family of *Lemnaceae* (Les et al, 2002).

Phytoremediation, metal and nutrient uptake studies, and bioassays usually use most species of the *Lemna* genus as model plants (Mkandawire and Dudel, 2005). The most studied species in the Lemnaceae family in ecotoxicology and phytoremediation are the *Lemna gibba L.* and the *Lemna minor L*. (Azizur Rahman et al, 2008). Arsenic accumulation in *L. gibba L.* in the tailing water of Lengenfeld and Neuensalz-Mechelgrün ranged between 0.54 and 110.8 mg/ kg in fresh mass, and 61.7 to 1966.48 mg/ kg in dry biomass.

Duckweed spreads very quickly, especially in water containing plenty of nitrogen and phosphates (Fcps.edu, n.d.). Great duckweed (*Spirodela polyrhiza L.*) has rapid growth, short life span, wide distribution and stability towards environmental changes (Azizur Rahman et al, 2008). From the research of Azizur Rahman, arsenic uptake in *S. polyrhiza L*. decreased by 3.18 folds when the plants are exposed to 4.1 μM of arsenate.

Common duckweed (*Lemna Minor L.)*, being one of the smallest flowering plants in the world has fronds that are usually pale green or sometimes a reddish-purple. They are no larger than 10 millimeters (Fcps.edu, n.d.). *L. minor* are able to uptake heavy metals from water and this has caused an increase in the occurrence of Heat Shock Proteins 70s (Hsp70s). Hsp70s play a primary role counteracting these toxic effects, protecting proteins from misfolding and proteolytic pathways, thus *L. minor* is considered a good bioaccumulator for heavy metals (Basile et al, 2015). The removal rate of arsenic for *L. minor* was 140 mg As/ ha.d with a removal recovery of 5 % (Alvarado et al, 2008).

2.4 Soil Microbes

Human activities, like land-use changes induce serious land degradation with potential effects on environment. Land degradation leads to the decline of microbial diversity and influences the environmental, economic and social sustainability. (Singh, 2015).

James J. Hoorman stated that 8-15 tons soil microbes (bacteria, fungi, actinomycetes, nematodes, protozoa, earthworms and arthropods) are found in 1 gram of soil as shown in **Table 2.3** (The Othio State University, 2010). Soil pH, humidity, soil, temperature or container medium composition, and target plant tissue (tuber, root, etc.) will affect the establishment of microbes in the soil (Hayes and Krause, 2010).

Table 1.3: The Abundance and Biomass of Major Soil Microorganisms (Tojo and Hirasawa, 2014).

Soil Microorganisms	Population		Biomass
	per m^2	per g-soil	wet kg/ ha
Bacteria	$10^{13} – 10^{14}$	$10^{8} – 10^{9}$	300 – 3000
Actinomycetes	$10^{12} – 10^{13}$	$10^{7} – 10^{8}$	300 – 3000
Fungi	$10^{10} – 10^{11}$	$10^{5} – 10^{6}$	500 –5000
Algae	$10^{9} – 10^{10}$	$10^{3} – 10^{6}$	10 – 1500
Protozoa	$10^{9} – 10^{10}$	$10^{3} – 10^{5}$	5 – 2000
Nematodes	$10^{6} – 10^{7}$	10 – 100	1 – 100
Earthworms	30 – 300		10 – 1000
Arthropods	$10^{3} – 10^{5}$		1 – 200

Below are some of the examples of microbial technologies for soil fertility which can be defined as the quality of a soil that enables it to provide nutrients in adequate amounts and in proper balance for the growth of specified plants or crops (Soil Science Glossary Terms Committee, 2008):

i. Effective microorganisms (EM) are one of such microbial technologies for agriculture and environmental stability. A constantly renewable biomass

source that is released to the environment is represented by the cyanobacteria (blue green algae or BGA). The extracellular polysaccharides (EPS), which get mineralized by the associated micro-flora, along with the BGA are beneficial in maintaining or restoring soil fertility (Singh, 2015). Cytobacteria produce bio-active compounds which increases growth of crops and attenuate soil pathogens.

ii. Various plant species show that under saline conditions, the ameliorative effects of microbes on soil fertility is clear. This is because plant growth-promoting rhizobacteria (PGPRs) have the potential to accumulate salts. Due to the synthesis of bio-active compounds, the enhancement in soil PGPR diversity may bring a few direct and indirect mechanisms for soil functioning and improved plant stress tolerance (Egamberdieva et al., 2013). Interaction of arbascular mycorrhizal fungi (AMF) and potential PGPR and their symbiotic with crop plants could be considered an emerging new tool for restoration of many degraded lands (Singh, 2015).

2.4.1 Effective Microbes (EM)

Effective microbes (EM) have the ability to improve the soil fertility and quality by attracting the nutritious elements (Sparling, 1992). There are 5 families of microorganisms in EM which are lactic acid bacteria, yeast, actinomycetes, photosynthetic bacteria and fungi. The functions of these microorganisms are shown in **Table 2.4** (EMRO, 2015).

Table 2.4: The Functions of Microorganisms in Effective Microbes (EMRO, 2015)

Families of Microorganism	Function
Lactic acid bacteria	Powerful sterilising properties.
Yeast	Manufacturing of anti-microbial and useful substances for plant.
Actinomycetes	Overcome harmful fungi and bacteria.
Photosynthetic bacteria	Enhance the production of the metabolites for the plant.
Fungi	Increases degradation speed of organic substances.

CHAPTER 3

METHODOLOGIES

3.1 Introduction

This project aims to investigate the performance of phytoremediation plant (duckweed) and effective microbes' application in reducing arsenic concentration in paddy plant. The phytoremediation plant was chosen as the companion plant with paddy while the microbe was applied every 2 weeks. Twelve 5.5 L bottles were used for paddy plantation. The heights of soil were maintained at about 15 cm. In this study, there were 4 sets of samples with triplicated which were:

i. The plantation of paddy without the application of effective microbes and also without the companion of duckweed (O1, O2, O3).
ii. The plantation of paddy with the companion of duckweed but without the application of effective microbes (D1, D2, D3).
iii. The plantation of paddy with the application of effective microbes but without the companion of duckweed (M1, M2, M3).
iv. The plantation of paddy with the application of effective microbes and with the companion of duckweed (MD1, MD2, MD3).

Another study was conducted by using six 1.5 L mineral bottles which were cut into 15 cm height with 8 cm height of soil and 3 cm of water level. There were 2 sets of samples with triplicated which were:

i. The plantation of duckweed with the application of effective microbes (md1, md2, md3).

ii. The plantation of duckweed without the application of effective microbes (d1, d2, d3).

The last study was to observe and record the reproduction rate of duckweed with and without contaminated soil. Twenty 500 mL water bottles were used in this study. The As in the soils from different samples were analysed using Inductively Coupled Plasma Mass Spectrometer (ICPMS) test in the lab of University Tunku Abdul Rahman.

3.2 Methodologies of the Plantation of Paddy

In this experiment, twelve 5.5L water bottles and 1 trial box were needed.

i. The paddy seeds were soaked in tap water for 24 hours.
ii. The seeds were dried in room temperature thereafter for 48 hours.
iii. The paddy seeds were sowed in one trial box as nursery.
iv. The soil from the water bottles was plowed.
v. The paddies were transplanted to water bottles 14 days after sowing.
vi. Totally 4 nurseries were transplanted into 5.5 L water bottles.
vii. The heights of soil were maintained at about 15 cm.
viii. Water level was maintained lower than 3cm during the first 10 days after transplanted.
ix. The paddies were irrigated once per 2 days in water level not over 5 cm 10 days and before 55 days after transplanting.
x. The water levels were dried from 55 days until 65 days.
xi. The water levels were maintained at the minimum level of 5cm 65 days after transplanting.
xii. The water levels were dried for 10 days from 85 days been transplanted.
xiii. During the 95 days after transplanted, the paddies grains were harvested.
xiv. The samples were sent to laboratory for further testing for their arsenic concentration.

3.2.1 Phytoremediation

There were totally 6 boxes used in this experiment. For the experiment of phytoremediation, coding D1, D2, D3, MD1 MD2 and MD3 were labelled. The paddies were planted with 40 duckweeds after transplanting. 20 duckweeds were collected and arsenic concentrations in duckweed were tested every 2 weeks.

3.2.2 Effective Microbes' Application

For microbes, there were 6 boxes used for the experiments. Codes M1, M2, M3, MD1, MD2 and MD3 were labelled to the samples. The samples were applied effective microbes once per two weeks. The grains were collected and the arsenic concentrations were tested.

3.3 Methodologies of Two Treatments without Paddy

There were total six 1.5 L water bottles needed in this experiment.

3.3.1 Phytoremediation

i. 3 mineral water bottles were cut into 15 cm height.
ii. The bottles were filled with soil in height of 8 cm.
iii. 3 cm of water levels were maintained for both of the samples.
iv. The bottles were labelled as d1, d2, and d3, respectively, which contain 40 duckweeds each.
v. 20 duckweeds were collected and the arsenic concentrations were tested by using ICPMS every 2 weeks.

3.3.2 Phytoremediation and Effective Microbes' Application

i. 3 mineral water bottles were cut into 15 cm height.
ii. The bottles were filled with soil in height of 8 cm.
iii. 3 cm of water levels were maintained for both of the samples.
iv. The bottles were labelled as md1, md2 and md3, respectively, which contain 40 duckweeds and 2 ml of effective microbes each.
v. The effective microbes were added every 2 weeks.
vi. 20 duckweeds were collected and the arsenic concentrations were tested by using ICPMS every 2 weeks.

3.4 Methodologies of the Reproduction Rate of Duckweed

In this experiment, twenty 500 mL of water bottles were needed.10 bottles were filled with 5 cm height of soil and the water levels were maintained at 3 cm, while another 10 bottles were filled with 8 cm height of water. One duckweed was put in each bottle. The number of duckweed that doubled its original amount against the number of days were observed and recorded.

3.5 Water Bath Extraction in HPLC-ICPMS Method (Raber et al., 2012)

Before carrying out this method, the samples need to be washed at least 3 times using ultra-pure water and dried in oven for 48 hours at 65 ^{o}C. About 0.015 g of powder samples were weighed. The samples were put into a 250 mL volumetric flask, and a 20 mL 0.02 M trifluoroacetic acid containing 50 μL of a 30% hydrogen peroxide (H_2O_2) solution was added. The samples were extracted by using shaking water bath at 95 ^{o}C for 1 hour. After cooled to room temperature, the samples were poured into 50 mL polypropylene tubes and were centrifuged for 15 min at 4700 rcf. 14.85 mL supernatants were added with 0.15 mL concentrated nitric acid and filtered using 0.45 μm syringe filter. The samples were ready for ICPMS analysis.

Adding trifluoroacetic acid to the sample during the water bath extraction HPLC-ICPMS method is because trifluoroacetic acid has the ability to remove t-butyl groups in protected amino acid by reacting with it and form t-butyl trifluoroacetate (Lundt et al., 1978). This will cause the extraction of arsenic from the protein. The duckweed samples will change from reddish orange to green colour after water bath. This is because anthocyanin pigments at the lower side of the duckweed were bleached by H_2O_2.

3.6 Preparation of Calibration Curve of As Concentration

i. 10 mL standard solution that contains 10 mg/ L As was taken by pipette.
ii. 89 mL of ultrapure water and 1 mL of concentrated nitric acid were added to the solution to dilute it into 1000 ppb As solution with 1 % of nitric acid.
iii. 50 mL of the solution prepared at (ii) was taken and added with 0.5 mL of concentrated nitric acid and 49.5 mL of ultrapure water to dilute the As solution into 500 ppb with 1 % of nitric acid.
iv. 2 mL of the solution prepared at (iii) was taken and added with 0.98 mL of concentrated nitric acid and 97.02 mL of ultrapure water to diluted As solution into 10 ppb with 1 % nitric acid.
v. All of the solution prepared was brought to ICPMS in order to prepare the calibration curve.

CHAPTER 4

RESULTS AND DISCUSSIONS

4.1 Arsenic Concentrations in Phytoremediation Plant

Figure 4.1 and **4.2** show the mean initial arsenic concentrations in duckweed were 74.601 ppb. The arsenic concentration in the duckweed dropped to the concentration of 0.256 ppb after transferring them to the paddy boxes. According to Prasad et al. (2001), the concentration of heavy metal in water will affect the concentration of heavy metal in duckweed by means of when the concentration is higher in water, concentration of heavy metal in duckweed will be higher.

The duckweed was collected from mining pond before it was transplanted into paddy boxes. The concentration of arsenic in mining pond is higher than the paddy soil provided. The decreasing concentration of arsenic can be explained as phytovolatilization and phytodegradation/ phyto-transformation of duckweed (Tangahu et al., 2011). There are two types of arsenic in the soil which are inorganic arsenic and organic arsenic. For inorganic arsenic, the duckweed accumulates the inorganic arsenic in its body and some of the arsenic will be released to the atmosphere. On the other hand, the duckweed will degrade the organic arsenic by the enzymes produced by itself and release the organic arsenic to the atmosphere (Tangahu et al., 2011).

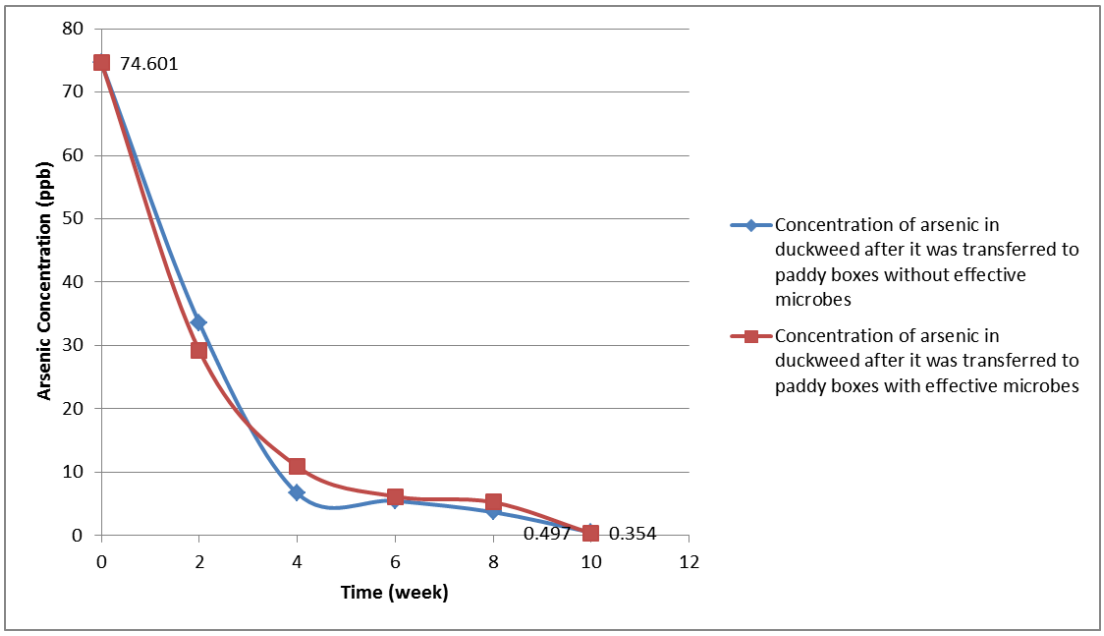

Figure 4.1: Concentration of Arsenic in Duckweed along The Time after It was Transferred to Paddy Boxes with and without Effective Microbes

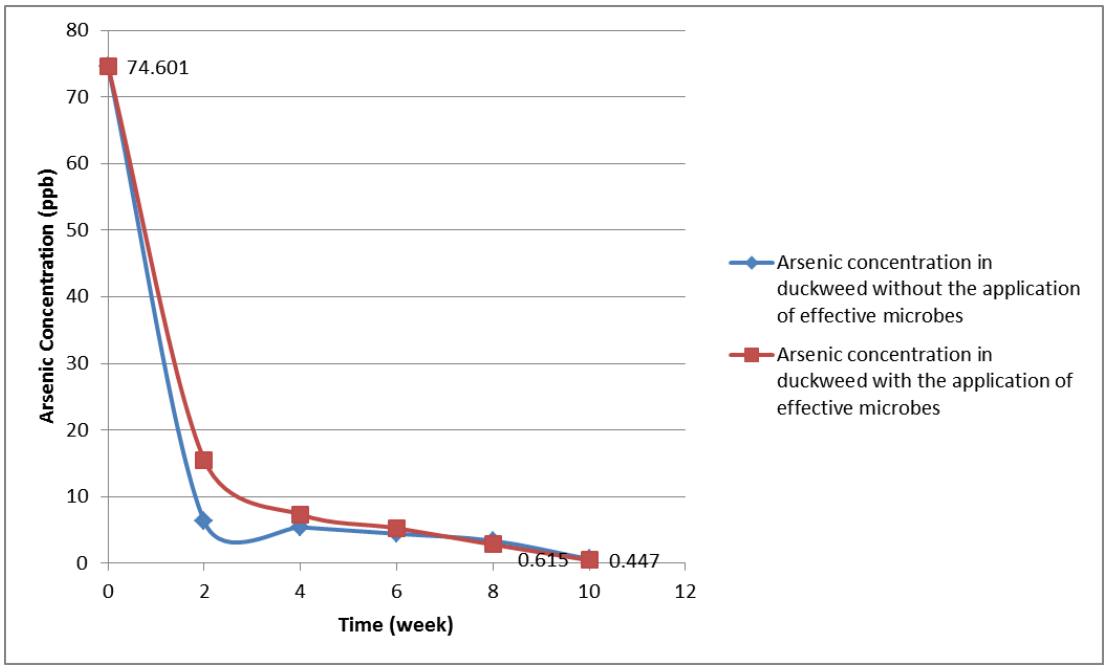

Figure 4.2: Concentration of Arsenic in Duckweed along The Time with and without Effective Microbes

Figure 4.1 shows that the initial arsenic concentrations in duckweed at paddy boxes with and without effective microbes were reduced from 74.601 ppb to 0.354 ppb and from 74.061 ppb to 0.497 ppb respectively. **Figure 4.2** shows that the concentrations of arsenic decreased to 0.447 ppb and 0.615 ppb for the duckweed applied with and without effective microbes. With the application of effective microbes, the arsenic concentrations in duckweed were slightly lower than the ones without effective microbes. This is because effective microbes attract other microbes which could produce nutritious elements and duckweeds would absorb nutrients instead of arsenic (Meharg, Naylor and Macnair, 1994).

Figure 4.1 shows the arsenic concentrations dropped significantly initially but became stable off in week 4 onwards. On the other hand, **Figure 4.2** shows the stabilisation of arsenic concentrations occurred in week 2 onwards. According to Zhao et al. (2015), the level of heavy metal in mixed culture plants will be higher than under monoculture plant when they were exposed to same low concentration of heavy metal. This is due to the different antioxidant enzyme concentrations produced by them. The enzymes produced by plants under mixed culture were higher than that of monoculture plant. This explains the results shown in **Figure 4.1** and **4.2**. The results as shown in **Figure 4.1** and **4.2** are produced from mixed culture plants consist of duckweed and paddy and monoculture plant which is duckweed only respectively.

However, at the end of this experiment, the arsenic concentrations in the duckweed as shown in **Figure 4.1** were slightly lower than results in **Figure 4.2**. It is because after a few weeks, the soil had become fertile and the paddy plants became the competitor. At this stage, duckweeds would absorb nutrients instead of arsenic. However, in monoculture condition where there was no competitor for the duckweed, the duckweeds would absorb nutrients and arsenic simultaneously (Crawley, 1986).

4.2 Arsenic Concentrations in Paddy

Figure 4.3 shows the arsenic concentrations in paddy grain and paddy husk. It shows that the arsenic concentrations in paddy husk are significantly higher than in paddy grain. Paddy absorbs arsenic through its roots accompanied by phosphate, and the uptake of nutrients (phosphate) is accompanied by the infiltration of water (Wan, Steudle and Hartung, 2004). The transportation of heavy metal from the roots to the grain of paddy is through the cohesion-tension theory of the plant (Wan, Steudle and Hartung, 2004). The water will flow from the root, and then followed by stem, leaf, husk and grain. Therefore, the arsenic concentrations are the highest in roots and the lowest in grain (H.K Das et al, 2002).

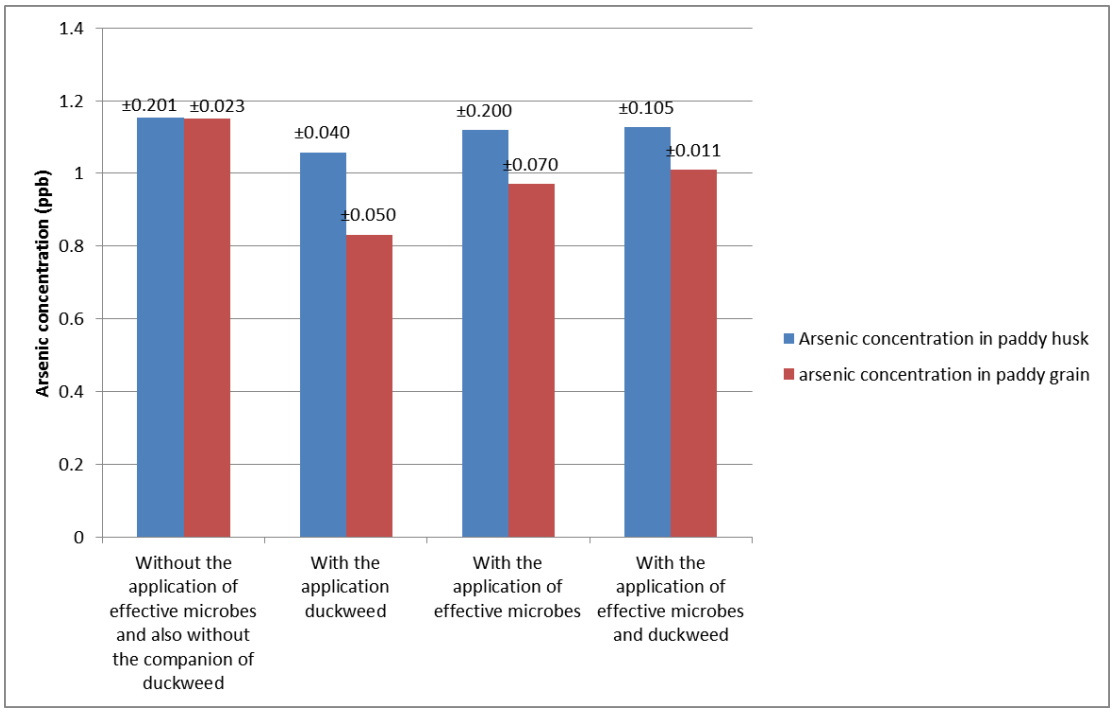

Figure 4.3: Arsenic Concentrations in Paddy Grain and Paddy Husk

From **Figure 4.3**, it was found that duckweed could reduce most the uptake of arsenic by paddy, followed by the effective microbes and lastly the mixture of effective microbes and duckweed. The arsenic in paddy grain reduced by 27.697 % and 8.268 % in paddy husk respectively as compared to the control sample (the plantation of paddy without the application of effective microbes and also without the companion of duckweed) which its arsenic concentrations are 1.149 ppb in paddy grain and 1.153 ppb in paddy husk.

Duckweed is a phytoremediation plant which can degrade the heavy metal into simpler molecular form. It can also accumulate and release the heavy metal to the atmosphere (Tangahu et al., 2011). In addition, duckweed can also act as a bio fertilizer for the paddy as duckweed will release nitrogen during decomposition (Sood et al., 2011). Effective microbes have many effective microorganisms which will improve the quality and fertility of soil by attracting nutritious elements (Sparling, 1992). When the arsenic concentrations of soil decrease and the soil fertility increase, paddy will absorb the nutrient instead of heavy metal (Meharg, Naylor and Macnair, 1994).

However, among the three methods used in this study, the method which is using the mixture of effective microbes and duckweed in reducing the arsenic in paddy had the least potential in reducing the arsenic concentration in paddy. The mixture could only reduce the arsenic concentration by 12.21 % in paddy grain and 2.313 % in paddy husk. In this case, the duckweed acted as the competitor of paddy plant (Crawley, 1986). The nutrients attracted by the effective microbes were be absorbed by the duckweed causing the nutrients in the soil to decrease. Therefore, the paddy absorbed less nutrients, resulting in the increasing of arsenic uptake by paddy.

4.3 Reproduction Rate of Duckweed

In this experiment, 10 duckweeds were observed of the reproduction rate under tape water with contaminated soil while 10 duckweeds were cultivated using tape water without contaminated soil. **Figure 4.4** shows the number of duckweeds could double

their initial amount over the number of days. At the tape water with contaminated soil, the duckweeds required a maximum of 4 days to increase their initial amount to ten plants. However, at the tape water without contaminated soil, the duckweeds required only 3 days to increase their amount to 10.

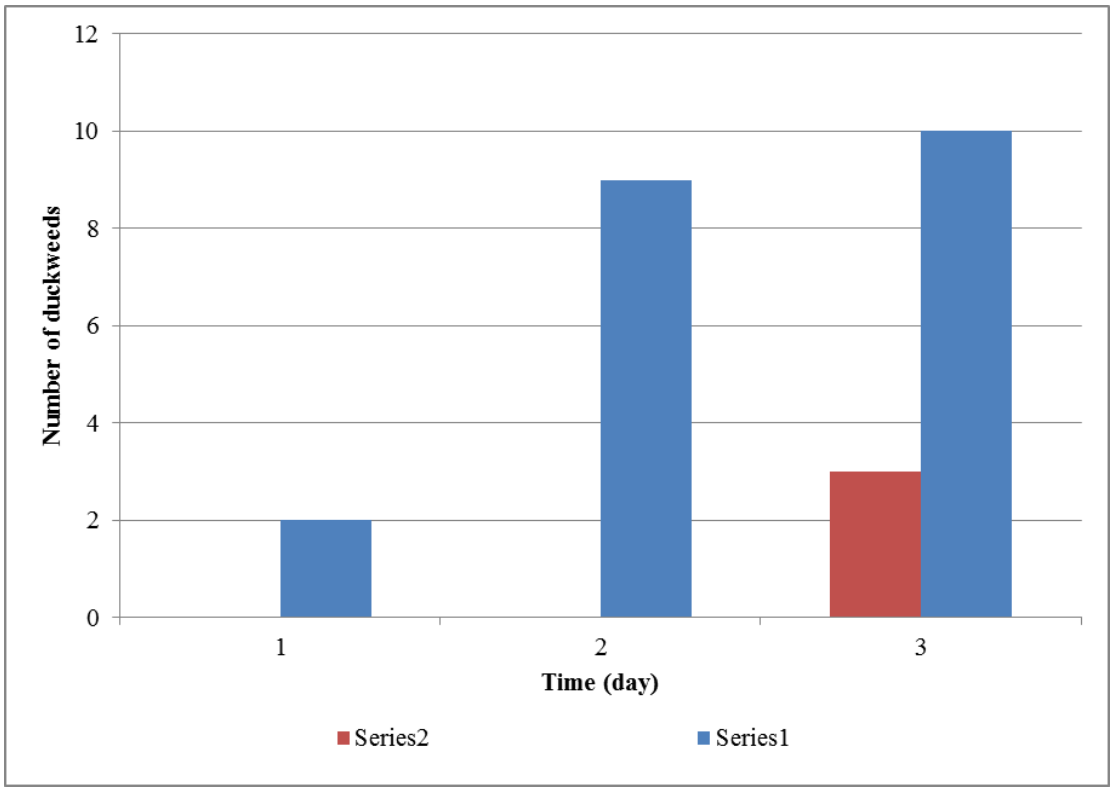

Figure 4.4: Number of duckweeds which doubled their initial amount against the number of days

Duckweed can tolerate to heavy metal exposure. However heavy metal can indicate the degree of metabolic damage of duckweed. Arsenic would damage the cytoplasm and mitochondrial structure, thus affect the impact of respiration on total oxygen exchange of duckweed (Prasad et al., 2001).

CHAPTER 5

CONCLUSIONS AND RECOMMENDATIONS

Duckweed is able to adsorb arsenic from the contaminated soil. The results show that the duckweed has the best performance in reducing the arsenic concentration in paddy. Besides that, the findings show that the concentration of arsenic inside the body of duckweed would be released but the releasing rate decreased with time. The duckweed planted without paddy as companion would stop releasing arsenic from its body in the fourth week. However, when the duckweed was planted side by side with paddy, the arsenic releasing rate from the body of duckweed would stop in the second week. It was found that the arsenic concentration in duckweed under monoculture was relatively higher than the duckweed in the environment of mixed-culture. The reproduction rate of duckweed was also investigated. It was found that duckweed requires more time to multiply its number at the contaminated soil and two days without the addition of contaminated soil compared to at the soil without contamination. This proves that the contaminant will affect the reproduction rate of duckweed.

This project needs a longer time to have comprehensive and complete result, because the duckweed takes a relatively long period to clean up a contaminated soil. The concentration of arsenic accumulated in duckweed will be affected by the concentration of water/soil. Therefore, duckweed should be pre-cultured in water that without any contaminant before carrying out the experiment. Besides that, the paddy should not be planted with duckweed when there is application of microbe or fertilizer because duckweed and paddy will become competitor.

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