UNIVERSITI PUTRA MALAYSIA

MUTAGENICITY ASSESSMENT OF PADDY WATER FROM TANJUNG KARANG, SELANGOR USING SALMONELLA TYPHIMURIUM TA 98 STRAIN: A PRELIMINARY STUDY

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Thesis submitted in fulfilment of the requirement for the degree of Bachelor Science (Environmental and Occupational Health) from the Faculty of Medicine and Health Sciences, Universiti Putra Malaysia.

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ABSTRACT

Introduction: Paddy plantation is one of the important agricultural activities because rice is a staple food in Malaysia. However, there is a possible exposure of mutagenic compounds to surface water via paddy plantation such as from heavy metals, pesticides, heterocyclic amines, and polycyclic aromatic hydrocarbons. This can cause a threat to the human health as the surface water are used as a primary source of drinking water, irrigation for agricultural purposes and other human activities. **Objective:** The study aimed to determine the mutagenic activities of paddy water at Tanjung Karang, Selangor by using *Salmonella typhimurium* TA 98 strain. **Method:** Ames test was chosen as it is widely used to screen a wide range of chemical substances that can produce genetic damage that leads to gene mutation. This study consists of three major methods which were sampling of water, solid phase extraction and Ames test. Firstly, the water was obtained from paddy field in Kampung Sawah Sempadan, Tanjung Karang at 9 respective sampling points. Next, solid phase extraction was conducted by using the 9 water samples to concentrate and purified the solution. Then, the concentrated solutions from solid phase extraction were exposed to the *Salmonella typhimurium* TA 98 strain in Ames test to evaluate the mutagenic activities. **Result:** From the result, it was found that there was mutagenic activity in 6 samples during sampling time 1 and 6 samples during sampling time 2. The positive samples were from inlets, points and also outlets of paddy blocks with a minimum total mean of 92 ± 87 revertants to maximum of 377 ± 222 revertants colonies per plate. Based on the statistical analysis, it was found that there was no significant difference in each paddy water samples in sampling time 1 and sampling time 2 and between the sampling time 1 and sampling time 2 since the $p>0.05$. **Conclusion:** It was concluded that there were positive mutagenic activities in some of the paddy field water samples from Tanjung Karang, Selangor. All in all, public need to be aware of the chemicals that they are using and exposed, to reduce the risk from the exposure to cancer agents.

Keywords: Paddy water, mutagens, Ames test

PENILAIAN MUTAGENISITI AIR SAWAH PADI DARI TANJUNG KARANG, SELANGOR MENGGUNAKAN STRAIN *SALMONELLA TYPHIMURIUM* TA 98: KAJIAN PERMULAAN

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ABSTRAK

Pengenalan: Penanaman padi merupakan salah satu aktiviti pertanian penting kerana beras adalah makanan ruji di Malaysia. Walau bagaimanapun, terdapat kemungkinan pendedahan bahan mutagen ke air permukaan melalui sawah padi seperti dari logam berat, racun perosak, amina heterosiklik, dan hidrokarbon aromatik polisiklik. Ini boleh menyebabkan ancaman kepada kesihatan manusia kerana air permukaan digunakan sebagai sumber utama air minuman, pengairan untuk tujuan pertanian dan aktiviti manusia yang lain. **Objektif:** Kajian ini bertujuan untuk menentukan aktiviti mutagen air padi di Tanjung Karang, Selangor dengan menggunakan strain *Salmonella typhimurium* TA 98. **Kaedah:** Ujian Ames dipilih kerana digunakan secara meluas untuk menyaring pelbagai jenis bahan kimia yang dapat menghasilkan kerosakan genetik yang membawa kepada mutasi gen. Kajian ini terdiri daripada tiga kaedah utama iaitu pensampelan air, pengekstrakan fasa pepejal dan ujian Ames. Pertama, air diperoleh dari medan padi di Kampung Sawah Sempadan, Tanjung Karang di 9 titik pensampelan masing-masing. Pengekstrakan fasa pepejal seterusnya dilakukan dengan menggunakan 9 sampel air untuk memekatkan dan membersihkan sampel air. Kemudian, hasil pengekstrakan fasa pepejal didedahkan kepada strain *Salmonella typhimurium* TA 98 dalam ujian Ames untuk menilai aktiviti mutagenik. **Hasil kajian:** Hasilnya didapati bahawa terdapat aktiviti mutagenik dalam 6 sampel semasa persampelan 1 dan 6 semasa persampelan 2. Sampel positif adalah dari inlet, point dan juga outlet cawangan blok padi dengan jumlah minimum 92 ± 87 dan maksimum 377 ± 222 koloni bagi setiap piring. Berdasarkan analisis statistik, didapati tidak terdapat perbezaan yang signifikan dalam setiap sampel air padi dalam masa persampelan 1 dan masa persampelan 2 dan antara masa persampelan 1 dan masa persampelan 2 dengan nilai $p > 0.05$. **Kesimpulan:** Secara kesimpulan, terdapat aktiviti mutagenik yang positif di beberapa sampel air padi dari Tanjung Karang, Selangor. Secara keseluruhannya, orang ramai perlu mengetahui tentang bahan kimia yang mereka gunakan dan terdedah, untuk mengurangkan risiko daripada pendedahan kepada agen kanser.

Kata kunci: Air sawah padi, mutagen, Ujian Ames

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%	percent
Hrs	Hours
℃	Degree Celsius
mL	Mililitre
mg	Microgram
L	litre
M	Mole
ha	Hectare area
GM	Glucose Minimal
RARE	Rare mutation cell collection
E.coli	*Estecheria coli*

CHAPTER 1

INTRODUCTION

1.1 Background

The agricultural sector in Malaysia is growing rapidly as a result of technology development. Agricultural also is one of the important sectors in Malaysian economy development. One of the important agricultural activities is paddy plantation as Malaysian consumed rice from paddy plant as a staple food. During paddy plantation, some mutagens are exposed to the surface water. Example of the mutagens that existed in surface water are heavy metals, pesticides, polycyclic aromatic hydrocarbons and aromatic amines (Ohe, Watanabe and Wakabayashi, 2004).

The mutagens that are exposed to the surface water can cause a threat to the human health. This is because the surface water sources are used as a primary source of drinking water, irrigation for agricultural purposes and other human activities (Guan et al., 2017). Mutagenicity of untreated water and

consumable water are turning into huge community health problems (Warren et al., 2015; Manasfi et al., 2016; Praveena et al., 2016; Xiao et al., 2016).

Agricultural workers are a group of people that are believed to be exposed to pesticides such as organophosphates frequently due to their daily working activities (Kim et al., 2016). Besides organophosphates, the agricultural workers are also being exposed to herbicides such as chlornitrofen during their daily working activities. This is very dangerous since it can cause mutagenic effect such as cancer to the agricultural workers for a long term exposure.The water sample from paddy field may have this mutagenic properties since the paddy plantation process involves pesticides and herbicides which are believed to contain mutagenic properties and can cause mutagenic effect to human.

Mutagen is defined as a physical or chemical substance that can alter the genetic materials of human. As example, when the DNA of a living organism is being altered by this mutagenic substance, this process is irreversible and lead to a cancer formation. Nowadays, it is broadly acknowledged that, mutually with the expanded lifespan, extended interaction to various artificial and pure substances in the nature is a leading source of cancer activation (Albreht et al., 2008).

Ames test is a method of determining the mutagenic activity of a substance by using bacterial system. It is a short term bacterial reverse mutation assay that is used to detect chemical substances that can damage the genetic material and causing genetic mutations (Moltermans and Zeiger, 2000). Example of bacteria strain that can be used in Ames test is *Salmonella typhimurium* TA 98 strain that carries the frame-shift mutation. During Ames test, the *Salmonella typhimurium* TA 98 strain is grown on a plate of agar that contains histidine and only the bacteria that transformed into histidine independence are able to grow on the media. The bacteria that revert to histidine independence are bacteria that exposed to mutagens and undergo the reverse mutations. Therefore, when there is a colony of bacteria formed on the agar plate during Ames test it is concluded that the substance contains mutagenic properties.

1.2 Study Justification

Nowadays, incident of cancer in our country has increased. One of the reasons for cancer incident is because of exposure to mutagenic substances. Paddy plantation is one of the largest agricultural activity in Malaysia and it may involve the use of several chemicals including heavy metal, polycyclic aromatic hydrocarbons, aromatic amines, pesticides and herbicides that have mutagenic properties. The paddy water field may have mutagenic activity due to the use those chemicals to the paddy plant. This study is important as mutagen is a very dangerous substance as it can change the genetic materials and in prolonged period may cause cancer. Paddy plantation also uses variety of pesticides and herbicides that are believed to contain mutagenic activity based on previous research.

At the end of this research, I will be able to determine the mutagenicity of paddy water at Tanjung Karang at different sampling place. Apart from that, I will also gain knowledge about the physical properties of water such as the dissolved oxygen, temperature and pH and how it affects the mutagenicity of paddy water. As a result, the findings from this research can be used as a baseline for future study in this field.

1.3 Objectives

The general objective of this study is to conduct mutagenicity assessment of paddy water from Tanjung Karang, Selangor using *Salmonella typhimurium* TA 98 strain.

And the specific objectives of this study are:

1) To determine the physicochemical parameters of paddy water samples.
2) To determine potential mutagenic activity of paddy water samples using Ames test, *Salmonella typhimurium* strain TA 98 from different sampling points.
3) To compare mutagenicity of paddy water samples from different sampling points in different sampling times.

1.4. Research Questions

1) Is there any mutagenic activity in paddy water in Tanjung Karang, Selangor?
2) Is there any difference in mutagenicity of paddy water in each different sampling points?

1.5 Hypothesis

1) There is a positive mutagenic activity in *Salmonella typhimurium* TA 98 strain from paddy water samples from Tanjung Karang using Ames Test.

2) There is a significant mean difference in mutagenic activity in Salmonella typhimurium TA 98 strain from paddy water samples in different sampling points and sampling times.

1.6 Conceptual Framework

Figure 1.1 shows a conceptual framework for this study.

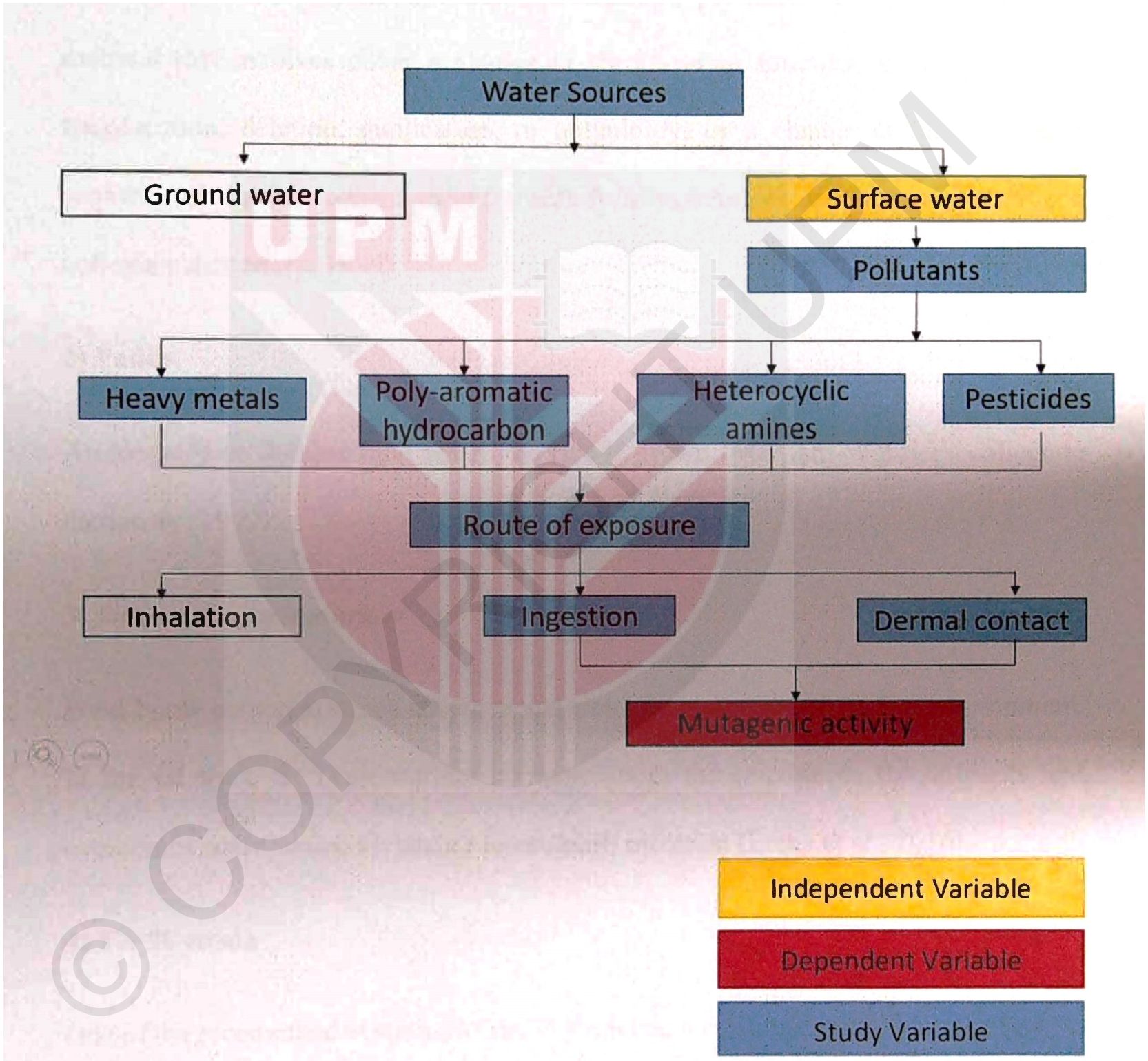

Figure 1.1: Conceptual Framework

1.7 Definition of Terms

1) Mutagenicity

Capacity to induce mutations which is a relatively permanent change in hereditary material that involves either a change in chromosome structure or number (as in translocation, deletion, duplication, or polyploidy) or a change in the nucleotide sequence of a gene's codons as in frameshift or missense errors (Merriam-Webster's collegiate dictionary, 1999).

2) Paddy

An irrigated or flooded field where the rice is grown (Merriam-Webster's collegiate dictionary, 1999).

3) *Salmonella typhimurium*

Food-borne pathogen which adapts to and alters the gastrointestinal (GI) environment. In the GI tract, S. Typhimurium competes with the microbiota for nutrients and overcomes colonization resistance to establish infection (Elena et al., 2016).

4) TA 98 strain

One of the recommended strain in OECD guideline 471 for use in the bacterial reverse mutation test (Sugiyama et al., 2016).

CHAPTER 2

LITERATURE REVIEW

2.1 Paddy Plantation in Malaysia

In Malaysia, plantation of paddy is common in rural community and usually involved in traditional farming. The organization of local paddy production has been assisted by the measures that were introduced by The Government of Malaysia through the establishment of incentives such as announcing a security crop to the rice crop, commencing the National Agriculture Act (1992-2010), advancement of current irrigation systems and constructing new irrigation systems, controlling market price and other measures to enhance the local (Mahmudul et al., 2011). Besides covering up to 86 % of local market demand and can sustained by itself, the residual unfulfilled market demand is eased by importing rice from Thailand, Indonesia and Cambodia which is the neighbouring countries that produce rice. The most important purpose of most local paddy farmers is to elevate the yield of crop by certain measures such as establishment of new cultivars, assessing current farming practices including fertilizing and pesticide cycle, amount and type of fertilizer and pesticide used and the strength and occurrence of the cycle (Toriman et al., 2013).

In order to enhance the system of irrigation for the whole region, Development Authority (MADA) and Kemubu Agriculture Development Authority (KADA) had performed an important part. Therefore, since 1988 and 1999 and until now, farmers could employ double and triple rice cropping per year, in comparison with the traditional irrigation system which only enables them to plant only once a year. Although there were a significant decrease in rainfall and water resources availability for irrigation in the country in 1980s, farmers still have the capability to enhance the output of total crop by approximate of 16 % over the decade. Nevertheless, because of the doubt on the production of economic and unpredictable climate, it has become a constant challenge for the government and paddy farmers to produce higher amount of rice. This comprises the effective used of water resources, good management practices and accurate data such as yearly effective rainfall, runoff, consumptive use and water discharge policy. Ideal water supervision can be accomplished through the distribution of accurate amount of irrigation water at the exact moment to enhance yield of crop (Alam et al., 2012).

For an average planting area of 651 600 ha for both main and off season in the years 1900's, paddy plantation has sustained been recorded as the main economic interest of the rural community in Malaysia. However, there is a maintain yearly decrease in terms of plantation area. Regarding to the use of land, the third most essential crop in Malaysia after the oil palm (2.3 million ha) and the rubber plant (1.8 million ha) is paddy plant (ICDC, 2002). Malaysia is a minor rice producing country in the world with a rice production output of 0.4 %. Majority of the paddy farmers in

Malaysia consists of minor scale farmers that have average planting area of 1.06 ha despite the total number of paddy farmers which is 286,000. Only 116,000 are full time paddy farmers who rely fully on the paddy production as their source of (Mahmudul et al., 2010).

2.2 Production of Rice

The second essential plantation in the world is rice, with wheat being the first one (Rashid and Yasmeen, 2018) and Asia's countries are the highest manufacturer and user for rice (Gumma et al., 2011). There is about 70 % increasing need for rice in Asia for upcoming years because of the increasing number of the population (Papademetrious, 2000). According to Norimah Jr, the adult population in Malaysia eats about two and half plates of rice everyday in average based on the food consumption model (Norimah Jr, 2008). Although previous study conducted shows that rice is a low quality food for the population (Tey, 2008), but it is still the most essential staple food in Malaysia (Brown, 1973).

Dasar Agromakanan Negara (DAN) was employed by the Ministry of Agriculture and Agro-based Industry (MOA) in order to make sure that food is adequately been supplied and to change the agro-based industry into a more economically sustained and high incomes industry. Since there was only 7 % of the world's rice production is been imported, Dasar Agromakanan Negara (DAN) 2011-

2012 to emphasize the local rice production to be increased. On the other hand, the plan of paddy manufacturing was organized by using the approach of system dynamics (Alias et al., 2011), though the consumption of rice is founded on a few ideas (Abdullah, Ito and Adhana, 2006) which indicates an elevated pattern until 2050.

Rice is an important part in Malaysian everyday food consumption. Malaysia generated about 1.66 million metric tons of rice in the year of 2011 and statistics show that global production was at 36.3 million metric tonnes (DOA, 2014). This indicates that the rice produce by Malaysian is only 0.4% estimated of the total global production of rice. Continuously until current years, Malaysia only manufacture about 70% of the amount required to maintain itself and need to import the other 30%, mostly from Thailand and Vietnam. About 82.3 kilograms of rice were consumed annually in average by Malaysian people. Meanwhile, the size of one hectare paddy field can produces about 3.7 metric ton (MT) of rice (Rajamoorthy, Rahim and Munusamy, 2015).

More study and technological improvement are needed in order to increase the production of rice to support the elevated number of populations that will consume rice. A lot of elements have been used to enhance the rice production including machineries, fertilizers and pesticides. However, all these methods can cause harmful effects to the environment. Example of harmful environmental impacts resulting from production of rice includes greenhouse gases emission, eutrophication and acidification (Brodt et al., 2014; Wang et al, 2010).

2.3 Mutagenicity of Surface Water

A practice of examining residues, surface water, and water used for consumption by the *Salmonella* assay is constantly being performed. For instance, in São Paulo State, Brazil, the governing organization consistently examined its waterways with the *Salmonella* assay beyond 20 years (Umbuzeiro et al., 2001). Researchers from Japan have conducted experiment by using the *Salmonella* assay to examine the periodic oscillation of the mutagenicity of stream water in Fukui, Japan (Watanabe et al., 2002).

One of the parts in the RARE (Rare Mutation Cell Collection) study is it is operated with the *Salmonella* mutagenicity assay to examine surface water, residues, and water used for human consumption for mutagenicity. The *Salmonella* mutagenicity assay has been applied widely to characterize genotoxic compounds in ecological specimens (Zwiener et al., 2007). The assay is beneficial in current situation since the capability to recognize mutagenic activity in surface waters (Ohe et al., 2003), residues, and water for consumption (Richardson et al.,2007).

The mutagenicity and carcinogenicity of fresh water and water that is being used for human consumption turn out to be a main community health problem. The problem rises as the harmfulness of pure water was being addressed due to specific derivatives that were produced from the process of cleansing water and were

acknowledged as mutagenic substances (Warren et al., 2015; Manasfi et al., 2016; Praveena et al., 2016; Xiao et al., 2016).

In the beginning of 1990s, Stahl, De Flora and Houk studied about the genotoxic and carcinogenic hazards of surface waters, the aquatic environment, waste from industrial companies and sewage. From the study conducted, Houk and Stahl concluded that genotoxic organic substances can contaminate the surface waters from many type of industrial and municipal sources by reviewing their genotoxic information with the used of short-term genetic bioassays. They also emphasized the usefulness of bioassays to identify mutagenicity and genotoxicity of surface water that result from the many type of genotoxic substances in the nature and the need of the detection of the sources of contaminants (Houk et al., 1992).

In 2010, Yangtze River and Taihu Lake which are the sources of drinking water in China were contaminated with mutagens and carcinogens. Many procedures have been carried out to reduce the level of contaminants. Besides closing the nearby industries that contributes in high amount of pollution and improving the tools of the wastewater treatment plant to lessen the movement of contaminants into the water, the pond's self-cleansing mechanism also has been implemented. Yangtze River is proposing a strategy called "The Water Diversion from Yangtze River to Taihu Lake" into Taihu Lake to improve the moderately constant lake water movement. Nevertheless, there is a huge number of manures that was obtained by Yangtze River waters that could contaminate the process of water diversion once again. Hence, there

must be an extra assessment on the influence of water quality of the water diversion (Li et al., 2013).

Meanwhile, there was also another study conducted regarding mutagenicity testing of surface water which took place at Besos and llobregat Rivers in Spain. Grifoll has conducted the mutagenicity identification of the suspended and particulate parts of the Besos and Llobregat Rivers, which move along inhabited and manufacturing basins near Barcelona, Spain. Together, the two rivers share domestic, industrial and agricultural uses and receives huge amount of untreated sewage. From the study result, it was confirmed that both rivers are frequently polluted by base substitution and frameshift mutagens (Grifoll et al., 1992).

A huge amount of waste water from manufacturing, farming and other resources together with municipal sewage treatment plants is deposited into the surface waters. Commonly, the initial phase in analysing quality of water is physicochemical evaluation. Nevertheless, it is insufficient to examine toxic and genotoxic potential in surface water by using the specific directed chemical investigation because the contaminating compounds is in such complicated combinations and are often existed in a large number and very small amount to enable their analytical purpose. In contrast, the risk for environment and human well-being can successfully be determine by using biological monitoring since it also includes long-term exposure at low doses of toxic compounds (Wadhia and Thompson, 2007).

One study was carried out by Radić in order to analyse the potential genotoxicity of microcontaminant in the surface water affected by wastewater from manufacturing of fertilizer and disposal of phosphogypsum. Problems arising from the incident of phosphogypsum disposal are elevated amount of fluorides, heavy metals and concentrations of radionuclide in soil or groundwater and significant health risk for human. The sewage from industrial, which is from wastewater channel, runs into Ilova River and contains a high amount of abundant ammonium and phosphate substances, fluorides and heavy metals from the substances utilized in maintaining production processes. Therefore, important evidence regarding the existence of genotoxic and toxic compounds in the surface water and the potential process of their toxicity can be obtained from the screening performed in the study (Radić et al., 2013).

2.4 Mutagenic Activity of Pesticides

Residues of pesticides can be available in crops and plants and can cause health risk to human. Previous studies had determined that long term exposure to low level of pesticides can cause teratogenic effects and is associated with carcinogenicity when exposed to pregnant mother (Siddiqui and Ahmad, 2003). There is also a study regarding the mutagenicity of wastewater that concluded that there is an association between mutagenicity and water in India (Jolibois and Guerbet, 2005). Besides, in another study about mutagenicity of wastewater it was determined that there is mutagenic activity in various liquid waste and sludges from industrial (Fatima and Ahmad, 2006, Şık et al., 2009).

Mutagenic activity of pesticides as well as their influence on non-target organisms is a global concern. In the last decade, problems of animal use and care in toxicology research and testing have become one of the key concerns of science and ethics. The carcinogenic and mutagenic effect of herbicides, insecticides and fungicides on animal is widely known .Various manufactured and indigenous substances must be examined using animal testing for their carcinogenic potentials. Unfortunately, determination of mutagenic activity by the aid of animal experiments consumes a lot of time and expensive. Thus, in vitro experiment which is less time consuming have been developed that use microorganisms or flora cells to determine the carcinogenic activity of substances (Bull et al., 2006, Karabay and Gunnehir, 2005).

Water that being polluted by pesticide waste is a huge problem. Pesticides are one category of substances that even though they have many advantages, they are also able to give a broad range of toxic unwanted secondary effects that is possible to create hazard to the ecosystems. The discharge materials from pesticide may have portrayed an essential role in the transportation of environmental carcinogens. Genotoxic materials in wastewater or soil also may cause a negative effect on human health when the population is being threaten with the pollutants such as from inhalation of dust, ingestion of plants that consumed the materials from soil, and compounds extract from soil to groundwater and surface water that is used to drink (Covacia et al., 2005, Watanabe et al., 2008).

2.5 Determination of Mutagenic Activity by Ames test

The *Salmonella* strain mutagenicity assay is a short period bacterial assay precisely construct to detect chemicals that can induced genetic alteration that brings to mutations and to determine the mutagenic properties of compound. Since the Ames test is broadly used as a primary screening to examine the mutagenic activities of water samples, the mutagenicity of the solution of water samples taken from the Yangtze River and Hanshui River in this study was examined by using the *Salmonella* bioassay (Lv et al., 2015).

The Ames *Salmonella* assay is highly accurate and broadly utilized as a screening test for the determination of environmental mutagens. This experiment is based on the capability of compounds to produce backwards mutations in certain histidine involving *Salmonella* typhimurium strains which can aid in the recognition of the groups of genotoxic chemicals existed in the sewerage water samples. The water extracts have to be concentrated and appropriate extraction of the mutagenic materials is needed for the chemical experiment and *Salmonella* test. Various planning can be used to extract organic genotoxicants from water samples and the Amberlite XAD resins have been the most practiced technique. The XAD resins approve the recovery of a broad range of compounds and are very effective to obtain all the polar and the non-polar chemicals potentially efficient in toxicity and genotoxicity assays (Siddiqui and Ahmad, 2003, Aleem and Malik, 2005, Ansari and Malik, 2009, Siddiqui et al., 2011).

Rehana et al. (1995) utilized five various *Salmonella* tester strains to analyze the mutagenic potential of water samples from four points of the Ganges river, India, performing the XAD resin extraction technique and the liquid–liquid extraction method. Their samples exhibit highest mutagenic activity for TA98 and TA100, both with and without S9 fraction. The highest action for each strain was >10,000 revertants per liter. Siddiqui and Ahmad (2003) and Aleem and Malik (2003) declared that TA98 (classified as extreme) was extraordinary high reactive strain with XAD concentrated water extracts (river Yamuna, Mathura, India) in comparison to TA100 (classified as high), both with and without S9 fraction. They also analyze that XAD concentrated water samples were more mutagenic than liquid–liquid extracts. They later indicated that water extracts obtained during the summer season displayed higher mutagenic activity in comparison with other seasons, and water samples also contained oxidative (TA102) mutagens. This maximal mutagenic contamination of the river water is probably obtained from a mixture of domestic, municipal, and industrial waste taken at the sampling site (Rehana et al., 1995).

Certainly, Bruce Ames had proposed a reasonably simple and quick in vitro assay that was used for determination of mutations in DNA cause by chemicals. Frame-shift mutations or base-pair substitutions are identified by exposure of chemical of interest to the histidine-dependent genetically engineered strains of *Salmonella typhimurium* in Ames test. The reverse mutations will bring back the bacteria's capability to produce histidine and as a result it will grow on a sample that is insufficient in amino acid when being exposed to a mutagen. A fundamental association between genetic destruction and cancer uprising had been verified with the application of the Ames test to huge number of chemicals since it showed that this

assay has elevated positive predictivity for DNA-reactive chemical carcinogens. Nowadays, Ames test is definitely the most frequently utilized, well-known in vitro test or screening of chemical mutagenicity (OECD, 1997) (Gadaleta et al., 2016).

The Ames Salmonella/microsome mutagenicity assay (Salmonella test; Ames test) is a temporary bacterial reverse mutation assay precisely invented to identify a broad scope of chemical compounds that can produce genetic damage that leads to mutation of gene. A numerous histidine dependent Salmonella strains, each involving different mutations in numerous genes in the histidine operon was used in the test. Through several mechanisms, these mutations portray as popular spots for mutagens that induce DNA destruction. Merely, only those bacteria that transformed to histidine independence (his^+) are capable to form colonies when the *Salmonella* tester strains are cultivated on minimal media agar plate containing a trace of histidine. The amount of naturally produced revertant colonies per plate is reasonably stable. Nevertheless, the amount of revertant colonies in a plate is elevated commonly in dose-related manner after a mutagen is inserted to the plate (Mortelmans and Zeiger, 2000).

The Ames test is globally utilized as a primary screening tools to identify the mutagenic properties of new compounds and derivatives. Besides, Ames test is also used to deliver information to government bodies for chemical registration or acknowledgement including derivatives and biocides. In order to certify the stability of test methods, international guidelines have been established to be used by companies and research laboratories. An enormous amount of naturally radiation and chemical-induced histidine mutants of *Salmonella typhimurium* LT-2 were created by

a research carried out to determine the genes in charge of histidine production. A few amounts of the mutants consisted of single base changes (base-pair substitution mutants) while a few consisted of additions or deletions of one or more bases (frameshift mutants). After a few moments, it was recognized that some of these mutant strains could be utilized to determine and describe mutagenic chemicals by their capability to change back to wild-type (histidine-independence) when there is an existence of mutagens. Back in 1966, Ames and Whitfield recommended a set of histidine mutant strains for examining chemicals for mutagens utilizing a spot test method that has been used before by Szybalski and Iyer for examining mutagen with *E.coli* strain. The immediate examination consists of smearing a little amount of the test compound straight to the centre of a selective agar medium plate seeded with the test organism. A concentration gradient is formed as the compound disseminate into the agar. A mutagenic compound will grow to a ring of revertant colonies surrounding the area where the compound was smeared. A zone of growth inhibition will also be detected if the chemical is mutagen (Mortelmans and Zeiger, 2000).

CHAPTER 3

RESEARCH METHODOLOGY

3.1 Study location

The water samples were obtained from paddy field in Kampung Sawah Sempadan, Tanjung Karang, Selangor. Water samples were collected in February and March, 2019.

3.2 Water Sampling

Composite paddy water sample of each paddy blocks were taken in 5 different points as shown in Figure 3.1. One litre of paddy water were collected from each sampling points and homogenized. There were 9 sampling points including inlet and outlet points. The water samples were collected using scotch bottle and kept in ice box at 0-4 degree ℃ upon transportation.

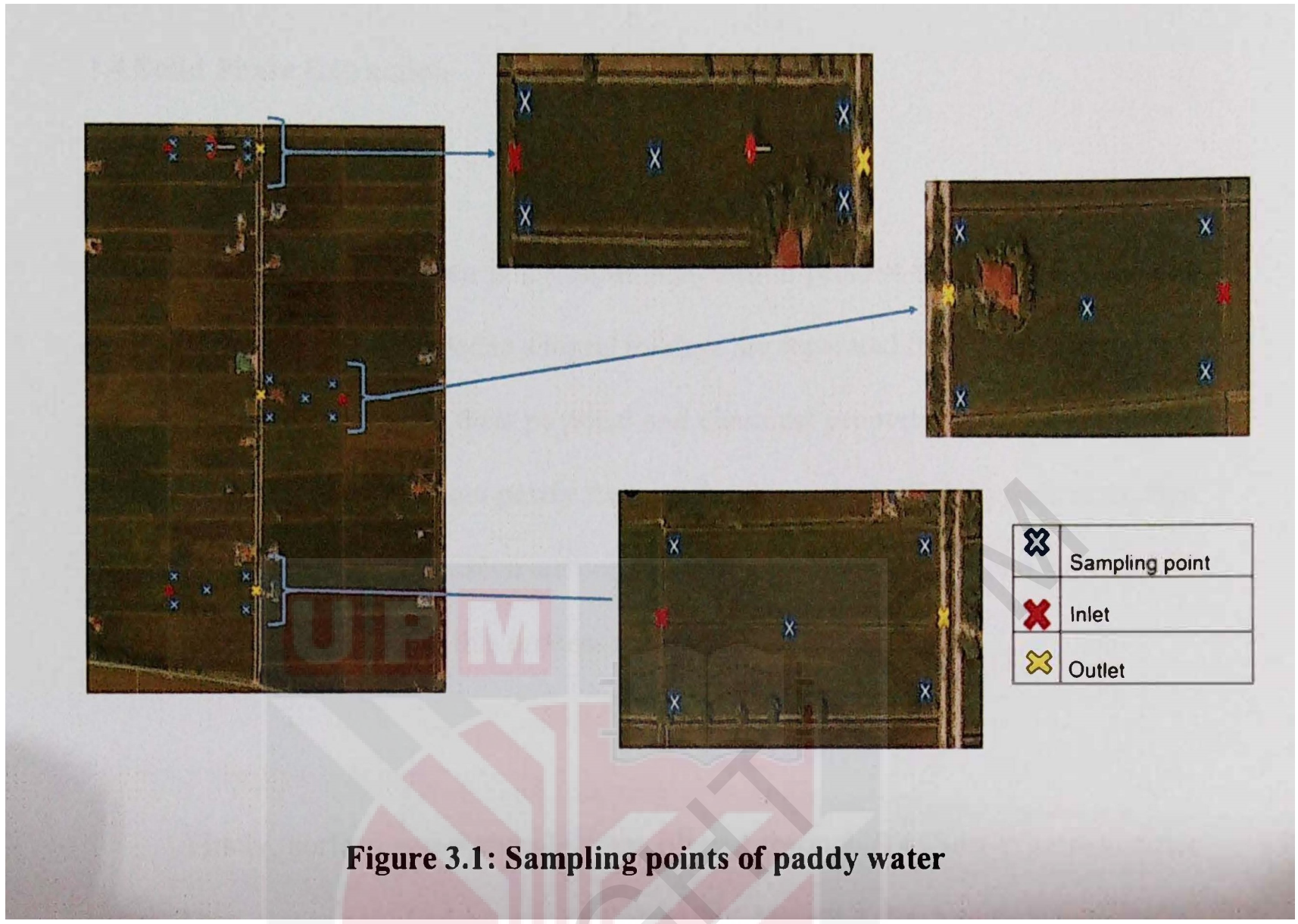

Figure 3.1: Sampling points of paddy water

3.3 Measuring Parameters of Water

The parameters of water that were measured includes dissolved oxygen, temperature and pH level of water. The dissolved oxygen was measured by using EUTECH DO 110. This device measured the amount of gaseous oxygen dissolve in water. Meanwhile, the pH level was measured by using EUTECH pH 450. This device measured the acidity and alkalinity of water as well as the temperature.

3.4 Solid Phase Extraction

Solid phase extraction is a sample preparation process by which compounds that are dissolved or suspended in a liquid mixture are separated from other compounds in the mixture according to their physical and chemical properties. The aims for this process was to concentrate and purify the samples for analysis. Solid phase extraction consists of four basic steps which are conditioning, loading, washing and elution. The cartridges that were used in this process were Oasis HLB cartridges.

Firstly, surface water sample were filtered through 0.45 µm Whatman filter paper. Then, it was acidified by using sulphuric acid to pH 3. Each sample were being added with five nanograms of each surrogate standard. The solid phase extraction was carried out with a Visiprep SPE manifold. Oasis HLB cartridges were being added sequentially with 10 mL of methanol and 10 mL pure water. Samples were applied to the SPE cartridges at a flow rate of 5mL/min. The SPE columns were washed by addition of 200 mL filtered surface water samples with 10 mL methanol. Then, it was eluted with 5 mL methanol and 3 mL ethyl acetate. The eluate was evaporated by using shaking incubator SI-300 until a final volume become 2.0 mL.

3.3 Ames Test

This test is also known as bacterial reverse mutation assay. Ames test uses several strains of bacteria that carry mutation in gene that encodes histidine (His^-). This process involves culturing (His^-) salmonella in a media containing certain chemicals, causes mutation in histidine encoding gene, such that they regain the ability to synthesize histidine (His^+) which is called reverse mutation.

3.3.1 Day 0

A twelve ml of nutrient broth was aliquoted in a sterile 100 ml conical flask. Then, 12 µl of bacteria was inoculated from frozen stock to 12 ml of nutrient broth. The conical flask containing the bacterial culture was incubated at 4 °C for 7 hrs. Next, the conical flask was transfer to 37 °C incubator for 12-16 hrs with shaking (100 rpm). After 12-16 hrs incubation at 37 °C, the bacterial culture can be used for Ames Test.

3.3.2 Day 1

A 0.5 ml of 0.1 M sodium phosphate buffer (for test without metabolic activation) was added in a sterile culture tube. Then, 0.05-0.1 ml of test article was added in the culture tube. After that, 0.1 ml of overnight bacterial suspension was added. The density of the bacterial suspension should be approximately 1-2 x 109

colony forming units (CFU)/ml). The mixture was incubated in 37 °C incubator for 20 mins with shaking (100 rpm). After 20 mins being incubated, 2 ml of melted top agar supplemented with histidine/biotin (the top agar temperature should be maintained at 43-45 °C prior to use) was added to the culture tube containing the mixture. The mixture was gently mixed and being poured onto GM agar plates. The plate was swirled thoroughly to ensure the melted top agar spread evenly on the surface of GM agar plate. The agar was left hardened for about 3-5 mins. The agar was incubated upside down in 37 °C incubator for 48 hrs. The positive control used in this test was 2-Nitrofluorene and the negative control was a mixture of methanol and ethyl acetate.

3.3.3 Day 3

The GM agar plates were being observed. The revertant colonies in each plate were counted.

3.4 Quality Control

All the glassware that is being used for water sampling, solid phase extraction and Ames test such as scotch bottle, beaker, measuring cylinder, filter funnel, test tubes, conical flasks and pipette tips were being washed by Decon 90 to decontaminate. As for the glassware used for Ames test, after being washed with

Decon 90, it was being autoclaved to 121 °C for 15 mins to ensure that the glassware were free from bacteria.

3.5 Statistical Analysis

The table 3.1 shows the statistical analysis that were run in this study. Firstly, one way analysis of variance (ANOVA) was run using data between samples in Time 1 and also between samples in Time 2. Next, paired t-Test was run using data between samples in Time 1 and Time 2.

Table 3.1: Statistical analysis

Category	Statistical Analysis
Between samples in Time 1	One way ANOVA
Between samples in Time 2	One way ANOVA
Between samples in Time 1 and Time 2	Paired t-Test

CHAPTER 4

RESULTS

4.1 Physicochemical Properties of Paddy Water

Table 4.1 shows the physicochemical parameters of the paddy water samples. It was found that the lowest pH in time 1 was 5.97 at Inlet 3 and the highest was 6.46 at Outlet 2. However, in time 2 the lowest pH was 6.65 at Outlet 3 and the highest was 7.35 at Outlet 2. For the temperature, it was found that the lowest temperature in time 1 was 27.5 at Inlet 1 and the highest temperature was 36.2 at Point 2. However, in time 2 the lowest temperature was 28.0 at Inlet 3 and the highest was 32.0 at Point 1. As for the dissolved oxygen, it was found that the lowest dissolved oxygen in time 1 was 4.84 at Outlet 1 and the highest was 6.58 at Inlet 2. However, in time 2 the lowest dissolved oxygen was 2.87 at Outlet 3 and the highest was 6.21 at Point 1.

Table 4.1: Physicochemical parameters of paddy water samples

	Time 1			Time 2		
Point of sampling	**pH**	**Temperature (°C)**	**Dissolved Oxygen (mg/L)**	**pH**	**Temperature (°C)**	**Dissolved Oxygen (mg/L)**
Inlet 1	6.15	27.5	5.05	7.27	31.4	6.06
Inlet 2	6.39	34.3	6.58	7.12	30.5	4.75
Inlet 3	5.97	33.9	5.66	6.8	28.0	4.48
Point 1	6.4	36.1	5.67	7.19	32.0	6.21
Point 2	6.16	36.2	5.66	7.31	31.2	4.57
Point 3	6.41	33.9	5.79	7.18	28.5	3.73
Outlet 1	6.39	35.9	4.84	7.26	28.5	4.71
Outlet 2	6.46	34.8	6.22	7.35	28.5	4.02
Outlet 3	6.42	33.6	5.55	6.65	28.5	2.87

4.2 Mutagenic Activities of Paddy Water in Time 1

Figure 4.1 shows a total mean of revertant colonies of *Salmonella typhimurium* TA98. There were 6 paddy water samples showed to be positive to induce mutagenic activities which were from Inlet 1, Inlet 3, Point 1, Point 2, Point 3 and Outlet 3. The data were presented in number of total mean of revertant colonies ± Standard Error of Mean (SEM). The highest total mean of revertant colonies is at Point 3 with total mean of 349 ± 157. Meanwhile, the lowest total mean of revertant colonies is Inlet 2 with total mean of 10 ± 2.8. Based on One Way Analysis of Variance (ANOVA) showed that $F(2,51) = 2.28$, $p>0.05$ which indicates there is no significant difference of mean of revertant colonies in different sampling points in Time 1.

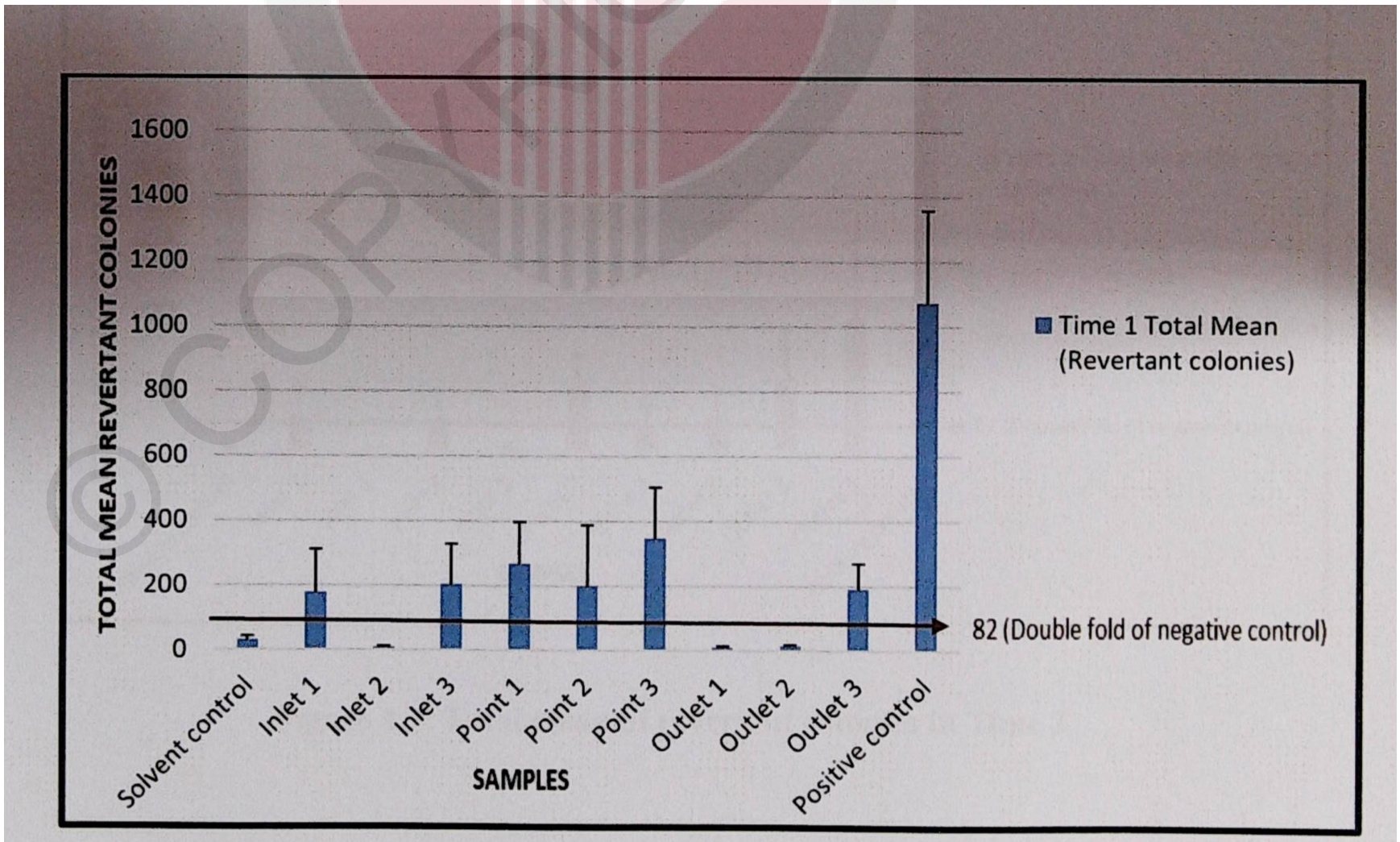

Figure 4.1: Total mean of revertant colonies in Time 1

4.3 Mutagenic Activities of Paddy Water in Time 2

Figure 4.2 shows a total mean of revertant colonies of *Salmonella typhimurium* TA98. There were 6 paddy water samples showed to be positive to induce mutagenic activities which were from Inlet 1, Inlet 3, Point 2, Outlet 1, Outlet 2 and Outlet 3. Point 3 and Outlet 3. The highest total mean of revertant colonies is at Outlet 3 with total mean of 377 ± 222. Meanwhile, the lowest total mean of revertant colonies is at Inlet 2 with total mean of 6 ± 1.8. Based on One Way Analysis of Variance (ANOVA) showed that $F(2,51) = 2.07$, $p>0.05$ which indicates there is no significant difference of mean of revertant colonies in different sampling points in Time 2.

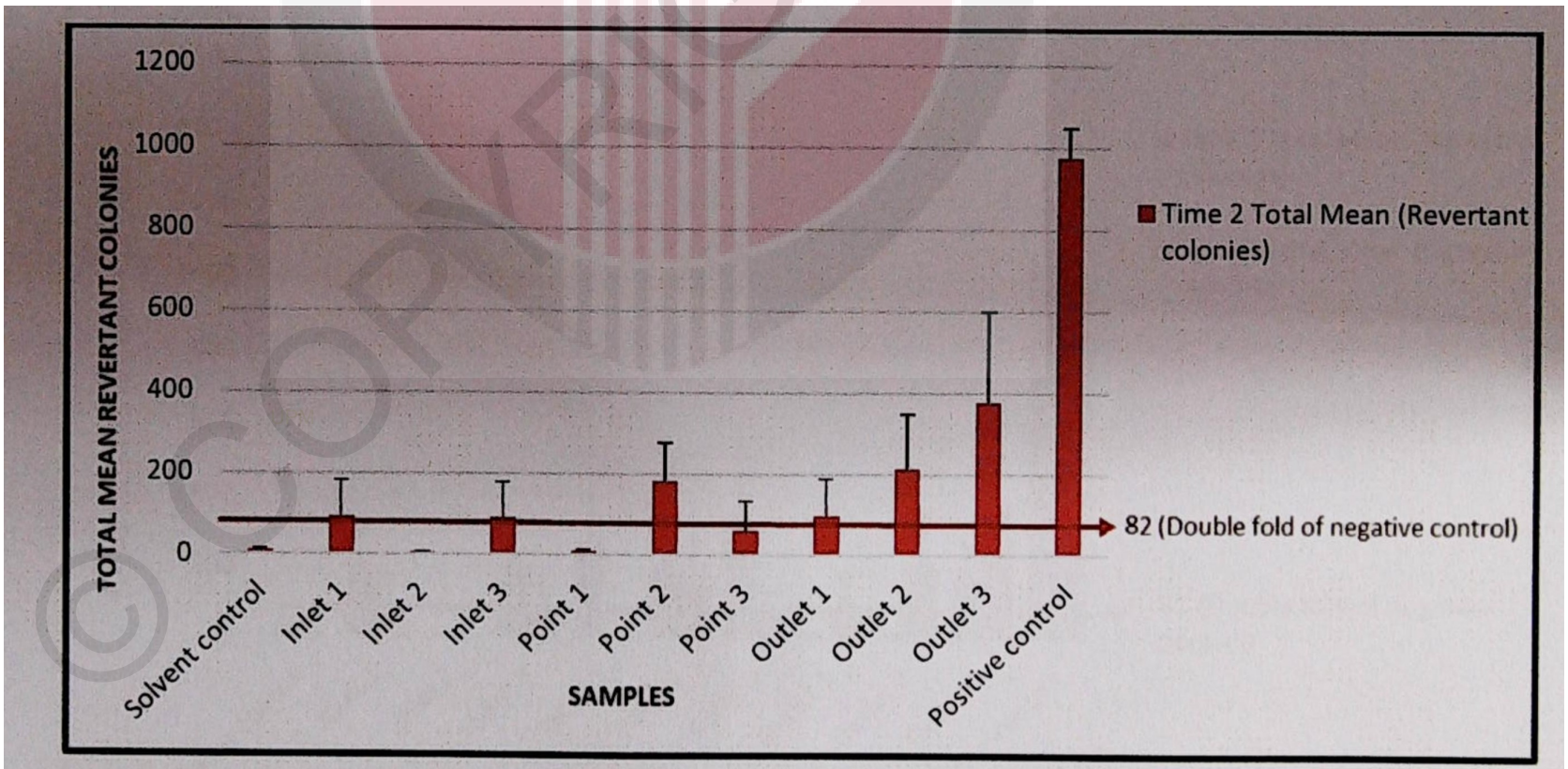

Figure 4.2: Total mean of revertant colonies in Time 2

4.4 Mutagenic Activities of Paddy Water in Time 1 and Time 2

When comparing the T1 and T2 (Figure 4.3), it was shown that there were difference in the total mean of revertant colonies. Some of the samples increase from time 1 to time 2 and some of the samples decrease from time 1 to time 2. The highest total mean for both of the sampling time is at Outlet 3 during Time 2 which has a total mean of 377 ± 222. Meanwhile, the lowest total mean is at Inlet 2 during Time 2 which has a total mean of 6 ± 1.8. Based on Paired t-Test showed that $t(53) = 0.92$, $p>0.05$ which indicates there is no significant difference of mean of revertant colonies in different sampling points in Time 1 and Time 2.

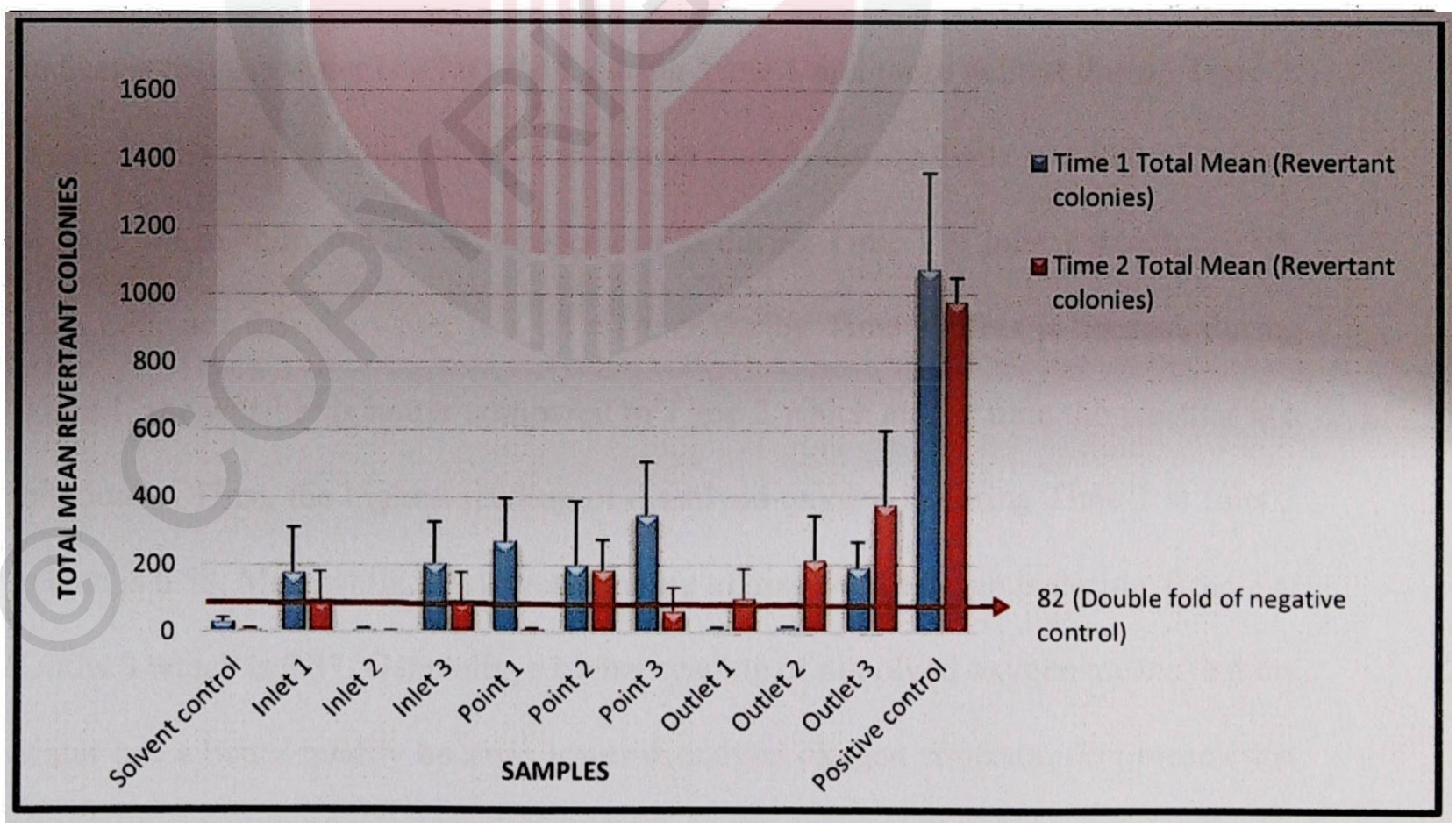

Figure 4.3: Total mean of revertant colonies in Time 1 & 2

CHAPTER 5

DISCUSSION

Table 4.1 shows the physicochemical properties of paddy water samples. For pH level, the highest reading of pH level is at Outlet 2 during Time 2 which is 7.35. Meanwhile, the lowest pH level is during Time 1 is at Inlet 3 which is 5.97. This indicates that the water is a bit acidic during Time 1 and more neutral during Time 2. Next, for the temperature, the highest temperature is during Time 1 at Point 2 which is 36.2. Meanwhile, the lowest temperature is during Time 1 at Inlet 1 which is 27.5. This indicates that the water is a bit warmer during Time 1. This is because during Time 1, the weather is hotter compared to Time 2 which at that time the weather is a bit cloudy. Then, the highest reading of dissolved oxygen is during Time 1 at Inlet 2 which is 6.58. Meanwhile, the lowest reading of dissolved oxygen is during Time 2 at Outlet 3 which is 2.87. Generally, a higher reading of dissolved oxygen means that the water has a better quality because lower dissolved oxygen concentration means that the water has higher algal growth (MacLean et al., 1981; Evans et al., 1996; Nicholls, 1997). This indicates that the water during Time 2 has a better quality than Time 1.

Based on Figure 3.3, there were 12 positive samples for both Time 1 and Time 2. For Time 1, the positive samples were Inlet 1, Inlet 3, Point 1, Point 2, Point 3 and Outlet 3. As for Time 2, the positive samples were Inlet 1, Inlet 3, Point 2, Outlet 1, Outlet 2 and Outlet 3. The double fold of negative control is used to determine which of the sampling point shows mutagenic activity. The double fold of negative control for this experiment is 82. All the samples that exceeds this value were considered as positive samples and shows mutagenic activities.

Point 1, 2 and 3 shows mutagenic activities because there were 5 difference points being taken at each paddy block and homogenized into 1 sample. The possible reason behind the positive induction of mutagenic activities is because during Time 1 and Time 2, the mutagenic substances that may have been used during paddy plantation such as heavy metals, pesticides, herbicides and polycyclic aromatic hydrocarbons was exposed into the paddy blocks. According to Ohe, Watanabe & Wakabayashi, example of the mutagens that existed in surface water are heavy metals, pesticides, polycyclic aromatic hydrocarbons and aromatic amines (Ohe, Watanabe & Wakabayashi, 2004). These mutagens can induce the positive effects in Ames Test.

Some efforts should be done by the governments, public sectors, private sectors and the authorities to prevent any mutagenic substances from polluting the paddy water samples that eventually will end up in our surface water sources. In previous study regarding mutagenicity of surface water at Yangtze River and Taihu Lake, it was determined that huge attempt must be conducted to lower the risk of mutagenicity. Besides closing the industrial company that contributes high pollution in its

surrounding and improving the wastewater treatment plant facilities to reduce the movement of pollutants into the water, improving the lake's self-purification ability and optimizing the drinking water treatment method is also important (Li et al., 2013).

The determination of possible mutagen in the surface water is important because it can give us a proper information about mutagenicity that we need in order to figure out appropriate actions to reduce and prevent the mutagenicity of surface water. By determination of possible mutagen, we also prevent the exposure of mutagenic substances towards the human and environment as it could lead to many health threats.

On the other hand, for the result of Ames test, it was observed that after 48 hrs of incubation the revertant colonies that grows on the medium plate is very small in size. According to Moltermans and Zieger, when the plates are inspected after 48 hrs of incubation and growth retardation is seen as evidenced by smaller than anticipated colony sizes, the plates should be incubated for an additional 12–24 hrs. Therefore, the incubation time of the plates were added to 12 hrs to ensure clearer sight of revertant colonies growth (Moltermans and Zeiger, 2000).

Meanwhile, one of the research gap is that the relationship between the physicochemical properties of the paddy water such as temperature, level of pH and amount of dissolved oxygen with the level of mutagenicity of paddy water has not yet

been clarified, so it is not confirmed whether it contributes to the mutagenic activity in the paddy water or not.

Besides, the specific chemicals that induce the mutagenic activity in the paddy water has also not yet been clarified. In order for us to take appropriate action on this matter, we need to know the exact type of chemicals that is being used so that the government or any related bodies can implement a new regulation based on the used of that chemicals in the paddy water in Malaysia.

In this current research, the sampling points were only 9 and only take into accounts a block of the village which is Block D. Because of this, the result that was obtained may not represent the whole region of the paddy blocks in Kampung Sawah Sempadan, Tanjung Karang, Selangor. In order to make the data represents the whole region, the sampling point should be increased and includes all blocks in Kampung Sawah Sempadan.

CHAPTER 6

CONCLUSION AND RECOMMENDATIONS

6.1 Conclusion

As a conclusion, the paddy water from several points were shown to induce mutagenic activities without the presence of metabolic activation system, S9. The difference of mutagenic activities in each sampling points may be caused from different chemicals that were present for during the sampling process and the difference of weather condition during both of sampling time. Although there were positive in Ames test, however, based on the statistical analysis showed that no significant difference in each paddy water samples in sampling time 1 and sampling time 2 and between the sampling time 1 and sampling time 2 ($p>0.05$).

6.2 Recommendations

For recommendations, the possible mutagens in the paddy water sample should be identified by using High Performance Liquid Chromatography (HPLC) in order to determine the specific chemicals that were causing the mutagenic effect in the paddy water. Besides, the samples also should be exposed with presence of metabolic activation system (S9 mix) in Ames test. This is to identify the possible health risk due to biotransformation process of chemical to human that can be caused by the mutagenic substances in the paddy water samples.

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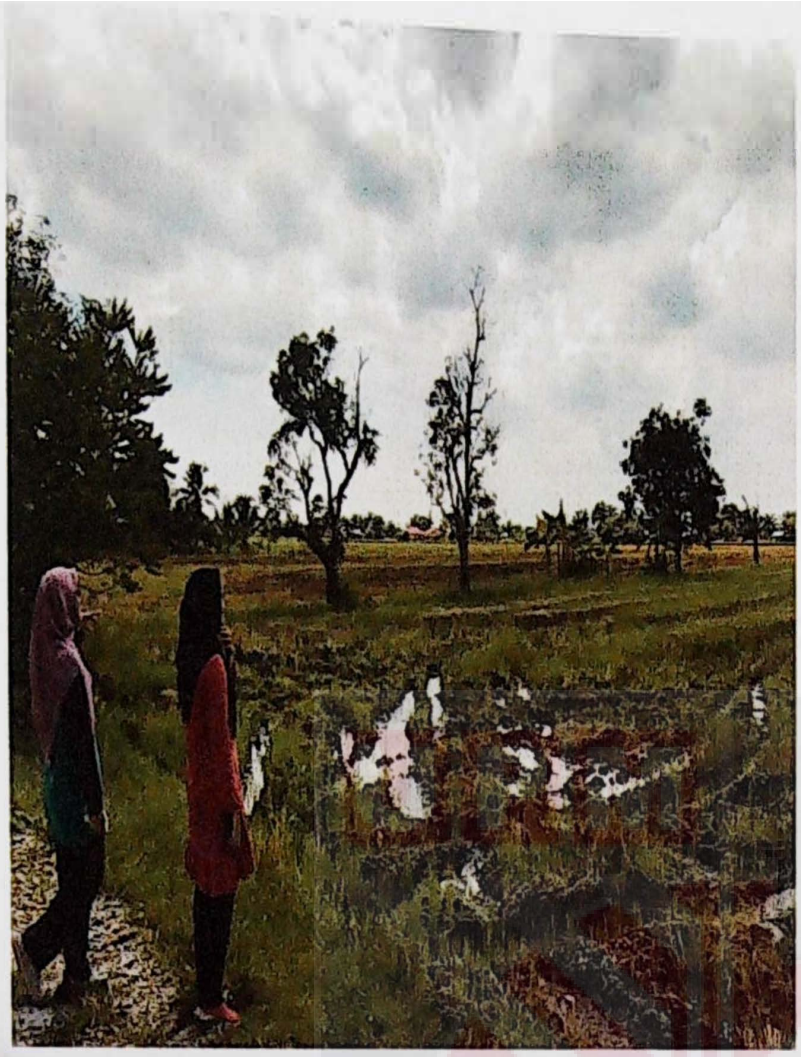
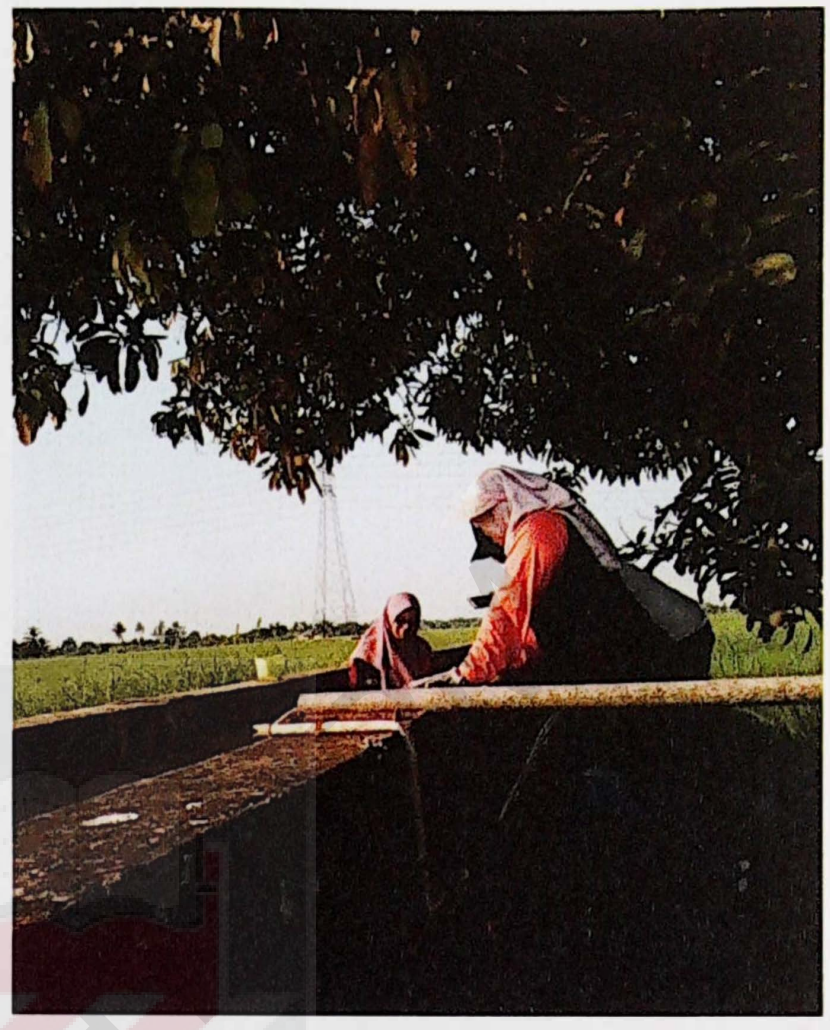
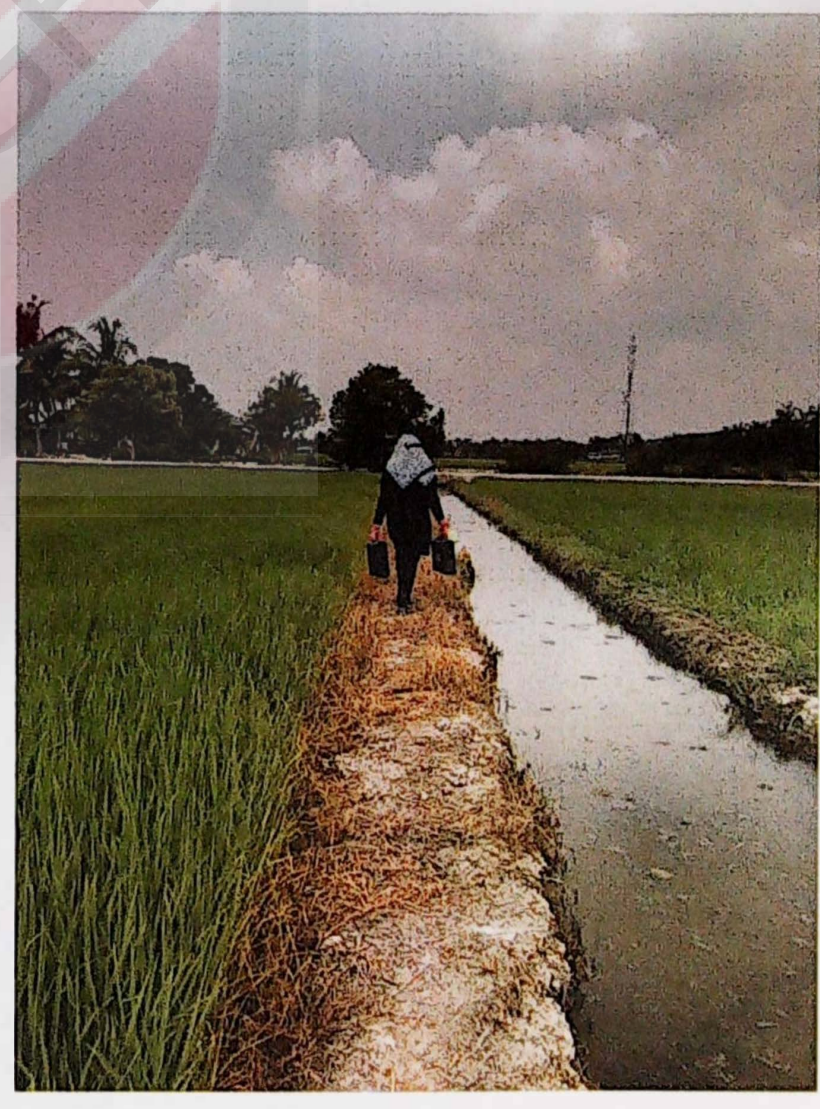
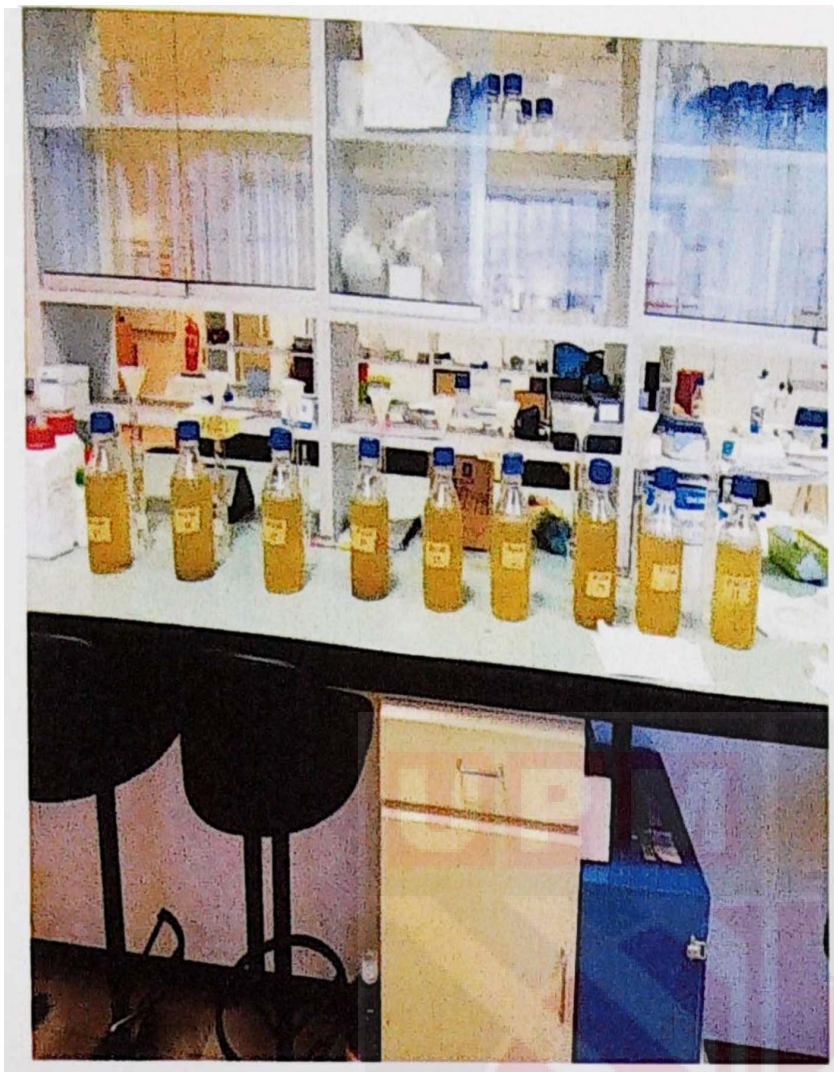
APPENDICES

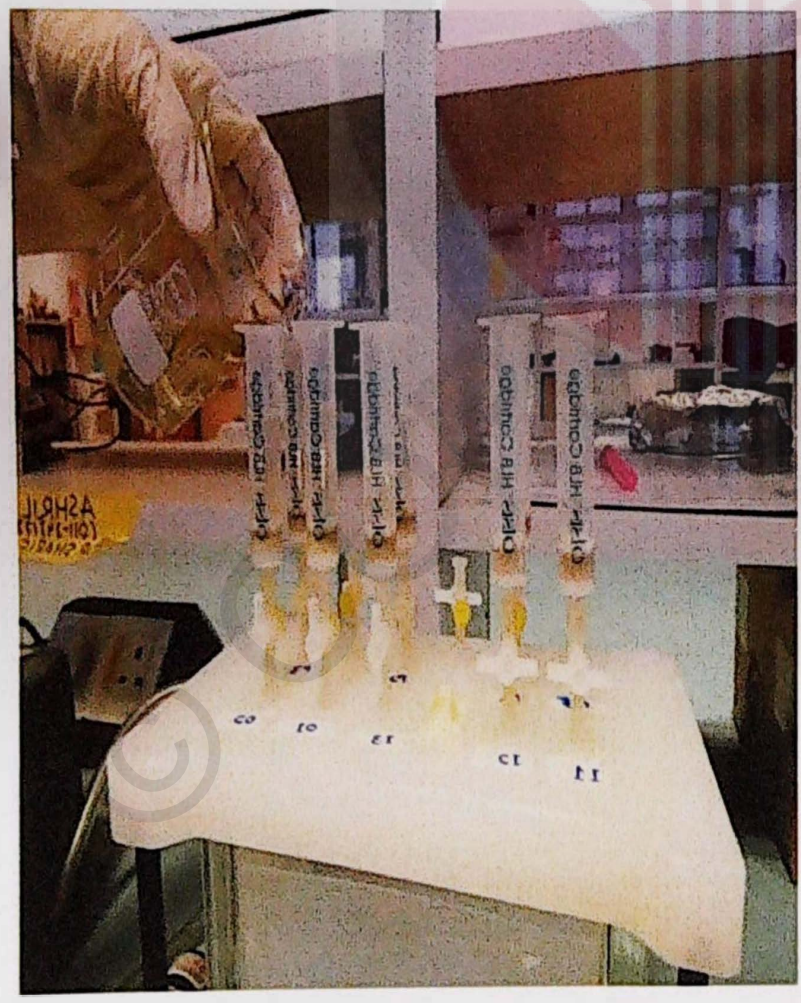


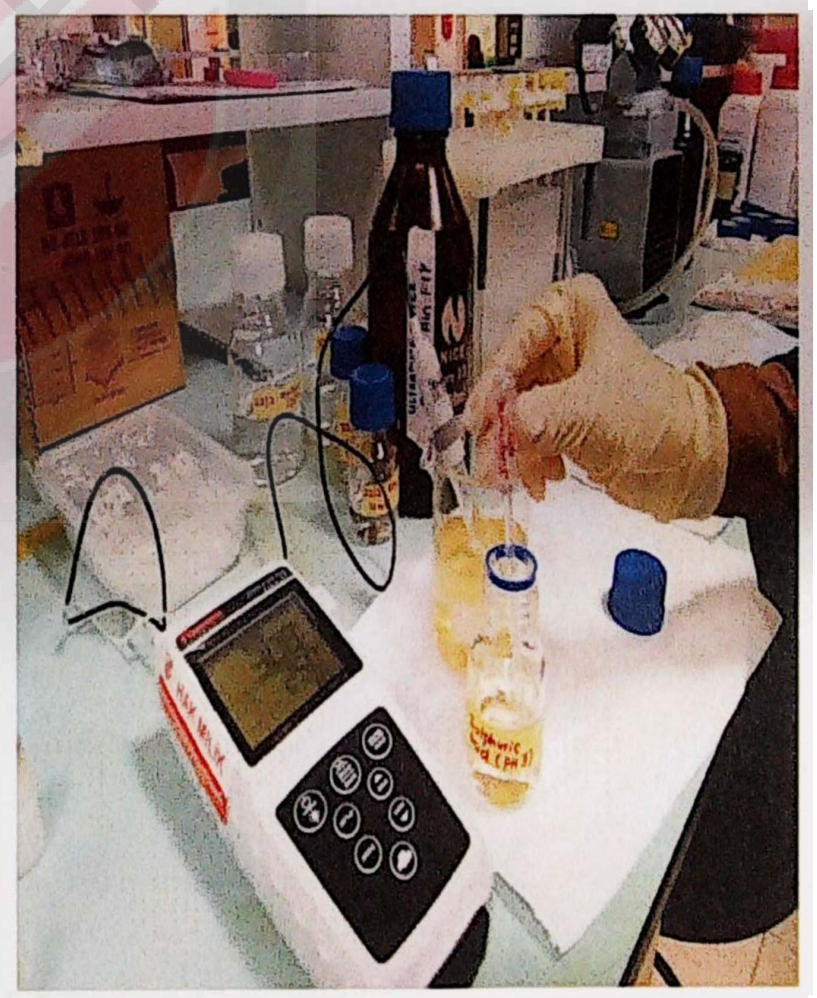




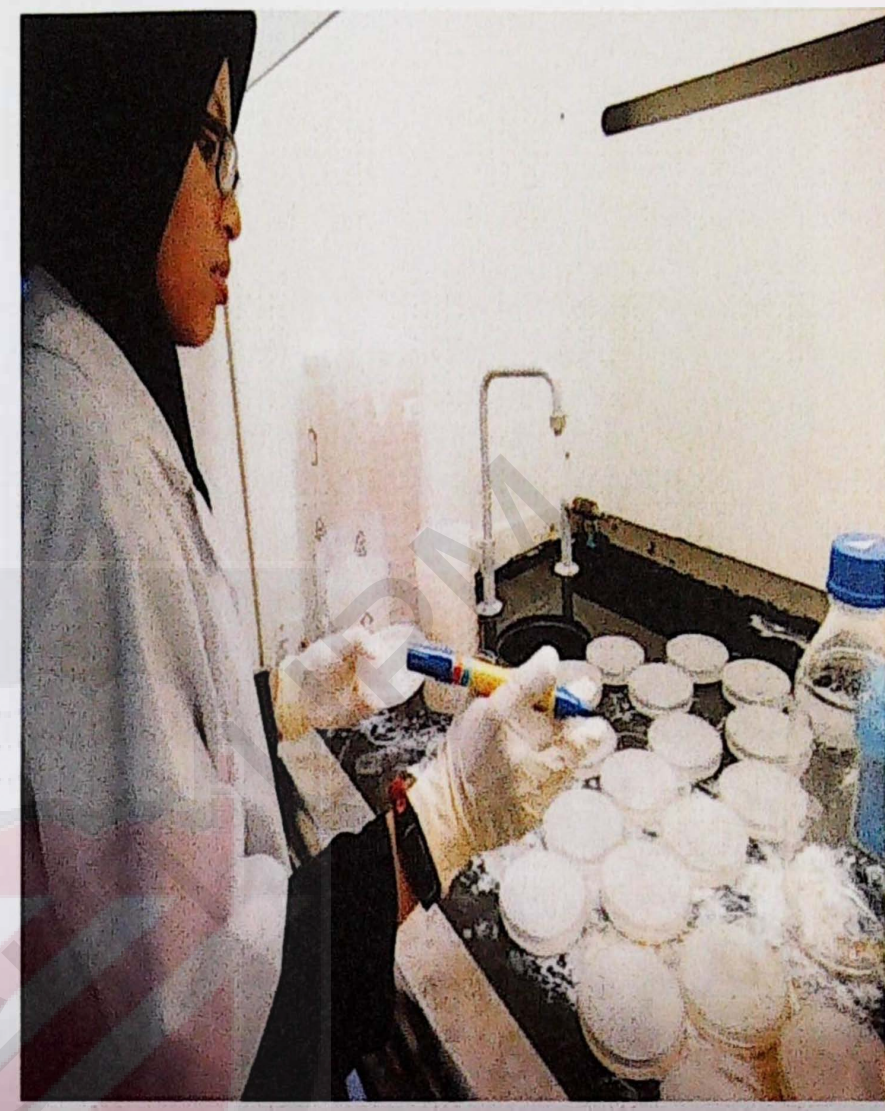
Appendix 1: Sampling of Paddy Water



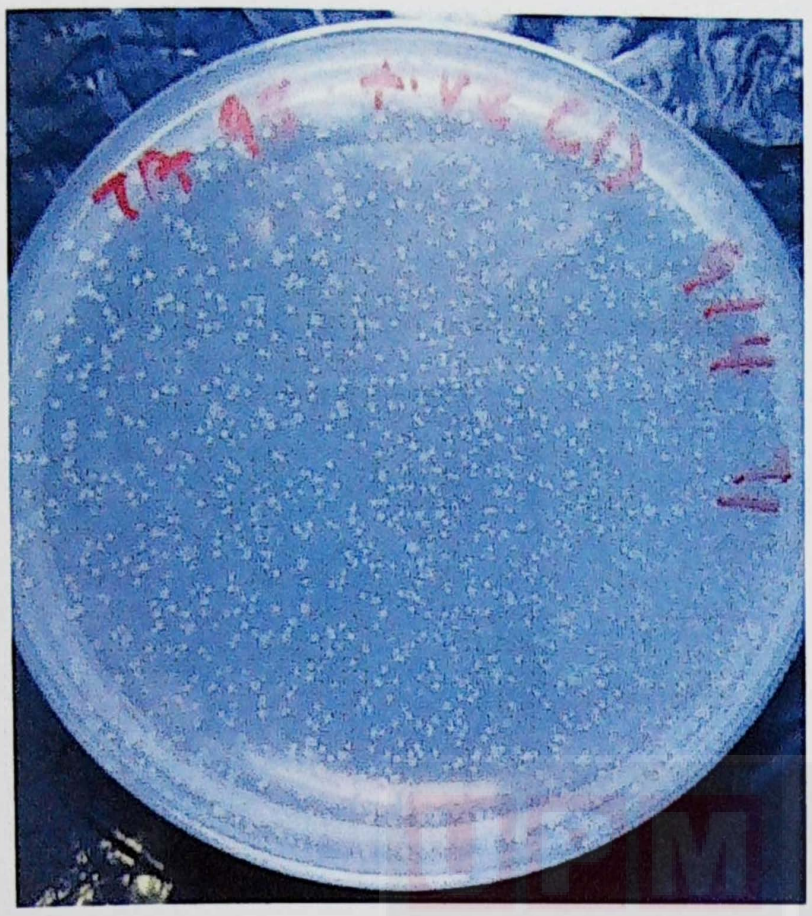




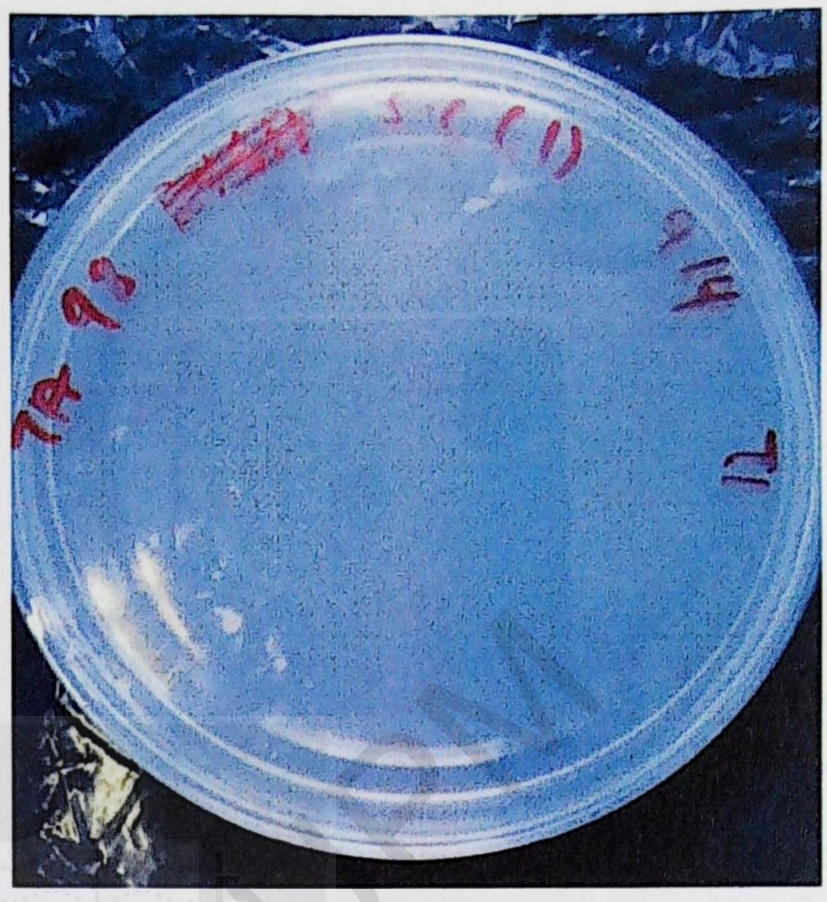
Appendix 2: Solid Phase Extraction



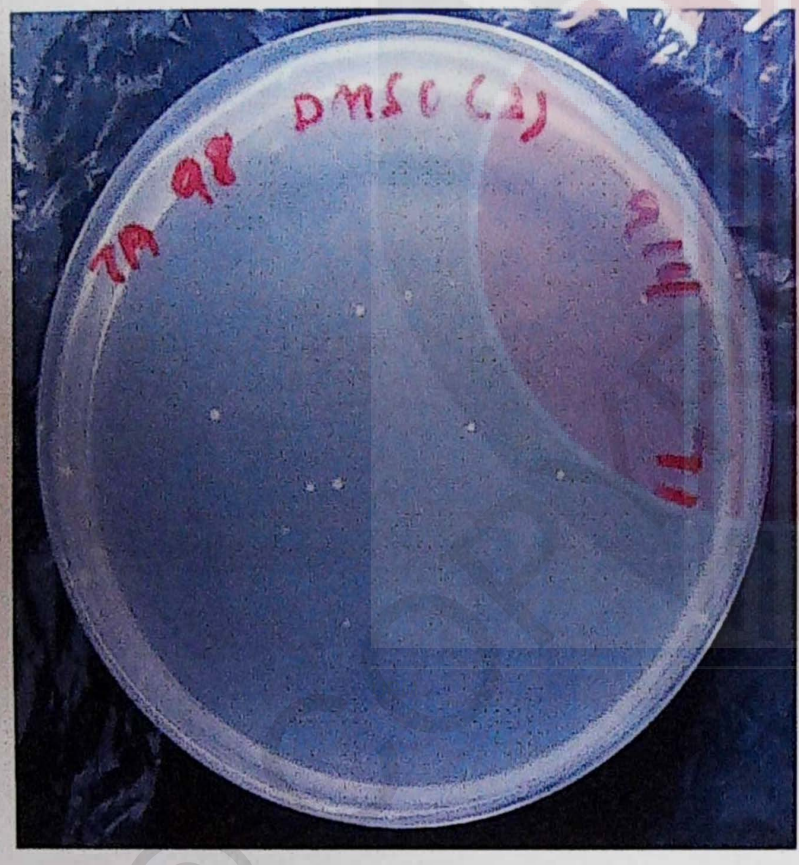
Appendix 3: Ames Test



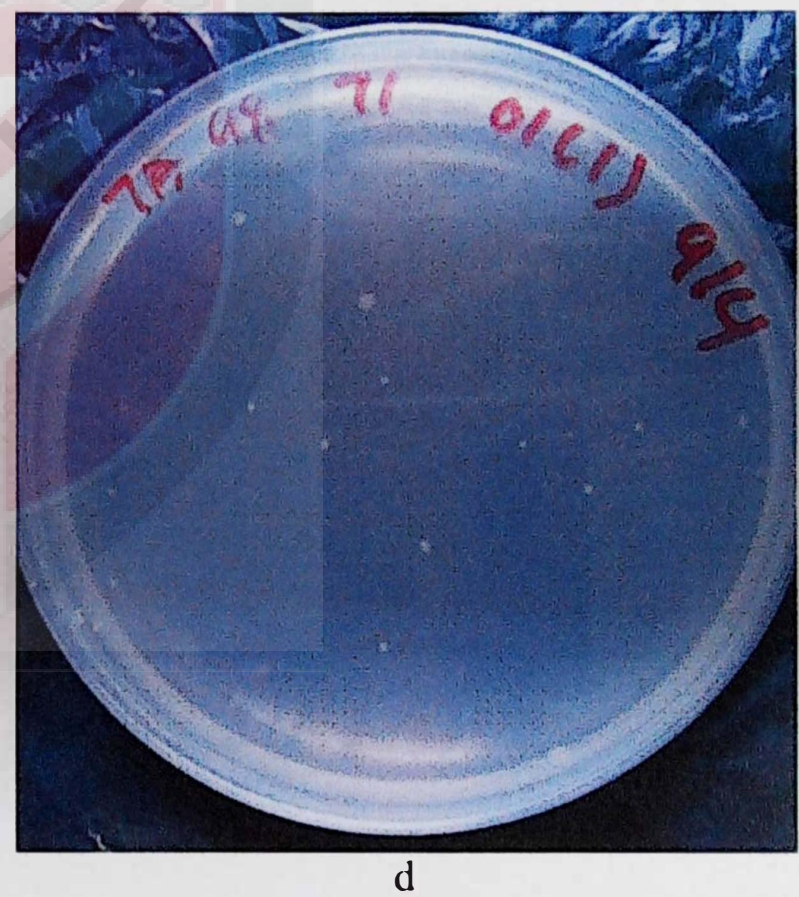
a



b



c



d

Appendix 4: Growth of Revertant Colonies

Remarks: a=positive control, b=negative control, c=DMSO, d=outlet