BIOSORPTION OF LEAD (Pb) USING *Pseudomonas putida* and *Pseudomonas fluorescens*

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"Biosorption of Lead (Pb) using *Pseudomonas putida* and *Pseudomonas flourescens*"

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"Success, like happiness,
is more than a destination – it
is a venture; more than achievement
it is attitude."

-William Arthur Ward

At night when we sleep, we dream, and the reason we wake up the next morning is to make that dream a reality. Dreams will be an imagination forever until we learn to strive to make it come true... Pisay life is a dry and boring one without a cluster of friends and amazing people who are ready to help you in times of need...

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ABSTRACT

The study was conducted using an experimental design to determine the ability of the two bacterial species namely, *Pseudomonas putida* and *Pseudomonas fluorescens* to reduce the initial concentration of lead in the broth medium at 6.54 ppm ± .0.5 ppm in each of the two bacterial culture set-ups. The independent variable is the presence of bacteria in the broth medium with lead and the response variable is the concentration of lead in the broth medium after three periods of exposure. The statistical procedure included the paired T-test at 5% confidence level.

Results showed that the inoculation of the two bacteria in the broth medium with lead has significantly decreased the concentrations of lead in the culture and thus greatly proved that the two bacteria has the potential to be used in bioremediation and biosorption of lead in the environment.

In conclusion, the bacteria that has the highest reduction of concentration of lead in the broth medium is *Pseudomonas fluorescens* that reduced lead concentration from 6.54 ppm to 1.16 ppm after twelve hours of exposure to the metal, compared to *Pseudomonas putida* that has only reduced the concentration of lead from 6.3 ppm to 1.4 ppm.

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CHAPTER 1

INTRODUCTION

A. Background of the Study:

Heavy metals are ubiquitous and persistent environmental pollutants that are introduced into the environment through anthropogenic activities, such as mining and smelting, as well as through other sources of industrial waste. Contamination and subsequent pollution of the environment by toxic heavy metals has become an issue of global concern due to their sources, widespread distribution and multiple effects on the ecosystem (Nriagu, 1990).

In recent times, it has been reported that heavy metals from waste dumpsites can accumulate and persist in soils at an environmentally hazardous levels (Alloway, 1996; Amusan et al., 2005). In Nigeria, leachates from refused dumpsites constitute a source of heavy metal pollution to both soil and aquatic environments (Odukoya et al., 2000 and Oni, 1987).

The interactions between heavy metals and microorganisms has specially focused on bacterial transformation and conservation of metallic ions by reduction in different polluted environments (Chang et al., 1993), the selection of metal-resistant microorganisms as indicators of potential toxicity to other forms of life (Doelman et al., 1994) as well as mechanisms, determinants and genetic transfer of microbial metal-resistance (De Rore et al., 1994). Different procedures for the removal of toxic metal species from contaminated environments have been developed; physiochemical methods, such as chemical precipitation, chemical oxidation or reduction, electrochemical treatment, evaporative recovery, filtration and membrane technologies have been widely

used to remove heavy metal ions from industrial wastewater; these processes maybe ineffective or expensive, especially when the heavy metal ions are in solutions containing in the order of 1-100 mg of dissolved heavy metal ions/L (Hussein et al., 2003 and Mellado et al., 1998).

Lead is an element that is harmful even if taken in small amounts and it is extremely difficult to clean up after dispersal in the environment. It widely contaminates the environment. It is a potent poison; in fact, lead at 500ppm in soil and solid waste qualifies the substance as hazardous waste (Detwyler 2000). Long-term exposure to lead can cause numerous health problems, like anemia, brain damage and kidney diseases. Lead is usually found in dumpsites because many materials that contain lead and its other compounds usually thrown there. Some of these materials are leaded paints, hair dyes, leaded gasoline and batteries (consume 83% of lead used in US in 1990s) (Detwyler 2000).

Biosorption of heavy metals by microbial cells must been recognized as a potential alternative to existing technologies for recovery of heavy metals from industrial waste streams. Most studies of biosorption for metal removal have involved the use of either laboratory-grown microorganism or biomass generated by from pharmacology and food processing industries or wastewater treatment units (Hussein et al., 2003).

Biosorption is considered to be a fast physical or chemical process. The biosorption rate depends on the type of process. Biosorption can be divided into two main processes: adsorption of the ions on cell surface and bioaccumulation within the cell. Biosorption can be defined as the ability of biological materials to accumulate heavy metals from wastewater through metabolically mediated or physio-chemical pathways of

uptake (Fourest and Roux, 1992). Algae, bacteria and fungi have proved to be potential metal absorbents.

Thus the aim of this study is to determine the effect of *Pseudomonas putida* and *Psedomonas fluorescens* on the biosorption of heavy metals in dumpsites using the atomic absorption method. Specifically the study aims to determine the ability of *Pseudomonas putida* and *Pseudomonas fluorescens* in utilizing or converting the harmful heavy metals into a harmless byproduct.

B. Statement of the Problem:

Are *Pseudomonas putida* and *Pseudomonas fluorescens* capable of decreasing the concentration of lead (Pb)?

C. Objectives of the Study:

The general objective of this study is to determine the ability of *Pseudomonas putida* and *Pseudomonas fluorescens* to reduce the concentration of harmful heavy metals in the environment.

Specifically this study aims to:

1.) To determine the concentration of lead in the nutrient broth at three different time intervals, (a) 4 hours, (b) 9 hours, (c) 12 hours, before and after exposure to *Pseudomonas putida* and *Pseudomonas fluorescens.*

2.) To compare the concentration of lead in the nutrient broth at three different time intervals (a) 4 hours, (b) 9 hours, (c) 12 hours, before

and after exposure to *Pseudomonas putida* and *Pseudomonas fluorescens.*

D. Hypothesis:

The presence of *Pseudomonas putida* and *Pseudomonas fluorescens* in the broth medium added with lead (Pb) cannot decrease or alter the concentration of the said metal.

E. Significance of the Study:

Pollution is one of the leading problems in the environment today. Very common of this pollution is caused by improper waste disposal of chemicals and substances in industries and households. Many of these chemicals were accumulated in time in the environment and later causes various environmental and health problems to humans and to other living organisms. Thus this study will contribute on the reduction or even removal of heavy metals specifically lead, in the environment by using two saprophytic, non-pathogenic bacterial species that will reduce the metal concentrations in lead-polluted places such as waste dumpsites.

F. Reasearch Paradigm:

Independent

Prescence of *P. putida* and *P. fluorescens* in solution with lead in different time intervals, (a) 4 hours, (b) 9 hours, (c) 12 hours

Dependent:

Concentration of lead in the nutrient broth solution

G. Definition of Terms

- **Analysis**

Analysis is the separation of something into its constituents in order to find out what it contains, to examine individual parts, or to study the structure of the whole. (Microsoft Encarta 2008)

- **Aseptic Technique**

Aseptic technique is a set of specific practices and procedures performed under carefully controlled conditions with the goal of minimizing contamination by pathogens. (Microsoft Encarta 2008)

- **Bacteria**

Bacteria are ubiquitous one-celled organisms, spherical, spiral, or rod-shaped and appearing singly or in chains, comprising the Schizomycota, a phylum of the kingdom Monera (in some classification systems the plant class Schizomycetes), various species of which are involved in fermentation, putrefaction, infectious diseases, or nitrogen fixation. (www.msn.com)

- **Bacteria Culturing**

Bacteria culturing is the growing of microorganisms especially bacteria, in a nutrient substance culture medium in specially controlled conditions for scientific, medical, or commercial purposes. (Microsoft Encarta 2008)

- **Centrifugation**

Centrifugation is a process where a centrifuge, a device that rotates rapidly and uses centrifugal force to separate substances of different densities, is used. (Microsoft Encarta 2008)

- **Concentration**

Concentration is the amount of a particular substance in a given amount of another substance, especially a solution or mixture. (Microsoft Encarta 2008)

- **Inoculation**

Inoculation is the process of introducing a microorganism or suspension of microorganism into a culture medium; to introduce microorganisms into a culture medium. (Microsoft Encarta 2008)

- **Nutrient Broth**

A general-purpose liquid basal medium composed of, e.g. beef extract and peptone, which allows many types of microorganisms to grow. (Microsoft Encarta 2008)

- **Pollutant**

A pollutant is a substance that pollutes something, e.g. a chemical or waste product contaminating the air, soil, or water. (Microsoft Encarta 2008)

- **Sample**

A sample is a small part or quantity of something such as blood or soil, for scientific or medical examination or analysis. (Microsoft Encarta 2008)

- **Sterilization**

Sterilization means destroying all forms of life on and in an object. A substance is sterile, from a microbiological point of view, when it is free of all living microorganisms. to kill all living microorganisms in something in order to make it incapable of causing infection. (Microsoft Encarta 2008)

- **Subculture**

An identifiably separate social group within a larger culture, especially one regarded as existing outside mainstream society. (Microsoft Encarta 2008)

- **T-test**

T-test is a test of whether a sample of observations comes from a larger sample with a standard distribution of statistical properties. (Microsoft Encarta 2008)

- **Water Sample**

A portion of a water body collected for the purpose of measuring water quality indicators. (Microsoft Encarta 2008)

CHAPTER 2

REVIEW OF RELATED LITERATURE

A. Heavy Metals

Heavy metals are elements having atomic weights between 63.546 and 200.590, and have a specific gravity greater than 4.0 (Kennish and Connell et al, 1984). Living organisms require trace amounts of heavy metals like cobalt, copper, iron, manganese, and zinc. Excessive levels of essential metals, however, can be detrimental to the organism. Non-essential heavy metals of particular concern to surface water systems are cadmium, chromium, mercury, lead, arsenic, and antimony (Kennish, 1992).

Heavy metals are introduced to the environment through anthropogenic activities, such as mining and smelting, as well as though other sources of industrial waste. In fact, over one-half of the superfund sites in the United States are contaminated with heavy metals (www.atsr.cdc.gov). Heavy metals on drinking water reservoirs and fresher habitats and can alter macro- and microbiological communities. The known mechanisms of heavy metal toxicity include inducing oxidative stress and interfering with protein folding and function.

The production of heavy metals has increased rapidly since the industrial revolution. Toxic metal species are mobilized from industrial activities and fossil fuel consumption and eventually are accumulated through the food chain leading to serious ecological and health problems. Since the natural mineralization of metals is a slow process, pollution by heavy metals constitutes one of the most important environmental problems of industrial societies.

The U.S. EPA (Environmental Protection Agency) in its six-year review of chemical contaminants and health effects issued June, 2003 provided the following water quality standards for heavy metals in river and seawater system:

Table 2.1. Recorded water quality of a typical natural aquatic system by the U.S. EPA (Environmental Protection Agency)

Water Quality of a Typical Natural Aquatic System		
Substance or Quality	**River Water**	**Sea Water**
pH	6.8	8.0
Dissolved Oxygen	6-8 ppm	6-8 ppm
Na^+	6.7 ppm	1.1×10^4 ppm
K^+	1.5 ppm	380 ppm
Ca^{2+}	17.5 ppm	400 ppm
Mg^{2+}	4.8 ppm	1.3×10^3 ppm
Cl^-	4.2 ppm	1.9×10^4 ppm
SO_4^{2-}/HSO_4^-	17.5 ppm	2.6×10^3
CO_3^{2-}/HC_3^-	33.0 ppm	142 ppm
Hg^{2+}	< 1 ppb	0.03 ppb
Cd^{2+}	< 1 ppb	0.1 ppb
Pb^{2+}	< 1 ppb	4-5 ppb

Source: http://www.ausetute.com.au/waterana.html

Contamination and subsequent pollution of the environment by toxic heavy metals have become an issue of global concern due to their sources, widespread distribution and multiple effects on the ecosystem (Nriagu, 1990).

In recent times, it has been reported that heavy metals from waste dumpsites can accumulate and persist in soils at an environmentally hazardous levels (Alloway, 1996; Amusan et al., 2005). In Nigeria, leachates from refused dumpsites constitute a source of heavy metal pollution to both soil and aquatic environments (Odukoya et al., 2000 and Oni, 1987).

B. Lead

Lead is a highly toxic metal found in small amounts in the earth's crust. Because of its abundance, low cost, and physical properties, lead and lead compounds have been used in a wide variety of products including paint, ceramics, pipes, solders, gasoline, batteries, and cosmetics. Since 1980, federal and state regulatory standards have helped to minimize or eliminate the amount of lead in consumer products and occupational settings. Today, the most common sources of lead exposure in the United States are lead-based paint in older homes, contaminated soil, household dust, drinking water, lead crystal, and lead-glazed pottery. (http://www.niehs.nih.gov/health/topics/agents/lead/index.cfm)

C. Mechanism of Bacterial Heavy Metal Resistance

Because heavy metals are increasingly found in microbial habitats from natural and industrial processes, microbes have evolved several mechanisms to tolerate the presence of heavy metals (by efflux, complexation, or reduction of metal ions) or to use them as terminal electron acceptors in anaerobic respiration. Thus far, tolerance and mechanisms for metals such as copper, zinc, arsenic, chromium, and nickel have been identified and described in detail. Most mechanisms studied involve the efflux of metal ions outside the cell, and genes for this general type of mechanism have been found on both chromosomes and plasmids. Because the intake and subsequent efflux of heavy metal ions by microbes usually includes a reduction-oxidation reaction involving the metal (that some bacteria can even use for energy and growth), bacteria that are resistant

to and grow on metals also play an important role in the biogeochemical cycling of those metal ions.

D. *Pseudomonas putida* and *Pseudomonas fluorescens* strains

D.1. *Pseudomonas putida*

Pseudomonas putida is a rod-shaped, flagellated, gram-negative bacterium that is found in most soil and water habitats. It grows optimally at 25-30 C and can be easily isolated. *Pseudomonas putida* has several strains including the KT2440, a strain that colonizes the plant roots in which there is a mutual relationship between the plant and bacteria. The surface of the root, rhizosphere, allows the bacteria to thrive from the root nutrients. In turn, the *Pseudomonas putida* induces plant growth and protects the plants from pathogens. Because *Pseudomonas putida* assist in promoting plant development, researchers use it in bioengineering research to develop biopesticides and to the improve plant health.

In 1982, the US National Institutes of Health designated *Pseudomonas putida* a safety strain which meant it could be used to clone genes from other soil-inhabiting bacteria. Certain strains of *Pseudomonas putida* are not pathogenic due to lack of certain genes including those for enzymes that digest cell membranes and walls of humans and plants.

Due to the bacteria's strong appetite for organic pollutants, researchers are attracted to using *Pseudomonas putida* as the "laboratory 'workhorse' for research on bacteria-remediated soil processes". This bacteria is unique because it has the most genes involved in breaking down aromatic or

aliphatic hydrocarbons which are hazardous chemicals caused by burning fuel, coal, tobacco, and other organic matter. There is great interest in sequencing the genome of *Pseudomonas putida* due to its strong effect in bioremediation.

Pseudomonas putida has important lipids that are developed as an adaptation mechanism to respond to physical and chemical stresses. The bacteria are able to change its degree of fatty acid saturation, the cyclopropane fatty acids formation, and the cis-trans isomerization. In different phases, the cell changes its characteristics to better respond to the environment. During the transition from growth to stationary phase, there is a higher degree of saturation of fatty acid and a higher membrane fluidity which improves substrate uptake, thus regulating the cell. All these characteristics allow *Pseudomonas putida* to survive deadly toxins in the soil and allow it to thrive in contaminated areas. Its metabolism allows these bacteria to convert harmful organic solvents to nontoxic composites which are so essential to bioremediation.

Pseudomonas putida is able to tolerate environmental stresses due to its diverse control of proteins including protein and peptide secretion and trafficking, protein modification and repair, protein folding and stabilization, and degradation of proteins, peptides, and glycopeptides. Some important proteins include the global regulatory proteins which link the pathway genes to the cell status. *Pseudomonas putida* exercises a very complex metabolism, the proteins control a particular pathway that not only depends on the signal received, but also the specific promoters and regulators in the pathway.

(http://microbewiki.kenyon.edu/)

D.2. *Pseudomonas fluorescens*

Pseudomonas fluorescens encompasses a group of common, nonpathogenic saprophytes that colonize soil, water and plant surface environments. It is an obligate aerobe that produces a soluble, greenish fluorescent pigment, particularly under conditions of low iron availability. It is motile by means of multiple polar flagella.

Some strains can use NO3 instead of O2 as the electron acceptor. *Pseudomonas fluorescens* also used siderophores to satisfy the need for iron. Strain Pf-5 possesses many extracellular hydrolytic enzymes that degrade polymers found in soil as well as hydrolases used on plant-derived carbohydrates. They are also capable of degrading and using components of plant tissues such as hydrocarbon molecules, fatty acids and oils. *Pseudomonas fluorescens* produces viscosin which is a peptidolipid that enhances antivirality. They also use a sulfate transport system that is competitively inhibited by chromate, which may be associated to *P. flurorescens*'s sensitivity to chromate. (http://microbewiki.kenyon.edu/)

Pseudomonas fluorescens has simple nutritional requirements and grows well in mineral salts media supplemented with any of a large number of carbon sources. Genetic techniques such as conjugation, transposition, and gene replacement are well established. (DOE Joint Genome Institute)

E. Biosorption

The search for new technologies involving the removal of toxic metals from wastewaters has directed attention to biosorption, based on metal binding capacities of various biological materials. Biosorption can be defined as the ability of biological materials to accumulate heavy metals from wastewater through metabolically mediated or physico-chemical pathways of uptake (Fourest and Roux, 1992). Algae, bacteria and fungi and yeasts have proved to be potential metal biosorbents (Volesky, 1986).

The biosorption process involves a solid phase (sorbent or biosorbent; biological material) and a liquid phase (solvent, normally water) containing a dissolved species to be sorbed (sorbate, metal ions). Due to higher affinity of the sorbent for the sorbate species, the latter is attracted and bound there by different mechanisms. The process continues till equilibrium is established between the amount of solid-bound sorbate species and its portion remaining in the solution. The degree of sorbent affinity for the sorbate determines its distribution between the solid and liquid phases.

F. Atomic Absorption Spectrometry (AAS)

The principle used in atomic absorption spectroscopy was discovered in 1802 by Wollaston when he observed the "Fraunhofer lines" or absorption lines in the spectrum of the sun, yet this principle was only applied in 1955 by an Australian physicist, Alan Walsh. The principle states that "Matter absorbs light at the same wavelength at which it emits light". Basically this means that atoms in the ground state absorb the same radiation as they emit in the excited state. An atom in the ground state will absorb an amount of energy equal to the energy difference between the energy

level of the electron in the excited state and the energy level that the electron occupies in the excited state.

In Atomic Absorption Spectrometry, the sample solution is first vaporized and atomized in a flame, transforming it to unexcited ground state atoms, which absorb light at specific wavelengths. A light beam from a lamp whose cathode is made of the element in question is passed through the flame. Radiation is absorbed, transforming the ground state atoms to an excited state. The amount of radiation absorbed depends on the amount of the sample element present. Absorption at a selected wavelength is measured by the change in light intensity striking the detector and is directly related to the amount of the element in the sample.

This process is employed in both qualitative and quantitative use. AAS is a rapid method for the former, if only a few elements are being tested. However if many elements are of interest the process can be too time consuming and uneconomical. The usual quantitative method brackets the sample's absorption spectrum with that of standard concentrations to produce a linear calibration curve.

G. Centrifuge

A centrifuge is a mechanical device that uses the principle of centrifugal force to separate substances of different densities. A common centrifuge is a container that is spun rapidly. The only limit to the centrifugal force is the strength of the metal of which the device is made. Centrifugal forces may be thousands of times as great as the force of gravity.

Centrifuges may be used for rapid separation of substances that would normally separate slowly under the influence of gravity. For example, the draining of water from a wet solid may be accelerated by spinning the solid. This principle is used in the spin cycle of an ordinary automatic washing machine. The smaller the diameter of a centrifuge, the greater the forces and accelerations exerted on the contents and the more rapidly it may be spun without breaking. The most powerful centrifuges, known as ultracentrifuges, are long, narrow tubes rotated at enormous speeds.

The rotor, the spinning part of the centrifuge, in the Beams ultracentrifuge is magnetically suspended in a vacuum and electrically driven. Friction is thus reduced to a negligible amount. (Microsoft Encarta 2008)

CHAPTER 3

MATERIALS AND METHODOLOGY

A. Materials

1 analytical balance

1 autoclave

2 gallon distilled water

10 disposable inoculating loop

15 test tubes

20 100mL test tubes

3 test tube racks

1 250mL graduated cylinder

1 1000mL graduated cylinder

2 250mL Erlenmeyer flasks

1 oven

1 AA Spectrophotometer

1 incubator

3 1000mL beaker

2 250mL beaker

1 antibacterial soap

1 pack of cotton

1 bottle of liquid soap

2 bottles of ethyl alcohol

2 magnetic stirring rods

Bacteria:

Pseudomonas putida

Pseudomonas fluorescens

Chemicals:

1 50mL Lysol concentrate

10 grams of $Pb(NO_3)_2$

METHODOLOGY

B. Sterilization of glass wares

All of the glass wares that had been used in this study were washed thoroughly with soap and water. It was then sterilized in the autoclave at 120 degrees Celsius at 15psi for 90 minutes.

C. Preparation of Nutrient Broth (NB) medium

Nutrient broth powder was weighed according to the manufacturer's instructions. Three and a quarter grams of nutrient broth powder were weighed using an analytical balance. After weighing the powder, it was dissolved in 250ml distilled water in a 250mL beaker. The broth medium was properly distributed in 4 test tubes (two for each bacteria) each containing 10ml of broth. The test tubes were sealed with a cotton plug and wrapped again with an aluminum foil. The prepared test tubes were sterilized in an autoclave at 15psi at 121°C for 1 hour and 20 minutes. After the sterilization process the test tubes were cooled for awhile and were refrigerated for storage, for preparation for the next phase.

D. Sub-culturing of Specimen

The two bacterial cultures (P. *putida* and *P. fluorescens*) were obtained from BIOTECH in University of the Philippines, Los Baños, Laguna. The specimen was expected to be in a agar medium and was inoculated to the nutrient broth prepared earlier.

In the inoculation process, the inoculating loop was heated until the wire of the inoculating loop is glowing red. It was then set aside to let it cool for awhile. The cotton plug from the test tube prepared earlier was removed. The open end of the test tube was heated using a Bunsen burner or an alcohol lamp, creating an updraft (taking air contaminants from the tube entrance). Also, the side lid of the agar plate containing the bacteria was heated before and after getting a bacterial colony from the culture for the removal and killing of the air contaminants that may affect the growth of the specimen. After heating the lid of the plate, a bacterial colony was scraped in the surface of the agar with the inoculating loop. The colony was then inoculated to the test tube prepared in the previous phase by simply dipping the loop wire with the colony to the broth. After the inoculation of the first test tube, the tube was then heated on the opening and also the lid of the agar plate. The same procedure was followed in the other remaining test tubes. Afterwards, there were two broth cultures for *P. putida* and two broth cultures for *P. fluorescens*. The broth cultures were incubated at 30°C for 24 hours using an incubator.

E. Preparation of Lead (Pb) in broth medium

Three 1000mL beakers was sterilized and filled with 13 grams of nutrient broth dissolved in 1000mL of distilled water. The first beaker was labeled as the culture of *P. putida* and the other one was labeled as the culture of *P. fluorescens*; the last beaker was labeled as the control.

Lead Nitrate ($Pb(NO_3)_2$) was obtained at the Chemistry SRA Department of the school. The concentration of lead in each of the beakers was computed by this equation:

concentration (in ppm)= milligram/Liter

The lead nitrate was weighed using the analytical balance. The weight of lead nitrate that was used was based on the reported lead concentration in dumpsites and other polluted wastewaters. The weighed lead nitrate was then sealed in a small paper to secure the poisonous chemical. There were three sealed papers after the weighing: one each for the two bacteria and one for the control.

The measured lead nitrate in grams was then dissolved to their respective beakers. It was then stirred using the magnetic stirrer. The prepared beakers with lead-broth solution were autoclaved at 15psi at 121°C for 1 hour and 20 minutes.

F. Inoculation of bacteria to the NB medium with lead

Inoculation of bacteria was performed by diluting 10mL each of the cultured bacteria to their labeled 1000mL beaker with broth-lead solution. It was then stirred using the stirring rod. The two samples were incubated at 30°C for later used.

G. Centrifugation of bacteria

The centrifugation process was performed to separate the bacteria form the broth. The centrifugation of the broth was performed after 4 hours, 9 hours, and 12 hours of exposure of bacteria to the lead, and every centrifuge, there will three (3) replicates for each of the bacterial culture.

After four hours of incubation, 78mL of each of the bacterial cultures was transferred to 6 different centrifugation bottles (6 centrifugation bottles for PP Culture, and 6 for PF Culture) containing 13mL of bacterial cultures using the pipette.

The prepared centrifugation bottles were placed in the centrifuge for 15 minutes to separate the bacteria from the broth. After the centrifugation, the supernatant in the centrifuge bottles were decanted and poured to the 100mL test tubes.

The same procedure was performed to the other bacterial culture and for the other centrifugation processes later.

H. Preparation of the broth for Atomic Absorption Spectroscopy analysis

After every centrifugation of the broth cultures, the test tubes containing the supernatant was sealed by cotton plug and was then covered by an aluminum foil at the mouth of the test tube. The test tubes were autoclaved, 15psi at 121°C for 1 hour and 20 minutes. The sterilization of the test tubes was performed in order to kill all the remaining bacteria and to prevent them to grow further.

After the sterilization process, the test tubes were refrigerated and later were packed carefully ready for transportation.

100mL of the control was placed in the sampling bottle and was sealed for with a tape ready for transportation.

I. Analysis of broth medium

The broth samples were analyzed using Atomic Absorption Spectrophotometer at UPV MIAGAO for the determination of the reduction of the concentration of lead in the broth medium. The results were measured in ppm.

J. Disposal of bacteria and used tools

All glassware and equipment used in contact of bacteria were sterilized by autoclaving it at 120 degrees Celsius at 15psi for 30 minutes. Before autoclaving, diluted Lysol was added to the broth cultures for proper and surely safe disposal.

All tools that were in contact with the heavy metal were also disposed. All broth with metals will be collected in one sealed container and was given to the school for them to dispose the chemical.

CHAPTER 4

RESULTS AND DISCUSSION

The ability of the two bacterial species specifically *Pseudomonas putida* and *Pseudomonas fluorescens* to reduce the concentration of lead in the nutrient broth was investigated. The samples of the broth containing each of the two bacteria together with the control were analyzed by using the Flame Atomic Absorption Spectrophotometer (FAAS) at University of the Philippines – Miag-ao Campus. The study was supposed to determine the availability of the said bacteria to be used in biosorption process and to reduce the toxic metals in the environment.

Initial concentration of lead in the broth medium was computed with 0.0085 mg ± 0.0005 mg of $Pb(NO_3)_2$, the calculated concentration of lead was 6.54 mg/L ± 0.5 mg/L.

A. Appearance of broth with lead after inoculation

After the inoculation of the two bacteria in the broth medium mixed with lead, the samples were incubated for four hours. After the four hours of incubation, the turbidity of the two broths was noticeably different than the previous observation. The broth became less clear than before and had an unpleasant odor.

Later after 9 succeeding hours of incubation the broth became more and more murky and more unpleasant than before. There were visible black sediments at the bottom of the container and when centrifuged, there were black residue separated to the broth together with the bacteria.

After the 12 hours of incubation, the broth became completely cloudy and the odor was also strong. The black residue separated at centrifugation has also become more evident.

B. Results

The broth, after undergoing centrifugation in order to separate the bacteria and its byproduct from the broth, were transported to UPV Miag-ao in order to determine the amount of lead remained in the broth. The broth samples were analyzed using the Flame Atomic Absorption Spectrophotometer and the mean concentration of lead for the specific time was gathered and shown in Table 4.1.

Table 4.1. Mean concentration (in ppm) of Lead (Pb) after three periods of exposure to the two bacterial strains

Bacterial Strains	**Hours of Incubation**			
	0 Hour	**4 Hours**	**9 Hours**	**12 Hours**
Pseudomonas fluorescens	6.5423	2.1623	1.7956	1.1609
Pseudomonas putida	6.3884	1.8874	1.6725	1.4097

It is evident in Table 4.1 that the concentration of lead decreases over time and the rate of reduction of the two bacteria are inconsistent.

Figure 4.1. Mean Concentration of Lead after three (3) different periods of interval to bacterial species

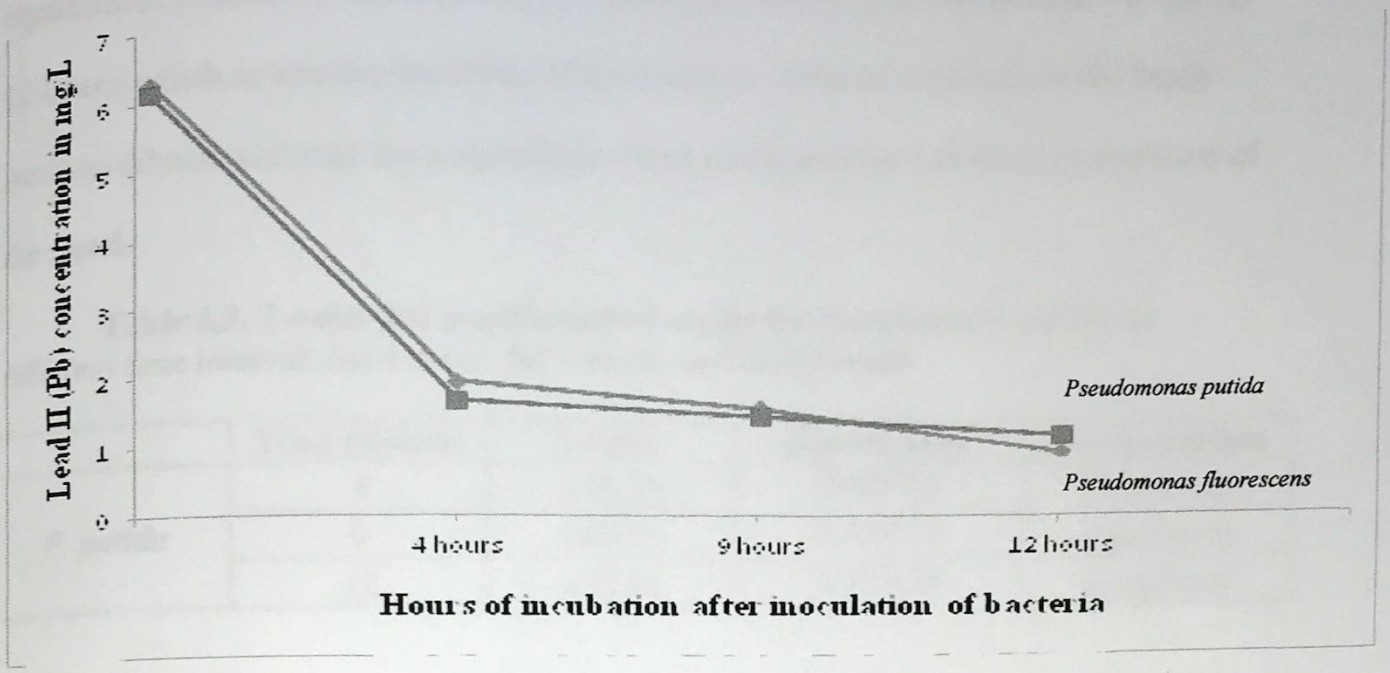

Figure 4.1 shows the concentration of lead versus the time of exposure to bacteria; the concentration of the metal decreases as the time of exposure increases. The graph also compares the rate of reduction of lead by the two bacteria.

C. Statistical analysis

The significance of the results in the reduction of lead by the two bacteria was determined statistically by the PAST. The statistical tool that was used was the one sample t-test.

Table 4.2. T-value and significance of results for *Pseudomonas fluorescens* in different time intervals (a) 4 hours, (b) 9 hours, and (c) 12 hours.

	Time (Hours)	t-value	Significance	Interpretation
P. fluorescens	4	±37.12	0.000725	Significant
	9	±70.12	0.000203	Significant
	12	±20.39	0.002396	Significant

Table 4.2 shows the one sample t-test results of *Pseudomonas fluorescens* at 5% significance yielded a T-value of ±37.12 for 4 hours, ±70.12 for 9 hours and, ±20.39 for 12 hours; which means that the effect of the presence of the said bacteria in the broth medium diluted with lead has a significant effect in the reduction of the concentration of the metal.

Table 4.3. T-value and significance of results for *Pseudomonas putida* in different time intervals (a) 4 hours, (b) 9 hours, and (c) 12 hours.

	Time (Hours)	**t-value**	**Significance**	**Interpretation**
P. putida	4	±36.30	0.000758	Significant
	9	±90.55	0.000122	Significant
	12	±74.87	0.000178	Significant

Table 4.3 shows the results of the statistical analysis for *Pseudomonas putida* at 5% level of significance yielded a t-value of ±36.30 for 4 hours, ±90.55 for 9 hours and, ±74.87 for 12 hours; which shows that the presence of the bacteria in the broth medium has a significant effect to the reduction of the concentration of lead in the medium.

D. Discussion:

The results in the statistical analysis that are shown in Table 4.2 and Table 4.3 clearly show that the two bacteria are very capable in reducing the amount of lead. It is shown in the tables that at 5% level of significance, the concentration of lead in the broth medium in the two cultures significantly decreased over time, as the time of exposure to bacteria lengthens.

The evidence that the bacteria are utilizing the lead in the broth cultures is that it produces visible byproducts at the bottom of the container. The odor and the turbidity of the broth have changed as the time of exposure of the broth to the bacteria lengthens.

The capacity of the two bacteria in reducing lead was determined by comparing the amount of the lead concentration reduced by the two bacteria in every time interval. The results reported that *Pseudomonas fluorescens* and *Pseudomonas putida* have the capability to reduce the concentration of lead in the broth medium.

CHAPTER 5

CONCLUSION AND RECOMMENDATION

This study aimed to find an alternative way of reducing the concentrations of toxic heavy metals in the environment by using bacteria. In this study, two saprophytic bacteria were used to determine their ability to degrade toxic lead concentration in broth medium.

A. Summary of Results

The results of this study proved that *Pseudomonas putida* and *Pseudomonas fluorescens* has the ability to reduce the concentration of lead in broth medium. The results indicate that the presence of the two bacteria in the broth medium with lead significantly decreases the concentration of lead as the time of exposure to the solution lengthens.

Results show that *Pseudomonas fluorescens* has higher capacity to reduce the concentration of lead in the broth medium than *Pseudomonas putida* in the given time duration of twelve hours. *Pseudomonas fluorescens* started rather slowly in the first 4 hours of exposure to lead than the *Pseudomonas putida* but has greatly reduced the concentration from 6.5 ppm to 1.1 ppm in twelve hours compared to *Pseudomonas putida* that reduced the concentration from 6.3 ppm to 1.4 ppm.

B. Conclusion

The results of this study proved that the two bacteria have significantly decreased the concentration of lead in the broth medium and it also proved that the two bacteria have the potential to be used in biosorption and bioremediation processes in the future.

The results proved that the usage of the two bacteria in the biosorption and remediation of toxic heavy metal in the environment is effective and it is convenient and cheaper than the conventional ways of removing the harmful pollutants in the environment.

C. Recommendations

Recommendations for further study are as follows:

1. Determine the by product produced by *Pseudomonas fluorescens* and *Pseudomonas putida* while on the process of reducing the amount of lead and determine if it is its harmful or not and.
2. Determine the highest concentration of lead the two bacteria can tolerate and can reduce.
3. Determine the effect of the two bacteria in the concentration of the other metals that are usually found in polluted areas like dumpsites.
4. Determine the ability of other species of bacteria to reduce the concentration of lead or other metals those are usually toxic and compare them to P. putida and P. fluorescens for its effectiveness as biosorbent.

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APPENDICES

APPENDIX A

A.1. Initial Concentrations of Lead (Pb) of PP broth and PF broth

PF:

$$\frac{0.0085\text{g PbNO3}}{1\text{ L}} \times \frac{1\text{mol PbNO3}}{269.2\text{g PbNO3}} \times \frac{1\text{mol Pb}}{1\text{ mol PbNO3}} \times \frac{207.2\text{g Pb}}{1\text{ mol Pb}} = 6.542\text{ppm Pb}$$

PP:

$$\frac{0.0083\text{g PbNO3}}{1\text{ L}} \times \frac{1\text{mol PbNO3}}{269.2\text{g PbNO3}} \times \frac{1\text{mol Pb}}{1\text{ mol PbNO3}} \times \frac{207.2\text{g Pb}}{1\text{ mol Pb}} = 6.388\text{ppm Pb}$$

A.2. Raw Data for Lead (Pb) Concentration Analyses

Table 1. Raw data for Lead (Pb) concentration analysis of the nutrient broth media after three different hours of exposure (4hrs, 9hrs, and 12hrs) to *Pseudomonas fluorescens*

Time(in hours)	Replication			Average	Std Dev
	1	2	3		
4	2.2217	2.3304	1.9348	2.1623	0.2044
9	1.8420	1.8826	1.6623	1.7956	0.1172
12	0.6536	1.2884	1.5406	1.1609	0.4570

Table 2. Raw data for Lead (Pb) concentration analysis of the nutrient broth media after three different hours of exposure (4hrs, 9hrs, and 12hrs) to *Pseudomonas putida*

Time(in hours)	Replication			Average	Std Dev
	1	2	3		
4	1.8362	2.1232	1.7029	1.8874	0.2148
9	1.6971	1.7478	1.5725	1.6725	0.0902
12	1.4710	1.4812	1.2768	1.4097	0.1152

APPENDIX B

Statistical Analysis for Lead (Pb) Concentration

Table set 1: Paired t-Test of 0 hours compared with 4, 9, and 12 hours

PF:

	PF			
Time (hours)	0	4	9	12
Pb Conc. (ppm)	6.5423	2.2217	1.842	0.6536
	6.5423	2.3304	1.8826	1.2884
	6.5423	1.9348	1.6623	1.5406
t-value	-	37.12	70.12	20.39

PP:

	PP			
Time (hours)	0	4	9	12
Pb Conc. (ppm)	6.3884	1.8362	1.6971	1.4710
	6.3884	2.1232	1.7478	1.4812
	6.3884	1.7029	1.5725	1.2768
t-value	-	36.3	90.55	74.87

APPENDIX C

Values for t-test to Determine Level of Significance

Table 1. Partial tabular value for t-test at 0.05, 0.025, 0.01, and 0.005 level of significance

∞ Df	0.05	0.025	0.01	0.005
1	6.314	12.706	31.821	63.657
2	2.920	4.303	6.965	9.925
3	2.353	3.182	4.541	5.841
4	2.132	2.776	3.747	4.604
5	2.015	2.571	3.365	4.604
6	1.943	2.447	3.143	3.707
7	1.895	2.365	2.998	3.499
8	1.860	2.306	2.896	3.355
9	1.833	2.262	2.821	3.250
10	1.812	2.228	2.764	3.169
11	1.796	2.201	2.718	3.106
12	1.782	2.179	2.681	3.055
13	1.771	2.160	2.650	3.012
14	1.761	2.145	2.624	2.977
15	1.753	2.131	2.602	2.947

Appendix D

List of Plates

Plate 1: Distribution of broth media using 10mL pipette

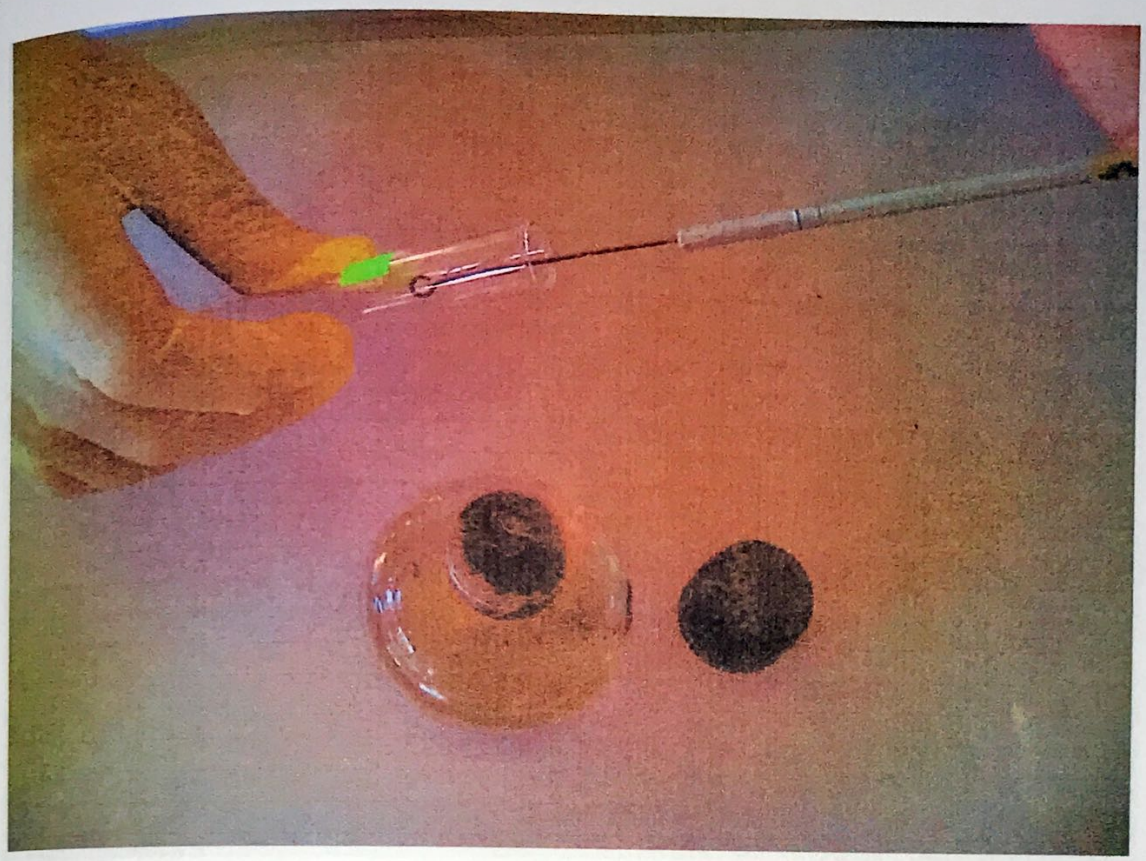

Plate 2: Subculturing of bacteria from slant agar medium to broth medium

Plate 3: Incubation of bacterial cultures at 25°-30° Celsius

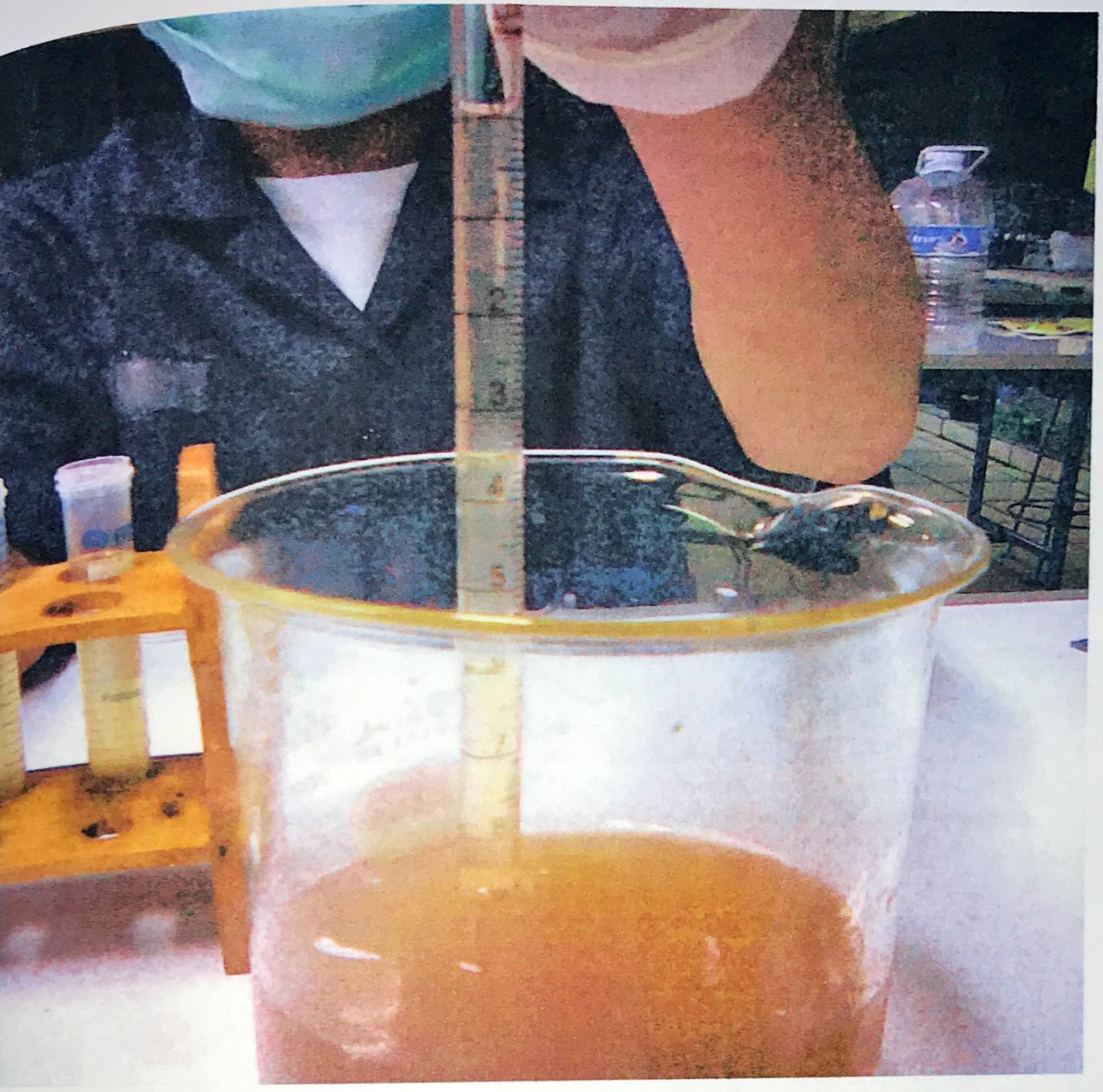

Plate 4: Preparation of bacteria culture for centrifugation

Plate 5: Distribution of bacteria culture to centrifugation tubes for centrifugation

Plate 6: Placing of centrifugation tubes in the centrifuge

Plate 7: Centrifugation of bacteria culture

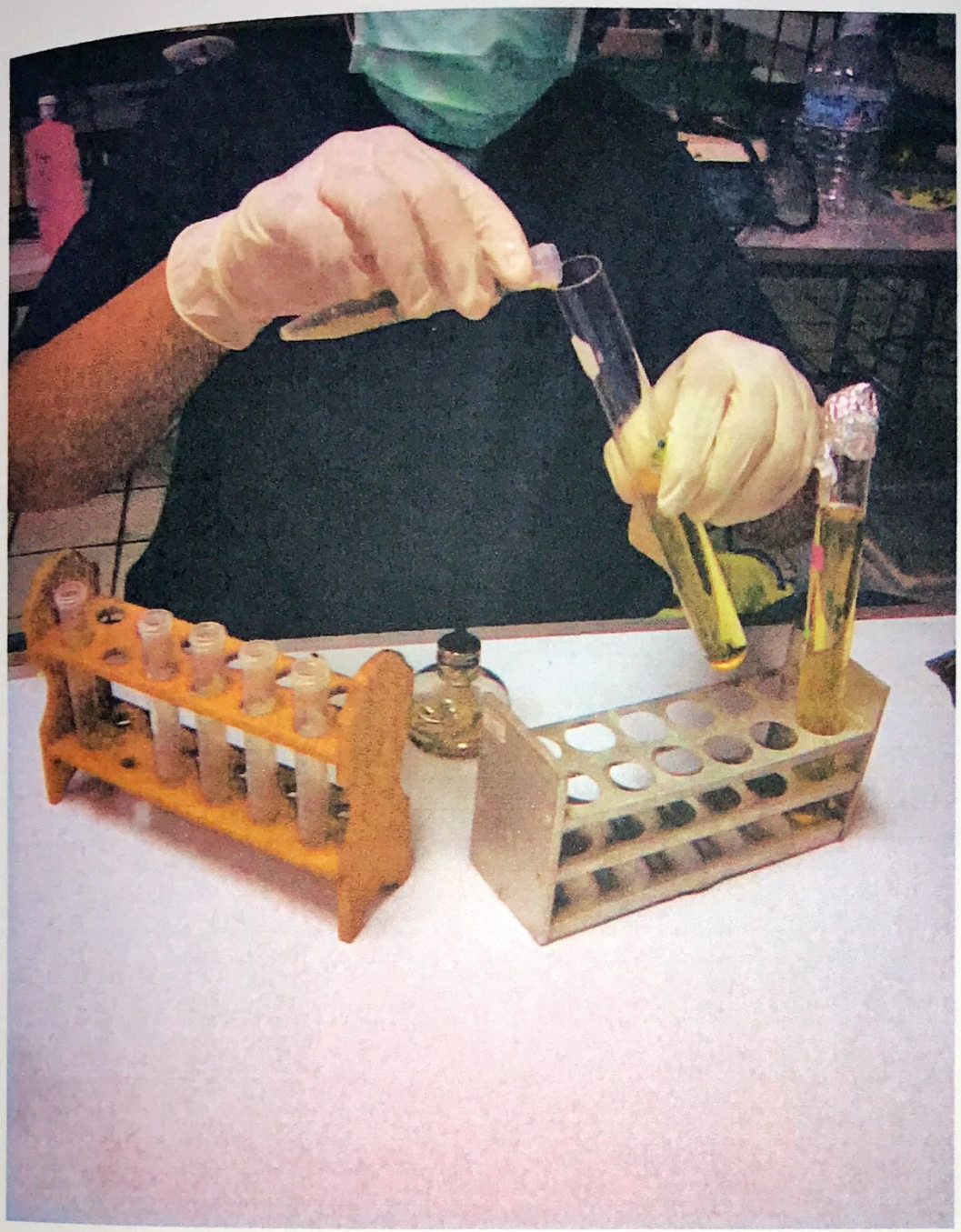

Plate 8: Bacteria culture after centrifugation