CHAPTER 3

MATERIALS AND METHODS

Laboratory experiments were conducted on soil/sludge samples so as to make a comparison between natural degradation and treated-soils and to estimate biodegradation rates.

3.1 Sampling site and soil contaminated oil

The sample soil was taken from Fang Petroleum Refinery, Chiang Mai Thailand. In order to get a representative sample, the pH of soils contaminated by oil were measured and when their pH levels were between 6 and 8 the soil was taken and transported to a laboratory to measure bulk density. There were 8 (eight) sites. Sampling site 1 was around the white oil barrel, site 2 was at the pond of waste water treatment, site 3 was opposite from site 2, site 4 was next to site 3, site 5 was up the front of site 4, site 6 was between post of waste water treatment and site 7 (small pit or lagoon), site 7 was at small pit or lagoon and site 8 was around of the white oil barrel (appendix 10 and appendix 11).

From the eight sampling sites two sites were chosen to be representative sites, these were site 3 and site 7.

The consideration of which these sites to choose was because site 7 was a pit or lagoon from which sludge oily from the oil refinery processing was dumped and its soil texture was mostly clay. This soil had a low soil porosity and poor nutrients. Site 3 was also soil contaminated oil but besides being rich in nutrients it also had high soil porosity

because its texture was sandy and a vegetation land (above this soil, the grasses found was growth well). So mixing the soil from site 3 would increase both soil porosity and nutrient to soil site 7, but pH still remained at the optimum for biodegradation.

The measurement of the bulk density was done at faculty of Civil Engineering, Chiang Mai University. The measurement of bulk density by using the thin wall sampler, a metal circular tube with dimensions are outside diameter (OD) (63.63 mm), inside diameter (ID) (60.53 mm), length (60 cm) and weight (1329.30 g). The tube was hammered into the soil until its depth was around 50 cm, then it was taken out and the length of soil in the tube was measured. And finally the tube and the soil were weighed.

3.2 Experiment model

The treatments were divided into 2 (two) groups were the first group, the tank 1 (the control group) contained a sample without additional nutrients and air. The inside for this tank is without aeration system. The tank shape and the inside of this tank are shown at figure 3.1. The second group, a treated-soil group where the soil was treated with nutrients and additional air. This group consisted of 5 (five) tanks with a varied aeration flow-rate. The aeration system for this group is perforated plastic pipes, an automatic timer, pressure regulator and an air flow rate regulator and air pump (figure 3.2). Thus, there were 6 tanks for the experiment. Each tank was made of glass with dimensions of 0.25 m x 0.25 m x 0.50 m.

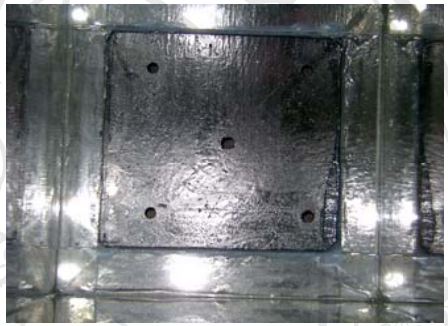

Figure 3.1 The tank shape (left) and the inside (top view) of tank 1 or control group

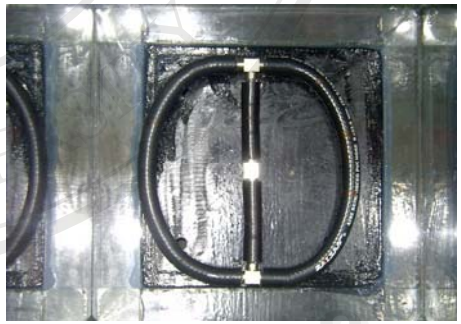

Figure 3.2. The tank shape (left) and the inside (top view) for treated-soil group (tank 2 through 6)

The air was pumped through plastic pipe and its flow was controlled by automatic timers. The plastic pipe with dimension (8 x 13.5 mm) was placed in a circular pattern at the base-middle of the tank (figure 3.2).

One end of the piping served as the inlet source while the other served as the exhaust. The plastic pipe was punched with 3 mm diameter holes as the entrance for air into the soil. Air flow rate was monitored with a Dwyer Rate-Master Flowrate series (RMB) on a daily basis. The air flow rate was varied and set to 2 hours intervals per day during experiment period and was controlled by the use of an automatic timer. Small stones with 1-cm diameter were used to cover and prevent plastic tubing from clogging with fine particles.

3.3 Adjustment of soil porosity

Before the soil was put in the reactor, the soil void volume was measured in order to get the soil void volume 25% (Von Fahnestock *et al*., 1996). The determination of soil porosity was done indirectly by calculation, after the measurement of water content was done. The method for measurement of the water content is shown in section 3.7.4. and this measurement and calculation was done by the faculty of Civil Engineering, Chiang Mai University.

3.4 Sample preparation

Before the sample was adjusted by soil nutrients and put in each tank, the materials such as macro fauna, large roots, stones and plastics that may disturb the

aerobic microorganism that degrade contaminants in the sample was removed.

In order to measure heterotrophic microorganism, physical and chemical, the samples was taken out and put in each container. Then each sample was stirred thoroughly until the sample was homogeneous. After the sample was homogeneous some amount of each sample was taken then the sample was put back into the original tank. These activities were done every time when the heterotrophic microorganism, physical and chemical was measured.

3.5 Adjustment of soil nutrients

Adjustment of soil nutrient is necessary when the compound of ratio nitrogen and phosphorous to total carbon is less than 100:10:1 (Von Fahnestock *et al*., 1996). Determination of soil nutrient was done by faculty of Agriculture, Chiang Mai University. The measurement results showed that the compound ratio of nitrogen and phosphorous to organic carbon was less than 100:10:1, so nitrogen and phosphorous was needed. The calculation is shown in the appendix 1. From this calculation, the total organic carbon, nitrogen and phosphorous in the 125 kg of soil sample was 21829 g, 275 g and 0.85 g, respectively. In order to achieve the ratio C:N:P (100:10:1) the amount of nitrogen and phosphorous added was 3.7 kg and 906 g, respectively. The sources of nitrogen and phosphorous were urea and diammoniun phosphate, respectively without the addition of water.

3.6 Aeration flow rate

After the concentration of total petroleum hydrocarbons was known and the adjustment of the nutrients was done then the amount of air to be injected into the soils was decided upon. Next, the formulation of the ratio of electron acceptors for bioremediation required the writing of a balanced reduction-oxidation (redox) equation. Because solid-phase treatment, polynuclear aromatic hydrocarbons (PAHs) usually control the cleanup time because of their low rate of degradation, thus the dominant contaminant of PAHs was identified. The equation for the half reaction for organic redox reaction is as shown below:

$$\frac{1}{Z}(C_aH_bO_cN_d) + (2a - \frac{c}{Z}(H_2O) = \frac{a}{Z}\ (CO_2) + \frac{d}{Z}\ (NH_3) + H^+ + e^-$$

Since that equation needs a number of atoms for organic contaminant, an analysis of dominant PAHs in the soil was identified by GC-MS technique at Department of Chemistry, Faculty of Science, Chiang Mai University (appendix 2). The result showed that the significant high contaminant in the soil was Naphthalene. Thus, the above equation can be solved. Since ammonia was chosen as the nitrogen source, the cell synthesis equation used was :

$$\frac{1}{8}\ CO_2 + \frac{1}{20}\ NH_3 + H^+ + e^- = \frac{1}{20}\ C_5H_7\ O_2N + \frac{2}{5}\ H_2O$$

Since the factors in the reaction for distribution of the energy between biomass synthesis and other needs is $f_e + f_s = 1$, for aerobic systems, the factors range between

0.12 and 0.6. In order to calculate the amount of oxygen in this study the factor f_s was varied 0.12, 0.25, 0.36, 0.5, and 0.6.

From the above equation the amount of oxygen needed to remediate the soil can be calculated, while the next engineering requirement is to determine the rate of oxygen to be supplied and the capacity of the air pump.

The degradation pattern is first-order as reported by most independent authors (Jorgensen *et al*., 1999 and Li *et al*., 2003), the equation is:

$$\frac{dL}{dt} = -kL \qquad \text{or} \qquad L_t = Le^{-kt}$$

where:

L = original concentration of organic compounds

L_t = concentration after degradation for time t in days

k = firs-order degradation rate constant, unit per day

And the rate of oxygen may be known by equation:

$$\frac{dN}{dt} = -k(R_N)L_t$$

where dN/dt = kg of oxygen required per day

R_N = mass ratio of the oxygen

One example of the calculation of the amount of oxygen that was forced into the tank may be shown at the appendix 3, and the result of that is as shown at table 3.1. The same way was done to tank 2, 3, 4, and 5; for f_s 0.25, 0.36, 0.5 and 0.6, respectively.

Table 3.1. The amount of forced air to the tank experiment

Treatment	fs	Amount of oxygen (kg/day)	Amount of air (l/min) ,(SCFH)	Notes
Tank 1	-	-	-	-
Tank 2	0.12	0.35	11.8, 24.9	Pump was run 2 hours/day
Tank 3	0.25	0.25	8.6, 18.1	Pump was run 2 hours/day
Tank 4	0.36	0.18	6.2, 13.2	Pump was run 2 hours/day
Tank 5	0.5	0.11	3.8, 8.1	Pump was run 2 hours/day
Tank 6	0.6	0.07	2.4, 5.2	Pump was run 2 hours/day

Note: 1 SCFH = 0.472 l/min

3.7 Physical and Chemical Analyses

Biodegradability of the six treatments were tested under aerobic conditions and the collected sample was examined periodically to measure soil temperature, soil pH, soil texture, soil moisture content, total nitrogen and phosphorous, organic carbon, total petroleum hydrocarbon (TPH) and the enumeration of the total heterotrophic microbial in the soil.

3.7.1 Measurement of soil temperature

Temperature was measured directly in places using a probe or thermocouple.

3.7.2 Measurement of soil pH

Measurement of soil pH was done by “Glass Electrode-Calomel Electrode pH meter Method” followed by the Method of Soil Analysis, part 2, Chemical and Microbiological Properties (2^{nd} ed.) (1982), ASA and SSSA. Measurement of soil pH was done by Faculty of Agriculture, Chiang Mai University.

A 20 g of air-dried ample were placed into a paper cup and then 20 ml distilled water was added using pipette. After mixing thoroughly and left standing for 10 min then the electrode (Beckman 34 pH meter series 015-247664-A) was inserted to record the pH of soil. This procedure is attached at appendix 4.

3.7.3 Determination of soil texture

Determination of soil texture was done by “hydrometer method” followed by the Method of Soil Analysis, part 1, Physical and Mineralogical Method, (1986), ASA and SSSA and its determination was done at the Faculty of Agriculture, Chiang Mai University.

A 40 g sample was weighed and 250 ml of distilled water and 100 ml of HMP were added. Then the sample was soaked overnight. After that the sample was stirred by Hamilton beach scovill (made in USA) series 936-31-1 for 5 min. After

the sample was well mixed then hydrometer was recorded. The soil texture was compared to USDA classification scheme (appendix 4).

3.7.4 Determination of soil moisture content

Determination of soil moisture content was done by "the gravimetric with oven drying" method followed the Method of Soil Analysis, part 1, Physical and Mineralogical Method (1986), ASA and SSSA. Determination of soil moisture content was done by Faculty of Agriculture, Chiang Mai University.

A sample between 10-100 g was put into metal cans and the sample was weighed (the balancing was AND HR 200 made in Japan series 12304150 max. 210 g, d=0.1 mg). The sample was placed into hot-air-oven (the oven was Melag made in Germany series 401) for 24 hours or until the sample reached a constant weight. After the sample reached a constant weight then the sample was weighed again and the tare weight of the sample container was determined and the moisture content calculated (appendix 4).

3.7.5 Determination of organic carbon

Determination of organic carbon was done by **"**Walkley-Black procedure" followed by the Method of Soil Analysis, part 1, Physical and Mineralogical Method, ASA and SSSA. Its determination was done by Faculty of Agriculture, Chiang Mai University.

The sample was grinded and sieved by a 100 mesh sieve. 5 ml of 1 N potassium dichromate was added to the sample containing 10 to 25 mg of organic C. After that it was swirled. The 7.5 ml of sulfuric acid was added and warmed at 150^0C for 30 min. Then 200 ml distilled water and 3 - 4 drops of 0.025 M phenanthroline indicator were added. The sample was titrated with 0.2 N ferrous sulfate heptahydrate until the color changed from blue to red (appendix 4).

3.7.6 Determination of total nitrogen

Determination of nitrogen was done by regular Kjeldah method and followed the Method of Soil Analysis, part 2, Chemical and Microbiological (Properties 2nd ed.), (1982), ASA and SSSA. Its determination was done by Faculty of Agriculture, Chiang Mai University.

The first step for determining nitrogen was digestion. After that a 2.2 g K_2SO_4-catalyst mixture and 6 ml of sulfuric were added into the sample (0.1-0.5 mg) in a micro-Kjedahl digestion flask, then the sample was heated by increasing temperature for 6 hours: started at 150^0C for 30 min., and finished at 345^0C for 120 min (the brand name of oven: DK heating digester Velp Scientifica).

The sample was allowed to be cool and 150 ml of distilled water was added. Then the contents transferred to distillation chamber (the brand name of distillation chamber and its control unit: Büchi 342/322 made in German). Kjedahl flask was rinsed with 15 ml of water then enough water was added until the volume was 250 ml and the stopcock was closed.

In a sequence of 15 ml of H_3BO_3-indicator and 20 ml 10N of NaOH was added then stopcock was opened. When the alkali about 1 ml, the funnel was rinsed and enough of water was transferred until a volume 80 ml then the funnel stopcock was closed and distillation was commenced immediately.

When the distillate reached 150-200-ml in the receiver flask, the distillation was stopped, then the condenser was rinsed. Then NH_4^+-N in distillate was determined by titration with 0.02 M H_2SO_4 until the color change from green to pink (appendix 4).

3.7.7 Determination of phosphorous

The determination of phosphorous was done by utilizing the phosphorous-availability indices method via soluble phosphorous diluted in acid-fluoride method followed by the Method of Soil Analysis, part 2, Chemical and Microbiological Properties (2^{nd} ed.) (1982), ASA and SSSA. Determination this parameter was done by Faculty of Agriculture, Chiang Mai University.

To 1 g of the sample 7 ml of extracting solution was added then the sample was shaken and filtered. To 2 ml of the filtrate 5 ml of distilled water, 2 ml of ammonium paramolybdate and 1 ml stannous chloride solution were added in sequence. After 5 or 6 min, the color was measured by Specthrophotometer (brand name: Genesis 10UV scanning thermo electron cooperation) and wave length was 660 nm (appendix 4).

3.7.8 Determination of total petroleum hydrocarbon (TPH)

To estimate the biodegradation rate of each tank, the changing hydrocarbon concentration was measured with the gravimetric method followed the USEPA 413.1 and the standard 5520 method B (gravimetry) (Gogoi *et al.*, 2003). Determination of TPH was done by Faculty of Chemistry, Chiang Mai University.

A 10 grams of soil was extracted by fluorocarbon-113, after which it was cooled and dried, then the sample was measured by gravimetry. The determination of TPH is attached at appendix 4.

3.8 Enumeration of heterotrophic bacterial

The media, preparation of the sample solution and preparation of serial dilutions followed the Methods of Soil Analysis, part 2, Microbiological and Biochemical Properties, (series 5), (1984) , Soil Science Society of America.

Peptone tryptone yeast extract glucose agar (PTYG) medium was chosen and its preparation is shown at appendix 4. The sample was weighted for 10 g and put in 90 ml of distilled water, then it was shaken for 20 min by automatic shaker. By automatic pipette, 1 ml of the sample was pipetted, then put in the series dilution (preparing ten fold series dilution is shown at appendix 4) and the sample was shaken vigorously for some seconds. 0.1 ml sample was pipetted by automatic pipette then delivered to the plate that contain PTYG medium. Once delivered to the plate, the inoculum was spread evenly across the plate using a flame bent glass spreader, then the dishes were incubated for 24 hours. Calculation of the microorganisms were conducted when the petri dishes

showed colonies between 30 to 300 that the results were reported as colony forming units per gram (CFU/g). Microorganism numbers from soil samples were corrected for the moisture of the soil and reported as colony forming units per gram of dry-weight soil (CFU/g-of-dry soil).

3.9 Statistical analyses

One-way analysis of variance (ANOVA) test was used to determine the statistical significance of soil pH, soil moisture content, soil nutrient (total nitrogen and phosphorous), total heterotrophic bacterial and TPH concentrations. Data were considered to be significantly different between two values if $p<0.05$. The statistics were calculated using SPSS release 6.1 for windows.

The selection of the best performance of biopile to removal soil contaminants from petroleum production was done by choosing of biodegradation rate. The determination of the biodegradation rate was done by the correlation between air flow rate and TPH degradation by using Linear Association with measured the goodness of fit of the linear line regression denoted by R^2 and the degradation rate was chosen when its R^2 was close to 1.

3.10 The flowchart of experiment

The flowchart of the experiment is shown at figure 3.3.:

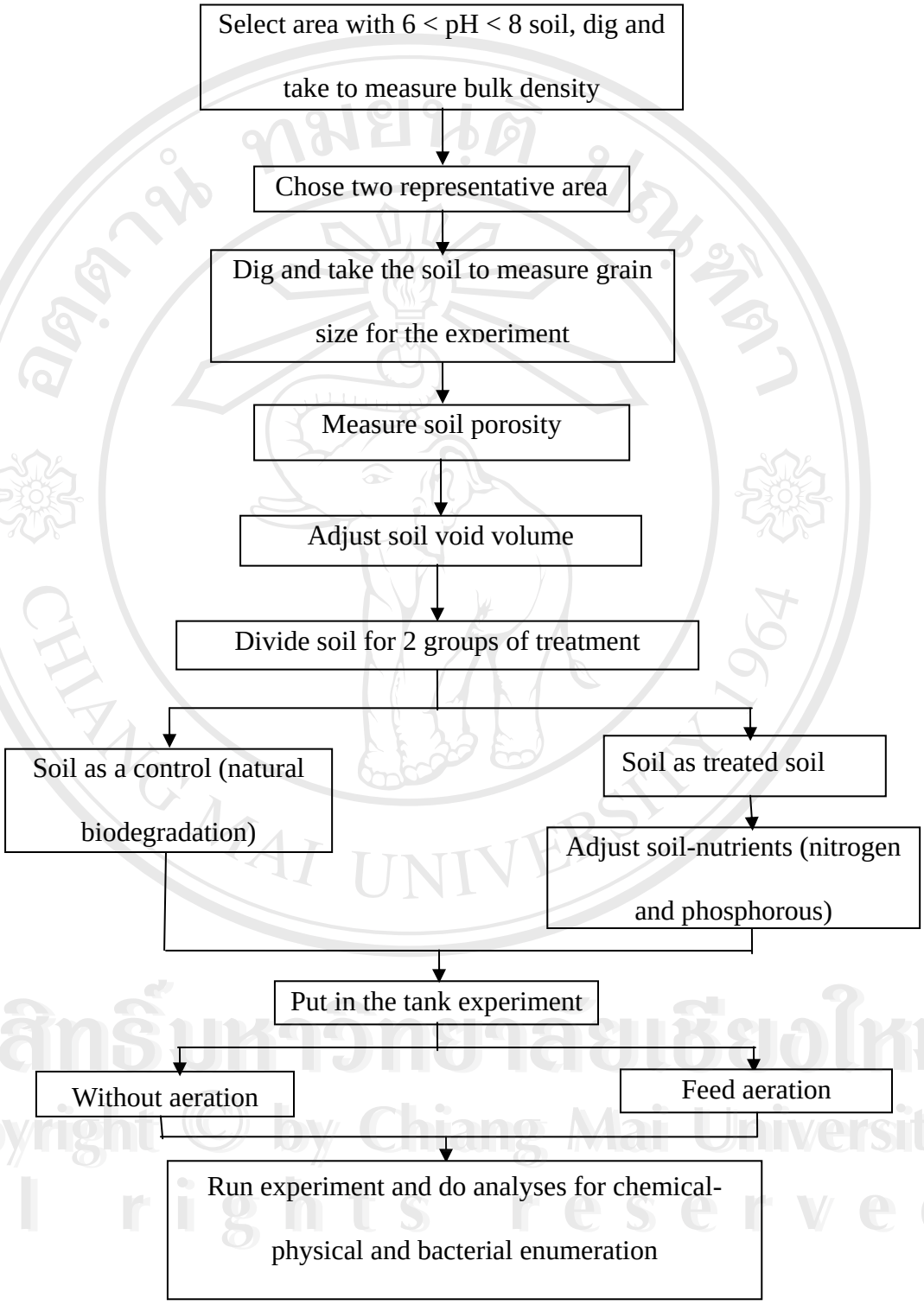

Figure 3.3. Flowchart of the biopile experiment