# CHAPTER III

# METHODOLOGY

## 3.1 Materials and Apparatus

### 3.1.1 Soil Sample Collection and Preparation

The experiments were done at Chiang Mai University therefore; soil sample was collected from an agriculture site in Chiang Mai province. Soil sample from agriculture site was not contaminated with PCE before the study. The sample was air dried for 48 hrs after that it was dried in an oven at 70°C. The dried soil was sieved through a U.S. standard sieve No.14 before used (Figure 3.1).

Selected soil properties were determined by the Department of Soil Science and Conservation, Faculty of Agriculture, Chiang Mai University. The properties were summarized in APPENDIX E. The soil sample composed with 85.3% sand: 11.6% silt: 3.1% clay.

Dried soil was analyzed to determine PCE background concentration and then spiked with PCE using gas-tight syringe to give a final PCE concentration of 100 mg/kg-soil. The contaminated soil was used throughout for the experiments.

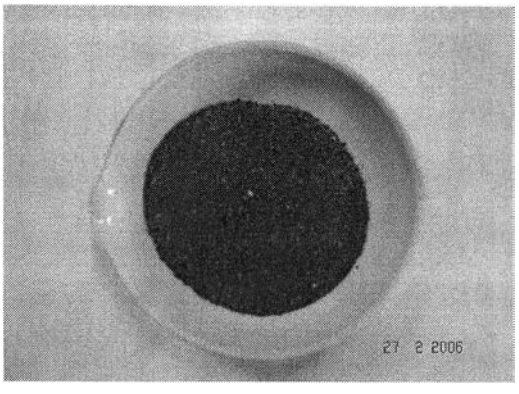


**Figure 3.1** Dried and sieved soil sample

### 3.1.2 Preparation of Granular Sludge Seed

Unacclimated granular sludge derived from the UASB waste water treatment plant of Leo Food Industrial, Chiang Mai Province (Figure 3.2) was used as seed for anaerobic bacteria in this study. The type of seed was selected based on the experiment done by Tungmee, 2002 which demonstrated that effective PCE dechlorination could be performed using unacclimated granular sludge.

Unacclimated granular sludge was dried at 70 °C for 2 days and then analyzed for PCE background concentration (APPENDIX E).

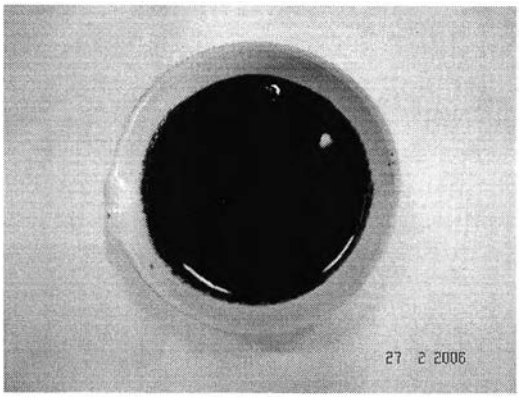


**Figure 3.2** Homogenized granular sludge seed

### 3.1.3 Wastes Preparation

Used edible oil was collected from two locations in Chiang Mai province, local market and industry. For local market, used lard and used soybean oil were obtained from pa tong go shop at Ton pa yom market. For industry, oil waste was brought from the screening process of Leo food Industrial that used palm oil to produce seasoning for instant noodle. Molasses were brought from sugar industry in Chiang Mai province. These wastes were used as carbon sources in anaerobic dechlorination process with concentrations related to the optimal concentration of glucose and soybean oil.

All wastes were filtered using GF/C membrane to remove impurities and then mixed with MS medium nutrient before adding to the soil (Figure 3.3).

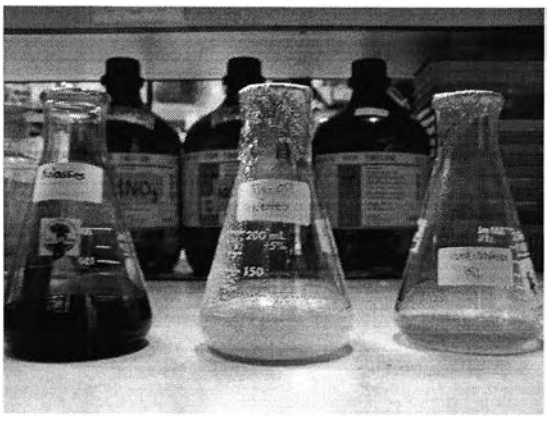

**Figure 3.3** Example of organic wastes

### 3.1.4 Soil Slurry Reactor.

40-ml glass vials connected with 10-ml plastic syringes was used as soil slurry reactor in the experiment (Figure 3.4). Each one was sacrificed for PCE and chloride ion ($Cl^-$) analysis at specific time point. Each 40-ml glass vials containing 10 grams dried soil and was applied with 5 ml of PCE/water solution to provide the final concentration of 100 mg-PCE/kg-soil using gas-tight syringe. After overnight, 10-ml of nutrient media solution containing different types and concentrations of carbon source and $NaHCO_3$ was filled into every syringe. $NaHCO_3$ was added as 5,000 mg/L to maintain pH 6-8. Approximately 0.1 g (60 mg-VSS/kg-soil) of granular sludge was later added into each syringes while continuously sparging with deoxygenated $N_2$ and $CO_2$ (80:20) for 5 minute in order to deplete oxygen in system (Leahy and Shreve, 1999; Tungmee, 2002; Kao et al., 2003). To close the reactor 10-ml plastic syringe septum was inserted and covered the connection with silicone glue and parafilm in

order to keep an anaerobic condition (Figure 3.5). The reactors were kept and shaken at 250 rpm in dark and room temperature as shown in Figure 3.6.

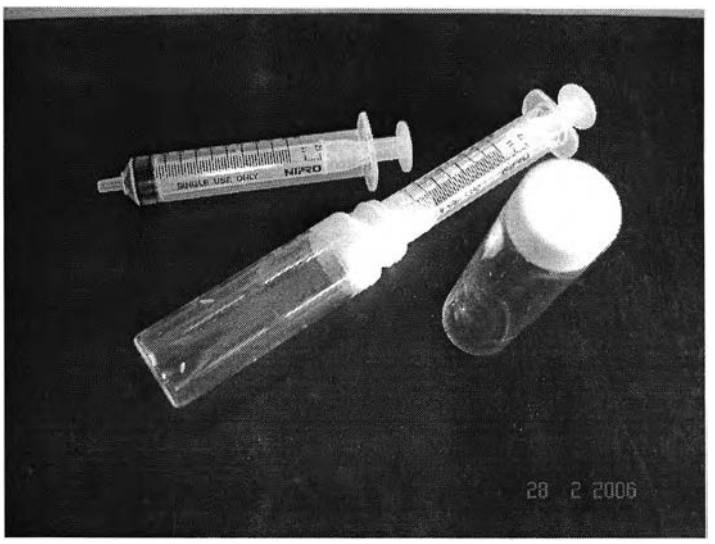


**Figure 3.4** Materials used for constructing the reactor

Figure 3.5 showed the example of test-reactors for each experiment before incubated in dark with the black box at room temperature (Tungmee, 2002; Kao et al., 2003). 24 days period was chosen as an appropriated incubation time period based on the suggestion from previous studies (Ferguson and Pietari, 1999; Leahy and Shreve, 1999; Kao et al., 2003). Buffer i.e. NaOH, HCl, $NaHCO_3$ was added into the reactors in order to maintain neutral pH. Every experiment was carried out in triplicate in order to confirm the precision of result.

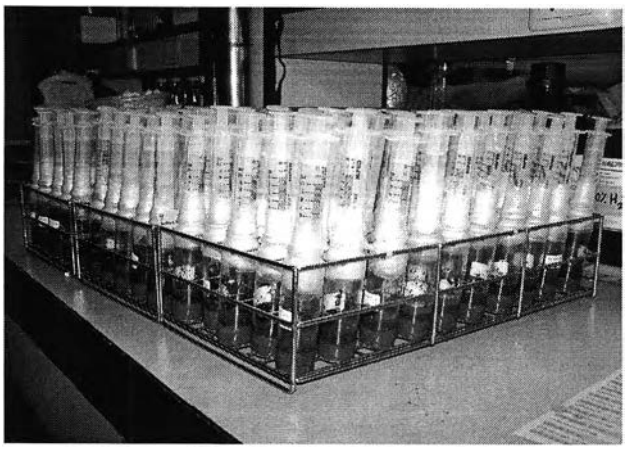

**Figure 3.5** Example of soil slurry reactors

Figure 3.6 showed that the reactors when incubated and shaken at 250 rpm in dark at room temperature.

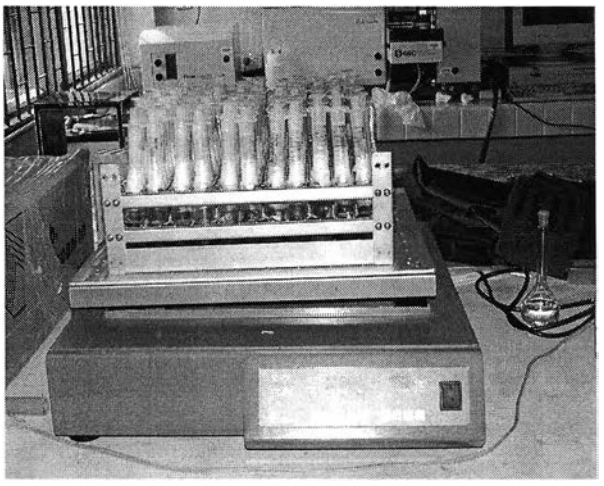

**Figure 3.6** Soil slurry reactors on orbital shaker. The reactors were shaken continuously.

### 3.1.5 Nutrient Media

Mineral salts (MS) medium was used to supply essential nutrients for the bacteria in soil slurry reactor. MS medium used in all experiment was prepared after Focht (1994) and its composition was shown in Table 3.1

**Table 3.1** Composition of MS medium used for this study

| Stock solution | Additions, mL | Final concentration, mM |
|---|---|---|
| $K_2HPO_4$, 1M | 10 | 10 |
| $NaH_2PO_4$, 1M | 3 | 3 |
| $(NH_4)_2SO_4$, 1M | 10 | 10 |
| $MgSO_4$, 1M | 1 | 1 |
| $Ca(NO_3)_2$, 1M | 0.1 | 0.1 |
| $Fe(NO_3)_3$, 1M | 0.01 | 0.01 |
| **Trace minerals** | 1 | |
| $MnSO_4$ | 0.001 | 1 |
| $ZnSO_4$ | 0.001 | 1 |
| $CuSO_4$ | 0.001 | 1 |
| $NiSO_4$ | 0.0001 | 0.1 |
| $CoSO_4$ | 0.0001 | 0.1 |
| $Na_2MoO_4$ | 0.0001 | 0.1 |

### 3.1.6 Chemicals

All chemicals used in this study were obtained commercially from Merck, USA. They are:

Tetrachloroethene (99.5% purity).

Trichloroethene.

1,1-dichloroethene.

cis-1,2-dichloroethene.

trans-1,2-dichloroethene.

Vinyl chloride.

95% n-hexane, solvent for organic residue analysis.

### 3.1.7 Gas Chromatography.

An electron capture detector (ECD) gas chromatography (Agilent 6890 N) equipped with a RTX-624 capillary column (30m x 0.25mmID x 1.4μm df) was used for PCE analysis (Figure 3.7). The PCE intermediates were observed from the peaks of TCE, DCE and VC in chromatogram. From the chromatogram, the retention time of PCE, TCE, 1,1-DCE, cis-DCE, trans-DCE and VC were 8.830, 6.426, 2.429, 4.416, 3.166 and 1.321 minute, respectively. Every experiment was carried out in triplicate.

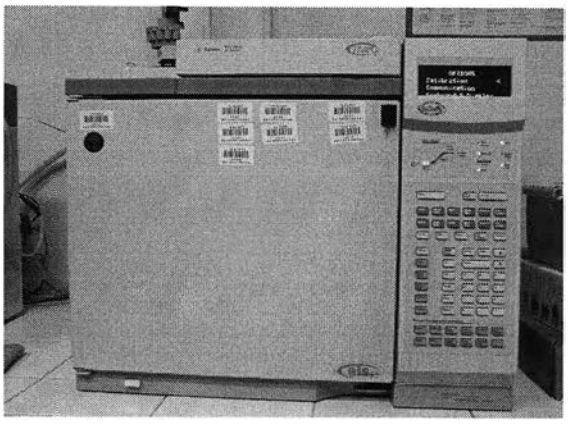

**Figure 3.7** Gas chromatography

### 3.1.8 Ion Chromatography.

Ion chromatography analysis was performed with a DIONEX ASI-100 Automated Sample injector (Figure 3.8). The ion chromatography was used for $Cl^-$ analysis.

The ion chromatography (Dionex DX500) equipped with a Dionex AG9-HC / AS9-HC (2mm). The Detector was the Suppressed Conductivity Detector, Dionex CD20 and the Suppressor was ASRS-I, external source electrolytic mode and100 mA current. 9.0 mM $Na_2CO_3$ of Eluent flow 0.40 mL/min. The retention time of chloride was 4.67 minute which presented in the chromatogram. Every experiment was carried out in triplicate.

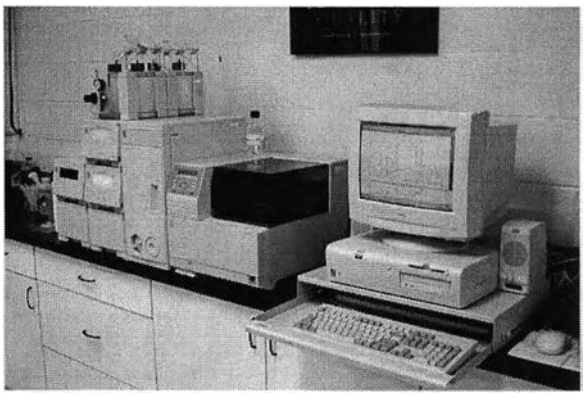

**Figure 3.8** Ion chromatography

## 3.2 Preliminary Experimental Design

The objective of this study was to investigate the effects of granular sludge, mineral nutrients and carbon source on PCE reductive dechlorination. Five sets of experiment were prepared as shown in Figure 3.9, which were; 1) sludge addition which aimed to observe the effectiveness of sludge, 2) nutrient addition which goal to observe the nutrient effectiveness, 3) sludge and nutrient addition, 4) sludge, nutrient and glucose as carbon source addition and 5) control set that nothing was added. Set (3) and (4) were prepared to investigate the effective of carbon source.

All sets were filled with 10 g soil which were contaminated by PCE/water solution and provided 100 mg-PCE/kg-soil for final concentration. After overnight, added sludge 60 mg-VSS/kg-soil (approximately 0.1 g) into set (1), (3) and (4). Nutrient was added into set (2), (3) and (4). Glucose 250 mg/kg-sludge act as carbon

source was added into set (4) only. The reactors were kept and shaken at 250 rpm in dark and room temperature. The triplicate samples were collected every six-day.

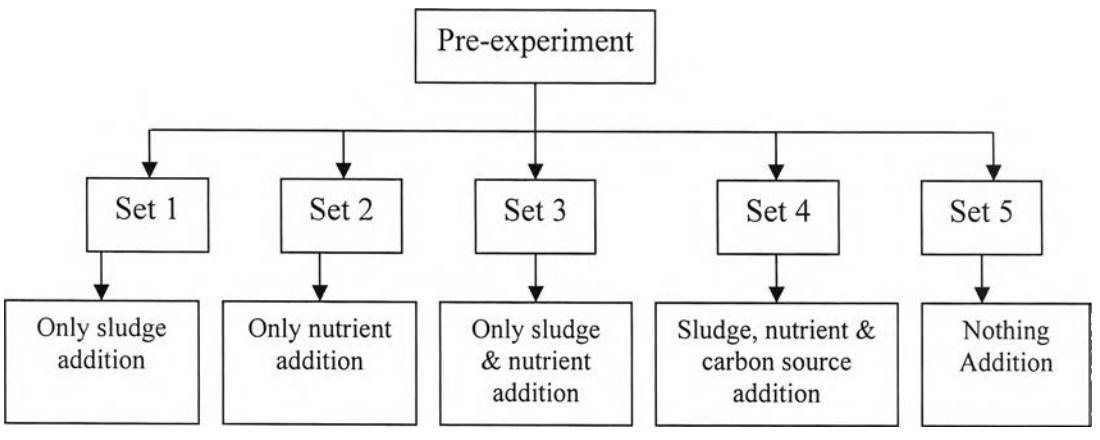


**Figure 3.9** Diagram of pre-experiment

## 3.3 Experimental Design

### 3.3.1 Investigation of the optimum concentration of glucose and soybean oil

PCE reductive dechlorination is depended on the appropriate type and concentration of electron donor. Therefore, we first identified the optimal concentration of electron donor i.e. glucose or soybean oil before start to utilize their relative wastes as electron donor. The experiment used sludge as 60 mg VSS/ kg-soil (approximately 0.1 g-sludge) in each reactor as seed of anaerobic bacteria (Wu et al., 1998). Figure 3.10 showed the varying final concentrations of glucose and edible oil at 500, 1,000, 5,000 and 10,000 mg/kg-sludge (Tungmee, 2002) in the soil slurry

reactors. These substrates were mixed in 1 liter of MS medium before added to soil slurry reactors. Final PCE concentration at 100 mg/ kg dry-soil was applied to contaminate soil sample. The study was carried out in triplicate.

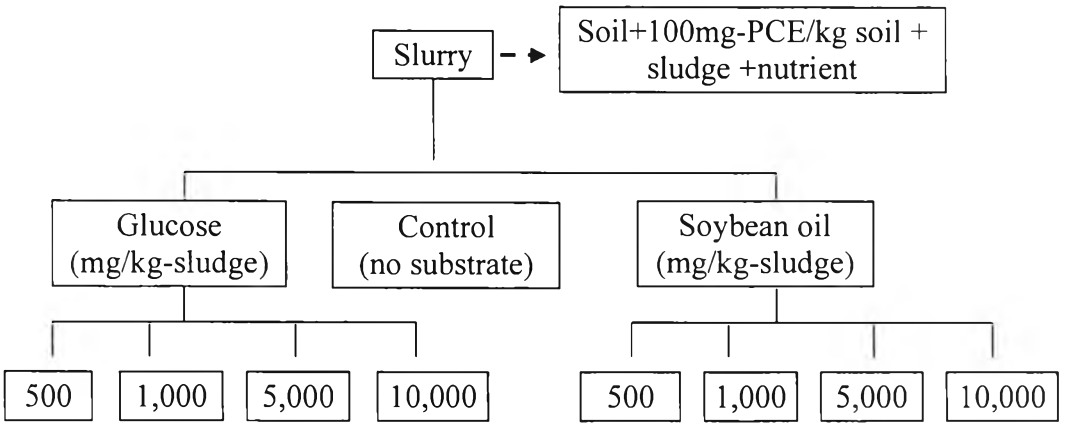


**Figure 3.10** Diagram of soil slurry reactors with various amount of glucose or soybean oil as electron donor

### 3.3.2 Determination the effectiveness of molasses and used-edible oil

From the first step (3.3.1), appropriate concentrations of glucose and soybean oil were obtained. Glucose and soybean oil concentrations were used to determine the amount of molasses and used edible oil in this step, respectively. Two concentrations of each waste were tested as alternative carbon source (figure 3.11). The amount of PCE contaminated soil and sludge was similar to the first experiment. Every experiment was carried out in triplicate in order to confirm the correct result.

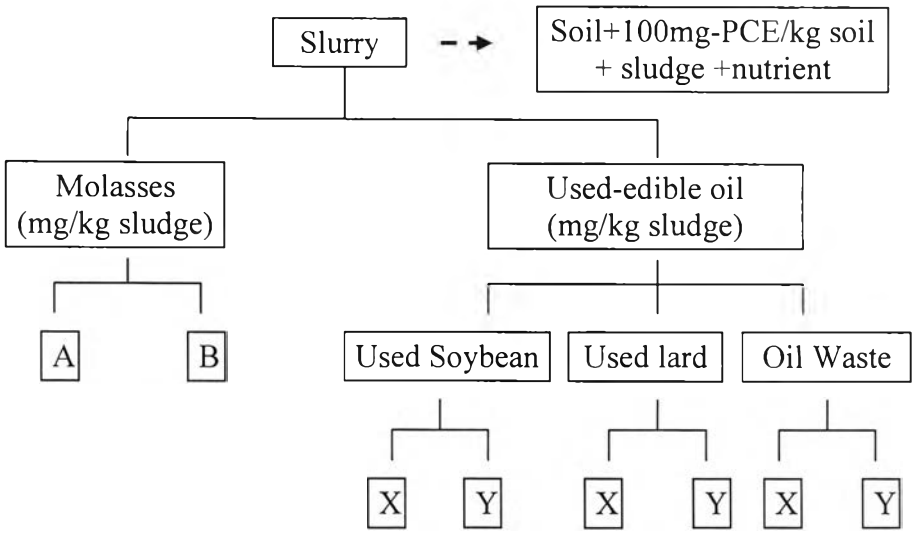


**Figure 3.11** Diagram soil slurry reactors that utilized waste as carbon source.

**Remark:** A & B are the two appropriate concentrations which were selected from glucose. X & Y are the two appropriate concentrations which were selected from soybean oil.

### 3.3.3 Determination the optimum concentration of PCE when applied molasses or used-edible oil as substrate

From the second step (3.3.2), the appropriate type and concentration of waste was obtained. To find the optimum PCE reductive dechlorination, five PCE concentrations ranging 100, 150, 200, 250 and 500 mg/kg-soil were applied with the selected type and concentration of waste from the second experiment (Figure 3.12). All sets were carried out in triplicate.

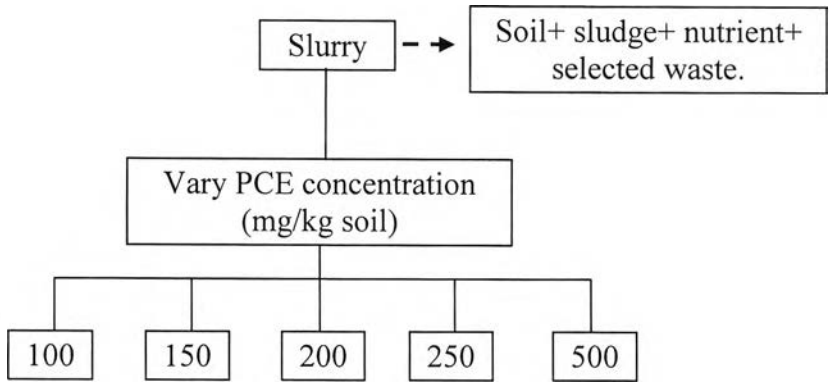


**Figure 3.12** Diagram for determination of the optimum concentration of PCE using an appropriated waste as carbon source supplementation

### 3.4 Time of Sampling

Incubation time period of soil slurry reactors was 24 days. Every 6 days, triplicate of each sample sets were collected and analyzed for PCE, its intermediates as well as chloride ion. For day 0, samples were collected immediately after finish setting up the experiment. The amount of gas production was recorded from the height of syringe septum. No PCE and its intermediates as well as chloride were measure in the gas. Silicone glue was used to seal the connection between glass-vial and plastic syringe in order to prevent the leakage of gas during incubation time period.

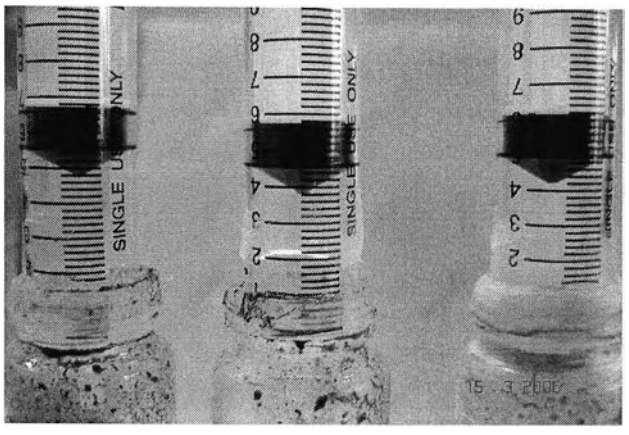

**Figure 3.13** Syringe septum was pushed up by gas production during organic digestion process in the soil slurry reactors after 18 days.

### 3.5 Soil Extraction Method

PCE extraction and analysis procedure was modified from Suttinun et at. (2004). Gas production was observed before PCE extraction. Cutter was used to slit silicone glue between syringe and vial. After that, PCE was extracted from soil and sludge by solvent extraction using mixed hexane and 15% triton X-100 and the vials were shaken at 250 rpm for 2 hrs. Then, the solvent was frozen at -4°C to solidify the lower aqueous layer, after that anhydrous sodium sulfate was added to dewater the solvent layer before injected to gas chromatography (GC). The extraction efficiencies were shown in APPENDIX D.

### 3.6 GC Analysis

This study gas chromatography was used to investigate PCE and its intermediates from soil slurry extraction. All PCE and its intermediates quantification was performed with 1,2-dichloropropane as internal standard. A volume of 1 µl of each sample extract was injected under the following conditions; injector temperature: 250°C, detector temperature: 280°C, initial column temperature: 50°C hold for 4 min to 100°C at a rate of 12°C/min and no hold then, programmed from 100°C to 230°C at a rate of 27°C/min and hold for 2 min. The carrier gas was helium (constant linear volume of 2.1 ml $min^{-1}$) and the make up gas was $N_2$ at 60 ml $min^{-1}$. Split ratio was kept at 40:1.