CHAPTER 2: MATERIALS AND METHODS

2.1 Sampling

Samples were collected from three extended aeration sewage treatment plants (STPs) in Klang Valley, Malaysia on 22 February, 9 June, 11 September, 3 December 2008 and 24 June 2009. An additional sampling was done in STP 1 on the 17 October 2008. STP 1 is located at a residential area (N 3°1.78', E 101°42.63') whereas STP 2 (N 3°1.75', E 101°42.47') and STP 3 (N 3°0.95', E 101°41.95') are located in a commercial and light industrial area. STP 1 served 5630 population equivalent (PE), and was designed for a wastewater flow rate of 1300 m^3 day^{-1} whereas STP 2 (4600 PE) and STP 3 (5630 PE) have a designed flow rate of 1000 m^3 $day^{-1}$and 1358 m^3 day^{-1}, respectively.

Some measurements were carried out in-situ such as pH, temperature, dissolved oxygen uptake rate (DOUR) and settled sludge volume (SSV). pH was measured with a pH paper, Universal indicator (Merck, Germany) whereas temperature was measured by a digital thermometer (Thermoworks, USA).

Samples were also kept in a cold box and brought back to the laboratory for further analysis (Table 2.1). Measurements such as total suspended solids (TSS), mixed liquor suspended solids (MLSS), mixed liquor volatile suspended solids (MLVSS), dissolved oxygen (DO), biological oxygen demand (BOD), chemical oxygen demand (COD), nutrients analysis [ammonia (NH_3), nitrite (NO_2^-), nitrate (NO_3^-), phosphate (PO_4^{3-})] and bacterial community composition analysis were carried out in the laboratory according to APHA standard (APHA, 2005).

Table 2.1: Types of wastewater analysis and measurements carried out in this study.

Test	Influent	Aeration tank	Effluent
Dissolved Oxygen Uptake Rate (DOUR)	-	✓	-
Total Suspended Solids (TSS)	-	-	✓
Mixed Liquor Suspended Solids (MLSS) and Mixed Liquor Volatile Suspended Solids (MLVSS)	-	✓	-
Settled Sludge Volume (SSV)	-	✓	-
Dissolved Oxygen (DO)	✓	✓	✓
Biological oxygen demand (BOD)	✓	✓	✓
Chemical oxygen demand (COD)	✓	✓	✓
Ammonia (NH_3)	✓	✓	✓
Phosphate (PO_4^{3-})	✓	✓	✓
Nitrite (NO_2^-)	✓	✓	✓
Nitrate (NO_3^-)	✓	✓	✓
Heavy Metal Analysis	✓	✓	-
Denaturing gradient gel electrophoresis (DGGE)	-	✓	-

The symbol (✓) denotes measurement whereas (-) was for measurements not done.

2.2 Dissolved Oxygen Uptake Rate (DOUR)

DOUR was measured using a calibrated DO probe (YSI 550A-12CC, USA). A 300 ml BOD bottle was filled with aeration tank sample. The DO probe was immediately inserted into the BOD bottle. The mixture was then mixed well by swirling the filled bottle constantly. DO measurements were recorded at intervals of 5 seconds until 60 seconds or until DO becomes rate-limiting i.e. no steady decrease.

2.3 Total Suspended Solids (TSS), Mixed Liquor Suspended Solids (MLSS), Mixed Liquor Volatile Suspended Solids (MLVSS)

A known volume of aeration tank and effluent sample were filtered onto Whatman GF/C filters. MLSS (mg l^{-1}) for aeration tank sample and TSS (mg l^{-1}) for effluent sample were measured as the filter weight gained after drying at 60ºC for about one week whereas MLVSS (mg l^{-1}) was the weight loss after combustion at 500°C for three hours (Vulcan A-130, UK).

2.4 Sludge Volume (SV)

Sample from aeration tank was transferred into a one-liter measuring cylinder. The sample was mixed well and then allowed to settle for 30 minutes. The volume of the settled sludge was recorded as settled sludge volume (ml l^{-1}).

Sludge volume index (SVI, ml g^{-1}) was calculated using the equation:

$$SVI = (V \times 1000)/MLSS$$

Where V= volume of settled sludge (ml), and MLSS = Mixed Liquor Suspended Solids (mg l^{-1}).

2.5 Dissolved oxygen (DO)

Dissolved oxygen was measured using modified Winkler's Titration Method. Samples were collected using 40 ml BOD bottles in triplicates, and fixed *in-situ* with 3 M manganese (II) chloride solution and alkaline-iodide solution (60 g of KI and 30 g of KOH in 100 ml distilled water). The samples were mixed well, and the precipitate that formed was allowed to settle. In the laboratory, sulfuric acid solution (50% v/v) was added, and mixed well before titrating with 0.01 M sodium thiosulfate solution. Starch was used as an indicator and the samples were titrated until colorless. Dissolved oxygen was calculated using the following formula:

Dissolved oxygen (mg l^{-1}) = $[(0.01 \times 250 \times 10^3 \times T) / (V - 0.6)] \times f \times (32/1000)$

Where T = volume of titrant used (ml), V = volume of sample (ml), and f = normality of sodium thiosulfate solution (0.01 M).

2.6 Biological oxygen demand (BOD)

BOD is the measurement of dissolved oxygen depletion over a five-day period at 25°C in the dark. Samples were diluted 10 times with aerated dilution water (Appendix A) before siphoning into BOD bottles and incubated. A set of BOD bottles containing only dilution water was also incubated as control. Dissolved oxygen concentration before and after incubation was measured, and BOD was calculated as:

BOD (mg l^{-1}) = ($DO_{0\ day}$ sample – $DO_{5\ days}$ sample) – ($DO_{0\ day}$ control – $DO_{5\ days}$ control)

2.7 Chemical oxygen demand (COD)

Samples were diluted 10× with distilled water before adding into conical flask in triplicates. Silver sulfate (0.2 g) was added before 10 ml of sulfuric acid reagent (22 g of Ag_2SO_4 in 2.17 l of concentrated H_2SO_4). After the mixture had cooled, 5 ml of 0.0208 M potassium dichromate solution was added. The mixture was then refluxed for 2 hours and then cooled to room temperature before titration with 0.125 M ferrous ammonium sulfate solution (FAS). Ferroin was used as an indicator. COD was then calculated using the following equation:

$$\text{COD (mg l}^{-1}) = [(A-B) \times N \times 8000] / V$$

Where A = volume of FAS used for blank (ml), B = volume of FAS used for sample (ml), N = normality of FAS, and V = volume of sample

Normality of FAS was determined by titrating 0.0125 M FAS with a cooled mixture of 5 ml of potassium dichromate and 1 ml of sulfuric acid solution. Normality of FAS was calculated as follows:

$$\text{Normality of FAS (N)} = (P \times 0.125) / F$$

Where P = volume of potassium dichromate (ml), F = volume of FAS used for titration.

2.8 Nutrients analysis

Samples were filtered through Whatman GF/C filter, and the filter was kept frozen (–20°C) until analysis. All the nutrients (NH_3, NO_2^-, NO_3^-, PO_4^{3-}) were measured according to APHA standard (APHA, 2005).

Ammonium was measured via phenate colorimetry where alkaline phenol and hypochlorite reacted with ammonium to form an indophenol blue dye which was further intensified with sodium nitroprusside. Absorbance was recorded at 640 nm with a spectrophotometer (Jenway 6300, UK). Nitrite was measured via reaction with sulfanilamide to form diazo compound that was then coupled with α-naphthyl-

ethylenediamine hydrochloride in an acidic medium to form azo dye. Absorbance was later recorded at 543 nm. For nitrate measurement, nitrate was first reduced by granulated copper-cadmium, before being measured as nitrite. The increase in nitrite was assumed to be proportional with nitrate concentration. Phosphate was under acidic condition to form 12-molybdophosphoric acid that was later reduced by ascorbic acid in the presence of potassium antimonyl tartarate to form phosphomolybdenum blue. The absorbance of the phosphomolybdenum blue was recorded at 880 nm.

2.9 Heavy metal analysis

Water samples from influent and aeration tank were collected and tested for arsenic (As), cadmium (Cd), chromium (Cr), copper (Cu), lead (Pb) and mercury (Hg) at Permulab Pte. Ltd. All heavy metals concentrations were measured using Inductive coupled plasma-atomic emission spectrometry (ICP-AES) (USEPA method 6010B, 2001) except arsenic and mercury which were analyzed using atomic absorption spectrophotometry (AAS) and cold-vapor atomic absorption (CVAA) methods, respectively.

2.10 DNA extraction

Activated sludge samples were collected in sterile bottles and filtered through a 0.2 μm pore size filter (47 mm diameter, Millipore) and the filter was kept frozen (–20°C) until DNA analysis. DNA extraction was carried out using a modified chemical-enzymatic lysis method (Yeates *et al.,* 1998). The filter was transferred into DNA extraction buffer using sterilized forceps. Cells trapped on the filter were lysed using lysozyme, SDS and p proteinase K before precipitating with polyethylene glycol. The pellet was resuspended in tris-EDTA (TE) buffer (pH 8.0) and was purified again using equal volume of phenol/chloroform/isoamyl alcohol (25:24:1). Subsequently, the

DNA was precipitated with 0.1 volume of NaCl and 2.5 volume of absolute ethanol before the DNA was resuspended in TE. The extracted DNA was examined in a 1% (w/v) Seakem LE agarose gel electrophoresis (Cambrex, USA) at 5.0 V cm^{-1} in 1× tris-borate-EDTA (TBE) buffer.

2.11 Amplification of the 16S rDNA

Partial 16S rDNA gene was amplified using a pair of universal primer, forward primer: 314F (5′-CCTACGGGAGGCAGCAG-3′) and the reverse primer: 907R (5′-CCGTCAATTCCTTTGAGTTT-3′) (Winter *et al*., 2007). A 40 bp long GC clamp (5′-CGCCCGCCGCGCCCCGCGCCCGTCCCGCCGCCCCCGCCCG-3′) (Muyzer *et al.,* 1995) was attached to the 5′ end of 314F primer. Polymerase chain reaction (PCR) was performed in a 15 µl reaction mixture containing: 1× PCR buffer, 2.0 mM of $MgCl_2$, 0.4 µM of each primer, 3 µl of DNA template (approximately 200 ng μl^{-1}), 0.4 mM of dNTPs and 2.0 U of *Taq* polymerase (Finnzymes DyNAzyme II, Finland). PCR amplification was carried out according to the following conditions: initial denaturation at 95°C for 1 min; 30 cycles of amplification (denaturation for 1 min at 95ºC, annealing for 1 min at 56ºC, extension for 1 min at 72ºC) and a final extension at 72ºC for 30 min. The amplicons were examined in a 1% (w/v) Seakem LE agarose gel electrophoresis (Cambrex, USA) at 5.0 V cm^{-1} in 1× (TBE) buffer with 100 bp Ladder (40 ng μl^{-1}, iNtRON Biotechnology, Korea).

2.12 Denaturing gradient gel electrophoresis (DGGE)

PCR amplicons were then resolved via DGGE using a DCode universal mutation detection system (Bio-Rad, USA) on a 8% polyacrylamide gels containing linear gradient of 20% to 70% denaturants [formamide (Sigma-Aldrich, USA) and urea (Bio-Rad, USA)]. Two stock solutions of 0 and 100% of denaturants with equal amount

of 40% Acrylamide / bis solution and 50× TAE buffer (Table 2.3) were prepared for the mixing of 20 and 70% denaturant solutions (Table 2.4). Before preparing the gel gradient, 100 µl of ammonium persulfate (Bio-Rad, USA) and 7 µl of N,N,N',N'-tetramethylethylenediamine (TEMED) were added to each of the 20 and 70% denaturant solutions (Winter et al., 2007). The polyacryalmide gel was allowed to polymerize for an hour before loading the samples.

Electrophoresis was performed at 80 volts and 60 ºC for 12 hours using 1× TAE (tris-acetate-EDTA, pH 8) buffer. The gel was stained with SYBR Gold (Invitrogen, USA) for 30 minutes before viewing with a gel documentation system (Major Science, USA).

2.13 DGGE bands extraction and re-amplification

Bands from the stained gel was excised and crushed in 50 µl of sterilized Milli-Q water. The mixture was then incubated at 37 ºC in a shaker incubator overnight. Supernatant containing the PCR fragments were obtained after centrifuging at 16 200× *g* for 1 min at 4ºC. The pellet was resuspended with 25 µl of sterilized Milli-Q water and mixed well before recentrifugation. The supernatant was added to the previous supernatant. The extracted product was examined in a 1% (w/v) Seakem LE agarose gel electrophoresis (Cambrex, USA) at 5.0 V cm^{-1} in 1× (TBE) buffer with 100 bp Ladder (40 ng μl^{-1}, iNtRON Biotechnology, Korea). The extracted PCR fragments were reamplified using 314F and 907R primers. PCR products were purified using QIAquick PCR Purification Kit (QIAGEN, Germany) prior to DNA sequencing at AITbiotech, Singapore. The nucleotide sequence of the samples was analyzed with both Basic Local Alignment Search Tool (BLAST) search program (Altschul *et al.,* 1990) at the National Center for Biotechnology Information (NCBI) and Ribosomal Database Project 10 (RDP-10) (Cole *et al*., 2009) for identification of the closest related bacteria. The

sequence data for the DGGE bands were deposited in GenBank with accession codes JF261089 − JF261093, JF433957 − JF433971, JF423919 − JF423928 and JF699653 − JF699671.

2.14 Phylogenetic and statistical analyses

All data unless otherwise noted, was reported as mean ± standard deviation. Selected variables were tested for differences among the STPs using analysis of variance (ANOVA) and Tukey's test. The DGGE banding patterns were analyzed by assuming the DGGE bands at the same vertical position as one unique operational taxonomic unit (OTU) to form a present and absent matrix to be analyzed in cluster analysis and analysis of similarities (ANOSIM). The cluster analysis was based on the similarity matrix calculated using the Morishita coefficient. Differences of bacterial community profiles among the STPs were analyzed using similarity percentage (SIMPER). The relationships between the distribution of the OTUs and the selected physio-chemical variables was illustrated with canonical correspondence analysis (CCA) with 1000 permutations. All statistical analyses were carried out by palaeontological statistics PAST (Hammer *et al*., 2001).

Phylogenetic tree of each STP was also constructed via analysis software Bosque (Ramírez-Flandes and Ulloa, 2008). The sequences were first aligned via MUSCLE 3.6 (Edgar, 2004), followed by construction of a maximum likelihood tree (Guindon and Gascuel, 2002), which was constructed from a matrix of pairwise genetic distances calculated by the Jukes-Cantor algorithm (Jukes and Cantor, 1969). Bootstrap analyses of 1000 replicates were also performed to assess the relative stabilities of the branches.