INTRODUCTION

Wastewater treatment is a process of treating domestic, commercial or industrial waste before it is released to open waters. The development of water and wastewater management dates back to the Industrial Revolution in Europe (Cooper, 2001). The main reasons that spark the development of water and wastewater management are initially for public health i.e. waterborne diseases (Table 1.1) and later to protect the environment (Feachem *et al*., 1982).

In 1854, Dr. John Snow linked a cholera outbreak to a contaminated source of water supply in London. The death rate of those receiving water downstream of the sewage outlets in Thames River was 8.5 times higher than those receiving water source upstream of the sewage outlets (Snow *et al.*, 1936). Steps were then taken to prevent pollution of water sources and to treat sewage.

1.1 Wastewater treatment

The purpose of wastewater treatment is to convert the waste material present in the wastewater into stable oxidized end product that is safe to be discharged to natural water source without any adverse ecological effects (Gray, 2004).

One of the earliest wastewater treatment methods is septic tank where sewage is collected in a tank and the scum, grease and settleable solids are removed from the liquid by gravity separation. It was patented in 1895 by Donald Cameron (Wolfe, 1999). The clarified effluent obtained is filtered with soil whereas the settled sludge is pumped out of the tank every three to four years (Canter & Knox, 1985).

Table 1.1: Waterborne pathogens and their associated disease.

Pathogen	Disease	Effects
	Bacteria	
Escherichia coli	Gastroenteritis	Vomiting, diarrhea, death in susceptible population
Legionella pneumophilia	Legionellosis	Acute respiratory illness
Leptospira sp.	Leptospirosis	Jaundice, fever (Weil's Disease)
Salmonella typhi	Typhoid fever	High fever, diarrhea, ulceration in small intestine
Salmonella sp,	Salmonellosis	Diarrhea, dehydration
Shigella sp.	Shigellosis	Bacillary dysentery
Vibrio cholera	Cholera	Heavy diarrhea, dehydration
Yersinia enterolitica	Yersinosis	Diarrhea
	Protozoa	
Balantidium coli	Balantidiasis	Diarrhea, dysentery
Cryptosporidium sp.	Crytosporidiosis	Diarrhea
Entamoeba histolytica	Amedbiasis	Diarrhea with bleeding, abscesses on liver and small intestine
Giardia lamblia	Giardiasis	Mild to severe diarrhea, nausea and indigestion
Naegleria fowleri	Amoeba meningo-encephalitis	Fatal disease, Brain inflammation
	Viruses	
Adenovirus (31 types)	Respiratory disease	
Enteroviruses (67 types)	Gastroenteritis	Heart anomalies, meningitis
Hepatitis A	Infectious Hepatitis	Jaundice, fever
Norwalk agent	Gastroenteritis	Vomiting, diarrhea
Reovirus	Gastroenteritis	Vomiting, diarrhea
Rotavirus	Gastroenteritis	Vomiting, diarrhea

(Metcalf and Eddy, Inc., 1991)

Later in 1901, trickling filter was developed for wastewater treatment (Vesilind, 2003). The initial stage of trickling filter uses gravel beds that allow formation of microbial slime when raw sewage flows into it. Other materials used as media beds for the trickling filter are rocks, slag, polyurethane foam, sphagnum peat moss, or plastic media. The incoming raw sewage causes splashing and diffusion which introduces air that is required for biochemical oxidation of organic compound into the system. Alternatively, some plants forces air into the media bed. As the slime on the media bed thickens, an inner anaerobic layer was formed and allows anaerobic reactions. The effluent will be collected together with the sloughed off slime from the media as sludge. The clear effluents and sludge will then be separated by settling in a separate tank (Vallero & Peirce, 2003; Gray, 2004).

By the end of 1930s, half of the sewage treatment plants in US were replaced with Imhoff tanks (Figure 1.1) (Wolfe, 1999). Imhoff tank is a modification of septic tank to two storey tank. The upper tank is designed for settling whereas the lower tank is designed for sludge digestion. The settled sludge from the upper tank will slide down the inclined bottom to the lower tank. Separate pipes are then employed to remove the effluent and sludge. The design avoided the mixing of the fresh sewage to settled sludge in the same tank as observed in septic tank (Liu & Liptak, 2000). However, sometimes the scum formed by the sludge will clog the connection of the two tanks.

At present, modern wastewater treatment is divided into five stages (Table 1.2) (Figure 1.2). A preliminary treatment to remove larger solids, grits, oil and grease present in the raw sewage. The preliminary treatment is to prevent interference in the later process or even damage to the sewage treatment plant equipment. The mechanism used in this part of treatment are mostly pump sump, screening bar or fine screen which traps the solids that are removed manually or by electric driven raking devices.

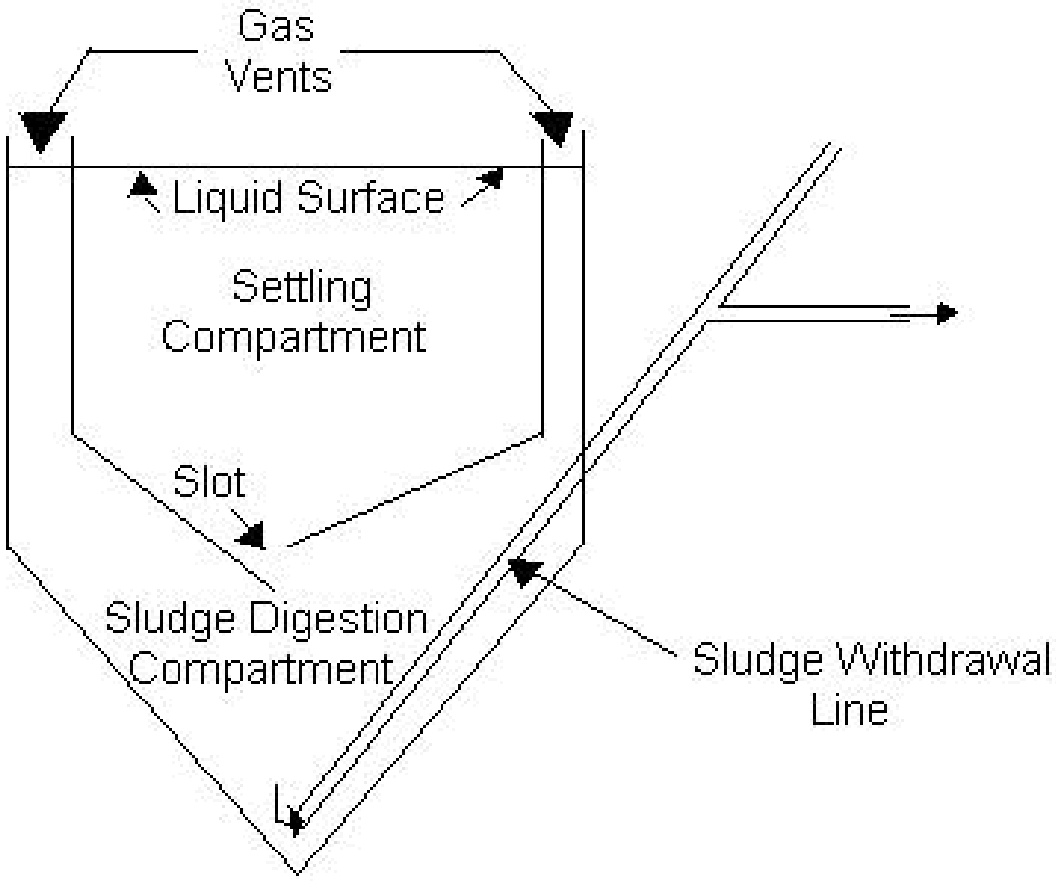

Figure 1.1: Design of Imhoff tank (modified from Negulescu, 1985).

Table 1.2: Different stages of wastewater treatment and its functions.

Treatment process	Functions
Preliminary Treatment	The removal and disintergration of gross solids and the removal of grit. Oil and grease were also removed in this stage if present in large amounts.
Primary (sedimentation) Treatment	Removal of settleable solids which are removed as sludge
Secondary (biological) Treatment	The dissolved and colloidal organics are oxidized in the present of microorganisms.
Tertiary Treatment	Further treatment to removed BOD_5, bacteria, suspended solids, specifics toxic compounds or nutrients
Sludge Treatment	The dewatering, stabilization and disposal of sludge.

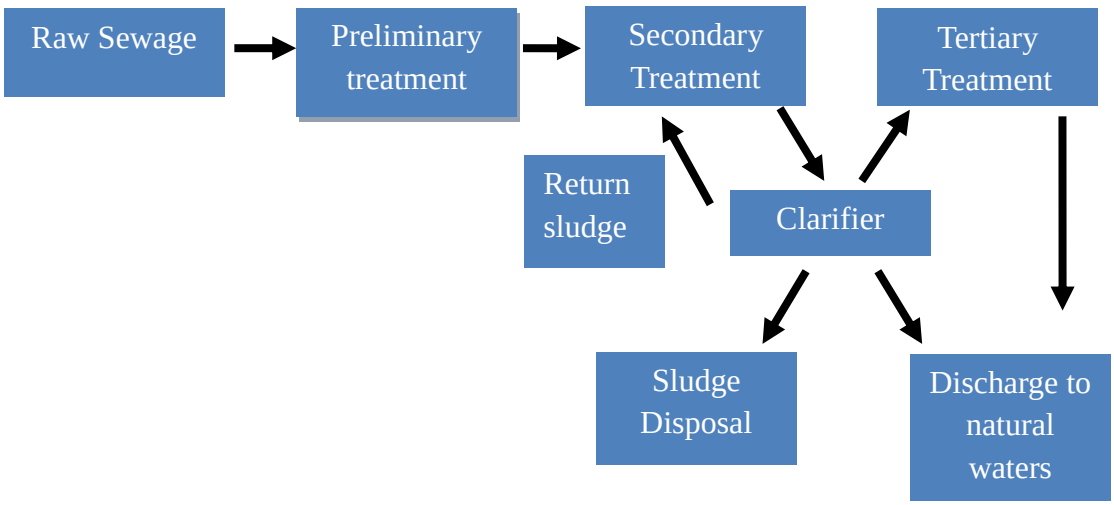

Figure 1.2: Outline of modern wastewater treatment process.

The second stage is primary treatment where the raw sewage is channeled into a sedimentation tank where it is allowed to settle. At this stage, a portion of suspended solids, scum and organic matter from raw sewage are removed as it settled to the bottom of the tank. It is estimated that 30-40% of biological oxygen demand and 50-70% of total suspended solids are removed at this stage (Barnes *et al.*, 1981). However the holding of raw sewage for a long period of time will produce unpleasant odor.

The next stage is the secondary treatment, and is also known as the biological treatment process or activated sludge because it involves microorganism (mainly bacteria) to breakdown and utilize the waste matter in the sewage to synthesize new cell material (Glymph, 2005). At present, secondary treatment is the main process of a wastewater treatment plant to produce effluent that is safe for discharge. Microorganisms produce exopolysaccharides and form flocs that separate from the wastewater by settling in a clarifier tank. The settled flocs are labeled as sludge and are removed. Some sludge is recycled back to the aeration tank as return sludge to facilitate the microbial community formation.

Tertiary treatment is referred to as advanced treatment with an aim of eliminating inorganic nutrients that are still present, and also to eliminate the microorganisms present in the effluent of activated sludge. There are a number of approaches adopted in tertiary treatment and one of it is channeling the effluent to a shallow lagoon and to hold for a few days (Hocking, 2005). The shallow lagoon allows good penetration of sunlight and efficient oxygen exchange to ensure aerobic bacterial action and photosynthetic nutrient utilization by rooted or free-floating plants. This method will also facilitate the decrease of suspended solids and bacterial counts by soil adsorption and coagulation or settling. However, it requires a large amount of space and time. Other alternatives include chemical methods where inorganic coagulant, such as

alum, is applied to promote agglomeration of residual colloidal matter to larger particles and removed through settling process. This process is costly and also burdens the effluents with inorganic fractions. Another option which is cheaper is hydrated lime which is used as a substitute for alum, but the problem of inorganic fractions is still unavoidable (Hocking, 2005). Chlorination is also employed in some of the tertiary treatment to kill potentially pathogenic bacteria and protozoa (Chiras, 2009).

The final stage in wastewater treatment is sludge treatment i.e. the dewatering, stabilization and disposal of sludge. Sludge collected is often laid on a sludge bed to be dried under the sun. After drying, the sludge will be disposed of or used as fertilizers.

In 2008, there are 9,525 public sewage treatment plants in Malaysia serving more than 21 million population equivalents (Table 1.3). These are managed by Indah Water Konsortium Pte. Ltd. (IWK), and are divided into communal septic tanks (38% of total treatment plants), Imhoff tanks (8%), oxidation ponds (5%), mechanical plants (42%) and Network Pump Station (7%) (IWK, 2007).

Septic tanks and Imhoff tanks were among the earliest treatment plants and were described earlier. Oxidation ponds or waste stabilization ponds are another method of treating waste where raw sewage is directed to a shallow pond construct (Tebbutt, 1998). This method is mostly adopted by tropical countries as it requires a warm sunny climate to promote the growth of both algae and bacteria. The bacteria in the pond utilize the organic matter and break it down to nutrients and carbon dioxide. The algae in turn utilize the carbon dioxide, nutrients and sunlight for photosynthesis, thus providing the bacteria with oxygen supply (Figure 1.3). It however requires a large area which results in the high cost of land and the quality of the effluent produced is not consistent as it is dependent on the weather.

Table 1.3: Types of wastewater treatment plant.

Type of STP	Total	Percentage	PE
Communal Septic Tank	3,635	38%	433,573
Imhoff Tank	760	8%	557,752
Oxidation Ponds	436	5%	1,824,403
Mechanical Plants	4,026	42%	15,099,139
Network Pump Stations	668	7%	3,558,108
Total	9,525	100%	21,472,975

(Indah Water Konsortium Sdn. Bhd., n.d.)

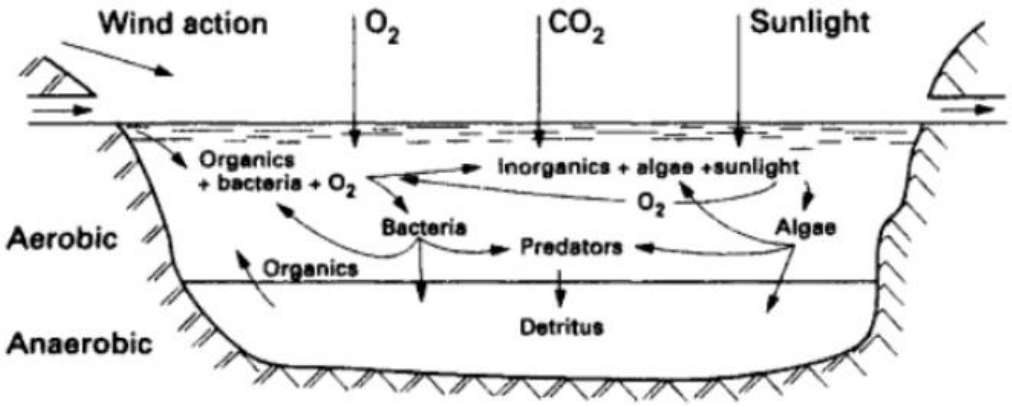

Figure 1.3: The relationship of bacteria and algae to treat wastewater in an oxidation pond (Tebbutt, 1998).

Mechanical plants with media include rotating biological contactors (Figure 1.4) which utilize a fixed-film biological reactor consisting synthetic media, mounted on a horizontal shaft and positioned in a tank (Wang *et al*., 2008). Raw sewage is directed into the tank to be treated by the microorganisms attached on medium. The medium is rotated slowly by mechanical drives or air motivation rotation to allow the medium to be exposed to the air and wastewater alternatively. Rotation of the media supplies the microorganism on the media with oxygen, provide mixing and shearing force to strip off additional biomass on the medium. The treated wastewater was then directed to a clarifier for sedimentation of sludge.

Other than communal septic tanks, most of the sewage treatment plants in Malaysia are mechanical plants without media especially extended aeration tanks and sequencing batch reactors. Extended aeration plants employ secondary treatment where raw sewage is directly channeled to aeration tank without passing primary treatment (Figure 1.5). Some of the plants have equalizer tank to ensure constant flow of influent or when aeration tank overflows during rainy seasons. The raw sewage in the aeration tank is aerated for at least 24 hours to allow the bacteria to fully utilize the organic matter in the raw sewage. When the food sources are low, the microorganisms undergo partial auto-oxidation utilizing their own cell structure for food. As a result, the effluent produced is high quality and there is low sludge production (Cheremisinoff, 1996).

Sequencing batch reactor (SBR) employs a series of process phases that take place in the same tank with each process having its own holding time (Wilderer *et al*., 2001). Table 1.4 explains the main processes involved in one cycle in a SBR. Precise control of timing, mixing and aeration is crucial for optimum efficiency of this process. Older sewage treatment plants are now being phased out, and replaced with sequencing batch reactor that serves more than 100,000 population equivalent each.

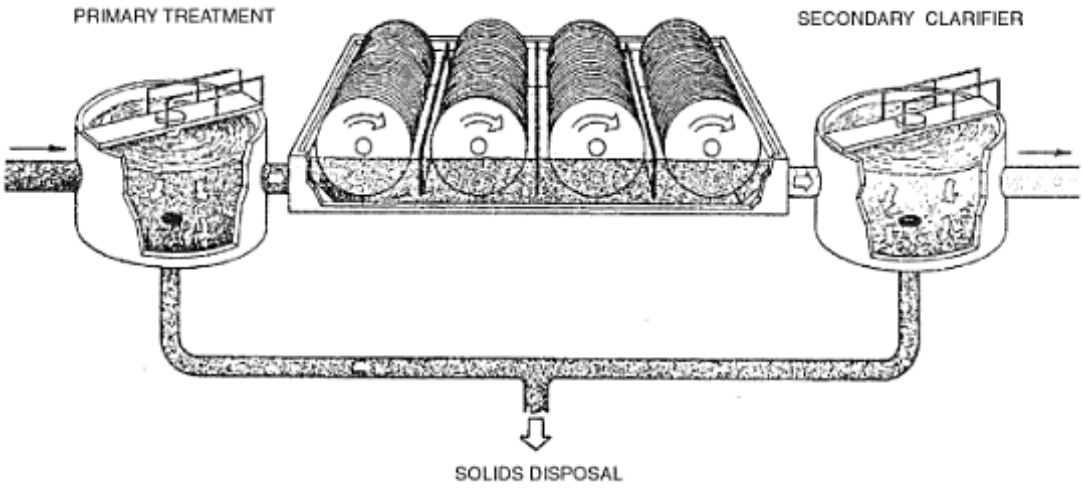

Figure 1.4: Design of Rotating Biological Contactor plant (Wang, 2008).

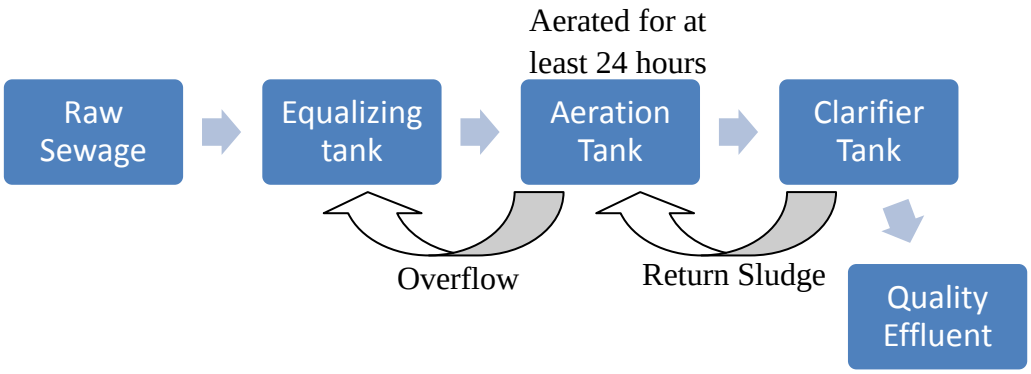

Figure 1.5: Extended aeration process (modified from Cheremisinoff, 1996).

Table 1.4: The main phase and function of Sequencing Batch Reactor in one cycle.

Phase	Function
Fill	The tank receives wastewater until designated volume. Nitrification or denitrification occurs depending on the amount of oxygen present.
React	Nitrification occurs during this phase where oxygen or air is pumped into the tank.
Settling	Sedimentation of the sludge takes place and the settled sludge provides an anaerobic condition for denitrification. Sludge can also be removed in the middle of this phase if required.
Decant	The clarified water is decanted and some of the sludge is removed. The leftover sludge is used as return sludge for the next cycle.
Idle	There are no aeration, no inlet and no outlet during this phase. Sludge can be removed during this phase if necessary while the tank prepares for the next cycle.

1.2 Microbiology of wastewater treatment

The modern wastewater treatment employs the principle of activated sludge where the main microorganism responsible for wastewater treatment is bacteria. Bacteria utilize wastewater in the presence of oxygen and multiply rapidly. In order to treat the wastewater and attain good effluent, good floc formation is essential. Flocs are loose clumps of bacteria brought together by pili or fibrils, sticky polysaccharides and poly-β-hydroxybutyrate or starch granules (Gerardi, 2006). Pili and fibrils have carboxyl grtoup and hydroxyl group that become ionized with the loss of hydrogen atoms. The ionized fibrils are able to join to each other through bivalent cations such as calcium thus forming flocs. Fibrils that are not joined will act as a broom that removes fine solids and heavy metals from the mixed liquor.

Sticky polysaccharides or glycocalyx are produced by bacteria and contributes to floc formation by sticking cells. The efficiency of these polysaccharides depends on the age of the cells where glycocalyx produced by young cells have weak bonds and are in large quantities and old cells have strong bonds and in small quantities. Poly-β-hydroxybutyrate granules are actually food reserves that are stored either inside or outside of the cells. When the granules are stored outside, it also acts as anchor that may attach to neighbouring bacteria and induce floc formation.

There are several factors that affect the formation of flocs e.g. sludge age, toxicity, surfactants and excessive shearing. If the sludge was not allowed to age enough, they are unable to experience the low nutrients condition and thus it cannot develop the components for floc formation (Figure 1.6). In contrast, toxicity kills the microorganism; retard its growth and its ability in removing nutrients. Adding surfactants would affect the stickiness of the slime and prevent the clumping of bacteria.

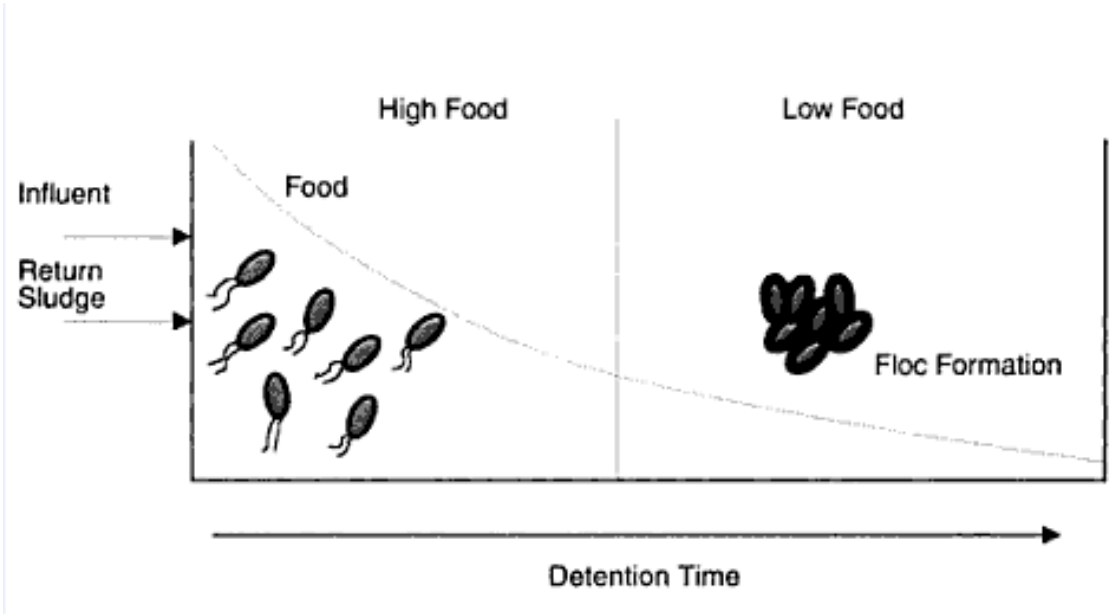

Figure 1.6: Relationship of microorganisms and the food concentration in activated sludge (Glymph, 2005).

Finally, excessive shearing by coarse air diffusers also causes the flocs to break (Glymph, 2005).

The microbial community present in activated sludge is not constant as raw sewage characteristics are different at each plant (Cloete & Muyima, 1997). In most cases, floc formers are the dominant group as they are retained in activated sludge whereas free growing cells are washed out. Flocs also protect the microbial cells from predators such as protozoa and metazoa.

Studies about the relationship of microorganisms and wastewater treatment are not new and its relationship was first reported in 1935. A bacterium, *Zoogloea ramigera*, was successfully isolated from activated sludge and is able to stabilize a liquid organic substrate and produces flocs (Butterfield, 1935). It was the first floc former isolated. Later, McKinney and Horwood found several other types of floc-formers i.e. *Escherichia intermedium*, *Paracolobactrum aerogenoides*, *Nocardia actinomorpha*, *Bacillus cereus* and a bacterium belonging to the genus *Flavobacterium* (Table 1.5) (McKinney & Horwood, 1952; McKinney & Weichlein, 1953). They also found that *Aerobacter aerogenes*, were able to form flocs even though it was not isolated from activated sludge. Dias and Bhat (1964) discovered that *Zoogloea* and *Comamonas* predominated in activated sludge. However none of the isolates worked well individually, suggesting the importance of microbial community in wastewater treatment processes. We now know individual floc formers are not the only microbes responsible for floc formation. Bacteria other than floc formers, ammonia oxidizing bacteria and denitrifiers are often blamed for increasing the turbidity of effluent and reducing the quality of effluent (Malik *et al.*, 2003). However, it was found that non-floc formers are also able to coaggregate with other intergeneric bacteria, and can help in floc formation e.g. coaggregation of *Acinetobacter johnsonii* S35 with other strains

Table 1.5: Significant genera of floc forming bacteria (Gerardi, 2006)

Achromobacter	*Citromonas*
Aerobacter	*Escherichia*
Alcaligenes	*Flavobacterium*
Arthrobacter	*Pseudomonas*
Bacillus	*Zoogloea*

Table 1.6: Group of bacteria and functions present in activated sludge (Cloete & Muyima, 1997)

Group	Species	Function
Oxic organotrophic microorganisms	Genera *Bacillus, Pseudomonas, Alcaligenes, Moraxella* and *Flavobacterium*	Degrade complex organic substrates
Fermentative bacteria	*Aeromonas punctata,* genera *Pasteurella* and *Alcaligenes*	Fermentative conversion of organic compound to volatile fatty acids
Anoxic organotrophic microorganisms (denitrifier)	Genera *Achromobacter, Alcaligenes, Arthrobacter, Bacillus, Flavobacterium, Moraxella* and *Pseudomonas*	Reduces nitrate to produce nitrogen gas under anoxic condition
Nitrifiers	*Nitrosomonas, Nitrococcus, Nitrosospira* and *Nitrosocytis*	Oxidation of Ammonia
	Nitrobacter, Nitrospina and *Nitrococcus*	Oxidation of nitrite to nitrate
Polyphosphate accumulation microorganism	*Acinetobacter, Aeromonas, Arthrobacter, Klebsiella, Moraxella* and *Pseudomonas*	Remove phosphate from wastewater by EBPR mechanism Some are able to denitrify
Sulfur bacteria	*Beggiatoa* and *Thiothrix*	Causes bulking problems

such as *Xanthomonas* spp. and coaggregation of *Acinetobacter junii* with four other isolates (Malik *et al.*, 2003).

Table 1.6 shows the function of bacteria other than floc formers in activated sludge systems. A group of bacteria responsible for removing ammonia are the ammonia oxidizing bacteria e.g. *Nitrosomonas eutropha*, *Nitrosomas oligotropha*, *Nitrosococcus mobilis*-like bacteria (Koops *et al.*, 1991; Juretschko *et al.*, 1998). Denitrifying bacteria are also important e.g. *Rhodobacter* group, *Rubrivivax* subgroup, *Pseudomonas* subgroup, *Brucella* isolates and *Acidovorax caeni* sp. Nov (Magnusson *et al.*, 1998; Heylen *et al.*, 2008).

Severe operational problems such as bulking and foaming that result in poor efficiency of the treatment process are also caused by excessive growth of filamentous bacteria. Common group of these filamentous bacteria are *Nocardioform* group, *Microthrix parvicella*, *Sphaerotilus natans*, *Nostocoida limicol*a, *Beggiatoa* etc. (Faheem & Khan, 2009).

1.3 Objectives of the study

In Asia, several efforts in identifying bacteria from wastewater treatment plants have already been done. For example in Singapore, nine species of bacteria belonging to the genera *Flavobacterium, Pseudomonas, Micrococcus, Aeromonas, Xanthomonas, Vibrio and Sphingomonas* were isolated (Fong & Tan, 2000). In Iran, the following isolates *Flavobacterium*, *Alcaligenes*, *Pseudomonas* and *Micrococcus* were discovered (Yazdi *et al.*, 2001). Research about wastewater treatment plant and activated sludge in Malaysia are far from being comprehensive. A study in Malaysia isolated 46 bacterial strains from four treatment plants but did not identify them (Jalal *et al.*, 2006).

As a tropical country, the microbial ecology of the activated sludge may differ from the well studied temperate countries. In this study, cultural-dependent methods were employed as it allows phenotypic characterization and the bacteria isolated are easier to manage, and have potential for further characterization. Due to the lack of microbial study of wastewater treatment plant in Malaysia, culture-dependent method may be able to obtain novel isolates and provide an initial understanding of microbial diversity in the activated sludge. Thus, the objectives of this study are to:

1. To determine the types of culturable bacteria isolated from selected activated sludge systems in Malaysia.
2. To relate the culturable bacterial community to the process and environmental conditions of the activated sludge system.

MATERIALS AND METHODS

2.1 Sampling

Samples were collected from three different sewage treatment plants (STP1, STP2 and STP3) located at Serdang, Klang Valley (Figs. 2.1, 2.2 and 2.3). These STPs were managed by Indah Water Konsortium Pte. Ltd and were all extended aeration sewage treatment plants. STP1 served 5630 population equivalent (PE), and was designed for a wastewater flow rate of 1300 m^3 day^{-1} whereas STP2 served 4600 PE and was designed for a wastewater flow rate of 1000 m^3 day^{-1}. STP3 served 5630 PE with a wastewater flow rate of 1358 m^3 day^{-1}. Some measurements were carried out in-situ (temperature, pH, sludge volume and diluted sludge volume) and samples were also brought back to the laboratory for further analysis (Table 2.1). pH was measured with a pH paper, Universal indicator (Merck, Germany) whereas temperature was measured by a digital thermometer (Thermoworks, USA).

For sludge volume measurement, 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 sludge volume (ml l^{-1}). For diluted sludge volume, three one-liter measuring cylinder were used. Aeration tank samples were diluted 2×, 4× and 10× with effluent, and allowed to settle for 30 minutes. Settled sludge was recorded in ml l^{-1}.

Sludge volume index (SVI, ml g^{-1}) and diluted sludge volume index (DSVI, ml g^{-1}) were calculated using the equations below:

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

$$DSVI = (V \times N)/MLSS$$

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

2.2 Dissolved oxygen

Dissolved oxygen was measured using modified Winkler's Titration Method (Grasshoff *et al.*, 1999). Samples were collected using 50 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 thiosulphate solution. Starch was used as an indicator and the samples were titrated until colorless. Dissolved oxygen was calculated using the following formula:

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

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

Figure 2.1: Map showing the location of STP1 (red star symbol) (N 3°1’47’’, E 101°42’38’’).

Figure 2.2: Map showing the location of STP2 (yellow star symbol) (N 3°1’45’’, E 101°42’28’’).

Figure 2.3: Map showing the location of STP3 (blue star mark) (N 3°0’57’’, E 101°41’59’’).

All maps were obtained from Google map (2009).

Table 2.1: Types of wastewater analysis and measurements carried out in this study. The symbol + denotes measurement carried out whereas – for measurements not carried out.

Type of measurement	**SAMPLING POINTS**		
	Influent	**Aeration tank**	**Effluent**
Mixed Liquor Suspended Solids (MLSS) and Mixed Liquor Volatile Suspended Solids (MLVSS)	–	+	–
Chemical Oxygen Demand (COD)	+	+	+
Biological Oxygen Demand (BOD_5)	+	+	+
Dissolved Oxygen (DO)	–	+	–
Total alkalinity	–	+	–
Ammonium (NH_4)	+	+	+
Nitrite (NO_2)	+	+	+
Nitrate (NO_3)	+	+	+
Phosphate (PO_4)	+	+	+
Isolation and enumeration of heterotrophic bacteria	–	+	–

2.3 Biological Oxygen Demand (BOD)

BOD is the measurement of dissolved oxygen depletion over a five-day period at 25°C in the dark (Tomar, 1999). Samples were diluted 10 times with aerated dilution water (Table 2.2) 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:

$$BOD = (DO_{0\ day}\ \text{sample} - DO_{5\ days}\ \text{sample}) - (DO_{0\ day}\ \text{control} - DO_{5\ days}\ \text{control})$$

2.4 Total Alkalinity

Total alkalinity was measured according to Gran (1952). Sample was collected using cleaned bottles and preserved *in-situ* by adding two drops of saturated $HgCl_2$ solution and mixed well. In the laboratory, the sample was titrated with 0.1N HCl, and changes in pH were measured with pH meter, Orion 4 Star (Thermo Fisher Scientific, USA). The measurement was carried out in triplicates. Total alkalinity was then calculated from the following formula:

$$\text{Total alkalinity } (\mu eq\ l^{-1}) = [(0.1 \times \text{x-intercept value}) / \text{sample volume (l)}] \times 10^6$$

Where the x-intercept value was obtained from a F1 against volume of HCl plot, and F_1 was calculated using the following equation:

$$F_1 = [\text{volume of sample (l)} + \text{volume of acid (l)}] \times 10^{-pH}$$

2.5 Mixed Liquor Suspended Solids (MLSS) and Mixed Liquor Volatile Suspended Solids (MLVSS)

A known volume of aeration tank sample was filtered onto Whatman GF/C filters. MLSS (mg l^{-1}) was measured as the filter weight gained after drying at 44ºC 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).

2.6 Chemical Oxygen Demand (COD) (Burns & Marshall, 1965)

Samples were diluted 10× with distilled water before adding into conical flask in triplicates. Mercury sulfate (0.2 g) was added before 10 ml of acid sulfuric reagent (22 g of $AgSO_4$ 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 equation below:

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

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

2.7 Nutrient analysis

For nutrient analysis, samples were filtered through Whatman GF/C filter, and the filtrate was kept frozen (−20°C) until analysis. Dissolved inorganic nutrients [nitrate (NO_3), ammonia (NH_3), phosphate (PO_4) and nitrite (NO_2)] were measured according to Nollet (2000).

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. All nutrient analyses were carried out in triplicates, and the average coefficient of variation was 5% for ammonium and phosphate, 6% for nitrate and 10% for nitrite.

Table 2.2: Preparation of the dilution water (Tomar, 1999).

Phosphate buffer	
Potassium dihydrogen phosphate, KH_2PO_4	8.50 g
Dipotassium phosphate, K_2HPO_4	21.75 g
Sodium phosphate, $Na_2HPO_4.7H_20$	33.40 g
Ammonium chloride, NH_4Cl	1.70 g
Distilled water	1 liter
pH	7.2

Dilution water	
Phosphate buffer	1 ml
Magnesium sulfate, $MgSO_4.7H_2O$ (22.5 g l^{-1})	1 ml
Calcium chloride, $CaCl_2$ (27.5 g l^{-1})	1 ml
Iron (III) chloride, $FeCl_3.6H_2O$ (0.25 g l^{-1})	1 ml
Distilled water	to 1000 ml

2.8 Isolation and enumeration of heterotrophic bacteria

Samples were kept in sterile bottles, and mixed well by a vortex machine. After serial dilution, 0.1 ml of the diluted sample was cultured by spread plating on Tryptone Glucose Yeast Agar (TGYA), Casitone Glyserol Yeast Agar (CGYA) and Reasoner's 2 Agar (R_2A) (Table 2.3). The plates were then incubated at 25°C for two days. Plates with colony counts between 25 cfu to 250 cfu were enumerated. Colony morphology was recorded for every unique colony on the plates, and every unique isolate was purified at least three times by dilution streaking.

2.9 Identification of bacteria

Purified isolates were identified via their 16S rDNA sequence. Genomic DNA of all isolates was extracted using standard protocols adapted from Ausubel *et al*. (2002). Isolates were initially lysed using SDS and lysozyme before genomic DNA extraction via buffered phenol: chloroform: isoamyl alcohol (25:24:1) extraction and ice-cold ethanol precipitation method. DNA was resuspended in Tris-EDTA (TE) buffer (pH 8.0) and stored at −20ºC. The extracted DNA was examined in a 1% (w/v) Seakem LE agarose (Cambrex, USA) at 5.0 V cm^{-1} for 30 minutes in 1× Tris-borate-EDTA (TBE) buffer.

2.10 Amplification of the 16S rDNA

The 16S rDNA gene was amplified using a pair of universal primer, forward primer: 27F (5'-AGAGTTTGATCMTGGCTCAG-3') and the reverse primer: 1525R (5'-AAGGAGGTGWTCCARCC-3') (Lane, 1991). Polymerase chain reaction (PCR) was performed in a 15 µl reaction mixture containing: 1× PCR buffer, 1.5 mM of $MgCl_2$, 0.4 µM of each primer, 3 µl of DNA template (approximately 200 ng μl^{-1}), 0.2 mM of dNTPs and 0.5 U of *Taq* polymerase (Finnzymes DyNAzyme^{TM} II, Finland).

Table 2.3: Composition of bacteriological media used in this study.

Tryptone Glucose Yeast Agar (TGYA)	
Casitone (Pancreatic digest of casein)	5.0 g
Glucose	1.0 g
Yeast extract	1.0 g
Agar	15.0 g
Distilled water	1000 ml

Casitone Glycerol Yeast Autolysate (CGYA)	
Casitone (Pancreatic digest of casein)	5.0 g
Glycerol	10.0 g
Yeast extract	1.0 g
Agar	15.0 g
Distilled water	1000 ml

Reasoner's 2 Agar (R_2A)	
Yeast extract	0.5 g
Peptone	0.5 g
Casein hydrolysate	0.5 g
Glucose	0.5 g
Soluble starch	0.5 g
Sodium pyruvate	0.3 g
Magnesium sulfate, $MgSO_4.H_2O$	0.024 g
Dipotassium hydrogen phosphate, K_2HPO_4	0.3 g
Agar	15.0 g
Distilled water	1000 ml

*** All media were autoclaved at 121°C and 15 psi for 20 minutes.**

PCR amplification was carried out according to the following conditions: initial denaturation at 95°C for 5 min; 30 cycles of (denaturation for 30 s at 95ºC, annealing for 40 s at 55ºC, extension for 90 s at 72ºC) and a final extension at 72ºC for 7 min. The amplicons were examined in a 1% (w/v) Seakem LE agarose (Cambrex, USA) at 5.0 V cm^{-1} for 30 minutes in 1× (TBE) buffer with DNA Marker λ DNA *Hind* III digest and ΦX174 DNA *Hae* III digest (Figure 2.4).

2.11 Restriction Fragment Length Polymorphism (RFLP)

For dereplication, RFLP was carried out. The restriction endonucleases, *Rsa*I and *Cfo*I (Roche, Germany) were used to digest the 16S rDNA amplicon separately and then resolved on a 3.0% (w/v) agarose gel [0.75% Seakem LE agarose (Cambrex, USA) plus 2.25% NuSieve Agarose gel (Lonza, USA)] at 5.0 V cm^{-1} for one hour in 1× Tris-borate-EDTA (TBE) buffer. Restriction patterns were analyzed using the AlphaEaseFC™ software (Alpha Innotech Corp., US), and strains showing different RFLP patterns were selected and their PCR product sent for sequencing.

PCR products were purified using QIAquick® PCR Purification Kit (QIAGEN, Germany) prior to sequencing at AITBIOTECH, Singapore. The nucleotide sequence of the samples was submitted to both the BLASTn search program (Altschul *et al.* 1990) of the National Center for Biotechnology Information (NCBI) and Ribosomal Database Project 10 (RDP-10) (Cole *et al.,* 2007) for identification of the closest related bacteria.

2.12 Phylogenetic analysis

The taxonomic position of the bacterial isolates was checked via a phylogenetic tree. The sequences were first aligned via MUSCLE 3.6 (Edgar, 2004), and any misalignments were corrected manually. A neighbour-joining tree (Guindon & Gascuel, 2002) was constructed from a matrix of pairwise genetic distances calculated by the

Kimura 2-Parameters algorithm using Bosque, a software system for phylogenetic analysis (Ramírez-Flandes and Ulloa, 2008). Bootstrap analyses of 1000 replicates were also performed to assess the relative stability of the branches.

2.13 Numerical analysis

Diversity statistics represents the species richness and evenness of a community (Magurran, 2004). In this study, Shannon's Diversity Index was adopted to analyze the diversity of the bacterial community. The equation for Shannon's Diversity Index is

$$H' = -\sum_{i=1}^{S}(p_i \ln p_i) - [(S-1)/2N]$$

n_i = The number of individuals in species *i*; the abundance of species *i*.

S = The number of species. Also called species richness.

N = The total number of all individuals

p_i = The relative abundance of each species, calculated as the proportion of individuals of a given species to the total number of individuals in the community: $\frac{n_i}{N}$

All data unless otherwise noted, was reported as mean ± standard deviation. Statistical tests such as ANOVA, Student's t-test, Tukey test and multivariate analyses including cluster analysis (with Morishita similarity coefficient) were carried out with PAST (Hammer *et al*., 2001).

RESULTS

In this study, temperatures measured ranged from 27°C to 33°C whereas pH ranged from pH 4.0 to pH 8.0 (Appendices A and B). pH measured at STP1 and STP2 was generally above 5 whereas at STP3, pH was below 5.

3.1 Influent characteristics

Influent BOD ranged from 200 to 500 mg l^{-1} (Figure 3.1) whereas influent COD ranged from 320 to 680 mg l^{-1} (Figure 3.2). The concentrations of both influent BOD and COD were not significantly different among the STPs. BOD:COD ratio which is sometimes used to indicate wastewater biodegradability (Celenza, 1999), ranged from 0.5 to 1.1 and was significantly different (F=4.56, df=24, p<0.05) among the STPs. BOD: COD ratio in STP1 (0.97) was significantly higher (q=3.96, df=24, p<0.05) than STP3 (0.60).

Nitrate (F=5.02, df=26, p<0.05) and nitrite (F=11.85, df=26, p<0.001) were significantly different among STPs, and both were highest at STP2. Average nitrate concentration in STP2 (4.20±3.90 mg l^{-1}) was higher than STP1 (1.10±0.55 mg l^{-1}; q=3.83, df=26, p<0.05) and STP3 (1.00±0.70 mg l^{-1}; q=3.93, df=26, p<0.05) (Figure 3.3) whereas nitrite in STP2 (0.34±0.02 mg l^{-1}) was also higher than STP1(0.25±0.04 mg l^{-1}; q=4.80, df=26, p<0.01) and STP3 (0.22±0.08 mg l^{-1}; q=6.60, df=26, p<0.001) (Figure 3.4). Similarly, ammonia (Figure 3.5) was also different among the STPs (F=44.09, df=26, p<0.001). Ammonia concentration was highest at STP2 (48±1.1 mg l^{-1}) relative to STP1 (41.0±1.0 mg l^{-1}; q=13.27, df=26, p<0.001) and STP3 (45.0±1.8 mg l^{-1}; q=6.24, df=26, p<0.001). The concentration of ammonia in the influent at STP3 was also significantly higher than STP1 (q=7.03, df=26, p<0.001).

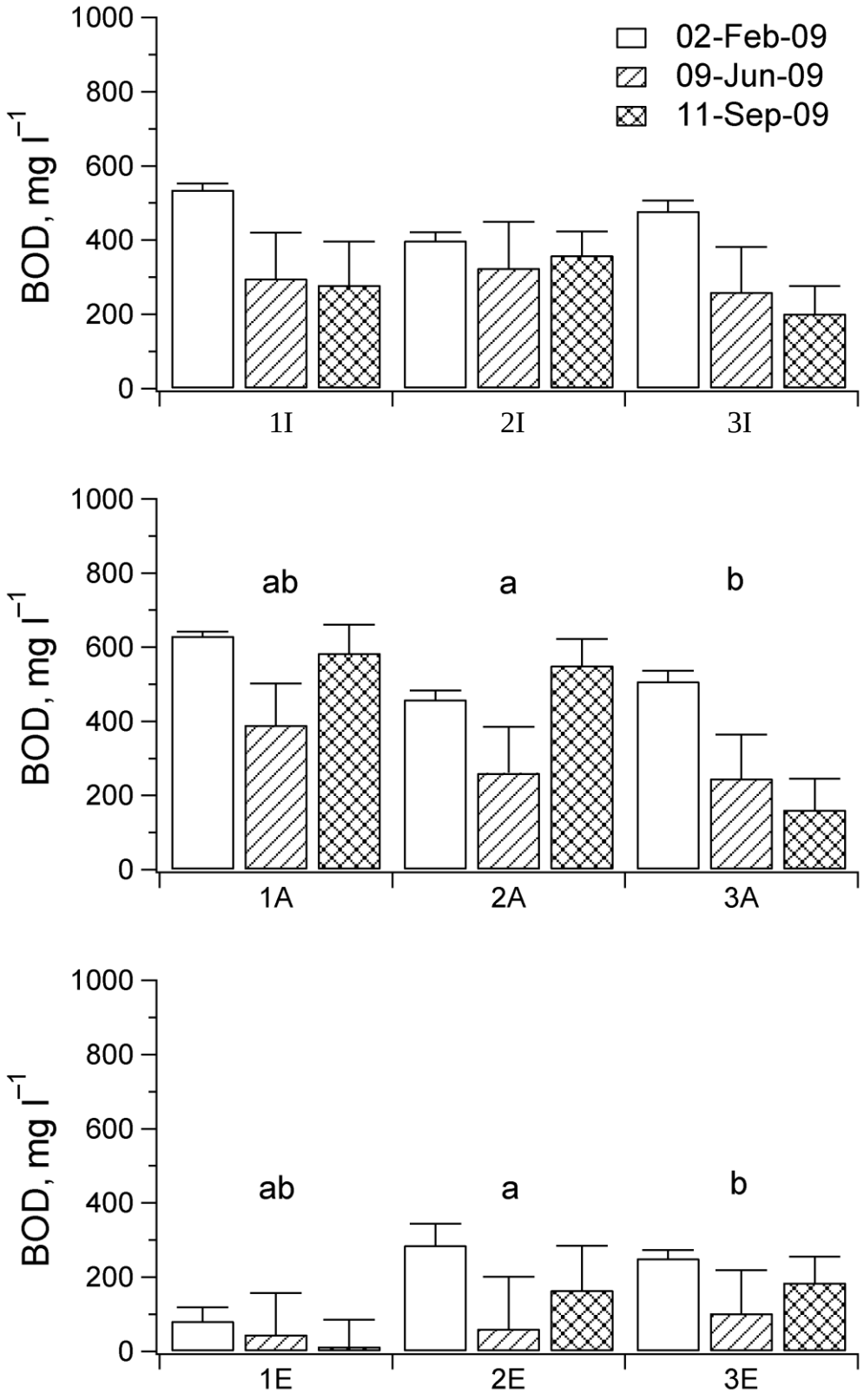

Figure 3.1: BOD_5 measured at each STP. The error bars represent the standard deviation of the values. The same letters of the alphabet are used to indicate values whose means were significantly different.

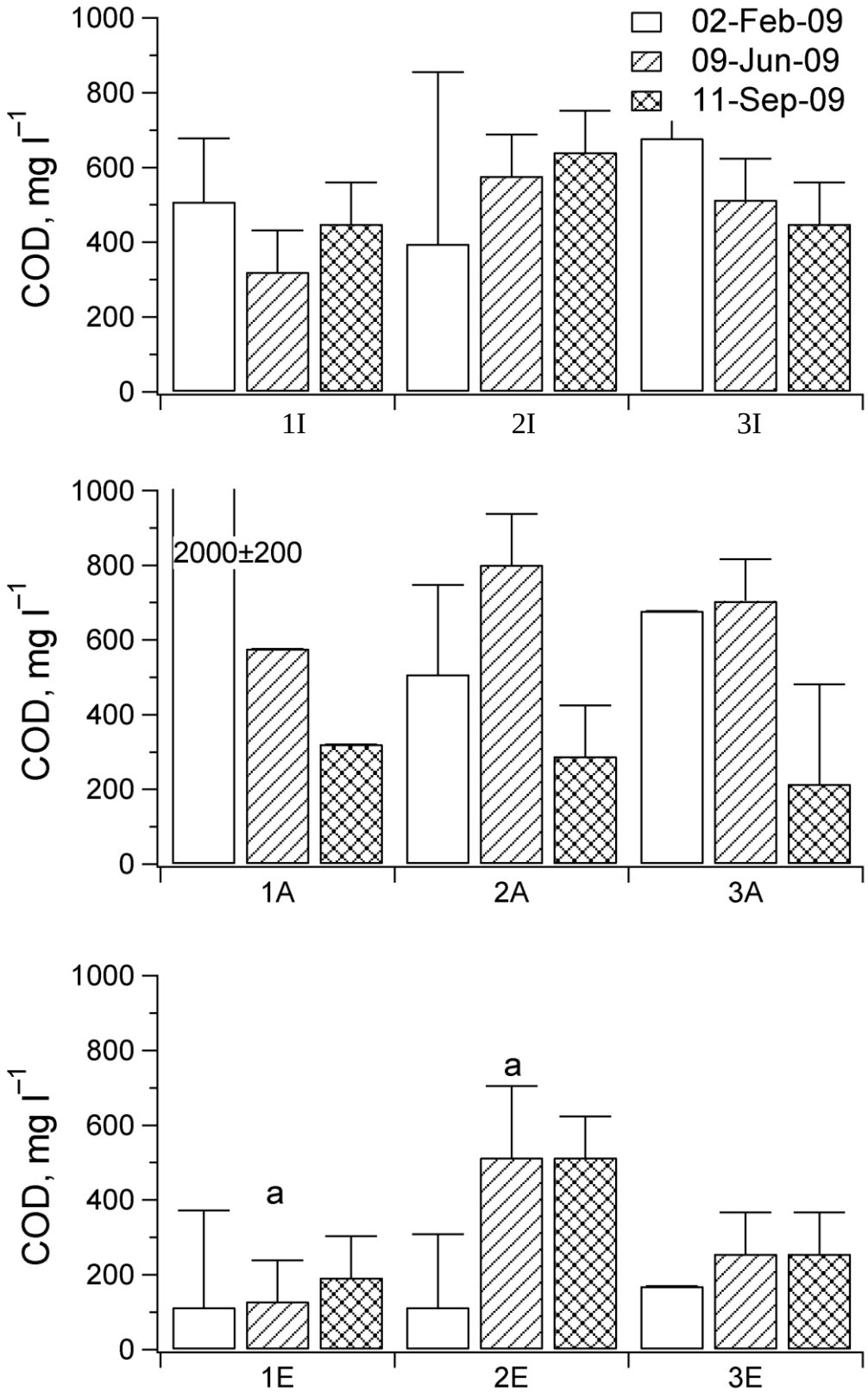

Figure 3.2: COD measured at each STP. The error bars represent the standard deviation of the values. The same letters of the alphabet are used to indicate values whose means were significantly different.

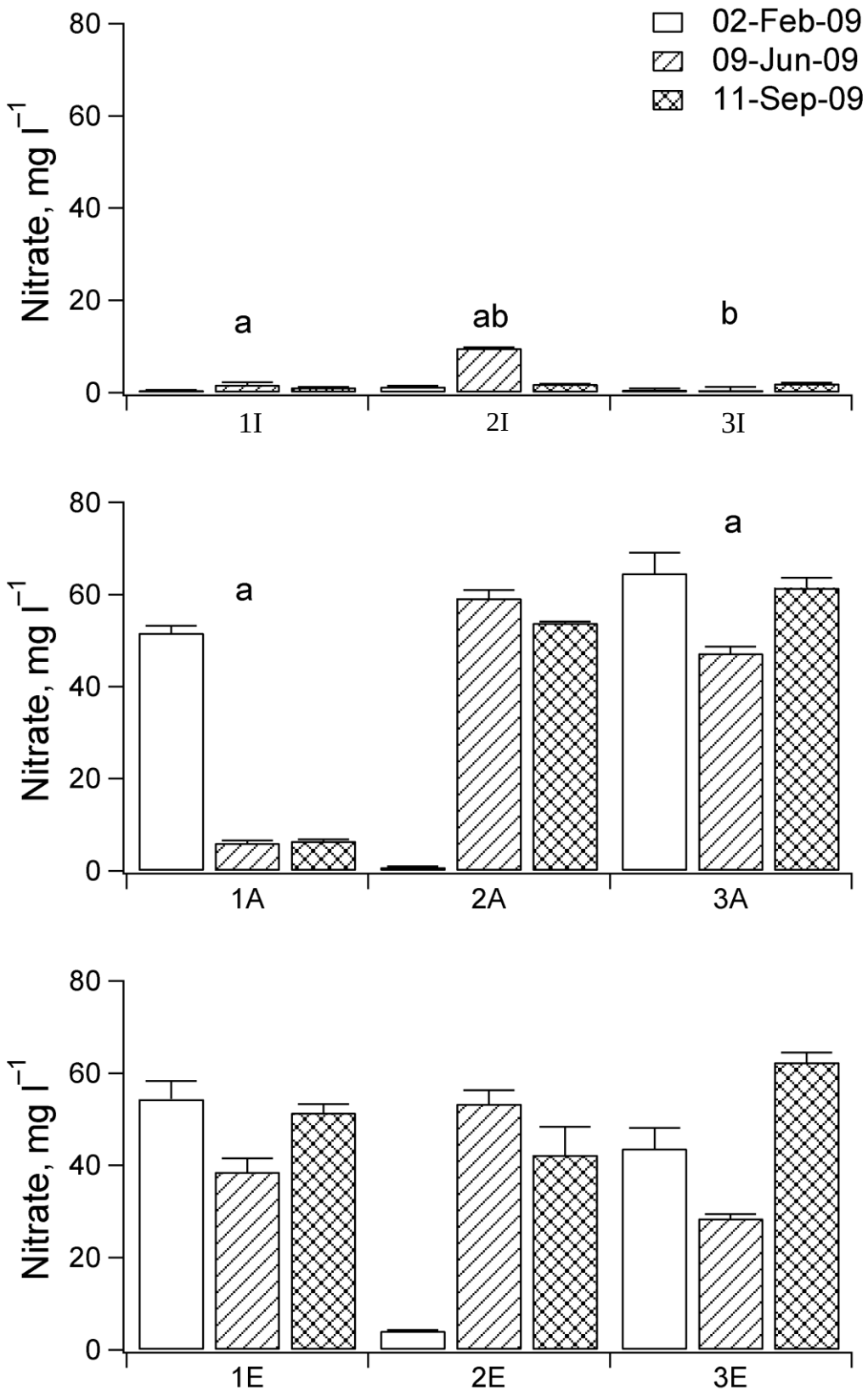

Figure 3.3: Nitrate concentration measured at each STP. The error bars represent the standard deviation of the values. The same letters of the alphabet are used to indicate values whose means were significantly different.

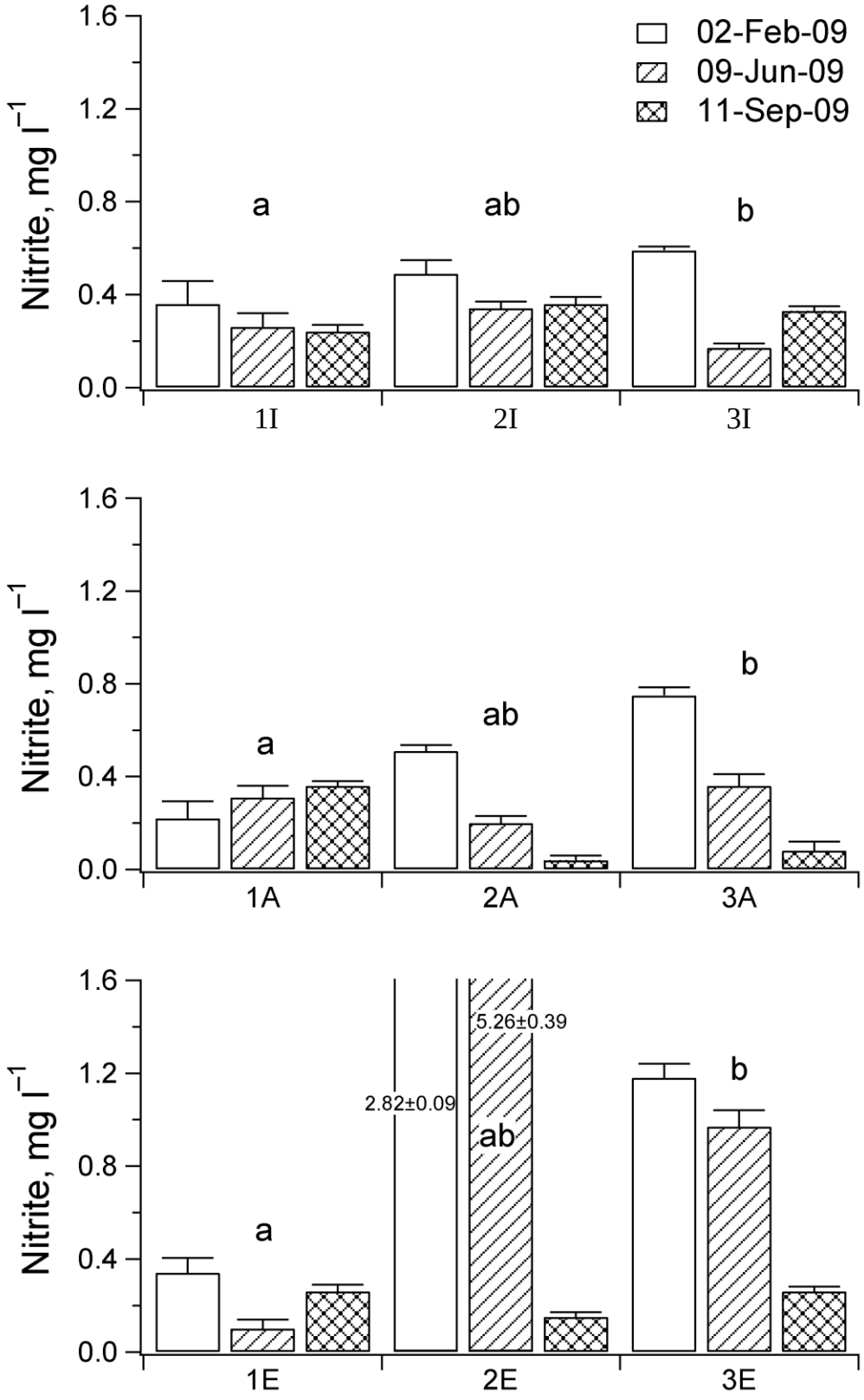

Figure 3.4: Nitrite concentration measured at each STP. The error bars represent the standard deviation of the values. The same letters of the alphabet are used to indicate values whose means were significantly different.

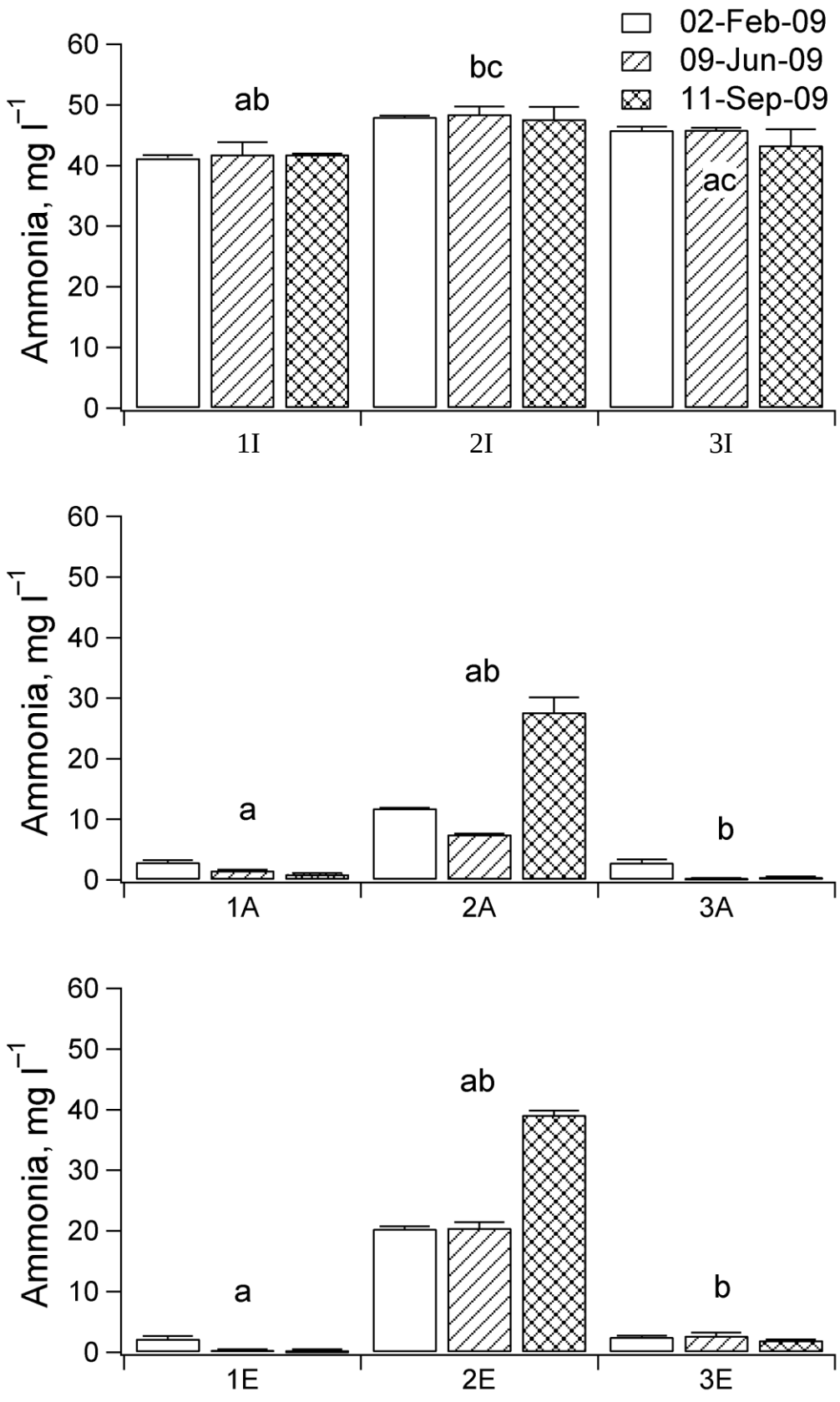

Figure 3.5: Ammonia concentration measured at each STP. The error bars represent the standard deviation of the values. The same letters of the alphabet are used to indicate values whose means were significantly different.

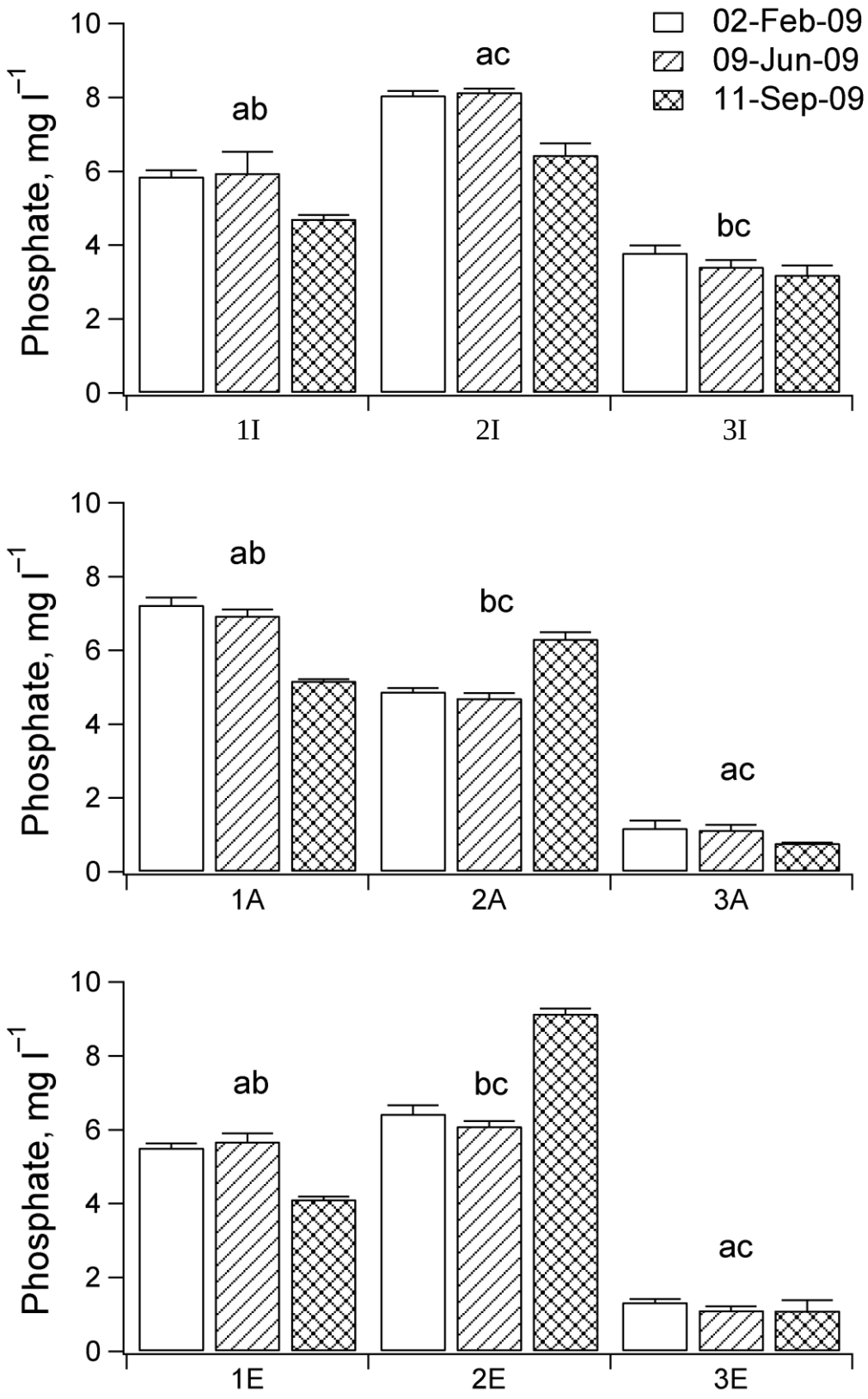

Figure 3.6: Phosphate concentration measured at each STP. The error bars represent the standard deviation of the values. The same letters of the alphabet are used to indicate values whose means were significantly different.

For phosphate, there was also a significant difference among the influent at the three STPs (F=87.95, df=26, p<0.001). The influent at STP2 had the highest phosphate concentration (7.54±0.80 mg l^{-1}), and was significantly higher than both STP1 (5.50± 0.60 mg l^{-1}; q=9.36, df=26, p<0.001) and STP3 (3.50± 0.30 mg l^{-1}; q=18.76, df=26, p<0.001) (Figure 3.6). Influent at STP1 also had higher phosphate concentration when compared to STP3 (q=9.40, df=26, p<0.001).

3.2 Physico-chemical characteristics of mixed liquor in aeration tanks

DO measured in the aeration tank ranged from non-detectable to 4.00 mg l^{-1} (Figure 3.7). Generally, DO was low with average DO of 2.3±1.0 mg l^{-1} at STP1, 2.1±1.4 mg l^{-1} at STP2, and 1.3±1.1 mg l^{-1} at STP3. DO was not detectable on one occasion at STP1. During our sampling, STPs 2 and 3 were also experiencing irregular mechanical disruptions in their air diffusers (*personal communication with Mr. Sarman, IWK Technichian*). In the aeration tank, BOD was significantly different (F=12.18, df=26, p<0.001) among the STPs (Figure 3.1). STP1 had the highest BOD at 560±50 mg l^{-1}, and was significantly higher than STP2 (430±100 mg l^{-1}; q=3.54, df=26, p<0.05) and STP3 (310±140 mg l^{-1}; q=6.98, df=26, p<0.001). On the other hand, COD did not show significant difference among the STPs. COD ranged from 200 to 900 mg l^{-1} except on one occasion when the COD reached 2000 mg l^{-1} (at STP1) (Figure 3.2).

Ammonia concentration was different among the STPs (F=20.53, df=26, p<0.001) (Figure 3.5). Ammonia concentration in the STP2 aeration tank (15.65±8.70 mg l^{-1}) was higher than STP1 (1.80±0.85 mg l^{-1}; q=7.67, df=26, p<0.001) and STP3 (1.15±1.20 mg l^{-1}; q=8.02, df=26, p<0.05). Other inorganic nitrogen species i.e. nitrate (F=6.50, df=26, p<0.01) and nitrite (F=7.39, df=26, p<0.01) also exhibited significant difference among the STPs (Figures 3.3 and 3.4). Nitrate concentration at STP3 (58.0±8.0 mg l^{-1}) was significantly higher than STP1 (q=5.09, df=26, p<0.01). Nitrate

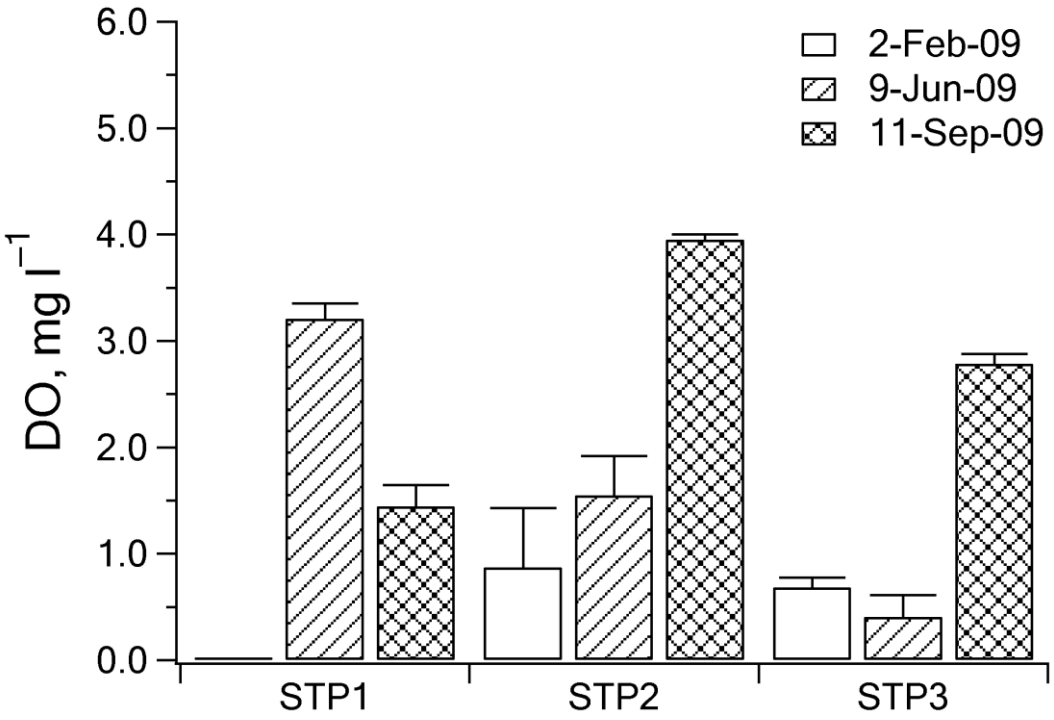

Figure 3.7: Dissolved oxygen (mg l^{-1}) measured in the aeration tank of each STP. The error bars represent the standard deviation of the values.

concentration increased considerably from influent to aeration tank at all STPs whereas ammonia decreased from influent to aeration tanks. Nitrite concentration in the aeration tanks were of similar range to influent. Nitrite lowest at STP2 (0.15±0.08 mg l^{-1}) when compared with STP1 (F=5.34, df=26, p<0.01) and STP3 (F=3.57, df=26, p<0.05).

Phosphate concentration (Figure 3.6) in aeration tanks was similar to influent where STP3 (1.0±0.2 mg l^{-1}) had the lowest phosphate concentration when compared to STP1 (q=22.29, df=26, p<0.001) and STP2 (q=17.57, df=26, p<0.001). STP1 was also significantly higher than STP2 (q=4.72, df=26, p<0.01). At STP3, phosphate concentration decreased from influent to aeration tank whereas no clear differences were observed at STP1 and STP2.

Alkalinity was also measured in aeration tank samples, and suggests the ability of the mixed liquor to neutralize acid (Spellman, 2008). Alkalinity (Figure 3.8) was significantly different (F=11.71, df=26, p<0.001) among the STPs, and was highest at STP1 (2250±1700 µeq l^{-1}) than both STP2 (560±460 µeq l^{-1}; q=5.04, df=26, p<0.01) and STP3 (60±35 µeq l^{-1}; q=6.53, df=26, p<0.001).

Sludge volume in an aeration tank is crucial for the operational efficiency of sewage treatment as it represents flocculation and settling characteristic of the activated sludge (Henze, 2002). Sludge volume at STP1 ranged from 440 to 980 ml l^{-1}, and was the highest (F=14.93, df=8, p<0.05) when compared with STP2 (q=6.25, df=8, p<0.05) and STP3 (q=7.06, df=8, p<0.01) (Figure 3.9). Although sludge volume at STP2 increased with each sampling, sludge volume at STP3 remained low. MLSS which represents the suspended solids in the mixed liquor or aeration tank were similar among the STPs (ranging from 390 to 6350 mg l^{-1}). However there was a significant

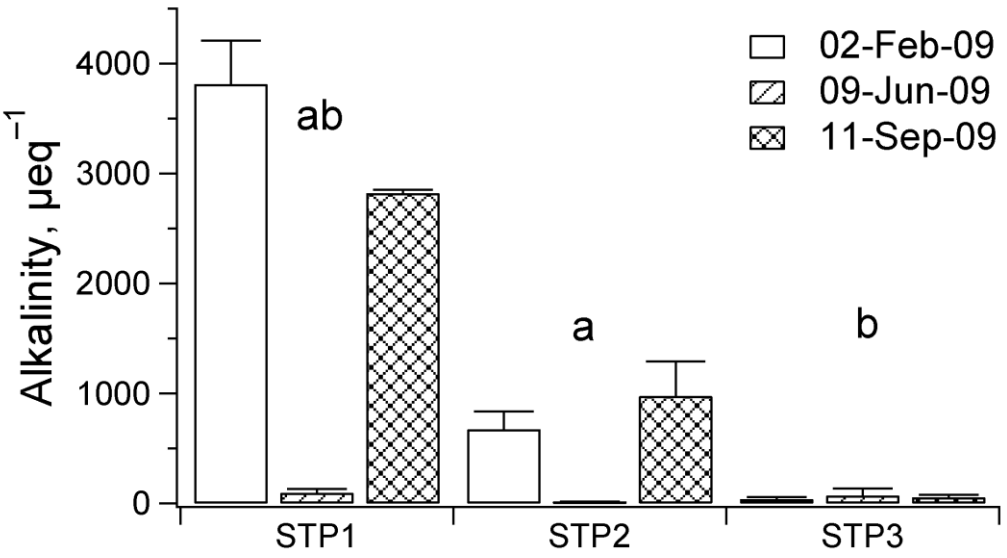

Figure 3.8: Alkalinity measured in the aeration tank of each STP. The error bars represent the standard deviation of the values. The same letters of the alphabet are used to indicate values whose means were significantly different.

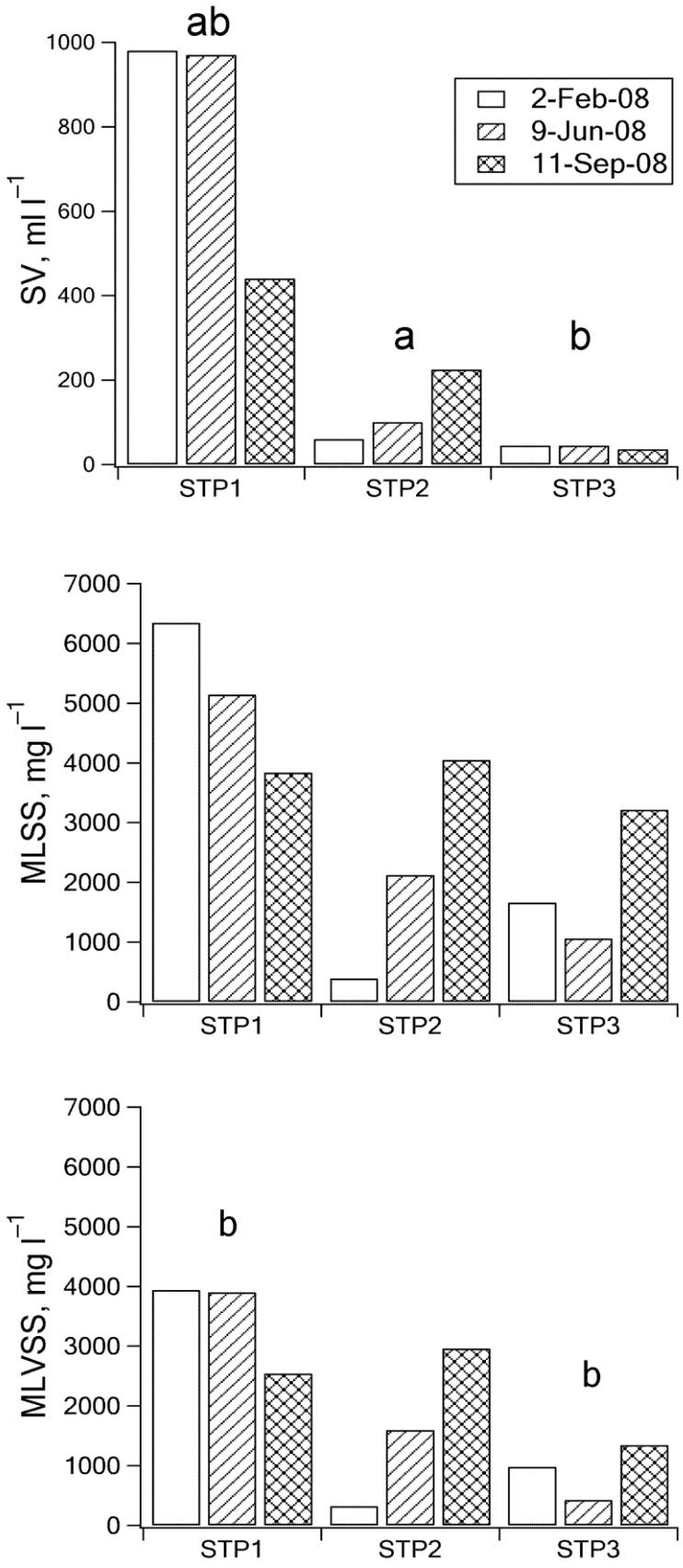

Figure 3.9: SV, MLSS and MLVSS measured from mixed liquor of aeration tank at each STP. The same letters of the alphabet are used to indicate values whose means were significantly different.

difference in MLVSS among the STPs (F=5.98, df=8, p<0.05), especially between STP1 and STP3 (q=4.74, df=8, p<0.05). The MLVSS for STP1 ranged from 2500 to 4000 mg l^{-1} whereas STP3 ranged from 420 to 1350 mg l^{-1}. This indicated that the suspended solids in the aeration tank at STP1 had a higher organic fraction (probably in the form of microorganism) than STP3. The MLVSS at STP2 also increased with each sampling (from 320 to 2960 mg l^{-1}).

3.3 Effluent characteristics

As effluent reenters the drainage system, the quality of the effluent is essential, and should not pollute the receiving waters. Effluent BOD was significantly different (F=12.70, df=26, p<0.001), and was lowest at STP1 (38±35 mg l^{-1}) (Figure 3.1). Effluent BOD at STP2 (161±90 mg l^{-1}; q=5.86, df=8, p<0.01) and STP3 (175±48 mg l^{-1}; q=6.45, df=8, p<0.001) were both significantly higher than STP1. Figure 3.2 showed that effluent COD was significantly different (F=4.81, df=26, p<0.05) among the STPs. COD at STP1 (145±130 mg l^{-1}) was significantly lower (q=4.32, df=26, p<0.05) than COD at STP2 (380±230 mg l^{-1}). The amount of BOD reduced was 345±100 mg l^{-1} at STP1, 210±100 mg l^{-1} at STP2 and 135±100 mg l^{-1} at STP3. BOD reduction rate at STP1 ranged from 84% to 95% whereas at STP3, it ranged from 8% to 60%. The BOD reduction rate at STP2 was initially 28% before improving to 81% and 54%. The percentage of COD reduction ranged from 50% to 80% at STP1 whereas at STP2, the reduction was high during first sampling (71%) but decreased to 11% and 20% for the second and third sampling. STP3 had a COD reduction rate that ranged from 40% to 75%.

Effluent inorganic nitrogen species such as ammonia showed significant differences (F=63.10, df=26, p<0.001) among the STPs. STP2 had the highest ammonia concentration (26.0±9.0 mg l^{-1}) compared to STP1 (q=14.15, df=26,

$p<0.001$) and STP3 ($q=13.33$, $df=26$, $p<0.001$) (Figure 3.5). Both STP1 and STP3 have high reduction percentage for ammonia which was above 90% as compared to STP2 which was below 60%. Effluent nitrite (Figure 3.4) was also different among STPs where STP2 (3.50±2.50 mg l^{-1}) was higher than STP1 (0.15±0.08 mg l^{-1}; $q=6.81$, $df=26$, $p<0.001$) and STP3 (0.70±0.30 mg l^{-1}; $q=5.66$, $df=26$, $p<0.01$). However, nitrate did not show significant difference among the STPs, and ranged from 4.00 to 65.00 mg l^{-1} (Figure 3.3). Phosphate (Figure 3.6) in effluent showed significant difference among the STPs ($F=92.74$, $df=26$, $p<0.001$) where STP3 at 1.20±0.18 mg l^{-1} was lower than STP1 ($q=12.32$, $df=26$, $p<0.001$) and STP2 ($q=18.98$, $df=26$, $p<0.001$) at 5.10±0.70 mg l^{-1} and 7.20±1.4 mg l^{-1}. Phosphate concentration in STP1 was also significantly lower than STP2 ($q=6.67$, $df=26$, $p<0.001$). The reduction of phosphate in STP3 was > 60% and was considerably higher than STP1 and STP2.

Effluent TSS at STP1 (40±9 mg l^{-1}) was the lowest among the three STPs whereas both STP2 (105±38 mg l^{-1}) and STP3 (105±51 mg l^{-1}) have relatively higher TSS values (Table 3.1).

3.4 Isolation and enumeration of bacteria

In this study, we cultured between 0.04 to 4.50 $\times 10^6$ cfu ml^{-1} in TGYA, 0.04 to 5.70 $\times 10^6$ cfu ml^{-1} in CGYA and 0.05 to 3.90 $\times$ 10^6 cfu ml^{-1} in R2A (Figure 3.10) from aeration tank samples. The number of culturable bacteria obtained on TGYA was significantly different among the STPs ($F=6.01$, $df=8$, $p<0.05$). STP1 (2.65±1.88 $\times 10^6$ cfu ml^{-1}) had a significantly higher cfu than STP3 (1.11±1.29 $\times 10^5$ cfu ml^{-1}; $q=4.76$, $df=8$, $p<0.05$) whereas at STP2, average cfu count was 1.82±2.58 $\times 10^6$ cfu ml^{-1}.

Table 3.1: TSS (mg l^{-1}) measured in the effluent of each STP.

Sewage Treatment Plant	**STP1**	**STP2**	**STP3**
02-Feb-08	48	80	64
09-Jun-08	30	85	90
11-Sep-08	40	148	163

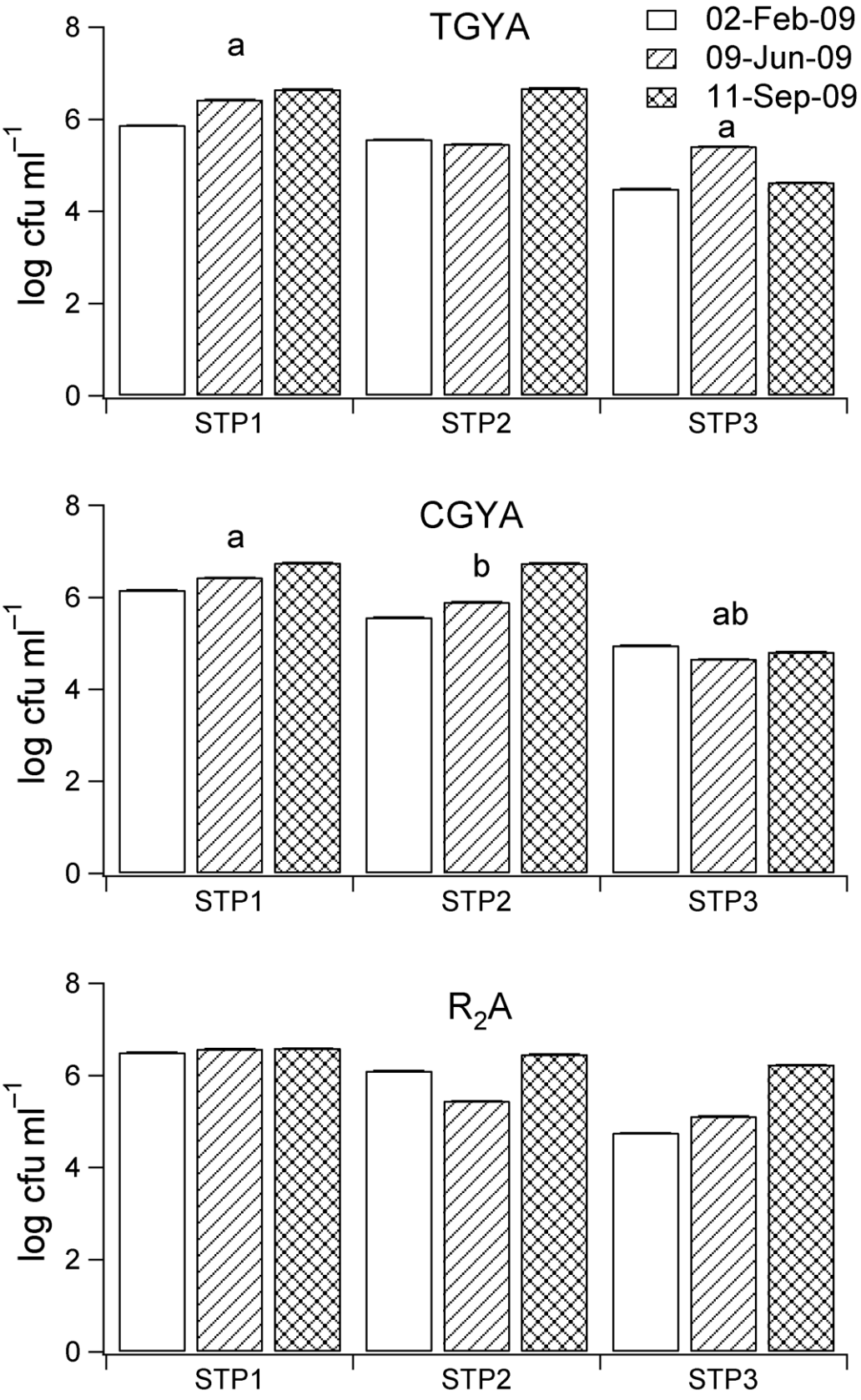

Figure 3.10: Enumeration of log cfu ml^{-1} according to media and STP. The same letters of the alphabet are used to indicate values whose means were significantly different.

On CGYA, the cfu obtained was significantly different among the STPs (F=13.79, df=8, p<0.01). STP3 (6.77±2.25×10^4 cfu ml^{-1}) had the lowest cfu when compared to STP1 (3.29±2.18×10^6 cfu ml^{-1}; q=7.09, df=8, p<0.01) and STP2 (2.26±2.90×10^6 cfu ml^{-1}; q=5.46, df=8, p<0.05). In contrast, cfu counts in R2A media ranged from 5.70×10^4 cfu ml^{-1} to 3.90×10^6 cfu ml^{-1} and did not show any significant difference among the STP.

Bacterial counts at STP1 generally increased with each sampling, especially on TGYA (from 7.50×10^5 cfu ml^{-1} to 4.50×10^6 cfu ml^{-1}) and CGYA (from 1.46×10^6 cfu ml^{-1} to 5.70×10^6 cfu ml^{-1}) media but not on R2A medium. On the other hand, STP2 counts on TGYA were low during the first two samplings but increased to 4.80×10^6 cfu ml^{-1} at the last sampling. STP2 counts on CGYA showed a gradual increase from 3.70×10^5 cfu ml^{-1} to 5.60×10^6 cfu ml^{-1}. However STP2 showed no clear pattern on R2A, fluctuating between 1.28×10^6 cfu ml^{-1} and 2.90×10^6 cfu ml^{-1}. Bacterial counts at STP3 were generally lowest when using TGYA and CGYA media whereas on R2A, there was a slight increase in cfu counts from 5.70×10^5 cfu ml^{-1} to 1.72×10^6 cfu ml^{-1}.

3.5 Bacterial identification

The genomic materials of each unique isolate were extracted using phenol chloroform method. The extracted suspension was then examined using gel electrophoresis (Figure 3.11). The 16S rDNA gene was amplified using PCR and the amplicon was around 1500 bp (Figure 3.12). RFLP analysis was then carried out for dereplication. The PCR product was initially digested by *Rsa*I to generate RFLP profile (Figure 3.13). Samples that exhibit similar RFLP profile in *Rsa*I digestion, were further differentiated with *Cfo*I digestion (Figure 3.14). Isolates with different RFLP profile was considered unique. The amplicons from these isolates were then sent to AITBiotech, Singapore for sequencing. DNA sequencing results were trimmed with

Bioedit Sequence Alignment Editor version 7.0.5.3 (Hall, 1999) before comparison to the National Center for Biotechnology Information (NCBI) database through NCBI nucleotide blast (Altschul *et al.*, 1990).

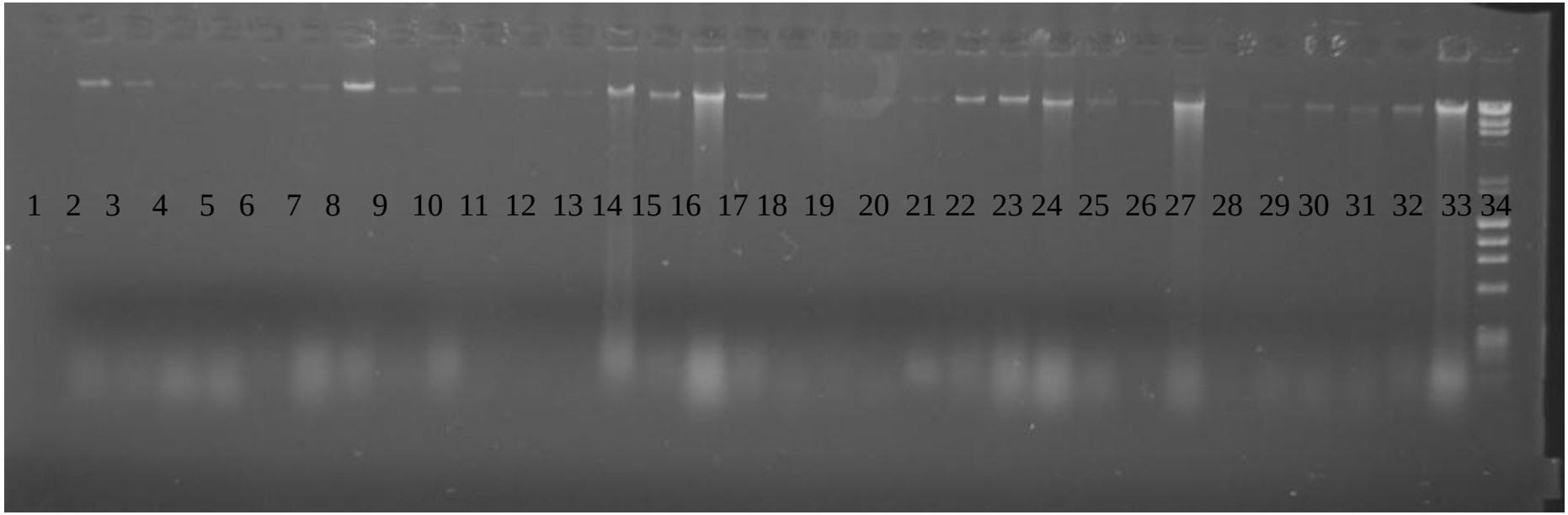

Figure 3.11: A sample of gel electrophoresis photo of extracted DNA. 3 µl of samples were loaded into the wells and the gel (1% w/v) was run at 5.0 V cm^{-1}. Lane 1 is negative control, lane 33 is positive control (*Escherichia coli* strain KA1) and lanes 2 to 32 are samples. Lane 34 was loaded with 3 µl of DNA Marker λ DNA *Hind* III digest and ΦX174 DNA *Hae* III digest (50 ng μl^{-1}). The samples are in the following order: 3R1-6, 3R1-22, 3R1-23, 3R2-2, 3R2-3, 3R2-7, 3R2-9, 3R2-10, 3R2-11, 3R2-12, 3R2-17, 3R3-1, 3R3-2, 3R3-4, 3R3-6, 3T1-1, 3T1-4, 3T1-5, 3T1-12, 3T1-13, 3T1-15, 3T2-12, 3T2-14, 3T2-17, 3T2-23, 3T2-24, 3T3-6, 3T3-7, 3T3-9 and 3T3-10.

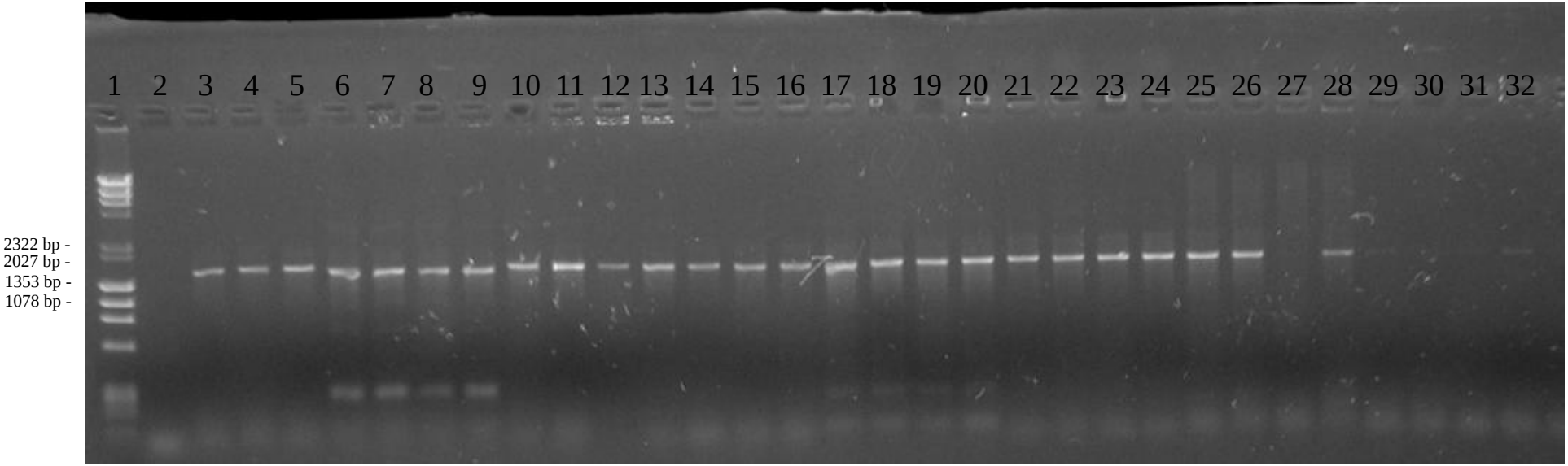

Figure 3.12: A sample of gel electrophoresis photo of sample after 16S rDNA PCR. 3 µl of samples were loaded into the wells and the gel (1% w/v) was run at 5.0 V cm^{-1}. Lane 1 was loaded with 3 µl DNA Marker λ DNA *Hind* III digest and ΦX174 DNA *Hae* III digest (50 ng μl^{-1}). Lane 2 is negative control, lane 3 is positive (*Escherichia coli* strain KA1) control and lanes 4 to 32 are samples. The samples are in the following order: 3R1-6, 3R1-22, 3R1-23, 3R2-2, 3R2-3, 3R2-7, 3R2-9, 3R2-10, 3R2-11, 3R2-12, 3R2-17, 3R3-1, 3R3-2, 3R3-4, 3R3-6, 3T1-1, 3T1-4, 3T1-5, 3T1-12, 3T1-13, 3T1-15, 3T2-12, 3T2-14, 3T2-17, 3T2-23, 3T2-24, 3T3-6, 3T3-7 and 3T3-9.

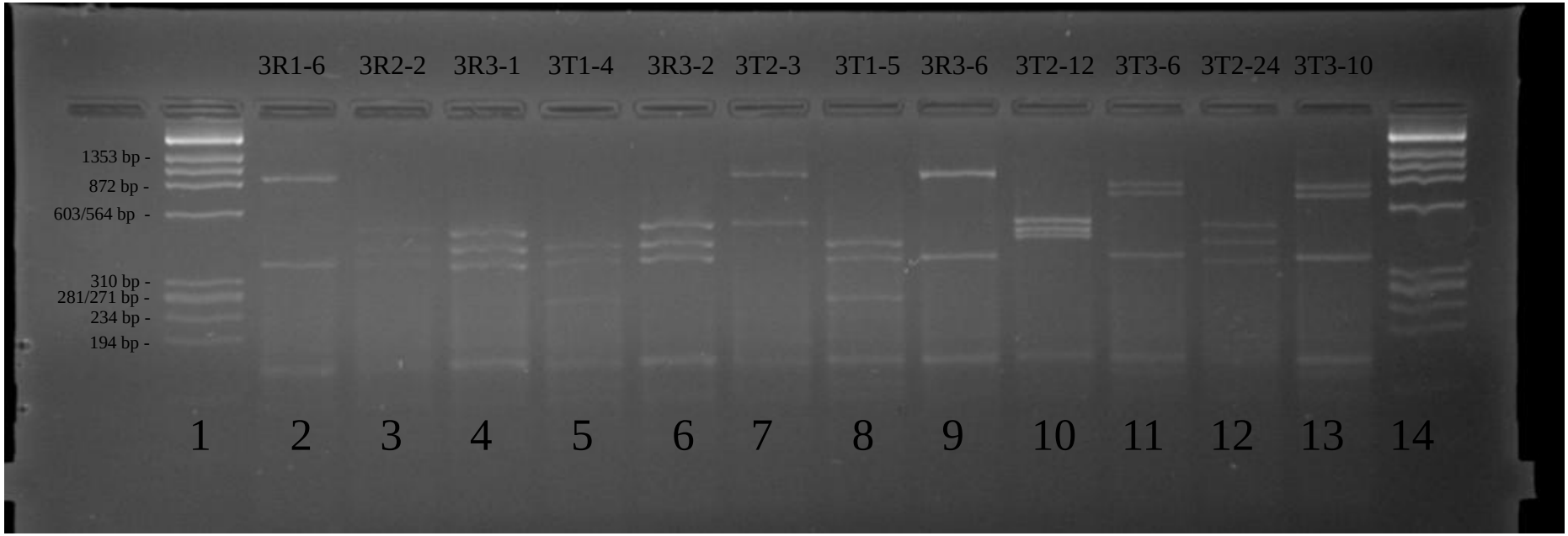

Figure 3.13: A sample of gel electrophoresis photo of sample obtained from RFLP using *Rsa*I. 5 µl of samples were loaded into the wells and the gel (3% w/v) was run at 5.0 V cm^{-1}. Lanes 1 and 14 were loaded with 3 µl molecular markers and lanes 2 to 13 are samples.

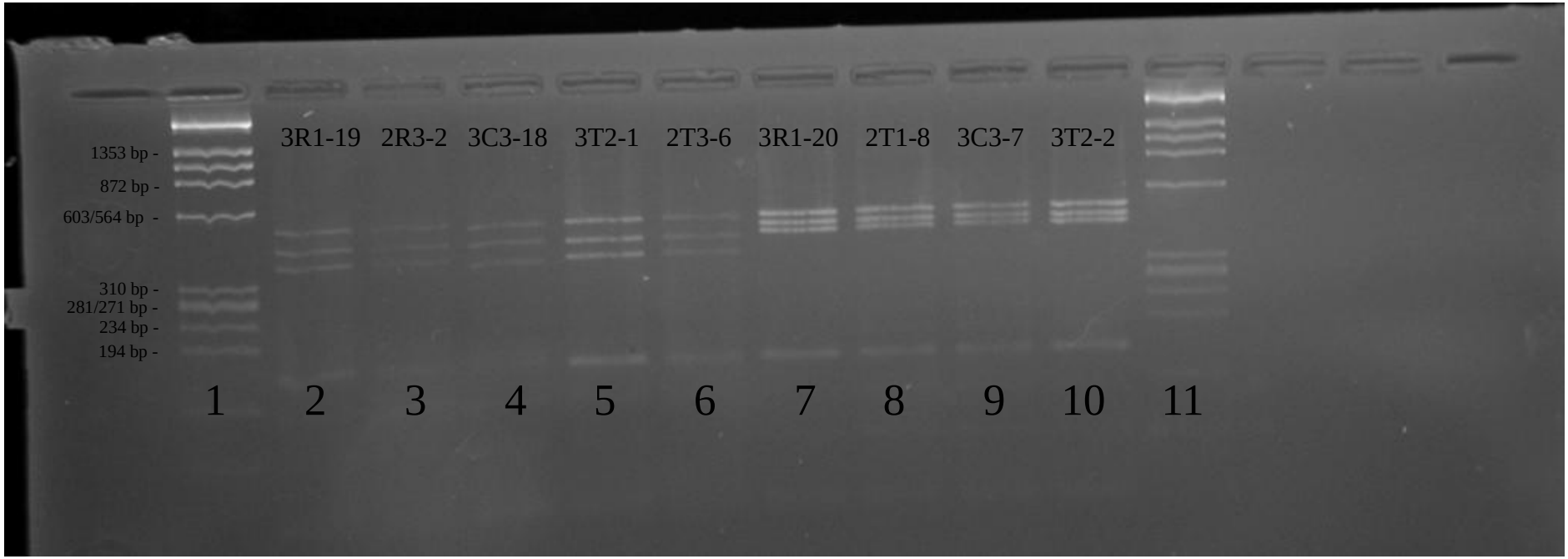

Figure 3.14: A sample of gel electrophoresis photo of sample obtained from RFLP using *Cfo*I. 5 µl of samples were loaded into the wells and the gel (3% w/v) was run at 5.0 V cm^{-1}. Lanes 1 and 11 were loaded with 3 µl molecular markers and lanes 2 to 10 are samples.

The sequence was also compared to the Ribosomal Database Project (RDPII) (Cole *et al.*, 2007) for further confirmation of the sequence identity. The sequence data was submitted to GenBank, and were assigned with accession numbers from GU126797 to GU300153 (Table 3.2).

Among the identified strains, some strains (*n*, %) were commonly found in all media and STPs i.e. *Stenotrophomonas maltophilia* (182, 11.48%), *Aeromonas* genus (138, 8.70%), *Acidovorax* genus (166, 10.47%), *Bacillus* genus (193, 12.17%), *Pseudomonas* genus (125, 7.88%) and *Staphylococcus* genus (98, 6.18%).

In this study, some bacteria were rarely cultivated e.g. *Achromobacter xylosoxidans* (10, 0.63%), *Bosea minatitlanensis* (5, 0.32%), *Brevundimonas diminuta* (6, 0.38%), *Ideonella dechloratans* (3, 0.19%), *Myroides odoratimimus* (1, 0.06%), *Comamonas testosteroni* (15, 0.95%), *Microbacterium deminutum* (1, 0.06%), *Paracoccus aminovorans* (90, 5.67%), *Xanthobacter autotrophicus* (1, 0.06%), *Thermomonas haemolytica* (3, 0.19%), *Zooglea oryzae* (13, 0.82%), *Enterobacter cloacae* (1, 0.06%), *Sphingomonas desiccabilis* (1, 0.06%), *Pandoraea pnomenusa* (3, 0.19), *Lysinibacillus sphaericus* (1, 0.06%) and *Moraxella osloensis* (1, 0.06%).

Some species were also unique to an STP e.g. *Citrobacter werkmanii* (1, 0.06%), *Ideonella dechloratans* (% as mentioned above), *Myroides odoratimimus*, *Klebsiella oxytoca* (2, 0.13%), *Xanthobacter autotrophicus* and *Moraxella osloensis* were found only in STP1. STP2 harboured several unique species such as *Comamonas testosteroni*, *Microbacterium deminutum*, *Diaphorobacter oryzae* (22, 1.39%), *Enterobacter cloacae*, *Sphingomonas desiccabilis*, *Variovorax* sp. and *Caulobacter* sp. whereas the species unique to STP3 were only *Lysinibacillus sphaericus*, and *Brevundimonas mediterranea* (2, 0.13%).

In this study, three types of media were used and some bacteria were found on only one type of media. *Bosea minatitlanensis, Brevundimonas diminuta, Microbacterium deminutum, Myroides odoratimimus, Pantoea agglomerans* (11, 0.69%), *Providencia alcalifaciens* (8, 0.50%), *Providencia vermicola* (19, 1.20%) and *Lysinibacillus sphaericus* (1, 0.06%) were only cultivated in TGYA. In R2A, the unique bacteria cultured was *Diaphorobacter oryzae, Paracoccus* sp. (29, 1.83%), *Achromobacter xylosoxidans, Comamonas testosteroni, Ideonella dechloratans, Pseudacidovorax intermedius* (3, 0.19%), *Sphingomonas desiccabilis* (1, 0.06%), *Thermomonas haemolytica, Xanthobacter autotrophicus* and *Zooglea oryzae*. In contrast, *Chromobacterium violaceum* (4, 0.25%), *Citrobacter werkmanii, Klebsiella oxytoca, Caulobacter* sp. (1, 0.06%), *Moraxella osloensis,* and *Variovorax* sp. (2, 0.13%) were exclusive to CGYA.

From plates with cfu between 25 to 250, we estimated the diversity for the culturable bacteria using the Shannon diversity index (Table 3.3). Shannon diversity indices were calculated for each media and STP separately. For R2A, the diversity index was 1.66, and was lower than TGYA and CGYA (1.90 and 1.80, respectively). Comparison among STPs showed that the diversity index for STP3 (1.59) was the lowest when compared to STP1 (1.87) and STP2 (1.91).

3.6 Phylogenetic trees and Cluster analysis

Phylogenetic trees were generated using the identified isolates from different STP (Figures 3.15, 3.16 and 3.17). The bacteria isolated in this study can be grouped into six classes of bacteria i.e. α-Proteobacteria, β-Proteobacteria, γ-Proteobacteria, Actinobacteria, Bacilli (Low GC) and Flavobacteria. Cluster analysis was also done to investigate the bacteria cultivated in different media (Figure 3.18)

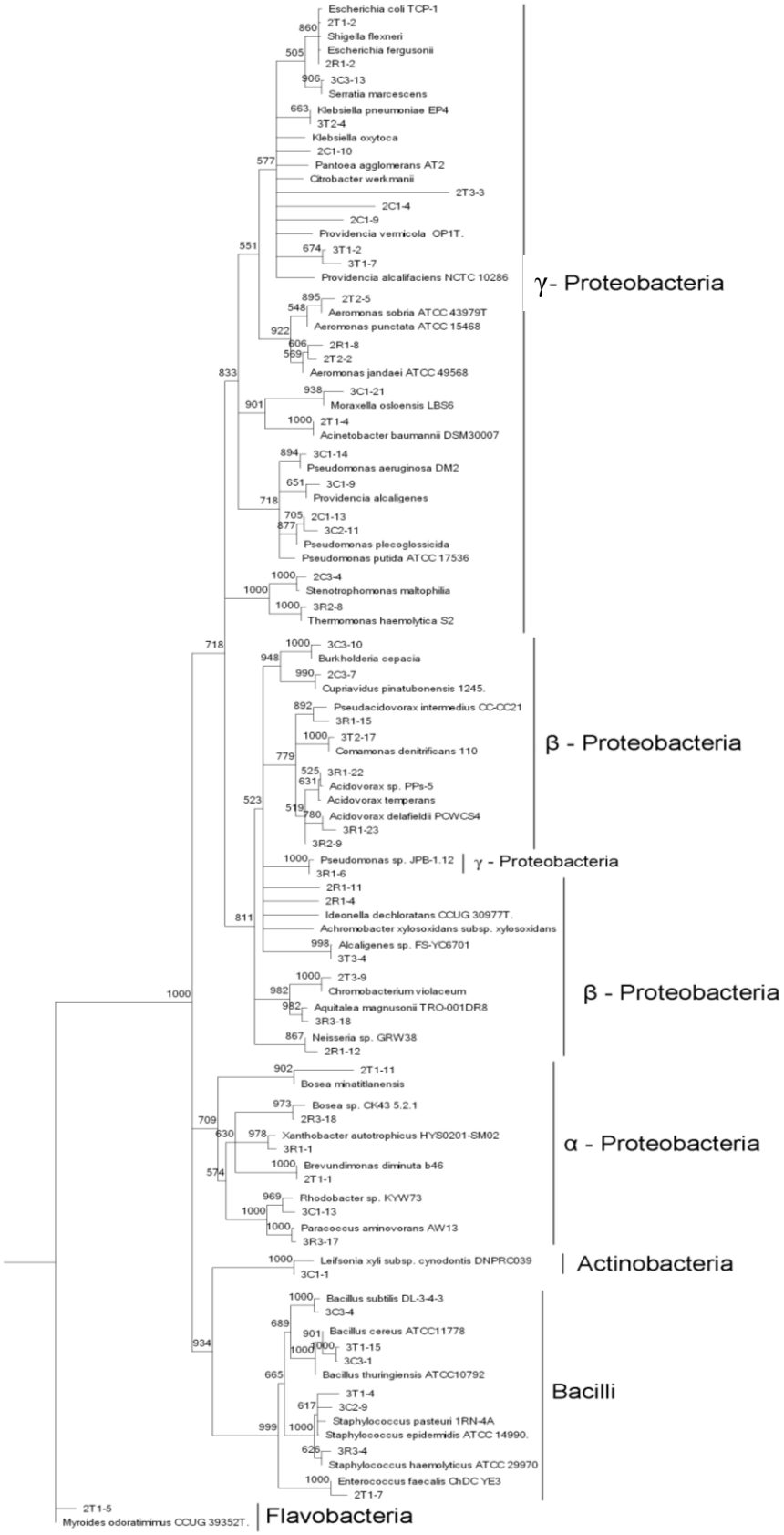

Figure 3.15: Neighbour-joining tree showing the phylogenetic relationship based on partial sequence of 16s rDNA derived from bacteria isolated from STP1. Bootstrap values (1000 replicates) >50% are shown on each branch.

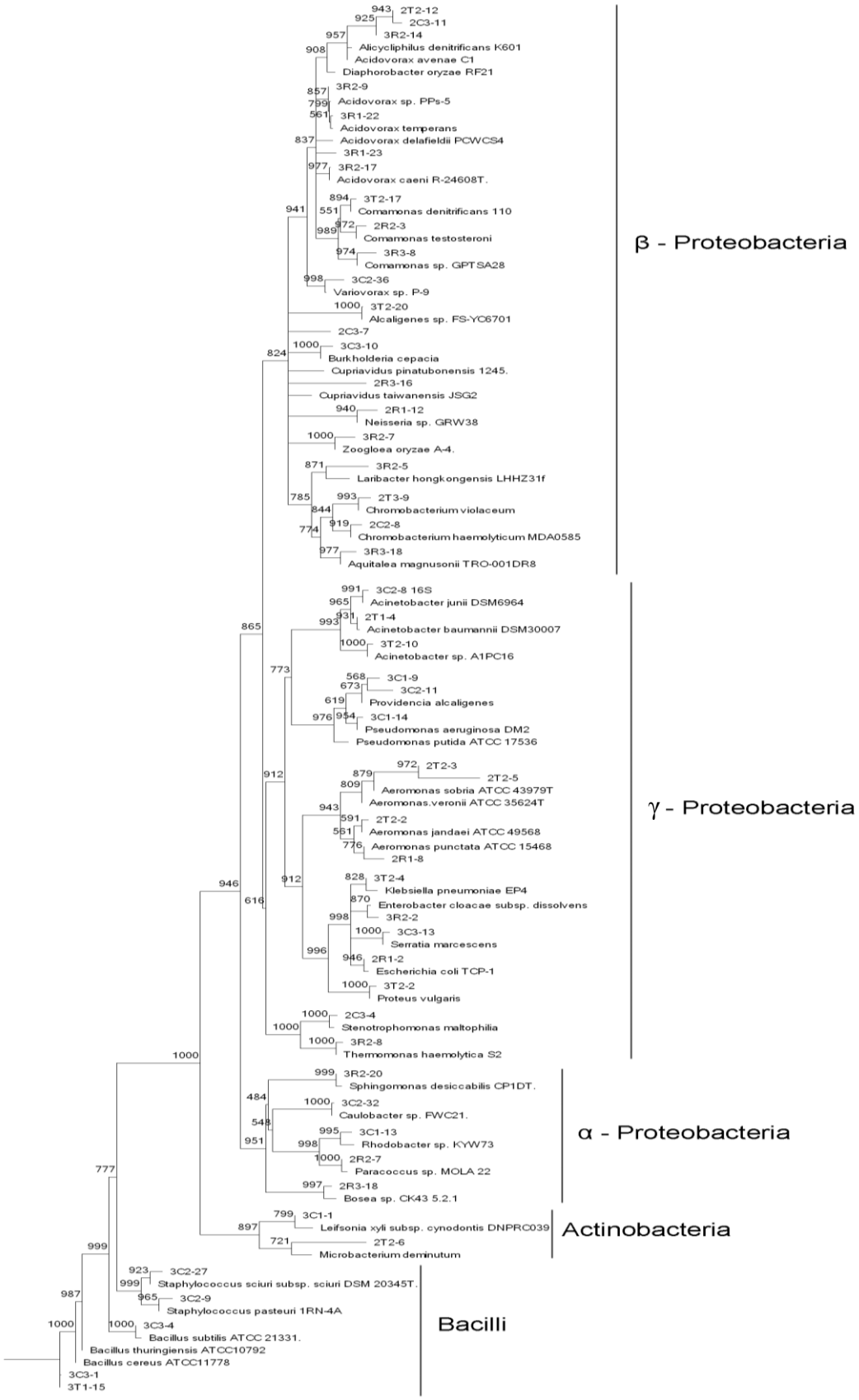

Figure 3.16: Neighbour-joining tree showing the phylogenetic relationship based on partial sequence of 16s rDNA derived from bacteria isolated from STP2. Bootstrap values (1000 replicates) >50% are shown on each branch.

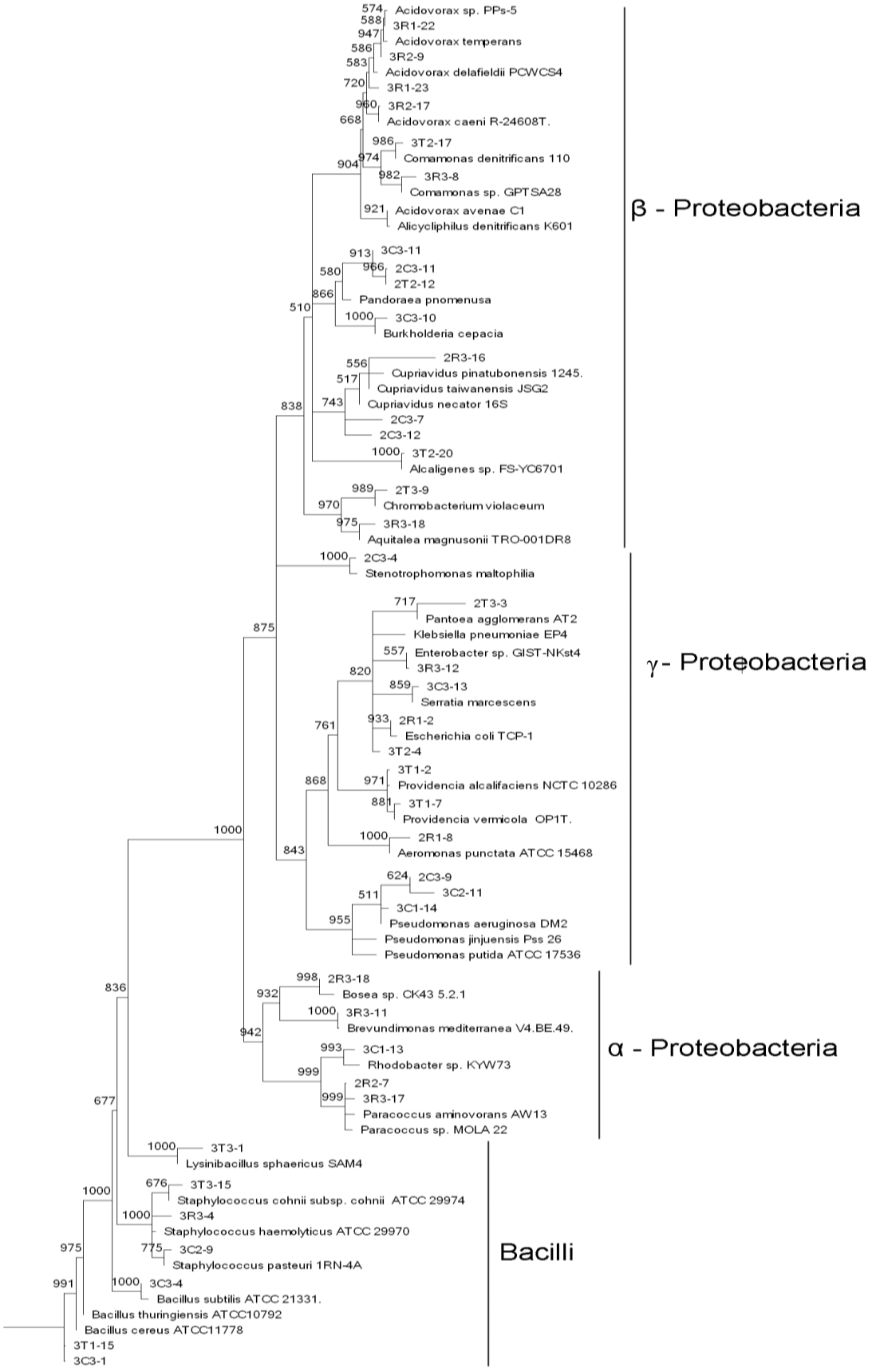

Figure 3.17: Neighbour-joining tree showing the phylogenetic relationship based on partial sequence of 16s rDNA derived from bacteria isolated from STP3. Bootstrap values (1000 replicates) >50% are shown on each branch.

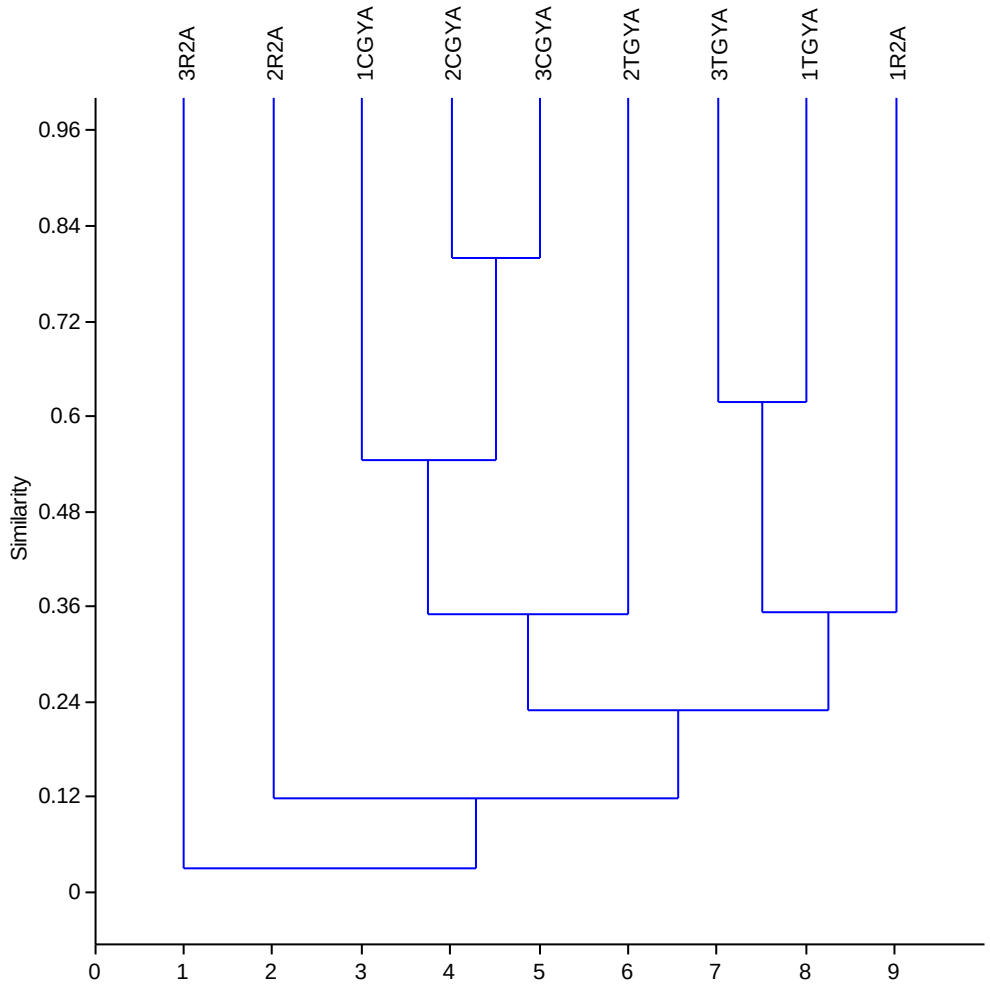

Figure 3.18: Dendrogram of similarity values between media used in each sampling. The cluster analysis was based on the similarity matrix calculated using the Morishita coefficient. The labeling represents sampling and media used.

Table 3.2: Identity of the isolates obtained in this study and their accession codes.

Accession code	Clone	Homolog	Homolog accession	Percentage Similarity, %
GU126797	2T2-11	*Bacillus thuringiensis*	AF290545	100
GU126798	2C2-6	*Acidovorax temperans*	AF078766	99
GU126799	2T1-1	*Brevundimonas diminuta*	X87274	100
GU126800	3C2-11	*Pseudomonas putida*	AY647158	100
GU126801	2R3-17	*Stenotrophomonas maltophilia*	AB194322	100
GU126802	3C3-1	*Bacillus thuringiensis*	AF290545	100
GU168980	2C3-2	*Cupriavidus taiwanensis*	AF300324	99
GU168981	2C3-6	*Bacillus cereus*	AJ577288	99
GU168982	2C3-7	*Cupriavidus pinatubonensis*	AB121221	99
GU168983	2C3-9	*Pseudomonas jinjuensis*	AF468448	98
GU168984	2C3-10	*Bacillus thuringiensis*	AF290549	99
GU168985	2C3-11	*Acidovorax avenae*	AF508114	99
GU168986	2C3-12	*Cupriavidus necator*	AM260479	98
GU168987	2C3-13	*Acidovorax avenae*	AF508114	100
GU168988	2C3-14	*Acidovorax* sp.	AM990765	99
GU168989	2C3-15	*Bacillus thuringiensis*	AF290545	100
GU168990	2R1-4	*Ideonella dechloratans*	NR_026108	98
GU168991	3R1-15	*Pseudoacidovorax intermedius*	EF469609	96
GU168992	2R1-8	*Aeromonas punctata*	X74674	99
GU168993	2R1-10	*Stenotrophomonas maltophilia*	AB008509	99
GU168994	2R1-12	*Neisseria* sp.	FJ502346	100
GU168995	3C3-10	*Burkholderia cepacia*	AY741344	100

Table 3.2, continued

Accession code	Clone	Homolog	Homolog accession	Percentage Similarity, %
GU168996	2R1-11	*Achromobacter xylosoxidans*	AB547225	100
GU168997	2R2-2	*Acidovorax temperans*	AF078766	99
GU168998	2R2-4	*Acidovorax temperans*	AF078766	100
GU168999	2R2-3	*Comamonas testosteroni*	GU296675	99
GU169000	2R2-7	*Comamonas testosteroni*	GU296675	99
GU169001	3C3-13	*Paracoccus* sp.	aM990798	99
GU169002	3T2-6	*Acidovorax* sp.	AM990764	100
GU169003	2T1-5	*Myroides odoratimimus*	AJ854059	99
GU169004	2R3-1	*Klebsiella pneumoniae*	GU373625	100
GU169005	2R3-3	*Cupriavidus taiwanensis*	EF114432	99
GU169006	2R3-5	*Acidovorax caeni*	AM084006	99
GU169007	2R3-7	*Acidovorax avenae*	FJ982928	99
GU169008	2R3-13	*Acidovorax temperans*	AF078766	99
GU169009	2R3-9	*Stenotrophomonas maltophilia*	AB008509	100
GU169010	2R3-14	*Cupriavidus taiwanensis*	EF114432	99
GU169011	2R3-15	*Alicycliphilus denitrificans*	AJ418042	99
GU169012	2R3-16	*Cupriavidus taiwanensis*	EF446928	99
GU169013	2R3-17	*Stenotrophomonas maltophilia*	AB008509	100
GU169014	2R3-18	*Bosea* sp.	FJ688405	95
GU169015	2R3-20	*Chromobacterium violaceum*	AE016825	100
GU169016	3R3-4	*Staphylococcus haemolyticus*	EU867334	99
GU169017	2C1-7	*Acidovorax delafieldii*	AF078764	99

Table 3.2, continued

Accession code	Clone	Homolog	Homolog accession	Percentage Similarity, %
GU169018	2C1-10	*Klebsiella oxytoca*	AB353045	99
GU169019	2C1-12	*Chromobacterium violaceum*	M22510	100
GU169020	2C1-13	*Pseudomonas plecoglossicida*	AB009457	99
GU169021	2R1-2	*Escherichia coli*	FJ823386	99
GU169022	2T2-6	*Microbacterium deminutum*	AB234026	100
GU169023	2T3-6	*Bacillus thuringiensis*	AF290545	99
GU169024	3R3-19	*Bacillus cereus*	AM419184	98
GU169025	3T2-4	*Klebsiella pneumonia*	FJ608656	99
GU169026	3T3-16	*Staphylococcus pasteuri*	AJ717376	97
GU169027	2C1-2	*Shigella flexneri*	X96963	100
GU169028	2C1-3	*Aquitalea magnusonii*	DQ018117	99
GU169029	2C1-4	*Escherichia fergusonii*	AF530475	100
GU169030	2C1-6	*Escherichia fergusonii*	AF530475	99
GU169031	2C1-8	*Bacillus thuringiensis*	AF290545	99
GU169032	2C1-9	*Citrobacter werkmanii*	AF025373	99
GU169033	2C3-4	*Stenotrophomonas maltophilia*	AB008509	99
GU169034	2R3-11	*Stenotrophomonas maltophilia*	AB008509	99
GU169035	2T1-11	*Bosea minatitlanensis*	AF273081	99
GU169036	2T1-2	*Shigella flexneri*	X96963	99
GU169037	2T1-3	*Bosea minatitlanensis*	AF273081	99
GU169038	2T1-4	*Acinetobacter baumanii*	X81660	100
GU169039	2T1-7	*Enterococcus faecalis*	AY942559	99

Table 3.2, continued

Accession code	Clone	Homolog	Homolog accession	Percentage Similarity, %
GU169040	2T2-12	*Alicycliphilus denitrificans*	NR_025510	99
GU169041	2T2-13	*Chromobacterium violaceum*	M22510	100
GU169042	2T2-15	*Aeromonas jandaei*	X74678	99
GU169043	2T2-1	*Bacillus thuringiensis*	AF290545	99
GU169044	2T2-2	*Aeromonas jandaei*	X74678	100
GU169045	2T2-3	*Aeromonas veronii*	X74684	99
GU169046	2T2-5	*Aeromonas sobria*	X74683	99
GU169047	2T2-8	*Acidovorax temperans*	AF078766	99
GU169048	2T3-3	*Pantoea agglomerans*	EF446899	100
GU169049	2T3-9	*Chromobacterium violaceum*	M22510	100
GU195125	3T1-1	*Staphylococcus pasteuri*	AJ717376	99
GU195126	3T1-2	*Providencia alcalifaciens*	DQ885261	99
GU195127	3T1-4	*Staphylococcus epidermidis*	D83363	99
GU195128	3T1-7	*Providencia vermicola*	AM040495	99
GU195129	3T1-12	*Aeromonas punctata*	X74674	99
GU195130	3T1-13	*Bacillus subtilis*	GQ199593	99
GU195131	3T1-14	*Aeromonas punctata*	X74674	100
GU195132	3T1-15	*Bacillus cereus*	AF290546	99
GU195133	3T1-17	*Chromobacterium haemolyticum*	DQ785104	99
GU195134	3T2-2	*Proteus vulgaris*	DQ499636	99
GU195135	3T2-3	*Acinetobacter* sp.	FN298236	100
GU195136	3T2-9	*Chromabacterium haemolyticum*	DQ785104.1	99

Table 3.2, continued

Accession code	Clone	Homolog	Homolog accession	Percentage Similarity, %
GU195137	3T2-10	*Acinetobacter* sp.	FN298236	100
GU195138	3T2-12	*Aeromonas punctata*	X74674	99
GU195139	3T2-14	*Acidovorax delafieldii.*	EU730925	99
GU195140	3T2-17	*Comamonas denitrificans*	AF233876	99
GU195141	3T2-20	*Alcaligenes* sp.	EU734659.1	100
GU195142	3T2-24	*Klebsiella pneumoniae*	FJ608656	100
GU195143	3T3-1	*Lysinibacillus sphaericus*	GQ202135	99
GU195144	3T3-2	*Alcaligenes* sp.	EU734659.1	100
GU195145	3T3-4	*Alcaligenes* sp.	AY346138	100
GU195146	3T3-6	*Bacillus cereus*	DQ298080	99
GU195147	3T3-9	*Burkholderia cepacia*	AY741344	100
GU195148	3T3-10	*Bacillus thuringiensis*	AF290545	99
GU195149	3T3-13	*Bacillus subtilis*	AB018487	100
GU195150	3T3-15	*Staphylococcus cohnii*	D83361	99
GU195151	3C1-1	*Leifsonia xyli subsp. Cynodontis*	DQ232615	99
GU195152	3C1-9	*Pseudomonas alcaligenes*	Z76653	99
GU195153	3C1-13	*Rhodobacter* sp.	FJ997595	96
GU195154	3C1-15	*Acidovorax delafieldii*	EU730925	99
GU195155	3C1-18	*Bosea* sp.	AJ313022	99
GU195156	3C1-20	*Stenotrophomonas maltophilia*	EU652101.1	100
GU195157	3C1-21	*Moraxella osloensis*	EU400648.1	99
GU195158	3C1-26	*Chromobacterium violaceum*	AE016825	100

Table 3.2, continued

Accession code	Clone	Homolog	Homolog accession	Percentage Similarity, %
GU195159	3C2-1	*Pseudomonas aeruginosa*	AY486350	100
GU195160	2C2-5	*Stenotrophomonas maltophilia*	EU244712.1	100
GU195161	3C1-14	*Pseudomonas aeruginosa*	GQ926937	100
GU195162	3C1-19	*Escherichia coli*	AB498890	99
GU195163	3C2-8	*Acinetobacter junii*	X81664	99
GU195164	3C2-9	*Staphylococcus pasteuri*	EU379258	100
GU195165	3C2-21	*Pseudomonas alcaligenes*	Z76653	99
GU195166	3C2-27	*Staphylococcus sciuri*	AJ421446	99
GU195167	3C2-32	*Caulobacter* sp.	AJ227767	100
GU195168	3C2-36	*Variovorax* sp.	AM411932	98
GU195169	3C3-4	*Bacillus subtilis*	FJ859701.1	99
GU195170	3C3-6	*Klebsiella pneumoniae*	DQ470487.1	100
GU195171	3C3-11	*Pandoraea pnomenusa*	AY2680170	100
GU195172	3C3-12	*Burkholderia cepacia*	AY741322	99
GU195173	3R1-1	*Xanthobacter autotrophicus*	CP000781	97
GU195174	3R1-6	*Pseudomonas* sp.	EU652471.1	99
GU195175	3R1-22	*Acidovorax temperans,*	AF078766	99
GU195176	3R1-23	*Acidovorax delafieldii*	GQ284437.1	98
GU195177	3R2-2	*Enterobacter cloacae*	DQ988523	98
GU195178	3R2-3	*Bacillus thuringiensis*	FJ772082.1	99
GU195179	3R2-7	*Zooglea oryzae*	AB201044	99
GU195180	3R2-9	*Acidovorax* sp.	FJ605421	100

Table 3.2, continued

Accession code	Clone	Homolog	Homolog accession	Percentage Similarity, %
GU195181	3R2-12	*Acidovorax avenae*	AF508114	98
GU195182	3R2-17	*Acidovorax caeni*	AM084006	99
GU195183	3R2-20	*Sphingomonas desiccabilis*	AJ871435	100
GU195184	3R2-21	*Escherichia coli*	CU928160.2	100
GU195185	3R3-8	*Comamonas* sp.	DQ851179	99
GU195186	3R3-9	*Comamonas denitrificans*	AF233876	97
GU195187	3R3-11	*Brevundimonas mediterranea*	AJ244709.1	100
GU195188	3R3-12	*Enterobacter* sp.	EF489446	95
GU195189	3R3-18	*Aquitalea magnusonii*	DQ018117	99
GU195190	3R2-18	*Comamonas denitrificans*	AF233876	97
GU195191	3R2-8	*Thermomonas haemolytica*	AF508107	100
GU300146	2C2-2	*Chromobacterium violaceum*	M22510	98
GU300147	2C2-7	*Acidovorax avenae*	NR_025510	100
GU300148	2C2-8	*Chromobacterium haemolyticum*	DQ785104	99
GU300149	3C3-3	*Bacillus subtilis*	EU722405	100
GU300150	3R2-10	*Paracoccus* sp.	GU169000.1	96
GU300151	3R2-5	*Laribacter hongkongensis*	EU421144.1	93
GU300152	3R2-14	*Diaphorobacter oryzae*	EU342380	98
GU300153	3R3-17	*Paracoccus aminovorans*	FJ172042	99

Table 3.3: Shannon Diversity Index of heterotrophic bacteria cultured from different media in different STP.

		TGYA	CGYA	R2A	Average by STP
STP1	Feb	2.27	1.43	1.17	
	Jun	2.07	1.78	2.07	
	Sep	1.59	2.19	2.27	
	Average	1.97	1.80	1.83	1.87
STP2	Feb	2.10	1.91	1.76	
	Jun	1.98	1.04	1.12	
	Sep	2.20	2.50	2.56	
	Average	2.09	1.81	1.81	1.91
STP3	Feb	1.44	1.37	1.55	
	Jun	1.28	2.11	1.03	
	Sep	2.19	1.87	1.45	
	Average	1.64	1.79	1.34	1.59
Average by media		1.90	1.80	1.66	

DISCUSSION

4.1 Physiochemical parameters

Activated sludge is a wastewater treatment method that utilizes bacteria to oxidize the organic compound in the wastewater. As bacteria are involved in the process, pH and temperature are two important factors. Being a tropical country, temperatures ranged from 20°C to 30°C throughout the year which are adequate for standard aerobic system (Sperling, 2007). In our study, the temperature recorded averaged at 30°C and did not fluctuate much among the readings. On the other hand, pH was not static. The optimum pH range for activated sludge system to maintain its bacterial community is pH 6 – 8 and the optimum range for carbonaceous oxidation is between pH 6.5 – 8.5 (Gray, 2004). The pH readings obtained at STP1 and STP2 were within the optimum pH range except for one sampling with pH 5. However, STP3 showed a persistently acidic condition where the pH in aeration tank was below pH 5.0 in all three sampling. This could be due to the light industrial waste channeled into STP3 where the pH for the influent was below pH 6.0.

4.2 Influent characteristic

In this study, influents COD ranged from 320 mg l^{-1} to 680 mg l^{-1} whereas influent BOD ranged from 200 mg l^{-1} to 500 mg l^{-1}. The sewage for STP1 and STP2 was fairly degradable and can be effectively treated biologically as suggested by a BOD: COD ratio of >0.6 whereas STP3 with a BOD: COD ratio of between 0.3 and 0.6 require seeding for biological treatment (Srinivas, 2008). If the ratio drops below 0.3, it is considered untreatable biologically.

Ammonia concentration at the STPs was under 50 mg l^{-1}. Phosphate level was higher at STP2 compared to other STPs. STP2 received sewage from a commercial area which consists of diners and hawker centers. These activities usually use large amounts of synthetic detergents and cleaning products that may contain phosphate thus elevating the raw sewage phosphate concentration (Seldak, 1991). STP1 manage waste from a residential area where detergents are also used but at a lesser volume than STP2. STP3 treated wastewater from a light industrial area such as auto servicing. The influent nitrite and nitrate concentration of all STPs were <0.4 mg l^{-1} for nitrite and <1.6 mg l^{-1} for nitrate, and are negligible except for one occasion at STP2 when the nitrate concentration for influent was slightly higher at 9.62 mg l^{-1}. Nitrite and nitrate are normally absent in raw sewage (Table 4.1) (Gray, 2004; Ministry of Housing and Local Government, 1998). Periodic increase of nitrate concentration has been attributed to fertilizer run off from land (Haughton and Hunter, 2003), but we did not investigate the source of nitrate elevation here.

4.3 Health of activated sludge in aeration tank

4.3.1 Dissolved oxygen

In order to facilitate microbial growth and oxidation of organic matter, oxygen must be present and dissolved oxygen represents oxygen level in the aeration tank. The minimum dissolved oxygen level that allows a proper development of biological sludge is 1−2 mg l^{-1} whereas inhibition of nitrification is observed at dissolved oxygen <1.0 mg l^{-1} (Gray, 2004; Haandel and Lubbe, 2007). STP1 showed a healthy dissolved oxygen level of above 1 mg l^{-1} except for the first reading when the sludge volume (SV) was too high for a reliable measurement of dissolved oxygen. Dissolved oxygen was low at STP2 due to the malfunction of the aerator during the first sampling. This caused the low SV, MLSS and MLVSS at STP2 at the first sampling. As the dissolved

oxygen level increased with subsequent sampling, these parameters also improved. This suggested that the problem at STP2 was due to insufficient dissolved oxygen level. STP3 also showed insufficient dissolved oxygen level in the first two samplings. The aerators in the STP3 were also found to function irregularly, and might have caused the poor condition at STP3 where the MLSS and MLVSS was lower than desirable in the first two samplings. Correlation analysis for all three stations showed that dissolved oxygen was significantly correlated with MLVSS (R^2=0.667, df=6, p<0.05), and suggested that dissolved oxygen was important for good floc formation.

4.3.2 Settleability of sludge

Sludge Volume Index (SVI) or Diluted Sludge Volume Index (DSVI) is calculated from SV to indicate the settleability of the sludge in mixed liquor (Gerardi, 2002). DSVI is calculated for mixed liquor samples with MLSS above 4000 mg l^{-1}. Good settling solids give a SVI/DSVI between 50 – 80 ml g^{-1} whereas a SVI/DSVI above 150 mg l^{-1} will give poor settling solids (Gray, 2005). In this study, STP1 has an acceptable sludge settleability that averaged 119.2±6.1 ml g^{-1} whereas STP3 had low SVI (26.9±15.6 ml g^{-1}) and a low amount of SV that indicated poor floc formation. STP2 showed an average SVI of 85.4±59.4 ml g^{-1} where the setteability was poor with SVI above 153.8 ml g^{-1} in the first sampling. The second and third sampling for STP2 showed lower SVI at 47.2 ml g^{-1} and 55.1 ml g^{-1} indicating improved sludge settleability.

4.3.3 Mixed Liquor Suspended Solids (MLSS) and Mixed Liquor Volatile Suspended Solid (MLVSS)

The MLSS and MLVSS are used to measure the concentration of microorganisms in the mixed liquor. The range of a healthy MLSS concentration in an extended aeration tank is 3000 to 5000 mg l^{-1} (Eckenfelder and Grau, 1998). In general,

STP1 had good MLSS concentration that averaged 5100±1250 mg l^{-1}. The MLSS concentration for the first sampling at STP1 was high at 6340 mg l^{-1} which resulted in a high SV and low settleability. STP2 and STP3 on the other hand have lower MLSS which averaged 2200±1850 mg l^{-1} and 2000±1100 mg l^{-1}, respectively. STP2 had extremely low MLSS on the first sampling (390 mg l^{-1}) that was probably caused by low dissolved oxygen. At STP2, subsequent MLSS increased with each sampling and increased with increasing dissolved oxygen. STP3 also suffered from low MLSS in the first two samplings at 1660 mg l^{-1} and 1060 mg l^{-1} and increased to 3216 mg l^{-1} similar with dissolved oxygen readings. The condition at STP3 might also be caused by low pH as activated sludge with low pH often faces problems such as pin floc and high effluent turbidity (Richard, 2003) which explained the low SV (<100 ml l^{-1}).

4.3.4 Alkalinity

Alkalinity serves as a buffer in the aeration tank to neutralize the acidity from the influent and nitrification process (Eckenfelder and Grau, 1998; Spellman, 2008). pH can fluctuate rapidly if the alkalinity is below 1600 µeq l^{-1}. In our study, STP1 had good alkalinity concentration above 1600 µeq l^{-1} except for second sampling. The STP2 and STP3 both had lower alkalinity that might cause the fluctuation of the pH especially for STP3 with <pH 6. The alkalinity at STP1 and STP2 during the second sampling plummeted before recovering. Although not investigated, this could be due to a sudden addition of acidic component into the tank (Spotte, 1992).

As a summary, the variables measured at STP1 suggested that it had the healthiest activated sludge among the STPs with consistent dissolved oxygen level, good sludge settleability, desirable MLSS and MLVSS and acceptable alkalinity level. STP2 on the other hand was disrupted by the inconsistent dissolved oxygen that probably caused the instability of the activated sludge in terms of SV, MLSS and

MLVSS. As dissolved oxygen increased with subsequent samlping, the condition of STP2 improved. STP3 encountered the most problem where dissolved oxygen disruption affected the MLSS and MLVSS concentration and poor floc formation that could have been caused by its acidic condition and low alkalinity.

4.4 Effluent and STP efficiency

BOD removal in STP1 was above 84% and STP1 was able to produce effluent BOD of 38±35 mg l^{-1}. The average BOD removal efficiency for an extended aeration system is 75−90% and STP1 removal efficiency was within acceptable range (Wang *et al*., 2008). STP2 and STP3 on the other hand produced effluent which exceeded the standard B level, with BOD loads of 161±90 mg l^{-1} and 175±48 mg l^{-1}, respectively. Both STP2 and STP3 suffered from low BOD reduction rate (<60%) except for the second sampling at STP2 where the BOD reduction rate was 81%.

COD reduction efficiency in all STPs was under par. The average COD removal efficiency for activated sludge process is 73−80% (Wang *et al.,* 2008) but in this study, the average COD removal efficiency for all STP was below 65% except for the first sampling where COD removal efficiency was above 70%. The average COD discharge by STP1 was 144±123 mg l^{-1}. However, STP2 and STP3 both produced COD loads higher than the standard, at 379±220 mg l^{-1} and 227±84 mg l^{-1}, respectively.

Monitoring ammonia concentration in the effluent is important in wastewater treatment as free ammonia is toxic to aquatic organism. Discharge of excessive ammonia in water could cause eutrophication and a potential health hazard when consumed (Sedlak, 1991). Removal of ammonia is facilitated by nitrification and assimilation by microorganism. Nitrification is a microbiological process that converts ammonium into nitrite and eventually to nitrate (Henze, 2002). The increase of nitrate in

the effluent suggested nitrification process. Effluent nitrate at STP1 increased by 47 mg l^{-1} and ammonia decreased by 40 mg l^{-1} whereas nitrate at STP2 increased by 29 mg l^{-1} whereas ammonia decreased by 21 mg l^{-1}. At STP3, nitrate increased by 43 mg l^{-1} whereas ammonia decreased by 42 mg l^{-1}. There was no significant increase (<4 mg l^{-1}) in nitrite concentration among the STPs. The percentage of ammonia removal at STP1 and STP3 were 97.8% and 94.7%, respectively. The average ammonia discharge loads for STP1 and STP3 were low at 0.89±1.0 mg l^{-1} and 2.4±0.4 mg l^{-1}. STP2 showed poor ammonia removal efficiency of 57.7% in the first two sampling and 17.7% in the third sampling, producing effluent with an average load of 26.6±9.4 mg l^{-1}. Low dissolved oxygen concentration at STP2 might have inhibited nitrification. Phosphate level reduction was only observed at STP3 with an average reduction of 65.9% whereas for STP1 reduction (7.6%) was minimal. A slight reduction of phosphate was observed at STP2 in the first two sampling at an average of 22% but phosphate concentration increased on the last sampling by 42% to 9.14 mg l^{-1}. However, there is no standard set for phosphate concentration for sewage discharge in Malaysia. Although ammonia was removed from the STPs, effluent nitrate concentration increased. The three STPs studied only employed a basic standard of preliminary, primary and secondary treatment, and did not include tertiary treatment for nutrient removal.

Table 4.1: Typical composition of Untreated Domestic waste.

No.	Parameter	Concentration(mg l^{-1})		
		Strong	**Medium**	**Weak**
1	Solids, Total	1,200	720	350
	Dissolved, Total	850	500	250
2	Fixed	525	300	145
	Volatile	325	200	105
	Suspended, total	350	220	100
3	Fixed	75	55	20
	Volatile	275	165	80
4	Settleable solids, ml l^{-1}	20*	10*	5*
5	Biochemical oxygen demand	400	250	110
6	Total organic carbon (TOC)	290	160	80
7	Chemical Oxygen Demand	1000	500	250
8	Nitrogen (total as N)	85	40	20
9	Organic	35	15	8
10	Free ammonia	50	25	12
11	Nitrites	0	0	0
12	Nitrates	0	0	0
13	Phosphorus (total as P)	15	8	4
14	Organic	5	3	1
15	Inorganic	10	5	3
16	Chlorides	100	50	30
17	Alkalinity (as CaCO3)	200	100	50
18	Grease	150	100	50

*All values except settleable solids are expressed in mg l^{-1}.

(Ministry of Housing and Local Government, 1998)

STP1 maintained excellent TSS level at 40±9 mg l^{-1} whereas STP2 and STP3 were higher. Apart from the dissolved oxygen disruption at STP2 that might cause the poor effluent TSS, it also had a malfunctioned scraper in the clarifier tank. Without the scraper, the sludge was not removed properly and caused the sludge blanket to spill into the effluent of STP2 (Jenkins *et al.*, 2004). STP3 suffered from low pH condition and developed a pin floc problem that caused high TSS in effluent (Richard, 2003).

4.5 Enumeration and characterization of heterotrophic bacteria

As suggested by the MLSS and MLVSS in this study, STP1 had higher cfu counts with an average of $3.19\pm1.21\times10^{6}$ cfu ml^{-1} as compared to STP2 ($1.85\pm2.00\times10^{6}$ cfu ml^{-1}) and STP3 ($2.72\pm5.40\times10^{5}$ cfu ml^{-1}). Khan and Kamal (2001) reported heterotrophic bacterial count that ranged from 5.5×10^{4} cfu ml^{-1} to 2.7×10^{6} cfu ml^{-1} in a wastewater treatment plant in Bangladesh whereas in Malaysia, Jalal *et al.* (2006) obtained $7-8\times10^{4}$ cfu ml^{-1} from several aeration tanks of wastewater treatment plants.

In this study, Shannon Diversity Index indicated the microbial population cultivated on R2A had lower diversity than TGYA and CGYA. There are always problems in finding a suitable media to culture bacteria in activated sludge. CGYA was selected here for its optimal count in cultivating aerobic heterotrophic bacteria (Gray, 2004) whereas TGYA is known to be a universal medium for isolating individual bacteria (McKinney, 2004). R2A is a low nutrient medium for cultivating bacteria that do not grow on rich nutrient medium and can increase the chance of cultivating novel bacteria (Bartram, 2003).

We found that bacterial diversity on the TGYA media was the highest among the three media but R2A was the media with higher average count. Ten strains were only found on R2A. When the bacterial diversity among the STP was compared, STP3 had

the lowest bacterial diversity compared to STP1 and STP2. This corroborated with the poor health of the activated sludge in STP3.

The phylogenetic tree generated from isolates cultivated in STP1 (Figure 3.15) showed that the isolates could be grouped into six classes of bacteria i.e. α-Proteobacteria, β-Proteobacteria, γ-Proteobacteria, Actinobacteria, Bacilli (Low GC) and Flavobacteria. The most dominant class was γ-Proteobacteria with 44% (*n*=22), followed by β-Proteobacteria with 26% (*n*=13). There was only one species in the class of Actinobacteria (2%, *n*=1) and Flavobacteria (2%, *n*=1) which was *Leifsonia xyli* and *Myroides odoratimimus*. Flavobacteria was only successfully cultured in STP1 and was not found in other STP. All isolates grouped according to their cluster except for strain 3R1-6 which was similar to the closer relative (*Pseudomonas* sp. Accession code: EU652471) from class γ-Proteobacteria but was clustered among β-Proteobacteria. Further work is required to clarify the identity of the strain 3R1-6.

Isolates in STP2 produced a phylogenetic tree (Figure 3.16) with five classes of i.e. α-Proteobacteria, β-Proteobacteria, γ-Proteobacteria, Actinobacteria and Bacilli (Low GC). In contrast to results from STP1, the most prevalent class in STP2 was β-Proteobacteria with 42% (*n*= 21) which was higher than γ-Proteobacteria with 34% (*n*= 17). There were five strains in α-Proteobacteria (10%) and some were found only in STP2 such as *Sphingomonas desiccabilis* and *Caulobacter* sp. Another unique strain to STP2 was *Microbacterium deminutum* which belonged to class Actinobacter (4%, *n*= 2).

STP3 had the lowest diversity among the STP according to Shannon Diversity Index. There were only four classes of bacteria in STP3 (Figure 3.17) which were α-Proteobacteria, β-Proteobacteria, γ-Proteobacteria and Bacilli (Low GC). The dominant

class was β-Proteobacteria (40%, *n*=16), followed by γ-Proteobacteria (30%, *n*=12), Bacilli (17.5%, *n*=7) and α-Proteobacteria (12.5%, *n*= 5).

From the cluster analysis, all three samplings (from all three STP) on CGYA grouped well under the same cluster with at least 50% similarity (Figure 3.18). Our results suggested that the types of bacteria cultivated on CGYA did not differ much between samplings. On TGYA, the first and third sampling gave a high similarity of 60%, and grouped with each other. However the second sampling was placed outside this group. In contrast, R2A gave very different results at each sampling with each sampling having low similarity with each other (< 15%). As each type of medium gave varying results, it was not advisable to use a single isolating medium for STP.

In this study, some species were commonly isolated from all STP and some rare bacteria were found only from specific STP or on specific media. Although the absence of certain bacteria could be due to the conditions of the plant or the type of media used, their absence could also be due to number of sampling carried out. Due to logistic and time constraints, only three samplings were carried out at each STP. More samplings are probably required to determine if the types of bacteria obtained from different STP were upheld. Alternatively, culture-independent methods such as Denaturing Gel Gradient Analysis or Amplified Ribosomal DNA Restriction Analysis could be employed to determine their absence or presence from an STP.

One of the most common bacteria found in this study was *Stenotrophomonas maltophilia*. *S. maltophilia* is a useful microorganism in wastewater treatment as it is capable of breaking down food components such as protein, fats and cellulose (Fong & Tan, 2000), monocyclic hydrocarbon (Urszula *et al.,* 2009) and is able to suppress filamentous growth that causes bulking (Wanner, 1994). *Aeromonas* was also prevalent in all STP, and its dominance in activated sludge systems was also reported by Neilson (1977). *Aeromonas* was also found to have bioflocculant-producing properties (Li *et al.*, 2007) which are essential for floc forming. *Aeromonas hydrophilia* also have the ability to accumulate phosphate which would aid in the removal of phosphate in wastewater (Cloete & Muyima, 1997; Sidat *et al*., 1999). *Aeromonas* also degrades food components such as protein, fats, starch and cellulose (Fong & Tan, 2000).

Another common genus, the *Acidovorax* is cultivated from various wastewater treatment plants, and plays a role of denitrifier (Hoshino *et al.* 2005; Heylen *et al.*, 2008; Nielsen *et al.,* 2009). The *Acidovorax* is nutritionally versatile as it can consume a wide array of carbon substance (Snaidr *et al.,* 1997) which explains their prevalence in the three different media used in this study. *Bacillus* and *Pseudomonas* are also common genera that were isolated from all STP and from all types of media. Both *Bacillus* and *Pseudomonas* are floc formers (Gerardi, 2006), and can also degrade complex organic substrates and carry out denitrification. In addition, *Pseudomonas* is also capable of removing phosphate from wastewater (Cloete & Muyima, 1997). Although *Staphylococcus* was also a common genus isolated in this study, it is often categorized as pathogens in wastewater (Gerardi & Zimmerman, 2005). *Staphylococcus* is also cultured from other municipal wastewater treatment plants (e.g. Mehandjiyska, 1995; Miyanaga *et al.*, 2007). However, McKenney *et al.* (1998) have reported that

Staphylococcus epidermidis is able to produce capsular polysaccharide that may contribute to floc forming.

Among the rare bacteria found in this study, *Myroides odoratimus* and *Citrobacter werkmanii* were cultivated from STP1 and both are known floc-formers (Gerardi, 2006). *Xanthobacter autotrophicus* is able to degrade a toxic and potentially carcinogenic compound, 1, 2-Dichloroethane (DCE) (Baptista *et al.*, 2006) whereas *Ideonella dechloratans* was able to remove chlorate and perchlorate in wastewater (Logan, 1998). Also found at STP1 was *Moraxella osloensis. M. osloensis* is a facultative organotrophic microorganism, as well as polyphosphate accumulating microorganism (Cloete & Muyima, 1997).

There were only a few rare species in STP3 which were *Lysinibacillus sphaericus* and *Brevundimonas mediterranea*. Influent at STP3 contained relatively higher heavy metal concentration. Coincidentally, *Lysinibacillus sphaericus* has metal binding properties with its cell wall that consist of S-layer protein that allow metal sequestration (Sherameti & Varma, 2009), and can reduce chromium *in vitro* (Pal *et al.* 2005). For *Brevundimonas mediterranea,* it is a novel species that was only reported in 2005 (Fritz *et al.*, 2005) and is capable of performing "enhanced biological phosphorus removal" (Ryu *et al.*, 2007). Although phosphate level reduction was the highest at STP3, we did not ascertain whether *B. mediterranea* played a major role in phosphate removal at STP3.

4.6 Possible factors affecting activated sludge at STP3

Our study showed that the influent of STP3 had poor pH condition that might have affected the wastewater treatment efficiency. Aeration system with low pH often faces problems such as pin floc and high effluent turbidity (Richard, 2003) which

explained the low SV in the aeration tank (<100 ml l^{-1}) and the high effluent TSS (average >100 mg l^{-1}) observed in STP3. Although DO improved at the last sampling, SV, MLSS and MLVSS remained depressed at STP3. As STP3 served a light industrial area, we also found heavy metal pollution (Copper and Lead) in the influent (unpublished data).

Copper (Cu) is known to inhibit the growth of nitrifiers in activated sludge system where copper is capable of binding to enzymes and membranes of cells which would cause the disruption of the enzyme structure in the cell. Formation of Cu(II)-amine species may also be one of the reason that inhibits the nitrification process (Lee *et al.*, 2009). The heavy metal pollution in the influent at STP3 could be the reason for the persistent poor health of the activated sludge at STP3.

CONCLUSION

In this study, STP1 showed the healthiest activated sludge condition among STPs with steady level of DO and other parameters such as SV, MLSS and MLVSS were well maintained. It exhibited good nutrient and organic matter removal efficiency. The bacterial abundance in STP1 was found to be higher and more diverse than other STPs. Among the bacteria isolated, there were floc formers i.e. *Achromobacter* sp., *Alcaligenes* sp., *Bacillus* sp., *Citrobacter* sp., *Escherichia* sp., *Pseudomonas* sp. and others (Gerardi, 2006).

STP2 suffered a series of equipment malfunction such as the aerator in the aeration tank and scraper in clarifier. This affected the functioning of the treatment system as it caused low dissolved oxygen level in the aeration tank and high TSS in the effluent. As the dissolved oxygen in STP2 increased gradually, the health of the activated sludge also improved as reflected by SV, MLSS, MLVSS and bacterial abundance. Despite the improvement of the parameters, the removal efficiency of nutrients and organic matter for STP2 was still below par and inconsistent. This may due to the high concentration of phosphate detected in STP2 that affected the workability of the sludge (Al-Tayyar, 1993).

In STP3, the aeration tank was constantly functioning irregularly with poor performing aerator. It caused depressed dissolved oxygen level and suppressed the microbial growth in STP3. The low alkalinity in STP3 offered limited buffering capacity and lead to acidic pH condition in aeration tank. The influent from the surrounding light industry was also found to contain significant concentration of heavy metal that could deteriorate the health of activated sludge. These conditions resulted in

lower bacterial diversity and abundance at STP3. Without decent activated sludge condition, nutrient and organic matter removal at STP3 were depressed.