CHAPTER 1: INTRODUCTION

Wastewater comprises approximately 99.9% water. The remaining 0.1% contains microorganisms, organic matter, inorganic matter, suspended solids, and dissolved solids. These contents in wastewater cause water pollution and public health complications when they enter into the open water and subsequently contaminate our water supply (Sperling, 2007). Contaminated water supply causes transmission of various types of infectious diseases. Among the most important infectious diseases are bacterial (typhoid, cholera, bacillary dysentery and gastro-enteritis), viral (hepatitis, poliomyelitis and various diarrhoeal infections), protozoal (crytosporidiosis, giardiasis and amoebic dysentery) and helminth (ascariasis, hookworm and schistosomiasis) infections (Gray, 2004).

Wastewater treatment plays an important role in public health and environmental protection by preventing the release of polluted water into open water and contamination of water supply. The type of treatment depends mostly on the composition of the wastewater. Wastewater treatment plant comprises different parts which function together to treat the complex wastewater from domestic, commercial, industrial and agricultural sectors. Wastewater treatment process can be classified into five categories: preliminary, primary, secondary, tertiary and sludge treatment (Figure 1.1) (Ramalho, 1977).

1.1 Wastewater treatment system

1.1.1 Preliminary treatment

Prior to treatment, the influent needs to be screened for large solids, grits, storm water and sometimes oil and grease to avoid damage to the pumps or blocking of the pipework (Godfree, 2003). Therefore, preliminary treatment such as screen, grit separation, dissolved air flotation and storm water is needed.

Large solids such as rags, paper, wood and plastic are first screened in preliminary treatment. There are a variety of patterns and a range of screens which come in four sizes: coarse, fine, very fine and micro screen (Table 1.1) (Gray, 2004). Coarse screen is normally used in domestic wastewater plants whereas fine screen is limited to certain industrial and food processing treatment plants. The screened materials contain very unpleasant and unhygienic materials. Hence, proper disposal is needed such as incineration and landfill. Some larger plants practice dewatering and compaction of screened materials to reduce the volume of screenings to be disposed (Gray, 2004).

After screening, the wastewater still contains grits which are mineral aggregate, mixture of slit, sand, gravel, fragments of metal, glass as well as plastic. Grits will settle from the wastewater when the flow rate is low as their gravity are higher than wastewater. There are four grit removal systems that are normally used for grit separation, i.e., horizontal-flow grit chambers, aerated chambers, detritus tanks, (Davis, 2010) and vortex separator (hydrocyclones) (Tchobanoglous *et al*., 2002). Horizontal-flow grit chamber is a velocity control channel where the velocity is controlled by proportional weir or Parshall flume whereas aerated grit chamber is designed with air flow introduced from a side at the bottom of the tank which causes a spiral roll pattern perpendicular to the flow through the tank (Davis, 2010). Both detritor and vortex separator are based on sedimentation technique. Detritor separator is a square shallow

tank designed to be shallow with a short retention time. To make sure that only dense particles are removed and all organic solids remain in suspension, flow rate of the wastewater is maintained low. The grit will then collect in a disposal unit. Another system i.e. vortex separator such as the Pista grit trap separates the grit by centrifugation (Gray, 2004).

There are also suspended solids that are less dense than water, which are removed by allowing them to float to the surface before removal by a mechanical skimming device (Kawamura, 2000). Dissolved air floatation (DAF) units are initially used to remove emulsified fats, oils and grease but they are now used to remove suspended solids and other contaminants from wastewater (Reay and Ratcliff, 1973). Compressed air fed into wastewater was mixed with the particles and formed bubbles. Thick solids formed by bubbles were known as float. Performance of DAF can increase by adding chemical flocculants such as polyacrylamide, iron and aluminium salts. For animal and human safety, chemicals such as chitosan, carrageenan or lignosulfonic acid are used. In some industrial applications, the use of flocculants in DAF systems has replaced conventional sedimentation tanks. DAF can be used either in preliminary or tertiary treatment to help remove such materials.

1.1.2 Primary treatment

Primary treatment is the process of removing settleable solids from screened sewage by sedimentation with gravity force (Shammas *et al.,* 2005). The minimum retention time is 2 hours. The settled sludge is then pumped as primary sludge to storage tanks. The remaining liquid is referred to as primary effluent enters secondary treatment (Sonune and Ghate, 2004). Reported removal efficiencies of incoming biological oxygen demand (BOD_5) and total suspended solids (TSS) are 70 ± 48% and 56 ± 53%, respectively (Steer *et al.,* 2002).

1.1.3 Secondary treatment

Primary effluent contains significant amount of dissolved and colloidal materials (Spellman, 2011). The secondary treatment process is the biological process was performed in a specially designed reactor suitable for the development of microbial population. Oxygen is supplied to the microbial population. After degradation of the colloidal organic matter present in the primary effluent, the microorganisms were then separated from the wastewater by settlement.

Treatment can be done either with fixed film or suspended microorganisms (activated sludge). There is also a hybrid system which includes both fixed film and suspended microorganisms (Wang *et al*., 2000). In fixed film system (Figure 1.2), the microbial biomass grows on a medium and degrades the sewage that passes over its surface (Figure 1.3). Activated sludge system is a system where the aerated sludge converts organic materials in primary effluent to inorganic materials which form biological floc. This biological floc comprises of saprophytic bacteria, protozoa and rotifers. The treated supernatant is removed and a portion of the settled sludge (or returned sludge) is brought to the start of the aeration system to be used again as biomass to degrade new sewage entering the tank. The accumulated sludge is then treated via thickening, stabilization and dewatering before disposal (Spellman, 2011).

1.1.4 Tertiary treatment

Tertiary treatment processes are normally referred to filtration, settlement and flocculation (Gray, 2004). The tertiary treatment is varies a lot and some can be used to reduce BOD_5 or suspended solids concentration, to eliminate bacteria or pathogens or nutrients, and to meet particular conditions as mentioned in Table 1.2.

One of the most efficient tertiary treatments is shallow lagoons as it gives better settlement and longer retention time compared with secondary settlement tank (Forster,

2003). Wastewater will stay in lagoon with retention time of less than 60 hours and then will be purified by flocculation and settlement, removing 30 to 40% suspended solids. With longer retention time of 14 to 21 days, lagoons can successfully remove 75 to 90% suspended solids, 50 to 65% BOD_5, and 99% coliform. Lagoon with an optimum depth of 1 m and at retention time of less than 60 hours will give maximum performance efficiency without excessive algal growths (Gray, 2004).

Land irrigation of grass plots is one of the most economical tertiary treatment. End products from secondary treatment are sprayed or channelled evenly to a slope grassland. Effluent will be percolated downhill and will be collected within a channel. At least two grass plots are needed for alternate use and better performance. According to Institute of Water Pollution Control (1987), a plot size range of 0.1 to 3.0 ha is needed. Loading of the effluent to grass plot from 2000 to 5000 $m^3ha^{-1}d^{-1}$ is also depending on the climate and soil conditions. Short grasses are needed to be mown regularly to avoid weeds. Suspended solids are greatly retained in the soil and some of the nutrients are absorb for plant growth. Maximum removal of 60 to 90% suspended solids and 70% BOD_5 are possible (Tebbutt, 1998).

A more efficient removal of suspended solids in the end product of secondary product is microstrainer. Microstrainer is made up of stainless steel fine mesh screener with mesh with aperture size from 20 to 40 μm for screening of relatively small solids (Tebbutt, 1998). The fine mesh is easily blocked by the treated effluent, and therefore, constant backwashing of the screen is needed. With the fine mesh screener, 70% of suspended solids removal is possible. However, microstrainer is very poor in removing enterobacteria compared to either lagooning or grass plot irrigation. From the collection chamber, about 2 to 5% of the filtrate is used for backwashing. Apart from clogging due to suspended solids in the effluent, the mesh is also clogged by bacterial growth. This bacterial growth is difficult to remove manually or chemically. Therefore, UV lamp is

used for the prevention of bacterial growth. The mesh requires high maintenance to keep the high performance of the screening.

Another tertiary treatment that is often used in wastewater treatment plants is gravity or pressure filtration through sands (Arceivala and Asolekar, 2008). There are three types of sand filter used to treat domestic wastewater, which are the slow sand filter, the rapid downward flow sand filter, and the rapid upward flow sand filter. Slow sand filters use gravity to operate and are suitable for small treatment plants as they only require periodically cleaning. Rapid downward flow sand filters have high loading rates, and therefore, the surface layer of sands is under pressure and bind rapidly. Backwashing is frequently needed to clean the sand medium and to reduce the bed compaction. With the regular backwashing, little biological activity occurs, and hence, BOD_5 removal is lower than that of slow sand filters. Rapid upward flow sand filters have similar removal of suspended solids and BOD_5 except they have higher hydraulic loadings. This makes them suitable for large treatment plants. The secondary effluent is forced upwards through a bed of increasingly fine sands as it progresses upwards under pressure or hydraulic head. This high pressured filtration is used to remove suspended solids and does not require frequent washing which makes rapid upward flow sand filters function efficiently.

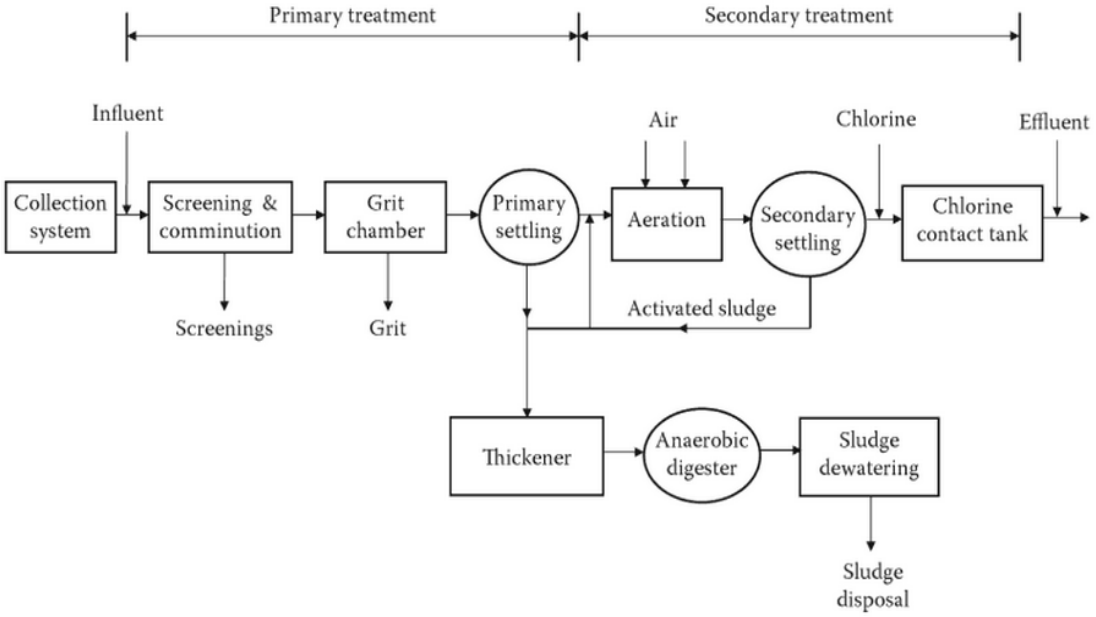


Figure 1.1: Schematic layout of standard wastewater treatment process with primary and secondary treatments using activated sludge process (taken from Spellman, 2011).

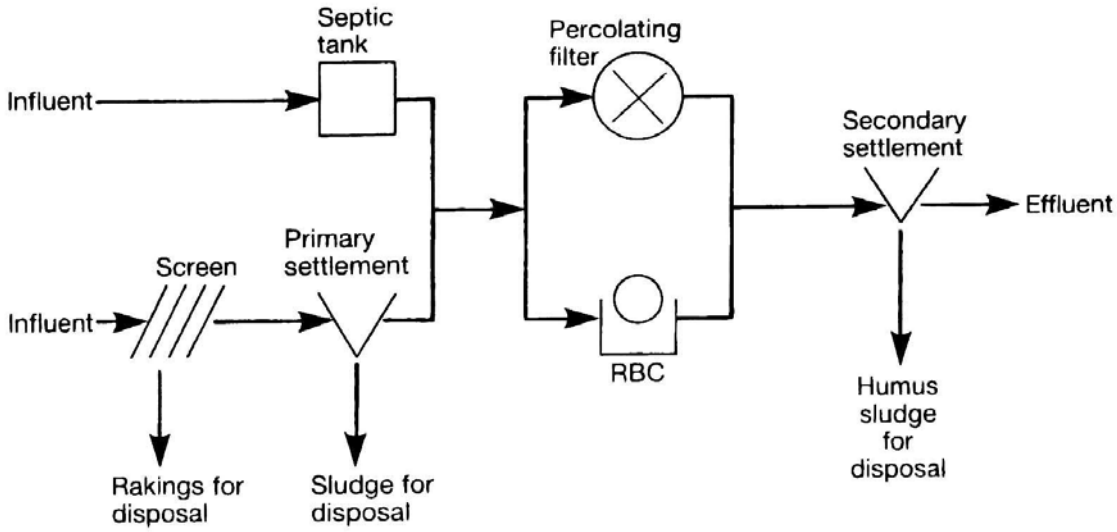


Figure 1.2: Schematic layout of sewage treatment with fixed film reactor (taken from Gray, 2004).

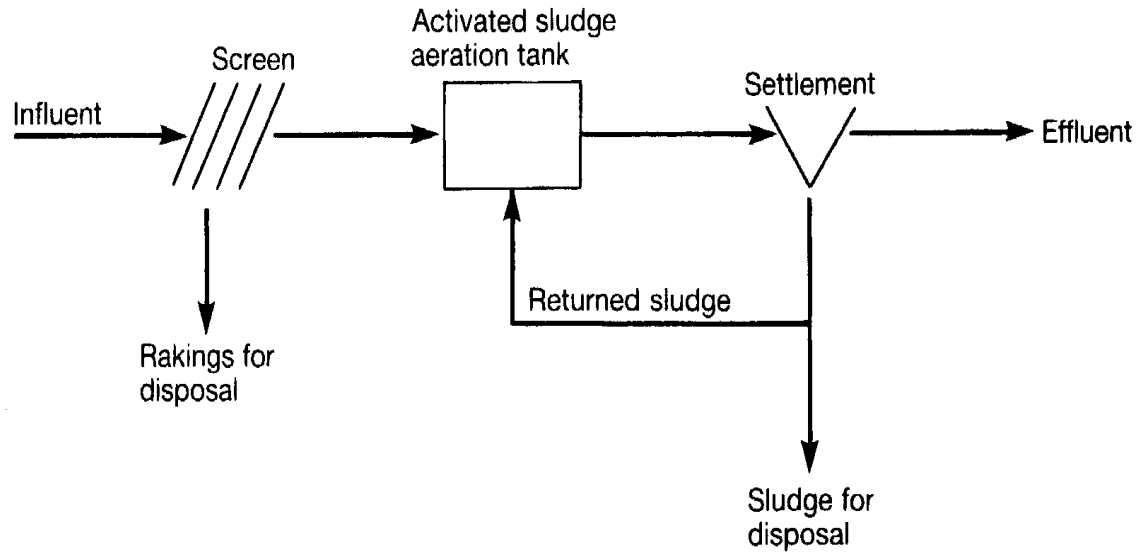


Figure 1.3: Schematic layout of sewage treatment at a small activated sludge plant (taken from Gray, 2004).

Table 1.1: Typical size range of screens used in wastewater treatment (taken from Gray, 2004).

Category	Spacing (mm)	Application
Coarse screen	> 10	Removal of large material
Fine screen	1.5 to 10	Used as substitute for primary sedimentation for extended aeration plants
Very fine screen	0.2 to 1.5	Used as substitute for primary sedimentation
		Used in conjunction with series of larger screens
Micro-screen	0.001 to 0.3	Effluent polishing. Treatment of inert quarry washings

Table 1.2: Parameter limits of effluent of Standard A and B in Environmental Quality (Sewage and Industrial Effluents) Regulations 1974.

Parameter	Unit	Standard	
		A	B
Temperature	°C	40	40
pH value	-	6.0 to 9.0	5.5-9.0
BOD_5 at 20°C	mg l^{-1}	20	50
COD	mg l^{-1}	50	100
Suspended solids	mg l^{-1}	50	100
Mercury	mg l^{-1}	0.005	0.050
Cadmium	mg l^{-1}	0.01	0.02
Chromium, Hexavalent	mg l^{-1}	0.05	0.05
Arsenic	mg l^{-1}	0.05	0.10
Cyanide	mg l^{-1}	0.05	0.10
Lead	mg l^{-1}	0.10	0.50
Chromium, Trivalent	mg l^{-1}	0.20	1.0
Copper	mg l^{-1}	0.20	1.0
Manganese	mg l^{-1}	0.20	1.0
Nickel	mg l^{-1}	0.20	1.0
Tin	mg l^{-1}	0.20	1.0
Zinc	mg l^{-1}	2.0	2.0
Boron	mg l^{-1}	1.0	4.0
Iron	mg l^{-1}	1.0	5.0
Phenol	mg l^{-1}	0.001	1.00
Free Chlorine	mg l^{-1}	1.0	2.0
Sulfide	mg l^{-1}	0.50	0.50
Oil and Grease	mg l^{-1}	Not detectable	10.00

1.2 Microbiology of activated sludge system

Aerobic activated sludge process is the most widely used biological wastewater treatment worldwide (Drysdale *et al*., 1999). Activated sludge process comprises two units, one is a biological reactor for conversion of pollutant, another one is a solid separation tank (gravity clarifier). These two units can be combined into a single unit called sequencing batch reactor (SBR).

Sludge contains a diverse microbial population for biodegradation process. The wastewater that flows into the wastewater treatment plant is the food source for the microorganisms in the aeration tank. The organic matter will be converted into new bacterial cells which will then be used as new active biomass (Stypka, 1998).

There are three main criteria of bacterial population in activated sludge for efficient removal of BOD, fine solids and heavy metals (Gerardi, 2002). Firstly, the bacterial population must be large enough for higher BOD and fine solids removal. Secondly, a great diversity of bacteria is required for diverse wastes to be degraded. Thirdly, bacteria population is under active condition, which is free of toxic and inhibitory substances. Hence, it is essential for the efficiency of the process, that the bacteria in the activated sludge process form firm and dense floc particles.

The structures of flocs in the activated sludge function as absorber of soluble substances, colloidal matters, and macromolecules (Stypka, 1998). Floc formation is initiated by floc forming bacteria, which agglutinate with increasing sludge age. With increasing sludge age, floc forming bacteria form three cellular components for floc formation, which are cellular fibrils, sticky starches (or polysaccharides), and granular starches polyhydroxybutyrate. Young flocs have weaker interactions because the polymer production is low. On the other hand, old flocs have stronger interactions as the polymers surrounding the cells form a compact region at the centre of the flocs. Although the bondings in the center of the flocs are strong, the interactions at the outer

parts of the flocs are still weak (Zita and Hermansson, 1994). Primary floc forming bacteria that commonly found in activated sludge are *Achromobacter, Alcaligenes, Arthrobacter, Citromonas, Escherichia, Flavobacterium, Pseudomonas,* and *Zoogloea* (Dias and Bhat, 1964; Pike, 1972).

Besides floc forming bacteria, there are also non-floc forming bacteria and filamentous bacteria. These three groups of bacteria help in BOD, fine solids, and heavy metal removal. Floc forming bacteria initiate floc forming whereas filamentous bacteria strengthen the floc particle and help in floc enlargement (Gerardi, 2002). The relationships between floc forming and filamentous bacteria can be divided into three types. The first type is normal floc, where there is a balance between floc-forming and filamentous bacteria. This results in a strong floc that keeps its integrity in aeration basin and is able to settle well in the sedimentation tank. The second type is pin-point floc. In this floc, filamentous bacteria are absent or occur in low number which results in a small floc that does not settle well (Figure 1.4). Therefore, the secondary effluent is turbid despite the low sludge volume. The third type is bulk filaments. This is caused by the domination of filamentous organisms in the tank. The filaments interfere with sludge settling and flocs campaction. Poor sludge settling is observed when the length of extended filamentous exceeds 10^7 μm mg^{-1} of suspended solids (Sezgin, 1982).

The structure of the sludge floc and the concentration of filamentous bacteria in the active sludge can vary considerably at each plant, and this reflects the differences in plant types, operations and wastewater compositions.

Examples of filamentous bacteria that are most often present in wastewater treatment plants are *Microthrix parvicella* [Figure 1.5(a)], *Nostocoida limicola*, 0041/0675 [Figure 1.5(b)], and *Nocardia,* whereas microorganisms that may be present in wastewater treatment plants with a high load of industrial wastewater are

Haliscomenobacter hydrossis, 0914, *Sphaerotilus natans*, *Thiothrix*, and 0803 (Jenkins *et al.*, 2004).

Microthrix parvicella belongs to the group of bacteria that can form foam or floating sludge on the surface of both the process basin and the clearing basin, which are water-repellent (hydrophobic). They only exhibit this behaviour during spring and autumn, when the temperature is around 12ºC (Jenkins *et al.*, 2004).

The non-biological components of activated sludge flocs are organic and inorganic particles, and extracellular microbial polymers. These non-biological components are generally made up largely of carbohydrates and glycoproteins (Figure 1.6) (Jenkins *et al*., 2004).

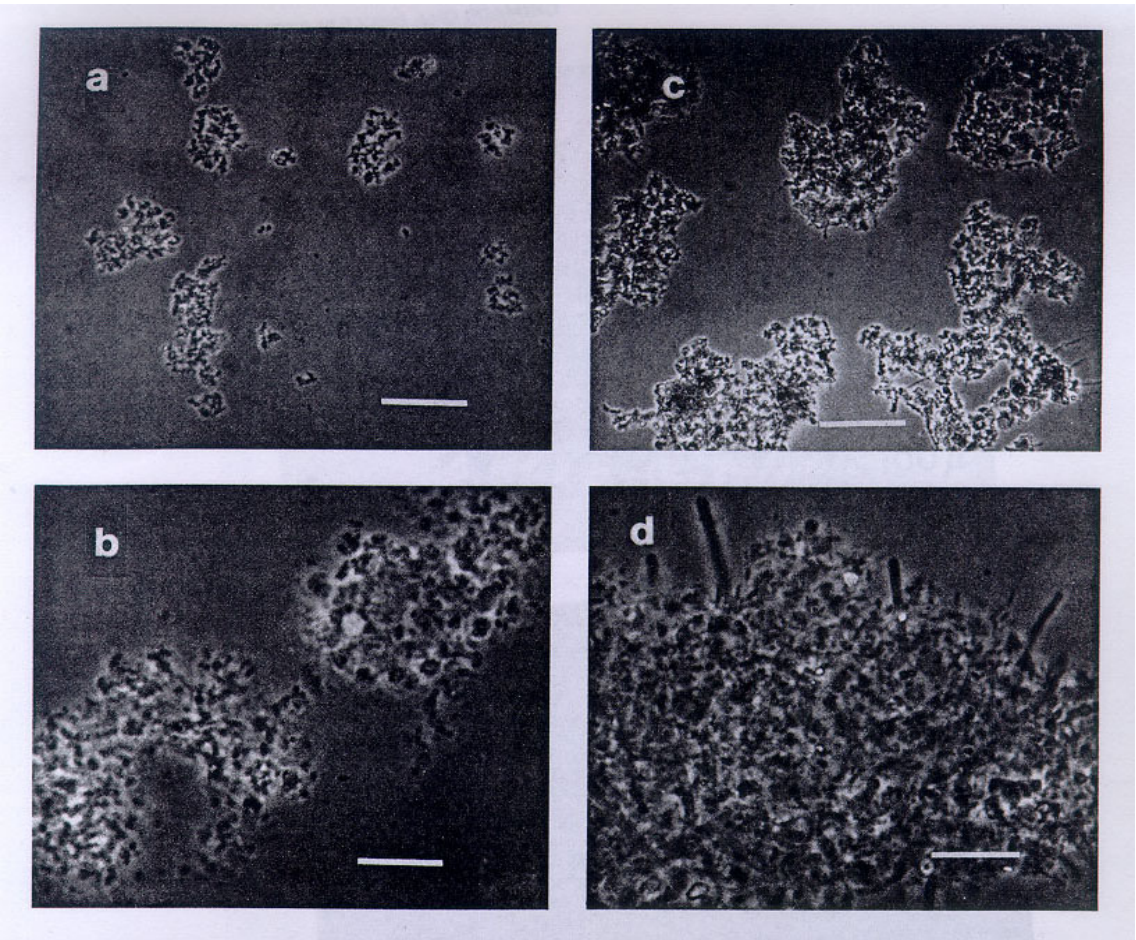
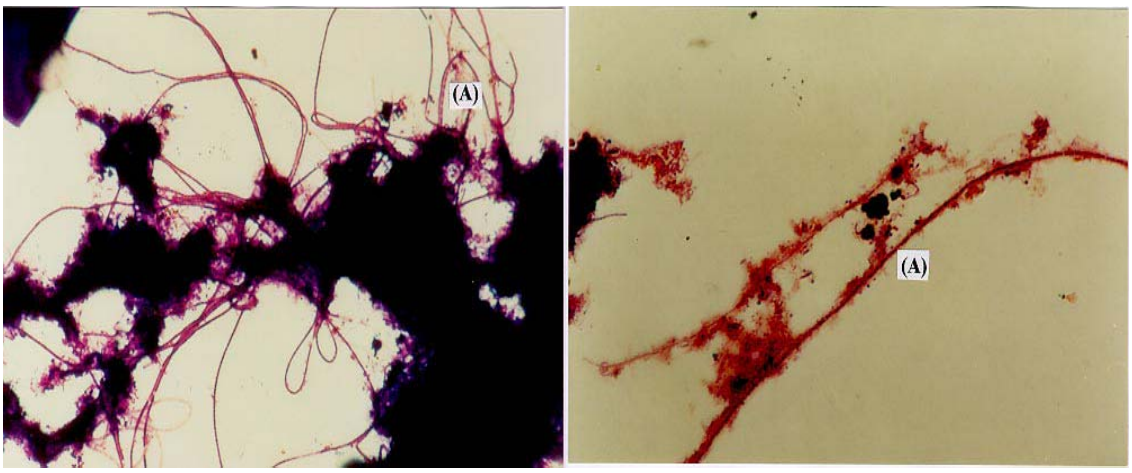


Figure 1.4: Activated Sludge Flocs II: (a) pin flocs; (b) small, weak flocs; (c) flocs containing filamentous organisms; (d) flocs containing filamentous organism “network" or “backbone." Bar = 10 µm (Jenkins *et al*., 2004).



(a) (b)

Figure 1.5: (a) *Microthrix parvicella*, a strongly Gram positive organism. It can be distinguished by the presence of spaces between the cells, indicating the presence of a sheath and (b) 0675, Gram negative with heavily attached growth (Lacko *et al.*, 1999).

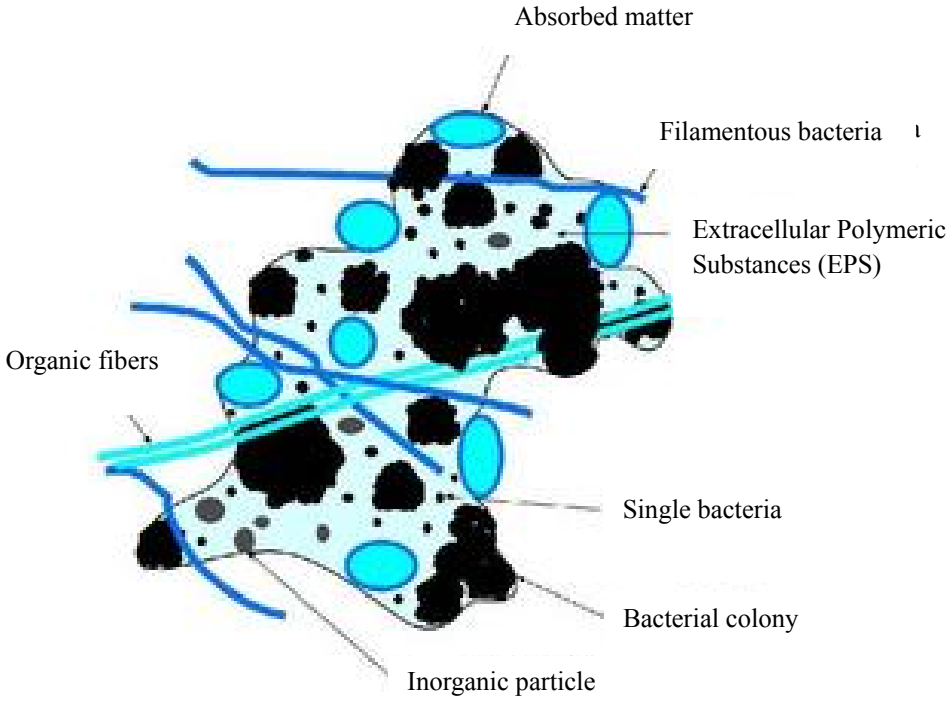


Figure 1.6: Filamentous bacteria on organic fibres forming floc (Jenkins *et al.*, 2004).

1.3 Molecular approaches for microbial community studies

Staley and Konopka (1985) coined the term "the great plate count anomaly" in their review, on the difference in the degree of abundance of cells from environmental samples, which can be cultivated and countable by microscopic examination. In the observation, vast majority of the microbial cells that can be observed under microscope cannot form colonies on common nutrient agar media. This finding shows that studies of microbial communities in natural environments using cultivation methods have reached a limitation. Generally, it is accepted that only less than 1% of total microorganisms present in the environmental samples can be recovered by cultivation methods (Ward *et al*., 1990; Amann *et al.,* 1995). Another limitation of cultivation methods are many of microbial species that dominate in natural environments cannot adapt to grow in media containing high concentration of complex organic carbon. Therefore, microbial studies using only cultivation methods are not considered precise in terms of representing the microbial diversity present in the natural environments.

In 1990s, culture-dependent techniques have been developed and since then, identification of single bacterial species is realized not only with cultivation of microorganisms (Ward *et al.,* 1990; Muyzer *et al.,* 1993; Ludwig *et al.,* 1994; Amann *et al.,* 1995). Culture-independent community analysis can be performed using phenotypic or genotypic methods. The most popular phenotypic method is phospholipids fatty acid (PLFA) analysis, where it characterized the phospholipids present in the periplasma of cells. Almost all the genotypic methods are DNA-based, where a total community genome DNA is extracted from environmental samples and analyzed directly through techniques such as "shot-gun cloning", "shot-gun sequencing" or "DNA probe hybridization". Besides analyzing the whole genome, specific genetic markers can also be amplified using polymerase chain reaction (PCR) and the PCR products are

subsequently analyzed by sequencing, hybridization, DNA melting behavior or length polymorphism (Nocker *et al.,* 2007).

Various techniques and technologies are used in different applications. Each approach has different specificity and limitation. Choice of approach in every research is based on the technologies available, stage of technologies development, and logistics.

Phospholipids are essential membrane components of all living cells; just like fatty acids in phospholipids. Mainly, different taxa have their own unique types of predominant fatty acids (Zelles, 1999). Fatty acid component in phospholipid membrane also adapts to the change of environmental conditions such as physical and chemical stress (Loffhagen *et al.,* 2004). PLFA is extracted by organic solvent or solid phase extraction and separated using gas chromatography to show PLFA profiles, which classifies the fatty acids and their molar abundance. Besides being used in estimating microbial biomass in environmental samples, PLFA are also used in detection of heavy metal pollution (Pennanen *et al.,* 1996), and monitoring shift of microbial population along environmental gradients (Frostegard *et al.,* 1993; Izbicki *et al.,* 2009). Due to the lack of accessible fatty acid pattern database, interpretation of the PLFA profiles is normally based on experience and literature. Therefore, the specificity and accuracy of PLFA method in profiling environmental microbial community are comparatively low.

Genotypic fingerprinting which is based on the 16S rRNA has been extensively used with accessibility of databases such as Ribosomal Database Project (RDP) (Maidak *et al*., 2000), and Basic Local Alignment Search Tool (BLAST) search program at the National Center for Biotechnology Information (NCBI) (Johnson *et al*., 2008). However, genotypic methods have their own limitations such as DNA extraction method efficiency, PCR bias and sequencing accuracy. Genotypic fingerprinting such as terminal restriction fragment length polymorphism (TRFLP) is based on the length polymorphism of PCR products from total community DNA extracted from

environmental samples. Fluorescent dye is labeled on either one or both of the forward and reverse primers. The terminal labeled PCR products are then digested with restriction enzymes at specific sites. The cut sites of the restriction enzymes on different PCR samples are located at different position due to different gene sequences of different microorganisms. Hence, the restriction enzyme produced fluorescent labeled terminal restriction fragments (TRFs) of various sizes. The abundance of the various sizes of TRFs is then sorted by electropherogram. TRFLP have been widely used in ecological samples to analyze microbial community structures and dynamics (Osborn *et al*., 2000), and to analyze changes in the bacterial community structures (Lukow *et al*., 2000).

Another tool that generates genotypic fingerprinting is denaturing gradient gel electrophoresis (DGGE), which is based on electrophoresis separation of PCR products in denaturants (urea and/or formamide) gradient gel (Muyzer *et al.*, 1993). PCR primer with specially designed CG clamp is used to hold the separated double stranded nucleic acid during the electrophoresis. The separation of PCR products are based on the melting behaviors of different sequences along the denaturant gradient gel. Along the linear gradient of denaturant, the separations on the polyacrylamide gel are based on the decreased electrophoretic mobilities of the partially melted double-stranded DNA fragments. DNA fragments melt at their specific melting temperatures, known as melting domains. Each stretch of base pair has its own melting domain. Once the DNA fragment reaches its melting domain at a particular position in the denaturing gradient gel, the helical molecule becomes partially melted and the migration of the molecule will halt. Therefore, DNA fragments with different sequences will migrate and stop at different positions in the polyacrylamide gel (Muyzer and Smalla, 1998). DGGE has been applied widely in analysis of environmental samples diversity and monitor changes in microbial community (Muyzer 1999; Gurtner *et al*., 2000; Díez *et al.,* 2001;

Ogino *et al.,* 2008). DGGE is able to detect 50% of the variations of microbial diversity in environmental samples (Myers *et al.,* 1985). Another similar technique is known as temperature gradient gel electrophoresis, which is based on temperature gradient instead of denaturant gradient, is also used to determine environmental microbial diversity (Felske *et al*., 1998; Heuer *et al*., 1999).

A higher degree of phylogenetic community analysis is obtained through cloning followed by sequencing or direct sequencing, which are widely used in microbial ecology. Cloning and sequencing analyze microbial community diversity (Britschgi and Giovannoni, 1991; Dunbar *et* al., 1999) and is used to compare the microbial community in different conditions (Skirnisdottir *et* al., 2000). However, this technique is time consuming and costly for routine work.

The latest technique for microbial community profiling is high-throughput pyrosequencing which allow simultaneous analysis of multiple microbial diversity. The numbers of species of bacteria per gram of soil vary between 2000 to 10 billion (Schloss and Handelsman, 2006), which makes it impractical to test by amplification and sequencing of the conserved 16S rRNA gene. This problem can be solved with high-throughput DNA pyrosequencing of different soil samples (Roesch *et* al., 2007). The high sensitivity of this high-throughput method provides very comprehensive information of the microbial composition (Quince *et al.,* 2008), is sensitive and gives highly reproducible results (Sogin *et* al., 2006) and novel organisms to be detected (Cardenas and Tiedje, 2008). This helps in better understanding of microbial community structure. However high-throughput method has its limitation where sequencing error can occur and cause false estimation of diversity (Kunin *et al.,* 2010).

In Asia, a number of studies on microbial communities in wastewater treatment plants using DGGE have been done. The studies of wastewater using DGGE were to profile the microbial communities (Xia *et al.,* 2005; Shin *et al.,* 2010), determine

dominant microbial population (Lui *et al.,* 2010) or monitor shift of bacterial group in different conditions (Mao *et al.,* 2008). Due to lack of microbial study in wastewater treatment plant in Malaysia, investigation of bacterial community diversity by DGGE (Muyzer *et al*., 1993) is needed. Although recent studies using pyrosequencing and other next generation sequencing approaches provide more comprehensive bacterial community diversity (Youssef *et al.,* 2009; Szczepanowski *et al.,* 2009; Bibby *et al.,* 2010; Nocker *et al.,* 2010), DGGE provides preliminary, quick and comparatively cheaper method for preliminary bacterial community screening.

1.4 Objectives of the study

Thus, the objectives of this study were:

1. To characterize the activated sludge systems in different locations.
2. To characterize the bacterial community structures in the activated sludge systems.