MOLECULAR IDENTIFICATION OF MICROBIAL COMMUNITIES IN WASTEWATER TREATMENT SYSTEMS

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1025224

This thesis was prepared under the supervision of Dr. Khaled Swaileh and has been approved by all members of the examination committee.

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The findings, interpretations and conclusions expressed in this study do not necessarily express the views of Birzeit University, the views of the individual members of the MSc committee or the views of their respective employers.

DEDICATION

In memory of my mother

To my father for his continuous encouragement.

To my wife Suhair and children: Ala', Dana and Ahmad for their support and patience.

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ABSTRACT

The microbial communities in samples from three wastewater treatment systems have been examined using modern molecular techniques. The samples included a scum layer of Al Bireh Wastewater Treatment Plant secondary sedimentation tank, Pilot-scale Upflow Anaerobic Sludge Blanket (UASB) sludge and a biofilm of a Biofilter. The three samples origin is a municipal wastewater. The goal of the study was to identify the microbial content of the scum sample in order to control scum formation and to improve the efficiency of the UASB and Biofilters. The molecular techniques applied to achieve these goals were PCR, DGGE and 16S rRNA sequencing. DNA was extracted from the three samples and small regions of the 16S rRNA genes of both Archaea and Bacteria were amplified via the polymerase chain reaction (PCR). Thereafter, samples were analyzed via Denaturing Gradient Gel Electrophoresis (DGGE), a technique that allows for the separation and visualization of individual PCR products that are of the same size, based on differences in their sequence. About 500bp of the 16S rRNA genes were amplified for the determination of dominant PCR product by DGGE. The whole 16S rRNA genes were amplified and cloned in pCRII® – TOPO vector. Dominant clones were sequenced and compared to a published database for identification using Basic Local Alignment Search Tool (BLAST search).

Several filamentous bacteria were identified including *Microthrix parvicella*, *Nocardia sp.*, *Hyphomicrobium facilis*, *Chloroflexi*, Candidates TM7 and *Nocardioides oleivorans*. These filamentous bacteria are thought to be the reason

behind scum formation in the BWWTP. The low F/M ratio and high grease and oil load in the aeration tank could be the main reasons behind the spread of these groups of bacteria in the system. These groups of microorganisms exhibit seasonal variation depending on temperature and oil load. Solving the problem of scum formation could be based on the idea that the F/M ratio must be increased and the oil/grease loads in the influents must be reduced.

The methanogenic community of the UASB analyzed by PCR based DGGE and FISH analysis showed the presence of highly diverse methanogens including *Methanospirillum hungatei*, *Methanosarcina barkeri*, *Methanosarcina mazei*, *Methanobrevibacter arboriphilus* and other methanogen clones. The abundance of these methanogens is low and this may result from high oil and grease load (municipal wastewater) which contains long chain fatty acids (LCFA) that are toxic to methanogens. All these conditions result in 60% removal for COD which is lower than other studies done at the same conditions and achieved 88% COD removal for domestic wastewater.

The chemical analysis of the UASB shows that the reactor removal efficiency for COD is 60% and that for BOD is 56%. The reactor needs further adjustment to increase the microbial content and achieve higher removal efficiency.

The microbial community in the Biofilter composed of mostly uncultured bacteria of soil origin. This could originate from the packing material of the system (rocks, sand and PVC). In addition, the biofilm was not yet developed as the system was in its startup phase. The presence of *Zoogloea ramigera* is promising as this microorganism plays a role in the biofilm formation.

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LIST OF ABBREVIATIONS

AOB	Ammonia Oxidizing Bacteria
BLAST	Basic Local Alignment Search Tool
BOD	Biological Oxygen Demand
bp	Base pair
BWWTP	Al Bireh Wastewater Treatment Plant.
ºC	Degree Centigrade
COD	Chemical Oxygen Demand
DAPI	4'-6-Diamidino-2-phenylindole
DGGE	Denaturing Gradient Gel Electrophoresis
DNA	Deoxyribonucleic Acid
DO	Dissolved Oxygen
EPA	Environmental Protection Agency
F	Forward primer
FISH	Fluorescent *In Situ* Hybridization
F/M	Food to Microorganisms ratio
GTE	Glucose, TRIS, EDTA
HEM	Hexane Extractable Material
HRT	Hydraulic Retention Time
ICSP	International Committee on Systematic of Prokaryotes
In	Inch
LCFA	Long Chain Fatty Acid
µM	Micromolar

MCRT	Mean Cell Residence Time
mg	Milligram
mM	Millimolar
NCBI	National Center for Biotechnology Information
NCDENR	North Carolina Department of Environment and Natural Resources
NOB	Nitrite Oxidizing Bacteria
NRC	National Research Council
PCR	Polymerase Chain Reaction
PVC	Polyvinyl Chloride
R	Reverse Primer
RAS	Return Activated Sludge
RBC	Rotating Biological Contactors
RDP	Ribosomal Database Project
RNA	Ribonucleic acid
SBR	Sequencing Batch Reactor
SOD	Superoxide Dismutase
TAE	Tris, Acetic acid, EDTA
Tm	Melting Temperature
TGGE	Temperature Gradient Gel Electrophoresis
TE	Tris – EDTA buffer
UASB	Upflow Anaerobic Sludge Blanket
UV	Ultra Violet
WWTPs	Wastewater Treatment Plants

CHAPTER ONE

INTRODUCTION AND LITERATURE REVIEW

1.1. WASTEWATER TREATMENT

Wastewater treatment has gained increased importance worldwide due to increasing demand on fresh clean water supplies. Biological treatment achieved by the activities of micro-organisms, is one of the most widely used processes in the treatment of wastewater. High numbers and activities of some groups of micro-organisms are required to treat wastewater in biological processes. In contrast, some groups of micro-organisms must be absent from the system since their presence and activities cause problems in the treatment of wastewater (Cofikuner, 2002).

Wastewater treatment is composed of multi-stage process to clean wastewater before it reenters a body of water, applied to the land or is reused. The aim of wastewater treatment is to remove organic matter, nutrients, solids and pathogens and other pollutants from wastewater.

The main stages of wastewater treatment systems include: primary treatment, secondary treatment and tertiary treatment.

Primary treatment is used to remove oil, grease, fats, sand, grit and coarse solids. Secondary treatment is designed to degrade the biological content of wastewater which includes human wastes, food wastes, detergents and soaps. Most of the municipal and industrial wastewater treatment systems are aerobic where

microorganisms need both oxygen and substrate to live. Other methods include the anaerobic treatment systems where microorganisms degrade the organic matters in the absence of oxygen to stable products.

In tertiary treatment, unit operations and chemical unit processes are used to further remove BOD, nutrients, pathogens, and parasites, and sometimes toxic substances.

1.1.1. ANAEROBIC WASTEWATER TREATMENT:

In anaerobic systems, microorganisms convert organic materials to stable products like carbon dioxide and methane. The process of conversion includes two separate but interrelated phases: acid formation (Acetogenesis) and methane production (Methanogenesis).

Acetogenesis:

In acid phase, bacteria convert complex organic compounds (carbohydrates, fats, and proteins) to simple organic compounds, mainly short-chain volatile organic acids (acetic, propionic, and lactic acids). The anaerobic bacteria involved in this phase are called “acid formers,” and are classified as non methanogenic microorganisms (EPA, 2002). During this phase, little chemical oxygen demand (COD) or biological oxygen demand (BOD) reduction occurs, because the short-chain fatty acids, alcohols, etc. still a carbon source which could be used by many microorganisms, and thereby exert an oxygen demand.

Methanogenesis:

Mehanogenesis is the formation of methane by some types of microorganisms. This is an important form of microbial metabolism and it's the final step in the decomposition of organic matter.

The microorganisms capable of producing methane are called methanogens. They are an old group of microorganisms which belongs to the Archaea domain or Archaebacteria (Figure 1).

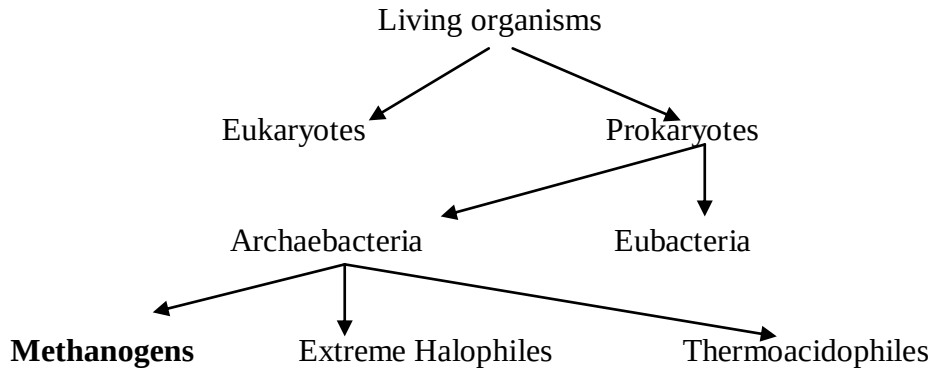


Figure 1: Classification of Methanogens.

Methanogenesis is an anaerobic respiration where the methane-producing Archaebacteria do not use oxygen to breath but the presence of oxygen is toxic to them.

Methanogens use the products of the acetogenesis (acetate, hydrogen and carbon dioxide) to produce methane through one of the following pathways:

1- Breakdown of acetic acid to produce methane and carbon dioxide:

$CH_3COOH \Longrightarrow CH_4 + CO_2$

2- The reduction of carbon dioxide by hydrogen gas to produce methane.

$$CO_2 + 4H_2 \Longrightarrow CH_4 + 2H_2O$$

During this phase, waste stabilization occurs, represented by the formation of methane gas.

In anaerobic wastewater treatment, the two phases of degradation occur simultaneously in dynamic equilibrium. That is, the volatile organic acids are converted to methane at the same rate that they are formed from the more complex organic molecules. The growth rate and metabolism of the methanogenic bacteria can be adversely affected by small fluctuations in pH substrate concentrations, and temperature, but the performance of acid-forming bacteria is more tolerant over a wide range of conditions. When the process is stressed by shock loads or temperature fluctuations, methane bacteria activity occurs more slowly than the acid formers and an imbalance occurs. Intermediate volatile organic acids accumulate and the pH drops. As a result, the methanogens are further inhibited and the process eventually fails without corrective action. For this reason, the methane formation phase is the rate-limiting step and must not be inhibited (EPA, 2002).

The physiology of cultured methanogenic Archaea is related to their phylogenetic relationships based on 16S rRNA sequences. For example, while most species of the *Methanobacteriaceae* and *Methanomicrobiaceae* prefer H_2 and CO_2 (or formate) as substrates for methanogenesis, *Methanosaeta*, a genus within the Methanosarcinaceae, is known to generate energy only from acetate fermentation.

Most of the other *Methanosarcinaceae* preferentially use methanol and related methyl-substrates for the generation of CH_4 (Kleikemper *et al*., 2005).

The diversity of methanogenic Archaea in the environment may be monitored or identified by using laboratory molecular methods such as Fluorescence *In Situ* Hybridization (FISH) or denaturing gradient gel electrophoresis (DGGE) with the subsequent cloning and sequencing of 16S rRNA gene.

UPFLOW ANAEROBIC SLUDGE BLANKET (UASB).

Anaerobic wastewater treatment is widely used all over the world. It needs lower energy input and gives less surplus sludge as compared to aerobic wastewater treatment. The Upflow Anaerobic Sludge Blanket (UASB) bioreactor (Figure 2) is the favorite anaerobic treatment system used (Roest and Heilig, 2005).

The UASB reactor was developed in the Netherlands (Lettinga *et al*., 1980). The wastewater flows in the bottom of the anaerobic reactor and through a layer of naturally forming, dense biological sludge granules. The sludge particles range in size from flocculants to granules with a diameter of 0.25 in. Gases formed in the digestion process generate mixing action and promote granule formation in the sludge layer. The methane gas is captured in a reservoir at the top of the reactor and may be used for energy reclamation (Lettinga *et al*., 1980).

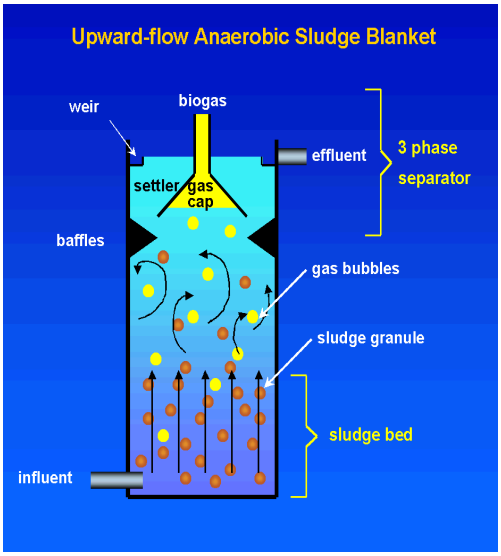


Figure 2: Upflow Anaerobic Sludge Blanket (UASB).

Anaerobic systems do not require aeration, which is typically a significant cost for aerated systems. In addition, the amount of sludge produced is less than that produced by aerobic systems. However, the treatment process is more sensitive to the presence of toxic compounds and changes in temperature than aerobic systems. Despite these advantages, the UASB reactors have difficulties in producing effluents that can comply with the environmental standards. Therefore, the post-treatment step is of great importance as a manner of adapting the treated effluent to the environmental discharge standards. The main objective of the post-treatment is to complement the organic matter removal, as well as to promote the removal of components which are rarely affected by the anaerobic treatment like nutrients and pathogens (Chernicharo and Nascimento, 2001).

1.1.2. AEROBIC WASTERWATER TREATMENT:

Aerobic systems treat wastewater using natural processes that require oxygen. Bacteria that thrive in oxygen-rich environments work to break down and digest the wastewater inside the aerobic treatment unit. Aerobic systems treat the wastewater in stages. Sometimes the wastewater receives pretreatment before it enters the aerobic unit. Treated wastewater leaving the unit requires additional treatment (passage through a soil absorption field) before being returned to the environment.

There are a variety of designs for aerobic systems, but they do have some common features. These include pretreatment to reduce the amount of clogging solids, an aeration process, settling for suspended growth systems, and final treatment/disinfection. The most common kind of aerobic system is "suspended growth." Air is forced into an aeration compartment in which sewage-digesting bacteria are freely suspended in the liquid/air mixture. The other method is "attached growth," in which a surface is provided for bacteria to attach themselves. The surface is alternately exposed to the liquid and air.

1.1.2.1. ATTACHED GROWTH BIOFILTER:

In a fixed-film biological process, microorganisms are attached to a solid substratum where they reach relatively high concentrations. The support materials include gravels, stones, plastic, sand, or activated carbon particles. Two important factors that influence microbial growth on the support material are the flow rate of

wastewater as well as the size and geometric configuration of particles (Bitton, 2005).

Biofilm reactors comprise trickling filters, rotating biological contactors (RBC), and submerged filters (downflow and upflow filters). These reactors are used for oxidation of organic matter, nitrification, denitrification, or anaerobic digestion of wastewater (Harremoes, 1978)

Reactors with fixed biomass (biofilters with solid filling in form of packets or loose plastic profiles) offer less possibilities with regard to biological nutrient removal. Primarily, as the biomass is fixed to biofilter's filling; it is not possible to integrate all processes in one biofilter like in the reactors with activated sludge. Polish operational experience shows that two-stage biofilter system with recirculation: 1- anaerobic contact bed, denitrifying, 2- aerobic trickling filter, nitrifying is a good solution (Kurbiel, 1998).

The removal efficiency of the biofilters is lower than activated sludge systems while the cost of operation is low and the system is very simple.

There are two types of biofilters used in wastewater treatment systems:

1-Trickling filters, where wastewater flows from the top to the bottom through porous media. Such biofilters at low loads can remove not only BOD_5 but also sustain nitrification.

2- Contact beds, where the filling in form of packets or loose plastic profiles is submerged in wastewater. Loose filling is suspended by wastewater flow from the bottom to the top. Contact beds may be aerated, and then the removal efficiency is similar to that obtained by trickling filters (removal of BOD_5 and possible

nitrification), or non-aerated, and then they can sustain denitrification. Contact bed used for denitrification in first stage reduces nitrates contained in recirculation from second stage (aerated) utilizing organic carbon contained in primary effluent (Kurbel, 1998).

Anoxic processes are typically used for the removal of nitrogen from wastewater. The process of biological nitrogen removal is known as denitrification. Denitrification requires that the nitrogen must be first converted to nitrate, which occurs in an aerobic treatment process such as a trickling filter or aerated suspended growth system. This type of water (nitrified water) is then processed in an environment without free oxygen. Organisms in this anoxic system use the nitrate as an electron acceptor and release nitrogen in the form of nitrogen gas or nitrogen oxides. A readily biodegradable carbon source is also needed for efficient denitrification processes to occur. It should be noted that sulfate can also be used as an electron acceptor, resulting in the formation of hydrogen sulfide. The research conducted in the filtration of the primary septic tank and settler effluent by (Buuren *et al*., 1999) reported that, in a single stage biofilter, the removal of nitrogen compounds can be achieved by nitrification and denitrification.

The recycling of nitrogen in the environment is essential to life's existence. Nitrification, a multi-step biological process, is an important part of the nitrogen cycle and also an integral component of water treatment systems. The overall reaction that defines nitrification is the conversion of ammonia to nitrate. Nitrate is a more oxidized form of nitrogen that can be utilized by plants and other bacteria. The reaction occurs as shown in Figure 3. The first step is the oxidation

of ammonia to nitrite, with hydroxylamine formed as an intermediate. This reaction is carried out by chemolithoautotrophic ammonia-oxidizing bacteria (AOB). The nitrite is then converted to nitrate by nitrite-oxidizing bacteria (NOB).

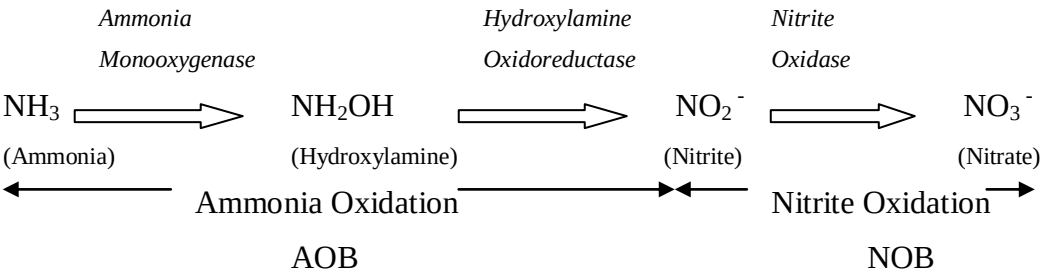


Figure 3: The Process of Nitrification

1.1.2.2. ACTIVATED SLUDGE PROCESS.

Activated sludge is a suspended-growth process that began in England at the turn of the century. This process has since been adopted worldwide as a secondary biological treatment for domestic wastewaters. This process consists essentially of an aerobic treatment that oxidizes organic matter to CO_2 and H_2O, NH_4, and new cell biomass. Air is provided by using diffused or mechanical aeration.

The activated sludge process has found vast application as an effective means of wastewater treatment. The objective of the activated sludge process is to remove soluble and insoluble organics from the wastewater and to convert this material into a flocculent microbial suspension that settles well in a conventional gravity

clarifier. Basically, activated sludge comprises a microbiological enrichment culture consisting of a mixed, and largely uncontrolled, consortium of micro- and macro-organisms that remove wastewater inorganics and organics and transform them into environmentally acceptable forms (Ramothokang *et al.,* 2003).

The success of the activated-sludge process depends on establishing a mixed community of microorganisms that will remove and consume organic waste material, that will aggregate and adhere in a process known as bioflocculation, and that will settle to produce a concentrated sludge (Return Activated Sludge) for recycling and clean effluent.

It is important to maintain the growth of floc-forming bacteria on wastewater organics, which will settle under gravity in the final clarifier so as to obtain or sustain a clarified supernatant (final effluent) and a thickened return sludge. However, not all bacteria in the activated sludge process are floc-formers. Many different types of filamentous bacteria have been identified in activated sludge and play important roles in wastewater treatment. Filamentous bacteria directly affect sludge settling as they make provision for the rigid support network or backbone upon which floc-forming bacteria can adhere and grow into suitable activated sludge flocs (Richard, 1989).

ACTIVATED SLUDGE BULKING:

Since the introduction of continuous-flow reactors, sludge bulking has been one of the major problems affecting biological waste treatment (Sykes, 1989). There are several types of problems regarding solid separation in activated sludge. Of these problems are the filamentous bulking and foaming.

Filamentous bulking is caused by the overgrowth of filamentous bacteria in activated sludge. These bacteria are normal components of activated sludge flocs but may out compete the floc-forming bacteria under specific conditions.

Biological foaming in activated sludge wastewater treatment systems can be described as the formation of a scum layer on the surfaces of aeration basins and secondary clarifiers due to the presence of large quantities of hydrophobic filamentous (Jenkins *et al*., 1993) and possibly non-filamentous microorganisms (Davenport and Curtis, 2002). This problem is widespread around the world, and 20–60% of wastewater treatment plants experience biological foaming from time to time (Pitt and Jenkins, 1990).

Filamentous microorganisms can be good indicators of conditions prevailing in an activated sludge system on a microbiological level. The indications given by the filamentous bacteria could be of low dissolved oxygen (DO) low food-to-micro-organism (F/M), presence of septic waste, nutrient deficiency and low pH in the system (Jenkins *et al.*, 1986).

Microorganisms identified in foams are *Nocardia* (now *Gordona*) *amarae* (Klatte *et al*., 1994), *N. rhodochrous, N. asteriodes, N. caviae, N. pinensis* now called *Skermania piniformis* (Chun *et al*., 1997), *Streptomyces sp, Microthrix parvicella,*

Micromonospora, Type 0675 and *Rhodococcus* (Blackall *et al*., 1989; Goddard and Forster, 1987; Lechevallier and Lechevallier, 1974; Lemmer and Kroppenstedt, 1984; Pujol *et al*., 1991; Seviour *et al*., 1990; Sezgin and Karr, 1986; Sezgin *et al*., 1988). *Nostocoida limicola*, Type 0041 (Goddard and Forster, 1987), *Sphaerotilus natans*, *Hyphomicrobium sp*., *Thiothrix nivea* (Jenkins *et al*., 1993; Layton *et al*., 2000), Type 1851 (Seviour and Blackall, 1999), member of chloroflexi phyla ((Beer *et al*., 2002) and candidate phylum TM7 (Hugenholtz *et al*., 2001) of the filamentous bacteria that can cause activated sludge foaming, *Nocardia* and *Microthrix parvicella* (commonly), and type 1863 (rarely).

Nocardial foam occurs as thick, stable, brown foam or "scum" inches to many feet thick on aeration basin and final clarifier surfaces. Description of filamentous bacteria and causes of activated sludge foaming are summarized in Tables 1 and 2 (Richard *et al.,* 2003).

Filamentous micro-organisms traditionally have been identified by their morphology and simple staining reactions (Eikelboom and van Buijsen 1983). The majority of filamentous bacteria in sludges, however, are still unidentified beyond these simple characteristics (Lindrea *et al*., 1999). Recently molecular methods have been used to identify and monitor filamentous micro-organisms (Blackall, 1994, Bradford *et al*., 1996, Erhart *et al.,* 1997, Kanagawa *et al*., 2000).

Table 1: Description and causes of activated sludge foaming (Richard *et al*., 2003).

Foam Description	**Cause (s)**
thin, white to gray foam	low cell residence time or "young" sludge (startup foam)
white, frothy, billowing foam	once common due to non-biodegradable detergents (now uncommon)
pumice-like, grey foam (ashing)	excessive fines recycle from other processes (e.g. anaerobic digesters)
thick sludge blanket on the final clarifier(s)	Denitrification
thick, pasty or slimy, grayish foam (industrial systems only)	nutrient-deficient foam; foam consists of polysaccharide material released from the floc
thick, brown, stable foam enriched in filaments	filament-induced foaming, caused by *Nocardia, Microthrix* or type 1863

1.2. MOLECULAR TECHNIQUES FOR THE IDENTIFICATION OF MICROBIAL COMMUNITIES IN WASTEWATER TREATMENT SYSTEMS.

Today, most modern wastewater treatment processes rely on the action of complex microbial communities. These microbial communities functioning in wastewater treatment plants have been accepted as ‘’Black Boxes’’ for a long time (Cofikuner, 2002).

Table 2: Causes of filaments in activated sludge foaming (Richard *et al*., 2003).

Cause	**Filaments**
low dissolved oxygen concentration	*Sphaerotilus natans*, type 1701 *Haliscomenobacter hydrossis*
low F/M	type 0041, type 0675, type 1851, type 0803 *Microthrix parvicella*
septicity	type 021N, *Thiothrix* I and II *Nostocoida limicola* I,II,III, type 0914 type 0411, type 0961, type 0581, type 0092
grease and oil	*Nocardia sp*., *Microthrix parvicella* type 1863
nutrient deficiency: nitrogen: phosphorus:	 type 021N *Thiothrix* I and II *Nostocoida limicola* III *Haliscomenobacter hydrossis* *Sphaerotilus natans*

In wastewater treatment bioreactors, microorganisms are present and active in biofilms and aggregates. Understanding the structure and function of these complex communities would be very useful for improving the design and operation of plants

The structure and function of micro-organisms in wastewater treatment plants (WWTP) have been investigated for decades. However these microorganisms can not be estimated easily by classical methods and most of them are still not isolated and identified. The reason that these microorganisms can not be estimated is the

lack of information about the natural habitat they grow in. A second reason is that a bacterium is often part of a larger more complex community or ecosystem with possible co-dependence on other members; consequently, this explains why classical culture techniques fail to accurately reflect the large microbial diversity in an environmental sample.

Growth-based methods do not give very reliable results on the microbial ecology of bacteria because of media selectivity. Growth media tend to contain high nutrient sources which encourage the growth of bacteria that can grow rapidly under these conditions. Bacteria present in relatively low numbers under normal conditions may proliferate rapidly in media and out-compete more abundant organisms. Many bacteria have not yet been cultured and such organisms cannot be characterized by growth-based methods (Cofikuner, 2002). Quantitative discrepancies are also significant. For example, it has been shown that results from plate count techniques can range from 1% to 15% of the total number of cells determined by direct microscopic counts (Manz *et al*., 1994)

Beside conventional culture dependent techniques, modern molecular biological techniques have been used to study the diversity and ecology of microorganisms in wastewater treatment processes since the mid-1980s. These techniques enabled researchers to better understand the aerobic and anaerobic microbial wastewater process and control. Since that time, molecular techniques have become increasingly popular in the detection and characterization of bacteria since these techniques do not require prior isolation and enrichment of these bacteria.

Several molecular techniques which are nucleic acid based methods have been developed to detect and determine bacteria. In bioreactors, where stability and performance is strongly dependent on the complex microbial interactions, this development can provide an opportunity to establish the connection between the microbial structure and the functional characteristics of the system (Pereira *et al.*, 2002)

With the rapidly growing knowledge of DNA sequences of homologous genes of different bacteria species, these methods become increasingly powerful tools, not only in academic research, but also for microbiological routine analysis. Recently, the development of culture-independent molecular techniques, like fluorescence *in situ* hybridization (FISH), polymerase chain reaction (PCR) or denaturing gradient gel electrophoresis (DGGE) improved the analysis of environmental samples (Eschenhagena *et al.*, 2003). Figure 4 summarizes the new molecular methods used to study microbial communities in natural habitats.

In several studies, these techniques were applied to investigate different kinds of wastewater treatment plants: laboratory-scale sequencing batch reactor (SBR) (Crocetti *et al.,* 2000) and pharmaceutical wastewater treatment plants (LaPara *et al.*, 2000).

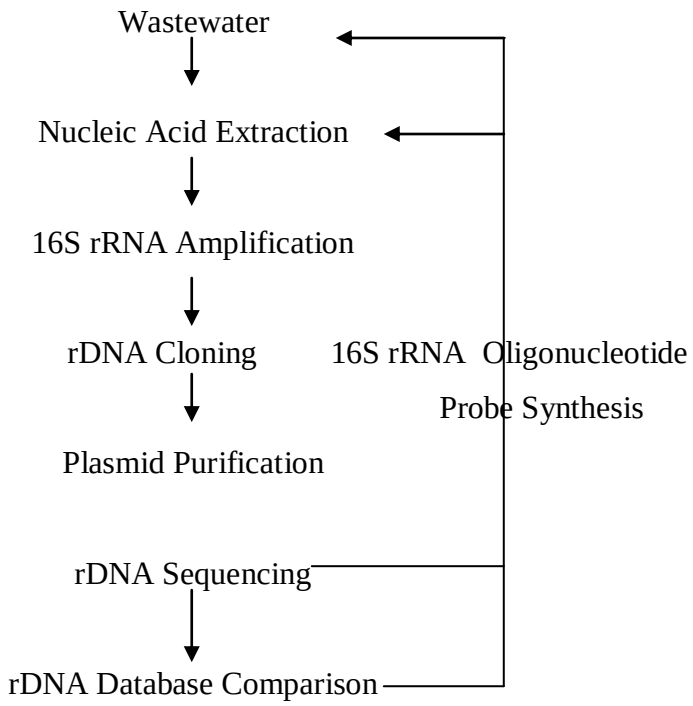


Figure 4: Characterization of Microbial Community by 16S rDNA Analysis.

1.2.1. POLYMERASE CHAIN REACTION (PCR):

Only a decade ago, the prospect of producing billions copies of a specific nucleic acid sequence by performing successive rounds of *in vitro* nucleic acid replication would have been considered science fiction (Persing, 1993).

The basic ingredients for an *in vitro* nucleic acid amplification method were described in a report by Kleppe *et al*. (1971). PCR became a reality in a relatively short time, leading to the publication by Saiki *et al*., of its first application in 1985.

Polymerase Chain Reaction (PCR) provides a sensitive tool of amplifying small quantities of DNA. The technique was become possible after discovery of *Taq* polymerase, the DNA polymerase that is used by the bacterium *Thermus*

auquaticus discovered in hot springs. This DNA polymerase is stable at the high temperatures of the PCR process for the amplification, whereas other DNA polymerases and enzymes are denatured.

The reason that makes PCR so useful is that the genetic material of each living organism-plant or animal, bacterium or virus-possesses sequences of its nucleotide building blocks (usually DNA, sometimes RNA) that are uniquely and specifically present only in its own species. Indeed, complex organisms such as human beings possess DNA sequences that are uniquely and specifically present only in particular individuals. These unique variations make it possible to trace genetic material back to its origin, identifying with precision at least what species of organism it came from, and often which particular member of that species. For species identification enough DNA under study must be available for analysis and this is the function of PCR which assemble the natural function of the enzymes known as DNA polymerases. These enzymes are present in all living things, and their function is to copy the genetic material. Small quantities from blood, hair, or tissue specimens, microbes, animals, or plants, PCR can characterize, analyze, and synthesize any specific piece of DNA or RNA even in even thousands or millions of years old. PCR requires a template molecule of the DNA you want to copy and two primer molecules to get the copying process started. The primers are short chains of the four different nucleotide components that make up any strand of genetic material. These four components are the building blocks that are used to construct genetic molecules. DNA itself is a chain of nucleotides. Under most conditions, DNA is double-stranded, consisting of two such nucleotide chains that

wind around each other in the double helix shape. Primers are single-stranded DNA. They consist of specific order of nucleotides that will bind a complementary sequence of the required single stranded DNA or RNA under optimal conditions. There must be two primers of nucleotide sequence that flanking the piece of DNA of interest. There are three basic steps in PCR:

1- **Denaturation**, the target genetic material must be denatured-that is, the strands of its helix must be unwound and separated-by heating to 90-96°C.
2- **Hybridization or Annealing**, in which the primers bind to their complementary bases on the now single-stranded DNA by lowering the temperature below the melting point (Tm) of the primers.
3- **Extension**, where DNA synthesis starts by a DNA polymerase. Starting from the primer, the polymerase can read a template strand and match it with complementary nucleotides very quickly with or without proofreading. The result is two new double stranded DNA, each composed of one of the original strands plus its newly synthesized complementary strand. Repeating the process for just 35 cycles can generate millions of copies of a specific DNA strand.

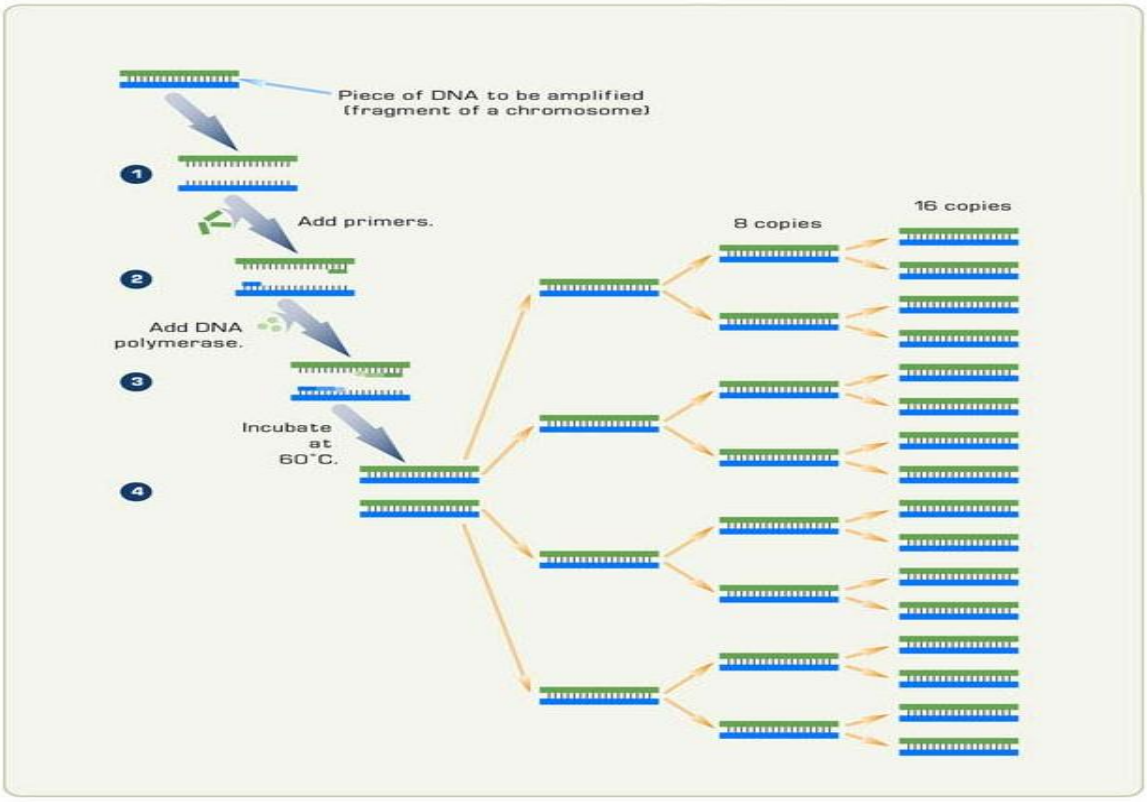


Figure 5: PCR process: 1) Denaturation. 2) Annealing. 3) Extension. 4) Repeating the process.

1.2.2. THE 16S rRNA GENE:

The ribosomal RNA (rRNA) approach is becoming a widely used method for studying the microbial community structure of natural and man-made environments in a truly cultivation-independent way. Ribosomal RNA genes have particular advantages as a molecular marker in molecular methods. Firstly, all living cells contain ribosomes, which are part of the cells' apparatus for translating deoxyribonucleic acid (DNA) into protein. rRNA is a dominant cellular macromolecule (Figure 6). Most bacterial cells have somewhere between 10^3 and 10^5 ribosomes. This natural amplification results in excellent sensitivities of hybridization assays. Secondly, the cellular RNA content varies depending on

the general metabolic activity or growth rate of a given species. Thirdly, rRNA are excellent molecules for discerning evolutionary relationships among bacteria because RNA molecules contain conserved and variable regions which make it possible to find general as well as specific target sites for probes. These regions are used for identification purposes. A practical reason for using rRNA is the public availability of large databases. They have enough sequence information to be used as a phylogenetic marker (Cofikuner, 2002).

The traditional methods for identification of bacteria depend on isolation and propagation in the laboratory. Biochemical, morphologic, and serologic tests usually require growth of the organism. Reliance on these procedures may have significantly limited our awareness of true bacterial diversity and is impractical in many situations. The rapidly expanding use of 16S rRNA sequence for phylogenetic, evolutionary, and diagnostic studies offers an opportunity for alternative approaches (Persing, 1993).

Over the past 20 years, more than 78,000 16S rRNA gene sequences have been deposited in GenBank and the Ribosomal Database Project, making the 16S rRNA gene the most widely studied gene for reconstructing bacterial phylogeny (Schloss and Handelsman, 2004)

16S rRNA genes are found in all bacteria and they have slow mutations rate over time. Highly polymorphic regions of the 16S rRNA provide unique signature to any bacterium and useful information about the relationship between them. On the other hand, since 16S rRNA have a certain conserved regions found in all known bacteria, PCR primers may be then designed to recognize these conserved

bacterial 16S rRNA gene sequences and used to amplify intervening, variable, or diagnostic regions. This procedure avoids the need to grow the bacterium and requires no preexisting phylogenetic information (Persing, 1993).

The average size of a 16S rRNA gene is 1500 bp, which is sufficient for preliminary phylogenetic analysis (Woese, 1987)

The highly defined characteristics of the 16S rRNA compensate for the difficulties with culture-based methods. To obtain preliminary information, bacteria no longer need to be cultured in the lab. Instead, they can be taken directly from their natural environment, DNA is extracted from the mixed bacteria and a universal primer is used to amplify the 16S rRNA variable regions.

The result of these PCR reactions is a mixed DNA product with equal size but having a species – specific variations. Different molecular methods can then subsequently be used to analyze and manipulate the PCR products, thereby allowing for the characterization of an unknown bacterial population.

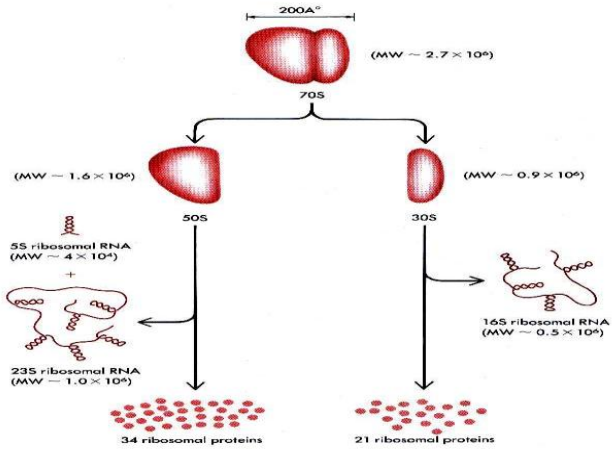


Figure 6: Ribosomal RNA.

1.2.3. DENATURING GRADIENT GEL ELECTROPHORESIS (DGGE):

Denaturing Gradient Gel Electrophoresis (DGGE) is a method that allow the separation of fragments of DNA which differ as a little as a single nucleotide. The method includes the PCR products double stranded DNA electrophoresis through a polyacrylamide gel containing a linear increase of denaturing agents (Figure 7) like formammide and urea, although increasing temperature have also bee successfully applied.

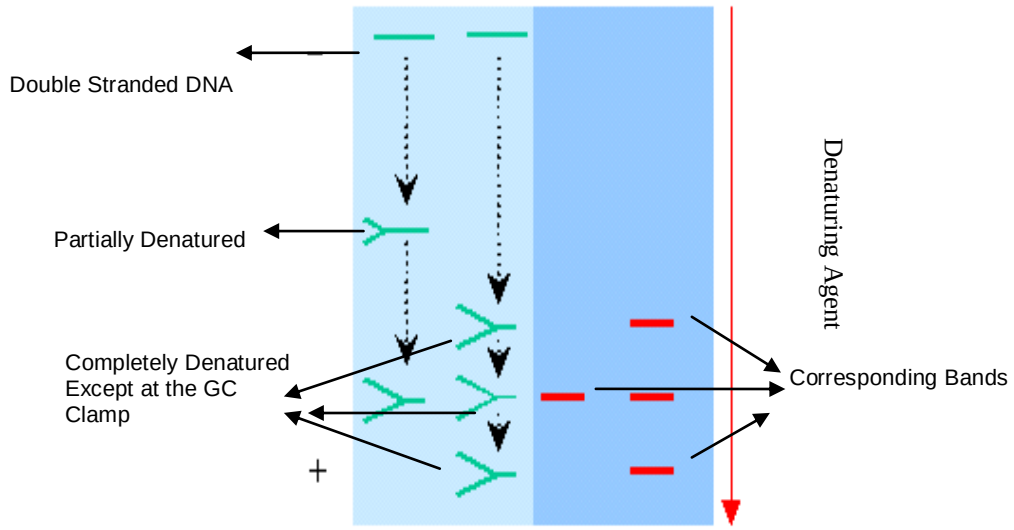


Figure 7: Schematic Diagram of DGGE Technique.

The separation of the DNA fragments by DGGE is based on the melting properties of double stranded DNA. The melting temperature of a double stranded DNA fragment is influenced by hydrogen bonds formed between complementary base pairs and also by the attraction between neighboring bases on the same strand (known as stacking interactions). The order of bases on a strand determines

the degree of stacking. A DNA molecule may therefore have several melting domains with characteristic melting temperatures (Tm) determined by the nucleotide sequence. Changes in the base sequence as small as a single base may alter the stacking significantly enough to modify the Tm by over 1°C. When separated by electrophoresis through a gradient of increasing temperature (TGGE) or chemical denaturant (DGGE), the mobility of the molecule is retarded at the concentration or temperature at which the DNA strands dissociate, forming a partially single stranded molecule with no further movement in the gel. Complete denaturation is prevented by the presence of a high melting domain, which is usually artificially created at one end of the molecule by incorporation of a GC clamp. This clamp is simply a long string of GC-repeats that gives one section of the fragment a high melting point, prohibiting it from completely denaturing into two single-stranded molecules during electrophoresis.

In principle, this means that DNA fragments of the same length are separated on the basis of differing sequences, even if only by a single base.

Denaturing electrophoresis quickly provides qualitative information regarding the diversity of the bacterial composition of a mixed culture, but identifying the bacterial components requires further manipulation. Theoretically, each distinct band is indicative of one unique species. A potential problem that must be given consideration is multiple fragments having similar mobility. In order to demonstrate that a band of interest only contains one species of PCR product, it may be excised and the nucleotide sequence analyzed (Fouratt, 2001).

The aim of doing DGGE is to monitor the microbial composition and activity during operation of the reactors. Profiles generated by DGGE can be analyzed by comparing the presence or absence of individual bands as well as measuring the intensity of a band within a profile. The intensity can relate to the relative abundance of a sequence within a sample, although at best this is considered a semi-quantitative measure that it allows the simultaneous analysis of multiple samples, making it feasible to monitor shifts in populations over periods of time or different environmental conditions.

Before the arrival of DGGE this was more commonly achieved by cloning and sequencing - an approach that is both labors intensive and relatively costly, especially when dealing with numerous samples. The DGGE method was first used to profile microbial communities and bacterial biofilms by Muyzer *et al*., (1993) and since then has been used to analyze microbial communities from extremely diverse microbial environments like wastewater treatment reactor, soil and marine microbial communities. DGGE has proved to be an exceptional tool to study species diversity and bacterial community dynamics (Hope, 2004).

1.2.4. FLUORESCENT *IN SITU* HYBRIDIZATION (FISH):

Fluorescent *in situ* hybridization (FISH) also is one of the most commonly used molecular methods for the identification of microorganisms in WWTP. With FISH, the target nucleic acids are detected directly in the cells. To achieve *in situ* detection, cells should be permeabilized to allow the probe access to the inside of cells. At the same time, the morphological integrity of the examined cells should

be maintained. This is usually achieved by fixing the cells with alcohols or aldehydes. Probes labeled with a fluorescent dye bind to a specific sequence in the ribosomal RNA of the target organism (s) of interest during the hybridization procedure. Figure 8 summarize the overall FISH process.

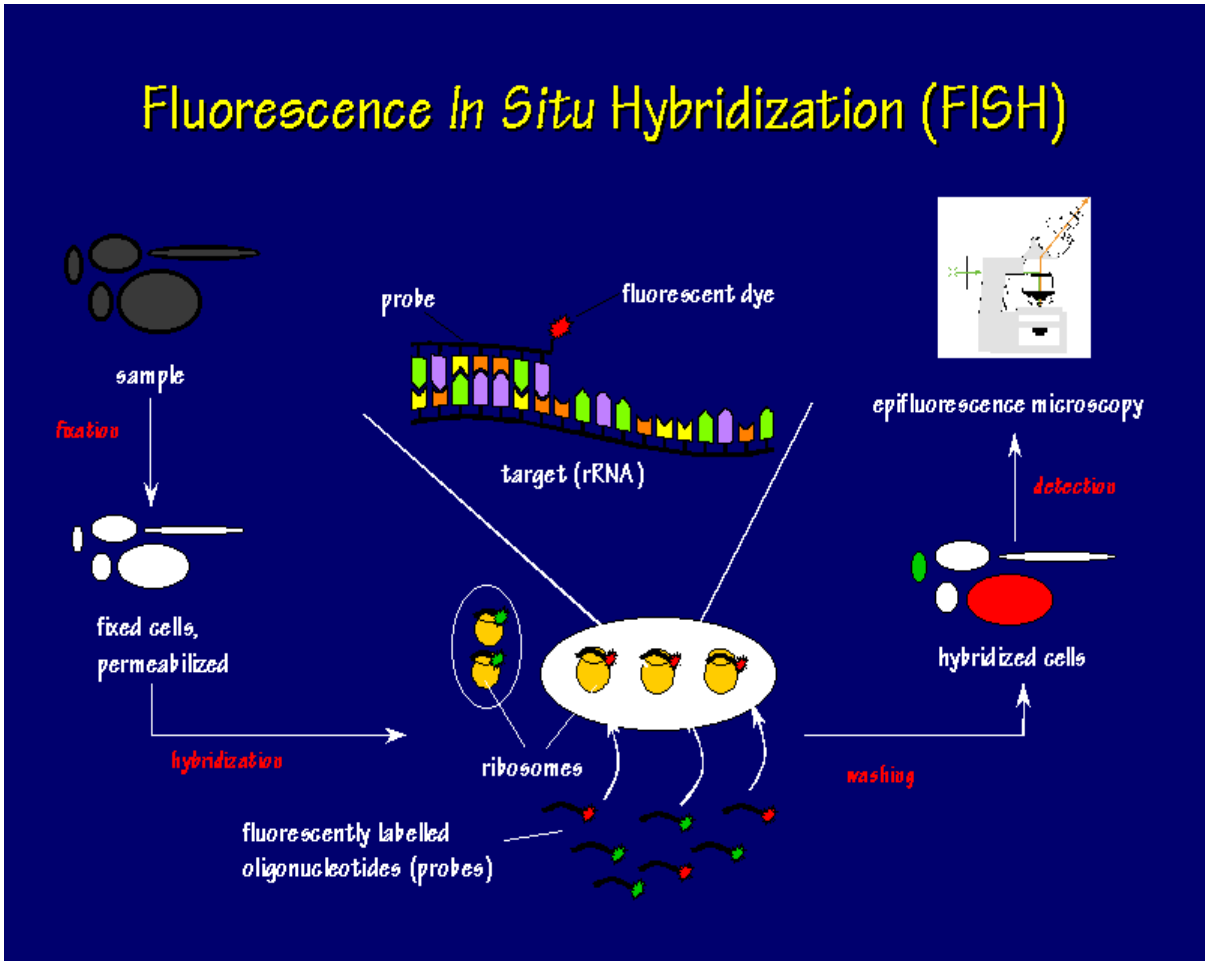


Figure 8: Fluorescent *In Situ* Hybridization (FISH).

The natural amplification of ribosomes and because rRNA have excellent discerning evolutionary relationship among bacteria, all give excellent sensitivities of hybridization assays and specific target sites for probes in FISH analysis. Over the past decade, FISH has become an appropriate tool to detect and

study microorganisms in their natural habitats (Amann *et al.,* 1995). FISH has been used in different studies to detect specific groups of Bacteria and Archaea in order to characterize the microbial population located in anaerobic biofilms (Amann *et al*., 1992); (Araujo *et al.*, 2000) and granular sludges (Sekiguchi *et al.*, 1999).

Using fluorescent *in situ* hybridization and other microscopy techniques for identification and localization of microorganisms could be achieved in different environment especially UASB for monitoring the development of the interested microorganisms in the reactor during the startup phase of the reactors. Methanogens are obligate anaerobic bacteria that are difficult to culture in the laboratory. The simplest method to identify and monitor the type of bacteria is the whole cell fluorescent *in situ* hybridization with 16S rRNA targeted probes.

The methanogens were the first microbial group to have their taxonomy based on the phylogeny inferred from 16S rRNA sequence divergence (Kurbiel, 1998) and there are many probes used for methanogens identification and localization.

OBJECTIVES:

- Application of molecular techniques (PCR, DGGE, Cloning and 16S rRNA sequencing) to identify bacteria causing scum formation in the secondary sedimentation tank of BWWTP.
- Recommendation of ways to control this phenomenon that causes deterioration of the effluent and operational problems.
- Molecular identification of microbial communities of a UASB-Multimedia Biofilters System in order to optimize the operation for efficient treatment.

CHAPTER TWO

MATERIALS AND METHODS

2.1. UASB INSTALLATION:

A 0.77 m^3 pilot-scale reactor UASB attached to two down flow biofilters packed with porous stones, sands and aggregates was installed at Al Bireh Wastewater Treatment Plant (BWWTP). The two Biofilters volumes were 0.7 m^3 (No. 1) and 0.44 m^3 (No. 2) volume respectively as seen in Figure 9. The average temperature of Al Bireh City is 23ºC during the month of sampling (August 2005). The flow Rate to the system was 200L/d and the hydraulic Retention Time (HRT) was 11.6 hrs for the UASB and 3hrs for both biofilters. Together the overall treatment efficiency of the system is 97% for COD and 29% for ammonia oxidation. The methane production was about 10 L/day.

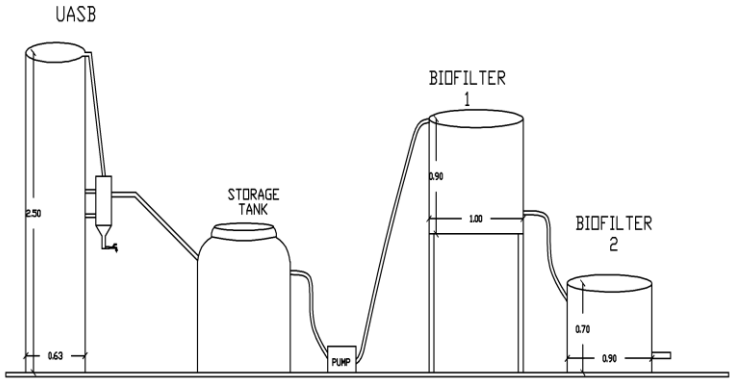


Figure 9: A schematic diagram of the pilot scale treatment system.

2.2. SAMPLING:

Three types of sample were taken during August 2005 in 50 ml sterile tubes in an ice box and transferred directly to Birzeit University laboratories. The samples were upper layer of a UASB sludge sample, Biofilters samples and the third sample was from the scum layer of secondary sedimentation tank of BWWTP. Gram stain was done from the fresh samples and the other part was preserved and stored in freezer until use. The preservation solution contains 6ml sample, 9.5 ml 96% ethanol and 0.5 ml of 0.8 M NaCl.

2.3. GENOMIC DNA PURIFICATION:

Genomic DNA was extracted according to Oude Elferink *et al.,* 1997 (with minor modifications). About 1 ml of preserved sample was dissolve in 400 µl sterile TE (10 mmol/L TRIS/HCl + 1 mmol/L EDTA, pH=8) in a 2.0 ml tube with 300 µl glass/zirconium beads (diameter: 0.11 mm). Thereafter, 200 µl TRIS/HCl buffered phenol pH=8 were pipetted into the tube. Then, the tube was bead beated for a total of 5 minutes with cooling on ice after each minute. The tube was centrifuged for 10 minutes at maximum speed (in a pre-cooled centrifuge) and the water phase (top) was transferred to a sterile 1.5 ml tube. DNA was extracted with 500 µl phenol/chloroform/isoamylalcohol 25:24:1 (v:v:v). If there was still a big inter phase (still proteins in the water phase), another extraction was done as above. Thereafter, another extraction with 500 µl chloroform/isoamylalcohol 24:1 (v:v) was done through centrifugation for 5 minutes at maximum speed. Then, the

water phase was transferred to a new tube and the volume was adjusted to 0.5 ml and the DNA was precipitated with 1 ml of 96 % ice-cold ethanol, 40 µl sodium acetate (3 mol/L pH=5.2) and stored overnight at -20°C.

DNA was collected by centrifugation for 15 minutes at maximum speed (in a pre-cooled centrifuge). The pellet was washed with 70 % ice-cold ethanol, dried, dissolved in 100 µl TE and stored either at -20°C for later analysis or at 4^{o}C for immediate use.

2.4. PRIMERS DESIGN:

Table 3 describes the sequence, specificity and the references of the primers used in this study for both PCR (DGGE and whole 16S rRNA gene).

Table 3: Primers used in this study.

No	Primer	Sequence (5'→ 3')	Specificity	Reference
1.	0007F	AGA GTT TGA T**YM** TGG CTC AG	Bacteria	Lane. (1991)
2.	0109F	AC**K** GCT CAG TAA CAC GT	Archaea	Großkopf *et al*. (1998)
3.	1492R	CGG CTA CCT TGT TAC GAC	Universal	Lane. (1991)
4.	0109F-T	ACT GCT CAG TAA CAC GT	Archaea	Großkopf *et al*. (1998)
5.	0515R	CGC CCG GGG CGC GCC CCG GGC GGG GCG GGG GCA CGG GGG G ATC GTA TTA CCG CGG CTG CTG GCA C	Universal	Lane. 1991
8.	0968F	CGC CCG GGG CGC GCC CCG GGC GGG GCG GGG GCA CGG GGG G AAC GCG AAG AAC CTT AC	Bacteria	Nübel *et al* (1996)
9.	1401R	CGG TGT GTA CAA GAC CC	Bacteria	Nübel *et al* (1996)
M=A, C **K**=G, T **Y**=C, T				

2.5. PCR AMPLIFICATION OF PARTIAL SEQUENCE OF THE 16S rRNA GENE FOR DGGE ANALYSIS:

For DGGE analysis the 16S rRNA was partially (V6–V8 region for Bacteria and V2–V3 region for Archaea) amplified from genomic DNA by PCR using *Taq* DNA Polymerase (Bio-Rad. USA). The amplification was done in a reaction mixture of 50 µl containing 1 µl genomic DNA, 0.2 mM deoxynucleoside triphosphates, 0.2 µM of primers (968 F– GC and 1401 R for Bacteria and 109 F-T and 0515-GC for Archaea (Table 2)), 1.25U *Taq* DNA Polymerase, 20 mM Tris-HCl (pH=8.4), 50 mM KCl, and 3 mM $MgCl_2$. Samples were subjected to the following thermocycling program: predenaturation at 95°C for 5 min followed by 35 cycles of 95°C for 30 sec, annealing at 52°C for 40 sec (Archaea) and 56°C for 20 sec (Bacteria), elongation at 72°C for 1 min (Bacteria) and 40 sec (Archaea) followed by post-elongation for 7 min at 72°C.

Finally, PCR products were subjected to agarose gel (1%) electrophoresis against 100bp DNA marker, stained with SYBR Green and visualized using UV transluminator.

2.6. 16S rRNA GENE AMPLIFICATION:

The 16S rRNA gene (~1500 bp) was amplified from genomic DNA by PCR in a reaction mixture of 50 µl containing 1 µl genomic DNA, 0.2 mM deoxynucleoside triphosphates, 0.2 µM of primers 07-f and 1492- r (Bacteria) and 0109-f and 1492-r (Archaea), 1.25 U *Taq* DNA Polymerase, 20 mM Tris-HCl (pH=8.4), 50 mM KCl, and 3 mM $MgCl_2$. Then, Samples were subjected to the

following thermocycling program: predenaturation at 95°C for 5 min followed by 35 cycles of 95°C for 30 sec, annealing at 52°C for 40 sec (Archaea) and at 48°C for 20 sec (Bacteria), elongation at 72°C for 1 min (Archaea) and 40 sec (Bacteria) and post elongation for 7 min. The PCR product was separated on 1% agarose, stained and visualized by UV transluminator.

2.7. DENATURING GRADIENT GEL ELECTROPHORESIS (DGGE):

DGGE analysis of the amplified (~500bp) portion of the 16S rRNA was performed on 8% (w/v) polyacrylamide gels containing denaturant gradients of 20–60%. The 100% denaturing solution containing 7 M urea and 40% (v/v) formamide. Gelbond film (Amersham Biosciences, Little Chalfont, England, UK) was used as a physical support for the gel during staining and drying. 10 µl of the PCR product were loaded and electrophoresis was performed in 0.5-X TAE buffer (20 mM Tris, 10 mM acetic acid and 0.5 mM EDTA, pH= 8) at 85 V and 60°C for 16 h using a DCodeTM System (BioRad, Hercules, CA, USA). During the first 5 min of electrophoresis, a voltage of 200 V was applied.

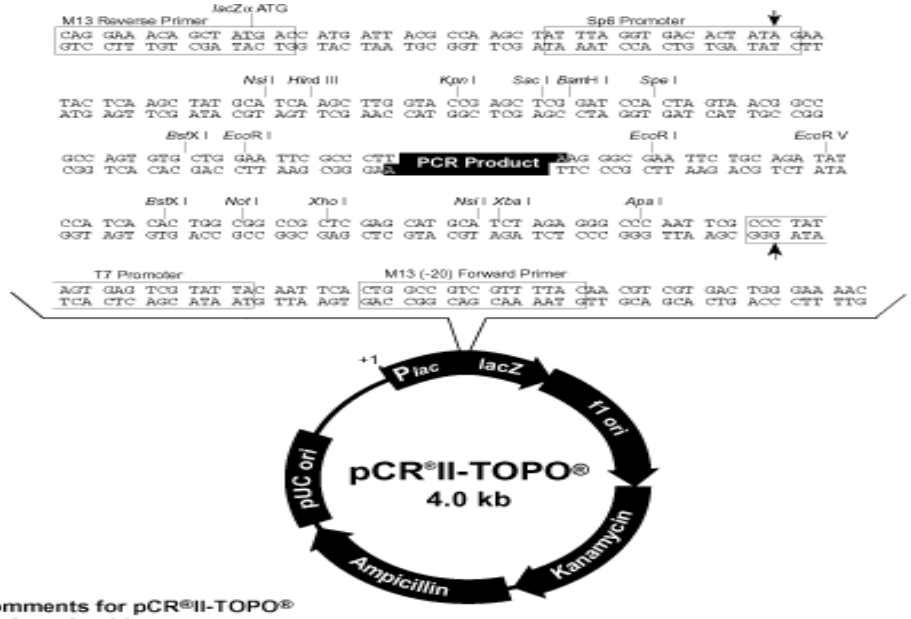
GEL SILVER STAINING:

Staining and development of the gels was performed as described by Sanguinetti *et al.,* (1994). Briefly, the gel was fixed in Carlos fixation solution (9.6 % ethyl alcohol and 0.5% acetic acid) for 3 min, staining for 10 min (0.2 % silver nitrate in Carlos fixation solution), color developing (0.005 % sodium borohydrate, 0.3 % formaldehyde and 1.5 % NaOH) till the bands were clear and the gel was

preserved using a preservation solution (24 % ethyl alcohol and 10 % glycerol). The gel was placed on a glass plate and covered by cellophane foil and dried over night at 45°C.

2.8. CLONING:

The amplified 16S rRNA gene products were purified with a QIAquick PCR purification kit (Qiagen GmbH, Hilden, Germany) according to manufacturer instructions, cloned in One shot ® OmniMax™ 2–T1® chemically competent *Escherichia coli* (Invitrogen, Breda, The Netherlands) by using the pCRII® – TOPO® plasmid (Invitrogen) (Figure 10). Thereafter, 50 µl and 100 µl of the cloning mixture were plated in LB Agar with ampicillin and Blue/White selection.



Comments for pCR®II-TOPO®
3973 nucleotides

LacZα gene: bases 1-589
M13 Reverse priming site: bases 205-221
Sp6 promoter: bases 239-256
Multiple Cloning Site: bases 269-383
T7 promoter: bases 406-425
M13 (-20) Forward priming site: bases 433-448
f1 origin: bases 590-1027
Kanamycin resistance ORF: bases 1361-2155
Ampicillin resistance ORF: bases 2173-3033
pUC origin: bases 3178-3851

Figure 10: pCRII® – TOPO® plasmid.

2.9. SCREENING:

About 30 white colonies of each sample type were picked up with sterile tooth stick and dissolved in 20 µl TE Buffer (pH=8), with the same tooth stick, the surface of the LB media was streaked (for storage). The microcentrifuge tubes with TE buffer and bacteria were boiled for 10 min at 94°C. Then, 1 µl of boiled TE Buffer was used as a target DNA for PCR reaction for DGGE analysis (V6–V8 region for Bacteria and V2–V3 region for Archaea) as described earlier.

For DGGE Screening 10 µl were run against the total DGGE profile of each sample type and by comparing the cloned PCR product with the Total profile, the dominant Bacteria and Archaea in each reactor was determined.

2.10. PLASMID ISOLATION FOR SEQUENCING:

Plasmids of about 37 different clones (Bacteria and Archaea) from the three sample types (UASB, Biofilter and Scum Layer) were isolated using QIA prep spin kit from QIAGEN and using Alkaline Lysis protocol (Sambrook. *et al.*, 1989) as follows: a single colony was picked and inoculated in 5 ml of LB broth containing 200 g/L ampicillin and incubated at 37^{o}C overnight. 1.5 mL cells were centrifuged in 1.5 mL Eppendorf tube at 12000g for 1 min and the supernatant was aspirated. The cells pellet was suspended in 100 µl of ice-cold GTE buffer (50 mM glucose, 25 mM Tris-HCl, 10 mM EDTA, pH=8). 200 µl of NaOH/SDS lysis solution (0.2 M NaOH, 1% SDS) were added and the tube was inverted 6-8 times. Immediately, 150 µl of ice-cold 5 M potassium acetate solution (pH=4.8) were added and the tube was spined at max speed for 1 min. The supernatant was

transferred to a new tube and the nucleic acids were precipitated with two volumes of ethyl alcohol on ice for 10 minutes, centrifuged at max speed for 5 min and all of the ethyl alcohol supernatant was aspirated. DNA pellet was washed with 1 ml 70 % alcohol, dissolved in 50 µl TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH=7.5) containing RNAse A solution (20 µg/ml) and stored at -20 ^{o}C until use.

2.11. SEQUENCING AND BLAST SEARCH:

16S rRNA genes were partially sequenced in Hy Laboratories Ltd – Israel, using pCRII® – TOPO targeted Sp6 Primer. Similarity searches of 16S rRNA gene sequences derived from clones against sequences deposited in publicly accessible databases were performed using the NCBI Blast search tool at http://www.ncbi.nlm.nih.gov.

2.12. FLUORESCENT *IN SITU* HYBRIDIZATION ANALYSIS (FISH):

Eight probes were used in this study, Five probes were used to describe the methanogens in the UASB, two for *nitrosomonas* and *nitrobacter* of the Biofilters and one for all samples eubacteria. The probes were designed complimentary to conserved sequences of 16s rRNA gene (Table 4).

Table 4: Probes used in Fluorescent *In Situ* Hybridization Analysis.

Probe	**Specificity**	**Target site 16S rRNA**	**Sequence 5' ⟶ 3'**	**Reference**
EUB 338	Bacteria	338 – 355	GCTGCCTCCCGTAGGAGT	Amann *et al* ., 1990
NEU 23a	*Nitrosomonas*	653 – 670	CCCCTCTGCTGCACTCTA	Wagner *et al*., 1996
NIT3	*Nitrobacter*	1035 – 1048	CCTGTGCTCCATGCTCCG	Wagner *et al*., 1996
MB1174	*Methanobacteriaceae*	1174 - 1195	TACCGTCGTCCACTCCTTCCTC	Raskin *et al*., 1994
MC1109	*Methanococcaceae*	1109 – 1128	GCAACATAGGGCACGGGTCT	Raskin *et al*., 1994
MG1200	*Methanomicrobiaceae* *Methanocorpusculaceae* *Methanoplanaceae*	1200 – 1220	CGGATAATTCGGGGCATGCTG	Raskin *et al*., 1994
MX825	*Methanosaeta*	825 – 847	TCGCACCGTGGCCGACACCTAGC	Raskin *et al*., 1994
MSMX860	*Methanosarcinaceae*	860 – 880	GGCTCGCTTCACGGCTTCCCT	Raskin *et al*., 1994

Briefly, FISH protocol was as follows:

Cells were fixed using 3 volumes of 4% paraformaldehide, incubated for 4 hrs or overnight at 4 °C. 10 µl of fixed sample were spotted on gelatine coated slides and dried at 46°C for 10 min and Sonicated for 20 sec. Dehydration was carried using increasing ethanol series (50, 80 and 96%) . 10 µl of hybridization buffer and 1 µl of probes were added to wells, The slides were incubated for 1.5 hrs at 46 °C in the hybridization tube, washed with washing buffer and incubated in washing buffer with 100 µl of 0.1% 4'-6-Diamidino-2-phenylindole (DAPI) for 15 min in a water bath 48 °C. After that the washing buffer was removed with distilled water and dried with air compressor.

The slides were embedded with vectashield, the cover slip sealed with nail polish and examined under fluorescent microscope.

2.13. N – HEXANE EXTRACTABLE MATERIAL (HEM; OIL AND GREASE).

Oil and grease was determined in BWWTP aeration and secondary Settling tank according to EPA 1664 Standard Method which is summarized below:

A 1L sample was acidified with hydrochloric acid to pH <2 and extracted with n-Hexane in a separatory funnel. The extract was dried over sodium sulfate. The solvent was distilled and the HEM was dried and weighed.

CHAPTER THREE

RESULTS

DNA was isolated from the samples and the V6 – V8 region of the bacterial and V2 – V3 region of Archaeal 16S rRNA genes were amplified (Figure 11). The amplified products (~500 bp) were used in DGGE analysis (Figure 12).

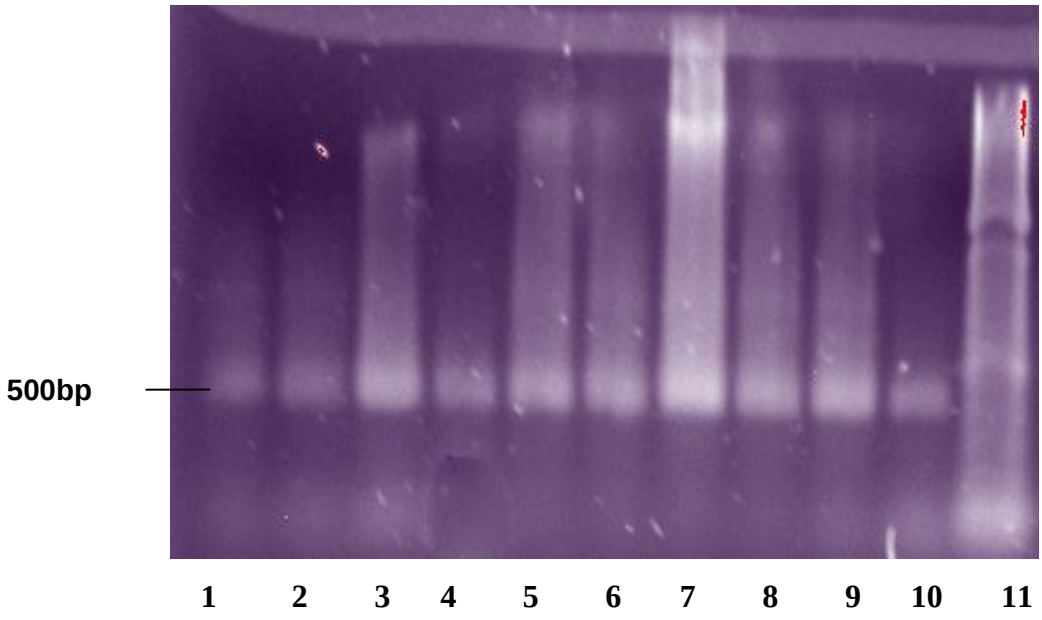


Figure 11: amplified V6-V8 of Bacteria 16S rRNA gene (~500bp). Lanes 1 to 10 are different DNA concentrations for the three samples. Lane 11 is a ladder marker.

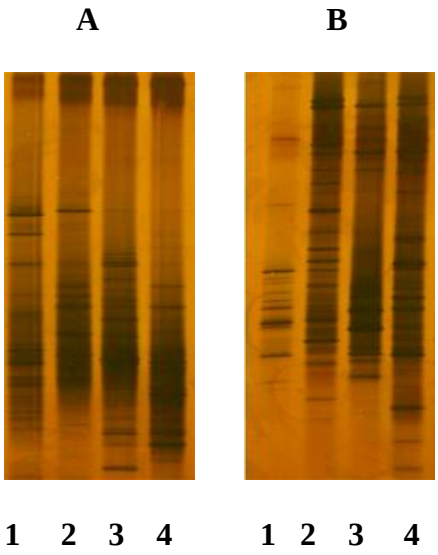


Figure 12: Dominant Archaeal (A) and Bacteria (B) community of UASB, Biofilter and Scum layer. Lanes 1, 2, 3, and 4 are positive control, UASB, Biofilter and scum layer, respectively.

The 16S rRNA gene (~ 1500bp) was amplified for both Bacteria and Archaea (Figure 13) for cloning in pCRII® – TOPO® plasmid. The positive clones were chosen according to blue white selection (Figure 14). The dominant clones in each sample were sequenced for identification.

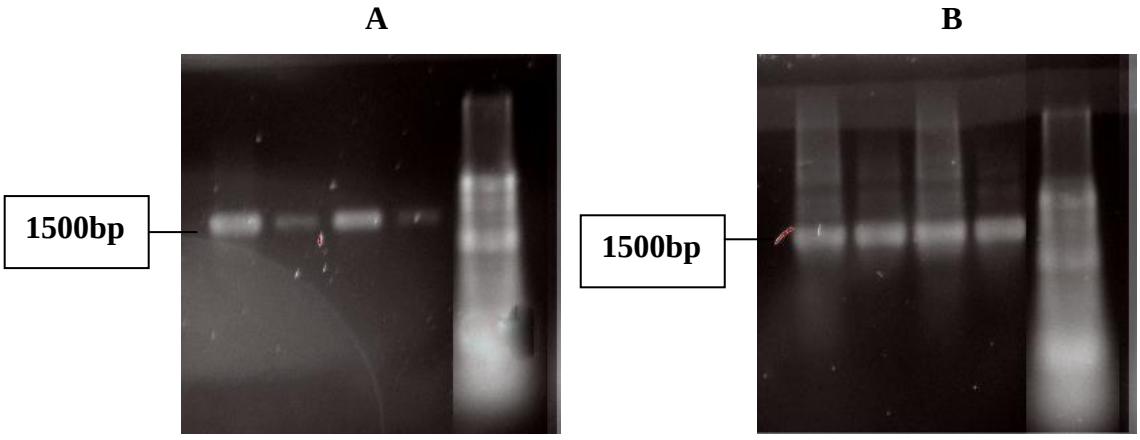


Figure 13: Archaeal (A) and Bacterial (B) 16S rRNA amplified genes (~1500bp).

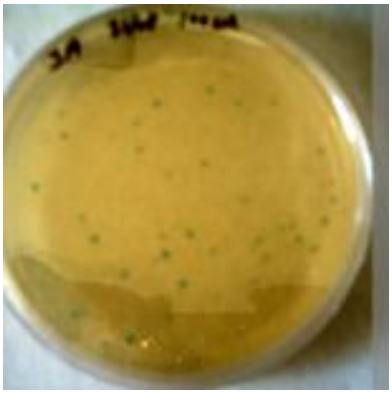


Figure 14: Blue white selection of 16S rRNA gene cloning.

In the three types of samples, the sequences of all dominant clones were compared to the published databases and the results are listed below for each sample type separately.

3.1. ACTIVATED SLUDGE FOAMING:

The dominant clones of both Bacteria and Archaea was determined by comparing the clones to the total profile of the scum sample. A total of 13 clones were chosen for 16S rRNA sequencing using forward SP6 primer and ABI prism sequencer. The sequences obtained were trimmed, aligned and compared to available databases by the use of the Basic Local Alignment Search Tool (BLAST) (Altschul *et al*., 1997) and Ribosomal Database Project-II (RDP). Sequences (from the BLAST search) with the greatest similarity to the clone sequences were selected (Table 5).

Table 5: BLAST Result for dominant microbial content of the scum layer of BWWTP.

CLONE	MICROORGANISM	% SIMILARITY	ACCESSION
PL 2- 3B	*Hyphomicrobium facilis*	97	Y14312
PL 3- 3B	*Nocardioides oleivorans*	99	AJ698724.1
PL 4- 3B	Uncultured bacterium FukuS110	96	AJ289986
PL 10- 3A	*Methanobrevibacter arboriphilus*	96	AB065294.1
PL14- 3B	Uncultured candidate division TM7 bacterium clone SM1G12	97	AF445701
PL 17- 3B	*Microthrix parvicella*	94	X89560
PL 20- 3A	activated sludge foam clone 47	97	AF513095.1
PL 24- 3B	Bacteria: phylum *Chloroflex*i: clone SHA-147.	95	AJ306749
Pl 26- 3B	*Nocardioides sp*. str. ND6	95	AJ511294.1
PL 33- 3A	Candidate division OP11 clone LGd2	90	

The microscopic examination of the Scum layer revealed the presence of *Nocardia sp*. Even it was not detected by the 16S rRNA analysis (Figure 15), also *M. parvicella* was clear in the gram stain analysis as shown in Figure 16.

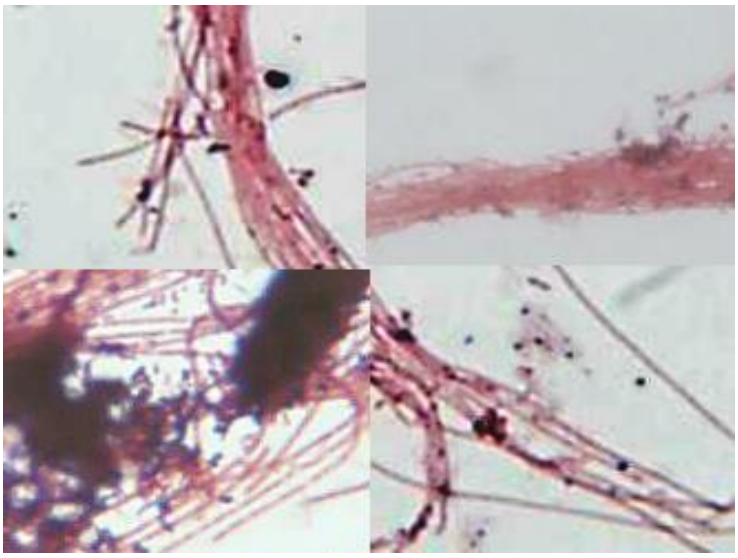


Figure 15: Gram Stain of scum bacteria in BWWTP (November 2005), showing the filamentous bacteria including *Nocardia sp*.

other sample examined in different periods of the year showed differences in the dominancy of these filamentous bacteria during these different periods as shown in Figures 15, 16, 17, and 18.

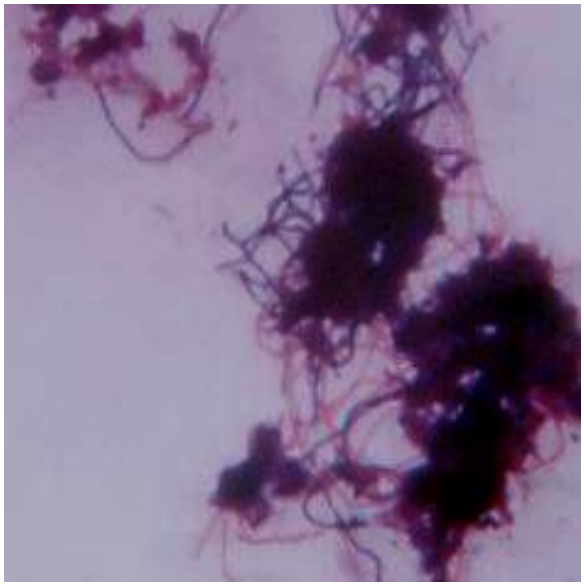


Figure 16: Gram Stain of scum bacteria in BWWTP (February 2006), showing the gram positive *M. parvicella.*

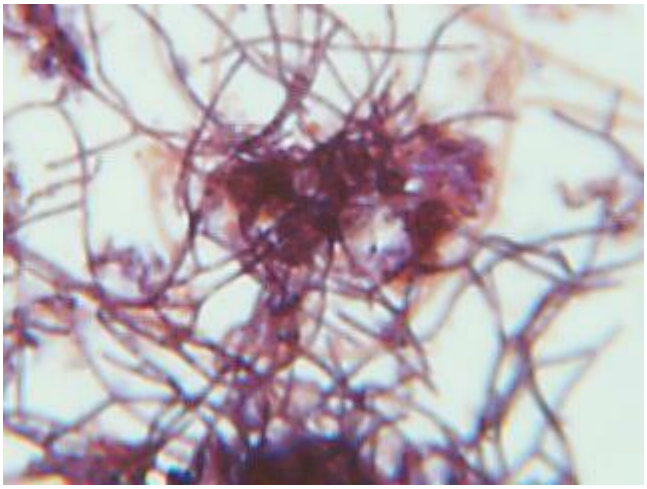


Figure 17: Gram Stain of scum bacteria in BWWTP (March 2006), showing the dominancy of *M. parvicella.*

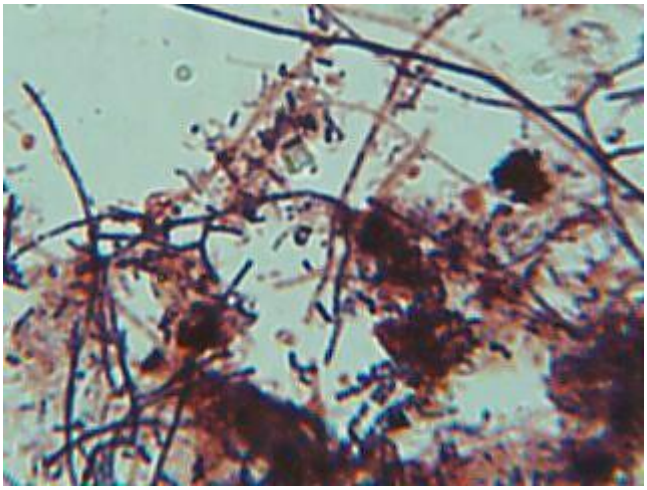


Figure 18: Gram stain of filamentous bacteria in BWWTP during May 2006 showing the dominancy of *Nocardia sp*.

The dominant clones that represent the filamentous bacteria and other bacteria found in the scum layer are shown in Figure 19.

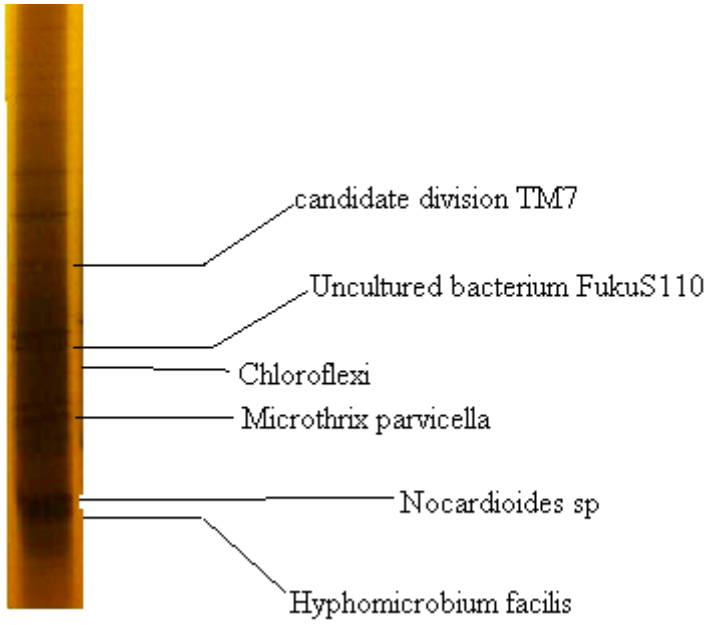


Figure 19: DGGE fingerprint of scum forming bacteria.

3.2. UPFLOW ANAEROBIC SLUDGE BLANKET (UASB):

The methane production of the UASB was 10.3 L/ Day (0.05L/L) and the DGGE profile of both Archaea and Bacteria of the UASB sample shows the dominancy and diversity of many microbial consorts (Figure 20). Fourteen dominant clones of the UASB reactor were sequenced and compared to the 16S rRNA database for identification.

The result shows the presence of many methanogens (Figure 21) with different substrate dependency (Table 7) including *Methanospirillum hungatei*, *Methanosarcina barkeri* fusaro, *Methanosarcina mazei* and *Methanobrevibacter arboriphilus*. In addition to methanogens, denitrifiers were detected in the reactor like *Rhodobacter sp*., *Arcobacter sp*. and *Chryseobacterium sp*. (Figure 22). The results are summarized in Table 6.

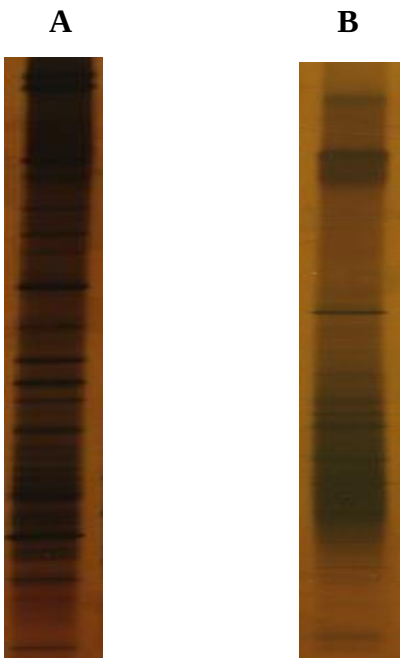


Figure 20: DGGE fingerprint of both Archaea (A) and Bacteria (B) of the UASB.

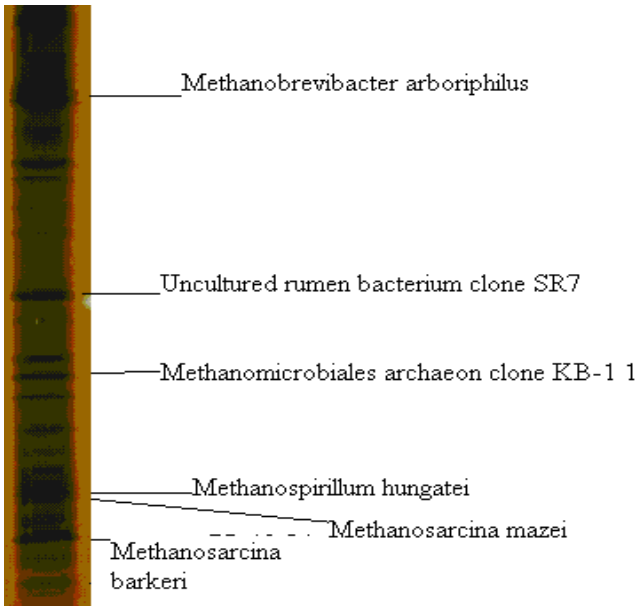


Figure 21: The Archaeal community of the UASB reactor

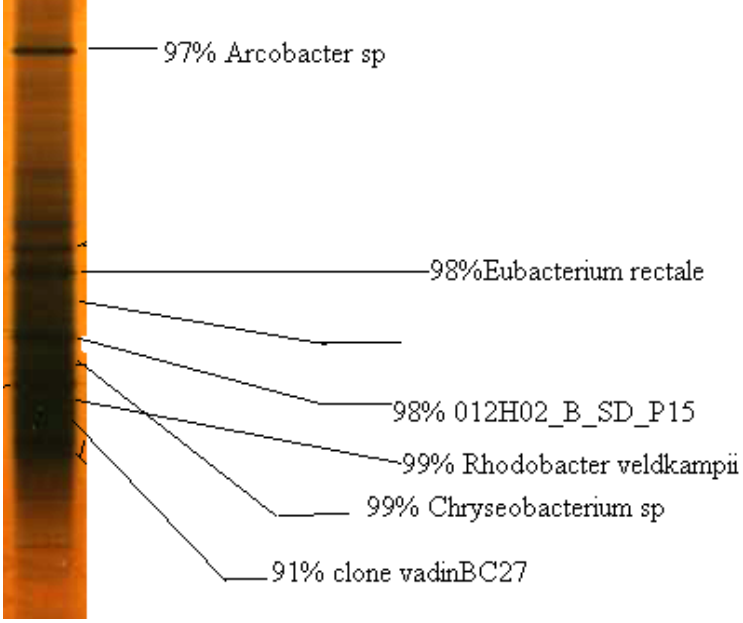


Figure 22: The bacterial community of the UASB Reactor.

Table 6: BLAST result of Bacterial and Archaeal contents of the UASB (August 2005).

CLONE	MICROORGANISM	% SIMILARITY	ACCESSION
PL 1-1B	*Rhodobacter veldkampii*	99	D16421
PL 3 -1B	*Unidentified eubacterium clone vadinBC27*	91	U81676
PL 4 -1B	*Clostridiales: Eubacterium rectale strain S2Ss2/7*	98	AY804151.1|
PL 11- 1B	Uncultured bacterium: clone 012H02_B_SD_P15	98	CT573893
PL13- 1B	*Chryseobacterium sp. R-25053*	99	AM084097
PL14- 1B	*Campylobacteraceae: Uncultured Arcobacter sp. clone DS031*	97	DQ234115
PL4- 1A	*Methanospirillum hungatei*	99	M60880
PL11- 1A	*Uncultured rumen bacterium clone SR7*	96	DQ394637
PL12- 1A	*Methanosarcina barkeri* fusaro	99	CP000099

Table 6: *Continued.*

CLONE	MICROORGANISM	% SIMILARITY	ACCESSION
PL18- 1A	*Methanosarcina barkeri*	99	AF028692
PL22- 1A	Uncultured *Methanomicrobiales* archaeon clone KB-1 1	99	AY780566
PL24- 1A	*Methanosarcina mazei*	97	AB065295
PL25- 1A	*Methanosarcina barkeri*	99	AF028692
PL34- 1A	*Methanobrevibacter arboriphilus str. SA*	97	AB065294.1

Table 7: Classification of methanogens found in the UASB and their substrate(s).

Order	Family	Genus, species	Substrate
Methanobacteriales	*Methanobacteriaceae*	*Methanobrevibacter* *M. arboriphilus*	H_2
Methanomicrobilaes	*Methanomicrobiaceae*	*Methanospirillum* *M. hungatei*	H_2, Methanol
Methanomicrobilaes	*Methanosarcinaceae*	*Methanosarcina* *M. barkeri*	Methanol, Acetate, H_2
Methanomicrobilaes	*Methanosarcinaceae*	*Methanosarcina* *M. mazei*	Acetate, H_2 Methylamines, Methanol

3.3. BIOFILTERS MICROBIAL COMMUNITY:

Two Biofilters were installed as a post treatment process for the UASB effluent polish. The microbial community of these Biofilters was studied using PCR – based DGGE and 16S rRNA sequencing and the sequence result was compared to 16S rRNA published data. The results show the dominance of soil clone and other bacterial consorts including *Zooglea ramigera* that play a role in the biofilm formation. The complete clones are listed in Table 8.

Table 8: BLAST result of biofilters microbial contents (August 2005).

CLONE	MICROORGANISM	% SIMILARITY	ACCESSION
PL 16-2B	Uncultured bacterium clone CJRC123	99	DQ202185.1
PL 34 -2B	Uncultured bacterium strain LMG 20242	97	AJ316319.1
PL 28-2B	Uncultured soil bacterium clone L1A.13C12	93	AY989463.1
PL 24-2B	Uncultured soil bacterium clone C129	93	AF507687.1
PL 57-2B	*Staphylococcus sp.*	99	AY553115.1
PL 44-2B	Uncultured methanotrophic bacterium clone FH2-8	92	AY599191.1
PL 47-2B	*Zoogloea ramigera*	98	X74913
PL 27-2B	bacterium rJ14	96	AB021332

3.4. IDENTIFICATION OF METHANOGENS USING FISH TECHNIQUE.

Five 16S rRNA directed probes were used to describe the methanogens consortium within the Pilot –Scale UASB under study. The probes names, sequence, target and origin were previously described in Table 3. These probes cover the three orders of methanogens (*Methanobacteriales, Methanococcales and Methanomicrobiales*) and the six families *Methanobacteriaceae, Methanococcaceae, Methanomicrobiaceae, Methanocorpusculaceae, Methanoplanaceae and Methanosarcinaceae* . The aim of this experiment was to test qualitatively for the presence of methanogens in the reactor. Eubacterium probe (EUB 338) was used as a control and to calculate the Eubacteria percentage in the reactor (Figure 23) and probe MX825 was used to detect the genus *Methanosaeta* which can use only acetate which is very important in granule formation.

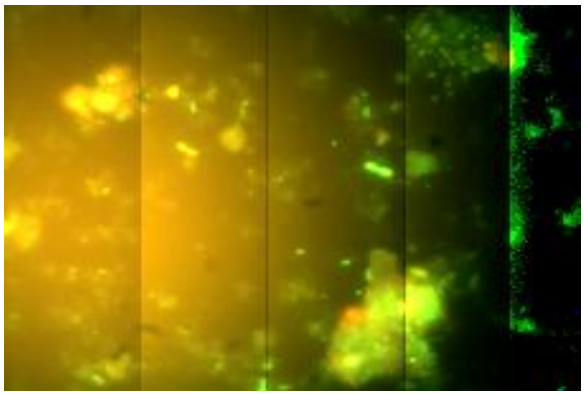


Figure 23: Eubacteria detected in the UASB by probe EUB 338 as a control.

The results of FISH analysis show the presence of the orders and families of methanogens tested with different concentrations as shown in Figures 24- 27.

The percentage of Eubacterium in the reactor was about 30% of the total reactor microbial community.

The Probe MB1174 was used to detect these three genera of the family *Methanobacteriacea*. The result obtained by FISH shows that about 16% of the microbial community in the reactor belongs to the family *Methanobacteriacea* and the dominance of Methanobrevibacter genera of this family (Figure 24).

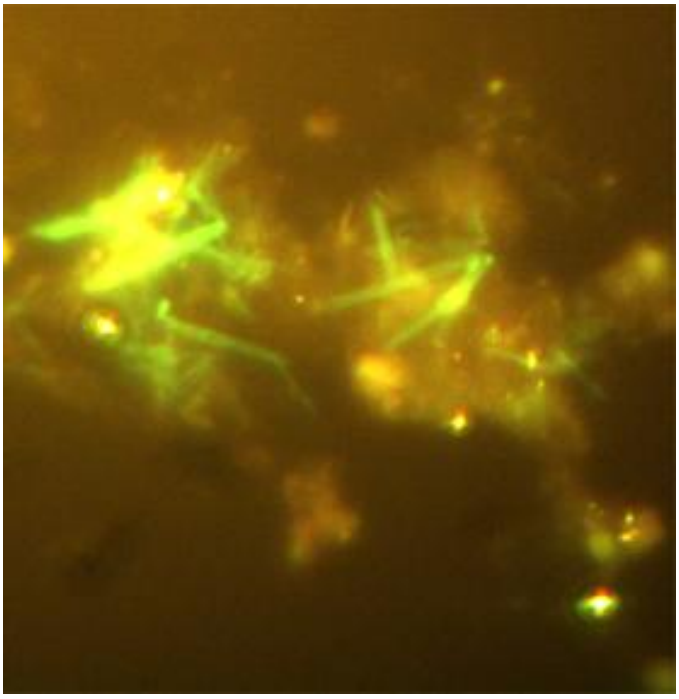


Figure 24: The presence of *Methanobacteriaceae* in the UASB detected by probe MB1174 showing the presence of *Methanobrevibacter* within the sludge granule.

The families of the order *Methanomicrobiales* (*Methanomicrobaoceae*, *Methanocorpusculaceae* and *Methanoplanaceae*) were detected using the probe MG1200. MG1200 revealed that about 17% of the microbial community in the UASB reactor that belong to *Methanomicrobiales* order (Figure 25).

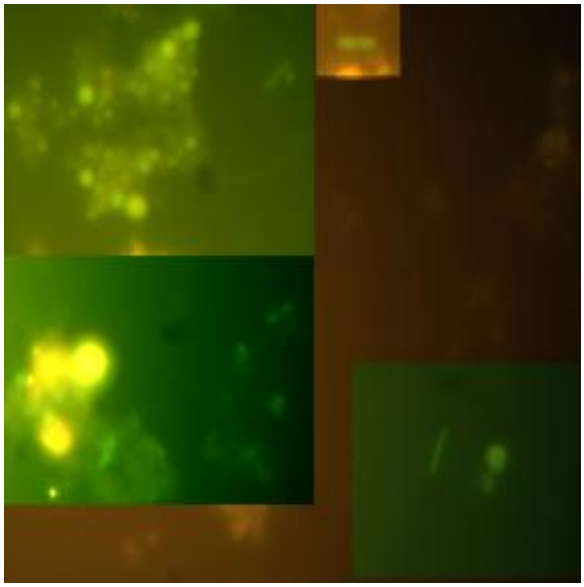


Figure 25: Different cell shapes of methanogens belonging to order *Methanomicrobiales.*

The fourth family *Methanosarcinaceae* of the order *Methanomicrobiales* was included in the probe MSMX860 (Figure 26). They constitute about 14% of the microbial population in the reactor. The genus *Methanosaeta* which belongs to this family was detected by probe MX825 and the result shows that it constitutes 42% of this family. The filamentous *Methanosaeta* which is important in granule formation is shown in Figure 27.

Methanosarcina barkeri was one of the most dominant species in the reactor detected by the 16S rRNA sequencing. It was detected by FISH (Figures 26 and 32A) although it was not clear from FISH analysis that it is one of the dominant methanogens showed by DGGE analysis.

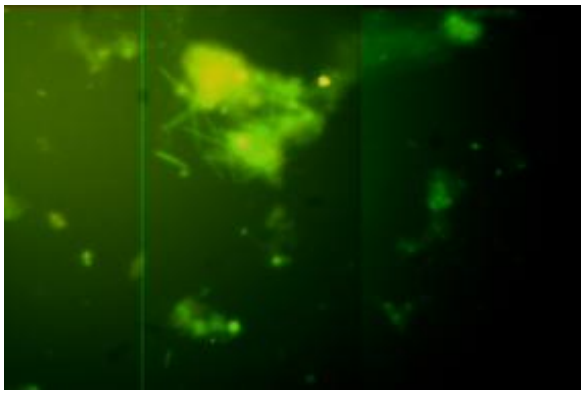


Figure 26: Different genera shapes of the family *Methanosarcinaceae* detected by probe MSMX860 including *M. barkeri.*

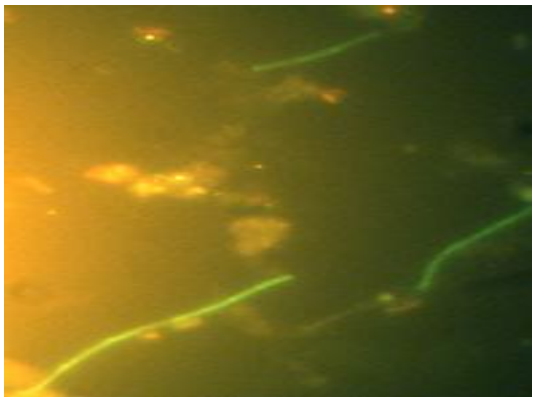


Figure 27: *Methanosaeta* detected by probe MX825.

The family *Methanococcaceae* of the *Methanococcales* order was studied in the UASB by the probe MC1109. It was found that this family composes about 14% of the total microbial community.

CHAPTER FOUR

DISCUSSION:

The efficiency of a waste treatment plant depends on the microbial composition of that Plant. Therefore, studying the microbial composition is important to understand and control the treatment process. New molecular methods give a good alternative for the detection of these microbial communities especially those that are difficult to be identified by culture techniques.

The analysis of 16S rRNA genes, aided by using PCR to amplify target sequences in environmental samples, has enabled microbial ecologists to identify and characterize microorganisms in a natural community, like activated sludge, without prior cultivation (Schuppler *et al*., 1995). Moreover, the taxonomic position of an organism can be determined by comparing the DNA sequence with those of other bacteria (Amann *et al*., 1995).

4.1. ACTIVATED SLUDGE FOAMING:

The analysis of the microbial community in activated sludge is important to understanding and possible control of separation problems in sewage treatment plants (Schuppler *et al*., 1995). Control of filamentous bulking remains a challenge facing all engineers, chemists and microbiologists working in the field of wastewater treatment (Beccari and Ramadori, 1996).

Foaming and sludge Bulking are the solid separation problems experienced in activated sludge systems. These problems are widespread around the world, and 20–60% of wastewater treatment plants experience biological foaming from time to time (Pitt and Jenkins, 1990). Microorganism morphology is a poor descriptive attribute that can vary widely depending upon nutritional conditions. Employing molecular biological methods provide an alternative approach for the detection of microorganisms that are difficult to identify by conventional culture techniques or microscopy (Blackall, 1994).

Microorganisms identified in foams of WWTPs are *Nocardia* (now *Gordona*) *amarae* (Klatte *et al*., 1994*), Sphaerotilus natans, Hyphomicrobium sp., Thiothrix nivea* (Jenkins, *et al*., 1993), *N. rhodochrous, N. asteriodes, N. caviae, N. pinensis* now called *Skermania piniformis* (Chun *et al*., 1997), *Streptomyces sp, Microthrix parvicella, Micromonospora,* Type 0675 and *Rhodococcus* (Blackall *et al*., 1989; Goddard and Forster, 1987; Lechevalier and Lechevalier, 1974; Lemmer and Kroppenstedt, 1984; Pujol *et al*., 1991; Seviour *et al*., 1990; Sezgin and Karr, 1986; Sezgin *et al*., 1988). *Nostocoida limicola* and Type 0041 can also cause foaming (Goddard and Forster, 1987).

Recently, less studied bacterial phyla such as *Chloroflexi* (Beer *et al*., 2002; Bradford *et al*., 1996), *Planctomycetes* (Liu *et al*., 2001) and candidate phylum TM7 (Hugenholtz *et al*., 2001) have also been shown by molecular methods to have filamentous representatives in sludge.

However, many of filamentous bacteria are still uncharacterized and need further investigation.

Filamentous microorganisms can also be good indicators of conditions prevailing in an activated sludge system on a microbiological level. The indications given by the filamentous bacteria could be of low dissolved oxygen (DO) (e.g. *Sphaerotilus natans*), low food-to-micro-organism (F/M) ratio (e.g. *Microthrix parvicella*, Type 0092), presence of septic waste (e.g. *Thiothrix sp*.), nutrient deficiency (e.g. *Haliscomenobacter hydrossis*) and low pH in the system (e.g. fungi) (Jenkins *et al*., 1986).

In this study the filamentous bacteria found in a scum layer of the secondary settling tank of the BWWTP were studied using 16S rRNA, DGGE and Sequencing.

From the results in Table 5, it appears that the famous filamentous bacteria mainly responsible for scum forming in BWWTP belong to the *Microthrix* genera, chlorofelxi, Candidate TM7 and *Hyphomicrobium facilis.*

The trials to identify scum bacteria with conventional methods in samples taken at different periods through the year based on morphological and staining techniques was very difficult. *Nocardia sp*. is clear from the microscopic investigation that its present with low concentration during cold months and it was increased to be one of the dominant bacteria later as the temperature increased after April (Figure 18). This is in spite of the fact that, it was not one of the sequenced clones. This may be due to the DNA extraction protocol which might be not efficiently enough to break up all gram-positive cells (Wagner and Cloete, 2002). Other reason could be that not all of the clones were sequenced or due to screening difficulties.

The characteristics of filamentous bacteria may vary and that same bacteria could have different "morphotypes". Recent work performed by Eikelboom and Geurkink (2002) showed that a large number of "new" filamentous species were encountered in industrial WWTPs and the authors still don't know if they were new morphotypes of species previously known.

Other bacteria found in the scum sample were filamenotus *Nocardiodes sp*. including *Nocardioides oleivorans*, obligate aerobic, gram-positive, non-endospore forming, crude-oil-degrading bacterium (Schippers *et al.*, 2005) which may be a good indicator for high grease and oil content.

Microorganisms that were identified in the scum sample of BWWTP are discussed below.

Microthrix parvicella

Microthrix parvicella (Figure 28), a common cause of foaming in wastewater treatment plants where low food-to-micro-organisms ratio is recorded (Ramothokang *et al*., 2003). A report by Seviour *et al*. (1990) indicated that 62% of the foam samples in Australia contained *M. parvicella*, and in 24% of those samples it was predominant. Hwang and Tanaka (1998), showed that the application of chlorination had no effect on *M. parvicella* reduction or foaming suppression and that a more effective and economical method for the control needs to be established.

Andreasen and Nielsen (1997) found *M. parvicella* to thrive on the long chain fatty acids oleic and palmitic acid and on trioleic acid with oleic acid being utilized at aerobic, anoxic and anaerobic conditions. However, for *Microthrix parvicella* fatty acids are toxic at high concentrations and this means, in order to utilize high amounts of fatty acids, *M. parvicella* must already have constituted a high biomass concentration. The ground of some *Microthrix parvicella* problems might therefore well be prepared by other scum bacteria, be it filaments or not, and further on stabilized by *M. parvicella* (Lemmer *et al.*, 2000).

Microthrix parvicella seems to be restricted to strongly substrate-limited conditions below F/M ratio of 0.15 kg BOD (kg dry weight · d)$^{-1}$. And these conditions cause the increase of *M. parvicella* biomass and the reactors develop thick stable scum layers (Lemmer *et al*., 2002).

In our sample, *M. parvicella* was one of scum bacteria (Clone PL 17- 3B) with 94% similarity. Microscopic examination shows that it was less dominant than other gram negative bacteria found in samples after April. This result may be due to high temperature in Al Bireh City (August avg. temp. 23^{o}C) (Figure 18) and *M. parvicella* proved to favors temperature below 15 ^{o}C (Lemmer *et al.*, 2000). As the temperature decreases, *M. parvicella* concentration in the scum increases as clear from the microscopic examination of another sample taken at the end of March 2006 (Figure 17).

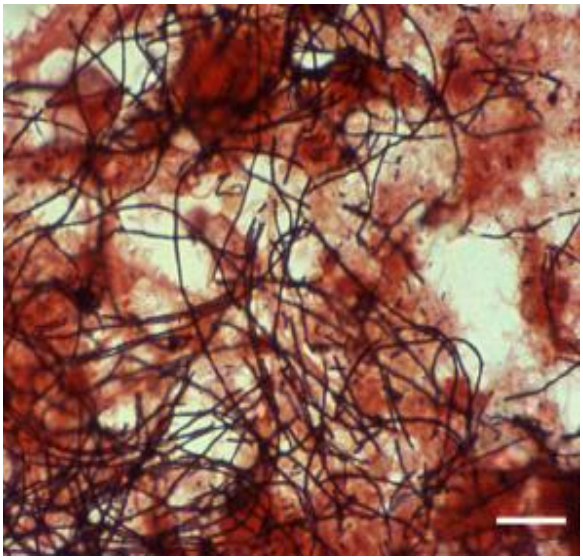


Figure 28: Gram-positive *M. parvicella* (Rossetti *et al.*, 2005)

Hyphomicrobium facilis:

One microorganism of particular interest routinely monitored in wastewater treatment systems is a gram-negative bacterium *Hyphomicrobium facili*s. They can be used in denitrification of drinking water (Liessens, 1993) or sewage (Nyberg *et al.*, 1992) and industrial activated sludge. *Hyphomicrobium* in industrial activated sludge is important due to its ability to degrade C-1 compounds such as methanol, which is found in the influent wastewater (Kloos *et al.*, 1995). In recent years, Hyphomicrobia became of special interest because of their versatility and ability to use toxic waste compounds that are not metabolized by other methylotrophs (Hanson, 1992).

However, hypertrophic growth (i.e. hyphal elongation) of *Hyphomicrobium* can lead to poor sludge settling and compaction.

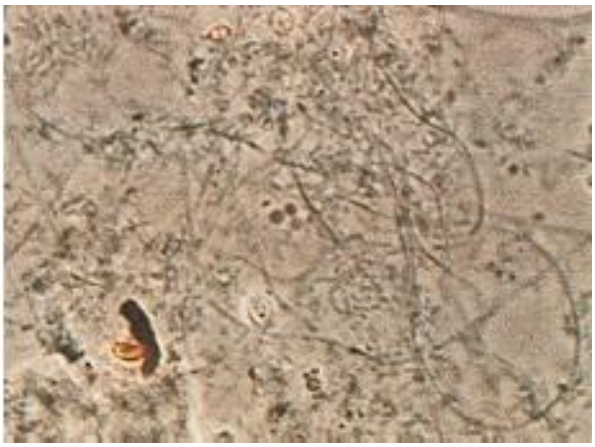


Figure 29: Phase-contrast microscopic image of *Hyphomicrobium facilis*. X920. (Layton *et al.*, 2000)

In BWWTP, *Hyphomicrobium facilis* is one of the dominant filamentous bacteria found in the scum layer formed in the secondary settling tank. Clone PL 2- 3B is 97% similar to *Hyphomicrobium facilis* when comparing the 16S rRNA gene sequence to published data (BLAST Search). The F/M ratio and temperature in AL Bireh W.W.T.P. are a good conditions for *Hyphomicrobium facilis* to live, since the most important factor that limit the presence of this filamentous bacteria is the low F/M ratio as in BWWTP (below 0.1 kg BOD (kg dry weight · d)$^{-1}$) .

Phylum *Chloroflexi* (green non-sulfur bacteria)

Chloroflexi and candidate phylum TM7 have also been shown by molecular methods to have filamentous representatives in sludge and cause serious operational disorders of bulking and foaming in activated sludge wastewater treatment plants *(*Beer *et al.*, 2002; Bradford *et al.*, 1996; Hugenholtz *et al.*, 2001)

Beer *et al* (2002) showed that the 16S rDNA sequence of Eikelboom Type 1851 (Figure 30) from a bulking activated sludge plant is very close to `*Roseiflexus castenholzii*', a member of the phylum `Chloroflexi', class `Chloroflexi', previously called the green non-sulfur bacteria. Type 1851 which belongs to the C*hloroflexi* group, is associated with high mean cells residence time MCRTs (>10 days) and low food-to-microorganism ratios (<0.2 Kg BOD_5 / kilogram of mixed-liquor VSS) and is often seen when simple sugars are present in the wastewater (Jenkins *et al* 1993). These conditions are similar to the present conditions in BWWTP since the F/M ratio range between 0.06 – 0.08 (kilograms of 5-day biochemical oxygen demand/ kilogram of mixed-liquor VSS (Tommalaih, Personal communication). Filamentous *chloroflexi* (green non-sulfur bacteria) are abundant in biological nutrient removal W.W.T.Ps. In a US survey, type 1851 was the 13th most common filament found in WWTPs (Jenkins *et al* 1993).

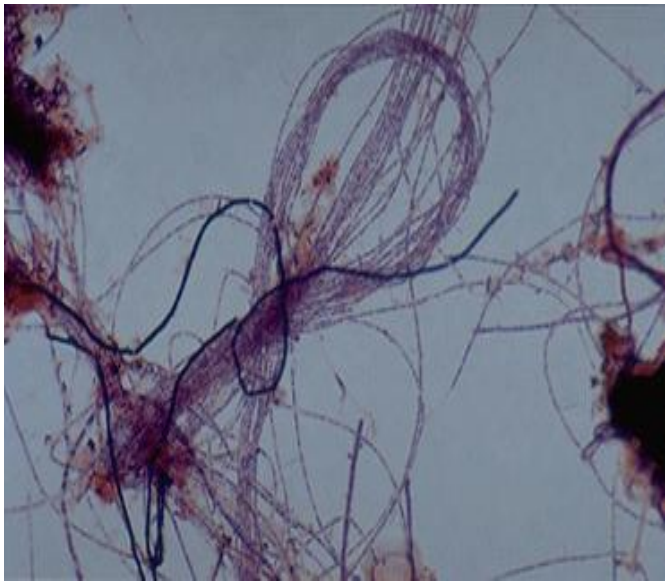


Figure 30: Type 1851 bacteria (Gram stained) (ASSIS, 2000).

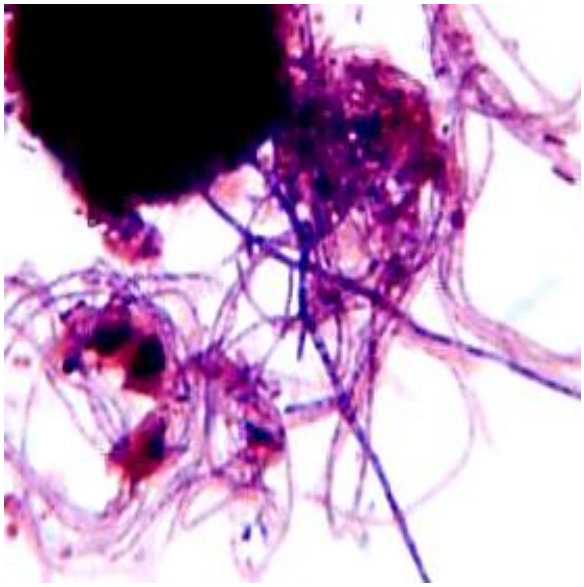


Figure 31: Gram Stain of scum bacteria in Al Bireh WWTP (April 2006), (150X).

In our scum sample, clone (PL 24-3B) 16S rRNA gene sequence have a similarity of 95 % to Chloroflexi phylum and this result support the primary results shown in the gram staining (Figures 15 and 31) which is very close to published type 1851 gram stain (Figure 30).

Candidates TM7:

The TM7 group is a recently recognized phylum-level lineage in the bacterial domain and has no known cultured representatives to date (Rheims *et al*., 1996; Hugenholtz *et al*., 2001). Candidates TM7 and Chloroflexi are less studied bacterial phyla that have also been shown by molecular methods to have filamentous representatives in sludge (Bjornsson *et al*., 2002). Identification and enumeration of filaments using FISH with group-specific 16S rRNA-targeted probes revealed that 14–16% is of filaments of the Type 0041 morphotype

hybridized with TM7-specific probes in two WWTPs (Thomsen *et al*., 2002). Thomsen also found that no significant physiological differences between TM7–positive and TM7–negative Type 0041 filaments and TM7 filamentous bacteria can uptake carbon substrates under aerobic and anaerobic conditions. Type 0041 ranks 4th in number of predominance, common in over 50% of sludge in low abundance and can be beneficial at low abundance as a backbone structure for the floc formation. This filament is usually found in environments with low F/M ratio and a long MCRT. It can be controlled by increasing the F/M ratio which can be achieved through increasing sludge wasting. These conditions are similar to the conditions of the filamentous bacteria present in BWWTP sample.

From the previous results we obtained from the scum layer of BWWTP we can notice that most of the filamentous bacteria found in the sample have the same abundance conditions in activated sludge systems. Most of them are the result of the low F/M ratio, high MCRT and High lipid and grease content.

In BWWTP the F/M ratio is between 0.06 – 0.08 and the oil content is about 100 mg/L measured by EPA1664 standard method in the aeration tank and 30 mg/L in the secondary sedimentation tank.

The F/M ratio is low enough (< 0.2) and the grease and oil concentration is high enough (100 mg/L) for the filamentous bacteria to cause foaming or scum layer. The average oil and grease content of domestic wastewater is 16-65 mg/L (NCDENR, 2002).

The oil concentration in BWWTP is high compared to domestic wastewater and this may be because the separation awareness of oil is not practiced among the

Palestinian community, the leakage of runoff water through the sewer system and the industrial wastewater discharged into BWWTP. All these reason may increase the oil and grease concentration of the influent and effluent of BWWTP. Most of the WWTPs that do not have primary settling tank exhibit the problems of sludge bulking and scum formation since most of the lipid are usually removed in this primary tank. The foam-forming microbial population is specialized in consuming lipids, substrates classified as slowly degradable. When the temperature increases, the rate of lipid hydrolysis becomes sufficiently high for this population to become abundant, accumulate on the surfaces of the aeration basins, and cause biological foaming (Frigon *et al.*, 2006). Communities with enforced grease and oil ordinances appear to suffer less from foaming problems. Also, treatment of septage, which contains substantial grease and oil, has been associated with foaming problems (Jenkins *et al*., 1993).

The scum layer of BWWTP exhibit similar behavior since the problem of the scum increases in summer as the temperature and the strength of the wastewater increases. The presence of the crude oil degrading bacteria *Nocardioides oleivorans* may support the presence of high lipid load and the result in scum formation in the secondary settling tank.

The presence of methanogens *Methanobrevibacter arboriphilus* in the sample suggests the anoxic condition of the activated sludge. Low dissolved oxygen in the activated sludge also result in the formation of foam in the aeration and secondary settling tank.

4.2. UPFLOW ANAEROBIC SLUDGE BLANKET (UASB):

In the UASB reactor, sludge develops in a particular granular form and the success of the anaerobic process relies on the formation of active and settleable granules (Hulshoff Pol, *et al*., 2004). The active granules then form a blanket through which the effluent flows and then diffuses into the sludge granules where it is degraded by the bacteria (Gerardi, 2003). The result of this anaerobic degradation process is bio gas production, methane and carbon dioxide.

The biologically mediated formation of methane (methanogenesis) is the terminal step in the carbon cycle in many anaerobic environments and is exclusively mediated by members of the domain Archaea.

The efficiency of the UASB depends on addition to granular formation on the diversity of the methanogenic granule population. Methanogenic population depends mainly on the composition of the substrate (Le'vesque and Guiot, 2004) and a sufficient quantity of active methanogenic populations should be maintained within an anaerobic reactor so that the required COD removal efficiency can be Obtained (Ince *et al*., 2005). It is, therefore, necessary to monitor any changes in the numbers and activities of the methanogenic species and other microorganisms in the anaerobic reactor using available techniques such as FISH, DGGE and 16S rRNA analysis.

Methane is produced by various methanogenic Archaea present in the (UASB) bioreactors and can be used to predict and improve UASB bioreactor efficiency (Keyser *et al*., 2006). Approximately 70% of the methane formed during the

UASB process is produced by members of the acetoclastic *Methanosarcina* and *Methanosaeta* species (Conklin *et al*., 2004; Gerardi, 2003)

Application of 16S rRNA analysis of the UASB gave an idea about the activity and microbial community within the UASB. For example, denitrifiers were found in the UASB (Figure 22) even there role in nitrogen removal is neglicable because denitrification is not taking place in the UASB. The denitrifiers isolated were *Rhodobacter sp*. which belongs to denitrifying *Rhodobacteraceae*, *Arcobacter sp*. which are microaerophilic, gram-negative, spiral-shaped bacteria (Heylen, *et al.,* 2006), Chryseobacterium *sp*. which also contain denitrifiers isolates (Wang and Skipper, 2004) was also identified in the UASB reactor.

The nitrogen removal in the UASB reactor is about 2.6% (Al Saed and Swaileh, 2006) and that was one of the reasons to adopt two biofilters for polishing the effluent of the UASB by achieving nitrification denitrification.

In addition to the denitrifiers identified, other clones like Clone PL 3-1B was 90% similar to a clone (Unidentified Eubacterium Clone VadinBC27) isolated from anaerobic digestor (Godon *et al*., 1997) and Clone PL 4-1B gave 98% similarity to *Eubacterium rectale* which ferment glucose to butyrate, hydrogen, ethanol, carbon dioxide and formate, a necessary step in methane production.

Our reactor receives municipal wastewater. This gives the opportunity of a wide range of substrates that support the presence of different methanogenic bacteria. The results of the 16S rRNA phylogenetic analysis indicated that methanogens (Figure 21) belonging to *Methanosarcina*, *Methanomicrobiales*,

Methanobrevibacter and *Methanospirillum* groups were present in the UASB reactor.

This diversity of methanogens in the reactor is a good indication of the diversity of substrates present in the influent. The UASB COD removal is 60% (Al Saed and Swaileh, 2006). A study by Mahmoud, (2002) reported that 44% COD removal was achieved under low temperature (15 ºC). Mahmoud suggested that for an efficient use of UASB in Palestine, the HRT must be more than 22 hrs. Another study by Lew *et al*., (2003) within the same climatic region (Israel, 28 ºC) result in 82% COD removal and 1.1 L methane /L / Day compared to 0.05 L in our reactor. The decrease in the UASB effluent quality may be due to difference of wastewater quality between the Palestinian (high strength municipal wastewater) and Israeli (low strength domestic wastewater) people. Another reason could be due to the presence of high oil content of BWWTP that contains Long Chain Fatty Acid (LCFA) mainly oleic acid which are toxic to methanogens (Pereira *et al*., 2002). A decline in removal efficiency results in a decline in the gas production rate. In addition to that, part of methane is discharged with the effluent.

The presence of *Methanobrevibacter* and *Methanosaeta* is a good indication for the granular formation and methane production since these two genera play a role in granular formation and contribute to 70% of methane produced.

In general, the microbial community of the reactor exhibits diversity in thedenitrifies, methanogens and in the acid formers. Besides, the reactor efficiency is acceptable compared to other studies for onsite treatment.

The methanogens identified in the reactor include in the present work included: *Methanosarcina barkeri*: Clone PL 12-1A shows 99% similarity to *Methanosarcina barkeri* and this methanogen is the highly dominant methanogen in the reactor as shown in Figure 33 where the high frequency of picking the colonies belonging to this clones are clear.

Methanosarcina barkeri is one of the versatile *Methanosarcina* species that can utilize methylated compounds, acetate and H_2/CO_2 for growth. Physiological studies have demonstrated that *M. barkeri* can reduce methanol to methane. *Methanosarcina barkeri*, can maintain its viability under aerobic conditions for more than 24 h, which could be due to the formation of cell clots (Kiener and Leisinger, 1983).

The Fluorescent *In Situ* Hybridization (FISH) using *Methanosarcinaceae* targeted probe (MSMX860) could also detect the presence of *Methanosarcina barkeri* as can be seen in Figure 32 where it is compared to another *Methanosarcina barkeri image* (B) (ICSP, 2005)

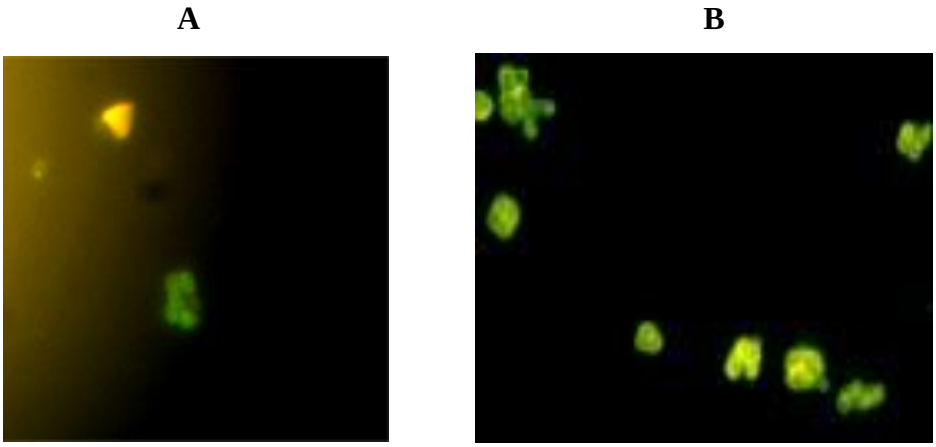


Figure 32: *Methanosarcina barkeri* detected by probe MSMX860 (A) compared to another published image (ICSP, 2005).

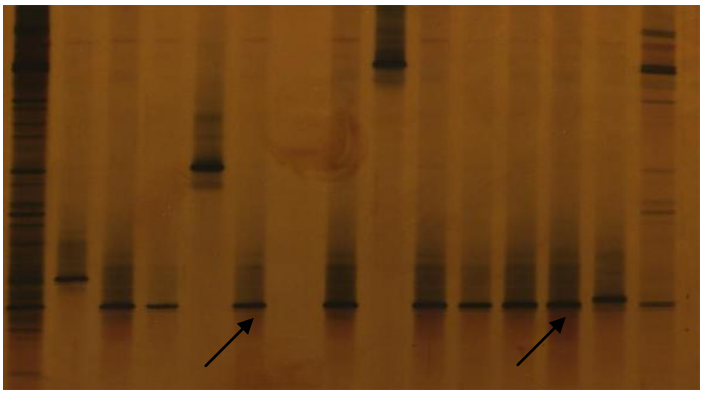


Figure 33: The abundance of Clone PL 12 1A *(Methanosarcina barkeri)* in the UASB reactor.

Methanosarcina mazei: Another species of *Methanosarcinaceae* was also found to be one of the UASB reactor communities. It is represented by clone PL 24- 1A which gives 97% similarity to *Methanosarcina mazei*. It is mesophilic and of great ecological importance along with other related species- as they are the only organisms fermenting acetate, methylamines and methanol to methane, carbon dioxide and ammonia (in case of methylamines) (Deppenmeier *et al*., 2002). Because they use the entire range of methanogenic substrates, they can adapt to a wide range of habitats (Hovey *et al.*, 2005). This type of methanogens again shows the diversity and wide range of substrate methanogens which gives a good microbial community indication for the reactor.

Methanospirillum hungatei: from the result in Table 6, Clone PL 4- 1A shows high similarity (99%) to *Methanospirillum hungatei* which belongs to the highly specialized methanogen group of bacteria that are strict anaerobes and obtain energy for growth via formation of methane by reduction of carbon dioxide (Beveridge *et al*., 1985). It is a member of the relatively newly-discovered domain of life – 'Archaea'. This novel organism was isolated from human sewage and

produces large amounts of methane gas during the natural process of waste disposal (NRC, 2005).

Methanobrevibacter arboriphilus: is represented by Clone PL 34- 1A which shows 97% similarity. *Methanobrevibacter arboriphilus* is an aerotolerance methanogen which has an exceptionally high catalase and superoxide dismutase (SOD) activity for the rapid elimination of toxic oxygen derivatives (Brioukhanov *et al.*, 2002). It can maintain its activity in the presence of oxygen for several hours (Kiener and Leisinger 1983). *Methanobrevibacter arboriphilus* uses H_2 as a substrate for methane production in addition to CO_2.

4.3. BIOFILTERS MICROBIAL COMMUNITY:

As shown in Table 7, the 16S rRNA analysis shows the presence of uncultured bacterial clones. In the system, most of these clones were belonging to soil bacteria. The two stages Biofilter was designed to polish the UASB effluent to achieve nitrification denitrification. The system is composed of two passive aerated biofilters operated in series, comprised of multilayers fixed film media.

The presence of uncultured bacteria from soil is due to presence of rocks and sand as a packing material in the Biofilters. The absence of nitrifiers and other autotrophs responsible for water treatment is due to the period of sampling which was at the startup phase of the system which suffered many operational problems like the homogenous distribution of the surface flow and the result of undeveloped microbial biofilm. The other clone (PL 16-2B) which was 99% similar to Uncultured bacterium clone CJRC123, was isolated from a denitrifying fluidized

bed reactor treating groundwater contaminated with nitrate and uranium (Cardenas, *et al.*, 2005). Clone (PL 28-2B) which is 96% similar to bacterium rJ14 isolated from a phenol-digesting activated-sludge system found by Watanabe et al., (1999) was also isolated.

Clone PL 47-2B is 98% similar to *Zoogloea ramigera*. This bacterium play role in floc formation in activated sludge process and help anchoring the biofilm bacteria to the filter media by formation of polymer–containing matrix. This step is necessary for biofilm formation in the biofilter (Bitton, 2005).

4.4. UASB METHANOGENS IDENTIFICATION USING FISH TECHNIQUE.

The probes described by Raskin *et al*., (1994) used in this study, revealed the presence of highly diverse methanogens in the reactor as describe early by 16S rRNA analysis. The abundance of these methanogens by FISH analysis and from the analytical data of the reactor reported by Al Sa'ed and Swaileh (2006), it is clear that the reactor is working properly but additional operational changes may be needed to increase the treatment efficiency. The methanogens which help in the granular formation like *Methanobrevibacter* and *Methanosaet*a are presence in the reactor. Presence of *Methanosaeta* gives an indication that acetate is present in the reactor and the acetogenesis is taking place.

Methanobacteriaceae detected by Probe MB1174 contains three rod shaped genera; *Methanobrevibacter, Methanobacterium,* and *Methanomicrobium*. They

represent about 16% of the microbial community of the UASB reactor. *Methanobrevibacter* was present in the reactor and detected by FISH and 16S rRNA sequencing. This genus along with *methanosaeta* is a good indicator for the performance of the reactor from the microbial point of view.

The order *Methanomicrobiales* contains three families; *Methanomicrobaoceae*, *Methanocorpusculum* and *Methanoplanaceae* were detected using the probe MG1200. Its abundance is relatively more than the orders *Methanobacteriles* and *Methanococcales* since they represent about 17% of the UASB microbial community.

The family *Methanomicrobiaceae* contains H_2-utilizing cocci (*Methanogenium* and *Methanoculleus*), as well as disc-shaped (*Methanoplanus*), rod-shaped (*Methanomicrobium*, and *Methanolacinia*), and spiral-shaped (*Methanospirillum*) methanogens. They all grow by reducing CO_2, using H_2 and formate as electron donors. Acetate is generally required as a growth factor and peptones are often required or stimulatory as well; many strains require tungstate and nickel (Stackebrandt**,** 1999).

The Family *Methanocorpusculaceae* contains only one genus, *Methanocorpusculum*, and is more closely related to the *Methanomicrobiaceae* than any other families of methanogens. The five species of the genus are very small in size, irregular cocci that utilize H_2, alcohols and formate as substrates for methanogenesis. The different shapes of member of these three families are clear in Figure 25.

The fourth family Methanosarcinaceae of the order *Methanomicrobiales* was identified by the probe MSMX860. All of the aceticlastic methanogens belong to this family, as well as all methanogens that disproportionate methanol, methylamines, or other methyl-containing compounds. The only species that catabolizes methanol are *Methanosphaera* species, which require H_2 to reduce it to methane. The family *Methanosarcinaceae* can be divided into three physiological and morphological types: 1) the genus *Methanosarcina*, which contains coccoid and pseudosarcinal cells and can disproportionate methanol and catabolises acetate, H_2 and CO_2; 2) the genus *Methanosaeta*, which grows only by the aceticlastic reaction; 3) the halophilic, methylotrophic organisms of the genera *Methanolobus*, *Methanococcoides*, and *Methanohalophilus*. Phylogenetically, only the first of these groups is composed of closely related species (Whitman, *et al*., 1999). The five genera including *Methanosaeta* were detected by MSMX860 and they represent 14% of the reactor microbial community and as mentioned earlier they contribute to granular formation and 70% of methane production.

Probe MX825 was specific for *Methanosaeta*. The *Methanosaeta* genera are important for the efficiency of the UASB since the efficiency depends on the formation of settleable granules and *Methanosaeta* play a major role in sludge granulation. (Zheng *et al*., 2006).

Methanosarcina species can form methane from acetate but preferentially form methane via energetically more favorable pathways when substrates other than acetate (H_2-CO_2, methanol, or methylamines) are available (Harper and Pohland, 1986).

Methanosarcina barkeri was one of the most dominant species in the reactor detected by the 16S rRNA sequencing. It was detected by FISH as shown in figure 32A even though it was not clear that it is one of the dominant methanogens in the UASB by FISH analysis like 16S rRNA and DGGE analysis. Probe MX825 targeted the genus *Methanosaeta*. The long-filament type of *Methanosaeta* cells (Figure 27) are usually found in mesophilic UASB and seldom observed in thermophilic UASB granules (Sekiguchi *et al*., 2001). Our reactor is working under a mesophilic conditions and by FISH analysis it was clear that this genus share 6% of the reactor microbial community. This genus is a very good indicator for the presence of settling sludge and the presence of acetate as a substrate in the reactor.

The family *Methanococcaceae* of the *Methanococcales* order was studied in the UASB by the probe MC1109. *Methanococcaceae* contains only one genus, *Methanococcus,* which is composed of six species of mesophilic and thermophilic organisms. There shape is irregular cocci and they can not easily be distinguished from other coccal methanogens (Stackebrandt, 1999). They utilize H_2 and CO_2 for methanogensis, they can not utilize a acetate, methyl compounds or alcohol for methane production.

CHAPTER FIVE

CONCLUSIONS AND RECOMMENDATIONS

5.1. CONCLUSIONS

- Molecular techniques (PCR, DGGE, Cloning, FISH and 16S rRNA) were successfully applied to study and construct a basic profile for the dominant microbes in wastewater treatment systems. They can be used to monitor any change in the microbial content of the system.
- Analysis of the scum layer revealed the presence of *Hyphomicrobium facilis, Microthrix parvicella*, candidate division TM7 bacterium, *Chloroflex*i : clone SHA-147, Candidate division OP11, *Nocardioides sp., Nocardioides oleivorans*, *Methanobrevibacter arboriphilus* and other uncultured activated sludge clones.
- *Hyphomicrobium facilis, Microthrix parvicella*, candidate division TM7 bacterium, *Chloroflex*i : clone SHA-147, *Nocardioides sp*. and *Nocardia sp*. are the cause of scum formation in the secondary sedimentation tank of BWWTP.
- The high grease and oil content along with the low F/M ratio were the main reasons behind the out growth of scum forming bacteria in the system.

- Analysis of the UASB sample revealed that the dominant methanogens were: *M. arboriphilus, M. hungatei, M. barkeri, M. mazei* and *Methanomicrobiales* archaeon clone KB-1 1. In addition to that denitrifiers were also detected.
- These methanogens play a role in methane production and granule formation which is an important step for the UASB efficiency.
- The UASB system has 60% COD removal efficiency, which is acceptable for onsite treatment. The type of wastewater is municipal and the presence of oil and consequently the presence of long chain fatty acid (LCFA) could be one of the reasons for the low removal efficiency.
- Analysis of the two stages Biofilters revealed the presence of soil bacterial clones and other uncultured clones because the Biofilters were at the startup phase and the microbial Biofilm was still undeveloped.

5.2. RECOMMENDATIONS:

- The application of molecular techniques to monitor and study the microbial community in wastewater treatment systems is highly recommended as it provides quick and reliable information about the dominant bacteria which helps in optimizing and monitoring the efficiency of the system.
- In order to alleviate the scum formation problem in Al Bireh Wastewater Treatment Plant, the following is recommended:
    1. Increasing the F/M ratio by increasing sludge wasting and reducing sludge age helps.
    2. If possible decrease grease and fat content of the influent by primary sedimentation tank.
    3. Raising the awareness of oil separation among the Palestinian community and controlling the industrial oil discharge.
- For the UASB, the microbial quality and the removal efficiency are generally acceptable. The control of oil and long chain fatty acids in the influent to prevent the possible toxicity of these fatty acids to methanogens is recommended.
- The use of Biofilters for UASB effluent polishing is recommended with a modification in the system operational parameters and backing material to achieve a good microbial biofilm development.

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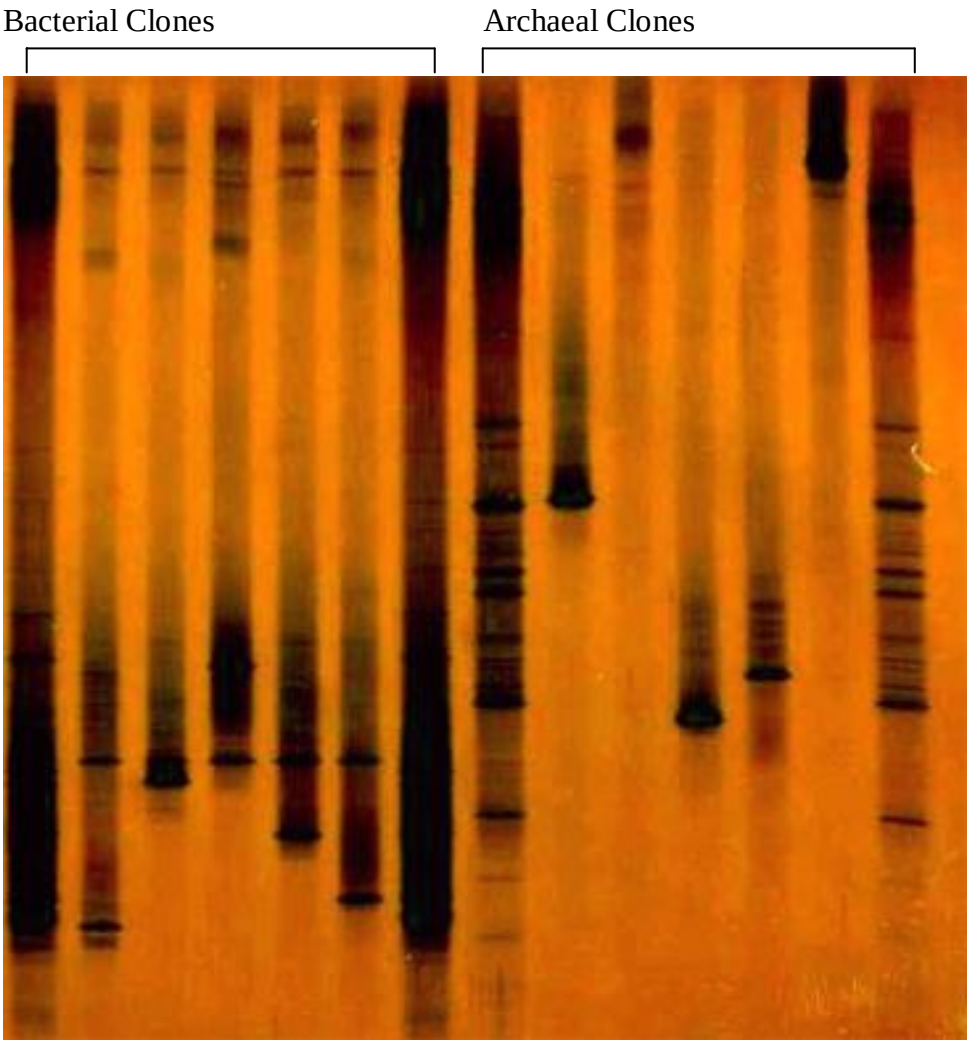
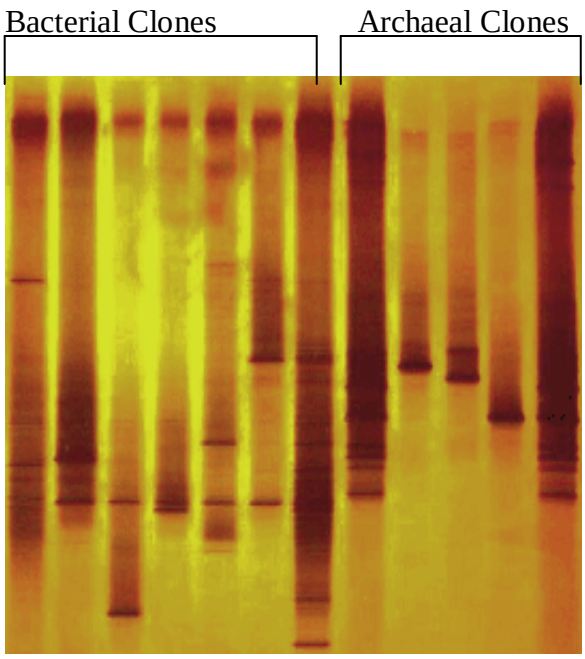
APPENDICES

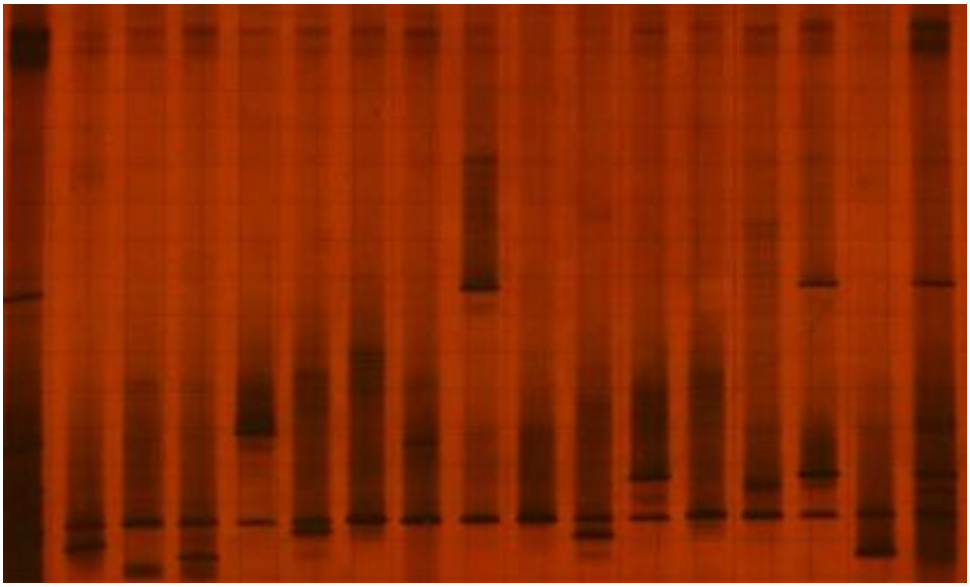
Appendix 1: DGGE of dominant clones of Scum Layer of BWWTP.



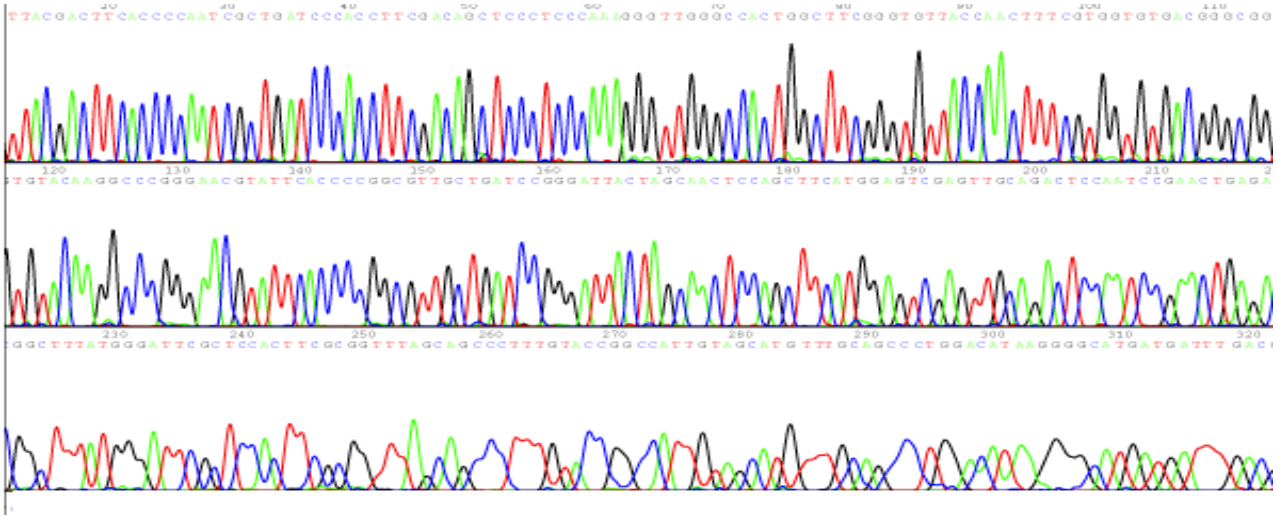
Appendix 2: DGGE of the dominant clones of the Biofilter.



Appendix 3: DGGE of dominant bacterial clones of the UASB reactor.

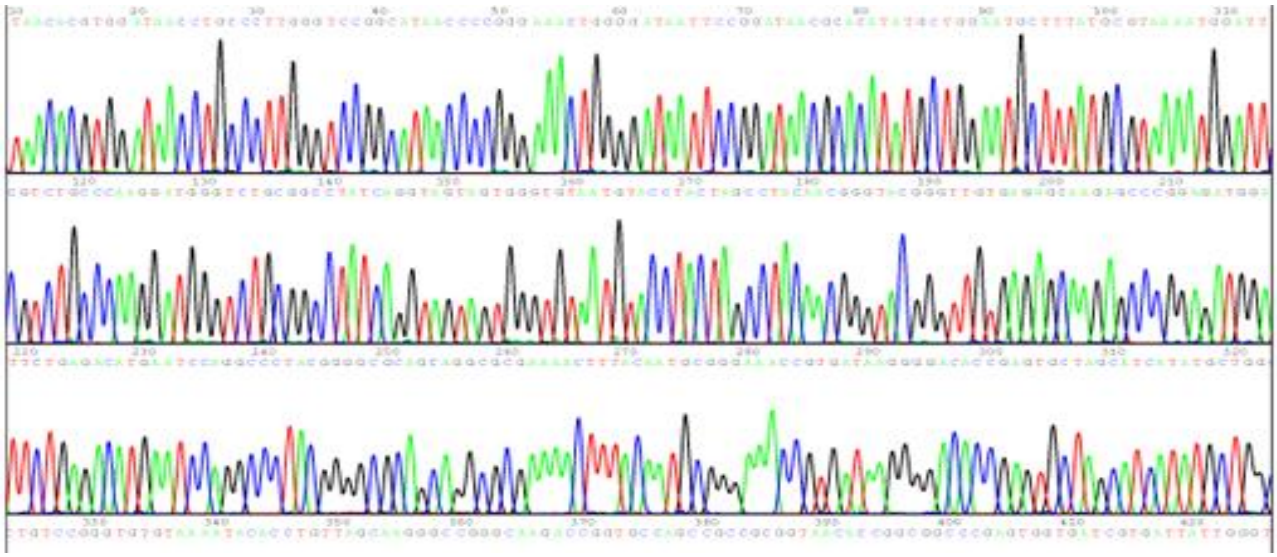


Appendix 4: Partial sequence of *Microthrix parvicella* 16S rRNA gene



CGGCTACCTTGTTACGACTTCACCCCAATCGCTGATCCCACCTTCGACAGCTCCCTCCCAAAGGGTTGGGCCACTGG
CTTCGGGTGTTACCAACTTTCGTGGTGTGACGGGCGGTGTGTACAAGGCCCGGGAACGTATTCACCCCGGCGTTGCT
GATCCGGGATTACTAGCAACTCCAGCTTCATGGAGTCGAGTTGCAGACTCCAATCCGAACTGAGACCGGCTTTATGG
GATTCGCTCCACTTCGCGGTTTAGCAGCCCTTTGTACCGGCCATTGTAGCATGTTTGCAGCCCTGGACATAAGGGGCA
TGATGATTTGACGTCGTCCCCACCT

Appendix 5: Partial sequence of *M. barkeri* 16S rRNA gene.



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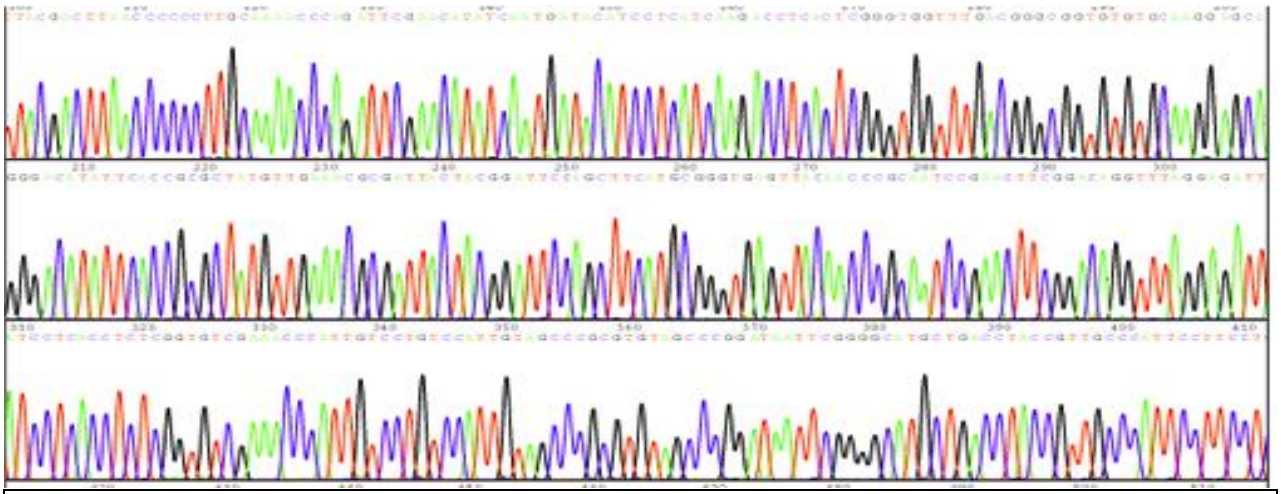
TGCTGGAATGCTTTATGCGTAAAATGGATTCGTCTGCCCAAGGATGGGTCTGCGGCCTATCAGGTAGTAGTGGGTGTAATGTACC
TACTAGCCTACAACGGGTACGGGTTGTGAGAGCAAGAGCCCGGAGATGGATTCTGAGACATGAATCCAGGCCCTACGGGGCGCA
GCAGGCGCGAAAACTTTACAATGCGGGAAACCGTGATAAGGGGACACCGAGTGCTAGCATCATATGCTGGCTGTCCGGGTGTGT
AAAATACACCTGTTAGCAAGGGCCGGGCAAGACCGGTGCCAGCCGCCGCGGTAACACCGGCGGCCCGAGTGGTGATCGTGATTA
TTGGGTCTAAAGGGTCCG

Appendix 6: Partial sequence of *M. hungatei* 16S rRNA gene.



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ATGCGGGTGAGTTACAACCCGCAATCCGAACTTCGGACAGGTTTAGGAGATTATCCTCACCTCTCGGTGTCGAAACCCATTGTCC
TGTCCATTGTAGCCCGCGTGTAGCCCGGATAATTCGGGGCATGCTGACCTACCGTTGCCCATTCCTTCCTCCCCTTTAGCAGAGGC
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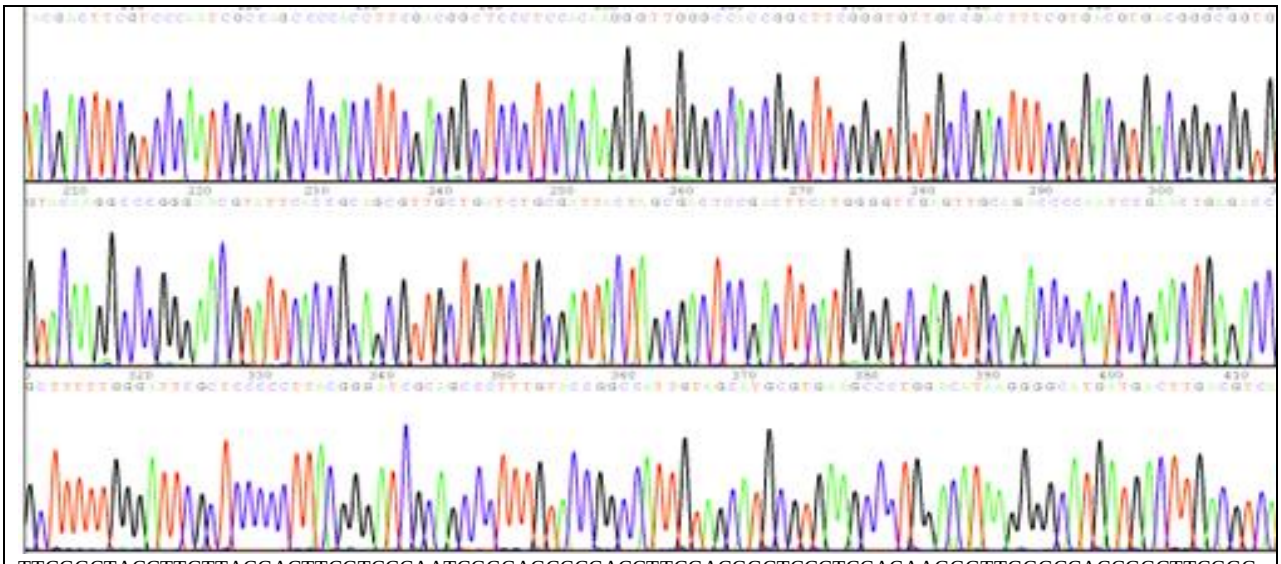
Appendix 7: Partial sequence of *N. oleivorans* 16S rRNA gene.



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TGTTGCCGACTTTCGTGACGTGACGGGCGGTGTGTACAAGGCCCGGGAACGTATTCACCGCAGCGTTGCTGATCTGCGATTACTA
GCGACTCCGACTTCATGGGGTCGAGTTGCAGACCCCAATCCGAACTGAGACCGGCTTTTTGGGATTCGCTCCCCCTTACGGGATC
GCAGCCCTTTGTACCGGCCATTGTAGCATGCGTGAAGCCCTGGACATAAGGGGCATGATGACTTGACGTCATCCCCACCTTCCTC
CGAGTTGACCCCGGCAGTCTCCTATGAGTCCCCACCATTACGTGCTGGCAACATAGGAC

الخلاصة

لقد تم استخدام تقنيات ألأحياء الجزيئية الحديثة للكشف عن الميكروبات الموجودة في ثلاثة عينات مختلفة من محطات معالجة المياه العادمة في فلسطين. هذه العينات تشمل أولاً: طبقة الرغوة المتشكلة على سطح حوض الترسيب في محطة البيرة، ثانياً: نظام المعالجة اللاهوائي (Up Flow Anaerobic Sludge Blanket - UASB)، ثالثاً: الميكروبات المتشكلة في المرشح البيولوجي الموصول بنظام المعالجة اللاهوائي السابق.

لقد كان الهدف من هذه الدراسة هو التعرف على البكتيريا الخيطية التي تشكلت في حوض الترسيب وأدت إلى مشاكل في عملية الترسيب وبالتالي سوء نوعية المياه المعالجة الخارجة من المحطة. بالإضافة إلى ذلك، التعرف على أنواع الميكروبات الموجودة في نظام المعالجة اللاهوائي (UASB) والمرشح الموصول به بغرض تحسين فعالية النظام ككل.

التقنيات الجزيئية التي استخدمت هي PCR, DGGE, 16S rRNA و Cloning. لهذا الغرض تم عزل المادة الوراثية (DNA) من العينات الثلاثة وتم مضاعفة حوالي 500 نيوكليوتيد من المناطق التي تحتوي على اختلافات بين الكائنات الحية من الجين المسئول عن إنتاج ال 16S rRNA بواسطة ال PCR لاستخدامها في عملية فصل DNA بواسطة تقنية ال DGGE. هذه التقنية تسمح بفصل ال DNA بناء على التتابع في القواعد النيتروجينية وليس بناء على الوزن الجزيئي.

لقد تم مضاعفة الجين ككل(16S rRNA gene) واستنساخه داخل بكتيريا ال *E. coli* ومن ثم معرفة التتابع للقواعد النيتروجنية للميكروبات الموجودة بكثرة في أنظمة المعالجة الثلاث ومقارنتها بالجينات المخزنة في قواعد بيانات ال 16S rRNA.

لقد تم التعرف على أعداد كثيرة من البكتيريا الخطية المسببة لمشكلة الترسيب في محطة البيرة ومن بينها: *Microthrix parvicella, Nocardia sp., Hyphomicrobium facilis, Chloroflexi, Candidates TM7* و *.Nocardioides oleivorans*.

إن انخفاض نسبة F/M وزيادة كميات الزيوت الداخلة إلى محطة المعالجة في مدينة البيرة قد يكون السبب وراء كثرة هذه البكتيريا الخيطية. لقد تم ملاحظة وجود تغير فصلي في كثافة هذه الأنواع في الأوقات المختلفة من السنة وذلك بسبب التغير في درجات الحرارة خلال السنة ونوعية المياه العادمة خلال الفصول المختلفة. حيث أن جزء من هذه البكتيريا يفضل درجات حرارة منخفضة وأخرى عالية. إن التغير في نسبة F/M (زيادتها) والتقليل من كمية الزيوت الواردة للمحطة قد يكون من الحلول الممكنة لهذه المشكلة.

لقد استخدمت تقنيات PCR و DGGE و FISH في الكشف عن الميكروبات المنتجة لغاز الميثان وتبين أن هناك عدة أنواع منها: *Methanospirillum hungatei* و *Methanosarcina barkeri*

Methanosarcina mazei, *Methanobrevibacter arboriphilus*

إن كمية هذه الميكروبات قليلة نسبياً وهذا قد يعود إلى وجود الأحماض الدهنية ذات السلسلة الكربونية الطويلة (LCFA) والتي تعتبر سامة لهذه الميكروبات. هذه الظروف أدت إلى انخفاض نسبة المعالجة لهذه المياه نوعا ما.

أما بالنسبة للعينة الثالثة والتي تم فيها فحص تجمعات البكتريا الموجودة في المرشح، فقد أظهرت الفحوصات وجود أنواع بكتيرية موجودة عادة في التربة وهذا عائد إلى أن المرشح يتكون من الصخور والرمل أساسا. بالإضافة لذلك، تم الكشف عن وجود *Zoogolea ramigera* التي تلعب دور مهم في عملية تكون والتصاق البكتيريا لمكونات المرشح. قد يكون السبب في عدم وجود أنواع كثيرة من الميكروبات في المرشح هو أن المرشح كان في المرحلة الأولى لتشغيله وما زال يعاني كثيرا من المشاكل.