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FLUORESCENT IN SITU HYBRIDIZATION FOR THE DETECTION OF BACTERIAL COMMUNITY IN ACTIVATED SLUDGE FROM TEXTILE FACTORIES

by

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by

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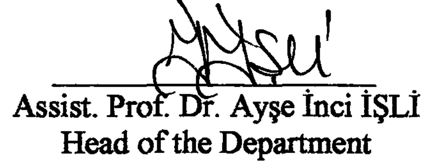
I certify that this thesis satisfies all the requirements as a thesis for the degree of Master of Science.



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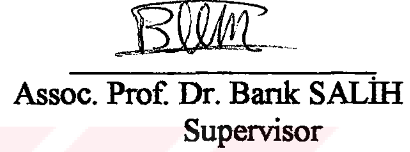

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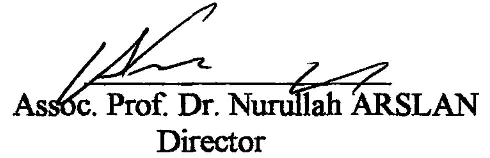

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ABSTRACT

The bacterial community structure in the activated sludge samples from wastewater treatment plant has been studied by conventional methods. The technical details of these techniques are well known, however, the microbiological details, e.g. the compositions of the bacterial populations that are responsible for bioremediation, is still not clear and treatment plants vary in their quality performance in this regard.

Activated sludge samples from 3 textile factories were analyzed by fluorescent in situ hybridization (FISH) using 16S rRNA probes for the domain bacteria (EUB, ALF, BET, GAM, CF, HGC) and the domain Archea. Samples were also examined by phase-contrast microscopy for floc determination and by conventional methods (culture and biochemical tests).

All groups of bacteria were detected by the FISH technique in the activated sludge samples of the 3 factories but at variable concentrations. In Factory I, the predominant groups were the beta subclass of Proteobacteria and the cytophaga-flavobacterium cluster followed by gamma, high G-C gram-positive bacteria and alpha subclass. Factory II showed a similar pattern but at a lower concentration, while Factory III showed a predominant alpha and beta subclasses, and cytophaga-flavobacterium cluster followed by gamma subclass and high G-C gram-positive bacteria that was still at a much lower concentrations than in the previous two factories. The domain Archea was detected in samples of the 3 factories. The Floc characteristics determined by phase-contrast microscopy revealed normal floc category with some filamentous bacteria for

factory I and at a lesser degree for factory II. However, factory III had diffuse and atypical flocs with abundant filaments, which might be one of the reasons for the operation problems in this factory.

The analysis of bacterial community in the activated sludge samples from the 3 textile factories by the FISH technique provided an evaluation scheme for the quality performance among these treatment plants.

Keywords: Activated Sludge, Fluorescence in situ hybridization (FISH), Phase-contrast microscopy, floc.

TEKSTİL ENDÜSTRİSİ AKTİF ÇAMURUNDAKİ MİKROORGANİZMALARIN FLUORESCENT IN SITU HYBRIDIZATION (FISH) METODUYLA BELİRLENMESİ

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ÖZ

Bu çalışmada, atıksu arıtma tesisi aktif çamurundaki bakteri topluluğunun yapısı konvensiyonel metodlarla incelenmiştir. Bu metodların teknik detayları iyi bilinmekle beraber biyolojik arıtmada rol oynayan bakteri topluluğunun kompozisyonu gibi mikrobiyolojik detayları hala net değildir ve bu bakımdan arıtma tesislerinin kalite performansları değişim gösterir.

3 tekstil fabrikasından alınan aktif çamur örnekleri "fluorescent in situ hybridization" (FISH) metodu ile 16S rRNA probları kullanılarak Bacteri (EUB, ALF, BET, GAM, CF, HGC) ve Archea grupları analiz edildi. Örnekler ayrıca "phase-contrast" mikroskopi ile flok tanımlanması için ve kültür ve biyokimyasal testler gibi konvensiyonel metodlarla incelendi.

3 fabrikadan alınan aktif çamur örnekleri FISH tekniği ile incelenerek yukarıda adı geçen bütün bakteri grupları değişen konsantrasyonlarda bulunmuştur. 1. fabrikadan alınan numunelerde Beta altgrup ve Cytophaga-Flavobacterium (CF) grup ağırlıkta olmakla beraber, Gama altgrup ve yüksek G-C gram positive bakteriler ikinci sırada ve Alfa alt grup en az miktarda bulunduğu tespit edilmiştir. 2. fabrikadaki bakteri dağılımı 1. ile benzer sonuçlar vermekle beraber bakteri popülasyonu daha düşük gözlenmiştir. 3. fabrikadan alınan örneklerde ise Beta altgrup, Alfa altgrup ve Cytophaga-Flavobacterium (CF) grup ağırlıktaki bakterileri Gama altgrup ve yüksek G-C gram pozitif bakteriler izlemekle beraber diğer iki fabrikaya oranla çok daha az konsantrasyonda gözlenmiştir. Ayrıca Archaea grup bakterilerinin varlığı da 3 fabrikanın örneklerinde tespit edilmiştir. Phase-contrast mikroskobu ile yapılan incelemelerde ise flok ve filament yapısının 1. fabrikanın örneklerinde orta derecede olduğu, 2.fabrikada nadir olduğu görülmüştür. 3. fabrikada ise filament yoğunluklu

içiçe geçmiş, atipik bir flok yapısı gözlenmiş ve bunun bu fabrikadaki işletme problemlerinin sebeplerinden biri olabileceği düşünülmüştür.

3 farklı fabrikaya ait aktif çamur örneklerinin bakteri toplulukları açısından analizleri FISH yöntemi ile gerçekleştirilmiş ve bu bilgiler yardımıyla, arıtma tesisleri arasındaki verim performasları karşılaştırılmıştır.

Anahtar Kelimeler: Aktif Çamur, Fluorescence In Situ Hybridization (FISH), phase-contrast mikroskopi, flok.

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INTRODUCTION

Biological wastewater treatment systems like activated sludge or anaerobic digesters are essentially a specialized aquatic ecosystem, where microorganisms play a major role. In order to fully characterize, understand, and, in the long run, control those microbial communities, knowledge of both their structure and function is necessary. Attention should, therefore, be given to identification, enumeration, and spatial distribution (structural parameters) as well as to in situ activities (functional parameter) of the community members. Furthermore, from an ecological and from an engineering point of view stability or dynamics of these microbial communities are important both for theory and practice. Until recently, identification of microorganisms required the isolation of pure cultures and the investigation of physiological and biochemical traits. Enumeration had to be done by plate counts or most probable number (MPN) techniques. However, since all cultivation-dependent techniques are not only time-consuming and labor intensive but select for certain organisms, they are inadequate for determining reliable cell numbers (Staley and Konopka, 1985), and in many cases even for the identification of the main catalysts of a system (Wagner et al., 1993a, 1994b).

To circumvent these limitations, identification techniques based on nucleic acids have been developed and successfully applied to wastewater treatment systems during the last ten years. Molecular methods are more rapid and more reliable than microbiological or biochemical methods since they are not affected by growth conditions and culture media. In Turkey, no detailed study on the use of molecular based techniques for the identification of bacterial community in the activated sludge is being reported. Our objectives were:

- In order to reveal factors influencing the efficiency and stability of biological wastewater treatment plants and to develop strategies for improved process performance.

- Activated sludge samples from 3 textile factories were collected to determine and compare the bacterial community structure and count using:
  1. Fluorescent in situ hybridization (FISH) technique.
  2. Phase-contrast microscopy.
  3. Culture-dependent methods (attempted).

CHAPTER 1

1.1. REVIEW OF LITERATURE

1.1.1. Activated Sludge

The activated sludge is a biological process of wastewater treatment that has significant ecological and economical impact (Wagner et al., 2002d, Kampfer et al., 1996, Wagner et al., 1994b, Snaidr et al. 1997). It has been applied for more than a century to ameliorate damage to the environment. Activated sludge is currently the most widely used process for the treatment of both domestic and industrial wastewaters and, at least by scale, one of the most important microbiological technologies (Wagner et al., 2002d, Whiteley and Bailey, 2000, Wagner et al., 1994b). It is well known that prokaryotic microorganisms catalyze the most important transformations in wastewater treatment plants and are responsible for the observed conversions that relies primarily on the degradation and uptake of organic matter under oxic conditions (Juretschko et al., 2002, Wagner et al., 2002d, Wagner and Loy, 2002e). Processes at modern plants are often supplemented with anoxic reactor stages to enhance nitrogen and phosphorous removal.

In general, the activated sludge treatment process consists of the following three basic components: 1) a reactor in which the microorganisms responsible for treatment are kept in suspension in an aeration basin by mechanical mixers or diffused air 2) liquid-solids separation, usually in a sedimentation tank; and 3) a recycle system for returning solids removed from the liquid-solids separation unit back to the reactor (Tchobanoglous et al., 2003). The biomass is finally separated from the purified water by gravitational settling prior to the re-circulation of part of the sludge back into the aeration basin. The formation of flocculent settleable solids that can be removed by gravity settling in sedimentation tanks is an important feature of activated sludge process. The process, therefore, selects for microorganisms that remain in the system

due to their growth in flocs, which constitute a complex consortia of various microorganisms (Kampfer et al., 1996, Wagner et al., 1994b, Schramm et al. 1999).

In most cases, the activated sludge process is employed in conjunction with physical and chemical processes that are used for the preliminary and post treatment, including disinfections and possibly filtration (Tchobanoglous et al., 2003). With greater frequency, activated sludge processes used in today's plant design may incorporate nitrification, biological nitrogen removal and/or biological phosphorus removal. These designs employ reactors in series, operated under aerobic and anaerobic conditions and may use internal recycle pumps and piping (Kampfer et al., 1996). Aerobic biological strategies are commonly used to treat wastewater containing soluble and particulate organic material (LaPara et al., 2000). Several variations of this process have been developed in order to optimize carbon, nitrogen, and phosphorous removal from the wastewater. The technical details of these techniques are well known, however, the microbiological details, e.g., the compositions of the microbial populations that are responsible for the mineralization processes, are far from clear (Kampfer et al., 1996).

Activated sludge consists of a mixed community of microorganisms that metabolize and transform organic and inorganic substances into environmentally acceptable forms. The typical microbiology of activated sludge consists of approximately 95% Bacteria and 5% higher organisms (protozoa, rotifers, and higher forms of invertebrates) (Wisconsin, Department of Natural Resources, Activated sludge microbiology and process control, http://www.dnr.state.wi.us/org/water/wm/ww/tech /asludge.htm, 2003). The composition of these microorganisms depends on both the chemical composition of the wastewater and the specific characteristics of the organisms in the biological community (Benedict and Carlson, 1971, Curds and Cockburn, 1970, Curds and Fey, 1969, Curtis, 1969, Grabow, 1968, Jenkins et al., 2003, Laubenberger and Hartmann, 1971, Painter, 1970, Siebert and Toerien, 1969, Spellman, 1997, Toerien, 1967, Toerien, 1970).

Microorganisms present in activated sludge plants have always been of central interest to microbiologists. The structure of a microbial community is mainly defined by two parameters: identity and abundance of its members. A broader definition of structure would also include the spatial arrangement of species relative to each other, an information, that might be especially important in stratified habitats like biofilms (Schramm and Amann, 1999). It is well accepted that the number of bacterial species in

the environment by far exceeds the number of described species and for many years, the magnitude of microbial diversity was underestimated (Moter and Gobel, 2000, Guan et al. 2003). The efficiency and robustness of a wastewater treatment plant mainly depend on the composition and activity of its microbial community, however, the role of microbial consortia in activated sludge is still not completely understood because culture-based techniques were too selective to give a comprehensive and authentic picture of the entire microbial community (Wagner et al., 2002d, Moter and Gobel, 2000). Our current knowledge of microbial community structure-function correlations and consequently a microbiological understanding of the activated sludge process are still very limited (Juretschko et al., 2002, de los Reyes and Rothauszky, 2002, Wagner and Loy, 2002e).

Although the bacterial composition in activated sludge have been analyzed for decades by cultural methods, the low percentage of culturable bacteria on the routinely used cultivation media and the biases caused by the cultivation process made it impossible to decrypt the in situ microbial community composition (Juretschko et al., 2002). These methods have several limitations: i; they are quite time-consuming; often identification requires additional testing of physiological and biochemical traits, ii; there is a large discrepancy between the total direct microscopic counts and viable plate counts obtained for sludge samples, iii; not only underestimate the number of active cells quantitatively but also cause strong qualitative shifts in bacterial community composition (Kampfer et al., 1996, Wagner et al., 1994b, Wagner et al. 1993a). Only 1-15% of the bacteria in the microbial composition of activated sludge can be detected and are selective for certain species. Therefore, these conventional methods miss a high percentage of the microorganisms actually present and cause heavily biased shifts in community composition (Wallner et al., 1995).

1.1.2. Molecular-Based Techniques

The frequent discrepancy between direct microscopic counts and numbers of culturable bacteria from environmental samples is just one of several indications that we currently know only a minor part of the diversity of microorganisms in nature. Recent developments in cultivation-independent techniques, such as the ribosomal ribonucleic acid (rRNA) approach, now permit considerably more detailed and accurate analysis of mixed microbial communities. The differently conserved primary structure regions of

rRNAs provide target sites for specific hybridization probes which have been proven to be powerful tools for the identification of microbes on the basis of their phylogenetic relationships (Ludwig and Schleifer, 1994). These studies have confirmed the presence of complex microbial communities that are likely the underlying reason for the functionally robust nature of biological wastewater treatment systems (LaPara et al., 2000). A combination of direct retrieval of rRNA sequences and whole-cell oligonucleotide probing can be used to detect specific rRNA sequences of uncultured bacteria in natural samples and to microscopically identify individual cells (Ludwig and Schleifer, 1994).

Molecular biology offers new methods for direct quantitative determination of species mixtures in samples. Ribosomal DNA (rDNA) sequences are highly conserved, well-characterized gene families with high copy numbers and distinct variable regions. It has been used in a variety of techniques, including directed polymerase chain reaction (PCR), fluorescent in situ hybridization (FISH), in situ PCR, slot blotting, most probable number (MPN) PCR, flow cytometry, Southern hybridizations, and colony hybridizations for the identification of microorganisms at several taxonomic levels (DuTeau et al, 1998). A databank of rDNA sequences has been created to aid in designing probes for single species or groups of related species, and a collection of oligonucleotide probe sequences has also been developed for organized distribution of published probes and conditions for their use (Cottrell and Kirchman, 2000; Loy et al. 2003). Because these species-specific sequences can be used in more than one quantitative molecular technique, once developed, they can be used for a variety of environmental samples (Amann et al. 2001b). The rRNA genes contain conserved regions of sequences that are the same within a kingdom and variable regions that contain differences between taxonomic groups down to the species level (Cottrell and Kirchman, 2000; Loy et al. 2003). The comparative analysis of homologous nucleic acid sequences, most notably of rRNA molecules and the genes encoding them has profoundly changed our view of microbial systematics over the past 25 years. Large databases of sequence information exist, and rRNA gene fragments are today routinely retrieved without prior cultivation (Amann et al. 2001b).

1.1.3. Ribosomal Ribonucleic Acid (rRNA)-Based Methods

The applications of rRNA-based nucleic acid techniques to the analysis of wastewater treatment systems today range from a simple identification of isolates over

the detection of bacterial diversity and population dynamics to attempts at fully and quantitatively describing the complex microbial communities. For several reasons 16S rRNA sequence comparison is currently considered the most powerful tool for the classification of microorganisms (Woese, 1987):

1. Ribosomes and consequently rRNA molecules are present in all organisms. As an essential component of the protein synthesis apparatus they have a homologous origin and show functional constancy. No lateral gene transfer has been shown for rRNA genes so far, therefore it is a valid assumption to reconstruct the phylogeny of the organisms based on these molecules.

2. Some positions of the rRNA molecules are evolutionary more conserved than others. Consequently, sequence regions can be found that allow differentiation at any taxonomic level from species and genera up to kingdoms or domains.
3. 16S rRNA sequences have been determined for many of the described bacterial species and deposited in public databases (e.g., Maidak et al., 1997).
4. The natural amplification of rRNA within microbial cells (usually more than 1,000, frequently several 10,000 copies) makes it easier and more sensitive to assay this molecule and gives, e.g., the opportunity for identification of single bacteria by fluorescent oligonucleotide hybridization.
5. The presence and abundance of ribosomes and, consequently, rRNA in individual cells is connected to their viability and general metabolic activity, or at least their metabolic potential. Cells in a rapidly growing *E. coli* culture need and have more ribosomes than those in a slowly growing culture.

The rRNAs are the main target molecules for FISH for several reasons: they can be found in all living organisms; they are relatively stable and occur in high copy numbers (usually several thousands per cell); and they include both variable and highly conserved sequence domains. Signature sequences unique to a chosen group of microorganisms, ranging from whole phyla to individual species, can therefore be identified by comparative sequence analysis. In general the domain Bacteria and Archaea possess 5S, 16S, and 23S rRNAs with approximately 120, 1500 and 3000 nucleotides in length, respectively. The public databases now include 16S rRNA sequences for most cultured microbial species and they are the most widely used in FISH methods (Amann et al. 2001b).

Considering the high microbial diversity in many wastewater treatment plants the risk for false-positive or false-negative assignments based on single probes is rather high. Consequently, "one probe is no probe" and probes should always be applied as sets of probes. One possibility is the so-called "top-to-bottom" approach. As it is rather laborious and time-consuming to evaluate the abundance of all the bacteria that may be important in a certain sample by specific probes on the species or genus level, a pre-selection of the necessary probes has to be performed. In this regard, analysis starts with probes specific for the highest taxonomic levels, the two domains or kingdoms, Bacteria and Archaea, followed by intermediate levels such as the alpha, beta, and gamma subclasses of Proteobacteria. The Cytophaga-Flavobacterium and Bacteroides (CFB) cluster, the gram-positive bacteria with high G-C content. Genus-, species-, and subspecies-specific probes are then selected based on the information obtained with the more general probes.

1.1.4. Fluorescent In Situ Hybridization (FISH)

Fluorescent in situ hybridization (FISH) is currently a well-recognized tool for phylogenetic identification of bacteria at the level of individual cells (Whiteley and Bailey, 2000, Amann et al., 2001b, Bidnenko et al., 1998, DeLong et al., 1999, Wallner et al., 1995, Moter and Gobel, 2000, Behrens et al., 2003). Since the FISH technique provide a detailed picture of the microenvironments without any selective purification or amplification steps, it has been extensively used in the field of environmental diversity research, such as that of aquatic habitats, e.g. populations in fjords, bacterio-plankton in rivers or seawater and lake snow of a high mountain lake, and in soils and on root surfaces (Moter and Gobel, 2000). The method is based on hybridization of fixed cells with oligonucleotide probes targeted to rRNA. Generally, these probes are of 15–30 nucleotides in length and are covalently labeled at the 5' end with a fluorescent dye. After stringent washing, specifically stained cells are detected via epifluorescence microscopy or flow cytometry (Wagner et al., 2003f).

1.1.5. Fluorescent dyes

Fluorochromes with different excitation and emission maxima allow simultaneous detection of two or more microorganisms. For simultaneous microscopic observation of multicolor FISH, multi-band pass filters may be used. The combined

fluorochromes should have sharp emission peaks to prevent spectral overlap between probes, thereby eliminating background problems and bleed-through. The brightest, most photo-stable dye should be used for low-abundance targets (Moter and Gobel, 2000). The commonly used dyes for FISH are:

1. Fluorescein-derivates
   a. Fluorescein-Isothiocyanate (FITC).
   b. 5-(-6-) carboxyfluorescein-N-hydroxysuccimide-ester (FluoX))
2. Rhodamine-derivates
   a. Tetramethyl-Rhodamine-Isothiocyanate (TRITC)
3. Carbocyanine dyes
   a. Cy3
   b. Cy5

The latter ones have been shown to be superior to the classical dyes because they provide brighter staining and are very stable to photo bleaching. Table 1 shows some of the fluorochromes characteristics.

Table 1.1. Different types of fluorochromes used for the FISH method

Fluorochrome	**Wavelenght (nm)**	**Applicable excitation***	**Color**
FITC	490	B	Green
Cy3	552	G	Red
Cy5	650	IY	Infrared
DAPI	372	U	Blue
TRITC	541	G	Red

*The selection of a wideband, superwide band or narrow band filter cube depends on the condition of the specimen and observation purpose. Generally, a wide band filter cube is suitable in most cases. For some specimens where more brightness is desired, a superwide filter cube may be applied at some expense to contrast. If more contrast for a specific wavelength band is repaired for a sufficiently bright specimen, a narrow band filter cube may be appropriate.

Staining with DAPI (4',6'-diamidino-2-phenylindole) enhances the fluorescence activities (Schweitzer et al., 2001). DAPI is known to form fluorescent complexes with natural double-stranded DNA, showing a fluorescence specificity for AT, AU and IC clusters. Because of this property DAPI is a useful tool in various cytochemical

investigations. When DAPI binds to DNA, its fluorescence is strongly enhanced, what has been interpreted in terms of a highly energetic and intercalative type of interaction, but there is also evidence that DAPI binds to the minor groove, stabilized by hydrogen bonds between DAPI and acceptor groups of AT, AU and IC base pairs (Fluorescence microscopy of DAPI stained cell, http://www.celldeath.de/apometh/dapi.html, 2003). Quantitative whole-cell hybridization experiments using oligonucleotide probes accounted for 90-100% of the total DAPI-stained cells in most samples (DeLond et al. 1999).

The use of horseradish peroxidase (HRP)-labeled probes, followed by treatment with fluorescent tyramide, was recently reported to increase the sensitivity of FISH by 10-20 fold, when compared with probes labeled directly with fluorochrome. With either type of probe, cells are specifically colored according to their phylogenetic affiliation and can be visualized by epifluorescence microscopy (Bidnenko et al., 1998). Although quantitative FISH has provided novel insights into the structure and dynamics of microbial communities, it suffers from tediousness and limited accuracy for samples containing densely aggregated cells like activated sludge flocs or biofilms (Daims et al., 2001a).

The first applications of FISH were in less diverse systems: for example, the identification of bacterial symbionts in situ or the identification of magnetotactic bacteria that could be physically removed from the environment before hybridization. Over the past few years, more and more applications to complex samples, such as activated sludge or soil, have been reported (Amann et al. 2001b). As a result, new rDNA sequences, substantially different from any previously known sequences, have led to the discovery of new phyla of still uncultured microorganisms (Casamayor et al., 2002). However, groups of closely related rRNA sequences (with similarities ranging from 90-99.9%) also indicate high diversity but at a much smaller scale (microdiversity). Such microheterogeneity in the ribosomal sequences has been observed in a wide range of environments and in genetic libraries obtained from Bacteria, Archaea, and eukaryotic microorganisms. Therefore, this might be a widespread characteristic of microbial populations (Casamayor et al., 2002). With selective targeting of regions of rRNA, which consist of evolutionarily conserved and variable nucleotide regions, any taxonomic level ranging from kingdom to species should be able to be specifically identified (Lange et al., 1997).

To take full advantage of engineering efforts in rational design and optimization of activated sludge systems, results from oligonucleotide probe hybridizations remain to be interfaced with representations of microbial biomass and activity used in mathematical models of microbial growth and substrate utilization. This will only become possible when rapid and reliable methods for quantifying changes in the activity of microbial populations become available. One approach was based upon determining the relationship between cellular ribosome content and growth rate of a target population. When this information is available, FISH signal intensity can be quantified with digital microscopy to determine the in situ cellular growth rate in environmental samples (Oerther et al., 2000).

There have been two types of rRNA-based studies on activated sludge in the last few years. Group- and genus specific oligonucleotide probes were used to directly analyze the community structure of activated sludge by in situ hybridization (Snaidr et al. 1997, Wagner et al, 1993a). These studies indicated dominance of the beta subclass of the class Proteobacteria. Whereas members of this group were underestimated by cultivation techniques, members of the gamma subclass of Proteobacteria, most notably the genus Acinetobacter, were overestimated. The probes used in these studies were still based on publicly available rRNA sequences and therefore mainly on sequences of cultured bacteria. Other investigators have used direct retrieval and subsequent comparative analysis of sequences of genes coding for 16S rRNA to analyze the microbial diversity present in activated sludge (Snaidr et al. 1997). Based on 16S rRNA gene library analyses, several general microbial diversity surveys of activated sludge and biofilm systems have been performed since 1995. In total, more than 750 16S rRNA gene sequences were analysed and sequences affiliated with the beta-, alpha- and gamma-Proteobacteria as well as the Bacteroides and the Actinobacteria were most frequently retrieved (Wagner and Loy, 2002e).

Despite the overall impressive number of 16S rRNA gene clones that have been obtained from wastewater treatment reactors, sufficient data are still not available for the analysis of full-scale wastewater treatment plants (WWTPs). Furthermore, the actual community composition of complex systems cannot be inferred from 16S rRNA gene libraries alone, but must be deduced by the use of quantitative FISH with probes targeting the environmentally retrieved sequences. Unfortunately, the so-called, full-cycle rRNA approach, has rarely been used to at least partially characterize microbial

community structures in WWTPs. This is probably because of the tediousness and technical difficulties of microscopic counting of the different probe-labeled microbial populations (Wagner and Loy, 2002e). The development of semi-automatic digital image analysis routines has significantly accelerated quantitative FISH experiments and enabled microbiologists for the first time to obtain encompassing, high-resolution insights into the microbial community composition of activated sludge (Wagner and Loy, 2002e).

Comparison of a newly retrieved rRNA sequence to the continuously growing rRNA databases allows the direct design of oligonucleotide probes complementary to more or less variable target regions. Intermediate group-specific oligonucleotides complementary to sequence regions characteristic of phylogenetic entities at the genus or subclass level have been used successfully for rapid identification of bacteria. Molecular taxonomists found significant rRNA homologies between Actinomycetes and a group of related bacteria, all characterized by gram-positive staining and a high G-C content of DNA, and consequently proposed a group named the "Actinomycetes branch" or gram-positive bacteria with high G-C content of DNA. Many species of this phylogenetic group have biotechnological or medical importance (Roller et al., 1994). The use of FISH probing and cloning of 16S rDNA to describe activated sludge bacterial communities has shown that Actinobacteria (gram-positive bacteria with high G-C content) and beta-Proteobacteria are dominant in enhanced biological phosphorus removal (EBPR) mixed communities (Bond et al., 1999).

Microorganisms which are of major importance for processes such as wastewater treatment, microbial leaching or methane production can be detected and quantified in situ within a complex microbial community. For certain processes, such as nitrification or biological phosphate removal, new microorganisms have become the focus of interest and have led to an improved understanding of these bioremediation techniques (Lipski et al., 2001). Recently, it has been reported that a group of bacteria in activated sludge with enhanced biological phosphorus removal were identified. Another group focused on mycolic-acid containing Actinomycetes that are thought to be involved in filamentous foaming in sewage treatment plants (Moter and Gobel, 2000). Also members of the domain Archaea, particularly methanogens, have been found by FISH in anaerobic digesters and within anaerobic sludge granules, despite their strong autofluorescence (Moter and Gobel, 2000). Members of the CF group were revealed by

in situ hybridization as an important constituents of sludge flocs and characteristic colonizers of filamentous bacteria (Wagner and Loy, 2002e).

Mass occurrence of certain bacterial species can also be detrimental for sewage treatment by negatively influencing the settling properties of activated sludge in the secondary clarifiers, by contributing to the formation of foam or by out competing microorganisms required for nutrient removal. Consequently, a thorough knowledge of the ecology of the microbial communities is required to reveal factors influencing the efficiency and stability of biological WWTPs and to develop promising strategies for improved process performance (Wagner and Loy, 2002e). Often the microscopically most prominent microorganisms like the filamentous bacteria in foaming activated sludge cannot be found by standard cultivation procedures. It enables only the identification of predominant filaments in bulking activated sludge, whereas filaments occurring in low numbers are easily missed (Wagner et al., 1993a).

Due to intricate interactions within the microbial community, process control of wastewater treatment plants can be difficult. Population shifts within the microbial community may result from changes in the plant operating conditions and cause sludge quality problems such as poor sludge settling, compaction, and dewatering. Some operational problems with activated sludge can often be detected microscopically at the microorganism level. For example, poor sludge settling due to filamentous bulking is due to excessive growth of bacterial filaments (e.g., *Sphaerotilus natans*, *Microthrix parvicella*, *Hyphomicrobium* spp., *Thiothrix nivea*, etc.). Diagnosis and correction of such activated sludge problems require the correct identification of the responsible microbial population(s) and institution of appropriate process changes to select for or against specific organisms (Layton et al., 2000).

One of the major operational problems of these systems is the excessive growth of filamentous bacteria that causes poor settlement of activated sludge flocs, a problem commonly referred to as bulking (Kanagawa et al., 2000). Deficiency in the nutrient supply such as nitrogen usually results in activated sludge bulking and this phenomenon often takes place in the industrial wastewater treatment plants with activated sludge process (Peng et al., 2003). On the other hand, it has been suggested that a certain number of filamentous bacteria are required for proper floc formation. Thus, depending upon their identity and abundance, filamentous bacteria can be either beneficial or

detrimental for efficient separation of the treated wastewater from the biomass in the settling tanks (Wagner and Loy, 2002e). Therefore, the ability to unambiguously identify filamentous bacteria is crucial for proper control of wastewater treatment systems. Since cultivation of most filamentous bacteria is difficult and time-consuming, their classification is traditionally achieved by microscopic observation of morphological traits and simple staining reactions of the filaments within activated sludge using the keys proposed by Eikelboom (Wagner and Loy, 2002e). Since the majority of filamentous bacteria in activated sludge are morphologically different from previously identified bacteria, type numbers provisionally classified them (Wagner and Loy, 2002e).

Many of the filamentous bacteria associated with the serious operational disorders of bulking and foaming in activated sludge of WWTPs have never been grown in axenic culture. Most are still referred to as morphological types (e.g. Type 021N, Type 1863), using a naming system taken from the original proposals that is based exclusively on their microscopic features (Beer et al., 2002). Of those gram-positive and gram-negative filamentous bacteria that have been successfully cultured, many have been shown to represent novel organisms (Beer et al., 2002). In situ hybridization of gram-positive filamentous bacteria often resulted in an irregular distribution of fluorescence signals over the whole filaments. While some parts gave bright signals, other parts of the same filament exhibited no fluorescence. The borderlines of single cells in the filaments are invisible. Consequently, it remains unresolved whether different parts of the filaments belong to the same cell or not. A possible explanation for the staining pattern observed would be that parts showing no signals represent dead cells or at least cells with a low metabolic activity. Variation in permeability of the cell envelope within the filament is another possible explanation of this phenomenon (Schuppler et al., 1998). It is also clear now that relying solely on morphological criteria for their identification is inappropriate. For example, while some like *Microthrix parvicella* appear to be single genetic entities based on their 16S rRNA gene sequences, regardless of where they occur in the world, other morphotypes like Type 1863 and *Nostocoida limicola* have now been shown to contain several phylogenetically unrelated bacteria, which coincidentally appear indistinguishable under the microscope (Beer et al., 2002).

More than 30 different filamentous morphotypes have been observed in domestic WWTPs and approximately 40 additional morphotypes were recently described in industrial activated sludge plants (Wagner and Loy, 2002e). Using microautoradiography (MAR) to investigate the in situ physiology of *Microthrix parvicella*, one of the few filaments that can unambiguously be identified on the basis of its morphology. This gram-positive organism, which is difficult to maintain in pure culture, possesses a hydrophobic cell surface and is the causative agent for worldwide foaming and bulking problems in WWTPs with nutrient removal (Wagner and Loy, 2002e). MAR analyses showed that *M. parvicella*, in contrast to most other activated sludge bacteria, is able to take up and store long-chain fatty acids under anaerobic conditions and subsequently metabolize them under aerobic conditions. This physiological potential offers *M. parvicella* a competitive advantage in nutrient-removal plants with dynamic anaerobic-aerobic conditions against most other activated sludge bacteria (Wagner and Loy, 2002e).

Foaming of activated sludge due to an extensive multiplication of filamentous bacteria causes serious separation problems in sewage treatment plants throughout the world. In addition to *Microthrix parvicella*, a filamentous bacterium belonging to the group of high G-C gram-positive bacteria, generally considered to be the cause of foaming in sewage treatment plants (Schuppler et al., 1998). The reasons for the extensive multiplication of these filamentous bacteria are still unknown. Classical approaches fail to differentiate foam-causing species such as *Gordona amarae*, *Rhodococcus rhodochrous* and *Tsukamurella paurometabolum* due to their variable morphology, staining behavior and fastidious nature (Schuppler et al., 1998).

1.1.6. Phase Contrast Microscopy

Unpigmented living cells are not clearly visible in the brightfield microscope because there is little difference in contrast between the cells and water. Thus microorganisms often must be fixed and stained before observation to increase contrast and create variations in color between cell structures. A phase-contrast microscope converts slight difference in refractive index and cell density into easily detected variations in light intensity and is an excellent way to observe living cells. (Presscott et al., 2002).

Phase contrast is preferable to bright field microscopy when high magnifications (400x, 1000x) are needed and the specimen is colorless or the details so fine that color does not show up well. Cilia and flagella, for example, are nearly invisible in bright field but show up in sharp contrast in phase contrast. Amoebae look like vague outlines in bright field, but show a great deal of detail in phase. Most living microscopic organisms are much more obvious in phase contrast. (Experimental Biosciences, Phase Contrast Microscopy, http://www.ruf.rice.edu/~bioslabs/methods/microscopy/phase html, 2003).

1.1.7. Bacterial Groups

1.1.7.1. Proteobacteria

The Proteobacteria are the largest and most diverse group of bacteria; currently there are over 380 genera and 1.300 species. Although the 16S rRNA studies show that they are phylogenetically related, Proteobacteria vary markedly in many respects. The morphology of these gram-negative bacteria ranges from simple rods and cocci to genera with prosthecae, buds, and even fruiting bodies. Physiologically these bacteria are just as diverse. Photoautotrophs, chemolithotrophs, and chemoheterotrophs are all well represented. The phylum Proteobacteria, which has five classes: Alpha, beta, gamma, delta and epsilon subclasses, is sometimes called the purple bacteria because of the purple photosynthetic bacteria scattered among the alpha, beta and gamma subclasses. This has led to the proposal that the Proteobacteria arose from a photosynthetic ancestor, presumably similar to the purple bacteria. Subsequently photosynthesis would have been lost by various lines and new metabolic capacities would have been acquired as these bacteria adapted to different ecological niches (Prescott et al., 2002).

1.1.7.1.1. Alpha subclass

The alpha subclass of Proteobacteria include most of the oligotrophic bacteria (those capable of growing at low nutrient levels). Some have unusual metabolic modes such as methylotrophy (Methylobacterium), chemolithotrophy (Nitrobacter), and the ability to fix nitrogen (Rhizobium) (Prescott et al., 2002).

1.1.7.1.2. Beta subclass

The beta subclass of Proteobacteria overlap the alpha subclass metabolically but tend to use substances that diffuse from organic decomposition in the anaerobic zone of habitats. Some are chemoheterotrophs, photolithotrophs, methylotrophs or chemolithotrophs. Sphaerotilus, one of the members of this group prefers slowly running freshwater polluted with sewage or industrial waste. It grows so well in activated sewage sludge that it sometimes forms tangled masses of filaments and interferes with the proper settling of sludge. Nitrosomonas and Nitrosospira are also members of this group (Prescott et al., 2002).

1.1.7.1. 3. Gamma subclass

The gamma subclass of Proteobacteria constitutes the largest subgroup of Proteobacteria with an extraordinary variety of physiological types. Many important genera are chemoorganotrophic and facultatively anaerobic. Other genera contain aerobic chemoorganotrophs, photolithotrophs, chemolithotrophs, or methylotrophs. According to some DNA-rRNA hybridization studies, the gamma Proteobacteria are composed of several deeply branching groups. The genera Pseudomonas, Azotobacter, Moraxella, Xanthomonas, and Acinetobacter belong to this superfamily. Leucothrix is an aerobic chemoorganotroph that forms long filaments. It is usually marine and is attached to solid substrates by a holdfast. Thiothrix is a related genus that forms sheated filaments. In contrast to Leucothrix, Thiothrix is a chemolithotroph that oxidizes hydrogen sulfide and deposits sulfur granules internally. Thiothrix grows in sulfide-rich flowing water and activated sludge sewage systems. Nitrococcus and Nitrosococcus are also members of this group. The pseudomonads have a great practical impact in several ways, including the following: Many can degrade an exceptionally wide variety of organic molecules. Thus they are very important in the mineralization process (the microbial breakdown of organic materials to inorganic substances) in nature and in sewage treatment.

1.1.7.2. Nitrifying Bacteria

Nitrifying bacteria are within the alpha, beta, and gamma subclasses of Proteobacteria. Nitrobacter (alpha), Nitrosomonas and Nitrosospira (beta), Nitrococcus and Nitrosococcus are in the gamma subclasses (Prescott et al., 2002). Nitrifying

bacteria are aerobic chemolithotrophic that are divided into three groups based on the inorganic compounds they prefer to oxidize: 1. Nitrifiers, 2. Colorless sulfur bacteria (sulfur oxidizers), 3. Iron- and manganese-oxidizing bacteria.

These bacteria are very important ecologically and can be isolated from soil, sewage disposal systems, freshwater and marine habitats. The genera Nitrobacter and Nitrococcus oxidize nitrite to nitrate; Nitrosomonas, Nitrosospira, and Nitrosococcus oxidize ammonia to nitrite. When two genera such as Nitrobacter and Nitrosomonas grow together in a niche, ammonia is converted to nitrate, a process called nitrification. Nitrification occurs rapidly in soils treated with fertilizers containing ammonium salts. Nitrate nitrogen is readily used by plants, but it is also rapidly lost through leaching of the water soluble nitrate and by denitrification to nitrogen gas.

1.1.7.3. Cytophaga

Members of the genera Cytophaga and Sporocytophaga are aerobes that actively degrade complex polysaccharides. Cytophagas play a major role in the mineralization of organic matter. They are a major component of the bacterial population in sewage treatment plants and presumably contribute significantly to this waste treatment process (Prescott et al., 2002).

1.1.7.4. High G-C Gram-Positive Bacteria

The high G-C gram-positive bacteria are commonly called Actinomycetes. The Actinomycetes are aerobic, gram-positive bacteria that form branching filaments or hyphae and asexsual spores. It may be a nuisance that pollute water supplies, or grow in sewage treatment plants where they form thick clogging foams. Nocardia is distributed worldwide in soil and also is found in aquatic habitats. Nocardia are involved in the degradation of hydrocarbons and waxes and can contribute to the biodeterioration of rubber joints in water and sewage pipes (Prescott et al., 2002).

1.1.8. Textile Industry

The textile industry is one of the most important and developed industries worldwide. For example in Turkey, the textile industry comprises nearly 35% of the total exports comprising a cost of approximately $7 billion per year (Arslan, 2000). At the same time, this industry is one of the major water consumers among all industrial

sectors with a typical process water consumption of 2000 ton/day/plant (Nemerow, 1978, Correia, et al., 1994). Hence its parallel contribution to environmental pollution should not be underestimated (Arslan, 2000). Like many other industrial effluents, textile industry wastewater varies significantly in quantity, but additionally in composition (Besseliewre and Schwarz, 1976, Correia et al., 1994). Textile wastewater is characterized by its high chemical oxygen demand (COD), medium biochemical oxygen demand (BOD_5), high alkalinity and total solids content (Masselli et al. 1959, Nemerow, 1978). It is highly colored which creates environmental as well as aesthetic problems.

As regulations are becoming ever more stringent, the need for technically and economically more efficient means of decolourization and mineralization grows more acute. At present time, there are no economically attractive technologies to achieve colour removal. Existing physicochemical technologies such as membrane filtration or activated carbon adsorption are expensive and commercially unattractive. Furthermore, these processes are rather transferring pollutants from one phase to another than eliminating them from the water matrix. Recovery and reuse of certain chemical compounds present in dye-bath effluents is under investigation (Grau, 1991). In addition, the problem of color has become particularly identified with the dyeing of cotton fibers, that contributes to almost 40 % of total fiber consumption annually worldwide, and the use of reactive dyestuffs since as much as 50 % of these dyes end up in the exhausted dye-bath in their hydrolyzed and unfixed form (Easton, 1995, Reife, 1996).

Textile wastewater is characterized by high volume and extreme variability in composition, which can include non-biodegradable, potentially toxic dyestuffs and a variety of other chemicals (waxes, resins, fixing agents, fire and water proofing agents) (Nemerow, 1978). It is known to contain high total dissolved solid (TDS) (1000-10000 mg/L), total alkalinity (300-900 mg/L) and COD (600-40000 mg/L), mid-strength BOD_5 (200-1800 mg/L), and varying pH (4-12; mostly alkaline) (Nemerow, 1978). Organic substances such as dyes, starches and detergents in textile wastewater undergo chemical and biological changes that consume dissolved oxygen from the receiving stream and so destroy aquatic life. Such organic contaminants should be removed to prevent septic conditions and obnoxious odors, and to avoid rendering the stream waters unsuitable for municipal, industrial, agricultural, and residual use. High concentrations

of soluble inorganic salts may make the stream unsuitable for industrial and municipal use, and may have a corrosive effect on boats and other structures (Arslan, 2000).

The wastes can be treated in various ways, and the best combination of methods differs from plant to plant. Each treatment plant must evaluate possible treatment procedures according to the existing type of treatment facility, type of wastewater, and degree of treatment needed. The main problems caused by textile processing effluents in the operation of treatment include foaming, colour persistent, high pH, temperature, heavy metals originating from dyestuffs and variations in the flow rate. Conventional treatment of textile effluents consists of segregation (seperation of process effluents that need special treatment), equalization, screening, mechanical filtration and neutralization as the primary steps followed by chemical coagulation, flocculation, flotation and biological tratment or their combination as secondary treatment technique, by which the reduction of organic load is aimed, and finally optional tertiary treatment (polishing) namely chemical oxidation (disinfection) via chlorination or ozonation (Arslan, 2000).

1.1.9. Dyes

Synthetic organic colorants (e.g., azo dyes) are used universally in manufacturing processes ranging from food and textile production to the printing and pharmaceutical industries. By design, the majority of these dyes are recalcitrant so that they can confer color on various raw materials; moreover, certain dyes and dye precursors and some aromatic amines produced through biotransformation of dye compounds have been shown to be carcinogenic. In addition, very low concentrations of dyes (<1 mg/liter) can be highly visible in solution and are therefore aesthetically unacceptable. Some 10,000 dyes are currently manufactured; many of these are azo dyes due to their [--N═N--] bond structure, and it is estimated that at least 15% of them are released into the environment, predominantly by textile and dye manufacturing industries. Wastewaters produced by dye manufacturers typically comprise mixtures of the various dyes produced by the manufacturers and their intermediate precursors (Plump et al., 2001).

The textile industry and dyestuff manufacturing industry are two major sources of released azodyes (Zissi and Lyberatos, 1996). Azo dyes are consumed and otherwise utilized in varying quantities in many parts of the world. Such widely used chemicals

are of great concern with regard to their potential toxicity and carcinogenic properties. Reduction of the carcinogenic dyes usually leads to loss of carcinogenic activity. By contrast, most of the highly charged water-soluble dyes become mutagenic only after reduction. Many problems regarding mutagenic and carcinogenic activation remain to be solved. At the present time, it is apparent that both oxidative and reductive pathways yield toxic products. This is critical in the treatment of waste from chemical plants where there is a great need for soil bacteria, which catalyze reduction aerobically (Levine, 1991). Most reactive dyes are not readily biodegraded in aerobic sewage treatment processes. Some, however, may be adsorbed intact by the sludge at sewage treatment works and hence removed from the aquatic compartment. It is possible that preferential adsorption of these latter chromophore types from a colored dyehouse effluent during biological treatment leads to the characteristic straw or pink tones often observed in sewage treatment works outfall (Easton, 1995).

CHAPTER 2

MATERIALS AND METHODS

2.1. TEXTILE FACTORIES

Three textile factories located in the Istanbul city (European region) were selected based on the fabric coloring agents (using reactive dyes as the general coloring agents) and the general setting of the treatment plants (equalization basin, neutralization basin, aeration basin and sedimentation basin). All factories seems to meet the rules under the federal regulations of Istanbul Water and Sewage Administration (IWSA) "İstanbul Su ve Kanalizasyon İdaresi" (İSKİ). In addition, only factory I has an ISO-14000 certificate. The differences in performance among the 3 factories was calculated according to the efficiency equation (table 2) (Tchobanoglous, 2003).

Table 2.1. Performance differences among the 3 textile factories

Factory	**Parameters**	**Influent**	**Effluent**	**Performance (Influent - Effluent /Influent) %**
I	COD pH Temp.	1400-1600 mg/L 10-11 40-60 °C	300-350 mg/L 6,5- 8 37-38 °C	78%
II	COD pH Temp.	1100-2000 mg/L 11-12 or 2-3 40-60 °C	500-700 mg/L 6 - 9 25-30 °C	61%
III	COD pH Temp.	1200-1800 mg/L 10-11 40-60 °C	550-750 mg/L 7 - 9 38-42 °C	43%

COD: Chemical Oxygen Demand, Temp: Temperature

Below are the general schemes of the wastewater treatment plants of the 3 factories.

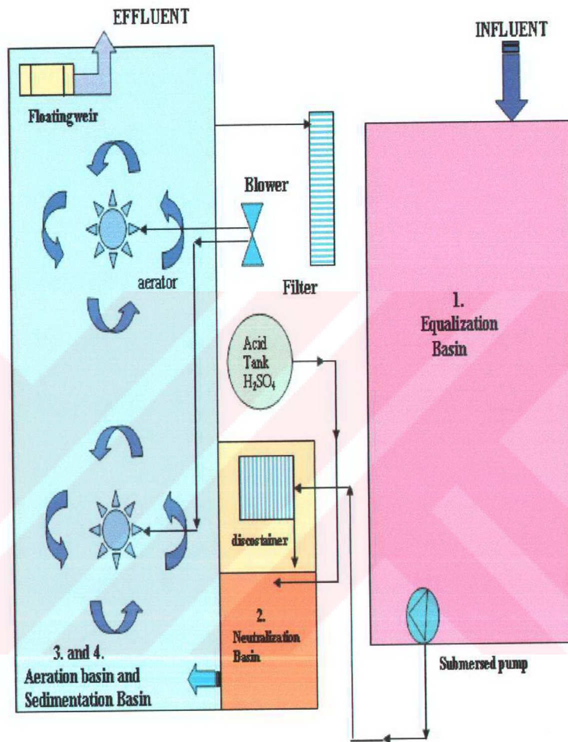


Figure 2.1. Schematic presentation of the wastewater treatment plant of factory I, showing:

1. Equalization basin, 2. Neutralization basin, 3. Aeration basin, 4. Sedimentation basin.

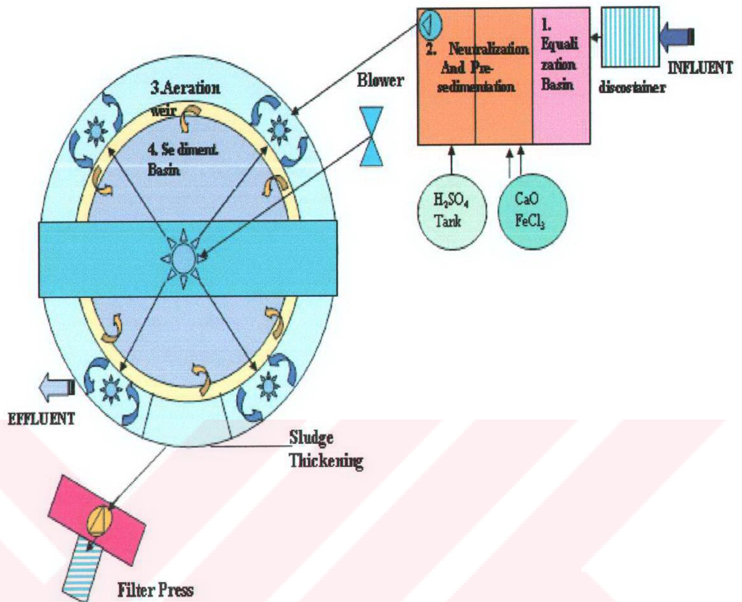


Figure 2.2. Schematic presentation of the wastewater treatment plant of factory II, showing:

1. Equalization basin, 2. Neutralization basin, 3. Aeration basin, 4. Sedimentation basin.

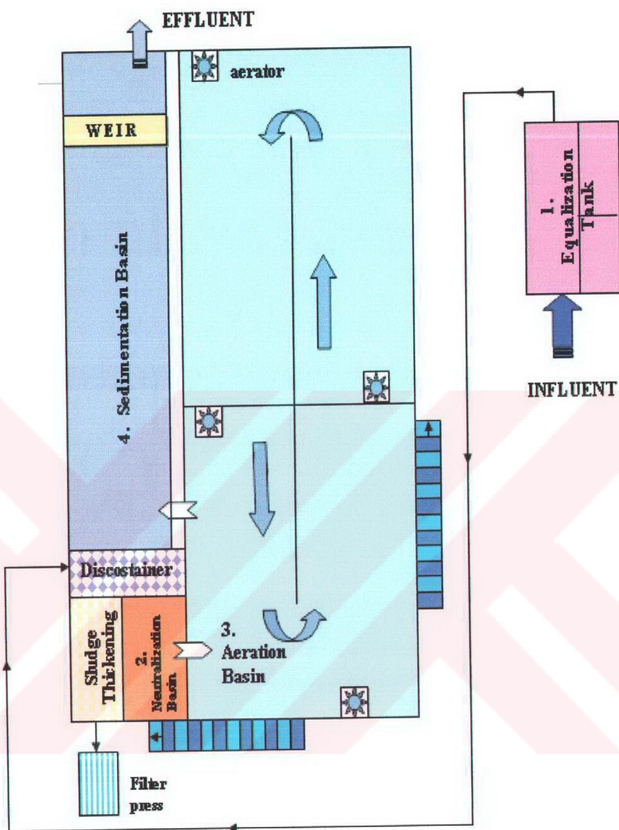


Figure 2.3. Schematic presentation of the wastewater treatment plant of factory III, showing: 1. Equalization basin, 2. Neutralization basin, 3. Aeration basin, 4. Sedimentation basin..

2.2. SAMPLES

A 300 mL of activated sludge sample from each factory was collected from the aeration basin by a bucket (pail) and placed into a sterile glass flasks. Samples were brought to the laboratory within 1-2 h and processed immediately.

2.3. FLUORESCENT IN SITU HYBRIDIZATION (FISH)

The principles of the FISH technique:

- Fixation of the specimen.
- Hybridization with the respective probes.
- Washing steps to remove unbound probes.
- Mounting, visualization and documentation of results.

2.3.1. Fixation

One mL of the activated sludge samples was fixed with fresh (not older than 24 h) cold (4%) paraformaldehyde solution in phosphate-buffered saline (PBS) pH 7.2. The fixative solution was prepared by the addition of 1 drop of 10 M NaOH, 2 g of paraformaldehyde, and 16.5 ml of 3X PBS buffer to 33 ml of double-distilled H_2O. After paraformaldehyde was dissolved, the solution was cooled on ice, the pH was adjusted to 7.2 and the solution was filtered through 0.2 µm pore-size filter. One volume of sample suspension was mixed with 3 volumes of the fixative solution, and the mixture was incubated at 4°C for 3 h or overnight. The suspension was centrifuged at 14.000 rpm (Hettich microcentrifuge, Germany) for 2 min at 4°C. The supernatant was removed and the pellet was washed 3 times with PBS and then finally resuspended to the original volume with PBS and stored at -20°C (Juretschko et al., 2002, Amann et al., 1990).

2.3.2. Hybridization

A 10 µL of the fixed sample was then spotted separately on precleaned gelatin-coated [0.1% gelatin, 0.01% $KCr(SO_4)_2$] of 10 well microscopic slide (Novakemi; AB, Enskede, Sweden). The slides were then air dried, and dehydrated by immersing into

several concentrations of ethanol (50, 80, and 100%) for 3 min each. (Wagner et al., 1993a).

10 of μl of the hybridization buffer [0.9 M NaCl, 20 mM Tris/HCl, pH 7.4, 0.01% sodium dodecyl sulfate (SDS) and different formamide concentrations containing (5 ng/μL) of each probe was added to the slides and incubated at 46°C for 90 min (Snaidr et al., 1997). The probes were labeled with a Cy3 and FITC reactive fluorescent dye at the 5' end (Thermo BioSciences, Ulm, Germany). Hybridization was carried out in a properly sealed moisture chamber to prevent evaporative concentration of the hybridization solution, which might result in nonspecific binding of fluorescent probe to the cell.

2.3.2.1 Probes

The following probes complementary to specific regions of the 16S rRNA were used (table 3) (Snaidr et al., 1997).

a. EUB 338 probe: specific for the domain Bacteria.
b. ALF1b probe: specific for the Alpha subclass of Proteobacteria.
c. BET42a probe: specific for the Beta subclass of Proteobacteria.
d. GAM42a probe: specific for the Gama subclass of Proteobacteria.
e. CF319a probe: specific for the Cytophaga-Flavobacterim (CF) cluster.
f. HGC69a probe: specific for the high G-C gram-positive Bacteria.

Table 2.2. The properties of the oligonucleotide probes used in this study and their target

Probe	Label	F (%)	Sequence 5'-3' Target position	Specificity
EUB338	Cy3	0	GCTGCCTCCCGTAGGAGT	338-355 domain Bacteria
ALF 1b	FITC	20	CGTTCGYTCTGAGCCAG	19-35 alpha subclass of Proteobacteria
BET42a	FITC	35	GCCTTCCCACTTCGTTT	1027-1043 beta subclass of Proteobacteria
GAM42a	Cy3	35	GCCTTCCCACATCGTTT	1027-1043 gamma subclass of Proteobacteria
CF319a	Cy3	35	TGGTCCGTGTCTCAGTAC	303-319 Cytophaga Flavobacterium cluster
HGC69a	FITC	35	TATAGTTACCACCGCCGT	1901-1918 high G-C gram-positive bacteria

F: Formamide concentration

2.3.3. Washing

After hybridization, unbound oligonucleotides were removed by rinsing with washing buffer (1 mM NaCI, 20 mM Tris/HCI, pH 7.4, 0.01% SDS at 48°C for 15 min). NaCl was adjusted according to the concentration of formamide. At 0, 20 and 35% concentration of formamide a 900, 225, and 80 mM NaCl was used. To enumerate the total bacteria number in the samples, the samples were stained with DAPI (33 μg/mL). Washing buffer was removed with distilled water, and the slides were air-dried (Snaidr et al., 1997). Citiflour (Citifluor Ltd., London, United Kingdom) was used to prevent photoleaching (Wagner et al., 1993a).

2.3.4. Visualization

The Olympus BX50 fluorescent microscope (Olympus Cooperation, Japan) was used in this study. It has three dichroic mirror/filter combinations. The cube U gives an excitator beam of about 350 nm, and is used for DAPI stained specimen. The cube B gives an excitator beam of 435-490 nm, and is used for FITC, acridine orange and auramine stained specimens. The cube G gives an excitatory fluorescence of 546 nm, and is used for a number of different fluorescent antibody methods.

2.3.5. Bacterial Count by the FISH technique

The activated sludge samples from each factory were diluted in 10-fold serial dilutions (10^{-1} - 10^{-7}) and then, 10 μL from each dilution series was hybridized by the respective probe. The number of the bacterial in each dilution was counted by in a systematic manner that covers the entire field.

2.4. PHASE CONTRAST MICROSCOPY

One drop of well-mixed activated sludge sample using a loop or disposable pipette was placed onto a 25 x 75 mm microscopic slide. Covered with a cover slip and excess the fluid was removed by touching the edges of the slip with a tissue to make a thin preparation. The freshly made wet mount then examined under phase-contrast microscope at 20X magnification. The characteristics of the flocs and the abundance of filamentous bacteria in the activated sludge samples were examined together with other living microorganisms. The floc shape was characterized whether round or irregular, compact or diffuse. A subjective scoring system was used for the analysis of the

abundance of filamentous bacteria. An overall abundance scale from 0 (none), 1 (few), 2 (some), 3 (common), 4 (very common), 5 (abundant) to 6 (excessive) was used. Separation problems are encountered when the abundance of filamentous bacteria scores very common or greater.

2.5. CULTURE-DEPENDENT TECHNIQUES

2.5.1. Culture Media Preparation

In this study, Nutrient agar, Blood agar and MacConkey agar was used for culturing bacteria from the activated sludge samples. The pour plate technique was applied to differentiate and select bacteria according to their size, color and morphology. Selected colonies were then subcultured and tested by biochemical tests.

2.5.2. Bacterial Counts by Culture

The activated sludge samples were diluted into a 10-fold serial dilution series into PBS buffer and then mixed with nutrient agar for pour plate method. The nutrient agar was cooled to 50°C then 2 ml of the bacterial suspension from each dilution was added and mixed throughly with the agar and then poured into petri dishes. After being solidified, the plates were incubated at 37°C for 24-48 hours. The number of the colonies was counted in each plate for each dilution using the Gel Doc 2000 (BIO RAD, Italy). From the count it is a simple matter to calculate the number of organisms per milliliter of the original culture.

CHAPTER 3

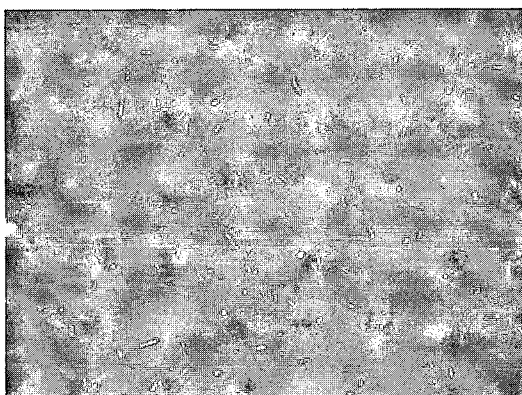
RESULTS

3.1. FLUORESCENCE IN SITU HYBRIDIZATION (FISH)

The results of the bacterial groups in the activated sludge samples detected by FISH were as follows: All the bacterial groups specified by the 16S rRNA probes of the domain Bacteria (alpha, beta, and gamma subclasses of Proteobacteria, CF, high G-C gram-positive bacteria) and the domain Archea were detected in the activated sludge sample of factory I (Figures 3.1, 3.2, 3.3, 3.4, 3.5, 3.6, 3.7). Similarly the presence of these groups was also detected in the activated sludge samples obtained from factory II (Figures 3.8, 3.9, 3.10, 3.11, 3.12, 3.13) and Factory III (Figures 3.14, 3.15, 3.16, 3.17, 3.18, 3.19).

3.1.1.Factory I.

The EUB338 probe visualization results are shown in figure 3.1. The domain Bacteria (a and b) appeared blue when stained with DAPI stain (a).



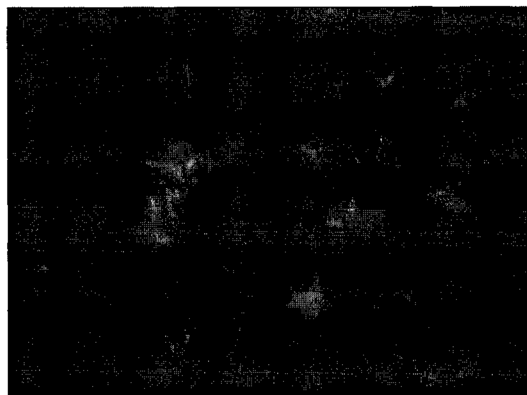


Figure 3.1 a b

The ALF1b probe visualization results are shown in figure 3.2. The alpha subclass of Proteobacteria (a and b) appeared blue when stained with DAPI stain (a).

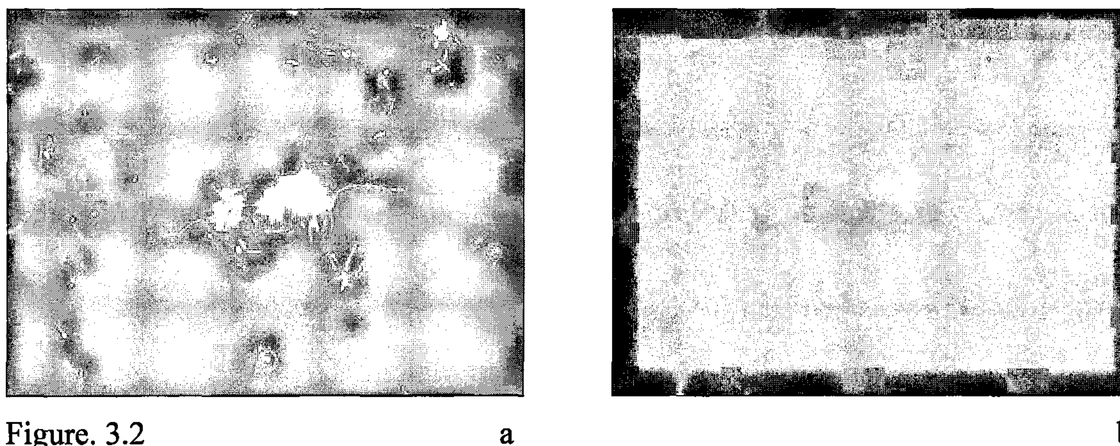


Figure. 3.2 a b

The BET42a probe visualization results are shown in figure 3.3. The beta subclass of Proteobacteria (a and b) appeared blue when stained with DAPI stain (a).

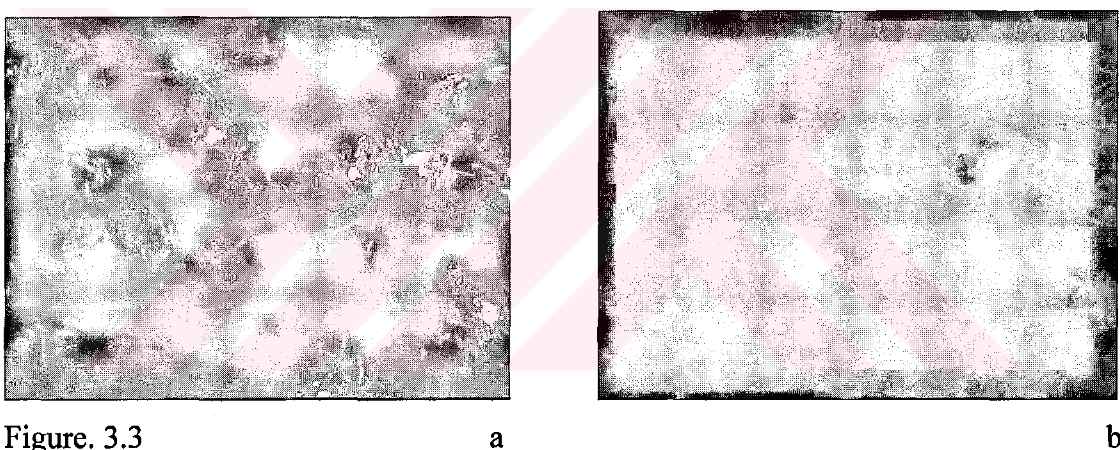


Figure. 3.3 a b

The GAM42a probe visualization results are shown in figure 3.4. The gamma subclass of Proteobacteria (a and b) appeared blue when stained with DAPI stain (a).

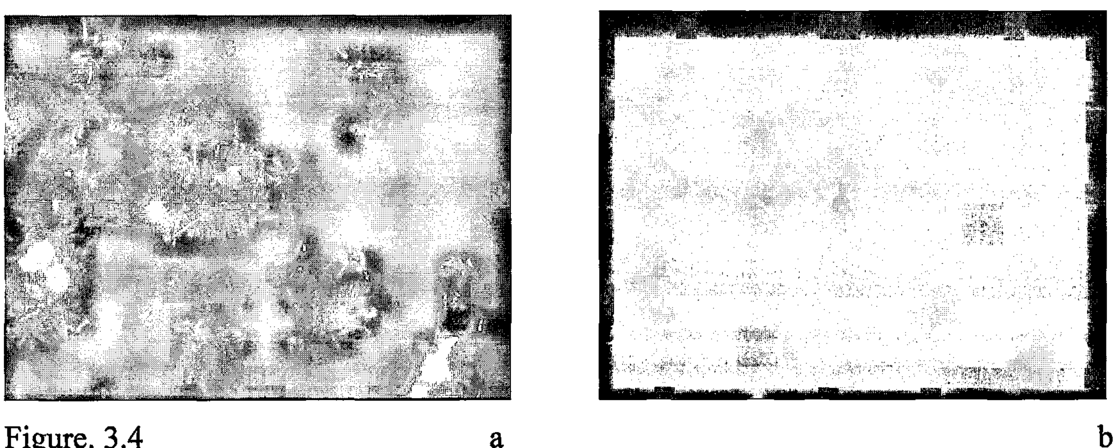
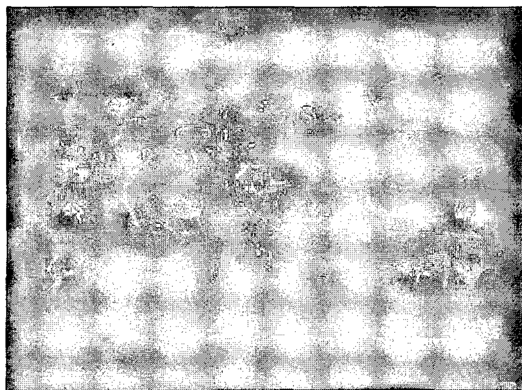


Figure. 3.4 a b

The CF319a probe visualization results are shown in figure 3.5. The Cytophaga-flavobacterium (a and b) appeared blue when stained with DAPI stain (a).



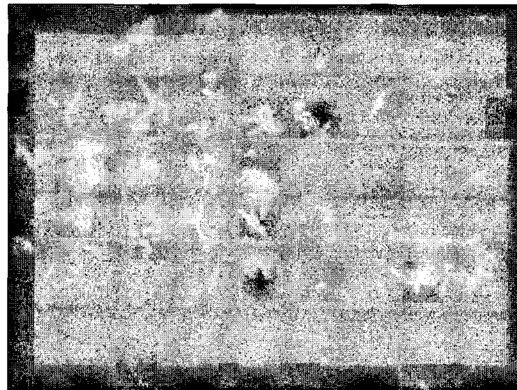
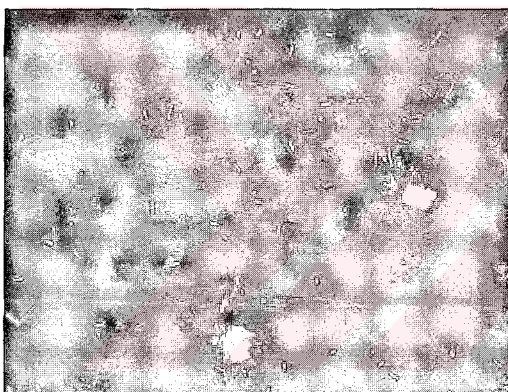


Figure 3.5 a b

The HGC69a probe visualization results are shown in figure 3.6. The high G-C gram-positive bacteria (a and b) appeared blue when stained with DAPI stain (a).



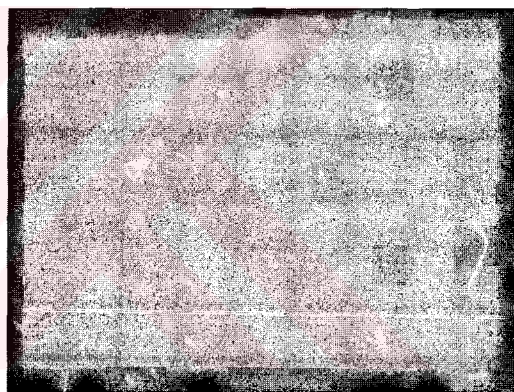
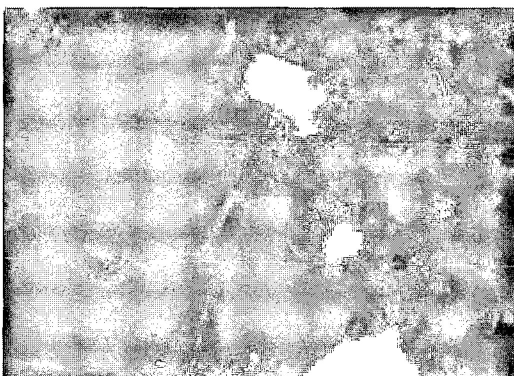


Figure 3.6 a b

The domain Archea detected by the probe (ARC 915) (a and b). a stained with DAPI, b stained with Cy3 (figure 3.7).



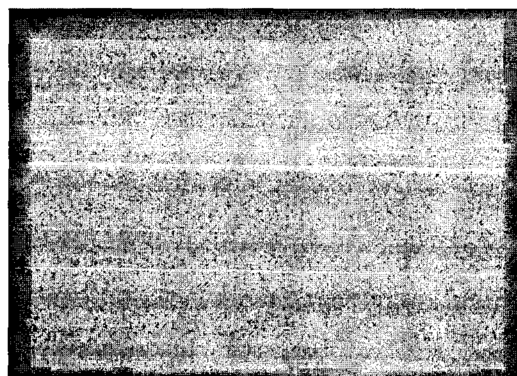


Figure 3.7 a b

3.1.2. Factory II

The ALF1b probe visualization results are shown in figure 3.8. The alpha subclass of Proteobacteria (a and b) appeared blue when stained with DAPI stain (a).

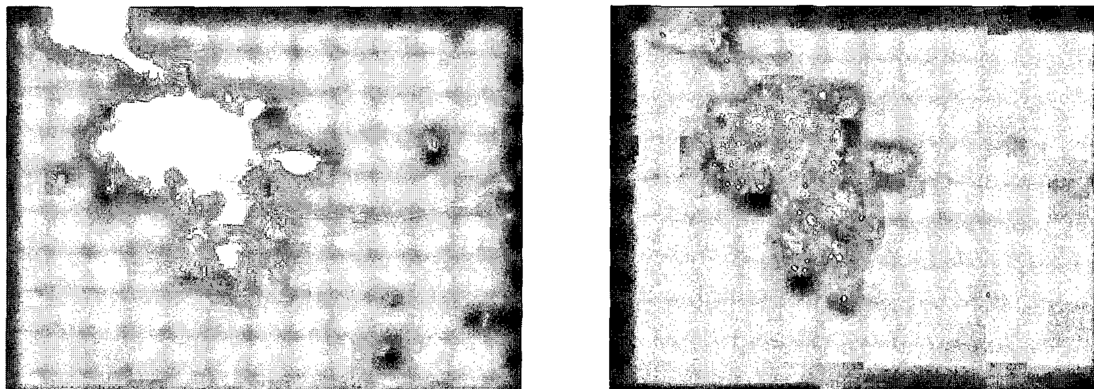


Figure 3.8 a b

The BET42a probe visualization results are shown in figure 3.9. The beta subclass of Proteobacteria (a and b) appeared blue when stained with DAPI stain (a).

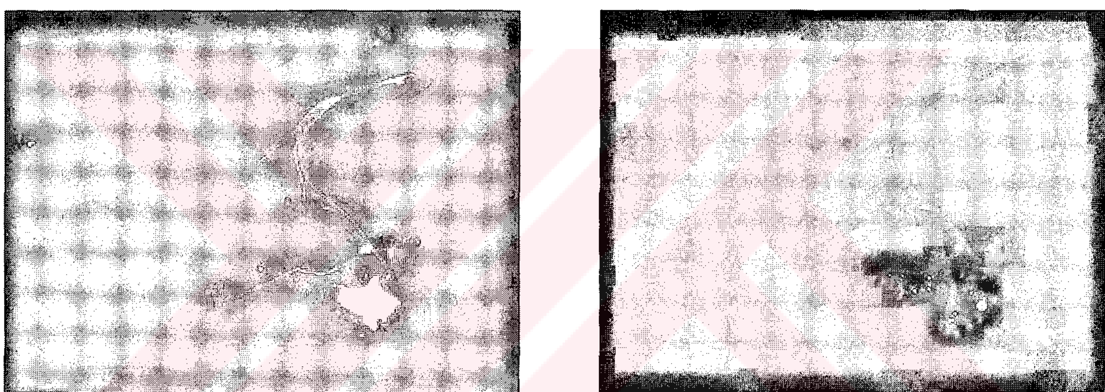


Figure 3.9 a b

The GAM42a probe visualization results are shown in figure 3.10. The gamma subclass of Proteobacteria (a and b) appeared blue when stained with DAPI stain (a).

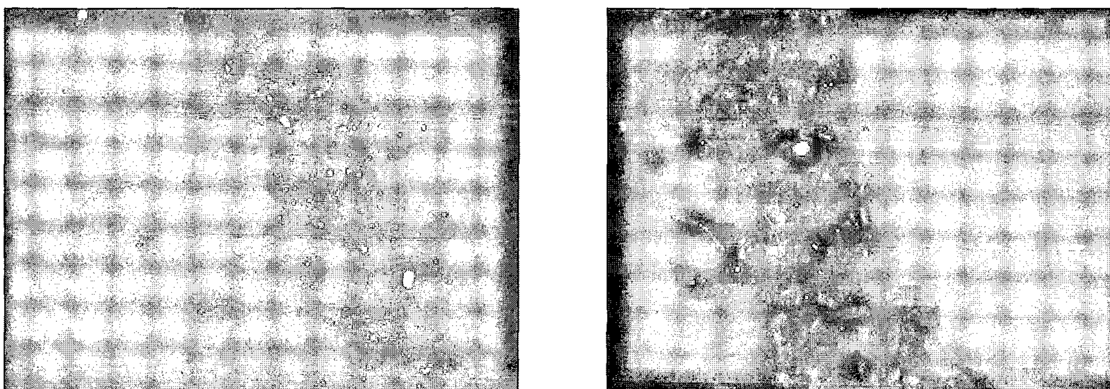


Figure 3.10 a b

The CF319a probe visualization results are shown in figure 3.11. The Cytophaga-flavobacterium (a and b) appeared blue when stained with DAPI stain (a).

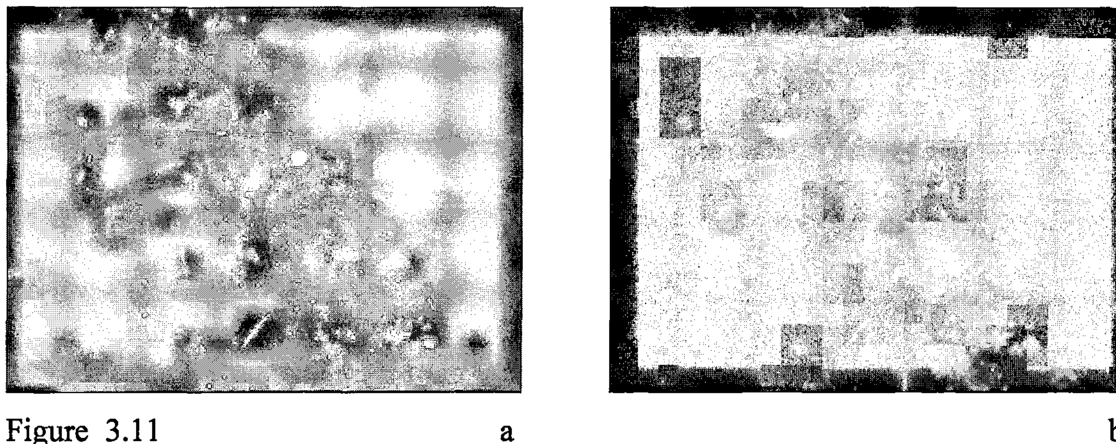


Figure 3.11 a b

The HGC69a probe visualization results are shown in figure 3.12. The high G-C gram-positive bacteria (a and b) appeared blue when stained with DAPI stain (a).

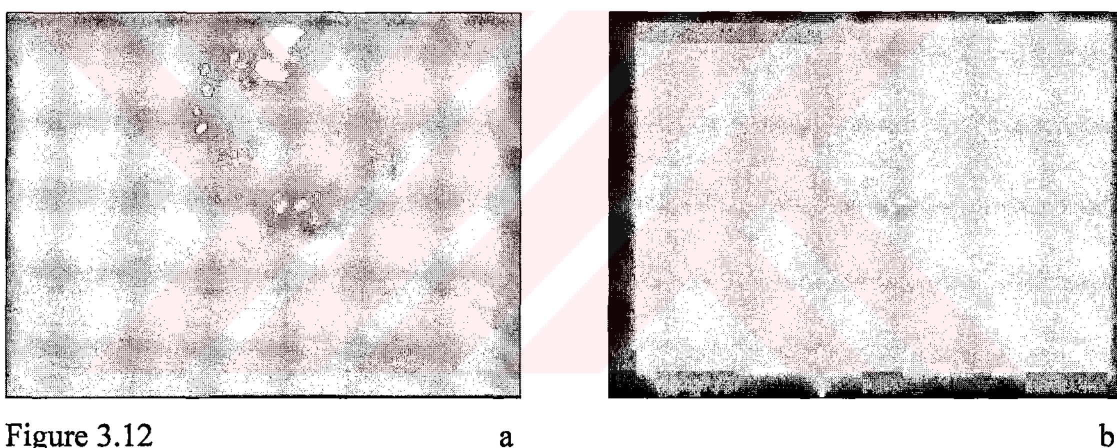


Figure 3.12 a b

The domain Archea detected by the probe (ARC 915) (a and b). a stained with DAPI, b stained with Cy3 (figure 3.13).

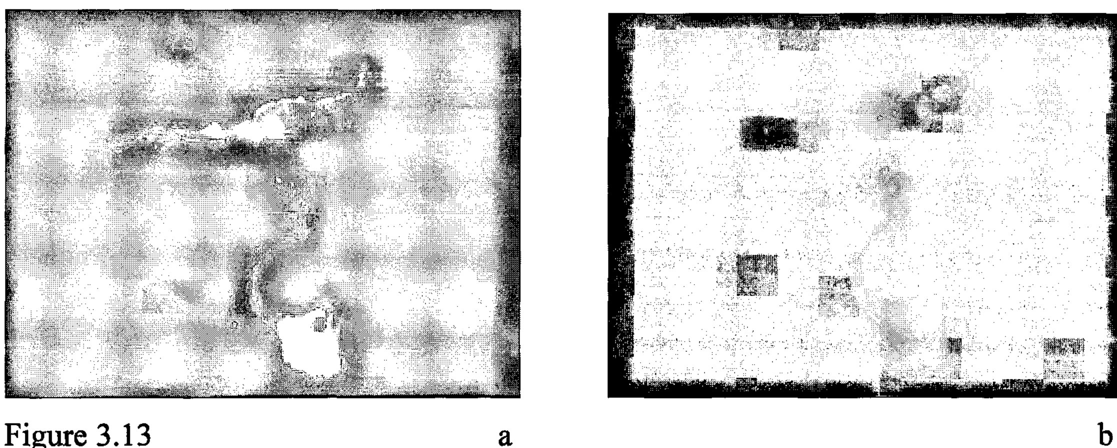


Figure 3.13 a b

3.1.3. Factory III

The ALF1b probe visualization results are shown in figure 3.14. The alpha subclass of Proteobacteria (a and b) appeared blue when stained with DAPI stain (a).

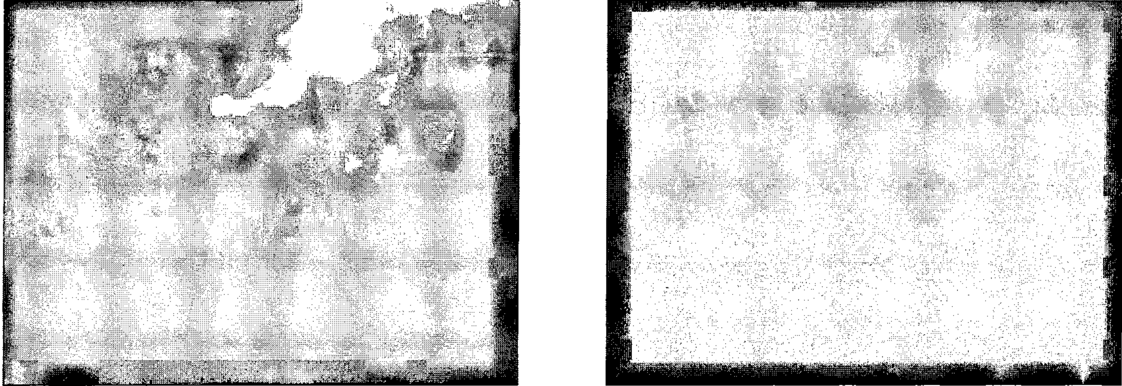


Figure 3.14 a b

The BET42a probe visualization results are shown in figure 3.15. The beta subclass of Proteobacteria (a and b) appeared blue when stained with DAPI stain (a).

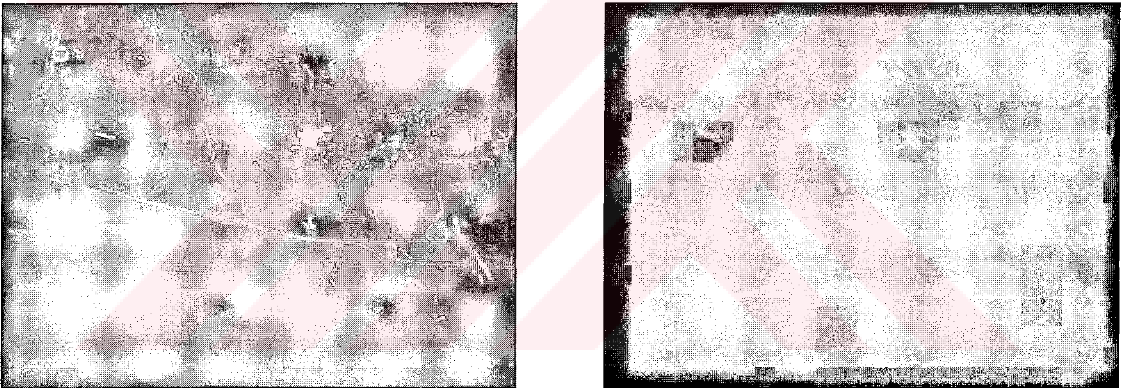


Figure 3.15 a b

The GAM42a probe visualization results are shown in figure 3.16. The gamma subclass of Proteobacteria (a and b) appeared blue when stained with DAPI stain (a).

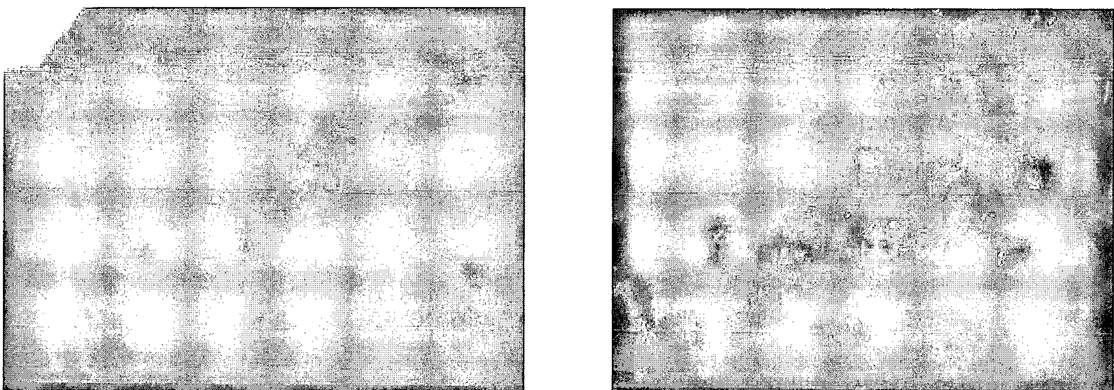


Figure 3.16 a b

The CF319a probe visualization results are shown in figure 3.17. The Cytophaga-flavobacterium (a and b) appeared blue when stained with DAPI stain (a).

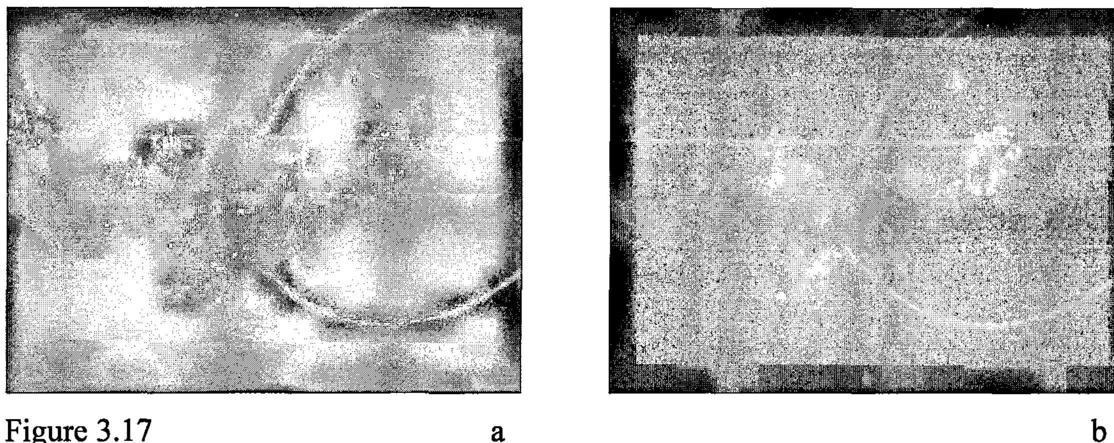


Figure 3.17 a b

The HGC69a probe visualization results are shown in figure 3.18. The high G-C gram-positive bacteria (a and b) appeared blue when stained with DAPI stain (a).

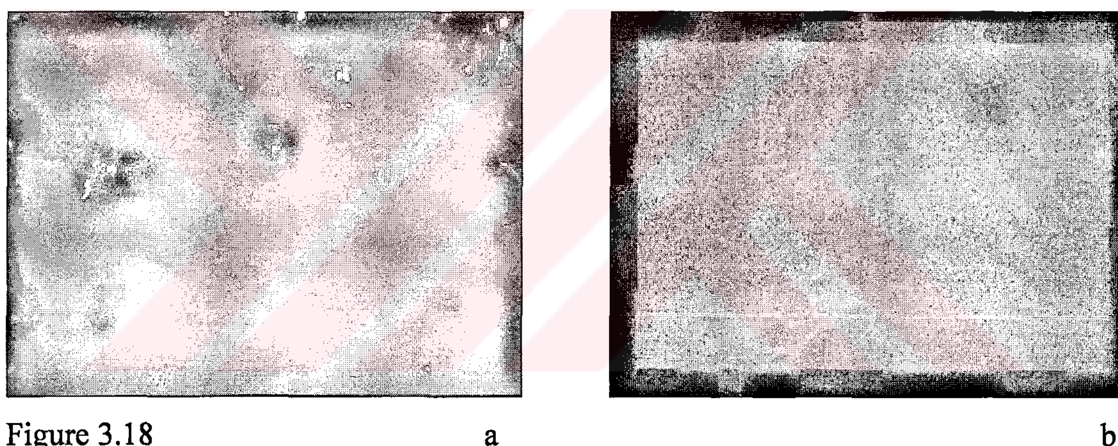


Figure 3.18 a b

The domain Archea detected by the probe (ARC 915) (a and b). a stained with DAPI, b stained with Cy3 (Figure 3.19).

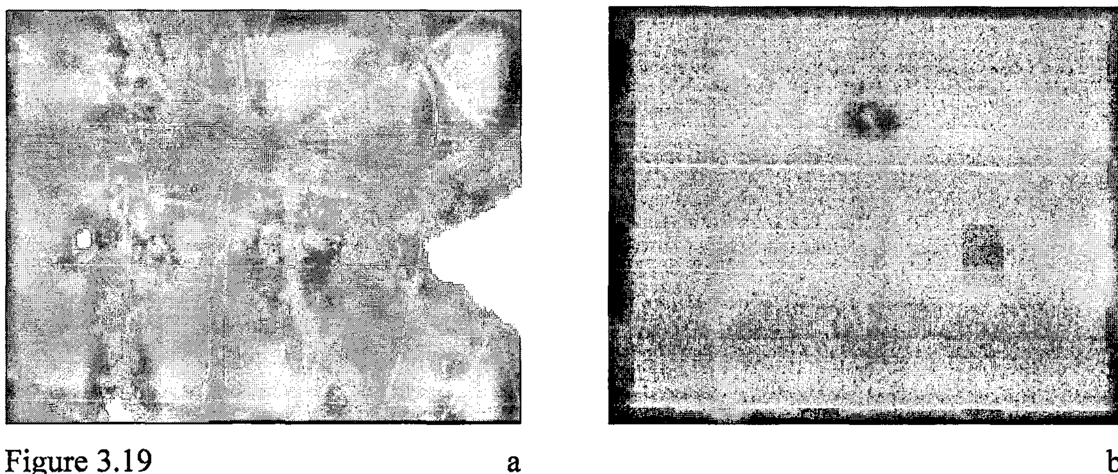


Figure 3.19 a b

3.2. BACTERIAL COUNT by the FISH TECHNIQUE

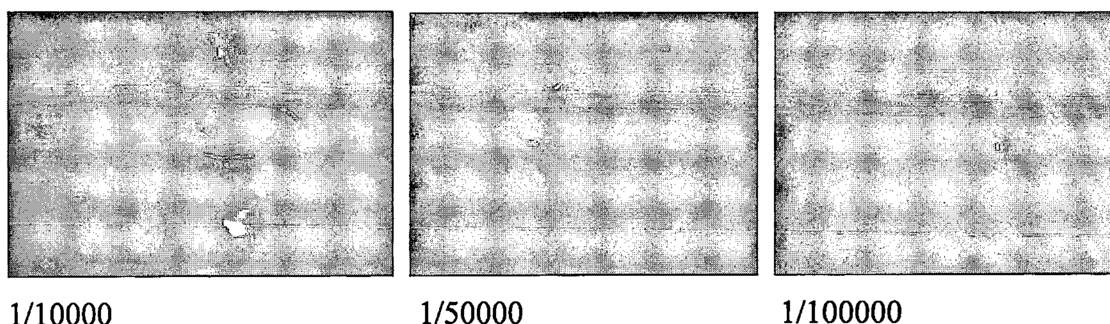
The determination of the predominant bacterial group in the activated sludge samples showed the following results (Table 3.1).

Table 3.1. Quantitative determination of the bacterial groups in the activated sludge samples from 3 factories

Factory	Alf	Bet	Gam	CF	HGC
I	$1x\ 10^{-5}$	$1x\ 10^{-6}$	$5x\ 10^{-5}$	$1x\ 10^{-6}$	$5x\ 10^{-5}$
II	$5x\ 10^{-2}$	$1x\ 10^{-4}$	$5x\ 10^{-3}$	$1x\ 10^{-4}$	$5x\ 10^{-3}$
III	$1x\ 10^{-3}$	$1x\ 10^{-3}$	$5x\ 10^{-2}$	$1x\ 10^{-3}$	$5x\ 10^{-2}$

Alf: Alpha subclass of Proteobacteria, Bet: Beta subclass of Proteobacteria, Gam: Gamma subclass of Proteobacteria, CF: Cytophaga-Flavobacterium cluster, HGC: High G-C gram-positive bacteria.

Table 3.1 shows that the beta subclass and CF cluster detected at a dilution of $1x10^{-6}$ were the predominant group in the activated sludge sample of factory I followed by the gamma subclass and the HGC group ($5x10^{-5}$) and finally the alpha subclass group ($1x10^{-5}$). Similarly the beta subclass and CF cluster detected at a dilution of $1x10^{-4}$ were the predominant group in the activated sludge sample of factory II followed by the gamma subclass and the HGC group ($5x10^{-3}$) and finally the alpha subclass group ($5x10^{-2}$). However the alpha and beta subclasses and the CF cluster ($1x10^{-3}$) were the predominant groups in the activated sludge sample of factory III followed by the gamma subclass and the HGC group ($5x10^{-2}$).



1/10000 1/50000 1/100000

Figure 3.20. The ALF1b probe dilutions

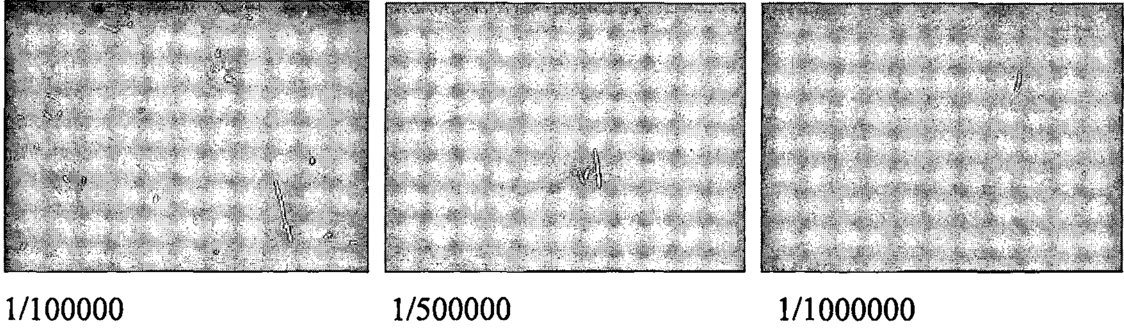


Figure 3.21. The BET42a probe dilutions

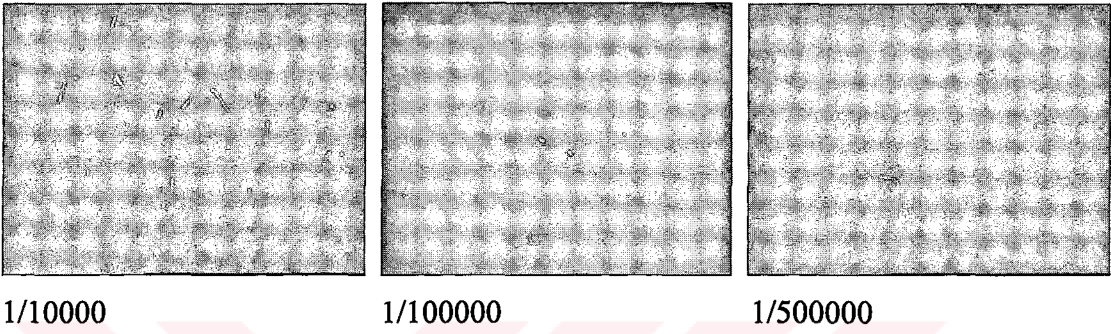


Figure 3.22. The GAM42a probe dilutions

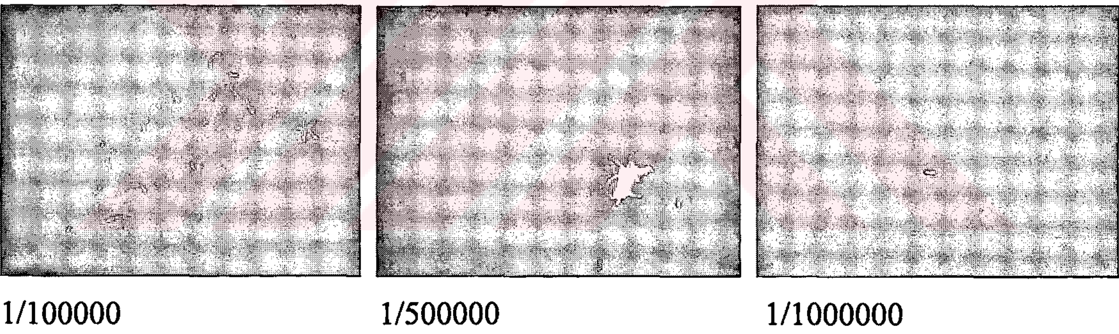


Figure 3.23. The CF319a probe dilutions

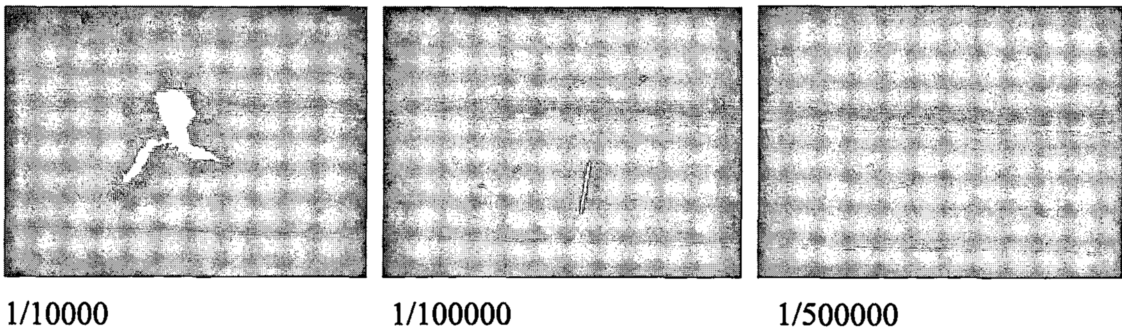


Figure 3.24. The HGC69a probe dilutions

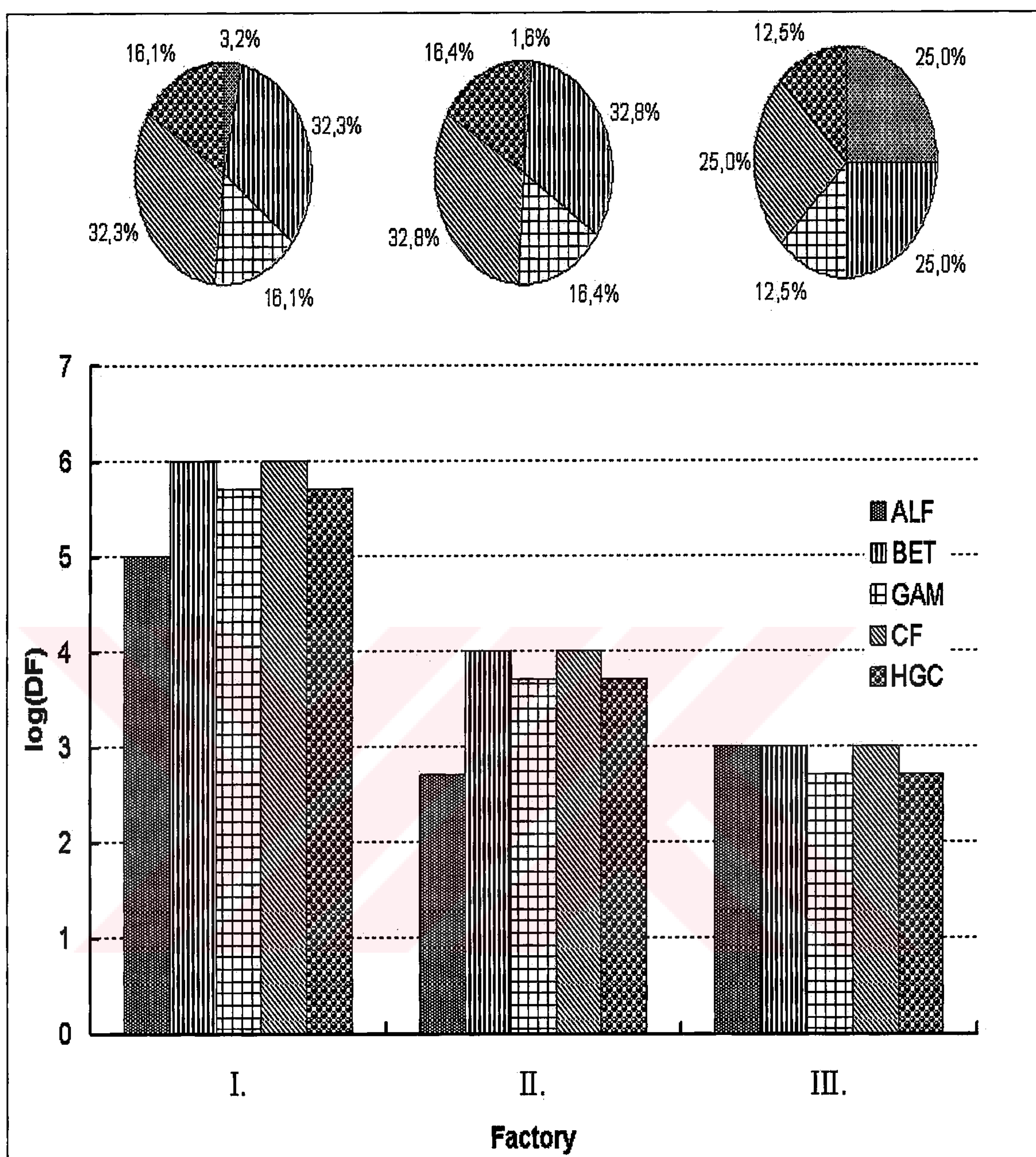


Figure 3.25. Comparison of the distribution percentages (pie) and the log (bar) of the bacterial groups in the activated sludge samples of the 3 factories.

3.3. IN SITU ANALYSIS of the CULTURED ACTIVATED SLUDGE SAMPLES

The activated sludge samples were cultured using the pour plate method. Single colonies were taken from the petri plate and prepared for FISH. The following pictures show the cultural uniformity of the bacterial groups detected by the specified probes.

3.3.1. Factory I

The EUB338 probe visualization results are shown in figure 3.26. The domain Bacteria (a and b) appeared blue when stained with DAPI stain (a).

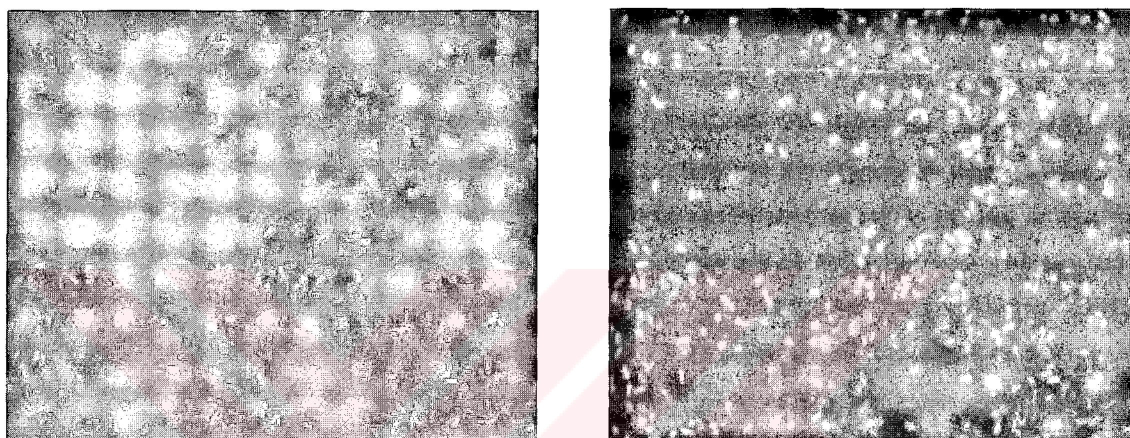


Figure 3.26 a b

The ALF1b probe visualization results are shown in figure 3.27. The alpha subclass of Proteobacteria (a and b) appeared blue when stained with DAPI stain (a)

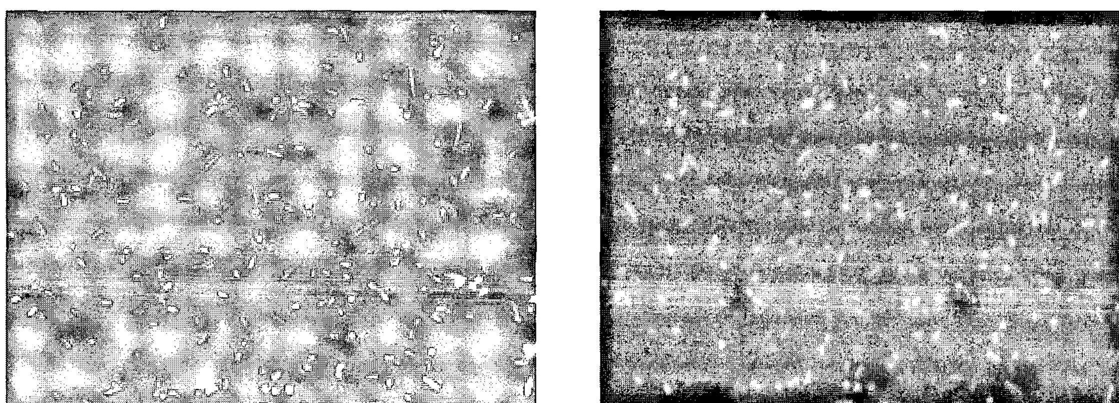


Figure 3.27 a b

The BET42a probe visualization results are shown in figure 3.28. The beta subclass of Proteobacteria (a and b) appeared blue when stained with DAPI stain (a).

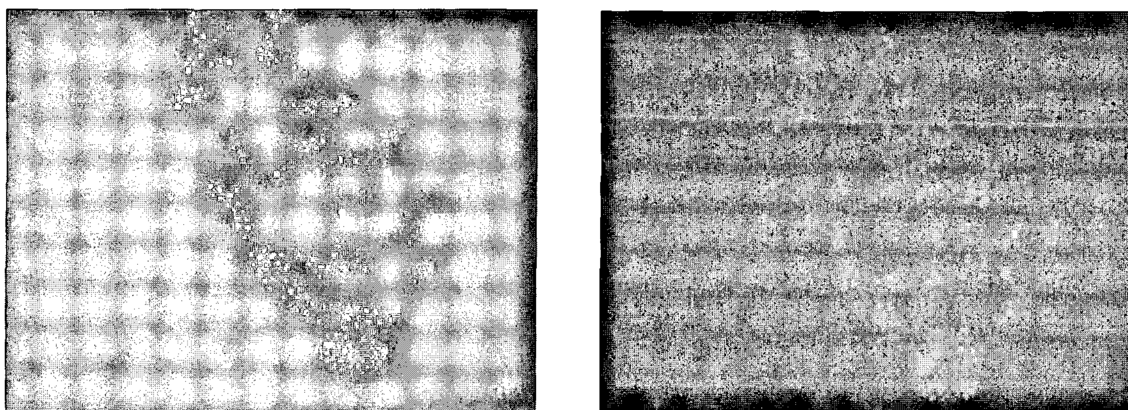


Figure 3.28 a b

3.3.2. Factory II

The EUB338 probe visualization results are shown in figure 3.29. The domain Bacteria (a and b) appeared blue when stained with DAPI stain (a).

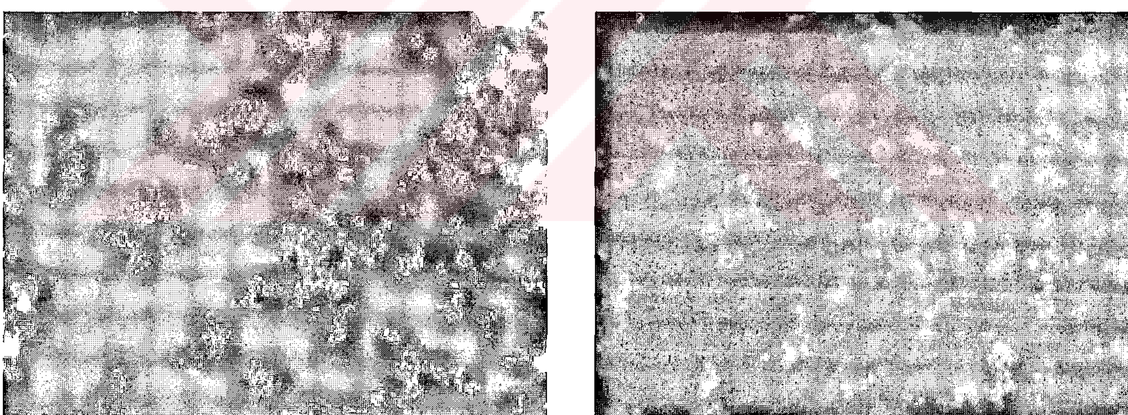
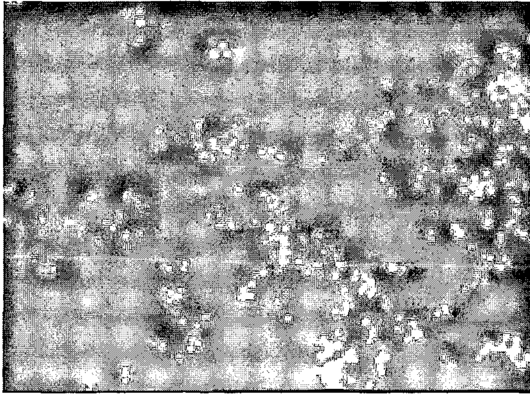


Figure 3.29 a b

3.3.3. Factory III

The EUB338 probe visualization results are shown in figure 3.30. The domain Bacteria (a and b) appeared blue when stained with DAPI stain (a).



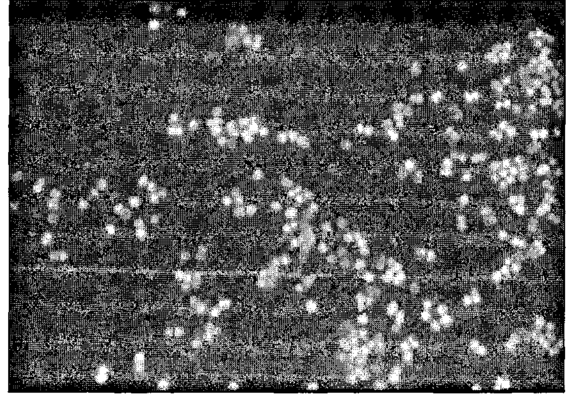


Figure 3.30 a b

3.4. PHASE-CONTRAST MICROSCOPY

The activated sludge samples were examined directly by phase-contrast microscopy. The floc shape characteristics and the abundance of filamentous bacteria in each sample from the 3 factories were determined.

3.4.1. Factory I

The floc shape in the activated sludge sample of factory I was characterized as irregular and diffuse with substancial free cells. The score for the abundance of filamentous bacteria was 2 (some) indicating that the filaments commonly observed but not present in all flocs (figure 3.31).

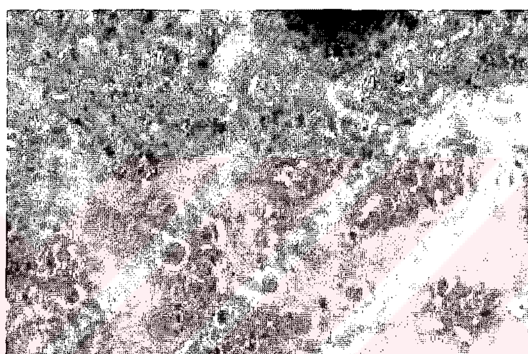


Figure 3.31. Phase-contrast micrograph (20X) of floc characteristics and the abundance of filamentous bacteria in the activated sludge sample from factory I.

3.4.2.Factory II

The floc shape in the activated sludge sample of factory II was characterized as irregular, compact and less in abundance. The score for the abundance of filamentous bacteria was 1 (few) indicating that the filaments present but only observed in an occasional floc (figure 3.32).

Figure 3.32. Phase-contrast micrograph (20X) of floc characteristics and the abundance of filamentous bacteria in the activated sludge sample from factory II.

3.4.3. Factory III

The floc shape in the activated sludge sample of factory III was characterized as diffuse and atypical. The score for the abundance of filamentous bacteria was 5 (abundant) indicating that the filaments observed in all flocs at high density (figure 3.33).

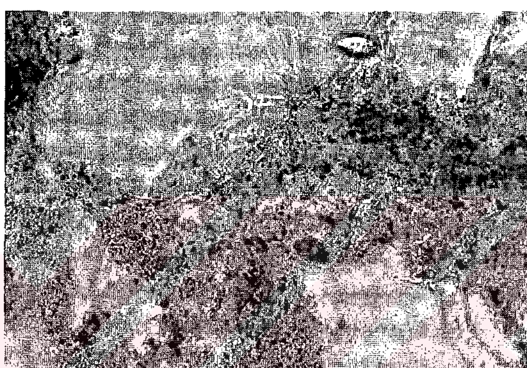


Figure 3.33. Phase-contrast micrograph (20X) of floc characteristics and the abundance of filamentous bacteria in the activated sludge sample from factory III.

3.4.4. Phase-contrast micrographs of higher organisms

The following higher organisms were observed in the activated sludge samples from factories I and II. No such organisms were observed in samples from factory III.

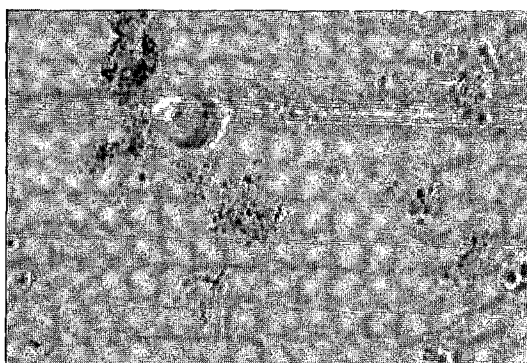


Figure 3.34. Phase-contrast micrograph (20X) of Vorticella sp.

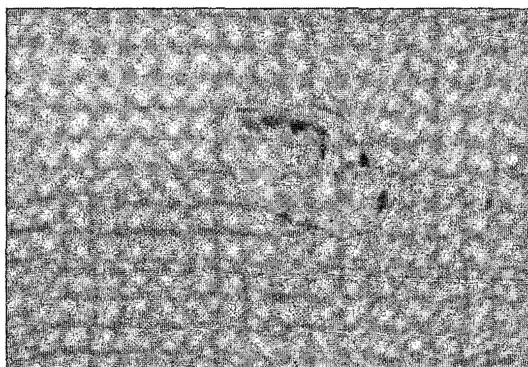
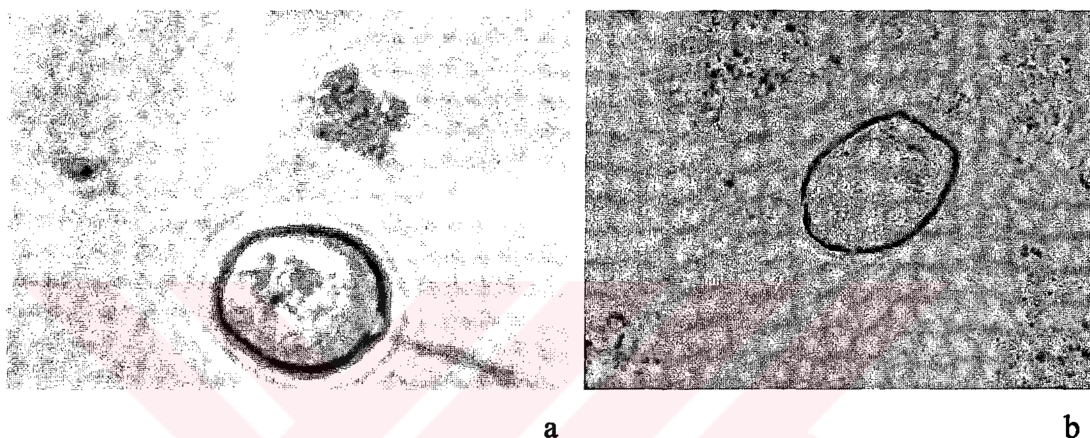


Figure 3.35. Phase-contrast micrograph (100X) of ciliate (Paramecium)



a b

Figure 3.36. Phase-contrast micrographs (40X) of rotifers (a and b)

3.5. COMPARISON of FLOC and FILAMENT CHARACTERISTICS using FISH and PHASE-CONTRAST MICROSCOPY

3.5.1. Factory-I

The floc shape was characterized as irregular and diffuse with some filaments. It is illustrared figure 3.37 with FISH method (a) and Phase-Contrast (b).

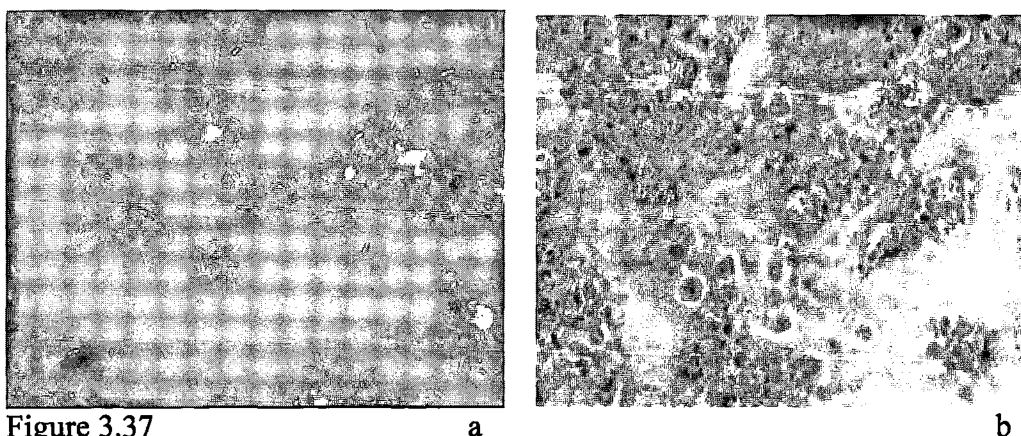


Figure 3.37 a b

3.5.2. Factory-II

The floc shape was characterized as irregular, compact and less in abundance with few filaments. It is illustrared figure 3.38 with FISH method (a) and Phase-Contrast (b).

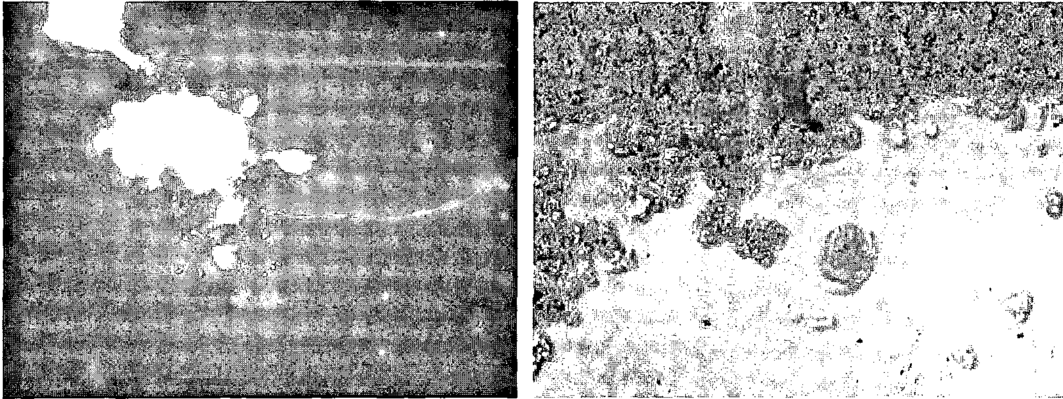


Figure 3.38 a b

3.5.3. Factory-III

The floc shape was characterized as diffuse and atypical abundance with abundant filaments. It is illustrared figure 3.39 with FISH method (a) and Phase-Contrast (b).

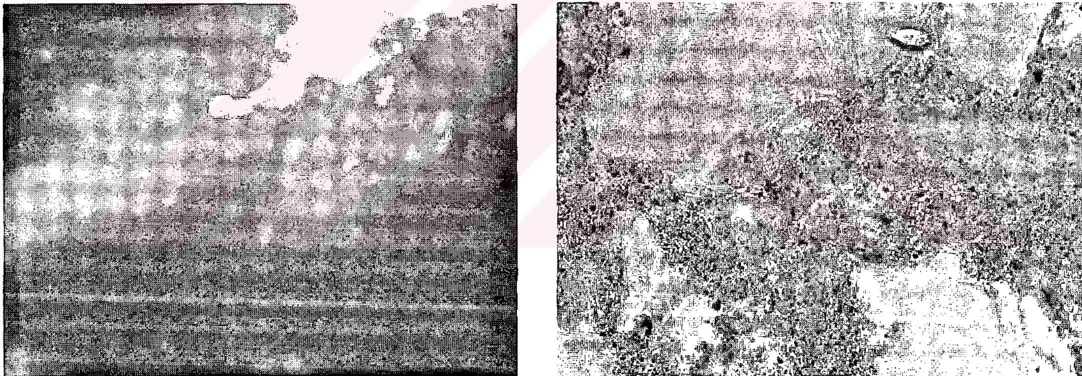


Figure 3.39 a b

3.6 CULTURE-DEPENDENT METHODS

3.6.1. Culture

The bacterial groups in the activated sludge samples from the 3 factories were also examined by the conventional methods using culture media. Samples were inoculated onto nutrient agar, blood agar and McConkey agar media and incubated at 37°C for 24 h. All these cultures supported the growth of different bacterial groups. Based on morphological differences, single colonies from each culture were selected and subcultured on fresh nutrient agar media and incubated at 37°C for 24 h. Pure cultures of single colonies were then examined by several biochemical tests. The figures below shows the bacterial colonies observed on different culture media from the 3 factories.

3.6.1.1. Factory I

Figure 3.40. Nutrient agar culture

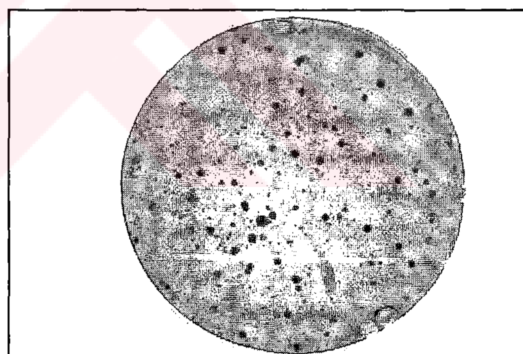


Figure 3.41. Blood agar culture

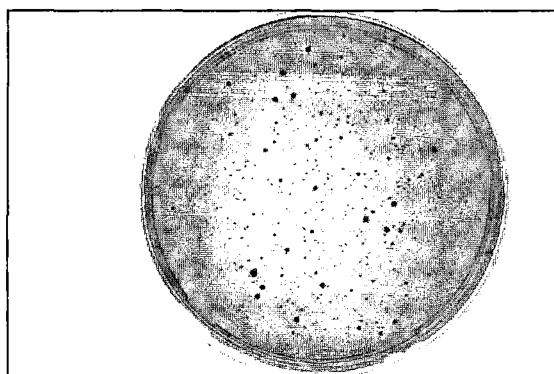


Figure 3.42. McConkey agar culture

3.6.1.2. Factory II

Figure 3.43. Nutrient agar culture

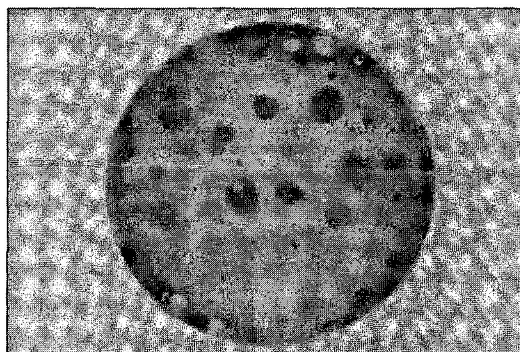


Figure 3.44. Blood agar culture

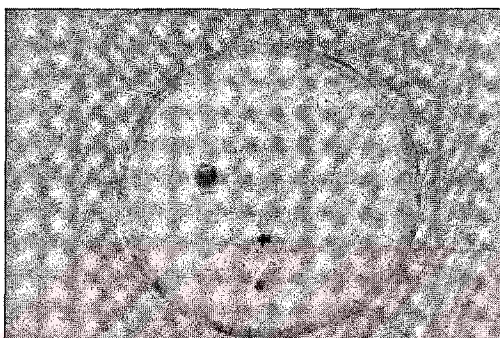


Figure 3.45. McConkey agar culture

3.6.1.3. Factory III

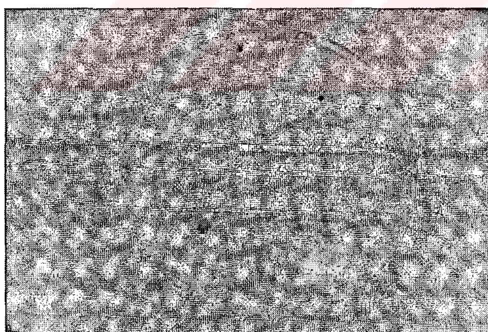


Figure 3.46. Nutrient agar culture

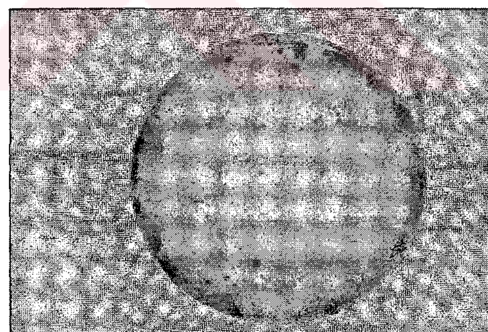


Figure 3.47. Blood agar culture

3.6.2. Bacterial Count Using Culture Methods

The pour plate technique was used to count the bacteria in the activated sludge samples. To count the colonies of bacteria, activated sludge samples were diluted in a 10-fold serial dilution in PBS then mixed with nutrient agar poured on plates. The figures obtained using Gel Doc 2000 (BIO RAD, Italy).

3.6.2.1. Factory I

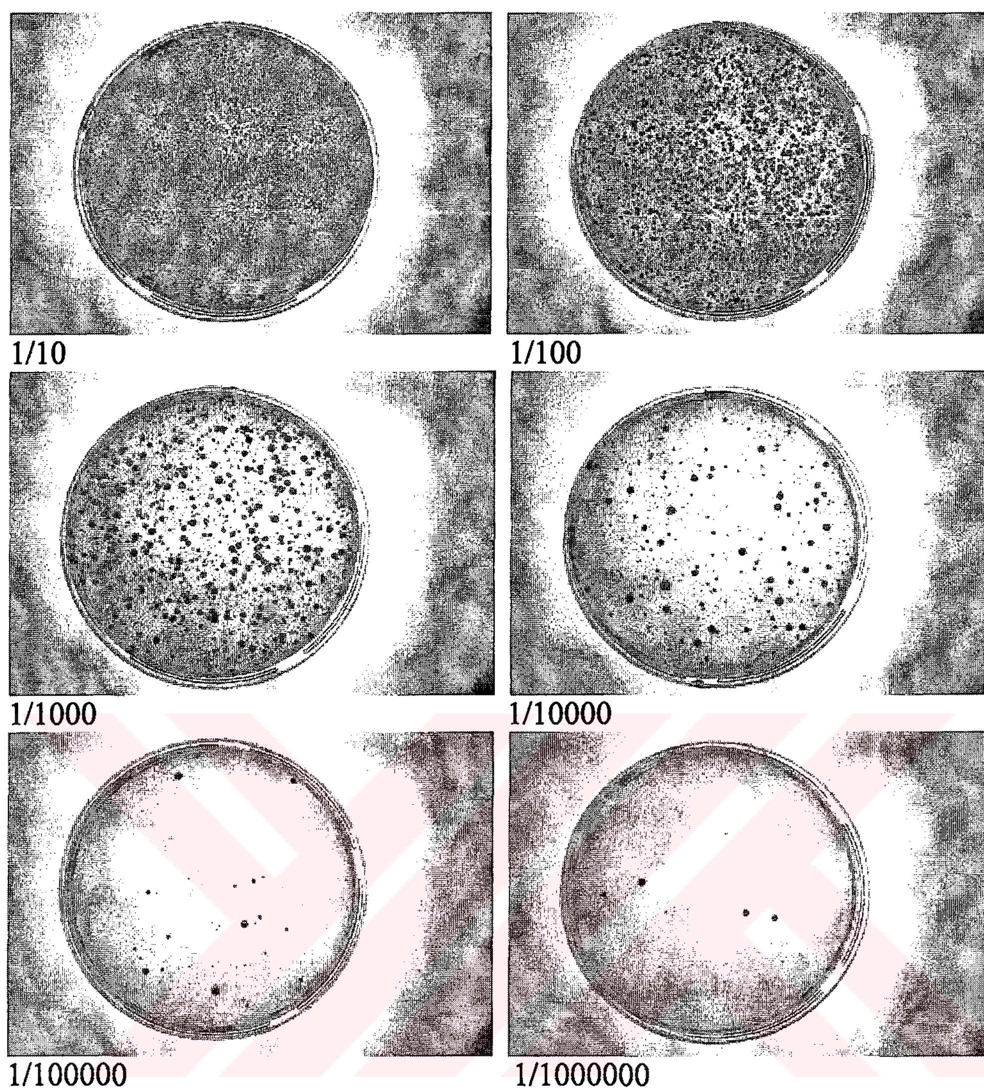


Figure 3.48 shows the 10-fold serial dilutions of bacterial colonies isolated from factory I.

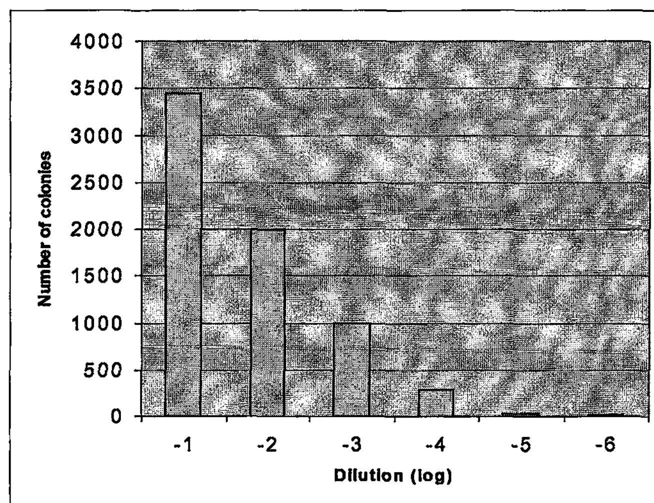


Figure 3.49.Bacterial count in each dilution of the activated sludge sample from factory I.

Gel Doc illustration files used for counting the bacterial colonies (factory I).

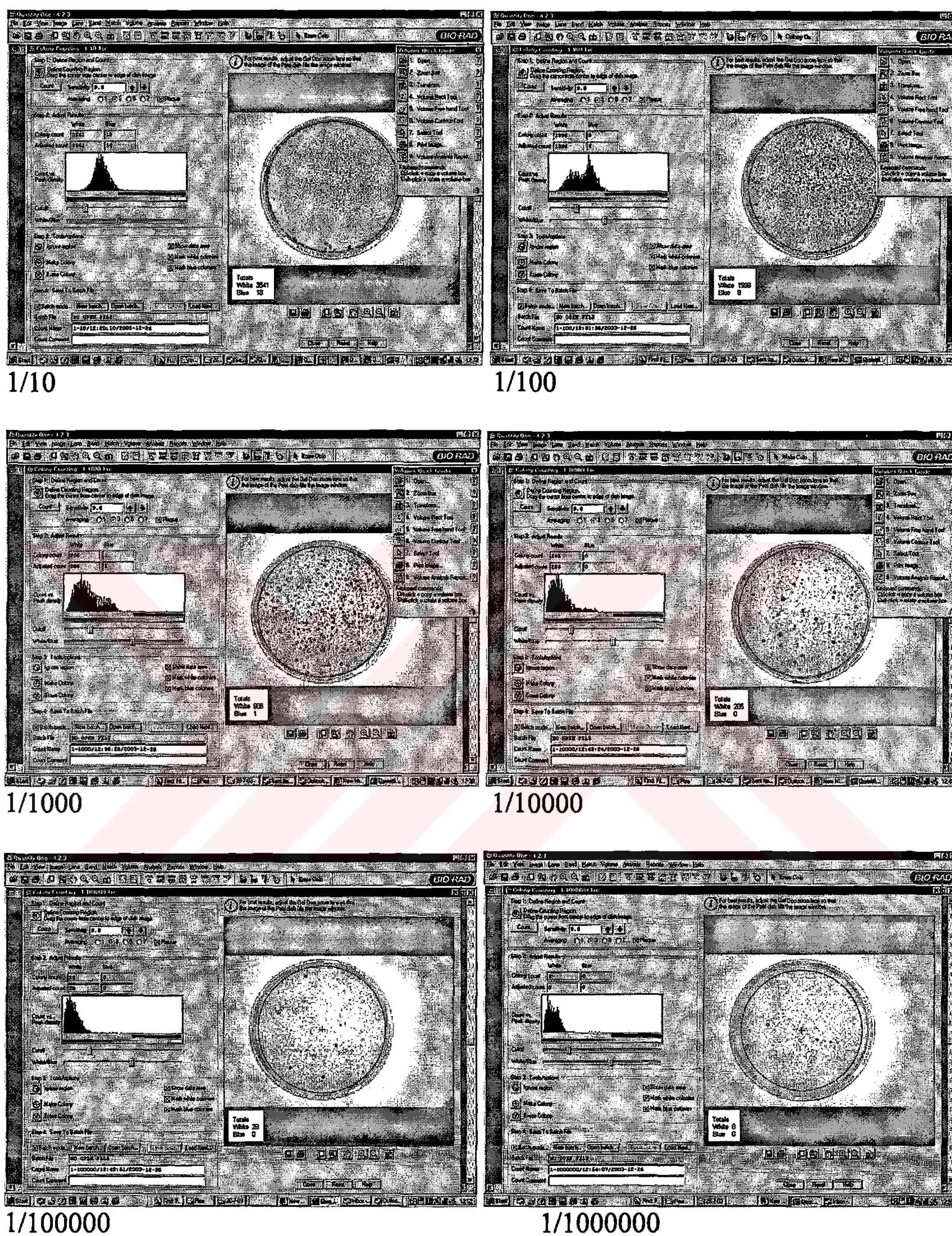


Figure 3.50. Gel Doc illustration for factory I.

3.6.2.2. Factory II

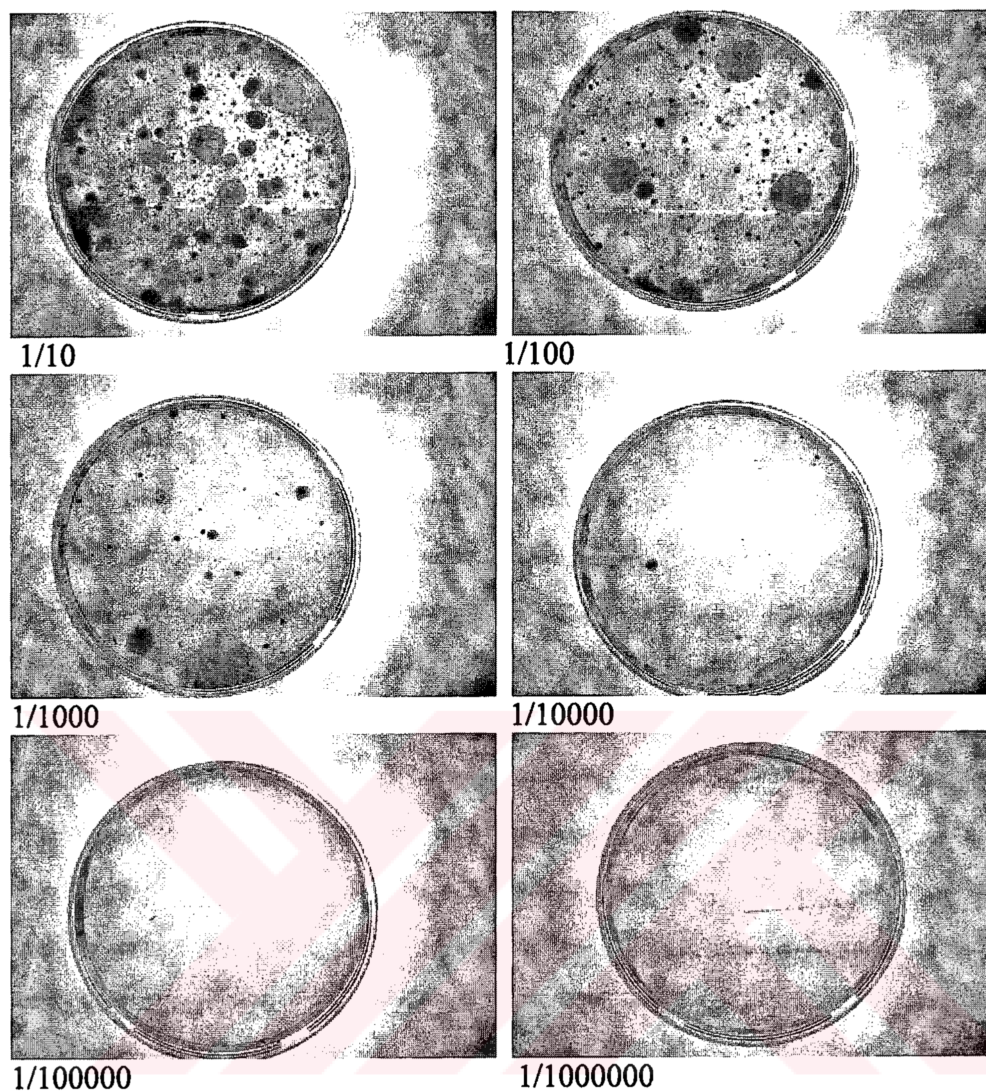


Figure 3.51 shows the 10-fold serial dilutions of bacterial colonies isolated from factory II.

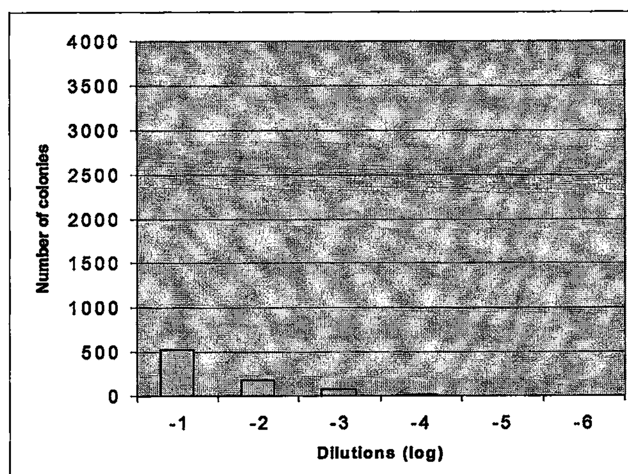
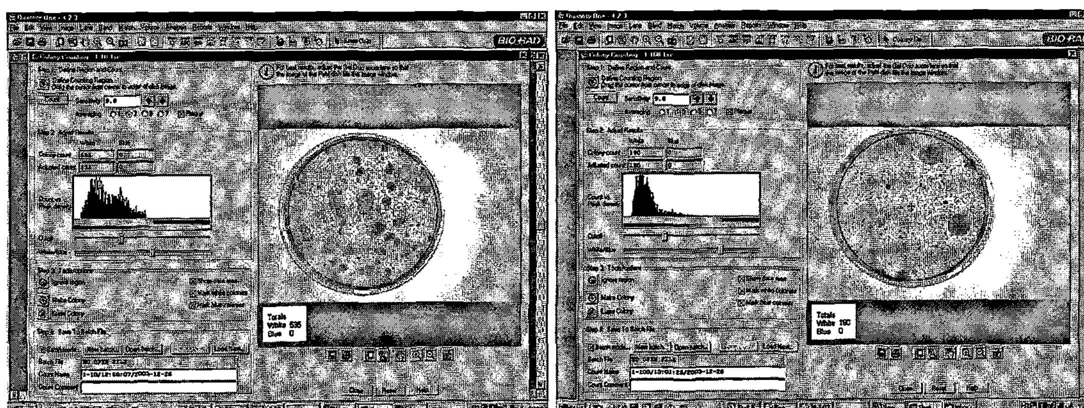
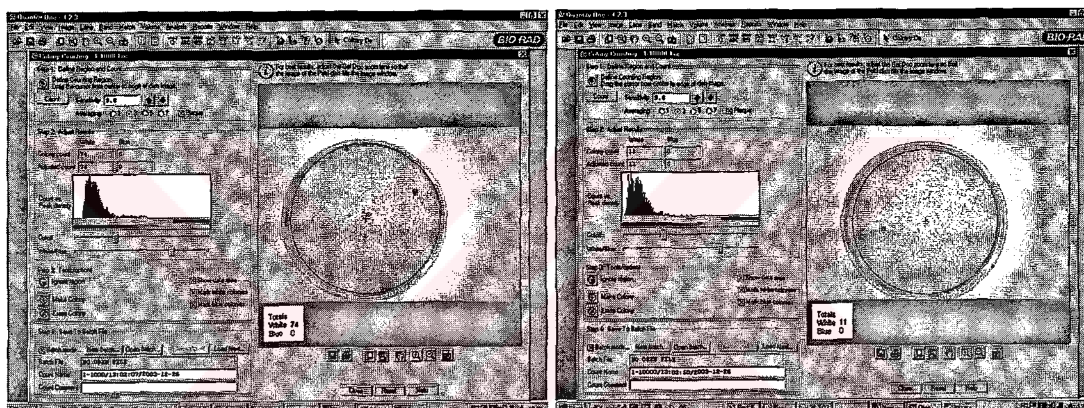


Figure 3.52. Bacterial count in each dilution of the activated sludge sample from factory II.

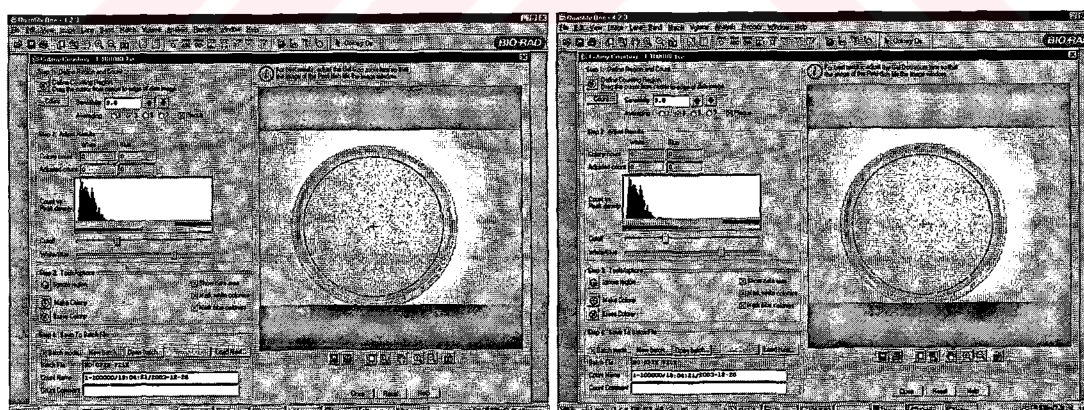
Gel Doc illustration files used for counting the bacterial colonies (factory II).



1/10 1/100



1/1000 1/10000



1/100000 1/1000000

Figure 3.53. Gel Doc illustration for factory II

3.6.2.3. Factory III

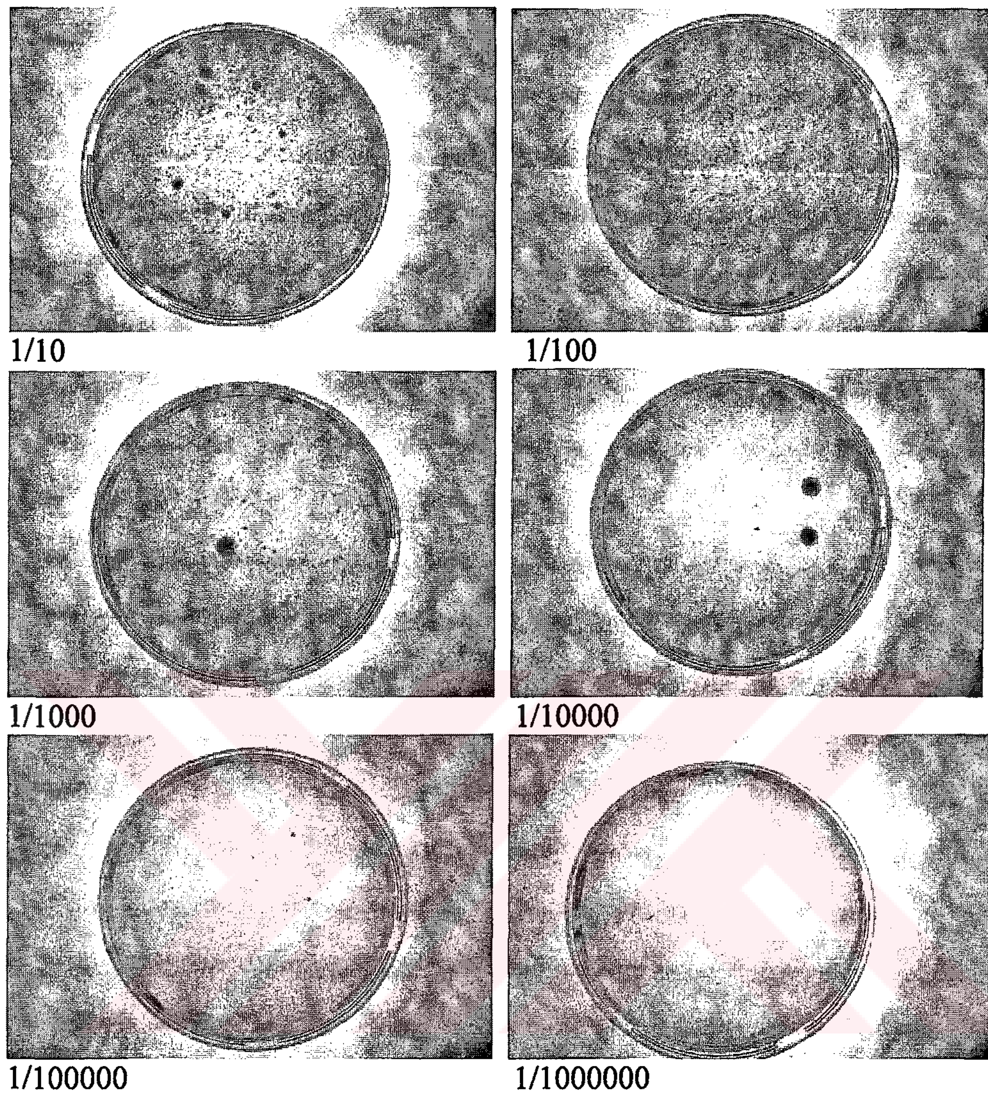


Figure 3.54 shows the 10-fold serial dilutions of bacterial colonies isolated from factory III.

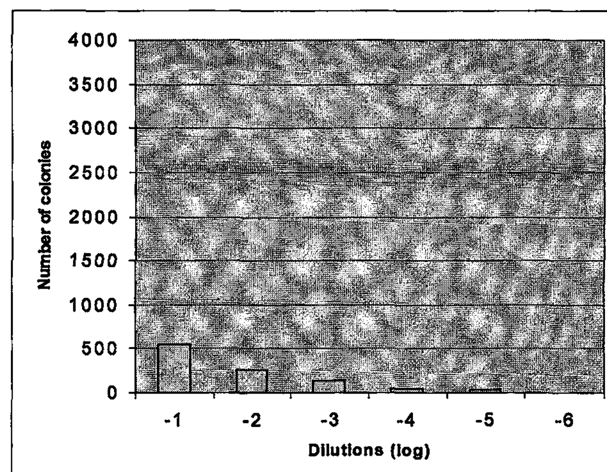


Figure 3.55. Bacterial count in each dilution of the activated sludge sample from factory III.

Gel Doc illustration files used for counting the bacterial colonies (factory III).

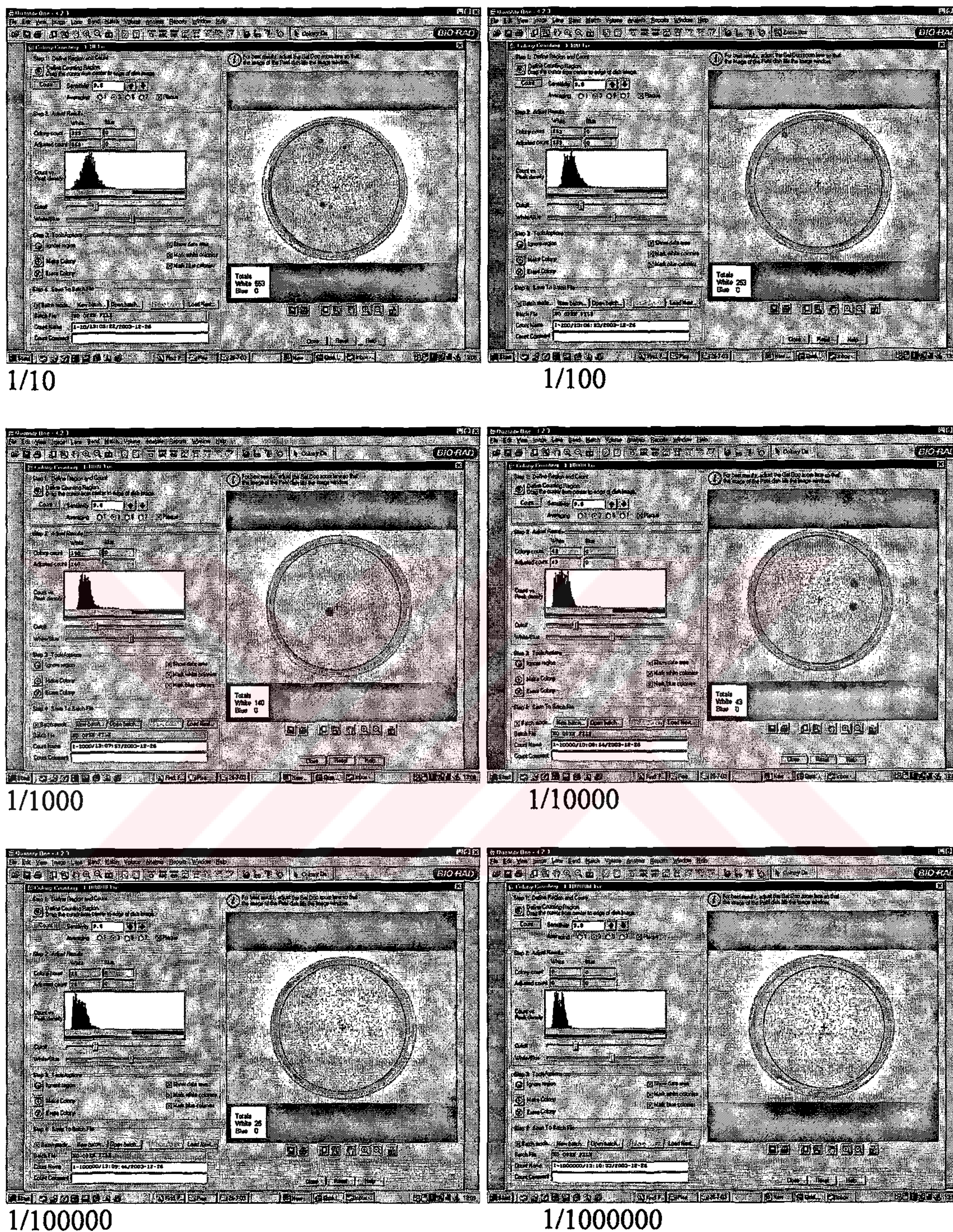


Figure 3.56. Gel Doc illustration for factory III.

3.6.3. Biochemical Tests

The results of the biochemical tests were shown in figures 3.57, 3.58, 3.59, 3.60, 3.61, 3.62, 3.63, 3.64, 3.65, 3.66 and table 3.2, 3.3 shows the results of the different biochemical tests used in this study.

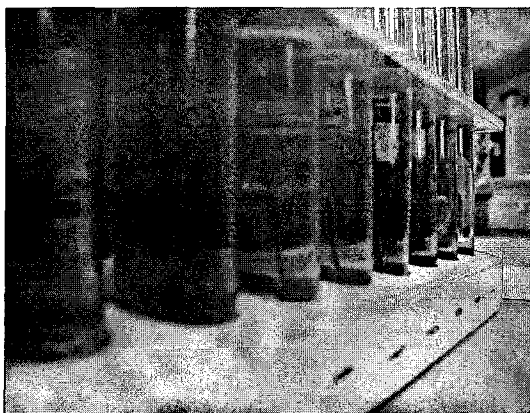


Figure 3.57 Ferrmentation Test

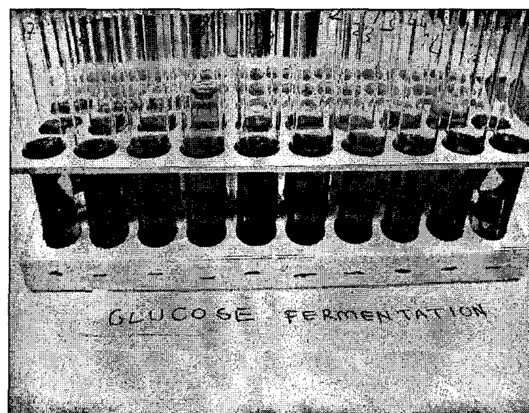


Figure 3.58. Glucose

Figure 3.59 Sucrose

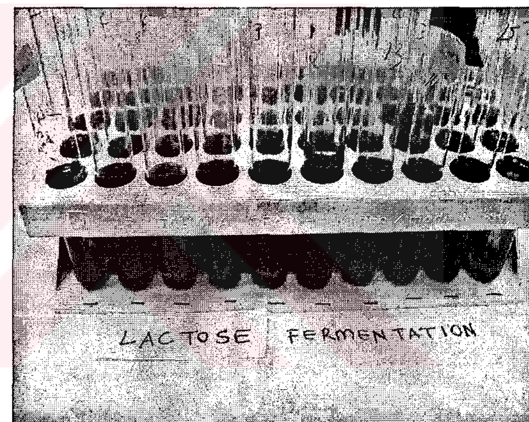


Figure 3.60. Lactose

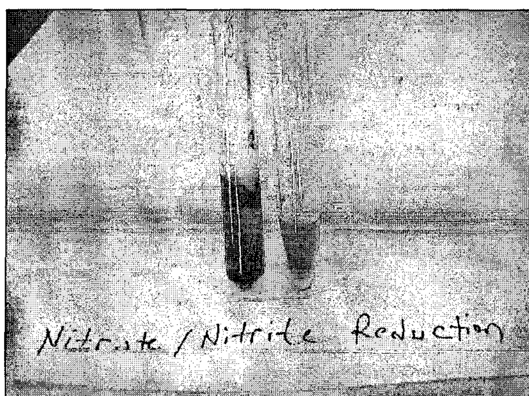


Figure 3.61. Nitrate-Nitrite Reduction

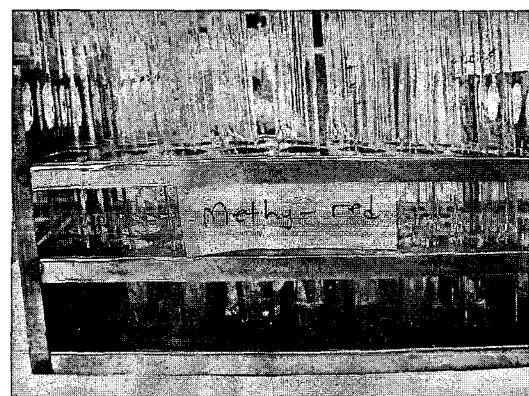


Figure 3.62. Methyl-Red

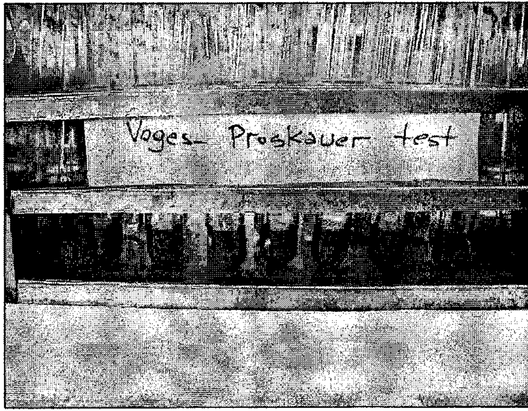


Figure 3.63. Voges-Proskauer test

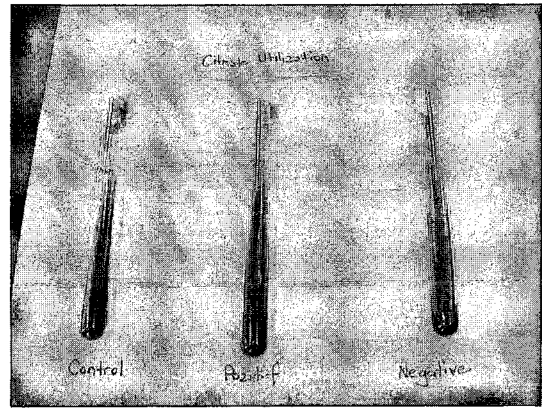


Figure 3.64. Citrate Utilization

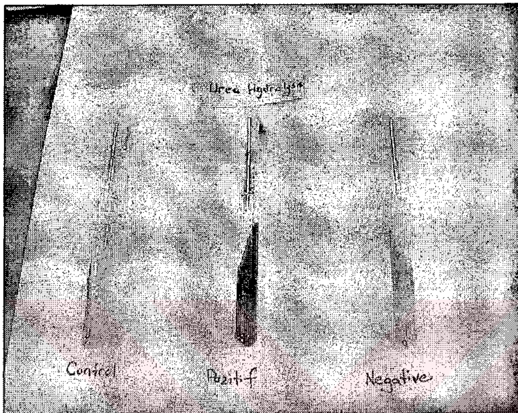


Figure 3.65. Urea Hydrolysis

Figure 3.66. Casein Hydrolysis

	IDENTIFICATION of BACTERIA																			
					Morphology															
		Types of agar			Gram staining		Fermentation							IMVIC TESTS				Hydrolysis		
no	Factory	BA	NA	McCA	G +	G -	Shape	Oxidase	Catalase	Glucose	Sucrose	Lactose	Nitrate/ nitrite reduction	Indole production test	Methyl-red	Voges-proskauer test	Citrate utilization	Casein	Gelatin	Urea
1	Factory I		x		x		Rod	(+)	(+)	(-)	(-)	(-)	(+)	(-)	(-)	(-)	(+)	(-)	(-)	(+)
2	Factory I		x		x		Cocci&rod	(+)	(+)	(-)	(-)	(-)	(+)	(-)	(-)	(-)	(+)	(-)	(-)	(+)
3	Factory I		x		x		Rod	(+)	(+)	(-)	(-)	(-)	(+)	(-)	(-)	(-)	(+)	(-)	(-)	(+)
4	Factory I	x			x		Rod	(+)	(+)	(-)	(-)	(-)	(+)	(-)	(-)	(-)	(+)	(-)	(-)	(+)
5	Factory I	x			x		Rod	(+)	(+)	(-)	(-)	(-)	(+)	(-)	(-)	(-)	(+)	(-)	(-)	(+)
6	Factory I	x			x		Cocci	(+)	(+)	(-)	(-)	(-)	(+)	(-)	(-)	(-)	(+)	(-)	(-)	(-)
7	Factory I	x			x		Rod	(+)	(+)	(-)	A	(-)	(+)	(-)	(-)	(-)	(+)	(-)	(-)	(+)
8	Factory I			x	x		Rod	(+)	(+)	(-)	(-)	(-)	(+)	(-)	(-)	(-)	(+)	(-)	(-)	(-)
9	Factory I			x	x		Rod	(+)	(+)	(-)	(-)	(-)	(+)	(-)	(-)	(-)	(+)	(-)	(-)	(+)
10	Factory I			x	x		Rod	(+)	(+)	(-)	A	(-)	(+)	(-)	(-)	(-)	(+)	(-)	(-)	(+)
11	Factory I			x	x		Rod	(-)	(+)	(-)	A	(-)	(+)	(-)	(-)	(-)	(+)	(-)	(-)	(+)
12	Factory II		x		x		Rod	(+)	(+)	(-)	A	(-)	(-)	(-)	(+)	(-)	(-)	(+)	(-)	(-)
13	Factory II		x		x		Rod-chain	(+)	(+)	(-)	A	(-)	(-)	(-)	(+)	(-)	(-)	(+)	(-)	(-)
14	Factory II		x		x		Rod-chain	(+)	(+)	(-)	A	(-)	(-)	(-)	(+)	(-)	(-)	(+)	(-)	(-)
15	Factory II		x		x		Rod	(+)	(+)	(-)	(-)	(-)	(+)	(-)	(-)	(-)	(-)	(+)	(-)	(-)
16	Factory II		x			x	Coccobacilli	(+)	(+)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)
17	Factory II	x			x		Rod-chain	(+)	(+)	(-)	A	(-)	(-)	(-)	(+)	(-)	(-)	(+)	(-)	(-)
18	Factory II	x			x		Rod-chain	(+)	(+)	(-)	(-)	(-)	(+)	(-)	(+)	(-)	(-)	(+)	(-)	(-)
19	Factory II	x				x	Coccobacilli	(+)	(+)	(-)	(-)	(-)	(+)	(-)	(-)	(-)	(-)	(-)	(-)	(-)
20	Factory II			x	x		Cocci	(+)	(+)	(-)	(-)	(-)	(+)	(-)	(-)	(-)	(-)	(-)	(-)	(-)
21	Factory II			x	x		Rod	(+)	(+)	(-)	(-)	(-)	(+)	(-)	(-)	(-)	(+)	(-)	(-)	(+)
22	Factory III		x		x		Rod	(+)	(+)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(+)	(+)	(-)	(-)
23	Factory III		x		x		Coccobacilli	(+)	(+)	(+)	A	(-)	(+)	(-)	(-)	(-)	(-)	(+)	(-)	(-)
24	Factory III		x		x		Coccobacilli	(+)	(+)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)
25	Factory III		x		x		Coccobacilli	(+)	(+)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(+)
26	Factory III	x			x		Coccobacilli	(+)	(+)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(+)
27	Factory III	x			x		Coccobacilli-thin	(+)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)
28	Factory III	x			x		Coccobacilli	(+)	(+)	(-)	(-)	(-)	(+)	(-)	(-)	(-)	(+)	(-)	(-)	(+)

Table 3.2. The results of the biochemical tests

no	Factory	G st.	Shape	Genera (presumed)
1	Factory I	G+	Rod	Arthrobacter, Aureobacterium, Curtobacterium, C.streum or C.plantarum, Microbacterium, M.lactium, Rubrobacter, Sphaerobacter
2	Factory I	G+	Cocci&rod	Deinococcus, D.radiopugnans, Marinococcus, M.albus, Salinicoccus, Arthrobacter, Curtobacterium, C.streum, Microbacterium, Rubrobacter, Sphaerobacter
3	Factory I	G+	Rod	Arthrobacter, Aureobacterium, Curtobacterium, C.streum or C.plantarum, Microbacterium, M.lactium, Rubrobacter, Sphaerobacter
4	Factory I	G+	Rod	Arthrobacter, Aureobacterium, Curtobacterium, C.streum or C.plantarum, Microbacterium, M.lactium, Rubrobacter, Sphaerobacter
5	Factory I	G+	Rod	Arthrobacter, Aureobacterium, Curtobacterium, C.streum or C.plantarum, Microbacterium, M.lactium, Rubrobacter, Sphaerobacter
6	Factory I	G+	Cocci	Deinococcus, D.radiopugnans, Marinococcus, M.albus, Salinicoccus
7	Factory I	G+	Rod	Aureobacterium, Cellulomonas, Rubrobacter, Sphaerobacter,
8	Factory I	G+	Rod	Arthrobacter, Aureobacterium, Curtobacterium, C.streum or C.plantarum, Microbacterium, M.lactium, Rubrobacter, Sphaerobacter
9	Factory I	G+	Rod	Arthrobacter, Aureobacterium, Curtobacterium, C.streum or C.plantarum, Microbacterium, M.lactium, Rubrobacter, Sphaerobacter
10	Factory I	G+	Rod	Aureobacterium, Cellulomonas, Rubrobacter, Sphaerobacter
11	Factory I	G+	Rod	Listeria, L.murrayi, Aureobacterium, Brachybacterium, Cellulomonas, Jonesia, Rubrobacter
12	Factory II	G+	Rod	Lactobacillus, Aureobacterium, Brevibacterium, Microbacterium, M.imperiale, M.laevaniformans
13	Factory II	G+	Rod-chain	Lactobacillus, Brevibacterium, Microbacterium, M.imperiale, M.laevaniformans
14	Factory II	G+	Rod-chain	Lactobacillus, Brevibacterium, Microbacterium, M.imperiale, M.laevaniformans
15	Factory II	G+	Rod	Arthrobacter, Aureobacterium, Brevibacterium, Curtobacterium, C.albidum, C.flaccumfaciens,C.luteum, Microbacterium, M.lactium,
16	Factory II	G-	Coccobacilli	Alcaligenes, Brucella, Deleya, D.pacifica,Comanonas, Hydrogenophaga, Moraxella cocci=M.(B)cuniculi, Oligella, Pseudomonas, Sinorhizobium, Taylorella
17	Factory II	G+	Rod-chain	Lactobacillus, Brevibacterium, Microbacterium, M.imperiale, M.laevaniformans,
18	Factory II	G+	Rod-chain	Arthrobacter, Brevibacterium, Curtobacterium, C.albidum, C.flaccumfaciens,C.luteum, Microbacterium, M.lactium, Rubrobacter
19	Factory II	G-	Coccobacilli	Alcaligenes, Alteromonas, Aminobacter, Brucella, Cupriavidus, Comanonas, Halomonas, Hydrogenophaga, Lampropedia, Moraxella cocci=M.(B)caviane, Taylorella
20	Factory II	G+	Cocci	Deinococcus, D.radiopugnans, Marinococcus,M.albus, Micrococcus, M.nishinomiyaensis, Salinicoccus, Microbacterium, M.lactium,
21	Factory II	G+	Rod	Arthrobacter, Aureobacterium, Curtobacterium, C.streum or C.plantarum, Microbacterium, M.lactium, Rubrobacter, Sphaerobacter
22	Factory III	G+	Rod	Lactobacillus, Arthrobacter, Aureobacterium, Brevibacterium, Curtobacterium, C.albidum, C.flaccumfaciens, C.luteum
23	Factory III	G+	Coccobacilli	Marinococcus, M.hispanicus, Salinicoccus, Brevibacterium, Cellulomonas, Propionibacterium, Rubrobacter
24	Factory III	G+	Coccobacilli	Salinicoccus, Arthrobacter, Curtobacterium, C.streum or C.plantarum, Sphaerobacter
25	Factory III	G+	Coccobacilli	Salinicoccus, Arthrobacter, Curtobacterium, C.streum or C.plantarum, Sphaerobacter
26	Factory III	G+	Coccobacilli	Salinicoccus, Arthrobacter, Curtobacterium, C.streum or C.plantarum, Sphaerobacter
27	Factory III	G+	Coccobacilli-thin	Aerococcus, Peptococcus, Ruminococcus, Sarcina, Butyrivibrio, Curtobacterium, C.streum or C.plantarum, Thermoanaerobacter
28	Factory III	G+	Coccobacilli	Salinicoccus, Arthrobacter, Curtobacterium, C.streum or C.plantarum, Microbacterium M.lactium, Rubrobacter, Sphaerobacter

Table 3.3. Genera lists according to the results of the biochemical tests

CHAPTER 4

DISCUSSION

The applications of the FISH technique for the analysis of the bacterial community structure in the activated sludge samples are well documented. Samples from a wastewater treatment plant hybridized with fluorescein-labeled oligonucleotide probes specific for members of the domains Bacteria and Eucarya showed that the majority of the detected cells (approximately 40%) were of the beta subclass of Proteobacteria (Wallner et al., 1995). Similarly, Manz et al. (1994a) found that the beta subclass of Proteobacteria was the dominant group in the aerobic reactors treating municipal wastewater. Wagner et al. (1993a) studied the bacterial community structures in activated sludge samples from aeration tanks of a two-stage system with a high-load first stage (B1) and a low-load second stage (B2) by oligonucleotide probes. The activated sludge samples were dominated by the alpha, beta, or gamma subclass of Proteobacteria. These Proteobacteria accounted for about 80% of all the active bacteria found in the activated sludge. In the B1 sample, the ALF probe hybridized to 10% of the cells hybridizing with probe EUB, the BET probe hybridized with 42%, and the GAM probe hybridized with 34% of the cells. In the B2 sample, the ALF and BET counts (37%) dominated over the GAM counts (7%). They concluded that the community structures in the two differently loaded aerated basins B1 and B2 were quite different.

In this study we have detected the presence of several bacterial groups that varied in their concentration percentages among the 3 factories. The beta subclass of Proteobacteria and the CF cluster were the predominant bacterial groups in the activated sludge samples from the 3 factories but at different concentrations. The highest concentration of these groups was found in factory I, which also has the highest performance. Mudaly et al. (2001) in their study on 16S rRNA also showed that the beta subclass were dominant (22%), followed by alpha (19%), gamma (17%), HGC (11%) and CF (8%).

The composition of the microbial community present in the nitrifying-denitrifying activated sludge of an industrial wastewater treatment plant connected to a rendering facility was investigated by the full-cycle rRNA approach (Juretschko et al., 2002). Eighty nine percent of all bacteria detectable by FISH with a bacterial probe set could be assigned to specific divisions. Consistent with the 16S rRNA gene library data, members of the beta subclass of Proteobacteria dominated the microbial community and represented almost half of the biovolume of all bacteria detectable by FISH. (Juretschko et al., 2002). Bond et al. (1999) investigated that identification of some of the major groups of bacteria in efficient and non-efficient biological phosphorus (P) removal activated sludge systems and found that bacteria inhibiting P removal overwhelmed the reactor, and according to FISH, bacteria of the beta subclass of Proteobacteria were dominant in the sludge (58% of the population). In another study, the characterization of bacterial communities from activated sludge by culture-dependent numerical identification versus in situ identification using group- and genus-specific rRNA-targeted oligonucleotide probes revealed that the in situ beta subclass of Proteobacteria and the high G-C gram-positive bacteria were dominant, while the two gamma Proteobacterial genera Aeromonas and Acinetobacter constituted less than 5% of all bacteria (Kampfer et al., 1996).

The composition of the microbial population in the anaerobic and aerobic zones of treatment plant, as determined by fluorescent oligonucleotide probing in the aerobic zone showed that 80% of the cells visualized by DAPI were also detected by the bacterial probe EUB (Kampfer et al, 1996). Cells belonging to the beta subclass were dominant and the high G-C gram-positive bacteria accounted for approximately 16% in the aerobic-, and 14% in the anaerobic zone of the basin. In the aerobic zone BET and HGC (33% and 17%, respectively) dominated over ALF (13%) and GAM (10%) counts. The CF counts were very low and accounted for only 1% of the total.

Members of the CF cluster within the CFB phylum are frequently isolated from many natural and man-made ecosystems (soil, fresh and marine waters, clinical specimens, air-conditioning, sewage-treatment plants) and exhibit a broad range of phenotypic diversity (Manz et al., 1996b). Whiteley et al. (2000), studied the bacterial community structure within an industrial phenol bioremediation system and found that at the whole-cell level, cells assigned to the CF cluster and to the gamma subclass of Proteobacteria numerically dominated the treatment compartments. The alpha subclass of Proteobacteria were of low relative abundance throughout the treatment system

whilst the beta subclass of the Proteobacteria exhibited local dominance in several of the processing compartments. This means that the beta and gamma Proteobacteria comprise a large fraction of the bacteria in wastewater treatment plants. The striking observation was the prevalence of the CF cluster in the majority of the processing compartments. Based on their numerical abundance, assessed by culture-independent methods, the CF cluster appeared to play an important process role or occupy a common niche within industrial phenol bioremediation wastewater treatment plant. Similarly, Lapara et al. (2000) found that the CF cluster dominant in the activated sludge of an industrial treatment facility.

An industrial bioremediation system designed for the removal of phenolic compounds was also shown by FISH to be dominated by members of the CF cluster and gamma subclass of Proteobacteria. Of these two groups, only the latter was positively correlated with phenol degradation (Amann et al., 2001b). Simultaneous in situ visualization of seven distinct bacterial genotypes (such as the filamentous bacterium *Sphaerotilus natans* and bacteria formerly classified as pseudomonads; *comamonas* spp. and *acidovorax* spp.), all affiliated with the phylogenetically narrow group of beta-1 Proteobacteria, was achieved in activated sludge. These findings indicated that the high diversity found in the same sample by direct rRNA sequence retrieval was indeed present in this complex community (Amann et al., 1996a). In this respect, members of the beta 2 group of Proteobacteria have been linked to enhance biological phosphate removal (Amann et al., 2001b).

Members of the CF cluster have been shown by in situ hybridization as an important constituent of sludge flocs and characteristic colonizer of filamentous bacteria (Wagner and Loy, 2002e). Interestingly, two different types of cell morphology could be distinguished in activated sludge samples. The first type, which was detected in variable percentages, was characterized by tightly packed, small spindle-shaped cells, typically forming the cores of activated sludge flocs. The second type morphology exhibited by cells that hybridizing with probes CF319a and CF319b characteristically colonized the surfaces of inorganic and organic structures (Manz et al., 1996b). In situ hybridizations of activated sludge samples obtained from different wastewater treatment plants showed that members of the CF group were always present, in amounts ranging from 10% to 50% of the cells which could be hybridized with the bacteria-specific probe EUB338 (Manz et al., 1996b). These results confirmed earlier findings that CF bacteria may have a direct role as floc-forming organisms and, due to the known

importance of bacterial floc formation, affect the performance of the activated sludge process (Manz et al., 1996b). One of the major operational problems of these systems is the excessive growth of filamentous bacteria that causes poor settlement of activated sludge flocs, a problem commonly referred to as bulking (Kanagawa et al., 2000). It has been suggested that a certain number of filamentous bacteria are required for proper floc formation. Thus, depending upon their identity and abundance, filamentous bacteria can be either beneficial or detrimental for efficient separation of the treated wastewater from the biomass in the settling tanks (Wagner and Loy, 2002e). Large filamentous populations were still present in about 60% of the industrial activated sludge plants, which means that bulking of activated sludge, which is no longer a serious problem in modern domestic nutrient removal plants, still occurs frequently in industrial treatment plants (Elkelboom and Geurkink, 2002).

In our study, the floc characteristics detected by phase-contrast microscopy were of the normal category with some filamentous bacteria for factory I and at a lesser degree for factory II. However factory III had diffuse and atypical flocs with abundant filaments, which might be one of the reasons for the operation problems in this factory.

Nitrification, the oxidation of ammonia to nitrate catalyzed by bacteria, is a key part of global nitrogen cycling. In the first step of nitrification, chemolithoautotrophic ammonia oxidizers transform ammonia to nitrite, which is subsequently oxidized to nitrate by the nitrite-oxidizing bacteria. All isolated chemolithoautotrophic, nitrite-oxidizing bacteria, belong to one of four different genera Nitrobacter (alpha subclass of Proteobacteria), Nitrococcus (gamma subclass of Proteobacteria), Nitrospina (delta subclass of Proteobacteria), and Nitrospira (phylum Nitrospira) (Daims et al., 2001b). While species of the genus Nitrobacter have been isolated from a variety of environments, including soil and fresh water, it was long assumed that the other three genera were confined to marine environments. In recent studies, however, bacteria related to the genus Nitrospira were also found to occur in different non-marine habitats (Watson et al., 1986). Initial studies revealed that Nitrosomonas populations predominated, often in close association with Nitrobacter spp. In addition, quantitative comparisons made by using this hierarchical collection of probes suggested a yet-undescribed diversity of ammonia oxidizers within the beta subclass of the Proteobacteria (Wagner et al., 1995c). These observations have considerable significance to our understanding of the microbiology of nitrification and the process control of engineered systems. For example, the close spatial contiguity of ammonia-

and nitrite-oxidizing populations could be important. The availability of culture-independent tools for the characterization of nitrifying populations will provide an important research tool and a potentially powerful monitoring tool for the study of these bacteria in engineered and natural systems (Mobarry et al., 1996).

The microbial community present in activated sludge plants plays a major role in the bioremediation process. The efficiency and robustness of a wastewater treatment plant mainly depend on the composition and activity of its microbial community. However, the role of microbial consortia in activated sludge is still not completely understood because culture-based techniques were quite time-consuming, often requires numerous testing of physiological and biochemical traits for identification and being too selective to give a comprehensive and authentic picture of the entire microbial community (Wagner et al., 2002d, Moter and Gobel, 2000). This fact was appreciated in our study were we were able only to reach identification at the group level and thus additional testing is demandable in order to achieve the final identification at the genus and species level.

In conclusion, we have shown that the FISH technique provided comprehensive information on the microbial consortia of activated sludge samples and their concentrations among the 3 textile factories. The beta subclass of Proteobacteria and the CF cluster were the predominant bacterial groups in these samples but at different concentrations. The highest concentration of these groups was found in factory I, which was also the best factory in performance. Factory I also had normal floc characteristics and some filamentous bacteria and similarly for factory II but at a lesser degree. However factory III had diffuse and atypical flocs with abundant filaments, which might be one of the reasons for being the lowest in operation performance.

Future Plans

- This is a pilot study were 3 factories were compared and their performance efficiencies were elaborated.
- Several other factories could also be checked for their performance efficiencies.
- As a result a detection system using FISH will be set and recommendations for strategy improvement could be given.
- A collaborative work could then be established between our university and textile factories with biological wastewater treatment plant.

APPPENDIX

Fluorescence In Situ Hybridization (FISH)

Figure A.1 illustrates the principles of the technique.

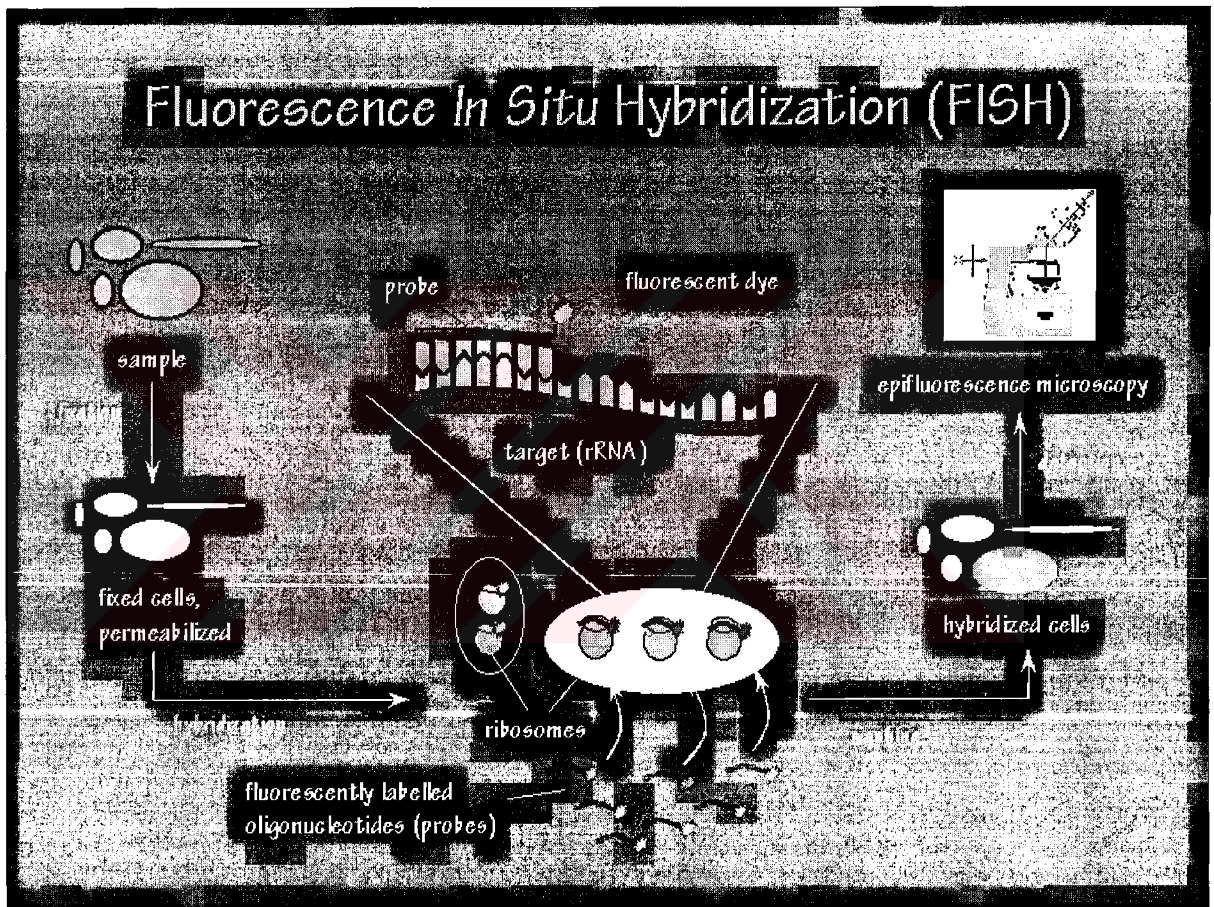


Figure A1.FISH procedure

Fluorescence microscopy is used mainly to detect and localize minute amounts of substances in the sample, where the fluorescence is observed as luminosity against a black backgrounds. Fluorescence is exhibited by molecules, which are usually fairly complex. The molecules absorb light energy from an excitatory beam, and are raised to a higher energy state. This is called the excited state, and from here the molecules re-

emit the energy as light of a different longer wavelength. For optimal fluorescence detection, the excitation and emission filters should be centered on the dye's absorption and emission peaks. To maximize the signal, one can choose excitation and emission filters with wide bandwidths.

The important criteria to be considered in selecting a probe are; specificity, sensitivity and ease of cell penetration. A typical oligonucleotide probe is of 15-30 base pair (bp) in length and is generated on an automated synthesizer. Short probes have easier accessibility to their target cells than longer ones, but they might carry fewer labels. There are different ways of labeling. Direct fluorescent labeling is most commonly used and is also the fastest, cheapest and easiest way because it does not require any further detection steps after hybridization. One or more fluorescent dye molecules are directly bound to the oligonucleotide either chemically during synthesis through an amino-linker at the 5'-end of the probe (Fig. A.2a), or enzymatically using terminal transferase to attach fluorescently labeled nucleotides at the 3'-end (Fig. A.2b). Coupling of Fluorescein- Isothiocyanate (FITC) to the oligonucleotide via an 18 carbon spacer may increase signal intensity as compared to a directly conjugated probe. An increase in fluorescence signal has also been reported by labeling probes at both ends, one fluorescent molecule at the 3'-end and four molecules at the 5'-end using appropriate spacers to prevent quenching of fluorescence. Fluorescence labeled oligonucleotides is now commercially available. Probes can be stored at -20°C for several months (Moter and Gobel, 2000).

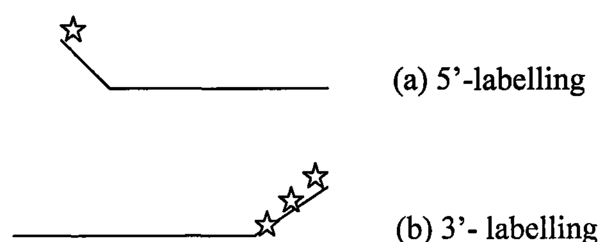


Figure A.2. Direct fluorescent labelling (a) and (b)

Phase Contrast Microscopy

The condenser of a phase-contrast microscope has an annular stop, an opaque disk with a thin transparent ring, which produces a hollow cone of light. As this cone passes

through a cell, some light rays are bent due to variations in density and refractive index within the specimen and are retarded by about ¼ wavelength. The deviated light is focused to form an image of the object. Undeviated light rays strike a phase ring in the phase plate, a special optical disk located in the objective, while the deviated rays miss the ring and pass through the rest of the plate. If the phase ring is constructed in such a way that the undeviated light passing through it is advanced by ¼ wavelenght, the deviated and undeviated waves will be about ½ wavelenght out of phase and will cancel each other when thay come together to form an image. The background, formed by undeviated light, is bright, while the unstained object appears dark and well defined (Presscott et al., 2002).

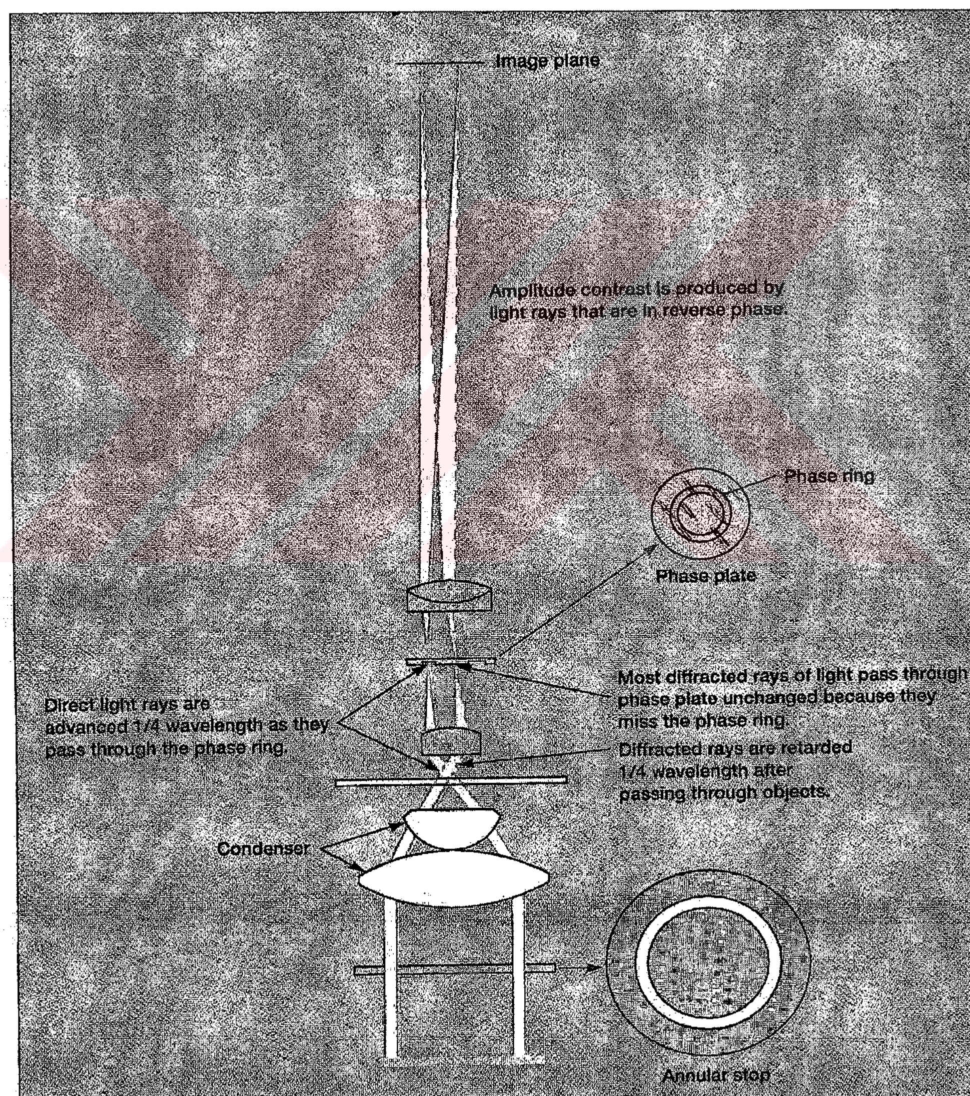


Figure A.3. Phase- contrast Microscopy (Prescott et al., 2002)

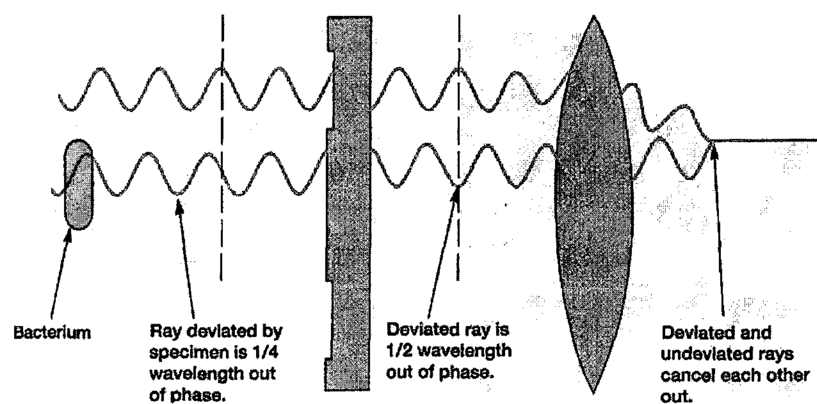


Figure A.4. The production of contrast in phase microscopy. The behavior of deviated and undeviated or undiffracted light rays in the dark-phase-contrast microscope. Because the light rays tend to cancel each other out, the image of the specimen will be dark against a brighter background (Prescott et al., 2002)

Highly refractive structures bend light to a much greater angle than do structures of low refractive index. The same properties that cause the light to bend also delay the passage of light by a quarter of a wavelength or so. In a light microscope in bright field mode, light from highly refractive structures bends farther away from the center of the lens than light from less refractive structures and arrives about a quarter of a wavelength out of phase.

Light from most objects passes through the center of the lens as well as to the periphery. Now if the light from an object to the edges of the objective lens is retarded a half wavelength and the light to the center is not retarded at all, then the light rays are out of phase by a half wavelength. They cancel each other when the objective lens brings the image into focus. A reduction in brightness of the object is observed. The degree of reduction in brightness depends on the refractive index of the object (Experimental Biosciences, Phase Contrast Microscopy, http://www.ruf.rice.edu/~bioslabs/methods/microscopy/phase.html, 2003).

Floc Characteristics and Overall Filament Abundance

The general size and shape of flocs

The floc size range (maximum dimension or diameter if approximately spherical) from small ≤ 150 μm, medium 150-500 μm, to large ≥ 500 μm. The floc shape was

characterized whether round or irregular, compact or diffuse; and whether the texture is firm or weak (Figure A.5 and A.6).

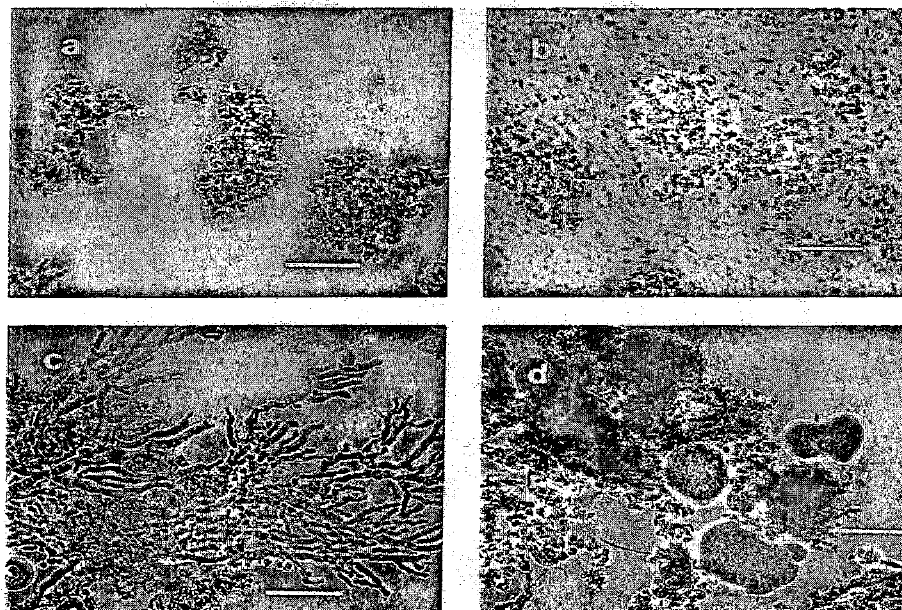


Figure A.5. Floc "texture" in activated sludge: a. rounded, firm, and compact; b. irregular and diffuse with substantial free cells. Appearance of zoogloeal organisms in activated sludge: c. fingered; d. amorphous. (all 100X phase contrast; bar = 100 μm).

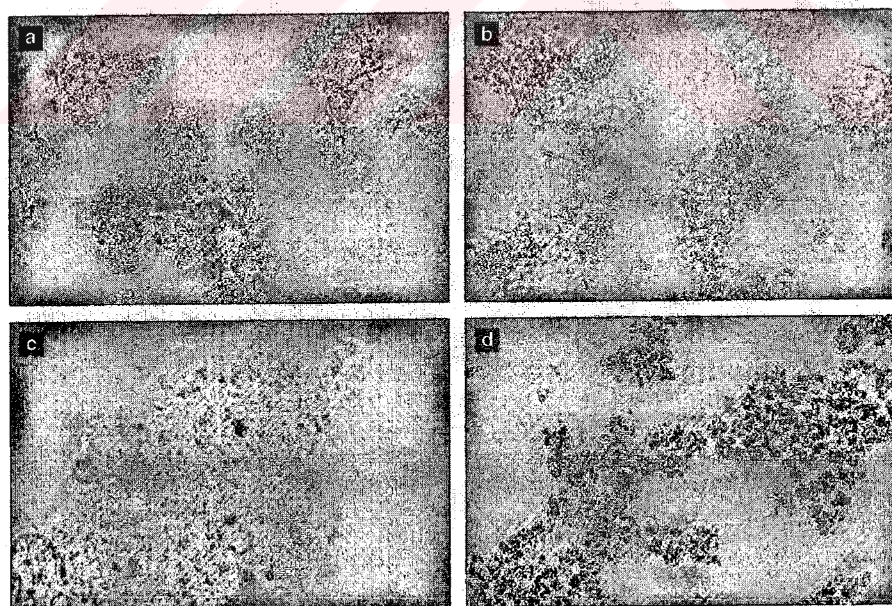


Figure A.6. Phase contrast micrographs of floc characteristics: (a) compact, (b) diffuse, (c) firm, (d) weak. (Original magnifications 100X, (a) and (b); 1000X, (c) and (d).)

The abundance of filamentous organisms

This can be measured by several methods. For this type of analysis a subjective scoring system is used. Filamentous organisms are observed first at 100X and then at 1000X and on the basis of these observations are subjectively rated for overall abundance on a scale from 0 (none) to 6 (excessive) (Table A.1) and (Figure A.7). Determine a sample abundance rating for all filament types together and an abundance rating for each filamentous organism type. Consider individual filamentous organisms dominant and most likely responsible for solids separation problems if they scored " very common" or greater. Consider individual organisms secondary (present but not in sufficient abundance to account for solids separation problems) if they are scored "common" or less. This method is rapid and suitable for establishing whether a filamentous organism is dominant or secondary and for determining filament response to remedial actions.

Table A.1. Subjective Scoring of Filament Abundance

Score	Abundance	Explanation
0	None	No filaments observed
1	Few	Filaments present but only observed in an occasional floc
2	Some	Filaments commonly observed but not present in all flocs
3	Common	Filaments observed in all flocs, but at low density (1 to 5 filaments per floc)
4	Very common	Filaments observed in all flocs at medium density (5 to 20 per floc)
5	Abundant	Filaments observed in all flocs at high density (>20 per floc)
6	Excessive	Filaments observed in all flocs (more filaments than floc and/or filaments growing in high abundance in bulk solution)

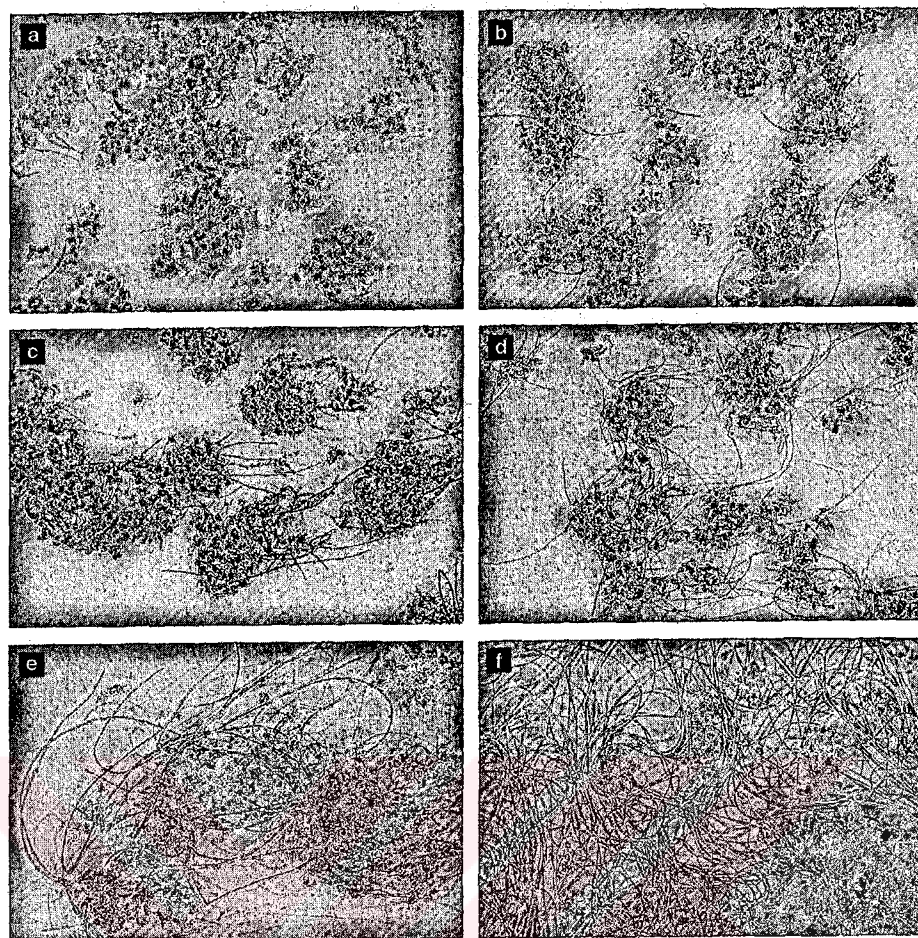


Figure A.7. Phase contrast micrographs of filament abundance categories using the subjective scoring system: (a) few, (b) some, (c) common, (d) very common, (e) abundant, and (f) excessive. (Original magnification 100X.)

An abundance rating is determined for the sample as a whole (all filament types together) and for each filamentous organism observed. Individual filamentous organisms are considered dominant (and likely most responsible for solids separation problems) if they are scored "very common" or greater. Organisms are considered secondary, i.e., they are present but not in sufficient abundance to account for solids separation problems, if they are scored "common" or less. This method is both rapid and suitable for establishing whether a filamentous organism is dominant or secondary and for determining their response to remedial actions. Abundance categories generally are reproducible to within ± one abundance category between observers (Jenkins et al., 2003).

Culture Media Preparation

A microbiological medium (liquid and solid) is the food that we use for culturing bacteria, molds, and other microorganisms. The liquid media include nutrient broth, citrate broth, glucose broth, litmus milk, etc. These media are used for the propagation of large numbers of organisms, fermentation studies, and various other tests. Solid media are made by adding a solidifying agent is one that is not utilized by microorganisms, does not inhibit bacterial growth, and does not liquefy at room temperature. Agar and silica gel do not liquefy at room temperature and are utilized by very few organisms. Gelatin, on the other hand, is hydrolysed by quite a few organisms and liquefies at room temperature. Nutrient agar, blood agar, and Sabouraud's agar are examples of solid media that are used for developing surface colony growth of bacteria and molds (Benson et al., 2002).

Nutrient Agar

Nutrient Agar is suspended 23 g of nutrient agar in 1000 mL of cold distilled water, heated to dissolve and sterilize by autoclaving at 121°C for 15 minutes, adjusted final pH 7.0 and poured into sterile petri dishes.

Blood Agar

Blood agar base is suspended 40 g in 1000 ml of cold distilled water, heat to boiling and autoclave at 121°C for 15 minutes, cooled to about 50°C, aseptically add 5% (v/v) of sterile defibrinated blood; mixed well and poured into sterile petri dishes, incubated at 37°C for at least 24 hours before use, to test the sterility, adjusted final pH 7.4.

MacConkey Agar

MacConkey agar is suspended 50g in 1000ml of cold distilled water, heat to boiling and sterilise by autoclaving at 121°C for 15 minutes, Dried the surface of the medium before inoculation, adjusted final pH 7.1.

Pure Culture Techniques

Several different methods of getting a pure culture from a mixed culture are available to us. The two most frequently used methods involve making a streak plate or a pour plate. Both plate techniques involve thinning the organisms so that the individual species can be selected from the others (Benson et al., 2002).

Streak Plate Method

For the streak plate technique, 20 mL of the medium was transfered to sterile petri dishes and left to dry. It is important that the surface of the medium is thoroughly dry before use. Inoculate the plates by surface streaking the medium with the material to be examined, incubate at 37°C and examine the colonies after 16-18 hours. A longer incubation period alters the growth characteristics of the microorganisms (Benson et al., 2002).

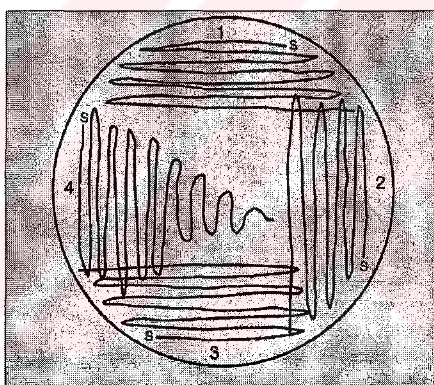


Figure A.8. (Benson et al., 2002).

1. Streak one loopful of organisms back and forth over area 1, starting at point designated by "s". Apply loop lightly. Don't gouge into the medium.
2. Flame the loop, cool 5 seconds and touch the medium in sterile area momentarily to insure coolness.
3. Rotate the dish 90 degrees while keeping the dish closed. Streak Area 2 with several back and forth strokes, hitting the original streak a few times.
4. Flame the loop again. Rotate the dish and streak area 3 several times, hitting last area several times.
5. Flame the loop, cool it, and rotate the dish 90 degrees again. Streak Area 4, contacting Area 3 several times and drag out the culture as illustrated.
6. Flame the loop before putting it aside.

A properly executed streak plate will give as good an isolation as is desired for most work. Figure A.9 illustrates how colonies of a mixed culture should be spread out on a properly made streak plate. The important thing is to produce good spacing between colonies.

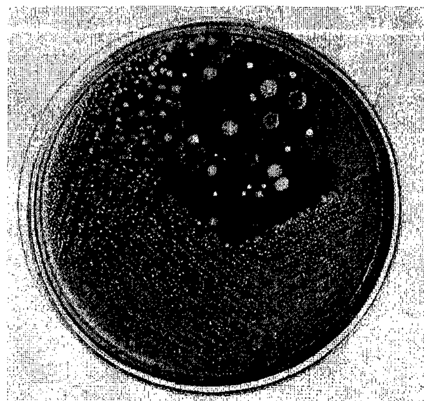


Figure A.9. If the plate reveals well-isolated colonies of three colors (red, white, and yellow), then the plate suitable for subculturing (Benson et al., 2002).

Pour Plate Method

This method of separating one species of bacteria from another consists of diluting out one loopful of organisms or 1 mL of activated sludge samples with three tubes or 15 mL tubes of liquefied nutrient agar in such a manner that one of the plates poured will have an optimum number of organisms to provide good isolation. Figure A.10illustrates the general procedure.

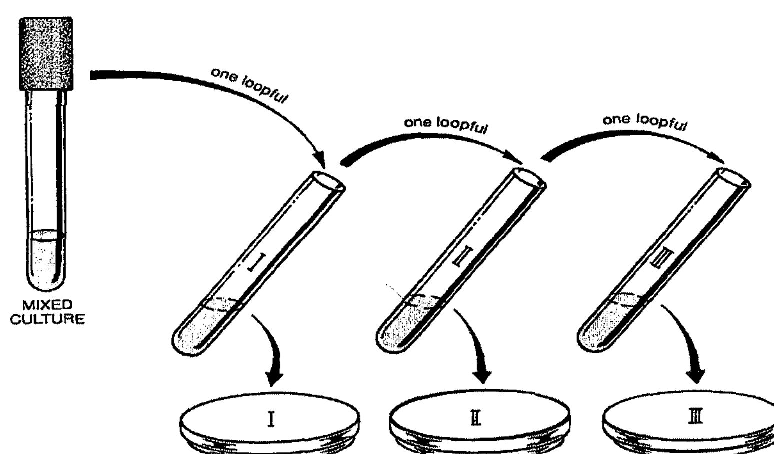


Figure A.10. Three steps in the loop dilution technique for separating out organisms (Benson et al., 2002).

Bacterial Population Counts

The procedure consists of diluting the organisms with a series of sterile PBS blanks as illustrated in figure A.11. By using the dilution procedure indicated here, a final dilution of 1:1,000,000 occurs in blank C. From blanks B and C, measured amounts of the diluted organisms are transferred into empty petri plates (Benson et al., 2002).

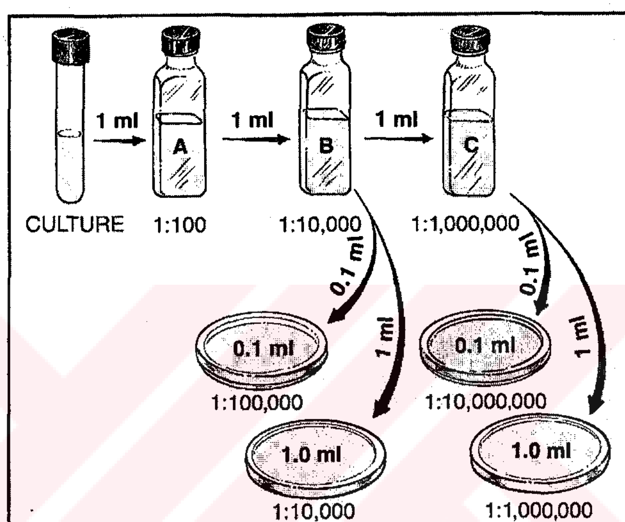


Figure A.11. Quantitative plating procedure

Gram Staining

Gram Staining technique seperates bacteria into two groups: those that are gram-positive and gram negative.

Bacterial identification, regardless of the method used, first requires the cultivation, isolation, and staining of a pure culture. After colony characteristics, cell morphology, and Gram reaction have been determined, conventional identification methods require microbiologists to perform biochemical tests specified in an identification scheme, which is a key of bacterial characteristics (Alexander and Strete, 2001).

Biochemical Tests

Oxidase Test

Prepared oxidase reagent (1 % Tetramethyl-p-phenylenediamine) and used wooden rods. Picked an amount of inoculum from a plate culture and placed it on a piece of filter paper. Added one drop of the reagent (if it is dark blue, it is old and should not be used). A positive reaction will usually occur within 10-15 seconds, and will be a bluish-purple color that progressively becomes more purple. (Oxidase test, http://www.rlc.dcccd.edu/mathsci/reynolds/micro/lab_manual/oxidase.html, 2003)

The production of oxidase is one of the most significant tests we have for differentiating certain groups of bacteria (Benson, 2002). Bacteria that contain cytochrome oxidase, such as pseudomonas species are oxidase positive, while those that lack this enzyme, such as *Escherichia coli* and other enterics, are oxidase negative. The oxidase test requires the use of a reduced chemical reagent. This reagent does not interact directly with cytochrome oxidase, but instead interacts with the enzyme's product, oxidized cytochrome c. Cytochrome c. changes the reduced reagent to an oxidized form (Alexander and Strete, 2001).

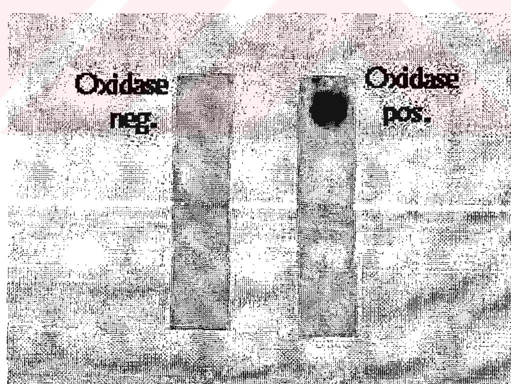


Figure A.12. Result of oxidase test on a whatman filter (no.2), with *Pseudomonas aeruginosa* (oxidase positive) on the right and *E .coli* (oxidase negative) on the left. (Oxidase test, http://medic.med.uth.tmc.edu/path/oxidase.htm,2003)

2.4.4.2. Catalase Test

The catalase test is performed by adding 3% hydrogen peroxide to an 18-24 hour culture on an agar slant or glass slide. The culture is observed for the immediate apperance of bubbles (Alexander and Strete, 2001).

Bacterial cells produce hydrogen peroxide during aerobic respiration. If hydrogen peroxide accumulates in the cell, it becomes toxic. For this reason, most aerobic and facultatively anaerobic bacteria posses an enzyme called catalase, which breaks down hydrogen peroxide. However, some bacteria, such as streptococcus and enterococcus, lack this enzyme. These bacteria are easily distinguished from catalase-positive bacteria, such as staphylococcus and micrococcus.

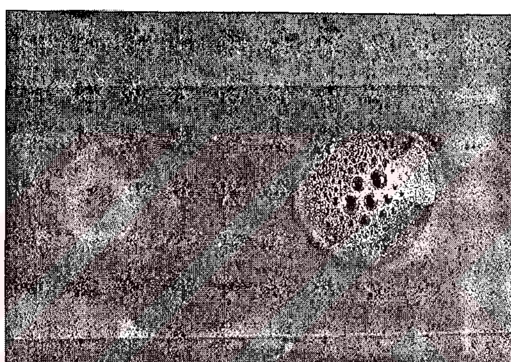


Figure A.13. Results of catalase tests on a glass slide. Each culture suspension received several drops of hydrogen peroxide. Enterococcus faecalis(at left) is catalase negative, while Staphylococcus epidermidis (at right) is catalase positive.

Carbonhydrate Fermentation

Phenol Red broth contains peptone, a single carbonhydrate, plus the pH indicator phenol red. This indicator is red at an alkaline pH, but yellow at an acidic pH. A small, inverted glass tube called a durham tube, is placed at the bottom of each tube containing carbonhydrate fermentation media. The tube collects gas, which is often a product of carbonhydrate fermentation. An isolate is inoculated into a phenol red broth tube with a sterile transfer loop. The tube is incubated at 35°C for 24-48 hours before examination. The broth is observed for color change and gas production (Alexander and Strete, 2001).

Carbonhydrate fermentation media, such as phenol red broth, is used to determine whether an organism has the ability to ferment various carbonhydrates. Carbonhydrates typically tested include adonitol, arabinose, glucose, lactose, sucrose etc. In this study, glucose, lactose and sucrose were used. Bacteria that ferment a carbonhydrate produce acid, or acid and gas, as end-products. Acids lower the pH of the medium, causing the phenol red pH indicator to turn yellow, while gases(if produced)collect to form bubbles in the durham tube. If bacteria do not ferment a carbonhydrate, teh medium will remain red. In addition, no bubbles will be present in the durham tube. A range of results for phenol red broth with lactose are shown in Figure A.14.

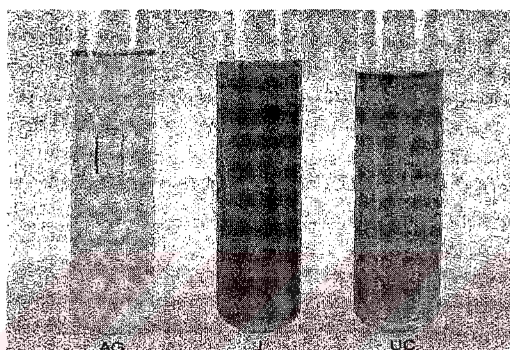


Figure A.14. Carbonhydrate utilization test in phenol red lactose broth tubes(left to right): *E.coli*, acid and gas (AG); Alcaligenes faecalis, inert (I); uninoculated control(UC).

Nitrate Reduction

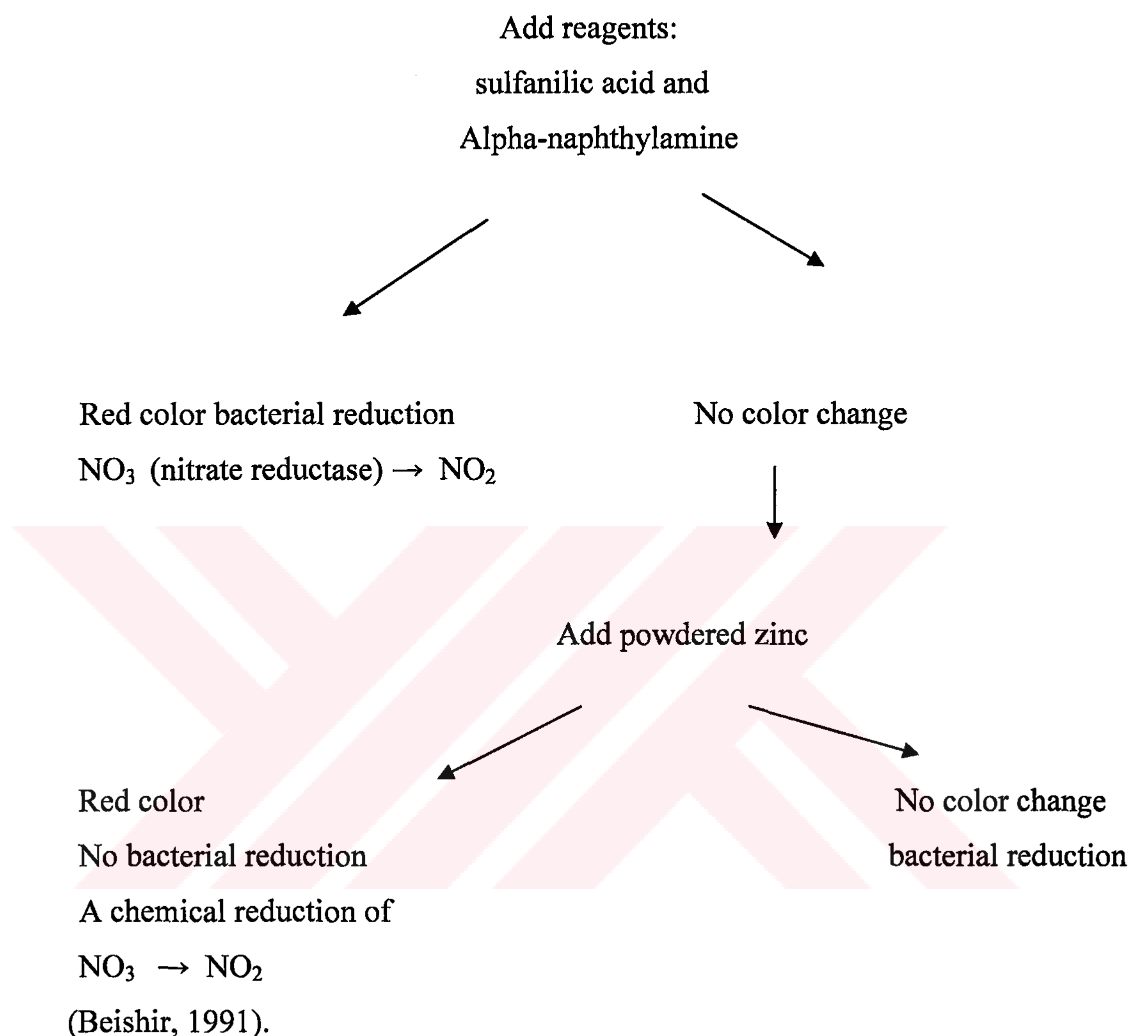
To determine the ability of an organism to reduce nitrate to nitrites or free nitrogen gas. If a microbe is growing in nitrate broth, one of the following results may occur, dependeing on which enzymes the microbe is capable of producing, if any.

1. Nitrate remains unaltered.
2.Nitrate is reduced to nitrite
3.Nitrate has been rapidly reduced to nitrite, then further reduced to ammonia or free nitrogen.

Below is generalized reaction of nitrate reduction to nitrite and subsequently to nitrogen gas.

NO_3 nitrate reductase $\rightarrow$ NO_2 nitrite reductase $\rightarrow$ NH_3 or N_2

It is beneficial tı test first for result 2, the reduction of nitrate (NO_3) to nitrite (NO_2), because if a positive nitrite test is obtained , no further testing is necessary. If the test is negative, it must be determined if nitrate remains unaltered or has been reduced to ammonia or free nitrogen.



(Beishir, 1991).

Trypticase-nitrate broth is used to detect an organisms ability to reduce nitrate to nitrite or a further reduced nitrogenous compound such as nitrous oxide or nitrogen gas. Nitrate may be reduce to multiple compounds by two processes. Anaerobic respiration and denitrification. In anaerobic respiration the bacterium uses nitrate as it's terminal electron acceptor, reducing nitrate to a variety of compounds, while denitrification reduces nitrate solely to molecular nitrogen. Sulfanilic acid and dimethyl 1-naphthylamine are added to detect nitrite, which will complex with these molecules forming a red color. If no red color is observed there are two possibilities; the nitrate has not been reduced, or it has been reduced further than nitrite. To differentiate

between these two possibilities, zinc powder is added, which will complex with nitrate forming a red color. Thus if the tube turns red after zinc, nitrate has not been reduced and the result is negative. If no red color is observed then the nitrate has been reduced further than nitrite and the result is positive.

The IMVIC Test

The IMViC test series consists of four different tests; they are: Indole Production, Methyl-red test, Voges-Proskauer test and Citrate Utilization Test. The name IMViC stands for the first letter of the name of each test in the series with the lowercase (i) added for ease pronunciation. The test is designed to determine specific physiological properties of microorganisms (Beishir, 1991).

Indole is produced in tryptone broth by the enzyme systems of certain organisms. Tryptone broth is rich in the amino acid trytophan,which can used by some bacteria as a source of carbon and nitrogen as well as energy. As these organisms grow in trytone broth, they attack the trytophan and degrade it to indole , pyruvic acid, and ammonia.

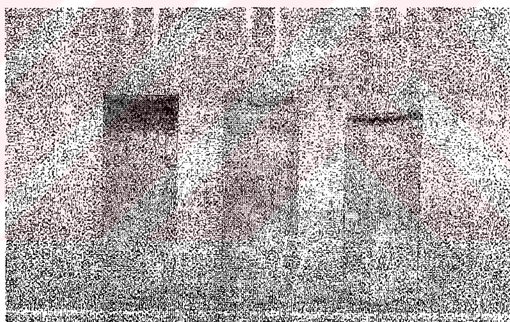


Figure A.15. Results for indole production in Trytone broth tubes. Inoculated tubes received five drops of Kovak's reagent after incubation. The reagent appears as a layer on the agar surface. Left: *E Coli* (indole positive), center: *Enterobacter aerogenes* (indole negative); right : uninoculated (Alexander and Strete, 2001).

The methy-red and Voges –Prokauer tests must be considered together since they are physiological related and are inoculated into the same medium, MR-VP broth. MR-VP medium contains peptone, dextrose (glucose), and dipotassium phosphate. The dextrose is a significant ingredient designed to determine what types of end products an organism forms from the degradation of glucose.

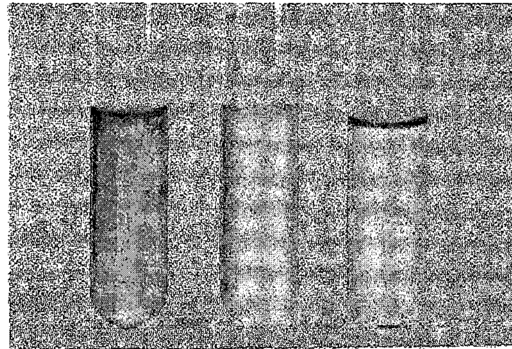


Figure A.16. Methyl red test results in MR-VP medıum tubes. The inoculated tubes received five drops of methyl red after incubation. Left: *E.coli* (methyl-red pozitive); center: *Enterobacter aerogenes* (methyl-red negative); right: uninoculated (Alexander and Strete, 2001).

The Voges-Proskauer test reagents react chemically with acetyl methyl carbinol to produce a pink or red color. The MR-VP tests demonstrate the practical use of good knowledge of microbial metabolism.

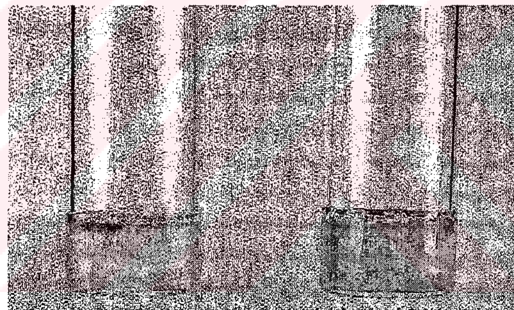


Figure A.17. Results of the Voges-Proskauer test in MR-VP tubes. Tubes have received six drops of Barritt's Reagent B. *E.coli* (left) is VP negative, and *Enterobacter aerogenes* (right)is VP positive. (Alexander and Strete, 2001).

The citrate test is performed by inoculating the microorganisms into an organic synthetic medium in which sodium citrate is the only source of carbon and energy. An organic synthetic medium is one that is chemically defined, containing known amounts of mineral salts and known amounts of simple organic compounds as the sole nutritional substrates.

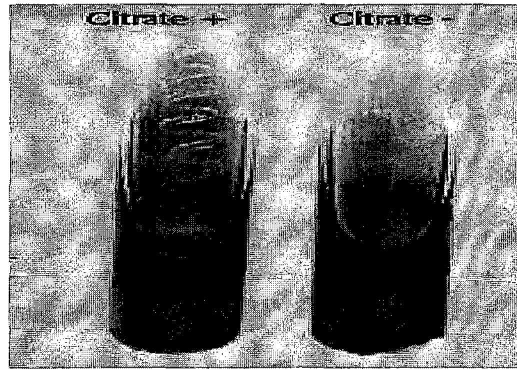
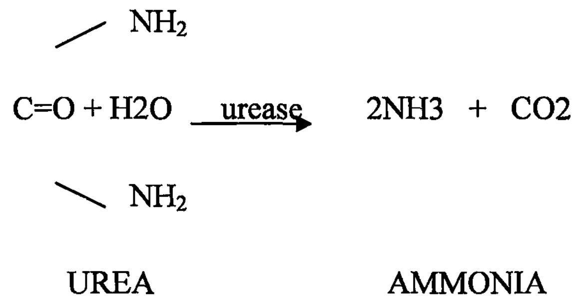


Figure A.18. Simmon's citrate slant inoculated with citrate-nagative E.coli (at left) and citrate pozitive Enterobacter aerogenes (at right). Citrate-pozitive organisms display growth and blue color. (Alexander and Strete, 2001).

Urea Hydrolysis

The conventional method of testing for urea hydrolysis is to grow bacteria in rehydrated, commercially prepared urea broth. Urea broth is a highly buffered medium that contains yeast extract, which supports bacterial growth: urea, which is the enzyme substrate: and phenol red, which is a pH indicator. When Proteus is grown in this broth, proteal urease is produced, which hydrolyzed the urea. This results in the accumulation of sufficient ammonia to make the environment quite alkaline. As the pH of the broth becomes more alkaline (that is, if the pH is 8,1 or higher), the phenol red (which is salmon-colored at pH 6.8 before inoculation) turns a cerise or pinkish-red color. The appearance of the cerise color is a positive test for urea hydrolysis. When a lactose-nonfermenting, Gram-negative bacillus gives a positive test for urea hydrolysis, no further testing is necessary to eliminate it from the prominent enteric pathogens, and genetic name, Proteus, has been determined (Beishir, 1991).

Some bacteria produce urease, an enzyme capable of breaking down urea. The breakdown of urea within 24 hours is a trait used to distinguish species of Proteus from other enteric bacteria. Urea broth contains yeast extract, urea, and pH indicator phenol red. Phenol Red is yellow yellow-orange at the initial pH of 6.8 but changes to pinkish-red at of 8.4 (Alexander and Strete, 2001).



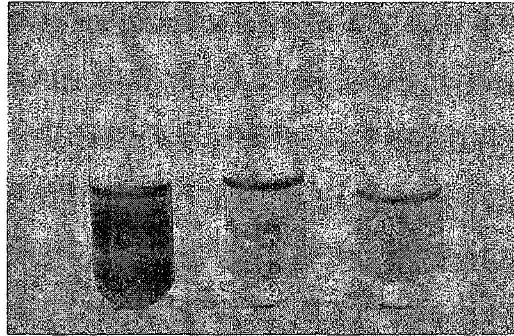


Figure A.19. Results of urea broth tubes inoculated with *Proteus vulgaris* on the left (urease pozitive) and *E.Coli* in the center (urease nagative). The tube on the rigth is uninoculated (Alexander and Strete, 2001).

Gelatin Utilization

Nutrient gelatin tubes are used to determine whether an organism produces gelatinases. Nutrient gelatin contains beef extract and peptone to support growth, and enough gelatin(120 g/l)to cause the medium to gel. An isolate is inoculated into a nutrient gelatin tube with a sterile transfer needle. The tube is incubated at 35 ^{o}C for 24-48 hours. The medium is chilled thoroughly in a refrigerator before examination. Chilling is essential because gelatin is liquid at temperatures above 20 ^{o}C. After chilling, the medium is observed for gelling by carefully tilting the tube to the side.

If gelatinases are produced, the medium will not gel when chilled because has been broken down into individual amino acids. A liquid medium after chilling represents a positive test for gelatin utilization. A gelled medium after chilling represents a negative test (Alexander and Strete, 2001).

Gelatin is a protein that is digested by bacterial extracellular enzymes called gelatinases. The end products of this reaction are amino acids that are transported into

the cell for utilization. Some bacteria, such as *Pseudomonas aeruginosa*, produce gelatinases, while others, such as *Alcaligenes faecalis*, do not.

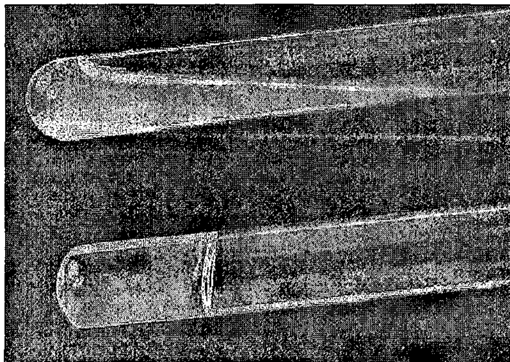


Figure A.20. Nutrient gelatin tubes inoculated with *Pseudomonas aeruginosa* at the top (gelatin positive) and with *Alcaligenes feacalis* at the bottom (gelatin negative). Nutrient gelatin tubes must be thoroughly chilled before examination (Alexander and Strete, 2001).

Skim Milk Utilization

Skim milk agar is used to test for teh presence of caseinase, this medium contains peptone to support growth, 10% skim milk, and agar. An isolate is inoculated onto a plate with a sterile transfer loop. The plate is incubated at 35 °C for 24-48 hours before examination. Skim milk agar appears white due to the presence of casein. The zone around growth of caseinase-positive organisms will be clear because of casein breakdown. The zone around growth of caseinase-negative organisms will remain white(Alexander and Strete, 2001).

Casein is the primary protein in the skim milk. Only bacteria that produce the extracellular enzyme caseinase, such as Basillus cereus, can break down this protein, which gives milk its white color. The breakdown products are single amino acids that are transported into the cell and used in metabolism.

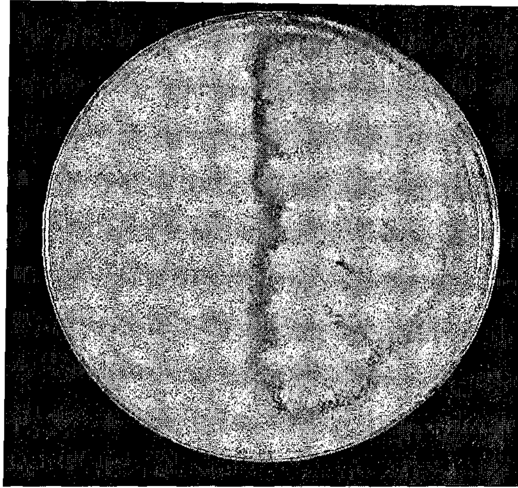


Figure A.21. Results on skim milk agar plate inoculated with Alcaligenes faecalis on the left (caseinase negative) and Basillus cereus on the right (caseinase positive).

REFERENCES

Alexander, S. K., Strete D., *Microbiology: A Photographic Atlas for the Laboratory*, Benjamin Cummings, USA, 2001.

Amann, R. I., Binder, B. J., Olson, R. J., Chisholm, S. W., Devereux, R., Stahl D. A., "Combination of 16S rRNA-targeted oligonucleotide probes with flow cytometry for analyzing mixed microbial populations", *Applied and Environmental Microbiology*, Vol. 56(6), pp.1919-1925,1990.

Amann, R., Snaidr, J., Wagner, M., Ludwig, W., Schleifer, K. H., "In situ visualization of high genetic diversity in a natural microbial community", *Journal of Bacteriology*, Vol. 178(12), pp. 3496-3500, 1996.

Amann, R., Fuchs, B. M., Behrens, S., "The identification of microorganisms by fluorescence in situ hybridization" *Current Opinion in Biotechnology*, Vol. 12, pp. 231-236, 2001.

Arslan, I., *Treatment Of Reactive Dye-Bath Effluents By Heterogeneous and Homogeneous Advanced Oxidation Processes*, Ph. D. Thesis, Boğaziçi University, 2000.

Beer, M., Seviour, E. M., Kong, Y., Cunningham, M., Blackall, L. L., Seviour, R. J., "Phylogeny of the filamentous bacterium Eikelboom Type 1851, and design and application of a 16S rRNA targeted oligonucleotide probe for its fluorescence in situ identification in activated sludge", *FEMS Microbiological Letters*, Vol. 207(2), pp. 179-183, 2002.

Behrens, S., Ruhland, C., Inacio, J., Huber, H., Fonseca, A., Spencer-Martins I., Fuchs, B. M., Amann, R., "In situ accessibility of small-subunit rRNA of members of the domains Bacteria, Archaea, and Eucarya to Cy3-labeled oligonucleotide probes", *Applied and Environmental Microbiology*, Vol. 69(3), pp. 1748-1758, 2003.

Beishir, L., *Microbiology In Practice*, Harper Collins, New York, 1991.

Benedict, R. G. and Carlson, D. A., "Aerobic Heterotrophic Bacteria in Activated Sludge", *Water Research*, Vol. 5, pp. 1023-1030, 1971.

Benson H. J., *Microbiological Applications Laboratory Manual in General Microbiology*, eighth edition, Mc Graw Hill, New York, 2002.

Besseliewre, E. E., and Schwarz, M., *The treatment of Industrial Waste*, Mc Graw Hill, 1976.

Bidnenko, E., Mercier, C., Tremblay, J., Tailliez, P., Kulakauskas, S., "Estimation of the state of the bacterial cell wall by fluorescent In situ hybridization", *Applied and Environmental Microbiology*, Vol. 64(8), pp. 3059-3062, 1998.

Bond, P. L., Erhart, R., Wagner, M., Keller, J., Blackall, L. L., "Identification of some of the major groups of bacteria in efficient and nonefficient biological phosphorus removal activated sludge systems", *Applied and Environmental Microbiology*, Vol. 65(9), pp. 4077-4084, 1999.

Casamayor, E. O., Pedros-Alio, C., Muyzer, G., Amann, R., "Microheterogeneity in 16S ribosomal DNA-defined bacterial populations from a stratified planktonic environment is related to temporal changes and to ecological adaptations", *Applied and Environmental Microbiology*, Vol. 68(4), pp. 1706-1714, 2002.

Correia, V. M., Stephenson, T., and Judd, S. J., "Characterization of Textile Wastewaters-A Rewiev", *Environmental Technology*, Vol.15, pp. 917-929, 1994.

Cottrell, M. T., Kirchman, D. L., "Community composition of marine bacterioplankton determined by 16S rRNA gene clone libraries and fluorescence in situ hybridization", *Applied and Environmental Microbiology*, Vol. 66(12), pp. 5116-5122, 2000.

Curds, C. R. and Fey, G. J., "The Effect of Ciliated Protozoa on the Fate of *Escherichia coli* in the Activated-Sludge Process," *Water Research*, Vol. 3, pp. 853-867, 1969.

Curds, C. R. and Cockburn, A., "Protozoa in Biological Sewage-Treatment Processes-I. A Survey of the Protozoan Fauna of British Percolating Filters and Activated-Sludge Plants", *Water Research*, Vol. 4, pp. 225-236, 1970.

Curtis, E. J. C., "Sewage Fungus: Its Nature and Effects," *Water Research*, Vol. 3, pp. 289-311, 1969.

Daims, H., Ramsing, N. B., Schleifer, K. H., Wagner, M., "Cultivation-independent, semiautomatic determination of absolute bacterial cell numbers in environmental samples by fluorescence in situ hybridization", *Applied and Environmental Microbiology*, Vol. 67(12), pp. 5810-5818, 2001.

Daims, H., Nielsen, J. L., Nielsen, P. H., Schleifer, K. H., Wagner, M., "In situ characterization of Nitrospira-like nitrite-oxidizing bacteria active in wastewater treatment plants", *Applied and Environmental Microbiology*, Vol. 67(11), pp. 5273-5284, 2001.

DeLong, E. F., Taylor, L. T., Marsh, T. L., Preston, C. M., "Visualization and enumeration of marine planktonic archaea and bacteria by using polyribonucleotide probes and fluorescent in situ hybridization", *Applied and Environmental Microbiology*, Vol. 65(12), pp. 5554-5563, 1999.

de los Reyes, F. L. 3rd, Rothauszky, D. Raskin, L., "Microbial community structures in foaming and nonfoaming full-scale wastewater treatment plants", *Water Environmental Res.*, Vol. 74(5), pp. 437-449, 2002.

DuTeau, N. M., Rogers, J. D., Bartholomay, C. T., Reardon, K. F., "Species-specific oligonucleotides for enumeration of Pseudomonas putida F1, Burkholderia sp. strain JS150, and Bacillus subtilis ATCC 7003 in biodegradation experiments", *Applied and Environmental Microbiology*, Vol. 64(12), pp. 4994-4999, 1998.

Easton, J. R., "The Dye Maker's View", in Cooper, P. (Editor), *Colour in Dyehouse Effluent*, The Society of Dyers and Colorists, Alden Press, Oxford, 1995.

Elkelboom, D. H., Geurkink, B., "Filamentous micro-organisms observed in industrial activated sludge plants", *Water Science and Technology*, Vol. 46(1-2), pp. 535-542, 2002.

Fluorescence microscopy of DAPI stained cells, 2003, http://www.celldeath.de/apometh/dapi.html.

Grabow, W. O. K., "The Virology of Waste Water Treatment," *Water Research*, Vol. 2, pp. 675-701, 1968.

Grau, P., " Textile Industry Wastewaters Treatment" *Water Science and Technology*, Vol. 24, pp.97-103, 1991.

Guan, Y., Kurisu, F., Satoh, H., Mino, T., "A quantitative method for measuring the mass concentration of the filamentous bacterium Type 021N in activated sludge using fluorescence in situ hybridization", *Letters in Applied Microbiology*, Vol. 37(2), pp. 100-104, 2003.

Ivanov, V., Tay S. T., Tay, J. H., "Monitoring of microbial diversity by fluorescence in situ hybridization and fluorescence spectrometry", *Water Science and Technology*, Vol. 47(5), pp. 133-138, 2003.

Jenkins, D., Richard, M. G., Daigger, G. T., *Manual On The Causes And Control Of Activated Sludge Bulking, Foaming and Other Solids Seperation Problems*, Lewis Publishers, New York ,U.S.A., 2003.

Juretschko, S., Loy, A., Lehner, A., Wagner, M., "The microbial community composition of a nitrifying-denitrifying activated sludge from an industrial sewage treatment plant analyzed by the full-cycle rRNA approach", *Systematic and Applied Microbiology*, Vol.25, pp. 84-99, 2002.

Kampfer, P., Erhart, R., Beimfohr, C., Bohringer, J., Wagner, M., Amann, R., "Characterization of Bacterial Communities from Activated Sludge: Culture-Dependent Numerical Identification Versus In Situ Identification Using Group- and Genus-Specific rRNA-Targeted Oligonucleotide Probes" *Microbial Ecology*, Vol. 32(2), pp. 101-121, 1996.

Kanagawa, T., Kamagata, Y., Aruga, S., Kohno, T., Horn, M., Wagner, M., "Phylogenetic analysis of and oligonucleotide probe development for eikelboom type 021N filamentous bacteria isolated from bulking activated sludge", *Applied and Environmental Microbiology*, Vol. 66(11), pp. 5043-5052, 2000.

Lange, J. L., Thorne, P. S., Lynch, N., "Application of flow cytometry and fluorescent in situ hybridization for assessment of exposures to airborne bacteria", *Applied and Environmental Microbiology*, Vol. 63(4), pp.1557-1563, 1997.

LaPara, T. M., Nakatsu, C. H., Pantea, L., Alleman J. E., "Phylogenetic analysis of bacterial communities in mesophilic and thermophilic bioreactors treating pharmaceutical wastewater", *Applied and Environmental Microbiology*, Vol. 66(9), pp. 3951-3959, 2000.

Laubenberger, G. and Hartmann, L., "Physical Structure of Activated Sludge in Aerobic Stabilization," *Water Research*, Vol. 5, pp. 335-341, 1971.

Layton, A. C., Karanth, P. N., Lajoie, C. A., Meyers, A. J., Gregory I. R., Stapleton, R. D., Taylor, D. E., Sayler, G. S., "Quantification of Hyphomicrobium populations in activated sludge from an industrial wastewater treatment system as determined by 16S rRNA analysis", *Applied and Environmental Microbiology*, Vol. 66(3), pp. 1167-1174, 2000.

Levine, W. G., "Metabolism of azo dyes: implication for detoxication and activation", *Drug Metab. Rev.*, Vol. 23(3-4), pp. 253-309, 1991.

Lipski, A., Friedrich, U., Altendorf, K., "Application of rRNA-targeted oligonucleotide probes in biotechnology", *Applied Microbiology and Biotechnology*, Vol. 56(1-2), pp. 40-57, 2001.

Loy, A., Horn, M., Wagner, M., "probeBase: an online resource for rRNA-targeted oligonucleotide probes" *Nucleic Acids Research*, Vol. 31(1), pp. 514-516, 2003.

Ludwig, W., Schleifer, K. H., "Bacterial phylogeny based on 16S and 23S rRNA sequence analysis", *FEMS Microbiological Review*, Vol. 15(2-3), pp. 155-173,1994.

Maidak, B. L., Olsen, G.J., Larsen, N., Overbeek, R., McCaughey, M. J., Woese, C. R. , "The RDP (Ribosomal Database Project)", *Nucleic Acids Research*, Vol. 25(1), pp. 109-111, 1997.

Manz, W., Wagner, M., Amann, R., and Schleifer, K. H., "In situ characterization of the microbial consortia active in two wastewater treatment plants", *Water Resources*, Vol. 28, pp. 1715-1723, 1994.

Manz, W., Amann, R., Ludwig, W., Vancanneyt, M., Schleifer, K. H., "Application of a suite of 16S rRNA-specific oligonucleotide probes designed to investigate bacteria of the phylum cytophaga-flavobacter-bacteroides in the natural environment", *Microbiology*, Vol. 142(5), pp.1097-1106, 1996.

Masselli, J. L., Masselli, N. W., and Burfordi, M. G., *A simplification of textile Waste Survey Treatment*, New England Interstate Water Pollution Control Commission, 1959.

Mobarry, B. K., Wagner, M., Urbain, V., Rittmann, B. E., Stahl, D. A., "Phylogenetic probes for analyzing abundance and spatial organization of nitrifying bacteria", *Applied and Environmental Microbiology*, Vol. 62(6), pp.2156-2162, 1996.

Moter, A., Gobel, U. B., "Fluorescence in situ hybridization (FISH) for direct visualization of microorganisms", *Journal of Microbiological Methods*, Vol. 41(2), pp. 85-112, 2000.

Mudaly, D. D., Atkinson, B. W., Bux, F., "16S rRNA in situ probing for the determination of the family level community structure implicated in enhanced biological nutrient removal" *Water Science and Technology*, Vol. 43(1), pp. 91-98, 2001.

Nemerow, N. L., *Industrial Water pollution: Origins , Characteristics, and Treatment*, Addison-Wesley Publishing Company, Inc., 1978.

Nitrate Reduction, http://www.cdc.gov/ncidod/dastlr/gcdir/NeIdent/Nitrate.html, 2003.

Oerther, D. B., Pernthaler, J., Schramm, A., Amann, R., Raskin, L., "Monitoring precursor 16S rRNAs of Acinetobacter spp. in activated sludge wastewater treatment systems", Applied and Environmental Microbiology, Vol. 66(5), pp. 2154-2165, 2000.

Oxidase test, 2003, http://www.rlc.dcccd.edu/mathsci/reynolds/micro/lab_manual/oxidase.html,

Oxidase test, 2003, http://medic.med.uth.tmc.edu/path/oxidase.htm,

Painter, H. A., "A Review of Literature on Inorganic Nitrogen Metabolism in Microorganisms," *Water Research*, Vol. 4, pp. 393-450, 1970.

Peng, Y., Gao, C., Wang, S., Ozaki, M., Takigawa, A., "Non-filamentous sludge bulking caused by a deficiency of nitrogen in industrial wastewater treatment", *Water Science and Technology*, Vol. 47(11), pp. 289-295, 2003.

Phase Contrast Microscopy, 2003, http://www.ruf.rice.edu/~bioslabs/methods/microscopy/phase.html.

Plumb, J. J., Bell, J., Stuckey, D. C., "Microbial populations associated with treatment of an industrial dye effluent in an anaerobic baffled reactor" , *Applied and Environmental Microbiology*, Vol. 67(7), pp. 3226-3235, 2001.

Prescott, L. M., Harley, J. P., Klein D. A., *Microbiology*,fifth edition, Mc Graw Hill, New York, 2002.

Reife, A. and Freeman, S., *Environmental Chemistry of Dyes and Pigments*, John Wiley&Sons, Inc., Canada, 1996.

Roller, C., Wagner, M., Amann, R., Ludwig, W., Schleifer, K. H., "In situ probing of gram-positive bacteria with high DNA G-C content using 23S rRNA-targeted oligonucleotides" *Microbiology*, Vol. 140, pp. 2849-2858, 1994.

Schaechter, M., Maaloe, O., Kjeldgaard, N. O., "Dependency on medium and temperature of cell size and chemical composition during balanced grown of Salmonella typhimurium", *J. Gen. Microbiology*, Vol. 19(3), pp. 592-606, 1958.

Schramm, A., Santegoeds, C. M., Nielsen H. K., Ploug, H., Wagner, M., Pribyl, M., Wanner, J., Amann, R., de Beer, D., "On the occurrence of anoxic microniches, denitrification, and sulfate reduction in aerated activated sludge", *Applied and Environmental Microbiology*, Vol. 65(9), pp. 4189-4196, 1999.

Schramm, A., and Amann, R., "*Nucleic acid-based techniques for analyzing the diversity, structure, and dynamics of microbial communities in wastewater treatment*", In Biotechnology: a multi volume comprehensive treatise, H.-J. Rehm, G. Reed, A. Pühler, and P.I.W. Stadler (eds.), Environmental Processes I, J. Winter (ed.), Vol. 11a, pp. 85-108. Wiley-VCH Verlag GmbH, Weinheim, Germany, 1999.

Schuppler, M., Wagner, M., Schon, G., Gobel U. B., "In situ identification of nocardioform actinomycetes in activated sludge using fluorescent rRNA-targeted oligonucleotide probes", *Microbiology*, Vol. 144(1), pp. 249-259, 1998.

Schweitzer, B., Huber, I., Amann, R., Ludwig, W., Simon, M., "Alpha- and beta-Proteobacteria control the consumption and release of amino acids on lake snow aggregates" *Applied and Environmental Microbiology*, Vol. 67(2), pp.632-645, 2001.

Siebert, M. L. and Toerien, D. F., "The Proteolytic Bacteria Present in the Aerobic Digestion of Raw Sewage Sludge," *Water Research*, Vol. 3, pp. 241-250, 1969.

Snaidr, J., Amann, R., Huber, I., Ludwig, W., Schleifer, K. H., " Phylogenetic analysis and in situ identification of bacteria in activated sludge" *Applied and Environmental Microbiology*, Vol. 63(7):2884-2896, 1997.

Spellman, F. R., *Microbiology for Water/Wastewater Operators*, Ph.D., Lancaster, PA: Technomic Publishing Co. Inc., 1997.

Stackebrandt, E., and Goebel, B. M., "Taxonomic note: a place for DNA-DNA reassociation and 16S rRNA sequence analysis in the present species definition in bacteriology", *International Journal of Systematic Bacteriology*, Vol. 44, pp. 846-849, 1994.

Staley, J. T., Konopka, A., "Measurement of in situ activities of nonphotosynthetic microorganisms in aquatic and terrestrial habitats", *Annual Review of Microbiology*, Vol. 39, pp. 321-346, 1985.

Toerien, D. F., "Direct-Isolation Studies on the Aerobic and Facultative Anaerobic Bacteria Flora of Anaerobic Digesters Receiving Raw Sewage Sludge", *Water Research*, Vol. 1, pp. 55-59, 1967.

Toerien, D. F., "Population Description of the Non-methanogenic Phase of Anaerobic Digestion-I. Isolation characterization and identification of Numerically Important Bacteria," *Water Research*, Vol. 4, pp. 129-148, 1970.

Tchobanoglous, G., Burton, F. L., Stensel H. D., Metcalf&Eddy, Inc., *Wastewater Engineering Treatment*, and Reuse, pp. 661-663, Mc Graw Hill, pp. 661-663, New York, 2003.

Wagner, M., Amann, R., Lemmer, H., Schleifer, K. H., "Probing activated sludge with oligonucleotides specific for proteobacteria: inadequacy of culture-dependent methods for describing microbial community structure", *Applied and Environmental Microbiology*, Vol. 59(5), pp.1520-1525, 1993.

Wagner, M., Assmus, B., Hartmann, A., Hutzler, P., Amann, R., "In situ analysis of microbial consortia in activated sludge using fluorescently labelled, rRNA-targeted oligonucleotide probes and confocal scanning laser microscopy", *Journal of Microscopy*, Vol. 176, pp.181-187, 1994.

Wagner, M., Rath G., Amann R. , Koops H. P., and Schleifer K. H. "In situ identification of ammonia-oxidizing bacteria", *Systematic and Applied Microbiology*, Vol. 18, pp. 251-264, 1995.

Wagner, M., Lay, A., Nagueira, R., Purkhald, U., Lee, N., and Daims, H. "Microbial community composition and function in wastewater treatment plants", *Microbial Ecology Group*, Vol. 81, pp. 665-680, 2002.

Wagner, M., Loy, A., "Bacterial community composition and function in sewage treatment systems", *Current Opinion in Biotechnology*, Vol. 13(3), pp. 218-227, 2002.

Wagner, M., Horn, M., Daims, H., "Fluorescence in situ hybridisation for the identification and characterisation of prokaryotes", *Current Opinion in Biotechnology*, Vol. 6(3), pp. 302-309, 2003.

Wallner, G., Erhart, R., Amann, R., "Flow cytometric analysis of activated sludge with rRNA-targeted probes", *Applied and Environmental Microbiology*, Vol. 61(5), pp. 1859-1866, 1995.

Watson, S. W., Bock, E., Valois, F. W., Waterbury, J. B., and Schlosser, U., "Nitrospira marina gen. nov. sp. nov.: a chemolithotrophic nitrite-oxidizing bacterium", *Archives of Microbiology*, Vol. 144, pp. 1-7, 1986.

Wisconsin, Department of Natural Resources, Activated sludge- microbiology and process control, 2003, http://www.dnr.state.wi.us/org/water/wm/ww/tech/asludge.htm.

Whiteley, A. S., Bailey, M. J., "Bacterial community structure and physiological state within an industrial phenol bioremediation system", *Applied and Environmental Microbiology*, Vol. 66(6), pp. 2400-2407, 2000.

Woese C.R., "Bacterial evolution", *Microbiol Rev.*, Vol. 51(2), pp. 221-271, 1987.

Zissi, U., and Lyberatos. G. "Azo-dye biodegradation under anoxic conditions" *Water Science and Technology*, Vol.34, pp. 495-500, 1996.