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Isolation and characterization of the genomic variability in activated-sludge: a comparative analysis between bacterial isolates and operation parameters

Dissertação de Mestrado em Genética Molecular

Trabalho efectuado sob orientação da
Doutora Ana Nicolau

Trabalho efectuado sob co-orientação de
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Abril de 2012

Declaração

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Título da tese de mestrado:
Isolation and characterization of the genomic variability in activated-sludge: a comparative analysis between bacterial isolates and operation parameters

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Ano de conclusão: 2013

Designação do Mestrado ou do Ramo de Conhecimento do Doutoramento:
Ciências - Mestrado em Genética Molecular

1. É AUTORIZADA A REPRODUÇÃO INTEGRAL DESTA TESE/TRABALHO APENAS PARA EFEITOS DE INVESTIGAÇÃO, MEDIANTE DECLARAÇÃO ESCRITA DO INTERESSADO, QUE A TAL SE COMPROMETE;

Universidade do Minho, 30 de Abril de 2012
Assinatura:_____

The present work acknowledges the Project “PROTOFILWW – Establishment of relationships between protozoa, metazoa and filamentous bacteria of activated sludge and physical-chemical and operational parameters of plants” (PTDC/AMB/68393/2006) supported by the Foundation for Science and Technology (FCT).

Abstract

Isolation and characterization of the genomic variability in activated-sludge: a comparative analysis between bacterial isolates and operation parameters

Activated-sludge is one of the most important biotechnological processes of our times supported by a mixture and variable set of micro and macro organisms that, in complex association, are able to remove and/or transform not only particulate pollutants but also particles dissolved in the mix. Bacteria play an essential role in these transformations that are carried mainly in aerobic conditions.

As in any other ecosystem, the microbiological community of activated-sludge is determined by the operational and physical-chemical variables prevalent in the aerated tank of these systems. Over the years, wastewater treatment was engineered with none or little knowledge about microorganisms, being the main information on the process provided by chemical and physical analyses. The difficulty of identifying the prevailing microorganisms, especially the bacteria, has been one of the reasons for its withdrawal. Molecular methods brought some advantages to this scenario enabling the identification of the prokaryotic microorganisms.

The aim of the present project was the study of the prokaryotic community of 8 wastewater treatment plants (WWTP) in the south region of Portugal, using molecular and bioinformatic approaches. A polymerase chain reation (PCR) was carried on and the primer M13 was chosen to discriminate the bacterial isolates previously obtained from the samples. The software Bionumerics was used to analyse the data building a dendrogram of the isolates based on the genetic profile and enabling the subsequent analyses of the relations between the microorganisms and the physical-chemical and operational parameters of the WWTP. This work is a exploratory work and, to the knowledge of the team, it was never done before.

The results showed a tendency for an aggregation of the microrgamisms of only one of the studied WWTP. In fact, the isolates that showed the highest similarity belong to that plant. The other isolates do not seem to show any pattern of similarity, probably indicating low variability among the remaining systems. This can be due to the fact that all the studied WWTP came from one limited geographic region and are explored by one enterprise. The present results show some interesting clues about the potentialities of these techniques to be use in the project PROTOFILWW that aimed at studying thirty-seven WWTP, all over the country over two years.

In conclusion, molecular techniques together with bioinformatics can have a significant contribution to the study and comprehension of the complex communities of activated-sludge systems, namely the prokaryotic component.

Resumo

Isolamento e caracterização da variabilidade genómica nos processos de lamas activadas: uma análise comparativa entre os isolados bacterianos e os parâmetros de operação

O processo de lamas activadas é um dos mais importantes processos biotecnológicos dos nossos tempos e não é nada mais do que uma mistura e um conjunto variável de organismos que, numa complexa associação, são capazes de remover e/ou transformar os poluentes. As bactérias desempenham um papel essencial nestas transformações que ocorrem principalmente em condições aeróbias. Como em qualquer outro ecossistema, a comunidade microbiológica de lamas activadas é determinada pelas variáveis operacionais e físico-químicas prevalecentes no tanque de arejamento dos sistemas de tratamento. Ao longo dos anos, o tratamento das águas residuais foi levado a cabo sem ter em atenção os microrganismos que o levavam a cabo, tendo sido as principais informações para a sua monitorização fornecidas por análises químicas e físicas. A dificuldade em identificar os microrganismos que prevalecem nas lamas activadas, especialmente as bactérias, tem sido uma das razões para este facto. Os métodos moleculares vieram melhorar em grande parte este cenário ao permitir a identificação dos microorganismos procarióticos não distinguíveis por métodos microscópicos.

O objetivo do presente trabalho foi o estudo da comunidade procariótica de 8 estações de tratamento de águas residuais (ETAR) na região sul de Portugal, através de abordagens moleculares e bioinformática. Uma reacção de polimerização em cadeia (PCR) foi realizada, usando o primer M13, com o objetivo de discriminar as bactérias isoladas a partir das amostras dessas ETAR. O software Bionumerics foi utilizado para analisar os dados e para a construção de um dendrograma com base no perfil genético, permitindo análises posteriores das relações entre os microorganismos e os parâmetros físico-químicos e operacionais das ETAR. O presente trabalho é um trabalho exploratório, na medida em que não se conhece nenhum feito nos mesmos moldes em ETAR.

Os resultados mostraram uma tendência para agregação dos microrganismos de apenas uma das ETAR estudadas. De facto, os isolados que mostraram mais semelhança entre si pertencem a esta ETAR. Os demais isolados não parecem mostrar qualquer padrão de similaridade, provavelmente indicando baixa variabilidade entre as ETAR. Este facto pode ocorrer porque as ETAR estudadas se encontram numa região geográfica limitada e são exploradas pela mesma empresa. Os resultados demonstram algumas pistas interessantes sobre as potencialidades desta técnica que pode ser explorada no projeto PROTOFILWW que teve como objetivo estudar 37 ETAR em todo o país durante dois anos.

Em conclusão, as técnicas moleculares, juntamente com a bioinformática, podem ter uma contribuição significativa no estudo e na compreensão das complexas comunidades de lamas ativadas, nomeadamente a componente procariótica.

Acknowledgement/Agradecimentos

Porque nada na vida se faz sem ajuda, agradeço:

À Professora Doutora Ana Nicolau por ter aceitado a orientação da minha tese, especialmente por o ter feito nas condições em que fez. Por mais anos que viva e por mais voltes que a vida dê, jamais esquecerei o que ela fez por mim. Mais do que uma tese de mestrado ela permitiu que eu terminasse os meus estudos depois de tanto esforço e dedicação. Deixo aqui um agradecimento também à Doutora Rosário por ter encontrado a Doutora Ana e lhe ter explicado a situação.

Agradeço também à Marta Neto por tudo. Por me acompanhar em todas as etapas. Ensinou-me sempre com paciência e atenção vezes e vezes sem conta. Não podia ter tido melhor pessoa a meu lado. Obrigada por tudo, Marta.

Agradeço à Liliana, à Vânia e aos restantes colegas de laboratório por me ajudarem sempre e por criarem um excelente ambiente a cada dia. Dava gosto trabalhar lá.

Ao Pedro Lopes deixo aqui um agradecimento especial por ter sido o meu grande apoio neste momento. Sem ti não teria conseguido. Obrigada por me ajudares com tudo. Por leres, por ajudares com o vocabulário, formatação etc, obrigada por te preocupares comigo, por acreditares em mim. Obrigada pela força, obrigada por me acalmares, obrigada por simplesmente me abraçares quando eu mais precisei, obrigada pelo carinho. Desculpa as noites mal dormidas, o mau humor que aturaste, o stresse, o tempo trancado em casa e a falta deste para tudo… Prometo que te compenso. Agradeço também à tua família o carinho com que me acolheu.

À Sónia Duarte que juntamente com o Pedro foi, também ela, o meu grande apoio. Obrigada Soninha por sempre te preocupares comigo, por sempre me animares, por me tirares de casa, por me telefonares quase todos os dias só para saber se estou bem. Muito obrigada por seres minha amiga.

Obrigada à Paula e à madrinha Cândida por estarem sempre presentes e acreditarem em mim.

Obrigada à Filipa por me mostrar que não importa o tempo, a distância, ou as zangas que temos na vida. Quando somos amigos, corremos atrás e estamos lá quando e preciso. Jamais esquecerei.

Agradeço à Edite por ter estado presente e ao Pedro Rodrigues pelas horas que passou a dizer que eu havia de conseguir. Agradeço também à Marlene pelo apoio especialmente nesta fase final, agradeço também ao Flávio a amizade sempre demonstrada e ao Nuno Sousa as conversas e as idas ao Porto.

Agradeço à Isabel a amizade para além do tempo e por sempre torcer por mim. Falta pouco para entregar a tua tese Belita, ficarei muito feliz nesse dia.

Por último mas não menos importante, agradeço a Casa Do Menino Jesus por me terem educado e por me terem ensinado a importância de estudar. Obrigada pelo apoio e por terem permito que estudasse. Hoje estou pronta para assumir as rédeas da minha vida.

Obrigada a todos que não mencionei mas que de um modo ou de outro estiveram presentes na minha vida e no meu trabalho. Sozinha jamais teria conseguido. Muito obrigada.

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Symbols and Abbreviations

% - percentage

µl – microliter

ARDRA - Amplified ribosomal DNA restriction analysis

BOD - Biochemical oxygen demand

CO_2 - Carbon dioxide

CSLM - confocal scaning laser microscopy

DGGE - Denaturing gradient gel electrophoresis

DNA - DesoxiribonucleicAcid

EDTA - Ethylenediamine tetraacetic acid

FISH - Fluorescent in situ hybridization

H_2O - water

L - Liter

Mg - Magnesium

mg - Milligram

ml - Milliliter

NH_4 - ammonium

NO_2 - Nitrogen Oxide

O_2 - Oxygen

ºC - Celsius degree

PCR - Polymerase chain reaction

pH - Potential of Hydrogen

RISA - Ribosomal RNA intergenic spacer analysis

RNA - RibonucleicAcid

Rpm - rotations for minute

SDS - Sodium Dodecil Phospate

TAE - Tris base, acetic acid and EDTA.

TE - Tris-EDTA

TSA - Tryptone Soy Agar

TSB - Tryptic Soy Broth

UV- Ultra Violet

WWTP - Waste Water Treatment Plant

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CHAPTER I

Background and Objectives

1) Background and Objectives

This work part of the project PROTOFILWW (PTDC/AMB/68393/2006) funded by the Foundation for Science and Technology (FCT) entitled PROTOFILWW - Establishment of relationships between protozoa, metazoa and filamentous bacteria of activated sludge and physicochemical and operational parameters of plants. All samples in this work came from this project.

This part of the project was elaborated with the intention of isolating microorganisms from activated sludge samples from various wastewater treatment plants (WWTP). Then, the relation between the microorganisms and the WWTP characteristics would be made in achieve a better understanding of what determines the prokaryotic community of activated-sludge systems. To accomplish this, polymerase chain reaction PCR with the M13 primer was applied to the isolates previously made. Then, there was a verification of the strand patterns in an agarose gel followed by an analysis ran by a computer program in order to group and correlate the isolates, whether this is with each other or with the physical and chemical parameters of the wastewater treatment plants.

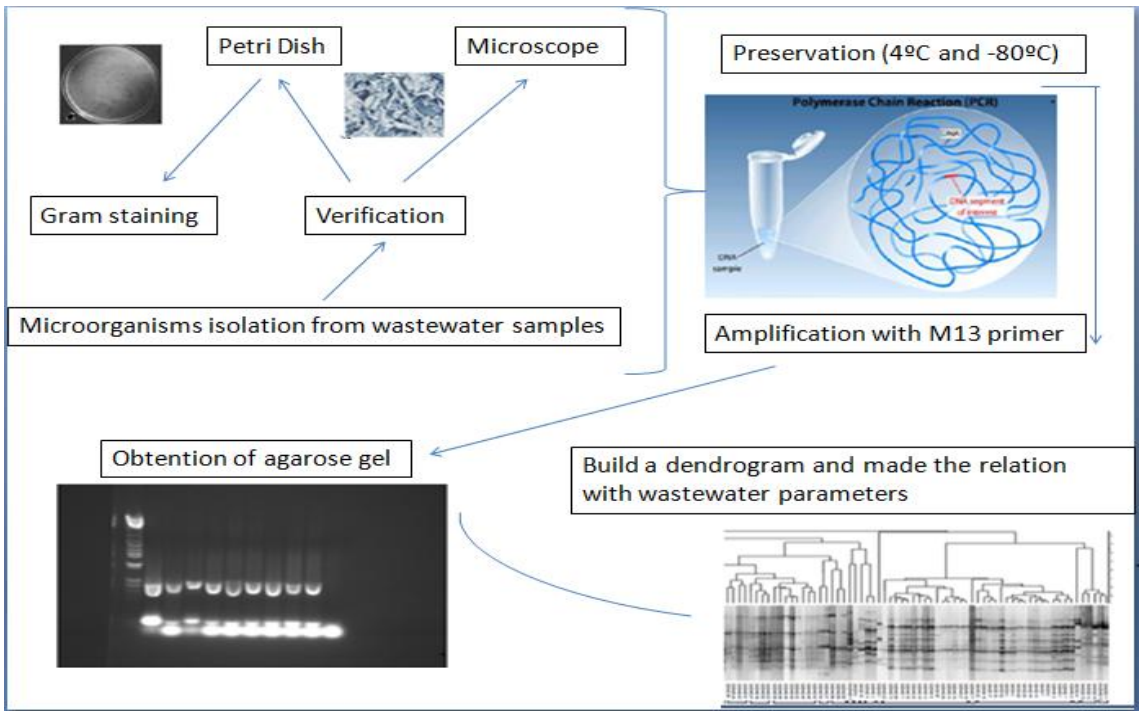

Figure 1: **Goals of this work**

CHAPTER II

Introduction

2.1) General Introduction

Water is one of the most valuable resources in the planet and, when people started to realize that it was becoming polluted, they began trying to clean it up. In the past, people have tried to create their own sewage systems (Lens *et al*., 2004) but it was only in the nineteenth century (1820 to 1850) that people started to understand that some diseases can infect humans through contaminated water (Chartered Institute Environmental Health, 1998). The treatment of water originated from the need to reduce human disease, followed by the environmental issues and finally because pure water is needed for human activities (Vesilind, 1998). It also finally came to light that the water cannot just be deposited into the sea or any other course of water. The technology to treat water exists and, in most cases, enables the direct re-introduction into its natural cycle or even the re-use of water to replace potable water in domestic use, such as toilet water supply. Microorganisms play the fundamental role in the majority of urban wastewater treatment systems. Although, over the years, wastewater treatment was engineered with none or little knowledge about microorganisms, it is important to understand which microorganisms exist and in what quantities in order to see what works better in these treatment systems. The main information was provided from chemical and physical parameters. Not surprisingly, proliferation of some microorganisms with undesirable effects, causing settling problems like bulking and foaming, often grow in the aeration tank of wastetwater treatment plants. Besides, the existence of pathogenic microorganisms in the final effluent can be a threat to public and environmental health (Gilbride *et al*., 2006).

2.2) Residual Water

Residual water is one of the many residuals that human beings produce every day, individually or as a group for industrial, agricultural or individual purposes. The residual water is easily identified because of its characteristic smell due to its provenience which is a mix of domestic waters, sanitary waters, urban waters infiltration water and water seepage. Its aspect resembles a much diluted suspension of different materials (Prescott *et al*., 2005). In this residual water, carcinogenic and/or mutagenic substances, such as toxic compounds, can exist, being able to cause serious disturbances to the ecosystems and to human health (Metcalf and Eddy, 2003). In table 1, physical and chemical characteristics of residual water are shown

It is not possible to estimate the volume of residual water produced *per capita* since this number depends on the referred country and also depends on the water availability and of the level and quality of life of the population (Water UK, 2006).

Table 1: Characteristics of residual water (Adapted from Tandoi *et al*, 2006)

Physical	**Chemical**			**Microbiologic**
Odor	Organic	Inorganic	Gases	Microbiological characteristics will be described above in section 1.7
Temperature	Proteins	Ph	Oxygen	
Suspended solids	Carbohydrates	Chloride	Hydrogen	
	Lipids	Alkalinity	Sulphide	
	Surfactants	Nitrogen	Methane	
	Phenols	Phosphorus		
	Pesticides	Heavy metals		
		Toxic materials		

The characteristic look of residual water is due to physical characteristics. The smell is due to two main groups of chemical substances: nitrogen and sulfur compounds, such as mines, ammonia, diamines, and skatole and, in a minor extension, to chlorine and phenol compounds like hydrogen sulfide, mercaptans, organic sulfide and sulfur dioxide. The size of suspended particles that float in water varies between 1µl and distinguishable organic matter.

2.3) Importance of the Water Quality to the Receptor Ecosystems

Organic material, such as food waste or fecal matter and other biological material, is naturally degradable in the rivers and in the sea. Bacteria and other microorganisms are responsible by this clean up; in order to do that, microorganisms need to use dissolved oxygen to break it down in the respiration process. If the pollution is too much, the consequences can be irreversible. For instance, organic compounds will serve as food for the bacteria which, in turn, will use most of the available oxygen

killing the aquatic animal and plant life. The components released as a result of bacterial activity and organism death, such as phosphorus and nitrogen, can lead to a huge growth of green algae and cyanobacteria which produce toxic products (Codd, 1995). This will cause a domino effect and directly it will be difficult for other animals to survive with little oxygen. Besides organic waste, another big issue is the chemicals used in modern life including heavy metals which are not biodegradable and may accumulate in river sediments or worst in fish and plants. These toxic compounds came from industrial and domestic sources, and can be toxic to animals and humans (Water UK, 2006).

If the water goes through the appropriate treatment, the same water can reenter in its normal cycle without harmful consequences. In fact, the objective of water treatment is the removal of unwanted components in wastewaters providing a safe discharge into the environment. This is not simple but can be made by physical, chemical and biological means, either alone or in combination (Cooper, 2004).

2.4) WWTP (Waste Water Treatment Plant)

The biological wastewater treatment is one of the most important biotechnological processes of our times and differs from the conventional biotechnological process because it does not require pure cultures or controlled aerobic fermentations of economically important metabolites. Its importance can be seen when it is taken into account that this process has been used for over a century (Gray, 1990; Matsui *et al.*, 1991).

In WWTP, an artificial ecosystem is built, consisting in one abiotic component (the plant structure and the sewage) and the biotic component comprising the living organisms such as the bacteria, the fungi, the protozoa and the little metazoan, the latter feeding on the bacteria inhabiting the same mixture. The bacteria extract the energy necessary for their metabolism from organic matter and from the oxygen that enters within wastewater (Madoni *et al.*, 1993): as a result, at the same time as new biomass is produced, soluble organic material is removed from the waste treatment (Bonde, 1977). The engineering of this system is almost perfect because it gives the microbes all the nutrients and necessary oxygen and maintains them in intimate contact. In this way, most of the time, water with high degree of purity is obtained (Hawkes, 1983; Megank and Faup, 1988; Seviour and Blackall, 1999). In the end only biomass, carbon dioxide

and water would be obtained. Of course, its main product is the excess of sludge consisting of microbial biomass (Rocher *et al*., 1999).

2.4.1) Treatment of Water in WWTP

First, coarse solids and oils are removed with the goal of preventing the equipment from clogging. This is called preliminary treatment.

In the primary treatment, screens and sedimentation tanks are used with the aim of removing a significant proportion of the suspended solids.

The secondary treatment comprises the removal of soluble organic matter by bacteria in the aerated tank of activated-sludge systems. Oxygen is provided and flocculation of the biomass is favored to enable subsequent separation from the liquid fraction in the secondary sedimentation tank.

If the tertiary treatment is used, recalcitrant organic compounds can be removed as well as excessive nutrients like nitrogen and phosphorus and finally eventual pathogens, generally using physical and/or chemical treatments. The result is the reduction of BOD, nutrients, pathogens and toxic substances (Cooper, 2004). Figure 1 is a scheme of the operation of a WWTP system.

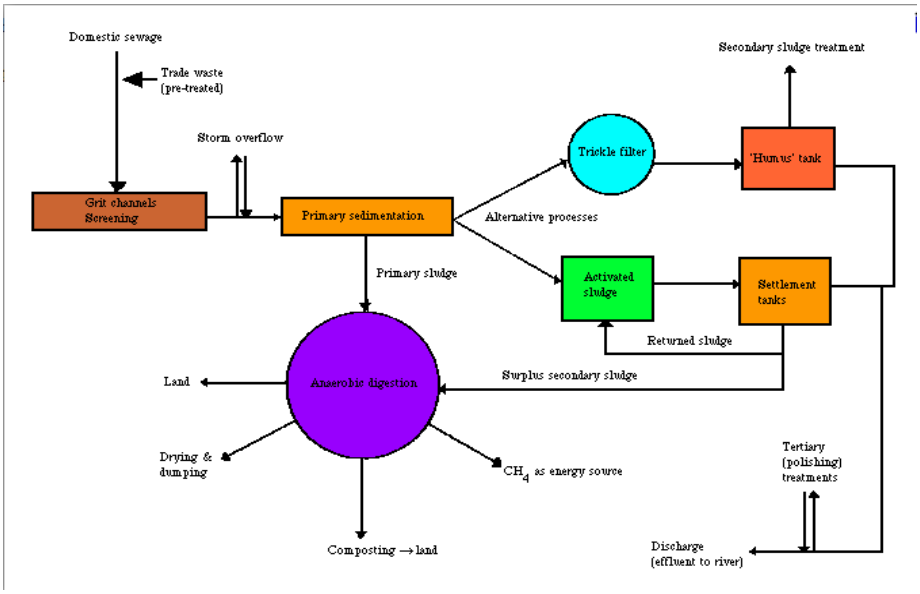

Figure 2: Waste Water Treatment Plant

Available in <http://weather.nmsu.edu/Teaching_Material/SOIL350/waste_water_treatment_plant.htm>, acessed in April, 1.

2.5) Activated Sludge

Activated Sludge is nothing more than a mixed and variable set of micro and macro organisms in one complex association that are able to remove and/or transform not only particulate pollutants but also particles that remain dissolute in the mix. This is mainly operated by bacteria present in flocs under aerobic conditions (Lens and Stuetz, 2004).

Activated sludge needs to deal with a diversity of organic and inorganic compounds with irregularities of the system and the microorganisms need enough time to metabolize the biodegradable compounds (Painter, 1983).

In summary, this is an operation developing through two steps: first, the biomass removes the soluble organic matter with the help of the oxygen provided through several ways in the aeration tank and after that, the separation of the liquid portion in the secondary sedimentation is achieved (Painter, 1978).

The objectives of the activated sludge treatment are:

- the reduction of the sludge volume reducing this way its fermentation capacity which leads to a better smell and to a diminution of pathogenic microorganisms in the sludge;
- the removal of soluble organic matter of the wastewater so it cannot cause any important damage to receptor ecosystems;
- the removal of the substances that have a demand for oxygen from the system like nitrogen and phosphorus in order to make sure that photosynthetic organisms in receiving waters stay with their growth limited.

A short way to resume how an activated sludge system works is mentioning the essential factors of its operation: it needs suspended biomass, oxygen (1 - 2 mg/l, ideally) and posterior separation by gravity (figure 2). The retention time varies with the effluent characteristics and with the desired depuration degree (Santana *et. al*., 2009).

On the other hand, sludge production depends on several factors like the degradability of organic compounds, mass loading of the treatment plant (Eckenfelder, 1978), cellular lyses (Hamer, 1984) or deregulation of the ecosystem for instance, with excessive growth of the bacteria grazers (Lee and Welander, 1996).

The biomass is organized into a discrete spatial entity. In the aerated tank, constant aeration and suspension provided by the agitation or by the bubbles rising from

diffusers in the basin floor is needed (Hawkes, 1983). The mixing also enables that the microbes stay in intimate contact and grow in a three-dimensional way in order to form flocs. These flocs shall have settling properties that allow an easy separation from the liquid mixing (Frey, 1992). This way of operation has not actually changed much in one century: from the beginning, most of the aerobic reactors consisted of a rectangular basin with submersed mechanical diffusers or mechanical surface agitators (Gray *et al.*, 1999).

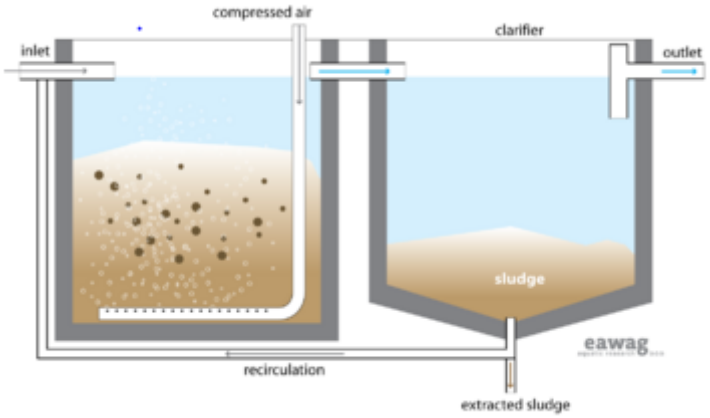

Figure 3: Mix of activated sludge and posterior separation by gravity

Availabel in <http://www.akvo.org/wiki/index.php/Activated_Sludge>, acessed in April, 1.

Aeration allows the continuous entrance of oxygen which is indispensable so nutrients can be oxidized, enabling the growth of biomass in size and cell number and the non-soluble particles being incorporated in the flocs (Wanner, 1994). The resultant water after aeration contains low content of dissolved organic compounds but has a lot of suspended solids that will be removed in a secondary decanter (Santana *et. al.*, 2009).

Then, a part of the solids separated from the liquid by gravity and the biomass, now enriched with microbes, can be recycled and used to re-inoculate the incoming raw sewage. Some of this mass is wasted in determined time intervals because of the age of the sludge since, over time the sludge becomes less and less efficient (Hansen *et al.*, 1993). If it is possible and the oxygen is enough, the activated sludge systems should have microbes capable of removing some compounds like nitrogen and phosphorus because of its toxicology (Megank and Faup, 1988). In the end of the second stage, there was a reduction of the biochemical oxygen demand (BOD), suspended solids and, of course, if everything goes according to plan, a huge reduction in toxicity and a low concentration of nutrients (Sahlstrom *et al.*, 2004).

In the end, as a result of the flocculation process, a mixture of organic and inorganic particles and live cells in a colloidal solution are obtained (Santana *et. al.*, 2009). It is impressive that all this process is carried out by resident microorganisms but, in the end, a reduction in the number of pathogenic organisms present is observed (Betancourt and Rose, 2004).

2.5.1) Problems Associated With the Process

Some problems affect the separation process of the solids in activated sludge. There is a double goal in the activated sludge system, one is to metabolize organic substances and the other is to form flocks that allow posterior filtration and elimination of the system (Nicolau, 2009). Some microorganisms promote some undesirable effects such as disperse growth, pin floc, bulking and foaming. The problem is that the floc does not compact correctly which causes problems in the following steps (Metcalf and Eddy, 2003; Wanner, 1994). There are two phases in floc formation: flocculation of bacterial cells due to extracellular polymers of a viscous nature and the formation of a filamentous skeleton. This latter is important because the flocks can increase the size and exhibit better resist mechanic aggression resulting from turbulence. This phase corresponds to the formation of the macrostructure (Nicolau, 2009). It is extremely important that the sludge that comes out of the aeration tank should be easily separated from the liquid phase. If the separation goes well and the compression goes correctly performed too, a good quality of the effluent is assured (Flores-Alsina *et al.*, 2009).

There are a lot of reasons that lead to problems in solids separation such as low dissolved oxygen content, oxygen demand (chemical and biological), nitrogen (nitrite and ammonia), phosphate and metals (heavy and trace), lack of nutrients, presence of septic waters, low food/microorganisms ratio, old sludge, configuration of the biologic reactor, temperature and pH. As a consequence, different phenomena can be observed:

- Dispersed growth: if there is a lack in exopolymer bridges, the microorganisms are free in the medium individually (Wanner, 1994; Larsdotter, 2006); other causes can be a high relation between monovalent cations/divalent cations (Higgins and Novak, 1997) and the presence of substances which decrease the tension between two biodegradable liquids (Bott and Love, 2002).
- Pin-point flock: sometimes, when the sludge is old, flocks are not exposed to exogenous metabolism. This occurs when bacteria form small flocs that, although small and round, have difficulty in sedimenting (Wanner, 1994). If

flock formation is not well succeeded, in the aeration tank total destruction of these kinds of flocks can occur (Wanner, 1994).

- Filament bulking: if filamentous organisms grow excessively in the system, they produce a diffused structure of the flock, interfering with sedimentation and with the compression of the sludge, leading to bad quality of the final products: the final effluent and the sludge (Jenkins *et al.*, 2004).
- *Bulking zoogleal*: if there is excessive zoogleal growth, because of the excessive growth of exocellular material, a viscous sludge is formed also leading to a bad quality of both final effluent (Wanner, 1994).
- Foaming: organisms like *Nocardia* and *Microthrix parvicella* have hydrophobic cells that are less dense than the water, accumulating in the surface of the aerated tank as a scum (Jenkins *et al.*, 2004).

2.5.2) Possible Solutions

In order to solve one or more of these problems, some adjustments can be made (Nicolau, 2009; MetCalf and Eddy, 2003).:

- Increase or decrease the dissolved oxygen;
- Variation in flux of the sewage;
- Adjustment in recirculation flow;
- Addition of chemical compounds in order to improve the flocculation and/or to decrease the amount of filamentous microorganisms;
- Addition of nutrients;
- pH alteration especially if it favors alkalinity.

2.6) Environmental Conditions in the WWTP

The environmental conditions that prevail in the aeration tank of the WWTP determine the microbial community in these systems. Some of the main characteristics are reflected by physical-chemical parameters, often determined in a routine way in the WWTP laboratories. There are six frequent parameters used to measure the quality of the WWTP. It is possible to start with the Biochemical Oxygen Demand (BOD) which is the quantity of oxygen consumed when organic matter is heated at 20ºC due to biological oxidation (Apha, 1995). Chemical Oxygen Demand (COD) is more or less the same as the previous parameter but it is faster to measure. It represents the quantity of oxygen needed to decompose organic matter using a chemical agent that is used with

the intent of replacing O_2 in the reaction in organic matter oxidation. This parameter is complete because it measures the total organic matter, not biodegradable and degradable, and the toxic substances, including bacteria and other microorganisms, that oxidize organic matter (Apha, 1995).

Suspended material can also be measured by two ways. Suspended Solid Total (*SST*) which is the solids that get retained after a filtration by glass fiber filters with a porosity of 0.45 mm, the percentage of this value is high which means a good operation in the system. As the quantity of SST lowers, the quality of the resulting effluent increases which suggests that the diversity lowers (Apha, 1995) and for Volatile Suspended solids (*SSV*) which is the approximation of the organic matter in the suspended solid fraction in the residual water expressed by the amount of the same that is incinerated at 550 ºC (Apha, 1995).

Maybe the most basic measure is pH which expresses the basicity or acidity of any solution which means that the concentration of hydrogen (H^+) in any solution varies in a scale between zero and fourteen at 25ºC. pH alterations have several causes such as the decomposition of organic matter. This decomposition creates carbon hydrates that will be used by microorganisms as food, liberating CO_2 and increasing the amount of H^+ in water which leads to a decrease in pH. A pH close to being neutral is better. Also, pH is a very important parameter in several stages of water treatment such as coagulation, disinfection, control of corrosion and removal of hardness (Wanner, 1994).

Finally, it is possible to measure the Oxygen. The quantity of oxygen in water indicates a normal operation of the system. If the rate of oxygen is low it means that microorganisms used all the oxygen present in the system and they started to do anaerobic respiration which generally shows an increase of pathogenic microorganisms. The tank needs to be aerated in order to solve this problem since in these systems, there are no plants that undergo photosynthesis so, it is normal that the demand of oxygen is higher than the maximum solubility of oxygen in water (approximately 0 to 19 mg/L in surface waters but a value of 5 to 6 mg/L is enough to support marine life). The increase on the tax of respiration of the microorganisms leads to a huge quantity of CO_2 and methane gas which results in a stampede of oxygen that presents a low solubility in water (Madoni, 1994).

Besides these parameters, nowadays the WWTP managers pay attention to the microbiological communities and try to use parameters that assess the overall state of

the community. The Sludge Biotic Index (SBI) is the most used microbiological parameter used in the WWTP.

SBI is a measure of the "health of the sludge" since it is an objective value based on an objective calculation. This value can illustrate the operational conditions of the WWTP and can be used to compare different WWTP or the performance of one WWTP along time, since the result is a numerical value. Anyway, the SBI only give us information about the aeration tank and not about the sedimentation tank performance. SBI is calculated using a two entry table; the right side has four classes relative to the number of microfauna taxa (excluding the flagellates and the Fuchs-Rosenthal chamber counting on flagellates, and in the left side there are the different dominant groups found founded in the samples and the total density of the samples.

The four classes proposed by Madoni are shown in table 3.

Table 2: SBI Classes (Madoni, 1994)

SBI Value	Class	Evaluation
8-10	I	Stable and well colonized Sludge; optimal biologic activity; high purifying efficiency
6-7	II	Stable and well colonized Sludge; sub-optimal biologic activity; sufficient purifying efficiency
4-5	III	Insufficient biologic activity; mediocre purifying efficiency
0-3	IV	Very low biologic activity; low purifying efficiency

This value, proposed by Madoni in 1994, is based on the specific diversity of the community as well as its abundance, and in different sensibilities revealed by that specific population to different physical chemical parameters (Santos, 2008).

There are a lot of advantages of using this method like the use of several simultaneous criteria such as the numeric and specific richness and the indicator concept (Santos, 2008).

2.7) Organisms Living in WWTP

There is a set of microorganisms living in it like bacteria, yeasts, fungi, algae, protozoa, metazoa, rotifers, larvae and insects (Oliveira, 1982) and, since this came from sewage, some of them are pathogenic organisms.

Since protozoa feed by active grazing on bacterial cells, the appear in large numbers in the system (Madoni, 1994). Some of protozoa are indicators of the good quality of water treatment (Madoni, 1994). Most protozoa inhabiting the aerating tank of WWTP are ciliate protozoa. The main function of ciliate protozoa is to be the predator of the system and they exist in large number (Curds, 1982). Also, flagellate can be in high numbers. These classes of protozoa possess small number of flagella which leads to the belief that movement is harder for them then for the previously mentioned organisms (Seviour and Blackall, 1999).

Fungi are not important members of the common activated sludge process. They cannot compete with bacteria unless in very low pH (Jenkins *et al.*, 1993).

Referring to metazoan, a large amount of these are nematodes, rotifers and oligochaete worms, and it is believed that they are bacteria grazers, although, their real role is not yet truly understood (Ratsak *et al.*, 1993).

In wastewater treatment, bacteria are the dominant group corresponding to 95% of total microbial population (Martins *et al.*, 2004), in both biomass and number, being also responsible for the most part of the mineralization and elimination of organic and inorganic compounds (Amann, 1998, Bitton, 1978).

Bacteria are unicellular prokaryotic organisms and can present different types of morphology: sphere (cocci) or cylinders (rods or bacilli), rigid blades (vibrios, spirillum) and flexible helices (spirochetes) (Lopes and Fonseca, 1996). Like all prokaryotic organisms, there is no nucleolus in the cells and the DNA is located in the cytoplasm like all the other cell components such as carbohydrates and other organic complexes that have Ribonucleic acid (RNA) and can synthesize their own proteins, its dimensions are located between 1 and 3μm in size and 1,5 μm of diameter (Metcalf and Eddy, 2003). The most common reproductive process is the binary division (Figure 3). In some cases, the daughter cells stays together which leads to the formation of a "chain" resulting in the formation of the filamentous forms of bacteria (Nicolau, 2009).

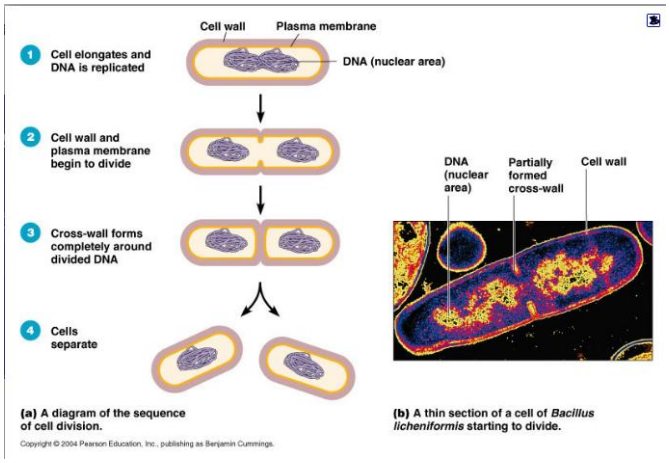

Figure 4: bacteria division

Available in <http://science.nayland.school.nz/graemeb/yr11%20work/microbes/bacteria.htm>, acessed in April, 1.

This group includes many fecal commensally bacteria but also pathogenic bacteria (Grant *et al*., 1996).

2.7.1) Metabolism of Bacteria

Bacteria are without a doubt very important in chemical changes that occur like the metabolizing process of the wide diversity of organic compounds present and, in some advanced plants, bacteria are responsible for the removal of nitrogen and phosphorus (Seviour and Blackall, 1999); in fact, 91% of all organisms that exist in activated sludge are bacteria (Nicolau, 2009). In the end, bacteria convert organic and inorganic nutrients into bacterial cells and inorganic products such as carbon dioxide, water, ammonia and phosphate (Copper, 2004).

Considering the metabolism of bacteria, they are classified according to the energy source or the carbon source that they use in the conversion of the substrate (Nicolau, 2009).

Chemoheterotrophs make up most of the bacteria present in activated sludge systems. They are aerobic and are responsible for the degradation and utilization of organic compounds which later turn in cell biomass and CO_2 (Painter, 1983).

Chemoautotrophic nitrifying bacteria are the de-nitrificants by excellence (Robertson and Kuenen, 1992). Nitroso bacteria oxidize NH_4^+ into NO_2^- and Nitro bacteria oxidize NO_2^- into NO_3^- (Bock *et al.,* 1992). Their growth rates are low and the energy they release is also low which means that they can be quickly washed out of the activated sludge system (Painter 1986).

Photoautotrophic and photoheteretrophic bacteria are purple and metabolically versatile and can denitrify (Hiraoshi *et al*., 1995).

Table 3: Metabolic groups in activated sludge (Nicolau, 2009)

Metabolic Group	Carbon Source	Energy Source	Electron Acceptor	Growth Structure
Organotrophs	Organic	Aerobic Oxidation	O_2	FF,FIL
Fermentative Anaerobes	Organic	Fermentation	Organic Compound	FF
Denitrifiers	Organic	Anoxic Oxidation	$NO_3 - N$	FF,FIL
Nitrifiers	Inorganic	Aerobic Oxidation	O_2	Adhered
Poli-P	Organic	Aerobic Oxidation	O_2	Clusters,FIL
S-oxidizers	Inorganic	Aerobic Oxidation	O_2	FF,FIL
SO_4**-Reducers**	Organic	Anaerobic Oxidation	$SO_4 - S$	FF

In the treatment systems, carbon is the main energy source, so, the dominant microorganisms are the ones responsible for the metabolism of carbonate compounds. There are various carbon sources available for organisms, so, its characterization is measured by the biochemical oxygen demand (BOD) and can be subdivided in Biodegradable BOD, biochemically modified by the enzymatic system of the organisms and can be used as a substrate and carbon source, and non biodegradable BOD, either because it is toxic or the enzymatic system of the organisms cannot degrade them. These bacteria can be divided in subgroups. They can be organotrophs aerobic and are bacteria that can remove the most part of the organic compounds in depurating systems because of its enzymatic system that allows the quick utilization of soluble biodegradable compounds.

Can be fermentative bacteria and this fermentative process occurs in the absence of oxygen and nitrates but it is difficult for this process to occur in conventional systems. These bacteria are responsible for the removal of phosphorus and the conversion of organic compounds into volatile fatty acids such as acetic acid.

Another subgroup is the anoxic bacteria. An example of these is denitrifying bacteria. These kinds of bacteria have as final electron acceptors nitrates or analogue substances.

Talking about nitrifying bacteria, these bacteria are responsible for the oxidation of nitrito to nitrate.

Finally, bacteria can accumulate polyphosphates bacteria: these are of extreme importance in advanced depuration of residual water, however, their metabolism or identification is not well understood. It is believed that these bacteria are responsible for the removal of phosphates (Nicolau; 2009).

2.8.) Advantages of Molecular Methods Vs Classic Methods in the Identification of Microorganisms

When the research, in the field of wastewater microbiology, first wanted to identify microorganisms from WWTP, they used very basic methods based on coloration and microscopic observation. Nowadays, with the advances in molecular methods, there are a lot of other options available (Gilbride *et al.,* 2006). In order to use molecular approaches, the very first step is to extract and purify DNA/RNA (deoxyribonucleic acid/ribonucleic acid) with the nucleic acid it is possible, later, to obtain pieces of this DNA/RNA belonging to all members of the community regardless of special growth needs (Talbot *et al.,* 2008). Over the time, molecular approaches have been very useful and have allowed the identification of new species and has confirmed older species (Gilbride *et al.,* 2006) and information about the composition, structure, activity of the microbial community and other kinds of information is always welcome (Gilbride *et al.,* 2006). Table 4 shows classic and molecular methods of microorganisms identification. Cultures of microorganism are sometimes done, in order to subsequently identify them, an appropriate medium is difficult to find. If the aim is to obtain cultures of all existing microorganisms, the task is increasingly difficult. For example, the average cell culture count, with the help of a microscope, is 10^{10} cells/ml (Victoria *et al.,* 1996) while the number of cells resulting from lab cultures is only 10^{2} or 10^{8} cells/ml (Fulthorpe *et al*. 1993). In fact, when the number of microorganisms grown in lab provided from activated sludge is compared with the estimates provided from direct observation of wastewater prior to cultivation using methods like direct cell counting or immunochemical techniques, the numbers do not match (Howgrave-Graham and Steyn, 1988).

Most of the times, growing cells is time consuming (Kampfer and Dott, 1989) and can be limited to growing a certain number of bacteria (Amann *et al.,* 2001). Some molecular methodologies allow cells to be collected right away, cultures grown in labs show differences in structure and metabolic activity also, one of the most important advantages is due to molecular approaches the samples can be frozen in order to keep the metabolic status and microbial composition intact (Widada *et al.,* 2002). Also, with direct extraction of DNA, information about microorganisms that are not able to grow in labs but may be responsible for some of the biodegrading activity can be obtained, which is an important issue (Brockman, 1995). With the use of these techniques it is possible to understand the diversity and interaction of microorganisms present in the wastewater.

Table 4: Different methods of microorganism identification (Adapted from Gilbride *et al.*, 2006)

Technique	Benefits	Limitations	Examples of usage
Microscopy	Fast Direct observation of microbial cells	Majority of bacterial population cannot be identified	Association of filamentous bacteria with sludge bulking(Eikelboom, 1975; Seivour *et al.*, 1997)
Media-Based Methods	Easy to perform Identification of individual microorganisms	Majority of bacteria cannot be easily cultivated on general purpose media	Domination of aerobic and facultative anaerobic heterotrophs in kraft pulp treatment systems (Liss and Allen, 1992) Common bacterial isolates from kraft bleached pulp mill (Fulthorpe *et al.*, 1993)
Indicator microorganism based pathogen estimation	Easy to perform Current standard for coliforms	Labor intensive and time consuming Indirect estimation of pathogens rather than direct detection	Fecal coliform/fecal streptococcus ration to differentiate human vs. non-human pollution (Scott *et al.*,2002) F+ RNA and DNA coliphage densities as an indicator of water quality (Cole *et al.*, 2003)
Amplified ribosomal DNA restriction analysis (ARDRA)	Culture-independent Suitable for analysis of a wide range of microorganisms	DNA extraction and PCR biases Not quantitative	Microbial diversity of activated sludge (Blackall *et al.*,1998; Pellegrin *et al.*, 1999)
Ribosomal RNA intergenic spacer analysis (RISA)	Culture-independent Suitable for analysis of a wide range of microorganisms Significant heterogeneity in length	DNA extraction and PCR biases Not quantitative Significant heterogeneity in length and sequence	Bacterial diversity and community analysis from different pulp and paper wastewater treatment systems (Baker *et al.*, 2003)

	and sequence among bacteria	among bacteria	
Denaturing gradient gel electrophoresis (DGGE)	Culture-independent Suitable for analysis of a wide range of microorganisms Use of rRNA gene sequence heterogeneity	DNA extraction and PCR biases Not quantitative Specificity can be an issue because of short target sequences	Population shift (Ferris *et al.*, 1997) Succession of bacterial population (Simpson *et al.*, 2000)
Terminal-restriction fragment length polymorphism (t-RFLP)	Culture-independent suitable for analysis of a wide range of microorganisms Fast and semi-quantitative	DNA extraction and PCR biases	Composition of pulp mill microbial community (Gilbride and Fulthorpe, 2004) Bacterial community composition from sewage treatment plants (Hiraishi *et al.*, 2000)
Fluorescent in situ hybridization (FISH)	Quantitative Direct visual resolution of microbial cells including non-culturable	Inactive cells may not be detected	In situ analysis of microbial community structure in activated sludge (Wagner *et al.*, 1993) Observation of sludge floc forming microorganisms (Rosselló-Mora *et al.*,1995)
Fluorescent in situ hybridization (FISH) and confocal scaning laser microscopy	Quantitative Direct visual resolution of microbial cells including slow growing and non-culturable	Expensive instrumentation	Visualization of nitrifying bacteria (Wagner *et al.*, 1998; Juretschko *et al*,. 1998)

(CSLM)			
Multiplex PCR	Rapid and simultaneous detection of several target microorganisms	Combinations of primer pairs must function in a single PCR reaction	Detection of bacterial pathogens in wastewater (Ibekwe *et al*,. 2002) and viruses (Beuret, 2004)
Nucleic acid microarrays	High throughput design Various applications	Low sensitivity for environmental samples Sample processing complexities	Pathogen detection in water (Straub and Chandler, 2003), Foods (Call *et al.*, 2001) and wastewater (Lee *et al.*, in press)
On-chip technology	Combination of PCR with nucleic acid hybridization on a single chip Less interference between parallel reactions	Integration and packaging	Detection of bacterial pathogens (Lagally *et al.*, 2004; Blaskovic and Barak, 2005)

If classic microbiology is combined with molecular methods, the information will lead to a more comprehensive perspective and a better interpretation of the *in situ* microbial community and its response to engineered bioremediation is possible (Brockman, 1995).

2.9) Contribution of Bioinformatics to the Identification

When the goal is to identify microorganisms, after all the lab work and after compiling the results, these same results need to be analyzed in order to establish conclusions. Bioinformatics gives us a huge help in this step.

Bioinformatics made the bridge between lab work and the computers. With bioinformatics, the management and analysis of biological data is done and stored via computers. The next step is to analyze and merge biological data. Computers are, nowadays, in charge of sequence generation, storage, interpretation and analyses. (http://www.bioinformatics.nl/webportal/background/techniques.html).

In the end, with bioinformatics organized information and the right program that can help analyze the data, a clearer insight into the biology of microorganisms is obtained (http://www.bioinformatics.nl/webportal/background/techniques.html).

Finally, molecular techniques together with bioinformatics can be a huge contribution in microorganism identification and in their comprehension. With all the information together and available, it is possible to study microorganism characteristics and learn which environmental factors determine their prevalence, frequency and possible dominance (Figure 5).

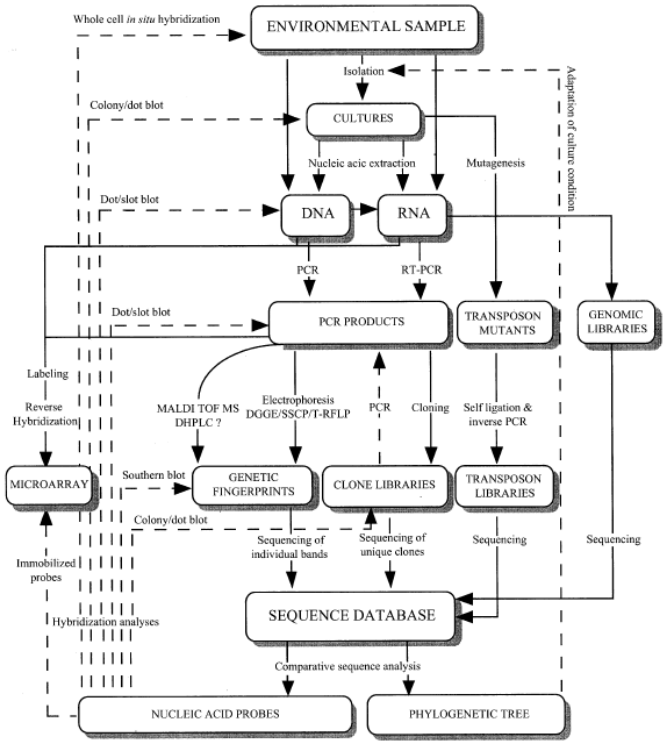

Figure 5: Molecular approaches for detection and identification of xenobiotic-degrading bacteria and their catabolic genes from environmental samples (Adapted from Muyzer and Smalla, 1998)

CHAPTER III

Methodology

In order to achieve the goal of the present work, a methodology was thought and applied step by step starting by the sampling of the biological material passing through the extraction of the DNA and finally the assemblage of the dendrogram.

3.1) Samples

In order to study the microbial biodiversity of the activated sludge process of six WWTP (Wastewater Treatment Plant), samples were taken from the aeration tank in the period between and subsequent, then the plating was made in order to obtain a series of isolates of each sample. A first set of samples, from 2 WWTP, was used to get acquainted with the methodology: these were samples 2.2MON, 3.2MON, 2.2VZI, 3.2VZI. Next, 4 more WWTP were chosen to complete the study and these latter were chosen with one year of interval between the two samples of each of the WWTP. Samples are named as:

2.2.MON	2.2.CUC	2.2.SEI
3.2.MON	3.3.CUC	3.3.SEI
2.2.VZI	2.2.LAG	2.2.ZIA
3.2.VZI	3.3.LAG	3.3.ZIA

3.1.1) Culture of Samples and Isolation of Morphotypes

The samples were cultivated in Tryptone Soy Agar (TSA) (Liofil Chen, Bacteriology products, Roseto DA, Italy) (Constitution on the appendix II). This medium was used according to the specifications of the manufacturer and it was chosen because it possesses a large spectrum of grown organisms. The microorganisms were plated directly from the samples to a TSA petri dish and grown at 37ºC over 24 hours. The cultures were observed and the different colonies were tagged and later subculture to a new Petri dish for another 24 hours in the same conditions. The colonies to subsequent isolation were chosen among isolated colonies and based on the apparent differences of colony morphotype. The procedure was repeated as many times as necessary until the culture appeared to be isolated.

3.2) Isolates Preservation

The next step was storing the isolates. In order to do that, the cells were places from the petri dish directly into a TSB (Tryptic Soy Broth) (Liofil Chen, Bacteriology products, Roseto DA, Italy) medium (constitution in the appendix II).

The growth was performed during 24 hours to 37ºC with constant agitation and then 1 ml was taken and centrifuged (Centurion Scientific Ltd) 5 min at 10000 rpm. The supernatant was rejected. This procedure was performed continuously until a reasonable quantity of cells was reached.

In order to preserve bacteria, these pellets were suspended in 1ml of TSB (Liofil Chen, Bacteriology products, Roseto DA, Italy) with 15% of glycerol. In the end, these preparations were frozen at -80ºC and -20ºC in duplicate.

3.3) Gram Staining

The Gram staining technique is a differential staining technique that allows bacteria split into two main groups: the Gram-positive and Gram-negative. The differences in staining are due to the difference in chemical composition the cell wall. In this study gram staining was performed according to steps in appendix I. Figure 5 Shows a overview of all technique.

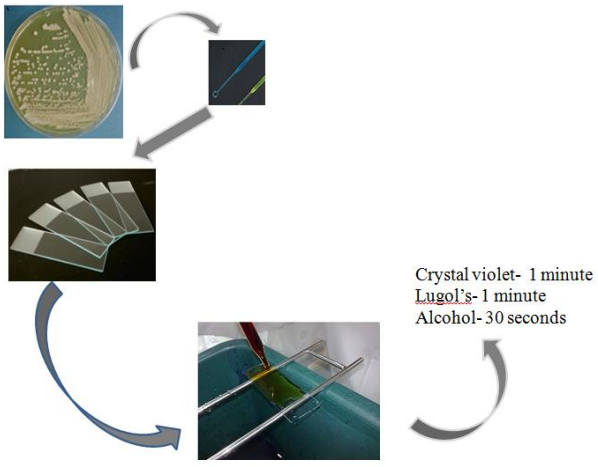

Figure 6: Gram staining procedure

The bacteria that remain blue are called Gram-positive. On the other hand, the bacteria that are decolorized and took the safranin remaining red are Gram-negative. It is believed that the absence of lipids in cell wall of Gram-negative cells and its major abundance in gram positive cells can be an explanation for these results. Crystal violet

is positively charged. When enters in cells, binds to negatively charged compounds. With mordant the process is exactly the opposite (James and Mittwer, no year). Sometimes it is not so easy to say if a bacteria is Gram-negative or Gram-positive because some organisms have no consistence in results. These organisms are called gram variable. The results were observed under a microscope (Olympus, CX 41), both Gram staining results and new fresh preparation, and the results were compared (see results) using a 100X amplification.

When an isolate had one more morphology than it was assumed that it was not properly isolated and all the procedure to get an isolate was carried on again.

3.4) Molecular Approaches

The frozen cells were unfrozen and later cultivated again such as in step 2.1.1 conditions in order to move on to a molecular approach. This step was repeated for every isolate.

3.5) DNA Extraction

In order to obtain DNA to perform PCR the cell lyses were performed. The cells were directly removed with a loop from the petri dish to 400 µl of a aqueous solution. The aqueous solution used was made with one solution consisting of 200 µl of 0,5% SDS, sterilized with a filter (with 0,2µl), plus 200µl of TE buffer (composition in the appendix II) and were heated at 65ºC for 20 minutes (protocol adapted from Laboratory for Environmental Pathogens Research, Department of Environmental Sciences University of Toledo DNA, no year).

3.6) PCR

PCR-fingerprinting is a generic term applied to PCR-based methodologies that originate a fingerprint of each microorganism. There is a variety of PCR fingerprinting methodologies; nonetheless, some are based on amplification of different regions of the genome by PCR, using only one primer. In this work, M13 primer (Invitrogen) was used. Without question, the major advantage of the PCR-based typing is the fact that the technique requires very few starting materials, so this makes the technique cheap, has a universal application and it is rapid to perform (Diaz-Guerra *et al.*, 1997). PCR DNA-

fingerprinting consists in applying, ideally, just one specific primer to create a fingerprint that will be unique to each organism. In order to achieve that goal, it is important to have low restringing conditions and direct the primer to repetitive sequences of the genome.

M13 primer has the required conditions. It belongs to the core of M13 phage mini-satellite that is able, due to low restringing conditions and due to its high affinity to genome, to amplify several regions of the genome. In fact, this methodology is employed to amplify hyper-variable genomic DNA sequences (Vassart G. *et al.*, 1987). M13 primer has the sequence 5'-GAGGGTGGCGGTTCT-3' (Neto, 2008).

The PCR reaction occurred with the help of the high-fidelity enzyme *Taq DNA Polimerase* (Invitrogen). The PCR mixtures were done according to the description below.

Table 5: Composition of DNA mix

Compounds	Initial concentration	Final concentration	Quantity
Taq	5U	1,25U	0,25 µl
Buffer	10X	1X	2,5 µl
Mg	3mM	1,5mM	1,5 µl
dNTP´s	10mM	4mM	0,5 µl
Primer	50pmol	0,5µM	1,25 µl
DNA template	X	1:10 dilution	1 µl
Water	X	X	18 µl

All the reagents used belong to Invitrogen.

The following PCR program was used:

Table 6: PCR program

Steps	Conditions	Nº of Cycles
Step 1: Initial Denaturation	3min, 95ºC	1
Step 2:		
Denaturation	1min, 95ºC	
Annealing	2min, 50ºC	40
Extention	2min, 72ºC	
Step 3: Final Extension	5min, 72ºC	1
	4ºC	∞

The reaction mix was made for 25 µl each tube plus one for user errors.

DNA was diluted in a proportion of 1:10 (9µl of water plus 1µl of DNA from damaged cells. This step was performed with intuit of have the perfect amount of DNA in DNA electrophoresis.

3.7) Electrophoresis Gel

Electrophoresis gel was performed with 1,5% agarose (BioRon) gel prepared with 1X TAE (Tris-acetate-EDTA) buffer (constitution in appendix II) and PCR samples were run over 90 minutes at 180 Volts.

Several run times and others buffers were experimented but these were the conditions that showed the best resolution and the best migration of the pattern band. The conditions were maintained for all the isolates in order to obtain a platform where it is possible to compare the results.

Before the polymerization of the agarose (BioRon) 10 mg/ml of ethidium bromide was added in order to observe the results under UV light.

Table 7: Quantities of ladder and sample for the electrophoresis gel

Ladder	5 µl NZYDNA Ladder III	
Sample	10 µl sample	2 µl loading

TAE buffer is useful in the separation of nucleic acids such as DNA and RNA and was used in the container before the agarose was there and then was added again until it covered the gel. The ladder used belongs to NZYTech, Lda and is ready to use. This molecular weight marker produces a pattern of 14 bands ranging from 200 to 10000 base pair (see appendix III).

3.8) Bioinformatics

To perform the analysis of the isolates obtained, Bionumerics (Applied Maths) software was the chosen tool. This type of analysis allows the establishment of correlations between the microbiology, the physical-chemical prevailing parameters and the performance of the WWTP.

Bionumerics (Applied Maths) software is the only program that can integrate several techniques like 1D electrophoresis gels, all kinds of spectrometric profiles, 2D protein gels, phenotype characters, microarrays and sequences in the same platform and better, this program is able to combine information from various genomic and

phenotypic sources in one global database and lead to a conclusive analysis (http://www.biosystematica.com/bionumerics.pdf). Cluster analysis, is, with no question, an indispensible tool in bioinformatics, the connections and the flexibility of relational database with the contribution of multiple techniques (http://www.applied-maths.com/bionumerics/modules/bn_tn.htm). It is possible to detect and analyze mutations, make epidemiological typing of bacteria, fungal and virus, bacterial source tracking, plant and animal breeding and generate the phylogenetic inference and evolution (Bryan *et al.*, 2004).

The typification of the isolates was done with a M13 primer. This technique was applied in the analysis of the 113 isolates. After the DNA extraction and amplification by PCR the M13-PCR fingerprinting profiles were revealed by electrophoresis. Each profile consists of a complex pattern of bands that categorize each microorganism and that can be used to differentiate the isolates. The primer used determines the loci amplified and consequently defines a particular genome sample.

In silico analysis of the densitometry profiles that corresponds to the fingerprints obtained allowed the construction of the dendrograms applying a coefficient of correlation “Dice” and a clustering method based on Unweight Pair Group method with Arithmetic Mean (UPGMA), an optimization of 0,5%, a band Matching Tolerance of 0,5% and a branch quality with cophenetic correlation. The purpose in the analysis of the dendrograms obtained after the *in silico* treatment of the M13-PCR fingerprinting profiles were to group the isolates by cluster similarity.

CHAPTER IV

Results and Discussion

In this chapter, the results obtained will be shown and discussed. First, the characterization of the bacterial isolates will be presented and a relationship between this characterization and the plant operation is essayed. Then, the results on the M13 PCR fingerprinting will be displayed and the dendogram obtained after clustering analysis will be presented. The composition of the groups assembled in the dendogram will be discussed taking in consideration the physical-chemical and operational parameters of the studied WWTP.

4.1) Characterization of the Bacterial Isolates

A total of 113 isolates were selected from the 12 studied samples. Each sample contributed with 8 to 13 isolates. Table 8 shows the characteristics of each of the isolate considered.

Table 8: bacteria isolates generated from each sample

Name of the sample	Number of isolates generated	Gram stain and form of the cells
2.2 MON	12	6 Gram negative rod-shaped cells 3 Gram positive rod-shaped cells 1 Gram negative spherical cells 1 Gram positive spherical cells
3.2 MON	8	5 Gram negative rod-shaped cells 1 Gram positive rod-shaped cells 1 Gram negative spherical cells 1 Gram variable spherical cells
2.2VZI	9	3 Gram negative rod-shaped cells 3 Gram positive rod-shaped cells 2 Gram variable rod-shaped cells 1 Gram negative spherical cells
3.2 VZI	10	9 Gram negative rod-shaped cells 1 Gram positive rod-shaped cells
2.2 CUC	9	3 Gram negative rod-shaped cells 6 Gram positive rod-shaped cells
3.3 CUC	9	2 Gram negative rod-shaped cells 5 Gram positive rod-shaped cells 2 Gram negative spherical cells
2.2LAG	9	4 Gram negative rod-shaped cells 2 Gram positive rod-shaped cells 2 Gram positive spherical cells 1 Gram negative spherical cells
3.3 LAG	13	6 Gram negative rod-shaped cells 1 Gram negative spherical cell 5 Gram positive spherical cells 1 Gram variable spherical cells
2.2 SEI	8	1 Gram negative rod-shaped cells 6 Gram positive rod-shaped cells 1 Gram negative spherical cells
3.3 SEI	9	6 Gram negative rod-shaped cells 2 Gram positive rod-shaped cells 1 Gram variable rod-shaped cells
2.2 ZIA	9	3 Gram negative rod-shaped cells 6 Gram positive rod-shaped cells
3.3 ZIA	8	3 Gram negative rod-shaped cells 5 Gram positive rod-shaped cells 1 Gram positive spherical cells

It is possible to see than Gram negative as well as rod-shaped cells are predominant in the set of samples: 52,2% of the isolates are Gram negative, 43,4% are Gram positive and 4,4% are Gram variable; 83,2% are rod-shaped and 16,8% are spherical. In fact, Gram negative bacteria dominate quantitatively the WWTP microbiota, but this is not visible in these analyses because the isolates were not selected to represent the distribution of colonies in the plates. That is, a sample can be dominated by a gram positive morphotype and only one isolate is selected: the distribution of bacterial isolates reflects the plating only qualitatively. Gram negative have a thin cell wall, although they have one second lipid layer which confers an extra protection to these bacteria making them more resistant to adversities (Prescott *et al.*, 2009). That can be the reason why gram negative are in higher number than gram positive that only have one cell wall made of peptidoglycan. In fact, Carvalho and Fernandes, in 2010, demonstrated that in the sea, Gram negative bacteria respond well to stressful conditions such as high salinity conditions. The aerated tank of WWTP is also a much stressed ecosystem and this can be one of the reasons of this slight dominance.

Gram negative appears when there are a lot of inorganic compounds like sulfates, nitrites, methane or CO_2 (Madoni, 1994).

Out of all the WWTP, the one that has the highest nitrogen load is 3.3ZIA with a nitrogen load of 209 mg/l, other high values belong to 2.2MON (175 mg/l), 3.3MON (160mg/l) and 3.2VZI (146mg/l) WWTP.

3.2MON, 2.2LAG, 3.3SEI and 2.2MON, have the highest values of BOD which means there is a lot of organic matter available to feed microorganism, these WWTP possesses a majority of gram negative. The COD of these samples are higher than the BOD because in WWTP not only is biodegradable matter oxidized but non biodegradable matter is also oxidized (Sawyer, 2003). BOD diminishes in VZI, CUC, LAG and ZIA WWTP, these systems improved from one year to another.

According to Madonni (1994), the SBI values of the samples 2.2MON, 2.2VZI, 3.2VZI, 2.2CUC, 3.3CUC, 2.2LAG, 3.3LAG, and 2.2ZIA belong to the class I which means that these systems are very well colonized. Belonging to the class II there are 3.2MON, 3.3SEI and 3.3ZIA. Class II is also a very sufficient system. Belonging to the class III, there is only 2.2SEI. Besides the low SBI value, WWTP 2.2SEI, as well as 3.3SEI, has a bigger amount of organisms of *Opercularia* sp. that is one genus that is particularly resistant to stressful situations such as the presence of certain toxins (such

as salts and heavy metals), acidity and lack of oxygen which is the case. This species is associated with huge amounts of NH_4^+ that results from catabolism. Looking at the N concentration on exit, the N has a huge concentration (Seviour and Blackall, 1999). But, from one year to another (2.2SEI to 3.3SEI), the SBI increased which lead to a conclusion that the system improved from one year to another.

4.2) Genomic characterization by M13-PCR fingerprinting

With the images obtained after the M13-PCR and subsequent electrophoresis, a dendrogram was built. It is expected to determine the relationship among samples considering the clusters obtained. After that, the relationships of the assembled groups with the characteristics of each of the WWTP are discussed.

The following figure shows an example of a gel with M13-PCR fingerprinting profiles obtained with *Chemi Doc*, Biorad, California, for some of the strains in study:

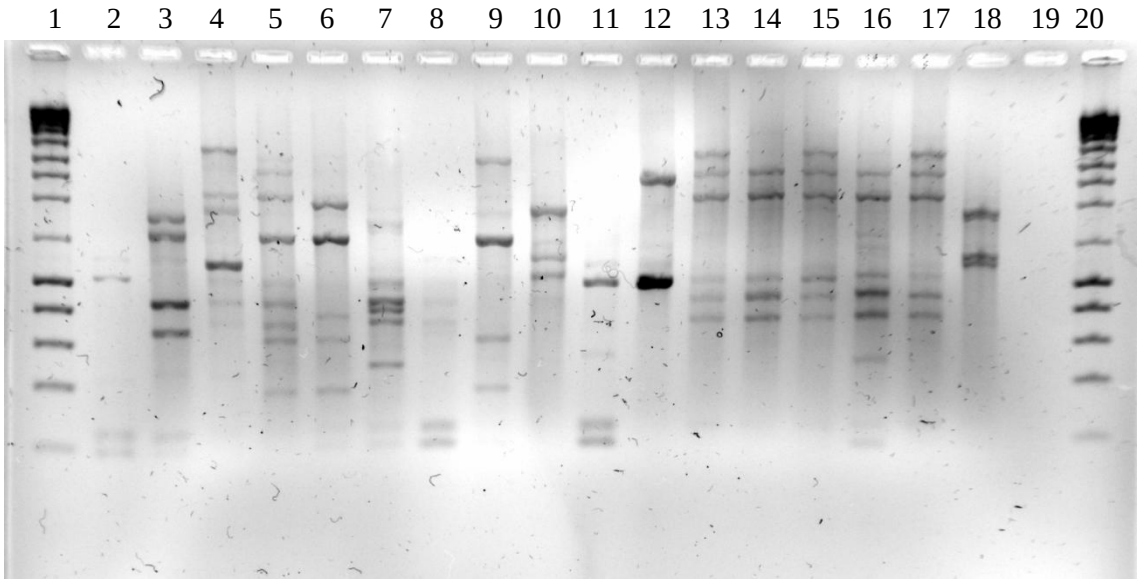

Figure 7: Agarose gel with M13-PCR fingerprinting profiles obtained for some of the strains in study

(Samples: 1-Ladder, 2-Negative control, 3-3.3CUC2.1, 4-3.3CUC3.1, 5-3.3CUC4.1, 6-3.3CUC5.1, 7-3.3CUC7.1, 8-3.3CUC7.2, 9-3.3CUC8.1, 10-2.2LAG1.2, 11-2.2LAG2.2, 12-2.2LAG4.1, 13-2.2LAG6.1, 14-2.2LAG8.2, 15-2.2LAG8.1, 16-3.3LAG1.1, 17-3.3LAG2.1, 18-3.3LAG3.1.

Figure 8 shows the dendogram obtained after clustering analysis by the Bionumerics (Applied Maths) software. The following table (Table 9) shows the composition of all the groups defined in the dendrogram.

Figure 8. Dendogram obtained after clustering analysis.

Table 9: Numbers given to the groups of isolates present in dendrogram

Group		Isolates
I		2.2VZI4.1, 3.2VZI1.1, 2.2MON4.1, 3.2 VZI6.1, 2.2MON5.2, 2.2MON4.3, 2.2MON5.1
II		2.2MON1.1, 2.2SEI6.1, 2.2CUC1.1, 2.2SEI7.1
III		2.2SEI8.1, 3.3SEI7.1, 3.3SEI6.1
IV		2.2ZIA6.1, 3.2VZI5.2
V		2.2ZIA3.1, 2.2ZIA 4.2, 2.2ZIA4.1, 2.2ZIA1.1, 2.2MON4.2, 3.3SEI8.2, 2.2MON5.1, 3.3CUC1.1
VI		2.2VZI1.1, 3.3SEI8.1, 2.2SEI3.1, 2.2ZIA2.1, 3.3SEI1.1
VII		2.2LAG1.2, 2.2ZIA8.1, 3.3CUC8.1, 3.3LAG6.1, 3.3LAG7.1, 3.2VZI3.1, 3.3CUC4.1, 3.3CUC3.1
VIII		2.2CUC3.1, 3.3LAG3.4, 2.2LAG7.1, 2.2VZI2.1
IX		2.2ZIA7.1, 3.3CUC5.1, 2.2MON1.2, 3.3ZIA3.2, 2.2CUC8.1, 3.3SEI3.2, 3.3SEI3.1
X		3.3ZIA3.1, 3.3ZIA5.1, 3.3LAG2.1, 3.3ZIA5.3, 3.3LAG1.1, 3.3ZIA1.1, 2.2LAG6.1, 3.3ZIA5.2
XI		2.2SEI1.2, 3.3SEI2.1, 2.2CUC1.2, 3.3LAG3.3, 3.3LAG4.1, 3.3LAG3.5, 3.3LAG5.1, 3.3LAG4.2, 3.3LAG5.2
XII	**a**	2.2MON4.4, 3.2VZI5.3, 3.3LAG3.1, 2.2LAG2.2, 2.2LAG4.1, 3.3ZIA7.1
	b	2.2CUC7.1, 2.2VZI5.1, 2.2MON3.1, 2.2SEI1.1, 3.2MON3.1, 3.3CUC6.1
	c	3.2MON1.1, 3.3CUC7.1, 2.2SEI1.3, 3.3LAG3.2, 3.3ZIA2.1, 3.2MON2.1
XIII	**d**	2.2LAG1.1, 3.3CUC2.1, 2.2CUC2.1, 2.2SEI2.1, 2.2MON2.1, 2.2VZI5.2, 2.2VZI5.3
	e	2.2VZI3.1, 3.2MON5.1, 2.2LAG8.1, 2.2LAG8.2
XIV		2.2VZI1.2, 2.2VZI4.2, 2.2VZI4.1
XV		3.2MON1.2, 3.3CUC7.2, 3.2VZI3.2
XVI		3.2MON3.2, 3.2MON3.3, 2.2CUC6.1, 2.2MON3.2
XVII		2.2LAG3.1, 3.2MON2.2, 3.2VZI2.1

Analyzing the dendrogram obtained with the M13-PCR fingerprinting profiles (Fig.8), it is possible to define seventeen main groups of isolates. In groups XII and XIII, due to their dimension, subgroups were defined in order to simplify the subsequent analysis.

The lowest level of similarity was obtained in the group XVII (with about 10% similarity) and higher level of similarity was among the isolates 3.3LAG3.3 and 3.3LAG4.1 belonging to group XI and 2.2LAG8.1 and 2.2LAG8.2 belonging to group XIII, specifically in the group XIIIe.

The number of isolates in each cluster defined by this technique varied between two in group IV and eighteen in group XII. In some cases, despite the low level of similarity (which sometimes is only 25%) is enough to group the bacteria in the same cluster. The clusters vary in size but all isolates are, in one way or another, related with the isolates that belong to the same group. In the dendrogram, clusters can be distinguished from one another and each cluster shares one or several characteristics with the ones that are related to them. In one first observation it is possible to observe that the similarity among the isolates is very low, emphasizing the low level of similarity (21%). In the dendrogram it is possible to verify a significant heterogeneity in the set of isolates which makes it difficult to create the relation between the groups and with the characteristics of the WWTP.

Some groups, due to the characteristics of its results, will be presented and discussed by numerical order.

Group I

The cluster that belongs to group I is composed by seven isolates but these seven isolates belong to two WWTP: 2.2MON, 2.2VZI and 3.2VZI, although in one WWTP the samples belongs to different years. The most related isolates belong to different WWTP. The isolates 2.2MON4.1 and 3.2VZI6.1 share a similarity of 62%. These isolates (2.2MON4.1 and 3.2VZI6.1) are more closely related with one another than to the other isolates belonging to the same group and the same WWTP. These samples share characteristics such as: the range of SSV (228 mg/l in 2.2MON and 292 mg/l in 3.2VZI) and nitrogen that is loaded in the system (175 mg/l in 2.2MON and 146 mg/l in 3.2VZI) and occurrences, although with very different numbers, of *Arcella* sp. (techaete amoeba) (2.2MON has 360 ind/ml and3.2VZI has 20 ind/ml), of the crawler ciliate *Acinera uncinata* (2.2MON has 2080 ind/ml and 3.2VZI has 60 ind/ml) and of two attached ciliates: *Epistylis* sp. (5260 ind/ml in 2.2MON and 4320 ind/ml in 3.2VZI ind/ml) and *Vorticella microstoma* (60 ind/ml in 2.2MON and 680 ind/ml in 3.2VZI). Taking into consideration the Gram staining results, it is possible to see that both WWTP mostly contain Gram negative rod-shaped cells. More importantly, both WWTP have an SBI value belonging to a class I category. Maybe all these similarities were enough allow for the growth of bacteria with a certain similarity.

Group V

Another big group formed is the group V. In this group there are 8 isolates. Half of these isolates belong to the same WWTP, 2.2ZIA. The isolates 2.2ZIA3.1 and 2.2ZIA4.2 are the most related of the group where the similarity percentage between them is about 62%. In this group of 8 isolates, the four isolates obtained from the sample 2.2ZIA are the ones that share a higher similarity in the group (2.2ZIA4.1 shares 57% of similarity with the first two and 2.2ZIA1.1 that shares about 42% similarity with the previous three). The other four isolates, two of them belong to sample 2.2MON. Nonetheless, the isolate 2.2MON4.2 is more related to 3.3SEI8.2 isolate than to the other isolate from the same WWTP. These isolates share a similarity of approximately 50%, the same similarity shared by 2.2MON5.1 and 3.3CUC1.1 isolates. All the isolates are related, in one way or another. This is possible since three of the four WWTP present in this cluster have an SBI value that belongs to a category of class I. Only the sample 3.3SEI has a lower SBI which belongs to a category of class II. The sample of the WWTP 3.3SEI belongs to category of class II and it is more related to WWTP sample 2.2MON. Both share the same flagellates genera, like *Peranena* sp. (3.3SEI has 80 ind/ml and 2.2MON also has 80 ind/ml) and the same kind of ciliate crawlers *Acineria uncinata* (3.3SEI has 4940 ind/ml and 2.2MON has 2080 ind/ml), the same attached ciliates *Epistylis sp*. (3.3SEI has 1800 ind/ml and 2.2MON has 5260 ind/ml) but 2.2MON has about three times more than 3.3SEI. BOD values are also similar between 2.2MON, 3.3SEI and 3.3CUC (400 mg/l for 2.2MON, 422 mg/l for 3.3SEI and 341 mg/l for 3.3CUC), COD values (890 mg/l for 2.2MON, 1028 mg/l for 3.3SEI and 1005 mg/l for 3.3CUC), pH values (7,46 for 2.2MON, 7,12 for 3.3SEI and 6,88 for 3.3CUC).

Related to 2.2ZIA WWTP samples, it has an SST loaded similar to 3.3SEI and 2.2MON WWTP samples (382 mg/l for 2.2ZIA, 428 mg/l for 3.3SEI and 305 mg/l for 2.2MON) and SSV (330 mg/l for 2.2ZIA, 382 mg/l for 3.3SEI and 228 mg/l for 2.2MON) and nitrogen load similar to sample 3.3CUC (49.8 mg/l in 2.2ZIA and 53 mg/l in 3.3CUC). All the WWTP except 3.3CUC have the same flagellates genera, *Peranema sp.,* in number and specie (80 ind/ml in 2.2MON; 80 ind/ml in 3.3SEI and 60 ind/ml in 2.2ZIA).

The Gram staining showed that in 3.3SEI and 2.2MON there are predominantly gram negative rod-shaped cells (Table 8) and in 2.2ZIA and 3.3CUC positive rod-shaped cells prevail. The same conditions along with some shared microorganisms can

lead to this similarity. If one WWTP shares microorganisms with another, the isolation of samples can hook the same microorganisms. One reason for these microorganisms not being exactly the same can be because in different WWTP the conditions are not the same. Liu *et al*., (2001), took several samples during the same day and verified that depending of the time of the day when the samples were collected, the isolates where not the same. Certain populations that were present in freshly collected sludge decrease in abundance over time and can no longer be detected. If this happened during only one day, in the space of one year, this can also be true. Conversely, other populations are initially minor members of the community and cannot be detected in freshly collected activated sludge, but increase in number to become dominant members of the community after a while (Liu *et al.,* 2001). Maybe the time of collection is grouping the bacteria form different WWTP in the same group or depend of other such as reproduction that not always is clonal (Prescott, 2002). That way, if there is sexual reproduction with plasmids, phages or even transposons, the individuals will be not the same.

Group VII

Group VII also has eight isolates but shows a greater diversity in terms of WWTP. The two isolates that show a higher similarity belong to the same WWTP, 3.3LAG (3.3LAG 6.1 and 3.3LAG7.1 sharing 54% similarity. With a slightly lower similarity there is 2.2LAG1.2 and 2.2ZIA8.1 sharing 50% similarity; these two WWTP have several characteristics in common such as pH (7.07 in 2.2LAG and 6.88 in 2.2ZIA), the flagellates genera *Peranema sp.,* (80 ind/ml in 2.2LAG and 60 ind/ml in 2.2ZIA), techaete amoeba genera, *Arcella sp.,* (20 ind/ml in 2.2LAG and 740 ind/ml in 2.2ZIA), crawlers ciliate specie, *Aspidisca cicada*, (100 ind/ml in 2.2LAG and 6020 ind/ml in 2.2ZIA), attached ciliate, *Epistylis sp.*, (5160 ind/ml in 2.2LAG and 1800 ind/ml in 2.2ZIA) and carnivore, *Acineta sp.*, (20 ind/ml in 2.2LAG and 40 ind/ml in 2.2ZIA) although in different quantities. As referred before, these characteristics can be enough to favor some bacteria over another. These two isolates share a higher level of intimacy but, are only separated from isolate 3.3CUC8.1 with a similarity of approximately 43%. The sample 3.3CUC has characteristics in common that make it possible to put these samples in the same group, such as pH (7,41 in 3.3CUC), nitrogen load 2.2ZIA (53 mg/l in 3.3CUC and 49.8 mg/l in 2.2ZIA), COD that is similar to 2.2LAG (1562 mg/l in 3.3CUC and 1435 mg/l in 2.2LAG) and the three WWTP have

the same crawlers ciliates, *Aspicidisca cicada*, (940 ind/ml in 3.3CUC). These similar characteristics may be conducive to the development of similar microorganisms. 3.3VZI3.1 and 3.3CUC4.1 isolates are more closely related than 3.3CUC3.1 and 3.3CUC4.1 isolates, although the three are related. It is important to point out that the SBI of all the samples in this group belong to a category class I.

Group IX

Group IX has one less isolate in the same cluster than the clusters previously mentioned. Beyond these seven isolates the ones that are more related, with 61% of similarity, are the isolates 2.2ZIA7.1 and 3.3CUC5.1. These two isolates were obtained from a WWTP belonging to an SBI category class I. With about 54% of similarity from these isolates is 2.2MON1.2. These isolates can be related because the SBI of this WWTP also belongs to a category class I and the SST load value is similar between the WWTP samples 2.2MON and 2.2ZIA (305 mg/l in 2.2MON and 382 mg/l in 2.2ZIA). Looking at the microbiology, 2.2MON and 2.2ZIA have flagellates genera in common, *Peranema* sp., (80 ind/ml in 2.2MON and 60 ind/ml in 2.2ZIA), techaete amoeba, *Arcella* sp., (360 ind/ml in 2.2MON and 740 ind/ml in 2.2ZIA) and *Euglypha* sp., (460 ind/ml in 2.2MON and 260 ind/ml in 2.2ZIA), attached ciliate *Epistylis sp.,* (5260 ind/ml in 2.2MON and 220 ind/ml in 2.2ZIA).

In the same sub-group but with low similarity (about 38%), there is 3.3ZIA3.2 isolate. The WWTP 3.3ZIA has an SBI value that belongs to a class II category. Although the low similarity of clustering, there is some similarities what concerns with the operational parameters. This isolate shares similar pH with the samples 2.2MON, 2.2ZIA and 3.3CUC (7.54 in 3.3ZIA, 7.46 in 2.2MON, 6.88 in 2.2ZIA and 7.41 in 3.3CUC), SSV; 2.2MON (177 mg/l in 3.3ZIA and 228 mg/l in 2.2MON), COD; 2.2ZIA (989 mg/l in 3.3ZIA and 1005 mg/l in 2.2ZIA), nitrogen load; 2.2MON (209mg/l in 3.3ZIA and 175 mg/l in 2.2MON mg/L). The gram staining results showed that, in a general way, this set of samples presents a mix between gram negative and positive rod-shaped cells as well as some gram variable, but in all the cases, rod-shaped cells prevail.

The isolates 2.2CUC8.1 and 3.3SEI3.2 have a similarity of 54%. They are distant from the isolate 3.3SEI3.1. These isolates have a similar SST (427 mg/l in 2.2CUC and 428 mg/l in 3.3SEI) and SSV (340 mg/l in 2.2CUC and 382 mg/l in 3.3SEI). Although, they belong to different SBI classes; 3.3SEI belongs to a class II category and 2.2CUC belongs to a class I category. In the Gram staining results it is

possible to see that the rod-shaped cells are mainly gram positive in 2.2CUC and 3.3SEI has mainly negative rod-shaped cells.

Group X

In group X the isolates 3.3ZIA3.1 and 3.3ZIA5.1 are related with one another with a 71% similarity and the isolate 3.3LAG2.1 is related with these two with 68% similarity, having then a fourth isolate, 3.3ZIA5.3, that is related with these three with a level of similarity of approximately 59%. In this group, samples that come from WWTP 3.3ZIA are "interrupted" by isolates from 3.3LAG. 3.3ZIA and 3.3LAG can be related since they show a similar pH (7.54 in 3.3ZIA and 7.13 in 3.3LAG) and a SST (177 mg/l in 3.3ZIA and 224 mg/l in 3.3LAG). To what concerns to microfauna, there is crawler's ciliate *Acineria uncinata*, (20 ind/ml in 3.3ZIA and 320 ind/ml in 3.3LAG) and a attached ciliate *Epistylis* sp. (4260 ind/ml in 3.3ZIA and 80 ind/ml in 3.3LAG). The next two samples also belong to 3.3LAG and 3.3ZIA and the isolates are related between themselves with 62% of similarity and these last two are related with the previous isolates with a similarity of 53%. The next two isolates are related with the isolates presented before with a percentage of 44% but these two are more similar between each other, sharing a similarity of 56%, and belong to the WWTP 3.3ZIA and 2.2LAG samples. The curious thing is that between these two WWTP there is not much in common. Gram staining results between these WWTP shows a prevalence of negative rod-shaped cells. Contrarily to Wagner *et al.*, 2002, that in geographical areas, found a better relation between the organisms.

Group XI

The group XI relates two isolates that belong to the same WWTP (SEI) in different years. This WWTP as modified and that can be seen by the change in SBI value that changes from five to seven which means a rise from class III to class II. The similarity is not too high, it is about 53%, but this is enough to group these isolates that changed a lot in the same cluster. Liu *et al.* in 2001 verified that in the same WWTP in different days, not years, the changes in terms of microorganisms were significant. The result was surprising because sludge from the same wastewater treatment plant were operated under identical conditions.

Those changes can be possible because the waste continues to come from the same sources. There is another isolate closely related to these two samples, 2.2CUC7.1.

What this sample has in common with the other two isolates is the SST load value which is similar to 3.3SEI (427 mg/l in 2.2CUC and 428 mg/l in 3.3SEI). In a microbiological level this last isolate has flagellates protozoa in common with 2.2SEI and 3.3SEI , *Peranema* sp., (40 ind/ml in 2.2CUC, 40 ind/ml in 2.2SEI and 80 ind/ml in 3.3SEI), one species of techaete amoeba in common with 3.3SEI, *Centropyxis* sp., (240 ind/ml in 2.2CUC and 20 ind/ml in 3.3SEI), two crawlers ciliate species in common with 3.3SEI , *Aspidisca cicada,* (380 ind/ml in 2.2CUC and 60 ind/ml in 3.3SEI), and *Acineria uncinata* (20 ind/ml in 2.2CUC and 4940 ind/ml in 3.3SEI) and share attached ciliates with 3.3SEI ,*Epistylis* sp., (40 ind/ml in 2.2CUC and 1800 ind/ml in 3.3SEI). This can be enough to group these isolates. The next isolates form new group which includes 3.3LAG. These isolates, as shown in this work, have a tendency of staying together. In this group it is possible to see one of the two most related samples 3.3LAG3.3 and 3.3LAG4.1, which belong to the same WWTP and to the same year, and have a similarity percentage of about 90%. With the exception of 2.2CUC, which Gram staining results showed mostly Gram negative rod-shaped cells.

Group XII

The biggest group that can be found in this dendogram is the group XII. Because the cluster is so big and it was decided to divide this cluster into three smaller clusters named XIIa, XIIb and XIIc.

The cluster XIIa includes 6 isolates. The first two isolates belong to different WWTP and show a 57% similarity. These two isolates 2.2MON4.4 and 3.2VZI5.3, join with the isolate 3.3LAG3.1 with 32% of similarity possesses an SBI value that belongs to a category class I which indicates a good level of operation and provides similar conditions between the WWTP (Santos, 2008). Maybe the reason why different WWTP are grouped so often next to each other is because of this very fact. The next isolates belong to the 2.2LAG WWTP (2.2LAG2.2 and 2.2LAG4.1 are related between each other with 50% of similarity) and, in a smaller level of similarity, these are related with 3.3ZIA7.1 (45% of similarity). This relation already occurred above and the explanation is the same (see group X). Although the relation between 3.3ZIA and 2.2LAG is already known, its relation with the rest of the subgroup XIIa is as follows: The pH and nitrogen entrance of 3.3ZIA (7,54 for pH and 209 mg/l for nitrogen) is similar to 2.2MON (7,46 for pH and 175 mg/l for nitrogen). The BOD of 3.3ZIA (261 mg/l) is similar with 3.3LAG (225mg/l) and 3.2VZI (240mg/l). 3.3ZIA shares one type of

species of techaete amoeba (*Arcella* sp.*)* which it has 20 ind/ml, 3.2VZI has 20 ind/ml and 2.2MON has 360 ind/ml, and shares one species of crawler ciliate (*Acineria uncinata*) with 3.3LAG, 3.2VZI and 2.2MON. In 3.3ZIA the quantity is 20 ind/ml, in 3.3LAG, 320 ind/ml in 3.2VZI, 60 ind/ml and in 2.2MON 2080 ind/ml. All these similarities could provide some conditions where some microorganisms survive and for this reason, they look similar in a molecular point of view (Tabka *et al.*, 1993). Considering the Gram staining results of the WWTP from which these isolates originate it is possible to see a higher inclination towards negative rod-shaped cells with some WWTP showing a balance between negative and positive rod-shaped cells.

In the subgroup XIIb there are two different isolates that share a similarity of 50%, 2.2CUC7.1 and 2.2VZI5.1, this value is not too high. Up until this point, when similarities are not too high, it is more and more difficult to find similarities in physico-chemical characteristics but these two samples have an SBI value that belongs to the category of class I and this stability can be favorable for the development of similar microorganisms. 2.2MON3.1 is the most related isolate with these two samples (40% of similarity) and its SBI value is also of a category class I. Related with these isolates there is 2.2SEI1.1 and 3.2MON3.1 with 35% of similarity with the previous isolates and 46 % with each other. These WWTP are different in many aspects and even their SBI value (5 class II for 2.2SEI and 7 which means class III for 3.2MON WWTP) is different. 3.3CUC6.1 is least similar of the group with a similarity of only 25% and it has an SBI value of 9 meaning it is in a category of class I. The Gram staining results for this Group demonstrate a preference for positive rod-shaped cells although 2.2VZI has a balance of negative and positive rod-shaped cells and 2.2CUC, 3.3CUC and 2.2SEI seem to have more positive rod-shaped cells.

In the subgroup XIIc, 3.2MON1.1 and 3.3CUC7.1 are related with 2.2SEI1.3. The SBI class of this last one is of a category class III while 3.2MON belongs to a class II category and 3.3CUC belongs to a class I category. All these WWTP belong to a different SBI class. The similarity percentage is not too high (53% between 3.2MON1.1 and 3.3CUC7.1 and 37% between these two and 2.2SEI1.3). This percentage is low but, even so, these samples are in the same group. Sometimes, in genetic terms, microorganisms that are different are related in small way since all organisms, following the evolutionary trail, are related. When organisms are found with a low similarity between them, this can be the reason. The other three isolates are less related with the previous three and these are 3.3LAG3.3, 3.3ZIA2.1 that are related between

each other with 50% of similarity and share 41% of similarity with 3.3MON2.1. Only the 3.3LAG isolate has a WWTP that has an SBI belonging to a category of class I. These three samples share with the previous three samples a similarity of only 24%. The Gram staining results for this group do not vary much from the other XII groups showing a higher affection for negative rod-shaped cells with one example (2.2SEI) of mostly positive rod-shaped cells and two examples (3.3CUC and 3.3LAG) of a balance between positive and negative rod-shaped cells.

Group XIII

The group XIII was also divided into two subgroups (XIIId and XIIIe). In XIIId the isolates 2.2LAG1.1 and 3.3CUC2.1 belong to different WWTP but they share a 40% similarity. Related to these two isolates there are another two slightly better related, sharing a 42% similarity. These isolates are 2.2CUC2.1 and 2.2SEI2.1. These last pair isolates share a 30% similarity with the first pair. Truth is, at this stage, similarities are rare. Related with these four isolates is 2.2MON2.1 with a similarity close to 22%. This is a very heterogeneous group. All these isolates, except from 2.2SEI, have an SBI value that belongs to a category of class I. Related to these isolates, with 20% of similarity are the isolates 2.2VZI5.2 and 2.2VZI5.3 that show a 100% similarity with one another. Taking into consideration how the samples were isolated and these two came from the same Petri dish it is possible that they are indeed the same organism. Visually the band pattern is very similar.

In XIIIe the isolates 2.2LAG8.1 and 2.2LAG8.2 share 90% of similarity because they came from the same WWTP and are related with a 43% similarity to the isolates 2.2VZI3.2 and 3.2MON5.1 which share a similarity with one another of 50%. The Gram staining results for this group XIII (XIIId and XIIIe) show a balance between positive and negative rod-shaped cells having two WWTP with a higher affection for negatives, two with a higher affection for positives and two with a balance between both negative and positive rod-shaped cells.

Initially, it was expected that the samples would group according to their geographical localization like in the study of Liu *et al.* (2001) that in its results indicate that the microbial community structure of activated sludge varies between geographically distinct wastewater treatment plants. In this study a pattern like this was not verified. When someone studies patterns of species, they get the idea that it is easy to establish a correlation between species and how certain species relate to each other.

But when a set of samples originate from a heterogeneous group, an obvious relationship is not expected in the assemblage (Sugihara *et al.,* 2003). In this work the heterogeneity originates from geographical parameters as well as from different seasons and years of the sample collection was verified. In work of Liu *et al*., 2001, they assumed that all the disparity between the groupings is attributed, despite the reasons due to differences in wastewater composition and plant operation that affect the structure of the indigenous microbial communities. In this case, the reason cannot be this one because the same WWTP are separated from the isolates that belong to the same WWTP. Residuals can change with geographical location but also depend on the level of life of the people that inhabit certain areas; once again, the geographical region of these samples is almost the same which exclude this hypothesis. Also, Portugal is such a small country and microorganisms have a tendency to be similar. In this study this was not verified because, despite of the similarity level, the similarity was not very high in any isolate.

Tabka *et al*., (1993) and Liu *et al*., (2001), found that activated sludge from different wastewater treatment plants does vary from one wastewater treatment plant to other but the reason why the microorganisms are so different between the same WWTP remains unknown.

Liu *et al*, 2001 and Wagner (1993) assumed that WWTP in different continents clustering together suggesting that there is a microbial community in geographical regions. The isolates in my results are mixed together. It would be interesting take another geographical region and do the same treatment with the goal of see if that isolates form two different populations based on the geographical regions

Different clusters can yield different dendrograms in aspect (Legendre and Legendre, 1983) and for single linkage methods like this one whose only parameter is a genetic parameter (Sugihara *et al.,* 2003) advocate UPGMA as the best method.

CHAPTER V

Conclusions and Future Work

Upon finishing the present work some conclusions can be achieved. Being a exploratory work, similar work to relate with the present results was difficult to find, making the discussion even harder.

Beside the fact that the microorganisms grouped, they grouped with low similarity. Even when isolates grouped with a higher similarity, the similarity was never really that high. Even in Gram staining results, all samples showed Gram negative and Gram positive isolates with no indication of a pattern.

The isolates did not show any pattern in their distribution. Even the Geographical regions did not group together frequently.

In conclusion, molecular techniques together with bioinformatics can have a significant contribution to the study and comprehension of the complex communities of activated-sludge systems, namely the prokaryotic component.

In the future, the same analyses can be made with different primers in order to confirm and see better the relations between the microorganisms. Also, different molecular techniques can be used. As an example it is possible enumerate; random amplification of polymorphic DNA (RAPD), restriction fragment length polymorphism (RFLP) detection and southern hybridizations with various repetitive sequence-based probes. Techniques like these are able to group microorganisms and assure that the isolates are in the right group. In recent years, methods that couple PCR and rRNA based microbial phylogeny have been developed and used to assess the diversity within microbial communities in terms of the kinds and relative abundance of various phylogenetic groups of organisms.

Some authors thought in better ideas that can be applied in the future. While the data obtained cannot be directly translated into taxonomic terms (i.e., species), the concept of an operational taxonomic unit (OTU) can be employed to provide a way to overcome this problem (Moyer *et al*., 1996). Instead of comparing communities based on the analysis of cloned 16S rDNA, the OTU richness and evenness (of numerically dominant members of the community) are estimated from restriction site polymorphisms or differences in the melting behavior of the 16S rDNA genes. Both of these methods provide insight to the diversity of 16S rDNA genes (or OTUs) present in a community, while obviating the need for extensive sequence analysis. However measures of microbial diversity based on differences in 16S rDNA genes are also limited because, while they provide an estimate of the number and kinds of

phylogenetically distinct groups of organisms in the community, they do not provide direct measures of genetic diversity *per se*.

Another interesting approach, just with the intention of seeing how Portuguese microorganisms behave, another geographical region could be chosen and its results compared with the results from this work in order to try and find a pattern in Portuguese population.

CHAPTER VI

Bibliography

Amann, R., Fuchs, B.M. and Behrens, S. (2001). **The identification of microorganisms by fluorescence *in situ* hybridization.** *Current Opinion in Biotechnology*, 12:231–236.

Amann, R., Lemmer, H. and Wagner, M. (1998). **Monitoring the community structure of wastewater treatment plants: a comparison of old and new techniques.** *FEMS Microbiology Ecology*. 25, 205-215.

Apha (1995). **Standard methods for the examination of water and wastewater.** *19th edn American public health association*, Washington DC.

Betancourt, W.Q. and Rose, J.B. (2004). **Drinking water treatment processes for removal of Cryptosporidium and Giardia.** *Vet. Parasitol*., 126(1–2), 219–234.

Bitton, G. (1978). **Survival of enteric viruses.** *In Water Pollution Microbiology ,Vol. 2 (ed. R. Mitchell),* pp. 273–300, Wiley Interscience, New York, USA

Bock, J. K., Loebell, H. and Morey, R. (1992). **From conceptual roles to structural relations: Bridging the syntactic cleft.** *Psychological Review,* 99, 150-171.

Bonde, G.J. (1977). **Bacterial indication of water pollution.** *In Advances in AquaticMicrobiology (eds. M.R. Droop and H.W. Jannasch),* pp. 273–364. AcademicPress, London.

Bott, C.B. and Love, N.G. (2002). **Investigating a mechanistic cause for activated sludge deflocculation in response to shock loads of toxic electrophilic chemicals.** *Water Environmental Research,* 74 (3), 306-315.

Brockman, F.J. (1995). **Nucleic acid based methods for monitoring the performance of in situ bioremediation.** *Mol. Ecol.* 4: 567-578.

Bryan, A., Shapir, N. and Sadowsky, M.J. (2004). **Frequency and distribution of tetracycline resistance genes in genetically diverse, nonselected, and nonclinical Escherichia coli strains isolated from diverse human and animal sources.** *Appl Environ Microbiol.* 70:2503–7.

Carvalho, C.R. and Fernandes, P. (2010). **Production of Metabolites as Bacterial Responses to the Marine Environment. Mar. Drugs. Vol.** *8*. pp.705-727.

Codd, G.A. (1995). **Cyanobacterial toxins: occurrence, properties and biological significance.** *Wat. Sci. Tech.,* 32, 4: 149-156.

Cooper, S. (2004). **Bacterial Growth and Division.** *In Encyclopedia of Molecular and Cellular Biology and Molecular Medicine*. *Meyers, R.A. (ed),* Weinheim, Germany: Wiley-VCH, pp. In Press.

Curds, C.R. (1982). **The ecology and role of protozoa in aerobic sewage treatment processes.** *Annu. Rev. Microbiol.,* 36, 27-46.

Diaz-Guerra T.M., Martinez-Suarez J.V., Laguna F. and Rodriguez-Tudela J.L. (1997). **Comparison of four molecular typing methods for evaluating genetic diversity among *Candida albicans* isolates from human immunodeficiency viruspositive patients with oral candidiasis.** *J Clin Microbiol 35*:856-861.

Eckenfelder, W.W. (1978). **Technical consideration of biological, physical and chemical treatment combination for improved efluent quality from industrial wastewater.** *Prog Water Technol.,* 10: 434±441.

Flores-Alsina, X., Comas, J., Rodriguez-Roda, I., Gernaey, K.V. and Rosen, C. (2009). **Including the effects of filamentous bulking sludge during the simulation of wastewater treatment plants using a risk assessment model.** *Water Research,* 43: 4527-4538.

Frey, W. (1992). **A comparison of different aeration systems.** *Water Sci. Technol.,* 25, 143-149.

Fulthorpe, R.R., Liss, S.N. and Allen, D.G. (1993). **Characterization of bacteria isolated from a bleached Kraft pulp mill wastewater treatment system.** *Can. J. Microbiol.* 39, 13–24.

Gilbride, K.A., Lee, D.Y. and Beaudette, L.A. (2006). **Molecular techniques in wastewater: Understanding microbial communities, detecting pathogens, and real-time process control.** *Journal of Microbiological Methods*, 66, 1–20.

Grant, S.B., Pendroy, C.P., Mayer, C.L., Bellin, J.K. and Palmer, C.J. (1996). **Prevalence of enterohemorrhagic *Eschichia coli* in raw and treated municipal sewage.** *Appl. Environ. Microbiol.,* 62, 3466-3469.

Gray, N.F. (1990). **Activated Sludge: Theory and Practice.** *Oxford: Oxford University Press*.

Gray, N.F. (1999). **Water Technotogy-an Introduction for Scientists and Engineers.** *Oxford: Boston Elsevier/Butterworth-Heinemann*.

Guide Water UK. (2006).

Hansen S, Mehlum JE and Bakken LR (1993). **N20 and CH4 fuxes in soil influenced by fertilization and tractor traffic.** *Soil Biol Biochem.,* 25,621-630.

Hawkes, H. A. (1983). **Activated Sludge.** *in Ecological aspects of used-water treatment, 2,* Londres, Academic Press, 1983, pp. 77-162.

Higgins, M.J. and Novak, J.T. (1997). **Dewatering and Settling of Activated Sludges: The Case for Using Cation Analysis.** *Water Environment Research. Vol. 69, No. 2* (Mar. - Apr., 1997), pp. 225-232.

Hiraoshi, A., Muramatsu, K. and Urato, K. (1995). **Characterization of new denitrifying *Rhodobacter* strains isolated from photosynthetic sludge to wastewater treatment.** *J. Ferm. Biogen.*, 79, 39-44.

Howgrave-Graham, A.R. and Steynm, P.L. (1988). **Application of the fluorescent-antibody technique for the detection of *Sphaerotilus natans* in activated sludge.** *Appl Environ Microbiol.*, 54, 799-802**.**

Jenkins, D., Ramadori, R. and Cingolani, L. (1993). **Prevention and Control of Bulking Activated Sludge.** *Centro Luigi Bazzucchi, Perugia.*

Jenkins, D., Richard, M.G. and Daigger, G.T. (2004). **Manual on the Causes and Control of Activated Sludge Bulking, Foaming, and Other Solids Separation Problems.** *London: Lewis Publishers*.

Kampfer, P. and Dott, W. (1989). **Numerische Identiflzierung aquatischer Mikroorganismen mittels automatisierter Methoden am Beispiel von Bakterien aus dem belebten Schlamm.** *Zbl Bakt Hyg. B,* 187, 216-229**.**

Larsdotter, K. (2006). **Microalgae for phosphorus removal from wastewater in a Nordic climate.** *A Doctoral Thesis from the School of Biotechnology, Royal Institute of Technology, Stockholm, Sweden,* ISBN: 91-7178-288-5.

Lee, N.M. and Welander, T. (1996). **Use of protozoa and metazoa for decreasing sludge production in aerobic wastewater treatment.** *Biotechnol Lett* 18: 429±434

Legendre, P. and Legendre L. (1983). **Numerical ecology.** *Develop. Environ. Model. V. 3.* Elsevier.

Lens, P. and Stuetz, R. (2004). **Water and Environment Management Series.** *Alliance House Street, London UK. 3º Edition*.

Liu, W.T., Marsh, T.L., Cheng, H. and Forney, L. J. (1997). **Characterization of microbial diversity by determining terminal restriction fragment length polymorphisms of genes encoding.** *16S rRNA. 63,* 4516-4522.

Lopes, A. M. and Fonseca, Á. (1996). **Biologia Microbiana.** *Universidade Aberta.*

Madoni, P. (1994). **A sludge biotic index (SBI) for the evaluation of the biological performance of activated sludge plants based on the microfauna analysis.** *Water Res.*, 28:67-75.

Madoni, P., Davoli, D. and Chierici, E. (1993). **Comparative analysis of the activated sludge microfauna in several sewage treatment works**. *Water Res.*, 27:1485–91.

Martins, A.M., Pagilla, K., Heijnen, J.J. and Loosdrecht, M. (2004). **Filamentous bulking sludge - a critical review.** *Water Res.*, 38:793-817.

Matsui, S., Barrett, F.D. and Banerjee, J. (1991). **Guidelines of Lake Management. Toxic Substances Management in Lakes and Reservoirs.** *International Lake Environment Committee Foundation, Otsu. Vol. 4.* 170 pp.

Megank, M.T.J. and Faup, G.M. (1988). **Enhanced biological phosphorus removal from WasteWaters.** *Biotreatment Syst.*, 3, 111-204.

MetCalf and Eddy, I. (2003). **Wastewater engineering: treatment and reuse.** *4ª Edition. McGraw-Hill Higher.*

Moyer, C. L., Tiedje, J. M., Dobbs, F. C., and Karl, D. M. (1996). **A computer-simulated restriction fragment length polymorphism analysis of bacterial small-subunit rRNA genes: Efficacy of selected tetrameric restriction enzymes for studies of microbial diversity in nature.** *Appl. Environ. Microbiol. 62*, 2501-2507.

Muyzer, G. and Smalla K. (1998). **Application of denaturing gradient gel electrophoresis (DGGE) and temperature gradient gel electrophoresis (TGGE)**. *in microbial ecology.*

Neto, M. (2008). **Alterações na Comunidade Microbiana de um Solo Contaminado com Gasóleo.** *Tese de Mestrado, Faculdade de Ciências e Tecnologia, Universidade Nova de Lisboa.*

Nicolau, A. (2009). **Identificação de problemas ao Nível do Decantador e Controlo do Crescimento Filamentoso.** *1º Nível. Biotempo, Consultoria em Biotecnologia*.

Oliveira, J.S. (1982). **Operações e processos fundamentais em engenharia sanitária. Fundamentos da depuração biológica.** *Faculdade de Ciências e Tecnologia. Universidade Nova de Lisboa*.

Painter, H.A. (1978). **Biotechnology of wastewater treatment. In the oil industry and microbial ecosystems.** *Chater, K. and Someville, H* (ed.), London, Heyden and son.

Painter, H.A. (1983). **Metabolism and physiology of aerobic bacteria and fungi.** *In Ecological Aspects of Used Water Treatment, Vol. 2 (eds. C.R. Curds andH.A. Hawkes),* pp. 11–75. Academic, London.

Painter, H.A. (1986). **Nitrification in the treatment of sewage and wastewater.** *Nitrification (ed. J.I. Prosser),* IRL Press, Aberdeen, pp. 185-211.

Prescott, L., Harley, J. and Klein, D. (2005). **Microbiology**. *6th edition McGraw-Hill*

Prescott, L. et al,. (2009). **Microbiology.** *7th edition Wm. C. Brown Publishers. (Unit – 1, 4)*

Ratsak, C.H., Koojiman, S.A.L.M. and Kooi, B.W. (1993). **Modelling the growth of an oligochaete on activated sludge.** *Water Res.* 27, 739-747.

Robertson, L.A. and Kuenen, J.G. (1992). **Nitrogen removal from water and waste.** *Microbial Control of Pollution*. Cambridge University Press, Cambridge, pp. 227-267.

Rocher, M., Goma, G., Pilas Begue, A., Louvel, L. and Rols, J.L. (1999). **Towards a reduction in excess sludge processes: Biomass physicochemical treatment and biodegradation.** *Addlied Microbiology Biotechnology,* 51, 883-890.

Sahlstrom, L., Aspen, A., Bagge, E., Danielsson-Tham, M.L. and Albihn, A. (2004). **Bacterial pathogen incidences in sludge from Swedish sewage treatment plants.** *Water Res*. 38, 1989–1994.

Santana, F., Amaral, L. and Maurício, R. (2009). **Técnicas de Drenagem e Tratamento de Águas Residuais.** *Faculdade de Ciências e Tecnologia,* Universidade Nova de Lisboa.

Santos, L. (2008). **Avaliacao do desempenho de estacoes de tratamento de aguas residuais atraves da observacao da comunidade de protozoarios nos sistemas de lamas activadas.** *Tese de Mestrado, Escola de Engenharia, Universidade do Minho.*

Sawyer, C.N. (2003). **Chemistry for Environmental Engineering and Science.** *5th Edition. NewYork. Tata McGraw-Hill*

Seviour, R.J. and Blackall, L.L. (1999). **The Microbiology of Activated Sludge.** *Kluwer Academic Publishers.*

Sneath, P. H. A., Mair, N. S., Sharpe, M. E., Holt, J. G. (1986). **Bergey's manual of systematic bacteriology.** *Vol. 2. Williams and Wilkins Co.*, Baltimore.

Sugihara, G., Bersier, L.F., Southwood, T.R.E., Pimm, S.L. and May, R.M. (2003). **Predicted correspondence between species abundances and dendrograms of niche similarities**. *PNAS vol. 100 No. 9.*

Tabka, H., Seignez, C., Adler, N., Pulgarin, C. and Peringer, P. (1993). **Inoculum standardization for biodegradability tests.** *Biotechnol. ¹echn. 7*, 217-222.

Talbot, G., Topp, E., Palin, M.F. and Masse, D.I. (2008). **Evaluation of molecular methods used for establishing the interactions and functions of microorganisms in anaerobic bioreactors.** *Waterresearch*. 42, 513 – 537.

Tandoi V., Jenkins D. and Wanner J. (2006). **Activated sludge separation problems.** *IWA Publishing, London.*

Vassart, G., Georges, M., Monsieur, R., Brocas, H., Lequarre, A.S. and Christophe, D. (1987). **A sequence in M13 phage detects hypervariable minisatellites in human and animal DNA.** *Science,* 235, 683-684.

Victoria, L., Gilbride, K.A., Allen, D.G. and Liss, S.N. (1996). **Phenotypic fingerprinting of microbial communities in wastewater treatment systems.** *Water Res.*, 30, 1077–1086.

Wagner, M., Brumelis, D. and Gehr, R. (2002). **Disinfection of wastewater by hydrogen peroxide or peracetic acid: Development of procedures for measurement of residual disinfectant and application to a physicochemically treated municipal effluent.** *Water Environ. Res*., 74: 33-50.

Wanner, J. (1994). **Activated sludge population dynamic.** *Water Sci. Technol.* 30, 159-169.

Widada, J., Nojiri, H. and Omori, T. (2002). **Recent developments in molecular techniques for identification and monitoring of xenobiotic-degrading bacteria and their catabolic genes in bioremediation.** *Appl Microbiol Biotechnol.* 60, 45–59.

Websites:

http://science.nayland.school.nz/graemeb/yr11%20work/microbes/bacteria.htm. Accessed in December, 2011.

http://weather.nmsu.edu/Teaching_Material/SOIL350/waste_water_treatment_plant.htm. Accessed in January, 2011.

http://www.akvo.org/wiki/index.php/Activated_Sludge. Accessed in December, 2011.

http://www.bioinformatics.nl/webportal/background/techniques.html. Accessed in January , 2011.

http://www.piercenet.com/browse.cfm?fldID=5558F7E4-5056-8A76-4E55-4F3977738B63. Accessed in December, 2011.

http://www.purdue.edu/rem/hmm/ethidbr.htm. Accessed in February, 2011.

http://www.biosystematica.com/bionumerics.pdf. Accessed in December, 2011.

http://www.applied-maths.com/bionumerics/modules/bn_tn.htm. Accessed in January, 2011.

Appendix

Appendix I

Gram staining process:

First, a colony was selected and a sterilized loop was used in order to prepare a thin layer of sample in a glass slide glass.

- The previous colony was resuspended in a water drop previously placed in the slide.
- The smear was air dried

Then, the coloration was made:

- The smear was placed inside a recipient containing Crystal violet by 1 minute.
- The slide glass was cleaned with running water.
- The smear was placed inside a recipient containing Lugol's solution for 1 minute.
- The slide glass was cleaned with running water.
- 30 seconds dropping alcohol drop by drop in to the slide glass
- The smear was placed inside a recipient containing safranine solution for 1 minute (see better detailed description in the appendix).

Gram staining consists in the following steps;

- Primary stain; (Crystal violet, methyl violet or Gentian violet). The primer dye is a stable solution and may contain a mordant with the goal of making the constancy of the solution. Sodium bicarbonate can also be added with the objective of intensifying the color. Alkaline pH is better (Sheppe and Constable, 1923).
- Mordant; Gram's iodine (James and Mittwer, with no year).
- Decolorize; (ethyl alcohol, acetone or 1:1 ethanol-acetone mixture). Most of the times, the alcohol used is 95%, but acetone can also be used or a mixture of alcohol and acetone (Lillie, 1928). This is the most critical step in all the process because there is a danger for both over and under discoloration (Neide, 1904)
- Counterstain; (Dilute carbol fuchsin, safranin or neutral red) (James and Mittwer, no year).

Appendix II:

Mediums and buffers composition

TSB

Formula / Liter

Enzymatic Digest of Casein ..... 17.0 g

Enzymatic Digest of Soybean Meal ..... 3.0 g

Sodium Chloride ..... 5.0 g

Dipotassium Phosphate ..... 2.5 g

Dextrose ..... 2.5 g

Final pH: 7.3 ± 0.2 at 25°C

TSA

Trypticase peptone.....1.5%

Phytone peptones.....0.5%

NaCl.....0.5 %

Agar.....1.5%

Enzymatic Digest of Casein and Enzymatic Digest of Soybean Meal are nitrogen sources in TSB. Dextrose is the carbon energy source that facilitates organism growth. Sodium Chloride maintains osmotic balance and Dipotassium Phosphate is a buffering agent. This medium was weighted just like the TSA medium.

TE buffer

For 1 liter of 1x TE solution

1M Tris-HCl pH 7.5 or 8.0……10ml/10mM

0.5M EDTA pH 8.0……2ml/1mM

ddH_2O……..998ml

TAE

In its composition there is Tris-acetate buffer, usually at pH 8.0, and EDTA, which sequesters divalent cations (Ogden and Adams, 1987).

Buffer was weighed in an analytic scale in a 1L shot, was agitated with a magnetic agitator and then was put inside the electrophoresis

10X TBE Electrophoresis Buffer

Tris Base…….108g

Boric Acid……55g

0.5M EDTA…….20ml

Water to 1.0 L

Appendix III

DNA ladder:

BAND SIZE (bp)	ng/BAND
10000	100
7500	75
6000	60
5000	50
4000	40
3000	30
2500	25
2000	20
1400	14
1000	100
800	80
600	60
400	40
200	20

Figure 8: Ladder NZYDNA ladder III with corresponding band size

Appendix IV

These tables belong to the project PROTOFILWWWW and are not results of my work, although, these parameters are essentials for the relation between different results:

Table 10: Physico-chemical characteristics

WWTP ID	0_2 mg/L (AT)	pH	SST mg/L (AT)	SSV mg/L (AT)	SST (In)	SST (Out)	SST%	SSV (In)	SSV (Out)	SSV %	N mg/L (In)	N mg/L (Out)	Ntotal%	BOD (In)	BOD (Out)	BOD%	COD (In)	COD (Out)	COD%
2.2.MON	2,86	7,46	2605	1900	305	5	98	228	3	99	175	18	90	400	6	99	890	48	95
3.2.MON	1,53	7,24	3820	2960	423	12	97	363	9	98	160	8	95	800	14	98	1080	37	97
2.2.VZI	0,51	7,54	2680	1930	156	2	99	102	1	99	12	10	17	257	8	97	359	24	93
3.2.VZI	2.00-5.00	6-9	2690	2030	456	4	99	292	3	99	146	50	66	240	9	96	560	61	89
2.2.CUC	6,35	8,43	4400	2833	427	6	99	340	4,4	99	77	18	77	203	8,7	96	815	27	97
3.3.CUC	0,22	7,41	4633	3132	1107	8	99	737	5	99	53	3,6	93	161	4	98	1562	30	98
2.2.LAG	1,26	7,07	5600	4200	668	4,2	99	563	2,4	100	82,5	5	94	483	8	98	1435	35	98
3.3.LAG	1,15	7,13	2933	1933	264	8	97	224	5	97,8	67,4	15	77,74	225	21	91	508	45	91
2.2.SEI	0,45	7,18	4467	3367	236	21	91	198	17	91	50	21,7	57	296	8	97	699	64	91
3.3.SEI	0,16	7,12	3433	2834	428	23	95	382	20	95	92	21	77	422	15	96	1028	53	95
2.2.ZIA	3,27	6,88	3922	2844	382	8,8	98	330	6,8	98	49,8	9,33	81	341	8	98	1005	26,9	97
3.3.ZIA	0,14	7,54	4567	3732	207	33	84	177	26	85	209	194	7	261	46	82	989	593	40

Table 11: Microbiologic Characteristics (ind/mL). Protozoa (part 1)

	Flagellates		Techaete amoeba			Free-swimmers		
WWTP ID	*Peranema* sp.	Flagelado not identified 1	*Arcella* sp.	*Euglypha* sp.	*Centropyxis* sp.	*Colpidium* sp.	*Cinetochilum margaritacium*	*Spirostomum teres*
2.2.MON	80		360	460				
3.2.MON								
2.2.VZI	20		260				60	
3.2.VZI			20					
2.2.CUC	40	1		1780	240		200	
3.3.CUC								
2.2.LAG	80		20			40		60
3.3.LAG	40							140
2.2.SEI	40	60	380					
3.3.SEI	80				20			
2.2.ZIA	60		740	260	400			
3.3.ZIA			20		40			

Table 12: Microbiologic Characteristics (ind/mL). Protozoa (part 2)

	Crawlers ciliates									
WWTP ID	*Chilodonella uncinata*	*Trithigmostoma cucullulus*	*Trochilia minuta*	*Aspidisca cicada*	*Aspidisca lynceus*	*Euplotes patella*	*Oxytricha* sp.	*Acineria uncinata*	*Acineria incurvata*	*Microthorax* sp.
2.2.MON							20	2080		
3.2.MON									780	
2.2.VZI										15420
3.2.VZI				7280		60		60		
2.2.CUC	20			380	1			20		
3.3.CUC	40		100	940	380			460		
2.2.LAG				100		20				
3.3.LAG								320		
2.2.SEI										
3.3.SEI	80	340		60				4940		
2.2.ZIA				6020						
3.3.ZIA					120			20		

Table 13: Microbiologic Characteristics (ind/mL). Protozoa (part 3)

	Attached ciliate					
WWTP ID	*Vorticella microstoma*	*Vorticella aquadulcis*	*Vorticella convallaria*	*Carchesium* sp.	*Epistylis* sp.	*Opercularia* sp.
2.2.MON	60				5260	
3.2.MON					440	
2.2.VZI			2240		440	
3.2.VZI	680		80		4320	
2.2.CUC	180		340		40	
3.3.CUC		560	200			
2.2.LAG			680	120	5160	40
3.3.LAG		1060	280		80	20
2.2.SEI						2300
3.3.SEI		80			1800	2640
2.2.ZIA		160			220	
3.3.ZIA					4260	

Table 14: Microbiologic Characteristics (ind/mL)ç. Protozoa (part 4)

	Carnivorous								**SBI**
WWTP ID	*Coleps hirtus*	*Litonotus* sp.	*Acineta* sp.	*Tokophrya* sp.	*Discophrya* sp.	*Plagiocampa rouxi*	*Carnivoro (not identified)*	Suctoria (not idenified. 1)	0
2.2.MON									9
3.2.MON				60					7
2.2.VZI		20							9
3.2.VZI									9
2.2.CUC	40				40	400			10
3.3.CUC									9
2.2.LAG			20						9
3.3.LAG									9
2.2.SEI			20						5
3.3.SEI							220		7
2.2.ZIA	520		40	1	240			1	10
3.3.ZIA									7

Appendix V

Gram staining results:

2.2CUC

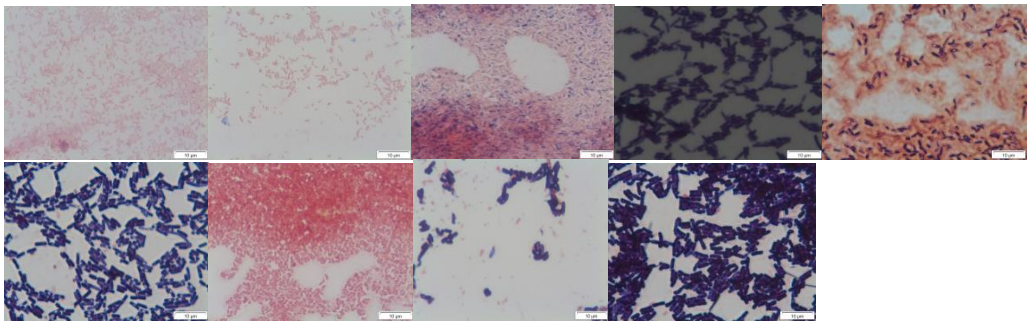

Figure 9: 2.2CUC

(2.2CUC1.1- Negative bacillus. This cells are different from every cells obtained. Seemed like it have litle ball inside the cells ; 2.2CUC1.2- Gram negative bacillus; 2.2CUC2.1- Gram positive bacillus in a red background; 2.2CUC3.1- Bacillus gram positive; 2.2CUC4.1- gram positive in a red background. The cells have spors; 2.2CUC5.1- Bacillus positive with spors; 2.2CUC6.1- Gram negative bacillus; 2.2CUC7.1- Gram positive bacillus; 2.2CUC8.1-Gram positive bacillus).

3.3CUC

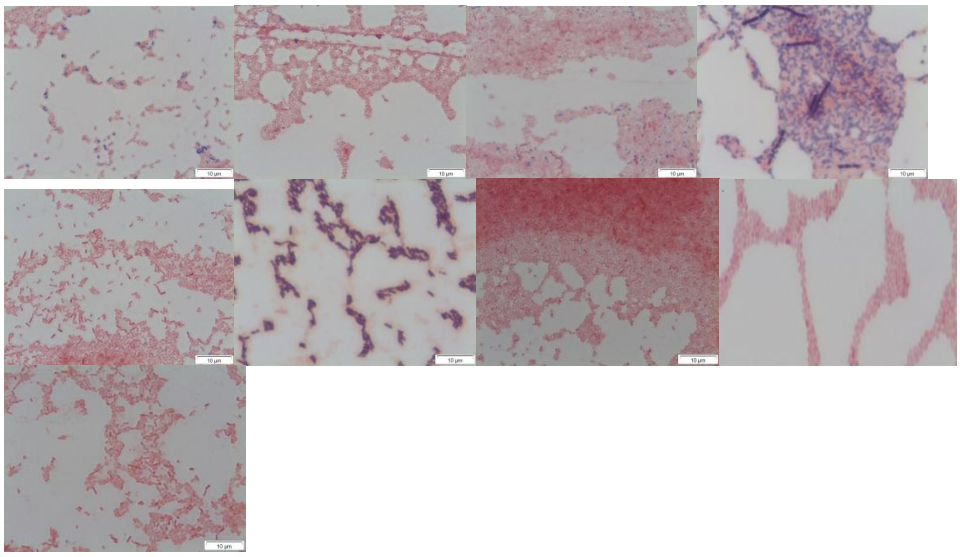

Figure 10: 3.3CUC

(3.3CUC1.1-Gram positive with some negative cells; 3.3CUC2.1- Coccus gram negative; 3.3CUC3.1- Bacillus gram positive but a few gram negative; 3.3CUC4.1- Bacillus some gram positive and others gram negative; 3.3CUC5.1-Bacillus gram

negative; 3.3CUC6.1- Mainly bacillus gram positive but it is possible that bacillus gram, negative exists too; 3.3CUC7.1- Gram negative coccus; 3.3CUC7.2- Gram negative bacillus; 3.3CUC8.1- Gram positive bacillus).

2.2LAG

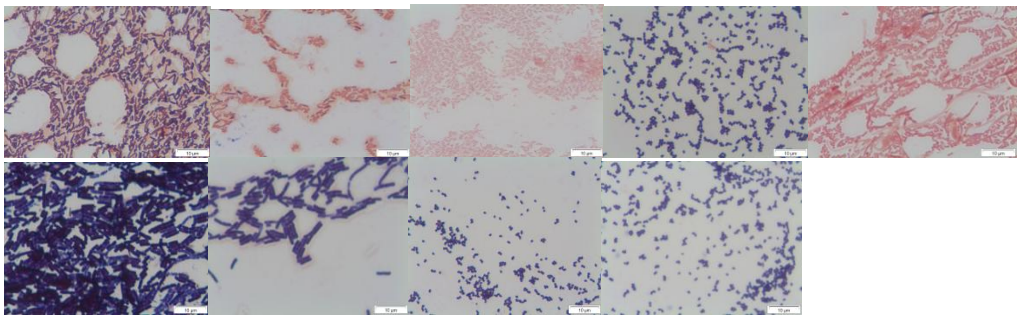

Figure 11: 2.2LAG

(2.2LAG1.1Gram negative bacillus; 2.2LAG1.2- Gram negative bacillus. Some of them; 2.2LAG2.2-Gram negative bacillus; 2.2LAG3.1- Gram positive coccus; 2.2LAG4.1-Gram positive bacillus; 2.2LAG6.1-.Gram negative bacillus; 2.2LAG7.1-Gram positive bacillus; 2.2LAG8.1-Gram positive coccus; 2.2LAG8.2- Gram negative coccus).

3.3LAG

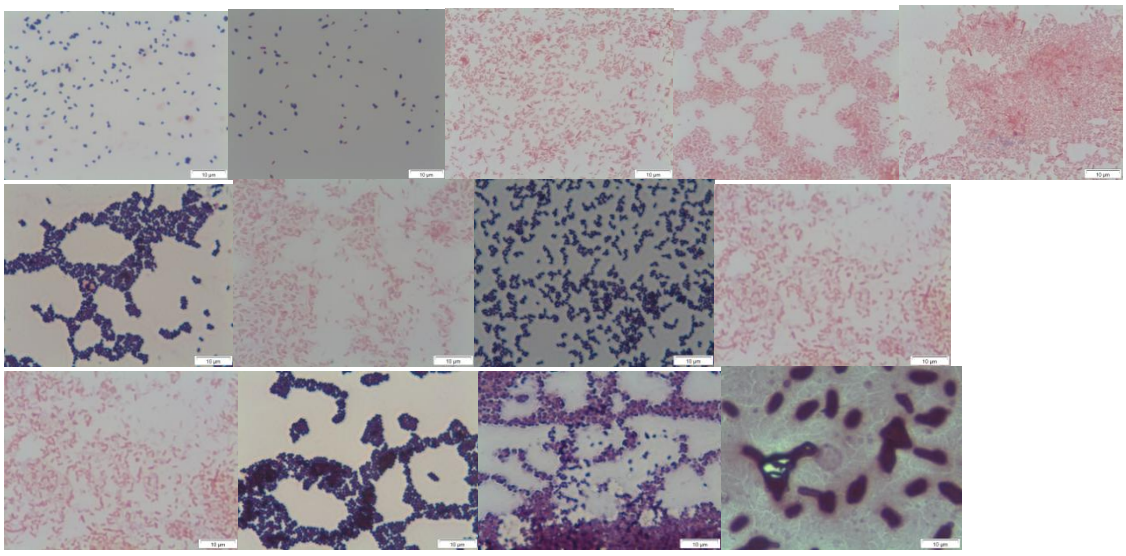

Figure 12: 3.3LAG

(3.3LAG1.1- Gram positive coccus; 3.3LAG2.1- Coccus gram variable; 3.3LAG3.1-Gram negative bacillus; 3.3LAG3.2-Gram negative bacillus; 3.3LAG3.3- Gram negative bacillus; 3.3LAG3.4- Gram positive coccus; 3.3LAG3.5- Gram negative,

coccus; 3.3LAG4.1-Gram positive coccus; 3.3LAG4.2- Gram negative bacillus; 3.3LAG5.1- Gram negative bacillus; 3.3LAG5.2- Gram positive, coccus; 3.3LAG6.1-Gram positive coccus; 3.3LAG7.1- Gram negative bacillus).

2.2MON

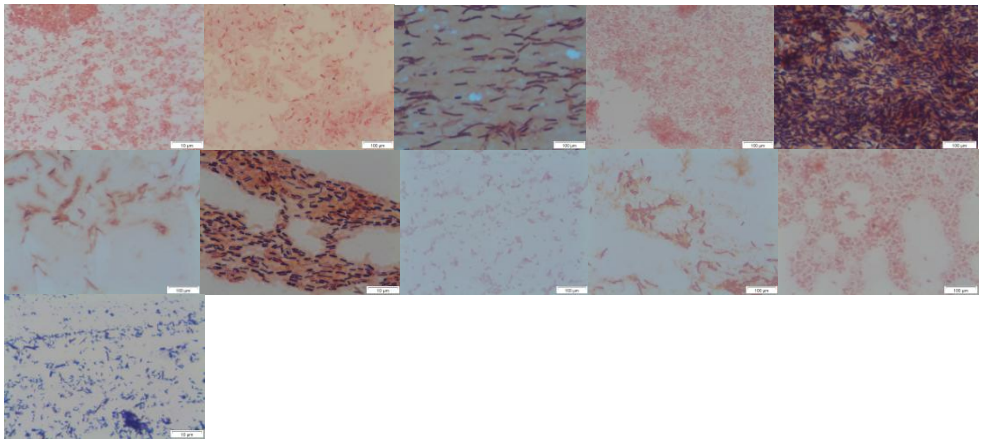

Figure 13: 2.2MON

(2.2MON1.1-Bacillus gram negative; 2.2MON2.1-Bacillus gram negative; 2.2MON3.1-Gram positive bacillus in a negative background; 2.2MON3.2- Bacillus gram negative; 2.2MON4.1-gram negative in a positive background; 2.2MON4.2-Gram positive bacillus; 2.2MON4.3-gram positive bacillus in a positive background; 2.2MON4.4-Coccos gram negative; 2.2MON5.1-gram negative bacillus; 2.2MON6.1-Bacillus gram negative; 2.2MON6.2-positive coccus).

3.2MON

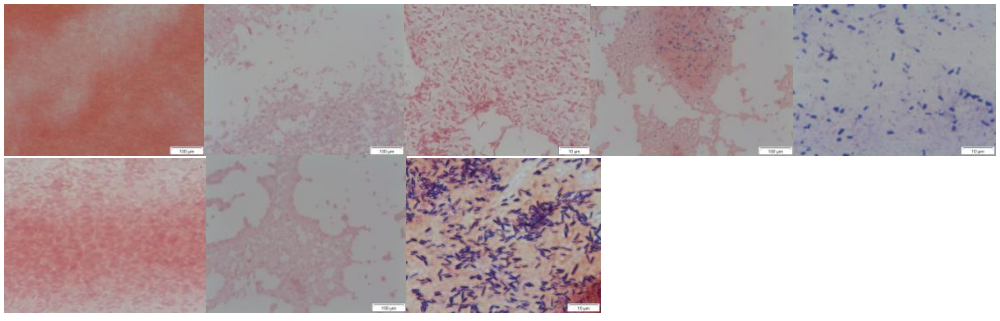

Figure 14: 3.2MON

(3.2MON1.1- Bacillus gram negative; 3.2MON1.2- Gram negative, Bacillus; 3.2MON2.1- Gram negative bacillus; 3.2MON2.2- Mainly gram negative bacillus but

there is some positive cells; 3.2MON3.1- Negative and positive Coccus; 3.2MON3.2- Gram negative bacillus; 3.2MON3.3- Coccus gram negative; 3.2MON5.1- Gram positive in a negative background).

2.2SEI

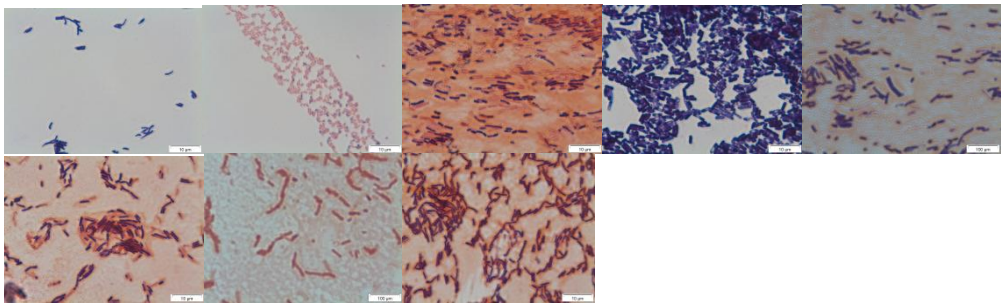

Figure 15: 2.2SEI

(2.2SEI1.1-Bacillus gram positive; 2.2SEI1.2-Cocos gram negative; 2.2SEI1.3-Bacillus with red background gram positive; 2.2SEI2.1-Bacillus gram positive; 2.2SEI3.1-Big bacillus ; 2.2SEI6.1-Bacillus with red background gram positive; 2.2SEI7.1-Bacillus gram negative; 2.2SEI8.1-Bacillus with red background gram positive).

3.3SEI

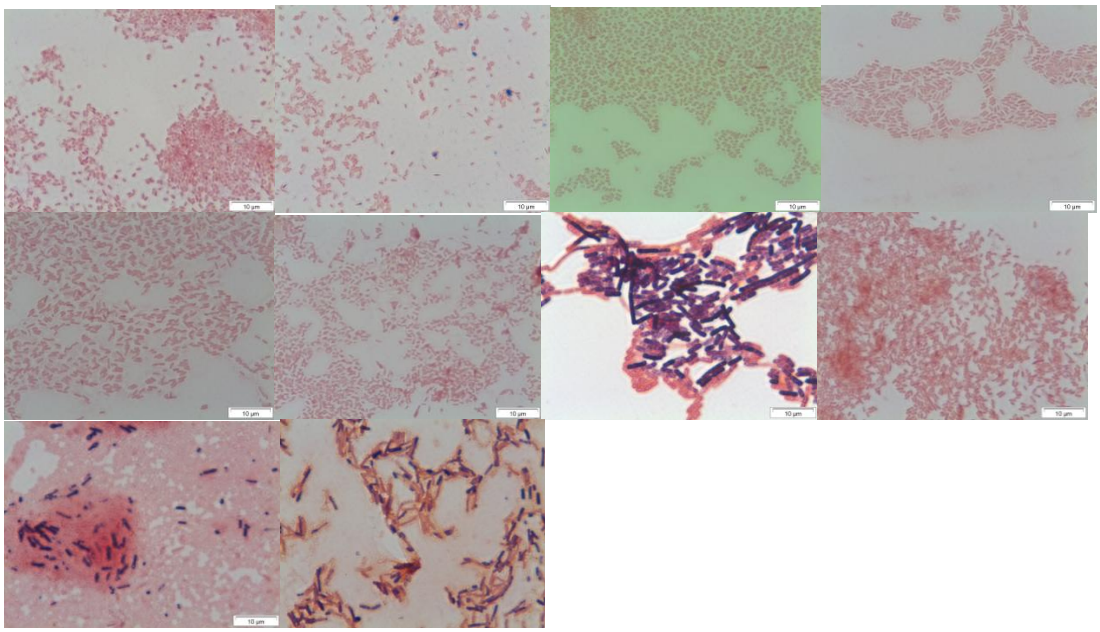

Figure 16: 3.3SEI

(3.3SEI1.1-Bacillus gram negative; 3.3SEI2.1-Bacillus gram negative; 3.3-SEI3.1-Bacillus gram negative; 3.3SEI3.2-Bacillus gram negative; 3.3SEI5.1-Bacillus gram negative; 3.3SEI6.1-Bacillus gram variable; 3.3SEI7.1-Bacillus gram negative; 3.3SEI8.1-Bacillus gram positive and coccos gram negative; 3.3SEI8.2-Bacillus with red background gram positive).

2.2VZI

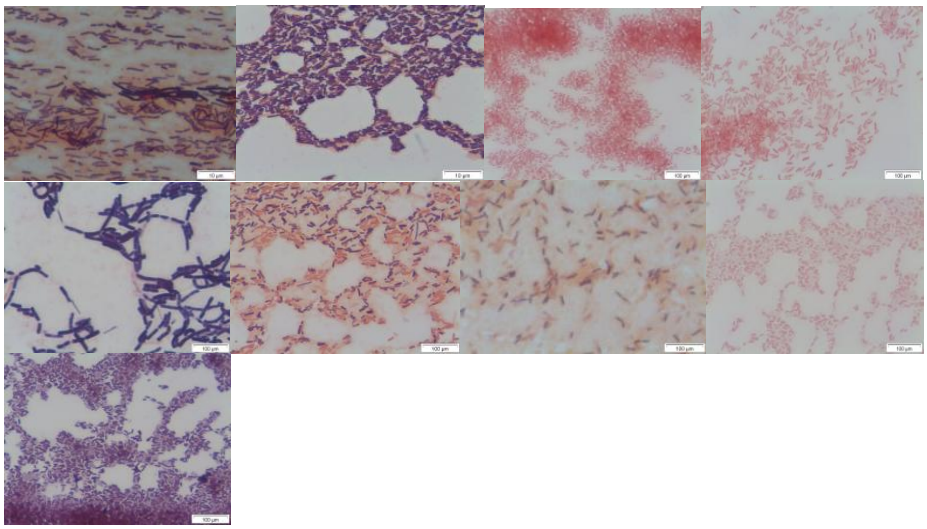

Figure 17: 2.2VZI

(2.2VZI1.1-Bacillus with red background gram positive; 2.2VZI1.2-Bacillus gram variable; 2.2VZI2.1-Bacillus gram negative; 2.2VZI3.1-Bacillus gram negative; 2.2VZI4.1-Bacillus gram positive; 2.2VZI4.2-Bacillus gram variable; 2.2VZI5.1-Cocos gram negative and Bacillus gram positive; 2.2VZI5.2-Bacillus gram negative; 2.2VZI5.3-Bacillus gram positive).

3.2VZI

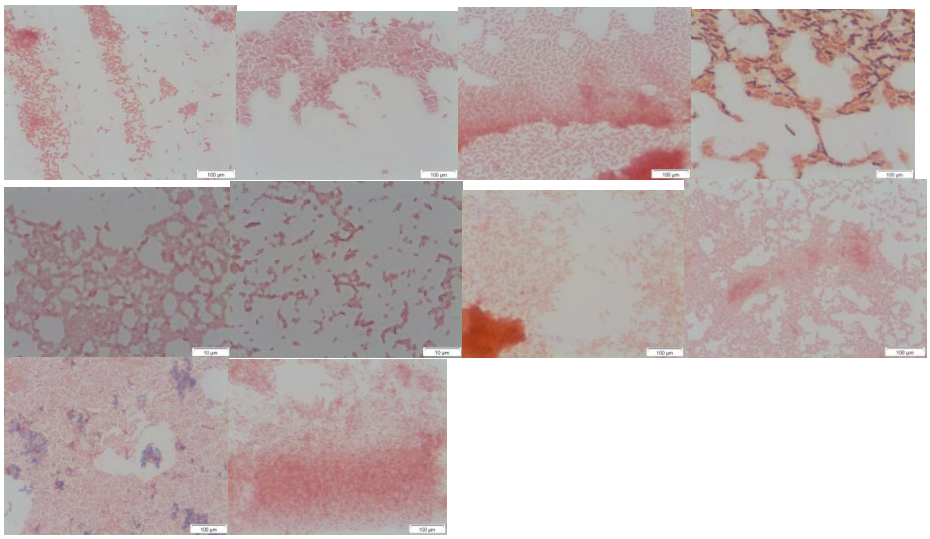

Figure 18: 3.2VZI

(3.2VZI1.1-Short bacillus gram negative; 3.2VZI2.1-Bacillus gram negative; 3.2VZI2.2-Short bacillus gram negative; 3.2VZI3.1-Bacillus gram negative; 3.2VZI 3.2-Bacillus gram negative; 3.2VZI4.1-Bacillus gram negative; 3.2VZI5.1-Bacillus gram negative with blue spots; 3.2VZI5.2-Bacillus with red background gram positive with some gram negatives; 3.2VZI5.3Bacillus predominantely gram negative with some blue spots; 3.2VZI6.1-Bacillus gram negative).

2.2ZIA

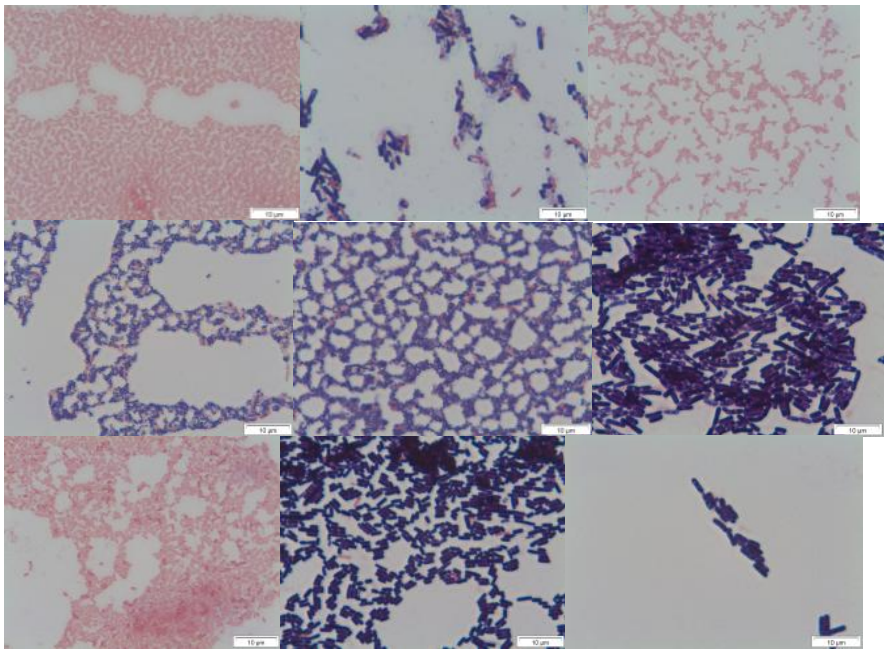

Figure 19: 2.2ZIA

(2.2ZIA1.1-Bacillus gram negative; 2.2ZIA2.1-Bacillus gram positive; 2.2ZIA3.1-Bacillus gram negative; 2.2ZIA4.1-Bacillus gram positive; 2.2ZIA4.1-Cocos gram positive; 2.2ZIA5.1-Bacillus gram positive; 2.2ZIA6.1-Bacillus gram negative; 2.2ZIA7.1-Bacillus gram positive; 2.2ZIA8.1-Bacillus gram positive)

3.3ZIA

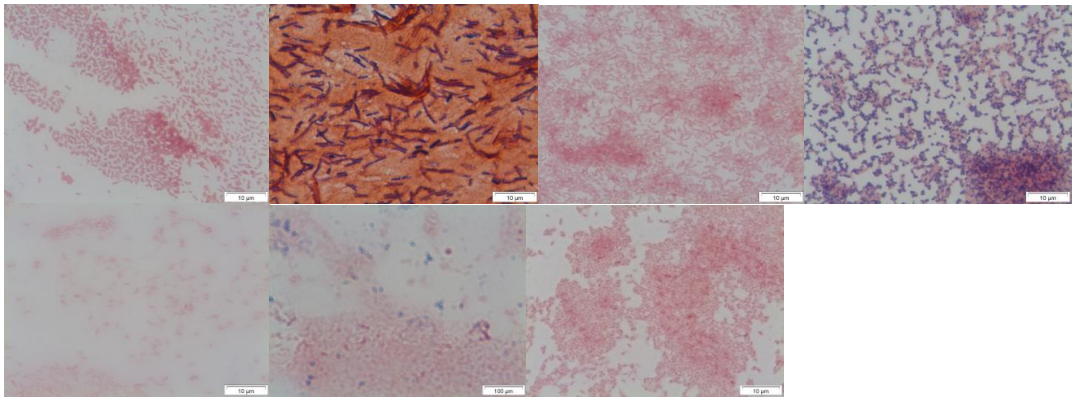

Figure 20: 3.3ZIA

(3.3ZIA1.1-Bacillus gram negative; 3.3ZIA2.1-Bacillus gram negative; 3.3ZIA3.1-Bacillus gram negative; 3.3ZIA3.2-Bacillus gram negative; 3.3ZIA5.1Cocos with red background gram positive; 3.3ZIA5.2-Bacillus gram negative; 3.3ZIA5.3-Bacillus gram negative; 3.3ZIA7.1-Bacillus gram negative).