Microbial Population Dynamics in Indigenous Olive Wastewater Biofilms

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ABSTRACT

The olive industry in South Africa, although small compared to the rest of the world, is rapidly expanding and producing increased volumes of wastewater on an annual basis that could in future develop into a major environmental problem. Olive mill wastewater (OMWW) and table olive wastewater (TOWW) are characterised by high chemical oxygen demand (COD), biological oxygen demand (BOD) and phenolic content that are toxic to the environment. Due to the nature of olive wastewater (OWW), its irresponsible and unregulated environmental release will result in oxygen depletion, nutrient enrichment and accumulation of toxic compounds in receiving water bodies that ultimately disrupts aquatic and terrestrial ecosystems. An estimated 3500 - 4500 tons of olives are processed on an annual basis by 51 farmers in the Western Cape. Economic forecasts predict a steady growth, i.e. increased production and processing of olives in the South African olive industry, in the future due to consumer demand. These production increases will consequently lead to increased volumes of wastewater production, which would, in turn, require an expansion of treatment capacity of the wastewater prior to release. Two South African olive factories were chosen for this study: Buffet Olives, situated in Dal Josefat (Paarl), that produces table olives and Vesuvio Estate on Sorento farm (Wellington) that produce extra-virgin olive oil.

Preliminary COD determinations showed that indigenous OWW biofilms within a rotating biological contactor set-up reduced the COD from TOWW and OMWW by 47% and 32%, respectively, over a 10-day period. These preliminary results strongly suggested that biofilms indigenous to OWW have the potential to remediate the pollution problems of OWW. However, the overall aim of this study was to determine how sustainable the application of indigenous biofilms in the OWW are over two production seasons and whether it would be feasible to apply and develop these naturally occurring biofilms as an effective bioremediation tool to reduce the COD and polyphenol content of OWW.

In this study, the polymerase chain reactions (PCR) and denaturing gradient gel electrophoresis (DGGE) techniques, in combination with statistical techniques were used to fingerprint the OWW (both for TOWW and OMWW) microbial biofilms over time. With these techniques it was possible to monitor the changes in microbial diversity profiles within OWW biofilms in response to seasonal activities over two 6-month production seasons. The biofilms were periodically harvested from OWW streams over two consecutive production periods of approximately 6 months each. The total genomic DNA was then extracted from the biofilms followed by amplification of fragments of the 16S or 18S rDNA by PCR. To determine the total number of operational taxonomic units

(OTU's) present in the OWW biofilms, the 16S and 18S rDNA amplified products were separated using DGGE, followed by multivariate statistical analysis. Furthermore, the DNA of prominent OTU's was subjected to cloning, DNA sequence determination and preliminary phylogenetic analysis.

DGGE and following PCA statistical analysis revealed that both the bacterial and fungal populations in TOWW biofilms were able to re-establish itself from the 2004 to the 2005 production season at Buffet. A similar deduction could be made from bacterial and fungal populations in OMWW biofilms at Vesuvio although the re-establisment of the microbial communities was less pronounced in this case than with the TOWW biofilms. Therefore, the microbial populations from both TOWW and OMWW biofilms compared well over the 2004 and 2005 production seasons and in most cases, the microbial population in the OWW biofilms re-established with similar species diversity after two consecutive seasons of olive production. Phylogenetic analysis of the OMWW biofilms revealed the presence of key bacterial and fungal species that could play a significant role in the bioremediation of OWW, for example the utilisation of polycyclic aromatic compounds. Also of note was the presence of fungi related to *Geotrichum candidum* and bacterial species related to *Pectinatus* and *Pseudomonas* species in OMWW that have previously been shown to contribute to COD and phenol degradation in OMWW.
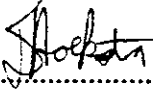
The sensitivity of the indigenous OWW biofilms to various chemicals used during olive processing was also evaluated by artificially treating cultured OWW biofilms with NaOH and other detergents used at the olive factories. In TOWW, NaOH had a severely negative effect on the microbial biodiversity resulting in a 50% reduction in biodiversity, while the antimicrobial properties of the detergents, Contrabac and Robot, reduced the biodiversity by 17% and 4%, respectively. Similarly, in OMWW, the highly alkaline detergents (pH 11 – 12) Removal and Limex, resulted in a 27% and 9% reductions in microbial biodiversity, respectively.

From the good preliminary COD reductions; the slight, but significant, re-establishing of similar microbial populations over two production seasons; as well as the presence of phenol-degrading species according to phylogenetic analysis, it was deduced that OWW biofilms showed a good potential to be used as a future bioremediation tool to treat OWW.

DECLARATION

I, DIRK TJALLING HOEKSTRA hereby declare that the contents of this work represent my own work and that opinions expressed are mine and not necessarily that of the Cape Peninsula University of Technology.

I also herewith state that this work has not previously been submitted for academic examination towards any qualification at any other academic institution.

Signed:......................................

At:....CPUT.............................

on the ...31.... day of ..MARCH............. 2008

BIOGRAPHICAL SKETCH

Dirk Hoekstra was born in Bellville, in the Western Cape in 1981 and went to school in Ladismith (Western Cape) until Grade 6. He continued his education in Robertson until Grade 12. In 2000 he enrolled for the National Diploma in Food Technology at the Cape Technikon, now the Cape Peninsula University of Technology, and graduated in December 2002. He continued his studies towards a Bachalaureus Technologiae in Food Technology in 2003 where he started to investigate the characteristics of the indigenous biofilm organisms growing in olive wastewater. He graduated at the end of 2003.

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CHAPTER 1

GENERAL INTRODUCTION AND PROJECT AIMS

1.1 Background and problem statement

The global olive industry produces vast amounts of wastewater with environmental pollution potential. This has long been the case for Mediterranean countries that contribute to 95% of world olive production and produce 30 million tons of waste residues annually (Bas Jimenez *et al.*, 2000). The South African olive industry, of which 90% of the production is located in the Western Cape, is still in its early growth phase, but expands rapidly annually. There are an estimated 51 commercial olive farmers in the Western Cape that produced and processed approximately 3500 - 4500 tons of olives per year by 1999 (Wesgro's Sector Research Section, 1999). Approximately 40% of the domestic olives are processed as table olives, while the remainder is used for olive oil extraction. Domestic demand increases for table olives and olive oil started in the early 1990's as consumers became aware of the health benefits of olive products and during 1999, in South Africa there was a 10% growth in demand for table olives and 20% for olive oil, annually (Wesgro's Sector Research Section, 1999). An increase in demand will lead to increased production and processing volumes of olives. This increase will concomitantly lead to an increase in the volumes of wastewater produced by the olive processing plants. Regulatory bodies are becoming more concerned with wastewater released and therefore impose evermore stringent restrictions and guidelines (Wesgro's Sector Research Section, 1999). It is therefore necessary for the South African olive industry to proactively develop and implement more effective technologies to treat olive wastewater prior to it becoming an environment hazard.

Olive mill wastewater (OMWW) and table olive wastewater (TOWW) are characterised by high values of chemical oxygen demand (COD). The organic content of OMWW, for example, is often 200 – 400 times higher than typical municipal sewage (Al-Malah *et al.*, 1999). As microorganisms in the environment consume the organic materials, oxygen will be depleted from the water and hence can have an adverse effect on life in aquatic environments. A significant fraction of the COD content of OMWW and TOWW represent a complex mixture of aromatic compounds (i.e. simple phenolic and polyphenolic compounds). Four classes of phenolic compounds are found in olive fruit including phenolic acids, phenolic alcohols, flavonoids and secoiridoids (Vinha *et al.*, 2005). Apart from causing a strong black/brown colour in these wastewaters that could cause discolouration of natural waters (Zouari and Ellouz, 1995), these phenols are also considered toxic to the environment. Phenols have been shown to be phytotoxic and have antimicrobial activity (Alburquerque *et al.*, 2005). Al-Malah *et al.* (1999) showed that some polyphenols, like methylcatechol, tyrosol, hydroxytyrosol and *o*-quinone, which are naturally present in OWW, have

toxic effects on some strains of gram-positive and gram-negative bacteria. Another negative property of OMWW is the presence of a high total solids content that is considered problematic, especially for the treatment purposes. If the wastewater is used for irrigation purposes, a loss of soil fertility can result (Lee, 2001). High COD concentrations in the wastewater can also be a source of objectionable odour when in contact with chlorinated waters. This results in the formation of chlorinated phenols that are highly toxic, poorly biodegradable and carcinogenic (Ozkaya, 2005). Despite existing laws, these wastewaters are routinely dumped without proper treatment and the potential risk exists that the high organic load, phytotoxic and antimicrobial effects could cause severe problems in natural watercourses, including rivers and underground water.

Two factories in the Western Cape were chosen for this study: Buffet Olives situated in Dal Josefat that produces table olives and Vesuvio Estate on Sorrento Farm close to Wellington that produces extra virgin olive oil. Although no official surveys have been conducted, it is known that most olive processing plants in the Western Cape execute some minimal treatment of olive wastewater prior to disposal into the environment (Smit, 2004; Van Dyk, 2004). The two major role players in the industry, Buffet Olives and Vesuvio Estate, mainly implement an evaporation pond method, which requires long treatment times, and could also result in bad odours, proliferation of insects and contamination of groundwater resources (Kyriacou et al., 2004, 2005; Stolting and Bolle, 2000). Worldwide the most frequent method of OWW treatment used is anaerobic degradation or the detainment of the wastewater in evaporation ponds.

1.2 Aims of the study

In an attempt to develop a new olive wastewater treatment system, this study sets out to characterise the indigenous biofilms that exist within olive wastewater. The characterisation of naturally-occurring biofilms from olive wastewater could offer a better understanding of the factors that need optimisation in an attempt to develop an alternative bioremediation approach and contribute to the development of a more effective biological treatment system for olive wastewater. The specific aims for the project were the following:

i) To determine the population complexity and microbial species distribution within biofilms at various stages of growth in table olive wastewater and olive mill wastewater.

ii) To monitor population shifts in the bacterial and fungal communities within the biofilms in response to changes in wastewater over time and under certain operational conditions, for example high phenol levels, NaOH/NaCl and presence of detergents.

iii) To investigate the sustainability of these biofilms over two production seasons in an attempt to predict the feasibility of using these biofilms as a bioremediation tool. Sustainability in this context is defined as the occurrence of similar microbial species and population complexities in the indigenous OWW biofilms at similar time points over a prolonged production period.

To accomplish the aims of this study, well-known molecular biology techniques were employed. The biofilms were cultured on glass slides that were immersed in the wastewater streams for several months and harvested by means of sonication. The total genomic DNA was then extracted followed by polymerase chain reaction (PCR) amplification of the 16S rRNA fragment (bacteria) or the 18S rRNA fragment (fungi). To determine the total number of species present in the biofilm, the amplified products were analysed with denaturing gradient gel electrophoreses (DGGE). The distribution of the biofilm organisms over an extensive time period (± 12 months) was analysed by Shannon-Weaver indexes of microbial diversity and Principal Component Analysis to obtain the sustainability profiles of the biofilms in response to seasonal changes and other external factors. It is important to state that these methods cannot provide a comprehensive analysis of population complexity and species distribution, however it can still address the aims of the study to a good extend.

Lastly, this study contributes to the ultimate research goal, i.e. to develop an eco-friendly, easy-to-manage, biological bioreactor that reduces COD and phenolic content of olive wastewater. This study is a proactive step that will ensure the sustainable growth of the South African olive industry and international competitiveness through sound environmental waste management practices.

1.3 Thesis outline

The first chapter of this thesis provides the background to the research problem as well as the research aims. Chapter 2 covers the current literature on table olive and olive oil production, the dangers and characteristics of the respective toxic wastewaters as well as bioremediation processes to treat the wastewater. This chapter also focuses on biofilm structures and its adherence mechanisms as well as the application of biofilms in bioremediation. Chapter 3 covers the literature theory and applications for the molecular biology techniques (PCR-DGGE) used in this study and is discussed in detail with the rest of the methods in the Materials and Methods chapter (Chapter 4). Chapter 5 presents and discusses the results while the conclusions are in Chapter 6.

CHAPTER 2

LITERATURE REVIEW

2.1 Table olive and olive oil production and its wastewater

The olive industry is notorious for producing high volumes of wastewater. This is especially true for the Mediterranean countries that account for ± 95% of world olive production and produce 30 million tons of solid and liquid waste residues annually (Bas Jimenez *et al.*, 2000). The South African olive industry, of which 90% of production is localised in the Western Cape, is still in its early growth stages, but expands relatively fast as an industry. There are an estimated 51 commercial olive farmers in the Western Cape that produce approximately 3500 - 4500 tons of olives per year (Wesgro, 1999). According to the Wesgro (1999) report, approximately 40% of locally produced olives are processed for table olives and the rest processed into olive oil, of which both types of processing produce significant volumes of wastewater. The typical table olive and olive mill wastewater (TOWW and OMWW, respectively) composition by weight is: 83-94% water, 4-16% organic compounds and 0.4-2.5% mineral salts (Buldini *et al.*, 2000). The organic fraction contains 2-15% phenolic compounds with concentrations between 3-10 g/l (Cabrera *et al.*, 1996; Fiestas Ros de Ursinos and Borja-Padilla, 1996). The mineral fraction contains 47% potassium salts and 7% sodium salts. The following sections of this chapter will discuss the production steps and wastewater characteristics of table olives and olive oil in more detail.

2.1.1 Table olive processing and wastewater production

The main purpose of table olive processing is to remove, at least partially, the natural bitterness of the olive fruit caused by the secoiridoid, oleuropein (Fig. 2.1). Oleuropein, usually a glucoside, is required to be removed from the fruits to improve the organoleptic quality. In unripe green olives, oleuropein is present as the major *o*-diphenolic compound, while in ripe olives demethyloleuropein predominates (Briante *et al.*, 2002; Lo Scalzo and Scarpati, 1993; Servili *et al.*, 1999).
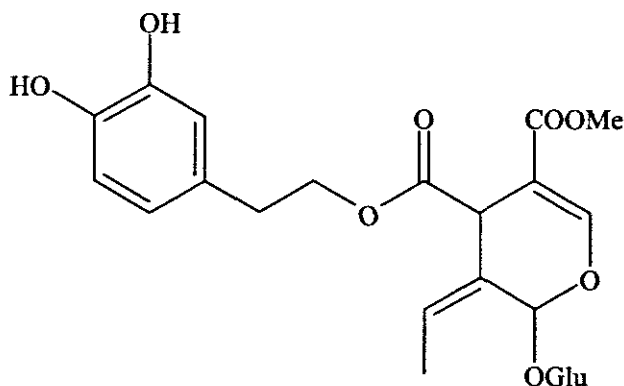
Figure 2.1: The molecular structure of oleuropein, a 3,4-dihydroxy-phenylethanol (hydroxytyrosol) ester with a β-glucosylated elenolic acid (Savournin *et al.*, 2001; Briante *et al.*, 2002).

Bitterness in the fruit can be eliminated quickly and completely by alkaline hydrolysis; that is, by treating the olives with a sodium hydroxide solution (usually 1.8-2.5% w/v) before fermentation, and by further storage in brine or dry salt (Fernandez *et al.*, 1997; Chartzoulakis *et al.*, 2002; Kyriacou *et al.*, 2004). Any olives that are treated with sodium hydroxide are referred to as pickled or treated olives. Treated olives need to be washed 2 to 3 times with excess water to remove lye solution before the brine fermentation can commence and therefore produce high amounts of wastewater (Sanchez *et al.*, 2000). Bitterness may also be removed slowly and partially during the acid fermentation process, without prior lye treatment. These olive products are known as untreated olives. According to Fernandez *et al.* (1997) and Colmagro *et al.* (2000), three important processing styles are generally used for table olive production:

- Spanish-style, untreated, green olives in brine. During this style of processing, lactic acid fermentation in brine is the principal feature and wastewater generated in the production process has a reduced pollution impact, because no alkali treatment is used.

- Californian-style black olives, pickled in brine. During this style of processing no fermentation is needed, and the olives are directly treated with lye, oxidised, washed, placed in brine (NaCl), packed in cans and heat sterilised. This method produces the most wastewater because of the lye treatments and associated washings.

- Greek-style, naturally black olives in brine. For this style of processing the main organisms responsible for fermentation are yeasts, while the lactic acid bacteria form a smaller percentage of the total microflora. No lye treatment is used during this process, and debittering relies solely on yeast fermentation to remove bitterness. The production of these olives is spread uniformly throughout the year resulting in the production of large quantities of effluent.

According to Beltran-Heredia (2000), the whole process requires large amounts of water mainly for the washing stages. The lye treatment alone gives rise to approximately 8.5 liters of wastewater (1.5 liter lye solution and 7 liter washing water) for every kilogram of olives produced. Therefore, the approximate 1400 tons of table olives produced annually in the Western Cape will result in approximately 11.9×10^6 liters of lye wastewater. This excludes the fermentation brine volumes. Furthermore, very little, if any, research has focused on the treatment of such high volumes of table olive wastewater, while most literature available only discusses the characteristics and treatment of OMWW. Although, the TOWW is similar in nature and characteristics, but somewhat weaker in organic strength than OMWW (Aggelis *et al.*, 2001), it still has the same environmental concerns than OMWW.

The composition of table olive wastewater (TOWW) is highly variable since various types of cultivars are often processed within the same production plant. As a general rule, wastewater from treated olives contains considerably higher concentrations of phenols compared to wastewater from untreated olives. Characteristics of TOWW vary widely depending on the type of the product and the process, but it generally has a pH between 3.8 and 12 (Table 2.1) and contains suspended solids, dissolved solids, and sodium chloride (Kyriacou et al., 2004).

Table 2.1: A summary of the main characteristics of TOWW in the Mediterranean countries (Fernandes et al., 1997).

Characteristic	Lye	Washing water	Fermentation brine
pH value	9.5 - 12.0	9.0 - 11.5	3.8 - 4.2
Free NaOH (g/l)	11	1.5	-
Free acidity (lactic acid g/l)	-	-	6-15
Polyphenols (tannic acid g/l)	2.5 - 4.0	2.5 - 4.0	4.0 - 6.0
Reducing sugars (tannic acid g/l)	6 – 9	6 - 9	-
Dissolved organic solids (g/l)	20 – 30	20 - 30	15 - 25
Dissolved inorganic solids (g/l)	20 – 35	7 - 25	90 - 110
COD (g O_2/l)	15 – 35	12 - 35	10 - 35
BOD (g O_2/l)	9 – 20	9 - 20	8 - 20

The first main pollution product from table olive processing is the alkaline waste streams as a result of lye treatment, which is characterized by high COD and alkalinity values, including being rich in polyphenols and organic acids (Table 2.1) (Aggelis et al., 2002). As a result of the lye treatment, the fruit tissue are softened while organic compounds in the olives, including phenolic compounds, are hydrolysed and partially extracted from the fruit and ultimately end up in the wastewater. The phenols present in TOWW that undergo hydrolysis during contact with sodium hydroxide are: oleuropein, hydroxytyrosol-4-β-glucoside, diglucoside, apeginin-glucoside, luteolin-7-glucoside and caffeic acid derivatives such as verbascoside (Fernandez et al., 1997). According to Blekas et al. (2002), sodium hydroxide treatment hydrolyses the ester bonds of these phenols. Oleuropein produces hydroxytyrosol and elenolic acid glucoside, while verbascoside is hydrolysed to caffeic acid and hydroxytyrosol-1-o-rhamnosylglucoside.

The second main pollution product is generated during the fermentation of the olives in NaCl brine. During the fermentation process, sugars and various other carbohydrates within the olive as well as in the brine are broken down and fermented to smaller organic acids, for e.g. lactic acid, resulting in increased acidity within the fermentation brine. Apart from containing high NaCl levels, this type of wastewater also has a relatively high organic content. A substantial increase of phenols in the brine solution can also usually be detected as the result of phenol extraction from the fermenting fruit. The rate of phenol extraction during fermentation is however slower compared to lye extraction. Briante et al. (2002) have also found that, during further olive fermentation, β-glucosidase and esterase activity started to play a significant role in phenol release. Hydroxytyrosol, tyrosol, caffeic, vanillic and p-coumaric acid are among the non-hydrolysable phenols within olive flesh. When the brine is acidified the caffeic and p-coumaric acids are not easily dissolved and remains in the olive flesh (Fernandez et al., 1997). Also, the aglycones of flavonoids are slightly insoluble in water and therefore remain in the oily flesh of the fruit (Fernandez et al., 1997). Since small oil droplets and olive solids can be present in the wastewater, it is possible for aglycones and flavonoids to also be present in the wastewater.

Romero et al. (2004) have determined the changes that occur in anthocyanins, hydroxytyrosol-4-β-glucoside, oleuropein and hydroxytyrosol in brine solution during the Greek-style fermentation stage of naturally black olives (no lye treatment). The concentration of monomeric anthocyanins (black colour) decreased as a result of dilution and the polymerisation reactions during this period; however, an increase in polymeric and total anthocyanins was measured. Anaerobic conditions produced higher concentrations of total anthocyanins in the brine than aerobic conditions. Romero et al. (2004) determined that a steady state in anthocyanin diffusion between the olive fruit and the brine solution was reached during the third fermentation month. Hydroxytyrosol-4-β-glucoside and oleuropein in the brine increased in concentration during the first 3 months of anaerobic fermentation due to diffusion from the olive flesh, however the concentration started to decrease later in the process. This decrease was caused by an acid hydrolysis reaction from hydroxytyrosol-4-β-glucoside and oleuropein into hydroxytyrosol under anaerobic conditions. Under aerobic conditions, a further decrease in hydroxytyrosol-4-β-glucoside occurs due to oxidation of these orthodiphenols (Gianfreda et al., 2003). A gradual increase in hydroxytyrosol was measured over the 12-month fermentation period, which was a direct effect of acid hydrolysis of oleuropein and hydroxytyrosol-4-β-glucoside (Romero et al., 2004).

Since the scope of this study focuses on South African olive wastewater, it is necessary to discuss and compare the composition of South African TOWW to international literature. Burton (2004)

measured the various characteristics in TOWW (Western Cape, South Africa) and compared black and green olive fermentation wastewater as well as lye wastewater to each other (Table 2.2).

Table 2.2: The physico-chemical characteristics of various TOWW fractions in the Western Cape, South Africa (Burton, 2004).

Property[a]	Black olive brine	Green olive brine	NaOH treatment
pH	4.53	4.08	9.91
Conductivity (mS.cm^{-1})	83.1	79.0	16.3
Total Solids	114.2	114.3	59.8
Dissolved Solids	113.9	113.9	58.5
Suspended Solids	0.3	0.4	1.3
COD	58.7	50.4	66.3
TOC	18.5	18.3	20.6
Reducing sugar	0.26	1.18	9.31
Lipids	0.83	1.08	1.39
Total Phenols (GAE)[b]	4.05	2.14	1.06
Simple Phenolics[c]	1.87	1.64	0.51
Na$^+$	22.35	-	-
Cl$^-$	30.00	-	-

a) All units in g/l, unless otherwise stated
b) Gallic acid equivalents
c) As determined by HPLC

If the Table 2.1 and 2.2 are compared, differences and comparisons can be observed between South African wastewater and those of the Mediterranean areas. The pH of the fermentation brines as well as that of the lye wastewater fell within range of the previously published values, but the measured COD values of the South African wastewaters were higher in comparison with international values. Total organic carbon (TOC) values and reducing sugar values were also relatively similar for both Mediterranean and South-African wastewaters. Total phenolic compounds in both wastewaters were still in close proximity, although South African values are slightly lower.

2.1.2   Olive oil production and wastewater

Spain, Greece and Italy are the three main producers of olive oil in the world and represent 80% of the world's olive oil production. Olive oil extraction is mainly carried out by the traditional

discontinuous press procedure or by the continuous centrifugation of a mixture of milled olives and hot water. In both systems, three phases from the olive raw product are produced, i.e. 20% olive oil, 30% solid residue and 50% aqueous liquor. It is important to dispose of the highly polluted olive by-products correctly, especially the aqueous liquor that comes from the vegetation water and the soft olive tissue. The mixture of this by-product with the wash water used in all stages of the process constitutes olive mill wastewaters (OMWW) (Mantzavinos et al., 2004).

Vossen (1997) describes the oil processing steps in detail and shows the wastewater resulting from each step. The steps are as follows: Washing, grinding, malaxing (mixing with 100% hot water), oil extraction (3-phase centrifuge), extraction of residual oil and oil purification. The quantity of wastewater produced depends on the milling process which ranges from approximately 50 kg water per 100 kg olives using a traditional batch mill to about 110 kg water per 100 kg olives using continuous processes (Vitolo et al., 1999). Between 100 and 150 l of water are used per ton of olives during washing and grinding. Oil extraction with a 3-phase centrifuge can produce up to 1200 kg wastewater and 500 kg solid wastes per ton of olives. In the purification process, between 100 and 150 l water per ton of oil are added to remove impurities.

Olive mill effluents could therefore be considered diluted solutions of crushed olive juice after the extraction of the oil. The main constituents are sugars, nitrogenous compounds (proteins), salts, volatile organic acids, polyalcohols, pectins, gums, fats, water-soluble polyphenols and polysaccharides (Fiestas Ros de Ursinos and Borja-Padilla, 1996; Paredes et al., 1999; Buldini et al., 2000; Mulinacci et al., 2001). OMWW is characterised by high COD levels due to its high organic load caused mainly by carbohydrates, proteins and fats. The COD can reach values of up to 220 g/l and a pH range of 3 to 6. The high content of phenols, polyphenols, and tannins in OMWW are phytotoxic (toxic to plants) and has antimicrobial activity against soil microorganisms (Capasso et al., 1995; Filidei et al., 2003). In one case, an excessively high content of polyphenols of up to 80g/l with a high solid matter content (total solids of up to 20g/l) has been measured in the past (Vitolo et al., 1999). The quantities and composition of OMWW vary considerably and are influenced by various factors. It is important to know that OMWW quality is largely dependent on a variety of fruit culturing methods (planting and harvesting season, olive cultivars, location, soil quality, time of harvest, type of pesticides) as well as fruit processing methods (machinery, oil extraction procedures, brine fermentation methods, storage, olive maturity) (Balis and Antonakou, 2000; Garcia et al., 2003; Stolting and Bolle, 2000).

The main phenols found in OMWW are catecol, 4-methylcatecol, tyrosol, and hydroxytyrosol. The phenolic compounds are present in a range of different molecular weights, starting from low-molecular weight substituted phenols with one single aromatic ring and one or more substituting groups, to complex high-molecular weight phenolic compounds, which are usually condensed aromatic rings with several substituting groups (Mulinacci *et al.*, 2001). Polymeric phenols, which display a lignin-like structure, are the most recalcitrant fraction within OMWW and are responsible for the dark brown/black colour within the water (D'Annibale *et al.*, 2004; Tsioulpas *et al.*, 2002).

During the crushing and malaxing process, oleuropein and demethyloleuropein are hydrolysed by glycosidase enzymes to the dialdehyde form of decarboxymethyloleuropein aglycone and aldehydic form of oleuropein aglycone. The aglycones are hydrophobic and dissolve in the oil, while the hydrophilic glycosides remain dissolved in the wastewater. The crushing process also causes the opening of the rings of secoiridoids that result in the generation of isoforms of oleuropein (Shahidi and Naczk, 2004).

The malaxation process also causes loss of both secoiridoids and phenyl alcohols from the olive flesh. It is believed that endogenous oxidoreductases, non-enzymatic oxidation and complexation with certain sugars may contribute to this loss. The addition of cell wall-degrading enzymes during malaxation, which is the practice in some cases, increases the concentration of phenols in virgin olive oil. The acidity of the oil in the filtration step influences the amount of secoiridoids aglycones that will be hydrolysed. The phenols in the olive pulp after crushing are sensitive to high temperatures. Heating olive oil and/or OMWW above 180°C results in a decrease in the polyphenolic content and this is due to thermal destruction and/or oxidative degradation at elevated temperatures (Shahidi and Naczk, 2004).

OMWW also contains inorganic compounds such as chloride, sulphate and phosphoric salts of potassium as well as calcium, iron, magnesium, sodium, copper and traces of other elements (Turano *et al.*, 2002). Table 2.3 shows the composition of OMWW in South Africa and also compares it to the values for South African TOWW. It is clear from Table 2.3 that OMWW contains significantly higher suspended solids levels, consisting of olive pulp, mucilage, pectin and oil in a relatively stable emulsion.

Table 2.3: Comparison between the composition of TOWW and OMWW (Burton, 2004).

	OMWW (Mill wastes)	TOWW (Olive waste)
pH	4.53	4.86
Conductivity (mS.cm^{-1})	9.55	62.31
Total Solids (g/l)	135.5	114.2
Dissolved Solids (g/l)	58.72	113.9
Suspended Solids (g/l)	60.28	0.19
Volatile Suspended Solids (g/l)	?	0.15
COD (g(O_2)/l)	262.1	58.7
TOC (g/l)	?	119.5
Total Phenols (g/l)	5.97	4.17
Phenol fraction of COD (%)	11	32
Lipids (g/l)	74	0.35
Reducing Sugar (g/l)	13.82	0.26
NH_4^+ (g/l)	0.03	0.31
Na^+	1.90	22.35
Cl^-	0.50	30.00
NO_2^-	0.20	0.0

Comparing the COD levels between OMWW and TOWW, it is evident from the data in Table 2.3 that OMWW (262.1 g/l) has significant higher organic loads than TOWW (58.7 g/l). Because of this, OMWW might need a longer retention time in a bioreactor system than TOWW in order to obtain similar bioremediation efficiencies. OMWW is higher in reducing sugars than TOWW. It is likely that bioremediation organisms will first use these sugars as their primary carbon sources and once depleted, will start to metabolise lipids and phenols as secondary carbon sources. Total phenols for both types of wastewaters are also relatively high, but it is interesting that phenols account for 32% and 11% of the COD in TOWW and OMWW, respectively. Thus, although the COD for TOWW is generally lower, the ratio of phenols to other organic compounds is significantly higher.

The oil fraction present in the OMWW is indicative of inefficient extraction, because of the formation of an oil-water emulsion that is difficult to separate with centrifugation. Lipids can inhibit biodegradation as well as physical processes such as filtration. However, oil in the wastewater can be minimized with centrifugal feed adjustments. As expected, TOWW contain very little oil, since the olive cells are not disrupted in the fermentation process. Both wastewaters are low in nitrogen and whether the bioremediation organisms will need supplemented nitrogen to metabolise the waste, will have to be determined by experimentation. TOWW is, as expected, high in sodium and chlorine ions due to the salt used in the fermentation process (Burton, 2004).

2.2   General environmental impacts associated with olive wastewater

One of the most studied environmental effects of most wastewaters is excessive nutrient enrichment. An oversupply of nutrients, such as oxidisable carbon, phosphorus and nitrogen will stimulate a rapid increase in growth in all aquatic ecosystems that could lead to the formation of large algal blooms and weed beds. Such a process is referred to as eutrophication and can deteriorate ecosystems in a variety of ways (Welch, 1992). Eutrophication will contribute to oxygen depletion in aquatic systems as large quantities of organic compound respired by bacteria will consume dissolved oxygen faster than the rate at which new oxygen can dissolve into water. The amount of oxygen consumed during the decay processes over a period of days is known as the wastewater's biological oxygen demand (BOD). Any oxygen that is consumed through chemical oxidation reactions within the receiving water is known as its chemical oxygen demand (COD). Reduced oxygen levels within rivers contaminated with olive wastewater will have a large effect on many fish species as well as plankton (Hvitved-Jacobsen, 1982). Furthermore, increases in water temperature and salinity levels could further exacerbate the oxygen depletion phenomenon.

Impacts on ecosystems resulting from toxic substances can either be acute or they can be cumulative and appear only after an extended period of time (Harremoës, 1988). High levels of ammonia and chlorine, high loads of oxygen-demanding materials or toxic concentrations of heavy metals normally cause acute effects on ecosystems. Cumulative effects result from a gradual increase of toxins in very low concentration. Toxins can accumulate within sediments or biomass and only become apparent when the concentration exceeds a certain threshold (Welch, 1992). Substances that are normally found in very low concentrations in wastewater can sometimes accumulate in the fatty tissues of plants and animals. This process is known as bioaccumulation and is very stable since many of the chemicals are not easily degraded. Through another process called bio- magnification, some of these toxins can even be passed up in the food chain from prey to predator, resulting in an increased toxin concentration within the top predator (e.g. herring gull or fish eagle) of millions of times that of the original wastewater (Environment Canada, The Green Lane, 2005).

Despite existing South African laws, olive mill and table olive wastewaters are regularly dumped without proper treatment. No official surveys have been done in South Africa regarding the environmental impacts of olive wastewater (Smit, 2004; Van Dyk, 2004). The potential risk exists that phytotoxicity, antimicrobicity, bad odours, proliferation of insects and severe contamination of rivers and underground waters could occur (Filidei *et al.*, 2003). This can also lead to an increase in salinity and decrease in the permeability of the soil if the wastewater is used for irrigation purposes

(Lee, 2001). Olive producers in South Africa risk the chance of penalties, since the COD and BOD levels in untreated olive wastewater are substantially higher than specifications (75 mg/l COD and pH 5.5 − 7.5) and can therefore not be discharged into sewage systems (Coetzee *et al.*, 2003). If wastewater effluents are therefore disposed of into rivers or onto open soil, it will be responsible for irreversible damage to ecosystems. An increase in nutrient content, decrease in dissolved oxygen and presence of toxic substances can disrupt the balance in natural ecosystems and can bio-accumulate and bio-magnify in aquatic life. Non-aesthetic physical changes to the environment can also occur, causing the temperature, flow rate and suspended solids concentration of receiving waters to increase (Environment Canada, The Green Lane, 2005).

Apart from ground water pollution, increased toxins within olive wastewater that comes into contact with soil can also diminish soil fertility. Soil is the foundation of any terrestrial community and need to be protected. Soil bacteria are responsible for the decomposition of organic matter and the recycling of nutrients and minerals back into plants. Olive wastewater toxins can cause "burning" of the soil, in other words, destroying the soil bacteria and changing the pH of the soil. Lower bacterial counts within soil affect the rates of decomposition, the return of nutrients into the soil and thus the viable regeneration of new plants.

2.2.1 A formula for quantification and prioritisation of negative environmental impacts in the olive industry

According to national legislation and foreign markets, all processes that have potential impacts on the environment, such as wastewater disposal, must be responsibly managed by means of an effective environmental management system (EMS). Prior to the implementation of an EMS, various risk factors need to be identified and prioritised. Although the majority of environmental risk factors will differ slightly from one olive farm to the other, the severity of such impact may vary considerably.

A formula for calculating the significance of each environmental factor has been developed as an EMS tool for the wine industry, but can also be applied to olive production. The formula also includes social and aesthetic factors as well as requirements for the integrated production of olives (IPO). The IPO is a regulatory system by which natural processes are favoured and employed above human intervention in the olive industry, resulting in environmentally friendly, profitable and safe olive oil and table olive production (IOBC Technical Guideline III, 2002). By using this significance formula, olive production management could list and prioritise negative environmental

impacts. By doing an environmental assessment study for many olive oil and table olive producers in South Africa, it will be possible to determine the most efficient methods necessary for wastewater treatment, thereby keeping production and wastewater treatment costs to a minimum. A complete environmental assessment study needs to be done at the olive processing plant before implementing an bioremediation strategy. The formula is expressed in the following terms:

$$S = [(fd + int + sev + ext + loc) \times (leg + ipo + pol + ia + str) \times P] \text{ (Van Schoor, 2000)}$$

S is the significance value of the environmental impact (0 – 100). The first variable (fd) measures the frequency and duration of the impact (rated as 1, 1.5, 2, 2.5 or 3). The second variable (int) represents the intensity of the impact and is a measure of the pollutant concentration and pollutant quantity (rated as 1, 1.5, 2, 2.5 or 3). The·third variable (sev) describes the severity of the environmental impact and is an indication of the reversibility of the environmental changes (rated as 1 – 3). The fourth variable (ext) gives an indication of the extent of the impact (rated as 1 – 3). The fifth variable (loc) shows the sensitivity of the location to pollution (rated as 1 – 3). If the company is complying with legal (leg) and integrated production of olive (ipo) requirements (leg) and has an environmental policy (pol), it is also incorporated into the equation (rated as 0 or 1). Wastewater pollution can affect the communities in the vicinity to some degree and therefore the impact on interested and affected parties (ia) is determined (rated as 1 – 3). Finally, the last two factors involved in the equation deals with whether there is a strategy in place (str) to solve the issue (rated as 0, 0.5 or 1) and what the probability of the occurrence of the impact (P) is (rated as 0, 0.25, 0.5, 0.75 or 0.95).

In an attempt to apply a strategy to address wastewater minimisation, it is important to identify the different processing points where wastewater is produced and estimate the volumes and characteristics of each point. With these data, the production manager can usually introduce some wastewater reduction measures to minimise water usage that could contribute to substantial reductions in total wastewater volumes (IOOC, 1990). Some general considerations to introduce cleaner production practises have been suggested, including the re-use of water, optimisation of the amount of chemicals added in processing as well as the regeneration of fermentation brines for re-use in packaging of the final product (Fernandez *et al.*, 1997). Internal control measures have been adopted, such as the re-use of the lye for treatment of olives; the reduction of the lye concentration to a minimum effective value; and the reduction, or even the complete elimination of washing operations, by neutralisation of the residual free lye which remains in the fruit after the alkali

treatment (Fernandez *et al.*, 1997). Although these processes can minimise the wastewater volume, they cannot solve the problem of environmental toxicity.

2.3 Remediation of olive wastewater

The treatment processes used for the remediation of olive wastewater can be separated into five different categories: physical, thermal, physico-chemical, biological and combined processes. Physical processes involve the separation of different phases through mechanical means. The main physical processes are: dilution, sedimentation, filtration, flotation, centrifugation and the use of membrane technology (Iorio *et al.*, 2002). Thermal processes are designed to concentrate the olive wastewater through evaporation and/or distillation. This reduces the water content and eventually total volume of wastewater. Physico-chemical processes involve the use of additional chemicals for the neutralisation, flocculation, precipitation, adsorption, chemical oxidation (chlorine and ultra-violet light), ozonation, cryogenesis and ion exchange treatment of olive wastewater (Grady *et al.*, 1999; Niaounakis and Halvadakis, 2004).

Unfortunately, many of these treatments and the complex technology involved are too expensive for most small-to-medium sized olive mills, especially in South Africa. Additional treatment methods include physico-chemical systems such as aerobic treatment combined with Fenton's reagent (Rivas *et al.*, 2001), ozonation (Beltran-Heredia, 2000) and bleaching of OMWW by clay in the presence of hydrogen peroxide (Oukili *et al.*, 2001).

Biological processes employ the use of microorganisms to break down the pollutants present in olive wastewater. The type of microorganisms involved will depend on the conditions in which the wastewater is treated, i.e. aerobic or anaerobic. Anaerobic processes are used to treat concentrated wastewater streams using a 3-step process, i.e. hydrolysis, acidogenesis and methanogenesis (Marques, 2000), while aerobic processes are mostly used as a finishing or polishing step to remove residual organic matter (Beccari *et al.*, 1999; Niaounakis and Halvadakis 2004). The use of specific microorganisms for bioremediation has been limited to one or two individual bacterial or fungal isolates (refer to section 2.4). Another process that combines the use of aerobic and anaerobic biodegradation is reed bed wetland systems in which plants play a major role in absorption or degradation of COD (Skerratt and Ammar, 1999).

The main treatment processes currently in use in South Africa for treating olive wastewater are lagooning or evaporation ponds (thermo-biological combination process) and anaerobic digesters

(biological process). Rotating biological contactors (aerobic biological process) are a relative "old" technology that has recently been tested in South Africa to treat wine wastewater (Viljoen-Bloom *et al.*, 2003) and will be discussed later in more detail.

In recent years, olive wastewater has also become the focus of research as a possible source of valuable products. If the phenolic fraction of the wastewater could be harvested or bio-transformed efficiently, it will be advantageous to the overall biodegradation process. According to Allouche *et al* (2004), biotransformation is the process of enzymatic conversion of one compound into another compound for use as an economically valuable product. Through hydroxylation reactions of various aromatic, phenolic compounds in OMWW, using tyrosinase enzyme (purified extracts from mushrooms or in whole cells), hydroxytyrosol can be synthesized. Hydroxytyrosol has powerful antioxidant properties and presents interesting advantages to human health. It has also been demonstrated that hydroxytyrosol acts *in vitro* as an antibacterial agent against both gram-positive and gram-negative bacteria (Allouche *et al.*, 2004).

2.3.1 The use of biofilms in bioremediation of olive wastewater

2.3.1.1 Introduction to biofilms. Microorganisms can be divided into two groups depending on their growth characteristics. Microbial cells can either be planktonic or sessile. If cells are planktonic, they are free-floating and individual organisms. Sessile organisms form part of a complex, closely integrated, biofilm community attached to a surface (Maukonan *et al.*, 2002). Wimpenny *et al.* (1999) describes biofilms as either homogenous or heterogeneous microbial cells, i.e. either single or multiple species of bacteria, fungi, algae, and protozoa (Trachoo, 2003), embedded in an extracellular polymeric substance (matrix) attached to a substratum (liquid-solid interface). Aerobic microorganisms predominate mainly in biofilms, but anaerobic microorganisms are also present (Poulsen, 1999). Most microorganisms form biofilms and more than 99% of all microorganisms are living in such aggregates in nature (Flemming, 2002). If conditions are suitable and nutrients are available, all microorganisms can form biofilms on most types of surfaces (biological and abiological).

Biofilms are not composed solely of microorganisms; in fact, microorganisms generally make up about 2-5% of the total biomass. The greatest fraction of biofilm is composed of extracellular polymeric substances (EPS) excreted by microbes or resulting from cell lyses. The exopolymer matrix is mainly composed of water (97%) and highly heterogeneous polysaccharides (1 − 2%), but other substances are also present, such as proteins (< 1%), nucleic acids (< 1%), lipids,

phospholipids and peptidoglycan (Sutherland, 2001). The EPS of biofilms vary with different bacterial strains, physiological states and environmental conditions (Lazarova and Manem, 1995). The exopolysaccharides are responsible for the morphology, architecture, coherence, physico-chemical properties and biochemical activity of these microbial aggregates (Flemming *et al.*, 2003; Wuertz *et al.*, 2003) and also acts as a protective barrier against environmental dangers such as detergents and other antimicrobial agents (Poulsen *et al.*, 1999). The exopolysaccharides are therefore considered as a key component that determines the functional integrity of biofilms (Evans, 2000).

The structural details of any biofilm formed under a specific set of parameters will be unique to the environment and the microflora. A large amount of microorganisms (99%) are capable of forming biofilms either as single species (relatively rare) or as interacting multiple species. Because of the large range of polysaccharides produced, an infinite number of permutations in structural detail usually exist (Sutherland, 2001).

2.3.1.2 Mechanism of attachment and detachment. The distribution of living cells in the cellular aggregate, their extracellular polymers and three-dimensional water channel networks vary constantly. The species within the biofilm interact and communicate with each other to facilitate their adaptation to changing environmental parameters (Liu and Tay, 2001). Bacteria in a multi-species biofilm are not randomly distributed, but they are rather organised to best meet the needs of each other. The architecture of single-species biofilms is species-specific, but even in a single culture, adherence can introduce some heterogeneity as cells may adhere in different ways. For multi-species biofilms the architecture is more substrate-specific. When more than one species is present, colonisation patterns may differ as some organisms will join existing biofilms, while other will attach to non-colonised areas where it will start a new biofilm. After initial colonisation, heterogeneity will increase even more because of the differences in each individual species' growth rate, metabolism, EPS production, adherence patterns, etc. (Genigeorgis, 1995; Costerton *et al.*, 1995). These will result in irregular, three dimensional and highly complex biofilms.

According to Schachter (2003), microbial attachment can be defined as a 5-step process. The process starts (step 1) with the transport of a few nutrients, inorganic and organic matter to a solid surface. This stage is reversible. Following this stage (step 2), the absorption of more nutrients, inorganic and organic matter to the surface occurs, resulting in the formation of a conditioning film. This stage then becomes irreversible as more organic and inorganic molecules attach to the surface. The accumulation of nutrients occurs because most solid surfaces assume a net negative charge

when immersed in water (Zottola and Sasahara, 1994). As a result, cations and a variety of macro-molecular and colloidal materials will be electrostatically attracted to such surfaces.

Figure 2.2 shows a diagrammatic representation of biofilm formation as described above and below the figure and also shows an approximate timeline in which these various steps occur.
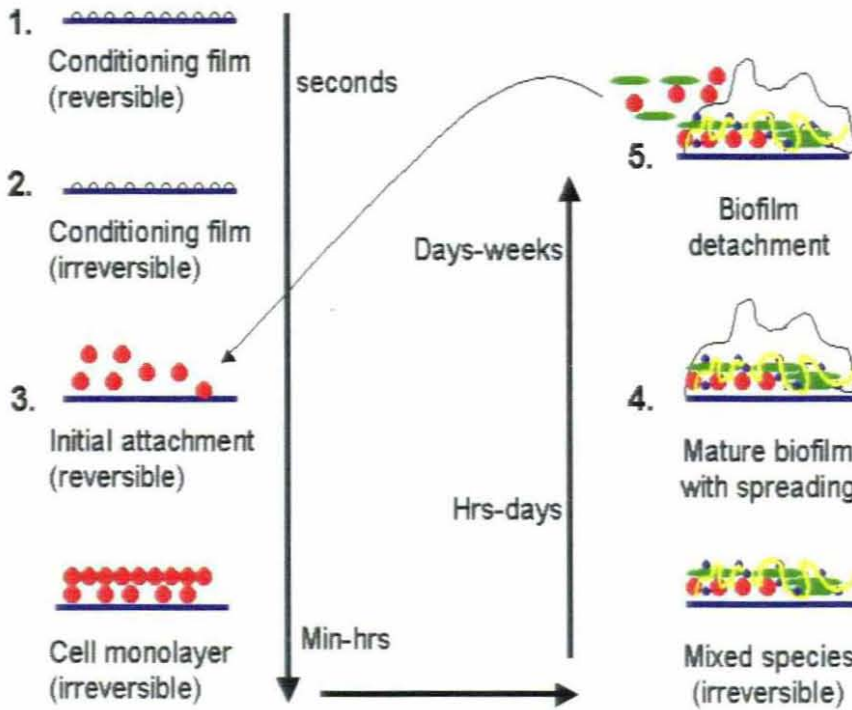
Figure 2.2: A diagrammatic representation of biofilm formation in an aquatic environment (adapted from Singh *et al.*, 2006).

During the next phase (step 3), the first cells start to attach to the moist surface and growth is initiated. During this phase, a faster flow rate in the bulk fluid, surrounding the biofilm, will cause faster attachment, but a too high flow rate can have the opposite effect. Chemical signalling begins to occur between individual cells in order to determine the current population density and gene transfer occurs, to a small degree, in order to change phenotypic structures (Wilson, 2001). Adhesion of the microorganisms to the conditioned surface continues and can either be reversible or irreversible (Denyer *et al.*, 1993). During the reversible phase, the organisms are near, but not in actual contact with the surface. They are attracted to the surface with short-range interactions like electrostatic, hydrophobic and Van der Waals forces. When organisms bound reversibly, they scan the surface to determine whether the nutrients are sufficient to permanently attach (Zottola, 1994). These initial biofilm cells exhibit Brownian movement from the surrounding bulk fluid and can result in cells detaching from the surface in case nutrient conditions are not optimal.

When microorganisms attach irreversibly, they are bound to the surface with short-range interactions including dipole-dipole, hydrophobic, ion-dipole, ion-ion, covalent bonds and hydrogen bonds. These interactions occur on the surface of flagella and fimbriae of microorganisms, as these are the specific part of the cell used for attachment. After irreversible attachment, the growth phase will start (step 4) in which the biofilm will reach maturity. As mentioned before, the biofilm organism will start excreting exopolysaccharides (EPS) as a protective slime barrier that also aids in attaching the cells firmly to the surface and to each other (Poulsen, 1999). According to Singh *et al.* (2006), the matured attached biofilm is able to spread across the surface and is therefore motile. Many microorganisms within the biofilm move in relation to nutrient or toxin concentrations within the bulk fluid and this phenomenon is referred to as chemotaxis.

Many factors affect the way microorganisms adhere to surfaces (Poulsen, 1999; Genigeorgis, 1995). In general, nutrient-limiting conditions enhance microbial attachment efficiency, while microbial cells will detach from a surface as the nutrient concentration increases. Furthermore, the hydrophobic or hydrophilic properties of a surface will determine the number and type of cells attaching. More cells attach to surfaces with better "wettability". In environmental situations it has been found that the more soil present on the surface, the more cells tend to attach to the surface as soil provides a larger surface area for the cells to attach and also contains nutrients that organisms can utilise. The presence of certain proteins, for example, bovine serum albumin (BSA), gelatine, fibrinogen and pepsin inhibit bacterial surface adhesion as these proteins adsorb to the bacterial surface, fimbriae or EPS and prevent adhesion to the surface. However, the above-mentioned proteins are not expected to be present in most natural biofilms. Microbial cell physiology also affects the attachment in biofilm. In general, the highest amount of attachment normally occurs in the lag phase followed by a decrease in the number of cells attaching through the stationary and death phase. Shear stress (frictional forces between adjacent cells) affects initial attachment, with fewer numbers of microorganisms attaching as the shear stress (or flow velocity) increases. Furthermore, the temperature of the environment will also affect the rate of surface attachment. The colder the biofilm environment, the less cells will attach, although the strength of the EPS decreases as the temperature increases (Schachter, 2003). The pH of the bulk water in which the surfaces are immersed, has a major influence on adherence to surfaces, since acidic and alkaline water will create opposite ionic charges. When cell motility is impaired, for example when the flagella are damaged, attachment is also slower.

In a review article by Rickard *et al.* (2003), the coaggregation model for multi-species biofilm development is described in more detail (Fig. 2.3). Coaggregation is a term used for a process by

which genetically distinct microorganisms become attached to surfaces and to each other via specific molecules. The development of biofilms on surfaces proceeds as a succession of adhesion and multiplication events. The first organisms that attaches are called the primary colonisers and bind through specific and non-specific physico-chemical interactions to components of an adsorbed, organic conditioning film (Fig. 2.3a) (Marsh and Bradshaw, 1995; Van Loosdrecht *et al.*, 1995; Dang and Lovell, 2000). Primary colonisers will then multiply to form micro-colonies if conditions are favourable (Fig. 2.3b). As environmental conditions change and more primary cells cover the surface, secondary colonisers will start to attach to the primary cells (Fig. 2.3c) thereby creating a more complex multi-species biofilm (Fig. 2.3d).
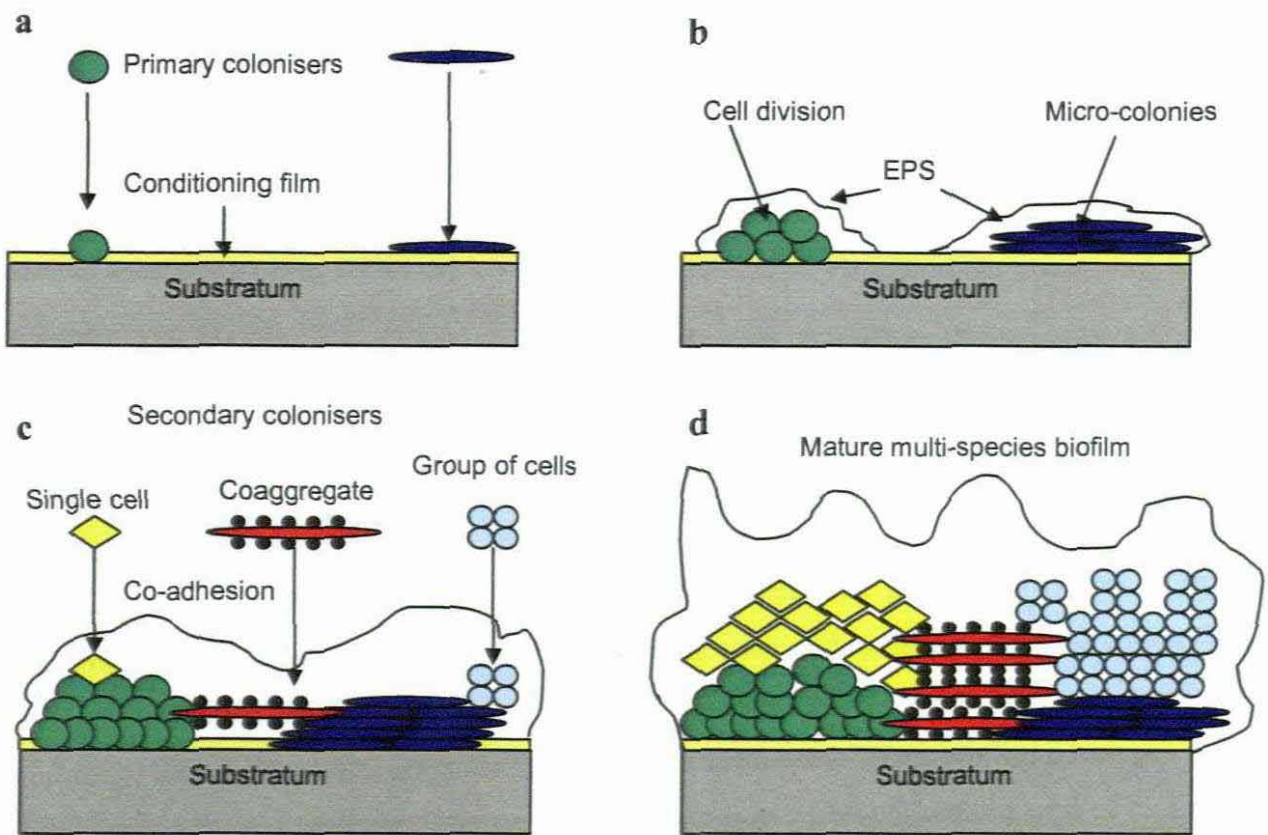
Figure 2.3: A diagram illustrating the roles that coaggregation can play in the development multi-species biofilm (Rickard *et al.*, 2003).

The specific mechanism of coaggregation can be described along two routes through a process called coadhesion. The first route is when single cells in the bulk fluid specifically recognize through chemical signalling and adhere to genetically similar cells in the developing biofilm. In the second route, secondary colonisers will first coaggregate with each other while still in suspension and then adhere to the biofilm as a unit (Fig 2.3c) (Busscher *et al.*, 1995; Bos *et al.*, 1994). The coaggregation pattern is thought be crucial for the ordered succession of more microorganisms and

mutually beneficial pairing of species (Marsh and Bradshaw, 1995). Coaggregation allows for more efficient transport of nutrients to all cells as well as quick removal of toxic substances. It is during this stage that EPS is produced which strengthens adhesion and act as receptors for coaggregation interactions. As a result of the coaggregation, cohesion, cell division and EPS formation, genetically identical microorganism cluster together, a phenomenon referred to as clonal mosaics (Molin, 1999; Moller et al., 1998; Gilbert et al., 2002).

The fifth and final step in the attachment model from Schachter (2003) is called detachment (refer to Fig. 2.2). If the microorganisms starve, they will detach from the biofilm. Normally the cell size will decrease as a result of poor nutrition, but this is reversible when nutrients become available again. A study by Horn et al. (2002) explains the importance of detachment of microorganisms from biofilm surfaces immersed in flowing water systems. Detachment from biofilms is caused by a combination of processes, including abrasion, erosion, sloughing and predator grazing. There are two mechanisms that can lead to detachment or erosion of cells from surfaces: Increase in the external shear forces (e.g. flow rate) and/or decrease of the internal strength (e.g. through hydrolysis of exopolysaccharides) (Rittmann, 1989).

As will be discussed later, the architecture (outward structure) of the biofilm can also affect the metabolic state of microorganisms and improve its ability to survive and resist many unfavourable conditions. Research revealed that there exist 3 types of biofilm architectures that are closely related to nutrient availability. The first type can be described as simple stalked or irregular branching structures that are well separated from each other and exist in water distribution systems with very low substrate concentrations (Walker and Keevil, 1994). The second type of biofilm has mushroom-shaped structures penetrated by large and small pores. This biofilm developes in media containing significant amounts of nutrients (Costerton et al., 1995). The third type of biofilm has a more or less flat, dense, homogenous structure consisting of small transportation channels. These biofilms have been found in environments where nutrient levels are generally high or periodically extremely high (Wood et al., 2000).

There is constant competition between organisms for nutrients and oxygen within a biofilm community (Nivens et al., 1995). To simplify diffusion within biofilms, it consist of a porous structure with capillary water channels also referred to as interstitial voids (Lewandowski et al., 1999) to allow the transport of nutrients, water and oxygen to all cells, as well as to remove toxic cellular waste. The architecture and thickness of a biofilm will mainly depend on the flow rate of the surrounding liquid and the biodiversity of species. The biofilm can maintain maximum

thickness between laminar and turbulent flow. If the flow rate is laminar (constant flow rate in one direction), the thickness is substrate-dependant. If the flow rate is turbulent, the thickness is erosion-dependant (Costerton *et al.*, 1995). Substrate transport can either take place through passive diffusion or through movement of the water in the capillary channels. Exchange of substrates mostly occurs on the outside of the biofilm, therefore various gradients exist across the biofilm.

2.3.1.3 Functions of exopolysaccharides (EPS) in biofilms. Exopolysaccharides (EPS), as the main chemical component of a biofilm, acts as the "cement" for all the cells and products, and is excreted by the cells within the biofilm (Sutherland, 2001). Biofilm exopolysaccharides capture inorganic and organic molecules from the liquid phase, making it available as nutrients for microorganisms inside the biofilm. Furthermore, they act as an ion-exchange resin where strongly charged molecules are actively removed from solution as they pass through (Schwartz *et al.*, 2003). It is not surprising that many researchers have also suggested that the EPS matrix might physically prevent the access of antimicrobials to cell surfaces. In addition to their potential as a diffusion barrier, polysaccharides and cellular materials at the periphery of biofilms may react chemically with and neutralise the disinfecting agent and thereby further reduce its availability and effectiveness (Evans, 2000; Sutherland 2001).

Different strains of bacteria produce different amounts of EPS and any one bacterium can produce various types of EPS at different times. For example, it has been shown that a strain of *Pseudomonas* uses one type of EPS to attach to surfaces and another to resist harsh conditions (Bjorklund *et al.*, 2004). Therefore different biofilm microorganisms equipped with various EPS molecules will result in an increased degradation capability and will be more resistant to harsh conditions than planktonic cells with no EPS. It has been shown that biofilm species that do not produce EPS can also gain extra resistance from harsh conditions if they are in the presence of EPS-producing bacteria (Sutherland, 2001).

According to Wingender *et al.* (1999), the molecular mass of EPS molecules ranges from a few thousand to several million Daltons. The prominent functional groups on EPS molecules are carboxyl, amino and phosphate groups. The type of biofilm microorganisms and wastewater can be correlated with the different functional groups and physical surface properties of EPS (Sponza, 2003). For example, the protein content of EPS is influenced by the amount of biodegradable matter in the wastewaters. Sponza (2003) concluded that the protein levels in the EPS increased, while DNA levels decreased as the ratio of inert COD to soluble COD decreased. It was also found

that EPS molecules from microbial flocs from winery and municipal wastewater were predominantly hydrophilic, whereas EPS from paper pulp and petrochemical wastewaters were more hydrophobic.

2.3.1.4 Mechanisms of biofilm resistance and survival. According to Morris *et al.* (1996), the physiological properties of biofilm bacteria are often markedly different from those of their planktonic counterparts, because of some basic properties of many biofilms. Biofilms can become more resistant to heat, light, drying and antimicrobial agents by using a variety of mechanisms. Because of these protective mechanisms, many organisms can live synergistically and therefore exchange metabolites and even genetic material when closely in contact with each other. Cloete (2003) describes seven possible mechanisms that biofilms use to gain resistance to unfavourable situations.

Firstly, the limited diffusion of antimicrobial agents through the biofilm plays a role. The EPS creates a diffusion barrier to antimicrobial agents and slows down the transport of chemicals to the enclosed and protected cells. The interaction with poly-anionic charges of the EPS molecules, distance of diffusion, viscosity of EPS and molecular sieving are important factors for limited diffusion of chemicals to cells. Because of the anionic nature of EPS, microbial polysaccharides can also bind toxic metal ions outside the cells, thus helping the organism to resist the toxic effects of metal cations (Bridge *et al.*, 1999).

Secondly, antimicrobial agents can interact with the biofilm cells and become neutralized. Gram-positive cells are more susceptible to antimicrobial agents since the cell walls do not contain receptor molecules or permeases to block bactericide penetration. However, gram-negative cell envelopes have evolved to regulate the transport of substances into the cells and thereby protecting the cells from bactericides (Cloete, 2003). **Thirdly,** biofilm microorganisms can excrete enzymes that transform bactericides into non-toxic substances. This is referred to as enzyme-mediated resistance. Certain bacteria used for bioremediation purposes are able to degrade toxic aromatic and phenolic compounds in such a way (Ma *et al.*, 1998), and this is the reason why indigenous olive wastewater biofilms are excellent candidates for olive wastewater bioremediation.

Fourthly, the species arrangement and architecture of the biofilm will determine the metabolic state of the microorganisms present. The metabolic state of the various species will then determine how susceptible it is to antimicrobial agents (Cloete, 2003). Many chemical gradients exist within a biofilm that can influence the metabolic state of microorganisms and consequently its resistance to

toxic chemicals. Nutrient gradients exist because of respiration in the outer layer, fermentation in the middle layer, and the release of methanol. Rapid utilisation of oxygen by the aerobic microorganisms at the water-biofilm interface, are responsible for the development of an oxygen gradient. This lack of oxygen causes anoxic zones where anaerobic conditions prevail and bacteria, carrying out fermentation or anaerobic respiration, can proliferate. At this stage, biofilms are considered more mature, with a higher species diversity, stability and resistance (Wilson, 2001).

The **fifth** resistance mechanism stated by Cloete (2003), is the ability of biofilm microorganisms to undergo genetic adaptation. Specific gene expression in cells can happen while the cell is firmly attached to a surface, other cells, the EPS matrix or dirt. Genetic exchange, i.e. horizontal gene transfer, between microorganisms could also result in the acquisition of antibiotic or other resistance genes that are usually carried on mobile plasmids in microorganisms. Because a change in genotype will result in a change in phenotype of a specific microorganism, it might therefore be enabled to produce toxin-degrading or repair enzymes (Morris *et al.*, 1996; Farr and Kogoma, 1991). Intrinsic factors, such as gene expression, do not only produce resistance mechanisms in microorganisms, but can also affect bacterial colony structures. Genetic sequences, in conjunction with other physico-chemical factors, can also determine the morphology of the cell, mode of reproduction, the presence or absence of flagella and fimbriae, production of EPS, motility, energy metabolism, pigment formation, etc (Winpenny 1999, 2000).

The **sixth** resistance mechanism of biofilms is in the structure of the outer membranes (cell envelope) of the microbial cells. For microbicides to be effective, they must be able to penetrate through the cell envelope into the cytoplasm of the cell and accumulate at high concentrations at the specific attack site. Hydrophilic antimicrobial agents are blocked by the lipopolysaccharides as well as the underlying phospholipids within the hydrophobic outer membranes of microorganisms, while hydrophobic chemicals are blocked by the presence of outer membrane proteins.

The **seventh** and final resistance mechanism discussed by Cloete (2003) is referred to as the presence of efflux pumps. Multi-drug resistance efflux pumps are a system of protein reactions within cell and cell envelopes to remove antimicrobial agents. Pumps may be specific for one substrate or may transport a range of structurally dissimilar compounds. Efflux pumps in bacteria can be divided into five main groups of protein transporter mechanisms: MF, MATE, RND, SMR and ABC. The ABC mechanism uses ATP hydrolysis as its energy source, while the MF, MATE, RND and SMR use the proton motive force to drive the export of substrates (Webber and Piddock, 2003).

In the Gram positive bacterium, *Staphylococcus aureus*, the QAC efflux system have been characterised that encodes for resistance against ethidium bromide and benzalkonium chloride. The genes *qacA* and *qacB* encode for high resistance while *qacC* and *qacD* encode for low resistance (Rouche *et al.*, 1990). In the Gram- negative bacterium, *Pseudomonas aeroginosa*, three efflux systems have so far been identified, i.e. MexA-MexB-OprM, MexC-MexD-OprJ and MexE-MexF-OprN. These systems remove many antibiotic compounds from the cells such as tetracycline, chloroamphenicol, fluoroquinolones, β-lactams, novobiocin, erythromycin, fusidic acid and rifampin (Schweizer, 1998; Ong *et al.*, 2007).

A study by Brooun *et al.* (2000) also suggests that *Pseudomonas aeruginosa* biofilms exposed to antibiotics (ciprofloxacin and ofloxacin) may express multidrug resistance mechanisms (MDR) responsible for extrusion of unrelated antimicrobials from the majority of bacterial cells in the biofilm. Brooun *et al.* (2000) also hypothesises that it is possible that biofilms might contain a small subpopulation of super-resistant cells that are responsible for biofilm re-growth after treatment with an antibiotic. Although Brooun *et al.* (2000) only mentions antibiotics and *Pseudomonas aeruginosa* biofilms, it is suggested that it might be possible for other mixed-species biofilms to have even more complex resistance mechanisms not only against antibiotics, but also against common cleaning detergents and disinfectants used in industry.

2.3.1.5 Identification of putative antimicrobial agents in the olive industry. Olive wastewater contains many substances that have the ability to either kill or inhibit growth of many bacterial and fungal species. The most important antimicrobial molecules in olive wastewater are a variety of phenolic compounds. Olive wastewater may also contain high levels of sodium hydroxide (NaOH) and sodium chloride salt (NaCl) that increase osmotic stress within microbial cells. Lastly, the wastewater contains variable levels of detergents that have been used in the factories to clean the equipment and the floors. It might be possible that the presence of these antimicrobial agents has a "hurdle" effect on microorganisms, meaning that the chemicals could work synergistically against the formation of biofilms.

The phenolic compounds in olive wastewater have the potential to denature membrane proteins of cells and block transport of nutrients through the cell membrane. It can react with nucleic acids, interfere with DNA replication and cause leakage of nucleic acids and proteins out of the cell. Phenolic compounds can also chelate metal ions, resulting in the lowering of the metal ion activity in the cell. By chelating metal ions, such as iron and copper, it reduces the bioavailability of these ions that is necessary for enzyme functioning (Wong and Kitts, 2006). A correlation exists between

the structure of the phenolic compounds, especially flavonoids, and its antimicrobial activity. Antimicrobial activity can increase or decrease depending on the positions of the hydroxyl groups on the ring structure of flavonoids. It was also shown that 2', 4' – dichloro derivatives of flavonoids had a 4-fold increase in antimicrobial properties compared to its non-chlorinated counterparts (Cushnie and Lamb, 2005). A recent review from Cushnie and Lamb (2005) also describes three antimicrobial mechanisms in bacteria resulting from phenolic compounds, such as flavonoids, that include the inhibition of nucleic acid synthesis, disruption of proper cytoplasmic membrane functioning and energy metabolism. The disruption of energy metabolism is suggested to be a result of the inhibition of CoQ and NADH – cytochrome c reductase within the bacterial respiratory electron transport chain (Haraguchi et al., 1998).

Important antimicrobial chemicals present in olive wastewater are sodium hydroxide and sodium chloride. When NaOH and NaCl dissolve in the wastewater, it dissociates into Na^+, OH^- and Cl^- ions. The negatively charged hydroxide ions can bind to or hydrolyse the outer membrane proteins of the cell envelopes thereby disrupting its function. Sodium hydroxide will therefore weaken the outer membrane structure and make the cell more acceptable to other antimicrobial agents, such as phenolic compounds. A high concentration of extracellular sodium and chloride ions will create two osmotic gradients (for water and salt ions) between the cytoplasm and the wastewater outside the cell. Depending on the salt concentration in the wastewater, water will be removed from the cells at a faster rate than it can be absorbed. There could also be uncontrolled movement of ions into the cells. This will disturb the functions of ion efflux pumps such as the sodium/potassium ATPase ion pump that regulate the concentration of ions within the cell. The lack of water will also disturb or prevent many water-dependant biochemical reactions within the cells.

Both table olive as well as olive oil processing uses detergents in order to clean and sanitise working surfaces and floors. These detergents are aimed at eliminating the microorganisms present. The different properties (concentration, type and organic content) of each detergent play a role in removing the unwanted organisms. The antimicrobial property of these solutions is anticipated to have a negative effect on the development of indigenous olive wastewater biofilms. Detergents used inside the factory to clean working areas and surfaces are generally bacteriostatic and/or bacteriocidal as well as fungistatic and/or fungicidal (Mah and O'Toole, 2001). These detergents will significantly affect the microbial community structure within indigenous biofilms. Therefore the influence of the detergents has to be evaluated as well as the time taken to recover from the shock of the detergents. A quicker recovery time for the biofilms will result in a more effective and

rapid bioremediation process, since the process of bioremediation relies completely on the efficiency of the biofilms.

On the other hand, sub-inhibitory concentrations of detergents in the wastewater might result in increased acquired resistance over long periods of application (Geesey *et al.*, 1994). The probability of acquired resistance in olive wastewater biofilms is high, since high volumes of wastewater resulting from lye treatment, washing steps and fermentation brines follow the same disposal route as the detergent water. This means that detergent concentrations in the bioreactor system will be diluted and therefore stimulate possible acquired resistance in biofilm microorganisms. At Buffet Olives, a table olive producer, six cleaning chemicals are routinely used: Robot, Contrabac, Alkaliser 485, Sanitiser HA, Order SC and Tetrasheen (trade names). Sodium hydroxide is used as a chemical in the debittering process of the olives, while NaCl is used during the fermentation step and these could also have an antimicrobial effect on specific microorganisms. At Vesuvio Estate, an olive oil producer, two cleaning chemicals are routinely used: Removil™ and Limex™. These chemicals and their physical properties are further discussed in Chapter 4.

According to Prescott *et al.* (1999), many conditions can influence the effectiveness of antimicrobial agents. Firstly, the size and composition of the microbial population is important. A larger population would require a longer time to die than a smaller population. If the population is composed of multiple species, certain species will have more resistance than others and therefore take longer to die. The concentration of the antimicrobial chemicals and duration of exposure will also determine the killing rate of the cells. Environmental factors, for example temperature and pH, will play an important role in the antimicrobial activity of these chemicals. Higher temperatures and lower pH values will generally enhance the effectiveness of the antimicrobial agents. The most well known antimicrobial chemicals can be classified according to their functional groups in five categories: Alcohols, halogens, heavy metals, quaternary ammonium compounds (QAC's) and aldehydes.

Alcohols are bactericidal and fungicidal, but not sporicidal. The most popular used alcohols are ethanol and isopropanol at a concentration between 70 and 80%. They act by denaturing proteins and dissolving lipids in the cell membranes. Important halogens used are chlorine and iodine. Iodine kills by oxidizing cell constituents and iodinating proteins. At high concentrations it may even kill spores. Chlorine can be applied in many forms and acts in the same way as iodine, although spores are resistant to this chemical. Most heavy metals are more bacteriostatic than bactericidal, but there are exceptions. Heavy metals combine with the sulfhydryl groups of proteins

and inactivate or even precipitate them. Aldehydes, like formaldehyde and glutaraldehyde, are highly effective sterilants. They are bactericidal, fungicidal and sporicidal and inactivate proteins. Quaternary ammonium compounds are cationic detergents and are characterised by positively charged quaternary nitrogen (e.g. NH_4^+) and a long hydrophobic aliphatic chain. They disrupt microbial membranes and may also denature proteins. Detergents containing chelating agents such as EDTA help in biofilm removal probably through the chelation of calcium and magnesium ions that cause the destabilisation of the exopolysaccharides matrix. In addition, it has been found that acidic and alkaline detergents affect the viability of *Staphylo aureus* and *Pseudomonas aeruginosa* biofilms, respectively (Prescott *et al.*, 1999).

Russel *et al.* (1997) identified 27 active chemicals found in most antimicrobial detergents and showed where the attack sites are in bacterial spores, vegetative bacteria and fungi. This is represented in Figure 2.4.
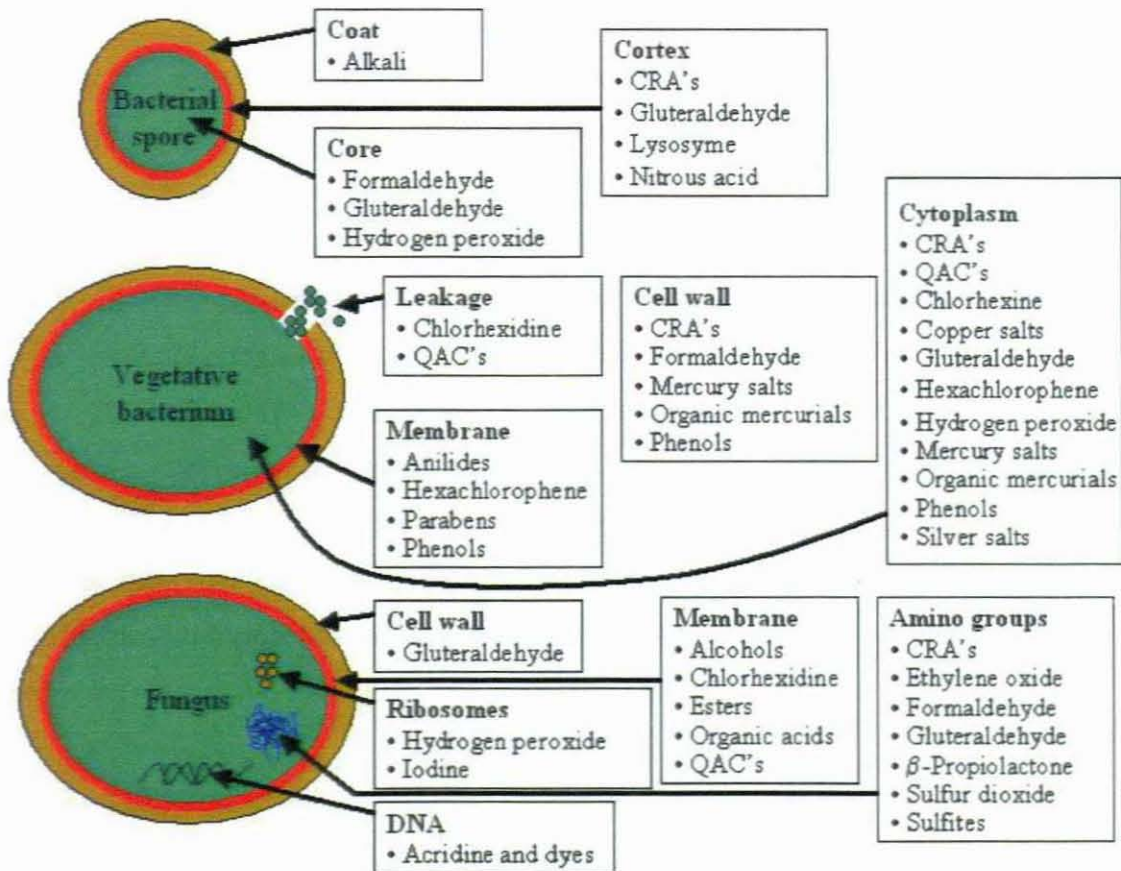
Figure 2.4: A diagrammatic representation of the attack sites of a variety of detergent chemicals on bacterial spores, vegetative bacterial cells and fungi (QAC's and CRA's are abbreviations for quaternary ammonium compounds and chlorine-releasing agents, respectively) (Russel *et al.*, 1997).

2.3.1.6 Application of biofilms in bioremediation of wastewater. Bioremediation is a rapidly changing and expanding area of environmental biotechnology and can be defined as the use of microbes to degrade biodegradable organic wastes that are present in polluted environments (Lazarova and Manem, 1995). There has been a significant amount of interest in the last few years in bioremediation based on the introduction of bacteria able to catabolise recalcitrant compounds deposited in the environment (Molin and Tolker-Nielsen, 2003).

The use of bioremediation processes is of interest to many researchers because of its attractive advantages. The bioremediation process is flexible and adaptable to variable environmental conditions and, over time, microorganisms can evolve to degrade novel synthetic (i.e. man-made) chemicals that were previously hard to degrade (Mandelbaum *et al.*, 1995). Bioreactors for bioremediation purposes can be implemented on site, often directly into the waste effluent system, and can be used with dilute or widely diffused contaminants (Iwamoto *et al.*, 2001).

Biofilm reactors are desirable in biological treatment processes because a very high biomass concentration can be reached in the treatment systems (Liu and Tay, 2001). This high biomass concentration means that contaminant degradation is rapid and highly concentrated or large volumes of waste can be treated in reactors, which take up limited space. An important advantage of using biofilms for bioremediation is the fact that they are relatively inexpensive in comparison with other methods such as physico-chemical and advanced oxidation treatments of wastewater (Schachter, 2003; Grommen *et al.*, 2002). Some areas where biofilms are applied in bioremediation processes are ground water treatment, sewage treatment (Gilbert *et al.*, 2003), microbial-enhanced oil recovery, mine effluent treatment and treatment of various other industrial wastewaters. Other processes include a variety of solid-state fermentation processes (Vandenberghe *et al.*, 2000), as well as biological nitrogen fixation in which olive mill waste has been used as a substrate (Balis *et al.*, 1996).

The common objective in the various bioremediation processes is to create the necessary environment to facilitate growth and contaminant degradation by the appropriate microorganisms (Ward *et al.*, 2003). The duration of bioremediation processes may range from 5 to 25 years for natural attenuation processes, 0.5–3 years for *in-situ* subsurface processes, 1–18 months for soil pile/composting processes, 1–12 months for land-farming and slurry phase systems and 15 days for accelerated slurry phase systems. Natural attenuation is the natural breakdown processes that occur within untreated wastewater under uncontrolled conditions. With accelerated slurry phase processes, nutrients or bio-enhancers are added to increase the metabolism of bioremediation

microorganisms. Average daily rates of contaminant degradation can range from 5 ppm to 10,000 ppm for natural attenuation processes to accelerated slurry phase systems, respectively (Ward *et al.*, 2003).

Complete degradation of industrial wastewaters is difficult and complex. None of the individual species in biofilms are usually capable of completely degrading the complex pollutants in wastewater (Liu *et al.*, 2002). As mentioned previously, the advantages of using biofilms are that the species in biofilms can resist stressful environmental conditions, such as hydrodynamic shear force, heat, cold, starvation, pH, metabolic products and toxic substances by increased production of exopolysaccharides, gene expression and plasmid transfer to adjacent cells (Danese *et al.*, 2001; Lazarova and Manem, 1995). Other resistance mechanisms include the alteration of the membrane-protein composition in response to antimicrobial agents, which result in decreased permeability of the cell to these compounds (Mah and O'Toole, 2001).

Piperidou (2000) listed six factors that affect the rates and degrees of contaminant degradation in soils, i.e. the nature of the contaminants, properties of the soil, nature of the process, size and make-up of the microbial composition, temperature and pH play an important role in the bioremediation process. The nature of contaminants can be subdivided into the structure, solubility, bioavailability, biodegradability, co-metabolism potential, substrate/metabolite concentration and toxicity of the contaminants. The nature of the process involves factors such as homo- or heterogeneous environments, contents of the water, nutrients, oxygen and presence of bioavailability enhancing agents.

Although microbial bioremediation provides a promising way for improving environmental impacts from various wastewaters, researchers still have a limited understanding of the intricate and complex interactions present within multi-species biofilms as well as its full impact on the ecosystem (Iwamoto *et al.*, 2001). Another challenge for microbiologists is that an estimated 99% of microorganisms are unculturable with conventional laboratory techniques (Kuske *et al.*, 1997). For a direct analysis of the microorganisms in treatment processes, a combination of conventional and modern molecular techniques, such as the polymerase chain reaction (PCR) and DNA fingerprinting techniques is normally used (Stams and Oude Elferink, 1997).

2.4   The microbiology of olives and olive processing

In order to study the seasonal changes in the microbial populations of indigenous biofilms growing in olive wastewater, it is important to know the organisms that are associated with olive fruits and olive processing in general. It is highly expected that many of these microorganisms associated with olives and olive processing will be present within the biofilms. The microbial ecology of olive fruits and olive processing has been studied in detail over the last few years.

From the literature it is clear that olives and its processing stages are rich in biodiversity. For example, Nychas *et al.* (2002) reported that the majority of the yeast population on olive fruits is present on the skin surface and the stomal openings of olives, whereas bacteria are present in the intercellular spaces of the sub-stomal cells. Faid *et al.* (1994) have done microbial studies on green olives before the fermentation process and showed that there is a possible interaction between yeasts, pseudomonads and coliforms causing post-harvest alterations to olive fruits (Marchi *et al.*, 2006). Yeasts isolated and identified in this study were *Debaryomyces hansenii, Rodothorula glutinus, Pichia membranefaciens, Pichia anomala* and *Candida bacarum*. The gram-negative fermenting bacteria were represented by the species: *Erwinia carotovora, Hafnia alvei, Enterobacter agglomerans, Enterobacter aerogenes, Serratia marcescens, Serratia liquefaciens* and *Shigella flexneri*. The oxidase-positive bacteria were most abundant and mainly dominated by *Pseudomonas* species including *Pseudomonas aeruginosa, Pseudomonas alcaligenes* and *Pseudomonas syringae*. Faid *et al.* (1994) also found that most of these microorganisms on green olives are cellulolytic and lipolytic, however isolated *Bacillus* species (*Bacillus megaterium, Bacillus pumilus, Bacillus cereus* and *Bacillus olei*) were found to not be involved in olive fruit deterioration.

The predominant microorganisms found in fermented Spanish style treated olives are lactic acid bacteria and yeasts responsible for the fermentation of olives in natural processing (Fernandez et al., 1997). The predominant lactic acid bacteria (LAB) found on fermented olives are *Lactobacillus plantarum*, however other species, such as *Lactobacillus pentoses* and *Leuconostoc mesenteroides* are also present (Campaniello *et al.*, 2005). These microorganisms consume the sugars of the olive fruit, which are present in the brine, and produce mainly lactic acid and to a lesser extent other acids, resulting in a drop in pH (Adams, 1990). The majority of LAB involved in olive fermentations are homofermentative (utilise a single sugar as carbon source) in order to ensure best quality and maximum preservative effect. Lactic acid bacteria predominate on fermented olives, but predominant bacteria on the raw materials are the mesophilic *Bacillus* species. During the first

phase of fermentation *Enterobacter cloacae*, *Enterobacter amnigenus* and *Chryseobacterium* species can also be isolated. During latter fermentation stages, the mesophilic microorganism, *Bacillus subtilis*, can also be detected in low numbers but are soon inhibited by the proliferation of lactic acid bacteria and yeasts (Campaniello *et al.*, 2005). Campaniello *et al.* (2005) found no significant difference in yeast and bacterial species of Spanish style treated olives compared to naturally fermented olives.

High NaCl concentrations, low pH and low oxygen tension are the important factors that inhibit the further growth of mesophilic microorganisms as well as preventing pathogen contamination. If the olives lack adequate fermentable sugars (especially after lye treatment) or if the pH drop during the first stage of fermentation is too slow, the LAB might not dominate the rest of the microflora. This will result in spoilage or the presence of pathogens. To overcome these problems, the olives can be pasteurised and a starter culture, additional sugars, extra salt or acid can be added to the brine. The starter culture usually consist of *Lactobacillus plantarum*, *Lactobacillus pentoses* or *Enterococcus casseliflavous*. The most affordable sugars that can be added to brines are sucrose and glucose and are normally used at a concentration of at least 0,5% in brines (Chorianopoulos *et al.*, 2005).

Hernández *et al.* (2007) identified 72 strains of 17 yeast species that was present in the brine (pH 3.96 – 4.18 and 7.8 – 9.5% w/v NaCl) of fermented green olives. The following yeast species were identified in the brine and those indicated with an asterix (*) were also present on the fresh, unfermented green olives: *Pichia anomala, Pichia guilliermondii*, *Candida maris*, *Candida rugosa, Candida humicola*, *Candida zeylanoides, Candida inconspicua, Candida glabrata, Candida parapsilosis, Kluyveromyces marxianus*, *Cryptococcus laurentii*, *Saccharomyces cerivisae, Trichosporum cutaneum, Debaryomyces hansenii, Rhodotorula glutinis*, *Rhodotorula minuta* and *Torulaspora delbrueckii*.

A limitation to the above-mentioned studies is that these studies only used culture-dependant identification methods and microscopy. It is therefore possible that un-cultureable species may exist that can also play a functional role on olive skins and in the fermentation process. For the purpose of this study, it is important to realise that all of these bacteria and fungi are likely to be present in either the wastewater from olive oil producers (fresh fruit microorganisms) or table olive producers (fresh and fermented fruit and brine microorganisms) and that a significant fraction of the microorganisms present on the raw materials and the products could also be present in the biofilm that develop in biological wastewater treatment systems (Viljoen-Bloom *et al.*, 2003). They can therefore play an integral part in the bioremediation of the wastewater. As the wastewater and

biofilms are exposed to external environmental factors, such as contact with soil and other debris, many other microorganisms that are not associated with olives and the fermentation brine, could also be present in the wastewater and participate in biofilm formation.

In previous studies, researchers evaluated single species of microorganisms that shows good potential for degrading phenol-containing wastewaters. Fungi and yeast play an important role in the biodegradation of this type of wastewater and are essential in natural biofilm communities (Elvers *et al.*, 1999). The yeasts *Candida krusei, Saccharomyces chevalierie* and *Saccharomyces rouxii* have been used successfully to degrade olive mill wastewater. The wastewater contains reducing sugars and organic acids that were both readily utilised by the yeasts when aerated in shaker flasks (Gharsallah, 1993). According to Viljoen-Bloom *et al.* (2003), previously 'hard to degrade' wastewaters are now being successfully degraded by various yeasts and fungal species. Lyberatos *et al.* (2001) studied the removal of phenolics from olive mill wastewater by using the white-rot fungus *Pleurotus ostreatus*. The phenols, similar to lignin, are difficult to degrade. Lignin can only be broken down to CO_2 and water by certain microorganisms, such as *P. ostreatus* and *Phanerochaete chrysosporium* that produce manganese peroxidase, lignin peroxidase and laccases that are responsible for the oxidation of lignin (Reddy *et al.*, 1992). This process is called mineralisation (Sayadi *et al.*, 1999). When cellulose is broken down to sugar, hydrogen peroxide, hydroxyl and superoxide radicals are formed that are required to initiate a snowball reaction in the oxidation of lignin and polyphenolic compounds. This has been shown with a brown rot fungus *Gloeophyllum trabeum* (Balis and Antonakou, 2000). Other microorganisms such as *Asperigillus terreus* and *Asperigillus niger* were studied in similar ways by Hoyos *et al.* (2002).

Other microorganisms that were studied for their possible olive wastewater bioremediation purposes were *Lentinula edodes* (D'Annibale *et al.*, 1999), *Phanerochaete flavido-alba* (Lopez *et al.*, 2001), *Chalara paradoxa* (Galvez *et al.*, 1999), *Geotricum candidum* (Hamdi et al., 2001) and bacterial strains of *Comamonas, Ralstonia, Pseudomonas* and *Sphigomonas* (Di Gioia *et al.*, 2001). All these studies were done to evaluate the microorganisms for efficiency of COD and phenol breakdown and colour reduction in olive mill wastewater. Decolourisation of olive wastewater corresponds to the depolymerisation of high molecular mass aromatics combined with mineralisation of a wide range of mono-aromatics (Perez, 1997). These studies showed significant reductions depending on temperature, whether the water was sterilised or non-sterile, diluted or concentrated and heat-treated.

Naturally occurring biofilms have been used to bioremediate olive wastewater for many years in the technologies discussed earlier. However, research describing the changes in microbial populations over time and the effect of external factors on biofilms population dynamics is lacking. Understanding biofilm population dynamics within olive wastewater could potentially contribute to a better understanding of the conditions and mechanisms involved to optimise or maximise the bioremediation efficiency of these indigenous olive wastewater biofilms.

CHAPTER 3

LITERATURE REVIEW OF METHODOLOGIES

This chapter will give a literature overview of the techniques and methods employed during this study in order to establish the relevance of these techniques and their applicability in this type of study.

3.1 The Rotating Biological Contactor (RBC) as a bioremediation tool for treating wastewater

3.1.1 General description

Several criteria should be considered when deciding on a treatment system for wastewater. These include the eco-friendliness of the process as well as its flexibility to handle various concentration loads. The treatment option should have low capital and operating costs, require minimal personal attention and a setup that does not occupy too much land or space. Furthermore, the desired degree of degradation must be achieved without the need for dilution of the wastewater (Viljoen-Bloom *et al.*, 2003). Many other biological systems, for example anaerobic and aerobic digesters, result in good COD removal, but are costly in terms of installation, operation and maintenance, while they still requires long retention times, which adds to the total cost of treatment (Beccari *et al.*, 1999).

The RBC is potentially suitable for the treatment of olive wastewater. This system is based on microbial biofilms that develop on the surface of discs (also called bio-discs) mounted on a horizontal shaft with at least 40% of the discs submerged in the wastewater. Rotation of the shafts results in alternating contact with water and air that allows for aerobic growth. Various factors can be controlled, such as rotation speed, wastewater re-circulation and flow speed, i.e the hydraulic retention time. RBC's are easy to operate, have short start-up times, require little maintenance and is easily oxygenated, while sloughing-off of the biomass is limited and well controlled by reactor design. Fast growers are on the outside of the film protecting the slow growers on the inside from shear stress (Viljoen-Bloom *et al.*, 2003). For larger industrial-scale treatment plants, individual RBC units (called shafts) are arranged in series to maximise capacity and treatment efficiency. Baffles are used to separate groups of shafts into a series of completely mixed bioreactors, each referred to as a stage. Baffles can be repositioned to change the size of the stages in response to long-term variations in process loadings. A series of stages is called a "treatment train" and several "trains" can be placed parallel to each other depending on the volume of wastewater being treated (Grady *et al.*, 1999).

The biomass that grows in the tank of the contactor is of dual form. Some microorganisms are attached to the disc surfaces as biofilms, while other organisms form sludge granules suspended from the discs in the trough area. This suspended biomass grows inside the wastewater and not on the disc surfaces. Although the suspended biomass can contribute to bioremediation, it can easily grow to large quantities over time and result in clogging of the system, which could result in high capital and maintenance costs. To keep these costs to a minimum, multiple contactor systems (treatment trains) should be used for the bio-treatment of large volumes of wastewater (Niaounakis and Halvadakis, 2004).

3.1.2 Applications of the RBC

Rotating biological contactors have typically been used to provide secondary treatment (organic matter removal) to municipal wastewater as well as nitrification and carbon oxidation (e.g. polysaccharide breakdown) of municipal wastewater. In the United States, 70% of RBC treatment plants are solely used for organic matter removal, 25% for combined carbon oxidation and nitrification and 5% for separate nitrification of municipal wastewater (Hyned and Iemura, 1980). RBC's have been used successfully at plants with a wastewater flow rate less than 40 000 m^3/day and moderate hydrogen sulphide levels. Although many RBC's have provided acceptable performance, many others have not met the performance expectations. A survey indicated that 80% of RBC's designed before 1980 experienced operational problems (Water Pollution Control Federation, 1988). Improving construction techniques and the use of appropriate organic and hydraulic loading has solved many of these problems. New approaches are being investigated for upgrading existing RBC facilities to accomplish higher levels of treatment (Neu, 1993).

3.1.3 Factors affecting the performance of a RBC plant

Many factors affect the performance of a RBC treatment plant such as surface organic loading, hydraulic retention time, rotational speed, number of stages, temperature of water, wastewater-specific characteristics (e.g. COD or BOD concentration and pH) and biofilm-specific characteristics (e.g. microbial species and biofilm architecture on surfaces) (Grady et al., 1999). The surface organic loading (SOL) of a RBC system is the amount of COD or BOD in 1 m^2 of wastewater that passes over the total media surfaces per day and is measured in COD / (m^2 x day). The COD removal rate generally increases as the SOL increases, but at a decreasing rate. Therefore, substrate removal efficiency decreases as the SOL increases. The hydraulic retention time is the total volume of the fluid at a specific moment in the RBC divided by the inflow rate.

Longer retention times will generally increase the removal rate of COD and BOD from the wastewater and *vice versa*.

A faster rotational speed of the RBC discs will decrease biofilm attachment (high shear stress) and hence decrease the COD removal efficiency of the biofilm. However, if the rotational speed is too slow, the biofilm will become too thick and detach due to weight stress. This can result in clogging of the system and higher maintenance costs. A higher number of stages present in a RBC system will result in an increase in the surface organic loading (SOL) of the reactor as well as the surface area of the biofilm in contact with the wastewater and will consequently increase the COD or BOD removal capacity of the plant. Finally, the temperature of the wastewater also affects RBC performance. Higher temperatures will generally improve the removal rate of COD and BOD, however changes are relatively small between 15°C and 25°C (normal temperatures of wastewater). In colder countries where wastewater temperatures can reach below 15°C, removal rates are significantly lower (Grady *et al.*, 1999).

A laboratory-scale RBC unit can be used to study and model the efficiency of naturally occurring biofilms to remove COD and to degrade toxic compounds. It can also be used to predict the effect that biocides will have on the ability of larger industrial-scale RBC's to effectively treat higher volumes of wastewater. This is important if the accumulation of biocides in the environment is to be avoided. According to a study by Laopaiboon *et al.* (2003), glutaraldehyde, a biocide present in many food wastewaters such as poultry processing, were used to determine the effectiveness of the RBC. The maximum concentration that did not adversely affect treatment efficiency and operation of the RBC was found to be 80 ppm. Laopaiboon *et al.* (2003) observed that the system remained in steady-state with high COD and gluteraldehyde removal rates. Biofilm appearance and thickness were good and there was a balance between attachment and detachment of organisms shown by constant viable counts in the biofilm and the wastewater. Gluteraldehyde at higher concentrations than 80 ppm caused the system to fail after 3 weeks. The biofilm became too thick, detached and started to block the RBC system.

3.2   Fingerprinting of environmental microbial communities

Several modern molecular techniques are used for understanding the dynamics of microbial communities. Methods such as polymerase chain reactions (PCR) and denaturing gradient gel electrophoresis (DGGE), can be applied as powerful tools in bioremediation processes to detect and quantify the target microorganisms that are directly or indirectly related to the degradation of

contaminants. The 16S or 18S ribosomal RNA (rRNA) of microorganisms can be isolated and used to monitor changes in bacterial or fungal communities. Advanced molecular methods can play a significant role in the optimisation, validation and impact of bioremediation processes in future and will ensure that the technology is reliable and safe.

3.2.1 The polymerase chain reaction (PCR)

The main purpose of PCR is to make a large amount of copies of a specific gene of interest in order to have enough DNA template for further reactions. There are three major steps in a PCR, which are repeated for 30 or 40 cycles. This is done in an automated cycler, which can heat and cool the sample tubes with the reaction mixture in a very short time. The 3 basic steps are: denaturation at 94°C, annealing between 48 and 56°C and extension at 72°C (Vierstraete, 1999).

During the denaturation step, the double strands melts to single stranded DNA, all enzymatic reactions stop, for example the extension from a previous cycle. During the annealing of the primers, the primer will shift around constantly with Brownian motion. Hydrogen bonds are constantly formed and broken between the single stranded primer and the single stranded template. If the primers are highly complimentary to the template, it will hybridise to the template with stable H-bonds. After hybridisation, the polymerase can start attaching single bases. Once there are a few bases built in, the hydrogen bond is so strong between the template and the primer that it does not break anymore. During the extension step the temperature is raised to 72°C to provide the ideal working temperature for the DNA polymerase to attach single bases between the forward and reverse primers on the template DNA. The primers with the build-in bases attract to the template with strong hydrogen bonds and are stronger than the forces that break the attraction. Primers that do not fit precisely on the template will detach at 72°C, because of the higher temperature and cannot extend the fragment. The bases or deoxynucleotide phosphates (dNTP's), which are complementary to the template bases, are joined to the primer on the 3' side (the polymerase adds dNTP's from 5' to 3' to the primer, reading the template from the 3' to 5' side.)

Bacterial and fungal identification based on molecular methods, especially PCR and sequencing of the rRNA gene loci, has become a very important and sensitive technique in studying bacterial communities in environmental samples (Amman et al., 1995; Head et al., 1998). Culture-independent methods became more attractive to researchers, because it overcomes problems associated with selective culturing and isolation of bacterial and fungal species from natural samples. The main reason why culture-independent methods are so attractive is that very little

information is known about the habitat and growth conditions of most microorganisms. Developing growth media for environmental microorganisms can be a difficult process because growth conditions in media can't always accurately resemble conditions in their natural habitat. Molecular methods are also much more rapid and reliable than cultivation of microorganisms, since only 1 % of all microorganisms are culturable on media (Kuske *et al.*, 1997).

Other applications of PCR are used for e.g. the generation of hybridisation probes for Southern Blotting and cloning, genetic fingerprinting for forensic analysis, DNA sequencing, analysis of ancient DNA, earlier detections of viral diseases and quantification of a specific DNA fragment of interest within a food or blood sample.

3.2.2 Denaturing gradient gel electrophoresis (DGGE)

One of the most important culture-independent methods used to study microbial profiles is denaturing gradient gel electrophoresis (DGGE). It is based on the separation of polymerase chain reaction amplicons of the same size, but different base pair sequences. According to Ercolini (2004), the first researchers to introduce PCR-DGGE of ribosomal DNA into microbial ecology were Muyzer *et al.* (1993). With DGGE it is possible to assess a significantly larger fraction of the microbial community structures within environmental samples and to determine the dynamics of microbial communities in response to environmental variations without cultivation. There are many applications to DGGE of which the most recent application focused on the structure and evolution of microorganisms in soil (Avrahami *et al.*, 2003), river and lake water (Crump *et al.*, 2003), gastrointestinal tracts (Zoetendal *et al.*, 2002), wastewater treatment bioreactors (Stamper *et al.*, 2003), insect ecology (Reeson *et al.*, 2003) as well as clinical samples (McBain *et al.*, 2003). PCR-DGGE can also be used for identification and typing of microbial species from virtually any surface (Nielsen *et al.*, 2000).

As mentioned earlier, with DGGE it is possible to detect differences between DNA fragments of the same size, but with different sequences. The reason for this is that these fragments can be separated in a denaturing gel based on differences in its denaturation or melting profile (Fisher and Lerman, 1983). In an acrylamide gel, urea and formamide are used in combination to ensure melting of double-stranded DNA fragments. A solution of 100% denaturant consists of 7 molar urea and 40% formamide in water. A gradient from low to high denaturing solutions in acrylamide are prepared, mixed with a gradient maker, and pumped slowly into a gel casting to create a gel with a linear denaturing gradient. Electrophoresis is normally carried out at a constant temperature between 55

and 65°C, mostly 60°C. In a DGGE gel, the double-stranded DNA fragments are subjected to an increasing denaturing concentration as they progress through the gel and will partially melt in a very small region in the gel normally referred to as melting domains. The melting temperature (T_m) depends on the sequence of the DNA fragments travelling through the gel. Once the T_m of the lowest melting domain is reached, the DNA fragment will partially melt, resulting in branched "breaking" molecules. The primers for the amplified gene fragments are designed to contain a 30 to 40 bp GC-tail ensuring that the DNA remain partially double-stranded and that the region screened falls in the lowest melting point domain (Sheffield *et al.*, 1989). This partial melting is essential to ensure optimal resolution (effective separation) of DNA fragments.

Partial melting of the DNA causes a drastic decrease in mobility in the gel thereby fixing the position of the specific fragment within the acrylamide matrix. Other fragments with different base pair sequences will respond differently to the denaturing gradient and will find other melting domains to denature. Hypothetically, based on rRNA sequence variation, each PCR product produced should have a unique sequence composition and because of this phenomenon, DNA fragments of the same size but with different sequences can be separated.

DGGE can be performed in either perpendicular or parallel denaturing gradient gels. In perpendicular gradient gels, the denaturing gradient is perpendicular to the direction of electrophoresis. Broad gradient ranges are normally prepared for such gels (0 – 100% or 20 – 100%). These gels are used to detect the melting behaviour of DNA fragments and optimise the denaturing gradient to be used in parallel electrophoresis experiments. Only one sample or a mixture of amplicons for which the melting behaviour needs to be determined can be loaded in a perpendicular gel. For parallel DGGE, the gradient is in the same direction as that of the electric field and denaturing gradient ranges is narrower (30 – 50% or 40 – 60%) to allow for a better separation (Myers *et al.*, 1987). Multiple samples can be loaded on a single parallel gel and are therefore the most commonly used.

The optimal time for electrophoresis must be determined beforehand to allow good separation of the DNA fragments. A study by Sigler *et al.* (2004) indicated that under a constant voltage-time set-up (e.g. 100 V for 10 hours), shorter electrophoresis times might minimise instability of the denaturing gradient and result in a more complete band separation than can be achieved following longer runs. Sigler *et al.* (2004) ran four gels with 200 bp DNA fragments at a constant Volt-hour setup of 1000 V.h., but altered the time for each gel (25 V, 40 hours; 60 V, 16.7 hours; 100 V, 10 hours; 200 V, 5 hours) in order to observe the differences in band separation. If the voltage and time values for

each gel are multiplied, it is found that all four gels gave a constant Volt-hour value of 1000 V.h. The gels that ran for 40 and 16.7 hours gave poor separation while the gels that ran for 10 and 5 hours gave good separation even though all gels had the same Volt-hour regime. It is also of utmost importance that gel images cannot be compared unless all the parameters were the same for all gels, including the run time.

According to Ercolini (2004), the target for PCR amplification prior to DGGE is in most cases the ribosomal DNA. The reason for this being that the ribosomal DNA is a highly conserved area of the genome that includes variable regions. Therefore primers can be designed by hybridising to conserved regions, but spanning variable regions in order to obtain PCR amplicons with species-specific differences in base pair composition. These amplicons can then be separated on a DGGE gel. When targeting gene fragments from bacteria, primers are usually designed to amplify regions on the 16S rRNA. If fungi or other eukaryotic cells are used, primers are employed to amplify regions on the 18S rRNA or the 26S rRNA. In this study, the bacterial primers were designed to amplify the highly conserved V3 area of 16S rRNA of the bacteria. The fungal primers that were used, bound to and amplified the V9 region of the 18S rRNA of fungi.

In order to view the DNA pattern in the gel after electrophoresis, the polyacrylamide gel is stained in a solution of ethidium bromide and viewed under an ultraviolet light. Other, more sensitive, staining methods are also available for example silver staining (Felske *et al.*, 1996) and SYBR Green or SYBR Gold (Muyzer *et al.*, 1997). Silver staining is highly sensitive, but gels cannot be used for hybridisation experiments afterwards. Single-stranded DNA fragments are also detected with the silver staining method. SYBR stains are also very sensitive, does not give background staining and DNA can be detected at very low concentrations.

As with most techniques, DGGE has a few intrinsic problems that need to be understood in order to prevent misinterpretation of results. It is possible to introduce bias in many of the steps of the process from sample collection, sample handling, DNA isolation methods, PCR programs and DGGE itself. A good source of variability is introduced during the DNA extraction method, because of the difficulty of purifying nucleic acids from complicated matrixes, for example food. Certain species are less sensitive to lytic agents or might be less abundant and this will affect the efficiency of the DNA extraction. The different amount of species can also affect the concentration of the DNA and its detectability, and if this step is not optimised it could result in potentially very important species escaping detection later during PCR-DGGE. If matrix residues, such as humic acid in soil samples or phenols in olive wastewater biofilms, are present in the extracted DNA it is

possible for those residues to inhibit the PCR (Wilson, 1997). The DNA extraction usually requires prior optimisation in order to obtain highest species representivity and highest degree of purification possible.

The PCR itself can also influence the outcome of DGGE results. Reysenbach *et al.* (1992) reported that preferential or biased amplification might occur. This is when the template DNA re-anneals to itself thereby compromising the hybridisation of the primers (Suzuki and Giovannoni, 1996). Denaturation enhancing solvents can be added to the PCR mixture to overcome this problem. Preferential amplification may present serious problems for PCR-DGGE analysis in mixed microbial communities, because a lack of amplification of certain templates in a DNA mixture will result in a substantial percentage of the microorganisms not being detected in the sample. Therefore the choice of primer pairs and target genes is fundamentally important.

Other problems are the formation of chimeric or heteroduplex molecules that can affect the distribution of the bands in the DGGE profile (Kopczynski *et al.*, 1994; Ferris and Ward, 1997). This problem can be overcome by PCR optimisation (increasing the annealing temperature to prevent non-specific binding) or gel gradient optimisation. The fragments to be analysed by DGGE cannot be longer than 500 bp. This limits the sequence analysis and makes it sometimes difficult to achieve reliable identification of microbial species. This problem can be overcome by amplifying larger areas (1500 bp) of the rRNA, using the original gDNA as template, and then subsequent cloning and sequencing of the longer rRNA fragments. Another problem is the presence of multiple copies of 16S rRNA with sequence micro heterogeneity. A single species can display multiple bands in a DGGE gel, which overestimates the community diversity detected (Nübel *et al.*, 1996; Blaiotta *et al.*, 2003). It has been suggested that this phenomenon is the result of incomplete elongation of some of the rRNA gene fragments by the *Taq* DNA polymerase enzyme and that by optimising the final elongation time of the PCR, this effect can be limited.

Another problem that can occur is that bands that contain a similar GC-content, but with different base pair sequences can also position themselves in the same position on the DGGE gel. By cloning and sequencing the bands, the problem can however be overcome.

CHAPTER 4

MATERIALS AND METHODS

4.1 Preliminary evaluation of indigenous olive wastewater biofilm's potential to bioremediate South African olive wastewater

Prior to analysing the population dynamics of microbial biofilms that form in table olive wastewater (TOWW) and olive mill wastewater (OMWW), a preliminary study was executed in a small-scale RBC to determine the potential of these indigenous biofilms to reduce the COD concentration within a short (10-day) period. Preliminary visualisation of the biofilms in TOWW and OMWW with fluorescent and electron microscopy was executed to confirm the development of biofilms under these conditions. These results will indicate whether biofilms are formed in the TOWW and OMWW and whether the biofilms that form naturally in olive wastewaters can be used as a possible bioremediation strategy to treat olive wastewater.

4.1.1 Collection of olive wastewater

Two olive processing plants situated within the Western Cape of South Africa were selected for this study: Buffet Olives, producer of table olives and Vesuvio Estate, producing olive oil products. Collection of wastewater and biofilms samples spanned over a two-season production period at both facilities during 2004 and 2005. Fresh olive wastewater (25 l) from Buffet Olives and Vesuvio Estate was collected from the effluent streams during the active production stages of the 2004 season and used as effluent for the rotating biological contactor (RBC) experiment described in the next paragraph.

4.1.2 The rotating biological contactor set-up

A small-scale laboratory RBC (batch flow system) of 80 cm long were designed and built from PVC plastic for evaluation of the indigenous olive wastewater biofilm's potential to bioremediate olive wastewater. The RBC (Fig. 4.1) consisted of a trough with a 4000 ml total capacity, effluent depth of 6 cm and 16 equally spaced polystyrene discs (diameter 17.5 cm and thickness 2.7 cm) mounted onto a horizontal stainless steel shaft with at least 40% of the discs submerged in the wastewater at all time. The systems were supplied with continuous electricity by a 12V power generator motor and the discs were set at a fixed rotational speed of 15 rpm. TOWW and OMWW were pumped into two separated RBC systems continuously by means of a peristaltic pump at a constant flow rate of ca. 41.5 ml/min, resulting in a theoretical hydraulic retention time of 1 hour and 37 minutes.
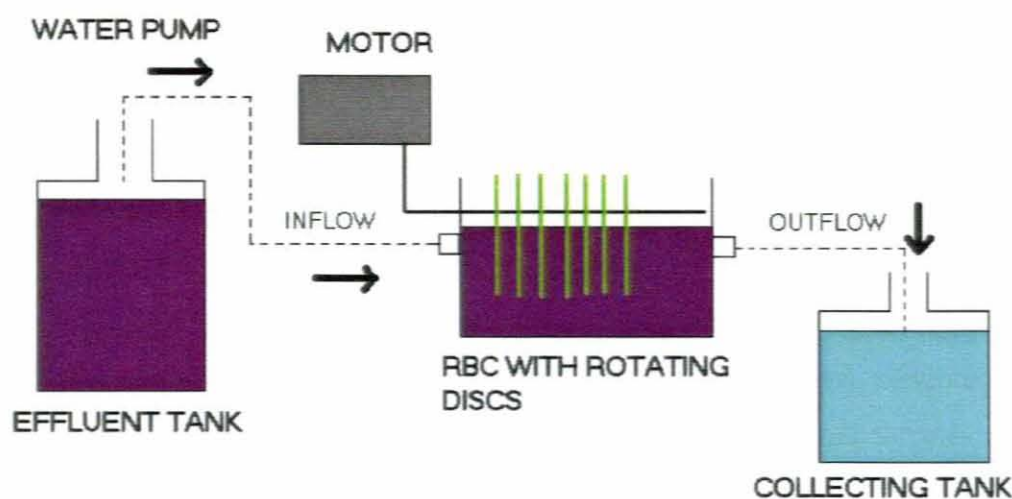
Figure 4.1: The schematic representation of the set-up for the small-scale evaluation of the single stage RBC.

4.1.3 Chemical Oxygen Demand (COD) removal

The COD determinations were carried out over a 10–day period using a spectrophotometric test kit (Merck, Spectroquant COD test kit). The COD measurement with this kit expresses the amount of oxygen originating from the potassium dichromate that reacts with the oxidizable substances contained in 1 l of water under the working conditions of the manufacturer's specified procedure.

The COD of the fresh olive wastewater was measured immediately after collection as the starting COD level. To determine the rate of COD removal, triplicate samples of approximately 10 ml of olive wastewater were collected daily into sterile Falcon tubes from the wastewater reservoir (effluent tank) and from the outflow collection tank of both RBC's, i.e. TOWW and OMWW. COD measurements were obtained using a sample of 1 ml of 20 X diluted olive wastewater. The sample was pipetted and added into 3 differently labelled COD Cell Test tubes, containing 2.2 ml COD solution A and 1.8 ml COD solution B. Samples were then mixed gently and inserted into a preheated Spectroquant Nova TR420 (Merck) digester set at 148°C for 2 hours. After the digestion, samples were allowed to cool down to room temperature for 10 minutes. After 10 minutes samples were analysed for COD using the Spectroquant Nova 60 (Merck) according to the manufacturer's guidelines. The COD reading obtained was measured in triplicate from each COD cell tube and the readings were averaged. Data was plotted using Sigma Plot 2001 (Windows Version 7.0), using the simple error bar option and standard deviation as the error calculation formula.

4.1.4 Microscopic examination of biofilm samples

As part of the preliminary study, it was necessary to visualise the formation of indigenous biofilms in TOWW and OMWW. Microscope slides taken from the modified Pederson devices (described in 4.2.1) were examined under an epifluorescent microscope to verify the presence of microbial biofilms. One to three week old slides, containing biofilm growth, were flushed with 1 ml 0.1% acridine orange and incubated in the dark for 1-3 minutes to allow optimal binding of the dye. Acridine orange was used, because it visually differentiates between metabolically active and inactive microorganisms. Following incubation, the slides were rinsed three times with distilled water and examined under an epifluorescent microscope (Zeiss, Germany). Images were photographed under 600 X magnification using the Axiocam MRc5 digital camera (Zeiss, Germany). The 0.1% acridine orange was prepared as follows: Formalin-phosphate buffer was prepared by adding 34 g KH_2PO_4 to 500 ml of milliQ-water. The pH of the buffer was adjusted with 40 g/l NaOH to pH 7.2 (+/- 0.05). A volume of 70 ml of 37% formalin (formaldehyde dissolved in water, HCHO) was added to complete the buffer. Acridine orange powder (160 mg) was added to the formalin-phosphate buffer and topped up with milliQ-water to a final volume of 1 litre.

The scanning electron microscope (Leo S440) was also used to observe the development of the biofilm on the RBC discs over a 10-day growth period. After 7 days of reactor operation, a 1 cm^2 square of polystyrene containing the biofilm was carefully sliced from disc 1, 3, 5, 7, 9 and 11 with a sterile scalpel and placed in sterile 2 ml eppendorf tubes. The biofilm squares were then exposed to an alcohol dehydration process where all the water in the biofilm samples was replaced with alcohol. The samples were first immersed in 30% ethanol for 10 minutes followed by immersion into 50%, 70%, 80%, 90%, 95% and 100% ethanol for 10 minutes each. After the 100% ethanol exposure, samples were critical point dried (CPD) in order to replace the alcohol with liquid and eventually gaseous carbon dioxide. The samples were then mounted on small aluminium SEM stubs and sputter coated with gold / palladium in order to be examined.

4.2 PCR-DGGE characterisation of the microbial communities from olive wastewater biofilms

After the preliminary results have shown that indigenous biofilms develop in South African TOWW and OMWW and exhibit the potential to significantly reduce the COD levels in olive wastewater, the characterisation of the biofilm microbial community over time was commenced. The first step

in this process was to cultivate the biofilms, followed by genomic DNA isolation and PCR-DGGE analysis.

4.2.1 Cultivation of olive wastewater biofilms for PCR-DGGE analysis

Perspex holders, also known as modified Pederson devices (Wolfaard, 2004), containing 12 standard microscope slides were used as culturing chambers for the TOWW and OMWW biofilms. The holders (7.8 cm x 8.5 cm x 3.7 cm) were designed in such a way that water can flow straight through it and over the slides. It had an open lid that held the slides in place but still allowed water to flow through. The modified Pederson devices were immersed in the wastewater pipe systems at the beginning of the 2004 production season as follows: At Vesuvio Estate, two Pederson devices were placed inside the inlet pipe, at an open evaporation pond (Fig. 4.2) that are exposed to seasonal climate changes. Since industrial scale RBC systems would also be exposed to such weather factors, it was found necessary to cultivate the biofilms outside the facility at a point in the stream prior to any treatment process that already exist and that might influence the final composition of the biofilm communities.
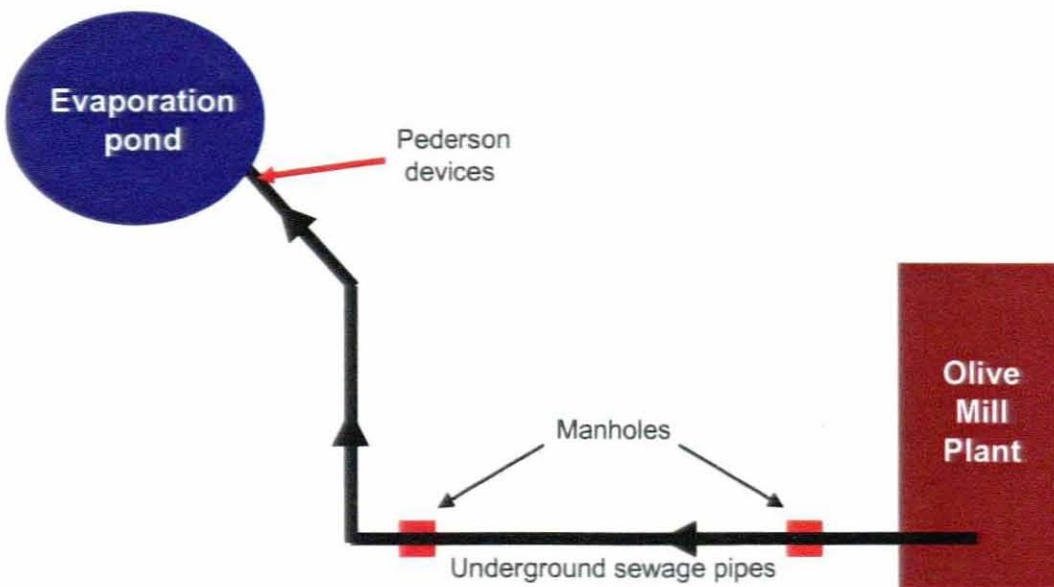
Figure 4.2. A diagram of the placement of the modified Pederson devices in the wastewater outflow pipes at Vesuvio Estate. Only a single evaporation pond is used for disposal of wastewater.

At Buffet Olives, wastewater from the processing plant runs into an anaerobic underground chamber from which it was either pumped into a freshwater dam or into a series of three evaporation ponds. The water was aerated inside the evaporation ponds by pumping air into the

wastewater to induce aerobic digestion and evaporation. Two modified Pederson devices were placed inside a closed manhole in front of an anaerobic digester (Fig. 4.3). This was the only possible position to place the modified Pederson devices, since the wastewater would have had no prior treatment. This position was outdoors and was therefore exposed to seasonal climate changes.
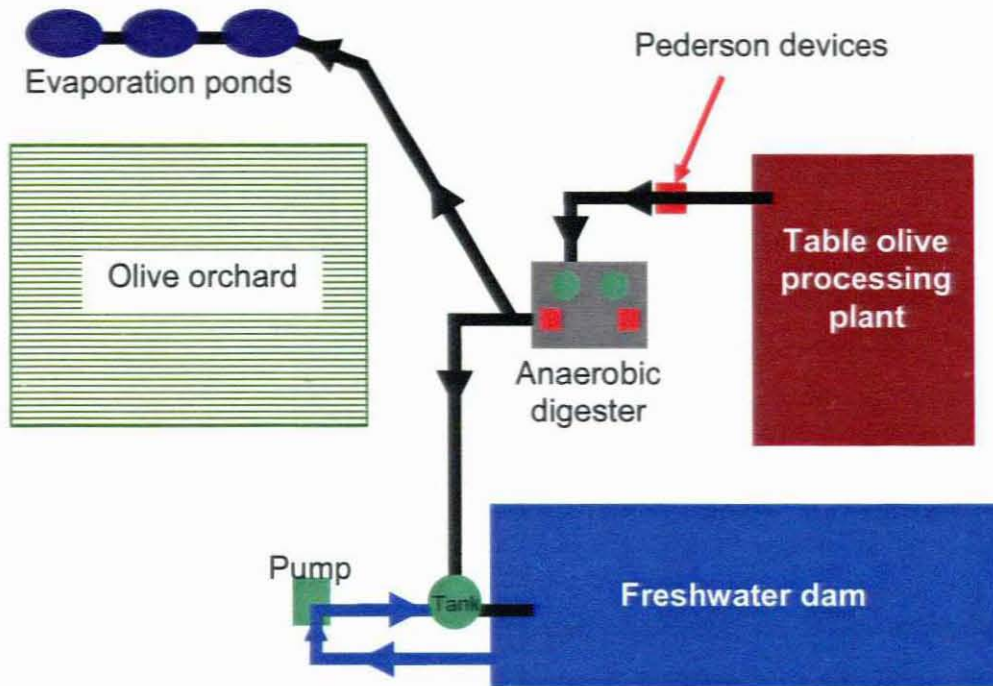
Figure 4.3: A diagram of the placement of the modified Pederson devices in the wastewater outflow pipes at Buffet Olives.

For both TOWW and OMWW, a total of 24 biofilm slides could be sampled and analysed over an extended period of time. The slides were examined every month during the production seasons of 2004 and 2005 for biofilm growth and samples (slides) were collected aseptically in 50 ml Falcon plastic tubes containing the wastewater from the environment it was collected in.

In order to compare biofilm profiles from the farms to biofilms growing under more controlled environmental conditions (not exposed to seasonal climate changes etc.), an additional two modified Pederson's devices were also incubated in the laboratory in 2 l beakers filled with 1.5 l of TOWW and OMWW, respectively. Each beaker contained a magnetic stirrer rotating at a speed that ensured a steady flow of wastewater over the slides. Fresh olive wastewater was added every 14 days from each farm to allow the laboratory-cultivated biofilms to adapt to wastewater conditions in approximately the same manner as it would adapt to wastewater conditions at the farms. The laboratory slides were examined every two weeks for biofilm growth and were also collected aseptically in 50 ml Falcon tubes for further analysis.

4.2.2 Sample timing with production operations at Buffet Olive and Vesuvio Estate

The table olive harvesting seasons at Buffet started at the end of February 2004 and 2005 and were completed at the end of July 2004 and 2005. During this time, green olives were processed according to the Spanish style method with NaOH treatment (February to March) followed by 3 washing steps (March to July) and brine fermentation until December. During the months of February to July, the concentrations of sodium (NaOH) in the wastewater effluent as well as the volume of the wastewater were at its maximum during the year. During the same period, the black olives were transferred directly to fermentation tanks without any initial NaOH treatment. At the end of August, NaOH was added to the fermentation tanks containing the black olives (Kalamata cultivar) in order to correct the pH of the brine in the fermentation process. This process was repeated 3 months later in November. In December, all the fermentation water (NaCl and NaOH) from black and green olives was drained at one time and the olives were allowed to oxidise for a period of time before packaging. The packaging line ran throughout the year that also resulted in normal wash water containing disinfectants.

Harvesting of biofilm samples at Buffet started in February 2004 with the following sample times: week 2 (February), week 3 (March), week 5 (March), week 6 (March), week 7 (April), week 16 (June) and week 25 (August). During 2005, the sample order was as follows: week 9.3 (February), week 12.4 (March), week 16.4 (April), week 19.4 (May), week 21.4 (May) and week 23.4 (June).

At Vesuvio Estate, olives were harvested and processed for oil extraction as described by Vossen (1997) between April and July of 2004 and 2005 resulting in increased amounts of wastewater during that part of the year. The steps, as described by Vossen (1997), are as follows: Washing, grinding, malaxing (mixing with 100% hot water), oil extraction (3-phase centrifuge), extraction of residual oil and oil purification. Other wastewater generated in the process was wash water from the factory floor and equipment and was present throughout the whole year. During August and September of 2004 and 2005, milling of olives was stopped and all equipment, walls and floors were systematically cleaned resulting in an increase in detergent residues in the wastewater outflow. Between April and July 2004, 250 tons of olives were milled resulting in approximately 2×10^6 l of wastewater. From April to July of 2005, 800 tons of olives were milled which resulted in the release of approximately 7×10^6 l of wastewater.

The harvesting of the farm samples at Vesuvio commenced as follows during 2004: week 2 (April), week 3 (April), week 4 (April), week 5 (May), week 14 (July) and week 23 (September). During

2005, the harvesting of the samples at Vesuvio started in April and commenced as follows: week 3 (April), week 5 (May), week 7 (May), week 9.3 (June), week 12.4 (July) and week 16.4 (August). The last samples of the year were taken during the cleaning months.

4.2.3 Isolation of olive wastewater biofilms

Biofilms can be removed from biological and abiological surfaces with surfactants, scraping of surfaces, sonication or homogenisation. According to a comparative study by McDaniel and Capone (1985), sonication appears to be an efficient and safe method to remove biofilms from biological surfaces, break up clumps of microorganisms, while providing a high yield of intact cellular material. Sonication has been shown to be less harmful to microorganisms than chemical elution or scraping of surfaces (Sugarman, 1982).

Microscope slides isolated from modified Pederson devices were immediately sonicated in a UMC-5 sonication water bath (Instrulab, USA) after collection to remove the surface-attached cells. The slides were first sonicated in 25 ml sterile distilled water (ddH$_2$O) for three minutes at maximum setting, after which the water was collected in a second sterile 50 ml Falcon tube. The sonication step was repeated for an additional three minutes after adding 25 ml of fresh ddH$_2$O to the slide. After the second sonication step, the water sample was combined with the first 25 ml sample. The dispersed biofilm samples were centrifuged (Biofuge Pico, Kendro, Germany) for 10 minutes at 13000 rpm at room temperature. The supernatant was discarded and the cell pellets were briefly resuspended with a vortex mixer (Zx^3, Velp Scientifica) to disperse the pellet into the remaining liquid. All the liquid (the dispersed pellets) were pooled into a single 2 ml eppendorf tube with a pipette and stored at 4°C for no longer than 24 hours before further analysis.

4.2.4 Simulated sodium hydroxide and detergent shock treatments of olive wastewater biofilms

In order to observe the effect of NaOH and cleaning detergents on biofilm population diversity, a series of artificial shock treatments were conducted on the TOWW and OMWW cultured biofilms. The purpose of these studies was to give an indication of the effect of the NaOH and cleaning chemicals used at Vesuvio Estate and Buffet Olives on biofilm development and population complexity and could provide essential information regarding the subsequent study of microbial population changes during the actual production seasons.

Fresh TOWW and OMWW (5 l) were collected from Buffet Olives and Vesuvio Estate olive farms. Polystyrene slides (6 cm x 2.5 cm x 0.5 cm) were cut to size, inserted into modified Pederson devices and completely submerged in 2 l glass beakers containing 1 l of both TOWW and OMWW, respectively. The olive wastewater was constantly mixed by means of a magnetic stirrer, while the biofilms were allowed to develop at room temperature on the polystyrene discs. Biofilms in the Buffet Olive wastewater were allowed to grow from 29 April 2005 until 14 June 2005, equalling 44 days, while biofilms in the Vesuvio Estate wastewater were allowed to grow for 21 days from 24 May 2005 until 14 June 2005, before commencement of shock treatments.

A 2.5% (v/v) final concentration of NaOH and each detergent was freshly prepared in 100 ml of either TOWW or OMWW in separate beakers. To evaluate the effect of detergents on the biofilm microbial populations, Robot™ and Contrabac™ were used for TOWW, while Removil™ and Limex™ were tested in OMWW. A polystyrene disc containing the TOWW or OMWW cultured biofilms, was immersed in each of the treatment solutions for approximately 3 hours and slowly stirred. A negative control polystyrene disc was also treated the same way, but the 100 ml wastewater in which it was immersed contained no additives (NaOH or detergents). After the shock treatment, the biofilms were isolated from the polystyrene discs using the sonication method as described in 4.2.3. Subsequently, genomic DNA isolation, PCR-DGGE and image analysis were performed as mentioned in the following sections.

The following description of the detergents used in this study was obtained from the manufacture's information sheets. Robot is a white liquid that is composed of neutralized sulphamic acid. It is effective at a pH of 7-8 and has a slight alcoholic odour but has no polluting effect on the environment. Contrabac is a broad-spectrum antimicrobial agent that is generally used as hand soap. It is mostly effective against Gram-positive and Gram-negative bacteria, viruses, fungi and algae. Furthermore, it is not damaging to the environment, as it is completely biodegradable. Limex is a clear, pale yellow liquid with a mild lemon smell. Its optimum pH is 11.8 as it has an alkaline (NaOH) reaction. It is biodegradable and therefore will not have a negative effect on the environment. Removil is a high alkalinity descaling detergent (pH 12.8 in 1% solution) that contains anionic and non-ionic synthetic detergents, which is 90% biodegradable. It is a colourless and slightly opalescent liquid. Unfortunately, the exact composition of these chemicals could not be obtained from the manufacturers, because the chemical formulation is protected by patents.

4.2.5 Genomic DNA isolation from biofilm samples

Several DNA isolation methods are available for bacterial and fungal species. Millar *et al.* (2000) describes different methods and compared the DNA extraction efficiency of each method. The main methods investigated were as follows: Qiagen QIAmp Blood kit, Roche High Pure PCR template preparation kit, Puregene DNA extraction kit, the boiling method, glass beads/sonication method, phenol-chloroform method and wash-alkali-heat lyses method. The results indicated that a simple wash-alkali-heat lyses method was the most sensitive, reproducible, simple and cost-effective extraction method. This was the only method that also removed any PCR inhibitors and contaminant DNA. Yeates *et al.* (1998) determined that bead beating has a lysis efficiency of more than 90% and a reduced co-extraction of inhibitors. Therefore, it is more likely that bead beading will break open all cells in biofilm samples.

The DNA isolation method used in this study was an adaptation of the normal glass bead and phenol-chloroform methods as described by Ausubel *et al.* (1995). This method is ideal for isolating DNA from robust cells surrounded by protective EPS. The cell pellet aliquots obtained in the sonication procedure described in section 4.2.3 were split into 200 µl aliquots in 2 ml eppendorf tubes and resuspended in 200 µl breaking buffer (2% Triton X-100, 1% SDS, 100 mM NaCl, 10 mM Tris-Cl [pH 8], 1 mM EDTA [pH 8]). To this mixture, 0.3 g glass beads (=106 µm, acid-washed, Sigma Aldrich, USA) and 200 µl phenol/chloroform/isoamyl alcohol (PCI in 25:24:1 ratio) were added and vortexed at maximum speed for 3-5 minutes. An additional 200 µl of TE buffer (10 mM Tris-HCl, 1 mM EDTA [pH 8]) was added and briefly mixed. The samples were centrifuged for 10 min at 13000 rpm at room temperature after which the clear top aqueous layer (TE buffer) was removed and transferred to a clean 1.5 ml eppendorf tube. Genomic DNA (gDNA) was precipitated by adding 1 ml 100% ethanol and mixing by inversion. The samples were centrifuged again for 3 min at 13000 rpm at 4°C, the supernatant was discarded and the DNA pellets resuspended in 400 µl TE buffer. After DNA resuspension, 3 µl of a 10 mg/ml RNAse A solution (Roche Diagnostics, Germany) was added, mixed and incubated for 5 minutes at 37°C. After RNA removal, 10 µl of 4M filter-sterilised ammonium acetate (NH_4OAc) (0.22 µm acetate Cameo filter, Whatman, USA) and 1 ml 100% ethanol were added and mixed gently. gDNA was precipitated by centrifugation for 3 minutes at 13000 rpm at room temperature, the supernatant was discarded and the DNA pellet air-dried at 45°C. The DNA pellet was finally resuspended in 50 µl TE buffer. Agarose gel electrophoresis was executed (0.8% agarose in 1 X TAE buffer) to confirm the presence and quality of gDNA. The loading dye used in all electrophoreses steps was Type 2 and

was prepare as follows: 300 mM NaOH; 6 mM EDTA; 0.25% (w/v) bromophenol blue; 0.25% (w/v) xylene cyanol FF and 15% (w/v) Ficol (Type 400, Pharmacia) in water.

The 0.8% agarose gels were prepared by adding 0.8 g of agarose powder (Agarose D1 Low EEO, Conda) to 100 ml of 1 X TAE buffer. The agarose powder were dispersed in the buffer and boiled for a minimum of 3 minutes in a microwave oven to allow the agarose to dissolve completely. Ethidium bromide (2 µl of 10 mg/ml solution) was added to the 100 ml cooled agarose, mixed and poured into the cast tray to allow solidifying. Loading buffer (1 µl) was added to 10 µl gDNA, mixed and loaded into the wells. The gels were exposed to 80 Volts for 60 minutes and viewed on a UV source to confirm the presence of DNA in the sample. Large agarose gels (250 ml) were run at 120 Volts for 60 minutes. The 1 X TAE buffer was prepared by diluting a 50 X TAE solution. One litre of 50X TAE contained 242 g Tris-base, 57.1 ml glacial acetic acid, 100 ml of 0.5 M EDTA and distilled water to make a final volume of 1 l.

4.2.6 PCR amplification of 16S and 18S rRNA fragments

Two PCR strategies have been used for the amplification of the desired 16S and 18S rRNA fragments. The first strategy involved a double PCR step in which a 1500 bp fragment is first amplified from the gDNA and then used as template in a nested-PCR reaction to obtain the final DGGE PCR DNA fragments. After unsuccessful initial attempts with this strategy, a second strategy was used. The desired bacterial and fungal DGGE fragments of 232 bp (16S rRNA) and 340 bp (18S rRNA), respectively, were directly amplified by using gDNA from all the biofilm samples directly as PCR template.

The *Taq* DNA polymerase enzyme used in all PCR reactions was Takara Ex Taq (Takara Inc., Japan) with proof reading activity, while 2% dimethyl sulfoxide (DMSO, Sigma-Aldrich, USA) was included in all PCR reactions to eliminate any template or primer self-annealing artefacts. The thermocycler used for all PCR reactions was the Thermo Hybaid (USA). The bacterial PCR program was as follows: 1) 94°C for 4 minutes; 2) 94°C for 30s, 50°C for 1 minute, 72°C for 1 minute [35 cycles]; 3) 72°C for 10 minutes as a final extension. Primers I-341F-GC (5' CGC CCG CCG CGC GCG GCG GGC GGG GCG GGG GCA CGG GGG GCC **TAC GGG IGG CIG CA** 3') and I-533R (5' TIA CCG III CTI CTG GCA C 3') were used as primer set targeting the conserved V3 region (232 bp) of the 16S rRNA gene fragment (Watanabe *et al.*, 2001).

The fungal PCR program was as follows: 1) 94°C for 4 minutes, 2) 94°C for 30s, 48°C for 1 minute, 72°C for 1 minute [35 cycles], 3) 72°C for 10 minutes as final extension. Primers F1Ra (5' CTT TTA CTT CCT CTA AAT GAC C 3') and NS7-GC (5' CGC CCG GGG CGC GCC CCG GGC GGG GCG GGG GCA CGG GGG **GAG GCA ATA ACA GGT CTG TGA TGC** 3') were used as primer set targeting the V9 region of the 18S rRNA gene fragment (340bp) (White *et al.*, 1990; de Souza *et al.*, 2004). The typical preparation of bacterial and fungal PCR reactions is shown in Table 4.1 below.

Table 4.1: Preparation of 100 µl PCR reaction mixtures

PCR reagents	Bacteria	Fungi
MilliQ water ·	68.5 µl	64.5 µl
10 X Ex Taq buffer	10 µl	10 µl
dNTP's	8 µl	8 µl
Primer 1 (20 µM stock)	3 µl	5 µl
Primer 2 (20 µM stock)	3 µl	5 µl
DMSO	2 µl	2 µl
Template DNA (gDNA or PCR product) (ca. 1 µg)	5 µl	5 µl
Takara Ex Taq polymerase	0.5 µl	0.5 µl

For both the bacterial and fungal primer sets, the sequences in bold are homologous to the 16S or 18S rRNA genes fragments, respectively, while the rest of the primer (only I-341F-GC and NS7-GC) comprises the GC-tail. The purpose of the GC-tail is to prevent the screened gene fragments to completely denature within the denaturing polyacrylamide gel and thereby remain partially double-stranded, ensuring optimal resolution. The GC-tail also slows the migration of the DNA through the gel matrix. Before DGGE analysis, the PCR products were verified on a 2% agarose gel by adding 2 µl loading buffer to 20 µl of PCR product. Agarose gels were run at 120 Volts or 80 Volts for 60 minutes depending on the size of the gel. Where required, a second PCR reaction with the 232 bp 16S rRNA or 340 bp 18S rRNA PCR products as template was executed on samples with similar programs and primer sets to obtain higher concentration of 16S and 18S rRNA.

4.2.7 DGGE gel preparation.

For the bacterial DGGE analysis, a 9% polyacrylamide gel (acrylamide/bis-acrylamide ratio = 37.5:1) was used with a 40 – 60% urea-formamide denaturing gradient. For the fungal DGGE analysis, a 9% polyacrylamide gel was used with a 30 – 50% denaturing gradient. Polyacrylamide gels were prepared in 50 ml Falcon tubes according to the following method in Table 4.2.

Table 4.2: DGGE polyacrylamide gel preparation for bacterial and fungal analysis (Adaptation of Ausubel *et al.*, 1995)

	Bacteria		Fungi	
Reagents (added in this order)	60%	40%	50%	30%
Urea powder (U)	6.048 g	4.032 g	5.04 g	3.024 g
Formamide (F)	5.76 ml	3.84 ml	4.8 ml	2.88 ml
50X TAE	480 µl	480 µl	480 µl	480 µl
40% Acrylamide	5.4 ml	5.4 ml	5.4 ml	5.4 ml
MilliQ water	Adjust to 24 ml	Adjust to 24 ml	Adjust to 24 ml	Adjust to 24 ml
20% ammonium persulfate (0.2 g/ml)	65 µl	65 µl	65 µl	65 µl
TEMED	6.5 µl	6.5 µl	6.5 µl	6.5 µl

The purpose of ammonium persulfate (APS) and TEMED are to polymerise the polyacrylamide. When DGGE gels were prepared, the TEMED was only added to the polyacrylamide mix after the gel cassette had been assembled and the gradient mixer had been set-up correctly.

The gel cassette (with glass plates) and buffer tank (with 18 l of 1 X TAE) for DGGE analysis were assembled according to the instruction manual from the Ingeny PhorU system (Netherlands). Before gels were poured into the gradient mixer, the tube connecting the two filling columns was closed and the magnetic stirrer switched on. The gel with the highest urea/formamide concentration was poured into the first column (closest to the outlet) of the gradient mixer containing the relatively slow-rotating stirrer bar. The tube connecting the two filling columns of the gradient mixer was carefully opened and immediately closed to allow air to be removed between the columns. The second gel with the lower urea/formamide concentration was poured into the second column. After the gels were poured into the gradient mixer (Ingeny PhorU, Netherlands) it was pumped with a peristaltic pump (Watson Marlow 205S, England) into the gel cassette at a flow rate

of 5 ml/min. A syringe needle was first inserted between the two glass plates on the side of the cassette and the spacer between the plates was pulled in the upward position in order seal the bottom. The pump was started at the exactly the same time as the valve between the columns of the gradient mixer was opened. Care was taken not to allow the rising level of the gel to touch the comb between the two glass plates, since this would disrupt the gel gradient. After the gel was poured between the plates, the gradient mixer and tubes were rinsed with ddH_2O using the peristaltic pump at maximum speed.

A layer of 100 % butanol (± 1 ml) was carefully pipetted onto the surface of the poured acrylamide gel to prevent uneven gradient formation during the next 2 hours while the gel polymerised. After the gel had set, the butanol was rinsed off thoroughly with distilled water. The stacking gel was prepared in the following order with 2.25 ml acrylamide, 200 μl of 50 X TAE, milliQ water to a final volume of 10 ml, 90 μl of 20% APS, and finally 9 μl TEMED to polymerise the constituents. It was carefully, but quickly injected with a 10 ml syringe on top of the gradient gel and allowed to polymerise for 5 minutes. Care was taken not to allow air bubbles to be trapped between the wells of the comb.

PCR products (±15 μl DNA of equimolar concentrations) and 2 μl loading buffer (Type 2) were loaded in a chronological order of biofilm age onto the gels and were categorised into the following sample groups: Buffet's 2004 and 2005 farm samples, Vesuvio's 2004 and 2005 farm samples, Buffet's 2004 laboratory samples and Vesuvio's 2004 laboratory samples. The Ingeny phorU system was used to execute the DGGE for 16 hours at 100 V and at 60°C using 1 X TAE buffer. For the preparation of the staining solution, 50 μl of 10000 X SYBR gold in DMSO was diluted with 1 X TAE to a final volume of 500 ml in order to obtain a 1 X SYBR gold staining solution. The DGGE gels was stained for 45 minutes with 1 X SYBR gold staining solution, rinsed with ddH_2O and photographed using the UV Gel Doc system.

4.2.8 Analyses of DGGE banding patterns

The PCR-DGGE banding pattern images were converted to 8-bit images and analysed with AlphaEase[R] FC Stand Alone software (version 4.0, Alpha Innotech). The lane and band detection functions of the software were used to generate a table of results showing the intensity value or area (n_i) of each band as well as the total intensity value (N) of each sample lane. From the molecular (PCR-DGGE) community fingerprints analysed by the AlphaEase[R] FC Stand Alone software, the Shannon–Weaver diversity index (H′) (Shannon and Weaver, 1963) was calculated to compare

changes in the diversity of the bacterial and fungal community structure within a sample, on the basis of the relative dominance of the bands in the patterns. H´ was calculated by using the function $H' = -\Sigma\ P_i \log P_i$, where $P_i = n_i/N$, where n_i is the intensity of band i in the lane and N is the total intensity of all bands in the lane. H´ is the Shannon-Weaver index of microbial diversity of that lane representing one sample. The band intensity values (n_i) within the band detection data table from AlphaEase[R] FC were copied onto an Excel spreadsheet. The two above-mentioned equations: were used as formulas on the spreadsheet to calculate the Shannon-Weaver index (H') of each sample. After calculating the H´ value for all the samples, the Shannon-Weaver index for each sample was plotted against the biofilm age (in weeks) of the samples. The resulting graph was an indication of how the biodiversity changed in the microbial communities within the biofilms over time.

The next step in analysing the DGGE patterns was to match the bands in one lane with bands in adjacent lanes in order to generate a comparative table that can be exported to an Excel spreadsheet and used for Principle Component Analysis (PCA). This comparative table, which formed a binary matrix of the bands from the DGGE gel photographs, was used as an input matrix for the PCA analysis. Absence of bands was indicated as a 0, while presence of bands was indicated by its intensity values (P_i). According to Sei *et al.* (2004), PCA is a mathematical technique that generates a spatial configuration map where the distance between data points reflects the relationship between individual variables in the underlying data set.

The closer two sample points are to each other on the PCA axis, the higher the similarity between the two samples regarding the positions of the DNA bands and the intensity (concentration) of the bands. The individual biofilm samples were selected to represent the data points that were visible on the graph. The positions of the P_i-values (positions of the DNA bands) within the binary data point (sample lane or column on gel), as well as the magnitude of the P_i-values itself, were the two important variables that determined the position of the data point on the PCA graph. This quantitative binary input matrix, with these two variables, was then analysed with Statistica 7.1 in order to generate two-dimensional data points from the first and the second principle component values (x and y-axis on the PCA graph) that could then be plotted on a scatter graph.

The analysis involved an intricate procedure (eigen analysis) that transformed a number of (possibly) correlated variables (in the above-mentioned input matrix) into a number of uncorrelated variables called *principal components*. The principle components were referred to in mathematical terms as eigenvectors and eigenvalues that were the sum of the squares and cross products of the all

the P_i-values in the square symmetric input matrix. The highest and second highest eigenvector for each data point were referred to as the first and second principle component, respectively. The first principal component accounted for most of the variability in the whole dataset, and each succeeding component accounted for as much of the remaining variability as possible.

4.3 Preliminary isolation, cloning and sequencing of prominent DGGE operational taxonomic units (OTU's)

4.3.1 Excision and elution of operational taxonomic units from DGGE gels

DNA bands (OTU's) were randomly chosen from the DGGE images, and were excised from the polyacrylamide gels with a sterile scalpel blade. Excision of the DNA bands was carried out rapidly to prevent UV light-induced.DNA mutations. The individual excised bands were placed in 1.5 ml eppendorf tubes, rinsed with 1 ml of sterile water, vortexed for 5 seconds and centrifuged at 13000 rpm for 1 minute. The supernatant were removed, leaving only the gel piece inside the tube. This procedure removed contaminant DNA from the surface of the gel piece as well as some of the urea and formamide within the gel (Ausubel *et al.*, 1995; Stafford, 2005). Following the cleaning procedure, 50 µl of TE buffer was added to each gel piece, vortexed briefly and allowed to elute at room temperature or 37°C for 24 – 48 hours. A higher temperature increases the diffusion rate of the DNA from the gel. The TE buffer acted as protection against possible endonuclease contamination that might have been introduced.

The bacterial and fungal DGGE bands that were excised and eluted in TE buffer were amplified by PCR using similar cycling temperature profiles and primer sets as described in Table 4.1. After PCR, a 2% agarose gel electrophoreses step verified the quantity and quality of these PCR products.

4.3.2 Ligation and bacterial transformation of DNA

The re-amplified excised DGGE bands were cloned into the pGEM®-T Easy Vector System II (Promega, USA) according to the manufacturer's instructions. The molar ratio of PCR product to vector used for the ligation reactions was approximately 1:1. The ligation reaction mixture (10 µl) was prepared by adding 5 µl of 2 X Rapid ligation buffer, 2 µl pGEM®-T Easy vector, 2 µl insert PCR DNA and 1 µl T4 DNA ligase enzyme. The ligation mixtures were incubated for 16 hours at 8°C to allow for maximum number of ligation events.

The next step in the cloning protocol was to perform bacterial transformation using competent *Escherichia coli DH5α* cells. The genotype for these cells are: F⁻ φ80d*lac*ZΔM15 Δ(*lac*ZYA-*arg*F) U169 *rec*A1 *end* A1 *hsd*R17(r$_K$⁻, m$_K$⁺) *pho*A *sup*E44 λ⁻ *thi*-1 *gyr*A96 *rel*A1. Competent cells were prepared by growing a 5 ml culture of the *E. coli* cells overnight in Luria Bertani (LB) broth at 37°C. The cells were then diluted 1:100 (5ml into 500 ml LB broth) and shaken at 37°C for 1.5 – 2 hours, after which 1 ml of culture were pipetted into a plastic cuvette for OD measurement (600 nm). When the OD reached between 0.5 and 0.6, 2 x 250 ml of the culture were transferred to two pre-cooled sterile GSA centrifuge bottles (Beckman-Coulter) for centrifugation at 5000 rpm at 4°C for 10 minutes. The supernatant was removed and the cells were placed on ice. The cells were gently resuspended in 100 ml of ice cold 100 mM MgCl$_2$ and incubated on ice for 30 minutes, after which it were centrifuged at 4000 rpm for 10 minutes at 4°C. The supernatant was once again discarded and the cells kept on ice. The cells were then resuspended in a 10 ml of ice cold 100 mM CaCl$_2$ - 15% glycerol solution and aliquoted into pre-cooled 1.5 ml eppendorf tubes (240 μl/eppendorf tube). The competent cells tubes were stored at -80°C until further use.

Ligation mixtures were transformed into competent *E. coli* cells as follows. The competent cells were allowed to thaw on ice followed by adding 10 μl of the ligation mix to 80 μl competent cells. After an incubation period of 30 minutes on ice, the cells were heat shocked at 37°C for 40 seconds, placed back into the ice for 10 seconds and then kept at room temperature for 2 minutes. A volume of 400 μl of sterile Luria Bertani (LB) broth was added to the competent cells, incubated for 1 hour at 37°C and centrifuged at 13000 rpm for 10 seconds to collect the transformed cells. The supernatant (ca. 300 μl) was removed and the remaining 150 μl of the resuspended cells were plated on prepared selective LB-agar plates containing IPTG, X-gal and ampicilin. The plates were incubated overnight at 37°C to allow for the growth of ampicilin-resistant *E. coli* transformants.

The stock concentration of IPTG used was 24 mg/ml dissolved in milliQ water and the solution was filter sterilised through a 0.22 μm filter. The LB agar contained 5 ml IPTG/l (final concentration was 120 mg/l). An X-gal stock solution of 50 mg/ml dissolved in N'N'-dimethyl formamide was also prepared and 1.60 ml X-gal from the stock was added per litre autoclaved LB agar (final concentration was 80 mg/l). The concentration of ampicilin used was 100 mg/ml dissolved in milliQ water and the solution was filter sterilised through a 0.22 μm filter. The LB agar contained 1 ml ampicilin/l. The LB agar (1 l) was prepared according to manufacturer's instructions and sterilised. After it was allowed to cool down the IPTG, X-gal and ampicilin were added and 60

plates were poured. All stock of IPTG, X-gal and ampicilin were stored at -20°C, while extra plates were stored at 4°C.

4.3.3 Small-scale plasmid isolation

After 24 hour incubation at 37°C, white colonies were selected from each LB agar plate (6-12 colonies per plate) and inoculated into 5 ml of LB broth containing 5 μl ampicilin (100 mg/ml). The cultures were incubated overnight in a shaking incubator at 37°C. Plasmids were isolated from the cultures using the High Pure Plasmid Isolation Kit (Roche Diagnostics, Germany) according to the manufacturer's instructions. For restriction enzyme analysis of cloned inserts, a restriction enzyme digestion mixture (20 μl) was prepared containing 12 μl isolated plasmid DNA, 2 μl Buffer H, 0.5 μl EcoR1 enzyme (10 U/μl) and 5.5 μl milliQ water and incubated at 37°C for 2.5 hours. During this step the DNA insert of interest (232 bp of bacterial 16S rRNA or 340 bp of fungal 18S rRNA) was digested from the circular pGEM T-Easy plasmids. The complete reaction mixture (20 μl) was loaded with 4 μl loading buffer onto a 2 % agarose gel to verify the presence of cloned DNA inserts.

4.3.4 Sequencing and alignment of the cloned operational taxonomic units (OTU)

Plasmid DNA of the clones that gave proper insert sizes, as determined from the agarose gel to verify cloning, was sequenced using the universal M13 forward and M13 reverse primers. At least three clones from the same isolated DGGE band were submitted for sequencing, to establish any potential ambiguities of the OTU's. After the sequencing results were received, the forward and reverse sequences were aligned against each other using the computer software, DNAMAN Lynnon Biosoft©. Alignment was also done between the sequences of different clones of the same DGGE band, to determine whether the clones have similar sequences. Theoretically, three clones from one DGGE DNA band should match, resulting in one species to be identified from one OTU.

After the sequences had been aligned with DNAMAN, insert sequences were edited by removing all the pGEM vector sequences and used for homologous sequence searches using a basic local alignment search tool (BLAST) (National Centre for Biotechnology Information). Bacterial and fungal species could then be provisionally identified on the degree of homology (percentage identity) to the known database sequences. The NCBI database was found on the following website: http://www.ncbi.nlm.nih.gov/

4.4 Phylogenetic tree construction

All the DNA sequences were converted from the original FASTA format into the correct format using the text editor function in MEGA 3.1 (Kumar *et al.,* 2004). The FASTA format is a text-based format for representing nucleic acid sequences, in which base pairs are represented using single-letter codes. All the aligned and closely related sequences were imported from the GenBank database and manually refined in MEGA 3.1. The 16S and 18S rRNA sequences were compared to the sequences in the GenBank database by using the program BLASTN 2.0, which is available through the National Centre for Biotechnology Information website. All bacterial and fungal clones were assembled and aligned to their closely related BLAST sequences using the CLUSTALW algorithm. The BLAST sequences were first trimmed to approximate the start point and length of the OTU rRNA sequences. To test the robustness of the tree topologies, the same dataset was first tested with most of the common phylogenetic algorithms to obtain the best representation of the results. Evolutionary distances between the various clones and there corresponding BLAST results were determined using the Jukes and Cantor (1969) algorithm, followed by phylogenetic tree construction using the neighbour-joining (NJ) tree method from Saitou and Nei (1987).

The robustness of inferred tree topologies (branching patterns) was evaluated by 1000 bootstrap re-samplings of the data. Bootstrapping is a technical statistical procedure for estimating the variability of a measurement. In phylogenetics, bootstrapping involves the production of a new, pseudo-dataset by randomly extracting data points from the original dataset. For each pseudo-dataset, a new phylogeny is created. Rounds of this provide an estimation of the well- and poorly-supported regions of the original phylogeny. As a general rule, if the bootstrap value for a given interior branch is 95% or higher, then the topology at that branch is considered "correct". For the bacterial phylogenetic tree construction, the *E. coli* 16S rRNA gene was used as an "out group" control, while the yeast *Saccharomyces cerevisiae*'s 18S rRNA gene was used during the fungal phylogenetic tree construction.

CHAPTER 5

RESULTS AND DISCUSSION

Results and Discussion

5.1 Preliminary evaluation of the potential of indigenous olive wastewater biofilms to bioremediate olive wastewater

In an attempt to evaluate the potential of indigenous olive wastewater biofilms to reduce the polluting impact of South African olive wastewater, small-scale RBC's were setup in 2004 to measure the COD concentration in both TOWW and OMWW over time. Preliminary results were obtained and showed significant reductions in COD. These COD measurements were obtained over a 10-day period and recorded in Fig. 5.1. In TOWW, a fast degradation of COD was observed during the first 2 days, with a 31% drop in COD from ± 9000 mg/l to ± 6200 mg/l. After 2 days, the COD reduction rate slowed down slightly until day 8 when a COD level to ± 5500 mg/l was observed. From day 8 to 10 another reduction in COD was observed and a COD level of ± 4800 mg/l was measured on day 10. At the end of the 10-day period there was an overall reduction of ± 47% in COD within TOWW.
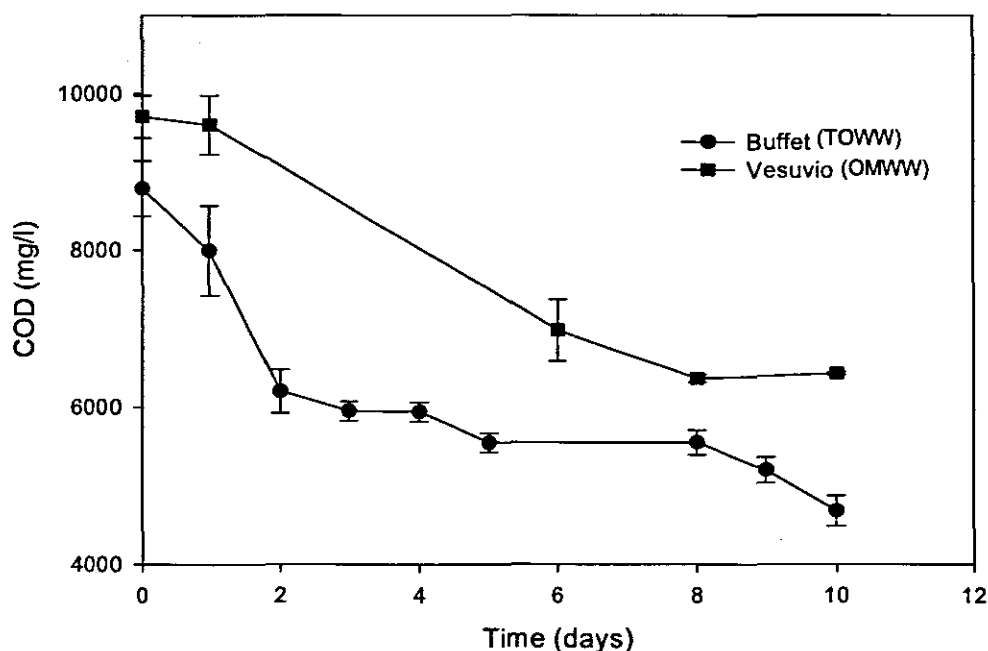
Figure 5.1: Preliminary data for COD degradation (measured in mg/l) in TOWW (Buffet Olives) and OMWW (Vesuvio Estate) over a 10-day period using biofilms that developed spontaneously in these two types of wastewater.

In OMWW, the COD concentration remained at ± 9600 mg/l for the first day, after which it started to decrease with a constant rate over a period of 5 days to a concentration of ± 7000 mg/l. After day 5, the COD degradation rate slowed down and the COD were recorded on day 8 at ± 6500 mg/l. During the final 2 days of the experiment, the COD concentration remained relatively stable at ± 6500 mg/l, resulting in a final overall COD reduction of ± 32% in OMWW.

These promising results indicate that the indigenous biofilms from TOWW and OMWW, respectively, had the potential to degrade the COD from these wastewaters and could therefore be developed as a possible bioremediation strategy to treat olive wastewater in a RBC setup. The results are preliminary, as the biofilms in this experiment did not even reach maturity, and one would predict even more significant COD removal rates with an older biofilm community. Because of the potential bioremediation capabilities of TOWW and OMWW biofilms, it was therefore worthwhile to study the microbial population fingerprints of these biofilms throughout the entire olive production season. A more detailed evaluation of the bioremediation effect of indigenous olive wastewater biofilms on TOWW and OMWW and optimisation of the RBC setup is part of another study in our research group.

5.2   Microscopic evaluation of biofilm formation

Fluorescent microscopy revealed typical microbial matrixes in which bacteria (rods and cocci), yeast cells and fungal hyphae were visible in both TOWW and OMWW biofilms (Fig. 5.2 A-H). Fungal hyphae with visible septa could be observed (Fig. 5.2 A & D). Typical 3D biofilm morphology showing microbial clusters with the origins of water channels was clearly visible (Fig. 5.2 A, C & F). In some fields of the biofilms yeast cells, with and without buds could also be observed (Fig. 5.2 B & D). Fluorescent microscopy also revealed that if plant debris was present (Fig. 5.2 E), microbial clusters often seem to surround or engulf the plant material, indicating that the plant debris possibly acts as a nutrient source for these biofilms. Protozoa (Fig. 5.2 G) was also regularly observed within the microbial clusters. Although the exact role of protozoa in biofilms remains unclear, research has shown that these predators are present in natural biofilms and that there is a significant positive relationship between them and biofilms. Protozoa also have a marked influence on the population dynamics within biofilm communities as they often become integrally associated with biofilms and graze on bacteria present in the biofilm (Huws *et al.*, 2005).

Acridine orange staining revealed metabolically active cells throughout the biofilms. However, some of the cells in the biofilms (Fig. 5.2) stained metabolically inactive (green). The majority of the cells were stained; therefore the oily nature of the biofilm did not seem to have a significant effect on the efficiency of Acridine orange penetration. The presence of metabolically inactive cells could be an indication of the harsh conditions, i.e. low or alkaline pH, high concentrations of NaCl and or NaOH and the presence of phenolic compounds, long chain fatty acids and detergents in olive wastewater, rendering the microorganisms metabolically inactive at the beginning stages of biofilm development.
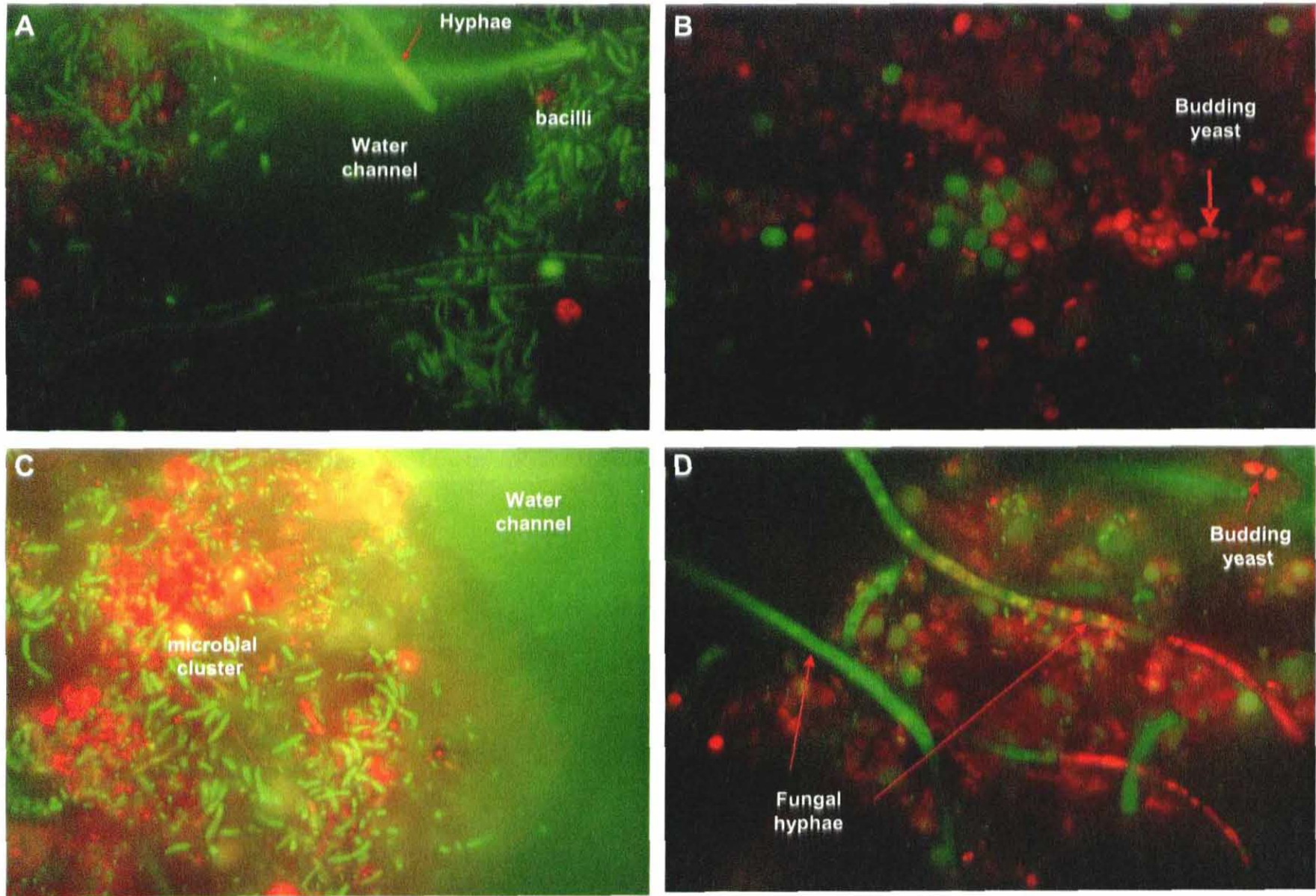
Figure 5.2: Biofilm micrographs (600x magnification) depicting fungal hyphae (A) and (D), pronounced yeast cells (B) and (D), bacterial rods and cocci (A) & (C) , and the 3D-structure of biofilm (A) & (C).
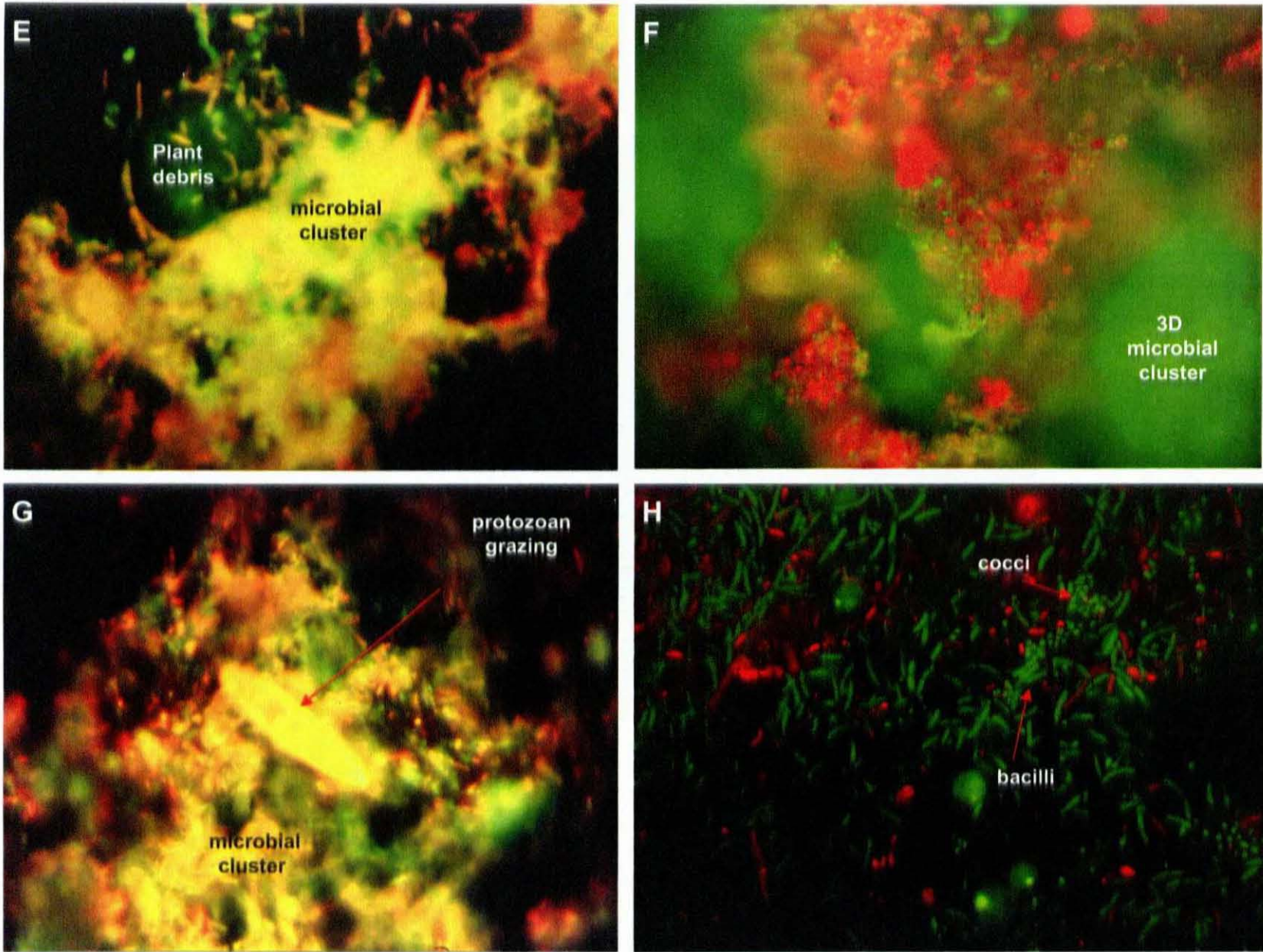
Figure 5.2: Biofilm micrographs (600x magnification) depicting bacterial rods and cocci (H), interaction with plant debris (E), complex 3D-structure of biofilm (F) and the presence of protozoa (G).

A substantial amount of exopolysaccharides (EPS) were also observed with scanning electron microscopy within 7-day old biofilms in both TOWW and OMWW that attached to the RBC's polystyrene discs (Fig. 5.3). The EPS had the form of a web or network of threads, bridging the microscopic spaces between irregular parts of the polystyrene surface. Within the network of threads, microorganisms were suspended. Microorganisms were also attached directly on the surface of the polystyrene surface. No further analysis was done on EPS identification, since it falls out of the scope of this study.

Figure 5.3: A SEM micrograph showing a 9000 X magnification of a 7 day-old biofilm from TOWW attached to polystyrene surface. Bacterial cells attached to the EPS and to the polystyrene surface are clearly visible.

In summary, from the microscopic examinations, typical biofilm structures were visualised in both TOWW and OMWW. Further detailed morphological and architectural analyses of biofilm structure were not included in this study.

5.3   PCR-DGGE fingerprinting of microbial consortia in olive wastewater

5.3.1   DGGE analysis of bacterial species in OMWW biofilms from Vesuvio Estate (2004 – 2005)

The DGGE analysis of biofilms in OMWW from Vesuvio Estate farm samples revealed a relative high biodiversity early in the biofilm development, i.e. after 2 weeks old, at the start of the production season in April 2004. Approximately 18 – 25 dominant operational taxonomic units (OTU) per sample were visible on the gel images. From the images in Fig. 5.4 it can be seen that

the biofilm community was sensitive to the changes in the olive mill wastewater conditions (such as pH, salt concentration and toxic phenols) at Vesuvio as there was small changes in the bacterial community from time point to time point. Appearances and disappearances of OTU's on the DGGE profile during the two seasons indicated that the bacterial species migrated to and from the biofilm community on a weekly base according to wastewater conditions at that specific stage. The 16S rDNA samples from 2004 and 2005 were run on the same DGGE gel to accurately align the OTU's.
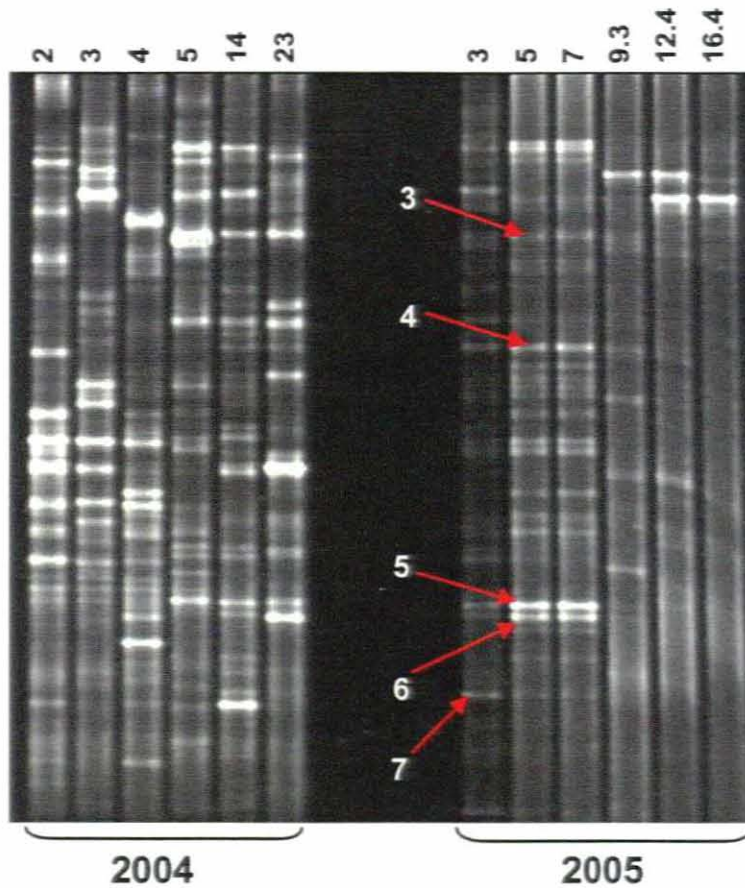
Figure 5.4: DGGE analysis of bacterial microbial population from OMWW biofilms (2004 – 2005) collected from Vesuvio Estate farm.

In Fig. 5.4, the grouping on the left represents Vesuvio 2004 farm samples and that on the right Vesuvio 2005 farm samples. Samples were loaded chronologically according to biofilm age in weeks. From left to right: 2, 3, 4, 5, 14, 23 weeks (2004) and 3, 5, 7, 9.3, 12.4, 16.4 weeks (2005). Week 1 was in beginning of the pressing season at the start of April. The DGGE gel images from 2004 and 2005 were compared and revealed more than 10 operational taxonomic units that were present in both seasons. To determine whether these are similar species, bacterial sequencing needed to be done. Bands 3 – 7, marked with red arrows in Fig. 5.4, were selected for cloning, sequencing and BLAST analysis.

Table 5.1 contains an extract of the Shannon-Weaver index calculations for Lane 1 in Fig. 5.4 (Vesuvio 2004, 2 week old biofilm). Complete tables of the raw data are included in the Appendix section at the end of the thesis. The highlighted value at the bottom right corner represents the Shannon-Weaver index of that sample and the 29 peaks represent 29 bands in that lane. The first 3 columns (peak, area and percentage area) have been generated by AlphaEase[R] FC and the last 2 using the Shannon-Weaver equations in an Excel-spreadsheet. Similar tables were generated for all the other sample lanes on Fig. 5.4 and the Shannon-Weaver indexes were calculated and displayed in Table 5.2.

Table 5.1: Extract of bacterial DGGE profile calculations for lane 1 (Fig. 5.4) of the OMWW biofilm farm samples from Vesuvio for 2004.

Week 2 (2004)	Peak (Band)	Area (n_i)	% Area	P_i	H'
	1	852	6.9	0.073077	-0.08303
	2	165	1.3	0.014152	-0.02617
	3	170	1.4	0.014581	-0.02677
	4	529	4.3	0.045373	-0.06094
	5	248	2	0.021271	-0.03557
	↓	↓	↓	↓	↓
	29	296	2.6	0.025388	-0.0405
		11659			**1.456373**

All the Shannon-Weaver indexes for bacterial OMWW biofilms from Vesuvio 2004 farm samples are on the left and Vesuvio 2005 farm samples are on the right in Table 5.2. All the Shannon-Weaver values were plotted and compared on a graph to see how the 2004 bacterial community diversity differed from the 2005 community.

Table 5.2: Summary of Shannon-Weaver indexes for OMWW biofilms from Vesuvio farm samples.

2004		2005	
Biofilm age (weeks)	SW indexes	Biofilm age (weeks)	SW indexes
2	1.456	3	1.298
3	1.333	5	1.379
4	1.395	7	1.365
5	1.313	9.3	1.268
14	1.454	12.4	1.301
23	1.312	16.4	0.981

The graph in Fig. 5.5 shows the changes in bacterial biodiversity as represented by Shannon-Weaver indexes in the biofilm formed in OMWW during the olive oil production seasons of 2004 and 2005 that started in April and ended in August. At the start of the second season, new slides

were inserted in the system to allow new biofilm growth and to observe whether the same diversity profile appears as with the first season. Although biofilm bacterial diversity fluctuated during the two seasons, it was more constant during 2004. At the end of the 2004 production season, a ± 10 % drop in biodiversity was observed until week 23.
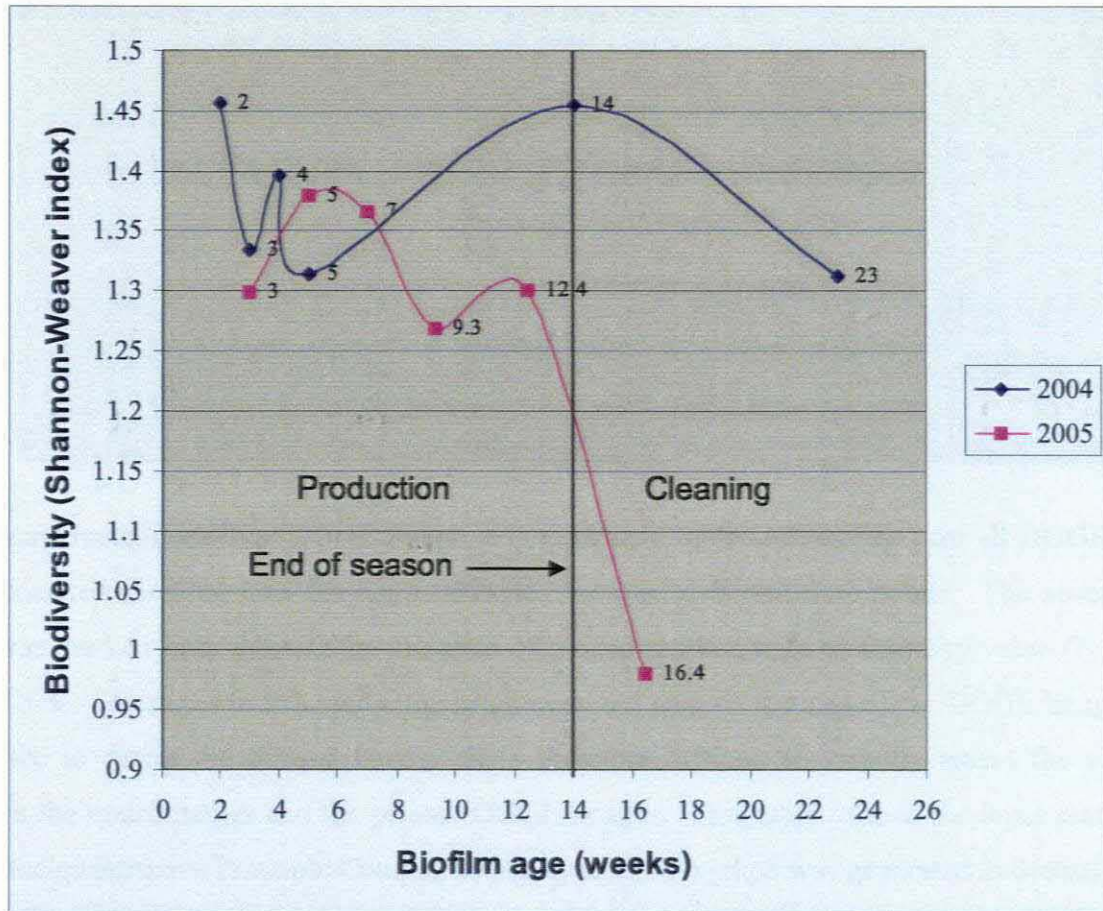
Figure 5.5: Bacterial biodiversity in OMWW biofilms from Vesuvio farm samples during 2004 and 2005.

A significant (± 25 %) drop in bacterial biodiversity was observed at the end of the 2005 season (week 12 –16) compared to the 2004 season. At the end of the production seasons (beginning of August – week 14), all machines and working surfaces were systematically washed with descaling agents with high alkalinity (pH 11 – 12). This alkaline pH shock might have contributed to the significant drop in bacterial biodiversity in the OMWW biofilms at the end of the 2005 season.

It can also be noted that the majority of the 2005 biodiversity levels, apart from week 16.4, fell within a close range to the 2004 samples and the differences were relatively small (within 1,25 – 1.45). The next step in analysis was to do band matching between all the samples on the DGGE gel (Fig. 5.4) in order to generate a binary matrix of data that could be used for PCA.

Table 5.3: Binary matrix results for the bacterial population in OMWW biofilms from Vesuvio farm samples derived from Fig. 5.4.

Weeks in 2004						Weeks in 2005					
2	3	4	5	14	23	3	5	7	9.3	12.4	16.4

After band matching of the DGGE image, a comparative table (spreadsheet) for all DGGE OTU's was generated showing how the bands matched between different time points. The absence of a band was marked with a 0 and the presence of a band marked with its intensity value (P_i values). For all binary matrixes in this study, the brightness and gamma settings of the DGGE images were optimised to detect the faintest bands. It is therefore difficult to visually assess the similarity between the matrix tables and the printed DGGE images. This table formed the input matrixes on which the quantitative Principle Component Analysis (PCA) graph was generated in Statistica 7.1.

Figure 5.6: Principle Component Analysis of the bacterial population from OMWW biofilms from Vesuvio farm samples during 2004 and 2005.

The Principle Component Analysis graph (Fig. 5.6) generated from the binary matrix in Table 5.3 revealed that the bacterial community within most of the OMWW biofilm samples of Vesuvio stayed relatively similar during the two production seasons and that no drastic shifts in the biodiversity of the bacteria have been observed. All PCA graphs presented within this study have a very low variance percentage (10 – 20%) on its x and y-axes, indicating that the first and second principle components (factors) represent only between 10 and 20% of all the variance between biofilm samples. PCA of the bacterial biofilm community from Vesuvio farm samples revealed a small grouping (inner circle) of samples from 2005 that were significantly similar in its bacterial species-profile. The biofilms samples were 3, 9.3, 12.4 and 16.4 weeks old. If the timeline between the inner group is taken into consideration, an interesting observation can be made. Between week 3 (inner group) and week 5 there was a small, bacterial population shift which remained relatively stable for 2 weeks until week 7, when it reverted back to its "original" population (inner circle) at week 9.3. Furthermore, although there was a drop in microbial diversity during the 2005 season from week 9.3 until week 16.4 according to the Shannon-Weaver indexes (Fig. 5.5), the bacterial population stayed relatively similar according to the PCA analysis (Fig. 5.6). A possible explanation for these differences is the fact that Shannon-Weaver indexes does not compare between different time points as does PCA analysis. Thus although the biodiversity index dropped, in terms of band density, the bacterial population did in fact not vary significantly.

Although all 2004 biofilms samples were absent from the inner circle, most of them (except week 3) was grouped within a bigger outer circle. Since all inner group samples from 2005 could potentially be loosely grouped within the outer group, it can be deduced that the biofilm formed in OMWW during 2004 and 2005 are relatively closely related in bacterial species composition. Although one would have preferred a clearer spatial grouping of the biofilms populations of 2004 and 2005 to confirm without a doubt that the biofilms re-establish with similar population profiles from one season to the next. Thus, although the PCA results are not as clear, one could make the broad assumption that a similar bacterial population from 2004 was re-established in the OMWW biofilms during the 2005 season, showing promising indications for future biofilm bioremediation applications.

The 2004 olive oil pressing season was a month shorter (8 April – 2 June) than the 2005 season (4 April – 1 July), which could have contributed to the slightly uncorrelated 2004 and 2005 biofilm communities. During 2004 an approximate output of 250 tons of olives were processed, while 800 tons of olives were processed during 2005. The shorter production period in 2004 resulted in a reduced wastewater outflow, compared to 2005. The large increase in wastewater volumes from

2004 to 2005 could have influenced the biofilm population significantly, but as already mentioned, the PCA graph showed that the biofilm populations could be loosely grouped between the two seasons, despite small changes that had occurred.

5.3.2 DGGE analysis of bacterial species in TOWW biofilms from Buffet Olives farm (from 2004 – 2005)

Approximately 10 – 20 operational taxonomic units (OTUs) per time point were visible on the DGGE gel images from TOWW biofilms from Buffet farm samples. From the images in Fig. 5.7 it could also be seen that the biofilm community were sensitive to the changes in the TOWW conditions at Buffet Olives as there was small changes in the bacterial population visible from time point to time point. OTU's had appeared, disappeared and reappeared during the two seasons indicating that the bacterial species migrated to and from the biofilm community on a weekly basis according to wastewater conditions at that specific stage. During 2004 it was found that one specific bacterial species, as indicated on Fig. 5.7, remained in the biofilm for the whole season and was also present, in lower numbers, during 2005. Therefore, this bacterium had the ability to re-establish itself over the two seasons. The 16S rDNA samples from 2004 and 2005 biofilm samples were analysed on the same DGGE gel to accurately align the bands.
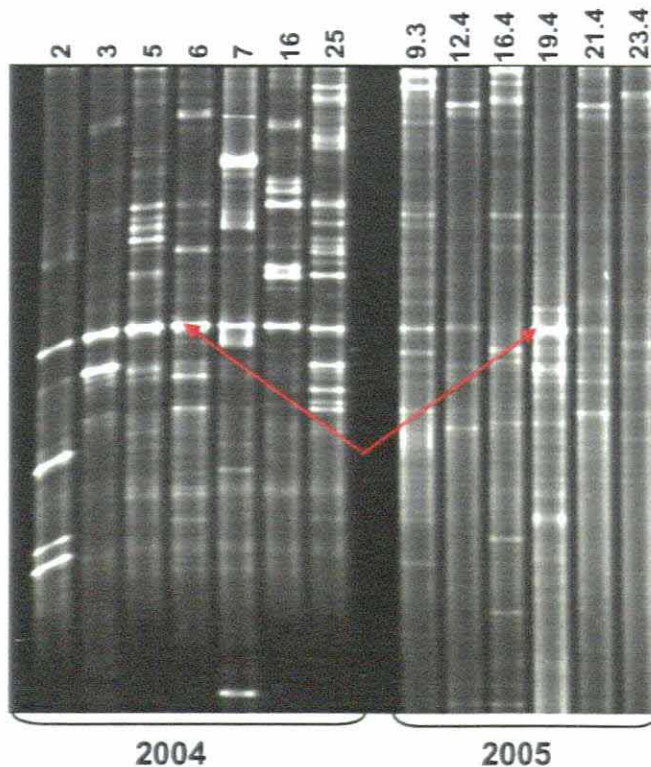
Figure 5.7: The DGGE banding pattern for TOWW biofilms from Buffet Olives, with 2004 bacterial farm samples (grouping on the left) and the Buffet 2005 bacterial farm samples (grouping on the right). The samples were loaded chronologically according to biofilm age. From left to right: 2, 3, 5, 6, 7, 16, 25 weeks (2004) and 9.3, 12.4, 16.4, 19.4, 21.4, 23.4 weeks (2005).

At the start of the second season, new slides were inserted in the system to allow new biofilm growth and to observe whether the same diversity profile appears as with the first season. According to the Shannon-Weaver index over time graph (Fig. 5.8), the bacterial biodiversity of Buffet farm biofilms during 2004 showed a strong increase up to week 6. After a small drop in biodiversity during week 7, it recovered quickly and continued to increase until week 25.

Table 5.4: Shannon-Weaver indexes of bacterial species in TOWW biofilms from Buffet farm samples.

2004		2005	
Biofilm age (weeks)	SW index	Biofilm age (weeks)	SW index
2	1.063	9.3	1.322
3	1.124	12.4	1.166
5	1.194	16.4	1.274
6	1.353	19.4	1.107
7	1.243	21.4	1.164
16	1.284	23.4	1.311
25	1.398		
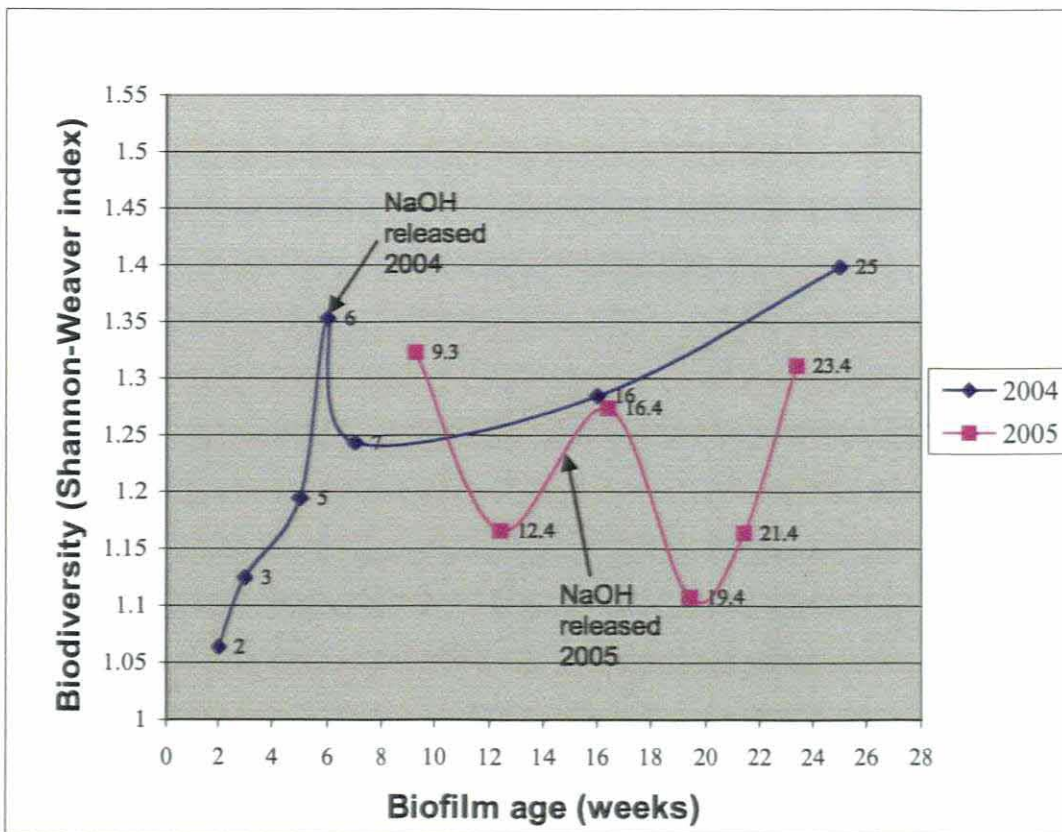
Figure 5.8: Bacterial biodiversity in terms of the Shannon-Weaver indexes for TOWW biofilms from Buffet farm samples during 2004 and 2005 (February to August).

The drop in biofilm biodiversity, at week 7, occurred at the exact stage in the production at the end of March 2004, when concentrated lye solution was drained. This however had a minimal impact on the biofilm, since a continued increase in the biodiversity was observed during the subsequent olive washing steps until week 25 (August). The bacterial community from Buffet farm biofilms in 2005 did not show the same trend as in 2004. The development of the biofilm from week 1 to week 9.3 (not shown on graph) occurred between December 2004 and January 2005. The release of all the brine solution in December did not seem to affect the biofilm significantly since it was able to redevelop until week 9.3 in February. After week 9.3, no visible and clear pattern was observed in the biodiversity, because of fluctuations. Between week 12.4 (March) and 16.4 (April), lye solution was released for 2005. The resulting decrease in biodiversity from the lye solution only occurred after week 16.4, but the biofilm recovered again, resulting in the biodiversity to increase until week 23.4. The following step in analysis of the DGGE images of Buffet bacterial biofilms was to do band matching of the OTU's to obtain a binary matrix for further PCA analysis.

Table 5.5: Binary matrix results for bacterial population in TOWW biofilms from Buffet Olive farm samples (derived from Fig.5.7).

Weeks in 2004							Weeks in 2005					
2	3	5	6	7	16	25	9.3	12.4	16.4	19.4	21.4	23.4

This comparative table (Table 5.5) formed a binary matrix of the OTU's from the DGGE gel photograph (Fig. 5.7), as generated by gel image analysis software (AlphaEase FC) and was used as an input matrix for the PCA analysis (Fig. 5.9). Absence of bands was indicated as a 0 within the matrix, while presence of bands was indicated by its intensity values (Pi-value in Shannon-Weaver equation).
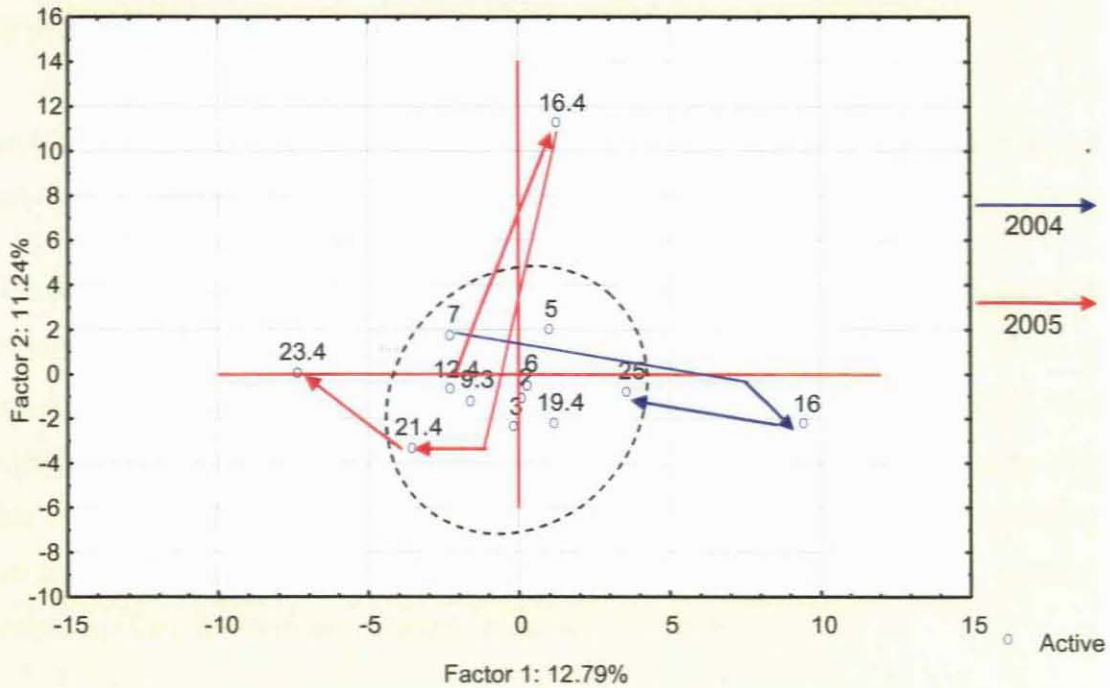
Figure 5.9: Principle Component Analysis graph generated from the band matching results of DGGE profiles of bacterial species in TOWW biofilms during 2004 and 2005.

The Principle Component Analysis graph (Fig. 5.9) generated from the binary matrix in Table 5.5 revealed that bacterial species within the TOWW biofilm population for most of the sampling times at Buffet stayed relatively similar (within dashed circle) during the two production seasons. The bacterial populations from the biofilm samples that were outside the group (week 16 from 2004, week 16.4 from 2005 and week 23.4 from 2005) appear to be later in the season while all of the younger biofilm samples were situated inside the group. This indicated that a significant microbial shift occurred during the aging of the biofilm (refer to arrows on Fig. 5.9). There were however also older samples (19.4 and 21.4 from 2005) present within the group, indicating that the microbial population shifted back to the original group. This trend was visible during both the olive seasons.

Similar to the findings for the bacterial biofilm community in OMWW from Vesuvio farm samples, the bacterial biofilm community in TOWW from Buffet farm samples were also able to re-establish from 2004 to 2005. As mentioned earlier, it was important to recognise that the variance between

the samples on the PCA graph was low (11 – 13%). This means that the biofilms samples that were positioned outside the main cluster were not drastically different in its bacterial community than those within the cluster. However, the differences were still significant. The NaOH released from week 12.4 to 16.4 during 2005 could explain the shift on the PCA graph (Fig. 5.9), however this effect was not so pronounced during 2004. The significant biofilm population shift at week 16 during 2004 cannot be explained, as the NaOH treatment occurred already during week 6 of that particular production season.

5.3.3 DGGE analysis of bacterial species from TOWW and OMWW biofilms cultured in the laboratory during 2004

The DGGE analysis of the bacterial population in laboratory-grown biofilm samples in TOWW and OMWW suggests that a higher bacterial biodiversity developed in the biofilms in the artificial laboratory condition compared to the biofilms that developed at the farm site. Both Buffet and Vesuvio biofilms that were cultivated in the laboratory had 20 to 30 bacterial OTU's per time point. A possible explanation for this could be that the various external conditions in the laboratory, such as climate (±22°C), water temperature (±22°C) and flow rate, were kept constant, and this would not have been the case for biofilms cultured at the two farm sites.
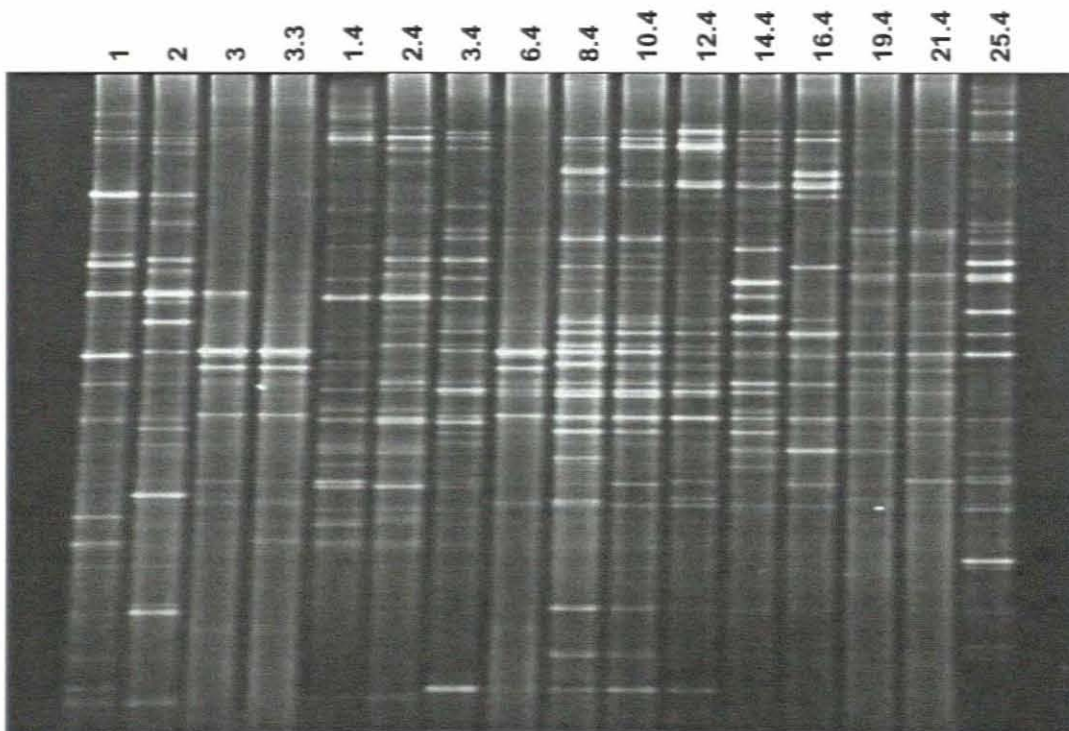
Figure 5.10. The DGGE profile of the bacterial population from TOWW biofilms (Buffet Olives) cultured in the laboratory. The samples were loaded chronologically from left to right: 1, 2, 3, 3.3. The first 4 samples were used as a test run (the Pederson's device was changed afterwards). After the restart of biofilm growth, the sample order was as follows: 1.4, 2.4, 3.4, 6.4, 8.4, 10.4, 12.4, 14.4, 16.4, 19.4, 21.4, 25.4 weeks (2004).
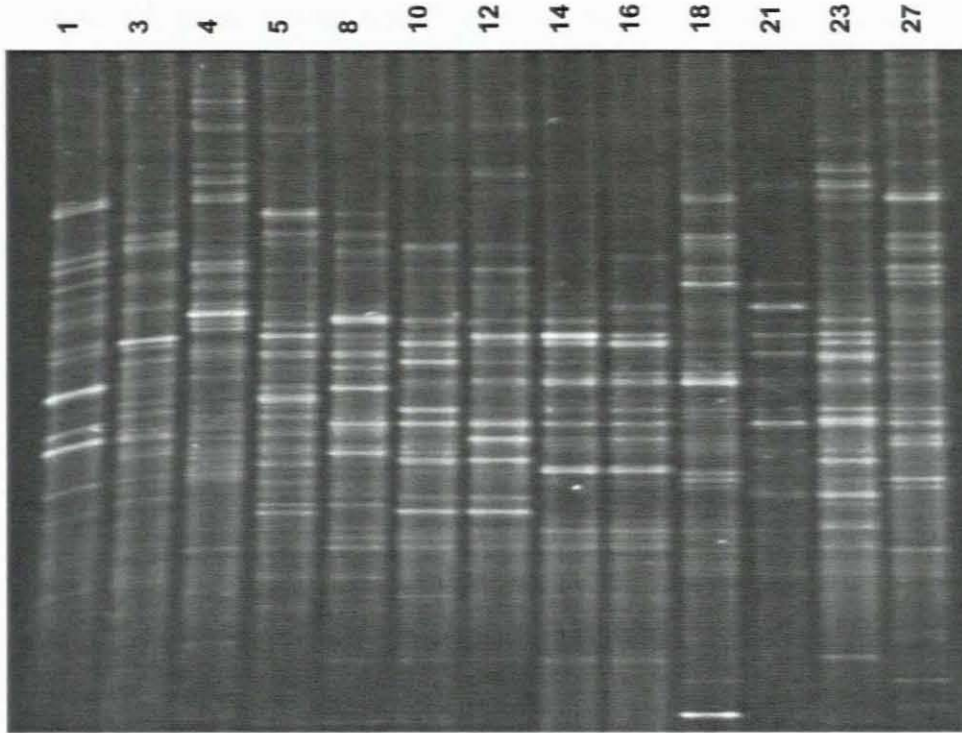
Figure 5.11: The DGGE profile of the bacterial population from OMWW biofilms (Vesuvio Estate) cultured in the laboratory. The samples were loaded chronologically from left to right were: 1, 3, 4, 5, 8, 10, 12, 14, 16, 18, 21, 23, 27 weeks (2004).

Table 5.6: Shannon-Weaver indexes of bacterial species from OMWW and TOWW biofilms cultured in the laboratory during 2004.

OMWW 2004		TOWW 2004	
Biofilm age (weeks)	SW indexes	Biofilm age (weeks)	SW indexes
1	1.254422	1	1.4345238
3	1.186703	2	1.44484212
4	1.354237	3	1.29645183
5	1.417688	3.3	1.32619551
8	1.303633	Restart	-
10	1.34361	1.4	1.49729311
12	1.335246	2.4	1.45303896
14	1.267854	3.4	1.44737105
16	1.266254	6.4	1.26054533
18	1.293503	8.4	1.47588977
21	1.075617	10.4	1.46745091
23	1.348823	12.4	1.40312403
27	1.462551	14.4	1.4322197
		16.4	1.47413118
		19.4	1.37563421
		21.4	1.47184817
		25.4	1.26412676

From week 1.4 to 6.4, there was an initial drop of 16% in biodiversity (Fig 5.12). During the beginning stages of biofilm development at Buffet Estate there was lye solution that had been released at the end of March, which could have reduced the bacterial diversity significantly. Between week 6.4 and 8.4 there was a sharp increase of 15% in biodiversity showing that biofilm were able to recover over a 2-week period after the lye had been released in the wastewater. From week 8.4, biodiversity started to fluctuate around a high level of biodiversity (average 1.45) and fluctuation occurred within a small range (0.1). This pattern repeated itself for 13 weeks (±3 months of olive washing) until week 21.4 (August), after which the biodiversity suddenly dropped by 14% over the following month until it reached a similar value (1.25) as week 6.4. The possible reason for the decrease in biodiversity could be the result of an increased concentration of NaCl in the wastewater, because of fermentation solution that had been released. However, uncertainty remains with the final decrease in biodiversity since the majority of fermentation water (NaCl) was released after this stage in December. The period during December is not shown on the graph (Fig. 5.12). Another possibility for the final decrease in bacterial biodiversity could be an increased concentration of cleaning chemicals as a result of a large cleaning operation within the factory, although this should have also had an influence on the biofilm samples at the farm. It is also possible that during the final bacterial population decrease, there was a significant reduction in the volume of TOWW produced.
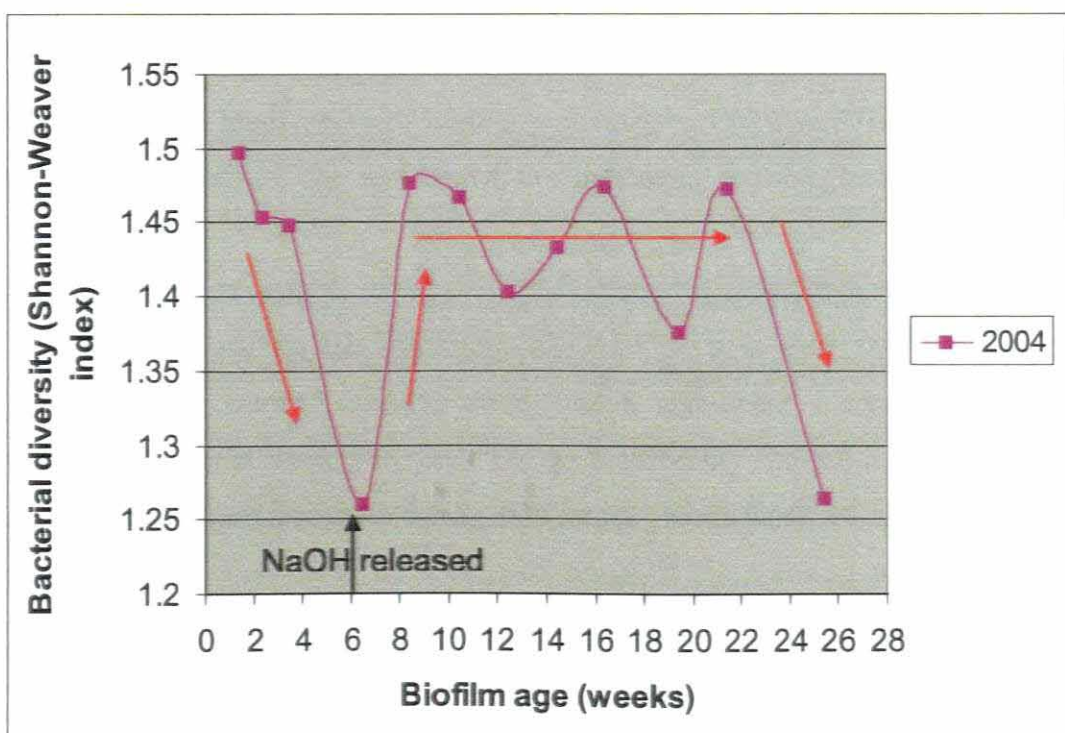
Figure 5.12: Bacterial biodiversity of biofilms grown in TOWW (Buffet Estate) in the laboratory (February to August 2004). The samples from the test run (1, 2, 3, and 3.3) were removed from the graph. The red arrows indicate trends that are visible during the season.

In comparison, the bacterial diversity within the farm-cultivated biofilms in TOWW during 2004 (Fig. 5.8), showed a gradual increase, while the bacterial biodiversity of the laboratory-cultivated samples fluctuated constantly (Fig. 5.12). These fluctuations in bacterial biodiversity were still relatively small and according to the red trend arrow between week 6.4 and 21.4, it indicated that the bacterial biodiversity within the laboratory biofilms were still relatively stable.
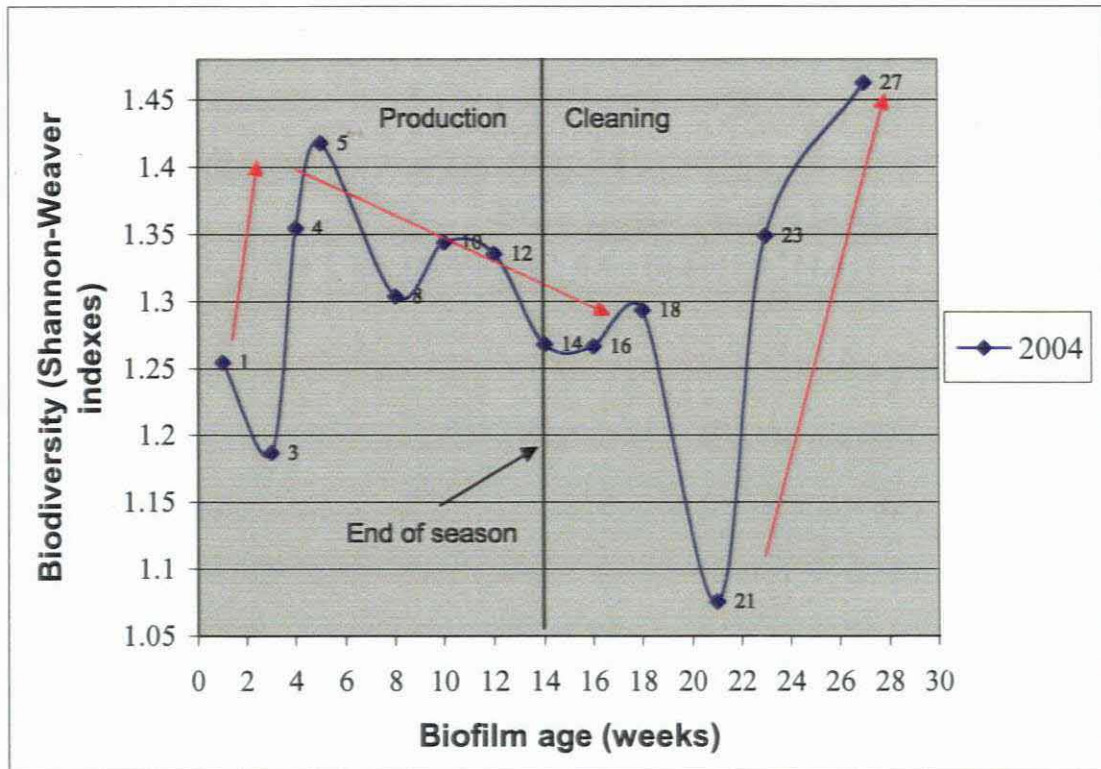
Figure 5.13: Bacterial biodiversity of biofilm cultivated in OMWW (Vesuvio) in the laboratory (April to November 2004). The red arrows are indicating possible trends within the bacterial biodiversity.

For the biofilms cultivated in the laboratory in OMWW, it is clear that early in the biofilm development, between week 3 and 5, there was a significant increase in biodiversity by approximately 20%. From week 5 to week 18, the biodiversity showed a downward trend of 9% although small fluctuations occurred during this stage. From week 18 to 21 a drop of 17% in biodiversity occurred which could be a delayed result of the cleaning processes that started at Vesuvio a month earlier (week 14). It is interesting to note that the biodiversity had also dropped significantly at the farms at the start of the cleaning process, but the drop in biodiversity at the farm site occurred immediately. The delayed effect observed here is due to the wastewater being replaced only every two weeks. It is possible that the impact of some activities between the sampling times could have been missed, i.e. any peaks in wastewater composition that could influence the microbial population of the biofilms.

At week 21, which was at the end of the cleaning process, the bacterial biodiversity started to increase quickly over the following 6 weeks by 36% until week 27 where it reached the highest level (1.45) during the whole season. This indicated that bacterial biodiversity within the laboratory-cultivated biofilms recovered significantly at the end of the cleaning stage.

After the Shannon–Weaver graphs were interpreted, a binary matrix pattern of bacterial species DGGE profiles for both TOWW and OMWW laboratory-cultivated biofilms was created and used for Principle Component Analysis.

Table 5.7: Binary matrix results for bacterial species in TOWW biofilms cultured in the laboratory during 2004 (derived from Fig. 5.10).

Table 5.8: Binary matrix results for bacterial species in OMWW biofilms cultured in the laboratory during 2004 (derived from Fig. 5.11).

The open blocks within the matrix contains the value 0, while the coloured blocks contain the band intensity values or the Pi-values from Shannon-Weaver equation. These matrixes (Tables 5.7 and 5.8) were then used as input data for the Principle Component Analysis (Fig. 5.14 and 5.15)

PCA analysis (Fig. 5.14) of the bacterial population with TOWW biofilms cultured in the laboratory during 2004, revealed that the bacterial population was unstable during the early stages of biofilm development (week 1.4 to week 3.4) since none of the earlier biofilms samples was present within any cluster. However, as the biofilms reached maturity, the bacterial community stabilized, and cluster formation started to occur. This cluster is marked with the dashed ellipse on the PCA graph (Fig. 5.14).

Figure 5.14: The PCA graph of the bacterial population within TOWW biofilms cultured in the laboratory during 2004. The main group (dashed ellipse) contained the following, highly similar, consecutive samples: 8.4, 10.4, 12.4, 14.4, 19.4 and 25.4 week old biofilms. The test samples (1, 2, 3 and 3.3) were removed to prevent misinterpretation.

Bacterial population changes from week 6.4 and onwards were minimal with the 16.4 week old biofilm slightly away from the cluster, but it reverted back within 3 weeks to the stabilized cluster population. The laboratory conditions for the growth of TOWW biofilms were better controlled, while biofilms cultured at the farm were exposed to constant temperature changes as well as variable flow volumes and flow rates of the wastewater. This could be a possible reason for the good stabilization of the bacterial communities within the laboratory-cultivated biofilms. This then clearly indicates the importance of studying biofilms *in situ* in their actual environment as this is a true reflection of their development over time. Laboratory conditions that remain constant do not produce a true reflection of how the biofilms will behave in nature.

The PCA graph (Fig 5.15) of the bacterial population in OMWW biofilms cultured in the laboratory during 2004, revealed that a loose degree of grouping could be observed during the season, however the bacterial population of samples within the group was still relatively variable if the timeline is taken into consideration (refer to the red arrows). During the olive oil production stage, from week

1 to 14, changes in the bacterial biofilm population occurred only within the demarcated group, with the exception of week 4.
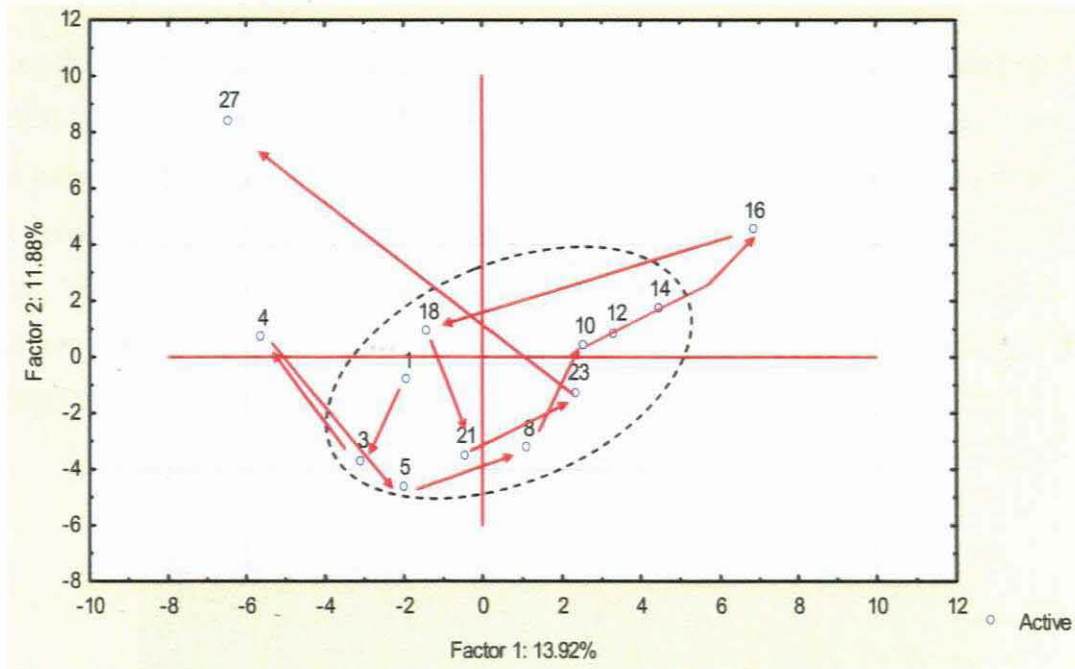
Figure 5.15: The PCA graph of the bacterial population in OMWW biofilms cultured in the laboratory during 2004.

Week 14 marked the end of the production season and the start of the 2-month cleaning period in which higher concentrations of detergents were used in the plant. This resulted in the bacterial population to shift even more drastically from week 16 to 18 as well as from week 18 to 27. The sensitivity in the OMWW biofilm's bacterial population could also be observed in the farm samples of 2004 where no significant clustering occurred (Fig. 5.6). A possible reason for this is that the biofilm was very sensitive to environmental changes and to changes in olive mill wastewater composition, especially cleaning chemicals. The huge population shift on the PCA graph (Fig. 5.15) from week 21 to 27 also aligns with the rapid increase in bacterial biodiversity according to the Shannon-Weaver indexes (Fig. 5.13). This indicated that the change from the more acidic, production wastewater to the more alkaline, cleaning wastewater might have been the reason for the significant population shifts during the cleaning phases.

5.3.4 DGGE analysis of the fungal population from OMWW biofilms cultivated at the Vesuvio farm from 2004 to 2005

The fungal DGGE images of Vesuvio (Fig. 5.16) indicated that the average fungal population in the farm-cultivated biofilms were significantly smaller compared to average bacterial population. A careful count of the bright and faint operational taxonomic units indicated the presence of 8 – 10 fungal species at any specific time point compared to the 18 – 25 bacterial species found at the same sampling point. The samples were loaded onto the same DGGE gel in order to do a band comparison between 2004 and 2005. Bands 1 and 2 were present throughout most of 2004 as well as during 2005 and was therefore excised from the gel for cloning and sequencing.
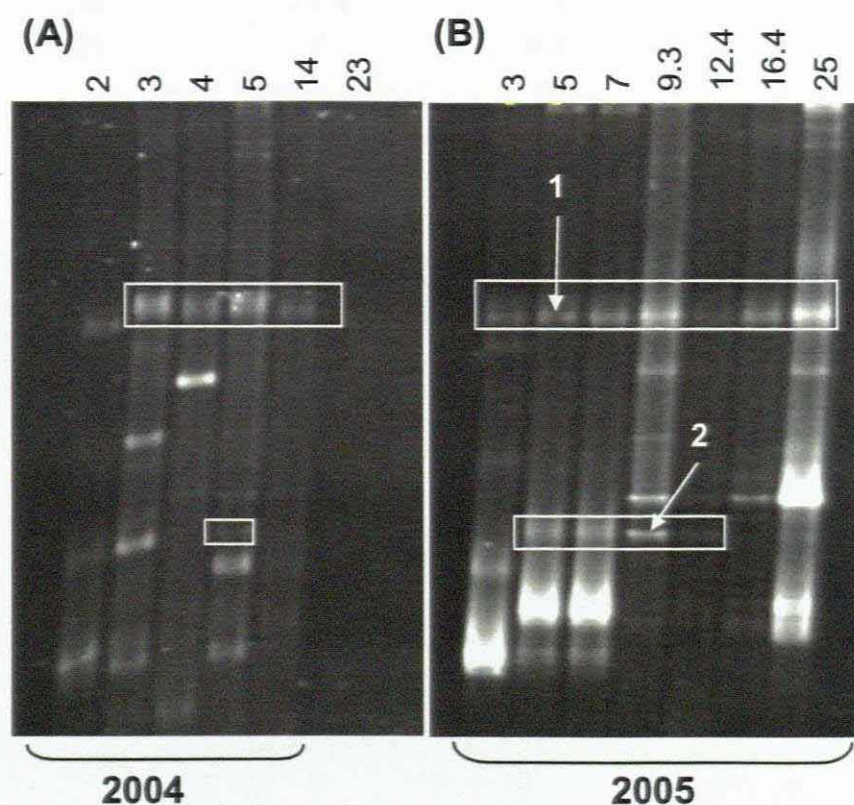
Figure 5.16: DGGE analysis of the fungal population in OMWW biofilms cultured at Vesuvio farm during (A) 2004 and (B) 2005.

Samples were loaded chronologically according to biofilm age in weeks. From left to right: 2, 3, 4, 5, 14, 23 weeks (2004) and 3, 5, 7, 9.3, 12.4, 16.4, 25 weeks (2005). Band 1 and 2 (arrows) were used for further cloning, sequencing and BLAST analysis. Because of the smaller number of OTU's in the fungal population, visual inspection of the DGGE analysis clearly suggests that some fungal species were present throughout 2004 and 2005, while other species appeared and disappeared from the biofilm. The DGGE image analysis with AlphaEase[R] FC software and calculations for the

Shannon-Weaver index was executed in Excel-spreadsheet as described for the bacterial population DGGEs.

Table 5.9: Shannon-Weaver biodiversity indexes for the fungal population in OMWW biofilms from Vesuvio farm.

2004		2005	
Biofilm age (weeks)	SW indexes	Biofilm age (weeks)	SW indexes
2	0.425	3	0.532
3	0.724	5	0.625
4	0.446	7	0.496
5	0.912	9.3	0.819
14	0.274	12.4	0.523
23	0	16.4	0.594
		25	0.749
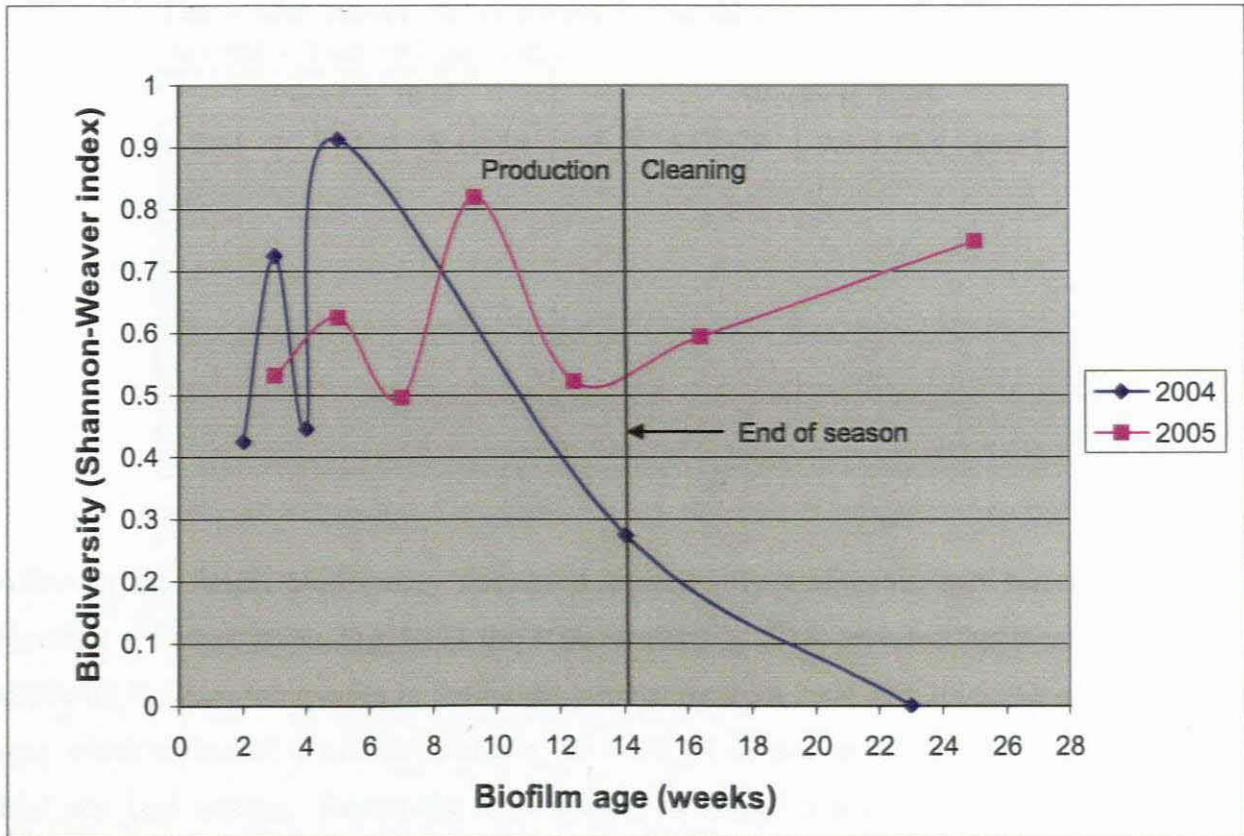
Figure 5.17: The biodiversity of fungal communities in OMWW biofilms at Vesuvio farm during 2004 and 2005 (April to August).

During the first four weeks of the production season of 2004, the fungal biodiversity was relatively unstable and fluctuations occurred between the values of 0.4 and 0.9. After week 5, a rapid decline in biodiversity was observed until week 23, where no fungal species could be detected on the DGGE gel. The fact that no fungal species were detected on the DGGE gel does not necessary

indicate that the fungal species were completely absent from the biofilm at that stage, but rather that the low number of fungal cells were over-shadowed by the bacterial population and that possibly detection of the fungal cells were outside the limits of detection for the techniques used (i.e. cell disruption, PCR and DGGE). PCR amplification of week 23 was repeated, with no success. Although gDNA was purified, it might also be that excess phenolic compounds contaminated the gDNA and resulted in PCR inhibition.

The fungal species were more stable during 2005 since fluctuations were less dramatic than 2004. During the cleaning period of the 2005 season, there was a gradual increase in diversity from week 12 to 25, but during 2004 the exact opposite was observed. The Shannon-Weaver values for all fungal samples were in a lower range (0 – 1) compared to the bacteria (1 – 2). This was understandable, because fungi formed a small percentage of the whole biofilm community.

Table 5.10: Binary matrix results for fungal species in OMWW biofilms cultured at Vesuvio Estate farm.

Weeks in 2004					Weeks in 2005						
2	3	4	5	14	3	5	7	9.3	12.4	16.4	25

Although the fungal biodiversity fluctuated significantly during the two seasons (refer to the Shannon – Weaver graph, Fig. 5.17), the PCA results (Fig. 5.18) revealed that there was still a good similarity between the species in the fungal population from 2004 and 2005. One cluster could be seen which contained three 2004 samples (2, 4 and 14 weeks) and six 2005 samples (3, 5, 7, 9.3, 12.4 and 16.4 weeks). During the 2004 season the fungal population shifts within the younger biofilm samples (week 3 – 5, 2004) was more severe, but as the biofilms reached maturity the fungal population became more stable. However, during 2005 the opposite were observed. The younger biofilms were clustered together within the same group (week 3, 5 and 7), but as the biofilm matured from week 9.3 to week 25, the fungal population became less stable and shifted away from the cluster.
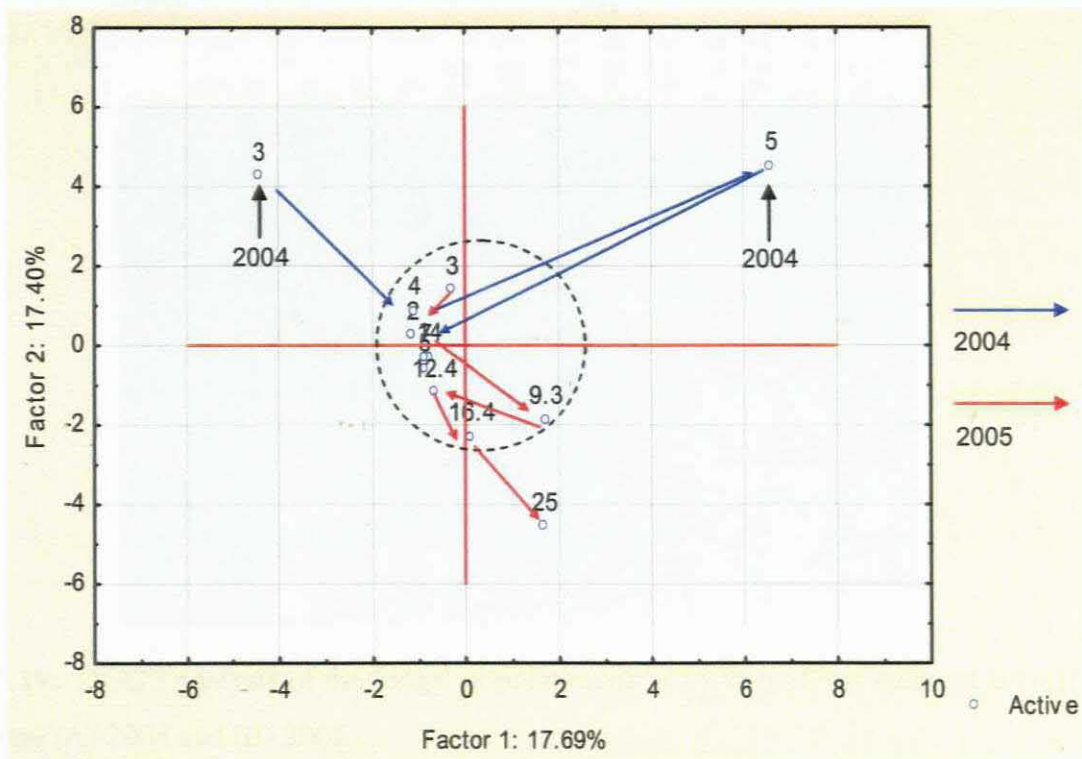
Figure 5.18: The PCA results for fungal species in OMWW biofilms (Vesuvio farm) for 2004 and 2005.

5.3.5 DGGE analysis of the fungal population in TOWW biofilms cultured at Buffet farm from 2004 to 2005

The fungal DGGE images of Buffet (Fig. 5.19) indicated that the average fungal population in the farm-cultivated biofilms were also significantly smaller in number of species compared to average bacterial population from the same biofilm sample. The number of fungal operational taxonomic units was between 8 and 10 compared to the 10 – 20 OTU's for the bacterial population. The samples were loaded onto the same DGGE gel in order to do a band comparison between 2004 and 2005. Samples were loaded chronologically according to biofilm age in weeks. From left to right: 2, 3, 5 (gap), 6, 7, 16, 25 weeks (2004) and 9.3, 12.4, 16.4, 19.4, 21.4 (gap), 23.4 (gap), 25 weeks (2005). Once again due to the limited number of OTU's, visual inspection of the DGGE gel photograph could clearly identify specific patterns in the fungal population within TOWW biofilms. Week 6 (2004) as well as week 16.4 (2005) revealed 2 similar bands (white squared) as indicated on the image, while week 16 (2004) also revealed a repeating pattern (red squared) in week 25 (2005). Furthermore, week 16 and 25 during 2004 showed that the fungal population stayed almost identical (blue and red squares), except for the absence of one OTU during week 25.
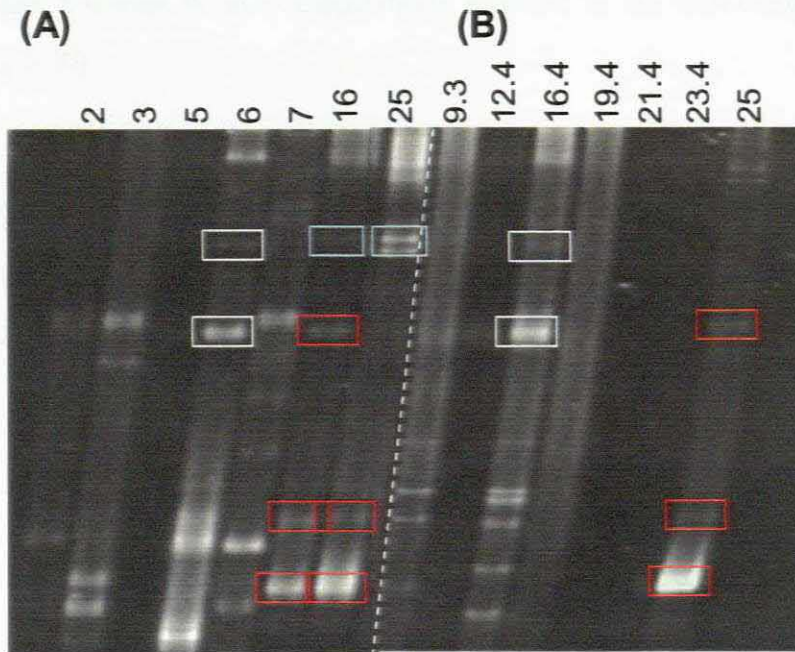
Figure 5.19: DGGE analysis of the fungal population of TOWW biofilms cultured at Buffet Olive farm during (A) 2004 and (B) 2005.

Week 5 (2004) and weeks 21.4 and 23.4 (2005) did not give any DGGE PCR result and thus no OTU's were visible on the DGGE gel (Fig. 5.19), while week 19 (2005) produced a smear and only 1 very faint band was visible on closer inspection. Various reasons could explain the results absent fungi from the biofilms. Firstly, it could be explained by the assumption that there were no fungi present in the TOWW biofilm at that point in time. As this is the least likely explanation, it is more likely that low cell numbers of fungi was present at that time(s) and that the gDNA extraction and PCR-DGGE methods were not sensitive enough ensure the detection of the fungal 18S rDNA. It is interesting to note at this point that the release of NaOH from the processing plant correlates well with the 2005 biofilm analysis (week 19, 21.4 and 23.4 followed after NaOH release during week 12-16), while this is not true for the 2004 fungal population, i.e. week 5 is prior to the NaOH release at week 6. Lastly, this could be due to experimental errors, with contaminating DNases responsible for the absence of any DGGE-PCR DNA.

Image analysis and Shannon-Weaver calculations resulted in the following biodiversity profile (Table 5.11 & Fig 5.20).

Table 5.11: Shannon-Weaver biodiversity indexes for the fungal population in TOWW biofilms cultured at Buffet Olives farm.

2004		2005	
Biofilm age (weeks)	**SW indexes**	**Biofilm age (weeks)**	**SW indexes**
2	0.303	9.3	0.816
3	0.848	12.4	0.339
5	0	16.4	0.661
6	0.944	19.4	0.004
7	0.696	21.4	0
16	0.705	23.4	0
25	0.456	25	0.569

The Shannon-Weaver biodiversity graph for the fungal population in TOWW biofilms (Fig. 5.20) revealed that, during 2004, there was an overall increase in fungal biodiversity during the earlier stages of biofilm development (week 2 – 6). This deduction is made if the Shannon-Weaver index data for week 5, which was believed to be an error, is ignored. Directly after NaOH release (week 7) the fungal biodiversity decreased again, after which it stayed relatively stable until week 16 during 2004. Towards week 25, the fungal biodiversity started to slowly decline.
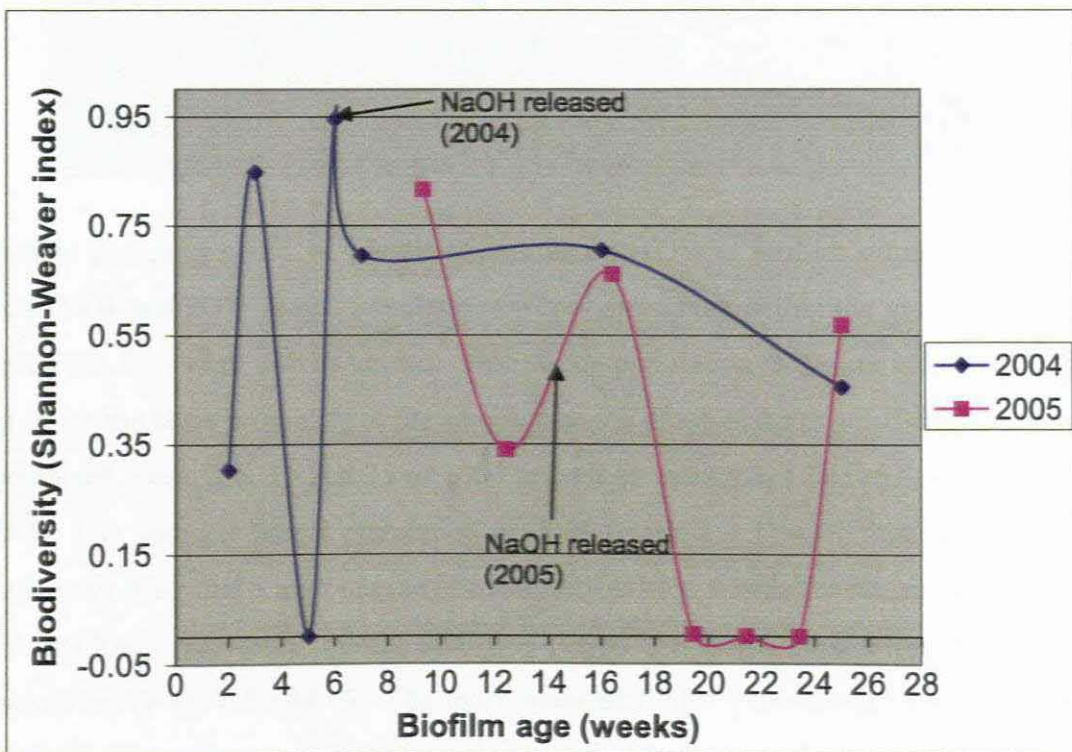
Figure 5.20: The biodiversity of fungal communities cultivated in TOWW at Buffet farm during 2004 and 2005 (April to August).

During 2005, no stability was observed within the biodiversity of the fungal population because of rapid fluctuations that occurred between week 9.3 and 19.4. From week 19.4 to week 23.4, the fungal biodiversity was extremely low, but managed to recover again until week 25. This data could be interpreted in such a way to explain that the NaOH release in 2005 had a much more severe impact on the fungal communities compared to 2004. A possible explanation for this could be that either the NaOH concentration or the volume of the wastewater, or both, was higher during 2005.

This comparative table (Table 5.12) formed a binary matrix of the fungal OTU's from the DGGE gel photograph (Fig. 5.19), as generated by gel image analysis software (AlphaEase FC) and was used as an input matrix for the PCA analysis (Fig. 5.21). Absence of bands was indicated as a 0 within the matrix, while presence of bands was indicated by its intensity values (P_i-value in Shannon-Weaver equation).

Table 5.12: Binary matrix results for the fungal population in TOWW bioflims cultured at Buffet Olives (derived from Fig 5.19).

Weeks in 2004							Weeks in 2005						
2	3	5	6	7	16	25	9.3	12.4	16.4	19.4	21.4	23.4	25.4

Only a single grouping could be identified within the TOWW biofilm's fungal population over time. Both 2004 and 2005 fungal populations were represented within the group, but the majority of the group (63.6%) were 2005 samples. Not all the population shifts are indicated by the arrow timelines, since the close proximity of the samples on the graph make this difficult to visualise. The cluster contained week 2, 5, 16 and 25 of 2004 as well as week 9.3, 12.4, 16.4, 19.4, 21.4, 23.4 and 25 of 2005. The younger fungal population of 2004 (week 2 – 7) were randomly shifting on the PCA graph, indicating that a high degree of instability existed within the fungal population (refer to the arrow timeline). However, during this time period the fungal population shifted back to the cluster twice during week 5 and 16. The more matured fungal populations of week 16 and 25 fell together within the main cluster, thereby indicating that the population might have started to stabilise. The Shannon-Weaver graph (Fig. 5.20) revealed a similar trend as the PCA graph and

indicated that biodiversity was also variable during the younger stages of biofilm growth, but as maturity was reached, a degree of stability set in.
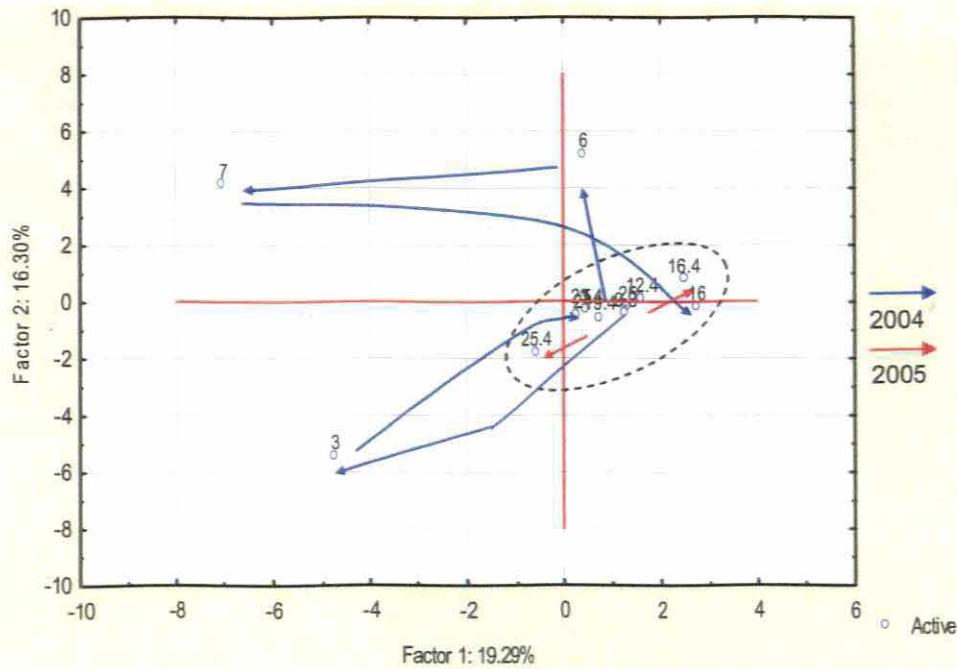
Figure 5.21: The PCA results for the fungal population in TOWW biofilms cultured at Buffet Olives during 2004 and 2005.

The fungal population of 2005 clustered together with 2004 samples, thereby indicating that similar fungal population within the biofilm re-established within the TOWW biofilms during the second production season (2005). Although the Shannon-Weaver graph (Fig. 5.20) showed significant fluctuation in the biodiversity of the fungal species in 2005 as well as a very low biodiversity from week 19 to 23.4, the PCA (Fig. 5.21) revealed a similar fungal population at most of the time points.

5.3.6 DGGE analysis of the fungal population from OMWW and TOWW biofilms cultured in the laboratory during 2004

The fungal DGGE analysis of OMWW laboratory-cultivated biofilms showed that the laboratory biofilms produced approximately similar amount of fungal operational taxonomic units (8 – 10) compared to the biofilms cultured at the farm (Fig. 5.22). As indicated by block 2 (white rectangular) on, a prominent species was present throughout the season (week 1 to 18). It could also be seen that some fungal species appeared, disappeared and reappeared during the season as indicated by block 1 and 3. Furthermore, visual inspection of the DGGE results show a marked

reduction in fungal OTU's after week 16, which coincided with the end of the production season at Vesuvio Estate and the start of the cleaning regime.

Figure 5.22: DGGE analysis of the fungal population of OMWW biofilms cultured in the laboratory during April to August 2004. The samples were loaded chronologically as follows: 1, 3, 4, 5, 8, 10, 14, 16, 18, 21, 23 weeks.
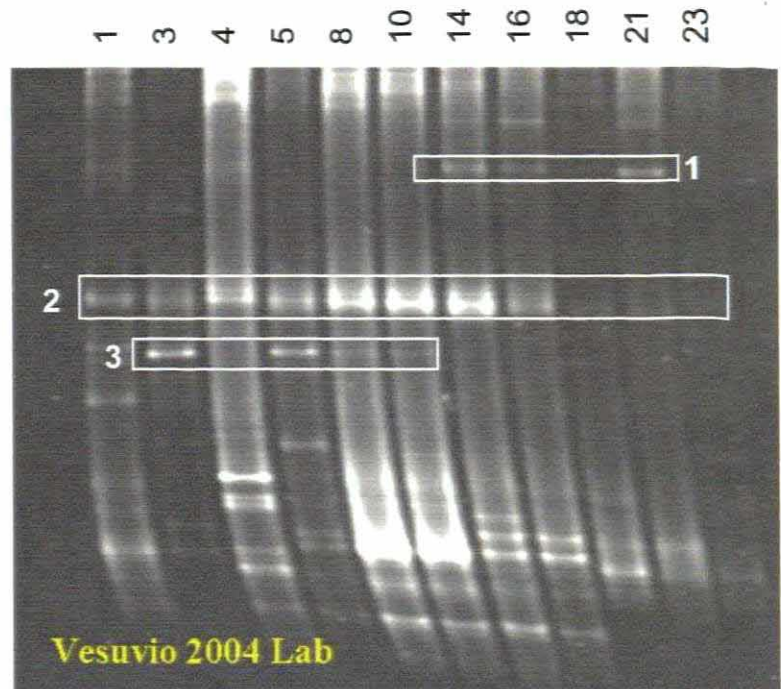
Figure 5.23: DGGE analysis of the fungal population of TOWW biofilms cultured in the laboratory during April to August 2004. The fungal DGGE profile of Buffet's laboratory-cultivated biofilm samples were loaded chronologically from left to right as follows: 1.4, 2.4, 3.4, 6.4, 8.4, 10.4, 12.4, 14.4, 16.4, 19.4, 21.4, 25.4 weeks (2004).
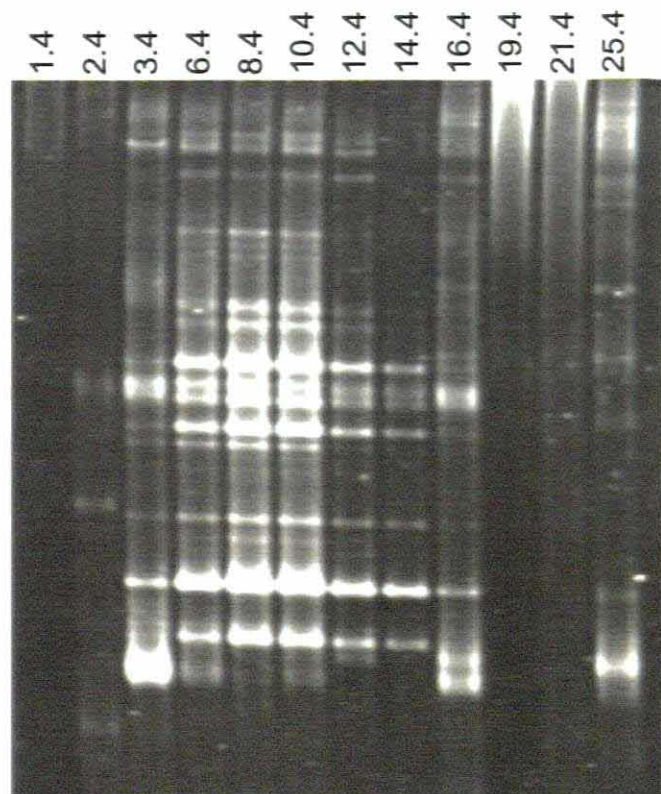
The DGGE fungal population analysis in TOWW laboratory-cultivated biofilms (Fig. 5.23) showed that the laboratory biofilms produced more operational taxonomic units (12 – 17) compared to the farm biofilms (8 – 10). A similar phenomenon were also observed with bacterial OTU's, indicating that laboratory conditions were also selecting for a higher fungal biodiversity than those at the farms (*in situ*). Prominent species were present from week 3.4 to 16.4 and the biofilm seemed to be relatively stable during this time as indicated by the repetitive patterns. On visual inspection of the DGGE photograph, a clear reduction in fungal OTU's is again visible after week 16.4.

Table 5.13: Shannon-Weaver indexes of fungal biodiversity in TOWW and OMWW biofilms cultured in the laboratory during 2004.

OMWW 2004		TOWW 2004	
Biofilm age (week)	SW indexes	Biofilm age (weeks)	SW indexes
1	0.958	1.4	0
3	0.594	2.4	0.575
4	0.860	3.4	0.701
5	0.836	6.4	1.179
8	1.039	8.4	1.128
10	0.995	10.4	1.213
14	0.936	12.4	1.030
16	0.851	14.4	0.858
18	0.698	16.4	1.041
21	0.774	19.4	0.270
23	0.429	21.4	0.279
		25.4	0.950

Plotting of the Shannon-Weaver index values (Fig. 5.24) revealed that the fungal biodiversity in OMWW biofilms changed significantly during the season. The fungal biodiversity increased during the first week of biofilm growth after it dropped by 38.5% to week 3. From week 3 to 8 the fungal biodiversity recovered by 42.3%, after which it started to gradually decrease again by 33.7% over the following 10 weeks. This decline in biodiversity might be explained by the increased concentration of alkaline cleaning chemicals within the wastewater after week 14. During the next 3 weeks (week 18 – 21) the biodiversity seemed to have stabilised, but this stage was immediately followed by a second decrease in biodiversity of 37.7% until week 23.
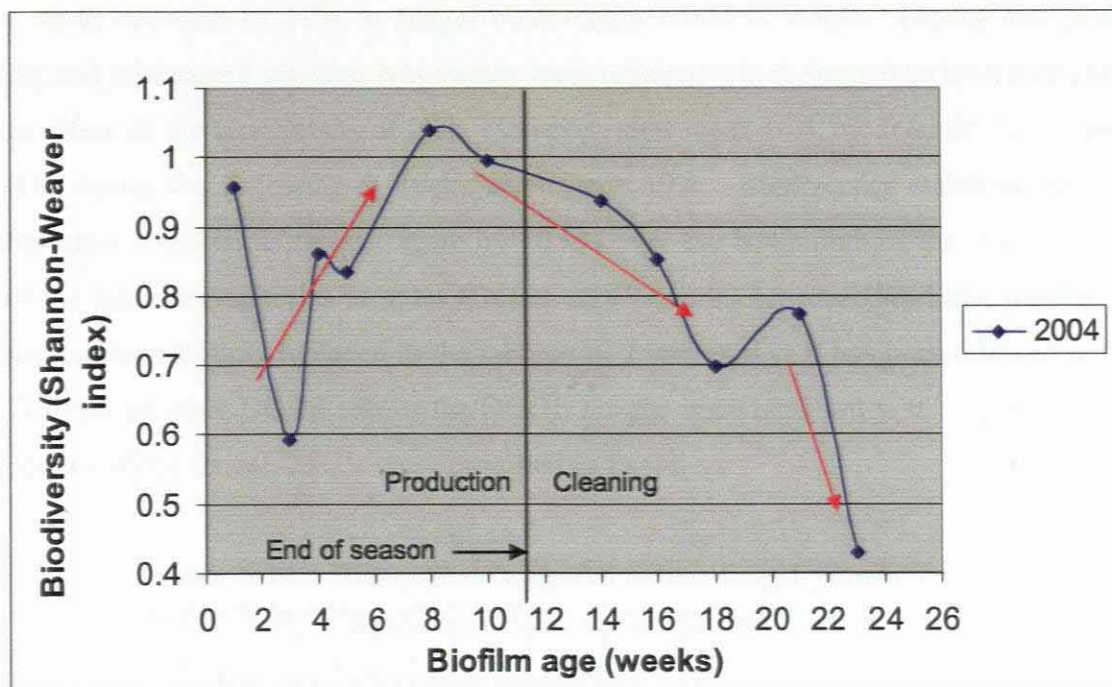
Figure 5.24: Shannon-Weaver graph depicting the biodiversity of laboratory-cultivated fungal communities within OMWW (Vesuvio) during April to August 2004.
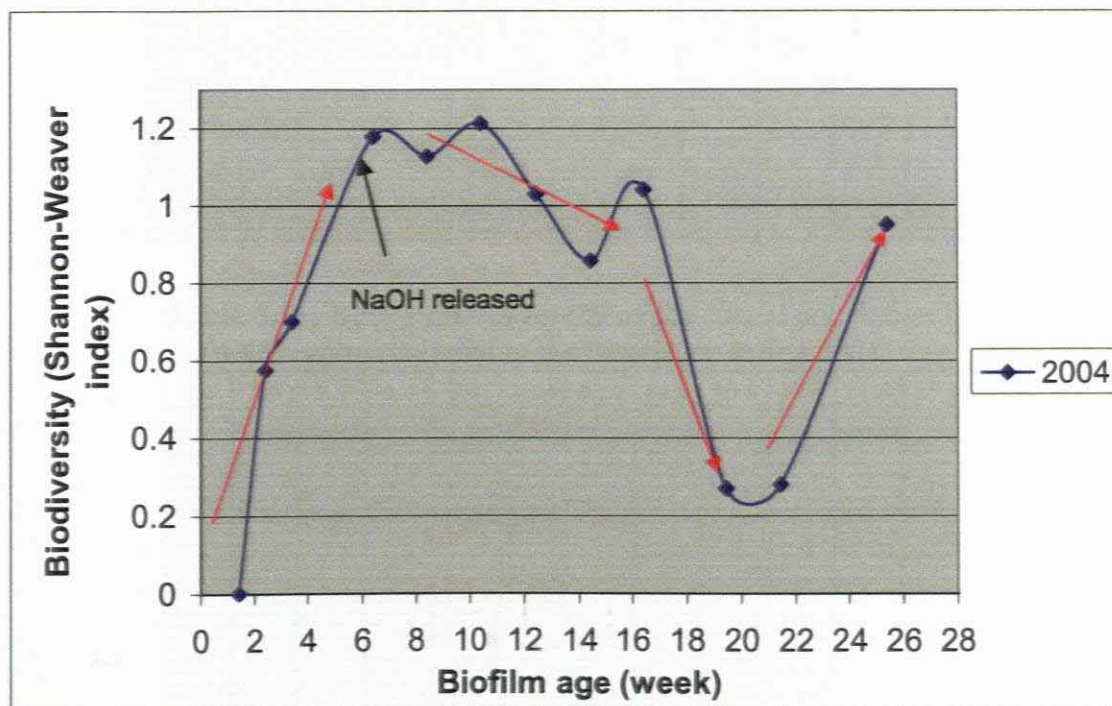
Figure 5.25: Shannon-Weaver graph depicting the biodiversity of laboratory-cultivated fungal communities within TOWW (Buffet) during April to August 2004.

Plotting the Shannon-Weaver indexes for the laboratory-cultivated fungal population in TOWW biofilms (Fig. 5.25) revealed that there was a significant increase in fungal species from week 1 and it was able to stay at high biodiversity levels for ±10 weeks. During these 10 weeks (week 6.4 to

16.4) a small decrease of 11% in fungal biodiversity could be noted. During this period, lye treatment and subsequent washing wastewater were released which seemed to have a relative small decrease effect on the biodiversity at first. However, after week 16.4, biodiversity fell considerably with 74% during the following 3 weeks until week 19.4. Biodiversity stabilised for 2 weeks thereafter until it started to recover again by 70.5% over the last month of the season. The data from all the laboratory biofilm samples (Buffet and Vesuvio) however produced delayed results, since wastewater was only replaced in the laboratory 2 weeks after it has been released at the farm sites. As with all other DGGE results the DGGE images were analysed with AlphaEase FC and a binary matrix of the fungal OTU's was developed in Excel.

Table 5.14: Binary matrix results of the fungal population in OMWW biofilms cultured in the laboratory during 2004.

1	3	4	5	8	10	14	16	18	21	23

Table 5.15: Binary matrix results of the fungal population in TOWW biofilms cultured in the laboratory during 2004.

1.4	2.4	3.4	6.4	8.4	10.4	12.4	14.4	16.4	19.4	21.4	25.4

As with the other binary matrixes, the open blocks within the matrix contains the value 0, while the coloured blocks contain the band intensity values or the P_i-values from Shannon-Weaver equation.

These binary matrixes (Tables 5.14 and 5.15) couldn't be interpreted independently and were only used as input data for the quantitative Principle Component analysis graphs to follow (Fig.s 5.26 and 5.27).
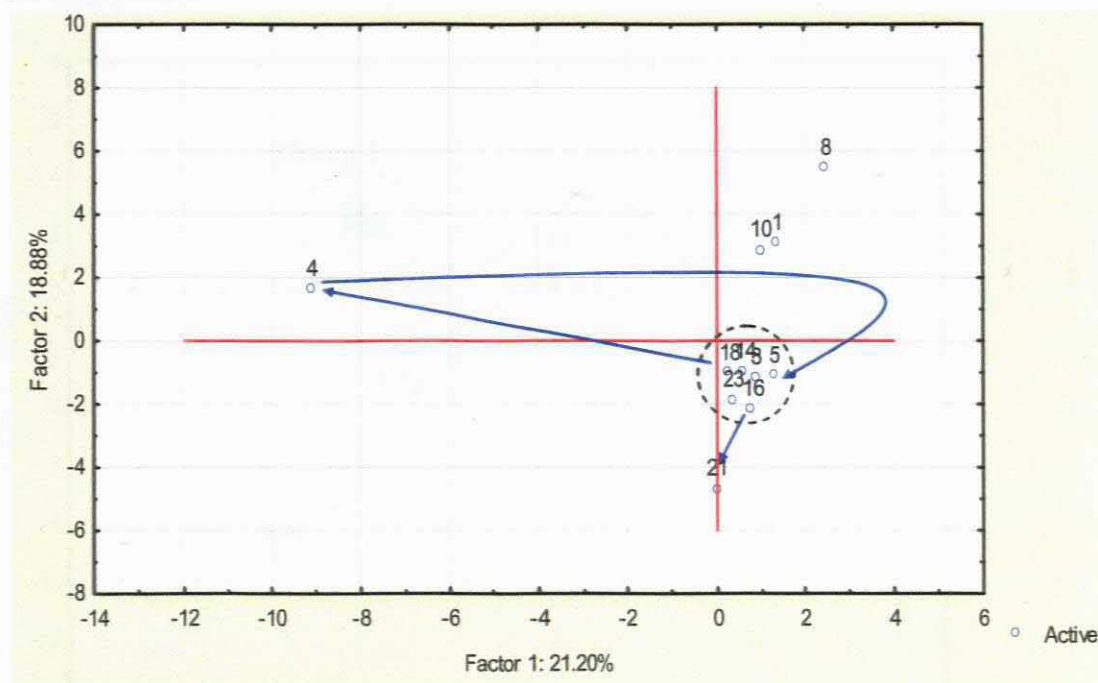
Figure 5.26: The PCA graph for the fungal population in OMWW biofilms cultures in the laboratory during 2004.

According to the PCA data on Fig. 5.26, a single group was identified on the graph that revealed similarity between the laboratory-cultivated fungal populations during the Vesuvio season. However, this was not an indication that the biofilm communities were stable over the 23-week period. Large fungal species shifts occurred between the younger populations during weeks 1, 3, 4, 5, 8 and 10. The only relative stability in the community occurred during weeks 14, 16 and 18 (consecutive samples within group) as the biofilms started to reach maturity. A small population shift occurred away from the group towards week 21, but managed to recover again to its original community within the inner circle (week 23).

As mentioned before, week 14 marked the start of the 2 month cleaning period and did not seem to have a large effect on the fungal population in OMWW biofilms cultured in the laboratory. This could be seen by the close proximity of the samples following week 14 on the PCA graph. The Shannon–Weaver graph (Fig. 5.24) revealed however that the fungal biodiversity of the laboratory-cultivate biofilms dropped significantly during the cleaning time (week 14 to 22) at Vesuvio. These two, seemingly contrary, results showed that it was possible for two or more biofilm samples with closely related species profiles (PCA graph), to have variable biodiversity values (Shannon –

Weaver graph). The PCA data however, would be regarded as a more accurate and statistically correct representation of the data, since Shannon-Weaver measures biodiversity only in term of band intensities at one time point. PCA uses band intensity and band positions and compares this with all the time points.
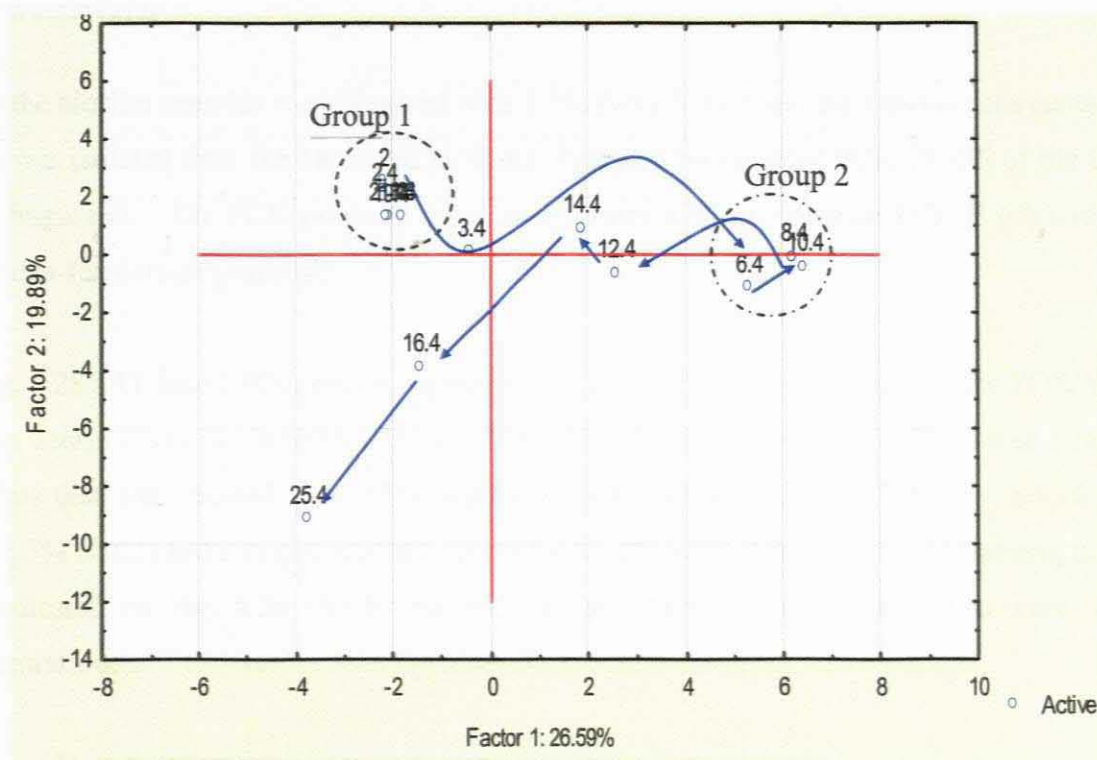
Figure 5.27: The PCA graph of the fungal population in TOWW biofilms cultured in laboratory during 2004. This is the only microbial population that started to display a 2-cluster profile.

The PCA data for the fungal population in TOWW biofilms cultured in the laboratory (Fig. 5.27), revealed that from the first time point the younger fungal population (week 1.4, 2.4, and 3.4) were grouped together within group 1. As the older biofilm started to develop during the next 3 weeks (week 6.4, 8.4 and 10.4), the population shifted to group 2. Week 6 was marked as the time point where lye solution and washing wastewater were released in the table olive process and this could have played a significant role during the fungal population shift that occurred after week 6.4.

From week 10.4 to week 25.4 the changes in the fungal biofilm community was much more significant than directly after the start of the release of the lye and wash waters at week 6. This can be contributed to the delayed effect of the fungal community in the laboratory, due to wastewater that was replaced only two weeks after the changes had occurred at the farms. If the data from Fig. 5.27 is compared to the fungal population in TOWW cultured at Buffet farm in 2004 (Fig. 5.21) it

can clearly be seen that in the latter the main fungal population shifts started to occur immediately after the lye and wash water release in week 6.

5.4 Shock treatment effects of NaOH and olive factory detergents on the biofilms in olive wastewater

After the biofilm samples were shocked with 2.5% (v/v) NaOH and the various detergents, genomic DNA was isolated from the harvested biofilms, followed by bacterial PCR-DGGE of the 16S rRNA gene fragments. The PCR products were loaded onto a 9% acrylamide DGGE gel with a 60% - 40% urea-formamide gradient.

In Fig. 5.28 (A), lane 1 (Control) is representative of all the bacteria present in the TOWW biofilm, as this sample was not treated with any NaOH or detergent solutions. The other lanes contain biofilms that were treated with 2.5% NaOH or detergent solutions for 3 hours. Shock treatment with 2.5% NaOH had a negative effect on the bacterial profile (lane 2), as most bacteria disappeared (as indicated on Fig. 5.28 (A) by the red dashed boxes), while other OTU's were reduced in concentration.
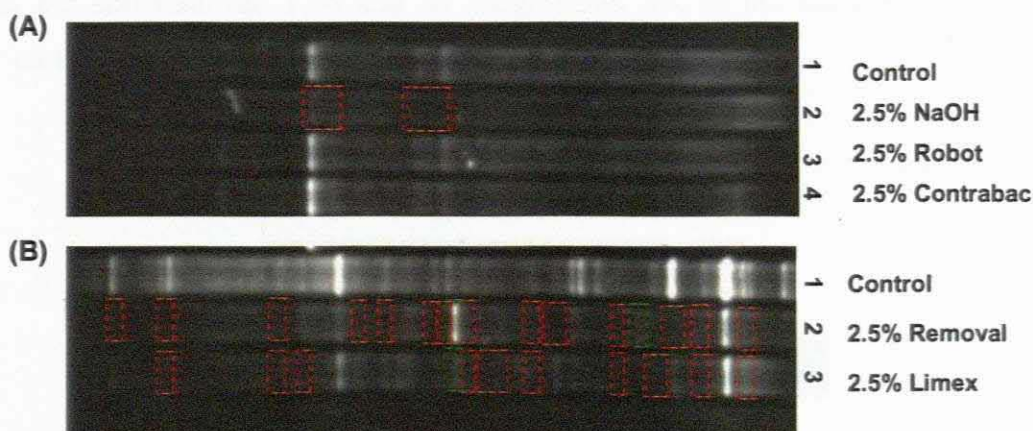
Figure 5.28: DGGE analysis of bacterial biofilms in (A) TOWW, Lane 1 represents the control sample. Lane 2, 3 and 4 represent biofilm samples treated with 2.5% NaOH, 2.5% Robot and 2.5% Contrabac, respectively. Photo (B) is the bacterial biofilm samples from OMWW where lane 1 represents the control. Lane 2 is the sample treated with 2.5% Removal and lane 3 is the sample treated with 2.5% Limex.

From Fig. 5.28 (B), it can be seen that the control biofilm in OMWW (lane 1) was an indication of all the bacteria present in the biofilm before shock treatment. The two detergents had a strong killing or removal effect on the bacteria in the OMWW biofilms, as various bands disappeared or

reduced in intensity when compared to the control lane (dashed red boxes). There were bands (green dashed boxes) present in lanes 2 and 3 that were not present in lane 1 (Control). A possible explanation for this could be that the specific detergents increased the detachment of the cells from the polystyrene surfaces during the sonication stage, resulting in an increased isolation of some bacterial species, previously non-detectable by PCR-DGGE. The fact that the species band could only be detected in the sample treated with the detergent does not necessarily imply that the species was not present in the control biofilm. Because of the lack of detergent in the control sample, there exist the slight possibility that the specific bacteria present in the chemically treated samples, but absent in the control, did not detach from the control polystyrene slide during sonication and might not have been available for genomic DNA isolation and PCR.

The Shannon Weaver biodiversity index for the bacteria in TOWW biofilms obtained in Fig. 5.29 was plotted and showed that the control gave the highest peak and therefore contained the highest biodiversity. The biofilms that were treated with NaOH and detergents gave lower peaks and it can therefore be deduced that the detergents removed bacterial species form the TOWW biofilm community. The NaOH seemed to be the most effective removal agent as it gave the lowest peak with a 50% reduction in biodiversity. The Contrabac and Robot detergents seemed to be less effective, but still removed 17% and an insignificant 4% of the bacterial population from the TOWW biofilm, respectively.
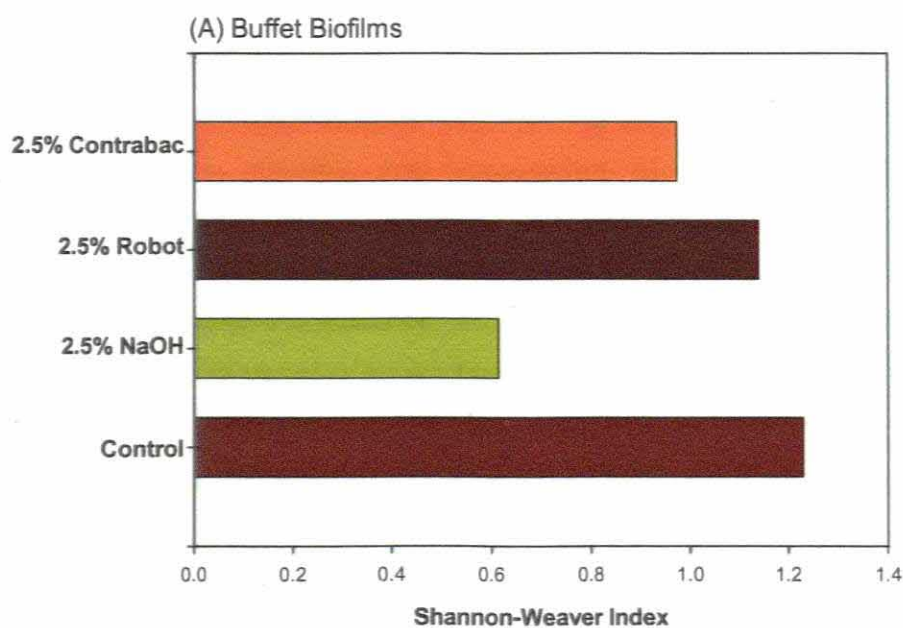
Figure 5.29: The Shannon-Weaver indexes of bacterial diversity in TOWW biofilms treated with NaOH and detergents.

(B) Vesuvio Biofilms
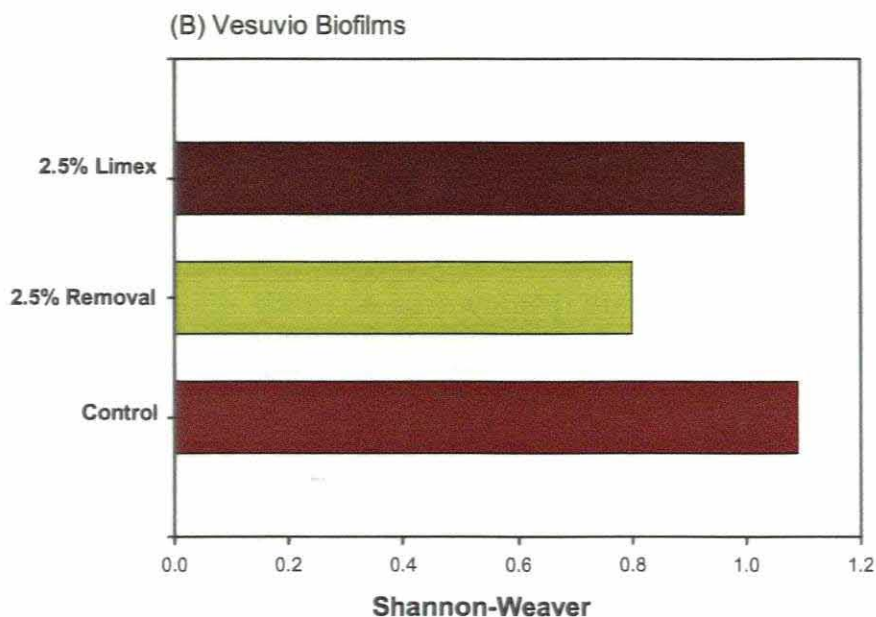
Figure 5.30: Shannon-Weaver indexes of bacterial diversity in OMWW biofilms treated with detergents.

The Shannon-Weaver index graph of OMWW biofilms treated with detergents (Fig. 5.30) again showed the control producing the highest biodiversity index, indicating that most of the organisms were present before detergent treatment. In this case, Removal detergent seemed to be the most effective as it gave the lowest bacterial peak with a 27% reduction in biodiversity. Limex did not seem to be as effective, but it showed a slight, insignificant reduction of 9% in the bacterial biodiversity when compared to the control.

5.5 Preliminary isolation, cloning and sequencing of prominent DGGE operational taxonomic units (OTU's)

The preliminary sequencing of the bacterial and fungal DGGE OTU's were only performed on OMWW biofilms cultured at Vesuvio Estate farm during 2005 as a preliminary test to observe whether species could be identified. In Fig. 5.4 (bacterial population in OMWW biofilms) and Fig. 5.16 (bacterial population in OMWW biofilms), the seven OTU's selected for cloning are marked with arrows. It is of utmost importance to note that the identification of microorganisms in this study is only preliminary and that microorganisms could only be identified to its genus level. Although species names are given in the phylogenetic trees, a significant degree of uncertainty remains with regard to its accuracy. The most important reason for this can be contributed to the fact that the 16S and 18S rDNA sequences used in the BLAST analysis were spanning a very short

and limited section (232 – 340 bp) of the complete 16S and 18S rDNA fragments on the GenBank database.
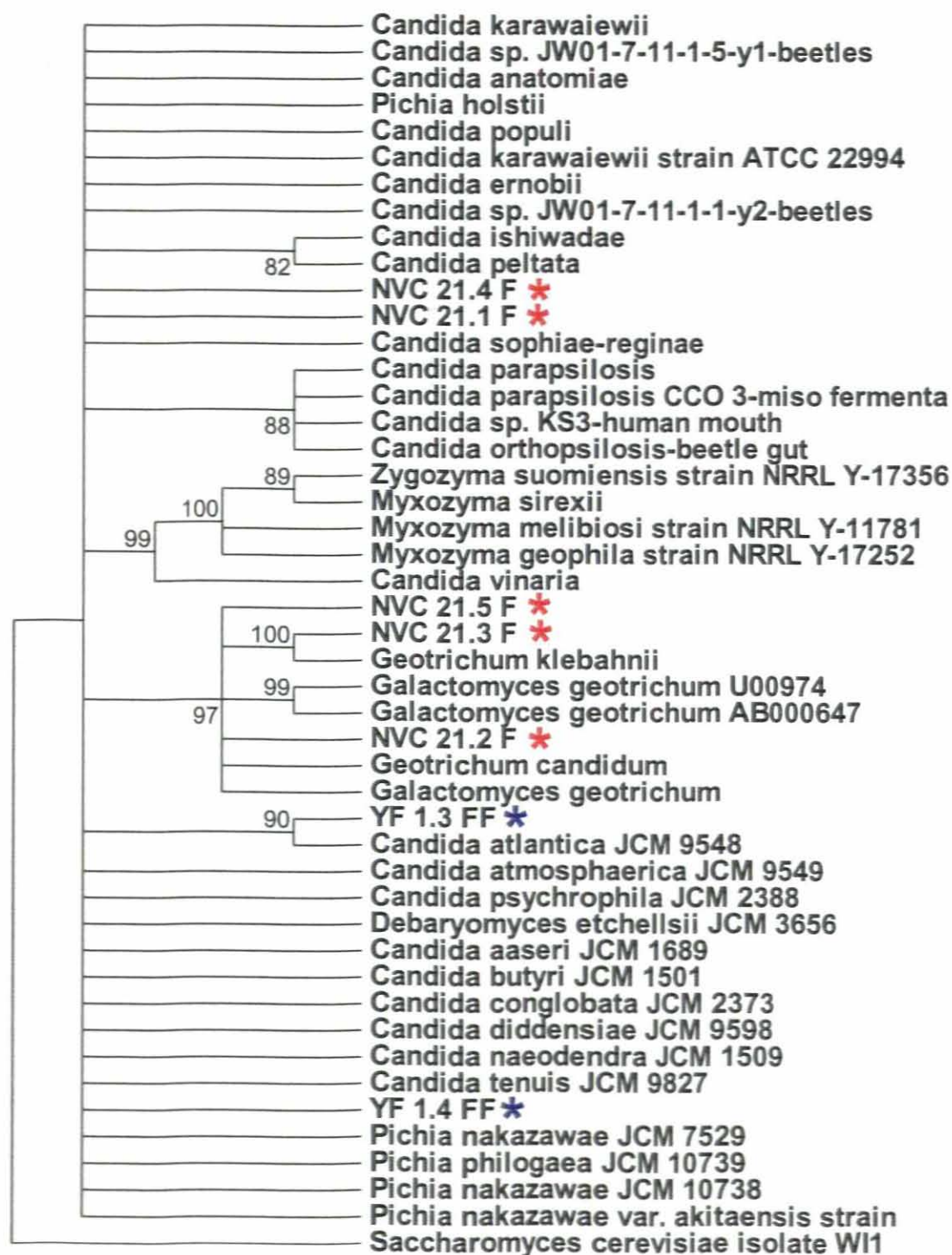
Figure 5.31: Phylogenetic tree for the fungal isolates identified from OMWW biofilms. (OTU 1* = YF 1.3 FF and YF 1.4 FF; OTU 2* = NVC 21.1 F, NVC 21.2 F, NVC 21.3 F, NVC 21.4 F and NVC 21.5 F)

The fungal species that were identified in this study were from two operational taxonomic units. According to the BLAST results in Fig. 5.31, **OTU 1** (Fig. 5.16) contained a variety of *Candida* and *Pichia spp* (98-99% homology) all belonging to the order *Saccharomycetales*. The *Candida* (family: *Saccharomycetaceae*) and *Pichia* (family: *Endomycetaceae*) genera are closely related and small differences occur with its spore-forming genotypes. **OTU 2** (Fig. 5.16) contained the fungus *Geotrichum* (99% homology) belonging to the *Dipodascaceae* family (sub-group: *Galactomycetes*) and is widely distributed in environmental samples. *Geotrichum candidum* have previously been found to have the ability to reduce COD, TOC, and to a smaller degree, low molecular weight phenols in OMWW and is therefore considered an important microorganism for bioremediation purposes (Garcia *et al.*, 2000). All the yeast and fungal genera detected within this study were also recently detected within table olive products by Arroyo-Lopez (2006) using molecular techniques.

The following phylogenetic tree (Fig. 5.31) reveals the positions of the genera within related family groups. OTU 1 consisted of 2 clones on the yeast and fungi phylogenetic tree (YF 1.3 FF and YF 1.4 FF), while OTU 2 consisted of 5 clones (NVC 21.1 F, NVC 21.2 F, NVC 21.3 F, NVC 21.4 F and NVC 21.5 F). It is noted in Fig. 5.31 that OTU 2 also contained a variety of *Candida* species, as with OTU 1, however the bands are visibly well separated from each other on the DGGE gel (Fig. 5.16). It was therefore possible for some clones from OTU 1 to move together with clones in OTU 2 in a DGGE gel. A possible explanation for this was that the GC-content of the base pair sequences was similar.

It was interesting to note that not all clones from OTU 2 fell within a single cluster. A possible explanation for this unexpected phenomenon can be that the OTU bands within a DGGE gel do not always separate clearly and that more that one species can therefore overlap on the gel. The reason for this is that the two sequences have similar melting points due to similar GC content within the sequences. Even if the G and C base pairs are slightly different from within two sequences, it can still have the same melting point on a DGGE gel, hence overlapping positions. Some bootstrap values for this tree are rather low (below 95%), which reduces the accuracy of the branching patterns (topology). Again, the main reason for this was the small sizes of the sequences that were used. This can be improved by rather using 1500 bp sequences in order to obtain higher homology over a longer length of sequence.

The next phylogenetic tree (Fig. 5.32) shows the variety of bacterial species that were identified in the OMWW biofilms. Five bacterial OTU's (band 3 – 7) (Fig. 5.4) were selected from the Vesuvio farm samples for sequence identification and were marked on Fig. 5.32. Certain problematic

BLAST sequences have been removed from the tree to improve the bootstrap values, however many of those sequence also had homologies ranging between 95% and 98%. **OTU 3** (marked red) contained an uncultured *Arcobacter spp.* with a 98% homology belonging to epsilon-proteobacteria (sub-groups: *Campylobacterales, Campylobacteraceae*). Another possibility (removed from tree) was an uncultured *Tolumonas spp.* (96%) from the gamma-proteobacteria group (sub-groups: *Aeromonadales, Aeromonadaceae*). Sequences from **OTU 4** (marked blue) revealed with a 99% homology *Pantoea agglomerans* (Group: gamma-proteobacteria, *Enterobacteriaceae*). It might also contain *Pectinatus haikarae* (Group: *Firmicutes, Clostridiales, Acidaminococcaceae*) (96%) and *Flavobacterium spp* (96%). **OTU 5** (marked yellow) was predominantly *Pseudomonas spp* (Group: gamma-proteobacteria, *Pseudomonadaceae*), including *P .fluorescence* and *P. putida* (99% homology).

OTU 6 (marked green) revealed many *Enterobacteriaceae spp.* and *Pseudomonas spp.* from the gamma-proteobacteria group with a 98% homology. In this group a variety of *Enterobacter* species were found as well *Klebsiella oxytoco* and an unidentified *Pantoea* species. Another unidentified biocide degrading bacteria (no phylogenic group name given) were found with a 95% percent homology. A final species identified in OTU 6 was *Agrobacterium tumefaciens* (99% homology). This species belong to the group alpha-proteobacteria (sub-groups: Rhizobiales, Rhizobiaceae, Rhizobium / agrobacterium group). The last band, **OTU 7** (marked purple), contained *Pseudomonas* sequences (99%), *Pectinatus spp* (96%) and *Pantoea agglomerans* (98%).

Some of the above mentioned bacterial genera associated with plants or with bioremediation was found in the olive wastewater biofilms. *Agrobacterium tumefaciens*, a soil bacterium, express mutation genes into the damaged root and stem cells of plants and cause cancer tumors. They are single or pairing rods, non-spore forming, gram – negative and motile cells that grow aerobically. Some strains can grow anaerobically, thereby using nitrogen as an electron acceptor. It is known for its large amounts of EPS that it produces; therefore could potentially play an important role in the protection of the OMWW biofilm against antimicrobial compounds such as the phenols and fatty acids present in OMWW. It utilises carbohydrates, salts and amino acids as it energy source. *Enterobacter agglomerans* (gram – positive, facultative anaerobe) is also found on many plants and human faeces and is used to fight fire blight disease in tree fruit.
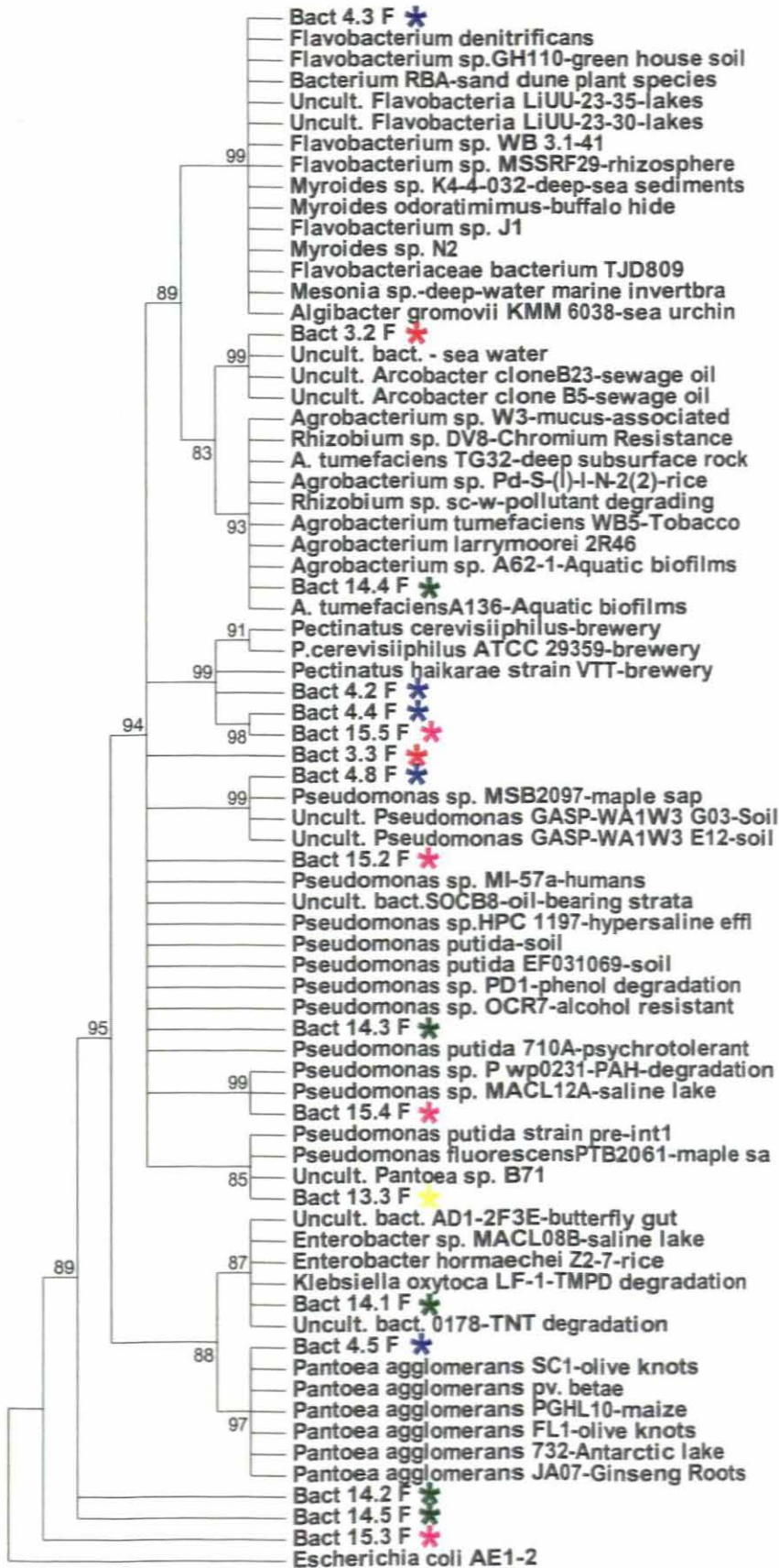
Figure 5.32: Phylogenetic tree diagram of the bacterial species identified from the OMWW farm-cultivated biofilms at Vesuvio.

All bacterial clones from OTU 3 to 7 are indicated on the phylogenetic tree (Fig. 5.32) as follows: OTU 3* (Bact 3.2 F, 3.3 F), OTU 4* (Bact 4.2 F, 4.3 F, 4.4 F, 4.5 F, 4.8 F), OTU 5 (Bact 13.3 F), OTU 6* (Bact 14.1 F, 14.2 F, 14.3 F, 14.4 F, 14.5 F) and OTU 7* (Bact 15.2 F, 15.3 F, 15.4 F, 15.5 F).

A similar phenomenon was observed with the bacterial phylogenetic tree (Fig. 5.32) when compared to the fungal tree (Fig. 5.31). Species from a single OTU were not grouped together within a single cluster as expected. This might again be the result of co-migration of sequences on the DGGE gel, as a result of similar melting points or inaccurate alignments to database sequences.

The following microorganisms on the bacterial phylogenetic tree are considered to be of importance: Bact 4.2, 4.4 and 15.2 were found to be related to the relative fast-growing *Pectinatus portalensis* (sequence removed from tree). This has previously been isolated from a winery wastewater treatment plant (Gonzalez *et al.*, 2004). Olive mill wastewater, as with winery wastewater, is high in phenolic compounds and COD and it is therefore possible that Bact 4.2, 4.3 and 15.2 might play a role in the degradation of OMWW in a reactor setup. Bact 4.5 was found to be related to *Pantoea agglomerans* strains that are commonly found in the inner tissues of the olive knots which is a disease sometimes found on olive trees. Bact 13.3 compared with *Pseudomonas putida N6* that has been studies for its aromatic phenol degradation pathways by Tropel and Van der Meer (2004). Bact 14.3 and 15.4 was also related to *Pseudomonas* sp. PD1 and *Pseudomonas* sp. pwp0231, respectively. Both these species are known for it capability to degrade phenol as well as other polycyclic aromatic hydrocarbons (PAH's) (Daane *et al.*, 2001)

It should be noted that the above results are only a preliminary identification of the bacterial and fungal isolates and that species identification of all the DGGE bands are currently underway in a different research project. An important reason for the possible uncertainty of the results was that the sequence sizes were too small to align accurately to the database sequences (±340 bp for fungi and ±230 bp for bacteria). In future, PCR primers will be used that selects for 1500 bp segments of the 16S and 18S rRNA genes in order to obtain more accurately base pair alignment to database sequences.

CHAPTER 6

GENERAL CONCLUSIONS AND RECOMMENDATIONS

General Conclusion and Recommendations

6.1 Conclusions

Preliminary COD determination of table olive wastewater (TOWW) and olive mill wastewater (OMWW), treated separately within two Rotating Biological Contactors (RBC's), indicated that the indigenous biofilms that developed within both the RBC's had the potential to degrade the COD from these wastewaters. At the end of the 10-day growth period, it was found that the COD within TOWW and OMWW was reduced by 47% and 32%, respectively. Scanning electron microscopy and fluorescent microscopy revealed that a typical biofilm structure, comprising of bacteria, fungi, yeast and EPS developed in both TOWW and OMWW. These results were a good indication of the biofilm's bioremediation potential and it was decided to further investigate the microbial population profiles of these biofilms as well as to study the seasonal changes that occur within the bacterial and fungal populations.

Detergent and NaOH shock treatments of bacterial populations within olive wastewater biofilms from Buffet and Vesuvio gave an indication of the efficiency of the cleaning chemicals used at both farms to reduce the bacterial numbers. At Buffet, the 2.5% NaOH had severely negative effects on the bacterial biodiversity resulting in a 50% reduction in biodiversity. The antimicrobial properties of the detergents, Contrabac and Robot, reduced the biodiversity by 17% and 4%, respectively. At Vesuvio, the highly alkaline detergents (pH 11 – 12), Removal and Limex, gave 27% and 9% reductions in bacterial biodiversity, respectively. These results also provided preliminary information regarding the subsequent study of how the detergents used at the olive factories might affect the microbial changes during the production seasons.

6.1.1 Farm-cultivated biofilms from TOWW at Buffet Olives

The methods used in this study, such as DGGE, Shannon-Weaver indexes and PCA could not provide a full and comprehensive analysis of the bacterial and fungal populations. Therefore, the term "bacterial population" or "fungal population" used in the discussion did not refer to the complete microbial structure of the biofilms, but only to a representative fraction of the microbial structure. Environmental biofilm samples can contain hundreds to thousands of species and even the best gDNA isolation and PCR-DGGE strategy can only visualise approximately 40 – 50 16S rRNA amplicons on the DGGE image.

In order to determine whether reproducible bacterial populations can develop in TOWW between two production seasons, farm-cultivated biofilms of 2004 and 2005 were compared. The Shannon-Weaver biodiversity index for bacterial populations in TOWW biofilms (Figure 5.8) revealed that the biodiversity of the 2004 farm-cultivated biofilms were more stable compared to 2005, although both biodiversity profiles remained within the same range (1 – 1.4). From the principle component analysis of DGGE data, (Figure 5.9), a weak assumption can be made that the bacterial population from 2004 re-established itself within the biofilm during 2005. As the biofilm from both seasons aged, the shifts in total bacterial population became more pronounced. This could be correlated to the NaOH (lye and wash water) being released at week 6 (2004) and week 14 (2005) of the production seasons.

The biodiversities of fungal populations from both seasons at Buffet showed opposite trends as the bacterial populations regarding biofilm maturity. During 2004, biodiversity (Shannon-Weaver) as well as the total population profile (PCA) of the fungal population during early biofilm development were highly unstable, but as maturity were reached, the fungal populations stabilised and clustered together with 2005 populations. During 2005, the biodiversity fluctuated significantly, however the populations remained related to each other as well as related to 2004 populations as indicated by PCA analysis. It could therefore be concluded that the majority of fungal species in 2004 were re-established within the biofilm in 2005.

6.1.2 Farm-cultivated biofilms from OMWW at Vesuvio Estate

The comparison of 2004 and 2005 bacterial populations in OMWW biofilms cultured at Vesuvio Estate, revealed a smaller degree of reproducibility compared to the results for biofilms in TOWW. According to the Shannon-Weaver profiles on Figure 5.5, the bacterial biodiversity of 2004 was more stable during the production season and cleaning months. The bacterial biodiversity of the 2005 season ended approximately 28% lower than 2004 and could have therefore been more sensitive to the alkaline cleaning chemicals. Although biodiversities (Shannon–Weaver indexes) were relatively stable during 2004, the PCA revealed a significant degree of shifting in the total bacterial population profiles. Bacterial populations from 2005 were more related to each other (close grouping of time points) and were more stable than those of 2004. However, both 2004 and 2005 were still in relative close proximity to each other. It can therefore be deduced that the bacterial profile from 2004 re-establised itself during 2005 in OMWW biofilms, but to a lesser degree than with Buffet's bacterial populations.

The fungal biodiversity as indicated by the Shannon-Weaver data (Figure 5.17) in OMWW at Vesuvio Estate fluctuated considerably during 2004, and after the onset of the cleaning months, was reduced to a very low, undetectable level. During the start of 2005, fungal species were introduced to the biofilm and as the season commenced, biodiversity were showing an averaged increase until the end of the cleaning month. This gave the indication that fungal species might have adapted to the cleaning chemicals or that the concentration of the cleaning chemicals was lower for the second season. PCA analysis (Figure 5.18) of the fungal populations in OMWW during 2004 indicated that during the younger biofilms stages, more population shifts occurred. However, with maturity came stability and this resulted in clustering later in the season. The 2005 fungal population revealed opposite results and was more stable during earlier biofilm development. As the biofilm aged, the fungal population became less stable and shifted away from the clustered group. Most of the fungal populations of 2005 were in very close proximity with 2004 populations and therefore indicated that similar fungal species were present in both seasons.

6.1.3 Farm-cultivated biofilms (2004) compared to laboratory-cultivated biofilms in TOWW

The laboratory and farm-cultivated bacterial population in TOWW showed remarkable opposite trends as the biofilms matured. In the laboratory, younger biofilm samples had an unstable bacterial population, but as the biofilm matured, stability set in and significant clustering was observed within the older populations. However, the biofilms that were cultivated at the farm showed more stable populations earlier in biofilm development and started to shift away from the cluster closer to end of the season. A possible reason for this could have been the delayed replacement of olive wastewater to the biofilms in the laboratory as well as the fact that the environment of the laboratory-cultivated biofilms was controlled compared to the natural environment of the farm-cultivated biofilms.

A comparison of the fungal populations of the laboratory and farm-cultivated TOWW biofilms also revealed significantly opposite development patterns regarding biofilm maturity. As with the bacteria, the laboratory-cultivated fungal populations during 2004 were also unstable during early biofilm development, but started to cluster together close to the end of the season. The farm-cultivated fungal populations during 2005 were stable for the first 3 weeks of biofilm growth after which it shifted to a completely different cluster and stabilised for 3 weeks. As the biofilm matured further, more pronounced population shifting started to occur.

6.1.4 Farm-cultivated biofilms (2004) compared to laboratory-cultivated biofilms in OMWW

The shifts in the laboratory and farm-cultivated bacterial populations in OMWW biofilms were both irregular and no clear groupings of time points could be observed on the PCA graphs (2004 data on Figure 5.6 and Figure 5.15). It was noted however, that the laboratory-cultivated bacterial population were in slightly closer proximity to each other than the farm-cultivated bacterial populations of 2004. The shifts in the fungal populations of both laboratory and farm-cultivated OMWW biofilms were much more pronounced between earlier biofilm time points when compared to the closely grouped fungal populations from the more matured biofilms.

6.2 Future recommendations

The research presented within this study can be regarded as the initial groundwork for studying the indigenous biofilms developing in olive wastewater, however there are still many aspects of this work that can be improved upon. Firstly, sampling should take place at least twice a month over 3 seasons in order to be more representative. Biofilms should rather be grown continuously over all three seasons and not re-grown on new media at the beginning of each season. Secondly, sampling times must be rigorously correlated with the daily/weekly production schedule at the factories in order to keep track of wastewater outflow. Each biofilm sample must be compared to a water sample that was collected at the same time. This water sample should undergo COD, phenol and pH determination tests. Thirdly, Shannon-Weaver determination of biodiversity as well as Principle Component Analysis for all three seasons must be presented on single graphs. This will provide a higher degree of accuracy when determining whether the biofilms in the wastewater are really showing repeatability in its microbial communities and growth patterns over the seasons.

As mentioned in Chapter 5, the sequencing of 16S and 18S rRNA of bacterial and fungal species included in this thesis is only preliminary. Research in constructing full length 16S and 18S rRNA libraries of all DGGE operational taxonomic units, with phylogenetic tree analysis, is recommended.

CHAPTER 7

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CHAPTER 8

APPENDIX

8.1 DGGE analysis of bacterial species from Vesuvio Estate farm biofilms (from 2004 – 2005)

Shannon-Weaver calculations				
Lane: 1				
Peak	Area	%	Pi	H'
1	852	6.9	0.073077	-0.08303
2	165	1.3	0.014152	-0.02617
3	170	1.4	0.014581	-0.02677
4	529	4.3	0.045373	-0.06094
5	248	2	0.021271	-0.03557
6	270	2.2	0.023158	-0.03787
7	559	4.5	0.047946	-0.06325
8	629	5.1	0.05395	-0.06841
9	188	1.5	0.016125	-0.0289
10	191	1.5	0.016382	-0.02925
11	487	3.9	0.04177	-0.05761
12	186	1.5	0.015953	-0.02867
13	190	1.5	0.016296	-0.02914
14	743	6	0.063728	-0.0762
15	954	7.7	0.081825	-0.08895
16	403	3.3	0.034566	-0.05051
17	972	7.9	0.083369	-0.08995
18	656	5.3	0.056266	-0.07032
19	421	3.4	0.036109	-0.05208
20	574	4.7	0.049232	-0.06438
21	265	2.1	0.022729	-0.03735
22	236	1.9	0.020242	-0.03428
23	264	2.1	0.022643	-0.03725
24	328	2.7	0.028133	-0.04363
25	549	4.5	0.047088	-0.06249
26	291	2.4	0.024959	-0.04
27	339	2.7	0.029076	-0.04467
28	377	3.1	0.032336	-0.04819
29	296	2.6	0.025388	-0.0405
	11659			1.456373
Lane: 2				

Peak	Area	%	Pi	H'
1	460	4.6	0.045554	-0.06111
2	279	2.8	0.027629	-0.04306
3	692	6.9	0.068528	-0.07978
4	248	2.5	0.024559	-0.03954
5	1223	12.1	0.121113	-0.11104
6	316	3.1	0.031293	-0.04708
7	247	2.4	0.02446	-0.03942
8	138	1.4	0.013666	-0.02548
9	271	2.7	0.026837	-0.04217
10	632	6.3	0.062587	-0.07532
11	572	5.7	0.056645	-0.07063
12	707	7	0.070014	-0.08085
13	453	4.5	0.04486	-0.06048
14	510	5.1	0.050505	-0.06549
15	483	4.8	0.047831	-0.06315
16	248	2.5	0.024559	-0.03954
17	415	4.1	0.041097	-0.05697
18	230	2.3	0.022777	-0.03741
19	262	2.6	0.025946	-0.04115
20	226	2.2	0.022381	-0.03693
21	374	3.7	0.037037	-0.05301
22	567	5.6	0.05615	-0.07022
23	235	2.3	0.023272	-0.03801
24	185	1.8	0.01832	-0.03182
25	125	1	0.012379	-0.02361
	10098			1.333263
Lane: 3				
Peak	Area	%	Pi	H'
1	570	6	0.060235	-0.0735
2	227	2.4	0.023988	-0.03886
3	190	2	0.020078	-0.03408
4	145	1.5	0.015323	-0.02781
5	993	10.5	0.104935	-0.10274
6	409	4.3	0.043221	-0.05897

	Peak	Area	%	Pi	H'
	7	277	2.9	0.029272	-0.04489
	8	207	2.2	0.021875	-0.03631
	9	293	3.1	0.030963	-0.04673
	10	136	1.4	0.014372	-0.02648
	11	163	1.7	0.017225	-0.03038
	12	190	2	0.020078	-0.03408
	13	597	6.3	0.063088	-0.07571
	14	237	2.5	0.025045	-0.0401
	15	493	5.2	0.052098	-0.06685
	16	612	6.5	0.064673	-0.07691
	17	423	4.5	0.0447	-0.06033
	18	232	2.5	0.024517	-0.03948
	19	162	1.7	0.017119	-0.03024
	20	249	2.6	0.026313	-0.04157
	21	246	2.6	0.025996	-0.04121
	22	481	5.1	0.05083	-0.06577
	23	609	6.4	0.064356	-0.07667
	24	289	3.1	0.03054	-0.04627
	25	189	2	0.019973	-0.03394
	26	152	1.6	0.016063	-0.02882
	27	192	2	0.02029	-0.03434
	28	388	4.1	0.041002	-0.05688
	29	22	0.2	0.002325	-0.00612
	30	90	1.1	0.009511	-0.01923
		9463			1.395281

Lane: 4

Peak		Area	%	Pi	H'
	1	485	5.4	0.053562	-0.06808
	2	521	5.8	0.057537	-0.07135
	3	232	2.6	0.025621	-0.04077
	4	691	7.6	0.076311	-0.08527
	5	462	5.1	0.051022	-0.06593
	6	270	3	0.029818	-0.04549
	7	484	5.3	0.053451	-0.06799
	8	1342	14.8	0.148205	-0.12288

	Peak	Area	%	Pi	H'
	9	415	4.6	0.045831	-0.06136
	10	332	3.7	0.036665	-0.05264
	11	109	1.2	0.012038	-0.02311
	12	400	4.4	0.044174	-0.05985
	13	130	1.4	0.014357	-0.02646
	14	224	2.5	0.024738	-0.03974
	15	258	2.8	0.028493	-0.04403
	16	150	1.7	0.016565	-0.0295
	17	195	2.2	0.021535	-0.0359
	18	78	0.9	0.008614	-0.01779
	19	615	6.8	0.067918	-0.07933
	20	99	1.1	0.010933	-0.02144
	21	142	1.6	0.015682	-0.0283
	22	438	4.8	0.048371	-0.06363
	23	191	2.1	0.021093	-0.03535
	24	226	2.5	0.024959	-0.04
	25	475	5.2	0.052457	-0.06716
	26	91	0.9	0.01005	-0.02008
		9055			1.313429

Lane: 5

Peak		Area	%	Pi	H'
	1	548	6.2	0.06174	-0.07467
	2	276	3.1	0.031095	-0.04687
	3	225	2.5	0.025349	-0.04046
	4	577	6.5	0.065007	-0.07717
	5	178	2	0.020054	-0.03405
	6	526	5.9	0.059261	-0.07273
	7	360	4.1	0.040559	-0.05645
	8	164	1.8	0.018477	-0.03203
	9	158	1.8	0.017801	-0.03114
	10	302	3.4	0.034024	-0.04995
	11	132	1.5	0.014872	-0.02718
	12	178	2	0.020054	-0.03405
	13	143	1.6	0.016111	-0.02888
	14	174	2	0.019603	-0.03348

Peak	Area	%	Pi	H'
15	172	1.9	0.019378	-0.03319
16	332	3.7	0.037404	-0.05338
17	156	1.8	0.017575	-0.03085
18	439	4.9	0.049459	-0.06458
19	287	3.2	0.032334	-0.04819
20	244	2.7	0.02749	-0.04291
21	221	2.5	0.024899	-0.03993
22	462	5.2	0.05205	-0.06681
23	393	4.4	0.044277	-0.05994
24	197	2.2	0.022195	-0.0367
25	238	2.7	0.026814	-0.04214
26	236	2.7	0.026589	-0.04189
27	210	2.4	0.023659	-0.03847
28	626	7.1	0.070527	-0.08122
29	350	3.9	0.039432	-0.05537
30	101	1.1	0.011379	-0.02212
31	162	1.8	0.018251	-0.03173
32	89	1	0.010027	-0.02004
33	20	0.4	0.002253	-0.00596
	8876			1.454538

Lane: 6

Peak	Area	%	Pi	H'
1	421	5	0.049658	-0.06475
2	481	5.7	0.056735	-0.0707
3	479	5.6	0.056499	-0.07051
4	225	2.7	0.026539	-0.04183
5	522	6.2	0.061571	-0.07454
6	341	4	0.040222	-0.05613
7	495	5.8	0.058386	-0.07203
8	143	1.7	0.016867	-0.0299
9	358	4.2	0.042227	-0.05804
10	138	1.6	0.016277	-0.02911
11	246	2.9	0.029016	-0.04461
12	1281	15.1	0.151097	-0.12401
13	230	2.7	0.027129	-0.0425

Peak	Area	%	Pi	H'
14	306	3.6	0.036093	-0.05207
15	501	5.9	0.059094	-0.07259
16	643	7.6	0.075843	-0.08495
17	220	2.6	0.02595	-0.04115
18	201	2.4	0.023708	-0.03853
19	169	2	0.019934	-0.0339
20	172	2	0.020288	-0.03434
21	268	3.2	0.031611	-0.04742
22	258	3	0.030432	-0.04615
23	243	2.9	0.028662	-0.04422
24	34	0.4	0.00401	-0.00961
25	24	0.3	0.002831	-0.00721
26	40	0.5	0.004718	-0.01098
27	39	0.4	0.0046	-0.01075
	8478			1.312544

Lane: 7

Peak	Area	%	Pi	H'
1	371	13.2	0.132123	-0.11614
2	136	4.8	0.048433	-0.06368
3	270	9.6	0.096154	-0.09779
4	165	5.9	0.058761	-0.07233
5	199	7.1	0.070869	-0.08147
6	151	5.4	0.053775	-0.06826
7	122	4.3	0.043447	-0.05918
8	232	8.3	0.082621	-0.08947
9	144	5.1	0.051282	-0.06616
10	78	2.8	0.027778	-0.04323
11	62	2.2	0.02208	-0.03656
12	100	3.6	0.035613	-0.05158
13	29	1	0.010328	-0.02051
14	46	1.6	0.016382	-0.02925
15	92	3.3	0.032764	-0.04864
16	55	2	0.019587	-0.03346
17	72	2.6	0.025641	-0.0408
18	64	2.3	0.022792	-0.03743

19	87	3.1	0.030983	-0.04675
20	115	4.1	0.040954	-0.05683
21	42	1.5	0.014957	-0.0273
22	61	2.2	0.021724	-0.03613
23	55	2	0.019587	-0.03346
24	23	0.8	0.008191	-0.01709
25	37	1.2	0.013177	-0.02477
	2808			1.298267
Lane: 8				
Peak	Area	%	Pi	H'
1	856	13.8	0.138198	-0.11878
2	369	6	0.059574	-0.07297
3	262	4.2	0.042299	-0.0581
4	146	2.4	0.023571	-0.03836
5	111	1.8	0.017921	-0.0313
6	126	2	0.020342	-0.03441
7	149	2.4	0.024056	-0.03894
8	165	2.7	0.026639	-0.04194
9	88	1.4	0.014207	-0.02625
10	243	3.9	0.039232	-0.05517
11	122	2	0.019696	-0.03359
12	129	2.1	0.020827	-0.03502
13	133	2.1	0.021472	-0.03582
14	124	2	0.020019	-0.034
15	228	3.7	0.03681	-0.05279
16	258	4.2	0.041653	-0.0575
17	127	2.1	0.020504	-0.03461
18	167	2.7	0.026962	-0.04231
19	181	2.9	0.029222	-0.04483
20	211	3.4	0.034065	-0.05
21	153	2.5	0.024701	-0.0397
22	508	8.2	0.082015	-0.08908
23	436	7	0.070391	-0.08112
24	127	2.1	0.020504	-0.03461

25	198	3.2	0.031966	-0.0478
26	152	2.5	0.02454	-0.03951
27	200	3.2	0.032289	-0.04814
28	118	1.9	0.019051	-0.03277
29	107	1.6	0.017275	-0.03045
	6194			1.379902
Lane: 9				
Peak	Area	%	Pi	H'
1	656	9.9	0.099229	-0.09956
2	487	7.4	0.073665	-0.08344
3	229	3.5	0.034639	-0.05059
4	153	2.3	0.023143	-0.03785
5	88	1.3	0.013311	-0.02497
6	198	3	0.02995	-0.04563
7	200	3	0.030253	-0.04596
8	118	1.8	0.017849	-0.03121
9	275	4.2	0.041597	-0.05744
10	339	5.1	0.051278	-0.06615
11	162	2.5	0.024505	-0.03947
12	299	4.5	0.045228	-0.06081
13	154	2.3	0.023295	-0.03803
14	256	3.9	0.038723	-0.05468
15	207	3.1	0.031311	-0.0471
16	224	3.4	0.033883	-0.04981
17	269	4.1	0.04069	-0.05658
18	257	3.9	0.038875	-0.05483
19	444	6.7	0.067161	-0.07877
20	398	6	0.060203	-0.07347
21	151	2.3	0.022841	-0.03749
22	253	3.8	0.03827	-0.05423
23	176	2.7	0.026622	-0.04192
24	333	5	0.050371	-0.06537
25	196	3	0.029648	-0.0453
26	89	1.3	0.013462	-0.02519
	6611			1.365871

Lane: 10

Peak	Area	%	Pi	H'
1	620	10.8	0.108411	-0.10461
2	266	4.7	0.046512	-0.06197
3	409	7.2	0.071516	-0.08193
4	161	2.8	0.028152	-0.04365
5	125	2.2	0.021857	-0.03629
6	341	6	0.059626	-0.07302
7	115	2	0.020108	-0.03412
8	133	2.3	0.023256	-0.03799
9	294	5.1	0.051408	-0.06626
10	226	4	0.039517	-0.05545
11	107	1.9	0.01871	-0.03233
12	188	3.3	0.032873	-0.04876
13	152	2.7	0.026578	-0.04187
14	544	9.5	0.095122	-0.09719
15	406	7.1	0.070991	-0.08155
16	437	7.6	0.076412	-0.08534
17	257	4.5	0.044938	-0.06055
18	252	4.4	0.044064	-0.05975
19	316	5.5	0.055254	-0.06949
20	211	3.7	0.036895	-0.05287
21	159	2.7	0.027802	-0.04326
	5719			1.26824

Lane: 11

Peak	Area	%	Pi	H'
1	636	7.8	0.078066	-0.08646
2	353	4.3	0.043329	-0.05907
3	318	3.9	0.039033	-0.05498
4	649	8	0.079661	-0.08753
5	704	8.6	0.086412	-0.09189
6	401	4.9	0.049221	-0.06437
7	264	3.2	0.032405	-0.04826
8	142	1.7	0.01743	-0.03065
9	276	3.4	0.033878	-0.0498
10	170	2.1	0.020867	-0.03507
11	275	3.4	0.033755	-0.04968
12	299	3.7	0.036701	-0.05268
13	453	5.6	0.055603	-0.06978
14	303	3.7	0.037192	-0.05317
15	294	3.6	0.036087	-0.05206
16	295	3.6	0.03621	-0.05218
17	276	3.4	0.033878	-0.0498
18	520	6.4	0.063827	-0.07627
19	334	4.1	0.040997	-0.05687
20	549	6.7	0.067387	-0.07894
21	537	6.6	0.065914	-0.07785
22	99	1.3	0.012152	-0.02327
	8147			1.30064

Lane: 12

Peak	Area	%	Pi	H'
1	500	19.7	0.196696	-0.13891
2	191	7.5	0.075138	-0.08447
3	136	5.4	0.053501	-0.06803
4	570	22.4	0.224233	-0.14559
5	167	6.6	0.065696	-0.07768
6	53	2.1	0.02085	-0.03505
7	86	3.4	0.033832	-0.04976
8	154	6.1	0.060582	-0.07377
9	173	6.8	0.068057	-0.07943
10	167	6.6	0.065696	-0.07768
11	261	10.3	0.102675	-0.1015
12	84	3.1	0.033045	-0.04894
	2542			0.980803

Binary matrix for PCA analysis

2004						2005					
2	3	4	5	14	23	3	5	7	9.3	12.4	16.4
0	0	0	0.054	0	0	0	0.138	0.099	0.108	0.078	0.197
0	0	0	0	0	0.05	0.132	0	0	0	0	0
0	0	0	0	0.062	0	0	0	0	0	0	0
0.073	0	0	0	0	0	0	0	0	0	0	0
0	0	0.06	0	0	0	0	0	0	0	0	0
0	0	0	0	0.031	0	0	0	0	0	0	0
0	0	0	0.058	0	0	0.048	0	0	0	0	0
0	0	0	0	0.025	0	0.096	0	0	0	0	0
0	0.046	0	0.026	0	0	0	0	0	0	0.043	0
0.014	0	0.024	0	0	0	0.059	0	0	0	0	0
0.015	0	0	0.076	0.065	0	0.071	0.06	0.074	0	0	0
0	0.028	0	0	0	0	0	0	0	0	0	0
0.045	0	0	0.051	0	0.057	0	0	0	0.047	0	0.075
0	0	0	0	0	0	0.054	0.042	0.035	0	0.039	0
0	0.069	0	0	0	0	0	0	0	0	0	0
0.021	0.025	0.02	0.03	0.02	0.056	0.043	0	0	0.072	0.08	0
0	0	0.015	0	0	0	0	0	0	0	0	0.054
0.023	0.121	0	0.053	0.059	0	0.083	0	0	0	0	0
0	0	0	0	0	0	0	0.024	0.023	0.028	0.086	0.224
0	0	0	0	0	0.027	0	0	0	0	0	0
0.048	0	0	0	0	0	0.051	0.018	0.013	0.022	0	0.066
0	0	0.105	0	0	0	0	0	0	0	0	0
0	0	0	0.148	0.041	0.062	0.028	0.02	0.03	0	0.049	0
0	0	0	0	0	0	0	0	0	0.06	0	0
0	0	0	0	0	0	0	0	0	0	0.032	0
0.054	0	0.043	0	0	0	0	0	0	0	0	0.021
0.016	0	0.029	0	0	0	0.022	0.024	0.03	0.02	0	0.034
0	0	0	0	0	0	0	0	0.018	0	0	0
0	0	0.022	0	0.018	0	0.036	0	0	0.023	0.017	0
0.016	0.031	0	0	0	0	0	0	0	0	0	0
0	0	0	0	0.018	0	0	0	0	0	0	0
0	0.024	0	0	0	0.04	0	0	0	0	0	0
0	0.014	0.031	0.046	0.034	0.058	0.01	0.027	0.042	0.051	0	0.061
0	0	0	0	0.015	0	0	0.014	0	0	0	0
0	0.027	0	0	0	0	0	0	0	0	0	0
0	0	0	0	0	0	0	0.039	0	0	0	0
0.042	0	0	0	0.02	0.017	0.016	0	0.051	0.04	0.034	0
0.016	0	0	0	0	0	0	0.02	0.025	0.019	0	0.068
0	0	0.014	0	0	0.042	0	0.021	0.045	0	0	0
0	0	0	0	0	0	0	0.021	0	0	0	0
0	0	0.017	0	0	0	0	0	0	0	0	0
0.016	0.063	0	0.037	0.016	0	0	0	0	0	0	0
0	0.057	0	0	0.02	0	0.033	0	0	0.033	0.021	0
0.064	0	0.02	0.012	0	0	0	0.02	0.023	0	0.034	0
0	0	0	0	0.019	0	0	0	0	0	0	0

Binary matrix for PCA analysis

2004						2005					
0	0	0	0	0	0.016	0.02	0	0	0.027	0	0
0	0	0	0	0.037	0	0	0	0	0	0	0
0.082	0	0	0	0	0	0	0	0	0	0	0
0	0.07	0.063	0	0	0	0	0.037	0.039	0	0	0
0.035	0	0	0.044	0.018	0.029	0.026	0.042	0.031	0	0.037	0
0.083	0	0	0	0	0	0	0	0	0	0	0
0	0.045	0	0	0	0	0	0	0	0	0	0
0	0	0.025	0	0.049	0.151	0	0	0	0.095	0.056	0.066
0	0	0	0	0	0	0	0.021	0	0	0	0
0	0	0.052	0	0	0	0	0.027	0.034	0	0.037	0
0.056	0.051	0.065	0.014	0	0	0	0	0	0	0	0
0	0.048	0	0	0.032	0.027	0	0.029	0.041	0.071	0.036	0.103
0.036	0	0	0	0	0	0	0	0	0	0	0
0	0	0.045	0.025	0	0	0.023	0.034	0.039	0	0.036	0
0	0.025	0	0	0	0	0	0	0	0	0	0
0	0	0	0	0.027	0	0	0	0	0	0	0
0	0	0	0.028	0	0	0	0	0	0	0.034	0
0	0	0	0.017	0.025	0.036	0	0.025	0	0	0	0
0.049	0.041	0.025	0	0	0	0.031	0	0	0.076	0	0
0	0	0	0	0	0	0	0	0	0	0.064	0
0	0.023	0	0.022	0	0	0	0	0	0	0	0
0	0	0.017	0	0	0	0	0	0	0	0	0
0	0	0	0.009	0	0	0	0	0	0	0	0
0.023	0.026	0.026	0	0	0	0	0	0	0	0.041	0
0	0	0	0.068	0	0	0	0	0	0	0	0
0.02	0.022	0.026	0	0.052	0.059	0	0.082	0.067	0.045	0	0
0	0	0	0	0	0	0.041	0	0	0	0	0
0	0	0	0	0	0	0	0	0	0	0	0.033
0	0	0.051	0.011	0.044	0.076	0.015	0.07	0.06	0	0.067	0
0	0.037	0	0	0	0	0	0	0	0	0	0
0.023	0	0	0.016	0	0	0	0.021	0.023	0	0	0
0.028	0	0.064	0	0.022	0	0	0	0	0.044	0.066	0
0	0	0.031	0	0.027	0	0	0.032	0.038	0	0	0
0	0	0	0.048	0	0	0	0	0	0.055	0	0
0.047	0.056	0	0	0.027	0.026	0	0	0	0	0	0
0	0	0.02	0	0	0	0	0	0	0	0	0
0	0.023	0	0.021	0	0	0	0	0	0	0	0
0	0	0	0	0.024	0.024	0.022	0.025	0.027	0.037	0	0
0.025	0.018	0.016	0.025	0.071	0.02	0	0	0	0	0	0
0	0	0.02	0	0	0.02	0	0.032	0.05	0	0	0
0.029	0	0	0.052	0	0	0	0	0	0	0	0
0	0	0	0	0.039	0	0	0	0	0	0	0
0	0	0	0	0	0.032	0	0	0	0	0	0
0.032	0	0.041	0	0	0	0	0	0	0	0	0
0	0	0	0	0.011	0	0	0	0	0	0	0
0	0	0	0	0	0.03	0	0	0	0	0	0
0.025	0	0	0	0	0	0	0	0	..0	0	0

Binary matrix for PCA analysis

	2004						2005					
0	0	0	0	0	0.029	0	0	0	0	0	0	0
0	0	0	0	0.018	0	0	0	0	0	0	0	0
0	0.012	0	0	0	0	0	0	0	0	0	0	0
0	0	0	0.01	0	0	0.02	0.019	0.03	0	0	0	0
0	0	0.002	0	0.01	0	0	0	0	0	0	0	0
0	0	0	0	0.002	0	0.008	0	0	0	0	0	0
0	0	0	0	0	0	0.013	0.017	0.013	0	0	0	0
0	0	0	0	0	0.004	0	0	0	0.028	0	0	0
0	0	0	0	0	0.003	0	0	0	0	0	0	0
0	0	0	0	0	0.005	0	0	0	0	0.012	0	0
0	0	0	0	0	0.005	0	0	0	0	0	0	0
0	0	0.01	0	0	0	0	0	0	0	0	0	0

8.2 DGGE analysis of bacterial species from Buffet farm biofilms (from 2004 – 2005)

Shannon-Weaver calculations

Lane 1

Peak	Area	%	Pi	H'
1	178	2.6	0.02602	-0.04123
2	120	1.8	0.017541	-0.0308
3	423	6.2	0.061833	-0.07474
4	731	10.7	0.106856	-0.10378
5	163	2.4	0.023827	-0.03867
6	348	5.1	0.05087	-0.0658
7	306	4.5	0.04473	-0.06036
8	299	4.4	0.043707	-0.05942
9	1255	18.3	0.183453	-0.13511
10	326	4.8	0.047654	-0.06299
11	196	2.9	0.028651	-0.0442
12	678	9.9	0.099108	-0.09949
13	1277	18.7	0.186669	-0.13607
14	288	4.2	0.042099	-0.05792
15	253	3.5	0.036983	-0.05296
	6841			1.063549

Lane 2

Peak	Area	%	Pi	H'
1	352	4.9	0.049224	-0.06438
2	249	3.5	0.03482	-0.05077
3	207	2.9	0.028947	-0.04453
4	195	2.7	0.027269	-0.04266
5	240	3.4	0.033562	-0.04948
6	343	4.8	0.047965	-0.06327
7	1392	19.5	0.194658	-0.13835
8	200	2.8	0.027968	-0.04344
9	1476	20.6	0.206405	-0.14145
10	365	5.1	0.051042	-0.06595
11	331	4.6	0.046287	-0.06177
12	274	3.8	0.038316	-0.05428
13	228	3.2	0.031884	-0.04771
14	285	4	0.039855	-0.05578
15	334	4.7	0.046707	-0.06215
16	106	1.5	0.014823	-0.02711
17	218	3	0.030485	-0.04621
18	356	5	0.049783	-0.06486
	7151			1.124151

Lane 3

Peak	Area	%	Pi	H'
1	580	8.6	0.08649	-0.09194
2	329	4.9	0.049061	-0.06423
3	74	1.1	0.011035	-0.0216
4	205	3.1	0.03057	-0.0463
5	177	2.6	0.026394	-0.04166
6	124	1.8	0.018491	-0.03205
7	469	7	0.069937	-0.0808
8	300	4.5	0.044736	-0.06036
9	622	9.3	0.092753	-0.09578
10	598	8.9	0.089174	-0.09361
11	232	3.5	0.034596	-0.05054
12	471	7	0.070236	-0.08101
13	159	2.4	0.02371	-0.03853
14	81	1.2	0.012079	-0.02317
15	1227	18.3	0.18297	-0.13496
16	312	4.7	0.046525	-0.06199
17	165	2.5	0.024605	-0.03959
18	57	0.8	0.0085	-0.0176
19	165	2.5	0.024605	-0.03959
20	63	0.9	0.009395	-0.01904
21	296	4.4	0.04414	-0.05982
	6706			1.194183

Lane 4

Peak	Area	%	Pi	H'
1	500	5.6	0.056256	-0.07031
2	299	3.4	0.033641	-0.04956
3	482	5.4	0.05423	-0.06864

	Area	%	Pi	H'
4	219	2.5	0.02464	-0.03963
5	183	2.1	0.02059	-0.03472
6	391	4.4	0.043992	-0.05968
7	166	1.9	0.018677	-0.03229
8	268	3	0.030153	-0.04585
9	517	5.8	0.058168	-0.07186
10	203	2.3	0.02284	-0.03749
11	202	2.3	0.022727	-0.03735
12	254	2.9	0.028578	-0.04412
13	1389	15.6	0.156278	-0.12598
14	227	2.6	0.02554	-0.04068
15	103	1.2	0.011589	-0.02244
16	534	6	0.060081	-0.07337
17	507	5.7	0.057043	-0.07095
18	133	1.5	0.014964	-0.02731
19	461	5.2	0.051868	-0.06666
20	331	3.7	0.037241	-0.05322
21	223	2.5	0.02509	-0.04016
22	112	1.3	0.012601	-0.02394
23	127	1.4	0.014289	-0.02636
24	254	2.9	0.028578	-0.04412
25	289	3.3	0.032516	-0.04838
26	239	2.7	0.02689	-0.04223
27	182	2	0.020477	-0.03458
28	93	0.8	0.010464	-0.02072
	8888			1.352586

Lane 5

Peak	Area	%	Pi	H'
1	670	6.8	0.067574	-0.07908
2	332	3.3	0.033485	-0.04939
3	2007	20.2	0.202421	-0.14043
4	349	3.5	0.035199	-0.05116
5	232	2.3	0.023399	-0.03816
6	359	3.6	0.036208	-0.05218
7	628	6.3	0.063338	-0.0759
8	266	2.7	0.026828	-0.04216

	Area	%	Pi	H'
9	248	2.5	0.025013	-0.04007
10	244	2.5	0.024609	-0.03959
11	819	8.3	0.082602	-0.08946
12	680	6.9	0.068583	-0.07982
13	162	1.6	0.016339	-0.02919
14	282	2.8	0.028442	-0.04397
15	113	1.1	0.011397	-0.02215
16	206	2.1	0.020777	-0.03496
17	170	1.7	0.017146	-0.03028
18	157	1.6	0.015835	-0.02851
19	319	3.2	0.032173	-0.04802
20	426	4.3	0.042965	-0.05873
21	481	4.9	0.048512	-0.06375
22	229	2.3	0.023096	-0.0378
23	536	5.5	0.05406	-0.0685
	9915			1.243245

Lane 6

Peak	Area	%	Pi	H'
1	726	6.8	0.068175	-0.07952
2	222	2.1	0.020847	-0.03504
3	688	6.5	0.064607	-0.07686
4	192	1.8	0.01803	-0.03144
5	136	1.3	0.012771	-0.02419
6	492	4.6	0.046202	-0.06169
7	687	6.5	0.064513	-0.07679
8	953	8.9	0.089492	-0.09381
9	105	1	0.00986	-0.01978
10	167	1.6	0.015682	-0.0283
11	319	3	0.029956	-0.04564
12	937	8.8	0.087989	-0.09288
13	900	8.5	0.084515	-0.09069
14	214	2	0.020096	-0.0341
15	896	8.4	0.084139	-0.09045
16	155	1.5	0.014555	-0.02674
17	172	1.6	0.016152	-0.02894
18	190	1.8	0.017842	-0.0312

Peak	Area	%	Pi	H'
19	219	2.1	0.020565	-0.03469
20	365	3.4	0.034276	-0.05021
21	747	7	0.070147	-0.08095
22	686	6.4	0.064419	-0.07672
23	185	1.7	0.017373	-0.03058
24	296	2.7	0.027796	-0.04325
	10649			1.28447

Lane 7

Peak	Area	%	Pi	H'
1	760	5.3	0.053435	-0.06798
2	1089	7.7	0.076566	-0.08544
3	419	2.9	0.029459	-0.0451
4	315	2.2	0.022147	-0.03665
5	808	5.7	0.056809	-0.07076
6	383	2.7	0.026928	-0.04227
7	229	1.6	0.016101	-0.02887
8	780	5.5	0.054841	-0.06915
9	419	2.9	0.029459	-0.0451
10	517	3.6	0.03635	-0.05233
11	562	4	0.039513	-0.05545
12	283	2	0.019897	-0.03385
13	601	4.2	0.042256	-0.05806
14	252	1.8	0.017718	-0.03103
15	190	1.3	0.013359	-0.02504
16	843	5.9	0.05927	-0.07273
17	177	1.2	0.012445	-0.02371
18	857	6	0.060255	-0.07351
19	289	2	0.020319	-0.03438
20	223	1.6	0.015679	-0.0283
21	717	5	0.050411	-0.06541
22	363	2.6	0.025522	-0.04066
23	617	4.3	0.04338	-0.05911
24	377	2.7	0.026506	-0.04179
25	551	3.9	0.03874	-0.05469
26	807	5.7	0.056739	-0.0707
27	302	2.1	0.021233	-0.03552

Peak	Area	%	Pi	H'
28	493	3.6	0.034662	-0.05061
	14223			1.398206

Lane 8

Peak	Area	%	Pi	H'
1	1627	9.9	0.099135	-0.09951
2	1526	9.3	0.092981	-0.09592
3	431	2.6	0.026261	-0.04151
4	315	1.9	0.019193	-0.03295
5	589	3.6	0.035888	-0.05186
6	413	2.5	0.025165	-0.04024
7	234	1.4	0.014258	-0.02632
8	475	2.9	0.028942	-0.04453
9	646	3.9	0.039361	-0.0553
10	455	2.8	0.027724	-0.04317
11	320	1.9	0.019498	-0.03334
12	265	1.6	0.016147	-0.02893
13	834	5.1	0.050816	-0.06576
14	438	2.7	0.026688	-0.042
15	834	5.1	0.050816	-0.06576
16	651	4	0.039666	-0.0556
17	658	4	0.040093	-0.05601
18	1118	6.8	0.068121	-0.07948
19	1084	6.6	0.066049	-0.07795
20	666	4.1	0.04058	-0.05647
21	1193	7.3	0.072691	-0.08276
22	612	3.7	0.03729	-0.05327
23	572	3.5	0.034853	-0.05081
24	456	2.8	0.027785	-0.04324
	16412			1.322669

Lane 9

Peak	Area	%	Pi	H'
1	408	7.1	0.070649	-0.08131
2	646	11.2	0.111861	-0.10642
3	431	7.5	0.074632	-0.08412
4	177	3.1	0.030649	-0.04639
5	135	2.3	0.023377	-0.03813

Peak	Area	%	Pi	H'
6	167	2.9	0.028918	-0.0445
7	396	6.9	0.068571	-0.07981
8	498	8.6	0.086234	-0.09178
9	352	6.1	0.060952	-0.07406
10	351	6.1	0.060779	-0.07392
11	425	7.4	0.073593	-0.08339
12	654	11.3	0.113247	-0.10713
13	252	4.4	0.043636	-0.05935
14	235	4.1	0.040693	-0.05658
15	407	7	0.070476	-0.08119
16	241	4	0.041732	-0.05757
	5775			1.165644
Lane 10				
Peak	Area	%	Pi	H'
1	199	5.3	0.052883	-0.06752
2	536	14.2	0.14244	-0.12056
3	173	4.6	0.045974	-0.06149
4	165	4.4	0.043848	-0.05955
5	152	4	0.040393	-0.0563
6	223	5.9	0.059261	-0.07273
7	131	3.5	0.034813	-0.05077
8	241	6.4	0.064045	-0.07644
9	111	2.9	0.029498	-0.04514
10	73	1.9	0.019399	-0.03322
11	428	11.4	0.113739	-0.10738
12	294	7.8	0.078129	-0.0865
13	44	1.2	0.011693	-0.02259
14	44	1.2	0.011693	-0.02259
15	129	3.4	0.034281	-0.05022
16	50	1.3	0.013287	-0.02493
17	59	1.6	0.015679	-0.0283
18	98	2.6	0.026043	-0.04126
19	105	2.8	0.027903	-0.04337
20	19	0.5	0.005049	-0.0116
21	143	3.8	0.038002	-0.05397
22	71	1.9	0.018868	-0.03253

Peak	Area	%	Pi	H'
23	123	3.3	0.032687	-0.04856
24	152	4.1	0.040393	-0.0563
	3763			1.273794
Lane 11				
Peak	Area	%	Pi	H'
1	48	1	0.010031	-0.02005
2	56	1.2	0.011703	-0.02261
3	167	3.5	0.034901	-0.05086
4	193	4	0.040334	-0.05624
5	151	3.2	0.031557	-0.04736
6	128	2.7	0.02675	-0.04207
7	412	8.6	0.086102	-0.0917
8	3	0.1	0.000627	-0.00201
9	6	0.1	0.001254	-0.00364
10	176	3.7	0.036782	-0.05276
11	34	0.7	0.007106	-0.01527
12	43	0.9	0.008986	-0.01839
13	83	1.7	0.017346	-0.03054
14	28	0.6	0.005852	-0.01307
15	15	0.3	0.003135	-0.00785
16	46	1	0.009613	-0.01939
17	51	1.1	0.010658	-0.02102
18	124	2.6	0.025914	-0.04111
19	376	7.9	0.078579	-0.08681
20	988	20.6	0.206479	-0.14146
21	450	9.4	0.094044	-0.09655
22	564	11.8	0.117868	-0.10945
23	643	13.3	0.134378	-0.11713
	4785			1.107332
Lane 12				
Peak	Area	%	Pi	H'
1	250	5.1	0.051345	-0.06621
2	127	2.6	0.026083	-0.04131
3	22	0.5	0.004518	-0.0106
4	107	2.2	0.021976	-0.03644
5	113	2.3	0.023208	-0.03793

6	139	2.9	0.028548	-0.04409
7	176	3.6	0.036147	-0.05212
8	417	8.6	0.085644	-0.09141
9	563	11.6	0.115629	-0.10834
10	301	6.2	0.06182	-0.07473
11	334	6.9	0.068597	-0.07983
12	418	8.6	0.085849	-0.09154
13	329	6.8	0.06757	-0.07907
14	286	5.9	0.058739	-0.07231
15	492	10.1	0.101047	-0.10059
16	444	9.1	0.091189	-0.09484
17	351	7	0.072089	-0.08233
	4869			1.163685

Lane 13				
Peak	Area	%	Pi	H'
1	611	5.9	0.05857	-0.07218
2	130	1.2	0.012462	-0.02373
3	672	6.4	0.064417	-0.07672
4	566	5.4	0.054256	-0.06866
5	320	3.1	0.030675	-0.04642
6	278	2.7	0.026649	-0.04195
7	231	2.2	0.022143	-0.03664
8	592	5.7	0.056748	-0.07071
9	864	8.3	0.082822	-0.0896
10	226	2.2	0.021664	-0.03605
11	276	2.6	0.026457	-0.04173
12	303	2.9	0.029045	-0.04464
13	292	2.8	0.027991	-0.04347
14	546	5.2	0.052339	-0.06706
15	314	3	0.0301	-0.04579
16	794	7.6	0.076112	-0.08513
17	396	3.8	0.03796	-0.05393
18	454	4.4	0.04352	-0.05924
19	339	3.2	0.032496	-0.04836
20	819	7.9	0.078508	-0.08676
21	366	3.5	0.035084	-0.05104

22	836	8	0.080138	-0.08784
23	207	2	0.019843	-0.03378
	10432			1.311464

Binary matrix for PCA analysis

	2004							2005					
2	3	5	6	7	16	25	9.3	12.4	16.4	19.4	21.4	23.4
0	0	0	0	0	0	0	0.099	0	0	0	0	0
0	0	0	0	0	0.068	0	0	0	0	0	0	0
0	0	0.086	0.056	0.068	0	0	0	0	0.053	0	0	0
0	0	0	0	0	0.021	0	0	0	0	0	0	0
0	0	0	0	0	0	0.053	0.093	0.071	0	0	0.051	0
0	0	0	0	0	0	0	0	0	0	0	0	0.059
0.026	0	0.049	0	0	0	0.077	0	0	0.142	0	0	0
0	0	0	0	0	0	0	0	0	0	0	0	0.012
0	0	0	0.034	0	0	0	0	0.112	0	0	0.026	0
0	0	0	0	0	0	0	0	0	0	0	0.005	0
0	0	0	0.054	0.033	0	0.029	0	0.075	0.046	0	0	0.064
0	0.049	0	0	0	0	0	0.026	0	0	0	0	0
0	0	0	0.025	0	0.065	0.022	0	0	0	0	0	0
0	0	0	0	0	0	0	0	0.044	0	0	0	0
0	0	0	0	0	0	0	0.019	0.031	0	0	0	0.054
0.018	0	0.011	0	0	0	0.057	0	0	0	0	0	0
0	0	0	0	0	0	0	0.036	0	0	0	0	0
0	0	0	0	0	0	0.027	0	0	0	0	0	0
0	0	0	0	0	0.018	0	0	0	0	0	0	0
0	0	0.031	0	0	0.013	0	0	0	0.040	0	0	0
0	0	0	0	0.202	0	0.016	0	0	0	0	0	0.031
0	0	0.026	0	0	0	0	0.025	0	0	0	0	0
0	0	0	0	0.035	0	0	0	0	0	0	0	0
0	0.035	0	0	0	0	0	0	0	0	0	0	0
0	0	0.018	0.021	0	0	0	0	0	0	0	0	0.027
0	0	0	0	0	0.046	0	0	0	0	0	0	0
0	0	0	0	0.023	0	0	0.014	0	0	0	0	0.022
0	0	0	0	0	0.065	0	0	0	0	0	0	0
0	0	0.070	0.044	0	0.089	0.055	0	0	0	0	0	0
0	0.029	0	0	0	0	0	0	0	0	0	0	0
0	0.027	0.045	0.019	0.036	0.010	0	0.029	0	0.059	0.010	0	0
0	0	0.093	0.030	0.063	0	0.029	0.039	0	0.035	0	0	0.057

Binary matrix for PCA analysis

	2004							2005					
0	0	0	0	0	0.016	0	0	0	0	0	0	0
0	0	0.089	0	0	0	0	0	0	0	0	0	0
0	0	0	0	0	0	0.036	0	0	0	0	0	0
0	0	0.035	0	0	0	0	0.023	0	0	0	0	0
0.062	0	0	0.058	0.027	0.030	0	0.028	0	0.064	0	0	0.083
0	0	0	0	0	0	0.040	0	0.029	0	0.012	0	0
0	0	0	0	0	0	0	0.019	0	0	0	0	0
0	0.034	0	0.023	0.025	0	0.020	0	0	0	0	0	0
0	0	0	0	0	0	0	0.016	0	0	0	0	0
0	0	0	0	0	0.088	0	0	0	0	0	0	0
0	0	0.070	0	0	0	0	0	0	0	0.035	0	0
0	0	0	0.023	0	0.085	0.042	0	0	0.029	0.040	0	0
0	0	0	0	0	0	0	0	0.069	0.019	0.032	0	0.022
0	0	0	0	0	0.020	0	0.051	0	0	0	0	0
0	0	0	0	0	0.025	0	0	0	0	0	0	0
0	0	0	0	0	0	0.018	0	0	0	0	0	0
0	0	0.024	0	0	0	0	0	0	0	0	0	0
0	0	0	0	0	0	0	0	0	0	0	0	0.026
0	0	0	0	0	0	0	0	0	0	0	0.022	0
0	0	0	0.029	0	0	0	0	0	0	0	0	0
0	0	0	0	0	0	0	0.027	0	0	0	0	0
0	0.048	0	0	0	0	0	0	0	0	0	0	0
0	0	0.012	0	0	0	0	0	0	0	0	0	0
0	0	0	0	0	0	0	0	0	0	0.027	0	0
0	0	0	0	0	0	0	0	0	0	0	0	0.029
0	0	0	0	0	0	0.013	0	0	0	0	0	0
0.107	0.195	0.183	0.156	0.083	0.084	0.059	0.051	0.086	0	0.086	0.023	0.028
0	0	0	0	0	0	0	0	0	0	0.001	0	0
0	0	0	0	0.069	0	0	0	0	0	0	0	0
0.024	0.028	0	0.026	0	0	0	0	0	0	0	0	0.052
0	0	0	0	0	0	0.012	0	0	0	0	0	0
0	0	0	0.012	0	0	0	0	0	0	0	0	0
0	0	0	0	0	0	0	0.040	0	0.114	0.001	0	0
0	0	0	0	0	0.015	0	0	0	-0	0	0	0

Binary matrix for PCA analysis (left)

2004							2005					
0	0	0	0	0	0	0	0	0	0	0	0	0.030
0	0	0	0	0	0	0	0	0	0.078	0	0	0
0.051	0.206	0.047	0.060	0.016	0.016	0.060	0.040	0.061	0	0.037	0.029	0
0	0.051	0	0	0	0	0.020	0	0	0	0	0	0
0	0	0	0	0	0	0	0	0	0.012	0	0	0
0	0	0	0.057	0	0	0	0	0	0	0	0	0
0	0.046	0.025	0	0.028	0	0.016	0	0.061	0	0.007	0.036	0.076
0.045	0	0	0.015	0	0.018	0.050	0	0	0	0.009	0	0
0	0	0	0	0	0	0.026	0	0	0.012	0	0	0
0	0	0	0	0	0.021	0	0	0	0	0	0	0
0	0.038	0	0.052	0.011	0	0.043	0.068	0.074	0	0	0.086	0.038
0	0	0	0	0	0	0	0	0	0	0	0	0.044
0	0	0	0	0	0	0	0	0	0.034	0	0	0
0	0	0	0	0	0	0	0	0	0	0.017	0	0
0.044	0.032	0.008	0	0	0	0	0	0	0	0	0	0
0	0	0	0	0.021	0	0	0	0	0	0	0	0
0	0	0.025	0.037	0	0.034	0	0	0.113	0	0	0	0
0	0	0	0	0	0	0	0.066	0	0	0	0	0
0.183	0	0	0	0.017	0	0.027	0	0	0	0.006	0.116	0.032
0	0	0	0	0	0	0	0	0	0	0.003	0	0
0	0	0	0	0	0	0	0	0	0	0	0.062	0
0	0.040	0.009	0	0.016	0	0	0	0	0.013	0	0	0.079
0	0	0	0	0	0	0	0	0	0	0	0.069	0
0	0	0	0	0	0	0	0	0	0	0.010	0	0
0	0	0	0.025	0	0	0	0	0	0	0	0	0
0	0	0	0	0	0.070	0.039	0.041	0	0.016	0	0	0
0.048	0	0	0	0	0	0	0	0	0	0	0	0
0	0	0	0.013	0	0	0	0	0	0	0.011	0	0
0	0	0	0	0.032	0	0	0	0.044	0	0	0.086	0
0	0.047	0	0.014	0	0	0	0	0	0	0	0	0
0.029	0	0	0	0	0	0	0.073	0.041	0	0.026	0.068	0.035
0	0	0	0.029	0	0	0	0	0	0	0	0.059	0
0	0	0	0	0	0	0	0	0	0.026	0	0	0
0	0	0	0	0.043	0	0	0	0	0	0	0	0

Binary matrix for PCA analysis (right)

2004							2005					
0	0	0	0	0	0	0	0	0	0	0.079	0	0
0	0.015	0.044	0.033	0	0.064	0.057	0	0	0	0	0	0
0	0	0	0	0	0	0	0	0	0	0	0.101	0
0	0.030	0	0	0	0.017	0	0.000	0	0	0	0	0
0	0	0	0	0	0	0	0.037	0.070	0	0	0.091	0.080
0	0	0	0	0	0	0	0	0	0	0.206	0	0
0.099	0	0	0.027	0	0.028	0.021	0.035	0	0	0	0	0
0.187	0	0	0	0	0	0	0	0	0.028	0	0	0
0	0.050	0	0.020	0.049	0	0.035	0	0	0.005	0	0	0
0	0	0	0	0	0	0	0	0	0.094	0	0	0
0.042	0	0	0	0	0	0	0	0.028	0	0	0	0
0	0	0	0.010	0.023	0	0	0	0	0	0	0	0
0.037	0	0	0	0	0	0	0	0	0	0.118	0	0
0	0	0	0	0	0	0	0	0	0	0	0	0.020
0	0	0	0	0	0	0	0	0	0	0	0.072	0
0	0	0	0	0	0	0	0	0	0	0.134	0	0
0	0	0	0	0	0	0	0	0	0.038	0	0	0
0	0	0	0	0	0	0	0	0	0.019	0	0	0
0	0	0	0	0	0	0	0	0	0.033	0	0	0
0	0	0	0	0.054	0	0	0	0	0	0	0	0
0	0	0	0	0	0	0	0	0.042	0.040	0	0	0

8.3 DGGE analysis of bacterial species from Buffet and Vesuvio wastewater biofilms isolated in the laboratory during 2004

Shannon-Weaver calculations of bacterial laboratory samples from Vesuvio

Lane 1

Peak	Area	%	Pi	H'
1	232	2.7	0.02668814	-0.042
2	548	6.3	0.06303923	-0.07567
3	610	7	0.0701714	-0.08097
4	278	3.2	0.03197975	-0.04781
5	333	3.8	0.03830668	-0.05427
6	220	2.5	0.02530772	-0.04041
7	719	8.3	0.08271023	-0.08953
8	402	4.6	0.0462441	-0.06173
9	416	4.8	0.0478546	-0.06317
10	725	8.3	0.08340044	-0.08998
11	319	3.7	0.03669619	-0.05267
12	422	4.9	0.04854481	-0.06378
13	218	2.5	0.02507765	-0.04014
14	1010	11.6	0.11618544	-0.10862
15	184	2.1	0.02116646	-0.03544
16	470	5.4	0.05406649	-0.06851
17	822	9.5	0.09455884	-0.09686
18	300	3.5	0.03451053	-0.05046
19	218	2.5	0.02507765	-0.04014
20	147	1.7	0.01691016	-0.02996
21	100	1.1	0.01150351	-0.02231
	8693			1.254422

Lane 2

Peak	Area	%	Pi	H'
1	156	3.3	0.03268385	-0.04856
2	181	3.8	0.03792164	-0.05389
3	207	4.3	0.04336895	-0.0591
4	372	7.8	0.0779384	-0.08638
5	318	6.7	0.06662476	-0.07837
6	392	8.2	0.08212864	-0.08915
7	515	10.8	0.1078986	-0.10434
8	388	8.1	0.08129059	-0.0886
9	538	11.3	0.11271737	-0.10686
10	697	14.6	0.14602975	-0.12202
11	223	4.7	0.04672114	-0.06216
12	75	1.6	0.01571339	-0.02834
13	18	0.4	0.00377121	-0.00914
14	74	1.6	0.01550388	-0.02806
15	197	4.1	0.04127383	-0.05714
16	151	3.2	0.03163629	-0.04745
17	18	0.4	0.00377121	-0.00914
18	0	0	0	0
19	4	0.1	0.00083805	-0.00258
20	25	0.5	0.0052378	-0.01195
21	38	0.8	0.00796145	-0.01671
22	62	1.3	0.01298973	-0.0245
23	19	0.4	0.00398072	-0.00955
24	38	0.8	0.00796145	-0.01671
25	67	1.2	0.01403729	-0.02601
	4773			1.186703

Lane 3

Peak	Area	%	Pi	H'
1	582	5.4	0.05420509	-0.06862
2	495	4.6	0.04610226	-0.06161
3	303	2.8	0.02822017	-0.04373
4	154	1.4	0.01434293	-0.02644
5	265	2.5	0.02468101	-0.03968
6	258	2.4	0.02402906	-0.03891
7	483	4.5	0.04498463	-0.06059
8	649	6	0.06044519	-0.07366
9	228	2.1	0.02123498	-0.03553
10	253	2.4	0.02356338	-0.03836
11	359	3.3	0.03343578	-0.04934
12	819	7.6	0.07627829	-0.08525
13	551	5.1	0.05131787	-0.06619
14	423	3.9	0.03939648	-0.05533
15	1110	10.3	0.10338083	-0.10189
16	711	6.6	0.06621961	-0.07807

17	583	5.4	0.05429822	-0.0687
18	292	2.7	0.02719568	-0.04257
19	212	2	0.01974481	-0.03366
20	177	1.6	0.01648505	-0.02939
21	623	5.8	0.05802366	-0.07174
22	262	2.4	0.0244016	-0.03935
23	319	3	0.02971035	-0.04537
24	405	3.8	0.03772003	-0.05369
25	26	0.2	0.00242153	-0.00633
26	70	0.7	0.00651951	-0.01425
27	69	0.6	0.00642638	-0.01409
28	56	0.9	0.00521561	-0.01191
	10737			1.354237

Lane 4

Peak	Area	%	Pi	H'
1	516	4.5	0.04458654	-0.06023
2	179	1.5	0.01546704	-0.028
3	225	1.9	0.0194418	-0.03327
4	206	1.8	0.01780005	-0.03114
5	177	1.5	0.01529422	-0.02777
6	167	1.4	0.01443014	-0.02656
7	756	6.5	0.06532446	-0.0774
8	355	3.1	0.03067485	-0.04642
9	259	2.2	0.02237968	-0.03693
10	259	2.2	0.02237968	-0.03693
11	362	3.1	0.0312797	-0.04707
12	265	2.3	0.02289812	-0.03756
13	224	1.9	0.0193554	-0.03316
14	483	4.2	0.04173507	-0.05757
15	739	6.4	0.06385553	-0.07629
16	631	5.5	0.05452346	-0.06889
17	315	2.7	0.02721853	-0.0426
18	365	3.2	0.03153893	-0.04734
19	1048	9.1	0.0905556	-0.09446
20	505	4.4	0.04363605	-0.05935
21	545	4.7	0.04709237	-0.06249
22	539	4.7	0.04657392	-0.06203

23	530	4.6	0.04579625	-0.06133
24	359	3.1	0.03102048	-0.04679
25	313	2.7	0.02704571	-0.04241
26	444	3.8	0.03836516	-0.05433
27	211	1.8	0.01823209	-0.03171
28	395	3.4	0.03413117	-0.05007
29	123	1.1	0.01062819	-0.02098
30	32	0.3	0.00276506	-0.00707
31	46	0.4	0.00397477	-0.00954
	11573			1.417688

Lane 5

Peak	Area	%	Pi	H'
1	204	2	0.01973875	-0.03365
2	158	1.5	0.01528786	-0.02776
3	136	1.3	0.01315917	-0.02475
4	102	1	0.00986938	-0.0198
5	149	1.4	0.01441703	-0.02654
6	275	2.7	0.02660861	-0.04191
7	336	3.3	0.03251089	-0.04838
8	471	4.6	0.04557329	-0.06113
9	345	3.3	0.03338171	-0.04929
10	1198	11.6	0.11591679	-0.10848
11	399	3.9	0.03860668	-0.05456
12	222	2.1	0.02148041	-0.03583
13	582	5.6	0.0563135	-0.07036
14	569	5.5	0.05505564	-0.06933
15	796	7.7	0.07701984	-0.08575
16	1396	13.5	0.13507499	-0.11744
17	872	8.4	0.08437349	-0.0906
18	247	2.4	0.02389937	-0.03876
19	287	2.8	0.02776971	-0.04322
20	182	1.8	0.01761006	-0.03089
21	262	2.5	0.02535075	-0.04046
22	370	3.6	0.03580068	-0.05177
23	356	3.4	0.03444606	-0.05039
24	107	1	0.01035317	-0.02055
25	108	1	0.01044993	-0.0207

Peak	Area	%	Pi	H'
26	124	1.2	0.01199806	-0.02305
27	67	0.6	0.00648283	-0.01419
28	15	0.3	0.00145138	-0.00412
	10335			1.303633

Lane 6

Peak	Area	%	Pi	H'
1	133	1.3	0.01301879	-0.02455
2	204	2	0.01996868	-0.03394
3	122	1.2	0.01194205	-0.02296
4	127	1.2	0.01243148	-0.02369
5	277	2.7	0.02711433	-0.04248
6	116	1.1	0.01135474	-0.02208
7	556	5.4	0.05442443	-0.0688
8	253	2.5	0.02476507	-0.03978
9	263	2.6	0.02574393	-0.04092
10	503	4.9	0.04923649	-0.06439
11	238	2.3	0.02329679	-0.03804
12	660	6.5	0.06460454	-0.07686
13	707	6.9	0.06920517	-0.08027
14	457	4.5	0.04473375	-0.06036
15	691	6.8	0.067639	-0.07912
16	800	7.8	0.07830854	-0.08662
17	620	6.1	0.06068912	-0.07385
18	650	6.4	0.06362569	-0.07612
19	330	3.2	0.03230227	-0.04816
20	342	3.3	0.0334769	-0.04939
21	630	6.2	0.06166797	-0.07461
22	532	5.2	0.05207518	-0.06683
23	508	5	0.04972592	-0.06481
24	274	2.7	0.02682067	-0.04215
25	115	1.1	0.01125685	-0.02193
26	108	1.1	0.01057165	-0.02089
	10216			1.34361

Lane 7

Peak	Area	%	Pi	H'
1	254	2.3	0.02317095	-0.03789
2	192	1.8	0.01751505	-0.03077
3	357	3.3	0.03256705	-0.04843
4	177	1.6	0.01614669	-0.02893
5	101	0.9	0.00921365	-0.01876
6	112	1	0.01021711	-0.02034
7	273	2.5	0.02490421	-0.03994
8	422	3.8	0.03849662	-0.05446
9	292	2.7	0.02663747	-0.04194
10	197	1.8	0.01797117	-0.03137
11	263	2.4	0.02399197	-0.03887
12	350	3.2	0.03192848	-0.04776
13	962	8.8	0.08775771	-0.09273
14	340	3.1	0.03101624	-0.04679
15	654	6	0.05966065	-0.07304
16	233	2.1	0.02125525	-0.03555
17	1027	9.4	0.09368728	-0.09634
18	945	8.6	0.0862069	-0.09176
19	745	6.8	0.06796205	-0.07936
20	412	3.8	0.03758438	-0.05356
21	771	7	0.07033388	-0.08108
22	478	4.4	0.04360518	-0.05932
23	360	3.3	0.03284072	-0.04872
24	544	5	0.04962598	-0.06473
25	366	3.3	0.03338807	-0.04929
26	135	1.1	0.01231527	-0.02352
	10962			1.335246

Lane 8

Peak	Area	%	Pi	H'
1	435	5	0.05030065	-0.06531
2	147	1.7	0.01699815	-0.03008
3	127	1.5	0.01468548	-0.02692
4	281	3.2	0.03249306	-0.04836
5	304	3.5	0.03515264	-0.05111
6	239	2.8	0.02763645	-0.04307
7	408	4.7	0.04717854	-0.06257
8	1260	14.6	0.14569843	-0.12188
9	367	4.2	0.04243756	-0.05823
10	241	2.8	0.02786772	-0.04333

11	839	9.7	0.09701665	-0.09829
12	243	2.8	0.02809898	-0.04359
13	422	4.9	0.04879741	-0.064
14	493	5.7	0.0570074	-0.07092
15	783	9.1	0.09054117	-0.09445
16	282	3.3	0.0326087	-0.04848
17	200	2.3	0.02312673	-0.03783
18	334	3.9	0.03862165	-0.05458
19	393	4.5	0.04544403	-0.06101
20	404	4.7	0.046716	-0.06216
21	257	3	0.02971785	-0.04538
22	189	2.1	0.02185476	-0.03629
	8648			1.267854

Lane 9

Peak	Area	%	Pi	H'
1	189	3.1	0.03058747	-0.04632
2	131	2.1	0.02120084	-0.03548
3	249	4	0.04029778	-0.0562
4	318	5.1	0.05146464	-0.06631
5	178	2.9	0.02880725	-0.04438
6	349	5.6	0.05648163	-0.07049
7	683	11.1	0.11053569	-0.10573
8	92	1.5	0.01488914	-0.0272
9	177	2.9	0.02864541	-0.0442
10	485	7.8	0.07849167	-0.08675
11	245	4	0.03965043	-0.05558
12	346	5.6	0.05599612	-0.0701
13	443	7.2	0.07169445	-0.08206
14	575	9.3	0.09305713	-0.09597
15	210	3.4	0.03398608	-0.04992
16	481	7.8	0.07784431	-0.08631
17	310	5	0.05016993	-0.0652
18	179	2.9	0.02896909	-0.04456
19	145	2.3	0.02346658	-0.03824
20	218	3.5	0.03528079	-0.05124
21	176	2.9	0.02848357	-0.04402
	6179			1.266254

Lane 10

Peak	Area	%	Pi	H'
1	142	1.6	0.01554972	-0.02812
2	315	3.4	0.03449409	-0.05044
3	538	5.9	0.05891371	-0.07245
4	763	8.4	0.08355234	-0.09007
5	395	4.3	0.04325449	-0.059
6	647	7.1	0.07084976	-0.08145
7	422	4.6	0.04621113	-0.0617
8	228	2.5	0.02496715	-0.04001
9	192	2.1	0.02102497	-0.03526
10	208	2.3	0.02277705	-0.03741
11	548	6	0.06000876	-0.07332
12	977	10.7	0.10698642	-0.10385
13	341	3.7	0.03734122	-0.05332
14	490	5.4	0.05365747	-0.06816
15	450	4.9	0.04927727	-0.06442
16	386	4.2	0.04226894	-0.05808
17	466	5.1	0.05102935	-0.06594
18	253	2.8	0.02770477	-0.04315
19	475	5.2	0.05201489	-0.06678
20	259	2.8	0.0283618	-0.04388
21	141	1.5	0.01544021	-0.02797
22	496	5.5	0.0543145	-0.06871
	9132			1.293503

Lane 11

Peak	Area	%	Pi	H'
1	117	6.2	0.06213489	-0.07498
2	58	3.1	0.03080191	-0.04655
3	65	3.5	0.03451938	-0.05047
4	315	16.7	0.16728625	-0.1299
5	34	1.8	0.01805629	-0.03148
6	105	5.6	0.05576208	-0.06991
7	23	1.2	0.01221455	-0.02337
8	136	7.2	0.07222517	-0.08243
9	70	3.7	0.03717472	-0.05315
10	80	4.2	0.0424854	-0.05828

11	412	21.9	0.21879979	-0.1444
12	77	4.1	0.04089219	-0.05677
13	37	2	0.0196495	-0.03353
14	150	8	0.07966012	-0.08753
15	59	3.1	0.03133298	-0.04712
16	145	7.7	0.07700478	-0.08574
	1883			1.075617

Lane 12

Peak	Area	%	Pi	H'
1	150	1.3	0.01260292	-0.02394
2	391	3.3	0.03285162	-0.04873
3	417	3.5	0.03503613	-0.05099
4	273	2.3	0.02293732	-0.0376
5	113	0.9	0.0094942	-0.0192
6	214	1.8	0.01798017	-0.03138
7	273	2.3	0.02293732	-0.0376
8	191	1.6	0.01604772	-0.0288
9	419	3.5	0.03520417	-0.05117
10	554	4.7	0.0465468	-0.06201
11	409	3.4	0.03436397	-0.05031
12	870	7.3	0.07309696	-0.08305
13	679	5.7	0.05704924	-0.07095
14	375	3.2	0.03150731	-0.04731
15	1224	10.3	0.10283986	-0.10159
16	719	6	0.06041002	-0.07363
17	678	5.7	0.05696522	-0.07089
18	356	3	0.02991094	-0.04559
19	739	6.2	0.0620904	-0.07494
20	460	3.9	0.03864897	-0.05461
21	697	5.9	0.05856159	-0.07217
22	502	4.2	0.04217779	-0.05799
23	509	4.3	0.04276592	-0.05854
24	441	3.7	0.0370526	-0.05303
25	170	1.4	0.01428331	-0.02636
26	36	0.3	0.0030247	-0.00762
27	43	0.3	0.00361284	-0.00882
	11902			1.348823

Lane 13

Peak	Area	%	Pi	H'
1	200	1.9	0.01910037	-0.03283
2	288	2.8	0.02750454	-0.04292
3	368	3.5	0.03514469	-0.05111
4	210	2	0.02005539	-0.03405
5	131	1.3	0.01251074	-0.0238
6	128	1.2	0.01222424	-0.02338
7	201	1.9	0.01919587	-0.03296
8	704	6.7	0.06723331	-0.07883
9	505	4.8	0.04822844	-0.0635
10	566	5.4	0.05405405	-0.0685
11	447	4.3	0.04268933	-0.05847
12	206	2	0.01967338	-0.03357
13	477	4.6	0.04555439	-0.06111
14	538	5.1	0.05138	-0.06624
15	256	2.4	0.02444848	-0.0394
16	284	2.7	0.02712253	-0.04249
17	234	2.2	0.02234744	-0.03689
18	295	2.8	0.02817305	-0.04367
19	425	4.1	0.04058829	-0.05648
20	463	4.4	0.04421736	-0.05989
21	547	5.2	0.05223952	-0.06697
22	392	3.7	0.03743673	-0.05341
23	442	4.2	0.04221182	-0.05802
24	474	4.5	0.04526788	-0.06085
25	320	3.1	0.0305606	-0.04629
26	158	1.5	0.01508929	-0.02748
27	202	1.9	0.01929138	-0.03308
28	297	2.8	0.02836405	-0.04389
29	377	3.6	0.0360042	-0.05198
30	123	1.2	0.01174673	-0.02267
31	30	0.3	0.00286506	-0.00729
32	62	0.6	0.00592112	-0.01319
33	66	0.6	0.00630312	-0.01387
34	35	0.3	0.00334257	-0.00828
35	20	0.4	0.00191004	-0.00519

	10471			1.462551

Binary matrix for PCA of bacterial laboratory samples from Vesuvio

1	3	4	5	8	10	12	14	16	18	21	23	27
0	0	0	0	0	0	0	0	0	0	0	0	0.019
0	0	0	0	0	0	0	0.05	0	0	0	0	0
0	0	0	0	0	0	0	0	0	0	0	0	0.028
0	0	0	0	0	0	0	0	0.031	0	0	0	0
0	0	0.054	0.045	0	0	0	0	0	0	0	0	0.035
0	0	0	0.015	0	0	0	0	0	0	0	0	0
0	0	0	0	0	0	0.023	0	0	0	0	0	0
0	0	0.046	0	0.02	0.013	0	0	0	0	0	0	0.02
0	0	0	0	0.015	0	0	0	0	0	0	0	0
0.027	0.033	0.028	0.019	0	0.02	0.018	0	0	0	0	0	0.013
0	0	0	0	0	0	0	0	0	0	0	0	0.012
0	0	0.014	0	0	0	0	0	0	0	0	0	0
0	0.038	0	0.018	0	0.012	0	0	0	0.016	0	0.013	0
0	0	0.025	0	0.013	0	0	0	0	0	0	0	0
0.063	0.043	0.024	0.015	0.01	0	0	0	0	0.034	0	0.033	0.019
0	0	0.045	0	0	0.012	0.033	0	0	0	0.062	0.035	0
0	0	0	0	0.014	0	0	0	0	0	0	0	0
0.07	0.078	0.06	0.014	0	0	0.016	0.017	0	0.059	0	0.023	0.067
0	0	0.021	0	0	0	0	0	0	0	0	0	0
0.032	0.067	0	0.065	0.027	0.027	0.009	0.015	0.021	0	0	0.009	0
0	0	0	0	0	0.011	0	0	0	0	0	0	0
0	0	0	0	0	0	0	0	0	0	0	0	0.048
0	0	0	0	0	0	0.01	0	0	0	0	0	0
0	0	0.024	0	0	0	0	0	0	0	0	0	0
0.038	0.082	0	0.031	0.033	0	0	0	0	0.084	0	0	0
0	0	0	0	0	0	0	0	0	0	0.031	0	0
0.025	0.108	0	0.022	0	0	0	0	0	0	0	0	0.054
0	0	0	0	0	0	0	0	0	0	0	0.018	0
0	0	0	0	0.046	0	0	0	0	0	0	0	0
0.083	0	0.033	0	0	0.054	0.025	0	0	0	0	0	0
0	0	0.076	0	0	0	0	0.032	0.04	0	0	0	0
0	0	0	0.022	0	0	0	0	0	0	0	0	0
0	0.081	0	0	0	0	0	0	0	0	0	0	0
0	0	0	0	0	0.025	0	0	0	0	0	0	0

Binary matrix for PCA of bacterial laboratory samples from Vesuvio

1	3	4	5	8	10	12	14	16	18	21	23	27
0.046	0	0	0	0.033	0	0.038	0	0	0.043	0	0.023	0.043
0	0	0	0	0	0	0	0	0	0	0	0	0.02
0	0	0	0	0	0	0	0	0	0	0	0.016	0
0	0	0	0	0	0	0	0	0	0	0.035	0	0
0.048	0	0.051	0.031	0	0.026	0	0	0	0.071	0	0	0.046
0	0	0	0	0	0	0.027	0	0	0	0	0	0
0	0	0	0	0	0	0	0.035	0	0	0	0	0
0	0	0	0.023	0	0	0	0	0	0	0	0	0
0.083	0.113	0.039	0	0	0	0.018	0	0	0	0	0	0
0	0	0	0	0	0	0.024	0	0	0	0	0	0
0	0	0	0	0	0	0	0	0.051	0	0	0	0
0	0	0	0	0	0	0.028	0	0	0	0	0	0
0	0	0.103	0.019	0	0	0	0	0	0	0.167	0	0.051
0	0	0	0	0	0	0	0	0	0.046	0	0	0
0	0	0.066	0.042	0.116	0.049	0.032	0.047	0	0.025	0.018	0.035	0.024
0	0	0	0	0	0	0	0	0.029	0	0	0	0
0.037	0.146	0	0.064	0	0.023	0.088	0.146	0.056	0	0.056	0.047	0
0	0	0	0	0.039	0	0	0	0	0	0	0	0
0	0	0	0	0	0	0	0	0	0	0.012	0	0
0.049	0	0	0	0.021	0.065	0	0	0.111	0	0	0.034	0.027
0	0	0	0	0	0	0	0	0	0.021	0	0	0
0	0	0.054	0	0	0	0	0	0	0	0	0	0
0.025	0.047	0	0.055	0.056	0	0	0.042	0	0.023	0.072	0.073	0.022
0	0	0	0	0	0.069	0.031	0.028	0.015	0	0	0	0.028
0	0	0	0.027	0	0	0	0	0	0	0	0	0
0	0	0	0	0.055	0	0	0	0	0	0	0	0
0.116	0.016	0.027	0	0	0	0	0	0	0.06	0	0	0
0	0	0	0	0	0	0	0	0.029	0	0	0	0
0	0	0	0.032	0.077	0.045	0.06	0.097	0.078	0.107	0.037	0.057	0.041
0.021	0.004	0.02	0.091	0	0	0	0	0	0	0	0	0
0	0	0.016	0	0	0	0	0	0	0	0	0	0
0	0.016	0	0	0	0	0	0	0	0	0	0	0
0	0	0	0	0	0	0	0	0	0.042	0	0	0
0.054	0	0	0.044	0	0.068	0.021	0.028	0.04	0.037	0	0.032	0.044

Binary matrix for PCA of bacterial laboratory samples from Vesuvio

1	3	4	5	8	10	12	14	16	18	21	23	27
0	0	0.058	0	0	0	0	0	0	0	0	0	0
0	0	0	0	0	0	0	0	0.056	0	0	0	0
0.095	0.041	0	0	0.135	0.078	0.094	0.049	0	0	0.219	0.103	0.052
0	0	0	0.047	0	0	0	0	0	0	0	0	0
0	0.032	0.024	0	0	0	0.086	0.057	0.072	0	0	0	0.037
0	0	0	0	0	0	0	0	0	0	0	0	0.042
0	0.004	0.03	0.047	0.084	0.061	0	0	0	0.054	0	0	0
0	0	0	0	0	0	0	0	0	0	0	0.06	0
0	0	0	0	0	0	0	0	0	0	0.041	0	0
0	0	0	0.046	0	0.064	0.068	0	0	0	0	0.057	0
0	0	0	0	0	0	0	0	0	0	0.02	0	0
0.035	0	0.038	0	0	0.032	0	0.091	0.093	0	0	0	0
0	0	0	0	0	0	0	0	0	0.049	0	0	0
0	8E-04	0	0.031	0	0	0	0	0	0.042	0	0.03	0.045
0	0	0	0	0	0	0	0	0	0	0	0	0.031
0	0	0	0	0	0	0	0.033	0	0	0	0	0
0	0	0	0	0	0	0	0	0	0	0	0.062	0
0	0	0	0	0	0	0	0	0	0	0.08	0	0
0.025	0.005	0	0.027	0.024	0.033	0.038	0	0	0	0	0	0
0	0	0	0	0.028	0	0	0	0	0	0	0	0
0	0	0	0	0	0	0	0	0	0	0	0.039	0
0	0	0	0	0	0	0	0	0	0	0	0	0.015
0	0.008	0	0.038	0	0.062	0.07	0	0	0	0	0	0
0.017	0	0	0	0.018	0	0	0.023	0.034	0	0	0.059	0
0	0	0	0	0.025	0.052	0.044	0.039	0.078	0.051	0.031	0	0.019
0	0	0.002	0.018	0.036	0.05	0.033	0.045	0.05	0.028	0	0.042	0.028
0	0.013	0	0	0	0	0.05	0.047	0.029	0	0	0	0
0	0.004	0	0	0	0	0	0	0	0	0	0	0
0.012	0	0	0.034	0.034	0	0	0	0	0.052	0.077	0.043	0.036
0	0	0	0	0	0	0	0	0.023	0	0	0	0
0	0	0.007	0	0.01	0.027	0.033	0.03	0.035	0.028	0	0.037	0.012
0	0.008	0	0.011	0.01	0	0	0	0	0	0	0	0
0	0.014	0	0	0	0	0	0	0	0	0	0	0.003
0	0	0.006	0	0	0	0	0	0	0	0	0	0.006

Binary matrix for PCA of bacterial laboratory samples from Vesuvio

1	3	4	5	8	10	12	14	16	18	21	23	27
0	0	0	0	0.012	0.011	0.012	0.022	0.028	0	0	0.014	0
0	0	0	0	0	0	0	0	0	0.015	0	0	0.006
0	0	0	0	0	0	0	0	0	0	0	0	0.003
0	0	0.005	0	0	0	0	0	0	0.054	0	0	0
0	0	0	0	0	0	0	0	0	0	0	0.003	0
0	0	0	0.003	0.006	0.011	0	0	0	0	0	0	0
0	0	0	0	0	0	0	0	0	0	0	0	0.002
0	0	0	0.004	0.001	0	0	0	0	0	0	0.004	0

Shannon-Weaver calculations of bacterial laboratory samples from Buffet			
Lane 1			
Area	%	Pi	H'
499	3.4	0.0340266	-0.0499572
293	2	0.0199795	-0.0339535
480	3.3	0.032731	-0.0486069
191	1.3	0.0130242	-0.0245539
1437	9.8	0.0979884	-0.0988532
360	2.5	0.0245482	-0.0395222
391	2.7	0.0266621	-0.041969
356	2.4	0.0242755	-0.0392008
195	1.3	0.013297	-0.0249484
612	4.2	0.041732	-0.0575706
1006	6.9	0.0685987	-0.0798272
875	6	0.0596659	-0.0730474
449	3.1	0.0306171	-0.0463554
267	1.8	0.0182066	-0.0316753
389	2.7	0.0265257	-0.0418134
988	6.7	0.0673713	-0.0789272
539	3.7	0.0367542	-0.052731
596	4.1	0.040641	-0.0565331
245	1.7	0.0167064	-0.0296893
459	3.1	0.031299	-0.0470884
371	2.5	0.0252983	-0.0403991
203	1.4	0.0138425	-0.0257302
354	2.4	0.0241391	-0.0390397
268	1.8	0.0182748	-0.0317643

Area	%	Pi	H'
402	2.7	0.0274122	-0.0428194
569	3.9	0.0387999	-0.0547532
741	5.1	0.0505285	-0.0655083
304	2.1	0.0207296	-0.0348964
326	2.2	0.0222298	-0.0367473
211	1.4	0.014388	-0.0265027
145	1	0.0098875	-0.0198236
144	0.8	0.0098193	-0.0197164
14665			1.4345238
Lane 2			
Area	%	Pi	H'
307	2	0.0199727	-0.0339448
358	2.3	0.0232906	-0.0380294
323	2.1	0.0210136	-0.0352503
311	2	0.0202329	-0.0342734
649	4.2	0.0422224	-0.0580328
479	3.1	0.0311626	-0.0469423
427	2.8	0.0277796	-0.0432327
175	1.1	0.0113851	-0.0221288
785	5.1	0.0510702	-0.0659741
704	4.6	0.0458005	-0.0613328
1450	9.4	0.0943335	-0.0967233
593	3.9	0.0385791	-0.0545373
947	6.2	0.0616095	-0.0745692
581	3.8	0.0377985	-0.0537693
378	2.5	0.0245918	-0.0395733
301	2	0.0195823	-0.0334493
298	1.9	0.0193872	-0.0332002
519	3.4	0.0337649	-0.0496862

Area	%	Pi	H'
602	3.9	0.0391647	-0.0551088
460	3	0.0299265	-0.0456063
293	1.9	0.0190619	-0.0327833
951	6.2	0.0618698	-0.0747709
341	2.2	0.0221846	-0.0366922
228	1.5	0.0148331	-0.0271263
422	2.7	0.0274543	-0.0428669
572	3.7	0.0372129	-0.0531887
274	1.8	0.0178258	-0.0311764
214	1.4	0.0139223	-0.0258438
298	1.9	0.0193872	-0.0332002
711	4.6	0.0462559	-0.0617439
131	0.9	0.0085225	-0.0176368
289	1.9	0.0188016	-0.0324479
15371			1.44484212

Lane 3

Area	%	Pi	H'
250	2.5	0.024505	-0.0394713
317	3.1	0.0310723	-0.0468455
363	3.6	0.0355813	-0.0515494
285	2.8	0.0279357	-0.0434076
868	8.5	0.0850814	-0.0910511
644	6.3	0.0631249	-0.0757372
600	5.9	0.058812	-0.0723702
1379	13.5	0.1351696	-0.1174787
883	8.7	0.0865517	-0.0919806
533	5.2	0.0522447	-0.0669755
383	3.8	0.0375417	-0.0535151
691	6.8	0.0677318	-0.0791925

Area	%	Pi	H'
284	2.8	0.0278377	-0.0432978
223	2.2	0.0218585	-0.0362934
415	4.1	0.0406783	-0.0565688
223	2.2	0.0218585	-0.0362934
221	2.2	0.0216624	-0.0360526
167	1.6	0.0163693	-0.0292351
356	3.5	0.0348951	-0.0508504
170	1.7	0.0166634	-0.0296315
266	2.6	0.0260733	-0.041295
232	2.3	0.0227406	-0.0373674
103	1	0.0100961	-0.0201502
346	3.1	0.0339149	-0.0498417
10202			1.29645183

Lane 4

Area	%	Pi	H'
327	2.5	0.0247278	-0.039733
410	3.1	0.0310042	-0.0467723
494	3.7	0.0373563	-0.0533312
329	2.5	0.024879	-0.0399101
234	1.8	0.0176951	-0.0310044
305	2.3	0.0230641	-0.0377574
335	2.5	0.0253327	-0.0404391
322	2.4	0.0243497	-0.0392884
400	3	0.030248	-0.0459559
482	3.6	0.0364489	-0.052425
1859	14.1	0.1405777	-0.119784
1005	7.6	0.0759982	-0.0850569
946	7.2	0.0715366	-0.0819432
406	3.1	0.0307018	-0.0464467

Area	%	Pi	H'
446	3.4	0.0337266	-0.0496464
829	6.3	0.0626891	-0.0754029
319	2.4	0.0241228	-0.0390204
419	3.2	0.0316848	-0.0475003
253	1.9	0.0191319	-0.0328732
456	3.4	0.0344828	-0.0504275
655	5	0.0495312	-0.0646442
419	3.2	0.0316848	-0.0475003
802	6.1	0.0606473	-0.0738192
173	1.3	0.0130823	-0.0246381
599	4.4	0.0452964	-0.0608755
13224			1.32619551
Lane 5			
Area	%	Pi	H'
701	6.6	0.0662008	-0.0780598
528	5	0.0498631	-0.0649327
262	2.5	0.0247427	-0.0397504
177	1.7	0.0167155	-0.0297014
686	6.5	0.0647842	-0.076998
291	2.7	0.0274813	-0.0428973
123	1.2	0.0116158	-0.022476
87	0.8	0.0082161	-0.0171333
126	1.2	0.0118991	-0.0228997
122	1.2	0.0115214	-0.0223342
254	2.4	0.0239872	-0.0388597
202	1.9	0.0190764	-0.0328019
176	1.7	0.016621	-0.0295745
128	1.2	0.012088	-0.0231805
144	1.4	0.013599	-0.0253825

Area	%	Pi	H'
253	2.4	0.0238927	-0.0387476
727	6.9	0.0686562	-0.0798691
144	1.4	0.013599	-0.0253825
136	1.3	0.0128435	-0.0242911
157	1.5	0.0148267	-0.0271174
238	2.2	0.0224762	-0.037047
143	1.4	0.0135046	-0.0252471
167	1.6	0.0157711	-0.0284217
305	2.9	0.0288035	-0.0443733
419	4	0.0395694	-0.0555016
411	3.9	0.0388139	-0.0547669
133	1.3	0.0125602	-0.023877
506	4.8	0.0477854	-0.0631104
199	1.9	0.0187931	-0.0324369
229	2.2	0.0216262	-0.0360081
131	1.2	0.0123713	-0.0235993
579	5.5	0.0546794	-0.069015
417	3.9	0.0393805	-0.0553185
183	1.7	0.0172821	-0.030458
446	4.2	0.0421192	-0.0579358
113	1.1	0.0106715	-0.0210417
451	4.3	0.0425914	-0.0583791
95	0.4	0.0089716	-0.018366
10589			1.49729311
Lane 6			
Area	%	Pi	H'
468	2.6	0.0264079	-0.0416786
800	4.5	0.0451416	-0.0607346
582	3.3	0.0328405	-0.0487219

434	2.4	0.0244893	-0.0394529
439	2.5	0.0247715	-0.0397842
550	3.1	0.0310349	-0.0468052
322	1.8	0.0181695	-0.0316269
493	2.8	0.0278185	-0.0432763
388	2.2	0.0218937	-0.0363365
672	3.8	0.037919	-0.0538883
321	1.8	0.0181131	-0.0315531
796	4.5	0.0449159	-0.0605287
601	3.4	0.0339127	-0.0498393
1224	6.9	0.0690667	-0.0801679
505	2.8	0.0284957	-0.0440321
433	2.4	0.0244329	-0.0393865
736	4.2	0.0415303	-0.0573797
617	3.5	0.0348155	-0.0507689
878	5	0.0495429	-0.0646544
525	3	0.0296242	-0.0452762
534	3	0.030132	-0.04583
1315	7.4	0.0742016	-0.0838171
694	3.9	0.0391604	-0.0551046
404	2.3	0.0227965	-0.0374349
582	3.3	0.0328405	-0.0487219
507	2.9	0.0286085	-0.0441574
471	2.7	0.0265771	-0.0418721
620	3.5	0.0349848	-0.0509421
518	2.9	0.0292292	-0.044843
139	0.8	0.0078434	-0.0165142
154	0.8	0.0086898	-0.0179095
17722			1.45303896

Lane 7			
Area	%	Pi	H'
561	4.3	0.0432237	-0.0589691
256	2	0.0197242	-0.0336297
259	2	0.0199553	-0.0339229
303	2.3	0.0233454	-0.038095
326	2.5	0.0251175	-0.0401886
395	3	0.0304338	-0.0461572
427	3.3	0.0328993	-0.0487835
628	4.8	0.0483859	-0.063641
486	3.7	0.0374451	-0.0534194
595	4.6	0.0458433	-0.0613715
649	5	0.0500039	-0.0650548
461	3.6	0.0355189	-0.0514861
479	3.7	0.0369058	-0.0528825
320	2.5	0.0246552	-0.0396478
237	1.8	0.0182603	-0.0317453
564	4.3	0.0434548	-0.0591838
1027	7.9	0.0791278	-0.0871728
586	4.5	0.0451499	-0.0607421
515	4	0.0396795	-0.0556082
282	2.2	0.0217274	-0.0361325
468	3.6	0.0360582	-0.0520319
197	1.5	0.0151784	-0.027606
184	1.4	0.0141767	-0.0262046
284	2.2	0.0218815	-0.0363216
226	1.7	0.0174127	-0.0306313
208	1.6	0.0160259	-0.0287693
225	1.7	0.0173357	-0.0305292

Area	%	Pi	H'
300	2.3	0.0231143	-0.0378177
590	4.5	0.045458	-0.0610224
193	1.5	0.0148702	-0.027178
748	6	0.0576316	-0.0714251
12979			1.44737105

Lane 8

Area	%	Pi	H'
376	4.1	0.0407588	-0.0566457
284	3.1	0.0307859	-0.0465375
279	3	0.0302439	-0.0459514
222	2.4	0.024065	-0.038952
228	2.5	0.0247154	-0.0397185
507	5.5	0.0549593	-0.0692465
292	3.2	0.0316531	-0.0474665
284	3.1	0.0307859	-0.0465375
373	4	0.0404336	-0.0563344
492	5.3	0.0533333	-0.0678934
1554	16.8	0.1684553	-0.1303028
762	8.3	0.0826016	-0.0894585
369	4	0.04	-0.0559176
593	6.4	0.0642818	-0.0766183
740	8	0.0802168	-0.0878963
411	4.5	0.0445528	-0.0601964
393	4.3	0.0426016	-0.0583887
339	3.7	0.036748	-0.0527248
238	2.6	0.0257995	-0.0409796
179	1.9	0.0194038	-0.0332215
139	1.5	0.0150678	-0.0274527
171	1.8	0.0185366	-0.0321048

Area	%	Pi	H'
9225			1.26054533

Lane 9

Area	%	Pi	H'
460	2.5	0.024502	-0.0394677
405	2.2	0.0215724	-0.0359418
1004	5.3	0.0534782	-0.0680148
521	2.8	0.0277511	-0.0432007
534	2.8	0.0284436	-0.0439742
756	4	0.0402685	-0.0561759
449	2.4	0.0239161	-0.0387753
464	2.5	0.024715	-0.039718
396	2.1	0.021093	-0.035349
506	2.7	0.0269522	-0.0422989
1026	5.5	0.05465	-0.0689907
895	4.8	0.0476723	-0.0630101
869	4.6	0.0462874	-0.0617723
1011	5.4	0.0538511	-0.0683265
981	5.2	0.0522531	-0.0669826
515	2.7	0.0274316	-0.0428412
442	2.4	0.0235432	-0.0383315
752	4	0.0400554	-0.055971
654	3.5	0.0348354	-0.0507893
820	4.4	0.0436774	-0.0593901
420	2.2	0.0223714	-0.0369196
432	2.3	0.0230105	-0.037693
510	2.7	0.0271652	-0.0425404
446	2.4	0.0237563	-0.0385854
393	2.1	0.0209332	-0.0351503
642	3.4	0.0341962	-0.0501324

Area	%	Pi	H'
453	2.4	0.0241291	-0.0390278
229	1.2	0.0121977	-0.023343
548	2.9	0.0291893	-0.0447991
580	3.1	0.0308938	-0.0466536
356	1.9	0.0189624	-0.0326553
305	1.6	0.0162459	-0.029068
18774			1.47588977

Lane 10

Area	%	Pi	H'
673	4	0.0404569	-0.0563567
699	4.2	0.0420198	-0.0578422
859	5.2	0.0516381	-0.0664598
432	2.6	0.0259693	-0.0411754
614	3.7	0.0369101	-0.0528868
245	1.5	0.014728	-0.0269796
261	1.6	0.0156898	-0.0283104
202	1.2	0.0121431	-0.0232621
782	4.7	0.0470093	-0.0624197
388	2.3	0.0233243	-0.0380697
342	2.1	0.0205591	-0.0346831
359	2.2	0.021581	-0.0359524
267	1.6	0.0160505	-0.0288028
365	2.2	0.0219417	-0.0363953
618	3.7	0.0371506	-0.0531266
788	4.7	0.04737	-0.0627414
840	5	0.0504959	-0.0654803
785	4.7	0.0471897	-0.0625807
813	4.9	0.0488729	-0.064069
1278	7.7	0.076826	-0.0856219

Area	%	Pi	H'
839	5	0.0504358	-0.0654284
504	3	0.0302976	-0.0460096
295	1.8	0.0177337	-0.0310553
299	1.8	0.0179742	-0.0313712
182	1.1	0.0109408	-0.0214544
400	2.4	0.0240457	-0.0389291
373	2.2	0.0224226	-0.0369819
425	2.6	0.0255485	-0.0406895
311	1.9	0.0186955	-0.0323108
251	1.5	0.0150887	-0.0274817
444	2.7	0.0266907	-0.0420016
300	1.8	0.0180343	-0.03145
402	2.4	0.0241659	-0.0390714
16635			1.46745091

Lane 11

Area	%	Pi	H'
394	3	0.0301039	-0.0457994
1047	8	0.0799969	-0.0877508
1505	11.5	0.1149908	-0.1080151
1058	8.1	0.0808374	-0.0883058
285	2.2	0.0217757	-0.0361918
268	2	0.0204768	-0.0345799
294	2.2	0.0224633	-0.0370314
190	1.5	0.0145171	-0.0266842
151	1.2	0.0115373	-0.0223581
279	2.1	0.0213172	-0.0356268
401	3.1	0.0306388	-0.0463788
266	2	0.020324	-0.034388
504	3.9	0.0385086	-0.0544681

Area	%	Pi	H'
438	3.3	0.0334658	-0.0493754
561	4.3	0.0428637	-0.0586337
407	3.1	0.0310972	-0.0468721
500	3.8	0.0382029	-0.0541681
661	5.1	0.0505043	-0.0654875
759	5.8	0.0579921	-0.0717148
252	1.9	0.0192543	-0.0330302
333	2.5	0.0254432	-0.0405673
283	2.2	0.0216229	-0.0360039
196	1.5	0.0149756	-0.0273246
328	2.5	0.0250611	-0.0401228
276	2.1	0.021088	-0.0353428
356	2.7	0.0272005	-0.0425803
355	2.7	0.0271241	-0.0424938
299	2.3	0.0228454	-0.0374938
218	1.7	0.0166565	-0.0296222
65	0.5	0.0049664	-0.0114423
159	1.2	0.0121485	-0.0232702
13088			1.40312403

Lane 12

Area	%	Pi	H'
452	3.2	0.0320159	-0.047852
356	2.5	0.025216	-0.0403034
434	3.1	0.0307409	-0.046489
344	2.4	0.0243661	-0.0393077
455	3.2	0.0322284	-0.048077
732	5.2	0.0518487	-0.0666392
241	1.7	0.0170704	-0.0301763
253	1.8	0.0179204	-0.0313007

Area	%	Pi	H'
539	3.8	0.0381782	-0.0541437
563	4	0.0398782	-0.0558001
218	1.5	0.0154413	-0.027969
1016	7.2	0.0719649	-0.0822472
697	4.9	0.0493696	-0.0645034
995	7	0.0704774	-0.0811865
424	3	0.0300326	-0.0457218
451	3.2	0.031945	-0.0477769
771	5.5	0.0546111	-0.0689585
574	4.1	0.0406573	-0.0565487
473	3.4	0.0335033	-0.0494145
563	4	0.0398782	-0.0558001
616	4.4	0.0436322	-0.0593482
638	4.5	0.0451905	-0.0607791
254	1.8	0.0179912	-0.0313936
153	1.1	0.0108372	-0.021296
318	2.3	0.0225244	-0.0371055
321	2.3	0.0227369	-0.0373629
285	2	0.020187	-0.0342155
658	4.7	0.0466072	-0.0620596
125	0.9	0.0088539	-0.0181759
85	0.6	0.0060207	-0.013368
114	0.7	0.0080748	-0.0168995
14118			1.4322197

Lane 13

Area	%	Pi	H'
544	3.4	0.0340383	-0.0499693
659	4.1	0.0412339	-0.0570984
566	3.5	0.0354148	-0.0513804

1049	6.6	0.0656363	-0.0776383
1219	7.6	0.0762733	-0.0852451
565	3.5	0.0353523	-0.0513167
212	1.3	0.0132649	-0.0249022
671	4.2	0.0419847	-0.0578091
618	3.9	0.0386685	-0.0546248
344	2.2	0.0215242	-0.0358824
203	1.3	0.0127018	-0.0240843
287	1.8	0.0179577	-0.0313496
283	1.8	0.0177074	-0.0310207
441	2.8	0.0275935	-0.0430236
1027	6.4	0.0642598	-0.0766016
521	3.3	0.0325992	-0.0484682
375	2.3	0.0234639	-0.0382368
547	3.4	0.034226	-0.0501631
288	1.8	0.0180203	-0.0314317
494	3.1	0.0309098	-0.0466708
240	1.5	0.0150169	-0.0273821
312	2	0.019522	-0.0333723
940	5.9	0.0588162	-0.0723735
503	3.1	0.0314729	-0.0472743
569	3.6	0.0356026	-0.051571
311	1.9	0.0194594	-0.0332925
254	1.6	0.0158929	-0.0285881
403	2.5	0.0252159	-0.0403032
290	1.8	0.0181454	-0.0315954
377	2.4	0.023589	-0.0383862
233	1.5	0.0145789	-0.0267709
346	2.2	0.0216494	-0.0360365

Area	%	Pi	H'
101	0.6	0.0063196	-0.0138988
113	0.7	0.0070705	-0.0152054
77	0.4	0.0048179	-0.0111638
15982			1.47413118
Lane 14			
Area	%	Pi	H'
216	2.1	0.0206916	-0.034849
287	2.7	0.0274931	-0.0429105
164	1.6	0.0157103	-0.0283385
295	2.8	0.0282594	-0.0437692
272	2.6	0.0260561	-0.0412753
315	3	0.0301753	-0.045877
249	2.4	0.0238529	-0.0387003
506	4.8	0.0484721	-0.0637169
428	4.1	0.0410001	-0.056876
396	3.8	0.0379347	-0.0539038
630	6	0.0603506	-0.0735866
439	4.2	0.0420538	-0.0578743
464	4.4	0.0444487	-0.0601009
339	3.2	0.0324744	-0.0483368
723	6.9	0.0692595	-0.0803078
489	4.7	0.0468436	-0.0622715
395	3.8	0.0378389	-0.0538092
526	5	0.050388	-0.0653871
638	6.1	0.061117	-0.0741861
481	4.6	0.0460772	-0.0615828
435	4.2	0.0416707	-0.0575126
375	3.6	0.035923	-0.0518953
538	5.2	0.0515375	-0.0663739

421	4	0.0403295	-0.0562346
418	4.2	0.0400421	-0.0559582
10439			1.37563421

Lane 15

Area	%	Pi	H'
330	2.8	0.0275943	-0.0430245
352	2.9	0.0294339	-0.0450678
361	3	0.0301865	-0.0458891
275	2.3	0.0229952	-0.0376745
162	1.4	0.0135463	-0.0253069
889	7.4	0.0743373	-0.0839115
201	1.7	0.0168074	-0.0298248
463	3.9	0.0387156	-0.0546709
392	3.3	0.0327787	-0.0486569
393	3.3	0.0328623	-0.0487447
428	3.6	0.0357889	-0.0517598
774	6.5	0.0647211	-0.0769504
470	3.9	0.0393009	-0.0552413
380	3.2	0.0317752	-0.0475965
553	4.6	0.0462413	-0.0617308
372	3.1	0.0311063	-0.0468819
518	4.3	0.0433147	-0.0590537
322	2.7	0.0269253	-0.0422684
284	2.4	0.0237478	-0.0385754
216	1.8	0.0180617	-0.0314859
576	4.8	0.0481646	-0.0634458
372	3.1	0.0311063	-0.0468819
315	2.6	0.02634	-0.041601
352	2.9	0.0294339	-0.0450678

286	2.4	0.023915	-0.0387741
279	2.3	0.0233297	-0.0380762
266	2.2	0.0222427	-0.036763
390	3.3	0.0326114	-0.0484811
238	2	0.0199013	-0.0338545
191	1.6	0.0159712	-0.0286949
332	2.8	0.0277615	-0.0432124
227	1.9	0.0189815	-0.0326799
11959			1.47184817

Lane 16

Area	%	Pi	H'
352	5.7	0.0565099	-0.0705173
282	4.5	0.0452721	-0.0608534
443	7.1	0.071119	-0.0816456
330	5.3	0.052978	-0.0675949
375	6	0.0602023	-0.0734701
206	3.3	0.0330711	-0.0489635
94	1.5	0.0150907	-0.0274846
187	3	0.0300209	-0.0457091
106	1.7	0.0170172	-0.0301053
221	3.5	0.0354792	-0.0514458
60	1	0.0096324	-0.0194214
684	11	0.109809	-0.1053466
864	13.9	0.1387061	-0.1189966
10	0.2	0.0016054	-0.0044861
449	7.2	0.0720822	-0.0823303
137	2.2	0.0219939	-0.0364592
419	6.7	0.067266	-0.0788495
2	0	0.0003211	-0.0011217

4	0.1	0.0006422	-0.00205
6	0.1	0.0009632	-0.0029054
0	0	0	0
81	1.3	0.0130037	-0.0245241
27	0.4	0.0043346	-0.0102428
6	0.1	0.0009632	-0.0029054
61	1	0.0097929	-0.0196748
110	1.8	0.0176593	-0.0309573
135	2.2	0.0216728	-0.0360654
10	0.2	0.0016054	-0.0044861
430	6.9	0.0690319	-0.0801426
83	1.3	0.0133248	-0.0249885
40	0.6	0.0064216	-0.0140784
15	0.2	0.0024081	-0.0063052
0	0	0	0
6229			1.26412676

Binary matrix for PCA of bacterial laboratory samples from Buffet

1	2	3	3.3	1.4	2.4	3.4	6.4	8.4	10.4	12.4	14.4	16.4	19.4	21.4	25.4
0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0.057
0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0.045
0	0	0	0	0.066	0	0	0	0	0	0	0	0	0	0	0
0.034	0	0	0	0	0	0	0	0	0.03	0	0	0	0	0	0.071
0	0	0.025	0.025	0.05	0	0	0	0	0	0	0	0	0	0	0
0	0	0.031	0	0.025	0.026	0	0	0	0	0	0	0	0	0	0
0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0.053
0.02	0.02	0	0	0.017	0	0	0	0	0.04	0.08	0.032	0.034	0	0.028	0
0.033	0.023	0.036	0.031	0.065	0.045	0.043	0.041	0.025	0.042	0	0.025	0.041	0.021	0.029	0.06
0.013	0.021	0	0	0.027	0	0	0	0	0	0	0	0	0	0	0
0	0	0	0	0	0.033	0.02	0	0.022	0.052	0.115	0.031	0	0.027	0.03	0
0	0.02	0	0	0	0	0	0	0	0	0	0	0	0	0	0
0	0	0	0	0	0	0	0	0	0	0	0.024	0.035	0.016	0	0
0	0	0	0	0.012	0.024	0	0	0	0.026	0	0	0	0	0	0
0	0	0	0	0.008	0	0.02	0	0.053	0	0	0.032	0	0.028	0	0
0	0	0	0	0	0	0	0	0	0	0	0	0.066	0	0	0
0	0	0	0	0.012	0	0	0	0	0	0	0	0	0	0	0
0	0	0	0	0	0	0	0	0.028	0.037	0.081	0.052	0.076	0	0.023	0.033
0	0	0.028	0	0	0.025	0.023	0	0.028	0	0	0	0	0	0	0
0.098	0.042	0	0.037	0.012	0	0	0	0	0.015	0.022	0	0.035	0.026	0.014	0.015
0.025	0.031	0	0	0	0	0	0	0	0	0	0.017	0	0	0	0.072
0	0	0	0.025	0	0	0	0	0	0.016	0	0	0	0	0	0
0	0	0	0	0.024	0.031	0.025	0	0	0	0.02	0	0.013	0.03	0	0
0	0	0	0	0	0	0	0	0	0	0	0	0	0	0.074	0
0	0	0	0	0	0	0	0	0	0.012	0	0.018	0	0	0	0
0.027	0.028	0	0	0	0	0	0	0	0	0	0	0	0.024	0	0.03
0.024	0.011	0	0	0.019	0.018	0.03	0	0	0	0	0	0	0.048	0.017	0.017

1	2	3	3.3	1.4	2.4	3.4	6.4	8.4	10.4	12.4	14.4	16.4	19.4	21.4	25.4
0.013	0	0	0	0	0	0	0	0	0	0	0	0.042	0	0	0
0	0	0	0	0	0	0.033	0.031	0.04	0.047	0.022	0	0	0.041	0	0.035
0	0	0	0	0	0	0	0	0	0	0	0.038	0	0	0	0
0	0	0	0	0	0.028	0	0	0	0	0	0	0	0	0	0
0	0	0	0	0.017	0.022	0	0.03	0	0	0	0.04	0	0	0	0
0.042	0	0	0	0	0	0	0	0.024	0.023	0.015	0	0	0	0.039	0.01
0	0.051	0	0	0	0.038	0.048	0.024	0	0	0	0	0	0.038	0	0.11
0	0	0	0	0.012	0	0	0	0	0	0	0	0	0	0	0
0	0	0	0.018	0	0	0	0	0	0	0	0	0	0	0	0
0	0	0	0	0	0	0	0	0	0	0.015	0	0	0	0	0
0.069	0	0	0	0.014	0.018	0	0.025	0.025	0.021	0.012	0	0.039	0	0	0
0	0.046	0	0	0	0	0	0	0	0	0	0	0	0	0	0
0	0	0	0	0	0	0	0	0	0	0	0	0.022	0	0	0
0	0	0	0.023	0	0.045	0	0	0	0	0	0	0	0.06	0.033	0.139
0	0	0	0	0	0	0	0.055	0	0	0	0	0	0	0	0
0	0	0	0	0.024	0	0.037	0	0.021	0.022	0.021	0	0	0	0	0
0	0	0	0	0	0	0	0	0	0	0	0.072	0.013	0	0	0
0	0	0	0	0	0.034	0	0	0	0	0	0	0	0	0	0
0	0	0	0	0	0	0	0	0.027	0.016	0.031	0	0	0	0	0
0.06	0.094	0.085	0	0	0	0	0	0	0	0	0.049	0.018	0	0	0
0	0	0	0.025	0	0	0	0	0	0	0	0	0	0	0	0
0	0	0	0	0.069	0.069	0.046	0.032	0	0.022	0.02	0	0.018	0	0	0.002
0	0.039	0	0.024	0	0	0	0.031	0	0	0	0	0	0.042	0.033	0
0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0.072
0.031	0	0	0.03	0	0	0	0	0	0	0	0.07	0	0.044	0	0
0	0	0	0	0	0.028	0	0	0	0	0.039	0	0.028	0	0.036	0
0.018	0.062	0	0	0	0	0	0	0.055	0.037	0	0	0	0	0	0
0	0	0	0	0	0	0.05	0	0	0	0	0	0	0	0	0
0	0	0.063	0.036	0	0	0	0.04	0	0	0	0	0	0	0	0
0	0	0	0	0.014	0.024	0.036	0.053	0.048	0.047	0.033	0.03	0.064	0.032	0.065	0.022

0	0	0.059	0	0	0	0	0	0	0	0	0	0	0	0	0
0	0	0	0	0.013	0.042	0	0	0.046	0	0	0	0	0	0	0
0	0	0	0	0.015	0	0	0	0	0	0	0	0	0	0	0
0.027	0.038	0.135	0.141	0	0	0.037	0.168	0.054	0.05	0.043	0.032	0	0.069	0.039	0.067
0.067	0	0	0	0	0	0	0	0	0	0	0.033	0	0	0	1E-03
0	0	0	0	0.022	0	0.025	0	0	0	0	0	0	0	0	0
0	0	0	0	0	0	0	0.052	0.047	0.031	0	0.023	0	0	0	0
0	0.025	0.087	0.076	0.014	0.035	0.018	0.083	0	0	0	0	0	0.047	0.032	3E-04
0	0	0	0	0.016	0	0	0	0	0	0	0	0	0	0	0
0.037	0.02	0.052	0.072	0	0.05	0.043	0.04	0.027	0.049	0.038	0.055	0.034	0.038	0.046	6E-04
0	0	0	0	0.029	0.03	0.079	0.064	0.024	0.077	0.051	0.041	0	0.05	0.031	1E-03
0	0	0.038	0.031	0	0	0	0	0.04	0	0	0	0	0	0	0
0	0.019	0	0	0	0	0	0	0	0	0	0	0.018	0	0.043	0
0	0	0	0.034	0	0	0	0	0	0	0	0	0	0	0	0
0	0	0	0	0.04	0.03	0	0	0	0	0	0.034	0	0	0	0
0	0	0.068	0.063	0	0	0	0	0	0	0	0	0	0	0	0
0.041	0.034	0	0	0	0	0.045	0.08	0.035	0.05	0.058	0.04	0.031	0.061	0.027	0.013
0	0	0	0	0.039	0.074	0	0	0	0	0	0	0.015	0	0	0
0	0	0	0	0	0.04	0	0	0	0	0	0	0	0	0	0
0.017	0.039	0	0	0	0	0	0.045	0.044	0.03	0.019	0.044	0.02	0.046	0.024	0
0	0	0.028	0	0	0	0	0	0	0	0	0	0	0	0	0
0	0	0	0.024	0	0	0	0	0	0	0	0	0	0	0	0
0	0	0	0	0	0.022	0	0	0	0	0	0	0	0	0	0
0	0	0	0	0.013	0	0	0	0	0	0	0	0	0	0	0
0	0	0	0	0	0	0	0	0.025	0	0	0	0	0	0	0
0.031	0.03	0.022	0	0	0	0.036	0.043	0.022	0.018	0	0	0	0	0	0
0	0	0	0	0.048	0	0	0	0	0	0.045	0.059	0.042	0	0	0
0	0	0.041	0	0	0	0	0	0	0	0	0	0	0	0	0
0	0	0	0	0	0	0	0	0	0	0	0	0	0	0.018	0

0	0	0	0.032	0	0	0	0	0	0	0	0	0	0	0	0
0	0	0	0	0.019	0.039	0.015	0	0	0	0.022	0	0	0	0	0.004
0	0	0	0	0	0	0	0	0.023	0	0	0	0	0	0	0
0	0	0	0	0	0	0	0	0.018	0	0	0	0.036	0	0	0
0.025	0	0	0	0.022	0.023	0	0	0	0	0	0.018	0.031	0	0	1E-03
0	0	0	0	0	0	0	0	0	0	0	0	0	0	0.048	0
0	0	0.022	0.019	0	0	0.014	0	0.027	0.011	0.015	0	0	0	0	0
0	0	0	0.012	0	0	0	0	0	0	0	0	0	0	0	0
0.014	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0.01
0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0.018
0	0.019	0.022	0.034	0.055	0	0.022	0	0	0	0.025	0	0	0	0.031	0
0	0	0	0	0	0	0	0	0	0	0.011	0	0	0	0	0
0	0	0	0	0	0.033	0	0	0.024	0.024	0	0	0.036	0.052	0	0
0.024	0	0	0	0.039	0.029	0.017	0	0	0	0	0	0	0	0	0
0	0	0	0	0	0	0	0	0	0	0	0	0	0.026	0	0
0	0	0	0	0	0	0	0	0.021	0	0	0	0	0	0	0
0	0	0	0	0	0	0	0	0	0	0.023	0	0	0	0	0
0	0.062	0	0	0	0	0	0	0	0	0	0	0	0	0	0
0.018	0	0.016	0	0	0	0.016	0	0	0	0.021	0	0	0.04	0.029	0
0	0.022	0.035	0.05	0	0	0	0.037	0.034	0	0	0	0	0	0	0
0	0	0	0	0	0	0	0	0.022	0.027	0.023	0.019	0.04	0.024	0	0
0.027	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0.022
0	0.015	0	0	0.017	0.027	0.017	0	0	0	0	0	0	0	0	0
0.039	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
0	0	0	0	0.035	0	0	0.024	0.026	0.027	0.02	0.016	0	0	0	0.002
0	0	0.017	0.032	0.042	0	0.023	0.026	0	0	0	0	0	0	0	0
0	0.027	0	0	0	0	0	0	0	0	0	0	0	0	0.023	0
0	0	0	0	0.011	0	0	0	0.012	0	0	0	0	0	0	0
0	0	0	0	0	0	0	0	0	0	0	0	0.025	0	0.022	0

0.051	0.037	0.026	0.061	0.043	0.029	0.045	0	0.029	0	0	0	0	0	0.033	0
0	0	0.023	0	0	0	0	0	0	0	0	0	0	0	0	0
0	0	0	0.013	0	0	0	0.019	0	0	0	0	0	0	0	0
0	0	0	0	0	0	0	0	0	0	0	0	0.018	0	0	0.069
0.021	0.018	0	0	0	0.008	0.015	0	0	0.019	0.023	0	0.024	0	0	0.013
0	0	0	0	0	0	0	0	0	0.015	0	0	0	0	0	0
0	0	0	0	0	0	0	0	0	0	0.047	0	0	0	0	0
0	0	0	0	0	0	0	0	0	0	0	0	0	0.02	0	0
0	0	0	0	0	0	0	0	0	0	0	0.015	0	0	0	0
0	0.014	0	0	0	0	0	0	0	0	0	0	0	0	0	0
0	0.019	0	0	0.009	0	0	0	0.031	0.027	0.017	0.009	0.022	0	0.016	0.006
0.022	0.046	0.01	0	0	0	0	0	0	0	0.006	0	0	0	0	0
0	0	0	0	0	0	0	0.015	0	0	0	0	0	0	0	0
0	0	0.034	0	0	0	0	0	0	0	0	0	0	0	0	0
0	0	0	0.045	0	0	0	0	0	0	0	0	0	0	0	0
0	0	0	0	0	0	0	0	0	0	0	0	0.006	0	0	0.002
0	0	0	0	0	0	0	0	0	0	0	0	0	0.028	0	0
0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
0.014	0.009	0	0	0	0	0	0	0.019	0.018	0.005	0.008	0.007	0	0.019	0
0.01	0	0	0	0	0	0.058	0.019	0.016	0.024	0.012	0	0.005	0	0	0
0.01	0.019	0	0	0.009	0	0	0	0	0	0	0	0	0	0	0

8.4 **DGGE analysis of the fungal population from OMWW biofilms cultivated at the Vesuvio farm from 2004 to 2005**

Shannon-Weaver calculations				
Lane: 1				
Peak	Area	%	Pi	H'
1	292	31.9	0.319125683	-0.15829854
2	132	14.4	0.144262295	-0.12130254
3	491	53.7	0.536612022	-0.14506748
	915			0.424668565
Lane: 2				
Peak	Area	%		
1	587	25	0.250426621	-0.15058641
2	122	5.2	0.052047782	-0.06680842
3	435	18.6	0.185580205	-0.13574605
4	19	0.8	0.008105802	-0.01695089
5	129	5.5	0.05503413	-0.06930822
6	662	28.2	0.282423208	-0.15507848
7	390	16.7	0.166382253	-0.12959397
	2344			0.724072425
Lane: 3				
Peak	Area	%	Pi	H'
1	276	20.8	0.208144796	-0.14187866
2	819	61.8	0.617647059	-0.12924859
3	57	4.3	0.042986425	-0.0587482
4	174	13.1	0.131221719	-0.11573681
	1326			0.445612258
Lane: 4				
Peak	Area	%	Pi	H'
1	412	15.1	0.151081775	-0.1240061
2	392	14.4	0.143747708	-0.12109294
3	259	9.5	0.094976164	-0.09710224
4	75	2.8	0.02750275	-0.04292145
5	104	3.8	0.038137147	-0.05410333
6	344	12.6	0.126145948	-0.11342119
7	80	2.9	0.029336267	-0.04496062
8	113	4.1	0.041437477	-0.05729173
9	615	22.6	0.225522552	-0.14587025
10	333	12.2	0.122112211	-0.11151787
	2727			0.91228771
Lane: 5				
Peak	Area	%	Pi	H'
1	405	79.9	0.798816568	-0.0779269
2	34	6.7	0.067061144	-0.0786982
3	68	13.4	0.134122288	-0.11702157
	507			0.27364667
Lane: 6				
Peak	Area	%	Pi	H'
1	82	1.1	0.010684039	-0.02106107
2	490	6.4	0.063843648	-0.07628565
3	163	2.1	0.021237785	-0.03552849
4	255	3.3	0.033224756	-0.04912407
5	1043	13.6	0.135895765	-0.11779364
6	4810	62.7	0.626710098	-0.12718035
7	832	10.8	0.108403909	-0.1046049
	7675			0.531578176
Lane: 7				
Peak	Area	%	Pi	H'

Peak	Area	%	Pi	H'
1	330	5.8	0.057662065	-0.07144963
2	364	6.4	0.063603005	-0.07610242
3	537	9.4	0.093831906	-0.09642631
4	1189	20.8	0.207758169	-0.14178288
5	2946	51.5	0.514764983	-0.14845359
6	264	4.6	0.046129652	-0.06163013
7	93	1.5	0.016250218	-0.02907393
	5723			0.624918883

Lane: 8

Peak	Area	%	Pi	H'
1	242	3.8	0.037972697	-0.0539413
2	324	5.1	0.050839479	-0.06577606
3	649	10.2	0.10183587	-0.10103129
4	437	6.9	0.068570532	-0.07980667
5	4368	68.5	0.685391495	-0.11244621
6	263	4.1	0.041267849	-0.05713072
7	90	1.4	0.014122078	-0.02612728
	6373			0.496259529

Lane: 9

Peak	Area	%	Pi	H'
1	433	11.1	0.11142563	-0.10619029
2	150	3.9	0.038600103	-0.05455783
3	154	4	0.039629439	-0.05555976
4	1328	34.2	0.341739578	-0.15935478
5	446	11.5	0.114770973	-0.10790399
6	290	7.5	0.074626866	-0.0841123
7	626	16.1	0.161091096	-0.12773372
8	422	10.9	0.108594956	-0.10470621
9	37	0.8	0.009521359	-0.01924553

Peak	Area	%	Pi	H'
	3886			0.819364402

Lane: 10

Peak	Area	%	Pi	H'
1	18	7.8	0.077922078	-0.08636411
2	129	55.8	0.558441558	-0.14129815
3	6	2.6	0.025974026	-0.0411808
4	27	11.7	0.116883117	-0.10896408
5	51	22.1	0.220779221	-0.1448404
	231			0.522647539

Lane: 11

Peak	Area	%	Pi	H'
1	169	10	0.100059207	-0.10003349
2	108	6.4	0.063943162	-0.0763613
3	912	54	0.539964476	-0.14451329
4	88	5.2	0.052101835	-0.06685431
5	318	18.8	0.188277087	-0.13653902
6	94	5.6	0.055654233	-0.06981834
	1689			0.594119745

Lane: 12

Peak	Area	%	Pi	H'
1	1742	15.5	0.154679453	-0.12537812
2	1118	9.9	0.099271888	-0.09958695
3	194	1.7	0.01722607	-0.03038358
4	71	0.6	0.006304386	-0.0138719
5	832	7.4	0.073876754	-0.08359097
6	657	5.8	0.058337773	-0.07199174
7	320	2.8	0.028414136	-0.04394148
8	409	3.6	0.036316818	-0.0522923
9	5248	46.6	0.465991831	-0.154533

10	671	6.1	0.05958091	-0.07298022
	1262			0.748550261

Binary matrix for PCA

	2004				2005						
2	3	4	5	14	3	5	7	9.3	12.4	16.4	25
0	0	0	0.151	0	0	0	0	0	0	0	0
0	0	0	0.144	0	0.011	0	0	0	0	0	0
0	0	0	0	0	0	0.058	0.038	0	0.078	0	0.155
0	0.06	0	0.095	0	0	0	0	0.111	0	0.1	0.099
0	0	0	0	0	0	0.064	0.051	0	0	0	0
0	0	0	0	0	0	0	0	0	0	0.064	0.017
0	0	0	0.028	0	0	0	0	0	0	0	0.006
0	0	0	0.038	0	0	0	0	0.039	0	0	0
0	0	0	0	0	0	0	0	0.04	0	0	0
0	0.25	0.208	0.126	0	0	0	0	0	0	0	0
0	0	0	0	0.799	0.064	0.094	0.102	0.342	0.558	0	0.074
0	0	0	0	0	0	0	0	0	0	0.54	0
0.319	0	0	0	0	0	0	0	0	0	0	0
0	0	0	0	0	0.021	0	0	0	0	0	0
0	0.052	0	0	0	0	0	0	0	0	0	0
0	0	0	0	0	0	0	0	0.115	0	0	0.058
0	0	0.618	0	0	0	0	0	0	0	0	0
0	0	0	0	0	0	0	0	0	0	0	0.028
0	0	0	0	0	0	0	0	0	0	0	0.036
0	0	0	0	0	0	0	0	0.075	0	0	0
0	0.186	0	0	0	0	0	0	0	0	0	0
0	0	0	0	0	0.033	0	0	0	0	0	0
0	0.008	0	0	0	0	0	0	0	0.026	0.052	0
0	0	0.043	0.029	0.067	0	0	0	0.161	0.117	0.188	0.466
0	0.055	0	0	0	0	0	0	0	0	0	0

0	0	0	0	0	0	0.208	0.069	0.109	0.221	0	0
0	0	0	0.041	0	0	0	0	0	0	0	0
0	0.282	0	0	0	0	0	0	0	0	0	0
0.144	0	0	0	0.134	0.136	0	0	0	0	0	0
0	0	0	0.226	0	0	0	0	0	0	0	0
0	0	0	0	0	0	0.515	0.685	0	0	0	0
0	0	0	0	0	0	0	0	0.01	0	0.056	0
0	0	0	0	0	0	0	0	0	0	0	0.06
0	0	0	0	0	0.627	0.046	0.041	0	0	0	0
0	0	0	0.122	0	0.108	0.016	0.014	0	0	0	0
0.537	0	0	0	0	0	0	0	0	0	0	0
0	0.166	0	0	0	0	0	0	0	0	0	0
0	0	0.131	0	0	0	0	0	0	0	0	0

8.5 DGGE analysis of fungal species from Buffet biofilms cultivated at the farm from 2004 to 2005

Shannon-Weaver calculations

Lane: 1

Peak	Area	%	Pi	H'
1	44	20.6	0.205607	-0.14124
2	11	5.1	0.051402	-0.06626
3	159	74.3	0.742991	-0.09586
	214			0.303361

Lane: 2

Peak	Area	%	Pi	H'
1	301	14.4	0.143675	-0.12106
2	256	12.2	0.122196	-0.11156
3	320	15.3	0.152745	-0.12464
4	6	0.3	0.002864	-0.00728
5	38	1.8	0.018138	-0.03159
6	52	2.5	0.024821	-0.03984
7	83	4	0.039618	-0.05555
8	128	6.1	0.061098	-0.07417
9	138	6.6	0.065871	-0.07781
10	712	34	0.339857	-0.15929
11	61	2.8	0.029117	-0.04472
	2095			0.847523

Lane 3

Lane: 4

Peak	Area	%	Pi	H'
1	681	11.4	0.113614	-0.10732
2	460	7.7	0.076743	-0.08557
3	22	0.4	0.00367	-0.00894
4	65	1.1	0.010844	-0.02131
5	95	1.6	0.015849	-0.02853
6	791	13.2	0.131965	-0.11607
7	290	4.8	0.048382	-0.06364
8	550	9.2	0.091758	-0.09519
9	955	15.9	0.159326	-0.1271
10	983	16.4	0.163997	-0.12876
11	985	16.4	0.164331	-0.12888
12	117	1.9	0.01952	-0.03337
	5994			0.944659

Lane: 5

Peak	Area	%	Pi	H'
1	57	3	0.029518	-0.04516
2	2	0.1	0.001036	-0.00309
3	18	0.9	0.009322	-0.01893
4	2	0.1	0.001036	-0.00309
5	28	1.5	0.0145	-0.02666
6	19	1	0.009839	-0.01975
7	575	29.8	0.297773	-0.15666
8	96	5	0.049715	-0.0648
9	88	4.6	0.045572	-0.06113
10	20	1	0.010357	-0.02056
11	769	39.8	0.398239	-0.15924
12	257	13.2	0.133092	-0.11657
	1931			0.695636

Lane: 6

Peak	Area	%	Pi	H'
1	558	18.2	0.182293	-0.13476
2	323	10.6	0.105521	-0.10306
3	66	2.2	0.021562	-0.03593
4	67	2.2	0.021888	-0.03633
5	347	11.3	0.113362	-0.10719
6	499	16.3	0.163019	-0.12842
7	1201	39.2	0.392355	-0.15942
	3061			0.705103

Lane: 7

Peak	Area	%	Pi	H'
1	219	27.6	0.276166	-0.15433
2	0	0	0	0
3	0	0	0	0

Peak	Area	%	Pi	H'
4	0	0	0	0
5	190	24	0.239596	-0.14867
6	384	48.4	0.484237	-0.15251
	793			0.455511
Lane: 8				
Peak	Area	%	Pi	H'
1	366	19.8	0.197518	-0.13913
2	387	20.9	0.208851	-0.14205
3	243	13.1	0.131139	-0.1157
4	203	11	0.109552	-0.10521
5	252	13.6	0.135996	-0.11784
6	304	16.4	0.164058	-0.12879
7	98	5.2	0.052887	-0.06752
	1853			0.816235
Lane: 9				
Peak	Area	%	Pi	H'
1	673	64.3	0.643403	-0.12322
2	328	31.4	0.313576	-0.15793
3	45	4.3	0.043021	-0.05878
	1046			0.339938
Lane: 10				
Peak	Area	%	Pi	H'
1	645	37	0.370264	-0.15976
2	425	24.4	0.243972	-0.14947
3	50	2.9	0.028703	-0.04426
4	332	19.1	0.190586	-0.1372
5	0	0	0	0
6	0	0	0	0
7	0	0	0	0
8	237	13.6	0.136051	-0.11786
9	43	2.5	0.024684	-0.03968
10	10	0.5	0.005741	-0.01286
	1742			0.66111
Lane: 11				
Peak	Area	%	Pi	H'
1	1790	99.9	0.998884	-0.00048

Peak	Area	%	Pi	H'
2	2	0.1	0.001116	-0.00329
	1792			0.003779
Lane 12				
Lane 13				
Lane: 14				
Peak	Area	%	Pi	H'
1	214	12.3	0.123343	-0.1121
2	178	10.3	0.102594	-0.10145
3	37	2.1	0.021326	-0.03564
4	173	10	0.099712	-0.09984
5	126	7.3	0.072622	-0.08271
6	1007	58	0.580403	-0.13713
	1735			0.568875

Binary matrix for PCA

	2004						2005						
2	3	5	6	7	16	25	9.3	12.4	16.4	19.4	21.4	23.4	25.4
0	0.144	0	0	0.03	0	0	0	0	0	0	0	0	0
0.206	0	0	0	0	0	0	0.198	0	0	0.999	0	0	0
0	0	0	0.114	0	0	0	0	0	0.37	0	0	0	0
0	0	0	0	0.001	0.182	0	0	0	0	0	0	0	0
0	0.122	0	0	0.009	0	0	0	0	0	0	0	0	0.123
0	0	0	0.077	0	0.106	0	0.209	0	0.244	0	0	0	0
0	0	0	0	0	0	0	0	0.643	0	0	0	0	0
0	0	0	0	0	0	0.276	0	0	0	0	0	0	0.103
0	0	0	0	0.001	0	0	0	0	0	0	0	0	0
0	0	0	0.004	0	0	0	0	0	0	0	0	0	0.021
0	0	0	0	0.015	0	0	0	0	0	0	0	0	0
0	0	0	0.011	0.01	0	0	0	0	0	0	0	0	0
0	0	0	0	0	0.022	0	0	0	0	0	0	0	0
0	0	0	0.016	0	0	0	0	0	0	0	0	0	0
0	0	0	0	0	0.022	0	0	0	0.029	0	0	0	0
0.051	0.153	0	0	0.298	0	0	0	0	0	0	0	0	0.1
0	0	0	0.132	0	0.113	0	0.131	0.314	0.191	0	0	0	0
0	0.003	0	0	0	0	0	0	0	0	0	0	0	0
0	0	0	0	0.05	0	0	0	0	0	0	0	0	0
0	0.018	0	0	0	0	0	0	0	0	0	0	0	0
0	0	0	0	0	0	0	0.11	0	0	0	0	0	0
0	0	0	0	0.046	0	0	0	0	0	0	0	0	0
0	0.025	0	0.048	0.01	0	0	0.136	0	0	0	0	0	0
0	0	0	0	0	0	0	0	0	0	0	0	0	0
0	0.04	0	0	0	0	0	0.164	0	0	0	0	0	0.073
0	0	0	0.092	0	0	0.24	0	0	0.136	0	0	0	0
0	0	0	0	0	0.163	0	0	0	0	0	0	0	0
0.743	0.061	0	0	0	0	0	0	0	0	0	0	0	0
0	0	0	0.159	0.398	0	0	0	0	0	0	0	0	0
0	0	0	0	0	0	0	0	0.043	0.025	0	0	0	0
0	0.066	0	0	0	0	0	0.053	0	0	0	0	0	0.58
0	0	0	0	0	0.392	0.484	0	0	0	0	0	0	0

Binary matrix for PCA

	2004						2005						
2	3	5	6	7	16	25	9.3	12.4	16.4	19.4	21.4	23.4	25.4
0	0.34	0	0	0.133	0	0	0	0	0.006	0	0	0	0
0	0	0	0.164	0	0	0	0	0	0	0	0	0	0
0	0	0	0.164	0	0	0	0	0	0	0	0	0	0
0	0.029	0	0	0	0	0	0	0	0	0	0	0	0
0	0	0	0.02	0	0	0	0	0	0	0	0	0	0
0	0	0	0	0	0	0	0	0	0	0.001	0	0	0

8.6 DGGE analysis of fungal species from Buffet and Vesuvio biofilms isolated in the laboratory during 2004

Shannon-Weaver calculations (Buffet)				
Lane: 1				
Lane: 2				
Peak	Area	%	Pi	H'
1	73	11.1	0.11145	-0.1062
2	192	29.3	0.29313	-0.15622
3	211	32.2	0.322137	-0.15848
4	179	27.4	0.273282	-0.15396
	655			0.574866
Lane: 3				
Peak	Area	%	Pi	H'
1	512	9.8	0.097859	-0.09878
2	149	2.8	0.028479	-0.04401
3	295	5.6	0.056384	-0.07041
4	51	1	0.009748	-0.0196
5	464	8.9	0.088685	-0.09331
6	0	0	0	0
7	6	0.1	0.001147	-0.00337
8	78	1.5	0.014908	-0.02723
9	444	8.5	0.084862	-0.09091
10	558	10.7	0.106651	-0.10367
11	2675	51.1	0.511277	-0.14896
	5232			0.700261
Lane: 4				
Peak	Area	%	Pi	H'
1	1084	13.7	0.137024	-0.11828
2	275	3.5	0.034762	-0.05071
3	227	2.9	0.028694	-0.04425
4	316	4	0.039944	-0.05586
5	177	2.2	0.022374	-0.03692
6	92	1.2	0.011629	-0.0225
7	244	3.1	0.030843	-0.0466
8	115	1.5	0.014537	-0.02671
9	95	1.2	0.012009	-0.02306
10	550	7	0.069523	-0.0805
11	317	4	0.040071	-0.05599
12	954	12.1	0.120592	-0.11079
13	442	5.6	0.055872	-0.07
14	241	3	0.030464	-0.04619
15	625	7.9	0.079004	-0.08709
16	75	0.9	0.00948	-0.01918
17	14	0.2	0.00177	-0.00487
18	253	3.2	0.031981	-0.04781
19	1123	14.2	0.141954	-0.12036
20	560	7.1	0.070788	-0.08141
21	132	1.5	0.016686	-0.02966
	7911			1.178741
Lane: 5				
Peak	Area	%	Pi	H'
1	623	8.5	0.085051	-0.09103
2	53	0.7	0.007235	-0.01549
3	215	2.9	0.029352	-0.04498
4	116	1.6	0.015836	-0.02851
5	178	2.4	0.0243	-0.03923
6	74	1	0.010102	-0.02016
7	179	2.4	0.024437	-0.03939
8	331	4.5	0.045188	-0.06078
9	292	4	0.039863	-0.05579
10	866	11.8	0.118225	-0.10963
11	853	11.6	0.116451	-0.10875
12	114	1.6	0.015563	-0.02814
13	561	7.7	0.076587	-0.08546
14	69	0.9	0.00942	-0.01908
15	55	0.8	0.007509	-0.01595

16	306	4.2	0.041775	-0.05761
17	406	5.5	0.055427	-0.06963
18	1036	14.1	0.141433	-0.12014
19	998	13.8	0.136246	-0.11794
	7325			1.127688

Lane: 6

Peak	Area	%	Pi	H'
1	826	6.6	0.066122	-0.078
2	148	1.2	0.011848	-0.02282
3	288	2.3	0.023055	-0.03775
4	348	2.8	0.027858	-0.04332
5	270	2.2	0.021614	-0.03599
6	150	1.2	0.012008	-0.02306
7	360	2.9	0.028818	-0.04439
8	233	1.9	0.018652	-0.03225
9	268	2.1	0.021454	-0.0358
10	298	2.4	0.023855	-0.0387
11	666	5.3	0.053314	-0.06788
12	665	5.3	0.053234	-0.06781
13	1633	13.1	0.130724	-0.11551
14	397	3.2	0.03178	-0.0476
15	674	5.4	0.053955	-0.06841
16	1024	8.2	0.081972	-0.08905
17	121	1	0.009686	-0.01951
18	53	0.4	0.004243	-0.01007
19	526	4.2	0.042107	-0.05792
20	224	1.8	0.017931	-0.03132
21	1992	15.9	0.159462	-0.12715
22	1186	9.5	0.094941	-0.09708
23	142	1.1	0.011367	-0.0221
	12492			1.213493

Lane: 7

Peak	Area	%	Pi	H'
1	476	13.3	0.132775	-0.11643

2	144	4	0.040167	-0.05608
3	130	3.6	0.036262	-0.05224
4	118	3.3	0.032915	-0.0488
5	123	3.4	0.03431	-0.05025
6	184	5.1	0.051325	-0.06619
7	409	11.4	0.114086	-0.10756
8	82	2.3	0.022873	-0.03753
9	232	6.5	0.064714	-0.07695
10	170	4.7	0.04742	-0.06279
11	7	0.2	0.001953	-0.00529
12	87	2.4	0.024268	-0.03919
13	34	0.9	0.009484	-0.01919
14	974	27.2	0.271688	-0.15376
15	304	8.5	0.084798	-0.09087
16	111	3.2	0.030962	-0.04673
	3585			1.029823

Lane: 8

Peak	Area	%	Pi	H'
1	316	13	0.130041	-0.11521
2	44	1.8	0.018107	-0.03155
3	52	2.1	0.021399	-0.03573
4	285	11.7	0.117284	-0.10916
5	175	7.2	0.072016	-0.08228
6	140	5.8	0.057613	-0.07141
7	163	6.7	0.067078	-0.07871
8	116	4.8	0.047737	-0.06307
9	841	34.6	0.346091	-0.15948
10	298	12.3	0.122634	-0.11177
	2430			0.858364

Lane: 9

Peak	Area	%	Pi	H'
1	955	16	0.160396	-0.12748
2	587	9.9	0.098589	-0.0992
3	273	4.6	0.045852	-0.06138

4	156	2.6	0.026201	-0.04144
5	141	2.4	0.023682	-0.0385
6	139	2.3	0.023346	-0.0381
7	139	2.3	0.023346	-0.0381
8	890	14.9	0.149479	-0.12338
9	75	1.3	0.012597	-0.02393
10	77	1.3	0.012932	-0.02442
11	63	1.1	0.010581	-0.0209
12	334	5.6	0.056097	-0.07018
13	876	14.7	0.147128	-0.12246
14	900	15.1	0.151159	-0.12404
15	253	4.2	0.042492	-0.05829
16	96	1.7	0.016124	-0.0289
	5954			1.040686

Lane: 10

Peak	Area	%	Pi	H'
1	1133	31.2	0.311949	-0.15782
2	2499	68.8	0.688051	-0.11173
	3632			0.269546

Lane: 11

Peak	Area	%	Pi	H'
1	713	34.4	0.34378	-0.15942
2	1361	65.6	0.65622	-0.12006
	2074			0.279473

Lane: 12

Peak	Area	%	Pi	H'
1	780	19	0.190337	-0.13713
2	531	13	0.129575	-0.115
3	646	15.8	0.157638	-0.12648
4	286	7	0.06979	-0.08069
5	362	8.8	0.088336	-0.09309
6	113	2.8	0.027574	-0.043
7	26	0.6	0.006345	-0.01394

8	329	8	0.080283	-0.08794
9	124	3	0.030259	-0.04597
10	84	2	0.020498	-0.03461
11	101	2.5	0.024646	-0.03964
12	716	17.5	0.174719	-0.13238
	4098			0.949867

Binary matrix for PCA (Buffet)											
1.4	2.4	3.4	6.4	8.4	10.4	12.4	14.4	16.4	19.4	21.4	25.4
0	0	0	0.137	0.085	0.066	0.133	0.13	0.16	0.312	0.344	0.19
0	0	0	0	0	0.012	0	0	0	0	0	0
0	0.111	0	0	0	0	0	0	0	0	0.656	0
0	0	0	0	0	0	0	0	0	0.688	0	0
0	0	0	0	0	0	0	0	0	0	0	0
0	0	0	0	0	0	0	0	0	0	0	0
0	0	0	0	0	0	0	0	0	0	0	0.13
0	0	0.098	0	0	0.023	0	0	0.099	0	0	0.158
0	0	0	0.035	0	0	0	0	0	0	0	0.07
0	0	0.028	0.029	0.007	0.028	0.04	0	0.046	0	0	0.088
0	0	0.056	0.04	0.029	0.022	0.036	0	0	0	0	0
0	0	0	0.022	0.016	0.012	0.033	0	0.026	0	0	0.028
0	0	0	0	0	0	0	0	0	0	0	0.006
0	0	0	0.012	0	0	0	0	0	0	0	0
0	0	0	0.031	0.024	0.029	0	0	0	0	0	0
0	0	0	0.015	0.01	0.019	0	0	0	0	0	0
0	0	0	0.012	0.024	0.021	0	0	0	0	0	0
0	0	0	0	0	0.024	0	0	0	0	0	0
0	0	0	0	0	0	0	0	0.024	0	0	0.08
0	0	0	0.07	0.045	0.053	0.034	0.018	0	0	0	0
0	0	0	0.04	0.04	0.053	0.051	0.021	0	0	0	0
0	0	0	0	0	0	0	0	0.023	0	0	0.03
0	0	0.01	0.121	0.118	0.131	0.114	0.117	0.023	0	0	0
0	0	0	0	0	0	0	0	0	0	0	0
0	0.293	0.089	0.056	0.116	0.032	0.023	0.072	0.149	0	0	0
0	0	0	0.03	0.016	0.054	0.065	0.058	0	0	0	0
0	0	0	0	0	0	0	0	0	0	0	0.02
0	0	0	0.079	0.077	0.082	0.047	0.067	0.013	0	0	0
0	0	0.001	0.009	0.009	0.01	0.002	0	0.013	0	0	0
0	0	0	0.002	0.008	0.004	0	0	0	0	0	0
0	0.322	0	0	0	0	0	0	0	0	0	0
0	0	0.015	0.032	0.042	0.042	0.024	0.048	0.011	0	0	0
0	0	0	0	0.055	0.018	0	0	0	0	0	0

Binary matrix for PCA (Buffet)											
1.4	2.4	3.4	6.4	8.4	10.4	12.4	14.4	16.4	19.4	21.4	25.4
0	0	0	0	0	0	0.009	0	0	0	0	0
0	0	0.085	0.142	0.141	0.159	0.272	0.346	0.056	0	0	0.025
0	0	0	0	0	0	0	0	0	0	0	0
0	0	0.107	0.071	0.136	0.095	0.085	0.123	0	0	0	0
0	0	0	0	0	0	0.031	0	0.147	0	0	0.175
0	0	0.511	0.017	0	0.011	0	0	0.151	0	0	0
0	0	0	0	0	0	0	0	0	0	0	0
0	0.273	0	0	0	0	0	0	0	0	0	0
0	0	0	0	0	0	0	0	0.042	0	0	0
0	0	0	0	0	0	0	0	0.016	0	0	0

Shannon-Weaver calculations (Vesuvio)				
Lane: 1				
Peak	Area	%	Pi	H'
1	1039	27.2	0.272417	-0.15385
2	331	8.7	0.086786	-0.09213
3	322	8.4	0.084426	-0.09063
4	147	3.9	0.038542	-0.0545
5	372	9.8	0.097535	-0.09859
6	200	5.2	0.052438	-0.06714
7	263	6.9	0.068956	-0.08009
8	197	5.2	0.051652	-0.06647
9	348	9.1	0.091243	-0.09487
10	474	12.4	0.124279	-0.11255
11	121	3.2	0.031725	-0.04754
	3814			0.95837
Lane: 2				
Peak	Area	%	Pi	
1	565	37.5	0.374917	-0.15974
2	171	11.3	0.11347	-0.10724
3	348	23.1	0.230922	-0.14699
4	401	26.6	0.266092	-0.15299
5	22	1.5	0.014599	-0.0268
6	0	0	0	0
	1507			0.593765
Lane: 3				
Peak	Area	%	Pi	
1	1964	14.2	0.142339	-0.12052
2	6110	44.3	0.442818	-0.15666
3	292	2.1	0.021162	-0.03544
4	663	4.8	0.04805	-0.06335
5	133	1	0.009639	-0.01943
6	178	1.3	0.0129	-0.02437
7	996	7.2	0.072184	-0.0824
8	752	5.5	0.054501	-0.06887
9	219	1.6	0.015872	-0.02856
10	31	0.2	0.002247	-0.00595
11	402	2.9	0.029135	-0.04474
12	200	1.4	0.014495	-0.02665
13	643	4.7	0.046601	-0.06205
14	908	6.6	0.065807	-0.07777
15	137	1	0.009929	-0.01989
16	170	1.2	0.012321	-0.02352
	13798			0.860163
Lane: 4				
Peak	Area	%	Pi	
1	937	28.8	0.2876	-0.15565
2	393	12.1	0.120626	-0.1108
3	804	24.7	0.246777	-0.14997
4	354	10.9	0.108656	-0.10474
5	121	3.7	0.037139	-0.05312
6	71	2.2	0.021793	-0.03621
7	313	9.6	0.096071	-0.09774
8	93	2.9	0.028545	-0.04409
9	94	2.9	0.028852	-0.04443
10	78	2.2	0.023941	-0.03881
	3258			0.835549
Lane: 5				
Peak	Area	%	Pi	
1	1102	8.2	0.082319	-0.08927
2	235	1.8	0.017554	-0.03082
3	584	4.4	0.043624	-0.05934
4	1041	7.8	0.077762	-0.08626
5	242	1.8	0.018077	-0.03151
6	985	7.4	0.073579	-0.08338
7	922	6.9	0.068873	-0.08003
8	173	1.3	0.012923	-0.02441
9	375	2.8	0.028012	-0.04349

Peak	Area	%	Pi	
10	318	2.4	0.023754	-0.03858
11	354	2.6	0.026444	-0.04172
12	1930	14.4	0.14417	-0.12126
13	563	4.2	0.042056	-0.05788
14	3677	27.5	0.274669	-0.15414
15	361	2.7	0.026966	-0.04232
16	525	3.8	0.039217	-0.05516
	13387			1.039567

Lane: 6

Peak	Area	%	Pi	
1	1217	13	0.129827	-0.11511
2	410	4.4	0.043738	-0.05945
3	372	4	0.039684	-0.05561
4	335	3.6	0.035737	-0.05171
5	128	1.4	0.013655	-0.02546
6	1239	13.2	0.132174	-0.11616
7	1580	16.9	0.168551	-0.13034
8	423	4.5	0.045125	-0.06072
9	474	5.1	0.050565	-0.06554
10	446	4.8	0.047578	-0.06293
11	449	4.8	0.047898	-0.06321
12	2088	22.3	0.222744	-0.14527
13	66	0.7	0.007041	-0.01515
14	147	1.3	0.015682	-0.0283
	9374			0.994957

Lane: 7

Peak	Area	%	Pi	
1	1324	14.8	0.148347	-0.12294
2	2473	27.7	0.277087	-0.15444
3	513	5.7	0.057479	-0.0713
4	164	1.8	0.018375	-0.0319
5	428	4.8	0.047955	-0.06326
6	717	8	0.080336	-0.08798
7	1193	13.4	0.133669	-0.11682

Peak	Area	%	Pi	
8	306	3.4	0.034286	-0.05022
9	585	6.6	0.065546	-0.07757
10	623	7	0.069804	-0.0807
11	599	6.8	0.067115	-0.07874
	8925			0.935874

Lane: 8

Peak	Area	%	Pi	
1	992	18.1	0.180956	-0.13435
2	273	5	0.049799	-0.06488
3	1816	33.1	0.331266	-0.15895
4	243	4.4	0.044327	-0.05999
5	178	3.2	0.03247	-0.04833
6	85	1.6	0.015505	-0.02806
7	109	2	0.019883	-0.03383
8	748	13.6	0.136447	-0.11803
9	498	9.1	0.090843	-0.09463
10	500	9.1	0.091208	-0.09485
11	40	0.8	0.007297	-0.01559
	5482			0.851491

Lane: 9

Peak	Area	%	Pi	
1	306	17.4	0.173666	-0.13204
2	268	15.2	0.1521	-0.1244
3	310	17.6	0.175936	-0.13277
4	4	0.2	0.00227	-0.006
5	9	0.5	0.005108	-0.01171
6	92	5.2	0.052213	-0.06695
7	87	4.9	0.049376	-0.06451
8	686	39	0.38933	-0.1595
	1762			0.697871

Lane: 10

Peak	Area	%	Pi	
1	1052	22.1	0.220684	-0.14482

	2	512	10.7	0.107405	-0.10407
	3	1869	39.2	0.39207	-0.15943
	4	375	7.9	0.078666	-0.08686
	5	276	5.8	0.057898	-0.07164
	6	154	3.2	0.032305	-0.04816
	7	130	2.7	0.027271	-0.04266
	8	271	5.7	0.056849	-0.07079
	9	117	2.5	0.024544	-0.03952
	10	11	0.2	0.002308	-0.00608
		4767			0.774038
Lane: 11					
Peak		Area	%	Pi	
	1	436	59.9	0.598901	-0.13334
	2	210	28.8	0.288462	-0.15574
	3	48	6.6	0.065934	-0.07786
	4	34	4.7	0.046703	-0.06215
		728			0.429093

Binary matrix for PCA (Vesuvio)

1	3	4	5	8	10	14	16	18	21	23
0.272	0.375	0.142	0.288	0.082	0.13	0.148	0.181	0.174	0.221	0.599
0	0	0	0	0	0	0	0.05	0.152	0.107	0.288
0	0.113	0	0.121	0.018	0.044	0	0	0	0	0
0	0	0	0	0	0	0.277	0.331	0.176	0.392	0
0	0	0.443	0	0	0	0	0	0	0	0
0.087	0	0	0	0.044	0.04	0	0	0	0	0
0.084	0	0	0	0.078	0	0.057	0.044	0.002	0.079	0
0	0	0	0	0	0.036	0	0	0	0	0
0	0	0.021	0	0	0	0	0	0	0.058	0
0.039	0	0.048	0	0	0.014	0	0	0.005	0	0
0	0	0	0	0.018	0	0	0	0	0	0
0	0	0	0	0	0	0	0	0	0.032	0
0	0	0	0	0	0	0.018	0.032	0	0.027	0
0	0	0.01	0	0	0	0	0	0	0	0
0	0	0.013	0	0	0	0.048	0.016	0	0.057	0
0	0	0	0	0	0	0	0.02	0	0	0
0	0	0.072	0	0.074	0.132	0.08	0	0	0	0
0.098	0.231	0.055	0.247	0.069	0.169	0.134	0.136	0.052	0	0.066
0	0	0	0	0	0	0	0	0	0.025	0
0.052	0	0	0	0	0	0	0	0	0	0
0	0.266	0	0.109	0.013	0.045	0	0	0	0	0
0	0	0	0.037	0	0	0	0	0	0	0
0	0	0	0	0.028	0	0	0	0	0	0
0.069	0	0	0.022	0.024	0	0	0	0	0	0
0	0	0	0	0.026	0	0	0	0	0	0
0	0	0.016	0	0	0	0	0	0	0	0
0	0	0	0.096	0	0	0	0	0	0	0
0	0	0	0	0	0.051	0	0	0	0	0
0	0	0.002	0	0	0	0	0	0	0	0
0.052	0	0	0	0.144	0.048	0	0	0.049	0	0
0	0	0.029	0	0	0	0	0	0	0	0
0	0	0	0	0.042	0.048	0	0	0	0	0
0	0	0.014	0	0	0	0	0	0	0	0

0.091	0	0	0	0	0	0.034	0	0	0	0
0	0.015	0	0	0	0	0	0	0	0	0
0	0	0	0.029	0	0	0.066	0.091	0	0	0
0	0	0	0	0	0	0	0	0	0.002	0
0	0	0	0.029	0	0	0	0	0	0	0
0.124	0	0.047	0	0.275	0.223	0.07	0.091	0	0	0
0	0	0	0	0	0	0	0	0.389	0	0
0	0	0	0	0	0	0	0	0	0	0.047
0	0	0.066	0	0	0	0	0	0	0	0
0.032	0	0	0	0.027	0.007	0	0	0	0	0
0	0	0.01	0	0	0	0	0	0	0	0
0	0	0.012	0.024	0.039	0.016	0.067	0.007	0	0	0