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# Response of microbial communities to synthetic winery wastewater in biological sand filters

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A thesis submitted in partial fulfilment of the requirements for the degree of the Magister Scientiae in the Department of Biotechnology, University of the Western Cape.

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**March 2017**

**Keywords:** biological sand filters, winery wastewater, constructed wetlands, terminal restriction length polymorphism

## ABSTRACT

**Response of microbial communities to synthetic winery wastewater in biological sand filters**

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There has been a significant increase in the use of constructed wetlands globally for the bioremediation of wastewater (Welz *et al*., 2011). In South Africa, the wine industry generates more than one billion litres of wastewater annually and this is disposed of by irrigation (Burton *et al*., 2007). A more cost effective and simple system need to be employed for the treatment of winery effluent and a possible solution would be the use of biological sand filters (BSFs). The microbial communities present in these BSFs play an important role in the biodegradation of the organic wastewater pollutants. Physicochemical and microbiological studies have been used to determine the performance of BSFs for the remediation of winery effluent since 2009.

In this study, changes in the bacterial and fungal communities in different spatial niches was analysed through the use of molecular fingerprinting techniques [terminal restriction fragment length polymorphism (T-RFLP)] of the 16S rRNA gene and fungal ITS gene as a response to the exposure to synthetic winery wastewater and to perform functional studies using q-PCR on selected genes. Changes in the bacterial and fungal community profiles were observed at the different niches after amendment with synthetic winery wastewater. This result was confirmed by performing the phylogenetic analysis on the bacterial population present within the BSF systems. Sand organisms including *Clostridium*, *Sarcina*, *Streptomyces*, Actinobacteria were among the expected species present in the sand samples at the deep inlet of the BSFs. Through the study by Burton *et al*, (2007), one could hypothesise which organisms mentioned above were able to metabolise the components of the synthetic winery wastewater to secondary metabolites. Amplification of the functional gene through the use of qPCR of catechol 2,3-dioxygenase was successful. Increase in the amount of copy numbers between the samples showed that the increase in expression of the catechol 2,3-dioxygenase meant that there was an increase in the amount of organisms degrading the catehol build-up in the BSF systems.

The outcome of this study, provided insight that amendment with synthetic winery wastewater indeed had an effect on the bacterial and fungal population present in the BSFs and that the expression of functional genes by the microbial communities present in the BSFs also aid in the degradation or build-up of components of the synthetic winery wastewater, confirmed by the metabolite profile of sugars, phenolics and alcohols of each BSF system. The results obtained in this study will aid in the implementation of a full scale system being placed at a small winery for the treatment of winery wastewater.

**DECLARATION**

I declare that *response of microbial communities to synthetic winery wastewater in biological sand filters* is my own work, that it has not been submitted for any degree or examination in any other university, and that all the sources I have quoted have been indicated and acknowledged by complete references.

**Name** Zaida Palmer_____

**Date** _____20 March 2017_____

**Signed** ____Z.Palmer_____

**ACKNOWLEDGEMENTS**

My experiences over the past three years and the write up of this thesis would not have been made possible without the help of my support system. Many individuals have contributed to keeping me “sane” during this time period.

First and foremost Shukran to my creator Allah for guiding me and providing me with the knowledge that I have gained and experienced through this time. Sabr (patience) was the biggest life lesson I learnt. Shukr for providing me with the people that I hold close in my life, new people that I met and that have helped me.

To my supervisors: Marilize Le Roes-Hill, Pamela Welz and Bronwyn Kirby- thanks so much for advice, the motivation, the help with regards to lab work and the editing of all the chapters. I really do appreciate it all.

To my family: although we have had our days I do appreciate everything that my family especially my mother and brother have done for me. The evenings where you allowed me to vent and just lose it, made persevering in the lab so worthwhile.

To my friends: Ni-shaat one of my biggest supporters and voice in my head saying YOU CAN DO IT!!! Shukran to you for all that you do and I keep making duah that Allah grant you all the best Insha Allah Ameen.

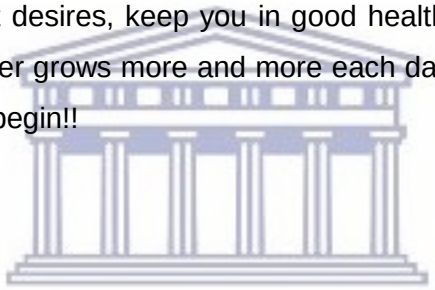
Shafieka Isaacs, I met you in 2013 and in more ways than one you became a good influence and help to me. Had I met you before this and you told me that Masters would have caused so much chaos in my life I might have taken your advice. Shafieka shukran for all the hours of trying to figure out peak scanner, T-REX and primer 6. Both the buckets and the BSFs got the better of us but the results we got at the end of the day. Weeks of struggling with you sitting on the ottoman next to me and shukran for always being the extra pair of hands that we needed. You are an amazing person and a very warm hearted person. My duah for you will be that you prosper further in life Insha Allah Ameen and be given all that your heart desires.

Dr Jean Baptiste Ramond, Gerda, Mapula: thanks so much for all the assistance with the T-RFLP analysis.

Prof Laurenz and Mrs Corrie Eys: thank so much for taking out the time to assist with the statistics and Primer 6.

Mrs Anne Swenchgnewaan and Ms Levanya Reddy: thank you for the assistance with the reagents for qPCR and helping with the use of the LC480 and all the days of optimization during the analysis

Last but certainly not least, to my husband Nadeem Anthony. As I sit here typing this, the tears can't help from falling. Words cannot describe how much I appreciate all the things you have done for me. You have been my biggest fan since I have met you. The countless days I came home crying telling you I can't anymore baby, your words still linger in my head; "Zaida you are almost there, keep focused.....Im proud of you, Im here to support you and hold your hand through all of this". All the saturdays we spent in the lab with you making media or filling tips while I explain all the science and geeky terms to you. Shukran for taking off your weekends to help me complete my experiments. This has been one long adventure for the both of us but Alghamdulillah each day a new life lesson has been learnt and brought us closer together. Shukran for your patience, your love, all your motivation and support. I make duah, that Allah provide you with all that your heart desires, keep you in good health and make sure that our bond and love for each other grows more and more each day. Let our future be bright and let the new adventure begin!!



## PUBLICATIONS AND CONFERENCES

### List of Publications

1. Welz PJ, Ramond, J-B., Palmer, Z., Isaacs, S., Kirby, B., Le Roes-Hill, M. (2014) The effect of redox status on the bacterial community structure and biodegradation rate of organic fractions of agri-industrial effluent in sand bioreactors. Proceedings of the 9th IWA International Symposium on Waste Management Problems in Agro Industries, Volume II.
2. Welz, P.J., Palmer, Z., Isaacs, S., Kirby, B., Le Roes-Hill, M. (2014) Analysis of substrate degradation, metabolite formation and microbial community responses in sand bioreactors: a comparative study. Journal of Environmental Management 145:147-156.

### Conference Proceedings

Oral Presentation

1. Palmer, Z., Le Roes-Hill, M., Isaacs, S., Kirby, B., Welz, P.J. Response of microbial communities to synthetic winery wastewater in sand bioreactors (SASEV, Somerset West, 2014).

**CONTENTS**

**CHAPTER THREE: RESULTS AND DISCUSSION**

## LIST OF ABBREVIATIONS

| | |
|---|---|
| AS | Activated Sludge |
| BOD | Biological Oxygen Demand |
| BSFs | Biological sand filters |
| C23O | Catechol 2,3-dioxygenase |
| COD | Chemical oxygen demand |
| CWs | Constructed wetlands |
| DO | Dissolved Oxygen |
| DGGE | Denaturing gradient gel electrophoresis |
| FWSF | Free water surface flow |
| HSSF | Horizontal sub-surface flow |
| ITS | Internal transcribed spacer |
| LB | Luria Bertani |
| LB-amp | LB-ampicillin |
| mDNA | Metagenomic DNA |
| NGS | Next Generation Sequencing |
| q-PCR | Quantitative PCR/ real time PCR |
| *rpoB* gene | Ribosomal RNA polymerase β subunit gene |
| TOC | Total organic carbon |
| T-RFLP | Terminal-restriction fragment length polymorphism |
| T-RFs | Terminal-restriction fragments |
| SWW | Synthetic winery wastewater |
| VFA | Volatile Fatty Acid |
| VSSF | Vertical subsurface flow |

## LIST OF FIGURES

## LIST OF TABLES

# CHAPTER ONE

# LITERATURE REVIEW

## 1.1 General introduction

Natural wetlands have been found to purify wastewater. In an attempt to mimic this, researchers have designed and created constructed wetlands to treat wastewater. Some of these systems are planted (traditional) while others are unplanted. Among the unplanted constructed wetlands (CWs), those that are filled with sand are termed biological sand filters (BSFs). Wineries and other grape processing industries generate variable volumes of wastewater (Petruccioli *et al*., 2000) and the various systems for the treatment of wastewater effluent have their own advantages/disadvantages in relation to cost, efficacy and reliability (Andreottola *et al*., 2011; Mosse *et al*., 2011). This thesis reports on the use of molecular fingerprinting techniques to monitor changes in microbial communities present in BSFs after amendment with synthetic winery wastewater. The presence of functional genes in the microbial populations was assessed through quantitative PCR (qPCR). This work formed part of the Water Research Commission (WRC) funded Project entitled "Treatment of winery wastewater in unplanted constructed wetlands" (Welz *et al*., 2015). Previous studies by Ramond *et al*. (2010) and Welz *et al*. (2011) have shown that amendment with synthetic winery wastewater has an impact on the bacterial community structure in BSFs. However, at present, little is known of the role that the fungal communities play in bioremediation or the expression of functional genes (both aspects are covered in this thesis). The following literature review has been divided into two sections. Firstly, CWs and BSFs will be discussed, while the second section relates to the molecular fingerprinting techniques used in this study and the importance of assessing the expression of functional genes in these systems.



### 1.1.1 Current state of the art

The production of wine results in the generation of large volumes of wastewater. With increasing environmental restrictions for the disposal of this effluent, a manageable wastewater treatment system is required (Mosse *et al*., 2011). Several systems for the treatment of winery wastewater e.g. biological treatment systems, activated sludge systems, membrane based reactors; are currently available and ongoing advancements offers access to promising new technologies (Andreottola *et al*., 2011).

Biological systems used for the treatment of wastewater provide eco-engineered systems containing different micro-environments where numerous physical, chemical, and biological processes can take place. The biological processes assist in the recycling, removal, transformation or immobilization of nutrients and other wastewater pollutants through aerobic or anaerobic systems using suspended biomass or biofilms attached to inert media/supports (Andreottola *et al*., 2011; Imfeld *et al*., 2009; White *et al*., 2006).

With the variability in amounts of winery wastewater generated depending on season, the choice of treatment system is not simple. Many factors need to be taken into account such as retention time and pollutant removal efficiency (Jourjon *et al*., 2005). Several biological treatment systems are available such as aerobic, anaerobic and a combination of the two systems. Examples of aerobic systems include activated sludge, jet-loop reactor (JLR), sequential batch reactor (SBR), fixed bed biofilm reactor (FBBR) system, air micro-bubble bioreactor (AMBB), aerated submerged biofilters (ASBs), rotating biological contactor (RBC), aerated lagoons, biological sand filter (BSF), membrane bioreactor (MBR), anaerobic digestion, upflow anaerobic sludge blankets (UASB), anaerobic fluidized bed reactor (AFBR), upflow anaerobic filter (UAF), and anaerobic moving bed biofilm reactor (AMBBR) (Iannou *et al*.,2015). For the purpose of the study Figure 1.1 shows the examples of aerobic systems for the treatment of winery wastewater (Lofrano and Meric, 2016).



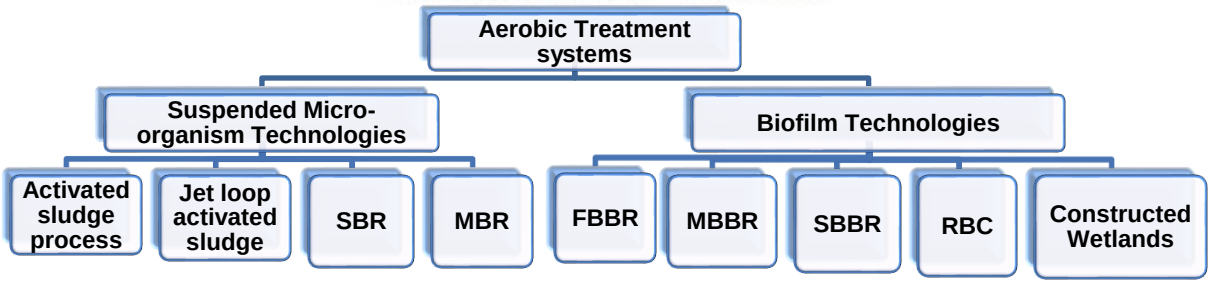



**Figure 1.1:** Examples of Aerobic treatment systems for winery wastewater. Systems can either be based on suspended microorganism technologies or biofilm technologies. Abbreviations: Fixed bed biofilm reactor (FBBR), Membrane bioreactors (MBR), Moving bed biofilm reactors (MBBR), Rotating biological contactor (RBC), Sequencing batch reactor (SBR), Sequencing batch biofilm reactors (SBBR) (Lofrano and Meric, 2016).

Aerobic treatment systems are the most commonly used systems for the treatment of winery wastewater, due to their high efficacy and ease of use. For the biological treatment of winery wastewater the most conventional method is activated sludge which provides a simple, flexible and economical treatment system amounting for the high variability levels of winery effluents (Ioannou *et al*., 2015). The advantages and disadvantages of the biological system highlighted in Figure 1.1 is summarised in Table 1.1.

Anaerobic digestion systems for the treatment of winery wastewater have potential benefits such as low sludge production and can be profitable by the generation of biogas (Andreottola *et al*., 2009, Keyser *et al*., 2003, Satyawali and Balakrishnan, 2008). Examples of anaerobic systems include: continuous stirred tank reactors, anaerobic lagoons, upflow anaerobic sludge blanket reactor, anaerobic filters, upflow sludge blanket filtration, fluidized bed and anaerobic sequencing batch reactors. However, the disadvantage of using such systems is the production of volatile fatty acids (VFAs) which can accumulate in the reactors resulting in a decrease in pH. In addition, in many anaerobic digestion systems the anaerobic processes need to be followed by an aerobic treatment step. The most promising anaerobic process resulted in the study by Moletta, (2005) that demonstrated the use of a granular up-flow anaerobic sludge blanket reactor (UASB) for the treatment of winery wastewater. The success of the system depended on the formation of active granules which were further innoculated with selected bacterial strains. One reactor was seeded with granular sludge enriched with *Enterobacter sakazakii* and this system resulted in a 90% COD removal (Keyser *et al*., 2003). Literature has stated that common drawbacks with anaerobic systems are problems associated with clogging and channelling (Moletta, 2005).

A review by Ioannou *et al*., 2015 assessed that constructed wetlands (CWs) with plants can tolerate and detoxify wastewater, and these systems have also been used for the treatment of agro-industrial waste. This method of remediation combines physical, chemical and biological process to remove contaminants. Iannou *et al*., 2015 discussed the categories of COD, biological oxygen demand (BOD), total nitrogen, as the most frequent parameters for the determination of the efficiency of the biological processes for the treatment of winery wastewater. Biological sand filters treatment systems are considered environmentally friendly and cost effective. However, these systems are not able to adequately remove the organic matter that exists in high concentration levels in winery wastewater, which may later result in the buildup of toxic compounds which may degrade slowly or become recalcitrant. Winery effluents contain phenolic compounds, which have a high COD and generally are acidic, and thus care needs to be taken when selecting the microorganisms employed in the adaptation of treating these effluents in BSFs (Strong and Burgees, 2008).

**Table 1.1:** Advantages and disadvantages of biological treatment systems for winery wastewater (Lofrano and Meric, 2016) (modified from Andreottola *et al*., 2005)

| Biological Aerobic treatment system | Advantages | Disadvantages |
|---|---|---|
| **Activated sludge (AS)** | • High COD and BOD removal efficiency<br>• Short retention time | • pH control<br>• Floc disintegration, bulking<br>• High operational costs<br>• Inhibition of biomass due to polyphenolic compounds<br>• Nutrient addition may be required |
| **Jet loop reactor** | • Absence of mechanical devices for aeration<br>• Reduced energy consumption<br>• No bulking problems | • pH control<br>• Poor sludge settlement can occur<br>• Limited implementation at different scales and especially at full-scale<br>• Nutrient addition may be required |
| **Membrane bioreactor (MBR)** | • Great improvements in treated water quality<br>• Effluents free of suspended solids and bacteria (with UF)<br>• Possibility of direct reuse on site<br>• No sedimentation tanks<br>• Small amount of wasted sludge<br>• Quick start-up<br>• Low footprint | • pH control<br>• Fouling<br>• High capital and managing costs<br>• Inhibition of biomass due to polyphenolic compounds<br>• Nutrient addition may be required |
| **Sequencing biofilm reactors (SBR)** | • Operational flexibility<br>• Less land required than AS<br>• Lower cost than AS<br>• Biomass cannot be washed out<br>• Low maintenance | • pH control<br>• Storage basin to reduce shock loading<br>• Nutrient addition may be required |
| **Fixed bed film bioreactor (FBBR)** | • Reduction of the required volume with respect to the conventional AS system<br>• Reduction in bulking problems<br>• Absence of return flow and backwashing<br>• Easier management with respect to AS | • pH control<br>• Plastic media costs<br>• Nutrient addition may be required |
| **Moving bed biofilm reactors (MBBR)** | • High empty space of plastic media<br>• Backwashing not required<br>• Simple management<br>• No bulking problems | • pH control<br>• Additional cost of plastic media compared to activated sludge<br>• Nutrient addition may be required |
| **Sequencing batch biofilm reactors (SBBR)** | • High empty space of plastic media<br>• Backwashing not required<br>• Simple management<br>• No bulking problems | • pH control<br>• Additional cost of plastic media compared to activated sludge<br>• Nutrient addition may be |

| | | |
|---|---|---|
| | | required |
| **Rotating biological contactors (RBC)** | • Easy to operate<br>• Short start-up<br>• Little maintenance<br>• Effectively oxygenated<br>• Little sloughing of biomass<br>• No bulking problems | • pH control<br>• Low rate of treatment<br>• Nutrient addition may be required |
| **Constructed wetlands (CWs)** | • Low-energy consumption and costs<br>• Capacity to treat record high organic loads<br>• High rate of treatment in relatively short adaptation time<br>• | • High retention time<br>• Large area<br>• pH and TSS influence<br>• Odour problem<br>• Feasible only in low population density areas |

Currently, numerous winery wastewater treatments are available but the development of alternative process combinations is required in order to increase the efficiency of removal of both recalcitrant organic compounds, as well as ecotoxicity. These systems could also simultaneously reduce operational costs. Literature has also stated that physicochemical processes (e.g. Coagulation/ flocculation) have been found to be effective for the pre-treatment of winery wastewater, and more specifically, lowering the total suspended solids for further treatment by other biological, membrane filtration or separation processes (Ioannou *et al*., 2014; Ioannou *et al*., 2015).

Biological treatment is particularly appropriate for the treatment of winery wastewater showing high removal rates of organic compounds. For the residual removal of organics, a further pre- or post-treatment system is needed for the safe disposal of the effluent into the environment.

### 1.2 Constructed wetlands (CWs) and biological sand filters (BSFs)

Natural wetlands are present worldwide and in a range of different settings. These environments are described as being in- between a terrestrial and aquatic system (Haberl *et al*., 2003), and are the most biologically diverse of all ecosystems (Ramsar Convention Secretariat, 2013). Inadvertently, they have been used for the treatment of various types of wastewaters. CWs and BSFs are also known as treatment wetlands and are engineered wastewater treatment systems that mimic the bioremediation processes associated with a natural wetland (Vymazal, 2005).

According to Juwarkar *et al*. (2012) and Semple *et al*. (2007) in order to establish a functional microbial consortium and the successful bioremediation of effluent/organic compounds, the environmental conditions should be favourable for growth of microorganisms and their metabolic activities. With this being said, conventional reactor-type biological treatment systems generally fit these given criteria. Reactor based technologies are usually expensive to install, require lots of energy and skill for operation, making this type of technology unsuitable in small wineries (Christen *et al*., 2010). CWs are reactor-type biological treatment systems that have been shown to be suitable for the treatment of winery wastewater as reports have shown that wine producing countries use these systems for the reduction of the chemical oxygen demand (COD) in winery wastewater (De la Varga *et al*., 2013, Mulidzi, 2010).

CWs generally consist of a physical substrate (usually sand or gravel) that serves as a support for plants and microbial communities that work synergistically to treat wastewaters (Kiviasi, 2001; Glick, 2010; Vymazal, 2005). Plants metabolize available nutrients and are able to accumulate heavy metals or directly degrade certain organic contaminants (Glick, 2010). While BSF systems are similar to CWs in configuration, they do not contain any plants. Research has shown that despite CWs being slow filtration systems for the treatment of winery wastewater, the resulting effluent has shown to be toxic to the plants used in CWs (Arienzo *et al*., 2009; Christen *et al*., 2010). As BSF do not contain plants, they rely solely on the substrate and microorganisms for the treatment of effluent. The engineering and design of these systems allows for the structural and flow mechanisms to be tailored to the requirements of the treatment of specific target waste streams. CWs and BSFs are of particular interest as they represent a cost-effective, ecologically-friendly and aesthetically attractive option for wastewater treatment (Welz *et al*., 2015).

### 1.2.1 Hydraulic flow regime of CWs and BSFs

There are three basic hydraulic regimes employed in the design of CWs; free water surface flow (FWSF), horizontal sub-surface flow (HSSF), and vertical subsurface flow (VSSF) (Figure 1.2) (Vymazal, 2007). In CWs and BSFs, the flow of wastewater may take place predominantly over the surface (surface flow) or within the substratum (subsurface flow). Subsurface flow may be from the top of the CWs and BSFs to the bottom (vertical subsurface flow) or from the inlet to the outlet (horizontal subsurface flow) (Vymazal, 2005). The combination of different hydraulic regimes, however, have become more popular, either by using a series of CWs each with a different mode of operation or by combining more than one hydraulic flow regime in the same CW setup (Burton *et al*., 2011; Toscano, 2009).

Faulwetter *et al*., (2009) describes the use of treatment systems stating that VSSF systems will typically favour aerobic microbial populations and pollutant removal while HSSF systems will favour anaerobic populations. The Feeding mode (batch, intermittent or continuous) appears to have a secondary influence on the performance of the systems, with batch mode favouring more aerobic processes and continuous mode favouring more anaerobic processes. More research into microbial population density and diversity heterogeneity, both spatially and temporally, between feeding modes will help to further optimize design of HSSF systems.

The BSFs used in this study have been identified as being suitable for the efficient treatment of winery effluent (Ramond *et al*., 2013; Welz *et al*., 2014). Through the study by Ramond *et al*., (2013), showed that through the use of BSFs for the treatment of winery effluent the microbial communities were responsive to the impact of the winery effluent and that as the community structure changed over time the remediation capacity remained high with a COD and phenolic removal rates of >98% throughout the experimental period. The study by Welz *et al*., (2014) found that when the COD influent was at 2027 mg/L the COD removal rate was at 76% with an excellent removal of organics being achieved. These studies became the basis for the work in the thesis. These were designed in the configuration of a subsurface flow system with both vertical and horizontal flow. Subsurface flow CWs are either operated in batch or continuous mode. Batch mode applies to VSSF CWs, where the surface is flooded and followed by a period of drainage. If the period of drainage is more than 24 hours it is termed ‘tidal’ and if it is a shorter time period it is termed ‘pulsed’ (Austin, 2009).

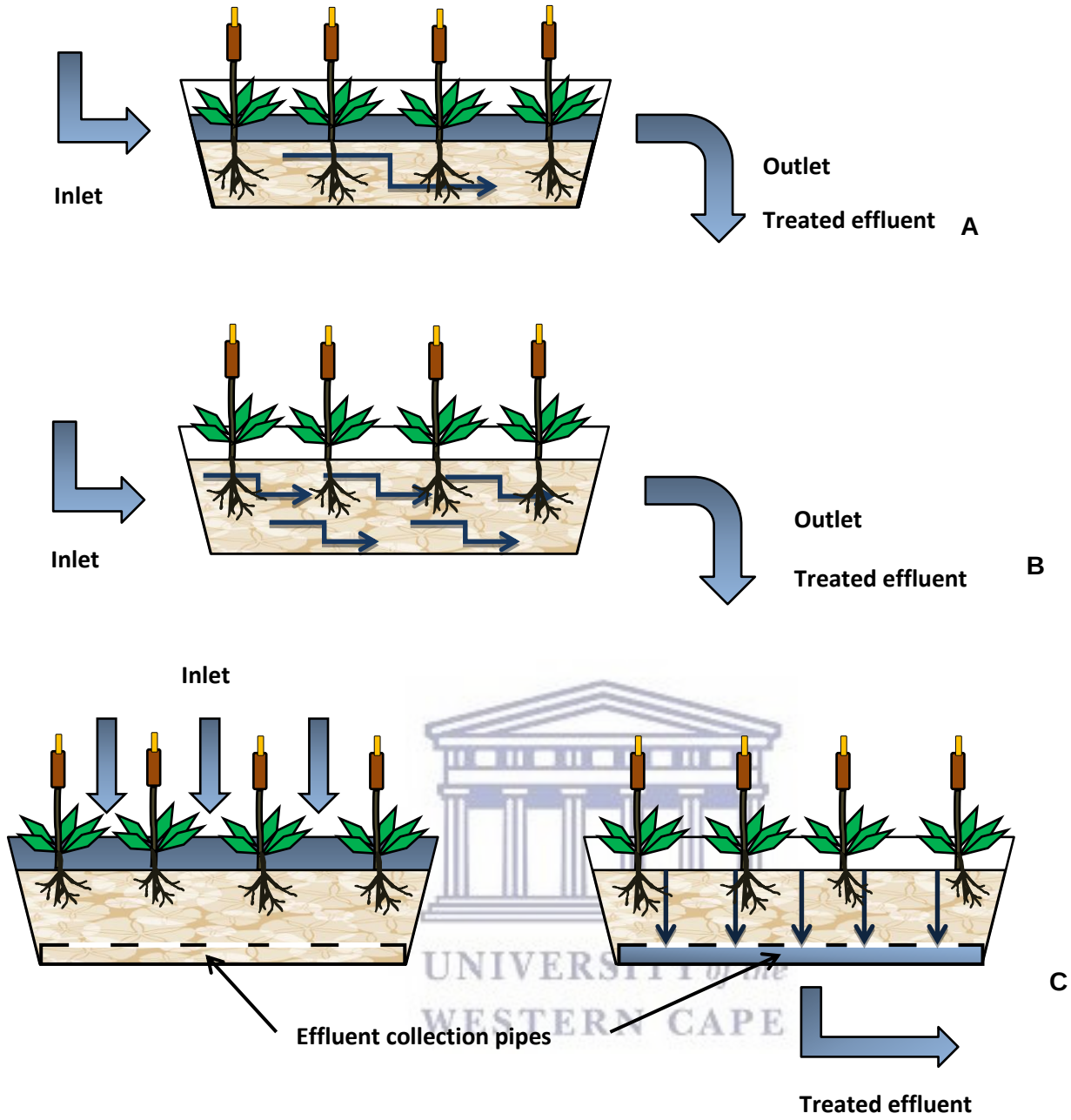



**Figure 1.2:** Schematic diagram of the FWSF (A), HSSF (B) and VSSF (C) operated in batch mode. A "fill" period (left) is followed by a "drain" period (right) (Welz, 2010).

### 1.2.2 Substrate composition of CWs and BSFs

Removal rates of carbon (C), nitrogen (N) and phosphorus (P) are closely related to the chemical and physical properties of the substrate medium in CWs (Zhang, 2007). Examples of substrate media which have been employed include sand, soil, gravel, zeolite, slag, compost, and alum sludge. Sand and gravel are the most common substrates used in CWs (Aslam, 2007; Li, 2008, Babatunde, 2011).

Sand particles provide a large surface area for biofilm attachment. The shape and size of the sand particles plays a role in biofilm formation and subsequent removal performance. It has been demonstrated that natural sand presents a superior biofilm attachment surface to crushed sand, resulting in enhanced removal of C and ammonia ($NH_3$) (Torrens, 2009). One of the major disadvantages of sand-filled CWs and BSFs is that the small grain size can result in clogging of the matrix pores by suspended solids and/or biofilm. Clogging can be prevented by the use of pre-filters or clarifiers to remove suspended solids. The use of filters in conjunction with batch mode could ensure complete substrate degradation between treatments (Knowles, 2010). A study by Girvan *et al*. (2003) indicated that the chemical composition of the support matrix is a determining factor in shaping the microbial communities present within sand samples. Similarly, in BSFs, the composition of the sand has been shown to be the most important factor for the selection of bacterial species (Welz *et al.,* 2014).



### 1.2.3 Treatment of industrial wastewater in CWs and BSFs

In many developing countries, waste streams such as untreated sewage, industrial and domestic wastewater, as well as agricultural run-off may contaminate aquatic environments. The water being released into the environment could be seen as unsuitable for human consumption, fish production or recreation (Haberl *et al*., 2003). It is important to treat contaminated water before discharge either via irrigation or directly into the environment. CWs and BSFs have been used to treat municipal wastewater, landfill leachate, agricultural wastewater and industrial wastewater (Poach, 2003; Vymazal, 2005).

The environmental impact of winery wastewater is notable and this can cause eutrophication (nutrient enrichment) of water resources (e.g. natural streams, rivers, dams and wetlands). If untreated winery wastewater with high levels of BOD are allowed to flow into water streams the dissolved oxygen (DO) in the water streams may quickly be consumed, leading to the suffocation of aquatic and amphibious life (Chapman *et al*., 1995). Therefore, the adequate

treatment of the winery effluent is essential as it could cause significant environmental damage if released untreated (Strong and Burgees, 2008).

Chemical oxygen demand (COD) refers to the measurement of organic compounds within wastewater. In a literature survey Vymazal and Kröpfelova (2009) investigated the removal of organics in 292 horizontal flow CWs situated in 36 countries. The study concluded that municipal wastewater had the lowest COD inflow concentrations, while the highest COD concentration was observed for industrial wastewater (Table 1.2). The authors deduced that CWs could be utilised for the treatment of several waste streams. It has been reported that high COD removal rates can be achieved in VSSF CWs treating domestic wastewater by applying low loading rates and loading frequencies (Prochaska, 2007).

**Table 1.2:** Performance characteristics of 292 horizontal flow CWs (Vymazal and Kröpfelova, 2009)

| **Type of wastewater** | **Number of CWs** | **Average Influent COD (mg $L^{-1}$)** | **Average effluent COD (mg $L^{-1}$)** | **Average COD removal (%)** |
|---|---|---|---|---|
| Agricultural[a] | 17 | 871 | 327 | 63.0 |
| Industrial[b] | 25 | 1865 | 789 | 63.1 |
| Landfill leachate | 6 | 933 | 698 | 24.9 |
| Municipal | 244 | 287 | 76 | 63.2 |

[a]Farm effluent, chicken manure, shrimp aquaculture effluent, trout farm effluent and dairy parlour effluent

[b] Abbatoir and meat processing effluent, food processing effluent, distillery effluent, winery effluent, petrochemical effluent, lignite pyrolysis effluent, mixed industrial effluent



Welz *et al*. (2011) reported the use of BSFs for the treatment of winery effluent with a bi-weekly loading frequency, a hydraulic loading rate of 1.95 $L.m^3.day^{-1}$ and a high influent COD (ethanol) of 15 800 mg $L^{-1}$. During this study >98% COD removal was consistently achieved. These results strongly suggest that the presence of plants is not a prerequisite for effective organic removal. This was further supported by the findings of Tietz *et al*. (2008) and Melian (2010) who reported that plants had no significant influence on CW productivity, microbial biomass or total organic carbon (TOC) removal from pre-settled municipal wastewater, but rather that the type of substrate medium significantly affected COD and TOC removal. Additional research is needed to clarify which parameters (chemical (e.g. COD, BOD), and operational parameters would be HRT, OLR etc) are more important for the removal of specific organic wastes. A study by Welz *et al*. (2016) suggested the use of stabilisation ponds as a pre-treatment step for the treatment of winery wastewater prior to the use of a BSF.

According to the review by Wu *et al*. (2015) for the treatment of effluents from various industries (food, textile, tannery, oil and gas, cooking) through the use of CWs has been investigated. The use of CWs for the treatment of these various effluents has been shown to be cost effective and require little maintenance. Each kind of industrial effluent has a specific character which must be taken into consideration when designing and planning the CW. Wu *et al*. (2015) summarises each industrial effluent and as an example explains that food industry waste is often rich in organic compounds and salt, in contrast to the mining industry that might have little or no organic content, but would be high in metals and dissolved solids.

### 1.2.4 Treatment of agricultural wastewater in CWs and BSFs

#### 1.2.4.1 Winery wastewater

The South African Wine Industry consists of about 600 independent wineries located mostly in the Western Cape region. The industry is said to generate more than one billion litres of winery effluent per annum (Mosse *et al*., 2011; Sheridan *et al*., 2011) which needs to be treated before being discharged. Although wastewater is regarded as a significant environmental risk, it is used by most of the wineries to irrigate their land (Van Schoor, 2004).



Treated winey wastewater needs to be compliant with South African legislation (South Africa Water Act 96, 1998), which states that discharging biodegradable industrial waste into the environment should meet the maximum COD limits ranging between 30 mg $L^{-1}$ to 5000 mg $L^{-1}$ depending on the daily amount of wastewater being generated. Remediation is problematic for smaller wine cellars due to the fact that the composition and volume fluctuates depending on the season (Malandra *et al*., 2003; Saadi *et al*., 2007; Sheridan, 2007; Arienzo *et al*., 2009; Bolzonella and Rosso, 2013;). In addition, there is an increase in organic chemicals observed over the vinification period. Vinification refers to the process of making wines from grapes (Chambers and Pretorius, 2010). Acetic acid, ethanol, fructose, glucose, and sucrose are significant contributors to COD levels in winery wastewater (Malandra *et al*., 2003; Sheridan, 2007). Small to medium sized wineries in rural areas are not equipped to treat winery effluent and cannot afford sophisticated systems (Christen *et al*., 2010). In these wineries, a more cost effective, simple system such as CWs or BSFs would be ideal for the treatment of wastewater. Ideally these systems should be tolerant to seasonal input flux, and not require lengthy start up and shut down periods (Christen *et al*., 2010).

There has been an increase in successfully using CWs for the treatment of various industrial effluents (Wu *et al*., 2015) and the use of CWs for the treatment of winery wastewater have been highlighted in Table 1.2. A study by Welz *et al*. (2014) demonstrated the tolerance of BSFs to season input fluxes. In BSF systems, the substratum provides a large surface area for biofilm attachment and may contain co-factors required for microbial metabolism (Calvo-Bado, *et al*., 2003; Hunter *et al*., 2013; Welz *et al*., 2014). Given the benefits of BSFs (low cost and ease of operation) these systems have the potential to treat wastewater at small to medium size wineries.

From Table 1.3 it shows that laboratory scale and full scale CWs are quite different in size, exhibit different hydraulic flow and require different pre-treatment steps. However, no performance characteristics was shown the Table was just used to illustrate the use of CWs and BSFs currently being employed. CWs are directly used for the treatment of raw winery wastewater effluent, which has been generated by small rural wineries as indicated by in Table 1.3. One of the major limitations of CWs, is the clogging of the system by biofilm formation and clogging of substrates due to the accumulation of solids (Mosse *et al*., 2011). The solution to this problem is some sort of pre-treatment step that is outlined in Table 1.3. Therefore, it has been estimated that when the productivity levels of winery wastewater is lower than 2000 hL of wine per year the treatment scheme usually consists of a pre-treatment by septic or Imhoff tanks. This Imhoff tank serves as an equalization role and the winery wastewater is can then be treated by a single stage of vertical flow subsurface wetlands. For larger sized wineries, more robust models is set in place are required, such technologies being as anaerobic digestion systems or aerobic biological systems (Masi *et al*., 2014). The A study by Welz *et al*. (2016) suggested the use of continuous flow settling basins and waste stabilization ponds as a pre-treatment step for the treatment of winery effluent.

**Table 1.3**: Performance characteristics of CWs for the treatment of winery wastewater (Wu *et al*., 2015)

| **Location** | Scale | HLR | Pre-treatments | Wetland type and area ($m^2$) | References |
|---|---|---|---|---|---|
| Hopland, California | Pilot | 500 L/d | Upflow coarse-sand filter | HF, 14.9 | (Shepherd *et al*., 2001) |
| Bordeaux region, France | Pilot | | Upflow coarse-sand filter + intensive aeration in a first equalization basin | 2 VF in series, 157 + 174 | (Rochard *et al*., 2002) |
| Bordeaux region, France | Pilot | | Straw screening | 2 VF in series, 35.6 in total | (Rochard *et al*., 2002) |
| La Croce, Siena, Italy | Full | <8 $m^3/d$ | Imhoff tank | A single HF, 215 | (Masi *et al*., 2014) |
| Bolgheri (Leghorn province), Italy | Full | 42 $m^3/d$ | Septic tank | 2 HF in parallel þ VF + VF + VF + FWS + Ponds, 1316 in total. | (Masi *et al*., 2014) |
| Castellina in Chianti-Siena, Italy | Full | 35 $m^3/d$ | Imhoff tank | HF (480) + FWS (850) | (Masi *et al*., 2014) |
| Hopland, California | Full | 137 $m^3/d$ | Facultative lagoons and screening | HF, 4400 | (Grismer *et al*., 2003) |
| GlenEllen, California | Full | 21 $m^3/d$ | Facultative lagoons and screening | HF, 304 | (Grismer *et al*., 2003) |
| Stellenbosch, South Africa | Full | | | HF, 160 | (Sheridan *et al*., 2014) |
| Goudini, South Africa | Full | 4 $m^3/d$ | | HF, 180 | (Mulidzi, 2007) |
| California | Full | 6 $m^3/d$ | | 2 HF in parallel, each 58 | (Grismer and Shepherd, 2011) |
| California | Full | 22 $m^3/d$ | | 2 HF in parallel, 72 + 49 | (Grismer and Shepherd, 2011) |
| Galiza, (Northwest Spain), Spain | Full | 6.8 $m^3/d$ | HUSB anaerobic digester,6 $m^3$ | A VF (50) and 3 parallel HF (100 each) | (Serrano *et al*., 2011) |

### 1.2.4.2 The fate of organics and inorganics in CWs and BSFs

During the bioremediation of various waste streams, the substrata can become saturated with inorganic, non-volatile compounds which may require mechanical removal. One of the major advantages of using BSFs or CWs for the treatment of organic waste streams is that complete mineralization of many hydrocarbon pollutants may be achieved (Welz *et al*., 2011). However, with non-domestic wastewaters there is a risk of toxic metabolites and pollutants being formed in the CWs. The biodegradability of organic compounds also needs to be considered during CW and BSF design (David, 1999; Welz *et al*., 2011).

The microbial communities functioning in CWs are involved in many biological processes including hydrocarbon degradation, transformation, and mineralisation (Knowles, 2010). The results of these studies obtained are on the basis of the wastewater composition, microbial acclimation, and CW substrate type and plant species (Li, 2008; Burton *et al*., 2011; Welz *et al*., 2011; Rodriguez-Caballero *et al*., 2012).

With each litre of wastewater generated from wine cellars the effluent COD values typically range from 800-12 800mg/L, however some studies have reported peaks of 25 000 mg/L (Malandra *et al*., 2003; Saadi *et al*., 2007). Inorganics (sodium and potassium) are often found in these samples, due to the high acidity and high percentage of organics present in winery wastewater. These inorganics can sometimes become problematic to remove (Strong and Burgees, 2008).



Previous studies for the WRC Project K5/2104 demonstrated the excellent removal of sugars, ethanol and phenolics that can be achieved with the use of BSFs, however the removal of volatile fatty acids (VFA) is very poor. Irrespective of whether VFAs were present in the original influent, VFAs would be formed from other organic substrates and accumulate within the BSFs (Welz and Le Roes-Hill, 2014). Based on this study, it was concluded that a downstream process would be more suitable and the use of trickling filters was suggested. In inclusion of trickling filters would enhance aerobic processes such as nitrification and aerobic degradation pathways (Matthews *et al*., 2009). They have successfully been applied in the removal of organics present in anaerobic secondary biological treatment of domestic wastewater and municipal landfill leachate (Matthews *et al*., 2009; Pontes and De Lemos Chernicharon, 2011).

### 1.2.5 Role of microbial populations in CWs and BSFs

The presence of microorganisms in CWs is the most important factor that allows for many biotic processes to proceed; these include microbial degradation, transformation, and mineralization. Microbial communities are essential in the mineralization of organic matter and in nitrogen and phosphorous removal (Faulwetter *et al*., 2009; Truu *et al*., 2009; Glick, 2010). CWs become colonized over time by populations of organisms that adapt to specific wastewater environments. Colonization of CWs by microorganisms allows for biofilm formation and leads to a reduction of hydraulic conductivity (Knowles, 2010; Burton *et al*., 2011). Colonisation of CWs is dependent on how microorganisms adapt to the changing environment, a process called acclimation. Previous studies have shown that microbial communities acclimatize to grow in the presence of toxic chemicals such as *p*-nitrophenol and acrylonitrile (Zaida, 1996; Hu, 1997), cellulose substrates (Cheng, 2010), as well as harsh physical parameters (e.g. cold) (Koda, 2002). The concentration and toxicity of the chemicals, as well as the period of acclimation all influence the ultimate success of the acclimation process. Studies have shown that longer acclimation periods lead to higher substrate degradation rates (Zaida, 1996).

Ramond *et al*., (2013) demonstrated the use of BSFs that were operated in mixed vertical (VSSF) and horizontal (HSSF) subsurface flow mode (Stottmeister *et al*. 2003) and subjected to bi-weekly feeding followed by gradient-directed drainage. This type pf BSF is considered to favor aerobic microbial processes and thus increase COD and/or BOD removal potential (Faulwetter *et al*. 2009). Micro-organisms as we know, play an important role in any bioremediatory processes (Gadd, 2010), and when BSF systems are impacted by contaminants, the microbial communities either become resistant (i.e. the community structure remains unaltered) to the contaminant, or resilient (i.e. the community changes initially but restores at a later stage), or sensitive (i.e. the community structure is permanently changed) to the contaminant (Allison and Martiny, 2008).

For the study by Ramond *et al*., (2013), they hypothesized that for reliable organic removal efficiencies to be achieved, the microbial communities should be able to conserve the basic biogeochemical processes necessary for the bioremediation of the winery wastewater used (Yagi *et al*. 2010). Low pH and high organic (including ethanol and phenolic compounds) and salt contents, which characterize the winery wastewater used in the study, have all been shown to induce structural changes in environmental microbial communities (Lucassen *et al*. 2002; Moussa *et al*. 2006; Feris *et al*. 2008).

A study by Welz *et al*., (2012) demonstrated whether organisms would be able to utilize vanillin and gallic acid using microcosms. Kunc, (1970) showed that the incubation of soil samples with vanillin can increase the number of bacteria capable of utilizing this phenolic as a sole carbon source. Welz *et al*., (2012), clearly showed that a 9-week period was sufficient for the microbial population to acclimate to the presence of gallic acid and vanillin, resulting in significantly enhanced biotic degradation rates when compared to a non-acclimated population. These results support previous literature findings that low concentrations of phenolic acid mixtures stimulate the growth of phenolic acid-utilizing bacteria within the bulk soil, and that competitive selection of these bacteria enhances biodegradation of phenolic acids (Blum *et al*., 2000; Vaughan *et al*., 1983).

Ramond *et al*., (2013) previously showed that BSF microbial communities that were exposed to incremental concentrations of ethanol become more tolerant to the ethanol contaminant than communities that are exposed to higher concentrations of ethanol from the onset of study and that phenomenon is referred to as microbial acclimation. Rodriguez-Caballero *et al*., (2012) demonstrated that the addition of bioavailable nutrients to organic rich effluents (particularly nitrogen) enhanced the remediatory performances of treatment BSFs and that the nitrogen fixing bacteria are selected in organic contaminated environments suggesting that bioavailable nitrogen and /or diazotrophic communities are crucial in such environment.

Incremental priming refers to the exposure of CWs and BSFs systems to increasing concentrations of pollutants (Jacobs *et al*., 1995). It has been determined that “incremental priming” during the start-up period of BSFs enhances the acclimation of the microbial communities already present on the substrate (sand), and results in significantly superior degradative rates and capacities when compared to unprimed systems containing non-acclimated microbial populations (Burton *et al*., 2011; Welz *et al*., 2011). There are a wide range of techniques available to assess the presence of microorganisms, changes/fluctuations in response to the outside stress of these microbial communities and examples of these methods are discussed in section 1.3.

### 1.3 Molecular fingerprinting techniques

#### 1.3.1 Detection of microbial communities in environmental samples

A number of molecular methods, such as polymerase chain reaction (PCR)-based culture-independent techniques, have been employed to discover and characterise microorganisms that are found in diverse ecosystems (Singh *et al*., 2004). One promising approach in the detection of microorganisms is molecular fingerprinting which allows for the possibility of characterising and comparing microbial community structures to assess their similarity or differences (Edberg and Melson, 2001). The amplification of the 16S rRNA gene from sand DNA samples combined with molecular fingerprinting techniques such as denaturing gradient gel electrophoresis (DGGE) and T-RFLP provide detailed information about the bacterial community structures (Torsvik and Ovreas, 2002).

Over the years, several culture-independent techniques targeting the 16S rRNA gene to analyse microbial communities haves been established. Fingerprinting techniques such as DGGE (Muyzer *et al*., 1993), automated ribosomal intergenic spacer analysis (ARISA) (Fisher and Triplett, 1999), and T-RFLP analysis (Liu *et al*., 1997) have been developed. These techniques have been successfully employed in numerous studies to explore the bacterial diversity of the populations present in various habitats. The advantage of using these techniques is that they are high throughput compared to culture dependent methods (Rappe and Giovannoni, 2003; Weng *et al*., 2006).



#### 1.3.2 T-RFLP analysis of the bacterial 16S rRNA gene and fungal ITS region

T-RFLP is a popular high-throughput fingerprinting technique used to monitor changes in the structure and composition of microbial communities. Most frequently the technique includes steps used to amplify the small subunit (16S or 18S) rRNA genes from total community DNA using PCR wherein one or both primers are labelled with a fluorescent dye. The resulting mixture amplicons is digested with restriction endonucleases, and the sizes and relative abundance of the fluorescent labelled terminal restriction-fragments (T-RFs) is determined by an automated DNA sequencer. A different length T-RFs reflects differences in the sequences of the 16S rRNA genes (due to the presence/absence and location of restriction enzyme recognition sites), which ultimately correlates to distinct populations of organisms. Therefore, the patterns of the T-RFs are a composite of the DNA fragments with unique lengths that reflect the dominant populations in the community (Shütte *et al*., 2008). T-RFLP

has been used for the analysis of functional genes (Horz *et al*., 2000; Lueders and Friedrich, 2003) including the detection of genes involved in nitrogen fixation (Tan *et al*., 2003; Rösch and Bothe, 2004) and methane oxidation (Horz *et al*., 2001; Mohanty *et al*., 2006). Although like any PCR-based technique, problems may occur (e.g. poor primer specificity, mispriming, incorrect annealing temperature). Primer design is limited by the number of sequences found on the database and this becomes a disadvantage as to whether the sequences reflect the actual gene diversity found in nature. This would affect the overall T-RFLP profile for each sample. T-RFLP has been shown to provide a means to assess changes in microbial community structure on both a temporal and spatial scale by monitoring the appearance or disappearance of specific fragments in microbial profiles (Franklin and Mills, 2003; Mummey and Stahl, 2003). When T-RFLP analysis of the 16S rRNA gene is coupled with clone library construction and sequencing, a more detailed composition of the microbial communities can be obtained (Shütte *et al*., 2008).

A determining factor for the success of PCR relies greatly on the quality and purity of the nucleic acid extracted (Anderson and Cairney, 2004). In contrast to bacteria, taxonomic identification of fungi, based on sequences of the eukaryotic ribosomal small subunit (18S rRNA), is more problematic with identification commonly limited to the genus or family level. This is primarily due to the relative lack of variation within 18S rRNA genes between closely related fungal species as a result of the relatively short period of evolution of fungi compared to bacterial evolution (Hugenholtz and Pace, 1996). Given the limitations of the 18S, the internal transcribed spacer (ITS) region located between the 18S rRNA and 28S rRNA genes incorporating the 5.8S rRNA gene, has also been targeted (White *et al*., 1990; Gardes and Bruns, 1993; Larena *et al*., 1999). Fungal ITS sequences generally provide greater taxonomic resolution than sequences generated from coding regions (Anderson *et al*., 2003).

Primer design and selection plays a vital role in the successful application of oligonucleotide primers selected for the use in microbial community DNA-profiling methods and thus needs to be specific and universal to the target group. For analysis of bacterial communities, primers that amplify DNA from the 16S rRNA gene region are frequently used. The main reasons for this are that the 16S rRNA gene is found universally in bacteria and contains both highly conserved and variable sequence regions. For the analysis of fungi, the 18S, 5.8S and 28S rRNA genes, which are traversed by the hyper-variable internal transcribed spacer (ITS) 1 and ITS2 regions (Figure 1.3), are recognized as being useful for the same reasons (Hill *et al*., 2000).

Fungal-specific primer pairs that have been tested for analysis of fungal community DNA extracted from environmental samples include: ITS1F (Gardes and Bruns, 1993) and ITS4 (White *et al.*, 1990). Turenne *et al.* (1999) designed the ITS86F primer and coupled this with the ITS4 reverse primer (White *et al.*, 1990) for use in screening human blood for medically important fungal pathogens and maybe useful in fungal isolates. This primer pair amplifies the hypervariable ITS2 region, which straddles the 5.8S and 28S genes (Figure 1.3).

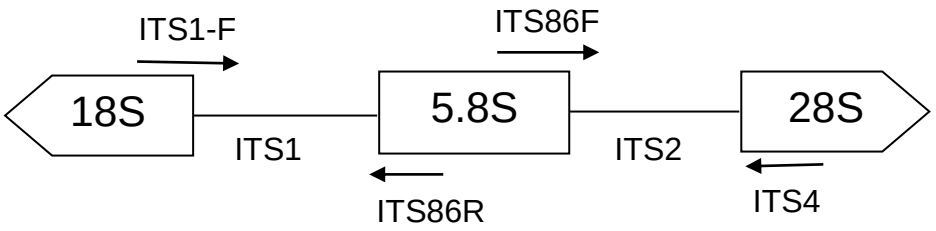



**Figure 1.3:** Schematic representation of fungal ribosomal genes bridged by ITS regions targeted by the two primer sets that were assessed by Vancov and Keen, (2009).

Studies conducted by Truu *et al*. (2009), and Weber and Legge (2011) showed that extended periods are required for microbial communities to stabilize and properly establish during operational periods of the CWs. The presence of a settled microbial community is generally considered to be a critical factor for maintaining the stability of the ecosystem, resilience after contamination and for long-term sustainability (Wohl *et al*., 2004). Studies performed by Ramond *et al*. (2013) assessed the changes in bacterial community structure through T-RFLP analyses after amendment with synthetic winery wastewater and concluded that through T-RFLP analyses they were able to detect changes in the community structures.

### 1.3.3 Denaturing gradient gel electrophoresis (DGGE)

DGGE is a popular molecular fingerprinting technique and was initially used for the detection of point mutations in clinical bacterial isolates. The technique has been adapted and is routinely used to study microbial genetic diversity including population structure in environmental settings (Muyzer *et al*., 1993). The methodology used in DGGE is relatively simple. The gene of interest is amplified from metagenomic DNA using modified gene-specific PCR primers. The The primers used have a 35-40 bp GC-clamp that is attached to

the 5'-end of the forward primer to ensure that that part of the DNA remains double stranded during electrophoresis under denaturing conditions (Kirk *et al*., 2004). Amplified PCR products are electrophoresed through a polyacrylamide gel with an increasing linear concentration gradient of chemical denaturants (formamide and urea) (Muyzer *et al*., 1993; Kirk *et al*., 2004). As double-stranded DNA melts in sequence specific domains it migrates differentially through the gel, allowing for separation based on differences in sequence (Muyzer *et al*., 1993; Muyzer, 1999.)

The addition of the GC-clamp to the 5'-end of the primer was shown to be effective through a study performed by Myers *et al*. (1985) and Sheffield *et al*. (1989). The GC-clamp ensures that almost 100% of all possible sequence variations can be detected by differential migration in DGGE gels. The GC-clamp was further explored by Muyzer *et al*. (1993) who showed that a 40 bp GC-clamp was more successful than a 30 bp one in the formation of stable, partially melted fragments. In addition, the GC-clamp was found to only be effective at increasing stability when it was incorporated on the 5' –end of the primer and not the 3' -end (Muyzer *et al*., 1993).

The different band patterns obtained by DGGE is a reflection on the species diversity within the environment (Muyzer *et al*., 1993). An advantage of DGGE analysis, is that the method is less time-consuming technique compared to conventional cloning techniques and displays a mixed community present within a sample in a qualitative manner (Muyzer *et al*., 1993).



### 1.3.4 Quantitative PCR

Quantitative PCR (qPCR), often referred to as real-time PCR, is a widely used technique in microbial ecology to determine gene or transcript numbers present within environmental samples. The target specificity of any qPCR assay is dependent on the design of the primers. Unlike many fingerprinting techniques which is limited to phylogenetic markers such as the 16S rRNA gene, due to its sensitivity and selectivity this technique allows for the quantification of functional genes present within a mixed community sample. qPCR has been shown to be a highly sensitive and reproducible technique that quantitatively shows gene expression level shifts across temporal and spatial changes within samples under different environmental or experimental conditions (Smith and Osborn, 2008).

Recent advances in the quantification of messenger RNA (mRNA) and gene expression has allowed for the development of reliable methods of reverse transcription followed by qPCR (RT-PCR) (Gibson *et al*., 1996). RT-PCR has benefits over conventional methods of quantifying RNA as the technique is more sensitive and accurate (Huggert *et al*., 2005). As RT-PCR allows for the generation of amplification curves it allows for the accurate quantification of the starting template (Wilhelm and Pingoud, 2003).

Quantitative analysis is used to compare transcription rates (gene expression levels), determined by the amount of RNA present for samples under different environmental conditions. Normalization of results obtained through RT-PCR becomes problematic and generally this is overcome through the use of genes that are constantly expressed (or non-regulated genes) known as reference genes (often housekeeping genes are targeted) (Wilhelm and Pingoud, 2003). These reference genes are used as internal standards for normalization of data by comparing the expression of functional genes within a given sample to the expression of the internal standard. Depending on the desired outcome of the research, the use of reference genes may vary when normalising data in different research conditions (Huggert *et al*., 2005).

**1.4 Functional bacterial genes**



Detection of functional genes within microbial communities has been extensively researched using gene-specific PCR (Henckel *et al.*, 1999). Gene-specific PCR has two major disadvantages as it requires the design of primers which is dependent on sequences found in databases. Secondly, a single fragment of a functional gene will be amplified requiring additional PCR steps to amplify the full length gene (Cowan *et al*., 2005). For profiling functional microbial communities the use of RNA is more effective than DNA, as RNA is a more sensitive biomarker and the presence of specific RNA shows that the gene is being expressed under the tested conditions (Manefield *et al*., 2002). RT-PCR has been used as a quantification tool for the detection of genes from environmental samples and has become an important aspect in microbial ecology, monitoring of microbial diversity and bioremediation (Sharkey *et al*., 2004) and this is method is to be used in this study for the detection of the functional genes.

Chemical analysis of winery wastewater has revealed that phenolics and trace amounts of ethanol are present. As catechol dioxygenase and alcohol dehydrogenase enzymes are necessary for the microbial degradation of many phenolics and ethanol, respectively, in the

present study it was decided to target two functional genes that are involved in the breakdown of these products, namely alcohol dehydrogenase and catechol 2,3-dioxygenase.

### 1.4.1 Bacterial alcohol dehydrogenase

Aerobic microorganisms break down ethanol via the Krebs cycle and through this pathway ethanol is oxidized to acetaldehyde by alcohol dehydrogenase (EC 1.1.1.1). Acetaldehyde is further converted to acetyl-CoA either directly by acetaldehyde dehydrogenase or through acetate by an acetaldehyde dehydrogenase and acetate-CoA ligase. This is followed by acetyl-CoA being oxidized to $CO_2$ in the Krebs cycle (Figure 1.4) (Campbell and Farrell, 2006). The intermediates in the Krebs cycle are not toxic as they are intracellularly metabolized and rarely excreted.

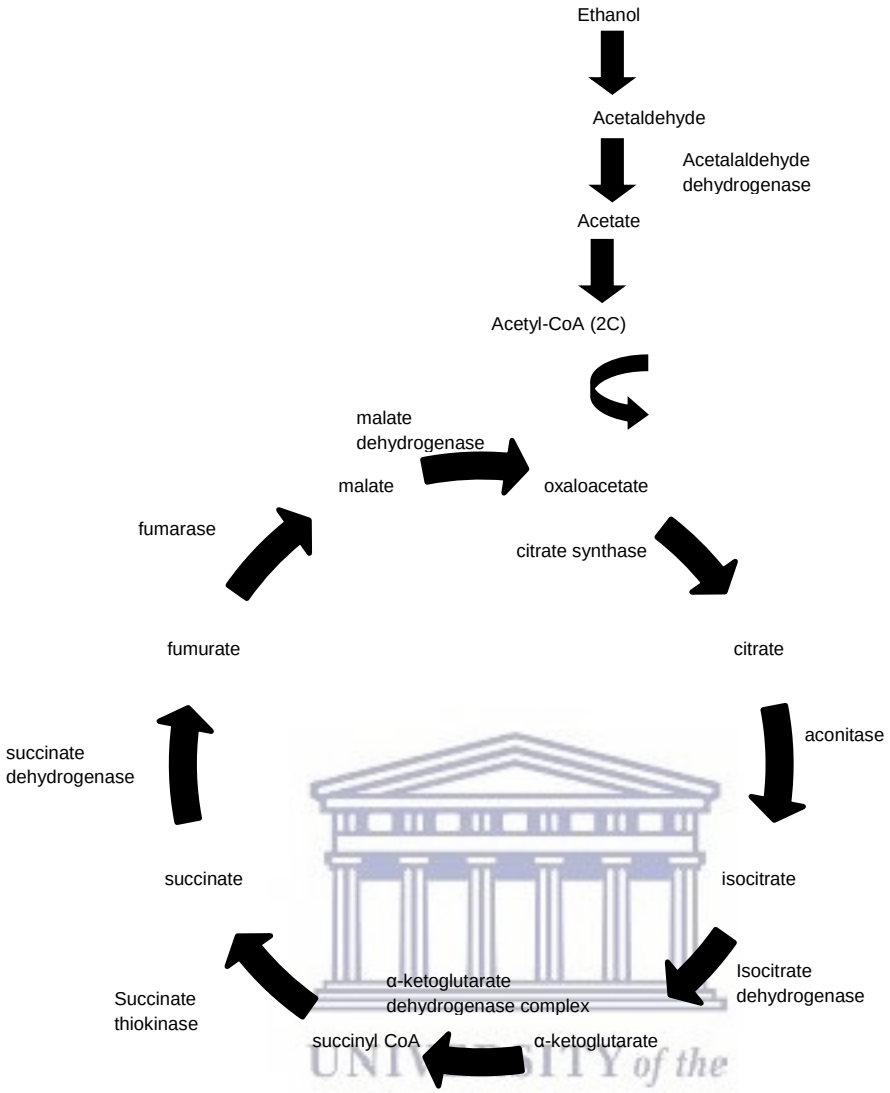





**Figure 1.4**: The Citric acid cycle (Krebs cycle) involving the use of alcohol dehydrogenase in the oxidation of ethanol (Campbell and Farrell, 2006).

The reaction mechanism of alcohol dehydrogenase requires $NAD^+$ and the alcohol molecule binds to the enzyme, in such a way so that it is attached to both the substrates. With the enzyme bound to the substrates, a hydrogen is transferred from the alcohol molecule to NAD resulting in the formation of NADH and a ketone or an aldehyde (Voet *et al*., 2008) as shown in Figure 1.5.

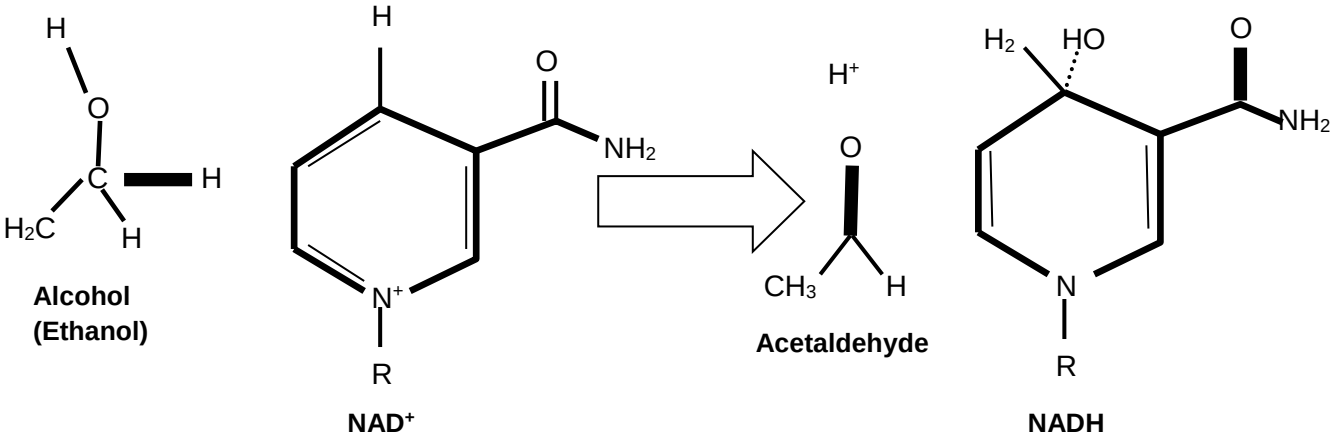



**Figure 1.5:** The mechanism of alcohol dehydrogenase (Metzler, 1977)

Studies by Gottschalk (1986) showed that *Gluconobacter* species are unable to oxidise acetate (Acetyl-CoA) in the Krebs cycle as it lacks the enzyme succinate dehydrogenase, while A*cetobacter* species can metabolise acetate through the Krebs cycle. They produce large amounts of acetic acid in the presence of ethanol. Chinnawirotpisan *et al*. (2003) reported that acetic acid bacteria are primarily involved in the oxidation of ethanol to acetic acid. These acetic acid producing bacteria have an additional alcohol dehydrogenase enzyme which is cytosolic, together with the membrane bound pyrroloquinoline quinone (PQQ) and NAD-dependent enzymes, all play a part in the accumulation of ethanol rather than the degradation of ethanol. Quintero *et al*. (2009) investigated the expression of the alcohol dehydrogenase gene within acetic acid bacteria and used this as a marker for analysing acetic acid bacteria's ability to oxidise ethanol.



### 1.4.2 Bacterial catechol dioxygenases

Bacterial degradation of phenolic compounds generally occurs in three phases and can also be extracellular (Dagley, 1986; Harayama and Timmis, 1992). The first step includes preparing the compound for ring cleavage by introducing hydroxyl groups by mono- or dioxygenases to result in the dihydroxyaromatic metabolites. Catechols are usually the metabolites in the breakdown of most aromatic substrates (Williams and Sayers, 1994). The second phase is catalysed by the dioxygenases that breaks one of the carbon-carbon bonds of the ring by adding an oxygen molecule and producing an unsaturated aliphatic acid. The third phase involves the conversion of the ring cleavage products into smaller aliphatic compounds that are further metabolised to enter the metabolic cycle (Fenner *et al*., 2005; Williams and Sayers, 1994).

Catechol molecules are also known as pyrocatechol or 1,2-dihydroxybenezene and were first discovered in the plant extract catechin (Naquet *et al*.,1868). Catechol dioxygenases catalyse the oxidation of catechol molecules, a key step in the breakdown of aromatic compounds by soil bacteria. Catechol dioxygenases can be divided into two major groups (Hayaishi *et al*., 1955): enzymes that cleave the aromatic ring between the vicinal diols (the intradiol enzymes) and those that cleave the ring to one side of the vicinal diols (the extradiol enzymes) (Figure 1.6). It is known that both types of catechol dioxygenases have an iron present in the active site which is required for enzymatic activity, with the intradiol enzymes containing Fe(III) and the extradiol form containing Fe(II) (Broderick, 1999).

The intradiol dioxygenases, typically the catechol 1,2-dioxygenase (EC 1.13.11.1), cleave the carbon-carbon bond between the hydroxyl group of the phenolic compound to result in muconic acid and require $Fe^{3+}$ as a co-factor (Hayaishi *et al*., 1955). The extradiol dioxygenases, typically catechol 2,3-dioxygenase (EC 1.13.11.2), cleave the carbon-carbon bond that is adjacent to the phenolic hydroxyl group to yield 2-hydroxymuconaldehyde and require $Fe^{2+}$ as the co-factor for the reaction (Kojima *et al*., 1961). The reactions catalyzed by the two families of ring cleavage enzymes; intradiol (or *ortho*) dioxygenases and extradiol (or *meta*) dioxygenases are shown in Figure 1.4 (Dagley, 1971).

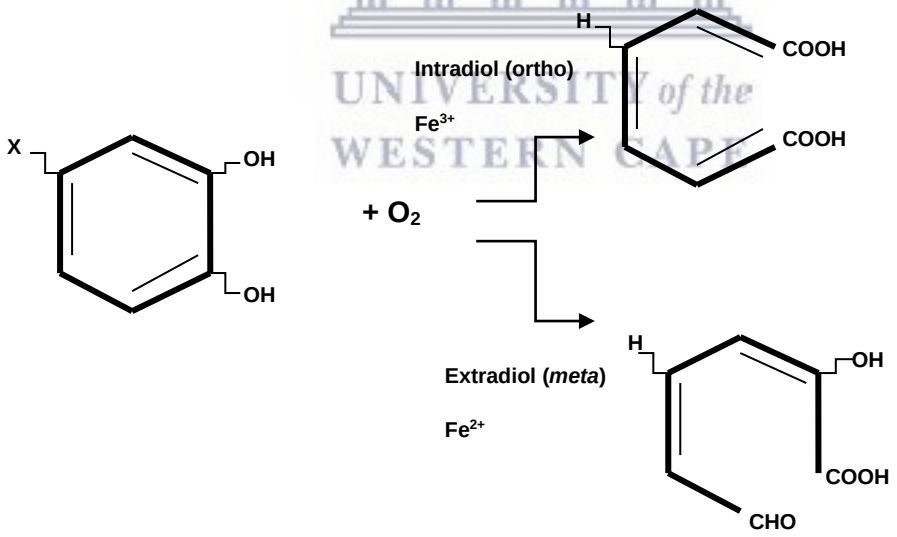



**Figure 1.6:** Schematic of the modes of ring cleavage by the catechol dioxygenases (Broderick, 1999; Bugg, 2003)

Reneike and Knackmuss (1988) showed that through the use of pure culture studies chlorocatechols were metabolised via a modified *ortho* cleavage pathway that involved the intradiol cleavage of the aromatic ring by the enzyme chlorocatechol 1,2-dioxygenase. Several studies (Nakatsu and Wyndham, 1993; Wieser *et al*., 1994; Koh *et al*., 1997; Mars *et al*., 1999) have shown that strains including *Alcaligenes eutrophus*, *Azotobacter* sp. GP1 and *Sphingomonas* sp. BN6 could transform chlorocatechols to chlorophenols via the *meta* pathway. It does however remain unclear which pathway (*ortho* or *meta*) is used by bacteria in their natural environments. Lillis *et al*. (2010) examined the abundance and expression of two functional genes that encodes for the intradiol and extradiol cleavage of the chlorocatechols present in soil samples. The expression of these two genes was assessed through qPCR and the results obtained provided insight into the relevance of the intradiol and extradiol cleavage of the chloroaromatic pathways. Phylogenetic analysis of the sequences obtained in the study by Lillis *et al*. (2010) showed that the chlorocatechol 1,2-dioxygnease gene sequences related to that of frequently isolated chlorophenol-degrading bacteria such as *Pseudomonas*, *Ralstonia* and *Burkholderia* species. The catechol 2,3-dioxygnease sequences clustered with known soil bacteria that express the gene, namely *Pseudomonas*, *Azoarcus*, *Novosphingobium* and *Burkholderia* species. Chlorocatechol dioxygenases have also been detected in eutrophic lake and soil samples (Kasuga *et al*., 2007).

In addition, the expression of the catechol 2,3-dioxygenase genes has been monitored using cassette PCR of genomic DNA extracted from samples taken from a phenol and crude oil-degrading bacterial consortium, (Okuta *et al*., 1998). Cowan *et al*. (2005) reported the use of gene-specific PCR to probe communities for microorganisms with specific metabolic or degradation capabilities. The biodegradative abilities of these microbial communities were assessed by screening the metagenomic DNA obtained for the presence of catechol 2,3-dioxygenase and chlorocatechol dioxygenase genes.

### 1.5 Next Generation Sequencing

The analysis of environmental DNA using specific gene markers has been a key application for next generation sequencing technologies in the field of ecology and environmental research. New technologies are being rapidly developed using next generation sequencing (NGS) and therefore allowing for more broad and robust applications in environmental DNA research (Shokralla *et al*., 2012).

The conventional method of DNA sequencing approach was introduced by Sanger sequencing in 1977 and can recover up to 1 kb of sequence data from a single specimen at a time. With the recent advances of NGS technologies, thousands to tens of millions of sequencing reads in parallel can be generated. This massive parallel sequencing capacity can generate sequence reads from fragmented libraries of a specific genome (i.e. genome sequencing); from a pool of cDNA library fragments generated through reverse transcription of RNA molecules (i.e. RNAseq or transcriptome sequencing); or from pooled PCR-amplified molecules (i.e. amplicon sequencing) (Shokralla *et al*., 2012). In relation to all three, sequences are generated without the need of a conventional, vector-based cloning methods that is typically used to amplify and separate DNA templates (Mardis 2008a).

Since the introduction of NGS in 2005, the high-throughput method has been faced with many challenges. The first being the being the total output of the sequencing experiment in relation to the cost and the labour used. A third challenge exists with regards to the amplification step prior to the sequencing This amplification step includes different sources of PCR bias, formation of chimeric sequences and secondary structure-related issues (Mardis 2008a; Shendure & Ji 2008).

New NGS technologies promise to fundamentally change the nature of genomics-based studies, especially when coupled with the computational algorithms necessary to analyse their vast sequencing output (Mardis 2008b).

## 1.6 Aims and objectives of study

The Water Research Commission (WRC) has funded many studies performed at the Biocatalysis and Technical Biology (BTB) Research Group in the use of BSFs for the treatment of synthetic winery wastewater. During the course of these projects, optimization of molecular fingerprinting techniques to monitor the effects of amendments with synthetic winery wastewater on the microbial communities was conducted (Ramond *et al*., 2012). The experiments performed together with members of the Institute for Microbial Biotechnology and Metagenomics (IMBM) at the University of the Western Cape (UWC), showed the presence of complex bacterial communities contributing to the remediation of winery wastewater in these BSFs.

This Masters project involved the use of pilot-scale BSFs that were amended with synthetic winery wastewater over a 31-week period. The main aims of the study were:

- To monitor the changes in bacterial and fungal communities in response to amendment with synthetic winery wastewater through the use of molecular fingerprinting techniques;
- To determine the degradation of organic and inorganic constituents in the synthetic winery wastewater;
- To perform a functional bacterial study for the biodegradation of ethanol and phenolics using qPCR



## 1.7 Outline of thesis

The thesis is arranged as follows: Chapter 1 covers the literature and background of this study; Chapter 2 provides a detailed description of the methods and materials used in this study; the results and discussion are presented in Chapter 3; and lastly the general discussion of the study is given in Chapter 4.

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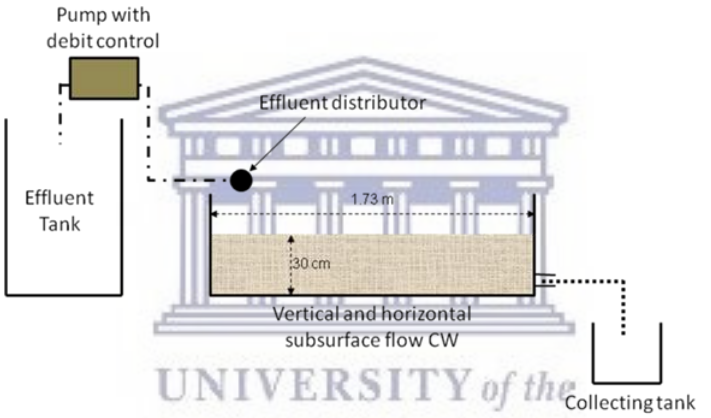
# CHAPTER TWO

# MATERIALS AND METHODS

## 2.1 Biological sand filters (BSFs)

### 2.1.1 Set-up and mode of operation of the BSFs

In this study, three BSFs containing Phillipi sand were used; two experimental systems (P1 and P2) and a control system (PC). Each polyethylene tank (173 cm in length, 106 cm in width) was packed to a depth of 30 cm (Figure 2.1). All BSFs were housed in an outdoor, undercover area in order to mimic environmental conditions while avoiding exposure to direct precipitation and sunlight that could influence the concentration of the feeding and amendment solutions.




A

B

**Figure 2.1:** Schematic of BSFs (A) (Ramond *et al*., 2013) and actual set-up of BSFs on site at CPUT (B) (Welz *et al.*, 2011)

All BSFs were operated in batch mode: Feed/wastewater was distributed over the surface of the inlet twice weekly via drip irrigation at a rate of 0.67 L $min^{-1}$. Before feeding/amendment, the outlet was plugged to ensure flooding of the surface and to increase the hydraulic retention time (HRT). Each BSF was unplugged 48 hours after the commencement of feeding. Once unplugged, flow through the BSF was allowed to proceed uninterrupted for 24 hours. In this project, the term “feeding” refers to the application of a dilute concentration of basal nutrients to the substrate of the BSFs in order to support the growth of consistent microbial communities in the BSFs during an establishment phase. The term “amendment” is used to describe the application of wastewater during an experimental phase.

### 2.1.2 Analyses of sand samples

Analyses on sand samples were performed (in triplicate) according to the methods described by the non-affiliated soil analysis committee (1990) at Bemlab (Strand, Western Cape). The following analyses were performed:

- $CaCO_3$ equivalents (eq.) by titration with HCl
- pH, measured in a KCl solution
- Mechanical fraction (%)
- Available micronutrients (P, K, Cu, Zn, Mn, B, Fe), heavy metals (Cd, Pb, As, Hg, Sb) and exchangeable cations and cation exchange capacity (Na+, K+, Ca++, Mg++) using a Varian® MPX ICP-OES spectrophotometer (Agilent Technologies, Santa Clara, USA) after extraction according to standard methods
- The major elements in the sand samples were determined by X-ray fluorescence (XRF).

### 2.1.3 Feeding/amendment and loading regime

When performing comparative studies with BSFs it is important that the microbial communities within the BSFs are equilibrated (i.e. formed stable populations, with similar representatives in all experimental BSFs) and previous studies have shown that a 90-day establishment period is required for microbial populations to be equilibrated (Ramond *et al*., 2013). Thus prior to the amendment with synthetic winery wastewater, all BSFs were fed with 1 g yeast extract (Biolab®, Midrand, RSA cat no: HG000BX6.500) and 0.3 g D(+) glucose (Merck®, Whitehouse station, USA, cat no: SAAR2676020EM) dissolved in 40 L tap water and fed twice a week for a 3 month equilibration phase.

During the experimental phase/s, the BSFs P1 and P2 were amended with synthetic winery wastewater (Table 2.1), while the control BSF (PC) was supplemented with glucose and yeast extract (identical to feed during the equilibration phase). Table 2.2 shows the COD concentrations of each of the components of the synthetic winery wastewater over the 31 week period.

The theoretical COD ($COD_a$) for each component of the feeding solution was calculated as follows:

Theoretical COD ($COD_t$) values were determined using the equation:

$$COD_t = \frac{8(4x+y-2z)}{(12x+y+16z)} \text{mg COD/ mg } C_xH_yO_z \text{..... Equation1}$$

From Eq. (1), the $COD_t$ values were calculated for each component of the synthetic winery wastewater (Appendix B). The operational parameters are given in Table 2.1.

**Table 2.1:** Composition in COD terms and final COD concentration of synthetic winery wastewater

| | Gallic acid (CODmg $L^{-1}$) | Vanillin (CODmg $L^{-1}$) | Ethanol (CODmg $L^{-1}$) | Acetic acid (CODmg $L^{-1}$) | Glucose (CODmg $L^{-1}$) | Final conc (CODmg $L^{-1}$) |
|---|---|---|---|---|---|---|
| **Week 1-4** | 25 | 25 | 250 | 200 | 0 | 527 |
| | 50 | 50 | 500 | 400 | 0 | 1027 |
| | 100 | 100 | 1000 | 800 | 0 | 2027 |
| **Week 5-7** | 25 | 25 | 250 | 200 | 27 | 527 |
| **Week 8-10** | 100 | 100 | 1000 | 800 | 27 | 2027 |
| **Week 26-28** | 25 | 25 | 25 | 0 | 227 | 527 |
| **Week 29-31** | 100 | 100 | 1000 | 0 | 827 | 2027 |

**Table 2.2:** Operational parameters applied during the experimental period

| | week | PC | P1, P2 |
|---|---|---|---|
| ***HLR (L/m³sand.day⁻¹)*** | all | 22.9 | 22.9 |
| ***OLR (gCOD/m³sand.day⁻¹)*** | 5-7, 26-28 | 0.6 | 12.0 |
| | 8-10, 29-31 | 0.6 | 48.0 |
| ***Influent COD (mg L⁻¹)*** | 5-7, 26-28 | 27 | 527 |
| | 8-10, 29-31 | 27 | 2027 |

**HLR = hydraulic loading rate** **OLR = organic loading rate** **COD = chemical oxygen demand**

**COD, OLR values include contributions by glucose**

### 2.1.4 System hydraulic conductivity (SHC)

The hydraulic conductivity was determined by collecting effluent from each BSF over the period of 90 s in a measuring cylinder and the results expressed as the volume collected per hour for each cubic metre of sand. These times were initially determined as the period taken to collect between 0.5 and 1 L effluent.

## 2.2 Sampling

### 2.2.1 Core sand samples

Sand samples were taken from pre-designated sites using a Perspex® corer (internal diameter of 2.5 cm) sealed with a rubber stopper (Figure 2.2) from the middle of each BSF before the amendment with synthetic winery wastewater (baseline, week 0). Three samples were taken from each BSF. Surface fractions were collected from a depth of 0 to 5 cm, while deep fractions were collected at a depth of 20 to 25 cm. Composite samples were created by pooling the three core samples, from each site (either the surface or depth) and thoroughly mixing. The core sand samples were replaced after each sampling procedure with new sand.

**Figure 2.2:** Core sample of sediment collected with the Perspex pipe (sealed with a rubber stopper) used to collect surface and depth samples.

The sampling areas were predetermined and demarcated with pipette tips prior to the start of the experiment. Figure 2.3 shows this demarcation diagrammatically. Samples were taken in order from a to c. Each small square measured 10 cm x 10cm. Sampling from the top right corner of each square allowed further samples to be taken from the bottom left of each square after the third sampling (in the same order). This manner of sampling allowed maximum recovery of adjacent sediment sites before any subsequent sampling. The template was applied close to the inlet as well as close to the outlet at each BSF.

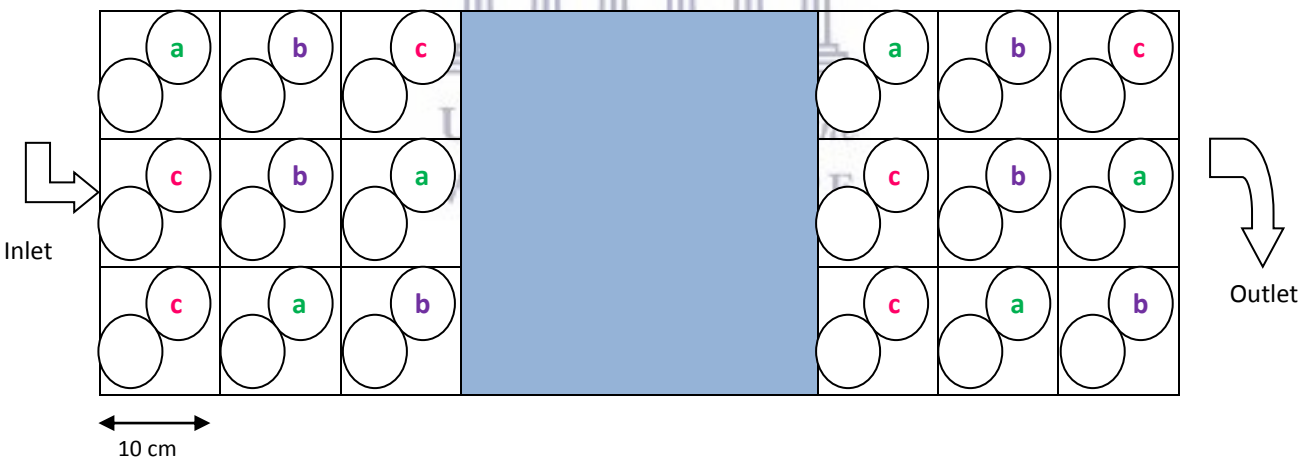



**Figure 2.3:** Template used for the determination of composite sampling order used in this study. Samples were taken at point 'a' (designated baseline samples), 'b' (designated samples after the amendment with synthetic winery wastewater containing acetate, 'c' (designated samples after the amendment with synthetic winery wastewater containing glucose- end of experiment).

The samples collected were used for the molecular studies. The samples were named according to the time periods during the experiment at which the core sand samples were collected: sampling points designated as ‘a’ are the baseline samples (0 weeks), samples designated as ‘b’ are samples collected during treatment with synthetic winery wastewater containing acetate (11 weeks), and samples designated as ‘c’ are samples collected during the treatment with synthetic winery wastewater containing glucose (31 weeks). Sand samples used in this study were at the start of the experiment (baseline) and the end of the experiment (synthetic winery wastewater containing glucose).

This study ran parallel in conjunction with a PhD candidate’s study that was trying to evaluate the biodegradative metabolic processes taking place in the BSFs, as well as the effect of winery wastewater composition on these processes, the treatment of two formulations of synthetic winery wastewater were compared. Both formulations contained ethanol and the phenolics, vanillin and gallic acid, which are all common components of winery effluent. In addition, the first formulation of synthetic winery wastewater contained acetate, and the second formulation contained glucose, which are typically major contributors to the organic fraction of winery wastewater (Bolzonella and Rosso, 2013; Malandra *et al*., 2003). Therefore acetate was replaced with glucose in the second formulation, on the hypothesis that the treatment of this effluent would be more effective than the treatment of the one containing acetate.



### 2.3 Molecular studies

#### 2.3.1 Metagenomic nucleic acid extraction

##### 2.3.1.1 DNA extraction

Metagenomic DNA was extracted from sand samples at the start and end of the experiment using the PowerSoil DNA isolation kit (MoBio Laboratories, Inc; Radnor Pennsylvania, US) according to the manufacturer’s instructions. 0.5 g of sand was used per extraction and all extractions were performed in duplicate. All metagenomic DNA samples were quantified using a Nanodrop spectrophotometer (section 2.3.2). Metagenomic DNA samples were stored at -20 °C until use.

#### 2.3.1.2 RNA extraction

RNA was isolated from sand samples at the start and end of the experiment using the RNA Powersoil Total RNA Isolation kit (MoBio Laboratories, Inc; Radnor Pennsylvania, US) according to manufacturer's instructions. RNA samples were stored at -20 °C. All RNA samples were quantified using a Nanodrop spectrophotometer (section 2.3.2) and immediately synthesised to cDNA (section 2.3.5.1).

### 2.3.2 Quantification and visualization of nucleic acids

A 2% (w/v) agarose gel (2.0 g in 100 ml 1x TAE) containing ethidium bromide (10 µg/ml) was prepared. Genomic DNA (5 µl) was mixed with 2 µl 6X loading dye (KAPA Biosystems (Pty) Ltd, Cape Town, South Africa). A KAPA universal ladder (5 µl) was included in all gels to determine the approximate size of the DNA bands. A horizontal BG-Subi MIDI Marine electrophoresis system (Baygene Biotech Limited, Beijing, China) was used for gel electrophoresis. Agarose gels were electrophoresed at 100 V for 40min and viewed under UV.

### 2.3.3 Microbial community fingerprinting

#### 2.3.3.1 PCR amplification of bacterial and fungal genes



The 16S rRNA gene and ITS region were amplified from metagenomic samples (mDNA) isolated from each BSF using the primer sets and conditions as indicated in Table 2.3. The basic PCR reaction mix for a 50 µl reaction consisted of1X Dream*Taq* PCR Mastermix, 0.5 µM of each primer, 0.1% (w/v) bovine serum albumin (BSA) and between 10 to 20 ng of metagenomic DNA. Amplification was performed in a Touchgene Gradient Thermal Cycler (Techne, Gaithersburg, USA). Negative controls containing all reagents except for template DNA were included for all PCR reactions. Genomic DNA (gDNA) from the following organisms was used as a positive control:

- *Streptomyces* strain GSIII#1 was used for the bacterial primer set
- *Trametes pubescens* strain CBS 696.94 was used for the fungal primer set

All bacterial PCR products were analysed on a 2% (w/v) agarose gel and the amount of amplified product present in each sample quantified using a Nanodrop (ND-1000) spectrophotometer. For the fungal primer set all PCR products were analysed on a 2% (w/v) agarose gel, and the fragments of the expected size were excised from the gel and purified using the Nucleospin Gel and PCR clean-up kit (Macherey-Nagel). The purified DNA products from BSFs P1, P2, and PC were re-amplified using the ITS primers and the PCR protocol in Table 2.3, and purified using the Nucleospin Gel and PCR clean-up kit (Macherey-Nagel).

**Table 2.3:** PCR parameters and conditions for amplification of target genes

| Primer set | Sequence (5'-3') | Amplification region | Target specificity | PCR cycles-parameters | Reference |
|---|---|---|---|---|---|
| **E9F** | GAGTTTGATCCTGGCTCAG | 16S rRNA | Universal for bacteria | 94 °C for 4 min<br><br>30 cycles: 94 °C for 30 s, 52 °C for 30 s and 72 °C for 105 s | Marchesi *et al.,* 1998; Reysenbach and Pace, 1995 |
| **U1510R** | GGTTACCTTGTTACGACTT | | | 72 °C for 10 min | |
| **ITS1F** | CTTGGTCATTTAGAGGAAGTAA | 5.8S, ITS1 and ITS2 | Specific for fungi | Touchdown PCR 94 °C for 5 min<br><br>10 cycles: 94 °C 45 s, 55 °C for 45 s, 72 °C for 1 min.<br>25 cycles: 94 °C 45 s, 55 °C for 45 s, 72 °C for 1 min.<br>72 °C for 20 min | Gardes and Bruns, 1993 |
| **ITS4** | TCCTCCGCTTATTGATATGC | | | | White *et al.*, 1990 |

### 2.3.3.2 Restriction digests and T-RFLP protocol

Purified PCR products (approx. 100 ng) were digested with the restriction enzyme *Hae*III (Sigma Aldrich, Seezle, Germany) The restriction digest protocol was carried out as follows: 2 µl of 10x restriction enzyme buffer, 10 µl purified PCR product, 5 U/ml (final conc.) restriction endonuclease and $dH_2O$ to the final volume of 20 µl. Digests were performed for 3 hours at 37°C. Thereafter the restriction enzyme was inactivated by heating at 80°C for 10 minutes. The digested products were analysed on a 2% (w/v) agarose gel and the size of the digested products used for the basis of the selection of internal standards to be used in T-RFLP analysis.

To perform T-RFLP analyses, the 16S rRNA gene forward primer (E9F) and the ITS forward primer ITS1F were 5'-end FAM-labelled (6-carboxyfluorescine). Prior to fragment analysis, digested samples were diluted with $dH_2O$ (1:2 dilution) and denatured at 95°C for 5 minutes and stored on ice for 5 minutes and analysed with a Applied Biosystems 3130xl DNA Sequencer (Applied Biosystems, Foster City, USA). Prior to analysis 4 µl of each digested product was mixed with the ROX500 internal size standard and formamide, and analysed directly by capillary electrophoresis (50 cm capillary, POP7 polymer). Samples were analysed by the Central Analytical Facility (CAF) at Stellenbosch University.



### 2.3.3.3 T-RFLP analysis using Peak Scanner and T-REX

The precise lengths of the terminal fragments (T-RFs) were determined by capillary electrophoresis. The molecular weight standard ROX500 was included in all analyses and an acceptable error of ±1 bp was allowed. T-RFLP patterns and quality were analysed using the freeware, PeakScanner™ (version 1.0) (Applied Biosystems, https://products.appliedbiosystems.com). The data table was exported in .csv format and converted to tab-delimited text format for the online software, T-REX (http://trex.biohpc.org/; Culman *et al.,* 2009), and a label file with sample names was created. These two tab-delimited text files were used to create large data matrices through the use of the T-REX software. Peak height was used to characterise each unique T-RF and only peaks at positions between 30 and 500 bp were considered in order to exclude T-RFs caused by primer-dimers and to obtain peaks within the linear range of the internal standard (Singh *et al*., 2006). The relative abundance of a T-RF in a T-RFLP profile was calculated by dividing the peak height of the sample (T-RF) by the total peak heights of all the samples (T-RFs in the profile).

T-RFs with intensities lower than 0.5%, which may have originated from background interference, were excluded from the matrices thereby minimizing the effect of the variations in the T-RFLP profiles caused by the starting quantities of DNA (Singh *et al*., 2006; Ding *et al*., 2013).

The above T-RFLP protocol was used for processing bacterial and fungal amplicons.

#### 2.3.3.4 Statistical analysis of terminal restriction fragment length polymorphism data

Data matrices obtained from T-REX were exported to Microsoft Excel and analysed by the software Primer 6 (Primer-E Ltd, UK). The community structures obtained from the T-RFLP data were square-root transformed to create Bray-Curtis similarity matrices used to establish the cluster analysis (group average linkage) and non-metric multidimensional scaling (NMDS) plots. The same analyses were performed for the T-RFLP data obtained for both fungal and bacterial communities. An analysis of similarity (ANOSIM), performed on the resemblance matrix, was used to test for differences in bacterial community structure between predefined groups (Clarke, 1993).

#### 2.3.3.5 Phylogenetic analysis of bacterial T-RFLP



Phylogenetic analysis was performed on the bacterial T-RFLP community profiles using the programme Microbial Community Analysis III (MICA 3) (Shyu *et al*., 2007). T-RFLP profiles from Peak Scanner were analysed in MICA 3: T-RFLP Analysis (PAT+) and the relevant parameters used for the T-RFLP analysis included primers and restriction enzyme used. The 16S rRNA gene bacterial sequence database was selected in MICA 3 for the phylogenetic analysis. The output file generated was presented graphically as pie charts.

### 2.3.4 Reverse Transcription for quantitative PCR (qPCR)

#### 2.3.4.1 RNA extraction and cDNA synthesis

Sand samples collected at the start and end of the experiment were used for RNA extraction (section 2.3.1.2). The RNA isolated from sand samples were used for reverse transcription PCR to obtain cDNA using the RETROscript® Kit from Applied Biosystems (AmbionLife Technologies-Thermo Scientific, California, USA) according to the manufacturer's

instructions. 1 µl of RNA was used to synthesise cDNA and the synthesised cDNA samples were stored at -20°C. All RNA and cDNA samples were quantified by Nanodrop.

### 2.3.4.2 Selection of primers and standards for qPCR

Different primer sets (Table 2.4) were selected for the detection of the three functional genes (alcohol dehydrogenase, catechol 1,2-dioxygenase and catechol 2,3-dioxygenase). Bioinformatic analysis of the genome sequence of *Streptomyces pharetrae* strain CZA14$^T$ (a laboratory strain) showed that it had all three genes present in its genome and therefore this strain was used as a positive control for qPCR. gDNA from *S. pharetrae* CZA14$^T$ was extracted using the method by Wang *et al*. (1996) and this was used for optimization of qPCR.

**Table 2.4:** Primer sets used in the amplification of the genes for qPCR

| Primer set | Sequence (5'-3') | Binding positions | Expected amplicon size | Amplification region | Reference |
|---|---|---|---|---|---|
| **ADH1** | CGGTGAATATGTCTGGCACTTC | Gene1:770 – 787bp | Approx 66 bp | Alcohol dehydrogenase | Quintero *et al*., 2009 |
| **ADH2** | ATCTGCTGAACCGAGGTGTAAT | Gene2: 255 – 275 bp | | | |
| | ****Note:** CZA14$^T$ has three ADH genes, therefore gene 1-3 | Gene3: 289 – 307bp | | | |
| | | Gene1: 117 – 136bp | | | |
| | | Gene2: 31 – 46 bp | | | |
| | | Gene3: 322 – 335bp | | | |
| **CCDbF** | GTGTGGCA(CT)TCGACGCCGGA(CT) | 194 – 205 bp | 270-279 bp | Catechol 1,2-dioxygenase | Leander *et al*., 1998 |
| **CCDbR** | CCGCC(CT)TCGAAGTAGTA(CT)TGGGT | 389 – 409 bp | | | |
| **C23OF** | AAGAGGCATGGGGGCGCACCGG TTCGATCA | 75 – 101 bp | Approx 300-400 bp | Catechol 2,3-dioxygenase | Sei *et al*., 1999 |
| **C23OR** | AACAAA(AGT)GCGC(GC)GTCATGCGG | 202 – 219 bp | | | Lillis *et al*., 2010 |

### 2.3.4.3 Optimization of qPCR standards

Each reaction contained the following (final concentrations): 1X KAPA SYBGR Readymix (KAPA Biosystems (Pty) Ltd, Cape Town, South Africa), 200 nM of each primer, 0.1 % (w/v) BSA (Sigma Aldrich, Seezle Germany), 2 µl template DNA and water to a final volume of 20 µl. A dilution series of gDNA from CZA14$^T$ was prepared at 10 ng, 50 ng and 100 ng. qPCR was performed on a Light Cycler LC480 (Roche Diagnostics, Basel, Switzerland). qPCR reactions were set up in white 96-well PCR plates and included 18 µl mastermix (included the specific primers), and 2 µl of genomic DNA. For the initial amplification of the alcohol dehydrogenase and the catechol 2,3-dioxygenase genes the following amplification conditions were used: denaturation (1 cycle) at 95 °C for 3 mins; amplification (45 cycles) at 95 °C for 10 secs, 60°C for 20 secs and 72 °C for 1 sec. Amplification was followed by melting curve analysis at 95 °C for 5 secs, 65 °C for 1 min and 97 °C (continuous) for 5 °C and cooling (1 cycle) at 40 °C for 10 secs.

Purified CZA14$^T$ amplicons were sequenced. The purified metagenomic DNA sample was cloned into a pJET 1.2/blunt cloning vector (section 2.3.4.7). The two samples were cloned and sequenced to confirm that the gene of interest had been amplified.

### 2.3.4.4 Amplification of catechol 2,3-dioxygenase genes using qPCR

Amplification of the catechol 2,3-dioxygenase gene was achieved using the primer set as indicated in Table 2.4 and the following PCR parameters: denaturation (1 cycle) at 95 °C for 4 mins; amplification (45 cycles) at 95 °C for 10 secs, 62°C for 20 secs and 72 °C for 1 sec; melting curve analysis at 95 °C for 5 secs, 65 °C for 1 min and 97 °C continuous for 5 °C and cooling (1 cycle) at 40 °C for 10 secs.

Once the optimised conditions were obtained for CZA14$^T$ gDNA, the conditions were used for the experimental samples.

### 2.3.4.5 Amplification of the *rpoB* gene using real-time PCR

Amplification of the β subunit of the ribosomal RNA polymerase (*rpoB*) gene through qPCR was achieved with each reaction containing the following: 1X KAPA SYBGR Readymix (KAPA Biosystems (Pty) Ltd, Cape Town, South Africa), 200 nM of each primer, 0.1 % (w/v) BSA (Sigma Aldrich, Seezle Germany), 2 µl template DNA and water to a final volume of 20 µl. qPCR reactions were set up in white 96-well PCR plates and included 18 µl mastermix, the specific *rpoB* primer set [rpoB2-F 5'-TGGCAACATCGTTCAAGGTG-3' and rpoB148-R 5'-ATCGATGGACCATCTGCAAGG-3'(Rodrigues and Tiedje, 2007)] and 2 µl of genomic DNA. For the amplification of the *rpoB* gene the following amplification conditions were used: denaturation (1 cycle) at 95 °C for 3 mins; amplification (45 cycles) at 95 °C for 10 secs, 60°C for 20 secs and 72 °C for 1 sec. Amplification was followed by melting curve analysis at 95 °C for 5 secs, 65 °C for 1 min and 97 °C continuous for 5 °C and cooing (1 cycle) at 40 °C for 10 secs.

### 2.3.4.6 Cloning of C23O PCR product

Competent cells were prepared based on a variation of the method described by Hanahan *et al.* (1991). 2x 250ml Super optimal broth (SOB) media (g/200mL: 4.0 tryptone, 1.0 yeast extract, 2ml of 1M NaCl, 500µl of 1M KCl; pH adjusted to 7.0; supplemented with 1ml each of 2M $MgCl_2$ and 2M $MgSO_4$ after autoclaving) were prepared. 2x 5ml of SOB medium was transferred aseptically to sterile 50ml flasks. The 5ml SOB medium was inoculated with 50µl of *E. coli* BL21(DE3) 20% glycerol stock and incubated at 37°C shaking at 160rpm overnight. 1ml of the overnight culture was transferred to the 200ml SOB medium and incubated at 37°C until $OD_{600nm}$ of 0.4 was reached. The cells were kept at 4°C for 20 minutes before harvesting by centrifugation in pre-chilled tubes at 5000rpm for 10 minutes (4°C). The cell pellets were gently resuspended in 80ml chilled CCMB80 buffer (10mM sodium acetate; 80mM $CaCl_2.2H_2O$, 20mM $MnCl_2.4H_2O$; 10mM $MgCl_2.6H_2$; 10% (v/v) glycerol, pH adjusted to 6.4, filter-sterilised with a 0.22µm filter). The cells were incubated on ice for 20 minutes, and centrifuged as described above. The cells were resuspended in 10ml CCMB80 buffer and diluted with CCMB80 buffer until $OD_{600nm}$ was between 1.0 and 1.5. 500µl aliquots were transferred into pre-chilled 1.5ml microfuge tubes. Aliquots were stored at -80 °C until use.

#### 2.3.4.7 Cloning, ligation and transformation of the amplified catechol 2,3-dioxygenase genes from the metagenomic sample

The catechol 2,3-dioxygenase gene from the mDNA sample was amplified using qPCR and the amplicons were purified using the Nucleospin Gel and PCR clean-up kit. Cloning of the purified catechol 2,3-dioxygenase gene from the metagenomic sample was performed using the CloneJET PCR cloning kit according to the manufacturer's specifications (ThermoFisher Scientific, Waltham, USA). Cloning of the catechol 2,3-dioxygnease gene was used to confirm that the correct gene of interest had been amplified.

Transformation of the ligation mixture into competent *E. coli* BL21cells was performed by adding 50 µl competent *E. coli* cells and 5 µl of the ligation mixture into a 1.5 ml microfuge tube and incubated on ice for 30 minutes. The cells were heat shocked at 42 °C for 2 minutes after which 950 µl of the Luria Bertani (LB) broth was immediately added to the 1.5 ml microfuge tube and incubated at 37 °C (shaking) for one hour. After the recovery period, 50 µl was spread onto Luria Bertani (LB) agar (g/L: 10 tryptone, 5 yeast extract, 5 NaCl and 15 agar) containing 100 µg/ml ampicillin. The remaining culture was centrifuged at 10 000 rpm for 5 minutes, 800µl of the supernatant was removed and the cells resuspended and 50 µl was plated onto LB agar plates. Agar plates were incubated at 37 °C overnight. Positive clones were selected and re-streaked onto LB agar containing ampicillin and analysis of recombinant clones were analysed by colony PCR using pJet 1.2 forward and reverse primers according to the manufacturer's instructions.



### 2.3.5 Denaturing gradient gel electrophoresis (DGGE)

#### 2.3.5.1 Optimization of nested PCR using DGGE primers

The amplified catechol 2,3-dioxygenase gene from CZA14$^T$ generated by qPCR was purified using the Nucleospin Gel and PCR cleanup kit (Macherey-Nagel). The purified product was amplified using the catechol 2,3-dioxygenase DGGE primers which included a GC-clamp (C23OF 5'CGCCCGCCGCGCGCGGCGGGCGGGGCGGGGGCACGGGGGG**AAGAGGCA TGGGGGCGCACCGG**TTCGATCA-3' and C23OR 5'-AACAAA(AGT)GCGC(GC)GTC ATGCGG-3') in a nested PCR. The section highlighted in bold is the gene specific portion. During the optimization, different *Taq* polymerases were used for the amplification of the C23O gene. Table 2.5 shows the conditions and parameters used for the nested PCR.

**Table 2.5:** Optimization of nested PCR with GC clamped catechol 2,3-dioxygenase-specific primers

| Taq polymerase | PCR conditions | PCR cycle parameters |
|---|---|---|
| DreamTaq | 1x DreamTag buffer, 1 µM DNTPs, 1 µM forward and reverse primer, 0.1% BSA, 1.25 U/rxn of DreamTaq DNA polymerase, and nuclease free water to 50 µl | 94 °C for 4 min<br><br>20 cycles: 94 °C for 45 s, 65 °C for 45 s and 72 °C for 60 s<br><br>20 cycles: 94 °C for 30 s, 55 °C for 30 s and 72 °C for 60 s<br><br>72 °C for 10 min |
| Phusion Taq | 1x Phusion HF, 200 µM DNTPs, 0.5 µM forward and reverse primer, <250 ng template DNA, 3% DMSO, 1 U/50 µL PCR rxn Phusion DNA polymerase and nuclease free water to 20 or 50 µl. | 94 °C for 4 min<br><br>20 cycles: 94 °C for 45 s, 64 °C for 45 s and 72 °C for 60 s<br><br>20 cycles: 94 °C for 30 s, 58 °C for 30 s and 72 °C for 60 s<br><br>72 °C for 10 min |
| Hifi Taq (Hotstart) | 1x KAPA HiFi buffer, 0.3 mM DNTPs, 0.3 µM forward and reverse primer, 0.1% BSA,10-50 ng template DNA, 0.5 U/rxn Kapa HiFi (hotstart) DNA polymerase and water to 25 µl | 95 °C for 4 min<br><br>20 cycles: 98 °C for 20 s, 60 °C for 15 s and 72 °C for 60 s<br><br>20 cycles: 98 °C for 20 s, 58 °C for 15 s and 72 °C for 60 s<br><br>72 °C for 5 min |



The nested PCR products for CZA14$^T$ were analysed on 2% agarose gel electrophoresis prior to loading it onto the DGGE gels. Once optimized PCR conditions were obtained for CZA14$^T$, the same conditions were used for the cDNA samples. qPCR amplification of the cDNA samples and the catechol 2,3-dioxygenase primer set was performed as described in section 2.3.6.2. The qPCR amplicons were purified using the Nucleospin Gel and PCR clean-up kit (Macherey-Nagel). The purified samples were amplified using conditions specified in Table 2.5. Prior to DGGE analysis amplicons were analysed by agarose electrophoresis.

### 2.3.5.2 Optimization and setup of DGGE

Optimisation of the catechol 2,3-dioxygenase DGGE was performed using CZA14$^T$ amplicons. DGGE gels were prepared as described by the manufacturer (Bio-rad). The gels were poured using a gradient former containing a 'high' (maximum amount of denaturants needed) and a 'low' (minimum amount of denaturants needed) gel solution. The 'high' and 'low' gel solutions were prepared by mixing a 0% and 100% denaturant gel stock solutions to give the required denaturant concentrations. The '0%' solution contained 40% acrylamide: *N,N'* bis-acrylamide (37.5:1) and 1 x TAE (40 mM Tris-HCl, 10 mM glacial acetic acid, 1 mM EDTA, pH8.0) only, while the '100%' solution included the denaturants 7 M urea and 40% (v/v)deionised formamide . The denaturing gradient for each set of primers used was determined by first using a broad denaturing gradient such as 30% to 70%, then adjusting the range depending on the level of separation achieved (10% and 40%). Electrophoresis was performed using the Bio-Rad DCode™ DGGE system. The PCR products were separated at a constant 100 V for 16 hrs in 1 x TAE at a constant temperature of 60°C. After electrophoresis the gels were stained in 1 x TAE containing ethidium bromide (0.5 mg $L^{-1}$) for 10–15 minutes and destained in 1 x TAE for 15 minutes.

Gels were viewed and the images captured under UV using the AlphaImage (AlphaInnotech) imaging system.



### 2.3.6 Cloning, ligation and transformation of the catechol 2,3-dioxygenase amplicons from the BSFs samples for phylogenetic analysis

The catechol 2,3-dioxygenase gene from the samples at the deep sediments of the BSFs was amplified using qPCR and the amplicons were purified using the Nucleospin Gel and PCR clean-up kit (Macherey-Nagel). Cloning of the purified catechol 2,3-dioxygenase gene from the samples at the deep sediments of the BSFs was performed using the CloneJET PCR cloning kit according to the manufacturer's specifications (ThermoFisher Scientific, Waltham, USA).

Prior to transformation, the ligation mixture was purified by drop dialysis. A Merck Millipore filter disk (Merck, Darmstadt, Germany) was placed in a petri dish base containing sterile water. 10 µl of the ligation mixture was added to the disk filter and allowed to stand at room temperature for one hour. After that time period the contents from the disk filter was transferred to a sterile 1.5 ml microfuge tube and stored at -20 °C.

Transformation of the ligation mixture into electrocompetent *E. coli* Epi 300 cells was performed by adding 40 µl competent *E. coli* cells and 1µl of the ligation mixture into a 1.5 ml microfuge tube. The contents were gently mixed and transferred to an electroporation cuvette. The cuvette was placed in the Gene Pulser (Bio-Rad, Philladelphia, USA) and pulsed as per the ECI programme for bacteria. Immediately after the electroporation 1 ml of LB broth was added to the cells and transferred to a sterile 1.5 ml microfuge tube and incubated at 37 °C for one hour. After the incubation period, 50 µl was spread onto LB agar containing 100µg/ml ampicillin. The remaining culture was spun down (10 000 rpm, 22 °C for 5 minutes), 800µl of the supernatant was removed and the cells resuspended and plated onto agar plates. Agar plates were incubated at 37 °C overnight. Positive clones were selected and restreaked onto LB agar containing ampicillin and analysis of recombinant clones were analysed by colony PCR using pJet 1.2 forward and reverse primers.

# CHAPTER THREE

# RESULTS AND DISCUSSION

## 3.1 Introduction

This chapter relates to assessing the impact that the amendment with synthetic winery wastewater had on the BSFs and this was achieved through using molecular fingerprinting techniques. Sand samples were collected from the BSFs at the start of the experiment and after the amendment with synthetic winery wastewater. The effect of the synthetic winery wastewater on the bacterial and fungal communities was assessed through amplification of the 16S rRNA gene and ITS region, respectively. The resultant amplicons were digested and analysed by T-RFLP. Bacterial and fungal community profiles were created using bioinformatics based programmes. The sand samples were also used to assess the expression of functional genes (alcohol dehydrogenase and catechol 2,3-dioxygenase) through the use of quantitative-PCR.



## 3.2 Biological sand filters (BSFs) set up and mode of operation

In the Cape Town area there are two types of inexpensive commercial sands that are readily available, namely Malmesbury and Phillipi sand. BSFs at the Cape Peninsula University of Technology (CPUT) consist of three polyethylene tanks (173 cm in length, 106 cm in width) which contain Phillipi sand packed to a depth of 30 cm. During weeks 1-4 of the experiments, the systems were fed/amended three times per week and plugged for a 24-hour period, however unsatisfactory removal performance warranted an increase in the hydraulic retention time (HRT). Therefore, from week 5, the regime was decreased to feeding twice per week with a plug time of 48 hrs. During weeks 5-10 the BSFs were amended with synthetic wastewater containing acetate and from weeks 26-31 the BSFs were amended with synthetic wastewater containing glucose instead of acetate. For the purpose of this study, T-RFLP analysis was performed on the sand samples taken at baseline (0 weeks) and after the amendment with the synthetic winery wastewater containing acetate (week 31).

Previous studies (Welz *et al.*, 2011, 2012) have shown that acetate is the most common metabolite produced during the degradation of many organics, including those present in winery wastewater (vanillin, gallic acid, acetate and ethanol). Acetate is also a major component of winery wastewater (Welz *et al.,* 2016). This resulted in the addition of acetate

to the synthetic winery wastewater used in this study. From literature it is clear that acetate plays a vital role in microbial pathways, especially pathways associated with co-enzyme A (Wolfe, 2005). Many microorganisms are able to utilize acetate for acetogenesis. *Clostridium* species are examples of organisms that are able to degrade or build-up acetate to intermediate fatty acids under anaerobic conditions.

### 3.3 Sand sample analyses

It is known that many factors affect the growth of microorganisms and this could be negatively or positively. The presence of micro-nutrients and heavy metal concentrations, even when present in trace amounts, could lead to differences in the bacterial community structures (Carson *et al.*, 2007; Gadd, 2010). Table 3.1 represents the physical and chemical properties of the sand samples. The major component of the Phillipi sand is silicone ($SiO_2$), comprising approximately 86% and was present as quartz in the samples. Based on mechanical fractionation the sand is predominately medium sand (41.8%). The micronutrients K, P and Fe were the most abundant metals in the sand, while heavy metals were detected in limited quantities.



**Table 3.1:** Phillipi sand sample composition

| **Mechanical fraction** | | | | | | |
|---|---|---|---|---|---|---|
| Clay | Silt | Fine sand | Medium sand | Coarse sand | | |
| 3.93% | 1.67% | 24.93% | 41.80% | 27.67% | | |
| **Major elements*** | | | | | | |
| $Al_2O_3$ | CaO | $Fe_2O_3$ | $K_2O$ | MgO | $SiO_2$ | $Na_2O$ |
| 0.31% | 7.66% | 0.07% | 0.15% | 0.15% | 85.59% | 0.21% |
| **Micronutrients present** | | | | | | |
| P (mg/kg) | K (mg/kg) | Cu (mg/kg) | Zn (mg/kg) | Mn (mg/kg) | B (mg/kg) | Fe (mg/kg) |
| 4.67 | 7.33 | 0.59 | 0.37 | 0.47 | 0.11 | 12.7 |
| **Heavy metals** | | | | | | |
| Cd (mg/kg) | Pb (mg/kg) | As (mg/kg) | Hg (mg/kg) | Sb (mg/kg) | | |
| 0.03 | 0 | 0.31 | 0 | 0.07 | | |
| **Exchangeable cations** | | | | | | |
| Na [cmol(+)/kg] | K [cmol(+)/kg] | Ca [cmol(+)/kg] | CEC [cmol(+)/kg] | Mg [cmol(+)/kg] | | |
| 0.09 | 0.02 | 12.23 | 1.65 | 0.17 | | |
| **Base saturation** | | | | | | |
| Na | K | Ca | Mg | | | |
| 0.77% | 0.16% | 97.67% | 1.40% | | | |

*$Cr_2O_3$, MnO not detected, $P_2O_5$ and $TiO_2$ detected in limited quantities

### 3.4 PCR amplification of 16S rRNA bacterial gene and ITS fungal region

#### 3.4.1 Optimization of PCR conditions: bacterial primers

PCR optimization was performed by firstly comparing two DNA *Taq* polymerases: KAPA *Taq* Readymix DNA polymerase [KAPA Biosystems (Pty) Ltd, Cape Town, South Africa] and Dream*Taq* PCR Mastermix (ThermoScientific, Waltham, USA). The 16S rRNA gene PCR products of six mDNA samples extracted from BSF P1 were selected to establish the optimal amplification conditions for the universal bacterial primers E9F and U1510R (Marchesi *et al*., 1998; Reysenbach and Pace, 1995). Amplification of the 16S rRNA gene was achieved as described in section 2.3.4.1. The approximate size of the gene of interest is in the region of 1200- 1600 bp.

Once the optimized conditions were obtained the remaining mDNA samples were amplified. Dream*Taq* (Figure 3.1 Gel B) performed better than Kapa*Taq* (Gel A), and was thus used for all future PCR reactions. A likely reason for Dream*Taq* polymerase performing better than the Kapa*Taq* is that Dream*Taq* is an enhanced DNA Polymerase that has been optimised for all types of PCR reactions including those which include PCR inhibitors (such as humic acid). The polymerase has been engineered to have enhanced yields, be more sensitive and amplify longer PCR products as compared to conventional *Taq* DNA polymerases (Life Technologies, 2015).

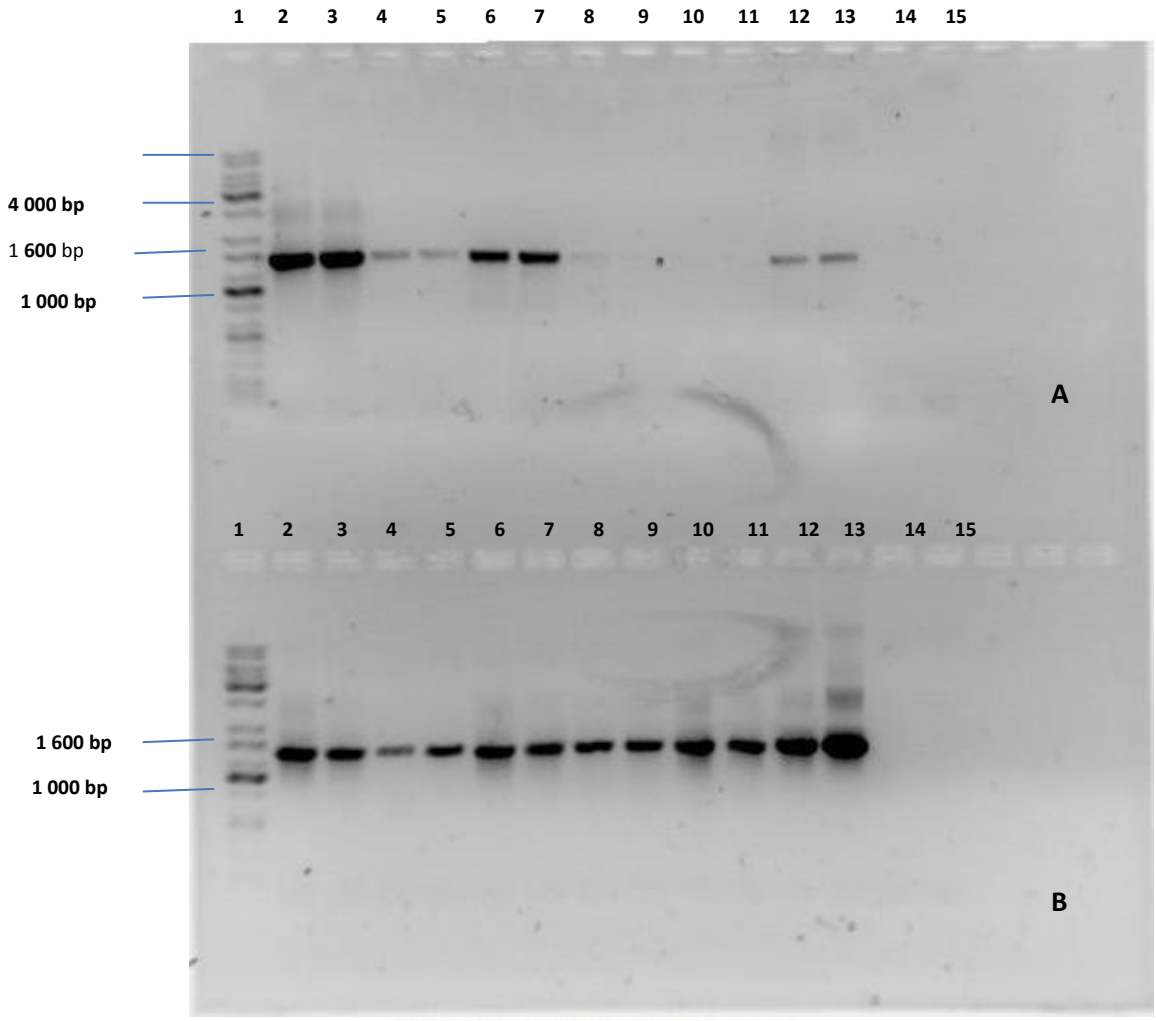





**Figure 3.1:** PCR products generated using two different DNA *Taq* polymerases. Gel A represents amplicons generated with Kapa*Taq* ReadyMix DNA polymerase and Gel B represents amplicons generated with Dream*Taq* Green PCR MasterMix. Lane 1: marker, lane 2 and 3: P1 superficial inlet, lane 4 and 5: P1 deep inlet, lane 6 and 7: P1 superficial outlet, lane 8 and 9: P1 deep outlet, lane 10 and 11: P2 superficial inlet, lane 12 and 13: positive control *Streptomyces* sp. GSIII #1 and lane 14 and 15: negative control

### 3.4.2 Optimization of PCR conditions: fungal primers

Fungi play a significant role in bioremediation (Frey-Klett *et al*., 2011). PCR using the ITS primers ITS1F and ITS4 required numerous optimization steps which included performing a gradient PCR by changing the annealing temperature over a temperature range of 40 to 60 °C increasing in 2 °C increments; and performing a Touchdown PCR (changing the amount of cycles at annealing temperature step of 10 cycles at 55 °C and 25 cycles at 55 °C). The presence of fungal communities was confirmed by the presence of amplified products, but in some instances two bands were detected (Figure 3.2).

PCR optimization was attempted through the method described by Manter and Vivanco (2007), and the authors suggested that the different fragment sizes (420 to 825 bp) represent three fungal classes: ascomycetes (420-806 bp), basidiomycetes (503-825 bp) and zygomycetes (653-788 bp). The positive control, *Trametes pubescens* forms part of the basidiomycete family and the presence of similar sized band (503-825 bp) observed on the agarose gel indicated that many of the sand samples are likely to contain basidiomycetes. For the samples containing two bands, each band was excised independently from the agarose gel and re-amplified separately by PCR. For several samples after the second round of PCR, additional bands were detected, probably indicating non-specific binding.



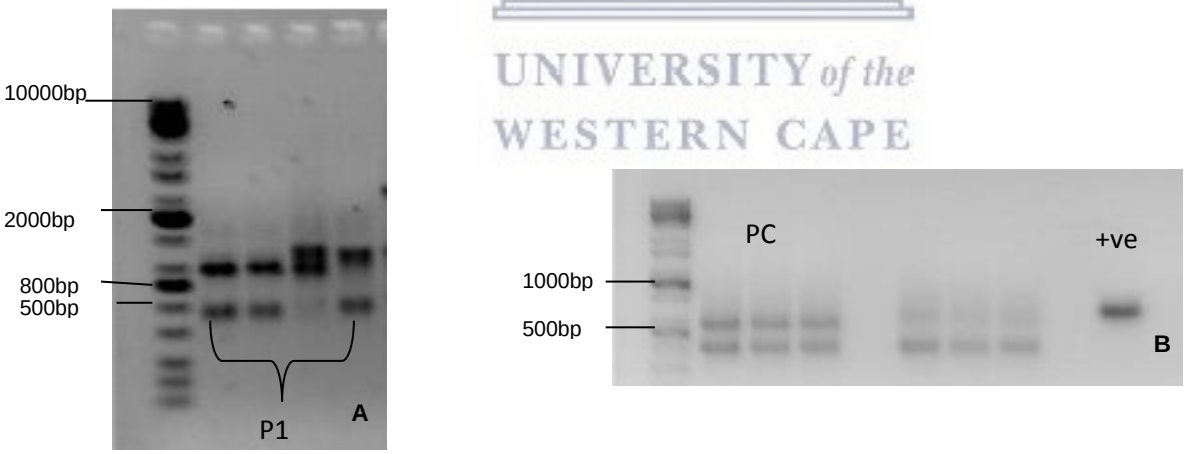



**Figure 3.2:** 2% agarose gels showing samples for BSFs P1 and PC. Gel A contains samples from BSF P1 and Gel B samples from BSF PC and the positive control used in the study, *Trametes pubescens.*

### 3.5 Terminal restriction fragment length polymorphism analysis

#### 3.5.1 Peak Scanner data

Bacterial and fungal community structures were assessed by T-RFLP fingerprinting using the 16S rRNA gene and ITS region as markers, respectively. Figure 3.3 represents the T-RFLP profile generated from PeakScanner. Each peak is characterised according to height and area, and represents a separate operational taxonomic unit (OTU) within each T-RFLP profile.

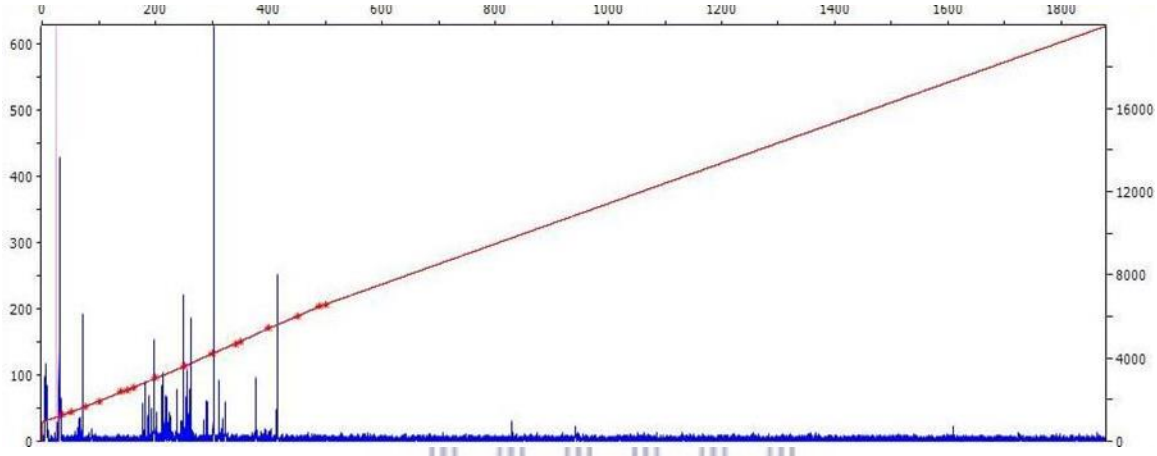



**Figure 3.3:** T-RFLP profile of a P1 bacterial sample using PeakScanner with each peak being characterised by height and area.



#### 3.5.2 Non-metric dimensional scaling (N-MDS) plots and phylogenetic analysis of bacterial populations

**3.5.2.1 Assessing the effect of synthetic winery wastewater on the bacterial population at the superficial sediments of the BSFs through T-RFLP and phylogenetic**

The evolution of the microbial community structure was assessed through T-RFLP analysis and visualised using multidimensional scaling (MDS) plots where the distance between each point shows the degree of similarity within the community profiles of the samples. Clarke (1993) suggested that if the stress value is >0.2, then non-multidimensional scaling (NMDS) representation is random, if <0.15 it is good and if <0.1, it is ideal. Therefore, if the 2D stress value is higher than 0.15, at least one more statistical analysis, such as analysis of similarity (ANOSIM) and/or Cluster analysis must be performed to infer the NMDS representation. It can be presumed that if R >0.75 the groups are well separated, if >0.5 the groups clearly differ (despite overlap), if <0.25 then the groups are not different (Clarke and Gorley, 2006).

In this study, the ANOSIM analyses of the T-RFLP profiles showed that significant different bacterial communities were found in the superficial sediments ($R = 0.407$ and $p < 0.05$) and the deep sediments ($R = 0.591$ and $p < 0.05$) when analysing the samples before and after the amendment with synthetic winery wastewater. The overall fungal model also showed differences at the superficial sediments ($R = 0.532$ and $p < 0.05$) and the deep sediments ($R = 0.356$ and $p < 0.05$). Differences were observed when comparing the R values for each niche, however the p value for each showed no significance between the niches (p value< 0.05).

To allow more thorough analyses of the effect of winery wastewater on the bacterial communities in the different sediments, separate NMDS plots were compiled for the superficial (Figure 3.4) and deep (Figure 3.8) sediments. The plot represents data at 0 (baseline or start of experiment) and 31 weeks after the amendment with synthetic winery wastewater, indicated with the letter 'F'. The experimental BSFs were compared to the control BSF PC. The stress value indicated is 0.08 and according to Clarke (1993) this is ideal.

Before amendment with synthetic winery wastewater (baseline), all points representing the bacterial communities in the superficial sediments share 40% similarity, expect for the outlying group the control (PC) which only shares an overall 20% similarity with the other baseline samples on the NMDS plot (Figure 3.4). After amendment with the synthetic winery wastewater, all points representing the bacterial communities shared 60% similarity, and only 40% similarity to the controls, as compared to the baseline communities showing that the amendment with synthetic winery wastewater had an effect on the bacterial communities.



After amendment with the synthetic winery wastewater, the points representing the bacterial communities from the inlet and outlet of each BSF cluster closer to one another than non-related samples. In contrast, at the start of the experiment the bacterial communities do not cluster together. The shift in bacterial communities after amendment with synthetic winery wastewater of the experimental systems P1 and P2 is evident by the distance between the points when looking at the arrows, confirming that indeed amendment with synthetic winery wastewater had an effect on the bacterial communities and showing that the organisms become more similar after amendment. Unexpectantly, when comparing the experimental systems to the control BSF PC at the start and end of the experiment, BSF PC bacterial community structure shifts drastically and groups with the experimental systems at the end of the experiment. The shift in the control BSF PC could be due to a constant seasonal shift in microbial communities. Taking this into consideration the shifts observed in microbial

communities in the experimental systems could have been due to the temporal/seasonal (or both) changes. However, as the experimental and control communities are different at the end of the experiment it is likely that community shifts is due to differences in the chemical milieu resulting from substrate degradation gradients from inlet to outlet (Appendix A) rather than temporal changes (or possibly a combination of both factors). From these results, one can deduce that the addition of synthetic winery wastewater had an impact on the bacterial community structure in the superficial sediments (Figure 3.4). The changes in bacterial communities were confirmed by the ANOSIM (superficial sediments global R value 0.407 and deep sediments R value 0.591). Figure 3.5 shows the position of each BSF system used in this study (P1, P2 and PC).

Several hypothesis could be made on why a shift between systems were observed, one being from Figure 3.4 it is clear that the control system changed the most as compared to the experimental BSFs, this could be due to the fact that there were some problems in establishing the population in this wetland at the start of the experiment (leakage in the system). Additionally, the two experimental systems P1 and P2 were situated on the opposite side as the control system PC (which was less protected than the experimental systems). The different positioning of the BSFs between the experimental and control systems could contribute to the shifts of the bacterial communities on the NMDS plots (Figure 3.5). This could therefore be the reason for the PC system being an outlying group as compared to BSF P1 and BSF P2 at the superficial sediments at the start of the experiment. Another hypothesis could be made that the shifts observed in all the NMDS could be on the basis of the phenomenon known as bacterial succession. Bacterial succession is when organisms changes over time or if they have been placed into a new environment where changes have been made to it (e.g. pH). Drastic changes could lead to one species outcompeting the present ones for nutrients leading to the demise of the primary species (Dini-Andreote *et al*., 2014). The dynamics of bacterial communities in engineered eco-systems are influenced by both deterministic and stochastic factors. Deterministic factors includes, competition and niche-specific variables while stochastic factors includes microbial dispersal by random events of colonization/extinction or fluctuations in the influent composition e.g. nitrogen and organics loads or presence of toxic compounds (Dini-Andreote *et al*., 2014; Cydzik-Kwiatkowska and Zielin`ska, 2016). A study by Zhang *et al*., 2011 assessed the removal of acetone, butanol and ethanol fermentation wastewater through the use of an anaerobic baffled reactor. The reactor yielded ~90% removal of COD of methane and through the spatial community succession patterns populations such as hydrogenotrophic methanogens like *Methanoculleus* sp. IIE1 (ABRA01)

and an acetogen like *Acetoanaerobium noterae* (ABRB10), were identified as possible key species for oxidization of solvents or VFAs by analysis of spatial community succession.

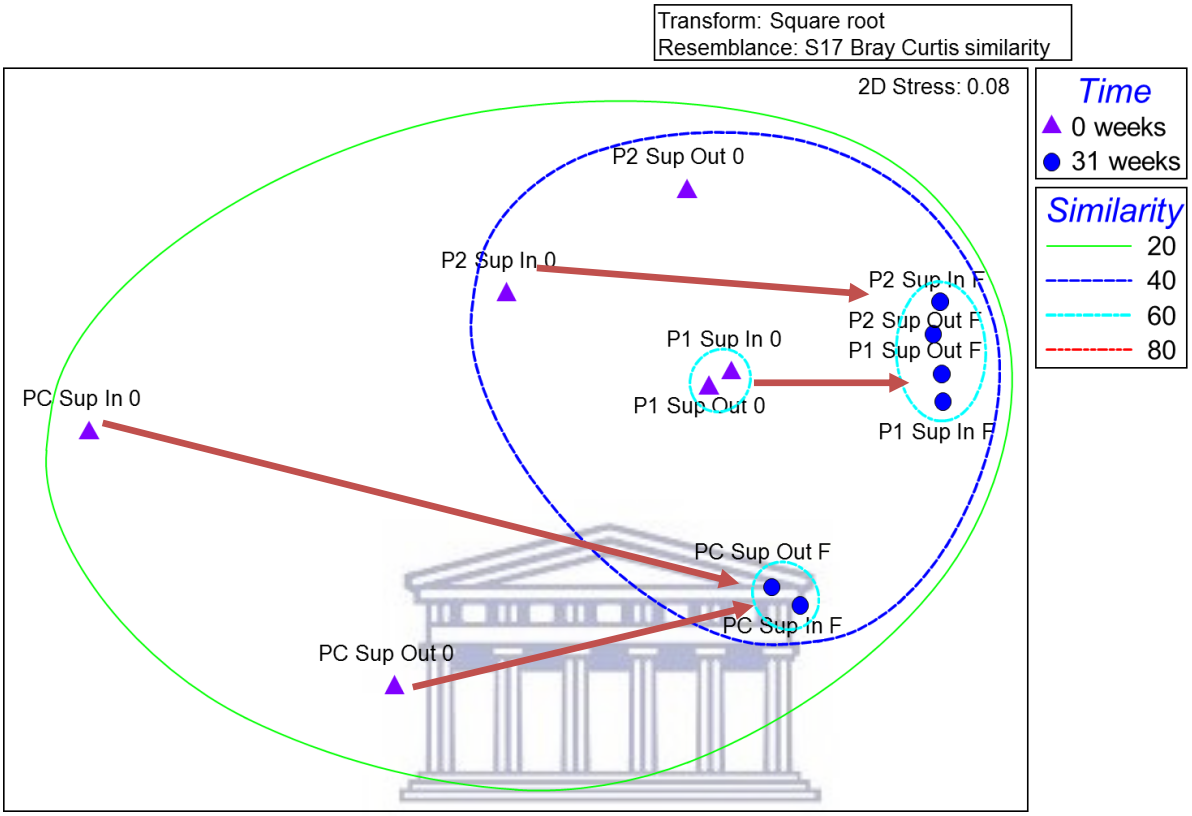



**Figure 3.4:** 2D-Nonmetric multi-dimensional scaling plot of Bray Curtis similarity of the microbial community structures in the superficial sediments of the BSFs. P1 and P2 denote the experimental systems and PC the control (stress value =0.08). Sup In: superficial inlet and Sup Out: superficial outlet of each BSF where the core sand samples were taken. Red arrows indicating the shift in similarity or dissimilarity between points on the NMDS plot.

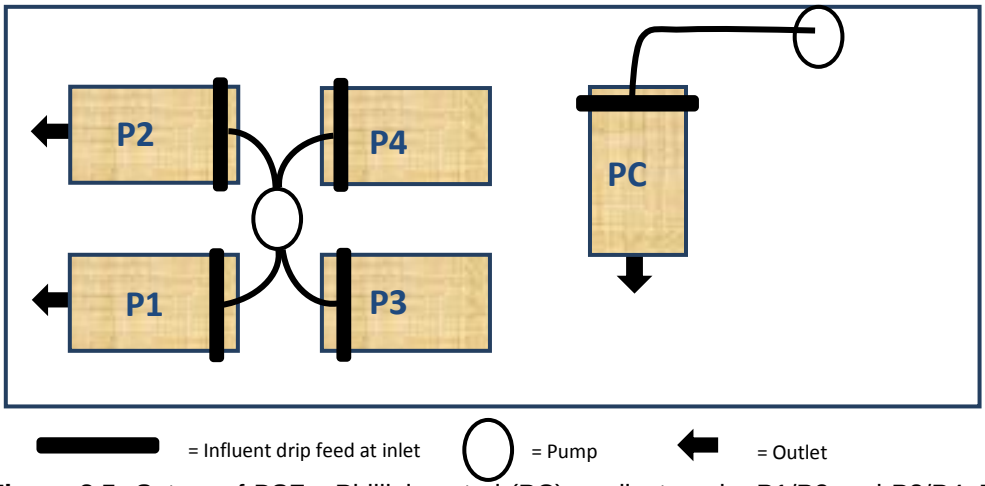



**Figure 3.5:** Set-up of BSFs: Phillipi control (PC), replicate pairs P1/P2 and P3/P4. Three BSFs used in study P1, P2 and PC (Welz *et al.*, 2011)

For the phylogenetic analysis of the bacterial communities' structure, the T-RFLP profiles for both the experimental systems and the control system were exported from PeakScanner and uploaded into the software MICA3. These profiles were compared to the MICA3 16S rRNA gene database. The output file generated from MICA3 represented the possible "hits" (species) that could be in the sand samples. This was performed for the two experimental systems (P1 and P2) and the control system (PC) at all four sites (superficial inlet and outlet, and the deep inlet and outlet). The phylogenetic data was compared to the T-RFLP data in order to corroborate the changes in community structure observed and confirming that amendment with synthetic winery wastewater resulted in changes in diversity of organisms present. Table 3.2 shows how many TRFs were detected before and after the amendment with synthetic winery wastewater. This TRFs data was used to establish the MICA3 pie charts and organisms possibly present in the BSFs. Each niche of the BSFs were shown below with 0 representing the start of the experiment and F at the end of the experiment. Table 3.2 shows an increase in TRFs from day 0 to at the end of the 31-week period.

**Table 3.2:** TRFs detected before and after the amendment with synthetic winery wastewater. Values represented after normalisation of data.

| BSF | Sup In | | Sup Out | | Deep In | | Deep Out | |
|---|---|---|---|---|---|---|---|---|
| | **0** | **F** | **0** | **F** | **0** | **F** | **0** | **F** |
| **P1** | 29 | 48 | 23 | 38 | 4 | 17 | 6 | 22 |
| **P2** | 26 | 35 | 29 | 35 | 14 | 27 | 12 | 18 |
| **PC** | 7 | 31 | 9 | 32 | 8 | 15 | 6 | 13 |

From the pie charts (Figure 3.6 and Figure 3.7) it is clear that after the amendment with synthetic winery wastewater there is a drastic change in the diversity of the bacterial population present within each sample at the superficial sediments of the BSFs. Figure 3.8 and Figure 3.9 shows the changes in diversity of the possible organisms present at the deep sediments of the BSFs. The segment labelled 'Other' in the pie charts represents any type of uncultured bacterium [including uncultured bacterium, proteobacterium (α, β, γ, δ), and uncultured rumen bacterium] that was identified by MICA3. Although a large percentage of the 'hits' of possible organisms were most related to unculturable organisms, many Gram negative organisms (*Enterobacter, Pantoea* species, *Vibrio*, and *Shewanella*), Gram positive organisms not associated with sand (*Psychrobacter, Serrodes*, *Deinococci, Lactobacillus,* and *Magnetococcus*) and marine organisms (*Marinobacter*, *Aeromonas*, *Pseudoalteromonas, Pseudomonas*, and *Roseobacter*) were identified. Table 3.3 and Table 3.4 highlights key organisms detected in this study that have previously been identified to play an important role as sand organisms. The MICA3 data identified Gram negative bacteria and marine organisms, and literature has shown that some of these organisms are involved in the degradation of compounds as discussed below.



Bacteria have the unique biochemical ability to oxidise compounds into smaller subunits which can enter various metabolic pathways. Each genera identified could contribute to a specific metabolic function within the BSFs to treat wastewater effluent. Complete degradation of the wastewater components may be achieved by the sequential metabolism of two or more organisms (Cloete and Muyima, 1997). A number of environmental factors affect the presence of microbial populations in wastewater and the rate of biochemical reactions. The most important factors being temperature, pH, availability of nutrients, and the toxicity and inhibitory compounds (Beltran *et al*., 1999). Studies have shown that microorganisms that occur naturally in winery wastewater are adapted to the conditions within the wastewater and should be useful in the biological treatment of that specific wastewater (Petruccioli *et al*., 2002). Microbial degradation of compounds present in several

wastewaters has been explained in literature (Pant and Adholeya, 2007). A study by Ghosh *et al*. (2004) showed bacterial strains that were isolated from soil exposed to distillery spent wash effluent were capable of degrading recalcitrant compounds. These organisms included *Pseudomonas*, *Enterobacter*, *Stenotrophomonas*, *Aeromonas*, *Acinetobacter* and *Klebsiella* species. Malandra *et al*. (2003) reported the presence of microorganisms in biofilms in a rotating biological contactor (RBC) that was used for the treatment of winery wastewater. A synthetic winery wastewater was prepared in the study and the biofilm communities were assessed through T-RFLPs. The fingerprinting and identification of bacteria present in an upflow anaerobic sludge blanket (UASB) bioreactor which was used for the treatment of canning wastewater was also performed (Keyser *et al*., 2007). Through the study, different granules that were collected contained a consortium of bacteria, each consisting of a different bacterial population and each with a specific metabolic function which contributed to the overall functionality and stability of the bioreactor system. A total of 68 different bacterial species were identified through sequencing. In addition, it was observed that each granule had a distinct PCR-based DGGE phylotype (unique band) and some phylotypes were consistent in all samples irrespective of wastewater type and the sequence information obtained indicated that only 35% of the identified bacteria were unculturable. The culturable bacteria identified included *Pseudomonas*, *Bacillus*, *Bacteroides*, *Enterococcus*, *Clostridium*, *Shewanella*, *Vibrio*, and *Rhodococcus* species (Keyser *et al*., 2007). The results obtained in this study (presented below) are comparable to those of Keyser *et al*. (2007).

The hits generated by MICA3 identified potential organisms which could be present in the BSFs. These hits could truly be misdirecting which novel organisms could possibly be present. From Table 3.3, it can be seen that at the surface the BSF P1 at the start of the experiment many marine organisms (*Aeromonas*, *Pseudoalteromonas*) were present, while after amendment with synthetic winery wastewater more sand organisms were present (*Streptomyces*, *Acidobacteria*). This shift may reflect that sand organisms are more suited to the nutrients available in the synthetic wastewater and are able to breakdown of the phenolic compounds present. Although the MICA3 data showed organisms not associated with the sand (*Paracoccus*, *Fibrobacter*, *Chloroflexi*,) it showed that the addition of the synthetic winery wastewater had an effect of the diverse microbial organisms present in the BSFs. Additional experiments (DGGE and clone sequencing) was performed in order to confirm the presence of these organisms detected through MICA3.

**Table 3.3:** Phylogenetic profiles of the possible organisms present at the superficial sediments of the BSFs

| BSF niches | Organism of interest/ associated with sand | Application | Reference |
|---|---|---|---|
| **Superficial Inlet**: Start of experiment (baseline) | P1: Bacteriodetes | This phylum composes of three large classes of Gram negative non-sporeforming, either anaerobic or aerobic bacteria. They are also found in diverse environments including soil, sea water, and guts and skins of animals. | (Krieg *et al*., 2010) |
| | P2: *Sarcina* | ***Sarcina*** is a genus of the Gram-positive cocci belonging to the *Clostidiaceae*. The genus *Sarcina* is known for its synthesis of cellulose. Species of *Sarcina* have been associated with soil, mud, and guts of rabbits and guinea pigs. | (Ross and Mayer, 1991) |
| | PC:Actinobacterium | ***Acinobacterium***: The class *Actinobacteria* represents a large group of bacteria that are morphologically complex. Actinobacteria have been discovered in a variety of environments that include composts, marine environments, manure, waste water and they have also been associated with buildings and artworks. | (Embley and Stackebrandt, 1994)<br><br>(Groth *et al*., 1999; Gottlieb, 1973) |
| After the amendment with synthetic winery wastewater (SWW) | P1:*Acidobacteria*, *Fibrobacter*, *Micromonospora*, *Propionibacterium*, *Rhodococcus*, *Streptomyces* | ***Acidobacteria***, members of this phylum of bacteria are diverse and found in many environments, especially in soils. Many of these organisms are acidophilic and first identified in 1991 (*Acidobacterium capsulatum*). These organisms have been identified to be a contributor to many ecosystems especially being abundant in soil. | (Barns *et al*., 2007; Rappe and Giovannoni, 2003; Eichorst *et al*., 2007) |

| | | | |
|---|---|---|---|
| | P2:*Propionibacterium*, *Rhodococcus*, *Streptomyces* | ***Fibrobacter***, organisms responsible for the degradation of plant based cellulose in rumen bacteria. | (Montgomery *et al*., 1988) |
| | | ***Micromonospora***, are Gram positive, spore forming, aerobic organisms that form branched mycelium. These organisms are predominantly found in soil and water with some species known for the production of secondary metabolites such as aminoglycoside antibiotics. | (Kroppenstedt *et al*., 2005) |
| | | ***Propionibacterium***, is a Gram positive, rod-shaped genus of bacteria that is well known for its unique ability to synthesise propionic acid by using transcarboxylase enzymes. | (Cheung *et al*., 1975) |
| | | Most species of ***Rhodococcus*** are pathogenic. Some strains of *Rhodococcus* are important for their ability to breakdown a wide variety of compounds to produce bioactive steroids, acrylamide and acrylic acid. They have been isolated from environments including soil, water and eukaryotic cells. They are also known for their use as biological systems to convert cheap starting material into more valuable compounds. This includes their ability to metabolize harmful environmental pollutants such as toluene and naphthalene and herbicides. | (McLeod *et al*., 2006)<br>(Treadway *et al*., 1999). |

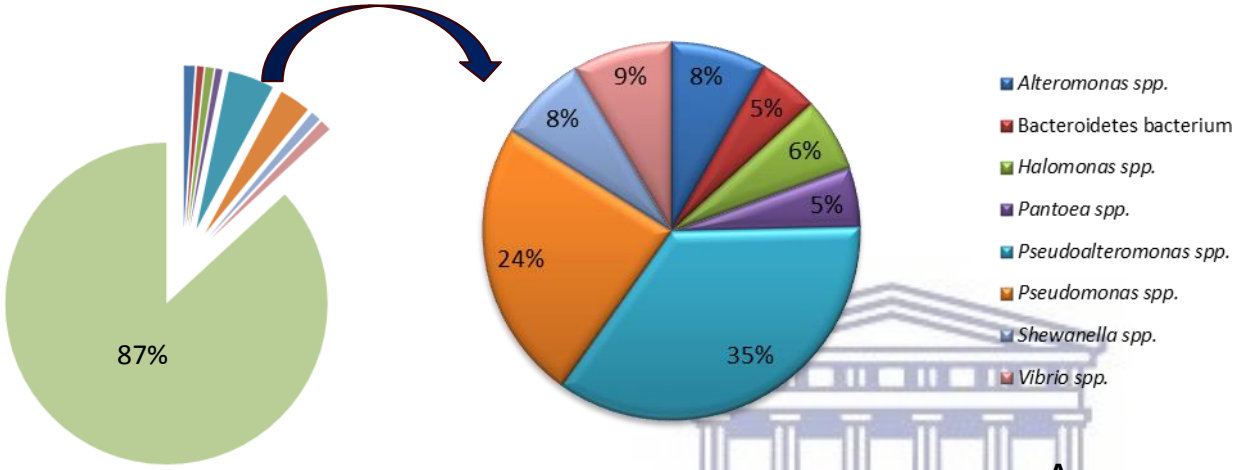
| | | | |
|---|---|---|---|
| | PC: No organisms identified. | Members of the genus ***Streptomyces*** specifically, is well-known for the production of antibacterials, anti-tumour agents, antifungals and herbicides.<br>The genus *Streptomyces* has become industrially important due to its ability to degrade or form complex polymers such as lignin, melanins, and many others. | (Willey *et al*., 2008)<br><br>(Claus and Decker, 2006) |
| **Superficial Outlet**: Start of experiment (baseline) | P1:Bacteriodetes<br><br>P2: Bacteriodetes, *Propionibacterium*<br><br>PC:*Azospira*, *Rhodococcus* | As above<br><br>As above<br><br>Only two species of the genus ***Azospira*** have been described namely *Azospira oryzae* and *Azospira restricta* which are both nitrogen fixing bacteria. | (Falkow and Dworkin, 2006) |
| After the amendment with synthetic winery wastewater (SWW) | P1: Acidobacteria, *Corynebacterium*, *Dietzia*, *Microbispora*, *Micromonospora*, *Nocardiopsis*, *Propionobacterium*, *Rhodococcus*, *Thermus* | The genus ***Corynebacterium*** was first described by Collins and Cummins, (1986) as Gram positive, non-spore forming, catalase positive, rod shaped bacteria. They occur | Collins and Cummins (1986) |

| | | | |
|---|---|---|---|
| | | commonly in soil, water, plants and food products. | |
| | | They are also important industrially for their production of amino acids and nucleotides; biological conversion of steroids; degradation of hydrocarbons; aging of cheese; and the production of enzymes. | (Constantinides, 1980)<br><br>(Cooper *et al*., 1979)<br>(Lee *et al*., 1985) |
| | | Some species of *Corynebacterium* can produce secondary metabolites similar to antibiotics and antitumor agents. | |
| | | L-lysine production is specific to *Corynebacterium* in which core enzymes are manipulated through genetic engineering to produce NADPH through the pentose phosphate pathway. | (Milas and Scott, 1978)<br>(Kjeldsen, 2008) |
| | | Multiple strains of ***Dietzia***, have been found in soil, deep sea sediments, marine aquatic life, and soda lakes. | (Gerday and Glansdorff, 2007) |
| | | Some of *Dietzia* species have been described as human pathogens. One of the strains<br>*D. cinnamea* is capable of degrading a wide variety of petroleum hydrocarbons which have been described as being beneficial to many environmental implications to the world's current situation. | (Von der Weid, 2006) |
| | | ***Microbispora*** is a genus belonging to the Class *Actinomycetales.* With one species *Microbispora corallina* being isolated from Thai soil. | (Nakajima *et al*., 1999) |
| | | ***Nocardiopsis*** is a genus of aerobic, | (*Nocardiopsis*, 2003) |

| | | | |
|---|---|---|---|
| | | Gram positive soil bacteria similar to *Nocardia* but differ in cell wall type. | |
| | | **Thermus** is a genus of thermophillic bacteria, belonging to the Deinococcus-Thermus group. This group consists of the well-known species namely, *Thermus aquaticus*, which is the source of the heat resistant enzyme called *Taq*-polymerase which is used in molecular biology. | Brock and Freeze, 1969; Chung *et al*., 2000)<br><br>(Brock and Freeze, 1969) |
| | P2:*Azospira*, *Clostridium* | ***Clostridium*** is a Gram positive bacteria. Some species are present as human pathogens and these include, *C. botulinum* which is capable of producing a botulinum toxin in foods and wounds *C. perfringens* which can cause a wide variety of symptoms from food poisoning to gas gangrene; as well as it can be used as a replacement for yeast in salt rising breads. Some species of *Clostridium* have commercial use and this includes *C. thermocellum* which can use lignocellulosic waste to generate ethanol thus making it a promising candidate for the production of bioethanol. The properties of *C. thermocellum* are that it requires no oxygen and is thermophillic. | (Wells and Wilkins, 1996a)<br><br>(Wells and Wilkins, 1996b)<br><br>(Demain *et al*., 2005) |
| | PC: Actinobacterium | As explained above | |

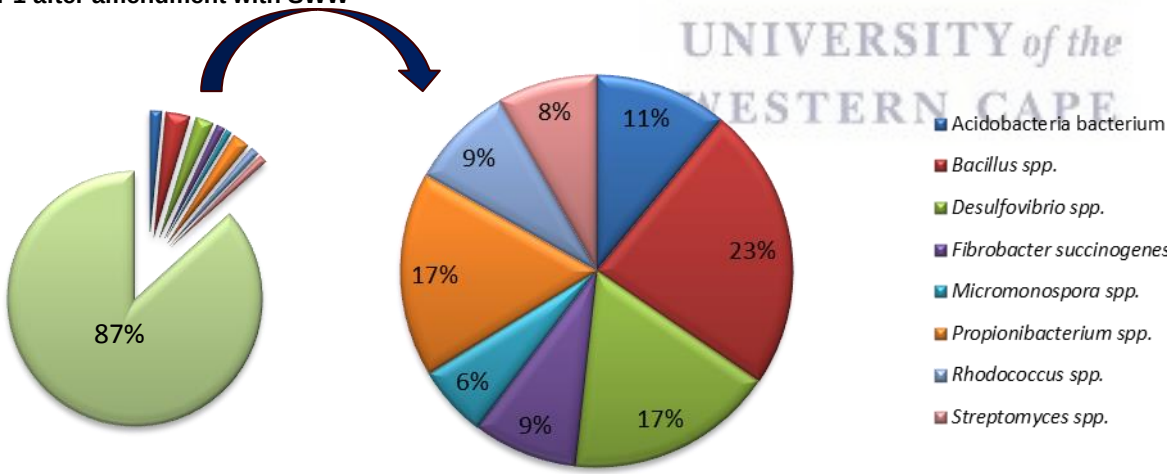
- **Superficial Inlet**

**P1 baseline:**




**A**

**P1 after amendment with SWW**



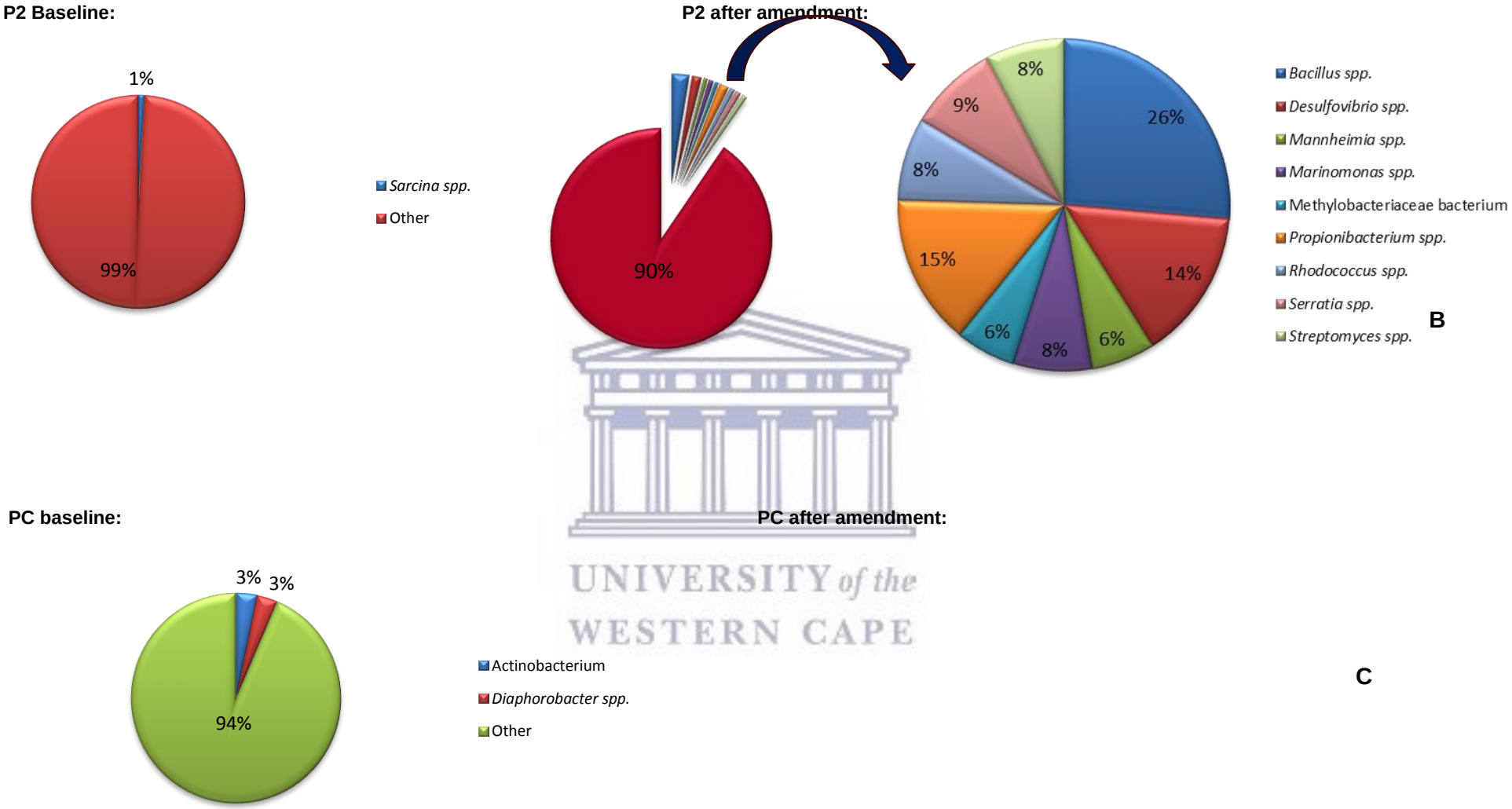
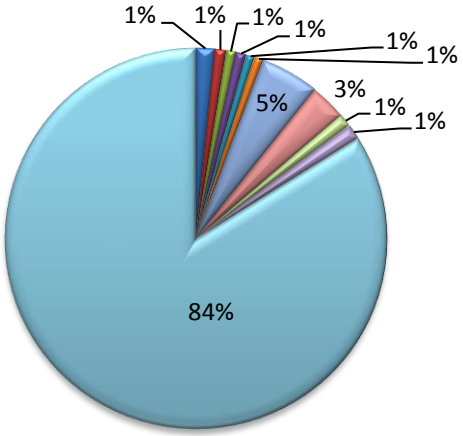
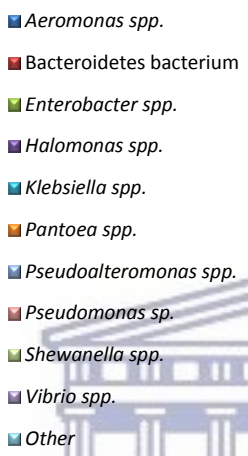



**Figure 3.6:** Phylogenetic analysis of the T-RFLP profiles of the bacterial community structure at the superficial inlet using the software MICA 3. Pie chart A represents the changes in the bacterial community structure of P1, P2 (B) and PC (C) before and after the amendment with synthetic winery wastewater.

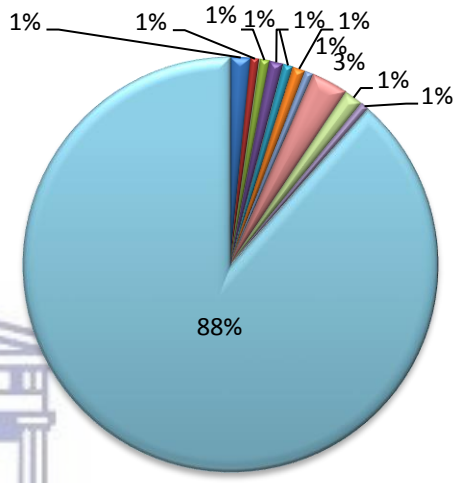
- **Superficial Outlet**

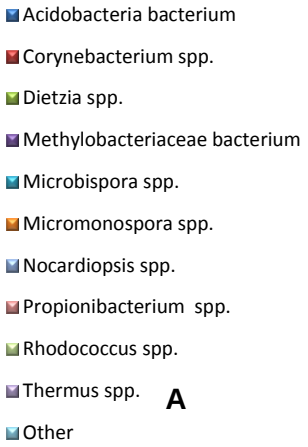
**P1 baseline**



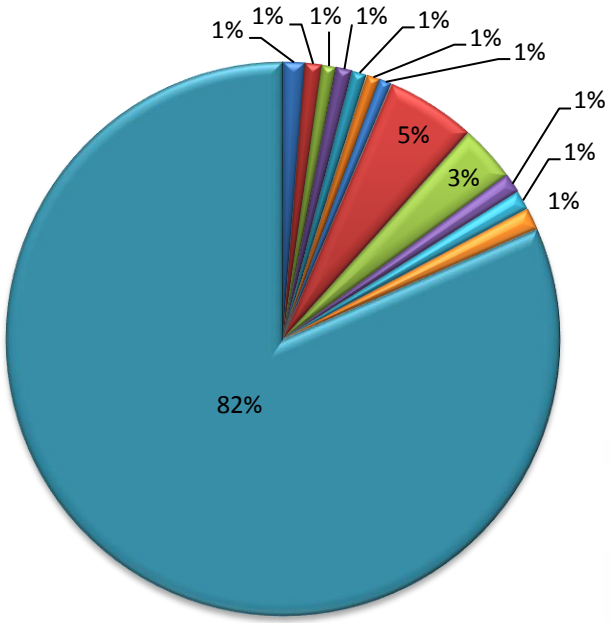

**P1 after amendment**




**A**

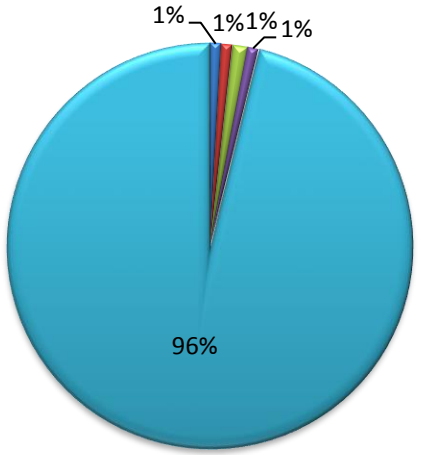
**P2 baseline**




- *Alteromonas spp.*
- *Bacteroidetes*
- *Enterobacter spp.*
- *Halomonas spp.*
- *Klebsiella spp.*
- *Pantoea spp.*
- *Propionibacterium spp.*
- *Pseudoalteromonas spp.*
- *Pseudomonas spp.*
- *Psychrobacter spp.*
- *Shewanella spp.*
- *Vibrio spp.*
- Other



**P2 after amendment**




- *Azospira spp.*
- *Chloroflexi bacterium*
- *Clostridium spp.*
- *Ochrobactrum spp.*
- Other

**B**

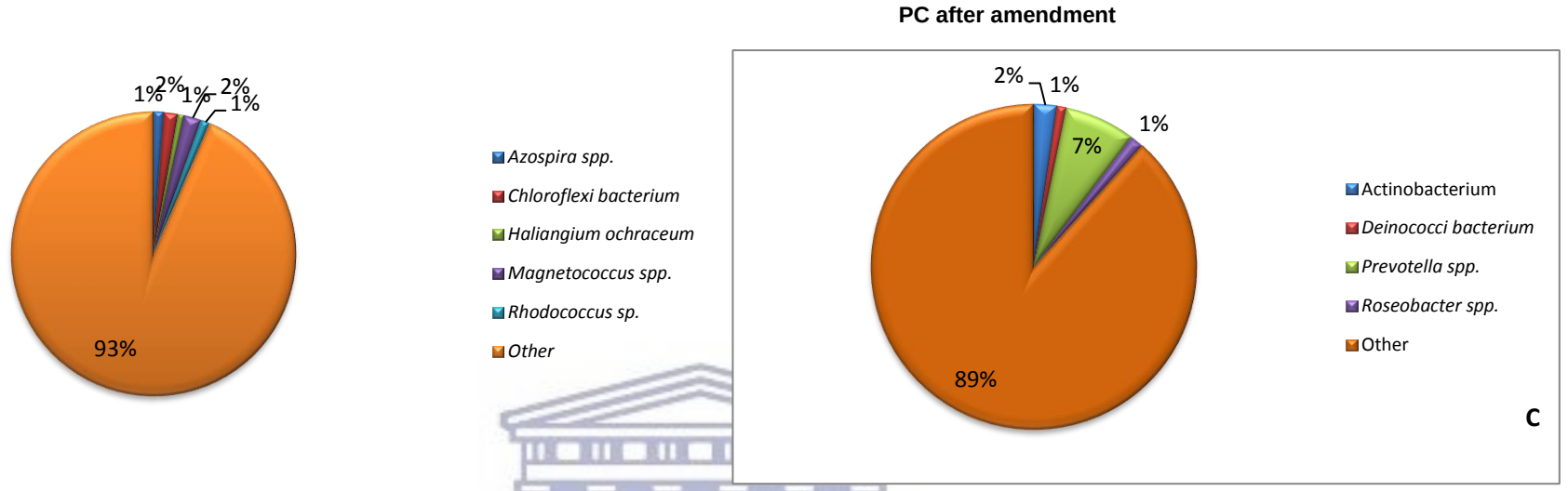



**Figure 3.7:** Phylogenetic analysis of the T-RFLP profiles of the bacterial community structure at the superficial outlet using the software MICA 3. Pie chart A represents the changes in the bacterial community structure of P1, P2 (B) and PC (C) before and after the amendment with synthetic winery wastewater.

### 3.5.2.2 Assessing the effect of synthetic winery wastewater on the bacterial population at the deep sediments of the BSFs through T-RFLP and phylogenetic analysis

The effect of synthetic winery wastewater on the bacterial community structures at the deep sediments is depicted in Figure 3.8. Before amendment with synthetic winery wastewater (baseline), all points representing the bacterial communities in the deep sediments shared 40% similarity, with the outlying group being BSF P2 sharing 20% similarity to the other baseline points on the NMDS plot (Figure 3.8). Despite the two experimental systems (P1 and P2) being treated identically during the experiment, slight changes in the bacterial community structure were observed. However, this is to be expected due to different bacterial populations being present at the start of the experiment. Another possibility is that some organisms were dormant in one wetland at the start of the experiment and were only revived when the BSF was treated with the synthetic winery wastewater. The differences in community structure also shows that the establishment phase (3 months) was successful.

The control BSF PC at the start of the experiment (baseline – 0 weeks) shared an overall 40% similarity with the experimental system P1 and a 20% similarity to P2. At baseline, points representing the bacterial communities from the inlet and outlet of each BSF cluster closer to one another compared to points from any of the other replicates. In contrast, after amendment with synthetic winery wastewater, the points representing the bacterial communities at the inlet and outlet of both experimental BSFs (P1, P2) group closer together (showing 40% similarity to one another) and only 20% similarity to the control. The two experimental systems P1 and P2 appear to be more similar after amendment with synthetic winery wastewater, showing that amendment had an effect on the bacterial communities and likely resulted in a shift towards organisms that are better adapted to synthetic wastewater. No change in bacterial community structure was observed for BSF PC. This is expected as it is the control systems and it was only fed with basal nutrients. Thus, similar trends were noted in the deep and superficial sediments, where the addition of the synthetic winery wastewater had an impact on the bacterial community structures and it was evident that the control samples are not as different as the superficial control BSFs.

Part of this study was to assess the presence of functional genes within the sand samples of the BSFs. The data obtained from the T-RFLP analysis showed that amendment with synthetic wastewater had an effect on the bacterial community structure. This was evident through the shift in the communities between experimental and control systems Changes observed in the bacterial populations after amendment with the synthetic winery wastewater as compared to when the systems were being fed basal nutrients could be due to the

addition of phenolics to the systems which could have enhanced the growth of specific bacteria or resulted in the germination of dormant species. Conversely, the phenolic compounds may have had a toxic/inhibitory effect on key species within the population. The degradation of the phenolic compounds present in the synthetic winery wastewater could have also led to the development of new populations involved in producing secondary metabolites through different metabolic pathways. Minor shifts in communities may also have been due to changes in environmental conditions.

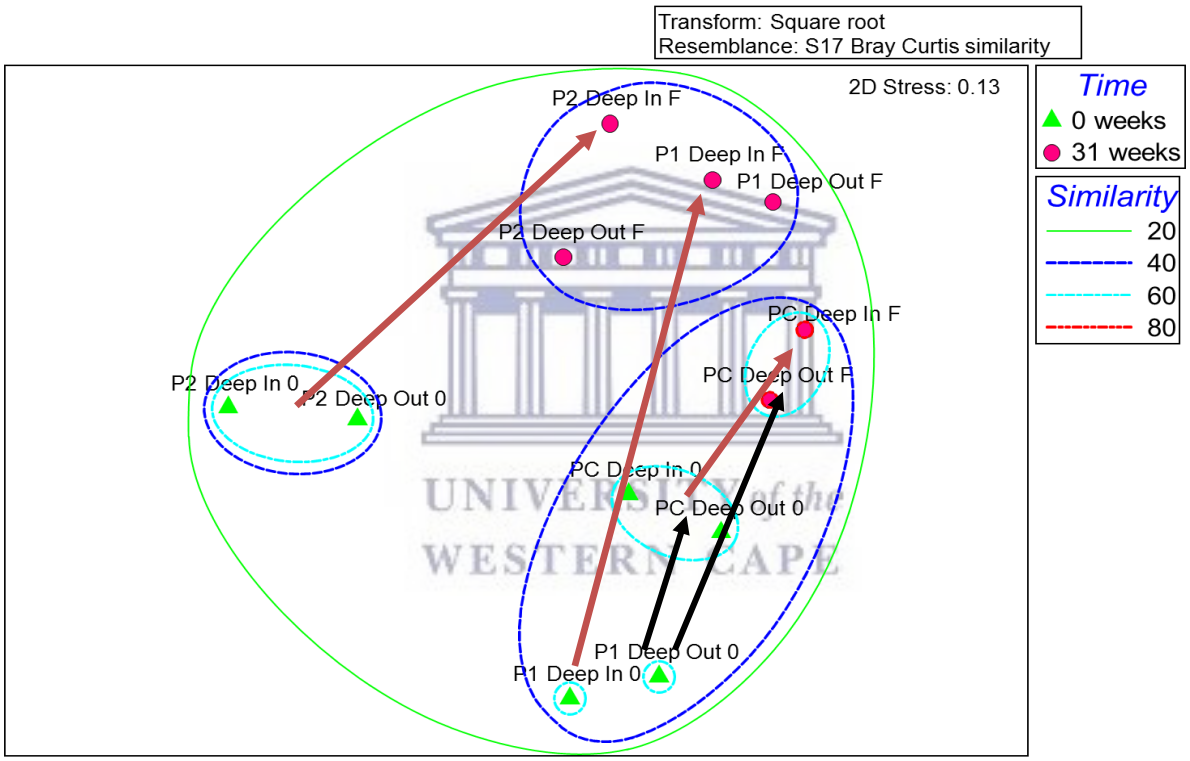



**Figure 3.8:** 2D-Nonmetric multi-dimensional scaling plot of Bray Curtis of the microbial community structure at the deep sediments of BSFs filled with Phillipi sand (P) with P1 and P2 the experimental systems and PC the control (stress value =0.13). Deep Inlet and Deep Outlet of each BSF where the core sand samples were taken. Red arrows indicating the shift in similarity or dissimilarity between points on the NMDS plot.

Table 3.4 represents the data generated from the phylogenetic profiles of the bacterial community at the deep inlet. It was observed that most degradation of phenolic compounds present in the BSFs were degraded at the deeper sediments of the BSFs (chemical data not shown). The reason for the 95% similarity cut off (regarded as 'other') was that we were only trying to identify organisms in degradation pathways and/or organisms that could be present in the sand. From Figure 3.8, after amendment with synthetic winery wastewater, radical changes in the bacterial population structure were observed as compared to the start (baseline) of the experiment.

At the deep inlet of the BSFs one can observe a change in the diversity of organisms from the experimental system to the control and this is a constant trend throughout the niches (superficial inlet and outlet and the deep outlet) (Figures 3.6, 3.7 and Figure 3.9, 3.10). Different organisms were present in the experimental BSF systems (P1 and P2). In addition, differences were observed when comparing the control BSF PC to the experimental system despite all three systems being fed with basal nutrients at the start of the experiment (baseline). Differences in the surface and deep NMDS plots could also be due to the surface of the BSFs being more exposed to elements (weather and wind), and is supported by the fact that the deeper samples which are not as exposed to changing weather/wind conditions and are likely to experience more stable environmental conditions are more similar.

Differences in diversity of species were observed between experimental systems after amendment with synthetic winery wastewater. Similar organisms (at the genus level) were detected between the niches (superficial and deep sediments), as the bacterial communities of the experimental BSF and control BSF PC systems group together on the NMDS plots (Figure 3.4 and Figure 3.8). For example, at the start of the experiment (baseline) the outlying group is BSF P2. When comparing the organisms present, *Clostridium* species are present at both the start of the experiment for BSF P2 and BSF PC and *Actinobacterium* was present in BSF P1 and BSF P2 after the amendment with the synthetic winery wastewater. It is also hypothesised that each genus present in the sand plays a vital role in either degradation of compounds to produce other secondary metabolites or the organisms could possibly be pathogenic. Each organism has a role as sand organisms in the degradation or build-up of compounds in the BSFs.

When assessing the metabolite profiles of the sugars, alcohols and acids present at the surface sediments and the deep sediments, differences were noted. When the BSFs were amended with the synthetic winery wastewater containing acetate (labelled 'b') as compared to the when they were amended with synthetic winery wastewater containing glucose (labelled 'c') (Figure 2.3) there were higher concentrations of ethanol and lower

concentrations of propionate. When the BSFs were amended with the synthetic winery wastewater containing acetate there were higher concentrations of lactate and residual glucose from the surface and deep sediments, respectively (refer to Appendix A).

The redox potential of each BSF was determined as part of a parallel study. The redox status (Eh) was consistently higher in the surface sediments [average: 102 ± 33 mV; range: 28 to 157 mV (n = 48)] than in the deep sediments [average: 25 ± 33 mV, range: -18 mV to 92 mV (n = 48)]. The conclusions for this separate study was that the degradation of non-phenolic compounds, with the exception of acetate, was more readily degraded under reduced redox conditions of the deep niches. However, these findings are contrary to what literature has stated. Literature states that when VSSF and HSSF CWs are operated in batch mode the oxygen transfer rates are enhanced due to the atmospheric gases that is drawn into the substrate as a result of a higher redox status during these cycles when compared to conventional HSSF systems operated in continuous mode (Fan *et al*., 2013, Tietz *et al*., 2008).

The changes observed in the metabolite profiles (Appendix A) of both the surface and deep sediments of the BSFs, correlates to the diversity of organisms detected after amendment with synthetic winery wastewater. It is pivotal to note that changes in the redox status of the BSFs also could relate to the selection of organisms found in the deep and surface sediments of the BSFs. Previous studies by Welz *et al*. (2011) and Ramond *et al*. (2013) showed that ethanol is readily degraded in the experimental systems. Therefore, in the present study one could hypothesize that the organisms present in the experimental systems are involved in the Krebs cycle (discussed in 3.4.3). Chemical analysis of the effluent taken from the BSFs (Figure S1 and S2), showed trace amounts of catechol build-up through the systems, therefore it was decided to focus on two functional genes, namely alcohol dehydrogenase and catechol dioxygenase and the expression of the genes encoding for these enzymes was determined by qPCR in this study.

**Table 3.4:** Phylogenetic profiles of the possible organisms present at the deep sediments of the BSFs

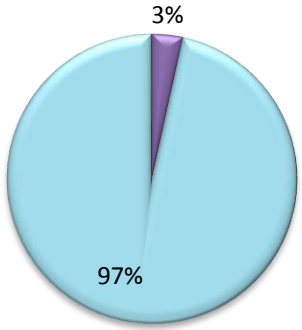
| BSF niches | Organism of interest/ associated with sand | Application | Reference |
|---|---|---|---|
| **Deep Inlet**: Start of experiment (baseline) | P1: *Carnobacterium* | ***Carnobacterium*** forms part of the family *Leuconostocaceae*. The following species namely *C. divergens* and *C. maltaromaticum* have been discovered in the wild as well as in food products that may grow anaerobically | (Leisner *et al*., 2007) |
| | P2: *Prevotella* and *Clostridium* species | ***Prevotella*** species are culturable microbes associated with rumen and gut of animals where they play a role in the breakdown of proteins and carbohydrates of food; and humans where they are present as opportunistic pathogens. | (Guillot and Mouton, 1997) |
| | PC: *Clostridium*, *Geobacillus*, *Rhodococcus* and *Sarcina* species. | ***Sarcina*** is a genus of Gram positive cocci bacteria that form part of the Class *Clostridia* and *Sarcina* species are able to synthesise cellulose. | (Ross *et al*., 1991) |
| | | ***Rhodococcus*** is a genus of aerobic, non-sporolating gram positive bacteria closely related to *Mycobacterium* and *Corynebacterium*. | (van der Geize and Dijkhuizen, 2004) ble |
| After the amendment with synthetic winery wastewater (SWW) | P1: *Alteromonas*, *Pseudomonas* and *Pseudoalteromonas*. | These species are predominantly found in sea water. ***Pseudoalteromonas*** was described as a new genus by Gauthier *et al*., (1995) and split from ***Alteromonas*** through rRNA-DNA analysis. ***Pseudomonas*** is the genus belonging to the family | (Gauthier *et al*., 1995) (Madigan and Martinko, 2005) |

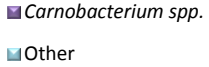
| | | | |
|---|---|---|---|
| | | *Pseudomonadaceae*. The genus has been excellent to study through its ability to create a great deal of metabolic functions. The most studied species include, *P. aeruginosa* as its role as a human pathogen; *P. syringae* described as the plant pathogen and the soil bacterium *P. putida.* | |
| | P2: Actinobacterium and *Streptomyces* species | As explained in the Superficial sediments (Table 3.3) | |
| | PC: *Acinetobacter* species | ***Acinetobacter*** is a genus of gram negative bacteria belonging to the Class Gammaproteobacteria. They are important soil organisms that contribute to the mineralization of aromatic compounds. | (Rokhbakhsh-Zamin *et al*., 2012) |
| Deep Outlet: Start of experiment (baseline) | P1: Bacteriodes | As explained in the Superficial sediments (Table 3.3) | |
| | | This group of bacteria contains isolates including aerobic thermophiles (grows in high temperatures and uses oxygen), anoxygenic phototrophs (uses light for photosynthesis) and anaerobic halorespirers (uses halogenated organics) | (Sutcliffe, 2010) |
| | P2: *Chloroflexi* | ***Chloroflexi*** bacteria also known as the green non sulphur bacteria are abundant in wastewater treatment process. They are classified as | (Björnsson *et al*., 2002) |

| | | | |
|---|---|---|---|
| | PC: Colwellia | filamentous bacteria and have been associated with many sludge filaments.<br><br>Not associated with soil | |
| After the amendment with synthetic winery wastewater (SWW) | P1:Actinobacterium, *Corynebacterium*<br><br>P2: *Paracoccus*<br><br>PC: Bacteriodetes, *Propioniobacterium* | As explained in the Superficial sediments (Table 3.3)<br><br>Not associated with soil<br><br>As explained in the Superficial sediments (Table 3.3) | |

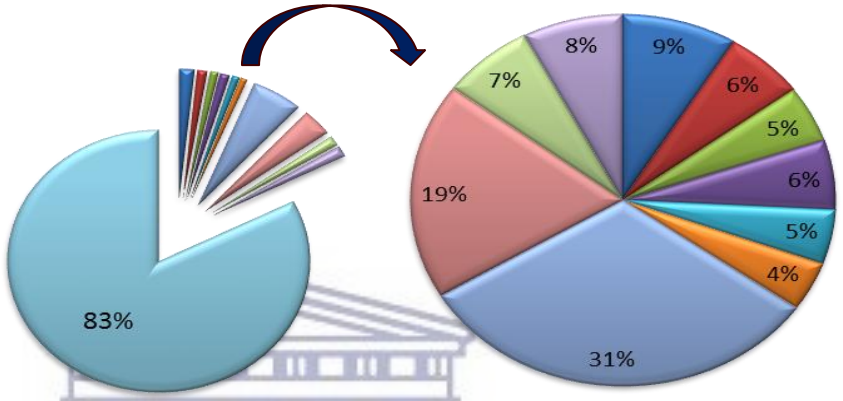
•

**Deep Inlet**
**P1 baseline**



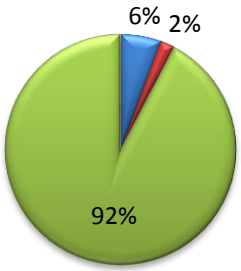



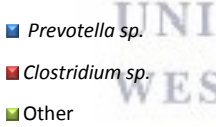

**P1 after amendment**




**A**

**P2 baseline**

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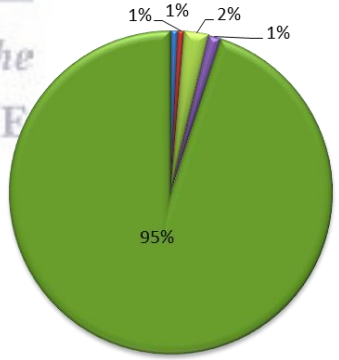



**B**

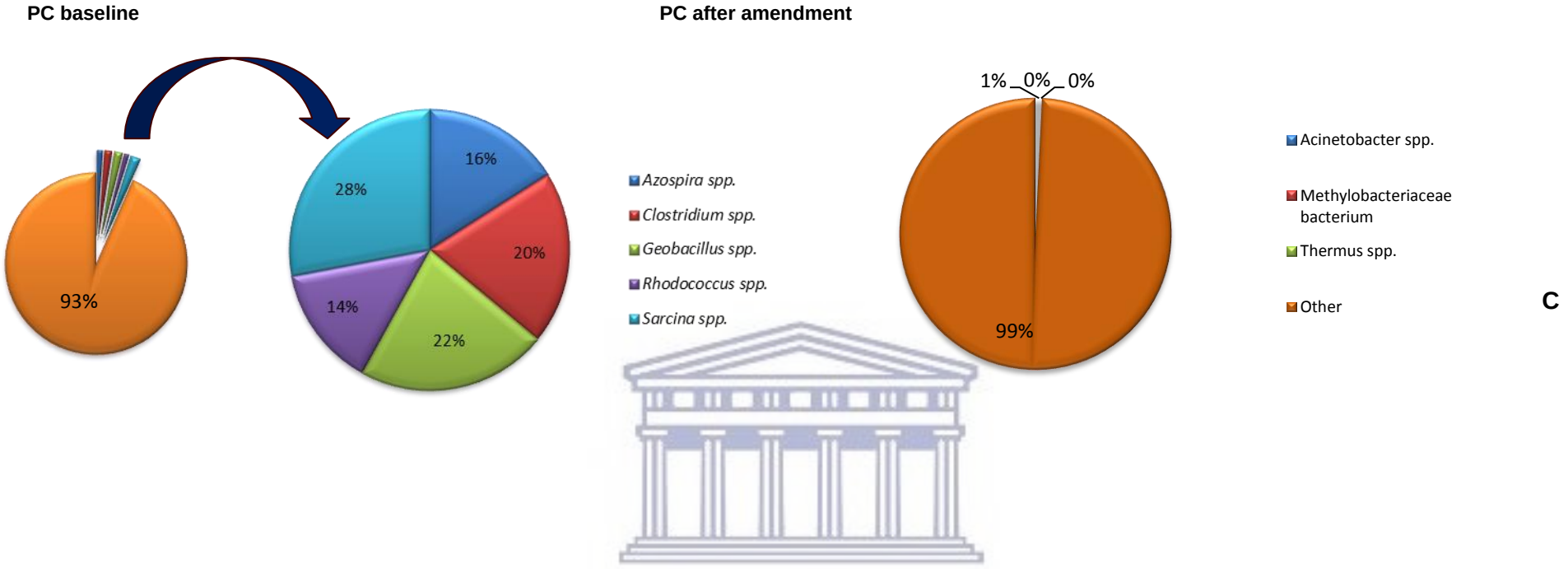
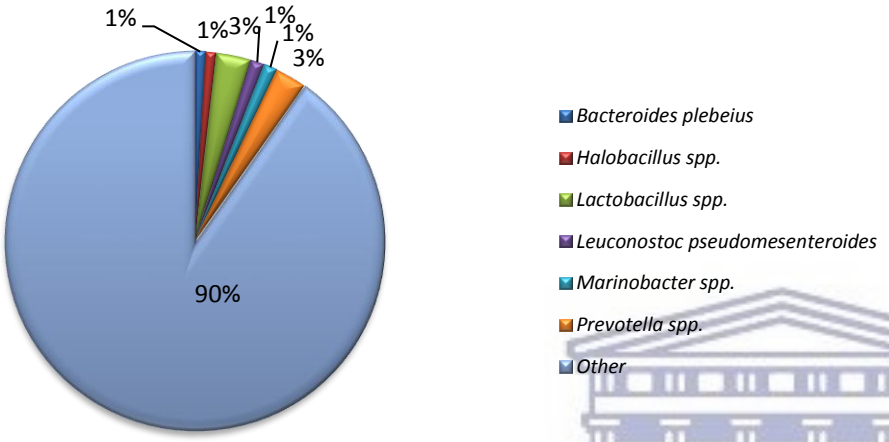


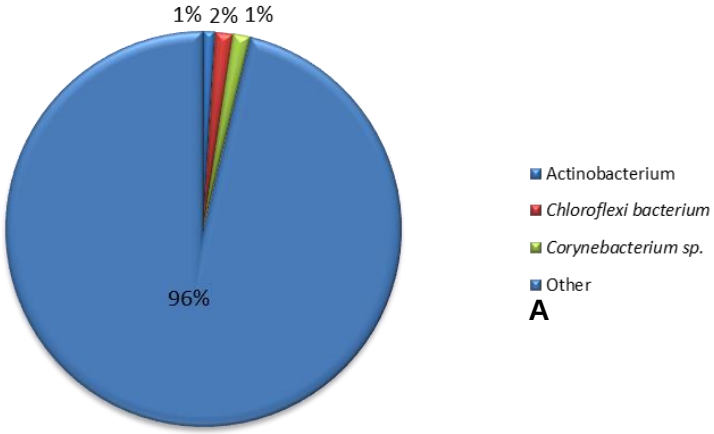

**Figure 3.9:** Phylogenetic analysis of the T-RFLP profiles of the bacterial community structure at the deep inlet using the software MICA 3. Pie chart A represents the changes in the bacterial community structure of P1, P2 (B) and PC (C) before and after the amendment with synthetic winery wastewater.

- **Deep Outlet**

**P1 baseline**



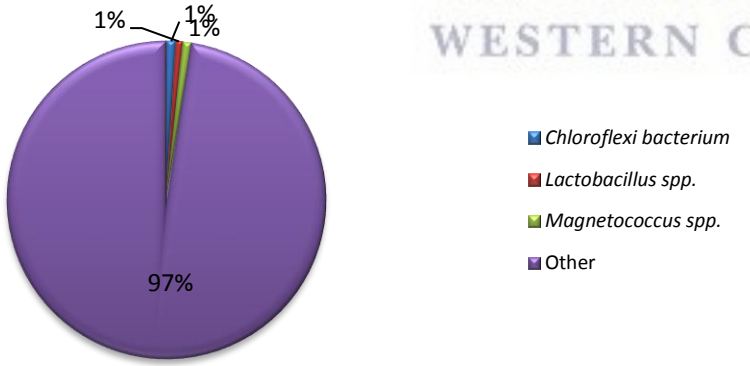

**P1 after amendment**



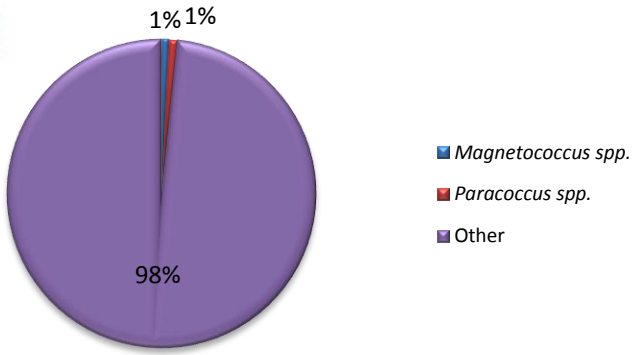

**A**



**P2 baseline**




**P2 after amendment**




**B**

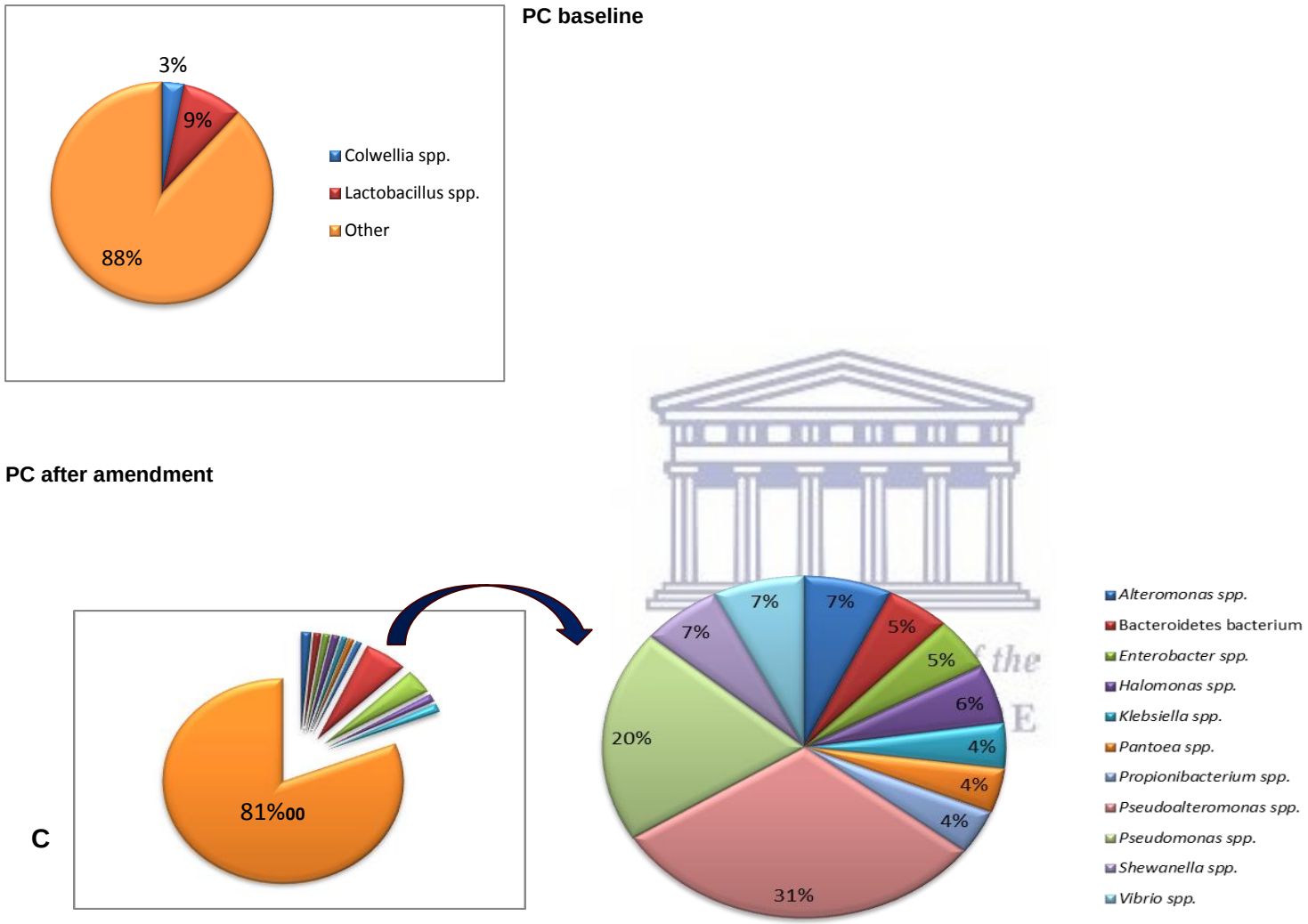



**Figure 3.10:** Phylogenetic analysis of the T-RFLP profiles of the bacterial community structure at the deep outlet using the software MICA 3. Pie chart A represents the changes in the bacterial community structure of P1, P2 (B) and PC (C) before and after the amendment with synthetic winery wastewater.

### 3.5.3 Metabolic pathways that bacterial populations could be involved in

Burton *et al*. (2007) demonstrated through a trial study that the use of constructed wetlands is a feasible and practical method for the treatment on winery effluent. The microorganisms identified in the study were able to degrade different classes of compounds, and chemical- and molecular phylogenetic analysis were used to determine the degradation rates in the wetland exposed to winery wastewater. Figure 3.11 shows a possible overview of the trophic interactions that are likely to take place under anaerobic conditions, together with the organisms possibly involved that was identified in the study by Burton *et al*. (2007). The phylogenetic data that was collected in the present study was compared to the findings of Burton *et al*. (2007). This analysis confirms the potential presence of microbial communities within the BSFs which are able to degrade the main components of winery wastewater including glucose, fructose, ethanol and phenolic substances. The microbial communities present in the BSFs could possibly be involved in many complex metabolic pathways for either the breakdown or build-up of compounds to produce secondary metabolites (Figure 3.11). When comparing the two figures one can observe that the organisms are able to breakdown the elements of the synthetic winery wastewater during different levels of fermentation. When linking the key organisms in the pie charts for both the experimental and control BSFs systems there was notably overlapping organisms in Tables 3.2 and 3.3. These organisms included *Clostridium*, *Acinetobacter* and *Propionibacterium* species which could potentially be involved in acetic acid, butyric acid and propionic acid production (indicated by red in Figure 3.11). *Clostridium* species are involved in both primary and secondary fermentation. Potential degradative processes which could occur in the wetlands include the breakdown of monosaccharides to butyric acid, $H_2$ and acetic acid; pectin to acetic acid; lactic acid to butyric acid and one important component of the synthetic winery wastewater, ethanol being converted to $H_2$. *Acinetobacter* species could possibly be involved in the degradation of monosaccharides to lactic acid and aromatic compounds to acetic acid (Rokhbakhsh-Zamin *et al*., 2012).

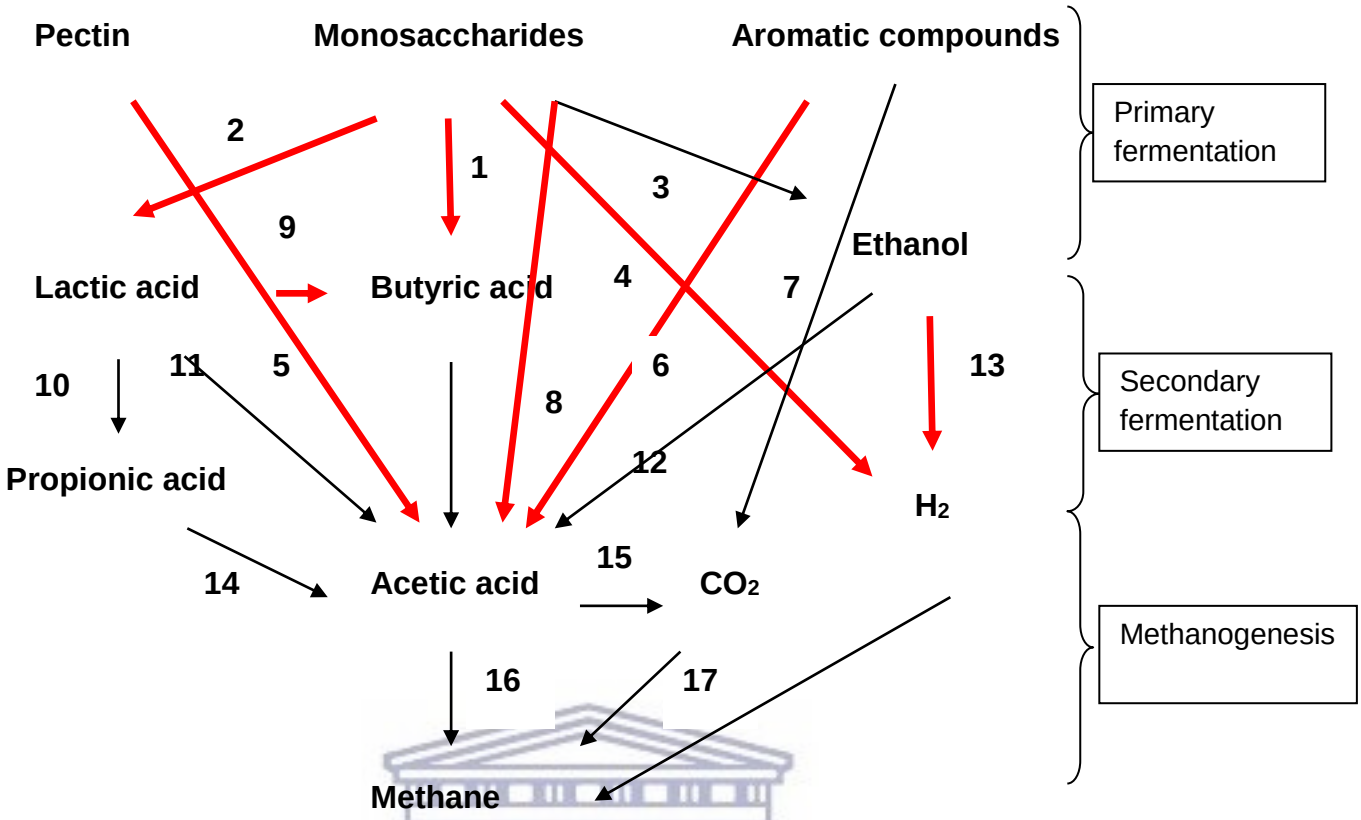



**Figure 3.11:** Schematic diagram of the trophic interactions likely to contribute to the anaerobic degradation of components of winery wastewater in CWs or BSFs. The following microorganisms that have been identified in the study by Burton *et al*. (2007) might be involved in the following steps: 1, *Clostridium*; 2, *Citobacter*, *Acinetobacter*, 4 and 5, *Clostridium*; 6, *Syntrophus*, *Acinetobacter*, *Rhodocyclus*; 7, *Syntropus*; 8, *Clostridium*, *Frateuria*; 9, *Clostridium*; 10, *Propionibacterium*; 11, *Citrobacter*; 12, *Pelobacter*, *Frateuria*; 13, *Clostridium*, *Pelobacter*; 14, *Syntrophobacter*; 15 and 16, *Methanosarcinales*; 17, *Methanomicrobiales*. Indicated by red arrows are *Clostridium*, *Acinetobacter* and *Propionibacterium* which could potentially be involved in acetic acid, butyric acid and propionic production.

### 3.5.4 Effect of synthetic winery wastewater on the fungal population at the superficial sediments of the BSFs

Figure 3.12 represents the NMDS plot of the fungal population at the superficial sediments of the BSFs. The points representing the baseline samples group separately from the points representing the samples taken after amendment with synthetic winery wastewater on the NMDS plot (indicated by the arrows). The baseline samples are more similar than the samples collected after amendment with synthetic winery wastewater. At baseline, the experimental systems BSF P1 and P2 share an overall 40% similarity to each other and 20% similarity to the control. The shift in bacterial communities between the experimental systems is evident by the arrows before and after amendment with the synthetic winery wastewater. After amendment with synthetic winery wastewater, the experimental systems are less similar to each other, however with the inlet of each system being most similar to each other (60% similarity) showing that the amendment had an effect of the bacterial communities. In this NMDS plot the control PC is an outlying group which is to be expected as the control system only received basal nutrients for the experimental period as compared to P1 and P2 that received synthetic winery wastewater. It is apparent from the NMDS plots that the fungal communities in the experimental systems clearly shifted in response to the treatment with winery wastewater.

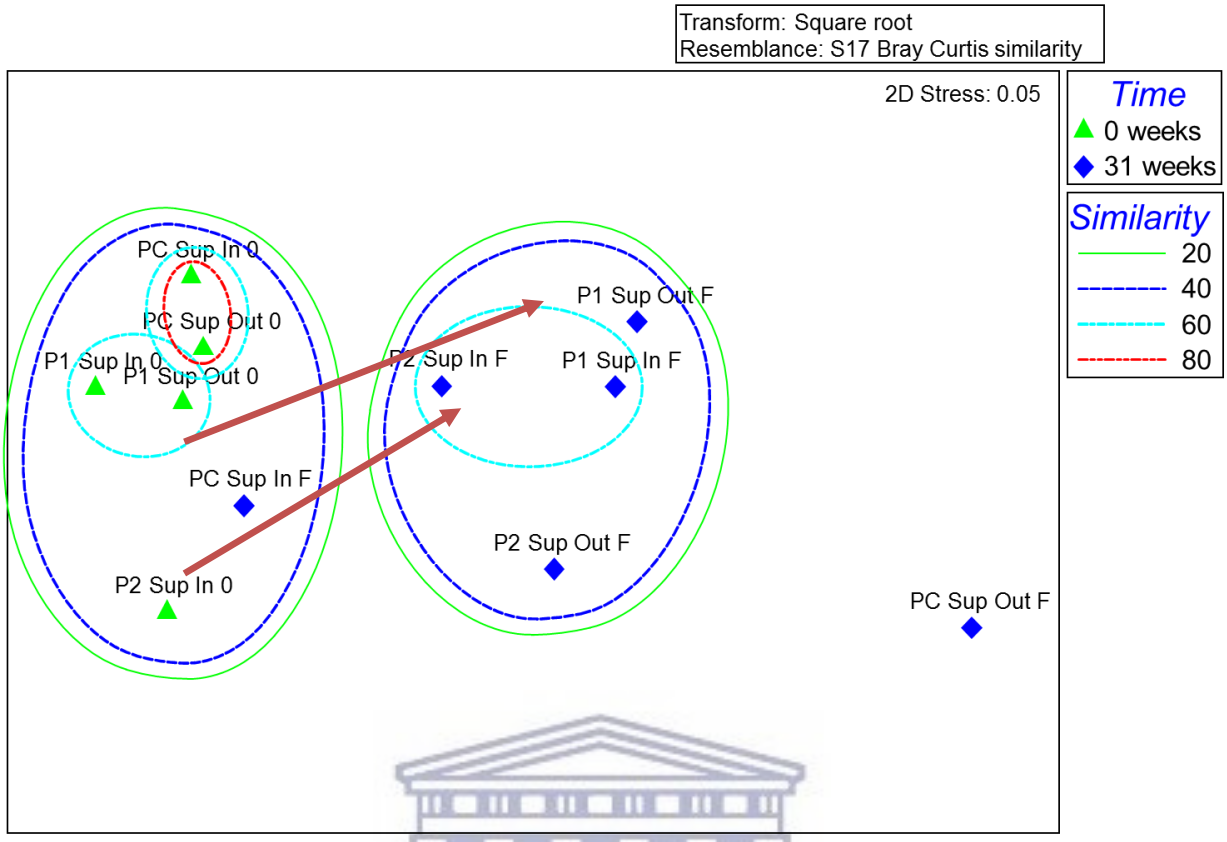



**Figure 3.12:** 2D-Nonmetric multi-dimensional scaling plot of Bray Curtis of the fungal community structure at the superficial sediments of BSFs filled with Phillipi sand (P) with P1 and P2 the experimental systems and PC the control (stress value =0.05). Sup In: superficial inlet and Sup Out: superficial outlet of the BSF where core sand samples were taken. Red arrows indicating the shift in similarity or dissimilarity between points on the NMDS plot.



### 3.5.4.1 Effect of synthetic winery wastewater on the fungal population at the deep sediments of the BSFs

Literature has stated that fungi play a vital role in bioremediation (Frey-Klett *et al*., 2011). In this study, several optimization steps were performed in order to optimise the fungal ITS primer set (section 3.4.2). These optimization steps included performing a gradient PCR (annealing temperatures over a range of 40 °C to 60 °C) and performing a touchdown PCR by changing the annealing temperature (amount of cycles) to 55 °C, for 10 cycles and 55 °C for 25 cycles. Despite several attempts, the baseline samples failed to amplify with the ITS primers.

Figure 3.13 shows the NMDS plot for the fungal population at the deep sediments of the BSFs. This NMDS plot represents points that were taken after amendment with synthetic winery wastewater. At time zero, the start of the experiment, despite numerous attempts

there was no amplification of the fungal gene in these samples and we could hypothesize that that the number of fungi present at the start of the experiment was very low (below detection levels) and treatment with synthetic winery wastewater favoured their growth. Another possibility was that fungi were present as spores/dormant forms at the start of the experiment, which only germinated after treatment. The relatively gentle DNA extraction method employed in this study is unlikely to be able to lyse spore coats.

After amendment with the synthetic winery wastewater the experimental systems P1 and P2 at both the inlet and outlet share 40% similarity as compared to 20% similarity to the control system. One of the experimental systems (P1) shared 60% similarity to the control. As was observed for the superficial samples, control BSF PC is the outlying group as compared to the experimental systems.

The T-RFLP profiles of the fungal population showed that changes were observed after amendment with synthetic winery wastewater at the superficial sediments of the BSFs, while at the deep sediments, fungi were only observed at the end of the experiment. Despite the fact that the phylogenetic analysis was only performed using the T-RFLP data from the bacterial community structure at the superficial and deep sediments of the BSFs, it is evident that fungi could also play a pivotal role in the breakdown or build-up of secondary metabolites. Similarities between the bacterial and fungal populations on the MDS plots are that the fungal populations in the deep sediments can be linked to the shifts that were observed in the bacterial communities.

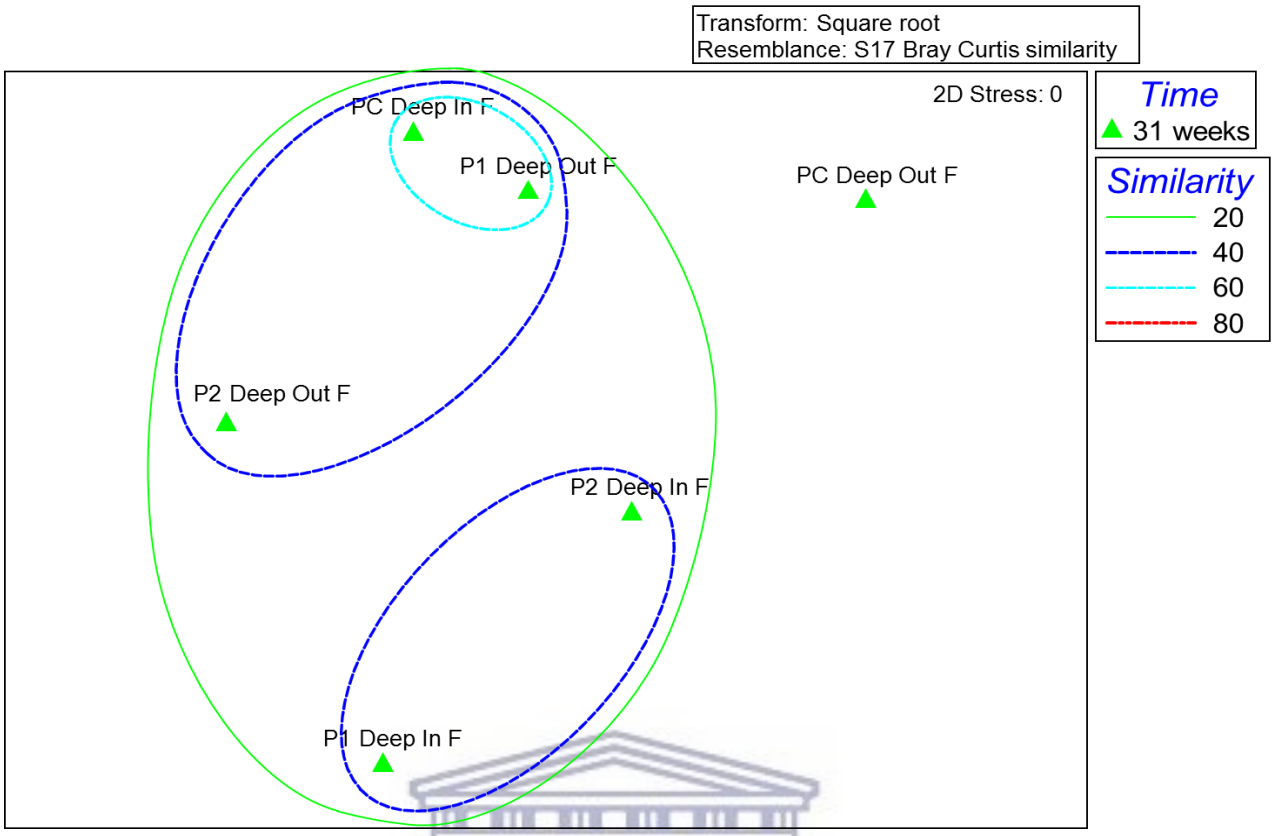



**Figure 3.13:** 2D-Nonmetric multi-dimensional scaling plot of Bray Curtis of the fungal community structure at the deep sediments of BSFs filled with Phillipi sand (P) with P1 and P2 the experimental systems and PC the control (stress value =0). Deep Inlet and Deep Outlet of each BSF where the core sand samples were taken. Red arrows indicating the shift in similarity or dissimilarity between points on the NMDS plot.



### 3.6 Optimization of standard using real time PCR and the primer sets

Assessing the bacterial community structure through the T-RFLP analysis above showed that indeed amendment with synthetic winery wastewater caused a significant change in the bacterial community structure, and this was evident at both the superficial and deep sediments of the BSFs. The change in bacterial populations prompted the further investigation to identify which organisms are involved in specific metabolic pathways by screening for specific metabolic genes (Figure 3.11). Complete degradation of ethanol was observed in the deep sediments of the BSFs (Figure A.1). In the superficial sediments, degradation of ethanol was improved when acetate was excluded from the synthetic winery wastewater composition. The metabolic pathways for microorganisms for both ethanol and acetate result in the production of acetyl co-A. One could suggest that ethanol degradation in the BSFs were repressed when higher concentrations of acetate were present. The

complete degradation of ethanol led to the decision to focus on the detection of alcohol dehydrogenase production via a molecular approach.

Figure A.2 shows the temporal changes of the phenolic compounds in the surface and deep sediments of the BSFs, monitored by HPLC analysis (performed by Dr P Welz as part of a parallel study). A number of unidentified peaks were present and with many phenolic compounds on the market, a complete phenolic characterisation was not within the scope of the project. Gallic acid and vanillin, the primary break down product of vanillic acid and the common toxic metabolite, catechol, was identified and quantified in the study. Vanillic acid and gallic acid amounted to half of the phenolics present in the samples and the other half being unidentified (other).

Results showed a build-up of catechol, as well as the breakdown of ethanol by the organisms present within the BSFs (Appendix A). Based on these findings it was decided to initially focus on three functional genes namely: alcohol dehydrogenase, catechol 1,2-dioxygnease, and catechol 2,3- dioxygenase.

Although published primer sets were used, extensive optimization was required for the amplification of the functional genes. For the alcohol dehydrogenase primer set optimization was performed according to Quintero *et al*. (2009) and for catechol 1,2-dioxygenase and catechol 2,3-dioxygenase optimization was performed according to the initial conditions described by Lillis *et al*. (2010). Optimization of the functional gene primer sets included adjusting the annealing temperature, performing a touchdown PCR, increasing the number of amplification cycles, and altering the ratios of gDNA to mastermix, The catechol 2,3-dioxygenase gene was successfully amplified from the synthesised cDNA samples taken at the start and end of the experiment. Despite several attempts both the alcohol dehydrogenase and catechol 1,2-dioxygenase primer sets did not work. This failure can be because the genes were present at low levels in the DNA, or the primers failed to bind due to differences in template DNA sequence. Therefore, the rest of the study was focused on catechol 2,3-dioxygenase.

### 3.6.1 qPCR and amplification of the catechol 2,3-dioxygenase gene

Amplification of the catechol 2,3-dioxygenase gene was achieved through qPCR. A standard curve was constructed using CZA14$^T$ gDNA as the template and this was used to determine the gene copy number. Amplification curves, melting curves and copy number was calculated using the slope of the standard curve and copy numbers are reported in Appendix D. Table 3.5 shows the crossing points (Cp values) and copy numbers of the samples from the start of the experiment (baseline) to the end of the experiment (after amendment). Due to budgetary limitations samples taken from the experimental systems P1 and P2 at the start of the experiment were pooled based on the hypothesis that the bacterial communities present in P1 and P2 would be similar after the initial establishment phase when they only received basal nutrients. Copy number refers to how many copies of the catechol 2,3-dioxygenase (C23O) genes were present/expressed within each sample. As expected, from Table 3.5 and Appendix D it can be seen that there is an increase in the copy number of C23O after amendment with synthetic winery wastewater. Despite P1 and P2 samples being pooled at the start of the experiment there was an obvious trend observed in that the copy numbers of all samples increased after amendment with synthetic winery wastewater. After testing a range of cDNA concentrations it was found that 100 ng template cDNA samples produced a more precise single peak for the amplification of the C23O gene and it was therefore used as the standard template concentration for all reactions. The increase in expression of C23O (Figure 3.14) is further supported by HPLC analysis (Appendix A), which clearly showed that there was an increase in the formation of catechol in the BSF systems.

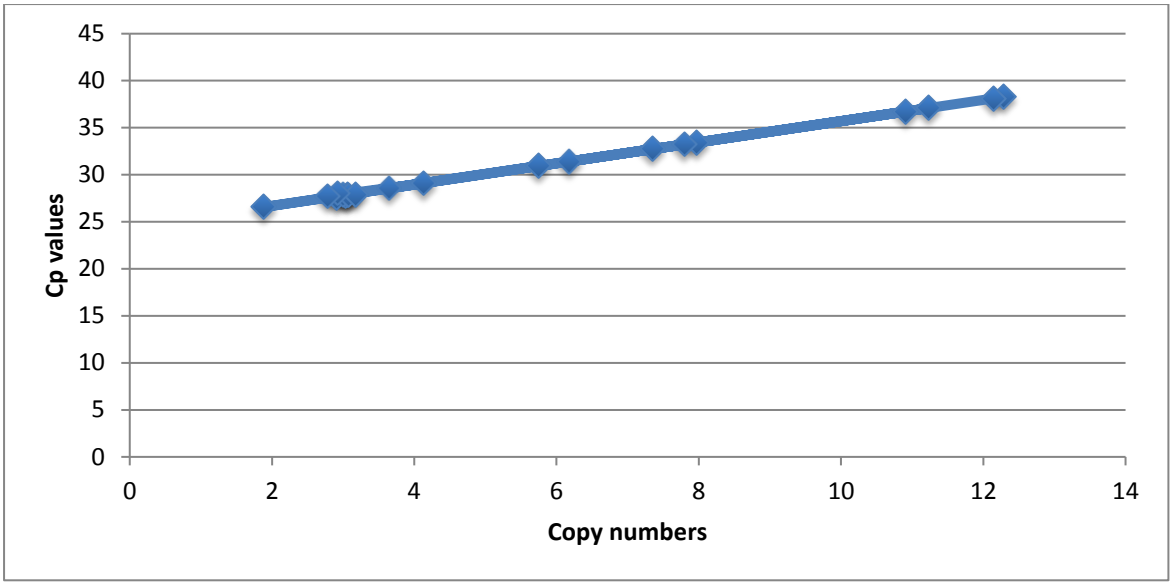



**Figure 3.14:** Graph depicting the copy numbers and Cp values of the BSF P1, P2 and PC samples at 100 ng DNA.

**Table 3.5:** Cp values and copy numbers of the C23O genes of the samples of 100 ng DNA at the start and end of the experiment. Copy numbers represented as log values of the C23O gene of CZA14$^T$ gDNA as indicated on standard curve (Appendix D). copy numbers represented before normailisation.

| Samples | Cp number concentration | Copy numbers |
|---|---|---|
| P1 and P2 Sup In 100 ng | 27.58 | 11.37 |
| P1 and P2 Deep In 100 ng | 27.72 | 11.44 |
| P1 and P2 Sup Out 100 ng | 27.87 | 11.51 |
| P1 and P2 Deep Out 100 ng | 27.90 | 11.53 |
| PC Sup In 100 ng | 27.82 | 11.49 |
| PC Deep In 100 ng | 27.91 | 11.53 |
| PC Sup Out 100 ng | 28.03 | 11.59 |
| PC Deep Out 100 ng | 27.74 | 11.45 |
| | | |
| **After amendment samples** | | |
| P1 Sup In 100 ng | 29.10 | 12.13 |
| P2 Sup In 100 ng | 28.56 | 11.86 |
| P1 Deep In 100 ng | 26.56 | 10.85 |
| P2 Deep In 100 ng | 37.08 | 16.15 |
| P1 Sup Out 100 ng | 30.92 | 13.05 |
| P2 Sup Out 100 ng | 33.42 | 14.31 |
| P1 Deep Out 100 ng | 36.72 | 15.97 |
| P2 Deep Out 100 ng | 32.72 | 13.96 |
| PC Sup In 100 ng | 31.40 | 13.29 |
| PC Deep In 100 ng | 33.23 | 14.21 |
| PC Sup Out 100 ng | 38.28 | 16.76 |
| PC Deep Out 100 ng | 38.12 | 16.68 |

### 3.6.1.1 Amplification of the *rpoB* gene using qPCR

Amplification of the ribosomal RNA encoding-gene (*rpoB*) that encodes for the bacterial RNA polymerase β-subunit (Morse *et al*., 1996) was achieved through real time PCR. This small subunit ribosomal RNA gene is used for estimating the phylogenetic diversity in microbial communities (Hugenholtz *et al*., 1998; Ward *et al.,* 1990). The gene is used as a universal marker and has the advantage of containing highly conserved regions allowing for the design of PCR primers, targeting all members of the bacterial community (Vos *et al*., 2012). The *rpoB* gene was also used in this study as it is a known housekeeping gene and is a single copy gene.

Due to the inherent instability of RNA it was decided to immediately convert the RNA to cDNA and this synthesised cDNA was used for the amplification of the catechol 2,3-dioxygenase gene and the *rpoB* gene. However, a housekeeping gene is needed for determining relative quantification. It was hypothesised that immediately synthesising to cDNA and storing at -20 °C would allow for the samples to be more stable. Although the RNA concentrations were quantified prior to cDNA conversion the RNA starting concentration for each sample was not normalised and this resulted in different starting amounts of RNA being used in each reaction which may account for the differences observed in copy numbers of catechol 2,3-dioxygenase genes. Therefore, it was decided to "normalize" the data to a housekeeping gene (*rpoB* gene) to account for the variability in the RNA and cDNA concentrations. Table 3.6 shows the copy numbers of the amplification of the rpo*B* gene. Figure 3.15 shows the amplification curves of the *rpoB* gene with the synthesised cDNA samples taken before and after the amendment with synthetic winery wastewater, while Table 3.6 shows the copy numbers and cp values. Appendix E shows the standard curve used to calculate the copy numbers of the gene present within each sample.

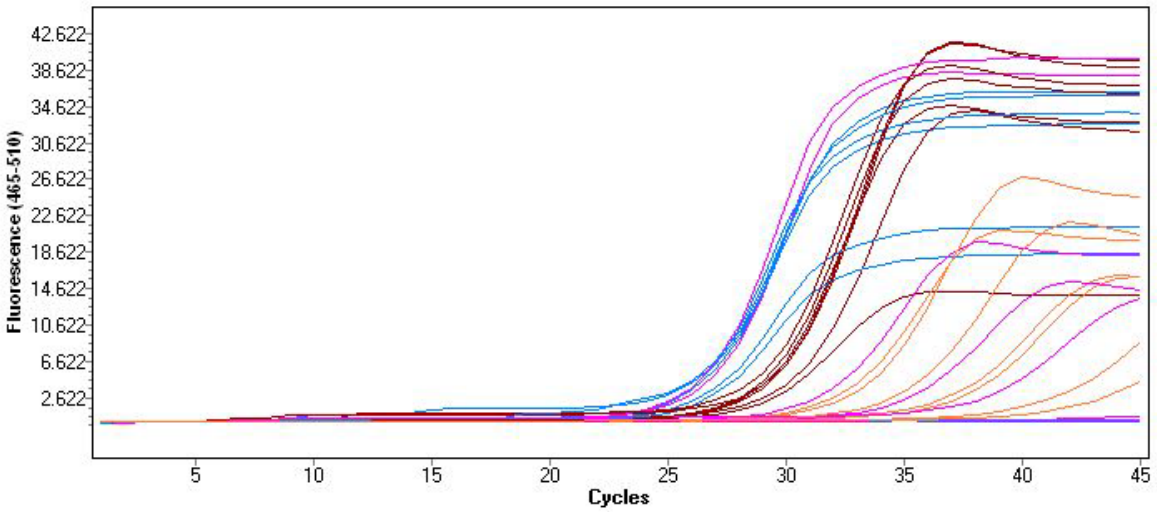



**Figure 3.15:** Amplification curves of synthesised cDNA samples at 100 ng with the *rpoB* gene using real time qPCR of the samples taken at the start of the experiment (baseline) and end of experiment

**Table 3.6:** Values and copy numbers of the *rpoB* genes of the samples of 100 ng DNA at the start and end of the experiment. Copy numbers represented as log values of the *rpoB* gene of CZA14$^T$ gDNA as indicated on standard curve (Appendix E)

| **Samples** | **Cp number concentration** | **Copy numbers** |
|---|---|---|
| P1 and P2 Sup In 100 ng | 34.93 | 15.09 |
| P1 and P2 Deep In 100 ng | 37.72 | 16.71 |
| P1 and P2 Sup Out 100 ng | 39.10 | 17.51 |
| P1 and P2 Deep Out 100 ng | 32.48 | 13.67 |
| PC Sup In 100 ng | 35.65 | 15.51 |
| PC Deep In 100 ng | 31.93 | 13.35 |
| PC Sup Out 100 ng | 30.86 | 12.73 |
| PC Deep Out 100 ng | 32.65 | 13.77 |
| | | |
| **After amendment samples** | | |
| P1 Sup In 100 ng | 41.47 | 18.88 |
| P2 Sup In 100 ng | 34.45 | 14.81 |
| P1 Deep In 100 ng | 36.95 | 16.26 |
| P2 Deep In 100 ng | 35.87 | 15.63 |
| P1 Sup Out 100 ng | 33.62 | 14.33 |
| P2 Sup Out 100 ng | 30.33 | 12.42 |
| P1 Deep Out 100 ng | 33.66 | 14.35 |
| P2 Deep Out 100 ng | 29.82 | 12.13 |
| PC Sup In 100 ng | 37.65 | 16.67 |
| PC Deep In 100 ng | 38.92 | 17.40 |
| PC Sup Out 100 ng | 39.77 | 17.89 |
| PC Deep Out 100 ng | 36.95 | 16.26 |

Table 3.7 shows the copy numbers of the C23O and *rpoB* gene used to normalise the gene expression obtained by the qPCR analysis. There are two ways of normalising data, one by normalising the RNA value by using a bio-analyser (the recommended method) or a reference gene. As we did not have access to a BioAnalyser in-house in this study normalisation was to a reference gene. Expression of the *rpoB* gene (reference gene) was normalised to non-targeted C23O gene (target gene) expression levels within each sample and this was done according to Haimes and Kelley (2014). Differences between the target and reference gene resulted in the ΔCq value and this serves to correct the variation in the values. In the case of biological samples, the ΔCq value was exponentially transformed to the ΔCq expression as indicated in Table 3.6. The results presented in Table 3.7, indicates that the higher the ΔCq value, the lower the expression of the target gene. Samples P1 Deep Out and P2 (after amendment) have negative expression values and this could be as a result that the amplification of the *rpoB* gene by qPCR was performed at a later time period than the isolation of the RNA or the RNA sample was degraded by the time the experiment was performed.

For future research, if the same method of quantification is employed using the above equation a second reference gene (eg. 16S rRNA) would be included. The determined ΔCq expression value could then be divided that by the ΔCq value for the 16S rRNA gene and relate this value as a percentage.

**Table 3.7:** Copy numbers of theC23O and *rpoB* genes of the samples of 100 ng DNA at the start and end of the experiment to normalise the data

| Samples | C23O copy number (Cq reference) | Normalise *rpoB* data (Cq target) | Cq (Target-Ref) (ΔCq) | ΔCq expression ($2^{-\Delta Cq}$) |
|---|---|---|---|---|
| P1 and P2 Sup In 100 ng | 11.37 | 15.09 | 3.72 | 0.08 |
| P1 and P2 Deep In 100 ng | 11.44 | 16.71 | 5.27 | 0.03 |
| P1 and P2 Sup Out 100 ng | 11.51 | 17.51 | 6.00 | 0.02 |
| P1 and P2 Deep Out 100 ng | 11.53 | 13.67 | 2.14 | 0.23 |
| PC Sup In 100 ng | 11.49 | 15.51 | 4.02 | 0.06 |
| PC Deep In 100 ng | 11.53 | 13.35 | 1.82 | 0.28 |
| PC Sup Out 100 ng | 11.59 | 12.73 | 1.14 | 0.45 |
| PC Deep Out 100 ng | 11.45 | 13.77 | 2.32 | 0.20 |
| **After amendment samples** | | | | |
| P1 Sup In 100 ng | 12.13 | 18.88 | 6.75 | 0.001 |
| P2 Sup In 100 ng | 11.86 | 14.81 | 2.95 | 0.13 |
| P1 Deep In 100 ng | 10.85 | 16.26 | 5.41 | 0.02 |
| P2 Deep In 100 ng | 16.15 | 15.63 | -0.52 | -0.70 |
| P1 Sup Out 100 ng | 13.05 | 14.33 | 1.28 | 0.41 |
| P2 Sup Out 100 ng | 14.31 | 12.42 | -2.18 | -0.23 |
| P1 Deep Out 100 ng | 15.97 | 14.35 | -1.62 | -0.33 |
| P2 Deep Out 100 ng | 13.96 | 12.13 | -1.83 | -0.28 |
| PC Sup In 100 ng | 13.29 | 16.67 | 3.33 | 0.09 |
| PC Deep In 100 ng | 14.21 | 17.40 | 3.19 | 0.11 |
| PC Sup Out 100 ng | 16.76 | 17.89 | 1.22 | 0.43 |
| PC Deep Out 100 ng | 16.68 | 16.26 | -0.06 | -0.96 |



## 3.7 Denaturing gradient gel electrophoresis (DGGE)

### 3.7.1 Optimization of nested PCR and method of DGGE

While qPCR analysis can be used to determine the abundance of a target gene, one is unable to determine what the “diversity” of the target gene is – in this study “diversity” would reflect how many different organisms are expressing catechol 2,3-dioxgenase. This was to be achieved by sequencing the dominant bands from the DGGE acrylamide gels. Figure 3.16 shows the principle behind DGGE. The DGGE gel uses a DNA denaturant or a linear temperature gradient to allow for the migration through the gel. Each lane on the gel represents a microbial community (Figure 3.16). The technique shows to an extent that microbial communities are different or the same in taxonomic composition. The number of bands at different horizontal positions on the gel can be used to estimate the level of

biodiversity in that sample. In order to know more about phylogenetic affiliation, the bands can be excised and sequenced (Ward *et al*., 1998; Muyzer, 1999).

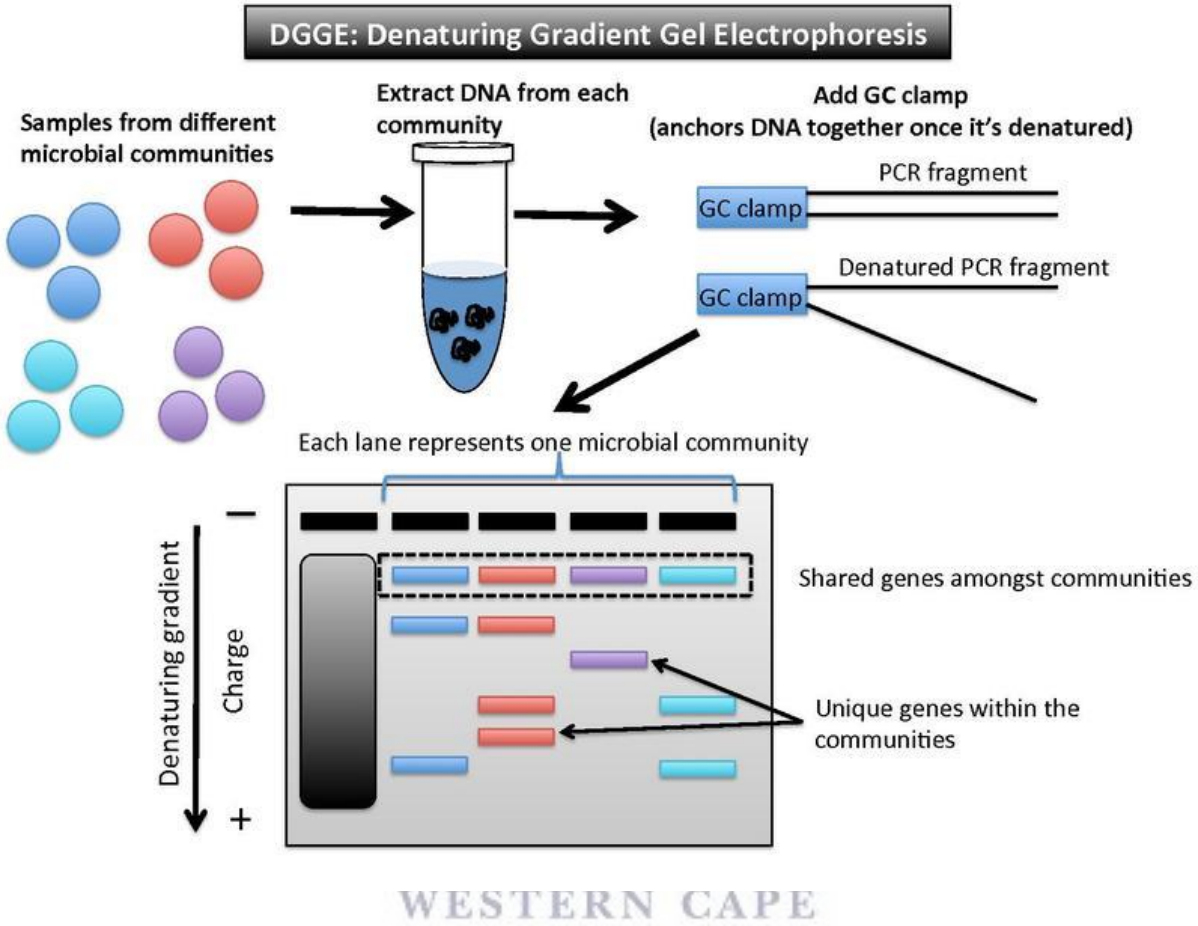





**Figure 3.16:** The principle of DGGE demonstrating the migration of samples through a DGGE gel and that each and represents different microbial communities.

The sequence data would provide insight into the organisms present expressing catechol 2,3-dioxgenase and this diversity data could be compared to the MICA3 phylogenetic data. Several optimization steps for the nested PCR using the GC-clamped catechol 2,3-dioxygenase primer sets was required. This included changing PCR parameters such as annealing temperatures, cycle conditions according to each *Taq* polymerase that was used, starting concentration of DNA for each sample and the addition of additives (e.g. BSA and DMSO). The positive qPCR products were analysed by agarose gel electrophoresis and a band of the expected size was observed on the gel. However, when the qPCR product was used as the template for a nested-PCR there was no amplification of the desired nested product. One possible reason for the failure is low copy number of the catechol 2,3-dioxygenase gene. It could also be that a more robust polymerase is required for the amplification of the gene in the nested PCR. It is possible that an inhibitor, such as residual SYBR Green, may be present which inhibited the second PCR reaction or too few amplification cycles in the PCR run or incorrect annealing temperatures were selected. Another possible reason is that the primers selected for the amplification of the specific genes did not bind to the template DNA. When quantifying the amount of RNA prior to synthesising to cDNA, low RNA concentrations were observed and high concentrations for cDNA was observed which could possibly result in an inaccuracy in the amount of copy numbers of the gene. It could also be that the GC clamp could be interfering in the amplification of the catechol 2,3-dioxygenase gene as it would be possibly 3 times more than the reverse primer in amounts of concentration and/number of moles when adjusting the amounts of primer (qPCR- 200 nM and for DGGE-PCR- 0.5 µM). There were very faint bands observed on the agarose gels and therefore it was decided to attempt DGGE, however no bands were observed on the acrylamide gels. For future research the nested PCR would require more optimization steps, possibly a more robust Taq polymerase, increasing the amount of amplification cycles, changing annealing temperatures or purifying the qPCR amplicon prior to the second nested PCR.



### 3.8 Cloning, ligation and transformation of the catechol 2,3-dioxygenase genes from the samples at the deep sediments of the BSFs for phylogenetic analysis

Several optimization steps were performed for DGGE analysis, however these experiments were unsuccessful, therefore it was decided to construct clone libraries. Cloning of the catechol 2,3-dioxygenase gene was initially performed using chemical transformation with the CloneJET PCR cloning kit. The qPCR products (which contain SYBR Green) were purified using the Nucleospin Gel and PCR kit and cloned using the blunt-end cloning

protocol. This was followed by chemical transformation and plating. However, the cloning failed as no colonies were observed on the LB-ampicillin plates but a lawn of growth on the LB plates which suggests that the strain of *E.coli* is growing, but without the insert. From these results, more optimization steps were required. This was followed by performing the cloning of the catechol 2,3-dioxygenase using the sticky-end cloning protocol, however, the results were the same. The CloneJET PCR kit suggested that if the PCR products were >3kb, the ligation procedure should be prolonged up to 30 minutes and therefore it was decided to extend the ligation to 15 minutes using both the blunt and sticky-end cloning protocol of the C23O gene, however this resulted in no colonies. In addition, a separate experiment with the extended ligation period and doing transformation by electroporation for both the blunt and sticky-end cloning protocol was also attempted, this too was unsuccessful. In addition, two different *E. coli* strains *E. coli* BL21 and electrocompetent *E. coli* Epi 200, were tested without success. The cloning may have been unsuccessful for several reasons including very low transformation efficiency of the competent *E. coli* cells, presence of proteins or salts in the ligation mixture that was not purified by the Nucleospin Gel and PCR cleanup kit that could have been inhibiting the electroporation and this could have resulted in the low transformation efficiency. The other possibility for the unsuccessful cloning of the C23O gene is that the target sequence of the gene cannot be cloned into *E. coli* as it is toxic to the host and therefore it was suggested that in the future a more robust *E. coli* host needs to be chosen.



### 3.9 Conclusions

This project started with the isolation of metagenomic DNA from sand samples that were amended with synthetic winery wastewater. Previous studies performed on the same BSFs (Welz *et al*., 2011; Raymond *et al*., 2010) showed the presence of diverse bacterial communities contributing to the remediation of winery wastewater in biological sand filters. From the non-metric dimensional scaling plots changes in the bacterial populations were observed when comparing the two experimental systems to the control system after amendment with the synthetic winery wastewater. Changes in the bacterial community structure, one could hypothesise that it is due to seasonal/ temporal shifts or based on the phenomenon known as bacterial succession (Dini-Andreote *et al*., 2014). Chemical data (a parallel study performed by Dr Pamela Welz) showed differences observed in COD removal efficiencies before the addition of the synthetic winery wastewater containing acetate (11 weeks) and adding the synthetic winery wastewater formulation replacing the acetate with the glucose (refer to section 2.2.2). HPLC metabolite profiles (Appendix A) also suggested

complete removal of ethanol by the BSF systems, breakdown of the phenolic compounds (gallic acid and vanillin) and build-up of the toxic compound catechol. The changes in the chemical milieu relate to the changes observed in the bacterial community structure of each BSF.

Similar trends were observed for the fungal populations. Bacterial community structures shifted in the deep and surface samples. Fungal populations were more prominent at the surface of the BSFs and only detected in the deep samples after amendment with the synthetic winery wastewater. These results for the bacterial community structures were confirmed when analysing the phylogenetic profiles of each BSF system. The MICA3 data showed differences in the bacterial community structure from the start of the experiment to after the amendment with synthetic winery wastewater. Diversity of organisms between BSFs included the presence of *Clostridium*, *Alteromonas*, *Pseudomonas*, *Pseudoalteromonas* and *Acinetobacter* species. Each of the identified species could play distinct roles in the breakdown or build-up of metabolites. The diversity of species can be linked to spatial changes in the NMDS plots. The phylogenetic analysis showed the diverse bacterial communities present in the sand samples and from the study by Burton *et al*. (2007) it could be hypothesised that each bacterial species present in these systems could possibly be involved in specific metabolic pathways. By comparing our phylogenetic data to the bacteria identified by Burton *et al*. (2007) we could hypothesise that these organisms convert simple compounds (aromatic compounds and monosaccharides) into secondary metabolites and this is possibly achieved through several metabolic pathways.

RNA was extracted from sand samples collected before and after amendment with synthetic winery wastewater and the total RNA was converted into cDNA. Attempts to amplify the alcohol dehydrogenase and catechol 1,2-dioxygenase genes were unsuccessful. The catechol 2,3-dioxygenase gene was successfully amplified and the changes in the expression levels of this gene were monitored by qPCR. An increase in the copy numbers of the C23O gene was observed after amendment with the synthetic winery wastewater as compared to the baseline samples, thus showing that with the addition of the synthetic winery wastewater could have increased the amount of C23O genes. Amendment with the synthetic winery wastewater resulted in the selection of microbial communities that would possibly have the ability to degrade catechol and were actively involved in the breakdown of waste. The amendment resulted in an increase in the number of organisms with the ability to produce the functional gene catechol 2,3-dioxygnease and therefore this resulted in an increase in the amount of catechol 2,3-dioxygenase genes being expressed. Attempts to investigate the diversity of C23O by DGGE analysis and cloning failed.

# CHAPTER FOUR

# GENERAL DISCUSSION

This study forms part of a larger WRC funded project that has been assessing the use of BSF for the bioremediation of winery wastewater. Studies by Welz *et al*. (2011) and Ramond *et al*. (2010) have shown that the bacterial communities present in the sand play a pivotal role in the breakdown of the components of the synthetic winery wastewater. This research project is therefore necessary in order to monitor changes in both the bacterial and fungal communities through the use of molecular fingerprinting techniques before and after the amendment with synthetic winery wastewater, as well as to assess the presence of functional genes by qPCR. The isolation of the metagenomic DNA from sand samples before and after amendment with synthetic winery wastewater was performed and followed by the amplification of the bacterial 16S rRNA and the fungal ITS regions. The PCR products were digested and used for T-RFLP analysis. The T-RFLP profiles were assessed through several bioinformatics based programmes. Non-metric scaling dimension plots were created using Primer 6. These T-RFLP profiles were used for phylogenetic analysis using MICA3. From the sand samples taken, RNA was extracted and synthesised to cDNA and this was used for the amplification of the functional genes of interest using qPCR.

*The key findings in the study included the following:*



The T-RFLP profiles for both the bacterial and fungal community structures showed distinct differences in the community profiles before and after amendment with synthetic winery wastewater when comparing the experimental system to the control system (with the control system only receiving basal nutrients). The NMDS plots showed differences at the surface and depths of the BSFs for both the bacterial and fungal community structures. The study by Ramond *et al*., (2013), showed that through the use of BSFs for the treatment of winery effluent the microbial communities were responsive to the impact of the winery effluent and that as the community structure changed over time the remediation capacity remained high with a COD and phenolic removal rates of >98% throughout the experimental period. The results from this study strongly suggest that there was a selection of functionally redundant microbial communities (Allison and Martiny, 2008).

The T-RFLP profiles of the bacterial community were used for the phylogenetic analysis using the programme MICA3. These phylogenetic profiles of each BSF system showed distinct differences in the microbial community structures between experimental systems and control. Organisms of interest identified by MICA3 included *Clostridum*, *Actinobacterium*, *Streptomyces*, *Acinetobacter*, *Rhodococcus* and *Sarcina* species, which are common sand organisms. The prevalence of these organisms showed that they play a pivotal role in either the degradation or build-up of secondary metabolites through metabolic pathways as has been reported by others (Burton *et al*., 2007). The study provided insight into which metabolic pathways these organisms identified by MICA3 could possibly be involved in. The degradation of monosaccharides, or aromatic compounds to secondary metabolites by these organisms mentioned above could be possibly deduced from the study. The diversity of organisms detected by the MICA3 phylogenetic data of the experimental BSF and control BSF system confirmed the spatial differences of the points on the NMDS plots between the experimental and control BSF systems.

Assessing the presence of functional genes was performed by extraction of RNA from the sand samples and synthesising it to cDNA. Amplification of the alcohol dehydrogenase and catechol 1,2-dioxygenase gene by real time PCR was unsuccessful. Amplification of the catechol 2,3-dioxygenase gene was successful. The catechol 2,3-dioxygenase gene was amplified by real time PCR for the cDNA from samples before (baseline) and after the amendment of synthetic winery wastewater. Normalisation of the C23O gene to a reference gene (*rpoB*) was performed. Relative quantification analysis revealed that there was an increase in the copy numbers from the start to the end of experiment



The amendment with synthetic winery wastewater indeed had an effect on the bacterial and fungal community profiles of the BSF systems. The spatial differences in the points between the experimental BSF systems and the control BSF system was observed on the MDS plots. The change in microbial community structure was confirmed through the phylogenetic data generated by MICA3. The change in the microbial communities present in the BSFs was shown by the diversity of organisms present when comparing the organisms at the start (baseline) and after the amendment with synthetic winery wastewater. These organisms present are involved in the degradation or build-up of the compounds of the synthetic winery wastewater into other secondary compounds. The change in the bacterial community structure was indeed through the amendment with synthetic winery wastewater, and the components of the synthetic winery wastewater were degraded by the organisms present in the sand samples. The presence of functional genes, namely C23O gene in these sand samples which was confirmed by qPCR analysis; however, the diversity of the organisms producing the C23O gene could not be confirmed by the DGGE or sequencing data.

Despite the negative result obtained through this project, the key points obtained from this study include: that the T-RFLP analysis results provided insight that both the bacterial and fungal communities play a role in the remediation of the synthetic winery wastewater (metabolite profiles, Appendix A) and that functional genes such as catechol 2,3-dioxygenase genes can be detected through qPCR, The increase in the expression of the C23O genes indicted that there was an increase in the microbial communities ability to degrade catechol. Cloning of catechol 2,3-dioxygenase gene using the CloneJET PCR cloning kit to confirm the diversity of organisms that was expressing the C23O gene by sequencing the positive colonies on the LB-amp plates, was unsuccessful. In future, more optimization would be required.

This research project was necessary to establish the role that bacterial and fungal species play in the degradation or build-up of compounds associated with the synthetic winery wastewater. Two formulations of synthetic winery wastewater was included; both formulations contained ethanol and the phenolics, vanillin and gallic acid, which are all common components of winery effluent. In addition, the first formulation of synthetic winery wastewater contained acetate, and the second formulation contained glucose, which are typically major contributors to the organic fraction of winery wastewater. The changes in the microbial communities can thus be compared to the chemical data of the effluent analysis (Appendix A), to assess whether the organisms present in the sand are involved in the remediation of the synthetic winery wastewater. In order to evaluate the biodegradative metabolic processes taking place in the BSFs, as well as the effect of winery wastewater composition on these processes, the treatment of two formulations of synthetic winery wastewater were compared (section 2.2.2, page 32). It has previously been shown that build-up of VFAs, particularly acetate, takes place in BSFs and other systems treating winery effluent or components of winery effluent (Ganesh *et al*., 2010; Welz *et al*., 2011, 2012). When assessing the superficial niches after the amendment with the synthetic winery wastewater containing acetate, both experimental BSFs P1 and BSF P2 group together, showing 60% similarity to one another and 40% similarity to the control (PC), and at had an impact on the bacterial community structure (Figure 3.4) which was greater at the outlet than the inlet. It was hypothesised that these differences observed from the changes in chemical milieu resulting from substrate degradation gradients between the inlet and outlet of the BSFs (Welz *et al*., 2014). After the amendment with the synthetic winery wastewater containing glucose, BSF P1 and BSF P2 group together showing 60% similarity to one another and 40% similarity to the control. It is inferred from these results that, unlike amendment with synthetic winery wastewater containing acetate, synthetic winery wastewater containing glucose had a similar impact on the bacterial community structure in

the superficial niches at the inlet and outlet (Welz *et al*., 2014). At the deep niches, similar trends were noted in the deep and superficial niches, where each wastewater had an impact on the bacterial community structures, with similar impacts being determined at the inlet and outlet after amendment with synthetic winery wastewater containing glucose, but a greater impact at the outlet after amendment with synthetic winery wastewater containing acetate. In each instance, temporal changes not related to wastewater amendment were also noted, as evidenced by shifts in points representing the control BSF PC. The two formulations of synthetic winery wastewater was included on the hypothesis that the treatment of this effluent containing the glucose would be more effective than the treatment of the one containing acetate. However in the deep niches, the synthetic winery wastewater containing acetate had more of an effect.

This project dealt with assessing the changes of the bacterial community structures after the amendment with synthetic winery wastewater, in addition metabolite profiles (including sugars, alcohols and acids; phenolic compound characterisation) was performed as a parallel study which revealed that during the treatment with synthetic winery wastewater many other factors played a vital role. In a previous study by Welz *et al*., (2012), it has been shown that phenolic removal in BSFs takes place via adsorption and biodegradation. In the parallel study, at the end of amendment with each wastewater formulation, the total phenolic concentrations in the effluent from BSF1 and BSF2 were 11.0 ± 0.0 and 5.0 ± 1.4 mg GAE/L (week 19) and 25.1 ± 0.0 and 19.7 ± 0.4 mg GAE/L (week 30), respectively. No phenolics were detected in samples from the control (BSFC). The influence of wastewater composition on phenolic removal in different spatial niches was accomplished by analysis of pore water samples taken at the end of amendment with synthetic winery wastewater containing acetate and containing glucose (week 20, week 31) (Appendix A).

The parallel study by Welz *et al*., (2014) showed that the overall organic removal, weekly COD concentration was measured during the course of the 31-week study. However, despite the mode of operation, set up and experimental protocol being exactly the same for each experimental system, during the amendment with the synthetic winery wastewater containing acetate, there were notable differences in the results obtained for BSF P1 and BSF P2, differences were also observed in the community structures. This confers that these should be replicated of a biological nature even if a larger study is conducted (Hurlbert, 2013; Morrison and Morris, 2000). As hypothesized, the effluent COD was higher during amendment with synthetic winery wastewater containing acetate, than with synthetic winery wastewater containing glucose at the end of the respective experimental periods (week 20 and 31). This finding strongly suggests that the microbial communities had rapidly adapted (acclimated) to synthetic winery wastewater containing glucose, but not to synthetic winery

wastewater containing acetate at this stage (Welz et al., 2012). The COD of the control system (BSFC) was consistently less than 25 mg/L.

The redox status of each BSF system resulted in the selection of the bacterial consortia with different organisms being present at the surface and deep sediments. Figure A.1 and A.2 showed the metabolite profiles and that the accumulation and biodegradation of organic substrates and metabolites also led to the different organisms being present which was strongly influenced by the redox potential of each niche. From the Figure A.1 and A.2, organics such as glucose, ethanol and phenolic were more readily degraded in the deep sediments. However, the accumulation of volatile fatty acids was more prominent in the deep sediments of the BSFs. For future research, it was suggested that the BSFs be configured and operated in a manner that the wastewater be treated under redox conditions in order to obtain complete degradation of compounds. One alternative that was suggested is a polishing aeration step for the wastewater that has been treated in the BSFs. However, trickling filters are a more feasible option and is currently being explored.

The presence of the functional gene (C23O gene) provided a new insight to previous studies by Welz *et al*. (2011) and Ramond *et al*. (2010), as it confirmed that presence of genes that play a role in the build-up or degradation of components of the synthetic winery wastewater. The cumulative findings from these studies provided the necessary information required to design (configuration and operation) a full scale sand bioreactor system to be utilised for the treatment of winery wastewater which has been successfully installed at a winery.



Despite that many of the experiments (i.e. DGGE, conclusive phylogenetic analysis, amplification of ADH gene), the chemical profile for each BSF and T-RFLP analysis, in conjunction with previous studies by Welz *et al*., (2011) and Ramond *et al*., (2013) provides the basis for the possible implementation of a full scale BSFs situated at a small winery.

For future studies, according to Cydzik-Kwiatkowska and Zielińska, (2016) one fundamental step to improve wastewater treatment technology an in depth of the microbial ecology is needed. Bacterial metabolism determines the effectiveness and running of the biological treatment of wastewater systems. Hence, it is important to define the relations between the species structure and the performance (operational parameters) of a treatment system. The relationship between microbial succession and operating parameters of the wastewater treatment system needs to be well defined and possibly coupled culture-dependant and PCR based methods could support the implementation of full-scale technologies.

NGS allows for the genomic scale DNA sequencing and data to be generated at a low cost and thus NGS is revolutionising the field of molecular ecology. Few molecular ecologists are yet to familiarise themselves with the use of NGS data, however an increase in the number of statisticians have begun developing models to incorporate NGS characteristics into molecular ecology analysis (Andolfatto *et al*. 2011; Gompert & Buerkle 2011). The excitement of using new NGS platforms has generated momentum among researchers to involve environmental DNA analysis, however optimization and benchmarking is required. Rapid progression of NGS technologies has provided optimism for a bright future for the field of next-generation environmental DNA analysis (Shokralla *et al*., 2011). In relation to this study, for future research NGS should be considered as a platform for DNA analysis of environmental research.

Even though the study has presented various results, there are some recommendations for future research:

- Optimization of the nested PCR using the catechol 2,3-dioxygenase GC clamped primers set, including changing parameters
- Further optimization for the alcohol dehydrogenase and catechol 1,2-dioxygenase primers sets
- Possibly re-design the functional genes primers according to available sequences on GenBank
- Cloning of the catechol 2,3-dioxygenase gene but using other *E. coli* strains as a host
- Normalisation of RNA to another reference gene and expressing this data as a percentage.
- Next generation sequencing (NGS)

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**APPENDIX A: METABOLITE PROFILES OF BSFs**

Figure A.1 and Figure A.2 shows the chemical analysis performed by HPLC of each metabolite profile of the BSFs. This analysis was performed by Dr Pamela Welz, during the course of her PhD candidacy. The total phenolics of each BSF was determined using the Folin-Ciocalteau micro method for total phenolics in wine, based on the method described by Slinkard and Singleton, (1997). Gallic acid monohydrate standards were prepared at the BTB laboratory and the results were expressed as mg/L in gallic acid equivalents (GAE) determined from a standard curve. Individual phenolics, sugars, acids and alcohols in the effluent samples were identified and quantified using reverse phase HPLC (described by Welz et al., 2012).

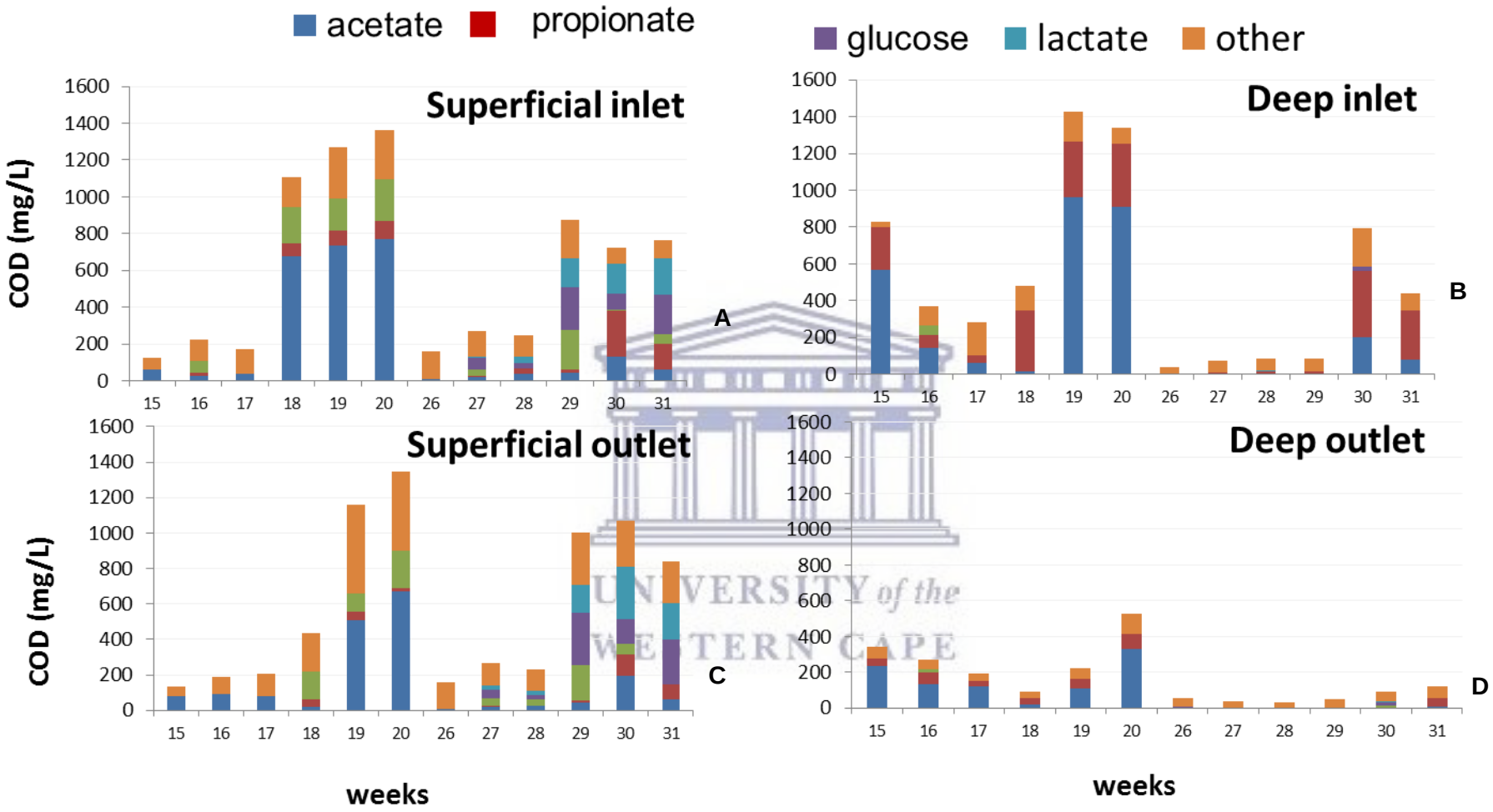



**Figure A.1:** The chemical oxygen demand/ metabolite profiles of the sugars, acids and alcohols of pore water samples taken from the superficial inlet (A) deep inlet (B), superficial outlet (C) and the deep outlet (D) of BSF P1 and P2 after the amendment with synthetic winery wastewater over the 31-week study period.

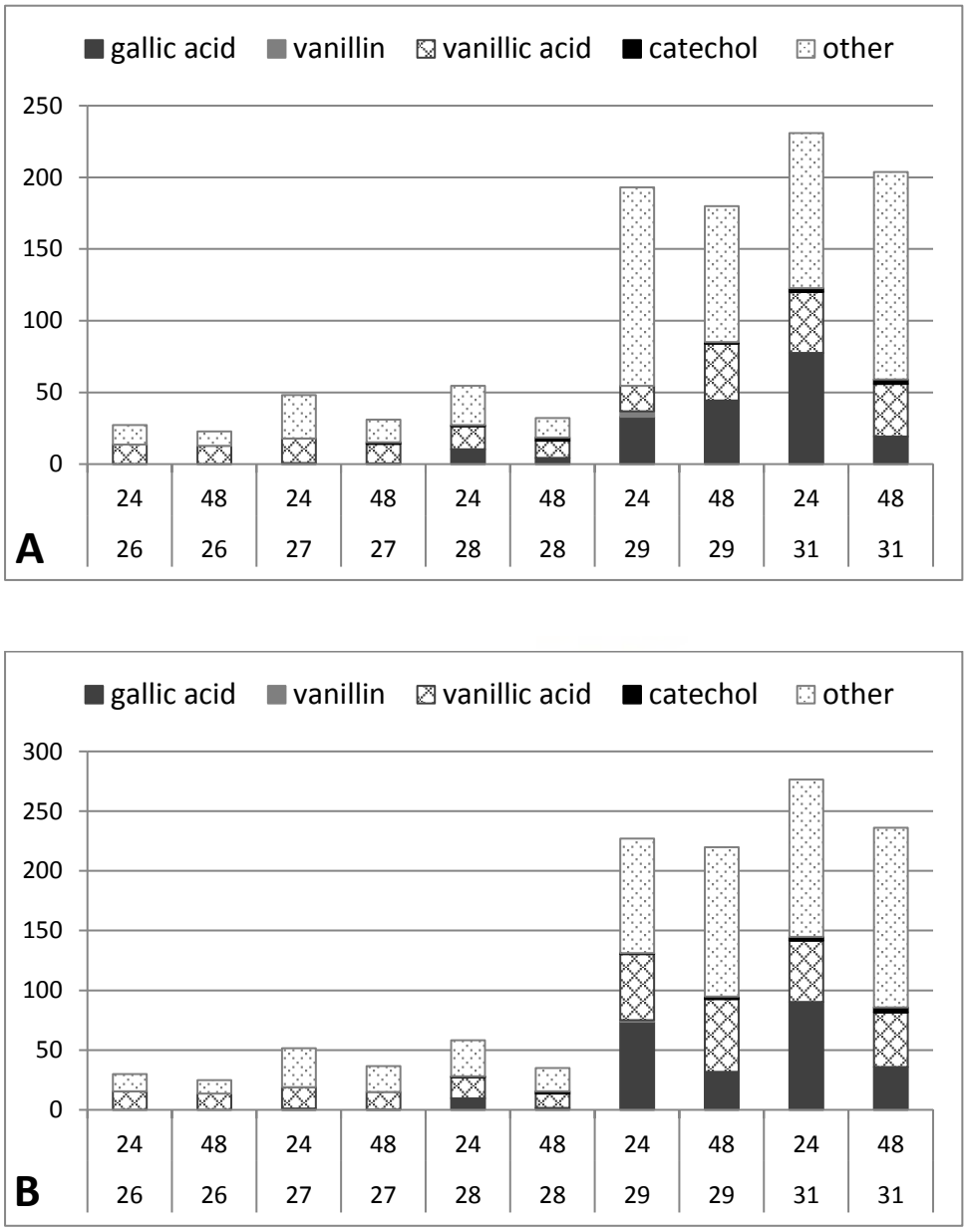



**Figure A.2**: The Ttemporal changes in the average concentration of total phenolics in the superficial (A) and deep (B) niches during amendment with synthetic winery with an influent phenolic concentration of 62.5 mg GAE/L (weeks 26-28), and 250.0 mg GAE/L (weeks 29-31).

## APPENDIX B: THEORETICAL COD CALCULATIONS

**100 mg/ L Vanilllin ($C_8H_8O_3$):**

$COD_t = \frac{8(4x+y-2z)}{(12x+y+16z)}$ mg COD/ mg $C_xH_yO_z$

$COD_t = \frac{8[4(8)+8-2(3)]}{[12(8)+(8)+16(3)]} = \frac{272}{152} =$ 1.79 mg COD/ mg

100 mg/L X $\frac{1}{1.79 \text{ mg COD/mg}} =$ 55 mg/L

55 mg → 1 L

X → 22 L

X = 1210 mg

X = 1210 mg ÷1000 = 1.21 g vanillin

**100 mg/L Gallic acid ($C_7H_6O_5$):**

$COD_t = \frac{8(4x+y-2z)}{(12x+y+16z)}$ mg COD/ mg $C_xH_yO_z$

$COD_t = \frac{8[4(7)+6-2(5)]}{[12(7)+(6)+16(5)]} = \frac{192}{170} =$ 1.12 mg COD/ mg

100 mg/L X $\frac{1}{1.12 \text{ mg COD/mg}} =$ 89.29 mg/L

89.28 mg → 1 L

X → 22 L

X = 1964.16

X = 1964.16 ÷ 1000 = 1.96 g gallic acid

**800 mg/L Glucose ($C_6H_{12}O_6$):**

$COD_t = \frac{8(4x+y-2z)}{(12x+y+16z)}$ mg COD/ mg $C_xH_yO_z$

$COD_t = \frac{8[4(6)+12-2(6)]}{[12(6)+(12)+16(6)]} = \frac{192}{180} =$ 1.07 mg COD/ mg

100 mg/L X $\frac{1}{1.07 \text{ mg COD/mg}} =$ 747.66 mg/L

747.66 mg → 1 L

X → 22 L

X = 16448.52 mg

X = 16448.52 mg ÷ 1000 = 16.45 g glucose

**1000 mg/L Ethanol ($C_2H_6O$):**

$COD_t = \frac{8(4x+y-2z)}{(12x+y+16z)}$ mg COD/ mg $C_xH_yO_z$

$COD_t = \frac{8[4(2)+6-2(1)]}{[12(2)+(6)+16(1)]} = \frac{96}{46} =$ 2.09 mg COD/ mg

100 mg/L X $\frac{1}{2.09 \text{ mg COD/mg}} =$ 478.47 mg/L

478.47 mg → 1 L

X → 22 L

X = 10438.34 mg

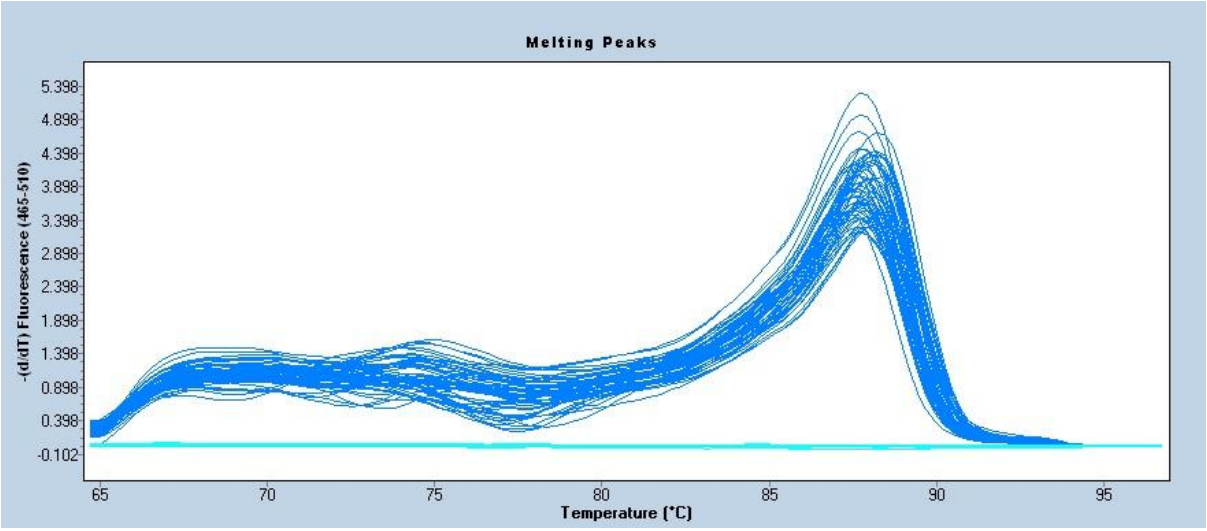
X = 10438.34 mg ÷ 1000 = 10.44 g ethanol

$\rho = \frac{m}{v}$

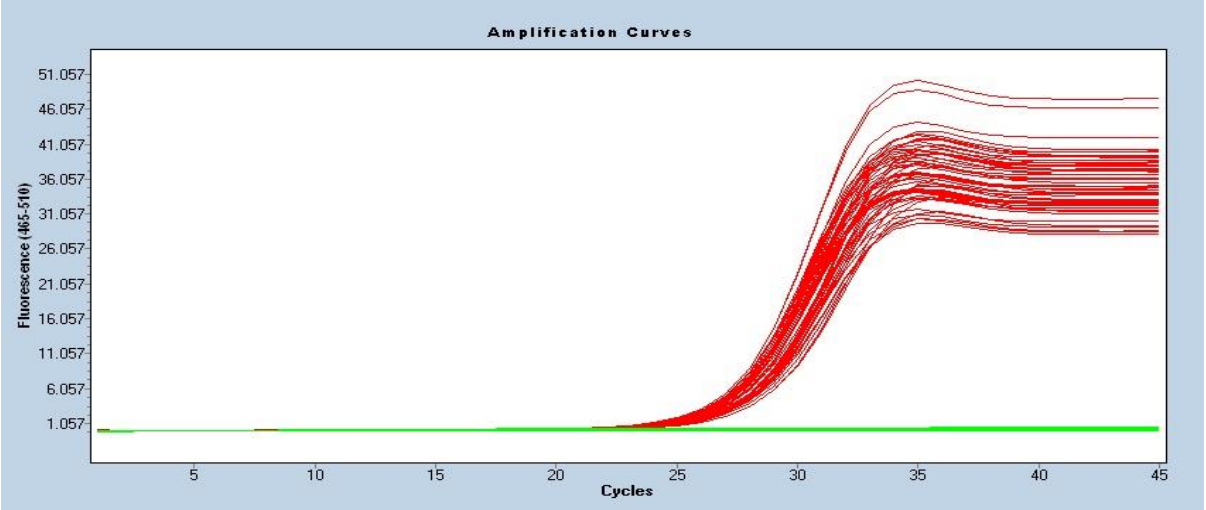
$v = \frac{m}{\rho} = \frac{10.44\ g}{0.790\ g/ml}$ = 13.22 ml ethanol

**APPENDIX C: AMPLICATION AND MELTING CURVES**

**STANDARD CURVE AND qPCR Cp VALUES AND COPY NUMBERS FOR C23O GENE**




A




B



**Figure C.1:** Melting curve analysis (A) and Amplification curves (B) of CZA14$^{T}$gDNA, synthesised cDNA samples with the catechol 2,3-dioxygenase gene using real time qPCR of the samples taken at the start of the experiment (baseline).

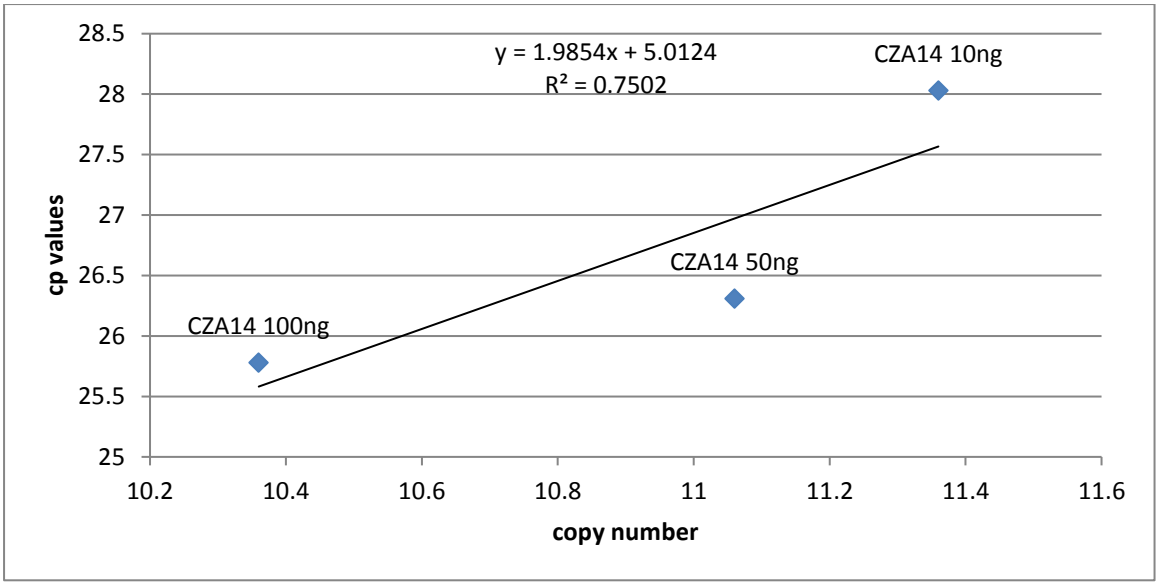



**Figure C.2:** Standard curve of CZA14$^T$ gDNA at different concentrations using the online calculator (copy number calculator for real time PCR – Science Primer, 2015) for the C23O gene.



**Table C.1:** Copy numbers of the C23O genes at the start and end of the experiment

| **Baseline Sample** | **Cp number concentration** | **Copy numbers** |
|---|---|---|
| CZA14$^T$10 ng | 27.64 | |
| CZA14$^T$50 ng | 27.47 | |
| CZA14$^T$100 ng | 27.61 | |
| P1 and P2 Sup In 10 ng | 27.64 | 11.40 |
| P1 and P2 Sup In 50 ng | 27.56 | 11.36 |
| P1 and P2 Sup In 100 ng | 27.58 | 11.37 |
| P1 and P2 Deep In 10 ng | 27.53 | 11.34 |
| P1 and P2 Deep In 50 ng | 27.60 | 11.38 |
| P1 and P2 Deep In 100 ng | 27.72 | 11.44 |
| P1 and P2 Sup Out 10 ng | 27.97 | 11.56 |
| P1 and P2 Sup Out 50 ng | 27.94 | 11.55 |
| P1 and P2 Sup Out 100 ng | 27.87 | 11.51 |
| P1 and P2 Deep Out 10 ng | 27.53 | 11.34 |
| P1 and P2 Deep Out 50 ng | 27.30 | 11.23 |
| P1 and P2 Deep Out 100 ng | 27.90 | 11.53 |
| PC Sup In 10 ng | 28.01 | 11.58 |
| PC Sup In 50 ng | 28.02 | 11.59 |
| PC Sup In 100 ng | 27.82 | 11.49 |
| PC Deep In 10 ng | 27.86 | 11.51 |
| PC Deep In 50 ng | 27.86 | 11.51 |
| PC Deep In 100 ng | 27.91 | 11.53 |
| PC Sup Out 10 ng | 27.92 | 11.54 |
| PC Sup Out 50 ng | 27.77 | 11.46 |
| PC Sup Out 100 ng | 28.03 | 11.59 |
| PC Deep Out 10 ng | 27.99 | 11.57 |
| PC Deep Out 50 ng | 27.27 | 11.21 |
| PC Deep Out 100 ng | 27.74 | 11.45 |
| **After amendment samples** | | |
| CZA14$^T$10 ng | 25.78 | |

| | | |
|---|---|---|
| CZA14$^{T}$50 ng | 26.31 | |
| CZA14$^{T}$100 ng | 28.03 | |
| P1 Sup In 10 ng | 27.68 | 11.43 |
| P1 Sup In 50 ng | 28.80 | 11.98 |
| P1 Sup In 100 ng | 29.10 | 12.13 |
| P1 Deep In 10 ng | 27.43 | 11.29 |
| P1 Deep In 50 ng | 26.70 | 10.92 |
| P1 Deep In 100 ng | 26.56 | 10.85 |
| P1 Sup Out 10 ng | 36.38 | 15.80 |
| P1 Sup Out 50 ng | 33.99 | 14.60 |
| P1 Sup Out 100 ng | 30.92 | 13.05 |
| P1 Deep Out 10 ng | 30.95 | 13.06 |
| P1 Deep Out 50 ng | 32.24 | 13.71 |
| P1 Deep Out 100 ng | 36.72 | 15.97 |
| P2 Sup In 10 ng | 29.29 | 12.23 |
| P2 Sup In 50 ng | 26.99 | 11.07 |
| P2 Sup In 100 ng | 28.56 | 11.86 |
| P2 Deep In 10 ng | 39.56 | 17.40 |
| P2 Deep In 50 ng | 37.73 | 16.48 |
| P2 Deep In 100 ng | 37.08 | 16.15 |
| P2 Sup Out 10 ng | 30.78 | 12.98 |
| P2 Sup Out 50 ng | 34.90 | 15.05 |
| P2 Sup Out 100 ng | 33.42 | 14.31 |
| P2 Deep Out 10 ng | 30.40 | 12.79 |
| P2 Deep Out 50 ng | 31.66 | 13.42 |
| P2 Deep Out 100 ng | 32.72 | 13.96 |
| PC Sup In 10 ng | 31.39 | 13.29 |
| PC Sup In 50 ng | 31.46 | 13.32 |
| PC Sup In 100 ng | 31.40 | 13.29 |
| PC Deep In 10 ng | 32.13 | 13.66 |
| PC Deep In 50 ng | 33.02 | 14.11 |
| PC Deep In 100 ng | 33.23 | 14.21 |
| PC Sup Out 10 ng | 37.41 | 16.32 |
| PC Sup Out 50 ng | 38.12 | 16.68 |
| PC Sup Out 100 ng | 38.28 | 16.76 |
| PC Deep Out 10 ng | 37.07 | 16.15 |
| PC Deep Out 50 ng | 37.41 | 16.32 |
| PC Deep Out 100 ng | 38.12 | 16.68 |

**APPENDIX D: STANDARD CURVE FOR *rpoB* GENE**

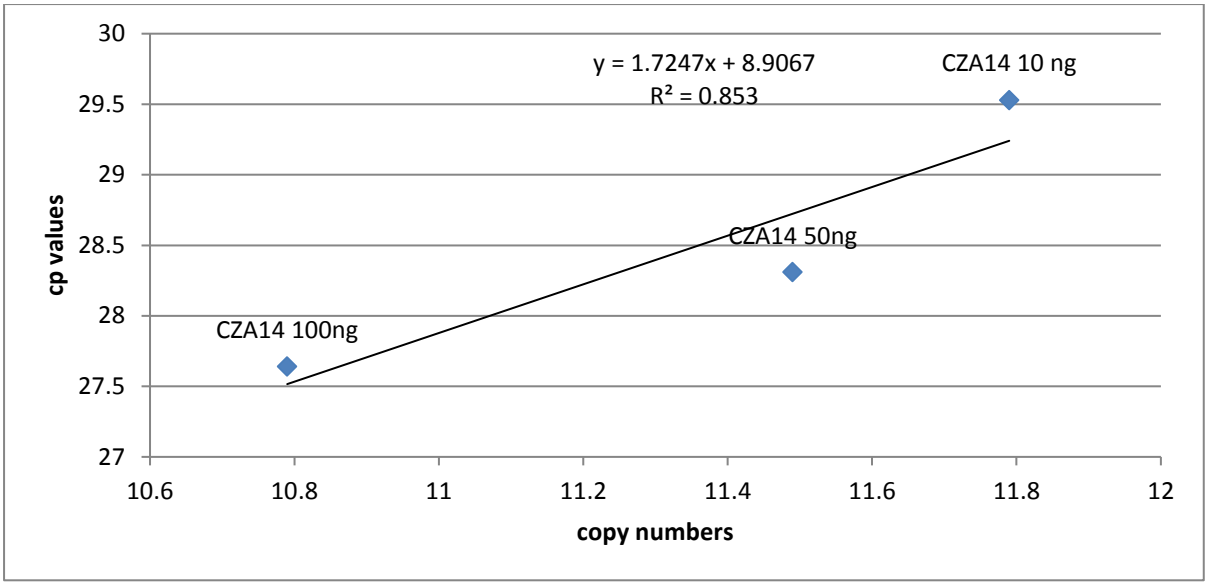



**Figure D.1:** Standard curve of CZA14$^T$ gDNA at different concentrations using the online calculator (copy number calculator for real time PCR – Science Primer, 2015) for the *rpoB* gene.