TREATMENT OF WINE DISTILLERY WASTEWATERS BY HIGH RATE ANAEROBIC DIGESTION AND SUBMERGED MEMBRANE SYSTEMS

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ABSTRACT

Experiences in treating wine distillery wastewaters (WDWs) contribute to the field of oenology as many oenologists are concerned with the selection, efficiency and economy of their wastewaters. Wine distillery wastewaters are strongly acidic, have high chemical oxygen demand (COD), high polyphenol content and are highly variable. Primary attention was focussed on sustainable biological treatment of raw wine distillery wastewater (RWDW) and fungally pre-treated wine distillery wastewater (FTWDW) by energy-efficient high rate anaerobic digestion (AD). This study also explored the development of a novel dual-stage anaerobic digestion ultrafiltration (ADUF) process, using a ceramic submerged membrane bioreactor (SMBR) in the treatment of both RWDW and FTWDW. The first stage was for the selection of microorganisms that were able to treat the toxic pollutants from WDWs. It was operated at a high feed-to-microorganism ratio. The second stage, a secondary digester, was operated like a typical membrane bioreactor at a low feed-to-microorganism ratio to sustain a stable efficient population for a long period. The characteristics of RWDW were as follows: pH 3.83, 15 000 mg/l soluble COD (COD_s) and 5229 mg/l of phenols. After pre-treatment of RWDW with *Trametes pubescens,* starting parameters for FTWDW were as follows: pH 6.7, 7000 mg/l soluble COD (COD_S) and 1440 mg/l of phenols. During operation of a high rate anaerobic digester for RWDW treatment, K_2HPO_4 was required for buffering the digester. Volatile fatty acid concentrations were <300 mg/l throughout the study, indicating degradation of organic acids present. Mean COD_S removal efficiency for the 130 day study was 87 %, while the mean polyphenol removal efficiency was 85 %. Addition of 50 mg/l Fe^{3+} increased the removal efficiencies of COD_S to 97 % and of polyphenols to 99 %. High removal efficiencies of COD_S and polyphenols were attributed to the addition of macronutrients and micronutrients that caused pH stability and stimulated microbial activity. The COD_S removal efficiency of high rate anaerobic digestion of FTWDW reached 99.5%. During FTWDW digestion, pH buffering was achieved using K_2HPO_4. A combination of a SMBR and a secondary digester was tested for the treatment of RWDW and FTWDW during a 30 day study. Results for RWDW showed that pH buffering was achieved by dosing the feed stream with $CaCO_3$ and K_2HPO_4. Buffering proved to be significant for optimum performance of the system in removal of soluble COD_S, and volatile fatty acids (VFAs). Different batches of RWDW used for feeding the reactor had variable compositions with respect to concentrations of nitrates, ammonium and total phenolic compounds. Ammonium accumulated in the secondary digester after 14 days of system operation, indicated the time required for the establishment of anaerobic conditions in the system. Dosing of the SMBR treating FTWDW with $CaCO_3$ and K_2HPO_4 buffered the pH;

this proved significant for optimum performance of the system in removal of COD_S. The system eliminated an average of 86 (± 4) % of COD_S present in the FTWDW. The residual COD_S levels in the effluent were approximately 400 mg/l, significantly lower than the concentrations observed when treating RWDW, indicating that fungal pre-treatment might have provided additional nutrients for removal of recalcitrant components of the wastewater. The resulting effluent was rich in nitrates and phosphates and might be used as a fertiliser. Alternatively, a membrane process, such as reverse osmosis (RO) or nanofiltration (NF) could be applied to raise the water quality to meet the levels required for reuse. Biomass samples were obtained from the four treatment systems and population shifts characterization using phospholipids fatty acids (PLFA) and 16S rRNA analysis to provide an indication of limitations within the microbial population. The values of the concentrations of the individual PLFAs detected in the samples indicated that ten bacterial species were present, with the GC content of the 16S rRNA increasing from 1 to 10. Analysis of denaturing gradient gel electrophoresis DGGE data indicated that the composition of the archeal community changed the consortia used for both RWDW and FTWDW treatment. Changes in band intensities indicated the presence of different components of the archeal communities. The results were not conclusive in terms of species identity as cloning, sequencing and phylogenetic analyses were not performed, but they did indicate microbial population shifts and species diversity for high rate anaerobic digestion. The results also confirmed prevalence of relatively few species during operation of SMBRs for treatment of RWDW and FTWDW, which suggested that the microorganisms that survived were either tolerant of toxic components of RWDW and FTWDW or they were able to remove polyphenols.

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LIST OF ABBREVIATIONS

AD	Anaerobic digestion
ADUF	Anaerobic digestion ultrafiltration
BOD	Biochemical oxygen demand in milligrams per litre
BOD_5	Five day test for biochemical oxygen demand in milligrams per litre
C	Carbon
COD	Chemical oxygen demand
COD_S	Soluble chemical oxygen demand
COD_T	Total chemical oxygen demand
DNA	Deoxyribonucleic acid
DGGE	Denaturing gradient gel electrophoresis
DWAF	Department of Water Affairs and Forestry
EC	Electrical conductivity
et al	And others
EMBR	External membrane bioreactor
FAMEs	Fatty acid methyl esters
FAU	Formazine attenuation units
F/M	Food to microorganism
FTWDW	Fungally pre-treated wine distillery wastewater
GC\MS	Gas chromatography\mass spectrophotometry
HRT	Hydraulic retention time
MBR	Membrane bioreactor
MCRT	Mean cell residence time
MF	Microfiltration
MLSS	Mixed liquor suspended solids
MS	Mixed solids
MSS	Mixed suspended solids
MW	Molecular weight
N	Nitrogen
NO_3^-	Nitrates
P	Phosphorus
PCA	Principal component analysis
PCR	Polymerase chain reaction

PLFA	Phospholipid fatty acid
PLFAs	Phospholipid fatty acids
PO_4^{3-}	Phosphates
N	Nitrogen
NF	Nanofiltration
NH_4	Ammonia
RO	Reverse osmosis
RWDW	Raw wine distillery wastewater
SD	Standard deviation
SMBR	Submerged membrane bioreactor
SMP	Soluble microbial products
SRT	Sludge retention time
SS	Suspended / settleable solids
SO_4^{2-}	Sulphates
TDS	Total dissolved solids
TOC	Total organic carbon
TN	Total nitrogen
TP	Total phosphorus
TS	Total solids
TSS	Total suspended solids
UASB	Upflow anaerobic sludge blanket
UF	Ultrafiltration
VFA	Volatile fatty acid
VFAs	Volatile fatty acids
viz.	namely
VLR	Volumetric loading rate
VS	Volatile solids
VSS	Volatile suspended solids
WDCS	Waste Discharge Charge System
WDW	Wine distillery wastewater
WDWs	Wine distillery wastewaters

LIST OF ABBREVIATED UNITS

COD/m^3	COD per cubic metres
d	days
Da	daltons
g/l	grams per litre
g	grams
g COD/l/d	grams of COD per litre per day
g COD/g/VSS/d	grams of COD per gram per VSS per day
g VSS/l	grams of VSS per litre
g VSS/g COD	grams of VSS per gram of COD
h	hour
kg/m^2	kilograms per square metres
kg $COD/m^3/d$	kilograms of COD per cubic metres per day
kg COD/kg VSS	kilograms of COD per kilograms of VSS
kg $COD/m^3/h$	kilograms of COD per cubic metres per hour
kg $TOC/m^3/d$	kilograms of TOD per cubic metres per day
l	litre
mg/l	milligrams per litre
m^3/kg VSS/kg feed COD	cubic metres per kilogram of VSS per kilogram feed COD
meq/l	milli equivalents per litre
m^2	square meters
mM	milli molar concentration
ml	millilitres
$S.cm^{-1}$	siemen per centimeter
µl	microlitres
v/v	volume per volume

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CHAPTER 1
Introduction and Synopsis

1.1 Introduction

The most widely used raw material in industrial processes, as well as the most abundant component of chemical, petrochemical, petroleum refining, food and drink, paper and pulp, wine distilleries and many other industrial sectors is water (Mack *et al*., 2004). In South Africa, water awareness and motivation campaigns for its sensible utilization are increasing (Van Schoor, 2005; DWAF, 1996). This is a result of a powerful economic driving force, as the costs of wastewater treatment are ever increasing, environmental standards becoming more stringent and the scarcity and price of good quality water resources escalating (Van Schoor, 2005; DWAF, 1996). Minimization of potable water usage can be achieved by reduction in the use of fresh water by replacing potable quality water with partially treated wastewater in applications where the required quality of the water is not high. Water can be reused on-site provided that the water will not adversely affect the process for which it is to be used. Use of treated but non-potable water that meets disposal standards for agricultural irrigation is also another method of minimization of water usage (Van Schoor, 2005; Mack *et al*., 2004; DWAF, 1996).

As South African industries are experiencing increasing international pressure to reduce the amount of freshwater use, wastewater is regarded as the most significant environmental hazard and also a significant resource, because freshwater water is a non-renewable natural resource (DWAF, 1996). Increase in industrialization and in particular wine production in South Africa exacerbates the pressure which the industry exerts on natural resources such as water, soil, land space and indigenous vegetation (Hayward, 2000; DWAF, 1996). This increase has occurred at a time when national legislation and foreign markets are becoming increasingly stringent, demanding that all factors which have potential to affect the natural environment should be controlled (Hayward, 2000; DWAF, 1996). For example, high concentrations of nutrients in wine distillery wastewaters (WDWs) (Eusébio *et al*., 2004;

Ramana *et al*., 2002a) make their discharge into water bodies problematic, causing eutrophication and other adverse environmental effects such as salination, contamination of groundwater, soil sodicity, waterlogging and anaerobiosis, loss of soil structure and increased susceptibility to erosion (Collins *et al*., 2005; Borja *et al*., 1993; DWAF, 1996). These adverse environmental effects may be exacerbated by process interruptions during treatment of wastewaters, especially WDWs. Process interruptions may stem from power failure, fire, floods, storms, overloading or underloading of wastewater treatment systems, temporal unavailability of wastewater holding dam capacity and the absence of trained operators. Therefore, it is important to manage winery wastes and their potential environmental impacts effectively and to make provision for emergency situations (Van Schoor, 2005).

Wine distilleries produce large volumes of waste, often called wine distillery wastewater, stillage or vinasse (Nataraj *et al*., 2006; Coetzee *et al*., 2004; Mendonca *et al*., 2004; Wolmarans and de Villiers, 2002; Benitez *et al*., 1999a; Jimenez and Borja, 1997). A high amount of wastewater is produced in the distillery industry; figures from 2 l per litre of wine produced (Eusébio *et al*., 2004; Benitez *et al*., 2000); to 20 l per litre of ethanol produced (Wilkie *et al*., 2000) are common. In molasses based distilleries, 13 - 15 l of wastewater is generated for every 1 l of ethanol produced (Ruiz *et al*., 2002). Further, the seasonal nature of distillery industries raises specific problems for the treatment processes in terms of WDW volume and composition (Van Schoor, 2005; Coetzee *et al*., 2004; Eusébio *et al*., 2004). As a result, treatment plants must be versatile in relation to the loading regime and must be able to cope with successions of start-ups and closedowns, and intervals of inactivity (Van Schoor, 2005; DWAF, 1996; Sales *et al*., 1987).

Environmental pollution due to the release of natural polyphenolic compounds from agroindustrial operations has become globally widespread (Van Schoor, 2005; DWAF, 1996). The structure of polyphenols present is similar in many industrial wastewaters, like those produced in wine distilling, olive oil extraction, green olive debittering, cork preparation, wood debarking and coffee production (Mendonca *et al*., 2004; Aggelis *et al*., 2003; Lesage-Meessen *et al*., 2001; Minhalma and de Pinho, 2001; Brand *et al*., 2000; Borja *et al*., 1993; Field and Lettinga, 1991). Therefore, environmental biotechnology today

is dominated by attempts to find ways of dealing with growing industrialization and the problems it causes, such as production of toxic wastewaters. Amongst solutions being attempted, bioremediation is the most popular (Sayler, 1997). Bioremediation encompasses all processes that occur in order to transform the environment altered by contaminants back to its original state (Sayler, 1997). The exact processes that can be used to achieve the desired outcomes differ, but they all have the same principle: to use microorganisms and the enzymes they produce to remove contaminants. Therefore bioremediation and waste treatment technologies are gaining momentum (Sayler, 1997).

Several problems have been encountered during biological treatment of WDWs, linked to its high toxicity and partial inhibition of biodegradation by the polyphenolic compounds present (Goodwin *et al.,* 2001) demonstrating their antibacterial activity (Borja *et al.,* 1993). Polyphenol concentrations in some distillery wastewaters range between 90 mg/l and 20 000 mg/l (Eusébio *et al*., 2004; Jimenez *et al*., 2003; Martin *et al*., 2002; Genovesi *et al*., 2000; Borja *et al*., 1993; Tofflemire, 1972). Polyphenols are responsible for strong inhibitory effects on microbial activity; therefore they must be removed during wastewater treatment as they pose environment and public health risks. Biological waste treatment methods have long been recognised as viable methods for the treatment of wastewaters with a high organic contamination load, such as those coming from agroindustry, brewing and wine distilleries in particular (Benitez *et al*., 1999a). Aerobic biological wastewater treatment processes such as activated sludge have been dogged by operational problems when treating high organic load wastewaters such as WDW (Vlissidis and Zoubalis, 1993). In the last few years the search for sustainable treatment systems capable of minimising energy consumption has encouraged the use of anaerobic biological wastewater treatment systems, even in cases where the main goal is to eliminate the biodegradable and dissolved fraction of carbonaceous substrates (Van Lier *et al*., 2001; Rajeshwari *et al.,* 2000). These anaerobic treatment systems have been used mainly for high strength organic wastewaters such as those from breweries and distilleries (Van Lier *et al*., 2001; Rajeshwari *et al.,* 2000). Although anaerobic digestion of this type of wastewater is feasible and appealing from an energy point of view, the presence of polyphenols slows down the treatment processes and thus hinders complete removal of the chemical oxygen demand (COD) exerted by the wastewater. Thus, improvements in anaerobic digestion (AD) efficiency during treatment of

wastewaters can be achieved by either modifying the digester design or incorporating appropriate advanced operating techniques (Rajeshwari *et al.,* 2000).

In this study, performance of a high rate anaerobic digester was investigated for the removal of COD and polyphenols from raw wine distillery wastewater (RWDW) and fungally pre-treated (FTWDW) in mesophilic temperatures. Use of a high rate digester should be advantageous because of longer sludge retention times and the fact that these digesters are able to withstand increased organic loading rates. Moreover, the advantages of AD of treatment of wastewaters include low production of stabilised excess sludge, low nutrient requirement, no energy input required for operation, production of methane as a potential energy source and therefore cost saving, the ability to operate with high hydraulic and organic loading rates, anaerobic sludge well adapted as bioreactors can operate for long periods with existing consortia and the fact that valuable compounds like ammonia are preserved, further confirming the choice of anaerobic digestion for WDW treatment (Rajeshwari *et al.,* 2000; Lettinga, 1995).

The disadvantages of AD of wastewaters are the susceptibility of anaerobic bacteria to perturbation (especially the methanogens), slow commissioning, the effluent often requires further (usually aerobic) treatment and little experience with AD in WDW treatment. These disadvantages are often outweighed by the advantages of AD, since a high rate digester can be operated for 130 d mean cell residence time (MCRT). A MCRT of 130 d will allow gradual growth of biomass and acclimatization of methanogens to the WDW, enabling them to withstand high organic loading rates. Therefore, development of a high rate digester for treatment technology for WDWs would also be advantageous for on-site treatment in distilleries. Considering the fact that operational anaerobic digesters are never without potential problems, that biomass developed in digesters during on-site treatment is unique, and the seasonal operations of wineries and distilleries, there is a need for a novel, portable design of technology that is capable of dealing with different kinds of high strength, recalcitrant generic wastewaters. Therefore this study also explores the development of a novel dual-stage ceramic membrane bioreactor (MBR) for the treatment of RWDW and FTWDW. The portability of this MBR will allow treatment of other wastewaters such as those arising from the manufacture of food and drink, paper and pulp and petrochemicals.

The development of appropriate operating protocols to achieve good productivity (short retention times), high efficiency in COD and polyphenol removal and low maintenance requirements due to the ability of an MBR to select for the desired biomass will make it easier to use. Selection occurs because the MBR is able to retain biomass which would be washed out of a gravity separation system and therefore retains all appropriate organisms through long term acclimatisation, rather than only organisms which settle well. The first digester unit will be for the selection of microorganisms that are able to treat the toxic pollutants from WDW. This can be operated at a high feed-to-microorganism ratio, and the opportunistic predatory microorganisms will be suppressed. The second digester (also called the hydrolysis tank) will be operated like a typical MBR, at a low feed-to-microorganism ratio and is expected to be able to sustain a stable efficient population for a long period. These advantages, in combination with conventional MBRs inherent limitations and advantages, make the design of the dual-stage SMBR novel.

1.2 Synopsis

The remainder of this thesis is set out as follows:

Chapter 2 reviews the recent literature available concerning the sources of distillery wastewater, its characteristics, a description of the treatment and disposal methods used currently and a comparison of the advantages and disadvantages of each with special reference to the use of AD and MBRs for the treatment of wine distillery wastewater (WDW).

Chapter 3 sets out the objectives of the study. Chapter 4 reports on initial operation of an anaerobic digester treating RWDW over a trial period of 130 days. The results include the impact on the major nutrients in the WDW, removal of COD and polyphenols, and overall quality of the treated effluent. The introduction of the K_2HPO_4 buffer helped to stabilise the removal efficiency for COD and polyphenols, even at increased organic loading rates. Soluble COD (COD_s) removal efficiency for the 130 day study was 87 % and polyphenol removal efficiency for the 130 day study was 63 %. The addition of K_2HPO_4 was essential

for buffering the bioreactor, and addition of Fe_3^+ as $Fe(NO_3)_3$ increased COD removal efficiency further to 95 %. Addition of 50 mg/l Fe^{3+} as $Fe(NO_3)_3$ between days 86 and 92 increased the removal efficiencies of COD_s to 97 % and of polyphenols to 65 %. Addition of 50 mg/l Co^{3+} as $Co(NO_3)_3$ decreased CODs removal efficiency from 97 % to 92 % between days 94 and 100; while polyphenol removal efficiency increased from 65 % to 93 %. Addition of 50 mg/l Ni^{3+} as $Ni(NO_3)_3$ decreased CODs and polyphenol removal efficiencies to 74 % and 70 % respectively. Soluble COD removal efficiency for the study period from day 108 to day 130 improved from 74 % to 92 % and from 70 % to 84 % for polyphenols, despite increased organic loading rates. However, a membrane was introduced to the digester in order to increase organic loading rates during treatment to further increase organic loading rates during treatment and to shorten sludge retention times.

Chapter 5 describes the wastewater treatment achieved using anaerobic digestion followed by ultrafiltration (ADUF) for RWDW. A more complex, four-reactor system was used for a short term test and showed that while the ADUF system was more robust in terms of the ability to withstand fluctuations in influent quality, the COD and polyphenol removals achieved did not meet the Department for Water Affairs and Forestry (DWAF) guidelines for irrigation (DWAF, 1996). The results indicated that the polyphenols present in the RWDW were exerting too strong an inhibitory effect on the bacterial consortia in the reactors, and that AD was not viable as the first treatment step.

Chapter 6 presents results observed when using the four-reactor ADUF system as a second treatment step after a pre-treatment of RWDW using a lignolytic fungus called *Trametes pubescens* to generate FTWDW. The two-step treatment system decreased the CODs of the final effluent from 1100 mg/l (in Chapter 5) to 400 mg/l. Phenol removal was not as consistent as COD removal, but a minimum effluent polyphenol concentration of 1 mg/l was achieved. The most striking result observed was that the reduced phenol concentration in the FTWDW compared to the RWDW enabled more stable digester operation and the requirement for the UF membrane became questionable.

The work presented in Chapter 7 details the treatment of FTWDW using AD without the use of a membrane. The CODs removal efficiency after fungal pre-treatment alone reached

53.3 %. During digestion, pH buffering was again achieved using K_2HPO_4. This provided a stable environment inside the digester for efficient and time-independent CODs removal. The total CODs removal efficiency reached 99.5 %, and the system proved able to eliminate shock CODs loads, as indicated by the concentrations of sludge and volatile fatty acids. The pH of the FTWDW was increased to 6.7 after high rate digestion.

Chapter 8 describes data obtained to assess the changes in microbial communities during the treatment of WDWs as a response to the selection pressures exerted by the low pH, and the concentrations of COD, polyphenols and nutrients found in the wastewater. While time constraints precluded full phylogenetic analyses of the consortia in all the digesters and other bioreactors used, phospholipids fatty acid (PLFA) analysis confirmed diversity of bacterial communities found in anaerobic systems as four and five species were identified in the sludge used to treat FTWDW and RWDW. Analysis of denaturing gradient gel electrophoresis (DGGE) data indicated that the composition of the archaeal community changed during both RWDW and FTWDW anaerobic treatment.

Chapter 9 presents a discussion and implication of the results reported in the preceding chapters. Chapter 10 summarizes the conclusions which could be drawn from the results. Recommendations for further work are also included.

CHAPTER 2
Literature Review

2.1 Wastewater generation in distillation industries

Wine production is one of the most important agricultural industries in Mediterranean countries such as Italy and Spain, and its importance to other parts of the world (e.g. Australia, Brazil, Chile, China, France, Germany, India, South Africa, and the United States of America) is increasing and impacting on their economies (Nataraj *et al*., 2006; Coetzee *et al*., 2004; Mendonca *et al*., 2004; Wolmarans and de Villiers, 2002; Benitez *et al*., 1999b; Jimenez and Borja, 1997). In South Africa the agricultural sector is an important earner of foreign exchange[1]. In the year 2000 the agricultural exports resulted in foreign exchange of 14 573 million rands with sugar and wine accounting for the largest exports, as well as citrus fruit, grapes, preserved fruits and nuts. Although South Africa represents 6 % of the world population and 4 % of the total surface area of the African continent, it nevertheless produces a significant proportion of the total agricultural production of Africa[1]. The other continent famous for its vineyards is Australia[2]. In Australia the gross open value of the state's farm output between 1983 and 1984 contributed 4 % to the state's total production, which indicated that vineyards which are regarded as rural economy had added value[2]. A high volume of wastewater is produced in these industries. Waste minimization is an important aspect to any industry, as it not only reduces the consumption of potable water but also decreases the volume of wastewater generated. During the production of wine from grapes, large quantities of liquid effluent are generated from various units of operation and processes. Musee *et al*. (2006) designed a system that identified waste-generating mechanisms, analyzed the causes, and then derived options of feasible waste minimization alternatives.

1 http://www.agriwriters.org.za/farm_industries.html/06062007

2 http://atlas.sa.gov.au/resources/atlas-of-south-australia-1986/productio/agriculture/06062007

Musee *et al*. (2007) identified 90 waste minimization strategies, which could yield considerable benefits to the wine industry if incorporated as an integral part of the entire vinification process. Waste minimization can prove deleterious to biological treatment systems though, as it can lead to a more concentrated wastewater.

2.2 Wastewater characteristics

Table 2.1 lists the characteristics of different distillery wastewaters from all over the world. Parameters such as the pH, alkalinity, electrical conductivity (EC), total chemical oxygen demand (COD_T), soluble chemical oxygen demand (COD_S), five-day biochemical oxygen demand (BOD_5), total organic carbon (TOC), phenol, volatile fatty acids (VFAs), volatile solids (VS), volatile suspended solids (VSS), total solids (TS), total suspended solids (TSS), mixed solids (MS), mixed suspended solids (MSS), total nitrogen (TN), ammonia (NH_4^+), nitrates (NO_3^-), total phosphorus (TP) and phosphates (PO_4^{3-}) are reported. In general, distillery wastewaters are acidic, have a brown colour and have a high content of organic substances that varies according to the raw material distilled e.g. wine type, lees etc. (Bustamante *et al*., 2005; Keyser *et al.,* 2003; Genovesi *et al*., 2000; Benitez *et al*., 1999b; Borja *et al.,* 1993; Vlissidis and Zoubalis, 1993). The average values for COD are 7 to 40 g/l and for BOD_5 5.5 to 20 g/l (Manisankar *et al*., 2004; Fumi *et al*., 1995; Racault and Lenoir, 1995). In other examples, the concentration of organic substances is very high, ranging from 20 to 150 g/l COD (Perez *et al*., 2004 Sheridan *et al.*, 2005; Martin *et al.,* 2002; Goodwin *et al*., 2001; Wilkie *et al*., 2000; Goodwin and Stuart, 1994). In studies conducted in South Africa, the COD (whether soluble or total was not specified) of wine distillery wastewater ranged from 20 g/l to 30 g/l (Wolmarans and de Villiers, 2002) while Driessen *et al*. (1994) reported COD between 22 and 48 g/l.

Wine distillery wastewaters are acidic and their high organic content can cause considerable environmental pollution (Keyser *et al.,* 2003; Borja *et al.,* 1993). The pH values of wine distillery wastewaters range from 3.5 to 5.0 as shown in Table 2.1 (Bustamante *et al*., 2005; Martin *et al*., 2002; Wolmarans and de Villiers, 2002; Goodwin *et al*., 2001; Genovesi *et al*., 2000; Rajeshwari *et al*., 2000; Benitez *et al*., 1999b; Harada *et al.,* 1996; Sheehan and Greenfield, 1980), which is also toxic for many life forms. In addition to COD and BOD pollution, wine distillery wastewaters contain phenolic compounds, mainly gallic and

gentisic acid, which provide them with high antibacterial activity, alone or in combination with low pH (Keyser *et al*., 2003; Seghezzo *et al*., 1998; Borja *et al.,* 1993). Moosbrugger *et al.* (1993) observed that South African wine distillery wastewater consists primarily of organic acids such as lactic acid (29 % v/v), tartaric acid (27 % v/v); succinic acid (26 % v/v), acetic acid (10 % v/v) and mallic acid (8 % v/v). In addition to these organic acids alcohol, hexose sugars and soluble proteins were found (Keyser *et al*., 2003; Seghezzo *et al*., 1998). Several problems have been encountered during biological treatment of wine distillery wastewater, linked to its high toxicity and inhibition of biodegradation due to the presence of polyphenolic compounds (Goodwin *et al.,* 2001), demonstrating the antibacterial activity reported in earlier literature (Borja *et al.,* 1993). Polyphenol concentrations in some distillery wastewaters vary considerably and can range from 29 - 474 mg/l (Bustamante *et al*., 2005). Polyphenols are responsible for strong inhibitory effects on microbial activity, and must be removed during wastewater treatment, owing to the environmental and public health risks they pose. Humans exposed to phenol at 1300 mg/l of concentration exhibited significant increases in diarrhoea, dark urine, mouth sores and burning of the mouth (Collins *et al*., 2005). Wine distillery wastewaters were also characterized for heavy metals, *viz.* iron and zinc, metal ions such as Ca^{2+}, K^{+} and Na^{+} (Nataraj *et al*., 2006; Ramana *et al*., 2002a; Harada *et al*., 1996) and sulphates (Ramana *et al*., 2002a; Harada *et al*., 1996). High concentrations of these constituents (Eusébio *et al*., 2004; Ramana *et al*., 2002a), plus other nutrients such as nitrate and phosphate make possible discharge of wine distillery wastewaters into water bodies problematic, causing eutrophication and other adverse environmental effects (Collins *et al*., 2005; Borja *et al*., 1993; DWAF, 1996).

2.3 Wine distillery wastewater disposal and use

Three popular methods are employed by distilleries to handle their wastewaters: (1) collection of wastewater in storage tanks followed by use for irrigation, (2) wastewater treatment in ponds primarily for settling of solids, evaporation processes and application of resultant sludge on land and (3) discharge of the wastewater to a local municipal treatment facility (Benitez *et al*., 1999b). These three methods have their associated problems and environmental risks. Treatment of wine distillery wastewaters at municipal facilities is very expensive and is often not a feasible, practical or viable option. In South Africa, the

Department of Water Affairs and Forestry (DWAF) began a project in 1999 to develop a Waste Discharge Charge System (WDCS) in order to manage wastewater and water resources efficiently and effectively (DWAF, 2003). It addresses the pricing of water used for waste disposal and proposes a system in which wastewater treatment costs are minimized when at least partial wastewater treatment occurs on the premises of the discharger as opposed to the release of raw, untreated wastewater to the sewer or receiving environment (DWAF, 2003).

Table 2.1: Chemical characteristics of distillery wastewaters.

	Type of wastewater					
Parameter	Distillery wastewater[1]	Wine distillery wastewater[2]	Vinasse[3]	Raw spent wash[4]	Molasses wastewater[5]	Lees stillage[6]
pH	3.0 - 4.1	3.53 - 5.4	4.4	4.2	5.2	3.8
Alkalinity (meq/l)	-	30.8 - 62.4	-	2	6000	9.86
EC ($S.cm^{-1}$)	346	-	-	2530	-	-
Phenol (mg/l)	-	29 - 474	477	-	450	-
VFAs (g/l)	1.6	1.01 - 6	-	-	8.5	0.248
COD_T (g/l)	100 - 120	3.1 - 48	-	37.5	80.5	-
COD_S (g/l)	-	7.6 - 16	97.5	-	-	-
BOD_5 (g/l)	30	0.21 - 8.0	42.23	-	-	20
TOC (mg/l)	-	2.5 - 6.0	36.28	-	-	-
VS (g/l)	50	7.340 - 25.4	-	-	79	-
VSS (g/l)	2.8	1.2 -2.8	-	-	2.5	0.086
TS (g/l)	51.5 - 100	11.4 - 32	3.9	2.82	109	68
TSS (g/l)	-	2.4 - 5.0	-	-	-	-
MS (g/l)	-	6.6	-	-	30	-
MSS (mg/l)	-	900	100	-	1100	-
TN (g/l)	-	0.1 - 64	-	2.02	1.8	1.53
NH_4^+ (mg/l)	0 - 45	55 - 900	-	1200 -12540	-	10 - 50
NO_3^- (mg/l)	4900	-	-	-	-	-
TP (g/l)	-	0.24 - 65.7	-	0.24	-	4.28
PO_4^{3-} (mg/l)	-	130 - 350	-	139	-	-

References:

[1] Nataraj *et al*., 2006; Harada *et al*., 1996
[2] Bustamante *et al*., 2005; Eusébio *et al*., 2004; Genovesi *et al*., 2000; Benitez *et al*., 1999a; Driessen *et al*. 1994; Borja *et al*., 1993
[3] Martin *et al*., 2002
[4] Ramana *et al*., 2002a
[5] Jimenez and Borja 1997
[6] Tofflemire, 1972.

Wine distillery wastewaters were thought to have some beneficial impacts on crop yields as land application or irrigation is a common method of disposal (Mulidzi *et al*., 2002). The wastewater is first screened, settled in ponds and then distributed over land containing trees, grass and crops using a sprinkler system or channels (Mulidzi *et al*., 2002). According to DWAF analysis (DWAF, 2003), winery wastewater disposal by irrigation has tremendous potential for polluting groundwater and other fresh water bodies due to the presence of high concentrations of phenolic compounds, salinity, phosphates, nitrates and ammonia which can lead to toxic effects and eutrophication. As a result, DWAF (2003) proposed that irrigation of fields by winery wastewaters can be done only if the concentration of nutrients is within set limits. These limits were established after research studies reported that the high salt concentrations in wine distillery wastewaters resulted in severe inhibitory effects on plants during irrigation. Investigations showed that there were differing responses to varying concentrations of wine distillery wastewater in irrigation water with regards to the percentage of seeds sown that germinated and the speed of germination (Ramana *et al*., 2002a). At low concentrations of wine distillery wastewater, all crops that were tested showed no inhibition of seed germination, except for tomatoes. However, percentage germination and the germination speed were inhibited by irrigation with water containing increased concentrations of wine distillery wastewater (Ramana *et al*., 2002a).

Thus the inhibitory effects of wine distillery wastewater on plant growth can be attributed to the high percentage of organic compounds and salts and thus high electrical conductivity, which made water uptake by seeds difficult and caused retardation of germination (Ramana *et al*., 2002a; Pandey and Sony, 1994). It was found that at concentrations of wine distillery wastewater >25 % (v/v) there was significant fungal growth on seeds, which was also inhibitory to seed germination (Ramana *et al*., 2002a). Conversely, Ramana *et al*. (2002b) later showed an increase in grain yield of maize, associated with larger cob sizes, higher numbers of seeds per cob and increased grain weight upon irrigation with wine distillery wastewater. It was found that the positive effect on maize crops upon irrigation was observed at low concentrations of wine distillery wastewater. At these low concentrations grain yields equivalent to those achievable when using the recommended NPK+FYM (nitrogen, phosphate, potassium and farm yard manure) level of fertilization could be obtained (Ramana *et al*., 2002b). The concentration of wine distillery wastewater used to irrigate maize crops could not be increased to greater than 25 %, as this would have resulted

in problems of salinity. Instead it was recommended that a non-saline fertilizer be used to supplement wine distillery wastewater for increased maize grain yields (Ramana *et al*., 2002b). A similar effect was observed for groundnut (Ramana *et al*., 2002c). It was concluded that soil and crop types are important if choosing to irrigate land with wine distillery wastewater, as its effect is both soil dependent and crop specific (Ramana e*t al*., 2002c).

This was corroborated by a study of irrigation of *Pennisetum clandestinum* (kikuyu grass) on sandy soil. The organic components of wine distillery wastewater leached through the sandy soil and reached the groundwater table, receiving at least partial treatment on the way (Mulidzi *et al*., 2002). Groundwater recharge by high rate infiltration is a common method of renewing water sources with wastewater in arid regions of the USA, and water shortages in areas surrounding alcohol and wine distilleries in South Africa could be partially ameliorated by the reuse of treated wine distillery wastewater to replace potable water for irrigation purposes wherever possible, for example in vineyard irrigation (Van Schoor, 2004; DWAF, 1996). However, distillery wastewater disposal by irrigation could potentially cause a large scale environmental problem to which little attention has been paid by this industry until recently (Van Schoor, 2004; Benitez *et al*., 1999b). One historical alternative to broad surface irrigation disposal of stillage was deep well disposal (Sheehan and Greenfield, 1980; Zajic 1971). Even though deep well disposal is a cheaper method than land disposal, limited underground storage and very specific geological formations interfere with any wide scale stillage disposal. Van Schoor (2004) summarized research advances and made recommendations on the use of wine distillery wastewater, the legal requirements in South Africa for winery wastewater irrigations and wine distillery wastewater storage. Again, ferti-irrigation and biocomposting with sugarcane press mud were also found to be popular methods for wastewater disposal (Noble and Stern, 1995).

However, these methods were highly energy intensive and hence financially and environmentally expensive. These disadvantages emphasized the need for further research using novel solid / liquid separation methods. As a result, membrane-based separation techniques, such as reverse osmosis (RO) and nanofiltration (NF) were investigated and yielded excellent results when applied to wine distillery wastewater (Noble and Stern, 1995). The effectiveness of NF membrane processes in water and wastewater treatment is generally acknowledged and has now become the most reliable standard technique in

combination with biological treatment (Bustamante *et al*., 2005; Trussell *et al*., 2006). Membrane based separation processes like NF, ultrafiltration (UF) and RO have been applied for treating a wide variety of industrial wastewaters (Nataraj *et al*., 2006). As the cost of wastewater disposal increases, more emphasis is being placed upon the recovery and recycling of the valuable chemicals and other components contained within these waste streams.

2.4 Traditional treatment practices

Most of the wastewaters from different distillery sources have been historically discharged directly into the soil or in groundwater. Reich (1945) proposed one of the first treatment systems, a continuous integrated method to concentrate the stillage by fermentation, where the fermentor discharge was centrifuged and the yeast that was not recycled was drum-dried for use as animal feed. The stillage was concentrated to 70 to 80 % solids and then neutralised with potassium carbonate (K_2CO_3). The concentrated, neutralized wastewater was then passed through low temperature carbonising retorts and activated at 870 ºC; the resultant carbon underwent aqueous extraction to produce potash fertilizer (potassium oxide (K_2O)), potash liquor and char. A decade later Montanani (1954) reported on the slightly more developed Tibrocal system, in which stillage was neutralized with lime (calcium oxide (CaO) or calcium hydroxide ($Ca(OH)_2$)) and then evaporated in 10 cm shallow containers, and also used as a fertiliser. Other similar schemes were proposed by Chakrabarty (1963) and again by Yamauchi (1977), with the difference that crystallised potassium sulphate was produced instead of potassium oxide. In Europe, distillery wastewater was incinerated, normally yielding 34.7 % of potash fertilizer and 2.2 % of phosphorus oxide (or ceramic oxide (P_2O_5)) (Sastry *et al*., 1964). Another disposal method was distillery wastewater concentration to 30 to 40 °Brix[*], followed by spray drying and combustion at 700 ºC, collecting the resultant ash at the column base (Gupta *et al*., 1968). Similar methods were performed with small variations such as concentration of stillage to 60 % solids and spray drying into fuel gases (Dubey, 1974). Tartrate removal has also been used as a pretreatment step (Tofflemire, 1972). Fluidized bed combustion of stillage followed by heat recovery was also suggested (Kujala *et al*., 1976). However, scale formation was reported as a problem in

[*] °Brix: A density scale used in the sugar industry. A Brix hydrometer has a scale calibrated in units equivalent to the percentage of sugar in a pure sugar solution.

some of the incineration and evaporation schemes and the energy costs were prohibitive. Jackman (1977) reported on Brazilian efforts to reduce scaling and to raise the ash fusion temperature by addition of other chemicals. The French practice was to concentrate the stillage to 60 % solids and then use it as a fertilizer at an application rate of 2.5 to 3.0 tonnes per hectare (Lewiki, 1978). Monteiro (1975) considered this method uneconomical in the Brazilian context. Extraction of specific chemicals from wine distillery wastewater for sale as by-products has been conducted to offset the costs of wastewater treatment and to improve subsequent treatment and disposal (Zabrodiskii *et al*., 1970). Gypsum ($CaSO_4.2H_2O$) was recovered by addition of seed crystals to the stillage at 80 ºC and stirring at 22 to 25 rpm for 60 minutes. This alleviated the problem of gypsum precipitation in cases where stillage was to be used for fodder yeast growth. Potassium and its double salt ($K_2SO_4 \cdot 5CaSO_4 \cdot H_2O$) can also be removed from wastewater concentrated to 30 to 60 ºBrix (Julsingha, 1970). Bass (1974) found that stillage concentrated to 60 to 80 ºBrix formed coagulate when soluble phosphate was added and the temperature raised again to 105 to 120 ºC. The coagulate was further dried and used as a fertiliser or ruminant fodder. Dubey (1974) stated that glycerol and germ oil were other chemicals that could be recovered from distillery wastewaters, but as late as 1980, distillery wastewater or stillage was still normally simply evaporated to provide animal feed or fertiliser, or incinerated with possible recovery of the potash (Sheehan and Greenfield, 1980).

2.5 Current treatment and disposal options

More recent wine distillery wastewater treatment includes methods to remove recalcitrant compounds from distillery wastewater and biologically treated distillery wastewater by physico-chemical processes (Pandey *et al*., 2003). In one case example, the physico-chemical treatment of biologically treated wastewater using conventional coagulant iron pickling wastewater supplemented with coagulant generated an effluent with COD in the range 940 to 1780 mg/l and BOD of 25 to 30 mg/l and colour in the range of 580 to 1100 platinum cobalt units. It was recommended that the waste sludge from this industry be utilized as a substitute for conventional coagulants. Wastewater generated after chemical coagulation could be further treated efficiently by using 8 g/l of activated carbon with a contact time of 45 minutes to reduce residual COD to < 250 mg/l to meet discharge limits (Pandey *et al*., 2003). Anodized graphite anodes were found to be suitable for the treatment

of wine distillery wastewater, especially in the presence of supporting electrolytes such as sodium halide, or sodium chloride which was found to be the most effective in degradation of polyphenols (Manisankar *et al*., 2004). Beltran de Heredia *et al*. (2005) later evaluated a combination of the Fenton coagulation/flocculation process (using H_2O_2/Fe^{2+}) for the treatment of wine distillery wastewaters and obtained a 74 % COD reduction under optimized conditions. Worldwide scarcity of water is a strong incentive for recovering clean water for reuse from wastewaters. Nataraj *et al*. (2006) investigated the treatment of distillery spent wash by removing the colour and the contaminants using a combination of NF and RO processes. High fluxes were obtained and significant rejection rates of total dissolved solids (TDS), COD, potassium and chloride were achieved. The absence of heat energy requirements in this application and the high rate of mass transfer generated by RO showed that a large amount of clean water could be permeated economically instead of being vaporized by energy intensive evaporation processes or steam distillation using tall towers. Water reclaimed by NF and RO is suitable for use in both municipal and industrial applications.

Chemical oxygen demand was considerably reduced in distillery wastewaters in India in order to reduce cost of wastewater disposal, emphasising the recovery and recycling of valuable chemicals contained in the wastewaters (Nataraj *et al*., 2006). Some methods of treatment of wine distillery wastewater result in single cell production, production of organic acids for sale in the industrial market and the production of viable biological products including enzymes, astaxanthia, plant hormones and biopolymers such as chitosan (Wilkie *et al*., 2000). Glycerol recovery, first suggested in 1974, was finally achieved towards the end of the 20^{th} century by concentrating wastewater to 60 % solids followed by the addition of quicklime (calcium oxide (CaO)) and ethanol, precipitating 90 % of the glycerol present. Germ oil was obtained by heating distillery wastewater, centrifuging at 6000 g and extracting the oil solvent from the lightest fraction (Dubey, 1974). As with the generation of fertilizer for direct land application, the economics of any treatment method rely heavily on the financial value that can be assigned to the resultant product (Sheehan and Greenfield, 1980).

Pretreatment of wine distillery wastewater with ozone improved its kinetic behaviour during anaerobic digestion but at the same time decreased COD removal efficiencies (Martin *et al*., 2002; Benitez *et al*., 1999a).Martin *et al*. (2002) investigated ozonation of vinasse in an

effort to reduce COD. Vinasse is known to be chemically very complex because of the high content of polyphenols that delay biological processes such as anaerobic digestion. As a result, ozonation is seen as a desirable chemical pretreatment prior to biological treatment because it is capable of converting the inhibitory and refractory compounds into simpler, low molecular weight compounds that are more readily degradable by microorganisms. Ozonation of aromatic compounds usually increases their biodegradability. Moreover, in many cases the chemical pretreatment used to make the waste biodegradable diminishes the COD of the wastewater, but intermediate compounds of higher microbial toxicity can be generated, depending on the type of ozonation used as pretreatment (Martin *et al*., 2002). In such cases an alternative chemical oxidant has been used, or treatment of wine distillery wastewater in a continuous reactor using a combination of ozonation and aerobic degradation in activated sludge systems was also investigated (Benitez *et al*., 2000).

In this combined system, oxidation by ozone achieved a reduction in the organic substrate concentration of 4.4 to 18 %, while a total phenol compounds content removal in the range of 50 to 60 % was reached. Aerobic degradation of these vinasses by activated sludge in experiments of varying hydraulic retention time (HRT) and substrate concentration provided organic substrate removals in the range of 12 to 60 % (Benitez *et al*., 2000). Ozonation of this aerobically pre-treated vinasse led to increases in COD removal efficiency from 16 to 21.5 %, as well as higher rate constants (Benitez *et al*., 2000). Schäfer *et al*. (2001) later applied membrane filtration with concomitant chemical treatment in the management of wastewaters containing natural organic problems. However, COD removal efficiencies were improved in aerobically pretreated and then ozonated wastewaters (Benitez *et al*., 1999a). Later, Benitez *et al*. (2000) pretreated wine distillery wastewater with activated sludge and then ozonated it, which improved substrate removal. The COD removal efficiencies were decreased in the presence of ozone, UV light or titanium dioxide, but methane yield increased (Martin *et al*., 2002).

2.6 Biological treatment

Tofflemire (1972) named pretreatment as the usual practice in nearly all major systems for treating wastewaters long before pre-ozonation of wine distillery wastewater began. On-site modifications such as water conservation are performed for the essential reduction of waste

and removal of solids. Relatively easy solid / liquid separation is desirable, because it reduces the volumetric load on the wastewater treatment system. Solid residues such as stems, pomace and lees can be removed from wastewaters by filtration, sedimentation, cycloning or screening. Solids have been disposed of by burying, spreading on fields or use as cattle feed. Neutralization by mixing wastewaters with each other or by base addition is still practised. Non-chemical pretreatment of wine distillery wastewater includes mechanical processes such as steam explosion (Wilkie *et al*., 2000), supercritical explosion by CO_2, ammonia freeze explosion, solvent delignification using alcohols and thermal mechanical processes to improve microbial access to the substrate (Zheng *et al*., 1998). All these methods can be used to improve subsequent biological treatment. Pretreatment was also recently investigated by Nataraj *et al*. (2006) working with wine distillery wastewater with a pH of around 3. It was pretreated by neutralization with sodium hydroxide (NaOH) and filtration was carried out to remove high concentrations of suspended solids, before using the wine distillery wastewater as secondary influent (Nataraj *et al*., 2006). For biological treatment of wine distillery wastewater, aerobic systems such as aerated lagoons or activated sludge plants are commonly used to remove the COD (Benitez *et al*., 1999a). However, aerobic processes have high operating costs and generate large quantities of waste sludge which must be disposed of (Benitez *et al*., 1999a).

Combinations of distillery wastewaters with municipal or other wastewater may allow for dilution of toxic components and facilitate treatment. Jackson *et al.* (2007) used a bioreactor system to treat mixed metal-contaminated river water and distillery wastewater with a two week HRT. The aluminium concentration decreased from 0.75 mg/l to 0.18 mg/l and nickel was completely removed (originally 0.19 mg/l, while the COD of the distillery wastewater was decreased from 2255 mg/l to a final value of <150 mg/l. In studies conducted by Benitez *et al*. (1999a) on WDW treatment by a combined processes consisting of aerobic degradation followed by anaerobic digestion was performed with the aim of evaluating the influence of the first stage, considered as a pretreatment, on the performance of the second stage. The pretreatment of the wine distillery wastewater by means of an aerobic process led to a significant increase in methane yield of the following anaerobic stage. Results of this research indicated that single aerobic treatment achieves an important reduction of the substrate (± 90 %) and significant removal of the total phenolic compounds (66 to 79 %). However, biological wastewater treatment processes such as activated sludge and aerated

ponds have been dogged by operational problems when treating high organic load wastewaters such as wine distillery wastewater (Vlissidis and Zoubalis, 1993). These aerobic treatment systems are used mainly to remove the BOD of these wastes. Partial reduction of BOD and COD is achieved in many distilleries using biological treatment (Coetzee *et al*., 2004; Wolmarans and de Villiers, 2002; Laubscher *et al*., 2001; Jawed and Tare, 1999).

Hybrid biological treatment systems include anaerobic treatment with recovery of biogas, followed by aerobic treatment for removal of residual BOD and COD. However, most of the biologically treated distillery wastewaters contain high COD due to the presence of recalcitrant compounds such as caramel, melanoids, a variety of sugar decomposition products, anthocyanins and tannins, and other xenobiotic compounds formed during yeast growth and processing of alcohols producing a characteristic dark brown colour (Benitez *et al*., 1999b). The biological treatment of industrial wastewaters usually depends upon the oxidative activities of microorganisms, and most bacteria are not able to degrade the recalcitrant xenobiotics mentioned above. Filamentous fungi can be important sources of phenolic-degrading organisms, as they frequently grow on wood utilizing lignin as a carbon source (Mendonca *et al*., 2004; Coulibaly *et al*., 2003; Benitez *et al*., 1999b). Fungi are not frequently used in wastewater treatment due to difficulties in their cultivation in liquid media and their slow growth rate in comparison with most microbial species (Mendonca *et al*., 2004; Coulibaly *et al*., 2003). However, organic compounds like phenol and its derivative have antibacterial effects that limit bacterial treatment, because they can be growth limiting even to species that have the metabolic ability to use phenolic compounds as substrates. Fungi have shown potential for the treatment of various specific pollutants and mixed wastewaters, including dark coloured, phenolic wastewaters such as molasses (Jimenez *et al*., 2003) and olive mill waste (Mendonca *et al*., 2004; Aggelis *et al*., 2003; Fenice *et al*., 2003; Ruiz *et al*., 2002; Perez *et al*., 1998), therefore, fungal treatment of these wastewaters could be used as a pre-treatment step for biological treatment. According to Coulibaly *et al*. (2003) fungi can be used to treat wastewaters in a liquid environment where bioreactors with wastewater can be exposed to the specific live fungus capable of degrading a single pollutant, or preferably a mixture of pollutants. Another approach would be to use an enzyme derived from fungi, to treat the wastewater (Coulibaly *et al*., 2003).

There has been considerable global scientific effort to investigate the use of fungi in bioremediation, especially the lignin degrading white rot fungi for degradation of wastes with phenolic content (Fernando *et al*., 1990). *Phanerochaete chrysosporium*, a white-rot fungus producing peroxidases is exceptionally versatile in degrading wastewaters with phenolic compounds (Coulibaly *et al*., 2003; Bumpus *et al*., 1987). Fungal pretreatment of wastewaters that exert some antibacterial activity under aerobic conditions has obtained complete phenol and colour removal and BOD reductions up to 85.4 % (Coulibaly *et al*., 2003). Aerobic pretreatment of molasses with *Penicillium decumbens* enhanced the rate of subsequent anaerobic degradation and kinetic coefficients doubled (Jimenez and Borja, 1997). Successful biodegradation of natural phenolic compounds such as phenol, catechol and resorcinol, prepared at concentration of up to 1 g/l was achieved in the presence of a filamentous fungus called *Fusarium flocciferum* (Mendonca *et al*., 2004). However, the search for sustainable treatment systems capable of minimizing energy consumption has encouraged the use of anaerobic bacterial systems, even in cases where the main goal is to eliminate the biodegradable and dissolved fraction of carbonaceous substrates (Rajeshwari *et al.,* 2000). These anaerobic treatment systems have been used mainly for high strength organic wastewaters such as beer brewing wastewaters (Benitez *et al*., 1999b; Sales *et al.,* 1987). Although anaerobic digestion of this wine distillery wastewater is feasible and appealing from an energy point of view, the presence of polyphenols slows down the digestion process and thus hinders COD removal. An improvement in digestion efficiency can be brought about by modifying the digester design, incorporating appropriate advanced operating techniques (Rajeshwari *et al.,* 2000) or using more robust microorganisms. Table 2.2 shows a summary of different digester configurations used for anaerobic digestion of distillery wastewaters. Anaerobic digestion offers significant advantages over aerobic systems, including lower energy consumption, reduced solids formation, lower nutrient requirements and potential energy recovery from the methane produced (Garcia-Calderon *et al*., 1998; Steward *et al*., 1995; Hall, 1992). This process is now widely used in many environmental applications, in different reactor configurations and modes of operation (Coetzee *et al*., 2004; Wolmarans and de Villiers, 2002; Goodwin *et al*., 2001; Borja *et al*., 1993). Genovesi *et al*. (2000) claimed that in the last few decades biological treatment processes have been proven effective and economical in dealing with highly polluted wastewaters, anaerobic digestion in particular.

Several technologies are applied for winery wastewater treatment including: free cells or flocs (anaerobic contact digesters, anaerobic sequencing batch reactors and anaerobic lagoons), anaerobic granules (Upflow Anaerobic Sludge Blanket), or biofilms on fixed support (anaerobic filter) or on mobile support as with the fluidised bed (Moletta, 2005). Anaerobic digestion is able to operate under severe conditions i.e. high strength influents and short hydraulic retention times, provided the sludge retention time is high. It is a process often used as a treatment for stabilization of primary and secondary sludges. Anaerobic digestion of high strength wastewater is a proven technology that has been widely applied (Wolmarans and de Villiers, 2002; Rajeshwari *et al*., 2000). The COD removal using anaerobic digestion for winery and distilleries wastewaters (vinasses) is very high, up to 95% removal, with organic loads between 5 and 15 kg COD/m^3 of digester/day. The biogas production is between 400 and 600 l per kg COD removed and has between 60 to 70% methane content (Moletta, 2005). However, a major concern is that digestion systems often do not perform well, since long start up periods in the order of one to two months have been reported (Austermann-Haunn *et al*., 1994), which is a major barrier to the use of such systems for seasonal wine distillery wastewater streams.

García-Bernet *et al*. (1998) studied a down-flow anaerobic fluidized bed treating red wine distillery wastewater over 1.5 years at a laboratory scale. The system attained carbon removal efficiency rates between 75 and 95 %, at an organic loading rate (OLR) of 17 kg TOC/m^3/d with an HRT of 0.35 days. It did however require a two-month start-up period with stepwise increases in OLR by reducing the HRT. Hickey *et al*. (1991) suggested that when commissioning a reactor for the first time on a particular wastewater it is advantageous to utilize sludge from a reactor treating a similar waste as the commissioning inoculum. If this is not possible, the sludge will have to be acclimatized to the specific influent, a process that can take several weeks or months. Several processes have thus been developed to operate anaerobic digestion reactors, each of them having several advantages. One of the most common is the upflow anaerobic sludge blanket (UASB), a process that has successfully been used to treat a variety of wastewaters but is often limited by poor biodegradability of complex organic substrates (Coetzee *et al*., 2004; Wolmarans and de Villiers, 2002; Goodwin *et al*., 2001; Seghezzo *et al*., 1998; Goodwin and Stuart, 1994). Keyser *et al*. (2003) improved the performance of an UASB during the treatment of winery wastewater by adding granular sludge enriched with *Enterobacter sakazaki* to the reactor.

The enriched bioreactor led to better wastewater treatment performance, as the reactor start up time was reduced and COD removal of > 90 % was achieved.

2.7 Membrane bioreactors in the treatment of distillery wastewaters

A membrane bioreactor (MBR) can be defined as a process that integrates biological degradation of wastewater when coupled with membrane filtration (Cicek *et al.,* 2001). The combination of membranes with biological treatment of wastewaters was first reported by Smith *et al*. (1969). In that study an UF membrane was used for the separation of activated sludge from the final effluent with recycling of biomass to the aeration tank (Smith *et al*., 1969). This led to the development of three generic membrane processes. The first is the solid - liquid membrane separation process that employs ultra/micro filtration modules for the retention of biomass for recycle to the bioreactor. Secondly, gas-permeable membranes can be used to provide diffused oxygen mass transfer to the degradative bacteria present in the bioreactor. This same membrane can act as support for biofilm development with direct oxygen transfer through the membrane wall in one direction and nutrient diffusion from the bulk liquid phase into the biofilm in the other direction (Brindle and Stephenson, 1996). The third MBR is an extractive membrane that was designed for the transfer of degradable organic pollutants from industrial wastewaters, via a non-porous silicone membrane to a nutrient medium for subsequent degradation (Schoeberl *et al*., 2005).

These three MBRs are not mutually exclusive and if necessary can be coupled together into one bioreactor (Brindle and Stephenson, 1996). Additionally, micro- and ultra-filtration membranes allow for the separation of the activated sludge (biomass) from the treated wastewater, which offers the advantages of complete removal of solids and bacteria, as well as most of the viruses and allows a much higher biomass concentration (Cornel and Krause, 2006).The coupling of a membrane to a digester offers several advantages over conventional biological wastewater treatment systems that employ gravity for solids separation from the effluent (Visvanathan *et al*., 2000). These advantages include: better biomass retention, higher organic loading rates, high quality effluent, compact plant configuration, complete solids removal, disinfection capability and nitrates removal. This makes MBRs attractive for water reclamation and meeting stringent effluent discharge

requirements (Schoeberl *et al*., 2005; Fan *et al*., 2000). Membrane bioreactor systems are therefore increasingly applied to industrial wastewater treatment (Schoeberl *et al.,* 2005; Enegess *et al*., 2003; Cicek *et al*., 2001). In solid-liquid separations, the membrane can be external to the bioreactor and be operated under pressure, called an external membrane bioreactor (EMBR), as illustrated in Figure 2.1A; or submerged into the bioreactor and operated under a vacuum, called a submerged membrane bioreactor (SMBR) as shown in Figure 2.1B (Trussell *et al*., 2006; Stephenson *et al*., 2000).

In EMBRs the mixed liquor is pumped from the aeration tank to the membrane at flow rates that are 20 to 30 times the product water flow, to provide adequate shear for controlling solids accumulation at the membrane surface (Trussell *et al*., 2006). Submerged membrane bioreactor systems have an advantage over EMBR, as the higher cost of pumping makes EMBR systems impractical for full scale wastewater treatment works which do not generate any financially valuable by-products (Gander *et al.,* 2000). Another advantage for MBRs in wastewater treatment is the long sludge retention time (SRT) that can be achieved (Huang *et al*., 2001). This leads to increased mixed liquor suspended solids (MLSS) concentrations, the ability to treat wastewaters with high organic loads, and the selective development of biomass with the ability to efficiently eliminate specific wastewater components. In SMBR systems the wastewater is driven through the membrane leaving solids behind, using a static head of mixed liquor or a low vacuum (Trussell *et al*., 2006; Gander *et al.,* 2000; Stephenson *et al*., 2000). The principal process limitation of SMBRs is membrane fouling, *i.e.* decrease in membrane permeability with time during system operation. Membrane fouling can be minimized by bubble aeration or by backflushing. submerged MBRs are often operated with 10 to 20 g/l of MLSS (Trussell *et al.,* 2000; Mourato *et al.,* 1999; Côte *et al*., 1998), thus minimizing or eliminating the need for sludge wasting and disposal. Trussell *et al.* (2000) maintained that regardless of operating conditions, SMBR effluents generated after treatment contain undetectable concentrations of TSS < 2 mg/l and a COD between 20 and 30 mg/l as a result of the filtration provided by the membrane. Since the 1980s MBR technology has been successfully applied to a range of industrial wastewaters including oily wastewater (Knoblock *et al*., 1994), food wastewater (Mallon *et al*., 1999) tannery wastewaters (Yamamoto and Win, 1991) and landfill leachates (Mirsha *et al*., 1996). In South Africa MBR technology has been applied for the treatment of maize wastewater (Ross *et al*., 1992) and brewery wastewater (Strohwald and Ross, 1992).

Table 2.2: Performance levels of anaerobic digestion of wine distillery wastewaters.

Reactor type	HRT	Organic loading rate	Temp. (ºC)	COD removal efficiency (%)	Waste type	Application	References
Anaerobic digester	3 d	-	35	-	Vinasse	Lab. scale	Martin *et al*., 2002
Anaerobic filter and UASB	1.3 d	3.0 -5.4 kg COD/m^3/d	37.5	90	Distillery wastewater	Lab. scale	Blonskaja *et al.,* 2003
Anaerobic granular sludge reactor	24 h	10.0 kg COD/m^3/d	15.0 - 18.0	80 - 90	Phenolic wastewater	Lab. scale	Collins *et al.,* 2005
Anaerobic up-flow fixed bed	-	0.2 - 18.0 kg COD/m^3/d	36	-	Winery wastewater	Pilot scale	Genovesi *et al*., 2000
Down flow fluidized bed	1.3 d	1.8 - 4.5 kg TOC/m^3/d	35	>95	Wine distillery wastewater	Lab. scale	Garcia-Calderon *et al*., 1998
Flasks	1.7 - 4.0 d	3.79 g/l/d COD	55	78.9	Vinasse	Lab. scale	Solera *et al*., 2002
Stirred anaerobic digester	3.1 -15.4 d	0.55 - 0.75 gCOD/gVSS/d	-	-	Molasses wastewater	Lab. scale	Jimenez and Borja, 1997
UASB	2.1d	5.46 - 20.0 kg COD/m^3/d	"Mesophilic" (actual range not given)	70 - 90	Distillery pot ale	Lab. scale	Goodwin *et al.,* 2001
UASB	-	0.46 - 0.75 kg COD/kgVS	35	90	Distillery pot ale	Lab. scale	Goodwin and Stuart, 1994
UASB	-	2.0 -18.0 kg COD/m^3/d	34 - 36	90	Distillery wastewater	Full scale	Wolmarans and de Villiers, 2002
UASB	48 h	6.1 - 18.0 kg COD/m^3/d	35	>90	Grain distillation wastewater	Lab. scale	Laubscher *et al*., 2001
UASB	-	19.0 - 24.0 kg COD/m^3/d	60 - 65	>95	Recalcitrant distillery wastewater	Lab. scale	Harada *et al*., 1996
UASB	2.2 d	5.1 - 10.12 kg COD/m^3/d	35	90	Winery wastewater	Lab. scale	Keyser *et al.,* 2003

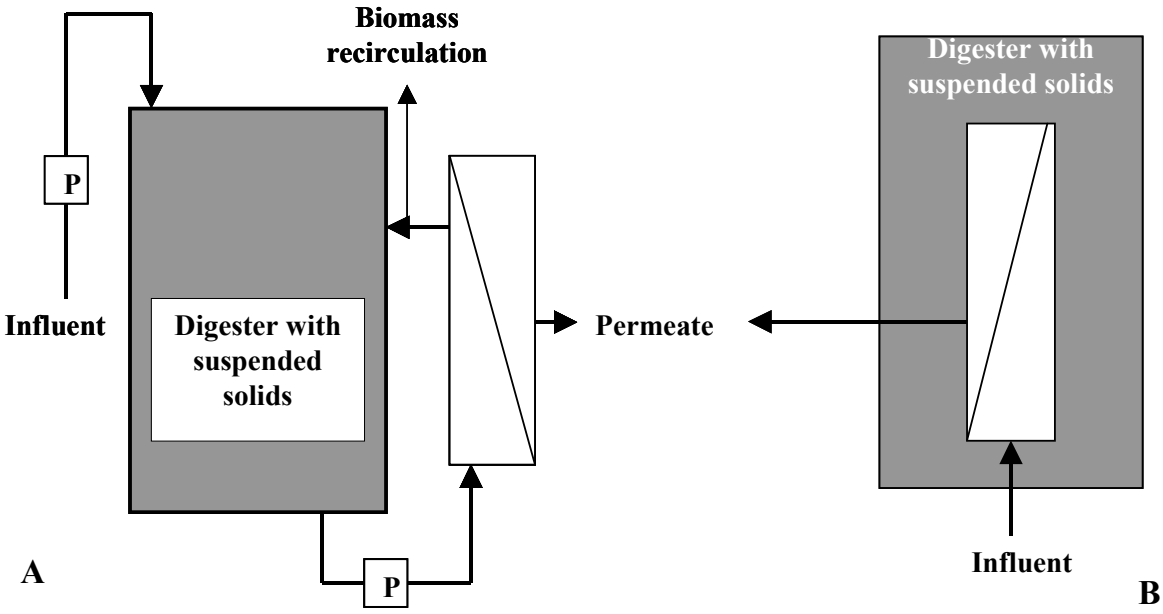


Figure 2.1: Configurations of a solid-liquid MBR: **A.** External MBR **B.** Submerged MBR **P**. Pump. (Stephenson *et al*., 2000).

A study of the molecular weight distribution of compounds in the supernatant inside an SMBR and in its permeate found that most of the permeate components had molecular weights of <30 000 Da. This portion constituted 60 to 70 % of the material, while 10 to 20 % originated from compounds with molecular weights of >100 000 Da. The relative proportion of the high molecular weight fraction in the permeate increased with operation time (Huang *et al*., 2001). Ultrafiltration and microfiltration (MF) membranes can prevent the loss of biological solids and high molecular weight solutes from the bioreactor. Complete mineralization of the organic matter is facilitated by maintaining a high biomass concentration and retention of high molecular weight compounds (Brindle and Stephenson, 1996).

As a result of membrane separation, SRT is independent of HRT, although both the SRT and HRT have enormous influence on process performance. Ren *et al*. (2005) investigated the impact of changing HRT on the removal of organic pollutants from domestic sewage by lab scale SMBRs. Results obtained demonstrated that when HRT was 3 h, COD removal efficiency was 89.3 to 97.2 %, when HRT was 2 h COD removal was 88.5 to 97.3 %, and at 1 h HRT, COD removal was 80 to 91.1 %. Results also showed that the optimum MLSS had to be maintained at 6000 mg/l. Membrane bioreactors are most attractive for situations where a long SRT is necessary to achieve the removal of slowly degradable pollutants. Due

to the high biomass concentrations that can be maintained in MBRs, minimum maintenance energy is required for biosynthesis and cell growth (Brindle and Stephenson, 1996). Maintenance of a low feed to microorganism (F/M) ratio in MBRs results in minimum sludge generation, reduced footprint and the development and retention of microorganisms that are wastewater specific.

At steady state, MBRs can remove organic pollutants over a wide range of concentrations, producing a high-quality permeate at high organic loading rates (Brindle and Stephenson, 1996). These loading rates range from 0.2 kg COD/m^3/d in aerobic MBRs, i.e. loading rates that are similar to conventional activated sludge, to 19.7 kg COD/m^3/ in anaerobic MBRs (Brindle and Stephenson, 1996). Removal efficiency of organic compounds is generally greater than 90 %, although COD removal efficiencies as low as 61 % have been reported. Aerobic MBRs have been investigated for the treatment of municipal and inorganic industrial wastewaters. Knoblock *et al*., (1994) demonstrated that aerobic MBRs operated at 54.2 h HRT and an organic loading rate of 6.3 kg COD/m^3/d were capable of treatment of high strength metal working wastewaters and achieved 94.4 % COD removal. In addition to oxygen demand reduction, significant removal of ammonia, fats, oils, greases and phosphorous have been confirmed. Brindle and Stephenson (1996) investigated the effect of organic loading rates on membrane fouling in an aerobic SMBR treating municipal wastewater. The study was carried out for 415 d at pilot scale. Steady state fouling rates were determined for 10, 5, 4, 3 and 2 d SRT that corresponded to F/M ratios of 0.34, 0.55, 0.73, 0.84 and 1.41 g COD/g VSS/d respectively. It was found that membrane fouling increased as F/M ratio was increased and that carbohydrate soluble microbial products (SMP) were responsible for increased fouling rates at high loading rates. Yamada *et al.* (2006) achieved >80 % COD removal in a pilot-scale multi-staged thermophilic (55 °C) UASB reactor with a working volume of 2.5 m^3 operated for a period of over 600 days using alcohol distillery wastewater. What was exceptional was the organic loading rate of 60 kg COD/m^3/day. From their studies it was concluded that the propionate degradation step was the most critical bottleneck regarding overall anaerobic degradation of organic matter under thermophilic conditions.

Synthetic wastewater was treated with a SMBR to investigate the organic removal performance and the behaviour of SMP during long term operation (Huang *et al.,* 2000). Chemical oxygen demand removal efficiency was 90 %, while removal efficiencies of TOC

and BOD were 94 % and 95 % respectively. Accumulation of TOC with a molecular weight > 100 000 Da was 34 %. Accumulation proved to be inhibitory towards metabolic activity of activated sludge, but it decreased from 34 to 16 %, while TOC of molecular weight < 30 000 Da increased from 33 to 52 % (Huang *et al.,* 2000). Trussell *et al.* (2006) demonstrated that slow-growing nitrifying bacteria were retained in a MBR at organic loading rates of 0.9 to 2.0 kg COD/m^3/d. The system could maintain 100 % nitrification and 90 % COD removal efficiency for 300 day SRT and 7.4 to 50.0 h HRT. Even at low HRTs such as 2 hours, organic removal and complete nitrification were achieved. A membrane biological reactor (Zenon ZW- 10) with a 220 L volume was used for 50 days to treat a synthetic wastewater similar to that generated in wineries. Chemical oxygen demand removal efficiency above 97 % was obtained and the COD concentration in the permeate varied between 60 to 80 mg/l. Biomass concentration, in terms of volatile suspended solids ranged between 0.5 and 15 g VSS/l and the apparent biomass yield was estimated at 0.14 g VSS/g COD (Artiga *et al*., 2005).

One of the earliest applications of MBRs in wine industry wastewater treatment was the use of an EMBR to treat Shochu distillery wastewater containing high strength organic compounds and ultra-high-strength suspended solids (Nagano *et al*., 1992). A pilot-scale EMBR was operated for 190 days with an UF membrane unit of 12 m^2, an operating pressure of 1.5 kg/cm^2, polysulphone membranes with molecular weight cut-off of 2 000 000 Da, an operation temperature of 37 ºC and a MCRT of infinity (no sludge removal). The MBR was capable of achieving 98 % COD and 99 % BOD removal efficiencies (Nagano *et al*., 1992). Suspended solids were decomposed at a high ratio of 85 % with little excess sludge discharged from the MBR. The conversion rate was 0.057 kg-VSS/kg-feed COD and the methane production rate was from 0.28 to 0.34 m^3/kg-feed COD (Nagano *et al*., 1992). Recently, Zhang *et al* (2006) monitored performance of a metallic SMBR treating simulated distillery wastewater at temperatures of 30 to 45 ºC. A stainless steel membrane of 0.2 μm pore size was used to treat this wastewater with a COD concentration of about 1 g/l. Results obtained showed that sludge settleability became poorer with increasing temperature. Mean COD and TN removal efficiencies at 10 to 30 h HRT and volumetric loading rate (VLR) of 0.6 to 2.8 kg COD/m^3/h were 94.7 % and 84.4 %, respectively (Zhang *et al*., 2006). Results concur with earlier work and support the idea

that MBRs could be much more widely used in the wine and associated distillery industrial sectors.

2.8 Concluding remarks

Although the wide variations in the composition of distillery wastewaters make them extremely difficult to bioremediate, some successful biological treatment of these wastewaters has been reported. This suggests that novel methods of treatment or improvement of established ones could be successful despite changes in wastewater volume and composition. In the evaluation and reporting of any treatment process, sufficient detail about the characteristics and concentration of species present in distillery wastewaters must be provided alongside the treatment performance in order to determine its applicability. However, this information is not readily available in public literature, as the chemical characteristics of distillery wastewaters are often not reported except for COD, pH, VFA, and occasionally BOD, TN and TP (Table 2.1). There is a lack of consistency in the characterization of distillery wastewaters as parameters like phenols / polyphenols, alkalinity, EC, VS, VSS, TS, TSS, NO_3^-, NH_4^+, PO_4^{3-} are commonly omitted. Harada *et al.* (1996) were the only researchers to include parameters such as SO_4^{2-}, K^+, Na^+, Fe^{3+}, Zn^{3+} and Ca^{2+} in their publications. This has been a trend despite the inhibitory characteristics of phenols, SO_4^{2-}, metal ions and heavy metals at low concentrations. Regulatory bodies such as the DWAF have established maximum allowable concentrations for these parameters that must be met prior to the reuse or disposal of effluents (DWAF, 1996). It is therefore recommended that local water quality studies are necessary in order to comply with standards of effluent disposal. Pretreatment of wine distillery wastewaters by either solids removal, neutralization with alkali or dilution of wastewater before treatment is often necessary. A number of unsuccessful digester trials suggest again that high organic loading rates adversely affected digester performance. At bioreactor configuration level, the existing information can thus be used to further improve performance. Phenols are the main toxic factor in wine distillery wastewaters, causing a pH increase of up to 2 – 3.5 units and thus must be closely monitored. At the bioreactor configuration level, the existing information can be used to further improve performance, although the role of inorganic ions in biological treatment processes has yet to be determined. Polyphenol, NO_3^-, NH_4^+ and PO_4^{3-} removal efficiencies also need to be profiled as indicators of performance in digesters.

Membrane bioreactors in the treatment of wine distillery wastewaters show potential but there is little recent research reported easily accessible. At the same time, amelioration of membrane fouling does not appear to pose a major problem, and with the increasing energy and wastewater disposal costs the most attractive treatment processes for wine distillery wastewaters are those with the lowest operational and maintenance, rather than the lowest capital costs.

2.9 Interim summary and conclusions

Chapter 2 summarized research efforts and case studies in the treatment of WDWs. Experiences in treating WDWs can contribute to the field of oenology as many oenologists are concerned with the selection, efficiency and economy of their wastewaters. Characteristics of wastewaters from different distilleries and various attempts methods for treating these wastes are discussed. Wine distillery wastewaters are strongly acidic, have high chemical oxygen demand, high polyphenol content and are highly variable. Primary attention was focussed on sustainable biological treatment of wine distillery wastewaters, mainly by energy-efficient anaerobic digestion in different reactor configurations from bench to pilot and full scale treatment. Finally areas where further research and attention are required were identified.

CHAPTER 3
Objectives of the Study

3.1 Characterization of RWDW and FTWDW.

In this study RWDW and FTWDW will be characterized for pH, COD, polyphenol concentration, colour, turbidity, total nitrogen, total phosphorus, $N\text{-}NH_4^+$, $N\text{-}NO_3^-$ and $P\text{-}PO_4^{3-}$. These parameters were chosen to be indicators of process performance, as that would facilitate control and further stability.

3.2 Treatment of RWDW and FTWDW in high rate anaerobic digester.

The objective of this study is to operate the high rate anaerobic digester for treatment of RWDW and then FTWDW. The digesters will be run for 130 dwith an infinite mean cell residence time (MCRT) at increasing feed strength from 5 % (v/v) until WDW shows inhibitory effects on the system. Parameters like COD_S removal efficiency and polyphenol removal efficiency will be monitored. Moreover, pH, VFAs, NO_3^-, NH_4^+ and PO_4^{3-} also will be profiled as indicators of performance during digestion. Enhanced performance of the digester will be investigated by addition of micronutrients *viz.* cobalt, iron and nickel at very low concentrations.

3.3 Treatment of RWDW and FTWDW in dual-stage anaerobic submerged membrane bioreactors.

A dual-stage submerged membrane bioreactor (SMBR) which uses ceramic membranes for biomass retention and cell recycle will be used. The first digester unit will be for the selection of microorganisms that are able to treat the toxic pollutants from WDW. Thus operated at high feed-to-biomass ratio and the opportunistic predatory microorganisms will be killed. The microorganisms selected in this digester will be concentrated by an

ultrafiltration membrane submerged into it and the sludge from this digester will be used to inoculate the second digester. The second digester (also calledthe hydrolysis tank) will be operated like a typical membrane bioreactor at a low feed-to-biomass ratio and will still be able to sustain a stable efficient population for a long period. Ceramic membranes of 0.2 micron pore size, used to construct this module will be chosen for their excellent chemical compatibility, durability, surface charge maintenance and ability to be operated dry (for air backflush). Increased organic loading and process parameters will be monitored asdescribed in Section 3.2. The MBR will be used for two types of WDW: a) the RWDW and b) the FTWDW.

3.4 Population shifts characterization using PFLA analysis and 16S rRNA

Samples will be obtained during operations of the high rate digester and the dual-stage SMBR for PLFA analysis and 16srRNA characterization. Rapid DNA characterisation of existing populations within high rate anaerobic digesters and SMBR will be performed. Analysis of PLFAs will give a measure of useful viable biomass content, microbial community population structure and the physiological status of the community. Changes in PLFA profiles would therefore be indicative of changes or stress responses as well as in situ metabolic status within microbial community. By combining 16s rRNA nucleic analysis with lipid biomarker identification and quantification, it is envisaged that stress responses experienced during bioreactors operation can be evaluated and correlated with process operational characteristics. Thus specific MBR operating conditions under which the metabolic status and stability of the membrane and tank associated populations subject to shifting may be identified.

CHAPTER 4

Performance of high rate anaerobic digester during treatment of raw wine distillery wastewater

4.1 Introduction

The search for sustainable treatment systems capable of minimizing energy consumption has encouraged the use of anaerobic biological systems, even in cases where the main goal is to eliminate the biodegradable and dissolved fraction of carbonaceous substrates (Rajeshwari *et al.,* 2000). These anaerobic treatment systems have been used mainly for high strength organic wastewaters such as distillery wastewaters (Sales *et al.,* 1987). Although anaerobic digestion of this type of wastewater is feasible and appealing from an energy point of view, the presence of polyphenols slows down the process and thus hinders complete removal of COD. An improvement in digestion efficiency can be reached by either modifying the digester design or incorporating appropriate advanced operating techniques (Rajeshwari *et al.,* 2000). Use of high rate digestion for the treatment of WDW at mesophilic temperature range should be advantageous, because of longer sludge retention times and the system's ability to withstand increased organic loading rates. Digesters are ideal for treating high strength organic waste like WDW. Anaerobic digestion is commonly used to kill pathogens and to reduce sludge volumes.

In this chapter a high rate anaerobic digester will be investigated for removal of polyphenols from a wine producing distillery, Olafbergh Distilleries Inc. (Worcester, South Africa). Raw wine distillery wastewater (RWDW) will be characterized and toxicity assays performed to determine RWDW's biodegradability; the performance of a high rate anaerobic digester will be monitored during treatment of RWDW for performance at stepwise increases feed concentration of 5 % (v/v) until WDW shows inhibitory effects on the system. Parameters like COD removal efficiency and polyphenol removal efficiency will be monitored. Moreover, NO_3^-, NH_4^+ and PO_4^{3-} will also be profiled as indicators of performance during digestion. The digester will be operated for treatment of RWDW for 130 d mean cell

residence time (MCRT) at increasing organic loading rates from 5 % (v/v). Enhanced performance of the digester will be investigated by addition of micronutrients *viz.* cobalt, iron and nickel at very low concentrations.

4.2 Materials and Methods

4.2.1 Set-up of a high rate anaerobic digester

A 10 l high rate anaerobic digester was set up in a controlled temperature environment of 30 ºC with mixing to suspend the sludge solids (Figure 4.1).

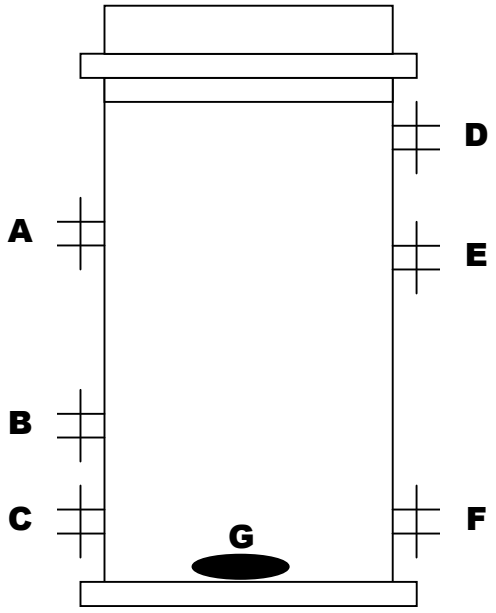


Figure 4.1: The high rate anaerobic digester made of PVC. A peristaltic pump (Watson Marlow 501RL2) was used to pump in the feed at C, with rubber tubing of 3.2 cm diameter. The digester had 15 l capacity and a height of 36.09 cm. A = supernatant outlet, B = sampling outlet, C = feed inlet, D = inlet for nitrogen gas, E = unused port, F = sludge inlet and G = stirrer bar to provide mixing.

The digester was inoculated with 2.5 l of methanogenic sludge obtained from an established laboratory digester originally inoculated with standard rate digester sludge from Grahamstown Municipal Wastewater Treatment Works, 0.5 l of WDW and 7.0 l nutrient broth, a general purpose medium used for the cultivation of a wide range of bacteria which are not fastidious in their food requirements (containing 1 g/l meat extract, 2 g/l yeast extract, 5g/l peptone and 8 g/l AnaLar grade sodium chloride, Merck Chemicals (Pty) Ltd, Johannesburg). The digester was operated at a 48 h hydraulic retention time (HRT) and 130

d mean cell residence time (MCRT). The digester feed was autoclaved and a mixture of nutrient broth and RWDW, and the concentration of RWDW was increased incrementally (see Table 4.1). Calcium carbonate ($CaCO_3$) at 2000 mg/l was added to buffer the pH value of the system from day 62 to day 73. From day 73 until day 84, this was replaced with a mixture of $CaCO_3$ and K_2HPO_4 (1 g/l each, UniLAB grade, Merck). Finally, only K_2HPO_4 (1 g/l) was used to buffer the system from day 84 until day 130. Micronutrient amendments were supplied as 50 mg/l of $Fe(NO_3)_3$ (UniLAB, Merck) added from day 86 to day 92; $Co(NO_3)_3$ (UniLAB, Merck) from day 94 to day 100, and $Ni(NO_3)_3$ (UniLAB, Merck) from day 102 to day 106.

Table 4.1: Incremental changes in digester feed strength.

Time (days)	Feed concentration of WDW (v/v)
0 to 52	5 %
108 to 112	10 %
114 to 120	15 %
122 to 124	20 %
126 to 130	30 %

4.2.2 Performance of a high rate anaerobic digester during treatment of RWDW

Digester performance was monitored by determination of feed and supernatant parameters. The digester agitation was switched off every 48 h and the solids allowed to settle for one hour (Figure 4.2). The supernatant was then removed from the digester and replaced with equal volume of fresh feed. Samples of the supernatant withdrawn were retained at 4 °C for a maximum of 48 hours before analysis. The feed and supernatant parameters analysed were pH, soluble COD (CODs) and the concentrations of phosphates, nitrates, ammonia, polyphenols and volatile fatty acids (VFAs). The pH values were measured using a Cyberscan 2500 pH meter (Eutech Instruments, Johannesburg, South Africa). Colorimetric reagent test kits (Merck Chemicals (Pty) Ltd, Johannesburg), based on the principles of (APHA *et al*., 1998), were used to measure COD_s (Spectroquant reagent test 14538/9 analogous to method number 5220-D), phosphates (14543 analogous to 4500-P-E), nitrates (14773, analogous to 4500-NO_3-E) and ammonia (14752, analogous to 4500-NH_3-F). Concentrations of total VFAs were determined according to a standard titration method (SCA, 1979). Colour and turbidity were measured using a Spectroquant Nova 60 spectrophotometer (Merck Chemicals Pty Ltd, Johannesburg).

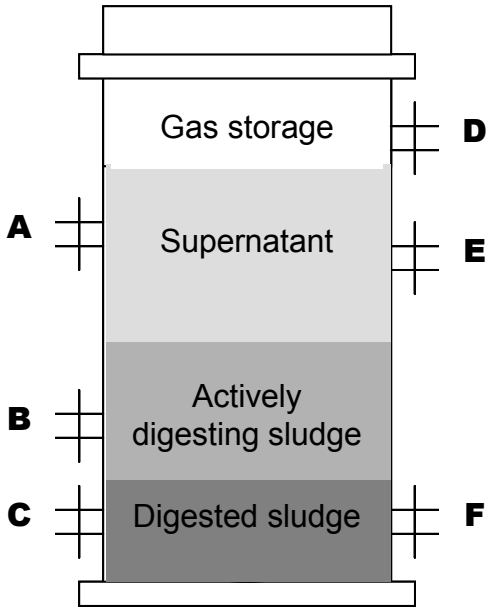


Figure 4.2: Anaerobic digester after settling.

Polyphenols were measured using the Folin-Ciocalteu's spectrophometric method by Box (1983). Polyphenols analysis was conducted by taking a 100 µl sample and mixing it with 1.6 ml of distilled water and 250 µl of Folin-Ciocalteu's reagent. The mixture was vortexed briefly and 1.5 ml (100 g/l) of an aqueous solution of Na_2CO_3 (Merck Chemicals (Pty) Ltd, Johannesburg) was added. Samples were made up to a volume of 10 ml with distilled water, vortexed briefly and incubated in the dark at room temperature for 2 hours. After incubation, absorbance at 765 nm was measured using multi-wavelength multi wellplate reader (PowerWavex, Bio-Tek Instrument Inc). Calibration curves were measured using phenol (AnaLAR, Merck) as a standard, and polyphenol concentrations were expressed as mg of phenol per litre (mg/l). Mixed liquor suspended solids (MLSS) in the digester were determined by withdrawal of a sample during digester mixing and analysed (APHA *et al.*, 1998).

4.3 Results and Discussion

Experimental data for raw and treated WDW are summarised in Table 4.2. Before anaerobic digestion, the RWDW-containing feed has an average pH of 3.83, 4185 mg/l COD_s and 674.6 mg/l of phenols. After digestion the pH increased to 7.05. The supernatant also showed improvement in colour, A_{500} decreased from 1.29 to 0.59 and turbidity from 0.74 to

0.33 FAU. Decreases in concentrations of phenols, and COD_s were indicative of the efficiency of the high rate anaerobic digester during treatment of WDW.

Table 4.2: Characterisation of digester influent and effluent.

Parameter	No of samples	Influent	Standard Deviation	Effluent	Standard Deviation
pH	3	3.83	±0.01	7.05	±0.01
Colour (A_{500})	3	1.29	±0.02	0.59	±0.03
Turbidity (FAU)	3	0.74	±0.03	0.33	±0.03
Phenol (mg/l)	3	674.6	±23.3	9.25	±0.6
COD_s (mg/l)	3	4185.0	±27.8	55.0	±8.7

The results in Table 4.2 are indicative of the robustness of the digester during operation. The observed pH and VFA profiles are shown in Figure 4.3. The pH of the digester supernatant increased from the initial value of 7.3 to 9.2 between day 0 and day 8. From day 8 until day 16, a gradual decrease in pH was recorded, with the minimum value of 6.0 recorded on day 16. Subsequently, pH increased again to reach 9.2 on day 20, and then a gradual decrease was recorded to the value of 6.2 on day 42. A sharp spike in pH to 12.3 recorded on day 52 suggested that there was a complete breakdown of the anaerobic system. The digester had to be recovered and pH buffering had to be introduced, which accounts for the relatively constant value of 7.05 recorded between days 54 and 130. The digester was revived by first removing half of its contents and replacing with fresh methanogenic sludge, fed at 5 % (v/v) of RWDW strength in nutrient broth and allowing recovery until day 62. Buffering was accomplished using addition of $CaCO_3$ or the $CaCO_3$ / K_2HPO_4 mixture (see Section 4.2.2).

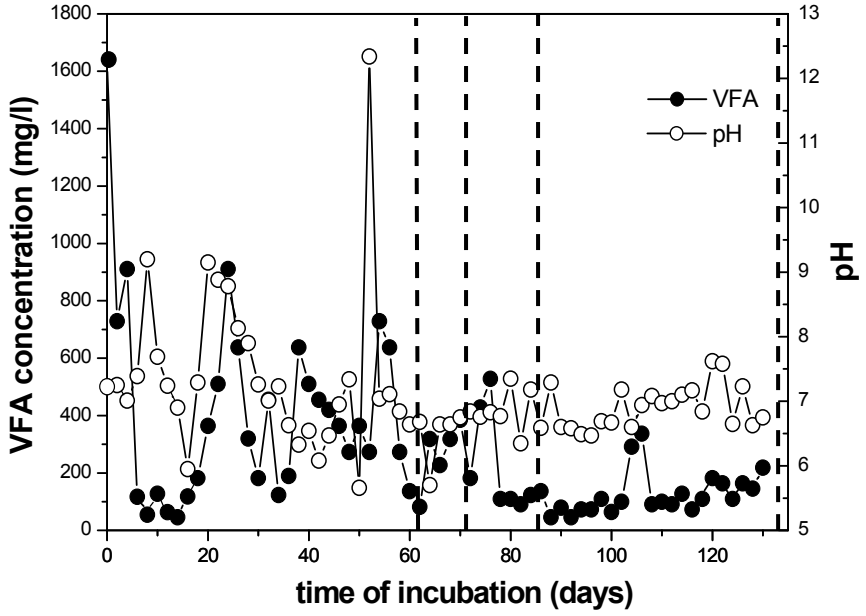


Figure 4.3: Volatile fatty acids concentration and pH in the digester during the trial. $CaCO_3$ (2000 mg/l) was added to buffer the pH value of the system from day 62 to day 73. From days 73 to 84, this was replaced with a mixture of $CaCO_3$ and K_2HPO_4 (1 g/l). K_2HPO_4 (1 g/l) was used to buffer the system from day 84 until day 130 as indicated by the vertical dotted lines.

The VFA concentration decreased from 1639.3 mg/l on day 0 to 45.5 mg/l on day 14, indicating breakdown of readily available COD_s in the digester. From day 16 until day 52, the VFA concentration increased to 900 mg/l. Peak values of 475 mg/l and 640 mg/l were recorded on day 32 and day 38, respectively. We hypothesise that increase of VFA concentration could be thereafter attributed to digestion of complex polyphenols thus releasing organic acids. Recommendations on testing this hypothesis are in Section 10.2 After recovery of the system and implementation of pH buffering, VFA concentration reached a maximum of 550 mg/l on day 76 and 340 mg/l on day 106. The residual concentration of VFA was 200 mg/l on day 130. Recovery of pH from acidic range in combination with the decrease in VFA concentration from day 16 to day 50 suggested successful treatment of RWDW. After digester recovery (between day 54 and 62), $CaCO_3$ was added as a buffer, followed by a combination of $CaCO_3$ and K_2HPO_4, and K_2HPO_4 alone. Buffer capacity in the digester was improved when K_2HPO_4 was added alone, compared to the use of a combination of $CaCO_3$ and K_2HPO_4 or $CaCO_3$ alone. VFA concentrations fluctuated less after the induction of pH buffering (Figure 4.2). Addition of

K_2HPO_4 alone also led to pH stability and robustness of the digester as VFAs were digested even at increased organic loading rates.

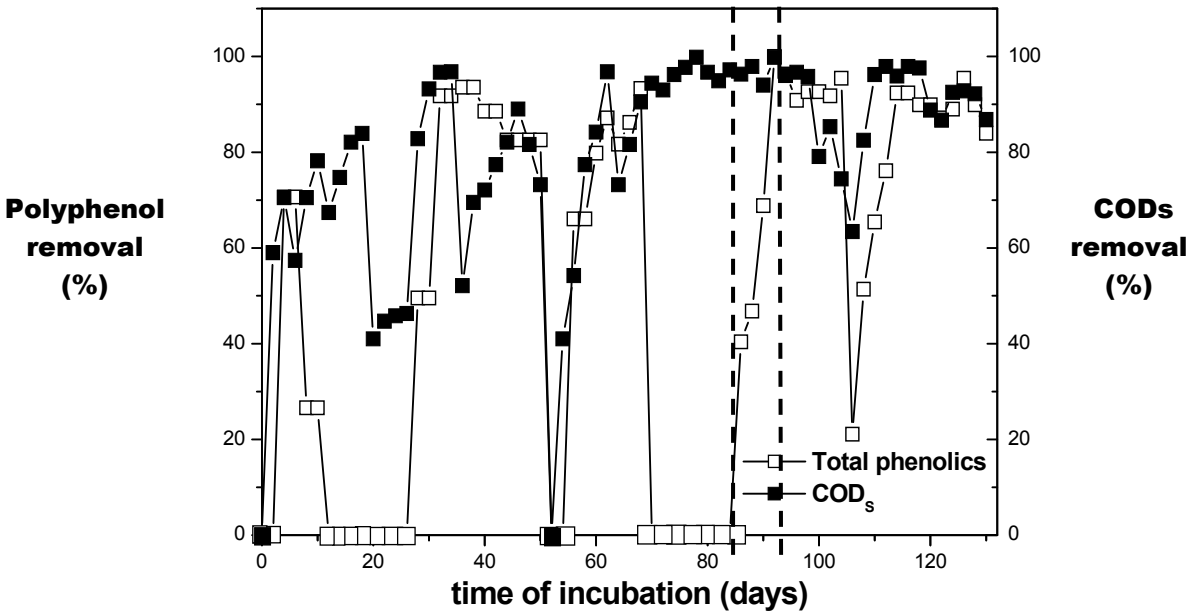


Figure 4.4: Removal efficiency of COD_S and polyphenols in the anaerobic digester as a function of the time of incubation, with vertical dotted lines marking addition of 50 mg/l Fe^{3+} between days 86 and 92.

The pH stability achieved (as in Figure 4.3) after addition of macro and micro nutrients led to better removal efficiencies of VFAs, CODs and polyphenols (Figure 4.4). However, no similar work was available such that the data can be compared to literature (Chapter 2 of this thesis covers the available literature in this field). Removal efficiencies of CODs and polyphenols as a function of the time of incubation are shown in Figure 4.4. For CODs the values fluctuated between day 0 and day 20, with the average value equal to 66 % and the minimum equal to 40 % recorded on day 20. Fluctuations continued until day 52, with two distinct minima of 52 % at day 38 and 40 % at day 52. The introduction of the K_2HPO_4 buffer helped to stabilise the removal efficiency for CODs and polyphenols, even at increased organic loading rates. Overall CODs removal efficiency for the 130 day study was 87 % and polyphenol removal efficiency for the 130 day study was 63 %. Addition of 50 mg/l Fe^{3+} as $Fe(NO_3)_3$ between days 86 and 92 increased the removal efficiencies of CODs to 99 %, on day 92 and of polyphenols to 90 % on the same day. Addition of 50 mg/lCo^{3+} as $Co(NO_3)_3$ decreased CODs removal efficiency from 97 % to 92 % between days 94 and 100; while polyphenol removal efficiency increased from 65 % to 93 %. Addition of 50 mg/l Ni^{3+} as $Ni(NO_3)_3$ decreased CODs and polyphenol removal efficiencies to 74 % and

70 % respectively. Similar results have been reported before (Sharma and Singh, 2001), and might indicate the possible physiological significance of ferric ions to sludge under the conditions prevailing in the digester. The actual mechanism of ferric salt improving overall efficiency is not fully understood although addition of all these micronutrients has been shown to improve overall efficiency of anaerobic digestion in previous studies (Sharma *et al*., 2001). It is suspected that ferric salts induced coagulation of colloidal materials, thus further studies like investigating molecular weight distribution on the bioreactor during addition of micronutrients is a recommendation for future work. Average CODs removal efficiency for the study period from day 108 to day 130 improved from 74 % to 92 % and from 70 % to 84 % for polyphenols, despite increased organic loading rates (see Section 4.2.2). The improvement of CODs and polyphenol removal efficiencies further confirmed the robustness of the digester. These results further confirmed methanogenic activity during WDW treatment. Removal efficiencies of CODs obtained in this study were higher than those obtained by Wolmarans and de Villiers (2002) who used an upflow activated sludge bed or blanket (UASB) reactor to treat distillery wastewater. Addition of macro and micronutrients caused pH stability and thus stimulated microbial activity. In the digester organic loading rates had to be gradually increased from 5 % WDW feed strength to 30 % strength as shock loading rates employed at the beginning led to dramatic decrease in pH and thus decrease in methanogenic activity.

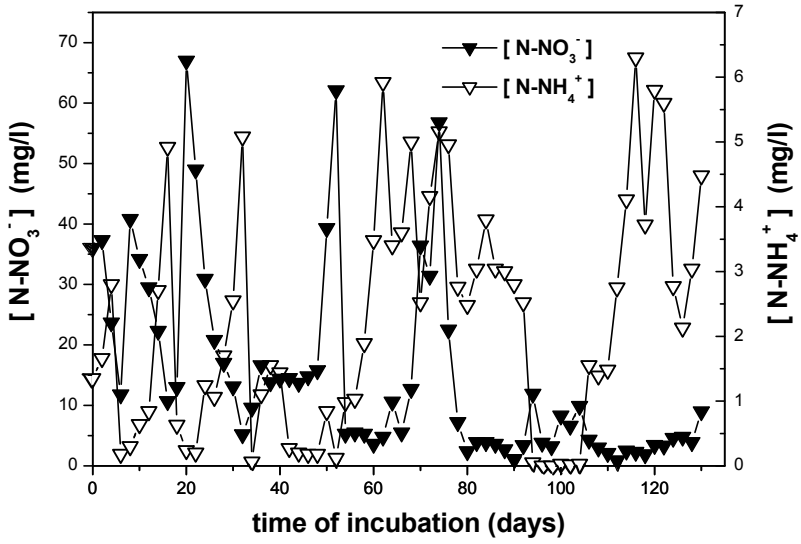


Figure 4.5: Nitrate and ammonium concentrations measured in the supernatant during WDW anaerobic digestion.

Nitrate ($N\text{-}NO_3^-$) and ammonium ($N\text{-}NH_4^+$) concentrations measured in the supernatant during WDW anaerobic digestion are shown in Figure 4.5. Nitrate concentration reached peak values of 37.3 mg/l on day 2, 40.8 mg/l on day 8, 67.0 mg/l on day 20, 62.1 mg/l on day 52, and 56.8 mg/l on day 74. When the organic loading rate was increased the volumetric fraction of RWDW reached 30 % of RWDW in the feed, nitrate concentration dropped below 10 mg/l. Data in Figure 4.5 indicates that when nitrate concentration peaked ammonium concentration was at its lowest and *vice versa* i.e. when nitrates were being used up, ammonium was being formed or released into the medium. This trend is specifically evident between days 40 to 50, days 80 to 90 and days 108 to 130, when ammonium concentrations were particularly low. Organic loading rates of RWDW were kept at 30 % (v/v) as any further increase had negative effects on all removal efficiencies. Mutual relationship of the nitrate and ammonium concentrations in an anaerobic digester originates from the possible occurrence of the following processes: nitrification/denitrification, dissimilatory nitrate reduction (anaerobic respiration with nitrate as the terminal electron acceptor), the reduction of $N\text{-}NO_3^-$ to NH_4^+ coupled with uptake into activated sludge cells and its utilization in protein synthesis and other physiological processes, followed by the release of NH_4^+ after cell lysis (Ruiz *et al*., 2006).

Although a detailed study of the significance of individual processes was not conducted for this experimental system, the observed temporal trends in nitrate and ammonium concentrations allowed the following to be postulated: nitrates were reduced into ammonium cations by denitrifying bacteria, or bacteria utilising nitrate as the terminal acceptor of electrons during anaerobic respiration. Ammonium cations produced were successively assimilated by the other microbial cells present in the activated sludge, and used to meet the needs of protein synthesis and other physiological processes. As the particular cells died off, the proteins were degraded, and the ammonium was released into the system, where it could be transformed into nitrate, or the nitrates were supplied by the replenished feed after supernatant removal for analyses. A mass balance of nitrogen over the experimental system was not conducted, so other processes mentioned could be occurring simultaneously, leading to the loss of nitrogen out of the experimental system via the evolution of N_2 or NO_x gases.

As mentioned before, micronutrients were added to the systems (Section 4.2); the addition of $Fe(NO_3)_3$ between days 86 and 92 did not lead to any significant changes in the

concentration of ammonium or nitrates, which ranged from 2.5 to 3.0 mg/l for ammonium and 1.1 to 3.4 mg/l for nitrates. On the other hand, additions of $Co(NO_3)_3$ and $Ni(NO_3)_3$ led to a significant decrease in ammonium concentrations to values below the limits of detection of the analytical method used, and an increase in the concentration of nitrates, with peak values of 11.4 mg/l on day 94 and 9.9 mg/l on day 104. This might point to the possible toxicity of Ni^{3+} to the microorganisms responsible for the denitrification or dissimilatory nitrate reduction to ammonium. A lot has been speculated and contradictory data have been published in the potential and proven toxicity of the ammonia / ammonium acido-basic equilibrium towards anaerobic wastewater treatment system (Calli *et al.,* 2005). After introduction of pH buffering and with the addition of iron as a micronutrient, the observed ammonium concentrations were not toxic to the sludge present in the experimental digester. This might be an indication of the suitability of the studied experimental system for the reduction of nitrogen loading of the studied WDW. Figure 4.6 shows the supernatant phosphate ($P\text{-}PO_4^{3-}$) and the MLSS concentrations measured during anaerobic treatment of WDW. From day 0 to 26 the concentration fluctuated between 113 and 96 mg/l. There was a major decrease in phosphate concentration from day 28 to day 50 which led to the digester failure on day 52, when the pH increased rapidly to 12.34, solubilising the contents of the digester.

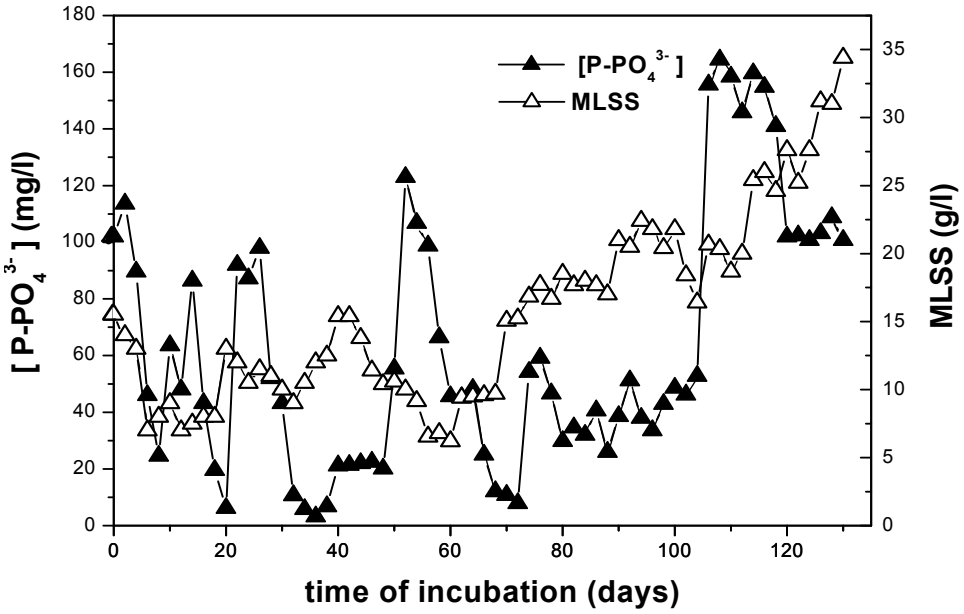


Figure 4.6: Phosphate and total suspended solids concentrations measured in the supernatant during WDW anaerobic digestion.

When phosphate concentration was measured during this period, a drastic decrease in phosphate concentration was observed up to day 72 even when $CaCO_3$ (2000 mg/l) was added as a buffer. Addition of a combination of K_2HPO_4 and $CaCO_3$ (1000 mg/l each) from day 74 to day 84 stabilised phosphate concentration at approximately 60 mg/l. As from day 86 to day 130 only K_2HPO_4 (1 g/l) was used to buffer resulting in increased phosphate concentration even at increased organic loading rates. The digester MLSS concentration fluctuated during the course of the anaerobic digestion with peak values of 9 g/l on day 10, of 13 g/l on day 20, of 15.4 g/l from day 40 until day 42, and continued to increase after introduction of pH buffering, MLSS continued to increase to the final value of 13.44 g/l. This seemed to be sufficient biomass to degrade polyphenols and CODs in the WDW.

4.4 Interim conclusions

Average CODs removal for the 130 day study was 87 ± 14 %. Average polyphenol removal efficiency for the 130 day study was 63 ± 21 %. Optimisation of anaerobic treatment for WDW was achieved at 30% wastewater strength. Additions of $CaCO_3$ (2000 mg/l), $CaCO_3$ and K_2HPO_4 (in combination with concentration of 1000 mg/l each), and K_2HPO_4, (1000 mg/l) were essential for buffering the bioreactor and addition of 50 mg/l Fe_3^+ increased CODs removal efficiency further to 95 %. It was not possible to increase the feed strength to more than 30 % due to the lack of an adequate concentration of biomass to break down the polyphenolic components. Therefore, an ultrafiltration membrane was included, in order to improve biomass retention and enable the treatment system to deal with higher organic loading rates.

CHAPTER 5

Treatment of raw wine distillery wastewater by submerged membrane bioreactor and secondary digestion

5.1 Introduction

This chapter builds on the operating parameters investigated in Chapter 4, such as feed strength and micronutrient / buffer addition, and focuses on the use of a dual-stage submerged membrane bioreactor (SMBR) with ceramic membranes for improved biomass retention. The purpose of this MBR is to select and retain the microorganisms that are able to treat the toxic pollutants from WDW. It will be operated at a high feed-to-biomass ratio in order to avoid the proliferation of opportunistic predatory microorganisms. During operation of conventional MBRs there is a shift in the dynamics of population of microbes from those that utilize toxic pollutants in wastewaters to microorganisms that are resistant to toxic pollutants but do not degrade them. The new population develops within a short period of operation and predates on the desired population that is able to degrade toxic pollutants. This occurs even when these conventional MBRs have been in operation for a short period. In this study, a new approach to the operation of MBRs will involve use of a dual-stage SMBR. The first unit will be for the selection of microorganisms that are able to treat the toxic pollutants from WDW. The microorganisms selected in this digester will be concentrated by a submerged UF membrane and the sludge from this digester will be used to inoculate the second digester. The second digester (also called the hydrolysis tank) will be operated at a low feed-to-biomass ratio and is hoped to be able to sustain a stable efficient population. Ceramic membranes of 0.2 μm pore size, used to construct this module were chosen for their excellent chemical compatibility, durability, surface charge maintenance and ability to be operated dry (for air backflush). Such MBRs can be used for on-site treatment of high strength industrial wastewater before discharge or to aid in water reuse programmes in industry. Increase in organic loading rates of WDWs and parameters will be monitored as in Section 3.2. The MBR will be used for two types of WDW: a) the RWDW and b) the FTWDW. For both experiments the operation time will be 30 d.

5.2 Materials and Methods

5.2.1 Biological wastewater treatment system

The wastewater treatment system consisted of four individual process units (Figure 5.1). The feed (a mixture of nutrient broth and 30 %(v/v) RWDW, the maximum practicable concentration of RWDW having been determined in Chapter 4). Two drums of RWDW, obtained at the same time from Olafbergh Distilleries Inc. (Worcester, South Africa), were used in the feed, the first from day 0 to day 13 and the second from day 14 to day 30. Feed was poured into reactor A, which operated as a balancing tank and supply of influent for reactor B. The influent was pumped into reactor B using a peristaltic pump (Watson Marlow 505S, Watson Marlow, Falmouth, UK). Reactor B was a 10 l SMBR which was operated for 30 days without sludge wasting, with a hydraulic retention time (HRT) of 12 hours. Reactor B was inoculated with 10 % (v/v) of methanogenic sludge from an anaerobic digester at Grahamstown municipal wastewater treatment works. The submerged UF membrane module consisted of four tubular ceramic membranes (surface area 55 cm^2, pore size 0.2 μm; Synexa Life Sciences, South Africa). During filtration, the permeate was withdrawn into reactor C (permeate balancing tank) using pump F (Watson Marlow 505S) and then fed to reactor D (the secondary digester) every 48 hours using pump G (Watson Marlow 505S) with outlet I open to drain the overflowing supernatant. Reactor D was a 10 l low rate digester with a 48 hour HRT and 8 day SRT, which was inoculated with sludge from reactor B on day 22 of the study using pump H (Watson Marlow 505S). Backflushing was employed for amelioration of fouling when it occurs.

During the start-up period, reactor A was filled with deionised water and this was continuously pumped into reactor B for 2 days to deplete all internal carbon sources of the methanogenic sludge in the reactor. The study was initiated by feeding reactor B after 2 days with RWDW as described above. The feed pH was buffered with 1000 mg/l $CaCO_3$ and 1000 mg/l K_2HPO_4 (both AnaLar grade, Merck Chemicals (Pty) Ltd, Johannesburg) for the first 10 days. On day 12, these concentrations were increased to 8000 mg/l $CaCO_3$ and 4000 mg/l K_2HPO_4 (see Section 5.3) and 50 mg/l $Fe(NO_3)_3$ (AnaLar grade, Merck) was added as a micronutrient, based on previous work (Section 4.2). The experimental study lasted 30 days. Samples were collected every 48 hours from reactors A, B, C and D and analysed to determine values of selected operation parameters.

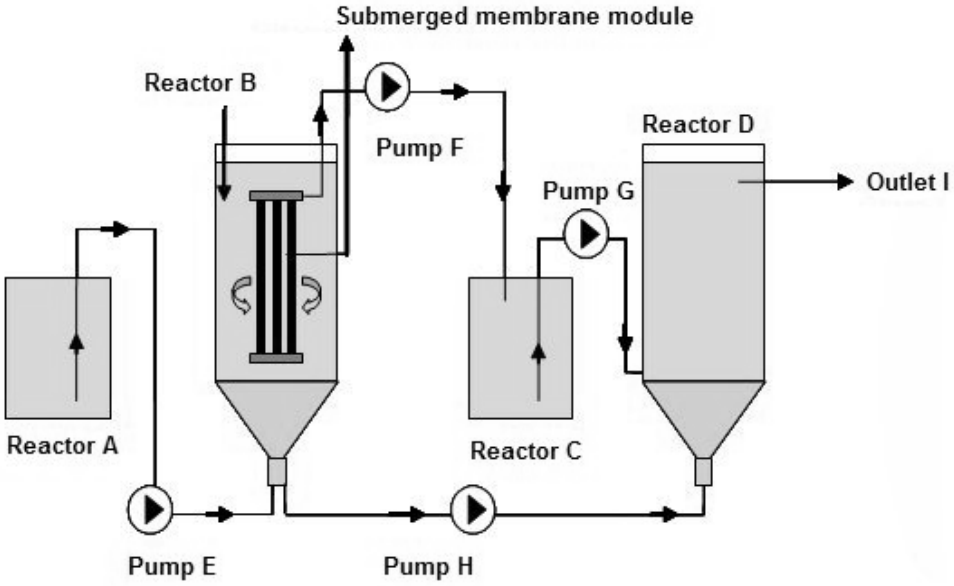


Figure 5.1: The biological wastewater treatment system used in the study, showing the flow paths between reactors A through D. A = influent tank, B = SMBR, C = permeate balancing tank, D = secondary digester.

5.2.2 Sample analysis

Several parameters in all four reactors were monitored, including pH and concentrations of COD_S, phosphate, nitrate, ammonia, total phenolics as phenol equivalents and total volatile fatty acids (VFAs). All analysis was performed off-line and after centrifugation of supernatant samples. Measurements of pH and electrical conductivity were made using a Cyberscan 2500 electrode (Eutech Instruments, Johannesburg, South Africa). Colorimetric reagent test kits (Merck) based on the principles of APHA *et al*. (1998) were used to measure concentrations of COD_S (Spectroquant reagent test 14538/9 analogous to APHA *et al*. (1998) number 5220-D), nitrates (14773, analogous to 4500-NO_3-E), phosphate (14543, analogous to 4500-P-E) and ammonium (14752, analogous to 4500-NH_3-F). Total VFAs were determined according to a standard titration method (SCA, 1979). The total concentrations of phenolics in individual samples were measured using a modified version of the Folin-Ciocalteau reaction (Khan 2005; Box, 1983). For individual samples, 100 µl aliquots were mixed with 1.6 ml of deionised water. The mixture was vortexed for 30 seconds and 250 µl of Folin-Ciocalteau reagent (Merck) added. After vortexing for 30 seconds, 1.5 ml of aqueous solution of Na_2CO_3 (100 g/l; Analar grade, Merck) was added to the mixture and vortexed again for 30 seconds. The mixture was then diluted to 10 ml with deionised water in volumetric glassware and incubated at 20 °C in the dark for

60 minutes. The total concentration of phenolics was then determined by measuring the absorbance of the sample at 765 nm, and converting it to phenol equivalents (mg/l). The calibration curve was measured under identical conditions as the samples, using phenol (Analar grade, Merck) as the standard. All absorbance measurements were performed on a multi-wavelength multi-well plate reader (PowerWaveX, Bio-Tek Instruments Inc., Winoski, VT). Removal efficiencies of CODs were calculated using equations (1) and (2):

$$COD_{\text{removal}}(SMBR;\%) = 100 \times \left(1 - \frac{COD_{\text{permeate}}}{COD_{\text{feed}}}\right) \quad (1)$$

$$COD_{\text{removal}}(total;\%) = 100 \times \left(1 - \frac{COD_{\text{effluent reactor D}}}{COD_{\text{feed}}}\right) \quad (2)$$

Where:

COD_{removal} = COD_S removal efficiency in the respective part of the system (%)

COD_{permeate} = COD_S in reactor C (mg/l)

COD_{feed} = COD_S in reactor A (mg/l)

$COD_{\text{effluent reactorD}}$ = COD_S of final effluent leaving reactor D (mg/l)

5.3 Results and Discussion

Degassing wastewater treatment systems contributes significantly to the commissioning costs of a full-scale system, so avoiding it and establishing anaerobic conditions during operation without prior degassing might lead to a decrease in operation costs of the system presented in this study (Zhuravlev and Matveev, 1985). Consequently, neither the feed nor the contents of any of the reactors were degassed prior to the test period. The pH values inside the reactors over 30 days of system operation are shown in Figure 5.2. During days 0 - 10, pH values in all four reactors ranged from 4.38 to 6.37, which is outside of the methanogenic range and indicated the need for better pH buffering. The pH in all four reactors increased after increasing the feed concentrations of $CaCO_3$ to 8000 mg/l, and K_2HPO_4 to 4000 mg/l, respectively on day 12, after which those concentrations were maintained for the remainder of the experiment.

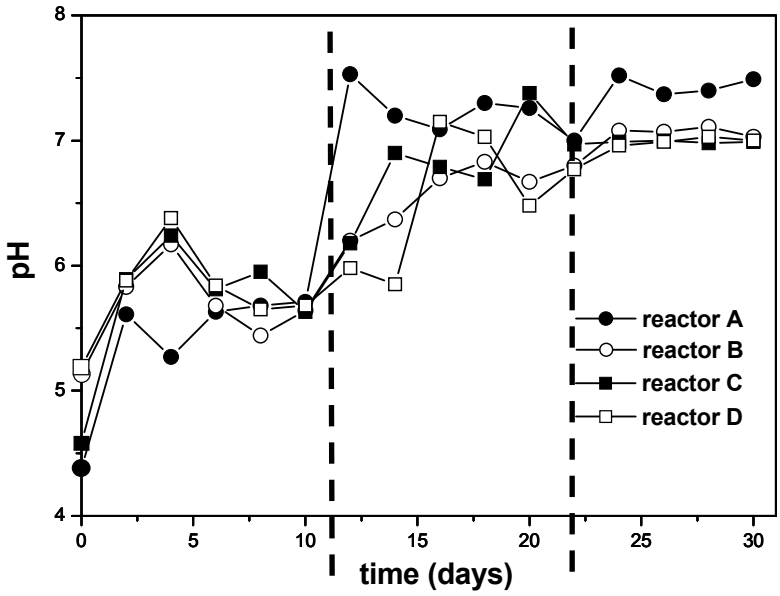


Figure 5.2: pH of the supernatant in the individual reactors of the bioreactor system as function of time of operation. A = influent tank, B = SMBR, C = permeate balancing tank, D = secondary digester. The first dotted line on day 12 indicates the time when extra $CaCO_3$ was added as buffer and the second dotted arrow on day 22 when Reactor B was inoculated using sludge from Reactor D.

Changes in pH were related to the concentrations of VFAs, shown in Figure 5.3. Changes in pH were related to the concentrations of VFAs as shown in Figure 5.3 and Figure 5.4. In Figure 5.3, pH levels fluctuated between 5.0 and 6.2 at the same time there was accumulation of volatile fatty acids. The pH levels in reactor B and D stabilized upon addition of $CaCO_3$ (as stated in Section 5.2.1). From Figure 5.3 it is observed that as soon as the pH of both reactors stabilized around seven VFA concentration decreased considerably. Organic material with MW <30 000 Da can pass through the ceramic membranes with pore diameters of 0.05 - 0.20 µm (Liu *et al*., 2005). Major components of total VFAs (e.g. acetate, propionate and butyrate) smaller than 30 000 Da can pass through the UF module inside reactor B. Also, major changes in VFA concentrations that have direct effects on the process performance can be expected to occur in reactors B and D. Based on the above-mentioned facts, the concentrations of VFAs are shown for these two reactors only. The initial total VFA concentrations were 730 mg/l in reactor B (SMBR) and 820 mg/l in reactor D (secondary digester). After 2 days of operation the concentrations of VFAs in reactor B dropped to 273 mg/l and in D increased to 1458 mg/l. Taking the pH values for that time interval into account, it appeared that conditions in the system were not yet favourable for methanogenesis. The initial concentrations of oxygen might have caused cell lysis of methanogenic microflora in reactor B, leading to diffusion of enzymes and other cell

components into the permeate in reactor C and then into reactor D. As a result, the concentration of VFAs in reactor D increased, while the lack of active biomass in reactor B might have led to the lack of hydrolysis and acidogenesis, i.e. lack of substrates for VFA production.

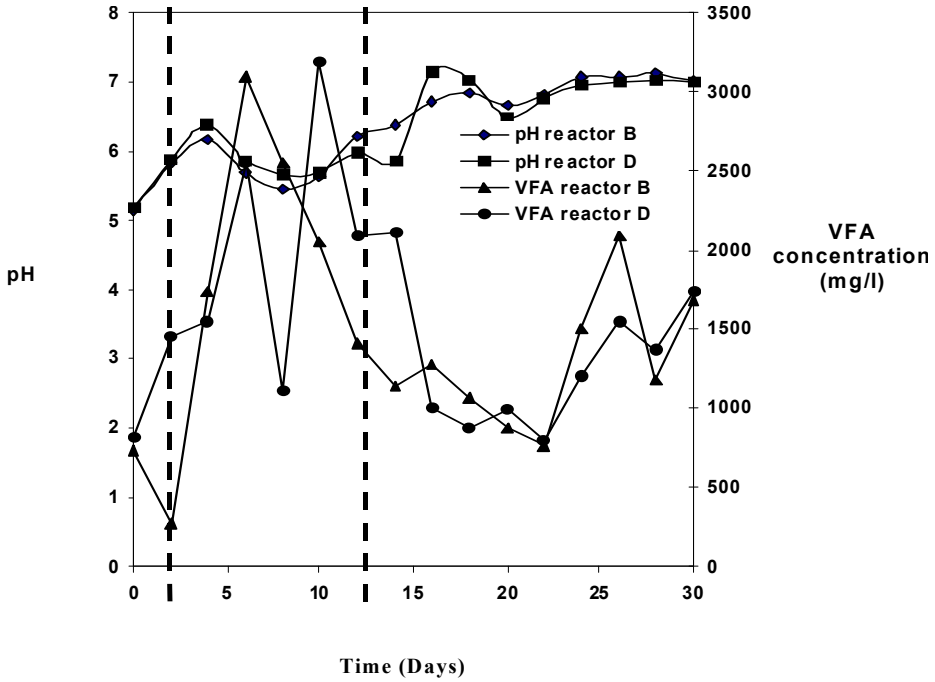


Figure 5.3: pH of the supernatant in the individual reactors of the bioreactor system as function of time of operation from B = SMBR and D = secondary digester. The dotted lines on days 2 and 12 indicate the period when the pH was <7.4 and VFA concentration was at its highest.

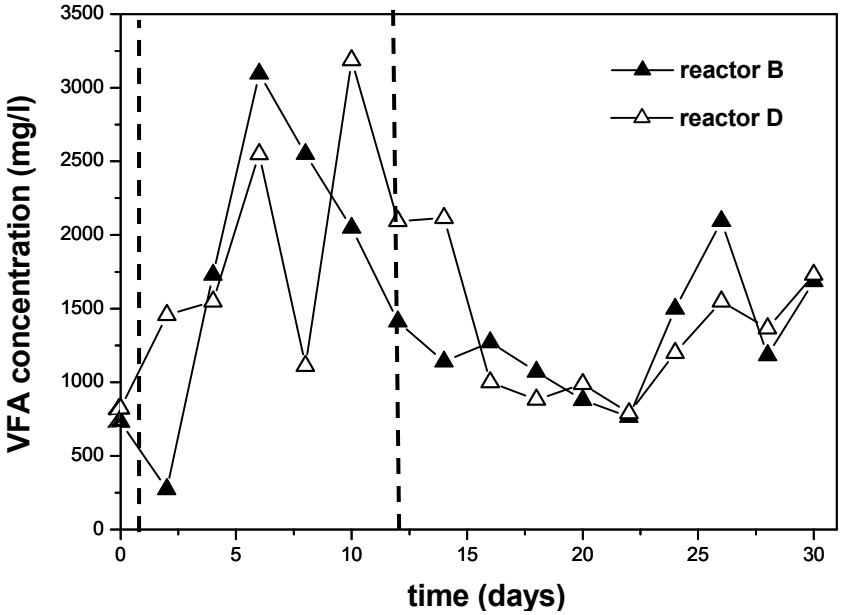


Figure 5.4: Volatile fatty acid concentrations in reactors B and D of the bioreactor system as function of time of operation. A = influent tank, B = SMBR, C = permeate balancing tank, D = secondary digester. The dotted lines on days 2 and 12 for reactors B and D bracket the period when the pH was <7.4 and [VFA] was highest.

From days 2 to 10 the concentrations of VFAs increased to peak values of 3100 mg/l in reactor B on day 6, and 3190 mg/l in reactor D on day 10. If anaerobic conditions were already established, then degradation of readily available RWDW compounds such as carbohydrates or proteins might have led to stimulation of the hydrolysis and acidogenesis pathways of anaerobic metabolism in reactor B, and thus to an increase in the VFA concentrations observed. After addition of 8000 mg/l of $CaCO_3$ and 4000 mg/l of K_2HPO_4 to the feed from day 12 onwards, pH values in all four reactors stabilised and ranged from 5.98 for reactor D on day 14 to 7.53 in reactor B on day 12. The concentrations of VFAs decreased to the minimum values of 764 mg/l in reactor B and 790 mg/l in reactor D on day 22. Decreases in the concentrations of VFAs might have indicated stabilisation of anaerobic conditions inside the system (Mourato *et al*., 1999). From this point until the end of the operation period, the concentrations of VFAs ranged from 1200 to 2100 mg/l in both reactors. Soluble COD values of the second batch of WDW used for the feed did not vary significantly from the first batch. As a result, the influence of the changes in composition of RWDW on the concentrations of VFAs in reactor B and reactor D could be deemed negligible. Changes in the composition of the microbial community in reactor B and D could therefore explain oscillations in the concentrations of VFAs. As individual components of the WDW became depleted sequentially, different portions of the microbial community became active. Therefore a breakdown of higher MW compounds from the RWDW led to successive accumulation of concentrations of VFAs in both reactors B and D. At the same time, death of some of the active biomass from reactor B might have provided additional nutrients to the sludge in reactor D. The average COD_S of the feed in reactor A was 4840 (± 950) mg/l. The COD_S of the original sample of RWDW was approximately 16 100 mg/l. This is on the lower end of the range for previously published data (Trussell *et al*., 2000). The SMBR and the total system COD_S removal efficiencies were calculated to establish whether secondary digestion improved the COD_S removal. During the initial 22 days of operation, before the secondary digester was inoculated, the SMBR COD_S removal efficiency fluctuated widely, between 0 and 76 % (Figure 5.5). From days 6 to 10 the removal efficiency ranged from 0 to 25 %, which coincided with the maximum concentrations of VFAs and pH values outside the methanogenic range. The system had probably not reached stability for anaerobic removal of COD_S, possibly because of residual oxygen concentrations in the system. A similar trend was observed for the total removal of COD_S, which ranged from 0 to 42 % for the same time period, suggesting that

COD_S removal was only marginally caused by the reaction of the individual components of the WDW and the methanogenic sludge lysis.

After pH stabilisation using 8000 mg/l $CaCO_3$ and 4000 mg/l K_2HPO_4 began on day 12, COD_S removal in the SMBR reached 76 % on day 14, while the total COD_S removal increased to 72 % on day 16. The lag phases between day 12 when the pH stabilisation was introduced and the actual peak values of COD_S removals could have been caused by the lag phase of the respective microflora and microfauna. After the maximum removal had been reached for reactor B (SMBR), there was a sharp drop in the removal efficiencies of COD_S. After inoculation of reactor D (secondary digester) on day 22, the total COD_S removal remained higher than that attained by the SMBR alone. This could be explained by the fact that prolonged biomass / substrate contact time was required for the removal of recalcitrant RWDW components in reactor D. The two batches of RWDW introduced into the feed between days 0 to 13 and 14 to 30 showed limited variability in the concentration of total soluble organic compounds as indicated by the lower degree of variability in the COD_S concentrations in comparison to other monitored parameters. The secondary digester (reactor D), together with the pH stabilisation of the system led to improved and more constant removal efficiencies for CODs.

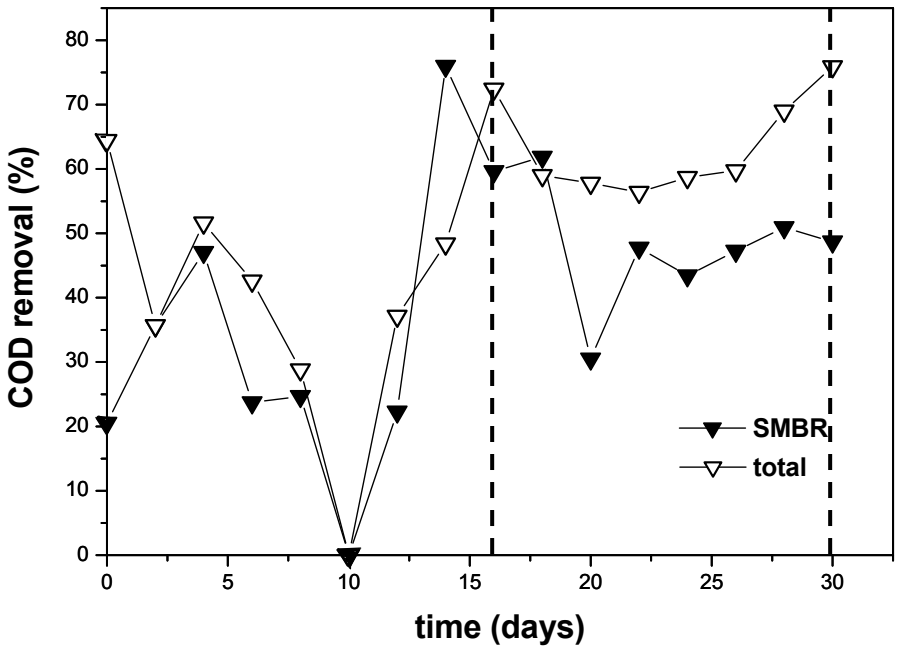


Figure 5.5: Removal efficiency of COD_S in the SMBR and the total system as a function of time. SMBR = reactor B, total = final effluent from secondary digester (reactor D). The first dotted line on day 16 and the second on day 30 for both reactor B and D bracket the period when COD_S removal efficiency began to stabilize and then increased slightly.

The concentrations of the total phenolic compounds in the system are shown in Figure 5.6 The concentration of phenolic compounds in the RWDW in the system varied widely, from 29 mg/l to 503 mg/l phenol equivalents. Several methods have been used to measure the total phenol content (or total polyphenol content) of wines and WDWs (Liu *et al*., 2005; Arnous *et al*., 2001). The Folin-Ciocalteau reaction has been shown to be sensitive towards phenol (with one hydroxyl group), as well as towards tannic acid (with 11 phenolic hydroxyl groups) (Thoss *et al*., 2002). Molecular weight and the respective value of the molecular absorption coefficient of the standard used influences the measured concentrations of the total phenolic compounds in a particular RWDW sample. The results obtained in this study are comparable to previously reported values (Liu *et al*., 2005) but lower than others (Beltrán *et al*., 1999) both of which used gallic acid as the standard. Gallic acid's MW of 170.89 g/mol is higher than that of phenol (94.11 g/mol). When expressing the results in mg/l, the slope of a calibration curve of the Folin-Ciocalteau method based on gallic acid as a standard will be lower than a curve based on phenol as a standard. Consequently, concentrations calculated based on gallic acid will be higher than those calculated based on phenol. Since the precise mechanism of the reduction of the Folin-Ciocalteau reagent by phenolic compounds is not known, the molar absorption coefficients of standards will be dependent on the particular protocol of the Folin-Ciocalteau method used in the measurements (Arnous *et al*., 2001).

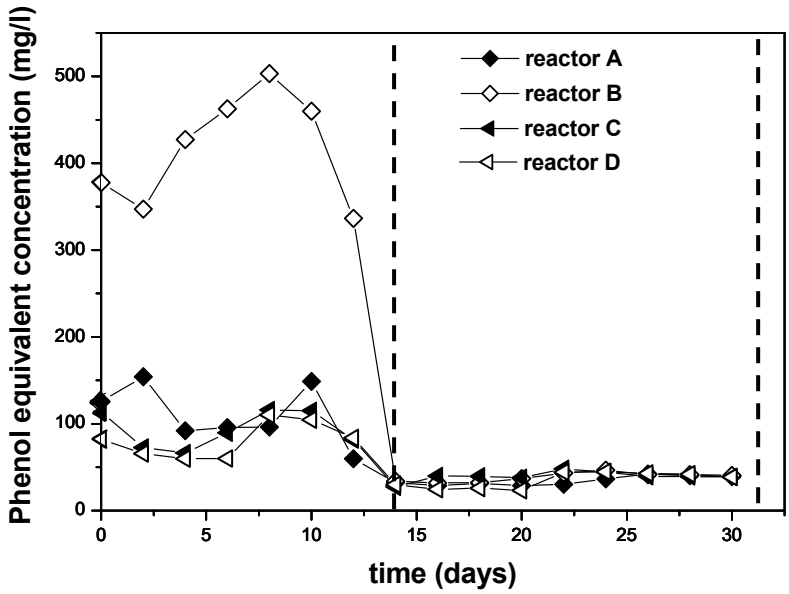


Figure 5.6: The total concentration of phenolic compounds in the bioreactor system expressed in phenol equivalents as a function of time. A = influent tank, B = SMBR, C = permeate balancing tank, D = secondary digester. The dotted lines indicate the beginning and end of the period when phenol levels became very low.

Published results therefore differ based on the exact protocol followed as well as the phenolic compound standard used for calibrations. The consequence of this is that comparison between published values of the total phenols / polyphenols measured by the Folin-Ciocalteau method should be carried out with caution. The total concentrations of phenolic compunds in reactor A ranged from 92 to 154 mg/l during days 0 to 10. After the increase in the concentrations of $CaCO_3$ and K_2HPO_4 on day 12, the total concentration of phenols in reactor A decreased to 59 mg/l. From day 13 onwards a new batch of WDW was used for the preparation of the feed in reactor A. There was an immediate decrease in the total concentration of phenolic compounds in reactor A to 29 mg/l, and the values ranged from 30 to 42 mg/l for the remainder of the experiment. The total concentration of phenolics in reactor B (SMBR) ranged from 347 to 503 mg/l between days 0 and 8 of operation. After that, a slight decrease in concentration was recorded with 337 mg/l recorded on day 12, coinciding with increasing concentrations of $CaCO_3$ and K_2HPO_4. After application of the second batch of WDW for feed, there was a sharp decrease in the total concentration of phenolics in reactor B, with individual values ranging from 31 to 46 mg/l. This was due in part to a decrease in the concentration of phenolic compounds in the RWDW, but this decrease did not account fully for the extent of the decrease observed in reactor B. The results were probably due in the main to a change in the distribution of specific phenolic compounds types and molecular weights; this could not be proven at the time and more detailed chemical analysis of the RWDW (recommended in Chapter 10) is required to support this hypothesis.

The second batch of feed had CODs that were slightly higher than the first batch (± 200 mg/l). As a result the better CODs removal efficiencies achieved afterwards could be attributed to the addition of $CaCO_3$ and K_2HPO_4 performed as from day 12. The total concentrations of phenolics in reactor C (permeate) and reactor D (secondary digester) followed similar trends as reactors A and B. For reactor C, the total concentrations of phenolics ranged from 66 to 116 mg/l between days 0 and 10, decreased to 82 mg/l on day 12, and decreased to 27 mg/l on day 14, when the second batch of WDW was used. The total concentration of phenolics in reactor C ranged from 37 to 48 mg/l for the remainder of the experiment. In the secondary digester (reactor D), the total concentrations of phenolics ranged from 60 to 111 mg/l between days 0 and 10, decreased to 84 mg/l on day 12, decreased further to 27 mg/l on day 14, and ranged between 23 and 45 mg/l from then onwards. During the initial 10 days of operation the total concentrations of phenolics

fluctuated in all four reactors. This can be explained by mutual transformations of the individual molecules with phenolic groups and detectable by the Folin-Ciocalteau method. These changes could have led to alterations in the number of hydroxyl groups available for the Folin-Ciocalteau reaction, i.e. the more phenyl hydroxyl groups available for the reaction, the higher the concentrations measured. In reactors A (feed), C (permeate) and D (digester) the phenolic molecules probably originated solely from the WDW. However, the concentrations in reactor B (SMBR) were much higher than in the other three reactors during this time. This observation could be explained through the release of additional phenolics from the biomass of the methanogenic sludge that was used as inoculum for reactor B. Increases in the total concentrations of phenolics were not observed in reactor D after its inoculation with mixed liquor from reactor B on day 22. Therefore the phenolic compounds originating from the methanogenic sludge biomass were degraded by the time reactor D was inoculated.

Changes in the total concentrations of phenolics in the feed in reactor A could be explained by intrinsic activity of native microorganisms present in the WDW. From day 10 to 14, the total concentration of phenolics in the system started to decrease, probably due to the depletion of the readily biodegradable phenolic compounds by the sludge biomass. After day 14, all the phenolic compounds remaining or introduced into the system were refractory and resistant to biodegradation, as noted by the fact that no significant differences between the concentrations of phenolics in all four reactors. Recommendations on experiments that can be performed to determine changes in MW distribution of phenolic compounds during biodegradation in the system and to ascertain the fate of the phenolic compounds in more detail are in Section 10.2.

The concentrations of nitrogen compounds over time in all four reactors are shown in Figures 5.7 (nitrates) and 5.8 (ammonium). The concentrations of nitrates in reactor A ranged from 34.0 to 94.0 mg/l from day 0 until day 12. After the application of the second batch of WDW for feeding the reactor system and the increase in concentration of $CaCO_3$ and K_2HPO_4, the concentrations decreased to the range 8.1 -13.7 mg/l from day 14 onwards. The concentrations of nitrates in reactor B ranged from 7.6 to 29.3 mg/l during days 0 - 10, and decreased to 6.6 mg/l on day 12. After day 14, nitrate concentrations decreased to values ranging from 6.3 to 15.5 mg/l.

The decrease in nitrate concentrations between days 0 and 10 in Reactor B (compared to Reactor A in the same period, Figure 5.7), in combination with the rate of oxygen mass transfer in the system (data not shown), demonstrated that anaerobic conditions were established in the bioreactor, since it was effective in reducing the nitrate concentrations in WDW through denitrification and dissimilatory reduction of nitrate to ammonium. The concentrations of nitrates in reactor C (permeate) fluctuated from 5.6 to 44.0 mg/l between days 0 to 12. After the application of the second batch of WDW for feeding the system and the increase in concentrations of $CaCO_3$ and 4000 mg/l on day 14, nitrate concentrations decreased to 9.6 - 19.3 mg/l. The nitrate concentrations in reactor D fluctuated from 5.3 to 68.0 mg/l from days 0 to 12 and were 8.0 - 11.3 mg/l after day 14.

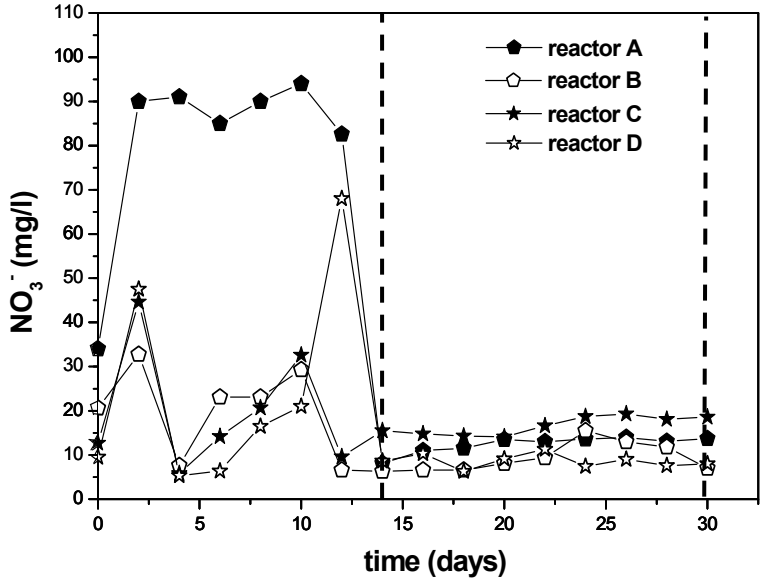


Figure 5.7: Concentration of nitrates in the system as function of time. A = influent tank, B = SMBR, C = permeate balancing tank, D = secondary digester. The dotted lines on days 14 and 30 bracket the period when nitrate levels became very low.

The concentrations of ammonium in the feed in reactor A ranged from 0.05 to 0.34 mg/l during days 0 to 12 (Figure 5.8). After day 14, ammonium concentrations increased from 4.4 to 7.0 mg/l, in reactor A. The ammonium in reactor B fluctuated between 1.7 and 9.8 mg/l from day 0 to 12, peaking at 9.8 on day 4. After day 14 the ammonium concentrations were 7.6 - 16.0 mg/l. The concentrations in reactor C were 1.3 - 8.0 mg/l during days 0 to 12 and 5.4 - 11.8 mg/l from day 14 onwards. Reactor D (secondary digester) ammonium was 3.0 - 12.8 mg/l during days 0 to 12 and 18.4 - 38.0 mg/l from day 14 onwards. Ammonium was observed to accumulate in reactor D after pH stabilisation and introduction of the new batch of WDW into the feed. This indicates that denitrification and

dissimilatory assimilation of nitrates (as the terminal acceptor of electrons in anaerobic respiration) was taking place, suggesting that anaerobic (anoxic) conditions were established in the system, i.e. residual oxygen was eliminated from the system within the first 14 days of operation.

Concentrations of phosphates in the system were virtually constant in all four reactors over the duration of the experiment, due to the addition of K_2HPO_4 at 1000 or 4000 mg/l into the feed for pH buffering. The overall average concentration of phosphates in the bioreactor system was 100 (± 20) mg/l (data not shown). Possibilities of effluent applications and decrease in concentrations of nitrates and phosphates will be discussed in Chapter 6 of this study.

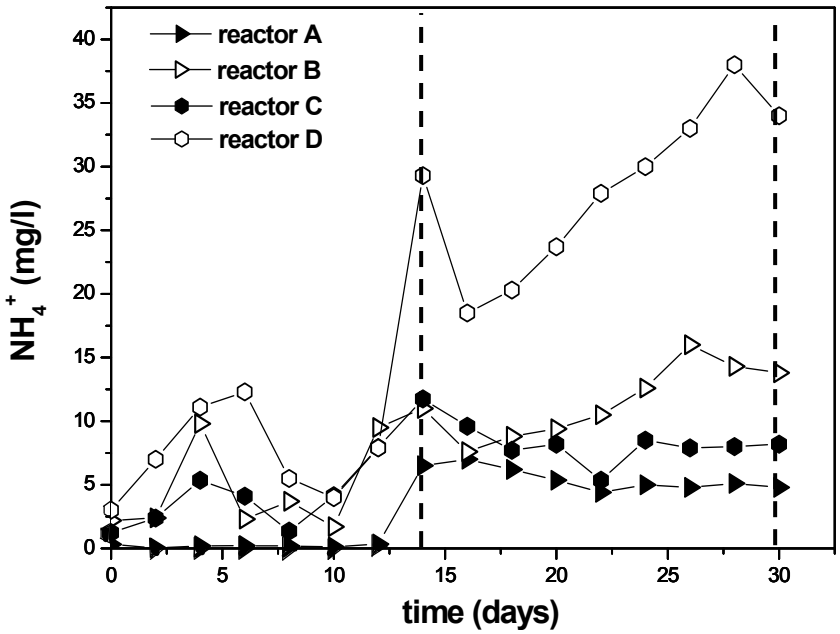


Figure 5.8: Concentration of ammonium in the system as function of time. A = influent tank, B = SMBR, C = permeate balancing tank, D = secondary digester. The first dotted line on day 14 and the second on day 30 for reactor A, B, C and D bracket the period when ammonium levels became very low.

5.4 Interim conclusions

The experimental system, comprising four reactors (balancing tank A, SMBR B, permeate balancing tank C and low rate anaerobic digester D) averaged 76 % CODs removal from WDW. The residual CODs levels in the system effluent were ≈1100 mg/l. Secondary digestion downstream of the SMBR, together with pH buffering using 8000 mg/l $CaCO_3$

and 4000 mg/l K_2HPO_4 stabilised CODs removal. Wine distillery wastewater showed variable composition in the concentrations of nitrates, ammonium and the total concentrations of phenolics. Readily biodegradable phenolics were probably removed from the WDW, and it is likely that only recalcitrant compounds with hydroxyl groups managed to pass through the experimental system without significant decrease in their concentrations. Molecular weight changes of the phenolic compounds are currently being evaluated to elucidate the nature of the fate of compounds with different numbers of phenolic hydroxyl groups in the molecule. However, the effluent quality did not meet the standards required for use of the treated wastewater for crop irrigation. Anaerobic digestion was not suitable as a first treatment step. To meet regulatory requirements and explore the full capabilities of the bioreactor system for RWDW treatment, fungal pre-treatment of the RWDW was chosen and experiments with the system were repeated to try to further decrease the effluent CODs.

CHAPTER 6

Treatment of fungally pretreated wine distillery wastewater by submerged membrane bioreactor and secondary digestion

6.1 Introduction

Fungi have been attracting a growing interest for the biological treatment of heavy metals, inorganic and organic compounds (Lacina *et al*., 2003). For WDWs, it was shown that fungal pre-treatment under aerobic conditions facilitates the reduction of phenol concentrations by 51 - 100 %; to induce decolourisation of 31-100 %; and reduce the five day biochemical oxygen demand (BOD_5) by up to 85.4 % (Lacina *et al*., 2003). In the present study a fungal pre-treatment step followed by a submerged membrane bioreactor (SMBR) and secondary digester were used in series to investigate the biological treatment of RWDW to obtain reusable water. Fungal pre-treatment of RWDW was tested for improvement of the performance of the biological treatment system used in Chapter 5 of this study.

6.2 Materials and Methods

6.2.1 Biological wastewater treatment system

Trametes pubescens was selected as the pre-treatment fungus using data from flask studenies in which four white-rot fungi were screened for their ability to biodegrade RWDW (Strong and Burgess, in press). Briefly, the method for treating RWDW using fungi was performed as follows: a bubble-lift bioreactor (Figure 6.1) was constructed from fibreglass. The bioreactor was sterilised by pumping 5 l of an aqueous solution of formaldehyde (4 $\%_{W/V}$) through the system. After sterilisation, the bioreactor was rinsed twice with 2 l of autoclaved distilled water, after which 5 l of autoclaved distilled water was circulated around the reactor system overnight to rinse out any residual sealant components

and to leak test the system. In the meantime, an inoculum of *Trametes pubescens* for fungal pre-treatment was grown in a liquid medium of the following composition: malt extract (2 $\%_{W/V}$), glucose (1 $\%_{W/V}$) and yeast extract (0.2 $\%_{W/V}$). The fungus was incubated in 500 ml Erlenmeyer flasks placed on a benchtop shaker (labcon SPL15, Laboratory Marketing Services (Pty) Ltd., Johannesburg) at 150 rpm at 28 ºC. Subsequently, the fungus was harvested in the late exponential phase of growth (based on preliminary experiments; see Appendix D).

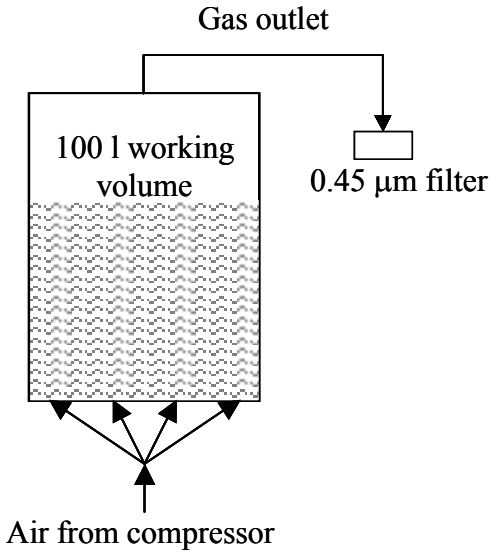


Figure 6.1: The bioreactor used for fungal pre-treatment of wastewater with floating *Trametes pubescens* mycelial balls. The working volume of the bioreactor was 102 l with the following dimensions: height 2.3 m (of which 1.2 m was the lower, V-shaped part), and a square 0.5 × 0.5 m cross-section. Two ports at the bottom were used for aeration (for both oxygenation and mixing) and for withdrawal of samples. (Adapted from Strong and Burgess, in press).

The fungal bioreactor was filled with 45 l of autoclaved WDW with the pH adjusted prior to autoclaving to 5.3 with solid Na_2CO_3 (AnaLar grade, Merck Chemicals (Pty) Ltd, Johannesburg). The bioreactor was inoculated with 5 l of the *T. pubescens* and pre-treatment was conducted at 25 ºC for 16 days (pre-treatment performance data are shown in Appendix D). Upon conclusion of the pre-treatment, the fungus was separated by gravity settling and the supernatant was transferred to the SMBR / secondary digester system described fully in Section 5.2.1 and Figure 5.1. As mentioned earlier, the biological treatment system comprised a balancing tank (reactor A) containing the influent (which consisted of 30 $\%_{V/V}$ FTWDW and 70 $\%_{V/V}$ deionised water), a SMBR (reactor B), permeate balancing tank

(reactor C) and a secondary low rate digester (reactor D), from which the final effluent escaped via a weir. The SMBR contained a module of four tubular ceramic membranes (surface area 55 cm^2, pore size 0.2 μm; Synexa Life Sciences, South Africa). Flow rates from reactor A through B and C to D were controlled using peristaltic pumps (Watson Marlow 505S, Falmouth, UK). Conditions of the study and system operating parameters were the same as described in Section 5.2.1, with one exception: for pH buffering, the concentrations of $CaCO_3$ and K_2HPO_4 were kept at 1000 mg/l for the whole duration of the study. This was a consequence of the higher pH of WDW after fungal pre-treatment (see Section 7.3). Samples were taken from each of the four reactors comprising the system every 48 hours and analysed according to the methods set out in Section 5.2.2.

6.3 Results and Discussion

The prevailing pH values inside reactors A, B, C and D as a function of time of operation are shown in Figure 6.2. The individual values ranged from 7.13 to 9.08. Fungal pre-treatment was effective in reducing the extent of pH buffering required, as indicated by the concentrations of $CaCO_3$ and K_2HPO_4 added to the feed for buffering; these were maintained at 1000 mg/l during the whole 30 day operation of the system. Fungal pre-treatment also improved the stability of the system, as indicated by lower concentrations of volatile fatty acids (VFAs) compared to treatment of raw WDW (Section 5.3). Concentrations of VFAs as a function of time are shown for reactors B and D, and the data are shown in Figure 6.3. Examination of the molecular weight (MW) distribution of the organic material passing through the ceramic membranes (pore diameter of 0.05 μm) in the permeate suggested that VFAs passed through the membrane, out of reactor B into reactors C and D (Liu *et al*., 2005). It is therefore reasonable to expect that there will be a mutual relationship between the concentrations of the VFAs in reactor B and reactor D. As in Section 5.3 of this study, the major changes in VFA concentrations that have direct effect on the performance of the process were expected to occur in reactors B and D. Therefore the concentrations of VFAs are shown for these two reactors only (Figure 6.3). The initial VFA concentrations were 228 mg/l in reactor B (SMBR) and 547 mg/l in reactor D (secondary digester), respectively. The concentration of VFAs in reactor B increased to the maximum value of 548 mg/l from day 6 until day 8 of bioreactor system operation. VFA

concentrations in reactor D fluctuated between 200 and 474 mg/l during the first 10 days of bioreactor system operation.

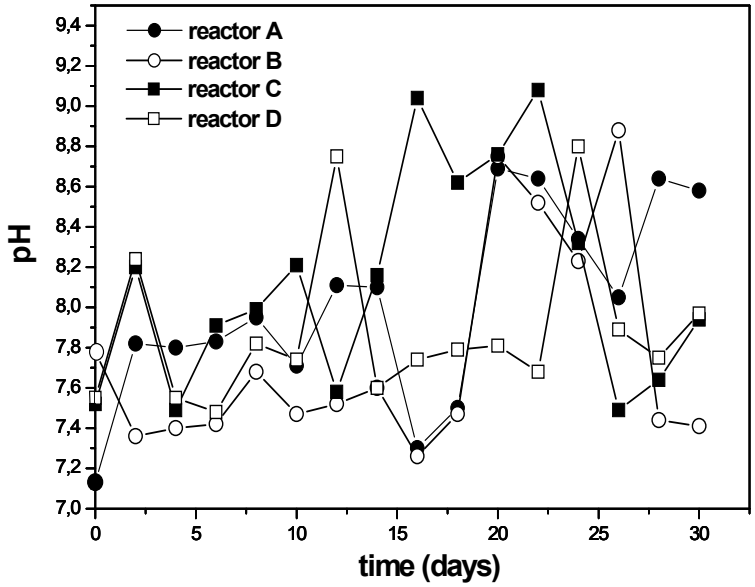


Figure 6.2: Prevailing pH of the bulk liquid in the individual reactors of the experimental system as function of time of operation of the bioreactor system. A = influent tank, B = SMBR, C = permeate balancing tank, D = secondary digester.

From days 10 to 12, the concentration of VFAs decreased in both reactor B (SMBR) and reactor D (secondary digester). In reactor B, the concentrations decreased from 365 to 63 mg/l, while the concentrations dropped from 274 to 51 mg/l in reactor D over the same period. From day 12 until the end of the experiment, the VFA concentrations fluctuated within the range 46 - 91 mg/l in reactor B, and 27 - 82 mg/l in reactor D. Only a single batch of the FTWDW was used in the study, and so the observed trends can be confirmed as a result of the WDW treatment and not variability in feed composition. Autolysis of the fungal biomass might have provided additional nutrients for the methanogenic sludge used to inoculate the SMBR. As a result, it might have been easier for the sludge microorganisms to degrade components of WDW, as indicated by the lower residual VFA concentrations in the bioreactor system.

The average COD_S of the feed was 4300 (± 1800) mg/l. This value is comparable to the feed used in the treatment of the diluted WDW without fungal pre-treatment in Section 5.3, but the composition of the COD_S was different due to enrichment of the fungally pre-treated WDW with components of fungal biomass. Removal efficiencies of COD_S were calculated

using the method described in Section 5.2.2. The COD_S removal efficiencies achieved by the SMBR and the total treatment system were calculated to establish whether secondary digestion improved the COD_S removal.

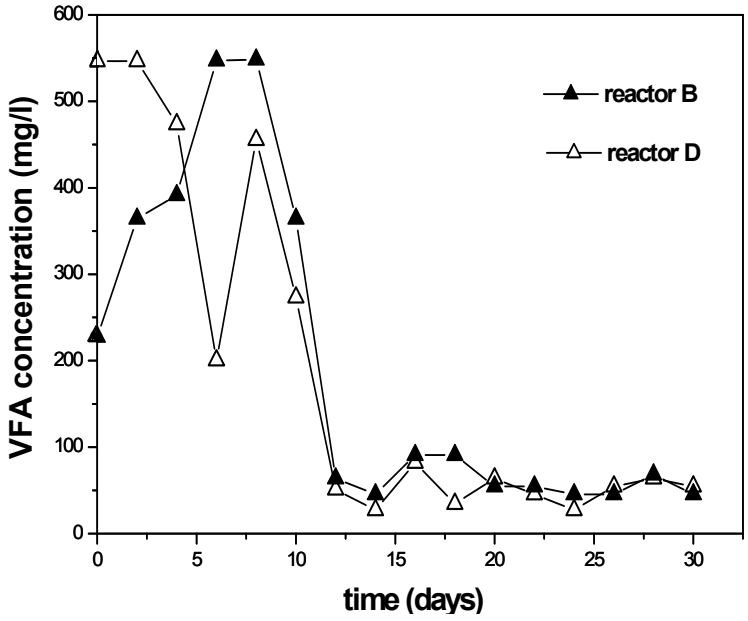


Figure 6.3: Total VFA concentrations in reactor B (SMBR) and reactor D (secondary digester) as function of time.

The data are presented in Figure 6.4. Soluble COD removal efficiencies fluctuated in the SMBR: from days 0 to 4 the COD_S removal increased from 0 % to 47 %. After day 4, a sharp decrease to the minimum, 0 %, was recorded on day 10. This could have been caused by the release of an inhibitory lower molecular weight compound from the breakdown of higher molecular weight components of the mixed liquor in reactor B. Subsequently, an increase in COD_S removal for the SMBR was recorded, with the maximum value of 76 % on day 14. From day 16 to 30 the COD_S removal efficiencies for the SMBR fluctuated between 43 and 62 %.

The total COD_S removal efficiency was equal to 0 % between days 0 and 2 of system operation. From day 2 onwards, COD_S removal stabilised and became practically independent of time, with the average value equal to 86 (± 4) %. The lack of removal of COD_S by both the SMBR and the bioreactor system as a whole could be explained by the acclimation of the biomass to the new medium. The results indicated that a combination of fungal pre-treatment, SMBR and the secondary digester stabilise the extent of COD_S removal.

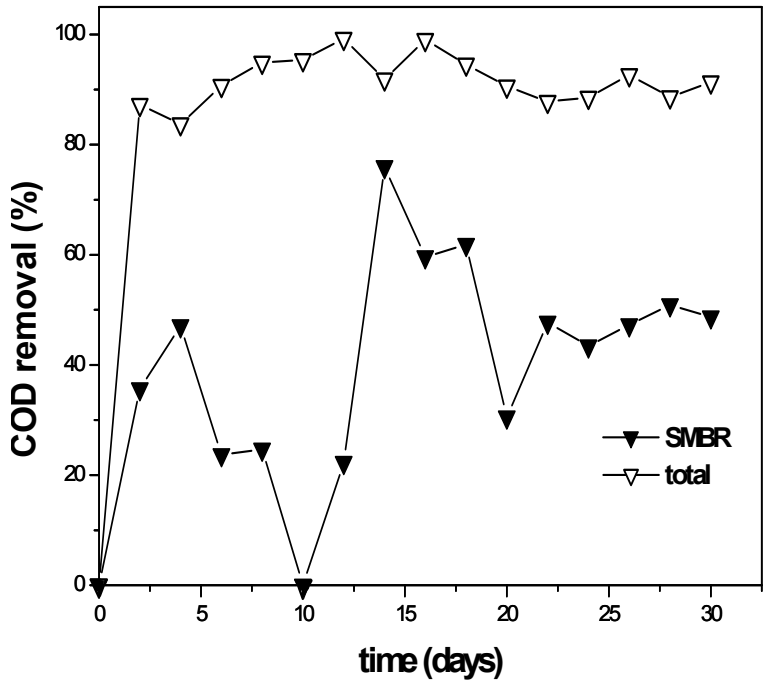


Figure 6.4: Removal efficiency of COD_S in the SMBR and over the total treatment system as a function of time.

Fungal pre-treatment and autolysis of fungal biomass probably provided additional nutrients required by the methanogenic sludge for the removal of WDW components as the feed was not filtered before use. Experiments using filtered FTWDW would confirm this, while the secondary digester prolonged the period of effective biodegradation, and thus increased the efficiency of COD_S removal. The residual COD_S levels were around 400 mg/l. These levels could be decreased by further treatment based on a membrane processes (see below). The total concentrations of phenolic compounds in the bioreactor system as a function of time of operation are shown in Figure 6.5.

The total concentration of phenolic compounds in the raw WDW in the bioreactor system ranged from 1 to 86 mg/l in phenol equivalents. The total concentrations of phenolics in reactor A fluctuated between 12 and 62 mg/l, and no clear trend with time could be established. At the beginning of the system operation, the total concentration of phenolics in reactor B was 52 mg/l. A decrease to 19 mg/l was recorded on day 2, and the values fluctuated between 9 and 27 mg/l from day 4 until day 14, followed by a sharp decrease to 1 mg/l was recorded on day 16, which constituted the minimum value in reactor B and the whole bioreactor system for the entire duration of the experiment. The total concentration of

phenolics ranged from 39 to 86 mg/l in reactor B from day 17 onwards. The total concentrations of phenolics in reactor C fluctuated between 12 and 30 mg/l, and no clear trend with time could be established from day 0 until day 14 of system operation.

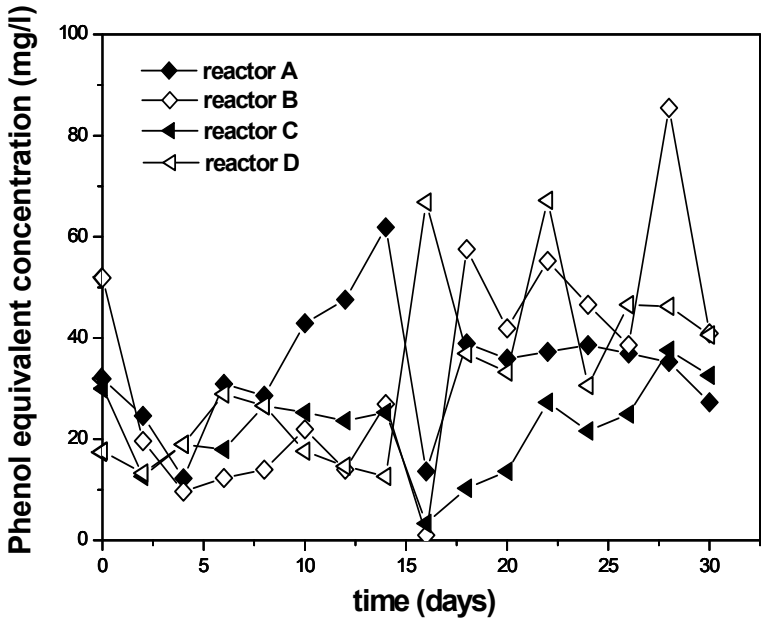
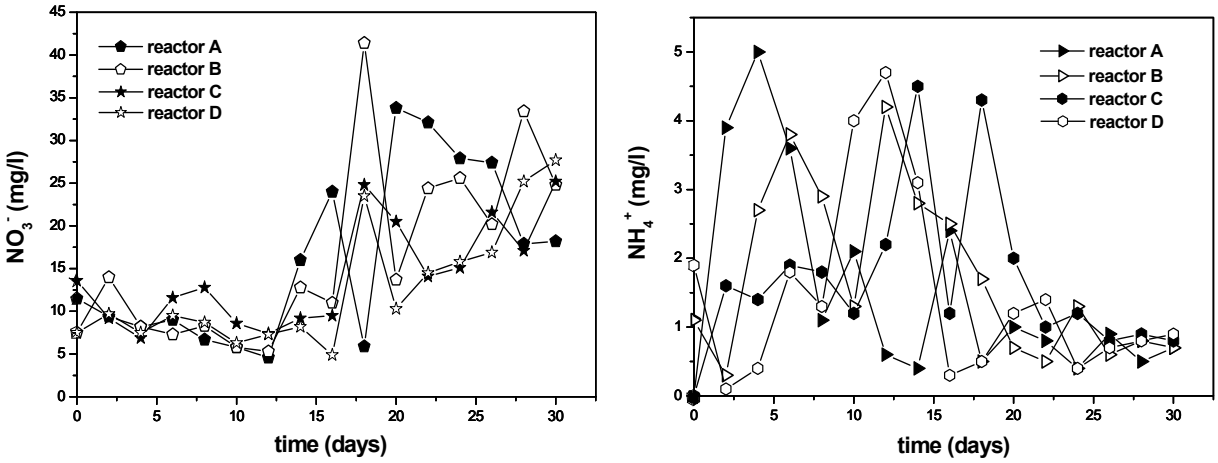


Figure 6.5: The total concentration of phenolic compounds in the treatment system expressed in phenol equivalents as a function of time operation. A = influent tank, B = SMBR, C = permeate balancing tank, D = secondary digester.

A sharp decrease to 3 mg/l was recorded on day 16, which constituted the minimum value of the total concentration of phenolics in reactor C. For the remainder of the experiment, the individual values were 10 - 38 mg/l. The total concentrations of phenolics in reactor D fluctuated within 13 - 67 mg/l, and no clear trend with time could be established during the entire study period. The trends in the total concentration of phenolics in the individual reactors of the bioreactor system indicate a complex series of (mutual) transformations of phenolic compounds in the experimental system. The total concentrations of phenolics did not vary significantly with time in the experimental system, but the molecular structure of particular compounds underwent changes in the system. The concentrations of nitrogen compounds as a function of incubation time in all four reactors of the bioreactor system are shown in Figure 6.6. The concentrations of nitrates in reactor A ranged from 4.6 to 33.8 mg/l. The concentrations of nitrates in reactor B fluctuated between 5.3 and 12.8 mg/l from day 0 until day 16. A sharp increase in the nitrate concentration to 41.4 mg/l was recorded on day 18, while the nitrate concentrations fluctuated between 13.7 and 33.4 mg/l for the

remainder of the experiment. The concentrations of nitrates in reactor C fluctuated between 6.9 and 13.6 mg/l from day 0 until day 16.



6.6a **6.6b**

Figure 6.6: Concentration of nitrates (6a,★ ⬟) and ammonium (6b, ▶ ⬢) in the system as function of incubation time. A = influent tank, B = SMBR, C = permeate balancing tank, D = secondary digester.

A peak in the nitrate concentration of 24.8 mg/l was recorded on day 18, while the nitrate concentrations were 14.1 - 25.2 mg/l for the rest of the experiment. The concentrations of nitrates in reactor D fluctuated between 4.9 and 9.7 mg/l from day 0 until day 16. A sharp increase in the nitrate concentration (23.5 mg/l) occurred on day 18. From day 20 onwards, nitrate concentrations increased from 10.3 to 27.8 mg/l. Nitrates were accumulating in the second bioreactor system after day 18 of operation. This could have been caused by the release of nitrates from the residual fungal components in the feed.

No systematic trends in the concentrations of ammonium in all four reactors of the bioreactor system were observed. The values of ammonium concentrations fluctuated with time, and ranged from 0 to 5 mg/l. Based on the data for nitrate and ammonium concentrations, no effective removal of nitrates or ammonium could be observed by SMBR treatment or digestion after fungal pre-treatment of WDW.

Concentrations of phosphates in the system were virtually constant in all four reactors of the bioreactor system throughout the duration of the experiment, due to the addition of K_2HPO_4 at 1000 mg/l to the feed stream for pH buffering. The average concentration of phosphates

in the bioreactor system was 107 (± 16) mg/l (no graph shown). Additional treatment of the bioreactor system effluent would be required to meet the water quality guidelines for use in crop irrigation (DWAF, 1996).

In previous, similar studies membrane processes have been successfully used for treatment of wastewaters (Van Voorthuisen *et al*., 2005). Na_2HPO_4 was present in a simulated wastewater at levels comparable to the residual concentrations in this study. Based on the process used for phosphate removal, the concentrations of phosphates in the wastewater could be reduced to 1.4 - 36.4 mg/l (Van Voorthuisen *et al*., 2005). These levels would allow for the application of the treated effluent as vineyard irrigation water, since phosphates are highly immobile in soils. As a result, the phosphate molecules do not percolate down the soil profile and the risk to the groundwater is reduced to a minimum. The residual COD levels in the final effluent could be reduced by nanofiltration, and the nitrates could be further treated using reverse osmosis (Ritchie and Bhattacharyya, 2002). The financial aspect of the resulting process design would be the overriding concern in choosing the optimum solution to the additional treatment, and a cost-benefit analysis has been included in the list of recommendations for further work (Chapter 10).

6.4 Interim conclusions

The experimental system using ADUF to treat FTWDW has been shown to eliminate up to an average of 86 (± 4) % of COD_S present in the WDW, after fungal pre-treatment. Secondary digestion, together with pH buffering using 1000 mg/l of $CaCO_3$ and K_2HPO_4, led to the stabilisation of COD_S removal. The residual COD_S levels were 400 mg/l, significantly lower than the concentrations obtained using ADUF for RWDW without fungal pre-treatment (1100 mg/l, Chapter 5), indicating that fungal pre-treatment might have provided additional removal of recalcitrant components of the wastewater. Phenol removal was not as stable as the COD_S removal, but the concentration of phenol in the effluent was reduced from a minimum of 23 mg/l without fungal pretreatment, indicating to one of 1 mg/l. The requirement for the UF membrane, with its associated capital cost, and a complex four-stage treatment system became questionable, and it was decided to test the performance of anaerobic digestion of FTWDW.

CHAPTER 7

Performance of a high rate anaerobic digester during treatment of fungally pretreated wine distillery wastewater

7.1 Introduction

Wine distilleries produce large volumes of WDWs (Nogales *et al.,* 2005). The composition of WDW is highly variable depending on the raw material distilled and production parameters (Benitez *et al*., 1999a). Discharge, irrigation, or reuse inside the plant cannot be undertaken without prior treatment of the particular WDWs or treatment processing (DWAF, 1996). Most distilleries practise biological treatment of the produced wastewaters, which can be conducted under anaerobic or aerobic conditions, both of which lead to reduction in BOD and COD (Pandey *et al*., 2003). Yeoh (1997) reported COD_S of WDW higher than 100 g/l. Membrane processes have been known for a long time to be highly effective in treating high strength wastewaters, but they are often associated to high capital and operating costs (Lacina *et al.,* 2003; Benitez *et al*., 1999b). The antibacterial activity of WDW has resulted in investigation of various methods for treatment and pre-treatment, for example chemical oxidation, anaerobic digestion in different reactor configurations, use of activated sludge systems, dilution of WDW before treatment and treatment of WDW with fungi (Lacina *et al.,* 2003) that have been used effectively as a pre-treatment for anaerobic digestion of materials with high phenolic content, such as molasses and olive mill wastewater (Lacina *et al.,* 2003). The phenolic compounds in such materials and WDWs exert antimicrobial activity inside wastewater, inhibiting the effectiveness of the treatment (Lacina *et al.,* 2003). In such cases, fungal pre-treatment under aerobic conditions makes it possible to obtain phenol concentration reduction between 51-100 %, good decolourisation (31-100 %), BOD reductions of up to 85.4 % and production of enzyme-degrading xenobiotics e.g., laccase (Lacina *et al.,* 2003).

The search for sustainable treatment systems capable of minimizing energy consumption has encouraged the use of anaerobic biological systems, even in cases where the main goal

is to eliminate the biodegradable and dissolved fraction of carbonaceous substrates (Rajeshwari *et al.,* 2000). This is as a result of the possibility of using the biogas produced for meeting the energy demands of the process and/or recovering part of the operating costs (Vlissidis and Zouboulis, 1993). Anaerobic treatment systems have been used mainly for high strength organic wastewaters, including distillery wastewaters (Sales *et al.,* 1987). Although anaerobic digestion of this type of wastewater is feasible and appealing from an energy point of view, the presence of polyphenols slows down the process and hinders complete removal of COD (Vlissidis and Zouboulis, 1993). In this study a high rate anaerobic digester was investigated for the removal of phenolic compounds from a lab-scale FTWDW treating WDW from Olafbergh Distilleries Inc. (Worcester, South Africa).

7.2 Materials and Methods

7.2.1 High rate anaerobic digester set-up

Fungal pre-treatment of RWDW using *Trametes pubescens* was conducted in the bubble-lift bioreactor, described in Section 6.2.1. Conditions of incubations and sludge inoculum preparation have been described previously (Section 6.2.1). Following the fungal pre-treatment, the FTWDW was transferred into a 10 l anaerobic digester set up in a controlled temperature room of 30 ºC. A schematic representation of the digester is shown in Figure 4.1. At the beginning of the anaerobic treatment step, the digester was seeded with 2.5 l of methanogenic sludge as described in Section 4.2.1, 0.5 l of fungally pre-treated WDW and 7.0 l of nutrient broth (containing 1 g/l meat extract, 2 g/l yeast extract, 5g/l peptone and 8g/l sodium chloride, from Merck Chemicals (Pty) Ltd, Johannesburg). Anaerobic digestion was carried out as described in Chapter 4 (Section 4.2.1). During anaerobic digestion, HRT was 48 hours and the SRT was 100 d. The contents of the digester were agitated for 48 h, after which the agitation was switched off for withdrawing of the supernatant. The solids were allowed to settle for one hour (Figure 4.2). Subsequently, the supernatant was removed from the digester and replaced with an equal volume of fresh feed as in Section 4.2.1.

7.2.2 Sample analysis

Samples of the supernatant were retained at 4 ºC for a maximum of 72 hours prior to analyses. The digester feed was a mixture of nutrient broth and FTWDW and its concentration was increased from 5 % (v/v) between days 0 - 36, to 10% (v/v) for days 38 - 44 to 15 % (v/v) for days 46 - 50, to 20 % and finally to 30% (v/v) for days 52 - 100. Additions of $CaCO_3$ (Merck Chemicals (Pty) Ltd, Johannesburg) for pH buffering were made by adding 2000 mg/l from day 0 to day 4. Due to the lack of sufficient buffering, a combination of $CaCO_3$ and K_2HPO_4 was introduced into the system from day 6 onwards. The concentration of $CaCO_3$ was kept at 2000 mg/l from day 6 until day 14, and decreased to 500 mg/l after this point until the end of the experiment (day 100). The concentration of K_2HPO_4 in the feed was kept at 1000 mg/l from day 6 until day 8, and changed to 500 mg/l from day 10 until the end of the experiment on day 100. Based on previously obtained data, micronutrient amendments were supplied as 50 mg/l of $Fe(NO_3)_3$ added from days 18 to 24; $Co(NO_3)_3$ from days 26 to 32, and $Ni(NO_3)_3$ from days 34 to 38 (Merck Chemicals (Pty) Ltd, Johannesburg).

7.2.3 Performance of a high rate anaerobic digester during treatment of FTWDW

Digester performance was monitored by determination of the feed and the supernatant parameters as described in Section 4.2.2 (pH, COD_s, the total concentration of phenolic compounds and VFAs, turbidity and colour). All parameters were measured off-line. The pH values were measured using a Cyberscan 2500 pH meter (Eutech Instruments, Johannesburg, South Africa). Colorimetric reagent test kits (Merck Chemicals (Pty) Ltd, Johannesburg), based on the principles of *Standard Methods* (APHA *et al*., 1998), were used to measure COD_s (Spectroquant reagent test 14538/9 analogous to *Standard Method* number 5220-D), phosphates (14543 analogous to 4500-P-E), nitrates (14773, analogous to 4500-NO_3-E) and ammonia (14752, analogous to 4500-NH_3-F). The concentration of VFAs was determined according to a standard titration method (SCA, 1979). The total concentration of phenolic compounds was measured using a modified version of the Folin-Ciocalteu's spectrophotometric method with phenol as the standard, and it was expressed in mg phenol equivalent/l (Khan, 2005; Box, 1985). Colour and turbidity were measured using a Specroquant Nova 60 (Merck Chemicals Pty Ltd, Johannesburg).

7.3 Results and Discussion

The influence of aerobic pre-treatment on the parameters of WDW can be seen from the data in Table 7.1. The total average concentration of phenolic compounds in the untreated WDW was 522.9 mg/l, while the COD_s value was 15000 mg/l. The total concentration of phenolics dropped to 144.0 mg/l after fungal pre-treatment, and the COD_s concentration decreased to 7000 mg/l.

Nollet and Verstraete (2004) reported that during anaerobic digestion reductive acetogens and methanogens compete for H_2 as a substrate. The domination of methanogens, i.e. higher productivity of methane than acetate indicates stability of the anaerobic digester (Vlissidis and Zouboulis, 1993).

Table 7.1: Characteristics of FTWDW and RWDW (average values).

Parameter	RWDW	Standard Deviation	FTWDW	Standard Deviation
pH	3.83	± 1.91	6.7	± 0.1
Colour (A_{500})	1.29	± 0.62	4.76	± 0.01
Turbidity (FAU)	0.74	0.37	1.29	0.01
Phenols (mg/l)	522.9	± 261.5	144.0	± 1.2
COD_s (mg/l)	15 000	± 7501	7000	± 520
Total nitrogen (mg/l)	4.2	± 2.1	3.3	± 0.1
Total phosphorus (mg/l)	40.0	± 20.6	10.2	± 0.3
NH_4 (mg/l)	0.24	± 0.12	0.24	± 0.01
NO_3 (mg/l)	124.8	± 62.4	98.8	± 1.3
PO_4^{3-}(mg/l)	163.6	± 81.7	18.8	± 0.7

According to Nollet and Verstraete (2004), methanogens had the competitive advantage over acetogens for H_2 as a substrate in the pH range from 7.0 to 7.5, while acetogens dominated at pH levels around 6.5. Therefore, pH of the fungally pre-treated WDW had to be increased by additions of $CaCO_3$ and K_2HPO_4 to ensure system stability by stimulating methanogenic activity, and at the same time encourage higher removal of organic components. Colour and turbidity of WDW increased after fungal pre-treatment. The pH values and the VFA concentration profile with time are shown in Figure 7.1. During the 100 day study period, pH values fluctuated between 6.98 and 8.63, with peak values above 8 on

days 16, 18, 38, 58, 84, 92, 96, and 98. The pH was very stable and mostly in the methanogenic range, especially between days 18 and 36.

At day 0 the VFA concentration was 614 mg/l, it increased to 729 mg/l on day 2. A gradual decrease in VFA concentrations was recorded until day 10 and onwards, with the minimum value reaching 72.9 mg/l on day 100. This coincides with a decrease in COD_S values (Figure 7.2), indicating the degradation of higher molecular weight compounds into new VFA molecules and their subsequent removal. As from day 6, VFA concentration was always below 300 mg/l except between day 54 and day 60 when it was slightly above. During the period of addition of micronutrients, (mentioned in Section 7.2.1), VFA was below 100 mg/l. There was VFA accumulation between days 56 and 62 with a maximum of 911 mg/l being recorded on day 62, at pH value of 7.04. As the pH on that day was within the methanogenic range this high concentration was probably not indicative of the digester failure. Volatile fatty acid accumulation during this period was due to increased organic rate from 5% (v/v) to 30 % (v/v).

When comparing data in Figures 7.1 and 7.2, it can be noticed that the COD_s value on day 62 was 1320 mg/l, and thus coincided with the maximum in VFA concentrations. This indicates that more refractory organic matter was loaded into the system during feed refills of this period and system needed time to degrade the respective organic components. As it can be seen from Figure. 7.1, VFA concentration dropped to 182.2 mg/l on day 64, suggesting robustness of the digester. Soluble COD values and the total concentration of polyphenols are summarized in Figure 7.2. Soluble COD dropped from the initial value of 1935 mg/l to 80 mg/l on day 10. The values increased to 340 mg/l on day 16, and subsequently fluctuated between 42 and 1320 mg/l until the end of the experiment, with peak values of 385 mg/l on day 30, 825 mg/l on day 54, 1320 mg/l on day 62 and 704 mg/l on day 80. The final CODs value was equal to 72 mg/l. The fungal pre-treatment led to removal of 53.3 % of the initial CODs. These values are comparable those observed by others (Lacina *et al.* 2003). From day 0 until day 8, the total concentration of phenolics decreased from the initial value of 58.9 down to 3.4 mg/l. For the remainder of the experiment, the concentrations fluctuated between 3.7 and 117.0 mg/l. The observed trend might be explained by the changes in the molecular weight of the individual phenolic compounds. The MLSS values fluctuated during the experiment, however, never decreased below the initial value of 13640 mg/l (Appendix E). The final MLSS concentration was

equal to 21800 mg/l. The ability of the system to handle high organic loadings of 30 % (v/v), and the MLSS data indicate suitability of the presented system for the treatment of WDW, except for the CODs removal efficiency that still needs to be improved.

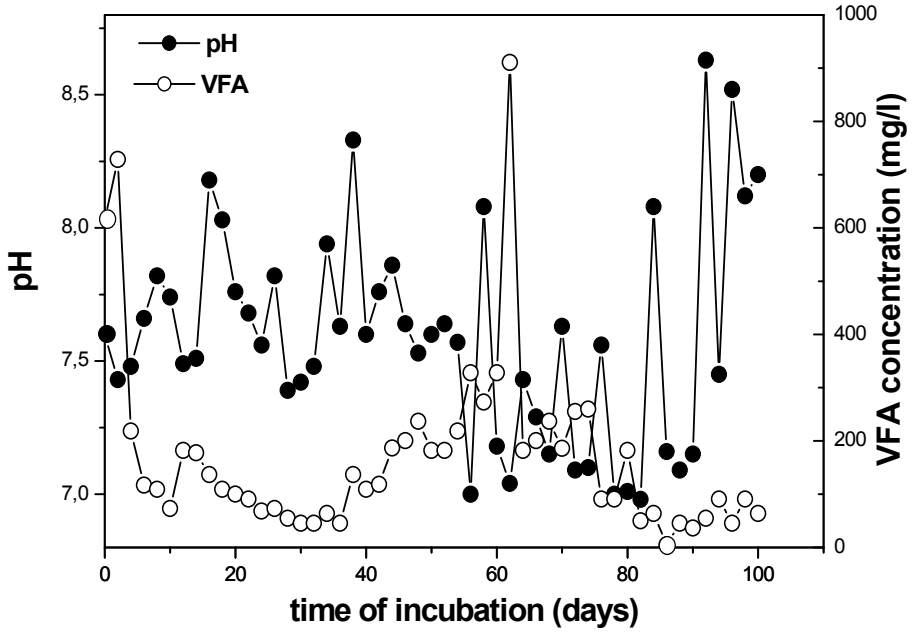


Figure 7.1: pH and VFA concentration as a function of time during anaerobic digestion.

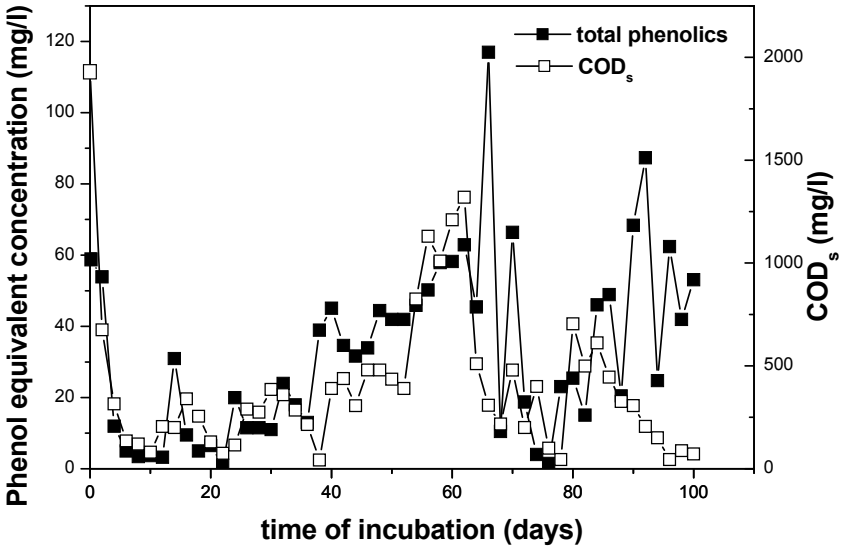


Figure 7.2: The total concentration of phenolics and COD_s as a function of time during anaerobic digestion.

7.4 Interim conclusions

Fungal pre-treatment of WDW led to a significant reduction in COD_s and polyphenols in the studied WDW. The COD_s removal efficiency after fungal pre-treatment reached 53.3 %. The pH of the fungally pre-treated wastewater reached 6.7, reducing the pH buffering requirements for anaerobic digestion. The latter was conducted under pH buffering using a mixture of $CaCO_3$ and K_2HPO_4, which provided stable environment inside the bioreactor system for efficient CODs. It was clear from the variable effluent quality obtained when comparing AD and ADUF for RWDW and AD and ADUF for FTWDW that both the fungal pre-treatment and the presence of UF membrane affected treatment performance. This could be a symptom of differences in microbial population dynamics, so biomass samples were taken to try to make a rapid scanning test to compare the biomass in each system.

CHAPTER 8
Population diversities in anaerobic digesters and submerged membrane systems

8.1 Introduction

Biological treatment processes are now widely used to achieve high quality effluent for environmental disposal of wastewater (Akarsubasi *et al.,* 2005; Coetzee *et al*., 2004; Wolmarans and de Villiers, 2002; Laubscher *et al*., 2001; Vlissidis and Zoubalis, 1993). Performance of anaerobic biological processes is, among other factors, related to the composition and activity of microbial populations they contain (Jawed and Tare, 1999). The types of microorganisms present and their relative population levels in the digester depend on wastewater characteristics and conditions maintained during operation (Jawed and Tare, 1999). At the same time, microbial communities within contaminated ecosystems tend to be dominated by organisms capable of utilizing and/or surviving toxic contaminants (McNaughton *et al*., 1999). As a result the microbial communities in contaminated ecosystems are typically less diverse than those in uncontaminated systems.

Microbial diversity may also be influenced by the complexity of chemical structures present and the length of time the populations have been exposed to the wastewater and/or its toxic component (McNaughton *et al*., 1999). As anaerobic digesters are also known to treat medium to high strength wastewaters (Coetzee *et al*., 2004; Wolmarans and de Villiers, 2002), the potential toxicity of chemicals present in these wastewaters can lead to severe problems in the efficiency of treatment. Thus composition, distribution and the dynamics of populations are of particular importance (Jawed and Tare, 1999). Maintenance of sufficient methanogenic populations in anaerobic digesters is important for their stable performance (Laubscher *et al*., 2001; Jawed and Tare, 1999). Therefore, improvements in the understanding of the microbial processes and anaerobic digestion are essential. Any imposed stress may lead to a change in species types and their relative population levels and this reflect in the digester performance (Jawed and Tare, 1999). Digester performance is usually evaluated in terms of process efficiency and stability through estimation of organic

substrate removal, VFA concentration, composition of biogas produced and biomass (Coetzee *et al*., 2004; Vlissidis and Zoubalis, 1993). Characterisation of the type of (active) biomass present in an anaerobic digester, as well as its temporal changes, is usually done by monitoring relative population levels of microoganisms under varied operational and or environmental conditions (Jawed and Tare, 1999; Zelles *et al*., 1992). Direct quantification of microbial of individual species/genera present can be done under *ex-situ* conditions, but usually provided unreliable results due to limited potential of culturable techniques isolating microorganisms from substrate, as well as additional stress being placed on the microbes (Van der Merve, *et al*., 2002; McNaughton *et al*., 1999; Zelles and Bai, 1993). Therefore, indirect methods such as phospholipids ester linked fatty acids have been developed. The application of "signature" chemicals to estimate microbial biomass and to recognise the microbial components of communities has been used by many researchers (Van der Merve *et al*., 2002; Lawlor *et al*., 2000; Roslev *et al*., 1998; Zelles and Bai, 1993). This quantitative measurement of PLFAs has been regarded as one of the most sensitive and reliable chemical measures of microbial biomass and community structure. (Lawlor *et al*., 2000; Roslev *et al*., 1998; Zelles and Bai, 1993).

Methods based on extraction and fractionations of microbial PLFAs have become increasingly popular because of their relative simplicity and good resolution. Phospholipids ester linked fatty acids (PLFAs) may be used as sensitive chemotaxonomic biomarkers and individual fatty acids or fatty acid signatures (Lawlor *et al*., 2000; Roslev *et al*., 1998; Zelles and Bai, 1993). A complementary method by which the shift in microbial community structure can be monitored in greater detail is denaturing gradient gel electrophoresis (DGGE). This method makes use of the 16SrRNA molecule carried by all bacteria, the sequences of which provide molecular markers for species identification (McNaughton *et al*., 1999). The method was used for profiling microbial populations in environmental samples by various authors (Kowalchuk *et al*., 1999; Stephen *et al*., 1999; Van Hanenn *et al*., 1999; Felske *et al*., 1998; Gillian *et al*., 1998; Ovreas and Torsvik, 1998; Muyzer *et al*., 1993).

The overall aim of the project was treatment of RWDW and FTWDW using methanogenic bacteria in different bioreactor configurations. It is general knowledge that microorganisms from the methanogenic sludge that were able to survive within these bioreactors were either

able to utilize the WDWs as COD or predadotory to other microorganisms as anaerobic sludges are made up of bacterial consortia (Section 3.4). The objective of this study was to profile microorganisms in laboratory scale anaerobic digesters both anaerobic high rate digesters, and two stage anaerobic submerged membrane bioreactors (SMBRs). These bioreactors were used in the treatment of raw wine distillery wastewater (RWDW) and fungally-pretreated wine distillery wastewater (FTWDW).

8.2 Materials and methods

8.2.1 Sample collection and lyophillization

Samples were collected on days 4, 10, 34, 68, 98 and 120 from high rate anaerobic digestion of RWDW, days 8, 10, 28, 30, 44, 50, and 60 from high rate anaerobic digestion of FTWDW and days 14, 24 and 28 from SMBR systems treating RWDW and FTWDW. The feed and other characteristics of each bioreactor correspond to those in Section 4.2.1; Section 5.2.1; Section 6.2.2 and Section 7.2.1 respectively. After collection samples were frozen in liquid nitrogen and then freeze dried into powder (Edwards Freeze Drier Modulyo), following a method by Chun *et al.* (1997). Samples were then stored at -80º C.

8.2.2 Phospholipid fatty acids extraction, transesterification and analyses

Analyses of the PLFA profiles were conducted for the samples taken during the anaerobic fermentation of the RWDW and FTWDW. The modified method of White *et al.* (1998) was used. Between 1.5 and 3.5 g of the lyophilized sample was mixed with 5 ml of phosphate buffer (pH = 7.4), and shaken overnight. The aqueous phase was decanted into a capped test tube, and an equal volume of the $CHCl_3$:MeOH (7:3, v/v) mixture was added. The contents of the tube were shaken at room temperature for 1 hour, and then centrifuged at 3800 rpm using a Hettich Universal Centrifuge (Labotec, Cape Town, RSA). The aqueous phase was discarded, and the organic phase was subsequently evaporated to dryness under a gentle stream of N_2 (Afrox, Johannesburg, RSA). The residue was redissolved in 200 µl of $CHCl_3$, and stored at -20 ºC until fractionation, transesterication and analyses.

For lipid fractionation, a protocol was kindly provided by Dr. Nicole Richoux from the Department of Zoology and Entomology of Rhodes University. Cotton wool plugs were placed in the constriction of the required number of Pasteur pipettes (Merck Ltd., Johannesburg, RSA), and pipettes were combusted in a muffle furnace at 450 ºC overnight. A 0.8 g portion of silica gel (200 µm particle diameter) was weighed and transferred into each Pasteur pipette, and care was taken so that no particles were seeping through the cotton wool plug. The silica gel particles were subsequently activated by combusting the Pasteur pipettes in the FSCI laboratory drying oven (Labcon via EC Labs, Port Elizabeth, RSA) at 100 ºC for 1 hour. The sample extracts were taken out of the freezer and equilibrated to room temperature. After the activation period the pipettes were allowed to cool to room temperature in a dessicator, and stored until fractionation of lipids. In the mean time, the $CHCl_3$:MeOH:formic acid (98.5:1.0:0.5, v/v) mixture was prepared for lipid separation procedure.

Thawed sample extracts were placed on a laboratory bench, and the pipettes for individual samples were clamped to a stand in an upright position. Two aliquots of 3 ml of methanol were carefully passed through the silica gel by pipetting down the side of the respective Pasteur pipette, in order to avoid disturbance of the levelled silica gel layer. The methanol wash was intended to activate the silica gel for extraction, and the silica gel was at all times under the methanol layer. As the methanol reached the top of the silica gel layer in the Pasteur pippette, 2 × 3 ml of $CHCl_3$ were passed through the column in the same manner as with methanol. The procedure was repeated with the $CHCl_3$:MeOH:formic acid (98.5:1.0:0.5, v/v) mixture with slight modifications. Therefore the first 3 ml of the mixture were passed through the pipettes without collection. Once the second 3 ml aliquot of the $CHCl_3$:MeOH:formic acid (98.5:1.0:0.5, v/v) mixture had been added onto the column, a 30 ml centrifugation tube (Fluka, Johannesburg, RSA) was placed under the Pasteur pipette and collection for lipid fractionation started.

For lipid fractionation, the concentrated sample extracts were applied on top of the silica gel layer in a Pasteur pipette, once the layer of liquid reached the top of the silica gel. Eight millilitres of $CHCl_3$:MeOH:formic acid (98.5:1.0:0.5, v/v) mixture was subsequently added, and care was taken to keep the silica gel layer under the liquid. The collection of the eluant continued until the liquid reached the top of the silica gel layer. At this point in time,

another 30 ml glass centrifugation tube was switched for the first one. The first fraction collected from the silica gel column contained neutral lipids, and was discarded. The second fraction collection was finished after rinsing the column with 2 × 3 ml of acetone, and this was also discarded. Three millilitres of $CHCl_3$ were used to recover neutrality of the silica gel packing inside the Pasteur pipette, before the phospholipid fraction was finally eluted with 2 × 3 ml of MeOH, collected into a clean 30 ml glass centrifugation tube, and used for further processing.

The methanolic eluate was concentrated to approximately 2 ml under a gentle stream of N_2, and mixed with 2 ml MeOH:HCl (1:1, v/v) in a capped test tube. The vial was allowed to stand at room temperature overnight, to achieve transesterification of PLFAs. After 12 h, 1 ml of *n*-hexane and 0.1 ml of $KHCO_3$ (both purchased from Sigma-Aldrrich Ltd, Johannesburg, RSA) were added to the contents of each tube, and the tubes were vortexed for 30 s. The organic layer was pooled, and the extraction was repeated twice times. The organic layers were pulled, concentrated under a gentle stream of N_2 to about 50 µl and 150 µl of isooctane were added (Sigma, Johannesburg, RSA). The liquid was pulled into an automatic pipette, the walls of the test tube were rinsed to prevent any losses of fatty acid methyl esters (FAMEs), and the liquid was then transferred into a 2 ml amberglass GC vial equipped with an inlet. The vial was sealed with a PTFE-lined silicone septum, and the extracts were stored at -20 ºC until analyses.

The samples were subjected to GC/MS analysis using a 6980 Agilent gas chromatograph equipped with a 5890 quadruple mass selective detector (Agilent Technologies, Seattle, USA). For qualitative analyses of the samples, the method of McNaughton *et al.* (1999) was used for separation and detection of the FAMEs. The sample extracts were brought to room temperature and 1 µl of the concentrated sample extracts was injected on a SPB1 fused silica capillary column (60 m × 0.25 mm × 0.25 µm; purchased from Supelco, Johannesburg, RSA). The injector was kept at 270 ºC, the transferline temperature was set to 280 ºC and the ion source temperature was kept at 150 ºC. The energy of the ion source was set to 70 eV. The column temperature was initially held at 60 ºC for 2 minutes, ramped to 150 ºC at 10 ºC/min, and then finally to 312 ºC at 3 ºC/min. The identities of the individual FAMEs were determined by comparison of the mass spectra, obtained by operating the system in full-scan mode, with the spectra from the WILEYs 1998 mass

spectral library, as well as by the analyses of the MATREIA standard FAME mixture (Sigma, Johannesburg, RSA). Results were evaluated by principal component analysis (PCA) (version 1.57 from http://folk.uio.no/ohammer/past/download.html) using the PAST freeware program.

8.2.3 Deoxyribonucleic acid isolation

Approximately 300 mg of biofilm or lyophilized samples were pre-treated using lysozyme. Nucleic acids were then extracted using the hot phenol:isoamyl alcohol:$CHCl_3$ first, and then using a second extraction step using isoamyl alcohol:$CHCl_3$ (Akarsubasi *et* al., 2005). Deoxyribonucleic acid (DNA) content was quantified spectrophotometrically, and 100 ng of template was used for polymerase chain reaction (PCR) amplification of 16S ribosomal DNA.

8.2.4 Polymerase chain reaction amplification

Polymerase chain reaction (PCR) was performed using the Hybaid Omne (UK) thermal cycler. The PCR method was a modified form from Akarsubasi *et* al.,(2005). The PCR master mix had the following composition: 2.5 U *Taq* DNA polymerase in 20 mM Tris-HCl buffer, 100 mM KCl, 3 mM $MgCl_2$, Brij 35, 0.01 % (v/v), 0.04 mM dNTP mix (dATP, dCTP, dGTP, dTTP), with the final pH value of 8.3 being reached at 20 ºC (PCR Master Roche, Germany). At the same time, 4 mM $MgCl_2$ and 50 ng BSA was added to 25 µl of the PCR mix. The PCR was run using universal bacterial primers GM5F (56 bp, 5'-GCC CGC CGC GCC CCG CGC CCG TCC CGC CGC CCC CGC CCG CCT ACG GGA GGC AGC AG-3') and 907R (20 bp, 5'-CCG TCA ATT CCT TTG AGT TT-3') for 35 cycles with the following conditions: initial one cycle at temperature 95 ºC for 300 seconds, 35 cycles of 94 ºC for 30 seconds followed by 65 ºC for 30 and finally 72 ºC for 1 minute. Then, finally, the temperature was held at 72 ºC for 300 seconds for one cycle, and dropped to 4 ºC.

8.2.5 Denaturing gradient gel electrophoresis analyses

Denaturing gradient gel electrophoresis (DGGE) analyses were performed on the D-Code system from BioRad (UK), and the conditions of Muyzer *et al.* (1993) were used. The

DGGE samples from the PCR were applied onto the 8 % (w/v) polyacrylamide gels prepared with 1.0 × TAE (20 mM Tris-acetic acid buffer with pH of 7.41, 10 mM sodium acetate, 0.5 mM Na-EDTA). The gradients in the gels were formed using 8 % (w/v) stock solutions of acrylamide with acrylamide-N,N'-methylenebisacrylamide ratio of 37:1). They contained 30 and 80 % of denaturants, i.e. 7 M urea and 40 % (v/v) formamide. These were deionized with AG501-X8 mixed-bed resin from Bio-Rad (UK). Electrophoresis was run at 100 V and 60 °C for 16.5 hours. Staining of the gels after electrophoresis was achieved by incubation in a solution of ethidium bromide in MilliQ water (0.0001 %, w/v). After staining, the gels were transluminated at 302 nm using the Cybertech CS 1 (UK). GeneGenius (UK) and the Syngene Synoptics software (UK) were used for gel documentation.

8.3 Results and Discussion

8.3.1 Phospholipid fatty acids results evaluation

The concentrations of the individual PLFAs detected in the samples from anaerobic digestion of the RWDW and FTWDW are summarised in Tables 8.1 and 8.2. For RWDW, the values of the PLFA concentrations ranged from 2196 pmol/g.d.w. observed for C20:1:w9 on day 10 up to 7000075 pmol/g.d.w. observed for 12Me-C14:0 on day 68. For the FTWDW, the values of the PLFA concentrations ranged from 1536 pmol/g.d.w. observed for C20:1:w9 on day 10 up to 395560 pmol/g.d.w. observed for 12Me-C14:0 on day 44. The data were transformed for the purposes of the PCA analyses, using the routine of Meglen (1992). As the first step the respective arithmetic averages X_{avg} were calculated using MS Excel according to Eq. (1).

$$X_{avg} = \frac{\sum_{i=1}^{n} X_i}{n} \tag{1}$$

Where X_i is the concentration of the particular PLFA at the particular time during anaerobic fermentation of RWDW or FTWDW, and n is the total number of samples for a particular PLFA. Subsequently, the standard deviations of the set of data for all PLFAs included in the PCA analyses were calculated using Eq. (2).

$$SD = \left[\frac{\left(X_i - X_{avg}\right)}{n(n-1)} \right]^{\frac{1}{2}} \tag{2}$$

Table 8.1: Concentrations of PLFAs during anaerobic digestion of RWDW (pmol/g.d.w).

PLFA	Day 4	Day 10	Day 34	Day 68	Day 98	Day 120
C14:1	37424	19319	24949	38758	3791	11676
C14:0	54653	65727	44746	73145	13875	24557
iC15:0	92854	65727	86646	85110	20825	51377
aC15:0	ND	339445	444694	700075	249180	298735
C15:0	121098	88153	121567	138775	64274	82753
2OH-C14:0	ND	ND	ND	ND	ND	ND
14Me-C15:0	38992	33086	39106	43836	15074	23026
13Me-C15:0	151796	129553	85063	247708	206177	188624
C16:1w9c	17654	8819	4362	38246	8756	11089
C16:1w7t	5140	5531	1709	2996	3255	4023
C16:0	220365	168866	194331	397536	291815	213027
15Me-C16:0	ND	13354	20359	17073	7379	14021
14Me-C16:0	111143	71193	103112	131589	76636	77725
C17:1	35159	27383	43429	37280	49356	77024
Cyc-C17:0	9486	ND	ND	11366	ND	ND
C17:0	62829	42082	64702	75430	48816	41972
C18:2w6c	45797	24587	3981	192884	74892	34804
C18:3w3	16087	15426	23275	7259	3448	14238
C18:1w9c	59911	32560	42753	216610	92165	48100
C18:1w7c	132395	94680	97122	225438	129069	100571
C18:1w7t	9299	6838	9324	4489	2393	5804
C18:0	72523	51402	75430	90672	60686	72257
C19:1w12	17307	9199	13035	10182	2206	6360
Cyc19:0	14482	8506	6611	16016	8370	7114
C20:1w9	ND	2196	14161	2379	2139	6750
C20:0	5628	3736	6919	5435	2255	3720
C21:0	3660	ND	ND	ND	ND	ND
C22:0	2896	ND	ND	ND	ND	ND

ND = not detected

Table 8.2: Concentrations of PLFAs during anaerobic digestion of FTWDW (pmol/g.d.w).

PLFA	Day 10	Day 30	Day 44	Day 50	Day 60
C14:1	17312	25859	29922	4632	38853
C14:0	35170	40860	46499	10059	55003
iC15:0	77959	79168	93877	35490	113179
aC15:0	337939	385745	395560	205399	ND
C15:0	91775	97425	103032	57838	125645
2OH-C14:0	ND	1546	ND	ND	ND
14Me-C15:0	55244	54925	35384	24234	49186
13Me-C15:0	85606	154330	91055	103101	ND
C16:1w9c	4266	25450	5837	5693	16556
C16:1w7t	8583	12547	7337	4484	11157
C16:0	213082	289488	143223	150355	247183
15Me-C16:0	19080	16597	18147	12238	18981
14Me-C16:0	88526	102254	80507	66912	93533
C17:1	30877	27060	32043	27186	31528
Cyc-C17:0	ND	ND	ND	ND	ND
C17:0	50109	47143	47776	39987	54276
C18:2w6c	61231	74119	12249	18375	42413
C18:3w3	14054	16814	20866	18583	18839
C18:1w9c	198640	104211	21650	28929	50190
C18:1w7c	77217	144553	ND	88680	137559
C18:1w7t	5898	6900	7441	7348	7487
C18:0	91124	81914	47402	45595	70732
C19:1w12	14810	8529	12968	8940	12198
Cyc19:0	5576	16955	8119	10644	18274
C20:1w9	1536	4367	ND	7546	2000
C20:0	7625	6880	5595	3509	4660
C21:0	ND	3018	3656	ND	2833
C22:0	ND	4297	2020	ND	2597

ND = not detected

Then the *z*-scores for the individual data points were calculated according to Eq. (3).

$$z = \frac{X_{\mathrm{i}} - X_{\mathrm{avg}}}{SD} \tag{3}$$

Principal component analysis (PCA) of the data for the RWDW was performed using the PAST freeware software package. Results are summarised in Tables 8.3 and 8.4.

Table 8.3: Results of the PCA analysis of RWDW PLFA data.

Principal component no.	Eigenvalue	Percentage of total variance (%)
1	11.0	47.1
2	6.6	28.0
3	3.6	15.6
4	1.5	6.2
5	0.8	3.6

Table 8.4: Results of the PCA analysis of FTWDW PLFA data.

Principal component no.	Eigenvalue	Percentage of total variance (%)
1	11.4	44.0
2	6.5	24.9
3	5.6	21.7
4	2.5	9.5

Several PLFAs were not detected in certain samples. Based on the criteria for missing data handling developed by Meglen (1992), the data for the following PLFAs were omitted from the PCA analyses: 2OH-C14:0 and cyc-C17:0 for RWDW and FTWDW, C21:0 and C22:0 for RWDW. Results in Table 8.3 show that five principal components explain 100 % of the variance in the PLFA data from the fermentation of RWDW. The Jollife cut-off was equal to 0.678. This indicated that five microorganisms were present in the fermentor during the fermentation of RWDW. Loadings of individual principal components were below 0.5, which would indicate moderate or strong influence of individual PLFAs on the variance of the particular principal component. That is why the cut off point of the significance for individual loadings was set to 0.15, taking the respective Eigenvalues into account. Any PLFA with a higher loading than 0.15, for a particular principal component, i.e. microorganism, was considered to be significant and used for analysis of microbial community dynamics. For principal component 1, the following PLFAs had loadings above the cut-off point: C14:1, iC15:0, C15:0, 14Me-C15:0, C16:1w9c, 14Me-C16:0, C17:0, C18:1w9c, C18:1w7c, C18:0 and Cyc-C19:0. For principal component 2, the following PLFAs had loadings above the cut-off point: 13Me-C15:0, C16:1w9c, C16:0, C18:2w6c, C18:1w9c and C18:1w7c. For principal component 3, the following PLFAs had loadings above the cut-off point: aC15:0, 15Me-C16:0, C17:1 and C20:1w9. For principal component 4, the following PLFAs had loadings above the cut-off point: C14:0, aC15:0 and 15Me-C16:0. For principal component 5, the following PLFAs had loadings above the cut-off point: C17:0.

Results in Table 8.4 show that 4 principal components explain 100 % of the variance in the PLFA data from the fermentation of FTWDW. The Jollife cut-off was equal to 0.7. This indicates that four microorganisms were present in the fermentor during the fermentation of RWDW. Loadings of individual principal components were below 0.5, which would indicate moderate or strong influence of individual PLFAs on the variance of the particular principal component. A cut off point of the significance for individual loadings was set to 0.15, taking the respective Eigenvalues into account. Again, any PLFA with a higher loading than 0.15, for a particular principal component, i.e. microorganism, was considered to be significant and used for analysis of microbial community dynamics. For principal component 1, the following PLFAs had loadings above the cut-off point: C14:0, C14:1, iC15:0, C15:0, 14Me-C15:0, C16:1w7t, C16:0, 15Me-C16:0, 14Me-C16:0, C17:0 and C18:0. For principal component 2, the following PLFAs had loadings above the cut-off point: 13Me-C15:0, C16:0, C18:2w6c, C18:1w9c, C18:1w7c, C18:0 and C20:0. For principal component 3, the following PLFAs had loadings above the cut-off point: C16:1w9c, C18:1w7t, C18:1w7c, Cyc-C19:0, C20:1w9 and C22:0. For principal component 4, the following PLFAs had loadings above the cut-off point: aC15:0, 13Me-C15:0 and C21:0.

The saturated and straight-chained PLFAs, i.e. C15:0, C17:0 and C18:0, have been shown to be widespread among different types of organisms (Zelles, 1999). Cyc-C19:0 PLFA was indicated in species 1 from the raw wine distillery wastewater digestion, and in species 3 from FTWDW. Phospholipids ester linked fatty acids containing a cyclopropyl ring in their structure have been reported in strictly anaerobic species, such as *Clostridium* spp., as well as the synthesized when bacterial species face stress factors, e.g., presence of toxic compounds in the extracellular medium. Strictly anaerobic species have also been suggested to contain vaccenic type of PLFAs (Zelles, 1999). These have been indicated in significant levels in species 1 and 2 from RWDW digestion by the presence of C18:1w7c. For the FTWDW, the same PLFA together with its trans counterpart C18:1w7t, were indicated to be significant in species 2 and 3. The presence of trans unsaturated PLFAs has been suggested as a protective mechanism when changes in the fluidity of biological membranes threaten the integrity of the cell (Yeom and Daugulis, 1999).

Branched PLFAs have been shown to originate mostly from *Actinomycetes*, which are Gram-positive, and able to thrive under anaerobic conditions (Stackebrandt *et al.,* 1997). Oleic type of PLFAs, i.e. C16:1w9c, C18:1w9c, and C20:1w9 have been shown to be present in Gram positive bacteria (Zelles, 1999). Their presence was indicated in species 1, 2 and 3 for the digestion of RWDW, and for species 3 in the digestion FTWDW. C18:2w6c is the linoleic acid, and is the only polyunsaturated PLFA (Zelles, 1999) detected in both samples from RWDW and FTWDW. The presence of polyunsaturated PLFAs has been reported in protozoa, fungi, cyanobacteria and algae (Zelles, 1999). At the same time, detection of both polyunsaturated PLFAs and cyclopropyl containing PLFAs in the same sample has been reported at the aerobic/anaerobic interfaces (Zelles, 1999).

The system did not have to be de-sludged during the entire operation. This could indicate that microaerophilic pockets could have been present in the digester system, as well as the presence of protozoa which preyed on the bacterial populations present. Its presence and fluctuations in the concentration of this PLFA, during the digestion of the FTWDW could be explained by the presence of fungal debri in the bioreactor. The logarithm of the 1-octanol/water partition coeffcient for linoleic acid has been calculated to be equal to 7.05 (Tandlich, 2006). This indicates that the compound is highly hydrophobic, and so might have been sorbed onto the rest of fungal cells present in the anaerobic digester. In the RWDW the other reason is that this fatty acid has been detected in eukaryotes and anaerobic system. Its presence in the RWDW can only constitute contamination of the samples during laboratory processing. Iso and anteiso PLFAs are widespread and can be indicative of both Gram positive and negative bacteria.

8.3.2 Denaturing gradient gel electrophoresis results evaluation

Thirteen samples were processed using DGGE analysis. The list of samples, with appropriate designation, is shown in Table 8.3. The DGGE gel is shown in Figure 8.1. As it can be seen 10 bacterial species were detected in the samples, with the GC content of the 16S rRNA increasing from one to ten. Species A was detected in sample 1. Species B was detected in samples 2 and 3, while species C was detected in samples 10, 11 and 12. Species D was detected in sample 12. Species E was detected in samples 6, 8 and 9. Species F was

detected in sample 2 while species G was detected in samples 2, 3, 7, 10 and 11. Species H was detected in samples 2 and 3 while species I was only detected in sample 12. Species J was detected in samples 1to 13 inclusive. The bands for Species H were not clear for samples 3, 4, 6 and 8. Species diversity for sample 3 obtained from day 10 of RWDW treatment was detected, equivalent to species B, G, H and J. Four species were observed on sample 12, equivalent to day 68 of anaerobic treatment of RWDW. The species were C, D, I and J. Three of the species from sample 12 were new, which indicated population shift during wastewater treatment. Sample 13 was taken on day 98 of anaerobic treatment of RWDW. Only one band was visible enough and is equivalent to species J. Five more bands were very faint on the lane thus could not be quantified.

For FTWDW treatment by high rate digestion, the number of species increased with time from two to five at the beginning i.e. day 8 to day 10. Species diversity had dropped again to three species found on days 28 and 44 during treatment of FTWDW. Species J was able to survive during the entire treatment, while species C and G needed about nine days to reach sufficient numbers, to be visible on DGGE gels or for sufficient weight of 16S rRNA for a band to be visible on the DGGE gel. For treatment of RWDW on SMBR one species prevailed in the ultra-filtration module and two on the secondary digester. Species shifted from J to species G and J in the secondary digester. For FTWDW in the SMBR, the number of species was constant over time between days 14 and 28. In the UF membrane module two species were identified from beginning to end i.e. species E and J. For the secondary digester, species numbers increased with time from one to three. Species J was detected after 14 days of operation and species E, I and J at day 28.

Since the WDW was autoclaved prior to treatment, the only source of all species was the methanogenic sludge inoculum used in the case of AD or ADUF of RWDW, and the fungal biomass was the only other source for FTWDW. The digester inoculum accounts for the bacteria present in FTWDW.

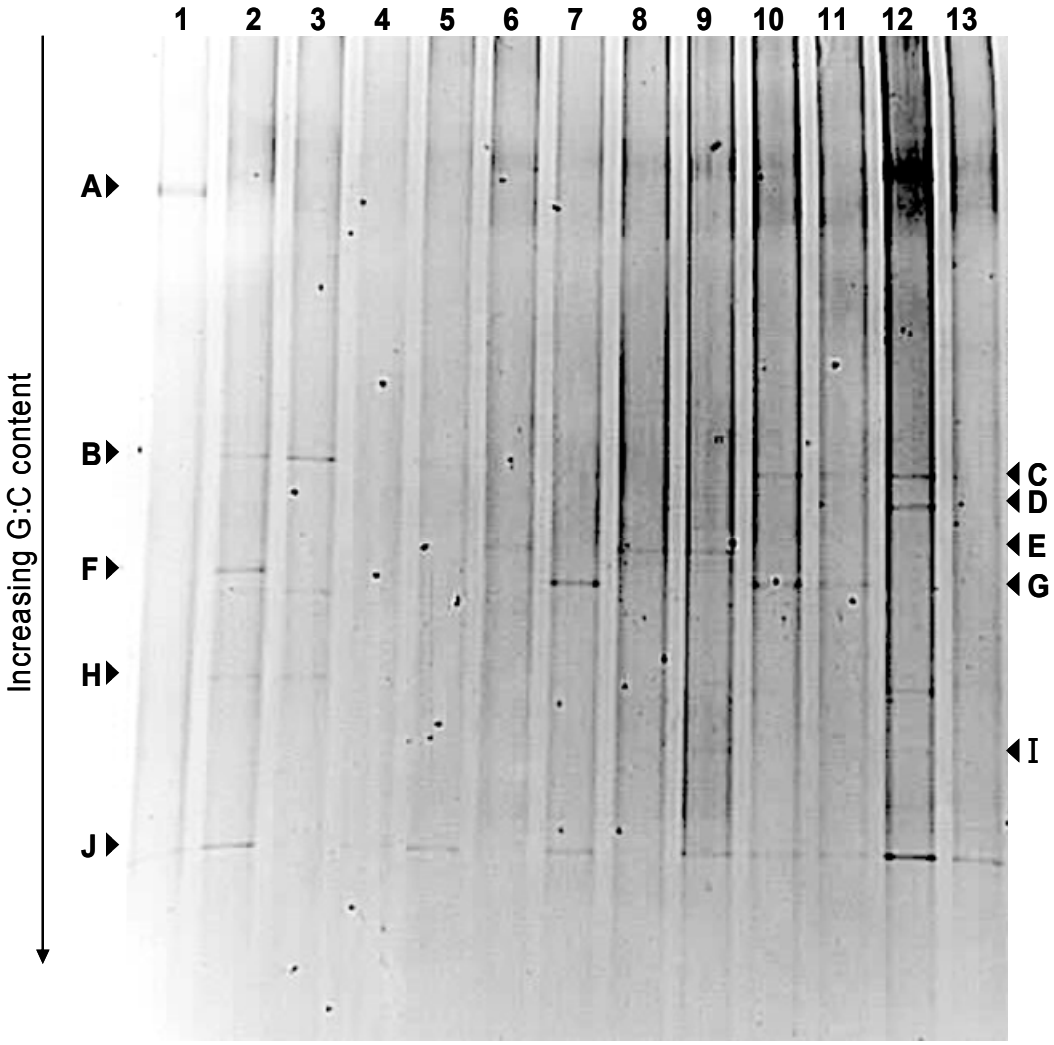


Figure 8.1: DGGE profiles of DNA extracted from samples taken during treatment of RWDW and FTWDW.

Table 8.5: Identification of samples in Figure 8.1, above.

Lane / sample number	Sample description (treatment system, wastewater, time)	Number of bands
1	AD, FTWDW, day 8	2
2	AD, FTWDW, day 10	5
3	AD, RWDW, day 10	4
4	4 SMBR, FTWDW, day 14	1
5	2 SMBR, RWDW, day 14	1
6	2 SMBR, FTWDW, day 14	2
7	2 SMBR, RWDW, day 24	2
8	2 SMBR, FTWDW, day 28	2
9	4 SMBR, FTWDW, day 28	3
10	AD, FTWDW, day 28	3
11	AD, FTWDW, day 44	3
12	AD, RWDW, day 68	4
13	AD, RWDW, day 98	1

8.4 Interim conclusions

Phospholipids ester linked fatty acids analysis confirmed diversity of bacterial communities found in anaerobic systems as four and five species of FTWDW and RWDW were identified. The PCR-DGGE method is considered as a rapid and reliable method for the relative comparison of different bacterial communities. The method also provides a comparison of the true sequences if DGGE bands are excised and sequenced. Analysis of DGGE data in Figure 8.1 indicated that the composition of the archeal community changed for both RWDW and FTWDW anaerobic treatment. Changes in band intensities were also indicative of the presence of different components of the archeal communities. Results from PCR-DGGE were not conclusive in terms of species identity as cloning, sequencing and phylogenetic analysis was not performed. However these results were able to indicate species diversity for high rate anaerobic digestion. The results also confirmed prevalence of few species during operation of SMBR for treatment of RWDW and FTWDW which suggested that the microorganisms that survived were either tolerant of toxic concentrations of RWDW and FTWDW or they were able to remove polyphenols.

CHAPTER 9
General Discussion

Anaerobic digestion of dissolved, suspended and solid organic matter has rapidly evolved but nevertheless still faces several scientific unknowns. The fundamentals of microbial shifts and diversity that occur during treatment of wastewaters, particularly WDW was identified as an issue a decade ago (Verstraete *et al.*, 1996) and still needs to be addressed today. Microbial population shifts from those in conventional digesters or municipal wastewater treatment systems are expected during the treatment of high strength, organic wastewaters. In order to verify these population shifts efficient design and operation of anaerobic digesters is essential. Thus for treating wastewaters a novel high performance bioreactor like a dual stage ceramic membrane bioreactor is necessary. This bioreactor requires anaerobic consortia to grow in a dense and eco-physiologically well organised way. Phospholipids ester linked fatty acids (PLFA) analysis and use of 16S rRNA sequences have the potential of explaining the different types of aggregates and population shifts during process operation. Due to long term operation of high rate anaerobic digestion, development of bacteria capable of degrading xenobiotics is necessary. Integration of UF in anaerobic digestion facilitates operation at higher VLRs and shorter SRTs.

As compared to conventional aerobic methods and in the light of the implementation of sustainable technologies, anaerobic processes solve problems in a much more holistic way. Instead of consuming energy, useful energy is produced in the form of biogas. Only a few percent of COD are converted into new biomass so that the volume of surplus sludge produced is significantly lower. Moreover, anaerobic sludge has a high dewatering capacity and is generally well stabilized. The sludge can be applied at any place and any scales subject to pathogen kill requirements. Biomass retention is very important for anaerobic processes, where the slow growth rate of anaerobic bacteria imposes the necessity to concentrate the methanogenic consortia in the bioreactor. Methanogenic biomass growth rate is very slow from about 7 to 12 days of lag phase (Verstraete *et al*., 1996; de Zeeuw, 1984).

High average COD and polyphenol removal efficiencies of 87 % and 63 % were obtained during high rate anaerobic digestion of RWDW (Chapter 4). These removal efficiencies were comparable to literature values (Wolmarans and De Villiers, 2002; Laubscher *et al.*, 2001; Harada *et al*., 1996; Driessen *et al.*, 1994), especially when considering the low pH of 3.8 in the RWDW. The microorganisms in the anaerobic digester also proved to be intolerant to RWDW as the digester was operated at low organic loading rates of 5 % (v/v) for the first 52 days of 130 days MCRT. During this period pH adjustment had to be performed every 6 days. The pH of the feed was adjusted to about 7 using 0.1 M NaOH to ensure operation of anaerobic bacteria as some authors maintain that the optimum pH of acidogenic bacteria is 5.2 to 6.5, and the growth rate is over 2 days (Solera *et al*., 2002). At the same time methanogens grow very slowly with a minimum doubling time of 3.6 days and the optimum pH of the bacteria is between 7.0 and 7.5. Thus it was also concluded that the anaerobic bacteria need time and a suitable pH to acclimatize to the organic RWDW to allow development of microorganisms that are toxic tolerant and mostly able to treat polyphenols.

There was a major decrease in phosphate concentration from day 28 to day 50 which led to the digester failure on day 52, when the pH increased rapidly to 12.34, solubilising the contents of the digester. The digester was revived successfully. To ascertain the necessity of phosphate to buffer the digester $CaCO_3$ was added, followed by a combination of $CaCO_3$ and K_2HPO_4, and K_2HPO_4 alone. Buffer capacity in the digester was improved when K_2HPO_4 was added alone, compared to the use of a combination of $CaCO_3$ and K_2HPO_4 or $CaCO_3$ alone. Volatile fatty acid concentrations fluctuated less after the induction of pH buffering (Figure 4.2). Addition of K_2HPO_4 alone also led to pH stability and robustness of the digester and VFAs were digested even at increased organic loading rates. Volatile fatty acid concentrations, which were mostly low throughout the study especially after addition of micronutrients, were also indicative of robustness, as well as MLSS concentrations. Theories postulated about the behaviour of nitrates and ammonia, as the trends were consistent throughout the high rate AD and SMBR need further investigations, as indicated in Chapter 10. The characteristics of RWDW listed in Table 7.1 confirm what had already been discussed in Chapter 2 regarding the variability of nutrients in WDWs. High levels of COD_s and polyphenols, as well as low pH of 3.8 were consistent with literature (Nataraj *et al*., 2006; Eusébio *et al*., 2004; Martin *et al*., 2002; Ramana *et al*., 2002a; Genovesi *et al*., 2000; Benitez et al., 1999b; Jimenez and Borja, 1997; Harada *et al*., 1996; Borja *et al*.,

1993; Tofflemire, 1972). Addition of Fe_3^+ increased COD removal efficiency further to a maximum of 97 % and polyphenol removal efficiency increased to 65 %. High rate anaerobic digestion of RWDW resulted in increased pH from 3.85 to 7.05, CODs decreased from 4185.0 mg/l to 55.0 mg/l; while phenols decreased from 674.6 mg/l to 9.25 mg/l.

Colour and turbidity also improved as indicated by Table 4.1. The effluent quality from this treatment met irrigation standards by the Department for Water Affairs and Forestry (DWAF, 1996) which states that an effluent of this quality can be used for irrigation at the wineries at 500 m^3 of volume daily (Table 9.1). As these results were significant it was concluded that a high rate digestion of RWDW at 30 % wastewaters strength can be implemented at wineries to increase pH, reduce COD and treat polyphenols. However, a membrane was introduced to the digester in order to increase organic loading rates during treatment further and also to shorten sludge retention times. For large wineries that generate WDW which once treated cannot all be used for irrigation, use of high rate anaerobic digestion can still be beneficial as it could lower tariffs on wastewater discharged to the municipal sewer. The other benefits of high rate anaerobic digestion of RWDW are the fact that OLR of RWDW on the digester improved with longer operation. Methanogenic sludge generated at these plants would also be unique to winery type, less pathogenic and would withstand seasonal operations. Having this anaerobic digester at a winery would also be beneficial as anaerobic digestion of sludge produces much less biomass than aerobic digestion, while at the same time it is able to stabilize organic matter and convert much of the solids to end products of liquids and gases. An anaerobic environment is also necessary for denitrification, as the bacteria which carry out this process require anaerobic conditions to reduce nitrate to nitrogen gas as many nitrogen removal technologies are designed to provide an anaerobic treatment chamber as part of their treatment process. Success obtained during treatment of RWDW was also made possible by dosing with 50 mg/l of Fe^{3+} and these results were similar to those obtained by Sharma and Singh (2001) at the same concentrations. South African guidelines for water disposal indicate that the maximum concentration for heavy metals such as iron, copper, manganese and zinc is 5 mg/l. It is therefore recommended that the high rate digester be either operated without dosing, as Fe^{3+} had a negative effect on polyphenol removal, or decrease the Fe^{3+} dose to <5 mg/l as these concentrations were still effective in improving COD_S removal efficiencies. The high phosphate concentrations on the effluent (100 mg/l -190 mg/l) were necessary to buffer the digester and improve methanogenic activity. These values were far too low when compared

to 1 g/l of phosphate needed for methanogenic activity in the upflow anaerobic sludge blanket (UASB) bioreactor investigated by Sharma and Singh, (2001).

Table 9.1: DWAF (1996) guidelines for irrigation water quality targets.

Parameter	Target water quality range	Parameter	Target water quality range
Aluminium	0 – 5 mg/l	Manganese	0 – 0.02 mg/l
Arsenic	0 – 0.1 mg/l	Molybdenum	0 – 0.01 mg/l
Beryllium	0 – 0.1 mg/l	Nickel	0 – 0.2 mg/l
Boron	0 – 0.5 mg/l	Nitrogen (inorganic)	0 – 0.5 mg/l
Cadmium	0 – 10 μg/l	pH	6.5 – 8.4
Chloride	0 – 1.0 mg/l	Selenium	0 – 0.02 mg/l
Chromium	0 – 0.1 mg/l	Sodium	0 – 70 mg/l
Cobalt	0 – 0.05 mg/l	Sodium absorption ratio	0 – 1.5
Faecal coliforms	< 1 counts/100 ml	Suspended solids	0 – 50 mg/l
Copper	0 – 0.2 mg/l	Total dissolved solids	0 – 40 mg/l
Fluoride	0 – 2 mg/l	Uranium	0 – 0.01 mg/l
Iron	0 – 5 mg/l	Vanadium	0 – 0.1 mg/l
Lead	0 – 0.2 mg/l	Zinc	0 – 1 mg/l
Lithium	0 – 2.5 mg/l		

Pre-treatment of RWDW with the characteristics listed in Table 7.1 improved its pH to 6.7, reducing the pH buffering requirements for anaerobic digestion, decreased COD_s from 15 000 mg/l to 7000 mg/l and decrease polyphenols from 5229 mg/l to 1440 mg/l. Pre-treatment of RWDW with *Trametes pubescens* led to COD removal of 53.3 % while treatment of this same wastewater diluted to 30 % (v/v) wastewater strength led to 99.5 % COD removal. It was concluded that fungal pre-treatment of RWDW is a viable method for pre-treatment of WDW characterized by high antibacterial activity and low pH. An extra biorector was necessary to carry out fungal pre-treatment of WDW, in this case bubble lift bioreactor. However, this was equivalent to employing an extra bioreactor to the main stream process. This extra biorector has guaranteed superior efficiency as already mentioned. However, to further confirm the effectiveness of incorporating the fungal pre-treatment step a control reactor operating with same hydraulic conditions of the bubble lift biorector should be employed. This control reactor should be operated aerobically without fungal treatment and then treatment efficiencies of both streams i.e. fungal pre-treatment coupled with main stream treatment and the control biorector coupled with main stream, should be compared. Although the performance of anaerobic digester during treatment of

FTWDW was better that in RWDW, colour removal was not obtained. Recommendations on necessary experiments are given in Section 10.2. Colour removal during treatment of wastewaters has to be monitored as it is a qualitative characteristic that can be used to assess the general conditions of the wastewater. Colour in the water may result from presence substances such as metallic ions, humus, weeds and industrial wastes and has to be removed to make effluent suitable for industrial use. Parameters like pH, COD_S and polyphenols also complied with irrigation standards (Table 9.1), except for colour and turbidity. This implied that a treatment step for colour removal from this effluent has to be incorporated before effluent disposal. Wine distillery wastewaters are known for a strong colour from reddish to brown (Bustamante *et al*., 2005; Borja *et al*., 1993) and treatment of these wastewaters with fungi further exacerbates the problem (Chapter 6) thus, colour removal during or after anaerobic becomes essential. An aerobic step must be incorporated after FTWDW high rate anaerobic digestion to remove colour as described by Singh and Thakur (2006). Singh and Thakur (2006) performed a similar experiment on pulp and paper mill wastewater that had been first treated anaerobically. A more complex, four-reactor system was used for a short term test in an attempt to increase OLR and shorten SRT. While this ADUF system was more robust in terms of its ability to withstand fluctuations in influent quality, the COD and polyphenol removals achieved did not meet DWAF guidelines for irrigation. The results indicated that the polyphenols present in the RWDW were exerting too strong an inhibitory effect on the bacterial consortia in the reactors, and further confirming that at AD was not viable as the first treatment step. Parameters monitored are listed in Appendix B.1 and B.2. Chapter 6 presents results observed when using the four-reactor ADUF system as a second treatment step after a pre-treatment of RWDW using lignolytic fungi called *Trametes pubescens* to generate FTWDW.

The two-step treatment system decreased the COD of the final effluent from ADUF treatment of RWDW from 1100 mg/l (in Chapter 5) to 400 mg/l. Phenol removal was not as consistent as COD removal, but a minimum effluent polyphenol concentration of 1 mg/l was achieved at one stage, and an average effluent concentration of 9.25 ± 0.64 mg/l was demonstrated by AD of RWDW. The most striking result observed was that the reduced phenol concentration in the FTWDW compared to the RWDW enabled more stable digester operation, and the requirement for the UF membrane became questionable. Thus it was concluded that, although treatment of RWDW and FTWDW was not optimized in the

SMBR; these MBRs have potential for treatment of WDWs and can offer insight to the secondary metabolites that accumulate during treatment as the membrane system separates low molecular weight polyphenols from high molecular weight. High COD_S and polyphenol removal efficiencies were obtained from high rate anaerobic digestion of RWDW compared to FTWDW digestion; while ADUF treatment of FTWDW obtained better CODs and polyphenol removal efficiencies than ADUF of RWDW. For high rate anaerobic digestion the success was because of the long start up periods with low OLR and pH stability. High rate anaerobic digestion of FTWDW was started at 30 % wastewater strength which led to removal of other CODs but not toxic phenols. Dosing with macronutrients in high rate anaerobic digestion only improved CODs removal but not phenols. The pH stability led to VFA digestion in ADUF of FTWDW. The success of ADUF of FTWDW can also be attributed to pre-treatment step of RWDW. Treatment of RWDW on SMBR was least efficient in terms of CODs and phenol removal efficiencies probably because of the efficiency of MBR in retaining the high molecular weight polyphenols, also operated at high M/F ratio and 30 % wastewater strength which resulted in the SMBR system struggling with pH and as a result high dosing of $CaCO_3$ and K_2HPO_4 to achieve pH stability and higher removal efficiencies. Operation of SMBR/ADUF for RWDW and FTWDW clearly indicated that short SRT times were not suitable for the processes and it was concluded that longer SRT or/ starting operations at low OLR of WDW would be suitable (Zhang et al., 2006; Schoeberl.*et al*.,2005; Trussell *et al*., 2004; Huang *et al*., 2001). However, before implementation of any of these systems in a winery, these results would have to be reproduced using non-sterile WDW.

In SMBR systems the UF resulted in the secondary digester performing better than the MBR. Microorganisms in the SMBR needed a longer period for acclimation than the short SRT recommended (Stephenson *et al*., 2000) which can be achieved as indicated by Haung *et al*., (2001). These SMBRs also showed high potential for development of microorganisms capable of tolerating and degrading the WDWs as they were highly selective. The selectivity of SMBRs was tentatively confirmed by the presence of few species as shown by DGGE analysis. While time constraints precluded full phylogenetic analyses of the consortia in the high rate digesters and SMBRs used, PLFA analysis confirmed diversity of bacterial communities found in anaerobic systems as four and five species in FTWDW and RWDW treatment were identified. Analysis of DGGE data indicated that the composition of archeal community changed for both RWDW and

FTWDW anaerobic treatment. Use of SMBR than high rate anaerobic digestion cannot be ruled out as these were able to concentrate biomass similarly to others (Bustamante *et al*., 2005). Fouling was not a factor in the operation of SMBRs as they were operated for a short time, with SRT equal to 30 days. Considerable and useful data were obtained during development of ceramic SMBR; further experiments listed in Section 10.2 can finalize this development. Characteristics of the effluents from all treatments are listed in Table 9.2 and for SMBR systems the effluent characterized is from the secondary digester, suggesting that the digesters were operating even though the rest of the SMBR systems found the effluents toxic. Results confirm that pre-treatment with fungi yields better COD_S and polyphenol removals. Thus it can be concluded that at a treatment facility the steps should be pre-treatment with fungi followed by anaerobic digestion and then post treatment step for phosphates removal before reuse or disposal.

Table 9.2: Mean characteristics of effluents from high rate anaerobic digestion and SMBR (secondary digester) systems treating RWDW and FTWDW.

Treatment	**AD**		**AD**		**SMBR**		**SMBR**	
Wastewater	**RWDW**		**FTWDW**		**RWDW**		**FTWDW**	
Parameter ↓	**(n=76)**	**SD***	**(n=51)**	**SD***	**(n=16)**	**SD***	**(n=16)**	**SD***
pH	7.05	± 0.01	8.1	± 0.1	7.05	± 0.02	8.0	± 0.1
Phenols (mg/l)	9.25	± 0.64	40.0	± 3.6	48.0	± 261.5	53.1	± 1.2
COD_s (mg/l)	55.0	± 8.7	70.0	± 5.0	1129.0	± 47.1	75	± 5.2
NH_4 (mg/l)	4.98	± 0.01	2.8	± 0.1	35.0	± 0.1	3.0	± 0.1
NO_3 (mg/l)	10.0	± 0.02	7.4	± 0.2	8.0	± 62.4	5.6	± 1.3
PO_4^{3-} (mg/l)	60.0	± 11.3	98.0	± 9.4	97.0	± 81.7	110.0	± 0.7
VFAs (mg/l)	220.0	± 13.2	63.0	± 7.2	53.0	± 62.4	55.0	± 1.3

SD* = standard deviation

CHAPTER 10
Conclusions and Recommendations

10.1 Conclusions

10.1.1 Characterization of RWDW and FTWDW.

Results obtained from characterization of RWDW and FTWDW (Table 4.1 and 7.1) confirmed presence of high COD, high polyphenol concentrations, low pH, high concentration of phosphates, high turbidity and colour of these wastewaters and thus variability in the composition of WDWs. It was concluded that the high variability of WDWs makes them extremely difficult to treat.

10.1.2 Treatment of RWDW and FTWDW in high rate anaerobic digester.

High rate anaerobic digestion of RWDW was achieved as high COD_S and polyphenol removals of 87 % and 85 % were obtained. The addition of K_2HPO_4, (1000 mg/l) was essential for buffering the bioreactor and addition of 50 mg/l Fe_3^+ as micronutrient increased COD_S removal efficiency further, to a maximum of 95 %. Optimization of anaerobic treatment for RWDW was achieved at 30% wastewater strength. Pre-treatment of RWDW to obtain FTWDW is beneficial as it further improved CODs removal to 99.5 %, and the system proved able to eliminate shock loads of high input CODs concentrations.

10.1.3 Treatment of RWDW and FTWDW in dual-stage anaerobic SMBRs.

Anaerobic digestion UF is valuable for treatment of WDWs as biomass retention was achieved during operation of SMBR for the treatment of RWDW and FTWDW. Secondary digestion downstream of the SMBR, together with pH buffering using 8000 mg/l $CaCO_3$ and 4000 mg/l K_2HPO_4 stabilised COD_S removal for RWDW and readily biodegradable phenolics were removed from the RWDW. Secondary digestion during treatment of FTWDW led to the stabilisation of COD_S removal as residual COD_S levels were 400 mg/l. As a result it was concluded that that fungal pre-treatment of RWDW is essential as it provided additional nutrients for removal of recalcitrant components of the wastewater and thus improved performance in high rate anaerobic digestion and ADUF. Thus the secondary

digester incorporated to the SMBR significantly improved performance of ADUF for both treatments.

10.1.4 Population shifts characterization using PFLA analysis and 16S rRNA.

Analysis of PLFAs confirmed diversity of bacterial communities found in high rate anaerobic digesters as four and five species of FTWDW and RWDW were identified. Results obtained also confirmed population shifts during high rate anaerobic digestion of RWDW and FTWDW. Analysis of PLFAs in anaerobic digesters indicated significant diversity as the microoganisms were Gram negative and positive. The analysis also confirmed the presence of viable biomass in SMBR systems. Analysis of PLFAs in SMBR systems confirmed ADUF's selectivity as two species were obtained upon characterization. It was therefore concluded that, microorganisms that survived the high organic loading rates in SMBR systems were toxic tolerant and able to degrade polyphenols.

10.2 Recommendations for Further Work

The effluent quality obtained after treatment by high rate AD and ADUF of RWDW and FTWDW did not meet the standards required for use for crop irrigation. Concentrations of nutrients such as nitrates, ammonia and particularly phosphates were too high. Therefore a post treatment step should be incorporated to further reduce COD_S and to meet regulatory requirements. The most suitable step for post treatment may be aerobic, e.g. activated sludge treatment, as it has shown the ability to remove both COD_S and colour during treatment of other wastewaters and has biological nutrient removal ability. An extra biorector was necessary to carry out fungal pre-treatment of WDW, in this case bubble lift bioreactor. However, this was equivalent to employing an extra bioreactor to the main stream process. This extra biorector has guaranteed superior efficiency as already mentioned. However, to further confirm the effectiveness of incorporating the fungal pre-treatment step, a control reactor operating with same hydraulic conditions of the bubble lift biorector should be employed. This control reactor should be operated aerobically without fungal treatment and then treatment efficiencies of both streams i.e fungal pre-treatment coupled with main stream treatment and the control biorector coupled with main stream, should be compared. Further studies need to be conducted on the theories postulated about the behaviour of nitrates and ammonia in anaerobic systems, such as monitoring MLSS, nitrite concentrations, redox potentials salinity and protein assays. An additional treatment step for

the removal of phosphates should be performed to allow for effluent use in irrigation or other application such as water reuse within the industrial site. Further studies need to be conducted on macro and micronutrient dosing in anaerobic digesters, as literature indicates performance of digester being unique to wastewater type.

The SMBR for the treatment of RWDW and FTWDW had low CODs removal efficiencies, probably due to low molecular weight changes of the phenolic compounds. These could be evaluated using GC-MS. This would lead to better insight into microbial conversions that occurred in the SMBR systems and to elucidating the fate of compounds with different numbers of phenolic hydroxyl groups in the molecule. The actual mechanism of ferric salt improving overall efficiency is not fully understood although addition of all these micronutrients has been proven to improve overall efficiency of anaerobic digestion in studies conducted by (Sharma and Singh, 2001). It is suspected that ferric salts induced coagulation of colloidal materials, thus further studies like investigating molecular weight distribution on the bioreactor during addition of micronutrients is a recommendation for future work.

Implementation of any AD or SMBR at full scale would still require a thorough cost-benefit analysis to offest the finanacial cost of building and operating an on-site wastewater treatment system with the socioeconomic gains to be made.

The PCR-DGGE results were not conclusive in terms of species identity as cloning, sequencing and phylogenetic analyses were not performed. These experiments need to be undertaken during treatment of RWDW and FTWDW, so that microbial diversity and identity can be established. Identification of the microorganisms responsible for wastewater treatment could lead to bioaugmentation of digesters and then monitoring for improved performance. Minimization of water usage can be achieved by reduction in the use of fresh water via the reuse of process water in areas where quality of the water is not key. Reuse of water within an industrial site can be done provided that the water will not adversely affect the process. Reduction in water treatment costs by implementing low cost biological treatments is another attempt at conserving freshwater. Use of treated water that meets disposal standards for ferti-irrigation purposes is also another method of minimization of freshwater usage (Van Schoor, 2005; Mack *et al*., 2004; DWAF, 1996).

Chapter 11

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Appendix A: Parameters monitored during high rate anaerobic digestion of RWDW.

Time (days)	pH	VFA (mg/l)	COD_s (mg/l)	Polyphenols (mg/l)	MLSS (mg/l)	NO_3^- (mg/l)	NH_4^+ (mg/l)	PO_4^{3-} (mg/l)
0	7.23	1639.34	2850	108.78	15500	36.0	1.35	102
2	7.25	728.60	1170	108.78	14000	37.3	1.66	113.6
4	7.01	910.75	840	31.94	13000	23.7	2.80	89.6
6	7.39	117.00	1215	31.94	7000	11.8	0.18	46.0
8	9.20	54.64	840	79.84	8000	40.8	0.30	24.6
10	7.69	127.50	620	79.84	9000	34.2	0.64	63.6
12	7.24	63.75	930	196.60	7000	29.5	0.84	48.0
14	6.90	45.54	720	196.60	7500	22.3	2.71	86.4
16	5.95	118.00	510	113.77	8000	10.7	4.92	43.2
18	7.29	182.15	460	113.77	8000	13.0	0.63	19.6
20	9.15	364.30	1680	161.67	13000	67.0	0.24	6.2
22	8.88	510.00	1575	161.67	12000	49.0	0.20	92
24	8.78	910.75	1545	122.75	10500	30.9	1.24	87.2
26	8.13	637.52	1530	122.75	11500	20.8	1.06	98.0
28	7.90	320.30	490	54.89	11000	17.0	1.70	52.0
30	7.26	182.15	195	54.89	10000	13.1	2.55	43.2
32	7.01	455.37	95	8.98	9000	5.2	5.08	10.7
34	7.23	122.90	90	8.98	10500	9.6	0.06	5.8
36	6.63	189.40	1365	6.99	12000	16.6	1.10	3.2
38	6.33	637.52	870	6.99	12500	13.8	1.55	6.8
40	6.54	510.00	795	12.47	15400	14.4	1.44	21.2
42	6.08	455.37	645	12.47	15400	14.5	0.27	21.4
44	6.47	420.00	510	18.96	13800	13.7	0.20	22.0
46	6.95	364.30	315	18.96	11400	14.8	0.18	22.5
48	7.34	273.22	525	18.96	10400	15.8	0.18	20.1
50	5.66	364.30	765	18.96	10600	39.3	0.84	55.4
52	12.34	273.22	3030	190.62	10000	62.1	0.12	123
54	7.04	728.59	1680	190.62	9160	5.3	0.98	106.8
56	7.11	637.52	1305	36.93	6540	5.5	1.03	98.8
58	6.84	273.22	645	36.93	6800	5.3	1.89	66.4
60	6.64	136.61	450	21.96	6200	3.6	3.48	45.6
62	6.68	81.97	90	13.97	9420	4.8	5.92	45.2
64	5.70	318.76	765	19.96	9500	10.6	3.40	48.2
66	6.64	227.69	525	14.97	9600	5.5	3.60	25.0
68	6.64	318.76	270	7.29	9700	12.7	5.00	12.1
70	6.75	385.12	159	209.58	15067	36.4	2.52	10.8
72	6.84	182.15	200	319.36	15233	31.4	4.16	7.9
74	6.76	428.05	109	361.27	16833	56.8	5.16	54.2
76	6.83	528.23	65	259.48	17700	22.5	4.96	59.2
78	6.77	109.29	5	274.45	16667	7.2	2.76	46.6
80	7.35	109.29	95	440.11	18500	2.4	2.48	29.8
82	6.35	91.07	145	198.60	17666	3.9	3.04	34.6
84	7.18	122.95	80	246.50	18000	3.9	3.80	32.0
86	6.59	136.61	105	64.87	17666	3.6	3.04	40.6
88	7.29	45.54	60	57.88	17000	2.7	3.00	26.0
90	6.6	78.86	170	33.93	21000	1.1	2.80	38.6
92	6.58	45.54	5	0.00	20500	3.4	2.52	51.2
94	6.49	72.90	115	3.99	22400	11.9	0.05	38.0

Time (days)	pH	VFA (mg/l)	COD_s (mg/l)	Polyphenols (mg/l)	MLSS (mg/l)	NO_3^- (mg/l)	NH_4^+ (mg/l)	PO_4^{3-} (mg/l)
96	6.47	72.90	95	9.98	21800	3.8	0.00	33.6
98	6.69	109.30	120	7.98	20400	3.2	0.00	42.9
100	6.67	63.80	595	7.98	21800	8.3	0.03	48.6
102	7.18	100.20	420	8.98	18400	6.6	0.02	46.2
104	6.6	291.40	730	4.99	16400	9.9	0.03	52.8
106	6.94	337.00	1044	85.83	20667	4.3	1.55	155.6
108	7.08	91.10	498	52.89	20333.3	3.0	1.39	164.4
110	6.97	100.20	108	37.62	18667	2.1	1.48	158.4
112	7.00	91.10	60	25.95	20000	0.9	2.75	145.8
114	7.10	127.50	120	8.28	25400	2.5	4.11	159.6
116	7.17	72.86	60	8.28	26000	2.3	6.30	154.8
118	6.84	109.30	69	10.98	24600	1.9	3.72	141.0
120	7.62	182.15	320	10.98	27600	3.44	5.80	102.0
122	7.58	163.93	380	13.97	25200	3.34	5.60	102.4
124	6.65	109.30	215	11.98	27600	4.6	2.77	100.8
126	7.23	163.93	205	4.99	31200	4.8	2.13	103.2
128	6.63	145.72	220	10.98	31000	3.9	3.04	108.8
130	6.75	218.58	375	17.46	34400	9.0	4.48	100.8
Max	12.34	1639.34	3030	108.78	34400	67.0	6.3	164.4
Mean	7.01	249.82	520.37	108.78	15208	13.4	2.11	61.2
Min	5.66	45.54	5	31.94	6200	0.9	0.00	3.2
SD*	0.94	273.52	624.99	31.94	6797	15.6	1.80	45.0

*SD = Standard Deviation

Appendix B

Table B.1: Parameters monitored during treatment of RWDW using SMBR.

Day	pH				VFA (mg/l)		COD_S (mg/l)		Polyphenol (mg/l)			
	Reactor A	Reactor B	Reactor C	Reactor D	Reactor B	Reactor D	Reactor B	Reactor D	Reactor A	Reactor B	Reactor C	Reactor D
0	4.38	5.13	4.58	5.19	730.42	819.67	2385	1195	28.94	21.62	26.61	19.29
2	5.61	5.83	5.89	5.88	273.22	1457.19	2520	3030	28.94	16.63	15.97	16.30
4	5.27	6.17	6.24	6.38	1730.04	1548.27	3030	2235	28.94	6.65	15.97	15.97
6	5.63	5.68	5.81	5.84	3096.54	2550.09	4710	2685	28.94	9.31	14.97	25.95
8	5.68	5.44	5.95	5.65	2550.05	1111.11	4785	3360	25.61	11.98	19.96	23.62
10	5.71	5.64	5.63	5.68	2049.18	3187.61	4920	5460	25.61	18.96	22.29	13.64
12	7.53	6.20	6.18	5.98	1411.58	2094.72	2940	5119	44.91	13.64	20.63	15.30
14	7.2	6.37	6.9	5.85	1139.56	2114.70	1140	2385	58.88	23.95	22.29	46.91
16	7.09	6.70	6.79	7.15	1270.60	1000.09	1980	1290	29.61	21.62	26.61	61.88
18	7.3	6.83	6.69	7.03	1070.44	882.02	2100	1935	31.27	26.95	11.31	37.59
20	7.26	6.67	7.38	6.48	879.47	988.22	2385	1950	24.95	14.64	33.27	21.29
22	7.00	6.80	6.97	6.77	764.50	789.53	2430	2040	25.95	21.29	48.24	157.35
24	7.52	7.08	6.99	6.96	1499.86	1200.54	2280	1950	36.59	40.58	44.24	35.26
26	7.37	7.07	7.00	6.99	2094.72	1548.27	2107	1892	29.61	35.26	25.95	47.24
28	7.40	7.11	6.98	7.03	1183.97	1366.12	2090	1475	27.61	38.59	38.59	47.24
30	7.49	7.03	6.99	7.00	1684.88	1730.40	2031	1129	28.94	33.60	38.59	52.89
Max	7.53	7.11	7.38	7.15	3096.54	3187.61	4920	5460	58.88	40.58	48.24	157.35
Mean	6.59	6.36	6.44	6.37	1464.31	1524.28	2800	2446	31.58	22.21	26.59	39.86
Min	4.38	5.13	4.58	5.19	273.22	789..53	1140	1129	24.95	6.65	11.31	13.64
SD*	1.02	0.65	0.73	0.64	724.41	675..90	1108	1273	8.77	10.39	11.03	34.91

*SD = Standard Deviation

Table B.2: Other parameters monitored during treatment of RWDW using SMBR.

Day	NO_3^- (mg/l)				NH_4^+ (mg/l)				PO_4^{3-} (mg/l)			
	Reactor A	**Reactor B**	**Reactor C**	**Reactor D**	**Reactor A**	**Reactor B**	**Reactor C**	**Reactor D**	**Reactor A**	**Reactor B**	**Reactor C**	**Reactor D**
0	34.0	20.6	12.7	9.5	0.32	2.20	1.24	3.00	57.9	60.4	47.6	35.7
2	90.0	32.8	44.6	47.5	0.04	2.40	2.38	7.00	118.4	83.1	95.2	92.4
4	91.0	7.6	5.8	5.3.0	0.2	9.80	5.36	11.10	112.4	96.0	95.6	100.4
6	85.0	23.1	14.2	6.4	0.21	2.30	4.10	12.30	109.0	134.4	98.0	96.0
8	90.0	23.1	20.7	16.5	0.19	3.70	1.36	5.50	117.0	135.6	91.2	97.2
10	94.0	29.3	32.6	21.0	0.12	1.70	4.14	4.00	139.6	106.0	115.6	101.2
12	82.6	6.6	9.6	68.0	0.34	9.50	7.92	7.90	112.4	113.6	116.8	125.6
14	8.1	6.3	15.5	8.6	6.50	11.00	11.75	29.30	104.4	118.0	104.0	103.2
16	10.99	6.6	14.8	10.3	7.00	7.60	9.60	18.50	105.2	122.8	111.6	104.4
18	11.56	6.6	14.3	6.3	6.20	8.80	7.70	20.30	103.2	114.0	104.0	101.2
20	13.5	8.1	14.1	9.1	5.35	9.40	8.20	23.70	105.2	108.0	102.0	106.4
22	13.0	9.4	16.6	11.3	4.40	10.50	5.35	27.90	107.0	116.0	94.0	101.6
24	13.6	15.5	18.7	7.4	5.00	12.60	8.50	30.00	103.2	99.6	120.0	99.6
26	14.0	13	19.3	9.0	4.80	16.00	7.90	33.00	103.7	101.0	119.0	91.4
28	13.1	11.8	18.1	7.6	5.10	14.30	8.00	38.00	104.0	100.4	121.0	93.0
30	13.7	7.0	18.6	8.0	4.80	13.80	8.20	34.00	103.8	102.6	120.3	89.0
Max	94.0	32.8	44.6	68.0	7.00	16.00	11.75	38.00	139.6	135.6	121.0	125.6
Mean	42.4	14.21	18.1	15.7	3.16	8.48	6.36	19.09	106.7	107.0	103.5	96.1
Min	8.1	6.3	5.8	5.3	0.04	1.70	1.24	3.00	57.9	60.4	47.6	35.7
SD*	37.6	8.9	9.1	17.3	2.77	4.72	3.05	11.97	16.0	18.5	18.3	18.2

*SD = Standard Deviation

Appendix C

Table C.1: Parameters monitored during treatment of FTWDW using SMBR.

Day	pH				VFA (mg/l)		COD_S (mg/l)		Phenol (mg/l)			
	Reactor A	Reactor B	Reactor C	Reactor D	Reactor B	Reactor D	Reactor B	Reactor D	Reactor A	Reactor B	Reactor C	Reactor D
0	7.13	7.78	7.52	7.55	227.70	546.40	387	467	28.94	21.62	26.61	19.29
2	7.82	7.36	8.2	8.24	364.30	546.40	455	501	28.94	16.63	15.97	16.30
4	7.80	7.4	7.49	7.55	391.60	473.60	433	453	28.94	6.65	15.97	15.97
6	7.83	7.42	7.91	7.48	547.10	200.40	374	287	28.94	9.31	14.97	25.95
8	7.95	7.68	7.99	7.82	548.30	455.40	502	479	25.61	11.98	19.96	23.62
10	7.71	7.47	8.21	7.74	364.30	273.20	319	296	25.61	18.96	22.29	13.64
12	8.11	7.52	7.58	8.75	63.80	51.00	57	54	44.91	13.64	20.63	15.30
14	8.10	7.60	8.16	7.6	45.50	27.30	36	32	58.88	23.95	22.29	46.91
16	7.30	7.26	9.04	7.74	91.00	82.00	87	84	29.61	21.62	26.61	61.88
18	7.50	7.47	8.62	7.79	91.00	34.60	63	49	31.27	26.95	11.31	37.59
20	8.69	8.75	8.76	7.81	54.60	63.80	59	62	24.95	14.64	33.27	21.29
22	8.64	8.52	9.08	7.68	54.60	45.50	50	48	25.95	21.29	48.24	157.35
24	8.34	8.23	8.32	8.8	45.50	27.30	36	32	36.59	40.58	44.24	35.26
26	8.05	8.88	7.49	7.89	45.50	54.60	50	52	29.61	35.26	25.95	47.24
28	8.64	7.44	7.64	7.75	68.30	63.80	66	65	27.61	38.59	38.59	47.24
30	8.58	7.41	7.94	7.97	45.50	54.60	50	52	28.94	33.60	38.59	52.89
Max	8.69	8.88	9.08	8.8	548.30	546.40	502	501	58.88	40.58	48.24	157.35
Mean	8.01	7.76	8.12	7.89	190.54	187.49	189	188	31.58	22.21	26.59	39.86
Min	7.13	7.26	7.49	7.48	45.50	27.30	36	32	24.95	6.65	11.31	13.64
SD*	0.48	0.53	0.53	0.39	187.84	201.55	182	189	8.77	10.39	11.03	34.91

*SD = Standard Deviation

Table C.2: Other parameters monitored during treatment of FTWDW using SMBR.

Day	NO_3^- (mg/l)				NH_4^+ (mg/l)				PO_4^{3-} (mg/l)			
	Reactor A	**Reactor B**	**Reactor C**	**Reactor D**	**Reactor A**	**Reactor B**	**Reactor C**	**Reactor D**	**Reactor A**	**Reactor B**	**Reactor C**	**Reactor D**
0	11.5	7.5	13.6	7.5	0.00	1.10	0.00	1.90	100.8	114.8	109.2	116.0
2	9.4	14.0	9.2	9.7	3.90	0.30	1.60	0.10	82.5	107.6	111.2	110.4
4	8.2	8.2	6.9	7.5	5.00	2.70	1.40	0.40	82.5	118.0	114	119.6
6	9.0	7.3	11.6	9.5	3.60	3.80	1.90	1.80	82.5	110.8	142.5	127.6
8	6.7	8.3	12.8	8.7	1.10	2.90	1.80	1.30	87.6	116.8	116.4	104.4
10	5.8	5.8	8.6	6.3	2.10	1.30	1.20	4.00	87.6	111.2	122.8	125.2
12	4.6	5.3	7.4	7.3	0.60	4.20	2.20	4.70	81.6	111.2	113.2	112.0
14	16.0	12.8.0	9.2	8.2	0.40	2.80	4.50	3.10	69.6	110.8	118.0	116.4
16	24.0	11.0.0	9.5	4.9	2.40	2.50	1.20	0.30	109.2	54.0	116.0	94.2
18	5.9	41.4	24.8	23.5	0.50	1.70	4.30	0.50	80.0	112.8	110.8	111.2
20	33.8	13.7	20.5	10.3	1.00	0.70	2.00	1.20	80.0	110.0	113.6	116.4
22	32.1	24.4	14.1	14.5	0.80	0.50	1.00	1.40	80.0	110.0	112.8	123.2
24	27.9	25.6	15.1	15.8	0.40	1.30	1.20	0.40	64.8	105.6	107.6	117.6
26	27.4	20.2	21.6	16.9	0.90	0.60	0.80	0.70	118.0	113.2	108.4	116.0
28	17.9	33.4	17.1	25.2	0.50	0.80	0.90	0.80	118.0	112.0	107.6	119.6
30	18.2	24.8	25.2	27.7	0.70	0.70	0.80	0.90	118.0	118.8	105.2	108.4
Max	33.8	41.4	25.2	27.7	5.00	4.20	4.50	4.70	118.0	118.8	142.5	127.6
Mean	16.2	16.5	14.2	12.7	1.49	1.74	1.68	1.47	90.17	108.6	114.3	114.9
Min	4.6	5.3	6.9	4.9	0.00	0.30	0.00	0.10	64.8	54.0	105.2	94.2
SD*	10.1	10.8	6.05	7.2	1.48	1.24	1.20	1.36	17.2	15.0	8.7	8.2

*SD = Standard Deviation

Appendix D: Performance of fungal unit for pre-treatment of RWDW.

Three white rot fungi were initially screened for bioremediation and enzyme production potential in RWDW by Strong and Burgess (in press), as described below.

Materials and Methods

Trametes pubescens MB 89 and *Ceriporiopsis subvermispora* were purchased from Centraalbureau voor Schimmelcultures (The Netherlands, cultures 696.94 and 347.63, respectively) while *Pycnoporus cinnabarinus* was kindly donated by Miss N. Khan (Khan, 2005). All four specimens were routinely subcultured on bacteriological agar (12 g/l, Biolab, Merck Chemicals (Pty) Ltd, Johannesburg) plates containing 2 % malt extract (Biolab, Merck), 1 % glucose (Saarchem, uniLAB, Merck) and 0.2 % yeast extract (Biolab, Merck).

Wastewater was obtained from a Olafbergh distillery near Worcester in the Western Cape province of South Africa (pH 3.9, total phenols of 540 mg/l and 25500 mg/l COD). Particulates were removed by centrifugation twice at 14300 *g* for 15 minutes in a J-10 Beckman centrifuge, followed by filtration through Whatman no. 1 filter paper. The pH was adjusted to 5.3 using sodium carbonate powder (Saarchem, uniLAB, Merck), as this was the lowest pH tested at which growth had occurred. Aliquots of 60 ml were placed in 250 ml Schott bottles, covered with aluminium foil (to prevent contamination) and sterilised by autoclaving for 15 minutes. Triplicate samples were inoculated with biomass of *C. subvermispora, UD4, P. cinnabarinus* or *T. pubescens* MB 89 from the liquid cultures described above. The wastewater samples were placed on a benchtop shaker (Labcon SP015+UPF75, Maraisburg) at 150 rpm at 28 ˚C for 14 days. Control inocula in distilled water were conducted in duplicate.

Results

Of the four species screened, *T. pubescens* MB 89 displayed the greatest potential for reducing the COD, total phenols and the colour of the untreated as well as the PVPP-treated wastewater (Table 1). This corroborated Fitzgibbon *et al*. (1998), who tested the effects of gallic acid, vanillic acid, and molasses spent wash concentration on decolourising ability of *Geotrichum candidum, T.* (*Coriolus*) *versicolor, Phanerochaete chrysosporium* and *Mycelia sterilia*, and also found the *Trametes* sp. to be superior to the other fungi tested.

Table D.1: Results for COD, total phenol, colour changes and laccase activities for the four white rot fungi in raw and PVPP-treated wastewater.

Removal efficiency (%)	***T. pubescens***	***C. subvermispora***	***P. cinnabarinus***
COD_S	78 ± 2	54 ± 4	50 ± 5
Total phenols	87 ± 3	45 ± 6	42 ± 7
Absorbance at 500 nm	89	35	54

These results led to the decision to use *T. pubescens* in the pre-treatment bioreactor employed in the present study. The fungal bioreactor achieved 60 ± 4 % COD_S removal, 83 ± 3 % removal of phenolic compounds and a decrease in absorbance at 500 nm of 84 %.

Appendix E: Parameters monitored during high rate anaerobic digestion of FTWDW.

Time (days)	pH	VFA (mg/l)	COD_S mg/l	Phenols mg/l	MLSS mg/l	NO_3^- mg/l	NH_4^+ mg/l	PO_4^{3-} mg/l
0	7.60	614.00	1935	58.88	13640	27.6	0.78	88.0
2	7.43	728.6	675	53.89	11020	17.5	0.66	134.8
4	7.48	218.58	315	11.98	8000	31.4	1.74	54.4
6	7.66	117.00	135	4.99	10280	41.5	4.44	45.6
8	7.82	109.29	120	3.39	13120	9.9	5.68	36.4
10	7.74	72.86	80	3.69	13880	27.1	2.00	35.4
12	7.49	182.15	205	3.19	11667	7.8	6.24	99.2
14	7.51	177.6	200	30.94	8833	6.9	6.68	81.6
16	8.18	136.61	340	9.48	10667	8.6	8.12	70.0
18	8.03	109.29	255	4.99	12000	9.0	7.72	94.8
20	7.76	100.18	130	6.49	17333	9.3	7.48	85.6
22	7.68	91.07	75	1.00	17167	6.5	7.56	67.6
24	7.56	68.31	115	19.96	18667	8.0	7.76	59.6
26	7.82	72.86	290	11.48	15600	4.6	0.03	72.0
28	7.39	54.64	275	11.48	22800	7.7	0.02	84.0
30	7.42	45.54	385	10.98	14200	13.1	0.08	78.9
32	7.48	45.54	360	23.95	16600	11.7	0.06	82.8
34	7.94	63.75	285	17.96	17200	26.0	0.14	84.6
36	7.63	45.54	215	12.97	17400	6.9	0.08	89.2
38	8.33	136.60	42	38.92	17333	2.5	2.30	125.4
40	7.60	109.30	390	45.11	20333	2.8	3.60	121.8
42	7.76	118.40	438	34.63	18667	2.8	1.57	177.6
44	7.86	186.70	306	31.64	20333	2.5	1.69	168.6
46	7.64	200.36	480	33.93	22800	3.2	1.08	135.6
48	7.53	236.79	480	44.41	21800	3.9	1.96	130.2
50	7.6	182.14	435	41.92	27600	4.2	1.38	156.0
52	7.64	182.14	390	41.92	27800	5.8	1.68	101.2
54	7.57	218.57	825	45.91	24600	5.6	1.56	120.0
56	7.00	327.87	1130	50.20	25600	19.7	1.49	94.4
58	8.08	273.22	1010	57.88	27400	20.2	0.94	97.6
60	7.18	327.87	1210	58.18	25800	21.9	0.97	113.6
62	7.04	910.75	1320	62.87	33600	24.9	0.77	120.0
64	7.43	182.15	510	45.41	27000	2.1	13.8	125.2
66	7.29	200.36	308	116.96	18600	3.6	12.1	112.4
68	7.15	236.79	217	10.48	21000	4.1	4.50	76.4
70	7.63	186.15	479	66.37	31000	0.1	8.10	43.5
72	7.09	255.00	200	18.76	18000	4.8	4.40	40.5
74	7.10	259.66	400	3.99	26400	3.3	4.90	37.5
76	7.56	91.07	100	1.50	23800	0.1	6.20	19.2
78	7.00	91.07	45	23.05	23400	3.8	6.60	100.0
80	7.01	182.15	704	25.45	33800	4.7	4.80	110.4
82	6.98	50.09	499	15.07	25000	5.1	1.90	80.7
84	8.08	63.75	612	46.01	34400	1.8	9.90	104.0
86	7.16	4.65	445	48.90	25200	2.7	14.30	105.6
88	7.09	45.54	327	20.46	25200	3.5	8.80	72.8
90	7.15	36.43	307	68.36	20400	3.7	14.50	71.2
92	8.63	54.64	206	87.32	16200	2.0	13.80	123.2
94	7.45	91.07	150	24.75	23800	2.8	11.50	119.6
96	8.52	45.54	45	62.37	19800	2.5	7.10	130.4

Time (days)	pH	VFA (mg/l)	COD_S mg/l	Phenols mg/l	MLSS mg/l	NO_3^- mg/l	NH_4^+ mg/l	PO_4^{3-} mg/l
98	8.12	91.07	88	41.92	21000	5.8	4.00	106.0
100	8.20	63.75	72	53.09	21800	7.4	2.80	96.0
Max	8.63	910.75	1935	116.96	34400	41.5	14.50	177.6
Mean	7.57	149.25	366	31.12	20309	8.3	4.54	93.2
Min	6.98	4.65	42	1.00	8000	0.1	0.02	19.2
SD*	0.40	169.23	368	24.87	6488	9.2	4.23	34.5

*SD = Standard Deviation