Evaluation of a ceramic membrane bioreactor functioning under non-steady state operational parameters for the removal of pollutants from municipal wastewater plant effluent

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ABSTRACT

In South Africa ill-managed municipal wastewater treatment plants limit the quality and quantity of already exploited surface water resources. Elevated nitrate levels cause eutrophication and chemicals (measured as chemical oxygen demand or COD) discharged through effluent water may even irreversibly alter the quality of potable water. Pathogens entering river systems cause high risk especially for rural communities. The status quo of membrane bioreactor technology for microorganism and solid retention is very broad and the development and application thereof is being driven by both fresh water shortage and anticipated stringent environmental regulations. Very little research is done on membrane bioreactors (MBRs) functioning under non-steady state parameters with spontaneous fluctuations in permeate flux. Basic models that provide a holistic understanding of the technology at a fundamental level are therefore of great necessity. It was hypothesized that a simple aerobic-anaerobic external circuit ceramic MBR operated under spontaneous parameter variance could reach high pollutant removal efficiencies, even when shock-loaded with nutrients and microorganisms. A ceramic membrane bioreactor system was constructed and operated under feed-and-bleed conditions (CNP=85:15:1) for 112 days and hydraulic retention time (HRT) of between 4 and 7 days, to determine whether the system could buffer nitrate and COD discharge into river systems. Transmembrane pressure was maintained at 10kPa and flux were allowed to change to obtain non-steady operating conditions. From stabilization (day 47) towards day 63 of operation, average nitrate and COD removal reached 47.64% and 86.55%, respectively the system stabilized after day 47 reaching a COD removal near 99% between days 85 and 101. Shock loading with nutrients (CNP=250:35:3) was done on day 64, where after nitrate and COD removing efficiency decreased to 9.41% and 80.29% respectively within 10 days. COD removal capacity recovered to 99.92% 5 days after the shock loading on day 69. This demonstrates the robustness of the system. However, it took 35 days for nitrate removal to recover to 25.12 %. A major challenge during the operation of the system was fouling which started on day 47. This could be ascribed to the extremely low average solid retention time (SRT) (0.086 days) values. A gas-liquid back flush regime of three times a week was necessary to overcome this problem. On day 146 the system was shock loaded with *E.*coli transformed with pBR322 for microbial retention analysis. Average retention was 89.15% in the first 5 days during the experiment after breakthrough occurred. Carbon breakthrough was measured as elevated COD. Microbial levels reached ~5.3×10^5 cfu/mℓ in the effluent. Biofilm samples were taken throughout MBR operation. DNA was extracted and the V3 region of 16S rRNA was amplified by PCR using primers for *Bacteria*. PCR products were subjected to SSCP and DGGE analysis to generate molecular fingerprinting profiles representative of the community structure associated with the non-steady operational conditions. Bands, representing various species were excised, reamplified and sequenced to determine identities of the bacteria.

The PCR-SSCP and PCR-DGGE banding patterns were subjected to Shannon Weaver diversity index analysis. Dendrograms for each of the SSCP and DGGE gel profiles were obtained using Ward's method and Euclidean distances. Maximum *H'* values of 1.11, 1.27 and 1.26 were reached on days 20, 40 and 81 with close correlation between days 40 and 81 with Euclidean linkage distances lower than 3.2. This is indicative of the increased vigor of specific organisms specializing in nutrient removal at high concentrations DGGE fingerprinting suggested a subsequent shift in diversity. Three distinctive shifts in diversity were evident throughout MBR operation. This may have been due to re-organization of the community as the species involved out-competed other species over time. A sudden shift in community was observed during 1) days 6-20 ; 2) days 27 to 40 and 3) days 63 to 81 with ultimate *H'* values exceeding 1.0 at the end of each phase with clear differences in SRT and flux showing a gradual drop in value for each of the three phases. Results suggest limitations in the surviving capacities of the mixed culture biofilm. PCR-DGGE as well as PCR-SSCP were useful methods to obtain a genetic fingerprint profile for MBR biofilm characterization. PCR-SSCP was the method of choice, due to its sensitivity and expediency. SCCP profiles showed that *Aeromonas hydrophila*, *Delftia* spp. and an uncultured bacterial species were the three most evident organisms present, potentially responsible for elevated nitrate and COD removal soon after nutrient shock loading with average nutrient removal (days 63 and 91) of 20.76% nitrate and 82.13% COD. Analyses of an MBR functioning under non-steady state conditions are complex with reference to spontaneous change in a variety of parameters. SSCP is less time consuming but the steps involved are nonetheless complex. We conclude that non-steady state MBRs have limited potential to be used as an add-on to existing municipal wastewater treatment plants to serve as buffer for reducing COD and nitrate levels in wastewater effluent due to the intricate and complex nature of operation. Therefore, steady state MBR operation remains the optimal method of operation for the purpose of studying biofilm characteristics.

Key words: wastewater treatment plants; chemical oxygen demand; nitrates; ceramic membrane bioreactor; non-steady state; biofilm characterization

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DECLARATION

I hereby declare that this dissertation is my own work, unless stated to the contrary in the text, and that it has not been submitted in part, or in whole to any other University.

A.G. Pienaar
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CHAPTER ONE

INTRODUCTION

1.1 GENERAL INTRODUCTION AND PROBLEM STATEMENT

1.1.1 SA water crisis

There have been numerous assumptions and perceptions over whether South Africa faces a potential water crisis or not (Davies & Day, 1998; Van Vuuren, 2008; Van Vuuren 2009; *See* DWAF, 2000; DWAF, 2010). Scientists are highlighting the possibility of a potential water scarcity while politicians are striving to understand the fundamental sources of the problem. The seasonal rainfall and overall high temperatures that flourish in most of South Africa mean that fresh water may become a rare commodity. Because stable bodies of standing fresh water are limited, the need to use river water is increasing on a daily basis (Dallas & Day, 2004). An increasing population growth rate in South Africa and a bigger demand for commercialization and the coupled increasing numbers of industries have further put a strain on the quality of river water in South Africa. Chemical and physical pollution due to anthropogenic activities result in intense pressure on the rivers, which also led to more strict measures through the amendment of the National Water Act (NWA) 36 of 1998 (Dallas & Day, 2004). In South Africa, both the availability as well as the exceedingly poor quality of natural water systems contributes to the present water crisis (John & Trollip, 2009; Van Vuuren 2009).

1.1.2 South African river resilience

As early as 1985, O'Keeffe stated that rivers are finite sources of water are also very dynamic and incredibly difficult to predict. Even so, it was stated that they have strong tendencies of resilience (O'Keeffe, 1985; DWAF, 2009). Polluting organic matter such as outfall from a sewage plant is processed by river organisms and the river will return to the initial condition before pollution occurred, as long as it's not disturbed in other ways. However, according to O'Keeffe (1985), South African rivers have dissimilar limited abilities of self purification in terms of pollutants are not infinitely adaptable and will become even more disturbed with population growth. The natural resilience of rivers is therefore increasingly being compromised by man-induced changes. These may include heavy metals, biocides and organic solvents that enter river systems prevent the normal functioning of the river's natural biota in purifying the water that they live in (O'Keeffe, 1985; Davies & Day, 1998). With reference to the Vaal River, the best quality water is found in the catchment of Vaal Dam and quality deteriorates downstream, independent of the natural microbiota present. This deterioration is fortunately in line with the general distribution of user water quality requirements in the catchment (Braune & Rogers, 1987). According to the first report of the National Microbial Monitoring Programme (NMMP) South Africa does not have a fundamental source of information for assessing the potential health risks associated with faecally polluted surface water (Mieta *et al.*, 2010). In 1994 it was stated by DWAF's minister that "Implementation of this programme has become a high priority as South Africa's water resources are coming under increasing threats from faecal contamination". The minister of DWAF also mentioned that this situation is primarily due to the swift demographic transformation that ultimately resulted in a variety of dense settlements that lack appropriate sanitation infrastructure.

1.1.3 Natural aquatic biota in South African river systems

According to the first report of the National Microbial Water Quality Monitoring Programme (NMMP), the change in the concentration of microorganisms in an aquatic habitat is independent of the initial concentration present (DWAF, 2000). This then complicates the monitoring of the levels or concentrations of these organisms which eventually lead to increased capital expenditures (DWAF, 2000). The Department implies that this behavior of faecal pollution indicators and/or pathogens, would limit the development of an "overall picture" of microbial quality of surface water resources in South African aquatic ecosystems. Instead, it is proposed to focus on high health risk areas where the possibility of faecal contamination would carry major health risks and then focus on that area when monitoring (DWAF, 2000). The human intestinal tract is home to a complex community of microbial species which serve as markers of gastro-intestinal health (Zhang et al., 2006). The occurrence and distribution of bacterial pathogens causing diarrhoea in humans has been shown in various studies (Mieta *et al.,* 2010). These pathogens may include *Vibrio cholerae, Shigella* and *Salmonella* spp. and are commonly associated with diarrhoea. Allan (2007) concluded that bacterial abundance, biomass and production demonstrate a distinct temporal pattern with the lowest values consistently recorded during the winter months in a single estuary. The issue regarding the complexity of monitoring faecal indicating organisms associated with faecal contamination within a river system supports the need for technology to limit the probability of disease transport.

1.1.4 Enteric bacteria and pollution

According to Diergaardt *et al.* (2004) there is little or no data regarding the occurrence of pathogens with specific reference to the aquatic pathogen, campylobacter, in South African environmental surface waters that should be seen as reservoirs thereof. They concluded that while *Campylobacter* sp. does occur, but not in abundance, *Alcobacter butzleri* is spreading in South African waters (Diergaardt *et al.*, 2004). *Alcobacter butzleri* is also an organism with elevated pathogenicity capacity.

1.1.5 Treatment facilities at SA local municipalities

Fresh water is an extremely limited natural resource in South Africa, therefore the small amount available should be treated effectively. The demand for effective local municipal waste water treatment and purification is being acknowledged by stakeholders throughout South Africa. Poor managerial skills, lack of expertise and degradation of small and local waste water treatment plants are not treating this water effectively (Frost & Sullivan, 2009). Existing wastewater treatment plants are failing to cope with the sharp increase in wastewater volumes and loads (Frost & Sullivan, 2009). The average service lifespan of a wastewater treatment plant ranges from 15 to 20 years, and this depends on the plant's maintenance routines. Unfortunately, routine maintenance has been lacking at most of the wastewater treatment plants of municipalities, causing further damage to the facilities (Frost & Sullivan, 2009). Fatoki *et al.* (2003) and Swart and Pool (2007) stated that reports on small treatment plants added to pollution due to non-compliance through regulations set out by DWAF.

South Africa runs a major waste water treatment business with a projected capital replacement value of more than R 23 billion and a projected operational expenditure exceeding R 3.5 b annually (South Africa, 2009). According to Van Rensburg (2008) total annual use of water has been estimated to exceed 16 billion cubic metres. The Department of Water Affairs and Forestry predicted an annual growth of four to six percent in coming years (Van Rensburg, 2008). Domestic or municipal use is responsible for at least 12 percent of this water consumption and it has been estimated that this figure will increase up to 19 percent in 2010. This may in turn lead to potential, constant elevation in pollution levels which emphasizes the demand for legal compliance of wastewater treatment plants.

The issue of ill-managed municipal wastewater treatment plants has raised much concern over the quality of the wastewater that enters the river systems and pollutes the environment downstream. The matter of wastewater treatment and compliance to legal standards was noted as a matter of urgency in the Green Drop (GD) Report issued by the DWAF in 2010 as a governmental intervention. Only 3.8 % of the total plants received the Green Drop status, which is roughly equivalent to international standards. In this way wastewater treatment plants are motivated and challenged to deliver better quality water. But, this incentive may not be adequate if one considers the future water demand in South Africa. As alternative, instead of undergoing huge capital expenditures in rebuilding, remodifying or upgrading existing plants, one needs to reflect on implementing newer technologies or utilize it as an add-on to existing technologies.

1.1.6 Membrane bioreactor technology in South Africa

Membrane technology in South Africa is not new (Ross *et al.*, 1990; Cicek *et al.*, 1998; Jacobs *et al.*, 2006). Offringa (2000) stated that South African research and development is being undertaken on most membrane processes, including reverse osmosis (RO), ultrafiltration (UF), microfiltration, (MF) and electrodialysis (ED). Research further focused on membrane materials (polymeric and ceramic), membrane bioreactors and de-fouling studies. The first Frost & Sullivan study (Frost & Sullivan, 2009) entitled 'South African Membrane Market' divided the market into three segments – industrial, municipal and commercial. South African wastewater treatment facilities usually follow conventional wastewater treatment approaches (Osifo, 2001; Hlophe & Venter, 2009). Although there have been studies conducted on the applicability of membrane technology in wastewater treatment (Ross *et al.*, 1990; Cicek *et al.*, 1998), literature available on MBR technology in wastewater treatment plants in South Africa is still limited and underdeveloped.

Wastewater treatment using submerged membranes has become an industry standard treatment technology in South Africa over the last 15 years (Wozniak, 2011). MBR systems have also gained acceptance as one of the best waste treatment technologies available. But, to date 60 % of all MBR plants that have been built in industrial applications are discharging their treated effluent into the sewers of the local municipality for further treatment (Wozniak, 2011).

Several negative perceptions may contribute and limit the implementation and use of MBR technology in South Africa. Mickley (2003) and Santos (2011) stated that a perception problem sometimes occurs in terms of MBR technology. This may include regulators that are not familiar with membrane technology when used in water treatment plants. Similar to the public, they are not aware of the differences between membrane concentrate and other industrial wastes. The public also usually regards industrial and municipal waste as environmentally toxic and hazardous. In this way membrane technology suffers from inclusion in this group (Mickley, 2003).

In contrast to some perceptions of MBR technology, Cicek *et al.* (1998) stated that compared to the conventional activated sludge process, the MBR system offers several advantages. These include removal efficiencies of 99.5% of influent COD, low sludge production and excellent heterotrophic microorganism retention. In South Africa, MBR development especially led to the commercialization of MBR technology for use on high-strength industrial effluent (Judd, 2006; Edwards *et al.*, 1999; Offringa, 2000). The application of membrane technology in terms of desalination has also influenced growth in the membrane market. The Emalahleni Desalination Plant in Witbank has revived much interest in the potential growth of membrane technology in the mining industry (Frost & Sullivan, 2009). The use of membrane technology (nanofiltration and reverse osmosis) for the removal of excess concentrations of nitrates, sulphates, phosphates, chlorides, calcium and magnesium in brackish groundwater was investigated by Hlophe and Venter (2009) in Madibogo village in the North West Province of South Africa for drinking water purposes. They found that the optimal technology for treating the brackish groundwater was a nanomembrane NF90 that produced drinking water that complied with SANS-241.

Recently, there has been a lot of interest in anaerobic nitrogen processes, specially referring to membrane bioreactors, as alternative technology to limit the occurrence of ill-managed municipal WWTPs. This application could also present an unconventional treatment process for the remediation of groundwater contaminated with nitrate (Wang *et al.*, 2009). Although MBR technology has been considered to be a new technology, it is becoming more popular as the technology of choice Reports show that the MBR market is growing faster than the larger market for advanced wastewater treatment equipment (Frost & Sullivan, 2009). The capital as well as the operating costs of membrane plants has decreased significantly in the last two decades due to improvements and advancements in technology (Frost & Sullivan, 2009). Although there has been a decline in the prices, it is still expensive and more expensive to maintain, replace and clean (Frost & Sullivan, 2009).

Worldwide MBR technology has not only attracted increasing interest for the set up of new wastewater treatment systems. MBR technology also has high potential looking at upgrading tasks of already existing municipal wastewater treatment plants (Brepols *et al.*, 2008; Yang *et al.*, 2006). The application of especially hybrid systems in which the conventional system is used as a backup to treat the inflow volume that exceeds the hydraulic membrane capacity is especially being considered in other countries. These include Germany, Italy, Netherlands, America and Switzerland (Wilf & Alt, 2000; Brepols *et al.*, 2008; Weiss & Reemtsma, 2008; Zanetti *et al.*, 2010).

1.1.7 Application of non-steady state membrane bioreactor operation

The majority of bioreactors usually operate under steady-state conditions (Defrance & Jaffrin, 1999). Under these conditions the transmembrane pressure and permeate flux are maintained at constant levels. Research conducted in the last 15 years employ steady state operating conditions, to investigate and evaluate a great variety of applications of membrane bioreactor technology (Burton *et al.*, 1998; Cicek *et al.*, 1999; Jang *et al.*, 2006; Edwards *et al.*, 2006; Bodzek *et al.*, 2006; Ng & Kim, 2007; Chang *et al.*, 2007).

Little research have been conducted on MBRs functioning under non-steady state conditions, in which the transmembrane pressure and permeate flux are allowed to fluctuate spontaneously within the system (Laspidou & Rittmann, 2004). Viero & Sant'Anna (2008) concluded that only when MBRs are operated under steady-state conditions, sound conclusions can be drawn about the reactor performance. According to Fenu *et al.*, (2010) the possibility of MBRs operating under non-steady-state conditions has not received much attention in the literature. Therefore, the statement made by Viero & Sant'Anna (2008) lacks credibility. Fenu *et al.* (2010) especially emphasized that a systematic overview of the best scientific work on MBRs in this area is missing, specifically regarding non-steady state conditions and modeling objectives. A perception persists that operating MBRs under non-steady state conditions is more energy-dependant and costly than steady-state MBRs (Chaize & Huyard, 1991; Tewari *et al.*, 2010). More research are necessary to serve as evidence for these strong statements.

1.2 Research Aim and Objectives

The aim of this study was to evaluate a ceramic membrane bioreactor functioning under non-steady state operational conditions to investigate the pollutant removing capacity and to characterize the associated biofilm in terms of bacterial diversity through PCR-SSCP and PCR-DGGE for potential implementation at local wastewater treatment plants.

Objectives were to:

i. Monitor MBR pollutant removing efficiency in terms of COD and nitrate under non-steady state conditions
ii. Monitor and evaluate MBR performance during elevated nitrate and COD concentrations in the event of nutrient shock-loading under non-steady state conditions
iii. To subject data obtained to singular and multiple regression analyses to acquire information on trends in pollutant removing capacity of the MBR operating under non-steady state conditions.
iv. Determine the degree of indicator bacteria retention in the MBR by inoculating the system with *E.coli* JM109 transformed with pBR322
v. Characterize and determine microbial diversity in the MBR biofilm through PCR-SSCP and PCR-DGGE as DNA fingerprinting tools for ultimate DNA sequencing and characterization.
vi. To perform cluster analysis by using Ward's method following calculation of Euclidean distances to compare banding patterns of PCR-SSCP and PCR-DGGE to determine whether microorganisms reveal a nonrandom pattern.

CHAPTER TWO

LITERATURE REVIEW

2.1 WASTEWATER TREATMENT SYSTEMS

2.1.1 Biological Wastewater Treatment

Biodegradation can be defined as: a) a minor change in an organic molecule leaving the main structure still intact; b) fragmentation of a complex organic molecule in such a way that the fragments could be reassembled to yield the original structure. Bacteria therefore has the ability to degrade pollutants (whether organic or inorganic) in aquatic ecosystems which they are naturally found in, from toxic to nontoxic form via natural metabolic pathways. Bacteria have the ability to degrade a large number of pollutants, but not all pollutants. Trois *et al.* (2007) postulated that by combining mechanical treatment steps and biological waste treatment would result in elevated organic carbon biodegradation which is suitable for South Africa, due to low expenditures.

2.1.2 Waste water technologies in South African

Tempelhoff (2009) stated that as early back as the 1950s, when South Africa experienced an industrial boom, authorities deliberately casting a blind eye on the deteriorating state of South African rivers, especially the Vaal river barrage. The author further stated that the Vaal River Barrage is primarily a storage facility of sewage and industrial waste water. As early as 1989 the need to upgrade sewage purification systems was seen as invaluable (Wiechers, 1989). During a survey covering 500 sewage works, four main process types were in use in South Africa, namely 1) oxidation ponds, 2) biological filtration and 3) activated sludge and biofiltration, and 4) activated sludge processes (Wiechers, 1989; Van Niekerk, 2000). A total of 20% of the treatment technologies are not specified (Van Niekerk, 2000). Most of wastewater treatment plants in South Africa treat the waste water by implementing conventional activated sludge treatment coupled with the harvesting or recycling of the sludge itself (Van Niekerk, 2000; Osifo, 2001; Trois *et al.*, 2007). During the process, wastewater undergoes primary (physical removal of settleable solids) and secondary (biological removal of dissolved organic matter) treatment, before it is returned to the river again (Trois *et al.*, 2007).

Recurring managerial problems at local municipal wastewater treatment facilities is the main cause of ineffective treatment of wastewater (DWAF 2010). The impact of this is enormous. It leads to poor quality wastewater entering South African rivers through return flow effluent discharge. This phenomenon strictly contradicts the NWA of 1998 in which the quality of any return flow effluent must meet specific effluent quality criteria (DWAF, 1998).

2.1.3 Assessment of effluent quality at local wastewater municipalities WWTPs

Municipal wastewater treatment plants (WWTPs) use guidelines set out by the Department of Water Affairs and Forestry (DWAF, 1996) in combination with the NWA to manage and optimize wastewater treatment processes (DWAF, 2009). Toxic effluent discharge into an environmental receiving water body should be limited through general and specific standards (DWAF, 1996). Water quality is therefore managed on the basis of these uniform effluent standards. Investigations confirmed that the situation with regard to waste water treatment and compliance with the respective Water Acts must be addressed as a matter of urgency (DWAF, 2010).

An incentive based programme - a Green Drop (GD) Report - was finalized in 2010 with reference to wastewater effluent discharge (DWAF, 2010). In this report, local municipalities were given a score regarding the quality of the effluent. The main aim of this report was to fundamentally address the gaps and raise the performance of municipal waste water service providers. The GD Report showed that most of South Africa's WWTPs had an average of 17 risk areas that needed urgent attention. Included in the GD Report was shocking statistical evidence of a substantial waste water management industry that comprises of about 850 municipal treatment plants. Only 55% of the systems scored between 0% and 49%. Furthermore, only 7.4% of all the systems in South Africa achieved green drop certification (DWAF, 2010). Based on cumulative risk ratings, 41.67 % of waste treatment facilities in the North West Province was identified as high risk profiles due to flow requirements exceeding design capacity. The average provincial GD Score in the North West was calculated to be a mere 33%. The need for technology that is easy to operate and maintain, to ultimately improve these scores, could therefore be invaluable.

2.1.4 Pollutants in the Vaal river

The Vaal River system is the most important South African water resource as it supplies water to the Pretoria-Witwatersrand-Vaal (PWV) metropolitan complex (Stephenson, 2002). In this area about 40% of South Africa's population resides and which accounts for more than 50% of the gross domestic product (GDP). Almost along its entire length the Vaal River is heavily utilized as a recreational resource (Bruwer *et al.,* 1985). These activities include camping, canoeing, boating, picnicking, bird watching, water-skiing and fishing (Du Preez, 2000). It also supports major economic activities (DWAF, 2009). The significant development within the system includes urbanisation, industrial growth, agricultural activities and mining activities. This development has led to deterioration in the water quality of the water resources in the system (DWAF, 2009).

Industries located upstream from the Parys area (Upper Vaal: downstream barrage sub-catchment) that may have lead to increased pollution through effluent discharge, include: Sasol I, II and III; Tutuka, Majuba and Lethabo power stations; Mittal Steel and Sappi and; a great variety of mines (DWAF, 2009). Major water quality issues that were identified of key concern in these areas were salinity and eutrophication (DWAF, 2009). The impact of non-compliant wastewater discharges from the wastewater treatment plants is considered to be a major contributor to salinity, eutrophication and microbiological problems currently observed (DWAF, 2009). In the Upper Vaal area the total effluent return flow from wastewater treatment plants to the river system is $295.5 \times 10^6 m^3/a$ (DWAF, 2002). Nitrate and phosphate discharges may lead to eutrophication and associated toxic algal blooms in dams (DWAF, 2009; Van Ginkel, 2001).

The persistent discharge of poorly treated sewage is one of the most obvious sources of degradation of urban freshwater ecosystems when exacerbated by intermittent spillages of raw sewage due to power failures (Luger & Brown, 2002). Acceptable levels of pollutants present in the Upper Vaal region are indicated as follows: nitrate 6 mg/ℓ, phosphate 0.26 mg/ℓ, electrical conductivity (EC) 61 mS/m; and total dissolved salts (TDS) 397 mg/ℓ (DWAF, 2009). Recently these pollutants were measured as ranging above acceptable levels as a result of an evitable increase over time (DWAF, 2009).

Natural river systems are rich in bacteria that are responsible for nutrient recycling maintaining the trophic state of such an aquatic ecosystems (Pace & Cole, 1994). In 2006 feacal coliform counts were measured in the Vaal river. Counts ranged between 10 and 130 cfu/100mℓ (DWAF, 2009). In January 2006 sewage that washed into the Vaal River barrage caused the biggest number of fish deaths in 30 years. In the process, rare species were also killed (Eliseev, 2006). The shock load of enteric bacteria such as *Escherichia coli* and other organisms deprived the system of oxygen leading to fish deaths. This is only one example of how sewage pollution can have devastating effects on the ecological integrity of surface water systems.

2.1.5 COD

The common water quality variables of concern in the municipal waste sector include chemical oxygen demand (COD) (Van Niekerk, 2000). The amount of COD found in a water sample indicates the amount of oxygen likely to be used in the degradation of organic waste (DWAF, 1996; Boyles, 1997; Wentzel *et al,* 2003). Influent COD levels entering wastewater treatment plants may range between 200 – 1200 mg/ℓ (Orhon *et al.,* 1997; Dulekgurgen *et al.,* 2006; Devi & Dahiya, 2008) with COD fractions in raw wastewater averaging between 60 – 65 % (Dulekgurgen *et al.,* 2006; Pasztor & Pulai, 2009). The COD is then reduced by the activated sludge process and averages about 50 mg/ℓ (Van Niekerk, 2000).

The South African guideline for COD in effluents to be discharged into the receiving water body is 30 mg/ℓ (Government Gazette, 1984). According to DWAF (1996) COD values of >75 mg/ℓ discharged is considered a category 4 industrial effluent. Effluent waters with high COD levels entering a natural resource may lead to dissolved oxygen depletion (Dallas & Day, 2004) and a decrease in ecological diversity and poor quality water (DWAF, 1996). Juveniles of many aquatic organisms are more sensitive to physiological stress arising from oxygen depletion caused by high COD levels. This is particularly due to secondary effects such as increased vulnerability to predation and disease (Ten Brink & Woudstra, 1991; Marklund *et al.,* 2001; Dallas & Day, 2004). The need for lower COD levels in wastewater effluent is therefore of ultimate importance.

2.1.6 Nitrates

Another common water quality variable of concern in the municipal waste sector is nitrate nitrogen (Van Niekerk, 2000). Chibi & Vinnicombe (1999) state that nitrate occur widely in South African waters, albeit in concentrations usually within the recommenced guidelines. However, in certain areas of the country, nitrate occurs in varyingly high concentrations above guideline standards such as they could be a threat to the health of the indigenous users (Chibi & Vinnicombe, 1999). High nitrate levels have a stimulatory effect on aquatic plant growth and algae (Morrison *et al.*, 2001). Surface runoff from the surrounding catchment area, the discharge of effluent streams containing human and animal excrement, agricultural fertilizers and organic industrial wastes are the major sources of inorganic nitrogen which enters aquatic systems (DWAF, 1996).

Nitrate levels higher than 15 mg/ℓ may cause spontaneous abortions, still births and infant mortalities. In higher concentrations there is some risk of death in older children and adults, especially from gastric and other cancers (Fourie, 2005). Elevated nitrate concentrations in water bodies, ranging from 150mg/ℓ to 850mg/ℓ is a threat to South African communities relying on this water for usage (Tredoux, *et al.*, 2001). Typical toxic responses to nitrate exposure are methaemoglobinaemia, abortion and still-born babies (Chibi & Vinnicombe, 1999; Tredoux *et al*, 2001).

In South Africa, inorganic nitrogen concentrations in unimpacted, aerobic surface waters are usually below 0.5 mg/ℓ but may increase to above 5 - 10 mg/ℓ in highly enriched waters (Ochse, 2007; DWAF, 1996). Unimpacted systems typically have an N:P ratio greater than 25-40:1, whilst most impacted (i.e., eutrophic or hypertrophic) systems have an N:P ratio of less than 10:1. High nitrate levels in an aquatic ecosystem, is usually also accompanied by high COD levels. Recent analysis of the Vaal River at three sampling sites, indicated average nitrate:phosphate levels of 11.75:1 (Van Niekerk, 2009). This result implies that the Vaal River is moving from being oligotrophic to eutrophic and highly enriched with organic and inorganic substances. In a study conducted by Igbinosa and Okoh (2009) the total nitrite levels exceeded the regulatory limits in the final effluent of a wastewater treatment plant in the Eastern Cape Province. This shows that wastewater discharge may consist and contribute to high nitrate levels in receiving water bodies. Nitrate is considered to pose a problem to communities when the receiving water body is used for domestic purposes (Igbinosa & Okoh, 2009). The South African guideline for nitrate in sewage effluent is 1.5 mg/ℓ NO_3N (Government Gazette, 1984; DWAF, 1996). Nitrate levels in secondary treatment plant effluent may average 7 mg/ℓ (Van Niekerk, 2000). Currently in South Africa nitrate levels are exceeding the compliance levels of the South African guidelines and World Health Organization tolerance limits for effluents intended for discharge into receiving watersheds (Van Niekerk, 2000; Igbinosa & Okoh, 2009).

From 1999 South Africa utilizes techniques for the removal of these contaminants. The technologies include ion exchange and other adsorptive processes, membrane processes (operating under steady-state conditions), chemical precipitation as well as the microbiological denitrification of nitrates (Chibi & Vinnicombe, 1999).

2.2 MEMBRANE BIOREACTOR SYSTEMS

2.2.1 History and development

The first commercial membrane bioreactors were developed by Dorr-Oliver in the late 1960s for wastewater treatment in North America (Bemberis *et al.*, 1971, as referenced by Judd, 2006). The Dorr-Oliver membrane sewage treatment (MST) process was based on flat-sheet ultrafiltration membranes operated at 3.5bar inlet pressure and fluxes around 171/(m^2h). This system succeeded in coupling normal activated sludge process with a membrane to concentrate the biomass. A purified, disinfected product was generated as end product. In late 1980s, an aqua renaissance programme was instigated by the government of Japan with founders Kazuo Yamamoto and co-workers (Trivedi, 2004). They requested many of their large corporations, including Kubota (an agricultural machinery company), to develop new wastewater treatment technologies that can produce high quality water with a small footprint (Trivedi, 2004; Churchouse & Wildgoose, 1999). Kubota then developed the submerged membrane unit using flat-sheet (FS) microfiltration membranes. In the 1990s a Kubota plant for sewage treatment was installed which lead to major domination of the membrane wastewater treatment market by displacing the older systems (Judd, 2006; Trivedi, 2004). Currently it is being used in more than 1,500 installations worldwide (Trivedi, 2004) to constantly produce water of high reuse quality.

In 2001, Enviroquip, Inc. of Austin, Texas partnered with Kubota to promote its membrane bioreactor technology in the United States, Japan and North America for municipal/domestic wastewater treatment (Trivedi, 2004; Buer & Cumin, 2010; Yang & Cicek, 2006). Simultaneously, the first immersed hollow fiber principle model was established in 1993. This was done by Mitsubishi Rayon who commercialized the ZeeWeed® in North America and Europe (Buer & Cumin, 2010). From the early 2000s USF commercialized Memjet (immersed unsupported hollow fibre). Puron (Germany) introduced a copy-like version of ZeeWeed®. Kolon and Para (Korea) introduced copies similar to ZeeWeed®. Toray introduced a copy-like version of the Kubota module and Mitsubishi Rayon replaced their fine hollow fibre with a braid based ZeeWeed® (Buer & Cumin, 2010). The collective capacity of both Zenon and Kubota has augmented dramatically since the immersed products were first introduced. To date, Kubota and Zenon are the two systems dominating the MBR market (Judd, 2006; Buer & Cumin, 2010).

Although MBR technology has been considered to be a new technology, it is becoming more popular as the technology of choice and reports show that the MBR market is growing faster than the larger market for advanced wastewater treatment equipment (Judd, 2006). In a market research study conducted by Frost & Sullivan (2009) it was predicted that the US and Canadian MBR market adds up to US$32.2 million and projected to reach US$89 million in 2010. Research (2006) states that the global MBR market is rising at an average annual growth rate of 10.9% and is expected to have reached US$363 million in 2010. Market drivers include increased funding, greater legislative requirements regarding water quality and incentives allied with decreasing costs as well as a growing confidence in the performance of the technology (Judd, 2006).

The application of MBRs has diversified to treat a wide spectrum of wastewaters (Churchouse & Wildgoose, 1999). In South Africa, conventional wastewater treatment plants producing liquid waste are faced with more and more problems (Pillay & Jacobs, 2008). These include final effluent that do not comply with regulations, large amounts of sludge being produced by conventional treatment, limited upgrading potential and limited space to build or expand new plants (Pillay & Jacobs, 2008). The option of MBR application is therefore receiving even more attention as the technology of choice for wastewater treatment.

2.2.2 Limitations and motivations for MBR technology in South Africa

Some draw-backs of using this new technology include that it is often seen as bearing high-risk with reference to the need for extensive knowledge to operate the system, especially when operating under anaerobic conditions (Pillay *et al.*, 2008; Pillay & Jacobs, 2008). Furthermore, it can be costly in comparison to the established conventional technologies (Whang *et al.*, 2009). Energy consumption needed to operate MBR processes may be six times higher than the energy required for conventional activated sludge processes (Thomas *et al.*, 2005). MBR technology is also facing major research and developmental challenges with membrane fouling being one of the key disadvantages which has retarded faster commercialization of the technology (Judd, 2006).

Factors that may advance the implementation of MBR technology at local municipalities in South Africa may be 1) the realization of legislation demanding higher water quality yield than those that can be attained by conventional technologies; 2) that MBRs offer the opportunity of a reduction in volume of point source discharges through recycling and improving the quality of point discharges to receiving waters and 3) the technology may also limit water stress that may lead to the deterioration of fresh water resources in terms of quality and quantity (Judd, 2006; Pillay & Jacobs, 2008).

Furthermore, conventional wastewater treatment processes do not allow for complete mineralization of influent matter due to the maintenance of exopolysaccharide-producing populations. On the other hand, membrane bioreactors maintain a high biomass concentration, and thus almost complete mineralization (Stephenson *et al.*, 2000).

2.2.3 MBR applications in South Africa

The first application of a membrane bioreactor in South Africa for waste treatment was reported when Smith *et al*. (1969) used a Dorr-Oliver ultrafiltration (UF) membrane to re-circulate activated sludge back to the aeration tank. Since then four major membrane bioreactor formats have evolved, which include 1) biomass retention bioreactors, 2) bubbleless oxygenation bioreactors, 3) fixed film reactors and 4) extractive membrane bioreactors.

In South Africa, MBR development led to the commercialization of anaerobic digester ultra filtration MBR for use on high-strength industrial wastewater (Judd, 2006). Commercial scale MBR plants that have been commissioned in South Africa by Weir EnVig, formerly Mebratek, are also actively treating industrial, domestic and landfill leachate (Edwards *et al.*, 2006). Ovivo South Africa is currently treating sewage effluent of the George Municipality in Cape Town. Water from the sewage plant is screened and then fed to an ultra filtration membrane plant. Permeate is then returned to a dam for further treatment to produce potable water (Ovivo, 2011).

Ross *et al.* (1992) state that as early as the 1990's ultrafiltration membrane technology was used for the treatment of wastewater in the food industry with up to 97% of COD removed from the water. Ultrafiltration membrane technology was also used for treating brewery effluent with up to 97% of total dissolved salts removed (Strohwald & Ross, 1992). Pillay *et al.* (1994) state that especially external microfiltration membranes were used in South Africa for sludge digestion during wastewater treatment processes.

Leukes (2000) stated in a presentation that intensive treatment systems such as MBRs can be considered appropriate technology for the provision of new water treatment services to rural communities. The modular nature of these reactors ultimately can cause the expansion of treatment in small communities. This author (Leukes, 2000) also mentioned that low cost ceramic membranes with various wall geometries and surface properties, with good consistency that is manufactured at the University of the Western Cape also holds much promise for treating harsh effluents. South Africa has established a strong research foundation for the development of membrane bioreactors for municipal applications (Offringa, 2006; Pillay *et al.*, 2008; Jacobs *et al.*, 1997).

An economic evaluation of a microfiltration MBR process for sludge treatment was performed at a wastewater treatment plant in Durban, South Africa. It was shown that the MBR system could reduce both the capital and operational cost of a conventional anaerobic digester (Pillay *et al.*, 1994). The reduction in cost in the use of membranes ought to support more widespread performance of this technology (Leukes, 2000). Foxon *et al.* (2006) conducted research to determine the appropriateness of an anaerobic baffled reactor for the treatment of domestic wastewater in low-income communities in KwaZulu Natal, South Africa. A pilot anaerobic baffled reactor was built and operated at two municipal WWTPs. Operation efficiency was characterized monitoring chemical and microbial performance using a number of different operating conditions. The ABR was found to be a robust treatment system, with biological and hydraulic advantages over septic tank systems (Foxon *et al.*, 2006). No literature could be found where MBR technology has been implemented as buffer system in conventional wastewater treatment before treated water is discharged into river systems.

2.2.4 International applications of MBR Technology

Yang *et al.* (2006) stated that MBR technology is progressing swiftly worldwide both in marketable applications and research with specific reference to municipal and industrial wastewater applications. Their aim was to review global academic research efforts with reference to MBRs. A total of 339 research papers published from 1991 to 2004 in peer-reviewed international journals were reviewed. From these research papers it was evident that Zenon occupied the majority of the MBR market, especially in North America, while Kubota and Mitsubishi-Rayon have a greater number of installations in other parts of the world (Yang *et al.,* 2006; Judd, 2006; Buer & Cumin, 2010).

From 2004 MBR technology is being used in more than 1,500 installations worldwide (Trivedi, 2004) to constantly produce water of high reuse quality. The application of MBRs has diversified to treat a wide spectrum of wastewaters (Churchouse & Wildgoose, 1999).

Fundamental MBR research is based on studies conducted on membrane fouling, sludge properties, operation and design parameters, microbiological characteristics, modeling and cost (Lu *et al.*, 1999; Daubert *et al.*, 2003; Choo & Lee, 1996; Ognier *et al.*, 2002; Park *et al.*, 1999;

Fan *et al.*, 2000; Xing *et al.*, 2003; Stamper *et al.*, 2003; Cicek *et al.*, 1998). Much of the ground-breaking research occurred in Japan, the United Kingdom and France, but countries such as South Korea, China and Germany have also notably added to the research collection with primary focus on water filtration (Yang *et al.*, 2006).

Applications include those associated with industrial, domestic and wastewater treatment. Some of the vast amount of advantages of MBR implementation in these sectors include: 1) ease of operation; 2) high pollutant removal capacities; 3) greater control over operational parameters; 4) limited sludge production; and 5) smaller environmental footprint (Leukes, 2000; Yang *et al.*, 2006; Ng and Kim, 2006; Judd, 2006). Yang *et al.* (2006) theorized that it may be expected that a significant increase in MBR plant capacity and increased applicability will occur in future. This theory specifically referred to nitrate removal in drinking water treatment and the removal of endocrine disrupting compounds from water and wastewater streams (Yang *et al.*, 2006).

2.3 MBR OPERATION

2.3.1 Materials

Membrane material is perm-selective to specific physical or chemical components (Figure 2.1) and allows some to pass more readily through it than others (Judd, 2006). Pressure is then applied to force the liquid through the membrane to extract a clearer product as permeate.

Usually polymeric or ceramic membranes are used in MBR operations (Fan *et al.*, 2000; Judd, 2006). Key features that a membrane must have include mechanical strength and structural integrity to endure thermal, chemical and operational stress. These include high temperatures, extreme pH, oxidant concentrations and high nutrient levels. Sudden high nutrient levels can occur during shock loading (Stamper *et al.*, 2003; Moharikar *et al.*, 2005). A membrane should also offer resistance to fouling – the major disadvantage of MBR technology (Yoon *et al.*, 1999; Wilf & Alt, 2000; Cho & Fane, 2002; Xing *et al.*, 2003; Thomas *et al.*, 2003).

The most used polymers that are suitable for membrane separation are polyvinylidene difluoride (PVDF), polyethylsulphone (PES), polyethylene (PE) and polypropylene (PP). Ceramic membranes are widely used for industrial water treatment (Cicek *et al.*, 1999; Sheldon & Small, 2005; Edwards *et al.*, 2006; Kim *et al.*, 2008; Fu *et al.*, 2009) and limited to domestic wastewater treatment (Chaize & Huyard, 1991; Brepols *et al.*, 2005; Devu & Dahiya, 2005; Mohammed *et al.*, 2005; Ng & Kim, 2007; Tewari *et al.*, 2010). Bacterial, COD and nitrate retention from municipal wastewater may thus be achieved if microfiltration through a ceramic membrane in an MBR process is followed.

2.5 PROCESS CONTROL AND PARAMETER OPTIMIZATION

2.5.1 Criteria for developing simulation liquid

The goals of the biological treatment of wastewater are to coagulate and remove the non-settleable colloidal solids and to stabilize the organic matter (Tchobanoglous & Burton, 1991). All of these processes are accomplished biologically using a variety of bacteria which are exploited to convert the dissolved and colloidal carbonaceous organic matter into various gases. For bacteria to function, they need a source of energy and carbon for cell synthesis, as well as elements or nutrients like phosphorus, potassium, calcium and nitrogen. A simulation liquid serves as the source of nutrients for the microorganisms to function properly. Adding to the importance of a simulation liquid for microbial nutrition, the success of simulating the hydrodynamic characteristics of the original liquid indicates the capability of the model (Kang *et al.*, 2008). Accurate predictions of how a system will perform can be made by using simulation liquid, without unnecessary alterations to the original setup (Ng & Kim, 2007). MBR simulation research can also be used to reach a global optimization for design criteria, operation protocol, and cost evaluation (Ng & Kim, 2007) through model predictions that may establish the accuracy, reliability, and utility of the model (Tsai *et al.*, 2004). Synthetic wastewater may also be used to control the variable nature of nutrient concentration in raw wastewater Mohammed *et al.*, 2008).

In MBR studies concerned with biofilm development, the carbon:nitrogen:phosphorus (C:N:P) ratio needed for microbial nutrition is usually adjusted to specific levels in the simulation liquid to achieve specific retention purposes and to meet the nutritional needs of the organisms involved. C:N:P ratios in municipal wastewater is about 100:20:5. However, during aerobic wastewater treatment, the C:N:P ratio should be in the range between 100:5:1 and 100:10:1 (San-Diego-McGlone *et al.*, 2000; Winkler, 2008; Somogyi *et al.*, 2010). Fu *et al.* (2009) achieved more than 95.0% organic matter removal efficiencies by following a specific C:N:P ratio in a wastewater treatment study.

For the sake of idealization simplicity, pilot studies are conducted before large scale implementation that can be used to predict process performance (Kang *et al.*, 2008). Bench or pilot scale experiments usually relate to the application in full-scale plants. The failure-rates of large membrane plants are also limited by research done on pilot scale (Churchouse & Wildgoose, 1999). Conclusions can therefore be drawn without exceptionally high capital expenditures. MBR studies conducted at pilot scale level usually involve the use of simulation liquid for especially pollutant retention purposes and microbial characterization (Bodzek *et al.,* 2006; Canziani *et al.,* 2006; Chang *et al,* 2007; Choo *et al.*, 1996; Leiknes & Ødegaard, 2005).

2.5.2 Aeration

High dissolved oxygen (DO) concentrations in an aerobic tank supports and maintain a viable micro-organism population in activated sludge processes coupled with membrane filtration (Canziani *et al.*, 2006; Judd, 2006; Choo *et al.*, 1996). Air is normally the critical and vital design parameter in MBR processes and is needed for floc agitation, membrane scouring and for biotreatment (Mohammed *et al.*, 2008; Shim *et al.*, 2002). Osifo (2001) states that oxygen uptake rate (OUR) is a way of controlling activated sludge systems and is based on the rate at which microorganisms use oxygen as they utilize substrate. High OUR levels therefore indicate high biological activity. Biological activity can therefore be calculated by measuring or monitoring dissolved oxygen decrease in the system over time. High microbiological activity within the MBR unit is needed for enhanced pollutant reduction (Shim *et al.,* Osifo, 2001).

2.5.3 Transmembrane pressure and flux

Membrane technologies with application to the municipal sector are primarily pressure driven (Chaize & Huyard, 1991; Brepols *et al.,* 2005; Judd, 2006; Tewari *et al.,* 2010). Mohammed *et al.* (2008) state that membrane bioreactors can replace the activated sludge process and the final clarification step in municipal wastewater treatment. The rejection of contaminants eventually places a primary constraint on all membrane processes and the rejected constituents in the retentate tend to build up at the membrane surface. This may then lead to a reduction in the flux at a given transmembrane pressure or even an increase in the TMP for a given flux, thereby reducing the permeability (the ratio of flux to TMP) (Brepols *et al.,* 2005). Net fluxes of between 25 $\ell/m^2/h^{-1}$ and 65 $\ell/m^2/h^{-1}$ for municipal wastewater is favored nowadays with critical flux levels reaching up to 100 $\ell/m^2/h^{-1}$ (Defrance & Jaffrin, 1999; Fan *et al.,* 2006; Tewari *et al.,* 2010; Zhang *et al.,* 2010). These levels are especially favoured in MBRs functioning under steady-state operating conditions. Extended knowledge on flux and TMP and the relationship of flux and TMP with flowrate becomes crucial in the operation of a MBR module functioning under non-steady state conditions. Any negligence with reference to flux, TMP and Flowrate ratios may increase fouling that may lead to higher operating cost (Defrance & Jaffrin, 1999).

2.5.4 Effect of fouling on flux and flowrate

For an environmental engineer the issue of advantageous and disadvantageous biofilm formation is a key element in operational design (Lewandowski & Beyenal, 2005). In addition, the issue of controlling flux and flowrate to optimize biofilm formation for the application of specific retention purposes has also received much attention in literature (Park & Lee, 2005). Thomas *et al.* (2005) described fouling as the increase in membrane resistance which manifests as a decline in the permeate flux and the presence of suspended and dissolved material have a dramatic influence on the permeate flux. Fouling may be associated with increased deposition of solid material onto the membrane surface and can take place through a number of physicochemical and biological mechanisms (Le-Clech *et al*,, 2006; Thomas & Judd, 2005).

Le-Clech *et al.* (2006) stated that studies conducted on optimizing flux to control fouling have been pursued since the mid-1980s. Fouling that occurs in the membrane structure is often referred to as "pore clogging" (Judd, 2006). Pore clogging may be irreversible and may lead to ultimate capital loss (Lee *et al.*, 2008). Much of the existing literature has been performed under constant pressure and flux with a rise in resulting transmembrane pressure for the purpose of monitoring fouling in complex fluids, such as municipal wastewater (Le-Clech *et al.*, 2006). According to Judd (2006) control of fouling is limited to five main strategies, including reducing the flux and increasing the aeration.

When fouling increases within the membrane lumen area, flux will decrease across the membrane ultimately leading to an increase in transmembrane pressure. This effect may contribute to biofilm formation changes which may lead to altered retention capacity of pollutants. Studies show that the occurrence of fouling is directly proportional to increases in flux and flowrate velocity although flowrate is kept constant (Le-Clech *et al.*, 2006; Osifo, 2008). Fouling increases almost exponentially with flux and maintenance operation. It is recommended that membrane bioreactor processes should be operated at modest fluxes and below critical flux (where no fouling occurs) (Osifo, 2008; Le-Clech *et al.*, 2006; Wang *et al.*, 2008). By understanding the relationship between flux and fouling, an environmental engineer can be able to study biofilm formation changes with reference to retention application, without the occurrence of extensive fouling.

2.5.5 Hydraulic retention time (HRT)

According to Metcalf and Eddy (1994) hydraulic retention time (HRT) is a process parameter which determines the time in which sewage will undergo aeration in a reactor. It governs the diversity and structure of the prokaryotic population which in turn controls the process performance i.e. of pollutant removal, biofilm structure and stability. It is depicted as:

$$Retention\ time\ (d) = \frac{Reactor\ volume(m\mathbf{3})}{Total\ daily\ flow\ \left(\frac{\mathbf{m3}}{\mathbf{d}}\right)} \qquad (1)$$

HRT of activated sludge is within the range of 4-8 hours will be the same value as the sludge age in the absence of sludge recycles (Metcalf & Eddy, 1994). Kim *et al.* (2008) developed a lab-scale anoxic-aerobic reactor that was continuously operated with coke wastewater. Their system experienced no operating problems and successfully achieved high COD levels at optimum HRT of 16.7h. Viero & Sant'Anna Jr. (2008) indicated that MBR performance depends on HRT. Biased conclusions may only be drawn if data is obtained under non-steady conditions (Viero & Sant'Anna Jr., 2008). To achieve steady-state does also not only depend on the extent of the operation time, but also on the sludge adapting to the assay conditions.

If a process requires very long HRT to produce the desired removal efficiency (usually when complex compounds need to be degraded), a completely different treatment process might be proposed (Viero & Sant'Anna Jr., 2008). A slight alteration in HRT may cause totally different results. When HRT increases suddenly, it may lead to increased pollutant removal efficiencies (Kargi & Konya, 2007). In the work of Ren *et al*. (2005) the effects of HRT (1–3 h) were evaluated after 32 days of operation. They treated a synthetic domestic sewage in a submerged membrane bioreactor. The influence of HRT was clearly observed when HRT changed from 2 to 1 h.

2.5.6 Solid Retention time (SRT)

According to Judd (2006) solid retention time implies the age of the sludge in wastewater treatment systems and is an essential design parameter used for suspended growth systems. The impact of SRT is also taking place via the control of the prokaryote population. Organic matter removal efficiency in MBRs is also associated with the solid retention time (Viero & Sant'Anna Jr., 2008). According to Osifo (2001) SRT is an approximation to the mean solids abiding time

in a reactor system. The importance of SRT in an MBR system is that all of the solids are retained by the membrane leading to better control over the SRT (Duan *et al.*, 2009).
SRT is expressed as follows:

$$SRT\ (\theta c) = \frac{(\textbf{Total solids in the reactor (kg)} \times \textbf{(d)})}{\left(\textbf{Total solids wasted daily} \left(\frac{\textbf{kg}}{\textbf{d}}\right)\right)} \qquad (2)$$

SRT is also usually in the range between 3 to 14 days, where an SRT of less than 3 days causes the biomass to be less dense and thus settleable, producing bulking sludge. When SRT is greater than 14 days, floc particles are too small to settle and the amount of living cells in the biomass is low (Osifo, 2001). Furthermore, sludge production from biodegradation in an MBR can be reduced by controlling the SRT (Massé *et al.*, 2006; Duan *et al.*, 2009). The change in SRT has the greatest impact on sludge production and mixed liquor suspended solids (MLSS) (Judd, 2006). Mixed liquor suspended solids (MLSS) (X g/m^3) in turn affects sludge production, membrane fouling and aeration demand. In turn, any alteration in fouling may change the biofilm capacity to help remove pollutants from the wastewater. Although MBR operation at high SRTs does not usually lead to greater fouling, the occurrence of fouling is still possible (Wang *et al.*, 2008).

Le Clech *et al.* (2006) stated that an optimum SRT value cannot be determined in an MBR system, because of the difficulty related to properly acclimatize a pilot plant to different SRTs. The authors also stated that this could explain the discrepancies in the SRT effects reported in the literature. Criteria recommended by the supplier of the membrane is more prone to define the working SRT. High SRT levels may result in differences in sludge characteristics and to deterioration of effluent quality (Massé *et al.*, 2006). A critical factor to keep in mind when operating MBRs for bioremediation applications is that the fouling rate may increase exponentially when operating at very low SRT levels (2 days). Le Clech *et al*. (2006) stated that there is no other reason, except pure research driven studies, to run MBRs at such extreme conditions. In contrast, Massé *et al.* (2006) stated that COD retention in MBRs may reach levels of 87% regardless of SRT. This may be invaluable information regarding MBR operation at non-steady state conditions with specific reference to biofilm development, monitoring and characterization.

2.5.7 Monitoring nutrient removal through effluent and permeate analysis

Usually membrane module overflow returns to a collecting tank (aerobic, anaerobic, denitrification tank or backflush) as membrane permeate (Arevalo *et al.*, 2009; Gil *et al.*, 2010). Membrane permeate can also be extracted by a pump that generates suction (Gil *et al.*, 2010) and sampled (Di Bella *et al.*, 2008) for a great variety of analysis. Such a system is also equipped with an internal recirculation mechanism driven by a peristaltic pump (Arevalo *et al.*, 2009). In studies concerned with nutrient or chemical removal in MBRs, samples are collected from influent (feed) liquid as well as effluent liquid (Arevalo *et al.*, 2009) to determine MBR effluent quality. In this manner, COD and nitrate levels can be monitored (Ng & Kim, 2007; Arevalo *et al.*, 2009). The effluent liquid is then defined as any liquid exiting a MBR system (normally associated with gravitational forces), after mechanical filtration has occurred (Judd, 2006) and that enters a collecting tank (Gil *et al.*, 2010). Gil *et al.* (2010) stated that by monitoring membrane permeate, with no effluent output, the cost of operating an MBR system may even be reduced. Differences in membrane permeate flux and effluent output flux may also effect MBR cost of operation (Ng & Kim, 2007; Di Bella *et al*., 2008).

Sahar *et al.* (2011) studied the removal of various organic micropollutants (OMPs) in two MBR pilot plants treating the same raw sewage of the Tel-Aviv WWTP. Each system's effluent constituted the feed for its coupled reverse osmosis process. They sampled the permeate and concluded that high removal rates (99 %) were achieved for the various OMPs.

Arevalo *et al.* (2009) characterized effluent quality in an MBR system equipped with polyvinylidenefluoride hollow fibre membranes. They applied the sludge biotic index (SBI) to conclude that there was no association between SBI and effluent quality.

Physical-chemical and microbiological effluent quality may also be determined by measuring the levels thereof in membrane permeate. Gil *et al.* (2010) monitored COD and faecal coliforms in a Kubota MBR pilot plant located in Southeast Spain by measuring levels in the membrane permeate. They found that COD removal reached levels up to 98%, while microbial removal reach values below 10 cfu/100mℓ in the membrane permeate.

Di Bella *et al.* (2008) acknowledged a difference in COD values measured in the MBR permeate and effluent output. They used a *total filtration factor f** to determine total COD removal in the third physical sub-model step.

Krzeminski *et al.* (2011) stated that in case of hybrid MBRs, the permeate produced by the MBR is mixed with the CAS (conventional activated sludge) effluent before final discharge. They found that mixing of the CAS effluent and MBR permeate had negligible effect on the quality of the total combined effluent produced. Krzeminski *et al.* (2011) also found that measured *N*-total can be less in the permeate liquid than in the effluent output of a hybrid MBR system. The levels of soluble microbial products (SMPs) may even differ between that in the effluent and that in the permeate liquid of an MBR system (Ng & Kim, 2007).

2.5.8 Multivariate statistical analysis

Levine *et al.* (2008) stated that in multiple regression the coefficient of multiple determination represents the proportion of the variation in Y that is explained by the set of independent variables. When considering multiple regression models, some statisticians suggest you use the adjusted r^2 to reflect both the number of independent variables in the model and the sample size. Reporting the adjusted r^2 is extremely important when you are comparing two or more regression models that predict the same dependent variable but have a different number of independent variables (Levine *et al.* 2008). Therefore, nutrient removal in an MBR system may be measured in feed water and permeate water. The two regression models being nutrient levels in the feed and permeate water respectively, each predicting the same dependent variable but with different independent variables.

Multivariate analysis may include the F test. The F test tests whether there is a significant relationship between the dependent variable and the entire set of independent variables (the overall multiple regression model) (Levine *et al.*, 2008). From F test results a p-value is calculated and the F statistic given in an ANOVA (analysis of variance) summary table. If the p-value is less than the level of significance, H_0 can be rejected and the conclusion can be made that at least one of the independent variables is related to the dependent variable (Levine *et al.*, 2008).

Ng & Kim (2007) conducted a review to provide an assessment of present efforts in modeling MBR systems for municipal wastewater treatment. They reviewed three models: biomass kinetic models, membrane fouling models and integrated models with light couplings. This was done to describe the complete MBR process. They used multiple regression analysis to determine the value of the three constants and to establish the relationship between sludge viscosity and suspended solid concentrations (Ng & Kim, 2007). They found that multiple regression analysis may be very useful for illustrating MBR hydrodynamics.

By using multivariate analysis it is even possible to compare different MBR systems to each other. Van den Broeck *et al.* (2011) sampled ten different MBR systems in Belgium and the Netherlands to compare single sludge parameters and activated sludge filterability. They concluded that filterability can be predicted by analyzing the bioflocculation state of the activated sludge, but it is dependent on the individual operational parameters of each MBR system.

Multivariate statistical analysis may predict operational changes that may occur when comparing two models of variance with dependent and independent variables (Levine *et al.*, 2008). It may therefore be used as a tool to add to existing MBR studies.

2.6 BACTERIAL RETENTION BY MICROFILTRATION

2.6.1 Biofilm formation

Microbial adhesion to surfaces and the ultimate biofilm formation has been documented in many different environments and that these biofilms represent a protected mode of growth that allows constitute survival in hostile environments (Simões *et al.*, 2010). The authors quoted several other authors stating that the main processes that have been identified as governing biofilm formation include: 1) the pre-conditioning of the adhesion surface; 2) transport of planktonic cells from the bulk liquid to the surface; 3) adsorption to the surface; 4) desorption of reversibly adsorbed cells; 5) irreversible adsorption of the cells at the surface; 6) production of cell-cell signaling molecules; 7) transport of substrates to and within the biofilm; 8) substrate metabolism by the biofilm-bound cells and product transport out of the biofilm. All of these processes are also convoyed by extracellular polymeric substance production, replication and cell growth.

Sheldon and Small (2005) noted that when designing a membrane bioreactor, the membrane morphology is a very important aspect. The membrane itself that is chosen should provide maximum surface area and a model environment for biofilm formation. Previous results indicate that especially heterogeneous biofilms develop on ceramic membranes in bioreactors and that the thickness of the biofilm is actually very uneven throughout the membrane unit (Sheldon & Small, 2005). Furthermore, bacterial cells attach to surfaces to form a biofilm only when there is a metabolic advantage. Certain bacterial strains have a higher tendency to attach to certain surfaces and might even dominate over others within a reactor (Emanaualsson & Livingston, 2002; Sheldon & Small, 2005). The formation of cake-layering also plays a role in nutrient removal or biodegradation of pollutants. Almost always, the pollutant in solution in the feed water must diffuse across the whole biofilm since only the surface of the biofilm is usually active (Emanualsson & Livingston (2002).

Other studies conducted by Emanualsson & Livingston (2002) indicated that high levels of nitrate cannot replace oxygen as electron acceptor. Nitrate can only replace oxygen in layers where nitrate reducing bacteria are present. Usually it is done in this manner for the nutrients to reach the inactive zones of bacteria in biofilms to decrease the flux and thus, decrease the fouling. The conclusion can be made that a developed biofilm that is too thin or too thick, will in effect not have optimum pollutant removal efficiency because nutrients do not reach the inactive zones of bacteria within a biofilm.

2.6.2 Microbial retention in MBRs

Membrane filtration is an excellent way to reduce the microbial population in solutions (Figure 2.1). Usually the filters are circular, porous and a little over 0.1mm thick. A wide variety of pore sizes are available, but usually membranes with pore size of about 0.2 μm in diameter are used to remove most vegetative cells from solutions ranging in volume from 1ml to liters, with viruses being the exception. Bacteria vary in shape and size, with the smallest being about 0.3μm in diameter.

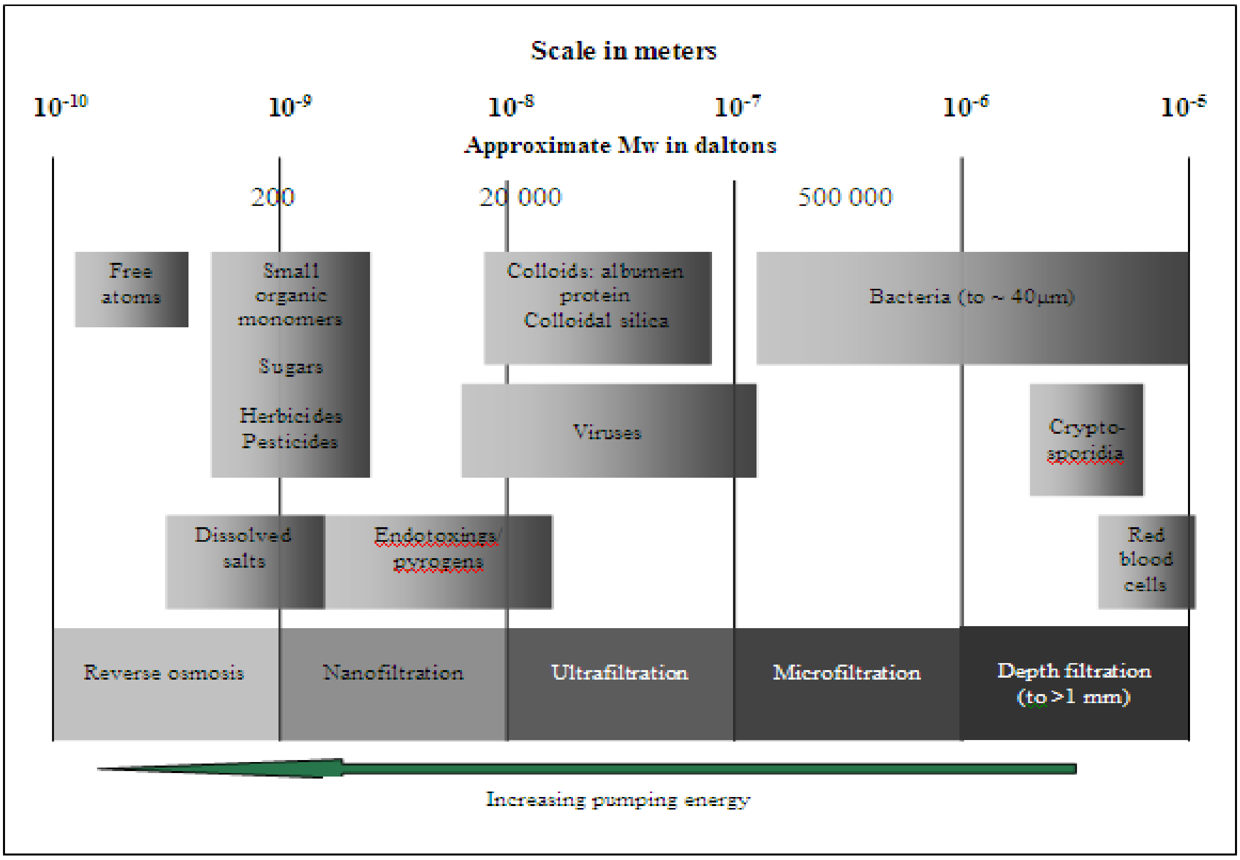

Figure 2.1: Membrane separation processes overview (Judd and Jefferson *In* Judd, 2006)

Figure 2.1 shows the microbial retention ability of membrane technology. Membrane science may thus be applicable to removing bacterial cells or pathogenic organisms from solutions (Figure 2.1). Yang *et al.* (2006) noted that one of the most attractive and promising areas of membrane bioreactor application, is that almost all of the microorganisms can be rejected by membrane processes such as microfiltration and ultrafiltration (Figure 2.1) of which the latter may be used for the retention of viruses. This is particularly advantageous in that it can replace many post-treatment processes in conventional biological waste water treatment.

With reference to pathogen removal MBR applications showed evidence of high removal efficiencies. All permeate samples analyzed indicated the absence of fecal coliform and *Escherichia coli* during municipal waste water treatment (Viana *et al.* 2005). *Escherichia coli* removal of over 98% was reached in hospital waste water treatment. The major disadvantage of using membrane technology for bacterial retention, is that it holds the potential for carbon source breakthrough and that there is little and limited knowledge of fouling potential (Judd, 2006).

2.6.3 *Escherichia coli* as model organism for bacterial retention studies

Escherichia coli is a gram negative bacillus of about 1.1 to 1.5µm wide and 2.0 to 6.0 µm long It is described as a neutrophile, growing optimum between pH 5.5 and 8.0. This species is mesophilic and is facultative anaerobic. Feacal coliform bacteria such as *E.coli* are used as a specific indicator of faecal pollution of water sources by human and warm-blooded animal faeces.

Naturally *E.coli* is not resistant to antimicrobial substances such as ampicillin and tetracycline antibiotics. The plasmid pBR322 can render the organism resistant to antimicrobial substances. *E.coli* could be transformed in the laboratory with pBR322 by using a calcium chloride heat shock technique (Wilson & Walker, 2005). When *E.coli* is transformed with pBR322, the antibiotic resistance genes incorporated in the plasmid render *E.coli* resistant to ampicillin and tetracycline. Transformed *E.coli* can then serve as model organism to measure bacterial retention capacity of membranes. The antibiotic resistance marker genes on pBR322 are exploited. Permeate from the membranes could then be plated on selective media containing either ampicillin or tetracycline. *E. coli* growing should all be transformants introduced into the system.

2.7 BIOFILMS AND MEMBRANE BIOREACTORS

2.7.1 Nitrification and denitrification in an MBR system

The nitrification process is carried out by two groups of bacteria: 1) the autotrophic ammonia oxidizing bacteria found in activated sludge (*Nitrosomonas* and *Nitrosospira*) and 2) the nitrite-oxidizing bacteria (*Nitrobacter* and *Nitrospira*) (Judd, 2006). These organisms need carbon dioxide, ammonia and oxygen as nutrients (Table 2.1). Nitrate oxidizing bacteria such as *Nitrococcus* spp. require nitrite and do not fancy elevated concentrations of ammonia, because it is toxic to them. Other studies conducted showed that ammonia oxidizing bacteria are system-specific and that long SRTs in MBRs are thus accepted as being highly advantageous for nitrification (Clara, 2005; Judd, 2006). On the other hand heterotrophs use organic carbon as an energy source (Table 2.1) and for syntheses of more cellular material and are responsible for denitrification. Autotrophs use inorganic reactions to derive energy and carry out nitrification processes. This is not limited to nitrification processes but applies to other lithotrophs as well. The energy required can be derived from light (photoautotroph) or chemical sources (chemoautotroph). These autotrophs grow more slowly that the heterotrophs with regards to their energy utilization, although both types of organisms prefer temperatures below 10 °C

(Table 2.1). The removal nitrogen by biochemical means demand that oxidation of ammonia to nitrate (Equation 4) take place under aerobic conditions with oxygen levels ranging between 1 1.5 mg/ℓ (Table 2.1). In addition, nitrate reduction to nitrogen is a process subjected to anoxic conditions (Table 2.1). Both these processes may be carried out by a great variety of phylogenetically-unrelated heterotrophs and the overall equation is depicted by:

$$NH_4^+ + 2O_2 \rightarrow NO_3^- + 2H^+ + H_2O \qquad (4)$$

Because the conversion from nitrite to nitrate proceeds at a much faster rate, nitrite does not accumulate in most bioreactors (Judd, 2006). The autotrophic nitrifiers are slow-growers which demand relatively long SRTs to provide almost for complete nitrification. This is seen as another advantage of MBRs where long SRTs are easily achieved.

The process of denitrification takes place under anoxic conditions (Table 2.1) where the nitrate ion is used to oxidize the organic carbon (Equation 5). Molecular nitrogen is generated as the primary end product (Equation 5). This process is depicted as:

$$C_{10}H_{19}O_3N \text{ (wastewater)} + 10NO_3^- \rightarrow 5N_2 + 10CO_2 + 3H_2O + NH_3 + 10OH^- \qquad (5)$$

For biofilm characterization purposes it is necessary to understand and acknowledge the nutritional needs of nitrifiers and denitrifiers to be able to exploit their metabolic inimitability to manipulate and operate MBR processes to optimal capacity. Nitrifiers need oxygen and ammonia to enhance nitrification, while denitrifiers only excel in anoxic conditions (Table 2.1).

Table 2.1: General requirement for nitrifying and denitrifying bacteria for nitrogen removal in MBR wastewater treatment (Adapted from Judd, 2006).

Organism	Process requirements	Nutrient requirement	Energy requirement	Temperature (°C)
Nitrifiers	Dissolved Oxygen 1.0-1.5mg/L	Carbon dioxide Ammonia Oxygen	Autotrophic obligate aerobic, uses carbon dioxide as carbon source	>10
Denitrifiers	Anoxic	Carbon	Heterotrophic, uses nitrate rich wastewater as carbon source	>10

Yoon *et al.* (1999) stated that most wastewaters that are treated by biological processes are carbon limited. Therefore, phosphorus is not significantly removed and it appears that membrane separation offers little or no advantage in terms of phosphorus removal. On the other hand, nitrate removal efficiencies of up to 98.5% have been reported in studies conducted on denitrification MBRs (Ramothokang *et al.*, 2006).

2.7.4 Biomass development

Microorganisms need certain metabolic nutrients for growth and biofilm formation. Over 95% of cell dry weight consists of the major elements, carbon, oxygen, hydrogen, nitrogen and iron. These macro-elements are required by microorganisms in very large amounts and are components of macromolecules, such as carbohydrates, proteins, lipids etc. The macronutrients are necessary for normal biosynthetic cell structures and normal metabolism. While some microorganisms incorporate carbon dioxide in anaplerotic reactions, carbon is usually needed for the skeletal structures of all organic molecules (Osifo, 2001). In addition, electrons are needed to move through the electron transport chains during oxidation and reduction reactions to provide the cell with energy. These electrons are also used to reduce molecules during biosynthesis. Accordingly, microorganisms employed in wastewater treatment, convert the colloidal and dissolved carbonaceous organic matter into gases. The biomass that was synthesized can be removed from the treated water through gravitational forces (Osifo, 2001).

2.7.5 Biofilm formation ecology

Biofilms are accumulations of single or multiple populations that are attached to biotic or abiotic surfaces through extracellular polymeric substances (Singh *et al.*, 2006). A biofilm matrix consists of water, microbial cells, and a biofilm matrix. A biofilm consists of secreted polymers, absorbed metabolites and nutrients, cell lysis products and particulate matter from the direct surrounding environment (Singh *et al.*, 2006). Another important factor in biofilm formation is that certain cell surface components, such as flagella, fimbriae and other surface associated protein or polysaccharide-like structures are important for surface attachment.

Simões *et al.* (2010) stated that microbial adhesion to surfaces and the ultimate biofilm formation has been documented in many different environments and that these biofilms represent a protected mode of growth that allows constitute survival in hostile environments. The main processes that have been identified as governing biofilm formation include 1) the pre-conditioning of the adhesion surface; 2) transport of planktonic cells from the bulk liquid to the surface; 3) adsorption to the surface; 4) desorption of reversibly adsorbed cells; 5) irreversible adsorption of the cells at the surface; 6) production of cell-cell signaling molecules; 7) transport of substrates to and within the biofilm; 8) substrate metabolism by the biofilm-bound cells and product transport out of the biofilm (Simões *et al.,* 2010). All of these processes are also convoyed by extracellular polymeric substance production, replication and cell growth. An organisms or species may therefore have a competitive advantage in a mixed culture biofilm. This may originate from free-living organisms found in wastewater that out competes other species within the biofilm.

2.7.6 Nutrient Gradients

According to Burton *et al.* (1998) nutrient gradients are established in biofilms. This is because when nutrients are supplied to an immobilized biofilm radial nutrient gradients are normally established. The cells that are the furthest away from the membrane surface are normally starved, while those closest the surface have first access to the nutrients. Additional diffusion resistance is then provided by extracellular product formation.

Burton *et al.* (1998) stated that a great variety of research has been undertaken to characterize the structural heterogeneity of biofilms resulting from these spatial gradients. This phenomenon can also be used to continuously produce secondary metabolites. The production of secondary metabolites can be triggered by nutrient starvation when new biomass is laid down at the lumen side of the biofilm. This will result in the new biomass pushing older biomass towards a region of low nutrient concentration. Judd (2006) stated that the high sludge concentration compared to the food available create an environment where bacteria are facing starvation.

2.7.7 Fouling

Membrane performance (fouling) is a function of the biofilm reactor effluent quality and varies with loading rates (HRT) (Leiknes & Ødegaard, 2005). Biofilms are usually maintained within the membrane at a constant rate determined by the flux (Judd, 2006). Because the fouling rate increases more or less exponentially with flux the sustainability of operating MBRs depends on fluxes that are maintained below the critical flux. Fluctuations between high and low TMP can also lead to elevated fouling, while reducing the flux reduces fouling but impacts directly on capital costs. It is crucial to gain knowledge fouling rates to be able to predict, monitor and even manipulate MBR processes. Such manipulation could be beneficial in nutrient removal applications.

Some factors influencing fouling may include physical cleaning regimes, adjusted SRTs and feed pretreatment (Judd, 2006). Meng *et al.* (2009) stated that operating conditions (HRT, SRT) and feedwater have indirect actions on membrane fouling by modifying sludge characteristics. Operating MBRs at extremely low HRTs and high SRTs, with no feed pretreatment and no physical cleaning regimes in place may increase fouling (Cho *et al.*, 2002; Zhang *et al.*, 2007; Ng & Kim, 2007; Liang *et al.*, 2008; Meng *et al.*, 2009).

2.8 MOLECULAR PROFILING BY NON-CULTURABLE METHODS AND BIOFILM CHARACTERISATION

Most membrane associated studies are normally conducted to characterize a membrane's hydraulic permeability or to investigate its behavior, seeking to improve its performance for treating wastewater (Viana *et al.,* 2005). But when it comes to biofilm characterization, most soil organisms cannot be cultured in laboratory environments. Culture-independent techniques do not rely on the cultivation of microorganisms on media in a controlled environment. Non-culturable methods can therefore be used as a more reliable way of investigating a biofilm (Felske *et al*., 1998). Many non-culturable methods have been used for the investigation of microbial community characteristics. These techniques include phospholipids fatty acid analysis (PFLA), nucleic acid examination such as terminal restriction fragment polymorphism (T-RFLP), single-strand conformation polymorphism (SSCP) and denaturing gradient gel analysis (DGGE) (Kowalchuck *et al.,* 2004). Biofilm characterization methods include scanning electron microscopy (SEM) technique. This entails cutting a representative piece of material from the membrane containing immobilized biomass. This is dried out through a gradient of alcohol concentrations. The samples are then critical point dried and Gold coated for SEM (Burton *et al.*, 1998).

For most culture independent methods, the microbial community DNA needs to be extracted. Nowadays, a great variety commercialized kits are available (peqLab DNA isolation kits, Wizard genomic DNA isolation kits) for DNA extraction with standardized reagents and quality control tested being the major advantages. These kits provide a high degree of reliability and the steps involved generally include cell lysis, removal of DNase and protein and eventual DNA precipitation with ethanol (Wilson & Walker, 2005; Duvenhage *et al.*, 2007). DNA molecules

are separated after which it can then be used preparative or analytically and can give qualitative or quantitative knowledge on the specific DNA that is analyzed (Wilson & Walker, 2005). .
The next step in microbial diversity analysis involves amplifying specific marker genes such as 16S rRNA genes for prokaryote diversity analysis or 18S rRNA genes for eukaryote diversity analysis. For this the polymerase chain reaction is used. The method is based on natural replication of DNA. By using this technique, sample or target DNA of high quality is amplified during a multi-step process consisting of specific cycle conditions to be used in other downstream applications. The PCR process is a multifaceted biotechnological tool that can also be applicable in microbial detection and diversity analyses, DNA sequencing, mRNA analysis, detection of unknown mutations and unlimited other applications (Wilson & Walker, 2005). According to Burmingham & Luettich (2003) "the role of PCR remains central in the setting of increasing importance of molecular techniques".

Agudelo *et al.* (2010) proved that by using the PCR technique (real-time PCR and multiplex PCR) for the detection of faecal pollution bacterial indicators, such as enterococci and *Bacteroides*, in untreated environmental water, detection levels and the feasibility of detection was much higher with the PCR technique than that of conventional microbiology, including plate culture methods. However, primer specificity is a major stumbling block, especially when attempting to quantify a mixture of homologous target sequences (Becker *et al.*, 2000). Constructing truly general primers has proven difficult, and even single mismatches in the middle of the primer can cause a preferential selection (Becker *et al.*, 2000). Lowering the annealing temperature allows for mismatches and increases diversity in the PCR product, but also increases the risk of unwanted by-products (Ishii & Fukui, 2001).

One distinctive advantage of DNA fingerprinting techniques is that it may provide a more representative view of a microbial community structure since no culture media and conditions are required (Dahllöf, 2002). Large numbers of samples can be analysed and compared (Smalla *et al.*, 2007). Potential limitations of DNA fingerprinting techniques include biases that may be introduced during sample collection. One of these biases includes the differences in opinions in sample sizes and that the specific size does not always represent the whole microbial community (Dahllöf, 2002). Factors may be present that influence the resolution of the fingerprinting technique, masking some of the information (Smalla *et al.*, 2007).

2.8.3 Single Strand Conformational Polymorphism (SSCP)

PCR-Single Strand Conformational Polymorphism (PCR-SSCP) is a powerful PCR-based technique for identifying sequence changes or mutations in amplified DNA (Fujita & Silver, 1994). According to Kerr & Curran (1996) the specific nucleotide sequence of interest (target sequence) is firstly amplified during the PCR process and eventually separated in single stranded form by electrophoresis on a 4-12% polyacrylamide gel and cross-linker bis-acrylamide. The SSCP technique is capable of identifying most sequence variations – up to between 150 and 250 nucleotides in length - in a single strand of DNA (Humphries *et al.* 1997). Kerr & Curren states that because mutation detection depends on the conformational changes of the single strand nucleic acid induced by the mutation, its efficacy is sensitive to the physical environment in the gel. Factors that may influence the efficacy of an SSCP gel to separate single strands include: 1) the level of cross linking between acrylamide and bis-acrylamide molecules; 2) temperature; and 3) the concentration of ions or solvents or additives. Bands can be excised from the SSCP gels, and sequenced. The SSCP process can thus provide diversity data as well as identifications of individual species.

According to Kerr & Curran (1996) most mutations will be detected in a fragment of less than 200 base pairs by electrophoresis at both room temperature and 40ºC, using a gel of 1% C containing 5% total acrylamide monomer, with 5% glycerol. Usually, [a-32P]dCTP or P^{32} is included in the PCR protocol after which the diluted PCR product is denatured by a brief boiling step (usually in the same thermocycler), after which the sample is loaded on a nondenaturing “sequencing” acrylamide gel. Samples thus adopt a single-stranded secondary structure because of the formation of interstrand base pairing (Humphries *et al*., 1997). Schwieger & Tebbe (1998) state that since no gradient gels and no GC clamp primers are required and since no specific and costly apparatus are necessary, the SSCP technique is very straightforward. A major application of SSCP is the one in which it is combined with DNA sequencing, whereby 16S rRNA genes attained from PCR with a variety of bacterial species could be differentiated and distinguished from each other (Schwieger & Tebbe, 1998).

SSCP is also known to be limited in terms of applicability in structural analyses of natural bacterial communities. According to Fujita & Silver (1994) the technique has been used to screen for inherited mutations or detect somatic mutations in cancer cells. They also mention that SSCP has also been used in microbiology to classify virus strains and to identify strongly selected molecular variants during molecular evolution analyses. The SSCP technique can also be used for biofilm detachment and oxygen uptake reactor studies (Derlon *et al.*, 2008). Kerr &

Curran (1996) state that some microbial applications for the PCR-SSCP technique includes: microbial identification of bacterial 16S ribosomal DNA as well as fungal 18S ribosomal DNA and enteroviruses. The technique can also be used to predict microbial susceptibility (*Bacillus subtilis, Staphylococcus aureus and Mycobacterium tuberculosis*) to chemotherapeutic drugs. Xingqing *et al.* (2007) characterized microbial communities of different depth sediment samples by SSCP. Their results showed that the sequencing of dominant bands demonstrated that the major phylogenetic groups identified, belonged to *Bacillus, Brevibacillus* and *Acinetobacter* sp. From their results they could conclude hat a highly diverse bacterial community existed in the lake sediment core. However, the heterogeneity of 16S between multiple copies within one species hampers pattern analysis (Klappenbach *et al.*, 2001). This can confuse the interpretation of diversity and sequences retrieved from banding patterns.

2.8.4 DGGE

DGGE analysis is used for the separation of double-stranded DNA fragments that are identical in length with different sequences. DGGE examines microbial diversity based upon electrophoresis of small PCR amplified DNA fragments (200-700 bp) (Justé *et al*., 2008). In the DGGE technique, a sample of heteroduplex DNA is amplified by the PCR process in which a 40bp G C sequence is attached to one end of the duplex (usually the 5' end of the forward primer). The mutated heteroduplex is then separated according to differences in sequences. This is due to changed melting point (T_m) properties. Separation is achieved by using a polyacrylamide gel that contains a gradient of the denaturants urea or formamide (Wilson & Walker, 2005; Justé *et al*., 2008). At a specific point in the gradient the heteroduplex will denature relative to a) the length of the product; b) its GC content; and c) its sequence. The GC clamp will not denature in the gel and it will prevent the denatured heteroduplex from further migration. Bands can be excised from the DGGE gels, and sequenced. The DGGE process can thus provide diversity data as well as identifications of individual species.

The DGGE technique, as well as SSCP has been widely adopted for genetic analyses of microbial communities because a comprehensive description of a specific community may be acquired (Justé *et al*., 2008). These techniques are also exceptionally appropriate to monitor microbial population dynamics or to compare microbial community compositions between different treatments, situations or environments. According to Ercolin *et al*., (2003) and Randazzo *et al*., 2002 (*In* Justé *et al*. 2008) a variety of regions of the 16S rRNA gene have been used for DGGE fingerprinting. But it is the species-specific heterogeneity and the length of the V3 region within this gene that makes this region the ideal choice for DGGE and SSCP analysis.

Despite the fact that DGGE can be used to study microbial communities from mats, biofilms, hot springs, rhizospheres and soil (Schwieger & Tebbe, 1998), it can also contribute immensely in food microbiology (Justé *et al*., 2008). Stamper *et al.* (2003) monitored the bacterial population of a greywater membrane bioreactor treatment system by means of PCR-DGGE profile analyses and found the bacterial population to be diverse and unstable during the experiment. Kowalchuk (1997) used DGGE to identify plant-infecting fungi. In their study, the 18S rRNA genes from 20 isolates of fungal species previously recovered from *A. arenaria* roots were cloned and partially sequenced to aid in the interpretation of DGGE data. DGGE patterns recovered from laboratory plants showed that this technique could reliably identify known plant-infecting fungi.

Liang *et al.* (2006) showed that DGGE fingerprinting may be used to characterize arbuscular mycorrhizal community structure in soil. Through the sequential amplification of 18S rRNA fragments by nested PCR followed by DGGE analysis, a high-resolution band profile was yielded, giving them insight into the arbuscular mycorrhizal community structure in the soil. Smit *et al.* (1999) also used DGGE for the analysis of fungal diversity in the wheat rhizosphere by sequencing cloned PCR-amplified genes encoding 18S rRNA. Smalla *et al.* (2007) used DGGE to assess the bacterial diversity of arable soil and found that The DGGE analysis of PCR-amplified 16S rRNA gene fragments displayed the typical characteristics of bulk soil patterns. Roling *et al*. (2000) used DGGE to study the anaerobic microbial community in an aquifer. They found that the DGGE technique was able to separate microbial communities from the polluted aquifer below a landfill site from those of aquifers located up or downstream of the landfill.

A major limitation of using DGGE as DNA fingerprinting tool is that it is a complex process in which the necessity of large primers increases the probability of non-identical strands annealing during PCR. The occurrence of co-migration of different fragments and the poor visibility of less abundant bands due to the low sensitivity of staining methods may also contribute to complex band pattern analysis. Separation by DGGE may therefore “hide” diversity within a sample (Dahllöf, 2002).

2.8.5 DNA Sequencing

In Applied Biosystems' Automated sequencing chemistry guide it is stated that DNA polymerases copy a single stranded DNA template by adding nucleotides to a growing chain in which elongation occurs at the 3' end of a primer (an oligonucleotide that binds to the template) during DNA replication. According to Wilson & Walker (2005) the determination of the sequence of bases alongside the length of DNA is a fundamental technique in molecular biology.

Two most popular techniques have been developed for DNA sequencing, namely Sanger Dideoxy sequencing and a Maxam and Gilbert chemical method of sequencing in which the Maxam and Gilbert method is more appropriate for the sequencing of shorter fragments. The only prerequisite for the Sanger method is that the DNA of interest to be sequenced is in a single-stranded form (Wilson & Walker, 2005).

In automated sequencing, the template DNA is prepared as single-stranded DNA followed by a short oligonucleotide that is annealed to the same position on each template strand. The oligonucleotide acts as primer for the synthesis of a new DNA strand complimentary to the template strand (Wilson & Walker, 2005; Primrose & Twyman, 2006; Hirsch *et al.,* 2010). This technique requires the addition of a template, a primer, four deoxynucleotides and one dideoxynucleotide to synthesize new DNA. After the first deoxynucleotide is added to the growing complementary sequence, DNA polymerase moves along the template and continues to add bases, while a dideoxynucleotide terminates elongation. During cycle sequencing, the dideoxynucleotides are tagged with different colored fluorescent dyes and as each labeled DNA fragment passes a detector the color is recorded and the sequence is reconstructed from the pattern of colors representing each nucleotide in the sequence (Wilson & Walker, 2005; Primrose & Twyman, 2006).

Automated sequencers also offer a variety of advantages, according to Primrose & Twyman (2006). These include 1) excellent data generation through manual sequencing and 2) the fact that the output from them is in machine-readable form which eliminates the errors that arise when DNA sequences are read and transcribed manually (Primrose & Twyman, 2006).

Molecular profiling techniques may be coupled to automated sequencing to give insight into a microbial community. Hirsch *et al.* (2010) described a range of molecular techniques used to investigate soil microbial communities by using metagenomics. They found that by using PCR coupled with automated sequencing, many different samples could be assessed simultaneously.

It has also improved the efficiency with which abundance of specific soil microbial populations can be measured.

DGGE and SSCP are two profiling techniques that may be coupled with automated sequencing. These two techniques may even give similar results (Hirsch *et al.*, 2010). SSCP is also less time-consuming and has high sample throughput (Talbot *et al.* 2008). In comparison, the shortcomings of especially DGGE arise from the relatively small number of microorganisms that can be distinguished. This may be due to a limited number of amplicons types that can be detected (Hirsch *et al.*, 2010). Also, gel-to-gel variability and limited sensitivity cause biotechnologists not choosing DGGE as the method of choice when automated sequencing is the following step in microbial identification in anaerobic reactors (Talbot *et al.* 2008). However these methods can be applied to investigate the structure of specific microbial communities if there is sufficient information to design primers that recognize conserved sequences that flank variable regions (Hirsch *et al.*, 2010).

In a study to elucidate the diversity of methanogens involved in anaerobic digestion, Leclerc *et al*. (2004) constructed 11 archeal small subunit (SSU) rRNA gene clone libraries from 11 anaerobic digesters. The digesters treated effluents from food-processing industries, pulp and paper plant, and pig slurry. An estimated amount of 45 clones from each library were screened by SSCP before sequencing a total of 170 clones. From the obtained sequences, phylogenetic analysis was done by using the region corresponding to *E. coli* positions 223–893 (a length of about 632 bp). Eight clones could not be related to any identified bacteria but the others presented at least the 93–95 % identity required for identification at the genus taxonomic level (Devereux *et al*., 1990) and a 97 % homology at the species taxonomic level (Stackebrandt & Goebel, 1994).

Conventional Sanger sequencing methods may be time consuming and may limit the number of samples that can be process. Nevertheless, as the technology improves, and longer fragments can be sequenced in each run, it will become even more useful as a tool (Hirsch *et al.*, 2010).

One of the most useful bioinformatics resources is BLAST which is a “basic local alignment search tool” that can be found at the NCBI website (www.ncbi.nlm.nih.gov). This useful tool makes it possible to identify sequence similarity that has already been identified by sequencing methods. Other sequencing methods include direct PCR pyrosequencing and PCR cycle sequencing (Wilson & Walker, 2005).

Padayachee *et al*. (2006) analyzed the genetic diversity of the community structure in activated sludge samples from the anoxic and aerobic zones of a lab-scale system by using DGGE techniques. They found that the profile for each of the zones revealed a variety of consistent bands throughout the duration of the experiment. Results also suggested that a diverse microbial community existed in both zones which provided a more precise understanding of the microbial community structure and genetic diversity that is present in domestic wastewater. They concluded that the microbial community structure seemed to be consistent under the stable steady state of the system and was unimpacted by the continuous established flow rate of the influent entering the system and the effluent exiting the system.

The use of DGGE methods to investigate bacterial community structures has gain even more attention in the last decade. The most popular and frequently used methods are the PCR followed by 16S rRNA gene sequencing which is a preliminary tool for bacterial identification (Moharikar *et al.*, 2005).

More fitting for bacterial community analyses over time within an MBR system can be evaluated using fluorescence PCR SSCP analysis. With these tools it is possible to analyze community structure at molecular level with specific reference to type and abundance of certain organisms without subculturing the microbial population (Moharikar *et al.*, 2005). Another benefit of employing molecular profiling techniques for bacterial community structure analysis is that it may be possible to discover new dominant species with specific reference to DGGE pattern or profile analysis. A key feature of monitoring microbial population over time is the understanding that it can evolve or change dramatically and continuously in terms of influent quantity and quality which provides it with a great variety of catabolic capacities at different times (Moharikar *et al.*, 2005).

2.9 SUMMARY OF THE LITERATURE

In the preceding literature review it was demonstrated that most WWTPs in South Africa experience managerial problems (DWAF, 2010). Only 7.4 % of all WWTPs in South Africa achieved green drop certification (DWAF, 2010). The persistent discharge of poorly treated sewage causes poor water quality wastewater effluent entering river systems. The main pollutants contributing to poor effluent quality are COD and nitrates. In effect increased levels cause eutrophication of river systems and contribute to elevated microbial levels within a river system. Nitrate levels exceeding 15 mg/ℓ may pose health risks to rural communities causing spontaneous abortions and infant mortalities. Nitrate levels averaging 12 mg/ℓ have been recorded in the Vaal River (Van Niekerk, 2009). WWTPs may reduce COD levels in feedwater from 200 – 1200 mg/ℓ to 50 mg/ℓ if managed properly. The DWAF guideline for COD and nitrate in effluent water is 30 mg/ℓ and 1.5 mg/ℓ (DWAF 1996). Driven by more stringent legislative requirements the use of membrane science as add-on technology to WWTPs may reduce pollutants entering river systems. Membrane bioreactors (MBRs) combine mechanical and biological waste treatment to treat wastewater. Most MBRs usually function under steady state operational parameters, but extensive knowledge is needed to operate such system for optimal nutrient removal. It is less complex to operate MBRs under non-steady state conditions. Also, when monitoring nutrient removal, samples can be taken from the membrane permeate liquid and/or the effluent output liquid, depending on the specific MBR operational parameters and design. In the event of elevated nutrients entering a WWTP, most MBR systems are robust and effective in reducing pollutants. The literature overview also dealt with MBR technology that can not only be used for nutrient removal purposes, but also for microbial retention purposes. To evaluate microbial retention in MBRs, transformed *E.coli* with marker PBR322 may be used as model organism in which culture dependant methods add to the simplicity of monitoring bacterial counts. Biofilm characterization may give insight into the microbial community structure within the developed biofilm in the MBR. For this, culture-independent methods may be used. Bacterial community analyses over time within an MBR system may be evaluated using PCR-DGGE and PCR-SSCP analysis. With these tools it is possible to analyze community structure at molecular level with specific reference to type and abundance of certain organisms without subculturing the microbial population (Moharikar *et al.*, 2005). DNA sequencing usually follows these methods. It may give insight into the presence of specific species and microbial population changes within a community contributing to nutrient removal.

CHAPTER THREE

MATERIALS AND METHODS

3.1 Inoculum preparation

An inoculum was prepared to determine whether natural aquatic bacteria could support and contribute to the total pollutant removing capacity of the MBR system by exploiting their nutritional needs. This inoculum was prepared by using 2 x 200mL Vaal river water. This water was inoculated into two 2L batch reactor systems containing simulation liquid. Simulation liquid constituents used to develop a stock solution with a C:N:P ratio of 85:15:1 as depicted in Table 3.1. Electrical conductivity (EC) and pH were adjusted to ± 75 $\mu S.cm^{-3}$ with 10% NaOH and 10% NaCℓ solutions. This was done to represent aquatic conditions from which the bacteria were originally isolated. The inoculum water was collected from 3 different areas in the vicinity of Parys twice weekly for a period of 8 weeks. River water temperatures varied between 18 and 20°C. These areas were chosen because raw sewage from the Parys wastewater treatment works flowed into the Vaal River adjacent to the centre of town (Van Riet & Tempelhoff, 2009; DWAF, 2009). The poor water quality was the direct result of poor management and infrastructure of the wastewater treatment plant. The water taken from these areas therefore gave representative samples to use for the specific objectives of this study. The coordinates for the sample sites were A) 26º55’17.58’’S 27 º25’26.97”E; B) 26 º53’59.37”S 27 º26’49.86”E; and C) 26 º52’59.45”S 27 º28’34.78”E. Best representative sample (*n*=10) was chosen, according to average physico-chemical results.

Table 3.1: Concentrations of constituents used to develop a stock solution of simulation liquid

Constituent	COD	NO_3	PO_4
Ratio	85	15	1
Source	1) Glucose 2) Acetate	KNO_3	KH_2PO_4
Concentration (mg/L)	1) 280 2) 788	245	14

The 2 ℓ batch reactors were operated under the following conditions at ambient temperature: HRT of 5 days and constant aeration of 8 mg/ℓ. Both batch reactors were fed and bled with simulation liquid on day 4. Stock solution with excess acetate was added to the feed water daily in which it was diluted by the feedwater to levels just below the C:N:P ratio (Table 3.1). After 28 days sufficient biomass (~$1x10^6$ cfu/mℓ) was available for use as an inoculum into a 20 ℓ reaction vessel. The 20 ℓ reaction vessel was filled with inoculum and the MBR process was started up.

3.2 MBR configuration

The lab-scale MBR system that was built consisted of an external circuit ceramic microfiltration membrane (Figure 3.2; 2) with surface area $6.923x10^{-3}m^3$ that was coupled to a 5 ℓ anaerobic tank (Figure 3.2; 10) and an 8 ℓ aerobic tank (Figure 3.2; 6), which yielded a total surface area of 0.01923 m^3. The aerobic tank (Figure 3.2; 6) was aerated with air at a constant pressure of 6-8 mg/ℓ air. The ceramic membrane consisted of 70% aluminium oxide (Al_2O_3), 29% zirconium dioxide (ZrO_2) and 1% yttrium oxide (Y_2O_3) (Edwards *et al.*, 2006). Pore size ranged from 1nm to 0.15µm. Figure 3.1 depicts the membrane lumen surface on to which microorganisms adhere to.

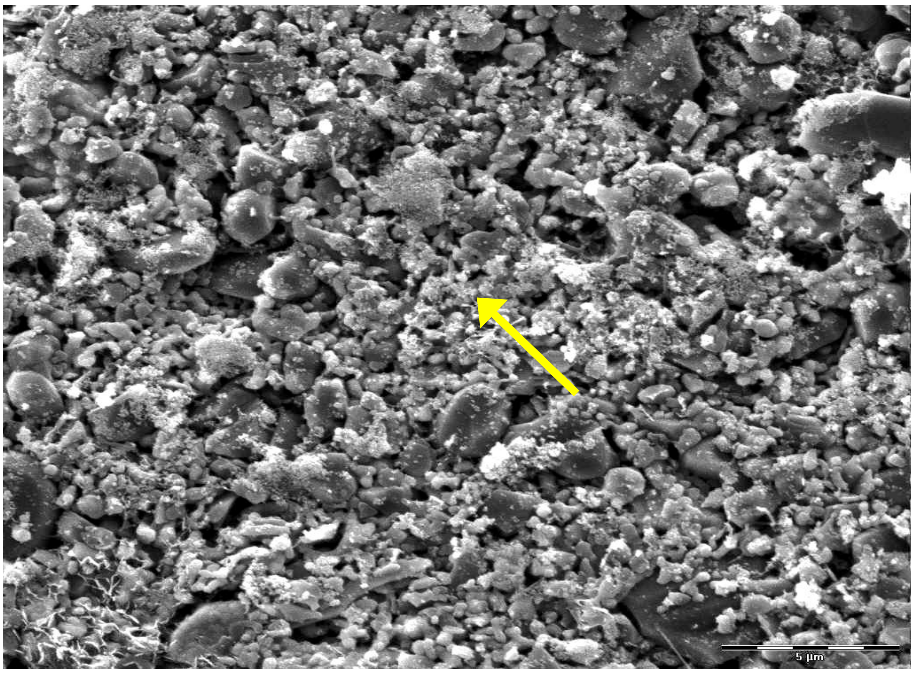

Figure 3.1: SEM microfigure of the lumen surface of ceramic membrane (The arrow indicates the smaller particles nested in or onto the support matrix)

The main processes that have been identified as governing biofilm formation (section 2.7.5) include the pre-conditioning of the adhesion surface and adsorption to the surface. By using a ceramic membrane with surface properties depicted in Figure 3.1, the biofilm will quickly and easily adsorb to the surface after which a relatively stable fixed biofilm will be established and aid in nutrient removal. The smaller particles nested into and onto the support matrix (Figure 3.1) supports the effective adsorption of organisms.

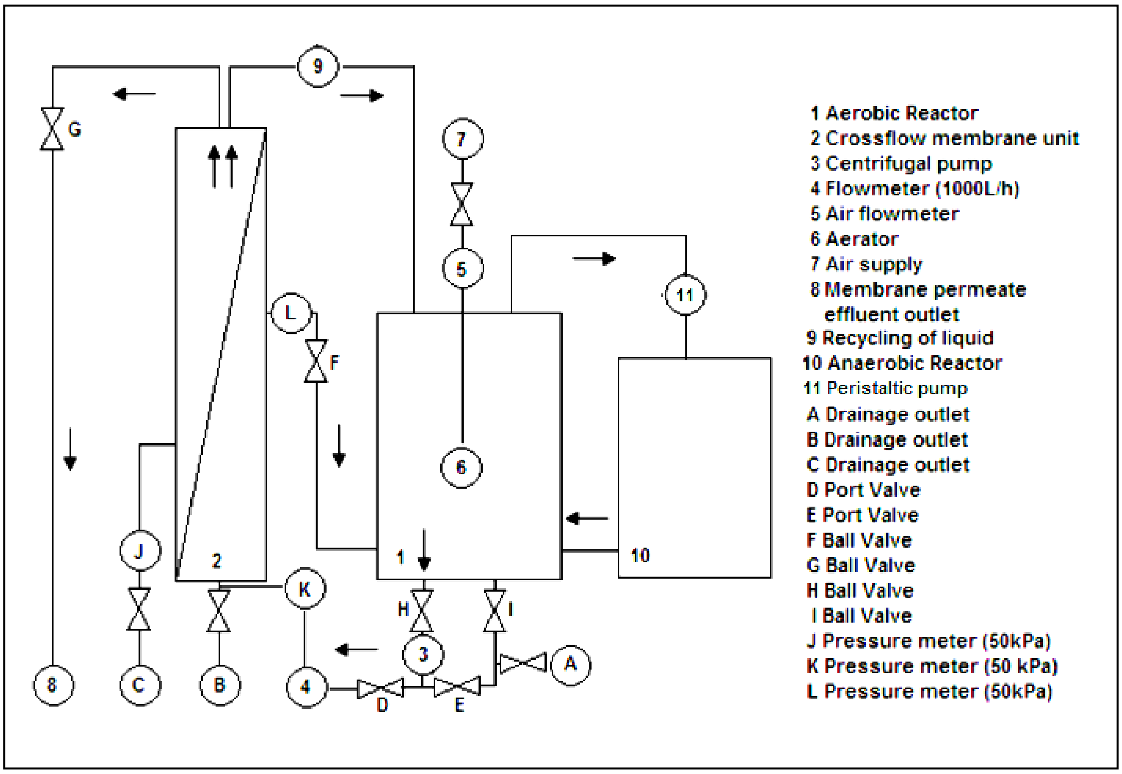

Figure 3.2: Schematic representation of the developed MBR model experimental setup.

Figure 3.2 shows the liquid flow arrows within the ceramic MBR system. A peristaltic pump (Figure 3.2; 11) (Watson Marlow 101U/R MKII peristaltic pump, England) was used to cycle water from the aerobic tank (Figure 3.2; 6) through the anaerobic reactor (Figure 3.2; 10) and back to the aerobic tank in 6h and 3h intervals. A March Pump (MARCH MDX-3 magnetic drive pump, Glenview, IL; Figure 3.2; 3) was used to pump water through the membrane lumen to the aerobic tank. Biofilm sampling took place at a drainage outlet (Figure 3.2; B), while effluent output liquid was collected at drainage outlet C at the bottom of the membrane unit (Figure 3.2; C). Because permeate from the membrane constitutes the treated effluent (STOWA, 2006), membrane permeate liquid was collected at outlet number 8 on Figure 3.2 at the top of the membrane unit. Wastewater return flow is indicated at 9. Flux and flowrate was measured at L and 4, respectively.

3.3 Operational conditions and associated physico-chemical analysis

A nitrate ion selective electrode (pHoenix Electrode Co., US) combined with a pH meter (Eutech eco scan ION 6 PH/ION/ºC, Thermo Fisher Scientific, USA) was used to measure nitrate concentrations. This was done in triplicate. For COD analyses (range: 10-150mg/L; 25-1500mg/L) 3ml of sample was mixed with acid and base solutions A (Cat. No 14538.0065) and B (Cat. No 1.14682.0495) from Merck© (Germany) and concentrations obtained by using a Spectroquant® Nova 60 spectrophotometer (Merck©, Germany) and instructions of the manufacturers. This was done in triplicate. A Multi 350i multimeter (WTW, Germany) was used to obtain pH, DO (Dissolved Oxygen), EC (Electrical Conductivity) and T (Temperature) readings in duplicate.

Figure 3.2 shows that feed was continuously circulating through the membrane module lumen and the aerobic reactor. The anaerobic reactor was kept airtight with silicone. COD and nitrate concentrations were used as parameters to analyze the efficiency of the MBR system by measuring pollutant concentrations in the influent (feed) and effluent (Figure 3.2; 8). Gas-air backflushing took place twice weekly (48 h) when effluent output reached levels below 2L. This was done by closing the valve and associated tubing (Figure 3.2; 9) that recycles lumen liquid back to the aerobic tank. It was necessary to increase pressure within the lumen side of the membrane, as well as the permeate, indicated by L and J on Figure 3.2. When transmembrane pressure increased to 30kPa outlet 9 was quickly opened to decrease the pressure in the membrane lumen. In the process membrane permeate was allowed to diffuse towards the lumen, causing the dislodging of the biofilm that adhered onto the surface to become sessile. The gas-air backflushing regime was also coupled with rinsing the aerobic and anaerobic tanks with distilled water and retracting two thirds of the total reactor volume and replacing it with distilled water when effluent output levels decreased below 2L. Cleaning of the MBR system was done on days 20, 60 and 80.

For the anaerobic reactor, two holes were drilled into the cap with tubing to and from the aerobic reactor. A volume of 5L simulation liquid was used to fill the anaerobic tank to the brim and the cap was sealed with silicone (Ng and Kim, 2007). It was envisaged that nitrate will be used as the electron acceptor for eventual pollutant removing abilities.

All experimental procedures were carried out at ambient temperature, ranging between 18 and 25 °C with difference in day (22-25 °C) and night temperatures (18-22 °C). In conventional aerobic biological wastewater treatment processes, oxygen is usually supplied as atmospheric air and oxygen transfer occurs from the surrounding air to the bulk liquid via a liquid/air interface. In the aerobic tank, air was pumped into the liquid at a constant rate of 6-8mg/L. Flowrate and flux values were measured on a daily basis but the system was allowed to operate under non-steady state conditions.

Non-steady state conditions include MBR operation functioning under spontaneous fluctuations in transmembrane pressure (TMP), flux, hydraulic retention time (HRT) and solids retention time (SRT) (Jang *et al.*, 2006; Ng & Kim, 2007; Tewari *et al.*, 2010). Solids retention time was allowed to fluctuate spontaneously with no mechanical input and to allow levels to change naturally. Hydraulic retention time alternated between 4 and 7 days. The system was kept running under these conditions for 112 days.

3.4 Statistical analysis of physico-chemical results

Pollutant (COD and nitrates) removal capacity of the MBR system was calculated by comparing percentage removal in feed and the membrane permeate by using multiple regression analysis (Gafan *et al.*, 2005). By analyzing data through multiple regression analyses it is possible to determine similarities between constant and non-constant variables through the *P*-test, the t-test or the *F*-test.

Sample size and values for specific data sheets of pollutant removal capacity in the MBR system were subjected to simple singular and multiple regression analyses (PH Stat-2, Levine *et al.*, 2008) with a cutoff for significance of 5% to optimize correlation trends between data values (Gafan *et al.*, 2005). Both the *F*-test and *p*-value were calculated for all simple singular regression while partial *F*-test values were calculated for the multiple regression analyses where HRT was chosen as the dependent variable. The adjusted coefficient of multiple determinations (R^2_{adj}) reflects both the number of independent variables in the model and the sample size. It may also be used as a tool to predict certain trends existing in a system (Levine *et al.*, 2008).

3.5 Challenging the system

3.5.1 Nutrient shock loading

Shock loading with nutrients (C:N:P of 250:35:3) was done on day 64 of operation (Moharikar *et al*., 2005). The system was then subjected to a stabilization period of 14 days.

Table 3.2: Concentrations of constituents used to develop a stock solution of simulation liquid for shock-loading purposes

Constituent	COD	NO3	PO4
Ratio	250	35	3
Source	1) Glucose 2) Acetate	KNO3	KH2PO4
Concentration (mg/L)	1) 83.33 2) 166.66	57.09	4.30

The constituents of the shock loading liquid (Table 3.2) was ± 2.75 X the strength of the initial stock solution (Table 3.1) made to serve as simulation liquid for the purpose of inoculating the system. The stock solution was added to the feed water and automatically diluted within the aerobic tank. By following this protocol, C:N:P ratios was kept relatively constant at high levels. This was done to determine the robustness of the system and the response to shock loading with high levels of nutrients.

3.5.2 Bacterial shockloading

3.5.2.1 Experimental Procedures

For inoculum preparation, *Escherichia coli* JM109 were transformed with plasmid pBR322 (Promega, Madison USA; Fermentas, Germany) using a calcium chloride heat shock method (Tu *et al.*, 2005). Transformants were plated on LB agar containing ampicillin (100µg/mℓ) (Zymo research, Inqaba, South Africa). Colonies were selected on the basis of attained resistance to ampicillin and inoculated into a starting volume of 20mℓ sterile LB broth by following sterile techniques and allowed to incubate 18h at 37ºC while shaking. This volume of 20 mℓ of inoculum was inoculated into a volume of 100mℓ of sterile LB broth containing ampicillin (100µg/mℓ) following incubation for 12h at 37ºC while shaking. The 100 mℓ inoculum was then inoculated into a volume of 1000mℓ of sterile LB broth containing ampicillin (100µg/mℓ).

After 12 hours of shaking incubation, the absorbance of the liquid was measured at 600nm by measuring the optical density of 1mℓ (Jarvis *et al.*, 2007). Sterile LB broth containing no cells was used as blank. A total volume of 1000mℓ LB broth containing ampicillin (~1 x 10^6 cfu/mℓ *E.coli*/pBR322 cells) were inoculated into the aerobic reactor on day 146 of operation. This resulted in a total concentration of ~53000 cfu/mℓ transformants flowing through the MBR system. Immediately after the inoculation permeate was collected and subjected to plate count by membrane filtration. This was done on a daily basis until day 154. The membrane filtration method is a standard method used to determine the microbial quality of water (levels of bacteria). It is based on filtering 100mℓ of water through a 0.45µm filter. The filter is aseptically removed from the filtration unit and placed on selective media. If the aim is to isolate faecal coliforms, mFC agar is a medium of choice (APHA, 1995; Shraft & Watterworth, 2005). In this case 100 µℓ effluent output samples were subjected to membrane filtration and placed on mFC agar containing ampicillin (100 µg/mℓ) as well as on mFC agar without ampicillin.

The plates were incubated (18h at 37ºC) and colony forming units counted. Only blue colonies present on the mFC agar containing 100 µg/mℓ ampicillin were considered as *E.coli* transformants. Single colonies of these were inoculated into 5mℓ of LB broth containing ampicillin (100 µg/mℓ) followed by incubation of 18h at 37 ºC. This was done in triplicate. The presence of plasmid pBR322 in each sample was determined by using a peqGOLD Plasmid Miniprep Kit 1 (Biotechnologie, GmbH, Germany) for low copy number plasmids. The presence of pBR 322 was determined by agarose gel electrophoresis as described previously.

3.6 Monitoring biofilm bacterial dynamics

3.6.1 DNA extraction, quantification and amplification

During back flushing of the system, both planktonic and sessile (loose biofilm) organisms were collected through extracting membrane lumen contents from the membrane module (Figure 3.2; B: Drainage outlet). Total DNA was extracted from the mixed-culture biofilm using a DNA extraction kit (peqLab, Biotechnologie, Germany), following the instruction of the manufacturer.

For amplification of the extracted DNA for both SSCP-PCR and DGGE-PCR an ICycler thermal cycler (Bio-Rad, UK) was used. A final reaction volume of 25µℓ consisted of 12.5 µℓ double strength PCR master mix (0.05U/µl *Taq* DNA Polymerase in reaction buffer, 0.4mM of each dNTP, 4mM $MgCl_2$) (Fermentas Life Science, US), 11.5 µℓ PCR grade water (Fermentas Life Science, US) 0.5 µℓ primer mix (50 pmol) and 0.5 µℓ extracted DNA (~10ng).

For amplification of the 16S rRNA gene, the primer mix contained 0.5 μℓ 27F (5'-AGAGTTTGATCMTGGCTCAG-3') and 0.5μℓ 1492R (5'-TACGGYTACCTTGTTACGACTT-3') primer mix was used. A total of 35 cycles were set with Cycling conditions set at 95°C for 300 sec denaturing; 30 sec at 95°C for melting, 30 sec at 52°C for annealing, 60 sec at 72°C for extension, these conditions were repeated for 35 cycles; final extension was done at 72°C for 10 minutes. The quality and quantity of the amplified products were determined by electrophoresis on 1% (w/v) agarose gels. The electrophoresis buffer was 1 x TAE (40mM Tris (Bio-Rad, UK), 20mM Acetic acid (Bio-Rad, UK), 1mM EDTA (Bio-Rad, UK), pH 8.0). The gel contained 1 μg/mℓ ethidium bromide. A molecular weight marker (MW 1kbp, Fermentas Life Science, US) was loaded in the gel and electrophoresis was performed for 45 min at 80V). Results were visualized with a GeneGenius Bio Imaging System and GeneSnap (version 6.00.22) software (Syngene, Synoptics, UK) (Bezuidenhout *et al.* 2006).

3.6.2 Sequencing through single stranded conformation polymorphism analysis (SSCP)

3.6.2.1 Experimental Equipment and Procedures

Single-strand-conformational polymorphism (SSCP) was one approach used to analyze the dynamics of MBR biofilm microorganisms. In this case a nested PCR approach was used. A fragment of the bacterial 16S rRNA gene was amplified by PCR with primers for *Bacteria* (27F: 5'-AGAGTTTGATCMTGGCTCAG-3' and 1492R: 5'-TACGGYTACCTTGTTACGACTT-3') to yield 1.4 kb fragments (Lane, 1991). This was followed by a second round of PCR using the 1.4 kb fragments as template and to amplify 200bp fragments (Schwieger & Tebbe, 1998; McGregor & Amman, 2005). The primer XXF (SEQUENCE) and ZZR (SEQUENCE) pair that was used for amplifying the latter.

PCR reactions were performed in a total volume of 25μl in microfuge tubes. For amplification of the extracted DNA for SSCP-PCR an ICycler thermal cycler (Bio-Rad, UK) was used. A total reaction volume of 25μℓ consisted of 12.5 μℓ double strength PCR master mix (0.05U/μl *Taq* DNA Polymerase in reaction buffer, 0.4mM of each dNTP, 4mM $MgCl_2$) (Fermentas Life Science, US); 11.5 μℓ PCR grade water (Fermentas Life Science, US); 0.5 μℓ primer mix (50 pmol)(no GC clamp) and 0.5 μℓ extracted DNA (~10ng).

The PCR protocol started with an initial denaturation cycle for 10 min at 95°C. A total of 35 cycles including 30s at 94ºC, 30s at 55ºC, and 60s at 72ºC was followed by a final primer extension step of 10 min at 72ºC (Schwieger & Tebbe, 1998; Justé *et al.*, 2008; Fujita & Silver, 1994).

The quality of the amplified product was analyzed by mixing 5 μℓ of product (200 bp fragments) with 3 μℓ loading dye and loading it onto a 1% w/v agarose gel containing ethidium bromide (1 μg/mℓ (Merck©, Germany)). A molecular weight marker (MW 100 bp; Fermentas Life Science, US) was loaded in lane 1 and electrophoresis was for 45 min at 80V. For SSCP analysisan 8% polyacrylamide gel was used (Kerr & Curran, 1996; McGregor & Amann, 2006). The running gel consisted of 40% acrylamide, 0.6 mℓ 50x TAE buffer, 7.2 mℓ formamide, 7.56 g urea and distilled water that added up to 30 mℓ. The stacking gel composed of 40% acrylamide (1.15 mℓ), TAE buffer (0.3 mℓ) and 8.55 mℓ dH_2O adding up to 10 mℓ. The gel solution was polymerized with 10 μℓ TEMED and 100μℓ and 75μℓ 10% ammonium persulfate (AMPS/APS) for the resolving and the stacking gel respectively. The same loading dye was used for denaturing purposes as well as sample loading onto SSCP gels with final concentration of 0.05% bromophenol blue, 0.05% xilene cyanol, 95% formamide and 0.5 M EDTA (20mM) adjusted to pH 8, and a total volume of 10 μℓ. All samples were subjected to denaturation at 95ºC (5min) in the thermocycler. Samples were then loaded onto polyacrylamide gels. The SSCP electrophoresis was for 3h at 500V, constant current and at 4ºC (Kerr & Curran, 1996; McGregor & Amann, 2006). After electrophoresis, the gel was stained with 1 μg/mℓ ethidium bromide (Merck®, Germany) for approximately 45 min and visualized using a Gene Genius Bio Imaging System (Syngene, Synoptics, UK) and GeneSnap software (version 6.00.22).

3.6.2.3 DNA Sequencing

Dominant bands were chosen in the SSCP gel using resolution and quality within the gel as selection criteria. By following sterile methods, these bands were excised from the SSCP gel and the DNA was allowed to diffuse into nuclease free sterile dH_20. Diffusion was at 4ºC overnight in microfuge tubes. Products were cleaned by following the an E.Z.N.A.® Gel Extraction Kit (Classic Line, peqLab), Germany) using the instructions from the manufacturer. Cleaned DNA was then subjected to PCR amplification. For amplification the PCR reaction was performed with a total reaction volume of 25μl in microfuge tubes and an ICycler thermal cycler (Bio-Rad, UK) was used. The total reaction volume of 25μℓ consisted of 12.5 μℓ double strength PCR master mix (0.05U/μl *Taq* DNA Polymerase in reaction buffer, 0.4mM of each dNTP, 4mM $MgCl_2$) (Fermentas Life Science, US), 11.5 μℓ PCR grade water (Fermentas Life Science, US), 0.5 μℓ primer mix (no GC clamp) and 0.5 μℓ extracted DNA (~10ng).

For amplification of the V3 region of the 16S rRNA gene, 0.5 μℓ 16S rRNA primer mix (w49 (5′- ACGGTCCAGACTCCTACGGG-3′; *E. coli* position, 331) and the reverse primer w34 (5′-TTACCGCGGCTGCTGGCAC-3′; *E. coli* position, 533) 5′) (Inqaba Biotech, SA) were used to yield 200bp fragments (Lane, 1991).

The PCR protocol started with an initial denaturation cycle for 10 min at 95°C. A total of 35 cycles including 30 sec at 94 ºC, 30s at 55 ºC, and 60s at 72 ºC was followed by a final primer extension step of 10 min at 72 ºC (Schwieger & Tebbe, 1998; Justé *et al.*, 2008; Fujita & Silver, 1994). The quality of the amplified product was analyzed by mixing 5 μℓ of product (200 bp fragments) with 3 μℓ loading dye and loading it onto a 1 % w/v agarose gel containing 10 μℓ ethidium bromide (1mg/mℓ). A molecular weight marker (Mw 1kbp) was loaded in lane 1 and run for 45 min at 80V) (Fermentas Life Science, US). The quality of the PCR cleaned product was determined using a NanoDropTM 1000 Spectrophotometer (Thermo Fischer Scientific, US) and only values between 1.7 and 1.9 was considered as acceptable.

Sequencing was done externally by the North West University. The PCR products obtained were prepared for ultimate DNA sequencing by using a kit (E.Z.N.A. Cycle-Pure Kit (Classic Line), peqLab, Germany) and following the manufacturer's guidelines to remove unwanted products. Second round amplification was done by using a Cycle Sequencing BigDye Terminator Kit (Zymo Research, US) with a final reaction mixture of 20 μℓ containing 4μℓ Ready Reaction Premix (2.5 X), 2 μℓ BigDye Sequencing Buffer (5X), 3.2 pmol of the 27F primer (Inqaba Biotech, SA), 1 μℓ Template (10-40 ng) and 9.8 μℓ PCR-grade water (Fermentas Life Science, US). Cycle conditions were set at at 96 °C for 60 sec with 25 cycles of 10 sec of denaturation at 96 °C, 5 sec annealing at 50 °C and 240 sec extension at 60 °C after which it was kept at 4 °C (600 sec). An ABI 3130 Genetic Analyzer (Applied Biosystems, UK) was used for sequencing purposes and chromatograms were viewed with Geospiza Finch TV (version 1.4) software. Sequences were then identified by blasting (BLAST Nsearch) the results using the NCBI database (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

3.6.3 Molecular profiling and diversity analyses through denaturing gel electrophoresis (PCR-DGGE)

3.6.3.1 Experimental Materials and Procedures

Denaturing gradient gel electrophoreseis of the amplified bacterial DNA within the MBR biofilm were used to investigate the microbial diversity of the biofilm through molecular fingerprinting. For amplification of the V3 region of the 16S rRNA 1.4 kb gene, 0.5 µℓ 16S rRNA primer mix containing a GC clamp (341F-GC: 5' CGCCCGCCGCGCGCGGCGGGCGGGGCGGGGGCACGGGCCTACGGGAGGCAGCAG-3' and 907R: 5'-CCGTCAATTCCTTTGAGTTT-3') (Inqaba Biotech, SA) were used to yield 550 bp fragments (Lane, 1991).

For amplification the PCR reaction was performed in a total reaction volume of 25µl using an ICycler thermal cycler (Bio-Rad, UK). The reaction mixes consisted of 12.5 µℓ double strength PCR master mix (buffer, 0.4mM dNTPs and DNA polymerase), 10.8 µℓ PCR grade water, 0.5 µℓ 50 pmol primer mix (with GC clamp) and 2µℓ extracted DNA (~2-5 ng), 1 µℓ MgCℓ and 0.2 µℓ 0.05 U/µℓ *Taq* polymerase (JM Holdings, UK). A total of 35 cycles were set with cycle conditions set as 30 s at 94 ºC, 30 sec at 56 ºC, and 60 sec at 72 ºC was followed by a final primer extension step of 10 min at 72 ºC (Schwieger & Tebbe, 1998; Justé *et al.*, 2008; Fujita & Silver, 1994).

The quality of the amplified product was analyzed by mixing 5 µℓ of product (550bp fragments ~1µg/µℓ) with 3 µℓ loading dye and loading it onto a 1% w/v agarose gel containing 1 µg/mℓ ethidium bromide (Merck®, Germany). A molecular weight marker (Mw 1kbp) was loaded in lane 1 and run for 45 min at 80V) (Fermentas Life Science, US).

DGGE analysis was performed as described by Ji *et al.* (2004). The Dcode™ Universal Mutation Detection System (Bio-Rad Laboratories, Germany) was used. Electrophoresis was performed at a constant voltage of 100V for 16h at 62ºC. Gels were stained with 1xTAE buffer containing 1µg/ml EtBr (Merck®, Germany). Staining was for 45 minutes and gels were visualized using a Gene Genius Bio Imaging System (Syngene, Synoptics, UK) and GeneSnap software (version 6.00.22).

3.6.4.1 Statistical Analyses of SSCP profiles

The SSCP banding patterns that were obtained were converted to a binary matrix using presence-absence data (Rôças *et al.*, 2004). Cluster analysis by using Ward's method (Dorigo *et al.,* 2005; Smalla *et al.,* 2007) following calculation of Euclidean distances (Pearson coefficient) was conducted. This was done to compare SSCP banding patterns and to determine whether the samples revealed a nonrandom pattern.

3.6.4.2 Statistical Analyses of DGGE profiles

The DGGE banding patterns obtained were converted to a binary matrix using presence-absence data (Rôças *et al.*, 2004). For non-culturable methods of calculating diversity analyses within the biofilm, the restraints of using univariate statistics were overcome (Grove, 2006) by using Shannon Weaver diversity indices. The use of the Shannon-Weaver diversity index was chosen as best possible option for bacterial diversity analyses by comparing band intensity to each other (Gafan *et al.* 2005). The Shannon-Weaver (also Shannon-Wiener) diversity index (Gafan *et al.* 2005) is described as:

$$\mathit{Shannon\ Weaver\ index}\ (H') = \sum_{i=1}^{s} (Pi)(\log_e Pi) \qquad (6)$$

where *s* is the number of species in the sample and P_i is the proportion of species *i* in the sample. If pi is the proportion of cover contributed by the ith species, diversity is therefore defined as the sum over all species of öpilog pi (Shannon & Weaver, 1949; Gafan *et al.*, 2005). The variability of different bands' migration lengths as well as their intensities would therefore give an excellent representation of the microbial diversity of a given set of samples run on a DGGE or SSCP gel. The lower the computed value calculated, the lower the bacterial diversity present in one sample (Kutako *et al.*, 2009). Hierarchical cluster analysis was calculated and expressed in dendograms to determine bacterial associations.

For logistic regression analysis, bands demonstrating significance were included as explanatory variables and used for dependent variables, with bands present, coded as 1 and bands absent, coded as 0. Cluster analysis were performed by using Ward's method or UPGMA (unweighted pair group method using arithmetic averages) following calculation of Euclidean distances (Pearson coefficient) to compare DGGE banding patterns in terms of similarities between single bands and to determine whether the samples revealed a nonrandom pattern. Similarities were displayed as a dendogram (Gafan *et al.*, 2005). The cluster analyses and dendogram generation were carried out by using Statistica version 9.0 (Statsoft, U.S.).

CHAPTER FOUR

RESULTS

4.1 MBR PARAMETER ANALYSES

4.1.1 COD Removal

For COD analyses (range: 10-150ml/L; 25-1500mg/L) 3ml of effluent output (Eou) and membrane permeate (Mp) samples were mixed with acid and base solutions A (Cat. No 14538.0065) and B (Cat. No 1.14682.0495) from Merck© (Germany). Mixes were put in a thermoreactor for 2 hours where after COD concentrations were read (after a 30 minute cooling period) by using a Spectroquant® Nova 60 spectrophotometer. Percentage COD removal are depicted in Figure 4.1.

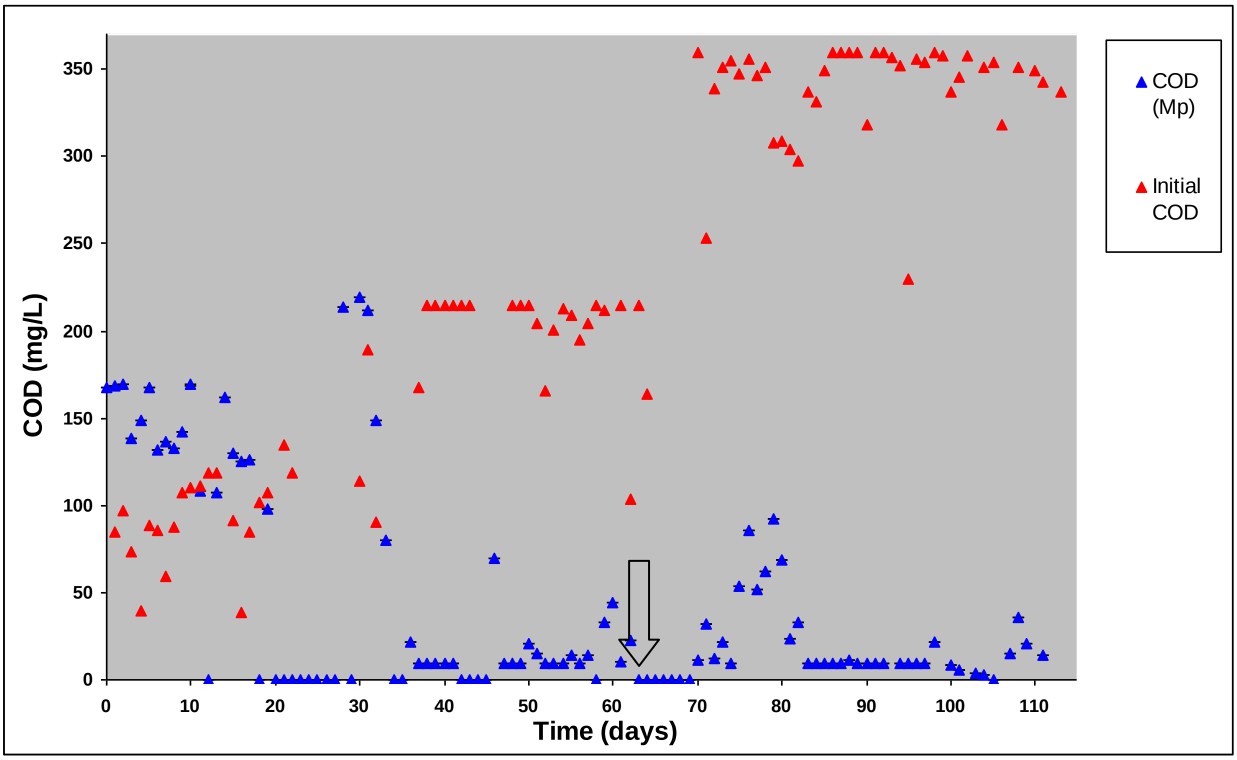

Figure 4.1: COD removal in the membrane permeate over the 112 days of MBR operation. Shock loading the system with nutrients occurred on day 64 (C:N:P ratio 250:35:3).

During the operational period of the MBR system, the average percentage COD removal was calculated as 47.44% (Eou) and 8.27% (Mp) before stabilization on day 51 (day 6 to 51) (Figure 4.2). The system was therefore very unstable with reference to COD removal within the first 30 days of operation. The system was rinsed with distilled water on days 20, 60 and 80. After day 47 of stabilization, the percentage removal efficiency increased to 99.92% (Eou) and 93.00%

(Mp) on day 57 of operation. Figure 4.2 shows that COD removal was stable after day 83. The occurrence of rinsing the system on day 80 could have attributed to stabilization. This trend was maintained until the MBR was shut down on day112.

Calculated average nitrate and COD removing efficiency decreased to 10.86% and 17.61% within 10 days of shock loading with a steady decline in especially nitrate removal towards day 20 (Figure 4.2) COD removal reached 99% after 5 days since the shock loading, which demonstrates the robustness of the system. However, it took nitrate removal efficiency longer (35 days) to recover to 33.57%. After day 64 of shock loading until the end of operation (day 112) COD removal reached 87.99% (Eou) and 84.33% (Mp), while nitrate removal reached 32.89% (Eou) and 17.08% (Mp).

4.1.2 Nitrate removal

A nitrate ion selective electrode (pHoenix Electrode Co.) combined with a pH meter (eco scan ION6 PH/ION/ºC, Eutech, Thermo Fisher Scientific, USA) was used to measure nitrate concentrations in the effluent output (Eou) as well as in the membrane permeate (Mp) on a daily basis. Results are depicted in Figure 4.2.

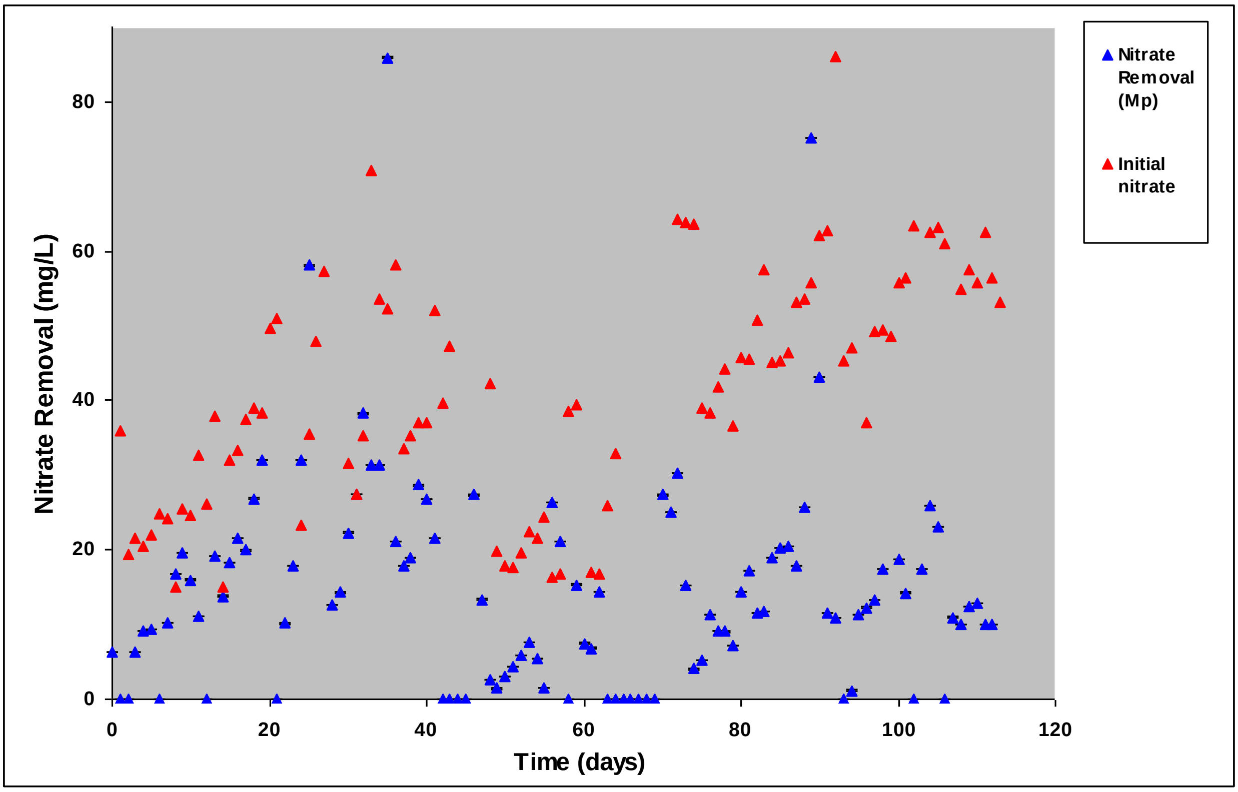

Figure 4.2: Nitrate removal in the membrane permeate liquid levels over the 112 days of MBR operation. Shock loading the system with nutrients occurred on day 64 (C:N:P ratio 250:35:3)

During the first 9 days of operation, average nitrate removal reached levels above 50 %. After 13 days (day 10 to day 33) little to no nitrate removal occurred (as depicted in Figure 4.1). In contrast nitrate was released into the system between days 25 towards day 40. The system showed a period of fairly constant nitrate removal after day 47 until day 64 (before shock loading) in terms of nitrate removal with average percentage removal of 47.64% (Eou) and 52.41% (Mp).

Table 4.1: Percentage COD and nitrate removal as measured in the effluent output (Eou) and the membrane permeate (Mp).

Operational period of MBR (days)	% COD Removal (Eou)	% Nitrate Removal (Eou)	% COD Removal (Mp)	% Nitrate Removal (Mp)
1-112 \(Average)	71.06	46.75	59.15	30.27
0-30	15.79	52.03	13.23	35.22
30-47	83.03	14.84	83.79	18.97
47-64 (Between fairly constant nutrient removal and shock loading)	86.55	50.54	85.22	51.21
64-112 (Shock loading to end)	87.99	32.89	84.33	17.08

Nitrate levels in the membrane permeate (Mp) was averaging lower than levels measured in the effluent output (Eou) (Table 4.1, days 0-30 and days 64-112). The same trend was evident when reviewing COD removal (days 0-30 and 47-112). Overall, nutrient removal was low between days 0 and 30. They system showed unsteady removal during this period and was unstable. With reference to average COD removal, a higher percentage removal was obtained in the effluent output (with a total difference of 3.58 %; Table 4.1, and correlation of 81.08%; Table 4.2).

Table 4.2 shows that a poor correlation (R^2 = 2.88%; p = 0.269) between nitrate and COD existed. There was also no correlation between COD and HRT. Nitrate removal as observed in effluent output was in fact best explained by HRT variable values (p: 0.058). All other independent variables had poor or no correlation with each other and did not correlate to HRT. HRT did therefore not influence nutrient removal.

Table 4.2: Statistical analyses results for MBR pollutant removal data sets (Effluent output (Eou); Membrane permeate (Mp))

Data set	Type of regression	Description	Dependant Variable	Independent Variable	*F*-test	*p*-value (< 0.05: good correlation)	R^2_{adj}	Combined Result
1	Simple singular	Nitrate removal (Eou and Mp)	% Nitrate removal (Eou)	% Nitrate removal (Mp)	216.3189 (*F* critical = 3.96)	1.14896E-25	70.29%	Good correlation: Independent variable explains dependent variable Good predictability
2	Simple singular	COD removal (Eou and Mp)	% COD removal (Eou)	% COD removal (Mp)	322.4401 (*F* critical = 3.98)	1.09251E-28	81.08%	Good correlation: Independent variable explains dependent variable Good predictability
3	Simple singular	Nitrate and COD removal (Eou)	% Nitrate removal (Eou)	% COD removal (Eou)	1.2342 (*F* critical = 3.97)	0.269915154	2.88%	Poor correlation: Independent variable doesn't explain dependent variable Poor predictability
4	Simple singular	Nitrate and COD removal (Mp)	% Nitrate removal (Mp)	% COD removal (Mp)	0.0033 (*F* critical = 3.98)	0.954223567	0.13%	Very poor correlation: Independent variable doesn't explain dependent variable Very poor predictability
5	Multiple	Nitrate and COD removal associated with HRT	HRT	- % Nitrate removal (Eou) - % Nitrate removal (Mp) - % COD removal (Eou) - % COD removal (Mp)	2.8166 (*F* critical = 2.52)	0.058671479 0.469892245 0.18626221 0.891267244	9.91%	- Fair correlation - Relative correlation - Poor correlation - Poor correlation

4.1.3 Change in Electrical Conductivity during MBR operation

A Multi 350i multimeter (WTW) was used to obtain daily EC (Electrical Conductivity) readings of the feed water and the permeate. Figure 4.3 indicates EC as measured in the effluent output (Eou), the membrane permeate (Mp) and the feed (aerobic reactor).

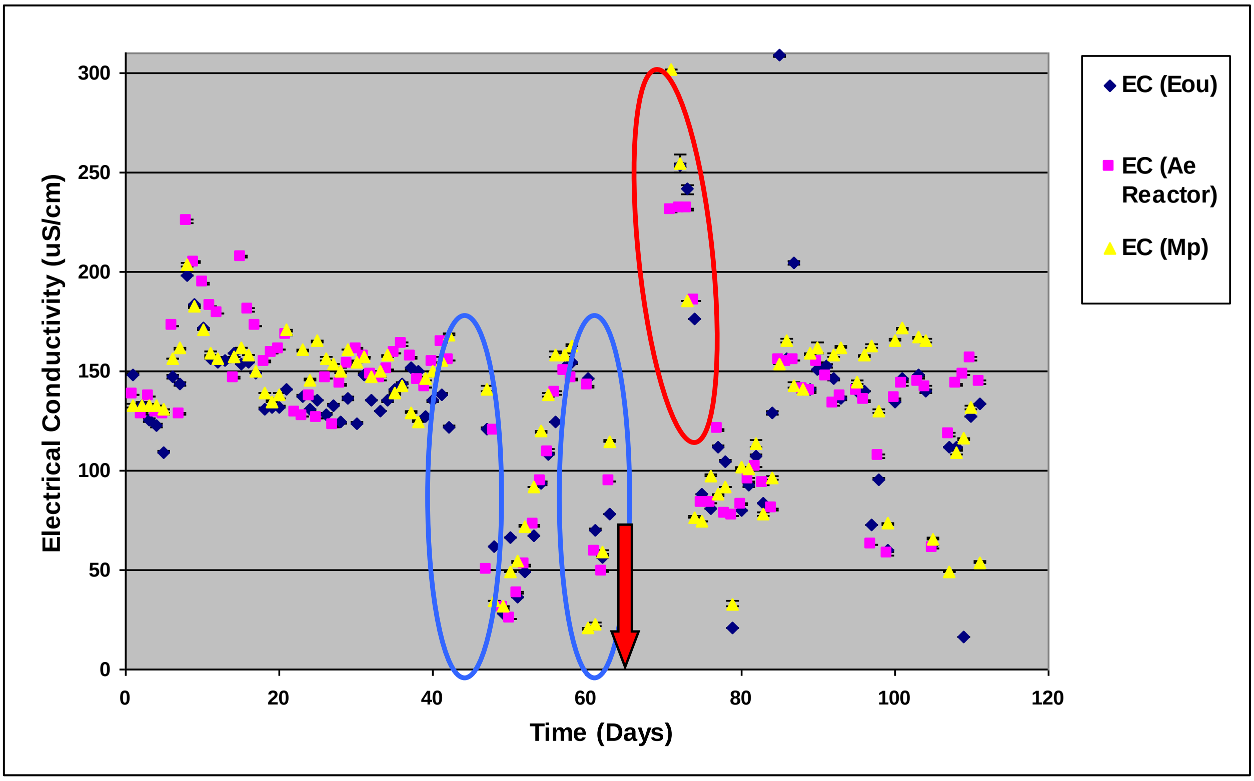

Figure 4.3: Electrical Conductivity values measured in the effluent output liquid (Eou), the aerobic reactor (ae reactor) and the membrane permeate (Mp). (Day 45: Cleaned aerobic reactor with distilled water. Replaced reactor liquid with dH_2O and simulation liquid CNP = 85:15:1 to limit excessive biofilm/sludge formation; Day 20, 60 and 80: Cleaned aerobic reactor with distilled water. Replaced reactor liquid with dH_2O and simulation liquid CNP = 85:15:1 to limit excessive biofilm/sludge formation; Day 64: Shock loaded with nutrients: CNP = 250:35:3)

EC values were at times higher in the Mp as in the feed liquid as depicted in Figure 4.3. Sudden drops in EC values are shown, which relates to cleaning the system with distilled water on days 20, 60 and 80. Similarities between EC in the reactor liquid and the effluent output are also indicated by Figure 4.3. EC values were relatively stable, dependent on rinsing and irrespective of nutrient removal.

4.1.4 Change in flux during MBR operation

Non-steady state conditions include MBR operation functioning under spontaneous fluctuations in flux (Jang *et al.*, 2006; Ng & Kim, 2007; Tewari *et al.*, 2010). MBR flux readings were noted on a daily basis to evaluate its effect on the system under non-steady state conditions. Figure 4.4 depict results obtained.

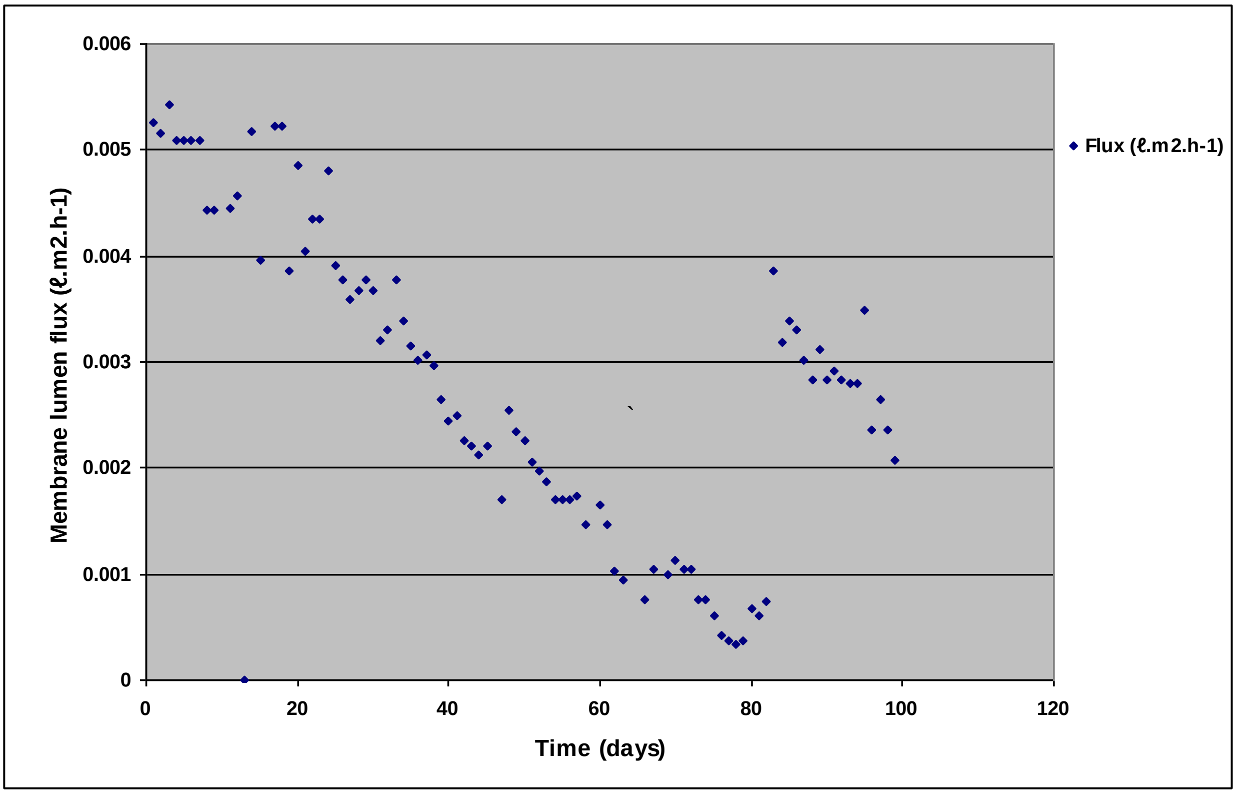

Figure 4.4: MBR lumen flux change over time of operation (dH_2O cleaning on day 80).

Figure 4.4 indicates a rapid flux decline over almost the complete period of MBR operation. An associated decrease in transmembrane pressure (TMP) with about 2 units every week was noted. After day 83, flux values increased, after which dramatic decreases in flux was evident towards the end of MBR operation. On days 20, 60 and 80 the system was rinsed with distilled water. That could explain why flux increased on day 83. Flux shown to be dependent on both rinsing and fouling (Figure 4.4).

4.1.5 Solid Retention Time and Hydraulic Retention Time

MBR systems functioning under spontaneous fluctuations in solid retentions time (SRT) (Tewari *et al.*, 2010) are also operating under non-steady state conditions. The effect of operating the MBR system under non-steady state conditions on the solid retention time was monitored. Results obtained are shown in Figure 4.5.

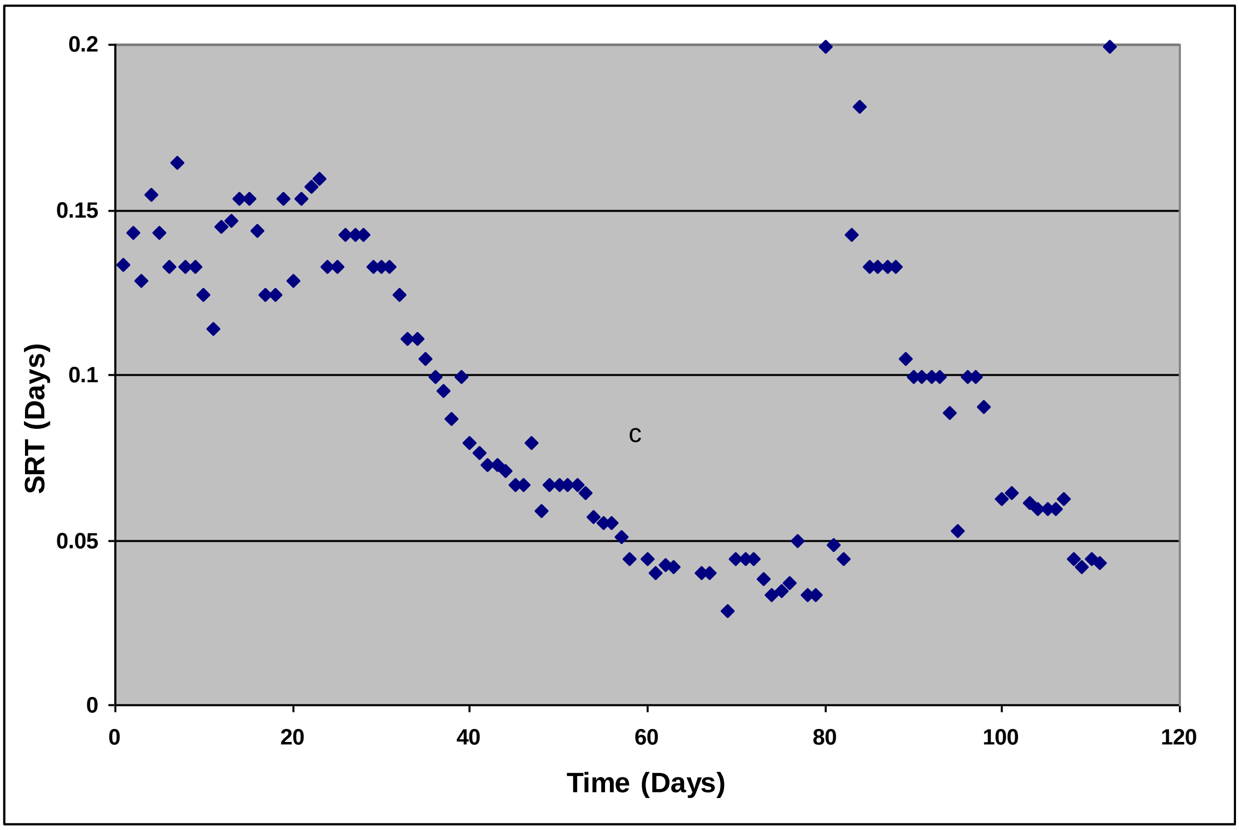

Figure 4.5: Solid Retention Time (SRT) of MBR during operation (periodic SRT values from day 83 to day 112 due to more frequent back-washing and system cleaning with distilled water to limit extensive fouling)

SRT values were measured during the whole period of MBR operation. SRT values decreased periodically over MBR operation. A gradual decline in SRT values were measured especially from day 30 towards day 63 of MBR operation (Figure 4.5). From day 101 towards day 108 of operation, SRT values were stable. The system was rinsed with distilled water on days 20, 60 and 80. Rinsing on day 20 and 80 may explain the dramatic increase in SRT. Solids were therefore not retained in the reactor. After day 60 no visible effect of rinsing could be seen and SRT showed to be relatively constant. Extremely high SRT values (days 0-30 and after day 80) shows that little solids exited the reactor.

Hydraulic retention times were monitored to evaluate the efficiency of the MBR system in terms of pollutant removal, holistically. HRT was kept relatively constant at 5 days. However, it was expected that the hydraulic retention time (HRT) would fluctuate spontaneously, because of the MBR system functioning under non-steady state conditions. Low HRT values may indicate increased fouling. HRT readings obtained are shown in Figure 4.6.

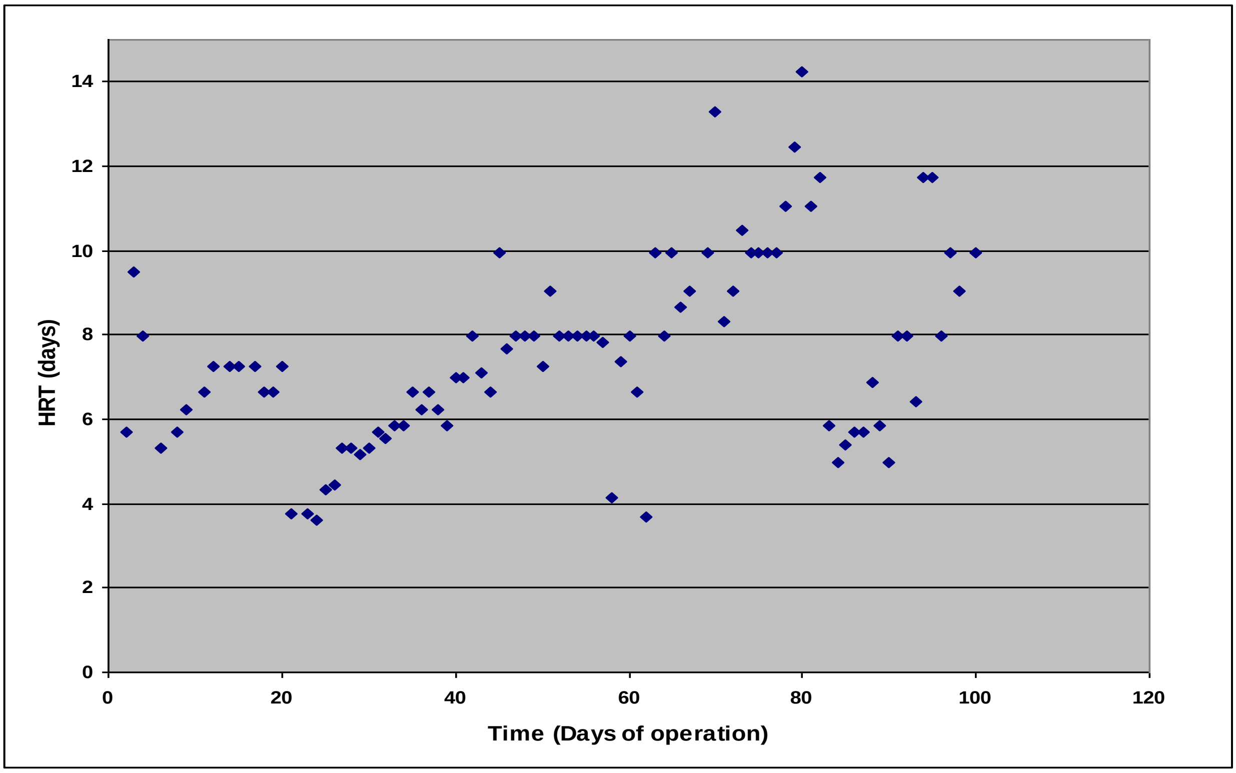

Figure 4.6: Hydraulic Retention Time (HRT) of MBR during operation

Maximum HRT values reached 14.2 days, while minimum values were measured as 3.8 days. Average HRT was noted as being 8 days during MBR operation (Figure 4.6). Periodic increases in HRT can be seen in Figure 4.6 with constant increases from days 20, 60 and 80 respectively. On these days the aerobic and anaerobic tanks were rinsed with distilled water by retracting two thirds of the total reactor volume and replacing it with distilled water when effluent output levels decreased below 2L. Around days 20, 60 and 80 HRT values were lower, indicating increased fouling within the membrane. Thereafter, HRT values increased, due to rinsing and decreased fouling.

4.2 BACTERIAL RETENTION THROUGH MICROFILTRATION

4.2.1 Retention efficiency: presence of plasmid pBR322 in *E.coli*

To ensure that the transformed *E.coli* have been retained by the MBR system during operation, it was necessary to test for the presence of plasmids. Figure 4.7 depicts plasmid pBR322 isolated from transformed *E.coli* colonies from day 1-7 (day 147 of total MBR operation) of MBR operation during retention studies. Colonies were isolated from effluent output. The plasmids were isolated using a peqGOLD Plasmid Miniprep Kit 1 (Biotechnologie, GmbH, Germany).

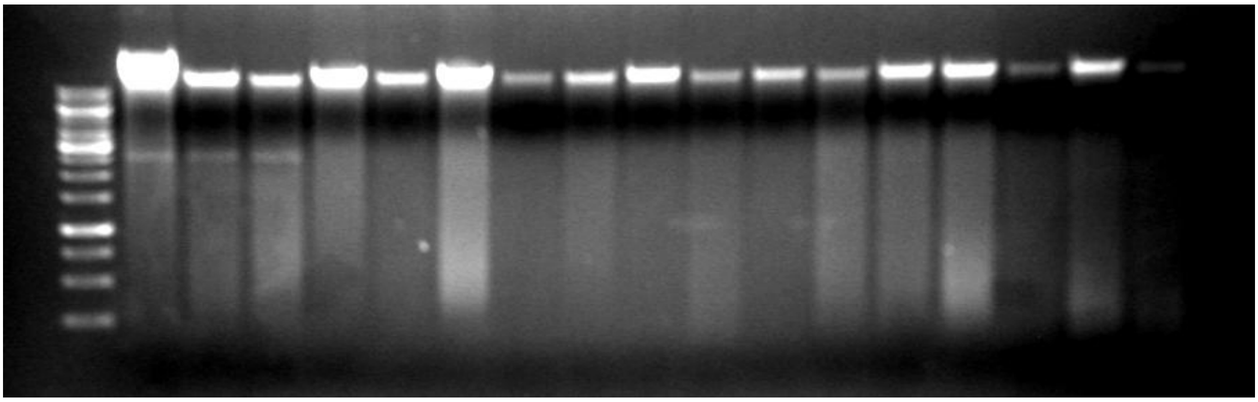

Figure 4.7: Analyses of extracted plasmid pBR322 by subjecting MBR lumen biofilm samples to 1% agarose gel electrophoresis. Lane 1: 100 bp molecular weight marker (Mw: 100 bp); Lanes 2-4: Inoculum (day 146) into aerobic reactor; Lanes 5-7: Effluent output (Eou) liquid (ℓ) (day 146); Lanes 8-10: Eou (ℓ) of day 148; Lanes 11 & 12: Eou (ℓ) of day 151; Lanes 13 & 14: Eou (ℓ) of day 152; Lanes 15 & 16: Eou (ℓ) of day 153; and Lanes 17 & 18: Mp (ℓ) of day 153. (Note the presence of the open circular and supercoiled DNA structures pBR322 in lanes 2, 3 and 4, and only open circular plasmid DNA present in the rest of the lanes)

4.2.2 MBR process performance during microbial shock-loading

A concentration of ~53000 CFU/mℓ *E.coli*/pBR322 cells was inoculated into the MBR system to evaluate the effect of microbial shock-loading on the ability of the system to retain indicator bacteria. The system was also evaluated for COD and nitrate removal during this period. Immediately after inoculation effluent was collected as effluent output and subjected to membrane filtration. Analysis for ampicillin resistant *E.coli* followed that continued on a daily basis until day 154. Retention percentages are depicted in Figure 4.8.

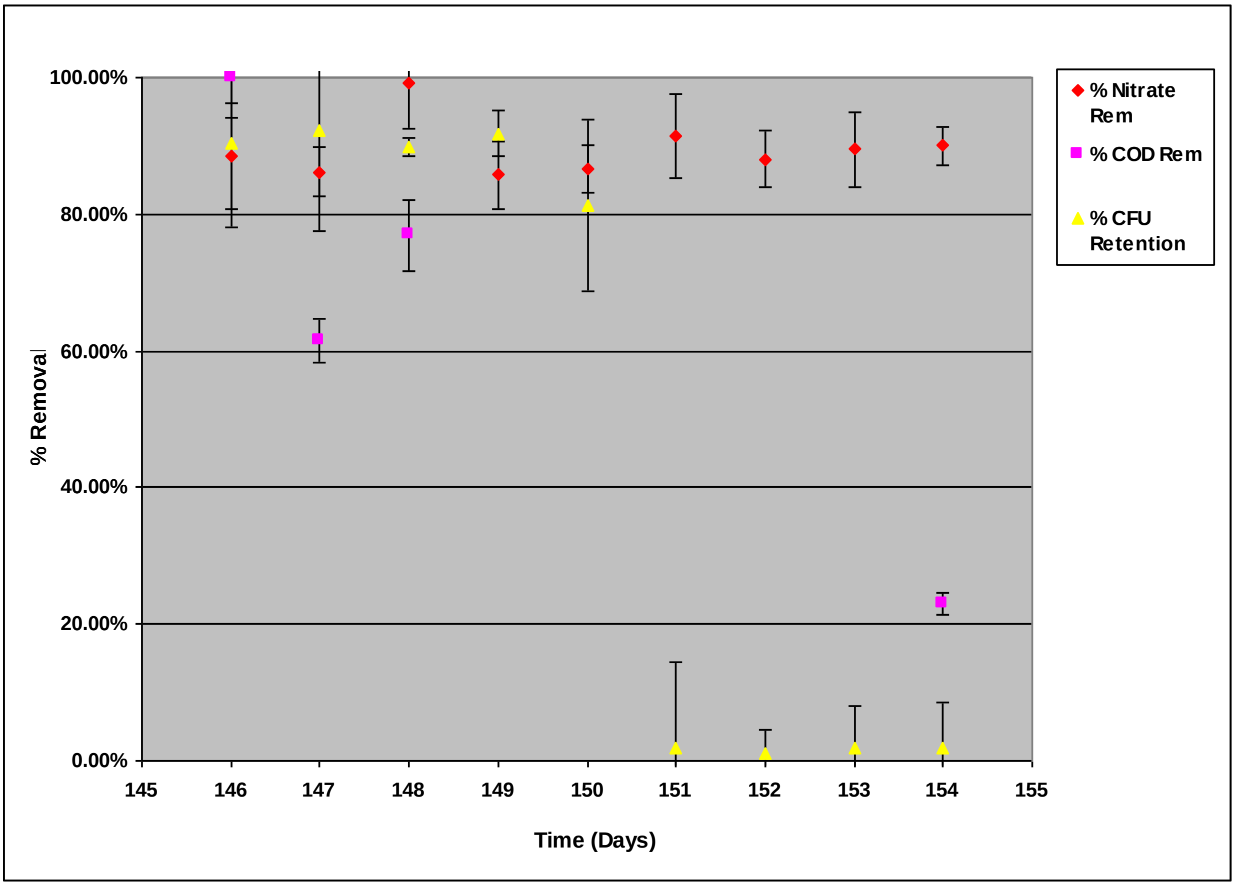

Figure 4.8: Percentage nitrate, COD and transformed *Escherichia coli* retention in the MBR over time

As shown in Figure 4.8, *E.coli* transformant retention was calculated to be between 80 % and 100 % from days 146 to 150. After the 5th day the retention percentage dropped to 81.38% and further decreased to 0 % retention.

4.3 MOLECULAR PROFILING BY NON-CULTURABLE METHODS AND BIOFILM CHARACTERISATION

4.3.1 Biofilm characterisation through SEM

Scanning electron microscopy (SEM) was done at the end of the experiment to analyze biofilm formation on the ceramic membranes. Figures 4.12a-e show SEM photos of the biofilm.

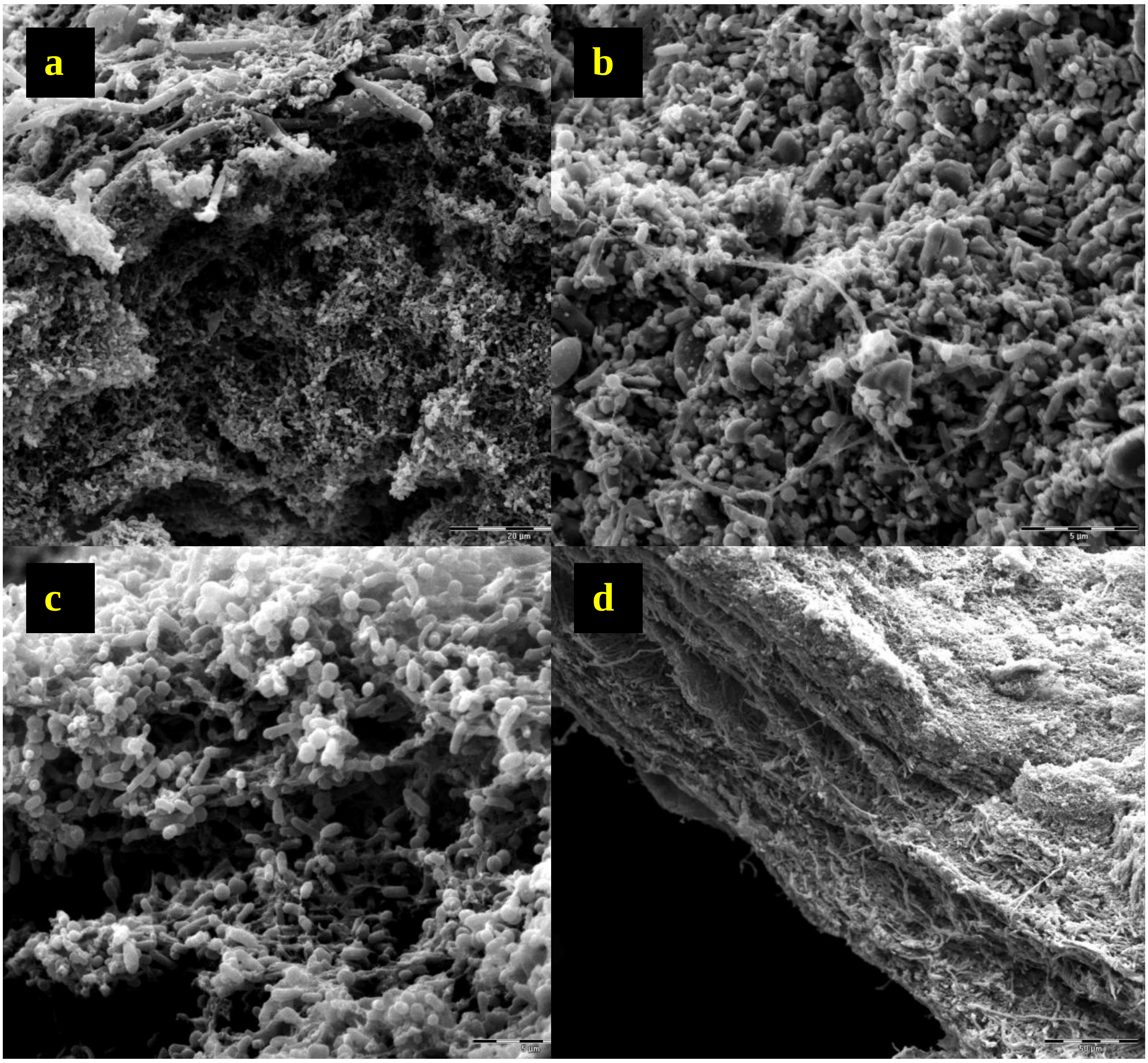

Figure 4.9a-d: SEM of biofilm sections on day 112 of operation: a) a cross section of biofilm with top layer of filamentous fungi and inner layer of bacteria (magnification 3000X; bar = 20µm); b) biofilm covering membrane lumen (*note* slimy matrix on support matrix); (magnification 4000X; bar = 5 µm); c) biofilm cross section indicating cocci and rod shaped cells (magnification 4000X; bar = 5 µm); d) biofilm indicating cake-layering (300X bar = 50 µm).

Figure 4.9a-d shows that the top layer of the developed biofilm is primarily dominated by active biomass (mainly bacilli, cocci connected with filamentous fungi and bacteria, Figure 4.9 a-e). Cake layering is clearly visible in Figure 4.9 d with alternating zones of active and inactive biomass.

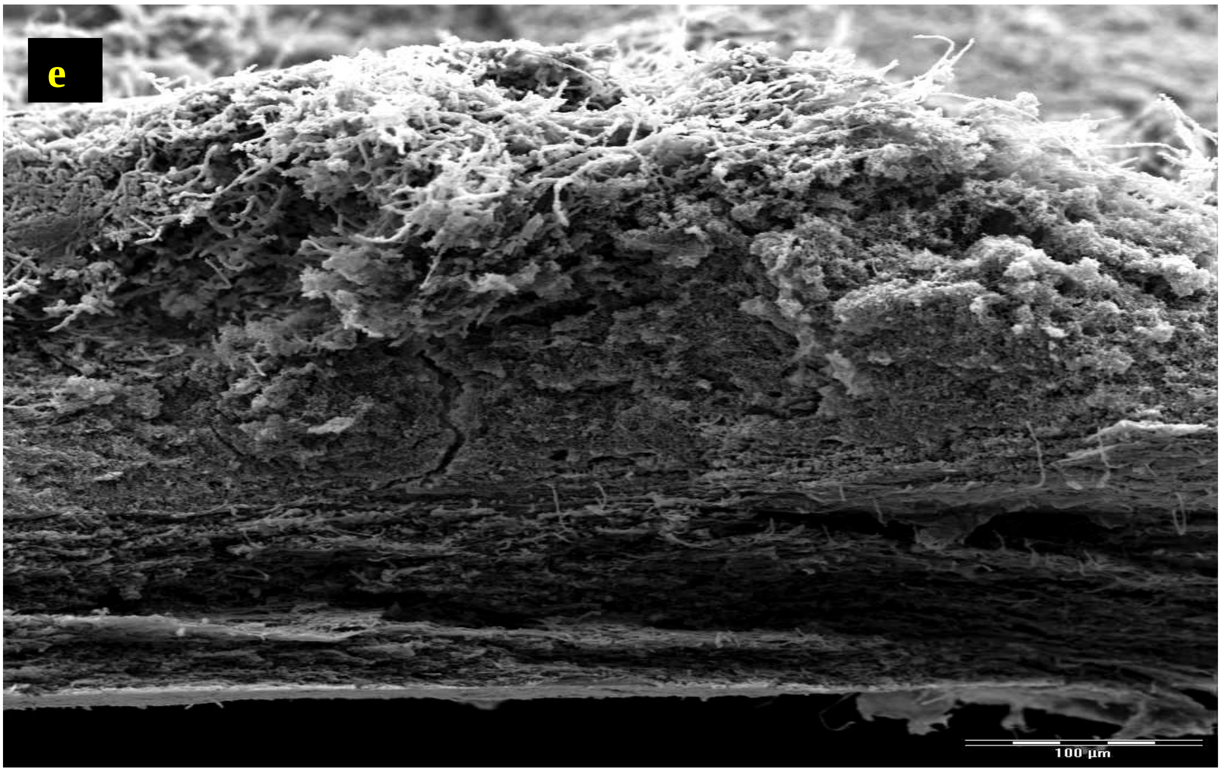

Figure 4.9e: SEM of biofilm sections on day 112 of operation: e) cross-section of biofilm indicating cake-layering (*note* filamentous fungi on the inside/top) (150 X; bar = 100 µm).

Figure 4.9e shows that the bottom layer of the biofilm was mainly dominated by residual inert biomass of which the thickness increased with time (Figure 4.9 e). The bottom layers' thickness is also denser than those layers near the top of the biofilm. At the end of operation (day 112) the membrane surface was covered with a thick cake layer (Figure 4.9e) consisting of sludge flocs and membrane particles congealed together.

4.3.2 Biofilm characterization through non-culturable methods

4.3.2.1 Genomic DNA isolation, quantification and quality analyses

DNA isolated with the peqLab extraction kit was of consistent lower concentration but of high quality. NanoDrop quality and quantity determination (A260:280) indicated band quality of between 1.75 and 1.9 with concentrations averaging at 7 ng/µℓ with RNA contamination being the major contminant. The method was quick, reliable and most suitable for the purpose intended.

4.3.2.2 DNA amplification by Polymeric Chain Reaction (PCR)

For PCR-SSCP analyses MgCℓ (4 mM) and Taq polymerase (1 U) was added to the reaction mixes. Community 16S rRNA was amplified using procedures described in section 3. NanoDrop quality and quantity determination (A260:280) indicated band quality of between 1.75 and 1.9 with concentrations averaging at 30 ng/µℓ.

4.3.2.3 Molecular profiling and microbial diversity analyses through non-denaturing gel electrophoresis (PCR-SSCP)

Polymerase chain reaction single-strand-conformational polymorphism (PCR-SSCP) was used for the analysis of MBR biofilm during the operational period. A fragment of about 200bp of the bacterial 16S rRNA gene was amplified by PCR using primers for *Bacteria*. This was done to identify the most dominant bands for ultimate microbial community analyses of the developed biofilm responsible for nutrient removal. The SSCP profile obtained is depicted in Figure 4.10.

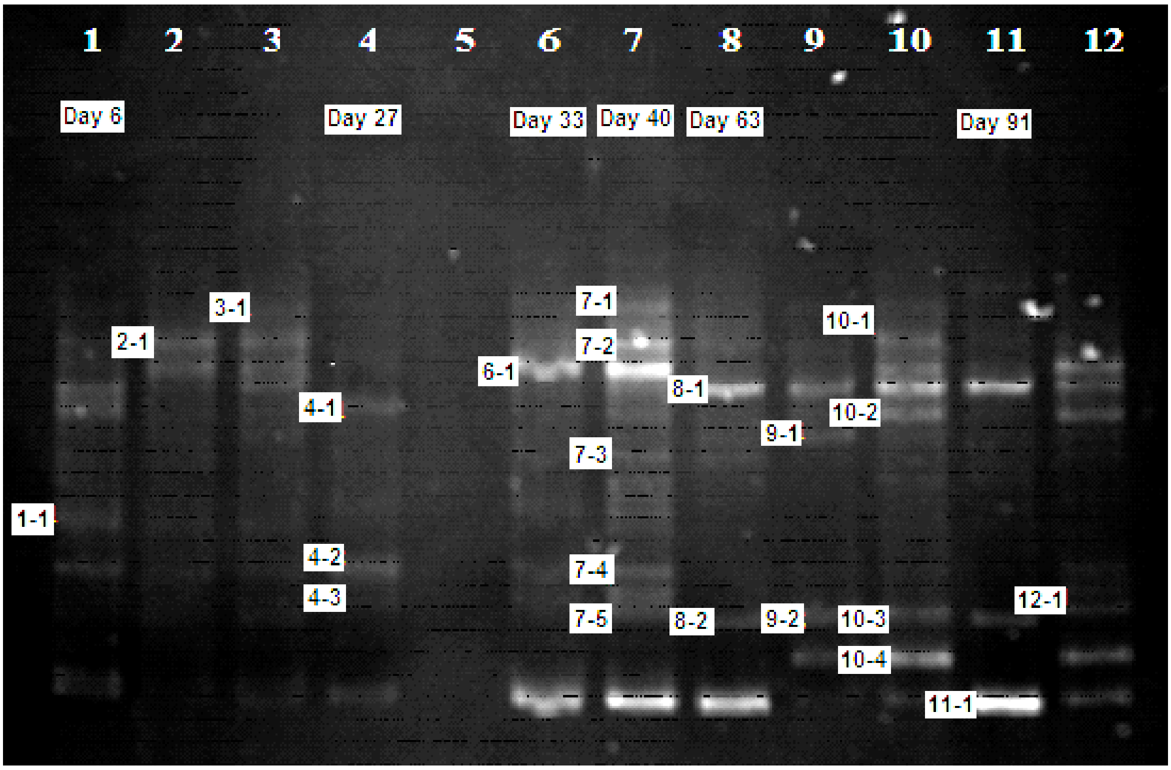

Figure 4.10: SSCP analysis of 16S rRNA PCR products on an 8% polyacrylamide gel indicating sampling days during MBR operation. Selected bands were chosen according to dominance. These bands were excised and then sequenced.

From the 11 biofilm samples a total of 16 different bands were identified on the SSCP gel. Bands with high band intensity were excised, reamplified and sequenced. The identities of sequences are presented in Table 4.2. SSCP analysis (Figure 4.10) revealed complex but readily resolved patterns for all biofilm samples taken. Band 1-1, lane 1) was present throughout the period of operation and decreased during the first 33 days. After day 33 band 1-1 (Figure 4.10, lane 1) increased gradually towards day 40 of operation (lanes 1, 2, 3, 4, 6 and 7) after which band intensities decreased towards the end of operation on day 112. A similar scenario was observed for bands 6-1, 7-4 and 8-2 (Figure 4.10 lanes 6, 7 and 8). The presence of a different organism (6-1, lane 6) was evident during the total period of operation. A third major organism (7-4, lane 7) was also present during the whole period of operation peaking in numbers soon after the acclimatization period. Another organism (8-2, lane 8) was present from day 63 to day 91 with similar concentrations. Band 2-1 was initially present with fairly high intensity (Figure 4.10, lanes 2 and 3).

4.3.3. DNA Sequencing

Purified bands amplified from pure cultures excised from the initial PCR-SSCP gel could be reamplified by PCR and only sequence identification results of greater than 80% were chosen as reference organisms for biofilm characterization over MBR time of operation as values less than that indicate the formation of non-specific products that influenced the sequencing of the DNA. All other identified bands (in which identification was less than 80%) were discarded during biofilm analyses.

Table 4.1.1: rRNA SSCP analysis of PCR product: sequencing results

Lane	**1**	**4**	**6**	**7**	**8**	**11**
Sample Date	26 Feb		25 Mar	1 Apr	24 Apr	22 May
Day of operation	6	27	33	40	63	91
Sequencing results	**1-1:** Bacteroidetes 92%/ (FJ828426) *Flexibacteraceae* 90% (FM209167)	**4-3:** Rhodocyclaceae 96%/ (AM268341) *Zoogloea oryzae* 97% (AB201044)	**6-1:** *Rhodocycloceae* 97%/	**7-4:** *Azoarcus denitrificans* 89%/ (AZORRDG) *Azoarcus tolulyticus* 89% (AF229876) *Rhodocycloceae* 97%/ **7-5**: *Rhodocycloceae* 97%	**8-1:** *Uncultured bacterial clone mdc38fo7* 95%/ (AY537655) *Delftia sp* RF-83 95% (GQ205102) **8-2:** *Aeromonas hydrophila* 322A 99% (GQ352498)	**11-1:** *Delftia sp* RF-83 94%/ *Aeromonas hydrophila* 322A 94%

The following organisms were identified: *Bacteroidetes, Zoogloea oryzae, Rhodocycloceae, Aeromonas hydrophila* and *Delftia sp.* (Table 4.1.1). Another key feature attributing to biofilm diversity is the narrow correlation between days 6 and 27 of MBR operation with exactly the same HRTs (5.31 days). Also, between days 17, 20 and 33 linkage distances of less than 2.0 can be observed.

Eleven biofilm samples were analysed for PCR-SSCP and 16 banding positions were identified. Present/absent analysis was conducted and the resulting matrix was analyzed using Statistica version 9.0 (statsoft, US). Euclidean distances and Wards method was used for the cluster analysis. The resultant dendrogram is presented in Figure 4.11. From Figure 4.11 it is evident that the profiles from the first 33 days formed a separate cluster from those from day 40 to 101. *Delftia sp.* was identified as the band with the highest intensity (Figure 4.10 lanes 8-2, 9-2, 10-3 and 11), although some bands were weaker but still characteristic in the lower half of the gel with dendogram linkage distances below 1.75 for days 63, 73 and 91 (Figure 4.11).

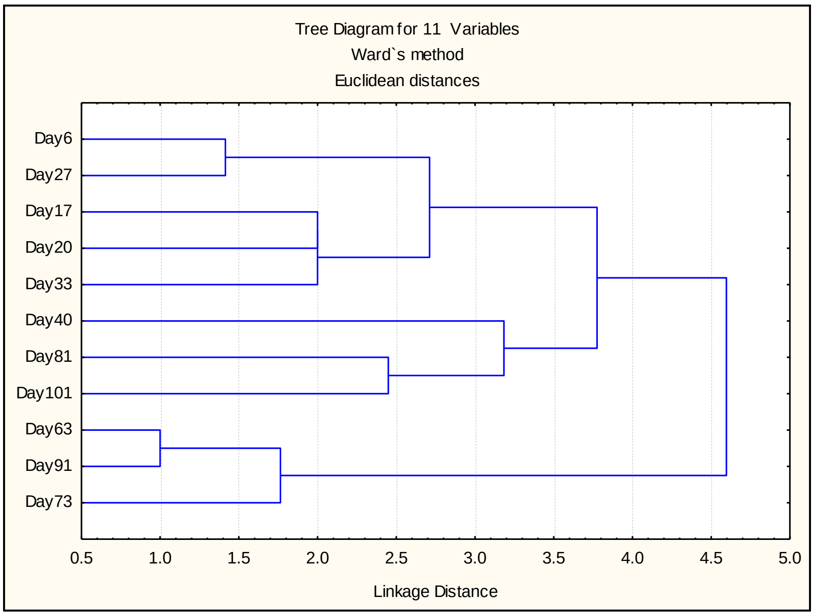

Figure 4.11 Dendogram obtained by Ward's method for clustering of SSCP patterns from MBR biofilm samples to determine bacterial diversity within the biofilm over the operational period

The profiles of days 17, 20 and 33 were very similar compared to day 6 and 27. Profiles of days 40, 81 and 101 formed a cluster separate from those of days 63, 91 and 73 (Figure 4.11). Cluster analysis is supported by the community structure (Figure 4.12).

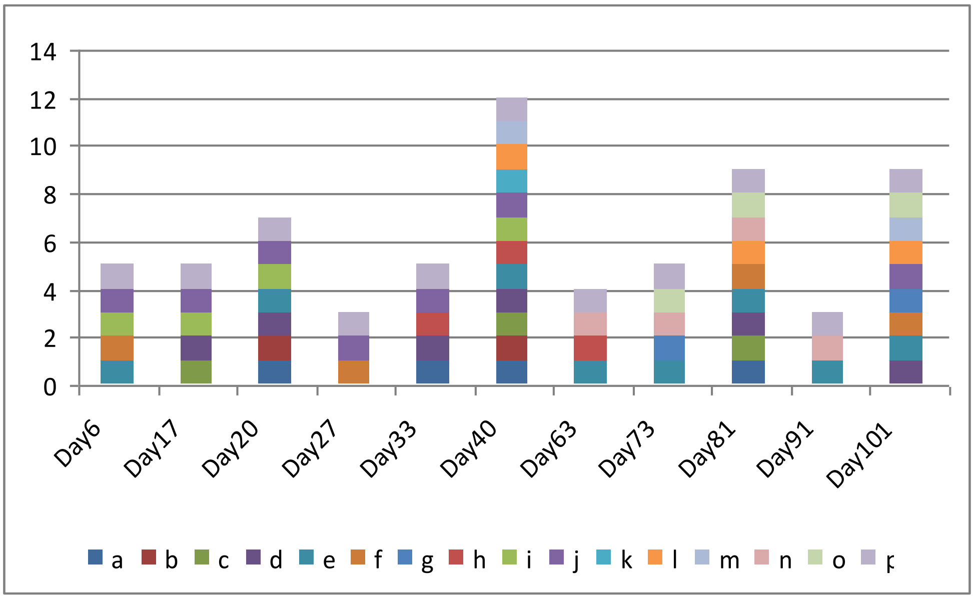

Figure 4.12 Biofilm community structure during MBR period of operation (112 days) as determined by SSCP profile analysis. Each type of organism is depicted as alphabetical letters.

It was observed that on day 63 and 91 of MBR operation, 3 organisms was mainly present: *Uncultured bacterial clone* mdc38fo7 (95%); *Aeromonas hydrophila* 322A (99%) and *Delftia sp.* RF-83 (94%) which attributed to a mere 30-40% of the total bacterial group present and linkage distance correlation of 1.0 – the closest correlation of all organisms present. The abundance of these organisms was evident soon after rinsing the system with distilled water on days 20, 60 and 80 when bacterial diversity decreased periodically as shown in Table 4.3.

Table 4.3: Bacterial diversity in biofilm samples calculated from SSCP patterns (Average *H'*=0.77)

Biofilm Sample (Day of operation)	Shannon-Weaver Diversity Index (*H'*)
Day 6	0.53
Day 17	0.60
Day 20	1.11
Day 27	0.32
Day 33	0.53
Day 40	1.27
Day 63	0.45
Day 73	0.80
Day 81	1.26
Day 91	0.39
Day 101	1.26

4.3.2.4 Molecular profiling and microbial diversity analyses through denaturing gel electrophoresis (PCR-DGGE)

Polymerase chain reaction denaturing gradient gel electrophoresis (PCR-DGGE) was used for the analysis of MBR biofilm during the operational period. A fragment of about 550bp of the bacterial 16S rRNA gene was amplified by PCR using primers for *Bacteria*. This was done to identify the most dominant bands for ultimate microbial community analyses of the developed biofilm responsible for nutrient removal. The DGGE profile obtained is depicted in Figure 4.13.

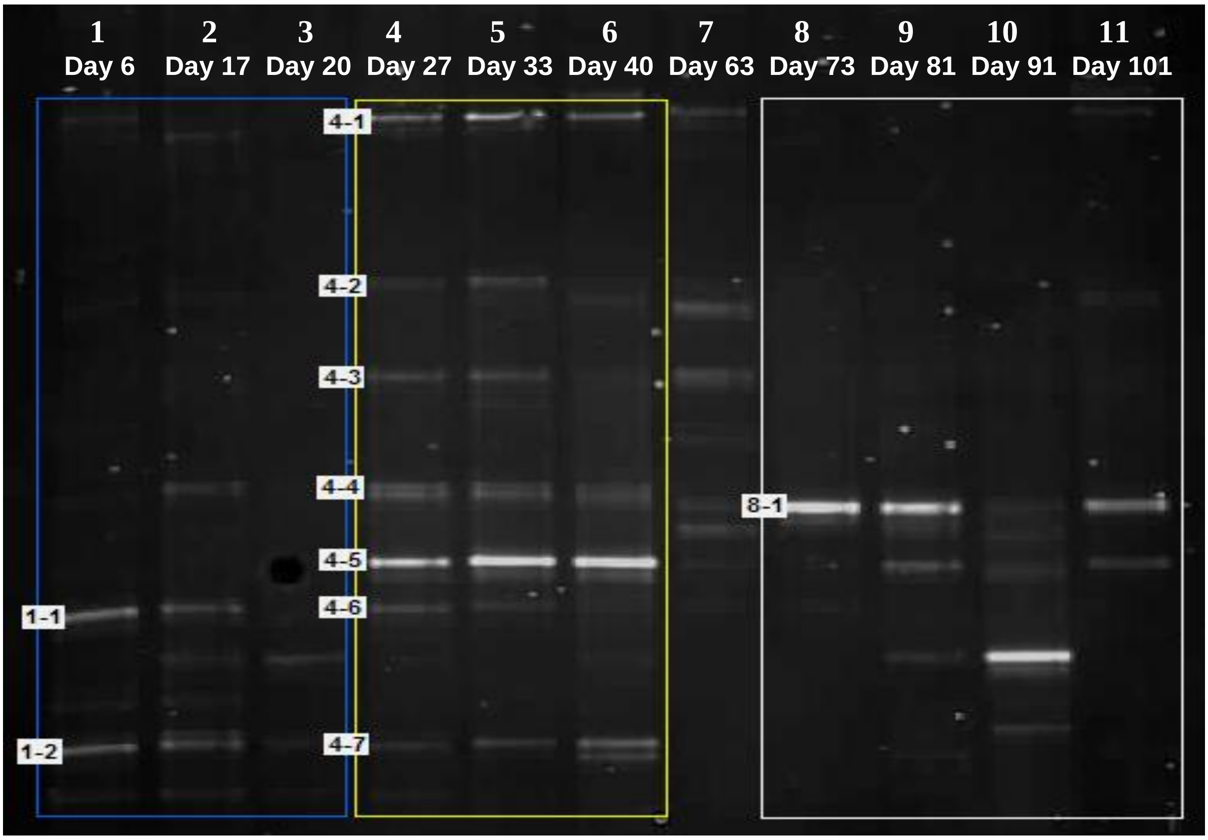

Figure 4.13: DGGE Profile of PCR amplified gene fragments 341 to 907 of the 16S rRNA gene from the MBR bacterial community depicting microbial diversity changes in the biofilm over time (*Note* the three distinctive banding pattern clusters).

Initial PCR-DGGE analysis without MgCℓ and Taq polymerase produced smears (non-specific products) and a poor quality gel with minimum band resolution. Nested PCR was performed on excised DGGE-PCR bands. Nested-PCR results delivered bands with high resolution and quality during normal agarose gel electrophoresis, but DGGE analyses of those products produced poor quality gels with little or no resolution. The optimization of the PCR-DGGE method was considered very problematic for ultimate DNA sequencing. These issues relates to microbial diversity studies conducted by Justé *et al.* (2007), Xingking *et al.* (2007) and Kirk *et al.* (2004) on MBR biofilms.

In Figure 4.13 three distinctive shifts in microbial biofilm diversity is evident. A sudden shift in community is observed during 1) days 6-20 (lane 1 to 3); 2) days 27 to 40 (lane 4 to 6) and 3) days 63 to 81 (lanes 8 to 11). Banding patterns of the PCR-DGGE gel also shows a small variety of double-banded heteroduplexes or chimeras (Figure 4.13; lane 2, 4, 5, 6 and 7).

Table 4.4: Lanes indicating dates of samples taken for PCR-DGGE diversity analysis during 112 day period of MBR operation

Lane	1	2	3	4	5	6	7	8	9	10	11
Date sample was taken	26 Feb	9 Mar	12 Mar	19 Mar	25 Mar	1 Apr	24 Apr	4 May	12 May	22 May	1 Jun
Day of operation	6	17	20	27	33	40	63	73	81	91	101

4.3.2.4.1 DGGE diversity analysis

A total of 17 different bands were identified on the DGGE gel, where biofilm samples (n = 11) produced a total of 17 bands respectively. Bands occurring in several replicate samples as well as bands occurring only once but with high band intensity were chosen for the similarity index calculations.

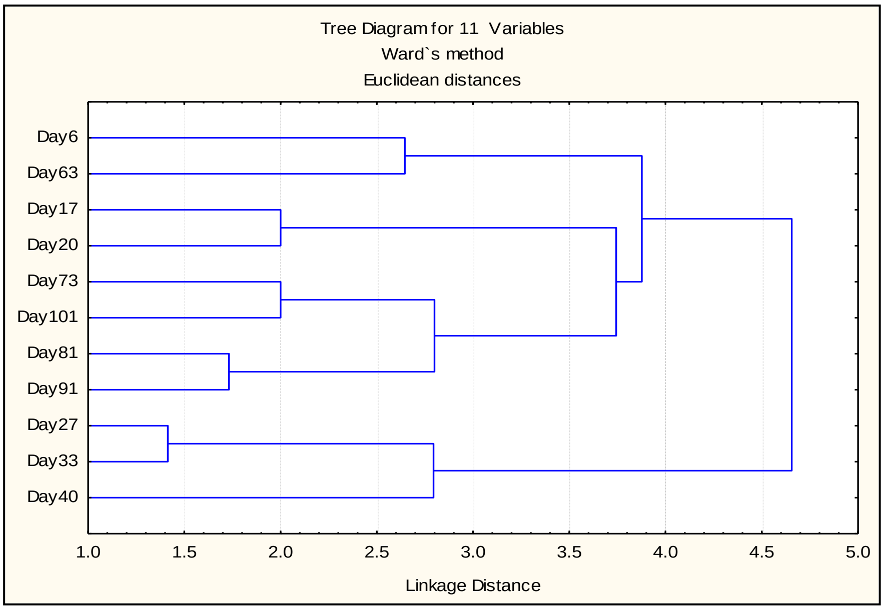

Figure 4.14: Dendogram obtained by Ward's method for clustering of DGGE patterns from MBR biofilm samples to determine bacterial diversity within the biofilm over the operational period

Results are shown in Figures 4.17 and 4.18 and were used to evaluate the microbial diversity within the MBR biofilm and its associated nutrient removing capacities.

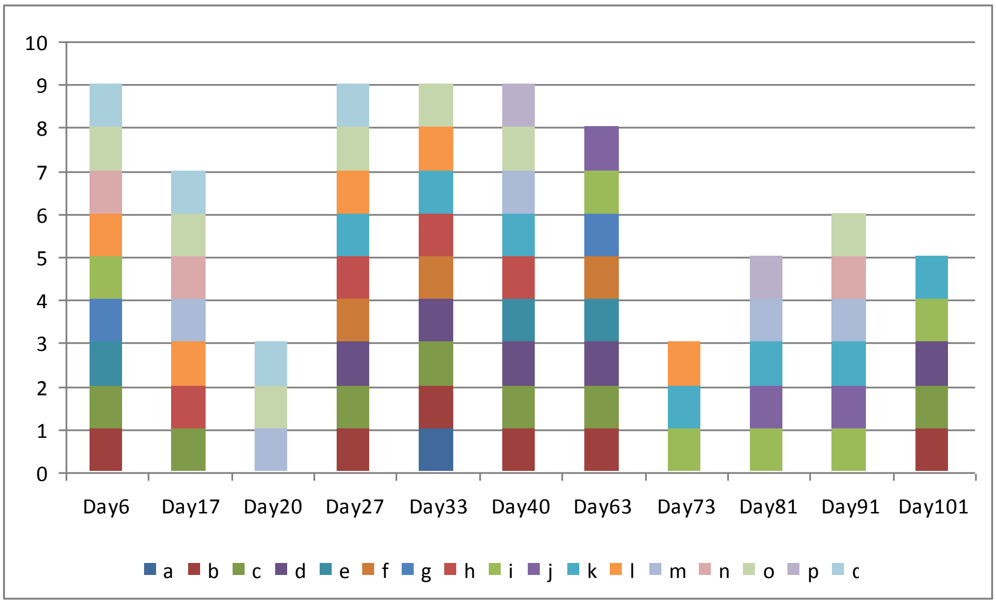

Figure 4.15: Biofilm community structure during MBR period of operation (112 days) as determined by DGGE profile analysis through Ward's method and Euclidean distances.

Table 4.5: Bacterial diversity in biofilm samples calculated from DGGE patterns (Average *H'*=0.79)

Biofilm Sample (Day of operation)	Shannon-Weaver Diversity Index (*H'*)
Day 6	0.95
Day 17	0.85
Day 20	0.48
Day 27	0.95
Day 33	0.95
Day 40	0.95
Day 63	0.90
Day 73	0.48
Day 81	0.70
Day 91	0.78
Day 101	0.70

4.3. Summary

DGGE analysis of the mixed MBR biofilm culture showed multiple banding patterns for single strains and species. Amplification of extracted DGGE DNA bands for sequencing purposes was not possible, due to the limitations and difficulty with optimizing the DGGE-PCR process. Limited amounts of primer-dimer complexes formed, which impeded interpretation of the results to an extent with reference to the total amount of species' DNA identified. In contrast, extracted SSCP-PCR bands could be amplified and sequenced with identification ranges of between 89% - 99%. SSCP results delivered Shannon Weaver indices reaching maximum diversity values of H'=1.27 whereas DGGE fingerprinting analysis delivered maximum values of H'=0.95. This demonstrated that PCR-SSCP is the more favorable between the two molecular methods for identifying microbial species within a mixed culture MBR biofilm in terms of identifying species richness more accurately than DGGE.

CHAPTER 5

DISCUSSION

5.1 MBR PARAMETER ANALYSES UNDER NON-STEADY STATE CONDITIONS

5.1.1 COD removal

No correlation existed between nitrate and COD removal. This contradicts findings in other studies in which high strength wastewater was treated. Fu *et al.* (2009) achieved COD removal of 90.5-90.6% at C:N ratios of 9.3:1. During operation, removal efficiencies of COD were at times above 95.0%, suggesting that the removal was irrespective of C:N ratios (Fu *et al.,* 2009).

It must be acknowledged that a strong relationship may exist between the nitrate and COD concentrations. Chiu & Chung (2003) state that denitrifying bacteria will dominate under higher nitrate concentration and less carbon, to satisfy the denitrification need. In this study, initial C:N:P of 85:15:1 was introduced to the system, in which the C:N ratio was 5.66:1 according to general C:N:P ratios of 100:10:1, which clearly influenced pollutant removal capacities of the system.

Also, COD removal was irrespective of HRTs with a *p*-value of 0.186. This phenomenon is supported by Viero & Sant'Anna (2008) who concluded in their study on HRT as essential MBR parameter, that only when MBRs are operated under steady-state conditions, sound conclusions can be drawn about the reactor performance. Our MBR operated under non-steady state conditions and sound conclusion cannot be drawn about reactor performance in terms of HRT and COD correlation, but according to Table 4.1.1 there was no clear relationship between HRT and COD removal. The major contributing factor may have been that the system was backwashed every second day and routinely rinsed on day 20, 60 and 80 with distilled water to prevent or limit microbial fouling of the membrane. This was done to investigate the possibility of the MBR system to operate naturally, without needing personal operation and heavy maintenance.

Between days 75 and 83 of operation, COD removal efficiency decreased to 51.81% on day 81 and gradually increased to 92.04% on day 80. This may be explained by a possibility of carbon source breakthrough. Judd (2006) mentions that carbon source breakthrough is one of the major disadvantages of using MBR technology for bacterial retention purposes.

5.1.2 Nitrate removal

Nitrate removal

Nitrate removal showed unstable and inconsistent results before stabilization (Figure 4.1). The system stabilized after day 47 until day 64 (before shock loading) with average nitrate removal of 47.64% (Eou) and 52.41% (Mp).

When Figures 4.1 and 4.2 are compared, no obvious correlation between nitrate and COD removal is clear, except for average removal percentages after day 87 towards the end of operation. Ramothokang *et al.* (2006) mentions that filamentous bacteria (such as *Zoogloe spp.*) can be grouped into five main groups of denitrifiers: true denitrifiers, sequential denitrifiers, nitrate respirers, non-denitrifiers and nitrate respierers at high concentrations and true denitrifiers at low concentrations. Nitrate respirers usually are abundant at nitrate levels of between 0.5 to 1g/ℓ, while true denitrifiers are most abundant at nitrate levels less than 0.2 g/ℓ (as imposed through normal operation as well as shock-loading the MBR sysem with nitrate levels up to 35mg/ℓ). At high nitrate levels nitrite reductase enzymes are inhibited, and the nitrate respirers – true denitrifiers will proliferate due to their affinity for low nitrate concentrations.

For denitrification to have been successful, nitrification must have occurred in the aerobic reactor that caused the system being able to achieve good total-N removal. As was revealed in studies conducted by Fu *et al.* (2009) oxidized nitrogen produced in the aerobic tank could be almost completely denitrified in the anaerobic tank and the membrane lumen, yielding relatively high nitrate removal percentages. Other studies concerned with COD/N ratios of 5.3 allowed for 71,2% total nitrogen removal (Fu *et al.*, 2009).

Rhamothokang *et al.* (2006) mentions that in acidic conditions, denitrification can take place in the presence of oxygen, which indicate the need for strict anoxic environments for the process to take place. This may be a contributing factor resulting in the low nitrate removal efficiency of the MBR system, in that the anaerobic reactor was not totally oxygen deprived with pH levels averaging at about 6. This then caused that the denitrification process was rendered incomplete at the onset of the aerobic phase within the membrane module. When this is the case, floc-forming and filamentous bacteria compete for mutually growth-limiting substrate that's influenced by the inhibition of substrate utilization of the floc-forming bacteria under aerobic conditions (Rhamothokang *et al.* 2006). Intracellular denitrification intermediates then inhibit the aerobic cytochrome *o* of the floc-forming bacteria and eventually inhibits their substrate utilization ability. The filamentous bacteria (such as *Zoogloea spp*.) can then use the substrate which may lead to bulking.

Another possibility for the poor nitrate removal efficiency of the MBR system may be that nitrate respirers that only respires nitrate, and do not possess nitrite reduction enzymes, were abundant (Rhamothokang *et al.* 2006). In Figure 4.9 a,b,d and e fungi-structures are clearly seen. With reference to nitrate removal efficiency of between 50.54% and 51.21% during the period of operation after acclimatization and before shock loading, the possibility of nitrate removal may have increased due to the presence of fungi. The fungal consortium could have supported nitrate removal, when unsteady-state operational parameters didn't support bacterial nitrate removal. This relates to studies conducted by Greben *et al.* (2007) in which filamentous fungi was responsible for up to 65 % nitrate removal in synthetic wastewater. In another study conducted by Sheldon & Small (2005) the white rot fungi *Phanerochaete chrysosporium* grown on a ceramic membrane for nitrate removal purposes, was more abundant on the surface of the ceramic membrane than on the lumen side indicating its favorability to lower nutrient concentrations.

The visible cake layering suggests inconsistent nutrient concentrations throughout MBR operation, that is explained by continuously back-flushing and rinsing the system with distilled water on days 20, 60 and 80 to limit fouling. Furthermore, SEM evidence suggested that the richness in terms of thickness of the biofilm layer was not formed due to consistent low nitrate levels, as suggested by Emanuelsson & Livingston (2002) since the event of shock loading increased nitrate levels dramatically.

5.1.3 Recovery of MBR after shock-loading

The event of shock loading the system with nutrients caused that nitrate and COD removal decreased to between 10% and 20% within 10 days, although COD removal reached 99% after 5 days. This demonstrated the robustness of the system to retain high nutrient levels. However, the system took longer to recover in terms of nitrate removal. Towards the end of operation, nutrient removal increased.

The three main organisms that may have attributed to this nutrient removal phenomenon was observed by SSCP diversity analysis (Figures 4.3; 4.4 and 4.5) as *Uncultured bacterial clone* mdc38fo7 (95%); *Aeromonas hydrophila* 322A (99%) and *Delftia sp.* RF-83 (94%). Before shock-loading the system pollutant removal efficiency reached average COD removal of

86.55%. Soon after shock-loading, COD removal reached averages of 87.99%. This phenomenon relates to studies conducted by a variety of other studies conducted that achieved COD percentage removal of 82,4% in a laboratory membrane bioreactor treating wastewater including municipal landfill leachate (Bodzek *et al.*, 2006; Yang *et al.*, 2006; Ng and Kim, 2006; Judd, 2006 & Leukes, 2000).

Studies conducted in Malaysia (Mohammed *et al.*, 2008) showed total COD and nitrogen removal efficiencies reached levels of up to 99,9% removal rates when treating synthetic liquid with COD concentrations of up to 2500mg/L with a laboratory scale MBR system developed for treating municipal wastewater at different operating conditions. The results also indicated the robustness of a potential MBR system for removing COD and nitrates such as found in our MBR results.

The potential of treating high concentrations pollutants can therefore be applicable to municipal waste, possibly without any failure. The robustness of the MBR system becomes clear when reviewing COD removal percentages before and after shock-loading. A minute difference of 1.44% average COD removal distinguishes pollutant removal abilities before and after shock-loading which demonstrates the roughness of the system to cope with such high levels of pollutants.

5.1.4 Comparison between removal efficiency with regards to effluent output and membrane permeate

There is substantial difference between nitrate removal according to values measured in the membrane permeate and the effluent output respectively. Nitrate levels in the membrane permeate (Mp) was averaging lower that levels measured in the effluent output (Eou). This difference in values may be due to the fact that the process of denitrification takes place under anoxic conditions, and that these conditions are most likely more achievable in the pipes carrying the membrane permeate to the effluent output collection vessel, rendering the eventual output more nitrate free than the initial membrane permeate liquid.

The same trend was evident when reviewing COD removal. With reference to average COD removal, a higher percentage removal was obtained in the effluent output. This may be due to potential biofilm formation in the tubing extending from the membrane module to the effluent output drum that removes or uses some of the remaining carbon sources found in the membrane permeate.

During the period after shock loading, more COD was removed as measured in the membrane permeate as in the effluent output. This may indicate the selectivity of the membrane module itself to remove pollutants in combination with the formed biofilm on the membrane lumen surface. The dislodging of biofilm in the tubing to the effluent output drum may also have contributed to less COD removal in the membrane permeate filtrate.

Towards the end of the operational period, pollutant removal was measured as being the highest in the effluent output. It is recommended that tubing carrying membrane permeate to the effluent output drum be replaced periodically during the period of operation, to limit variation in data collection between effluent output and membrane permeate values.

5.1.4.1 Statistical Analyses

Clear differences in nitrate and COD removal exist between the membrane permeate and effluent output values. Another key feature made evident from the statistical results, was that nitrate and COD volatility were not explained by HRT values as the dependant variable, except for nitrate removal in the effluent output. Nitrate removal as observed in effluent output was in fact best explained by HRT variable values. All other independent variables had poor or no correlation and did not explain HRT. These trends are supported by Viero & Sant'Anna (2008) who stated that only when an MBR system is operating under steady-state conditions, may the independent variables explain the dependant variable, such as HRT.

5.1.5 Change in electrical conductivity during MBR operation

EC values were at times higher in the Mp as in the Reactor liquid as depicted in Figure 4.3. This may suggest that some of the membrane particles may have dissolved into the Mp liquid during mechanical filtration (Judd, 2006). Because EC levels seem similar in the reactor liquid and the effluent output, one may conclude that little or no salts/ionic substances was removed/retained by the membrane. Another explanation of higher EC levels (especially between days 70 and 80), is the rapid introduction of ionic salts (KNO_3) during shock loading, which may have deteriorated the membrane filtration performance and may have lead to the eventual failure in terms of nitrate removal and bacterial retention efficiency (Cicek *et al.*, 1999). The zirconia used to manufacture a ceramic membrane, and that formed part of the filtration layer, was sheared off entirely and cationic abrasion took place according to SEM analyses (Cicek *et al*., 1999).

5.1.6 Change in flux during MBR operation

Visible decreases in flux could be seen during flux analyses. These decreases in flux were caused by rinsing the system on days 20, 60 and 80 due to fouling that limited effluent output with the combined effect of operation the MBR system under constant TMP. Le Clech *et al.* (2006) mentioned that current trends in MBR design are to operate at constant flux and constant TMP. In contrast very few recent studies report the operation of MBRs at constant TMP (Defrance & Jaffrin, 1999; Fan *et al.*, 2006; Zhang *et al*., 2010). At constant TMP, a rapid flux decline can be expected to occur during initial stages of operation and usually, the rate of fouling eventually decreases before reaching a plateau.

By backflushing the system with a gas-liquid regime twice weekly, it was expected that the rapid decline in flux would be limited and stable TMP could still be achieved. In reality, this proved to decrease the TMP with about 2 units every week, while a steady decline in flux was observed with fouling ever increasing and forming settleable solids and sludge.

By reviewing nutrient removal SEM results it was apparent that the rapid flux decline and increasing TMP was mainly due to surface cake layering. Zhang *et al.* (2010) conducted studies on the formation of dynamic membrane in an anaerobic membrane bioreactor for municipal wastewater treatment. They also suggest that rapid flux decline could be ascribed to the event of complex cake layering at the membrane surface.

It is speculated that foulant build-up occurred in two stages during this study: 1) initial flux decline caused by concentration polarization in which dissolved substances caused the accumulation of solutes on the retentate side of the membrane. The higher resulting osmotic pressure also further reduced the permeate flux. Long-term fouling was most probably caused by solute adsorption and particle deposition (cake-layering) which formed a gel layer.

Corresponding to the sudden drop in flux was the increase in concentration and diversity of major microorganisms present in the biofilm from days 70 to 80 of MBR operation. On day 70 there were 5 major organisms present and on day 80 there were 9 major organisms present which added to the degree of fouling. Also, this could have caused that suspended particles were transported to the membrane surface and deposited which reduced the hydraulic permeability as well as the permeate flux (Thomas *et al.*, 2005). According to Judd (2006) these increased hydraulic loads, coupled with feedwater quality fluctuations which represent one of the major challenges to especially side stream configurations which is more prone to fouling. This loss of permeability may even have caused severe deterioration in performance that may have been augmented by the misapplication of backflush and cleaning protocol.

In addition, fouling did not only cause a drop in permeate flux (day 20 to 80, Figure 4.4), but also instigated an increased pressure drop. This eventually meant that more pressure was needed for equal volumes of permeate to be obtained. Such a scenario holds enormous economic repercussions in terms of energy requirements of such a system. Thomas *et al.* (2005) states that in contrast, conventional activated sludge plants operates at about six times less energy consumption than that of membrane filtration techniques. Thus, considering the flux scenario as depicted here, it becomes economically not feasible to operate MBRs for waster treatment purposes under non-steady state conditions.

5.1.7 Solid retention time (SRT) and hydraulic retention time (HRT)

Higher species diversity can be expected at longer SRT values (Duan *et al.*, 2009). As can be seen in Figure 4.14 and Figure 4.15 species diversity decreased as the SRT values decreased over MBR operation, with an increase in fouling (Duan *et al.* 2009). However, high levels of nutrient removal can be obtained in MBR systems regardless of the SRT values (Duan *et al.*, 2009). The ratio between active biomass and total biomass increases with decreasing SRT and this may decrease microbial activity in an MBR. This is supported by Duan *et al.* (2009) and may explain why nitrate removal efficiency was so unpredictable, unsteady and low in the present MBR system.

Regardless of the extremely low SRT values under which the present MBR system spontaneously operated, it was anticipated that excellent quality effluent be obtained with specific reference to COD removal, as in studies conducted by Ng & Hermanowicz (2005). They investigated the performance and biomass characteristics of a membrane bioreactor (MBR) and a completely mixed activated sludge (CMAS) system operated at short solids retention times (SRT) ranging from 0.25 to 5 d and hydraulic retention times of 3 and 6 h. The lab-scale reactors were fed with synthetic wastewater to ensure consistency in feed composition. Their results showed that the MBR was capable of achieving excellent quality effluent regardless of the extremely short SRT.

It is postulated that increasing amounts of non-flocculating microorganisms at shorter SRT in the MBR system may have contributed to deteriorating sludge settling properties. Ng & Hermanowicz state that dispersed biomass and small flocs that are present in an MBR may contribute to better reactor performance probably due to less mass transfer resistance, when operating at steady-state conditions. During the present study this became evident when at SRT values of 0.07 days ensured removal efficiencies of up to 99% COD between days 40 and 60 of MBR operation with HRT averaging at 8 days. Overall, this trend was not always present, as our system operated under non-steady state conditions. Ng & Hermanowicz also mention that nitrification cease at SRT values of less than 0.25 days, with an ultimate decrease in denitrification as well. This may also have contributed to the non-steady and poor nitrate removal efficiency of the system, which operated at SRTs ranging between 0.025 and 0.2 days.

5.2 BACTERIAL RETENTION THROUGH MICROFILTRATION

5.2.1 Retention efficiency of indicator organism *E.coli* with marker pBR322

According to APHA (1995) coliform counts are used to monitor treated water supplies with the objective to determine adequacy of the water treatment process. Transformed *E.coli* was chosen as indicator organism and the plasmid pBR322 was chosen as marker in the bacterial retention experiment.

In this study, the efficacy of the MBR system to retain biological cells was tested by shock loading the system with an estimated total of $1 x 10^6$CFUs/mℓ that was inoculated into the aerobic reactor unit. The organisms were then diluted to ~ 53000 cfu/mℓ. Average *E.coli* transformant retention were calculated as 89.15% from days 1 to 5 during the transformant retention experiment that commenced on day 146 and ended on day 154 of total MBR operation. Churchouse & Wildgoose (1999) state that MBRs can retain up to 99.99 % faecal coliforms with initial sewage feed range of 0.9 – 64 x 10^6 CFUs/mℓ. After day 5 the *E.coli* retention percentage dropped to 81.38% after which no bacterial retention occurred. Cicek (2003) states that the retention of all microbial entities within a bioreactor allows for extensive biomass acclimation and enhanced reaction kinetics. Consequently, hybrid processes that combine microbial removal and contaminant retention receives and will receive much attention in the future. Furthermore, the opportunity to retain all bacteria and viruses can result in a sterile effluent, eliminating extensive disinfection steps (Cicek *et al*. 1998).

Under the bacterial shock loading conditions COD removal reached 20.31% and nitrate removal reached 89.25%. In terms of pollutant removal the system was not able to remove COD from the water with COD being totally discharged (100%) and nitrates being removed by 89.75%. It is speculated that nitrates were still absorbed and metabolized by breakthrough organisms present in the effluent output water, while the high levels of COD support this. Nitrate breakthrough could also have taken place, with high initial levels before entering the effluent output vessel. This phenomenon may be explained by the process of assimilatory nitrate reduction that took place in the effluent output discharge. During this process, nitrate assimilation takes place and is reduction to nitrite by nitrate reductase. This could have taken place in the cytoplasm of the transformants. It was further speculated that nitrite was then reduced to ammonia with a series of two electron additions catalyzed by nitrite reductase. The resulting ammonia was then incorporated into amino acids which may have contributed to the total high levels COD measured in the effluent output vessel.

In the present study, we can conclude that the system is ineffective in retaining microorganisms after 5 days in the event of shock loading, due to the extremely short-term efficacy. The sustainability of the filtration process was not successful referring to bacterial retention. The effect of carbon associated bacterial breakthrough due to mechanical sheering of the membrane and exponential increases in bacterial numbers needs more research.

5.2.2 MBR process performance during microbial shock-loading

Our study showed that 5.3×10^5 CFU/mℓ *E.coli* were present in the effluent output after 5 days, with no bacterial retention by the membrane. This indicates that the organisms grew and multiplied in the water during the MBR operational period. Mechanical abrasion of the ceramic membrane due to increased microbial cells as well as ionic mechanical shear could have been the reason for the failure of mechanical filtration. The zirconia dioxide filtering layer that contributed of 29% of the total membrane could have been abraded. This scenario relates to a study conducted by Cicek *et al.*, (1999). They used a pilot-scale ceramic membrane bioreactor (MBR) system for the treatment of simulated municipal wastewater. With the introduction of excess phosphorus to the bioreactor, a gradual deterioration of the membrane filtration performance and eventual failure of the pilot system was observed. A detailed investigation performed on the mixed liquor showed that phosphorus related calcium and magnesium complexes accumulated in the bioreactor and under excess phosphorus conditions, these crystals caused abrasion of the membrane active filtration layer, leading to higher permeability, extensive membrane fouling, and eventual replacement of the membrane unit.

Judd (2006) states that the major disadvantage of using membrane technology for bacterial retention is that it holds the potential for carbon source breakthrough as is evident from result obtained in Figure 4.1 with sudden increased amounts of COD was measured in the effluent output. Nevertheless, process performance increased with 2.25% with the onset of shock-loading and averaged at 89.15% bacterial retention within 5 days. Nitrate removal escalated to 89.25% and COD removal was measured as being low (20.31%) within the 5 days of maximum bacterial retention. This can be ascribed to the fact that the organisms present in the effluent output may have been responsible for removing some of the remaining nitrate present in the effluent output liquid.

In addition, the low COD removal could be due to transformants dying off because of the low survival rate of *E.coli* in mineral water microcosms (Messi *et al.*, 2002). The constituent cell matrix was thus being measured as COD, therefore attributing to total COD measured. This clearly demonstrates the rigidity and stability of the system and especially the membrane to manage effectively during processes on increased nutrient loads.

5.3 MOLECULAR PROFILING AND BIOFILM CHARACTERISATION

5.3.1 Biofilm characterization through SEM

Figure 4.9 depicts that the top layer of the developed biofilm is primarily dominated by more active biomass, while the bottom layer is dominated by residual less active biomass of which the thickness could have increased with time. The thickness of the bottom layer is also denser than those layers near the top of the biofilm. These images are similar to studies conducted by Laspidou & Rittman (2004) in which they evaluated the trends in biofilm density by following unified multiple-component cellular automation (UMCCA). As depicted in Figure 4.9 (a, b, and d) the MBR biofilm was examined by SEM. Towards the end of operation (day 112), the membrane surface was covered with a thick cake layer (Figure 4.9e). It was speculated that the cake layer consisted of sludge flocs and membrane particles congealed together. The major organisms were bacilli and cocci that were connected with filamentous fungi and bacteria. An important aspect of especially nitrate removal is that when a specific function is required such as denitrification, a more specific pool of microorganisms are required for the specialized task (Falk *et al.* 2009).

SEM results and DNA sequencing of PCR-SSCP products indicated that *Zoogloea oryzae* was one of the major isolates identified (97% probability; Tables 3.3 and 4.2). Their abundance in the biofilm is evident in Figure 4.9b in that they form bacilli-like filamentous chains which hold the sludge floc together as was also seen in sewage treatment and other MBR studies (Falk *et al.*, 2009; Roselló-Mora *et al.*, 1994). The SEM photos taken of the biofilm relate to studies conducted by Zhang *et al.* (2007). According to Judd (2006) the phenomenon of cake layering is usually ascribed to inconsistent MBR operational parameters, which became evident with regards to our MBR system operating under non-steady state operational conditions.

5.3.2 Biofilm characterization through non-culturable methods

5.3.2.1 DNA amplification by Polymerase Chain Reaction

PCR-SCCP gel analysis showed less banding patterns within single lanes, than that observed in the DGGE gel (Figures 4.13 and 4.16). This may indicate that the occurrence of heteroduplexes and chimeras was limited in the SSCP gel. Sequencing results suggested high quality analyses with results indicating identification percentages of between 89 and 99% for most of the bands isolated although overall gel resolution was not ultimately favorable. Banding patterns of the PCR-DGGE gel showed a small variety of double-banded heteroduplexes or chimeras which is one of the key disadvantages of DGGE analyses (Figure 4.13; lane 2, 4, 5, 6 and 7).

The possibility of contamination during the PCR process may also have caused a variety of band patterns (non-specific products) within one lane. Without the addition of MgCℓ and Taq polymerase, a great variety of smears or non-specific products were visualized with little or no resolution. Overall gel resolution was of high quality in contrast to the SSCP gel. The different banding pattern results obtained through SSCP and DGGE analyses makes it difficult to determine the exact number of species associated with the MBR biofilm.

5.3.2.2 Microbial diversity analyses through SSCP

The PCR products from 16 bacterial type strains could be differentiated from each other with *Azoarcus denitrificans* and/or *Azoarcus tolulyticus* (Figure 4.10, 7-4, lane 7) being the most difficult organism to differentiate from each other with 89% identification through sequencing forming one weak band on the SSCP profile. With template mixtures consisting of mixed culture DNA most of the single strains could be detected from the biofilm community after PCR-SSCP analyses. The concentration of the organisms Bacteroidetes (92%) and/or *Flexibactereceae* (90%) (Figure 4.10 band 1-1, lane 1) decreased during the first 33 days of operation and increased in band intensity towards day 40 of operation after which no Bacteroidetes and/or *Flexibactereceae* were present until the end of operation on day 112. The presence of *Rhodocycloceae* and *Zoogloeae oryzae* (6-1, lane 6) was evident during the whole period of operation, in which high band intensity could be seen on day 40 with corresponding COD removal of 99.92% and low nitrate removal efficiencies of 0.71%.

Azoarcus denitrificans (7-4, lane 7) was also present during the whole period of operation peaking in band intensity soon after the acclimatization period. *Aeromonas hydrophila* (Figure 4.10 band 8-2, lane 8) was present from day 63 to day 91. An *Uncultured bacterial clone* mdc38fo7 was present from the event of shock loading towards the end of the operational period. Shock loading took place on day 64 and it is evident that the concentration of *Aeromonas hydrophila* also increased dramatically soon after shock loading was introduced to the MBR system. From day 33 towards day 63 (30 days) the presence of *Delftia sp* showed high band intensity, as well as on day 91 of operation. This study demonstrates the potential of the selected PCR–single-stranded DNA approach for microbial community analyses.

Messi *et al.* (2002) investigated the survival ability of *Aeromonas hydrophila* in mineral water and found that *A. hydrophila* showed a quite strong survival capacity (150 days), while *E.coli* was the least well fitted to survive and was no longer detected after 70 days. They also found that when *A. hydrophila* was inoculated together with one or two other strains, its survival appeared to be dependent on interaction with other organisms – a type of commensialistic relationship existed between the organisms which were somewhat antagonistic. This may explain why *A.hydrophila* persisted towards the end of MBR operation in our study, especially after the event of shock-loading the system with nutrients. *A.hydrophila* has also been reported for remediation of metal because they are ubiquitous in fresh and marine water and tolerant towards heavy metals (Miranda & Castello, 1998).

In Figure 4.3, one strong band was detected in the gel pattern with electrophoretic mobility similar to *Delftia sp.* RF-83 (lane 8-2; 9-2; 10-3; 11). This may indicate the survival capacity of *Delftia sp.* throughout the MBR operation. In other studies it was found that *Delftia sp*. (especially *Delftia sp*. AN3) is able to degrade anilines which constitute a group of xenobiotics (pollutants which resemble naturally occurring compounds that may contain high levels of nitrates and CODs), that are used in the production of dyes, pesticides, plastics, and pharmaceuticals which is considered as major environmental hazards (Zhang *et al.*, 2007). Maximum H' values of 1.11, 1.27 and 1.26 were reached on days 20, 40 and 81 with close correlation between days 40 and 81 with linkage distances lower than 3.2 (Figure 4.4). On day 20, 60 and 80 the system was rinsed with distilled water. This was done when effluent levels decreased below 1 ℓ. In Figure 4.10 band intensity and band diversity decreases after these days, showing high band intensities for *Aeromonas hydrophila* and *Deltia sp.* This may indicate the increased vigor of these organisms to tolerate sudden fluctuations in operational parameters when operating MBRs. It may also be indicative of a mutual relationship between these organisms to co-exist in an MBR biofilm.

By following an SSCP protocol, we were able to analyze multiple samples simultaneously on a single gel which allowed direct comparisons between different samples. It was also possible to excise and sequence bands of interest to identify bacterial species within the MBR biofilm. It was demonstrated that by using 16S rRNA based PCR-SSCP fingerprinting methods the bacterial community associated with a ceramic membrane bioreactor can notably vary in composition and concentration over MBR operation.

In Figure 4.4 and 4.5 the average nitrate removal in the effluent output was calculated as 36.19% on day 63 and -147.16% on day 91, while COD removal was calculated as 60.27% COD removed on day 63 and 99.92% COD removed on day 91. Average nutrient removal between day 63 and 91 was calculated as being 20.76% nitrate and 82.13% COD removal. It is speculated that these three organisms were mainly responsible for most of the nutrient removal capacity under especially elevated nutrient conditions in our studies.

5.3.2.3 Microbial diversity analyses through DGGE

The experimental evidence available in Figure 4.11 shows that different process conditions occurring during the period of operation (Table 4.4). This may have favored the selection of different types of bacteria or may have caused a microbial population shift upon spontaneous parameter variations or operating conditions. Volcke *et al*. (2008) also observed such shifts when they tracked microbial diversity with two nitrifying inverse turbulent bed reactors (ITBRs) with different solid hold-ups. An acclimatization period of 30 days was observed before near-complete removal of COD (80.19%, day 30). Limited removal of nitrate (28.81%, day 30) was attained.

PCR-DGGE is a microbial diversity analysis tool mostly used when operating MBR systems at low solid retention times (Duan *et al.*, 2009; Zhang *et al.*, 2007). In this study, we strived to obtain qualitative estimations of bacterial diversity within the membrane module of the bioreactor. To be able to analyze biofilm change, we had to consider the fact that biofilm samples collected must integrate the level of heterogeneity within the MBR system. In this study, banding pattern similarity was analyzed by Ward's method and Euclidean distances.

Monitoring of the community through DGGE fingerprinting suggested a subsequent shift in diversity. In Figure 4.13 three distinctive shifts in diversity is evident which suggests that the initial acclimation period was due to biofilm formation and physical parameter changes within the MBR. Stamper *et al.* (2003) monitored bacterial population changes in a graywater treatment system that employed a MBR to process waste. In their study DGGE analysis indicated a diverse and unstable bacterial population throughout the 100-day period, with spikes in feed strength causing significant changes in community structure. The actual shift in diversity may also have been due to re-organization of the community that favored a selection of specialized species (Grove, 2006).

A sudden shift in community (Figure 4.13) is observed during 1) days 6-20 (lane 1 to 3); 2) days 27 to 40 (lane 4 to 6) and 3) days 63 to 81 (lanes 8 to 11) with ultimate H' values exceeding 1.0 at the end of each phase with clear differences in SRT and flux (Figure 4.4 and Figure 4.5) values measured, showing a gradual drop in value for each of the three phases. Especially the first two phases may reflect operational changes that may have led to starvation which may have resulted from washing the MBR system with distilled water on days 20, 60 and 80. It may also reflect phase 1 was associated with startup conditions, phase 2 with stable bioreactor functioning, and phase 3 with the system shock loading on day 64. Stamper *et al.* (2003) also found that peaks in nutrient levels may lead to significant changes in biofilm community structure. However, the results of the present study suggest that although the system was flushed with clean water the overall diversity of all three phases observed increased anyway over a period of about 20 days.

From day 27 towards day 40 of MBR operation, an average COD removal efficiency was calculated as 71.21% and nitrate removal decreased to 3.91%. Figure 4.4 depicts clear distinctive banding patterns in lane 4, 5 and 6 that may have been responsible for these high levels of pollutant removal. The same distinctive bands decreased in band intensity towards day 112 (end of operation). Soon after day 64 of shock-loading the system with COD and nitrates, the band intensity of these bands increased again towards the end of operation, with COD removal increasing to 87.99% and nitrate removal increasing to 32.89%. Similar trends were evident in Figure 4.13 band 8-1, lanes 8, 9 and 11 with distinctive, clear banding patterns with high intensities observed with increased pollutant removal. This may indicate that removal efficiency increases with elevated pollutant concentrations circulating the system. This increase may also be ascribed to specific bacterial strains outcompeting other species for nutrients and oxygen (Volcke *et al.*, 2008).

Evidence also suggests that the most robust organisms prevail in a system and that only a few species are responsible for ultimate nutrient removal. This can be seen in Figure 4.13 where especially one species is present towards the end of operation (band 8-1, lanes 7 to 11) by accepting that one band represents one species (Rôças *et al.*, 2004). However, some other bands were present throughout MBR operation (lanes 1 to 11, upper half of DGGE gel, Figure 4.13), buy they were always represented by faint bands. DGGE analysis indicated a diverse and relative unstable bacterial population throughout the MBR operational period of 112 days with nutrient shock loading causing significant changes in community structure and diversity (Stamper *et al.*, 2003).

We conclude that by combining DGGE with Ward's method of diversity analyses, it was revealed that the biofilm bacterial community was significantly different (Lear & Lewis, 2009) when different nutrient levels were employed in the system and overall more diverse (Table 4.5) at high nutrient levels (after shock-loading). By using PCR-DGGE as fingerprinting tool to analyze bacterial community diversity within the MBR biofilm, we conclude that clear differences in bacterial similarity exists within the same membrane biofilm with distinct phases correlating to operational conditions.

5.3.4 Summary

In this study, optimal nitrate removal reached an average of 47.64%, while COD removal averaged at 99.92% towards the end of operation. Differences in effluent output and membrane permeate was observed in terms of nutrient removal. During non-steady operation of the system, nutrient removal showed poor correlation to HRT values, while SRT values decreased. EC was relatively constant with decrease in flux, except for periods after days 20, 60 and 80 when the system was rinsed with distilled water. After shock loading the system with nutrients, COD and nitrate removal decreased to between 10% and 20% within 10 days, after which COD removal reached 99% in 5 days. It took nitrate removal 35 days to recover to 33.57%. After shock loading, COD and nitrate removal averaged at 87.99% and 32.89% towards the end of operation. These results indicate the robustness of the system to remove high levels of nutrients from wastewater.

During the first 5 days (day 146-150) of the microbial retention analysis study, the indicator bacteria *E.coli* was retained between 80% and 100%. After day 5, retention was calculated as 81.38% and decreased to 0% towards the end of the study.

SSCP and DGGE profiles revealed complex but readily resolved patterns for all biofilm samples taken. The DGGE technique, as well as SSCP has given a comprehensive description of the acquired biofilm community within the MBR.

Shannon Weaver diversity analyses conducted on SSCP results showed separate clusters during MBR operation (with *H’* close to 1.0). These results may reflect the three rinsing periods of the MBR system, on days 20, 60 and 80. From SSCP profile analysis, three organisms were mainly present throughout operation with high band intensities soon after the 3 periods of rinsing. These organisms were identified as an uncultured bacterial clone mdc38fo7, *Aeromonas hydrophila* and *Delftia sp.* with close correlation. The selection of these specialized species may have occurred within the MBR biofilm as result that mainly attributed to nutrient removal.

Clear population shifts was evident during DGGE profile analysis. Three distinctive shifts in diversity were present which may be associated with rinsing the system on days 20, 60 and 80. Shannon Weaver diversity analysis also showed three distinctive shifts associated with rinsing (with *H’* close to 1.0).

Since no gradient gels and no GC clamp primers were required for SSCP and since no specific and costly apparatus were necessary, the SSCP technique was more straightforward than DGGE. The SSCP technique was also exceptionally appropriate to monitor the microbial population dynamics and to compare microbial community compositions between different conditions within the MBR system.

CHAPTER 6

SUMMARY, CONCLUSION AND RECOMMENDATIONS

6.1 Summary

In this study a ceramic MBR system was operated under non-steady state conditions to investigate nutrient removal capacities and to characterize the associated biofilm. The system's robustness was also investigated during nutrient and bacterial shock loading. For biofilm characterization PCR-SSCP and PCR-DGGE techniques were used in combination with Shannon Weaver diversity indices for microbial diversity analysis.

This research confirmed that the membrane biofilm which formed on the lumen-side of the ceramic membrane can increase trans-membrane pressure and decrease effluent flux, while removing a limited amount of pollutants under non-steady state operational conditions. Nutrient removal results indicated that average nitrate removal was limited and not optimized under non-steady state conditions, although COD removal reached percentages of 99%. Nutrient removal may be optimized by following steady-state conditions. One key advantage is that under non-steady state operating conditions, the system is relatively easy to operate, maintain and to monitor. In contrast, under steady state conditions, a vast amount of knowledge is necessary to predict, manipulate and manage the system to optimize pollutant removal. This adds to the complexity of MBR operation. Particular care needs to be taken since the specific conditions present in non-steady-state MBR operations are reflected in some important discrepancies when compared to steady state parameter values.

The pollutant removing capacity of the MBR system increased in the event of shock-loading. Optimal COD removal occurred during shock loading, while average nitrate removal decreased and took longer to recover to average values. Associated changes in the biofilm were observed through PCR-SSCP and PCR-DGGE. It is speculated that this occurred due to species specialization, survival and natural phase shifting due to changes in physical parameters such as rinsing the system with distilled water. The robustness and capability of the system was proven through relative high nutrient removal during shock loading. The capacity of the system to handle sudden increases in nutrient concentrations that may occur at local wastewater treatment plants was relatively high.

In addition to the nutrient removal capacity of the biofilm it also functioned as a secondary barrier for filtering bacteria. In this case *E.coli* JM109 served as an indicator bacteria. The organism was transformed with pBR322 that served as marker for bacterial retention studies. The system retained the *E.coli* in the first 5 days of the bacterial retention study with percentages above 80%. After day 5 no *E.coli* was retained due to bacterial breakthrough. The system was therefore only capable of retaining the indicator organism for 5 days and was unsuccessful.

PCR-SSCP and PCR-DGGE profiles showed fairly high diversity of organisms within the biofilm during MBR operation. Shannon Weaver diversity results showed distinctive clusters and diversity shifts, especially after the three rinsing periods with average *H'* values below 1.0. DNA sequencing of SSCP results indicated that certain bacterial strains, such as *Delftia sp.* were present throughout MBR operation with high band intensity. This suggests the selection of the MBR for species-specific strains within the biofilm that may be associated with parameter fluctuations.

More research is needed to understand and manage MBRs functioning under non-steady state kinetics. This study gave unique insight into the positive roles of a biofilm associated with membrane processes and contributed to fundamental studies concerning biofilm development for nutrient removing membrane bioreactor processes.

6.2 Final conclusion

The aim of this study was to evaluate a ceramic membrane bioreactor functioning under non-steady state operational conditions to investigate the pollutant removing capacity and to characterize the associated biofilm in terms of bacterial diversity through PCR-SSCP and PCR-DGGE for potential implementation at local wastewater treatment plants. To achieve this, six well-formulated objectives were set for this study. The trends and conclusions for each objective are summarized in the discussion below.

i. Pollutant removal of the MBR under non-steady state conditions

The ceramic MBR system which was operated using under non-steady-state conditions and microfiltration was found to be relatively limited in removing nitrates but achieved high COD removal. The permeate quality was not of high standard which points toward the fact that it is more consistent to normalize against permeate product volume to produce specific energy demand components (Judd, 2006). The system was simple to operate, undemanding and cost-effective, especially with regards to aeration. The most fundamental relationships with respect to operating costs are those related with aeration. Physical and chemical backwashing requirements were dependent primarily on the membrane and process configuration and the feedwater quality (Judd, 2006).

In this study it was found that under non-steady state conditions, the necessity of gas-liquid back flushing twice weekly reduced the problem of fouling to an extent and limited the cost of chemical cleaning. It was also necessary to rinse the system with distilled water and to adjust the nutrient levels to limit fouling. The system is relatively uncomplicated in terms of operational management and would also be comparatively unproblematic to be automated. We conclude that this technology can indeed be considered as a possibility for implementation as an add-on technology at local municipal wastewater treatment plants to treat organic and inorganic pollutants. More research needs to be conducted to optimize nutrient removal within a non-steady-state system.

ii. Nutrient removal during shock-loading

A key aspect that was demonstrated was the robustness of the system to recover after shock loading it with elevated nutrient levels. COD removal increased during shock loading when compared to average nutrient removal during the entire MBR operation, while nitrate removal was constant. This elevated COD removing capacity was supported by clear differences in microbial population shifts, especially depicted by DGGE microbial fingerprinting profiles. Differences in physical parameters during this period of operation were also noted and included a sudden decline in flux and SRT values. Evidence suggests that pollutant retention may be optimized by following steady-state operational kinetics with the feed liquid consisting of pollutants with high nutrient levels, specifically referring to COD.

iii. Microbial/pathogen retention in the MBR

The system was only effective in retaining bacteria within 5 days of inoculation. After 5 days carbon breakthrough and biomass increases were measured as elevated COD. Elevated colony forming transformants was also measured with little or no nitrate being removed. Adding to this, he fact that *E.coli*'s surviving capacity in water microcosms is extremely low, may have led to the quick washout of the reference organism, especially when it needed to compete for space and nutrients within the already established MBR biofilm. Also, the instability of pBR322 in the absence of Amp/Tet may have attributed to loss of the plasmid with the consequence that no colonies would be present on amp containing solid media. This phenomenon may have resulted in ambiguities in results and does not necessarily mean that the MBR system was ineffective in retaining bacteria.

iv. Microbial diversity analysis through SSCP and DGGE as DNA fingerprinting tools

Through SSCP profile analysis and DNA sequencing results it was postulated that two major contributing organisms in terms of nitrate and COD removal were identified as *Aeromonas hydrophila* and *Delftia sp*. which was present throughout the MBR operation with high band intensities. It is speculated that these organisms were responsible for most of the nutrient removal capacity of the system, especially after the system was shock loaded with elevated nitrate and COD concentrations. Shannon Weaver diversity indices calculations showed three clusters of organisms that may be associated with rinsing the system on days 20, 60 and 80 to optimize effluent output volumes. In comparison, SSCP was more selective as DGGE, less time consuming in terms of PCR optimization and delivered well resolved amplicons which was easy to excise, isolate and identify by ultimate DNA sequencing.

DGGE analysis delivered well resolved profiles and three distinct shifts in microbial populations within the membrane biofilm could clearly be observed. This may be associated with rinsing the system on days 20, 60 and 80 to optimize effluent output levels. In this study, DGGE was more time-consuming with reference to PCR optimization for ultimate DNA sequencing for biofilm characterization and in due course not at all favorable. Nonetheless, it was possible to analyze the biofilm profile through Shannon Weaver diversity analysis combined with Ward's method and Euclidean distances, similar to SSCP. DGGE was found to be a more intricate technique as SSCP.

6.3 Recommendations

The summarized discussions and conclusions in the foregoing section have lead to the following recommendations:

1) Software can be developed to control a remote membrane system as add-on to local municipal wastewater treatment plants for serving as buffer for elevated nutrient levels, especially in terms of COD. This can be done by using Java and CORBA internet-based

architecture (Li *et al.*, 2002). Control via a desktop PC connected to the internet can be established in which communication between components from more than one unit can be achieved. This must only be applied to MBR systems operating under steady state kinetics and high nutrient levels to be retained.

2) In terms of operating an MBR under non-steady state operational parameters and spontaneous physical changes, we recommend feed water with high nutrient levels exceeding C:N:P ratios of 300:50:4 to optimize pollutant removing capacity of such a system, as evident in results obtain during shock-loading with nutrients and back flushing the system with a gas-liquid regime 3 times a week, after the initial acclimatization period of between 21 and 30 days, have been reached. It's not highly recommended to apply non-steady state operational parameters within a MBR as the method of choice as an add-on at wastewater treatment plants.

3) There is substantial evidence of MBR systems retaining biomass, whether bacterial, fungal or viral in origin. Our system was able to retain *E.coli* JM109 transformed with pBR322 for only 5 days. We recommend using another reference organism for this purpose, because of the low survival rate of *E.coli* in similar environments. The retention of viruses or pathogens can be investigated. Igbinosa et al. (2009) concluded in their study that rural wastewater treatment facilities in the Eastern Cape Province of South Africa are potential sources of *Vibrio* pathogens in the aquatic environment of the communities. Results from this study indicate that *Vibrio* and other related surface water pathogens may be removed from wastewater by using MBR technology. This research may prove to be invaluable in future. Structural degradation of the ceramic membrane can also be monitored through SEM analyses to investigate possible reasons for carbon breakthrough during microbial shock loading.

4) By evaluating PCR-SSCP profiles and DNA sequencing results, we recommend additional research in combining *Delftia sp*. with another biofilm associated organism in synergistic relationships. This may be done to monitor and evaluate pollutant removing capacities in MBRs under aerobic-anaerobic conditions. The ability of these organisms to assist in the removal of nutrients can also be studied under steady-state and non-steady state operations, to investigate the influence of physical changes on these organisms' nutrient removing

capacities. Overall, PCR-SSCP was more sensitive in differentiating between various strains and species of the biofilm mixture.

5) DGGE was not chosen as the method of choice for microbial diversity analysis of complex biofilms found in MBRs, because it has the disadvantage of requiring PCR amplification or DNA cloning prior to sequencing. This aspect makes DGGE time-consuming and complex.

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DEFINITIONS

COD	chemical oxygen demand	**DO**	dissolved oxygen
NO_4	Nitrate	**T**	temperature
MBR	membrane bioreactor	**TDS**	total dissolved solids
HRT	hydraulic retention time	**EC**	electrical conductivity
kPa	kilo pascal	**MST**	membrane sewage treatment
SRT	solid retention time	**PVDF**	polyvinylidene difluoride
E.coli	*Escherichia coli*	**PES**	polyethylsulphane
Cfu	colony forming units	**PE**	polyethylene
PCR	polymerase chain reaction	**PP**	polypropylene
rRNA	ribosomal ribonucleic acid	**TMP**	transmembrane pressure
SSCP	single strand conformational polymorphism	**MLSS**	mixed liquor suspended solids
DGGE	denaturing gradient gelelectrophoresis	**OMP**	organic micropollutants
SEM	scanning electron microscopy	**SBI**	sludge biotic index
NWA	national water act	**ANOVA**	analysis of variance
NMMP	national microbial monitoring programme	**DNA**	dioxyribonucleic acid
DWAF	department of water affairs and forestry	**bp**	base pair
RO	reverse osmosis	**Mp**	membrane permeate
UF	Ultrafiltration	**Eou**	effluent output
MF	Microfiltration	**SSU**	small subunit
WWTP	wastewater treatment plant	**LB**	Luria broth
EDTA	ethylenediaminetetraacetic acid		