

# **Investigating the removal of *Candida* and other potential pathogens from wastewater via an experimental rhizofiltration system**

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# Declaration

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## SUMMARY

Water is a requirement of life and its quality, as well as quantity, is very important for sustaining the world's economy and human population. However, microbial and chemical pollution of water systems is on the increase, and can mainly be attributed to urban runoff, domestic and industrial discharges, inadequate sanitation, as well as ageing water treatment facilities. Practical solutions for the treatment of runoff are necessary to ensure water systems remain clean and safe for both human use and crop irrigation. Consequently, the ability of a rhizofiltration system to remove chemical and microbial contaminants from urban runoff was evaluated. A rhizofiltration system was constructed at the Stellenbosch Sewage Works, into which settled sewage could be distributed to determine if the presence of reeds improved the removal of chemicals, indicator organisms, pathogens, and potentially pathogenic yeasts.

Indications were obtained that settled sewage from the Stellenbosch Sewage Works was microbiologically and chemically comparable to samples collected from the polluted Plankenburg River after a four day settling period in the storage tank constructed for the rhizofiltration system. This showed that the influent of the rhizofiltration system could be considered as urban runoff after four days of settling in the tank. The planted (experimental) and unplanted (control) side of the rhizofiltration system showed similar removal rates with regard to suspended solids, ammonium, Chemical Oxygen Demand, phosphates and sulphates in the influent, which percolated through the system within 45 min. Microbiologically, the experimental side was more effective than the control side in terms of faecal coliform, yeast and *Salmonella* removal but no difference was found between the two sides with regard to coliphage removal. The majority of yeasts that were isolated belonged to the genus *Candida*, and the experimental side was more effective than the control side in removing these opportunistic pathogens from wastewater. During the same experiments a number of antibiotic resistant bacteria were isolated which seemed to proliferate within the filter, the majority of which formed part of the *Burkholderia cepacia* complex. Additionally, the experimental side of the filter was significantly more effective at removing faecal coliforms, potentially pathogenic yeasts and *Salmonella* trapped within the sand, compared to the control side, six days after the wastewater percolated through the filter. *In vitro* sand filter experiments revealed that the presence of *B. cepacia* in the sand may be responsible for trapping some of the *Candida* species present in wastewater as it percolates through the sand, and thus may prolong the period in which these yeasts are subjected to the antagonistic effect of root exudates or other microbes.

## OPSOMMING

Water is 'n voorvereiste vir lewe, en die kwantiteit en kwaliteit daarvan is vir die instandhouding van die wêreld ekonomie en die menslike bevolking noodsaaklik. Mikrobiëse en chemiese besoedeling is egter aan die toeneem en kan hoofsaaklik aan stedelike afloop, huishoudelike en industriële afval, onvoldoende sanitasie en verouderende fasiliteite vir die behandeling van water toegeskryf word. Praktiese oplossings vir die behandeling van afloop is noodsaaklik om te verseker dat die waterstelsels skoon en veilig sal bly vir beide menslike gebruik en die besproeiing van gewasse, en daarom is die vermoë van 'n rhizofiltreerstelsel om chemiese en mikrobiëse kontaminante uit stedelike afloop te verwyder, geëvalueer. 'n Rhizofiltreerstelsel is by Stellenbosch se rioolwerke gebou, waarin besinkte riool gepomp kon word om te bepaal of riete die verwydering van chemikalieë, indikatororganismes, patogene en potensiële patogeniese giste kon verbeter.

Aanduidings is gevind dat besinkte riool van die Stellenboschse rioolwerke mikrobiologies en chemies vergelykbaar is met monsters wat vanuit die besoedelde Plankenburgrivier verkry is. Hierdie resultaat is verkry na vier dae besinking in die opgaartenk wat vir die rhizofiltreerstelsel gebou is. Dit dui aan dat die invloed van die rhizofiltreerstelsel as stedelike afloop beskou kan word na vier dae besinking in die tenk. Die beplante (eksperimentele) en onbeplante (kontrole) kante van die rhizofiltreerstelsel het vergelykbare verwyderingskoerse getoon ten opsigte van gesuspendeerde vastestowwe, ammoniak, "Chemical Oxygen Demand", fosfate en sulfate in die invloed, wat binne 45 min deur die sisteem geperkeer het. Mikrobiologies was die eksperimentele kant meer suksesvol as die kontrole kant in terme van die verwydering van fekale coliforme, giste en *Salmonella*, maar daar was geen verskil ten opsigte van die twee kante se verwydering van colifage gevind nie. Die meerderheid giste wat verwyder is, behoort aan die genus *Candida*, en die eksperimentele kant het dié oppertunistiese patogene meer suksesvol uit afvalwater verwyder. Gedurende dieselfde eksperimente is 'n aantal antibiotikumbestande bakterieë ook geïsoleer, wat bleikbaar binne die filter vermeerder en waarvan die meerderheid deel gevorm het van die *Burkholderia cepacia* kompleks. Daarby was die eksperimentele kant van die filter beduidend meer effektief vir die verwydering van fekale kolivorme, potensiële patogeniese giste en *Salmonella* wat binne die sand vasgevang was, vergeleke met die kontrole kant, ses dae nadat die afval water deur die filter geperkeer het. *In vitro* sand filter eksperimente het getoon dat die teenwoordigheid van *B. cepacia* in die sand verantwoordelik

mag wees om van die *Candida* spesies teenwoordig in die afvalwater vas te vang soos dit deur die sand perkoleer, en daardeur die tydperk te verleng waarin die giste aan die antagonisitiese effek van die wortelafskedings of ander mikrobes blootgestel is.

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# **CHAPTER 1**

## **Literature Review**

## **1.1 Introduction**

The availability of safe drinking water is a basic human right and is essential for health (Hodgson and Manus, 2006). It is however, becoming increasingly difficult to find safe groundwater and surface water sources due to the increased incidence of pollution in urban areas. Microbiological and chemical pollution are the two most prominent forms of water pollution that have an impact on human health. In both developed and developing countries, pathogenic microbes in water sources result in a range of diseases in both infants and adults which can prove fatal (WHO, 1996). The ultimate goal of water treatment is to remove such pathogens and provide a stable supply of potable water that meets the standards set by local governing authorities (Khan, 2004). However, the current physical, chemical and biological water treatment methods are often expensive to install and difficult to operate, thus not all water that enters these facilities is treated effectively (Shannon et al., 2008). Apart from groundwater and surface water, runoff from heavily populated and highly industrialised areas is also of concern as this water usually enters river systems directly. A promising technology for the treatment of urban runoff is rhizofiltration as it is a cost-effective and utilises an environmentally acceptable manner to remove contaminants from water before it enters water systems (Boyajian and Carreira, 1997; Singh et al., 2003). Rhizofiltration systems make use of plant roots to absorb and sequester metal pollutants or excess nutrients from wastewater (Dushenkov et al., 1995; Arthur et al., 2005). The roots also facilitate microbial activity by providing attachment sites for microbes as well as releasing organic carbon and oxygen in the rhizosphere. Ultimately, the process of rhizofiltration should produce an effluent that is cleaner and safer for both human use and crop irrigation.

## **1.2 Water and its associated problems**

### **1.2.1 Introduction**

The world faces a number of problems due to inadequate access to, and ineffective management of, water resources (Gleick, 1998). Water scarcity has quickly spread to many regions of the world due to increases in population size and consumption levels (Postel, 2000). South Africa is a largely semi-arid country with finite water resources, however greater urbanisation and industrial development are placing increasing pressure on an already depleted fresh water supply. Additionally, the development of informal settlements on the outskirts of major cities has increased the amount of urban runoff which flows into various water systems. This runoff increases the level of both pathogenic microbes and nutrients in

rivers and dams, the water of which is routinely used for drinking, bathing and irrigation. The use of polluted water has a detrimental effect on both the health of individuals and the ecosystem, and could lead to major world-wide epidemics in the future (Lee et al., 1996; Jackson, 2012).

### **1.2.2 The Importance of Water**

A safe, reliable and easily accessible water source is important for good health. For several years, numerous people in developing countries have not had access to adequate water provisions (Hunter et al., 2010). Howard and Batram (2003) estimated that a minimum of 7.5 litres of water per person per day is needed to meet the basic requirements for drinking, personal hygiene and the preparation of food. The availability of this basic water requirement is impacted by poor wastewater infrastructure, urban runoff as well as chemical and biological pollutants.

It is becoming increasingly rare to encounter a water source that can be used for potable-water supply prior to some form of treatment, due to the presence of both biological and inorganic matter which can cause problems for the people that consume it. The greatest threat to human health from drinking water derives from pathogenic microbes (Binnie et al., 2002). The number of outbreaks related to waterborne diseases that have been reported throughout the world demonstrates that pathogens in drinking water are a significant cause of illness. However, an estimate of illness based solely on reported outbreaks is likely to underestimate the problem as a significant proportion of waterborne illnesses are likely to go undetected due to ineffectual reporting systems. The symptoms of gastrointestinal illness; which include nausea, diarrhoea, vomiting and abdominal pain; are normally mild and usually only last a few days to a week, and as a result only a small percentage of those affected will seek medical attention.

Waterborne diseases are not only found in the developing world. Morris and Levine (1995) attempted to estimate the waterborne disease burden in the United States of America and found that approximately 7.1 million people may suffer from mild to moderate waterborne infection each year, leading to an estimated 1 200 deaths annually. Both the health and economic burden of waterborne diseases are considerable even for an industrialised society (Payment, 1997).

Several researchers have tried to estimate the total burden of waterborne disease world-wide. Huttly (1990) reported a total number of 1.4 billion cases of diarrhoea per year in children under the age of five, with an estimated 4.9 million children dying as a result of this

disease. However, not all these diarrhoea cases were waterborne. Additionally, Prüss and co-workers (2002) estimated that water, sanitation and hygiene were responsible for about 4 % of all deaths and 5.7 % of the total disease burden occurring globally. In countries where a major part of the population does not have access to safe drinking water, a large number of infections will be waterborne. Estimations of world-wide intestinal infections suggest that waterborne disease may account for one-third of all reported cases (Hunter, 1997). Such figures are likely to rise with the emergence of informal settlements in urban areas of developing countries, as they place increased pressure on already strained water treatment facilities.

### **1.2.3 Urbanisation and water quality**

In South Africa approximately 58% of the population is believed to live in urban areas with 11.5% of the households situated in informal settlements where basic services like sanitation and waste management are limited or lacking (Stats SA, 2005; DEAT, 2006). In the Western Cape there is an average of one toilet per 60 to 100 occupants within many of the informal settlements, while other settlements make use of uninhabited land or buckets for such purposes (Barnes, 2004; Britz et al., 2007). The lack of adequate infrastructure in these areas results in the runoff of contaminated water into rivers and other water sources where it threatens already fragile ecosystems. Additionally, the slowness of waste removal ensures that human faeces and urine remains in containers within these communities instead of being removed safely to specified dump sites (Stats SA, 2005). This has serious health implications for individuals within these settlements as well as creating elevated concentrations of contaminated material which may eventually flow into rivers (Lotter, 2010). Similarly, many residents living in unserviced informal settlements use the riverbanks, or even the river itself for ablutions, resulting in high concentrations of both chemical and biological pollutants in these water sources (Okafu et al., 2003). During rainfall events, the majority of the pollution in informal settlements flows into rivers and other water sources via urban runoff.

### **1.2.4 Urban runoff**

In the next forty years the number of people residing in urban areas within the developing world is estimated to increase by approximately three billion (UNPD, 2007). This places extensive pressure on water treatment systems to provide safe and clean water to the residents they serve. The lack of infrastructure and housing in developing urban areas usually forces poor communities to live in hazardous and unhealthy environments on the outskirts of these areas (Stephens et al. 1994). Residents use land and water systems for ablutions and cleansing

which compromises the health of aquatic ecosystems nearby as well as the livelihood of other water consumers (Nomqophu, 2005). Urban stormwater is a large contributor to elevated pollution levels in fresh and brackish receiving waters as this runoff transports a wide range of pollutants that may be detrimental to human health (ASCE, 1998; NOAA, 1998; Smith and Perdek, 2004). The pollutants in urban runoff include heavy metals, hydrocarbons and solids, as well as pathogenic microbes such as faecal bacteria, viruses and protozoans (Field et al., 1998; Mallin et al., 2009).

During rainfall events the effect of runoff is pronounced, resulting in an increased concentration of toxic substances and pathogens in water systems. Studies conducted in Australia have shown that waters receiving runoff in the Orange urban catchment contained moderate to high levels of pollution in terms of nitrogen, phosphorous, heavy metals and faecal coliforms (Bakri et al., 2008), while similar studies in North Carolina indicated that rainfall increased the levels of certain pollutants in water sources situated close to urban areas (Mallin et al., 2009). Australia and the United States are considered industrialised nations, however urban runoff is still regarded as a significant problem. A major concern is that most African countries lack the basic infrastructure and skills that these countries possess, meaning that urban runoff could present even greater health risks in these regions. Winter and Mgese (2011) have shown that urban runoff, particularly during rain events, increased the concentration of *Escherichia coli* and nutrients in the Berg River, South Africa. Similarly, studies conducted in the Umtata River catchment indicated excessive pollution of the river with regard to both chemical and microbiological characteristics (Fatoki et al., 2001). The concern for public health and safety with regards to the consumption of river water has resulted in an increased demand for monitoring water quality. As a result, indicator organisms have been identified that allow rapid detection of faecal and chemical pollution, allowing for a quicker response to any significant outbreaks.

### **1.2.5 The Use of Indicator Organisms**

Domestic and municipal sewage contains various pathogenic or potentially pathogenic microorganisms which, depending on species concentration, pose a potential risk to human health and whose presence must therefore be reduced in the course of wastewater treatment (Mulamoottil et al., 1999). The main objective of water treatment is to remove such organisms from the water prior to human consumption. Due to the difficulties and risks involved with cultivating specific disease-causing organisms, it is not always a viable option to analyse for them directly. A more suitable approach has been to look for the presence of easily identified bacteria that are known to reside in human faeces, and to view their presence

as an indication of possible faecal contamination of a water source (Binnie et al., 2002). If water is found to be free of such indicator organisms, then it is assumed that no human pathogens are present. To date no perfect indicator organism has been identified for wastewater, however the coliform bacteria group has long been regarded as the first choice among indicator organisms (Scholz et al., 2001).

The coliform group, that includes the genus *Escherichia*, are rod shaped bacteria that are widely found in the natural environment. These bacteria ferment lactose, forming gas and acid, in the presence of bile salts at a temperature ranging from 35°C to 37°C within 48 hours (Binnie et al., 2002). Bacteria that may originate either from the human gut or from the gut of other warm-blooded animals are of particular interest when testing water quality. These bacteria are able to grow in the human digestive system which represents an acidic environment at a temperature of around 37°C. Representatives of the coliform group thus serve as perfect indicator organisms of faecal contamination as they are able to survive under these conditions. If coliform bacteria are present, it is necessary to highlight those that are derived from the gut of warm-blooded animals. The *Escherichia coli* test can then be used to diagnose faecal contamination with *E. coli*, which usually constitutes 20-30% of the total coliforms present in wastewater (Scholz et al., 2001). The presence of coliforms can only provide an indication of bacterial pollution originating from faecal material, thus other indicator organisms are needed to reveal the presence of different disease causing agents.

Due to the omnipresence of bacteriophages in treated sewage, it was suggested that these viruses may also act as indicators of both faecal and viral pollution (Rosario et al., 2009; Ebdon et al., 2012). This is because their structure, morphology, and size, as well as their behaviour in the aquatic environment resemble that of enteric viruses. They are used to evaluate viral resistance to disinfectants, viral fate during water and wastewater treatment, and as tracers in surface and groundwater. Bacteriophages may also be used as indicators of faecal pollution because their presence in water samples would indicate the presence of its host (Tartera et al., 1989; IAWPRC, 1991; Payment and Franco, 1993; Grabow, 1996).

## **1.3 Microbes in the wastewater environment**

### **1.3.1 Introduction**

The drinking of contaminated water can lead to severe human health risks. The majority of diseases in developing countries can be linked to ineffective water treatment and the subsequent pollution of water sources (Khan, 2004). Microbial populations inhabiting both

surface water and groundwater can be divided into non-pathogens, opportunistic pathogens and pathogens (Prescott et al., 2002). The primary pathogens in terms of human infection include bacteria, viruses and protozoa which can result in diseases such as gastroenteritis, diarrhoea, hepatitis as well as typhoid fever (Khan, 2004).

### 1.3.2 Bacteria

Pathogenic bacteria are responsible for many human, animal and plant diseases and are generally transmitted through direct contact with an infected host or by ingestion of contaminated food or water (Schroeder and Wuertz, 2003). It has been estimated that several million bacterial species exist, but only a few thousand have been identified and characterized. Many of the important bacterial pathogens have been recognized for over 50 years and these organisms account for nearly all of the serious bacterial infections observed (Schroeder and Wuertz, 2003).

Bacterial genera usually associated with wastewater include the Gram-negative facultatively anaerobic bacteria, the Gram-negative aerobic bacteria and both the spore-forming and non-spore-forming Gram-positive bacteria (Dott and Kampfer, 1988). The major waterborne bacterial species and their resulting diseases are shown in Table 1.1 with gastroenteritis being the most commonly reported disease in humans (Payment, 2003). Diseases from pathogenic microbes only occur when a sufficient number of the pathogen has been ingested. The high infectious dose requirements for most bacterial pathogens make transmission through water difficult as firstly, enteric pathogens cannot usually multiply in water and secondly, water tends to increase the dispersal of these organisms (WHO, 1996; Schroeder and Wuertz, 2003).

*Salmonella* spp. are the most predominant pathogenic bacteria found in wastewater and the most common cause of gastroenteritis in humans in industrialised countries (Bitton, 1999; Baggensen et al., 2000; Bell and Kyriakides, 2002). It has been estimated that between two and four million human *Salmonella* infections occur annually in the United States with the majority of transmissions occurring through food contamination (Feachem et al., 1983; Sobsy and Olsen, 1983). Taxonomically, the genus *Salmonella* is made up of two species, *S. bongori* and *S. enterica*. *Salmonella enterica* can be further differentiated into six subspecies namely, *enterica*, *salamae*, *arizonae*, *diarizonae*, *indica* and *houtenae*, of which *S. enterica* subspecies *enterica* is most commonly associated with human and other warm blooded vertebrates (Levantsi et al., 2011). Members of the genus *Salmonella* are also clustered into

several serovars according to their flagellar and somatic antigens. Over 2400 serovars are currently described, of which only 50 are known to result in the infection of humans and warm blooded animals (Popoff, 2001). *Salmonella* is primarily considered a food pathogen but both drinking and natural waters can be a source of transmission for this bacterium to humans (LeClerc et al., 2002; Ashbolt, 2004).

*Salmonella* is spread by the faecal–oral route of contamination and usually enters water sources directly through the faeces of infected humans or indirectly via sewage discharge or agricultural land run off. Salmonellae are abundant in raw sewage ( $10^3$ - $10^4$  CFU/ml) and have been detected in wastewater effluent that may enter water systems after leaving treatment facilities (Maier et al., 2000; Wéry et al., 2008). This pathogen has been found in a variety of natural water sources including rivers, lakes, coastal waters and ground water where it poses a significant threat to human health (Polo et al., 1999; Martinez-Urtaza et al., 2004; Haley, et al., 2009; Levantesi et al., 2010). *Salmonella typhi*, the etiological agent for typhoid fever, is the most common serotype associated with waterborne outbreaks (Lloyd, 1983; Cohn et al., 1999). Globally, about 16 million cases of typhoid fever are reported annually with a mortality rate of between 12 and 30% when left untreated. Typhoid fever differs from salmonellosis in that *S. typhi* results in a systemic infection after initially colonising the intestines. The symptoms associated with this disease include high fever, fatigue, abdominal pain and diarrhoea. *Salmonella* related epidemics are occurring more frequently around the world particularly within developing countries. Reports of typhoid and paratyphoid fever are common in both Asia and Africa, where contamination of water has been suggested as the major reason for the outbreaks (Crump et al., 2004; Bhunia et al., 2009). Similarly, other diseases are increasing in prevalence around the world and include the potentially fatal cholera.

*Vibrio cholerae*, the causative agent of cholera, is a Gram-negative curved rod bacterium that is transmitted via water (Bitton, 1999; Sasaki et al., 2008; Hill et al., 2011). Cholera is primarily transmitted by the faecal-oral route and humans are the only known vertebrate host (Bishop and Camilli, 2011). These pathogens attach to the intestinal lining and secrete an enterotoxin, cholera toxin that results in severe diarrhoea, abdominal cramps, nausea, vomiting and finally hypovolemic shock which can prove fatal (Sterritt and Lester, 1988; Schroeder and Wuertz). Cholera occurs world-wide but is more prevalent in areas with inadequate protection of water supplies and is usually associated with major epidemics (Bitton, 1999; Schroeder and Wuertz, 2003). There have been seven pandemics of cholera since 1817 which has allowed this disease to spread globally (Bishop and Camilli, 2011). In 2008, the WHO

reported 190 130 cholera cases worldwide, including 5143 deaths (98 % in Africa), however cholera is usually under-reported with the true disease burden estimated to be in the millions (Sack et al., 2006; WHO, 2008). In addition to these endemic outbreaks, sporadic outbreaks can occur when water treatment systems and suitable infrastructure are lacking, such as in the case of the outbreak that occurred in Zimbabwe during 2008 and 2009 (WHO, 2008). However, wastewater also serves as a reservoir for other potentially fatal pathogens including the increasingly detectable pathogenic *E. coli*.

*Escherichia coli* is a normal inhabitant of the gut of warm-blooded animals where most strains are non-pathogenic (Caprioli et al., 2005; Rasko et al., 2011). However, there are several subtypes of this bacterial species that cause gastrointestinal disease in the form of haemorrhagic colitis. This disease results in nausea, abdominal cramps and vomiting as well as loose stools that may lead to bloody diarrhoea (Bitton, 1999). Pathogenic *E. coli* that are responsible for diarrhoea can be grouped into at least four classes including enteropathogenic, enteroinvasive, enterotoxigenic and enterohemorrhagic (Kaper et al., 2004; Prescott et al., 2005). Enterohemorrhagic *E. coli* (EHEC) are the most dangerous from a human health perspective as they are responsible for a number of illnesses that may be fatal in infants and the elderly (Khan, 2004; Nwachuku and Gerba, 2008). Serogroup O157:H7 is a representative of this class and is the most important pathogenic EHEC associated with human disease. This serogroup is responsible for haemolytic uremic syndrome, a systemic disease that primarily occurs in children under 10 years of age and is characterised by renal failure and haemolytic anaemia (WHO, 1996; Gerba et al., 1996; Mani et al., 2003). In 2005 it was estimated that there were a minimum of 20 000 *E. coli* O157:H7 cases and roughly 250 deaths in the United States each year (Prescott et al., 2005). Outbreaks of this organism have occurred around the world with incorrectly cooked meat providing the greatest source of infection (Rangel et al., 2005). However, contaminated water that is used for irrigation has also been implicated in the spread of this pathogen (Bitton, 1999; Islam et al., 2004; CDC 2006). The widespread occurrence of *E. coli* O157:H7 in surface waters has led to a number of outbreaks (Armstrong et al., 1996; Olsen et al., 2002). These outbreaks have arisen from a number of water sources including recreational water, drinking water as well as water that is in close proximity to farming practices (Chalmers et al., 2000; Rangel et al., 2005). Outbreaks are more pronounced after extreme rainfall events when high concentrations of pathogens are washed into water sources from sewage overflows and animal faeces (Curriero et al., 2001; Thomas et al., 2006). Gut-inhabiting bacteria are not the only pathogens which enter water sources as a result of human activities, enteric viruses are also known to be transmitted via faecally contaminated water.

### 1.3.4 Viruses

Enteric viruses are usually introduced into water sources by human activities such as urban runoff and leaking sewage (Fong and Lipp, 2005). It has been estimated that more than 100 types of pathogenic viruses are excreted in human and animal wastes but the number of viral particles found in wastewater is dependent on the health of the individuals that produce this waste (Melnick, 1984; Gilboa and Friedler, 2008). Direct contamination of water sources with faecal matter poses a significant health risk as enteric viruses are released in very high numbers in the faeces of infected individuals, usually ranging between  $10^5$  and  $10^{11}$  viral particles per gram of faeces (Farthing, 1989). The most commonly studied groups of enteric viruses include the families *Picornaviridae* (polioviruses, enteroviruses and hepatitis A virus), *Adenoviridae* (adenoviruses), *Caliciviridae* (noroviruses and caliciviruses), and *Reoviridae* (reoviruses and rotaviruses) (Fong and Lipp, 2005). The human noroviruses are responsible for the majority of episodes of non-bacterial gastroenteritis, accounting for more than 90% of viral gastroenteritis cases globally (Green et al., 2001). The Hepatitis A virus (HAV) is a major concern in densely populated areas, including informal settlements, as the virus is transmitted by contact as well as by the faecal-oral route (Koopmans and Duizer, 2004). Viral infections by enteric viruses are usually associated with diarrhoea and gastroenteritis but they have also been linked with respiratory infections, conjunctivitis, hepatitis and diseases that have high mortality rates, such as aseptic meningitis and encephalitis (Kocwa-Haluch, 2001). Enteric viruses can be transported in the environment by rivers, groundwater and wastewater thus suitable indicator organisms are necessary to indicate if water is contaminated with viruses derived from the faeces of affected individuals as bacterial indicators are ineffective (Bitton and Gerba, 1984; Sobsey et al., 1986; Straub et al., 1995; Savichtcheva & Okabe, 2006). Bacteriophages have been suggested as a solution to this problem as their detection would indicate the presence of a viral host (Payment and Franco, 1993; Grabow, 1996). Similarly, bacterial indicators are unable to predict concentrations of other potential pathogens such as fungi, including yeasts.

### 1.3.5 Yeasts

A significant number of yeast species have been isolated from aquatic environments, the majority of which belong to the genera *Candida*, *Cryptococcus*, *Pichia*, *Debaryomyces* and *Rhodotorula* (Medeiros et al., 2008). A large percentage of these yeasts originate from wastewater and terrestrial environments and thus have the potential to affect the safety of fresh water sources (Nagahama, 2006). Polluted water contains denser populations of yeasts when compared to clean water with opportunistic yeasts such as *Candida krusei*, *Candida*

*tropicalis* and *Candida parapsilosis* being regularly isolated from these environments (Hagler and Ahern, 1987). The presence of these organisms in polluted rivers could be an indication of faecal contamination (Kutty and Phillips, 2008). Medeiros and co-workers (2008) found a variety of *Candida* species in water samples collected from a river in south eastern Brazil, while a recent study of the Plankenburg River in South Africa revealed a possible external ecological niche for *Candida albicans* within the deeper zones of the water column (Stone et al., 2012). Both these rivers flow through under-developed urbanised areas with unsatisfactory sanitary services. Such living conditions could result in the continuous release of sewage into the neighbouring rivers. The isolation of human-associated yeasts from aquatic environments reveals an increased risk to public health as these species are known to cause invasive and life-threatening infections as well as showing resistance to antifungal drugs (Pfaller and Diekema, 2007; Nagahama, 2006, Medeiros et al., 2008). One of the most significant genera with regard to human health is *Candida*, a genus consisting of many opportunistic species which can become pathogenic when individuals are immunocompromised.

#### **1.4 The genus *Candida***

The genus *Candida* contains several members that may exist as commensals of the mucosal membranes of humans and other warm-blooded animals, where they pose no significant threat to the health of the host (Odds, 1988; Buck, 1990; Ramage et al, 2001; Sullivan et. al, 2004; Kumamoto et al., 2005, Cafarchia et al., 2006; Rao, 2012). However, in circumstances where the host is immunocompromised these yeasts can become pathogenic (Molero et al., 1998; Niewerth and Korting, 2002). This can be seen in countries with high numbers of HIV infected individuals such as South Africa (Korting et al., 1988; Rabeneck et al., 1993; Sharma et al., 2006). *Candida albicans* has long been considered the most important opportunistic pathogen in this genus. Recent evidence however, suggests that other *Candida* species, such as *C. tropicalis* and *Candida dubliniensis*, may also be causative agents of infection (Clancy, 2011; Rao, 2012).

The genus *Candida* contains ascomycetous yeasts which reproduce by holoblastic budding and do not form arthroconidia or ballistoconidia (Meyer et al., 2002). This genus includes white asporogenous yeasts that are able to form pseudohyphae and are usually characterised by colonial morphology, carbon utilisation and fermentation (Shepherd et al., 1985). Like other fungi, *Candida* species are non-photosynthetic, eukaryotic organisms with a cell wall that lies external to the cell membrane (Akpan and Morgan, 2002). The macroscopic and microscopic cultural characteristics of the different *Candida* species are very similar. They

can metabolise glucose both in the presence and absence of oxygen and can be found on or within the human body with the most significant areas for colonisation being the skin, gastrointestinal track and the vagina (Kurtzman and Fell, 2000). Candidiasis is a mycosis usually affecting humans and animals that results from infection by a *Candida* species (Epstein, 1990). The two most prominent forms of the condition are oral and vaginal candidiasis however cutaneous areas can also become infected.

Oral candidiasis (OC) is a collective term given to a group of oral mucosal disorders caused by a fungal pathogen belonging to the genus *Candida*. OC results primarily from infection by *C. albicans* with others such as *C. tropicalis* and *C. glabrata* also being implicated in the condition (Odds, 1988). It is usually found among the elderly, particularly those who wear dentures, but it can also be a sign of systemic disease (diabetes mellitus) and is the most common infection among the immunocompromised. The human immunodeficiency virus (HIV) leads to acquired immune deficiency syndrome (AIDS) which is usually accompanied by OC (Dupont et al., 1992). Interestingly, certain forms of candidiasis, particularly oral thrush, have been linked to immunodeficiency and have even been suggested as an indicator of immunodeficiency in AIDS patients (Klein et al., 1984; Sangeorzam et al., 1994; Calderone and Fonzi, 2001). *Candida* species have also been isolated in other areas of the body in immunocompromised individuals, including the vagina.

Vaginal candidiasis (VC) is a common mucosal infection caused by *Candida* species (Sobel, 1988; Kent, 1991; Sobel, 1992). Similar to OC, *C. albicans* is the major causative agent of VC having been isolated in approximately 85 to 95% of reported VC cases (Sobel, 1986; Odds, 1988; Landers et al., 2004). However, some non-albicans isolates have been identified, including *C. glabrata*, *C. krusei* and *C. tropicalis* (Singh et al., 2002; Corsello et al., 2003; Okungbowa, 2003; Buscemi et al., 2004; Nyirjesy et al., 2005). Symptoms of VC include itching, vaginal burning and vaginal discharge. Treatment is usually defined on an individual basis, but includes the use of antifungal treatments such as fluconazole and butoconazole (Sobel, 2001, 2007). VC has also been linked with HIV and AIDS with several studies indicating that vaginal colonisation with *Candida* is increased in HIV-positive women compared to those who are HIV-negative (Schuman et al., 1998; Duerr et al., 2003). Interestingly, there has been a tendency to isolate non-albicans *Candida* species in HIV-positive women which may be attributed to reduced sensitivity of these species to antifungal treatment (Vazquez et al., 2001; Sobel, 2007). Vaginal and oral candidiasis represent superficial, largely mucocutaneous infections but systemic infections do occur resulting in candidemia.

Candidemia is a significant problem in the health care setting with infections in intensive care units (ICUs) on the rise around the world (Ostrosky-Zeichner and Pappas, 2006). In the United States, candidemia is a very common bloodstream infection with similar trends being found in other countries (Jarvis, 1995; Rangel-Frausto et al., 1999; Garbino et al., 2002; Kullberg and Oude Lashof, 2002; Marchetti et al., 2004). *Candida albicans* is the most common cause of this systemic infection, accounting for between 40 and 60% of cases (Ostrosky-Zeichner and Pappas, 2006). However, the use of azoles has led to the emergence of non-*albicans* species that have acquired resistance to antifungal treatment, particularly *C. glabrata* and *C. krusei*. Blood stream infections by *Candida* species are a significant cause of morbidity and mortality in hospitalised patients (Giri and Kindo, 2012). Fatal *Candida* infections are often associated with life-prolonging measures; including catheters and other chronic invasive medical devices (Nguyen et al., 1995; Girishkumar et al., 1999), stem-cell transplants, cancer treatment (Bodey et al., 2002; Zollner-Schwetz et al., 2008), as well as anti-microbial treatments (Kurtzman and Fell, 2000; Navarathna et al., 2005; Indhumati et al., 2009).

Wastewater treatment facilities are responsible for the removal of pathogenic microorganisms, including *Candida* species from water before it is available for human use. These facilities, however, are not always maintained and operated correctly which can lead to the presence of *Candida* species in water systems.

## **1.5 Conventional wastewater treatment methods**

### **1.5.1 Introduction**

Conventional water treatment methods have been used to ensure that water leaving a treatment site is in a suitable condition for human usage, including agricultural applications and recreational uses. The processes used in water and wastewater treatment are classified according to the system changes that take place within each process, and can be grouped into physical, chemical and biological processes (Stuetz, 2009). Generally, these processes are combined in order to produce an effluent which is safe and of high quality.

### **1.5.2 Physical water treatment**

Physical wastewater treatment is used for the removal of suspended particles that are known to harbour a range of impurities (Stuetz, 2009). The major methods used in this process are screening, sedimentation and filtration. Screening is responsible for removing solids by passing water through openings that are smaller than the solid particles thereby removing both gross and abrasive solids, which may cause problems in downstream processes (Mara, 2004; Moharikar et al., 2005). These screens are usually classified according to the size of the opening as shown in Table 1.2. Initially, a coarse screen is used in order to prevent large objects from reaching the intake of treatment systems. This is usually followed by a continuous belt of mesh screens, with openings ranging between 5 and 15 mm that are slowly rotated in order to efficiently collect material in the water (Khan, 2004; Mara, 2004).

Following the removal of these larger solids, the water flows into a primary sedimentation tank which is routinely used for the preliminary treatment of water containing high concentrations of suspended solids (Moharikar et al., 2005). Sedimentation occurring in this tank involves the settlement of particles, which have a higher density than the liquid in which they are suspended, under the influence of gravity. The purpose of sedimentation is to allow floc to be deposited and thus reduce the amount of solids that must be removed by filtration (Zumstein et al., 2000; Gao et al., 2004; Mahajan, 2009). Factors which influence sedimentation include retention time, velocity of flow, as well as the size, shape and weight of the floc. Horizontal sedimentation tanks are simple rectangular tanks where water enters at one end and exits through a weir at the opposite end, allowing solids to settle to the bottom during its passage through the system (Stuetz, 2009). Vertical sedimentation tanks occupy less space than horizontal tanks and are commonly used for the settlement of screened wastewater. Water enters via a drum in the centre of the tank which directs the flow downwards towards the bottom. The water then flows upwards which reduces the liquid velocity and allows solids to settle. A large percentage of turbidity and colour are also removed during sedimentation, however, a small concentration of floc is usually carried over from these settling tanks which requires removal by filtration.

During filtration water passes through a granular bed of sand or other suitable medium, at low speed. Filtration is primarily used for potable water treatment and the tertiary treatment of domestic wastewater (Ellis, 1987). The two major techniques used during this stage are gravity filtration and continuous filtration. These processes differ with regards to the direction of the water flow through the filter medium. Gravity filtration is a batch process which is carried out for about 24 hours, after which the filter is removed for cleaning. Sand is the most common filter medium in such a system but anthracite, garnet and dolomite may

also be used under special circumstances. Slow sand filters have been used in the water treatment field for a long time (Baumann and Huang, 1974; Hamoda et al., 2004) and usually involve the filtering of wastewater through a bed of fine sand about one meter deep. A gelatinous layer will form on the surface of the sand due to the slow flow liquid velocity and is ultimately responsible for the removal of turbidity, colour and odours via a combination of filtration and biological activity (Steutz, 2009). This process results in a high quality effluent, however the capital cost of such a system is very high due to the amount of land required for its construction (Hamoda et al., 2004). An alternative to this process is continuous filtration where water enters the filter at the bottom of the filter and flows upward through the sand bed (Steutz, 2009). The water is filtered during its upward flow and is usually discharged from the top of the filter. The amount of water used is the same as in a gravity filter, but it is continuously discharged at a slow rate instead of being applied in large volumes.

The physical processes involved in wastewater treatment are used to remove debris and larger particles but are limited in their ability to remove harmful microbes from a water source (Steutz, 2009). It is thus critical to combine these physical methods with other forms of water treatment such as the addition of effective chemicals.

### **1.5.3 Chemical water treatment methods**

The chemical treatment of wastewater can occur during the physical processes or prior to release into a water body at the end of a water treatment system. Coagulation and flocculation, as well as precipitation, are used prior to some of the physical methods described above. Disinfection following these processes is however considered the most important step for the removal of pathogenic microbes.

Disinfection is not sterilisation, which implies the inactivation of all organisms; rather it is the killing of pathogenic organisms which results in a water source that is suitable for agricultural application or human consumption (Binnie et al., 2002). To achieve this, the water is firstly disinfected, to eliminate any pathogens that have passed through other treatment stages, and secondly to apply a residual treatment so that the water will remain safe after it has left a water treatment facility (Mahajan et al., 2009).

The disinfectant used within a treatment facility must remove any organisms of concern while not being toxic towards humans and animals (Binnie et al., 2002). Chlorination is the most common form of disinfection and can be applied either as gaseous chlorine ( $\text{Cl}_2$ ) dissolved in water or in the form of sodium hypochlorite. The use of sodium hypochlorite is usually more expensive than the application of chlorine gas, however many water treatment

facilities favour sodium hypochlorite due to the risks involved when handling  $\text{Cl}_2$  gas (Tchobanoglous and Schroeder, 1985; Mahajan, 2009). Chlorine and sodium hypochlorite are classified as oxidising biocides that kill pathogens by disrupting the cell wall and inhibiting enzyme activity (Binnie et al., 2002; Steutz, 2009). If bacteria and protozoa are to be removed, the concentration and contact time of chlorination need to be adjusted to ensure a safe effluent. An additional concern is the formation of by-products, such as trihalomethanes (THMs) when chlorine or sodium hypochlorite are applied to water that contains organic matter (Steutz, 2009). Trihalomethanes are the chemicals that result when halogens (usually chlorine) replace the three hydrogen atoms of methane to form potentially carcinogenic compounds such as chloroform ( $\text{CHCl}_3$ ). The formation of THMs has led to the development of alternative disinfection processes such as ozonation.

Ozone is a powerful oxidant that is able to oxidise organic matter and water without the formation of harmful by-products (Mahajan, 2009; Steutz, 2009). It is an efficient disinfectant which is useful in eliminating tastes and odours, as well as bleaching colour (Glaze, 1987). Ozone has long been used for water disinfection with systems currently operating in many countries around the world, including South Africa (Schalekamp, 1988; Pietersens et al., 1993). In practice, ozonation is usually only applied as a pre-treatment process or as a polishing step after filtration (Hoyer, 2006). The main application of this process is for the oxidation of organic pollutants, iron and manganese and the removal of algae. Ozonation is not without its disadvantages which include a high capital and operating cost, the possible formation of bromate and a short half-life, thereby rendering this process less efficient for residual disinfectant capacity (Khan, 2004; Hijnen et al., 2006).

The removal of pathogens from water is the primary goal of chemical treatments, however as mentioned above, the addition of chemicals to water may result in formation of toxic compounds. These concerns, combined with the expensive operation of some applications, have led researchers to search for a more natural alternative to both nutrient and pathogen removal. This has resulted in the development of biological processes that rely upon the activities of microbes and their associated growth surfaces for effective water treatment.

#### **1.5.4 Biological water treatment methods**

Biological methods are commonly used for the treatment of municipal and industrial wastewater. The microbes obtain energy and cellular material from either the aerobic or anaerobic oxidation of organic materials present in the wastewater. These processes can also be used to remove other wastewater components including suspended solids, ammonia and heavy metals (Pinney et al., 2000; Gernaey et al., 2004; Wu et al., 2011). Biological strategies

possess both advantages and disadvantages with the former being the fact that they are natural and odour reducing with a high removal efficiency potential. The major disadvantages of such processes, however, include a susceptibility to toxic chemicals, the production of noxious compounds and a prolonged treatment period when compared to the chemical treatment strategies mentioned previously (Steutz, 2009). The microbial action during biological processes can occur either on a fixed surface or in suspension, with both providing their own benefits for the removal of pathogens and organic matter (Mahajan et al., 2009).

Suspended growth processes utilise either free-living or flocculated microbes which are mixed with wastewater in an aeration tank to ensure that contact occurs between the water and the microbial population (Mahajan, 2009; Steutz, 2009). The most widely used suspended growth reactors are activated sludge systems (Figure 1.1) (Sykes, 1991; Hopkins et al., 1998). Activated sludge is formed by aerating biologically degradable wastes until settleable solids form, containing a variety of microbes. Wastewater is then fed into an aeration tank after being mixed with return sludge (Artan et al., 2004). Microbes stabilise the organic matter in the tank and the resulting mixture eventually flows into a sedimentation tank which allows the activated sludge to flocculate and settle out, resulting in a clear effluent with a low organic content (Gernaey et al., 2004).

A variety of microbes are usually found within activated sludge and include bacteria, fungi and protozoa. Bacteria are the most critical organisms for the stabilisation of organic matter and floc formation (McKinney, 1962). The dominating bacterial genera in activated sludge depends on the nature of the organic matter to be stabilised. A waste containing high protein concentrations will favour *Flavobacterium* and *Bacillus*, while *Pseudomonas* species will be more prevalent in carbohydrate-rich wastes. Similarly, bacteria are also dominant in water treatment strategies that use a fixed surface as they are necessary for the formation of effective biofilms.

Fixed film processes mainly rely on the formation of biofilms which develop on the surface of supporting material in the system. The microbial population present within the biofilm is in contact with wastewater that is passed over the surfaces on which the biofilm develops. A well-known example of a fixed film system is the trickling filter, which is the most commonly used aerobic biological waste treatment system (Mahajan, 2009; Steutz, 2009). It consists primarily of a rotating distributor allowing even water distribution over the surface of a filter bed. This filter bed originally consisted of sand, but the greater volumes of wastewater that are currently being treated have led to the use of rocks as the major substrate in this system (McKinney, 1962). The spaces between the rocks in the trickling filter allow

air to continuously move upward through the system resulting in a facultative environment (Moharikar et al., 2005). The microbes found within the filter reflect the facultative nature of it. Bacteria are the dominant microbes with aerobic spore-formers like *Bacillus* species found in the upper layers. Anaerobic zones exist at the interface between rocks where obligate anaerobes like *Desulfovibrio* are dominant. The majority of bacteria in such a system are facultative, existing aerobically when dissolved oxygen is present and anaerobically when the oxygen is removed (McKinney, 1962). The major facultative bacterial genera found in trickling filters include *Pseudomonas*, *Micrococcus*, *Flavobacterium*, as well as members of the family Enterobacteriaceae.

A relatively new biological technology for the treatment of wastewater is the use of constructed wetlands. These systems build on fixed film processes by incorporating vegetation which provides a greater surface area for the attachment of microorganisms. This may then lead to a cleaner effluent at the end of a treatment process as the influent comes into contact with a larger number of associated microbes.

## 1.6 Constructed Wetlands

### 1.6.1 Introduction

Constructed wetlands (CWs) are systems that have been designed to utilise the processes that occur in natural wetlands within a controlled environment (Vymazal, 2011). The properties and mechanisms of CWs make them highly desirable as a wastewater treatment technology and are a viable alternative to conventional water treatment methods that are often expensive. Specific emphasis is placed on phytoremediation which uses plant roots to remove contaminants and has many possible applications in all parts of the world.

### 1.6.2 Types of Constructed Wetlands

Constructed wetlands for wastewater treatment are classified according to Brix (1994) into three different groups, namely free-floating macrophyte-based systems, submerged macrophyte-based systems and rooted emergent macrophyte-based systems. Similarly, these different rooted emergent systems are further distinguished by water flow into (a) surface flow systems (SF), (b) horizontal subsurface flow systems (H-SSF), and (c) vertical subsurface flow systems (V-SSF).

Surface flow wetlands consist of basins or channels with soil or another suitable medium to support the rooted vegetation (if present) and water flowing at a low velocity. Plant stalks and litter regulate water flow and ensure plug-flow conditions, particularly in long, narrow channels (Reed et al., 1988). In nature, these wetlands are densely vegetated by a variety of plant species and typically have water depths of less than 0.4 m. In artificial systems, water flowing through the system is treated by physical, chemical and biological processes thereby ensuring the effective removal of organic material and suspended solids, through microbial degradation and filtration (Kadlec et al., 2000). Generally, SF wetlands are used for the advanced treatment of effluent from secondary or tertiary processes and are usually included as a polishing step once the water has passed through trickling filters and activated sludge systems. While surface flow wetlands are incorporated at the end of a treatment process, subsurface flow systems can be used for secondary and tertiary treatment of wastewater.

The SSF wetland technology is based on the work of Seidel (1967) and since then the technology has grown popular in many European countries and is currently applied worldwide. Subsurface flow wetlands employ a bed of soil or gravel as a substrate for the growth of rooted emergent wetland plants. Mechanically pre-treated wastewater flows by gravity, horizontally or vertically, through the bed substrate, where it comes into contact with a mixture of facultative microbes living in association with the substrate and plant roots. The bed depth in SSF wetlands is typically between 0.6 and 1.0 m, and the bottom of the bed is sloped to minimize overland water flow (Haberl et al., 2003). The ability of gravel alone to improve effluent quality might also be related to physical settling of suspended solids (Gersberg et al., 1984). The smaller size gravel used in the substrate of these systems may provide considerable filtering capacity, especially following substantial biofilm development (Coleman et al., 2001). Subsurface flow wetlands can be grouped according to the flow of water into either vertical or horizontal systems. Vertical subsurface and horizontal subsurface flow wetlands based on soil, sand and/or gravel can treat domestic and industrial wastewater (Rivera et al., 1995; Cooper et al., 1996, Decamp, 1999) by using different strategies based on oxygen transfer through the system.

In H-SSF wetlands (Figure 1.2A), the wastewater is fed in at the inlet and flows slowly through the porous medium under the surface of the bed following a horizontal path. The water will finally reach the outlet zone where it is collected before leaving via level control arrangement at the outlet. During this passage, the wastewater will come into contact with a network of aerobic, anoxic and anaerobic zones, with the aerobic zones occurring around roots and rhizomes that leak oxygen into the substrate (Brix, 1987; Cooper et al., 1996). Horizontal subsurface flow wetlands are primarily used for the secondary treatment of

municipal wastewater where they remove organic compounds, suspended solids, microbial pollution and heavy metals (Vymazal, 2011). The anaerobic conditions found within these wetlands make them unsuitable for the treatment of wastewaters with a high concentration of ammonia and phosphorous, as nitrification is limited due to the minimal oxygen content (Vymazal and Kröpfelová, 2008). However, this problem was overcome with the development of V-SSF wetlands (Figure 1.2B) which enable a greater transfer of oxygen throughout the system.

Vertical subsurface flow wetlands comprise of a flat bed of gravel topped with sand and planted with macrophytes (Vymazal, 2011). The gravel usually consists of different sized stones with larger stones being incorporated in the lower levels and fine stones making up the top layers. These systems are fed intermittently with large batches of wastewater resulting in surface flooding (Kadlec and Wallace, 2009). The water trickles slowly through the different layers and is expelled using a drainage network. The complete draining of the wetland allows air to enter the bed which provides greater oxygen transfer throughout the system. This unique ability of V-SSF wetlands allows for the oxidation of ammonia resulting in a nitrified effluent (Cooper, 1996; Mander and Jenssen, 2002). As a result, these systems are used for the treatment of wastes other than those associated with domestic or municipal wastewater (Kadlec and Wallace, 2009) such as food processing wastewaters and landfill leachates that are known to contain high levels of ammonia (Burgoon et al., 1999; Kadlec, 2003). The removal capacity of subsurface flow constructed wetlands however, is dependent on the type of vegetation incorporated into the system, as plants form the interface for contaminant removal while also preventing excessive clogging of the system.

### **1.6.3 Plants in Constructed Wetlands**

Plants found in natural wetlands are adapted to growth in water-saturated soils (Brix, 1994) with aquatic vascular plants, aquatic mosses and some larger algae being included in this group (Brix, 1997). Suitable plant species for constructed wetlands (Table 1.3) include the common reed and cattails (Gómez Cerezo et al., 2001; Stottmeister et al., 2003), which have been predominantly used in Europe and the United States, respectively (Du Plessis, 2006). These plants are favoured as they produce a significant amount of root biomass and are able to grow in the presence of high concentrations of heavy metals while requiring minimal maintenance (Dushenkov and Kapulnik, 2000).

Plants have become an indispensable component of constructed wetlands due to several key properties that they possess (Brix, 1994). These include physical effects brought about by the plant tissues, such as erosion control, filtration and surfaces for microbial attachment. Marsh plants also have certain physiological adaptations, including anaerobic respiration and oxygen transport to roots, which guarantee their survival even under extreme rhizosphere conditions (Stottmeister et al., 2003). Such conditions include an acidic or alkaline environment, high concentrations of toxic wastewater components (phenols, biocides and heavy metals) and salinity. The ability of wetland plants to overcome these difficulties provided scientists with a natural solution for pollutant removal. The processes necessary to remove contaminants from polluted water become integrated in the active zone of constructed wetlands, which is known as the root zone or rhizosphere (Stottmeister et al., 2003).

Root zone processes based on the microbial activity is used for both secondary and tertiary treatment of wastewaters, particularly in areas where conventional biological treatment processes are unavailable or not viable. The main features of the root zone method (RZM) treatments were given by Cooper and co-workers (1996) and can be summarised as follows: (i) The rhizomes of the reeds provide a hydraulic pathway (filter) through which wastewater can and must flow; (ii) roots and rock media are barriers to flow and disperse flow evenly, thus ensuring some form of mixing and the best possible mass transfer; (iii) atmospheric oxygen is supplied to the rhizosphere via the leaves and stems through hollow rhizomes and roots of the emergent macrophytes and lastly (iv) the wastewater is treated by microbial activity, where microbes are attached to plant roots.

Plant roots also provide a large surface area for the development of attached microbial growth and the resulting biofilm is thought to be responsible for the majority of the microbial processing that occurs within constructed wetlands (Hatano et. al., 1993; Brix, 1997). In SSF wetlands, the limited contact of the wastewater with the atmosphere, coupled with a high COD of the influent, results in anaerobic conditions predominating throughout the water column. While plant roots are usually ineffective in the bulk oxygenation of the wastewater stream, local oxidized environments on or near root surfaces harbor aerobic microbes which are thought to promote many treatment processes. Plants may thus play a critical role, both directly and indirectly, in the phytoremediation of contaminated water by removing various pathogens, heavy metals and nutrients.

Phytoremediation refers to the use of plants to bio-remediate contaminated soil and water and is considered to be a cost-effective and environmentally acceptable process (Boyajian and Carreira, 1997; Singh et al., 2003). Constructed wetlands have been used for various phytoremediation processes such as phytoextraction, phytostabilisation and rhizofiltration (Table 1.4). Phytoextraction involves the uptake of inorganic (primarily metal) contaminants by plants (McCutcheon and Schnoor, 2003). The roots of these plants absorb metals from the soil and translocate them to aerial shoots, where they accumulate (Jabeen et al., 2009). This process however, is only practical in sites containing low to moderate levels of metal pollution, as plant growth is not sustained in heavily contaminated sites (Padmavathamma and Loretta, 2007). During phytostabilisation, plants are responsible for the transformation of toxic soil metals to less toxic forms (Eapen and D'Souza, 2005). This process stabilises wastes, prevents exposure pathways and immobilises contaminants physically and chemically by root sorption (Cunningham et al., 1995; Berti and Cunningham, 2000). Of these, the most promising phytoremediation technology is rhizofiltration, which uses plant roots to absorb and concentrate metal contaminants and excess nutrients usually found in aqueous growth substrates, such as wastewater streams.

Rhizofiltration can be used to remediate metals like lead, cadmium and copper, as well as certain radionuclides (Jabeen et al., 2009). Plants influence the environment in the rhizosphere, providing suitable conditions for bacterial and fungal growth while their root systems provide suitable attachment sites for microbes, resulting in the formation of biofilms (Susarla et al., 2002). These biofilms are the main mechanism for the removal of pathogenic microorganisms during rhizofiltration, due to the increased activity in the rhizosphere.

#### **1.6.4 The use of constructed wetlands in water treatment**

Domestic wastewaters contain a variety of human pathogens including bacteria, viruses, protozoans, and helminths that can survive pre-treatment and thus enter treatment wetlands. Currently, the most commonly used regulatory measure for evaluating water quality is a test for faecal coliforms. However, faecal streptococci, *Salmonella*, *Yersinia*, *Pseudomonas* and *Clostridium* have all been studied in treatment wetlands as other possible indicators of faecal pollution (Herskowitz, 1986). Constructed wetlands are used in wastewater treatment to remove nitrate, biological and chemical oxygen demand (BOD and COD), enteric viruses and generally improve water quality (Pinney et al., 2000). It has been shown that both the SF and SSF wetland concepts are reliable and cost-effective technologies for the treatment of wastewater (Reed and Brown, 1992).

The ecology of microbes in a constructed wetland, as in any biological wastewater treatment system, is extremely complex. The organisms present in wastewater that affect human health include pathogenic bacteria and viruses. Protozoan pathogens and helminth worms are also of particular importance in tropical and subtropical countries (Rivera et al., 1995). In the aerobic environment of a V-SSF wetland and the colder partly aerobic environment of an H-SSF system, these organisms have minimal growth. Constructed wetlands are known to offer a suitable combination of physical, chemical and biological factors for the removal of pathogenic organisms. Physical processes include mechanical filtration, exposure to ultraviolet, and sedimentation while chemical factors include oxidation, exposure to biocides excreted by some plants and absorption by organic matter. Biological removal mechanisms include antibiosis, predation by nematodes and protists, attack by bacteria and viruses and natural die-off (Gersberg et al., 1989). The root excretions of certain aquatic macrophytes including *Scirpus lacustris* and *Phragmites australis* are known to kill faecal indicators and other pathogenic bacteria (Spratt and Morgan, 1990; Cooper et al., 1996). Additionally, the enhanced development of bacterial populations with antibiotic activity (e.g. *Pseudomonas*) in the rhizosphere may also account for coliform die-off (Mandernack et al., 2000).

Faecal bacterial removal efficiency in constructed wetlands is usually excellent, exceeding 95%, but this can vary with hydraulic residence time, wetland design, hydraulic and mass loading rate, substrate and temperature (Gersberg et al., 1987; Haberl et al., 1995; Potter and Karathanasis, 2001). Similarly, Neralla and co-workers (2000) found high removal of pathogens in CWs and reported a 99% removal of faecal coliforms observed in their systems, while Vacca and co-workers (2005) reported that bacteria removal in vertical sand filters planted with *Phragmites australis* is lower than that in non-planted ones. Interestingly, Wand and co-workers (2007) concluded that predation is the dominant mechanism of bacterial removal in planted and non-planted soil filters and CWs and attributed the higher removal efficiencies of the planted filters to the higher protozoa content. The improved aeration of the root zone in planted gravel beds would provide better conditions for protozoa development.

Constructed wetlands are excellent in terms of COD removal, with efficiencies in excess of 85% when reeds are present. In one pilot study conducted in South Africa, a treatment bed planted with *Pennisetum clandestinum* achieved a COD removal efficiency of 95% (Wood and Hensman, 1989). A study by Healy and O'Flynn (2011) found that the average removals of COD from primary and secondary wastewater were 88 and 72%, respectively, with average final effluent concentrations of  $53 \pm 18$  and  $45 \pm 27$  mg COD / L. The removal of ammonium ( $\text{NH}_4$ ) occurs as a result of nitrification, which is promoted due to the aerobic

nature of the V-SSF system. Once the water has percolated through the substrate, air can re-enter the system and provide the necessary oxygen for nitrifying bacteria to convert ammonia to nitrite and nitrate (Stottmeister et al., 2003). Tang and co-workers (2009) reported that planted wetlands removed 17% more  $\text{NH}_4$  than unplanted wetlands, while a study by Wu and co-workers (2011) showed an ammonium removal efficiency of between 70% and 80% when the organic load in the influent was kept low.

Unlike nitrogen and organic carbons, phosphorus is not biologically converted to a gaseous form. Removal of phosphorus occurs mainly through plant uptake, adsorption by the porous media and precipitation. During these processes, phosphorus reacts with the porous media and with minerals such as ferric oxyhydroxide and carbonate (Kadlec and Knight, 1996). Brooks and co-workers (2000) conducted a study on phosphorus removal by wallstonite as an alternative constructed wetland substrate. They found that phosphorus removal was greater than 80% and the concentrations of phosphorus in the effluent ranged from 0.14 to 0.50 mg/L. Gray and co-workers (2000) reported a 90% phosphorus removal for planted units, while another study reported 80% removal in a similar experiment (Brooks et al., 2000).

## 1.7 Aims of the study

From the preceding literature review it is obvious that constructed wetlands have the potential to provide a cost-effective and environmentally friendly alternative for conventional water treatment methods to remove unwanted microbes and nutrients from contaminated wastewater. With the ultimate goal of developing a rhizofiltration system which can be used for the treatment of urban runoff before it enters water systems, the objectives of this project were firstly to evaluate the influent of an experimental constructed rhizofiltration system as urban runoff using a polluted river as model (Chapter 2). Secondly, to determine the removal capacity of a constructed rhizofiltration system with regards to selected indicator organisms, bacterial pathogens, potentially pathogenic yeasts, and chemical pollutants associated with typical urban runoff (Chapter 3). The final aim of the study was to explore microbial interactions within the upper sand layer of the rhizofilter by periodically enumerating potentially pathogenic yeasts and selected proteobacteria after the wastewater has passed through the filter. The bacteria that were studied included faecal coliforms, *Salmonella*, as well as antibiotic resistant bacteria, primarily *Burkholderia* species. Then, binary interactions between *Burkholderia* and strains representing pathogenic *Candida* species were studied *in vitro* within sand columns (Chapter 4).

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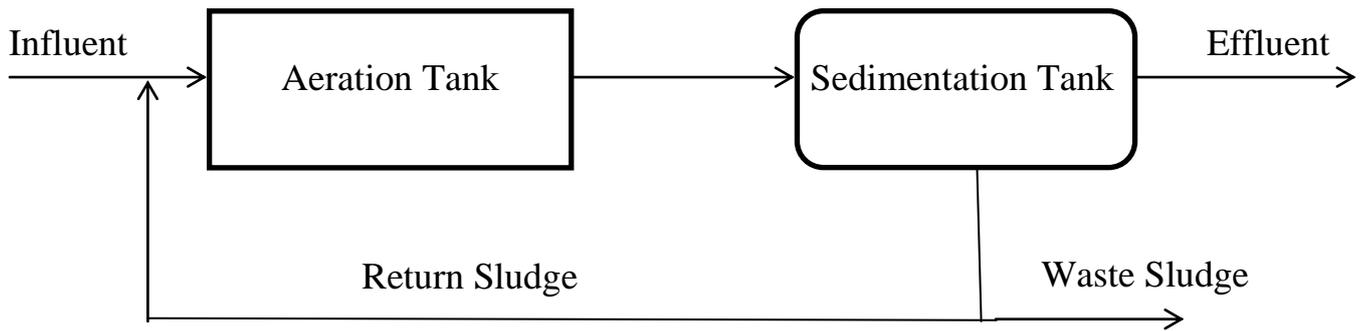
**Table 1.1 - Major waterborne bacterial pathogens which affect the gastrointestinal tract.**

<b>Bacterial Species</b>	<b>Major Disease</b>	<b>Source</b>
<i>Salmonella typhi</i>	Typhoid fever	Human faeces
<i>Salmonella paratyphi</i>	Paratyphoid fever	Human faeces
Pathogenic <i>E. coli</i>	Gastroenteritis	Human faeces
<i>Vibrio cholerae</i>	Cholera	Human faeces
<i>Campylobacter jejuni</i>	Gastroenteritis	Human/animal faeces
<i>Yersinia enterocolitica</i>	Gastroenteritis	Human/animal faeces

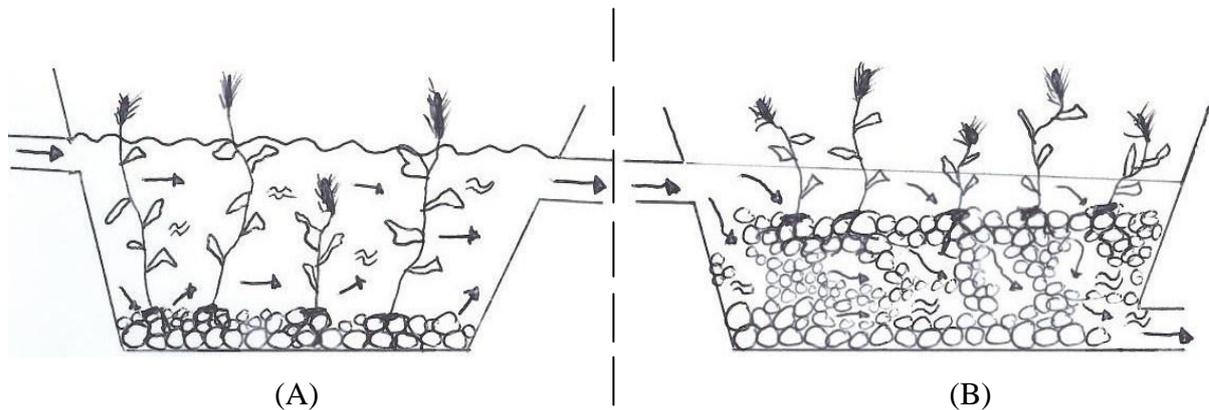
Adapted from: Sobsey and Olson (1983).

**Table 1.2 - Classification of screens used in wastewater treatment according to size (Stuetz, 2009).**

<b>Screen type</b>	<b>Opening (mm)</b>
Coarse	>50
Medium	15-50
Fine	3-15
Milli	0.25-3
Micro	0.025-0.2



**Fig. 1.1 - The various stages involved during the activated sludge process where microorganisms aerobically stabilise organic matter in the aeration tank, which is then allowed to flocculate in the sedimentation tank to produce a clear effluent. Adapted from McKinney (1962).**



**Fig. 1.2 - Constructed wetlands for wastewater treatment. Emergent macrophytes are used in (A) a horizontal subsurface flow system and (B) a vertical subsurface flow system. (Adapted from Stottmeister et al., 2003).**

**Table 1.3 - Plant species used in constructed wetlands and the parameters that they are conventionally used for.**

Scientific name	English name	Parameter
<i>Phragmites australis</i> (Cav.) Trin. Ex Steud.	Common reed	COD, BOD <sub>5</sub> , TSS, FC, NH <sub>4</sub> - N
<i>Juncus</i> spp.	Rushes	TP, TKN, NH <sub>4</sub> - N
<i>Scirpus</i> spp.	Bulrushes	BOD <sub>5</sub> , TSS, FC,
<i>Typha angustifolia</i> L.	Narrow-leaved cattail	Heavy metals
<i>Typha latifolia</i> L.	Broad-leaved cattail	COD, BOD <sub>5</sub> , TSS, FC, NH <sub>4</sub> - N
<i>Carex</i> spp.	Sedges	TP, TN

COD – Chemical Oxygen Demand; BOD<sub>5</sub> – Biological Oxygen Demand; TSS – Total Suspended Solids; FC – Faecal Coliforms; TP – Total Phosphorous; TKN – Total Kjeldahl Nitrogen; TN – Total Nitrogen, NH<sub>4</sub>-N – Ammonium nitrogen.

**Source: Adapted from Stottmeister et al., (2003).**

**Table 1.4 - Overview of some phytoremediation processes used within constructed wetlands.**

<b>Mechanism</b>	<b>Process Goal</b>	<b>Contaminants</b>	<b>Plants</b>
Phytoextraction	Hyper-accumulation, contaminant extraction and capture.	Metals: Ag, Cd, Co, Cr, Cu, Hg, Mn, Mo, Pb.	Indian mustard, sunflower, pennycress and alyssum.
Rhizofiltration	Rhizosphere accumulation, contaminant extraction.	Organics/Inorganics: Metals, radionuclides.	Sunflower, water hyacinth and Indian mustard.
Phytostabilisation	Complexation, contaminant destruction.	Inorganics: As, Cd, Cr, Cu and Pb.	Indian mustard, grasses and hybrid poplars.
Phytovolatilisation	Volatilisation by leaves, released into air.	Chlorinated solvents and some inorganics (Se, Hg).	Indian mustard.
Hydraulic Control	Contaminant degradation or containment.	Water-soluble organics and inorganics.	Cottonwood, willow and hybrid poplar.

Source: Adapted from Erakhrumen and Agbontalor (2007)

## **CHAPTER 2**

### **Evaluating settled sewage as a model urban runoff system for the Plankenburg River using a rhizofiltration system.**

Language and style used in this chapter are in accordance with the requirements of the journal *Water Research*.

## 2.1. Introduction

Water is a requirement of life and its quality, as well as quantity, is very important for sustaining the world's economy and human population. In South Africa, natural lakes are few and mostly small and therefore surface water is collected in dams and river systems. These water bodies are the main water resources that are primarily used for agricultural activities, industry, mining, energy production, as well as domestic and municipal needs (Jackson et al., 2009). However, microbial and chemical contaminants in river systems are on the increase. This can mainly be attributed to urban runoff, domestic and industrial discharges, inadequate sanitation, as well as ageing water treatment facilities. Urban runoff has increased dramatically in many developing countries due to urbanisation and the construction of informal settlements on the outskirts of major cities.

South Africa has not escaped the phenomenon of urbanisation with many people relocating to city centres in search of a better lifestyle (St. Louis and Hess, 2008). Upon arrival, they are faced with the reality that job opportunities are limited and are therefore forced to move into informal settlements where basic services, including sanitation, are limited. Residents are thus forced to use open land and water systems for ablutions, compromising the health of aquatic ecosystems and also the well-being and livelihood of water consumers (Nomqophu, 2005). During rainfall events, pollutants including excess nutrients are washed into rivers from land surfaces resulting in an increased concentration of toxic substances and pathogens in these water systems. This leads to a deterioration of river water quality and presents a major challenge for South Africa as it is perceived as an obstacle in the provision of adequate water supplies in order to meet the current and future needs of sustainable fresh water (CSIR, 2010).

Due to the difficulties and risks involved with cultivating specific disease-causing organisms, it is not always possible to assess their numbers directly in sewage polluted ecosystems. A more suitable approach has been to look for the presence of readily identifiable organisms that are known to reside in human faeces, and to regard their presence as an indication of possible faecal contamination of a water source (Binnie et al., 2002). Total coliforms (faecal and non-faecal), bacteriophages, protists and helminths are routinely used as indicators in order to assess the microbiological quality of both fresh and wastewater.

Coliforms can easily be detected in water samples by the formation of red metallic colonies on differential and slightly selective media containing bile salts such as MacConkey Agar (Atlas, 1993). Enumeration of bacterial pathogens, such as *Salmonella* spp., can be used alongside coliform enumeration to provide a more comprehensive analysis of water pollution.

The increased detection of *Salmonella* in water sources has led to this enteric pathogen gaining recognition as an indicator organism in water quality testing, but other pathogenic organisms such as fungi, including yeasts, should also be considered (Efstratiou and Tsirtsis, 2009).

Yeasts are diverse, unicellular fungi that are commonly found in food, soil and water. The primary ecological role of these organisms is to act as saprophytes that transform plant and animal organic material to yeast biomass and by-products (Kutty and Phillip, 2008). There are however, several yeast genera that exist as commensals within healthy individuals where they pose no significant threat (Ramage et al, 2001; Sullivan et. al, 2004). In circumstances where the host becomes immunocompromised, these yeasts could become pathogenic (Niewerth and Korting, 2002), as was revealed in numerous studies involving the genus *Candida* (Wingerd et al., 1979; Ostrosky-Zeichner and Pappas, 2006; Nucci and Colombo, 2007; Gasparoto et al., 2009). *Candida albicans* has long been considered the most important opportunistic pathogen in this genus but recent evidence suggests that other non-albicans *Candida* species, such as *Candida tropicalis*, *Candida krusei* and *Candida dubliniensis*, may also be causative agents of infection (López-Martínez, 2010). The presence of these organisms in polluted rivers could be an indication of faecal contamination (Kutty and Phillips, 2008). Toxigenic fungi or other pathogens cannot be predicted by using yeasts as indicator organisms, but the detection of pathogenic yeasts such as *Candida* species in water may suggest an increased risk to public health (Beuchat et al., 2001).

Water, however, may not only be biologically contaminated, but can also be polluted chemically with dissolved and toxic substances. In both developing and industrialized countries a growing number of these substances, ranging from excessive nutrient loads to emerging micropollutants, are entering water supplies as result of human activity (Shannon et al., 2008). The accumulation of excess nutrients results in eutrophication, favouring the formation of cyanobacterial blooms. This process has a negative impact on the composition and functioning of natural aquatic biota (Oberholster et al., 2009 a,b) and also has a detrimental effect on human health, as cyanobacteria produce potent neurotoxins. These toxins were found to block voltage-gated sodium channels and have been linked to neurodegenerative disease in humans, with no known antidote currently available (Aráoz et al., 2010).

The challenges described above increase public health and environmental concerns and drive efforts to purify water supplies previously considered clean and safe. Chlorine compounds, ozone (O<sub>3</sub>), UV radiation, sedimentation and membrane filtration are a few conventional

methods used to purify polluted water (Susarla et al., 2002). However, these methods are inadequate to remove cyanobacterial cells from the water and may cause lysis of these cells resulting in the release of detrimental neurotoxins. Additionally, many developing countries lack the infrastructure and funding to maintain and upgrade these water treatment facilities (Shannon et al., 2008). As a result, cheaper and more environmentally friendly options for water treatment are being investigated with rhizofiltration providing a promising alternative.

Rhizofiltration can be used to remediate metals like lead, cadmium and copper, as well as certain radionuclides (Jabeen et al., 2009). Plants influence the environment in the rhizosphere, providing suitable conditions for bacterial and fungal growth while their root systems provide suitable attachment sites for microorganisms, resulting in the formation of biofilms (Susarla et al., 2002). These biofilms are the main mechanism for the removal of pathogenic microorganisms during rhizofiltration, due to increased metabolic activity in the rhizosphere.

Although rhizofiltration has been widely used, little is known about the potential of this technology to purify urban runoff before it enters water systems. Previous studies that monitored water quality in the Plankenburg River reported high levels of faecal contamination as well as an elevated nutrient load (Ackerman, 2010; Lotter, 2010). Therefore, this study monitored the settling of sewage to establish the time required to obtain a microbiological and chemical composition similar to water from the Plankenburg River.

## **2.2. Materials and Methods**

To determine the water quality from both the Plankenburg River and settled sewage, samples were periodically collected at selected sites. Subsequently, the water was characterized regarding the presence of indicator microorganisms and physico-chemical composition.

### **2.2.1 Site description**

The Plankenburg River flows through Stellenbosch and is approximately 10 km long. It flows along an industrial area and informal settlement with an estimated population of 22 000 people who suffer from inadequate sewage facilities (Pulse et al., 2009). The first sampling site (PB1) (33°93'34"S, 18°85'13"E) was 1 km downstream of the Kayamandi informal settlement in a highly industrialised area (Figure 2.1) while the second sampling site (PB2) (33°92'74"S, 18°85'08"E) was 600 m upstream from PB1, at a substation within the Plankenburg industrial area. Barnes and Taylor (2004) sampled at this specific site in 2003

and reported that high numbers of *Escherichia coli* were present in the water. During our study, PB2 was visibly polluted with electrical waste, litter and also fresh human faeces. In addition to the sampling sites a 10 000 L tank was erected at the Stellenbosch sewage works (33°94'30"S, 18°82'42"E) into which settled sewage could be pumped prior to analysis. The settled sewage in this tank served as the influent for later experiments involving the constructed rhizofiltration system.

### **2.2.2 Sampling procedure**

River samples were collected weekly between March and October 2011 to obtain average values which represented all seasons of the year. A specialised stainless steel cup with a 1.5 m long handle was used to obtain the river samples. This sampling tool was thoroughly sterilised with 99% ethanol before and after each sampling event. Samples consisting of 250 ml clear flowing water, 250 ml rock-filtered water and 250 ml plant-debris filtered water were taken approximately 50 cm below surface level and stored in sterile 1 L glass jars. These three sampling zones were used to obtain an average which represented the majority of the water column in the river. The temperature and pH of the samples were measured directly after sampling by using a hand-held mercury thermometer and pH meter (Martini instruments).

In order to compare the quality of the river water and settled sewage, the latter was pumped into the 10 000 L tank and allowed to settle further for between one and seven days. After the desired number of days, the first 2000 L of sediment in the tank was flushed to remove large solids that might clog the rhizofiltration system in later experiments. The remaining wastewater was collected from the influent pipe of the rhizofiltration system at random intervals in sterile 250 ml bottles and transported on ice to the laboratory. The temperature and pH of the tank water were measured in a similar manner to the river samples.

### **2.2.3 Microbiological analyses**

Faecal coliform concentrations were determined by preparing a triplicate dilution series of each sample followed by plating 100 µl of each dilution on MacConkey Agar (Atlas, 1993). The plates were subsequently incubated at 44°C for 24 hours after which coliforms were enumerated by identifying colonies with a metallic red hue. Potential pathogenic yeasts were detected by filtering 100 ml aliquots of the samples collected from the settled sewage through 0.45 µm pore cellulose nitrate filter disks (Sartorius Stedim Biolab Products Augbane, France) in separate sterile polycarbonate filter systems. The filter disks were then transferred directly onto Sabouraud Glucose Agar (SGA) and incubated at 37°C for 48 hours until cream

colonies were clearly visible. After incubation, colony forming units were counted. *Salmonella* concentrations were determined by plating out 100 µl of each sample on *Salmonella-Shigella* Agar and incubating the plates at 37°C for 24 hours (Hassanein et al., 2011). *Salmonella* colonies were enumerated by identifying colonies which were colourless but contained a black centre.

#### **2.2.4 Physico-chemical analyses**

Samples were analysed by the Central Analytical Facility (CAF) at Stellenbosch University to determine the levels of various physico-chemical properties in the different water systems. The properties analysed included ammonium, chloride, chemical oxygen demand (COD), suspended solids and sulphates. The standard operating procedure of ion chromatography (Dionex DX-120, USA) was followed for the simultaneous determination of chloride and sulphate ions present in the water ([www.dionex.com/en-us/webdocs/4520-31183-03.pdf](http://www.dionex.com/en-us/webdocs/4520-31183-03.pdf)). Ammonium and COD concentrations were calculated according to the manufacturer's instructions of Merck Test Kits with a measuring range of 0.010 – 0.3 mg/L NH<sub>4</sub>-N; 0.013 – 3.86 mg/L NH<sub>4</sub><sup>+</sup> or 2.0 – 150 mg/L NH<sub>4</sub>-N; 2.6 – 193 mg/L NH<sub>4</sub><sup>+</sup> and 100 – 1500 mg/L or 500 – 1000 mg/L, respectively. The method of Wycoff (1964) was used to determine the total amount of solids per litre of sample.

#### **2.2.5 Statistical analysis**

Calculation of means and standard deviation for continuous variables were performed using Microsoft Excel Office 2007 version. The F-test, t-test and correlations were performed using the same software to evaluate differences between variables. All tests of variance, significance and correlations were considered statistically significant at *p* values of < 0.05.

### **2.3. Results and Discussion**

#### **2.3.1 Microbiological analysis**

Previous studies conducted by Barnes and Taylor (2004), as well as Ackermann (2010) and Lotter (2010) revealed high *E. coli* levels in the Plankenburg River. Therefore using the water quality of the Plankenburg River as a model, settled sewage was evaluated as experimental urban runoff. Consequently, it was decided to compare the levels of faecal pollution in settled sewage and the Plankenburg River. To determine the removal efficiency of settling with regard to indicator organisms, a 10 000 L tank was filled with settled sewage. Samples were collected after allowing this sewage to further settle for between one and seven days.

The results obtained for the river samples showed that the concentration of faecal coliforms and potentially pathogenic yeasts was lower at sampling site PB1 when compared to PB2 (Figures 2.2 and 2.3). This might have resulted from the downstream position of PB1 which allowed the water to become more diluted. The concentration of faecal coliforms was significantly lower in water sampled from the Plankenburg River (PB2) when compared with settled sewage allowed to further settle for one ( $p = 0.0002$ ), two ( $p = 0.0013$ ) and three days ( $p = 0.0004$ ) in the tank (Figure 2.2). Similarly, the concentration of potentially pathogenic yeasts were significantly lower in samples collected from the Plankenburg River (PB2) when compared to settled sewage allowed to further settle for one ( $p = 0.0000$ ), two ( $p = 0.0000$ ) and three days ( $p = 0.0000$ ) in the tank (Figure 2.3).

Following four days of settling in the tank, there was no significant difference in the concentration of faecal coliforms ( $p = 0.3249$ ) and yeasts ( $p = 0.9785$ ) between the settled sewage and river samples (PB2), indicating that this period of settling might be suitable for comparisons to be made between the influent of a rhizofiltration system and polluted rivers. Both coliform (Figure 2.2) and yeast numbers (Figure 2.3) were reduced by 2 log units following a seven day settling period. This log 2 reduction represents a 99.3% and 99.6% decrease in the number of coliforms and yeasts respectively, when these values were compared to the settled sewage originally pumped into the tank. These removal percentages can be deceiving, as the concentration of microbes per 100 ml is still considerably above the standards set for receiving water bodies (DWAf, 1996). Additionally, a large amount of sludge is generated from the settling process which must be discarded in a safe and affordable manner if this technique was to be used as a method for the removal of microorganisms from wastewater.

Previous studies suggest that a decrease in the concentration of pathogens in water results in an increase in their numbers in sludge, as most microorganisms are associated with settleable solids (Godfree and Farrell, 2005). A linear relationship was found between the number of coliforms and suspended solids (data not shown) in settled sewage ( $R^2 = 0.99$ ;  $p = 0.00$ ). This correlation could explain the decrease in indicator counts recorded in the sewage that was allowed to settle for an extended period of time. Furthermore, along with more time for sedimentation, the anaerobic conditions created from a longer period within the tank may have caused a greater volume of organic matter to be mineralized (Ruiz et al., 2010), resulting in fewer solids suspended in the water.

The concentration of *Salmonella* was significantly lower in water sampled from the Plankenburg River (PB 1) when compared with settled sewage allowed to further settle for

one ( $p = 0.0000$ ) and two days ( $p = 0.0025$ ) but no difference could be found between PB 1 and further settled sewage after three ( $p = 0.0570$ ) and four days ( $p = 0.0706$ ; Figure 2.4). No difference was found between the sewage and samples collected from PB 2 after one ( $p = 0.0506$ ), two ( $p = 0.5391$ ) and three days ( $p = 0.5232$ ) in the tank. However, a significant difference was found ( $p = 0.0089$ ) between PB 2 and settled sewage allowed to further settle for four days. *Salmonella* numbers dropped significantly after five days of settling and all culturable *Salmonella* was completely eliminated from the tank after six days.

Sewage sludge contains a large number of human pathogens including, *Salmonella* spp., *Listeria* spp., and *E. coli* (Jones and Matthews, 1975; Dudley et al. 1980; Jones, 1990), which have the ability to survive in the environment for a considerable period of time (Kearney et al. 1994). The elimination of *Salmonella* from the supernatant of settled sewage (Figure 2.4) is not surprising as many studies have shown that these microbes are frequently found in the sludge environment (Danielsson, 1977). Additionally, competition with other microbes, pH and temperature fluctuations, as well as predation can all be responsible for the reduction in the number of *Salmonella* following five days of settling during our study (Godfree and Farrell, 2005).

Analysis of two sites in the Plankenburg River in Stellenbosch indicated that a four day settling period of settled sewage in the storage tank resulted in equivalent concentrations of faecal coliforms, potentially pathogenic yeasts and *Salmonella*, when compared to this highly polluted river (Figure 2.5). There was no significant difference in faecal coliform numbers between the influent of the rhizofiltration system and both PB1 ( $p = 0.1417$ ) and PB2 ( $p = 0.3249$ ) following a four day settling period. Similarly, no difference was found in potentially pathogenic yeast numbers between the influent and sampling site PB2 ( $p = 0.9785$ ). However, a significant difference ( $p = 0.0002$ ) was found between the influent and PB1 with regard to yeast numbers. This difference may be explained by greater dilution of the river water as sampling site PB1 is situated downstream of PB2. Dilution is known to lower the concentration of contaminants by effectively distributing them throughout the water source (Herrick, 1995). No difference was found in the concentration of *Salmonella* between PB1 and the settled sewage after four days of settling ( $p = 0.0706$ ), but differences were found between the influent and PB 2 after this settling period.

The high levels of faecal pollution in the Plankenburg River are largely attributed to pollution entering the river from the Kayamandi informal settlement and Plankenburg industrial area. It has been reported (Barnes and Taylor, 2004) that the sanitation infrastructure in Kayamandi is minimal and that many of the existing facilities are not in working order. As a result of this,

public sanitation facilities overflow and effluent is washed either directly into the river or into storm water systems that drain into the Plankenburg River. It is also speculated that both industrial and sewage wastes are being released illegally into the Plankenburg River by some of the local industries due to expensive waste treatment procedures. The dumping of human waste increases the levels of faecal contamination, while industrial waste changes the chemical and nutritional properties of the river water. Settled sewage that is allowed to further settle for four days is microbiologically equivalent to sampling sites in the Plankenburg River with PB2 representing the most polluted site due to its close proximity to the Kayamandi informal settlement. Consequently, chemical comparisons were conducted using settled sewage that was allowed to further settle for a period of four days in the storage tank to ensure uniformity throughout the experiment.

### **2.3.2 Physico-chemical analyses**

To compare the Plankenburg River with settled sewage that had been allowed to further settle for four days, a range of physico-chemical analyses were conducted to determine if these two water sources were equivalent with regard to chemical oxygen demand (COD) and suspended solids as well as chloride, ammonium and sulphate concentrations (Table 2.1). There was no significant difference with regard to COD between the settled sewage and both PB1 ( $p = 0.1601$ ) and PB2 ( $p = 0.6253$ ). The COD concentration obtained at sampling site PB2 (477 mg/L) is significantly higher than the guideline set by the Department of Water Affairs and Forestry (75 mg/L) for water systems in South Africa (DWAF, 1996). Considerably lower COD concentrations were reported in previous studies at the same site, with a maximum value of 75.6 mg/L being obtained in June 2008 (Ackermann 2010; Lotter, 2010). The substantial increase in COD indicates that the water quality of the Plankenburg River has deteriorated over the previous three years. This might be attributed to increased chemical waste dumping from nearby industries as well as a population increase in Kayamandi informal settlement resulting in increased pressure on already stressed water facilities.

No significant difference was found between the settled sewage and both PB1 ( $p = 0.0752$ ) and PB2 ( $p = 0.9223$ ) with regard to chloride concentration (Table 2.1). The chloride concentration of PB2 (137 mg/L) was however higher than the guidelines set by the DWAF. Chlorine (the reactive form of chloride) is used as a disinfectant in wastewater treatment plants and as a bleaching agent in textile factories, laundromats and paper mills. The discharge of domestic and industrial waste or chemicals into the river might explain these high chloride levels. Such a level of chlorination, as found in the Plankenburg River, may

cause serious health damage as several studies have associated chlorinated by-products to an increased incidence of cancer (Flatten, 1992; Lopez-Cervantes et al., 2004).

Sulphate concentrations were statistically similar between the settled sewage and both PB1 ( $p = 0.3294$ ) and PB2 ( $p = 0.3990$ ). The sampled sites all had sulphate concentrations below the lower threshold for sulphate in water sources (200 mg/L) as stipulated by DWAF (1996). Ammonium concentrations were considerably lower in the river samples (PB1 and PB2) when compared with the settled sewage. The level of ammonium at PB2, however, was greater than the limit set by DWAF (1996). These elevated concentrations of ammonium can affect the disinfection of water and might result in nitrite formation in distribution systems, which can lead to both taste and odour problems (DWAF, 1996).

## **2.4. Conclusions**

Indications were obtained that settled sewage from the Stellenbosch Sewage Works was microbiologically comparable to samples collected from the Plankenburg River after a four day settling period in the storage tank. These comparisons could be applied to faecal coliform, yeast and *Salmonella* concentrations and could be used to show that the sewage influent of the rhizofiltration system was similar to what is usually found in a polluted river system in an urban area after this period of settling. Additionally, a four-day settling period also resulted in similar chemical concentrations between the influent of the rhizofiltration system and the Plankenburg River. The results obtained during this study were therefore used in a subsequent study to test the efficiency of a rhizofiltration system using settled sewage as an alternative for urban runoff.

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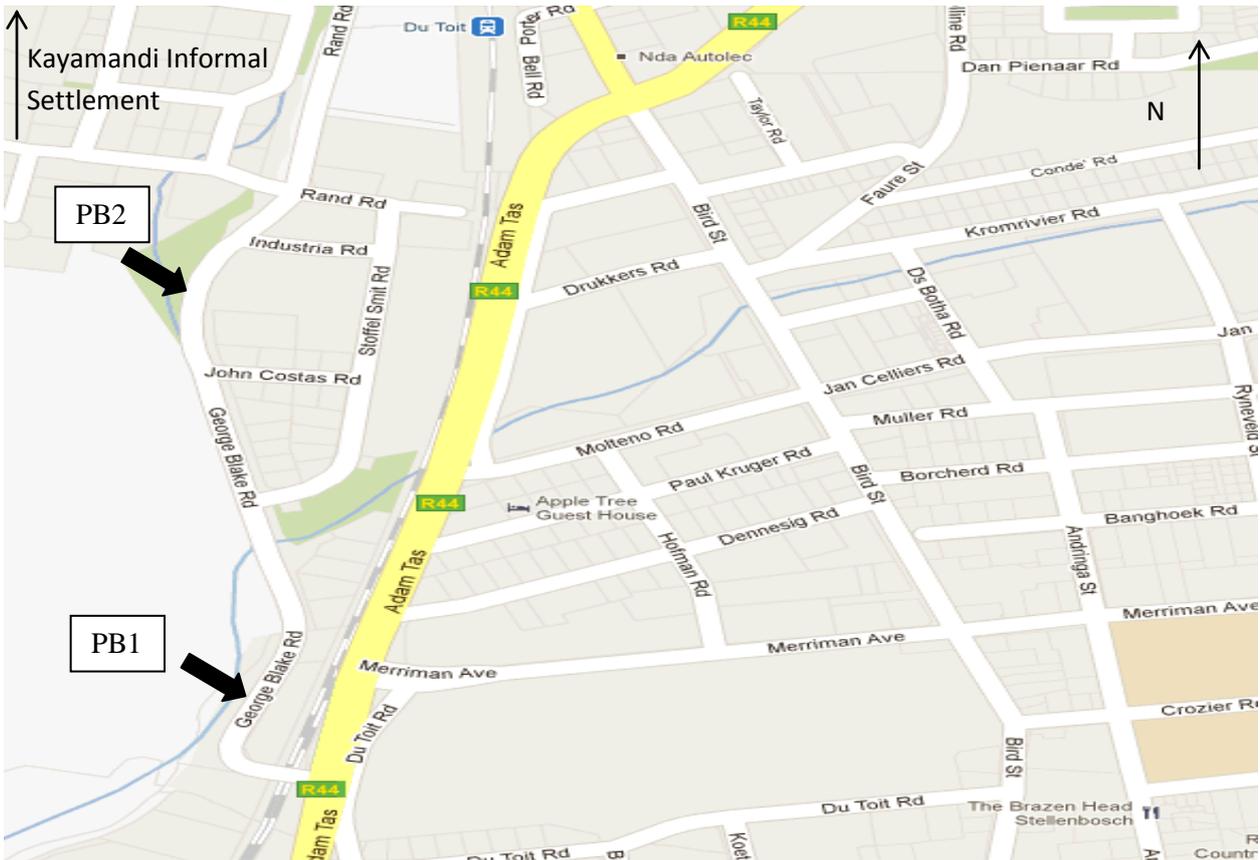


Fig. 2.1 – A street map of the Plankenburg industrial area in Stellenbosch with sites PB1 and PB2 indicated with black arrows.

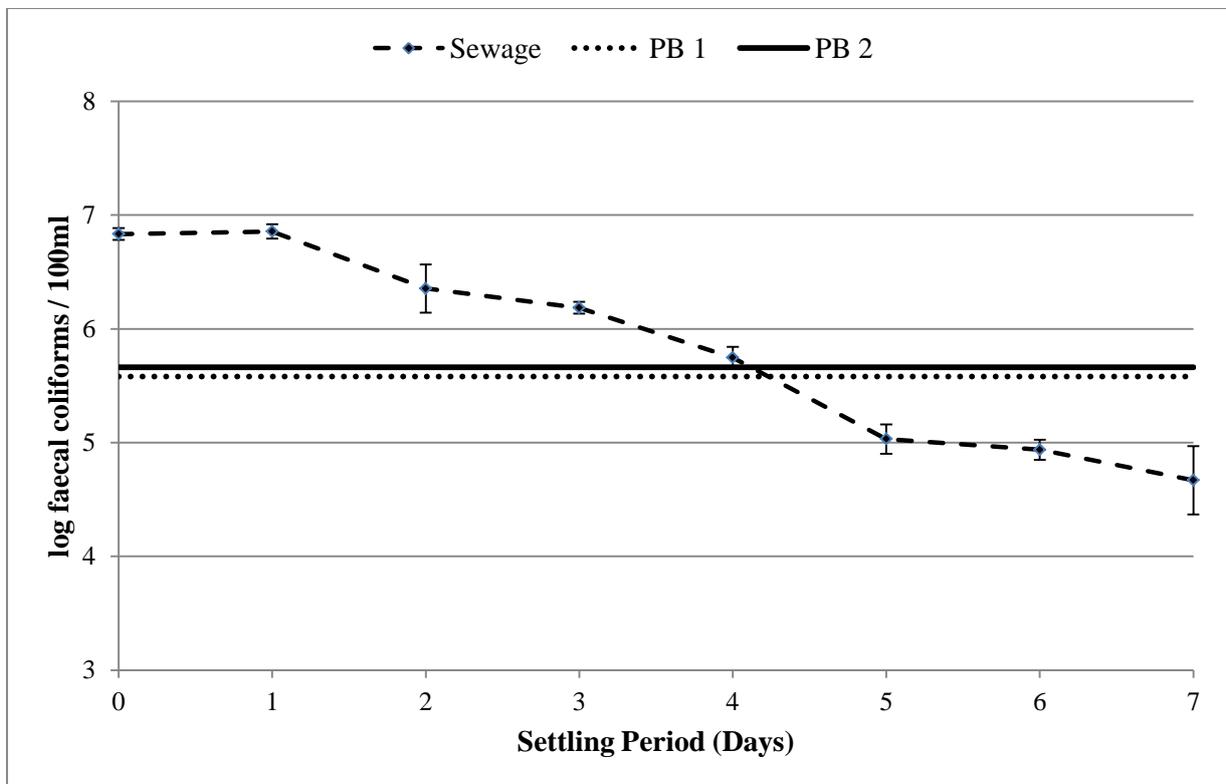


Fig. 2.2 – Decline of faecal coliform levels in sewage allowed to settle for seven days in a storage tank. Values are the means of six repetitions, while each error bar indicates the standard deviation for each value. The horizontal lines indicate the average level of faecal coliforms, calculated for twenty repetitions, in water collected at two sites in the Plankenburg River over a period of eight months.

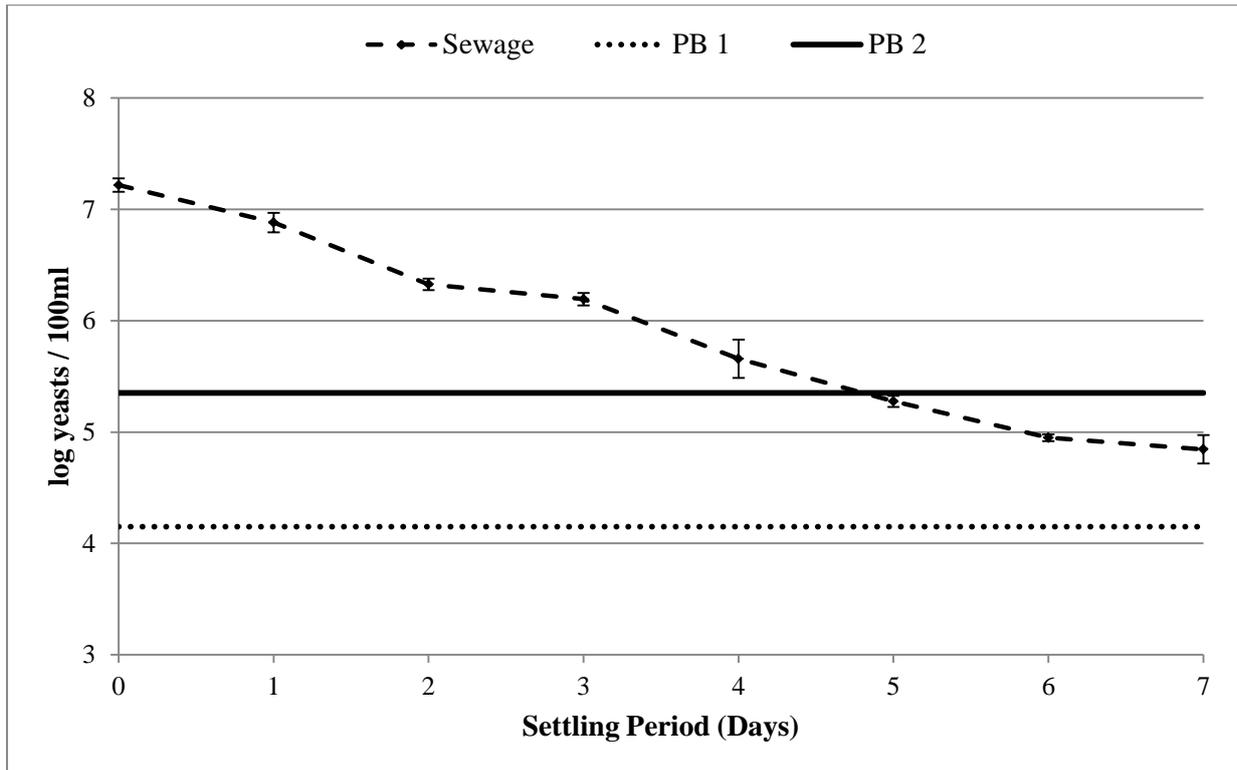


Fig. 2.3 – Decline of potentially pathogenic yeast levels in sewage allowed to settle for seven days in a storage tank. Values are the means of six repetitions, while each error bar indicates the standard deviation for each value. The horizontal lines indicate the average level of yeasts, calculated for twenty repetitions in water collected at two sites in the Plankenburg River over a period of eight months.

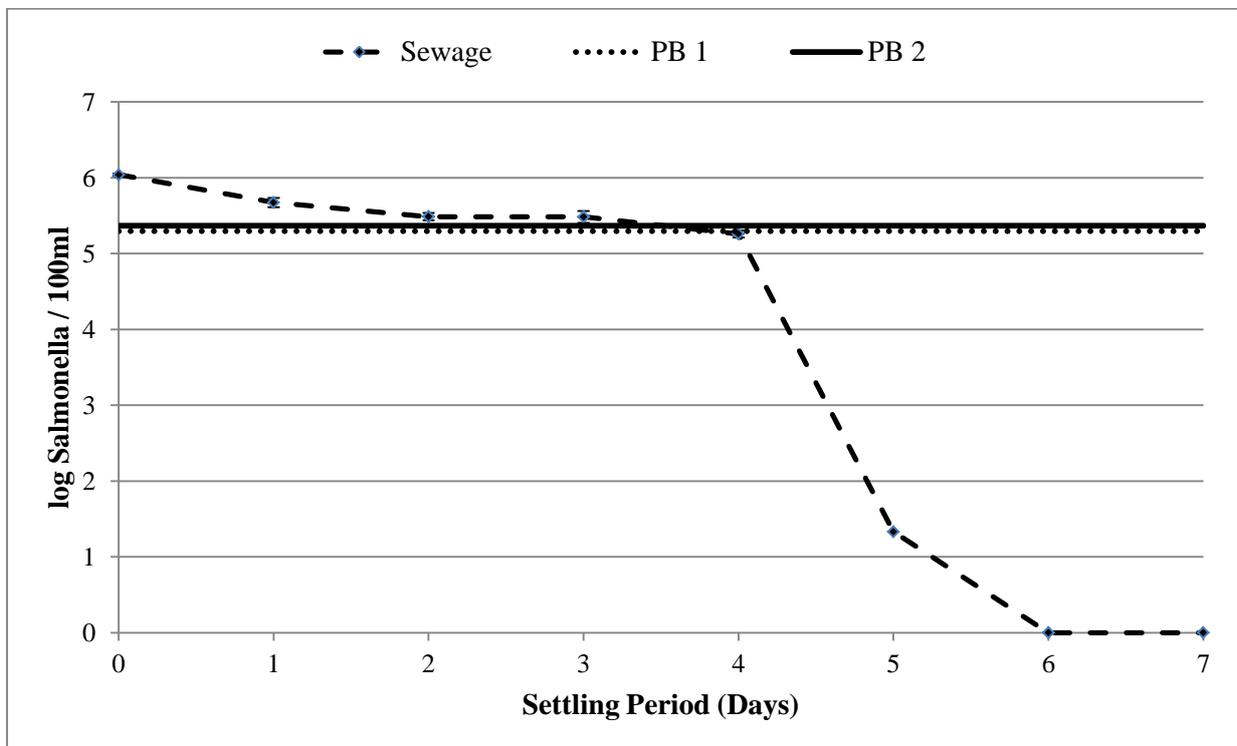


Fig. 2.4 – Decline of *Salmonella* levels in sewage allowed to settle for seven days in a storage tank. Values are the means of six repetitions, while each error bar indicates the standard deviation for each value. The horizontal lines indicate the average level of *Salmonella*, calculated for twenty repetitions of water collected in two sites in the Plankenburg River over a period of eight months.

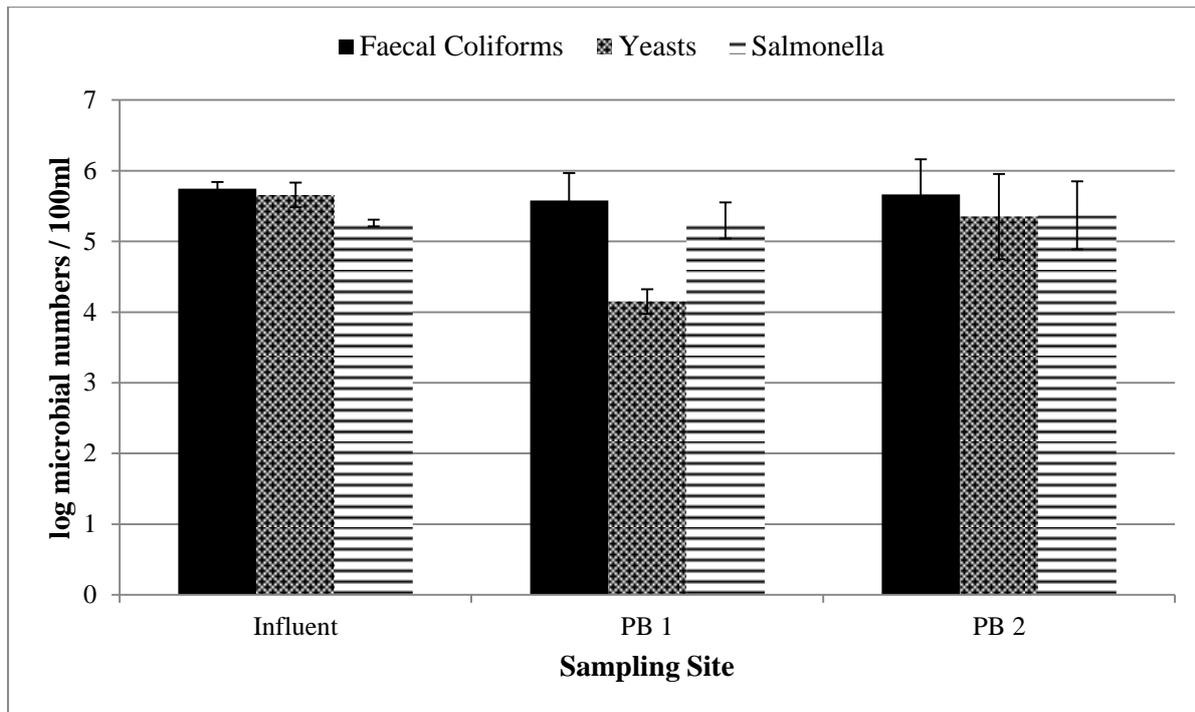


Fig. 2.5 - Comparison of faecal pollution levels as revealed by indicator organisms present in sewage settled for four days in a storage tank, and the Plankenburg River. Error bars indicate standard deviation values using six repetitions ( $n = 6$ ) for settled sewage and twenty repetitions ( $n = 20$ ) for the Plankenburg River over a seven month period.

Table 2.1 - Averages ( $n = 5$ ) of the physico-chemical compositions of water collected at selected sampling sites.

	Sewage (settled for four days)	PB1	PB2	DWAF <sup>A</sup> Water act no. 36 (1996)
<b>COD (mg/L)</b>	545 ± 186	322 ± 217	477 ± 164	75
<b>Suspended solids (mg/L)</b>	318 ± 139	17 ± 7	29 ± 3	25
<b>Chloride (mg/L)</b>	140 ± 62	76 ± 29	137 ± 24	100
<b>Ammonium (mg/L)</b>	46.24 ± 12	0.77 ± 1	10.07 ± 3	< 1.00
<b>Sulphate (mg/L)</b>	15.75 ± 10.1	10.35 ± 5.95	21.31 ± 6.96	< 200
<b>pH</b>	6.65 ± 0.25	7.41 ± 0.08	5.57 ± 2.84	5.5 – 9.5
<b>Temperature (°C)</b>	16.65 ± 1.08	16.25 ± 1.26	16.01 ± 0.82	N/A <sup>B</sup>

A = Department of Water Affairs and Forestry; B = Not applicable

## **CHAPTER 3**

**Comparison of the planted and unplanted side of a rhizofiltration system with regards to the removal of indicator organisms, pathogens and chemicals.**

Language and style used in this chapter are in accordance with the requirements of the journal *Water Research*.

### 3.1. Introduction

The availability of consumable fresh water is decreasing globally due to exponential population growth and deteriorating water treatment systems. Additionally, water sources are threatened by both point and non-point source pollution, which may have a considerable impact on the health of individuals. During the past decade a number of studies conducted on the water quality of South African rivers, have revealed an increase in pollution levels (DWA, 2000; Barnes, 2003; Matowanyika, 2010; Zuma, 2010). Urban runoff is the second most common form of non-point source pollution and impacts water quality through the addition of sediment, toxic chemicals, microbial pathogens and elevated levels of nutrients (Arnold and Gibbons, 1996; Jang et al. 2005).

The concern for public health and safety with regards to consumption of river water has resulted in an increased demand for monitoring the quality of water. According to the National Health and Medical Research Council (NHMRC) and Agricultural and Resource Management Council of Australia and New Zealand (ARMCANZ) (2001), it is important to evaluate dangerous pathogens in wastewater in order to predict the health risks associated with these microorganisms. To determine the concentration of pathogens in water, indicator organisms such as coliforms (faecal and non-faecal), bacteriophages, protists and helminths have been used to provide insights into the quality of a water source. Additionally, chemicals and nutrients are also routinely monitored during water testing with standards being set for a variety of parameters including Chemical Oxygen Demand (COD), suspended solids, ammonium and sulphate.

The removal of pathogens and chemical pollution from wastewater is usually achieved during conventional water treatment processes. However, these processes are often chemically, energetically and operationally intensive and usually focussed on large water systems. They require a combination of high capital, infrastructure and engineering expertise; all of which limit their use in developing countries (Shannon et al., 2008). There is therefore a need to develop alternative technologies that can effectively remove microbial and chemical contaminants from urban runoff before they enter various water systems.

Constructed wetlands (CW) are regarded as natural alternatives that have been used in wastewater treatments to remove biological and chemical oxygen demand (BOD and COD), enteric viruses and improve the overall quality of water (Pinney et al. 2000). These treatment systems have been applied previously to treat domestic, municipal (Kadlec and Knight, 1996; Toet et al., 2005; Iasur-Kruh et al., 2010), agricultural (Dubowy and Reeves, 1994; Diaz et al., 2010) and industrial wastewaters (Cheng et al., 2002; Yadav et al., 2010). Studies

revealed that up to 99 % of faecal coliforms were successfully removed by treating wastewater in a constructed wetland (Neralla et al., 2000; Steer et al., 2002). Interestingly, Wand and co-workers (2007) concluded that predation by protists is the dominant mechanism of bacterial removal in planted and non-planted soil filters. However, the efficiency of pathogen removal via predation varies with hydraulic residence time, hydraulic mass loading rate, wetland design, substrate type, as well as the temperature of the environment (Vymazal et al., 1995; Potter and Karathanasis, 2001). Constructed wetlands can be used for a variety of phytoremediation processes, of which the most attractive is rhizofiltration.

Rhizofiltration systems use hydroponically cultivated plant roots that absorb and sequester metal pollutants or excess nutrients from wastewater (Dushenkov et al., 1995; Arthur et al., 2005). Plant roots facilitate microbial activity by providing attachment sites for microbes, as well as producing organic carbon and oxygen in the rhizosphere. This release of oxygen increases the suitability of the system for aerobic bacterial decomposition and for supporting the growth of a wide range of aerobic aquatic organisms, some of which are capable of directly or indirectly consuming additional pollutants (Du Plessis, 2006). In addition, the released carbon promotes biofilm formation on plant roots and hosts a complex diversity of microorganisms including bacteria, fungi, protists and viruses. Although these microorganisms have a short life cycle and respond rapidly to the changes in energy sources, they are also responsible for a reduction in both pathogen numbers and harmful substances present in the wastewater (Funqua and Matthyse, 2001; Reddy et al., 2002).

With the ultimate goal of developing a low-cost and environmentally friendly alternative to the treatment of urban runoff, the objective of this study was to evaluate the removal capacity of a constructed rhizofiltration system with regards to selected indicator organisms, bacterial pathogens, potentially pathogenic yeasts and chemical pollutants associated with typical urban runoff.

## 3.2. Materials and Methods

### 3.2.1 Site description

The experimental filtration system (Figure 3.1) is located on site at Stellenbosch Sewage Works, Stellenbosch, South Africa. It consists of an above ground concrete construction approximately 9 m long, with a depth of 1 m and width of 2 m. The filter is made up of different layers of rocks and sand, ranging from coarse rocks (100 -120 mm) at the bottom to crushed rocks (19 - 25 mm) at the top, which is topped off with fine sand, on which a thin layer of the crushed rock is placed to protect the sand. The entire filter is divided lengthwise, in which one side contains sand only. The other side acts as the rhizofiltration system and was planted evenly with two different mature wetland plants, i.e. *Typha capensis* and *Phragmites australis*, four months prior to the start of the experimentation.

The influent to the filter was fed manually from an elevated 10 000 L storage tank via five sets of overhead taps (Figure 1, shows the position of the 1<sup>st</sup> and 3<sup>rd</sup> sets of taps), onto the top layers of the filter at a rate of ca. 7 L /sec. This release of influent from tank onto the filter was done to simulate pulse discharge of urban effluent. The effluent from the filter was collected in 10 wells, marked A to J in Figure 1. The hydraulic retention time of the filter system was found to be approximately 45 min.

### 3.2.2 Sampling procedure

Monthly experiments were conducted from November 2011 to August 2012 using settled sewage from the Stellenbosch sewage works, which was pumped into a 10 000 L tank constructed for use in the rhizofiltration system. This sewage was allowed to further settle for four days, after which the first 2000 L of sludge was flushed to remove solids which might result in clogging of the system. Subsequently, 2500 L of supernatant, henceforth referred to as wastewater, was fed into the system and distributed equally over both sides of the artificial wetland. Wastewater entered the system through the first and third sets of overhead taps on both sides of the system (Figure 1). This was done to effectively divide each side of the system into two equal parts. Wastewater from the first set of taps would move through the upper section of the system and flow out in Well B and Well G on the experimental and control side, respectively. The third set of taps provided water for the lower section and drained out into Well E and Well J. After the wastewater had filtered through the system for approximately 45 minutes, samples were collected from the inlet point and sampling wells B, E, G and J in sterile 250 ml Schott bottles. The temperature of both the influent and effluent

samples was recorded directly after sampling using a hand-held mercury thermometer. This sampling procedure was performed in triplicate while all collected samples were analysed in duplicate with regard to microbiological testing.

### 3.2.3 Physico-chemical analysis

Samples were analysed by the Central Analytical Facility (CAF) at Stellenbosch University to determine the physico-chemical properties of the influent and effluents of the rhizofiltration system. The properties analysed included ammonium, chloride, sulphates, chemical oxygen demand (COD), as well as suspended solids (SS).

The standard operating procedure of ion chromatography (Dionex DX-120, USA) was followed for the simultaneous determination of chloride and sulphate ions present in the water ([www.dionex.com/en-us/webdocs/4520-31183-03.pdf](http://www.dionex.com/en-us/webdocs/4520-31183-03.pdf)). Ammonium and COD concentrations were calculated according to the manufacturer's instructions of Merck Test Kits with a measuring range of 0.010 – 0.3 mg/L NH<sub>4</sub>-N; 0.013 – 3.86 mg/L NH<sub>4</sub><sup>+</sup> or 2.0 – 150 mg/L NH<sub>4</sub>-N; 2.6 – 193 mg/L NH<sub>4</sub><sup>+</sup> and 100 – 1500 mg/L or 500 – 1000 mg/L, respectively. The method of Wycoff (1964) was used to determine the total amount of solids per litre of sample.

### 3.2.4 Microbiological analysis

Faecal coliform concentrations were determined by preparing a triplicate dilution series of each sample followed by plating 100 µl of each dilution on MacConkey Agar (Atlas, 1993). The plates were subsequently incubated at 44°C for 24 hours after which coliforms were enumerated by identifying colonies with a metallic red hue.

*Salmonella* concentrations were determined by plating 100 µl of each sample in triplicate on *Salmonella-Shigella* agar and incubating the plates at 37°C for 24 hours (Hassanein et al., 2011). *Salmonella* colonies were enumerated by identifying colonies which were colourless but contained a black centre.

Coliphage concentrations were determined using the direct plaque assay method described by Baker et al. (2003). In brief, water samples were collected in 1 L sterile glass bottles and kept at 4°C for no longer than a week. Three dilutions (undiluted, 10<sup>-1</sup>, 10<sup>-2</sup>) of each sample were performed in triplicate. The positive control, T4 coliphage stock inoculated in sterile water, was also serially diluted. The negative control consisted only of sterile water. Of each serially diluted sample, 1.5 ml was transferred to a 2 ml micro-centrifuge tube to which three drops of chloroform was added. Tubes were centrifuged for 5 minutes at 11 752 g where after 1 ml of

the supernatant was transferred to a new tube. A 0.1 ml suspension of *Escherichia coli*, grown to stationary phase in Tryptic Soy Broth, was then added to each tube. The tubes were vortexed and incubated at 25°C for 5 min. Each mixture was then transferred to 5 ml melted top agar [0.7% (w/v); Luria-Bertani (LB)]. The tubes were gently mixed and poured onto a plate containing LB agar. Plates were incubated at 37°C for 18 hours. After 18 hours, the number of plaque forming units (PFU) was determined.

Potential pathogenic yeasts and antibiotic resistant bacteria were detected by filtering 100 ml aliquots of the samples collected from the influent and effluent through 0.45 µm pore cellulose nitrate filter disks (Sartorius Stedim Biolab Products Augbane, France) in separate sterile polycarbonate filter systems. The filter disks were then transferred directly onto Sabouraud Glucose Agar (SGA) supplemented with chloramphenicol (30µg/ml), tetracycline (10µg/ml) and kanamycin (50µg/ml), and incubated at 37°C for 48 hours until colonies were clearly visible. After incubation, colony forming units were counted.

### **3.2.5 Identification of *Candida* and bacterial species**

The identity of *Candida* and bacterial species were tentatively confirmed by spotting random isolates from the filter disks on Molybdate Agar (Atlas, 1993) followed by incubation at 37°C for four days. The colour, texture, margin and size of the resulting colonies was compared to a standardised table for tentative identification (Table 3.1; Addendum A, Figure A1).

The identity of the yeasts was confirmed by polymerase chain reaction (PCR) amplifying the D1/D2 region of the 26S ribosomal RNA gene, using the KAPATaq Ready Mix containing DNA polymerase (KAPA Biosystems, Cape Town, RSA). A modification of the standard colony PCR protocol was followed, where one reaction consisted of 10 µL Ready Mix, 1 µL of the forward primer, F63 (5'-GCATATACAATAAGCGGAGGAAAAG-3') and 1 µL of the reverse primer, LR3 (5'-GGTCCGTGTTTCAAGACGG-3') (Inqaba Biotechnical Industries, Pretoria, RSA), 3 µL of a single colony suspended in ddH<sub>2</sub>O and 10 µL ddH<sub>2</sub>O. The PCR was performed in an Applied Biosystems 2720 Thermal Cycler (California, USA) with an initial denaturation at 95 °C for seven minutes was followed by 30 cycles, each with a denaturation at 95 °C for 30 seconds, annealing at 57 °C for 30 seconds followed by an elongation step at 72°C for one minute (Fell et al., 2000). The final elongation step was carried out at 72 °C for two minutes and PCR products were visualised under UV light (GeneFlash Syngene Bioimaging Unit, Cambridge, UK) after electrophoresis in a 0.8 % (w/v) agarose gel (Horizon 11.4 GIBCO DRL Horizontal Gel Electrophoresis Apparatus, Life Technologies, California, USA).

The identity of the bacterial species was confirmed by polymerase chain reaction (PCR) amplifying the 16S ribosomal RNA gene, using the KAPATaq Ready Mix containing DNA polymerase (KAPA Biosystems, Cape Town, RSA). A modification of the standard colony PCR protocol was followed, where one reaction consisted of 12.5 µL Ready Mix, 1 µL of the forward primer (5-GAGTTTGATYMTGGCTCAG-3') and 1 µL of the reverse primer (5-GAAGGAGGTGWTCCADCC -3') (Inqaba Biotechnical Industries, Pretoria, RSA), 1.5 µL of a single colony suspended in ddH<sub>2</sub>O and 9 µL ddH<sub>2</sub>O. The PCR was performed in an Applied Biosystems 2720 Thermal Cycler (California, USA) with an initial denaturation at 94°C for four minutes was followed by 35 cycles, each with a denaturation at 95°C for 30 seconds, annealing at 50°C for 30 seconds followed by an elongation step at 72°C for 45 seconds. The final elongation step was carried out at 72 °C for five minutes and PCR products were visualised as described above.

Bacterial and yeast DNA were then sequenced with an ABI 37 PRISM (model 3100) genetic sequencer (Applied Biosystems, California, USA) while the online Basic Local Alignment Search Tool (BLAST) was used to detect homology with known species on the NCBI (National Centre for Biotechnology Information) database (<http://www.ncbi.nlm.nih.gov/BLAST/>).

### **3.2.6 Statistical analysis**

Microbial numbers were compared between the experimental and control side of the rhizofiltration system using one-way ANOVA to determine differences between the two sides on a monthly basis. The average microbial removal efficiencies between the separate sampling wells were compared using Box and Whisker plots while correlation matrixes were constructed to compare microbial numbers with temperature in the effluent samples. All statistical analyses were performed using Statistica Software version 10 (StatSoft, Tulsa, OK, USA) with all tests of variance, significance and correlations considered statistically significant at  $p$  values < 0.05.

## **3.3. Results and Discussion**

### **3.3.1 Physico-chemical analysis**

Suspended solids (SS) were removed efficiently on both sides of the system with similar removal rates found in the sand filters above Well B (experimental) and Well G (control) and Well E (experimental) and Well J (control) (Figure 3.2). Removal of suspended solids

reached a maximum in February with an approximate reduction of 90% on both the planted and unplanted side of the filter. The lowest removal rate was in June where both sides achieved a reduction of about 37% in suspended solids. Suspended solids removal in constructed wetlands is usually in excess of 80% but may decrease if the system operates with a short hydraulic retention time (Dong et al., 2011; Kurzbaum et al., 2012). Tanner and co-workers (1995) revealed that a hydraulic retention time of between two and seven days resulted in an 85% reduction in SS, while a similar study showed a 97% reduction in SS if the hydraulic retention time was ten days (Frossard et al., 2006). Interception and settling are the two major mechanisms involved in solids removal, but the presence of plants may create a better settling environment due to the release of plant litter (Kadlec and Wallace, 2009). The hydraulic retention time of our system (45 minutes) may have been too short to ensure effective settling of the solids, which may account for lower removal efficiencies when compared to other studies.

The increase in suspended solid removal in February, March and August compared to other months may be due to a higher concentration of suspended solids in the influent (Addendum B). Several studies have found a positive correlation between inlet concentration and suspended solids removal, where the removal of solids increases with a greater inlet concentration (Tanner et al., 1995; Dunne et al., 2012). A SS concentration of 349 mg/L and 365 mg/L in February and March, respectively, may thus have resulted in greater SS removal than in June where the influent only had a SS concentration of 102 mg/L.

The COD of the effluent was lower in all of the wells sampled over the entire sampling period, when compared to the influent. The greatest COD removal was found in November, ranging from 65% to 75%, after which removal decreased and stabilised between 30% and 45% from December to June (Figure 3.3). Interestingly, COD removal was generally similar on both sides of the system when they were compared with each other. This may indicate that the sand itself, and not the reeds, are responsible for the majority of removal. The planted sides of constructed wetlands are generally excellent in terms of COD removal with efficiencies in excess of 85% (Cerezo et al., 2001). In a pilot study conducted in South Africa, a treatment bed planted with *Pennisetum clandestinum* achieved a very high COD removal efficiency of 95% (Wood, 1989). A study by Healy and Flynn (2011) found that the average removal of COD from primary and secondary wastewater were 88 and 72%, respectively, with an average final effluent concentration of  $53 \pm 18$  and  $45 \pm 27$  mg/L respectively. The lower removal of COD found in this study may be attributed to the fact that a rhizofiltration system was used which had a shorter retention time than that of constructed wetlands.

Ammonium ( $\text{NH}_4$ ) levels dropped considerably on both the experimental and control sides of the system for the duration of the sampling period (Figure 3.4). However, both sides showed similar levels of ammonium removal, with an average removal of approximately 65% in samples from Well B and Well G, while the sand filters above Well E and Well J were able to remove about 55% of the ammonium in the influent. The removal of ammonium occurs as a result of nitrification, which is promoted due to the aerobic nature of the system. Once the water has percolated through the substrate, air can re-enter the system and provide the necessary oxygen for nitrifying bacteria to convert ammonia to nitrite and nitrate (Stottmeister et al., 2003).

Chloride levels decreased minimally on both sides of the system (Addendum B). This is in agreement with literature where chloride was considered conservative in wetland environments and interacted minimally with the ecosystem (Kadlec, 2003; Kadlec and Wallace, 2009). Sulphate concentrations increased evenly on both the planted and unplanted sides of the system for the majority of the sampling period (Addendum B). This probably resulted from oxidation of hydrogen sulphide to sulphate, which might have occurred due to increased oxygen content in the substrate following the movement of water through it. Wu et al. (2011) found that there was a simultaneous reduction and re-oxidation of sulphur compounds in constructed wetlands and that the concentration of the different sulphur states was dependent on the amount of oxygen present in the sand or soil. Less oxygen would result in sulphate reduction and the formation of sulphide which usually occurs in the anaerobic zones of the system.

A small decrease in phosphorous concentrations was found in some effluent samples when compared with the influent but the removal of phosphate did not differ between the experimental and control sides of the system (Addendum B). Removal of phosphorus mainly occurs through plant uptake, adsorption by the porous media and precipitation. During these processes, phosphorus reacts with the porous media and with minerals, such as ferric oxyhydroxide and carbonate (Kadlec and Knight, 1996). Brooks et al., (2000) conducted a study on phosphorus removal by wallostonite as an alternative constructed wetland substrate. They found that phosphorus removal was greater than 80% when the system had a hydraulic retention time greater than 1.7 days. Gray et al., (2000) reported a 90% phosphorus removal for planted units, while Drizo et al., (2000) reported 98% and 95% for planted and unplanted units, respectively. The retention times of both these systems was five days thus the reason for the relatively low removal rate of our rhizofiltration system may be ascribed to its short retention time compared to the other systems mentioned above.

### 3.3.2 Microbiological analysis

Faecal coliform reduction in the effluent samples was found to be significantly different between the experimental and planted side in December, and from April to August ( $p = 0.000$ ; Figure 3.5). However, no difference was found between the two sides of the system in November, February and March. The greatest level of reduction on the experimental side was seen in July when 62% of faecal coliforms were removed from the effluent as it passed through the rhizofilter. Interestingly, faecal coliform removal increased steadily from March to July on the planted side and also during the winter months (April to June) on the control side. This may be attributed to both the continued development of the reeds in the system or the temperature, which declined continuously over the same time period (Figure 3.6).

Additionally, statistical analysis revealed that faecal coliform removal and temperature were significantly negatively correlated ( $r = -0.2938$ ;  $p = 0.0020$ ), strongly indicating to greater removal rates at lower temperatures (Figure 3.7). Figure 6 also shows that there was a tendency for the experimental side to have a lower temperature when compared to the control side. However, the only significant difference between the two sides was seen in August ( $p = 0.00002$ ). The lower temperatures on the experimental side may have contributed to the greater removal efficiencies of this side when compared to the control side. Studies conducted in aquatic systems by both Schnabel et al. (2010) and Hennani et al. (2011) revealed that faecal coliform numbers increased during the warmer summer months when compared with lower concentrations in the cooler winter months.

Over the entire sampling period, significant differences were found between the planted and unplanted side with regard to faecal coliform removal (Figure 3.8). Samples from Well B contained 48% less faecal coliforms than the influent, while the sand filter above Well G only managed 24% removal efficiency, which resulted in values that were statistically different ( $p = 0.0000$ ) from each other. Faecal coliform reduction in Well E was significantly different ( $p = 0.0000$ ) from Well J with a removal efficiency of 48% and 22% for the two wells respectively, when compared with samples from the influent. Faecal bacterial removal efficiency in constructed wetlands is generally excellent, usually exceeding 95%, but varies with hydraulic retention time, wetland design, hydraulic and mass loading rate, substrate and temperature (Gersberg et al., 1987; Haberl et al., 1995; Potter and Karathanasis, 2001). Neralla et al., (2000) and Steer et al., (2002) also found that constructed wetlands were highly effective in removing pathogens and reported a 99% removal of faecal coliforms by these systems. The lower removal efficiency of our rhizofiltration system may be attributed to the young age of the system with more significant reductions expected in the future as the system

matures and stabilises, or to the shorter hydraulic retention time. The root exudates of certain aquatic macrophytes including *Phragmites australis*, which was used in our system, may also reduce faecal indicators and pathogenic bacteria (Cooper et al., 1996).

Additionally, the enhanced development of populations of bacteria with antibiotic activity (e.g. *Pseudomonas*) in the rhizosphere, may also account for coliform die-off (Mandernack et al., 2000). Decamp and co-workers (1999) stated that the improved aeration of the root zone of planted filters, when compared to non-planted ones, provided an explanation for higher removal efficiency in the former. Predation is probably the dominant mechanism of bacterial removal in constructed wetlands (Wand et al., 2007) and may be attributed to an increased protozoa concentration. Faecal coliform removal can also be enhanced by increasing the hydraulic retention time of the system or by decreasing the hydraulic loading rate (Kadlec and Wallace, 2009). The short residence time of our system may have contributed to lower removal rates as the water was not in contact with predators and/or competitors for an extended period of time.

There was no significant difference in the removal of coliphage between the planted and unplanted side of the rhizofiltration system between November and July, but the unplanted side did have a higher removal efficiency in August (Figure 3.9). Interestingly, the removal of coliphage decreased for both sides over the sampling period, with the highest removal in November where the experimental and control side removed 63% and 69% of coliphage respectively. In contrast, samples collected in June showed a substantial increase in coliphage concentrations on both sides of the system.

A significant positive correlation was found between temperature and coliphage removal ( $r = 0.6177$ ;  $p = 0.0000$ ) indicating that coliphage removal is reduced at lower temperatures (Figure 3.10). This may explain the drop in coliphage removal during autumn and winter when temperatures (Figure 2) also dropped continuously. The low removal rates of coliphage may be attributed to the ability of viruses to be hardier in natural environments than bacterial pathogens (Kadlec and Wallace, 2009). Due to their small size, viruses are not usually affected by physical removal treatments such as sedimentation and conventional filtration (Symonds et al., 2009; Thompson et al., 2003). Even when associated with particles, viruses can remain in suspension and pass through granular media filters (Rao et al., 1984; Templeton et al., 2005). Similarly, Lodder et al. (2010) showed that virus numbers were higher in water sources during the colder months of the year when compared to the warmer months, while other studies have shown that norovirus concentrations increase during the winter months in both river water (Kishida et al., 2012) and oysters (Lowther et al., 2012).

To determine the ability of the rhizofiltration system to lower pathogen concentrations, *Salmonella* removal was tested for from February to August (Figure 3.11). *Salmonella* numbers increased significantly in February ( $p = 0.0000$ ) and March ( $p = 0.0000$ ) when the effluent samples were compared to the influent. However, no significant difference was found between the planted and unplanted sides during these months. The removal of *Salmonella* improved on both sides of the system over the next five months with significant differences existing between the experimental side and control side in June, July and August (All  $p = 0.0000$ ). The planted side had a maximum removal of 59% in July while the unplanted side had its maximum removal (33%) in the same month. A study conducted by Sun et al. (2010) showed that *Salmonella* concentrations in the effluent were significantly lower than the influent in a constructed wetland, while a similar study by Hill and Sobsey (2001) reported a 96% reduction in *Salmonella* numbers after passing swine wastewater through a constructed wetland.

*Salmonella* removal was also found to be negatively correlated (Figure 3.12) to temperature during the sampling period ( $r = -0.8202$ ;  $p = 0.0000$ ) indicating that there is greater *Salmonella* reduction at low temperatures as was seen during the winter months. This is opposite to what is reported in literature where high temperatures usually result in a greater reduction of *Salmonella* when compared with lower temperatures. The performance of many microbes is dependent on the ecosystem in which they exist, thus it is not uncommon for many microbes to perform differently under varying environmental conditions.

The majority of studies involving yeasts in constructed wetlands focused on their identification and distribution within a system (Kacprzak et al., 2005; Bensultana et al., 2010), while the survival of yeasts in these systems has received little attention. During our study it was found that removal of potentially pathogenic yeasts was significantly different in all the effluent samples, when compared with the influent ( $p = 0.0000$ ). The planted side removed a greater number of yeasts in December and from April to August when compared to the unplanted side ( $p = 0.0000$ ) (Figure 3.13). The experimental side achieved 100% removal of yeasts from April to July while the control side had its highest removal efficiency in February (93%).

Over the entire sampling period, significant differences were found between the experimental and the control side regarding yeast removal (Figure 3.14). The sand filter above Well B had a removal efficiency of 93%, while the filter above Well G was only capable of removing

75% of potentially pathogenic yeasts. Similarly, the filter above Well E was better at removing yeasts (92%) when compared with Well J (73%). The differences in removal efficiencies between coliforms and yeasts may be due to differences in morphology and size between the two organisms. Yeasts are much larger than bacteria and thus may be retained more effectively by the filter and plants, where predators and microbes can remove them. It is also possible that there was a higher concentration of predatory protists in the planted filter, thereby resulting in the greater removal of yeasts on this side. Protozoan numbers are much greater in the presence of plants due to the improved aeration of the root zone in planted gravel beds, which provide better conditions for protozoa development (Wand et al., 2007).

Thus, potentially pathogenic yeasts may have been removed from the water by protists and other predatory microbial population associated with the roots of the plants (Mandernack et al., 2000; Wand et al., 2007). Also, biofilms that form on and around the roots may have increased the surface area for the removal of yeasts. Dense microbial populations may be more effective in trapping the larger yeasts as they filter through the sand substrate. Studies conducted to determine microbial populations within wetland systems revealed the presence of a wide variety of microbes. Ahn and co-workers (2007) showed that  $\alpha$ -proteobacteria, *Actinobacteria* and *Firmicutes* were dominant in wetland microcosms while another study indicated that *Bdellovibrio* species may play a role in pathogen removal in this ecosystem (Iasur-Kruh et al., 2010).

### 3.3.3 Identification of *Candida* and bacterial species

The analysis of microbial populations in the influent and effluent samples revealed dominant yeast species (Table 3.2). The majority of these yeasts belonged to the genus *Candida* including *Candida tropicalis* (99% homology), *Candida glabrata* (99% homology), *Candida krusei* (99% homology) and *Candida utilis* (99% homology). A study conducted by Biedunkiewicz and Ozimek (2009) also isolated these *Candida* species in the influent of a wastewater treatment plant. *Candida albicans* was also isolated during their research, but was never found during this study. The fifth major yeast species that was found was *Saccharomyces cerevisiae* (99% homology), which was most prevalent in February, a time during which wine farms harvest their crops. The presence of *S. cerevisiae* during this time period may indicate runoff or pollution from wine farms or industries in the nearby area. The numbers of the different *Candida* species varied during the sampling period with *C. tropicalis* only present in the influent samples in November, March and August. In contrast, *C. krusei* was present for the majority of the sampling period, while *C. utilis* and *C. glabrata* were found in influent samples for the duration of the study.

The effluent samples were also dominated by these five yeast species, but their presence was dependant on whether the water had filtered through the control or experimental side of the rhizofiltration system (Table 3.2). Effluent samples collected from November to February showed that both the planted and unplanted side contained *Candida* and *Saccharomyces* species, the majority of which were *C. glabrata*, *C. utilis* and *S. cerevisiae*. This indicated that the majority of yeasts in the influent were also present in the effluent if they were able to survive in the rhizofiltration system. Figure 3.13 revealed that there was a 100% reduction in potentially pathogenic yeast species on the experimental side from April to July and this is reflected in Table 3.2 where no yeast species could be identified over the same period. In contrast, potentially pathogenic yeasts were still identified in effluent samples from the control side indicating that the experimental side was more effective in the removal of these microbes. *Candida tropicalis*, *C. krusei* and *C. glabrata* are known commensals of human mucosal membranes but have also been isolated in cases involving both oral and vaginal candidiasis in immunocompromised individuals (Odds, 1988; Singh et al., 2002; Corsello et al., 2003; Okungbowa, 2003; Buscemi et al., 2004; Nyirjesy et al., 2005). Additionally, blood stream infections by these *Candida* species have also been reported and are a significant cause of morbidity and mortality in hospitalised patients (Giri and Kindo, 2012). The ability of the planted side to remove more of the isolated yeast species than the unplanted side may have a significant effect on the improvement of human health as these microbes will not be present to infect consumers of polluted water.

A number of antibiotic resistant bacteria were also isolated from samples collected at the influent and effluent of the constructed rhizofiltration system (Table 3.3). The majority of the bacteria formed part of the *Burkholderia cepacia* complex (Bcc), while *Citrobacter freundii* (98% homology) and *Escherichia coli* O157:H7 (97% homology) were also isolated in high concentrations. Samples collected from the influent between November and April had high numbers of *B. cepacia* and a *Burkholderia* species that could not be identified. During the later periods, *C. freundii* and *E. coli* O157:H7 became more dominant in the influent samples, but *Burkholderia* species were still present in many of the collected samples. Similarly, effluent samples on both the planted and unplanted side of the rhizofiltration system contained all of the bacteria mentioned above with *Burkholderia* species dominating in the earlier months and *C. freundii* becoming more prevalent as sampling continued. Members of *Burkholderia* occur naturally in soil and water, thus their presence in both the influent and effluent samples is to be expected (Balandreau et al., 2001; Fiore et al., 2001; Pallud et al., 2001, Parke and Gurian-Sherman, 2001; Miller et al., 2002; Coenye and Vandamme, 2003; Vermis et al., 2003;). They are more abundant in soil containing plant roots, especially if grasses, maize, rice and wheat are present (Parke and Gurian-Sherman, 2001; Vandamme et al., 2003; Ramette et al., 2005). Additionally, they serve an important role in the rhizosphere as antagonists of plant pests including fungi and yeasts (Lim et al., 1994). Studies have shown that these bacteria are capable of inhibiting the growth of *C. albicans*, *C. krusei*, *C. tropicalis* and *C. glabrata* and may thus play a significant part in the reduction of yeast species on both sides of the system (Kerr, 1994; 1999). However, bacteria that form part of this group have also been linked to the infection of cystic fibrosis patients and immunocompromised individuals (Speert, 2001; Chiarini et al., 2006).

### 3.4 Conclusions

From the results it is obvious that rhizofiltration does show potential to be used for the removal of pathogens from urban effluent. The short hydraulic retention time of the experimental filter at Stellenbosch Sewage Works, however, seemed to render the system inadequate to remove all the unwanted nutrients and pathogens from this effluent. The system should therefore be evaluated under different operating conditions in future.

The planted and unplanted side of the experimental rhizofiltration system showed similar removal rates with regard to suspended solids, ammonium, COD, phosphates and sulphates. This indicated that the substrate itself played a greater role in removing solids and nutrients than the rhizofilter. Microbiologically, the planted side was more effective than the unplanted side in terms of faecal coliform, yeast and *Salmonella* removal, but no difference was found between the two sides with regard to coliphage removal. Temperature and other environmental factors may play a significant role in the removal of microbes within this ecosystem and it is recommended that the system should be monitored for more than one year to accurately determine the effect of seasonal, and temperature, changes.

The majority of yeasts that were isolated belonged to the genus *Candida*, including *C. tropicalis*, *C. glabrata*, *C. krusei* and *C. utilis*. The experimental side was more effective than the control side in removing these opportunistic pathogens from wastewater. A number of antibiotic resistant bacteria were also isolated, the majority of which formed part of the *Burkholderia cepacia* complex which is found naturally in water and soil environments. The presence of *Burkholderia* species within the rhizosphere may be beneficial within a rhizofiltration environment due to their ability to remove potentially pathogenic yeasts and fungi. However, these opportunistic pathogens may also pose a risk to human health, thus further studies are needed to elucidate the specific role that these bacteria play within this environment.

Further studies are also needed to determine if microbial and chemical removal efficiencies increase on the planted side of the system after the reeds are allowed to grow for a longer period of time. Additionally, the effect of alternative plant species on pathogen and nutrient removal can be evaluated, while the mechanisms of pathogen removal within the rhizosphere also need to be elucidated.

### 3.5 References

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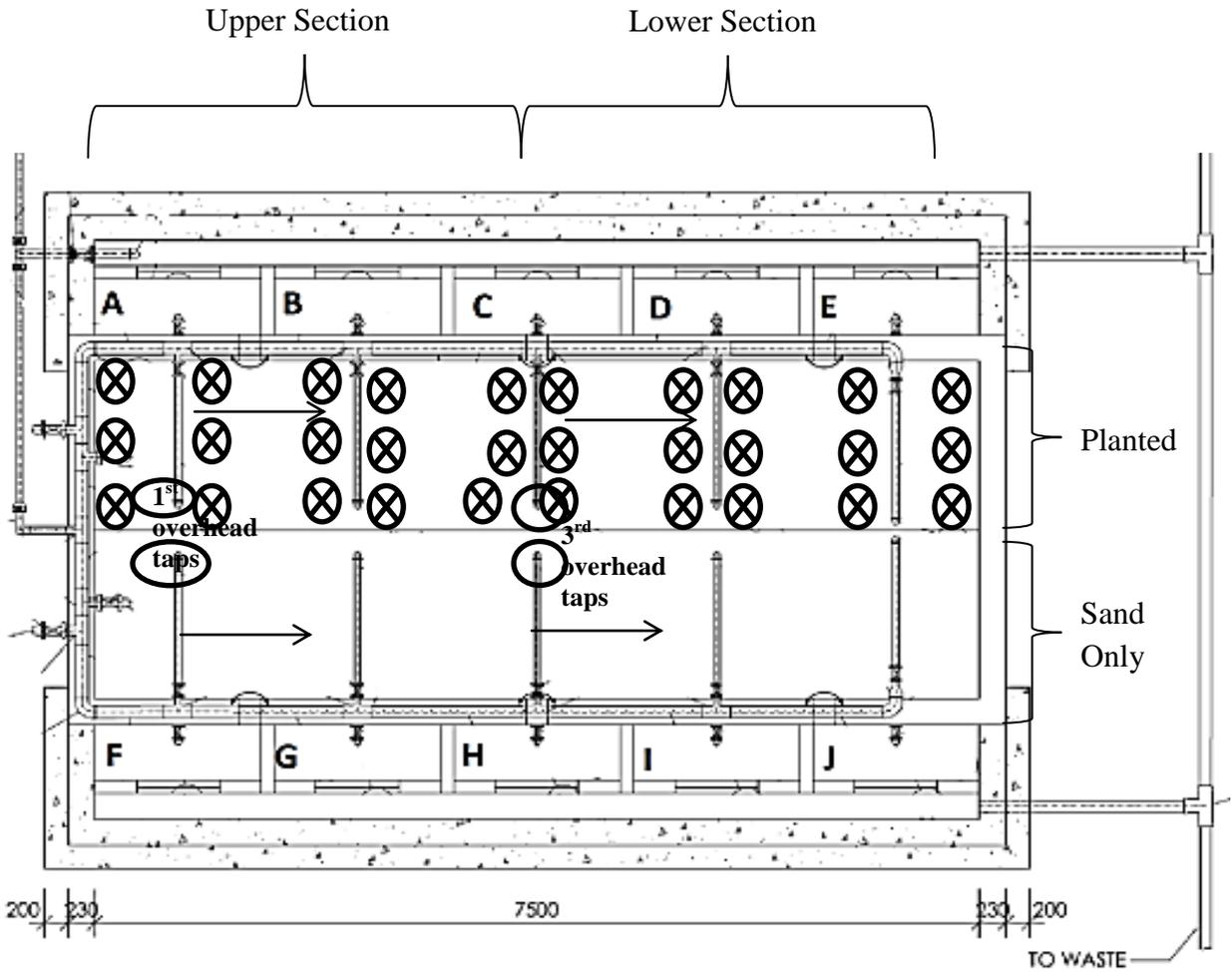
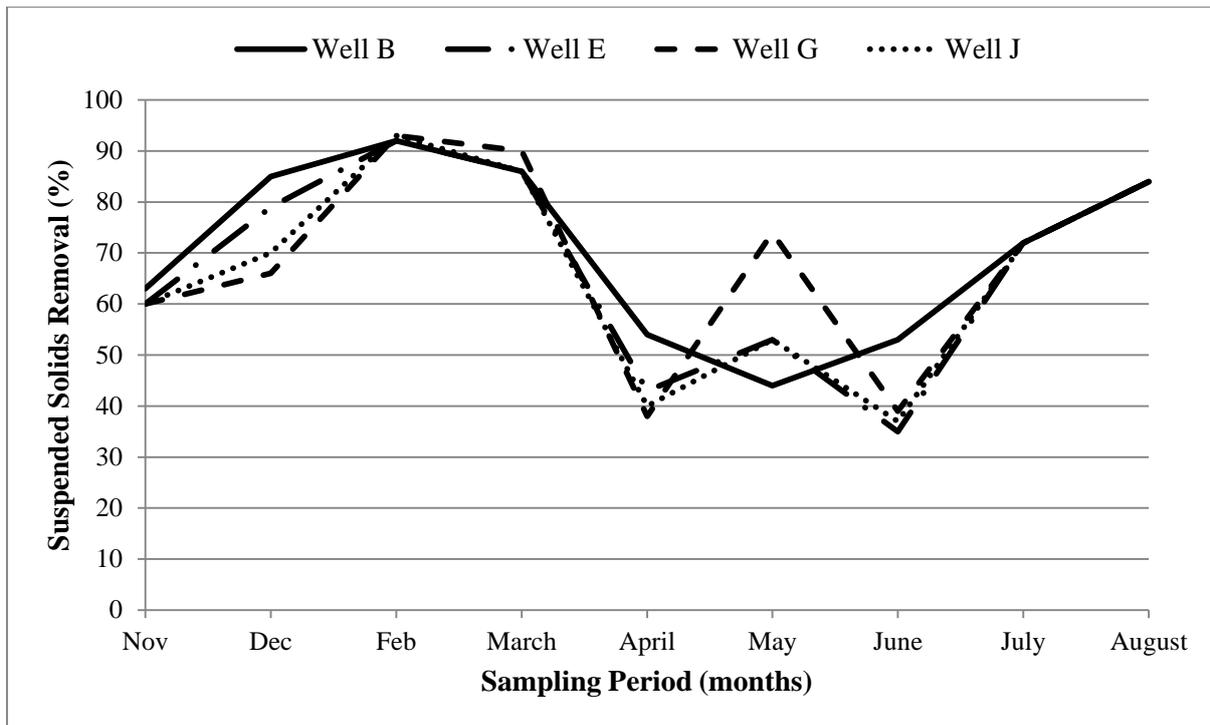
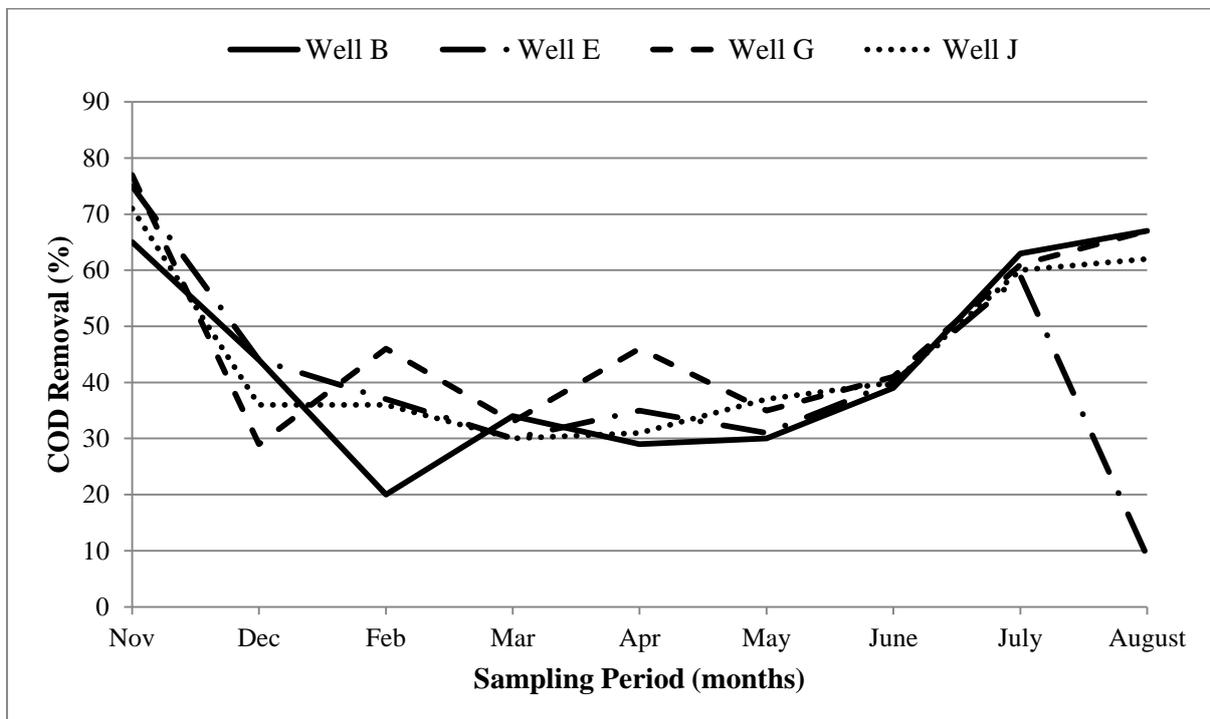


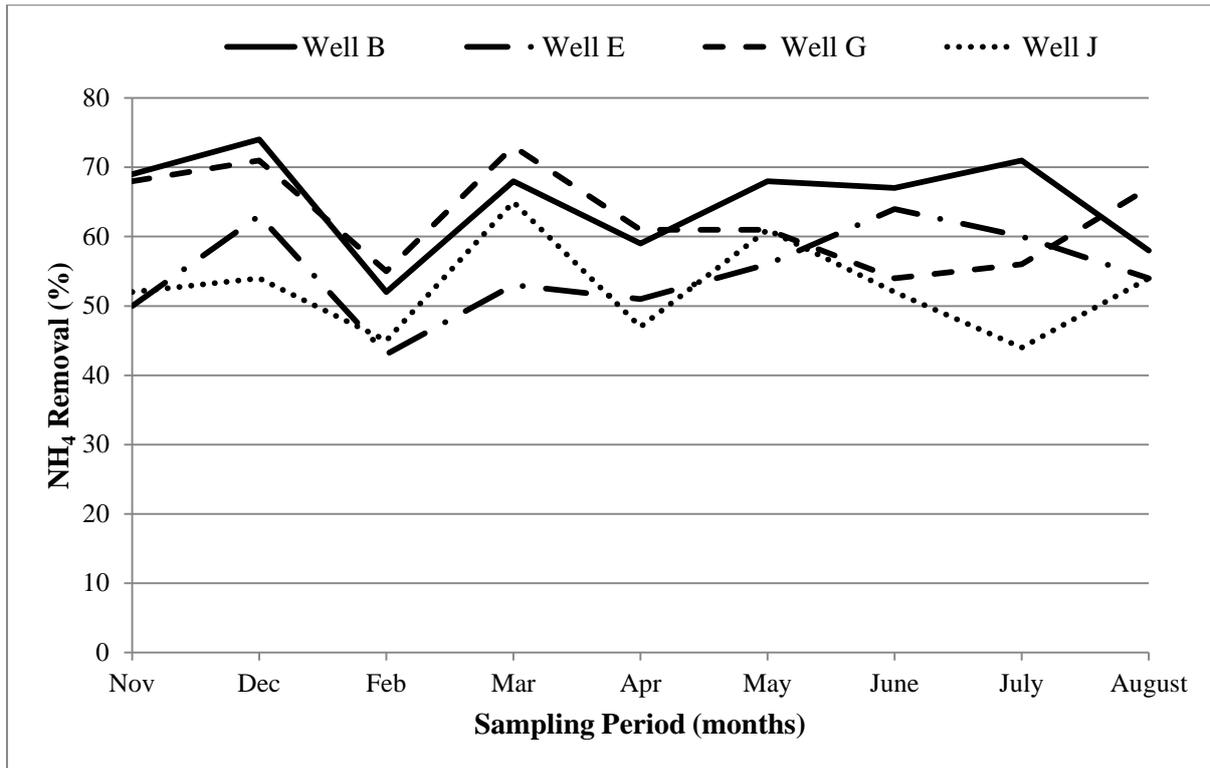
Fig. 3.1 – Plan of the experimental rhizofiltration system with sampling wells denoted by letters A-J. Rings represent the water influent points from the first and third overhead taps while crossed circles illustrate the position of the original reeds planted four months prior to testing.



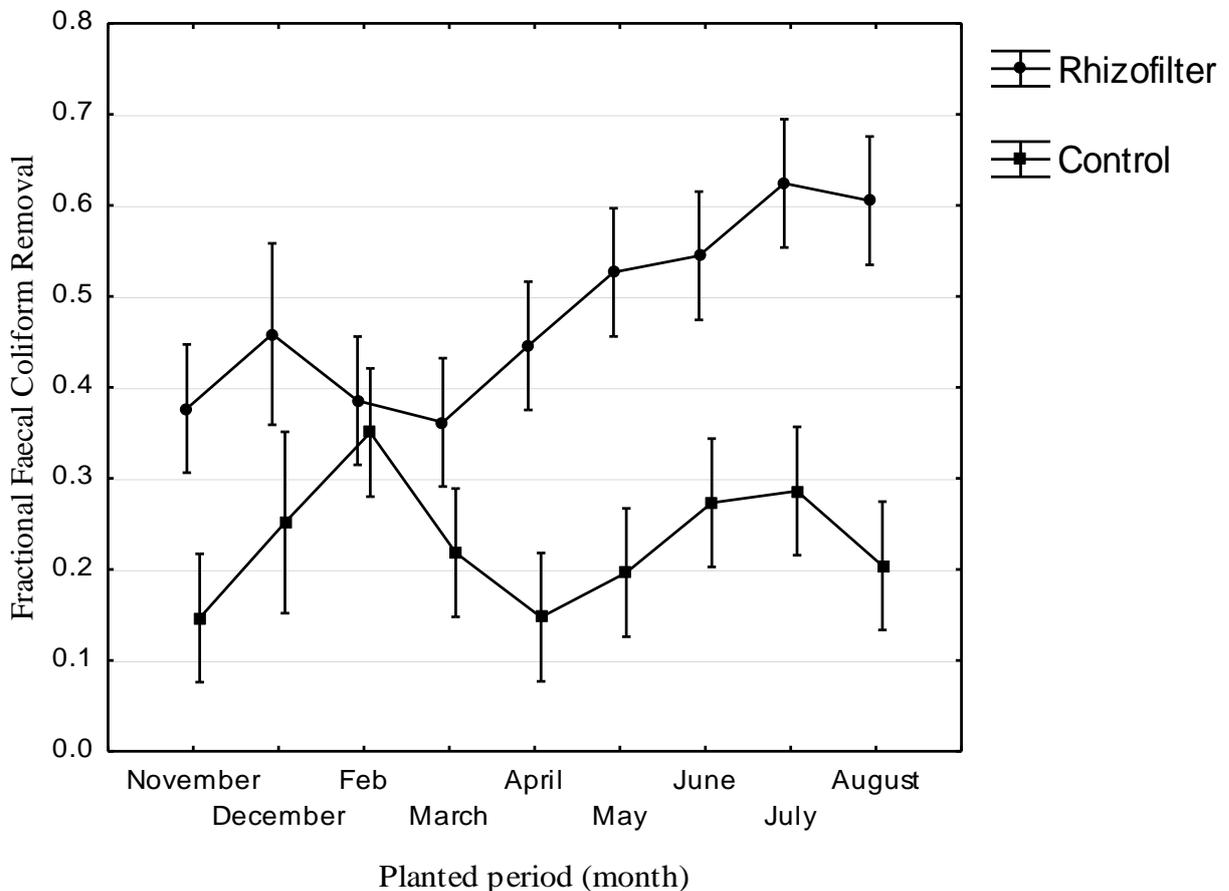
**Fig. 3.2** –Suspended solids removal on the planted (Well B and Well E) and the unplanted side (Well G and Well J) of the constructed rhizofiltration system after wastewater had passed through it. Monthly samples from each well were collected in triplicate and combined before analyses.



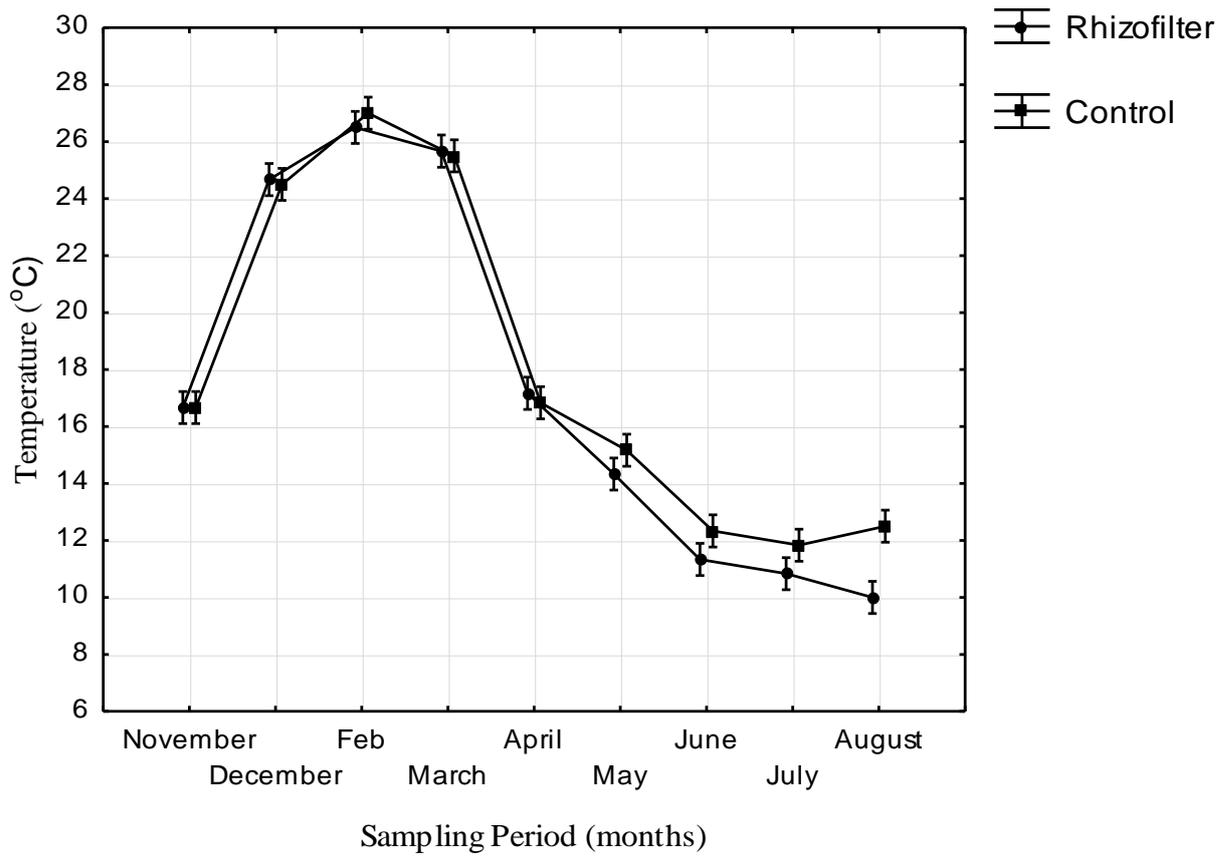
**Fig. 3.3** - Removal of Chemical Oxygen Demand (COD) after wastewater had passed through both the planted (Well B and Well E) and the unplanted side (Well G and Well J) of a constructed rhizofiltration system. Monthly samples from each well were collected in triplicate and combined before analyses.



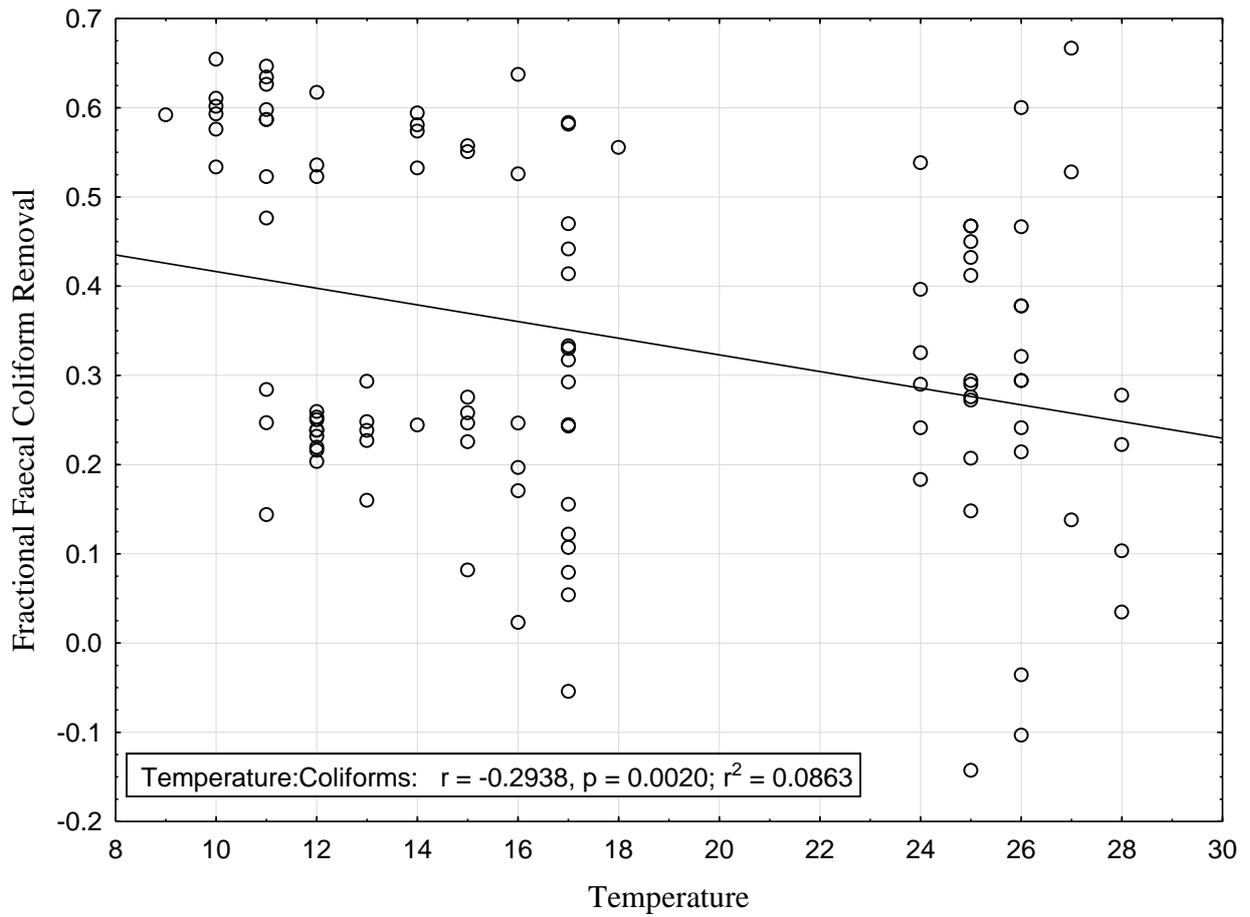
**Fig. 3.4 – Percentage ammonium removal on the planted (Well B and Well E) and the unplanted side (Well G and Well J) of a constructed rhizofiltration system after wastewater had passed through it. Monthly samples from each well were collected in triplicate and combined before analyses.**



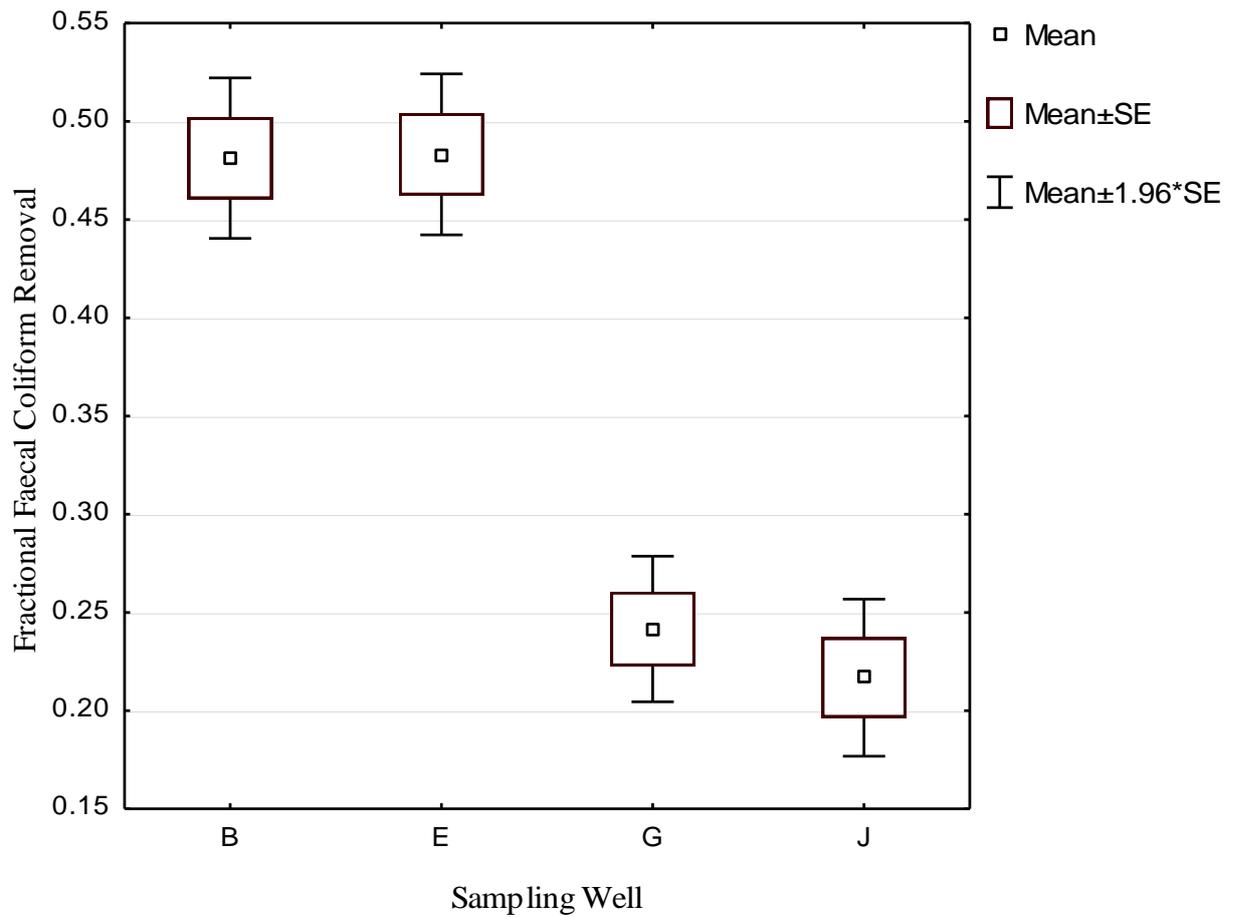
**Fig. 3.5 – Fractional faecal coliform removal after wastewater had passed through the planted (Rhizofilter) and the unplanted (Control) side of the filtration system. Values are means of six repetitions; vertical bars denote 0.95 confidence intervals.**



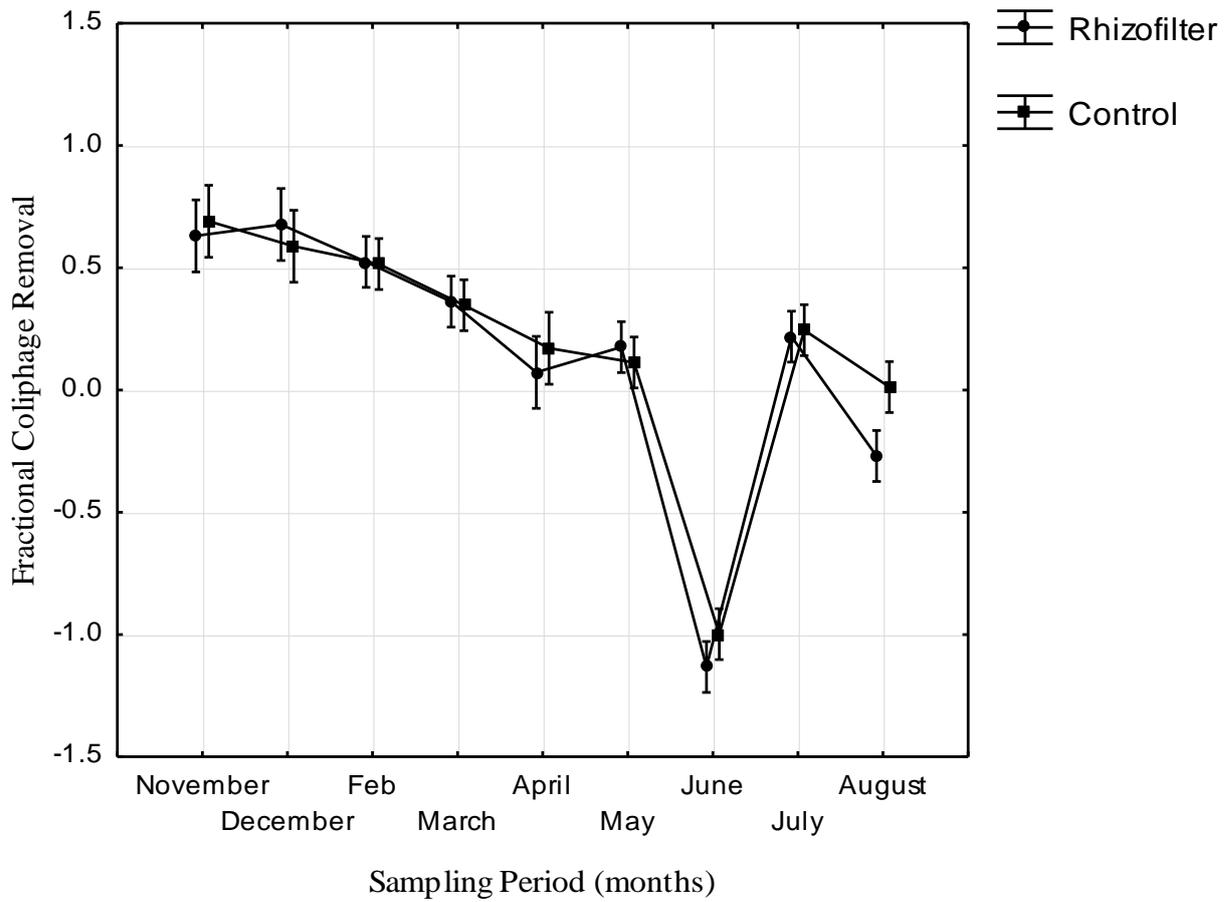
**Fig. 3.6 – Effluent temperature after passing through the planted (Rhizofilter) and unplanted (Control) side of the constructed rhizofiltration system. Values are the means of six repetitions; vertical bars denote 0.95 confidence intervals.**



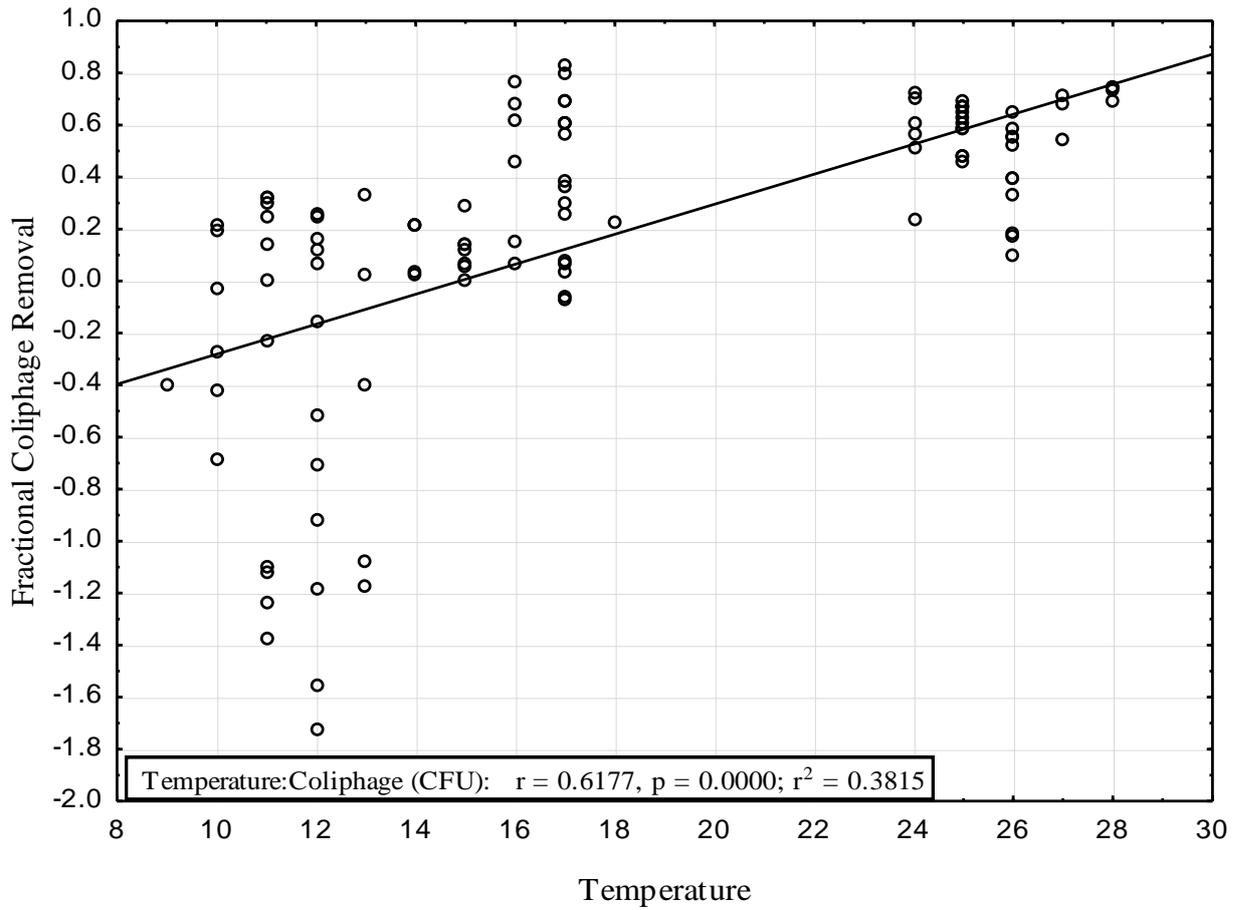
**Fig. 3.7 – Correlation between fractional faecal coliform removal and temperature using data collected over the entire sampling period from both the planted and unplanted sides of a constructed rhizofiltration system.**



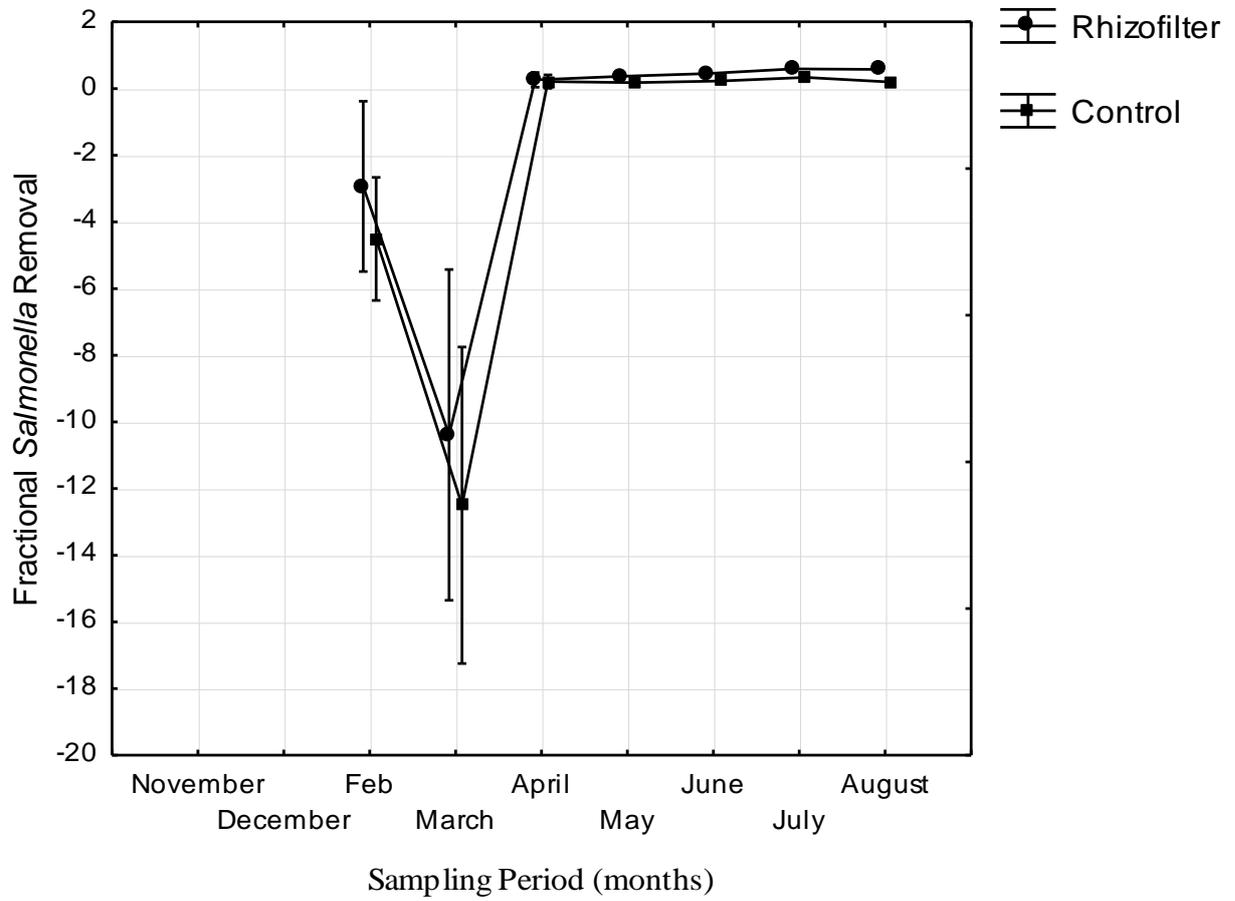
**Fig. 3.8** – Box and whisker plot showing average removal of faecal coliforms after wastewater had passed through the planted (Well B and Well E) and the unplanted side (Well G and Well J) of the rhizofiltration system. Values represent the means of 54 repetitions collected over the entire experimental period. SE refers to standard error of the means.



**Fig. 3.9 – Fractional coliphage removal after the wastewater had passed through the planted (Rhizofilter) and the unplanted (Control) side of a constructed rhizofiltration system. Values are the means of six repetitions; vertical bars denote 0.95 confidence interval. Negative values denote proliferation of coliphage.**



**Fig. 3.10 - Correlation between fractional coliphage removal and temperature using data collected over the entire sampling period from both the planted and unplanted sides of a constructed rhizofiltration system.**



**Fig. 3.11 - Removal of *Salmonella* after wastewater had passed through both the planted (Rhizofilter) and the unplanted (Control) side of a constructed rhizofiltration system using ANOVA analysis. Values are the means of six repetitions; vertical bars denote 0.95 confidence interval. Negative values denote proliferation of *Salmonella*.**

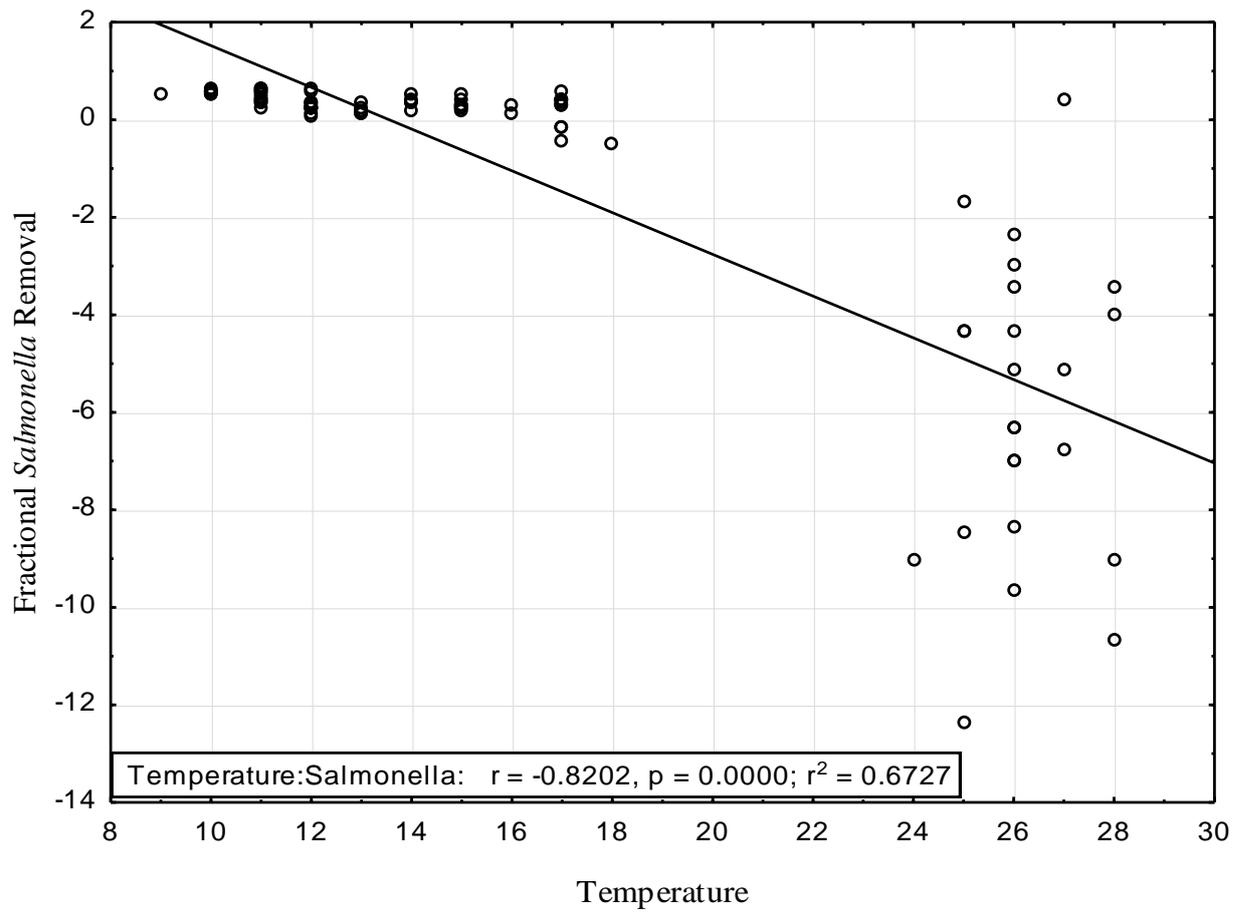
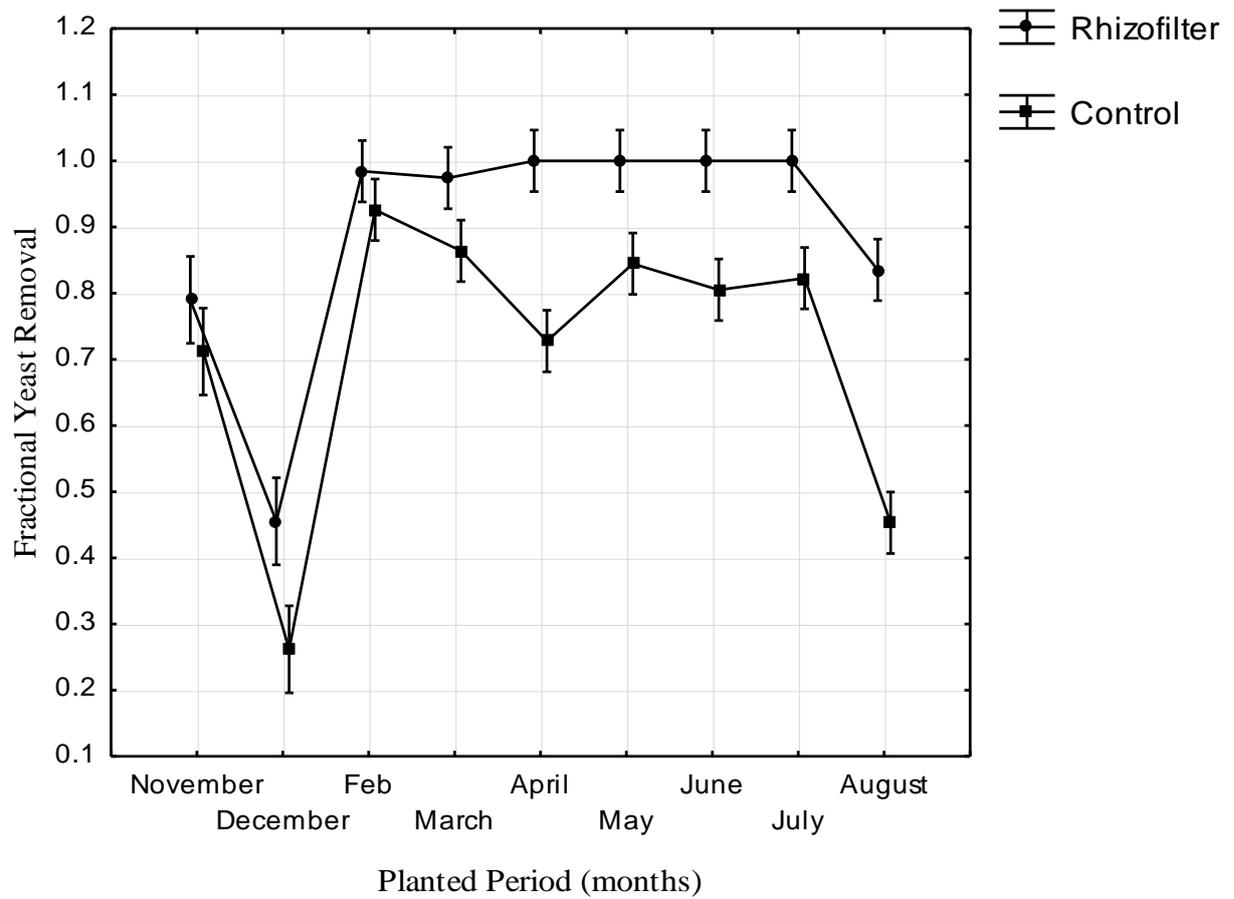
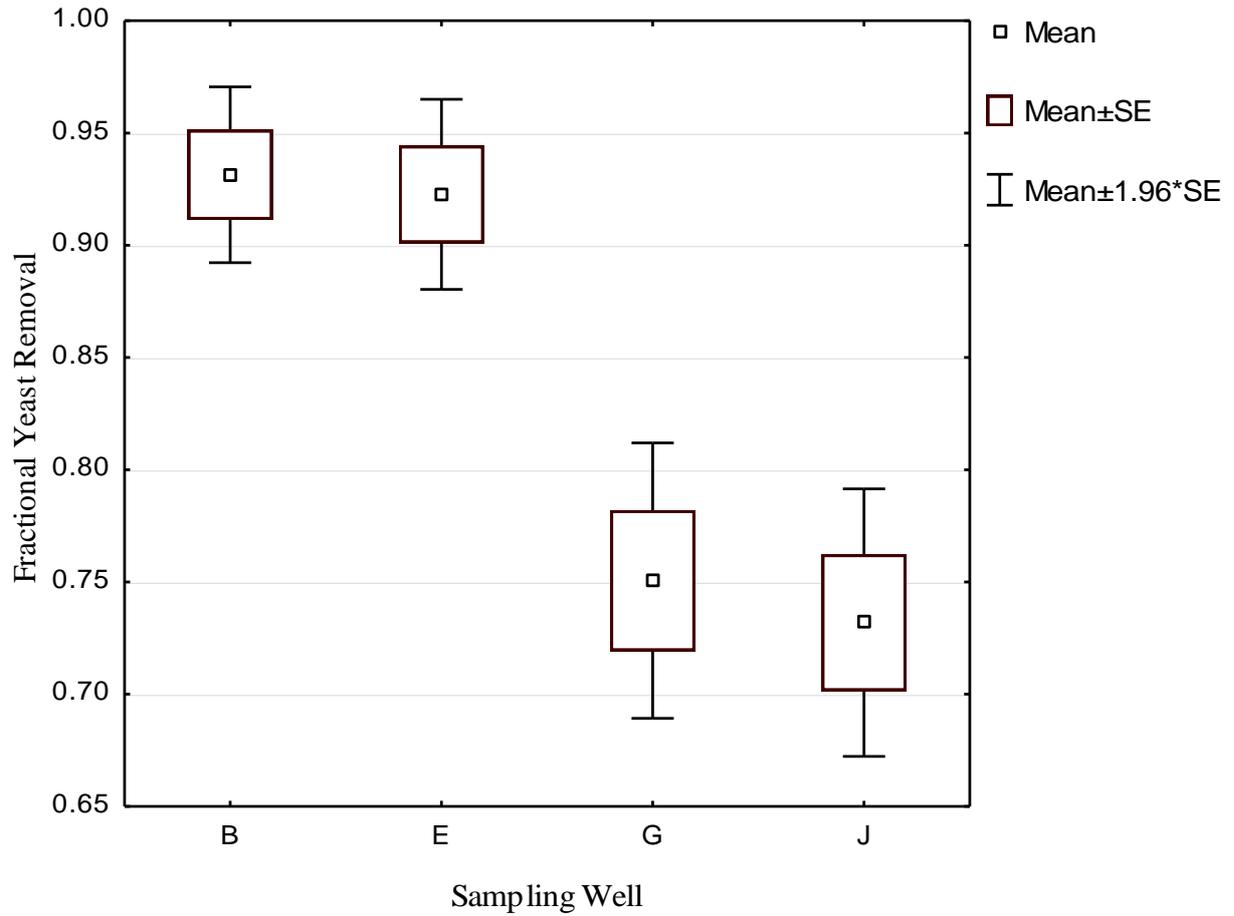


Fig. 3.12 - Correlation between fractional *Salmonella* removal and temperature using data collected over the entire sampling period from both the planted and unplanted sides of a constructed rhizofiltration system.



**Fig. 3.13 – Fractional potentially pathogenic yeast removal after wastewater had passed through the planted (Rhizofilter) and the unplanted (Control) side of a constructed rhizofiltration system. Values are the means of six repetitions; vertical bars denote 0.95 confidence interval.**



**Fig. 3.14** – Box and whisker plot showing the average removal of potentially pathogenic yeasts after wastewater had passed through both the planted (Well B and Well E) and the unplanted side (Well G and Well J) of the constructed rhizofiltration system. Values represent the means of 54 repetitions collected over the entire experimental period. SE refers to standard error of the mean.

**Table 3.1 - Appearance of yeast and bacterial species on Molybdate agar with regard to colour, texture and margin (see also Addendum A, Figure 1).**

<b>Organism</b>	<b>Colour (Top/Bottom)</b>	<b>Texture</b>	<b>Margin</b>
<b>Yeasts</b>			
<i>Candida tropicalis</i>	Brown/Brown	Rough	None
<i>Candida glabrata</i>	White/White	Smooth	None
<i>Candida utilis</i>	Brown/Turquoise	Smooth	White
<i>Candida lusitanae</i>	Brown/Brown	Smooth	None
<i>Candida krusei</i>	Green/Green	Rough	White (Fluffy)
<i>Saccharomyces cerevisiae</i>	Turquoise/Turquoise	Rough	None
<b>Bacteria</b>			
<i>Burkholderia cepacia</i>	Dark Yellow/Dark Yellow	Smooth	White
<i>Burkholderia</i> sp.	Light Green/Light Green	Slimy	None
<i>Burkholderia multivorans</i>	Light Yellow/Light Yellow	Smooth	White
<i>Citrobacter freundii</i>	Green/Blue	Rough	None
<i>Escherichia coli</i>	Light Blue/Light Blue	Slimy	White

**Table 3.2 –Percentage of the dominant culturable yeast species in the influent and effluent of a rhizofiltration system over a nine month (November 2011 to August 2012) sampling period.**

Month	Influent (%)					Rhizofilter (%)					Control (%)				
	<i>C. tropicalis</i>	<i>C. krusei</i>	<i>C. glabrata</i>	<i>C. utilis</i>	<i>S. cerevisiae</i>	<i>C. tropicalis</i>	<i>C. krusei</i>	<i>C. glabrata</i>	<i>C. utilis</i>	<i>S. cerevisiae</i>	<i>C. tropicalis</i>	<i>C. krusei</i>	<i>C. glabrata</i>	<i>C. utilis</i>	<i>S. cerevisiae</i>
November	49	2	40	0	9	33	3	54	2	8	30	0	57	4	9
December	0	0	44	38	18	0	0	17	13	70	0	0	40	0	60
February	0	0	5	15	80	0	0	20	40	40	0	0	40	40	20
March	22	30	18	20	10	0	0	0	100	0	0	0	25	75	0
April	0	30	10	60	0	0	0	0	0	0	0	10	10	80	0
May	0	31	46	23	0	0	0	0	0	0	0	38	24	38	0
June	0	25	58	17	0	0	0	0	0	0	0	25	25	50	0
July	0	12	38	50	0	0	0	0	0	0	0	23	23	54	0
August	19	19	19	43	0	33	0	33	34	0	40	10	30	20	0

**Table 3.3 - Percentage of dominant antibiotic resistant bacterial species isolated from the influent and effluent of a rhizofiltration system from November 2011 to August 2012.**

Month	Influent (%)					Rhizofilter (%)					Control (%)				
	BC	BM	B sp.	CF	EC	BC	BM	B sp.	CF	EC	BC	BM	B sp.	CF	EC
November	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
December	15	0	80	5	0	29	0	42	29	0	60	0	40	0	0
February	0	0	0	0	0	61	11	25	0	3	20	80	0	0	0
March	0	0	100	0	0	30	14	40	16	0	20	28	40	12	0
April	0	0	100	0	0	18	4	75	3	0	14	27	58	1	0
May	0	0	50	50	0	13	11	24	52	0	43	4	30	23	0
June	0	0	25	61	14	0	0	3	91	6	0	0	7	77	16
July	13	0	0	75	12	3	0	0	70	27	16	0	0	59	24
August	0	0	12	76	12	0	0	6	81	13	0	0	6	61	33

BC = *Burkholderia cepacia*; BM = *Burkholderia multivorans*; B sp. = *Burkholderia* species (not identified to species level); CF = *Citrobacter freundii*; EC = *E.coli* O157:H7.

## **CHAPTER 4**

### **Preliminary investigation into the interactions of selected Proteobacteria and yeasts within the upper sand layer of a rhizofiltration system.**

Language and style used in this chapter are in accordance with the requirements of the journal Water Research.

## 4.1. Introduction

Constructed wetlands and rhizofiltration systems are effective at reducing the concentration of nutrients, indicator organisms and pathogens usually found in wastewater (Wood, 1989; Gray et al., 2000; Neralla et al., 2000; Pinney et al., 2000; Wu et al., 2011). These water treatment technologies offer a suitable combination of physical, chemical and biological factors for the removal of pathogenic organisms. The major mechanisms for removal in such systems include filtration, sedimentation, antimicrobial root exudates, as well as predation by nematodes and protists, and finally attack by bacteria and viruses (Gersberg et al., 1989; Spratt and Morgan, 1990; Cooper et al., 1996; Wand et al., 2007). Additionally, the development of bacterial populations with antibiotic activity, such as proteobacteria belonging to *Pseudomonas* spp., in the rhizosphere may also account for microbial die-off (Mandernack et al., 2000). *Burkholderia* is another proteobacterial genus typically found in the rhizosphere which may also be responsible for the removal of pathogens within constructed wetlands and rhizofiltration systems (Fiore et al., 2001; Miller et al., 2002).

The genus *Burkholderia* contains more than 60 species that have been isolated from a variety of habitats. The majority of these species are associated with plants where they provide beneficial services within the rhizosphere, such as the degradation of aromatic compounds and nitrogen fixation (Suárez-Moreno et al., 2012). The most extensively studied cluster within this genus is the *Burkholderia cepacia* complex (Bcc), a group consisting of 17 defined species that may act as human, animal and plant pathogens (Isles et al., 1984; Speert, 2001; Vial et al., 2011). The Bcc has mainly been associated with infection in cystic fibrosis (CF) patients, the majority which result from *Burkholderia cenocepacia* and *Burkholderia multivorans*, but other species including *Burkholderia vietnamensis*, *Burkholderia dolosa* and *Burkholderia cepacia* have also been recovered (Reik et al., 2005). The increased recovery of *B. cenocepacia* and *B. multivorans* has been linked with the emergence of strains containing increased virulence, which may be attributed to antibiotic resistant nature of these organisms (Woods et al., 2004; Mahenthiralingam et al., 2005). Unlike infection caused by other CF pathogens such as *Pseudomonas aeruginosa*, Bcc infections usually result in a rapid, uncontrollable and fatal clinical decline in about 10 % of individuals (Mahenthiralingam et al., 2008). The Bcc was also shown to be capable of transmission through social contact which created a significant problem as CF patients are usually in close proximity to each other within treatment facilities (Lipuma et al., 1990; Govan et al., 1993). Members of the

Bcc group may pose a significant risk to human health, but recent studies have shown that *Burkholderia* species may also play a significant role in the natural environment.

Species within the Bcc have been isolated from numerous natural habitats including the plant rhizosphere, soil and river water (Fiore et al., 2001; Miller et al., 2002; Vermis et al., 2003). Within the rhizosphere, they serve as plant-pest antagonists, plant-growth-promoting rhizobacteria and degradative agents of toxic substances (Fries et al., 1997; Bevivino et al., 2000; Heungens and Park 2000; Tr n Van et al., 2000; Lee, 2003). Additionally, *B. cepacia* produces cepacidine A, a cyclic peptide which inhibits the growth of various animal and plant fungi (Lim et al., 1994), and pyrrolnitrin which inhibits the growth of *Candida albicans* and *Aspergillus niger* (Jayaswal, 1993). *Burkholderia cepacia* also inhibits the growth of other potentially pathogenic *Candida* species, including *C. tropicalis*, *C. glabrata* and *C. krusei* (Kerr, 1994). These yeasts exist as commensals of mucosal membranes in the majority of healthy individuals but can become pathogenic when the host is immunocompromised (Molero et al., 1998; Niewerth and Korting, 2002; Cafarchia et al., 2006; Rao, 2012). *Candida* infection can result in oral, vaginal or cutaneous candidiasis, as well as candidaemia, a systemic infection which often leads to death (Giri and Kindo, 2012).

Previously, we demonstrated that the numbers of proteobacteria such as faecal coliforms and *Salmonella*, as well as potentially pathogenic yeasts, were significantly reduced in settled sewage when percolating through a rhizofiltration system consisting of *Typha capensis* and *Phragmites australis* growing in the upper sand layer (Chapter 3). However, as this settled sewage, hereafter referred to as wastewater, passed through the filter, a significant increase in *Burkholderia* numbers was noted. The aim of this study was to explore the microbial interactions within this filter by periodically enumerating faecal coliforms, *Salmonella*, potentially pathogenic yeasts and antibiotic resistant bacteria, primarily *Burkholderia* species, within the sand of the filter after the wastewater passed through it. Then, binary interactions between *Burkholderia* and strains representing pathogenic *Candida* species were studied *in vitro* within sand columns.

## 4.2. Materials and Methods

### 4.2.1 Sample collection

To determine microbial interactions within the rhizofiltration system, after wastewater passed through it, the upper sand layer was periodically sampled. Sand was sampled at three points above Well B and Well E on the experimental side and above Well G and Well J on the control side (Figure 4.1) immediately after the wastewater had passed through the system. Additionally, similar samples were collected three and six days after the initial wastewater had been applied. Sand samples were collected 15 cm below the surface using a sterilised metal spoon and placed into autoclaved glass jars for transport back to the laboratory before being analysed.

### 4.2.2 Microbiological analysis

Faecal coliform concentrations were determined by preparing a triplicate dilution series of each sample followed by plating 100 µl of each dilution on MacConkey Agar (Atlas, 1993). The plates were subsequently incubated at 44°C for 24 hours after which coliforms were enumerated by identifying colonies with a metallic red hue. Potential pathogenic yeasts and antibiotic resistant bacteria were detected by preparing a dilution series and plating 100 µl of each dilution on Sabouraud Glucose Agar (SGA) supplemented with chloramphenicol (30µg/ml), tetracycline (10µg/ml) and kanamycin (50µg/ml). The plates were then incubated at 37°C for 48 hours until colonies were clearly visible and colony forming units were counted. *Salmonella* concentrations were determined by plating 100 µl of each sample on *Salmonella-Shigella* Agar and incubating the plates at 37°C for 24 hours (Hassanein et al., 2011). *Salmonella* colonies were enumerated by identifying colonies that were colourless but contained a black centre.

### 4.2.3 *In vitro* sand filter experiments

#### 4.2.3.1 Filter design

Polyethylene columns (diameter of 100 mm, length of 130 mm) were filled with an initial 15 mm layer of autoclaved coarse sand (500 µm), on top of which a 55 mm layer of autoclaved fine sand (250 – 355 µm) was placed. Polyethylene tubes were used to connect the columns to two peristaltic pumps (Watson Marlow 2058), one pump to supply the influent, and one pump to remove the effluent from each sand filter. The columns and tubes were sterilised in 3.5% (v/v) sodium hypochlorite for 24 hours prior to filling. The sand filters were held

upright by arms connected to a retort stand and each was inoculated with the appropriate microbe/s.

#### 4.2.3.2 Experimental procedure

The *in vitro* filters, employed as experimental and control filters, each received an inoculum of *B. cepacia* in wastewater. The latter was prepared by first growing *Burkholderia cepacia* (CAB 85) in Nutrient Broth for 16 hours at 37°C, followed by preparing 50 ml suspensions ( $2 \times 10^5$  cells/ml) of this bacterial strain in autoclaved wastewater. This suspension, of which the concentration of *B. cepacia* (CAB 85) was estimated using a Petroff-Hauser counting chamber (Hausser Scientific, Horsham), was used as inoculum for the *in vitro* filters. In each case the bacterial inoculum was transferred to the filter, using the above mentioned pumping system, until the sandy filter substrate was saturated with the suspension. The filters which received the *B. cepacia* inoculums were incubated at 22°C for 16 hours to allow for colonisation of the sandy substrate with the bacterium. Control filters containing no viable bacteria, but saturated with autoclaved wastewater, were also included in the experiment. These filters, called *Candida* control filters, would later be inoculated with different *Candida* strains to evaluate the movement of the yeasts through the substrate in the absence of viable bacteria.

The experimental filters, containing substrate colonised with *B. cepacia* (CAB 85), and *Candida* control filters, were subsequently inoculated with suspensions of *C. krusei* (CAB 83), *C. tropicalis* (CAB 80), *C. albicans* (CAB 629), *C. glabrata* (CAB 84) and *C. utilis* (CAB 78). These yeast suspensions were prepared by growing each yeast strain in Yeast Malt (YM) at 37°C for 16 hours, followed by preparing a 50 ml yeast suspension ( $1.5 \times 10^5$  cells/ml) of each yeast strain in autoclaved wastewater. Each of these suspensions, of which the yeast concentration was estimated using a haemocytometer (Marienfeld, Germany), was pumped into a sand filter until a 20 mm head of suspension was obtained. The suspension was then pumped from the bottom of each filter at a rate of 1.25 ml/min, creating a hydraulic retention time of approximately 45 minutes. The last 10 ml of effluent from all the sand filters was collected in autoclaved bottles for analysis.

#### 4.2.3.3 Effluent analysis

The concentration of *B. cepacia* (CAB 85) and *Candida* strains in the effluent of both the control and experimental filters was determined by preparing triplicate dilution series and plating 100 µl of each dilution on Nutrient Agar (NA). The plates were subsequently incubated at 37°C for 48 hours, after which *Candida* and *B. cepacia* were enumerated by identifying cream and yellow colonies respectively.

#### 4.2.3.4 Sand Analysis

To determine the growth and survival of *B. cepacia* (CAB 85) and the *Candida* strains in the sandy substrate of the filters, the substrate was sampled directly after the yeast was applied to the filter, as well as three and six days after the wastewater passed through the substrate. Sand was collected using a sterilised metal spoon, approximately halfway down the sand column, and placed into autoclaved bottles. Triplicate dilution series were prepared of each sand sample, followed by plating 100 µl of each dilution on NA. *Candida* and *B. cepacia* concentrations were determined by respectively enumerating cream and yellow colonies after incubation of the plates at 37°C for 48 hours.

### 4.3. Results and Discussion

#### 4.3.1 Microbiological analysis

To explore the microbial interactions within the constructed rhizofilter and to evaluate the effect of plants on the survival of faecal coliforms, *Salmonella*, yeasts and antibiotic resistant bacteria such as *Burkholderia*, substrate samples were periodically taken from the planted (experimental) and unplanted (control) side of the filter. The samples were taken immediately after the wastewater had percolated through the filter, and again after three and six days.

It was found that the decline of faecal coliform numbers, indicated as faecal coliform removal in Figure 4.2, was significantly more in the planted sand above Well B than in the unplanted sand above Well G. This difference was obvious after three ( $p = 0.0000$ ) and six days ( $p = 0.0000$ ) without wastewater application. Faecal coliform concentrations in the sand above Well B dropped by 94 % after six days while faecal coliform numbers in the sand above Well G dropped by 37 % over the same time. Similarly, faecal coliform removal in the planted sand above Well E was significantly more than in the unplanted sand above Well J. This was obvious after both three ( $p = 0.0000$ ) and six ( $p = 0.0000$ ) days without wastewater application. The greater reduction of faecal coliforms on the planted side may be attributed to

the plants themselves. The root excretions of certain aquatic macrophytes including *Scirpus lacustris* and *Phragmites australis* are known to kill faecal indicators and other pathogenic bacteria (Spratt and Morgan, 1990; Cooper et al., 1996). Additionally, the plant roots provide suitable attachment sites for other microbes which may be antagonistic towards indicator organisms.

Interestingly, immediately after application of the wastewater to the rhizofilter (Day 0) more faecal coliforms occurred in the planted sand of the filter than in the unplanted sand (Table 1). This supports our previous findings which revealed that coliform concentrations were significantly lower in wastewater that percolated through the planted side than in the wastewater which flowed through the unplanted side of the filter (Chapter 3). The substrate on the experimental side of the rhizofiltration system was thus much more effective at trapping and removing microbes. The planted side also reduced faecal coliform numbers more effectively than the unplanted side after three and six days (Figure 4.2; Table 4.1).

Similar to the faecal coliforms, the removal of potentially pathogenic yeasts was significantly more in the planted sand above Well B than in the unplanted sand above Well G (Figure 4.3). This difference was significant after three ( $p = 0.0265$ ) and six ( $p = 0.0460$ ) days without water application. The concentration of yeasts in the sand above Well B dropped by 91 % after six days while yeast numbers in the sand above Well G dropped by 52 % over the same period. Similarly, the sand above Well E and Well J achieved a 90 % and 47 % reduction in potentially pathogenic yeasts respectively, after six days. The removal of yeasts was significantly different between Well E and Well J both three ( $p = 0.0073$ ) and six ( $p = 0.0058$ ) days after water had been applied to the system. Certain root exudates and predatory bacteria may be responsible for the removal of yeasts within the rhizofiltration system. Additionally, predation in the substrate of the planted side may be responsible for the better yeast removal when compared to the unplanted side. The improved aeration of the root zone in planted gravel beds would provide better conditions for the development of predators, such as protozoa (Wand et al., 2007).

Yeasts in the wastewater percolating through the filter seemed, similar to the faecal coliforms, to be trapped more by the planted sand than the unplanted sand. Yeast concentrations on Day 0 were greater in sand collected from the planted experimental side when compared with unplanted sand collected from the control side of the filter (Table 4.1).

The planted side also seemed to reduce yeast numbers more effectively than the unplanted side after three and six days.

*Salmonella* removal was significantly more in the planted sand collected above Well B, than in the unplanted sand collected above Well G following three ( $p = 0.0020$ ) and six ( $p = 0.0359$ ) days without water application (Figure 4.4). *Salmonella* concentrations in the sand above Well B dropped by 92 % after six days while *Salmonella* numbers in the sand above Well G dropped by 38 % over the same time. Similarly, the sand above Well E and Well J resulted in a 91 % and 47 % reduction in *Salmonella* concentrations respectively, after six days. The removal of *Salmonella* was significantly different between Well E and Well J both three ( $p = 0.0002$ ) and six ( $p = 0.0000$ ) days after water had been applied to the system. The removal of these bacteria in the substrate of both the planted and unplanted side of the rhizofiltration system may be attributed to mechanisms that were similar for faecal coliform removal. These include predation, the release of root exudates and bacterial populations with antibiotic activity (Mandernack et al., 2000). The numbers of *Salmonella* in the upper sand layer of the rhizofiltration system, immediately after the application of the wastewater on Day 0 were greater in the planted sand compared with the unplanted sand (Table 4.1). The planted side also reduced *Salmonella* numbers more effectively than the unplanted side after three and six days.

The antibiotic resistant bacteria in the wastewater, able to grow on the selective medium, were also found to be trapped more by the planted sand than the unplanted sand (Table 4.1). The majority of these bacteria were represented by *Burkholderia* species (Chapter 3), which are known to be prolific in the rhizosphere where they are beneficial to plants (Suárez-Moreno et al., 2012). After three days, significantly more ( $p = 0.0250$ ) of these bacteria were removed in the planted sand above Well B than in the unplanted sand above Well G, but no difference could be found six days after the wastewater percolated through the system ( $p = 0.1349$ ), (Figure 4.5). Bacteria concentrations in the planted sand above Well B dropped by 80 % after six days while bacterial numbers in the unplanted sand above Well G dropped by 52 % over the same time. After six days, the sand above Well E and Well J resulted in a 82 % and 59 % reduction in bacteria, respectively. Bacterial removal from the sand was not significantly different between Well E and Well J, both three ( $p = 0.3686$ ) and six ( $p = 0.3505$ ) days after the wastewater was applied to the system.

### 4.3.2 *In vitro* sand filter experiments

#### 4.3.2.1 Water analysis

The effect of antibiotic resistant bacteria, i.e. *B. cepacia* (CAB 85) on the movement of *Candida* strains suspended in wastewater, through a sandy substrate was studied using *in vitro* sand filters. The experimental filters were inoculated with wastewater containing *B. cepacia* (CAB 85) while the control filters were treated with autoclaved wastewater, prior to inoculation of the sand with sewage containing different *Candida* strains. The concentration of all the *Candida* strains in the wastewater was significantly reduced in both the experimental and control filters, when compared to the initial concentration in the wastewater influent (Figure 4.6 A-E). The experimental sand filter, however, was significantly more effective ( $p = 0.0000$ ) at removing *C. krusei* (CAB 83) from the influent than the control filter (Figure 4.6 A). Similarly, the experimental filters were significantly more effective at reducing both *C. glabrata* (CAB 84) ( $p = 0.0008$ ) and *C. albicans* (CAB 629) ( $p = 0.0011$ ) concentrations when compared to the control sand filters (Figures 4.6 C and D). No significant difference was found between the experimental and control filters in the removal of both *C. tropicalis* (CAB 80) ( $p = 0.2051$ ) and *C. utilis* (CAB 78) ( $p = 0.1061$ ) from the influent (Figures 4.6 B and E). These results indicated that the presence of *B. cepacia* (CAB 85) in the sand may be responsible for trapping certain *Candida* species within this environment. The greater concentration of *Burkholderia* species and other antibiotic resistant bacteria found in the experimental side of the rhizofiltration system may thus have contributed to the greater reduction in the numbers of potentially pathogenic yeasts within water percolating through the experimental side of the filter compared to the control side. Additionally, the ability of these bacteria to trap yeasts and prevent them from moving through the substrate may prolong the period in which these unicellular fungi are subjected to the antagonistic effect of root exudates or other microbes.

#### 4.3.2.2 Sand analysis

The presence of *B. cepacia* (CAB 85) in the sand filter resulted in greater proliferation of *C. krusei* (CAB 83) as determined three and six days after the wastewater had flowed through the filter, when compared with sand sampled from the control (Figure 4.7A; Table 4.2). Similarly, *B. cepacia* (CAB 85) concentrations increased more in the experimental filters when compared with sand containing no *C. krusei* (CAB 83). This yeast may thus have an advantage within a wastewater contaminated rhizofiltration system if *Burkholderia* species

are present, since our experiments revealed that cooperation may exist between these two microbes. Additionally, it may also explain the high concentrations of *C. krusei* in many of the effluent samples from the rhizofiltration system collected during previous experiments (Chapter 3).

After six days no difference in the cell numbers of *C. tropicalis* (CAB 80) could be detected between the control and experimental sand filters (Figure 4.7B; Table 2). Interestingly, the increase in the concentration of this yeast after six days was more in the control filter than the experimental filter. These results indicate that *C. tropicalis* (CAB 80) competes for resources more effectively than *B. cepacia* (CAB 85) when the two microbes are together in this environment. The presence of *B. cepacia* (CAB 85), however, restricts the proliferation of *C. tropicalis* (CAB 80) substantially when compared with sand that does not contain bacteria.

The concentrations of *C. glabrata* (CAB 84) and *B. cepacia* (CAB 85) were significantly lower in sand samples collected from the experimental filters after six days, when compared with sand collected from the control filters (Figure 4.7C; Table 4.2). After six days, there was a 5-fold and 155-fold increase of *C. glabrata* (CAB 84) in sand collected from the experimental and control filters respectively, while *B. cepacia* (CAB 85) achieved a 326-fold increase in the presence of *C. glabrata* (CAB 84) over the same time period. Thus, while *B. cepacia* (CAB 85) appeared to be antagonistic towards *C. glabrata* (CAB 84), the bacterium benefitted from the presence of the yeast. The latter most likely occurred as a result of nutrients originating from the yeast.

The concentration of *C. albicans* (CAB 629) and *B. cepacia* (CAB 85) were similar in sand samples collected from the experimental filter after six days (Figure 4.7D; Table 4.2). However, the numbers of *C. albicans* (CAB 629) increased 467-fold in the absence of *B. cepacia* (CAB 85) but only managed to increase 23-fold in the presence of this bacterium. In contrast, *B. cepacia* (CAB 85) numbers increased more in the experimental filter (36-fold) when compared with the control filter (7-fold). Thus, similar to the interactions between *C. glabrata* (CAB 84) and *B. cepacia* (CAB 85), the bacterium seemed to be antagonistic towards *C. albicans* (CAB 629). In addition, it benefitted from the presence of *C. albicans* (CAB 629), possibly as a result of additional nutrients obtained from the yeast.

The interaction of *C. utilis* (CAB 78) with *B. cepacia* (CAB 85) in the *in vitro* sand filters was similar to the interaction of *C. glabrata* (CAB 84) and *C. albicans* (CAB 629) with this bacterium (Figure 4.7E). The presence of *B. cepacia* (CAB 85) in the sand filter resulted in

lower numbers of *C. utilis* (CAB 78), three and six days after wastewater had flowed through the filter, when compared with sand sampled from the control filter (Figure 4.7E, Table 4.2). In contrast, *B. cepacia* (CAB 85) concentrations increased more in the experimental filters when compared with sand containing no *C. utilis* (CAB 78).

#### 4.4. Conclusions

Compared to the unplanted side, the sand of the planted side of the rhizofiltration system was significantly more effective at removing proteobacteria such as faecal coliforms and *Salmonella* spp., as well as potentially pathogenic yeasts, from the wastewater. Additionally, there was a significant reduction in microbial numbers within the sand on the planted side which indicated that a rhizofiltration system would be effective at not only removing pathogens from wastewater during rain events, but would also lower their numbers in the sand after a period of time.

When exploring the potential interactions that may occur in the filter sand using *in vitro* experiments, it was found that *B. cepacia* in the sand may be responsible for trapping some of the *Candida* in the wastewater as it percolates through the substrate. The subsequent interactions of *B. cepacia* with the different *Candida* strains may differ. *B. cepacia* (CAB 85) was found to be antagonistic towards *C. tropicalis* (CAB 80), *C. glabrata* (CAB 84), *C. albicans* (CAB 629) and *C. utilis* (CAB 78) within this environment. In contrast, the numbers of *C. krusei* (CAB 83) increased in sand colonised with *B. cepacia* (CAB 85) when compared with the control. Whether intraspecific differences exist among the interactions of *B. cepacia* with different *Candida* species should be studied in the future. However, many complex interactions are known to occur in wetlands (Rivera et al., 1995) and future research should also aim to study the role of root exudates and microbial predators in eliminating pathogens from the rhizofiltration system.

## 4.5. References

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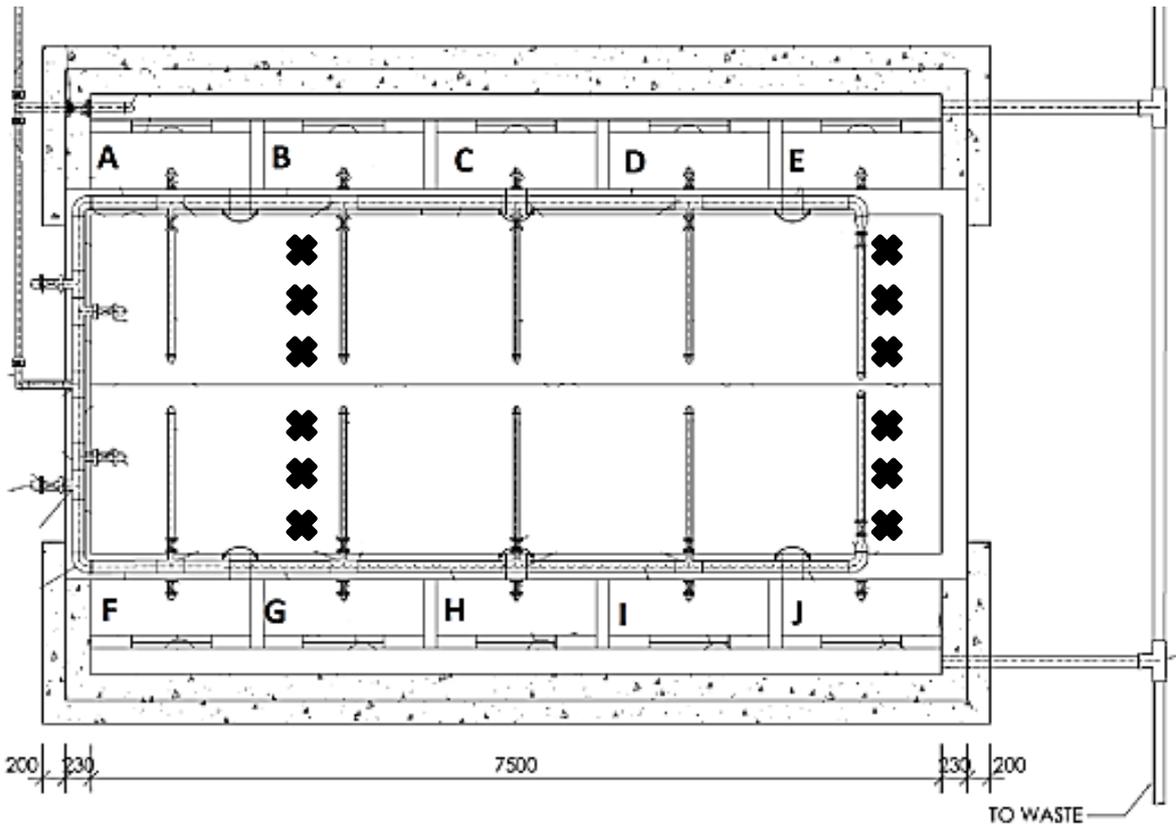


Fig. 4.1 - Rhizofiltration system with sampling wells denoted by letters A-J. Crosses represent the sites of sand collection above Well B and Well E on the experimental side and above Well G and Well J on the control side.

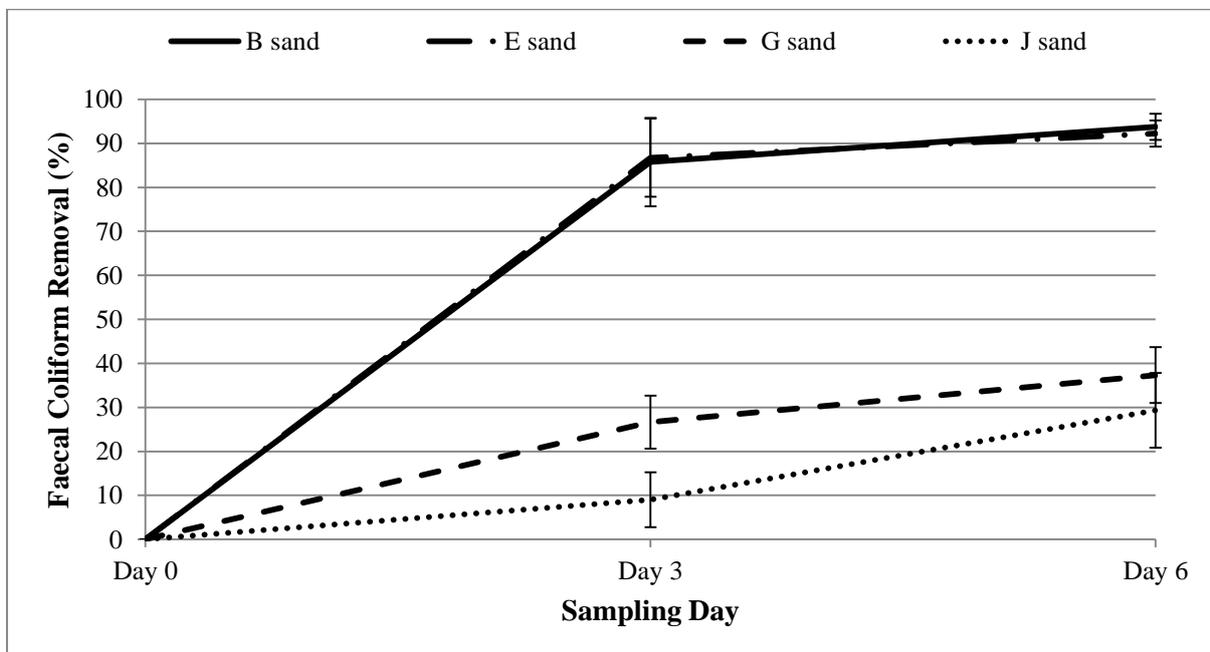


Fig. 4.2 – Faecal coliform removal in sand collected from both the experimental (B sand and E sand) and control (G sand and J sand) sides of the rhizofiltration system, three and six days after water had been applied to the system. Error bars indicate standard deviation values using nine (n = 9) repetitions for each sample site above the wells.

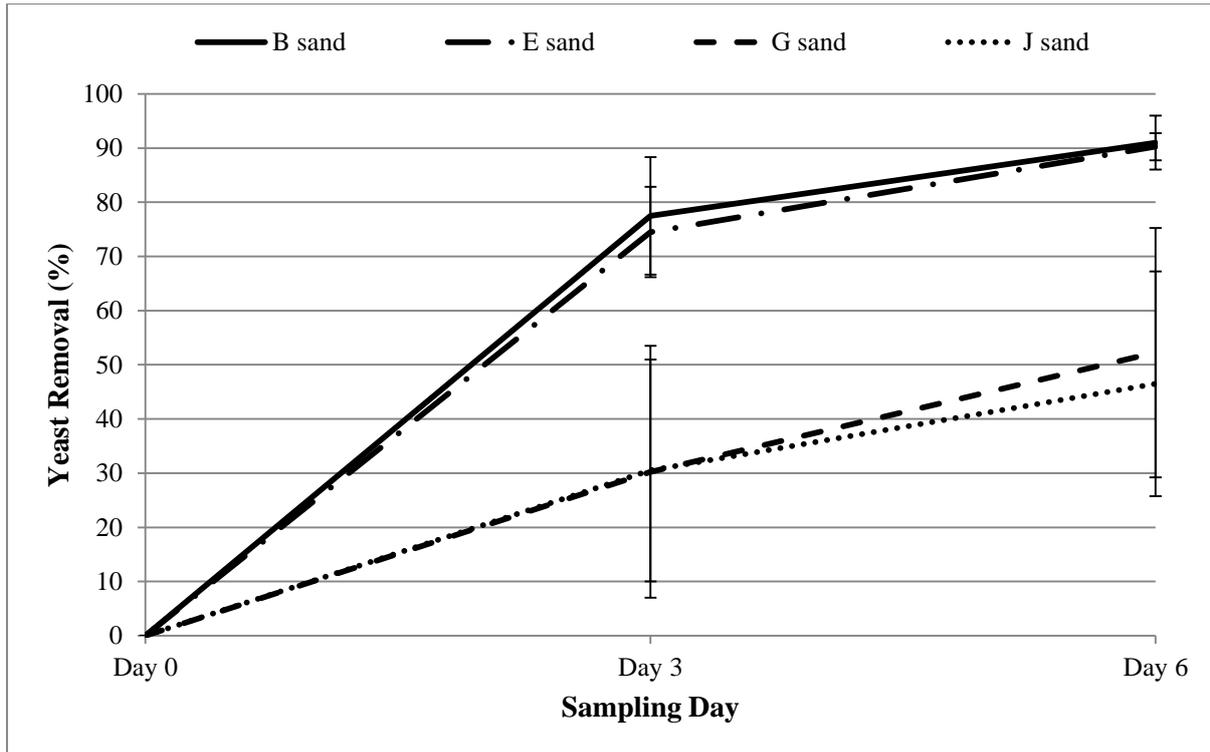


Fig. 4.3 – The removal of yeasts after three and six days in sand collected from both the experimental (B sand and E sand) and control (G sand and J sand) sides of the rhizofiltration system. Error bars indicate standard deviation values using nine (n = 9) repetitions for each sample site above the wells.

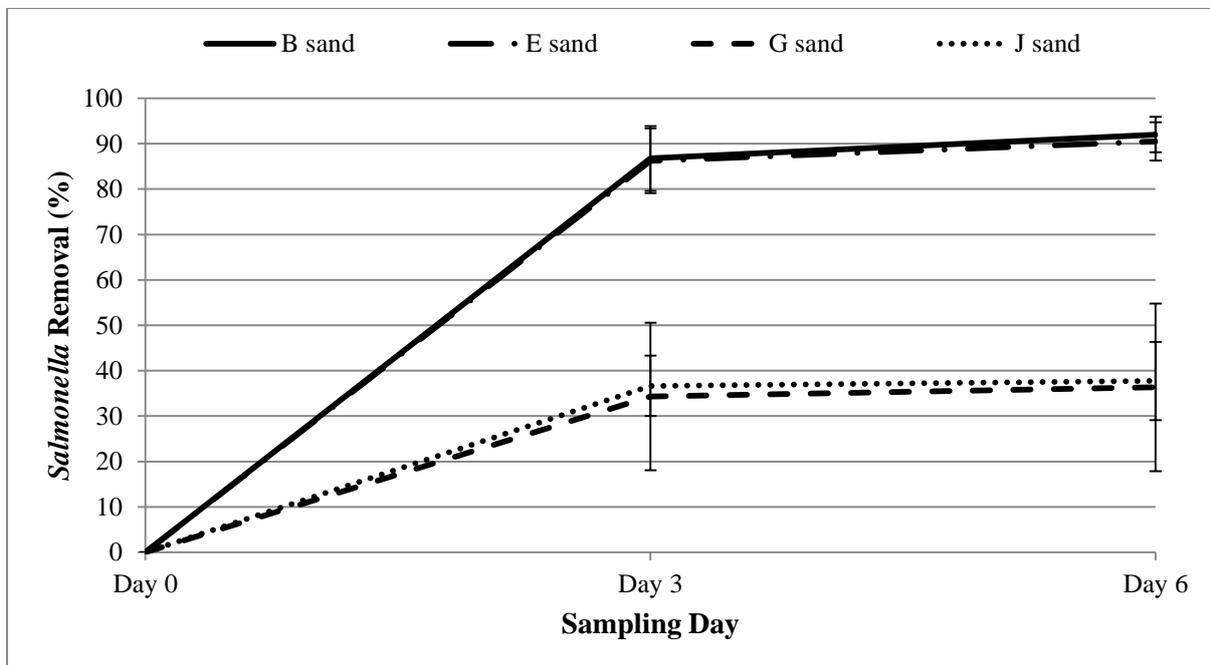
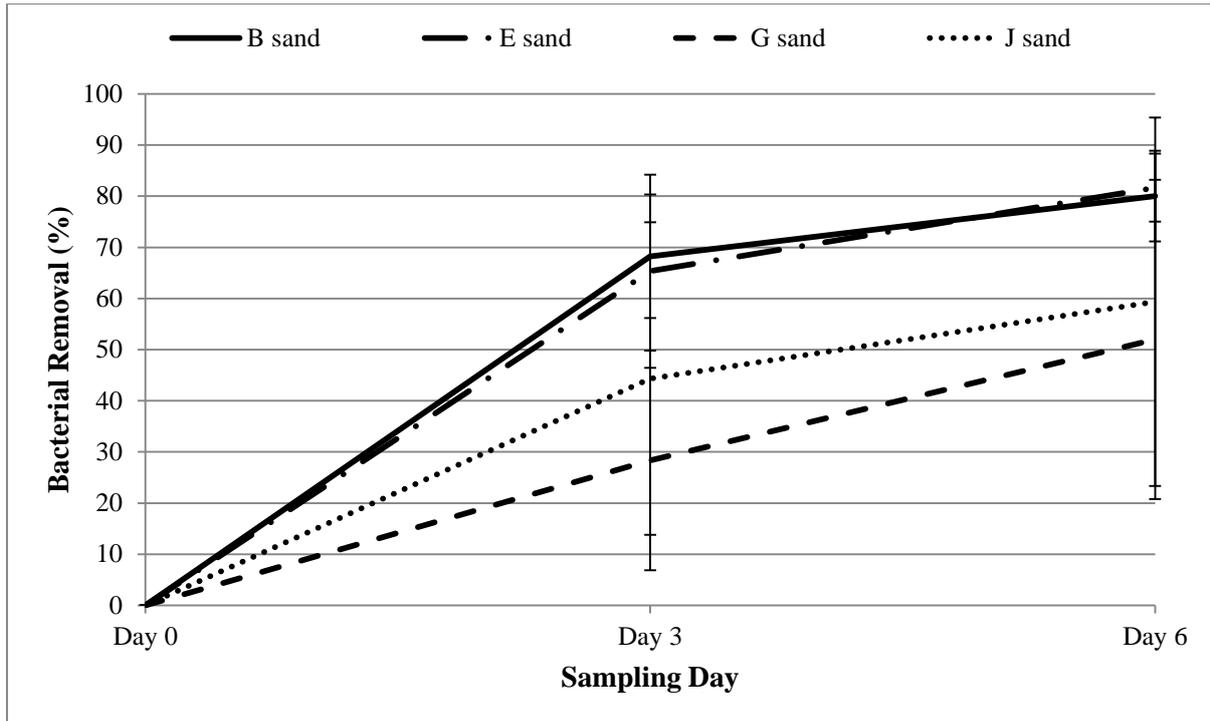


Fig. 4.4 – *Salmonella* removal in sand collected from both the experimental (B sand and E sand) and control (G sand and J sand) sides of the rhizofiltration system, three and six days after water had been applied to the system. Error bars indicate standard deviation values using nine (n = 9) repetitions for each sample site above the wells.



**Fig. 4.5 – Bacterial removal after three and six days in sand collected from both the planted (B sand and E sand) and unplanted (G sand and J sand) sides of the rhizofiltration system. Error bars indicate standard deviation values using nine (n = 9) repetitions for each sample site above the wells.**

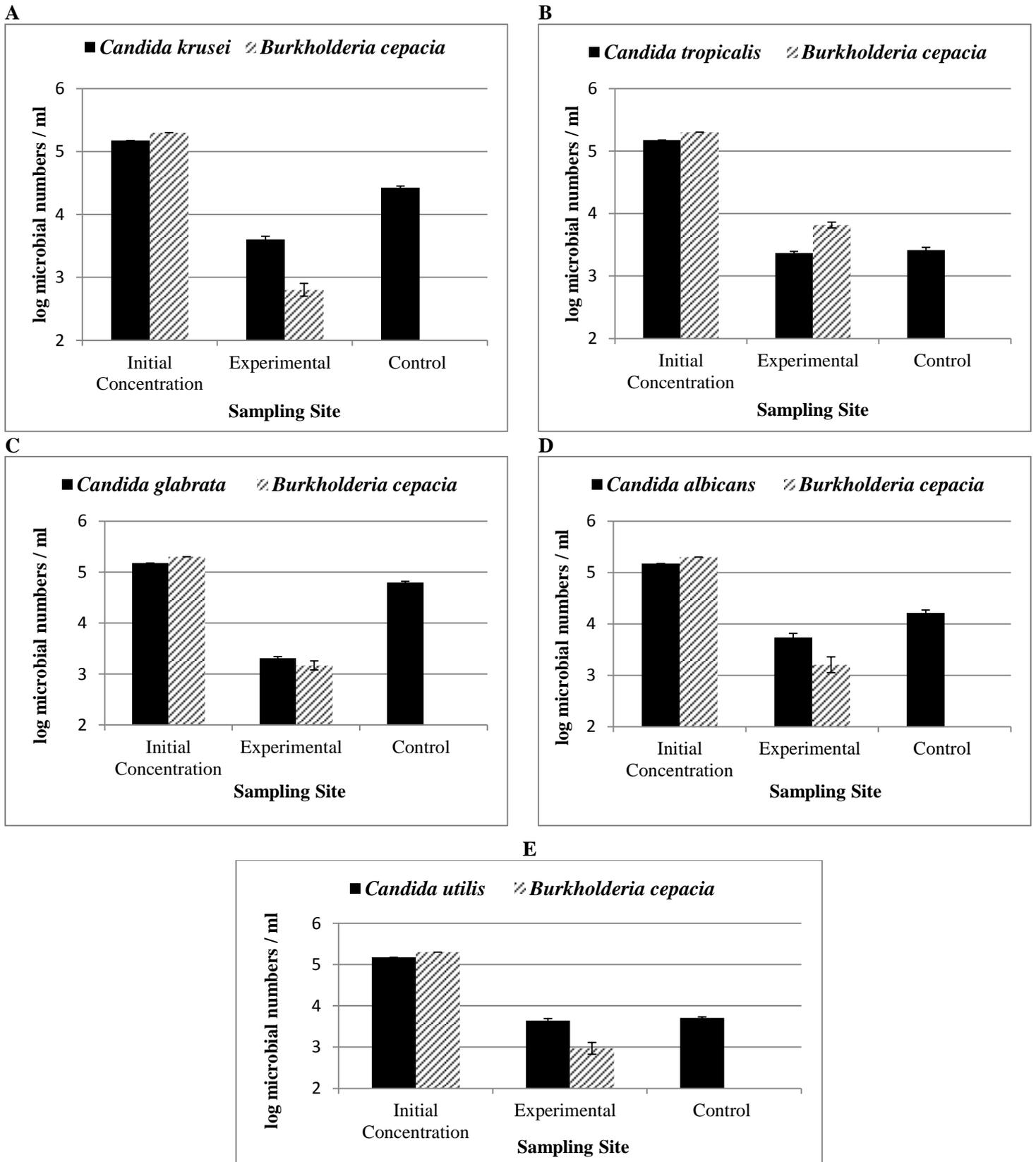
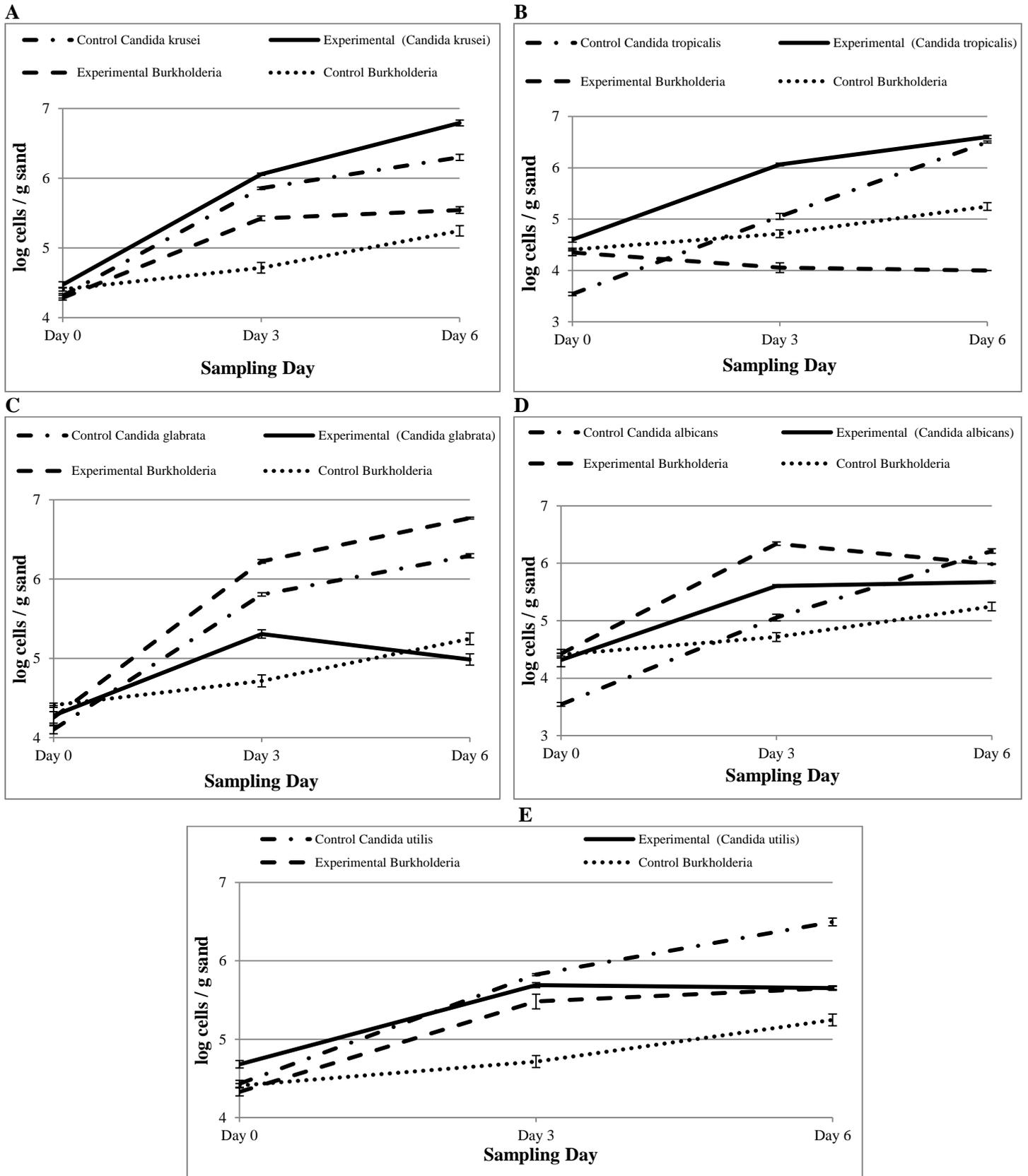


Fig. 4.6 – Reduction of *Candida* populations in water samples after wastewater containing either *C. krusei* (CAB 83), *C. tropicalis* (CAB 80), *C. glabrata* (CAB 84), *C. albicans* (CAB 629) or *C. utilis* (CAB 78) had moved through the sand filters. Experimental filters were inoculated with *B. cepacia* (CAB 85) prior to wastewater application while control filters received autoclaved wastewater. Error bars indicate standard deviation values using three (n = 3) replicates.



**Fig. 4.7** – Changes in *B. cepacia* (CAB 85) and *Candida* concentrations over a six day period after wastewater containing *C. krusei* (CAB 83), *C. tropicalis* (CAB 80), *C. glabrata* (CAB 84), *C. albicans* (CAB 629) and *C. utilis* (CAB 78) had percolated through the sand filters. Control filters received autoclaved wastewater while experimental filters were inoculated with *B. cepacia* (CAB 85) prior to wastewater application. Error bars indicate standard deviation values using three (n = 3) replicates.

**Table 4.1 – Concentration of different microorganisms in sand sampled above wells on the planted (Well B and Well E) and unplanted (Well G and Well J) side of the rhizofiltration system. Samples were collected directly after wastewater had percolated through the system, as well as three and six days after.**

Micro-organisms	Well B			Well E			Well G			Well J		
	Day 0	Day 3	Day 6	Day 0	Day 3	Day 6	Day 0	Day 3	Day 6	Day 0	Day 3	Day 6
<b>Faecal Coliforms (cells / g sand)<sup>a</sup></b>	3600	520	210	3000	400	240	2800	2050	1750	2156	1960	1536
<b>Yeasts (cells / g sand)<sup>b</sup></b>	3089	675	275	2867	730	300	1356	950	655	1211	830	640
<b><i>Salmonella</i> (cells / g sand)<sup>c</sup></b>	2700	350	225	2478	360	225	1122	742	720	1144	775	715
<b>Bacteria (cells / g sand)<sup>d</sup></b>	7967	2550	1600	6022	2100	1100	3144	2250	1515	3367	1900	1450

a – Faecal coliforms enumerated on MacConkey Agar; b – Potentially pathogenic yeasts enumerated on SGA supplemented with tetracycline, chloramphenicol and kanamycin; c – *Salmonella* enumerated on SS Agar; d - Antibiotic resistant bacteria enumerated on SGA supplemented with tetracycline, chloramphenicol and kanamycin.

**Table 4.2 – Concentration and increase of *B. cepacia* (CAB 85) and different *Candida* species in sand collected from filters allowed to stand for six days. Experimental filters contained a combination of the two species while control filters contained either *B. cepacia* or a *Candida* species.**

	<i>Candida krusei</i> (CAB 83)				<i>Candida tropicalis</i> (CAB 80)				<i>Candida glabrata</i> (CAB 84)				<i>Candida albicans</i> (CAB 629)				<i>Candida utilis</i> (CAB 78)			
	CC	CE	BC	BE	CC	CE	BC	BE	CC	CE	BC	BE	CC	CE	BC	BE	CC	CE	BC	BE
Day 0 (cells/ g sand)	2.1E +04	3.0E +04	2.6E +04	1.9E +04	3.1E +03	4.0E +04	2.6E +04	2.2E +04	1.3E +04	1.9E +04	2.6E +04	1.8E +04	3.5E +03	2.1E +04	2.6E +04	2.7E +04	2.7E +04	4.8E +04	2.6E +04	2.1E +04
Day 6 (cells/ g sand)	2.0E +06	6.2E +06	1.8E +05	3.5E +05	3.2E +06	4.0E +06	1.8E +05	1.0E +04	2.0E +06	9.7E +04	1.8E +05	5.9E +06	1.6E +06	4.7E +05	1.8E +05	9.7E +05	3.1E +06	4.5E +05	1.8E+ 05	4.5E+ 05
Fold Increase in numbers calculated from Day 0	97	209	7	18	1022	99	7	-2	155	5	7	326	467	23	7	36	116	9	7	21

CC - *Candida* control; CE - *Candida* experimental; BC - *Burkholderia* control; BE - *Burkholderia* experimental

## **CHAPTER 5**

### **General Conclusions and Future Research**

## 5.1 General Conclusions

Settled sewage, allowed to further settle for four days, in the storage tank resulted in concentrations of faecal coliforms, yeasts and *Salmonella* that were equivalent to samples collected from the Plankenburg River. Additionally, a four-day settling period also resulted in equivalent chemical concentrations between the influent of the rhizofiltration system and the Plankenburg River. Thus, settled sewage allowed to further settle for four days, can be used as experimental urban runoff to evaluate the efficiency of wastewater treatment systems such as the rhizofiltration system evaluated in this study.

The planted and unplanted side of the rhizofiltration system showed similar removal rates with regard to suspended solids, ammonium, COD, phosphates and sulphates. Microbiologically, the planted side was more effective than the unplanted side in terms of faecal coliform, yeast and *Salmonella* removal, but no difference was found between the two sides with regard to coliphage removal.

The majority of potential pathogenic yeasts that were isolated from the influent and effluent of the rhizofiltration system belonged to the genus *Candida*, including *C. tropicalis*, *C. glabrata*, *C. krusei* and *C. utilis*. The experimental side was more effective than the control side in removing these yeasts from wastewater. During the same experiments a number of antibiotic resistant bacteria were isolated which seemed to proliferate within the filter, the majority of which formed part of the *Burkholderia cepacia* complex. The role of these bacteria in the ecosystem of the filter is still largely unknown although *Burkholderia* is a well-known inhabitant of the rhizosphere.

When exploring the potential interactions that may occur in the filter sand using *in vitro* experiments, it was found that *B. cepacia* in the sand may be responsible for trapping some of the *Candida* in the wastewater as it percolates through the substrate. *B. cepacia* (CAB 85) was found to be antagonistic towards *C. tropicalis* (CAB 80) *C. glabrata* (CAB 84), *C. albicans* (CAB 629) and *C. utilis* (CAB 78) within this environment, while the numbers of *C. krusei* (CAB 83) increased in sand colonised with *B. cepacia* (CAB 85) when compared with the control. These interspecific differences in the interactions between *B. cepacia* and the yeasts may provide a clue to varying levels of survival of the different yeast species in sewage polluted water.

The sand filter on the planted side of the rhizofiltration system was significantly more effective at removing faecal coliforms, potentially pathogenic yeasts and *Salmonella* spp., when compared with the unplanted side. There was also a significant reduction in pathogen indicator numbers within the sand on the planted side which indicated that a rhizofiltration system would be effective at not only removing pathogens from wastewater during rain events, but also at destroying pathogens after a period of time.

To conclude, the rhizofiltration does show potential to be used for the removal of pathogens from urban runoff. Different operating conditions may render the system more efficient to remove unwanted nutrients and pathogens from wastewater. Also, the mechanisms of how the pathogens are trapped and destroyed within filter are largely unknown.

## 5.2 Future Research

Further studies are needed to determine if microbial and chemical removal efficiencies increase on the planted side of the rhizofiltration system after the reeds are allowed to grow for a longer period of time. Additionally, the effect of alternative plant species, and different operating conditions, including a longer hydraulic retention time, on pathogen and nutrient removal should be evaluated.

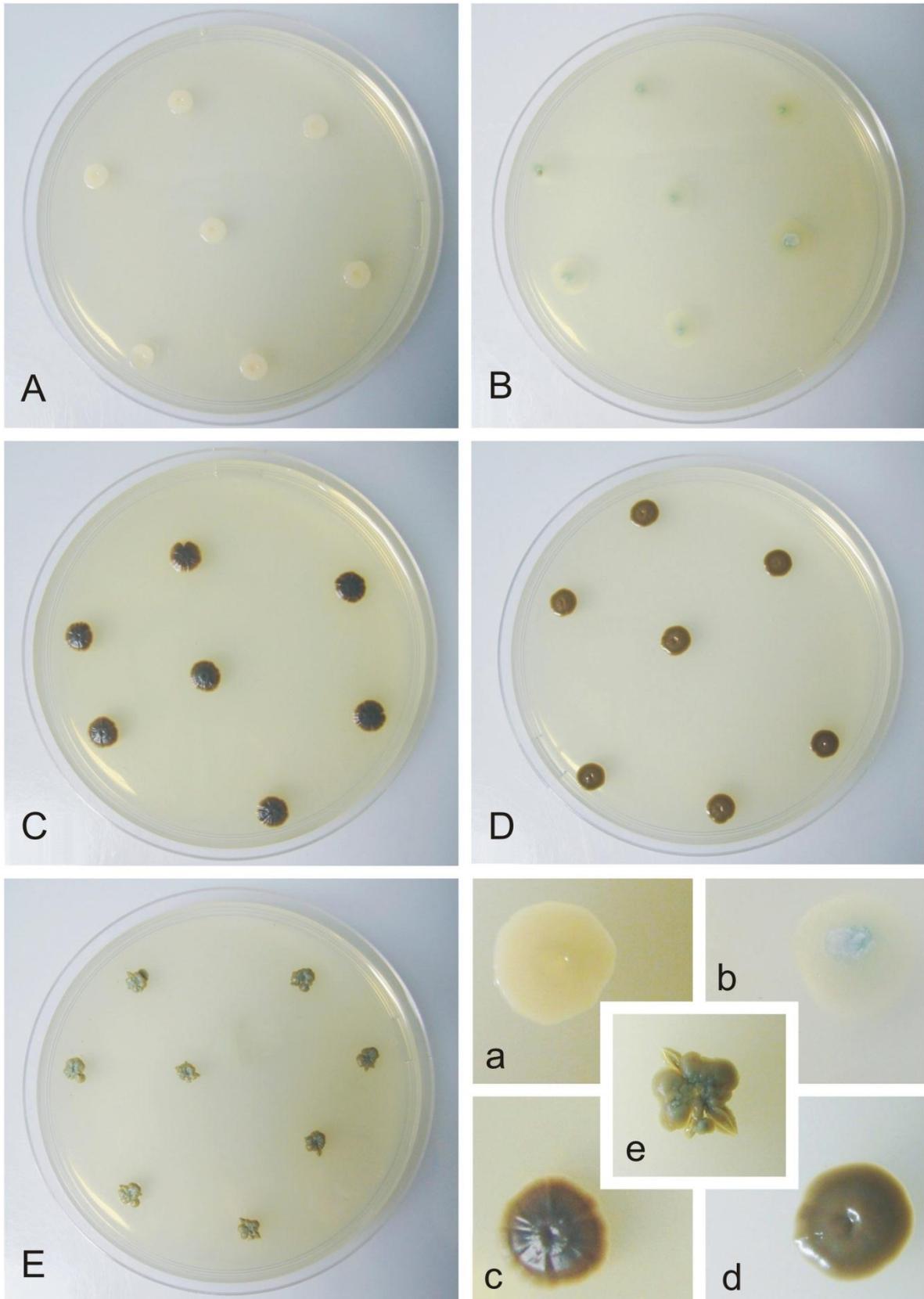
To optimally hone the rhizofiltration system for pathogen and nutrient removal from wastewater it is imperative that the mechanisms of pathogen removal within the rhizosphere are known. Therefore, research is also needed on the interactions of *B. cepacia* with different *Candida* species. Also, the role of root exudates and microbial predators, as well as bacteriophages, in eliminating pathogens from the rhizofiltration system should be studied. This work should be conducted with the knowledge that many complex interactions occur in wetlands.

# **Addendum A**

**Yeast and bacterial isolates.**

**Table 1 – Yeast and bacterial strains isolated from the influent and effluent of the rhizofiltration system.**

Strain number	Organism
CAB 76	<i>Candida lusitanae</i>
CAB 77	<i>Hanseniaspora opuntiae</i>
CAB 78	<i>Candida utilis</i>
CAB 79	<i>Saccharomyces cerevisiae</i>
CAB 80	<i>Candida tropicalis</i>
CAB 81	<i>Candida glabrata</i>
CAB 82	<i>Candida tropicalis</i>
CAB 83	<i>Candida krusei</i>
CAB 84	<i>Candida glabrata</i>
CAB 85	<i>Burkholderia cepacia</i>
CAB 86	<i>Burkholderia multivorans</i>
CAB 87	<i>Escherichia coli O157:H7</i>
CAB 88	<i>Burkholderia species</i>



**Fig. 1 – Colony morphology of yeast strains, representing different species, growing on Molybdate agar. The photographs illustrate differences in colour, texture and margin of the colonies. A, a: *Candida glabrata* (CAB 81); B, b: *Candida krusei* (CAB 83); C, c: *Candida tropicalis* (CAB 82); D, d: *Candida utilis* (CAB 78); E, e: *Saccharomyces cerevisiae* (CAB 79).**

## **Addendum B**

**Microbial and chemical concentrations in samples collected from the rhizofiltration system over the entire sampling period.**

**Table 1 – Chemical and microbial concentrations of samples collected on 14 November 2011 at the experimental side (Well B and Well E) and control side (Well G and Well J) of the rhizofiltration system.**

	<b>Faecal coliforms/ 100 ml</b>	<b>Yeasts /100ml</b>	<b>Coliphage / 100ml</b>	<b>COD (mg/L)</b>	<b>NH<sub>4</sub> (mg/L)</b>	<b>Cl (mg/L)</b>	<b>SO<sub>4</sub> (mg/L)</b>	<b>PO<sub>4</sub> (mg/L)</b>	<b>NO<sub>3</sub> (mg/L)</b>	<b>Suspended solids (mg/L)</b>	<b>Turbidity (NTU)</b>	<b>pH</b>	<b>Temp (°C)</b>
<b>Influent</b>	358 333	3 800 000	103 333	1058	62	104	n/d	45	n/d	139	127	5.93	19
<b>Well B</b>	<b>226 667</b> (37%)	<b>600 000*</b> (84%)	<b>35000*</b> (66%)	370	19	100	12.18	42	104	51	32	5.89	17
<b>Well E</b>	<b>220 000**</b> (39%)	<b>996 667</b> (74%)	<b>41333</b> (60%)	261	31	87	8.14	33	51	55	32	6.04	17
<b>Well G</b>	<b>275 000</b> (23%)	<b>890 000*</b> (77%)	<b>21000*</b> (80%)	246	20	84	14.04	42	79	56	33	6.22	17
<b>Well J</b>	336 667** (6%)	<b>1 300 000</b> (66%)	<b>43000</b> (58%)	307	30	85	8.14	37	49	55	30	6.25	17

**Bold** = Numbers are significantly different ( $p < 0.05$ ) to influent; % = Removal efficiency; \* = Well B is significantly different ( $p < 0.05$ ) to Well G; \*\* = Well E is significantly different ( $p < 0.05$ ) to Well J. Microbial values represent the average of six replicates.

**Table 2 – Chemical and microbial concentrations of samples collected on 5 December 2011 at the experimental side (Well B and Well E) and control side (Well G and Well J) of the rhizofiltration system.**

	<b>Faecal coliforms/ 100 ml</b>	<b>Yeasts /100ml</b>	<b>Coliphage / 100ml</b>	<b>COD (mg/L )</b>	<b>NH<sub>4</sub> (mg/L)</b>	<b>Cl (mg/L)</b>	<b>SO<sub>4</sub> (mg/L)</b>	<b>PO<sub>4</sub> (mg/L)</b>	<b>NO<sub>3</sub> (mg/L)</b>	<b>Suspended solids (mg/L)</b>	<b>Turbidity (NTU)</b>	<b>pH</b>	<b>Temp (°C)</b>
<b>Influent</b>	563 333	7 066 667	117 333	507	57.5	101.3	13.09	49.07	n/d	391	303	6.70	25
<b>Well B</b>	<b>300 000*</b> (47%)	<b>3 766 667*</b> (47%)	<b>35 100*</b> (70%)	286 (44%)	14.9	96.2	15.86	n/d	n/d	60	28.77	6.13	24
<b>Well E</b>	<b>310 000**</b> (45%)	<b>3 933 333*</b> (44%)	<b>40 667</b> (65%)	284 (44%)	21.4	98.0	22.72	19.27	n/d	83	27.47	6.39	25
<b>Well G</b>	<b>396 667*</b> (30%)	<b>5 666 667*</b> (20%)	<b>52 000*</b> (56%)	360 (29%)	16.7	100.7	30.1	n/d	126.62	133	20.72	5.16	24
<b>Well J</b>	<b>446 667**</b> (21%)	<b>4 766 667*</b> (33%)	<b>44667</b> (62%)	322 (36%)	26.4	109.3	16.42	23.03	n/d	117	30.83	6.42	24

**Bold** = Numbers are significantly different ( $p < 0.05$ ) to influent; % = Removal efficiency; \* = Well B is significantly different ( $p < 0.05$ ) to Well G; \*\* = Well E is significantly different ( $p < 0.05$ ) to Well J. Microbial values represent the average of six replicates.

**Table 3 - Chemical and microbial concentrations of samples collected on 7 February 2012 at the experimental side (Well B and Well E) and control side (Well G and Well J) of the rhizofiltration system.**

	Faecal coliforms /100 ml	Yeasts /100ml	Bacteria/100ml	Salmonella/100 ml	Coliphage (CFU) / 100ml	COD (mg/L)	NH <sub>4</sub> (mg/L)	Cl (mg/L)	SO <sub>4</sub> (mg/L)	PO <sub>4</sub> (mg/L)	NO <sub>3</sub> (mg/L)	Suspended solids (mg/L)	pH	Temp (°C)
<b>Influent</b>	335000	321 667	<b>0</b>	18 000	55 667	274	<b>61.5</b>	100.5	19	32	66	349	6.59	30
<b>Well B</b>	<b>201667</b>	<b>15 000</b>	<b>588 333</b>	83 333	25 717	219	29.5	106.5	18	31	<b>119</b>	<b>28</b>	5.99	28
<b>Well E</b>	218333	<b>10 000*</b>	<b>595 000</b>	58 333	<b>21 600</b>	173	35	101.5	19	20	<b>102</b>	<b>28</b>	6.33	27
<b>Well G</b>	<b>218333</b>	<b>36 667</b>	<b>1 035 000</b>	<b>88 333</b>	<b>21 217</b>	150	27.5	98	11	16	<b>98</b>	<b>25</b>	5.41	28
<b>Well J</b>	201667 <b>213333</b>	<b>46 667*</b>	<b>730 000</b>	<b>110 000</b>	<b>21 183</b>	176	34	104.5	23	18	<b>105</b>	<b>25</b>	5.87	27

**Bold** = Numbers are significantly different ( $p < 0.05$ ) to influent; % = Removal efficiency; \* = Well B is significantly different ( $p < 0.05$ ) to Well G; \*\* = Well E is significantly different ( $p < 0.05$ ) to Well J. Microbial values represent the average of six replicates.

**Table 4 – Chemical and microbial concentrations of samples collected on 12 March 2012 at the experimental side (Well B and Well E) and control side (Well G and Well J) of the rhizofiltration system.**

	Faecal coliforms /100 ml	Yeasts /100ml	Bacteria /100ml	<i>Salmonella</i> /100 ml	Coliphage (CFU) / 100ml	COD (mg/L)	NH <sub>4</sub> -N (mg/L)	Cl (mg/L)	SO <sub>4</sub> (mg/L)	PO <sub>4</sub> (mg/L)	NO <sub>3</sub> (mg/L)	Suspended solids (mg/L)	pH	Temp (°C)
<b>Influent</b>	355 000	110 000	260 000	15 000	120 333	450	66	90.3	12.3	n/d	nd	365	6.75	28
<b>Well B</b>	<b>210 000 (41%)</b>	<b>10 000</b>	<b>943 333*</b>	180 000	<b>75 000</b>	299	<b>21</b>	92.6	18.2	n/d	<b>97.1</b>	<b>51</b>	6.52	26
<b>Well E</b>	<b>233 333 (34%)</b>	<b>13 333**</b>	<b>713 333*</b>	161 667	<b>78 167</b>	317	<b>31</b>	92	21.9	n/d	<b>44.8</b>	<b>52</b>	6.88	26
<b>Well G</b>	<b>271 667 (23%)</b>	<b>20 000</b>	<b>638 333*</b>	<b>175 000</b>	<b>74 167</b>	303	<b>18</b>	93	23.3	n/d	<b>127</b>	<b>36</b>	6.27	26
<b>Well J</b>	<b>263 333 (26%)</b>	<b>25000**</b>	<b>631 667*</b>	230 000	<b>82 667</b>	313	<b>23</b>	94.3	13.7	n/d	<b>74.8</b>	<b>52</b>	6.50	26

**Bold** = Numbers are significantly different ( $p < 0.05$ ) to influent; % = Removal efficiency; \* = Well B is significantly different ( $p < 0.05$ ) to Well G; \*\* = Well E is significantly different ( $p < 0.05$ ) to Well J. Microbial values represent the average of six replicates.

**Table 5 – Chemical and microbial concentrations of samples collected on 16 April 2012 at the experimental side (Well B and Well E) and control side (Well G and Well J) of the rhizofiltration system.**

	Faecal coliforms/ 100 ml	Yeasts /100ml	Bacteria /100ml	<i>Salmonella</i> / 100 ml	Coliphage (CFU) / 100ml	COD (mg/L)	NH <sub>4</sub> -N (mg/L)	Cl (mg/L)	SO <sub>4</sub> (mg/L)	PO <sub>4</sub> (mg/L)	NO <sub>3</sub> (mg/L)	Suspended solids (mg/L)	pH	Temp (°C)
<b>Influent</b>	390 000	78 333	963 333	398 333	30 333	445	40	181	0	n/d	n/d	138	6.47	17
<b>Well B</b>	<b>221 667*</b> (43%)	<b>0*</b> (100%)	913 333	254 000 (36%)	26 333	314	<b>16</b>	159	22	n/d	n/d	64	5.67	16
<b>Well E</b>	<b>210 000**</b> (46%)	<b>0**</b> (100%)	861 667	294 000 (26%)	35 000	291	<b>19</b>	122	17	n/d	n/d	79	5.87	16
<b>Well G</b>	<b>325 000*</b> (17%)	<b>18 333*</b> (77%)	686 667	323 333 (19%)	29 667	239	<b>15</b>	198	17	n/d	n/d	46	5.59	16
<b>Well J</b>	335 000** (14%)	<b>23 333*</b> * (70%)	696 667	256 667 (36%)	<b>20 667</b>	307	<b>21</b>	143	16	n/d	n/d	83	5.61	16

**Bold** = Numbers are significantly different ( $p < 0.05$ ) to influent; % = Removal efficiency; \* = Well B is significantly different ( $p < 0.05$ ) to Well G; \*\* = Well E is significantly different ( $p < 0.05$ ) to Well J. Microbial values represent the average of six replicates.

**Table 6 – Chemical and microbial concentrations of samples collected on 7 May 2012 at the experimental side (Well B and Well E) and control side (Well G and Well J) of the rhizofiltration system.**

	Faecal coliforms /100 ml	Yeasts /100ml	Bacteria /100ml	<i>Salmonella</i> /100 ml	Coliphage (CFU) / 100ml	COD (mg/L)	NH <sub>4</sub> (mg/L)	Cl (mg/L)	SO <sub>4</sub> (mg/L)	PO <sub>4</sub> (mg/L)	NO <sub>3</sub> (mg/L)	Suspended solids (mg/L)	pH	Temp (°C)
<b>Influent</b>	608 333	115000	348 333	513 333	31 833	394	<b>41</b>	91	16	0	0	137	6.77	14
<b>Well B</b>	<b>305 000 (50%)*</b>	<b>0</b>	<b>555 000*</b>	<b>371 667 (28%)</b>	<b>24 217 (24%)*</b>	274	13	90	12	0	0	<b>77</b>	6.11	13
<b>Well E</b>	<b>268 333 (56%)**</b>	<b>0</b>	<b>528 333*</b>	<b>271 667 (47%)**</b>	<b>28 183 (11%)</b>	271	18	90	14	0	0	<b>65</b>	6.24	12
<b>Well G</b>	<b>486 667 (20%)*</b>	<b>16 667 (86%)</b>	340 000*	433 333 (16%)	<b>28 550 (10%)*</b>	256	16	101	11	0	0	<b>36</b>	6.21	13
<b>Well J</b>	<b>488 333 (20%)**</b>	<b>16 667 (86%)</b>	350 000*	<b>406 667 (21%)**</b>	<b>27 833 (13%)</b>	250	16	85	8	0	0	<b>64</b>	6.00	12

**Bold** = Numbers are significantly different ( $p < 0.05$ ) to influent; % = Removal efficiency; \* = Well B is significantly different ( $p < 0.05$ ) to Well G; \*\* = Well E is significantly different ( $p < 0.05$ ) to Well J. Microbial values represent the average of six replicates.

**Table 7 – Chemical and microbial concentrations of samples collected on 19 June 2012 at the experimental side (Well B and Well E) and control side (Well G and Well J) of the rhizofiltration system.**

	Faecal coliforms/100 ml	Yeasts /100ml	Bacteria /100ml	<i>Salmonella</i> /100 ml	Coliphage (CFU) /100ml	COD (mg/L)	NH <sub>4</sub> -N (mg/L)	Cl (mg/L)	SO <sub>4</sub> (mg/L)	PO <sub>4</sub> (mg/L)	NO <sub>3</sub> (mg/L)	Suspended solids (mg/L)	pH	Temp (°C)
<b>Influent</b>	880 000	91 667	551 667	628 333	27 833	400	46	67	0	0	0	102	7.00	13
<b>Well B</b>	<b>393 333*</b> (55%)	<b>0</b>	<b>785 000*</b>	<b>315 000*</b> (50%)	<b>54 167</b>	246	<b>15</b>	106	<b>20</b>	0	0	<b>48</b>	6.50	11
<b>Well E</b>	<b>405 000**</b> (54%)	<b>0</b>	<b>740 000**</b>	<b>370 000**</b> (41%)	<b>64 167</b>	240	<b>17</b>	69	<b>16</b>	0	0	<b>66</b>	6.80	11
<b>Well G</b>	<b>631 667*</b> (28%)	<b>20 000</b> (78%)	<b>498 333*</b>	<b>485 000*</b> (23%)	<b>52 500</b>	235	<b>21</b>	59	<b>15</b>	0	0	<b>62</b>	6.72	12.5
<b>Well J</b>	<b>645 000**</b> (27%)	<b>16 667</b> (82%)	503 333**	<b>476 667**</b> (24%)	<b>56 400</b>	242	<b>22</b>	61	<b>12</b>	0	0	<b>64</b>	6.61	12.5

**Bold** = Numbers are significantly different ( $p < 0.05$ ) to influent; % = Removal efficiency; \* = Well B is significantly different ( $p < 0.05$ ) to Well G; \*\* = Well E is significantly different ( $p < 0.05$ ) to Well J. Microbial values represent the average of six replicates.

**Table 8 – Chemical and microbial concentrations of samples collected on 17 July 2012 at the experimental side (Well B and Well E) and control side (Well G and Well J) of the rhizofiltration system.**

	Faecal coliforms/100 ml	Yeasts /100ml	Bacteria /100ml	<i>Salmonella</i> /100 ml	Coliphage (CFU) / 100ml	COD (mg/L)	NH <sub>4</sub> -N (mg/L)	Cl (mg/L)	SO <sub>4</sub> (mg/L)	PO <sub>4</sub> (mg/L)	NO <sub>3</sub> (mg/L)	Suspended solids (mg/L)	pH	Temp (°C)
<b>Influent</b>	800 000	221 667	418 333	510 000	43 333	604	48	64	0	0	0	89	6.64	12
<b>Well B</b>	<b>293 333*</b> (63%)	<b>0</b> (100%)	<b>611 667*</b>	<b>203 333*</b> (60%)	<b>33 833</b> (22%)	<b>221</b>	<b>14</b>	61	<b>18</b>	15	0	<b>25</b>	6.63	11
<b>Well E</b>	<b>306 667**</b> (62%)	<b>0</b> (100%)	<b>643 333**</b>	<b>211 667**</b> (58%)	<b>33 833</b> (22%)	<b>246</b>	<b>19</b>	57	<b>13</b>	0	0	<b>25</b>	6.46	11
<b>Well G</b>	<b>570 000*</b> (29%)	<b>20 000</b> (91%)	<b>378 333*</b>	<b>355 000*</b> (30%)	<b>33 667</b> (22%)	<b>234</b>	<b>21</b>	50	<b>12</b>	0	0	<b>25</b>	7.03	12
<b>Well J</b>	<b>571 667**</b> (29%)	<b>20 000</b> (91%)	<b>365 000**</b>	<b>323 333**</b> (37%)	<b>31 667</b> (27%)	<b>240</b>	<b>27</b>	56	<b>14</b>	15	0	<b>25</b>	6.89	12

**Bold** = Numbers are significantly different ( $p < 0.05$ ) to influent; % = Removal efficiency; \* = Well B is significantly different ( $p < 0.05$ ) to Well G; \*\* = Well E is significantly different ( $p < 0.05$ ) to Well J. Microbial values represent the average of six replicates.

**Table 9 – Chemical and microbial concentrations of samples collected on 14 August 2012 at the experimental side (Well B and Well E) and control side (Well G and Well J) of the rhizofiltration system.**

	Faecal coliforms /100 ml	Yeasts /100ml	Bacteria /100ml	<i>Salmonella</i> / 100 ml	Coliphage (CFU) / 100ml	COD (mg/L)	NH <sub>4</sub> -N (mg/L)	Cl (mg/L)	SO <sub>4</sub> (mg/L)	PO <sub>4</sub> (mg/L)	NO <sub>3</sub> (mg/L)	Suspended solids (mg/L)	pH	Temp (°C)
<b>Influent</b>	1 256 667	151 667	495 000	975 000	10 967	138	24	78	27	8	0	156	6.65	12
<b>Well B</b>	<b>500 000*</b> (60%)	<b>25 000*</b> (84%)	<b>700 000*</b>	<b>403 333*</b> (59%)	<b>15 050*</b>	<b>46</b>	10	60	26	19	0	<b>25</b>	6.87	10
<b>Well E</b>	<b>491 667*</b> (61%)	<b>25 000**</b> (84%)	<b>681 667**</b>	<b>428 333**</b> (56%)	12 517	126	11	65	25	12	0	<b>25</b>	6.44	10
<b>Well G</b>	<b>995 000*</b> (20%)	<b>73 333*</b> (52%)	453 333*	<b>791 667*</b> (19%)	11 050*	<b>46</b>	8	57	24	11	0	<b>25</b>	6.72	11
<b>Well J</b>	<b>1 003 333**</b> (21%)	<b>86 667**</b> (43%)	505 000**	<b>776 667**</b> (20%)	12 050	<b>52</b>	11	75	24	0	0	<b>25</b>	6.78	11

**Bold** = Numbers are significantly different ( $p < 0.05$ ) to influent; % = Removal efficiency; \* = Well B is significantly different ( $p < 0.05$ ) to Well G; \*\* = Well E is significantly different ( $p < 0.05$ ) to Well J. Microbial values represent the average of six replicates.

## **Addendum C**

**Statistical analysis of differences between the planted and unplanted side of the rhizofiltration system.**

**Table 1 - p – values obtained during Bonferroni test comparing coliform reduction in effluent samples collected from the planted and unplanted side of the rhizofiltration system.**

<b>Month</b>	<b>p value</b>
November	0.0014
December	0.6445
February	1.0000
March	0.7815
April	0.0000
May	0.0000
June	0.0000
July	0.0000
August	0.0000

**Table 2 - p – values obtained during Bonferroni test comparing effluent temperatures between the planted and unplanted side of the rhizofiltration system.**

<b>Month</b>	<b>p value</b>
November	1.0000
December	1.0000
February	1.0000
March	1.0000
April	1.0000
May	1.0000
June	1.0000
July	1.0000
August	0.0000

**Table 3 - p – values obtained during Bonferroni test comparing coliphage reduction effluent samples collected from the planted and unplanted side of the rhizofiltration system.**

<b>Month</b>	<b>p value</b>
November	1.0000
December	1.0000
February	1.0000
March	1.0000
April	1.0000
May	1.0000
June	1.0000
July	1.0000
August	0.0331

**Table 4 - p – values obtained during Bonferroni test comparing potentially pathogenic yeast reduction effluent samples collected from the planted and unplanted side of the rhizofiltration system.**

<b>Month</b>	<b>p value</b>
November	1.0000
December	0.0094
February	1.0000
March	0.1740
April	0.0000
May	0.0009
June	0.0000
July	0.0000
August	0.0000



