Community-level analysis of the microbiology in constructed wetlands treating distillery effluent

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DECLARATION

I, the undersigned, hereby declare that the work contained in this dissertation is my own original work and that I have not previously in its entirety or in part submitted it at any university for a degree.

Signature: ……

Date: ……

SUMMARY

Constructed wetlands have been widely used in the treatment of industrial and domestic wastewater to reduce biological and chemical oxygen demand (BOD and COD), to remove nitrate and enteric viruses as well as to generally improve water quality. Distillery wastewater has a complex character due to high concentrations of sugars, lignins, hemicellulose, dextrins, resins, polyphenols and organic acids, leading to a high COD that may exceed 100 000 mg/L. The potential application for the treatment of distillery wastewater by means of constructed wetlands is relatively unexplored.

In 1999 a study was initiated at Distell Goudini distillery, Western Cape, South Africa, to explore the possibility of using constructed wetlands to treat distillery wastewater. It was found that constructed wetlands do have the ability to treat distillery wastewater providing that the influent COD does not exceed 15 000 mg/L for extended periods and the correct substrate material is used. The present study expanded on the above-mentioned study and specifically aimed to provide information on the microbiological controls in wetland systems in an applied sense that may contribute to improved treatment efficiency. Furthermore, this project aimed to contribute to our fundamental understanding of the microbial ecology of constructed wetlands used for the treatment of distillery wastewater.

This study revealed that a highly dynamic microbial composition exists within wetlands. Furthermore it was found that wetlands can efficiently remove COD even though a low degree of similarity exists between microbial communities in various zones of the same wetland and those between different wetlands, as well as low similarity between communities sampled from the same zone over time. This demonstrates that it will be difficult to define the ‘ideal’ degradative community in terms of microbiological criteria and serves as a reminder that various indicators should be considered for monitoring system health. Furthermore the shifts in microbial community composition illustrate the ability of

microbial communities to adapt to changes in the environment without compromising their functional efficacy. When studying the attached microbial communities within wetland systems it was found that different morphotypes are detected at certain stages of biofilm development while some organisms are present at most phases of biofilm formation.

Measurement of CO_2 production and dissolved organic carbon (DOC) removal in laboratory scale columns showed that grazing protists had a notable effect on overall microbial activity and that organic loading influenced these predator-prey interactions. Interestingly, increased clogging of pores occurred in the presence of protists, resulting in reduced flow through the porous matrix. Terminal-restriction fragment length polymorphism (T-RFLP) analysis of biofilms on gravel in experimental wetlands indicated that the presence of protists and algae had an effect on the microbial community composition. Scanning electron microscopy (SEM) showed that the presence of algae also had an influence on biofilm structure suggesting that the algae provided labile nutrients that were utilized by the bacterial and yeast members of the community. Finally, augmentation with a commercial mixture or microbial populations isolated from distillery effluent demonstrated that the concentration at which supplements are applied influence degradative efficiency.

OPSOMMING

Kunsmatige vleilande word wêreldwyd gebruik in die behandeling van indusriële en huishoudelike afvalwater om biologiese en chemiese suurstof aanvraag (BSA en CSA) te verminder, om nitrate en ingewandsvirusse te verwyder asook om waterkwaliteit in die algemeen te verbeter. Distilleerafvalwater het komplekse eienskappe as gevolg van hoë konsentrasies suiker, lignien, hemisellulose, dekstrien, harpuis, polifenole en organiese sure, wat lei tot ‘n hoë CSA wat 100 000 mg/L kan oorskry. Daar is tot op hede relatief min studies gedoen oor die potensiële gebruik van kunsmatige vleilande vir die behandeling van distilleerafvalwater.

In 1999 is ‘n studie by Distell Goudini distilleeraanleg in die Wes Kaap van Suid Afrika onderneem om die moontlikheid van kunsmatige vleilande vir die behandeling van distilleerafvalwater te bestudeer. Daar was bevind dat kunsmatige vleilande die vermoë het om distilleerafvalwater te behandel gegewe dat die invloeiende CSA nie 15 000 mg/L oorskry nie en dat die regte substraat materiaal gebruik word. Die huidige studie het by die bogenoemde studie aangesluit met die doel om informasie oor die mikrobiologiese kontroles in vleilandsisteme op ‘n toegepaste wyse te voorsien, wat tot verbeterde behandeling doeltreffendheid kan lei. Hierdie studie het verder beoog om by te dra tot ons fundementele kennis van die mikrobiese ekologie van kunsmatige vleilande wat gebruik word vir die behandeling van distilleerafvalwater.

Dié studie het bevind dat daar ‘n hoogs dinamiese mikrobiese samestelling binne vleilande bestaan. Daar was verder bevind dat CSA steeds effektief deur vleilande verwyder kan word alhoewel daar ‘n lae graad van ooreenstemming is tussen mikrobiese gemeenskappe in verskeie sones van dieselfde vleiland en verskillende vleilande, asook ‘n lae graad van ooreenstemming tussen gemeenskappe wat in dieselfde sone oor tyd gemonster is. Dit demonstreer dat dit moeilik sal wees om die ‘ideale’ degraderende gemeenskap te vind in terme

van mikrobiologiese kriteria en dien as ‘n herinnering dat verkeie indikatore in ag geneem moet word om die welstand van ‘n ekologiese sisteem te monitor. Die verskuiwings in mikrobiese gemeenskapsamestelling illustreer verder die vermoë van natuurlike sisteme om aan te pas by veranderinge in die omgewing sonder om funksionele doeltreffendheid te verminder. Die studie van aangehegte mikobiese gemeenskappe het aangedui dat veskillende morfotipes bespeur kan word tydens sekere fases van biofilm formasie terwyl sekere organismes tydens meeste van die fases teenwoordig is.

Die bepaling van CO_2 produksie en die verwydering van opgeloste organiese koolstof in laboratoriumskaal kolomme het geïlustreer dat voedende protiste ‘n waarneembare effek gehad op die algehele mikrobiese aktiwiteit en dat die organiese lading hierdie predator-prooi interaksie beïnvloed het. Dit was interessant om te vind dat die teenwoordigheid van protiste die verstopping van porieë aangehelp het en dus tot verlaagde vloei deur die poreuse matriks gelei het. Terminale-restriksie fragment lengte polimorfisme (T-RFLP) analiese van biolfilm op klipgruis in eksperimentele vleilande het aangedui dat die teenwoordigheid van protiste en alge ‘n effek gehad het op die mikrobiese gemeenskapsamestelling. Skandeerelektronmikroskopie (SEM) het bewys dat die teenwoordigheid van alge ook ‘n invloed op biofilm struktuur gehad het wat daarop dui dat alge maklik afbreekbare voedingstowwe aan die bakterieë en giste van die mikrobiese gemeenskap beskikbaar gestel het. Laastens was bewys dat die konsentrasie van toevoeging van ‘n kommersiële mikrobiese mengsel of mikrobiese populasies wat uit afvoer geïsoleer was, die effektiwiteit van degradering kan beïnvloed.

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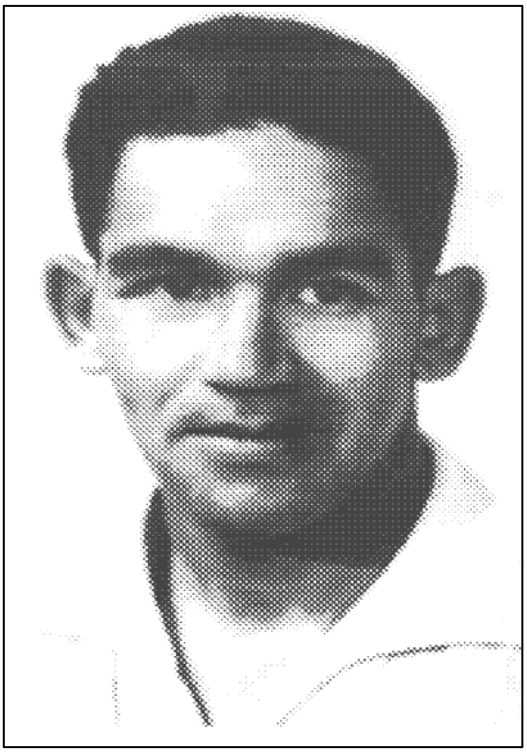
In loving memory of my late father, Henry William Sidwell du Plessis†



† 20/12/1931 – 01/02/1988

Genesis 1 – And God said; … and it was so.

The Holy Bible

TABLE OF CONTENTS

C H A P T E R

5 **99**

Microbial interactions during degradation of distillery effluent

CHAPTER

1

General introduction

GENERAL INTRODUCTION

1.1. GENERAL BACKGROUND

With the increasing pressure from environmental regulations and the growing awareness of the negative impacts of concentrated seasonal discharge of waters containing high nutrient and organic loadings in the nearby water courses, the wine and distillery industries are facing greater restrictions related to the discharge of their wastewater (Masi *et al*., 2002). South Africa has very strict laws concerning the irrigation of treated wastewater.

According to the National Water Act, 1998 (Act No. 36 of 1998) a person may–

(1) irrigate up to 500 cubic meters of domestic or biodegradable industrial wastewater on any given day, provided the –
   (a) electrical conductivity does not exceed 200 milliSiemens per meter (mS/m);
   (b) pH is not less than 6 or more than 9;
   (c) Chemical Oxygen Demand (COD) does not exceed 400 mg/l after removal of algae;
   (d) faecal coliforms do not exceed 100 000 per 100 ml; and
   (e) Sodium Adsorption Ratio (SAR) does not exceed 5 for biodegradable industrial wastewater.

(2) irrigate up to 50 cubic meters of biodegradable industrial wastewater on any given day, provided the –
   (a) electrical conductivity does not exceed 200 milliSiemens per meter (mS/m);
   (b) pH is not less than 6 or more than 9;
   (c) Chemical Oxygen Demand (COD) does not exceed 5 000 mg/l after removal of algae;
   (d) faecal coliforms do not exceed 100 000 per 100 ml; and

(e) Sodium Adsorption Ratio (SAR) does not exceed 5 for biodegradable industrial wastewater.

Furthermore, wastewater irrigation may also only take place –

(a) above the 100 year flood line, or alternatively, more than 100 meters from the edge of a water resource or a borehole which is utilized for drinking water or stock watering; and

(b) on land that is not or does not overlie a major aquifer (Government Gazette No. 20526, 8 October 1999).

Consequently, the treatment of winery and distillery wastewater requires particular attention and demands careful consideration of all available options (Masi *et al*., 2002). Treatment systems must have an ability to treat both vintage (during harvest season) and non-vintage flows (Goss, 2003). Considering the large differences in effluent volumes in the different seasons, this requirement needs careful consideration during the design of treatment facilities. For example, if design parameters are based on mean annual flow volumes, the wastewater will pass through the treatment system with too short retention time in vintage and thus not receive sufficient treatment, or retention time may be too long during non-vintage with the result that the wastewater turns anaerobic and generates malodours.

It is known that bacterial communities in nature play a key role in the production and degradation of organic matter, the degradation of many environmental pollutants, the cycling of nutrients such as nitrogen and sulfur, as well as the attenuation and cycling of metals (Davey & O'Toole, 2000). The size and diversity of microbial communities are directly related to the quality and quantity of the resources available in the system (Reddy *et al*., 2002). In natural wetlands, microorganisms colonise the water column, periphyton mats, plant detritus and the soil/gravel support matrix, making a notable contribution to the overall functioning and stability of wetlands. Furthermore, because of the short life cycles of microorganisms, they respond rapidly to changes in nutrient or

energy source status in wetlands, a factor that may be crucial for the maintenance of wetland systems that are exposed to the variation typically associated with industrial wastewaters. Extensive work has been done on the use of constructed wetlands to treat various types of wastewater. However, the use of these systems in the treatment of distillery wastewater is limited. Also, little is known of microorganisms within constructed wetland systems.

With the above as background it was the aim of this study to evaluate the microbial controls within constructed wetlands used for distillery effluent treatment.

1.2. HYPOTHESIS AND OBJECTIVES OF THIS STUDY

1.2.1. Hypothesis

A highly dynamic microbial distribution exists throughout a constructed wetland due to the variable distribution of effluent as well as decreasing effluent strength further down the system as a result of it being treated. There also exist a variation in the planktonic and attached microbial (biofilm) communities within these systems with both protists and algae having an impact on biofilm formation within wetland systems.

1.2.2. Objectives

The overall objective of this study was to evaluate the microbial controls within constructed wetlands used in the treatment of distillery wastewater.

Specific objectives were to:

a. determine the behavior of wetland microbial communities when challenged with the high organic loading associated with distillery effluents as well as to follow community structure and function in pilot scale constructed wetlands fed with distillery effluent;

b. investigate the impact of protistan grazing on nutrient flow as well as hydraulic properties in columns inoculated with the EPS secreting yeast *Cryptococcus laurentii*;
c. determine the impact of algae on the initial stages of biofilm formation on the gravel support matrix;
d. evaluate bio-augmentation as a means to improve COD removal by comparing a commercial product with three bacterial strains isolated from the raw distillery effluent.

Experimentation was conducted on three different scales:

i. Large scale (i.e. two 45 m long x 6 m wide wetlands of which one has a soil and the other a gravel substrate as well as three 6 m long x 3 m wide gravel wetlands with different retention times established at Distell Goudini distillery, South Africa);
ii. Pilot scale (i.e. two laboratory model-scale constructed wetlands that are 30 cm wide, 40 cm high and 50 cm long) and
iii. Mini scale (i.e. 50 ml laboratory columns containing 2 mm diameter glass beads).

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CHAPTER

2

Literature review

LITERATURE REVIEW

2.1. WINERY WASTEWATER

Over the past decade, the wine industry has experienced unprecedented growth (Brooker & Smyl, 2003). Along with production, problems of waste disposal have grown at a rapid rate. Approximately 2 to 4 kilo liters of waste effluent are generated per tonne of grapes crushed, up to 60% which is generated during vintage periods. In addition to water contamination by winery waste, the extensive use of caustic soda for washing equipment results in wastewater being moderately saline and highly alkaline. High-volume water use in cleaning also results in many land-based discharge systems being unable to cope with the volume of wastewater produced post vintage, especially during the cold, wet months. During these periods, producers often have to transport the excess to alternative disposal sites, at great cost, both financially and environmentally.

Winery wastes contain a rich cocktail of tannins, complex carbohydrates and protein dissolved or suspended in water (Brooker & Smyl, 2003). Although winery wastewaters are mainly characterized by the high content of organic material and nutrients, high acidity, and large variations in a seasonal flow production, (Masi *et al*., 2002) its characteristics depend on both the type of wine produced and the specific management practices at the winery (Shepherd *et al*., 2001). For example, the frequency with which winery floors are washed varies from once to four times a day. Other production variables affecting wastewater quantity and quality include methods of tank disinfection (steam sterilization versus chemical), type of disinfectant used, and filtering method (diatomaceous earth versus mechanical methods).

2.2. DISTILLERY WASTEWATER

Distillery wastewater (also termed stillage, distillery pot ale, distillery slops, distillery spent wash, dunder, mosto, vinasse and thin stillage) is the aqueous by-product from the distillation of ethanol following fermentation of carbohydrates (Wilkie *et al*., 2000). The production of ethanol from biomass, whether from sugar crops (sugar beets, sugar cane, molasses, etc.), starch crops (corn, wheat, rice, cassava, etc.), diary products (whey) or cellulosic materials (crop residues, herbaceous energy crops, wood, or municipal waste), results in the concurrent production of distillery wastewater which exhibits a considerable pollution potential (Sheehan and Greenfield, 1980; Callander *et al*., 1986). Up to 20 L of distillery wastewater may be generated for each liter of ethanol produced (van Haandel and Catunda, 1994) and the chemical oxygen demand (COD - a measure of the amount of oxygen required to oxidize the organic and inorganic matter in wastewater using a chemically oxidizing agent) can exceed 100 g/L (Yeoh, 1997). As a consequence, a medium-sized ethanol production facility producing 10^6 L ethanol/year can generate distillery wastewater with a pollution level equivalent to the sewage of a city with a population of 20 000.

The production and characteristics of distillery wastewater are highly variable and dependent on feedstock and various aspects of the ethanol production process (Wilkie *et al.*, 2000). Wash water used to clean the fermenters, cooling water blow down and boiler water blow down may all be combined with the distillery wastewater and contribute to its variability. Organic components of distillery wastewater have been studied by several researchers (Dowd *et al*., 1994; Benke *et al*., 1998; Pandiyan *et al*., 1999). The principle low molecular weight components of distillery wastewater from various feedstocks were found to be lactic acid, glycerol, ethanol, acetic acid, lactose, glucose, arabinitol, ribitol, trace amounts of amino acids as well as alanine and proline (Dowd *et al*., 1994).

Other important characteristics of distillery wastewater include colour, heavy metals content and the presence of organic priority pollutants (Wilkie *et al.*, 2000). Highly coloured effluents can have negative environmental impacts if released into surface waters, where it may disrupt the growth of normal aquatic flora. Phenolics (tannic and humic acids) from feedstock (Sierra-Alvarez *et al*., 1994), melanoidins from reaction of sugars with proteins (Chen and Chou, 1993), caramels from overheated sugars (Chen and Chou, 1993) and furfurals from acid hydrolysis (Rivard and Grohmann, 1991) can contribute to the colour of the effluent. In addition, these compounds are known to be inhibitory to biological treatment of the distillery wastewater (Field and Lettinga, 1987; Field *et al*., 1990; Ehlinger *et al*., 1992). Also, melanoidins are known to be mutagenic (Kitts *et al*., 1993a; 1993b).

Heavy metals including chromium, copper, nickel and zinc have been detected in effluents from several ethanol production facilities (USEPA, 1986). While some heavy metals may be introduced from the feedstock and chemicals used, corrosion of piping, tanks and heat exchangers is expected and may contribute to heavy metal levels in the effluent (Wilkie *et al.*, 2000). Organic priority pollutants, including chloroform, methylene chloride, pentachlorophenol, and phenol were also found in some ethanol production facilities (USEPA, 1986).

A variety of treatment methods and strategies like thermal pre-treatment, wet air oxidation, concentration-incineration, anaerobic treatment, etc., have been suggested or tested for the treatment of distillery wastewater (Sangave and Pandit, 2004). All these schemes on their own are either incomplete, or are impractical or unviable. The use of constructed wetlands is a relative unexplored method for distillery wastewater treatment.

2.3. CONSTRUCTED WETLANDS FOR DISTILLERY WASTEWATER TREATMENT

2.3.1. Wetlands

There is no single “correct” definition of wetlands for all purposes (Hammer and Bastian, 1989). Several definition and classification systems have been devised for differing needs and purposes. Just as the predominant types of wetlands vary from one region to another, names commonly used to describe them also vary regionally. Many types of wetlands often have above-ground water only after heavy rains or during one season of the year.

Wetlands are recognized as a transition between terrestrial and aquatic systems. In wetlands water is the dominant factor determining development of soils and associated biological communities and where, at least periodically, the water table is at or near the surface, or the land is covered by shallow water (Cowardin *et al*., 1979). This definition and classification system encompassed and systemically organized all types of wetland habitats for scientific purposes. Specifically, it required that wetlands meet one or more of three conditions: (1) Areas that, at least periodically, support predominantly hydrophytes. (2) Areas with predominantly undrained hydric soil (*i.e.* wet enough for long enough to produce anaerobic conditions that limit the types of plants that can grow there). (3) Areas with non-soil substrates (*e.g*. rock or gravel) that are saturated or covered by shallow water at some time during the growing season.

Important wetland functions include natural restorative processes, which improve water quality while being cost effective; conveyance and storage of storm water, which dampens the effects of flooding; reduction of flood flows and velocity of storm water, which reduces erosion and increase sedimentation; and modification of pollutants typically carried in storm water (Kao and Wu, 2000). Wetlands along coasts, lakeshores and riverbanks have a valuable role in stabilizing shorelands and protecting them from the erosive battering of tides,

waves, storms and wind (Hammer and Bastian, 1989). Perhaps the most important, but least understood function of wetlands is water quality improvement. Wetlands provide free treatment for many types of water pollution and can effectively remove or convert large quantities of pollutants including organic matter, suspended solids, metals and excess nutrients (*i.e.* >50% removal/conversion of various pollutants). Ye *et al.* (2001), for example, found a decrease of up to 99% for Fe, 91% for Cd and 63% for Zn while S and Mn were each reduced by more than 50%, while studying trace element removal from coal ash leachate by a 10-year-old constructed wetland.

Many wetlands are open systems that receive allochthonous inputs (microorganisms or substances not normally present) of organic matter, nutrients, metals and toxic organic compounds from adjacent agricultural watersheds, industrial and urban areas (Reddy and D'Angelo, 1997). Some wetlands are capable of attenuating pollutants while others are sensitive to contaminants (*i.e.* plants show signs of stress with a gas formation at the surface of the wetland – see Figure 4.7). Differences between wetland systems are largely due to differences in physical, chemical and biological conditions that affect transformations and transport processes and treatment efficiency in the soil-water-plant system.

Natural wetlands often do not function efficiently for purposes of permanent storage or controlled down-gradient discharge of nutrients or pollutants (Wetzel, 2000). Various conservation efforts have also further inhibited use of natural wetlands for applied purposes. These and many other factors have therefore resulted in rapid development of constructed wetlands in an attempt to simulate and potentially enhance optimal properties of natural wetlands in performing the desired functions.

2.3.2. Constructed wetlands

Economic growth has generally resulted in increases in the volumes of wastewater generated (Gui *et al.*, 2000). The pollution of rivers and lakes had been, and still is, a crisis for both developed and developing countries. It is still a difficulty to remove nutrients from wastewater in an economical way even though many wastewater technologies have been developed. Constructed wetlands may offer a low cost treatment option for domestic wastewater, which is especially suitable for developing countries (Haberl *et al.*, 1995; Kadlec, 1995).

A constructed wetland is a designed and man-made complex of saturated substrates, emergent and submergent vegetation, animal life, and water that simulate natural wetlands for human use and benefits (Hammer and Bastian, 1989). Constructed wetlands are emerging as a low-cost approach to improve treatment of point and diffuse discharges around the world (Reed and Brown, 1992; Gale *et al*., 1993). This includes Northern and Eastern Europe, South America, Canada, New Zealand, Australia and South Africa where COD removal efficiencies have been proven to be in excess of 85% (*e.g.* Cerezo *et al.*, 2001). Constructed wetlands are preferred above natural wetlands because they have more engineered systems and are easier to control (Ayaz and Akça, 2001). These low-tech treatment systems are often more economic than energy-intensive engineered treatment plants. In addition, they are easier to operate, and provide numerous secondary benefits (*e.g*. habitat enhancement) (Pinney *et al.*, 2000). At present, there are thousands of wetland-based wastewater treatment systems around the world (Reddy and D'Angelo, 1997).

There are basically two types of constructed wetlands, namely, free water surface (FWS) wetlands and subsurface flow wetlands (SF) (Polprasert *et al*., 1998). In FWS constructed wetlands the water level is above the ground surface and the majority of water flow is above it while SF systems are designed to conduct water through the bed of the system and the water surface lies below the ground surface. A FWS system consists of a basin or channels with a natural or

constructed subsurface barrier to minimize seepage (Crites, 1994). Emergent vegetation is grown and wastewater is treated as it flows through the vegetation and plant litter. A SF wetland system consists of channels or basins that contain gravel or sand media, which will support growth of emergent vegetation. The bed of impermeable material is sloped typically between 0 and 2 percent. Wastewater flows horizontally through the root zone of the wetland plants about 100 – 150 mm below the gravel surface. The treated effluent is collected in an outlet channel or pipe.

Constructed wetlands are widely 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). According to the study of Reed and Brown (1992) both the FWS and SF wetland concepts seem to be reliable and cost-effective methods for wastewater treatment. The SF constructed wetland can provide control of odors and insect vectors, and reduce concerns over public access issues which can be problematic for FWS wetland systems. For these reasons SF constructed wetlands are particularly well suited as small treatment systems in relatively close proximity to the public.

For optimal wastewater treatment to be achieved it is essential to have detailed knowledge when designing constructed wetlands. The effectiveness of various plant species, the colonization characteristics of certain groups of microorganisms and how biogenic compounds and particular contaminants (wastewater components) interact with the filter bed material should be studied in detail. Specific water quality entering wetlands will control design criteria (Pinney *et al.*, 2000). Consequently, existing wetlands are designed with a wide range of hydraulic retention times (HRTs), generally ranging from 2 to 20 days. Longer HRTs are generally employed to permit aeration via diffusion from the atmosphere for BOD removal and nitrification. Shorter HRTs are often employed when the wetlands receive higher quality treated wastewater and the wetland are

used for other design objectives *e.g.* contaminant polishing or habitat enhancement.

2.3.3. Wetland plants (Macrophytes)

Although bogs (poorly drained, peat-filled areas) and swamps (wetland characterised by the presence of trees growing on silty and organic soils) have been constructed for wastewater treatment, most constructed wetlands for wastewater treatment emulate marshes (treeless wetland where grasses, sedges and cattails predominate) (Hammer and Bastian, 1989). Marshes with herbaceous emergent and submergent plants show the most promise for wastewater treatment. Unlike bogs and swamps, marshes with cattail, bulrush, rush, or giant reed are adapted to fluctuating water and nutrient levels and are more tolerant of high pollutant concentrations.

The plants growing in wetlands, often called wetland plants or macrophytes, are adapted to growing in water-saturated soils (Brix, 1994). These include aquatic vascular plants, aquatic mosses and some larger algae (Brix, 1997). Emergent macrophytes selected for growth in artificial systems should be robust in habit, have a high biomass throughout the year and be readily available in the local area (Chambers and McComb, 1994). Species that propagate well from rhizome segments rather than seed are more desirable. Although all the plant species listed in Table 2.1 are suitable, the common reed and cattails are the emergent plants most used in constructed wetlands (Gómez Cerezo *et al*., 2001; Stottmeister *et al*., 2003). *Phragmites australis* and *Typha*, favoured in European and United States wetland systems respectively are both capable of adapting to different environments and waterloads, showing a high growth rate in a short time.

Macrophytes have several intrinsic properties that make them an indispensable component of constructed wetlands (Brix, 1994). The most important effects of the macrophytes, in relation to the wastewater treatment

processes, are the physical effects brought about by the plant tissues (*e.g.* erosion control, filtration effect and provision of surface are for attached microorganisms). Marsh plants possess specific characteristics of growth physiology that guarantee their survival even under extreme rhizosphere conditions (Stottmeister *et al*., 2003). The following extreme conditions can be found in the rhizosphere in wetlands used to treat wastewater:

- Highly reduced milieu (Eh up to < -200 mV) prompting the formation of H_2S and CH_4;
- Acidic or alkaline conditions;
- Toxic wastewater components such as phenols, biocides, heavy metals, etc.;
- Salinity.

Table 2.1. Selection of plant species used in constructed wetlands.

Scientific name	English name
Phragmytes australis (Cav.) Trin. Ex Steud.	common reed
Juncus spp.	rushes
Scirpus spp.	bulrushes
Typha angustifolia L.	narrow-leaved cattail
Typha latifolia L.	broad-leaved cattail
Iris pseudacorus L.	yellow flag
Acorus calamus L.	sweet flag
Glyceria maxima (Hartm.) Holmb.	reed grass
Carex spp.	sedges

Stottmeister *et al*., 2003

The active zone of constructed wetlands is the root zone (or rhizosphere) (Stottmeister *et al*., 2003). This is where physiochemical and biological processes take place that are induced by interaction of plants, microorganisms, the substrate (soil or gravel) and pollutants. Through absorption and assimilation, wetland plants remove nutrients for biomass production (Hammer and Bastian, 1989). One abundant by-product of the plant growth process is oxygen, which

increases the dissolved oxygen content of the water and also of the soil in the immediate vicinity of plant roots. This increases the capacity of the system for aerobic bacterial decomposition of pollutants as well as its capacity for supporting a wide range of oxygen-using aquatic organisms, some of which directly or indirectly utilize additional pollutants.

The macrophytes have other, site-specific, valuable functions such as providing a suitable habitat for wildlife and giving systems an aesthetically pleasing appearance, *e.g*. the Yellow Flag (*Pseudacorus* or *Canna*-lillies). In the study by Ayaz & Akça (2001) the best performances amongst *Phragmites*, *Cyperus*, *Rush*, *Iris*, *Lolium*, *Canna* and *Paspalum* were by *Iris* for COD (94%), total nitrogen (90%) and phosphorus (55%) removal and *Canna* for ammonia nitrogen (98%) removal. In another study by Lui *et al*. (2000) reductions of up to 96% for ammonium and 82% for phosphate have been reported for freshwater systems by these aesthetically pleasing plants.

Also, the stems and leaves of macrophytes that are submerged in the water column provide a large surface area for microbial biofilms (see section 1.5.1) (Brix, 1997). Dense communities of photosynthetic algae, as well as bacteria and protists colonize the plant tissues. The roots and rhizomes that are buried in the wetland soil also provide a substrate for attached growth of microorganisms. Thus, the biofilms present on the above and below ground tissue of macrophytes as well as the biofilms on all other immersed solid surfaces in the wetland system, are responsible for the majority of the microbial processing that occurs in wetlands. On the surfaces of the upper parts of the plants such as the leaves and flowers, the absence of water may limit the development of biofilms (Fuqua and Matthysse, 2001). However, extensive biofilms have been observed on leaves, especially on those in high humidity.

2.4. MICROORGANISMS IN WETLANDS

The diversity of microorganisms in the wetland environment may be critical for the proper functioning and maintenance of the system (Ibekwe *et al.*, 2003). Wetlands host complex microbial communities, including bacteria, fungi, protists and viruses (Reddy *et al*., 2002). The size and diversity of microbial communities are directly related to the quality and quantity of the resources available in the system. Microorganisms colonise the water column, periphyton mats, plant detritus and soil or gravel. Because of the short life cycles of microorganisms, they respond rapidly to any changes in nutrient or energy source status in wetlands. Microorganisms are the first to respond to external nutrient loading, thus providing an early warning signal of ecosystem health.

Microbial communities and their functions in elemental cycling are regulated by various biogeochemical controls including: the quality of organic substrates (DeBusk and Reddy, 1998), hydroperiod (Happel and Chanton, 1993), the supply of electron acceptors (D'Angelo and Reddy, 1994a; 1994b; Wright and Reddy, 2001) and the addition of growth limiting nutrients (McKinley and Vestal, 1992). Anthropogenic and natural perturbations such as hydrologic fluctuations and nutrient loading have resulted in the conversion of many oligotrophic wetlands into eutrophic wetlands, resulting in alteration in microbial communities (Reddy *et al*., 2002).

Wetlands have a large number of aerobic-anaerobic zones in the water column, soil water interface and the root zone of macrophytes (Reddy *et al*., 2002). The combination of aerobic (redox potential (Eh) ranging from about +600 mV to +350 mV) and anaerobic zones (Eh ranging from about -300 mV to +350 mV) (DeLaune *et al*., 1990; Masscheleyn *et al*., 1993) supports a wide range of microbial populations and associated processes mediated by microorganisms. The aerobic populations are restricted to the plant detritus layer and periphyton mats in the water column and to the surface soil/gravel (few millimeters), while

anaerobic populations dominate most of the substrate profile. In addition, macrophyte capacity to transport oxygen to the root zone also supports aerobic populations in the rhizosphere. Natural wetlands receive inputs from non-point sources such as runoff from agricultural lands that contain significant levels of nitrate and sulfate. Anaerobic bacteria can use nitrate and sulfate as alternate electron acceptors to support their respiration. However, in most freshwater wetlands, these electron acceptors of higher reduction potential are limited and the dominant microbial activity is methanogenesis, where microorganisms utilize HCO_3^- and organic substrate to produce methane.

Methanogenic bacteria are capable of obtaining energy from only a few end products such as acetate, hydrogen and formate (Oremland, 1988), whereas sulfate reducers are more versatile in their carbon metabolism and utilize numerous fatty acids, amino acids and even some aromatic compounds (Widdel, 1988). The facultative denitrifying bacteria are even more versatile, degrading a wide range of low molecular weight compounds (Tiedje, 1988; Beauchamp *et al*., 1989). As such the addition of electron acceptors will result in changes in carbon flow in wetlands, with much of the organic C forming CO_2 and CH_4.

Decomposition of standing dead material and possibly litter in aerobic zones of the water column may be dominated by fungi. Basidiomycetes (white-rot fungi) are the predominant decomposers of lignin in terrestrial ecosystems (Zeikus *et al.*, 1982), and may occur in wetlands under aerobic conditions (Reddy *et al*., 2002). In a study by Findlay and Arsuffi (1989) fungal biomass and production were found to exceed bacterial biomass and production on intact leaf litter, while Ellis *et al.* (1998) found that bacteria dominated heterotrophic biofilms on fine particulate organic matter. Sakamoto and Oba (1994) suggested that the ratio of fungal to bacterial biomass is related to the overall efficiency of substrate utilization by decomposers. The contributions of fungi and bacteria to organic matter degradation are, however, dependent on the type of detrital material as well as on the habitat (*e.g.* river, wetland, pool) (Findlay *et al*., 2002).

Oxygen depletion in wetlands forces a major shift in microbial metabolism of monomeric C compounds (*e.g*. glucose, acetate), from aerobic to anaerobic pathways (Westermann, 1993). The microbial growth rates are generally lower in anaerobic environments since the catabolic energy yields for bacteria utilizing alternate electron acceptors (NO_3^-, Mn^{4+}, Fe^{3+}, SO_4^{2-}, CO_2) are lower than for O_2 (Westermann, 1993; Reddy and D'Angelo, 1994; D'Angelo and Reddy, 1999). In addition, sulfate reducing and methanogenic bacteria must depend on fermenting bacteria (*e.g. Clostridium* spp.) to produce substrate in the form of short chain C compounds, such as volatile fatty acids, from the breakdown of mono- and polysaccharides (Howarth, 1993). Thus, although C metabolism occurs in the absence of O_2, and even in the complete absence of electron acceptors, the decomposition process for plant litter and soil organic matter is often significantly curtailed.

The removal of Biological Oxygen Demand (BOD) or Chemical Oxygen Demand (COD), and the oxidation of nitrogen substances generally occur under aerobic conditions, while anaerobic conditions are needed for denitrification (Marjaka *et al.*, 2003). The formation of biofilms or the immobilization of microorganisms provides both aerobic and anaerobic regions in an adjoining space (Noguera *et al*., 1999). Aerobic bacteria growing in the region near the aerobic bulk liquid consume dissolved oxygen (DO) and create an anaerobic region inside the biofilm or immobilizing carrier.

2.4.1. Biofilms

Microorganisms rarely occur on their own in nature or as free-floating organisms (Costerton *et al*., 1995). Direct observation of a wide variety of natural habitats has established that the majority of microorganisms persist attached to surfaces within a structured biofilm ecosystem. A biofilm is a complex layer of bacteria surrounded by an extra-cellular marix (Fuqua and Matthysse, 2001). Biofilms can be composed of a population that developed from a single species

or a community derived from multiple microbial species and can form on a vast array of abiotic and biotic surfaces (Davey and O'Toole, 2000). It is becoming clear that these natural assemblages of bacteria within the biofilm matrix function as a cooperative consortium, in a relatively complex and coordinated manner (Costerton *et al*., 1995).

Bacterial communities in nature play a key role in the production and degradation of organic matter, the degradation of many environmental pollutants and the cycling of nitrogen, sulfur and many metals (Davey & O'Toole, 2000). It is likely that bacteria residing within biofilm communities carry out many of these complex processes, as most of these processes require the concerted effort of bacteria with different metabolic capabilities. One of the key components of biofilms is the non-cellular matrix surrounding the cells, generally referred to as extracellular polymeric substances (EPS). EPS typically consist of a mixture of components, such as polysaccharides, proteins, nucleic acids and other substances.

Although Flemming (1993) reported that EPS play a critical role in the sequestering of metals, cations and toxins, as well as to provide protection from environmental stresses, such as UV radiation, pH shifts, osmotic shock and desiccation, the exact functions of EPS is not completely elucidated because of their extremely heterogeneous nature (Tsuneda *et al*., 2003). It has also been reported that EPS play significant roles in the formation and function of microbial aggregates, including adhesion phenomena, matrix structure formation and microbial physiological processes (Christensen, 1989; Buswell *et al.*, 1997). In particular, EPS significantly influence bacterial adhesion onto solid substratum surfaces, which is recognized as the initial stage in biofilm formation, because an EPS covering on a cell surface alters the physiochemical characteristics of the surface such as charge, hydrophobicity and the polymer property (Bruus *et al*., 1992; Rijnaarts *et al*., 1993; Suci *et al.*, 1995).

The interactions that occur between biofilms and their physical and chemical micro- and macro-environment determine, to a large extent, the manner and success whereby these biological systems cycle nutrients, degrade toxicants, survive in hostile environments and resist antimicrobial agents (Korber *et al*., 1999). The organisms within a biofilm community cooperate metabolically by means of highly permeable water channels interspersed throughout the biofilm in the areas surrounding the microbial microcolonies (Costerton *et al*., 1995). These channels provide an effective means of exchanging nutrients and metabolites with the bulk aqueous phase, enhancing nutrient availability as well as removal of potentially toxic metabolites. Hence, very efficient cooperations and mutual dependence can evolve within a biofilm (Davey and O'Toole, 2000). Biofilms provide an ideal environment for the establishment of syntrophic relationships. Syntrophism is a special case of symbiosis in which two metabolically distinct types of bacteria depend on each other to utilize certain substrates, typically for energy production.

Biofilms in illuminated aquatic environments consists of both algal and bacterial cells along with other microorganisms.

2.4.2. Algae

The algae are a diverse group of photosynthetic organisms comprising of eight major divisions (phyla) with sixteen classes (Sze, 1993). The algae may not necessarily be closely related to one another and may share only a few common characteristics, while ranging from unicellular types, through colonial and filamentous organisms, to complex multicellular types known as seaweeds (Round, 1973). Prokaryotic algae are divided into two groups (Sze, 1993). The majority of species are cyanobacteria (formerly called blue-green algae) belonging to the phylum Cyanophyta while only a few representatives of the second group, the prochlorophytes (phylum Prochlorophyta) are known. The other six divisions of algae are eukaryotic. These include the phyla Chlorophyta, Chrysophyta, Rhodophyta, Pyrrophyta, Cryptophyta and Euglenophyta.

Algae have a photosynthetic system based on chlorophyll *a* (Chl*a*), their reproductive structures lack sterile cells and they do not form embryos (Sze, 1993). The bryophytes and vascular plants also posses Chl*a*, but algae generally lack their structural complexity. While exceptions to these characteristics exist among species considered to be algae (*e.g.* some algae do not have photosynthetic pigments) they are clearly related to more typical algal species and therefore are classified with the algae. Algal divisions differ in their photosynthetic pigments, carbohydrate reserves and cell structures.

Algae require light for photsynthesis (Sze, 1993). Usable light for photosynthesis occurs in the range from 400 – 700 nm of the electromagnetic spectrum, which is referred to as photosynthetically active radiation (PAR). Biofilms in illuminated aquatic environments consist of both algal and bacterial cells, plus other microorganisms, held together by a matrix of EPS (Hartley *et al*., 1996). Algal biofilms form on surfaces such as submerged stones, aquatic plants and on the surface of the sediments (Hartley *et al*., 1996). Illuminated freshwater biofilms are usually composed of unicellular green algae and diatoms or filamentous green algae or cyanobacteria (Leadbeater and Callow, 1992). Algal EPS consists predominantly of carboxylated or sulfated acidic polysaccharides (Hoagland *et al*., 1993).

Important herbivores grazing on algae include rotifers and protists. The latter may be the principal consumers of picoplankton (0.2 – 2 µm) and constitutes an important source of energy in the microbial loop through these predator-prey interactions (Callieri and Stockner, 2002; Sze, 1993; Sieburth *et al*., 1978).

2.4.3. Protists

Protists are ubiquitous and abundant in all habitats and are important in the functioning of aquatic ecosystems (Sherr and Sherr, 2002). Protists vary greatly in size and shape, and these are often correlated with their ecological niche.

Protists has two subkingdoms: Adictyozoa, without Golgi dictyosomes, containing only the phylum Percolozoa (flagellates and amoeboflagellates); and Dictyozoa, made up of 17 phyla with Golgi dictyosomes (Cavalier-Smith, 1993). Dictyozoa are divided into two branches: (1) Parabasalia, a single phylum with hydrogenosomes and 70S ribosomes but no mitochondria, Golgi dictyosomes associated with striated roots, and a kinetid of four or five cilia; and (2) Bikonta (16 unicellular or plasmodial phyla with mitochondria and bikinetids and in which Golgi dictyosomes are not associated with striated ciliary roots).

Many protists are capable of encystation when environmental conditions become unfavourable (Cowling, 1994). Cysts serve three major functions: (1) protection against adverse changes in the environment; (2) sites for nuclear reorganization and cell division (reproductive cysts); and (3) a means of transfer from one host to another in parasitic species. These organisms excyst when favourable conditions return. In addition to specialized organelles, protists have organelles similar to those found in other eukaryotic cells. Most protists can move using one of three major types of locomotory organelles: pseudopodia, flagella and cilia.

Most protists are chemoheterotrophic which can either be holozoic (nutrients such as bacteria are acquired by phagocytosis and the subsequent formation of a food vacuole or phagosome) or saprozoic (nutrients such as amino acids and sugars cross the plasma membrane by pinocytosis, diffusion or carrier-mediated transport) (Cowling, 1994). Protistan grazing decreases bacterial numbers, stimulates mineralization of nutrients (Ekelund and Rønn, 1994), and is likely to affect the structures of the bacterial communities (Rønn *et al*., 2002). A number of reports demonstrate that grazing by protists is an important factor in shaping the morphological and taxonomical composition of bacterioplankton communities in marine and limnic ecosystems (Hahn and Höfle, 2001; Pernthaler *et al*., 2001; Simek *et al.*, 2001), as well as activated sludge (Güde, 1979). There is evidence that factors such as cell size (Jürgens and Güde, 1994; Hahn and Höfle, 2001),

cell surface properties (Monger *et al*., 1999), cell motility (Boenigk *et al*., 2001) and chemical composition (Verity, 1991; Jürgens and DeMott, 1995) may affect the susceptibility of bacteria to grazing. Generally medium-sized bacterial cells are most susceptible to predation by flagellates and ciliates, whereas smaller cells and large filamentous forms may be partly resistant to grazing (Jürgens and Güde, 1994; Hahn and Höfle, 2001). Grazing may, therefore, lead to a bidirectional shift in the relative abundance of large filaments and small cells (Hahn and Höfle, 2001). Rønn *et al*. (2002) found that protists reduced the average bacterial cell size in microcosms amended with a soluble resource (a solution of various organic compounds).

Changes in bacterial cell size and morphology within planktonic communities do not necessarily reflect changes in the taxonomic composition of the community, as individual bacterial strains may respond to increase grazing pressure by a change in these characteristics (Hahn and Höfle, 1998; Hahn *et al*., 1999). However, molecular-fingerprinting techniques have now confirmed that grazing affects the taxonomic composition of planktonic bacterial communities (Simek *et al*., 1997). In the study by Rønn *et al*. (2002), denaturing gradient gel electrophoresis (DGGE) (see section 1.7.3.1) of polymerase chain reaction (PCR) amplifications of 16S rRNA gene fragments, as well as community level physiological profiling (Biolog plates), suggested that a mixed protistan community had significant effects on the bacterial community structure. The DGGE gels indicated that high-G+C gram-positive bacteria increased in response to grazing, whereas a decrease in the abundance of gram-negative bacteria was observed.

Griffiths *et al*. (1999) used DGGE, community level profiling, and phospholipid fatty acid analysis to demonstrate that protistan grazing changed the microbial communities in soil microcosms. Phospholipid fatty acid analysis indicated that the proportion of gram-positive bacteria increased in response to protistan grazing. The lower edibility of gram-positive bacteria may be related to

a lower rate of digestion of the gram-positive cell wall (Gonzáles *et al*., 1990; Iriberri *et al*., 1994), which may enable survival during passage through the protistan cell (Iriberri *et al*., 1994). Although gram status may relate to the edibility, it is by no means an absolute factor, as many gram-positive bacteria are edible and many gram-negative bacteria are completely unsuitable as food for protists (Singh, 1941; Weekers *et al.*, 1993). Cordovilla *et al*. (1993) and Groscop and Brent (1964) found that representatives of both gram-positive and gam-negative bacteria produce substances that are toxic to protists.

Several other factors that affect the grazing resistance of bacteria (e.g. size, cell morphology and motility) are not related to gram-status (Rønn *et al*., 2002). Grazing may favour bacteria with high growth rates because they will be able to replace cells lost to predation (Gurijala and Alexander, 1987; Sinclair and Alexander, 1989).

2.5. MICROORGANISMS IN WASTE MANAGEMENT

2.5.1. Bacteria

It has long been recognized that certain microbial groups in animal waste are responsible for breaking down various organic compounds and for the suppression of pathogens in waste (Ibekwe *et al.*, 2003). Gersberg *et al.* (1986) found that the majority of water quality treatment in natural and constructed wetlands is due to bacteria. The authors found that nitrifying bacterial communities may develop around the microaerobic zones at the roots of the plants and convert ammonium to nitrate. As the water flows into the anaerobic region, the denitrifying bacteria convert nitrate to nitrogen gas. The complimentary microbial communities can nitrify ammonium-laden water under aerobic conditions or convert the nitrate to nitrogen gas by denitrifying bacteria under anaerobic conditions. The advantage of the SF wetland over the FWS wetland is that the water is being exposed to a far greater surface area of

bacterial biofilms, resulting in a higher level of nutrient removal over a shorter time period (Ibekwe *et al.*, 2003).

2.5.2. Algal biomass

Most efforts using algae for wastewater treatment were focused on tertiary treatment of municipal wastewater using suspended microalgae (Benemann and Oswald, 1996). Wastewater treatment using attached algae has also been reported and has potential advantages in the manner in which the algal biomass is removed and dried (Hoffman, 1998). Previous work by Mulbury and Wilkie (2001) and Wilkie and Mulbury (2002) have demonstrated that one method, using periphyton and termed algal turf scrubbers (ATS), can remove P and N from dairy manure effluents. ATS is essentially an artificial stream designed to promote biological wastewater treatment. The essential elements of the ATS system are a solid support for the growth and harvest of periphyton, a wave surge and optimal light (Craggs *et al*., 1996).

2.5.3. Protistan grazing as biomass control

Protists have been observed in biofilters and biotrickling filters for waste air treatment (Ottengraf, 1986; Diks, 1992). Also, in a study by Cox and Deshusses (1997), the predation of protists on bacteria was investigated as an approach to control the rate of biomass formation in trickling filters. Because protistan predation is coupled with CO_2 and heat generation, overall biomass production per amount of pollutant degraded should be less in the presence of protists. Ideally, protistan predation should balance bacterial production to obtain a stable biotrickling filter with a constant amount of biomass. Such an equilibrium has been shown to exist in natural ecosystems (Nagata, 1988; Bloem *et al*., 1989), although bacterial production rates in those systems is much lower than in biotrickling filters (Cox and Deshusses, 1997).

However, protistan predation increases carbon mineralization in systems with higher biomass production rates such as wastewater treatment plants, and

protists has been suggested as a means to decrease sludge production (Ratsak *et al*., 1994; 1996).

2.5.4. Bio-augmentation

Bio-augmentation, often referred to as bio-supplementation, is the addition of specific microorganisms to a bio-treatment system with the aim of providing sufficient quantity and diversity of microorganisms (Quan *et al*., 2004). Bio-augmentation is a means of maintaining sufficient biomass when adequate carbon substrates and nutrients are available (Ro *et al*., 1997). Bio-augmentation is said to be beneficial in many cases including the protection of the activated-sludge system against perturbations resulting from transient or continuous overloading (Beardsley and Coffey, 1985), and the increased degradability of previously hard-to-treat organics (Kennedy *et al*., 1990). The possibilities of operation at higher rates during cold weather, and in periods of reduction in sludge production, are additional benefits of bio-augmentation (Chong *et al*., 1997). Inconsistent results were, however, found when different tests of bio-augmentation were conducted under different conditions or situations (Wojnowska-Baryla and Young, 1983; Jones and Schroeder, 1989; Koe and Ang, 1992).

In activated sludge systems, bio-augmentation has been demonstrated to enhance degradation and removal of specific pollutants such as phenols, chloroaniline, chlorobenzoate, resin acid, chlorinated solvents and aromatic hydrocarbons (Quan *et al*., 2004 and references). However, due to a variety of reasons, bio-augmentation with special bacteria in a bio-treatment system does not always work (Quan *et al*., 2004). Although purchased cells is one method of bio-augmentation, Babcock *et al.* (1992) found no evidence of successful operation in experiments using purchased cells and well defined controls.

The survival and maintenance of the added bacteria in the system is a prerequisite to a successful bio-augmentation (Quan *et al*., 2004). Added

bacteria may, however, be washed out of the system or grazed upon by protists. Also, under operational conditions, target substances are in most cases mixed with a wide variety of other degradable materials. Many of these materials may be much more attractive to the added special culture than the target substances and may thus be preferentially biodegraded. It is, therefore, imperative to select suitable bio-augmentation microorganisms and to adopt an appropriate bio-augmentation strategy to achieve successful removal of the target substances.

2.6. TECHNIQUES TO OBSERVE AND ANALYZE MICROBIAL COMMUNITIES

2.6.1. Culture dependent techniques

It is a common misconception that microorganisms isolated in pure culture from an environment represent the numerically dominant and/or functionally significant species in that environment (Hugenholtz, 2002). Microorganisms isolated using standard cultivation methods are rarely numerically dominant in the communities from which they were obtained, since they are isolated by virtue of their ability to grow rapidly into colonies on high-nutrient artificial growth media, typically under aerobic conditions, at moderate temperatures. According to Hugenholtz (2002), easily isolated organisms are the ‘weeds’ of the microbial world and are estimated to constitute less than 1% of all microbial species. This figure was estimated by comparing plate counts with direct microscopic counts of microorganisms in environmental samples and has been called the “great plate-count anomaly” (Staley and Konopka, 1985).

The most of what is known about microbiology comes from the study of microbial weeds. Approximately 65% of published microbiological research from 1991 to 1997 was dedicated to only eight bacterial genera, *Escherichia* (18%), *Helicobacter* (8%), *Pseudomonas* (7%), *Bacillus* (7%), *Streptococcus* (6%), *Mycobacterium* (6%), *Staphylococcus* (6%) and *Salmonella* (5%) (Galvez *et al.,* 1998). All these organisms are relatively simple to grow on agar plates, but it seems unlikely that this handful of organisms can be representative of the

approximately 5000 validly described prokaryotic species (DSMZ Bacterial Nomenclature Up-to-date).

2.6.2. Culture independent methods

Biofilms on plants as on other surfaces are fragile, and disturbing them to make observations or measurements may give a misleading picture of the biofilm in the absence of disturbance (Fuqua and Matthysse, 2001). Therefore, it is important to attempt to confirm any observations made by techniques that disturb the biofilm with additional observations of undisturbed material. Technological and methodological advances, especially those enabling the nondestructive, direct analysis of native biofilms, have facilitated the task of delineating some of the processes driven by complex biofilm communities (Korber *et al*., 1999). Without these advances in technology it was previously difficult to identify constituent biofilm members *in situ*, as well as to quantify physical, chemical and spatial aspects of biofilms, and to link particular processes and activity with specific biofilm bacteria.

The maturation of the ribosome-directed oligonucleotide probe methodology, the refinement of fluorescent molecular probes sensitive to either the microbial condition or the microbial environment, as well as advances in microscopy and digital imaging, has been at the center of these developments (Korber *et al*., 1999). The application of molecular methods, including DNA-DNA hybridization and 16S and 23S rDNA probes, has revolutionized the routine identification of bacteria from environmental and industrial samples (Woese, 1987; Olsen and Woese, 1993).

However, techniques involving 16S rDNA have yet to fulfill their potential with respect to understanding and manipulating biological functions, especially in soils (O'Donnell and Görres, 1999). Such knowledge is important if we are to sustain agricultural productivity, to cope with changes in land use concomitant with global change or to remediate contaminated land.

2.6.3. Molecular techniques commonly employed

2.6.3.1. Denaturing-gradient gel electrophoresis (DGGE)

Denaturing-gradient gel electrophoresis is an electrophoretic technique that separates genes of the same size that differ in base sequence (Madigan *et al*., 2003). A gradient of DNA denaturant is used to 'melt' a double-stranded DNA fragment moving across the gel, which stops migration. The differences in melting properties are controlled largely by differences in base sequences. Therefore, each band observed on the gel, represents a specific sequence of a gene that may vary by as little as one nucleotide in their sequence.

This method gives a detailed picture of the number of phylotypes (distinct 16S rRNA genes) present in a sample (Meays *et al*., 2004). The bands can then be sequenced and compared with sequences of known species available in an appropriate database, thereby revealing the actual species present in a community. DGGE coupled with PCR amplification of rDNA genes has been used primarily to determine the genetic fingerprints of microbial communities (Chee-Sanford *et al*., 2001).

2.6.3.2. Reverse sample genome probing (RSGP)

Reverse sample genome probing makes use of the observation that the entire genome of a microorganism can be used as a specific probe for detection of the organism in the environment (Liu & Stahl, 2002). Reverse sample genome probing involves hybridization of labeled total community DNA with a master filter containing the denatured chromosomal DNAs of genomically distinct bacteria, isolated from the target environment (Voordouw, 1998). The denatured genomes of bacteria isolated from the target environment are spotted on a master filter in known amount. The genomes are pre-selected to have a limited degree of cross-hybridization. These pre-selected bacteria are referred to as standards.

Community analysis with RSGP involves isolation of total community DNA, spiking with an internal standard, labeling and hybridization with the master filter

(Voordouw, 1998). The prevalence of standards in the community can then be derived from the intensity of the hybridization signals. Because the genomic diversity of bacteria is huge it is not feasible to design a single filter to analyze all environmental microbial communities. This technique cannot analyze uncultured members of microbial communities.

2.6.3.3. Fluorescence *in situ* hybridization (FISH)

Specific nucleic acid sequences in eukaryotic and prokaryotic cells can be detected with *in situ* hybridization by binding oligonucleotide probes to their complementary target sequences (Manz, 1999). Detection of microorganisms by using FISH with rRNA-targeted nucleic acid probes is a very useful tool for rapid, reliable and cultivation-independent monitoring of phylogenetically defined populations in environmental samples (Nielsen *et al*., 1999). Amann *et al.* (1995) states that FISH in combination with advanced microscopic techniques, such as confocal laser scanning microscopy (CLSM) and digital image analysis, is an important part of a polyphasic approach in microbial ecology for the identification and localization of microorganisms within complex environments.

Comparative sequence analysis of small subunit rRNA provided the basis for the development of synthetic oligonucleotide probes and can be used as molecular tools for the determination of the bacterial phylogeny as well as the identification and *in situ* detection of bacteria (Manz, 1999). The first applications involved radioactively labeled oligonucleotides but were replaced by probes linked to fluorochromes or haptens such as digoxigenin. Fluorescent dye-labeled oligonucleotide probes combined with epifluorescence microscopy are advantageous because of their superior spatial resolution and their convenient detectability. Although rRNA targeted probes are extremely useful for identification it does not provide detailed information about the function of the microorganisms (Nielsen *et al*., 1999). This problem can, however, be solved by microautoradiography.

2.6.3.4. Microautoradiography (MAR)

The MAR technique determines cells that actively take up radiolabeled substrates e.g. mixtures of amino acids, glucose or acetate (Carman, 1990; Andreasen and Nielsen, 1997). The use of radiolabeled substrates in combination with MAR does allow analysis of the metabolic activity of prokaryotes under conditions that approach in situ conditions by direct visualization of microorganisms with active substrate uptake systems within a complex community (Brock and Brock, 1968).

Microautoradiography has been widely used in the medical field and in a range of studies of environmental microbiology (Rogers, 1979; Nielsen *et al*., 1999). The radiolabeled compound typically appears in the investigated cell or biological structure as a result of adsorption of a tracer or by an uptake of a labeled substrate (Nielsen *et al*., 1999). After an appropriate sample handling, the radiolabeled sample is placed in contact with a layer of radiosensitive emulsion. During an exposure time of hours to days or weeks the emissions from the radioactive sample interact with silver bromide crystals suspended in the emulsion. When the emulsion is developed using standard photographic procedures the silver grains appear on the top of the radioactive structure, which can be viewed clearly under the microscope.

Microautoradiography has been successfully used in many ecological studies to measure activities of members of different autotrophic and heterotrophic prokaryotic groups (Lee *et al.*, 1999). A major limitation of MAR has been an inability to correlate activity detected with identification and detection of a responsible organism. Another major disadvantage of using MAR is that there is no single substrate that targets all living microorganisms, thereby detecting all active cells (Nielsen and Nielsen, 2002). In the work of Andreasen and Nielsen (1997) it is shown that a substantial fraction of cells in activated sludge is not able to consume mixtures of amino acids or glucose that are often used as substrates. However, if the aim is to investigate the number of bacteria

consuming a specific substrate, the method is ideal (Nielsen and Nielsen, 2002). Other disadvantages of MAR are the laboriousness and tediousness of it. However, Nielsen and Nielsen (2002) demonstrated that it is possible to simplify the technique and still get reliable and useful results.

2.6.3.5. Combination of FISH and MAR (FISH-MAR)

Microautoradiography has recently been successfully combined with FISH as a tool to simultaneously determine phylogenetic identification and *in situ* nutrient uptake patterns of various cultivable and uncultivable bacteria (Lee *et al*., 1999; Ouverney and Fuhrman, 1999; Gray *et al.*, 2000; Nielsen *et al.*, 2000). Similarly, Ito *et al.* (2002) simultaneously determined the phylogenetic identification and *in situ* nutrient uptake patterns of sulfate-reducing bacteria (SRB) inhabiting a sewer biofilm with oxygen, nitrite or sulfate as an electron acceptor by FISH-MAR with family- and genus specific 16S rRNA probes.

2.6.3.6. Terminal-restriction fragment length polymorphism (T-RFLP) analysis

The T-RFLP technique (Figure 1) is a recent molecular approach that can assess subtle genetic differences between strains and may provide insight into the structure and function of microbial communities (Marsh, 1999). Because of its sensitivity and high throughput, it is an ideal technique for comparative community analyses. The T-RFLP technique is a quantitative molecular technique that was developed for rapid analysis of microbial community diversity in various environments (Liu *et al.*, 1997). These authors found a high species diversity in activated sludge, bioreactor sludge, aquifer sand and termite guts. As many as 72 unique ribotypes were found in these communities.

The T-RFLP technique permits an automated quantification of the fluorescence signal intensities of the individual terminal restriction fragments (T-RFs) in a given community fingerprint pattern (Lukow *et al*., 2000). As a general rule, a single species contribute a uniquely sized T-RF although several species

may have T-RFs of identical size (Marsh *et al.*, 2000). The T-RFLP seems to represent the superior tool for the biomonitoring of highly diverse communities and enables a more objective comparison of community fingerprint patterns because of the automated quantification of both size and relative abundance of individual T-RFs. The technique is a powerful tool for rapidly comparing the community structure and diversity of different ecosystems (Liu *et al.*, 1997).

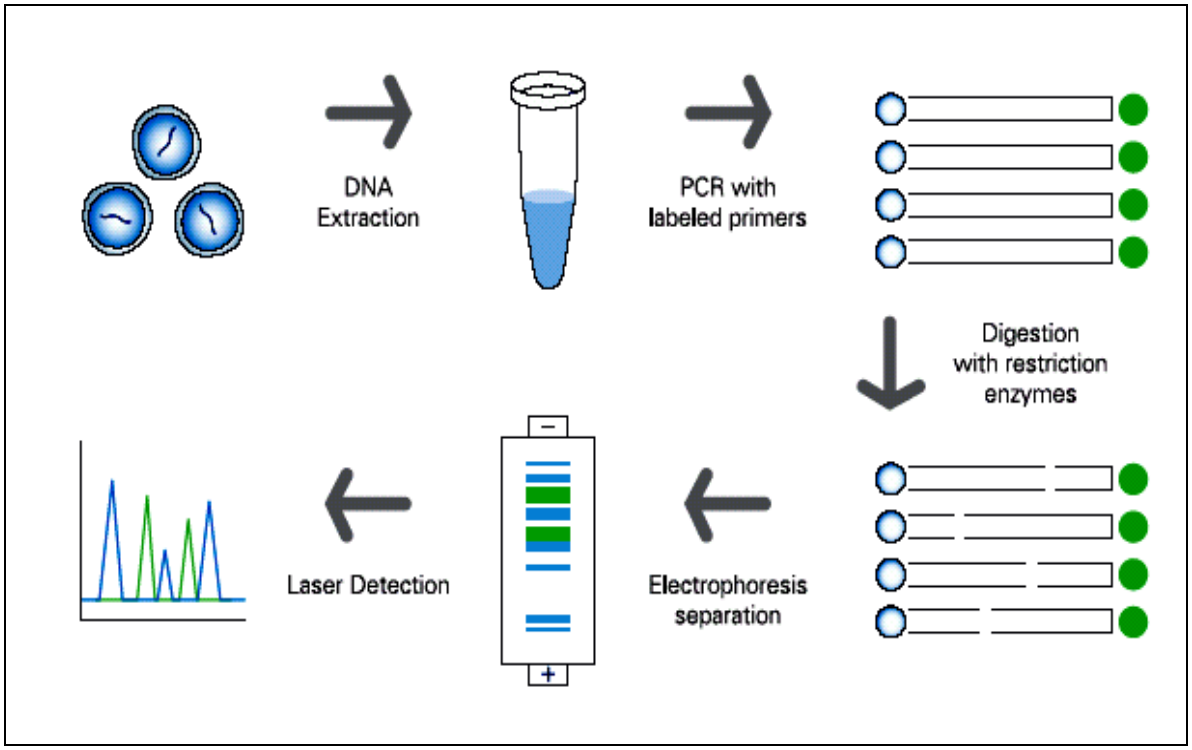

Figure 1. Steps of the T-RFLP technique.
(Applied Biosystems – http://docs.appliedbiosystems.com/pebiodocs/00115132.pdf)

Terminal-restriction fragment length polymorphism is a marriage of at least three technologies (Marsh, 1999): (1) *Comparative genomics* provide an insight into the design of primers for the amplification product (amplicon) of choice. (2) *Polymerase chain reaction (PCR)* amplifies the signal from a high background of unrelated markers. (3) *Nucleic acid electrophoresis* - Subsequent digestions with judiciously selected restriction endonucleases produces T-RFs appropriate for sizing on high resolution (± 1 base) sequencing gels. The latter is performed with automated systems such as the ABI gel or capillary electrophoresis systems that

provide digital output. The use of a fluorescently tagged primer limits the analysis to only the terminal fragments of the digestion. Size markers bearing a different fluorophore from the samples can be included in every lane thereby making the sizing extremely accurate. As the T-RF sizes can be compared to databases, the technique has potential in comparative community analyses.

2.7. THIS STUDY

With the above as background and after careful deliberations, a combination of culture dependent (i.e. general plate counts) and culture independent (i.e. T-RFLP) techniques together with scanning electron and epifluorescence microscopy was used to evaluate microbial controls within constructed wetlands used in the treatment of distillery wastewater.

2.8. REFERENCES

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CHAPTER

3

Microbiology and COD removal from distillery effluent in experimental constructed wetlands

MICROBIOLOGY AND COD REMOVAL FROM DISTILLERY EFFLUENT IN EXPERIMENTAL CONSTRUCTED WETLANDS

3.1. ABSTRACT

Microbial distribution and activity were evaluated in experimental systems that were constructed to evaluate the potential use of wetlands for the treatment of distillery effluent. Two 45 m-long and three 6 m-long wetlands were constructed at a distillery, and two pilot scale (laboratory model-scale) systems were built and operated under controlled conditions in a greenhouse. Of specific interest was to study the dynamics of attached and planktonic microbial communities at various points in the wetlands, and to evaluate COD removal efficiency. Terminal-restriction fragment length polymorphism analysis of whole-community DNA samples showed a high diversity among the microbial communities sampled at 14 locations in the 45 m-long wetlands. Analysis performed at the pilot scale models showed notable difference among the dominant planktonic populations (in terms of its presence throughout the experimental period and at various sampling points) at the inlet, middle zone and outlet of the wetlands. SEM observations showed the presence of distinct morphotypes during biofilm formation on the gravel support. The pilot scale systems demonstrated that an initial period of 3-6 weeks is needed to establish the biological system, where after a COD removal efficiency of up to 83% was recorded. However, the system rapidly (less than 2 weeks) became anaerobic at high influent COD > 20 000 mg/L, and required relatively long periods of flow with clear water to recover.

Key words: biofilm, COD, distillery effluent, wetlands

3.2. INTRODUCTION

Distillery wastewater has a complex character due to high concentrations of sugars, lignins, hemicellulose, dextrins, resins, polyphenols and organic acids,

leading to a high chemical oxygen demand (COD) that can exceed 100 000 mg/L (Yeoh, 1997). These effluents are therefore among the most complex to treat (Sangave and Pandit, 2004). A medium-size ethanol distillery producing 10^6 L ethanol/year can generate up to 20 L of wastewater per liter ethanol produced (van Haandel and Catunda, 1994). Water quality legislation and control on effluent discharge thus necessitate efficient treatment of distillery wastewater.

A number of treatment methods and strategies has been suggested or tested for the treatment of distillery wastewater (Sangave and Pandit, 2004). These efforts were met with varying success although no system has been demonstrated to meet most efficiency and financial requirements. A relatively unexplored method with potential application for the treatment of distillery wastewater is constructed wetlands. This approach has been demonstrated to be a low-cost alternative for improving treatment of point and diffuse discharges (Reddy and D'Angelo, 1997). Wetlands are often more economic and easier to operate than energy-intensive engineered treatment plants (Scholz and Xu, 2002), and also provide numerous secondary benefits such as habitat enhancement (Pinney *et al.*, 2000).

Gradients between aerobic and anaerobic zones in wetlands provide a diverse habitat range that supports complex microbial communities of various functional groups (Reddy *et al*., 2002). The role of microbial activity in the functioning of wetlands is widely recognized, including degradation of recalcitrant compounds and nutrient cycling. These processes are important determinants of ultimate water quality and ecosystem productivity. The aerobic populations are primarily found in the water column, outer regions of the plant detritus layer and periphyton mats in the water column, as well as at the surface of the geological medium. The capacity of macrophytes to transport oxygen to the rhizosphere supports aerobic populations in this otherwise anaerobic zone. Anaerobic populations dominate most of the remainder of the subsurface region of the wetland (Reddy *et al*., 2002), including interfaces where they exist as biofilms.

Microbial population size and community diversity in natural systems are related to the amount of available resources as well as its quality (Reddy *et al*., 2002). In addition to their key roles in the production and degradation of organic matter, the degradation of environmental pollutants and the cycling of nutrients and metals (Davey and O'Toole, 2000), microorganisms respond rapidly to changes in nutrient or energy sources because of their short life cycles. Microorganisms are therefore usually among the first to respond to external nutrient loading – a factor that has important consequences for the stability of biological systems. The role of biofilms as a survival mode under adverse environmental conditions has been recognized. Studying the distribution of attached and planktonic microbial populations and their response to various inputs in constructed wetlands may thus be important in understanding the role of microorganisms in these systems and potentially be used as a predictive and management tool during the implementation of wetlands for wastewater treatment.

The terminal restriction fragment length polymorphism (T-RFLP) technique can reveal subtle genetic differences between strains and provide insight into the structure and function of microbial communities (Marsh, 1999). T-RFLP analysis involves amplification of target genes from whole-community DNA extracts by using specific primer pairs, one of which is fluorescently labelled. Resulting amplicons are digested with restriction enzymes and labelled terminal fragments (T-RFs) are detected with an automated sequencer. Automated analysis of samples by capillary electrophoresis permits high sample throughput and precise determination of fragment lengths (Forney *et al*., 2004). The resulting community fingerprint can be used to analyse communities of bacteria and archaea (*e.g.* Braker *et al*., 2001) as well as fungi (*e.g.* Zhou and Hogetsu, 2002) from various samples.

We participated in a study on the feasibility of wetlands to treat winery and distillery effluent. According to the National Water Act, 1998 (Act No. 36 of 1998) of South Africa, it is permissible to irrigate up to 500 m^3 of domestic or biodegradable industrial wastewater with a maximum of 400 mg/L COD/day, and up to 50 m^3 of biodegradable industrial wastewater with a maximum of 5 000 mg/L per day (COD values taken after removal of algae). In view of these requirements, we were interested in the behavior of wetland microbial communities when challenged with the high organic loading associated with distillery effluents. Chemical, visual and molecular techniques were applied to follow community structure and function in experimental constructed wetlands irrigated with distillery effluent.

3.3. MATERIALS AND METHODS

3.3.1. Wetland construction, sampling and COD determinations

3.3.1.1. Experimental wetlands

Five wetlands were constructed at a distillery producing 8.8×10^6 L absolute alcohol (aa)/year in the Western Cape, South Africa, where rebate wine, distilling wine and crude spirits are distilled to produce 'A' spirits and pot-still brandy. Two 45 m-long by 6 m-wide wetlands (one with gravel and one with a soil substrate) with a 14 day retention time (RT) as well as three 6 m x 3 m gravel wetlands (4.5, 9 and 18 days RT, respectively) planted with *Phragmytes* and *Typha* spp were constructed and were fed with distillery effluent. The effluent was diverted from the final in a series of four holding dams that collects all discharge from the distillery and has a COD load ≤12 000 mg/L. The RT's of the experimental wetlands were taken into account when sampling wetland outflow to correspond with the respective influent dates. Major chemical properties of the wastewater for the first two months of operation are listed in Table 3.1. The COD was determined weekly by the Reflux Titrimetric method (American Public Health Association, 1998) over a period of 12 months. Operational factors beyond the

control of the current study could influence temporal studies on microbial behaviour. We therefore decided to study this aspect in pilot scale systems.

3.3.1.2. Pilot scale model systems

Pilot scale wetland models, constructed from Perspex were used to allow controlled experimentation in a greenhouse. Each system (0.5 m long × 0.3 m wide × 0.4 m high) was filled with gravel (~ 25 mm grain diameter) to 0.1 m from the top and *Typha* spp. planted. The plants were exposed to distillery effluent from the fourth holding dam at the distillery (COD range 1 500 – 10 000 mg/L) except when a COD shock was applied at weeks 27 and 28. For the latter, the COD was increased to 21 000 – 24 000 mg/L (effluent from the first of the four dams that receives effluent directly from the distillery and has a COD load varying between 5 000 – 40 000 mg/L). A timer-controlled peristaltic pump was used to deliver the effluent 3 × per day for 10 min. to result in a hydraulic retention time of 10 days. A COD load of 2 000 – 2 400 mg/L was fed through the systems after the shock treatment (during weeks 32 and 33) to determine the resilience of the systems.

3.3.2. Microbial community diversity in the experimental wetlands

3.3.2.1. Sampling and DNA extraction

DNA was extracted from the wastewater samples and biofilms on the surface of gravel particles according to the method of Zhou *et al.* (1996). Biofilm material was removed from gravel by vortexing 10 g of ~ 25 mm grain diameter gravel in 25 ml sterile distilled water for 1 min where after it was sonicated at 40 kHz in a 5.5 L water bath sonicator (Misonix) for 10 min. Following sonication, 4 ml of the sample was centrifuged and the supernatant discarded where after 1.0 ml of DNA extraction buffer (100 µM Tris-Cl, 100 µM EDTA, 100 µM sodium phosphate, 1.5 M NaCl, 1% [w/v] cetyltrimethylammonium bromide [CTAB], pH 8.0), was used to resuspend each sample. Following 3 freeze-thaw cycles (-70°C /+65°C), 200 µl of 0.5 mm diameter micro-glass (Sigma-Aldrich) beads were added to each tube and vortexed for 3 min. Each sample was incubated on a

rotary shaker for 30 min at 37°C after the addition of 5 µl of proteinase K (20 mg/ml). Subsequently, 150 µl of 20% (w/v) SDS was added. The samples were then incubated at 65°C for 2 hours, with agitation. Following centrifugation to remove cellular debris, the supernatant of each sample was mixed with an equal volume of chloroform:isoamyl alcohol (CI) (24:1, v/v). The nucleic acids were precipitated by the addition of 0.6 volume of isopropanol and ultimately resuspended in 100 µl TE buffer (10 mM Tris-Cl, 1 mM EDTA) at pH 8.0.

3.3.2.2. T-RFLP analyses

16S rRNA genes were amplified using the primer pair: 6-carboxyfluorescein (FAM)-labelled 341f (5'-CCT ACG GGA GGC AGC AG-3') (Muyzer *et al.*, 1993) and unlabeled rPP2 (5'-CGG ITA CCT TGG TTA CGA CTT-3') (Rawlings, 1995) for Eubacteria. The standard reaction mixture with a final volume of 50 µl contained 2 µl of template DNA (100 – 150 ng), 2.6 µl of each primer (0.5 µM) and 25 µl of 2X PCR Master Mix (Promega). Amplification was performed in a PCR Sprint Thermocycler (Hybaid™) and generated amplicons of ~ 1000 base pairs each. The thermal profile for the amplification of 16S rDNA was as follows: initial denaturation at 94°C for 2 min, followed by 30 cycles of denaturation (94°C for 30 s), annealing (50°C for 30 s) and extension (72°C for 90 s). Following a final extension step of 72°C for 4 min, the samples were stored at 4°C until further analysis. PCR products were purified with the GFX™ PCR DNA and Gel Band Purification kit (Amersham Biosciences).

For the removal of pseudo-terminal fragments (Egert and Friedrich, 2003), approximately 1 µg of PCR product was incubated for 10 min at 37°C with 0.1 – 0.2 U/µl mung bean nuclease (Promega). The reaction was terminated by the addition of 1 µl of 0.5 M EDTA pH 8.0 to the 50 µl reaction mixture, and the amplicons again purified with Microspin S-300 HR columns (Amersham Biosciences). The mung bean nuclease treated PCR products were digested with *Alu*I (Roche Molecular Biochemicals) for 1.5 h at 37°. As T-RFLP has proven to be a reproducible and accurate tool for community fingerprinting (Liu *et al.*, 1997;

Moeseneder *et al.*, 1999) and as it was not in the scope of this study to identify any organisms, only one restriction enzyme was used. The digested 16S rDNA was purified with Microspin S-300 HR columns (Amersham Biosciences) before being mixed with an internal size standard (GeneScan™ 500 ROX™; Applied Biosystems). The fluorescently labeled T-RFs were then detected on an ABI PRISM® 3100 automated gene sequencer (Applied Biosystems). T-RFLP electropherograms were analysed with GeneScan™ 3.7 software (Applied Biosystems).

3.3.3. Culturable microbial cell numbers

Total microbial and yeast counts were performed in late winter/early spring (August and September) on the inlet and outlet effluent of the experimental wetlands using tryptone soy agar (TSA) (Biolab™) and yeast malt extract agar (YMA) (3 g yeast extract, 3 g malt extract, 5 g peptone, 10 g glucose, 1 L distilled water), respectively. Triplicate plates were incubated at 22°C for 24 to 48 h.

3.3.4. Temporal studies of microbial distribution

Following the experimentation to assess microbial diversity and distribution in the experimental wetlands and 33 week study to assess the impact of COD on the pilot scale systems, an additional 11 week trial was performed to follow microbial dynamics under defined conditions in the pilot scale models. The models were first cleaned and new gravel and plants added. Fresh water (with no biocidal properties) was pumped through the systems for three weeks to allow the plants to re-establish. Subsequently distillery effluent with a COD ranging between 5 000 mg/L and 8 000 mg/L was pumped through the systems except when experiments were conducted to simulate a COD shock for which the COD load was increased to 16 000 – 24 000 mg/L for 3 weeks.

Table 3.1. Characteristics of wastewater discharged into the wetlands during experimentation. The samples were collected in late winter (August; sample 1) and spring (September; sample 2).

	Inlet		GO		SO		W1		W2		W3	
Sample	1	2	1	2	1	2	1	2	1	2	1	2
pH	4.9	4.8	6.0	6.3	6.2	7.3	6.6	6.4	5.2	5.2	7.3	7.2
*EC†	520	303	790	476	500	350	710	360	601	335	702	390
**Na	534	82	48	60	54	74	51	75	54	80	55	70
**K	1139	628	943	624	942	763	1068	599	1126	630	1036	564
**Ca	105	71	413	257	184	114	231	129	175	110	273	188
**Mg	66	36	207	135	97	70	151	75	106	59	195	111
**Fe	16	33	40	14	27	16	9	12	28	30	2	4
**Cl	90	115	88	110	91	107	93	115	90	119	98	107
**CO_3	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
**HCO_3	1607	879	2966	2022	1740	1392	2435	1409	1773	1069	2734	1512
**SO_4	114	97	22	6	32	30	150	116	100	120	14	2
**B	2.6	1.4	2.2	1.5	1.8	1.5	2.4	1.5	2.6	1.5	2.2	1.3
**Mn	1.5	0.9	5.1	2.2	1.7	0.6	1.1	0.8	1.6	1.0	0.7	0.7
**Cu	0.2	0.1	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
**Zn	0.7	0.2	0.0	0.0	0.0	0.0	0.0	0.0	0.1	0.2	0.0	0.0
**P	94	216	84	189	55	159	83	199	105	216	48	187
**NH_4-N	159	77	186	133	119	87	188	115	155	923	173	104
**NO_3-N	1.6	1.6	0.5	1.4	0.3	0.4	0.5	0.5	0.2	0.3	0.6	0.3
**COD	7428	6278	5868	3892	3565	3013	4679	5587	4605	3829	1857	1130

*mS/m; **mg/L ; †Electrical Conductivity

Inlet = Effluent obtained before entry into wetlands; W1 = Effluent at outlet of 6m wetland with 4.5 day RT; W2 = Effluent at outlet of 6m wetland with 9 day RT; W3 = Effluent at outlet of 6m wetland with 18 day RT; GO = Effluent at outlet of 45m gravel wetland with 14 day RT; SO = Effluent at outlet of 45m soil wetland with 14 day RT

Analyses included weekly monitoring of COD and microbial diversity in the influent, effluent and central zones of each system. T-RFLP as well as scanning electron and epifluorescence microscopy were applied for microbial analysis. To evaluate the behaviour of microorganisms in an established system when provided with fresh attachment surfaces, permeable holders containing sterile

(autoclaved) gravel (~2.5 mm diameter) were placed in the central regions of each system.

3.3.4.1 Microscopy

Gravel samples were collected from the holders on a weekly basis, mounted on stubs and viewed unfixed while still hydrated with a LEO 1430 VP scanning electron microscope, operated at 7 kV, after gold coating with an Edwards S150A sputter coater. Biofilms on the gravel were also stained with BacLight™ viability probe and viewed weekly with a Nikon Eclipse E400 epifluorescence microscope equipped with excitation/barrier filter sets of 465 – 495 / 515 – 555 nm (Texas Red) and 540 – 580 / 600 – 660 nm (FITC), was used. Fluorescent images were captured with a Nikon Coolpix 990 digital camera.

3.3.5. Data analysis

Data from T-RFLP analyses were processed with application of Statistica software (Statsoft®) using cluster analyses. For this application the presence or absence of T-RFs in each sample (with regard to the all the T-RFs of all the samples over the entire sampling period) was compared to determine the percentage disagreement between the various samples.

3.4. RESULTS AND DISCUSSION

3.4.1. COD removal efficiency

3.4.1.1. Experimental wetlands

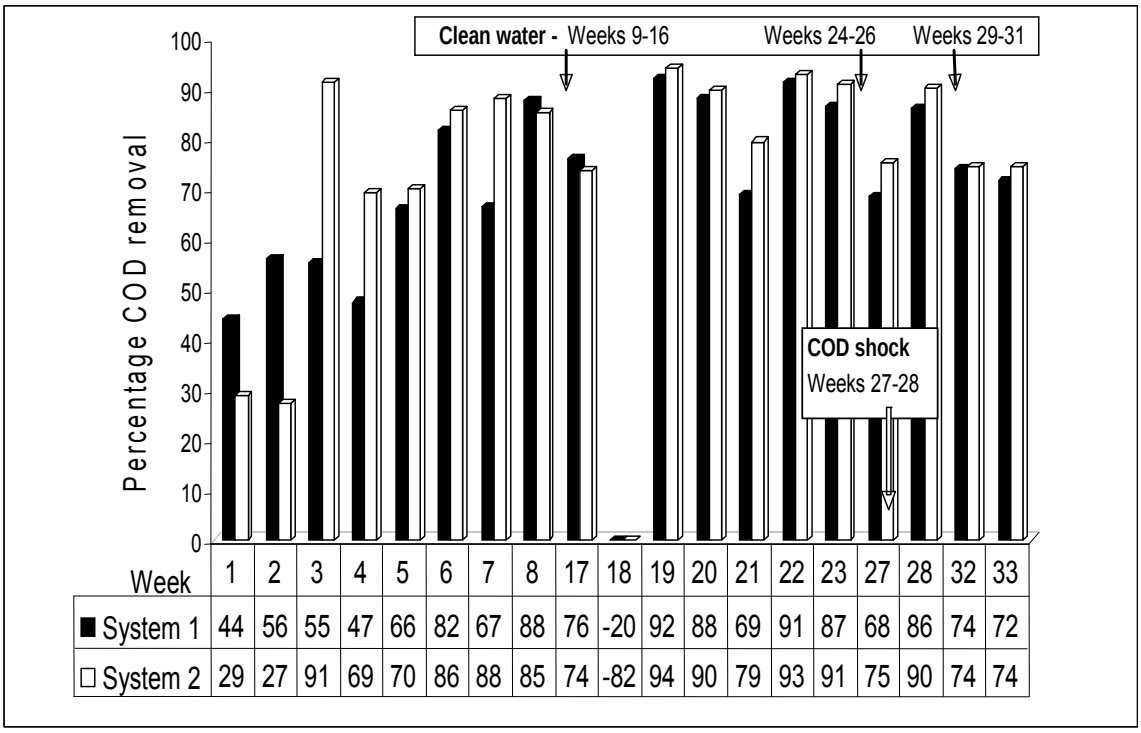
After 1 month, COD removal in the 45 m soil and gravel wetlands was ~50% and 20 - 40%, respectively. However, after 12 months the average COD removal in the gravel wetland was 84% while COD removal efficiency in the soil wetland decreased to < 40%, probably due to factors noted by Mattison *et al.* (2002), such as excessive plant growth, clogging of the soil matrix due to biomass accumulation, exopolymeric (EPS) production and/or insoluble biogas formation by soil microorganisms. COD removal efficiency was positively correlated with

retention time in the 6 m gravel wetlands, with an 18 day RT being most efficient (average 79%), followed by 9 (64%) and 4.5 days (39%) respectively over 12 months. Although the wetlands were effective in reducing most of the elements (*e.g.* K and N) they were not effective in reducing electrical conductivity.

3.4.1.2. Pilot scale model systems

Although COD removal was initially low (first 4 weeks in system 1; 2 weeks in system 2), both systems achieved COD removal that was similar to the 45 m gravel wetland (> 80% by week 6; Figure 3.1). Due to operational reasons, no distillery effluent was available between weeks 9 and 16. Therefore, fresh water was pumped through to the pilot scale systems for this period – a practice that may also occasionally be needed in practice. Despite the fact that the microbial communities were not exposed to effluent for this period, they responded rapidly to the re-introduction of effluent in week 17 and achieved a COD removal efficiency of > 70%. We do not have an explanation for the steep decline in COD removal capacity in week 18 (Figure 3.1). This may be due to some unknown characteristic in the effluent at that time since both model systems showed this result. The characteristics of distillery wastewater are highly variable depending on the various aspects of alcohol production process (Wilkie *et al*., 2000), wash water used to clean fermenters, cooling water blow down as well as boiler water blow down.

Although there was an average COD removal of 77% and 83% in pilot scale system 1 and 2, respectively during weeks 27 and 28, when effluent with a COD of 21 000 – 24 000 mg/L was passed through them, they became anaerobic and the plants showed signs of stress. The model systems were subsequently allowed 3 weeks to recover by letting only fresh water pass through them (weeks 29 to 31). The resilience of these systems was demonstrated by the recovery of their COD removal capacity to 73% and 74% at weeks 32 and 33 respectively.



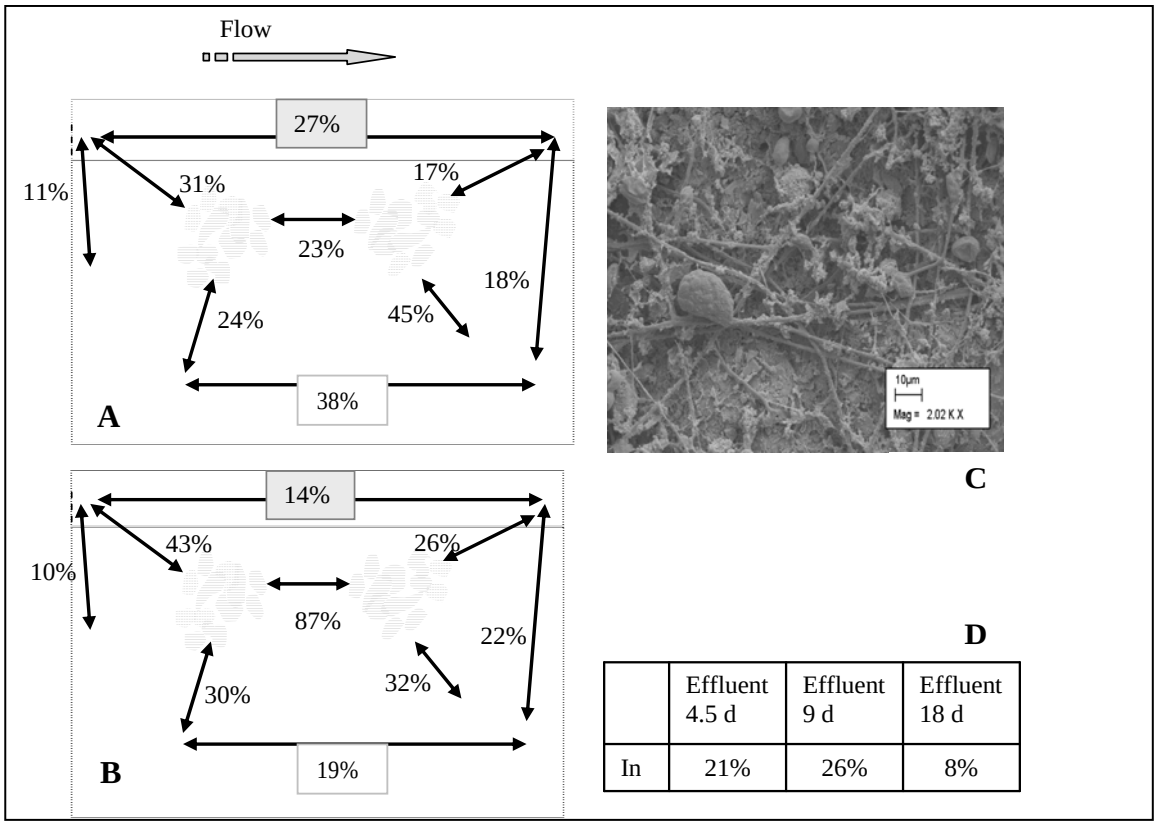
Week	1	2	3	4	5	6	7	8	17	18	19	20	21	22	23	27	28	32	33
■ System 1	44	56	55	47	66	82	67	88	76	-20	92	88	69	91	87	68	86	74	72
□ System 2	29	27	91	69	70	86	88	85	74	-82	94	90	79	93	91	75	90	74	74

Figure 3.1. COD removal over 33 weeks in the pilot scale model systems. Due to operational reasons, no distillery effluent was available between weeks 9 and 16 as well as weeks 24 and 26. Between weeks 29 and 31 the wetland systems were allowed to recover after a COD shock. Fresh water passed through the systems during these periods.

3.4.2. Microbial community structure in experimental wetlands

T-RFLP analyses of the 5 experimental wetlands revealed notable variation in their microbial communities. There was also little similarity between the community fingerprints for the respective sampling points over time (data not shown), *e.g.* there was only 6% corresponding fragments in the effluent at the outlet of the 45 m soil wetland between two consecutive months (August to September) while 39% of the fragments in the influent corresponded over this period. The highest correlation between samples collected on different dates was for those obtained from roots in the wetlands; *e.g.* 60% similarity between biofilm communities on the roots at the outlet of the 45 m soil wetlands. A possible explanation for the differences, especially in the aqueous phase is the change in effluent composition (Table 3.1). Overall these results are in agreement with the study by Ragusa *et al.* (2004), in which indicators of biofilm development was

monitored in constructed wetland microcosms, and it was found that micro-scale biofilm properties changed continuously.



	Effluent 4.5 d	Effluent 9 d	Effluent 18 d
In	21%	26%	8%

Figure 3.2. T-RFLP fingerprint data to compare the microbial communities in different regions of the experimental wetlands. Schematic diagrams of A (45 m wetland with gravel support) and B (45 m wetland with soil) show the percentage similarity, based on the number of corresponding T-RF's, between microbial communities present in the influent (top left corner of each diagram), effluent (top right corner of each diagram), biofilms on roots near the inflow and outflow, as well as biofilms on the support geologic material near the inflow and outflow, respectively. C shows a representative SEM image of the biofilms that developed on gravel. D shows the percentage similarity between microbial communities present in the influent and effluents of the three 6 m wetlands with retention times of 4.5, 9 and 18 days, respectively.

Figure 3.2 shows that there was little agreement between the T-RFs obtained from the planktonic communities of the influent and effluents of the five experimental wetlands. In fact, the highest percentage of correlation of T-RFs was between the influent and biofilms on soil near the inlet (43%; Figure 3.2B). It was also interesting to observe that there was no correlation between RT and

community profiles when the effluents of the five experimental wetlands were compared. These results suggest the possibility that various other biotic and abiotic factors influence microbial community composition in wetlands, and that unique micro-environments are established in response to the factors that facilitate microbial degradative processes.

The data in Figure 3.2 also show that planktonic communities differ markedly from attached communities within the same region of the wetland – *e.g.* there was only 17% and 18% correlation between the T-RFs obtained from the effluent and biofilm communities growing on the gravel and roots, respectively in the 45 m gravel wetland. Also, effluent from the 45 m soil wetland showed only 26% and 22% similarity with biofilms from soil and roots in the corresponding zones of the wetland. The attached communities on different surfaces within the same region of the wetland also varied markedly from each other.

Vacca *et al.* (2005) found that the microbial communities in wetland systems are strongly influenced by the movement of the wastewater through the systems, the substrate of the wetlands as well as the plants. Their study also revealed that fingerprinting of the rhizosphere of plants grown on sand or expanded clay exhibited many differences, which showed that different microbial communities exist depending on the soil type of the systems. Chiarini *et al.* (1998) showed that the rhizosphere of maize plants is markedly affected by the type and parameters of the soil. Similar results were found in this study with the molecular fingerprint patterns of the 45 m gravel wetland being completely different from that of the soil wetland, indicating the responsiveness of natural microbial communities. This adaptability suggests that microbial communities maintain metabolic function by modifying species composition in response to fluctuations in their environment. This corresponds to the concept of functional redundancy, which refers to the fact that different species in an ecosystem perform the same functional role so that changes in species diversity do not affect ecosystem functioning (Lawton and Brown, 1993). Furthermore, perfect functional redundancy means that

species are exactly equivalent in functional terms, so that losing any species from a mixture does not alter function (Johnson 2000). It is, therefore, not possible to say whether functional redundancy is being observed in our study, since it is not clear what role the organisms, detected by T-RFLP analyses, had in overall community functioning.

Retention time may play a role in the make-up of the microbial communities as the three 6 m wetlands all had different microbial fingerprint patterns at the outlets (Figure 3.2). Mayo and Mutamba (2004) found that improved nitrogen removal occurred with increase in hydraulic retention time in a high rate pond as well as in a subsurface-flow gravel bed constructed wetland. It is also known that the microbial nitrification–denitrification processes are responsible for the bulk of nitrogen removal (Sikora *et al*., 1995). Therefore, a change in retention time can alter the microbial community present within a system, which in turn impacts on the effluent amelioration efficiency.

3.4.3. Culturable microbial cell numbers

Frankenberg and Dick (1983) argued that plate counts correlate poorly with microbial biomass and enzymatic measurements of microbial growth. However, Harris and Birch (1992) provided evidence that plate count techniques are useful in comparative studies of specific microbial populations. The total culturable bacterial counts (cbc) on TSA at the inlet of the experimental wetlands were 1.8×10^3 to 2.4×10^3 cells/ml while the culturable yeast counts (cyc) ranged between 1.0×10^3 and 3.5×10^3 cells/ml. The cbc at the outlets of the wetlands ranged between 2.8×10^4 cells/ml in the 6 m wetland (RT 4.5 days) to 4.5×10^4 cells/ml at the outlet of the 45 m soil wetland (RT 14 days). The cyc were generally low ($< 5.0 \times 10^2$ cells/ml) at all the sampling points. The acidity of the distillery effluent may play a major role in the low bacterial counts in the influent of the wetland (Table 3.1) while the increase in bacterial counts within and after the wetlands may be as a result of exudates and root residues that enhance the underground C stock, which are readily available to microorganisms (Kuzyakov, 2002). These

conditions contribute to fast C turnover, and to higher microbial activity in the rhizosphere. Similar results were found in previous studies which showed that the bacterial populations are generally enhanced in the rhizosphere (Chiarini *et al*., 1998; Soto *et al*., 1999).

3.4.4. Temporal studies of microbial distribution

The T-RFLP analyses of the biofilm communities growing on gravel in the pilot scale systems showed that at week two of the 11 week trial most of the predominant organisms were already established within the biofilm (Figure 3.3A). Most of these organisms were detected throughout the experiment (*e.g.* organisms depicted by fragment sizes 88, 249 and 306 base pairs in Figure 3.3A). During the COD shock treatment between weeks 8 and 11, many of these organisms were still present (represented by fragments 88, 249 and 306 base pairs). Various organisms appeared after the initial COD shock (110 base pairs [weeks 9-11] and 270, 284, 402 and 478 base pairs [weeks 10-11]). These may be organisms that were either present in undetectable numbers due to unfavourable environmental conditions, or that were introduced into the system with the high concentration wastewater. It was also indicated by others (*e.g.* Davey and O'Toole, 2000) that biofilm structure is affected by both microbial species present and environmental parameters.

Cluster analysis of the data showed two distinct groupings (Figure 3.3B); the first group representing the samples collected in weeks 2 to 8, and the second group weeks 9 – 11. The sub-clustering of weeks 2 and 3, and subsequent changes between weeks 4 to 8 demonstrate shifts within the microbial community during these early developmental stages of the biological system. When compared to the COD removal data in Figure 3.1, it is clear that overall system performance (microbial community function) is not entirely dependent on a specific microbial community composition (community structure).

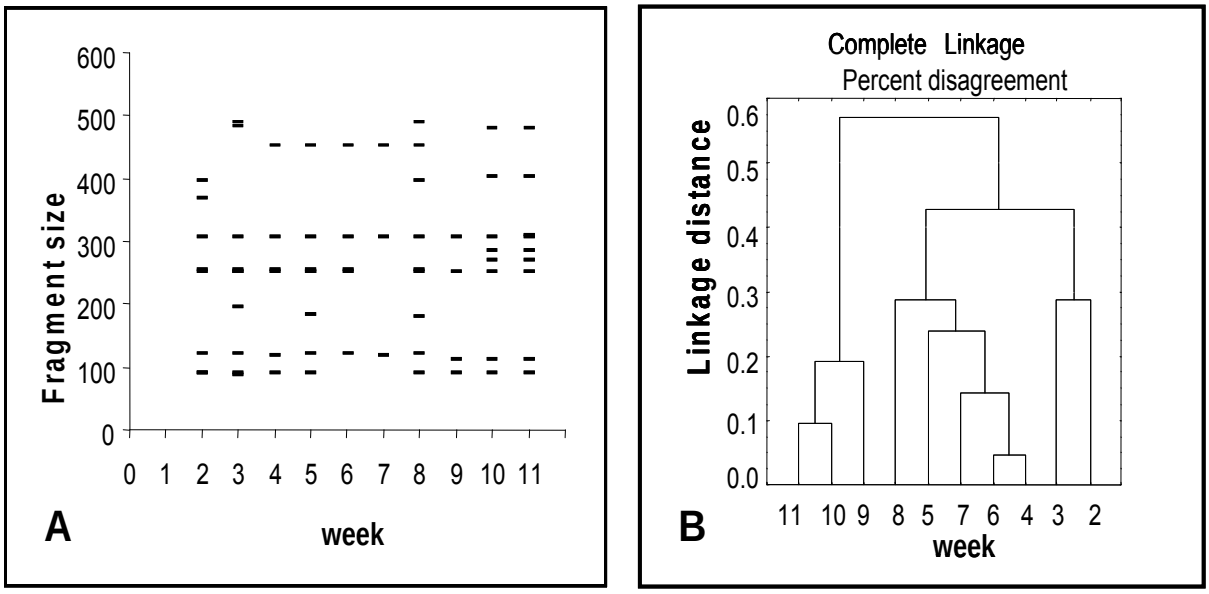


Figure 3.3. Biofilm community dynamics in the pilot scale systems. A. T-RFLP profiles were used to follow community shifts during the early stages of development and response to a COD shock during week 8. B. Cluster analysis of the data in A to demonstrate the sequential nature of community development.

Fundamentally, it therefore appears that similar to the emergence of individual cells where natural selection favours populations that are phenotypically distinct from their parental strains, degradative communities change in response to environmental stimuli with subsequent benefit to the community. This is illustrated by the fact that changes in the microbial communities over time (Figure 3.3) did not affect the COD removal efficacy of the system (data not shown). Community-level adaptation, *e.g.* to environmental stresses, occurs at the level of member (species) composition, similar to teams or consortia where members are 'selected' based on their potential contribution in a given situation. Selection at the community level is therefore a rapid process as illustrated in Figures 3.3 and 3.4 (and numerous other reports on microbial community dynamics in recent literature), as long as an extensive pool is maintained from which members are selected. This latter criterion is met in most environments, given the repeated mentioning in the literature of the described, as well as yet to describe species pool in natural and engineered environments. The

emergence of species with fragments 110, 270, 284, 402 and 478 base pairs (Figure 3.3) after the COD shock further provides support for this argument.

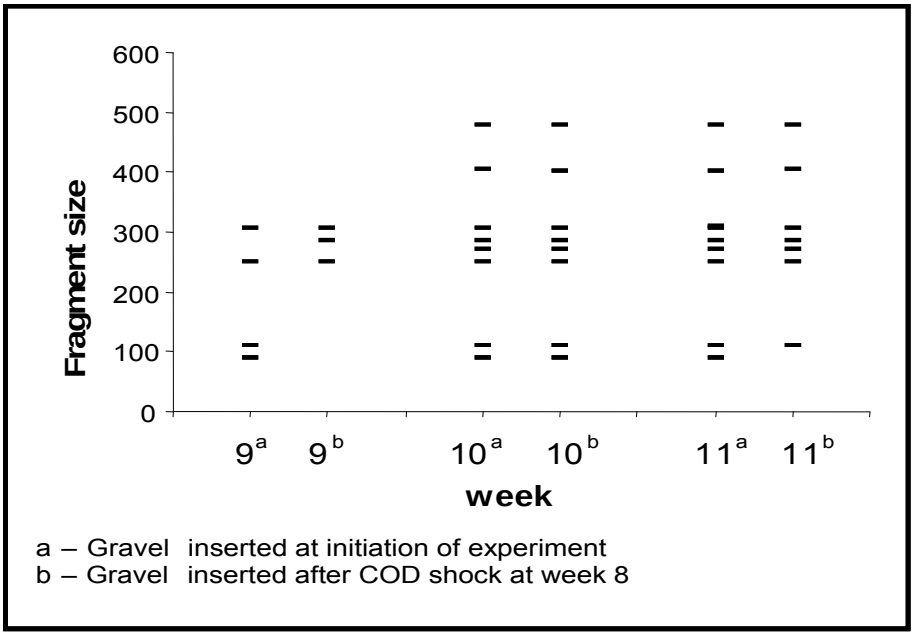


Figure 3.4. T-RFLP profiles to compare community dynamics of established biofilms after exposure to a COD shock with biofilms developing on gravel that was newly inserted after the COD shock.

Figure 3.4 suggests that despite notable differences one week after the COD shock was initiated there was a strong correlation between newly-formed and established biofilms, especially after 2 and 3 weeks of high COD effluent application. It was not within the scope of the present study to determine the time required by wetland communities to reach a steady state, and the system has not yet stabilized when the COD shock was inflicted during week 8. Ragusa *et al.* (2004) found that the total biomass can take upwards of 100 days to stabilize during batch operation of subsurface wetlands, however it is highly probable that a fair degree of flux will continuously occur in any facility used to treat industrial effluent because of seasonal and operational variants.

Similar to the biofilm communities, the planktonic microbial communities showed notable change in composition over time (Figure 3.5A). Furthermore,

there was also a notable reduction in dominant species (T-RF's) and an emergence of new species among the planktonic communities after the COD shock. This is due to the fact that wastewater treatment systems such as wetlands are likely to select different functional and phylogenetic groups of organisms (Liu *et al.,* 1997). Some organisms showed a strong dominance and persistence. For instance, the organism represented by the T-RF at 88 base pairs was present in the planktonic community at the inlet of pilot scale systems throughout the experiment and at most sampling dates in the other two zones (data not shown). The organism represented by the 249 base pair-fragment was also present in most samples. Although the microbial fingerprint patterns of the planktonic community at the inlet correlated strongly with that in the central zone in weeks 1 and 2 (70 and 100%, respectively) there was significant variation over time (Figure 3.5B).

3.4.5. Biofilm structure

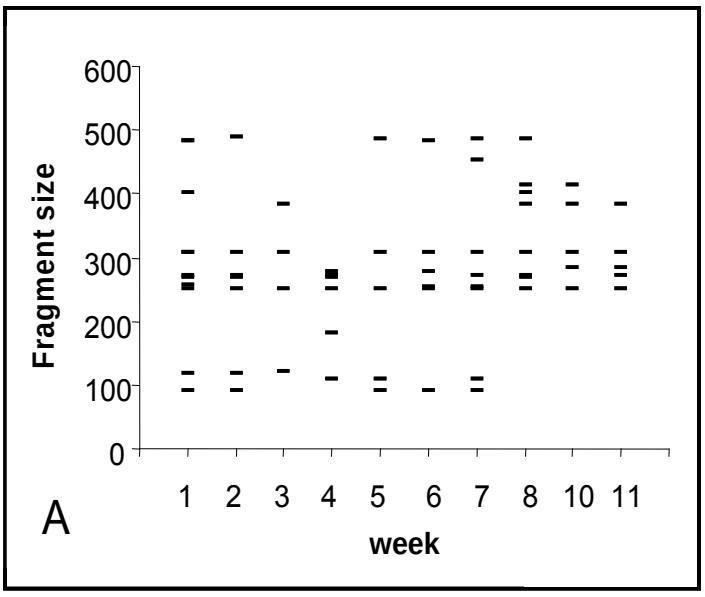
SEM and epifluoresence microscopy showed that colonization of the gravel occurred in less than one week, with subsequent progression from relatively thin biofilms to complex structures with notable heterogeneity in structure and cell morphotypes, typical of various stages of colonization. Due to the high degree of autofluorescence and the entrapment of non-cellular material in the biofilm matrix, it was not possible to determine the relative abundance of live vs. dead biomass. Nevertheless, investigations with the BacLight viability probes indicated that a large proportion of the bacterial community consisted of live (green) cells; these qualitative observations corresponded to the quantitative COD removal data and the T-RFLP results obtained from the gravel.

3.5. CONCLUSIONS

From this study the following conclusions can be drawn:

1. Distillery effluent can be treated effectively with constructed wetlands if the inflowing effluent COD is kept below 15 000 mg/L. However, higher

COD effluents may result in anaerobic conditions in the soil or gravel matrix, notable changes in microbial community composition, wilting of the plants, and finally a collapse in treatment efficiency. The distilling industry regularly produces effluents with COD that exceed the levels shown here to be tolerable to wetlands; therefore it is unlikely that wetlands can be applied as the sole treatment option.



B

Central

	Week 1	Week 2	Week 3	Week 4	Week 5	Week 6	Week 7	Week 8
Inlet	70%	100%	33%	41%	40%	50%	50%	9%
Outlet	44%	77%	67%	27%	33%	44%	44%	70%

Figure 3.5. A. T-RFLP profiles over time of planktonic microbial communities in the central zone of the bench-scale system. B. Percentage similarity between the planktonic communities of the central zone and those sampled at the inlet and outlet just before the COD shock at week 8 to show typical variation in the planktonic phase.

2. Wetlands have the ability to recover after a COD shock if the effluent is replaced with fresh water for a relatively short time (2-3 weeks).
3. A highly dynamic microbial composition exists within wetlands. It should also be noted that we only used absolute COD values, but admit that effluent make-up will also affect microbial response and overall system performance. This illustrates the need for pre-treatment before discharge into the wetland and a management plan that involves routine analyses to ensure that the pre-treatment is sufficient.
4. Wetlands can efficiently remove COD even though a low degree of similarity exists between microbial communities in various zones of the same wetland and those between different wetlands (Figure 3.2), as well as low similarity between communities sampled from the same zone over time. This raises the question whether the 'ideal' degradative community can be defined in terms of microbiological criteria and serves as a reminder that various indicators should be considered for monitoring system health.

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CHAPTER

4

Dynamics in the microbial distribution in experimental and pilot scale constructed wetlands used in the treatment of distillery wastewater

DYNAMICS IN THE MICROBIAL DISTRIBUTION IN EXPERIMENTAL AND PILOT SCALE CONSTRUCTED WETLANDS USED IN THE TREATMENT OF DISTILLERY WASTEWATER

4.1. ABSTRACT

The objective of this part of the study was to illustrate how the attached and planktonic microbial communities within various regions in experimental wetlands and a pilot scale system varied over time. Terminal-restriction fragment length polymorphism analyses (T-RFLP) revealed high diversity among the microbial communities sampled at 14 locations in the experimental wetlands on two sampling dates, one month apart (August and September). Similarly, T-RFLP analysis of microbial communities in pilot scale systems revealed notable difference among the dominant (in terms of its presence throughout the experimental period and at various sampling points) planktonic populations at the inlet, central zone and outlet of the wetlands, when monitored over an 11-week period. COD removal remained constant despite these shifts in the planktonic microbial community over time. SEM and epifluoresence microscopy showed the presence of distinct morphotypes during biofilm formation on the gravel support.

Key words: experimental wetlands, biofilm, pilot scale wetlands, microbial distribution, planktonic populations, T-RFLP

4.2. INTRODUCTION

In 1999 a study was initiated at Distell Goudini in the Western Cape, South Africa, to examine the feasibility of using constructed wetlands in the treatment of distillery wastewater (Mulidzi, 2005). The constructed wetlands, as described in chapter 3, became operational in 2001 and were monitored for its impact on various parameters (*e.g.* COD, electrical conductivity, pH, etc.) of distillery effluent. It was found that constructed wetlands had the capacity to treat distillery

wastewater (> 80% COD removal by the 45 m gravel wetland on average in 2004), providing the influent COD did not exceed 15 000 mg/L for extensive periods and the correct substrate material was used (Mulidzi, 2005). In our study the latter was verified over the first year of operation when the average COD removal for the 45 m gravel wetland was 84% while the average COD removal efficiency of the 45 m gravel soil wetland was < 40% (chapter 3). This was probably due to excessive plant growth, clogging of the soil matrix due to biomass accumulation, exopolymeric substance (EPS) production and/or insoluble biogas formation by soil microorganisms (Mattison *et al.,* 2002).

Using the T-RFLP technique we found notable variation in the microbial communities at respective sampling points in five experimental wetlands over time (chapter 3). The T-RFLP technique is a molecular approach that can assess subtle genetic differences between strains as well as provide insight into the structure and function of microbial communities (Marsh, 1999). Because of its sensitivity and high throughput, it is an ideal technique for comparative community analyses. Consequently, we decided to use periodic T-RFLP analyses, supported with epifluorescence and scanning electron microscopy to explore dynamics in the microbial populations within the experimental wetlands described in chapter 3.

This chapter describes experimentation in parallel with the research described in chapter 3. Here the use of T-RFLP to periodically generate DNA fingerprint patterns of microbial communities at different sampling points in the five constructed wetlands and one pilot scale system (chapter 3), used in the treatment of distillery effluent with varying COD content is reported. Furthermore, biofilm development in the pilot scale system was monitored via scanning electron and epifluorescent microscopy.

4.3. MATERIALS AND METHODS

4.3.1. Experimental wetlands and lab scale model systems

The same experimental wetlands (Figure 4.1) and pilot scale systems (Figure 4.2) were used as those described in chapter 3. Main chemical properties of the wastewater for the first two months of operation in the experimental wetlands are listed in Table 3.1. Distillery effluent with a COD ranging between 5 000 mg/L and 8 000 mg/L was pumped through the pilot scale systems except when experiments were conducted to simulate a COD shock for which the COD load was increased to 16 000 – 24 000 mg/L for 3 weeks.

4.3.2. Microbial community diversity in the experimental wetlands and pilot scale system

4.3.2.1. Sampling, DNA extraction and T-RFLP analyses

Monthly samples for two consecutive months (August and September) were collected at 14 sites in the experimental wetlands as indicated in Figure 4.1. For the pilot scale system (Figure 4.2) routine analyses included weekly monitoring of COD and microbial diversity in the influent and effluent as well as in the middle of the system. T-RFLP for experimental wetlands and pilot scale system as well as scanning electron and fluorescent microscopy for pilot scale system were applied for microbial analysis. To evaluate the behaviour of microorganisms from the established system when provided with fresh surfaces, a permeable holder containing sterile (autoclaved) gravel (~2.5 mm and ~25 mm diameter) were placed in the central region of the pilot scale system.

DNA was extracted from the wastewater samples and biofilms on the surface of gravel particles according to the method of Zhou *et al.* (1996) as previously described in chapter 3. For the T-RFLP analyses in the pilot scale wetland, ~25 mm diameter gravel was collected from the permeable holder. T-RFLP was performed as decribed in chapter 3 using the primer pair: 6-carboxyfluorescein (FAM)-labelled 341f (5'-CCT ACG GGA GGC AGC AG-3') (Muyzer *et al.*, 1993)

and unlabeled rPP2 (5'-CGG ITA CCT TGG TTA CGA CTT-3') (Rawlings, 1995) for Eubacteria. The restriction enzyme *Alu*I (Roche Molecular Biochemicals) was used to digest PCR products.

4.3.2.2. Microscopy

Gravel samples (~2.5 mm diameter) were collected from the permeable holder in the pilot scale system on a weekly basis and microscopically analyzed via scanning electron and epifluorescence microscopy as described in chapter 3.

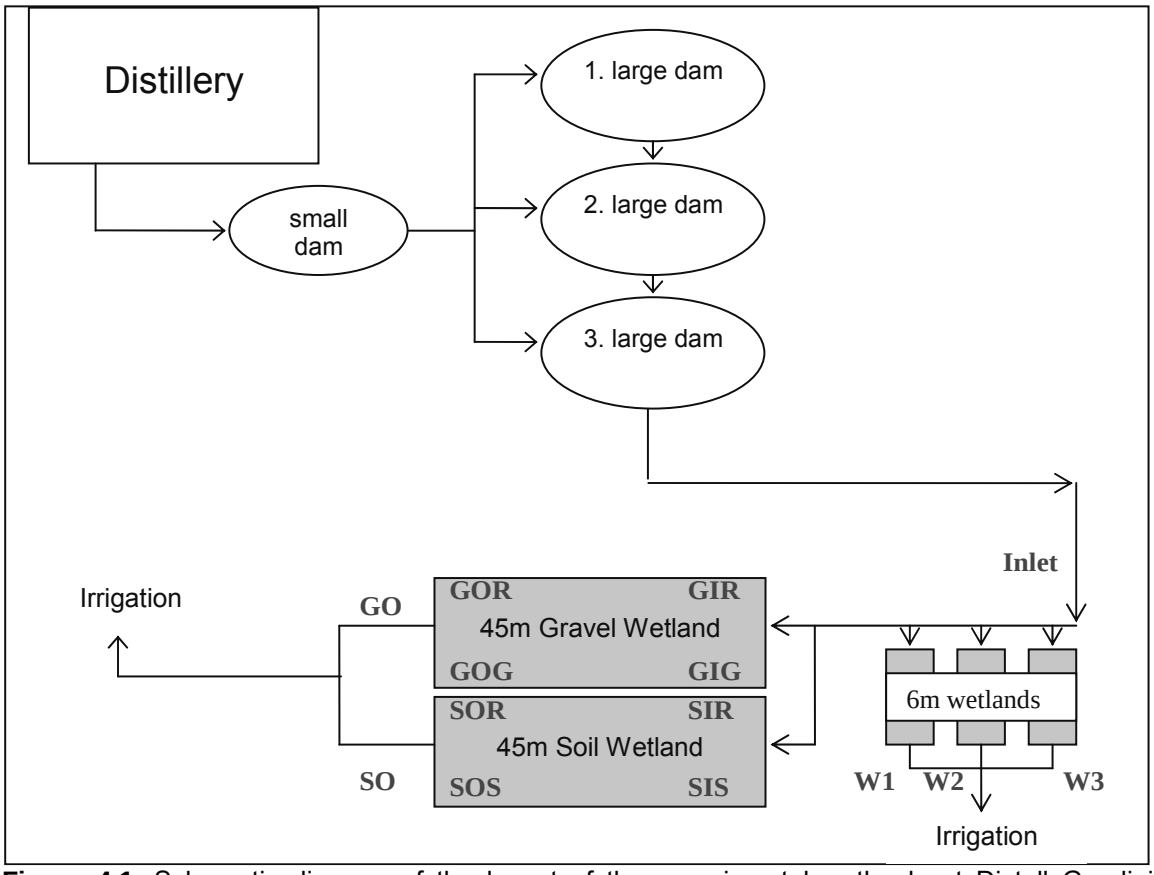


Figure 4.1. Schematic diagram of the layout of the experimental wetlands at Distell Goudini distillery in the Western Cape, South Africa. Samples were taken at the points indicated in red.

Inlet = Effluent obtained before entry into wetlands; W1 = Effluent at outlet of 6 m wetland with 4.5 day RT; W2 = Effluent at outlet of 6 m wetland with 9 day RT; W3 = Effluent at outlet of 6 m wetland with 18 day RT; GO = Effluent at outlet of 45 m gravel wetland with 14 day RT; SO = Effluent at outlet of 45 m soil wetland with 14 day RT; GIG = Gravel at inlet of 45 m gravel wetland with 14 day RT; GOG = Gravel at outlet of 45 m gravel wetland with 14 day RT; GIR = Roots at inlet of 45 m gravel wetland with 14 day RT; GOR = Roots at outlet of 45 m gravel wetland with 14 day RT; SIS = Soil at inlet of 45 m soil wetland with 14 day RT; SOS = Soil at outlet of 45 m soil wetland with 14 day RT; SIR = Roots at inlet of 45 m soil wetland with 14 day RT; SOR = Roots at outlet of 45 m soil wetland with 14 day RT.

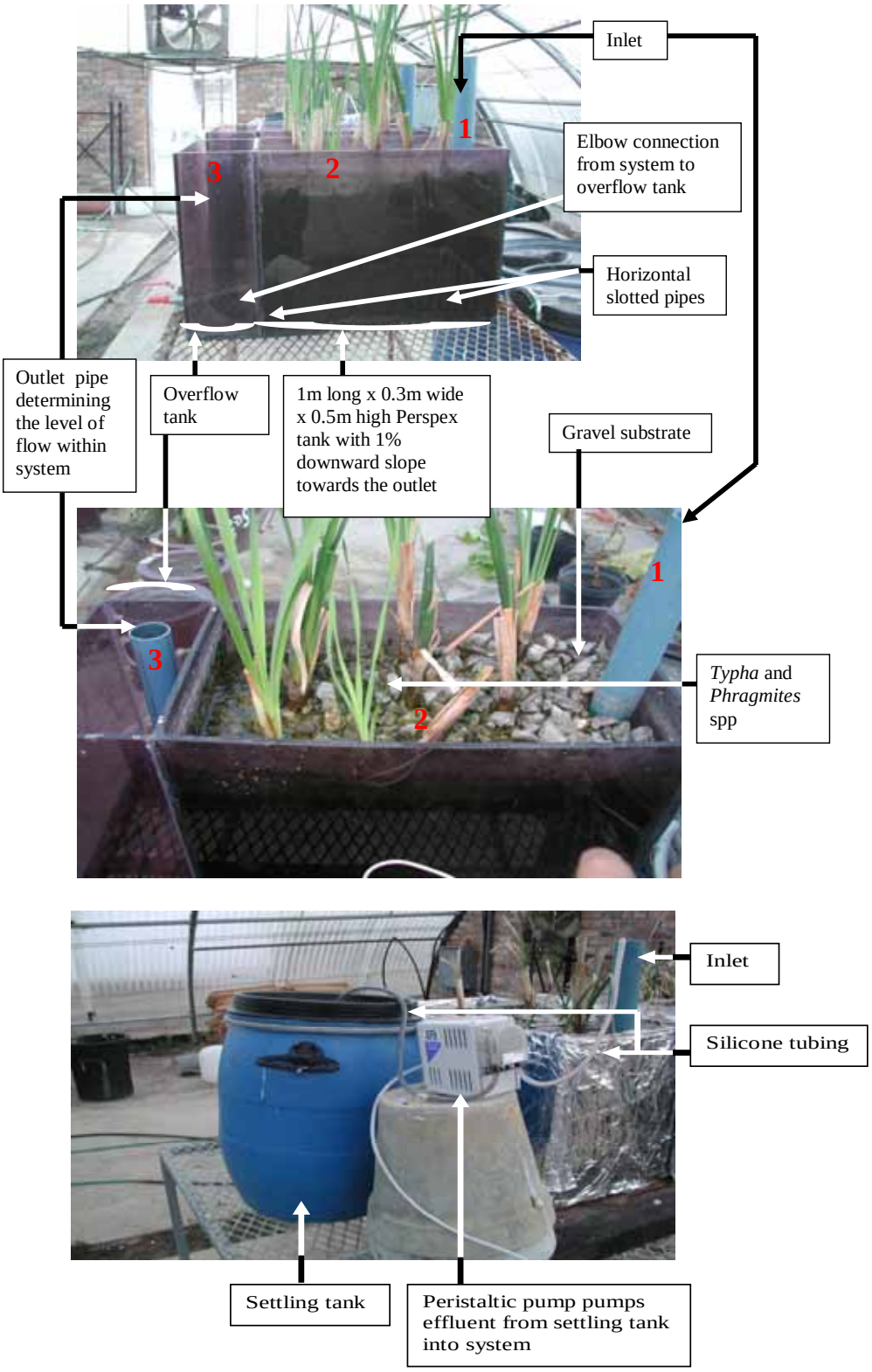


Figure 4.2. Pilot scale wetland system. Samples were taken at 1. the inlet, 2. the central zone and 3. the outlet.

4.3.3. Data analysis

Data from T-RFLP analyses were processed with application of Statistica software (Statsoft®) using cluster analyses. For this application the presence or absence of T-RFs in each sample (with regard to the all the T-RFs of all the samples over the entire sampling period) was compared to determine the percentage disagreement between the various samples.

4.4. RESULTS AND DISCUSSION

4.4.1. Microbial community diversity in the experimental wetlands

T-RFLP analyses of the 5 experimental wetlands revealed notable variation in their microbial communities (Figures 4.3 and 4.4). There was also little similarity between the community fingerprints for the respective sampling points between two consecutive months (Table 4.1). The highest correlation between samples collected at the same sampling points, but on different dates was for those obtained from roots in the wetlands. A possible explanation for the differences, especially in the aqueous phase is the change in effluent composition (Table 3.1).

There were consistently more organisms detected at all the respective sampling points in September compared to August (Table 4.2). This may also be due to change in effluent composition for the two months. There were 40 more organisms detected on the roots at the outlet of the 45 m gravel wetland in September than in August. This may be due to several factors including the fact that average daily temperatures are higher in September than in August. Overall these results are in agreement with the study by Ragusa *et al.* (2004), in which indicators of biofilm development was monitored in constructed wetland microcosms, and it was found that micro-scale biofilm properties changed continuously.

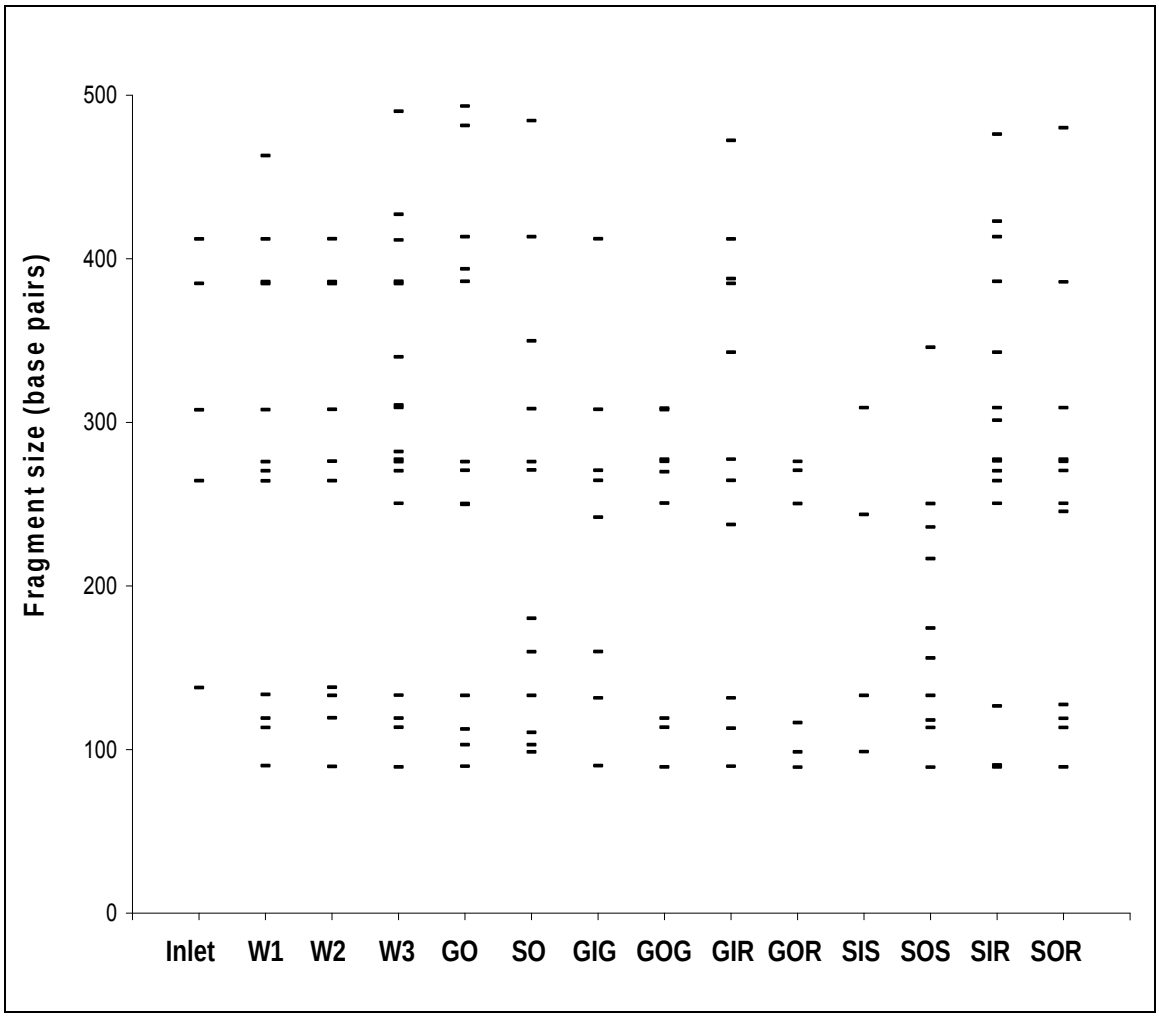


Figure 4.3. T-RFLP profiles of microbial communities in the various regions of the experimental wetlands on one sampling date in August.

Table 4.1. The % similarity between the community fingerprints for the respective sampling points in the experimental wetlands detected between two consecutive months (August and September).

Inlet	W1	W2	W3	GO	SO	GIG	GOG	GIR	GOR	SIS	SOS	SIR	SOR
39%	31%	31%	26%	41%	6%	14%	18%	15%	15%	20%	20%	31%	60%

Key for Figure 4.3. and Table 4.1.
Inlet = Effluent obtained before entry into wetlands; W1 = Effluent at outlet of 6 m wetland with 4.5 day RT; W2 = Effluent at outlet of 6 m wetland with 9 day RT; W3 = Effluent at outlet of 6 m wetland with 18 day RT; GO = Effluent at outlet of 45 m gravel wetland with 14 day RT; SO = Effluent at outlet of 45 m soil wetland with 14 day RT; GIG = Gravel at inlet of 45 m gravel wetland with 14 day RT; GOG = Gravel at outlet of 45 m gravel wetland with 14 day RT; GIR = Roots at inlet of 45 m gravel wetland with 14 day RT; GOR = Roots at outlet of 45 m gravel wetland with 14 day RT; SIS = Soil at inlet of 45 m soil wetland with 14 day RT; SOS = Soil at outlet of 45 m soil wetland with 14 day RT; SIR = Roots at inlet of 45 m soil wetland with 14 day RT; SOR = Roots at outlet of 45 m soil wetland with 14 day RT.

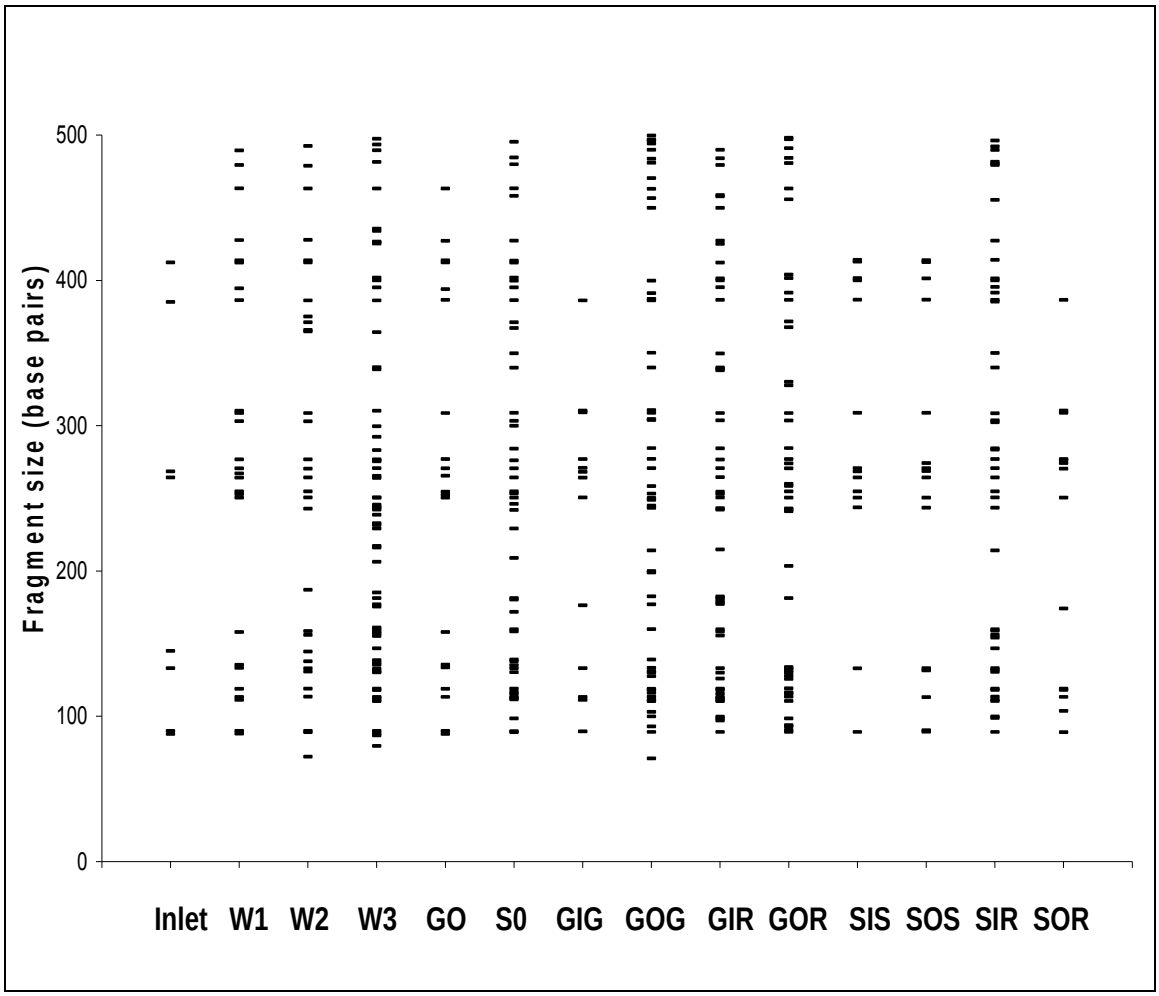


Figure 4.4. T-RFLP profiles of microbial communities in the various regions of the experimental wetlands on one sampling date in September.

Table 4.2. The amount of fragments detected for the respective sampling points in the experimental wetlands between August and September.

	Inlet	W1	W2	W3	GO	SO	GIG	GOG	GIR	GOR	SIS	SOS	SIR	SOR
August	5	12	10	17	13	12	8	9	11	6	4	10	15	12
September	7	23	29	47	19	41	12	45	42	46	14	14	33	14

Key for Figure 4.4. and Table 4.2.
Inlet = Effluent obtained before entry into wetlands; W1 = Effluent at outlet of 6 m wetland with 4.5 day RT; W2 = Effluent at outlet of 6 m wetland with 9 day RT; W3 = Effluent at outlet of 6 m wetland with 18 day RT; GO = Effluent at outlet of 45 m gravel wetland with 14 day RT; SO = Effluent at outlet of 45 m soil wetland with 14 day RT; GIG = Gravel at inlet of 45 m gravel wetland with 14 day RT; GOG = Gravel at outlet of 45 m gravel wetland with 14 day RT; GIR = Roots at inlet of 45 m gravel wetland with 14 day RT; GOR = Roots at outlet of 45 m gravel wetland with 14 day RT; SIS = Soil at inlet of 45 m soil wetland with 14 day RT; SOS = Soil at outlet of 45 m soil wetland with 14 day RT; SIR = Roots at inlet of 45 m soil wetland with 14 day RT; SOR = Roots at outlet of 45 m soil wetland with 14 day RT.

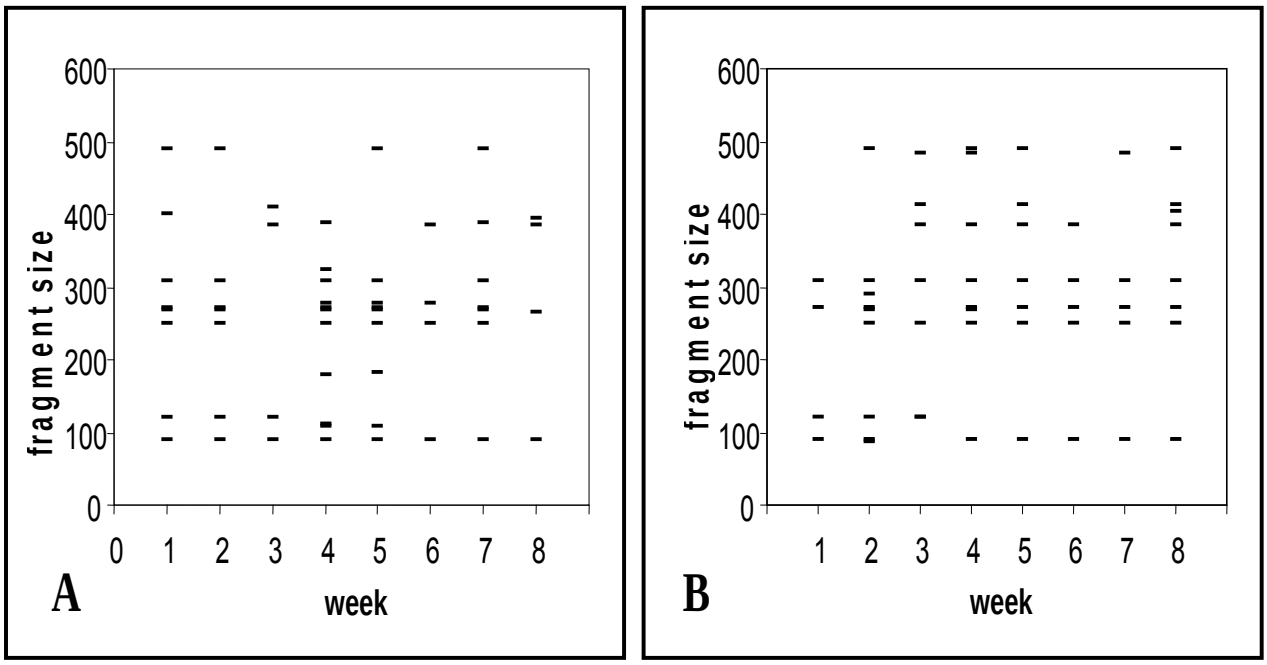
4.4.2. Microbial community diversity in the pilot scale systems

There was significant variation in the planktonic communities at the inlet and outlet respectively when compared to central zone of the pilot scale system over time (Figure 3.5B – previous chapter). Similarly, when the microbial fingerprint patterns of the planktonic community at the inlet (Figure 4.5A) were compared to that of the outlet (Figure 4.5B) very low similarity was detected (Figure 4.5C). The highest correlation was during week 2 when there was 78% similarity between the two sampling points. The T-RFLP profiles over time of planktonic microbial communities in the central zone of the pilot scale system are shown in Figure 3.5A (previous chapter).

The organism represented by the band at 88 base pairs in the planktonic community at the inlet and outlet (Figure 4.5A and B) of the pilot scale system was present during the entire 8 week testing period (accept week 2 at outlet), while the organism represented by the 249 base pair-fragment were present in most samples. The organisms represented by fragments at 88, 249, 270 and 306 base pairs were dominant in the planktonic community at the outlet of the pilot scale system (Figure 4.5B). These findings provides further support for the discussion and speculation in chapter 3; that a fair degree of flux will continuously occur in constructed wetlands used in distillery wastewater treatment because of seasonal and operational variants that will, subsequently, contribute to the highly dynamic microbial composition within these systems.

Cluster analysis of the data of the planktonic communities at the inlet, central region and outlet of the pilot scale system did not show gradual shifts in community composition, where samples collected on subsequent sampling dates typically cluster together, that would suggest a successional evolution of community composition (Figure 4.6A to C). The random clustering and sub-clustering of the microbial communities for the various sampling dates demonstrate that the composition of the planktonic microbial communities during the developmental stages of the biological system is solely not related to time,

but also other factors, in this case most probably the differences in COD along a concentration gradient from inlet to outlet. The planktonic microbial communities did not reach a steady state over the course of this experiment and appears to be influenced predominantly by the influent wastewater composition. As COD removal did not correlate with the shifts in the planktonic microbial community composition it reiterates the fact, as mentioned in chapter 3, that overall system performance is not entirely dependent on a specific microbial community composition.



C

	Inlet							
	Week 1	Week 2	Week 3	Week 4	Week 5	Week 6	Week 7	Week 8
Outlet	50	78	43	38	45	20	40	20

Figure 4.5. T-RFLP profiles over time of planktonic microbial communities at A. the inlet and B. the outlet of the pilot scale system. C. Percentage similarity between the planktonic communities at the inlet and those sampled at the outlet to show typical variation in the planktonic phase over time.

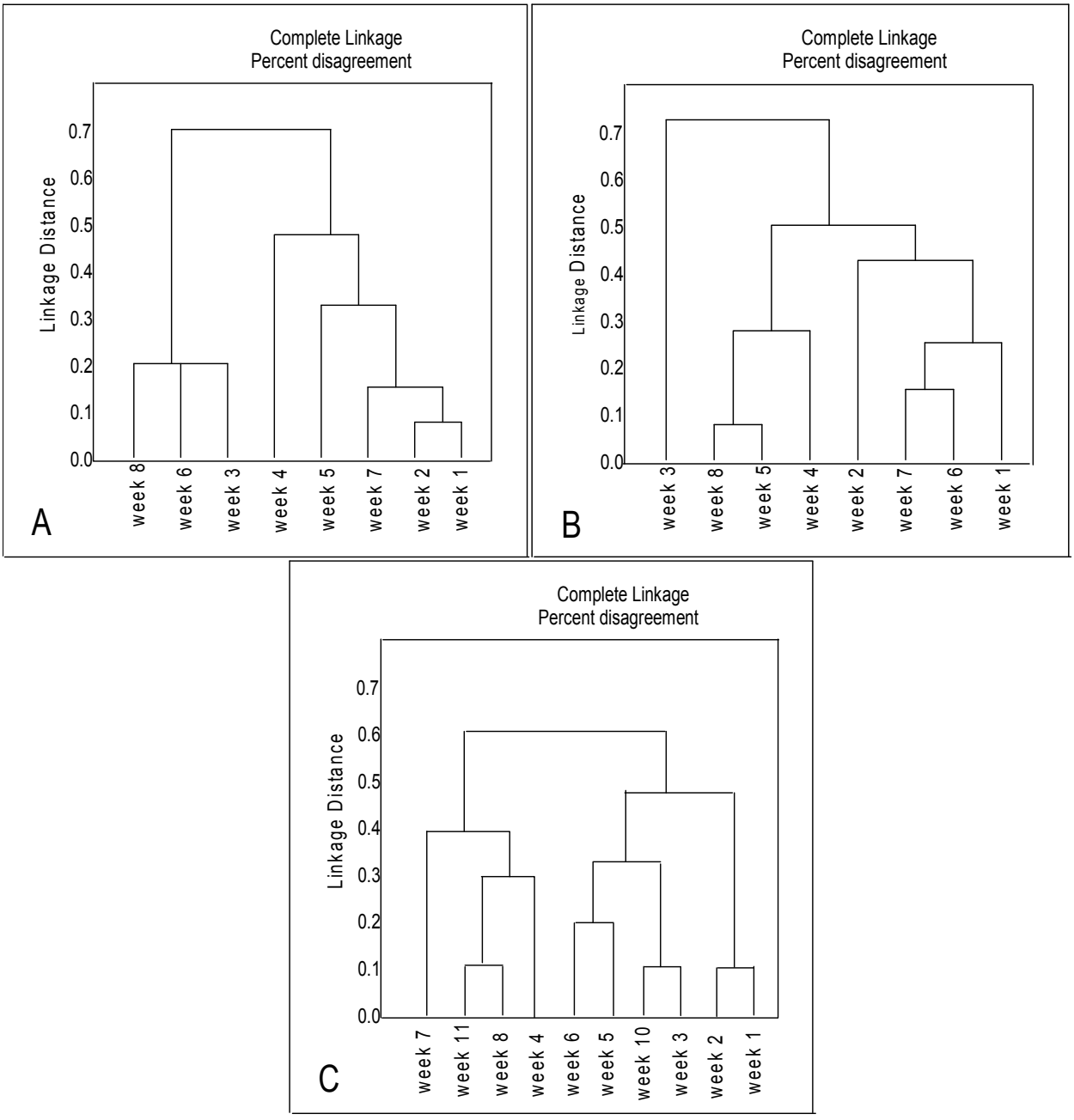


Figure 4.6. Cluster analysis of terminal restriction fragments (T-RFs) of the planktonic communities obtained at A. inlet, B. outlet and C. central region of the pilot scale wetlands over time.

4.4.2.1. Wetland response to increased COD treatment

Although the COD removal capacity of the pilot scale systems was > 60% (chapter 3) during the high COD application (16 000 mg/L – 24 000 mg/L) they

became anaerobic and the plants showed signs of stress (Figure 4.7). Figure 3.3 describes the change in the attached microbial community structure within the pilot scale systems after the COD was increased.

Figure 4.7. Pilot scale systems after 3 weeks of receiving distillery effluent with a COD in excess of 16 000 mg/l. Systems became anaerobic and plants showed signs of stress.

4.4.2.2. Microscopy

The scanning electron micrographs (Figure 4.8A to D) give an indication of biofilm development on the gravel over 8 weeks. From Figure 4.8C and D it is clear that microbial populations covered the gravel after 8 weeks. This is confirmed by micrographs obtained from fluorescence microscopy (Figure 4.8E to H). In Figure 4.8G and H the gravel sample is covered with green and red cells representing live and dead bacteria respectively, while no bacteria can be detected in Figure 4.8E and F.

A progression in biofilm development was observed (Figure 4.9A to H), with colonization of the gravel occurring in less than one week (Figure 4.9A and B). Several organisms could be detected throughout the 8 week period, with different morphotypes typical of various stages of colonization. A stratified biofilm development was observed, with a noticeable similarity in architecture between the specimens obtained three and four weeks after inserting the gravel into the wetlands (week 4 and 5 respectively – Figure 4.9D and E). These qualitative observations correspond to the quantitative T-RFLP results obtained from the gravel (Figure 3.3 – weeks 4 and 5).

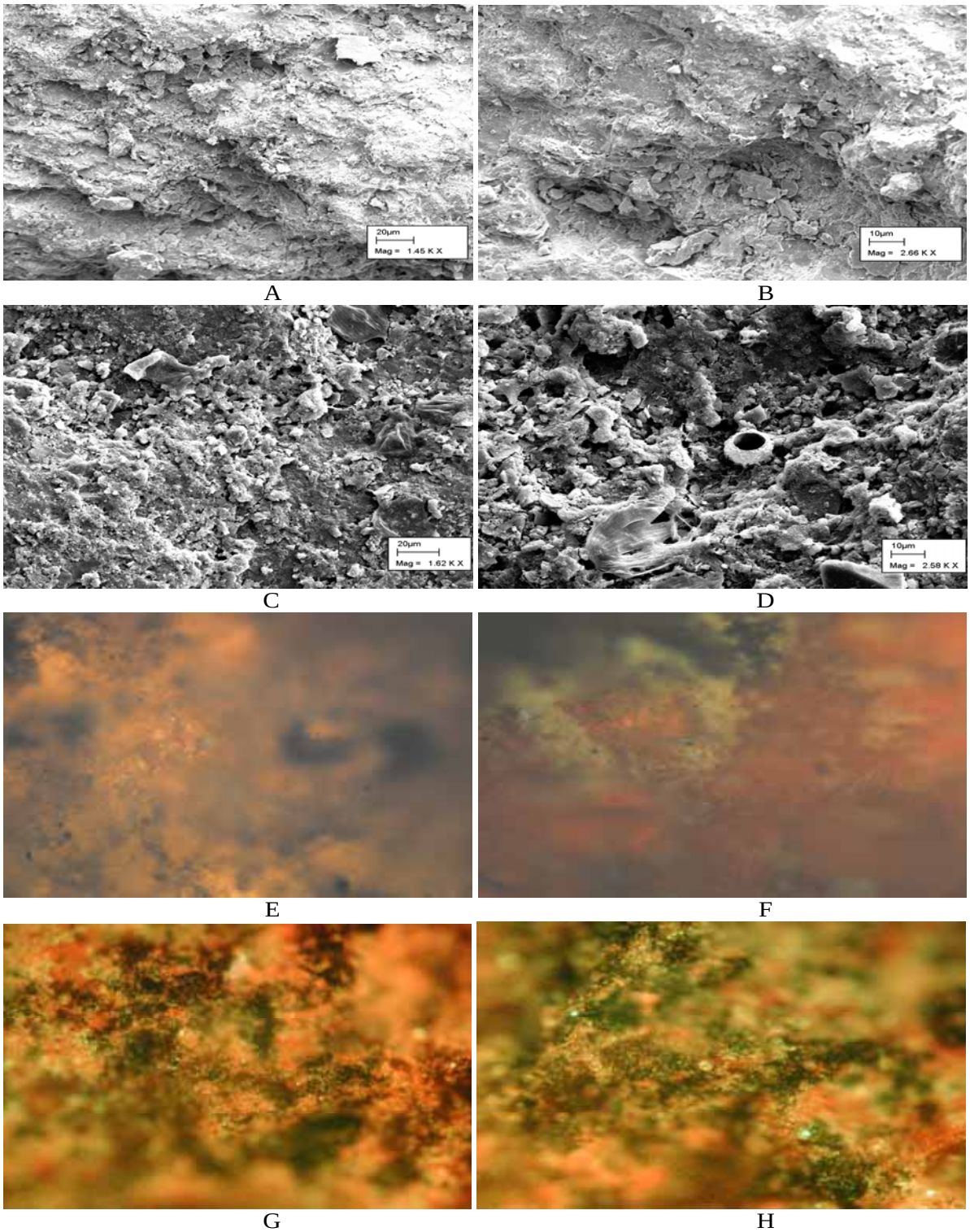


Figure 4.8. A and B, SEM images of a sterile piece of gravel before insertion into wetland. C and D, SEM images of gravel that was in the wetland for 8 weeks. E and F, Epifluorescent microscope image of sterile gravel before insertion into wetland. G and H, gravel that was in the wetland for 8 weeks showing numerous living (green) and dead (red) cells.

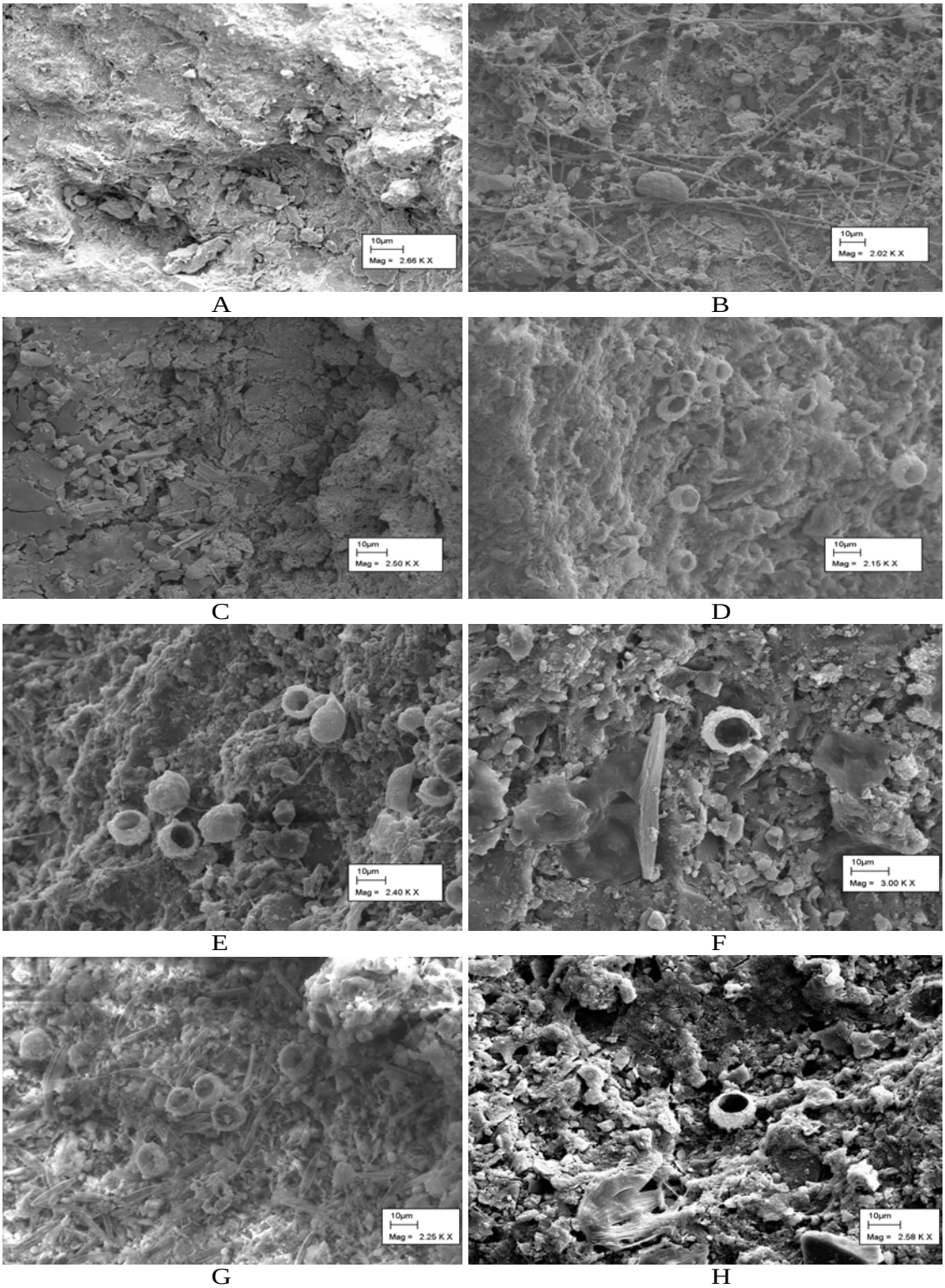


Figure 4.9. Scanning Electron micrographs of biofilm on gravel pieces: A. Week 1 (before insertion of gravel into wetlands); B to H. Week 2 (one week after insertion into the wetland) to Week 8 (seven weeks after insertion into the wetland) showing the progressive colonization of gravel by microorganisms.

4.5. CONCLUSIONS

In addition to the conclusions made in chapter 3 on microbial heterogeneity in the wetland systems, this section showed that:

1. Planktonic and attached microbial communities in various regions of the same wetland vary significantly from each other. Similarly, microbial communities at respective sampling points vary notably over time. This again illustrated the highly dynamic nature of microbial communities in constructed wetlands and corroborates our findings in the previous chapter.
2. The development of the planktonic microbial community composition at the inlet, central region and outlet of wetlands are not solely time-dependent. Furthermore, COD removal was not affected by the shifts in the planktonic microbial community over time. This corresponds to our previous findings that overall system performance is not entirely dependent on a specific microbial community composition.
3. Different morphotypes are detected at various stages of biofilm formation while some organisms are present at most phases of biofilm formation.
4. Constructed wetlands shows signs of stress when incoming effluent COD exceeds 15 000 mg/L.

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CHAPTER

5

Microbial interactions during degradation of distillery effluent

MICROBIAL INTERACTIONS DURING DEGRADATION OF DISTILLERY EFFLUENT

5.1. ABSTRACT

We participated in an evaluation of constructed wetlands as an option for the treatment of distillery effluent. Here we report on experimentation in laboratory model systems used to delineate microbial behaviour during the treatment of distillery effluent in constructed wetlands. Measurement of CO_2 production and DOC removal in laboratory scale columns showed that the presence of a grazing ciliate had a notable effect on microbial activity and that organic loading influenced these predator-prey interactions. Increased clogging of pores occurred in the presence of the protists, resulting in reduced flow through the porous matrix. Terminal-restriction fragment length polymorphism (T-RFLP) analysis of biofilms on gravel in experimental wetlands revealed that the presence of algae had an effect on microbial community composition, while SEM showed that biofilm structure was influenced by the presence of algae. Finally, augmentation with a commercial mixture or microbial populations isolated from distillery effluent showed that the concentration at which supplements are applied influence degradative efficiency.

Key words: distillery effluent, microbial community dynamics, predation, light, bio-augmentation

5.2. INTRODUCTION

Distillery effluent is among the most difficult to treat due to its complex character, including high concentrations of sugars, lignins, hemicellulose, dextrins, resins, polyphenols and organic acids, (Yeoh, 1997; Sangave and Pandit, 2004). Constructed wetlands are low-technology wastewater treatment systems that are often more economic and easier to operate than energy-intensive engineered treatment plants (Scholz and Xu, 2002). However, the final

treatment or ‘polishing’ of distillery effluent by microbial communities in the constructed wetlands is the relatively unexplored. Microorganisms play an important role in the functioning of wetlands where they participate in essential processes such as nutrient cycling and degradation of recalcitrant compounds. The water column and subsurface regions in wetlands are inhabited by aerobic and anaerobic microbial communities, respectively (Reddy *et al*., 2002). Water-plant and water-sediment interfaces are dominated by biofilms, which typically have steep redox gradients that facilitate co-existence by aerobic and anaerobic microbial communities in close proximity. Ultimately, the efficiency of wetlands in reducing the organic loading from the liquid phase is largely influenced by a complex array of microbe-microbe and plant-microbe interactions. Plant-microbe interactions include the transport of oxygen that allows aerobic microorganisms to grow in the rhizosphere, and the release of exudates that serve as microbial nutrients. Typical examples of microbe-microbe interactions include consortial activities such as sequential degradation and co-metabolism of complex molecules.

Microbial growth in natural porous media may lead to clogging through a combination of factors involving biomass accumulation, secretion of extracellular polymeric substances (EPS) and insoluble gas formation (Mattison *et al*., 2002). In wetlands this may lead to insufficient subsurface flow of water and reduced gas exchange. Therefore, protistan grazing on biomass and EPS may be an important natural mechanism whereby a sufficient porosity is maintained. In addition to the demonstrated role of grazing on bacterial community composition (Simek *et al*., 1997, 1999; Hahn *et al*., 1999), it has been indicated that protistan feeding of extracellular materials may also influence bacterial community morphology as showed in studies that focussed on the development of bacterial filaments and flocs (Jürgens *et al.*, 1999; Simek *et al*., 1999).

It can be expected that the presence of actively feeding protists will have an impact on microbial communities and consequently also on their resultant

micro-environment. Cox and Deshusses (1997) argued that because protistan predation is coupled with CO_2 and heat generation, overall biomass production per unit of a microbially degraded pollutant that is degraded by microbial activity should be less in the presence of protists. Others have indicated that although the impact of protists on *in situ* bioremediation is relatively unknown, the ability to selectively graze on and controlling the biomass of bacterial communities may have an impact on their physical environment (Sherr *et al*., 1992; Novarino *et al*., 1997). Because protists are usually present in natural and engineered systems where microbial activity contributes to the degradation of organic molecules, predation most probably plays a role in overall microbial activity and dynamics. It is possible that effluents related to the wine and brewing industries will favour the proliferation of yeasts, which may thus play an important role in the treatment of these effluents. Little work has been done on the impact of grazing on yeasts communities. Since it has been found that protists prefer medium-sized bacteria (Jürgens and Güde, 1994; Hahn and Höfle, 2001), the size of yeast may have an impact on their grazing.

The support matrix in constructed wetlands usually consists of coarse gravel that provides a large surface area for microbial colonization. Considering the demonstrated importance of biofilms in degradation processes, biofilms forming on gravel and plant roots and stems in wetlands most probably contribute largely to the system's overall activity. Biofilms are typically described in the literature as a population that developed from a single species or a community derived from multiple microbial species that can form on a vast array of abiotic and biotic surfaces (Davey and O'Toole, 2000). According to Costerton *et al*. (1995) the microbial assemblages within the biofilm matrix function as a cooperative consortium, in a relatively complex and coordinated manner. This description probably applies to wetland microbial communities. Because the water in wetlands are relatively shallow, biofilms on plant stems and the top regions of the support matrix are exposed to sunlight, conditions conducive for the growth of algae. Hartley *et al*. (1996) pointed out that biofilms in illuminated

aquatic environments consist of algal and other microbial cells held together by a matrix of EPS. Algal groups present in these biofilms include unicellular green algae, diatoms, filamentous green algae and cyanobacteria (Leadbeater and Callow, 1992).

In addition to the variety of naturally occurring microbial populations, microbial composition and activity can potentially be modified by the addition of specific microorganisms to a bio-treatment system with the aim of providing sufficient quantity and diversity of microorganisms. Such bioaugmentation or bio-supplementation (Quan *et al*., 2004) is considered a means of maintaining sufficient biomass when adequate carbon substrates and nutrients are available (Ro *et al*., 1997), and has been demonstrated to enhance the degradation and removal of many specific pollutants such as phenols (Selvaratnam *et al.*, 1997; Hajji *et al.*, 2000), chloroaniline (Boon *et al*., 2000), chlorobenzoate (Bouchez *et al*., 2000), resin acid (Yu and Mohn, 2001), chlorinated solvents (Ahring *et al*., 1992) and aromatic hydrocarbons (McClure *et al*., 1991; Nüßlein *et al*., 1992) in activated sludge systems.

Bioaugmentation may protect activated sludge systems from perturbations resulting from transient or continuous overloading (Beardsley and Coffey, 1985) and may facilitate the increased degradability of hard-to-treat organics (Kennedy *et al*., 1990). The possibilities of operation at higher rates during cold weather, and in periods of reduction in sludge production, are additional potential benefits of bioaugmentation (Chong *et al*., 1997). There are currently a number of bioaugmentation products on the market for the reduction of COD in industry. However, due to a variety of reasons, bioaugmentation with special bacteria in a bio-treatment system does not always have the desired results (Quan *et al*., 2004). In fact, Babcock *et al.* (1992) found no evidence of successful operation in experiments using commercial bio-supplements under well defined conditions. Possible reasons for the failure of bioaugmentation include washing out from the system, predation, and preferential feeding by the introduced microorganisms on

substrates more labile than the target pollutant. Therefore, to achieve successful bioaugmentation, it is necessary to select suitable microorganisms, adopt appropriate strategies, and have an overall improved knowledge of the ecology of naturally-occurring microbial communities.

We have described elsewhere (du Plessis *et al*., Submitted, Water Research) the presence of highly dynamic biofilm and planktonic microbial communities at various zones in experimental and laboratory-scale wetlands, and reported on COD removal efficiency of these systems when challenged with different strengths of distillery effluents. A particularly interesting observation made in the previous study was a weak relationship between COD removal efficiency and community composition as measured by T-RFLP, which gave rise to the question whether it is possible to define and thus manage an ideal degradative microbial community in terms of microbiological criteria. The purpose of the present study was to expand on that observation by further investigating the role of microbial interactions, which were mostly neglected so far, on community structure and degradative activity under controlled laboratory conditions. Specifically, we measured 1) carbon utilization, CO_2 production and pore flow to study the impact of protistan grazing on microbial degradation activity, 2) the impact of algae on microbial biofilm community structure, and 3) the impact of deliberate shifts in community composition by augmenting heterogeneous microbial communities with cell suspensions of autochthonous species or a commercial bio-supplement.

5.3. MATERIALS AND METHODS

5.3.1. Effect of predation

5.3.1.1. Isolation of protists

Isolation procedures based on the method described by Joubert *et al.* (2006) were carried out as follows: A growth medium was prepared by adding 1 L distilled water to 200 g of soil. Following mixing (gentle shaking for about 30 s),

the suspension was autoclaved and allowed to settle for 1 day. The supernatant was decanted into a sterile flask, ensuring transfer of as little soil as possible. Protists were cultivated by adding 10 ml of this growth medium to 1 g soil and allowing it to settle for 2 days, where after 1 ml was drawn from directly above the soil and added to 10 ml growth medium and 1 ml of an overnight culture of the yeast *Cryptococcus laurentii* serving as prey suspension. Samples were left for 2 to 3 days at room temperature to allow for the proliferation of protists, which were microscopically determined to consist of ciliates only.

5.3.1.2. Experimental set-up

Eighteen 50 ml polyethylene columns, each containing 20 g of 2 mm diameter glass beads were continuously fed with yeast malt extract (YM) by using a Watson-Marlow 205S peristaltic pump set at a rate of 20 $ml.hr^{-1}$ (hydraulic retention time = 0.75 hr) (Appendix – Figure A). Three series of 6 columns each received 10 %, 2% and 0.1% YM, respectively (where 100% YM consists of 10 g glucose, 5 g peptone, 3 g yeast extract and 3 g malt extract dissolved in 1 L distilled water). The medium was introduced at the top of each column, thereby allowing it to perfuse through the column without upstream contamination (Appendix – Figure B). The height of the outlets was adjusted to create a 10 mm saturation zone at the bottom of each column. The columns were immersed in a waterbath maintained at 25°C, with the glass beads directly below the waterline.

One ml of an overnight culture of *Cryptococcus laurentii* was used to inoculate the columns through the rubber stopper, using a 5 ml syringe. Half of the columns of each YM concentration was additionally inoculated similarly with 200 µl oligotrophic medium containing the protists (3233 ± 305 $protists.ml^{-1}$) while the other half was inoculated with filtered medium (0.3 µm pore-size filters) containing no protists. The filter pore size was large enough to let bacteria possibly associated with the protists through. The reservoirs furthermore

contained streptomycin (200 µl.ml^{-1}) to inhibit bacterial growth in the columns, thereby allowing only yeast, or yeast-predator activity.

5.3.1.3. Respiration measurements

Carbon dioxide production was determined by using the method of Smith (2003). On days 2, 4 and 7, triplicate columns with and without protists were each flushed with fresh outside air for 3 min by using a stopper assembly containing two 4 mm diameter stainless steel tubes (one to serve as an inlet that extended to just above the glass beads in the incubation tube and one to serve as an outlet) (Appendix – Figure C). After flushing with fresh air, the columns were sequentially connected to a closed-circuit gas exchange system, which included a buffer volume, a LI 6262 infrared CO_2 analyser (LI-COR Inc., Nebraska), a variable air pump, flow meter and a flow diverter that could divert the flow through a short tube of soda-lime (Appendix – Figure D). Air was circulated through the system at 1 L.min^{-1} when respiration was measured. The total volume of the system (pipes, analyser, sample column, buffer column, pump, etc.) was 162 ml. This volume was determined by injecting a known volume of pure CO_2 into the system when it contained CO_2-free air and subsequently measuring the CO_2 concentration once it had stabilised within the system. As the ideal gas law states that the volume of 1 mole of any gas at standard temperature and pressure (STP = 273K [0°C] and 101.3 kPa) is 22.4 L, the system contained 7.232×10^{-3} mol of air.

The CO_2 concentration in the closed-circuit gas exchange system was decreased to below 380 µmol by diverting the air through soda-lime using the flow diverter. Once the CO_2 concentration started to increase due to respiration in the columns and reached a concentration of 380 µmol it was measured every second for a period of 100 s. A blank column, containing 20 g of 2 mm diameter glass beads and receiving no flow was used to determine whether the system leaked CO_2 before respiration measurements. Respiration rates were calculated using Statistica 6.1 (Statsoft, Inc.) and converted to µmol CO_2 produced.h^{-1} per

reactor. Calibration of the LI 6262 infrared CO_2 analyser (LI-COR Inc., Nebraska) revealed that every reading should be corrected to obtain the true CO_2 production as follows: $[Co_2]_{true}$ = (CO_2 reading × 1.1072) – 52.6895. $[Co_2]_{true}$ was plotted against time (t) where the straight part of the slope depicted volumes per million CO_2 increase per second ($vpm.s^{-1}$). After the slope from the blank was subtracted from the sample slope the resulting answer was multiplied by 7.232 × 10^{-3} mol (mol amount of air in the system) and 3 600 s to get an answer in µmol CO_2 $produced.h^{-1}$ in each reactor.

5.3.1.4. Measurement of pore clogging

After completion of respiration measurements, the columns were gently disconnected without disturbing their porous matrices. The gravitational flow of 10 ml of sterile distilled water through the porous matrix was thereafter determined as indication of the extent of clogging by cellular and noncellular material in each column.

5.3.1.5. Dissolved organic carbon (DOC) measurements

The DOC at the inlets and outlets was measured with the persulphate–UV oxidation method (DWAF, 1992). To determine the DOC in the extracellular fraction of biofilms, a cell-free extract of 7-day old biofilms harvested from the outlet tubes of the respective columns were prepared according to the method of Zhang *et al.* (1999). Briefly, 1 g biofilm material was added to centrifuge tubes containing 25 ml MilliQ water (Millipore). After gentle shaking the tubes were centrifuged at 3 500 rpm (Biofuge® Fresca 24-well bench top centrifuge; Heraeus instruments) for 10 min, where after the liquid (containing loosely bound EPS) was decanted from the centrifuge tubes. Pellets were resuspended by vortexing for 1 min in 25 ml of 8.5% NaCl containing 0.22% formaldehyde to recover the capsule-bound material. The latter was added to the loosely bound EPS and centrifuged at 12 000 rpm (Heraeus instruments) for 30 min. The supernatant was filtered through a 0.22 µm acetate filter where after the DOC was determined as described above.

5.3.2. Effect of light

5.3.2.1. Growth conditions

Effluent was obtained from a distillery where rebate wines, distilling wine and crude spirits are distilled to produce spirits and pot-still brandy. Perspex mesocosms (20 cm long X 15 cm wide X 15 cm high) were constructed and filled about 1.5 cm high with sterile (autoclaved) 2.5 mm diameter gravel. Mesocosms were either painted black to prevent light penetration, or left translucent. A Chromalux® full spectrum plant light (100 W, 230 V), operated to simulate a normal daylight period, thereby stimulating algal growth in the translucent mesocosms The distillery effluent was pumped through the systems with a Watson-Marlow 205S peristaltic pump at a flow-rate of 20 $ml.h^{-1}$. Biofilm formation was monitored on a weekly basis by means of T-RFLP analysis and scanning electron microscopy (SEM).

5.3.2.2. Sampling, DNA extractions and T-RFLP analysis

DNA was extracted from 1 g of gravel, isolated from each microcosm, according to the method of Zhou *et al.* (1996). Biofilm material was removed from gravel by vortexing in 5 ml sterile distilled water for 1 min after which samples were sonicated for 10 min at 40 kHz in a 5.5 L water bath sonicator (Misonix). The T-RFLP analyses were performed as described earlier (du Plessis *et al*., Submitted, Water Research).

5.3.2.3. Scanning Electron Microscopy (SEM)

During 3 consecutive weeks gravel samples were collected once weekly from the microcosms, mounted on stubs and viewed unfixed and hydrated with a LEO 1430 VP Scanning Electron Microscope, operated at 7 kV, after gold coating with an Edwards S150A sputter coater.

5.3.3. Augmentation experiments

5.3.3.1. Isolation and characterization of test bacterial strains

Three dominant culturable bacterial strains (BY, BCF and BCR) present in the distillery effluent were isolated using agar plates supplemented with sterilized effluent and conventional spread plating. DNA was extracted from each of the bacterial test strains according to the method of Zhou *et al.* (1996). The resulting DNA samples were dried and dissolved in 100 µl Tris EDTA (TE) buffer (pH 8.0) and then subjected to electrophoresis on 0.8% (w/v) agarose gels containing ethidium bromide (10 $mg.ml^{-1}$). *Lambda* DNA digested with *Pst*I (Amersham Biosciences) was used as a molecular size marker.

5.3.3.2. Polymerase Chain Reaction (PCR)

The 16S rRNA genes were amplified using the primer combination fDD2 (5'-AGA TTT GAT CIT GGC TCA G -3') and rPP2 (5'-CGG ITA CCT TGT TAC GAC TT-3') for Eubacteria following standard protocol (Rawlings, 1995).

5.3.3.3. Cloning and sequencing of 16S rRNA genes

The ~1.5 kb 16S rDNA fragments, amplified from the three unidentified organisms, were cloned into the vector pGEM®-T Easy (Promega) at the *Sal*I and *Hind*III restriction enzyme sites. Recombinant plasmids were generated by sub-cloning to facilitate sequencing of the 16S rDNA inserts. All recombinant plasmids were transformed into E.coli DH5α and grown on Luria-Bertani medium containing 100 $\mu g.ml^{-1}$ ampicillin and 2 $\mu g.ml^{-1}$ X-gal (5-bromo-4-chloro-3-indolyl-ß-galactoside) for selection of transformants. Recombinant plasmids were purified using the High Pure Plasmid Isolation Kit (Roche). The 16S rDNA was partially sequenced by the dideoxy chain-termination method (Sanger *et al*., 1977) and an ABI PRISM 3100 genetic sequencer, and analyzed using the PC-based DNAMAN (version 4.1) package from Lynnon BioSoft. The gapped-BLAST program at the National Centre for Biotechnology Information (NCBI) [http://www.ncbi.nlm.nih.gov/BLAST/] was used to perform comparison searches (Altschul *et al*., 1997).

5.3.3.4. Effect of augmentation on COD removal

5.3.3.4.1. Effect of introduced bacterial strains. The three dominant culturable bacterial strains (BY, BCF and BCR) were grown overnight in tryptone soy broth (TSB) (Biolab^{TM}) in pure culture and as a mixture to between 10^6 and 10^7 cells.ml^{-1} before subsequently being added to the distillery effluent. Fifteen ml of each test strain, of the three strains grown in combination, or of a mixture prepared from equal volumes of the three strains grown individually, were added to a series of triplicate Erlenmeyer flasks each containing 150 ml effluent. The control received no bacterial augmentation. The initial COD was measured before the flasks were placed on a rotary shaker at 60 rpm at room temperature (23^{o}C ± 2^{o}C) followed by COD measurements after 1, 3 and 7 days. Microbial numbers in the effluent equalled 1.2 × 10^7 colony forming units.ml^{-1} before augmentation, as enumerated on tryptone soy agar (TSA) (Biolab^{TM}).

5.3.3.4.2. Effect of a commercial bio-supplement. A commercial bio-supplement was obtained from a local supplier. The product specifications are that 7.5 g of the powder should be added daily to 25 L of effluent for the first three days and subsequently once a week. The supplement was added to triplicate 0.83 L aliquots of distillery effluent in 2 L Erlenmeyer flasks on a rotary shaker at 60 rpm at the recommended, 2X and 3X the recommended concentration of 7.5 g per 25 L. Untreated distillery effluent served as control. The supplements were added on days 1, 2, 3, 8, 13 and 15. The COD was determined on days 1, 2, 3, 4, 8, 13, 15, 18 and 22. On each occasion, sampling for COD measurements was done before the supplement was added.

5.3.3.4.3. Effect of augment concentration. To test the possible inhibition of COD removal by augmentation if applied at inappropriate concentrations, removal efficiency was determined at a range of concentrations of the three test bacteria and the commercial bio-supplement. A mixture of the 3 bacterial strains were grown overnight to a concentration of 10^6 cells.ml^{-1} and mixed with the distillery effluent in Erlenmeyer flasks to a final volume of 200 ml,

diluted to ratio's of 1/10, 1/20, 10^{-2}, 10^{-3} and 10^{-4} (cell suspension: effluent), respectively. The commercial bio-supplement was added at $1/10^{th}$, equal and 10X the recommended concentration. After measurement of initial COD values, the flasks with the different augments and untreated controls were placed on a rotary shaker at 60 rpm at room temperature ($23^oC \pm 2^oC$) and the COD measured after 1, 3 and 7 days. To test the effect of augment concentration on community composition, DNA was extracted on the respective sampling dates from 0.85 ml of each flask, according to the method of Zhou *et al.* (1996) for performance of T-RFLP analysis.

5.4. RESULTS AND DISCUSSION

5.4.1. Effect of predation

Predation had a variable effect on carbon flow through the yeast columns. While predation had no effect on CO_2 production when the YM concentration was low (0.1%, data not shown), a general increase in CO_2 production occurred with predation at the higher YM concentrations (Figure 5.1A). Factorial analyses of variance (ANOVA) revealed that the differences in CO_2 release from the columns with and without predation in columns receiving 2% YM were not significant ($p < 0.05$). However, the differences between the respective columns that received 10% YM were significant ($p > 0.05$). As expected, there was an inverse relationship between CO_2 and DOC values at the column outlets (Figure 5.1B), i.e. predation that resulted in higher CO_2 production was accompanied by increased reduction in DOC values although the impact proved to be statistically insignificant under all nutrient concentrations applied ($p < 0.05$).

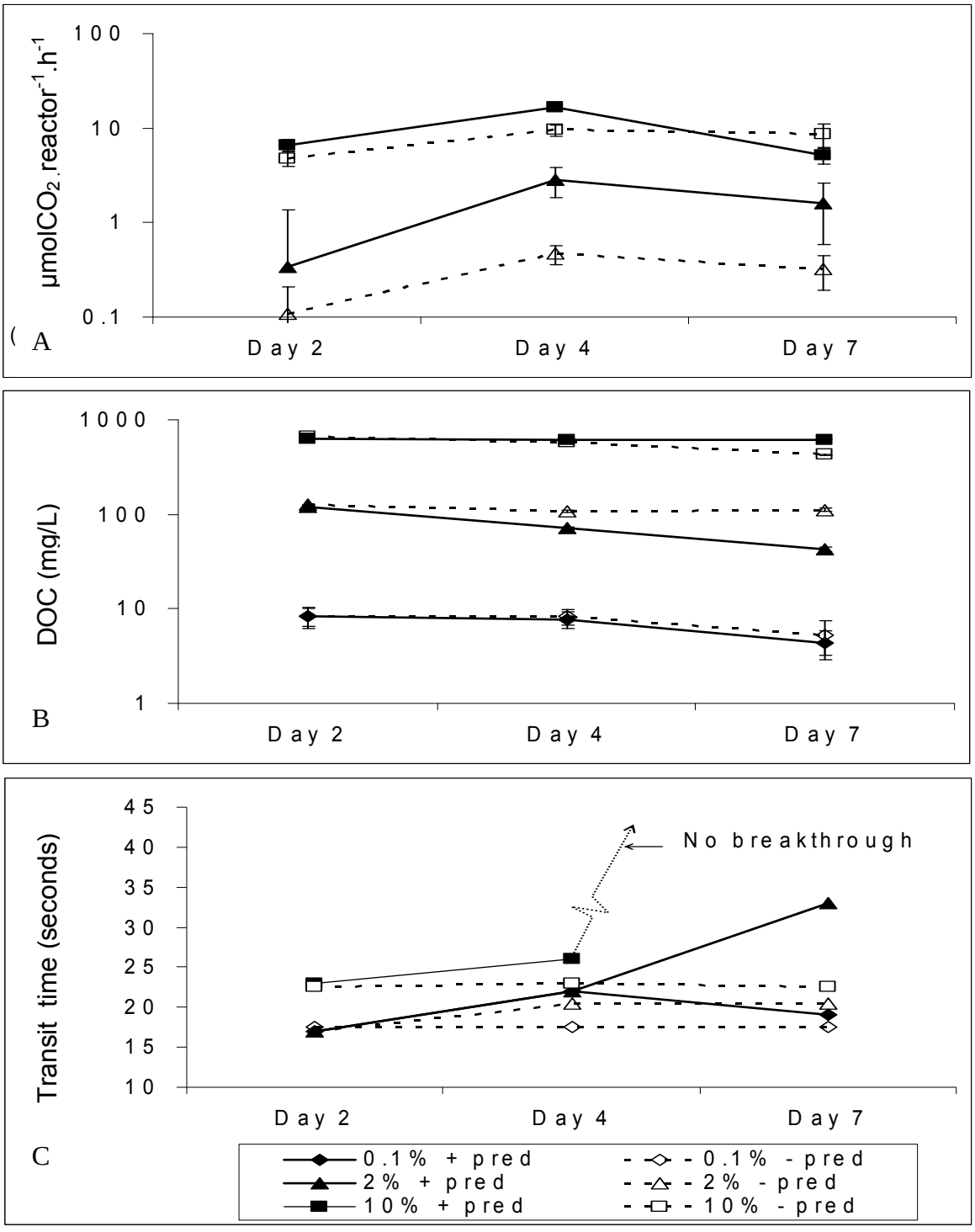

Figure 5.1. Effect of predation on carbon flow and biomass accumulation. The rates of CO_2 production in the columns receiving different concentrations of YM in the presence and absence of predation are shown in A, while B shows the DOC concentrations at the outlets of the columns, and C shows transit times of 10 ml water through the different columns as an indirect measurement of pore clogging.

In the subsequent evaluation of pore flow it was therefore expected that predation would serve as a mechanism to prevent pore clogging through constant removal of yeast biomass. However, measurements revealed the opposite (Figure 5.1C) in columns receiving 2% and 10% YM, respectively, where pore flow was increasingly restrained or completely prevented in the presence of predators. These results led us to postulate that predation may in fact stimulate increased EPS production and more extensive biofilm formation by the yeast, as indicated in a related study in our laboratory (Joubert *et al.,* 2006) where a general increase in biofilm biomass was repeatedly observed after introduction of protists to yeast biofilms. In that study it was shown that a more variable biofilm structure followed the addition of protists. Joubert *et al.* (2006) subsequently suggested that the observed fluctuation was caused by active dislodging of biofilm biomass by the ciliates feeding on the adhesive EPS matrix, followed by increased biofilm growth. Other studies have also demonstrated that grazing influence the production of EPS by bacterial cells (e.g. Liu and Buskey, 2000). Murray (1995) suggested that EPS can be utilized by flagellates as a food source.

To test potential implications of the hypothesis that predation stimulate increased EPS production in carbon flow during effluent treatment, we collected biofilms at the column outlets and used DOC values as an indirect measure of extracellular biofilm biomass. Surprisingly, there was no significant difference in DOC concentrations in the extracellular fraction of the biofilms collected from the different columns (Figure 5.2). DOC values in this fraction ranged between 170 and 190 $mg.L^{-1}$, irrespective of influent nutrient concentration, or the presence or absence of predation. This was despite the fact that there were notable differences in DOC concentrations in the surrounding liquid phase, ranging from 4.4 – 5.3 $mg.L^{-1}$ (0.1% YM influent), 43 – 111 $mg.L^{-1}$ (2% YM), and 616 – 425 $mg.L^{-1}$ (10% YM) in the presence and absence of predation, respectively (day 7 – Figure 5.1B). The columns used in this study were specifically configured to accurately measure CO_2 evolution, as it was not an objective to analyse biofilm

architecture. We can therefore only speculate on the similarity in DOC values in the extracellular fraction of the biofilms that developed in columns with large differences in influent nutrient concentration, or the presence or absence of predation. In addition to the classical view of biofilms as a survival mechanism, including slower growth rates of member cells (*e.g.* Spoering and Lewis, 2001) and serving as a storage reserve (Wolfaardt *et al*., 1998), it is possible that they are also important in the proliferation of populations when environmental conditions allow. A recent study in our laboratory (Bester *et al*., 2005) demonstrated high yields of cells from biofilms to the suspended phase and discussed this potential dual role of biofilms. It is possible that a range of environmental conditions dictates the relative amount of energy spent on cell maintenance, cell yield and non-cellular (EPS) yield. Based on the results we propose that labile substrates such as the YM used in our laboratory columns favour cell yield with little investment in storage materials such as EPS (thus a possible explanation for the results illustrated in Figure 5.2) while the opposite occurs when biofilm development is dependent on recalcitrant substrates. In fact, it was demonstrated in a related study (Lovis, 2003) that the biofilms that formed on the discs of a pilot scale rotating biological contactor (RBC), evaluated for treatment of the distillery effluent used in the experiments discussed below, consisted of copious amounts of EPS with relatively few bacterial and yeast cells. This may also be the reason why the five constructed wetlands, used in chapter 3, are still actively functioning, without clogging, after 7 years. To ensure minimal variation between the replicate experiments, a defined medium (YM) and a single binary microbial predator-prey pair were used in this study. Future studies using substrates with increasing recalcitrance and heterogeneous microbial communities should provide interesting additional information in this regard.

Though various possible roles of EPS, such as protection from environmental stress, has been reported (Flemming, 1993), the functions of EPS are still poorly described, partly because of their heterogeneous nature and diffuse character (Tsuneda *et al*., 2003). Improved methods for the analysis of

EPS are needed, not only for an enhanced fundamental understanding of the ecological role of EPS, but also to provide input parameters to predict carbon flow through biofilms in the bioprocesses that drive natural and engineered systems.

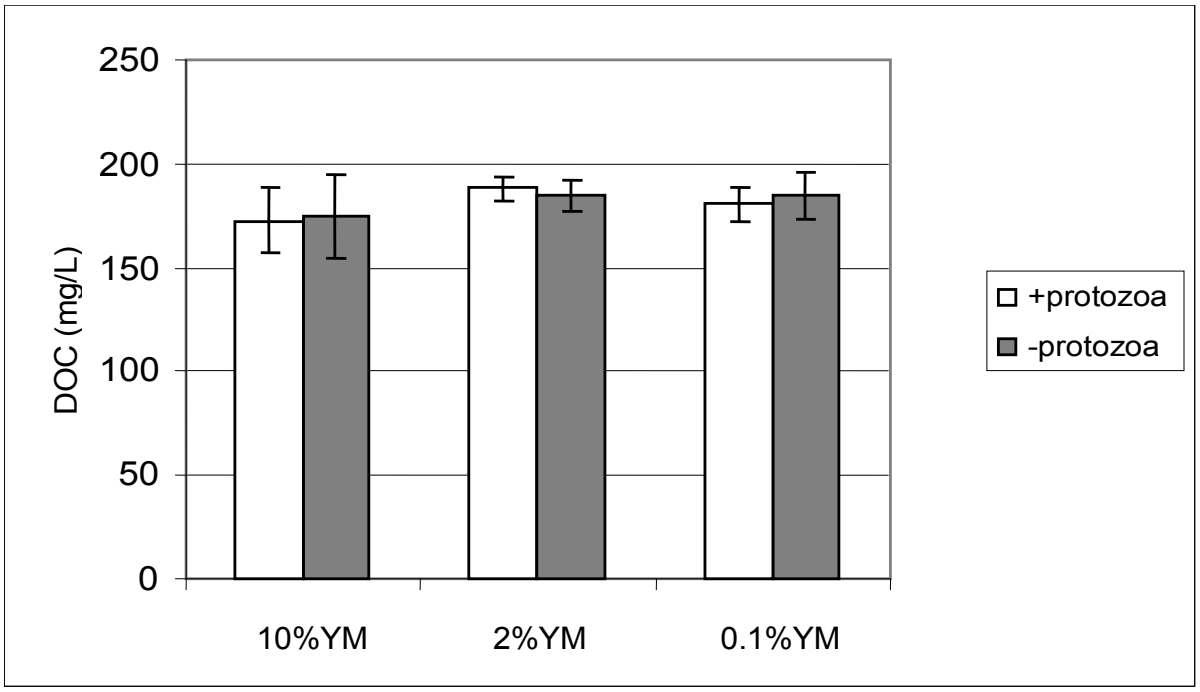

Figure 5.2. DOC values showing that the amount of carbon stored in the extracellular fraction of biofilms did not differ significantly even though there was a 100-fold difference in the influent concentrations.

5.4.2. Effect of light

The presence of light, and subsequent algal growth, had a notable effect on the whole-community molecular fingerprints as revealed by T-RFLP analysis. DNA digests obtained with both restriction enzymes *AluI* (Figure 5.3) and *RsaI* (not shown) revealed a relatively rapid increase in diversity over the 3 week experimental period. In the case of both enzymes there was initially a conspicuous difference between the communities cultivated in the dark and those cultivated in the light. For instance, there was only 18% similarity between the fragments that resulted from the communities grown in light and dark after 1 week following digestion with *AluI*. However, the similarity between these

communities increased to 73% and 81%, after weeks 2 and 3, respectively. Only one fragment (117 base pairs), presumed to represent a single microorganism, was present under both light and dark conditions, and in each weekly sample when digested with *AluI,* while two fragments were present in all samples when digested with *RsaI*, demonstrating the dynamic nature of microbial communities.

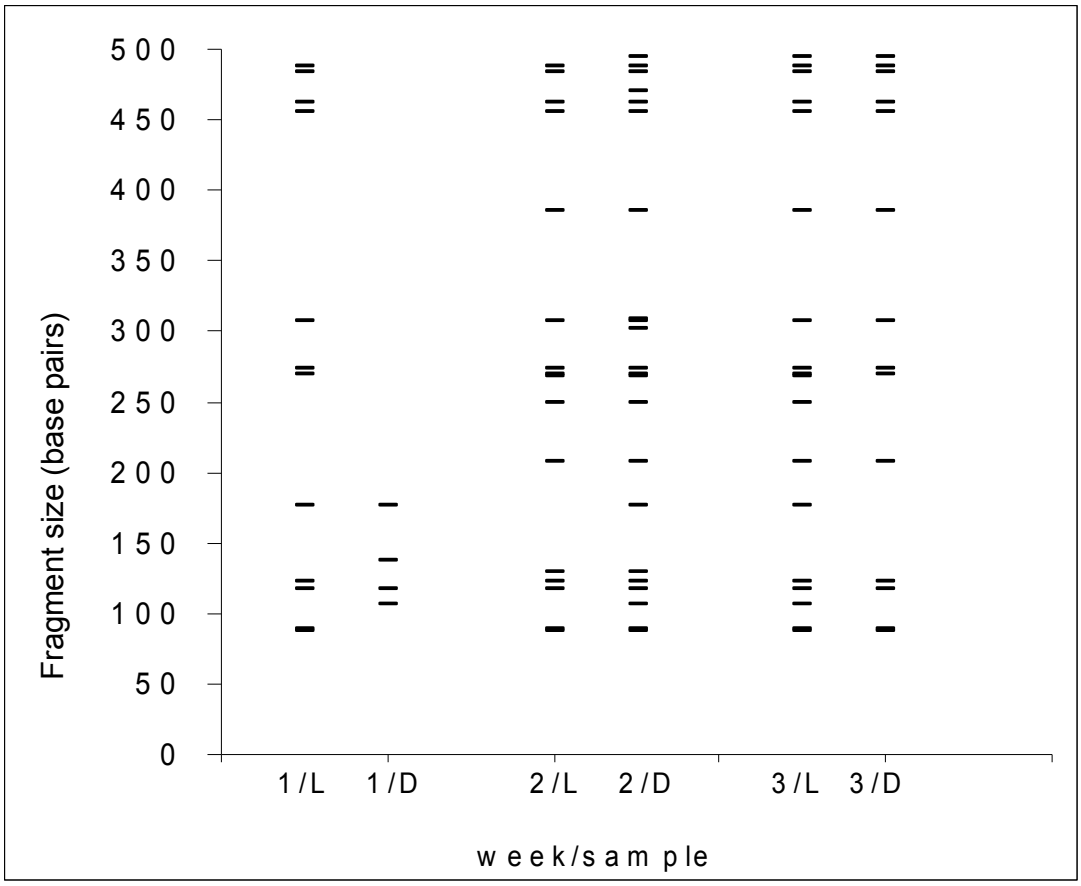

Figure 5.3. T-RFLP fingerprint patterns of distillery effluent microbial communities cultivated in the light (L) and dark (D) followed by digestion with *AluI*, showing shifts in community composition over a three week period.

Algae were present in biofilms exposed to light as evident by the green colour of the gravel surfaces and confirmed by SEM (Figure 5.4A). There were notable differences between biofilms that formed in the absence of light (Figure 5.4B) and those that formed on the shaded sides of gravel in the system that received light (Figure 5.4C), especially during the first week of colonization and growth. Algal EPS have a varied composition, including carboxylated or sulfated

acidic polysaccharides (Hoagland *et al.*, 1993). Barranguet *et al.* (2005) demonstrated a close coupling between autotrophic carbon production and EPS in water reservoirs, while it was demonstrated by Wolfaardt *et al*., (1994) that algal EPS stimulate the degradation of chlorinated hydrocarbons by a degradative bacterial consortium. The differences in biofilm structure shown in Figure 5.4B and C thus suggest that the effect exerted by algae on the biofilm community is probably related to the supply of nutrients to bacteria and yeast.

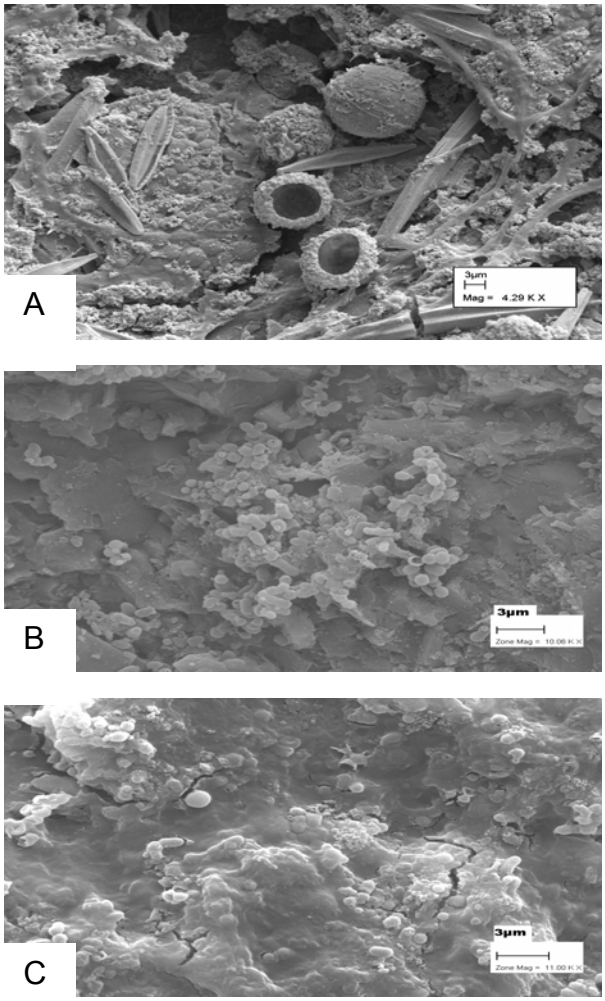

Figure 5.4. Representative SEM images illustrating: A. the presence of algae and diatoms in biofilms growing on the support gravel matrix exposed to light; B. early biofilm development was slower in the absence of light and after one week typically consisted of interspersed micro-colonies; and C. biofilms that developed in the deeper layers and shaded sides of the gravel in the system exposed to light already formed confluent structures within the first week.

5.4.3. Effect of augmentation

Partial sequencing of the 16S rDNA revealed that all three test bacteria (BY, BCF and BCR) were *Bacillus* strains. Despite their predominance in the distillery effluent and the fact that they were isolated from agar plates with sterile effluent as the sole nutrient, the addition of these strains resulted in reduced COD removal efficiency from the effluent, compared to the control over the 7 day period. The initial increase in COD due to the added biomass and associated residual growth medium was reduced by day 3 (data not shown); however after 7 days the removal efficiency in the flasks with augmentation was still 12 – 22% lower than the control (Figure 5.5).

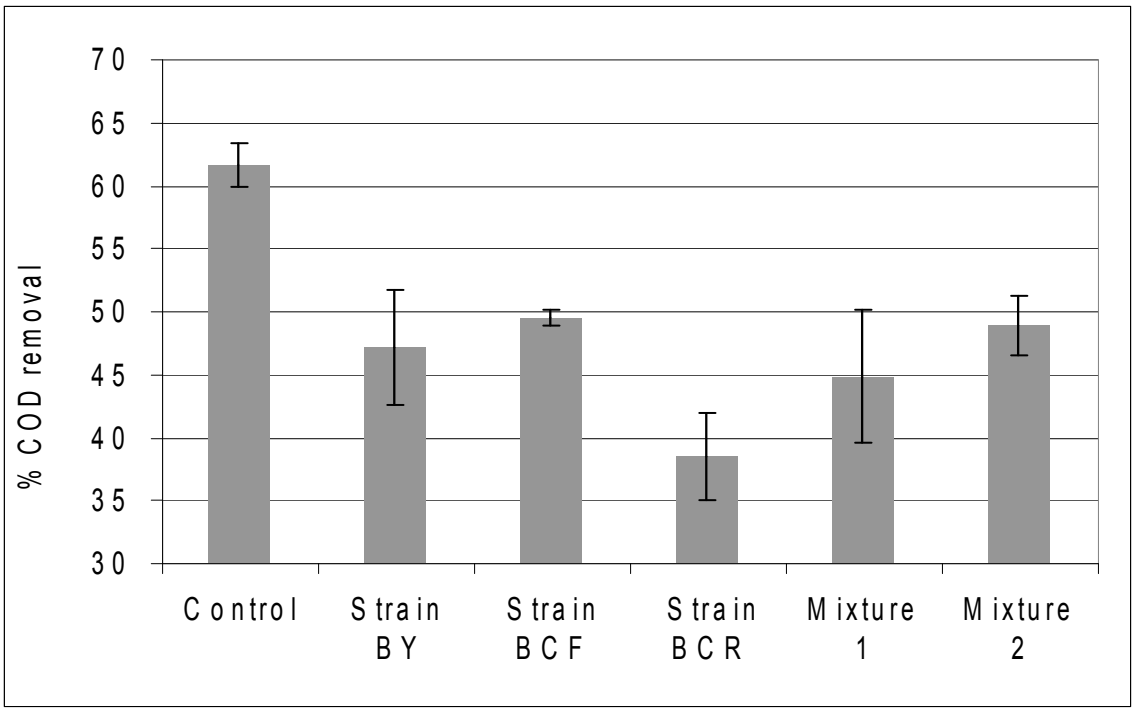

Figure 5.5. Effect of augmentation with three bacterial isolates originally isolates from the distillery effluent on COD removal after 7 days. The strains were added individually, or as a mixture grown together before addition (Mixture 1) or grown in pure culture, mixed together in equal ratios and then added (Mixture 2).

Addition of a commercial bio-supplement had no impact on COD removal, compared to the control, over the first 8 days (Figure 5.6). Thereafter, an increased COD removal was measured in the flasks that were supplemented at

the recommended concentration. In contrast, COD removal was less efficient in the flasks with 2X and 3X the recommended concentration of the bio-supplement. From these results we conclude that the complex nature of distillery effluent possibly leads to a highly specific microbial community structure and that disturbance of this structure has a negative impact on overall degradative efficiency. To test this, we conducted a further experiment by adding the commercial product, and a mixture of the 3 test strains at a wider range of concentrations to the effluent. Similar to the results shown in Figure 5.6 after the same incubation period (7 days), there was no significant difference in COD removal efficiency between the control flasks and those that were supplemented with the commercial product at the recommended concentration (Figure 5.7). There was a notable (but not significant) higher removal at 1/10 the recommended concentration of the commercial product.

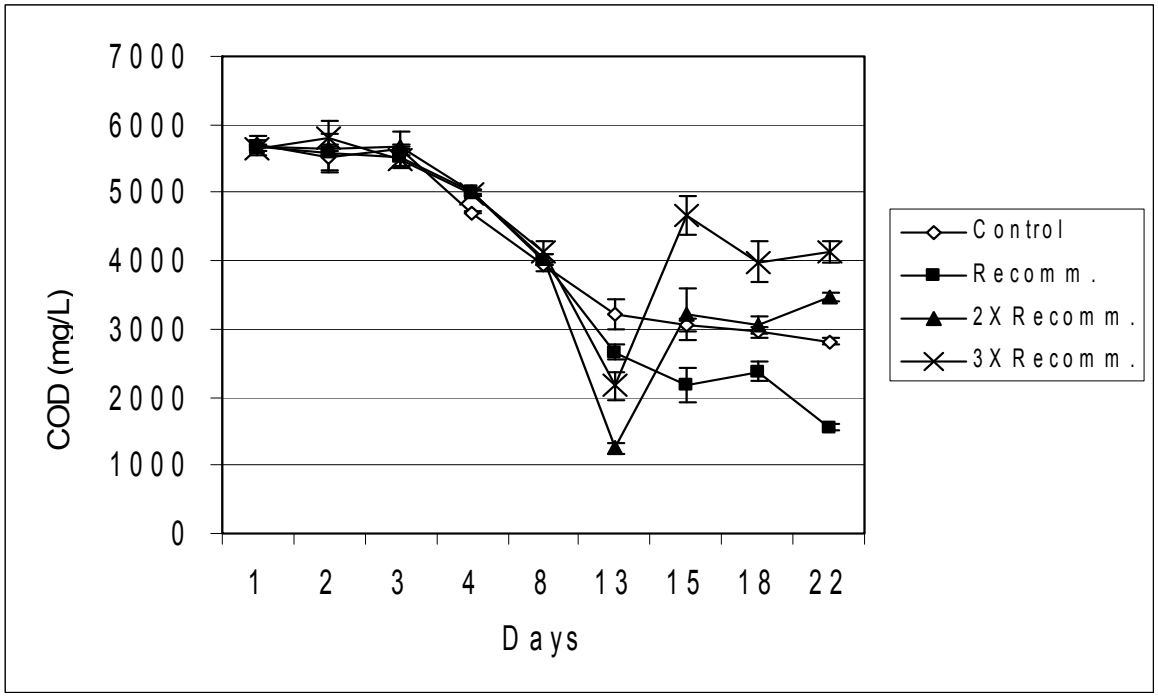

Figure 5.6. Effect of augmentation with a commercial bio-supplement on COD. The product was added at the recommended, as well as 2X and 3X recommended strength on days 1, 2, 3, 8, 13 and 15 after sampling for COD measurements.

The results shown in Figure 5.7 (1/10 Mixture 1) also corroborate those in Figure 5.5 (Mixture 1), since the 3 test strains grown in combination before being added to the effluent at a relatively high ratio resulted in reduced COD removal efficiency in both instances. However, when the mixture was added at 1/1000 dilution relative to the effluent, it indeed resulted in a significant increase in COD removal (Figure 5.7).

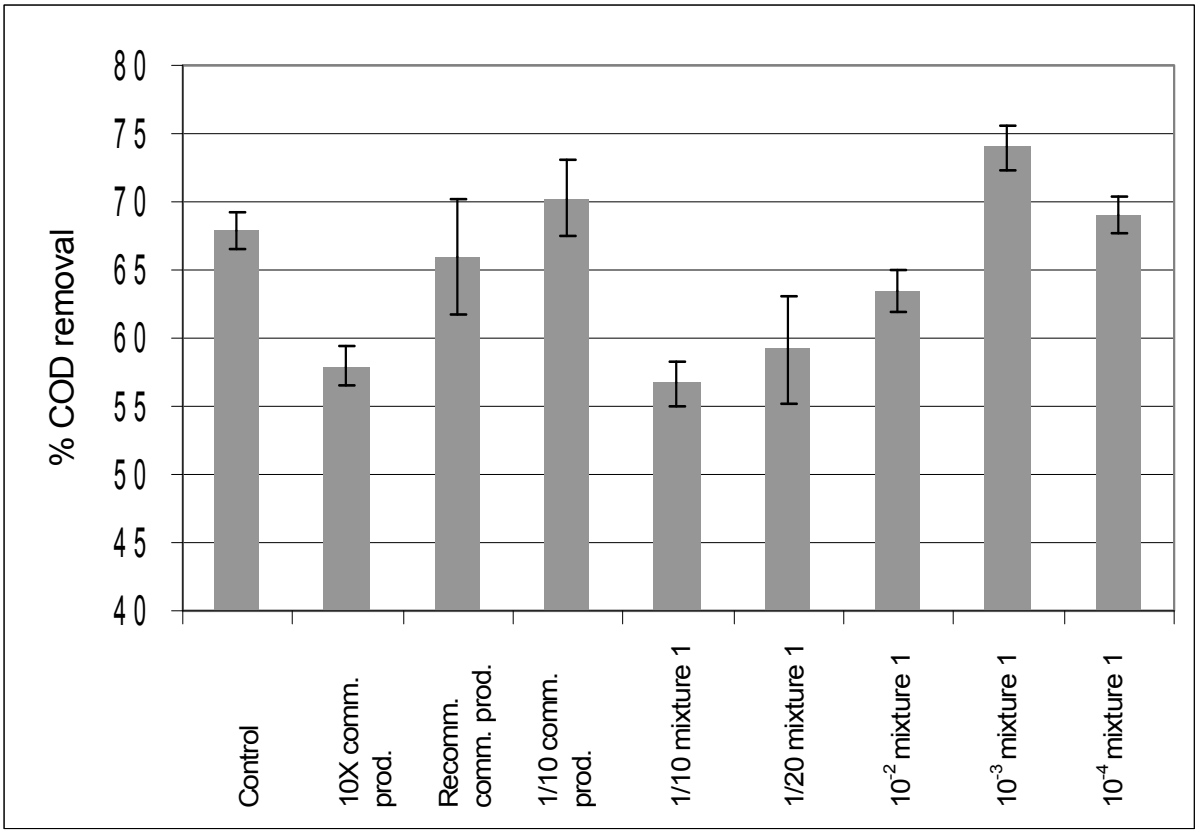

Figure 5.7. Effect of augment concentration on COD removal after 7 day incubation, compared to the untreated control. The commercial product was added at recommended, as well as 1/10 and 10X the recommended concentration. The bacterial mixture consisted of the 3 test strains grown individually overnight before they were added to the effluent as indicated (cell mixture: effluent).

Whole-community molecular profiles using T-RFLP analyses showed a direct relationship between variation in community structure and response to supplements. Most dominant fragments associated with the effluent microbial community (i.e. 110, 250 and 307 base pairs) were not altered by the supplements. One of the fragments (276 base pairs) generally disappeared 7

days after addition of the respective supplements, with the exception of the control flasks. An 87 base pair fragment which was absent from the controls, but found in all the flasks supplemented with the test strains and mixture, respectively, typically disappeared after 1 or 3 days. However, this was not the case with the flask to which the mixture was added at a 1/100 ratio, where this fragment was present at all sampling intervals.

Lovis (2003) demonstrated that the distillery effluent used in this study required long periods of aeration to be degraded and that neither nitrogen and phosphorus addition to the recommended COD/N/P ratio of 100/10/1 (Kargi and Eker, 2002), nor increased temperature, pH adjustment, dilution, or pre-treatment with ozone resulted in higher rates of degradation. The study by Lovis (2003) also showed that a pilot scale RBC typically removed <20% of the COD while the same RBC removed COD from winery effluents at an average efficiency of 43% (Malandra *et al*., 2003). The recalcitrance to degradation measured in the present study is thus in line with the previous work in our laboratory, and in general to other studies that reported on the complex nature of distillery effluent. Similar to other studies (e.g. Babcock *et al.,* 1992; Quan *et al*., 2004), the application of bioaugmentation has also been met with mixed success. An interesting result from the present study is that bioaugmentation most notably stimulated COD removal when added at recommended concentration (commercial supplement) or diluted (commercial supplement and the strains enriched from distillery effluent).

An interesting question is why the commercial bio-supplement, when applied at the recommended dose, improved COD removal efficiency, but had a negative impact at higher concentrations. It is possible that microbial strains in the supplement co-metabolized recalcitrant molecules or detoxified effluent components that were inhibitory to, or slowed down the activity of the indigenous microbial community, but when these added strains were present in high numbers competed for limiting nutrients. Whole-community analysis such as T-

RFLP provides a sensitive way to evaluate community composition in terms of the presence or absence of community members (represented by fragments with a specific number of base pairs), but lack the ability to quantify the members in the community. Therefore it was not possible to quantify the potential extent of effect on community structure exerted by the competition.

5.5. CONCLUSIONS

In addition to genetic determinants, microbial behaviour is influenced by numerous physical and chemical factors. In most fundamental research, microbial response to one or more of these factors is typically studied in pure culture. However, to better understand and control microbial activity in environmental and engineered processes require that interactions between different microbial species and trophic levels also be considered. The results of this study demonstrated that interactions between different microbial trophic levels influence metabolic activity, community composition and biofilm formation. Improved COD removal from the highly recalcitrant distillery effluent by bioaugmentation at the appropriate concentration is a promising result. Nevertheless, the observed reduction in efficiency when the commercial product or indigenous bacteria were applied at too high concentrations, indicate that the formulation and concentration of these supplements require careful consideration of the inherent properties of the effluent before addition, as well as routine monitoring during treatment.

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5.7. APPENDIX

Experimental set-up for respiration measurements

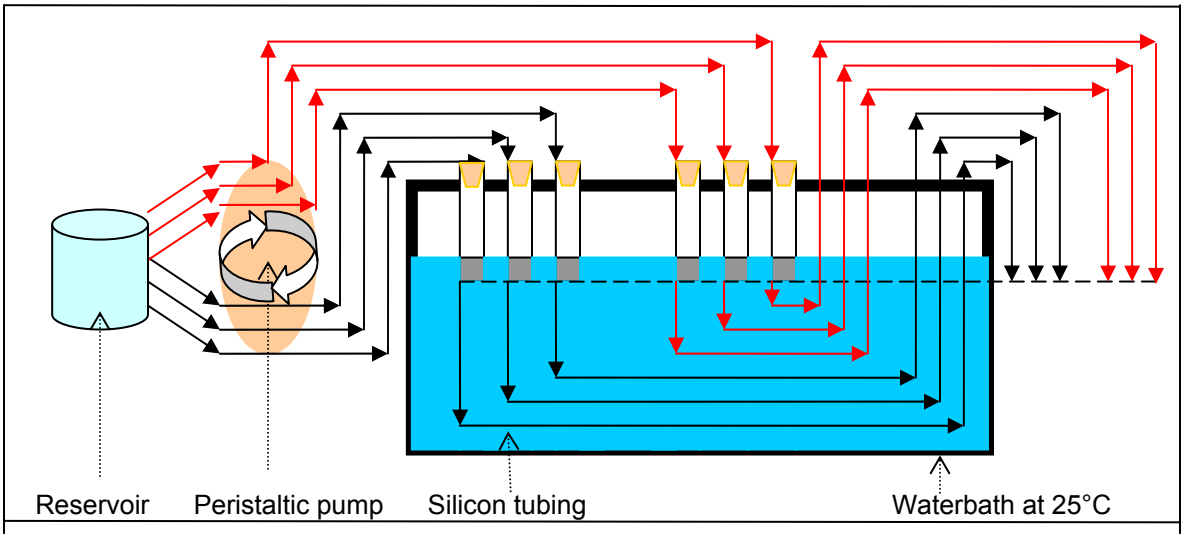

Figure A. Experimental set-up for 1 sampling day.
Triplicate columns with protists
Triplicate columns without protists

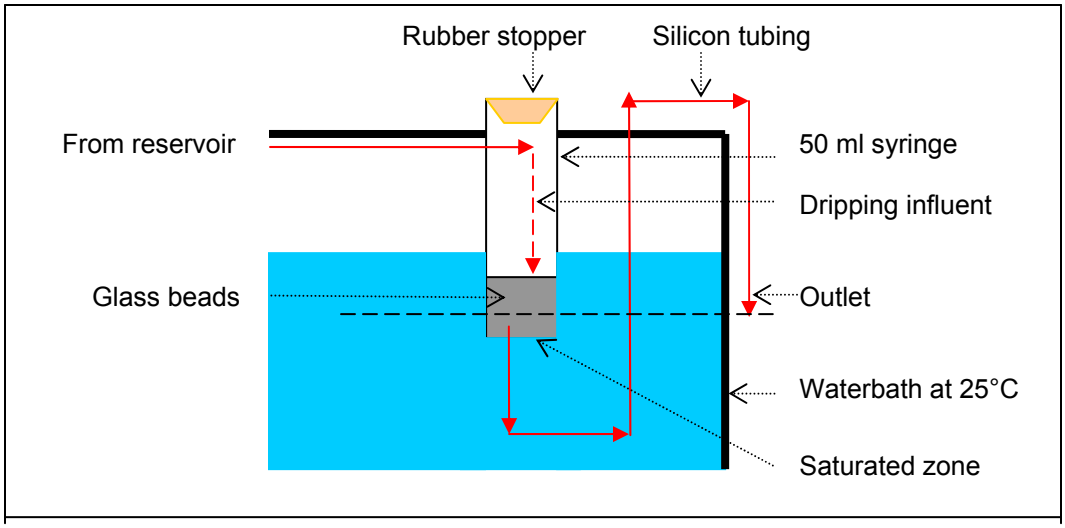

Figure B. Enlargement of one of the columns in the waterbath.

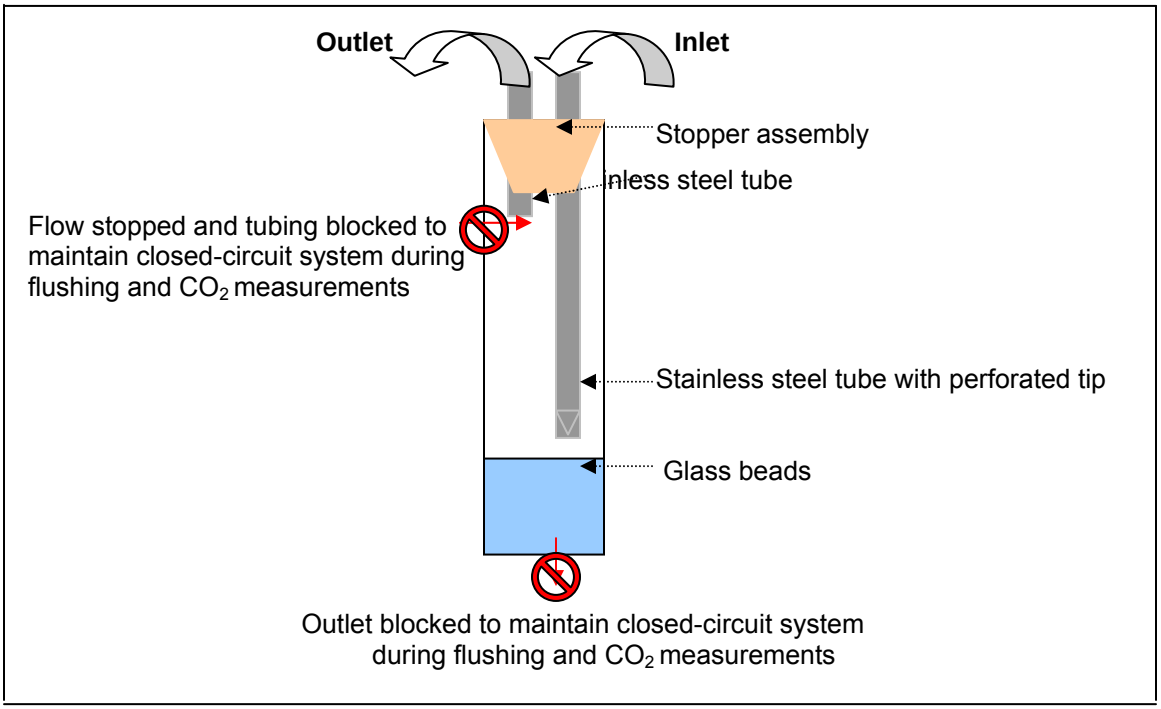

Figure C. Column with stopper-assembly.

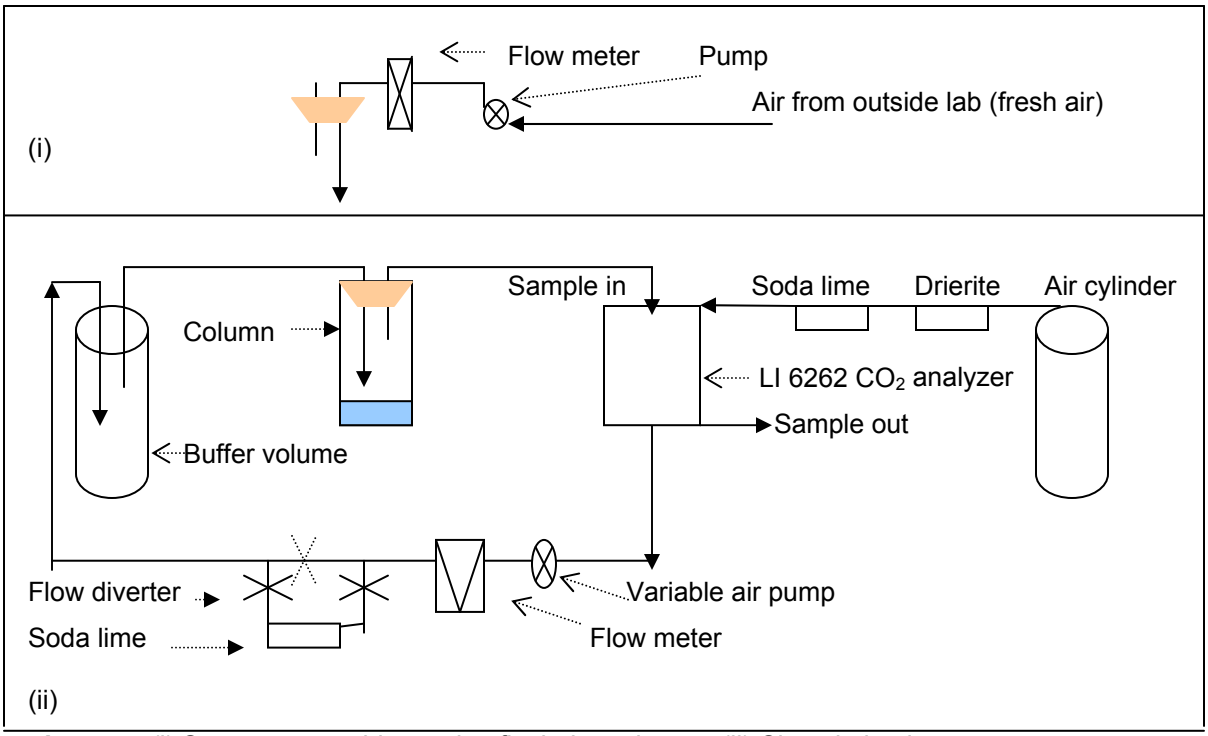

Figure D. (i) Stopper-assembly used to flush the columns. (ii) Closed-circuit system.

CHAPTER

6

General discussion and conclusions

GENERAL DISCUSSION AND CONCLUSIONS

The use of constructed wetlands for the improvement of water quality is a relatively new concept of the last two decades (Ibekwe *et al.*, 2003). The extent of water treatment in constructed wetlands depends upon the wetland design, microbial communities and types of plants involved. However, it has been found that the bulk of the water quality improvement in natural and constructed wetlands is due to bacteria (Gersberg *et al.*, 1986). Hence the diversity of microorganisms in the wetland environment may be critical for the proper functioning and maintenance of the system (Ibekwe *et al.*, 2003). The role of microbial activity in the functioning of wetlands is widely recognized, including degradation of recalcitrant compounds and nutrient cycling.

Alcohol distilleries are one of the most polluting industries generating an average of 8-15 L effluent per litre of alcohol produced (Saha *et al*., 2005) with a COD that can exceed 100 g/L (Yeoh, 1997). The production and characteristics of distillery wastewater are highly variable and dependent on the feedstock and various aspects of the ethanol production process (Wilkie *et al.*, 2000). Wash water used to clean the fermenters, cooling water blow down and boiler water blow down may all be combined with the distillery wastewater and contribute to its variability.

This study was initiated in 1999 at Distell Goudini, in the Western Cape, South Africa, to examine the feasibility of using constructed wetlands in the treatment of distillery wastewater (Mulidzi, 2005). Two 45 m-long by 6 m-wide wetlands (one with gravel and one with a soil substrate) with a 14 day retention time (RT) as well as three 6 m x 3 m gravel wetlands (4.5, 9 and 18 days RT, respectively) planted with *Phragmytes* and *Typha* spp were constructed and were fed with distillery effluent. Because of the successful treatment of distillery effluent by the gravel wetland over the first year and the known fact that wetlands host complex microbial communities (Reddy *et al*., 2002) that play an integral

part in the treatment of wastewater, it was decided to examine the microbial controls within gravel constructed wetlands.

The overall objective of this study was to evaluate the microbial controls within constructed wetlands used in the treatment of distillery wastewater. Specifically the following observations were made *en route* evaluating my hypothesis as stated on page 4:

- Terminal-restriction fragment length polymorphism (T-RFLP) analysis revealed that planktonic and attached microbial communities in various regions of the same wetland vary significantly from each other. Similarly, it was found that microbial communities at respective sampling points vary notably over time.

- The biofilm community on gravel did not stabilize after 8 weeks. It is highly probable that a fair degree of flux will continuously occur in any facility used to treat industrial effluent because of seasonal and operational variants and stabilization of biofilms may take months (if ever) to really stabilize. Ragusa *et al.* (2004) found that the total biomass can take upwards of 100 days to stabilize during batch operation of subsurface wetlands.

- Scanning electron microscopy detected different morphotypes at certain stages of biofilm formation while some organisms were present at most phases of biofilm formation which illustrates the highly dynamic nature of microbial communities in constructed wetlands.

- Even though a complex microbial composition exists within wetlands replicate samples showed similar T-RFLP profiles. This illustrates the value of the T-RFLP technique which has been proven to be a

reproducible and accurate tool for community fingerprinting (Liu *et al.*, 1997; Moeseneder *et al.*, 1999).

- Increased COD concentration caused notable changes in microbial community composition. Furthermore the shifts in microbial community composition illustrate the ability of microbial communities to adapt to changes in the environment without compromising their functional efficacy.

- The flow rates through the columns decreased significantly as biofilm development increased and decreased even more drastically in the presence of protozoa. Furthermore, an increased influent COD caused protozoa to become more active thereby stimulating the rate of CO_2 release.

- T-RFLP and SEM showed that biofilm structure was influenced by the presence of algae suggesting that the algae provided labile nutrients that were utilized by the bacterial and yeast members of the community.

- COD results revealed that augmentation, applied at correct concentrations, can be useful in COD removal. However, excessive augmentation can be detrimental as it would add to the COD.

6.1. FUTURE RESEARCH

In this study we only used absolute COD values, but admit that effluent composition will also affect microbial response and overall system performance. It would therefore be advisable to determine the impact of various harmful or potential harmful substances within the effluent on the microbial populations within the wetlands.

There is a need for pre-treatment before discharge of distillery wastewater into constructed wetlands. Further studies need to be performed to determine an economically viable option for pre-treating distillery wastewater before release into constructed wetlands. This will not only be an additional treatment step, but will also increase the longevity of the wetlands. Augmentation, at the correct concentrations, can be a useful pre-treatment option. This may be investigated on large-scale, by filling a 1000 L plastic container with water and a cheap carbon source. A few jugs of raw effluent can be poured into this container to act as a start-up culture. After a few days a certain amount of the contents of the 1000 L container, but not exceeding 10^{-3} (cell suspension: raw effluent), can be added to the raw effluent. This could be a cheap alternative to the relatively expensive commercial augmentation products currently available. Furthermore, a management plan will be required that involves routine analyses to ensure that the pre-treatment is sufficient.

Attempts should be made to characterize the dominant bacterial and yeast strains within typical wetland systems used in the treatment of distillery effluent. This will increase the possibility to identify indicator organisms that could aid in the rapid determination of system health. Furthermore it might shed some light on these complex systems on a level that is currently not really understood. Identification and characterization of “useful” organisms in these systems can also aid in formulating a supplement of microorganisms that is ideal for the specific wetland system. Although protists have been identified in this study as a possible problem through the fact that predation may cause clogging of the pores, it has been suggested as a means to decrease sludge production in wastewater treatment systems (Ratsak *et al*., 1994; 1996). It is therefore important that the role of these organisms be studied further in wetland systems. It would also be useful to monitor for the presence of bacteria from genera Pseudomonas, Enterobacter, Stenotrophomonas, Aeromonas, Acinetobacter and Klebsiella, as Ghosh *et al.* (2004) found that a combination of these organisms could reduce COD of distillery effluent by more than 40%.

Techniques such as fluorescence *in situ* hybridization (FISH) and denaturing-gradient gel electrophoresis DGGE could be used in the monitoring of phylogenetically defined populations in environmental samples in future research.

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