HEALTH FOR PURPOSE IN CONSTRUCTED WETLANDS:

ORGANIC REMOVAL EFFICIENCIES AND CHANGES IN MICROBIAL COMMUNITY DYNAMICS ASSOCIATED WITH EXPOSURE TO WINERY WASTEWATER

Report to the

WATER RESEARCH COMMISSION

by

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WRC Report No. 1936/1/11
ISBN 978-1-4312-0224-9

February 2012

Obtainable from

Water Research Commission
Private Bag X03
Gezina, 0031

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EXECUTIVE SUMMARY

BACKGROUND AND INTRODUCTION

Constructed wetlands (CWs) form low-maintenance, cost effective wastewater treatment systems that are environmentally friendly and aesthetically pleasing. Although CWs are used successfully for the treatment of a wide range of wastewaters, there is limited knowledge on their use for the treatment of high organic, industrial waste streams, such as winery wastewater. In South Africa, the wine industry accounts for the production of over one billion litres of wastewater per annum. To prevent adverse environmental consequences, this wastewater should be treated before discharge. This is especially important in the Western Cape, where most wineries are located; in this province, the peak wastewater production period coincides with the period of low precipitation, preventing natural dilution of discharged cellar waste in the catchment. CWs form ideal treatment systems for small to medium sized wineries as conventional systems are expensive and present operational difficulties, mainly related to the intermittent production and variable composition of winery wastewater. Notably, lengthy start-up times are required after seasonal periods of redundancy, a problem that may be overcome by the use of CWs.

RATIONALE

The original title of this project “Health for purpose in constructed wetlands” encapsulates the fundamental objectives of the research conducted, namely to maintain a viable “healthy” microbial population capable of degrading target pollutants within pilot-scale CWs. The need to understand the link between biology and chemistry is fundamental to understanding the biodegradative capacity of CWs. Enhanced knowledge of functional microbial community responses to target organic pollutants will ultimately improve design criteria applicable to selected waste streams.

OBJECTIVES AND AIMS

AIM 1

Development of molecular fingerprints of microbial communities and key degradative enzymes involved in the degradation of specific pollutants

Within a microbial community, not all the species are directly involved in the primary degradation process. The first aim of this project was to develop molecular fingerprinting technologies for wetland microbial communities, as well as for specific genes encoding for key degradative enzymes involved in pollutant remediation.

AIM 2

Development of methods to demonstrate microbial population changes resulting from impact of polluting waste streams

In a previous study (K5/1544), we successfully demonstrated that a combined molecular and biochemical approach was appropriate for studying the impact and fate of pollutants in wetlands. Further development of phenotypic methodologies to indicate changes in microbial populations constituted the second aim of the project.

AIM 3

Investigation of the effects of specific interventions (e.g. addition of fertilizer) on the "health-for-purpose" of the wetland microbial population

The third aim was to apply genotypic and phenotypic methodologies (see aims 1 and 2), to monitor the temporal and spatial changes taking place in the microbial population in response to selected interventions. It was hypothesized that the stability and/or recovery of CW function could be linked to the microbial responses to these interventions.

METHODOLOGY

Changes in microbial community dynamics in response to amendment was monitored by denaturing gradient gel electrophoresis (DGGE) and terminal restriction fragment length polymorphism (T-RFLP), which are molecular fingerprinting methods. Differences in microbial community responses in the deep sediments and the surface sediments at the inlet and the outlet of the CWs were compared. The hydraulic conductivity ($L/hr.m^{3-1}$) was monitored by measuring the volume of effluent discharged over the period of an hour, commencing one hour subsequent to amendment. Influent and effluent COD, total nitrogen, nitrate nitrogen, ammonia nitrogen and total phosphorus were quantified using relevant Hanna® kits according to the manufacturer's instructions. Sugars, phenolics and volatile fatty acids in the effluent were identified and quantified by HPLC. Where relevant, the concentrations of substrates and metabolites were converted into COD values to determine mass balance relationships and elucidate possible metabolic pathways. Comparisons between the removal of selected phenolics by sterilized and unsterilized soil columns and soil microcosms were used to determine abiotic and biotic removal rates.

RESULTS AND DISCUSSION

Aim 1

We successfully modified and applied two microbial fingerprinting methods (DGGE and T-RFLP), which allowed us to follow modifications in the microbial communities before and after amendments. These molecular tools were used to target the 16S rRNA gene of (i) the Eubacterial microbial community, the Archaeal community and (iii) the specific methanogenic Archael communities. By targeting the *nifH* gene, we studied the nitrogen-fixing (diazotrophic) communities.

Aim 2

Qualitative and quantitative influent and effluent analyses and enzyme assay methodologies were either developed or optimized. It was expected to find clear-cut differences in spatial and temporal trends in sediment microbial enzyme activities between control and test CWs (the latter amended with ethanol, winery wastewater and synthetic phenolic wastewater). However, assays chosen to indicate general microbial activity, e.g. fluorescein diacetate (FDA), were not useful, with activities from both control and test CW samples showing variations within similar ranges. Although methods for more specific activities (alcohol dehydrogenase, catechol dioxygenase) showed more promise, results were not reproducible.

The combined use of COD measurements and HPLC analysis to measure qualitative and quantitative degradation of organics was simple and applicable. During CW amendment with ethanol and phenolics, the identification and quantification of substrates and metabolites gave an indication of the aerobic and anaerobic catabolic pathways taking place as well as the advent of toxicity in the test CWs.

Aim 3

Using T-RFLP, DGGE and influent and effluent analyses, we demonstrated that autochthonous microbial communities within the CW were modified and were able to adapt to amendment with ethanol, winery wastewater and synthetic phenolic wastewater. We demonstrated both temporal and spatial adaptation of these communities. The addition of nutrients (nitrogen and phosphorus) prevented microbial community changes in a CW amended with ethanol, with biochemical results suggesting a link between this phenomenon and enhanced bioremediatory capacity. A second procedure, whereby increasing concentrations of ethanol was used for amendment (incremental priming), also resulted in an increase in biodegradative efficiency. Organic overloading by ethanol and phenolic

wastewater induced toxicity in the CW sediments. This was evidenced by accumulation of the toxic metabolites, butyrate and propionate (during amendment with ethanol) and catechol (during amendment with gallic acid and vanillin). Amendment with high concentrations of phenolics induced a loss of microbial diversity in the deep CW sediment, probably due to the accumulation of the toxic metabolite, catechol, under anaerobic conditions. Conversely, there was an increase in microbial diversity in the surface sediments, possibly due to the presence of a range of utilizable substrates from the preferential (aerobic) catabolism of vanillin and gallic acid.

GENERAL CONCLUSIONS

Development and use of molecular fingerprinting to monitor changes in microbial community structure

Two molecular fingerprinting methods to assess changes in the CW's microbial community structure were optimized: DGGE (Denaturing Gradient Gel Electrophoresis) and T-RFLP (Terminal-Restriction Fragment Length Polymorphism). We demonstrated that these rapid and relatively simple survey methods were effective tools to monitor both qualitative and quantitative changes in microbial community structure in CW systems. These fingerprinting methods were used to monitor both the compositional changes and the rate of change in a microbial community after chemical exposure. Using DGGE, it was demonstrated that after 89 days of feeding with low concentrations of yeast extract and glucose, the pilot-scale CWs were equilibrated. Microbial communitites which presented similar fingerprints in all CWs were established in both the surface and deep sediments. T-RFLP was used to follow the evolution of the microbial community structure in CWs amended with ethanol, winery wastewater and synthetic wastewater containing phenolics. As expected, all these amendments lead to modification of the microbial community structure in the CW sediments.

These results showed that microbial community fingerprinting with molecular tools (such as DGGE) is a key parameter for monitoring CW establishment before amendment with pollutants. The observation that microbial community structure is the slowest parameter to stabilize supports this view. The groundwork for the use of CW systems to investigate the relationships between microbial structure and system responses to environmental challenges was thus laid.

Measurement of hydraulic conductivity and correlation of this parameter with organic loading

Hydraulic conductivity was found to be a useful indicator of quantitative biotic changes within the CWs. There was a strong relationship between influent COD and hydraulic conductivity. During amendment with winery wastewater and high concentrations of ethanol, the hydraulic conductivity often decreased to such a level that the mode of operation inadvertently changed from subsurface to surface flow. This effect was reversible, with an increase in conductivity following a decrease in influent COD. We also noted that hydraulic conductivity may be useful as a determinant of microbial community development and that extractable DNA titres (by the procedure used in this project) is not a reliable parameter for monitoring microbial biomass.

Chemical oxygen demand and effluent chemical mass balances

Influent and effluent COD measurements (theoretical and actual) were utilized to indicate the overall operational efficiency and function of the CWs. Firstly, by expressing the concentration of influent organics in COD terms, comparisons of loading data could be made using industry norms. Secondly, a simple and novel method was employed whereby the concentrations of metabolic substrates and products were converted into COD terms for use in mass balance equations. This proved to be a valuable tool for elucidating potential metabolic pathways. The theoretical COD (COD_t) for a range of volatile fatty acids and phenolics correlated well with the measured values. By quantifying substrates and key metabolites in COD values, the percentage contribution of each to the total COD is possible. The "remaining" COD (not attributable to organics which have been identified and quantified) can then be calculated by this method. Furthermore, it can be hypothesized that if the COD contribution by unidentified organics is low, the metabolic assumptions made using effluent metabolic profiles are more relevant than those where the contribution is high**.**

Increased biodegradation of ethanol using incremental priming as a start-up measure

The metabolic profiles and removal efficiencies of a CW amended with ethanol in incrementally increasing concentrations (incremental priming) and a second CW, amended from inception with a moderate concentration of ethanol were compared. The incremental priming procedure enhanced COD removal and prevented the accumulation of toxic and malodorous volatile fatty acids. In addition, this procedure resulted in a selection of diazotrophic (nitrogen fixing) organisms which may have played a role in the success of the procedure due to the creation of favourable C:N ratio. This was borne out by N mass-balance calculations and the demonstration of numerically significant differences in the

number of diazotrophs cultured from sediment samples in comparison to the control CW. Similar microbial communities were selected during amendment with ethanol, irrespective of whether amendment was incremental or not. However, incremental priming resulted in an increase in the bioremediatory capacity, showing that this procedure increased the functionality of the microbial community via acclimation.

Nutrient balance

The addition of nutrients in a COD/N/P ratio of 300/5/1 to a second CW amended with ethanol significantly enhanced the degradative performance in comparison to a replicate amended with ethanol only. The addition of N and P helped to maintain the original microbial community structure in pilot-scale CWs amended with ethanol, which may have been the reason for superior COD removal efficiency. However, N and P addition resulted in increased nutrients in the treated effluent, and is therefore not recommended as a measure to increase CW performance.

This experiment demonstrated the importance of nutrient balance and added credence to the theory that part of the success of incremental priming was due to natural selection and acclimation of nitrogen fixing organisms; selection of a microbial population with the capacity for dealing effectively with the chemical composition of wastewater is a key process in all wastewater treatment systems.

Toxicity due to organic overload

At high loading rates, amendment of sand-filled pilot scale CWs with ethanol and synthetic phenolic wastewater resulted in increased effluent COD concentrations and the accumulation of potentially toxic metabolites. During amendment with high concentrations of gallic acid and vanillin, the toxic metabolite, catechol, was detected in the effluent in chronologically increasing amounts. This was accompanied by an increasing trend in hydraulic conductivity, which led to the assumption that the environment had become toxic for at least a portion of the microbial population. There was also a decrease in the bacterial diversity observed in the deep CW sediments when subjected to winery wastewater or synthetic phenolic wastewater, which suggested that these solutions were less toxic to the microbial communities at the surface. This is borne out by metabolic analysis, as the breakdown of toxic metabolites such as catechol is more likely to take place at a faster rate in the presence of oxygen.

Abiotic removal mechanisms

It must be borne in mind that non-biological (abiotic) factors may play a significant role in the removal of both organic and inorganic pollutants in CWs and sand filter systems. To prevent leaching of chemicals from CWs (and consequent rehabilitative requirements), it is essential that a fundamental biodegradative balance is achieved in CWs treating organic wastewater. During project K5/1936, phenolics were removed by both adsorption and biodegradation, with large amounts of gallic acid being adsorbed by the sediment matrix, probably by complexation with metals.

RECOMMENDATIONS FOR FUTURE RESEARCH

Building on the knowledge gained during project K5/1936, the recommendations outlined below form part of the study protocol for a new WRC funded project that commenced in May 2011.

- The use of **microbial community fingerprinting techniques** such as DGGE and/or T-RFLP, to monitor (i) the establishment and equilibration of microbial communities in pilot-scale CWs and (ii) their adaptive responses to various amendments has proven to be useful and is thus highly recommended. In addition, quantification of functional genes, together with chemical analyses is recommended to develop understanding of the microbial processes taking place in CWs during the treatment of organic wastewater.

- Prior to any chemical amendments, it is recommended that a **microbial community equilibration time** in the order 100 days is respected. Decreases in hydraulic conductivity measurements can be used to indicate the establishment process of the microbial communities.

- It is recommended that **organic loading maxima** for CWs with different media, modes of operation and waste streams are established and compared. COD or other appropriate chemical indicators can be used, depending on the waste type. It is further recommended that design criteria should incorporate features allowing pre-emptive measures to be taken during periods when maxima are exceeded. For example, systems that incorporate the option to return treated wastewater to the system head to dilute high-strength wastewaters is a practical measure that functions independently of external water supplies. It is envisaged that such measures will

prevent loss of CW function due to the advent of toxicity and/or septicity associated with overload.

- The use of **COD mass balances** to elaborate the metabolic processes occurring within CWs is scientifically relevant. During this project, the CWs were treated as "black boxes", with only influent and effluent analyses being conducted. To gain further insight into the metabolic sequence of biodegradation within CWs, it is recommended that proper collection of pore water from at least four sites (superficial inlet and outlet, deep inlet and outlet) be implemented in future studies. This will allow: (i) more accurate investigations on the specific connections between metabolic processes and microbial community dynamics; (ii) elucidation of the temporal and spatial locations where each metabolic step predominates, i.e. sequential, aerobic/anaerobic; and (ii) calculation of decay coefficients for inclusion in full-scale design parameters.

- The use of **incremental priming** to induce acclimation of microbial communities increased the ethanol removal capacity in the pilot-scale constructed wetlands and was a key finding of the project. It is recommended that comparative studies using appropriate wastewaters (whole and synthetic) are conducted. If previous results are reproducible, studies to determine the time-scales of acclimation necessary to exact the benefits without compromising the derived benefits are also recommended.

- There is a need to determine the importance of **media type** (gravels and sands) on the microbial community structure. During this project (K5/1936), it was hypothesized that the presence of high concentrations of enzymatic co-factors (iron, molybdenum, zinc), naturally present in the CW sand enhanced the efficiency of the CWs. It is recommended that the microbial community fingerprints in different media, exposed to a range of pollutants be determined. Thus, the relative importance of the pollutant source and/or CW media on the development of microbial communities can be ascertained. Such studies can be conducted in mesocosms, so that a large variety of media may be screened prior to piloting.

- During this project, it was found that addition of **nitrogen and phosphorus** to CWs amended with high COD wastewaters sustained the original microbial community structures and increased the CW degradative capacity. However, these nutrients are associated with eutrophication and their presence in the effluent was a concern. It is

thus recommended that research into the application of nutrient balances in CWs is continued. For example, incremental priming may be used to select diazotrophs, resulting in a “natural” nitrogen balance in CWs. To understand the role of diazotrophs in CWs, further molecular studies, quantifying copy numbers of nitrogen-fixing genes (e.g. *nifH*), which were initiated during this project, are suggested.

- The removal of organic molecules in CWs may take place via **abiotic mechanisms** (chiefly adsorption) or **biotic mechanisms** (mostly biodegradation). It is important that sufficient biodegradation of organic substrates takes place in CWs to prevent accumulation and subsequent leaching of pollutants into the treated effluent. It is thus recommended that microcosm experiments, such as those used during project K5/1936 are continued. The inclusion of adsorption/desorption experiments using target chemicals on CW media should also be considered.

ACKNOWLEDGEMENTS

The authors would like to thank the Reference Group of the WRC Project K5/1936 for the assistance and the constructive discussions during the duration of the project:

Prof A. Burger (University of Stellenbosch)

Ms L. Costa (OlivesInFact)

Ms V. Govender (eThekwini Municipality)

Mr D.J. Otto (Digby Wells Environmental)

Prof S.N. Venter (University of Pretoria)

Dr J.A. Wilsenach (Virtual Consulting Engineers)

We would also like to thank Dr Valerie Naidoo (WRC Research Manager) and Mr Benny Mokgonyana (WRC Proposal and Project Coordinator) for their guidance and assistance during the course of the project. The help and advice extended by Malilize le Roes Hill (Research Director, Biocatalysis and Technical Biology Research group) and Ms Kerry Horne (Laboratory Manager, Biocatalysis and Technical Biology Research group) is also gratefully recognized.

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LIST OF ABBREVIATIONS

AAS:	Atomic Absorption Spectrometry
AM:	Azotobacter medium
ANOVA:	Univariate Analysis of Variance
AOB:	Ammonium Oxidizing Bacteria
(A)RISA:	(Automatic) Ribosomal Intergenic Spacer Analysis
BOD:	Biochemical Oxygen Demand
BP:	Base Pair
BSA:	Bovine Serum Albumin
C:	Carbon
CFU:	Colony Forming Unit
CLPP:	Community level physiological profiling
COD:	Chemical Oxygen Demand
COD_a:	Actual COD
COD_e:	Effluent COD
COD_i:	Influent COD
COD_{it}:	Influent theoretical COD
COD_t:	Theoretical COD
cm:	Centimetre
CO_2:	Carbon dioxide
CPUT:	Cape Peninsula University of Technology
CW:	Constructed Wetland
D:	Day
D:	Deep/depth
DGGE:	Denaturing Gradient Gel Electrophoresis
DN:	Denitrification
DNA:	Deoxyribonucleic Acid
DNRA:	Dissimilatory Nitrate Reduction to Ammonia
DO:	Dissolved Oxygen
EC_{50}	Effective concentration 50
EDTA:	Ethylenediaminetetraacetic acid
EPA:	Environmental Protection Agency
EPS:	Exopolysaccharide
EtBr:	Ethidium Bromide
FC:	Folin-Ciocalteau
FDA:	Fluorescein diacetate
FP:	Finishing pond
FW:	Free water
FWSF:	Free Water Surface flow
g:	Gram
GAE:	Gallic Acid Equivalent
gCOD:	Gram of COD
gdwt	Gram dry weight
h or hr:	Hour
HC:	Hydraulic Conductivity
HF:	Horizontal flow
HPLC:	High Pressure Liquid Chromatography
HRT:	Hydraulic Retention Time
HSSF:	Horizontal Subsurface Flow
KBr:	Potassium bromide
kg:	Kilogram
L:	Litre
L:	Length
m:	metre
m^3:	Cubic Metre
MDS:	Multidimensional Scaling
mg:	Milligram
mgCOD:	Milligram of COD
min:	Minute

ml:	Millilitre
N:	Nitrogen
ND:	Not determined
NDMSA:	Nitrogen Deficient Mannitol Salt Agar
NH_3-N:	Ammonia Nitrogen
nm :	Nanometer
No:	Number
NOB:	Nitrite-Oxidizing Bacteria
NO_3^--N:	Nitrate Nitrogen
OD:	Optical Density
OLR:	Organic Loading Rate
OTU:	Operational Taxonomic Unit
O_2:	Oxygen
P:	Phosphorus
P:	porosity
PCA:	Principal Component Analysis
PCR:	Polymerase Chain Reaction
PTIS:	Partial tanks-in-series
PVPP:	Polyvinylpyrrolidone
Q:	Average flow
RNA:	Ribonucleic acid
RTD:	Retention Time Distribution
S:	Surface
SIP:	Stable isotope probe
SOM:	Soil Organic Matter
SRB:	Sulphate Reducing Bacteria
SS:	Suspended Solids
SSCP:	Single Strand Conformation Polymorphism
STIS:	Spatial tanks-in-series
TE:	Tris-EDTA
tHRT:	Theoretical hydraulic retention time
TIS:	Tanks-in-series
TN:	Total Nitrogen
TP:	Total phosphorus
T-RF:	Terminal-Restriction Fragment
T-RFLP:	Terminal-Restriction Fragment Length Polymorphism
USEPA:	United States Environmental Protection Agency
UV:	Ultraviolet
V:	Volt
VF:	Vertical flow
VFA:	Volatile Fatty Acid
Vol:	Volume
VSSF:	Vertical Subsurface Flow
W:	Width
W:	Week
Ww:	Wastewater
Wt:	Weight
WWTP:	Waste Water Treatment Plant
3-D:	Three Dimensional
µl:	Microlitre
µmol:	Micromole
°C :	Degrees Celcius

GLOSSARY OF COMMON TERMS USED IN TEXT

Acclimation
The process whereby organisms undergo a functional adaptation to changes in environmental conditions

Amendment (of CWs)
The addition of experimental chemicals in the influent

Amplification (of nucleic acid)
The use of purified enzymes to replicate specific nucleic acids

Baseline (of CWs)
The chemical and biological state of state of the CWs prior to amendment

Chemical oxygen demand (COD)
A common test used to measure the organic content of water based on the amount of oxygen required for oxidation of all organic matter to carbon dioxide ammonia and water under acidic conditions. The theoretical COD is the COD calculated from the molecular formula of an organic compound using the stoichiometric equation for the complete oxidation of that compound under acidic conditions

DGGE
Denaturing gradient gel electrophoresis is a molecular technique that separates different nucleic acid fragments in a gel by applying a denaturing agent

Diazotrophs
Bacteria and archaea capable of fixing atmospheric nitrogen

Dendogram
A form of tree diagram used to illustrate the clustering of genes or samples

Diversity index
A measure of the biodiversity within an ecosystem

Equilibration (of CWs)
A term used to describe the state when the microbial community structure no longer exhibits temporal adaptive responses to feeding or amendment

Feeding (of CWs)
A term used to describe the addition of low levels of nutrients in the CW influent during equilibration and amendment

Hydraulic conductivity (of CWs)
A measurement of the liquid transfer properties of the substratum calculated in this project as the volume of effluent collected for each CW system for the period of one hour after feeding/amendment per cubic metre of sand

Incremental priming (of CWs)
The addition of an influent chemical in chronologically increasing concentrations in order to induce acclimation of the resident microbial population

Metabolites
Microbial degradative products

Metagenomic
Referring to the genetic material recovered from environmental samples

Principle component analysis (PCA)
A multivariate statistical tool used to project variables on a plane in order to detect similarities and differences by analysing the proximity of data points (spatial variation) on a diagram

Restriction digest
The process by which nucleic acids are cut into fragments (digestion) using specific restriction enzymes

Species evenness
The relative abundance (proportion) of individuals amongst the species

Species richness
The number of species present in an ecosystem

LIST OF CONFERENCES AND PUBLICATONS

Publications

Welz, P.J., Ramond, J-B., Cowan, D., Prins, A., Burton S.G. (2011) Ethanol degradation and the effect of incremental priming on the removal efficiency in pilot-scale constructed wetlands. Ecological Engineering 37: 1453-1459.

Ramond, J-B., Welz, P.J., Cowan, D.A., Burton, S.G. (2011) Microbial community structure, a key parameter in monitoring the development and stability of constructed wetland mesocosms. *Manuscript submitted to Research in Microbiology (April 2011)*

Rodriguez Caballero, A., Ramond, J-B., Welz, P.J., Cowan, D.A., Odlare, M., Burton, S.G. (2011) Treatment of high ethanol concentration wastewater by constructed wetlands: enhanced COD removal and bacterial community dynamics. *Manuscript submitted to Journal of Environmental Management (April 2011).*

Welz, P.J., Ramond, J-B., Cowan, D., Prins, A., Burton S.G. (2011) Phenolic removal processes in constructed wetlands treating winery wastewater. *Manuscript submitted to Ecological Engineering (August 2011).*

Oral presentations

International congresses

Treatment of high ethanol concentration wastewater by constructed wetlands: enhanced COD removal and bacterial community dynamics. Rodriguez Caballero, A., Ramond, J-B., Welz, P.J., Cowan, D.A., Odlare, M., Burton, S.G. IWA congress: Microbes in wastewater and waste treatment, bioremediation and energy production. Goa (India), January 2011.

Soil column experiment to compare biotic and abiotic removal of plant phenolics in wetlands. P.J. Welz, A. Prins, J-B. Ramond, D.A. Cowan, S.G. Burton. SIL Congress. Cape Town (South Africa), July 2010.

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Molecular Survey of Constructed Wetland Microbial Communities impacted by Winery-Effluents. J-B. Ramond, P. J. Weltz, S.G. Burton, M. Tuffin, D.A. Cowan. *Cape Biotechnology* Forum *2010.* Somerset West *(South Africa), March 2010.*

Poster presentations

Microbial population changes is response to amendment with winery wastewater and ethanol in pilot-scale constructed wetlands. P. Welz, J-B. Ramond, D. Cowan, S. Burton. Cape Biotechnology Forum 2010. Somerset West (South Africa), March 2010.

Molecular survey of constructed wetland microbial communities impacted by winery-effluents. J-B. Ramond, P. Welz, S. Burton, M. Tuffin, D. Cowan. 22nd Congress of the South African Society for Biochemistry and Molecular Biology. Bloemfontein (South Africa), January 2010.

Microbial population changes is response to amendment with winery wastewater and ethanol in pilot-scale constructed wetlands. P. Welz, J-B. Ramond, D. Cowan, S. Burton. 22nd Congress of the South African Society for Biochemistry and Molecular Biology. Bloemfontein (South Africa), January 2010.

Impact of winery-effluents on microbial communities in constructed wetlands. J-B. Ramond, P. Welz, S. Burton, M. Tuffin, D. Cowan. Congress of the South African Society for Microbiology. Durban (South Africa), September 2009.

PART A GRAVEL-FILLED, PLANTED CONSTRUCTED WETLANDS

A1 OVERVIEW OF CONSTRUCTED WETLANDS

Constructed wetlands (CWs) are designed to mimic natural wetland systems and have been used since the 1950s to remediate several types of wastewater including domestic, (sewage and urban run-off), agricultural (crops and livestock), food processing and mining (Verhoeven et al., 1999; Shutes, 2001). The substrate medium of CWs is typically composed of sand, gravel, or crushed rock, often with different diameters and porosities. The CW microbial consortia, plants and substrate function synergistically to treat wastewater by a combination of processes: settlement and filtration of suspended solids, substrate sorption, nutrient uptake, biodegradation, mineralization and volatilization (McJannet et al., 1995; Verhoeven et al., 1999).

The CW medium provides a support for plants and microorganisms and can adsorb and filter organic material, nitrogen, phosphorus and heavy metals from the wastewater (Maloszewski et al., 2006; Vrhovšek et al., 1996). The physical parameters of the substrate (grain-size distribution, interstitial pore space, effective grain size, degree of irregularity and the coefficient of permeability) influence the CW flow rate, which ultimately impacts on the effectiveness of biodegradation (Stottmeister et al., 2003).

The presence of CW plants can increase the microbial diversity by the inclusion of rhizospheric flora to the microbial mix, and the symbiotic relationship between plants and microbes produces an appropriate environment for aerobic degradation. Oxygen flow rates of 126 µmol $O_2.h^{-1}$ per gram of root dry mass for *Juncus ingens* (Sorrell and Armstrong, 1994) and 120-200 µmol $O_2.h^{-1}$ per gram of root dry mass for *Typha latifolia* (Jespersen *et al*., 1998) have been measured.

The microbial communities in CWs are complex, with hundreds or thousands of species in multiple trophic layers of primary, secondary and tertiary degraders. Biological processes are responsible for the breakdown and consumption of organic matter and the transformation of nitrogenous constituents (Wallace and Knight, 2006). The distribution of functional species depends on environmental factors (temperature, pH, aeration) and the class of target pollutants. In degradative niches, such as those of wetlands, relatively few species are directly involved in primary degradation and a limited number of species may dominate.

CWs are broadly categorized into three types by the incumbent wastewater flow, namely horizontal surface flow (FWSF), horizontal subsurface flow (HSSF) and vertical subsurface flow.

Many CWs do not form stand-alone systems and are connected to additional CWs or other wastewater treatment systems to increase efficiency. For example, pre-treatment of winery wastewater by anaerobic digestion, sand pre-filters, Imhoff tanks and combined processes have been described (Grismer et al., 2003a; Masi et al., 2002; Serrano et al., 2010; Sheperd et al., 2001a). In contrast to standard horizontal-flow constructed wetlands, VSSF CWs are alternately flooded and drained. This allows atmospheric oxygen to enter the top layers during the drainage period; during the fill period, gases are absorbed by the incoming effluent and are transported to the deeper layers of the CW (Cooper et al., 1996; Gervin and Brix, 2001; Green *et al.*, 1998 Scholz, 2006; Stottmeister *et al.*, 2003). It has been recognized that these CWs usually have higher removal efficiencies with regard to organic pollutants and nutrients. However, in comparison to horizontal-flow wetlands, denitrification is less efficient (Luederits *et al*. 2001).

A2 EXPERIMENTAL PROCEDURES

A2.1 DESIGN BACKGROUND AND CRITERIA FOR EXPERIMENTAL GRAVEL-FILLED PILOT-SCALE CONSTRUCTED WETLANDS

In previous work (Burton et al, 2006 and Sheridan, 2006), a partial tanks-in-series (PTIS) model was developed which was found to adequately represent the functional hydraulics of a constructed wetland operating to treat winery waste. The dimensions of this wetland are shown in Figure A1 below. The mean residence time (τ, Tau), the dispersion number (D) and the number of tanks (N) for the PTIS model were determined and the average flow-rate for a residence time distribution experiment (using potassium bromide) was 0.74m^3/hour (standard deviation of 0.34). These previous investigations showed that the measured mean residence time deviated significantly from the ideal residence time (Figure A2), and that this deviation increased with increasing residence time. The correlation indicated that, for this system, the measured residence time was approximately 60% of the ideal residence time.

The relationship between the number of tanks (N) and the length, and the residence time (τ) and the length, is shown in Figure A3 (for the centre-line only). These correlations allow a standard tanks-in-series (TIS) model to be expanded for the design of CW systems provided that they are gravel based and the superficial velocity of the flow is not significantly different to this system (the superficial velocity is 4.4 m/day). The number of tanks was correlated to

the length of the wetland) and this relationship was used as the basis of a modified TIS model

($N = 0.0088 \cdot l^2 + 0.0538 \cdot l$).

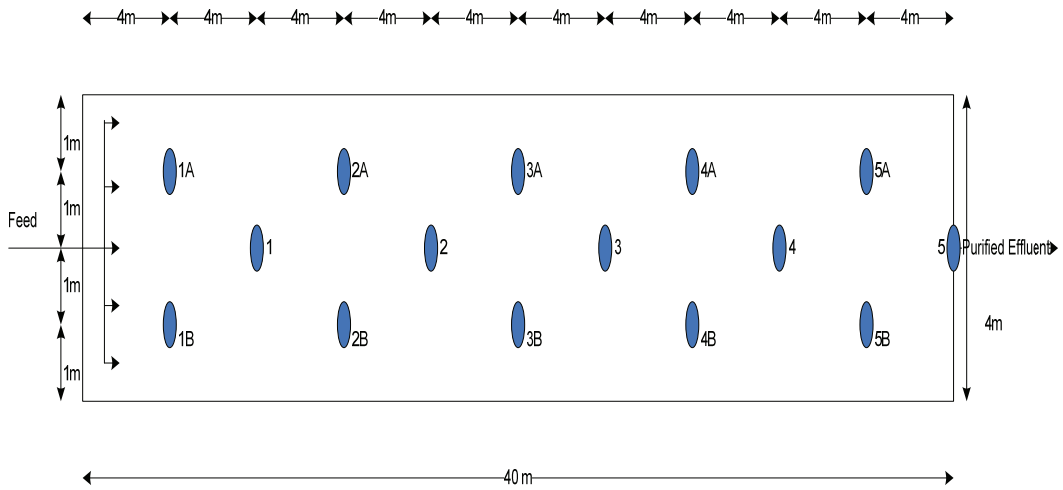

Figure A1 The dimensions and sample port locations of an experimental constructed wetland used in a previous study (not to scale) (Sheridan, 2006)

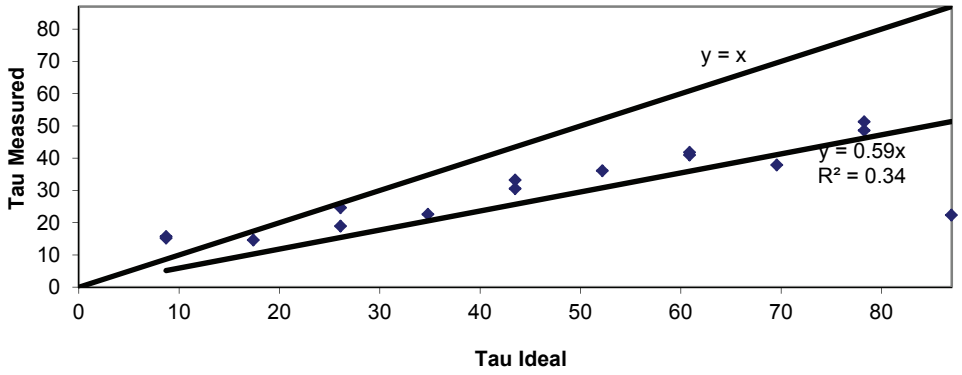

Figure A2 Graph showing the difference between measured and ideal residence time (Tau) obtained in an experimental constructed wetland used in a previous study

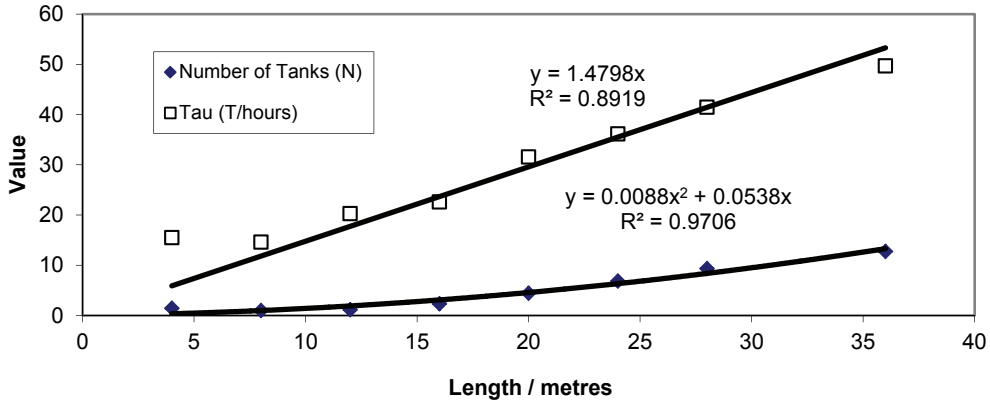

Figure A3 Graph showing the relationship between residence time, number of tanks and length of an experimental constructed wetland used in a previous study

Based on a spatial-tanks-in-series model, Sheridan (2007) developed a detailed design of an integrated wetland designed to have a maximum flow of 10m^3/day. The constructed wetlands were designed based on the second zone of this integrated wetland design, where a length of 2.8m could provide potential ethanol removal capacity of 4.1 ml.L^{-1} (k = 0.48/d) and COD removal capacity 7640 mg/L.d^{-1} (k = 0.089 / d)

A2.1.1 Flow rate, hydraulic conductivity and hydraulic retention time (HRT)

The flow rate required was based on Darcy's Law: $Q=k_s \cdot A_c \cdot S_w$

Q: average flow through wetland m^3/d

k_s: hydraulic conductivity m/d

A_c: cross-sectional area of bed m^2

S_w: slope of the hydraulic grade line

The hydraulic conductivity was based on a safety factor approach, with the recommendation that the clean bed hydraulic conductivity be reduced by a factor of 10 (or even a factor of 100). The clean bed hydraulic conductivity estimated was based on the grain size of the medium according to the Beyer relationship:

$k_s = d_{10}^2/100$ (Eq A1)

d_{10}: grain diameter at which 10% of the particles are finer by weight, mm

The hydraulic retention time was calculated using equation A2

$HRT = p \cdot L \cdot W \cdot D/Q$ (Eq A2)

p: porosity, %

L: length, m

W: width, m

D: depth, m

Q: average flow through wetland m^3/d

A2.1.2 Dimensions, fill material, effluent sampling system and operational parameters

Two wetlands were constructed from wood with PVC liner, with dimensions of 2.4 m (length) x 0.6 m (width) x 0.6 m (depth) (Figure A4). Gravel (14 mm), with a porosity of 0.35% was used as fill and the CWs were inoculated with ~ 40 L of soil from a natural wetland that had been impacted with winery wastewater. Prior to inoculation, leaves and other coarse plant material were removed by sieving (mesh size 8 mm). Sample wells were installed, with different lengths for taking samples within the upper, middle and lower depths of the bed.

A peristaltic pump was used to pump influent into an over fall basin which distributed the water evenly on the surface of the gravel at the inlet area. The peristaltic pump had been calibrated using a rota meter interconnected between the pump and the over fall basin. A constant flow rate (10 L/h) was chosen to maintain a continuous exchange of water in the wetlands. The site of the upper outlet (50 cm) ensured that the mode of operation was maintained as sub-surface flow.

A2.2 TRACER EXPERIMENTS TO DETERMINE FLOWPATHS OF GRAVEL-FILLED PILOT-SCALE CONSTRUCTED WETLANDS

Two separate tracer tests were performed using KBr and gallic acid. The difference between the density of the KBr solution (35 g in 2.5 L tap water) and water was < 5%. Due to solubility constraints (max. solubility of anhydrous gallic acid in water (20°C): 1.1 g/100 ml), the gallic acid solution was more dilute (25 g gallic acid in 2.5 L tap water. For both experiments, the flow rate was maintained at an elevated level of 25 L/h and tHRT of ~14 hrs. Samples (9 ml) were taken from each sampling port (1 to 6, see Figure A4) at the surface, middle and bottom, plus from the outlet, giving a total of 19 samples for each sampling instance. For the KBr study, samples were taken at baseline, then every hour for the first 6 hours, every 2 hours for the next 14 hours, twice per day for the following two days and once daily for the last three days, while for the gallic acid study, samples were taken at hourly intervals for the first 6 hours, at 2 hourly intervals for the next 10 hours, twice per day on the next day, once per day on the following day with the last samples being taken after 72 hours.

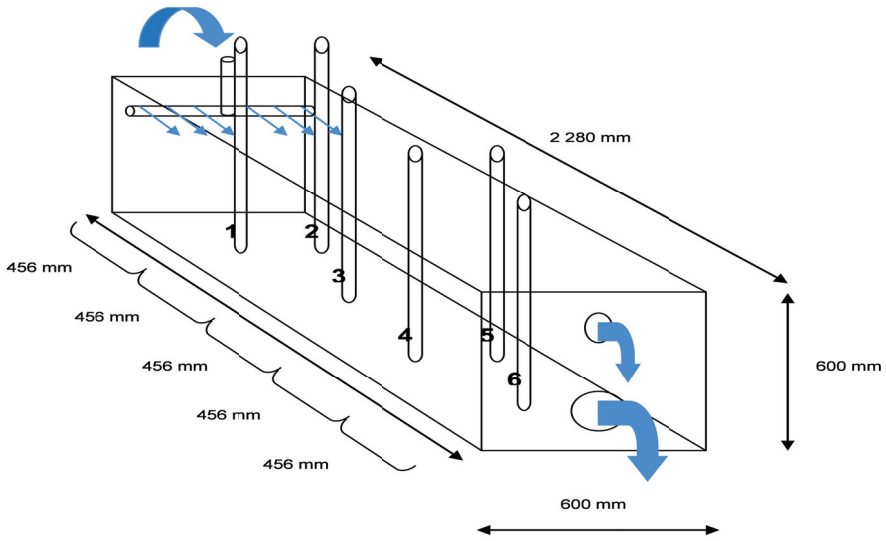

Figure A4 Diagram showing the design of gravel-filled pilot-scale constructed wetlands and the location of the sampling ports (labelled from 1 to 6)

The KBr study relied on the quantification of potassium (K^+) in the samples by Atomic Absorption Spectrometry (AAS) using a Varian spectrometer. The gallic acid tracer study was based on the quantification of gallic acid in centrifuged samples by reverse phase HPLC using a Merck Hitachi Lacrom instrument equipped with an L-7400 UV detector and a Waters Spherisorb® analytical cartridge. The mobile phase consisted of deionised water, methanol and acetic acid (80:20:2.5). The wavelength, flow rate and time were set at 280 nm, 0.5 $ml.min^{-1}$ and 60 min, respectively.

A2.3 METAGONOMIC ANALYSIS OF THE MICROBIAL COMMUNITY STRUCTURE IN A GRAVEL-FILLED PILOT SCALE CONSTRUCTED WETLAND

A2.3.1 Sample collection

For DNA extraction surface (S) soil/mud samples (0-2cm in deep) were collected with a sterile spatula, while deep (D) samples (approximately 20 cm below the surface) were composed of muddy water and were collected with a sterile 10 ml syringe. All samples were stored in sterile 2 ml screw capped Eppendorf tubes. Collected samples were placed on ice during transport to the laboratory and stored at -80°C within 2 hrs of sampling. Unless otherwise stated, samples (surface and deep) were collected at three transverse points along the wetland (Figure A5.). Four separate samples were collected at each sampling time.

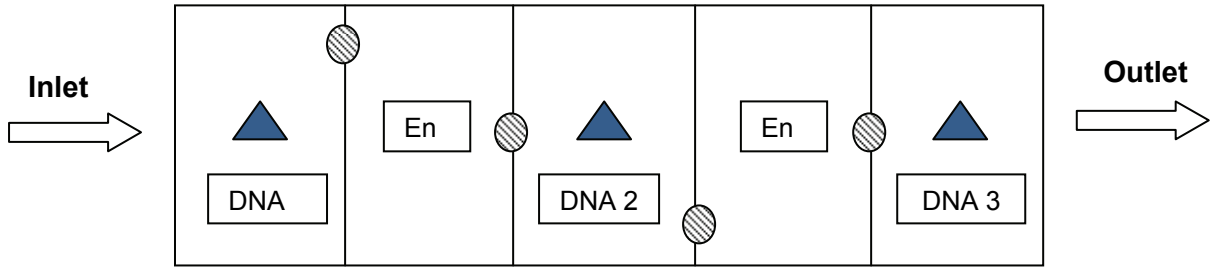

Figure A5 Schematic representation of a gravel-filled pilot-scale constructed wetland showing the sample sites for water- and enzyme analysis, and DNA extraction. Key: triangle, aurum lilies; hatched circle, samples collected from ports for water analysis; DNA (1-3), sediment collected for DNA extraction; Enz, sediment collected for enzyme analysis.

A2.3.2 Extraction of metagenomic DNA

Genomic DNA was extracted using a modified version of the method described by Wang *et al.* (1996). Soil samples were thawed on ice – approximately 500 mg of sediment (surface samples) or 500 µl of muddy water (deep samples). The depth samples were spun at 15000 x *g* for 2 min to remove excess water. The soil was re-suspended in 1 ml lysis buffer (25 mM Tris-HCl pH 8; 50 mM glucose; 10 mM EDTA; 25 mg lysozyme per ml) and 0.5 g quartz sand (Sigma S-9887) was added to each tube. Microbial cells were lysed by both mechanical and chemical methods. Mechanical shearing involved three cycles of vortexing at maximum speed for 1 min followed by rapid cooling on ice for 2 min. After vortexing the samples were incubated in lysis buffer overnight at 37°C. SDS was added to a final concentration of 1% and the samples were incubated at 65°C for 30 min. Samples were extracted twice with 1 vol equilibrated phenol (pH 7.6), followed by extraction with 1 vol chloroform:iso-amyl alcohol (24:1, vol/vol). Nucleic acid was precipitated with 1 vol ice-cold isopropanol at room temperature and harvested by centrifugation at 14 000 X g for 5 min. The resulting pellet was re-suspended in 50 µl 10 mM Tris-HCl; 1 mM EDTA (TE) buffer pH 7.8.

A2.3.3 Purification of DNA using self-constructed polyvinylpyrrolidone mini-columns

*S*elf-constructed polyvinylpyrrolidone (PVPP) mini-columns were used to purify DNA samples and remove humic substances. Columns were constructed using sterile P100 filter tips cut off approximately 2 mm below the filter. A suspension was prepared by re-suspending PVPP (100 g/L) in TE buffer and autoclaving. One hundred and fifty microlitres (150 µl) of the suspension was applied to the filter tip placed inside a 0.6 ml Eppendorf tube

and spun at 200 x g for 2 min. The flow-through is discarded and a second 150 µl aliquot of PVPP was applied and spun as before. The column was washed twice with 125 µl TE buffer (centrifuged as above). Columns were dried by centrifugation at 600 x g for 10 min. DNA (~20 µl) was loaded on the column and incubated at room temperature for 2 min. The DNA was eluted by centrifugation at 600 x g for 5 min, followed by 1700 x g for 10 min. The concentration of the eluted DNA was determined using a NanoDrop ND-100 spectrophotometer and DNA was diluted to a concentration of 10 ng/µl with sterile water. DNA samples were stored at stored at 4°C.

A2.3.4 PCR amplification with 16S rRNA gene and 16S rRNA gene-DGGE primers

PCR was carried out in 50 µl reaction volumes. Each reaction contained 1 X PCR buffer, 1.25 U Dream*Taq*™(Fermentas, USA) polymerase, 200 µM of each dNTP, 0.5 µM of each primer (1.25µM for primers A340F-GC and A533R), 0.1% BSA and approximately 20 ng metagenomic DNA. The PCR primers and amplification conditions are given in Table A1. PCR products amplified with the E9F/U1510R andA3Fa/Ab927R prime r combinations were electrophoresed on 1% agarose gels, while all nested PCR products were electrophoresed on 3% agarose gels. All gels contained 10 µg/ml ethidium bromide and were electrophoresed in 0.5 X TAE buffer (Sambrook *et al*., 1989) at 100 V for 1 h and visualized on an AlphaImager 3400 imaging system.

A2.3.*5* Denaturing gradient gel electrophoresis (DGGE) analysis

PCR amplicons obtained with the three nested primer sets (341F-GC/534R; A340F-GC/A533R and M340F-GC/M707R) were analysed by denaturing gradient gel electrophoresis (DGGE). Amplicons were separated on 16.5/16.5 cm, 1 mm 9% (wt/vol) polyacrylamide (37.5:1 acrylamide:bisacrylamide) gels with varying denaturing gradients (100% denaturant was 7 M urea and 40% (vol/vol) formamide). Gels were prepared using a Biorad gradient former and were cast according to manufacturer's specifications. Electrophoresis was performed using the DCode DGGE system (Biorad) and was carried out at 100 V for 16 hrs at 60°C (1600 Vh) in 1 X TAE buffer. Gels were stained in 0.5 µg/ml EtBr in 1 x TAE for 20 min and visualised on an AlphaImager 3400 imaging system. Bands of interested were excised from the gel and the gel fragments were soaked in 50 µl sterile water for ~16 hrs at 4°C. Eluted DNA was used as the template in a second PCR reaction (using the DGGE primers) to re-amplify the desired amplicon. The amplicons were rerun on a second DGGE to ensure the correct fragment had been obtained and cloned into *E. coli* as described below.

A2.3.6. Cloning of PCR-amplified DNA fragments

PCR amplicons were purified with an Illustra™ GFX PCR DNA and Gel Band Purification kit and cloned into pGEM®-T Easy (Promega) according to the manufacturer's instructions. Ligation reactions were desalted by drop-dialysis for 15 min with sterile water and reactions were transformed into electrocompetent *E. coli* GeneHogs™ (Invitrogen) using a BioRad Gene Pulser. Transformants were selected by blue-white screening on LA/ampicillin/IPTG/X-gal plates according to standard methods (Sambrook *et al*., 1989). All putative recombinant clones were screened by colony PCR using universal M13 primers (Table A1). Plasmid DNA was extracted using the silica mini-prep method described by Brown (1997). DNA sequencing was done by the DNA Sequencing Unit, University of Stellenbosch.

A2.3.7 16S rRNA gene sequence analysis

Chromatograms were edited in Chromas and the sequences were assembled in DNAMAN version 4.13 (Lynnon BioSoft). Local alignments were obtained by performing a standard nucleotide-nucleotide BLAST search (Altschul *et al*., 1997) of the GenBank database. Sequences were aligned using CLUSTAL_X, version 1.81 (Thompson *et al*., 1997) and checked manually for errors. Phylogenetic analyses were performed using MEGA version 3.1 (Kumar *et al*., 2004) and neighbour-joining (Saitou and Nei, 1987) trees were constructed. The robustness of the tree topology was evaluated by bootstrap analysis (Felsenstein, 1985) based on 1000 resamplings. Putative chimeric sequences were identified using the software of the Ribosomal Database Project II website (Cole *et al*., 2003).

Table A1 PCR primers sequences and cycling conditions used for the metagenomic analysis of the microbial community structure in a gravel-filled pilot-scale constructed wetland

Primer	Primer sequence	Target	Amplification conditions	Reference
E9F U1510R	GAGTTTGATCCTGGCTCAG GGTTACCTTGTTACGACTT	Most *Eubacteria*	94°C for 4 min 30 cycles – 94°C for 30 sec; 52°C for 30 sec; 72°C for 105 sec 72°C for 10 min	Hansen *et al*., 1998; Reysenbach and Pace, 1995
341F-*GC 534R	CCTACGGGAGGCAGCAG ATTACCGCGGCTGCTG	Most *Eubacteria* – used in nested PCR in combination with E9F/U1510R	94°C for 4 min 20 cycles – 94°C for 45 sec; 65°C for 45 sec; 72°C for 60 sec Additional 20 cycles -94°C for 30 sec; 55°C for 30 sec; 72°C for 60 sec 72°C for 10 min	Muyzer *et al*., 1993

Primer	Primer sequence	Target	Amplification conditions	Reference
A3Fa Ab927R	TCCGGTTGATCCYGCCGG CCCGCCAATTCCTTTAAGTTT C	Most *Archaea*	94°C for 4 min 20 cycles – 94°C for 45 sec; 55°C for 45 sec; 72°C for 75 sec Additional 10 cycles – 94°C for 30 sec; 55°C for 30 sec; 72°C for 75 sec 72°C for 10 min	Giovannoni *et al*., 1988; Mclnerney *et al*., 1995 (modified); Jurgens *et al*., 1997
A340F-*GC A533R	CCCTACGGGGYGCASCAG TTACCGCGGCKGCTG	Most *Archaea* – used in nested PCR in combination with A3Fa/Ab927R	94°C for 4 min 20 cycles – 94°C for 30 sec; 53°C for 30 sec; 72°C for 60 sec 72°C for 10 min	Øvreås *et al*., 1997
M340F-*GC M707R	CCTACGGGCGCAGCAG GGATTACARGATTTCAC	Most methanogenic *Archaea* – used in nested PCR in combination with A3Fa/Ab927R	94°C for 4 min 35 cycles – 94°C for 30 sec; 53°C for 45 sec; 72°C for 60 sec 72°C for 10 min	Watanabe *et al.,* 2004
M13Fwd M13Rev	CCCAGTCACGACGTTGTAAA ACG AGCGGATAACAATTTCACACA GG	Cloning vector pGem-T Easy	94°C for 4 min 35 cycles – 94°C for 30 s; 64°C for 30 s; 72°C for 90 s 72°C for 10 min	

*GC – For DGGE analysis a 40mer GC clamp was added to the 5' ends of the forward primers 341F, A340F and M340F. GC clamp – CGCCCGCCGCGCGCGGCGGGCGGGGCGGGGGCACGGGGGG. Primer binding sites corresponds to *E. coli* 16S rRNA gene numbering.

A3 RESULTS AND DISCUSSION

A3.1 KBR TRACER STUDY TO DETERMINE FLOW PATHS IN GRAVEL-FILLED PILOT SCALE CONSTRUCTED WETLANDS

Potassium bromide (KBr) was utilized as tracer, since it is reported to be non-reactive, relatively easy to analyse and widely used to label water flow in environmental studies, including studies of constructed wetlands (Ronkanen and Kløve, 2007; Muñoz *et al*., 2006; Maloszewski *et al*., 2006). The K^+ concentration naturally occurring in the tap water was measured and was found to be highly variable, complicating the creation of a single baseline. Thus 19 baselines were established, one for each sample port, including the outlet. However, even some of these individual baselines gave negative values when deducting the background K^+ concentration and consequently, it was decided to set the negative results to zero.

The samples collected from sample points at the surface showed very low concentrations of K^+ (average 1.05 mg/L) and indicated no clear trend in flow. On average, the samples taken

from the middle section, showed slightly higher concentrations of K^+ (average 1.56 mg/L), but again no definite flow paths were discernable. The much higher average concentration of K^+ detected in samples from the deep ports (184.15 mg/L) indicated that most of the flow occurred at the bottom of the CW. At all ports, maximal KBr concentrations were found during the first ten hours.

In the outlet samples, the tracer concentration rose to a maximum of 4.7 mg/L K^+/L after 26 hours, decreased to a local minimum of 2.41 mg/l after 74 hrs and then increased again until the end of the experiment (Figure A6). At that time (131 hrs), there was still a high tracer concentration detected in the deep samples from ports 4, 5 and 6. In order to ascertain how much tracer remained in the gravel matrix at the end of the study, a mass balance was carried out using the outlet data measurements. The total mass of tracer in the effluent was estimated using the area under the tracer curve (Figure A6), which was calculated using equation 3.

$$\int_0^{131} C\,dt = 641.66\ \frac{mg \cdot h}{l} \qquad \text{(Eq. 3)}$$

With a flow rate of 25 L/h, the total mass of tracer detected in the effluent was 16.04 g, which represented approximately half of the total mass of tracer injected into the CW (35 g KBr).

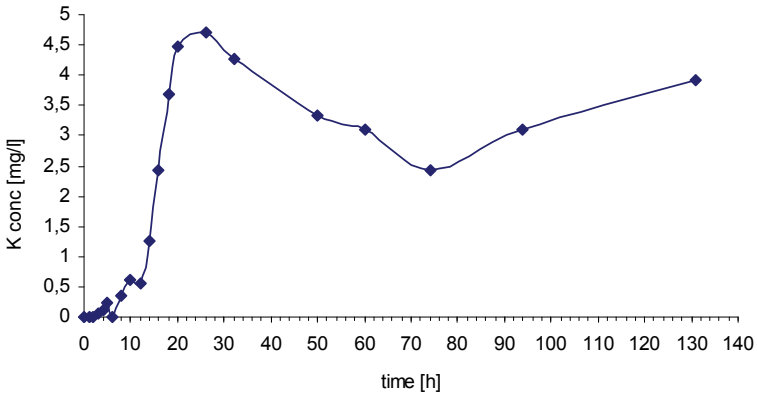

Figure A6 The amount of KBr (K^+) measured in the effluent of a gravel-filled pilot scale constructed wetland (outlet) during the course of a tracer experiment

These findings were supported by the progression displayed in graph in Figure A7, which indicated by extrapolation that under the same experimental conditions, KBr concentrations at sample ports 4, 5 and 6 would not reach zero within the next 190 hours.

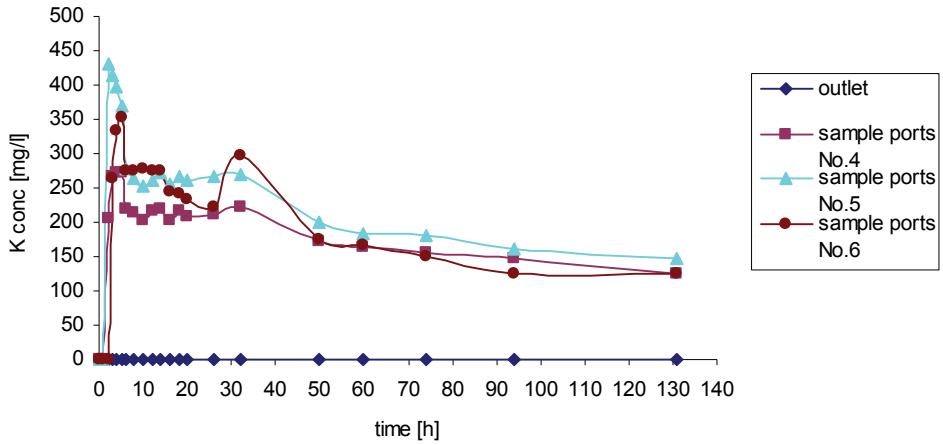

Figure A7 The amount of KBr (K^+) measured in the deep samples of a gravel-filled pilot scale constructed wetland taken from ports 4, 5 and 6 during the course of a tracer experiment

To obtain a general idea of how the tracer spread at the bottom of the CW, mass balances were performed for all sample ports of this layer (Figure A8). The mass of KBr (K^+) measured at each sample port was significantly higher than the tracer mass actually applied.

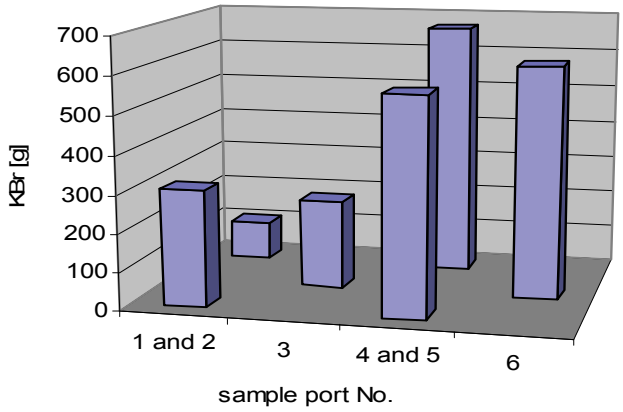

Figure A8 3-D diagram of mass balances of KBr (K^+) measured in the deep samples of a gravel-filled pilot scale constructed wetland during the course of a tracer experiment

A3.2 GALLIC ACID TRACER STUDY TO DETERMINE FLOW PATHS IN GRAVEL-FILLED PILOT SCALE CONSTRUCTED WETLANDS

Gallic acid was chosen to represent a phenolic found in a variety of agricultural wastewaters. The intention was to compare a phenol (gallic acid) to a salt (KBr) with regard to the hydraulic retention time and the flow properties in the wetland. Furthermore, rudimentary microbial cultures were performed to ascertain whether biotic degradation of gallic acid was possible in the CW (data not given). The gallic acid displayed a similar flow pattern to the KBr study. However, at the second study, no gallic acid was detected found in any samples and after 72 hours, the experiment was considered to be complete (Figure A9).

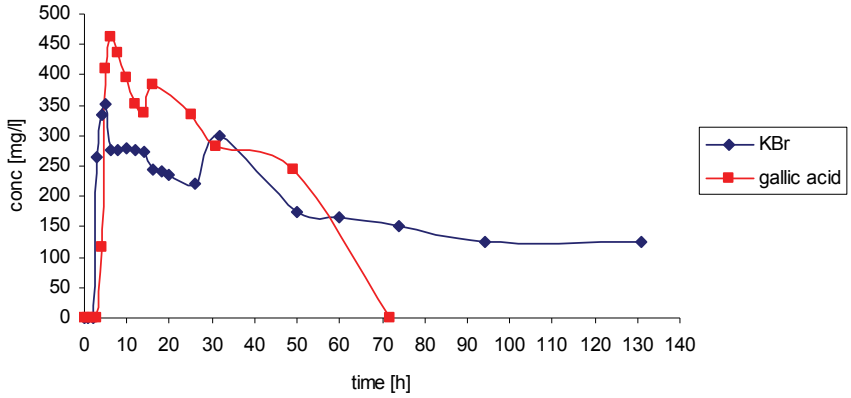

Figure A9 Progression of KBr and gallic acid concentration from deep samples taken from sample port no 6 of a gravel-filled pilot scale constructed wetland during the course of a tracer experiment

As described using KBr, the major portion of the tracer was detected in the bottom of the CW for the duration of the experiment, the highest concentration being detected at sample port 1 (Figure A10).

The fact that almost 100% of KBr was detected at the bottom of the CW was unexpected. Similar results were reported by Drizo *et al.* (1999), where no surface flow occurred and 66% of the Br^- tracer travelled through the bottom of the wetland (depth: 0.35 m). The authors explained this as arising from preferential flow paths with the location of the inlet pipe (0.2 m depth) or density gradients being postulated as the cause. In contrast, Headly *et al.* (2005) found that vertical mixing prevented depth preferences by LiCl, despite that fact that the inlet was located at a depth of 0.83 m. Most papers do not report on tracer depth distribution, only sampling from the surface (Ronkanen *et al.,* 2007) or from the outlet (Martinez and Wise, 2003) of the wetland.

According to Levenspiel (1999), the progression of tracer from a high maximum concentration followed by a long tail of relatively low concentrations signifies localized stagnation. As summarized by Holland *et al*. (2004) and many other authors, an ideal wetland flow behaviour can be described as plug-flow, in which the water runs uniformly and without dispersion from inlet to outlet. With plug-flow, if a tracer pulse is added, all of the tracer will exit the wetland at the same time, i.e. the ideal wetland has a single residence time. Since during both tracer studies, no single hydraulic retention time was observed, it could be concluded that the constructed wetland did not show ideal flow behaviour. According to the literature non-ideal flow properties are described more precisely with a retention time distribution (RTD) which comprises different flow paths and back mixing (Levenspiel, 1999).

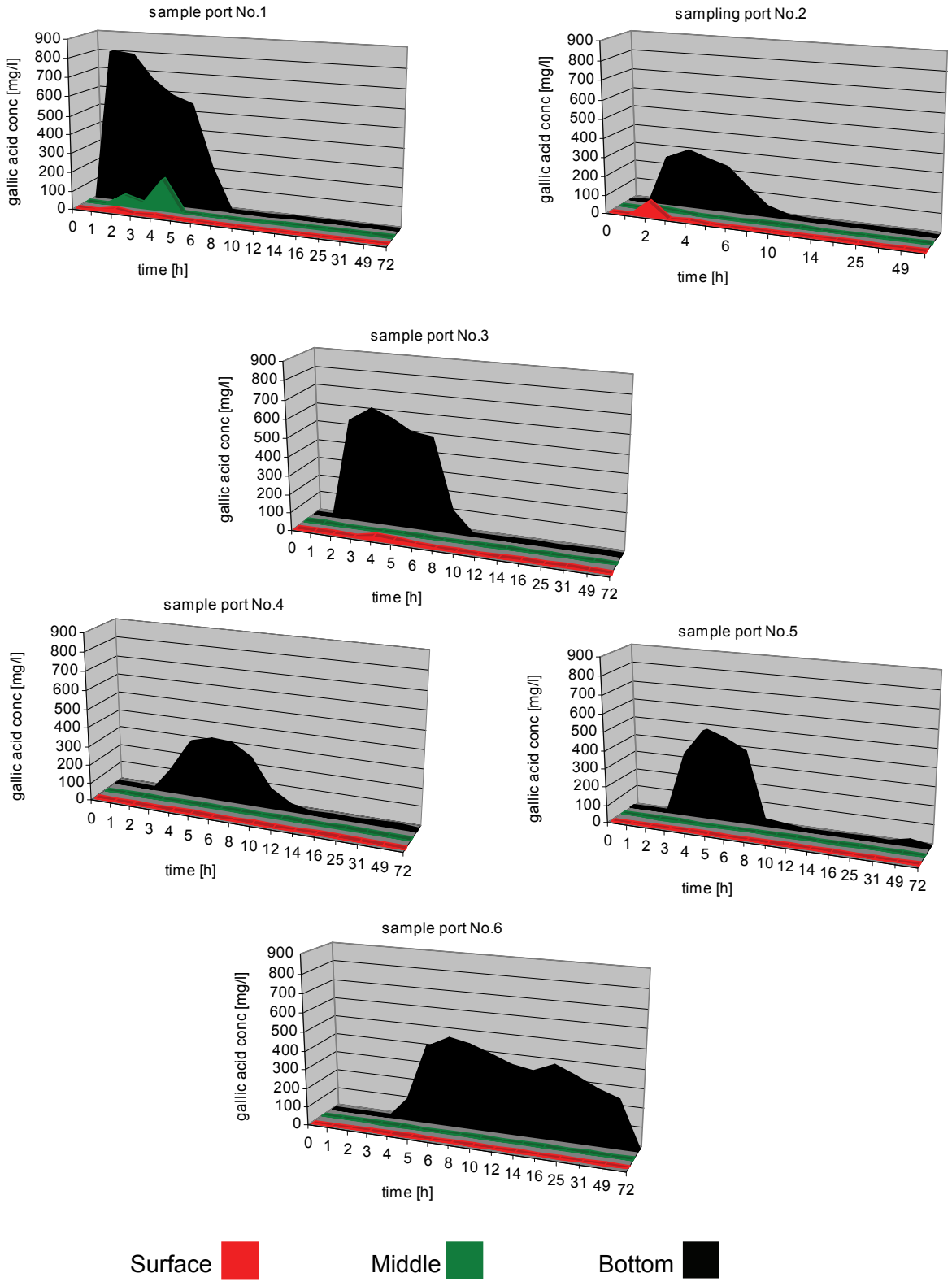

Figure A10 Distribution of gallic acid at all sample ports and sample depths of a gravel-filled pilot scale constructed wetland during the course of a tracer experiment

In the mass balances calculated using the individual sample port data, the calculated amount of tracers exceeded by far the injected mass. This discrepancy led to the presumption that in the bottom layer the degree of back mixing was very high, especially in the second half of the CW, where the level of the tracer concentration decreased only very slowly. Several factors have been linked with the presence of back mixing. Headly *et al*. (2005) associated the Reynolds number for flow through porous media with laminar and turbulent flow in wetlands. Using this premise, Holland *et al*. (2004) suggested that mixing mechanisms may be dominated by turbulent diffusions at higher flow rates. However, during the tracer study, a flow rate of 25 L/h was maintained, and the possibility of turbulent flow seemed doubtful. The incomplete mass balance from the outlet sampling indicated that only approx. 45% of the total tracer mass had left the wetland after 131 hours. This result was no surprise since at sample port No.4, 5 and 6 high KBr concentrations were still measured at the end of the tracer study.

It is noted that KBr is considered to be quite a conservative tracer when used in wetlands. Muñoz *et al*. (2006) and Matamoros *et al*. (2005) reported a KBr mass recovery of 48-186% and 100-108%, respectively. The latter discrepancy of 8% was attributed to evapotranspiration. Besides the always present analytical uncertainties, incomplete recovery of tracers can be explained by many reasons, such as (i) accumulation of tracer in the gravel matrix during the passage through the wetland, (ii) limited representativeness of data due to low sampling frequency or duration, resulting in bypassing of information and tracer curve interpolations, (iii) tracer concentrations which exceed the limit of detection, e.g. in the tailing part of the tracer curve (Małoszewski *et al.* 2006). It has been suggested that long HRTs may benefit treatment performance (Holland *et al*. 2004). However, accumulation of toxins in the CW sediments may have a detrimental effect on biodegradative efficiency (see section B3.4.5.6)

A3.3 MICROBIAL COMMUNITY ANALYSIS FROM SAMPLES TAKEN FROM A GRAVEL-FILLED PILOT SCALE CONSTRUCTED WETLAND

The objectives of this study, using PCR-based molecular phylogenetic techniques, were:

- To monitor the CW microbial populations.
- To gain an overview of the diversity of microorganisms within the wetlands.
- To identify key microorganisms present in the constructed wetlands.

- To monitor microbial populations during the equilibration of the constructed wetlands and to detect fluctuations in these populations during treatment with various chemical compounds.

A3.3.1 Planting and inoculation of gravel-filled pilot-scale constructed wetlands with sediment from a natural wetland impacted by winery effluent

Initially each wetland was inoculated with muddy sediment collected from a natural wetland located on a wine farm that had been seasonally impacted with winery effluent. The sediment was collected at the beginning of May which coincided with the end of the harvest, therefore the endemic microorganism within the sediment were already "primed" by the highly concentrated effluent flowing through the wetland. At this stage it was felt that plants were essential for the health of the constructed wetland – to supply nutrients and provide surfaces microbial attachment. The rhizophere (the interface between the roots and the surrounding soil) in particular is often a biologically active sites. Therefore, Arum lilies, *Zantedeschia aethiopica*, were planted in each wetland during the equilibration of the constructed wetlands (the location of the plants is shown in Figure A5).

Arum lilies were selected for a number of reasons: firstly, they grew along the flow channel of the winery effluent to the natural wetland and would thus be tolerant of concentrated effluent. Secondly, Arum lilies and the Bullrush (*Typha capensis*) are key endemic species in natural wetlands in the Western Cape Province. Thirdly, a previous study has linked Arum lilies to bioremediation processes within a lab scale constructed wetland (Belmont and Metcalfe, 2003).

A3.3.2 Optimization of DNA extraction, purification and PCR

Initially the bead beater method of Miller *et al*., 1999 was used to extract metagenomic DNA, followed by purification with PVPP- and Sephacryl S-500 HR columns (as outlined in previous reports). However, it was found that the yield of intact, high molecular weight DNA was very low using this method, less than 1 µg of DNA per g sediment. Therefore, three additional methods were tested on wetland samples: the inclusion of proteinase K and lysozyme during the lysis step using the Miller method, extraction with TENP buffer (Picard *et al*., 1992) and the chemical lysis method described by Wang *et al*. (1996). The latter method followed by purification with PVPP columns only was found to routinely yield 10 µg of purified DNA per g sediment (wet weight); a tenfold improvement compared to other extraction methods, and was used for all subsequent DNA extractions. As the further purification of PVPP purified DNA on Sephacryl columns resulted in a loss of DNA without any improvement in the purity, as assessed by the OD_{260nm}/ OD_{280nm} ratio this step was

excluded for the extraction process. While the yields of DNA from the surface samples were generally higher than the depth samples, this may be a reflection of the different sample types, not the size of microbial populations.

Initially, the success rate for PCR was approximately 40%. This was attributed to two factors. Firstly, in the equilibration phase (the first three months post inoculation) the microbial populations were relatively small as assessed by colony plate counts and low yields of DNA. However, once the microbial community was stabilised, the yields of good quality, high molecular weight DNA increased. Secondly, PVPP-purified genomic extractions were still slightly coloured and probably still contained humic substances, which are potent inhibitors of PCR. Therefore a range of PCR additives were tested (glycerol, betaine and BSA). Mills *et al.* (2003) reported that BSA at final concentration of 0.1% allowed for the amplification of DNA samples containing humic compounds. The inclusion of BSA was found to improve PCR amplification and was therefore routinely included in all PCR reactions. PCR with 16S rRNA gene primers can also be inhibited by high template DNA concentrations. Generally, it was found that PCR with 20 ng of template DNA amplified better than reactions containing greater than 50 ng DNA. Therefore, all DNA extractions were routinely diluted to10 ng/µl with sterile water.

A3.3.3 Optimization of the microbial community fingerprinting technique

Denaturing gradient gel electrophoresis (DGGE) was used to monitor the establishment of the microbial populations within the wetland, as well as the effects of feeding, increased flow rate and the addition of plants on these populations. During the equilibration phase, samples were collected every two weeks and analysed by DGGE. Based on these findings it appeared to take the microbial community approximately three months to stabilize and most of the dominant phylotypes were not overtly affected by subsequent treatment. Although five phylotypes were potentially introduced by the addition of the plants, only one (cloned plasmid 16) appeared to form a dominant member of the microbial population. Interestingly, sequence analysis of the GenBank database revealed that plasmid 16 was 96% similar to an uncultured clone from the rhizopheric soil of a wetland used to treat winery effluent.

From whole community analysis by DGGE it appeared that only the treatment with gallic acid affected the microbial population and unless stated otherwise all the results discussed in this report were obtained from sediment samples collected during this experiment.

Before DGGE can be employed to monitor temporal and spatial fluctuations in microbial communities it is essential to firstly ensure the reproducibility of the method. Several wetland samples, both surface and depth, were selected and duplicate DNA extractions (referred to

as DNA extractions A and B) were performed. PCR was carried out on all samples and the resulting amplicons were separated by DGGE. As PCR itself may introduce bias, due to slight differences in template DNA, cycling conditions and PCR components, two separate PCR reactions were set up for the DNA extractions B. From Figure A11 one can see that the banding patterns obtained for the each samples A and B DNA extractions were similar. This confirmed that the soil samples were homogenous and the micro-heterogeneity (the difference between replicate samples) was negligible. The products in lanes 2and3, 5and6, 8and9, 11and12 were obtained for duplicate B DNA extractions and the banding patterns were similar. This showed that the results were reproducible and that any differences in banding patterns were likely not due heterogeneity introduced by PCR.

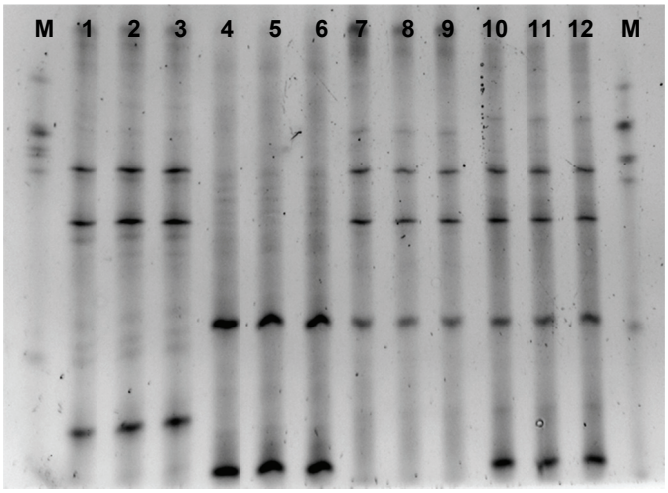

Figure A11 DGGE gel (gradient 40-60%) of partial 16S rRNA genes amplified with *Eubacteria* specific primers (340F-GC/A534R) from DNA extracts of samples taken from gravel-filled pilot-scale constructed wetlands

M, marker; 1, 3-1D-7 DNA extraction A; 2, 3-1D-7 DNA extraction B (duplicate extraction); 3, 3-1D-7 DNA extraction B (duplicate PCR reaction); 4, 72-1D-2 DNA extraction A; 5, 72-1D-2 DNA extraction B (duplicate extractions); 6, 72-1D-2 DNA extraction B (duplicate PCR reaction); 7, 72-1S-1 DNA extraction A; 8, 72-1S-1 DNA extraction B (duplicate extractions); 9, 72-1S-1 DNA extraction B (duplicate PCR reaction); 10, 72-1D-2 DNA extraction A; 11, 72-1D-2 DNA extraction B (duplicate extractions); 12, 72-1D-2 DNA extraction B (duplicate PCR reaction).

A3.3.4 DGGE analysis using Eubacterial-specific primers 341F-GC/534R

An operational taxonomic unit (OTU) is defined as “any unit that is convenient for the required purpose, be it an entire population, a species or a cohort of subspecies” (Rosselló-Mora and Amann, 2001). Therefore, for DGGE analysis one can consider each unique band on a gel to be an OTU. Both the surface and depth samples were found to be highly diverse (many OTUs). Generally, the presence of an OTU was consistent throughout the course of

an experiment with only the intensity of a band varying. However, one must be cautious when interrupting DGGE results as changes in band intensity may not be due to changes in the microbial population but rather due to differences in DNA extraction, PCR or sample loading. Therefore, all the phylotypes which were selected as increasing in intensity had to show an increase in more than three consecutive sampling instances. The DNA extraction and PCR amplification was repeated on these samples to confirm the result.

Fourteen phylotypes were identified as being dominant members of the microbial community within the wetland. These bands were all successfully excised from the gel, reamplified and cloned into *E. coli*. Figure A12 is a representative DGGE gel of amplicons generated with the eubacterial primers. Selected phylotypes are marked on the gel.

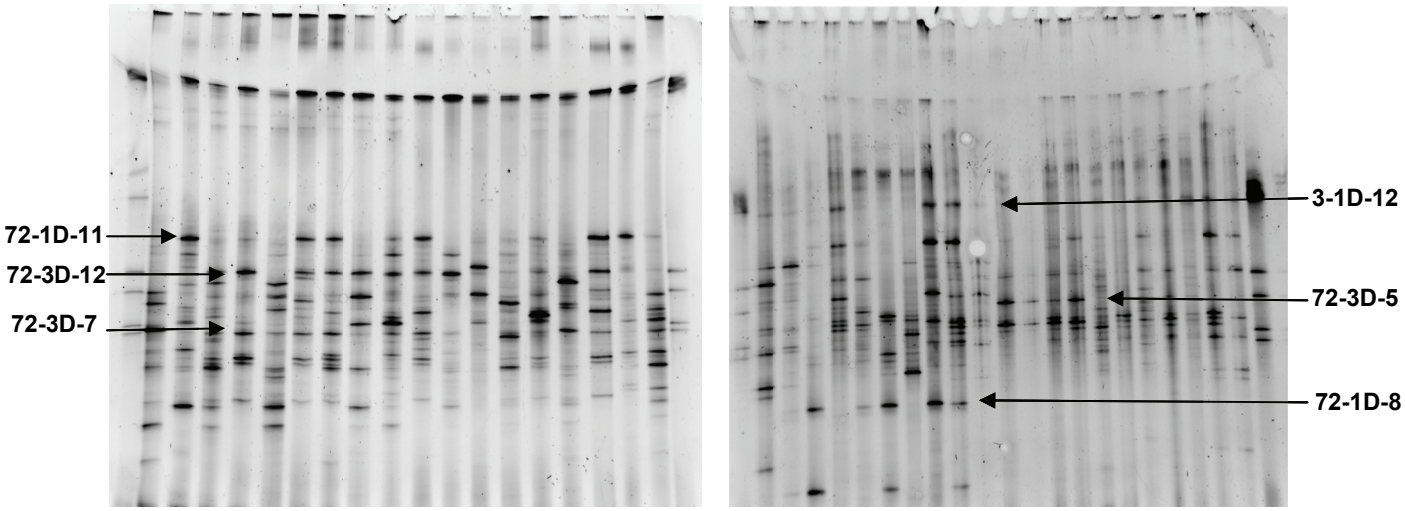

Figure A12 DGGE gel (gradient 40-60%) of partial 16S rRNA genes amplified with *Eubacteria* specific primers (340F-GC/A534R) from DNA extracted from the wetland during treatment with gallic acid

The BLAST results and phylogenetic trees for these 14 phylotypes are represented in Table A2 and Figures A13 and A14.

Seven clones were most closely related to various members of the phylum γ-*Proteobacteria*, predominantly the family *Enterobacteriaceae* (Figure A13). Four clones, 72-3D-1, 72-1D-8, 72-1D-9, 72-3D-12 were most closely related to *Citrobacter* species, with a high sequence identity (99%). *Citrobacter* species are Gram negative coliforms, commonly found in wastewater, soil and the human intestine were they are opportunistic pathogens. *Citrobacter* are obligate anaerobes and all four clones were derived from metagenomic DNA extracted from depth samples. Interestingly, some *Citrobacter freundii* strains produce extracellular

tannases and use tannic acid as a sole C-source. The potential of such strains to be used for the biodegradation of tannic acid in tanneries has been investigated (Kumar *et al*., 1999). Clone 72-1D-11 is most closely related to *Kluyvera ascorbata* and *Citrobacter* species. *Kluyvera* species are facultative anaerobes, belonging to the family *Enterobacteriaceae* (γ-*Proteobacteria*). They ferment sugar and alcohols. While they are reported to be human pathogens, most strains were isolated from food (which may explain their presence in faecal samples). Some *Kluvyera* strains have been found to promote plant growth in nickel-contaminated soil. Some studies have investigated their distribution in the terrestrial environment (Farmer *et al.,* 1981). Similarly, clone 72-3S-4 is most closely related to various *Enterobacter* species. *Enterobacter* are facultative anaerobe (clone 72-3S-4 was generated from metagenomic DNA extracted from a surface sample). Although *Enterobacter* are common commensal of the human digestive tract, they are also found in wastewater and soil.

Four clones were most closely affiliated to four divergent phyla, and have therefore been included in a separate tree (Figure A14). Clone 72-3S-2 is most closely related to acidobacterium species. The phylum *Acidobacteria* includes a number of genera with a single validly published representative, including the genus *Acidobacterium* (the type strain was isolated from acidic mineral soil, Kishimoto *et al.,* 1991), as well as the newly described genus *Edaphobacter* (both members of this genus were isolated from alpine and forest soils, Koch *et al*., 2008). Recently, Hartman *et al*. (2008) reported the dominance of *Acidobacteria* species in acidic wetlands, and the pH of our constructed wetlands was typically between 5 and 7. Clone 3-1D-12 is most closely related to the phylum *Bacteroidetes* which includes the classes *Bacteroidia*, *Flavobacteria* and *Sphingobacteria*. From the BLAST results 3-1D-12 is similar to *Flavobacteria* species (which are widely distributed in soil and freshwater, Bernardet *et al.*, 1996), however as the sequence similarity is low (93%) one cannot confidently assign this clone to a taxonomic group. Clone 72-3D-5 is most closely related to members of the genus *Chloroflexus.* The phylum *Chloroflexi* is a deep branching lineage of *Bacteria*. *Chloroflexi* are phototrophic, filamentous bacteria found in freshwater habitats and were formerly referred to as green non-sulphur bacteria. They can grow under both aerobic and anaerobic conditions, but do not produce oxygen during photosynthesis. Clone 3-1S-3 is most closely related to the genus *Burkholderia*, a group of obligately aerobic, Gram negative rods. Although *Burkholderia* species are pathogenic, particularly to horses and livestock, they have been investigated as potential biodegradation and biocontrol agents. Some species promote the growth of rhizobacteria (Mallik and Williams, 2008). Four clones 3-1D-1, 72-3D-4, 72-3S-9 and 72-3D-11 could not be included in any phylogenetic tree as there sequences were too divergent to align with ClustalX. They are included in Table A2.

Initially, eight phylotypes were identified where the intensity of band appeared to increase during the course of the gallic acid feeding trial (Table A3 and Figure A15). Only six DNA fragments could be successfully excised from a DGGE gel and reamplified to obtain a single band on a second DGGE gel. From Figure A15 one can see that although from BLAST analysis the clones are most closely related to γ-*Proteobacteria,* they form a separate clade. Members of the class *Actinobacteria* and *Deltraproteobacter* species were also included in the BLAST analysis and have therefore also been included in the tree. The sequence identities for all six clones were under 90%. Therefore, any relationships inferred from the phylogenetic tree would be purely speculative.

Table A2 BLAST results of the sequences of the 16S rDNA fragments recovered for the dominant phylotypes identified from a DGGE gel. DNA fragments were amplified with Eubacterial specific primers

Clone	Best Hit	% identity/ number of bp	Source	GenBank accession number	Closest bacterial species[a]	Source	% identity/ number of bp	GenBank accession number
72-3S-4	Uncultured bacterium clone ERF-1G5	99%/329	Rhizosphere	DQ906059	*Enterobacter cloacae* strain FR	Paper mill wastewater	99%/329	EU849019
3-1D-1	Uncul. actinobacterium	88%/324	Anoxic basin at a wastewater treatment plant	CU466695	Uncul. *Clostridium* sp.	Compost bioreactor core sample	84%/319	EU921200
3-1D-2	Uncul. bacterium	86%/320	Rhizosphere biofilm in a reed bed reactor	AB240350				
72-3D-4	Uncul. *Raoultella* sp. isolate BF25	90%/334	Biofilm on the surface of carrier used for treating wastewater	DQ839331	*Citrobacter freundii* strain 6			DQ444289
3-1S-3	Uncul. proteobacterium clone Amb_16S_435	97%/365	Trembling aspen rhizosphere	EF018102	*Burkholderia* sp. B7		96%/365	AY752916
3-1D-12	Uncul. bacterium clone Kas178B	94%/378	Sediment of Lake Kastoria, Greece	EF203208	Bacteroidetes bacterium Mo-0.2plat-K3	Freshwater bacterioplankton, Lake Mondsee	93%/378	AJ622888
72-1D-8	Uncultured *Citrobacter* sp. clone ASP-42	99%/359	Microbial fuel cell	EF679196	*Citrobacter freundii* strain YRL11	Radish leaf endophyte	99%/357	EU373418
72-3S-9	Uncultured bacterium clone cs71	89%/324	Shuangcheng moat sediment	DQ444169	*Klebsiella* sp. HBB9	Grapevine juice	89%/324	EU074057
72-3D-7	Uncultured bacterium clone 3.5.11	97%/357	Kentucky Lake, mudflat sediment	EU528229	*Citrobacter* sp. genomospecies 11		99%/357	AF025369
72-1D-9	Uncultured bacterium clone 3.5.11	99%/357	Kentucky Lake, mudflat sediment	EU528229	*Citrobacter* sp. genomospecies 11		99%/357	AF025369
72-3D-1	Uncultured bacterium clone CE2_g10	99%/354	Cheetah faeces	EU467996	*Citrobacter braakii*		99%/354	AF025368

Table A2 continued……

Clone	Best Hit	% identity/ number of bp	Source	GenBank accession number	Closest bacterial species[a]	Source	% identity/ number of bp	GenBank accession number
72-3D-5	Uncultured bacterium clone LaP15L05	98%/337	Anoxic river sediment	EF667640	Uncultured *Chloroflexi* bacterium clone ELC_30_7_bac	Gold mine tailings	97%/337	EF464632
72-3D-11	Uncultured *Klebsiella* sp. clone IITR RCP28	86%/317	Rhizospheric soil in the wetland ecosystem constructed to treat distillery effluent	FJ268989	*Enterobacter* sp. WAB1929	Tiete River water	86%/317	AM184268
72-3D-12	Uncultured bacterium clone 3.5.11	99%/357	Kentucky Lake, mudflat sediment	EU528229	*Citrobacter* sp. 'genomospecies 11'			AF025369

Table A3 BLAST results of the sequences of the 16S rDNA fragments recovered for the phylotypes which increased in intensity over time. DNA fragments were amplified with *Eubacteria*-specific primers

Clone	Best Hit	% identity/ number of bp	Source	GenBank accession number	Closest bacterial species[a]	Source	% identity/ number of bp	GenBank accession number
72-3S-7	Uncul. bacterium clone L2B_028	88%/330	Sulfate-reducing bioreactor treating mine drainage	EF551852	*Enterobacter aerogenes*		87%/373	AF395913
72-1D-10	Uncultured bacterium clone B12	88%/373	Biofilm reactor used to treat dye wastewater	EF655641	*Enterobacter aerogenes*		87%/373	AF395913
72-3D-3	Uncultured bacterium clone f6s1	89%/334	*Myrmeleon mobilis* (ant lion) GI tract	DQ068815	*Enterobacter aerogenes*		88%/334	AF395913
72-3D-8	Uncultured bacterium clone C80	89%/324	EBPR activated sludge system	FJ356046	*Citrobacter freundii*	Larvae of *Myrmeleon* bore	89%/324	AB244451
72-3D-1	Uncul. bacterium clone B11	89%/320	Biofilm reactor used to treat dye wastewater	EF655626	*Citrobacter werkmanii*[l] CDC 0876-58		89%/320	AF025373
72-3S-2	Uncultured bacterium clone s2s12	89%/393	*Myrmeleon mobilis* (ant lion) GI tract	DQ068897	*Klebsiella planticola*		88%/394	AF181574

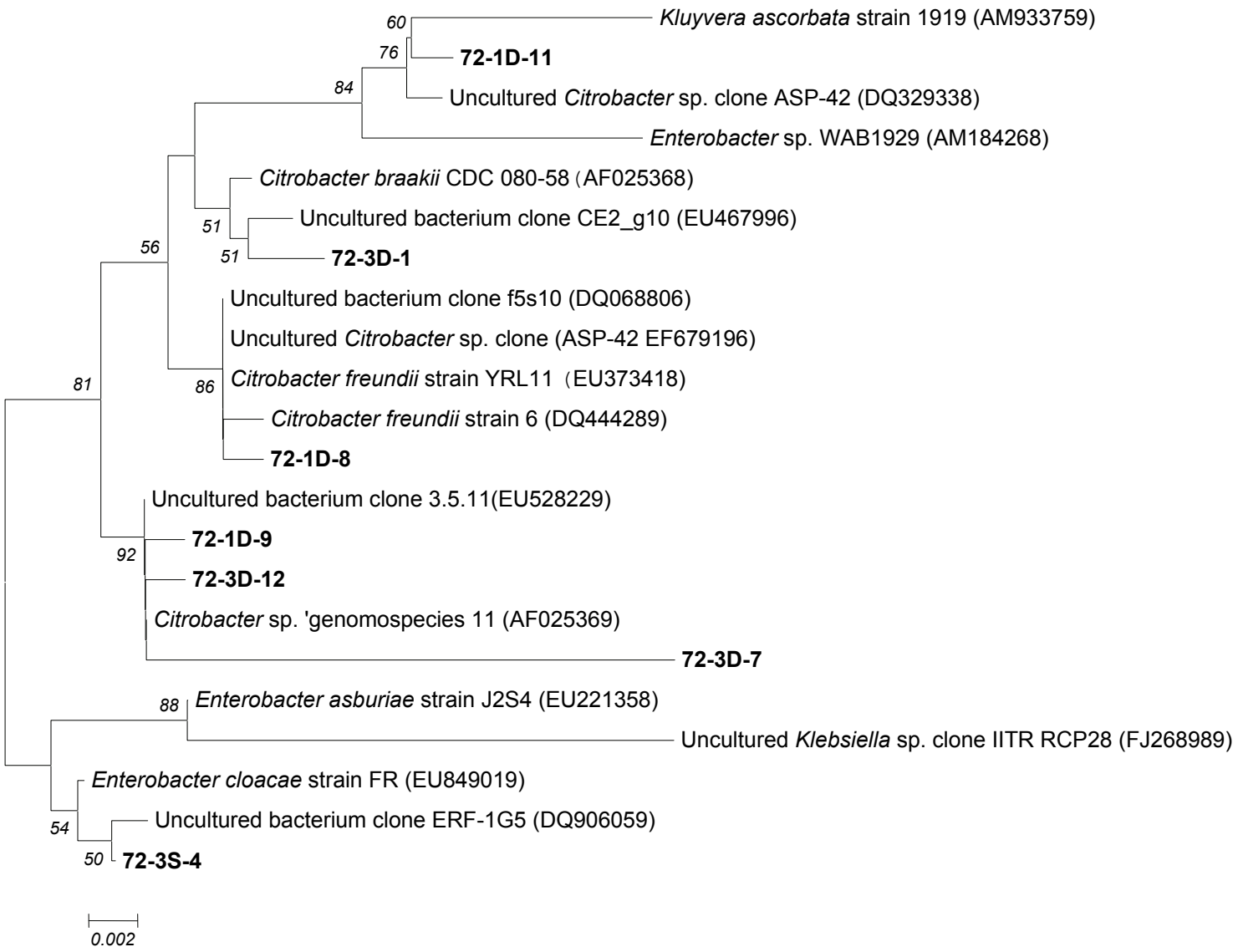

Figure A13 Neighbour-joining phylogenetic tree of partial 16S rRNA gene sequences for dominant *Eubacteria* clones most closely related to members of the family *Enterobacteriaceae*. The tree is based on 180 bp of sequence and was constructed using MEGA version 3. The scale bar represents 0.2% differences in nucleotide sequence. Numbers at nodes represent the bootstrap values (based on 1000 replicates) and only values greater than 50% are shown. Sequences obtained in this study are shown in bold

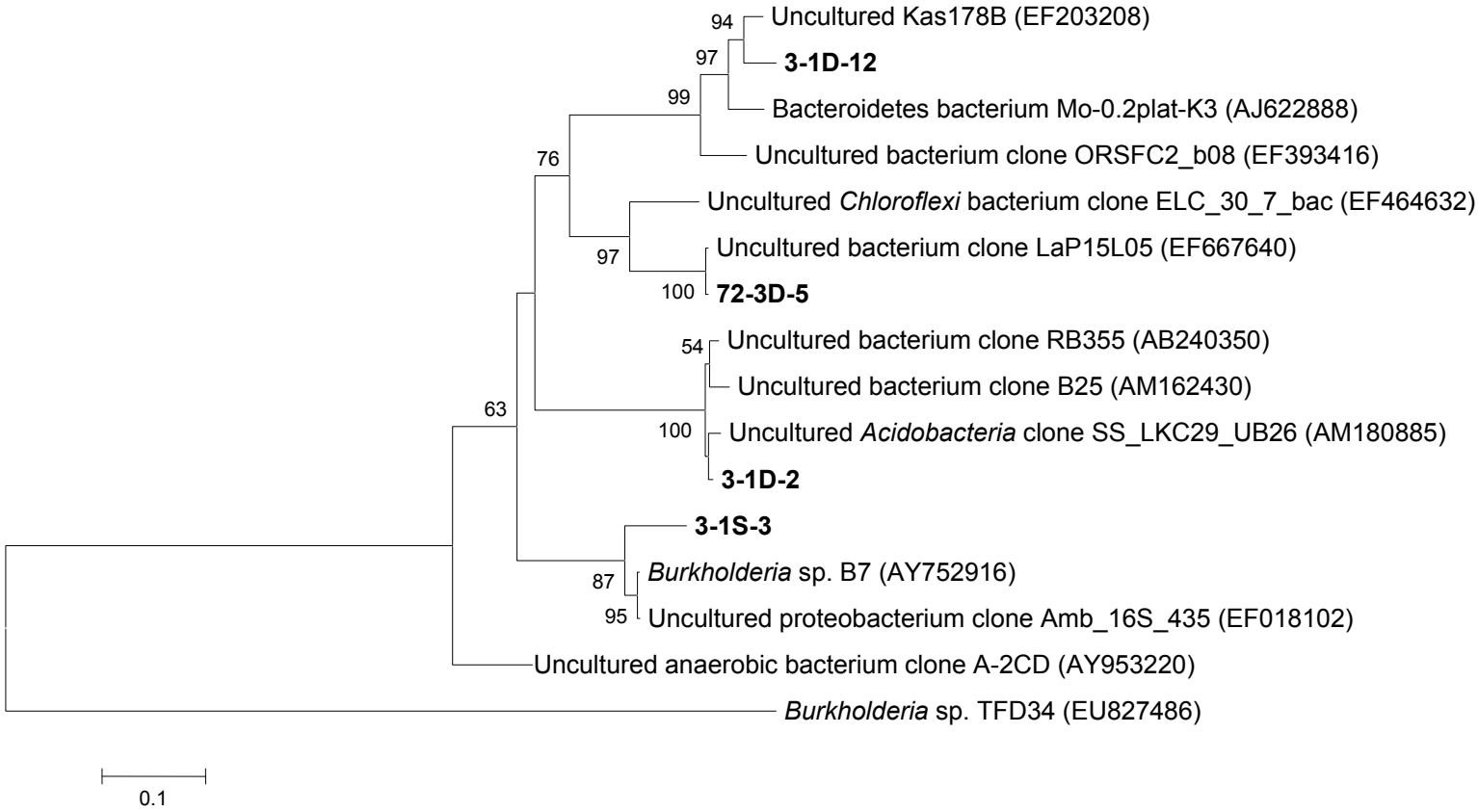

Figure A14 Neighbour-joining phylogenetic tree of partial 16S rRNA gene sequences for dominant *Eubacteria* clones most closely related to phyla other than the γ-*Proteobacteria.* The tree is based on 185 bp of sequence and was constructed using MEGA version 3. The scale bar represents 10% differences in nucleotide sequence. Numbers at nodes represent the bootstrap values (based on 1000 replicates) and only values greater than 50% are shown. Sequences obtained in this study are shown in bold.

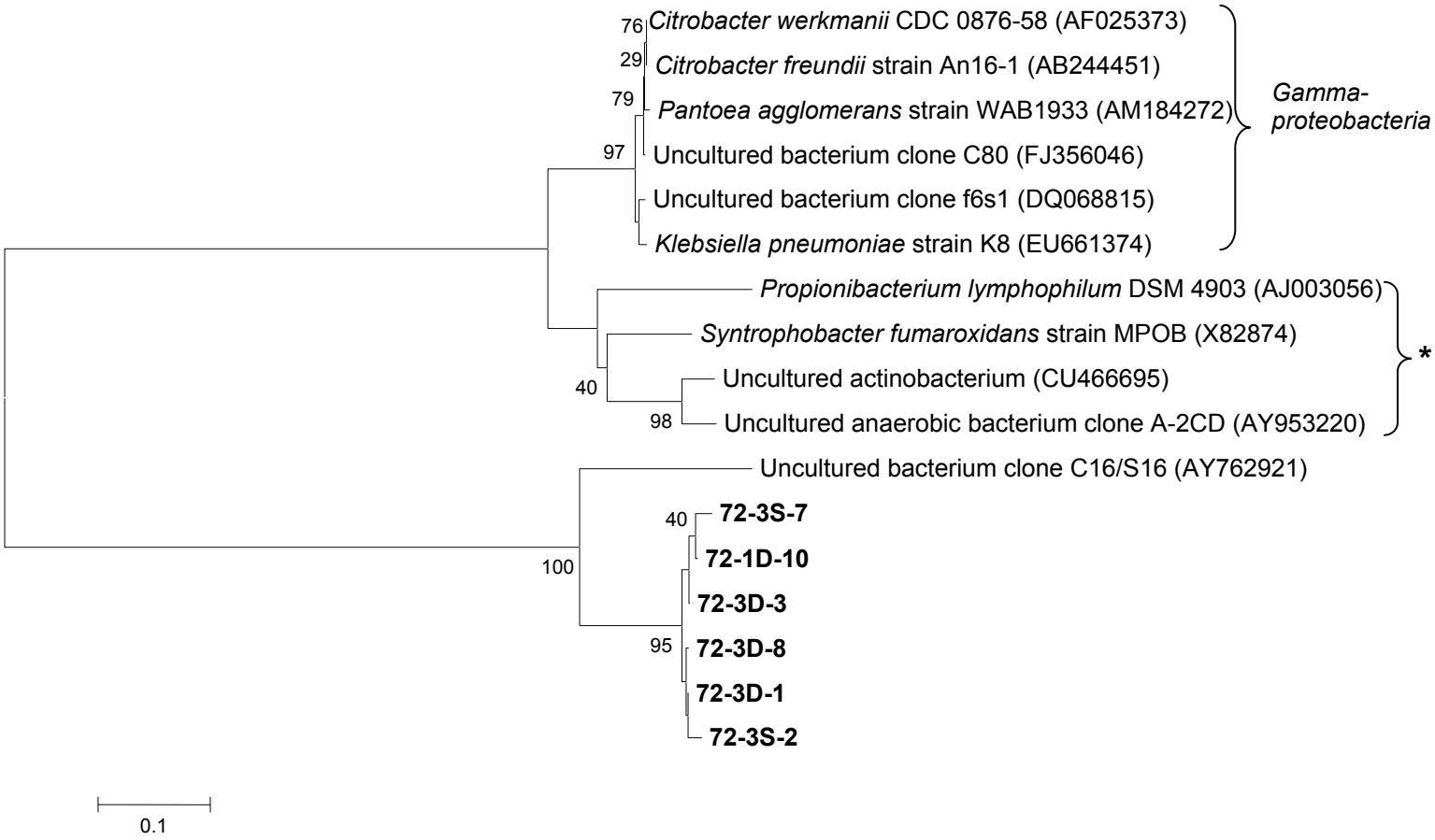

Figure A15 ***Eubacteria*** **clones whose band intensity increased during the gallic acid experiment. The neighbour-joining phylogenetic tree of partial 16S rRNA gene sequences is based on 190 bp of sequence and was constructed using MEGA version 3. The scale bar represents 10% differences in nucleotide sequence. Numbers at nodes represent the bootstrap values (based on 1000 replicates) and only values greater than 40% are shown. Sequences obtained in this study are shown in bold. *, contains members of the classes** ***Actinobacteria*** **and** ***Deltaproteobacteria***

A3.3.5 DGGE analysis of phylotypes amplified with Archaea- and Methanogenic Archaea specific primers

In natural wetlands, organic matter is decomposed anaerobically. Complex polymers are degraded in a multi-step conversion, finally being converted into methane and CO_2. Acetogenic and/or hydrogen producing bacteria and methanogenic play a key role in this process. Therefore, it was decided to focus on methanogenic *Archaea* specially.

As expected, DNA extracted from the surface samples amplified poorly with the *Archaea* specific primers A3Fa/Ab927R. As *Archaea* are predominantly anaerobic, it would be expected to find very few *Archaea* in the surface layer (0-2 cm) which is likely to be aerobic/micro-aerobic. On the other hand, the depth samples amplified well with these primers (Figure A16).

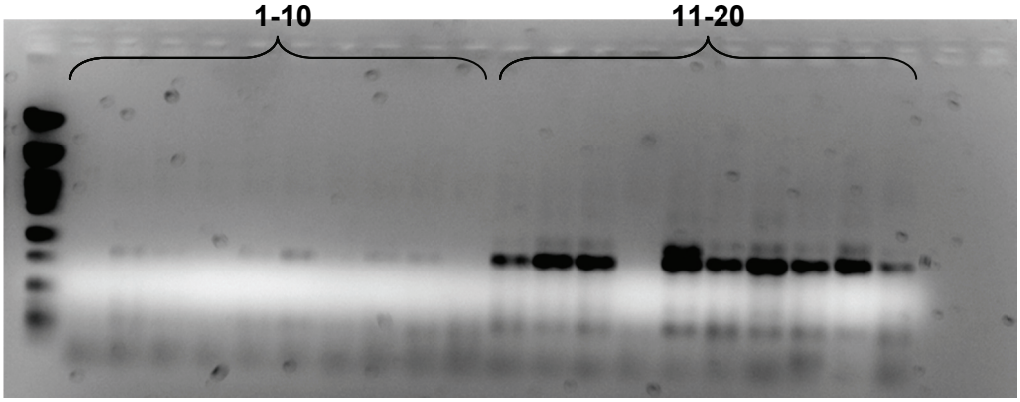

Figure A16 Agarose gel of PCR products generated with the *Archaea* specific primers A3Fa/Ab927R. M, molecular weight marker λ *Pst*I, Lanes 1-10, metagenomic DNA extracted from surface samples collected at time points 0, 3, 6, 12, 24, 36, 48, 72, control 0, control 72, respectively, Lanes 11-20, metagenomic DNA extracted from depth samples collected at time points 0, 3, 6, 12, 24, 36, 48, 72, control 0, control 72, respectively

From DGGE analysis with the *Archaea*-specific nested primers A340F-GC/A533R, four phylotypes were identified which formed stable, dominant members of the Archaeal community. However, only two bands could be successfully purified and cloned. These sequences were found to be identical to two clones generated with the Methanogenic specific primers and have therefore been included in the discussion below (Figure A17).

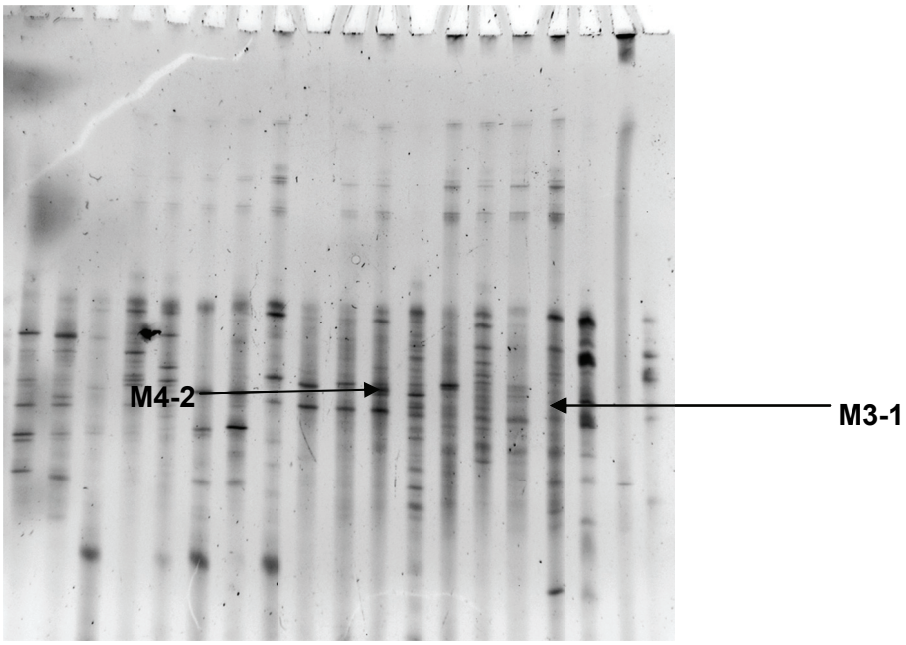

Figure A17 DGGE gel (gradient 45-65%) of partial 16S rRNA genes amplified with *Archaea* specific primers (A340F-GC/A533R) from DNA extracted from depth wetland samples during treatment with gallic acid. Bands successfully cloned and sequenced are marked on the gel

Generally, the DGGE profiles obtained for amplicons generated with the Methanogenic *Archaea*-specific primers (M340F-GC/M707R) was less diverse (fewer OTUs) than the profiles obtained with the Eubacterial and general *Archaea* primers. However, as primers M340F-GC/M707R are specific for methanogens, this is to be expected. Five dominant, stable phylotypes were identified and cloned (Figures A18 and A19).

Three clones, M3-1, M4-2 and M4-1, were found to be most similar to members of the Order *Thermoplasmatales* which includes the genus *Thermoplasma*, a cellwall-less Archaeal genus. While most *Thermoplasma* species are thermophilic acidophiles with optimum growth occurring at 60°C and pH 2, Edwards *et al*. (2000) have described a non-thermophilic species which was isolated from acid mine drainage. It must be noted that the sequence identities for all three clones is below 90% and no culturable species were identified from BLAST analysis (Table A4). Due to the inherit difficulty in obtaining sequences for *Archaea* (even “universal” Archael primers do not bind to all genera) most sequences on the GenBank database are short (less than 300 bp) and it is therefore difficult to make a proper comparison based on such short sequences.

The remaining two clones, M3-2 and M2-1, were most closely related to members of the Family *Methanomicrobiaceae*. Species belonging to the Family *Methanomicrobiaceae* reduce CO_2 to form methane and are strictly anaerobic. They are often found in anoxic

habitats such as marine sediments and anaerobic sewage digestors (Boone *et al*., 2001). *Methanomicrobiales* species are also hydrogenotrophic and hydrogenotrophy is an important nutritional pathway in anoxic environments such as wetlands and peatlands (Galand *et al.,* 2003).

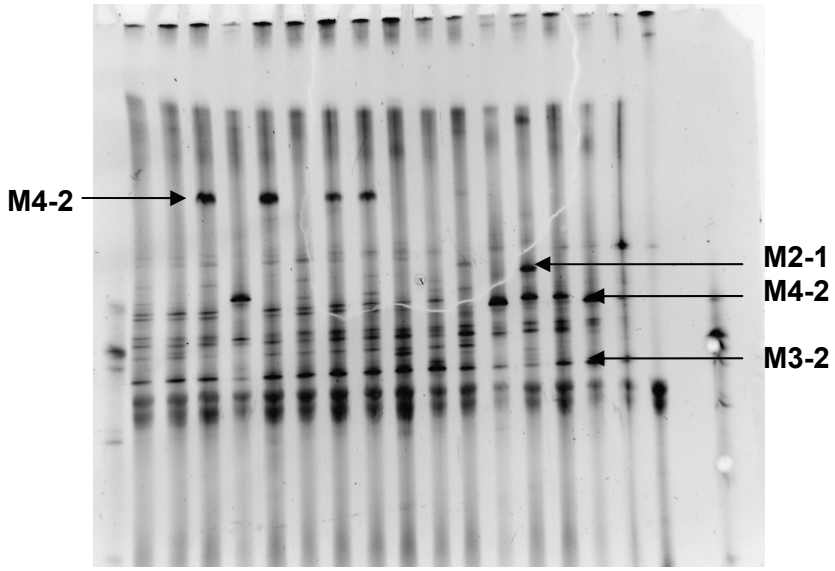

Figure A18 DGGE gel (gradient 45-55%) of partial 16S rRNA genes amplified with Methanogenic *Archaea* specific primers (M340F-GC/M707R) from DNA extracted from depth wetland samples during treatment with gallic acid. Bands successfully cloned and sequenced are marked on the gel

Table A4 BLAST results for the five partial 16S rDNA amplicons generated with the Methanogenic *Archaea*-specific primers

Clone	Best Hit	% identity/ number of bp	Source	GenBank accession number
M2-1	Uncul. archaeon 120A-4	98%/329	Wastewater sludge	AF424772
M3-2	Uncul. *Methanomicrobiaceae* archaeon clone LrhA51	96%/326	Rice rhizosphere	AJ879031
M4-1	Uncul. archaeon clone Amsterdam-1A-29	88%/338	Deep-sea mud volcanoes	AY592258
M4-2	Uncul. archaeon clone St_T_82	88%/329	Lake Stechlin sediment	AY531723
M3-1	Uncul. archaeon clone St_T_82	78%/216	Lake Stechlin sediment	AY531723

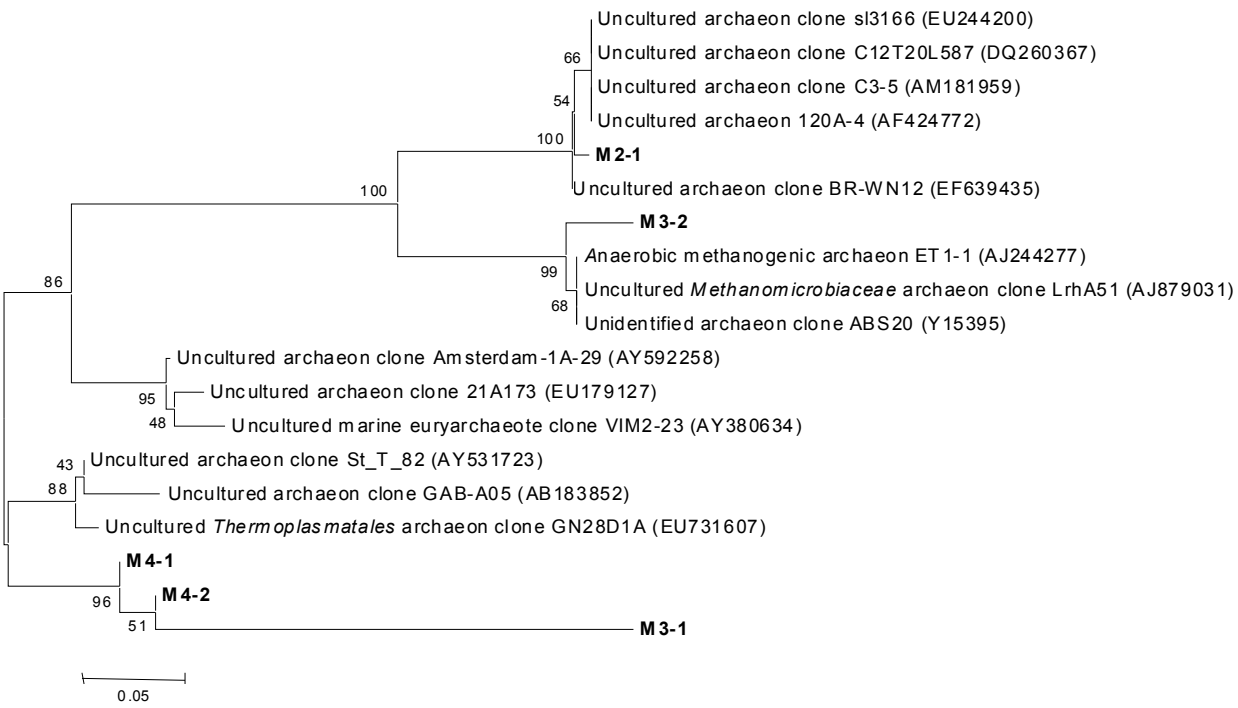

Figure A19 Clones obtained with the methanogenic *Archaea*-specific nested DGGE primers. All clones were derived from depth metagenomic DNA samples during the gallic acid experiment. The neighbour-joining phylogenetic tree of partial 16S rRNA gene sequences is based on 280 bp of sequence and was constructed using MEGA version 3. The scale bar represents 5% differences in nucleotide sequence. Numbers at nodes represent the bootstrap values (based on 1000 replicates) and only values greater than 40% are shown. Sequences obtained in this study are shown in bold

PART B: SAND-FILLED, PILOT SCALE CONSTRUCTED WETLANDS AMENDED WITH WINERY WASTEWATER, ETHANOL AND PHENOLICS

B1 INTRODUCTION AND BACKGROUND

B1.1 LITERATURE BACKGROUND

The South African wine industry, comprising some 600 independent wineries located almost exclusively in the Western Cape region, is a major contributor to national gross domestic product. Globally, the country is one of the top ten wine producers, and accounts for the production of around 8 million litres of wine per annum (SAWIS, 2010). The wine industry is also a significant producer of chemical wastes, mostly in the form of wastewater. Winery wastewater is typically dilute and production is highly seasonal, with major peaks occurring during the crush season (Saadi et al., 2007; Sheridan, 2003). The composition and degradation rates of cellar effluent exhibit both inter- and intra-winery variability, with the slowly biodegradable phenolic component often being antibacterial and/or phytotoxic in nature (Arienzo et al., 2009; Bustamante et al., 2007; Malandra et al., 2003).

South African legislation authorizes discharge of biodegradable industrial wastewater to the environment providing certain parameters are monitored and met (South African Water Act 96, 1998). Maximum chemical oxygen demand (COD) limits range from 30 $mg.L^{-1}$ to 5 000 $mg.L^{-1}$, depending on daily volume and whether the wastewater is to be used for irrigation or discharged directly into a watercourse. Treatment of the wastewater to ensure compliance with these standards before discharge is thus imperative.

Increased concentrations of organic chemicals responsible for the high COD of winery wastewaters are found around the period of vinification. Glucose, fructose, ethanol and acetic acid have all been identified as significant COD contributors (Malandra et al., 2003; Sheridan, 2007). Typical effluent COD values range from 800 to 12 800 $mg.L^{-1}$, but peaks as high as 25 000 $mg.L^{-1}$ have been reported (Malandra et al., 2003). Numerous aerobic and anaerobic suspended and attached growth systems for the treatment of winery wastewater have been evaluated (Andreottola *et al.*, 2009). For example, high COD removal performances (98% or more) were achieved in an aerobic activated sludge system and in an anaerobic sequencing batch reactor (Fumi *et al*., 1995; Ruiz *et al*., 2002) However, the stability of conventional biological wastewater treatment systems may be disrupted by the inherent variability in composition and volume of winery wastewater (Malandra et al., 2003;

Masi et al., 2002; Sheridan, 2007; Vymazal and Krőpfelová, 2009). As the production of winery wastewater is intermittent, conventional systems need to be periodically re-acclimated (usually bi-annually during the crush and bottling seasons) (Grismer *et al*., 2003; Eusébio *et al*., 2004). In addition, the installation and maintenance of expensive systems that require lengthy start-up periods and are operated for relatively short periods is not cost-effective for smaller wineries (Malandra et al., 2003; Saadi et al., 2007; Sheridan, 2007). Provided land requirements can be met, constructed wetlands (CWs) form economical, low maintenance, low energy wastewater treatment systems that demonstrate limited sensitivity to seasonal input fluxes (Masi et al., 2002).

Unlike the phenolic molecules, the simple sugars, organic acids and alcohols found in winery wastewater are readily biodegradable (Malandra et al., 2003; Serrano et al., 2010). Degradation of readily biodegradable COD, such as ethanol, in constructed wetlands is mediated predominantly by microorganisms (Caselles-Osorio and Garcia, 2006; Imfeld, 2009; Tietz et al., 2008; Vymazal and Krőpfelová, 2009). Aerobic ethanol biodegradation pathways are acetogenic (Hektor, 2000), and due to the inherent dependency on oxygen, are only expected to occur in the surface sediments of wetland systems, with anaerobic pathways dominating in the deeper sediments. Microorganisms capable of anaerobic ethanol fermentation are ubiquitous in nature and metabolic intermediates consist primarily of acetic acid, propionic acid and butyric acid (Schink, 1985).

The phenolic component of winery wastewater is generally slowly biodegradable or recalcitrant (Serrano et al., 2010). In addition, many aromatic chemicals are biologically toxic, an effect which may be magnified in the environment; for example, contact of certain phenolics with chlorine can result in the formation of priority pollutants such as polychlorinated biphenyls (Davi and Gnudi, 1999; Guerra, 2001; Chen et al., 2009). Winery wastewater has been shown to adversely affect the growth of a variety of aquatic and non-aquatic plants, including cash crops, a phenomenon related to the phenolic elements (Mosse et al., 2010; Arienzo et al., 2009; Bustamante et al., 2007; Shepherd et al., 2001). Plant phenolics may also be toxic to microbes, for example it has been demonstrated that tannins, which are abundant in red wines, are able to inhibit microbial digestion by precipitation of proteins (Arienzo, 2009; Sarni-Manchado et al., 1997). Treatment to reduce the phenolic content of winery wastewater before discharge is thus requisite. However, unlike biological wastewater treatment systems that incorporate sludge wasting, CWs can become saturated with recalcitrant organic chemicals. Hence, sufficient biodegradation and mineralization of a critical proportion of phenolics must occur to prevent accumulation and leaching of potentially harmful chemicals from CWs treating winery wastewater.

The resilience and performance of individual constructed wetland systems depend on a nutrient balance for the degradation of target pollutants. It has been found that nitrogen input can be achieved by bioaugmentation with diazotrophs; in experiments using diluted molasses with a TN:COD of 3:100 (nitrogen deficient), the addition of azotobacter cultures increased the rate and extent of COD removal in a similar fashion to nitrogen supplementation (Kargi and Özmíhçí, 2002). Also, the addition of nitrogen-deficient olive mill wastewater to soil has been shown to favour the proliferation of the diazotrophs, *Azotobacter chroococcum* and *Azotobacter vinelandii* (Balis et al., 1997).

The composition of wetland sediments is determined by the regional mineralogy and geochemistry and is also influenced by inputs from waterborne and atmospheric sources (Reynolds et al., 2009). Inorganic nutrients are an inherent component of natural wetlands and sand-filled constructed wetlands, a fact that sets them apart from most other biological wastewater treatment systems. In addition, phosphorus and other important inorganics can be re-circulated within the system as there is no sludge wasting. Some depletion due to leaching from the sediments may occur, but allochthonous inputs may counteract this. The resilience and performance of individual constructed wetland systems depend on a nutrient balance for the degradation of target pollutants. Nitrogen (N) and phosphorus (P) have been recommended as supplements in nutrient deficient biological treatment processes (Andreottola *et al.*, 2002; Bories *et al.*, 2005). However, N and P compounds released into the environment can lead to eutrophication, which impacts the dynamics and functioning of natural ecosystems due to changes in plant, animal and microbial community structures, pH fluctuations and oxygen depletion, among others (Smith *et al.*, 1999). Both N and/or P have been reported as key factors in eutrophication processes in different locations (Howarth, 1988). There is a general agreement on the fact that eutrophication is more often limited by N in terrestrial areas and marine environments, and by P in the case of freshwater ecosystems (Howarth, 1988; Smith *et al.*, 1999).

B1.2 RATIONALE FOR CHANGES IN MEDIUM (GRAVEL TO SAND) AND MODE OF OPERATION (HSSF TO VSSF) OF PILOT-SCALE CONSTRUCTED WETLANDS

Six new pilot-scale constructed wetlands were set up and operated at CPUT from April 2009. Drawing on the knowledge obtained from the operation of the gravel CWs, various changes were made. These changes were introduced with the overall aim of the K5/1936 “Health for purpose” project in mind, namely to gain maximum insight into biological degradation of organic pollutants within CWs. Consequently, the CWs were unplanted and the medium type, medium depth and mode of operation were altered.

It was deemed that collation of metabolic processes and community dynamics, intrinsic to the molecular studies, may have been confounded by the complexity added by the presence of plants; for example, different microbial populations occur in the rhizosphere and unrooted soil matrix. Thus, the decision was taken to not add macrophytes to the CWs.

Secondly, it was decided to change the medium from gravel to sand. Although gravel-filled HSSF CWs are popular in the United Kingdom and the United States, and sand media is often precluded because of its propensity for clogging (Knowles et al., 2010), sand-filled VSSFs are still common in Germany, Austria and Denmark; provided these systems are operated correctly, clogging can be avoided (Knowles et al., 2010). It was found that sampling of gravel for monitoring changes in microbial community dynamics and quantification of enzyme activities was extremely difficult. It was theorized that the use of sand would allow for homogeneity to be achieved more effectively with smaller sample sizes, thus adding to the accuracy of the results.

Thirdly, the mode of operation was changed from horizontal subsurface flow (HSSF) to a hybrid mode of HSSF and vertical subsurface flow (VSSF). It has been recognized that vertical-flow constructed wetlands usually have higher removal efficiencies with regard to organic pollutants (Luederits *et al*. 2001). The operation of the CWs at CPUT comprised intermittent feeding together with gradient-directed drainage, which ensured that the principal mode tended toward classical VSSF. The change in the mode of operation to VSSF was also goal directed, as the drainage cycle allows for draw-down of atmospheric gases into the superficial layers of CWs (Faulwetter et al., 2009; Knowles et al., 2010). High redox potentials promote aerobic processes such as nitrification, while low redox potentials are associated with anaerobic processes such as methanogenesis and sulphate reduction (Faulwetter et al., 2009). By employing VSSF, the development of a redox gradient (high to low) from the surface downwards was expected. Consequently, by sampling from both superficial and deep sand layers, it was hoped to discover differences in microbial community structures imposed by pollutant amendments, and also to improve removal efficiencies by promoting both aerobic and anaerobic catabolic processes.

B2 MATERIALS AND METHODS

B2.1 SET-UP AND MODE OF OPERATION OF PILOT-SCALE CONSTRUCTED WETLANDS

Six identical, sand-filled, pilot-scale constructed wetlands housed in polyethylene containers were set-up and operated at the Cape Peninsula University of Technology (CPUT) during the course of 2009, 2010 and 2011 (Figure B1).

Figure B1 Photograph of two of the six sand-filled hybrid vertical-horizontal subsurface flow pilot-scale constructed wetlands at the Cape Peninsula University of Technology

The CWs were filled with sediment taken from the gravel-filled CWs (originally from a wetland used to treat winery wastewater) and Malmesbury river sand, in a 1:4 ratio. The final volume of sand in the wetlands was 0.5 m^3, with a void fraction of 0.08 m^3. The CWs were operated in a hybrid mode of HSSF and VSSF, with a bias toward the latter achieved by bi-weekly feeding, followed by gradient-directed drainage. The dimensions and operational configuration of the CWs are shown in Figure B2. The characteristics of the CW medium were as follows:

Mechanical analysis: 1% clay, 7% silt, 4% fine sand, 12% medium sand; 76% coarse sand

pH 7.7 Resistivity 3 980 Ohm

Exchangeable cations: 0.07 cmol(+).kg^{-1} Na, 0.05 cmol(+).kg^{-1} K, 1.64 cmol(+).kg^{-1} Ca, 0.21 cmol(+).kg^{-1} Mg

Elemental analysis: 0.61 mg.kg^{-1} Cu, 1.0 mg.kg^{-1} Zn, 1.9 mg.kg^{-1} Mn, 0.10 mg.kg^{-1} B, 63.03 mg.kg^{-1} Fe, 6 mg.kg^{-1} P, 20 mg.kg^{-1} K

All CWs were maintained in an outdoor, undercover environment in order to mimic environmental fluctuations, but avoid exposure to precipitation events, which could influence the concentration of amendment solutions.

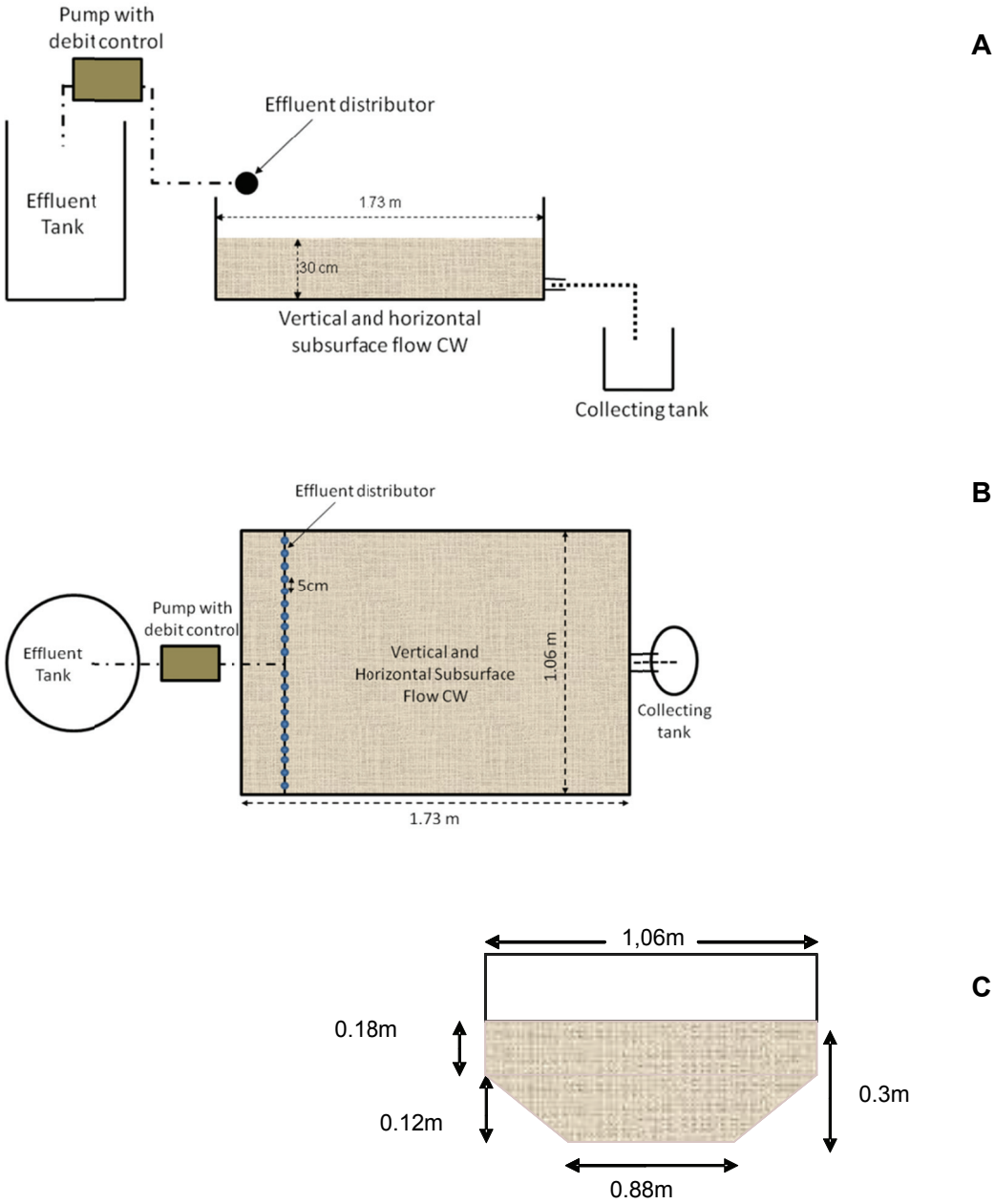

Figure B2 Schematic representation of the pilot-scale constructed wetlands

A Lateral view B View from above C Transverse view

B2.2 FEEDING AND AMENDMENT PROCEDURES APPLIED TO PILOT-SCALE CONSTRUCTED WETLANDS

Two of the six CWs (CW A and CW E), were designated as control CWs, while CW B, CW C, CW D and CW F were used as experimental systems. All CWs were used in only one experiment, except for CW C that was used in two experiments. After a trial period used to determine the optimal basal influent concentration, duration, frequency and flow rate, described in deliverable report no 8 (K5//1936), all CWs were fed with a bi-weekly basal influent of 0.3 g yeast extract (Biolab, RSA cat no: HG000BX6.500) and 0.3 g D (+) glucose (Merck, RSA, chemically pure cat no: SAAR2676020EM), dissolved in 12.5 L tap water at a rate of 0.68 $L.min^{-1,}$ for the duration of the equilibration (minimum three months) and experimental periods. In addition, the experimental systems were amended with either whole winery wastewater or synthetic wastewater containing ethanol, ethanol and nutrients or phenolics as outlined in table B1 below. The organic loading rate of ethanol at a theoretical COD (COD_t) concentration of 1 000 mg.L-1 was calculated as 7.17 g $COD/m^3.day^{-1}$. The theoretical COD of ethanol, vanillin and gallic acid were calculated using equation 2. These values were found to closely approximate measured values (see section B2.5.2).

$COD_t = 8(4x+y-2z)/(12x+y+16z)$ $mgCOD.mg^{-1}$ $C_xH_yO_z$ (Eq 2)

Table B1 Chemical oxygen demand concentrations of ethanol, phenolics and winery wastewater used for amendment of experimental constructed wetlands

Weeks	CW B Ethanol (COD_t $mg.L^{-1}$)	CW C Winery wastewater (COD $mg.L^{-1}$)	CW C Ethanol + N/P (COD_t $mg.L^{-1}$)	CW D Ethanol (COD_t $mg.L^{-1}$)	CW F Vanillin (COD_t $mg.L^{-1}$)	Gallic acid (COD_t $mg.L^{-1}$)
1-3	474	461 ± 126	-	-	136	105
4-6	474	461 ± 126	-	-	681	525
7	474	461 ± 126	-	-	3 404	2 625
8-9	948	461 ± 126	-	-	3 404	2 625
10-14	948	461 ± 126	-	-	-	-
15-16	1 896	461 ± 126	-	-	-	-
17-20	1 896	-	-	-	-	-
21-26	3 792	-	-	-	-	-
27-35	7 587	-	*7 587	7587	-	-
36-40	15 800	-	**7 587	7587	-	-
41-41	26 333	-	-	-	-	-
43-44	Rest	-	-	-	-	-
45-47	7 587	-	-	-	-	-

N and P added in the form of KNO_3 and KH_2PO_4. *COD:N:P ratio 300:5:1; **COD:N:P ratio 200:5:1. The “weeks” column denotes the time periods, not necessarily the relative timescales of amendment

B2.3 MEASURMENT OF THE PHYSICAL PARAMETERS OF THE PILOT-SCALE CONSTRUCTED WETLANDS

B2.3.1 Procedure for the measurement of sediment pH

The pH of the wetland soils was assessed according to the method used by Hartman *et al*. (2008). Equal quantities of de-ionised water and soil were mixed. Thereafter the pH of the slurries was taken using a Eutech Cyberscan pH 300 pH meter and probe.

B2.3.2 Procedure for the measurement of sediment temperature

From June 2009, daily temperatures were recorded in the early morning, at midday and again in the afternoon at a depth of 10 cm and 20 cm. This was gradually rationalized as the temperatures between replicate CWs stabilized in response to interventions, such as strategically placed shade-cloth and polystyrene barriers.

B2.3.3 Procedure for the measurement of hydraulic conductivity

Initially, the volume of effluent was measured every 15 minutes from initial discharge. After performing this procedure for three consecutive weeks, it was clear that the outflow stabilized after 15-30 minutes. Thus, the hydraulic conductivity for the wetlands under saturated conditions was taken as the volume of effluent measured between 30 and 90 minutes after initial effluent discharge subsequent to feeding/watering. This rate was expressed as $L/m^3 sand.hour^{-1}$. Weekly measurements of hydraulic conductivity proved invaluable and the procedure was continued for the duration of the project.

B2.3.4 Procedure for the measurement of void fraction

The volume of water capable of being contained in the CW medium before amendment was crudely determined. The sand was dried in a container for two months at room temperature, followed by one week at 37°C. Tap water (room temperature) was added to a measured amount of sand ($3.375 \times 10^{-3} m^3$) until saturation was achieved and the volume ($5.40 \times 10^{-1} L$) was recorded. Extrapolating this value to the CWs, gave a void fraction of approximately 0.08 m^3 in each CW.

B2.4 SEDIMENT SAMPLING PROCEDURE

Core samples were taken with minimal disturbance of sediment stratification using a sharpened Perspex corer with an internal diameter of 25 mm (Figure B3). Triplicate core samples were taken from predetermined areas at both the inlet and outlet areas of each CW. The upper 3 cm from triplicate samples were pooled and designated “surface inlet” and

“surface outlet”. Similarly, core samples from the 10-15 cm zone below the surface at the inlet and outlet were pooled and designated “deep inlet” and “deep outlet”.

Figure B3 Photograph of core sediment sample, together with the Perspex corer and rubber bung used during the sampling procedure

B2.5 METHODS USED FOR THE ANALYSIS OF THE INFLUENT AND EFFLUENT STREAMS OF THE PILOT-SCALE CONSTRUCTED WETLANDS

B2.5.1 Reagents

Chemicals: butyric acid (Aldrich, Germany, cat no: BIO350-0); (+)-catechin hydrate (Sigma, Germany cat no: C1251); catechol (Sigma, Germany, cat no: C9510); D (+) glucose (Merck, RSA, chemically pure cat no: SAAR2676020EM); ethanol (absolute) (Merck univAR, RSA, cat no: SAAR2233540LP); ferulic acid (Sigma-Aldrich, Germany, cat no: W51, 830-1); Folin-Ciocalteau reagent (Merck, Germany, cat no: 1.09001.0500); gallic acid monohydrate (Sigma-Aldrich, China, cat no: 27645); $NaHCO_3$ (Merck, Germany uniVAR cat no: 5822040); glacial acetic acid (Merck univAR, RSA, cat no: SAAR1021020LC), propionic acid (Fluka puriss, Germany, cat no: WB12794); vanillic acid (Sigma, Germany, cat no: V-2250); yeast extract (Biolab, RSA cat no: HG000BX6.500); vanillin (Sigma-Aldrich, Germany cat no: V1104).

Reagent kits: Hanna HI 93754A-25 for low range (LR) COD detection; Hanna HI 93754B-25 for medium range (MR) COD detection; Hanna HI 93754C-25 for high range (HR) COD detection; Hanna HI 93758B-50 and HI 93758C-45 for total phosphorus; Hanna HI 93767A-50 for total nitrogen; Hanna HI 93766-0 for nitrate; Hanna HI 93764A for low range ammonia detection and HI93764B for high range ammonia detection

B2.5.2 The calculation of theoretical COD and measured (actual) COD of substrates and metabolites in the influent and effluent of the constructed wetlands

The theoretical COD (COD_t) of gallic acid, vanillin, vanillic acid, catechol and acetic acid were calculated using equation 2. The purpose of this conversion was to determine the amendment concentrations according to industry norms and to quantitate the contribution of effluent organics to the total effluent COD (mass balances). The relationship between COD_t and the actual COD (COD_a), given below, was established by COD measurement of triplicate solutions of the following chemicals:

COD_t 1.12 mgCOD.mg and COD_a 1.02 ± 1.0 x 10^{-2} mgCOD.mg gallic $acid^{-1}$

COD_t 1.79 mgCOD.mg and COD_a 1.81 ± 0.5 x 10^{-2} mgCOD.mg $vanillin^{-1}$

COD_t 1.52 mgCOD.mg and COD_a 1.51 ± 1.4 x 10^{-2} mgCOD.mg vanillic $acid^{-1}$

COD_t 1.89 mgCOD.mg and COD_a1.92 ± 0.7 x 10^{-2} mgCOD.mg $catechol^{-1}$

COD_t 1.07 mgCOD.mg and COD_a 1.07 ± 0.3 x 10^{-2} mgCOD.mg acetic $acid^{-1}$

COD_t 2.09 mgCOD.mg and COD_a 2.02 ± 0.6 x 10^{-2} mgCOD.mg $ethanol^{-1}$

COD_t 1.81 mgCOD.mg and COD_a 1.81 ± 0.6 x 10^{-2} mgCOD.mg butyric $acid^{-1}$

COD_t 1.51 mgCOD.mg and COD_a 1.51 ± 0.9 x 10^{-2} mgCOD.mg propionic $acid^{-1}$

B 2.5.3 The identification and quantification of acids, alcohols and phenolics using HPLC chromatograms

Individual phenolics, sugars, acids and alcohols in the effluent were identified and quantified using reverse phase HPLC: samples were run using a Merck Hitachi Lachrom instrument equipped with a L-7400 UV detector. For the detection of phenolics, a Waters Spherisorb® S50DSI analytical cartridge was used with deionised water, methanol and acetic acid (80:20:2.5) as the mobile phase. The wavelength, flow rate and time were set at 280 nm, 0.5 $ml.min^{-1}$ and 60 min, respectively. Acids and alcohols were analysed by HPLC using a Phenomenex Rezex RHM-monosaccharide H+ (8% cross-linkage) column according the method of La'Zaro et al. (1989), with a L-7400 ultraviolet detector (210 nm) and an Agilent™ refractive index detector being used for the detection of acids and alcohols, respectively. Where possible, organic molecules were identified by spiking experiments and quantified using relevant standard graphs prepared from HPLC chromatograms.

B 2.5.4 Folin-Ciocalteau method for the determination of total phenolics

The total phenolics were determined using the Folin-Ciocalteau (FC) micro method for total phenol in wine, based on the method reported by Slinkard and Singleton (1977). Gallic acid standards were prepared in-house and results were expressed as $mg.L^{-1}$ in gallic acid equivalents (GAE) determined from a standard graph.

B 2.5.5 Hanna Instruments kit assays

These were all performed according to manufacturer's instructions using the kits stated in section B2.5.1 and a Hanna photometer C124 and a Hanna digestion block.

Chemical oxygen demand (COD)

The method used was an adaptation of the USEPA 410.4 approved method for the COD determination on surface waters and wastewaters. The principle is based on the fact that dichromate (orange) is reduced by oxidizable organic compounds to the chromic ion (green) and that both of these can be measured photometrically at specific wavelengths. In the case of the kit for low range COD (1-150 $mg.L^{-1}$) the amount of remaining dichromate is determined by a tungsten lamp with a narrow band interference filter at 420nm. In the case of the kit for medium range COD (0-1500 $mg.L^{-1}$) and high range COD (0-15000$mg.L^{-1}$), the amount of chromic ion formed is determined by a tungsten lamp with a narrow band interference filter at 610 nm.

Total phosphorus (TP)

The method is an adaptation of the EPA method 365.2 and *Standard methods for the examination of water and wastewater, 20th edition*, 4500-P E, ascorbic acid method. The principle is that organic and condensed inorganic forms of phosphates are converted to orthophosphate by persulphate digestion. Thereafter, orthophosphate reacts with a test reagent giving a blue colour that is detected by a tungsten lamp with a narrow band interference filter at 610 nm.

Total nitrogen (TN)

The chromotropic acid method includes a persulphate digestion is employed whereby all forms of nitrogen are converted to nitrate. Nitrate then reacts with the reagents to cause a yellow tint that is measured by a tungsten lamp with narrow band interference at 420 nm.

Nitrate nitrogen (NO_3^-–N)

Nitrate reacts with the reagents (chromotropic acid method) to cause a yellow tint that is measured by a tungsten lamp with narrow band interference at 420 nm.

Ammonia nitrogen (NH_3-N)

The method is an adaptation of the Nessler method taken from the *ASTM Manual of water and Environmental Technology*, D1426.

B2.6 MICROBIAL CULTURE

B2.6.1 Procedure for the selective culture of nitrogen-fixing bacteria

Media

Ringers solution 8.2 g NaCl, 4.18 g KCl, 3.32 g $CaCl_2$, 1.9 g KH_2PO_4 and 3.46 $MgSO_4 \cdot 7H_2O$ made up to 1L in distilled water (Umbreit et al.; 1972 described by Zuberer, 1996).

Nitrogen deficient mannitol salt agar (NDMSA) 10 g d-mannitol, 0.5 g K_2HPO_4, 0.1 g $MgSO_4 \cdot 7H_2O$, 0.2 g NaCl. 0.02 g $FeCl_3 \cdot 6H_2O$, 0.0025 g $(NH_4)6Mo \cdot 4H_2O$, 10.0 g $CaCO_3$, 12 g Agar made up to 1 L in distilled water.

Azotobacter medium (AM) 5.0 g glucose, 0.2 g $MgSO_4 \cdot 7H_2O$, 0.04 g $FeSO_4 \cdot 7H_2O$, 0.005 g $Na_2MoO_4 \cdot 2H_2O$, 0.15 g $CaCl_2$, 15 g agar made up to 1 L in distilled water (Brown et al. 1962; described by Knowles and Barraquio, 1996).

Method: One gram of soil was added to 95 ml Ringers solution and 20 g of 4 mm glass beads in 100 ml Erlenmeyer flasks. Flasks were shaken at 160 rpm for 30 min (Koebel-Boelke et al, 1988; described by Zuberer, 1996). 10 fold serial dilutions were made from 10^{-2} to 10^{-4} in Ringers solution. 1 ml, 2 ml, 3 ml, 4 ml and 5 ml from each dilution was spread onto NDMSA and AM giving 15 final dilutions ranging from 1 x 10^{-3} to 5 x 10^{-5} 10 -3 (Zuberer, 1996). Plates were left at room temperature and examined daily for 5 days. Plates with between 30 and 300 colonies were chosen for enumeration.

B2.6.2 Procedure for the enumeration of mesophilic heterotrophic bacteria

Fresh sediment samples (1 g) were diluted in 0.85% aq. NaCl and appropriate dilutions were plated on R2A agar for aerobes (superficial samples) and anaerobes (deep samples). The latter were incubated in anaerobic jars using the Oxoid Anaerogen™ system. All plates were incubated for 4 days at 28°C after which colony counts were performed manually. Data are represented as colony forming units (CFUs) per gram of sediment. Student's t-test was used to assess significant differences ($p < 0.05$) between CFU counts.

B2.6.3 Procedure for soil sterility testing

One gram of sediment oil was added to 5 ml physiological saline and vortexed for 5 minutes. One 5 ul loopful of each homogenate was plated for single colonies on R2A agar and incubated aerobically for 72 hours at 30ºC.

B2.7 SOIL COLUMN EXPERIMENT USED TO DETERMINE THE BIOTIC AND ABIOTIC REMOVAL FRACTIONS OF PHENOLICS BY CONSTRUCTED WETLAND SEDIMENT: EXPERIMENTAL PROCEDURE

Three irradiated and three unirradiated soil columns served to indicate abiotic ($abiotic_i$) and combined biotic and abiotic ($biotic_i$ and $abiotic_i$) removal of phenolics, respectively, with $biotic_i$ mechanisms accounting for the difference between the two. Six Perspex core samplers, 250 mm in length and 35 mm in diameter were used to extract core samples from the redundant test wetland previously used to treat winery wastewater (CW B). Care was taken to limit physical perturbations on sediment structure. The sediment mass (579 g ± 11.2 g) was calculated by deducting the mass of each corer from that of the entire column. The open ends of replicate columns A-C were sealed with duct tape after which they were irradiated at a dose of 30 kGy. All six columns were set up on retort stands with beakers positioned underneath to collect effluent. A phenolic cocktail consisting of gallic acid, (+)-catechin, ferulic acid and vanillin was prepared, filtered through 0.20 um syringe filters and made up with sterile deionised water to give a final concentration of 0.25 mM of each phenolic. 40 ml of the phenolic cocktail was delivered to the columns by injection through the duct tape using a sterile needle and syringe on days 1, 2 and 3, after which the sediments were sufficiently saturated and the volume was decreased to 20 ml per day for a further 6 days. The first two samples were collected after 24 hours and further samples were collected daily for a period of six hours after injection. Every effort was made to prevent contamination of the irradiated columns.

Phenolic removal rates were determined from influent/effluent analyses by the Folin-Ciocalteau assay. In order to correct for the small contribution of leached soil components to the assay, the removal efficiencies (%) of total phenolics in the unirradiated columns (UC) and irradiated columns (IC) were calculated using equations 3 and 4,

$$\% \text{ removal UC} = (100 - (TP_{EU}/TP_I \times 100)) \times (100/[(100 - (TP_{EU}/TP_I \times 100) \quad \text{(Eq 3)}$$

$$\% \text{ removal IC} = (100 - (TP_{EI}/TP_I \times 100)) \times (100/[(100 - (TP_{EU}/TP_I \times 100) \quad \text{(Eq 4)}$$

Where,

TP_I mean values of total phenolics in the influent (mg GAE.L^{-1})

TP_{EU} mean values of total phenolics (mg GAE.L^{-1}) in the effluent from the unirradiated columns

TT_{EI} mean values of total phenolics (mg GAE.L^{-1}) in the effluent from the irradiated columns

B2.8 PROCEDURE FOR SOIL MICROCOSM EXPERIMENTS USED TO DETERMINE THE ACCLIMATION OF MICROBIAL COMMUNITITES IN CONSTRUCTED WETLAND SEDIMENT TO COMMON WINERY PHENOLICS

Sediment samples were taken from the redundant test CW previously amended with vanillin and gallic acid (CW F) as well as the corresponding control (CW E). 10 g sediment aliquots from each CW were added to McCartney bottles together with 2 ml distilled water. Six such samples from each CW were autoclaved for 20 min at 121°C, once daily for three days, with interim incubation at 30°C. A further six samples were incubated at 30°C for 72 hrs. Following incubation, 5 ml of a sterile phenolic solution, consisting of 6.25 mM concentrations (951 mg.L^{-1} and 1 176 mg.L^{-1}) of vanillin and gallic acid, respectively, was added to half the replicates, while sterile distilled water was added to the remainder. A negative control (distilled water, no incubation) was included for each sediment type. All treated samples were incubated for 24 hrs at 30°C, after which the supernatant fluid was analysed for phenolic content by HPLC. $Biotic_i$ and $abiotic_i$ removal of phenolics was were calculated in the same theoretical manner as for the soil columns, with the autoclaved samples indicating $abiotic_i$ removal, and the difference in removal rates between the untreated and autoclaved replicates indicating $biotic_i$ removal. Soil sterility testing was performed on the autoclaved samples as the end of the experiment.

B.2.9 PROCEDURES FOR THE MEASUREMENT OF SODIUM AND CHLORIDE IN CONSTRUCTED WETLAND SEDIMENT AND EFFLUENT

Na and Cl in the sediments of CW A and CW C were determined at weeks 0, 8 and 16 at the Soil Science faculty CAF at Stellenbosch University using a EuroVector EA3000 instrument. To determine whether sodium and chloride was being eluted out of the CW C (amended with winery wastewater), effluent samples were sent to Bemlab (Pty) Ltd. located in the Strand, Western Cape. Cl was determined titrimetrically using a standardized 0.05M solution of silver nitrate. Na was determined using a Varian Inductively Coupled Plasma – Optical Emission Spectrometer (ICP-OES).

B.2.10 PROCEDURES FOR THE EXTRACTION OF DNA

B2.10.1 Procedures for the extraction and quantification of DNA from constructed wetland sediment

Different DNA extraction procedures have been used in this section of the study. For the establishment phase study (section B3.1), total DNA was extracted from 0.5 g sediment samples (wet weight) with a Bio-101 FastDNA Spin Kit and using the FastPrep FP 120 bead beating system (Bio-101, USA) according to the manufacturer's instructions.

To study the impact of ethanol (with or without GPP, section B3.3), winery waste effluents and phenolics on the microbial community structure, total DNA was extracted from 0.3 g sediment samples (wet weight) with the Powersoil DNA isolation kit (Mobio Laboratories, USA) according to the manufacturer's instructions.

For the nutriment experiment (section B3.2), metagenomic DNA was extracted using a modified version of the method described by Wang et al. (1996). Approximately 500 mg of sediment were thawed on ice. The soil was resuspended in 1 ml lysis buffer (25 mM Tris-HCl pH 8; 50 mM glucose; 10 mM EDTA; 25 mg lysozyme per ml) and 0.5 g quartz sand (Sigma S-9887) was added to each tube. Microbial cells were lysed by both mechanical and chemical methods. Mechanical shearing involved three cycles of vortexing at maximum speed for 1 min followed by rapid cooling on ice for 2 min. After vortexing the samples were incubated in lysis buffer overnight at 37°C. SDS was added to a final concentration of 1% and the samples were incubated at 65°C for 30 min. Samples were extracted twice with 1 vol equilibrated phenol (pH 7.6), followed by extraction with 1 vol chloroform:iso-amyl alcohol (24:1, vol/vol). Nucleic acid was precipitated with 1 vol ice-cold isopropanol at room temperature and harvested by centrifugation at 14 000 X g for 5 min. The resulting pellet was resuspended in 50 µl 10 mM Tris-HCl; 1 mM EDTA (TE) buffer pH 7.8.

The concentration of the DNA-samples was measured with a NanoDrop spectrophotometer (NanoDrop Technologies, Montchanin, DE, USA). The Student's t-test was used to assess significant differences ($p < 0.05$) between DNA concentrations.

B2.10.2 Procedures for the extraction and quantification of DNA from winery wastewater

DNA was extracted from the winery wastewaters used to contaminate the CWs (results given in deliverable 11, WRC Project K5/1725). 100mL of wastewaters were filtered using a 0.45 µm membranes (HPLV047, Millipore, Bedford, MA, EU) and a Sartorius filtration system. ½ of the filter was cut into small pieces using a sterile blade. The DNA from the filter

pieces was extracted using the Powersoil DNA isolation kit (MoBio laboratories, USA) according to the manufacturer's instructions.

B2.11 GENE AMPLIFICATION AND PURIFICATION PROCEDURES

B2.11.1 PCR amplification procedures

All polymerase chain reactions (PCRs) were carried out in a Perkin Elmer Thermocycler (Gene Amp PCR system 6700). Each reaction contained 1 X PCR buffer, 0.2 U Dream*Taq*™ polymerase (Fermentas, USA), 200 µM of each dNTP, 0.5 µM of each primer, 0.1% BSA and between 5 to 10 ng of metagenomic DNA. PCR amplification was carried out as described in table B2.

To perform DGGE, a nested-PCR was performed using 1 µL of the amplicon obtained with the 16S rRNA primer set E9F/U1510R with the primer set 341f-GC$^{(*)}$ (5'-CCTACGGGAGGCAGCAG-3') / 534r (5'-ATTACCGCGGCTGCTG-3') (Muyzer et al., 1993) as follow: 94°C for 4 min; 20 cycles – 94°C for 45 sec; 65°C for 45 sec; 72°C for 60 sec; additional 20 cycles -94°C for 30 sec; 55°C for 30 sec; at 72°C for 60 sec; and a final elongation step at 72°C for 10 min. PCR amplification with 341f-GC/534r was performed by using a 50-µl total volume mixture containing 0.2 U Dream*Taq*™ polymerase (Fermentas, USA), 1X PCR Buffer, 200 µM of each dNTP, 0.5 µM of each primer and 0.1% BSA.

B2.11.2 PCR purification and restriction procedures

The PCR amplicons were purified using the GFX^{TM} PCR DNA and gel band purification kit as directed by the supplier (GE Healthcare, UK). 100 ng of the purified PCR products were then digested using the restriction enzyme *Hae*III (during 3 h at 3°C). The resulting digested PCR products were then also purified using the same purification kit as directed by the supplier (GE Healthcare, UK).

Table B2 PCR primer sequences and cycling conditions used for the metagenomic analysis of the microbial community structure in constructed wetlands

Primer	Primer sequence	Target	Amplification conditions	Reference
E9F U1510R	GAGTTTGATCCTGGCTCAG GGTTACCTTGTTACGACTT	Most *Eubacteria*	94°C for 4 min 30 cycles – 94°C for 30 sec; 52°C for 30 sec; 72°C for 105 sec 72°C for 10 min	Hansen *et al.*, 1998; Reysenbach & Pace, 1995
341F-*GC 534R	CCTACGGGAGGCAGCAG ATTACCGCGGCTGCTG	Most *Eubacteria* – used in nested PCR in combination with E9F/U1510R	94°C for 4 min 20 cycles – 94°C for 45 sec; 65°C for 45 sec; 72°C for 60 sec Additional 20 cycles – 94°C for 30 sec; 55°C for 30 sec; 72°C for 60 sec 72°C for 10 min	Muyzer *et al.*, 1993
PolF PolR	TGCGAYCCSAARGCBGACTC ATSGCCATCATYTCRCCGGA	Bacterial *nifH* gene (nitrogenase reductase)	94°C for 5 min 35 cycles – 94°C for 30 sec; 50°C for 30 sec; 72°C for 80 sec 72°C for 10 min	Poly et al., 2001

* For DGGE analysis a 40mer GC clamp was added to the 5' ends of the forward primers 341f: GC clamp – CGCCCGCCGCGCGCGGCGGGCGGGGCGGGGGCACGGGGGG.

B2.12 PROCEDURES EMPLOYED FOR FINGERPRINTING OF MICROBIAL COMMUNITIES IN CONSTRUCTED WETLAND SEDIMENT SAMPLES

B2.12.1 Denaturing gradient gel electrophoresis (DGGE) analysis

PCR amplicons obtained with the nested primer sets (341f-GC/534r) were analyzed by denaturing gradient gel electrophoresis (DGGE). Amplicons were separated on 16.5x16.5 cm x1 mm 9% (wt/vol) polyacrylamide (37.5:1 acrylamide:bisacrylamide) gels with varying denaturing gradients (100% denaturant was 7 M urea and 40% (vol/vol) formamide). Gels were prepared using a BioRad gradient former and were cast according to manufacturer's specifications. Electrophoresis was performed using the DCode DGGE system (BioRad) and was carried out at 100 V for 16 hrs at 60°C (1600 Vh) in 1X TAE buffer. Gels were stained in 0.5 $\mu g.mL^{-1}$ EtBr in 1X TAE for 20 min and visualized on an AlphaImager 3400 imaging system.

DGGE gel pictures were processed with Gelcompar II 5.0 software (Applied Maths, Belgium). For the establishment phase study (section B3.1) the complete banding pattern was used for all comparisons. Similarity between fingerprints was calculated with the Cosine

coefficient. The clustering algorithm of Ward was used to calculate the dendrograms of a combination of all gels. Clustering analysis and non-metric Multi-Dimensional Scaling (MDS) were performed with Gelcompar II 5.0. For the nutriment experiment (section B3.2.) the DGGE banding patterns were compiled and band matching was performed in order to obtain large data matrixes based on the presence/absence of DGGE bands. The complete banding pattern of each profile was considered for comparison. The presence/absence matrix was used to perform Principal Component Analysis (PCA). On the PCA diagrams, the complex DGGE patterns of each sample was reduced to one point in a three dimensional space. In PCA diagrams, the relative importance of each axis is given by the percentage of variance explained by that axis. The proximity of two points on the diagram is related to the community structure: points which are close together represent similar communities and vice versa.

B.2.12.2 Terminal Restriction Fragment Length Polymorphism (T-RFLP) Analysis

PCR amplicons obtained with the E9F-FAM labeled / U1510R primer set were analyzed by terminal-restriction fragment length polymorphism (T-RFLP) in triplicate. The amplicons were purified using the GFX^{TM} PCR DNA and gel band purification kit as directed by the supplier (GE Healthcare, UK). 100 ng of the purified PCR products were digested using the restriction enzyme *Hae*III (during 3 h at 3°C). The resulting digested PCR products were purified using the purification kit recommended by the supplier (GE Healthcare, UK). The T-RFLP were performed by the DNA Sequencing Unit, University of Stellenbosch. Briefly, the digested products were denaturated for 2 min at 94°C with formamide and 0.5 µL of the molecular weight standard Rox 1.1. The size of the different fragments of the Rox 1.1 molecular weight standards were: 75, 100, 139, 150, 160, 200, 300, 340, 350, 400, 450, 490, 500, 583, 683, 782, 932, 991, 1121 bases. The preparation was denatured for 5 min at 95°C and then immediately put on ice before separation by capillary electrophoresis in a sequencer. The initial induction was for 15 sec at 1.6 kV. This migration separates the different fragments according to their size, and only the restriction fragments labelled at their terminal position (T-RFs) are detected. The precise length of the T-RFs (+/-1 base) were determined by comparison with the standards using the software Peak Scanner v1.0 (Applied Biosystems).

To analyse the T-RFLP profiles, only the T-RFs between 50 and 1000 bp were taken into account. The T-RFs detected by the software Peak Scanner (Applied Biosystem, USA) were compiled into large data matrixes and processed using the online software T-REX (http://trex.biohpc.org/index.aspx, Culman et al., 2009). The T-RFs which represented less than 1% of the total fluorescence were eliminated from the analysis as they could not be

distinguished from background fluorescence (Li *et al.*, 2007). The relative abundance of a true terminal restriction fragment within a given T-RFLP pattern was generated as a ratio of the respective peak height. MVSP v3.1. software (Multi-Variate Statistical Package, Rockware Inc., UK) was used to compare T-RFLP patterns with principal component analysis (PCA) using the relative intensity of each T-RF in the total T-RFLP profile and square root transformed data. Principal component analysis is a multivariate descriptive statistical analysis that can take into account a large amount of data. The principle of this analysis is based on the projection of all variables (in our case each T-RF and its relative abundance) in a plane, so as to understand the groups of points distant (or close to) each other, and then to look for a biological explanation for these observations. By using PCA, the different data of the complex T-RFLP patterns of one sample can be reduced to one point in a three dimensional space. Following the PCA, a planar projection of samples on a factorial plan is obtained, which corresponds to the projection plane that takes into account the maximum variance between samples. This plan is determined by two orthogonal axes (PC1 and PC2). The relative importance of each axis is given by the percentage of variance explained by that axis. It allows the significance of this axis with respect to the information displayed in the matrix to be determined. Typically, the first axis is much more informative than the second, and the closer the samples are represented on the PCA diagram, the more alike they are (and conversely, the more distant they are, the less alike they are). The Shannon ($H' = -\Sigma p_i \ln p_i$) and Eveness ($E = H'/\ln S$) diversity indexes were calculated from the combined 5'-end 16S rRNA encoding gene T-RFLP profiles. p_i corresponds to the relative abundance of T-RFs *i* in the combined T-RFLP profiles, and *S* to the richness, i.e. the total number of OTUs obtained with *Hae*III restrictions and detected in the compiled 16S rDNA T-RFLP profiles.

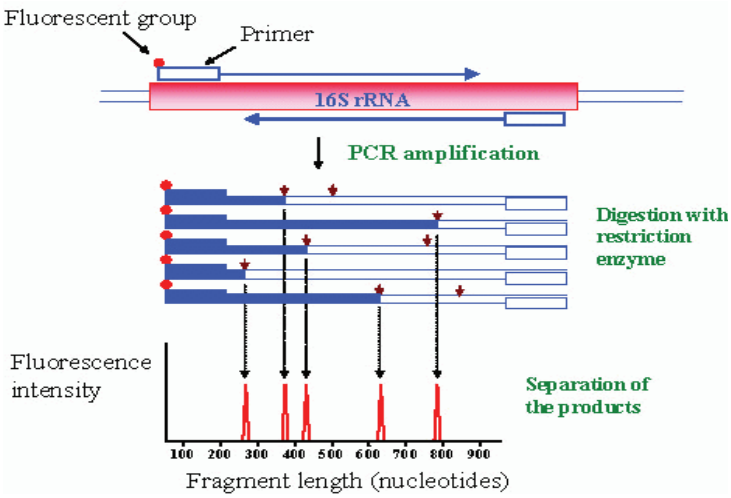

Figure B4 Schematic representation of the experimental steps performed to conduct the T-RFLP analysis

B3 RESULTS AND DISCUSSION

B3.1 EQUILIBRATION OF CONSTRUCTED WETLANDS PRIOR TO ORGANIC AMENDMENT

Some of the most important issues in operating CWs relate to response and equilibration kinetics. It has been shown that extended periods are required for microbial communities to stabilize during operational periods although the basis used to indicate community stability is not always consistent (Truu et al., 2009; Weber and Legge, 2010). In one report, using reactors with sand filters, the establishment of denitrifying bacteria populations required 75 days and ammonium- and nitrate-oxidizing bacteria populations required 95 days (Truu et al., 2009). Using community-level physiological profiling (CLPP), it has recently been shown in constructed wetland mesocoms that microbial communities in a start-up process reached a steady-state after a period of 75-100 days (Weber and Legge, 2010). The objective of this study was to use microbial fingerprinting to determine more accurately the kinetics of microbial population equilibration in pilot-scale CWs. We define an equilibrated system as one which demonstrates stabilized physical and biological patterns in terms of flow properties, biomass and microbial community structure. To avoid system heterogeneities introduced by the presence of plants (Caravaca et al., 2005; Calheiros et al., 2009; Weber and Legge, 2010), unplanted CWs were used.

The presence of a stable microbial community is generally considered to be a critical factor for maintaining ecosystem stability and resilience after contamination, nutrient cycling efficiencies and long-term sustainability (Torsvik and Ovreas, 2002; Wohl et al., 2004). To follow changes in microbial community composition and to establish the kinetics of community changes, we used Denaturing Gradient Gel Electrophoresis (DGGE) (Nicomrat et al., 2006; Baptista et al., 2008; Ruiz-Rueda et al., 2009). Bacterial diversity, rather than functionality (i.e. microbial related processes or metabolic activities such as microbial carbon source utilization patterns or (de)nitrification processes), was chosen as the principal assessment parameter. This choice was guided by the availability of reliable and rapid methods for environmental microbial community fingerprinting analysis (DGGE), whereas functional analysis can be limited by the choice and number of substrates studied such as the carbon sources in CLPP analysis, or by their price and availability in stable isotope probing analysis (DNA/RNA-SIP). Moreover, functional analysis can be confused by the functional redundancies in ecosystems, i.e. by the fact that two or more species can play the same role in ecosystems, where differently structured microbial communities may have a similar functionality. Bacterial counts and total extractable DNA titres were used as proxies for microbial biomass, and hydraulic conductivity was used to monitor changes in CW drainage processes.

Previous studies have suggested that equilibration times for achieving a stable microbial community are slow in terms of biomass or function stabilization (in the order of 75 to 100 days) for CWs of the dimensions used in this study (Figure B2) (Truu et al., 2009; Weber and Legge, 2010). On this basis we monitored system parameters only from 47 days from the beginning of the experiment. The CWs were operated in a hybrid mode of vertical and horizontal sub-surface flow (VSSF and HSSF) where VSSF was established as the principal mode of operation. This type of wetland is common, and is considered to produce substrate degradation rates superior to those of HSSF wetlands, probably due to the maintenance of a more oxic status (Truu et al., 2009).

To facilitate long term comparative analysis, the pilot-scale CWs must first be equilibrated over a period of time. A strong relationship between the composition of microbial communities and their function in CWs has recently been established (Truu et al., 2009). In parallel CW experiments, it is thus essential to establish that microbial communities in each CW have similar compositions before undertaking experiments involving perturbation of the systems (such as chemical challenge, etc.). In order to establish such consistency, nutrient supplements were added (feeding) throughout the experiment to three of the four CWs (namely A, B and C) (Table B3). This, to stimulate microbial growth and establish consistent microbial communities and hence to determine their necessary equilibration time (Wu et al., 1997), i.e. until the CWs reached similar, and stable, physical (hydraulic conductivity, Figure B7.A) and biological (microbial community structure and microbial biomass, Figures B5, B6, B7.B, B7.C) profiles. Feeding provided a C:N:P ratio of 32:7:1, with a low carbon supply (influent COD = 24 $mg.L^{-1}$) and non-limited nitrogen (N) and phosphorous (P) sources, with respectively 5.5 $mg.L^{-1}$ and 0.76 $mg.L^{-1}$ of total N and total P in the influent. This was done in order to maintain oligotrophic status, as oligotrophic systems are more reactive to changes in nutrient status such as wastewater contamination (Verhoeven et al., 2006).

We used DGGE to establish microbial community fingerprints, and to assess system factors such as stability of communities and their response/s to changes in nutrient status (Figures B5 and B6). The variation of the hydraulic conductivity in parallel with the microbial biomass was also studied in the pilot-scale CWs (Figure B7), as a decrease the hydraulic conductivity is a valuable indicator of production and accumulation of microbial biomass during the establishment of the microbial communities (Wu et al., 1997). Moreover, it is essential to monitor water flow in subsurface environments such as CWs because this will determine the transport rate, and therefore the fate, of the wastewater components in the sediment matrix.

Three of the wetlands (designated A, B and C) received identical treatments during the equilibration phase. They were fed bi-weekly with nutrients consisting of yeast extract

powder (Biolab, Gauteng, South Africa) and D (+) glucose (Merck Chemicals, Gauteng, South Africa) dissolved in tap water: volumes and concentrations are given in Table B3. The same solute concentrations were maintained from the 21st day of feeding, but the volumes were gradually decreased as a loss in the hydraulic conductivity of the wetland systems was observed. The fourth wetland (CW D), which was used as a negative control, received tap water without nutrient additions but in equal amounts and rate to CWs A, B and C.

Table B3 Feeding procedure used for three constructed wetlands (CW A, CW B and CW C) during the equilibration phase

Days (total number of feeds)	Yeast extract (g)	Glucose (g)	Volume (L)	Hydraulic loading rate ($L.min^{-1}$)
Day 1	10	2	30	0.27
Day 8 and day 13	5	1	50	0.27
Day 16 (1)	1	1	45	0.27
Day 21 (1)	1	1	40	0.68
Day 23 to Day 64 (12)	0.5	0.5	20	0.68
Day 67 to Day 96 (9)	0.3	0.3	12	0.68

B.3.1.1 The evolution of the microbial communities during equilibration

DGGE patterns were obtained for both surface and deep sediment samples, and across a temporal range from day 47 to day 96 for all four CW systems. DGGE patterns were subjected to cluster analysis, presented as dendrograms (Figures B5.A, B6.A) and multidimensional scaling (MDS) analysis (presented as three-dimensional distribution plots; Figures B5.B and B6.B), both methods being designed to establish the statistical relatedness between samples. In the three-dimensional MDS plots, each DGGE pattern is reduced to a single point and the distance between points in 3D space is indicative of the relatedness of the respective DGGE patterns and therefore the microbial communities. Unlike dendrograms, MDS does not provide clusters. The linkages between the samples in the 3D-MDS graphical representation are shown as an aid to interpretation (Figures B5.B and B6.B).

The dendrogram derived from the DGGE analysis of CW surface sediment samples (Figure B5.A) clearly distinguishes the microbial community profiles of two time periods: the initial sample point (day 47) and all subsequent sampling times (days 61, 75, 89, 96). These time periods only share 47.1% similarity with respect to their microbial community structures. Within the latter cluster, 3 coherent sub-clusters sharing over 91% similarity are evident. These are samples taken on (i) days 61 and 75 of the supplemented CWs (96.0% similarity), (ii) days 89 and 96 of the supplemented CWs (95.5% similarity) and (iii) days 61 to 96 of the

non-supplemented CW (98.0% similarity). These results can be interpreted as follows: the surface microbial communities in the three nutrient-supplemented CWs were highly consistent at any time point, but they evolved over the 49 day sampling period. The similarity of DGGE patterns at days 89 and 96 suggests that the communities had stabilized. In contrast, the consistency of the DGGE patterns from the control (non-supplemented) CW is strongly suggestive that the microbial community was equilibrated by day 61 and remained essentially stable for the duration of the experiment.

These observations are confirmed by the MDS analysis (Figure B5.B). The points representing the molecular fingerprints from day 47 sample patterns (represented as green spheres) are tightly grouped and well separated from all other samples. Through successive sampling points, all communities showed substantial compositional divergence. The most coherent group across all subsequent sample times (days 61 to 96; shown as a dotted ellipse) was that derived from CW D (control), a result which is consistent with the similarity values derived from the dendrogram (Figure B5.A). The other significant cluster is that formed by samples from days 89 and 96 of the nutrient-supplemented CWs (shown as the black ellipse, Figure B5.B). Together, these analyses indicate that surface sediment microbial communities were responsive to nutrient supplementation, and the 'new' community structure was stably equilibrated at 89 days from initiation of the experiment.

Similar analyses of the deep sediment microbial community structure (Figure B6) showed three relatively dispersed clusters. As for the surface samples, the day 47 samples (green ellipse, Figure B6.B) are spatially distinct, with a 91.5% similarity value within the group but only 47.9% similarity with the other samples (Figure B6.A). All other samples show substantial divergence from day 47 community patterns. A second dispersed cluster (yellow ellipse, Figure B6.B) represents the day 61 and 75 samples. The dispersed structure of this cluster suggests that all communities (in both supplemented and control CWs) are undergoing independent structural evolution. However, the third cluster (purple ellipse, Figure B6.B), which represents the days 89 and 96 samples from all CW experiments, is highly coherent. Two conclusions are possible from this result: firstly that all CW communities were stable after 89 days and, secondly, that the equilibrated state of these deep sediment communities was similar in control and supplemented CW experiments. These results might suggest that the nutrient-supplemented CW communities were responsive to nutrient addition, but the parallel behavior in the deep sediments of the non-supplemented control CW is inconsistent with this conclusion. We suggest that the parallel evolution of microbial communities in this particular CW compartment is indicative of the anoxic status of the microenvironment which would be expected to dominate changes in microbial community structure.

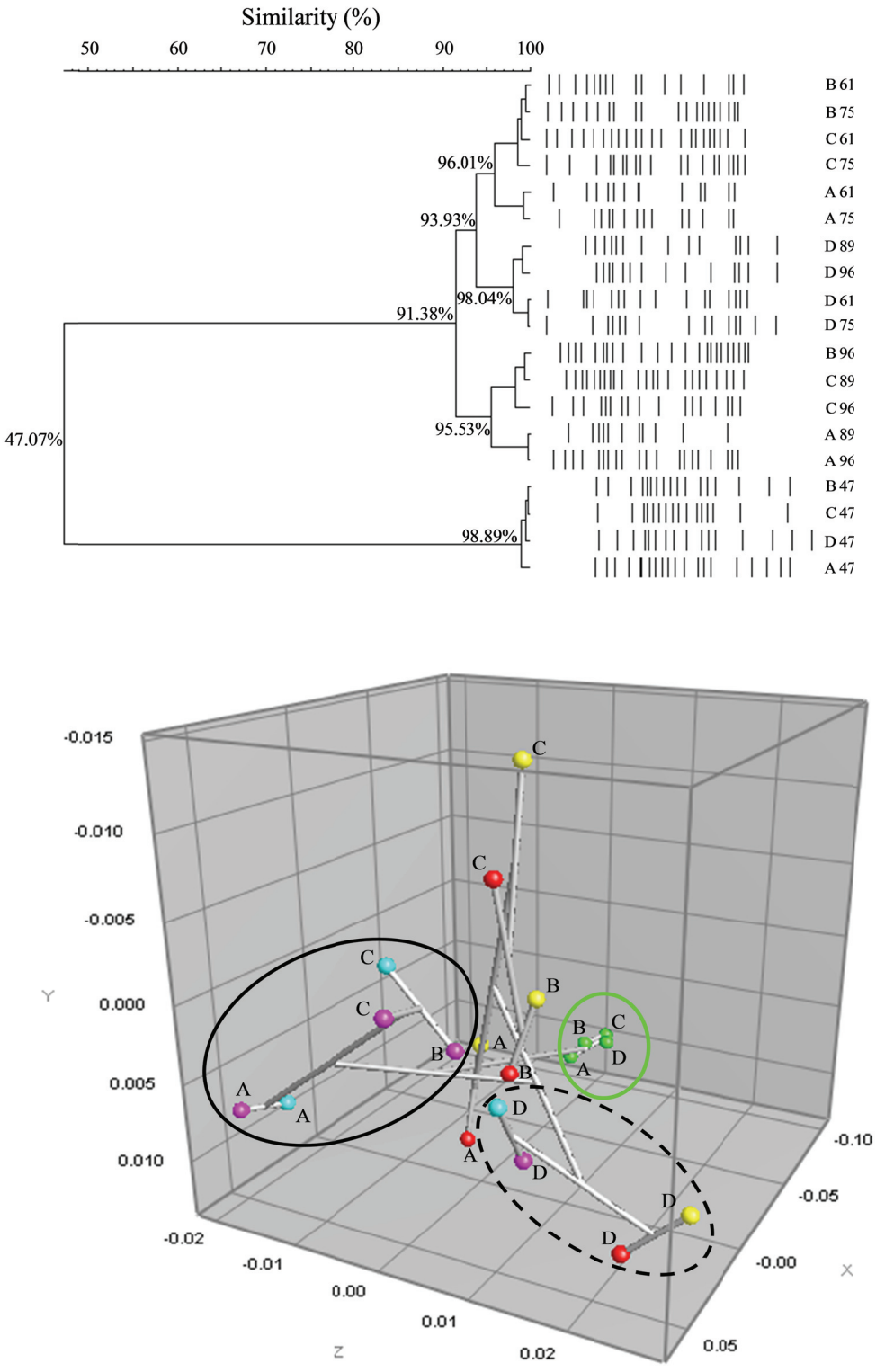

Figure B5 Dendrogram of DGGE pattern similarity (A) and three-dimensional multidimensional scaling (MDS) plot constructed from the matrix of similarity (B) showing the evolution of the microbial communities in the surface sediments during the equilibration phase. Ellipses around the samples indicate similarities in bacterial community fingerprints determined by cluster analysis. Letters designate the respective CWs. Data point colours are: Green, D47; Red, D61; Yellow, D75; Blue, D89; Purple, D96.

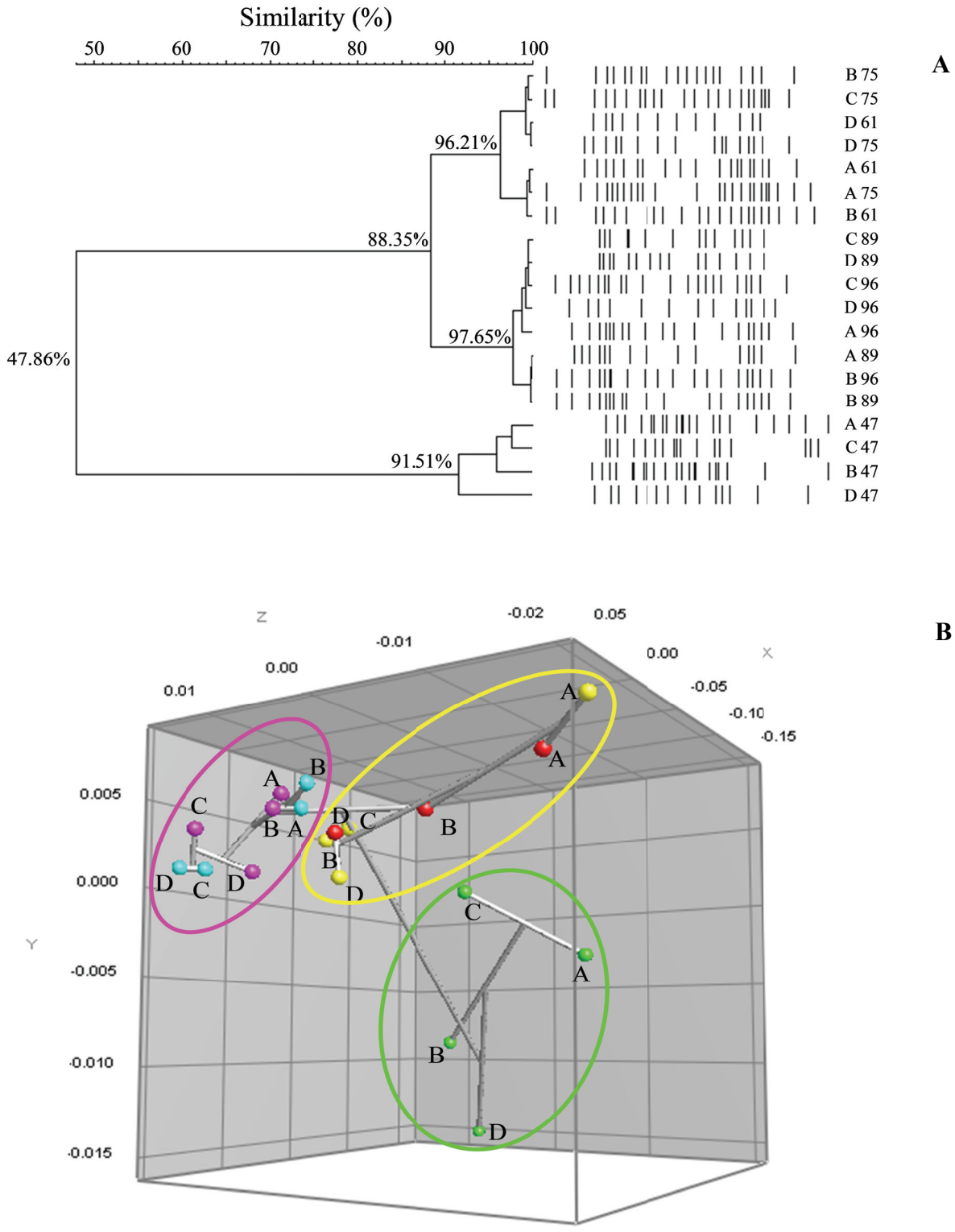

Figure B6 Dendrogram of DGGE pattern similarity (A) and three-dimensional multidimensional scaling (MDS) plot constructed from the matrix of similarity (B) showing the evolution of the microbial communities in the deep sediments during the equilibration phase. Ellipses around the samples indicate similarities in bacterial community fingerprints determined by cluster analysis. Letters designate the respective CWs. Data point colours are: Green, D47; Red, D61; Yellow, D75; Blue, D89; Purple, D96.

B.3.1.2 Changes in microbial biomass and hydraulic conductivity during equilibration

The concentration of solutes and volumes provided to the supplemented CWs were modified at intervals during the 96 day equilibration phase (Table B3); solute concentrations were maintained after day 21, but liquid volumes were adjusted to avoid protracted periods of surface flooding and the generation of anaerobic conditions. The occurrence of surface flooding was observed to be directly linked to reduced hydraulic conductivity, which decreased dramatically between days 47 and 61 for CWs A, B and C (69%, 59% and 56%, respectively; Figure B7.A). The hydraulic conductivity stabilized after day 61 and both volumes and solute concentrations were maintained thereafter. CFU and DNA concentration values are given, respectively, as the mean values ± S.D. of triplicate platings and measurements. ND: not determined.

The constructed wetlands were shown to be biologically stable in terms of the microbial community fingerprint. After 47 days, the hydraulic conductivity of CW D (control) was significantly higher than in the nutrient supplemented CWs ($p < 0.05$; Figure B7). Hydraulic conductivity values ranged from 2.91 to 2.28 $L.m^{-3}.h^{-1}$ in CW D, compared with values of 0.34 $L.m^{-3}.h^{-1}$ (CW A, day 96) and 2.19 $L.m^{-3}.h^{-1}$ (CW B, day 47). As it is highly unlikely that the influent caused clogging of interstitial spaces in the soil matrix due to its dilute nature, the observed decrease in hydraulic conductivity was attributed to an increase in *in situ* biomass (Wu et al., 1997; Weber and Legge, 2010). Accordingly, microbial biomass was investigated using culture independent (total DNA concentration) and culture-dependent (total CFU counts) methods (Figure B7).

Total metagenomic DNA concentrations are generally considered to be a reliable indicator of microbial biomass in soil or sediment environments (Marstorp et al., 2000; Agnelli et al., 2004; Ramond et al., 2009). From day 47, significantly more DNA was extracted from the surface and the deep sediments of CW D than from samples taken from the nutrient-supplemented CWs A, B and C ($p < 0.05$; Figure B7), suggesting that biomass loads were higher and/or that more biomass was produced in the unsupplemented control CW. This result is counter-intuitive, and is inconsistent with cell counts, hydraulic conductivity data and nutrient status. For both surface and the deep sediments, a Pearson's correlation coefficient analysis of the relationship between DNA concentration and hydraulic conductivity typically gave strongly positive values (CW A, $r = 0.91$ and $r = 0.86$; CW B, $r = 0.19$ and $r = 0.66$; CW C, $r = 0.70$ and $r = 0.58$, respectively for the surface and the deep sediments). These results were unexpected and inconsistent with the general concept that hydraulic conductivity is inversely related to biomass (Prochaska et al., 2007; Weber and Legge, 2010), and that nutrient supplementation stimulates microbial growth and biomass production (Wu et al.,

1997). We conclude that total extractable DNA, at least with the extraction procedure used in this study, is not a reliable indicator of microbial biomass in these systems, as also observed in forest humus (Leckie et al., 2004). One possible explanation may be in the physical nature of microbial communities developing in nutrient-supplemented CWs. For example, the formation of biofilms by resident microbial communities may play a role in changing the hydraulic conductivity in the nutrient supplemented CWs (Figure B5.A; Weber and Legge, 2010), as biofilms are typically found on solid substrates submerged in or exposed to an aqueous solution (Sutherland, 2001). Biofilm biomass contains a high proportion of extracellular polymeric material (exopolysaccharide, EPS), representing 85-90% of gross biofilm mass (Frølund et al., 1996; Jahn and Nielsen, 1998; Laspidou and Rittman, 2002), where microorganisms embedded in EPS matrices are less susceptible to lysis and DNA extraction processes. The development of biofilms could provide an explanation for the positive correlation between hydraulic conductivity and DNA concentrations in the nutrient supplemented CWs. The results suggest that the formation of biofilms by developing microbial communities in the nutrient-supplemented CWs may negatively impact on DNA extraction efficiency.

To confirm this hypothesis, microbial biomass was measured using culture-dependent methods (Figure B7.C). No differences were observed in the anaerobic CFU counts retrieved from the four CWs (data not shown). In contrast, from day 61 to the end of the experiment, aerobic CFU values were statistically higher in the nutrient supplemented CWs than in the control CW D ($p < 0.05$). For example, at day 96, CFU values were 7.7, 7.8 and 3.7 times higher in the CWs A, B and C, respectively, than in CW D. We have no immediate explanation for the anomalous data at day 75 where CFU values were similar in all CW systems. Nevertheless, using culture-dependent cell counts as a quantitative parameter of microbial biomass, the equilibration period of the supplemented CWs was estimated to be greater than 75 days. We note however, that culture-dependent methods are not reliable indicators of total microbial biomass or diversity. As the majority of the environmental bacteria are unculturable under laboratory conditions (Amann et al., 1995), such methods inevitably underestimate the genuine abundance of microorganisms present. Plate counts provide a valid comparative estimate of changes in the culturable population, but will be a valid indicator of changes in total biomass only if the culturable species are dominant members of the community. However, an expected inverse relationship between the hydraulic conductivity and cell counts was observed. Based on these results, it was concluded that the hydraulic conductivity of the system is a more useful indicator of CW equilibration in a start-up process, than microbial biomass. Using this parameter, it was concluded that the establishment phase for the CW mesocosms was approximately 61 days.

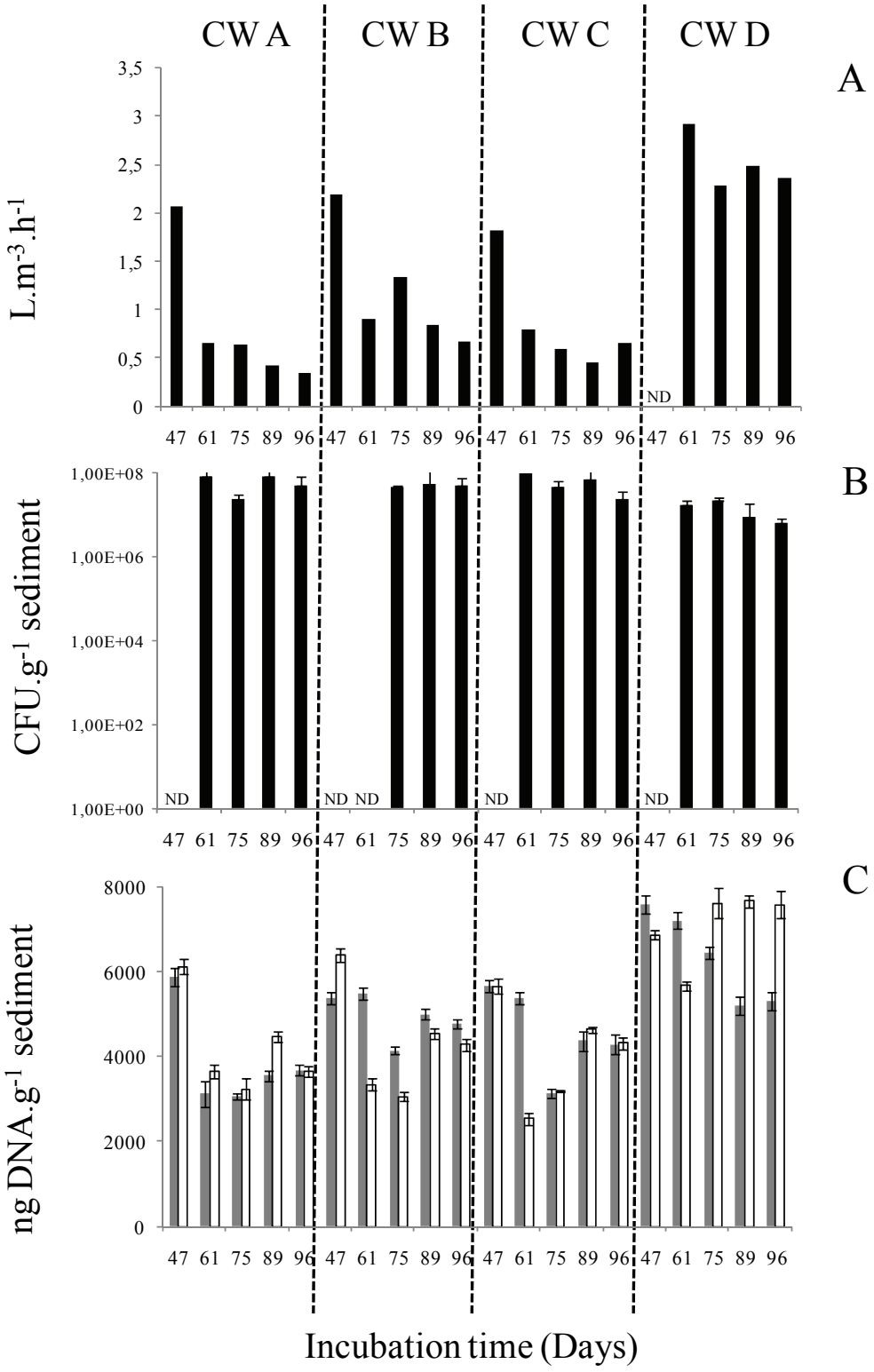

Figure B7 Histograms showing: (A) the changes in hydraulic conductivity (B) the abundance of culturable surface aerobic and mesophilic bacteria and (C) the total DNA extracted from sediment samples, with grey bars representing results from the deep sediment samples and white histograms representing results from the surface sediment samples

B.3.1.3 Discussion and Conclusions

The principal conclusion is that microbial community fingerprinting (using a rapid and relatively simple survey method such as DGGE) is an effective method in monitoring both qualitative and quantitative changes in microbial community structure in CW systems (Nicomrat et al., 2006; Baptista et al., 2008; Ruiz-Rueda et al., 2009). Alternative methods, such as T-RFLP (Sleytr et al., 2009) or ARISA (Kirk et al., 2004) could also be used to generate similar data sets. However, DGGE has the advantages of providing a visual pattern of the community structure and the possibility of assigning phylogenetic affiliations to major bands by re-amplification and sequence analysis. Detailed analyses of the strengths and weaknesses of DGGE and its attendant assumptions are presented elsewhere (Kirk et al., 2004).

The qualitative products of DGGE analysis allow changes in numerically dominant members of the microbial community to be monitored. While other, less dominant, phylotypes may be important functional components of the complex trophic structure, the assumption is made that dominant bands, which respond to an imposed change in the system, are directly affected by the change and that they probably constitute physiologically important components of the response (Niepceron et al., 2010). The value of DGGE in quantitative analyses derives from the ability to acquire kinetic data, in this case the rates of adaptation of the community to a new equilibrated state following some perturbation of the system. DGGE analysis, particularly coupled with post-electrophoretic phylogenetic analysis of dominant bands, can provide detailed physiological data.

It was demonstrated that after 89 days of feeding, the pilot-scale CWs were equilibrated, with an established microbial community presenting a similar fingerprint in the surface and the deep sediments. This emphasized the fact that microbial community fingerprinting with molecular tools (such as DGGE) is a key parameter for monitoring CW establishment before pollutant amendment. The observation that microbial community structure is the slowest parameter to stabilize supports this view. It was also noted that hydraulic conductivity was useful as a determinant of microbial community development and that extractable DNA titre was not a reliable parameter for monitoring microbial biomass.

B3.2 THE EFFECTS OF NUTRIENT ADDITION ON THE DEGRADATION OF ETHANOL IN PILOT-SCALE CONSTRUCTED WETLANDS

These experiments involved the use of pilot-scale CWs, which were amended with ethanol, chosen as a mono-pollutant because of its prominence in winery wastewater. The biodegradation and mineralization of ethanol within the CWs was assessed by effluent analyses, whereby COD, N and P were monitored and quantified. The effect of N and P addition was assessed by amending the CWs with high concentrations of ethanol (COD_t 7587 mg L^{-1}), with and without nutrient supplementation. The hypothesis was that the addition of nutrients (N and P) in an appropriate ratio would lead to an increase in the COD removal efficiency in CWs due to the enhancement of biodegradation. The COD removal performance and the biotransformation of N, as well as effluent N and P and the evolution of bacterial community structures were assessed.

B3.2.1 Analysis of nitrogen and phosphorus measured in the effluent of constructed wetlands amended with ethanol with and without nutrient supplementation

The breakdown and formation of nitrogenous (N) and phosphorus (P) compounds within the experimental CWs was assessed by means of chemical characterization of effluent samples, measuring the content of total nitrogen (TN), nitrate-nitrogen ($N0_3^-$-N), ammonia-nitrogen (NH_3^+-N) and total phosphorus (TP) (Figure B8).

Nitrate was added to the experimental CWs a week prior to ethanol amendment. This resulted in the $N0_3^-$-N (and thus TN) concentration in the effluent being high in the first week of the experiment. Subsequently, the $N0_3^-$-N in the effluent from both experimental systems was similar to that of the control CW. By day 28, a maximum of 6.7 $mg.L^{-1}$ TN was not accounted for by $N0_3^-$-N and NH_4^+-N, but this figure declined to 3.3 $mg.L^{-1}$ by the end of the experiment. The assumption was made that nitrite was accumulating in nutrient amended CW C.

TP concentrations in effluent samples from CW D (ethanol amended) and CW C (ethanol/nutrient amended) were significantly higher than those from CW A (Figure B8).

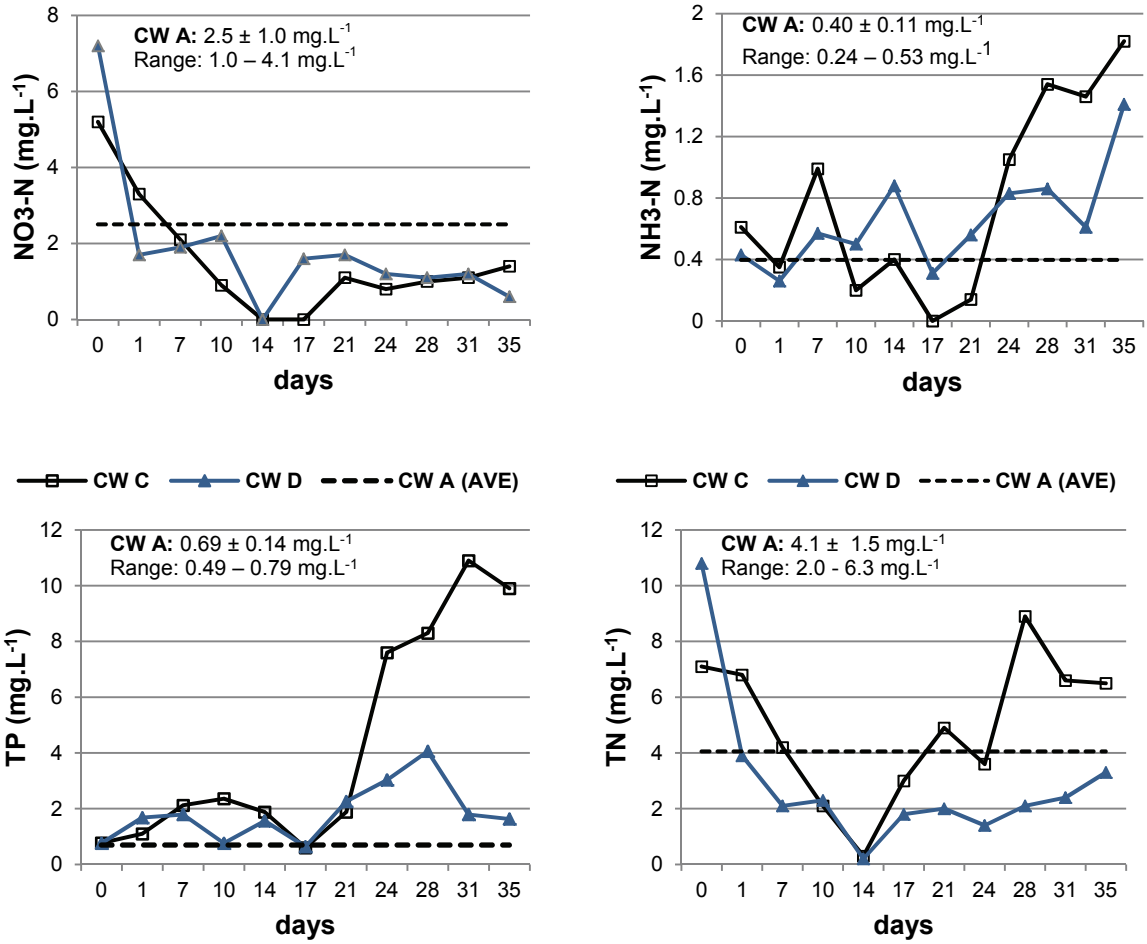

Figure B8 The nitrogen and phosphorus measurements in the effluent from ethanol amended CW (CW D) and ethanol/nutrient amended CW (CW C). The average control CW (CW A) figures are given on the charts as a means of comparison

B3.2.2 COD removal in constructed wetlands amended with ethanol, with and without nutrient supplementation

The COD removal efficiency (%) and effluent COD (COD_e) are shown in Figure B9. Both CW D (ethanol amended) and CW C (ethanol/nutrient amended) differed significantly from CW A (control) (Tukey, $p = 0.05$), in which the COD_e remained close to zero throughout the experiment. In both experimental CW systems, the COD increased after the addition of ethanol on day 1. The COD_e from CW C peaked at a value of 1076 $mg.L^{-1}$ on day 14, while that from CW D peaked at a value of 1471 $mg.L^{-1}$ on day 31. At the end of the experiment, the COD_e concentration from CW C was similar to that of CW A (control), reflecting close to 100% removal efficiency. The COD removal efficiency of nutrient-amended CW C was consistently greater than non-nutrient amended CW D (Figure B9).

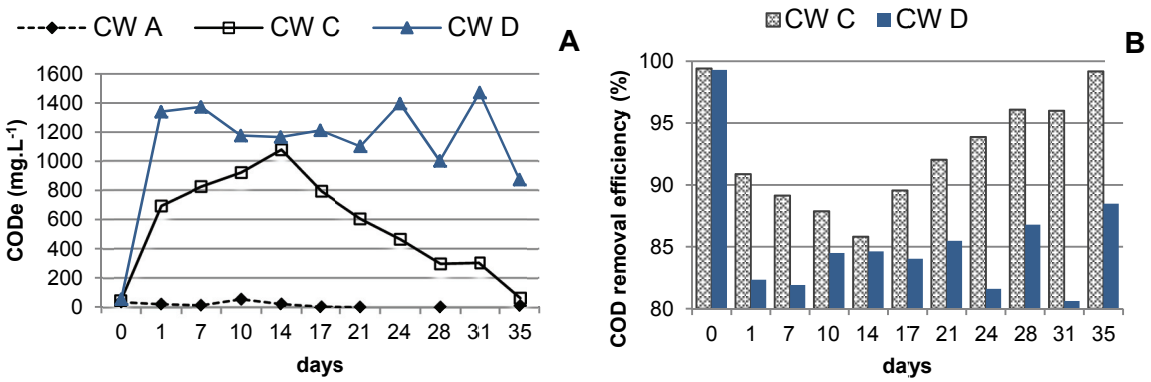

Figure B9 The (A) effluent COD (COD_e) and (B) the COD removal efficiency (COD_e) from control CW (CW A), ethanol/nutrient amended CW (CW C) and ethanol/no nutrients CW (CW D)

B3.2.3 Evolution of the bacterial community structure in constructed wetlands during amendment with ethanol, with and without the addition of nutrients

The evolution of the bacterial community structure was assessed by DGGE fingerprinting and PCA analysis of the CW sediments. Figures B10A and B10B are the 3D-PCA diagrams of the evolution of the bacterial community structure in the surface and the deep sediments respectively. In the surface sediment samples, 47.7% of the spatial variations were explained, with PC1, PC2 and PC3 explaining respectively 20.1%, 15.6% and 11.4% of the variance (Figure B10A). In the deep sediment samples, 41.1% of the spatial variations were explained, with PC1, PC2 and PC3 explaining respectively 16.1%, 14.4% and 10.6% of the variance (Figure B10B). Clustering of baseline communities in the superficial and deep sediments from both inlet and outlet samples showed that, following a three month equilibration period, communities from all CWs were similar (Figure B1A and B10B) (Ramond et al., submitted; section B3.1) These similarities in community composition allowed evaluation of subsequent community changes in response to amendment.

In the surface sediments of CW A (control) and CW C (ethanol/nutrients) the microbial communities evolved similarly throughout the experiment, (essentially along PC2 and PC3, explaining 15.6% and 11.4% of the total variance, respectively), and were closely located on the 3D-PCA representation at each sampling time (Figure B10A). In contrast, for the mid-term (day 28) and final (day 49) samplings, the microbial community fingerprints of CW B (ethanol) were well separated from those of CW A and CW C, and evolved essentially along PC1 and PC3 (explaining 20.1% and 11.4% of the variance respectively) (Figure B10A). It was concluded that at the surface, amendment with ethanol resulted in a severe modification

of the microbial community structure and that supplementation with nutrients (N/P) ameliorated this effect. This occurred independently from the nutrient load which changed during the course of the experiment in CW C. Points representing the inlet and outlet surface samples of each individual CW remained close on the 3D-PCA diagram throughout the experiment (Figure. B10A), indicating that amendment induced minimal spatial modification in the community structure.

In contrast, points representing the deep inlet and outlet sediment samples of each CW diverged on the PCA diagram (Figure B10B), indicating spatial modifications of the microbial community structure in all CWs during the course of the experiment. At the inlet, by day 28 (mid-term of the experiment), the community of CW A (control) was well separated along PC1/PC2 (30.7% of the variance) and PC3 (10.6% of the variance) from that of CW B (ethanol) and CW C (ethanol/nutrients) respectively (Figure B10B). These results suggest that at this stage of the experiment, ethanol amendment, with or without nutrient supplementation, modified the microbial community structure. However, by the end of the experiment (day 49), the community structure of CW C (ethanol/nutrients) was closer to that of CW A (control) than CW B (ethanol). These results suggest that at that stage, ethanol had impacted and modified the community structure, an effect that was not evident when nutrients were added simultaneously (COD/N/P ratio of 200/5/1). In the deep outlet sediments, the points representing CW A (control) and CW C (ethanol/nutrients) were very close on the 3D-PCA diagram throughout the experiment (Figure. B10B), indicating the presence of similar microbial community structures. In contrast, the points representing CW B (ethanol) were distant at each sampling, and evolved strongly along PC1 between day 0 and day 28 (16.1% of variance), and along PC1, PC2 and PC3 between day 28 and day 49 (41.1% of variance). This demonstrated that, throughout the experiment, in the deep outlet sediments ethanol induced a modification of the microbial community structure that was not apparent when nutrients were added.

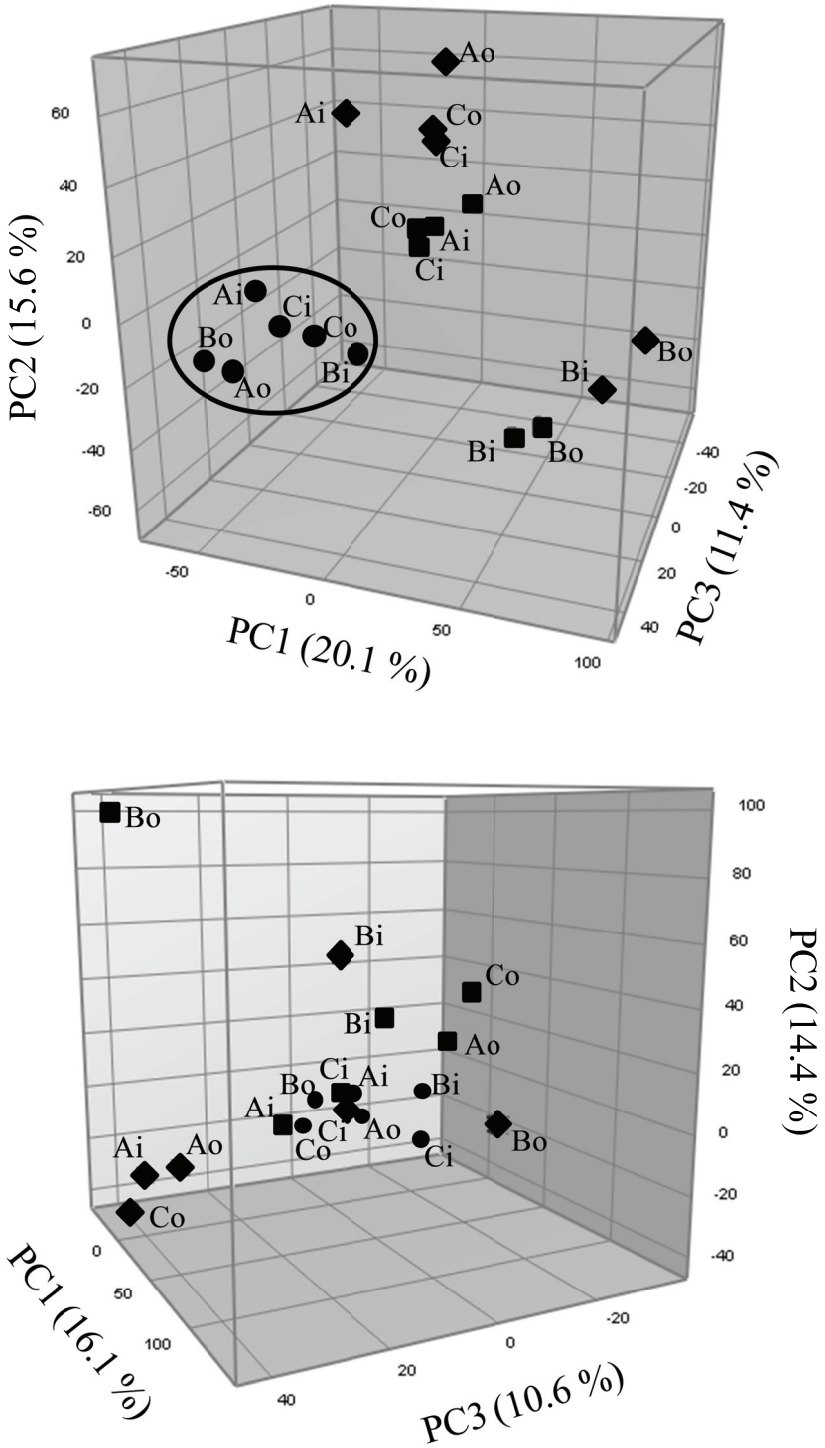

Figure B10 Three-dimensional principal component analysis (PCA) diagram representing the evolution of the microbial communities in the (A) surface sediments and (B) deep sediments of the constructed wetlands. Capital letters close to the points designate the respective constructed wetland: "i" and "o" respectively indicating inlet and outlet samples. ● Initial sampling (day 0) ♦ Sampled on day 28. ■ Final sampling (day 49)

B3.2.4 Discussion and Conclusions

COD removal

The results demonstrated that the addition of nutrients resulted in enhanced COD removal in CW C. After an initial acclimation period of ethanol/nutrient amended CW C, effluent COD (COD_e) values decreased steadily so that by 30 days of operation the removal capacity was similar to that achieved using incremental priming (section B3.3). COD removal by CW D, receiving the same concentration of ethanol but no nutrients, performed comparatively poorly and became septic. In addition, efficient NO_3^--N degradation was observed in CW C. This could be explained by the presence of ethanol as an electron donor and nitrate as an electron acceptor, enhancing the dissimilatory denitrification/nitrate reduction processes. However, the nitrate reductase activity measurements presented analytical difficulties and could not be used as an index of the performance of CW C (see section 3.6).

Nitrogen addition/removal/transformation

Water quality criteria related with NH_4^+-N toxicity in aquatic ecosystems has been recommended in order to protect sensitive aquatic animals as follows: 0.05-0.35 mg L^{-1} for short-term exposures and 0.01-0.02 mg L^{-1} for long-term exposures (US EPA, 1986). In this context, the effluent NH_4^+-N content in the experimental CWs of this study can be considered toxic if released into water-bodies and would need to be subjected to further treatment.

The most likely mechanisms for nitrate degradation in CW C were denitrification (DN) and dissimilatory nitrate reduction to ammonia (DNRA) possibly occurring simultaneously at varying rates (Stevens et al., 1998; Bonin et al., 1998; Fazzolari et al., 1998; Kelso et al., 1997). When $N0_3^-$-N is not limited, the dominance of DN or DNRA bacterial populations is dictated by the competition for carbon sources (Kelso et al., 1997). The DN and DNRA pathways share a reaction step in which $N0_3^-$-N is reduced to nitrite ($N0_2^-$-N) by the nitrate reductase enzymatic system. However, $N0_2^-$-N metabolism follows different reduction pathways, either being reduced to nitric oxide (NO) during DN, or to NH_4^+-N by DNRA (Zumft, 1997). Therefore, the NH_4^+-N in CWs D (ethanol amended) and C (ethanol/nutrient amended) may have been formed, at least to some degree, via the DNRA pathway. The maintenance of higher pH values in sediment samples from the nutrient supplemented CW C (up to 1.3 units higher than CW D, data not shown) supports the hypothesis of occurrence of DNRA, as this process has been suggested to be favoured by relatively high pH values (Stevens et al., 1998). In oxygen limited environments, ammonium oxidizing bacteria (AOB) can out-compete nitrite-oxidizing bacteria (NOB) for oxygen as the mutual electron acceptor

(Park et al, 2010). Results from a number of studies have shown that dissolved oxygen (DO) concentrations from as little as 0.5 mg/L (in a suspended growth system) to 5.0 mg/L (in an up-flow reactor), can cause an accumulation of nitrite (Park et al. 2010). Thus, oxygen deficiency may have contributed to the build-up of nitrite in CW C. Increased effluent concentrations of nitrite and/or ammonia due to oxygen limitation may require the addition of a finishing aeration step to CWs.

Phosphorus in sediments may be complexed with inorganic or organic matter. Co-precipitation with iron and manganese, absorption to oxyhydrides, clays, etc. and association with carbonates represent the three inorganic complexation mechanisms (Wetzel, 2001). The major route of phosphorus solubilization in sediments is by release from Fe(III) oxide under anoxic conditions (Richardson and Craft, 1993; Masscheleyn et al., 1992; Bostrom et al., 1982). Direct reduction by iron-reducing bacteria or indirect reduction by sulphate reducing bacteria (SRB) takes place. Indirect reduction involves the conversion of sulphates to sulphides that complex with iron and release phosphate (Wetzel, 2001). Drying and rewetting also caused desorption of P due to mineralization of organic P by the activation of phosphatase (Song et al., 2007).

Phosphorus addition/removal

The results from CW D suggest that no additional supply of this element was necessary as increased phosphorus solubilization occurred. Ultimately, due to solubilization and leaching, the supply of P would be expended. However, if a CW is situated to capture agricultural run-off, then theoretically the nutrients contained in the run-off should increase wastewater COD removal efficiency and vice versa. There was a sharp increase in the effluent TP from wetland C after 3 weeks. There are two possibilities for this spike, neither being mutually exclusive – breakthrough of influent nutrients and/or increased phosphate solubilization. It was beyond the scope of the study to perform in-depth analyses of complex changes in sediment chemistry, so further information on this topic cannot realistically be provided. However, the increased level of phosphorus in the effluent is of environmental concern and the need to attain the correct influent nutrient balance is important and will be taken into consideration in future studies.

Microbial community structure

In CWs, microbial communities play an important role in the removal of organic matter, nitrogen and phosphorous (Truu et al., 2009; Glick, 2010). Microbial activities induce biogeochemical transformations in natural, managed and engineered ecosystems, especially in response to contamination. Additionally, organic pollutants such as ethanol have been

proven to induce structural changes on microbial communities (Feris et al., 2008). However, in this study the addition of N and P with a COD/N/P ratio of 300/5/1 helped to maintain the microbial community structure in CW C (ethanol/nutrients) at all locations and sampling times except in the deep inlet sediments. These results thus confirmed that the addition of inorganic nutrients at an appropriate ratio, to nutrient deficient biological wastewater treatment processes, enhances the COD removal efficiency which could be linked to the maintenance of a stable microbial community structure.

B3.3 ETHANOL REMOVAL, INCLUDING THE EFFECT OF INCREMENTAL PRIMING ON THE DEGRADATION OF ETHANOL

These experiments involved the use of pilot-scale CWs amended with ethanol, which was chosen as a mono-pollutant because of its prominence in winery wastewater. The biodegradation and mineralization of ethanol within the CWs was assessed by effluent analyses, whereby the starting substrate (ethanol) as well as important metabolic intermediates (acetic acid, propionic acid and butyric acid) were identified and quantified, where appropriate. The primary aim of the experiments was to evaluate the benefits of diluting wastewaters during the start-up phase of CWs used to treat high strength wastewaters, such as winery wastewater. The effects of incremental and non-incremental ethanol amendments on CW stability and function were compared. During incremental amendment, the pollutant concentration was increased at regular intervals (incremental priming). This procedure was based on the hypothesis that exposure of key members of the resident microbial consortia to dilute concentrations of toxins would ultimately lead to tolerance at higher concentrations that may have proven lethal if employed from inception. The overall success of incremental priming was assessed using effluent COD values as the primary indicators of CW function and stability.

B3.3.1 Sediment pH

The pH in the deep sediments of all the CWs remained close to neutral throughout the experimental period. Respective average inlet and outlet values were 7.30 ± 0.43 and 7.38 ± 0.45 (CW A), 7.50 ± 0.29 and 7.61 ± 0.29 (CW B) and 7.79 ± 0.25 and 7.86 ± 0.20 (CW D). The pH from the superficial sediment samples of incrementally primed CW B remained between 7 and 8, except in the last sample from the outlet, where the pH was 6.7. From the initiation of ethanol amendment in unprimed CW D, acidification was observed in the inlet and outlet surface sediments, reaching a final pH of 6 at week 47.

B 3.3.2 Effluent analyses

Total effluent COD results were collated with the relative COD contributions of ethanol and selected metabolites. Mass balances were used to elucidate the metabolic processes involved in the biodegradation and mineralization of ethanol within the experimental CWs.

The COD_e values from the control (CW A) and incrementally primed CW B were low (< 100 $mg.L^{-1}$) for the first 40 weeks, except on two occasions, when the COD_e from CW B was 180 $mg.L^{-1}$ (at 14 weeks) and 104 $mg.L^{-1}$ (at 33 weeks) (Figure B11). The rise in COD_e from incrementally primed CW B to a maximum 365 $mg.L^{-1}$ between 41 and 45 weeks corresponded with an influent ethanol concentration increase from 15 800 $mg.L^{-1}$ COD_{it} to 26 333 $mg.L^{-1}$ COD_{it}. Small amounts of acetic acid, ranging between 13 $mg.L^{-1}$ (14 $mg.L^{-1}$ COD_a) and 96 $mg.L^{-1}$ (103 $mg.L^{-1}$ COD_a), were detected in the effluent at 33 weeks, and again between 38 and 45 weeks, while small amounts of propionic acid, ranging between 8 $mg.L^{-1}$ (13 $mg.L^{-1}$ COD_a) and 69 $mg.L^{-1}$ (118 $mg.L^{-1}$ COD_a) and ethanol, ranging between 13 $mg.L^{-1}$ (27 $mg.L^{-1}$ COD_a) and 106 $mg.L^{-1}$ (214 $mg.L^{-1}$ COD_a) were detected at a maximum influent ethanol concentration (26 333 $mg.L^{-1}$ COD_{it}), as well as one week after a rest period (Figure B12). During this period (41-45 weeks), propionic acid and butyric acid accumulated in the sediments, and the relative contribution of these volatile fatty acids (VFAs) to the total COD_e increased from 0% at 40 weeks (15 800 $mg.L^{-1}$ COD_{it}) to 48% at 45 weeks (26 333 $mg.L^{-1}$ COD_{it}) (Figure B13). CW B was rested (no feeding) during weeks 43 and 44, after which amendment was re-initiated with ethanol at the same concentration as unprimed CW D (7 587 $mg.L^{-1}$ COD_{it}) and at 47 weeks previous function was restored (COD_e of 48 $mg.L^{-1}$).

CW D was amended with a moderate concentration of ethanol from inception (7 587 $mg.L^{-1}$ COD_{it})(Table B1). This addition induced an immediate sharp increase in COD_e attributable mainly to acetic acid and ethanol (Figure B11). Between 34 and 40 weeks, there was a steady decline in COD_e, which generally corresponded to a reduction in the amount of acetic acid and ethanol in the effluent (Figure B12). However, the reduction in acetic acid was accompanied by an increase in the amount of propionic and butyric acid (Figure B12). Between 40 and 47 weeks, there was a second increase in COD_e (Figure B11). The proportion of propionic and butyric acid to the total COD_e increased over time for the first 9 weeks, from 2% to a maximum of 83% at week 43 (Figure B13). The CWs became malodorous due to accumulation of these VFAs in the sediments and the experiment was terminated after 47 weeks.

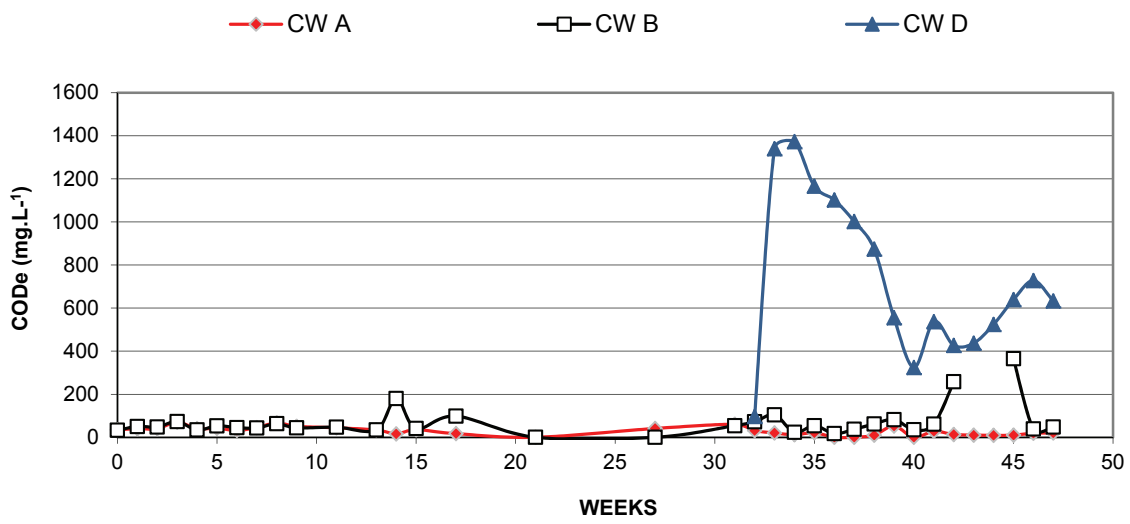

Figure B11 A comparison of the total effluent COD (COD_e) from the control (CW A), incrementally primed (CW B) and unprimed (CW D) constructed wetlands

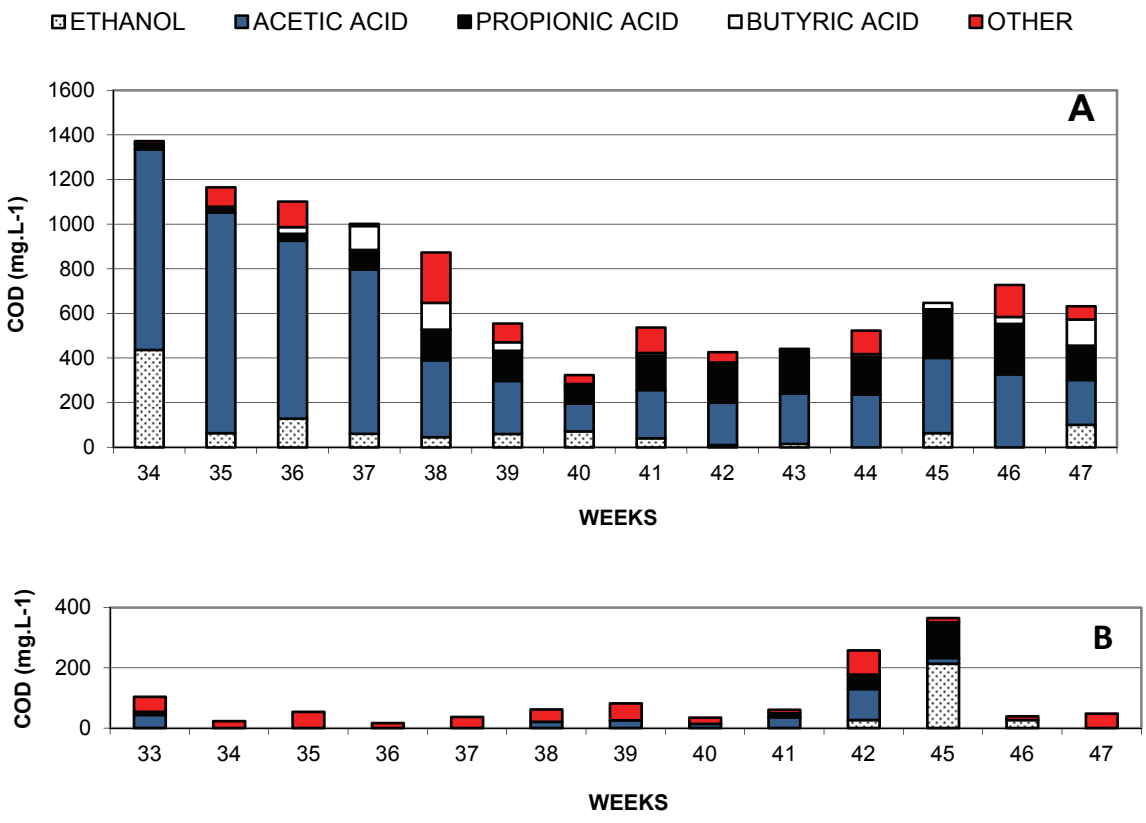

Figure B12 Metabolite profiles of effluent samples from unprimed CW D (A) and incrementally primed CW B (B). The "other" category represents the balance of COD_e once the contribution of ethanol and volatile fatty acids have been deducted

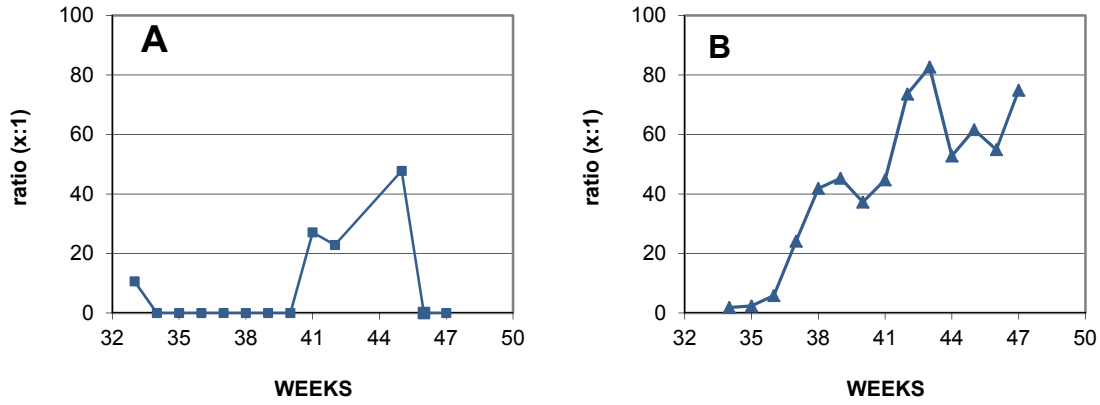

Figure B13 Ratio (%) of propionic acid and butyric acid (COD_a) to total effluent COD (COD_e) from incrementally primed CW B (A), and unprimed CW D (B)

B.3.3.3 The impact of incremental and non-incremental ethanol amendment on CW microbial community structures

B3.3.3.1 The use of T-RFLP to assess changes in the microbial community structure

Terminal-Restriction Fragment Length Polymorphism (T-RFLP) molecular fingerprinting methodology was optimized to assess the impact of contaminants (ethanol, winery wastewater wastes and phenolics) on the CW microbial community structure. Combined with capillary electrophoresis, T-RFLP is more sensitive than other fingerprinting methods requiring gel electrophoresis such as (D/T)GGE (Denaturing/Temperature Gradient Gel Electrophoresis), RISA (Ribosomal Intergenic Spacer Analysis) and SSCP (Single Strand Conformation Polymorphism) (Kirk et al., 2004; Marsh, 1999). Since its development it has widely been used to assess the microbial community structure of various environments, e.g. soils, sediments, contaminated waters, aquifer sediments, termite hind-gut, activated sludge and hypoliths (Ramond et al., 2011; Wong et al., 2010; Li et al., 2007; Calbrix et al., 2007; Bruneel et al., 2006; Braker et al., 2001; Osborn et al., 2000; Liu et al., 1997). Moreover, T-RFLP can be used to assess the diversity of any functional community using PCR amplification of a functional gene of interest, such as the ammonia-oxidizing (*amoA* gene) or the mercury-resistant bacterial community (*merA* gene) (Ramond et al., 2009; Horz et al., 2000).

In this section, two experiments studying the impact of ethanol on the microbial community structures are presented. The first, a 122 day experiment, involved the amendment of CW B with increasing ethanol concentrations ranging from COD_t values of 474 $mg.L^{-1}$ to 1 896 $mg.L^{-1}$. The second experiment compared the impact of ethanol on the community structure

in CW D, which was exposed to a moderate initial ethanol concentration (COD_t 7587 $mg.L^{-1}$) with that of CW B

T-RFLP optimization

The T-RFLP protocol used by Ramond et al. (2009) was optimized by the DNA sequencing unit of the University of Stellenbosch. Using the same PCR amplicons and restriction digests, more individual T-RFs were detected in sediment samples that included an additional purification step in comparison to those that did not (Figure B14, Table B4). It is likely that the additional step removed inhibitory substance/s present in the restriction solution. This clearly demonstrated the necessity of including an additional purification step after the restriction digest to obtain more comprehensive microbial community fingerprints. Also, as more T-RFs were detected than DGGE bands in the 3 samples tested (data not shown) it confirmed that T-RFLP is a more sensitive method for the assessment of CW microbial community fingerprints (Kirk et al., 2004).

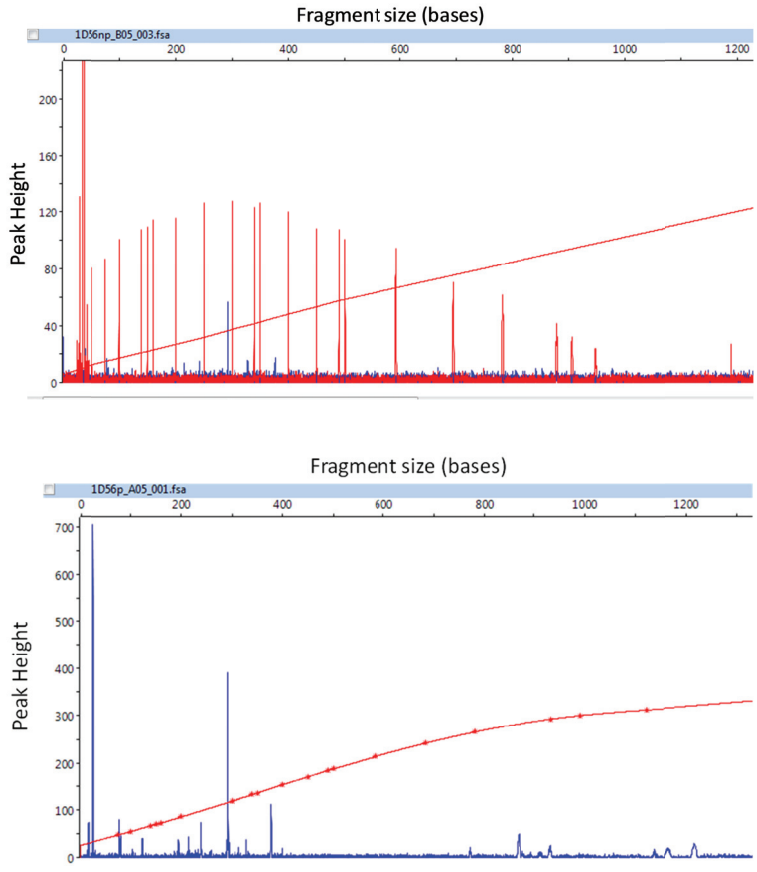

Figure B14 T-RFLP Chromatograms of the same CW sediment sample without (A) or with (B) a purification step after the restriction. In red: the Size ladder (Rox 1.1). In blue: 16S rRNA T-RFLP profile

Table B4 Number of T-RFs detected with or without the purification of the product of the restriction digestion

Inlet surface sediment sample	Control (CW A)		Ethanol amended (CW B)		Winery wastewater (CW C)	
Restriction digest	P	NP	P	NP	P	NP
No of T-RFs detected	25	3	22	0	31	3

P: purification step after restriction digest NP: no purification step after restriction digest

B3.3.3.2 The impact of ethanol on the surface sediment microbial community structure of CW B during incremental priming

The original microbial community structure in CW A (control) at the inlet and outlet was slightly different to that in CW B (ethanol amended), as a spatial variation was observed between these points along axis 2 at day 0 (Figure B15). These samples were taken 3 h after initial amendment. As this time frame is too short to observe microbial adaptive responses, it is suggested that the differences in the microbial community structure were related to ethanol-induced changes in the physico-chemical properties of the sediments which led to the release of attached bacterial populations, rendering them more accessible to DNA extraction (Héry et al., 2003; Ramond et al., 2009). This was confirmed by the fact that the diversity indices were higher in the ethanol amended sediments than in the controls (Table B5). Indeed, the consistent increase of H' and E diversity indices indicated that the number of rare, or less abundant, T-RFs (or OTUs) increased over the course of the experiment and that ethanol led to a selection of a more diverse microbial community in the surface sediments at both the inlet and outlet. Similar findings were encountered in the deep sediment samples (Figure B16, Table B5).

Evolution of the microbial communities at the surface inlet

The microbial community structure in the control (CW A) changed during the course of the experiment, evolving essentially along axis 1 during the first 35 days possibly due to changes in environmental factors such as temperature and/or pH (Figure B15). The community structure in CW B (ethanol amended) also evolved along axis 1 during this period. However, the control and ethanol-amended samples were spatially well separated on the PCA diagram, indicating that the addition of ethanol had modified the microbial community structure. Ethanol induced three community shifts along axis 2 (D35 to D49; D49 to D56; D84 to 112), with an interim stabilization from D56 to D84 (Figure B15). The influent ethanol concentration was doubled at D49 and following important shifts, after 63 days (D112), the community was similar to that at D49. Thus, it took between 49 and 63 days to

obtain a microbial community structure adapted to the presence of ethanol at the inlet surface sediments of CW B.

Evolution of the microbial communities at the surface outlet

At the outlet, all the samples taken from CW B after amendment with ethanol are well separated from those of the control (CW A) on the PCA diagram, clearly indicating the impact of ethanol on the microbial community structure (Figure B15). From D35, all samples from the ethanol impacted communities remain very close, indicating that after 35 days, a microbial community adapted to the presence of ethanol had been selected and that this community structure was not disturbed by increases in the concentration of ethanol applied during the study (Table B1).

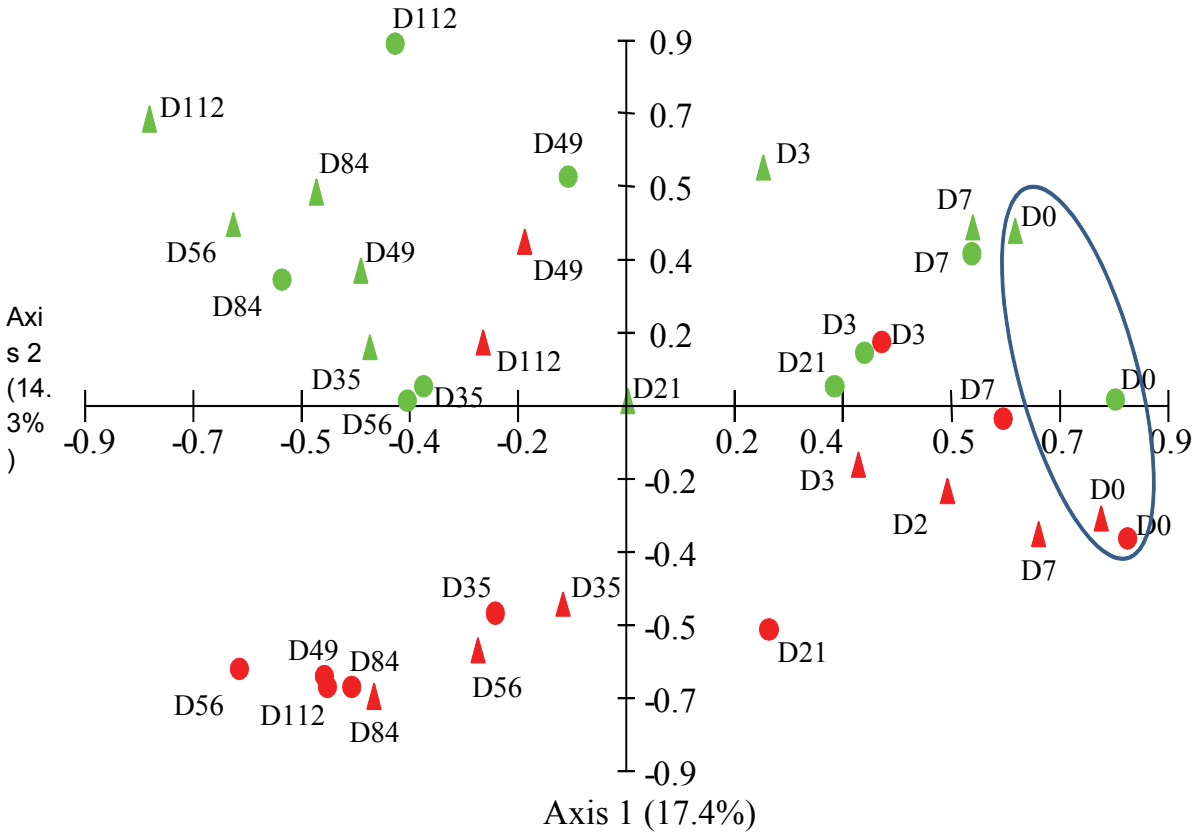

Figure B15 Principal component analysis (PCA) of *Hae*III digested 16S rRNA gene in the surface sediments (A) and deep sediments (B) of the control CW A (in green) and the ethanol amended CW B (in red) ▲ Inlet ● Outlet Numbers indicate the day of the sampling (D0 to D112)

B3.3.3.3 The impact of ethanol on the deep sediment microbial community structure of CW B structure during incremental priming

In contrast to the surface samples, the microbial communities in the samples from the inlet and outlet samples of the deep sediment communities responded differently to ethanol amendment and are well separated on the combined PCA diagram (Figure B16). These communitites are thus analysed using separate PCA diagrams (Figure B17).

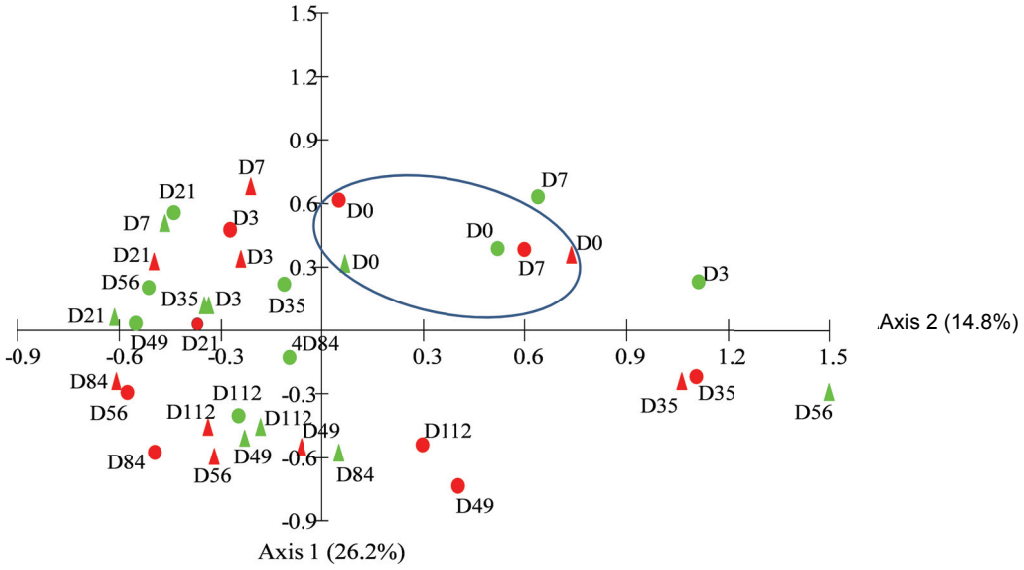

Figure B16 Principal component analysis (PCA) diagram of *Hae*III-digested 16S rRNA gene in the deep sediments of the control CW A (in green) and the ethanol amended CW B (in red) ▲ Inlet ● Outlet Numbers indicate the day of the sampling (D0 to D112)

Evolution of the microbial communities at the deep inlet

In the deep inlet samples, 45.7% of the spatial variation of the samples is explained on the PCA diagram, with the axis 1 and axis 2 contributing to 29.1% and 16.6% of the variance, respectively (Figure B17A). Although the samples from the control and ethanol amended CWs were close on D3, D7, D21, D49 and D112, a significant spatial variation was observed along axis 1 on D35 and D56, suggesting that the microbial community structure was impacted by ethanol after 35 days of exposure and again 7 days (D56) after doubling the influent ethanol concentration (Figure B17A). In addition, the increase in ethanol concentration led to a significant decrease in the microbial diversity, with only 13 T-RFs being present (in comparison to 48 T-RFs in the control at D56) (Table B5). However, the diversity increased again by D84, indicating that although the ecosystem had been impacted, it was able to adjust to the new environmental parameters (Table B5).

The D49 and D112 samples of both the control and the ethanol amended CWs are close on the PCA graphical representation (Figure B16 and B17A) suggesting a resilience of the original microbial community to ethanol amendment. Thus, the impacted microbial communities needed 49 days to adjust to the initial ethanol amendment concentration and between 35 and 63 days (D84 to D112), to adjust to an increase (doubling) in concentration (Table B1).

Evolution of the microbial communities at the deep outlet

In the deep outlet samples, 47.1% of the spatial variation of the samples is explained in the PCA diagram, with the axis 1 and axis 2 contributing to 29.1% and 18.0% of the variance, respectively (Figure B17B). An important spatial variation between the control and ethanol amended CW samples suggests that the microbial community structure was impacted by ethanol throughout the experiment at the outlet (Figure B17B).

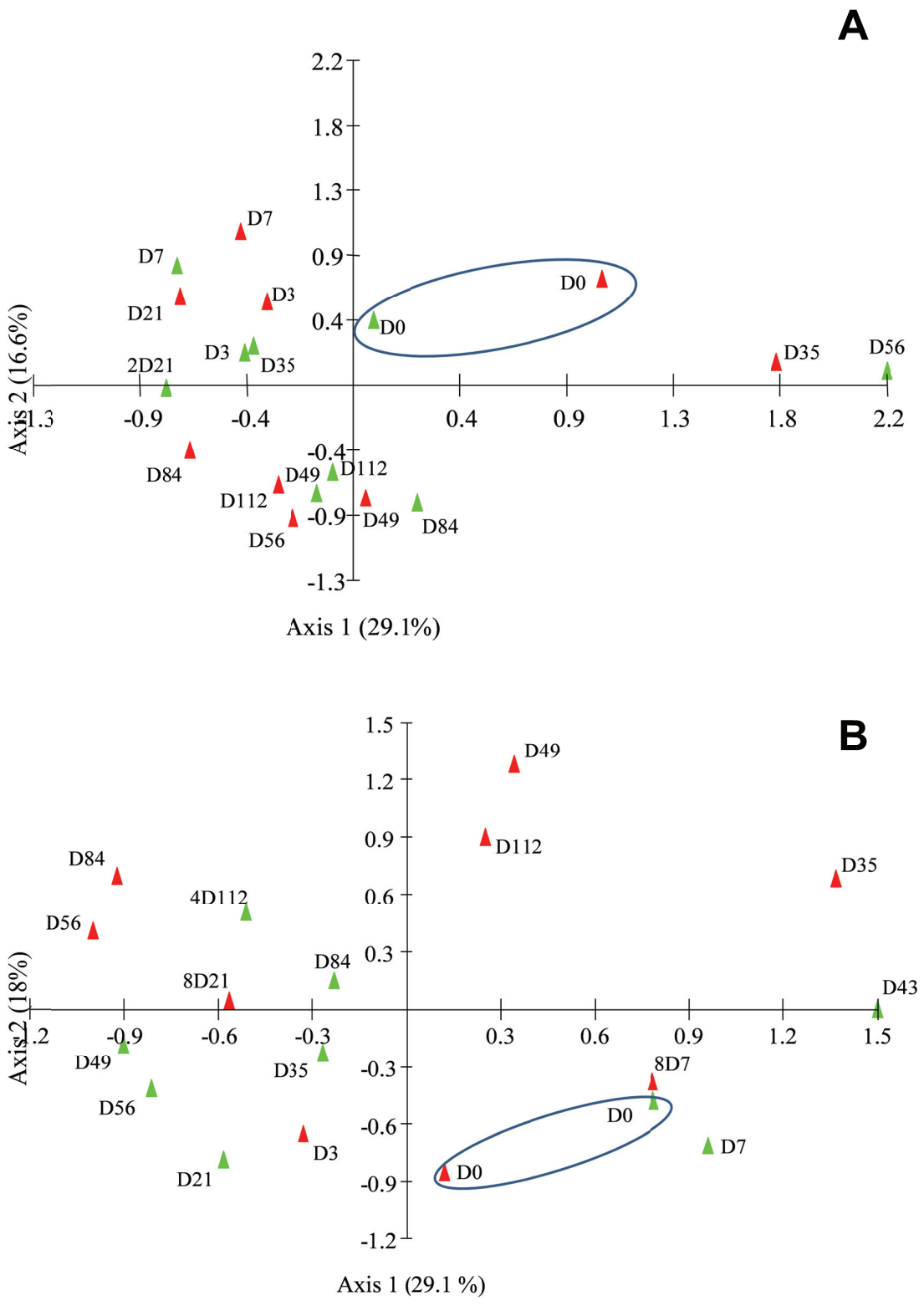

Figure B17 Principal Component Analysis (PCA) of *Hae*III-digested 16S rRNA gene in the deep sediments of the control CW A (in green) and the ethanol amended CW B (in red) at the inlet (A) and the outlet (B)
Numbers indicate the day of the sampling

Table B5 Diversity indices in the sediments of CW A (control) and CW B (ethanol amended via incremental priming)

Day (CW)	Surface inlet			Surface outlet		
	H'	E	S	H'	E	S
0 (CW A)	3,575	0,793	91	3,761	0,863	78
0 (CW B)	3,669	0,806	95	3,575	0,818	79
3 CW A)	3,959	0,823	123	4,265	0,916	105
3 (CW B)	4,301	0,894	123	4,169	0,831	151
7 (CW A)	3,692	0,878	67	3,073	0,943	26
7 (CW B)	3,295	0,811	58	4,123	0,877	110
21 (CW A)	3,792	0,890	71	4,011	0,889	91
21 (CW B)	3,853	0,835	101	3,635	0,874	64
35 (CW A)	3,970	0,880	91	3,609	0,850	70
35 (CW B)	4,125	0,888	104	3,894	0,910	72
49 (CW A)	3,910	0,873	88	3,005	0,673	87
49 (CW B)	3,518	0,741	115	4,164	0,948	81
56 (CW A)	3,634	0,877	63	3,906	0,939	64
56 (CW B)	4,157	0,966	74	3,857	0,976	52
84 (CW A)	3,057	0,950	25	3,634	0,944	47
84 (CW B)	3,781	0,913	63	3,851	0,933	62
115 (CW A)	3,055	0,823	41	3,378	0,893	44
115 (CW B)	3,615	0,825	80	4,075	0,915	86
Day (CW)	**Deep inlet**			**Deep outlet**		
	H'	**E**	**S**	**H'**	**E**	**S**
0 (CW A)	3,783	0,863	80	2,529	0,558	93
0 (CW B)	3,257	0,698	106	3,650	0,788	103
3 CW A)	3,524	0,857	61	3,238	0,762	70
3 (CW B)	4,024	0,860	108	3,862	0,867	86
7 (CW A)	3,711	0,862	74	3,144	0,704	87
7 (CW B)	3,504	0,905	48	3,514	0,754	106
21 (CW A)	3,798	0,913	64	3,998	0,884	92
21 (CW B)	3,730	0,884	68	4,011	0,889	91
35 (CW A)	3,963	0,943	67	3,939	0,867	94
35 (CW B)	3,259	0,757	74	2,786	0,720	48
49 (CW A)	3,292	0,905	38	3,965	0,908	79
49 (CW B)	3,650	0,911	55	3,151	0,901	33
56 (CW A)	2,493	0,644	48	3,859	0,899	73
56 (CW B)	2,356	0,919	13	3,818	0,918	64
84 (CW A)	3,506	0,916	46	3,536	0,934	44
84 (CW B)	3,575	0,866	62	3,543	0,906	50
115 (CW A)	3,623	0,859	68	3,726	0,874	71
115 (CW B)	3,685	0,900	60	3,408	0,805	69

B3.3.3.4 The impact of incremental ethanol amendment (CW B) compared to non-incremental amendment (CW D) on the surface sediment microbial community structure

Baseline surface sample points representing CW B (ethanol amended, incrementally primed) and CW D (ethanol, unprimed) were close to those of the control on the PCA diagram (Figure B18), indicating that the microbial community structures were similar prior to amendment. Baseline microbial community diversity at the surface was similar in all CWs, except at the outlet in CW D, where the diversity was increased. From week 37, in most of the samples from CW B and CW D, there was an increase in the microbial diversity in comparison to the control. The exception was in the surface outlet sample from CW B taken at 43 weeks, where there was a decrease in diversity, presumably associated with an increase in ethanol concentration from 15 800 $mg.L^{-1}$ to 26 333 $mg.L^{-1}$ COD_t.

In the surface sediment, 60.1% of the spatial variation of the samples is explained in the PCA diagram, with axis 1 and axis 2 contributing to 43.5% and 16.6% of the variance, respectively (Figure B18). Two distant, but distinct groups can be observed on axis 2 which consist of: (i) points representing the control CW samples and (ii) points representing ethanol amended CW samples (with the exception of those taken from CW D during the first five weeks after initial amendment). This shows that at concentrations ≥7 587 $mg.L^{-1}$ COD_t, ethanol led to the selection of an adapted microbial community. In the case of incrementally primed CW B, the community was functionally adapted to degrade high concentrations of ethanol (15 800 $mg.L^{-1}$ COD_t), while in the case of CW D, although the community composition was similar, functional adaptation to ethanol (7 587 $mg.L^{-1}$ COD_t) was not apparent and there was a build-up of VFAs in the effluent (Figure B18; section 3.3.2; section 3.3.3). Similar loss of degradative function also occurred in incrementally primed CW B when the ethanol amendment concentration was increased to 26333 $mg.L^{-1}$ COD_t, but prior function was restored at week 45, when the ethanol concentration was reduced to 7 587 $mg.L^{-1}$ COD_t (Figure B11, Table B1). In all cases, COD removal efficiency was not linked to changes in the microbial community composition (Figure B18; Table B6).

B3.3.3.5 The impact of incremental ethanol amendment (CW B) compared to non-incremental amendment (CW D) on the deep sediment microbial community structure

In the deep inlet sediment, 30.1% of the spatial variation of the samples is explained in the PCA diagram, with axis 1 and axis 2 contributing to 21.4% and 13.7% of the variance, respectively (Figure B19A). Although most of the points representing the control (CW A) are well separated from those representing the ethanol-amended CWs, an anomalous result

was obtained, whereby the baseline sample from CW D grouped with the ethanol amended sediment samples and not the control sediment sample, making it difficult to compare the impact of incremental priming on the microbial communities exposed to ethanol at the deep inlet. There was an increase in the diversity of both ethanol amended CWs in comparison to the control (Table B7). In the deep outlet, 38.2% of the spatial variation in the microbial community structure is explained in the PCA diagram, with axis 1 and axis 2 contributing to 24.3% and 13.9% of the variance, respectively (Figure B19B). Apart from the baseline samples, points denoting the microbial communities in the deep outlet sediments fall into 3 distinct groups: (i) samples taken from the control (CW A) from week 34 to 45; (ii) samples taken from ethanol-amended, unprimed CW D from baseline (week 30) to week 37; and (iii) samples taken from incrementally primed CW B and those taken from CW D from week 43 to the end of the experiment at week 45 (Figure B19B), showing that adaptation of the microbial communities had taken place. As with the microbial communities in the surface sediments, this adaptation was not related to incremental priming or functionality.

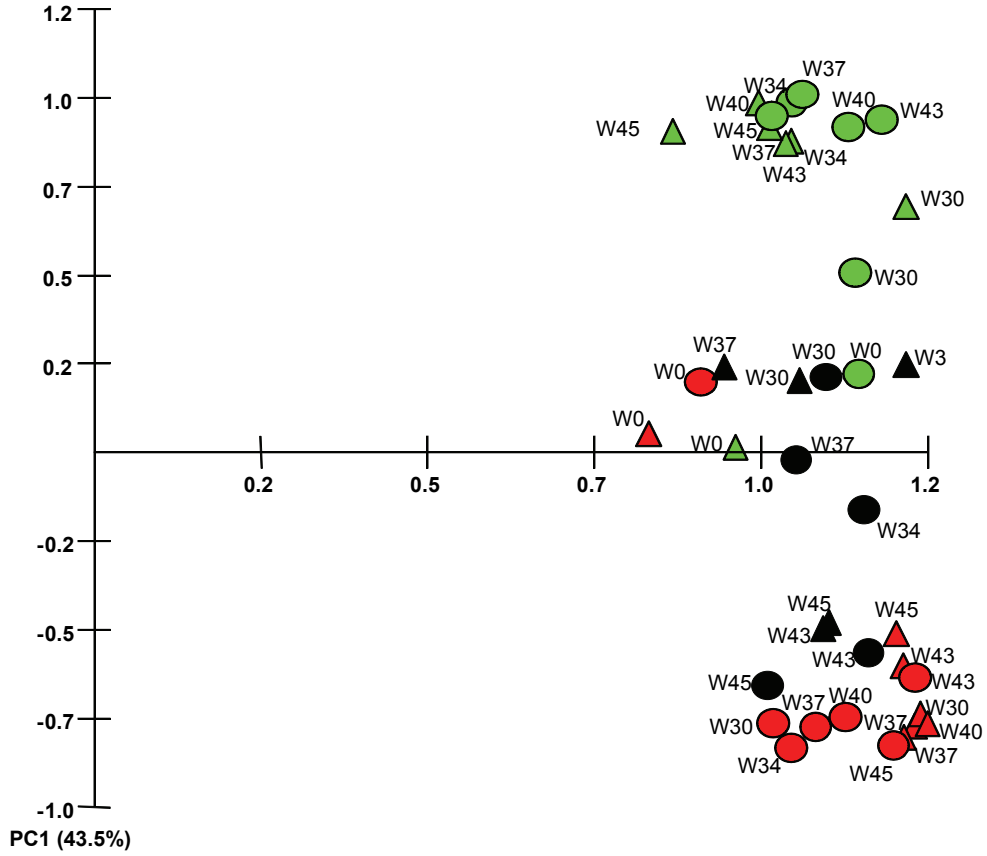

Figure B18 Principal component analysis (PCA) of *Hae*III-digested 16s rRNA gene in the surface sediments of the control CW A (in green) and ethanol amended, incrementally primed CW B (in red) and ethanol amended, unprimed CW D (in black) ▲ inlet ● outlet Numbers indicate the week (W) of sampling

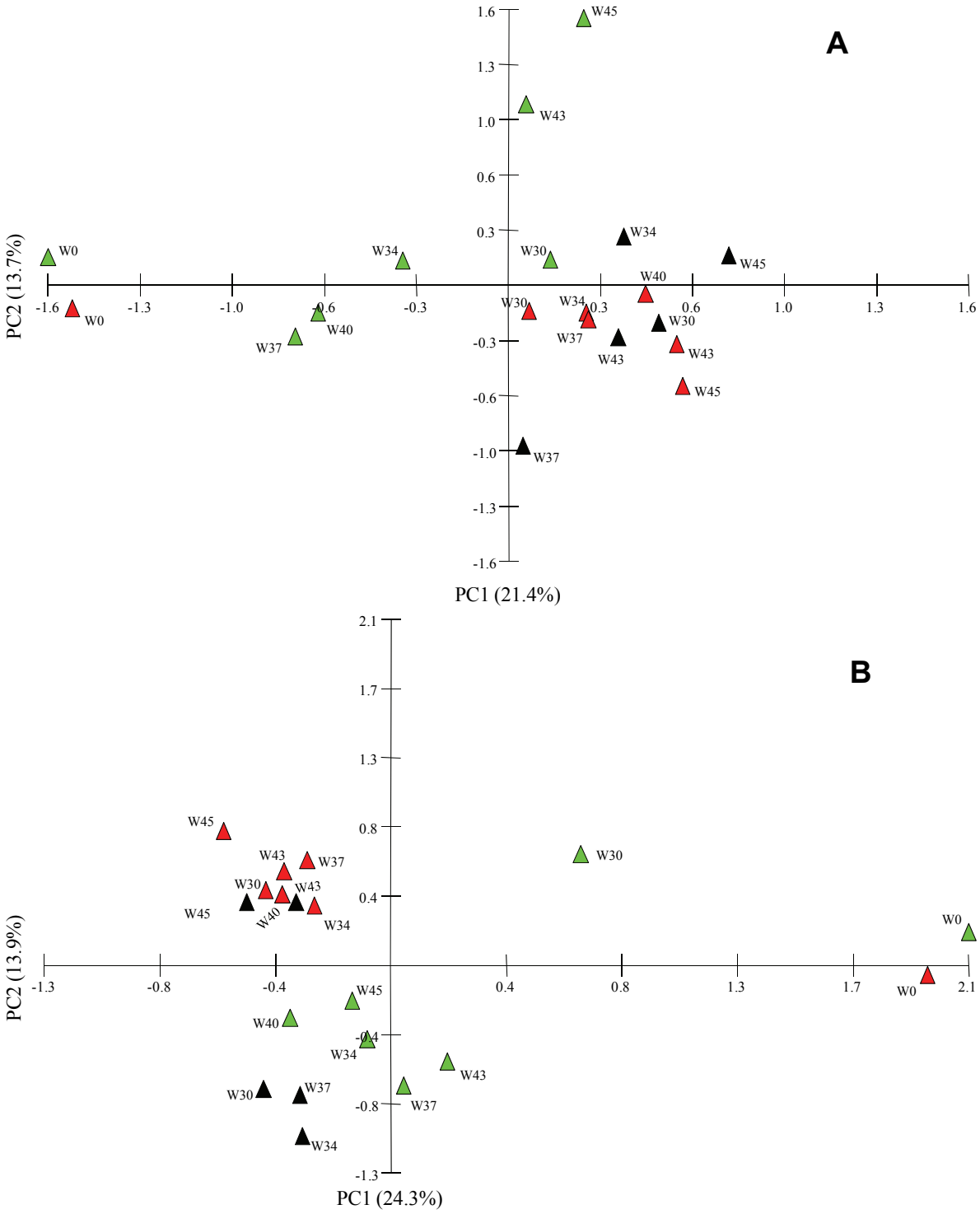

Figure B19 Principal component analysis (PCA) diagram of *Hae*III-digested 16S rRNA gene in the (A) deep inlet and (B) deep outlet sediments of the control CW A (in green), incrementally primed CW B (in red) and unprimed CW D (in black)
▲ inlet ● outlet Numbers indicate the week (W) of sampling

Table B6 Diversity indices in the sediments of CW A (control), CW B (ethanol amended via incremental priming) and CW D (ethanol amended, unprimed)

week (CW)	Surface inlet			Surface outlet		
	H'	E	S	H'	E	S
0 (A)	2.398	0.705	30	3.004	0.838	36
0 (B)	2.555	0.744	31	2.723	0.801	30
30 (A)	3.319	0.848	50	2.981	0.926	25
30 (B)	3.560	0.892	54	2.721	0.880	22
30 (D)	3.467	0.865	55	3.565	0.907	51
34 (A)	2.508	0.690	38	2.382	0.929	13
34 (B)	3.217	0.866	41	2.315	0.636	38
34 (D)	2.935	0.813	37	3.551	0.933	45
37 (A)	2.924	0.852	31	2.930	0.838	33
37 (B)	3.225	0.847	45	3.301	0.872	44
37 (D)	3.270	0.864	44	3.105	0.881	34
43 (A)	3.052	0.881	32	3.066	0.810	44
43 (B)	3.530	0.889	53	3.080	0.847	38
43 (D)	3.183	0.836	45	3.497	0.877	54
45 (A)	2.192	0.690	24	2.287	0.686	28
45 (B)	3.371	0.871	48	3.201	0.874	39
45 (D)	3.314	0.866	46	3.158	0.862	39
Week (CW)	**Deep inlet**			**Deep outlet**		
	H'	E	S	H'	E	S
0 (A)	2.681	0.737	38	1.304	0.508	13
0 (B)	2.652	0.834	24	2.205	0.835	14
30 (A)	3.216	0.905	35	2.681	0.911	19
30 (B)	3.447	0.866	49	2.933	0.832	34
30 (D)	3.452	0.912	44	3.458	0.958	37
34 (A)	3.202	0.862	41	3.379	0.893	44
34 (B)	3.580	0.935	46	3.345	0.889	43
34 (D)	3.420	0.874	50	3.400	0.904	43
37 (A)	2.195	0.759	18	3.184	0.852	42
37 (B)	2.858	0.825	32	3.208	0.943	30
37 (D)	3.113	0.890	33	3.487	0.945	40
43 (A)	3.207	0.858	42	3.022	0.978	22
43 (B)	3.434	0.908	44	3.403	0.916	41
43 (D)	3.140	0.851	40	3.335	0.938	35
45 (A)	2.578	0.811	24	3.469	0.917	44
45 (B)	2.917	0.850	31	3.282	0.902	38
45 (D)	3.163	0.852	41	3.368	0.933	37

In the case of CW C, the experimental procedure commenced after 30 weeks to allow for direct comparisons with incrementally primed CW using the same influent ethanol concentration (CODt 7 587 mg.L^{-1}). Week 0 serves as the baseline for CW A and CW B, while week 30 serves as the baseline for CW D. Data in Table B5 cannot be compared to the above data due to differences in T-RFLP methodology that was employed in each case.

B3.3.4 Discussion and conclusions

Bacterial species may adapt over time to new environmental factors, a process known as acclimation. Examples of successful acclimation to toxic chemicals (e.g. acrylonitrile and *p*-nitrophenol), substrates (e.g. cellulose) and physical parameters (e.g. cold) have been widely reported in the literature (Cheng et al., 2010; Koda et al., 2002; Hu et al., 1997; Zaida et al., 1996). Acclimation and degradation are influenced by the toxicity of a compound as well as the duration of acclimation with longer acclimation times leading to higher degradation rates (Chou et al., 1979). A link has also been demonstrated between acclimation and chemical concentration, with the period of acclimation being shortened at lower concentrations (Zaida et al., 1996). The acclimation phenomenon was used as a premise for the incremental priming procedure adopted in this study. It was hypothesized that exposure to incrementally increasing concentrations of ethanol would optimize the acclimation of the microbial consortium in CWs, and manifest in superior ethanol degradation when compared to a non-acclimated population. COD removal and the analysis of key metabolites were used as the primary means of assessing the success of incremental priming. This discussion includes a critical comparison of CW operation and function by comparison with literature data pertaining to CWs used in the treatment of winery wastewater. A summary of operational and performance parameters taken from the literature is presented in Table B7.

Data on influent composition show the considerable variation in influent COD (COD_i) that is attributed to both the characteristics of the winery wastewater and the pre-treatment steps involved (Table B7). Primary treatment, by means of anaerobic digestion, sand pre-filters or Imhoff tanks, and combined processes, have been reported as methods used to reduce suspended solids (SS) and the organic loading rate (OLR) (Serrano et al., 2010; Grismer et al., 2003a; Masi et al., 2002; Shepherd et al., 2001).

Wastewater with a high oxygen demand may be harmful to the environment, rendering it unsuitable for direct discharge. As such, the use of COD removal efficiency ($\%COD_i/COD_e$) as the sole criterion to assess the performance of biological systems treating highly variable wastewaters is debatable. For example, taking COD_i values realistic for winery wastewater of 20 000 $mg.L^{-1}$, 5 000 $mg.L^{-1}$ and 2 000 $mg.L^{-1}$, removal efficiencies of 95%, 75% or 50% respectively all result in the same effluent COD (COD_e) concentration of 1 000 $mg.L^{-1}$, an oxygen demand which may still render the water unsuitable for direct discharge.

Many wineries use treated wastewater for irrigation, and in most countries discharge limits apply (Christen et al., 2010; Mulidzi, 2010; Melamane et al., 2007; Sheridan, 2007; Masi et al., 2002). According to the National Water Act (no 36 of 1998), biodegradable industrial

wastewater in South Africa may be used for irrigation purposes provided the COD is either < 400 $mg.L^{-1}$ or < 5 000 $mg.L^{-1}$ for volumes of < 0.5 or < 0.05 $ML.day^{-1}$, respectively. If there is any danger of biodegradable wastewater contaminating a watercourse, even lower discharge limits apply. Thus, in circumstances where the wastewater is to be used directly for irrigation or discharged into the environment, COD_e is a critical parameter.

The COD removal performance of incrementally primed CW B and unprimed CW D confirmed that incremental priming substantially increased the biodegradative capacity of CW B (Figure B11, Table B7). COD removal in CW B was excellent (COD_i 58 ± 25) at a theoretical influent COD (COD_{it}) of 15 800 $mg.L^{-1}$ and OLR 112 $gCOD.m^{3-1}$. In contrast, unprimed CW D, amended with a comparatively moderate COD_{it} of 7 587 $mg.L^{-1}$ (OLR 54 $gCOD.m^{3-1}$), did not realize the same efficiency and became septic with the accumulation of VFAs (VFAs and or sulphides in wastewater provide evidence of septic conditions) (Bachman et al., 2007; Magro et al., 2005). There was an adaptation of the microbial communities to amendment with ethanol that was not related to incremental priming. Although the adapted community structures in the sediments of incrementally primed CW B and non-primed CW D, the former were functionally adapted to degrade ethanol while the latter were not.

VFAs were detected in the sediments of unprimed CW D (7 587 $mg.L^{-1}$ COD_{it}), from inception to the completion of the experiment. The pattern showed a decrease in the amount of acetic acid accompanied by an increase in the amount of propionic and butyric acids, with the latter two VFAs accounting for up to 83% of the effluent COD after 9 weeks (Figures B12 and B13). By comparison, neither of these VFAs were found in the effluent of incrementally primed CW B until the COD_{it} was at a maximum concentration (26 333 $mg.L^{-1}$). Even at this high influent COD, propionic and butyric acids only accounted for a maximum 48% of COD_e (Figures B12 and B13). The storage of high organic wastewaters of winery and distillery origin is known to result in the formation of malodorous VFAs (Bories et al., 2005). Although VFAs are usually readily biodegradable, when alternative electron acceptors such as oxygen and nitrates are unavailable, acidogenic accumulation of VFAs may cause inhibition of less tolerant organisms (Lasko et al., 1997). Heterotrophic respiratory pathways are energetically more favourable than fermentative pathways, occurring at faster rates (Atlas, 1997). The acidification of the surface sediments in unprimed CW D was thus almost certainly related to the faster aerobic production of acetic acid in comparison to the deep sediments. The pH trends in the sediments of the control (CW A) were not explained by amendment practices, which remained constant throughout (data not shown). There was also no significant correlation between sediment pH and temperature in CW A (Pearson's correlation coefficient $r < 0.2$), suggesting that biological processes were responsible for small changes in

sediment pH. In general, the pH values in all the sediments were considered optimal as most soil bacteria function best at intermediate and slightly alkaline pH (Verheye, 2002). The differences in metabolic profiles of the incrementally primed CW B and unprimed CW D suggest that acclimation of the microbial community in CW B resulted in superior ethanol mineralization, possibly as the result of enhanced interspecies hydrogen transfer (Figure B12) (Lasko et al., 1997). Furthermore, CW B proved resilient to a short period of overloading, as evidenced by the low COD_e, with no VFA accumulation, at 46 and 47 weeks (Figure B12).

COD_{it} concentrations between 15 800 and 26 333 $mg.L^{-1}$ resulted in decreased COD removal efficiency in CW B (Figure B11). The COD_{it} difference between these two amendment concentrations was thus considerable (close to 10 000 $mg.L^{-1}$), with the theoretical end point for stable CW function falling into this range. Process inhibition associated with high COD_i has been reported in CWs used to treat agricultural and industrial wastewaters (Vymazal and Krőpfelová, 2009). However, COD_i concentrations in winery wastewater from as low as 2 178 ± 1 715 $mg.L^{-1}$, have also been associated with poor treatment performance, with low influent pH (possibly due to VFAs) and/or high concentrations of inhibitory substrates such as polyphenols being suggested as contributing factors (Montalvo et al., 2010; Serrano et al., 2010; Masi et al., 2002). Recirculation of treated effluent to the system head, to dilute highly concentrated wastewater, has proved to be a successful mechanism to ameliorate the negative effects of organic overload (Serrano et al., 2010). Recirculation can neutralize acidic wastewaters and reduce the concentration of biological inhibitors (Serrano et al., 2010). Provided sufficient storage or CW capacity is available, this is a simple and cost-effective solution to maintain CW function.

Many conventional systems have been evaluated for the treatment of winery wastewater, with organic loading rates (OLRs) in a similar range to those applied to CWs used for the same purpose (Andreottola et al., 2009). However, the hydraulic retention times (HRTs) in CWs are in the order of days (Table B7), while those of most conventional systems are in the order of hours (Mulidzi, 2010; Andreottola et al., 2009; Mena et al., 2009; Grismer et al., 2003b; Petruccioli et al., 2002; Shepherd et al., 2001). In the case of CWs, the loading rate is usually expressed as the load applied to the surface area and does not take the depth of the CW into account. However, anaerobic carbon mineralization is an important pathway for degradation of organic matter in anaerobic soils and wetland sediments (Küsel and Drake, 1999; Segars and Kengen, 1998). Thus, CW depth has been included in loading calculations in this section of the report. Despite high COD_{it}, the COD_e concentrations from CW B were low when compared to literature values, signifying the success of incremental priming in improving CW biodegradative capacity (Table B7).

COD removal in CWs can be substantially enhanced by increasing the HRT (Table B7) (Mulidzi, 2010). HRT is dictated by flowpaths and the extent to which the wastewater interacts with the wetland porous medium and plants (Grismer et al., 2003 b). Apart from the demonstrated benefits of incremental priming in CW B, the COD removal process was almost certainly enhanced by the long HRT (Table B7), which was largely dictated by the slow hydraulic characteristics of the sand medium. Fundamentally, these hydraulic characteristics, together with the VSSF mode of operation, precluded the application of a higher OLR without increasing the COD_{it} to toxic levels. It can be hypothesized that a complex sand medium provides a better nutritional habitat than gravel for the growth of many microorganisms. Nevertheless, the additional capacity demanded by an inflexibly low hydraulic conductivity (HC) is a drawback that must be taken into consideration during CW design.

Amendment of the CWs with ethanol with and without the application of incremental priming, led to the selection and evolution of similar microbial community structures. However, the functional capability of the microbial population in terms of ethanol mineralization was increased by incremental priming. It is hypothesized that the incremental priming procedure facilitated the formation of biofilms. Biofilms can enhance the degradation of contaminants by bacterial consortia and provide protection from toxins (for example high concentrations of ethanol). This has been demonstrated with solvent-tolerant Gram-negative bacteria which successfully biofilter high concentrations of isopropanol (Bustard et al., 2001).

When both amended CWs were exposed to the same influent ethanol concentration (7 587 $mg.L^{-1}$ COD_t), the removal performance of unprimed CW D was significantly inferior to that of incrementally primed CW B (Figure B11). However, the microbial community structures were similar [determined by lack of spatial variation in the PCA diagram (Figure B18), and the fact that the most abundant OTUs in the community were identical (data not shown)]. Thus, in this study, amendment with ethanol played a similar selective role on the overall microbial community structure, but the functionality of the community, in terms of ethanol degradation and COD removal, was increased using incremental priming to facilitate adaptation of those communities.

Table B7 Selected data from constructed wetlands used to treat winery wastewater or synthetic winery wastewater

Reference	Mode of operation	COD_i ($mg.L^{-1}$)	COD_e ($mg.L^{-1}$)	COD removal efficiency (%)	OLR ($gCOD.m^3.day^{-1}$)	tHRT (days)
Serrano et al., (2010)	Full-scale Hybrid 2 stage (VF/HF)	1 558 ± 1 023	448 ± 541	Stage 1: 29 to 70 Stage 2: 23 to 79	ND/NG ND/NG	NG NG
Sheperd et al., (2001)	Pilot-scale HSSF	4 720	51 (at max. COD_i)	99 (at max. COD_i)	132.1 (at max COD_i)	10
Masi et al., (2002)	Full scale A: HSSF/FW B: VSSF/SF C: SF/FW/FP	 4 044 1 003 772	 91 79 90	 97.8 92.2 87.5	 ND/NG ND/NG ND/NG	 NG NG NG
Grismer et al., (2003 b)	Pilot-scale HSSF	Crush period: 7 406 ± 2 090 Non-crush: 1 721 439	3 748 ± 1 826 363 ± 676	49 79	ND/NG ND/NG	NG NG
Mena et al., (2010)	Pilot scale HSSF	ww1: 171 ww2: 84	12.0 ± 2.4 to 53.0 ± 16.4 12.3 ± 3.1 to 25.0 ± 5.5	ND	8.8 9.4	9.6 9.6
Mulidzi, (2010)	Full-scale HSSF	HRT A: ~2 000 to 12 000 HRT B: ~2 000 to 12 000	NG NG	60 80	~75 ~450	7 14
This study	Pilot-scale Hybrid (HSSF/VSSF)	CW B: 15 800 (COD_{it}) CW C: 7 587 (COD_{it})	58 ± 25 763 ± 331	99.5 to 99.8 81.9 to 95.7	112 54	22 22

COD_e = effluent COD COD_i = influent COD FW = free water FP = finishing pond HF = horizontal flow HSSF = horizontal sub-surface flow ND = not determined NG = not given OLR = organic loading rate (calculated from data published in cited articles) tHRT = theoretical hydraulic retention time VF = vertical flow VSSF = vertical sub-surface flow ww = wastewater

B3.4 TREATMENT OF WINERY WASTEWATER AND SYNTHETIC PHENOLIC-LADEN WASTEWATER IN PILOT-SCALE CONSTRUCTED WETLANDS

B3.4.1 Salt analysis, COD removal, total phenolics and the relationship between these parameters in constructed wetlands amended with winery wastewater and synthetic phenolic wastewater

This study was primarily designed to elucidate the microbial processes responsible for the biodegradation of winery wastewater, particularly phenolics, in sand-filled constructed wetlands. To our knowledge, the fate of winery phenolics within such environments has not been comprehensively described.

In addition, the high salt content (Na 102.14mg.L^{-1}; K 394.74mg.L^{-1}; Cl 214.99mg.L^{-1}) of the winery wastewater used for amendment of CW C was of concern because high levels of sodium and chloride may have a negative effect of the uptake of water by macrophytes, while at extreme levels, direct salt toxicity to root tissues of plants takes place (Table B8). Depending on the sodium absorption ratio, salts can accumulate in soil and ion toxicity of sensitive plants can be expected (Table B8)

Table B8 Salt toxicity of sensitive plants to Na and Cl

Ion	No toxicity	Slight to moderate toxicity	Severe toxicity
Na	0-70mg/L	70-210mg/L	>210mg/L
Cl	0-70mg/L	70-335mg/L	>335mg/L

Westcott and Ayers (1985)

This multi-dimensional study involved experiments conducted in unplanted, constructed wetlands, in soil columns and in soil microcosms, using either synthetic wastewater (of known phenolic content and concentration) or whole winery wastewater. The removal of phenolics and the formation of key metabolites were determined by influent and effluent analyses, while the biotic and abiotic phenolic removal mechanisms were expounded upon by comparing the chemical data obtained using untreated and sterilized soils. The response of the resident microbial consortia to phenolics was monitored using molecular fingerprinting tools.

The contribution of phenolic compounds to the overall COD in the effluent was determined by measuring the individual phenolics by HPLC and converting the values obtained into COD terms (measured COD: COD_m). The influent COD of the winery wastewater used to amend CW C was 461 ± 126 mg.L^{-1}, while the COD_m of the three successive synthetic influent concentrations used to amend CW F was 234 mg.L^{-1} (low), 1 176 mg.L^{-1} (moderate)

and 5 842 mg.L^{-1} (high) (Table B1). The quantity of phenolics detected in the effluent from CW C (2.2 ± 0.6 mg GAE.L^{-1}) and the corresponding unamended control, CW A (1.5 ± 0.17 mg GAE.L^{-1}), were low and not significantly different (student t-test, p = 0.83), reflecting complete removal of phenolics from the winery wastewater. Comparable values (1.4 ± 0.5 mg GAE.L^{-1}), were measured in the effluent from CW F during amendment with a low concentration of phenolics. These consistently low values (<3 mg.L^{-1}) were assumed to result from natural leaching of phenol polymers from the soil. However, amendment of CW F with moderate and high concentrations of synthetic wastewater generated higher effluent phenolic concentrations of 13.8 ± 7.3 mg GAE.L^{-1} and 480 ± 222 mg GAE.L^{-1}, respectively (Figure B20). The major contributors (%) to COD in the effluent from CW C were ethanol (0-78%) and acetic acid (13-99%), while the major contributors to COD in the effluent from CW F were phenolic in nature (Figure B20). This difference in the relative contribution of phenolics to the overall COD was confirmed by calculating the Pearson's correlation coefficient between COD and total phenolics in the effluent, which was r = 0.44 in the case of CW C (winery wastewater influent) and r = 0.99 in the case of CW F (synthetic wastewater influent).

The COD in the effluent from CW F increased concurrently with the concentration of the synthetic wastewater influent, and reached values close to 700 mg.L^{-1} at the highest influent COD_m concentration of 5 842 mg.L^{-1} (Figure B21).

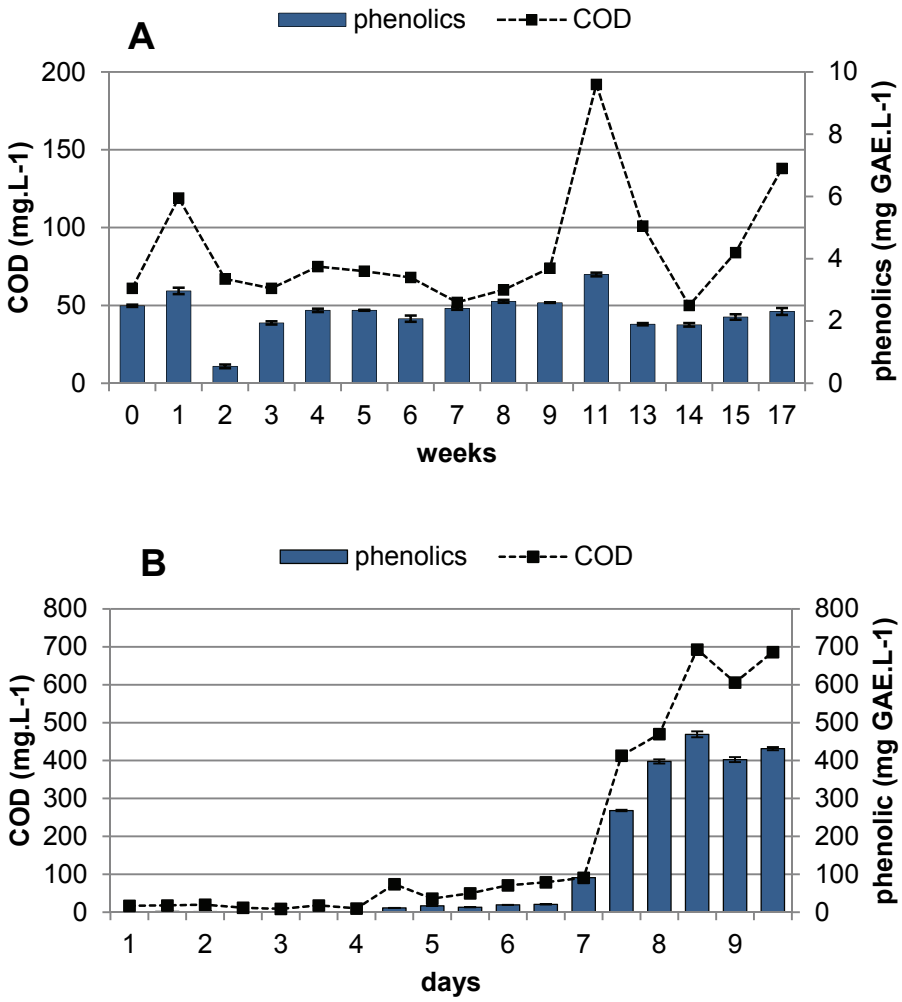

Figure B20 The total phenolics and COD measured in the effluent samples from CW C (A), and CW F (B)

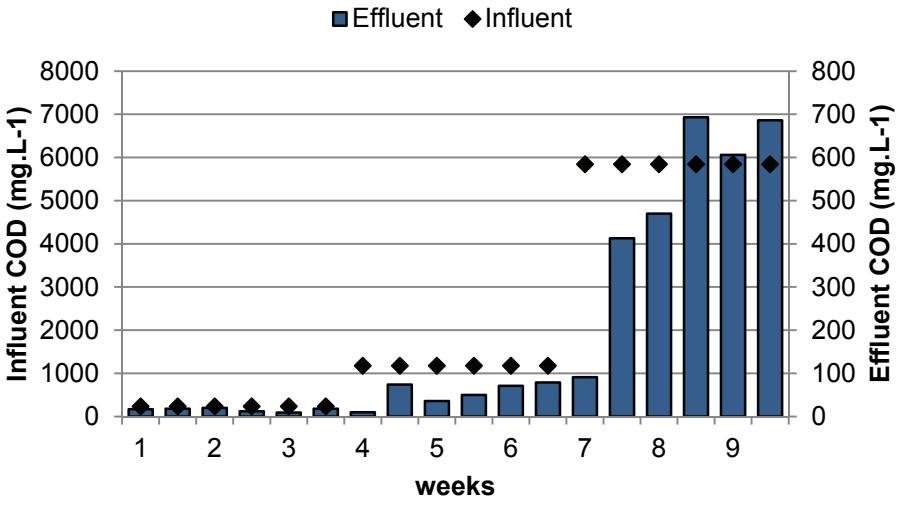

Figure B21 The relationship between the influent and effluent COD measured from CW F

B3.4.2 The biotic and abiotic removal of total phenolics in soil column experiments

Soil columns consisting of CW core samples were sterilized by irradiation, ensuring minimal disturbance of the sediment. A phenolic "cocktail" was allowed to permeate through three irradiated and three non-irradiated soil columns and the effluent was collected. The phenolic removal rates were calculated on a daily basis by measuring the total phenolics in the permeate. The removal rates in the irradiated and non-irradiated columns were attributed to $abiotic_i$ and combined biotic-abiotic mechanisms, respectively, with $biotic_i$ mechanisms accounting for the difference between the two.

The effluent flow stabilized after 2 days and the sample collection period was decreased from 24 hours to 6 hours to limit any breakdown of phenolics in the collection vessels. From days 4 to 9, there was a decrease in the $biotic_i$ removal fraction, and the phenolic removal rates calculated for the replicates were no longer consistent (Figure B22). Results indicated that both $abiotic_i$ and $biotic_i$ factors were involved in the removal of phenolics. No growth was observed on sterility plates from soil samples taken from irradiated columns before experimentation. However, further soil sterility tests indicated that after the 9 day operational period, the sterilized columns were either re-inoculated or contaminated with a microbial population capable of actively degrading phenolics (data not shown).

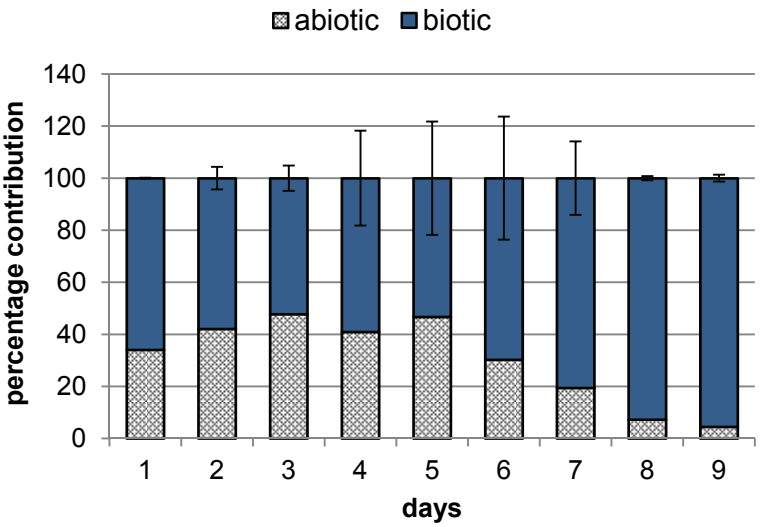

Figure B22 Bar chart showing the relationship between $biotic_i$ and $abiotic_i$ removal of phenolics in a soil column experiment

B3.4.3 Chemical profiles in wetland effluent

In order to understand the breakdown and retention of phenolics in the sediments of a CW, a pilot-scale CW (CW F) was amended with model phenolics (vanillin and gallic acid) and the effluent was characterized using HPLC. Apart from gallic acid and vanillin, two additional phenolics (vanillic acid and catechol) were identified in the effluent samples from CW F (Figure B23). The identity of some small peaks remained undetermined as they did not correspond to any of the phenolic standards employed (Figure B23). Acetate and lactate were identified in the effluent from week 7.5 using an HPLC method designed to detect sugars, acids and alcohols (La'Zaro et al. 1989). However, the lactate peak was not sufficiently resolved to perform accurate quantification (results not shown).

The concentrations of phenolics and acetate ($mg.L^{-1}$), determined by HPLC, were converted into COD values ($mg.L^{-1}$ COD_m) for use in mass balance calculations. No identifiable chemical entities were observed in the effluent during amendment with a low concentration of synthetic influent (weeks 1-3). During amendment with a moderate concentration of synthetic influent (weeks 4-6), small amounts of gallic acid (range: 0.8-1.0 $mg.L^{-1}$), vanillic acid (range: 1.7-6.9 $mg.L^{-1}$) and catechol were detected, the latter displaying an increased trend (from 3.5 to 15.0 $mg.L^{-1}$) (Figure B24). During CW amendment with a high concentration of synthetic influent, effluent analysis established that: (i) vanillin, acetate and lactate were detected for the first time; (ii) the highest concentration of vanillin (39.2 $mg.L^{-1}$) was quantified in the effluent samples at week 8.5; (iii) gallic acid was only present in small amounts (0.8 $mg.L^{-1}$ to 17.3 $mg.L^{-1}$), accounting for a maximum of 7% of effluent COD; (iv) high concentrations of the metabolites, vanillic acid (range: 104.2 $mg.L^{-1}$ to 157.6 $mg.L^{-1}$) catechol (range: 36.4 $mg.L^{-1}$ to 144.1 $mg.L^{-1}$) and acetate (32.5 $mg.L^{-1}$ to 152.6 $mg.L^{-1}$) were detected, with vanillin being the highest contributor to overall COD at week 8 (43%) and catechol at week 9.5 (40%); and (v) there was a steady increase in the contribution of the metabolites catechol and acetate, both in terms of concentration and contribution to overall COD (Figure B24).

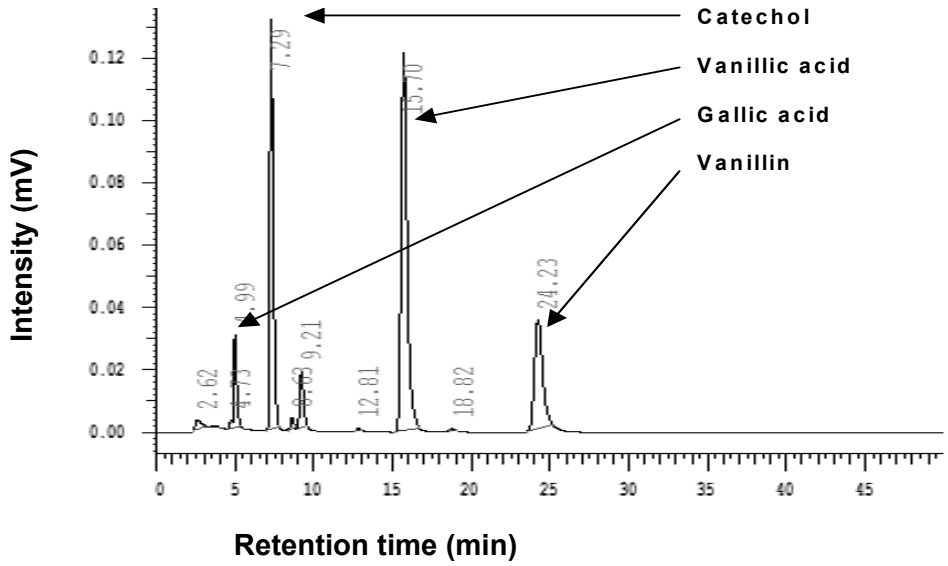

Figure B23 HPLC chromatogram (UV 280 nm) obtained using phenolic detection method of effluent from CW F at week 8.5

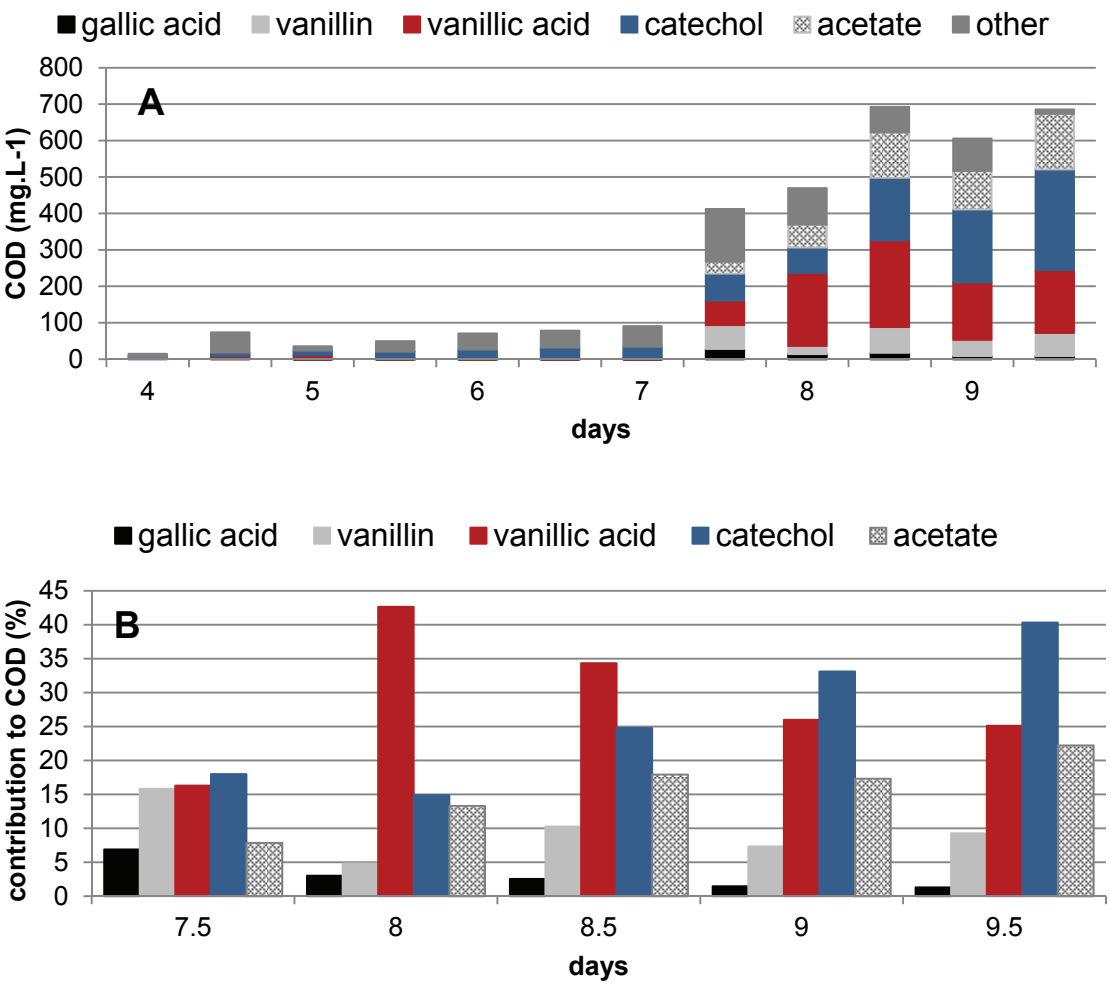

Figure B24 The COD_a contribution (A) and the % contribution (B) of known phenolics to the total effluent COD from CW F

B3.4.4 Phenolic profiles in soil microcosm supernatant

The microcosms contained (i) sediment taken either from CW D (acclimated by exposure to vanillin and gallic acid) or CW C (unexposed control) and (ii) a solution of vanillin and gallic acid. The $biotic_i$ and $abiotic_i$ removal fractions were calculated in the same theoretical manner as in the soil columns experiments. By comparing the post-incubation concentrations of the phenolic substrates and metabolites in the microcosms, the effect of acclimation on the microbial metabolism of the model phenolics was determined. Data analysis showed that $biotic_i$ removal of gallic acid and vanillin was respectively 6% and 12% higher in the microcosms containing acclimated sediment than in the control microcosms. $Biotic_i$ formation of vanillic acid was also higher in the CW D (acclimated) microcosms (44.1 $mg.L^{-1}$) than the CW C microcosms (9.3 $mg.L^{-1}$), with the concentration in both the autoclaved and non-autoclaved CW D microcosms (100.3 ± 29.4 $mg.L^{-1}$ and 144.5 ± 15.8 $mg.L^{-1}$, respectively), being higher than in the CW C microcosm counterparts (1.81 ± 0.03 $mg.L^{-1}$ and 11.1 ± 1.1 $mg.L^{-1}$, respectively). Catechol was only detected in the non-sterile CW D (acclimated) microcosms (Figure B25). In addition, the difference in phenolic concentrations between the autoclaved and non-autoclaved microcosms was statistically more significant (t-test) in the CW D microcosms (acclimated) (gallic acid: $p = 2.29 \times 10^{-2}$ and vanillin: $p = 2.99 \times 10^{-2}$) than in the CW C microcosms (gallic acid $p = 4.08 \times 10^{-2}$ and vanillin $p = 1.84 \times 10^{-2}$).

A set of control microcosm replicates were incubated with distilled water (no phenolics). Analysis of the HPLC chromatographs of the post-incubation supernatant of these microcosms showed the presence of catechol (a metabolic intermediate formed during the catabolism of phenolics) in the non-sterile microcosms containing acclimated sediment, indicating the presence of bioavailable phenolics in the CW sediment remaining from the acclimation process (data not shown). Pre and post-experimentation soil sterility testing proved that in contrast to the soil columns, the microcosms were not susceptible to contamination.

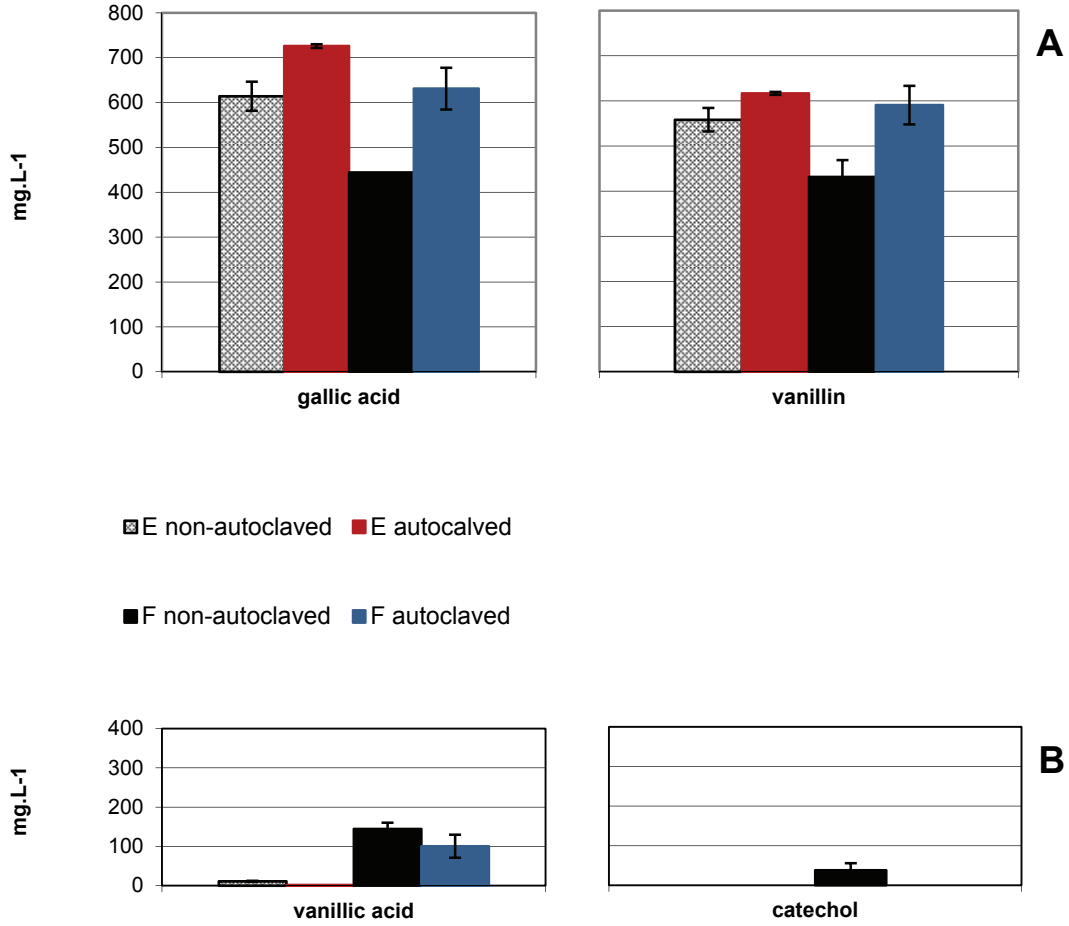

Figure B25 Concentrations of (A) substrate and (B) product phenolics identified in the microcosms containing acclimated sediment (F) and non-acclimated sediment (E) after incubation with vanillin (6.25 mM; 952 mg.L^{-1}) and gallic acid (6.25 mM; 1 176 mg.L^{-1})

B3.4.5 Salts (sodium and chloride) measurements in the effluent and sediment from CW C, amended with saline winery wastewater

After 16 weeks, there was a build-up of sodium at the superficial outlet only, while chloride levels diminished in the sediments at all sampling sites (Figure B26).

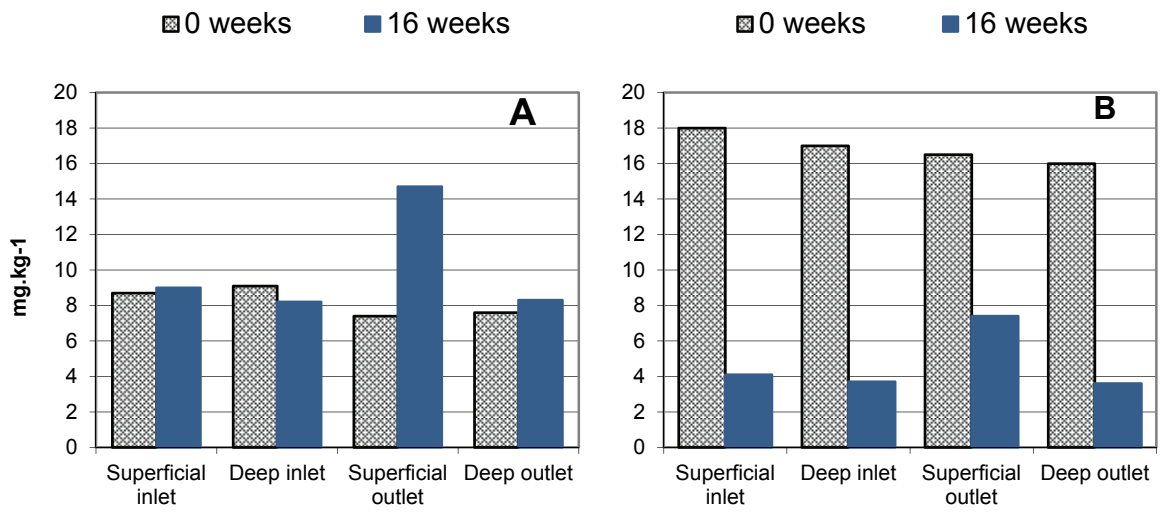

Figure B26 Graph of sediment sodium (A) and chloride (B) from wetland amended with winery wastewater before amendment (0 weeks) and after amendment (16 weeks)

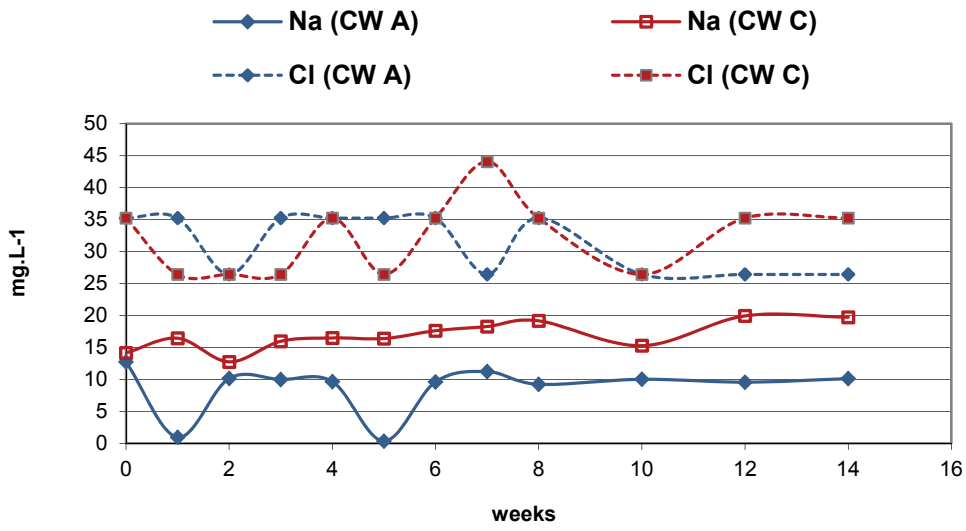

Figure B27 Effluent sodium (dotted lines) and chloride (solid lines) from the control (CW A) and the CW amended with winery wastewater (CW C)

B.3.4.6 The impact of winery wastewater on the microbial community structure

The interactions between different microbial consortia play an important role in maintaining ecosystem functionality (in this case the bioremediation of winery wastewater in a constructed wetland). Microbial activity induces biogeochemical transformations in natural, managed and engineered ecosystems, which is particularly relevant in contaminated systems. The converse is also true, where the introduction of solvents and other contaminants induces modifications in microbial community structure, often leading to the

selection of resistant organisms and the disappearance of sensitive ones (Feris et al., 2008, Bordenave et al., 2007). In the presence of complex pollutant mixtures, effective biodegradation often involves a combination of numerous tolerant organisms belonging to a variety of taxa and functioning as a microbial community. The ability of the community to tolerate the toxic components of the system and catabolise these components, and detoxify the environment is in itself a mechanism of resistance.

B3.4.6.1 Microbial fingerprinting of winery wastewater used for amendment of a constructed wetland

A high microbial diversity was observed in the winery wastewater, with 112 individual T-RFs being seen (Figure B28). Each unique 16S rRNA encoding gene T-RF was defined as an operational taxonomic unit (OTU), even though different taxa (or genotypes) can generate the same T-RFs with the same restriction enzyme (Blackwood et al., 2007).

The most abundant OTUs contributed to 12.8% and 10.9% of the total T-RFLP fluorescence profile, with sizes of 297 and 77 bp, respectively.

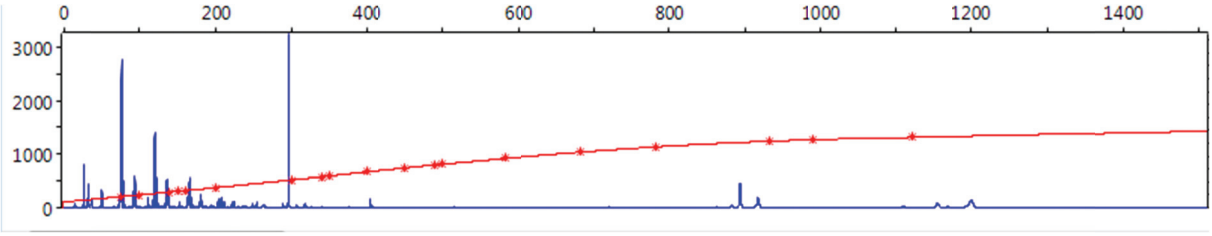

Figure B28 T-RFLP profile of the winery wastewater used for amendment of CW B

Putative identification of the selected OTUs was performed by *in silico* restriction using the MiCA3 program (available at http://mica.ibest.uidaho.edu/pat.php) and the Ribosomal Data Project dataset (RDP release 9.51) (Shyu et al., 2007). *In silico* restrictions were carried out with the *Hae*III enzyme for the 5'-end of the PCR amplifications. This predictive identification matched the OTU of 297 bp to *Clostridium tunisiense*, a bacterium that was previously isolated in olive mill wastewater (Thabet et al., 2004). Unfortunately, the OTU of 77 bp could neither be identified nor related to a single bacterial genus, as it showed high similarity to uncultured microorganisms. This result demonstrated that winery waste effluents may present an important microbial diversity which could modify the microbial community structure of constructed wetland sediments as observed in estuarine mudflat sediments contaminated by wastewater treatment plant (WWTP) effluent (Ramond et al., 2009, 2011).

3.4.6.2 The impact of amendment with winery wastewater on the surface sediment microbial community structure

In the surface sediments, 63.5% of the spatial variation of the samples is explained on the PCA diagram, with axis 1 and axis 2 contributing to 49.8% and 13.7% of the variance, respectively (Figure B29A). All the baseline microbial community fingerprints are closely clustered, confirming that after 96 days of equilibration, (section B.3.1) the microbial communities were similar to those of the control at the inlet and the outlet.

The samples from CW A (control) and CW C (amended with winery wastewater) evolved along axis 2 (13.7% of the variance) of the PCA diagram and 2 groups can be observed: (i) points representing samples from CW A after 21 days, located in the upper zone and (ii) points representing samples from CW C from day 0-84, located in the lower zone. This shows that amendment with winery effluent modified the microbial community structure of the surface sediments and that long-term amendment lead to a specialized, and presumably, adapted microbial community. However, the points representing samples taken from CW C at 112 days are located in the upper zone of the PCA representation, close to the points representing samples from the control CW (CW A), indicating a resilience of the original microbial communities to long-term exposure to winery effluent.

3.4.6.3 The impact of amendment with winery wastewater on the deep sediment microbial community structure

In the deep sediments, 58.6% of the spatial variations of the samples are explained on the PCA diagram, with axis 1 and axis 2 contributing to 45.4% and 13.2% of the variance, respectively (Figure B29B). Once again, all the baseline microbial community fingerprints are closely clustered, confirming that after 96 days of equilibration, the microbial communities were similar to those of the control at the inlet and the outlet.

T-RFs that were present in the winery wastewater were only intermittently detected in the sediment samples (data not shown), suggesting that the microbial population in the winery effluent did not proliferate in the deep sediments. At the end of the 112 days of experiment, a resilience of the microbial community to amendment with winery wastewater is seen in the deep outlet, as points are close to those of the control on the PCA diagram (Figure 29B). In contrast, the microbial community structure at the inlet of the winery impacted CW was significantly modified as sample points are well separated from those of the control (Figure 29B).

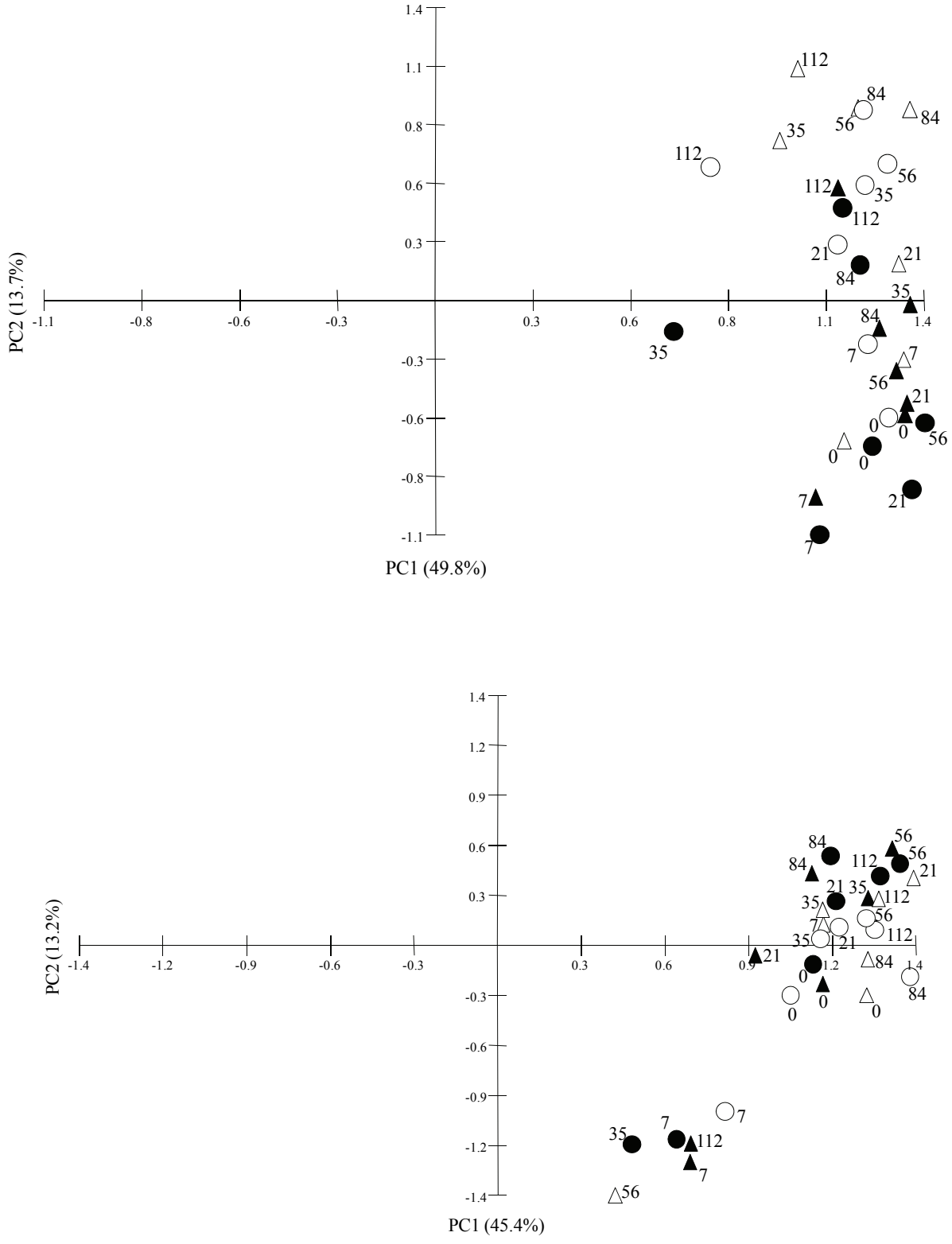

Figure B29 Principal component analysis (PCA) of *Hae*III digested 16S rRNA gene in the surface sediments (A) and deep sediments (B) of the control CW A and the winery effluent amended CW B. Numbers indicate the day of the sampling (D0 to D112)

○ outlet CW A ● outlet CW C △ inlet CW A ▲ inlet CW C

B3.4.7 Discussion and Conclusions

B3.4.7.1 Biodegradation and biotransformation of model phenolics in synthetic phenolic-laden wastewater

Experiments were designed to mimic the phenolic removal processes taking place in sediments during exposure to winery wastewater and common wine constituents were therefore chosen as model phenolics (Alén-Ruiz et al., 2009; Kallithraka et al., 2006; Rastija et al., 2009; Woraratphoka et al., 2007). Complete elimination of four model phenolics (gallic acid, vanillin, (+)-catechin, ferulic acid) was achieved in soil column experiments, of which ≥48% was attributed to $biotic_i$ mechanisms. During pilot-scale experiments conducted in constructed wetlands, vanillin and gallic acid were chosen as model phenolics.

Although sophisticated approaches (metaproteomics, metagenomics, metatranscriptomics) are currently being developed to unravel the complex metabolic processes taking place within environmental microbial consortia, the basic metabolic pathways which have been elucidated from *in-vitro* microbial cultures still provide plausible pathways for the biodegradation of vanillin and gallic acid (Abram et al., 2011; Copley, 2009; Finley et al., 2009; Wilmes and Bond, 2009).

Metabolic pathways for the biodegradation of vanillin

Vanillin and its oxidized product, vanillic acid, are major components of humus and many soil microbes have the ability to degrade these molecules (Souto et al., 2000). The characteristic vanillin degradation pathway is initiated by the oxidation of vanillin to vanillic acid, which is catalysed by vanillin dehydrogenase. Vanillic acid is then demethylated to form protocatechuic acid and formaldehyde, catalysed by vanillate demethylase (Fenner et al., 2005; Preifert et al., 1997). A number of pathways have been described for the breakdown of these products, including the formation of catechol by the decarboxylation of protocatechuic acid (Hara et al., 2007; D'Argenio et al., 1999; Doten and Ornston, 1987).

Metabolic pathways for the biodegradation of gallic acid

The microbial degradation of gallic acid to pyrogallol takes place either aerobically (by oxidation) or anaerobically (by decarboxylation). Pyrogallol is in turn degraded aerobically to simple aliphatic acids (that enter the citric acid cycle), or by a variety of complex anaerobic pathways to products such as volatile fatty acids (Bhat et al., 1998).

Metabolites identified from the biodegradation of vanillin and gallic acid in synthetic phenolic-laden wastewater

It was established that catechol, acetate and vanillic acid were formed as a result of microbial degradation/transformation in CWs and soil microcosms. Catechol accounted for up to 40% of the COD in the effluent from CW D (amended with synthetic phenolic wastewater) and was not detected in the "abiotic$_i$" soil microcosms, demonstrating that the formation of this dihydroxybenzene molecule was entirely biotic$_i$.

B3.4.7.2 Acclimation of microbial communities in a constructed wetland to vanillin and gallic acid

Bacterial species may adapt over time to new environmental factors, a phenomenon known as acclimation. During acclimation, biodegradation rates are influenced by the duration of acclimation and the degree of microbial toxicity imposed by the new environmental factors (Chou et al., 1979). It has been established that microbial acclimation to ethanol can be enhanced by exposing CW communities to increasing ethanol concentrations (Welz et al., 2011). It has also been shown that incubation of soil samples with vanillin can increase the number of bacteria capable of utilizing this phenolic as a sole carbon source (Kunc, 1970).

In this study, it was demonstrated that the microbial population in a test CW (CW F) had acclimated to the presence of gallic acid and vanillin, resulting in significantly enhanced biotic degradation rates when compared to a non-acclimated population. These results supported previous literature findings that low concentrations of phenolic acid mixtures stimulate the growth of phenolic acid-utilizing bacteria within the bulk soil, and that competitive selection of these bacteria enhances biodegradation of phenolic acids (Blum et al., 2000; Vaughan et al., 1983). Thus, the use of incremental priming, whereby wastewater is applied in incrementally increasing concentrations during the start-up phase, should enhance long-term phenolic removal performance in CWs (Welz et al., 2011).

B3.4.7.3 Accumulation of metabolites in CW sediments

Vertical flow CWs are operated in "fill and drain" cycles, with atmospheric gases being drawn into the substratum during drainage. It is expected that microbial oxidation of catechol to small readily biodegradable aliphatic compounds would be supported by presence of atmospheric oxygen in the upper CW sediments, but not in the deeper, anaerobic layers (Alva and Peyton, 2003; Bugg, 2003; Dagley, 1971). Indeed, anaerobiosis caused by waterlogging has been shown to decrease the diversity of bacteria carrying the *XylE* gene encoding for catechol 2,3-dioxygenase, a key enzyme involved in the aerobic catechol

degradation pathways (Fenner et al., 2005). Under anaerobic conditions, catechol is primarily mineralized by methanogens but if methanogenesis is hampered, the catabolic pathways become energetically unfavourable, resulting in accumulation of catechol (Bhat et al., 1998). Furthermore, the accumulation of catechol has been shown to impede methanogenesis (Hernandez and Edyvean, 2008). We therefore suggest that the systematic increase of catechol in the effluent from CW F emanated from the accumulation of catechol in the deeper, anaerobic layers of the substratum.

Similarly, the concentrations of acetate in the effluent exhibited an increasing trend, accounting for 22% of the effluent COD at the end of the experiment (Figure B24). Under favourable conditions this volatile fatty acid is readily biodegradable but it can accumulate in the environment when alternative electron acceptors such as oxygen and nitrates are unavailable (van Stempvoort et al., 2009; Whalen, 2005; Lasko et al., 1997). Since carbon dioxide, dihydrogen and acetate constitute the three primary substrates for the methanogenic bacterial community, any stresses on this population can also impede acetate mineralization (Whalen, 2005). We suggest that these factors were responsible for the accumulation of acetate in the deep, anaerobic sediments of CW F.

B3.4.7.4 The effects of catechol on microbial metabolism

In high concentrations, catechol can be highly toxic to microorganisms, often exhibiting greater levels of toxicity than "parent" phenolics, including benzene (Muñoz et al., 2007; Boyd et al., 1997). The catechol molecule has been shown to disrupt cell function by inducing changes in the fatty acid and protein composition of Gram positive and Gram negative bacterial cell membranes, but the exact mechanism responsible for these changes has not been described (Hernandez and Edyvean, 2008; Park et al., 2001). Furthermore, polymerized forms of catechol have been found to be more toxic than monomeric forms (Hernandez and Edyvean, 2008). In catechol bioassays using *Pseudomonas fluorescens* and *Vibrio fischeri*, low EC_{50} values of 0.77 $mg.L^{-1}$ and 0.268 $mmol.L^{-1}$, respectively have been reported (Isidori et al., 2005; Boyd et al., 1997).

However, catechol may function as a bacterial growth substrate, depending on the concentration of catechol and the organism/s involved (Chen et al., 2009; Alexivia et al., 2008; Muñoz et al., 2007; Park et al., 2001). Chen et al. (2009) found that in soil, low concentrations of catechol (0-400 $L/hr.m^{3-1}$ $\mu g.g^{-1}$) promoted microbial growth, while higher concentrations inhibited growth, with 60% inhibition being demonstrated at 3000 $\mu g.g^{-1}$.

High concentrations of catechol (36.4 $mg.L^{-1}$-144.1 $mg.L^{-1}$) were measured in the effluent from CW F from weeks 7-9, providing compelling evidence that inhibitory concentrations were reached in the substratum during this period. This is supported by the pattern observed in the hydraulic conductivity measurements: following a steady decline during the first six weeks of the experiment, there was an increased trend between weeks 7-9, which corresponded to an increasing catechol concentration in the effluent. Hydraulic conductivity may be retarded in CWs by entrapment of solids, the growth of vegetation and/or the formation of biofilm/biomass (Knowles et al., 2010). Since the experimental CWs were unplanted, the decreased conductivity could only have originated from the accumulation of polymerized forms of phenolics and/or biomass or biomass-related products (such as lipopolysaccharides). However, the trend reversed during weeks 7-9, discounting the possibility that clogging with polymerized phenolics was the primary mechanism for changes in conductivity. It is proposed that during the first 6 weeks, low levels of influent phenolics served as growth substrates, resulting in a proliferation of biomass/biofilm and a concomitant decline in conductivity. However, at high influent phenolic concentrations (week 7-9), the accumulation of catechol in inhibitory concentrations resulted in loss of biomass/biofilm with a consequent increase in hydraulic conductivity.

B3.4.7.5 Abiotic influences on the transformation of model phenolics in the substratum of constructed wetlands

In soil, the sorption of organics usually corresponds with the amount of soil organic matter (SOM), especially humic acids, kerogen and black carbons (Qualls, 2005; Huang et al., 2003). Binding of phenolics to the mineral surfaces of clay and SOM may occur via cation bridging or polymerization (Tharayil et al., 2006). Metallic interactions also play a fundamental role in the sorption/desorption of phenolics: (i) metal ions compete with phenolics for adsorption sites on humic acid; (ii) metal oxides and hydroxides can block the micropores of kerogen and black carbon; and (iii) phenolic acids may complex with the inner and outer spheres of hydroxylated iron and aluminium compounds on the surface of clay particles (Wang et al., 2009; Sun et al., 2008; Tharayil et al., 2006; Navia et al., 2006). However, sorption is not necessarily abiotic: polymerization and immobilization of phenolics, including vanillic acid, on the soil matrix can be microbially mediated by enzymes such as peroxidases (Bhandari and Xu, 2001).

Similarly, chemical transformations are not necessarily biotic: chemical transformation of phenolics can occur via the oxidation of phenolic acids coupled to iron and/or manganese reduction (Polubesova et al., 2010; Lehmann et al., 1986). The transformation of vanillic acid

into aromatic radical cations in Montmorillonite clay and Palouse soil has been described (Polubesova et al., 2010; Lehmann et al., 1986).

In soil microcosms, $abiotic_i$ mechanisms accounted for 38% and 46% of gallic acid elimination from acclimated and non-acclimated sediment, respectively. The higher removal rate achieved in the non-acclimated sediment was possibly related to higher saturation of gallic acid binding sites during the acclimation process. Due to the low carbon (1.9 $mg.g^{-1}$), low clay (1%), high Fe (63.0 $mg.kg^{-1}$) and Mn (1.9 $mg.kg^{-1}$) content in the CW sand, it is postulated that metal complexation and/or chemical transformation played a major role in this process. This is supported by the findings of Halvorson et al. (2009), who reported that gallic acid was not well adsorbed in loamy soils, suggesting that sorption onto SOM is not an important removal mechanism for this phenolic (Qualls, 2005; Huang et al., 2003). In soil microcosms, removal of 30% and 33% of vanillin from acclimated and non-acclimated sediment, respectively was $abiotic_i$, and vanillic acid originated from both $biotic_i$ and $abiotic_i$ oxidation of vanillin. This highlights the choice of suitable a substrate medium in CWs: providing the degradation rate of bound phenolics is sufficient, adsorption can enhance the overall removal of phenolics in CWs.

B3.5 NITROGEN FIXATION

Results obtained during this project showed that the addition of N and P enhanced the degradation of ethanol in pilot-scale constructed wetlands (section B3.2). During the course of experimentation using CW B (incrementally primed with ethanol), a mucilaginous layer appeared on the surface of the CW. The Gram stain and wet mount of the slime showed a monopopulation of organisms microscopically resembling *Azotobacter*. It was hypothesized that the N-deficient status of CW B leads to the selection of diazotrophic bacteria, and that the success of the incremental priming procedure was at least partially attributable to favourable nutrient balances imposed by nitrogen fixation. To support this hypothesis, selective culture, enumeration and identification (sequencing) of nitrogen fixing organisms from the superficial sediments of CW A (control) and CW B (incrementally primed) was performed, and results compared.

B3.5.1 Nitrogen mass balances

The total amount of ethanolic COD added to CW B over the study period amounted to 5.87 kg (calculated from the sum of influent volumes and theoretical COD values). Using the average baseline measurement of total nitrogen (TN) of the sediment from four sampling sites in CW B (0.018% wt.wt.) and taking the volume (502 L) and density (1.6 $kg.L^{-1}$) of the CW sand into account, the total amount of nitrogen in the system amounted to ~0.14 kg. At

least a portion of this TN would be expected to be biologically unavailable. Using these figures, the ratio of total COD to TN was 42:1. After sixteen weeks the average TN of the sediments decreased by only 0.001%.

B3.5.2 Selective culture, enumeration and sequencing of recoverable, viable, nitrogen-fixing organisms from CW sediment

The surface of CW B was covered with a rust and black mucilaginous layer. Wet mounts and Gram stains of this layer revealed a mono-population of organisms microscopically resembling the genus Azotobacter. The nitrogen-fixing organisms were enumerated on nitrogen deficient mannitol salt agar (NDMSA) and selective enumeration of *Azotobacter* spp. was performed on Azotobacter medium (AM). Results, expressed as colony forming units per gram dry weight sediment (cfu's.gdwt^{-1}) are presented in Table B9.

Three relevant observations were made from the growth patterns of sediment cultures (Table B9). These observations were confirmed by performing a Univariate Analysis of Variance (ANOVA). Firstly, there were significantly greater CFUs enumerated from the superficial sediment samples than from the complementary deep samples (p-value = 0.001). Secondly, there were significantly greater CFUs enumerated from the superficial sediment samples of CW B than CW A (p-value = 0.003). Thirdly, there were significantly more CFUs enumerated from the superficial sediment samples on AM than NDMSA (p-value = 0.18) and comparison of the number of CFUs cultured on both media types showed that the AM to NDMSA colony ratio was higher in CW B (4.5 to 1 and 4.0 to 1, from the inlet and outlet respectively) than in CW A (2.9 to 1 and 1.8 to 1, from the inlet and outlet respectively).

Table B9 The results of enumeration studies of recoverable, viable nitrogen-fixing organisms from wetland sediment samples taken at week 47

Sampling site	CW	NDMSA (cfu's/gmdwt^{-1})	AM (cfu's/gmdwt^{-1})
Superficial inlet	**A**	26 x 10^4	75 x 10^4
	B	86 x 10^4	39 x 10^5
Superficial outlet	**A**	24 x 10^4	44 x 10^4
	B	84 x 10^4	34 x 10^5
Deep inlet	**A**	23 x 10^3	47 x 10^4
	B	35 x 10^3	32 x 10^3
Deep outlet	**A**	22 x 10^3	24 x 10^4
	B	99 x 10^3	18 x 10^4

nitrogen deficient mannitol salt agar (NDMSA); Azotobacter medium (AM); colony forming units per gram dry weight sediment (cfu's.gdwt^{-1})

Two dominant, distinctive colony types, exclusive to cultures from the CW B sediments appeared on both NDMSA and AM. The colonial morphology of both was creamy and mucilaginous, one of which had large, extremely mucoid colonies > 10 cm in diameter that produced a brown/black pigment after 5 days. According to microscopic morphology (Gram stain and wet mount) together with the formation of cysts, growth on selective media and the development of a distinctive colonial morphology, these organisms were presumptively identified as the diazotrophic *Azotobacter* spp. (Knowles and Barraquio, 1996). These two strains, designated P1 and P2, were sequenced to confirm their identity. Nitrogenases are the primary enzymes responsible for diazotrophy and are encoded by the *nifH* gene and their phylogeny can be compared to that from ribosomal RNA (Zehr et al., 2003). Thus, the *nifH* gene sequences of strains P1 and P2 were studied. P1's 16S rRNA gene sequence presented 98% identity with *Azotobacter* sp. JDM1 (GenBank accession number: FJ824602.1) and *Azotobacter chroococcum* strain 10006 (GenBank accession number: EU930421.1). P2's 16S rRNA gene sequence presented 98% identity with *Azotobacter chroococcum* strain H-A (GenBank accession number: FJ032009.1), confirming that they are members of the genus *Azotobacter* (Table B10).

Table B10 BLASTn results of the *nifH* gene sequences of strains P1 and P2

Strain	Accession	Description	Max score	Total score	Query coverage	E value	Max ident
P1	FJ822995.1	*Agrobacterium tumefaciens* isolate gx-178 nitrogenase iron protein (nifH) gene, partial cds	322	322	100%	7,00E-85	87%
	GU117602.1	Uncultured bacterium clone RN3 dinitrogenase reductase gene, partial cds	316	316	100%	3,00E-83	86%
	EU693338.1	***Azotobacter chroococcum*** strain CGMCC 1.178 NifH (nifH) gene, partial cds	316	316	100%	3,00E-83	86%
P2	FJ822995.1	*Agrobacterium tumefaciens* isolate gx-178 nitrogenase iron protein (nifH) gene, partial cds	327	327	100%	2,00E-86	87%
	M73020.1	***A.chroococcum*** nifH gene complete cds, containing nifD gene 5'end	327	327	100%	2,00E-86	87%
	EU693341.1	*Acinetobacter* sp. Z21 NifH-like (nifH) gene, partial sequence	325	325	99%	6,00E-86	87%

B3.5.3 Discussion and Conclusions

Incremental priming (CW B) was as effective as nutrient addition (CW C) for the removal of COD (see sections B3.2 and B3.3) and CW B operated effectively for the removal of ethanol for almost eleven months without the addition of nitrogen. The COD:N ratios applied in the influent to CW C (60:1 and 40:1), were values taken from the operation of anaerobic systems) and proved adequate. However, any contribution of N from the sediment and atmosphere was not considered. Zhao et al. (2010) found that in unplanted, vertical-flow pilot-scale CWs, a ratio of 4.6:1 (approximately 10-fold greater than applied to nutrient-

supplemented CW C) was the most effective for COD removal. If the only N source in CW B (not supplemented with N) originated from the chemical make-up of the sediment, this would have resulted in a COD:N ratio of 42:1 over the experimental period. However, it was not known what proportion of the N was bioavailable and there was only a 0.001% decrease in TN over the 16 week experimental period. This strongly suggested that atmospheric nitrogen supplementation occurred in CW B.

The failure of unprimed, non-nutrient supplemented CW D to realize the same efficiency as incrementally primed CW B or nutrient supplemented CW C supported the hypothesis that the effectiveness of incremental priming was at least partly due to a gradual selection of nitrogen-fixing organisms. Thus, the nitrogen fixing microbial community may be important in environments subjected to organic contamination, and studies of these communities, both in terms of phylogeny (who is there?) and abundance (in what number?) may lead to enhanced CW management. As a basis for future studies, the direct amplification of the bacterial *nifH* gene was tested from sediment extracted metagenomic DNA. This proved successful, which indicated that T-RFLP and/or qPCR studies of the nitrogen fixing microbial community within CWs is possible (data not shown) and forms the basis for future studies.

B3.6 MICROBIAL ENZYME ACTIVITY ASSAYS

B 3.6.1 Background information

The determination of enzymatic activity on soil samples is fraught with problems:

(i) Abiotic components in the soil may interfere with spectroscopic methods by absorption of light at the test wavelength, enzyme immobilisation, substrate competition and/or quenching of product; (ii) The presence of more than one enzyme with different kinetics in the test assay class is distinctly possible; (iii) enzymes may be intracellular, membrane bound or extracellular; (iv) The growth kinetics of microorganisms may impact on assays that are conducted over longer periods; and (v) The disturbance of biofilms, changes in temperature and other physical and chemical factors present in a laboratory setting all contribute to the imperfection of these tests (Burns, 1982). Nevertheless, detection methods for a variety of enzymes have been well described and used as indicators of soil fertility and/or biogeochemical cycling. Two of the most widely cited reference books on soil analysis (Weaver et al., 1994; Alef and Nannipieri, 1995) include chapters on soil enzyme activity edited by experts in the field, Tabatabai and Nannipieri respectively.

The focus of project K5/1936, in terms of sediment enzyme assays was to develop the methodology for enzymes likely to be implicated in the degradation of winery wastewater

and individual components of winery wastewater. The enzymes were thus chosen for their function in carbohydrate or lipid metabolism or for their role as overall indicators of wetland microbial enzymatic activity. It was hoped to show spatial and temporal changes in sediment enzyme activities in the experimental CWs when compared to the control CWs, with the activities in the expected to show a more stable pattern. Preliminary testing of random soil samples for laccase and tyrosinase activity, together with the evaluation of results obtained from the gravel CW at UCT, indicated that further assay development would be prudent. The thrust to reflect the conditions encountered in the wetlands as accurately as possible without over-complicating the methodology was seen as a priority. Important aspects were identified, some of which are summarized below:

3.6.2 Factors affecting soil enzyme assays

3.6.2.1 The effects of the sample storage time on soil enzyme activities

A study by Verchot et al., (2005) found that many enzymes exhibited a decline after 6 day of storage at 4˚C, which was linked to both soil type and enzyme. Thus, testing was performed as soon as practically possible after collection (within a maximum of 48 hours). Although there are many literature references where samples are stored before testing, it was expected that the likelihood of obtaining accurate results would be improved by performing assays as soon as practically possible after collection.

3.6.2.2 The effects of incubation time and temperature on soil enzyme activities

Most of the assays were conducted at room temperature. Although the “field” temperatures often differed from laboratory temperatures due to the inherent nature of the wetlands and their exposure to climatic conditions, it was felt that incubation at room temperature would give a better approximation of *in-situ* conditions than incubation at elevated temperatures where activities of selected organisms are likely to increase.

Regarding the duration of incubation, unless agents such as toluene (Burns, 1982) are employed to prevent microbial growth, the contribution to enzymatic activity by bacteria and fungi most suited to the test conditions can skew the results to varying degrees. However, incubation times should be of sufficient duration to detect activity and for the purpose of this study, to detect temporal and spatial differences in the activities in the CWs. To optimize the assay procedures with special reference to determining optimal once-off incubation times, a variety of samples, which included a variety of soil types (sand, clay, humic) were collected and tested.

3.6.2.3 Additional factors affecting soil enzyme activities

The repeatability of the chosen assays, the method of inactivation of controls, the use of blanks and the preparation of standards graphs of substrate or products were tested in order to optimize the methodology prior to experimentation using the CWs.

B3.6.3 Results of fluorescein diacetate (FDA) activity, discussion and conclusions

In general, results were disappointing and thus the methodology and results are not discussed in detail in this report. Some of the assays proved to be unsuitable for soils and/or sediment, others showed erratic patterns and results from well-described soil assays, such as the FDA, were inconclusive. This assay relies on the enzymatic conversion of FDA (colourless) to fluorescein (yellow) by non-specific proteases, lipases and esterases (Figure B30). A lengthy literature search using FDA as a model was undertaken and the results of this assay are briefly discussed by way of example.

In 21 studies where the soil FDA assay was used as part of the experimental protocol, six different methods (most with some deviations) were used. Thus, it is almost impossible to make inter-study comparisons on FDA activity and there is a need to standardize the method. Only one of these studies (Hamdi et al., 2007) was conducted under mesocosm conditions, the rest taking place in outdoor environments subject to extraneous influences. FDA activity has been reported as useful for the quantification of biomass (D'Ascoli et al, 2006; Bandick and Dick, 1999). However, researchers have also reported poor correspondence with biomass estimated by the carbon-fumigation method (1999; Jorgenson et al., 1992), ATP quantification (Leon et al., 2006) and phospholipid fatty acid analysis (PLFA) (Gaspar et al., 2001).

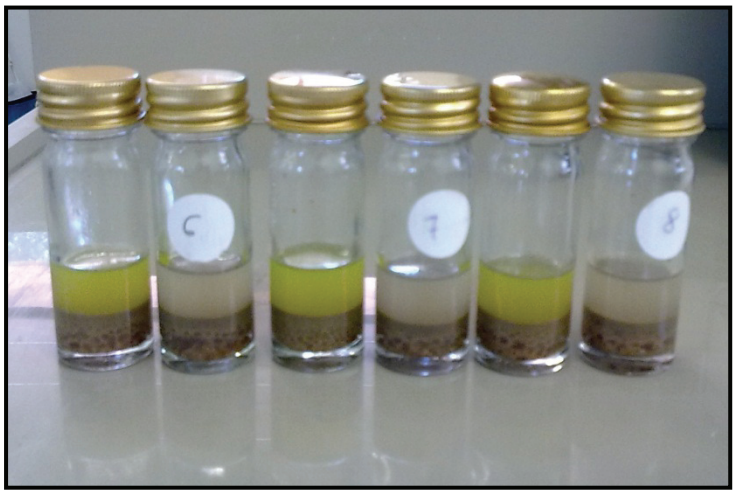

Figure B30 FDA assay showing three inactivated control samples together with three test samples with fluorescein (yellow) formation

During project K5/1936, the FDA assay was performed in accordance with the method described by Adam and Duncan, 2001. The FDA results obtained from sediment samples taken from the CWs at CPUT did not exhibit any trends, with erratic activity being shown in the control and experimental CW samples. Figure B21, as an example, shows the results obtained from the superficial inlet samples. Samples taken from other locations produced similar, erratic results, with no clear cut trends or distinctions between control and experimental CWs. Boissier and Fontvielle (1995) have also reported erratic activity with seepage water and Wang and Lu (2006) have reported decreased FDA activity with waterlogging, possibly related to the oxygen dependency of the enzymes involved in FDA hydrolysis.

In an effort to determine whether the failure to elicit meaningful results from the FDA assay was due to factors such as waterlogging or soil type, experiments are being conducted using indoor mesocosms with three different media (sand-based, clay-based and humic-based), under both saturated and unsaturated conditions and including control mesocosms. Once these results are available, a more informed strategy (if any) will be adopted regarding the use of enzyme assays to monitor metabolic activity in the CWs.

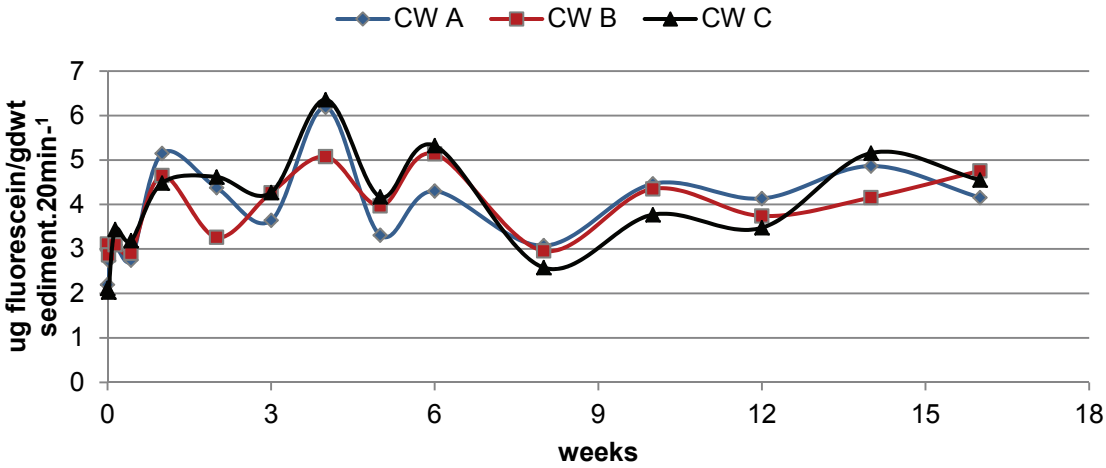

Figure B31 FDA activity from the samples taken from the surface inlet of the control (CW A) and experimental CWs (CW B and CW C). Results are expressed as the amount of fluorescein produced per gram dry weight of samples in 20 minutes

Specific enzyme assays, notably for alcohol dehydrogenase (during CW amendment with ethanol) and catechol dioxygenases (during amendment with phenolics), were generally higher in the test CWs. However, neither of these assays has previously been described for use in soil samples and both proved to be unreliable in terms of reproducibility, probably due to unwanted chemical reactions between target enzyme products and soil components and absorption interferences at spectroscopic assay wavelengths.

PART C: CONCLUSIONS AND RECOMMENDATIONS

C1 CONCLUSIONS

This project has culminated in a compilation of practical insights related to the biological function of constructed wetlands (CWs). A combination of molecular fingerprinting methods and chemical analyses were used to assess the impact of winery wastewater, ethanol and phenolics on the microbial communities in constructed wetlands. The capacity for functional adaptation of the microbial communities was enhanced by acclimation and the addition of inorganic nutrients to high COD wastewaters. However, the capacity for the microbial communities to adapt was compromised by organic overloading. At high concentrations, ethanol and phenolics induced toxicity due to accumulation of metabolic intermediates. In operating constructed wetlands, the need to maintain organic loading within operational parameters is key in maintaining a "healthy" microbial population and optimal biodegradative function.

C1.1 APPLICATION OF USEFUL TOOLS FOR MONITORING THE FUNCTION OF PILOT SCALE CONSTUCTED WETLANDS

C1.1.1 Molecular fingerprinting tools

During this project, two molecular fingerprinting methods were used to assess changes in the microbial community structure: Denaturing Gradient Gel Electrophoresis (DGGE) and Terminal-Restriction Fragment Length Polymorphism (T-RFLP). After optimization, it was demonstrated that these rapid and relatively simple survey methods were effective tools for monitoring both qualitative and quantitative changes in microbial community structure in CW systems (Ruiz-Rueda et al., 2009; Sleytr et al., 2009; Nicomrat et al., 2006; Baptista et al., 2008).

The DGGE method has the advantages of providing a visual pattern of the community structure and the possibility of assigning phylogenetic affiliations to major bands by re-amplification and sequence analysis (Part A). Detailed analyses of the strengths and weaknesses of DGGE and its attendant assumptions are presented elsewhere (Kirk et al., 2004). In DGGE analysis, it is assumed that dominant bands, which appear in relationship with an imposed change in the system (such as the various amendments used in this study), are directly affected by the changes, and that they probably constitute physiologically important components of the response (Niepceron et al., 2010; Part A). With the identification of these dominant responsive phylotypes for a range of different chemical inputs, it should be possible to build a catalogue of phylogenetic fingerprints of CW systems optimized for specific waste streams.

In contrast, T-RFLP, being associated with fluorescent labelling and capillary electrophoresis, is a more sensitive molecular fingerprinting method than gel-electrophoresis based methods, such as DGGE, which are limited by the resolution of the gels. In this study, T-RFLP was used to follow the evolution of the microbial community structure in CWs amended with ethanol, winery wastewater or synthetic wastewater containing phenolics (part B). As expected, all these amendments lead to modification of the microbial community structure in the CW sediments.

These fingerprinting methods were used very effectively to monitor both the compositional changes and the rate of change in a microbial community after chemical exposure in the constructed wetlands. This laid the groundwork for the use of CW systems to investigate the relationships between microbial structure and system responses to environmental challenges.

C1.1.2 Hydraulic conductivity measurements

The hydraulic conductivity of each CW system was measured weekly during each experiment. This simple tool was found to be a useful indicator of biotic changes within the CWs. There was a strong relationship between influent COD and hydraulic conductivity. During amendment with winery wastewater and high concentrations of ethanol, the hydraulic conductivity often decreased to such a level that the mode of operation inadvertently changed from subsurface to surface flow. This effect was reversible, with an increase in conductivity following a decrease in influent COD. As vertical subsurface flow CWs are known to degrade organics more efficiently than either free water surface flow or horizontal subsurface flow CWs, this loss of conductivity may present an operational challenge in full-scale systems.

It was also noted that hydraulic conductivity may be useful as a determinant of microbial community development and that extractable DNA titres (by the procedure used in this project) is not a reliable parameter for monitoring microbial biomass.

C1.1.3 The use of COD, including COD mass balances

During this project, influent and effluent COD measurements (theoretical and actual) were utilized to indicate the overall operational efficiency and function of the CWs. Firstly, by expressing the concentration of influent organics in COD terms, comparisons of loading data could be made using industry norms. Secondly, a simple and novel method was employed whereby the concentrations of metabolic substrates and products were converted into COD terms for use in mass balance equations. This proved to be a valuable tool for elucidating potential metabolic pathways. The theoretical COD (COD_t) for a range of volatile fatty acids

and phenolics correlated well with the measured values. By quantifying substrates and key metabolites in COD values, the percentage contribution of each to the total COD is possible. The "remaining" COD (not attributable to organics which have been identified and quantified) can then be calculated by this method. Furthermore, it can be hypothesized that if the COD contribution by unidentified organics is low, the metabolic assumptions made using effluent metabolic profiles are more relevant than those where the contribution is high.

C1.2 SUMMARY OF SIGNIFICANT RESULTS OBTAINED DURING THE COURSE OF PROJECT K5/1936

C1.2.1 Determination of the period required for equilibration of microbial communities in sand-filled constructed wetlands

Using DGGE, it was demonstrated that after a period of time (in this case, 89 days) of feeding with low concentrations of yeast extract and glucose, the pilot-scale CWs were equilibrated, with an established microbial community presenting similar fingerprints in the surface and the deep sediments. This emphasized the fact that microbial community fingerprinting with molecular tools (such as DGGE) is a key parameter for monitoring CW establishment before amendment. The observation that microbial community structure was the slowest parameter to stabilize supports this view.

C1.2.2 Incremental priming and the acclimation of microbial communities within CW sediments

The metabolic profiles in the effluent and the removal efficiencies obtained in a CW amended with ethanol in incrementally increasing concentrations (COD_t from 474 to 23 600 $mg.L^{-1}$) and a second CW, which was amended from inception with a moderate concentration of ethanol (COD_t of 7 587 $mg.L^{-1}$), were compared (Section B3.3). This procedure, termed incremental priming, resulted in enhanced COD removal. No volatile fatty acids (metabolites of ethanol degradation) were detected in the incrementally primed CW effluent up to and including an ethanol amendment concentration of 15 800 $mg.L^{-1}$ COD_t. In a comparative, "unprimed" system, where the CW was amended with a moderate concentration (7 587 $mg.L^{-1}$ COD_t) of ethanol from inception, COD removal was less efficient and the CW became septic, as evidenced by the presence of volatile fatty acids (including butyrate and propionate) in the effluent. In addition, the number of nitrogen-fixing bacteria (diazotrophs) were significantly greater in the sediment from the incrementally primed CW B than the control CW A, suggesting that nitrogen fixation was enhanced in CW B. It was theorized that nitrogen fixation redressed the unfavourable nutrient balance (high

carbon to nitrogen ratio) of the influent, allowing effective microbial biodegradation of ethanol. This was borne out by nitrogen mass-balance calculations.

Amendment with ethanol at moderate and high concentrations (≥7 587 mg.L^{-1} COD_t) led to the selection of similar, adapted microbial communities, irrespective of whether amendment was via incremental priming or not. However, in the case of incrementally primed CW B, the community became functionally adapted to degrade high concentrations of ethanol (15 800 mg.L^{-1} COD_t), while in the case of CW D, although the community composition was similar, functional adaptation was inadequate.

An incremental amendment procedure was also adopted for amendment in a CW used to treat a model synthetic phenolic wastewater consisting of a solution of gallic acid and vanillin (section B3.4). Microcosm investigations indicated that the microbial population became acclimated (functionally adapted) to the presence of these phenolics. Significantly enhanced biotic degradation of these aromatics occurred in phenol-amended CW sediment in comparison to unamended sediment from a control CW.

C1.2.3 Nutrient balance

The addition of nutrients in a COD/N/P ratio of 300/5/1 to a CW amended with ethanol significantly enhanced the degradative performance in comparison to a replicate amended with ethanol alone (COD_t of 7 587 mg.L^{-1}). This demonstrated the importance of nutrient balance and added credence to the theory that part of the success of incremental priming was due to natural selection and acclimation; selection of a microbial population with the capacity for dealing effectively with the chemical composition of wastewater is a key process in all wastewater treatment systems. However, the presence of increased amounts of N and P in the effluent was of concern and the addition of nutrients is thus not recommended unless additional downstream nutrient removal processes are incorporated into the system.

DGGE analysis showed that the addition of N and P helped to maintain the original microbial community structure in pilot-scale CWs amended with ethanol. Thus, the superior COD removal efficiency achieved by nutrient supplementation could be linked to the maintenance of a stable microbial community structure (section B3.2.3).

C1.2.4 Relationship between hydraulic conductivity, clogging and organic loading

Decreased loading capacity,y stemming from the clogging of sand-filled CWs with influent solids, plant roots or biomass/biofilm is well documented. However, there are also inherent problems associated with gravel-filled CWs. For example hydraulic retention times in these systems are often low, demanding higher physical capacities. During this project, potassium bromide and gallic acid tracer experiments showed that a portion of the influent stagnated at the bottom of a gravel-filled CW, probably as a result of backmixing.

Amendment of sand-filled CWs with winery wastewater resulted in a gradual loss of hydraulic conductivity which culminated in permanent inundation of the CW surface and concomitant loss of removal efficiency. Similar decreases in conductivity were also noted during amendment with ethanol and phenolics. Due to the lack of solids in the effluent and the fact that all CWs were unplanted, it was concluded the decreased conductivity was related to the accumulation of biomass/biofilm in the sediments. It was found that by decreasing the organic loading rate, the process was reversible.

C1.2.5 Organic overload and toxicity

At high loading rates, amendment of sand-filled pilot scale CWs with ethanol and synthetic phenolic wastewater resulted in increased effluent COD concentrations and the accumulation of potentially toxic metabolites in the effluent. It was shown that the loading rate at which this toxic effect occurred could be increased from <7 587 to >15 800 $mg.L^{-1}$ COD_t by using incremental priming during start-up to induce functional adaptation of the microbial communities. In a parallel experiment, this toxic effect was overcome by the addition of N and P, highlighting the importance of both acclimation and stable nutrient balances to prevent the accumulation of toxic metabolites in CWs amended with organic waste.

The most abundant form of slowly biodegradable COD in winery wastewater is the phenolic component. During CW amendment with whole winery wastewater, total removal of phenolics was accomplished. At low influent concentrations, phenolics were completely removed by a combination of biotic and abiotic influences in sand-filled pilot-scale constructed wetlands amended with vanillin and gallic acid. However, amendment with high concentrations of gallic acid and vanillin resulted in the accumulation of the metabolites catechol and acetate in the deep sediments. Increased hydraulic conductivity strongly suggested a concomitant loss of biomass/biofilm due to accumulation of inhibitory concentrations of catechol in the substratum. These results underscore the importance of

identifying the chemicals that are likely to be formed from an organic pollutant stream within CWs.

C1.2.6 Abiotic and biotic removal mechanisms

It must be borne in mine that abiotic removal may play a significant role in the removal of both organic and inorganic pollutants in CWs and sand filter systems. To prevent leaching of chemicals from CWs (and consequent rehabilitative requirements), it is essential that a fundamental biodegradative balance is achieved in CWs treating organic wastewater. This is not possible in systems treating predominantly inorganic waste streams such as acid mine drainage, where saturation is ultimately inevitable.

This study provided unequivocal evidence that the removal of plant phenolics in sediments is controlled by both abiotic and biotic factors. These natural phenomena can be exploited to achieve maximal removal efficiency in CWs. It is evident that abiotic removal is related not only to the physical structure, but also to the chemical composition of the substrate. The clay content, organic carbon content and metal content (particularly iron and manganese) are strongly associated with phenolic binding and/or chemical transformation reactions. CWs treating phenolic-laden wastewaters should therefore incorporate media which contain an abundance of one or more of these fractions.

To maintain an adequate biotic removal fraction, it is important to consider the character of the waste and to adhere to appropriate concentration limit/s to prevent accumulation of toxic metabolites. In addition, start-up techniques that enhance microbial acclimation can increase the biotic degradation fraction in CWs.

C2 RECOMMENDATIONS

This study has shown the importance of maintaining a stable, functional biodegradative microbial community in constructed wetlands treating organic wastewaters. The following guidelines are recommended for implementation in full-scale systems and/or in a research environment in order to maintain optimal microbial “health”.

- **Establish COD maxima for individual CWs**

  In order to maintain hydraulic conductivity and removal efficiencies within design parameters, it is recommended that COD maxima (including safety factors) are established for individual CWs treating high strength organic wastewaters. Sufficient hydraulic conductivity is especially important in VSSF based systems to allow attendant draw-down of atmospheric gases into the substratum. Regular monitoring of COD or

other relevant indicator parameters will allow for pre-emptive measures to be taken during periods when maxima are exceeded. For example, design features incorporating return of treated wastewater to the system head to dilute high-strength wastewaters is a practical measure that functions independently of external water supplies.

- **Monitor metabolic processes using COD mass-balances**

The use of COD mass balances to comprehend the metabolic processes occurring within CWs is scientifically relevant. During this project, the CWs were treated as “black boxes”, with only influent and effluent analyses being conducted. To gain further insight into the metabolic sequences of biodegradation within CWs, it is recommended that proper collection of pore water from at least four sites (superficial inlet and outlet, deep inlet and outlet) be implemented in future studies. This will allow (i) more accurate relationships to be formulated between metabolic processes and microbial community dynamics; (ii) elucidation of the spatial location where each metabolic step predominates, i.e. sequential, aerobic/anaerobic; and (ii) calculation of decay coefficients for inclusion in full-scale design parameters.

- **Apply incremental priming during the start-up phase of CWs**

The use of incremental priming to induce acclimation (functional adaptation) of microbial communities increased the ethanol removal capacity in the pilot-scale CWs. This was linked to an increase in microbial functionality, but not diversity. It is recommended that this procedure be adopted during the start-up phase of CWs to enhance the long-term functionality of the system. Further studies into the time-scale of acclimation necessary to exact the benefits without compromising the derived benefits are also recommended.

- **Assess acclimation in different substrata**

During this project, the effect of CW medium was not comparatively considered. It was hypothesized that the presence of high concentrations of metals (iron, molybdenum, zinc) may have enhanced the efficiency of the CPUT CWs. It is thus recommended that acclimation in different media, including gravel, be compared. Such studies can be conducted in mesocosms so that a large variety of media may be screened prior to piloting. It is also strongly recommended that the choice of substrate is related to the type of wastewater to be treated.

- **The importance of microbial fingerprinting in experimental CWs**

The use of microbial community fingerprinting techniques such as DGGE and/or T-RFLP, to monitor (i) the establishment and equilibration of microbial communities in pilot-scale CWs; and (ii) their adaptive responses to various contaminations has proven to be useful and thus is highly recommended.

- **The importance of equilibration of CWs prior to amendment**

Before any chemical amendments, for CWs of the dimensions used in this study (length 173 cm/ width 106 cm/ depth 30 cm), microbial community equilibration times in the order 100 days must be respected. Decreases in hydraulic conductivity measurements can be used to indicate the early equilibration of the microbial communities.

- **The elucidation of biotic and abiotic removal fractions in CWs**

Although microcosm and soil column experimental data can only be broadly extrapolated to larger systems, the data indicates whether removal mechanisms are biotic, abiotic or of combined origin. Such experiments can also be used to assess the acclimation of microbial communities in different substrata exposed to different wastewaters. It is thus recommended that experiments similar to those described in this report are conducted on a wider range of organic chemicals and media, including gravel. The use of adsorption-desorption experiments to compare abiotic sorptive capacities of various CW media is also recommended.

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