MANIPULATING BIOTIC AND ABIOTIC FACTORS TO ENHANCE THE REMEDIATION OF AGRI-INDUSTRIAL WASTEWATER IN PILOT-SCALE CONSTRUCTED WETLANDS

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A thesis submitted in fulfillment of the requirements for the degree of Doctor of Philosophy

at the
Institute for Microbial Biotechnology and Metagenomics,
University of the Western Cape

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September 2012
ABSTRACT

As a consequence of various cellar activities the wine industry produces copious volumes of potentially hazardous wastewater each year. South Africa is one of the top ten wine-producing countries, making the successful treatment of cellar effluent an important environmental obligation in this country. Constructed wetlands (CWs) are resilient to the seasonal input fluxes associated with agri-industrial waste and are ideal systems for the in-situ treatment of cellar effluent in small to medium-sized wineries not connected to municipal reticulation systems. In a project sponsored by the Water Research Commission of South Africa, a number of studies were undertaken to assess the remediation of winery wastewater and common components of winery wastewater in sand-filled pilot-scale constructed wetlands operated in batch mode.

This thesis contains the results of three studies. The first study evaluated the temporal aspects of CW equilibration as a basis for future studies of system response to amendment. Microbial biomass and hydraulic conductivity values were monitored and microbial community fingerprints were obtained using denaturing gradient gel electrophoresis. The study showed that microbial community fingerprinting provides a valuable tool to assess the time-scales of microbial equilibration, which was found to be in the order of 100 days.

In the second study, the biodegradation and mineralization of ethanol by acclimated and non-acclimated microbial populations in CWs were compared. By increasing the influent ethanol concentration at incremental intervals (incremental priming), the biodegradative capacity was significantly enhanced. At an influent COD concentration of 15 800 mg/L, no volatile fatty acids were detected in the effluent of an incrementally primed system and the maximum effluent COD measured was 180 mg/L. In contrast, an identical, unprimed system, amended with a lower concentration of COD (7587 mg/L), exhibited a maximum effluent COD concentration of 1 400 mg/L, with the metabolites butyrate and propionate accounting for up to 83% of the effluent COD. It was
conclusively demonstrated that the use of incremental priming, together with the batch mode of operation enhanced long-term function of the CWs.

In the third study, the removal of the phenolic component of winery wastewater was evaluated in CWs, as well as in sand columns and microcosms. It was found that at low influent phenolic concentrations in CWs, complete organic removal was accomplished, but at high concentrations, there was incomplete substrate removal and an accumulation of potentially toxic metabolites, including catechol. The sand provided a suitable substrate for the treatment of phenolic-laden waste, and both biotic (48%) and abiotic (52%) removal mechanisms effected the removal of model phenolics. Prior acclimation of microbial communities increased the biodegradation rate of phenolic acids significantly.
DECLARATION

I declare that “Manipulating biotic and abiotic factors to enhance the removal of organics from agri-industrial wastewater in pilot-scale constructed wetlands” is my own work, that it has not been submitted before for any degree or examination in any other university, and that all the sources I have used or quoted have been indicated and acknowledged as complete references.

Pamela Jean Welz

September 2012

Signed........................................
LIST OF ABBREVIATIONS

General

AMD  acid mine drainage
AS   activated sludge
Biotic i indicating biotic removal
BSA  bovine serum albumin
Abiotic i indicating abiotic removal
BSF  biological sand filter
BTEX benzene toluene ethylbenzene xylene
C    carbon
CAH  chlorinated aliphatic hydrocarbons
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 m measured COD
COD t theoretical COD
CW   constructed wetland
DDT  dichlorodiphenyltrichloroethane
DGGE denaturing gradient gel electrophoresis
DNA  deoxyribonucleic acid
dNTP deoxyribonucleotide triphosphate
DO   dissolved oxygen
é  electron
EDC  endocrine disrupting compound
EPS  exopolysaccharide
FC   Folin-Ciocalteau
FISH fluorescent *in-situ* hybridization
FP finishing pond
FW free water
FWSF free water surface flow
GAE gallic acid equivalents
HC hydraulic conductivity
HF horizontal flow
HLR hydraulic loading rate
HMN octahydro-1,3,5,7-tetranitro-1,3,5,7-tetraocine
HPLC high pressure liquid chromatography
HRT hydraulic retention time
HSSF horizontal subsurface flow
IC irradiated column
MBR membrane bioreactor
MDS multidimensional scaling
ND not determined
NG not given
NMDS non-metric multidimensional scaling
OLR organic loading rate
PAH polycyclic aromatic hydrocarbons
PCA principal component analysis
PCB polychlorinated biphenyl
PCP pentachlorophenol
PCR polymerase chain reaction
PVC polyvinyl chloride
PFLA phospholipid fatty acid
qPCR quantitative polymerase chain reaction
RBCOD readily biodegradable chemical oxygen demand
RDN hexahydro-1,3,5-trinitro-1,3,5-triazine
RNA ribonucleic acid
<table>
<thead>
<tr>
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<tr>
<td>SBCOD</td>
<td>slowly biodegradable chemical oxygen demand</td>
</tr>
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<td>SIP</td>
<td>stable isotope probing</td>
</tr>
<tr>
<td>SS</td>
<td>suspended solids</td>
</tr>
<tr>
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<td>subsurface flow</td>
</tr>
<tr>
<td>TAE</td>
<td>Tris acetate-ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>tHRT</td>
<td>theoretical hydraulic retention time</td>
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<td>TNT</td>
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</tr>
<tr>
<td>UC</td>
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<tr>
<td>VF</td>
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<tr>
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</tr>
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<td>Co</td>
<td>Co</td>
<td>cobalt</td>
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<td>hydrogen gas</td>
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<tr>
<td>$\text{Zn}$</td>
<td>zinc</td>
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</table>
ACKNOWLEDGEMENTS

The memory of my late father, Christopher Speight motivated me to contemplate a career U-turn at an age when many others consider slowing down. Desperately poor, with umpteen siblings and no financial support, my dad worked during the day and studied at night to obtain a BSc in Chemistry, followed by an MSc at the University of London. He never stopped learning nor lost his inquisitive nature or drive: in his retirement he set up a rudimentary computer system at home and taught himself programming (this was in the 70’s when systems bore no resemblance to those of today). He also taught himself Morse code and registered as a radio “ham”, activities which he continued until his death at the age of 80. He was a wonderful father, and I will always miss him.

My 83 year old mother continues to support me in my career decisions. This remarkable woman is still fit enough to dig out the odd tree that is “dropping too many leaves” and to my envy, completes the most challenging cryptic crosswords at lightning speed. Thanks for everything, mom, you are a phenomenon.

This thesis essentially began in 2006, when I spent two unpaid years pursuing an MSc in Environmental Science, something I could never have achieved without the emotional and financial support of my inimitable hubby, Deon. Deon also inspired me to complete my dissertation and find employment - every day he would appear in my makeshift office asking, in chronological order, “Have you finished writing yet?”, “How much longer?”, then “Have you got a job yet?” The latter was not so easy in a poor economic climate with no experience in my new field. About to reply to an advertisement for a cleaner for the crocodile enclosure at the zoo, the academic powerhouse couple, Professors Stephanie Burton and Don Cowan came to my rescue. Steph understood that I still had a good few years in me and gave me a chance, employing me to run a project funded by the Water Research Commission of South Africa. This was really serendipitous as the work suited my combined interests in wastewater
treatment and the environment like a glove. As the director of the Institute of Microbial Biotechnology and Metagenomics, our collaborating organization on this project, Prof Cowan was persuaded to become my PhD supervisor. During the last couple of years, he has taught me to write journal articles according to the “Cowan” formula, which has taken considerable effort and patience on his part. My sincere thanks to both of you, my appreciation is immeasurable.

I must also declare my heartfelt thanks to the present director of the Biocatalysis and Technical Biology research group, Dr Marilize le Roes-Hill, my colleague on the project since 2009, Dr Jean-Baptiste Ramond and my office-mate and sounding-board on all matters, Kerry Horne. These three have all played significant and integral roles in my journey and I could write pages on each. Thanks guys.
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1 BACKGROUND LITERATURE, AIMS AND SCOPE

The literature presented in the first three sections of Chapter 1 broadly deals with: The fundamentals and strategies of Bioremediation (Section 1.1), Environmental pollution and pollutants (Section 1.2), Microbial ecology (Section 1.3) and Biological wastewater treatment (Section 1.4). These topics are intrinsically related to the work presented in the chapters forming the body of this thesis (Chapters 2-4). Chapters 2-4 are presented in the form of published manuscripts and as such include more specific reviews of the literature, particularly in the introductory paragraphs. The format of the manuscripts has been changed, but the text has been included nearly verbatim from the originals which are also included as appendices.
1.1 BIOREMEDIATION

1.1.1 Introduction

Microorganisms, plants, animals and the inanimate (abiotic) environment are all involved to some degree in a complex cycle of degradation and synthesis of inorganic and organic chemicals (Faulwetter et al., 2009; Li et al., 2008; Tao et al., 2007; Li et al., 2008b). In the environment, degradation and/or detoxification of pollutants takes place via biological (biotic) and non-biological (abiotic) mechanisms, with the relative contribution of each mechanism being linked to the specific microenvironment and the pollutant involved (Fischer and Hahn, 2005; Jiang et al., 2010; Ojeda, et al., 2009; Rontani et al., 2009). Many physicochemical factors including redox status, temperature, pH and humidity influence pollutant degradation rates (Jung, et al. 2012; Owabor and Obahiaigbon, 2009; Taylor and McLoughlin, 2002).

1.1.2 Bioremediation fundamentals

Bioremediation is the intentional use of microorganisms or microbial processes to degrade and/or detoxify environmental contaminants (Atlas, 1997; Boopathy, 2000; Singh et al., 2008). Under most circumstances, the presence of pollutants in ecosystems results in the selection of microbial communities capable of proliferating under the amended conditions (Megharaj et al., 2011; Perelo, 2010). In addition, some species may be capable of utilizing the pollutant in metabolic reactions: for example, natural attenuation of aromatic hydrocarbon pollutants is effected by solvent-tolerant microorganisms that colonize sites contaminated with petroleum (Megharaj et al., 2011). However, not all pollutants are amenable to biodegradation, including radionuclides, some heavy metals and some chlorinated organics (Section 1.2.2) (Boopathy, 2000). In order for bioremediation to occur, there are three primary requirements:
(i) Microorganisms capable of actively degrading and/or transforming the target pollutant must be present,

(ii) The target pollutant must be bioavailable and in close proximity to the functional microbial species, and

(iii) The environmental conditions should support the growth and appropriate metabolic activities of the functional microbial species (Juwarkar et al., 2012; Semple et al., 2007).

Bioremediation has considerable cost benefits when compared to conventional techniques, with *in-situ* bioremediation strategies being generally simpler and more cost-effective than and *ex-situ* strategies (Megharaj et al., 2011; Perelo, 2010). *Ex-situ* strategies are applied when the contaminants are particularly toxic, the environment particularly sensitive or the waste stream is continuous and treated in a centralized location dedicated to this function (Megharaj et al., 2011).

### 1.1.3 Bioremediation strategies

Bioremediation strategies are used alone or in combinations to treat contaminated surface soils, subsurface soils, surface water, groundwater or marine environments either *in-situ or ex-situ* (Boopathy, 2000; Juwarkar et al., 2012; Megharaj et al., 2011). Each strategy relies on specific procedure/s to create conditions conducive to the remediation of a particular pollutant (Boopathy, 2000; Guimaraes et al., 2010; Megharaj et al., 2011; Park et al., 2011; Tyagi et al., 2011).

The choice of strategy is primarily incumbent on the desired pollutant remediation efficiency which is balanced against the cost, regulatory requirements, time constraints, space constraints and simplicity of the procedure/s (Boopathy, 2000; Megharaj et al., 2011). Brief explanations of the most commonly used bioremediation strategies and examples are given in Sections 1.1.3.1 - 1.1.3.5.
1.1.3.1 Addition of oxygen

The presence or absence of oxygen impacts on the degradation rate of some chemicals; for example, the degradation of highly chlorinated aliphatic hydrocarbons is enhanced under anaerobic conditions while the degradation of less chlorinated equivalents is enhanced under aerobic conditions (Table 1) (Hyun and Hayes 2009; Laturnus et al., 2005) (Ramsburg et al., 2010). However, in the case of most environmental pollutants, aerobic degradation pathways are typically more efficient than anaerobic degradation pathways or the latter may be limited or absent (Rittman and Perry, 2001). In these circumstances, the addition of oxygen as a terminal metabolic electron acceptor can result in an increase in biomass, microbial diversity and bioremediation capacity (Eyvazi and Zytner, 2009; Frutos et al., 2012; Kabelitz et al., 2009). Addition of oxygen can also be seen as a biostimulation strategy (Section 1.1.3.3) (Begley et al., 2012).

Table 1  Environmental degradation mechanisms and rates of selected chlorinated aliphatic hydrocarbons

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<th>Anaerobic biodegradation</th>
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0 No degradation  + (slow) to +++ (rapid) degradation rates

Bioventing

Bioventing is a physical strategy that relies on a vacuum being drawn through vents in the vadose zone adjacent to a contaminated site. The resultant negative pressure: (i) Accelerates the volatilization of hydrocarbons that are adsorbed on the soil matrix, and (ii) Increases the rate of diffusion of air into the subsurface, thereby enhancing aerobic hydrocarbon degradation (Frutos et al., 2012; Hoeppel et al., 1991). Bioventing is a cost-effective in-situ mechanism used
principally to remediate petroleum hydrocarbon spills (Eyvazi and Zytner, 2009; Frutos et al., 2012; Hoeppel et al., 1991).

**Air sparging**

Air sparging is used as an alternative to bioventing for the removal of volatilized contaminants and enhancement of petroleum hydrocarbon degradation (Bass et al., 2000; Kabelitz et al., 2009). In this case, air under pressure is actively pumped into the vadose zone through vents (Bass et al., 2000).

**Tilling and land farming**

Tilling of contaminated surface soil aerates the soil and encourages aerobic microbial biodegradation of pollutants (McCarthy et al., 2004). Land farming is a generic term used to describe the remediation of contaminated surface soils or excavated soils utilizing one or more strategies including tilling, air sparging, bioaugmentation and/or biostimulation (Chang et al., 2010; Komilis et al., 2010; Silva-Castro et al., 2012). Land-farming is applied either ex-situ on waste or sludge from remote sites or in-situ; in both cases bioremediation is stimulated by aeration and/or the addition of minerals, nutrients and moisture to contaminated soil which has been excavated soil and spread in a thin layer on the ground in order to stimulate appropriate microbial activity (Chang, 2008; Komilis et al., 2010; Silva-Castro et al., 2012).

**1.1.3.2 Addition of nutrients/ electron donors/surfactants**

**Biostimulation**

Biostimulation can be described as the intentional addition of oxygen, nutrients or other substances that stimulate the growth and metabolic functions of key members of the microbial population involved in the biodegradation of pollutants (Begley et al., 2012; Perelo, 2010). Oxygen can stimulate relevant aerobic pathways, but due to the low redox status in soils and sediments, most biodegradation naturally takes place using anaerobic metabolic pathways (Perelo, 2010). These pathways can be stimulated by the addition of non-oxygen electron acceptors such as ferric iron [Fe (III)] and nitrate (NO$_3^-$) (Perelo, 2010).
Other examples include: (i) The addition of oxygen and ethene to remediate sites contaminated with vinyl chloride (Begley et al., 2012), (ii) Addition of NO₃⁻, organic phosphorus (P) and persulphate to sites contaminated with benzene [persulphate increases the mineralization of organic P and benzene oxidation is coupled to NO₃⁻ and sulphate (SO₄²⁻) reduction] (Xiong et al., 2012), (iii) Addition of elemental iron (Fe⁰) to sites contaminated with polychlorinated biphenyls (PCBs) [hydrogen gas (H₂), which is generated from the reaction of Fe⁰ with water, accelerates anaerobic microbial dechlorination reactions] (Varadhan et al., 2011).

However, in instances where the on-site environmental status is already optimal for the functional microbial communities, biostimulation techniques have no benefit (Sudjarid et al., 2012).

**Addition of surfactants**

For biodegradation to take place, a pollutant must be (i) Bioaccessible: “freely available to cross an organism’s (cellular) membrane from the medium the organism inhabits at a given point in time” and (ii) Bioavailable: “available to cross an organism’s cellular membrane from the environment it inhabits, if the organism had access to it: however, it may be either physically removed from the organism or only bioavailable after a period of time” (Semple et al., 2007).

Hydrophobic organic compounds, in particular, bind to soil particles and are poorly soluble (Seo et al., 2008; Zoller and Reznik, 2006). Both the formation of biofilm and the addition of surfactants can increase the bioavailability of pollutants bound to soil minerals and organic matter (Boopathy, 2002; Seo and Bishop, 2007).

Surfactants can be synthetic or microbial in origin, with the latter being generally more biodegradable (Megharaj et al., 2011). Bioaugmentation (Section 1.1.3.3) with biosurfactant-producing microorganisms can aid in the bioremediation process (Megharaj et al., 2011; Semple, et al., 2007).
1.1.3.3 Addition of microorganisms

Bioaugmentation

Desirable catabolic reactions can be promoted by stimulating the activity of indigenous microbial consortia and/or augmenting the site with exogenous microorganisms (Chagos-Spinelli et al., 2012; Bastos and Magan, 2010; Mrozik and Piotrowska-Seget, 2010). The introduction of microorganisms with specific catabolic activities into a contaminated environment in order to speed up or enable the degradation of pollutants is known as bioaugmentation (Perelo, 2010). This can be useful in situations where the indigenous microbial communities lack the relevant catabolic genes for remediation of the target pollutant (Perelo, 2010). Introduced species may also impart catabolic capabilities to members of the indigenous microbiota through horizontal gene transfer (Pepper et al., 2002; Perelo, 2010).

Bioaugmentation strategies which are customized for the environmental conditions present at the contaminated site are more likely to succeed (Tyagi et al., 2011). For example, it is important that the degradative capabilities of the introduced species are not inhibited by any co-contaminants present at the site (Tyagi et al., 2011). Bioaugmentation can be ineffective if biotic and abiotic stress factors prevent successful colonization and dispersal of the introduced species in the new habitat, although long-term survival is not a requisite once (and if) horizontal gene transfer has taken place (Pepper et al., 2002; Perelo, 2010; Tyagi et al., 2011).

Without intervention, there is a lag phase known as the acclimation period, when the indigenous microbial populations adjust to the presence of pollutants in newly-contaminated sites; under these circumstances, bioaugmentation with material from similar, previously contaminated sites can speed up bioremediation if the material contains microbial consortia that are already functionally pre-acclimated (Tyagi et al., 2011). In sites with a long-term history of contamination, and therefore likely to contain pre-acclimated microbial
populations, biostimulation alone may be sufficient to increase remediation potential (Tyagi et al., 2011).

1.1.3.4 Controlling environmental parameters

Composting

In some cases, the addition of a bulking agent to a contaminated site can increase the biodegradation of contaminants via thermophilic microbial reactions (Juwarkar et al., 2012; Megharaj et al., 2011). The success of composting is dependent on the ratio and type of pollutant and the waste material that is applied (Megharaj et al., 2011). Composting improves the structure, the nutrient status and the microbial activity in soil, but there is a risk that pollutants that bind to the organic matter in the compost may be released at a later stage (Megharaj et al., 2011).

Bioreactors – anaerobic digestion

An extensive range of bioreactors have been designed and utilized for the treatment of liquid waste (Section 1.4.2), solid waste and environmental pollutants. Anaerobic digestion is used extensively for industrial, agri-industrial and domestic wastewater treatment, sludge reduction, solids digestion (e.g. kitchen waste, sewage) and co-digestion of pollutants in contaminated soils (McKeown et al., 2012; Moller and Muller, 2012; Scherr et al., 2012; Sayara et al., 2012). This process is being utilized more and more for the production of green energy from gas, principally methane, from the mineralization of organic material (Lv et al., 2010; McKeown et al., 2012). Bioreactors range from simple installations in domestic households to large, high-tech industrial plants (McKeown et al., 2012).

Hydrolytic acidogens, non-hydrolytic acidogens, syntropic acetogens and hydrogenotrophic and acetoclastic methanogens are the principal microorganism groups that sequentially drive methanogenesis (Lv et al., 2010). In one-step digestion, it is difficult to obtain optimal conditions for each group of microorganisms simultaneously (Bolzonella et al., 2012; Lv et al., 2010; Nasr et
It is therefore more efficient to split the process into two-steps: hydrolysis and methanogenesis (Bolzonella et al., 2012; Lv et al., 2010; Nasr et al., 2012). In two-step digestion, short hydraulic retention times (HRTs) and thermophilic conditions in a primary, hydrolytic reactor result in the production of high concentrations of volatile fatty acids (VFAs) which provide a feedstock for methanogenesis in a second digester (Bolzonella et al., 2012; Lv et al., 2010; Nasr et al., 2012).

### Table 2 Common bioremediation strategies, procedures and principles

<table>
<thead>
<tr>
<th>Strategy</th>
<th>Procedure</th>
<th>Principle</th>
</tr>
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<tbody>
<tr>
<td>Air-sparging</td>
<td>Active addition of air under pressure through vents</td>
<td>Increases functional aerobic microbial processes by delivery of oxygen to the vadose zone; physically removes volatile hydrocarbons</td>
</tr>
<tr>
<td>Bioaugmentation</td>
<td>Addition of functional microbial species</td>
<td>Enhances pollutant degradation by pre-selection of microbial species with known metabolic capabilities</td>
</tr>
<tr>
<td>Bioreactors</td>
<td>Control of environmental parameters</td>
<td>Stimulates pollutant degradation by creating conditions conducive for growth and catabolic functions of desirable microbial consortia</td>
</tr>
<tr>
<td>Biostimulation</td>
<td>Addition of nutrients or other amendments</td>
<td>Stimulates the growth and/or catabolic reactions of functional microbial species through provision of limiting nutrients or electron donors</td>
</tr>
<tr>
<td>Bioventing</td>
<td>Vacuum vents to enhance influx of air into vadose zone</td>
<td>Increases functional aerobic microbial processes by delivery of oxygen to the vadose zone; physically removes volatile hydrocarbons</td>
</tr>
<tr>
<td>Composting</td>
<td>Control of environmental parameters</td>
<td>Enhances decomposition of organic waste into stable material suitable for use as a soil conditioner</td>
</tr>
<tr>
<td>Land-farming</td>
<td>One or more strategies applied to surface soils</td>
<td>Dependent on strategy combination e.g. biostimulation, bioaugmentation, tilling</td>
</tr>
<tr>
<td>Tilling</td>
<td>Aeration of surface soil by tilling</td>
<td>Increase functional aerobic microbial processes by delivery of oxygen to subsurface or excavated piles</td>
</tr>
<tr>
<td>Natural attenuation</td>
<td>Monitor site</td>
<td>Allow effective natural processes to occur without intervention</td>
</tr>
<tr>
<td>Bioavailability</td>
<td>Addition of surfactant</td>
<td>Increase the biodegradation of pollutants by increasing the access of functional microbial populations to substrate</td>
</tr>
</tbody>
</table>

**Constructed wetlands (CWs) and biological sand filters (BSFs)**

See Section 1.4.3
1.1.3.5 Examples of additional bioremediation strategies

**Phytoremediation (plant remediation)**
Plants facilitate pollutant removal in one or more of the following ways: direct uptake, immobilization, enzymatic (plant) degradation in the rhizosphere and/or stimulation of functional rhizospheric microbial communities (Juwarkar et al., 2012; Megharaj et al., 2011).

**Vermistabilization and vermicomposting (earthworm remediation)**
The process by which organic material e.g. milk processing waste, textile mill sludge and primary sewage sludge is decomposed into compost and stabilized by the action of earthworms and microorganisms is known as vermicomposting and vermistabilization (Garg and Kaushik, 2005; Hait and Tare, 2011; Suthar 2012). These processes rely on bacteria and enzymes present in the gut of earthworms to decompose organic matter and detoxify organic pollutants (Juwarkar et al., 2012).

1.2 POLLUTION OF THE ENVIRONMENT

1.2.1 Point source and nonpoint source pollution

Anthropogenic generation of environmental pollutants emanates from consumption and production activities and tends to increase with economic advancement (Juwarkar et al., 2012). Pollution of the environment may be discrete (point source) or diffuse (nonpoint source) (American Waterworks Association 1990; Tong et al., 2011).

Examples of nonpoint source pollution include agricultural, highway and urban runoff (American Waterworks Association, 1990; Schulz, 2002; Tong et al., 2011), while examples of point source pollution include discharges and spills from industrial, agri-industrial and domestic wastewater treatment facilities, hazardous waste facilities, landfills, mine workings and mine tailings (American Waterworks Association, 1990; Chon et al., 2012; Fang et al., 2012). Diffuse
contamination is difficult to regulate because variables such as rainfall, soil type and microbial activity impact upon the chemistry and dispersal of contaminants (Albertin et al., 2012; O'Shea, 2002; Preston et al., 2011).

1.2.2 Environmental pollutants

Many organic pollutants are readily degraded, while others persist in the environment and are termed refractory or recalcitrant. The list of organic and inorganic chemical pollutants that are harmful to the environment and/or human health is extensive. The toxicity and biodegradability of the most important pollutants are discussed briefly in Sections 1.2.2.1 - 1.2.2.6

1.2.2.1 Pesticides

Pesticides typically enter the aquatic environment in agricultural run-off (Rittman and Perry, 2001). Chlorinated pesticides, e.g. dichlorodiphenyltrichloroethane (DDT) are particularly toxic to wildlife and humans (Galvao et al., 2012). These pesticides are recalcitrant, especially under aerobic conditions and are also hydrophobic, with a strong affinity for soil organic matter and fatty tissue (Weaver et al., 2012). Chlorinated pesticides are lipophilic and bioaccumulate in the fatty tissues of animals and humans, becoming more concentrated in higher trophic levels in a process known as biomagnification (Galvao et al., 2012; Jablonowski et al., 2012). In comparison to chlorinated pesticides, newer pesticides, including organophosphates, carbamates and triazines are less persistent in the environment (Buono et al., 2012; Rittman and Perry, 2001). However, they are not necessarily less toxic and it is concerning that they are commonly found in a variety of foodstuffs (Buono et al., 2012; Del Carlo et al., 2004; Fenoll et al., 2007; Rawn et al., 2006; Terada et al., 2008; Zhang, 2008). Atrazine is a popular triazine which is frequently detected in both surface and groundwater in South Africa and other countries (Pick et al., 1992).
1.2.2.2 Synthetic detergents (surfactants)

Synthetic detergents are classed as anionic, cationic and non-ionic (American Waterworks Association, 1990; Borghi et al., 2011; Gisslen and Magnusson, 1966). Cationic detergents are suitable as disinfectants because they are microbiologically toxic but not recalcitrant (Gheorghe et al., 2012).

1.2.2.3 Aliphatic hydrocarbons

In comparison to cationic detergents, non-ionic surfactants are less toxic to microorganisms and produce a synergistic effect during the biodegradation of hydrophobic organics (Song and Bieleveld, 2012). However, many non-ionic alklyphenolic detergents are classified as endocrine disrupting compounds (EDCs), can bioaccumulate in animal tissues and can become halogenated in chlorinated water (McAdam et al., 2011; Rittman and Perry, 2001). Biodegradation rates of alkylphenolics in activated sludge are inconsistent and tertiary treatment is required to ensure effective removal (McAdam et al., 2011). Chlorinated aliphatic hydrocarbons (CAHs) are widely used as solvents and have high dispersivity values; consequently, these toxic molecules are common groundwater contaminants (Hamonts et al., 2012; Randazzo et al., 2011; Scherr et al., 2011). Some CAHs, e.g. carbon tetrachloride and tetrachloroethane, are highly refractory, while others, e.g. chloromethane and dichloromethane are readily biodegradable (Kim and Lee, 2009; Rittman and Perry, 2001). Biodegradation of CAHs is typically related to the degree of chlorination and the prevailing redox status, with preferential degradation of highly chlorinated molecules taking place under anaerobic conditions (Section 1.1.1).

1.2.2.4 Aromatic hydrocarbons

The primary step in the environmental biodegradation of all aromatic hydrocarbons is an oxygenation reaction which is catalysed by oxygenase enzymes (Rittman and Perry, 2001). These reactions take place aerobically or
anaerobically, using molecular oxygen or oxygen derived from water, nitrates or carboxylates as a terminal electron acceptor (Rittman and Perry, 2001).

**BTEX compounds**

The BTEX (benzene, toluene, ethyl benzene and toluene) compounds are toxic, naturally occurring aromatic hydrocarbons found in petrol (Dou et al., 2008a; Dou et al 2008b; Farhadien et al., 2008). They are more water-soluble than other petroleum hydrocarbons, so they partition into the environment more easily (Dou et al., 2008a; Dou et al 2008b; Farhadien et al., 2008). However, microorganisms that degrade these aromatics are abundant in the environment making bioremediation a feasible option for cleaning up contaminated sites (Dou et al., 2008a; Dou et al 2008b; Farhadien et al., 2008; Seklemova et al., 2002).

**Polycyclic aromatic hydrocarbons (PAHs)**

PAHs are toxic, persistent, naturally-occurring molecules (Wang et al., 2010). With the exception of naphthalene, PAHs have a low solubility and contaminate soils more than groundwater (Rittman and Perry, 2001; Plaza et al., 2009; Yu et al., 2005). The low solubility reduces the bioavailability of these hydrocarbons, but smaller molecules are still readily biodegradable (Li et al., 2008; Silva et al., 2009). However, more complex molecules with many rings are generally recalcitrant (Li et al., 2008; Silva et al., 2009; Wang et al., 2010).

**Chlorinated aromatic hydrocarbons**

Of the chlorinated aromatic hydrocarbons, single-ringed molecules are readily biodegradable under aerobic conditions but more complex molecules are typically more recalcitrant (Anyasi and Atagana, 2011; Urbaniak, 2007). The priority pollutants, PCBs have been banned in most countries for decades (Anyasi and Atagana, 2011). However, they still exist in the built environment (e.g. in paint) and persist in soils because of their refractory nature (Anyasi and Atagana, 2011; Jartun et al., 2009; Rittman and Perry, 2001; Urbaniak, 2007). Being
lipophilic, PCBs bioaccumulate and biomagnify in the fatty tissues of animals and humans (Anyasi and Atagana, 2011; Urbaniak, 2007).

Pentachlorophenol (PCP), a common wood preservative disrupts the mitochondrial function of microorganisms, animals and humans at low concentrations and is also recalcitrant in the environment (Buono et al., 2012; Song and Huang, 2007; Liu et al., 2008). However, excellent biodegradation of PCP can be achieved in treatment facilities using pre-acclimated microbial consortia (Hickman and Novak, 1984; Liu et al., 2008). Dioxins are industrial by-products and are potent EDCs, carcinogens, neurotoxins and teratotoxins that partition to soils and sediments and are highly resistant to biodegradation (Chang, 2008; Hogue, 2012; Yoshioka et al., 2011).

1.2.2.5 Explosives

Soil, groundwater and marine environments have been contaminated by 2,4,6-trinitrotoluene (TNT), hexahydro-1,3,5-trinitro-1,3,5-triazine (RDN) and octahydro-1,3,5,7-tetranitro-1,3,5,7-tetrazocine (HMN) (Dalgren et al., 2009; Rosen and Lotufo, 2007; Steevens et al., 2002). These nitroaromatic molecules are mutagenic to microorganisms, plants and animals, with bioaccumulation of the parent molecules or metabolites occurring in particular species (Jarvis et al., 1998, Kolwzan et al., 2008; Meyers et al., 2007; Rosen and Lotufo, 2007). The amino transformation products of TNT interact with each other and with soil components but are only rarely mineralized in the environment, partly because of the low bioavailability afforded by adsorption onto soil particles (Casabe et al., 2003; Dalgren et al., 2009; Rittman and Perry, 2001). However, explosives are amenable to biodegradation under laboratory conditions and the provision of co-metabolites, usually in the form of compost, aids in-situ bioremediation by increasing the bioavailability and reducing the toxicity of the molecules (Dalgren et al., 2009; Clark and Boopathy, 2007; Hatzinger et al., 2009; Jarvis et al., 1998).
1.2.2.6 Inorganic pollutants

Metals/metalloids

The heavy metal(loid)s including As, Cd, Co, Cu, Hg, Mn, Ni, Pb and Zn are released as a consequence of geological activities and/or anthropogenic impacts (Luque-Garcia et al., 2011; Park et al., 2011). Bioaccumulation and biomagnification of metal(loid)s can take place in species with inadequate metal efflux mechanisms (Luque-Garcia et al., 2011; Lee et al., 2012; Jara-Marini et al., 2009; Patnaik et al., 2004). Heavy metals affect biochemical, nutritional and molecular processes in mammals (Patnaik et al., 2004). Other metals, while often not toxic per se, can have secondary toxicity effects: for example, oxidised Fe (Fe(III)) and S can acidify aqueous environments and increase the solubility of more toxic species (Matthies et al., 2012). In the environment, the degree of toxicity of metal(loid)s to microorganisms, plants and animals is affected by the solubility and speciation of the metal(loid)s, which is strongly dependent on redox status and pH (Giller et al., 1998).

Applicable bioremediation strategies for metal(loid) contaminated sites employ organic amendments and/or microbial processes to immobilize, volatilize or effect a change in metal(loid) speciation (Park et al., 2011). For example (i) Toxic, soluble, hexavalent chromium [Cr (VI)] is reduced to non-toxic Cr (III) which is immobilized in the soil as insoluble chromium hydroxide [Cr(OH)₃]; and (ii) SO₄²⁻ is reduced to sulphite (SO₃²⁻) and sulphide (S²⁻) to neutralize acid mine drainage (AMD) (Banerjee et al., 2011; Garg et al., 2012; Johnson, 2005; Park, et al., 2011; Polti et al., 2011)

Nutrients

Although the range of inorganic and organic nutrients required for the growth of microorganisms is extensive, in wastewater treatment terminology only N and P compounds are typically referred to as “nutrients” (Tchobanoglous and Burton, 1991). In most unpolluted waters, primary production is limited because these essential nutrients are naturally present in low concentrations and the aquatic
environment is oligotrophic or mesotrophic (Wetzel, 2001). However, increased concentrations can cause eutrophication, which is an overgrowth of aquatic organisms, particularly algae (Ekholm and Lehtoranta, 2012; Wetzel, 2001). When the pervading organisms senesce, they provide a substrate for microbial action which then depletes oxygen supplies, killing fish and other aquatic organisms (Atlas, 1997; Wetzel, 2001).

1.3 MICROBIAL ECOLOGY

1.3.1 Introduction

Microbial ecology is the study of the interrelationships between microorganisms and their living (biotic) and non-living (abiotic) environments. Sergei Winogradsky, who first described chemolithotrophy, the sulphur cycle and the nitrogen cycle, is considered the founder of this branch of science (Dworkin, 2012). Microbial ecology evolved from studies of microorganisms within soil and benthic environments, but many other ecological niches have since been studied: from wine grape berries (Barata et al., 2012) to shipwrecks (Bjordal, 2012) to the gut microbiota of a variety of species including humans, fish, chickens, pigs, mice and termites (Britton and Young, 2012; Czerwinski et al., 2012; Desai and Brune, 2012; Nava et al., 2011; Wong and Rawls, 2012; Zentek et al., 2012). Microbial ecology has been used to understand and compare amended, polluted, remediated and unperturbed environments and processes in a variety of geographical and biological settings (Guimaraes et al., 2010; Hanson et al., 2012; Semple et al., 2007).

1.3.2 Metagenomic approach to soil microbial analysis

Until the 1980’s, studies on microbial ecology were mostly dependant on culture techniques to identify and elucidate the metabolic behaviour of environmental microorganisms (Amman et al., 1995; Rosello-Mora and Amann, 2001). However, it has been estimated that more than 99% of microorganisms found in soil cannot be cultured using standard methods and culture-dependent methods are
thus biased towards the selective enrichment of microorganisms representing a minor fraction of the total microbial community (Amman et al., 1995; Lukow et al., 2000; Rosello-Mora and Amann, 2001). Thus, Winogradsky’s concept formulated in 1885, that organisms should be studied in conditions as close as possible to their natural environments in order to understand their role in nature has only been realized over the last twenty years with the wide-scale application of culture-independent methods (Dworkin, 2012; Su et al., 2012). Successful culture-independent methods for studying the distribution, abundance and function of microorganisms in the environment are molecular based polymerase chain reaction (PCR) and non-PCR based techniques such as phospholipid fatty acid (PFLA) analysis, stable isotope probing (SIP), microarray technologies and fluorescent in-situ hybridization (FISH) (Malik et al., 2008; Pratt et al., 2012; Su et al., 2012). There are inherent advantages and disadvantages with each approach, for instance, PCR-based methods are subject to errors emanating from sample handling, the presence of inhibitory substances in samples and nucleic acid extraction procedures (Malik et al., 2008; Stephen et al., 1999; Su et al., 2012). In some cases, differential amplification of target genes can lead to PCR bias, which can be overcome to some extent by the application of more than one method and/or the use of more than one set of primers (Su et al., 2012).

1.3.2.1 The use of DGGE and T-RFLP fingerprinting methods

PCR-based methods which have been used extensively for ecological studies of soil and sediment microorganisms are intergenic spacer analysis (ARISA), denaturing gradient gel electrophoresis (DGGE) and terminal restriction fragment length polymorphism (T-RFLP) (Enwall and Hallin, 2009; Marzorati et al., 2008; Okubo and Sugiyama, 2009). These techniques can be applied using a metagenomic approach, i.e. on composite genetic material extracted directly from environmental samples (Su et al., 2012). DGGE and T-RFLP, the methods discussed in more detail in this section, can both be used to compare microbial community structures using highly conserved sequences (typically in the 16S rRNA gene), or to perform studies limited to selected members of the microbial
populace associated with particular functions: for example, the methanogenic, ammonium oxidising and denitrifying communities have been targeted by employing primers for sequences in the methane monooxygenase genes (e.g. \textit{pmoA}, \textit{mcrA}), ammonium oxidising genes (e.g. \textit{amoA}) or nitrate reductase genes (e.g. \textit{nirS}), respectively (Chauhan et al., 2012; Lee et al., 2012; Vasileiadis et al., 2012). The principle of DGGE relies on the electrophoretic separation of denatured PCR-amplified products using gels which incorporate linear gradients of denaturing chemicals (Muyzer and Smalla, 1998; Okubo and Sugiyama, 2009). The double strands of AT base pairs are more easily dissociated than those of the GC base pairs allowing the differential separation of DNA fragments with different sequences and different AT/GC content (Okubo and Sugiyama, 2009).

Marker genes are amplified using primers that have been modified by the addition of a 35-40 bp GC-clamp which is attached to the 5'-end of the forward primer (Muyzer et al., 1993; Kirk et al., 2004). The GC clamp ensures that part of the DNA remains double stranded so that stable, partially melted fragments are formed. This allows close to 100% of all sequence variations to be detected by differential migration on the denaturing gel (Myers et al., 1985; Sheffield et al., 1989). The products migrate through the gel at different rates, each product forming a band, so that unique banding patterns, or fingerprints, are formed (Muyzer and Smalla, 1998).

Providing the methodology is reproducible, the banding patterns from more than one gel can be statistically compared to assess the similarity of the microbial community structures from different environmental samples (Epelde et al., 2012; Tourlomousis et al., 2010). For example, dendrograms can be compiled from the banding patterns (Figure 1A, 1B), which can be further analysed using multivariate statistical analyses such as nonmetric multidimensional scaling (NMDS) (Figure 1C) or principal component analysis (PCA) (Okubo and Sugiyama, Zheng et al., 2009). In addition, individual bands can be excised for further analysis e.g. by cloning into vectors to compile clone libraries (Kittelman and Janssen, 2011; Kusar and Avgustin, 2012; Lovell et al., 2008).
Figure 1  Dendrogram (A) of DGGE pattern similarity (B) and three-dimensional multidimensional scaling (MDS) plot (C) from the matrix of similarity, showing the similarities in the microbial community structure from different sand mesocosm replicates (A,B,C,D) taken at various chronological intervals (1: green, 2: red, 3: yellow, 4: blue, 5: purple). Ellipses around the samples indicate similarities in bacterial community fingerprints determined by cluster analysis.
In T-RFLP, 16s rRNA gene products are obtained using primers which are fluorescently labelled. The PCR products are then digested with specific restriction endonucleases to give a number of labelled terminal restriction fragments (T-RFs) which are then denatured, separated by electrophoresis and analysed in an automated system (Malik et al., 2008; Osborn et al., 2000). The intensity of fluorescence of each T-RF is detected by laser and the size of each T-RF is determined by comparison with internal standards using algorithms to produce an electropherogram that reflects the size and intensity of fluorescence of each T-RF (Figure 2) (Marsh, 1999; Osborn et al., 2000). The intensity of fluorescence is related to the abundance of each T-RF and can be used cautiously to quantify species within microbial consortia (Osborn et al., 2000). Similar to DGGE, multivariate statistical analysis of the data is performed to extract information relevant to a particular study (Enwall and Hallin, 2009; Horz et al., 2000; Lukow et al., 2000; Osborn et al., 2000). The most important advantages of T-RFLP over DGGE are that this method allows higher throughput and avoids gel-to-gel comparison bias (Enwall and Hallin, 2009).

![Figure 2](image-url)  
**Figure 2**  
Example of a T-RFLP electropherogram: T-RFs are shown in blue and the size ladder (Rox 1.1 standards) is shown in red.
1.4 BIOLOGICAL WASTEWATER TREATMENT

1.4.1 Introduction

To maintain satisfactory water quality, natural waters should not be overloaded with toxic substances or nutrients and the dissolved oxygen (DO), temperature, salinity, pH and turbidity should fall within acceptable ranges (Atlas, 1997) (Veissman and Hammer, 1998). The oxygen demand imposed by heterotrophic microbial activity during the degradation of waste products, e.g. sewage, leads to a depletion in the DO of receiving waters (Atlas, 1997; Mason, 2002). Oxygen depletion can result in the death of aerobic organisms, including microorganisms, fish and invertebrates and the microbial decomposition of these species creates an additional oxygen demand (Atlas, 1997; Mason, 2002).

The aim of wastewater treatment is to remove organic matter, human pathogens and toxic chemicals via physical, biological and/or chemical means (Atlas, 1997; Tchobanoglous and Burton, 1991). The oxygen demand can be reduced by primary physical separation followed by secondary microbial degradation of organic matter from wastewater (Atlas, 1997; Insel et al., 2011; Tchobanoglous and Burton, 1991). The configuration and operating parameters of wastewater treatment works can also be designed to remove N and P using microbial nitrification-denitrification and P accumulation (Tchobanoglous and Burton, 1991; Veissman and Hammer, 1998).

1.4.2 Conventional biological treatment processes

1.4.2.1 Suspended-growth processes

Suspended growth processes include the activated sludge (AS) process, aerated lagoons, sequencing batch reactors and more recently, membrane bioreactors (MBRs) (Ferrero et al., 2012; Jahan et al., 2011; Ni and Yu, 2012). The AS process was developed in England in 1914 and involves the natural evolution of an activated mass of microorganisms capable of stabilizing waste under aerated
conditions (Tchobanoglous and Burton, 1991). The process is continuous and organic matter is converted into biomass which is separated from the clarified supernatant fluid by settling in clarifiers (Jahan et al., 2011; Jenkins et al., 2004). The AS process and other aerobic processes for the removal of carbonaceous biological oxygen demand can be combined with anoxic and/or anaerobic processes for simultaneous removal of nutrients (Czerwionka et al., 2012; Fang et al., 2010; Gebremariam et al., 2011; Seviour et al., 2011).

The removal of biomass in most suspended growth systems is incumbent on the provision of optimal process conditions so that microorganisms grow together in settleable flocs (Eikelboom, 2000; Jenkins et al., 2004). However, maintaining desirable floc integrity can be problematic (Eikelboom, 2000; Jenkins et al., 2004). The use of membrane technology to separate the biomass from the treated water does not rely on floc formation and also obviates the need for clarifiers (Santos et al., 2011). MBRs have high energy costs and membranes are expensive and prone to fouling (Guo et al., 2012; Santos et al., 2011). However, the costs of membrane manufacture are decreasing and extensive research to find mechanisms of alleviating fouling problems is being conducted (Guo et al., 2012; Santos et al., 2011).

1.4.2.2 Attached growth processes

Attached-growth biological wastewater treatment processes include trickling filters, rotating biological contactors and packed bed reactors (Daigger and Boltz, 2011; Li and Qi, 2012; Singh and Mittal, 2012; Singh and Prema, 2009). Attached growth systems can be operated in conjunction with suspended growth systems (Veissman and Hammer, 1998). In attached growth processes, the microorganisms are present in the form of biofilm attached to various support matrices (Despland et al., 2012; Liu et al., 2012; Wesley et al., 2011). The redox status decreases from the exterior to the interior of the biofilm and the chemical status is not uniform throughout, so different organism groups with diverse metabolic functions can occupy different niches in the biofilm (Despland et al.,
Problems encountered with attached growth systems include mass transfer limitations, sloughing of microorganisms into the treated effluent and operational instability (Tchobanoglous and Burton, 1991).

1.4.3 CWs and biological sand filters (BSFs)

Natural wetlands are environments intermediate between terrestrial and aquatic systems, and have been used either deliberately or inadvertently to treat wastewaters for centuries (Haberl et al., 2003). CWs, also known as treatment wetlands (TWs), are engineered wastewater treatment systems that mimic the bioremediatory processes taking place in natural wetland ecosystems (Vymazal, 2005). BSFs are fundamentally unplanted CWs, and are often described as such. These two systems can be considered essentially as fixed-growth biological wastewater treatment systems. Typically, the physical substrate (substratum) supports the growth of microbial communities (and plants in the case of CWs), that function synergistically to remediate polluted water (Kiviasi, 2001; Vymazal, 2005). By incorporating a range of structural characteristics and hydraulic flow regimes in system design, they can be tailored for the treatment of different target waste streams. The ultimate design is dependent on a number of factors, including: the type of wastewater to be treated, the location, the effluent standards that must be attained and the costs of construction, operation and maintenance (Austin and Nivala, 2009; Kadlec, 2009; Van de Moortel et al., 2009; Verhoeven and Meuleman, 1999). Literature pertaining to specific aspects of CWs and BSTs pertinent to the work described in this thesis is included in Chapters 2-6, while this Section (1.4.3) describes general background information only.
1.4.3.1 Major factors influencing the performance of CWs and BSFs

Hydraulic flow regime, redox status and mode of operation

The predominant flow of wastewater in CWs takes place either over the surface of the substratum [free water surface flow (FWSF)] or within the substratum [subsurface flow (SSF)] (Figure 3) (Rosseau et al., 2004; Van de Moortel et al., 2009). Subsurface flow either proceeds from top to bottom [vertical subsurface flow (VSSF)] or from inlet to outlet [horizontal subsurface flow (HSSF)] (Vymazal, 2007).

In HSSF systems, the inlet may be located either above or below the surface, while the treated effluent exits below the surface (Suliman et al., 2006). In these systems, the flow paths and consequent removal efficiencies are affected not only by the substrate medium, but also by the relative positions of the inlet/s and outlet/s (Suliman et al., 2006). HSSF systems are typically operated in a continuous mode (Maier et al., 2009).

In conventional VSSF systems, the surface is flooded intermittently, followed by a period of drainage, i.e. a batch mode of operation (Giraldi and Iannelli, 2009; Maier et al., 2009; Molle, 2006). If the period of drainage is protracted (days), the flow is termed “tidal” with shorter periods being termed “pulsed” (Austin and Nivala, 2009). The inlet is located above the surface and effluent is generally collected via drainage pipes located at the bottom, ensuring a vertical hydraulic flow (Kantawanichul et al., 2009; Prochaska and Zouboulis, 2009). Hybrid systems are becoming popular; these combine the advantages of different hydraulic regimes, either by employing a series of reactors, each with different modes of operation, or by combining more than one hydraulic regime within the same system (Keffala and Ghrabi, 2005; Toscano et al., 2009).
Figure 3  Schematic diagrams of a free water surface flow (FWSF) CW (A), a horizontal subsurface flow (HSSF) CW (B) and a vertical subsurface flow (VSSF) CW showing the fill cycle (left) and drain cycle (right) (C)

The hydraulic regime and mode of operation have a significant impact on the redox potential of the substratum, which in turn affects the removal efficiency (Table 3) (Dusek et al., 2008; Tee et al., 2012) Aerobic processes, such as nitrification, are favoured in oxidised environments (high redox potential), while under reduced conditions (low redox potential), anaerobic processes, particularly sulphate reduction and methanogenesis, are more likely to occur (Faulwetter et al., 2009).
Due to continuous inundation of the surface, the substrata of FWSF systems are anoxic or anaerobic, with low redox potentials (Dusek et al., 2008; Faulwetter et al., 2009). Conversely, the highest redox potentials are encountered in VSSF systems (Faulwetter et al., 2009). This is because VSSF systems are typically operated in a batch mode, resulting in alternating periods of flooding and drainage with attendant draw-down of atmospheric gases into the substratum during the drainage period (Maier et al., 2009; Tietz et al., 2008; Torrens et al., 2009). Nitrification and organic degradation are enhanced under aerobic conditions; accordingly, the efficiency of these processes is highest in VSSF systems, especially near the surface (Dusek et al., 2008; Tietz et al., 2008; Van de Moortel et al., 2009).

On the other hand, because HSSF systems are mostly operated in continuous mode, a lower redox potential is found in the substratum than in VSSF systems, resulting in less efficient nitrification and organic degradation, but enhanced denitrification (Faulwetter et al., 2009; Verhoeven and Meuleman, 1999). It has been shown that organic removal rates in HSSF systems can be increased by changing the mode of operation from continuous to batch mode (Calheiros et al., 2009; Pedescoll et al., 2011).

The removal of organics in low-loaded FWSF systems may be comparable to that of some HSSF systems, but because the most important P removal processes typically take place in the substratum, the removal of P is lowest using this flow regime (Kadlec, 2009; Naz et al., 2009; Verhoeven and Meuleman, 1999). In addition, the formation of algal blooms, with resultant increased effluent chemical oxygen demand (COD) and possibly toxin formation may occur in FWSF systems (Naz et al., 2009).
Table 3  Comparison of the redox status and nutrient removal rates obtained with different hydraulic regimes and modes of operation in CWs

<table>
<thead>
<tr>
<th>Hydraulic regime/mode</th>
<th>Redox status</th>
<th>Nutrient removal rates</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>COD</td>
</tr>
<tr>
<td>FWSF/cont</td>
<td>low</td>
<td>low</td>
</tr>
<tr>
<td>VSSF/batch</td>
<td>high</td>
<td>high</td>
</tr>
<tr>
<td>HSSF/cont</td>
<td>mod</td>
<td>mod</td>
</tr>
<tr>
<td>cont: continuous</td>
<td>mod: moderate</td>
<td></td>
</tr>
</tbody>
</table>

Composition of the substrate

It has been shown that C, N and P removal rates are influenced by the chemical and physical properties of the substrate medium (Zhang et al., 2007). Soil, zeolite, slag, compost and alum sludge have been successfully employed as substrate matrices in CWs, but sand and gravel are most commonly used (Aslam et al., 2007; Babatunde et al., 2011; Li et al., 2008b).

Sand particles provide a large surface area for biofilm attachment and surface chemistry (Akratos and Tshrintziz, 2011; Torrens et al., 2009). The actual shape of the sand particle has been correlated with biofilm attachment/abundance and consequent C and N removal performance, with natural sand presenting a superior biofilm attachment surface to crushed sand (Torrens et al., 2009). In contrast to C and N removal, the major mechanisms responsible for P removal are abiotic and related to the chemistry and morphology of the substrate (Akratos and shrintziz, 2011; Korkusuz et al., 2005; Zhang et al., 2007). In sand of similar chemical composition, the removal of P has been shown to be affected by the geometry of the grains because particles with larger surface areas provide more space for P adsorption and precipitation (Akratos and Tshrintziz, 2011).

The major disadvantage of using sand as a substrate is that the small grain size can result in clogging of the matrix pores by suspended solids and/or biofilm (Knowles et al., 2010; Molle, 2006; Pedescoll et al., 2011; Prochaska, 2007; Turon...
et al., 2009). However, clogging can be prevented by: (i) The use of pre-filters or clarifiers to remove suspended solids and/or (ii) The use of batch mode to ensure complete substrate and biofilm degradation during the resting period and/or (iii) The application of low organic loading rates (Knowles et al., 2010; Prochaska, 2007; Tao et al., 2007; Torrens et al., 2009). The novel use of earthworms is a cost-effective, environmentally friendly approach which has recently been described for the restoration of clogged sand-filled CWs (Li et al., 2011).

**Microbial communities**

In the environment, microbial degradation rates are influenced by physical and chemical parameters, including temperature, redox status and pH, which can be selectively mimicked in CWs and BSFs to enhance pollutant removal (Truu et al., 2009). Sedimentary and epiphytic bacteria, as well as planktonic bacteria (in FWSF systems) are principally responsible for nitrification, denitrification, sulphate oxidation/reduction and hydrocarbon degradation, transformation and mineralization, which follow natural principles for the biogeochemical cycling of carbon, nitrogen and sulphur (Faulwetter et al., 2009; Li et al., 2008; Tao et al., 2007; Li et al., 2008b).

The abundance of microbial biomass is proportional to the organic loading rate and the heterotrophic growth rate is higher in SSF than SF systems due to superior oxygen mass-transfer (Truu et al., 2009). Similar to conventional biological wastewater treatment processes, the type of wastewater plays a selective role on the microbial community structure (Faulwetter et al., 2009; Nicromat et al., 2008).

**Plant/substrate/microorganism relationships**

It has been shown that various plant metabolites are excreted into the rhizosphere through a process known as rhizodeposition (Yao et al., 2012). Rhizodeposition has been shown to stimulate or inhibit the growth of different members of the microbial community but, with the exception of low loaded systems, plant metabolite secretions are low in comparison to most other
wastewater components (Stottmeister et al., 2003; Yao et al., 2012). Stottmeister et al. (2003) are of the opinion that plants have an insignificant impact on the functional microbial community and they are supported by research conducted by Baptista et al. (2003), who found that the functional microbial groups, including sulphate-reducing bacteria (SRB) and archaea, in planted (*Phragmitis australis*) and unplanted HSSF CWs treating beer influent were no more similar than if they had been randomly assembled from a common source community. However, (i) Sleytr et al. (2009) determined that the microbial communities differed in the rhizospheres of CWs planted with different emergent macrophytes, (ii) Calheiros et al. (2009) found that the type of substrate and the presence of plants, but not the process conditions, affected the microbial community structure in gravel and expanded clay aggregate-filled CWs treating tannery wastewater, and (iii) Weber et al., (2008) found that plants assisted in preventing detachment of microbial communities into the interstitial water when exposed to acid mine drainage (AMD).

Clearly, further research into the functional triad between microorganisms, plants and substrate is still required.

### 1.5 AIMS AND SCOPE

Worldwide, there is a lack of knowledge on the use of CWs/BSFs for the treatment of winery wastewater. Currently, many CWs/BSFs treating organic industrial waste streams do not perform according to expectations. Consequently, the widespread use of CWs/BSFs for the treatment of winery wastewater in South Africa has not been adopted. It is hypothesized that by exploiting the innate phenomena involved in organic adsorption/desorption, transformation and degradation in the design process, CWs/BSFs may provide feasible, efficient, cost effective options for the treatment of winery wastewater at small to medium sized wineries in this country.
The work presented in this thesis emanates from two four-year projects sponsored by the Water Research Commission (WRC) of South Africa focusing on understanding the factors contributing to the bioremediation of winery wastewater using CWs/BSFs. A number of experiments were designed to gain insight into the degradation of authentic winery wastewater and individual components of winery wastewater in sand-filled pilot-scale CWs/BSFs. It is envisaged that at the end of the study period, the knowledge that is gained will be incorporated into a set of design criteria for the construction of full-scale CW systems.

The proposal for the first project, (WRC Project K5/1936, 2007-2011), entitled “Health for purpose in constructed wetlands: organic removal efficiencies and changes in microbial community dynamics associated with exposure to winery wastewater” contained three principle aims:

(i) “To obtain molecular fingerprints of microbial communities and key enzymes involved in the degradation of specific pollutants”,
(ii) “To develop methods to demonstrate microbial population changes resulting from the impact of polluting waste streams”, and
(iii) “To investigate the effects of specific interventions (e.g. fertilizer) on the ‘health-for-purpose’ of the wetland microbial population”.

The second project, (WRC project K5/2104, 2011-2015), was designed to build on the knowledge obtained from the original (K5/1936) which clearly demonstrated that the removal of organics in CWs is naturally influenced by both biotic and abiotic factors but that there is inadequate integrated information available on how these functions can be optimized in CWs/BSFs treating winery wastewater. The aims of this project, as set out in the proposal document were to:

(i) “Understand the adaptations that can be implemented to increase the functionality of CWs treating agri-industrial waste”,


(ii) “Utilize microbial community fingerprinting to determine the ranges of physical and chemical parameters applicable to optimal functioning of CWs treating agri-industrial waste”,

(iii) “Identify a matrix of parameters through which to match the capacity of CWs to treat synthetic wastewater with the characteristics of authentic agri-industrial wastewater”,

(iv) “Investigate the reproducibility of CWs in varied environments by characterizing the microbial communities in these environments”,

(v) “Develop new understanding of the stability of microbial communities adapted to particular wastes under the variable conditions imposed by real world situations”,

(vi) “Understand the extent to which microbial communities in CWs can accommodate changes in the volume and composition of wastewater and the rates at which they can adapt”, and

(vii) “Understand the flexibility of microbial communities to accommodate different types of wastewater”.

The projects are of a collaborative nature between researchers based at the Institute of Microbial Biotechnology and Metagenomics (IMBM) at the University of the Western Cape (UWC) and the Biocatalysis and Technical Biology (BTB) Research group at the Cape Peninsula University of Technology (CPUT).

Eight sand-filled CW replicates are housed at BTB and all sampling and chemical analyses described in this thesis were conducted at CPUT. Dr J-B. Ramond, a post-doctoral research fellow employed at IMBM, assisted with sand sampling and performed all the molecular-based work described in this thesis. Mr Alaric Prins, a Biotechnology undergraduate student, assisted with selected HPLC analyses as part of his mini-thesis. The remainder of the work was conducted by the PhD candidate. In addition, the candidate, Mrs Welz, in consultation with Dr Ramond, designed all operational and experimental aspects pertaining to the projects. Mrs Welz is the designated project leader on WRC Project K5/2104.
1.6 OUTLINE OF THESIS

This body of this thesis is presented in the form of three published manuscripts. In the first two papers, given in Chapters 2 and 3, the systems are referred to as CWs. In the third, given in Chapter 4, the same systems are referred to as BSFs on the recommendation of a reviewer. Chapter 5 serves as a summation of the work.

Chapter 2 describes work which was undertaken to determine the recommended time-scales needed for the stabilization of microbial communities in experimental CW mesocosm replicates. The presence of stable (equilibrated) microbial communities is important to accurately compare perturbations on the microbial community structure and the bioremediatory capacity and function of CWs in response to the addition of chemicals.

Chapter 3 details work using ethanol as a model, common readily biodegradable fraction of winery wastewater. The experiments described in this paper describe the use of a procedure termed “incremental priming” to enhance the remediation of ethanol by prior acclimation of microbial communities. This was achieved by exposing CW microbial communities to increasing concentrations of ethanol (from low to high) and comparing CW removal efficiencies with non-acclimated microbial populations which were exposed to a moderate concentration of ethanol from inception.

Chapter 4 describes a series of experiments using BSFs, sand columns and sand microcosms to understand the removal of phenolics, a common slowly biodegradable fraction of winery wastewater, in BSFs. These experiments determined (i) The relative contributions of biotic and abiotic mechanisms to the removal of phenolics, (ii) The effect of prior acclimation of microbial communities on the biodegradation rate of phenolics, and (iii) The maximum concentration of influent synthetic wastewater above which a build-up of microbially toxic metabolites occurred.
1.7 REFERENCES


microbiota and bioaugmentation with isolated microbial consortia. Bioresource Technology, 100: 4669-4675.


2 MICROBIAL COMMUNITY STRUCTURE STABILITY, 
A KEY PARAMETER IN MONITORING THE 
DEVELOPMENT OF CONSTRUCTED WETLAND 
MESOCOSMS DURING START-UP

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Reference (APPENDIX 1):

2.1 ABSTRACT

Constructed wetlands (CWs) are known to be effective for treating waste streams, and pilot-scale CWs are useful for assessing the impact and remediation of pollutants. However, little is known with respect to the establishment of these mesocosm systems or the parameters which should be monitored to assess system equilibration, i.e. when stabilized physical and biological patterns are achieved. The aim of this study was to evaluate the temporal aspects of CW equilibration as a basis for future studies of system response to amendment. Microbial biomass and hydraulic conductivity values were monitored and microbial community fingerprints were obtained using Denaturing Gradient Gel Electrophoresis (DGGE). This study showed that microbial community fingerprinting provides a valuable tool for assessing the time scales of equilibration, being the last parameter to stabilize during the equilibration period. Hydraulic conductivity was also proved to be a useful indicator to determine the period necessary for equilibration to take place. For a CW of the dimensions used (1.73 m x 1.06 m / 0.30 m depth), community equilibration times demonstrated on the basis of similar microbial community structures were found to be in the order of 100 days.

2.2 INTRODUCTION

Wetlands are generally defined as areas that are inundated or saturated by surface or ground water at a frequency sufficient to support vegetation types adapted to the saturated conditions (Mitsch and Gosselink, 2000). Although constructed wetlands (CWs) have been used since the 1950s, they are now of particular interest globally, as they represent a cost-effective, ecologically-friendly and aesthetically attractive option for wastewater purification. Studies have shown that CWs can reduce the chemical oxygen demand (COD) levels in industrial or agricultural effluents by 60-90% and in sewage domestic effluents by 90-99% (Verhoeven and Meuleman, 1999; Vrhošek et al., 1996). In addition, the transformation of inorganics, including nitrogen (via microbial nitrification,
denitrification and annamox) and sulphur (via microbial sulphate reduction) provide important mechanisms for contaminant removal in CWs (for review, see Faulwetter et al., 2009). Moreover, CWs are already used to treat and purify various contaminated effluents such as municipal, industrial and/or agricultural wastewaters (Vymazal, 2009), and pilot-scale CWs are used to assess the feasibility of the technology before full-scale application (Chen et al., 2006).

In general, CWs comprise a substrate (e.g. soil) supporting plant and microbial communities that work synergistically to treat wastewaters. Plants metabolize available nutrients and are able to accumulate heavy metals (Glick, 2010). In addition, plants are capable of directly catabolizing certain organic contaminants via processes known as phytotransformation and phytodegradation (for review, see Singh and Jain, 2003). For example, the uptake and degradation of the toxic polycyclic aromatic hydrocarbon anthracene into anthrone, anthraquinone and hydroxyanthraquinone has been demonstrated in wheat and maize (Wild et al., 2005). Microbial communities are essential in the mineralization of organic matter and in nitrogen, sulphur and phosphorous removal (Faulwetter et al., 2009; Glick, 2010; Truu et al., 2009). The substrate assists in the removal of pollutants by natural sedimentation (Vrhošek et al., 1996). Studies have shown that the composition of the substrate can play an important role in the removal of pollutants. For example, the adsorption of phosphorus and the abiotic oxidation of phenolics are enhanced in CWs where iron and/or manganese forms part of the substrate (e.g. Lehmann et al., 1986; Polubesova et al., 2010; Sakadevan and Bavor, 1998).

It has been shown that extended periods are required for microbial communities in CWs to stabilize during operational periods, although the basis used to indicate community stability is not always consistent (Truu et al., 2009; Weber and Legge, 2011). In one report, using reactors with sand filters, the establishment of denitrifying bacteria populations required 75 days, while 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 CW mesocombs that microbial communities in a start-up process reached a steady-state after a period of 75-100 days (Weber and Legge, 2011).

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). In order to conduct reliable comparative experiments in CWs, it is essential that the microbial community composition and diversity is similar in all control and experimental CW replicates prior to amendment. The objective of this study was to determine suitable equilibration kinetics (i.e. the period necessary for the stabilization of microbial communities in CWs) of pilot-scale CWs and to ascertain whether microbial community fingerprinting is a reliable tool for assessing this equilibration process. 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 (Calheiros et al., 2009; Caravaca et al., 2005; Weber and Legge, 2011), unplanted CWs were used.

To monitor changes in the microbial community structures and to establish the kinetics of community changes, we used Denaturing Gradient Gel Electrophoresis (DGGE) (Baptista et al., 2008; Nicomrat et al., 2006; Ruiz-Rueda et al., 2009). In long-term amendment experiments in estuarine waters and time-lag experiments in fumigated soils, the fingerprints of microbial communities detected using DNA-based and RNA-based DGGE methods have been shown to exhibit similar patterns (Hoshino and Matsumoto, 2007; Niepceron et al., 2010). Thus, as the establishment of microbial communities in CWs has been proven to be a long process (Truu et al., 2009; Weber and Legge, 2011), a PCR-DGGE approach targeting the 16S rRNA genes in total extracted DNA rather than in total RNA was chosen to assess microbial community structure evolution and
stability in this study. 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 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. Total extractable DNA titers were used as proxy for microbial biomass, and hydraulic conductivity was used to monitor changes in CW flow rates. It has been demonstrated that a decrease in hydraulic conductivity is a valuable indicator of the production and accumulation of microbial biomass during the establishment of the microbial communities in soils and CWs (Knowles et al., 2010; Wu et al., 1997).

2.3 MATERIALS AND METHODS

2.3.1 Constructed Wetland set-up

Four polyethylene tanks were established as pilot-scale constructed wetlands, each containing an equal quantity of river sand (approx. 0.5 m³) obtained from Malmesbury (South Africa). The sand was thoroughly homogenized by hand-mixing in ratio of 1:4 with an inoculum of natural wetland sediment. The final CW sediment composition consisted of 1% clay, 7% silt, 4% fine sand, 12% medium sand and 76% coarse sand. The dimensions and operational set-up of the wetlands are shown in Figure 4. The wetlands were operated in a mixed vertical and horizontal sub-surface flow (VSSF and HSSF) mode, where VSSF was established as the principal mode of operation (Welz et al., 2011). During the drainage of VSSF CWs, atmospheric gases permeate the sediment matrix, which
ensures greater oxygenation of these systems when compared to HSSF CWs (where continual inundation leads to the presence of anoxic conditions in the substratum) (Faulwetter et al., 2009).

Three of the wetlands (designated A, B and C) received identical treatments during the establishment phase. They were fed bi-weekly with basal 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 4. 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), used as a negative control, received tap
water without nutrient addition but applied in equal amounts and rate to CWs A, B and C.

<table>
<thead>
<tr>
<th>Days (no. of feeds)</th>
<th>Yeast extract (g)</th>
<th>D-glucose (g)</th>
<th>Volume (L)</th>
<th>Rate (L/min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Day 1</td>
<td>10</td>
<td>2</td>
<td>30</td>
<td>0.27</td>
</tr>
<tr>
<td>Day 8 and 13</td>
<td>5</td>
<td>1</td>
<td>50</td>
<td>0.27</td>
</tr>
<tr>
<td>Day 16 (1)</td>
<td>1</td>
<td>1</td>
<td>45</td>
<td>0.27</td>
</tr>
<tr>
<td>Day 21 (1)</td>
<td>1</td>
<td>1</td>
<td>40</td>
<td>0.27</td>
</tr>
<tr>
<td>Day 23 to Day 12 (12)</td>
<td>0.5</td>
<td>0.5</td>
<td>20</td>
<td>0.27</td>
</tr>
<tr>
<td>Day 67 to Day 96 (9)</td>
<td>0.3</td>
<td>0.3</td>
<td>12.5</td>
<td>0.27</td>
</tr>
</tbody>
</table>

2.3.2 Sediment Sampling

A 30 mm diameter Perspex sediment corer was used to recover samples without gross disturbance of the sediment stratification. Duplicate core samples were taken from near the center of each wetland at 47, 61, 75, 89 and 96 days after the initiation of the experiment. Sampling sites were predetermined using a template so that samples were taken from areas which had not been previously disturbed. From each core, a surface (0-30 mm) and deep (150-200 mm) sub-sample fraction was retained for further analysis. After homogenization, sub-samples (1 g wet weight sediment) were frozen at −80°C for subsequent molecular analysis.

2.3.3 Hydraulic conductivity

The outflow of effluent induced by the twice weekly feeding/watering was recorded weekly for wetland A, B and C from Day 47 and for wetland D from Day 61. The hydraulic conductivity (HC) was determined by measuring the volume of effluent collected between 1-2 hours after the start of amendment. Initially, the volume of effluent was measured every 15 min from initial discharge but after
three consecutive readings it was evident that the outflow stabilized after 30 min (data not shown). This rate is expressed as L.m⁻³.solids.hour⁻¹.

2.3.4 DNA extraction and quantification

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. The concentration of duplicate 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.

2.4 PCR AMPLIFICATIONS

All polymerase chain reactions (PCRs) were carried out in a Perkin Elmer Thermocycler (Gene Amp PCR system 6700). Bacterial 16S rRNA encoding genes were amplified using the universal primers E9F (5’-GAGTTTGATCCTGGCTCAG-3’) and U1510R (5’-GGTTACCTTGTTACGACTT-3’). PCR was carried out in 50 μl reaction volumes. Each reaction contained 1 X PCR buffer, 0.2 U DreamTaq™ polymerase (Fermentas, USA), 200 μM of each dNTP, 0.5 μM of each primer, 0.1% BSA and between 5 to 10 ng of total extracted DNA. PCR amplification was carried out as follows: 4 min at 94°C for denaturation; 30 cycles of 30 s at 94°C, 30 s annealing at 52°C and 105 s at 72°C; and a final elongation step of 10 min at 72°C.

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. A 40 mer GC clamp was added to the 5’ ends of the forward primers 341f-GC (GC clamp – CGCCCGCCGCGCGGGCGGGCGGGCGGGGGGACGGGGG). PCR amplification
with 341f-GC/534r was performed by using a 50-µl total volume mixture containing 0.2 U DreamTaq™ polymerase (Fermentas, USA), 1X PCR Buffer, 200 µM of each dNTP, 0.5 µM of each primer and 0.1% BSA.

2.4.1 Denaturing gradient gel electrophoresis (DGGE) analysis

Equal amount of PCR amplicons obtained with the nested primer sets (341f-GC/534r) were analyzed by denaturing gradient gel electrophoresis (DGGE). Amplicons were separated on 165 x 165 mm x 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 1X TAE buffer. Gels were stained in 0.5 µg.mL⁻¹ ethidium bromide in 1X TAE for 20 min and visualized on an Alphalmager 3400 imaging system.

DGGE gel pictures were processed with GelCompar II 5.0 software (Applied Maths, Belgium). 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 (data not shown). Clustering analysis and non-metric Multi-Dimensional Scaling (MDS) were performed with GelCompar II 5.0. 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. MDS analyzes the matrix of similarities obtained using the Cosine coefficient. Thus, in Figure 5 and Figure 6, the linkages in the 3D-MDS graphical representation are shown as an aid to interpretation as the samples are connected by the similarity dendrogram branches (data not shown). This representation allows co-
evaluation and analysis using a dendrogram and a coordinate system such as MDS.

2.5 RESULTS AND DISCUSSION

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 high redox potentials that facilitate aerobic microbial processes (Faulwetter et al., 2009). Previous mesocosm studies have shown 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) (Truu et al., 2009; Weber and Legge, 2011). Moreover, to our knowledge, the equilibration of CW microbial community structures has never been addressed in such systems.

Our intention was to determine the period necessary for the equilibration of the microbial community structure and as such, the early phase microbial community succession was not seen as an integral part of the study. In addition, a decreasing trend in the HC of the nutrient-supplemented CWs stabilized after 47 days (data not shown). For these reasons, samples were taken for microbial community monitoring from 47 days after the initiation of nutrient-supplementation.

In parallel CW experiments, it is essential to establish that the composition of the microbial communities in CW replicates is similar before undertaking experiments involving perturbation of the systems (such as chemical challenge, etc.). In order to stimulate microbial growth and establish consistent microbial communities in each system, the same regime of nutrient supplements was added to three of the four CWs (CW A, B and C; Table 4 ) (Wu et al., 1997). The equilibration time, i.e. the time necessary for the CWs to reach similar, stable,
biological and physical profiles was determined by monitoring the microbial community structure using DGGE (Figure 5 and Figure 6) and microbial biomass (Figure 7B), estimated by total extracted DNA concentrations and HC (Figure 7B). The nutrient addition (Table 4), was designed to provide 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. Low nutrient concentrations were applied in order to maintain an oligotrophic state, as oligotrophic systems are more reactive to changes in nutrient status such as wastewater contamination (Verhoeven et al., 2006).

The HC measurements were compared on a temporal basis to the abundance of microbial biomass (Figure 7), as a decrease HC is a valuable indicator of the production and accumulation of microbial biomass during the establishment of microbial communities (Wu et al., 1997; Knowles et al., 2010).

2.5.1 Microbial community structure and dynamics

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 (data not shown). DGGE patterns were subjected to cluster analysis and multidimensional scaling (MDS) analysis (presented as three-dimensional distribution plots; Figure 5 and Figure 6), to establish the statistical relatedness between samples.

The MDS plot derived from the DGGE analysis of CW surface sediment samples (Figure 5) shows that points representing the molecular fingerprints from all samples taken at day 47 are tightly grouped and well separated from samples taken thereafter. This indicates that the microbial communities were highly similar at day 47, independent of the feeding regime (nutrients or water/unfed).

Through successive sampling points, all communities showed substantial structural divergence. The most coherent group across all subsequent sample times (days 61 to 96) was that derived from CW D (unfed control, Figure 5). This
shows that the oligotrophic nutrient amendment procedure used in this study elicited changes in the CW surface microbial communities between day 47 and day 61. The day 61 and 75 samples from the nutrient-supplemented CWs A, B and C were scattered on the 3D-MDS plot, suggesting that the surface communities evolved differentially during this time period, even though the same nutrient addition regime was adhered to (Table 4). It can be concluded that after 75 days, the surface microbial communities were not equilibrated.

In contrast, the day 89 and 96 samples are close on the MDS plot, indicating that the respective surface microbial communities were highly similar at this stage. Therefore, it can be interpreted that the microbial communities in the surface sediments of the three nutrient-supplemented CWs evolved over the 49 day sampling period. In contrast, the consistency of the DGGE patterns from the control (non-supplemented) CW is strongly suggestive that the microbial community in this CW was equilibrated by day 61 and remained essentially stable for the duration of the experiment. Together, these analyses indicate that the surface sediment microbial communities were responsive to nutrient supplementation. The ‘new’ community structure was stably equilibrated 89 days after the initiation of the experiment, and this equilibration took place between day 75 and 89.
Figure 5  Three-dimensional multidimensional scaling (MDS) plot from DGGE patterns showing the microbial community evolution in the surface sediments of the constructed wetlands. Ellipses around the samples indicate similarities in bacterial community fingerprints determined by cluster analysis. Letters designate the respective CWs and numbers indicate the sampling day.

Similar analyses of the deep sediment microbial community structure showed that, as for the surface communities, the day 47 samples were spatially distinct from the other samples. However, in contrast to the surface communities, points representing the microbial communities in the deep sediments were scattered on the MDS graphical representation (Figure 6). This suggests that the deep CW sediment microbial communities of the 4 CWs were different at this time. All other samples show substantial divergence from the day 47 community patterns.

A second dispersed cluster contains the day 61 and 75 samples. The dispersed structure of this cluster suggests that all communities (in both supplemented and
control CWs) underwent independent structural evolution. However, a third cluster (black ellipse, Figure 6), which represents the day 89 and 96 samples from all CW experiments, is highly coherent. Two conclusions are possible from this result: firstly, all CW communities were stable after 89 days and, secondly, the equilibrated state of these deep sediment communities was similar in control and supplemented CWs.

Figure 6  Three-dimensional multidimensional scaling (MDS) plot from DGGE patterns showing the microbial community evolution in the deep sediments of the constructed wetlands. Ellipses around the samples indicate similarities in bacterial community fingerprints determined by cluster analysis. Letters designate the respective CWs and numbers indicate the sampling day

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. It is also likely that the concentrations of supplemented nutrient components experienced by the deep sediment communities would be substantially lower than those experienced by surface microbial communities, being a function of nutrient depletion by surface communities.

Based on these results, we concluded that monitoring the evolution of the microbial communities in CW sediments using molecular fingerprinting methods such as DGGE is a valuable way to determine the time necessary for equilibration after start-up. In this study, DGGE results determined that the microbial communities in the surface and deep sediments of pilot-scale CWs required 89 days to equilibrate.

### 2.5.2 Nutrient supplementation, hydraulic conductivity and microbial biomass dynamics

As shown on Table 4, the concentration of solutes and volumes provided to the supplemented CWs were modified at intervals during the 96 day equilibration phase. Solute concentrations were maintained after day 21, but liquid volumes were adjusted to avoid protracted periods of surface flooding and the generation of permanent 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 7A).

The hydraulic conductivity stabilized after day 61, and the nutrient supplementation procedure was maintained until the mesocosms presented a stabilized and similar 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 7A). Hydraulic conductivity values ranged from 2.91 to 2.28 L.m\(^{-3}\).hr\(^{-1}\) in CW D, compared with values of 0.34 L.m\(^{-3}\).hr\(^{-1}\)
decreases in the HC of CW systems can be caused by the trapping of solids by the soil matrix, the growth of vegetation and/or the formation of biofilm/biomass (Knowles et al., 2010). The CW used in this study was unplanted, and due to the dilute nature of the influent, it is highly unlikely that the influent caused clogging of interstitial spaces in the soil matrix; the observed decrease in hydraulic conductivity was therefore attributed to an increase in in-situ biomass (Wu et al., 1997; Knowles et al., 2010; Weber and Legge, 2011).

Microbial biomass was investigated using total DNA concentration (Figure 7B), as this parameter is generally considered to be a reliable indicator of biomass abundance in soils and sediments (Marstorp et al., 2000; Agnelli et al., 2004; Faulwetter et al., 2009; Ramond et al., 2009). To allow comparisons between samples, the DNA extraction procedure was standardized using a commercial kit. 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 1B), suggesting that biomass loads were higher and/or more biomass was produced in the unsupplemented control CW. This result is counter-intuitive and is inconsistent with the HC 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 contradicted the general concepts that HC is inversely related to biomass abundance (Prochaska et al., 2007; Weber and Legge, 2011), 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. This has also been 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 HC in nutrient supplemented CWs (Weber and Legge, 2011), 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) and 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 HC and DNA concentrations in the nutrient supplemented CWs. We therefore suggest that the formation of biofilms by developing microbial communities in the nutrient-supplemented CWs may have impacted negatively impact on the DNA extraction efficiency.

Based on these results, we conclude that the HC of CW systems may be a useful indicator of early CW equilibration in a start-up process. Using this parameter, the establishment phase for the CW mesocosms was approximately 61 days. Within these systems, we also conclude that using total extractable DNA concentrations to monitor biomass is not appropriate. It is possible that the use of a molecular method such as quantitative PCR (qPCR), which specifically targets the bacterial 16S rRNA (Plassart et al., 2008; Niepceron et al., 2010), would provide a better indication of the amount of microbial biomass in CW sediments than the spectrophotometric method used in this study.

2.6 CONCLUSIONS

Microbial community fingerprinting is an effective method for monitoring changes in microbial community structure in CW systems (Nicomrat et al., 2006; Baptista et al., 2008; Ruiz-Rueda et al., 2009). This study lays the groundwork for the use of CW systems to investigate the relationships between microbial structure and system responses to environmental challenges. In particular, DGGE can be used to monitor both the compositional changes and the rate of change in a microbial community after chemical challenge, such as the addition of industrial waste streams, pollutant compounds, xenobiotics, etc (e.g. Niepceron et al., 2010). Other system changes, such as liquid flow rates, input C/N ratios, temperature regimes, etc. could also be addressed.

This study demonstrates that after 89 days of nutrient supplementation the pilot-scale CWs were equilibrated, with an established microbial community
presenting a highly similar fingerprint in the surface and the deep sediments. This study emphasizes that microbial community fingerprinting with molecular tools (such as DGGE) is a key parameter for monitoring CW establishment before pollutant amendment experiments. The observation that microbial community structure is the slowest parameter to stabilize supports this view. We also note that HC may be useful as a determinant of microbial community development and that quantifying extractable DNA is not a reliable method for monitoring the abundance of microbial biomass.

2.7 REFERENCES


3 ETHANOL DEGRADATION AND THE BENEFITS OF INCREMENTAL PRIMING IN PILOT-SCALE CONSTRUCTED WETLANDS

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Reference (APPENDIX 2):

3.1 ABSTRACT

There has been significant global growth in the use of constructed wetlands for wastewater treatment. The fundamental microbial processes involved in the biodegradation of organic wastewater pollutants determine the range of design and operational parameters relevant to individual constructed wetlands. In this study, the biodegradation and mineralization of ethanol by acclimated and non-acclimated microbial populations in pilot-scale constructed wetlands were compared. By increasing the pollutant concentration at incremental intervals (incremental priming), the biodegradative capacity of a sand-filled constructed wetland was significantly enhanced. At an influent COD concentration of 15 800 mg/L, no volatile fatty acids were detected in the effluent of an incrementally primed system and the maximum effluent COD concentration was 180 mg/L. In contrast, an identical, unprimed system, amended with a lower concentration of COD (7 587 mg/L), exhibited a maximum effluent COD concentration of 1 400 mg/L, with the anaerobic metabolites, butyrate and propionate accounting for up to 83% of the effluent COD. It was demonstrated that the use of incremental priming, together with a vertical subsurface flow mode of operation enhanced long-term function of the constructed wetlands. Future research should focus on determining the concentration gradients and incremental intervals necessary for optimal microbial acclimation to a range of organic pollutants and/or wastewaters, in order to minimize start-up times without significantly impairing the benefits derived from incremental priming.

3.2 Introduction

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 to 5000 mg/L, 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 essential.

Increased concentrations of organic chemicals are observed in winery wastewaters around the seasonal vinification period. 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, but peaks as high as 25 000 mg/L have been reported (Malandra et al., 2003). 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 Kröpfelová, 2009). 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) provide economical, low maintenance, low
energy wastewater treatment systems that demonstrate limited sensitivity to seasonal input fluxes (Masi et al., 2002).

In this study, two unplanted, experimental pilot-scale CWs were amended with ethanol, which was chosen as a singular pollutant because of its prominence in winery wastewater. Simple sugars, organic acids and alcohols found in winery wastewater are readily biodegraded (Malandra et al., 2003; Serrano et al., 2010). Mineralization of these organic molecules in CWs is mediated predominantly by microorganisms (Caselles-Osorio and Garcia, 2006; Imfeld, 2009; Tietz et al., 2008; Vymazal and Kröpfelevá, 2009). Oxygen is the preferred electron acceptor during the biodegradation of ethanol, rendering aerobic metabolic pathways energetically more favourable, and thus faster than anaerobic or anoxic pathways (Alvarez and Hunt, 1999). Aerobic biodegradation commences with the oxidation of ethanol to acetaldehyde, which is mediated by the enzyme alcohol dehydrogenase (Hektor et al., 2000; Österreicher-Cunha et al., 2009). Further oxidation results in the formation of acetyl-CoA, either directly from acetaldehyde or via acetate, both of which are mineralized intracellularly via the Krebs cycle or the glyoxylate shunt to CO\(_2\) (Alvarez and Hunt, 1999). Acetic acid bacteria sometimes lack the enzymes necessary to metabolize acetate and thus excrete the molecule; this may result in toxic levels of acetate accumulating in the environment (Gottschalk, 1986). Due to the inherent dependency on oxygen, these reactions 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 (Schink et al., 1985). The most common primary metabolites comprise acetaldehyde and the volatile fatty acids (VFAs), acetic acid, propionic acid and butyric acid, while carbon dioxide, methane and hydrogen gas constitute the major end-products (Alvarez and Hunt, 1999). Acetone and n-propanol may also be formed (Alvarez and Hunt, 1999). The anaerobic pathways rely on interspecies hydrogen transfers and as such are related to the sediment
composition and redox status (particularly whether conditions are methanogenic, nitrate reducing or sulphate reducing) (Stams et al., 2006; McKelvie et al., 2007). The concentration of H$_2$ is a critical parameter in methanogenic environments because at high H$_2$ concentrations, incomplete ethanol oxidation is energetically more favourable and the environment becomes acetogenic (acetate generating) (Dolfing, 2001). Consequently, VFAs can accumulate in CWs treating high ethanol wastewaters.

During this study, the biodegradation and mineralization of ethanol within the CWs was assessed by effluent analyses, with the starting substrate (ethanol) as well as important metabolic intermediates (acetic acid, propionic acid and butyric acid) being identified and quantified.

The primary aim of the study was to evaluate the benefits of diluting wastewaters during the start-up phase of CW’s 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 potentially toxic substances would ultimately lead to tolerance at higher concentrations that may have proven lethal if employed from inception. Furthermore, it was postulated that a gradual selection of efficient microbial degraders acclimated to the presence of ethanol, acetate, propionate and butyrate should theoretically protect against the accumulation of these organics in toxic concentrations. The overall success of incremental priming was assessed using effluent COD (COD$_e$) values and the accumulation of metabolites as the primary indicators of CW function and stability.
3.3 MATERIALS AND METHODS

3.3.1 Set-up and mode of operation of constructed wetlands

Three identical, unplanted, experimental CWs consisting of polyethylene containers filled with river sand to a volume ~0.5 m$^3$, void space of 0.08 m$^3$ and a depth of 0.3 m were inoculated in a ratio of 1:4 with sediment from a local wetland treating winery wastewater. The CWs were kept undercover to avoid exposure to precipitation events. Two CWs were used for comparative experiments and the third CW was used as a control system. The CWs were operated in a hybrid mode of vertical and horizontal subsurface flow (VSSF and HSSF), shown schematically in Figure 8. All CWs were subject to twice weekly inundation followed by gradient-directed drainage to ensure that the mode of operation was biased toward classical VSSF.

![Figure 8](image-url)  
**Figure 8** Schematic diagram of the constructed wetlands, showing the hybrid mode of operation with vertical (VSSF) and horizontal (HSSF) flow paths from the elevated inlet to the outlet at the bottom
3.3.2 Feeding/watering regime of the constructed wetlands

CW A was designated as a control and CW B and CW C as experimental systems. All CWs were equilibrated for a minimum of three months prior to experimentation, previously determined to as the period necessary for microbial community stabilization (Ramond et al., 2012). Due to the scale of the CWs and the need to generate data for a range of organic pollutants, the use of replicate CWs was excluded. However, replicate soil column and microcosm experiments using the CW sediments have shown excellent reproducibility (Welz et al., 2012) and the results presented in this paper are clear. All three CWs received a bi-weekly influent feedstock consisting 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 for the duration of the equilibration and 47 week experimental periods. The feedstock of the test CWs was supplemented with absolute ethanol (Merck uniVAR) as outlined in Table 5. CW B was initially amended with an ethanol load similar to that measured in cellar effluent from a South African winery (4.94 mM). Incremental priming, by amendment with ethanol at increasing theoretical COD (COD$_t$) concentrations, ranging from 474 mg/L to 26 333 mg/L (equivalent to 4.94 mM and 2.74 x $10^2$ mM ethanol), was applied to CW B. CW C was amended with moderate concentration of ethanol (COD$_t$ 7 587 mg/L; 54.4 mM) from inception to completion of the experiment, the concentration being an approximate average, based on literature for winery wastewater before primary treatment.
Table 5  Ethanol amendment in terms of theoretical influent COD and the applied loading rate to the experimental constructed wetlands

<table>
<thead>
<tr>
<th>Time (weeks)</th>
<th>CW B Incrementally primed</th>
<th>CW C unprimed</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>COD&lt;sub&gt;n&lt;/sub&gt; (mg/L)</td>
<td>OLR (gCOD/m&lt;sup&gt;3&lt;/sup&gt; day&lt;sup&gt;-1&lt;/sup&gt;)</td>
</tr>
<tr>
<td>1-7</td>
<td>474</td>
<td>3.4</td>
</tr>
<tr>
<td>8-14</td>
<td>948</td>
<td>6.7</td>
</tr>
<tr>
<td>15-20</td>
<td>1 896</td>
<td>13.5</td>
</tr>
<tr>
<td>21-26</td>
<td>3 792</td>
<td>27.0</td>
</tr>
<tr>
<td>27-32</td>
<td>7 587</td>
<td>54.0</td>
</tr>
<tr>
<td>33-35</td>
<td>7 587</td>
<td>54.0</td>
</tr>
<tr>
<td>36-37</td>
<td>15 800</td>
<td>112.4</td>
</tr>
<tr>
<td>38-40</td>
<td>15 800</td>
<td>112.4</td>
</tr>
<tr>
<td>41-42</td>
<td>26 333</td>
<td>187.3</td>
</tr>
<tr>
<td>43-44</td>
<td>Rest</td>
<td>Rest</td>
</tr>
<tr>
<td>45-47</td>
<td>7 587</td>
<td>54.0</td>
</tr>
</tbody>
</table>

COD<sub>n</sub> = theoretical influent COD  OLR = organic loading rate

3.3.3 Effluent analyses

Test samples comprised the total volume of effluent collected between 1 and 2 hours after the initiation of feeding. Acids and alcohols were analyzed by high performance liquid chromatography (HPLC) according the method of La’Zaro et al. (1989). A Merck La-Chrom instrument with a La-Chrom® D-7400 ultraviolet detector set at 210 nm and an Agilent™ refractive index detector were employed for the detection of acids and alcohols respectively. Sample components were separated using a Phenomenex Rezex RHM-monosaccharide
H+ (8% cross-linkage) column and a 1 mM H$_2$SO$_4$ solution at pH 2.52 (mobile phase). Sample acquisition time and flow rate were set at 60 min and 0.550 ml/min respectively. Peaks corresponding to ethanol and VFAs were identified by spiking effluent samples with ethanol (Merck univAR, RSA, cat no: SAAR2233540LP), glacial acetic acid (Merck univAR, RSA, cat no: SAAR1021020LC), propionic acid (Fluka puriss, Germany, cat no: WB12794) and butyric acid (Aldrich, Germany, cat no: BIO 350-0). Once identified, the concentrations were determined by comparing the peak areas to those of graphs prepared from standard concentrations of the same chemicals.

COD was quantified using a Hanna C214 multiparameter bench photometer, a Hanna C9800 digester and both low range (1-150 mg/L: HI93754A-25) and medium range (0-1 500 mg/L: HI93754B-25) COD kits, following the manufacturer’s instructions. Theoretical COD (COD$_t$) values were determined using the equation:

$$\text{COD}_t = \frac{8(4x+y-2z)}{(12x+y+16z)} \text{mgCOD}\cdot\text{mg}^{-1} \text{C}_x\text{H}_y\text{O}_z \quad \text{(Equation 1)}$$

From Equation 1, the COD$_t$ values were calculated to be 2.09 mgCOD/mg ethanol, 1.07 mgCOD/mg acetic acid, 1.51 mgCOD/mg propionic acid and 1.81 mgCOD/mg butyric acid. The relationship between COD$_t$ and the actual (COD$_a$) was established by COD measurement of 1000 mg/L COD$_t$ solutions of ethanol (Merck univAR, RSA, cat no: SAAR2233540LP), glacial acetic acid (Merck univAR, RSA, cat no: SAAR1021020LC), propionic acid (Fluka puriss, Germany, cat no: WB12794) and butyric acid (Aldrich, Germany, cat no: BIO350-0) (Table 6). The concentrations (mg/L) of ethanol and VFAs in the effluent were converted to COD$_t$ and ultimately to COD$_a$. COD$_a$ values were used for mass balance relationships between COD$_e$ and the relative contributions of ethanol and volatile fatty acids (VFAs) to COD$_e$. 
Table 6  Relationship between COD<sub>a</sub> (n=3) and standard COD<sub>t</sub> solutions

<table>
<thead>
<tr>
<th></th>
<th>Ethanol (mg/L)</th>
<th>Acetic acid (mg/L)</th>
<th>Propionic acid (mg/L)</th>
<th>Butyric acid (mg/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>COD&lt;sub&gt;t&lt;/sub&gt;</td>
<td>1000</td>
<td>1000</td>
<td>1000</td>
<td>1000</td>
</tr>
<tr>
<td>COD&lt;sub&gt;a&lt;/sub&gt;</td>
<td>966 ± 8</td>
<td>1002 ± 2</td>
<td>1126 ± 13</td>
<td>955 ± 8</td>
</tr>
</tbody>
</table>

3.4 RESULTS

3.4.1 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.

COD<sub>e</sub> values from the control (CW A) and incrementally primed CW B were low (< 100 mg/L) for the first 40 weeks, except on two occasions, when the COD<sub>e</sub> from CW B was 180 mg/L (at 14 weeks) and 104 mg/L (at 33 weeks) (Figure 9). The rise in COD<sub>e</sub> from incrementally primed CW B to a maximum 365 mg/L between 41 and 45 weeks corresponded with an influent ethanol concentration increase from 15 800 mg/L COD<sub>t</sub> to 26 333 mg/L COD<sub>t</sub>. Small amounts of acetic acid, ranging between 13 mg/L (14 mg/L COD<sub>a</sub>) and 96 mg/L (103 mg/L COD<sub>a</sub>), 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 (13 mg/L COD<sub>a</sub>) and 69 mg/L (118 mg/L COD<sub>a</sub>) and ethanol, ranging between 13 mg/L (27 mg/L COD<sub>a</sub>) and 106 mg/L (214 mg/L COD<sub>a</sub>) were detected at a maximum influent ethanol concentration (26 333 mg/L COD<sub>t</sub>), as well as one week after a rest period (Figure 10). During this period (41-45 weeks), propionic acid and butyric acid accumulated in the sediments, and the relative contribution of these VFAs to the
total COD$_e$ increased from 0% at 40 weeks (15 800 mg/L COD$_{in}$) to 48% at 45 weeks (26 333 mg/L COD$_{in}$) (Figure 11). 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 C (7 587 mg/L COD$_{in}$) and at 47 weeks previous function was restored (COD$_e$ of 48 mg/L).

A moderate concentration of ethanol (7 587 mg/L COD$_{in}$) was included in the feed of unprimed CW C from 33 weeks. This addition induced an immediate sharp increase in COD$_e$ attributable mainly to acetic acid and ethanol (Figure 9; Figure 10). 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. However, the reduction in acetic acid was accompanied by an increase in the amount of propionic and butyric acid (Figure 10). Between 40 and 47 weeks, there was a second increase in COD$_e$ (Figure 9). The proportion of propionic and butyric acid to the total COD$_e$ showed a temporal increase for the first 9 weeks, from 2% to a maximum of 83% at week 43 (Figure 11). The CWs became malodorous due to accumulation of these VFAs in the sediments and the experiment was terminated after 47 weeks.

3.5 DISCUSSION

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 (Zaida et al., 1996; Hu et al., 1997; Koda et al., 2002; Cheng et al., 2010). Acclimation and degradation are influenced by the toxicity of a compound, as well as the period 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. To our knowledge, comprehensive studies detailing the concentration-dependent acclimation of microbial communities within CW systems have not previously been published. 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 and from this study is presented in Table 7.

Data on influent composition highlight considerable variation in COD, that is attributable to both the characteristics of the winery wastewater and the pre-treatment steps involved (Table 7). 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) (Grismer et al., 2003a; Masi et al., 2002; Serrano et al., 2010; Shepherd et al., 2001a).

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/\text{COD}_e) as the sole criterion to assess the performance of biological systems treating highly variable wastewaters is debatable. For example, taking COD, values realistic for winery wastewater of 20 000 mg/L, 5 000 mg/L and 2 000 mg/L, removal efficiencies of 95%, 75% or 50% respectively all result in the same COD_e concentration of 1 000 mg/L, 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 (Masi et al., 2002; Melamane et al., 2007; Sheridan, 2007;
Christen et al., 2010; Mulidzi, 2010). 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 or <5 000 mg/L for volumes of <0.5 or <0.05 ML/day, 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.

High COD$_e$ values associated with peak-season overloading of CW treatment facilities have been reported in the literature (Table 7) (Grismer et al., 2003b; Serrano et al. 2010). The results obtained from this study showed that COD$_e$ from incrementally primed CW B complied with all South African legislative requirements for irrigation when the COD$_r$ fell in the range 474 mg/L to 15 800 mg/L (Figure 9). However, the effluent from unprimed CW C, amended with a COD$_r$ concentration of 7 587 mg/L, did not conform to legal limits for most of the study period, despite the fact that the COD removal efficiency was 81.9 to 95.7% (Figure 9; Table 7). Thus, only the effluent from incrementally primed CW B was rendered suitable for irrigation without further treatment.

![Figure 9](image.png)

**Figure 9**  
Comparison of total effluent COD (COD$_e$) from the control (CW A), incrementally primed (CW B) and unprimed (CW C) constructed wetlands
Unprimed CW C, amended with a comparatively moderate COD, did not realize the same removal efficiency as incrementally primed CW B and became septic with the accumulation of VFAs (VFAs and/or sulphides in wastewater provide evidence of septic conditions (Magro et al., 2005; Bachman et al., 2007).

![Figure 10 Metabolite profiles of effluent samples from incrementally primed CW B (A) and unprimed CW C (B). The “other” category represents the balance of COD once the contribution of ethanol and volatile fatty acids have been deducted.](image-url)

VFAs were detected in the sediments of unprimed CW C from inception to the completion of the experiment, with the pattern showing a decrease in the amounts 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 (Figure 11). 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 differences in metabolic profiles of the incrementally primed CW B and unprimed CW C suggest that acclimation of the microbial community in CW B resulted in superior ethanol mineralization, possibly as the result of enhanced interspecies hydrogen transfer (Lasko et al., 1997). Furthermore, CW B proved resilient to a short period of overloading.

![Figure 11](image)

**Figure 11** Ratio (%) of propionic acid and butyric acid (CODₐ) to total effluent COD (CODₑ) from (A, red) incrementally primed CW B and (B, blue) unprimed CW C

Process inhibition associated with high CODₑ has been reported in CWs used to treat agricultural and industrial wastewaters (Vymazal and Kröpfelová, 2009). However, CODₑ concentrations in winery wastewater from as low as 2 178 ± 1 715 mg/L, have also been associated with poor treatment performance, with low influent pH (possibly due to volatile fatty acids) and/or high concentrations of inhibitory substrates such as polyphenols being suggested as contributing factors (Masi et al., 2002; Montalvo et al., 2010; Serrano et al., 2010). 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 waste, with organic loading rates (OLRs) in a similar range to those applied to CWs used for the same purpose (Andreolella et al., 2009). However, the hydraulic retention times (HRTs) in CWs are in the order of days (Table 7), while those of most conventional systems are in the order of hours (Andreolella et al., 2009; Grismer et al., 2003b; Petruccioli et al., 2002; Shepherd et al., 2001; Mena et al., 2009; Mulidzi, 2010). 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 (Segers and Kengen, 1998; Küsel and Drake, 1999). Thus, CW depth has been included in loading calculations in this paper.

There is circumstantial evidence that aerobic processes are favoured by high redox potentials in CWs operated in a VSSF mode (Faulwetter et al., 2009). In addition, anaerobic degradation of organics such as ethanol in wetlands may result in the formation of the harmful greenhouse gas, methane (Sha et al., 2011). It may be deduced that the use of VSSF CWs for the treatment of high organic wastewaters is preferable, both from an efficiency and environmental perspective (Faulwetter et al., 2009; Sha et al., 2011).

COD removal in CWs can be substantially enhanced by increasing the HRT (Table 7) (Mulidzi, 2010). HRT is dictated by flow paths and the extent to which the wastewater interacts with the wetland porous medium and plants (Grismer et al., 2003b). Apart from the demonstrated benefits of incremental priming in CW B, the COD removal process was almost certainly enhanced by the long HRT and concomitant low hydraulic loading rates (HLRs), which were largely dictated by the slow hydraulic characteristics of the sand medium. Although low HLRs may
prevent clogging of CWs, the fundamental hydraulic characteristics, together with the VSSF mode of operation, precluded the application of a higher OLR without increasing the \( \text{COD}_{\text{it}} \) to toxic levels (Knowles et al., 2010). 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 HC is a drawback that must be taken into consideration during CW design.

Winery wastewater per se consists not only of readily biodegradable COD (RBCOD), but also a smaller fraction of slowly biodegradable COD (SBCOD) and unbiodegradable COD. Although ethanol, the pollutant used in the study, falls into the RBCOD category, the capacity of the CWs to effectively remove phenolics (SBCOD) from winery wastewater was demonstrated in a parallel experiment (Chapter 4).

### 3.6 CONCLUSIONS

The use of incremental priming as a start-up mechanism enhanced biodegradation of ethanol in a pilot-scale CW. The results supported the hypothesis that this technique would promote acclimation of key degradative members of the microbial consortia, leading to improved COD removal when compared to “unprimed” microbial communities. In this study, incremental priming enhanced both COD removal and wetland stability. To our knowledge, this is the first time that start-up procedures for CW’s have been explored in a structured manner. The use of this tool, together with the VSSF mode of operation to enhance the long-term biodegradative efficiency of CW systems treating wastewater with high organic loads was clearly demonstrated.

Further work is needed: firstly, to determine whether these results can be replicated with different wastewaters, and secondly, to determine whether prior function is retained after lengthy periods of redundancy.
<table>
<thead>
<tr>
<th>Reference</th>
<th>Mode of operation</th>
<th>COD$_{i}$ (mg.L$^{-1}$)</th>
<th>COD$_{e}$ (mg.L$^{-1}$)</th>
<th>COD removal efficiency (%)</th>
<th>OLR (gCOD.m$^{-3}$.day$^{-1}$)</th>
<th>tHRT (days)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Serrano et al., 2010</td>
<td>Full-scale Hybrid 2 stage (VF/HF)</td>
<td>1 558 ± 1 023</td>
<td>448 ± 541</td>
<td>29 to 70 (Stage 1)</td>
<td>ND/NG</td>
<td>NG</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>23 to 79 (Stage 2)</td>
<td>ND/NG</td>
<td>NG</td>
</tr>
<tr>
<td>Sheperd et al., 2001a</td>
<td>Pilot-scale HSSF</td>
<td>4 720</td>
<td>51 (at max. COD$_{i}$)</td>
<td>99 (at max. COD$_{i}$)</td>
<td>132.1* (at max COD$_{i}$)</td>
<td>10</td>
</tr>
<tr>
<td>Masi et al., 2002</td>
<td>Full scale A: HSSF/FW</td>
<td>4 044</td>
<td>91</td>
<td>97.8</td>
<td>ND/NG</td>
<td>NG</td>
</tr>
<tr>
<td></td>
<td>B: VSSF/SF</td>
<td>1 003</td>
<td>79</td>
<td>92.2</td>
<td>ND/NG</td>
<td>NG</td>
</tr>
<tr>
<td></td>
<td>C: SF/FW/FP</td>
<td>772</td>
<td>90</td>
<td>87.5</td>
<td>ND/NG</td>
<td>NG</td>
</tr>
<tr>
<td>Grismer et al., 2003b</td>
<td>Pilot-scale HSSF</td>
<td>7 406 ± 2 090 (crush period)</td>
<td>3 748 ± 1 826</td>
<td>49</td>
<td>ND/NG</td>
<td>NG</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1 721 ± 439 (non-crush period)</td>
<td>363 ± 676</td>
<td>79</td>
<td>ND/NG</td>
<td>NG</td>
</tr>
<tr>
<td>Mena et al., 2010</td>
<td>Pilot scale HSSF</td>
<td>ww1: 1.71</td>
<td>12.0 ± 2.4 to 53.0 ± 16.4</td>
<td>ND</td>
<td>8.8*</td>
<td>9.6</td>
</tr>
<tr>
<td></td>
<td>ww2: 84</td>
<td></td>
<td>12.3 ± 3.1 to 25.0 ± 5.5</td>
<td></td>
<td>9.4*</td>
<td>9.6</td>
</tr>
<tr>
<td>Mulidzi, 2010</td>
<td>Full-scale HSSF</td>
<td>~2 000 to 12 000 (HRT A)</td>
<td>ND</td>
<td>NG</td>
<td>60</td>
<td>~75*</td>
</tr>
<tr>
<td></td>
<td>~2 000 to 12 000 (HRT B)</td>
<td></td>
<td></td>
<td></td>
<td>80</td>
<td>~450*</td>
</tr>
<tr>
<td>This study</td>
<td>Pilot-scale Hybrid (HSSF/VSSF)</td>
<td>CW B: 15 800 (COD$^{2}$)</td>
<td>58 ± 25</td>
<td>99.5 to 99.8</td>
<td>112</td>
<td>22</td>
</tr>
<tr>
<td></td>
<td></td>
<td>CW C: 7 587 (COD$^{3}$)</td>
<td>763 ± 331</td>
<td>81.9 to 95.7</td>
<td>54</td>
<td>22</td>
</tr>
</tbody>
</table>

COD$_{i}$ = effluent COD  
COD$_{e}$ = influent COD  
FW = free water  
FP = finishing pond  
HF = horizontal flow  
HSSF = horizontal sub-surface flow  
ND = not determined retention time  
NG = not given  
OLR = organic loading rate (calculated from data published in cited articles)  
VSSF = vertical sub-surface flow  
tHRT = theoretical hydraulic retention time  
ww = wastewater
Clearly, the extended duration of the incremental procedure employed in this study is impractical in real terms. Therefore, concentration gradients and incremental intervals also need to be optimized to minimize the start-up period without compromising the derived benefits. The use of incremental priming in different geographical locations and thus different climatic conditions must also be considered.

In addition, the study demonstrated that CWs treating ethanol exhibit loading maxima after which performance becomes hampered. Similar findings have been reported in the literature for CWs treating winery wastewater. These maxima are highly individual and thus, in practical terms, it is recommended that CW-specific ranges for COD and/or important toxins are established (including safety factors). Subsequently, during periods of high influent concentration and/or low natural dilution (such as low precipitation), wastewater can be diluted to comply with established maxima. CW design incorporating features permitting return of treated wastewater to the system head provides an example of a mode of operation that functions independently from external water supplies (Serrano et al., 2010). This is an important factor in water-stressed regions such as South Africa.

3.7 REFERENCES


4 PHENOLIC REMOVAL PROCESSES IN BIOLOGICAL SAND FILTERS, SAND COLUMNS AND MICROCOSMS

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Reference (APPENDIX 3):

4.1 ABSTRACT

This study evaluated the removal processes involved in the removal of the phenolic component of winery wastewater in biological sand filters, sand columns and sand microcosms. It was found that at low influent phenolic concentrations, complete organic removal was accomplished, but at high concentrations, there was incomplete substrate removal and an accumulation of potentially toxic metabolites, including catechol. The sand provided a suitable substrate for the treatment of phenolic-laden waste, and both biotic (48%) and abiotic (52%) removal mechanisms effected the removal of model phenolics. Prior acclimation of microbial communities increased the biodegradation rate of phenolic acids significantly.

4.2 INTRODUCTION

The winemaking process generates copious amounts of cellar effluent: it has been estimated that South Africa produces one billion litres and Australia 5-9 billion litres of winery wastewater per annum (Mosse et al., 2011; Sheridan et al., 2011). Winery effluent requires treatment before discharge, but remediation is complicated by the fact that the composition and volume fluctuates on a seasonal basis, depending on cellar activities (Arienzo et al., 2009; Malandra et al., 2003; Mosse et al., 2011). Typical chemical oxygen demand (COD) values of 800 to 12 800 mg/L are found during the vinification period, resulting from the presence of high concentrations of organic molecules with variable degradation rates (Malandra et al., 2003). Simple sugars, organic acids and alcohols commonly found in winery wastewater are readily biodegradable, while the phenolic component is characteristically slowly biodegradable (Serrano et al., 2010).

Plant phenolics may be toxic to microbes. It has been demonstrated that tannins, which are abundant in red wines, can inhibit microbial activity by precipitation of key metabolic proteins (Arienzo, 2009). The levels of phenolic compounds in winery wastewater, particularly in the effluent emanating from the production of
red wine, are likely to inhibit microbial activity in soils, affecting soil and plant health (Mosse et al., 2011). It has been shown that the phenolic component of winery wastewater can adversely affect the growth of a variety of aquatic and non-aquatic plants, including cash crops (Arienzo et al., 2009).

Small to medium-sized wineries in rural areas are often not connected to municipal reticulation systems for the treatment of winery effluent and cannot afford to operate sophisticated biological treatment systems (Christen et al., 2010). In these wineries, constructed wetlands (CWs) and biological sand filters (BSFs), such as the FILTER system are ideal systems for the treatment and re-use of wastewater as they have low energy and maintenance requirements, are tolerant of seasonal input fluxes and do not require lengthy start-up and shut down periods (Christen et al., 2010).

In the environment, the fate of phenolics is influenced by both biotic and abiotic factors; it is important to understand these processes in order to apply appropriate design principles to BSFs and CWs used to treat phenolic-laden waste. Unlike biological wastewater treatment systems that incorporate sludge wasting, these systems can become saturated with recalcitrant organic chemicals with time. Hence, sufficient biodegradation and mineralization of a critical proportion of phenolics must occur to prevent accumulation and leaching of potentially harmful chemicals from BSFs and CWs treating cellar effluent.

There are a number of previous reports describing the overall removal of total phenolics from agri-industrial wastewaters including winery wastewater, olive mill wastewater and coffee processing wastewater in BSFs and CWs. This study was designed not only to quantify the overall removal of common winery phenolics from cellar effluent in BSFs, but also to gain insight into the biotic and abiotic removal processes taking place during the remediation of the phenolic component of winery effluent. BSFs were used is an alternative to CWs because of the potentially phytotoxic nature of winery effluent.
4.3 MATERIALS AND METHODS

4.3.1 Influent and effluent analyses

4.3.1.1 Chemical oxygen demand

Chemical oxygen demand (COD) was quantified using a Hanna\textsuperscript{*} instruments (Smithfield, USA) C214 multiparameter bench photometer, C9800 digester and low range (1-150 mg/L: HI93754A-25) and medium range (0-1 500 mg/L: HI93754B-25) COD kits, following the manufacturer’s instructions.

4.3.1.2 Phenolics, sugars, acids and alcohols

Total phenolics were determined using the Folin-Ciocalteau (FC) micro method for total phenolics in wine, based on the method reported by Slinkard and Singleton (1977), using Folin-Ciocalteau reagent (Merck\textsuperscript{*}, Whitehouse Station, USA, cat no: 1.09001.0500). Gallic acid monohydrate (Sigma-Aldrich\textsuperscript{*}, St. Louis, USA cat no: 27645) standards were prepared in-house and results were expressed as mg/L in gallic acid equivalents (GAE) determined from a standard graph.

Individual phenolics, sugars, acids and alcohols in the effluent were identified and quantified using reverse phase HPLC: samples were separated using a Merck\textsuperscript{*} Hitachi Lachrom instrument equipped with an L-7400 UV detector. For the detection of phenolics, a Waters\textsuperscript{*} (Milford, USA) Spherisorb\textsuperscript{*} S50DSI analytical cartridge was used with deionised water, methanol and glacial acetic acid (Merck\textsuperscript{*} uniVAR, cat no: SAAR1021020LC) (80:20:2.5) as the mobile phase. The wavelength, flow rate and time were set at 280 nm, 0.5 mL/min and 60 min, respectively. Acids and alcohols were analysed by HPLC using a Phenomenex\textsuperscript{*} Rezex RHM-monasaccharide H\textsuperscript{+} (8% cross-linkage) column according to the method described by La’Zaro et al. (1989), with a L-7400 ultraviolet detector (210 nm) and an Agilent\textsuperscript{*} 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.

The theoretical COD (\(\text{COD}_t\)) values of gallic acid, vanillin (Sigma-Aldrich\®, cat no: V1104), vanillic acid (Sigma-Aldrich\®, cat no: V-2250); catechol (Sigma-Aldrich\®, cat no: C9510); and acetic acid were calculated using Equation 2. The relationship between COD and \(\text{COD}_t\) was established by COD measurement of triplicate phenolic solutions to give a figure, termed “measured COD” (\(\text{COD}_m\)), which was used in subsequent mass balance calculations:

\[
\text{COD}_t = \frac{8(4x+y-2z)}{(12x+y+16z)} \text{mgCOD/mg C}_x\text{H}_y\text{O}_z \quad \text{(Equation 2)}
\]

Gallic acid: \(\text{COD}_t = 1.12 \text{mgCOD/mg}\) and \(\text{COD}_m = 1.02 \pm 0.01 \text{mgCOD/mg}\)
Vanillin: \(\text{COD}_t = 1.79 \text{mgCOD/mg}\) and \(\text{COD}_m = 1.81 \pm 0.005 \text{mgCOD/mg}\)
Vanillic acid: \(\text{COD}_t = 1.52 \text{mgCOD/mg}\) and \(\text{COD}_m = 1.51 \pm 0.014 \text{mgCOD/mg}\)
Catechol: \(\text{COD}_t = 1.89 \text{mgCOD/mg}\) and \(\text{COD}_m = 1.92 \pm 0.007 \text{mgCOD/mg}\)
Acetic acid: \(\text{COD}_t = 1.07 \text{mgCOD/mg}\) and \(\text{COD}_m = 1.07 \pm 0.003 \text{mgCOD/mg}\)

4.3.2 Experimental set-up, design and procedure

The relationship between the three experimental phases (BSFs, columns and microcosms) is shown schematically in Figure 12.

4.3.2.1 Biological sand filters

Four identical, unplanted, experimental BSFs, each consisting of river sand to a volume of \(~0.5 \text{ m}^3\), void space of \(0.08 \text{ m}^3\) and a depth of \(0.3 \text{ m}\) were inoculated in a ratio of 1:4 with sediment from a local wetland treating winery wastewater. The final BSF sediment consisted of 1% clay, 7% silt, 4% fine sand, 12% medium sand and 76% coarse sand. The elemental composition of the sediment per kilogram sand was as follows: 6 mg P/kg; 1.9 g C/kg; 0.07 cmol(+) Na/kg; 0.05
cmol(+) K/kg; 1.64 cmol(+) Ca/kg; 0.21 cmol(+) Mg/kg; 0.61 mg Cu/kg; 1.0 mg Zn/kg; 1.9 mg Mn/kg; 0.10 mg B/kg; 63.03 mg Fe/kg; 7.42 mg S/kg. The pH of the sediment was 7.7.

All BSFs were maintained in an outdoor, undercover environment in order to avoid exposure to precipitation events. The systems were operated in a hybrid mode of vertical and horizontal subsurface flow i.e. effluent was sprayed uniformly at a rate of 0.68 L/min over the inlet zone and allowed to gravitate longitudinally and vertically towards the outlet. Twice-weekly inundation, followed by gradient-directed drainage ensured that the mode of operation was biased towards classical vertical subsurface flow.

Two replicates (A and C) served as control BSFs and two replicates (B and D), served as test BSFs. All four BSFs received a twice weekly basal influent solution consisting of 0.3 g yeast extract (Biolab®, Midrand, RSA cat no: HG000BX6.500) and 0.3 g D (+) glucose (Merck® chemically pure cat no: SAAR2676020EM) dissolved in 12.5 L tap water, for the duration of the equilibration and experimental periods, the former continuing for a minimum of 16 weeks (Ramond et al., 2012). During the experimental period, BSF B was amended with winery wastewater diluted in a ratio of 1:5 (2.5 L in 12.5 L) for a period 17 weeks, while BSF D was amended with increasing concentrations of gallic acid and vanillin for 9 weeks (Table 8).

The hydraulic conductivity (HC) was determined by measuring the volume of effluent collected between 1-2 hrs. after the start of amendment and results were expressed as L/hr.m$^3$ sand$^{-1}$. It had previously been established that outflow was consistent during this period (data not shown).
Table 8  COD concentrations of synthetic wastewater and winery wastewater used for amendment of biological sand filters (BSF B and BSF D)

<table>
<thead>
<tr>
<th>Week</th>
<th>Synthetic wastewater</th>
<th>Winery wastewater</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Influent COD COD&lt;sub&gt;m&lt;/sub&gt; (mg/L)</td>
<td>OLR (gCOD/m&lt;sup&gt;3&lt;/sup&gt; day&lt;sup&gt;−1&lt;/sup&gt;)</td>
</tr>
<tr>
<td></td>
<td>Gallic</td>
<td>Vanillin</td>
</tr>
<tr>
<td>1-3</td>
<td>96</td>
<td>138</td>
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<tr>
<td>4-6</td>
<td>488</td>
<td>688</td>
</tr>
<tr>
<td>7-9</td>
<td>2400</td>
<td>3442</td>
</tr>
</tbody>
</table>

OLR organic loading rate

4.3.2.2 Sand columns

Without physically disrupting the sediment structure, six Perspex samplers (250 mm in length and 35 mm in diameter) were used to extract core samples (sediment mass 579 ± 11 g) from an experimental wetland that had previously been exposed to winery wastewater (BSF B) (Figure 12B). The open ends of three columns were sealed with duct tape after which they were gamma irradiated at a dose of 0.03 MGy at a commercial facility (Hepro Pty. Ltd.). All six columns were positioned vertically with the capacity to collect effluent fractions. A phenolic “cocktail” consisting of filter-sterilized solutions of gallic acid, (+)-catechin (Sigma-Aldrich®, cat no: C1251), ferulic acid (Sigma-Aldrich®, cat no: W51, 830-1); and vanillin (each at 0.25 mM) was prepared. Forty millilitres of the cocktail was delivered to each column by injection on days 1, 2 and 3, with the injected volume being reduced to 20 mL per day for a further 6 days. Effluent was collected over a 24 hour period for the first two days. Once the flow rate had stabilized (day 3), effluent was collected over a six hour period following injection. Sterile procedures were maintained throughout in order to prevent contamination of the irradiated columns.
The irradiated columns, served to indicate abiotic (abiotic) removal, while the non-irradiated columns served to indicate combined biotic and abiotic (biotic and abiotic) removal of phenolics, with biotic mechanisms accounting for the difference between the two. The removal rates were determined from total phenolic analyses of influent and effluent using the Folin-Ciocalteau assay. In order to correct for the small contribution to the assay of leached soil components, the removal efficiencies (%) of total phenolics in the non-irradiated columns (UC) and irradiated columns (IC) were calculated using Equations 3 and 4, respectively.

\[
\% \text{ removal UC} = (100 - (\frac{\text{TP}_{\text{EU}}}{\text{TP}_I} \times 100)) \times \frac{100}{100 - (\frac{\text{TP}_{\text{EU}}}{\text{TP}_I} \times 100)}
\]

(Equation 3)

\[
\% \text{ removal IC} = (100 - (\frac{\text{TP}_{\text{EI}}}{\text{TP}_I} \times 100)) \times \frac{100}{100 - (\frac{\text{TP}_{\text{EI}}}{\text{TP}_I} \times 100)}
\]

(Equation 4)

Where,

\(\text{TP}_I\) mean values of total phenolics in the influent (mg GAE/L)

\(\text{TP}_{\text{EU}}\) mean values of total phenolics (mg GAE/L) in the effluent from the non-irradiated columns

\(\text{TP}_{\text{EI}}\) mean values of total phenolics (mg GAE/L) in the effluent from the irradiated columns

### 4.3.2.3 Sand microcosms

Sand samples were taken from the experimental BSF previously amended with vanillin and gallic acid (BSF D), and from the corresponding control (BSF C) (Figure 12D). Aliquots (10 g) of sediment from each BSF were added to McCartney bottles together with 2 mL distilled water (to provide sufficient moisture for effective autoclaving). Six such samples from each BSF 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 (951 mg/L and 1176 mg/L) 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, and abiotic, removal of phenolics was calculated in the same theoretical manner as for the columns, with the autoclaved samples yielding abiotic, removal data. The difference in removal rates between the untreated and autoclaved replicates was used to calculate biotic, removal values.

4.3.3 Sterility testing of autoclaved and irradiated sand

One gram of sand from irradiated, autoclaved and untreated samples taken before and after experimentation was added to 5 mL 0.85% sterile NaCl and vortexed for 5 minutes. Five microliters of each homogenate was plated on R2A agar and incubated aerobically for 72 hours at 30°C, after which plates were examined for colony growth.
Figure 12  Schematic diagram of the experimental design showing the various experimental components: BSFs treating winery wastewater (A), and synthetic wastewater (C) and the relationship of these with column experiments (B) and microcosm experiments (D)

4.4 RESULTS AND DISCUSSION

4.4.1 Removal of total phenolics from winery wastewater in biological sand filters

The first part of this study focused on the removal of organics, including total phenolics, from winery wastewater in a BSF (BSF B). Holistically, newer technologies that result in the beneficiation of agri wastewaters are most desirable from an environmental perspective (Naqvi et al., 2010). However, these systems are sophisticated, expensive and not suited to the variable nature of winery wastewater. The key focus for small to medium sized wineries, in
particular, remains on the remediation of wastewater before discharge in a
reliable and cost-effective manner by employing systems such as CWs or BSFs.

Using a pilot-scale BSF, the COD and total phenolic concentration of the diluted
winery wastewater used for amendment was $461 \pm 126 \text{ mgGAE/L}$ and $78.8 \pm 22.6 \text{ mg GAE/L}$, respectively. COD and total phenolics were measured in effluent
samples each week (Figure 13A). The quantity of phenolics detected in the
effluent from BSF B ($2.2 \pm 0.6 \text{ mg GAE/L}$) and the corresponding unamended
control, BSF A ($1.5 \pm 0.17 \text{ mg GAE/L}$), were low and not significantly different
(student t-test, $p = 0.83$), reflecting complete removal of influent phenolics.
These low values (<3 mg/L) were assumed to result from natural leaching of
phenol polymers from the sand. COD removal rates were consistently > 90%
(Figure 13A) and the major contributors (%) to COD in the effluent from BSF B
were ethanol (0-78%) and acetic acid (13-99%) (data not shown).

Vertical flow BSFs and CWs are operated in batch mode, consisting of alternating
periods of flooding and drainage. In these systems, mass transfer of atmospheric
gases into the substratum via gravitational pull during the drainage period is the
primary mechanism responsible for oxygenation of the substratum (Petitjean et
al., 2012). Continuous, horizontal flow systems may achieve good COD removal in
the case of low COD wastewaters such as those produced by the aquaculture
industry (Shi et al., 2011). However, because the degradation of organic
molecules is enhanced under aerobic conditions, higher COD removal rates are
expected in batch-operated systems (Petitjean et al., 2012; Saeed et al., 2011).
Thus, vertical flow systems are recommended for the treatment of winery
wastewater with inherently high concentrations of organics.
Figure 13  Total phenolics and COD measured in effluent samples from (A) BSF B, amended with winery wastewater and (B) BSF D, amended with synthetic phenolic wastewater

4.4.2 Abiotic removal of model winery phenolics in sand columns

At this point of the study, the basic phenolic removal mechanisms were indeterminate. If the primary mechanism for the removal of phenolics in BSFs is
sorption onto substratum particles, eventual saturation of binding sites will necessitate the implementation of rehabilitative measures to prevent leaching of phenolics into the treated effluent. To establish the relative abiotic/biotic removal capacities of the substrate (sand) and the microbial population, respectively in BSF B, core samples were extracted and column experiments were set-up (Figure 12B): a phenolic “cocktail”, containing common winery phenolics was allowed to permeate through three irradiated and three non-irradiated columns for nine days and the total phenolic removal rates were calculated on a daily basis. The removal rates in the irradiated and non-irradiated columns were attributed to abiotic, and combined biotic-abiotic mechanisms, respectively, with biotic mechanisms accounting for the difference between the two.

Results obtained between days 3 and 5 of the experiment indicated that both abiotic, and biotic, factors were proportionately involved in the removal of phenolics (Figure 14). During this experiment, the permeate flow from the columns stabilized after 2 days and the collection period was decreased from 24 hours to 6 hours to limit degradation of the phenolics in the collection vessels. From days 5 to 9, there was a decrease in the abiotic removal fraction, and the phenolic removal rates calculated for the replicates were no longer consistent (Figure 14). No growth was observed on sterility plates from soil samples taken from irradiated columns before experimentation. However, growth was obtained on the plates from samples taken at the end of the experiment, indicating that either (i) the columns were contaminated during the experiment with an exogenous microbial population capable of actively degrading phenolics, or (ii) dormant microbes in the irradiated columns resumed growth, despite the fact that the gamma irradiation dose applied (0.03 MGy) is one recommended for sterilization of soil samples (Wolf and Skipper, 1994). The resurgence of microbial growth in the irradiated samples can explain the temporal increase in the biotic removal fraction seen after day 5 (Figure 14).
Figure 14  The relationship between biotic, and abiotic, removal efficiencies of phenolics in a soil column experiment calculated using Equations 3 and 4

In soil environments, the sorption of organics usually corresponds with the amount of soil organic matter (SOM), especially humic acids, kerogen and black carbons (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 (Tharayil et al., 2006). Abiotic chemical transformation of phenolics can also occur via the oxidation of phenolic acids coupled to iron and/or manganese reduction (Polubesova et al., 2010).

Due to the low carbon (1.9 mg/g), low clay (1%), high Fe (63.0 mg/kg) and Mn (1.9 mg/kg) content in the BSF sand, it is likely that metal complexation and/or chemical transformation played a major role in the abiotic removal of phenolics in the columns, and by inference, the BSFs.
4.4.3 Removal of phenolics from synthetic wastewater in a biological sand filter

To understand the primary metabolic processes taking place during the biodegradation of phenolics in BSFs and the effect of phenolic loading on these metabolic processes, synthetic wastewater containing common winery phenolics, was introduced to BSF D in incrementally increasing concentrations and the effluent was collected and analysed (Figure 12C; Table 8).

4.4.3.1 Determination of COD removal efficiency at increasing organic loading rates

During amendment with a low concentration of synthetic wastewater (Table 8), the concentrations of total phenolics measured in the effluent from BSF D (1.4 ± 0.5 mg GAE/L) were similar to those measured in the effluent from BSF B (winery wastewater amended) and the control BSFs (BSF A and BSF C). Thus, with both winery wastewater and synthetic wastewater, complete removal of phenolics was achieved. Amendment with moderate concentrations of synthetic wastewater (1 176 mg/L CODm) generated higher effluent phenolic concentrations of 13.8 ± 7.3 mg GAE/L, but the effluent COD remained <100 mg/L, with >90% COD removal efficiency (Figure 13B, Figure 15). During amendment with high concentrations of synthetic wastewater (5 842 mg/L CODm), COD removal efficiency remained high (>88%), but the effluent COD concentrations reached values close to 700 mg/L (Figure 15). At this stage, the major contributors (%) to COD were phenolics, with total phenolic concentrations of 480 ± 22 mg GAE/L being measured in effluent samples (Figure 15).
4.4.3.2 Microbial degradation of model phenolics in a biological sand filter

To gain insight into the microbial utilization and degradation of the model winery phenolics, vanillin and gallic acid, the effluent from BSF D was analysed using HPLC. 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. The microbial degradation of gallic acid to pyrogallol takes place either aerobically (by oxidation) or anaerobically (by decarboxylation) (Bhat et al., 1998).

No identifiable chemical entities were detected in the effluent during amendment with a low concentration of synthetic influent (weeks 1-3) (Table 8). During amendment with a moderate concentration of synthetic influent (weeks 4-6), small amounts of vanillic acid (range: 1.7 – 6.9 mg/L), gallic acid (range: 0.8 – 1.0 mg/L), and the microbial metabolite, catechol were detected, the latter displaying an increasing trend (from 3.5 to 15.0 mg/L). A well-described microbial
degradation pathway for the formation of catechol is via decarboxylation of protocatechuic acid, which is a product derived from the demethylation of vanillic acid (Fenner et al., 2005). Effluent analysis showed that during BSF D amendment with a high concentration (1 176 mg/L COD) of model phenolics: (i) vanillin, and the metabolites, acetate and lactate were detected in the effluent for the first time. The lactate peak was not sufficiently resolved to perform accurate quantification and the identity of some small peaks remained indeterminate as they did not correspond to any of the standards employed. Nonetheless, much of the organic fraction was accounted for: the COD contribution from unidentified entities ranged from 35% at week 7.5 to only 2% at week 9.5 (Figure 16B). High concentrations of the metabolites, vanillic acid (range: 104.2 mg/L to 157.6 mg/L), catechol (range: 36.4 mg/L to 141.1 mg/L) and acetate (range: 32.5 mg/L to 152.6 mg/L) were measured in the effluent. These results indicate that although microbial degradation was taking place, the biodegradative capacity of the BSF microbial community was exceeded, (ii) substantially higher concentrations of vanillin (< 39 mg/L) than gallic acid (<17.3 mg/L) were measured in the effluent, indicating preferential removal of the latter (iii) vanillin was the highest contributor to overall COD at week 8 (43%) and catechol at week 9.5 (40%) (Figure 16B). In contrast to catechol, the concentrations of vanillin remained within a narrow range, suggesting that the combined biotic and abiotic removal rate of this molecule was stable under the prevailing conditions (Figure 16A).

4.4.3.3 Accumulation of metabolites at high influent concentrations of model winery phenolics

During amendment of BSF D with a high concentration of synthetic wastewater (Figure 12C), there was a temporal increase in the contribution of the metabolites catechol and acetate, both in terms of concentration and contribution to overall COD, accounting for 40% and 22% of the effluent COD, respectively by the end of the experiment (Figure 16B).
Vertical flow CWs are operated in “fill and drain” cycles, with atmospheric gases being drawn into the substratum during drainage. In this study, the BSFs were operated in batch mode, with the predominant flow being vertical. 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 BSF layers, but not in the deeper, more anoxic/anaerobic layers (Bugg, 2003). 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 BSF D emanated from the accumulation of catechol in the deeper, anaerobic layers of the substratum.

Under favourable conditions, acetate is readily biodegradable but it can accumulate in the environment when alternative electron acceptors such as oxygen and nitrates are unavailable (Lasko et al., 1997; van Stempvoort et al., 2009; Whalen, 2005). 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 BSF D.
4.4.3.4 Toxic effects of catechol on microbial metabolism leading to loss of biofilm/biomass and hydraulic conductivity

In high concentrations, catechol can be highly toxic to microorganisms, often exhibiting greater levels of toxicity than “parent” phenolics, including benzene (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).

However, catechol may also function as a bacterial growth substrate, depending on the concentration of catechol and the organism/s involved (Alexivia et al., 2008; Chen et al., 2009; Park et al., 2001). Chen et al. (2009) found that in soil, low concentrations of catechol (0-400 µg/g) promoted microbial growth, while higher concentrations inhibited growth, with 60% inhibition being demonstrated at 3000 µg/g.

The high concentrations of catechol (36.4 mg/L - 144.1 mg/L) measured in the effluent from BSF D from weeks 7-9 provide 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 (from 1.6 to 0.20 L/hr.m³.sand⁻¹) there was an increased trend which corresponded to an increasing catechol concentration in the effluent (from 0.2 to 1.1 L/hr.m³.sand⁻¹). In CWs, hydraulic conductivity may be retarded by entrapment of solids, the growth of vegetation and/or the formation of biofilm/biomass (Knowles et al., 2010). Since the experimental BSF could be considered to be an unplanted CW, the decreased conductivity could only have originated from the adsorption/accumulation of polymerized forms of phenolics and/or an increase in biomass/biofilm. The fact that the hydraulic conductivity trend reversed when the phenolic components were increased after week 7 discounts the former option, as adsorption and polymerization of phenolics would be expected to increase, not decrease at higher influent phenolic concentrations.

It has previously been shown that there is a rapid loss of hydraulic conductivity stemming from an increase in heterotrophic biomass/biofilm after the application of organic wastewaters, including winery wastewater, to soil (Christen et al., 2010; Knowles et al., 2010; Welz et al., 2011). This phenomenon may be reversed by increasing the periods between infiltrations or by decreasing
the organic load (Christen et al., 2010; Knowles et al., 2010; Welz et al., 2011). It is thus proposed that during this study, low to moderate 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 levels, accumulation of catechol in inhibitory concentrations resulted in loss of biomass/biofilm with a consequent increase in hydraulic conductivity.

**4.4.4 Acclimation of microbial communities exposed to vanillin and gallic acid: microcosm studies**

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 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 order to determine whether the microbial communities in CW D had become acclimated to vanillin and gallic acid, microcosm studies were undertaken (Figure 12D). The microcosms contained (i) sediment taken either from BSF D (acclimated by exposure to vanillin and gallic acid) or BSF C (unexposed control) and (ii) a solution of vanillin and gallic acid. The biotic, and abiotic removal fractions were calculated in the same theoretical manner as in the column experiments: the removal rates in the autoclaved and non-autoclaved microcosms were attributed to abiotic, and combined biotic-abiotic mechanisms, respectively, with biotic mechanisms accounting for the difference between the two.

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 removal of gallic acid and vanillin was respectively 6% and 12% higher in the microcosms containing acclimated sediment than in the control microcosms. Biotic formation of vanillic acid was also higher in the acclimated microcosm sand (44.1 mg/L) than the control microcosms (9.3 mg/L), with the concentration in both the autoclaved and non-autoclaved acclimated microcosms (100.3 ± 29.4 mg/L and 144.5 ± 15.8 mg/L, respectively), being higher than in the control microcosm counterparts (1.81 ± 0.03 mg/L and 11.1 ± 1.1 mg/L, respectively). The metabolite, catechol, was only detected in the non-sterile, acclimated microcosms. In addition, the difference in phenolic concentrations between the autoclaved and non-autoclaved microcosms was statistically more significant (t-test) in the acclimated microcosms (gallic acid: \( p = 0.0229 \) and vanillin: \( p = 0.0299 \)) than in the control microcosms (gallic acid \( p = 0.0408 \) and vanillin \( p = 0.0184 \)).

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 in the non-autoclaved microcosms containing acclimated sediment, indicating the presence of bioavailable phenolics in the BSF sediment remaining from previous amendment with synthetic wastewater (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.

It was clearly demonstrated that a period of nine weeks was sufficient for the microbial population in a BSF to acclimate to the presence of gallic acid and vanillin, resulting in significantly enhanced biotic degradation rates when compared to a non-acclimated population. These results support 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, could enhance long-term phenolic removal performance in BSFs (Welz et al., 2011).

In sand microcosms, abiotic 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. Removal of 30% and 33% of vanillin from acclimated and non-acclimated sediment, respectively was abiotic, and vanillic acid originated from both biotic and abiotic oxidation of vanillin.

### 4.5 CONCLUSIONS

Both biotic and abiotic phenomena can be exploited to maximize removal efficiency in BSFs. Abiotic removal is related to the physical structure and the chemical composition of the substrate with clay, organic carbon and metals being strongly associated with phenolic binding and/or chemical transformation reactions. However, abiotic phenolic attachment sites are finite and biotic removal must also occur to ensure the longevity of BSFs. Start-up techniques that enhance microbial acclimation can be employed to optimize biotic removal. In addition, the accumulation of metabolites which are toxic to the microbial communities can be prevented by adhering to appropriate influent concentration limits.

### 4.6 REFERENCES


5 SUMMATION, RECOMMENDATIONS AND FUTURE WORK
5.1 INTRODUCTION

The remediation of wastewater in CWs is influenced by a variety of biological (biotic) and non-biological (abiotic) factors. CWs can be designed for optimal wastewater treatment performance by (i) Assessing the importance of each factor for the treatment of specific effluents (ii) Selecting the most important factors and comparing available options related to these factors, and ultimately, (iii) Incorporating this information into the design process.

Knowledge gaps associated with one or more critical design component/s of CWs treating particular waste streams should be identified during the design process. Shortcomings stemming from inadequate understanding of the fundamentals may lead to unsatisfactory system performance. It is therefore prudent to bridge these gaps before constructing full-scale systems. Wastewater is regarded as the most significant environmental risk at wineries and more than 95% of the South African wineries utilize their wastewaters to irrigate land (Van Schoor, 2004). The WRC of South Africa has identified that cellar effluent discharged from many wineries in this country does not comply with legislative requirements stipulated in the South African Water Act no. 96 of 1998. The WRC has also recognized that CWs may offer a simple and feasible solution to this problem and have therefore sponsored research pertaining to the use of CWs for the treatment of winery wastewater in this country. The researchers at CPUT and IMBM have identified specific knowledge gaps and have focused research on these key areas, some of which are outlined in this thesis (Chapters 3-4).

5.2 ABIOTIC CONSIDERATIONS FOR CW DESIGN

The type of substrate, the composition and volume of the wastewater, the climate, the location, the energy requirements (mostly for pumping), the construction and maintenance costs and the operational parameters [flow regime, hydraulic retention time, hydraulic load, organic loading rate and
frequency of wastewater application in the case of batch operated systems] are important abiotic factors to be considered.

The substrate should be chosen carefully for (i) Conductivity properties (porosity, grain size distribution, grain geometry), (ii) Chemical composition, and (iii) Local availability (Akratos and Shrintziz, 2011; Korkusuz et al., 2005; Zhang et al., 2007).

The climate and the wastewater (composition and volume) are inflexible parameters which must nonetheless be taken into account when deciding on the operational parameters of CWs. The design of CW treatment systems should take into account existing knowledge on the treatment of specific wastewater types. For example, CWs have been used to treat domestic wastewater since the 1960s and there are now thousands of systems being used successfully for the secondary and tertiary treatment of domestic effluent in individual households and smaller communities (Garcia et al., 2010; Jóźwiakowski, 2009; Scholz and Lee, 2005; Vymazal, 2005). It is therefore generally feasible to apply existing design principles for the construction of CWs treating domestic effluent. Conversely, there are only a handful of reports pertaining to CWs which have been used for the treatment of winery effluent (Table 7). It is therefore logical that more background data should be generated before designing such systems, which should ideally contain some generic components and some components that have been individualized for each winery. In addition, the design operational parameters should allow operational flexibility to compensate for seasonal fluctuations in wastewater composition and volume, temperature changes and the addition of rainfall to the hydraulic load.

5.2.1 Consideration of climate and selected operational parameters in the design of CWs treating cellar effluent in the Western Cape region of South Africa

5.2.1.1 Temperature and rainfall

Approximately 80% of South African wine is produced in the Western Cape region which experiences a Mediterranean climate with winter rainfall and hot,
dry summers (SAWIS, 2010). The highest load of cellar effluent in terms of both volume and COD occurs in the summer crush season during February to April of each year (Sheridan et al., 2011). During this period, precipitation is minimal and ambient temperatures are relatively high (Sheridan et al., 2011). In addition to these seasonal events, it has been shown that CWs treating common components of winery wastewater exhibit organic loading maxima after which there is a build-up of metabolites that may be toxic to the microbial population (Chapters 3 and 4). Consequently: (i) The possibility of hydraulic overload due to precipitation events is minimal, obviating the need for additional capacity to accommodate rainfall, (ii) Dilution from rainwater cannot be relied upon to mitigate the deleterious effects of organic overload from high-strength wastewater and it is therefore recommended that alternative wastewater dilution systems are investigated, and (iii) As wastewater treatment performance is only compromised in CWs at sustained ambient temperatures less than 15°C, temperature can be discounted during the design process (Chang et al., 2012; Stefanakis and Tshrintzis, 2012).

Ideally, potential wastewater dilution systems should not rely on water drawn from local resources, which are stressed. A feedback loop whereby treated effluent exiting the CW is returned to the CW inlet during COD overload provides a feasible solution. To lessen the environmental burden, the use of alternative energy sources for pumping, including wind and solar power could be explored. To optimize the efficiency of a dilution-feedback loop, periodic monitoring of effluent COD or another indicator parameter would be required to determine (i) The COD treatment limits of individual CWs, as each system will be unique in terms of influent composition and (ii) The actual COD of the influent so that appropriate dilution ratios can be calculated and applied.

5.2.1.2 Operational parameters and substrate

Winery wastewater is typically high in organic content, but low in nutrients (N and P). For the studies outlined in this thesis, a vertical subsurface flow mode of
operation was considered as appropriate without the need for additional research (Table 3).

The volumetric capacity of the CW should be sufficient to increase the retention time during high organic and/or high volumetric loading periods. The hydraulic retention time should be of sufficient duration to allow complete degradation of the organic load applied (Table 7). In substrates with high porosities, it may be necessary to prolong this period by mechanically controlling the outflow rate. However, in some instances the substrate can impede the flow to such an extent that the substrate itself becomes the primary determinant of retention time (Chapter 2).

The Malmesbury sand described in this thesis exhibited a slow retention time, obviating the need for manipulation of outflow, but decreasing the hydraulic flexibility (Chapter 3). Further studies are being conducted using sand obtained from a quarry site at Phillipi, Cape Town as well as a mixture of this sand with Malmesbury sand. These two substrates exhibit hydraulic conductivities \(-50\) and \(-10\) times faster than the Malmesbury sand (unpublished results). In the case of the Phillipi sand, the retention time is being manipulated by closing the outlet, which allows increased flexibility as the outlet can be closed for a predetermined period. It is not yet known whether the removal rates will be comparable to those achieved using Malmesbury sand. However, if good removal efficiencies are achieved, it may be possible to increase the weekly organic loading rate significantly.

5.3 BIOTIC CONSIDERATIONS

It is generally accepted that in CWs treating domestic wastewater, microorganisms, plants and substrate all work synergistically to remediate wastewater (Kiviasi, 2001; Vymazal, 2005).
5.3.1 Consideration of microbial community ecology and microbe-substrate interactions in the design of CWs treating cellar effluent in the Western Cape, South Africa

Sand affords a large surface area for the attachment of microbial biofilm (Section 1.4.3.1) and hence was the medium of choice for use in the experimental systems described in this thesis. Accessible, cost-effective, locally-available sand was sourced. In the studies presented in this thesis, excellent remediation of winery wastewater and common components of winery wastewater was achieved (Chapters 2 and 3). Clearly, the Malmesbury sand provided a suitable habitat for the growth of appropriate functional microbial species. It is hypothesized that because the physical and chemical properties of the substrate play a significant role in determining the microbial community structure (Li et al., 2008) if effective bioremediation can be achieved in experimental CWs, the same substrate can be confidently used in full-scale CWs.

During this project, ecological studies applying molecular fingerprinting tools showed that ethanol and phenolics significantly affected the microbial community structure in the CWs (Rodriguez-Calballaro, 2012). Furthermore, increasing CW depth and/or the addition of N and P ameliorated the magnitude of these changes (Rodriguez-Calballaro, 2012). Hence, further ecological studies are being conducted to determine whether other locally available sand will also support the growth of functional microbial species for the treatment of winery wastewater (Section 5.2.2.2).

In the studies described in this thesis (Chapters 3 and 4), the application of incremental priming as a start-up procedure increased the bioremediation of common readily and slowly biodegradable fractions of winery wastewater. This was attributed to acclimation of the microbial communities to steadily increasing organic concentrations. Further work to determine suitable, practical incremental concentrations and periods is needed to fully understand this phenomenon for application in full-scale systems. In addition to incremental
priming, bioaugmentation with sediment from areas previously exposed to winery wastewater may also assist in establishing appropriate functional microbial communities capable of degrading winery organics.

Results on the research pertaining to the effects of plants on the degradation of high organic content wastewater are conflicted (Section 1.4.3.1). In addition, the overall aim of the project (WRC: K5/1936) was to understand the role of the substrate, the microbial communities and the microbe-substrate interactions on the remediation of agri-industrial wastewater in CWs (Section 1.5). Therefore, the CWs described in this thesis did not contain plants and no research was conducted to assess any differences in removal performance between planted and unplanted systems. Adopting a bottom-up approach, experiments including plants may be considered once the fundamentals have been properly investigated in unplanted systems.

It was also recognized that for ecological studies, plants would impact on the microbial community structure and add another dimension to an already complex system (Section 1.4.3.1). In addition, roots and senescing above-ground biomass of plants block CWs and add to maintenance requirements, which could be exacerbated by the fact that the phenolic component of agri-industrial effluent is typically phytotoxic (Section 3.2).

5.4 SUMMARY OF FINDINGS

The studies outlined in this thesis showed that in the pilot-scale CWs at the Cape Peninsula University of Technology:

- Microbial communities required approximately 100 days to equilibrate (stabilize) following a new feeding/amendment regime.

- Below a certain COD threshold, ethanol was completely mineralized. However, if the threshold was exceeded, there was a build-up of VFAs.
Below a certain threshold, phenolics were completely removed from winery wastewater and synthetic wastewater, with biotic and abiotic mechanisms both playing significant roles. However, if the threshold was exceeded, there was a build-up of metabolites including the toxic molecule, catechol.

Prior acclimation of microbial communities to ethanol and phenolics resulted in superior degradation of these organics when compared to non-acclimated populations.

5.5 REFERENCES


GLOSSARY

All the terms are explained in the context in which they are used in this thesis. Many may have additional meanings which are not included in the glossary.

Acclimation: The successful functional adaptation of organisms to changed environmental parameters. In the context of this thesis, the word “organisms” pertains to microbial communities in the constructed wetland sediments.

Amendment: the addition of authentic or synthetic wastewater to experimental constructed wetland systems.

Bioaccumulation: The accumulation of a chemical in an organism through uptake by ingestion, respiration or direct contact so that the concentration becomes greater than that of the surrounding medium.

Bioaugmentation: The addition of specific microorganisms or microbial consortia to an environment in order to increase the rate of biodegradation of a particular pollutant.

Biomagnification: The processes by which particular chemicals accumulate in the tissues of organisms and reach higher concentrations through predator-prey relationship mechanisms in higher tropic levels.

Biostimulation: The addition of nutrients (usually nitrogen and phosphorus) to increase bioremediation by stimulating the growth of microorganisms.

Chemolithotrophic: Deriving energy from the oxidation of reduced inorganic compounds.

Heterotrophic: Deriving energy from the breakdown of organic compounds.
Community level physiological profiling: Evaluation of multiple physiological attributes of entire microbial communities for the purpose of discriminating spatial, temporal and/or experimental effects.

Conserved (nucleic acid sequences): Sequences of DNA or RNA (e.g. in 16s RNA gene) that are similar in multiple species.

Dendrogram: A genealogical tree with the trunk representing the oldest common ancestor.

Equilibration: The stabilization of microbial communities after exposure to altered environmental parameters.

Fouling (membranes): The deposition of microbial products or other solutes/particles onto the membrane surface or into the membrane pore, leading to impaired membrane permeability.

Incremental priming: The intentional addition of wastewater or wastewater components in incrementally increasing concentrations in order to increase bioremediation via acclimation of microbial communities.

Kerogen: Fossilized organic matter.

Macrophyte: Aquatic plant large enough to be seen with the naked eye.

Mesocosm: "Cosms" are experimental units designed to contain important components and to exhibit important processes of whole ecosystems. As a rule of thumb, the prefixes micro, meso and macro refer to units < 1 m$^3$, from 1m$^3$ to 1 000 m$^3$ and > 1000 m$^3$, respectively.

Oligotrophic: An environment (usually aquatic) with low primary productivity due to low nutrient availability.

Phytotoxic: Toxic to plants.
**Recalcitrant**: Resistance to environmental degradation (chemicals).

**Refractory**: as recalcitrant

**Rhizosphere**: The small region of soil around plant roots that is influenced by plant secretions and associated microorganisms.

**Sloughing**: The detachment of microorganisms and/or biofilm from attachment sites in attached growth biological wastewater treatment systems.

**Stable isotope probing**: The incorporation of $^{13}$C-labeled substrate into cellular biomarkers such as nucleic acids, followed by separation of labelled from unlabelled nucleic acids by density gradient centrifugation, and molecular identification of active microbial populations carrying labelled nucleic acids.

**Surfactant**: A surface active agent, or wetting agent, capable of reducing the surface tension of a liquid; typically organic compounds having a hydrophilic "head" and a hydrophobic "tail".

**Teratotoxin**: A substance that is toxic to an unborn foetus.

**Vadose zone**: The area between the land surface and the water table.
Microbial community structure stability, a key parameter in monitoring the development of constructed wetland mesocosms during start-up

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Received 12 April 2011; accepted 15 September 2011
Available online 13 October 2011

Abstract

Constructed wetlands (CWs) are known to be effective for treating waste streams, and pilot-scale CWs are useful for assessing the impact of pollutants and their remediation. However, little is known with respect to the establishment of these mesocosm systems or the parameters which should be monitored in assessing system equilibration, i.e. when they present stabilised physical and biological patterns. The aim of this study was to evaluate the temporal aspects of CW equilibration as a basis for future studies of system response to amendment. Microbial biomass and hydraulic conductivity values were monitored and microbial community fingerprints were obtained using denaturing gradient gel electrophoresis (DGGE). This study showed that microbial community fingerprinting provides a valuable tool for assessing the time scales of equilibration, as it was the last parameter which stabilised during the equilibration period. Hydraulic conductivity was also an important parameter in determining the time scale for initiation of the equilibration process during the study. For a CW of the dimensions used (173 cm long/106 cm large/50 cm depth), community equilibration times demonstrated on the basis of similar microbial community structures were found to be on the order of 100 days.

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Keywords: Constructed wetland mesocosm; Microbial community structure; Hydraulic conductivity; Denaturing gradient gel electrophoresis (DGGE); Start-up process

1. Introduction

Wetlands are generally defined as areas that are inundated or saturated by surface or ground water at a frequency sufficient to support vegetation types adapted to the saturated conditions (Mitsch and Gosselink, 2000). Although constructed wetlands (CWs) have been used since the 1950s, they are now of particular interest globally, as they represent a cost-effective, ecologically-friendly and aesthetically attractive option for wastewater purification. Studies have shown that CWs can reduce the chemical oxygen demand (COD) levels in industrial or agricultural effluents by 60–90% and in sewage domestic effluents by 90–99% (Vitsoeck et al., 1996; Verhoeven and Meuleman, 1999). In addition, the transformation of inorganics, including nitrogen (via microbial nitrification, denitrification and anammox) and sulphur (via microbial sulphate reduction) provide important mechanisms for contaminant removal in CWs (for review, see Faulkweather et al., 2009). Moreover, CWs are already used to treat and purify various contaminated effluents such as municipal, industrial, and/or agricultural wastewaters (Vymazal, 2009), and pilot-scale CWs are used to assess the feasibility of the technology before full-scale application for treating wastewaters (Chen et al., 2006).

In general, CWs comprise a substrate (e.g. soil) supporting plant and microbial communities that work synergistically to treat wastewaters. Plants metabolise available nutrients and are
able to accumulate heavy metals (Glick, 2010). In addition, plants are capable of directly catalysing certain organic contaminants via processes known as phytotransformation and phytodegradation (for review, see Singh and Jain, 2003). For example, the uptake and degradation of the toxic polycyclic aromatic hydrocarbon anthracene into anthrione, anthraqui-none and hydroxyanthraquinone has been demonstrated in wheat and maize (Wild et al., 2005). Microbial communities are essential in the mineralisation of organic matter and in nitrogen, sulphur and phosphorous removal (Fauplchtert et al., 2009; Truu et al., 2009; Glick, 2010). The substrate assists in the removal of pollutants by natural sedimentation (Vrbováek et al., 1996). Studies have shown that the composition of the substrate can play an important role in the removal of pollutants. For example, the adsorption of phosphorus and the abiotic oxidation of phenolics is enhanced in CWs where iron and/or manganese form part of the substrate (e.g. Lehmann et al., 1986; Sakadevan and Bavor, 1998; Polubesova et al., 2010).

It has been shown that extended periods are required for microbial communities in CWs to stabilise during operational periods, although the basis used to indicate community stability is not always consistent (Truu et al., 2009; Weber and Legge, 2011). In one report, using reactors with iron and manganese removal, the establishment of denitrifying bacteria populations required 75 days, while 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 CW mesocosms that microbial communities in a start-up process reached a steady-state after a period of 75–100 days (Weber and Legge, 2011).

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 Overaa, 2002; Wohl et al., 2004). In order to conduct reliable comparative experiments in CWs, it is essential that the microbial community composition and diversity is similar in all control and experimental CW replicates prior to amendment. The objective of this study was to determine suitable equilibration kinetics (i.e. the period necessary for the stabilisation of microbial communities in CWs) of pilot-scale CWs and to ascertain whether microbial community fingerprinting is a reliable tool for assessing this equilibration process. We define an equilibrated system as one which demonstrates stabilised 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; Calheiro et al., 2009; Weber and Legge, 2011), unplanted CWs were used.

To follow modifications in microbial community structures and to establish the kinetics of community change, we used denaturing gradient gel electrophoresis (DGGE) (Nicomrat et al., 2006; Baptista et al., 2008; Ruiz-Rueda et al., 2009). In long-term amendment experiments in estuarine waters and time-lapse experiments in nutrient soils, the fingerprints of microbial communities detected using DNA-based and RNA-based DGGE methods have been shown to exhibit similar patterns (Hoshino and Maizumoto, 2007; Nöpceron et al., 2010). Thus, as the establishment of microbial communities in CWs has been proven to be a long process (Truu et al., 2009; Weber and Legge, 2011), a PCR-DGGE approach targeting the 16S rRNA genes in total extracted DNA rather than in total RNA was chosen to assess microbial community structure evolution and stability in this study. 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 methods for environmental microbial community fingerprinting analysis (DDGE), 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. Total extractable DNA titres were used as proxy for microbial biomass, and hydraulic conductivity was used to monitor changes in CW drainage processes. It has been demonstrated that a decrease in hydraulic conductivity is a valuable indicator of the production and accumulation of microbial biomass during the establishment of the microbial communities in soils and CWs (Wu et al., 1997; Knowles et al., 2010).

2. Materials and methods

2.1. Constructed wetland set-up

Four polyethylene tanks were established as pilot-scale constructed wetlands, each containing an equal quantity of river sand (approx. 1.5 m³) obtained from Malmsbury (South Africa). The sand was thoroughly hand-mixed in a 1:4 ratio with an inoculum of natural wetland sediment for homogenisation. The final CW sediment composition consisted of 1% clay, 7% silt, 4% fine sand, 12% medium sand and 76% coarse sand. The dimensions and operational set-up of the wetlands are shown in Fig. 1. The wetlands were operated in a mixed vertical and horizontal subsurface flow (VSSF and HSSF) mode, where VSSF was established as the principal mode of operation (Welz et al., 2011). During the drainage of VSSF CWs, atmospheric gases permeate the sediment matrix, which ensures greater oxygenation of these systems when compared to HSSF CWs (where continual inundation leads to the presence of anoxic conditions in the substratum; Fauplchtert et al., 2009).

Three of the wetlands (designated A, B and C) received identical treatments during the establishment phase. They were fed bi-weekly with nutrients consisting of yeast extract powder (Biolab, Gauteng, South Africa) and D (+)-glucose (Merek Chemicals, Gauteng, South Africa) dissolved in tap water; volumes and concentrations are given in Table 1. 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), used as a negative control, received tap water without nutrient additions but in amounts and rate equal to CWS A, B and C.

2.2. Sediment sampling

A 30 mm diameter PVC sediment corer was used to recover samples without gross disturbance of the sediment stratification. Duplicate sample cores were taken from near the centre of each wetland at 47, 61, 75, 89 and 96 days after initiation of the experiment. From each core, a surface (0–3 cm) and a deep (15–20 cm) subsample fraction were retained for further analysis. After homogenisation, subsamples (1 g wet weight sediment) were frozen at −80 °C for subsequent molecular analysis.

2.3. Hydraulic conductivity

The outflow of effluent induced by the bi-weekly feeding/watering was recorded weekly for wetland A, B and C from day 47 and for wetland D from day 61 (Fig. 4A). The hydraulic conductivity (HC) was determined by measuring the volume of effluent collected between 1 and 2 h after the start of amendment. Initially, the volume of effluent was measured every 15 min from initial discharge but after three consecutive readings it was evident that the outflow stabilised after 30 min (data not shown). This rate is expressed as L m⁻¹ solids h⁻¹.

2.4. DNA extraction and quantification

Total DNA was extracted from 0.5 g sediment samples (wt weight) with a Bio-101 FastDNA spin kit and using the Fast-Prep FP 120 bead beating system (Bio-101, USA) according to the manufacturer’s instructions. The concentration of duplicate DNA samples was measured with a NanoDrop spectrophotometer (NanoDrop Technologies, Montchanin, DE, USA). Student’s t-test was used to assess significant differences (p < 0.05) between DNA concentrations.

2.5. PCR amplifications

All polymerase chain reactions (PCRs) were carried out in a Perkin-Elmer thermocycler (Gene Amp PCR system 6700). Bacterial 16S rRNA encoding genes were amplified using universal primers E9F (5’-GGGTTACCTTGTTACGACTT-3’) and U1510R (5’-GGTCTACCTGGCTGTTCGACT-3’). PCR was carried out in 50 μl reaction volumes. Each reaction contained 1 X PCR buffer, 0.2 U DreamTaq polymerase (Fermentas, USA), 200 μM of each dNTP, 0.5 μM of each primer, 0.1% BSA and between 5 to 10 ng of total extracted DNA. PCR amplification was carried out as follows: 4 min at 94 °C for denaturation; 30 cycles of 30 s at 94 °C, 30 s annealing at 52 °C and 105 s at 72 °C and a final elongation step of 10 min at 72 °C.

To perform DGGE, a nested PCR was performed using 1 μL of the amplicon obtained with the 16S rRNA primer set E9F/U1510R and with the primer set 341f-GC (5’-CCTACGGG-GAGGCAGCAG-3’)/534r (5’-ATTACCGCGGCTGCTG-3’) (Muyzer et al., 1993) as follow: 94 °C for 4 min; 20 cycles – 94 °C for 45 s, 56 °C for 45 s, 72 °C for 60 s; additional 20 cycles – 94 °C for 30 s, 55 °C for 30 s; at 72 °C for 60 s; and a final elongation step at 72 °C for 10 min. A 40 mer GC clamp was added to the 5’ ends of the forward primers 341f-GC (GC clamp — CGCCCGCGCGCGCGCGCGCGCGCGCGCGAC GGGGGG). PCR amplification with 341f-GC/534r was performed by using a 50-μl total volume mixture containing 0.2 U DreamTaq polymerase (Fermentas, USA), 1X PCR Buffer, 200 μM of each dNTP, 0.5 μM of each primer and 0.1% BSA.

2.6. Denaturing gradient gel electrophoresis (DGGE) analysis

Equal amounts of PCR amplicons obtained with the nested primer sets (341f-GC/534r) were analyzed 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% denaturation 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 h at 60 °C (1600 Vb) in 1X TAE buffer. Gels were stained in 0.5 μg mL⁻¹ ethidium bromide in 1X TAE for 20 min and visualised on an Alphalmager 3400 imaging system.
DGGE gel pictures were processed with GelCompar II 5.0 software (Applied Maths, Belgium). 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 (data not shown). Clustering analysis and non-metric multi-dimensional scaling (MDS) were performed with GelCompar II 5.0. 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. MDS analyzes the matrix of similarities obtained using the cosine coefficient. Thus, in Figs. 2 and 3, the linkages in the 3D-MDS graphical representation are shown as an aid to interpretation as the samples are connected by the similarity dendrogram branches (data not shown). This representation enables co-evaluation and analysis of a dendrogram and a coordinate system such as MDS.

3. Results and discussion

The CWs were operated in a hybrid mode of vertical and horizontal subsurface flow (VSSF and HSSF) where VSSF

<table>
<thead>
<tr>
<th>Days (number of feedings)</th>
<th>Yeast extract (g)</th>
<th>Glucose (g)</th>
<th>Volume (L)</th>
<th>Rate (L min⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Day 1</td>
<td>10</td>
<td>2</td>
<td>30</td>
<td>0.27</td>
</tr>
<tr>
<td>Day 8</td>
<td>5</td>
<td>1</td>
<td>50</td>
<td>0.27</td>
</tr>
<tr>
<td>Day 13</td>
<td>1</td>
<td>1</td>
<td>45</td>
<td>0.27</td>
</tr>
<tr>
<td>Day 21</td>
<td>1</td>
<td>1</td>
<td>40</td>
<td>0.68</td>
</tr>
<tr>
<td>Day 25 to Day 64 (12)</td>
<td>0.5</td>
<td>0.5</td>
<td>20</td>
<td>0.68</td>
</tr>
<tr>
<td>D 67 to Day 96 (9)</td>
<td>0.3</td>
<td>0.3</td>
<td>12</td>
<td>0.68</td>
</tr>
</tbody>
</table>
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 high redox potentials that facilitate aerobic microbial processes (Faulkner et al., 2009).
In mesocosms, previous studies have shown that equilibration times for achieving a stable microbial community are slow in terms of biomass or function stabilisation (in the order of 75 to 100 days) (Trua et al., 2009; Weber and Legge, 2011).
Moreover, to our knowledge, the equilibration of CW microbial community structures has never been addressed in such systems. Our intention was thus to determine the period necessary for the equilibration of the microbial community structure and as such, the early phase microbial community succession was not seen as an integral part of the study. In addition, a decreasing trend in the hydraulic conductivity of the nutrient-supplemented CWs stabilised after 47 days (data not shown). For these reasons, samples were taken for microbial community monitoring from 47 days after the initiation of nutrient supplementation.

In parallel CW experiments, it is 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 throughout the experiment to three of the four CWs (namely A, B and C; Table 1). This was done 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, stable, biological (microbial community structure (Figs. 2 and 3) and microbial biomass (Fig. 4B)), estimated by total extracted DNA concentrations and physical (hydraulic conductivity, Fig. 4A) profiles. The nutrient amendments, corresponding to a solution of glucose and yeast extract (Table 1), were designed to provide 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 an oligotrophic state, as oligotrophic systems are more reactive to changes in nutrient status such as wastewater contamination (Verhoeven et al., 2006).

We used DGGE to assess the stability of the pilot-scale CW microbial communities and their response to different nutrient status (Figs. 2 and 3). The variation in hydraulic conductivity in parallel with the microbial biomass was also studied (Fig. 4), as a decrease in hydraulic conductivity is a valuable indicator of production and accumulation of microbial biomass during the establishment of the microbial communities (Wu et al., 1997; Knowles et al., 2010).

### 3.1. Microbial community structure and evolution

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 (data not shown). DGGE patterns were subjected to cluster analysis and multidimensional scaling (MDS) analysis (presented as three-dimensional distribution plots; Figs. 2 and 3), to establish the statistical relatedness between samples.

The MDS plot derived from DGGE analysis of CW surface sediment samples (Fig. 2) shows that points representing the molecular fingerprints from day 47 sample patterns for the 4 CWs are tightly grouped and well separated from all other samples. This indicates that the microbial communities were highly similar at this sampling time, independently of the feeding regime. Through successive sampling points, all communities showed substantial structural divergence. The most coherent group across all subsequent sample times (days 61 to 96) was that derived from CW D (unfed control, Fig. 2). This shows that the oligotrophic nutrient amendment procedure used in this study changed the CW surface microbial communities between day 47 and day 61. The day 61 and 75 samples from the nutrient-supplemented CWs A, B and C were scattered on the 3D-MDS plot (Fig. 2), suggesting that the surface communities evolved differentially during this time period, even though the same nutrient supplementation procedure was observed (Table 1). Thus, it can be concluded that after 75 days of experiment, the surface microbial communities were not equilibrated. In contrast, the day 89 and 96 samples are close on the MDS plot, indicating that the respective surface microbial communities were highly similar at this stage. Therefore, it can be interpreted that the microbial communities in the surface sediments of the three nutrient-supplemented CWs evolved over the 49-day sampling period. In contrast, the consistency of the DGGE patterns from the control (non-supplemented) CW strongly suggests that the microbial community in this CW was equilibrated by day 61 and remained essentially stable for the duration of the experiment. 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, and that this equilibration took place between day 75 and 89.

Similar analyses of the deep sediment microbial community structure showed that, as for the surface communities, day 47 samples were spatially distinct from the other samples. However, in contrast to the surface communities, points representing the microbial communities in the deep sediments were scattered on the MDS graphical representation (Fig. 3). This suggests that the deep CW sediment microbial communities of the 4 CWs were different at that time. All other samples show substantial divergence from the day 47 community patterns. A second dispersed cluster (Fig. 3) contains the day 61 and 75 samples. The dispersed structure of this cluster suggests that all communities (in both supplemented and control CWs) underwent independent structural evolution. However, a third cluster (black ellipse, Fig. 3), which represents the day 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 CWs.
These results might suggest that the nutrient-supplemented CW communities were responsive to nutrient addition, but the parallel behaviour 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 microenviroment which would be expected to dominate changes in microbial community structure. It is also likely that the concentrations of supplemented nutrient components experienced by the deep sediment communities would be substantially lower than those experienced by surface microbial communities, being a function of nutrient depletion by surface communities.

Based on these results, we concluded that using molecular fingerprinting methods such as DGGE to monitor the evolution of CW microbial communities is a valuable tool to determine their equilibration in a CW start-up process. In this study, DGGE results determined that the microbial communities in the surface and deep sediments of pilot-scale CWs required 89 days to equilibrate.

3.2. Nutrient supplementation, hydraulic conductivity and microbial biomass evolution

As shown in Table 1, the concentration of solutes and volumes provided to the supplemented CWs were modified at intervals during the 96-day equilibration phase. Solute concentrations were maintained after day 21, but liquid volumes were adjusted to avoid protracted periods of surface flooding and the generation of permanent 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; Fig. 4A). The hydraulic conductivity stabilised after day 61, and the nutrient supplementation procedure was maintained until the mesocosms presented a stabilised and similar microbial community fingerprint (Figs. 2 and 3). After 47 days, the hydraulic conductivity of CW D (control) was significantly higher than in the nutrient supplemented CWs (p < 0.05; Fig. 4A). Hydraulic conductivity values ranged from 2.91 to 2.28 L m⁻² h⁻¹ in CW D, compared with values of 0.54 L m⁻² h⁻¹ (CW A, day 96) and 2.19 L m⁻² h⁻¹ (CW B, day 47). Decreases in the HC of CW systems can be caused by the trapping of solids by the soil matrix, the growth of vegetation and/or the formation of biofilm/biomass (Knowles et al., 2010). The CW used in this study was unplanted, and due to the dilute nature of the influent, it is highly unlikely that the influent caused clogging of interstitial spaces in the soil matrix; the observed decrease in hydraulic conductivity was therefore attributed to an increase in in situ biomass (Wu et al., 1997; Knowles et al., 2010; Weber and Legge, 2011).

Microbial biomass was investigated using total DNA concentration (Fig. 4B), as it is generally considered to be a reliable indicator of this parameter in soils and sediments (Marston et al., 2009; Agnelli et al., 2004; Faulsatter et al., 2009; Ramond et al., 2009). To allow comparisons between samples, the DNA extraction procedure was normalized by using a commercial kit. 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; Fig. 4B), suggesting that biomass loads were higher and/or that more biomass was produced in the unsupplemented control CW. This result is counterintuitive and is inconsistent with the hydraulic conductivity data and nutrient status. For both surface and deep sediments, 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 = 0.79 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 contradictory with the general concept that hydraulic conductivity is inversely related to biomass (Prochaska et al., 2007; Weber and Legge, 2011), 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. This has also been 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 hydraulic conductivity in the nutrient supplemented CWs (Weber and Legge, 2011), 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 (Frolund et al., 1996; Iahn and Nielsen, 1998; Laspidou and Ritman, 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. We therefore suggest that the formation of biofilms by developing microbial communities in the nutrient-supplemented CWs may negatively impact on DNA extraction efficiency.

Based on these results, we conclude that the hydraulic conductivity of the system is a useful indicator of early CW equilibration in a start-up process. Using this parameter, the establishment phase for the CW mesocosms was approximately 61 days. Within these systems, we also conclude that using total extractable DNA concentrations to monitor biomass is not appropriate. It is possible that the use of a molecular method such as quantitative PCR (qPCR), which specifically targets the bacterial 16S rRNA (Plassart et al., 2008; Nieperzon et al., 2010), would provide a better indication of the amount of microbial biomass in CW sediments than the spectrophotometric method used in this study.

In conclusion, microbial community fingerprinting is an effective method for monitoring changes in microbial community structure in CW systems (Nicomrat et al., 2006;
Bapista et al., 2008; Ruiz-Rueda et al., 2009). This study lays
the groundwork for the use of CW systems to investigate the
relationships between microbial structure and system
responses to environmental challenges. In particular, DGGIE
can be used very elegantly to monitor both compositional
changes and the rate of change in a microbial community after
chemical challenge, such as the addition of industrial waste
streams, pollutant compounds, xenobiotics, etc (e.g. Nieper
et al., 2010). Other system changes, such as liquid flow rates,
input C/N ratios, temperature regimes, etc could also be
addressed.
This study demonstrates that after 89 days of nutrient
amendment the pilot-scale CWs were equilibrated, with
an established microbial community presenting a highly
similar fingerprint in the surface and the deep sediments. This
study emphasizes that microbial community fingerprinting
with molecular tools (such as DGGIE) is a key parameter for
monitoring CW establishment before pollutaot challenge
experiments. The observation that microbial community
structure is the slowest parameter to stabilise supports this
view. We also note that hydraulic conductivity may be useful
as a determinant of microbial community development and
that extractable DNA titre is not a reliable parameter for
monitoring microbial biomass.

Acknowledgements

The authors gratefully acknowledge the South African
Water Research Commission for financial support. J-BR and
PJW would also like to thank Dr C. Bressa for his assistance
in establishing the pilot-scale CWs.

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APPENDIX 2

Ethanol degradation and the benefits of incremental priming in pilot-scale constructed wetlands

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ABSTRACT

There has been significant global growth in the use of constructed wetlands for wastewater treatment. The fundamental microbial processes involved in the biodegradation of organic wastewater pollutants determine the range of design and operational parameters relevant to individual constructed wetland systems. In this study, the biodegradation and mineralization of ethanol by acclimated and non-acclimated microbial populations in pilot-scale constructed wetlands were compared. By increasing the pollutant concentration at incremental intervals (incremental priming), the biodegradative capacity of a sand-filled constructed wetland was significantly enhanced. At an influent COD concentration of 15,400 mg L⁻¹, no volatile fatty acids were detected in the effluent of an incrementally primed system and the maximum effluent COD concentration was 1896 mg L⁻¹. In contrast, an identical unprimed system, amended with a lower concentration of COD (7587 mg L⁻¹), exhibited a maximum effluent COD concentration of 1640 mg L⁻¹, with the anaerobic metabolites, butyrate and propionate accounting for up to 83% of the effluent COD. It was demonstrated that the use of incremental priming, together with a vertical subsurface flow model of operation, enhanced long-term function of constructed wetlands. Future research should focus on determining the concentration gradients and incremental intervals necessary for optimal microbial activation to a range of organic pollutants and/or wastewaters, in order to minimize start-up times without significantly impairing the benefits derived from incremental priming.

1. Introduction

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, 2007). 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; Malandria et al., 2003).

South African legislation authorizes discharge of biodegradable industrial wastewater to the environment providing certain parameters are monitored and met (South African Wastewater Act 96, 1998). Maximum chemical oxygen demand (COD) limits range from 30 mg L⁻¹ to 5000 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 essential. Increased concentrations of organic chemicals are observed in winery wastewaters around the seasonal vinification period. Glucose, fructose, ethanol and acetic acid have all been identified as significant COD contributors (Malandria 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 (Malandria et al., 2003). The stability of conventional biological wastewater treatment systems may be disrupted by the inherent variability in composition and volume of winery wastewater (Malandria et al., 2003; Maiz et al., 2002; Sheridan, 2007; Vymazal and Kröpfl, 2009). 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 (Malandria et al., 2003; Saadi et al., 2007; Sheridan, 2007). Provided
land requirements can be met, constructed wetlands (CWs) provide economical, low maintenance, low energy wastewater treatment systems that demonstrate limited sensitivity to seasonal input fluxes (Masi et al., 2002).

In this study, two unplanted, experimental pilot-scale CWs were amended with ethanol, which was chosen as a singular pollutant because of its prominence in winery wastewater. Simple sugars, organic acids and alcohols found in winery wastewater are readily biodegraded (Malandra et al., 2003; Serrano et al., 2010). Mineralization of these organic molecules in constructed wetlands is mediated predominantly by microorganisms (Caselles-Osorio and Garcia, 2006; Tietz et al., 2008; Imfeld et al., 2009; Vymazal and Krápešová, 2009). Oxygen is the preferred electron acceptor during the biodegradation of ethanol, rendering aerobic metabolic pathways energetically more favourable, and thus faster than anaerobic or anoxic pathways (Alvarex and Hunt, 1999). Aerobic biodegradation commences with the oxidation of ethanol to acetaldehyde, which is mediated by the enzyme alcohol dehydrogenase (Hektor et al., 2000; Österreicher-Cunha et al., 2009). Further oxidation results in the formation of acetyl-CoA either directly from acetaldehyde or via acetate, both of which are mineralized intracellularly via the Krebs cycle or the glyoxylate shunt to CO₂ (Alvarex and Hunt, 1999). Acetic acid bacteria sometimes lack the enzymes necessary to metabolize acetate and thus excrete the molecule; this may result in the toxic levels of acetate accumulating in the environment (Gottschalk, 1986). Due to the inherent dependency on oxygen, these reactions are only expected to occur in the surface sediments of wetland systems, with anaerobic pathways dominating in the deeper sediments (Hektor et al., 2000).

Microorganisms capable of anaerobic ethanol fermentation are ubiquitous in nature (Schink et al., 1985). The most common primary metabolites comprise acetaldehyde and the volatile fatty acids (VFAs), acetic acid, propionic acid and butyric acid, while carbon dioxide, methane and hydrogen gas constitute the major end products (Alvarex and Hunt, 1999). Acetone and n-propanol may also be formed (Alvarex and Hunt, 1999). The anaerobic pathways rely on interspecies hydrogen transfers and as such are related to the sediment composition and redox status (particularly whether conditions are methanogenic, nitrate reducing or sulphate reducing) (Stams et al., 2006; McKelvie et al., 2007). The concentration of H₂ is a critical parameter in methanogenic environments because at high H₂ concentrations, incomplete ethanol oxidation is energetically more favourable and the environment becomes aetheogenic (acetate generating) (Dolfing, 2001). Consequently, under unfavourable conditions, volatile fatty acids can accumulate in CWs treated with high ethanol wastewater.

During this study, the biodegradation and mineralization of ethanol within the CWs was assessed by effluent analyses, with the starting substrate (ethanol), as well as important metabolic intermediates (acetic acid, propionic acid and butyric acid) being identified and quantified.

The primary aim of the study 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 polluted 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 potentially toxic substances would ultimately lead to tolerance at higher concentrations that may have proven lethal if employed from inception. Furthermore, it was postulated that a gradual selection of efficient microbial degraders acculturated to the presence of ethanol, acetate, propionate and butyrate should theoretically protect against the accumulation of these organics in toxic concentrations. The overall success of incremental priming was assessed using effluent COD (CO₂) values and the accumulation of metabolites as primary indicators of CW function and stability.

2. Materials and methods

2.1. Set-up and mode of operation of constructed wetlands

Three identical, unplanted, experimental CWs consisting of polyethylene containers filled with river sand to a volume ~0.5 m³, void space of 0.08 m³ and a depth of 0.3 m were inoculated in a ratio of 1:4 with sediment from a local wetland treating winery wastewater. The CWs were kept undercover to avoid exposure to precipitation events. Two CWs were used for comparative experiments and the third CW was planted. The CWs were operated in a hybrid mode of vertical and horizontal sub-surface flow (VSSF and HSSF), shown schematically in Fig. 1. All CWs were subject to bi-weekly inundation followed by gradient-directed drainage to ensure that the mode of operation was biased toward classical VSSF.

2.2. Feeding/watering regime of the constructed wetlands

CW A was designated as a control and CW B and CW C as experimental systems. All CWs were equilibrated for a minimum of three months prior to experimentation, previously determined as the period necessary for microbial community stabilization (Ramondo et al., unpublished results). Due to the scale of the CWs, and the need to generate data for a range of organic pollutants, the use of replicate CWs was excluded. However, replicate soil column and microcosm experiments using the CW sediments have shown excellent reproducibility (unpublished results) and the results presented in this paper are clear. All three CWs received a bi-weekly influent feedstock consisting of 0.3 g yeast extract (Biobal, RSA cat no: HKG000860.500), and 0.3 g o (+) glucose (Merck, RSA, chemically pure cat no: SMAR26760209EM) dissolved in 12.51 tap water for the duration of the equilibration and 47 week experimental periods. The feedstock of the test CWs was supplemented with absolute ethanol (Merck univar) as outlined in Table 1. CW B was initially amended with an ethanol load similar to that measured in celler effluent from a South African winery (4.94 mM). Table 1 Incremental priming, by amendment with ethanol at increasing theoretical COD (μmol) concentrations, ranging from 474 mg L⁻¹ to 26,333 mg L⁻¹ (equivalent to 4.94 mM and 2.74 to 105 mM ethanol), was applied to CW C. CW C was amended with moderate concentration of ethanol (COD, 7.587 mg L⁻¹, 5.44 mM) from inception to completion of the experiment, the concentration being an approximate average, based on literature for winery wastewater before primary treatment (see Table 3).

2.3. Effluent analyses

Test samples comprised the total volume of effluent collected between 1 and 2h after the initiation of feeding. Acids and alcohols were analysed by high performance liquid chromatography (HPLC) according the method of La Zaro et al. (1989). A Merck La-Chrom instrument with a La-Chrom® D-4400 ultraviolet detector set at 210 nm and an Agilent® G1310A diode array detector UV-VIS employed for the detection of acids and alcohols, respectively. Sample components were separated using a Phenomenex® Zorbax® RX-18 C18 (cross-linkage) column and a 1 mM H₃PO₄ solution at pH 2.52 (mobile phase). Sample acquisition time and flow rate were set at 60 min and 0.550 ml min⁻¹, respectively.
Fig. 1. Schematic diagram of the constructed wetlands, showing the hybrid mode of operation with vertical (VSSF) and horizontal (HSSF) flowpaths from the elevated inlet to the outlet at the bottom.

<table>
<thead>
<tr>
<th>Table 1</th>
<th>Ethanol amendment in terms of theoretical influent COD and the applied loading rate to the experimental constructed wetlands.</th>
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<tbody>
<tr>
<td>TIME (weeks)</td>
<td>CW B incrementally primed</td>
</tr>
<tr>
<td></td>
<td>COD(_\text{in} ) (mg( \text{L}^{-1} ))</td>
</tr>
<tr>
<td>1-7</td>
<td>474</td>
</tr>
<tr>
<td>8-14</td>
<td>986</td>
</tr>
<tr>
<td>15-20</td>
<td>1096</td>
</tr>
<tr>
<td>21-26</td>
<td>1370</td>
</tr>
<tr>
<td>27-32</td>
<td>7567</td>
</tr>
<tr>
<td>33-37</td>
<td>7567</td>
</tr>
<tr>
<td>38-42</td>
<td>15,800</td>
</tr>
<tr>
<td>43-47</td>
<td>15,800</td>
</tr>
<tr>
<td>45-47</td>
<td>7187</td>
</tr>
</tbody>
</table>

COD\(_\text{in} \): theoretical influent COD; OLR: organic loading rate.

<table>
<thead>
<tr>
<th>Table 2</th>
<th>Relationship between COD(_X) ((X = 1 )) and standard COD(_X) solutions.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ethanol (mg( \text{L}^{-1} ))</td>
<td>Acetic acid (mg( \text{L}^{-1} ))</td>
</tr>
<tr>
<td>COD(_X)</td>
<td>1000</td>
</tr>
<tr>
<td>COD(_X)</td>
<td>996 ± 8</td>
</tr>
</tbody>
</table>

COD\(_X\): theoretical COD; COD\(_X\): actual COD.

<table>
<thead>
<tr>
<th>Table 3</th>
<th>Selected data from CWs used to treat winery wastewater or synthetic winery wastewater.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Reference</td>
<td>Mode of operation</td>
</tr>
<tr>
<td>Serrano et al. (2010)</td>
<td>Full-scale Hybrid 2 stage (VF/RF)</td>
</tr>
<tr>
<td></td>
<td></td>
</tr>
<tr>
<td>Shepherd et al. (2001)</td>
<td>Pilot-scale HSSF</td>
</tr>
<tr>
<td></td>
<td></td>
</tr>
<tr>
<td>Masi et al. (2002)</td>
<td>Full-scale HSSF</td>
</tr>
<tr>
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<td></td>
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<tr>
<td>Gruenter et al. (2003b)</td>
<td>Pilot-scale HSSF</td>
</tr>
<tr>
<td></td>
<td></td>
</tr>
<tr>
<td>Mena et al. (2010)</td>
<td>Pilot scale HSSF</td>
</tr>
<tr>
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<tr>
<td></td>
<td></td>
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<tr>
<td>Mulkid (2010)</td>
<td>Full-scale HSSF</td>
</tr>
<tr>
<td></td>
<td></td>
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<tr>
<td>This study</td>
<td>Hybrid</td>
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COD\(_\text{in} \): influent COD; COD\(_X\): influent COD; VF: free water; FP: fixed 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; w.w.: wastewater.
Peaks corresponding to ethanol and VFAs were identified by spiking effluent samples with ethanol (Merck univar, RSA, cat no: SAAR221340L), gluconic acid (Merck univar, RSA, cat no: SAAR102100L), propionic acid (Fluka puriss, Germany, cat no: W812794) and butyric acid (Aldrich, Germany, cat no: B80350-0). Once identified, the concentrations were determined by comparing the peak areas to those of graphs prepared from standard concentrations of the same chemicals.

COD was quantified using a Hanna C214 multiparameter bench photometer, a Hanna C8800 digestor and both low range (1-150 mg/L; H93754A-25) and medium range (0-1500 mg/L; H93754B-25) COD kits, following the manufacturer’s instructions. Theoretical COD (CODT) values were determined using the equation:

\[
\text{CODT} = \frac{\text{mgCOD} \times \text{mg} \times 10^{-2}}{(12 \times \text{mg} - 16 }) \text{g} C_2 H_4 O_2
\]

(1)

From Eq. (1), the CODT values were calculated to be 2.09 mg COD/mg ethanol, 1.07 mg COD/mg acetic acid, 1.51 mg COD/mg propionic acid and 1.81 mg COD/mg butyric acid. The relationship between CODT and the actual (CODA) was established by COD measurement of 1000 mg L^-1 COD solutions of ethanol (Merck univar, RSA, cat no: SAAR221340L), gluconic acid (Merck univar, RSA, cat no: SAAR102100L), propionic acid (Fluka puriss, Germany, cat no: W812794) and butyric acid (Aldrich, Germany, cat no: B80350-0) (Table 2). The concentrations (mg L^-1) of ethanol and VFAs in the effluent were converted to COD, and ultimately to CODA. COD values were used for mass balance relationships between COD and the relative contributions of ethanol and volatile fatty acids (VFAs) to CODA.

3. Results

3.1. 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.

COD 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 CODA from CW B was 180 mg L^-1 (at 14 weeks) and 104 mg L^-1 (at 33 weeks) (Fig. 2). The rise in CODA from incrementally primed CW B to a maximum of 365 mg L^-1 between 41 and 45 weeks corresponded with an influent ethanol concentration increase from 15,800 mg L^-1 to 26,333 mg L^-1 CODA. Small amounts of acetic acid, ranging between 13 mg L^-1 (14 mg L^-1 CODA) and 68 mg L^-1 (116 mg L^-1 CODA) and ethanol, ranging between 13 mg L^-1 (27 mg L^-1 CODA) and 166 mg L^-1 (214 mg L^-1 CODA) were detected at a maximum influent ethanol concentration (26,333 mg L^-1 CODA), as well as one week after a rest period (Fig. 3). During this period (41-45 weeks), propionic acid and butyric acid accumulated in the sediments, and the relative contribution of these VFAs to the total CODA increased from 0% at 40 weeks (15,800 mg L^-1 CODA) to 48% at 45 weeks (26,333 mg L^-1 CODA) (Fig. 4). 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 C (7587 mg L^-1 CODA) and at 47 weeks previous function was restored (CODA of 48 mg L^-1).

A moderate concentration of ethanol (7587 mg L^-1 CODA) was included in the feed of unprimed CW from 33 weeks (Table 1). This addition induced an immediate sharp increase in CODA, attributable mainly to acetic acid and ethanol (Fig. 2). Between 34 and 40 weeks, there was a steady decline in CODA, which generally corresponded to a reduction in the amount of amount of acetic acid and ethanol in the effluent (Fig. 3). However, the reduction in acetic acid was accompanied by an increase in the amount of propionic and butyric acid (Figure 3). Between 40 and 47 weeks, there was a second increase in CODA (Fig. 2). The proportion of propionic and butyric acid to the total CODA increased over time for the first 9 weeks, from 2% to a maximum of 83% at week 47 (Fig. 4). The CVs became malodorous due to accumulation of these VFAs in the sediments and the experiment was terminated after 47 weeks.

4. Discussion

Bacterial species may 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 (Zaidi et al., 1996; Hu et al., 1997; Koda et al., 2002; Cheng et al., 2010). Acclimation and degradation are influenced by the toxicity of a compound as well as the period of acclimation, with longer acclimation times leading to higher degradation rates (Chou et al., 1978). A link has also been demonstrated between acclimation and chemical concentration, with the period of acclimation being shortened at lower concentrations (Zaidi 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. To our knowledge, detailed studies detailing the concentration-dependent acclimation of microbial communities within CW systems have not previously been published. 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 and from this study is presented in Table 3.

Data on influent composition highlight the considerable variation in COD, that is attributable to the characteristics of the winery wastewater and the pre-treatment steps involved (Table 1). Pre-treatment, by means of anaerobic digestion, sand pre-filters or Screening tanks, and combined processes, have been reported as methods used to reduce suspended solids (SS) and the organic loading rate (OLR) (Grissmer et al., 2003a; Masi et al., 2002; Serrano et al., 2010; 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 (ECOD/CODA) as the sole criterion to assess the performance of biological systems treating highly variable wastewaters is debatable. For example, taking COD values realistic for winery wastewater of 20,000 mg L^-1, 5000 mg L^-1 and 2000 mg L^-1, removal efficiencies of 95%, 75% or 50% respectively all result in the same COD concentration of 1000 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 (Masi et al., 2002; Melanisi et al., 2007; Sheridan, 2007; Chrisek et al., 2010; Mulitzi, 2010). According to the National Water Act (no 36 of 1998), biodegradable
Fig. 2. Comparison of total effluent COD (CODe) from the control (CW A), incrementally primed (CW B) and unprimed (CW C) constructed wetlands. CW C was amended with ethanol at 33 weeks. The immediate increase in COD is shown by the arrow on the figure.

Fig. 3. Metabolite profiles of effluent samples from unprimed CW C (above) and incrementally primed CW B (below). Note that no ethanol or VFAs were detected in the effluent from CW B until 33 weeks, and as such, previous weeks have not been included in this figure. The “other” category in the figure represents the balance of COD once the contribution of ethanol and volatile fatty acids have been deducted.

Fig. 4. Ratio (%) of propionic acid and butyric acid (CODa) to total effluent COD (CODe) from incrementally primed (CW B, left) and unprimed (CW C, right) constructed wetlands.
industrial wastewater in South Africa may be used for irrigation purposes provided the COD is either <400 mg L\(^{-1}\) or <5000 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 is a critical parameter.

High COD; values associated with peak-season overloading of CW treatment facilities have been reported in the literature (Table 3) [Grismer et al., 2003b; Serrano et al., 2010]. The results obtained from this study showed that COD\(_{B}\) from incrementally primed CW B complied with all South African legislative requirements for irrigation when the COD\(_{B}\) fell in the range 474 mg L\(^{-1}\) to 15,800 mg L\(^{-1}\). However, the effluent from unprimed CW C, amended with an COD\(_{B}\) concentration of 7587 mg L\(^{-1}\), did not conform to legal limits for most of the study period, despite the fact that the COD\(_{B}\) removal efficiency was 81.9–95.7%. Thus, only the effluent from incrementally primed CW B was rendered suitable for irrigation without further treatment.

Unprimed CW C, amended with a comparatively moderate COD\(_{B}\), did not realize the same efficiency as incrementally primed CW B and became septic with the accumulation of VFAs (VFAs and sulphides) in wastewater provide evidence of septic conditions (Magro et al., 2005; Bachman et al., 2007). VFAs were detected in the sediments of unprimed CW C from inception to the completion of the experiment, with the pattern showing 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 81% of the effluent COD after 9 weeks. 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 differences in metabolic profiles of the incrementally primed CW B and unprimed CW C suggest that accumulation of the microbial community in CW B resulted in superior ethanal mineralization, possibly as the result of enhanced interspecies hydrogen transfer (Lasko et al., 1997). Furthermore, CW B proved resilient to a short period of overloading.

Process inhibition associated with high COD has been reported in CWs used to treat agricultural and industrial wastewaters (Vymazal and Krupínek, 2009). However, COD concentrations in winery wastewater from as low as 2178 ± 1715 mg L\(^{-1}\) have also been associated with poor treatment performance, with low influent pH (possibly due to volatile fatty acids) and/or high concentrations of inhibitory substrates such as polyphenols being suggested as contributing factors (Masi et al., 2002; Montalvo et al., 2010; Serrano et al., 2010). 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 waste, with organic loading rates (OLRs) in a similar range to those applied to CWs used for the same purpose (Andréoleita et al., 2009). However, the hydraulic retention times (HRTs) in CWs are in the order of days (Table 3), while those of most conventional systems are in the order of hours (Andréoleita et al., 2009; Grismer et al., 2003b; Petruccio et al., 2002; Shepherd et al., 2001; Mena et al., 2009; Mulidži, 2010). 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 soils and wetland sediments (Segars and Rengen, 1998; Kinsel and Drake, 1999). Thus, CW depth has been included in loading calculations in this paper.

There is circumstantial evidence that aerobic processes are favoured by high redox potentials in CWs operated in a VSSF mode (Faubwater et al., 2000). In addition, anaerobic degradation of organics such as ethanol in wetlands may result in the formation of the harmful greenhouse-gas, methane (Sha et al., 2011). It may be deduced that the use of VSSF CWs for the treatment of high organic wastewaters is preferable, both from an efficiency and an environmental perspective (Faubwater et al., 2009; Sha et al., 2011).

COD removal in CWs can be substantially enhanced by increasing the HRT (Table 3) (Mulidži, 2010). HRT is dictated by flowpaths and the extent to which the wastewater interacts with the wetland porous medium and plants (Grismer et al., 2003b). Apart from the demonstrated benefits of incremental priming in CW B, the COD removal process was almost certainly enhanced by the long HRT and concomitant low hydraulic loading rates (HLRs), which were largely dictated by the slow hydraulic characteristics of the sand medium. Although low HLRs may prevent clogging of CWs, the fundamental hydraulic characteristics, together with the VSSF mode of operation, precluded the application of a higher OUR without increasing the COD\(_{B}\) to toxic levels (Knowles et al., 2010). It can be hypothesized that a less compact sand medium provides a better nutritional habitat than gravel for the growth of many microorganisms. Nevertheless, the increased capacity demanded by an inflexibly low HC is a drawback that must be taken into consideration during CW design.

Winery wastewater, per se, consists not only of readily biodegradable COD (RBCOD), but also a smaller fraction of slowly biodegradable COD (SBCOD) and unf utilizable COD. Although ethanol, the pollutant used in the study, falls into the RBCOD category, the capacity of the CWs to effectively remove phenols (SBCOD) from winery wastewater was demonstrated in a parallel experiment using whole winery wastewater (Welz et al., unpublished results).

5. Conclusions

The use of incremental priming as a start-up mechanism enhanced biodegradation of ethanol in a pilot-scale CW. The results supported the hypothesis that this technique would promote acclimation of key degradable members of the microbial consortia, leading to improved COD removal when compared to "unprimed" microbial communities. In this study, incremental priming enhanced both COD removal and wetland stability. To our knowledge, this is the first time that start-up procedures for CWs have been explored in a structured manner. The use of this tool, together with the VSSF mode of operation to enhance the long-term biodegradable efficiency of CW systems treating wastewater with high organic loads was clearly demonstrated.

Further work is needed: firstly, to determine whether these results can be replicated with different wastewaters, and secondly, to determine whether prior function is retained after lengthy periods of redundancy. Clearly, the extended duration of the incremental procedure employed in this study is impractical in real terms. Therefore, concentration gradients and
APPENDIX 3

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Phenolic removal processes in biological sand filters, sand columns and microcosms

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HIGHLIGHTS

• At low influent concentrations, phenolics in winery wastewater and model synthetic wastewater were completely removed by a combination of biotic and abiotic influences in biological sand filters.
• High influent concentrations of model phenolics resulted in accumulation of metabolites in biological sand filters. Increased hydraulic conductivity strongly suggested a concurrent loss of biomass/biofilm due to accumulation of toxic concentrations of catechol.
• Acclimation of microbial populations to vanillin and gallic acid resulted in enhanced biodegradation of these phenolics when compared to a non-acclimated population.

ARTICLE INFO

Article history:
Received 29 February 2012
Received in revised form 20 April 2012
Accepted 21 April 2012
Available online 18 May 2012

Keywords:
Biological sand filter
Constructed wetland
Organic
Phenolic
Winery wastewater

ABSTRACT

This study evaluated the removal processes involved in the removal of the phenolic component of winery wastewater in biological sand filters, sand columns and microcosms. It was found that at low influent phenolic concentrations, complete organic removal was accomplished, but at high concentrations, there was incomplete substrate removal and an accumulation of potentially toxic metabolites, including catechol. The sand provided a suitable substrate for the treatment of phenolic-laden waste, and both biotic (48%) and abiotic (52%) removal mechanisms affected the removal of model phenolics. Prior acclimation of microbial communities increased the biodegradation rate of phenolic acids significantly.

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1. Introduction

The winemaking process generates copious amounts of cellar effluent: it has been estimated that South Africa produces one billion liters and Australia 5–9 billion liters of winery wastewater per annum (Mossie et al., 2011; Sheridan et al., 2011). Winery effluent requires treatment before discharge, but remediation is complicated by the fact that the composition and volume fluctuates on a seasonal basis, depending on cellar activities (Arienzio et al., 2006; Malandra et al., 2003; Mossie et al., 2011). Typical chemical oxygen demand (COD) values of 800 to 12 800 mg/L are found during the vinification period, resulting from the presence of high concentrations of organic molecules with variable degradation rates (Malandra et al., 2003). Simple sugars, organic acids and alcohols commonly found in winery wastewater are readily biodegradable, while the phenolic component is characteristically slowly biodegradable (Serrano et al., 2010).

Plant phenolics may be toxic to microbes. It has been demonstrated that tannins, which are abundant in red wines, can inhibit microbial activity by precipitation of key metabolic proteins (Arienzio et al., 2009). The levels of phenolic compounds in winery wastewater, particularly in the effluent emanating from the production of red wine, are likely to inhibit microbial activity in soils, affecting soil and plant health (Mossie et al., 2011). It has been shown that the phenolic component of winery wastewater can adversely affect the growth of a variety of aquatic and non-aquatic plant species, including cash crops (Arienzio et al., 2009).

Small to medium-sized wineries in rural areas are often not connected to municipal reticulation systems for the treatment of winery effluent and cannot afford to operate sophisticated...
biological treatment systems (Christen et al., 2010). In these wineries, constructed wetlands (CWs) and biological sand filters (BSFs), such as the FILTER system are ideal systems for the treatment and re-use of wastewater as they have low energy and maintenance requirements, are tolerant of seasonal input fluxes and do not require lengthy start-up and shut down periods (Christen et al., 2010).

In the environment, the fate of phenolics is influenced by both biotic and abiotic factors; it is important to understand these processes in order to apply appropriate design principles to BSFs and CWs to treat phenolic-laden waste. Unlike biological wastewater treatment systems that incorporate sludge wasting, these systems can become saturated with recalcitrant organic chemicals with time. Hence, sufficient biodegradation and mineralization of a critical proportion of phenolics must occur to prevent accumulation and leaching of potentially harmful chemicals from BSFs and CWs treating cellular effluent.

There are a number of previous studies reporting the overall removal of total phenolics from agri-industrial wastewaters including winery wastewater, olive mill wastewater and coffee processing wastewater in BSFs and CWs. This study was designed not only to quantify the overall removal of common winery phenolics from cellular effluent in BSFs, but also to gain insight into the biotic and abiotic removal processes taking place during the remediation of the phenolic component of winery effluent. BSFs were used in an alternative to CWs because of the potentially phytotoxic nature of winery effluent.

2. Methods

2.1. Influent and effluent analyses

2.1.1. Chemical oxygen demand

Chemical oxygen demand (COD) was quantified using a Hanna® instruments (Smithfield, USA) C214 multiparameter bench photometer, C3800 digester and low range (1-150 mg/L; H90374A-25) and medium range (0.1-50 mg/L; H90375A-25) COD kits, following the manufacturer’s instructions.

2.1.2. Phenolics, sugars, acids and alcohols

Total phenolics were determined using the Folin–Ciocalteu (FC) micro method for total phenolics in wine, based on the method reported by Slinkard and Singleton (1977), using Folin–Ciocalteu reagent (Merck®, Whitehouse Station, USA, Cat No: 1.09001.0500), Gallic acid monohydrate (Sigma–Aldrich®, St. Louis, USA Cat No: 27645) standards were prepared in-house and results were expressed as mg/L in gallic acid equivalents (GAE) determined from a standard graph.

Individual phenolics, sugars, acids and alcohols in the effluent were identified and quantified using reverse phase HPLC: samples were separated using a Merck® Hitachi Lachrom instrument equipped with an L-7400 UV detector. For the detection of phenolics, a Waters® (Milford, USA) Shimadzu® 550DSI analytical cartridge was used with deionized water; methanol and glacial acetic acid (Merck® univar, Cat No: SAA10210200LC) (90:20:2.5) as the mobile phase. The wavelength, flow rate and time were set at 280 nm, 0.5 mL/min and 60 min, respectively. Acids and alcohols were analyzed by HPLC using a Phenomenex® Rezex RHM-monoacetylated HPAEC column according to the method described by Lazerot 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.

The theoretical COD (CO₂) values of gallic acid, vanillin (Sigma–Aldrich®, Cat No: V1104), vanillic acid (Sigma–Aldrich®, Cat No: V-2250); catechol (Sigma–Aldrich®, Cat No: C9510); and acetic acid were calculated using Eq. (1). The relationship between COD and CO₂ was established by COD measurement of triplicate phenolic solutions to give a figure, termed “measured COD” (CODₐ₉), which was used in subsequent mass balance calculations:

\[
\text{COD} = \frac{8(4x + y - 2z)}{112x + y + 16z} \text{mgCO₂/mgC₆H₅O₆} \quad (1)
\]

Gallic acid: CODₐ₉ = 1.12 mgCO₂/mg and CODₐ₉ = 1.52 ± 0.001 mgCO₂/mg;

Vanillin: CODₐ₉ = 1.79 mgCO₂/mg and CODₐ₉ = 1.81 ± 0.005 mgCO₂/mg;

Vanillic acid: CODₐ₉ = 1.52 mgCO₂/mg and CODₐ₉ = 1.51 ± 0.004 mgCO₂/mg;

Catechol: CODₐ₉ = 1.89 mgCO₂/mg and CODₐ₉ = 1.92 ± 0.007 mgCO₂/mg;

Acetic acid: CODₐ₉ = 1.07 mgCO₂/mg and CODₐ₉ = 1.07 ± 0.003 mgCO₂/mg.

2.2. Experimental set-up, design and procedure

The relationship between the three experimental phases (BSFs, columns and microcosms) is shown graphically in Fig. 1.

2.2.1. Biological sand filters

Four identical, unplanted, experimental BSFs, each consisting of river sand to a volume of ~0.5 m³, void space of 0.28 m³ and a depth of 0.3 m were inoculated in a ratio of 1:4 with sediment from a local wetland treating winery wastewater. The final BSF sediment consisted of 15% clay, 7% silt, 4% fine sand, 7% medium sand and 76% coarse sand. The elemental composition of the sediment per kilogram sand was as follows: 6 mg P/kg; 1.9 g C/kg; 0.07 cmol(+)* Na/kg; 0.05 cmol(+)* K/kg; 1.64 cmol(+)* Ca/kg; 0.21 cmol(+)* Mg/kg; 0.61 mg Cu/kg; 1.0 mg Zn/kg; 1.5 mg Mn/kg; 0.10 mg B/kg; 63.03 mg Fe/kg; 7.42 mg S/kg. The pH of the sediment was 7.7. All BSFs were maintained in an outdoor, undercover environment in order to avoid exposure to precipitation events. The systems were operated in a hybrid mode of vertical and horizontal subsurface flow i.e. effluent was sprayed uniformly at a rate of 0.68 L/min over the inlet zone and allowed to gravitate longitudinally and vertically towards the outlet. Bi-weekly irrigation, followed by gradient-directed drainage ensured that the mode of operation was biased towards classical vertical subsurface flow.

Two replicates (A and C) served as control BSFs and two replicates (B and D) served as test BSFs. All four BSFs received a bi-weekly basal influent solution consisting of 0.3 g yeast extract (Boilor®, Midbrand, RSA Cat No: HG000005.500) and 0.3 g n(-) glucose (Merck® chemically pure Cat No: 5420676028EM) dissolved in 12.5 L tap water, for the duration of the equilibration and experimental periods, the former continuing for a minimum of 16 weeks (Ramond et al., 2012). During the experimental period, BSF B was amended with winery wastewater diluted in a ratio of 1:5 (2.5 L in 12.5 L) for a period 17 weeks, while BSF D was amended with increasing concentrations of gallic acid and vanillin for 9 weeks (Table 1).

The hydraulic conductivity (HC) was determined by measuring the volume of effluent collected between 1–2 h after the start of amendment and results were expressed as L/h m⁻² m⁻¹. It had previously been established that outflow was consistent during this period (data not shown).

2.2.2. Sand columns

Without physically disrupting the sediment structure, six Perspex samplers (250 mm in length and 35 mm in diameter) were used to extract core samples (sediment mass 579 ± 11 g) from an
experimental wetland that had previously been exposed to winery wastewater (BSF B) (Fig. 1B). The open ends of three columns were sealed with duct tape after which they were gamma irradiated at a dose of 0.03 MGy at a commercial facility (Hevron Pty Ltd.). All six columns were positioned vertically with the capacity to collect effluent fractions. A phenolic “cocktail” consisting of filter-sterilized solutions of gallic acid, (+)-catechin (Sigma–Aldrich10, Cat No. C1251), ferulic acid (Sigma–Aldrich10, Cat No. W51, 830-1); and vanillin (each at 0.25 mM) was prepared. Forty milliliters of the cocktail was delivered to each column by injection on days 1, 2 and 3, with the injected volume being reduced to 20 mL per day for a further 6 days. Effluent was collected over a 24 h period for the first two days. Once the flow rate had stabilized (day 3), effluent was collected over a six hour period following injection. Sterile procedures were maintained throughout in order to prevent contamination of the irradiated columns.

The irradiated columns, served to indicate abiotic (abiotic) removal, while the non-irradiated columns served to indicate combined biotic and abiotic (biotic, and abiotic) removal of phenolics, with biotic, mechanisms accounting for the difference between the two. The removal rates were determined from total phenolic analyses of influent and effluent using the Folin–Ciocalteau assay. In order to correct for the small contribution to the assay of leached soil components, the removal efficiencies (%) of total phenolics in the non-irradiated columns (UC) and irradiated columns (IC) were calculated using Eqs. (2) and (3), respectively.

\[
\% \text{ removal } UC = \left(100 - \frac{(TP_{0}/TP_{1} \times 100)}{100/(100-(TP_{0}/TP_{1} \times 100))}\right)
\]

(2)

\[
\% \text{ removal } IC = \left(100 - \frac{(TP_{0}/TP_{1} \times 100)}{100/(100-(TP_{0}/TP_{1} \times 100))}\right)
\]

(3)

Where, \(TP_0\) mean values of total phenolics in the influent (mg GAE/L), \(TP_1\) mean values of total phenolics (mg GAE/L) in the effluent from the non-irradiated columns, \(TP_{01}\) mean values of total phenolics (mg GAE/L) in the effluent from the irradiated columns.

2.2.3. Sand microcosms

Sand samples were taken from the experimental BSF previously amended with vanillin and gallic acid (BSF D), and from the corresponding control (BSF C) (Fig. 1D). Aliquots (10 g) of sediment from each BSF were added to McCartney bottles together with 2 mL distilled water (to provide sufficient moisture for effective
autoclaving). Six such samples from each BSF 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 h (Fig. 1). Following incubation, 5 mL of a sterile phenolic solution, consisting of 6.25 mM (951 mg/L) and 1176 mg/L) 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 h at 30 °C, after which the supernatant fluid was analyzed for phenolic content by HPLC. Biotic and abiotic removal of phenolics was calculated in the same theoretical manner as for the columns, with the autoclaved samples yielding abiotic removal data. The difference in removal rates between the untreated and autoclaved replicates was used to calculate biotic removal values.

2.3 Sterility testing of autoclaved and irradiated sand

One gram of sand from irradiated, autoclaved and untreated samples taken before and after experimentation was added to 5 mL 0.85% sterile NaCl and vortexed for 5 min. Five microliters of each homogenate was plated on R2A agar and incubated aerobically for 72 h at 30 °C, after which plates were examined for colony growth.

3. Results and discussion

3.1 Removal of total phenolics from winery wastewater in biological sand filters

The first part of this study focused on the removal of organics, including total phenolics, from winery wastewater in a BSF (BSF B). Holistically, newer technologies that result in the beneficiation of agri wastewaters are most desirable from an environmental perspective (Naqvi et al., 2010). However, these systems are sophisticated, expensive and not suited to the variable nature of winery wastewater. The key focus for small to medium sized wineries, in particular, remains on the remediation of wastewater before discharge in a reliable and cost-effective manner by employing systems such as CWs or BSFs.

Using a pilot-scale BSF, the COD and total phenolic concentration of the diluted winery wastewater used for amendment was 461 ± 126 mg/L and 78.8 ± 22.6 mg GAE/L, respectively. COD and total phenolics were measured in effluent samples each week (Fig. 1A; Fig. 2A). The quantity of phenolics detected in the effluent from BSF B (2.2 ± 0.3 mg GAE/L) and the corresponding unamended control, BSF A (1.5 ± 0.17 mg GAE/L), were low and not significantly different (student t-test, p = 0.83), reflecting complete removal of influent phenolics. These low values (<3 mg/L) were assumed to result from natural leaching of phenol polymers from the sand. COD removal rates were consistently >90% (Fig. 2A) and the major contributors (8) to COD in the effluent from BSF B were ethanol (97-78%) and acetic acid (11-3%) (data not shown).

Vertical flow BSFs and CWs are operated in batch mode, consisting of alternating periods of flooding and drainage. In these systems, mass transfer of atmospheric gases into the substrate via gravitational pull during the drainage period is the primary mechanism responsible for oxygenation of the substratum (Petitjean et al., 2012). Continuous, horizontal flow systems may achieve good COD removal in the case of low COD wastewaters such as those produced by the aquaculture industry (Shi et al., 2011). However, because the degradation of organic molecules is enhanced under aerobic conditions, higher COD removal rates are expected in batch-operated systems (Petitjean et al., 2012; Saeed and Sun, 2011). Thus, vertical flow systems are recommended for the treatment of winery wastewater with inherently high concentrations of organics.

At this point of the study, the basic phenolic removal mechanisms were indeterminate. If the primary mechanism for the removal of phenolics in BSFs is sorption onto substratum particles, eventual saturation of binding sites will necessitate the implementation of rehabilitative measures to prevent leaching of phenolics into the treated effluent. To establish the relative abiotic/biotic removal capacities of the substrate (sand) and the microbial population, respectively in BSF B, core samples were extracted and column experiments were set-up (Fig. 1B): a phenolic “cocktail”, containing common winery phenolics was allowed to percolate through three irradiated and three non-irradiated columns for nine days and the total phenolic removal rates were calculated on a daily basis. The removal rates in the irradiated and non-irradiated columns were attributed to abiotic, and combined biotic-abiotic mechanisms, respectively, with biotic mechanisms accounting for the difference between the two.

Results obtained between days 3 and 5 of the experiment indicated that both abiotic and biotic factors were proportionately involved in the removal of phenolics (Fig. 3). During this experiment, the permeate flow from the columns stabilized after 2 days and the collection period was decreased from 24 h to 6 h to limit degradation of the phenolics in the collection vessels. From days 5 to 9, there was a decrease in the abiotic removal fraction, and the phenolic removal rates calculated for the replicates were no longer consistent (Fig. 3). No growth was observed on sterility plates from soil samples taken from irradiated columns before experimentation. However, growth was obtained on the plates from samples taken at the end of the experiment, indicating that either (i) the columns were contaminated during the experiment with an exogenous microbial population capable of actively degrading phenolics, or (ii) dormant microbes in the irradiated columns resumed growth, despite the fact that the gamma irradiation dose applied
(0.03 M Gy) is one recommended for sterilization of soil samples (Wool and Skipper, 1994). The resurgence of microbial growth in the irradiated samples can explain the temporal increase in the biotic removal fraction seen after day 5 (Fig. 3).

In soil environments, the sorption of organics usually corresponds with the amount of soil organic matter (SOM), especially humic acids, kerogen and black carbons (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 (Tharayil et al., 2006). Abiotic chemical transformation of phenolics can also occur via the oxidation of phenolic acids coupled to iron and/or manganese reduction (Polubeshova et al., 2010).

Due to the low carbon (1.9 mg/g), low clay (1%), high Fe (0.3 mg/g) and Mn (1.9 mg/g) content in the BF sand, it is likely that metal complexation and/or chemical transformation played a major role in the abiotic removal of phenolics in the column, and by inference, the ISFs.

3.3. Removal of phenolics from synthetic wastewater in a biological sand filter

To understand the primary metabolic processes taking place during the biodegradation of phenolics in ISFs and the effect of phenolic loading on these metabolic processes, synthetic wastewater containing common wineyard phenolics, was introduced to BSF D in incrementally increasing concentrations and the effluent was collected and analyzed (Fig. 1C; Table 1).

3.3.1. Determination of COD removal efficiency at increasing organic loading rates

During amendment with a low concentration of synthetic wastewater (Table 1), the concentrations of total phenolics measured in the effluent from BSF D (1.4 ± 0.5 mg GAE/L) were similar to those measured in the effluent from BSF E (wineyard wastewater amended) and the control ISFs (BSF A and BSF C). Thus, with both wineyard wastewater and synthetic wastewater, complete removal of phenolics was achieved. Amendment with moderate concentrations of synthetic wastewater (1.176 mg/L COD$_{eq}$) generated higher effluent phenolic concentrations of 13.8 ± 7.3 mg GAE/L, but the effluent COD remained <100 mg/L, with >90% COD removal efficiency (Fig. 2B, Fig. 4). During amendment with high concentrations of synthetic wastewater (5.842 mg/L COD$_{eq}$), COD removal efficiency remained high (>98%), but the effluent COD concentrations reached values close to 700 mg/L (Fig. 4). At this stage, the major contributors (%) to COD were phenolics, with total phenolic concentrations of 480 ± 22 mg GAE/L being measured in effluent samples (Fig. 2B).

3.3.2. Microbial degradation of model phenolics in a biological sand filter

To gain insight into the microbial utilization and degradation of the model winery phenolics, vanillin and gallic acid, the effluent from BSF D was analyzed using HPLC. Vanillin and its oxidized product, vanillic acid, are major components of humus and many soil microorganisms have the ability to degrade these molecules (Scotto et al., 2000). The characteristic vanillin degradation pathway is initiated by the oxidation of vanillin to vanillic acid, which is catalyzed by vanillic dehydrogenase. The microbial degradation of gallic acid to pyrogallol takes place either aerobically (by oxidation) or anaerobically (by decarboxylation) (Bhat et al., 1998).

No identifiable chemical entities were detected in the effluent during amendment with a low concentration of synthetic influent (weeks 1–3) (Table 1). During amendment with a moderate concentration of synthetic influent (weeks 4–6), small amounts of vanillic acid (range: 1.7–6.0 mg/L), gallic acid (range: 0.8–1.0 mg/L) and the microbial metabolite, catechol were detected, the latter displaying an increasing trend (from 3.5 to 15.0 mg/L). A well-described microbial degradation pathway for the formation of catechol is via decarboxylation of protocatechuic acid, which is a product derived from the demethylation of vanillic acid (Fenner et al., 2005). Effluent analysis showed that during BSF D amendment with a high concentration (1.176 mg/L COD$_{eq}$) of model phenolics: (i) vanillin, and the metabolites, acetate and lactate were detected in the effluent for the first time. The lactate peak was not sufficiently resolved to perform accurate quantification and the identity of some small peaks remained indeterminate as they did not correspond to any of the standards employed. Nonetheless, much of the organic fraction was accounted for: the COD contribution from unidentified entities ranged from 35% at week 7.5 to only 2% at week 9.5 (Fig. 5B). High concentrations of the metabolites, vanillic acid (range: 104.2 to 157.0 mg/L), catechol (range: 36.4 to 141.1 mg/L) and acetate (range: 32.5 to 152.6 mg/L) were measured in the effluent. These results indicate that although microbial degradation was taking place, the biodegradative capacity of the BSF microbial community was exceeded, (ii) substantially higher concentrations of vanillin (<39 mg/L) than gallic acid (<17.3 mg/L) were measured in the effluent, indicating preferential
3.3.3. Accumulation of metabolites at high influent concentrations of model winery phenolics

During amendment of BSF D with a high concentration of synthetic wastewater (Fig. 1C), there was a temporal increase in the contribution of the metabolites catechol and acetate, both in terms of concentration and contribution to overall COD, accounting for 40% and 22% of the effluent COD, respectively, by the end of the experiment (Fig. 5B).

Vertical flow CWs are operated in "fill and drain" cycles, with atmospheric gases being drawn into the substratum during drainage. In this study, the BSFs were operated in batch mode, with the predominant flow being vertical. 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 BSF layers, but not in the deeper, more anaerobic/anoxic layers (Bugg, 2003). Indeed, anaerobiosis caused by waterlogging has been shown to decrease the diversity of bacteria carrying the XyE 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 unfavorable, 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 BSF D emanated from the accumulation of catechol in the deeper, anaerobic layers of the substratum (Fig. 5).

Under favorable conditions, acetate is readily biodegradable but it can accumulate in the environment when alternate electron acceptors such as oxygen and nitrates are unavailable (Lasko et al., 1997; van Sierpontwort et al., 2009; Whalen, 2005). 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 BSF D.

3.3.4. Toxic effects of catechol on microbial metabolism leading to loss of biofilm/biomass and hydraulic conductivity

In high concentrations, catechol can be highly toxic to microorganisms, often exhibiting greater levels of toxicity than "parent" phenolics, including benzene (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).

However, catechol may also function as a bacterial growth substrate, depending on the concentration of catechol and the organism(s) involved (Alexiou et al., 2008; Chen et al., 2009; Park et al., 2001). Chen et al. (2009) found that in soil, low concentrations of catechol (0-400 μg/g) promoted microbial growth, while higher concentrations inhibited growth, with 60% inhibition being demonstrated at 3000 μg/g.

The high concentrations of catechol (36–4.144 μM) measured in the effluent from BSF D from weeks 7–9 provide 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 (from 1.6 to 0.20 L/h/m²/s) there was an increased trend (from 0.2 to 1.1 L/h/m²/s) which corresponded to an increasing catechol concentration in the effluent. In CWs, hydraulic conductivity may be retarded by entrapment of solids, the growth of vegetation and/or the formation of biofilm/biomass (Knowles et al., 2010). Since the experimental BSF could be considered to be an unplanted CW, the decreased conductivity could only have originated from the adsorption/accumulation of polymerized forms of phenolics and/or an increase in biomass/biofilm. The fact that the hydraulic conductivity trend reversed when the phenolic components were increased after week 7 discounts the former option, as adsorption and polymerization of phenolics would be expected to increase, not decrease at higher influent phenolic concentrations.

It has previously been shown that there is a rapid loss of hydraulic conductivity stemming from an increase in heterotrophic biomass/biofilm after the application of organic wastewaters, including winery wastewater, to soil (Christen et al., 2010; Knowles et al., 2010; Welz et al., 2011). It is thus proposed that during this study, low to moderate 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 levels, accumulation of catechol in inhibitory concentrations resulted in loss of biomass/biofilm with a consequent increase in hydraulic conductivity.
3.4. Acclimation of microbial communities exposed to vanillin and gallic acid: microcosm studies

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 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 order to determine whether the microbial communities in CW-D had become acclimated to vanillin and gallic acid, microcosm studies were undertaken (Fig. 1D). The microcosms contained (i) sediment taken either from BSF D (acclimated by exposure to vanillin and gallic acid) or BSF C (unacclimated control) and (ii) a solution of vanillin and gallic acid. The biotic, and abiotic, removal fractions were calculated in the same theoretical manner as in the column experiments: the removal rates in the autoclaved and non-autoclaved columns were attributed to abiotic, and combined biotic-abiotic mechanisms, respectively, with biotic mechanisms accounting for the difference between the two.

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 removal of gallic acid and vanillin was respectively 6% and 12% higher in the microcosms containing acclimated sediment than in the control microcosms. Biotic formation of vanillic acid was also higher in the acclimated microcosms (441 mg/L) than the control microcosms (53.7 mg/L), with the concentration in both the autoclaved and non-autoclaved acclimated microcosms (100.3 ± 20.4 mg/L and 14.5 ± 15.8 mg/L, respectively). 

The biotic, cateloh, was only detected in the non-stere, acclimated microcosms. In addition, the difference in phenolic concentrations between the autoclaved and non-auto-
claved microcosms was statistically more significant (t-test) in the acclimated microcosms (gallic acid: p = 0.0229 and vanillin: p = 0.0698) than in the control microcosms (gallic acid p = 0.0048 and vanillin p = 0.0318).

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 cateloh in the non-acclimated microcosms containing acclimated sediment, indicating the presence of bioavailable phenolics in the BSF sediment remaining from previous amendment with synthetic wastewater (data not shown). Pet and post-experimentation soil sterility testing proved that in contrast to the soil columns, the microcosms were not susceptible to contamination. It was clearly demonstrated that a period of nine weeks was sufficient for the microbial population in a BSF to acclimate to the presence of gallic acid and vanillin, resulting in significantly en-
hanced biodegradation rates when compared to a non-acclimated population. These results support 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 protoning, whereby wastewater is applied in incrementally increasing concentrations during the start-up phase, could enhance long-term phenolic removal performance in BSFs (Welz et al., 2011).


