# TREATMENT OF WINERY WASTEWATER IN UNPLANTED CONSTRUCTED WETLANDS

Report to the
**WATER RESEARCH COMMISSION**

by

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# EXECUTIVE SUMMARY

## Introduction

Building on experience from a previous WRC-funded project (Project K5/1936)** which focussed on the treatment of winery wastewater in constructed wetlands, Project K5/2104 commenced in 2011. The research team consisted of a multidisciplinary group with collective expertise in biological wastewater treatment, environmental science, civil engineering, biochemistry, molecular biology, and microbial ecology and metagenomics.

The research approach was both fundamental and applied, and has culminated in increased knowledge on the subject. This information has been applied to the design of a wastewater treatment system which is presently being set-up for "real world" testing at a local winery.

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## Project aims

The original aims of the project, summarized from the proposal, were:

1. To exploit knowledge generated from a previous WRC-funded project (K5/1936) to understand how constructed wetlands may be adapted for "real world applications"
2. To utilize microbial community fingerprinting techniques to define which natural process parameters allow effective treatment of wastewater in constructed wetlands
3. To identify a matrix of parameters through which the capacity of constructed wetlands can be measured, and relate these to the characteristics of "real" wastewaters
4. To investigate the reproducibility of constructed wetlands "adapted" for specific wastewaters by characterizing the microbial communities in varied environments impacted by wastewaters
5. To develop an understanding of the stability of microbial communities "adapted" to specific wastewaters under the variable conditions imposed in "real world" situations
6. To understand the extent to which microbial communities in constructed wetlands can accommodate changes in waste impacts and the rate at which they can "adapt"
7. To understand the flexibility of microbial communities to "adapt" to changes in the wastewater environment in a given constructed wetland

8. The original aims set out in the proposal were refined during the course of the project, both in response to experimental outcomes and advice from members of the reference group. For examples, at the first reference group meeting in 2011, members advised that the research group should continue to focus on winery effluent as the model wastewater, as there was already expertise in this field within the research group. It was also decided to exclude plants from the systems because of the cumulative phytotoxic nature of the phenolics and salts found in winery wastewater

***By definition, constructed wetlands contain plants. This strict definition is debateable because many natural wetlands do not contain plants. Nevertheless, to avoid confusion, the systems used in the project are referred to as biological sand filters.***

## Project summary

This report is presented in the form of eight chapters, of varying extents, but each detailing particular elements explored during the course of Project K5/2104. The first chapter provides background information. A brief synopsis of the rationale and outcomes of the remaining seven chapters is given in this executive summary.

### Summary Chapter 2: Bacterial community structure & function: Reproducibility between two groups of biological sand filter replicates exposed to acid mine drainage

#### Rationale

Studies conducted during Project K5/1936 did not make use of BSF replicates. Instead, they relied on the results from non-replicated test and control BSF systems. This led to questions being raised about the statistical validity of results. To ascertain the need to replicate (or not), it was decided to conduct a short-term study to assess the reproducibility of physicochemical and microbiological results between 'identical' biological sand filter replicates.

Four biological sand filters (one control and three experimental replicates), containing one batch of Malmesbury sand, and two biological sand filters (experimental replicates) containing a second batch of Malmesbury sand, were used in the study. The experimental replicates were amended with synthetic acid mine drainage for three weeks. The choice of wastewater was not in line with previous or future work, but was selected for this study

because, according to literature, it was expected to have a large selective impact on the microbial community structure.

### Outcomes

The two batches of sand exhibited highly similar physicochemical make-up and results from replicates containing the same batch of sand displayed the same trends. However, there were significant differences in the effluent chemistry and bacterial community structures in the replicates containing different batches of sand. Surprisingly, and contrary to literature findings, the sand batch had a greater influence over the bacterial community structure and function than the presence of acid mine drainage. Results showed that iron reduction dominated in biological sand filters containing the first batch of sand, while sulphate reduction dominated in the biological sand filters containing the second batch of sand. In the former, the presence of acid mine drainage was highly selective for the iron-reducing Gram positive bacillus *Clostridium beijerinkii,* which comprised up to 65% of the operational taxonomic units.

- *Reproducibility between replicates containing the same batch of sand was good, but the magnitude of differences were enough to warrant the use of duplicate systems for future studies*

## Summary Chapter 3: Analysis of locally available sand types for use in biological sand filters

### Rationale

It is imperative that biological sand filters are filled with a substrate that is inexpensive and readily available on a long-term basis. Therefore, the performance of biological sand filters containing different sand types that are locally available in the Cape Town environs was compared. Between 2009 and 2011, four existing biological sand filters contained Malmesbury sand. Excellent remediation of synthetic wastewater was achieved in these systems, but the hydraulic conductivity was extremely low. It became apparent that the theoretical size of biological sand filters containing Malmesbury sand would be too large for 'real world' applications.

In response to these findings, in 2012, the sand in the biological sand filters was changed, and four new systems were set-up, to give a total of eight replicates. Of the eight, five systems were filled with Phillipi sand and three with a mixture of Phillipi and Malmesbury

sand, referred to as Mix sand. This mix is available in a pre-determined mix ratio directly from the quarry. The sands were well characterised in terms of physicochemical attributes. In addition, column experiments were performed in order to calculate the intrinsic hydraulic conductivity of each sand type, and to assess the effect of biomass clogging on this value.

### Outcomes

The chemical composition and mechanical fractions of Phillipi, Malmesbury and Mix sand were similar. However, there were highly significant differences in the hydraulic conductivity of each sand type, which was most likely attributable to grain geometry.

The hydraulic conductivity of Phillipi and Mix sand fell well within the range suggested for use in slow sand filters, even after induced biomass clogging. Although differences were noted in the microbial community structures in biological sand filters containing Phillipi or Mix sand, the performance was shown to be similar when treating synthetic winery wastewater. Phillipi sand exhibited the highest hydraulic conductivity, thereby imparting potentially greater flexibility on systems filled with this sand. It was also least affected by biomass clogging.

- *Phillipi sand is a good candidate for use in biological sand filters treating winery wastewater from small cellars in the Cape Town environs*

## Summary Chapter 4: Treatment of winery wastewater in biological sand filters: analysis of organic degradation and microbial community structure and function

### Rationale

The organic composition of winery wastewater is directly related to cellar activities, including must production, fermentation processes, maturation/stabilization processes, and decanting, and is thus prone to seasonal variation. Each grape varietal also has a unique organic fingerprint, so that, for example, wastewater generated during crushing of late harvest grape varietals has a comparatively high sugar and low phenolic content, while the converse is true for wastewater generated during the crushing of early harvest red grape varietals.

In many countries, including South Africa, effluent chemical oxygen demand is used as a benchmark by regulatory authorities when determining wastewater discharge requirements for biodegradable industrial wastewater, and in most instances, no further characterization of the organic fraction is required. However, in terms of biodegradability, there is

considerable variation in the ratios of the different fractions of winery effluent, including readily biodegradable sugars, moderately biodegradable alcohols and slowly biodegradable/recalcitrant phenolics.

This study was conducted to gain a holistic understanding of biological organic winery wastewater treatment processes, particularly concerning the degradation and formation of different substrates and metabolites, respectively. Biological sand filters were amended with two different formulations of synthetic winery wastewater and results compared. The primary aim of the study was to determine how differences in wastewater composition would affect the metabolic processes of the functional microbial consortia. This was achieved by (i) chemically characterizing pore water samples taken at different spatial points in the systems, and (ii) determining the similarities in the microbial community structures in the same spatial niches before and after amendment with two different formulations of synthetic winery wastewater.

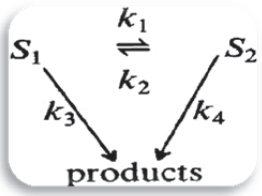
### Outcomes

During the treatment of winery effluent in biological sand filters the redox status played a vital role in the selection of the bacterial consortia, with different communities being found in the deep and superficial niches. The spatial niche played a greater role on bacterial community selection than the presence of different formulations of synthetic winery effluent. The accumulation and biodegradation of organic substrates and metabolites was therefore strongly influenced by redox status.

Some organics, including glucose, ethanol and phenolics, were degraded preferentially in the deep niches when compared to the superficial niches. However, accumulation of short-chain volatile fatty acids was favoured in the deep niches. Irrespective of whether acetate was present in the synthetic winery wastewater or not, it was invariably the highest contributor to effluent chemical oxygen demand. The presence of a readily biodegradable substrate (glucose), led to a decrease in the biodegradation of slowly biodegradable substrate (phenolics) in the superficial niches, and vice versa. Lactate was only formed in detectable amounts when glucose was present in the synthetic winery wastewater.

- *To optimize organic removal, including removal of acetate, it is recommended that winery wastewater is subjected to alternating redox environments. A polishing aeration step using gravity fed trickling filters provides a simple, low cost, low energy example of how this may be achieved (Chapter 6)*

## Summary Chapter 5: Biodegradation kinetics and correlation with physicochemical parameters in biological sand filters treating winery wastewater




### Rationale

To ensure the successful operational design of full-scale biological sand filters, an understanding of the rates of biodegradation, biotransformation and mineralization of the various fractions of winery effluent is required.

To achieve these aims, it is important to determine spatio-temporal and physicochemical associations with long term biodegradative performance and the degradation kinetics of different fractions of winery effluent. This study served to introduce and discuss these complex interactions. To this end, biological sand filter replicates were amended with different concentrations of a complex synthetic winery wastewater.

In order to establish (i) the effect of hydraulic retention time on system performance, (ii) temporal performance, and (iii) the degradation kinetics, three different, concurrent strategies were used: The systems were amended, then plugged for 24 hr (three times weekly), 48 hr (twice weekly), and for a protracted period (226 hr). Pore water samples from four environmental niches were periodically extracted for analysis.

### Outcomes

Long term, there was an improvement in removal efficiency, attributed to acclimation of the microbial communities to the synthetic winery wastewater. Approximately 75% COD removal of winery wastewater with an influent concentration of 2027 mg COD/L took place within the first 24 hr. The removal rate was five times higher than during the subsequent 24 hr. The highest degradation rates were measured during the first 2 hr after amendment and most of the residual organic fraction was attributable to acetate.

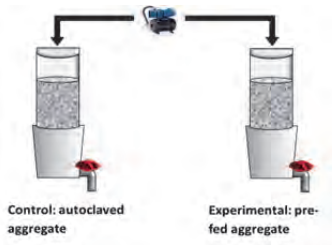
Substrate concentration gradients were found from inlet to outlet. This, together with the results of tracer studies, reflected a degree of plug flow of influent: (i) the highest COD concentrations were measured in pore water taken from the deep inlet, and the lowest in samples taken from the deep outlet; (ii) although COD concentrations and degradation kinetics were similar in pore water samples from the superficial inlets and outlets, higher concentrations and types of substrates were found at the inlets, while higher concentrations and types of metabolites were found at the outlets.

As described in Chapter 4, ethanol and phenolics were degraded preferentially in the deep niches. However, in previous experiments using ethanol as the sole influent organic, it was degraded preferentially in the surface niches. It is clear that the complexity of wastewater

has an influence on the functional microbial communities and predictions made using singular 'pollutants' cannot necessarily be extrapolated further. Although the removal of phenolics was significantly enhanced in the deep niches, removal continued in the superficial niches, so that by 336 hr after amendment, total phenolic concentrations were similar in the superficial and deep niches.

- *Degradation gradients caused by plug flow of influent ensure that the most 'treated' wastewater exits the biological sand filters first. If a polishing step to remove acetate is incorporated (Chapter 6), a nominal hydraulic retention time of 2 hr may be sufficient.*

## Summary Chapter 6: Trickling filters as a polishing step to remove residual acetate from wastewater treated in biological sand filters




### Rationale

During the course of Project K5/2104, it was demonstrated that excellent removal of sugars, ethanol and phenolics can be achieved in biological sand filters, but that the removal of volatile fatty acids, particularly acetate, is poor. Even when not present in the influent, these may be formed from other organic substrates and accumulate in biological sand filters, particularly in the reduced redox environments encountered in the deep niches (Chapters 4 and 5). It was hypothesised that acetate removal could be enhanced by utilising strategies to increase the redox potential.

Initially, the use of active intermittent aeration in-situ was considered. However, three factors led the team to re-consider: (i) In-situ aeration may negatively affect degradation of organics where the degradation kinetics favour lower redox conditions, (ii) There is competition for substrate within the systems and acetate is a central metabolite which is both formed and utilised during autotrophic and heterotrophic metabolism, and (iii) There is an energy requirement attached to active aeration. A low energy, downstream process

would therefore be more suitable. A 'proof of concept' laboratory scale trickling filter experiment was therefore set up to determine the feasibility of using such a system as a polishing step for the removal of acetate from winery wastewater treated in biological sand filters.

### Outcomes

In this experiment, over 96% of removal of acetate was achieved. It is highly likely that the major removal mechanism was volatilisation. However, there was also a significant improvement in biotic removal after 6 weeks of operation, demonstrating that both biotic and abiotic mechanisms played a functional role.

- *The results of this investigation showed that trickling filters may indeed present a feasible option to 'polish' wastewater containing short chain volatile fatty acids. Currently, experimental columns have been filled with either aggregate, clay balls or open cell polyurethane foam, in an effort to determine which of these substrates would be best for acetate removal.*

## Summary Chapter 7: Response of biological sand filters to shut-down and toxic events

### Rationale

The character and volume of winery wastewater is seasonal and differs from winery to winery. This is discussed extensively in Chapters 3 and 4 of this report. In reality, there is almost always a supply of effluent from most wineries, but it may be extremely dilute during periods when there is minimal cellar activity.

Conventional wastewater treatment systems used for the treatment of winery effluent require lengthy start-up periods to re-acclimate microbial populations during peak seasons; they are also vulnerable to changing influent character and toxic events. This study was performed to determine the effects of shut down and toxic events on the performance of biological sand filters treating synthetic winery effluent.

Biological sand filter replicates were shut down for periods of 2 months or 8 months and replicates were then either subjected to high concentrations of hypochlorite or basal nutrient solution for two weeks. All systems where then amended as previously with synthetic winery wastewater and COD removal results compared.

### Outcomes

Overall, the results showed that resting, and the duration of resting did not adversely affect system performance and in fact, enhanced system functionality. This demonstrated that systems with sufficient capacity for peak season flows are capable of coping with variable loads.

The results of this study are most encouraging, as upsets in conventional suspended and fixed growth biological wastewater treatment systems can take weeks to rectify. Microbial communities in soil are adapted to different moisture and nutritional conditions and many possess mechanisms to survive dry spells and/or varying nutritional supplies (e.g. the ability to sporulate). The sand substrate closely mimics a natural environment. This factor may have contributed to the resilience of the systems to desiccation (resting), lack of nutrients (resting), and toxicity (chlorination).

- *Biological sand filters are promising systems for the treatment of wastewater with variable qualitative and quantitative flows*

## Summary Chapter 8: Analyses of bacterial community structure and chemical analyses in sites impacted by winery wastewater

### Rationale

This study was conducted in order to gain insight into the impact of winery wastewater, location and seasonality on benthic bacterial community structures. Three sampling locations were identified: two winery wastewater irrigation storage dams of similar volumetric capacity, and one farm dam receiving waste from a nearby cellar. One of the storage dams was shallow, with a large surface area, while the other was deep with a relatively small surface area. The primary aims were to determine: (i) Whether there were inter-environmental similarities in the microbial communities impacted with winery wastewater, (ii) The impact of seasonality (before/after crush season) on the bacterial communities, and (iii) The primary chemical composition of the winery wastewater impacting the chosen sites.

Sediment samples were taken for microbial community fingerprinting from environments impacted with winery wastewater, and where possible, nearby control sites that had not

been impacted. Wastewater samples were collected from the same sites for chemical analysis. Samples were taken both before and after the crush season.

### Outcomes

Although the composition of the wastewater in the irrigation storage dams was similar during the crush season, there was significantly greater degradation of organics (including phenolics) in the irrigation storage dam with a larger surface area. This suggested that wind mixing and/or photodegradation played a pivotal role in the remediation process. Winery wastewater in high concentrations had a significant impact on the benthic bacterial communities, while low concentration wastewater did not. Seasonal bacterial community shifts were similar in magnitude to the impact of winery wastewater. In addition, site specific differences, not related to the concentration of winery wastewater, were apparent.

## Capacity building

One Biotechnology Work Integrated Learning (WIL) student was appointed to work on the project and successfully completed her mini-dissertation in 2013. This student has continued with research and will be commencing with MTech studies in 2015.

One MSc (Biotechnology) student and one MTech (Civil Engineering) student were appointed to work on the project. Both of these candidates have completed the practical aspects of their work and are currently engaged in writing their respective theses.

The project leader obtained a PhD, using work performed during the course of the project as material for her thesis. An NRF intern, a post-doctoral research fellow and experienced advisors provided invaluable additions to the project.

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# TABLE OF CONTENTS

# LIST OF FIGURES

# LIST OF TABLES

# LIST OF CONFERENCES AND PAPERS

## Publications and conference proceedings

1. Welz PJ, Ramond, J-B., Palmer, Z., Isaacs, S., Kirby, B., Le Roes-Hill, M. (2014) The effect of redox status on the bacterial community structure and biodegradation rate of organic fractions of agri-industrial effluent in sand bioreactors. *Proceedings of the 9th IWA International Symposium on Waste Management Problems in Agro Industries, Volume II.*
2. Welz, P.J., Palmer, Z., Isaacs, S., Kirby, B., Le Roes-Hill, M. (2014) Analysis of substrate degradation, metabolite formation and microbial community responses in sand bioreactors: a comparative study. *Journal of Environmental Management* 145: 147-156.
3. Welz, P.J., Le-Roes, M. (2014) Biodegradation of organics and accumulation of metabolites in experimental biological sand filters used for the treatment of synthetic winery wastewater: a mesocosm study. *Journal of Water Process Engineering* 3C: 155-163.
4. Welz, P.J., Ramond, J-B., le Roes-Hill, M., Burton, S.G., Cowan, D.A. (2014) Minor differences in sand physicochemistry lead to major differences in bacterial community structure and function after exposure to synthetic acid mine drainage. *Biotechnology and Bioprocess Engineering* 19: 211-220.
5. Ramond, J-B., Welz, P.J., le Roes-Hill, M., Tuffin, M., Burton, S.G., Cowan, D.A. (2014) Selection of Clostridium sp. in biological sand filters neutralizing acid mine drainage. *FEMS Microbial Ecology* 87: 678-690.
6. Ramond J-B., Welz P.J., Tuffin, M., Burton S.G., Cowan D.A. (2013) Selection of diazotrophic bacterial communities in biological sand filter mesocosms used for the treatment of phenolic-laden wastewater. *Microbial Ecology* 66: 563-570.
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8. Welz P.J., Ramond J-B., Cowan D.A., Burton S.G. (2012) Phenolic removal processes in biological sand filters used to remediate winery wastewater. *Bioresource Technology* 119: 262-269.
9. Rodriguez Caballaro, A., Ramond, J.B., Welz P.J. (2012) Treatment of high ethanol concentration wastewater by constructed wetlands: enhanced COD removal and bacterial community dynamics. *Journal of Environmental Management* 109: 54-60.
10. Ramond J-B., Welz P.J., Cowan D.A., Burton S.G. (2012) Microbial community structure stability, a key parameter in monitoring the development of constructed wetland mesocosms during start-up. *Research in Microbiology* 163: 28-35.
11. Welz, P.J., Ramond, J-B., le-Roes-Hill, M., Prins, A., Cowan, D.A., Burton, S.G. (2012) Treatment of winery wastewater and synthetic wastewater in pilot-scale vertical subsurface flow constructed wetlands: removal of slowly biodegradable and readily biodegradable COD (*WISA 2012*).
12. Ramond, J-B., Welz, P.J., Burton, S.G., Tuffin, M. and Cowan, D.A. (2012) Constructed wetlands as a bioremediatory process to treat organic-contaminated effluent: impact on and roles of microbial communities (*WISA 2012*).

13. Rodriguez Caballaro, A., Ramond, J.B., Welz P.J., Cowan, D.A., Odlare, M., Burton, S.G. (2011) Treatment of high ethanol concentration wastewater by constructed wetlands: enhanced COD removal and bacterial community dynamics: *Proceedings of the IWA Microbes in wastewater and waste treatment conference.*

## Oral conference presentations

**International**

**1.** Welz PJ, Ramond, J-B., Palmer, Z., Isaacs, S., Kirby, B., Le Roes-Hill, M. (2014) The effect of redox status on the bacterial community structure and biodegradation rate of organic fractions of agri-industrial effluent in sand bioreactors. *9th IWA International Symposium on Waste Management Problems in Agro Industries*: Kochi, Japan 2014)
2. Welz P.J., Ramond J-B., Prins A., le Roes-Hill, M., Cowan, D.A., Burton, S.G. Treatment of winery wastewater and synthetic wastewater in pilot-scale constructed wetlands: removal of slowly biodegradable and readily biodegradable COD. *13th IWA International congress on wetland systems for pollution control*: Perth Australia 2012)
3. Rodriguez Caballaro, A., Ramond, J.B., Welz P.J., Cowan, D.A., Odlare, M., Burton S.G. Treatment of high ethanol concentration wastewater by constructed wetlands: enhanced COD removal and bacterial community dynamics. *IWA Microbes in wastewater and waste treatment conference*: Goa, India 2011)

**National**

1. Palmer, Z., Le Roes-Hill, M., Isaacs, S., Kirby, B., Welz, P.J. Response of microbial communities to synthetic winery wastewater in sand bioreactors (SASEV, Somerset West, 2014).
2. Welz, P.J., Ramond, J-B., Cowan, D.A., Burton, S.G., le Roes-Hill, M. Minor differences in sand physicochemistry lead to major differences in bacterial community structure and function after exposure to synthetic acid mine drainage (SASM, Warmbaths 2013) ****Plenary presentation****
3. Welz P.J., Palmer, Z., Isaacs, S., Smith, I., Haldenwang, R., Kirby, B., le-Roes Hill, M. Treatment of winery wastewater and synthetic wastewater in biological sand filters (CPUT Water research day, Cape Town 2013)
4. Welz, P.J., Ramond, J-B., le-Roes-Hill, M., Prins, A., Cowan, D.A., Burton, S.G. Treatment of winery wastewater and synthetic wastewater in pilot-scale vertical subsurface flow constructed wetlands: removal of slowly biodegradable and readily biodegradable COD (WISA, Cape Town 2012)
5. Ramond, J-B., Welz, P.J., Burton, S.G., Tuffin, M. and Cowan, D.A. Constructed wetlands as a bioremediatory process to treat organic-contaminated effluent: impact on and roles of microbial communities (WISA, Cape Town 2012)
6. Ramond, J-B., Welz, P.J., Burton, S.G., Tuffin, M. and Cowan, D.A. Constructed wetlands as a bioremediatory process to treat organic-contaminated effluent: impact on and roles of microbial communities (SASM, Cape Town 2011)

## Poster presentations

**International**

1. Ramond, J.B., Welz, P.J., Cowan, D.A., Burton, S.G., Tuffin, M.I. Selection of highly specialized microbial communities in biological sand filters treating acid mine drainage (ISME, Copenhagen, Denmark, 2012).

**National**

1. Smith, I., Haldenwang, R., Terblanche, G., Weels, S., Le Roes-Hill, M., Welz, P.J. Hydraulic properties of different sand types used in the design of sand bioreactors for the treatment of winery wastewater (SASEV, Somerset West, 2014).
2. Weels, S., Terblanche, G., Le Roes-Hill, M., Welz, P.J. Comparison of two local sand type for the bioremediation of synthetic winery effluent (SASEV, Somerset West, 2014)
3. Terblanche, G., Welz, P.J, le Roes-Hill, M. Biochemical analysis of effluent generated from sand columns amended with synthetic winery wastewater (SASM, Warmbaths, 2013)
4. Isaacs, S., Welz, P.J., le Roes-Hill, M. The effect of micronutrients/metal(loids) on the microbial community structure in sand mesocosms (SASM, Warmbaths, 2013)
5. Welz, P.J., Ramond, J-B., Le Roes Hill, M., Prins, A., Cowan, D.A., Burton, S.G. Removal of slowly biodegradable and readily biodegradable organics in pilot-scale constructed wetlands (SASBMB, Drakensburg 2012)
6. Welz P.J. Ramond J-B., le-Roes-Hill M., Cowan D.A., Burton S.G. Acclimation of microbial communities to gallic acid and vanillin in constructed wetlands (SASM, Cape Town 2011)

## Technical reports

1. **Welz, P.J**., Haldenwang, R., Ramond, J-B., le Roes-Hill, M. (**2013**) Winery wastewater treatment – keeping it cheap and simple. *Wynboer* 10: 122-124.

# LIST OF ABBREVIATIONS

| | |
|---|---|
| AMD | Acid mine drainage |
| ANOSIM | Analysis of similarity |
| BSF | Biological sand filter |
| BSF1, BSF2 | Experimental biological sand filter replicates no 1 and 2 |
| BSFC | Control biological sand filter |
| CEC | Cation exchange capacity |
| COD | Chemical oxygen demand |
| CW | Constructed wetland |
| DNA | Deoxyribonucleic acid |
| Eh | Redox potential |
| HC | Hydraulic conductivity |
| HLR | Hydraulic loading rate |
| HPLC | High pressure liquid chromatography |
| HRT | Hydraulic retention time |
| HSF | Horizontal surface flow |
| HSSF | Horizontal sub-surface flow |
| ISD | Irrigation storage dam |
| ICP-OES | Inductively coupled plasma optical emission spectrometry |
| NMDS | Non-metric multi-dimensional scaling |
| NRF | National Research Foundation |
| OLR | Organic loading rate |
| PCR | Polymerase chain reaction |
| RSD | Rainwater storage dam |
| sAMD | Synthetic acid mine drainage |
| SHC | System hydraulic conductivity |
| SRB | Sulphate reducing bacteria |
| SW | Synthetic winery wastewater |
| SW1, SW2 | Synthetic winery wastewater formulation 1 and 2 |
| TF | Trickling filter |
| tHRT | Theoretical hydraulic retention time |

| | |
|---|---|
| T-RFLP | Terminal restriction fragment length polymorphism |
| VFA | Volatile fatty acid |
| VSSF | Vertical sub-surface flow |
| WIL | Work integrated learning |
| XRD | X-ray diffraction |
| XRF | X-ray fluorescence |

# GLOSSARY

**Acclimation:** The process by which organisms adapt to a change in the environment

**Amendment**: Application of synthetic wastewater

**Chemical oxygen demand**: A measure of the capacity of water to consume oxygen during the decomposition of organic matter and the oxidation of inorganic chemicals such as ammonia and nitrite

**Community fingerprinting**: A set of molecular biology techniques that can be used to profile the diversity of a community

**Feeding**: Application of a low concentration basal nutrient solution

**Gallic acid equivalent**: A measure of the total phenolic concentration expressed using gallic acid as a spectrophotometric standard

**Hydraulic conductivity**: A measure (co-efficient) of the ability of soil to transmit water under a hydraulic gradient

**Multidimensional scaling**: A data analysis technique that condenses large amounts of data into a relatively simple spatial map that relays important relationships

**System hydraulic conductivity**: The outflow rate of effluent from a system. In this report it is expressed as the flow per unit time per volume of sand

**Terminal restriction fragment length polymorphism:** A molecular biology technique used to profile microbial communities. It is based on the position of a restriction site closest to a fluorescent-labelled end of an amplified gene. A mixture of amplified variants of a single gene are digested using restriction enzymes and the size of each of the individual digested terminal fragments are detected using a DNA sequencer. The result is a graph image (electropherogram) where the X axis represents the sizes of the fragment and the Y axis represents their fluorescence intensity**.**

# CHAPTER 1:
# INTRODUCTION AND BACKGROUND

## 1.1 WHAT ARE CONSTRUCTED WETLANDS?

Natural wetlands have been used either inadvertently or deliberately to treat wastewater for centuries. Constructed wetlands (CWs) are engineered and controlled ecosystems which are designed to mimic the bioremediatory processes occurring in natural wetlands. 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 or directly degrade certain organic contaminants (Glick, 2010). Microbial communities are essential in the mineralization of organic matter and in nitrogen and phosphorous removal (Faulwetter et al., 2009; Truu et al., 2009; Glick, 2010). The substrate provides a habitat for functional microbial communities and assists in the removal of pollutants by natural sedimentation of organic matter and the sorption of (in)organic matter (Vrhošek et al., 1996). CWs are of particular interest as they represent a cost-effective, ecologically-friendly and aesthetically attractive option for wastewater remediation.

## 1.2 FACTORS AFFECTING THE FUNCTIONALITY OF CONSTRUCTED WETLANDS

### 1.2.1 Redox status and hydraulic flow regime

Many different combinations of configurations, operating modes and physical and biotic characteristics (plants and media types) have been applied to CWs. The redox potential (Eh) is one of the most critical parameters to be considered during CW design. High redox conditions promote aerobic processes, such as nitrification, and are associated with an oxidized environment (Faulwetter et al., 2009). In more reduced conditions (lower Eh), anaerobic processes such as sulphate reduction and methanogenesis are favoured (Faulwetter et al., 2009). The redox potential which is achieved is closely related to the hydraulic regime of CWs.

There are three basic flow regimes: free water surface flow (FWSF), horizontal sub-surface flow (HSSF) and vertical subsurface flow (VSSF) (Vymazal, 2007)(Figure 1). FWSF systems are the most anoxic (lowest Eh), consequent to continual inundation of the CW matrix. On the other end of the spectrum, VSSF provides the most aerobic environments. These systems are usually operated in batch mode, and alternating periods of flooding and drainage allows for the draw-down of atmospheric gases into the substratum during drainage, increasing the redox status (Herouvim et al., 2011; Pedescoll et al., 2011; Tietz et al., 2008; Torrens et al., 2009). It has been shown that nitrification and organic degradation of domestic wastewater

is enhanced in the more aerobic environment provided by VSSF systems (Van de Moortel et al., 2009).

Consequent to a continuous mode of operation, the redox conditions are lower in HSSF systems. Accordingly, HSSF systems are associated with less efficient organic degradation, but enhanced denitrification when compared to VSSF systems. However, a recent study has shown that organic removal rates can be increased by operating HSSFs in batch mode (Pedescoll et al., 2011). However, at this point, there are no published studies comparing organic removal efficiencies of VSSF and HSSF CWs operated in batch mode.

Hybrid CW systems are also becoming popular; these combine the advantages of different hydraulic regimes, either by employing a series of CWs, each with different modes of operation, or by combining more than one hydraulic regime within the same CW (Burton et al., 2011; Toscano et al., 2009).

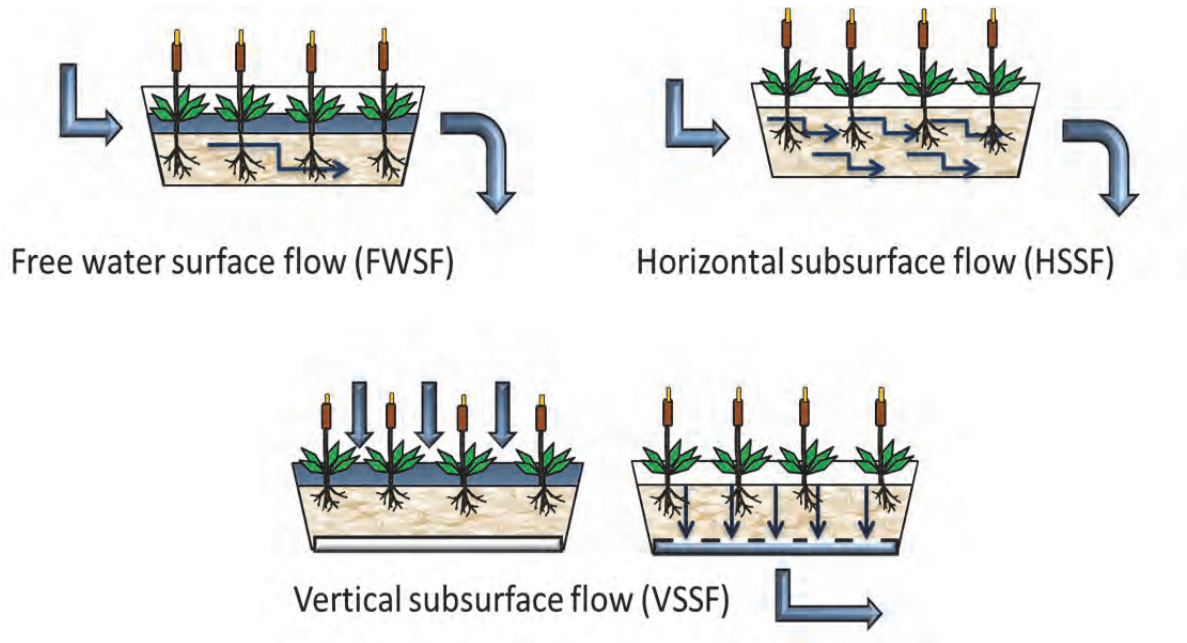


**Figure 1: The three basic configurations of constructed wetlands**

### 1.2.2 Choice of substratum medium

The popularity of different CW types is often influenced by the geographical location. This is because local expertise is accrued through practical experience in working with particular systems (Knowles et al., 2010; Vymazal and Kröpfelova, 2009). The media used in CWs is usually sand/soil, gravel or a combination of these two, but zeolite, slag, compost and alum sludge have also been used (Aslam et al., 2007; Babatunde et al., 2011; Li et al., 2008). The physical size and shape and the chemical nature of the particles affects the removal efficiencies of CWs (Akratos and Tshrinktzis, 2007; Torrens et al., 2009; Zhang, 2007). The

substrate, together with the redox status, the presence/absence of plants, and the plant species also plays a selective role on the microbial community composition (Lasur-Kruh et al., 2010; Sleytr et al., 2009; Welz et al., 2014).

An advantage of soil and sand is that the small particles form a large area for biofilm attachment and surface chemistry (Knowles et al., 2010). One of the major disadvantages of these smaller grain sizes is the propensity for clogging due to the accumulation of biofilm and/or suspended solids (Knowles et al., 2010). However, suspended solids can be removed in pre-filters or clarifiers and the problems associated with biofilm build-up can be overcome by intermittent operation (to allow complete biodegradation between treatments), or by employing low hydraulic loading rates (to accommodate the reduced hydraulic conductivity) (Knowles et al., 2010). The novel use of earthworms to restore clogged VSSF CWs in China has also been described (Li et al., 2011).

The type of substrate affects the hydraulic properties of CWs. It is accepted that chemical oxygen demand (COD) reduction can be improved by increasing the hydraulic retention time (HRT) in CWs (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., 2003). In HSSF systems, the flow paths are also affected by the inlet and outlet positions (Suliman et al., 2006). In the case of highly porous gravel-based systems, shortened HRTs can lead to poor removal rates, which must be taken into consideration during the design process (Ghosh and Gopal, 2010). Accumulation of biofilm can result in variable HRTs in both sand and gravel based systems, a factor which complicates design models and poses an engineering dilemma (Langergraber et al., 2008).

## 1.3 THE SOUTH AFRICAN WINE INDUSTRY AND WINERY WASTEWATER

South Africa is one of the top ten wine producers in the world, accounting for the production of around 1 100 million litres of wine, wine for brandy, and distilling wine in 2013, mostly in the Western Cape region (SAWIS, 2013). Each litre of wine accounts for the generation of 1-4 L of winery wastewater, which is regarded as the most significant environmental risk from wine cellars. Effluent COD values typically range from 800 to 12 800 mg/L, but peaks greater than 25 000 mg/L have been reported (Malandra et al., 2003; Saadi et al., 2007; Malandra et al., 2003). Inorganics, including sodium and potassium are often encountered in high concentrations.

95% of South African wineries utilize their wastewaters to irrigate land (Van Schoor, 2005). However, as winery wastes vary significantly in terms of chemical composition (both inter- and intra-winery), with peak flows occurring during the crush season in dry summers, this practice has been questioned (van Schoor, 2005).

Numerous aerobic and anaerobic suspended and attached growth systems for the treatment of winery wastewater have been evaluated (Andreottola et al., 2009). For

example, high COD removal performances (98% or more) were achieved in an aerobic activated sludge system and in an anaerobic sequencing batch reactor (Fumi et al., 1995; Ruiz et al., 2002). These processes are costly, energy intensive and require lengthy start-up periods. As the production of winery wastewater is intermittent, conventional systems need to be periodically re-acclimated (usually bi-annually during the crush and bottling seasons) (Grismer et al., 2003; Eusébio et al., 2004).

Provided sufficient land is available, CWs provide an attractive wastewater treatment alternative for small to medium sized wineries, with proven efficiencies in different countries and a variety of conditions (Shepherd et al., 2001; Masi et al., 2002; Grismer et al., 2003; Mulidzi, 2010).

## 1.4 AIMS OF PROJECT K5/2104

The original aims of the project, summarized from the proposal, were:

1. To exploit knowledge generated from a previous WRC-funded project (K5/1936) to understand how CWs may be adapted for "real world applications"
2. To utilize microbial community fingerprinting techniques to define which natural process parameters allow effective treatment of wastewater in CWs
3. To identify a matrix of parameters through which the capacity of CWs can be measured, and relate these to the characteristics of "real" wastewaters
4. To investigate the reproducibility of CWs "adapted" for specific wastewaters by characterizing the microbial communities in varied environments impacted by wastewaters
5. To develop an understanding of the stability of microbial communities "adapted" to specific wastewaters under the variable conditions imposed in "real world" situations
6. To understand the extent to which microbial communities in CWs can accommodate changes in waste impacts and the rate at which they can "adapt"
7. To understand the flexibility of microbial communities to "adapt" to changes in the wastewater environment in a given CW

The original aims set out in the proposal were refined in response to engagement with members of the reference group, and experimental outcomes. For example, at the first reference group meeting in 2011, it was advised that the research group should focus on winery effluent as the model wastewater. It was also decided to exclude plants from the systems because of the cumulative phytotoxic nature of the phenolics and salts found in winery wastewater. By definition, CWs contain plants. The systems used in this project are therefore not referred to as CWs, but as **biological sand filters (BSFs)**.

## 1.5 LAYOUT OF DOCUMENT AND COLLATION WITH AIMS OF THE PROJECT

Much of the background information and experimental results reported in the various interim deliverable reports have been excluded from this final report. The content has been rearranged and limited to those results which may be of practical value to other stakeholders. The content of each chapter is divided into Introduction, Materials and Methods, and Results and Discussion sections. The basis for each chapter is summarized below:

**Chapter 2:** This chapter deals with work performed primarily to assess the **reproducibility of microbial community composition and function** in BSFs, firstly when fed with a solution previously shown to have a minimal impact on the bacterial community structure (low concentration of basal nutrients), and secondly when amended with a high impact solution [synthetic **acid mine drainage** (AMD)]. This work also demonstrated how minor differences in sand physicochemistry can lead to major differences in bacterial community structure and function. The latter is re-iterated in Chapters 3 and 5.

**Chapter 3:** This chapter deals with work related to the **physical (sand) substrate** (medium). Five different aspects of locally available sand types are compared: (i) **Physicochemical properties** (available nutrients, mineralogy, elemental composition, porosity, and grain size distribution), (ii) Hydraulic **flow rates in** BSF systems containing different sand types, (iii) Intrinsic **hydraulic conductivity** (HC) of different sand types determined using custom-built columns, (iv) **COD removal** rates from synthetic winery wastewater in BSFs containing two different sand types, and (v) The **change in HC** associated with **biomass accumulation** with two different sand types.

**Chapter 4**: This chapter deals with work performed to assess the effect of the organic **composition of wastewater** and **redox status** on the performance of BSFs amended with synthetic winery wastewater. The degradation of wastewater organic substrates and formation of metabolites in different niche environments of the BSFs are discussed and aligned to the bacterial community structures in these niches.

**Chapter 5:** This chapter primarily focuses on the **degradation mechanisms and rates** of synthetic winery wastewater in the different environmental niches of the BSFs.

**Chapter 6:** This chapter reports on a proof-of-concept laboratory-scale experiment to determine whether the use of **trickling filters** (TFs) could be used as polishers to remove **residual volatile fatty acids** (VFAs) from winery wastewater treated in BSFs.

**Chapter 7:** This chapter describes experiments used to ascertain the extent to which the addition of **antimicrobial chemicals** and lengthy periods of **shut-down** affect the performance of BSFs.

**Chapter 8:** This chapter deals with work performed comparing the microbial community structure and chemical parameters in three **environmental sites impacted by winery wastewater**. The effect of wastewater and substrate (sand/clay) are discussed.

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# CHAPTER 2:
# BACTERIAL COMMUNITY STRUCTURE & FUNCTION: REPRODUCIBILITY BETWEEN TWO GROUPS OF BIOLOGICAL SAND FILTER REPLICATES EXPOSED TO ACID MINE DRAINAGE

## 2.1 INTRODUCTION

### 2.1.1 The need to replicate large-scale biological experiments

In the environment, the presence of a stable microbial community is generally considered to be a critical factor for maintaining ecosystem stability, nutrient cycling efficiencies, long-term sustainability, and resilience after contamination (Torsvik and Ovreas, 2002; Wohl et al., 2004). When studying the impact of effluents on microbial communities in replicate systems such as BSFs, it is crucial to establish similar communities for valid comparative analyses between replicates. In addition, many researchers feel that the sheer scale of large mesocosm, pilot-scale and full-scale experiments precludes the need for replication.

This study was performed in order to establish the reproducibility of the physicochemical parameters, and bacterial community structure and function in experimental BSFs and by inference, the need to replicate/not replicate future experiments. Six BSFs containing two batches of sand from the same quarry site were fed with a dilute solution of basal nutrients for an equilibration period of 3 months (Ramond et al., 2012). Feeding was intended to have minimal impact on the bacterial community structure, but result in the establishment of similar microbial communities in each system. Thereafter, the systems were amended with synthetic acid mine drainage (AMD), intended to have a major selective impact on the bacterial community structure. To prevent variations likely to arise with the use of complex sources of carbon, glucose was included as a defined, non-limiting and consistent source of readily available carbon known to be suitable for sulphate reduction by sulphate reducing bacteria (SRB). The study was not designed to assess the effect of heavy metals on microbial sulphate and/or iron reduction of the treatment of acid mine drainage (AMD) over a protracted period.

To assess the effect of feeding and amendment on the bacterial communities, molecular community fingerprinting was performed using terminal restriction fragment length polymorphism (T-RFLP) analysis of the bacterial 16S rRNA gene (Nicomrat et al., 2006; Baptista et al., 2008; Ruiz-Rueda et al., 2009). Physicochemical analyses were conducted to determine the reproducibility in the physicochemical environment and the bacterial community function.

### 2.1.2 Biological remediation of acid mine drainage

AMD emanates from sulphide-rich ores of abandoned mines flooded with groundwater, or mine tailings oxygenated with rainwater (Potgieter-Vermaak et al., 2006). AMD is characterised by a low pH and high concentrations of sulphate, iron and other dissolved metals (Ritcey, 2005). AMD is toxic and presents a major environmental problem in many countries, especially in aquatic environments where, in oxygenated surface waters, a toxic precipitate known as 'yellowboy' is formed by the oxidation of dissolved Fe(II) and hydrolysis of Fe(III) (Senko et al., 2008).

Conventional treatment of AMD typically consists of three basic processes which result in the production of calcite-rich sludge: dosing with lime slurry, aeration and flocculation. Many of the latest technologies focus on optimizing these processes to reduce the quantity of sludge production (Kalin et al., 2006). Systems requiring minimal intervention have been dubbed "passive" treatment systems and include treatment (constructed) wetlands (Kalin et al., 2006; Mayes et al., 2009; Reifler et al., 2008), permeable reactive barriers (Bartzas and Komnitsas, 2010; Gilbert et al., 2011), and other bioreactors designed to enhance microbial sulphate reduction (Choudhary and Sheoran, 2012). Wetland-type systems and permeable reactive barriers may include limestone to aid in the neutralisation of AMD through dissolution of carbonates (Reifler et al., 2008; Gilbert et al., 2011), but in most passive treatment systems, microbial reduction of sulphates to sulphides and the concomitant production of bicarbonate is fundamental to the neutralisation of AMD (Mayes et al., 2009; Refiler et al., 2008; Sheoran et al., 2012). The subsequent formation of sulphide-metal precipitates is also an important mechanism for the removal of iron and other metals from AMD in passive treatment systems (Reifler et al., 2008).

Biological iron and sulphate reduction relies on the supply of electron donors, usually in the form of an organic substrate such as compost, manure or municipal sludge (Bilgin et al., 2005; Lovely and Phillips, 1988; Matthies et al., 2012; Refiler et al., 2008; Sheoran et al., 2012). The degradation of complex materials is performed by microbial consortia, including cellulose-degrading, acetogenic, methanogenic and sulphate-reducing bacteria (Matthies et al., 2012; Mulopo et al., 2011; Mayes et al., 2009; Sheoran et al., 2012; Wijekoon et al., 2011). It is therefore difficult to achieve a steady concentration of utilizable, simple substrates for SRB and sulphate reduction may be rate-limited under field conditions, especially when the substrate has high cellulose content (Mayes et al., 2009; Logan et al., 2005; Utigar et al., 2002). In addition, AMD may be characterised by high concentrations of heavy metals which can negatively impact SRB communities (Utigar, 2002).

## 2.2 MATERIALS AND METHODS

### 2.2.1 Set-up, mode of operation, feeding and amendment of biological sand filters

Six identical BSFs (length 1.73 m/ width 1.06 m), each containing ~0.5 $m^3$ of river sand with a void volume of ~0.08 $m^3$ and depth of 0.3 m were operated in batch mode: influent was drip-fed onto the surface inlet at a rate of 0.68 L/min and allowed to gravitate vertically and longitudinally towards the outlet for the duration of equilibration and experimental periods (Figure 2).

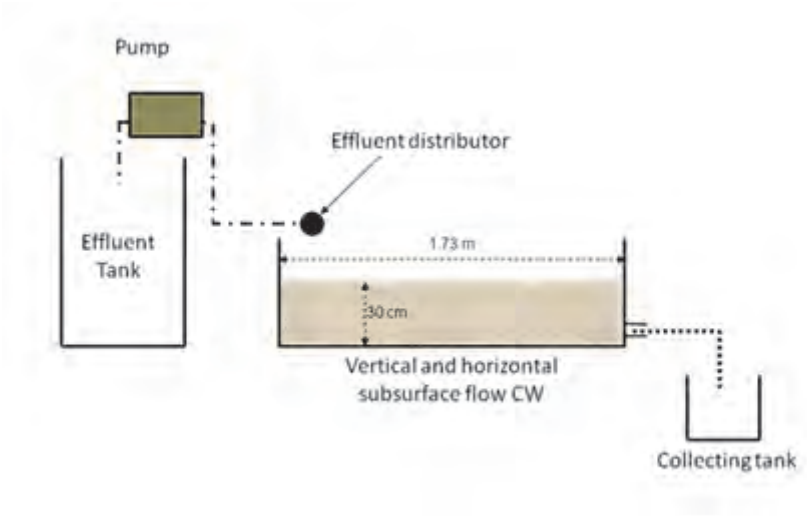


**Figure 2: Set up of biological sand filters**

For the study described in this chapter, the six BSFs that were used were divided into two groups. The first group was designated as group (A)BCD, which contained a control (BSFA) and three experimental systems (BSFB, BSFC and BSFD), and group EF, which contained two experimental systems (BSFE and BSFF). Each group contained different batches of Malmesbury sand obtained from the same quarry site. The mechanical fractions and major elements of each batch before (pre) and after (post) amendment with AMD are given in Table 1.

When initiating studies using microbial consortia it is important that the microbial communities within experimental replicates are equilibrated (i.e. present similar microbial community structures). It has been found that ~90 days is a suitable period for equilibration in these systems when fed twice weekly with a basal nutrient solution of 0.3 g yeast extract (Biolab®, Wadeville, Gauteng, RSA cat no: HG000BX6.500) and 0.3 g D (+) glucose (Merck®, Darmstadt, Germany, chemically pure Cat no: SAAR2676020EM), dissolved in 12.5 L tap water (Ramond et al., 2012). This procedure was therefore applied prior to amendment with AMD.

Thereafter, all BSFs were fed on a weekly basis with the same basal nutrients. In addition, the five experimental BSFs (B-F) were amended simultaneously with highly acidic (pH<2) synthetic AMD based on that used by Potgieter-Vermaak et al (2006), containing 500 mg/L magnesium ions ($Mg^{2+}$), 1500 mg/L Fe(II), 500 mg/L Fe (III) and 6000 mg/L sulphate ions ($SO_4^{2-}$). In order to provide sufficient carbon electron donors for the SRB, a high concentration of glucose (8000 mg/L) was also added to the synthetic AMD.

The amount of glucose was estimated by using the stoichiometric requirements for total sulphate reduction and doubling this to ensure adequate substrate for both the sulphate-reducing and non-sulphate reducing heterotrophic requirements, according to Equation 1. The COD/sulphate ratio (1.4, wt.wt), was thus considerably higher than suggested by other authors for sulphate reduction (0.67-1) (Mulopo et al., 2011; Reifler et al., 2008; Rodriguez et al., 2012).

$$C_6H_{12}O_6 + 3SO_4^{2-} \rightarrow 3H_2S + 6HCO_3^- \qquad \text{Equation 1}$$

### 2.2.2 Sampling procedures

#### 2.2.2.1 Sand samples

Composite (3x) core samples were extracted from each BSF using a Perspex pipe with an internal diameter of 25 mm and a rubber bung (Figure 3). Samples from each BSF were divided into four environmental niches [surface (0 to -30 mm) and deep (-100 to -150 mm) environments at the inlet and the outlet] and thoroughly mixed. Samples taken before and after the experiment were used for bacterial community fingerprinting.

Composite (3x) samples were also extracted from the centre of each BSF before amendment and at the end of the experimental period. The three cores from each of the (A)BCD BSFs were thoroughly mixed together as were those from group EF. These samples were used to compare changes in the elemental composition and concentration of available micronutrients of the sand before and after amendment with synthetic AMD.

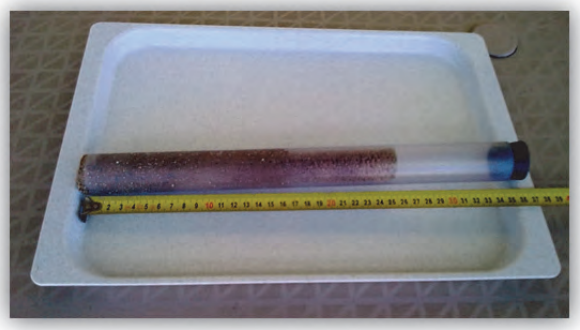

**Figure 3: Perspex corer with core sand samples extracted from a biological sand filter**

#### 2.2.2.2 Effluent samples

Beginning one hour after feeding/amendment commenced, effluent was collected for an additional hour. The volume of effluent emanating from each BSF was recorded (Section 2.2.3.1) and chemical analyses were performed on the samples (Section 2.2.5).

### 2.2.3 Measurement of physical parameters

#### 2.2.3.1 System hydraulic conductivity

The flow of effluent from the outlet can be measured. This flow was defined as the (saturated) system hydraulic conductivity (SHC). The SHC was determined by measuring the volume of effluent emanating from a given BSF between one and two hours after the commencement of feeding/amendment [L/hr/($m^3$ sand)].

### 2.2.4 Analysis of physical substrate (sand)

#### 2.2.4.1 Major element analysis

Major elements in the sand samples were determined at the Central Analytical Facility at the University of Stellenbosch (Stellenbosch, South Africa) by x-ray fluorescence (XRF) spectrometry on a PANalytical Wavelength Dispersive spectrometer (Almelo, Netherlands) fitted with an Rh tube and a gas-flow proportional counter using an argon (90%) and methane (10%) mixture. Prior to analysis, samples were crushed into a fine powder with a jaw crusher and milled in a tungsten Zib mill after which a fused glass disc was prepared using 10 g of high purity trace element and REE element free flux ($LiBO_2$ = 32.83%, $Li_2B_4O_7$ = 66.67%, Li = 0.50%) mixed with 1 g of sample. Correction for sample matrix effects was performed by applying theoretical alpha factors and measuring line overlap factors to the raw intensities measured with SuperQ PANalytical software. Control standards used for calibration were NIM-G (Granite from the Council for Mineral Technology, South Africa) and BHVO-1 (Basalt from the United States Geological Survey, Reston).

Differences in the elemental and mechanical properties of each sand batch were not significant (Table 1). The most abundant major element detected in both sands was silica (95.72% and 96.59% in group BCD and EF, respectively).

#### 2.2.4.2 Determination of mineralogy

Mineralogy was determined by X-ray diffraction (XRD) at the Department of Geological Sciences at the University of Cape Town (Cape Town, South Africa) using a Philips PW 1390 XRD instrument (Almelo, Netherlands) with a Copper K-α X-Ray tube with x-ray wavelength of 1.542 A, accelerating voltage of 40 kV and current of 5 mA. Bragg 2Θ angles between 2 and 70° were used for analysis. A continuous scan step (size 0.02° and time 0.4 s) was applied. The resultant XRD spectra of 2 theta vs. intensity were input into X'Pert software

and the d-spacing of the most intense peaks calculated by solving for the Bragg equation. The dominant mineral was quartz. No aluminosilicates or carbonate minerals were detected.

**Table 1: Mechanical fraction and major element analysis of two batches (BCD and EF) of Malmesbury sand before (pre) and after (post) amendment with acid mine drainage**

| | Mechanical fraction | | | | |
|---|---|---|---|---|---|
| | clay | silt | fine sand | medium sand | coarse sand |
| **BCD pre** | 0.4% | 1.0% | 34.4% | 24.0% | 40.2% |
| **EF pre** | 2.4% | 1.0% | 36. 6% | 22.6% | 37.4% |

| | Major elements [a] | | | | | |
|---|---|---|---|---|---|---|
| | $Al_2O_3$ | CaO | $Fe_2O_3$ | $K_2O$ | $P_2O_5$ | $SiO_2$ |
| **BCD pre** | 0.82% | 0.20% | 0.33 | 0.02% | 0.01% | 96.51% |
| **EF pre** | 0.37% | 0.84% | 0.17 | 0.03% | 0.01% | 96.59% |
| **BCD post** | 0.90% | 0.30% | 0.42 | 0.02% | 0.01% | 95.72% |
| **EF post** | 0.45% | 0.61% | 0.36 | 0.02% | 0.01% | 96.26% |

**No $Cr_2O_3$ or MgO detected. 0.02% MnO only detected in EF post. $Na_2O$ below detection limit**

#### 2.2.4.3 Quantification of available micronutrients

Analysis of available micronutrient elements was performed using a Varian® MPX ICP-OES spectrophotometer (Agilent Technologies, Santa Clara, USA) after extraction according to standard methods (1990) at Bemlab (Pty) Ltd. (Strand, South Africa). Mechanical fractions were determined according to standard methods (1990).

### 2.2.5 Effluent analysis

#### 2.2.5.1 Redox potential and pH

The redox potential and pH of freshly collected samples were determined using a pH700 meter and relevant probes (Eutech Instruments, Singapore).

#### 2.2.5.2 Sulphate ($SO4^{2-}$), Fe (II) and Fe (III)

The concentrations of $SO_4^{2-}$, Fe (II) and Fe(III) were determined immediately after sample collection using the Merck Spectroquant® test kit for SO4- (Cat no: 1.14791.000) and Fe (Cat no: 1.00796.0001) and a Merck Spectroquant® Pharo instrument, together with all system (in-built), instrument and reagent controls and standards as stipulated by the manufacturer. Samples falling out of range of the assays were diluted timeously and appropriately and the assay repeated.

#### 2.2.5.3 Chemical oxygen demand (COD)

COD concentrations were determined immediately after sample collection using a Merck Spectroquant® Pharo instrument and Merck Spectroquant® cell tests for a range of COD concentrations (cat no: 1.14895.0001, 1.14541.0001 and 1.14691.0001) according to the manufacturer's instructions.

#### 2.2.5.4 Identification and quantification of organic substrate (glucose) and metabolites using high performance liquid chromatography (HPLC)

Organic compounds in the fresh effluent samples were identified and quantified by reverse phase HPLC using a Merck® Hitachi Lachrom instrument and a Phenomenex® (Torrance, USA) Rezex RHM-monosaccharide H+ (8% cross-linkage) column according to the method described by La'Zaro et al. (1989). An L-7400 ultraviolet detector (210 nm) and an Agilent® (Santa Clara, USA) refractive index detector were used for the detection of acids and alcohols, respectively. Where possible, organic molecules were quantified using relevant standard graphs prepared from HPLC chromatograms and identified by spiking selected samples with relevant standard solutions. The theoretical COD and actual COD values of the organic molecules were calculated and used for subsequent mass balance analysis and the compilation of metabolic profiles as previously described (Welz et al., 2011).

### 2.2.6 Analysis of the microbial community structure

#### 2.2.6.1 Extraction of DNA from composite sand samples

Total DNA from the BSF sand was extracted from 0.5 g (wet weight) using the Powersoil® DNA isolation kit, MO BIO laboratories (San Diego, USA) according to the manufacturer's instructions.

#### 2.2.6.2 PCR amplification

All polymerase chain reactions (PCRs) were carried out in a Perkin Elmer Thermocycler (Gene Amp PCR system 6700). Bacterial 16S rRNA genes were amplified using the universal primers E9F (5'-GAGTTTGATCCTGGCTCAG-3') and U1510R (5'-GGTTACCTTGTTACGACTT-3'). Each PCR reaction contained 1X 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 and 10 ng of total 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 terminal restriction fragment length polymorphism (T-RFLP), the primer E9F was 5'-end FAMTM-labelled and the PCR products were purified using the GFXTM PCR DNA and gel band purification kit as directed by the supplier (GE Healthcare, UK). Purified PCR products (200 ng) were digested with the restriction enzyme *Hae*III at 37°C for 3 hr.

#### 2.2.6.3 Terminal- restriction fragment length polymorphism analysis

The microbial community structure was assessed by T-RFLP fingerprinting using the 16S rRNA gene as a phylogenetic marker. The precise length of terminal restriction fragments (T-RFs) was determined by capillary electrophoresis using the Applied Biosystems DNA Sequencer 3130 (Applied Biosystems, Foster City, USA) and according to the molecular weight standard Rox1.1 (with an acceptable error of ±1 bp). T-RFLP patterns and quality were analysed using the freeware PeakScanner™ (version 1.0) (Applied Biosystems, https://products.appliedbiosystems.com). Peak height was used to characterise each unique T-RF, and valid T-RF peaks (between 35 and 1000 bp) from triplicate T-RFLP profiles were identified, compiled and aligned to produce large data matrices using the online software T-REX (http://trex.biohpc.org) (Culman et al., 2009). T-RFs with intensities lower than 0.5% may have originated from background interference and were thus excluded from the matrices. Non-metric multidimensional scaling (NMDS) plots were created using Bray-Curtis similarity matrices with the software Primer 6 (Primer-E Ltd, UK). Two-dimensional NMDS plots were used, where the distance between points reflects the degree of similarity between the microbial community profiles in the samples.

## 2.3 RESULTS AND DISCUSSION

AMD poses an environmental hazard in many countries, including South Africa. There is a drive towards finding cost-effective solutions to this problem and bioremediation presents a feasible option. In this study, the neutralisation of AMD, the associated (biological) carbohydrate utilization patterns, the hydraulic properties and the similarities/differences in the microbial community structures in BSFs containing two different batches of sand were compared before and after exposure to AMD.

### 2.3.1 Characteristics of biological sand filters before exposure to acid mine drainage

* For reference, intra-group similarity is defined as comparisons between BSF B, C and D as separate entities or between BSF E and F as separate entities. Inter-group similarity is defined as comparisons between group BCD BSFs as a whole with group EF BSFs as a whole.

#### 2.3.1.1 Physicochemical characteristics of sand

The sand used to construct the BSFs was obtained from the same quarry site in two separate batches. BSFs designated A, B, C and D contained sand from one batch and those designated E and F contained sand from a second batch. Experimental systems were grouped (group BCD and group EF), based on sand batch.

## Mineralogy

In soil/sand environments, the primary substrate-dependent physicochemical processes responsible for increasing the pH of AMD are dependent on the rapid dissolution of carbonates and/or the slow dissolution of aluminosilicates (Jambor et al., 2002; Miller et al., 2010; Sherlock et al., 1995). In this study, no carbonate (e.g. calcite, siderite) or aluminosilicate-based minerals were identified in any of the sand samples. Dissolution of sand constituents was thus not expected to play a significant role in the neutralisation of AMD in the experimental BSFs.

## Available nutrients and micronutrients

Different concentrations of available nutrients and micronutrients were detected in each batch of sand, with available Fe and K being significantly higher and Zn significantly lower in the EF sand in comparison to the BCD sand (Figure 4). It is suggested that these nutrients/micronutrients played a role in the selection of microbial communities in the BSFs (Section 2.3.2).

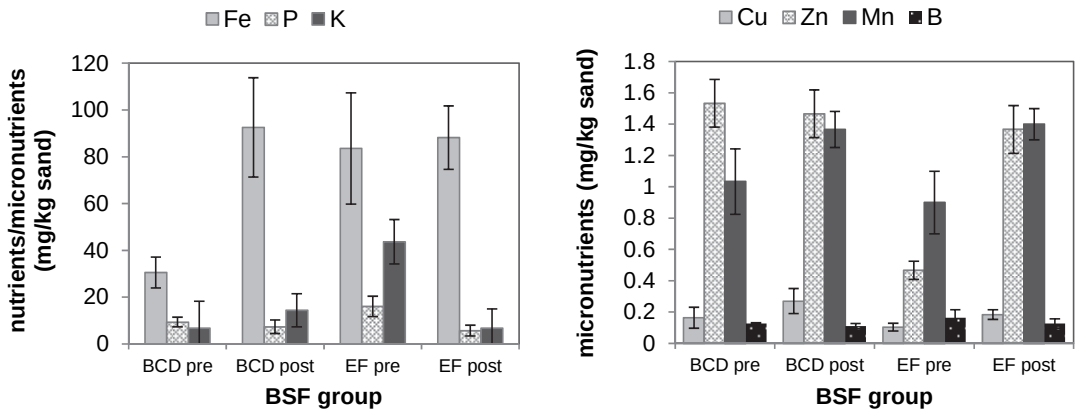


**Figure 4: Available nutrients/micronutrients in the sediment from group BCD and group EF biological sand filters before (pre) and after (post) amendment with acid mine drainage**

## Redox and pH

There were no inter-group differences in the effluent redox potential (Figure 5). The pH of the effluent from the group EF BSFs was slightly more alkaline (7.65 ± 0.21) than that from the group BCD BSFs (7.13 ± 0.15) (Figure 6).

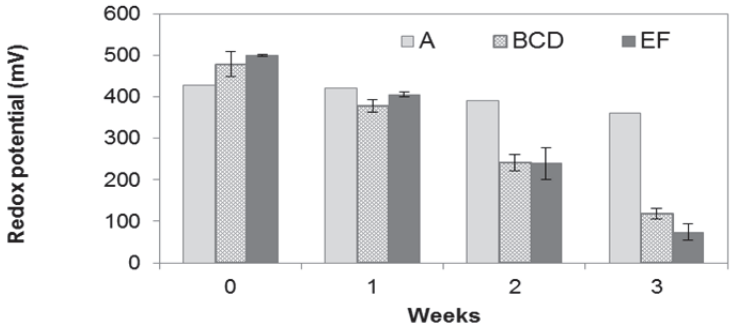


**Figure 5: Redox potential measured in the effluent from group BCD and group EF before (week 0) and during (weeks 1, 2, 3) amendment with acid mine drainage**

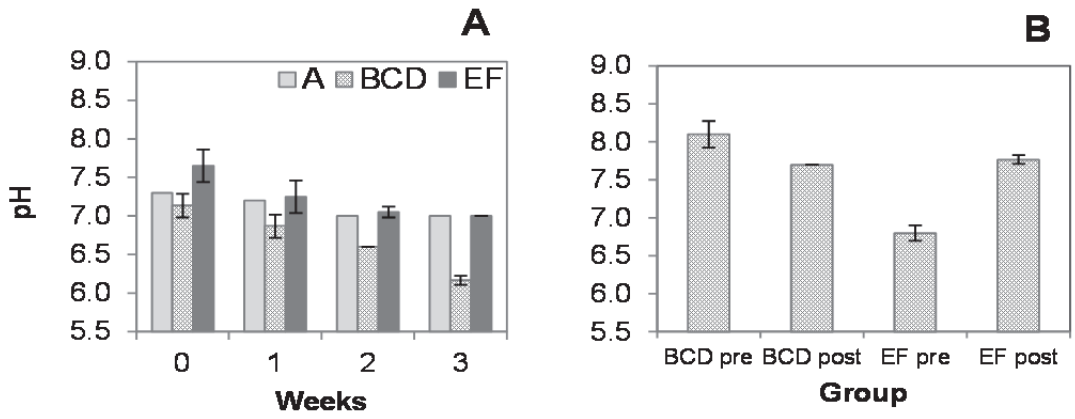


**Figure 6: pH measured in the biological sand filter effluent (A) and sediment (B) before (week 0, pre) and after (post) amendment with acid mine drainage**

### 2.3.1.2 Analysis of the pre-amendment bacterial community structure

Bacterial community structures were analysed using T-RFLP and are presented in the form of 2D NMDS plots with low (<0.1) 2D-stress values (Figure 7) (Clarke, 1993). The plots provide a visual representation of the similarity (%) in the communities from each BSF (including the control BSF) in four experimental niches [inlet, outlet: surface (0 to -3 cm), deep (-10 to -15 cm)]. The proximity of the points on the plot is indicative of the similarity of the communities with distant points being dissimilar and vice versa. When comparing the bacterial community structures prior to amendment with AMD [(triangles and circles designated (0) in Figure 7], it was found that there were significant intra-group, but not inter-group similarities. The intra-group similarity in the surface and deep sediments, respectively of the control (A) and BSFs B, C and D was 70% and 40% and that of BSFs E and F, containing a different batch of sand, 70% and 80%. In contrast, the inter-group similarity of groups BCD and EF was only 40% and ≤20% in the surface and deep sediments, respectively.

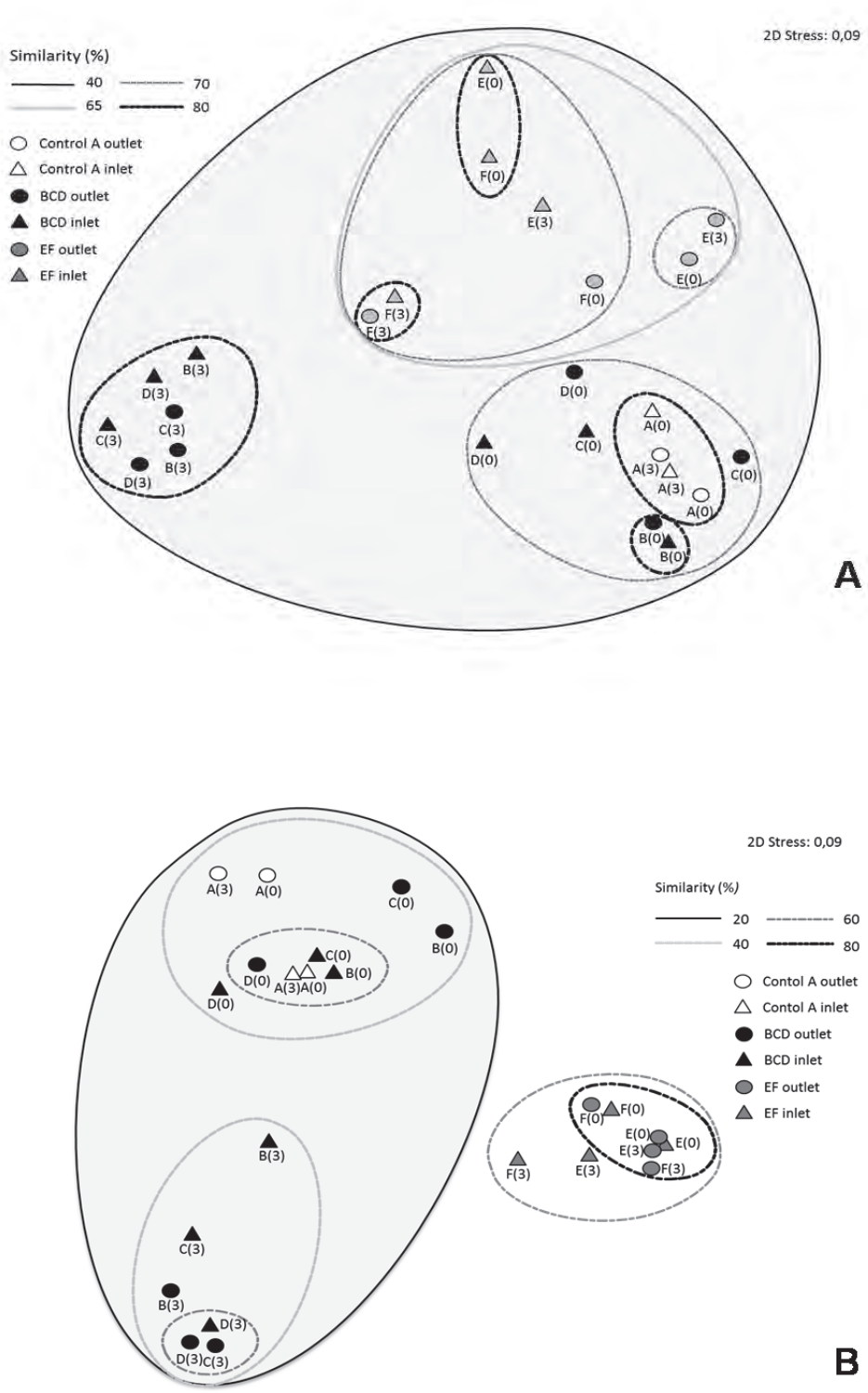


**Figure 7: Non-metric multidimensional-scaling plots depicting the similarity between the bacterial 16S community structure in the surface (A) and deep (B) sediment samples after feeding with basal nutrients (0) and after amendment with acid mine drainage (3)**

These results showed that (i) the sand batch was an important determinant of bacterial community composition after feeding with basal nutrients (ii) the 90 day equilibration period was sufficient to establish similar intra-group bacterial communities, despite differences in previous chemical treatments in the BSFs (Rodriguez Caballaro, 2012; Welz et al., 2011, 2012) and (iii) minor physicochemical differences (Section 2.3.1.1) in the sand had a significant influence on the bacterial community structure.

### 2.3.1.3 Hydraulic properties

It has been shown that when BSFs are fed, it takes around 90 days for the microbial communities to stabilise to the prevailing environmental conditions. Furthermore, during the acclimation process, the SHC undergoes constant changes, the degree of which is related to the amount of available nutrients to produce biomass. This is because biofilm/biomass clogs the interstitial spaces in the sand to some degree, causing a decrease in SHC. Once the microbial communities stabilise, so too does the SHC (Ramond et al., 2012).

A large disparity in the hydraulic flow rates was observed during the equilibration period, with the SHC of group BCD being lower than that of group EF (Figure 8). It has been shown that the hydraulic conductivity (HC) in wetlands increases with increased particle size, decreases as uniformity of particle size increases, and is more uniform in saturated sands (Giraldi et al., 2009; Suliman et al., 2002).

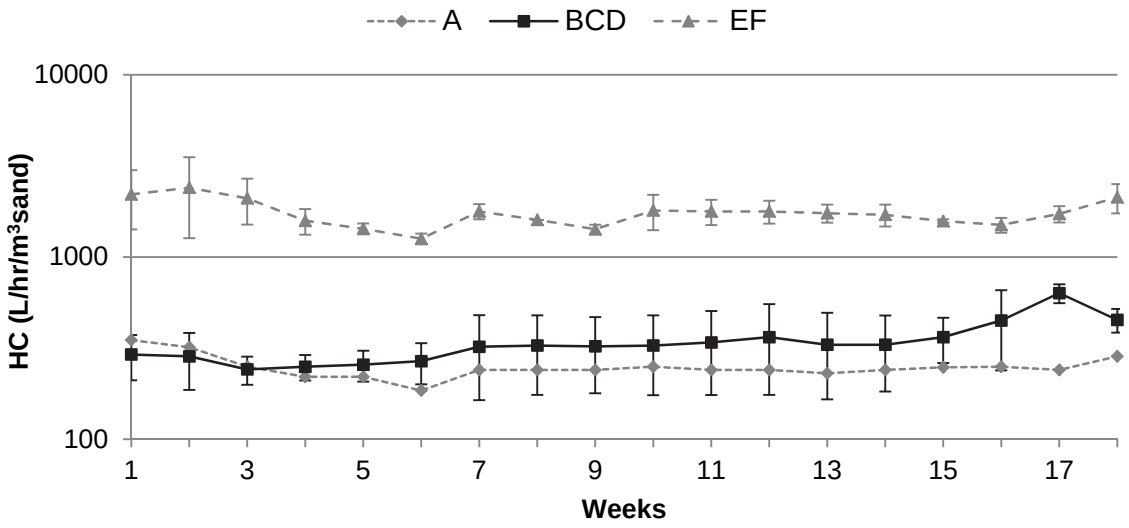


**Figure 8: System hydraulic conductivity measurements from the control A, group BCD and group EF during feeding with basal nutrients, amendment with acid mine drainage and a recovery period (weeks 1-13, 14-16 and 17-18, respectively)**

Difference in particle size was discounted as the primary basis for the disparities in SHC because, apart from the fact that each sand batch was highly similar, group EF sand particles were slightly smaller than group BCD (2% more clay, 2.2% more fine sand, 2.6% less medium sand and 2.8% less coarse sand), but the SHC of group BCD was lower than that of group EF. There were significant differences in the microbial community structure related to sand-batch (Section 2.3.1.2). It is therefore suggested that that the disparities in SHC were primarily related to biological clogging as previously described (Molle et al. 2006; Serrano et al., 2010; Welz et al., 2011).

### 2.3.2 Characteristics of biological sand filters after exposure to acid mine drainage

#### 2.3.2.1 Physicochemical characteristics of sand

There were no significant differences in the elemental composition of either sand batch associated with exposure to AMD. Amendment with AMD caused an expected increase in available Fe in each batch of sand, so that after 3 weeks, similar amounts (~90 mg/kg sand) were measured in group BCD and EF (Figure 4). The increase was marked (~ 50 mg/kg sand) in the group BCD sand and subtle (<10 mg/kg sand) in the group EF sand, demonstrating that Fe from the AMD accumulated preferentially in the Group BCD BSFs.

#### 2.3.2.2 Analysis of the bacterial community structures after exposure to AMD

After 3 weeks of exposure to AMD [triangles and circles designated (3) in Figure 7], the inter-group similarity of group BCD and EF remained low at 40% (surface sediments) and ≤ 20% (deep sediments). The intra-group similarity remained high (80% and 40% between BSF B, C and D and 70% and 60% between BSFs E and F in the surface and deep samples, respectively).

Comparison of similarity patterns obtained from before and after amendment with AMD [triangles and circles designated (0) compared with those designated (3) in Figure 7] indicated that the AMD had a significant impact on the bacterial communities in group BCD BSFs. In these BSFs, the pre-amendment communities only displayed 40% (surface) and 20% (deep) similarity with the post-amendment communities. By comparison, the impact of AMD on the bacterial communities in group EF BSFs was significantly lower, with 80% (surface) and 60% (deep) similarity between pre- and post-amendment bacterial community structures.

These results clearly demonstrate that the sand batch and exposure to synthetic AMD both had significant effects on the group BCD bacterial community structure. However, the effect of the physical substrate (sand) on the group EF community structure was considerably more pronounced than the effect of AMD.

It has previously been shown that the micro-topography, surface composition, surface charge and hydrophobicity of mineral particles play important roles in microbial colonization, biofilm formation and microbial community development (Carson et al., 2009; Gadd et al., 2010). However, it has also been shown that microbial communities in AMD-impacted environments are primarily influenced by the presence of AMD (Senko et al., 2008). This study demonstrated that the relative contributions of the physical substrate and AMD to the evolution of the bacterial communities are not always consistent.

#### 2.3.2.3 Neutralisation of acid mine drainage in biological sand filters

In the environment, the three most important processes that buffer AMD are: (i) abiotic dissolution of carbonate materials, which generates alkalinity and consumes protons, (ii) abiotic and microbial reduction of iron oxides and (oxy)hydroxides, and (iii) microbial sulphate reduction (Bilgin et al., 2005; Kalin et al., 2006; Lovely and Phillips, 1988; Mayes et al., 2009). Bioremediation systems harness one or more of these mechanisms, in particular bacterial sulphate reduction, to detoxify AMD.

In this study, highly acidic synthetic AMD (pH <2) was neutralized in all five BSFs (B-F). Average effluent pH values differed between group BCD and EF (Figure 6A). Although there was a steady temporal decline in effluent pH, near neutral values of 6.2 ± 0.1 and 7.0 ± 0 were still obtained from group BCD and EF, respectively at week 3. Different inter-group trends in the physical substrate (sand) pH measurements were also noted (Figure 6B): In group EF, the pH increased by close to one unit, while that of group BCD decreased slightly during the experimental period. However, the final pH of the sand was similar in both groups. Substrate dissolution was discounted as a major neutralisation mechanism (Section 3.3.1.1).

Despite a high influent concentration of sulphate (6000 mg/L), only negligible concentrations were detected in the effluent of all BSFs at week 1. In previous studies, a similar effect was seen, where plug flow forced the existing water in the substrate through the outlet, essentially creating a lag in the emergence of effluent and resulting in an anomalously low concentration of influent chemicals being detected after the first amendment with wastewater (Welz et al., 2011).

In group BCD BSFs, no dissolved sulphate removal occurred during week 2 and only 33% removal took place during week 3 (Figure 9). In contrast, 86% and 82% dissolved sulphate removal was achieved in group EF BSFs in weeks 2 and 3, respectively, strongly suggesting the functional evolution of sulphate reduction capacity. The sulphate-reducing and neutralizing capacity of group EF BSFs was superior to that of group BCD despite the consistently lower average nominal HRT (HRT group EF=1.9 days; HRT group BCD 11.1 days). There are two possible explanations for this finding: (i) the original bacterial communities in group EF were inherently more resilient to the presence of AMD and/or (ii) the combination of batch operating mode and low HRT minimized exposure of the bacterial communities,

particularly the SRB, to the toxicity of AMD. This is supported by the fact that the impact of AMD on the bacterial communities in group EF was significantly lower than the impact on the group BCD communities (Section 2.3.2.2).

Only small amounts of Fe were detected in the effluent during week 1. From week 2, the ratio of Fe(II) to Fe(III) measured in the effluent was considerably higher in group BCD than group EF replicates (Figure 9). These results suggest that abiotic and/or microbial iron reduction was enhanced in the group BCD BSFs in comparison with the group EF systems.

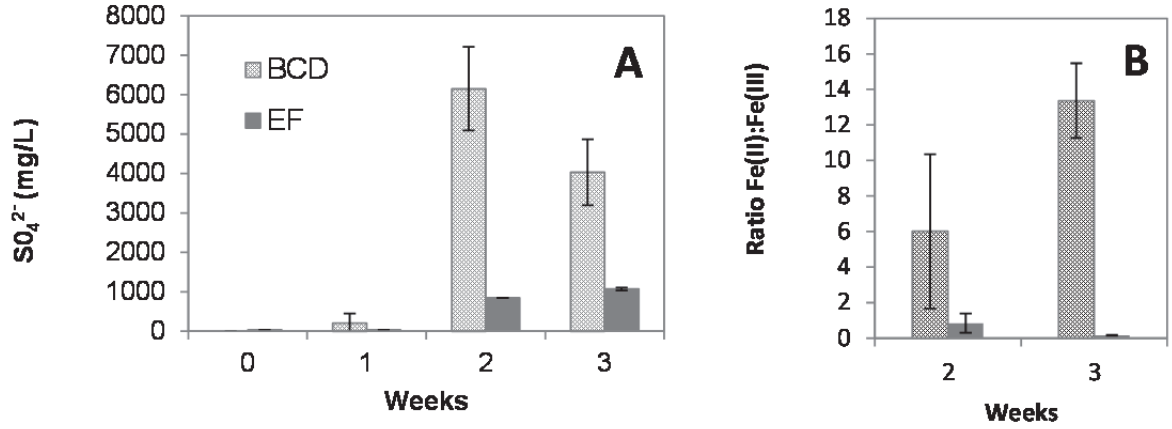


**Figure 9: Effluent sulphate concentration (A) and Fe (II) to FE (III) ratio (B) from group BCD and group EF before (week 0) and after (weeks 1, 2, 3) amendment with acid mine drainage**

### 2.3.2.4 Carbohydrate metabolism

In a real-world setting, locally available, cheap sources of carbon wastes are typically used as electron donors. However, these carbon sources do not provide a chemically consistent flow of electron donors. This adds complexity to the interpretation of experimental results. In this study, a simple source of carbon was used to limit temporal and spatial variation between replicates. To ensure that sulphate reduction was not rate limited by lack of a readily available carbon source, the stoichiometric COD:sulphate ratio applied (1.4) was higher than previously suggested for microbial sulphate reduction (0.67 to 1) (Mulopo et al., 2011; Reifler et al., 2008). The ratio of 0.67 is a theoretical value which does not take into account other carbon-dependent metabolic processes and it has been demonstrated that sulphate reduction can be increased with larger COD to sulphate ratios (Rodriguez et al., 2012). The utilization of the carbon source (glucose) was monitored by quantifying organic metabolites in the effluent and by measuring effluent COD. The glucose utilization patterns were related to sulphate and iron reduction (Figure 10).

There was a temporal increase in readily biodegradable substrates, mostly volatile fatty acids, as well as residual glucose measured in the effluent from group BCD BSFs and average effluent COD concentrations reached approximately 3500 mg/L by week 3. In contrast, only negligible amounts of substrates and metabolites were detected in the effluent from group EF BSFs, with average effluent COD concentrations of approximately 400 mg/L by week 3. This substantiates the hypothesis that microbial iron reduction dominated in the group BCD BSFs. The results are consistent with the fact that the organic carbon requirement for microbial sulphate reduction is substantially higher than that for microbial iron reduction (Chapelle et al., 2009).

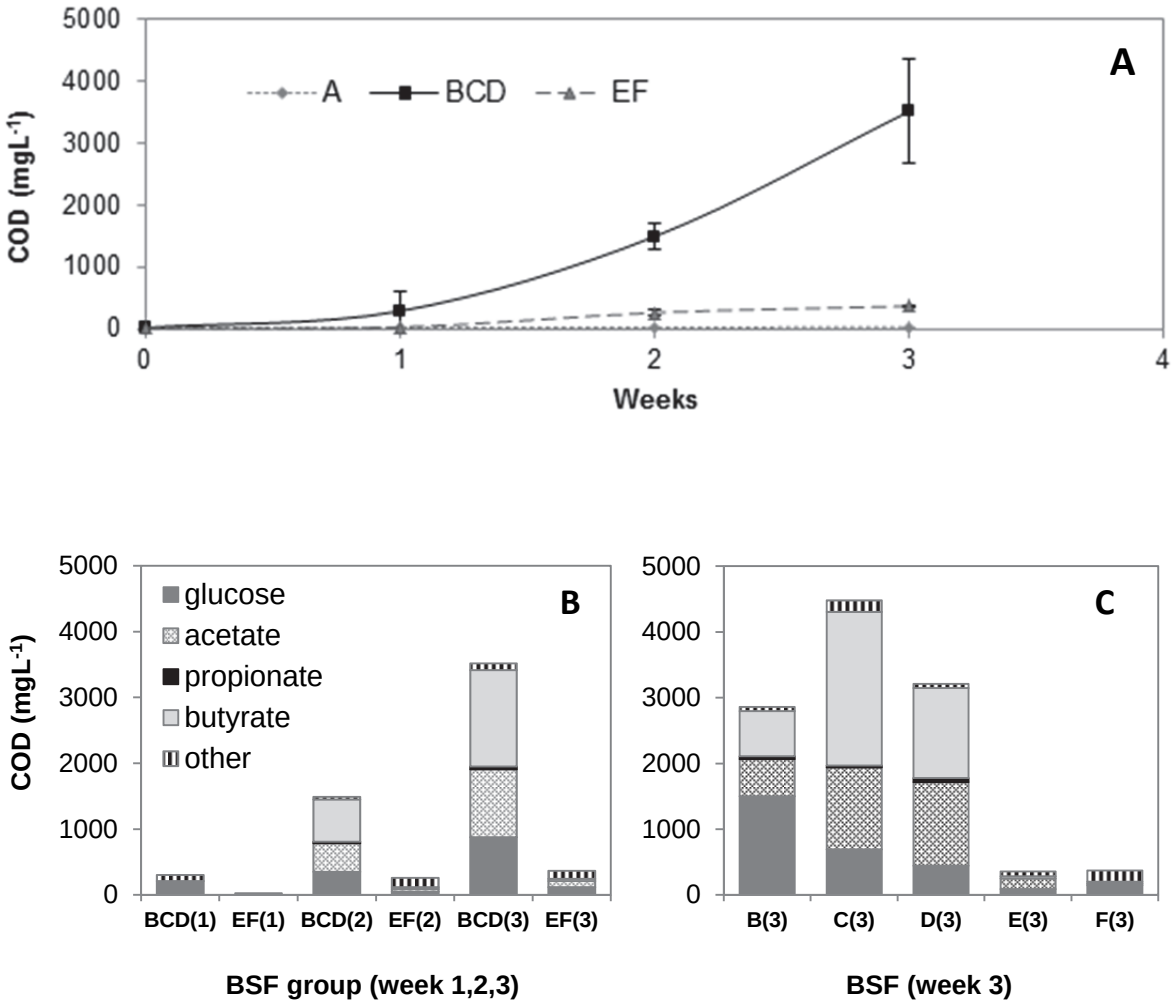


**Figure 10: Total chemical oxygen demand concentrations measured in the effluent from the control and group BCD and EF (A); Effluent substrate/metabolite profile from group BCD and EF from week 1-3 (B); Effluent substrate/metabolite profiles from individual BSFs from week 3 (C)**

### 2.3.3 Conclusions

There were significant differences the results obtained from the BSFs containing different batches of the same sand. From replicates containing the same sand, trends were well reproduced. However, it was felt that there were sufficient differences in the magnitude of the results obtained to merit the use of two replicates for further studies. Therefore, most of the experiments described in Chapters 3 and 4 of this report have made use of replicate BSFs and columns.

Each environment, whether natural or artificial, has a unique set of interrelated physicochemical and biological properties and the composition of the physical substrate can have substantial effects on the bacterial community composition and functionality. Nevertheless, it was expected that the impact of AMD on the bacterial community structure would over-ride any other physicochemical effects. In this study, however, the sand played a significant role in the determination of bacterial community structure. Subtle differences in the physical substrate (sand) led to significant differences in microbial community composition and consequent biogeochemical processes in BSFs, both after feeding with basal nutrients and after amendment with AMD. The physical substrate and the influent AMD had similarly significant effects on the bacterial community structure in three BSFs, while in additional replicates containing a different batch of sand from the same quarry site, the effect of the physical substrate was more pronounced. The importance of the physical substrate on the selection of functional microbial communities in systems remediating AMD should therefore not be under-estimated. In treatment systems relying on microbiological processes, the physical substrate should be carefully selected and it may be prudent to include small-scale comparative studies in each particular setting prior to full-scale implementation.

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# CHAPTER 3:
# ANALYSIS OF LOCALLY AVAILABLE SAND TYPES FOR USE IN BIOLOGICAL SAND FILTERS

## 3.1 INTRODUCTION

Apart from inter- and intra-cellar variability in the composition and volume of winery wastewater, some countries generate greater quantities of wastewater per volume of wine than others (McIlroy, 2011). It is therefore difficult to calculate the average volume of wastewater produced at different sized wineries. However, to give a broad contextual example, the volume of wine produced in 2013 by one medium sized winery in South Africa was 6.5 ML. According to literature estimates, this would result in the generation of between 6.5 and 32.5 ML of wastewater (Bories, 2010; McIlroy, 2011; Mosse, 2011).

It is imperative that if BSFs are filled with a substrate that is inexpensive and readily available on a long-term basis. Therefore, the sand that is locally available in the Cape Town environs was used during this project. Between 2009 and 2011, the BSFs at CPUT contained **Malmesbury sand**. Excellent remediation of synthetic wastewater was achieved in these systems, which were able to successfully mineralise wastewater with at a high organic loading rate (OLR) of > 110 g COD/$m^3$sand/day. However, the SHC was low, and decreased further due to the build-up of biomass, so that nominal HRT reached 22 days (Welz et al., 2011). The HRTs reported in literature from CWs treating winery waste range between 7 and 14 days (Welz et al., 2011). By virtue of the low SHC, the hydraulic loading rate (HLR) was restricted to approximately 7.1 L/$m^3$ sand/day. Thus, even at a lower range of production (1:1 wine:wastewater), and assuming that winery wastewater is generated evenly throughout the year, this would require the capacity of a BSF treating effluent from a typical medium-sized winery to be in the order of 2 500 $m^3$ (2.5 ML) if filled with Malmesbury sand.

It was thus apparent that the theoretical size of BSFs containing Malmesbury sand would be too large for “real world” applications. In response to these findings, in 2012, the sand in the BSFs was changed, and four new systems were also set-up. Five systems were filled with **Phillipi sand** and three with a mixture of Phillipi and Malmesbury sand, referred to as **Mix sand**. A number of comparative experiments were performed in order to ascertain the most suitable sand for use in the BSFs: (i) The physicochemical properties of the different sand types were analysed, (ii) The SHC of replicates before and after feeding/amendment was determined, (iii) The organic degradation performance was determined, (iv) the microbial community structures in the BSFs were analysed, and (iv) The true HC of the sand before and after biomass accumulation was determined in dedicated column experiments.

## 3.2 MATERIALS AND METHODS

### 3.2.1 Set-up, mode of operation and feeding/amendment of biological sand filters

The BSF set-up is described in Section 2.2.1 and shown in Figure 2. However, the mode of operation was changed because of the high SHC, which did not afford sufficient HRT. The systems were thus plugged before feeding/amendment. *In this document, the term feeding refers to the application of a dilute concentration of basal nutrients in order to induce the growth of consistent microbial communities in the CWs during an equilibration phase (Ramond, et al. 2012). The term amendment is used to describe the application of wastewater or wastewater components during an experimental phase (Welz et al., 2012, 2011; Rodriguez-Caballaro et al., 2012).*

During the 3 month equilibration phase, all CWs were fed with 1 g yeast extract (Biolab®, Midrand, RSA cat no: HG000BX6.500) and 0.3 g D (+) glucose (Merck®, Whitehouse station, USA, chemically pure cat no: SAAR2676020EM) dissolved in 40 L tap water. The addition of yeast extract and glucose at the same concentration was continued during the amendment phase.

During the experimental phase/s, two of the BSF replicates containing Phillipi sand (P1, P2) and two of the replicates containing Mix sand (M1, M2), were amended with synthetic winery wastewater (Table 2), while the control BSFs (PC and MC) were fed as per the equilibration phase. The actual COD (CODa) for each component was calculated according to the method described by Welz et al. (2011).

**Table 2: Composition of synthetic winery wastewater**

| Final conc* (COD mg/L) | Vanillin (COD mg/L) | Ethanol (COD mg/L) | Acetic acid (COD mg/L) | Gallic acid (COD mg/L) |
|---|---|---|---|---|
| **2027** | 100 | 1000 | 800 | 100 |

*** Includes contribution from glucose (27 mg COD/L) in basal nutrient solution**

The BSFs were operated in plug-fill-drain cycles, i.e. the outlets were plugged and then the systems were fed/amended. After 48 hr, the plugs were removed and the effluent was allowed to drain from the systems (Table 3). In the case of the BSFs filled with Phillipi sand and Malmesbury sand, bulk flow ceased after approximately one hour and two hours, respectively.

**Table 3: Operational parameters applied during the experimental period**

| | PC, MC | P1, P2 | P3, P4 | M1, M2 |
|---|---|---|---|---|
| ***HLR (L/m$^3$sand/.day)*** | 22.9 | 22.9 | 22.9 | 22.9 |
| ***OLR (g COD/m$^3$sand/day)*** | 0.6 | 48.0 | 48.0 | 48.0 |
| ***Influent COD (mg/L)*** | 27 | 2027 | 2027 | 2027 |
| ***Feeding frequency (times per week)*** | 2 | 2 | 2 | 2 |
| ***Plug time (hr)*** | 48 | 48 | 48 | 48 |

**HLR = hydraulic loading rate OLR = organic loading rate COD = chemical oxygen demand**
**COD, OLR values include contributions by glucose in basal nutrient solution**

### 3.2.2 Collection of effluent samples from biological sand filters

The total effluent flow emanating from the systems for 90 s and 180 s was collected, from the BSFs containing Phillipi and Mix sand, respectively. The period of collection from the BSFs containing Mix sand (180 s) was longer than the period of collection from the BSFs containing Phillipi sand, so that similar volumes could be collected from all BSFs (the outflow from the BSFs containing Phillipi sand was faster; see Section 3.2.2.1). Effluent samples were collected immediately upon unplugging of the systems. Further samples were collected 0.5 hr and 1.0 hr after unplugging, from the BSFs containing Phillipi and Mix sand, respectively.

### 3.2.3 Effluent analyses

Total phenolics were determined using the Folin-Ciocalteau method as described by Welz et al. (2012). Other analyses were performed as described in Section 3.2.5

### 3.2.4 Determination of sand physicochemical properties

Major elements and mineralogy were determined as described in Section 2.2.4.1 and 2.2.4.2, respectively.

### 3.2.5 Determination of sand porosity and density

Polypropylene containers were weighed and then filled with sand to an approximate volume of 2 L. The sand was compacted by the addition of water to emulate the procedure used to compact the sand in the CWs i.e. no mechanical interventions. The sand was then thoroughly dried at 80°C for 2 weeks. After allowing the sand to adjust to room temperature, the containers were weighed, and water (room temperature) was added until the sand was saturated. The volume of water used to obtain the saturation point was noted. The level of sand was marked on the container and the containers were emptied. Water was

then added to the containers to the marked level and measured to calculate the exact volume of sand that had been added to each container.

The effective porosity of the sand was determined from the volume of water that was added to obtain the saturation point for a given volume of sand.

The density of the sand was calculated by determining the weight of the dry sand in the measured volume by deducting the weight of the containers from that of the containers filled with dry sand. All procedures were conducted in triplicate for each sand type.

### 3.2.6 Determination of system hydraulic conductivity

The SHC was monitored according to the schedule given in Table 4. The first measurement was taken immediately upon unplugging the outlet. Once particular SHC patterns were established (at week 11), the measurement regime was rationalized. The final procedure that was adopted was as follows: Effluent was collected from the outlet over a fixed period of 90 s and the volume was measured. Results were expressed as the volume collected per hour for each cubic metre of sand. Due to the slower HC obtained with the Mix sand, the collection period was extended to 180 s for the BSFs containing this sand type.

**Table 4: Timing and intervals of system hydraulic conductivity measurements**

| **week** | **Effluent collection (minutes from t = 0)** | | |
|---|---|---|---|
| | **PC** | **P1-4** | **MC, M1-2** |
| ***5*** | ND | 0, 15, 30, 45, 60, 120 | ND |
| ***6-8*** | 0, 15, 30, 45, 60 | 0, 15, 30, 45, 60 | 0, 15, 30, 45, 60 |
| ***9-10*** | 0, 15, 30, 45 | 0, 15, 30, 45 | 0, 30, 60 |
| ***11*** | 0, 15, 30 | 0, 15, 30 | 0, 30, 60 |
| ***12-15*** | 0, 15, 30 | 0, 15, 30 | 0, 30 |

### 3.2.7 Set-up of sand columns for hydraulic conductivity studies

Three acrylic columns (300 mm ø x 1500 mm high) were fitted on prefabricated galvanized mild steel stands (Figure 11). The prefabricated stands were constructed with 100 mm x 50 mm channel sections (acting as legs) and welded to 2 x 300 mm ø rolled flat bars (60 x 6 mm). The rolled flat bars supported the sides of the acrylic columns and flatex 362 mild steel mesh was welded to the bottom of the rolled flat bars to support the columns at the bottom. The space between the rolled flat bars and columns was sealed using purpose made rubber o-rings and silicon (Sika Flex 11 FC+).

A purpose-made mild steel bucket with flange fitted with a 40 mm galvanized nipple was bolted to a flanged connection that was welded to the bottom of the stand. All the bolts

were also sealed with silicon to prevent leakage. A 40 mm ø isolating valve was connected to the nipple to drain fluids and regulate head conditions. The bottom 60 mm section of the column was filled with 19 mm aggregate. The columns were filled with sand to a height of 600 mm. Two peristaltic pumps were used to pump water from containers through 6 mm tubing to the columns. Water was dissipated on sand through a purpose-made perforated bucket fitted at the top of the columns.

### 3.2.8 Amendment of sand columns with synthetic winery wastewater to induce biomass formation

Two experimental columns 0.3 m in diameter, designated experimental columns 2 and 3, and containing sand to a height of 0.63 m were inoculated with an aqueous extract from sand samples taken from BSFs previously amended with synthetic winery wastewater. Thereafter, they were fed with glucose and yeast extract for a period of two weeks to establish functional microbial communities. The third replicate, column 1, was designated as a control and was not fed or amended.

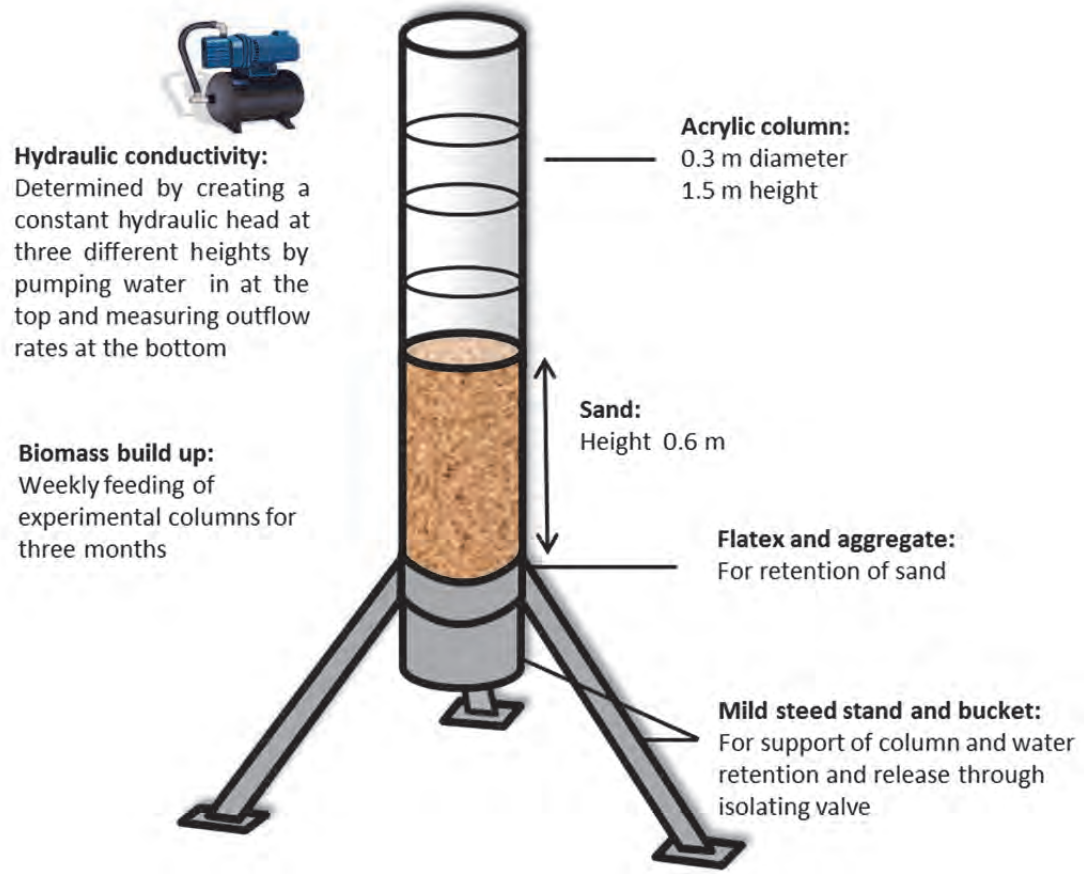


**Figure 11: Design and set-up of column for hydraulic conductivity experiments**

After 2 weeks, the columns were amended weekly for a period of 14 weeks with synthetic winery wastewater containing ethanol, glucose, and the phenolics gallic acid and vanillin. To ensure that the microbial population was not nutrient limited, inorganic nutrients ($KNO_3$ and $KH_2PO_4$) were also added in a COD:N:P ratio of 200:5:1 (Table 5).

Before feeding/amendment, taps located under the sand columns were closed to retain the effluent in the sand columns. The effluent was applied manually using volumetric cylinders/beakers. The entire experiment was first conducted using Phillipi sand and then repeated with Mix sand.

**Table 5: Composition of synthetic winery wastewater applied to sand columns**

| weeks | Vol. (L) | Influent organics (COD mg/L) | | | | Influent inorganics (mg/L) | | Total COD (mg/L) | OLR (g COD/$m^3$ sand/$day^{-1}$) |
|---|---|---|---|---|---|---|---|---|---|
| | | Ethanol | Glucose | Gallic acid | Vanillin | $KNO_3$ | $KH_2PO_4$ | | |
| ***1-3*** | 22.5 | 1000 | 800 | 100 | 100 | 700 | 1.9 | 2000 | 141.2 |
| ***4-10*** | 22.5 | 1000 | 800 | 100 | 100 | 640 | 1.8 | 2000 | 128.3 |
| ***11-14*** | 22.5 | 500 | 400 | 50 | 50 | 320 | 0.9 | 1000 | 64.2 |

**OLR: organic loading rate**

### 3.2.9 Procedure for constant head measurements of hydraulic conductivity in sand columns

The HC was determined from hydraulic experiments by determining the actual flow of water through the sand under constant head conditions. A product of Darcy's Law presented by

$$Q = kiA \qquad \text{Equation 2}$$

was used to calculate the HC. Through mathematical manipulation this formula can be re-written as

$$k = \frac{VL}{tAH} \qquad \text{Equation 3}$$

Where ($k$) is the coefficient of permeability, ($V$) is the volume of water collected in a certain time at constant head conditions, ($L$) is the length of the sample including constant head, ($t$) is the time taken to collect a certain amount of water under constant head conditions, ($A$) is the area of the sand and ($H$) is the constant head obtained.

The length and area were premeasured and calculated. Constant head conditions were obtained by pumping water through the sand and controlling the flow with pumps fitted with a variable speed drive. When the inflow and outflow rates were balanced under, the time taken to collect 20 L of water was recorded. Triplicate experiments were performed under three different constant head heights and the results calculated and recorded using

Darcy's formula. Three readings were taken from each column at each hydraulic head. There was no discernible difference between these replicate readings. These initial readings also only showed negligible differences at different hydraulic head for each column. The same was observed for the final readings for each column.

To determine the influence of microbial growth on HC, the measurements were repeated after the columns were fed with synthetic winery effluent (Section 3.2.6).

### 3.2.10 Microbial community fingerprinting

T-RFLP analyses were performed as described in Section 2.2.8

## 3.3 RESULTS AND DISCUSSION

### 3.3.1 Comparison of physicochemical properties of different sand types

#### 3.3.1.1 Mechanical fraction

It has been shown that the HC in wetlands generally (i) increases with increased particle size, (ii) decreases as uniformity of particle size increases, and (iii) is more uniform in saturated sands (Giraldi et al., 2009; Suliman, 2006). In soil and sand, the HC is primarily dependent on two factors: the porosity and the way in which the porosity is distributed in the substrate (Morin et al., 2010). In environments with similar grain size distribution, differences in the geometry of the grains can account for differences in HC because the porosity is dependent on inter-particle contact (packing of particles) (Narsilio et al., 2010). On a macro-scale, the distribution and connectivity of the porosity is important where topography is a contributing factor (Morin et al., 2010), but is unlikely to play a significant role in the HC in relatively homogenous systems such as the BSFs used in these studies.

The analysis of the mechanical (size) fraction of Malmesbury, Phillipi and Mix sands showed that sand constituted over 95% of all samples, with all three exhibiting highly similar grain size distributions (Figure 12). It is thus unlikely that the large disparity in HC (Section 3.3.3.2), was significantly related to particle size. The differences, on a micro-scale, were most likely attributable to variances in bulk porosity afforded by differences in grain geometry.

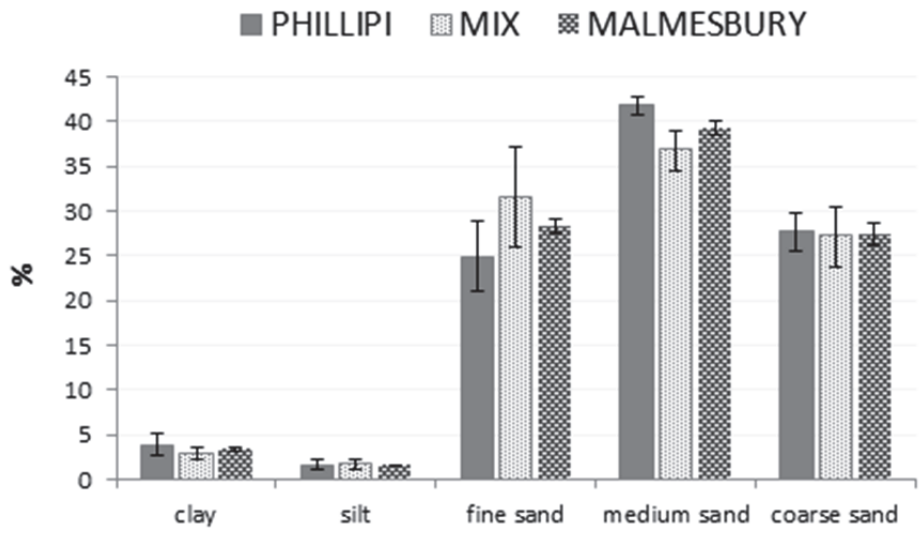


**Figure 12: Comparison of the mechanical fractions of three locally available sand types**

### 3.3.1.2 Concentrations of available micronutrients and heavy metals

The growth of microorganisms may be positively or negatively affected by the concentrations of available micronutrients and heavy metals, so large disparities in these fractions could lead to differences in the microbial community structures (Carson, 2007; Gadd, 2010). Fe, followed by K and P were the most abundant available micronutrients/heavy metals in all samples. However, the Mix and Malmesbury sand contained significantly more available Fe and K than the Phillipi sand in all samples (Figure 13). Apart from those elements included in Figure 13, B, Cd, Pb, As and Hg were also measured, but all were only present in concentrations of < 0.2 mg/kg sand.

### 3.3.1.3 Exchangeable cations, cation exchange capacity and major elements

Clay, organic matter and sesquioxides of soil particles carry negative charges, which may be variable (affected by ambient conditions, i.e. pH and salts) or permanent in nature. These negative charges are neutralized by an excess of cations (negative adsorption) and a deficit of anions (anion repulsion) (Sumner & Miller, 1996). The cation exchange capacity (CEC) gives an indication of the capacity of a soil to hold cations and should ideally be measured under field conditions (Sumner & Miller, 1996). The CEC should comprise the total of the measured cations.

The concentrations of exchangeable cations and the CEC showed that the amount of calcium extracted was significantly greater than the CEC in the Phillipi and Mix sands (Figure 14). The extraction method used did not account for the mineralogy of the sand, which on XRF and XRD analysis, was found to contain > 5% CaO in the form of calcite. The high Ca to CEC ratio was thus likely an experimental artefact due to dissolution of calcite during laboratory extraction (Sumner & Miller, 1996).

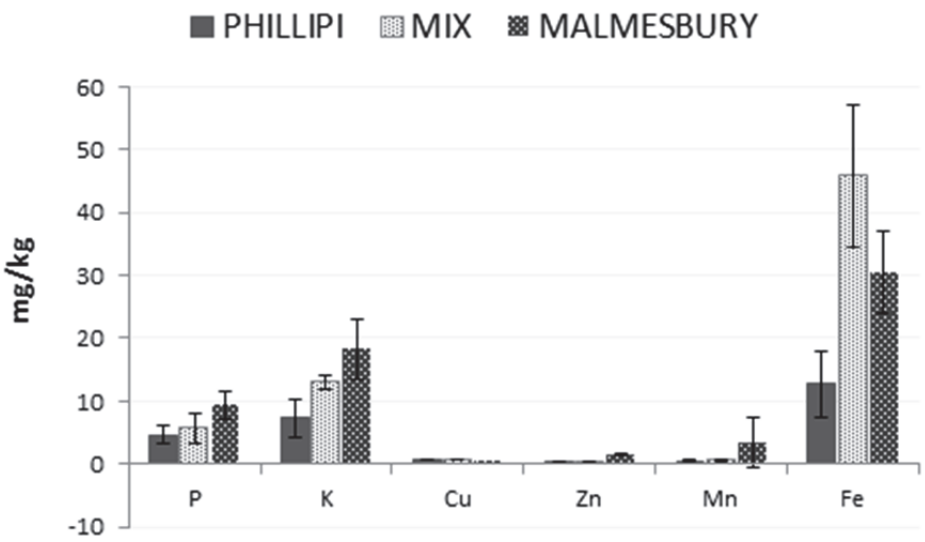


**Figure 13: Available micronutrients and heavy metals in concentrations > 0.2 mg/kg sand**

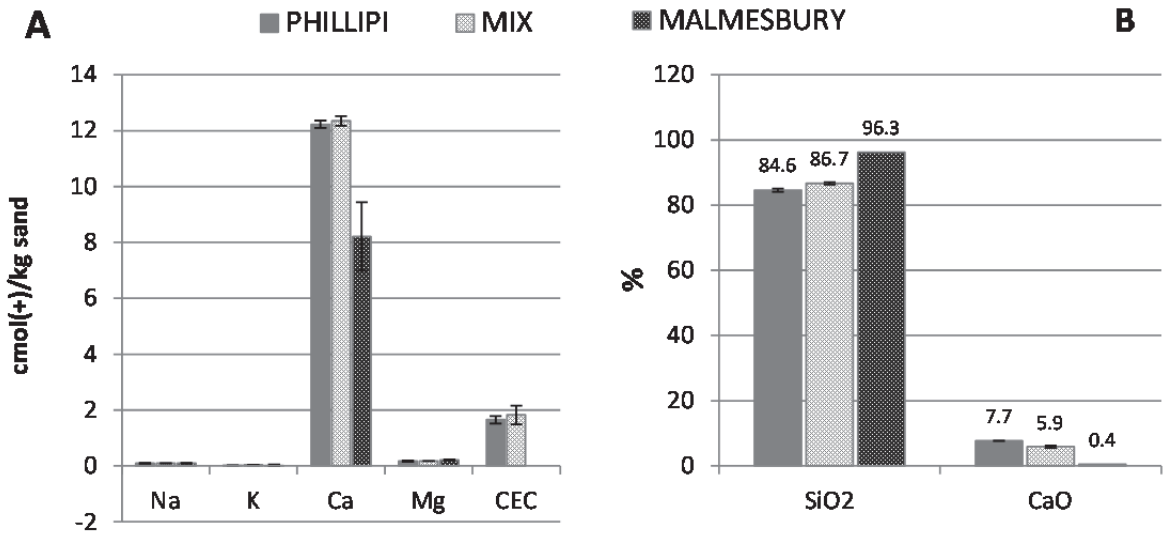


**Figure 14: Concentrations of exchangeable cations and the cation exchange capacity of locally available sand types (A), Percentage of oxides of major elements > 1% (B), cation exchange capacity of Malmesbury sand not determined**

The most abundant major element in all sand types was silica, with the highest concentration being determined in the Malmesbury sand and the lowest in the Phillipi sand. The converse was true for the second most abundant major element, calcium. This reflects the differences in origin of the sand. Unlike the Malmesbury sand, from an inland site, the

Phillipi quarry site is close to the ocean and the remnants of shells are clearly visible when viewing this sand type microscopically. The content of all other elements were low (Figure 15), but the samples containing Malmesbury and Mix sand showed significantly higher concentrations of Al and Fe, albeit still < 1%.

#### 3.3.1.4 Mineralogy

The qualitative mineralogy of the sand types was determined by XRD on three samples of each sand type. Silica, the most abundant major element was present as quartz in all samples. The second most abundant major element, calcium was present as calcite and aragonite in all Phillipi and Mix samples, one Malmesbury sample, and also as calcium aluminium fluoride in one sample of Mix. No other minerals were detected in the Phillipi samples and those that were detected in the Mix samples and Malmesbury samples (albite, microcline, hematite, chabazite, offretite, kayanite and sillimanite) were not common to more than one sample.

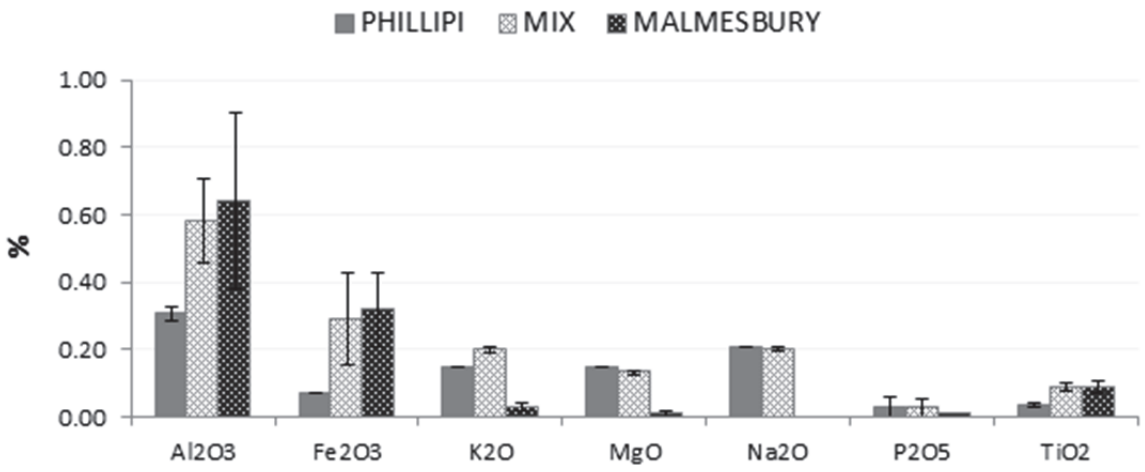


**Figure 15: Percentage of oxides of major elements < 1%. No MnO detected, $Cr_2O_3$ below detection limit**

### 3.3.2 Comparison of hydraulic properties of different sand types

From 2009, the BSFs at CPUT contained Malmesbury sand and the inherent SHC of the systems restricted the HLR of the CW systems to approximately 7.1 L/m$^3$ sand/day (Table 6). *Ad-hoc* testing showed that the HC afforded by Phillipi sand was far greater than Malmesbury sand and the systems were thus replaced with either Phillipi sand or Mix sand in 2012. Further testing revealed a vast difference in the SHC of the BSFs containing different sand types (Table 6). In comparison to the first batch of sand (Malmes. 1), the SHC of the BSFs containing a second batch of Malmesbury sand (Malmes. 2), Mix sand and Phillipi sand were approximately 6, 73 and 322 times faster, making both the Phillipi and

Mix more suitable candidates for use in BSFs from a hydraulic perspective. Literature confirms that the origins of hydraulic properties of sand are extremely complex, far beyond mere particle size considerations (Jarvis, 2008; Morin, 2009; Narsillo, 2010; Zeleke, 2005). In this case, it is hypothesised that the differences in hydraulic properties are most likely, on a micro-scale, to arise from differences in bulk porosity afforded by differences in grain geometry (Section 3.3.1.1). Indeed, the porosity that was determined for the Malmesbury sand was only 45% that of the Phillipi sand (Table 6). Further studies to confirm the hypothesis fell way beyond the scope of this project. Focus was placed only on the practical aspects of the HC phenomena that may impact the design and functional aspects of BSFs treating winery effluent.

**Table 6: Physical and operational parameters of biological sand filters and various locally available sand types**

| | Malmes. 1 | Malmes. 2 | Phillipi | Mix |
|---|---|---|---|---|
| ***Porosity (L/m$^3$sand)*** | 160 ± 16 | ND | 292 ± 20 | 259 ± 22 |
| ***Density (kg/L)*** | ND | ND | 1.67 ± 0.04 | 1.60 ± 0.03 |
| ***SHC (L/hr/m$^3$sand$^1$)*** | 0.29 ± 0.1 (n=48, 4 BSFs) | 1.84 ± 0.78 (n=24, 2 BSFs) | 93.32 ± 29.02 (n=16, 4 BSFs) | 21.12 ± 2.70 (n=8, 2 BSFs) |
| ***Hydraulic flow regime/mode*** | Flood-drain/batch | Flood-drain/batch | Flood-plug-drain/batch | Flood-plug-drain/batch |
| ***HLR (L/m$^3$sand/day)*** | ~7.1 | ~7.1 | ~22.9** | ~22.9** |
| ***Operational HRT (days)*** | ~22 | ND | 13 ± 1** | 11 ± 1** |
| ***tHRT (hr)**** | ~528.0 | ND | ~1.6 | ~6.2 |

***Calculated from maximum flow rate of unplugged systems under saturated conditions**
**** Rates applied during study period. These may be increased if required and functional**
**ND = not determined**

### 3.3.2.1 System hydraulic conductivity of biological sand filters filled with different sands

In the case of the BSFs filled with Phillipi sand, the magnitude of the hydraulic head had a significant effect on the outflow rate. The highest rates were found immediately after unplugging (t = 0, Figure 16A) and decreased notably after each subsequent reading. In contrast, the outflow from the BSFs filled with Mix sand remained stable, irrespective of hydraulic head, provided the systems were completely saturated (Figure 16B). This was also the case with outflow from the 'old' systems containing Malmesbury sand.

Previously, in the BSFs filled with Malmesbury sand, a large COD concentration-dependent decrease in SHC was demonstrated during feeding/amendment. There was close to 50%

reduction in SHC in the systems filled with Phillipi sand after 30 weeks amendment, but no reduction in the systems filled with Mix sand (Figure 17). During the operation of the latter, considerable amounts of clay particles washed out in the effluent. Is it possible that this countered the reduction in SHC due to the accumulation of biomass. Column experiments were used to gain absolute measures of HC and changes in HC in response to the build-up of biofilm/biomass (Section 3.3.2.2).

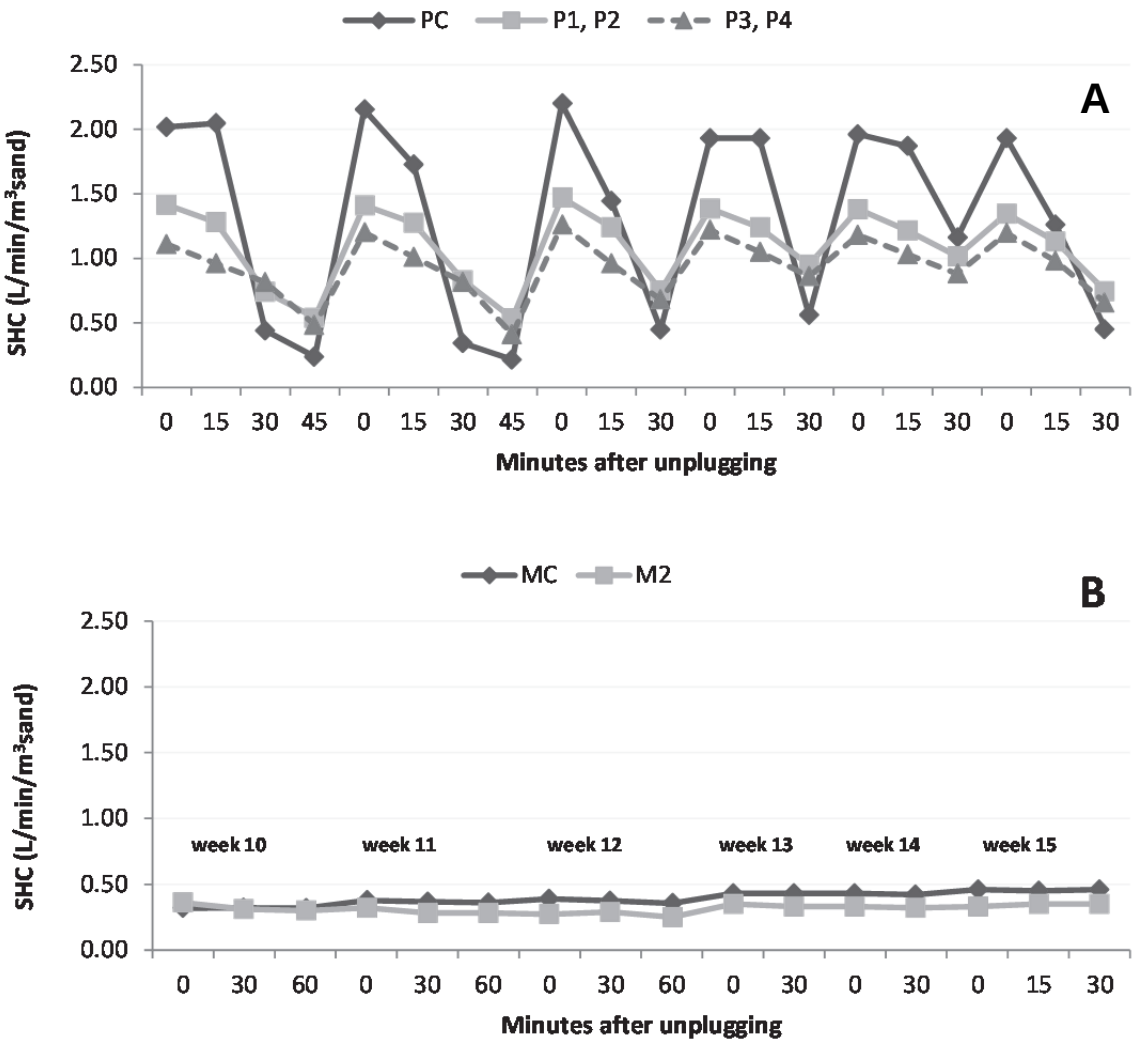


**Figure 16: System hydraulic conductivity measurements from biological sand filters containing Phillipi sand (A) and Mix sand (B) demonstrating the different effects of hydraulic head on each**

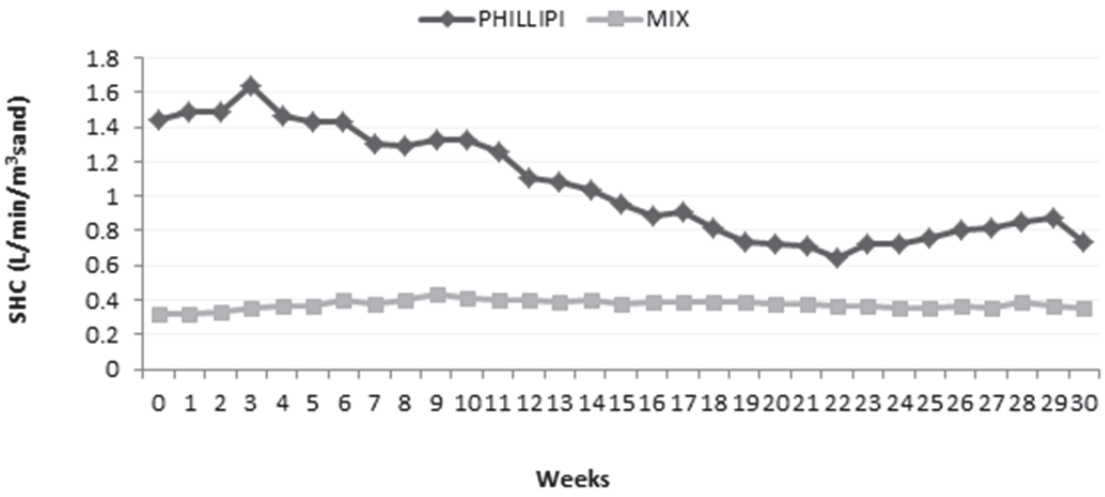


**Figure 17: Changes in system hydraulic conductivity during amendment of biological sand filters**

### 3.3.2.2 Determination of absolute hydraulic conductivity in sand columns before and after amendment to induce microbial growth

HC is an absolute value that is determined by the physical and chemical properties of the sand (porosity, mineralogy, particle size, and particle shape). It has been shown that the flow of water through sand can be retarded by biomass clogging (Serrano et al., 2010; Welz et al., 2011). It has also been demonstrated that bacterial strains that produce high amounts of exopolysaccharides contribute to clogging far more than those that do not (Vandeviviere and Baveye, 1992). Biomass clogging decreases the hydraulic loading of sand-filled (and other) CWs and BSFs and should be accounted for during the design of such systems.

The initial HC of three locally available sands in three experimental columns were compared. The reduction in HC associated with microbial biomass clogging in two of the sand types was also determined, using two of the three columns. The third column was designated as a control and was not fed/amended to induce microbial growth. To maximise biomass/biofilm accumulation in the two test columns, glucose and sources of nitrogen and phosphate were added to the synthetic wastewater to prevent nutrient limitation on microbial growth and exopolysaccharide production.

Over the three month experimental period, the HC of the Phillipi sand and Mix sand in the test columns was reduced by approx. 50% and 68%, respectively after feeding/amendment. In the control column, there was a comparatively small reduction in the initial HC of 8.35% and 29.20%, for the Phillipi and Mix sand, respectively after 3 months resting. The reduction in the control columns may have been influenced by settling of the sand over time, although this is unlikely because, to assist settling, water was run through the systems for at least 4 hours before the initial readings were taken. In addition, no more water was added during the three month resting period. Although the systems were unfed, they remained relatively

moist for some time over the three month period between the initial readings and the final readings. There was some visible algal growth on the exterior and it is highly likely that microbial biomass grew within the systems, taking advantage of the moist conditions and utilising residual nutrients within the sand environment. This would also explain why the column containing Mix sand was more affected than the column containing the Phillipi sand, as the slower HC of Mix sand would have allowed the column contents to remain saturated for longer. It is therefore hypothesised that the reduction in HC in the control systems was due to biomass growth.

The amount of biomass clogging is not only related to the wastewater composition, but also to the microbial community composition. In addition, even with pre-settled influent, a degree of clogging with suspended solids can be expected. Fortunately, in winery wastewater, most of this would be expected to be organic in nature and therefore potentially biodegradable. It is envisaged that these solids would be naturally degraded during off-peak seasons, or during scheduled resting of the BSFs. Nevertheless, to account for potential solids clogging, it is recommended that a safety margin of at least 10% over and above the experimental values is included when designing BSF systems for treatment of winery wastewater.

The expected ranges of HC for coarse, medium and fine sands, respectively are $9 \times 10^{-4}$ mm/s to 6 mm/s; $9 \times 10^{-4}$ mm/s to 5 mm/s, and $2 \times 10^{-4}$ to 2 mm/s (Domenico and Swartz, 1990). The ideal grain sizes of sand particles used in slow sand filters are 0.13 mm to 0.92 mm ($d_{10}$), which typically results in HC values of between 0.041 mm/s to 0.37 mm/s (Hendricks, 2011). For CWs containing sand and pea gravel, Sanford et al. (1995) reported HC ranges of between 1.7 mm/s and 5.3 mm/s, well above those described for slow sand filters.

The BSFs are intended to operate with a hydraulic loading in the range of slow sand filters, which are in essence BSFs. As expected from BSF system outflow readings that were determined in 2011/2012 (Welz et al., 2011), the HC of the Malmesbury sand was extremely low (0.0151 mm/s), precluding this sand for practical use in the systems. Biomass clogging experiments were therefore not conducted with Malmesbury sand. However, the HC of both the Mix sand and Phillipi sand fell within the expected range, both before and after induced biomass clogging (Figure 18).

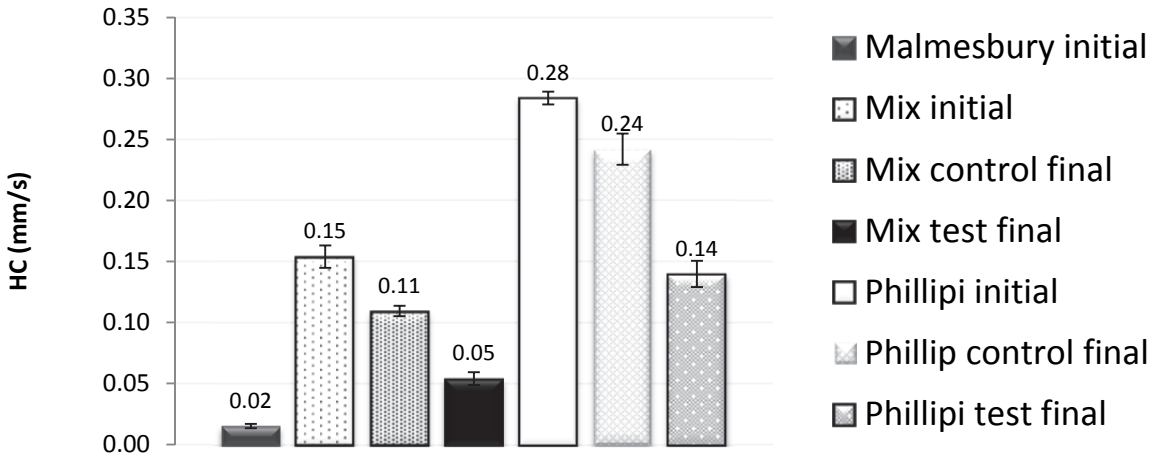


**Figure 18: Average hydraulic conductivity measurements determined from column experiments using three different sand types before (initial), and after either resting (control final) or feeding to induce biomass accumulation (test final)**

The results of the columns experiments show that, from a hydraulic perspective, the Phillipi sand is a good candidate for use as a substrate in BSFs treating winery wastewater in the Stellenbosch/Paarl/Cape Town winelands. As well as being the most readily available sand type in the area, (i) It has the highest HC of the three sand types tested, falling well within the operational range of slow sand filters, and (ii) It is the least affected by biomass clogging.

### 3.3.3 Comparison of organic removal performances in biological sand filters containing different sand types and amended with synthetic winery wastewater

The bulk flow of effluent from the BSFs containing Phillipi sand (PC, P1, P2) and Mix sand (MC, M1, M2) lasted for approximately one and two hours, respectively. Therefore, for comparative purposes, effluent samples were taken when all systems were unplugged (48 hr after amendment) and then 30 min thereafter in the case of the replicates containing Phillipi sand (48.5 hr) and 60 min thereafter (49 hr) in the case of the replicates containing Mix sand. With the exception of samples taken after the first amendment with synthetic wastewater, good intra-replicate reproducibility was demonstrated in all samples taken from M1 and M2. However, in P1 and P2, intra-replicate reproducibility was satisfactory in samples taken immediately after unplugging (48 hr, Figure 19), but often poor in those taken 30 min after unplugging. These results confirm that it is prudent to include replicates for such studies (Chapter 1). This phenomenon was almost certainly linked to SHC disparities in P1 and P2. During the first 3 weeks of the experiment, the COD removal performance in P1 and P2 was significantly better than that in M1 and M2, but by week 4 the performance of all systems was similar (Figure 19).

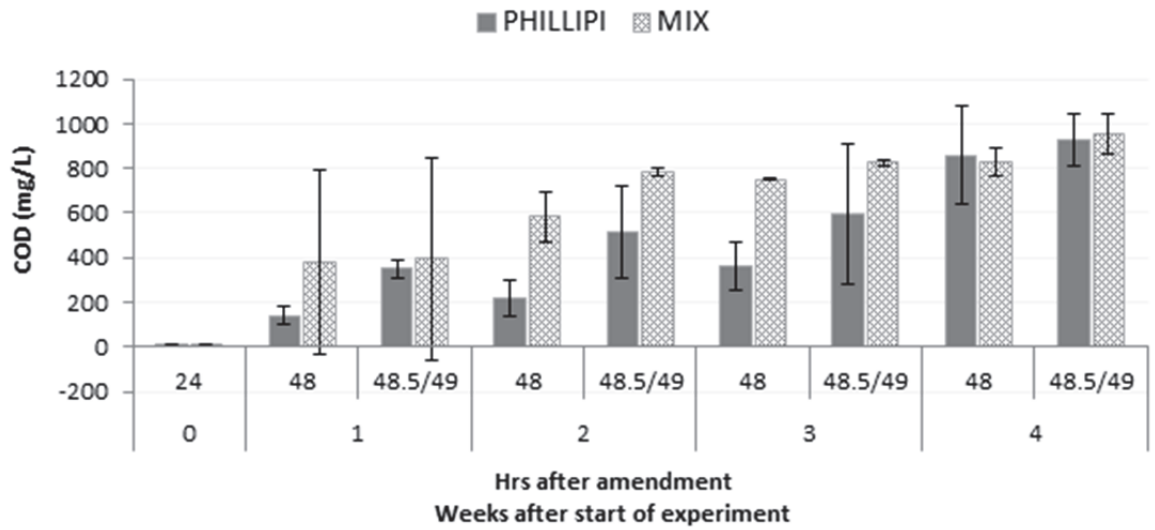


**Figure 19: Average effluent chemical oxygen demand from biological sand filter replicates containing Phillipi sand and Mix sand taken immediately after unplugging (48 hr after amendment) and 30 min after unplugging (48.5 hr after amendment, Phillipi) and 60 min after unplugging (49 hr after amendment, Mix)**

In the case of the non-phenolic organics that were identified in samples taken immediately after unplugging, acetate was the predominant substrate/metabolite in the replicates containing both Phillipi and Mix sand (Figure 20A). The degradation of acetate and other volatile fatty acids (VFAs) in the BSFs is discussed in further detail in Chapter 4. Ethanol was readily removed. Phenolic removal can take place by abiotic (chiefly adsorption) and biotic mechanisms (Welz et al., 2012). No vanillin (substrate) or vanillic acid (primary metabolite) was detected in the effluent samples. Small quantities of the substrate, gallic acid were detected in all systems after 3 weeks, but none thereafter. The presence of catechol, a metabolite formed during the microbial degradation of (poly)phenolics provided evidence that the biodegradation of phenolics occurred in all the BSFs (Figure 20B).

### 3.3.4 Effect of sand type on the microbial community structure in biological sand filters

Bacterial community fingerprinting using PCR and T-RFLP has been optimized during the course of this project (K5/2104) and a previous project (K5/1926). However, fungi can also play significant roles in bioremediation (Frey-Klett et al., 2011). This important sub-section of the microbial population was included in this study.

The methodology required protracted optimization. It was shown that fungal communities were present within the BSFs when performing PCR using the ITS primers (ITS1F and ITS4) described by White et al. (1990) and Gardes and Bruns (1993). Various PCR parameters were tested and in many instances, two specific bands were visualized on agarose gels.

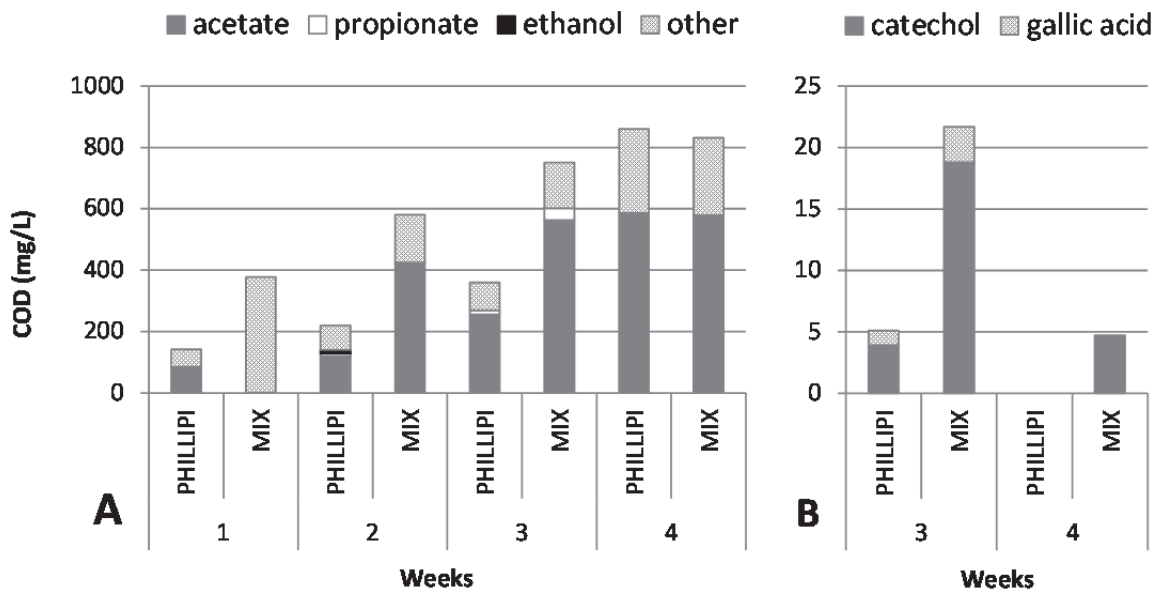


**Figure 20: Non-phenolic (A) and phenolic (B) substrate/metabolite profiles from effluent samples taken from biological sand filters containing Phillipi and Mix sand 48 hr after amendment**

According to Manter and Vivanco (2007), fragment sizes ranging from 420 to 825 bp represent the three fungal classes: ascomycetes (420-806 bp), basidiomycetes (503-825 bp) and zygomycetes (653-788 bp). The positive control, *Trametes pubescens* forms part of the basidiomycete family and the presence of similar sized bands observed on the agarose gel indicated that many of the sand samples contained basidiomycetes. The samples containing two bands were excised independently from the agarose gel and re-amplified by PCR. After the second round of PCR, more bands were present, probably indicating that additional fungal groups were present in the BSFs but were not sufficiently abundant to amplify initially.

Soil composition is considered to be a key factor that will determine which microbial communities will be present in the environment (Girvan et al., 2003). This has been confirmed by studies performed during the course of Project K5/2104 (described in Chapters 1 and 5). In this study, ANOSIM analyses of T-RFLP profiles showed that sand type (Phillipi or Mix) also influenced the microbial community composition in the BSFs. Significantly different bacterial assemblages were found in the surface sediments ($R = 0.704$ and $p < 0.05$), the deep inlet ($R = 0.486$ and $p < 0.05$), and the deep outlet ($R = 0.444$ and $p = < 0.1$) of the BSFs replicates containing Phillipi or Mix sand. The overall fungal model also showed significant differences ($R = 0.205$ and $p = < 0.05$) in the composition of the fungal communities present in the BSFs containing Phillipi or Mix sand.

### 3.3.5 Conclusions

There is little difference in the chemical composition or mechanical fractions of Phillipi, Malmesbury and Mix sand. However, there are enormous differences in the HC, which is most likely attributable to grain geometry. The HC of Malmesbury sand renders this substrate impractical for “real world” application in BSFs.

Although differences were noted in the microbial community structures, the performance of BSFs containing Phillipi or Mix sand was shown to be similar when treating synthetic winery wastewater. Even after induced biomass clogging, the HC of Phillipi and Mix sand fell well within the range suggested for use in slow sand filters. However, Phillipi sand is easier to obtain, does not need to be mixed, exhibits a higher (and thus more flexible) HC, and is less affected by biomass clogging. This sand is therefore seen as a good candidate for use as a substrate for use in BSFs in the Cape Town environs.

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# CHAPTER 4:
# TREATMENT OF SYNTHETIC WINERY WASTEWATER IN BIOLOGICAL SAND FILTERS: ANALYSIS OF ORGANIC DEGRADATION AND MICROBIAL COMMUNITY STRUCTURE AND FUNCTION

## 4.1 INTRODUCTION

The bulk of winery wastewater emanates from cleaning equipment, vats and floors of wine cellars during seasonal activities associated with winemaking (Bories and Sire, 2010; Vlyssides et al., 2005). Winery effluent is typically characterised by a high COD and low pH. However, the volume, COD range, and organic composition is directly related to cellar activities, including must production, fermentation processes, maturation/stabilization processes, and decanting, and is thus prone to seasonal variation (Bolzonello and Rosso, 2013; Vlyssides et al., 2005). Each grape varietal also has a unique organic fingerprint, so that, for example, wastewater generated during crushing of late harvest grape varietals has a comparatively high sugar and low phenolic content, while the converse is true for wastewater generated during the crushing of early harvest red grape varietals (Bolzonella and Rosso, 2013; Devesa-Rey et al., 2011; Malandra et al., 2003).

In many countries, effluent COD is used as a benchmark by regulatory authorities when determining wastewater discharge requirements for biodegradable industrial wastewater, and in most instances, no further characterization of the organic fraction is required (Andreoletta et al., 2009; Aybar et al., 2007; Mosse et al., 2011). However, in terms of biodegradability, there is considerable variation in the ratios of the different fractions of winery effluent, including readily biodegradable sugars, moderately biodegradable alcohols and slowly biodegradable/recalcitrant phenolics (Devesa-Rey et al., 2011; Malandra et al., 2003; Vlyssides et al., 2005).

There is a gap in the knowledge quest for a holistic understanding of biological organic winery wastewater treatment processes, particularly concerning the degradation and formation of different substrates and metabolites, respectively. There are scant literature descriptions which detail the chemical composition of winery wastewater during or after treatment. Ganesh et al. (2010), distinguished between VFA and non-VFA COD in conventional activated sludge and membrane bioreactor systems treating winery effluent, and de la Varga et al. (2013), measured methane and carbon dioxide emissions in subsurface flow CWs. Other studies have limited the analyses to COD and/or total phenolic removal when assessing the performance of biological systems used for the treatment of winery wastewater.

In this study, BSFs were amended with two different formulations of synthetic winery wastewater and results compared. The primary aim of the study was to determine how differences in wastewater composition would affect the metabolic processes of the functional microbial consortia. This was achieved by (i) chemically characterizing pore water samples taken at different spatial points in the systems, and (ii) determining the similarities in the microbial community structures in the same spatial niches before and after amendment with two different formulations of synthetic winery wastewater.

## 4.2 MATERIALS AND METHODS

### 4.2.1 Set-up, mode of operation and feeding/amendment of biological sand filters

The set-up and mode of operation of the BSF were as described previously in Sections 2.2.1 and 3.3.1, respectively. In this study, three BSFs containing Phillipi sand were used. Two of the BSFs were designated as experimental replicates (BSF1, BSF2) and one was designated as a control (BSFC) (Figure 21). The average saturated permeation rate of effluent flow from the three systems before feeding/amendment was 93 ± 29 L/$m^3$ sand/day (n = 12).

After three months equilibration, synthetic winery wastewater was added to the influent to BSF1 and BSF2 (amendment). An incremental priming procedure was adopted, whereby the synthetic winery wastewater was added in increasing concentrations in order to induce acclimation of the microbial communities as previously described (Welz et al., 2011).

This study compares the results obtained for two different synthetic winery wastewater formulations, the first consisting of ethanol, acetate, vanillin and gallic acid in a 10:10:1:1 ratio (SW1; Table 7) and the second consisting of ethanol, glucose, vanillin and gallic acid in the same respective ratio (SW2; Table 7). The final COD for both formulations was 2027 mg/L.

### 4.2.2 Sampling

#### 4.2.2.1 Effluent and sand sample collection

Sand and effluent samples were collected as described in Sections 2.2.2.1 and 2.2.2.2, respectively. Sand samples were extracted from each BSF (baseline) and after amendment with SW1 and SW2 (Figure 21).

#### 4.2.2.2 Pore water sample collection

Pore water samples were extracted 24 hr and 48 hr after feeding/amendment, the latter before unplugging the systems. The pore water samples were taken using syringes to extract samples from the bottom of in-situ polyvinyl chloride pipes, perforated at appropriate depths (Figure 21). The in-situ pipes were located longitudinally 0.3 m from the inlet and outlet and either perforated between 0 and -50 mm (surface samples) or between

-200 and -250 mm (deep samples). All samples were analysed immediately after collection. Each pipe was fitted with a bung at the bottom so that effluent could only enter at the desired depths, but collected within the pipes for aspiration sampling. Before sampling, any residual effluent was extracted by aspirating and discarding the aspirate four times. Thereafter, distilled water was aspirated four times to 'clean' the syringes. Samples were analysed immediately after collection.

**Table 7: Operating parameters applied to the biological sand filters**

| Parameter | Week | BSFC | BSF1, BSF2 |
|---|---|---|---|
| ***HLR (L/m$^3$sand/day)*** | 1-37 | 22.9 | 22.9 |
| ***OLR (g COD/m$^3$sand/day)**** | 1-14 | 0.6 | 0.6 |
| | 15-17 | 0.6 | 12.0 |
| | 18-20 | 0.6 | 46.4 |
| | 21-25 | 0.6 | 0.6 |
| | 26-28 | 0.6 | 12.0 |
| | 29-31 | 0.6 | 46.4 |
| ***Influent COD (mg/L) **** | 1-14 | 27 | 27 |
| | 15-17 | 27 | 527 (SW1) |
| **SW1/SW2: COD ratio 10:10:1:1** | 18-20 | 27 | 2027 (SW1) |
| **Ethanol:acetate:vanillin:gallic acid/** | 21-25 | 27 | 27 |
| **Ethanol:glucose:vanillin:gallic acid** | 26-28 | 27 | 527 (SW2) |
| | 29-31 | 27 | 2027 (SW2) |

**HLR = hydraulic loading rate OLR = organic loading rate COD = chemical oxygen demand**
**SW1/SW2 = synthetic winery wastewater first formulation/second formulation**
***Includes contribution from glucose (27 mg COD/L) in basal nutrient solution**

### 4.2.3 Characterization of influent, effluent and pore water

COD concentrations, identification and quantification of sugars, acids, alcohols and phenolics, as well as the conversion of concentrations into COD equivalents and compilation of metabolic profiles was performed using the same methods, calculations and equipment as previously described (Section 3.2.3). The redox potential (Eh) and pH of freshly collected pore water samples were determined using a pH700 meter and relevant probes (Eutech Instruments, Singapore).

### 4.2.4 Bacterial community fingerprinting

Total metagenomic DNA extraction, PCR amplification, purification and restriction digest, T-RFLP, and statistical analyses were performed as described in Section 2.2.6.

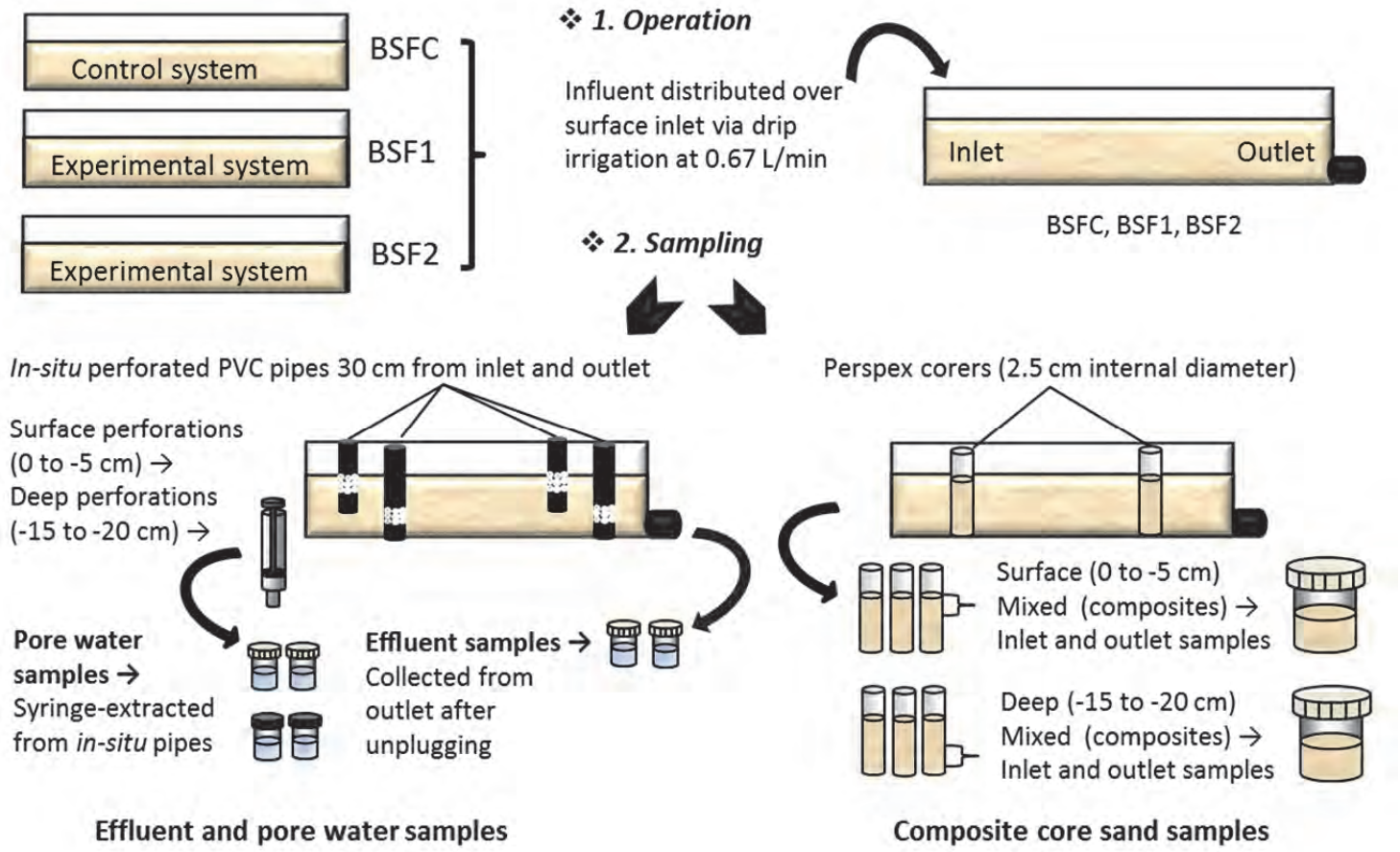



**Figure 21: Schematic diagram showing the set-up, operation and sampling procedures used during the study**

## 4.3 RESULTS AND DISCUSSION

### 4.3.1 COD removal in biological sand filters

#### 4.3.1.1 Overall COD removal

To ascertain overall organic removal, the weekly effluent COD concentration was measured. The same temporal trends were seen in both experimental replicates (BSF1 and BSF2) (Figure 22A). However, although the set-up, mode of operation and experimental protocol was identical, during amendment with high concentrations of SW1 (containing acetate), there were notable differences in the results obtained from BSF1 and BSF2. Differences were also found in the microbial community structures. This reiterates the need to replicate experiments of a biological nature, even if conducted on a relatively large scale (Hurlbert, 2013; Morrison and Morris, 2000).

As hypothesized, the effluent COD was higher during amendment with SW1 (containing acetate), than with SW2 (containing glucose) at the end of the respective experimental periods (week 20 and 31). Although the highest COD concentration was measured during amendment with SW2 (during week 30), a dramatic decrease was seen during week 31. In contrast, during amendment with SW1, there was an increase in effluent COD from week 19 to 20. This finding strongly suggests that the microbial communities had rapidly adapted (acclimated) to SW2, but not SW1 at this stage (Welz et al., 2012). The COD of the control system (BSFC) was consistently less than 25 mg/L.

#### 4.3.1.2 COD removal in spatial niches of sand bioreactors

In order to link qualitative and quantitative organic degradation taking place in four different spatial niches of the BSFs, pore water samples were extracted from the surface and deep substrata at the inlet and outlet (Figure 22B to 22E). The COD concentrations measured during amendment with SW2 were typically lower than those measured during amendment with SW1 in all niches, particularly at high influent concentrations. This clearly demonstrated that, as expected, the wastewater containing acetate was more recalcitrant than that containing glucose. From week 18 until the end of the experiment, the lowest COD concentrations were measured at the deep outlet, irrespective of the wastewater composition. During amendment with SW1, the highest COD concentrations were found at the deep inlet (Figure 22C), while during amendment with SW2, the highest concentrations were found at the superficial outlet (Figure 22D). Further qualitative analyses and discussion on possible reasons for these differences are presented in section 4.3.3.

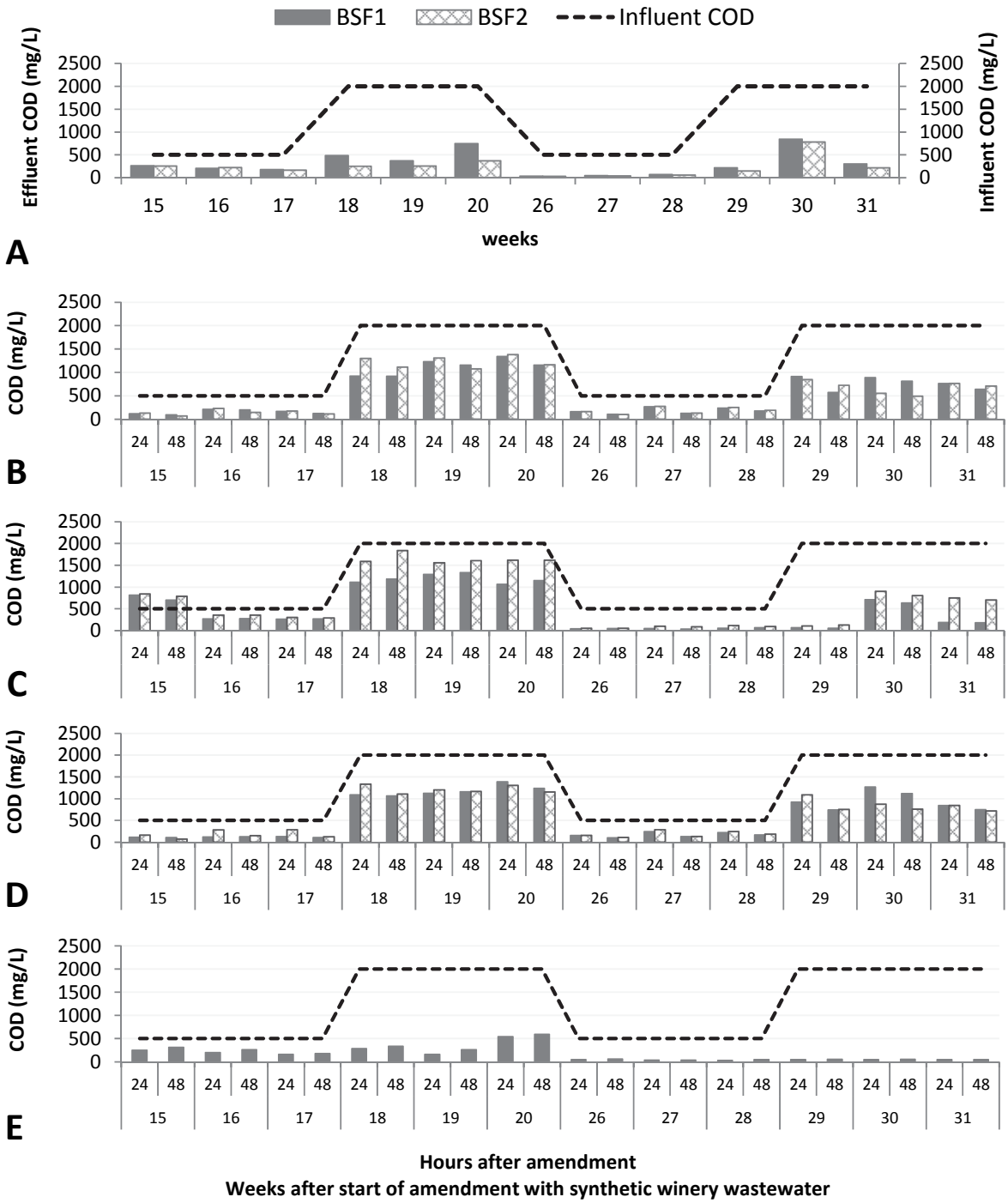



**Figure 22: Chemical oxygen demand measured in effluent samples (A) and pore water samples samples (B=superficial inlet; C=deep inlet; D=superficial outlet; E=deep outlet) of two BSF replicates during amendment with synthetic winery wastewater with respective overall COD concentrations of 527 mg/L (weeks 15-17 and 26-28) and 2 027 mg/L (weeks 18-20 and 29-31), and containing either acetate (200 mg COD/L: week 15-17; 800 mg COD/L: week 18-20) or glucose (200 mg COD/L: week 26-28; 800 mg COD/L: week 29-31), in the formulations**

### 4.3.2 Non-phenolic metabolism in spatial niches of biological sand filters

During amendment with increased concentrations of SW1 and SW2 (2 027 mg COD/L), notable qualitative and quantitative differences were found in the substrate and metabolite profiles compiled from analysis of pore water samples taken from the different spatial niches: superficial inlet (Figure 23A), deep inlet (Figure 23B), superficial outlet (Figure 23C) and deep outlet (Figure 23D).

#### 4.3.2.1 Spatial differences between superficial inlet and outlet

Spatial qualitative and quantitative chemical differences were noted between the two superficial niches (inlet and outlet; Figure 23A, 23C). In particular, during amendment with SW1, there was less acetate and more 'other' (unidentified) metabolites at the outlet than at the inlet. The 'other' was assumed to be primarily phenolic in origin because the detection method was able to identify all of the non-phenolic organics expected in winery wastewater (as characterised by Malandra et al., 2003). Tracer experiments have shown that the hydraulic operation of the BSFs creates a plug flow of wastewater both vertically (from surface to depth) and horizontally (from inlet to outlet). The hydraulic flow patterns in the SBs gave rise to degradation gradients from inlet to outlet. During this study, concentrations of substrates and metabolites were so low at the deep outlet (Figure 23D), that this niche has largely been excluded from this discussion.

#### 4.3.2.2 Spatial differences between superficial and deep niches

Differences were noted between the profiles compiled from the superficial and deep niches of the BSFs. During amendment with SW1, there were higher concentrations of ethanol and lower concentrations of propionate, while during amendment with SW2, there were higher concentrations of lactate and residual glucose from the superficial and deep niches, respectively (Figure 23). In addition, the redox status (Eh) was consistently higher in the superficial spatial niches [average: 102 ± 33 mV; range: 28 to 157 mV (n = 48)] than in the deep spatial niches [average: 25 ± 33 mV, range: -18 mV to 92 mV (n = 48)]. In this study, the degradation of all the non-phenolic organic substrates, with the possible exception of acetate, took place preferentially under the reduced redox environments of the deep niches. These results are contrary to expectations deduced from literature. Oxygen mass transfer rates are enhanced in VSSF and HSSF CWs operated in batch mode because atmospheric gases are drawn into the substratum during drainage cycles; it has been consistently reported that organic degradation is enhanced in these systems (with higher redox status) when compared to conventional HSSF systems operated in continuous mode (Dusek et al., 2008; Fan et al., 2013; Pedescoll et al., 2011; Tietz et al., 2008; Van der Moortel et al., 2009).

#### 4.3.2.3 Metabolism of volatile fatty acids

During amendment with SW1 at high concentrations, VFAs typically accounted for the major fraction of the COD, in the order of deep inlet > superficial inlet > superficial outlet (Figure 3). At the deep inlet, this held true irrespective of whether acetate was present in the wastewater (SW1) or not (SW2), suggesting higher formation and/or lower degradation in this niche. Propionate consistently accumulated in the BSFs, especially in the deep niches, albeit in lower concentrations than acetate. Traces of butyrate were also detected in the deep niches.

Acetate is a pivotal substrate/metabolite involved in the central and subsidiary microbial metabolic pathways, particularly in association with co-enzyme A (Wolfe, 2005). It is arguably the most common metabolite that is produced during the biodegradation of many organics, including those present in synthetic winery wastewater (vanillin, gallic acid, acetate and ethanol) (Thauer et al., 1977; Welz et al., 2011, 2012). Many organisms within microbial consortia are capable of both assimilatory acetogenesis and dissimilatory acetate utilization (Wolfe, 2005). The 'switch' from excretion (during acetogenesis) and adsorption (during utilization) takes place when readily available acetogenic carbon sources are expended (Wolfe, 2005). In saturated anaerobic environments, acetate is also utilized exergonically by acetoclastic methanogens to produce methane and carbon dioxide. Alternatively, syntropic, endergonic acetate oxidation can take place provided the partial pressure of hydrogen is kept at a low level (largely by hydrogenotrophic methanogens) (Botch and Conrad, 2011; Conrad and Klose, 2011). Acetate is also the main substrate involved in the formation of propionate and other intermediary fatty acids under anaerobic conditions. When a high flux of electrons is produced during the fermentation of organics, the proton reducing and/or hydrogen transfer reactions become saturated; under these circumstances, electron sink products, including acetate and propionate, are formed (Cohen et al., 1982; Thauer et al., 1977).

It is clear that the accumulation and biodegradation of VFAs is influenced by the redox status and the availability of acetogenic carbon sources. It is therefore unlikely that significant biodegradation of acetate will take place in BSFs until preferred substrates have been depleted. It is possible that alternating the redox status may enhance mineralization of residual organics triggering functional members of the microbial consortia to 'switch' to dissimilatory acetate utilization, while still supporting acetoclastic methanogenesis.

#### 4.3.2.4 Metabolism of ethanol and glucose

Total degradation of ethanol took place in the deep niches. In the superficial niches, degradation was typically better during amendment with SW2 (no acetate), than during amendment with SW1 (Figure 23). The catabolic pathways for both ethanol and acetate culminate in the production of acetyl coenzyme A. It is therefore suggested that ethanol degradation in the BSFs was repressed by feedback inhibition mechanisms when higher

amounts of acetate were present. Higher concentrations of ethanol were also detected at the surface inlet than at the outlet, a factor related to plug flow and the presence of degradation gradients (see section 4.3.2.1).

As expected, the substrate, glucose, was only found in the pore water samples during amendment with SW2. In addition, the metabolite, lactate (up to 300 mg/L) was also only detected during amendment with SW2 and only in the surface niches. Due to plug flow and associated degradation gradients, higher concentrations of glucose (substrate) and lower concentrations of lactate (metabolite) were found in the surface inlet samples, than in the surface outlet samples (Figure 23).

During glucose catabolism, pyruvate produced from classical and alternative glycolytic pathways will enter the energetically more favourable tricarboxylic acid respiratory pathway if electron acceptors such as oxygen and acetyl coenzyme A are readily available (Atlas, 1996). If electron acceptors are limited, fermentation of pyruvate to lactic acid and/or ethanol and/or volatile fatty acids, and/or mixed acids occurs (Atlas, 1996). The fact that significant concentrations of lactate were detected in the surface niches only strongly suggests that the drive to fermentative metabolism was not due to lack of electron acceptors, as higher concentrations of oxygen and higher redox conditions occur at the surface.

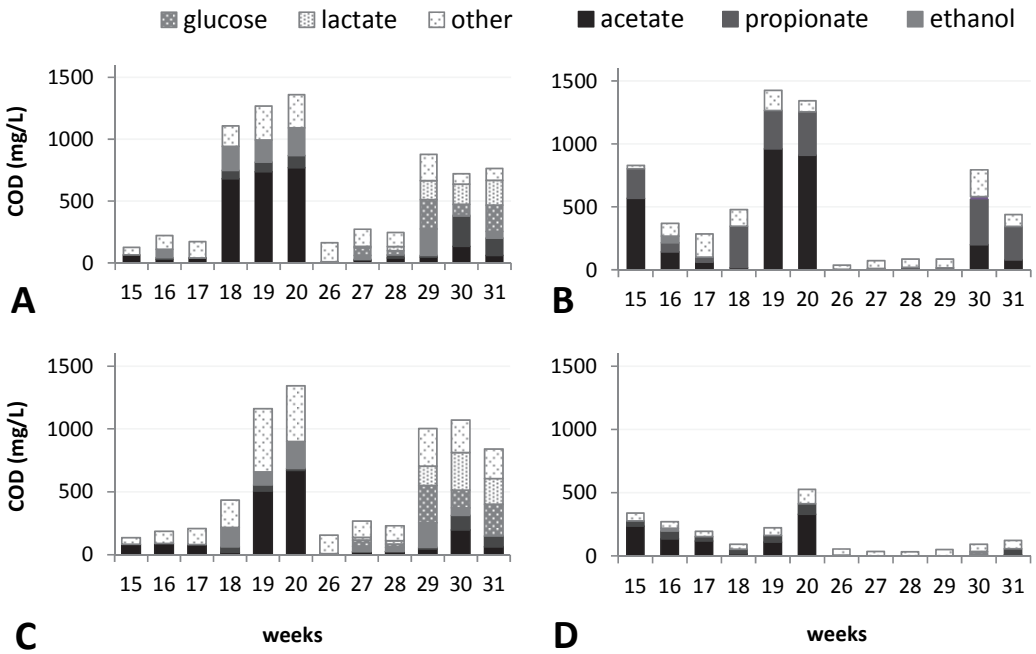



**Figure 23: Chemical oxygen demand substrate/metabolite profiles for sugars, acids and alcohols of pore water samples taken from the superficial inlet (A), deep inlet (B), superficial outlet (C) and deep outlet (D) of two BSF replicates 24 hours after amendment with synthetic winery wastewater**

Results strongly suggest that the competition for co-enzyme A by the acetoclastic and ethanol degradation pathways was the primary reason for the drive toward energetically less favourable fermentation. This is supported by the fact that other products of pyruvate fermentation reactions (e.g. propionate) were also found in higher concentrations in the surface samples during amendment with SW2 than during amendment with SW 1.

### 4.3.3 Phenolic removal in sand bioreactors

In a previous study, it has been shown that phenolic removal in BSFs takes place via adsorption and biodegradation (Welz et al., 2012). In this study, at the end of amendment with each wastewater formulation, the total phenolic concentrations in the effluent from BSF1 and BSF2 were 11.0 ± 0.0 and 5.0 ± 1.4 mg GAE/L (week 19) and 25.1 ± 0.0 and 19.7 ± 0.4 mg GAE/L (week 30), respectively. No phenolics were detected in samples from the control (BSFC). The influence of wastewater composition on phenolic removal in different spatial niches was accomplished by analysis of pore water samples taken at the end of amendment with SW1 and SW2 (week 20, week 31) from the superficial inlet (Figure 24A), the deep inlet (Figure 24B), the superficial outlet (Figure 24C) and the deep outlet (Figure 24D). In addition, analyses of pore water samples taken during amendment with SW2 were used to (i) assess temporal trends in phenolic removal (week 26 to 31; Figure 5), and to (ii) characterise the phenolics in the samples.

#### 4.3.3.1 Spatial differences between superficial and deep niches

Excellent removal of total phenolics was achieved in the deep niches of the BSFs, irrespective of wastewater composition (Figure 4B, 4D), but was markedly reduced in the superficial niches (Figure 4A, 4C). This was contrary to expectations that the higher redox conditions in the surface niches would translate into higher phenolic biodegradation rates. This pattern was also noted with the non-phenolic substrates (Section 4.3.2).

#### 4.3.3.2 Influence of wastewater composition in superficial niches

In both superficial niches, moderate removal of phenolics was seen during amendment with SW1, with pore water total phenolic concentrations ranging between 41.5 and 76.5 mg GAE/L (influent: 250 mg GAE/L). However, extremely poor removal was seen during amendment with SW2, with pore water concentrations ranging between 207.2 and 290.8 mg GAE/L. Although residual, un-degraded phenolics and phenolics with comparatively high GAE values may have impacted on the results, it was clear that minimal phenolic degradation took place. The difference in phenolic concentrations during amendment with SW1 and SW2 could not be attributed to residuals because, following amendment with SW1, the BSFs were fed with basal nutrients until no residual wastewater components were detected in effluent or pore water samples (week 21 to 25). In addition, glucose was degraded more readily than acetate in the BSFs (Section 4.3.2).

It is therefore proposed that substrate competition between phenolics and glucose (SW2) had a greater inhibitory effect on phenolic degradation than substrate competition between phenolics and acetate (SW1).

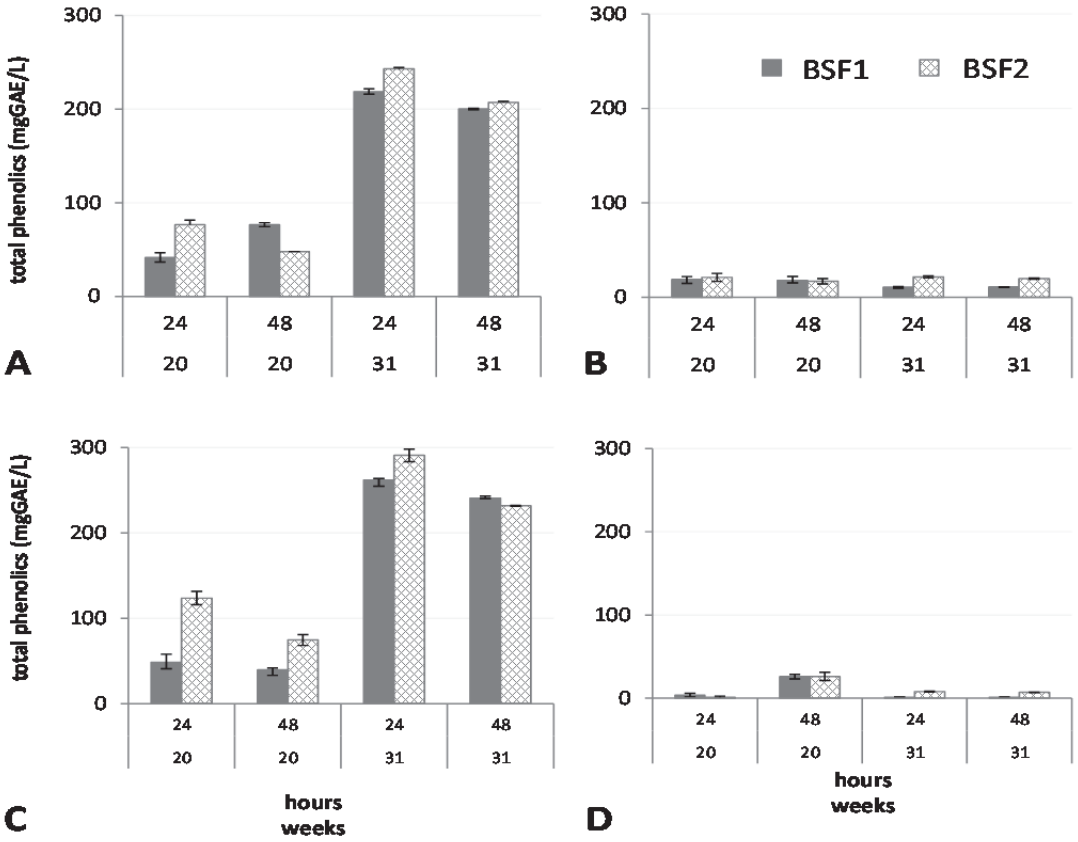



**Figure 24: Comparison of the total phenolics concentrations in the four environmental niches at the end of amendment with synthetic winery wastewater containing either acetate (week 20) or glucose (week 31) in the formulation. A = superficial inlet; B = deep inlet; C = superficial outlet; D = deep outlet. Error bars show the standard deviation from the mean of triplicate assays**

### 4.3.3.3 Temporal trends in superficial niches

The highest phenolic concentrations were obtained in the superficial niches during amendment with SW2. To assess whether there were any noteworthy temporal trends in phenolic removal, pore water samples were taken each week 24 and 48 hr after amendment with SW2 (Figure 25). Week 30 is excluded because analyses were delayed and phenolics in the samples polymerized before completion.

The total phenolic concentrations measured in the superficial samples were similar to influent values (Figure 25A). These results do not infer that no removal was taking place because (i) residual phenolics from previous amendments also contribute to effluent concentrations, and (ii) in all instances, the phenolic concentration decreased between 24 and 48 hr after amendment (Figure 25A).

#### 4.3.3.4 Characterisation of phenolic substrates and metabolites in superficial niches

A number of unidentified peaks were present in the chromatogram derived from phenolic analyses using HPLC. This is in contrast to the non-phenolic organics, where all peaks in the respective HPLC chromatograms were identified. Due to the overwhelming number of phenolic compounds in existence, complete phenolic characterisation was beyond the scope of this study. However, the substrates (gallic acid and vanillin), the primary breakdown product of vanillin (vanillic acid) and the common potentially toxic metabolite, catechol were identified and quantified. All concentrations were converted into gallic acid equivalents to evaluate the relative contribution of each the total phenolics in the samples. Vanillic acid and gallic acid accounted for approximately half of the phenolics, with the other half being unidentified (other) (Figure 25B, 25C).

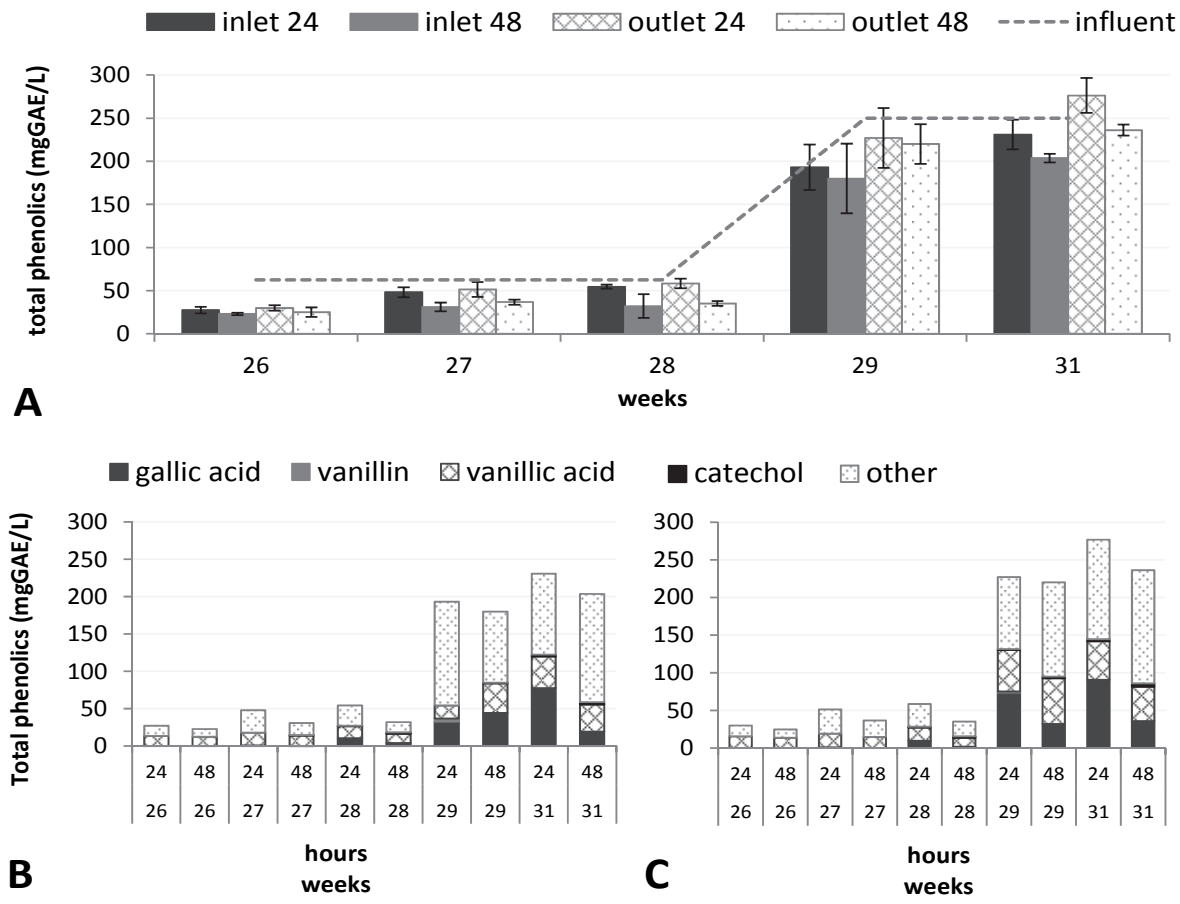



**Figure 25: Temporal changes in the average concentration of total phenolics in the superficial (A) and deep (B) niches during amendment with synthetic winery with an influent phenolic concentration of 62.5 mg GAE/L (weeks 26-28), and 250.0 mg GAE/L (weeks 29-31). Error bars indicate the standard deviation from the mean of triplicate assays of samples taken from each biological sand filter replicate**

### 4.3.4 Analysis of the bacterial community structure

#### 4.3.4.1 Effect of spatial niche (superficial/deep) on the bacterial community structures

In order to focus on the impact of spatial niches on the bacterial community structure, no distinction has been made between samples taken before and after wastewater amendment on the first NMDS plot (Figure 26A). On this plot, most of the points representing the bacterial community structures from the superficial niches (unfilled points) group separately from those representing the deep niches (black filled points), irrespective of whether the BSFs were amended with SW1, SW2 or not amended. The clustering of the sample points strongly suggests that the spatial niche (superficial versus deep) had more of an impact on the bacterial community structure than the presence of synthetic winery wastewater. The results also confirm that different physicochemical conditions attributable to redox status influenced both the microbial community structure and function (Sections 4.3.1, 4.3.2, 4.3.3).

#### 4.3.4.2 Effect of synthetic winery wastewater on the bacterial community structure

The effect of synthetic winery wastewater on the bacterial community structures is depicted in Figure 26B. As with Figure 26A, the points representing the samples taken from the superficial niches (distinguished by an 'S') generally cluster on the opposite side of the NMDS plot to those taken from the deep niches (distinguished by a 'D'). To allow more thorough analyses of the effect of winery wastewater on the bacterial communities in the different niches, separate NMDS plots were compiled for the superficial and deep niches.

##### Effect of synthetic winery wastewater on the bacterial community structures at the surface

Before amendment with synthetic winery wastewater (baseline), all points representing the bacterial communities in the superficial niches share 20% similarity (Figure 26C). After amendment with SW1 and SW2, all points share 20% and 40% similarity, respectively.

At baseline, the points representing the bacterial communities from the inlet and outlet of each BSF cluster closer to one another than to points from any of the other replicates. The points representing the control BSF are well separated from those of BSF1 and BSF2. A transient leak had developed in the control system the previous week resulting in the surface becoming dry. This may explain the anomalous result.

After amendment with SW1, points representing the bacterial communities at (i) the inlet of both experimental BSFs (BSF1, BSF2) group together, showing 60% similarity to one another and 40% similarity to the control, and at (ii) the outlet of both experimental BSFs (BSF1, BSF2) group together and are well separated from all other data points, showing 40% similarity to one another, but only 20% similarity to the control. It is inferred from these results that SW1 had an impact on the bacterial community structure, which was notably

greater at the outlet than at the inlet. It is suggested that this stemmed from differences in the chemical milieu resulting from substrate degradation gradients from inlet to outlet.

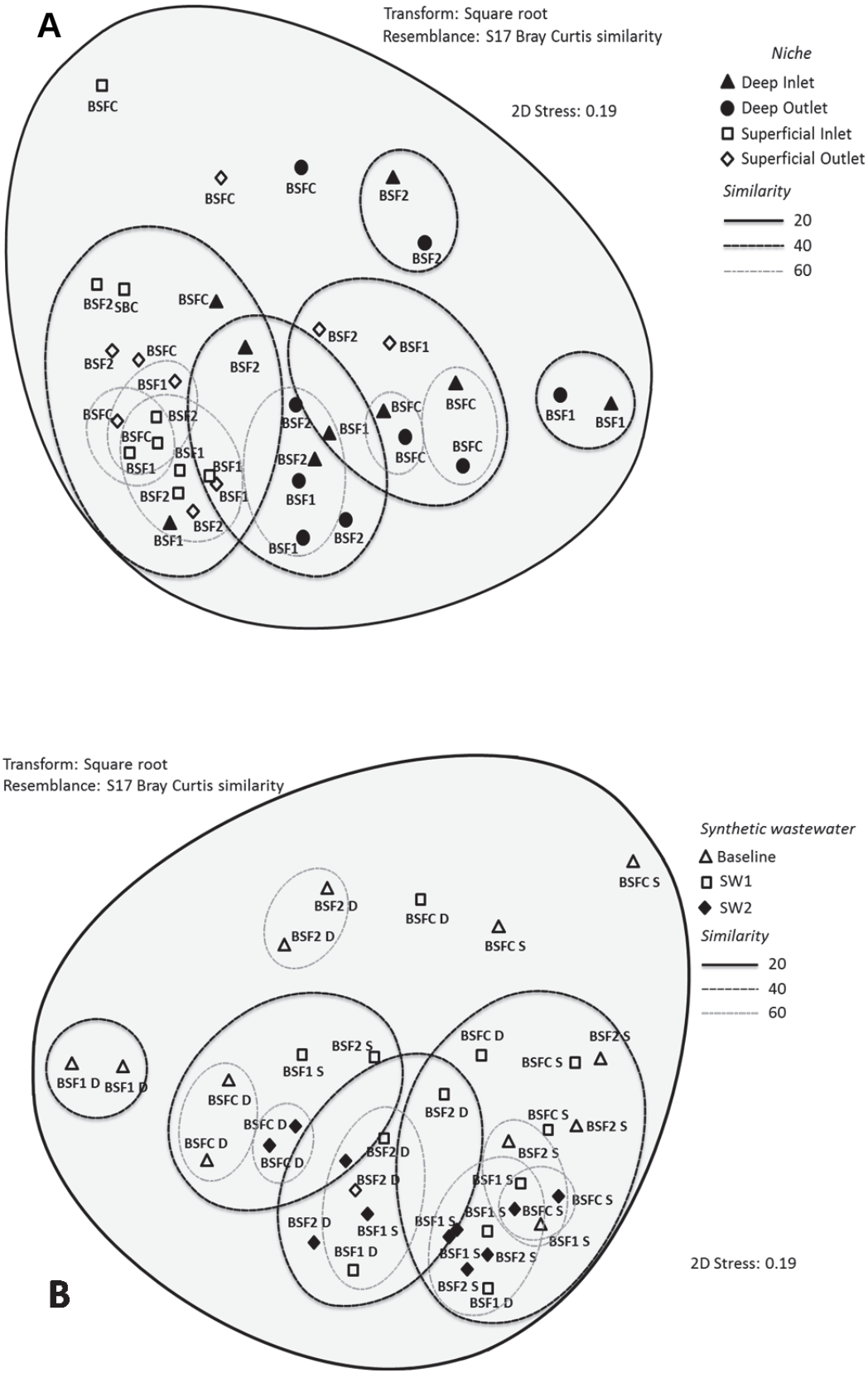
After amendment with SW2, points that represent the bacterial communities at the inlet and outlet of both experimental BSFs (BSF1, BSF2) group together, showing 60% similarity to one another and 40% similarity to the control. It is inferred from these results that, unlike amendment with SW1, SW2 had a similar impact on the bacterial community structure in the superficial niches at the inlet and outlet.

### Effect of synthetic winery wastewater on the bacterial community structures at depth

Before amendment with synthetic winery wastewater, and after amendment with SW1 and SW2, all points representing the bacterial communities in the deep niches shared 20% similarity on the NMDS plot (Figure 26D).

At baseline, points representing the bacterial communities from the inlet and outlet of each BSF cluster closer to each other than to points from the other replicates. In contrast, after amendment with SW1, points representing the bacterial communities at (i) the inlet of both experimental BSFs (BSF1, BSF2) group closer together, showing 40% similarity to one another and only 20% similarity to the control, and at (ii) the outlet of both experimental BSFs (BSF1, BSF2) group closer together, showing 60% similarity to one another, but only 20% similarity to the control. After amendment with SW2 points representing the bacterial communities at the inlet and outlet of both experimental BSFs (BSF1, BSF2) group together, showing 40% similarity to one another and 20% similarity to the control.

Thus, similar trends were noted in the deep and superficial niches, where each wastewater had an impact on the bacterial community structures, with similar impacts being determined at the inlet and outlet after amendment with SW2, but a greater impact at the outlet after amendment with SW1. In each instance, temporal changes not related to wastewater amendment were also noted, as evidenced by shifts in points representing the control BSF.


A
Transform: Square root
Resemblance: S17 Bray Curtis similarity
2D Stress: 0.19
Niche
Deep Inlet
Deep Outlet
Superficial Inlet
Superficial Outlet
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2D Stress: 0.19

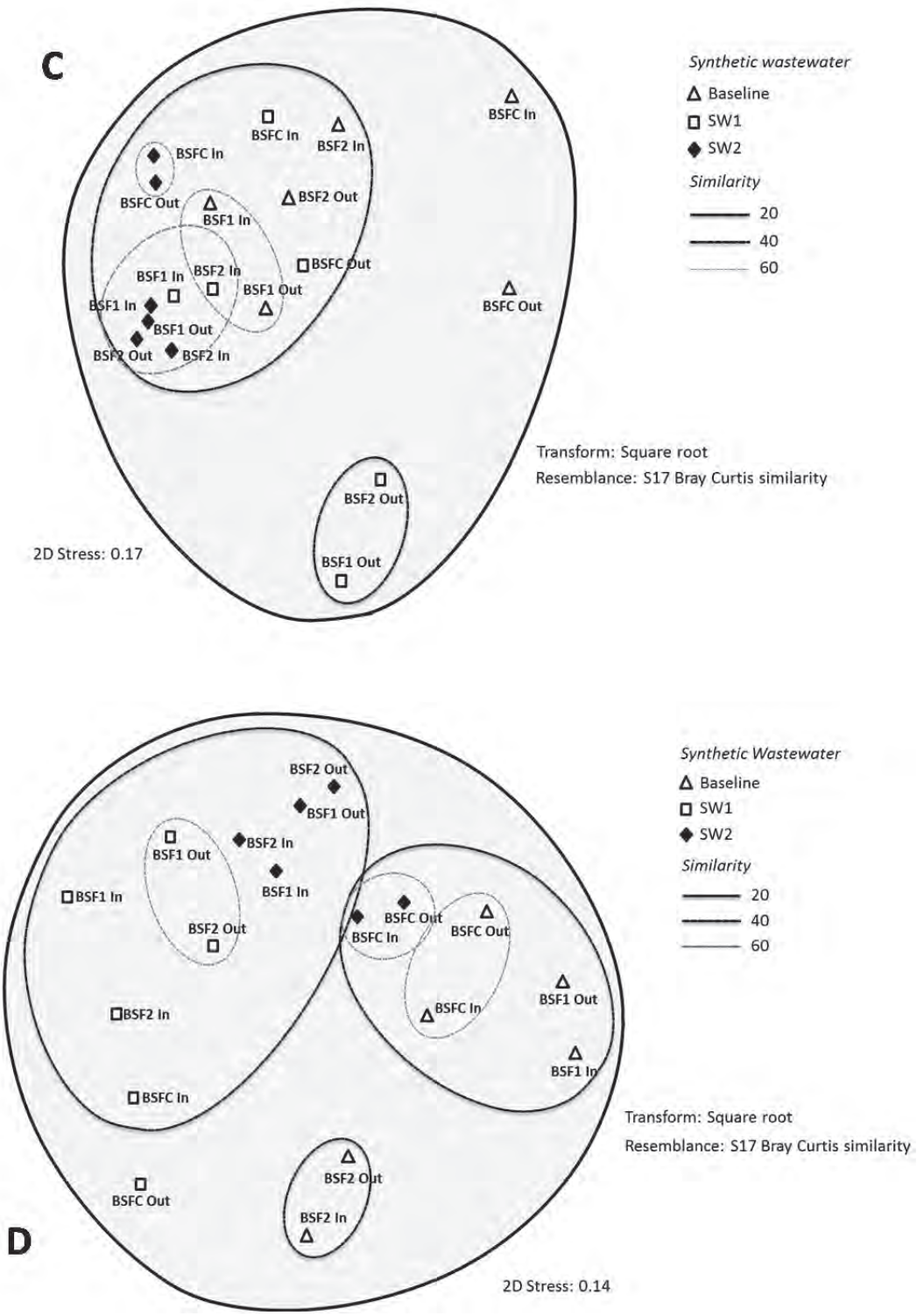


**Figure 26: Non-metric multi-dimensional scaling plots showing the effect of (A) spatial niche (deep/superficial); (B) synthetic winery wastewater in all niches; (C) synthetic winery wastewater in surface niches; (D) synthetic winery wastewater in deep niches, on the bacterial community structure in biological sand filters**

### 4.3.5 Conclusions

During the treatment of winery effluent in BSFs the redox status plays a vital role in the selection of the bacterial consortia, with different communities being found in the deep and superficial niches. The accumulation and biodegradation of organic substrates and metabolites is therefore strongly influenced by redox status. Some organics, including glucose, ethanol and phenolics, are degraded preferentially in the deep niches. However, accumulation of VFAs is also favoured in the deep niches. It is therefore recommended that BSFs are configured and operated in such a manner that the wastewater is subjected to alternating redox environments in order to stimulate complete biodegradation of organics. Alternatively, a polishing aeration step could be included for wastewater that has been treated in BSFs. Trickling filters provide a feasible option which is being explored (see Chapter 6).

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# CHAPTER 5:
# BIODEGRADATION KINETICS AND CORRELATION WITH PHYSICOCHEMICAL PARAMETERS IN BIOLOGICAL SAND FILTERS TREATING SYNTHETIC WINERY WASTEWATER

## 5.1 INTRODUCTION

To date, the in-situ use of BSFs at wine cellars, which is the ultimate goal of this cumulative work, has not been described. To ensure the successful operational design of full-scale BSF systems, an understanding of the rates of biodegradation, biotransformation and mineralisation of the various fractions of winery effluent is required.

To achieve these aims, it is important to determine spatio-temporal and physicochemical associations with long term biodegradative performance and the degradation kinetics of different fractions of winery effluent. Building on the knowledge from previous work, this study serves to introduce and discuss these complex interactions. To this end, results of amendment of BSF replicates with different concentrations of complex synthetic winery wastewater with a similar composition to that found in settled pond effluent is described.

## 5.2 MATERIALS AND METHODS

### 5.2.1 Set-up, mode of operation and feeding/amendment of biological sand filters

The set-up and mode of operation of the BSFs were as described previously in Sections 2.2.1 and 3.3.1, respectively. In this study, one BSF was designated as a control (BSFA) and two as experimental replicates (BSFB, BSFC).

The systems were operated in batch mode with the outlets being plugged prior to feeding/amendment. This prevented the bulk flow of wastewater from one area of the systems to another once plugged and filled with wastewater. Consequently, the batch mode of operation was suited to the calculation of degradation kinetics, determined from analysis of pore water samples taken at different time periods after amendment (Section 5.2.3.5).

In order to establish (i) the effect of hydraulic retention time on system performance, (ii) long-term performance, and (iii) the degradation kinetics, four different, concurrent strategies were used: The systems were plugged (i) for a period of 24 hr, three times weekly, for the first four weeks, (ii) for a period of 48 hr, twice weekly from week 5 to 19, (iii) for a protracted period (226 hr) at week 20/21, and (iv) for a period of 48 hr, twice weekly until week 24, at which time the experiment was terminated (Table 8).

After the designated time of plugging (24, 48 or 226 hr), the systems were unplugged and allowed to drain and rest according to the schedule given in Table 8.

After three months feeding with basal nutrients, as previously described, the two experimental systems (BSFB, BSFC) were also amended simultaneously with synthetic winery wastewater for 20 weeks and fed with yeast extract and glucose for a further four weeks according to the schedule and operating parameters given in Table 8. Feeding with basal nutrients alone (no synthetic winery wastewater) was continued in the control system (BSFA) throughout the experimental period.

It has previously been demonstrated that by incrementally increasing the concentrations of ethanol and phenolics to induce functional adaptation (acclimation) of the microbial communities, the biodegradative performance of BSFs can be improved (Welz et al., 2011, 2012). Therefore, a low influent COD concentration was used at the start of the experiment (527 mg/L at week 1, increasing to 1 027 mg/L at week 2, and 2 027 mg/L from week 8 to 20) (Table 8).

**Table 8: Operational parameters applied to the biological sand filters during the experimental period**

| | Week/s | BSFA (Control) | BSFB, BSFC |
|---|---|---|---|
| ***HLR (L/m$^3$sand/day)*** | 1-4 | 34.3 | 34.3 |
| | 5-20 | 22.9 | 22.9 |
| | 21 | 0 | 0 |
| | 22-25 | 22.9 | 22.9 |
| ***OLR (g COD/m$^3$sand/day)*** | 1 | 0.9 | 18.1 |
| | 2-4 | 0.9 | 35.2 |
| | 4.5 | 0.6 | 0.6 |
| | 5-7 | 0.6 | 12.0 |
| | 8-20 | 0.6 | 46.4 |
| | 21 | 0 | 0 |
| | 22-25 | 0.6 | 0.6 |
| ***Influent COD (mg/L)*** | 1 | 27 | 527 |
| | 2-4 | 27 | 1 027 |
| | 4.5 | 27 | 27 |
| | 5-7 | 27 | 527 |
| | 8-20 | 27 | 2 027 |
| | 21 | 0 | 0 |
| | 22-25 | 27 | 27 |
| ***Feeding frequency (times per week)*** | 1-4 | 3 | 3 |
| | 5-20 | 2 | 2 |
| | 21 | 0 | 0 |
| | 22-25 | 2 | 2 |

**HLR = hydraulic loading rate** **OLR = organic loading rate** **COD = chemical oxygen demand**

#### 5.2.1.1 Formulation of synthetic winery wastewater

The variable and unstable nature of winery wastewater does not lend itself to exacting long-term comparative experiments. Therefore, a synthetic effluent was formulated (Table 9). To determine the likely chemical composition of pond effluent, six samples were taken from two storage dams shortly after the crush season. No sugars or alcohols other than ethanol were detected in any of the samples. Ethanol concentrations ranged from 3 000 to 8 000 mg COD/L (to nearest 1000 mg/L) and acetate concentrations from 300 to 1 000 mg COD/L (to nearest 100 mg/L). The concentrations of total phenolics ranged between 30 and 70 mg GAE/L (gallic acid equivalents/L). It has previously been shown that ethanol with COD concentrations as high as 15 000 mg/L can be completely degraded in BSFs, but overload causes accumulation of short chain volatile fatty acids (Welz et al., 2011). To extract analytical data regarding the degradation and/or accumulation of acetate and phenolics, the concentrations of these organics in the formulation was purposefully high.

**Table 9: Composition of the synthetic winery wastewater used in the study**

| **Final conc*** (COD mg/L) | **Vanillin** (COD mg/L) | **Ethanol** (COD mg/L) | **Acetic acid** (COD mg/L) | **Gallic acid** (COD mg/L) |
|---|---|---|---|---|
| 527 | 25 | 250 | 200 | 25 |
| 1027 | 50 | 500 | 400 | 50 |
| 2027 | 100 | 1000 | 800 | 100 |

### 5.2.2 Sample collection

In total, 320 pore water samples and 78 effluent samples were collected as described in Sections 4.2.2.2 and 2.2.2.2 respectively. Effluent samples were collected from the outlet port immediately after unplugging the BSFs and again 30 minutes thereafter, with the exception of week 4, where samples were collected every 30 minutes for 2 hours in order to ascertain whether major temporal changes occurred.

At each sampling instance, four pore water samples were collected from each experimental BSF, one from each niche (superficial, deep; inlet, outlet). For the first four weeks of the experimental period, samples were collected 2 hr, 4 hr, 6 hr and 24 hr after amendment. From week 5 to week 19, samples were collected 24 and 48 hr after amendment. Between week 20 and 21, the systems were left plugged and samples were taken every 24 hr (up to 144 hr) and thereafter every 48 hr (up to 336 hr). At week 24 and 25, samples were collected after 24 hr.

### 5.2.3 Characterisation of influent, effluent and pore water

COD concentrations, identification and quantification of sugars, acids, alcohols and phenolics, as well as the conversion of concentrations into COD equivalents and compilation of metabolic profiles was performed using the same methods, calculations and equipment as previously described (Section 3.2.3). The redox potential (Eh) and pH of freshly collected pore water samples were determined using a pH700 meter and relevant probes (Eutech Instruments, Singapore).

### 5.2.4 Calculation of degradation kinetics

Degradation rates (k) were calculated assuming zero order kinetics

$$\boldsymbol{k} = \sum_{i=2}^{n} \left(\frac{c_n - c_{n-1}}{t_n - t_{n-1}}\right)$$ **Equation 4**

Where, C = COD concentration (mg $O_2$/L) in pore water samples taken at specific times instances (t), and t = hr after amendment ($t_0$ = 0 hr, $t_1$ = 24 hr, $t_2$ = 48 hr, $t_3$ = 72 hr, $t_4$ = 96 hr, $t_5$ = 120 hr, $t_6$ = 144 hr, $t_7$ = 192 hr, $t_8$ = 240 hr, $t_9$ = 288 hr)

Degradation rates were calculated for three periods: (i) 0-24 hr, using samples taken at 9, 10, 11 and 20 weeks after the start of the study (*n* = 1) (ii) 24-48 hr, using the samples taken at 9, 10, 11 and 20 weeks after the start of the study (*n* = 2), and (iii) from 48-288 hr, using samples taken daily 20/21 weeks after the start of the study (*n* = 3,4,5…9). It was not possible to extract pore water samples from the unsaturated sand. The influent COD concentration was therefore assumed as the starting (influent) concentration. The BSFs are undercover and protected from the prevailing winds and there is no above-surface effluent flow. Evaporative losses were therefore discounted.

### 5.2.5 Statistical analyses

Two-tailed Spearman correlation coefficients were calculated using IBM SPSS statistics 21 software, using results obtained from all temporal pore water samples taken during weeks 20 to 21 (*n* = 18 for each niche).

To calculate significant spatial and/or temporal differences in results, two-tailed t-tests were performed for two samples of unequal variance using Microsoft Excel (2010 version) to calculate p values.

## 5.3 RESULTS AND DISCUSSION

### 5.3.1 Aims of study

This study was designed to determine (i) The effect of retention time on the removal of overall COD and selected organics from (synthetic) winery wastewater in BSFs, (ii) The long term performance of BSFs, i.e. whether removal performance increases, decreases or remains static over time, (iii) The relative degradation rates of selected organics from (synthetic) winery wastewater, (iv) The effect of spatial niches, and by inference physicochemical parameters, on the degradation rates of selected organics from (synthetic) winery wastewater.

### 5.3.2 Overall COD removal in biological sand filters

#### 5.3.2.1 Analysis of effluent to determine the influence of influent concentration and retention time on overall COD removal performance

After 3 weeks of amendment with synthetic winery wastewater, the average overall COD removal rate in the experimental BSFs (76%) was less than expected. The influent COD concentration was thus reduced from 1 027 mg/L to 527 mg/L, and the following week the plugging period was increased to 48 hr and the feeding/amendment frequency decreased from three times to twice per week. Despite the increased retention time, decreased feeding/amendment frequency, and decrease in influent COD, the average effluent COD concentration did not decrease concurrently [(263 mg/L after 24 hr (week 4), 261 mg/L after 48 hr (week 5)], which reflected a substantial deterioration of the removal rate (from 76% to 60%). However, the removal rate improved over the next three weeks and the influent COD was increased to a more industry-related value of 2 027 mg/L (Figure 27A). Over the following four weeks, there was an improvement in performance, and the COD removal rate increased to 76% again, with an average effluent COD of 558 mg/L. It is recognized that the traditional calculation of removal performance using influent and effluent concentration ratios is somewhat flawed because it discounts accumulation of residual substrates and metabolites from previous amendments. In addition, high removal rates at low concentrations and *vice versa* cannot be realistically compared. However, the improved removal rate at high COD concentration was notable.

The values obtained from all samples taken from the control system (BSFA) were < 25 mg COD/L.

#### 5.3.2.2 Analysis of pore water samples to compare COD removal in different environmental niches

##### Effect of wastewater ingress on COD concentrations

There were no significant differences in the COD concentrations measured in samples taken from the superficial niches (inlet and outlet) ($p > 0.5$; Figure 27B). In contrast, there were

significant differences in the COD values determined from the samples taken from the deep inlet and outlet ($p < 0.01$), and of the four niches, the highest and lowest COD values were found at the deep inlet and outlet, respectively (Figure 27B, 27C). When the influent COD was decreased from 1 027 mg/l to 527 mg/L during week 4, the COD measured in deep inlet samples did not decrease concurrently. For two weeks, the concentration remained higher than that of the influent (Figure 27C).

These findings demonstrate that non-degraded organics accumulated at the deep inlet and that there was a degradation gradient from inlet to outlet. If substrate accumulation in this niche is to be prevented, the reasons for this occurrence need to be established. From a practical perspective pertaining to BSF design and operation, the presence of sequential degradation gradients can be seen as ideal because the most 'treated' wastewater exits from the outlet first. The COD concentrations determined in the effluent samples were consistently lower than those in samples from the superficial niches and the deep inlet, even when effluent samples were taken every 30 min (Figure 27A, week 3.5). For example, between weeks 8 and 11, the COD concentrations in samples taken from the deep inlet and effluent samples were, respectively, 1 413 ± 254 mg/L (range 1 066 to 1 840 mg/L), and 510 ± 194 mg/L (range: 250 to 859 mg/L).

### COD degradation kinetics

To assess temporal and spatial differences in COD degradation kinetics, degradation constants were calculated assuming zero order kinetics for three periods after amendment (Table 10). In all niches, the COD degradation rate was highest in the first two hours following the start of amendment. It is therefore plausible that the hydraulic retention time could be considerably reduced, with a concomitant increase in organic loading capacity in the BSF systems (Figure 2B, 2C). Removal rates were highly similar in both superficial niches [inlet: 33 ± 9 mg COD/L/hr (0-24 hr), 6 ± 4 mg COD/L/hr (24-48 hr)] and [outlet: 31 ± 8 mg COD/L/hr (0-24 hr)], 6 ± 6 mg COD/L/hr (24-48 hr)], and were approximately 5 fold higher in the first 24 hr (32 ± 9 mg COD/L/hr) than in the 2nd 24 hr (6 ± 5 mg COD/L/hr).

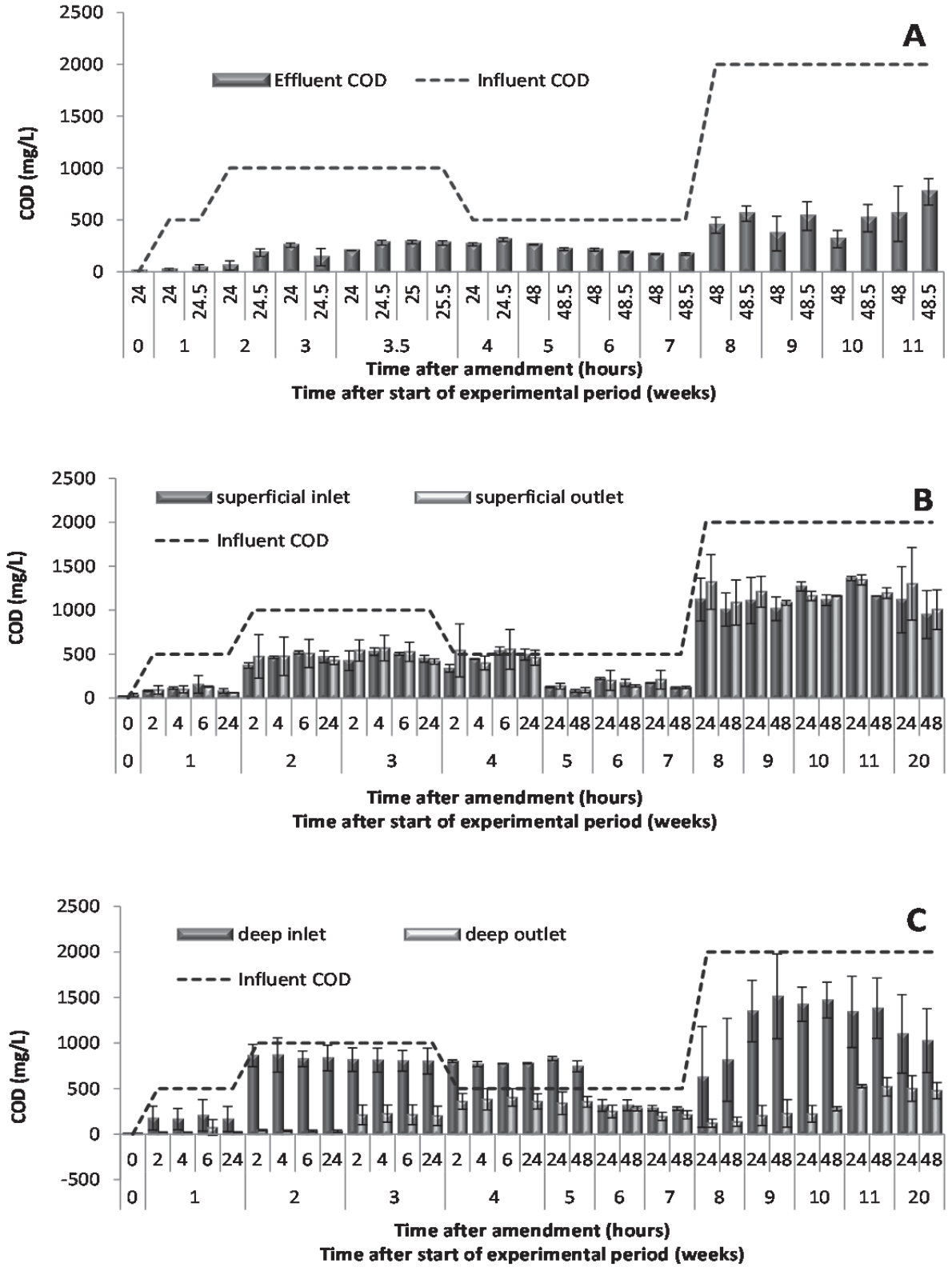


**Figure 27: Chemical oxygen demand in effluent samples (A) and pore water samples extracted from the superficial niches (B) and deep niches (C) of the experimental biological sand filters. Error bars show the standard error from the mean of COD concentrations from BSFB and BSFC.**

It is expected that the more readily biodegradable substrates would be degraded first, and at a faster rate i.e. sequential removal of the influent organics and metabolites in order of degradability. In samples taken from the superficial inlet, similar COD degradation rates were determined in both BSF replicates (BSFB, BSFC) between 48 and 228 hr, despite the COD range being higher in BSFC. However, at the superficial outlet, rates were > 3 times higher in BSFC (10.3 mg COD/L/.hr) than in BSF B (2.8 mg COD/L/hr). In this study, the systems were filled with the same volume of sand, pre-inoculated with the same volume of extract from BSFs previously amended with winery wastewater, and equilibrated using the same protocol. The set-up and operation were identical. These differences therefore highlight the need to include replicates for biologically based studies of this nature.

**Table 10: Degradation kinetics calculated from analysis or pore water samples taken from the experimental biological sand filters**

| | SI | DI | SO | DO |
|---|---|---|---|---|
| **0-24 hr** | | | | |
| Degradation rate (mg COD/L/hr) | 33 ± 9 | 29 ± 12 | 31 ± 8 | 68 ± 7.4 |
| COD range (mg COD/L) at 24 hr | 922 -1386 | 795-1588 | 1006-1333 | 126-600 |
| **24-48 hr** | | | | |
| Degradation rate (mg COD/L/hr) | 6 ± 4 | -2 ± 4.5 | 6 ± 6 | 0 ± 1.6 |
| COD range (mg COD/L) at 48 hr | 920-1161 | 778-1840 | 846-1236 | 121-592 |
| **BSF B: 48-228 hr** | | | | |
| Degradation rate (mg COD/L/hr) | 1.91 | 1.7 | 2.8 | -0.26 |
| COD range (mg COD/L) 72-228 hr | 379-757 | 365-778 | 186-846 | 371-414 |
| $R^2$ value | 0.982 | 0.996 | 0.994 | 0.628 |
| **BSF C: 48-228 hr** | | | | |
| Degradation rate (mg COD/L/hr) | 2.1 | 2.2 | 10.3 | 0.67 |
| COD range (mg COD/L) 72-228 hr | 497-1142 | 712-1274 | 340-1165 | 349-540 |
| $R^2$ value | 0.937 | 0.985 | 0.980 | 0.788 |

**Results from < 48 hr are the average of samples taken from P1 and P2 (n=8 for each sampling time). $R^2$ value calculated from regression analysis [hours v/s COD (mg/L)]. SI = superficial inlet; DI = deep inlet; SO = superficial outlet; DO = deep outlet. Anomalous results due to flow differences and metabolite accumulation are shaded light grey**

Between 24 and 288 hr after amendment, significant negative correlations were found between time (hr) and COD concentration in all niches, demonstrating that biodegradation was continuous during this period (Table 11). This was accompanied by increases in pH, and reduction in redox potential, with significant correlations being found in the superficial niches (Table 11).

There were anomalies in the degradation rates calculated using samples taken from the deep niches over the first 48 hr. It is suggested that this was due to accumulation of substrates and metabolites at the deep inlet and the fact that plug flow of remediated effluent resulted in low substrate/metabolite concentrations at the deep outlet. Thereafter, degradation rates at the deep inlet were similar to those calculated for the superficial inlet in both BSFs. However, at the outlet, the rates were lower (BSFC: 0.67 mg COD/L/hr) or there was a nett COD production (BSFB: -0.26 mg COD/L/hr). This strongly suggests that organics gradually diffused into the deep outlet driven by a concentration gradient.

### 5.3.3. Analysis of non-phenolic substrate/metabolite profiles in pore water samples taken from experimental biological sand filters

The concentrations of organics were converted into COD values and substrate/metabolite profiles were compiled to gain insight into the metabolic processes taking place in the BSFs.

#### 5.3.3.1. Substrate/metabolite profiles at low influent COD concentrations (week 1 to 7)

Analysis of HPLC chromatograms showed that acetate, propionate and residual ethanol were the only non-phenolic organics present during the first seven weeks of the experimental period (Figure 28). Acetate is a common metabolite that is produced during the biodegradation of many organics, including those present in the synthetic winery wastewater (vanillin, gallic acid, acetate and ethanol) (Thauer et al., 1977; Welz et al., 2011, 2012). It is also the main substrate involved in the formation of propionate and other intermediary fatty acids under anaerobic conditions. When a high flux of electrons are produced during the fermentation of organics, the proton reducing and/or hydrogen transfer reactions become saturated; under these circumstances, electron sink products, including acetate and propionate, are formed (Cohen et al., 1982; Thauer et al., 1977). In this study, acetate was not labelled, so it was not possible to determine the substrate (residual) to metabolite (production) ratio.

Clear qualitative and quantitative trends in the non-phenolic substrate/metabolite profiles were found. These trends differed in each of the four environmental niches of the BSFs [superficial inlet (Figure 28A), superficial outlet (Figure 28B), deep inlet (Figure 28C), deep outlet (Figure28D)]. There were four noteworthy findings, which are outlined and discussed. Firstly, it was established that ethanol was the preferred non-phenolic substrate because acetate was the major contributor to COD in the pore water samples taken from all environmental niches, despite ethanol being present in higher concentrations in the influent.

Secondly, it was established that ethanol was degraded preferentially in the deep niches. It is accepted that batch mode of operation allows atmospheric gases to be drawn into the substratum of constructed wetlands and BSFs during the drainage cycle and that higher oxygen saturation is found in the superficial layers (Maier et al., 2009; Torrens et al., 2009).

Organic degradation is typically more rapid when oxygen is used as the terminal electron acceptor (Maier et al., 2009; Torrens et al., 2009). It was therefore expected, as determined in previous experiments using ethanol as the sole 'pollutant', that ethanol degradation would be higher at the surface. It has also been shown that the bacterial community structure and function differs significantly in the superficial and deep niches of BSFs (Ramond et al., 2013a, 2013b; Rodriquez-Caballaro et al., 2012). The results of this study suggest that the complexity of wastewater has a profound influence on the functional microbial communities and that predictions made using singular 'pollutants' cannot necessarily be extrapolated to complex wastewaters. This was discussed more thoroughly in Chapter 4 of this report.

Thirdly, although COD concentrations were similar in both superficial sites, less ethanol (substrate) and more propionate (metabolite) was detected at the superficial outlet than at the superficial inlet. The presence of this degradation gradient establishes that plug flow of influent occurred at the surface, as well as at depth (alluded to in Section 5.3.2).

Lastly, significantly more propionate was detected in samples from the deep inlet than the superficial niches ($p < 0.01$), while similar concentrations were found in both superficial sites ($p = > 0.05$). This was not unexpected, because less oxygen, and therefore terminal electron acceptors, would have been present in the deeper layers. Lower redox measurements were indeed found in samples from the deep substrata (Section 5.3.5).

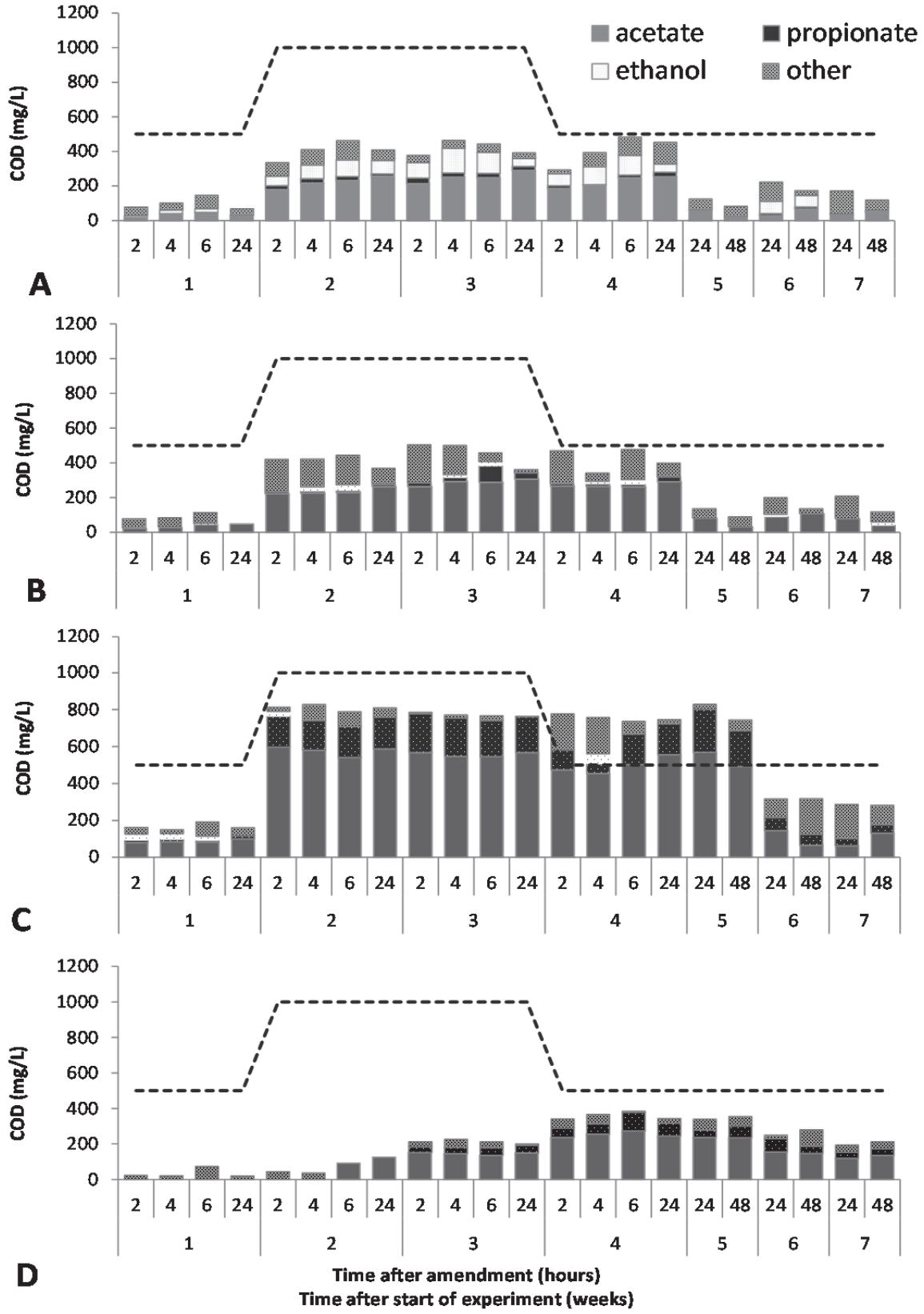


**Figure 28: Non-phenolic substrate/metabolite profiles compiled from samples taken from the superficial inlet (A), superficial outlet (B), deep inlet (C), and deep outlet (D) at low influent COD concentrations of 5000-1000 mg/L. The dotted lines on the graphs represent the influent COD values.**

#### 5.3.3.2 Non-phenolic substrate/metabolite profiles at high influent COD concentrations

From week 8, when the influent COD concentration was increased to 2 027 mg COD/L, the trends found during the first 7 weeks (outlined in Section 5.3.3.1), were mirrored (Figure 29). During the first amendment with more concentrated wastewater, there is typically a dilution effect, which produces anomalous results in effluent COD measurements. Therefore, the measurements from week 8 have been discounted from this discussion.

The BSF systems were amended for 12 weeks with 2 027 mg COD/L influent to allow equilibration of the microbial communities to the concentration and composition of the wastewater and assess the long-term performance of the systems. Three noteworthy functional changes were noted when comparing results from weeks 9-11 with those from week 20.

Firstly, there was generally an improvement in overall removal performance, and the acetate concentration was lower in all environmental niches (Figure 29). This indicated that the functional microbial communities had acclimated to the environmental parameters, as demonstrated in previous studies (Welz et al., 2011, 2012).

Secondly, there was an increase in ethanol concentration and a decrease in propionate concentration in samples taken from the superficial niches. Without carbon labelling, it is uncertain whether this was a reflection of decreased biodegradation of ethanol and/or phenolics to acetate and propionate, or *de novo* ethanol formation.

Lastly, there was also an increase in the propionate concentration in samples taken from the deep niches. Again, without labelling it is not possible to speculate whether this reflected increased conversion of acetate to propionate or increased accumulation of propionate.

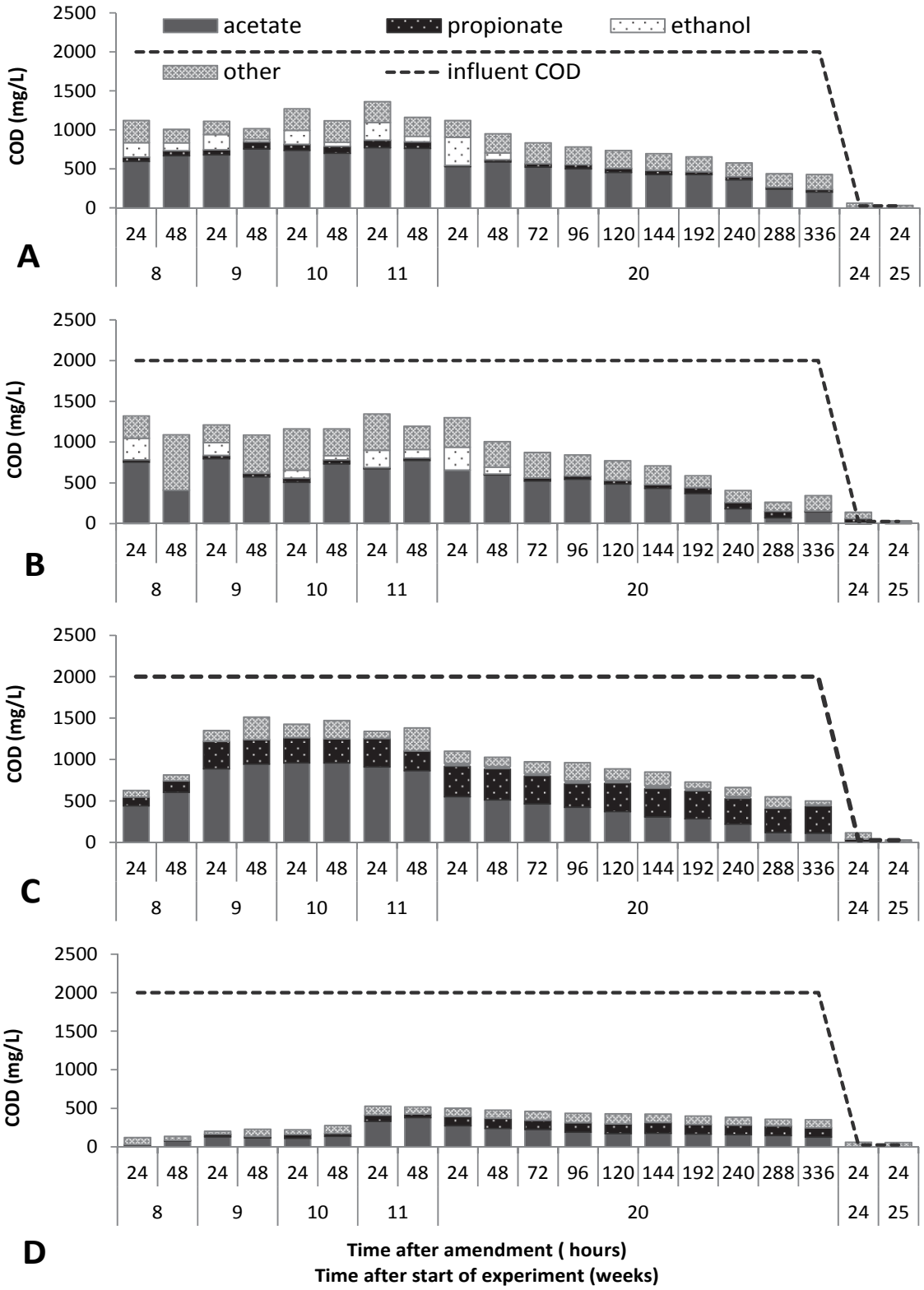


**Figure 29: Non-phenolic substrate/metabolite profiles compiled from samples taken from the superficial inlet (A), superficial outlet (B), deep inlet (C), and deep outlet (D), at high influent chemical oxygen demand concentration (2 027 mg/L) as well as during expended hydraulic retention time (week 20) and subsequent feeding period (week 24 to 25). The dotted lines on the graphs represent the influent COD values.**

### 5.3.4 Analysis of phenolic removal and metabolite formation

In BSFs, phenolics can be removed by both biotic and abiotic mechanisms (Welz et al., 2012). In this study, the chromatograms generated from HPLC analyses using a protocol for the detection and quantification of phenolics showed numerous peaks, reflecting a number of degradation and/or formation products. To determine in which niche environments the phenolics were removed, the total phenolic concentration was determined, the results from weeks 11 and 20 being shown in Figure 30A. Almost complete removal of phenolics occurred in the deep substrata and no significant ($p > 0.05$) temporal changes were demonstrated between week 11 and week 20. However, there was a significant ($p < 0.01$) temporal increase in the concentration of total phenolics in the superficial niches. In all samples, including those taken after 48 hr during week 20/21 (Figure 30A, 30B), the total phenolic concentrations were significantly higher ($p < 0.01$) in the surface, than in the deep niches, but not significantly different in both surface niches ($p > 0.5$). It was thus demonstrated that removal of phenolics was favoured under lower redox conditions. It was hypothesized that accumulation of phenolics in the superficial sites was related to the saturation of phenolic binding sites and/or abiotic catalytic sites, and/or the influent phenolics exceeded the degradative capacity of the systems. However, when the systems were plugged for 2 weeks and regular samples were taken to ascertain, amongst other parameters, the probable mechanism responsible for accumulation of phenolics, consistent removal took place, so that by 336 hr, most of the phenolics had been degraded, strongly suggesting that biodegradation was the dominant removal mechanism (Figure 30B).

### 5.3.5 Neutralization of acidic wastewater

Winery effluent is usually acidic and is pH adjusted prior to treatment or use as irrigation water. In this study, the pH of the influent was 4.0 ± 0.0 (527 mg $O_2$/L), 3.6 ± 0.2 (1 027 mg $O_2$/L) and 3.5 ± 0.1 (2 027 mg $O_2$/L). The pH of the baseline samples (t = 0 hr) and the control samples at all sampling instances was 7.9 ± 0.1.

The winery effluent was effectively neutralized in the BSFs without pH adjustment, despite the fact that there was an accumulation of VFAs. The average effluent values over the duration of the study were 7.1 ± 0.2 (range: 6.9 to 7.6). At the highest influent COD, the average pH either increased between 24 and 48 hr: from 6.5 ± 0.2 to 6.9 ± 0.1 (superficial inlet); 6.3 ± 0.1 to 7.1 ± 0.2 (superficial outlet); 7.0 ± 0.2 to 6.9 ± 0.1 (deep outlet), or remained the same (6.9 ± 0.2, deep inlet).

In the environment, abiotic neutralization of acid water takes place chiefly by the rapid dissolution of carbonates or slow dissolution of aluminosilicates (Miller et al., 2010). In the presence of suitable electron donors, neutralization can also take place via biotic mechanisms (such as the utilization of protons by methanogens) (Montalvo et al., 2010). In this case, the BSF sand contained the carbonate based mineral, calcite, which may have

contributed to the neutralization process. However, neutralization of winery effluent has also been achieved in BSFs containing quartz-dominated sand with no aluminosilicates or carbonate minerals (Ramond et al., 2013a).

In addition, positive correlations were found between pH and time (hours after amendment) and negative correlations between pH and COD, which were significant in the surface niches, strongly suggesting biotic neutralization mechanisms (Table 11).

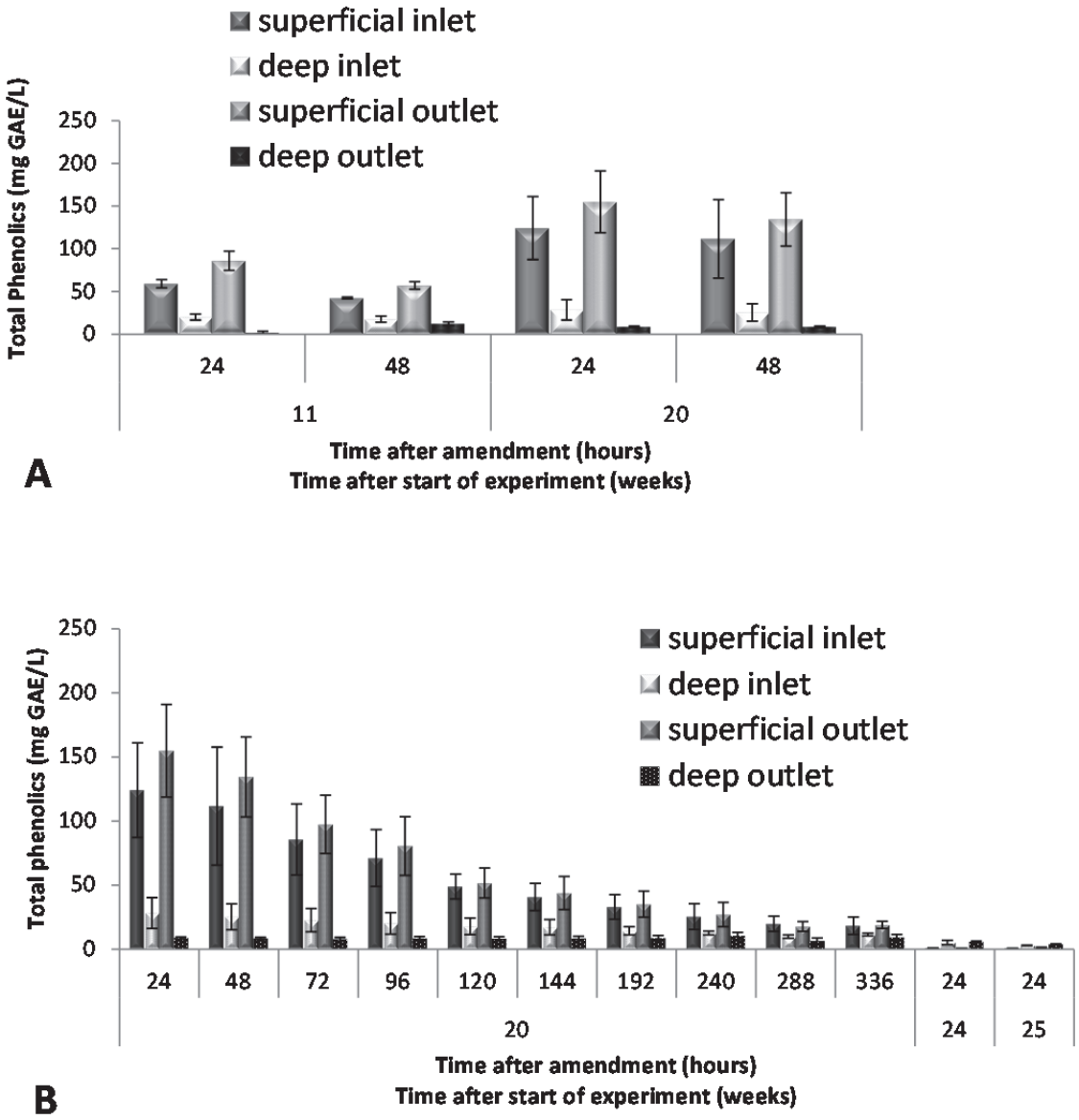


**Figure 30: Total phenolic concentrations in the four environmental niches: (A) at week 11 and week 20, showing temporal accumulation in the superficial niches, and (B) daily (24 to 144 h) and bi-daily (144 to 336 hr) measurements, showing sequential removal after amendment. Error bars show the standard deviation from the mean for samples taken from BSF B and BSF C.**

### 5.3.6 Spatio-temporal changes in redox status in response to amendment with synthetic winery wastewater in BSFs

Weekly redox measurements decreased over the first three weeks after initial amendment (Figure 31) and also decreased for the first 120 hr after amendment at week 20, but not thereafter (Figure 6B). From the second week, the Eh values determined in samples from the deep niches were consistently and significantly lower ($p < 0.01$) than those from the superficial niches. A significant correlation was found between COD and Eh in both BSFs in the surface niches (Table 11). The results are consistent with the fact that, during microbial activity, electron transfer reactions lead to the creation of more reduced environments, especially when the electron acceptors oxygen and nitrate are expended (Schink, 2006).

**Table 11: Two-tailed Spearman correlation co-efficients calculated for selected physicochemical parameters**

| | Hr/Eh | Hr/pH | Hr/COD | Eh/pH | Eh/COD | pH/COD |
|---|---|---|---|---|---|---|
| **SI** | -.894** | .815** | -.780** | -.811** | .781** | -.639** |
| **SO** | -.865** | .652** | -.879** | -.645** | .754** | -.388 |
| **DI** | -.701** | .730** | -.581* | -.462 | .185 | -.718** |
| **DO** | -.448 | .420 | -.722** | -.389 | .066 | -.026 |

**SI: superficial inlet; SO: superficial outlet; DI: deep inlet; DO: deep outlet**
**** Correlation is significant at the 0.01 level (2-tailed)**
*** Correlation is significant at the 0.05 level (2 tailed)**

### 5.3.7 Conclusions

In this study, it was found that most COD removal took place within the first 24 hr in the BSFs, with excellent removal of organics being achieved. Ethanol and phenolics were degraded preferentially in the deep niches. However, long-term performance of the systems was marred by the fact that there was an accumulation of acetate, usually considered to be a readily biodegradable substrate, especially in the deep niches of the biological sand filters. This is a feature that has previously been demonstrated in these systems.

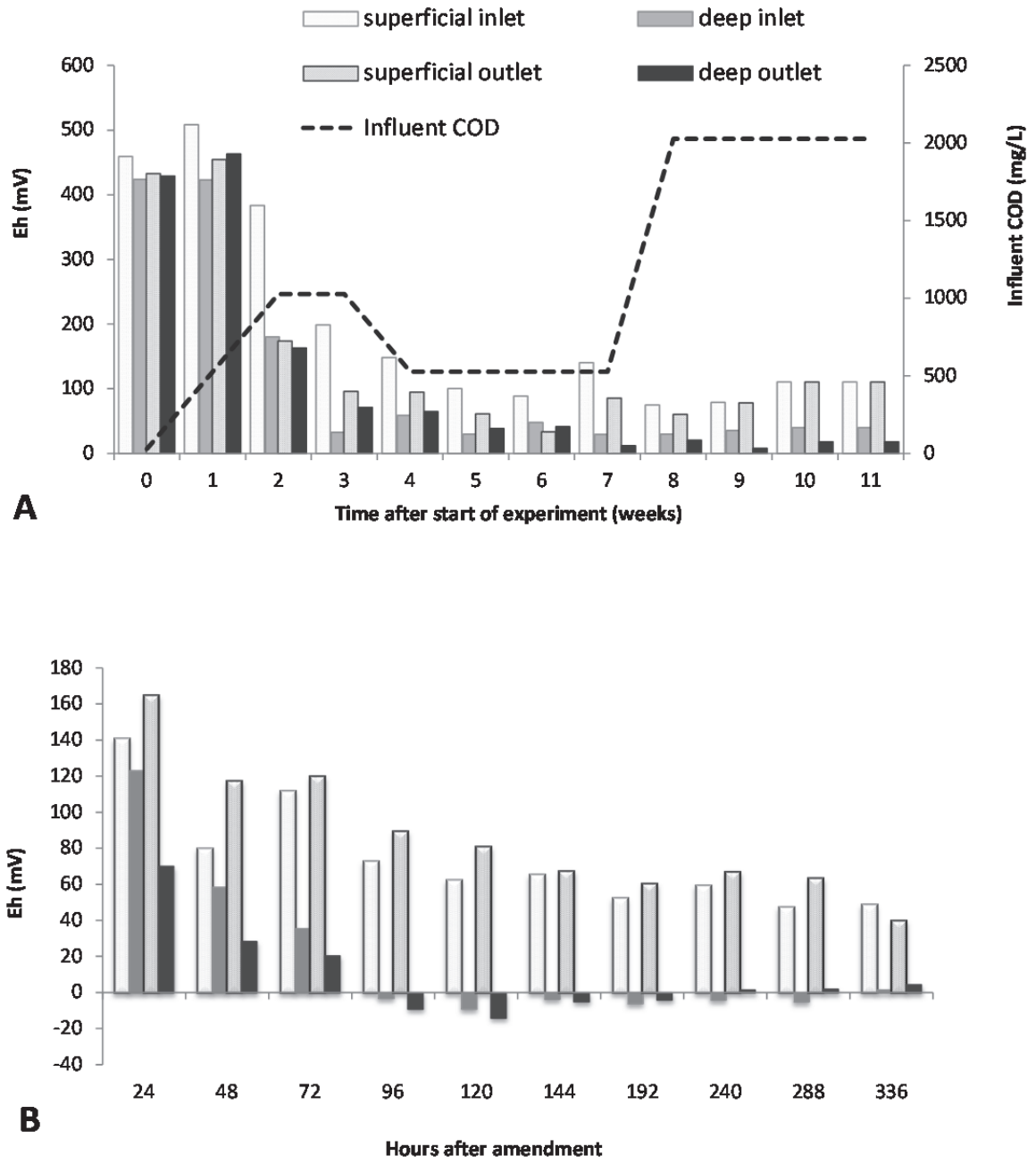


**Figure 31: Weekly redox measurements in pore water samples 24 hr after amendment (A) and regular temporal redox measurements in pore water samples taken after amendments at week 20 (B)**

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# CHAPTER 6:

# TRICKLING FILTERS AS A POLISHING STEP TO REMOVE RESIDUAL ACETATE FROM WASTEWATER TREATED IN BIOLOGICAL SAND FILTERS

## 6.1 INTRODUCTION

During the course of Project K5/2104, it was demonstrated that excellent removal of sugars, ethanol and phenolics can be achieved in biological sand filters, but that the removal of VFAs is poor. Even when not present in the influent, VFAs may be formed from other organic substrates and accumulate in BSFs, particularly in the reduced redox environments encountered in the deep niches (Welz and Le Roes-Hill, 2014). It was hypothesised that acetate removal could be enhanced by utilising strategies to increase the redox potential.

Initially, the use of in-situ active intermittent aeration was considered. However, three factors led the team to re-consider: (i) In-situ aeration may negatively affect degradation of organics where the degradation kinetics favour lower redox conditions, (ii) There is competition for substrate within the systems and acetate is a central metabolite which is both formed and utilised during autotrophic and heterotrophic metabolism (Cohen et al., 1982; Conrad and Klose, 2011; Wolfe, 2005), and (iii) There is an energy requirement attached to active aeration. A downstream process would therefore be more suitable.

Simple trickling filters (TFs) form passive treatment systems which can enhance aerobic process such as nitrification and aerobic organic degradation pathways (Matthews et al., 2009). TFs have been applied to remove residual organics present after anaerobic secondary biological treatment of domestic wastewater, piggery wastewater or municipal landfill leachate has been effected (Garzon-Zuniga et al. 2007; Matthews et al., 2009; Pontes and De Lemos Chernicharon, 2011). Although there is no literature evidence of TFs being used specifically for the removal of VFAs, the project team felt it was worth exploring. A ‘proof of concept’ laboratory scale TF experiment was therefore set up to determine the feasibility of using TFs as a polishing step for the removal of acetate from winery wastewater treated in BSFs.

## 6.2 MATERIALS AND METHODS

### 6.2.1 Set-up and operation of lab-scale trickling filters

Two acrylic columns 0.5 m in length, with a diameter of 0.3 m were suspended in bucket systems (Figure 32). The bottom of the columns were fitted with mesh and filled to a height of 0.32 m and volume of 2.26 x $10^{-2}$ $m^3$ with 30 kg of 13 mm builder's stone as the filter medium. Effluent was pumped at the rate of 5L/hr into a perforated bowl located at the top of the columns, from where it dripped onto the surface of the aggregate and trickled down into a collection bucket.

### 6.2.2 Establishment of microbial biofilm

In order to induce the formation of functional biofilm on the aggregate for the experimental TF, for two weeks prior to the set-up of the TFs, the aggregate was immersed in a bucket containing 10 g glucose and 5 g yeast extract dissolved in 20 L tap water. Every 3-4 days, 5 ml glacial acetic acid and 1 g yeast extract was added (5 times in total). In addition, a combined extract from the BSFs was used to seed the aggregate with functional microbial communities.

### 6.2.3 Sterilisation of aggregate

On the first day of the experiment, the aggregate used for the control system was autoclaved at 121$^{o}$C at 100 kPa for 25 minutes, stirred and autoclaved twice again under the same conditions.

### 6.2.4 Amendment and sample collection

On day 1 (week 1), the control and experimental TFs were fed with 15 L of a 315 mg/L solution of acetate. This was in the range determined in the effluent of the BSFs when amended with synthetic winery wastewater with an influent COD of 2 027 mg/L. Over the last 45 minutes, 15 ml samples were taken each 15 minutes from the tap at the bottom of the bucket. The TFs were then fed with 15 L of a 945 mg/L, followed by a 1 890 mg/L solution of acetate, and sampled as previously. The control experiment was then terminated before substantive microbial colonisation could take place.

The study was continued with the experimental system for a further 6 weeks to ascertain whether any temporal changes could be noted. During this time, from Monday to Friday, the TF was fed with 5 L of a 315 mg/l solution of acetate, also containing 0.5 g of yeast extract. In the second week, and sixth week the experimental TF was amended as previously, with 15 L of 315 mg/L, 945 mg/l and 1890 mg/L solution of acetate, respectively, and 15 ml samples were taken for analysis each 15 minutes for the last 45 minutes.

### 6.2.5 Statistical analyses

A one-tailed, paired student t-test was used to determine the level of significance of removal of acetate by the TFs using Microsoft Excel 2010.

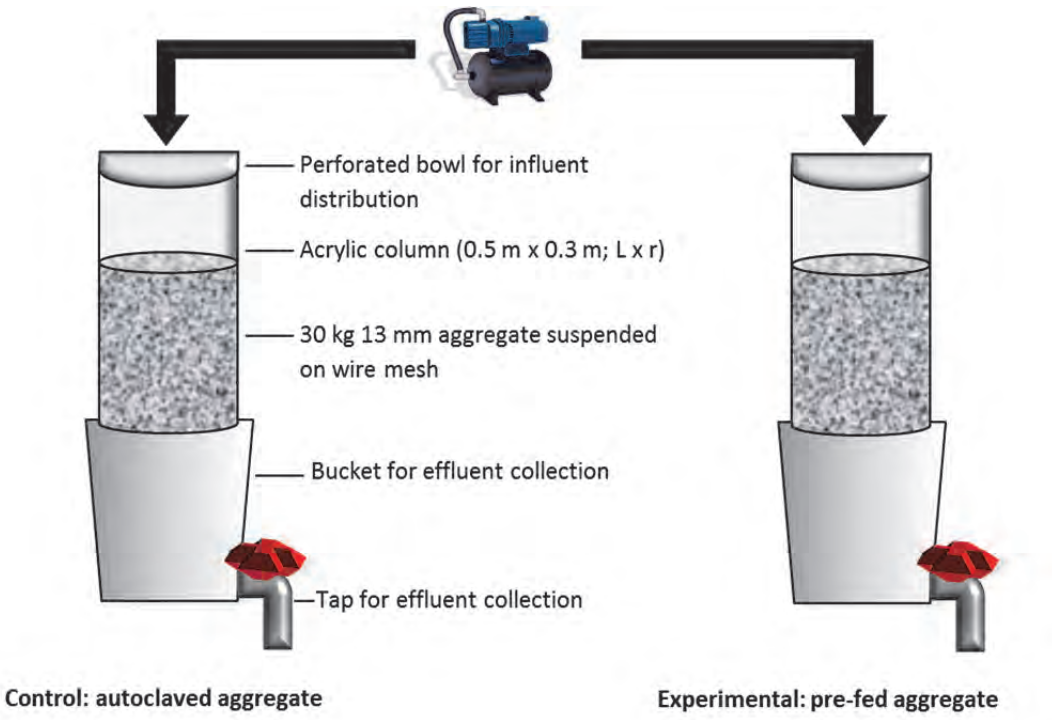


Figure 32: Set-up of laboratory scale trickling filters

## 6.3 RESULTS AND DISCUSSION

### 6.3.1 The use of trickling filters as a polishing step to remove residual acetate from winery wastewater

Abiotic removal of organics in wastewater treatment systems can occur via mechanisms such as adsorption, volatilisation, photo-degradation, and a host of non-biotic chemical transformation and mineralisation reactions. For example, it has been shown that abiotic adsorption can account for approximately 40% of phenolic removal in BSFs filled with Malmesbury sand (Welz et al., 2012). In this investigation, acetate removal in a laboratory scale TF containing aggregate which had been sterilized by autoclaving was assumed to be abiotic in nature. Similarly, it was assumed that in a non-sterilised replicate, removal was due to a combination of biotic and abiotic mechanisms. Because it is extremely difficult to maintain sterile conditions in such systems for any length of time, acetate removal rates were only determined in the control TF containing the autoclaved aggregate on one occasion, immediately after the aggregate had cooled after autoclaving (week 1). In this

investigation, significant removal ($p < 0.001$) of acetate occurred in both TFs. Greater than 96% abiotic removal of acetate was achieved, with influent concentrations ranging from 315 mg/L to 1890 mg/L (Figure 33).

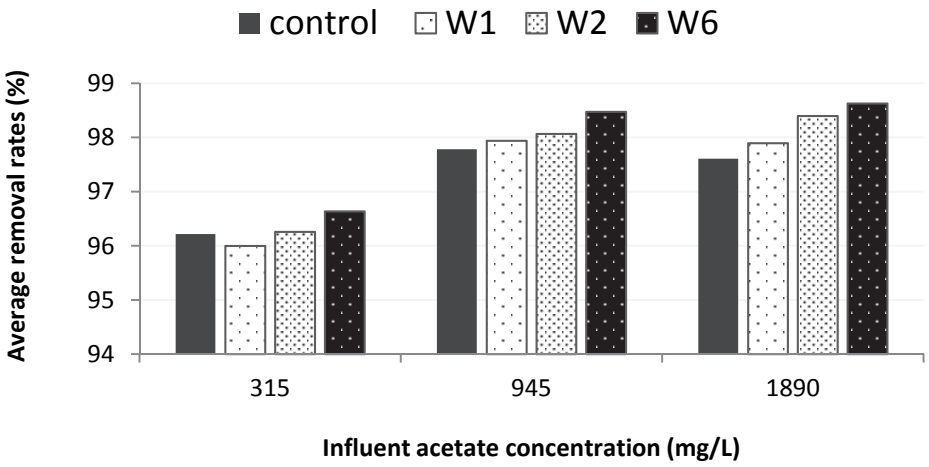


**Figure 33: Acetate removal rates in laboratory scale trickling filters at week 1, 2 and 6 (W1, W2, W6). Results are averages from all samples taken at each instance (*n*=3)**

There was no statistically significant difference in the concentrations of acetate measured in the effluent from the experimental and control TF at the start of the experiment, and there was also no significant decrease in the concentrations of acetate measured in the experimental TF at the start of the experiment, and after a week of operation ($p > 0.05$). However, there was a statistically significant difference in the concentrations of acetate measured in the effluent from the experimental and control TF after operation of the experimental TF for 2 weeks ($p < 0.05$ and $> 0.01$) and 6 weeks ($p < 0.01$), and the experimental system itself after 6 weeks of operation ($p < 0.01$) (Figure 34).

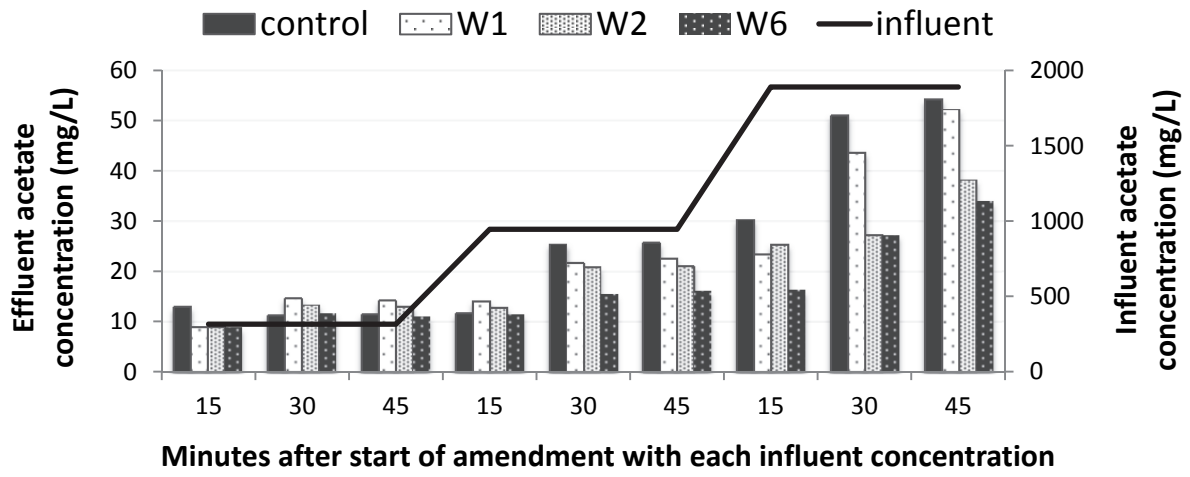


**Figure 34: Influent and effluent acetate concentrations measured from laboratory scale trickling filters filled with aggregate**

### 6.3.2 Conclusions

The results of this investigation showed that TFs may indeed present a feasible option to 'polish' wastewater containing short chain VFAs. In this experiment, over 96% of removal was abiotic in nature. It is highly likely that the major mechanism was volatilisation. However, there was significant improvement in biotic removal in the experimental TF after 6 weeks, demonstrating that both mechanisms played a role.

It is currently unknown whether these results will be replicable when the systems are scaled-up. It is also unknown whether aggregate is the best substrate. Currently, further studies are being conducted using the sand columns which were previously utilized for HC experiments. The columns have been filled with either aggregate, clay balls or open cell polyurethane foam, in an effort to determine which of these substrates would be best for acetate removal in TFs.

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# CHAPTER 7:
# RESPONSE OF BIOLOGICAL SAND FILTERS TO SHUT-DOWN AND TOXIC EVENTS

## 7.1 INTRODUCTION

The character and volume of winery wastewater is seasonal and differs from winery to winery. This has been discussed in the introduction sections to Chapters 3 and 4 of this report. In reality, there is almost always a supply of effluent from most wineries, but it may be extremely dilute during periods when there is minimal cellar activity.

Conventional wastewater treatment systems used for the treatment of winery effluent require lengthy start-up periods to re-acclimate microbial populations during peak seasons; they are also vulnerable to changing influent character and toxic events (Grismer et al., 2003; Eusébio et al., 2004).

This study was performed to determine the effects of shut down and toxic events on the performance of biological sand filters treating synthetic winery effluent.

## 7.2 MATERIALS AND METHODS

### 7.2.1 Set-up, mode of operation and feeding/amendment of biological sand filters

The set-up and mode of operation of the BSFs were as described previously in Sections 2.2.1 and 3.3.1, respectively. Four systems containing Phillipi sand were used in the experiments discussed in this report (P1-4).

The effects of shut down (resting) and toxicity (chlorination) on the microbial community function in BSFs was determined by (i) Neither feeding nor amending two BSFs for either two months (P1, P2), or eight months (P3, P4), and (ii) Amending two of these BSFs (P1, P3) with a chlorine solution (250 mg/L) and two BSFs (P2, P3) with tap water for two weeks (Table 12).

**Table 12: Experimental protocol applied to determine the effect of toxicity and resting (shut-down) on the performance of biological sand filters**

| BSF | Resting period | Chlorination 250 mg/L |
|---|---|---|
| **P1** | 2 months | Yes |
| **P2** | 2 months | No |
| **P3** | 8 months | Yes |
| **P4** | 8 months | No |

After chlorination, the systems were all amended according to the same protocol (Table 13). The synthetic winery wastewater applied was the same as that described in Chapter 5 (ethanol, acetic acid, vanillin and gallic acid) so that a comparison could be made between systems which had been equilibrated prior to amendment (previous study), systems that had been rested for either a short period (2 months) or a long period (8 months), and systems where the microbial communities had been exposed to a toxic chemical.

**Table 13: Operational parameters applied during the experimental period**

| | Week | P1 | P2 | P3 | P4 |
|---|---|---|---|---|---|
| ***Amendment (per week)*** | 2 x | 2 x | 2 x | 2 x | 2 x |
| ***HLR (L/m$^3$sand/day)*** | 1-6 | 22.9 | 22.9 | 22.9 | 22.9 |
| ***OLR (g COD/m$^3$sand/day)*** | 1-2 | 0 | 0 | 0 | 0 |
| | 3-6 | 48 | 48 | 48 | 48 |
| ***Influent COD (mg/L)*** | 1-2 | 0 | 0 | 0 | 0 |
| | 3-6 | 2027 | 2027 | 2027 | 2027 |
| ***Chlorine (mg/L)*** | 1-2 | 250 | 0 | 250 | 0 |
| | 3-6 | 0 | 0 | 0 | 0 |

**HLR = hydraulic loading rate OLR = organic loading rate COD = chemical oxygen demand**
**COD, OLR values include contributions by glucose**

### 7.2.2 Sampling

Effluent and pore water samples were taken as described in Sections 2.2.2.2 and 4.2.2.2, respectively.

### 7.2.3 Characterization of influent, effluent and pore water

The COD was determined as described previously (Section 3.2.3).

## 7.3 RESULTS AND DISCUSSION

### 7.2.1 The effect of long-term resting on the performance of biological sand filters

To determine whether resting affected COD removal performance, results obtained from a BSF that had been rested for 2 months (P2) was compared with previous results obtained from the same system during 'normal' operation (P2 pre) (Figure 35). Surprisingly, in all niches apart from the deep inlet, the COD concentrations were notably lower than in the systems which had not been rested. This would suggest that 2 months resting had a positive impact on the biodegradative performance of the BSFs. Two hypotheses for this are presented: Firstly, the experiments were not performed in parallel and therefore different

seasonal shifts in the microbial populations may have played a role in the disparate performances. Secondly, the second set of experiments was conducted using systems that had been rested, but had also previously been exposed to synthetic winery wastewater. Therefore, microorganisms which survived the desiccation and lack of nutrients during resting were already pre-acclimated to the synthetic winery wastewater. It is therefore possible that resting after amendment with synthetic winery wastewater resulted in the selection of organisms that were particularly robust and functionally adapted.

To determine whether the duration of resting had any effect on COD removal performance, results from BSFs which had been rested for either two months or eight months were compared. The results obtained from the two systems that had been chlorinated and rested for 2 months (P1) or eight months (P3) were similar. In addition, the results obtained from the two systems that had not been chlorinated, but were rested for 2 months (P2) or eight months (P4) were similar (Figure 35).

### 7.1.3 The effect of a toxic event on the performance of biological sand filters

To determine the effect of toxicity on COD removal performance, results obtained from BSFs amended with either chlorine (P1, P3) or tap water (P2, P4), were compared.

Chlorination is highly toxic to microbial communities at the concentrations used for this experiment. Chlorination had a definite effect on system bioremediatory performance at the superficial inlet. However, the adverse effects were localised to this particular niche. This can be seen by comparing results obtained from chlorinated P1 with non-chlorinated P2 (both rested for 2 months), and results obtained from chlorinated P3 with non-chlorinated P4 (both rested for 8 months) (Figure 35).

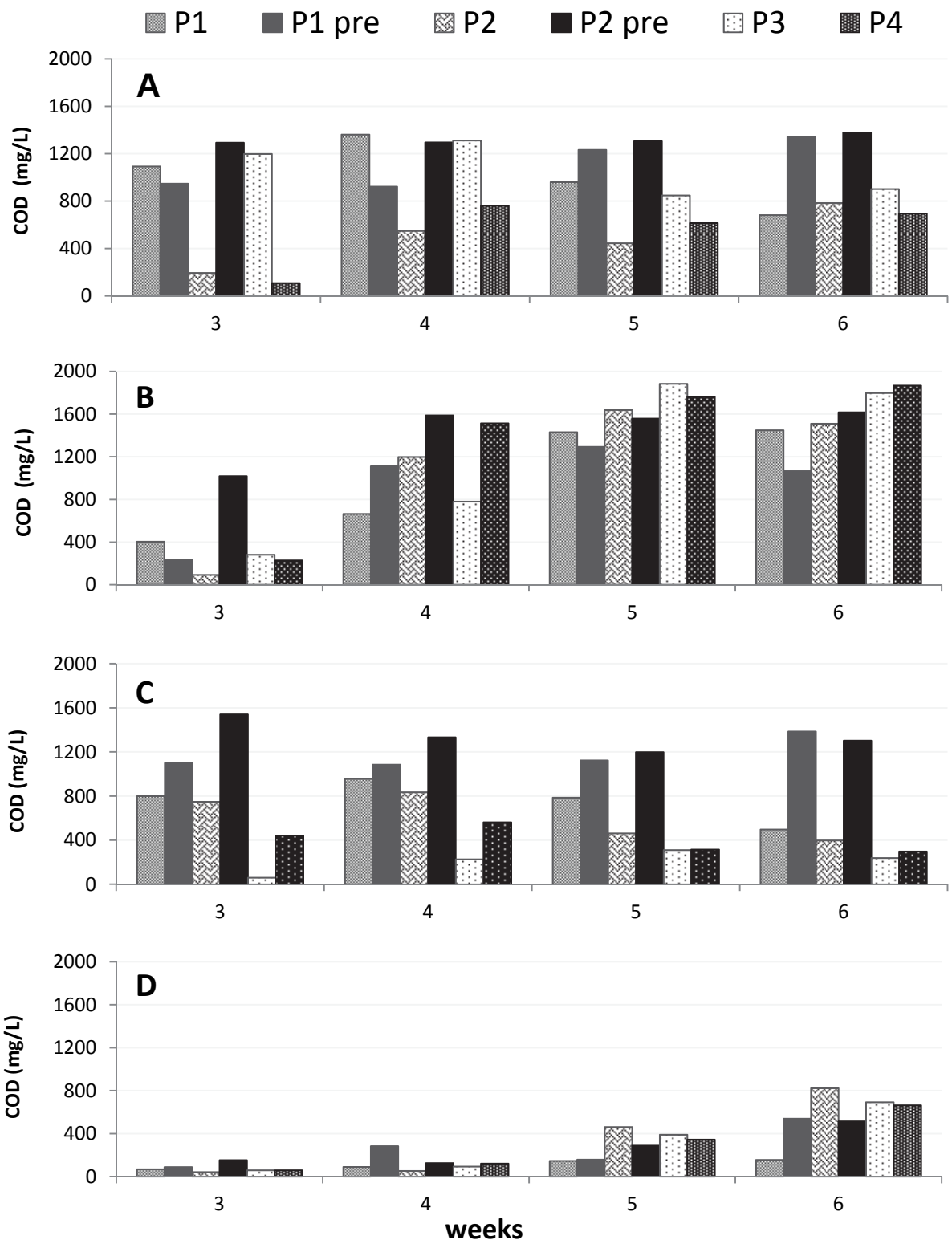


**Figure 35: Chemical oxygen demand concentrations in pore water samples taken from biological sand filters at the superficial inlet (A), deep inlet (B), superficial outlet (C), and deep outlet (D). During week 1 and 2, systems were amended with either chlorine or tap water and from week 3, with synthetic winery wastewater**

The adverse effect of chlorination at the superficial inlets had almost completely abated after four weeks, demonstrating excellent resilience of the systems to toxic events.

### 7.1.4 Conclusions

Overall, the results showed that resting, and the duration of resting did not adversely affect system performance and may, in fact, have enhanced system functionality. This demonstrated that systems with sufficient capacity for peak season flows are capable of coping with variable loads.

The results of this study are most encouraging, as upsets in conventional suspended and fixed growth biological wastewater treatment systems can take weeks to rectify. Microbial communities in soil are adapted to different moisture and nutritional conditions and many possess mechanisms to survive dry spells and/or varying nutritional supplies (e.g. the ability to sporulate). The sand substrate closely mimics a natural environment. This factor may have contributed to the resilience of the systems to dessication (resting), lack of nutrients (resting), and toxicity (chlorination).

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# CHAPTER 8:
# ANALYSES OF BACTERIAL COMMUNITY STRUCTURE AND CHEMICAL ANALYSES IN SITES IMPACTED BY WINERY WASTEWATER

## 8.1 INTRODUCTION

The primary objective of the work described in this chapter was to gain insight into the effect of winery waste on benthic bacterial communities. It was envisaged that these results would be used to broaden our knowledge on the use of BSFs for the treatment of agri-industrial wastewater. To this end, the impact of location (impacted with winery effluent/not impacted) and seasonality (before crush season/after crush season) on the benthic bacterial community structures was determined.

Three sampling locations were identified: two irrigation storage dams and one farm dam receiving waste from a nearby cellar. The primary aims were to determine:

- Whether there were inter-environmental similarities in the microbial communities impacted with winery wastewater
- The impact of seasonality (before/after crush season) on the bacterial communities
- The primary chemical composition of the winery wastewater impacting the chosen sites

## 8.2 MATERIALS AND METHODS

### 8.2.1 Sampling points

Three locations in the Stellenbosch environs were identified as suitable sites for the study. Sediment and wastewater from 2 co-operatives and one wine farm designated respectively A, B and C were sampled before (9$^{th}$ and 13$^{th}$ of February) and after (16$^{th}$ of May) the 2012 crush season.

The first location, Site A, consisted of an irrigation storage dam (ISD) that contained large quantities of winery effluent emanating from processing activities at the nearby co-operative. Kikuyu grass surrounded this site, but no macrophytes were present. The depth of the ISD was estimated to be around 3 m. Disposal of the wastewater from the ISD was accomplished via rotational irrigation on a dedicated field adjacent to the ISD. The site was ideal for studying the long-term adaptation of microbial communities to winery wastewater as it was continually inundated with winery wastewater. However, there was no ideal non-impacted control site on the premises.

At the second site (B), there were 2 adjacent dams that were both lined with clay and constructed in an almost identical manner. As in the case of site A, these dams did not

contain macrophytes. Wastewater was pumped into the wastewater ISD after filtration through gravel to remove suspended solids. As with site A, final disposal of the effluent took place via rotational irrigation on an adjacent field dedicated to this purpose. There was no physical connection between the adjacent rainwater storage dam (RSD) and the ISD. The former was used as a control and the latter as a test sampling site. The physical similarities between these dams made this site ideal for the purposes of this study. The surface area of the ISD at site B was far greater than that at site A, but the depth a lot shallower, estimated at 0.5 to 1.0 m, dependant on season.

The third location (site C) was a farm dam that received effluent from a small cellar nearby and agricultural run-off from the surrounding catchment. This area has been studied extensively in the past (WRC projects K5/1936 and K5/1544) as the flow of wastewater from the cellar is intercepted in a wetland area that has evolved naturally over time. It has previously been shown that the flow of waste through this wetland area (which is also diluted with water from a perennial spring), results in complete remediation of the wastewater. Although the wastewater flows through a wetland which contains macrophytes, the farm dam itself is open water with no emergent or floating vegetation. The test (impacted) sampling points were situated on the effluent flow path, which was dry before and wet after the crush season. The control (non-impacted) sampling sites were situated on the opposite side of the dam from the effluent discharge point, one of which was inundated and two dry both before and after the crush season (Figure 36).

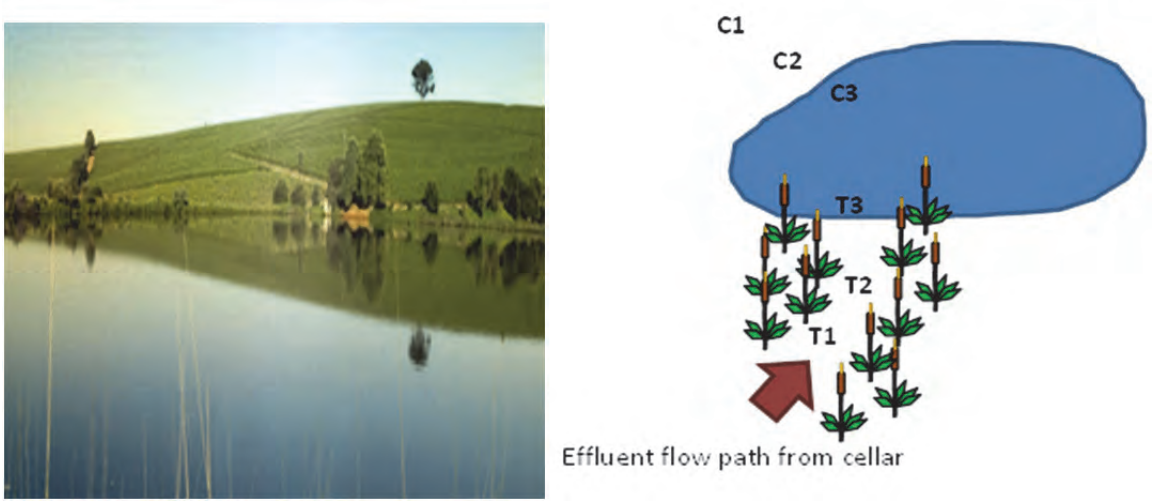


**Figure 36: Photograph and schematic representation of the farm dam at site C, showing the control sampling sites (1-3) and test sampling sites (1-3)**

### 8.2.2 Sampling procedures

Sediment samples from the first 3 cm were collected into sterile sampling bags, placed on ice and taken to the laboratory. Aliquots (~1 g) were immediately stored at -80°C for molecular characterisation. The remainder of the sediment was stored at 4°C. Composite water/effluent samples were taken from the inundated areas before the crush season. After the crush season, 3 further samples were taken from each inundated sample site. All samples were frozen at -20°C until analysis.

### 8.2.3 Molecular community fingerprinting

DNA extraction, PCR amplification, purification, restriction digestion, and T-RFLP analyses were performed as described in Section 2.2.6.

### 8.2.4 Chemical analyses

COD concentrations, identification and quantification of sugars, acids, alcohols and phenolics, as well as the conversion of concentrations into COD equivalents and compilation of metabolic profiles was performed using the same methods, calculations and equipment as previously described (Section 3.2.3). The pH of freshly collected pore water samples was determined using a pH700 meter and relevant probes (Eutech Instruments, Singapore).

## 8.3 RESULTS AND DISCUSSION

### 8.3.1 Chemical analyses of dam water impacted/not impacted by winery wastewater

#### 8.3.1.1 Chemical oxygen demand and total phenolic concentrations

Quantitative analysis of the COD concentrations in the water/effluent samples revealed two distinct groups: Samples from the ISDs fell into one group and were orders of magnitude higher than those from site C and the control dam at site B (Figure 37). In the case of site C, the results confirmed previous findings that the wastewater is remediated during passage through a naturally evolved (acclimated) wetland. The COD measurements from the ISDs ranged from 2 509 mg/L at site B prior to the crush season to 9 083 ± 201 mg/L at site A after the crush season. In both ISDs, the concentrations increased from before to after the crush season. The concentrations in the samples taken from three points at the site B ISD were similar, at around 3 800 mg/L. However, there were significant differences in those from the site A counterpart, ranging from 5 967 ± mg/L at point 3 to 9 083 ± 200 mg/L at point 1. Site A is a lot deeper than site B, with less surface area for convection mixing, which is the most likely explanation for this finding (Wetzel, 2001).

The COD concentration of a fresh effluent sample which was taken at the influent to the ISD at site B was more than double that in the ISD itself. It is known that the composition of

winery wastewater can vary almost on an hourly basis, and the COD concentration range extremely variable. For example, the COD range of 15 samples taken over a six month period from a medium-sized winery in Stellenbosch was 675 mg/L to 28 400 mg/L. However, it is also likely that reasonable organic degradation rates are achieved in this ISD by virtue of the dimensions (shallow, large surface area) which maximizes the opportunity for photodegradation (particularly of toxic phenolics), and aeration by wind-mixing.

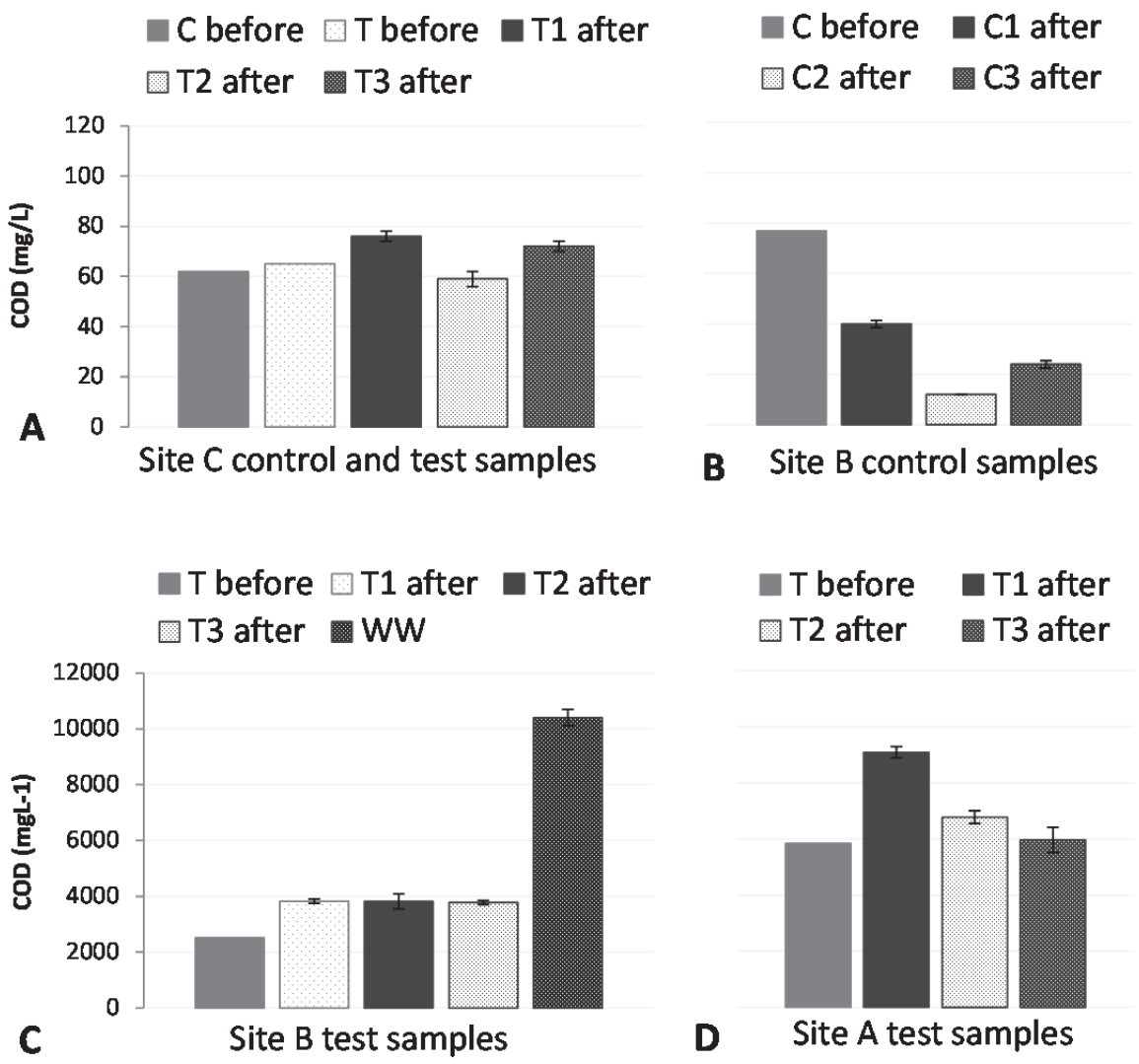


**Figure 37: Chemical oxygen demand concentrations measured before and after the crush season from: (A) A wetland and dam from areas either impacted (test area, T) or not impacted (control area, C) by winery effluent; (B) A rainwater storage dam used as a control at site B; (C) A winery effluent irrigation storage dam and influent to this dam at site B; and (D) An irrigation storage dam at site A. Samples were taken before and after the crush season**

The total phenolic concentrations in the samples were measured and the trends matched those of the COD measurements, except in the sample taken from the ISD at site B before the crush season (Figure 38). In this sample, the total phenolic concentration was low (~10 mg GAE/L) and similar to the concentrations (~1 mg GAE/L) found at the RSD at the same site, as well as the dam at site A, than in the other samples taken from the ISDs (~30 to 80 mg GAE/L). These results show that that almost complete degradation of phenolics took place in the ISD at site B between February and May. Furthermore, the high COD concentration in the same sample suggests that either (i) low concentrations of phenolics occur in the wastewater out of the crush season, and/or (ii) that there are more recalcitrant molecules than phenolics, either inherent in the winery wastewater, or metabolic products which were formed in the ISD.

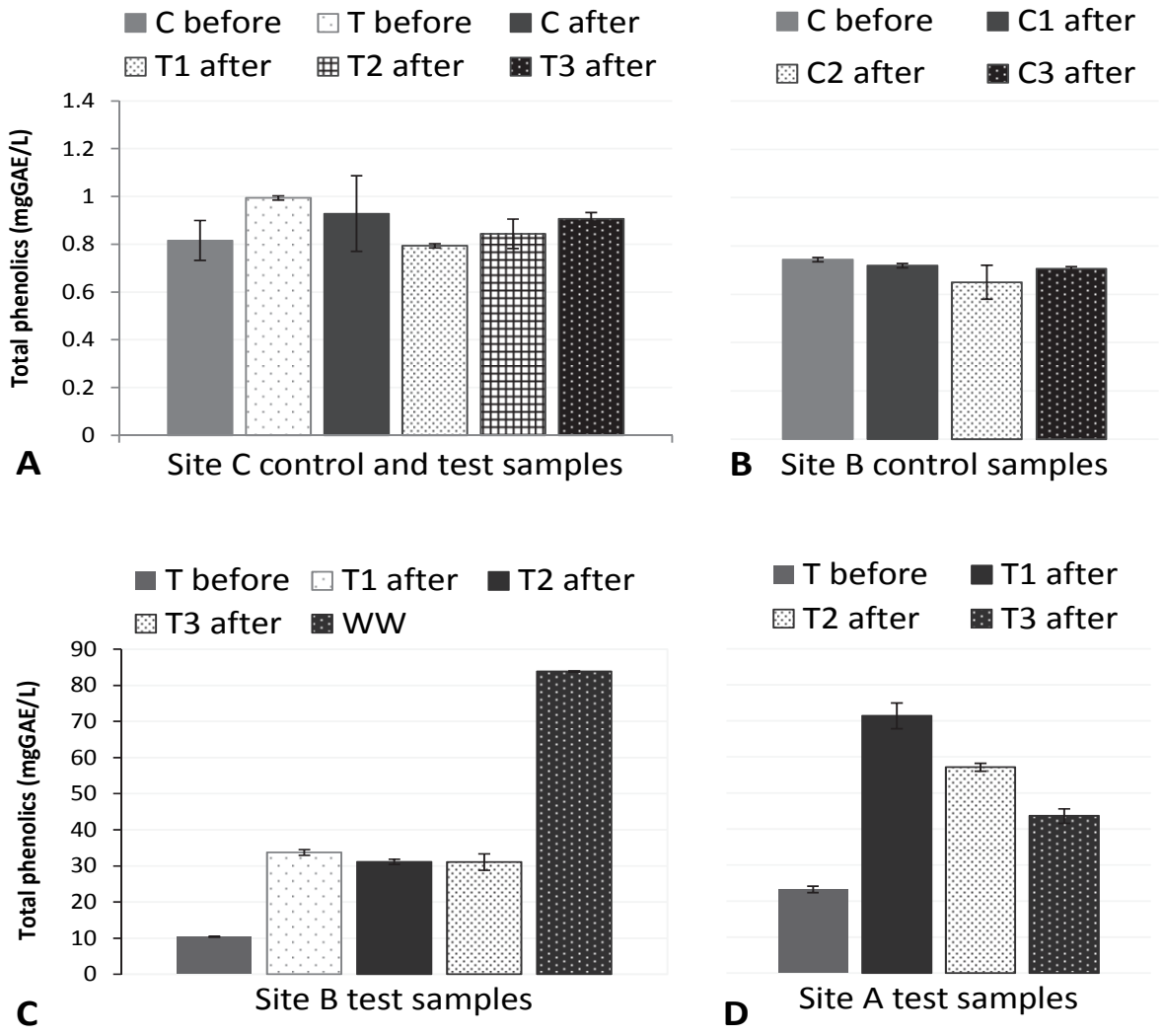


**Figure 38: Total phenolic concentrations measured before and after the crush season from: (A) A wetland and dam from areas either impacted (test area, T) or not impacted (control area, C) by winery effluent; (B) A rainwater storage dam used as a control at site B; (C) A winery effluent irrigation storage dam and influent to this dam at site B; and (D) An irrigation storage dam at site A. Samples were taken before and after the crush season**

### 8.3.1.2 Characterization of irrigation storage dam organics

The major contributors to COD in the samples taken from the ISDs were ethanol and the volatile fatty acids, acetate, propionate and butyrate (Figure 39). As well as exhibiting similar COD and total phenolic concentrations, the three samples from site B (shallow, with a large surface area for wind mixing), displayed similar concentrations of ethanol and volatile fatty acids. Conversely, the samples from the three sites at site B (deep, with small surface area) showed different concentrations and relative concentrations of these organic molecules.

The dominance of ethanol and acetate supports the work conducted at CPUT over the past 3 years, where ethanol and phenolics have been used as a proxies for readily and slowly biodegradable COD, respectively (Rodriguez Caballaro et al, 2012; Welz et al., 2012, Welz et al., 2011).

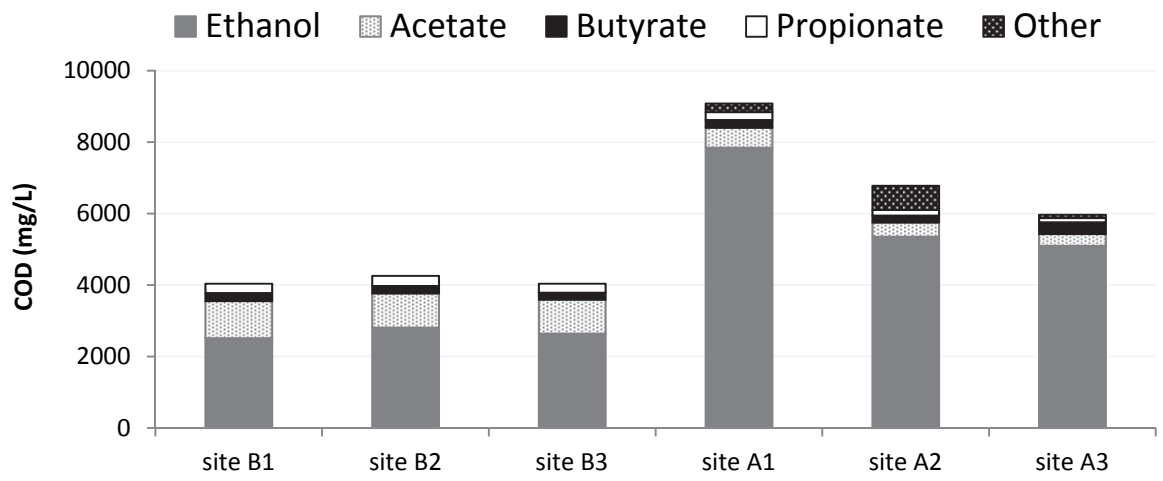


**Figure 39: The organic composition of winery wastewater samples taken from irrigation storage dams at site A and B after the 2012 crush season**

### 8.3.2 Analysis of the sediment bacterial community structures in sites impacted by winery wastewater

DNA extracted from sediments before and after the crush season was analysed using T-RFLP. NMDS and ANOSIM analyses were performed to evaluate statistical (dis)similarities between samples. The absence of plants decreased the complexity of the system, so that only the physical substrate and wastewater need be considered as primary determinants of the bacterial community structure.

In the NMDS plot comparing the bacterial communities from all of the samples, 2 groups were distinguished (Figure 40). The first group represents samples taken from site B, and the second group represents the remainder of the samples, irrespective of whether they were impacted by winery wastewater or not. ANOSIM pairwise tests confirmed that the bacterial communities in these two groups were significantly different (R = 0.745 to 0.939).

### 8.3.2.1 Detailed evaluation of site A irrigation storage dam

At site A, despite the continual contact of the bacterial communities with winery effluent in the irrigation dam, there were significant differences in the bacterial community structures before and after the crush season, sharing only 20% similarity with each other. This is in contrast to the similarity shared between the communities from the three samples (A1-3) taken before the crush season (minimum 70%) as well as the three samples taken after the crush season (minimum 70%). These results show that seasonal differences in the dam sediments had a significant impact on the bacterial communities. However, it is not known how much of this could be attributed to differences in the winery wastewater chemistry and how much to natural seasonal succession.

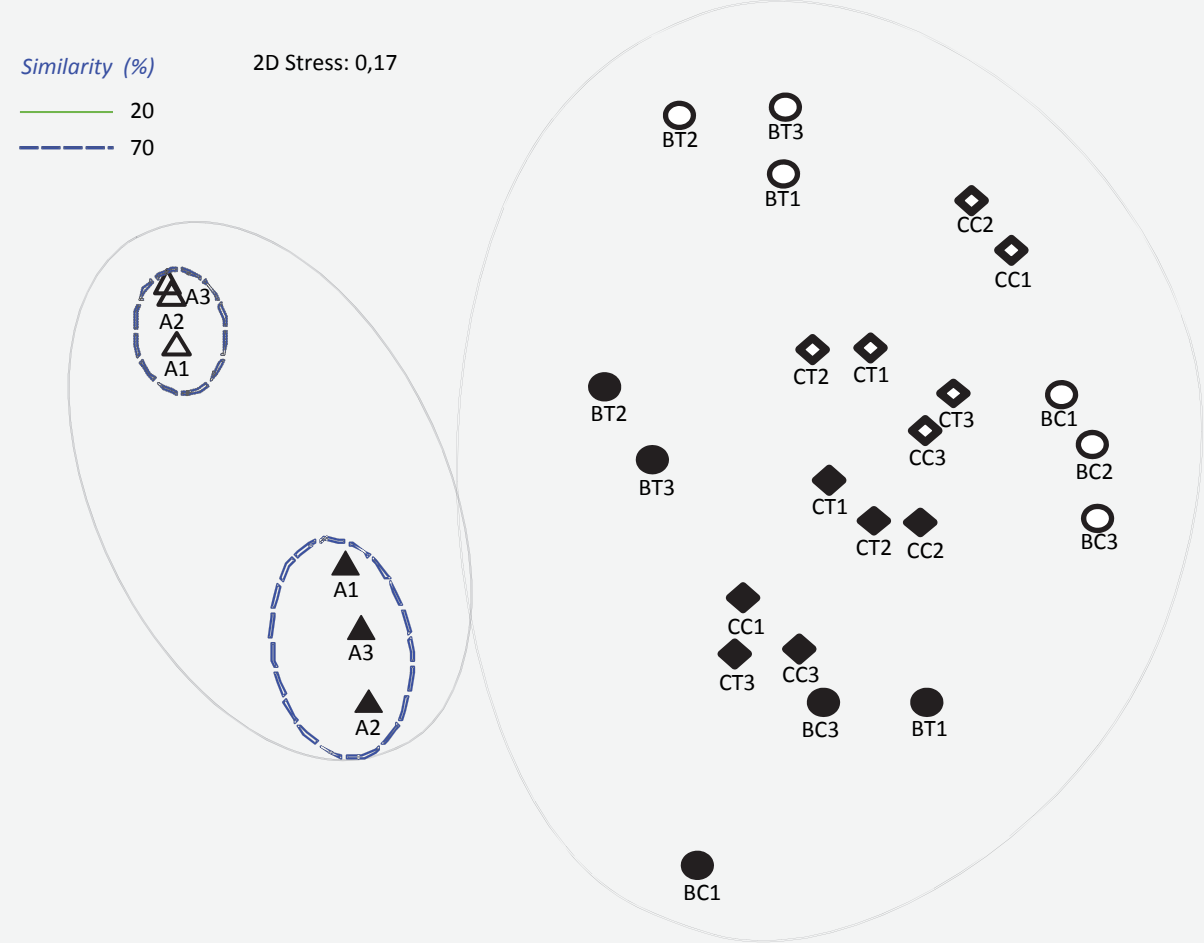


**Figure 40: Non-metric multi-dimensional scaling plot representing the evolution of bacterial community structure before (black) and after (white) the crush season. Letters represent the site (A, B, C) and whether the sample was from a test site (T) or control site (C), and numbers represent the replicates from each site at each sampling instance. Sites are also distinguished by shape.**

### 8.3.2.2 Detailed evaluation of site C wetland and farm dam

The bacterial communities from site B and C were evaluated further. In the case of site C, test sampling points were in wetland areas impacted by winery wastewater, while the

control sampling points were on the opposite side of the same dam. In the NMDS plot, 2 groups were distinguished, representing samples taken from before and after the crush season (Figure 41). The difference between the groups was statistically significant (ANOSIM R = 0.429). However, the difference between the bacterial communities at the test and control sites was not significant (ANOSIM R = 0.178), showing that the winery wastewater had little or no effect on the bacterial communities. This could be explained by the fact that the wastewater was rapidly remediated (as evidenced by the low COD and total phenolic concentrations) and was continually diluted by perennial spring water. The changes seen from before to after the crush season could thus be attributed to natural seasonal succession.

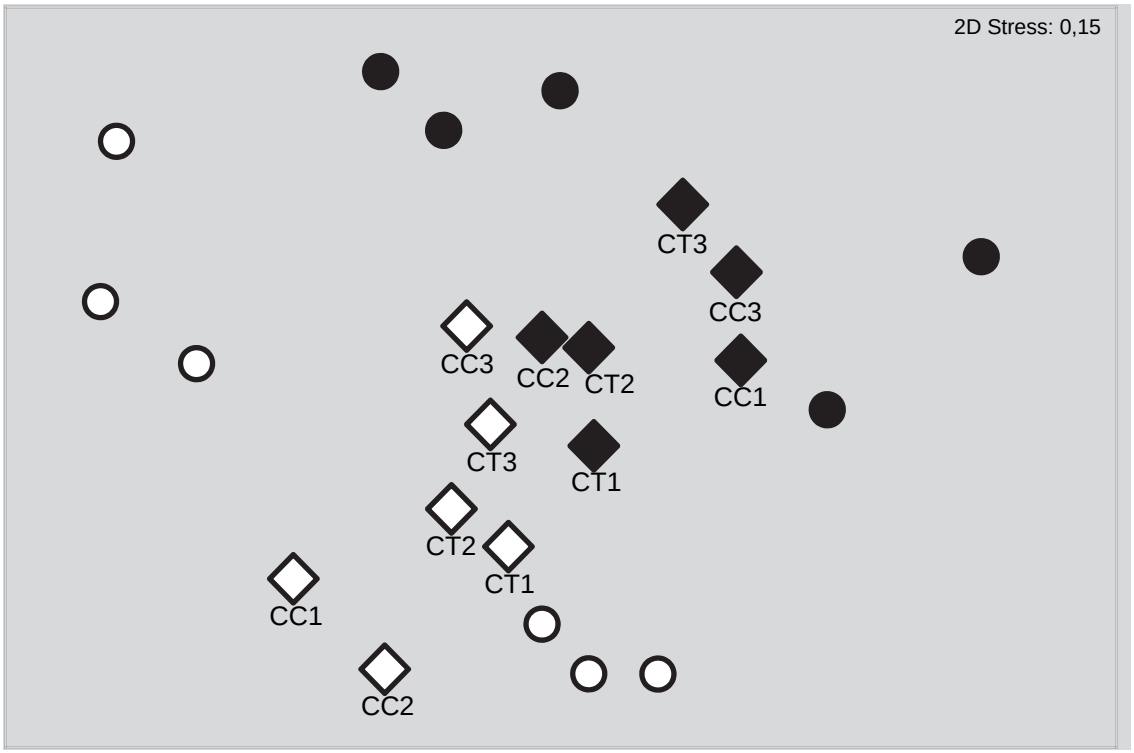


**Figure 41: Non-metric multidimensional-scaling plot representing the evolution of bacterial community structures before (black) and after (white) the crush season from site B (represented by circles) and C (represented by diamonds). The focus is on site C, the points of which are labelled as controls (CT) or tests (CT)**

### 8.3.2.3 Detailed evaluation of site B, with irrigation storage and rainwater storage dam

The bacterial communities from the control (RSD) and test (ISD) sample points at site B were evaluated further. On the NMDS plot, samples clustered into four distinct groups: (i) The control sampling points before the crush season, (ii) The control sampling points after the crush season, (iii) The test sampling points before the crush season, and (iv) The test sampling points after the crush season (Figure 42). Two-way crossed ANOSIM analysis was used to ascertain whether seasonality (before and after crush) or location/exposure to winery wastewater (control/unexposed and test/exposed) had a greater effect on the evolution of the bacterial community structures at this site. It was found that R = 1 for both groups, proving that both of these factors played a significant role in the evolution of the bacterial communities.

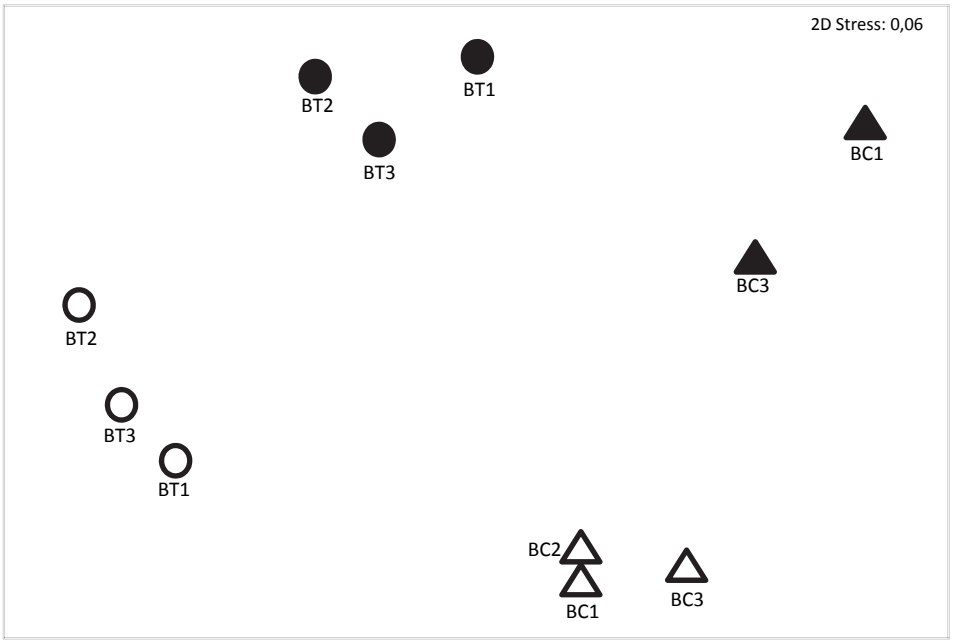


**Figure 42: Non-metric multidimensional-scaling plot representing the evolution of the bacterial community structures before (black) and after (white) the crush season from all sampling sites at site B. Samples from the irrigation storage dam impacted by wastewater are represented by circles, while those from the rainwater dam are represented by triangles**

### 8.3.3 Conclusions

Wind mixing and photo-degradation may play a role in the remediation of winery effluent, including phenolic degradation in irrigation dams. Winery wastewater in high concentrations has a significant impact on benthic bacterial communities, while low concentration effluent does not. Seasonal differences appear to be as significant as the impact of winery wastewater. In addition, site specific differences, not related to the concentration of winery wastewater, can occur.

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