INTEGRATED RESEARCH FOR USE IN CONSTRUCTED WETLANDS FOR TREATMENT OF WINERY WASTEWATER

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EXECUTIVE SUMMARY

Background

As a direct result of winemaking activities, the South African wine industry disposes of approximately a billion litres of wastewater annually. This effluent is normally irrigated on pastures or, in some cases, dumped directly in the nearest river. Some cellars do have water treatment facilities, but at present this is the exception rather than the rule. Most cellars are small, which means that the effect of each cellar's effluent disposal practices on the environment is negligible. However, when examined collectively, the industry certainly contributes to environmental degradation in the drainage basins of the main water catchments of the Western Cape Province – namely the Breede River, the Olifants River, and the Berg River.

Winery effluent is seasonal in nature and can be highly concentrated in terms of Chemical Oxygen Demand (COD) and levels of sodium and potassium ions (amongst other ionic species). The effluent is particularly concentrated from January to April (the harvest period) with subsequent smaller peaks later in the year, particularly coinciding with the dry season. The Berg, Breede and the Olifants rivers are normally brackish and have low flow at this time of the year. This means that effluent entering these rivers has a greater impact than it would otherwise, and as the river water levels decrease towards their mouths, the effluent can be strongly concentrated. The impact of the effluent is compounded by the non-point source of agricultural pollution that arises from the use of fertilizers and pesticides. These factors combined contribute significantly to eutrophication and deoxygenation of rivers and ground waters in these drainage basins.

The problem of winery effluent can be remedied by means of conventional effluent treatment plants. However, this can be expensive and additional trained personnel may be required to operate them. Smaller and marginal cellars are especially exposed to the increased cost because they may not have the funds to purchase effluent treatment plant and/or they may not be able to afford personnel to operate it. Conventional effluent treatment (anaerobic or aerobic digesters) is not cost efficient for this industry and is difficult to control, due to the seasonal variation of the effluent. Operational difficulties are not uncommon for these plants when they are used for winery effluent treatment.

For these reasons, constructed wetlands have been identified as a suitable means of treating winery effluent. They may provide a low-cost alternative treatment method with significant additional marketing potential for wineries, e.g. conservation and recreational areas. There are additional benefits accrued by the cellars from using constructed wetlands. These include: the establishment of a water treatment facility that requires little or no labour, no chemicals and very little maintenance, the establishment of recreational areas which can add value to the winery and demonstrate commitment to environmental causes, and provision of habitat for wildlife

species (especially birds and indigenous flora) through the creation of an environment suited to them.

Constructed wetlands can provide a cheap, sustainable means of effluent treatment for the wine industry in South Africa. Wetlands are known to be amongst the most biologically active of all living systems, but they are susceptible to high strength effluent. In order to find the limits of effluent strength that constructed wetlands can safely treat, it is necessary to characterise the wastewater entering the wetland. There are also certain other problems associated with constructed wetlands, which include: sensitivity of many plants to high COD levels, an inadequate understanding of the contribution of wetland micro-organisms and plants to the process of effluent biodegradation, an inadequate understanding of the hydraulics of constructed and natural wetlands, and a lack of suitable mathematical design models with which to design constructed wetlands.

Wetland microbiology has great significance with respect to the performance of constructed wetlands. Of particular interest are the plant communities and the microbial communities of constructed wetlands. Since constructed wetlands are complex biological systems, it may not be possible to include all these parameters in any mathematical model developed, but the linkage between biological activity and chemical changes is clearly relevant and important. Thus, this project included a preliminary approach to correlating biochemical, microbial and chemical variation over the seasonal course of wetland operation.

Objectives of the study

The study was initiated in order to address some of these considerations, and therefore the objectives of the project were set as follows:

1. To demonstrate the feasibility of modelling a constructed wetland, based on information correlating microbial end enzyme activities with organic pollutant removal.
2. To access and use available existing relevant information on wetlands to support the research model.
3. To identify soil, water and plant root microbes present in wine wastewater wetlands using phlyogenetic techniques.
4. To characterise their metabolic / enzyme activities in the biodegradation process.
5. To demonstrate the linkage between microbial diversity, biochemical activity and the bioremediation process.

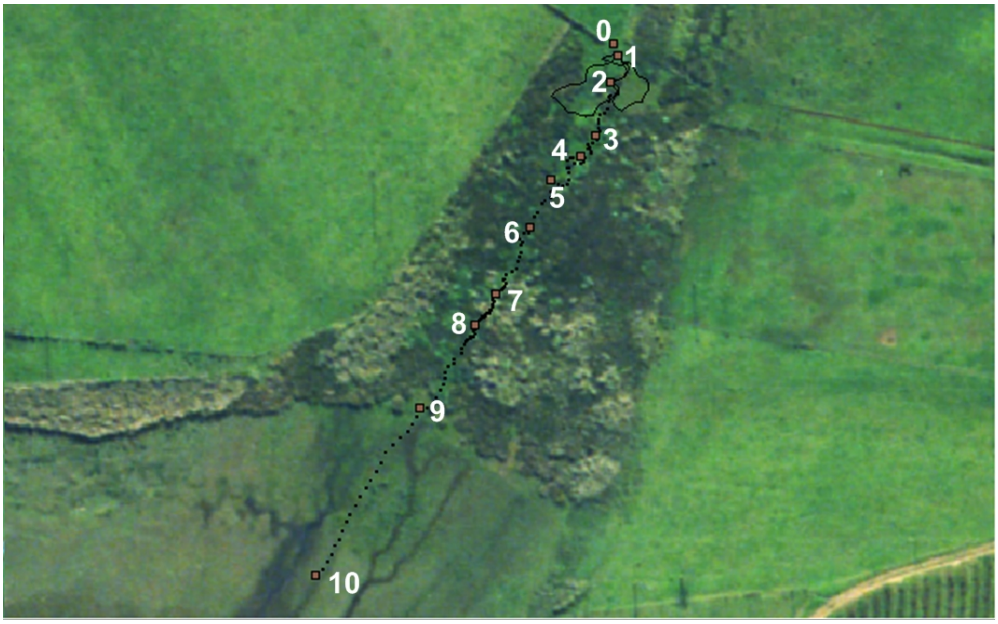
The intended outcome of the two-year feasibility study would be a body of data which will demonstrate feasibility of using a molecular / biochemical approach in developing a preliminary model of the wetland. This would yield new understanding of the contributions and complex interactions of microorganisms and plants in wetland bioremediation systems and the data

would be built into a novel predictive model of a wetland customised for winery effluent treatment.

Research process and results

(a) Wetland test sites

The study was conducted using wetlands of three different types: one at a local winery in the Stellenbosch area where a wetland has been established for many years and is operational as an effluent disposal facility (shown below), one at a research station which was constructed and could be used for tracer studies, and small-scale laboratory bench artificial wetlands set up to measure certain physical parameters.



Established wetland at Stellenbosch winery, showing sampling points

The small-scale laboratory "wetlands"

Constructed wetlands at ARC Nietvoorbij, Stellenbosch

(b) Findings from chemical characterisation and modelling of wetland performance

The results obtained confirm that the biodegradation of winery waste taking place in the wetland can be effectively characterised. The degradation of the main constituents of winery effluents (glucose, fructose, ethanol, and phenolic substances) was characterised in detail and this information has been used as the basis for the model of the wetland biodegradation.

In the initial characterisation of winery wastewater, ethanol is the major constituent of winery effluent and contributes approximately 86% to 91% of the COD; ethanol levels were shown to rise to above 4 ml/ℓ at certain times. Acetic acid is the second organic component that contributes significantly to the COD of winery effluent. Acetic acid may occur at concentrations as high as 0.4 ml/ℓ., and the pH of the effluent during the harvest period is roughly 4.5. The quantity of potassium was strongly dependent on the time of year and the intensity of the harvest season, ranging from 25 mg/ℓ and 225 mg/ℓ. Sodium levels in the effluent are less dependent on the harvest than potassium, ranging between 25 mg/ℓ and 125 mg/ℓ. Whilst other components such as longer (C_3, C_4, C_5) carboxylic acids, other organic acids and phenolic

components are present in winery effluent, they were found to be not significant as modelling parameters. However, they did contribute significantly to the organic loading which would supply carbon nutrient for microbial growth.

A mathematical model of a constructed wetland was developed in order to predict the trends whereby the wastewater components would be removed in a constructed wetland. The purpose of the model was to provide engineers with information about the size required for a constructed wetland as a treatment system, and to provide information on the expected final effluent specification. The model needed to incorporate aspects of time (temporal aspects), space (spatial aspects) and the degradation kinetics of the chemical species present in the effluent. Thus, the model should (within acceptable limits) describe the surface plots for different chemical species within the effluent. The model was set up as a set of continuous stirred tank reactors, and tracer studies provided sufficient information to calculate the degree of dispersion, the residence time of each tank, as well as the number of tanks needed to be modelled. Variables incorporated in the model included ethanol, acetic acid, sodium and potassium, and COD. The model was developed using one set of experimental data and then tested using a second set obtained later in the study.

The data generated by the chemical characterisation was combined into the mathematical model, and generated the following chemical information:

- The pH rises rapidly as wastewater enters a wetland, and then stabilises at approximately 7.5 to 8. This is a useful value in that effluent may be released into a river at this pH.
- Propanol, propionic acid, and butyric acid, minor components of the organic constituents of the effluent, are removed well before ethanol and acetic acid are, and can thus be neglected from modelling.
- Ethanol is the critical parameter and the model was found to be accurate in determining the rate of removal. If anything, the ethanol conversion in the wetland was under predicted which is positive in terms of design purposes because it implies that the model is conservative and constructed wetlands designed on this basis will be oversized.
- Acetic acid was found to be a significant but non-critical parameter. Further down the wetland, the acetic acid was fully consumed. This was to be expected as the acetate would be a suitable carbon source for microbial activity.

(c) Botanical survey results

Based on the findings of the botanical survey, it is recommended that the constructed wetland be sub-divided into regions. The length of the wetland should be divided into four equal regions. The first region should be planted with a combination of *Carex* (common sedge) and *Zantedeschia aethiopica* (Arum Lily). The second region should be planted with *Zantedeschia aethiopica* (Arum Lily) and *Typha capensis* (Bullrush). Region three should be planted with Arum lilies and Region 4 should be planted with *Pesicaria decipiens*. This would provide for a greater

degree of plant biodiversity, which would in turn provide increased bio-remediation. These plants are known to be resistant to winery effluent and are indigenous. Any other indigenous wetland plants may also be interspersed within this system as an experiment during the construction of CWs especially for winery effluent remediation.

(d) Microbiological and Biochemical analyses

It was demonstrated that the microbial community present in the wetlands, and its seasonal variation, could be effectively characterised using phylogenetic techniques. The anaerobic metabolic (enzymatic) activities occurring were detailed and correlated with the microbial population analysis. The direct detection of enzyme activities proved to be problematic. However, much has been learned and the second part of the project involved a new round of sampling, as new effluent entered the wetlands in January 2006, and modified enzyme assays were completed on fresh samples. These same samples were also used for confirmatory phylogenetic analysis to provide the correlation in the way that we now know is necessary. The project demonstrated that standard enzyme assays can be manipulated to allow for the detection of specific enzyme activities in soil samples, and key enzymes that may be involved in wine wastewater degradation can be detected by using these modified assays.

In the molecular (phylogenetic) investigations, ccommunity DNA was successfully extracted from winery wetland sediment samples taken at regular intervals throughout a full yearly cycle. The primary objective of the molecular phylogenetic analyses was to assess the feasibility of different systems as a means of determining microbial diversity, and in particular the fraction of the population with a functional role in degradation of winery waste stream components. This objective has been effectively satisfied.

Analysis of bacterial molecular phylogenetic markers (16S rRNA gene sequences) identified a wider range of aerobic and anaerobic microbial phylotypes. Also, amplification with archaeal-specific 16S primers indicated that the anaerobic sediments (all but the upper few mm of the sediment cores) contained a wide variety of methanogenic archaea. Organisms known to have a major role in degrading ethanol (e.g. *Pelobacter propionicus*) were identified in core samples. While 16S libraries did not provide sufficient data for significant differences in species distribution to be detected between samples recovered during harvest, and in the ‘off-season’, analysis of microbial species distribution using DGGE proved to be a reliable and effective means of comparing seasonal changes. Importantly, while most phylotypes were visible through the annual sampling cycle, significant changes in a number of phylotypes, associated with the seasonal harvest, were identified. Changes in archaeal populations suggest that these anaerobic organisms play a major role in secondary degradation of winery wastes, and may be a major contributor to carbon loss (as methane) from the system. It is important to note that since an open wetland is a multi-variable system, the population changes indicated are suggestive, rather than direct proof, of a role in degradation of winery waste products.

Trials using both 16S library analyses and 16S DGGE analyses demonstrated clearly that the latter method can be applied effectively to determine the dominant members of the microbial community. In addition, this method showed considerable promise as a technique for monitoring temporal changes in microbial populations. The effectiveness of this method thus allowed us to identify specific microorganisms which responded (both positively and negatively) to the seasonal changes in winery waste releases. We have thus been able to identify certain genera (target organisms) which are implicated in the direct metabolic degradation of winery wastewater chemical constituents, and which together constitute trophic components in the mineralization process.

Correlation with chemical and microbial data

Preliminary genera identification results from the natural wetland are presented in the full report and the tentative correlation of enzyme activity observed to genera detected in the NW samples was shown to be feasible. All of the phylogenetic information given in this report came from samples taken from the established functional wetlands area. It would be interesting to determine (in a future project) whether similar types of bacterial/archaeal populations would be observed in constructed wetlands, and this would give answers to the questions of whether we would need to seed artificial wetlands with specific bacteria or whether other populations have the ability to perform a similar role in the constructed wetlands where existing populations have adapted to the changing environment.

Discussion

This project was conducted as a feasibility study, to determine whether it is feasible to use the type of data generated in this study, to facilitate the modelling and effective design of wetlands. The study has demonstrated that the approach is successful, but there is a great deal more that can now be achieved using the technology and understanding that have been developed. The direct detection of enzyme activities has proved to be more difficult than anticipated. However, much has been learned. The last part of the project involved a new round of sampling, as new effluent entered the wetlands in January 2006, and modified enzyme assays were completed on fresh samples. These same samples were also used for confirmatory phylogenetic analysis to provide the correlation in the way that we now know is necessary. By January 2006 the monthly soil samples collected from the wetland inflow area and the respective extracted DNA samples, covered a full year. Using this sample set, the methods developed were applied to study the microbiological changes occurring in the wetland, which may be caused by the seasonal load with winery effluents. DGGE was used to detect qualitative or semi-quantitative changes in the composition of the bacterial community. The ideal method to identify the most abundant microbial species in the wetland would be to sequence a large number (>500) of amplified 16S rDNA. Within the current feasibility study, clone libraries containing ca. 40 clones

each were prepared from February and October samples, of which at least half are being sequenced. This would yield more data to use for correlations.

A major problem, of course, is that many other factors apart from the winery effluents may induce changes in the bacterial composition and activity in the wetland soil during the year (temperature, rain, sun, changing vegetation, birds etc.). It would therefore be very interesting and highly valuable for future research, to determine whether at least some of the data obtained from the wetlands could be reproduced in experiments with the lab-scale wetland. Soil from the wetlands could be applied to the model, from which samples would then be taken before, during and after the application of winery effluents. We do have a model wetland set up and samples could be taken for analysis in a future project.

The success of this trial study has many implications for further work. In the molecular work, using DGGE analysis of amplified 16S rRNA marker genes as a basic identification tool, the following are possible lines of investigation aimed at further understanding the details of the degradative process, the specific enzymes involved and the process kinetics.

- Identified organisms can be isolated and cultured, allowing the opportunity to investigate their specific enzymology, degradation capacity, reaction kinetics, response to concentration changes, etc.
- Quantitative changes in target organism populations can be more accurately monitored using qPCR (quantitative PCR).
- A knowledge of specific key organisms leads directly to the identification of key enzymes. In some instances, detailed information on these enzymes may be available in the scientific literature or through sequenced genomes. In either case, it is possible to specifically track the expression and activity of such organism-specific enzymes, using either specific assays of enzyme gene-specific qPCR.
- An understanding of which organisms play a key role in degradative processes permits a higher level of monitoring and control of the system. For example, the impact of potentially deleterious processes (such as intermittent releases of high or low pH water streams) could be monitored and understood.
- These techniques can potentially be applied to any wastewater system, particularly wastewaters containing where high concentrations of known chemical constituents.

Further studies using laboratory scale model wetland would be very useful. This is a controlled environment which lends itself to testing the effect of microbial action on effluent, without extraneous factors which might be found in either the natural or constructed wetlands. Studies of changes in bacterial community and enzyme activity before, during and after the addition of effluent may be carried out using this small-scale wetland. Various concentrations of effluent may be tested, as well as various components of the effluent. Once the changes in bacterial community and enzyme activity have been correlated it would be possible to seed the wetland

with various strains of bacteria in order to determine whether or not it is feasible to develop a starter culture which would increase the efficiency with which a constructed wetland degrades winery effluent.

Conclusions

This feasibility study has demonstrated an approach to monitoring wetlands used to reduce the pollutant load of wastewaters in wetlands. Chemical and biochemical analyses and molecular phylogenetics were used in preliminary characterization of the active microbial population, and in the development of a predictive model of degradation rates in a wetland exposed to winery wastewater. It is clear from this study that the impact of pollutant addition on natural microbial populations can be demonstrated by molecular methods. These methods allow us to monitor the survival, and more importantly, the health of the microbial population responsible for the biodegradation of the impacting pollutant. However, natural microbial communities are complex, containing hundreds or thousands of individual species, in multiple trophic layers of primary, secondary and tertiary degraders. Different microorganisms degrade different classes of compounds, and not all the species are necessarily directly involved in the primary degradation process. It is recognised that in degradative niches (such as wetland sediments) relatively few species are directly involved in primary degradation of pollutants and a very limited number of species may dominate and this study supports this. Thus, definitive correlation of chemical composition with microbial and/or enzymatic activity is extremely challenging.

Constructed wetlands have been demonstrated to be both feasible and practical method of treating winery effluent. A conservative semi-empirical model has been developed that predicts the effluent treatment efficiency of the constructed wetlands. The correct plants have been identified for use in constructed wetlands for use in the Western Cape Province and include primarily sedge species and Arum Lilies. This model, in conjunction with the botanical data, can be used for the design of new constructed wetlands for the wine industry

The success of this trial study has many implications for further work. We conclude that while this project has established useful methodologies and the approaches are useful, more in-depth study is required in order to effectively design wetlands for optimal microbial activity, as follows:

(1) The effect of pollutants on wetland microbial population distributions must be better understood in order to manage wetlands' effectiveness in reducing pollution of local water resources.

(2) The effect of pollutants on the metabolism of microbial populations in wetlands must be better understood in order to ensure that the requisite enzymes are expressed and active when xenobiotics are present.

(3) Constructed wetlands provide suitable cheap, low-tech, environmentally sustainable systems for reducing the impact of the pollutants on surface and ground water but we need to

know how more about how they can be adapted, to effectively remediate the pollutants impacting them

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TABLE OF CONTENTS

EXECUTIVE SUMMARY ..... **III**
ACKNOWLEDGEMENTS ..... **XIII**
TABLE OF CONTENTS ..... **XIV**
LIST OF FIGURES ..... **XVII**
LIST OF TABLES ..... **XXIII**

CHAPTER 1 BACKGROUND INFORMATION AND LITERATURE REVIEW **1**

1.1 Literature review **1**
1.1.1 Background information 1
1.1.2 The problem – effluent characteristics and the potential problems associated with constructed wetlands 3
1.1.3 Mathematical modelling of constructed wetlands 5
1.1.4 Wetlands Biology – botanical and microbiological 8
1.2 Objectives of the project **9**
1.3 References **10**

CHAPTER 2 SAMPLING AND MONITORING OF CHEMICAL COMPOSITION IN WETLANDS OVER TWO SEASONS **12**

2.1 Introduction **12**
2.2 Analytical methods for effluent characterisation **12**
2.3 Sampling methods **13**
2.4 Results **13**
2.4.1 Effluent composition 13
2.4.2 Critical parameter assessment 16
2.4.3 Composition over time for wineries and trends observed for various components 16
2.5 Conclusions on methods and trends observed from the data obtained **26**
2.6 References **26**

CHAPTER 3 MODELLING OF CHEMICAL REMOVAL PROCESSES IN WETLANDS **28**

3.1 Introduction **28**
3.2 Model development **28**
3.2.1 Determination of hydraulic parameters 30
3.2.2 Residence time distribution study 35
3.2.3 K-value determination 35
3.3 Testing of the model **38**
3.4 Generalisation of model **40**
3.5 Conclusions **41**
3.6 References **44**

CHAPTER 4 GENERAL SURVEY OF BOTANICAL FEATURES OF A WINERY WETLAND **46**

4.1 Introduction **46**
4.2 Methods **46**
4.3 Results **47**
4.3.1 Plant distribution 47
4.4 Discussion – implications for wetland design **47**
4.5 Conclusions **49**
4.6 References **50**

CHAPTER 5 INVESTIGATION OF BIOCHEMICAL ACTIVITY DETECTION IN WETLANDS51

5.1 Introduction 51
5.2 Methods 52
5.2.1 Methods for sampling, sample preparation and culture 52
5.2.2 Methods of enzyme activity measurement 54
5.3 Results of measurements over two seasons 55
5.3.1 Laccase 56
5.3.2 Alcohol dehydrogenase 60
5.3.3 Esterase 63
5.3.4 Glucose-6-phosphate dehydrogenase 65
5.3.5 Peroxidase 68
5.3.6 Tyrosinase 68
5.3.7 Glucose oxidase 69
5.3.8 Catechol 1,2-dioxygenase and catechol 2,3-dioxygenase 69
5.4 Conclusions 71
5.4.1 Trends observed 71
5.4.2 Correlation with chemical and microbial data 72
5.4.3 Key findings and recommendations for further research 73
5.5 References 73

CHAPTER 6 INVESTIGATION OF TRENDS IN MICROBIAL POPULATIONS IN WETLANDS 78

6.1 Introduction 78
6.2 Methods 79
6.2.1 Sampling site and sample collection 79
6.2.2 DNA extraction from soil samples and purification 82
6.2.3 Polymerase chain reaction (PCR) 82
6.2.4 Cloning of PCR-amplified DNA fragments into *Escherichia coli* 83
6.2.5 Amplified rDNA restriction analysis (ARDRA) 84
6.2.6 Denaturing gradient gel electrophoresis (DGGE) 84
6.2.7 Nucleotide sequence determination and analysis 84
6.3 Results 84
6.3.1 DNA extraction and purification from wetland soil samples 84
6.3.2 Construction of a “mini” 16S rDNA clone library 85
6.3.3 Identification of key microorganisms of the methanotrophic consortium in the winery wetland soil 89
6.3.4 Comparative bacterial phylogenetics from 16S rDNA libraries 96
6.3.5 Microbial diversity and (micro-) heterogeneity in the wetland soil 100
6.3.6 Temporal changes in wetland soil community composition 103
6.3.7 Overview of trophic interactions in the winery wetland 114
6.4 Conclusions and trends observed 114
6.5 References 115

CHAPTER 7 INTEGRATION OF RESULTS FOR DESIGN OF WETLANDS EFFECTIVE IN TREATING WINERY WASTEWATER 120

7.1 Correlation of chemical, biochemical and microbial information 120
7.2 Conclusions on relationships between chemical, biochemical and microbial data and implications for wetland design 124

CHAPTER 8 CONCLUSIONS AND RECOMMENDATIONS 125

8.1 Key findings 125
8.2 Results arising from phylogenetics studies 126
8.3 Recommendations for further research 126

APPENDIX 1 COD CALCULATIONS 128
APPENDIX 2 AGE DISTRIBUTION GRAPHS AND DATA TABLES 129
APPENDIX 3 DECAY CURVES FOR RTD TAIL ESTIMATION 149
APPENDIX 4 DERIVATION OF EQUATION OF THE GENERAL MODEL 154

LIST OF FIGURES

Figure 2.1 Winery effluent chromatogram 15

Figure 2.2 Changes in COD values at Winery 1 over the time period of June 2004 to September 2005 17

Figure 2.3 Effluent pH at Winery 1 as measured over the time period of June 2004 to September 2005 17

Figure 2.4 Raw effluent ethanol levels at Winery 1 as determined for the time period of February 2005 to September 2005....18

Figure 2.5 Calculated COD vs. actual COD for Winery 1 19

Figure 2.6 Raw effluent carboxylic acids detected for Winery 1 over the time period of September 2004 to September 2005 20

Figure 2.7 Raw effluent metals in solution detected for the time period of June 2004 to September 2005 20

Figure 2.8 Raw effluent COD at Winery 2 calculated for the time period of January 2005 to March 2005....21

Figure 2.9 Calculated COD vs actual COD for Winery 2 22

Figure 2.10 Raw effluent pH at Winery 2 measured over the time period of January 2005 to March 2005....23

Figure 2.11 Raw effluent alcohol levels detected at Winery 2 over the time period of January 2005 to March 2005....23

Figure 2.12 aw effluent acetic acid at Winery 2 as detected for the time period of January 2005 to March 2005....24

Figure 2.13 Raw effluent carboxylic acids detected in Winery 2 over the time period of January 2005 to March 2005....25

Figure 2.14 Effluent metallic species levels detected at Winery 2 over the time period of January 2005 to March 2005....26

Figure 3.1 Graphical description of the model 31

Figure 3.2 Sample port location 32

Figure 3.3 Different variables as a function of length 41

Figure 4.1 Predominant plant species observed in natural wetlands 48

Figure 4.2 Arum lilies growing along the nutrient path/flow channel 49

Figure 5.1 The change in laccase activity, with changing enzyme concentration, shown by ABTS and guaiacol....56

Figure 5.2 Comparison of the change in absorbance for the laccase assay between samples collected on 14/06/2005 and those collected on a) 17/03/2005 and 28/03/2005 (NW) and b) 31/03/2005 (CW). The substrate used was ABTS. 57

Figure 5.3 The changes in absorbance observed for ABTS and guaiacol in the laccase assay using various ratios of assay reaction mixture and sample 58

Figure 5.4 The change in absorbance observed for the laccase assay, using ABTS and guaiacol, before and after concentration of the effluent sample 59

Figure 5.5 Comparison of the change in absorbance per minute for the laccase assay (ABTS as substrate) between samples collected from CW and NW over the time period of 10 February 2006 to 03 April 2006, where R = reference sample collected on 10 February 2006, a = collected on 17 February, b = collected on 25 February 2006 (except for NW1b and NW2b which was collected on 03 April 2006), c = collected on 03 March 2006.....59

Figure 5.6 Comparison of the change in absorbance per minute for the laccase assay (guaiacol as substrate) between samples collected from CW and NW over the time period of 10 February 2006 to 03 April 2006, where R = reference sample collected on 10 February 2006, a = collected on 17 February, b = collected on 25 February 2006 (except for NW1b and NW2b which was collected on 03 April 2006), c = collected on 03 March 2006.....60

Figure 5.7 Comparison of the change in activity for the alcohol dehydrogenase assay between samples collected on 14/06/2005 and those collected on a) 17/03/2005 and 28/03/2005 (NW) and b) 31/03/2005 (CW) 62

Figure 5.8 Comparison of the change in absorbance per minute for the alcohol dehydrogenase assay between samples collected from CW and NW over the time period of 10 February 2006 to 03 April 2006, where R = reference sample collected on 10 February 2006, a = collected on 7 February, b = collected on 25 February 2006 (except for NW1b and W2b which was collected on 03 April 2006), c = collected on 03 March 2006.....63

Figure 5.9 Comparison of the change in activity for the esterase assay between samples collected on 14/06/2005 and those collected on a) 17/03/2005 and 8/03/2005 (NW) and b) 31/03/2005 (CW) 64

Figure 5.10 The changes in activity observed for the esterase assay using various ratios of assay reaction mixture and sample 65

Figure 5.11 Comparison of the change in activity for the glucose-6-phosphate dehydrogenase assay between samples collected on 14/06/2005 and those collected on a) 17/03/2005 and 28/03/2005 (NW) and b) 31/03/2005 (CW) 66

Figure 5.12 The changes in activity observed in the glucose-6-phosphate dehydrogenase assay using various ratios of assay reaction mixture and sample 67

Figure 5.13 Comparison of the change in absorbance per minute for the glucose-6-phosphate dehydrogenase assay between samples collected from CW and NW over the time period of 10 February 2006 to 03 April 2006, where R = reference sample collected on 10 February 2006, a = collected on 17 February, b = collected on 25 February 2006 (except for NW1b and NW2b which was collected on 03 April 2006), c = collected on 03 March 2006.....67

Figure 5.14 Comparison of the change in absorbance per minute for the peroxidase assay between samples collected from CW and NW over the time period of 10 February 2006 to 03 April 2006, where R = reference sample collected on 10 February 2006, a = collected on 17 February, b = collected on 25 February 2006 (except for NW1b and NW2b which was collected on 03 April 2006), c = collected on 03 March 2006 68

Figure 5.15 Comparison of the change in absorbance per minute for the tyrosinase assay between samples collected from CW and NW over the time period of 10 February 2006 to 03 April 2006, where R = reference sample collected on 10 February 2006, a = collected on 17 February, b = collected on 25 February 2006 (except for NW1b and NW2b which was collected on 03 April 2006), c = collected on 03 March 2006 69

Figure 5.16 Comparison of the change in absorbance per minute for the glucose oxidase assay between samples collected from CW and NW over the time period of 10 February 2006 to 03 April 2006, where R = reference sample collected on 10 February 2006, a = collected on 17 February, b = collected on 25 February 2006 (except for NW1b and NW2b which was collected on 03 April 2006), c = collected on 03 March 2006 70

Figure 5.17 Comparison of the change in absorbance per minute for the catechol 1,2-dioxygenase assay between samples collected from CW and NW over the time period of 10 February 2006 to 03 April 2006, where R = reference sample collected on 10 February 2006, a = collected on 17 February, b = collected on 25 February 2006 (except for NW1b and NW2b which was collected on 03 April 2006), c = collected on 03 March 2006 70

Figure 5.18 Comparison of the change in absorbance per minute for the catechol 2,3-dioxygenase assay between samples collected from CW and NW over the time period of 10 February 2006 to 03 April 2006, where R = reference sample collected on 10 February 2006, a = collected on 17 February, b = collected on 25 February 2006 (except for NW1b and NW2b which was collected on 03 April 2006), c = collected on 03 March 2006 71

Figure 6.1 Surroundings of the selected wetland in Stellenbosch (including dam, left) and close-up of the sampled main inflow, photographed during the harvest period in March (right) 79

Figure 6.2 Agarose gel (1%) of total DNA (125 ng) extracted from soil samples (selection) Marker: PstI-restricted λ-DNA 85

Figure 6.3 Unrooted neighbour-joining tree based on 488-bp 16S rDNA fragments amplified from wetland soil samples. The alignment was done with the ClustalX software (34) using the standard parameters as proposed by Hall (12). Regions containing mainly gaps were excluded from the analysis. The scale bar refers to 10 substitutions per 100 bp. The sequences were attributed to the different taxonomic groups using the Classifier software of the Ribosomal Database Project II (6) 89

Figure 6.4 Overview on processes and microbial groups involved in the anaerobic digestion of organic matter [adapted from (31)] 90

Figure 6.5 ARDRA patterns (using restriction enzyme AluI) of 16S rDNA PCR products of methanogenic Archaea (selection) 91

Figure 6.6 Phylogenetic tree of partial 16S rRNA gene sequences of methanogenic Archaea amplified from wetland soil. The tree was constructed with the software Phylo_win 2.0 using the neighbour-joining algorithm. The scale bar represents a 10% difference in nucleotide sequences. The sequences obtained in the present study are shown in bold, accession numbers of the remaining sequences are indicated. Numbers at nodes represent the bootstrap values (from 1000 replicates; only values >50% are shown). T26, a bacterial sequence, was used as an outgroup 92

Figure 6.7 Phylogenetic tree of partial 16S rRNA gene sequences from obtained with the primer pair Syn682F/Syn1196R. The tree was constructed with Phylo_win 2.0 using the neighbour-joining algorithm. The bar indicates 10% sequence divergence. The sequences obtained in the present study are shown in bold, accession numbers of the remaining sequences are indicated. Numbers at nodes represent the bootstrap values (from 1000 replicates; only values >50% are shown). letters. Arthrobacter globiformis was used as an outgroup 95

Figure 6.8 Unrooted phylogenetic neighbour-joining tree based on 43 sequences of a comparative 16S rDNA library from wetland samples (main inflow area, surface). A, sequences retrieved from Autumn samples (2 March 2005, winery effluent-impacted); S, sequences from Spring samples (6 October 2005, non effluent-impacted). The alignment was done with the ClustalX software (34) using the standard parameters as proposed by Hall (12). Regions containing mainly gaps were excluded from the analysis. The scale bar refers to 10 substitutions per 100 bp. The sequences were attributed to the different taxonomic groups using the Classifier software of the Ribosomal Database Project II (6) 99

Figure 6.9 Representation of phylogenetic groups in the surface mud of the winery wetland, as inferred from a total of 51 partially sequenced rRNA genes amplified with universal bacterial primers 100

Figure 6.10 DGGE gel (gradient, 40-60%) of PCR products amplified from wetland clones using primer pair 341F-GC/534R. Lanes, 1-13, clones Mid-2a, 2b, 2c, 2d, 2h, 2i, 2l, 3c, 3d, 3e, 3h, 3l, 3q; 14, E. coli; 15, mixture of samples loaded in lanes 1-14 101

Figure 6.11 DGGE gel (gradient, 30-70%) of partial 16S rRNA genes amplified from DNA extracted from wetland samples using primer pair 341F-GC/534R. Lanes: 1-4, replicate DNA extractions from sample 32 (wetland surface); 5, marker; 6-9, replicate DNA extractions from sample 75 (ca. 25 cm depth) 102

Figure 6.12 Micro-heterogeneity analysis. DGGE gel (gradient, 30-70%) of partial 16S rRNA genes amplified from DNA extracted from wetland samples using primer pair 341F-GC/534R. Lanes: 1-5, samples 11-15 (wetland surface, 15 Feb 2005); 6, marker; 7-11, samples 36-40 (ca. 25 cm depth, 23 Feb 2005); 12, negative Control 103

Figure 6.13 Agarose gel (1%) of partial 16S rRNA genes amplified from DNA extracted from wetland samples using primer pair 341F-GC/534R (selection). Lanes: 1-5, samples 12, 14, 17, 19, and 34; 7-16, samples 35, 37, 40, 41, 43, 46, 48, 53, 54, and 55; 18-28, samples, 57, 61, 62, 63a, 63b, 72, 73, 74, 76, 81, and 83; 30-31, samples 85b and 86; 32, neg. control; 33, pos. control (E. coli); 6, 17 and 29, PstI-restricted λ-DNA 103

Figure 6.14 Temporal changes in bacterial community composition (wetland surface). DGGE gel (gradient, 40-60%) of partial 16S rRNA genes amplified from DNA extracted from wetland samples using universal bacterial primers. Lanes: 2, 15 Feb 2005; 3, 23 Feb; 4, 2 Mar; 5, 18 Mar; 6, 2 Apr; 7, 29 Apr; 8, 25 May; 10, 29 Jun; 11, 3 Aug; 12, 8 Sep; 13, 6 Oct; 14, 16 Nov; 15, 22 Dec; 16, 2 Feb 2006; 17, 16 Feb; 1, 9 and 18, marker. The band labels correspond to those used in Figure 6.17 and Figure 6.18.....104

Figure 6.15 Temporal changes in bacterial community composition (at 20-28 cm depth). DGGE gel (gradient, 40-60%) of partial 16S rRNA genes amplified from DNA extracted from wetland samples using universal bacterial primers. Lanes: 2, 15 Feb 2005; 3, 23 Feb; 4, 2 Mar; 5, 18 Mar; 6, 2 Apr; 7, 29 Apr; 8, 5 May; 9, 29 Jun; 11, 3 Aug; 12, 8 Sep; 13, 6 Oct; 14, 16 Nov; 15, 22 Dec; 16, 2 Feb 2006; 17, 16 Feb; 19, negative control; 1, 10 and 18, marker.....105

Figure 6.16 Dendrogram derived from the DGGE gel shown in Figure 6.14 (temporal profile of the bacterial community in the wetland surface). The dendrogram based on the presence or absence of corresponding bands was obtained using the DGGEstat software (Erik van Hannen; available from http://www.sb-roscoff.fr/marine_microbes). The distance matrix was calculated using the Dice coefficient, and the clustering was done with the UPGMA (Unweighted Pair Group Method with Arithmetic Mean) method.....106

Figure 6.17 Intensity changes of selected DGGE bands of Figure 6.14, which were obtained by amplification of bacterial 16S rDNA from the wetland surface between February 2005 and February 2006. The analysis is based on the peak area (Y axis) calculated from the digitalised gel image.....107

Figure 6.18 DGGE gel (gradient, 30-60%) reflecting the bacterial community in the wetland surface at different time points (selection of samples depicted in Figure 6.14). All labelled bands were cut out from the gel and cloned. Sequences were obtained from the bands labelled in bold. N.B.: More bands are visible in this gel compared to Figure 6.14 because of the wider denaturing gradient range applied.....108

Figure 6.19 Temporal changes in archaeal community composition (wetland surface). DGGE gel (gradient, 45-65%) of partial 16S rRNA genes amplified from DNA extracted from wetland samples using universal archaeal primers. Lanes: 2, 15 Feb 2005; 3, 23 Feb; 4, 2 Mar; 5, 18 Mar; 6, 2 Apr; 7, 29 Apr; 8, 25 May; 9, 29 Jun; 11, 3 Aug; 12, 8 Sep; 13, 6 Oct; 14, 16 Nov; 15, 22 Dec; 16, 2 Feb 2006; 17, 16 Feb; 19, negative control; 1, 10 and 18, marker. The band labels correspond to those used in Figure 6.22.....111

Figure 6.20 Temporal changes in archaeal community composition (at 20-28 cm depth). DGGE gel (gradient, 45-65%) of partial 16S rRNA genes amplified from DNA extracted from wetland samples using universal archaeal primers. Lanes: 2, 15 Feb 2005; 3, 23 Feb; 4, 2 Mar; 5, 18 Mar; 6, 2 Apr; 7, 29 Apr; 8, 25 May; 9, 29 Jun; 11, 3 Aug; 12, 8 Sep; 13, 6 Oct; 14, 16 Nov; 15, 22 Dec; 16, 2 Feb 2006; 17, 16 Feb; 19, negative control; 1, 10 and 18, marker.....111

Figure 6.21 Dendrogram derived from the DGGE gel shown in Figure 6.19 (temporal profile of the archaeal community in the wetland surface). The dendrogram based on the presence or absence of corresponding bands was obtained using the DGGEstat software (Erik van Hannen; available from http://www.sb-roscoff.fr/marine_microbes). The distance matrix was calculated using the Dice coefficient, and the clustering was done with the UPGMA (Unweighted Pair Group Method with Arithmetic Mean) method.....112

Figure 6.22 Intensity changes of selected DGGE bands of Figure 6.19, which were obtained by amplification of archaeal 16S rDNA from the wetland surface between February 2005 and February 2006. The analysis is based on the peak area (Y axis) calculated from the digitalised gel image 113

Figure 6.23 Overview on the trophic interactions likely to contribute to the anaerobic degradation of winery wastewater constituents in the wetland. The following microorganisms that have already been identified in this study might be involved in the specific steps: 1, Clostridium; 2, Citrobacter, Acinetobacter; 3, Citrobacter; 4 and 5, Clostridium; 6, Syntrophus, Acinetobacter, Rhodocyclus; 7, Syntrophus; 8, Clostridium, Frateuria; 9, Clostridium; 10, Propionibacterium; 11, Citrobacter; 12, Pelobacter, Frateuria; 13, Clostridium, Pelobacter; 14, Syntrophobacter; 15 and 16, Methanosarcinales; 17, Methanomicrobiales 114

Figure 7.1 Correlation of enzyme activities and microbial groups 123

LIST OF TABLES

Table 1.1 Chemical characteristics of winery wastewater 4

Table 1.2 Chemical composition of winery wastewater samples taken from various wineries 4

Table 1.3 Inorganic Species in winery Effluent 4

Table 2.1 Initial effluent component determination 14

Table 3.1 Bromide concentration profiles for the various ports indicated 34

Table 3.2 Tau and N values 35

Table 3.3 K-value calculations 36

Table 3.4 Average k-values 36

Table 3.5 Model test – Port 2a and b 38

Table 3.6 Model test – Port 2 39

Table 3.7 Error% ± Standard Deviation% 40

Table 3.8 Model Predictions at each Sample Port 43

Table 5.1 Lists the samples used in the assay manipulations. The code shows which enrichment medium was used and the amount of sample and assay reaction mixture 53

Table 5.2 Lists the samples produced from the enrichments. The code shows which wetland the sample was collected from, which port and which enrichment medium was used 55

Table 5.3 The rates of activity observed for different concentrations of guaiacol and enzyme. No difference in activity was observed between the different substrate and enzyme concentrations 58

Table 5.4 The activities observed for the various assays, using the intracellular enzyme extract from the enrichment cultures of the water samples from CW and NW. Due to the bleaching of ABTS during the laccase assay, the assay was repeated using guaiacol 61

Table 6.1 Soil samples collected from a winery wetland in Stellenbosch 80

Table 6.2 PCR primers and cycling conditions 83

Table 6.3 (next page) - BLAST results of 20 partial 16S rDNA sequences obtained from wetland soil samples. Clone IDs containing the number “2” refer to sample A (retrieved from Wetland surface), whereas “3” refers to sample B (from ca. 25 cm depth) 88

Table 6.4 BLAST results of 22 partial 16S rDNA sequences obtained from wetland soil samples, using the primer set M340/M707R. Clone IDs containing the letter “T” were retrieved from the wetland surface, whereas “B” refers to a depth of ca. 25 cm 91

Table 6.5 BLAST results of 29 partial 16S rDNA sequences obtained from wetland soil samples, using the primer pair Syn682F/Syn1196R, which targets syntrophic bacteria. Clone IDs containing the letter “T” were retrieved from the wetland surface, whereas “B” refers to a depth of ca. 25 cm. 94

Table 6.6a BLAST results of 21 partial 16S rDNA sequences obtained from wetland soil samples (surface mud) collected in spring (6 October; non effluent-impacted), using universal bacterial PCR primers E9F/U1510R 97

Table 6.6b BLAST results of 22 partial 16S rDNA sequences obtained from wetland soil samples (surface mud) collected in autumn (2 March 2005; effluent-impacted), using universal bacterial PCR primers E9F/U1510R 98

Table 6.7 BLAST results of the sequences of 16S rDNA fragments recovered from a DGGE gel. The numbers correspond to those used in Figure 6.18 109

Table 6.7 (continued) - BLAST results of the sequences of 16S rDNA fragments recovered from a DGGE gel. The numbers correspond to those used in Figure 6.18.....110

Table 7.1 Correlation of bacterial/archaeal genera tentatively identified to be present in soil samples analysed by DGGE and enzyme activity detected in samples ...121

CHAPTER 1
BACKGROUND INFORMATION AND LITERATURE REVIEW

1.1 Literature review

1.1.1 Background information

The South African (SA) wine industry is situated primarily in the Western Cape Province. Worcester is the largest wine-producing region, followed by the Olifants River, Robertson and Paarl (18). This means that the bulk of wineries occur in the three main water catchments of the Western Cape Province – namely the Breede River, the Olifants River, and the Berg River.

The SA wine industry disposes of approximately a billion litres of wastewater annually (17), as a direct result of winemaking activities. This effluent is normally irrigated on pastures or in some cases dumped directly in the nearest river. Some cellars do have water treatment facilities, but at present this is the exception rather than the rule. Most cellars are small, which means that the effect of each cellar's effluent disposal practices on the environment is negligible. However, when examined collectively the industry certainly contributes to environmental degradation in all three drainage basins (17).

Winery effluent is seasonal in nature, and is highly concentrated in terms of Chemical Oxygen Demand (COD) and levels of sodium and potassium ions (amongst other ionic species). The effluent is particularly concentrated from January to April (harvest period) with subsequent lesser peaks later in the year. This effluent peak coincides directly with the dry season. The Berg, Breede and the Olifants rivers are normally brackish and have low flow at this time of the year. This means that effluent entering these rivers has a greater impact than it would otherwise, and as the river water levels decrease towards their mouths, the effluent can be strongly concentrated. The impact of the effluent is compounded by the non-point source of agricultural pollution that arises from the use of fertilizers and pesticides. These factors combined contribute significantly to eutrophication and deoxygenation of rivers and ground waters in these drainage basins.

The Department of Water Affairs and Forestry (DWAF) has in the last five years put much effort into remedying the problem of winery effluent, but has been largely ineffectual for a number of reasons. These include:

- a continuing lack of awareness of the latest water legislation
- a continuing effort to encourage pro-active behaviour rather than prosecute offenders
- a lack of visible policing
- a lack of manpower to properly enforce the legislation
- industrial resistance because of:

- fear of prosecution
- cost implications
- lack of willingness to conform because of added work load

The problem of winery effluent can be remedied by means of conventional effluent treatment plants. Unfortunately, this can be expensive and additional trained personnel may be required to operate them. Smaller and marginal cellars are especially exposed to the increased cost because they may not have the funds to purchase effluent treatment plants. If they are able to purchase such a plant, they may not be able to afford personnel to operate it. Conventional effluent treatment (anaerobic or aerobic digesters) is not particularly cost efficient for this industry and is difficult to control, due to the seasonal variation of the effluent. Operational difficulties are not uncommon for these plants when they are used for winery effluent treatment.

For these reasons, constructed wetlands have been identified as a suitable means of treating winery effluent. They may provide a cheap alternative treatment method with significant additional marketing potential for wineries, e.g. conservation and recreational areas. There are additional benefits accrued by the cellars from using constructed wetlands. These include:

- the establishment of a water treatment facility that:
  - requires little or no labour
  - no chemicals
  - very little maintenance
- the establishment of recreational areas – adds marketing value
- demonstrating commitment to environmental causes
- provision of habitat for wildlife species (especially birds and indigenous flora) through the creation of an environment suited to them

There are certain problems associated with constructed wetlands, which include:

- sensitivity of many plants to high COD levels which cause death
- an inadequate understanding of the contribution of wetland micro-organisms and plants to the process of effluent biodegradation
- an inadequate understanding of the hydraulics of constructed and natural wetlands
- a lack of suitable mathematical design models with which to design constructed wetlands

The bulk of the wine industry occurs in the Cape Floristic Kingdom in which there are over three hundred species of Restios, twelve hundred geophytes and many others (2), many of which may be used as the basis of plants in a constructed wetland. Many of these species may be tolerant to winery effluent and may harbour useful microbes for the remediation of the effluent. It is envisaged that by studying both the natural and constructed wetlands that are

currently being used for effluent treatment, a clearer understanding of the processes that occur in wetlands can be achieved.

This knowledge can be combined with traditional reactor analysis and design to aid in the formation of better design equations for constructed wetlands. There are opportunities here for many parties to be involved, from the wineries to Cape Nature Conservation. It is foreseen that this project will provide a means of sustainable water treatment at a lower cost than traditional treatment and will also provide cellars with additional marketing opportunities and contribute to the aesthetics of the wine industry in general.

1.1.2 The problem – effluent characteristics and the potential problems associated with constructed wetlands

Constructed wetlands can provide a cheap, sustainable means of effluent treatment for the wine industry in South Africa. Wetlands are known to be amongst the most biologically active of all living systems, but they are susceptible to high strength effluent. In order to find the limits of effluent strength that constructed wetlands can safely treat, it is necessary to characterise the wastewater entering the wetland. There are many studies on the composition of winery effluent, but there is disparity in the results reported. This is a consequence of comparing data from different wineries with different winemaking styles in different countries. In addition to this, winery effluent has orders of magnitude of variation between the off-season, and the late harvest peak [Table 1.1 (11)].

Other authors to characterise effluent, include Levay (8), Sheridan (17), Radford (13) and Chapman (1). Malandra *et al.* (9) analysed winery wastewater from three different wineries during the harvest season. They provide data on organic acids and other organic components in the effluent, which have not been found elsewhere in the literature (Table 1.2). Since alcohol has the highest concentration of any chemical component in wine, the above results may seem counter intuitive (considering the lack of alcohols in the effluent), but this can be explained by the microbial detoxification of the alcohols to their volatile fatty acid and associated esters *prior* to the effluent leaving the cellar. Unfortunately, in this study no mention was made of inorganic species in the effluent. In most literature, the individual inorganic species are not measured – the Sodium Adsorption Ratio or the Total Dissolved Solids are quoted. Sheridan (17) quotes values found in three different cellars over the 2002 harvest season (Table 1.3).

Table 1.1 – Chemical characteristics of winery wastewater

Parameter	Unit	Range
COD*	mg/ℓ	800 – 12800
TSS	mg/ℓ	200 – 1300
Phosphorous	mg/ℓ	5.0 – 77.0
Ammonia N ($N\text{-}NH_3$)	mg/ℓ	0.001 – 2.0
Nitric N ($N\text{-}NO_3$)	mg/ℓ	0.1 – 0.9
Total protein	mg/ℓ	5.2 – 20.9
Total polyphenols	mg/ℓ	13.1 – 247.0

Table 1.2 – Chemical composition of winery wastewater samples taken from various wineries

Parameter	Concentration in mg/ℓ (Except pH)
pH	3.9 – 5.2
COD*	320 – 5280
Acetic acid	0 – 491
Propionic acid	0 – 13.6
n-Butyric acid	0.5 – 8.8
2-Phenylethanol	0.5 – 4.5
Decanoic acid	0.5 – 7.5
All components less than 4 mg/ℓ not shown	

Table 1.3 – Inorganic Species in winery Effluent

Parameter	Range
Potassium / mg/ℓ	10 – 350
Magnesium / mg/ℓ	5 – 10
Sodium / mg/ℓ	50 – 225
Electrical Conductivity mS/m	35 – 225

There is insufficient data on the specific components of winery effluent. In particular, there is minimal information on both organic and inorganic species. This implies that data must be gathered on the composition of the specific effluent being assessed for the next phase of this project, i.e. modelling of the parameters in a constructed wetland. This sampling should be done during peak harvest period and should be analysed for all inorganic and organic species, in addition to the standard set of tests that are normally performed to winery effluent (COD, SAR, etc.).

* According to Shepherd[15] COD can be as high as 45 500mg/ℓ

1.1.3 Mathematical modelling of constructed wetlands

Constructed wetlands are an emerging field of study, and it is fascinating to observe the progression of science as early models fail, and more sophisticated models replace them. This is well catalogued in a review paper by Rousseau *et al.* (14).

Historically, wetlands are notoriously difficult to model. Initially, simple rules of thumb were developed by Wood (21) and Kadlec & Knight (6). They prescribe a range of hydraulic detention times, hydraulic loading rates and max effluent strength (such as BOD). These rules of thumb describe an enormous range of conditions. This led to the development of regression equations, which normally focus on input/output correlations (21). Pearson's coefficients for these correlations range from 0.33 to 0.96. However, none of these regression correlations work when extrapolated to other wetlands. This is typical of the early black box modelling attempts.

Because of this, certain assumptions were made, and the wetland came to be modelled as an ideal plug flow reactor (the assumptions being no spatial variations in conditions and material properties within a reactor) (3). Goulet *et al.* (4) give the equation as:

$$C_{out} = C_{in} \cdot e^{k \cdot \tau} \qquad [1]$$

where C_{out} is the exit concentration of the COD, C_{in} is the influent concentration, k is the rate constant and τ is the residence time. k has been shown to be an Arrhenius function of temperature (16). The authors modelled a wetland using this equation and found regression coefficients of 0.47. Ideally, any correlation should have a much higher coefficient of regression (approaching a maximum of 1) and should have low variance. Since there is no indication of the variance and the regression coefficient is low, it is believed that this is inadequate for a design equation.

Kadlec & Knight (6) developed the COD-residual model to deal with the inadequacy of the ideal plug flow reactor, and this is given by equation 2:

$$\frac{C_{out} - C^*}{C_{in} - C^*} = e^{k \cdot \tau} \qquad [2]$$

This equation was developed in an attempt to describe the occurrence of COD in wetland effluents regardless of influents. C^* is defined as this 'residual' COD value. The residual COD is the COD that is always found in the wetland outlet, and is due to decomposition of plant matter etc. The system will never degrade the effluent to a final COD value of zero. Shepherd *et al.* (16) refine this concept further because they noticed that the rate of degradation of BOD

was not constant, so they proposed a new model based on the hypothesis that more easily biodegradable components are broken down first, leaving less biodegradable ones in the wetland. This implies that the rate 'constant' becomes a function of time. Their model is given by equation 3:

$$C_{out} = C_{in} \cdot \exp\left[\frac{k_o}{b} \cdot \ln(b \cdot \tau + 1)\right] \qquad [3]$$

where k_o is the initial rate constant and b is the time-based retardation co-efficient. A strong advantage of all work performed by Shepherd *et al.* (16) is that the wetland was designed to treat winery effluent. The flaw with all three models given by equations [1] to [3] is that they all assume first order, ideal plug flow reactor conditions. It is very unlikely that wetlands will operate as first order reactors. This is shown by Rousseau *et al.* (14) who performed a case study and for their design specifications, the design equations gave a range of wetland sizes from $0.1m^2$ to $950m^2$ using rules of thumb, regression models, first order models or retardation models.

Mitchell & McNevin (10) proposed that Monod kinetics could be used to model a wetland. Monod kinetics assumes that the system is first order at low concentrations but zero order above a certain threshold concentration. This is given by equation [4]:

$$r = k_{0,v} \cdot \frac{C}{K + C} \qquad [4]$$

where K is the half saturation constant (dimensions $M\ell^{-3}$). Furthermore, this shows that there is an absolute maximum removal line and give excellent graphics. They also show the BOD loading rates for a selection of sub surface flow wetlands in the United States of America (USA) and calculate values for the maximum removal rates for BOD and TDS. Unfortunately, by not showing all the wetlands in the US EPA database, it is not possible to determine if they have selected only those wetlands that give credence to their modelling.

Wynn & Liehr (22) developed a computer simulation of a subsurface flow constructed wetland. This model uses Monod kinetics and takes into account carbon, nitrogen and oxygen cycles, auto- and heterotrophic bacterial growth rates and includes a water balance. This simulation has 15 differential equations, 42 parameters and requires the estimation of 15 initial conditions. Unfortunately, the complexity of this model (even with kinetic simplicity) is its own downfall, as the knowledge of 15 boundary conditions by a design engineer is unlikely. It serves as a warning that it is necessary to try to model complex processes, but that the models must retain an element of 'simplicity'.

Kincanon & McAnally (7) published a paper describing their attempts to enhance commonly used model predictions. Their model included a full water and chemical budget, but like all the other models, it ultimately relies on poor assumptions – those of ideal plug flow conditions.

In non-ideal reactors, where there may be dead spots or channelling, it is necessary to measure residence time distribution (RTD). The RTD is measured by adding an impulse of tracer to the reactor and then measuring the concentration of the impulse leaving the reactor. The mathematics of this is well described by Fogler (3).

Werner & Kadlec (20) best describe the ways of modelling this in a wetland, which include plug flow with dispersion, plug flow reactors in parallel, and CSTRs (tanks) in series. The CSTR in series models are the simplest, whilst providing equal levels of modelling accuracy to the plug flow in parallel models. They found that plug flow with dispersion was inadequate for modelling purposes. Werner & Kadlec (20) also developed a model called the zone of diminished mixing (ZDM - a computer based model) which appears to be accurate. This model should be able to be mixed with kinetic models.

Kadlec (5) published the most comprehensive paper seen to date on the topic of combined wetland modelling. He is the only author that has attempted to unite the hydraulic residence time distribution and a k value distribution (KVD) into a model called the Apparent Tanks in Series Model (PTIS). He hypothesised that as there are different chemical species in the effluent, and that each chemical species should have its own degradation rate, there must be a distribution of k values. In addition, he eliminated the assumption of using ideal plug flow reactors, and treats the wetland as a series of tanks (CSTR reactors) in series. He showed that for N tanks in series:

$$\frac{C_{out}}{C_{in}} = \frac{1}{(1 + k \cdot t/N)^N} \qquad [5]$$

He then defined a k value distribution and integrated to get an average concentration given in equation [6]:

$$\frac{C_{ave}}{C_{in}} = \frac{1}{(1 + k_o \cdot t/n)^n} \qquad [6]$$

where k_o is the average rate constant, n is the number of rate constants, and C_{ave} is the average outflow concentration. He also asserted that the average k value at any time in the process is

$$k = \frac{k_o}{(1+\beta \cdot t)^n} \qquad [7]$$

and showed that this is what Shepherd *et al.* (16) were showing with their retardation model, even if they were not supporting the concept of a k value distribution (KVD). β is a scaling factor. Kadlec (5) then defined the relaxed TIS (tanks in series) model where parameters are relaxed to become fitting parameters. This is given by equation [8]:

$$\frac{C_{out}}{C_{in}} = \frac{1}{(1+k_{app} \cdot {}^{t}\!/\!_{P})^P} \qquad [8]$$

where k_{app} is the apparent TIS rate constant and P is the apparent number of TIS. Kadlec (5) does solve the equations explicitly and these are given by equations [9] to [11] where g (t) is the gamma distribution, and $\overline{\overline{C}}$ is the double average concentration.

$$\frac{\overline{\overline{C}}}{C_{in}} = \int_0^\infty g(t)\left\{\int_0^\infty e^{-kt} \cdot f(k)\right\}dt$$

$$\frac{\overline{\overline{C}}}{C_{in}} = \int_0^\infty \frac{g(t)}{(1+\beta t)^n}dt \qquad [9, 10, 11]$$

$$g(t) = \frac{1}{t_i(N-1)!}\left(\frac{t}{t_i}\right)^{N-1}\exp\left(-\frac{t}{t_i}\right)$$

Kadlec (5) also gives indications of what to do if the KVD is discretised and shows the fitting of the PTIS model with actual data. The COD-residual model is the only model of all the simpler models shown above that has anywhere near the accuracy of the PTIS model. For his data, the PTIS model has R^2 values of 1.000 and the PF model has values of 0.941. When looking at a comparison of three different compounds, the PTIS model had an R^2 value of 0.999 whilst the COD-residual model had 0.990.

1.1.4 Wetlands Biology – botanical and microbiological

Wetlands biology has a great significance on how constructed wetlands perform. Of particular interest are:

- The plant communities of constructed wetlands
- The microbial communities of constructed wetlands

Since constructed wetlands are complex biological systems, it may not be possible to include all these parameters in any model developed.

Petroccioli *et al.* [12] described high rate aerobic treatment of winery wastewater in Portugal using different types of sludge reactors. Sludge bed reactors are substantially different to constructed wetlands, but this paper is useful to describe some of the organisms that are found in winery effluent and its associated sludge. Malandra *et al.* [9] described the microbiology of a biological contactor for winery effluent in South Africa. They also discuss the role of bio-films and their properties. They indicate that yeast may play a large role in the aerobic reduction of the effluent COD, especially *Candida krusei.* Since these yeasts are present in the vineyard and the cellar, it could be expected to find them in the wetland to be studied.

Stottmeister *et al.* (19) presented a paper discussing many aspects of how the plant communities affect wetland dynamics. They showed that wetlands prevent blockage of the substrate by root death. As roots grow, they create channels in the gravel matrix, and when the roots die and decompose, they create flow channels. Over the whole wetland, this should reduce or eliminate the blocking in the long term. Root death is also an important aspect for wetland microbiology. The decomposition of roots provides a source of carbon for the microbial communities, as well as vitamins and amino acids.

Plants also provide the matrix (and associated microorganisms) with oxygen – either through aerenchyma cells, or through active transport. *Phragmites australis* has been shown to be able to transport up to 10 mℓ/min of oxygen anywhere in the plant. Plants are also responsible for the absorption of inorganic elements in the effluent. These inorganic components include nitrogen (in all its associated forms), phosphorous and ionic species. Plants have also been shown to consume organic components of the effluent – phenols are degraded via catechol and further meta-ring cleavage.

Plants also seem to be responsible for some of the inhibition of pathogen growth. It remains unclear as to how this occurs, since certain microbes are encouraged, and others are discouraged – simultaneously.

1.2 Objectives of the project

Based on the information summarised above, the objectives of the project were set as follows:

5 To demonstrate the feasibility of modelling a constructed wetland, based on information correlating microbial end enzyme activities with polyphenolic pollutant removal.

6 To access and use available existing relevant information on wetlands to support the research model.

7 To identify soil, water and plant root microbes present in wine wastewater wetlands using phlyogenetic techniques.
8 To characterise their metabolic / enzyme activities in the biodegradation process.
9 To demonstrate the linkage between microbial diversity, biochemical activity and the bioremediation process.

The intended outcome of the two-year project would be a body of data which will demonstrate feasibility of using a molecular / biochemical approach in developing a preliminary model of the wetland. This would yield new understanding of the contributions and complex interactions of microorganisms and plants in wetland bioremediation systems and the data would be built into a novel predictive model of a wetland customised for winery effluent treatment.

1.3 References

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CHAPTER 2
SAMPLING AND MONITORING OF CHEMICAL COMPOSITION IN WETLANDS OVER TWO SEASONS

2.1 Introduction

This section of the report seeks to provide information that would allow the system to be modelled by an engineer in the field. This model should be able to be used to describe current constructed wetlands, as well as allow for the design of new constructed wetlands.

The original system to be studied was a soak-away at a winery in the Devon Valley in Stellenbosch. Over the last few decades, this system has gradually adjusted under the selective pressure applied every harvest as winery effluent passes through it on route to a dam. It is a healthy, intact eco-system that reduces the Chemical Oxygen Demand (COD) of the effluent to non-detectable levels during harvest time, although there is a zone of death at the effluent entry point during the peak harvest period. Unfortunately, the system is too 'wild' to be accurately quantified. There are multiple liquid entry points due to natural springs entering the marsh, and flow measurement is not practicably possible. For this reason, a constructed wetland, owned by the Agricultural Research Council (ARC) at Nietvoorbij, also in Stellenbosch, was utilised primarily for modelling purposes.

Thus, two systems were studied, and each system yields different types of data.

- The soak-away marsh (winery 1) provides detailed information on the characteristics of winery effluent at a typical working cellar in the primary winemaking region of South Africa, for the duration of a full year. It also provides relatively detailed information on which plant species may be utilised when designing a constructed wetland.
- The constructed wetland at the ARC (winery 2) provides comprehensive data on the characteristics of winery effluent over the duration of a harvest. This data, together with a measurement of the bio-remedial kinetics, and a comprehensive study of the hydraulics allows for a model to be developed to explain both temporal and spatial degradation, and allows for the design of constructed wetlands for the treatment of winery effluent.

The findings are presented under the following sections:

- Effluent characterisation (Section 2)
- Model development (Section 3)
- Botanical survey (Section 4)

2.2 Analytical methods for effluent characterisation

In order to accurately model the breakdown of winery effluent in the constructed wetland, the composition of winery effluent has to be known. This must be known over the course of the

harvest, as well as over the course of a year. This information is needed in order to prevent the modelling of lumped parameters such as Biological Oxygen Demand (BOD) or COD.

- The COD was measured using Merck COD Cell test (1.14555.001) and the Merck Spectroquant spectrophotometer.
- The effluent was initially analysed using an HPLC as described below. This method was unable to detect ethanol, so GC-FID was used for the routine analysis of effluent components, and GC-MS for the identification of components.
  - HPLC – Waters Fast Fruit Column, Column T = 60^{o}C, 553 PSI, 0.7 mℓ per minute, mobile phase; 0.1% phosphoric acid, UV detector at 215nm
  - HPLC – Waters Fast Fruit Column, Column T = 60^{o}C, 553 PSI, 0.7 mℓ per minute, mobile phase; 0.1% phosphoric acid, UV detector at 254nm
  - GC-MS – Restek RTX Capillary column (60m by 250µm), initial of 70°C, ramped at 5^{o}C per minute to 170^{o}C, second ramp of 10^{o}C per minute to 280^{o}C
  - GC – FID (J & W Scientific DB-Wax Column (60m by 250µm), initial of 40^{o}C for 10 minutes, ramped at 10^{o}C per minute to 163^{o} C)
  - Total phenols – Analysed using the Folin Ciocalteau method with a spectrophotometric analysis at a wavelength of 765nm
- Metallic ions were measured using a Varian SpectrAA spectrophotometer. Gas mixtures of acetylene/air are used for sample combustion and detection wavelengths for each of the species are specified in the user's manual.
- Inorganic anions such as nitrates, phosphates and chlorides were analysed using HPLC coupled to an anion column. These were found to be insignificant as other authors have reported (4, 8).
- HPLC – Anion Column, Column T = ambient, 1.5 mℓ per minute, mobile phase; 3.6 mM sodium carbonate, conductivity

2.3 Sampling methods

Grab samples were taken, approximately monthly, for a year at Cellar 1, and samples were taken daily at Cellar 2 during the 2005 harvest (from Jan 31 2005 to March 31 2005). These samples were taken from the cellars at a point where the entire cellar effluent stream had converged. These samples were analysed as indicated above.

2.4 Results

2.4.1 Effluent composition

Initial determinations of the effluent yielded the results given in Table 2.1. Na, K, Ca, Mg and Fe are the dominant metallic species that are most important and more specifically, Na and K were identified as being the most important parameters. This is in agreement with data obtained by Sheridan (8).

The initial organic analysis provided data with carboxylic acid concentrations in the range of approximately 500 mg/ℓ. Whilst the values for acetic acid agree with those obtained by Malandra *et al.* (4), the results for propionic acid and butyric acid or orders of magnitude greater. Furthermore, no glucose was detected in any of the samples tested over the course of this study, although other authors (2, 4) found both fructose and glucose.

The primary problems with the use of COD for modelling is that it is a lumped parameter and, therefore our interest was in determining what constitutes the COD in winery effluent. The mass spectral analysis indicated the following components to be the primary constituents in the effluent study (shown in Figure 2.1).

- Ethanol
- Acetic acid
- *n*-propanol
- Propionic acid
- Butyric acid
- Valeric acid

Table 2.1 - Initial effluent component determination

	Cellar 1	**Cellar 2**
Date Sampled	12 March 2004	24 February 2004
Inorganic Components		
Zn (mg/ℓ)	0.7	1.1
Mg (mg/ℓ)	12.3	7.2
Ca (mg/ℓ)	59.3	18.3
Na (mg/ℓ)	54.5	43.9
K (mg/ℓ)	82.9	285.0
Cu (mg/ℓ)	0.0	0.0
Fe (mg/ℓ)	21.4	5.3
Mn (mg/ℓ)	0.3	0.4
Cr (Total) (mg/ℓ)	Not Detected	Not Detected
Pb (mg/ℓ)	0.1	Not Detected
B (mg/ℓ)	Not Detected	Not Detected
Organic components	As measured by HPLC	
Glucose	Not Detected	Not Detected
Fructose	Not Detected	Not Detected
Citric acid	Not Detected	Not Detected
Acetic acid (mg/ℓ)	433.7	353.2
Propionic acid (mg/ℓ)	637.0	50.4
Butyric acid (mg/ℓ)	561.9	371.2

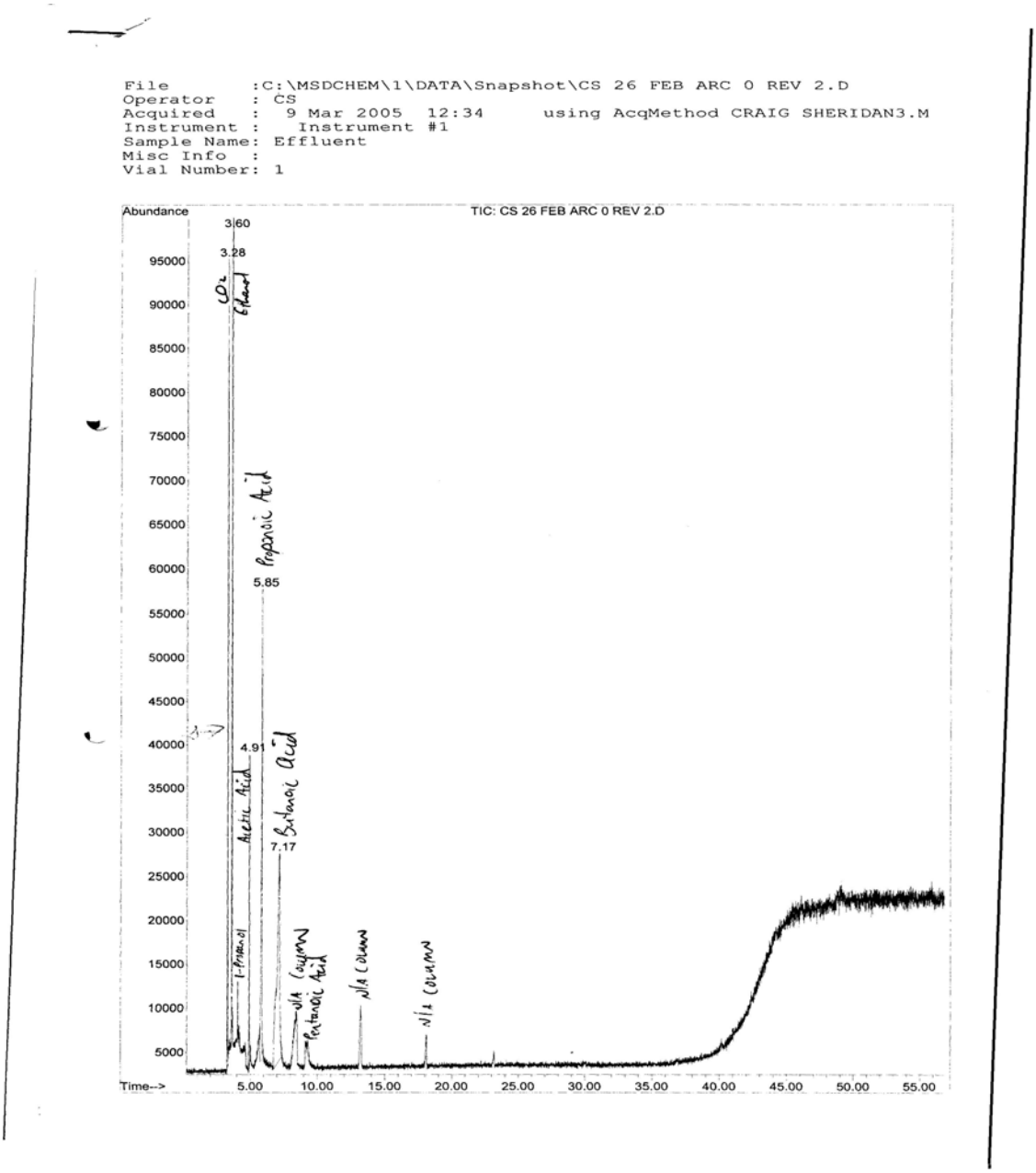

Figure 2.1 - Winery effluent chromatogram

This is in agreement with data found by Colin *et al.* (2) and these results were confirmed by GC-FID and the quantities of the above organic acids were also determined (using dioxane as an internal standard).

The total polyphenol content of both wineries' effluent was measured and was found to be between 17.72 ± 2.10 mg/ℓ and 37.08 ± 10.29 mg/ℓ. This is in agreement with data presented by Malandra *et al.* (4) and Petruciolli *et al.* (5), although in relation to components such as ethanol, the levels are insignificant. Based on this preliminary data, the following parameters were selected as being significant in winery effluent:

- pH
- Na
- K
- COD
- Ethanol
- Propanol
- Acetic acid
- Propionic acid
- Butyric acid

2.4.2 Critical parameter assessment

After the selection of these components as important parameters for modelling, two cellars were used for testing and analysis for different periods of time. Winery 1 was sampled approximately once a month outside of the harvest period over the course of a year and Winery 2 was sampled daily over the course of the 2005 harvest season (February and March). Some days of data are missing, normally on weekends or public holidays. The two wineries will be discussed separately.

2.4.3 Composition over time for wineries and trends observed for various components

2.4.3.1 Winery 1

Chemical Oxygen Demand (COD)

The COD at Winery 1 has been plotted for the duration of a full year (Figure 2.2). Data is shown as a COD value, and as a calculated COD value. The calculated COD value is calculated by summing the theoretical COD contributions from each of the organic components (simple combustion reactions). GC analyses on the sample were not performed until the method had been developed which occurred in mid February. As has been shown in other studies (1, 4, 6), the COD of the effluent peaks in mid March (which is mid harvest) and decreases for the rest of the year. The given peak of 3800 mg/ℓ is also in the expected range for a cellar of this size as given by Sheridan *et al.* (8).

pH

The pH of the raw effluent over the course of the year has been plotted in Figure 2.3. The pH is expected to be lower during harvest because grape juice and wine have a low pH (typically 3-4). This means that the effluent should also be acidic and this data bears this out, where the effluent has a pH of approximately 5 during the peak harvest period. What is surprising is that the pH was particularly low in August. This is ascribed to a pulse of high strength effluent that the measurement was sampled and is not representative of this period.

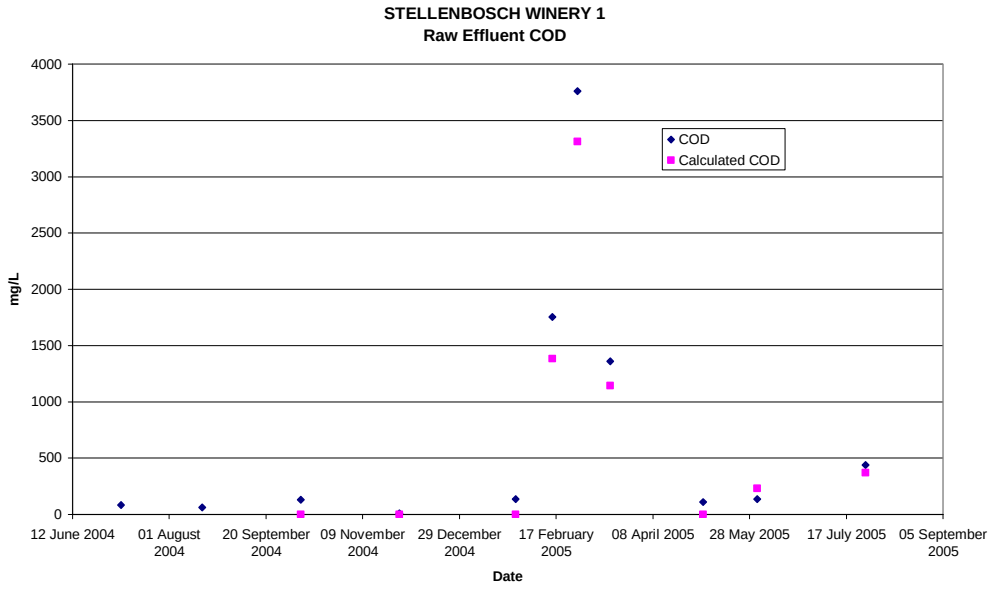

Figure 2.2 – Changes in COD values at Winery 1 over the time period of June 2004 to September 2005

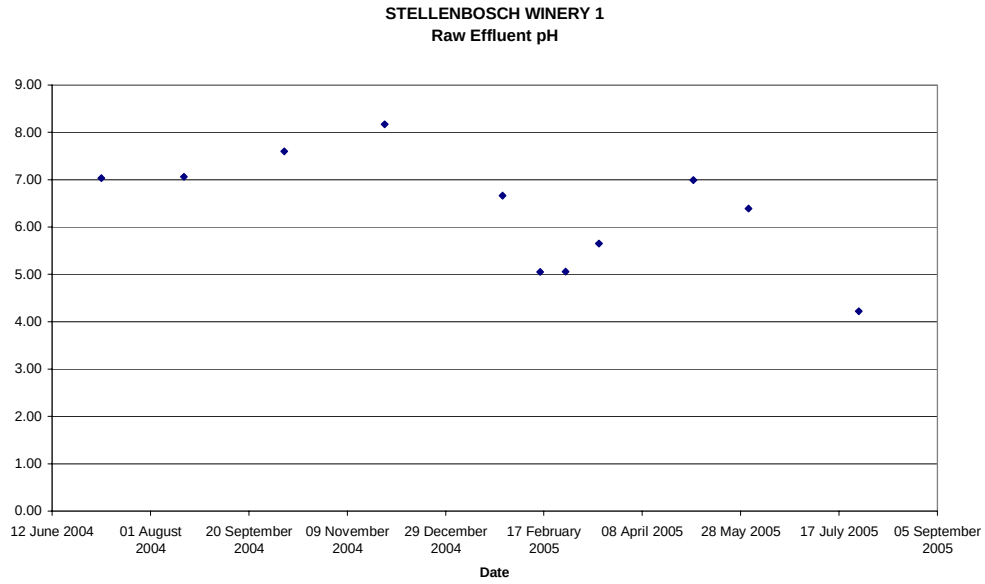

Figure 2.3 - Effluent pH at Winery 1 as measured over the time period of June 2004 to September 2005

Alcohols

The levels of ethanol in the effluent have been plotted (Figure 2.4) for the period running from early February to August 2005. Earlier data is not available because methods were still being developed. Propanol was not detected at any stage. The values range from 0 mℓ/ℓ to 1.7 mℓ/ℓ. The amount of ethanol is approximately one third the amount found by Colin *et al.* (2), although the COD is also roughly one third that found by Colin *et al.* (2) for the 2002/2003 harvest. This equates to 92% of the COD, which is different from that quoted by Colin *et al.* [they quote 79.5% (2)]. The following harvest they quote the value of ethanol as being 78.5% of the COD but a quick calculation of the COD of the ethanol shows that it should be 86% of the overall COD.

To remedy this problem, a plot (shown in Figure 2.5) of calculated COD vs. COD has been produced. In this case, the calculated COD is calculated by summing the contribution from ethanol, propanol, acetic acid, propionic acid and butyric acid. From this effluent stream, only ethanol and acetic acid were detected. These two components contribute 86% of the COD as is given by the correlation. The ethanol contributed 84% of the COD in this sample, and the acetic acid contributed approximately two percent of the COD. This agrees with the values quoted by Colin *et al* (2) if they are recalculated.

It is apparent, that whatever the contribution of all the other parameters, that ethanol is the most important parameter in winery effluent when discussing COD.

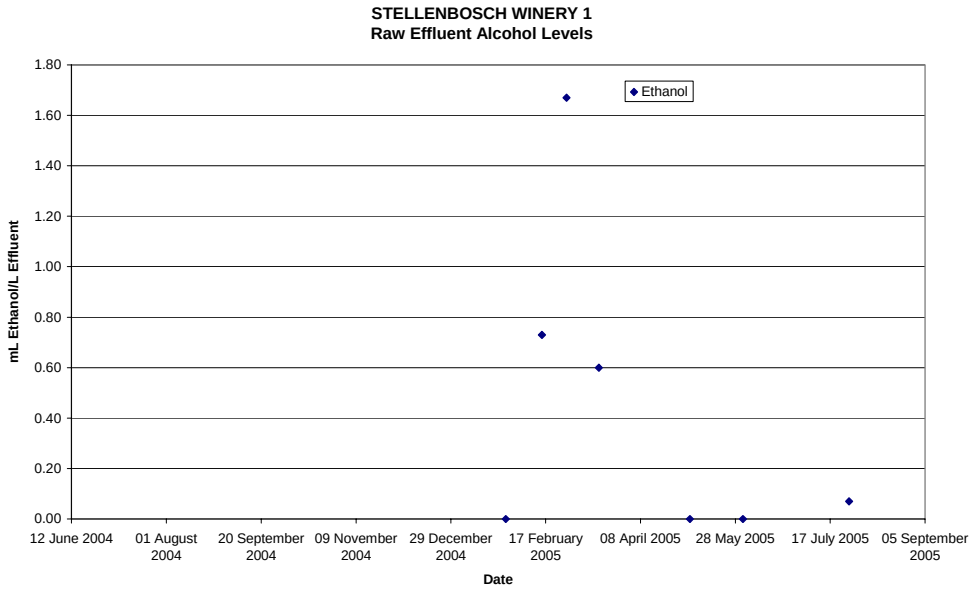

Figure 2.8 - Raw effluent ethanol levels at Winery 1 as determined for the time period of February 2005 to September 2005

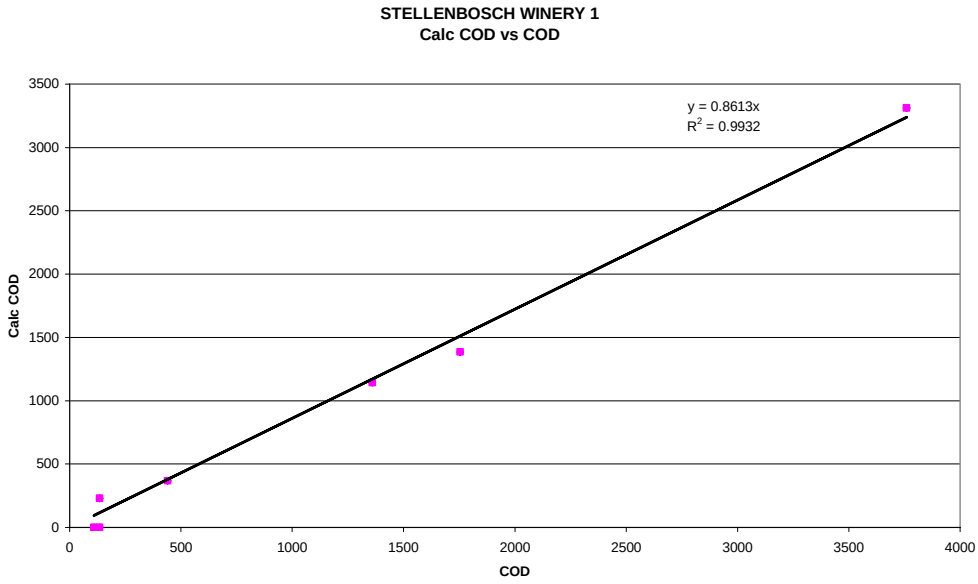

Figure 2.9 – Calculated COD vs. actual COD for Winery 1

Carboxylic Acids

Carboxylic acid levels are given in Figure 2.6, and rarely go above 0.2 mℓ/ℓ, which implies that their maximum contribution to the COD is approximately 2% or less. Their contribution to the pH is significant however, which is shown by the peak on 17 July 2005, which brought the pH of the effluent down to 4.2 (see Figure 2.3). Whilst acetic acid is relatively biodegradable, at these levels, it contributes to an extent that it makes the effluent pH too low for general disposal under SA law (3). Propionic and butyric acid are primarily important because they contribute significantly to the malodour of winery effluent.

Metallic Species

Sodium and potassium were chosen as being the most important metallic species and were investigated. The results are shown in Figure 2.7 and show two clear trends. Firstly, the potassium values rise significantly during harvest and subside to a base level after the harvest, and secondly, the sodium levels remain relatively unchanged over the course of a year. At this cellar, the sodium levels in the fresh water are 37.70 mg/ℓ, and Na_2CO_3 is used for cleaning throughout the year. Potassium is the primary ion in grapes and is very important for grapevine metabolism. This explains the rise of potassium levels in the effluent during harvest period, and the subsequent drop once juice and wine spillage ceases after harvesting. As was indicated in the pH and levels of acetic acid, there is a large spike of potassium in the effluent on the 17th of July 2005, and all of these factors point towards there being a spill prior to sampling.

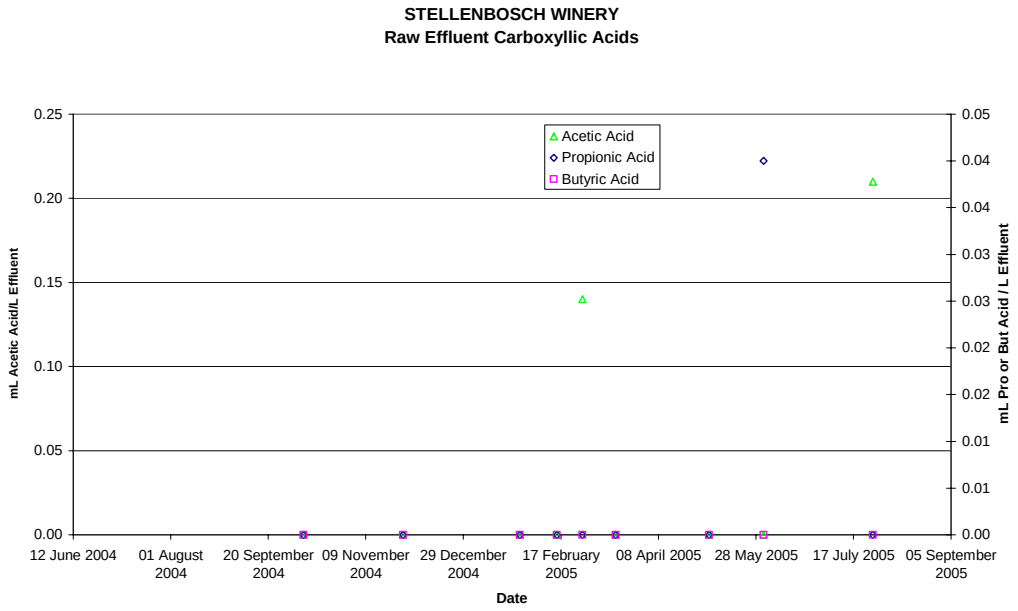

Figure 2.10 - Raw effluent carboxylic acids detected for Winery 1 over the time period of September 2004 to September 2005

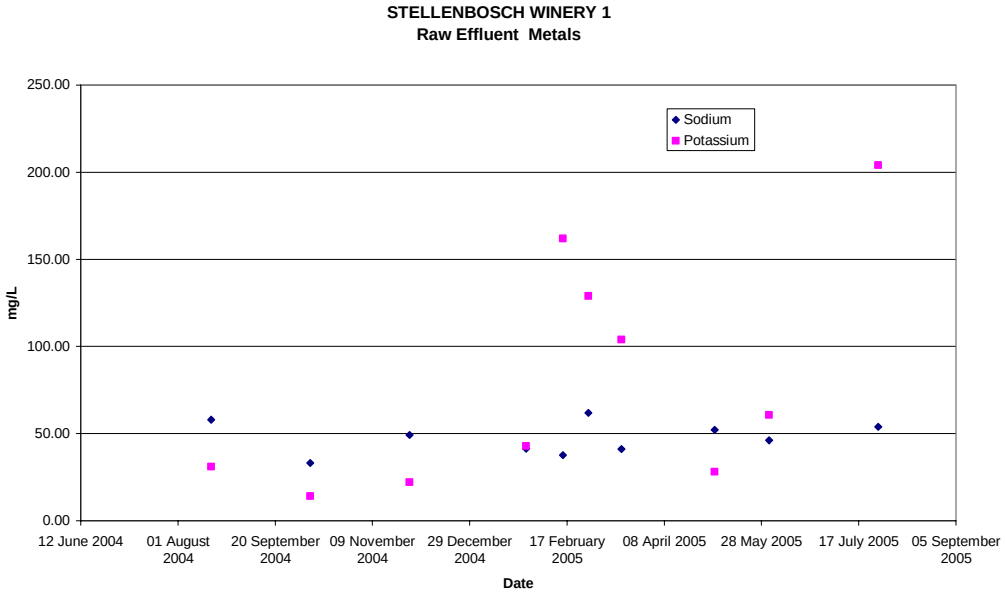

Figure 2.7 - Raw effluent metals in solution detected for the time period of June 2004 to September 2005

2.4.3.2 Winery 2

Samples were taken at this winery on a daily basis over the course of a harvest rather than over a whole year in an attempt to understand the dynamics of the harvest, rather than the dynamics of a year.

COD

The COD of the raw effluent has been analysed and is presented in Figure 2.8. The blue arrow depicts the rise in COD from pre-harvest until the middle of the harvest, and the red arrow depicts the decline in the levels of the COD from the middle to the end of the harvest. This is to be expected as the rate of processing increases and then decreases towards the end of the harvest. Those values circled in red need circumspection because the calculated COD was significantly higher than the measured COD using the formula described in Appendix 1 of this report. This *double-measurement* of COD provides a very useful check of the integrity of the data presented in this project. This data has been excluded from data analysis based on the lack of agreement.

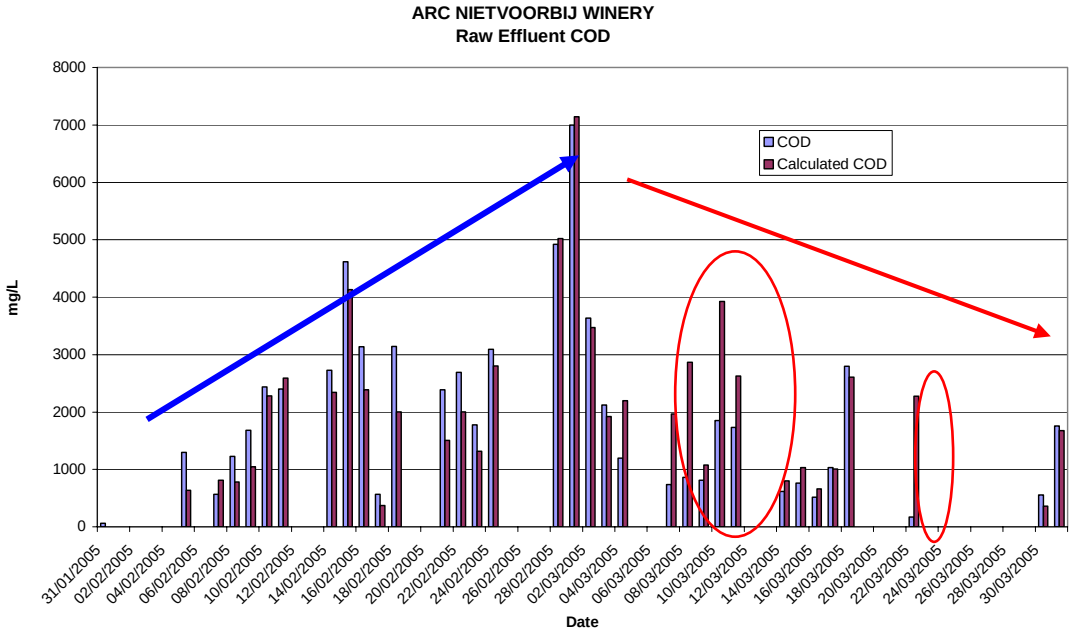

Figure 2.8 - Raw effluent COD at Winery 2 calculated for the time period of January 2005 to March 2005

In Figure 2.9, a plot of calculated COD vs. COD is given. The calculated COD includes the contributions from all the organic components, and the accounting system leads us to believe that we can account for approximately 91% of the COD using our methods. Rogue points like those shown in Figure 2.8 have been excluded (5 points in total). This implies that one can reliably guess a COD value for a winery, given one has a simple alcohol and carboxylic acid profile of the effluent.

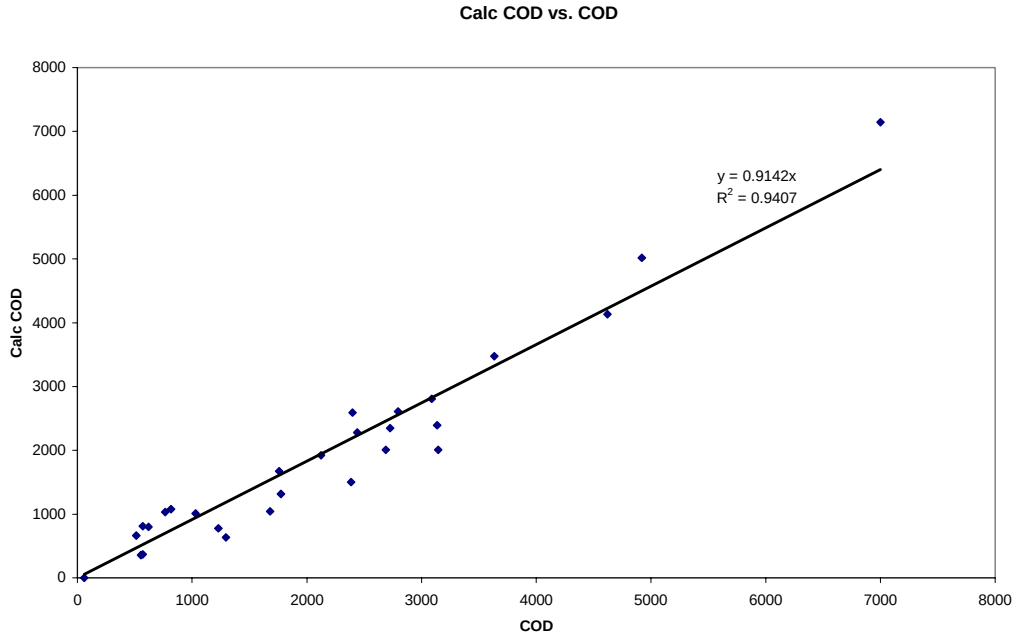

Figure 2.9 - Calculated COD vs actual COD for Winery 2

pH

The pH of the raw effluent is presented in Figure 2.10. The line shows that the pH is roughly constant and not dependent on the position in the harvest period. The average pH is 4.94 with a standard deviation of 0.61. The lack of harvesting over the long weekend (at the end of March) and subsequent lack of high strength effluent, coupled to ambient biodegradation and oxygenation can explain the high pH reading that was found on the 22nd of March.

Alcohols

The concentration of ethanol in the effluent has been plotted (Figure 2.11) for the period running from early March to late March 2005. Propanol was detected occasionally with values ranging from 0 mℓ/ℓ to 0.2 mℓ/ℓ. The ratio of propanol to ethanol never rises above 0.11, which implies that the quantity is insignificant in relation to the ethanol. The concentration of ethanol ranges from 0.2 mℓ/ℓ to 3.75 mℓℓ, which is significantly more than found in winery 1. Colin *et al.* (2) detected up to 4.9 g/ℓ, which is about 50% more than that detected in this study.

The alcohols follow the same trend as the COD shown in Figure 2.8, which is to be expected, i.e. the values rise to the middle of the harvest and then decrease from the middle to the end of the harvest, and confirm the conclusions drawn from the trends of the COD.

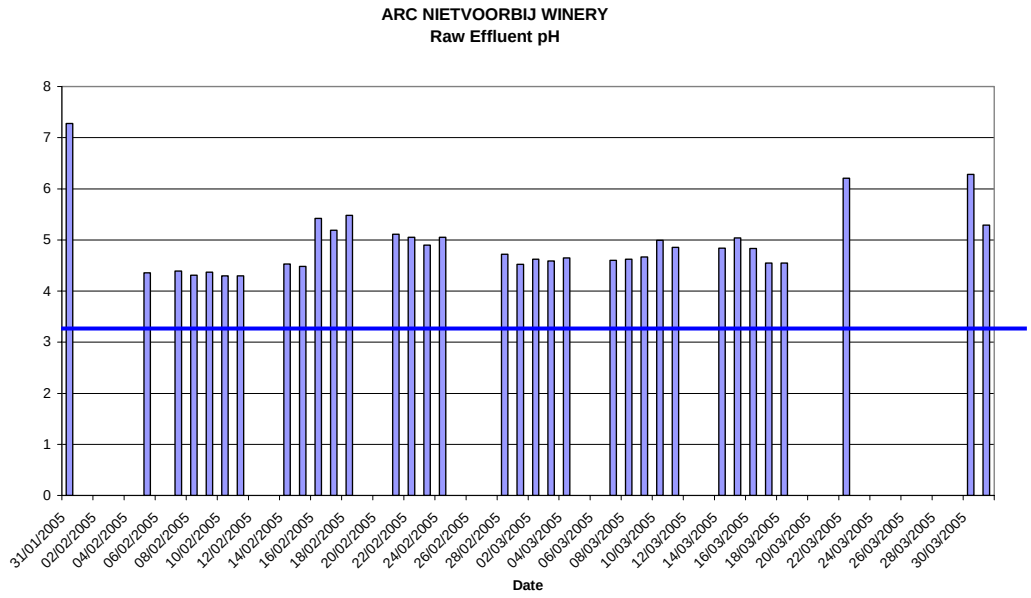

Figure 2.10 - Raw effluent pH at Winery 2 measured over the time period of January 2005 to March 2005

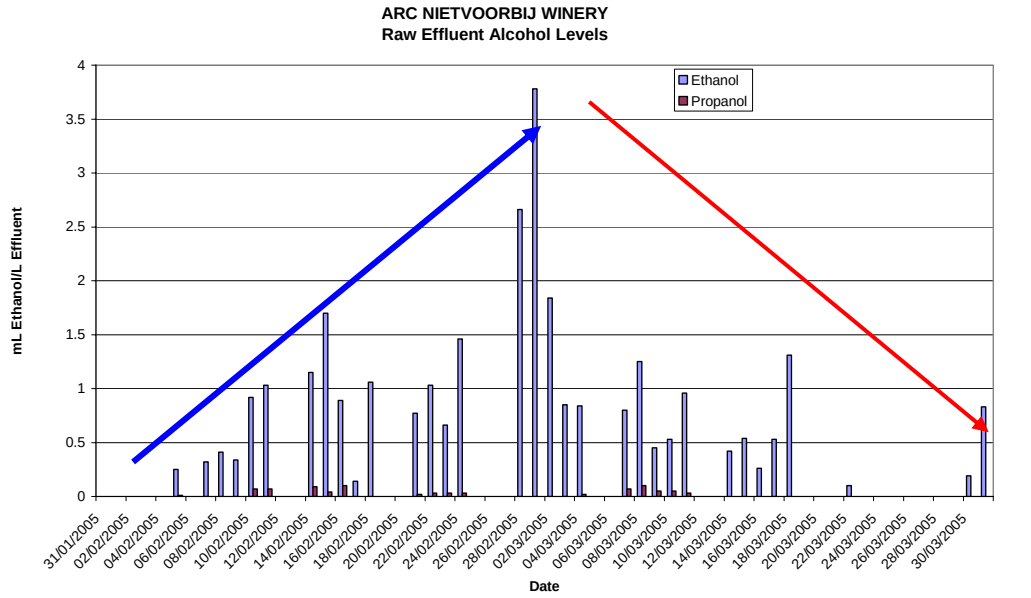

Figure 2.11 - Raw effluent alcohol levels detected at Winery 2 over the time period of January 2005 to March 2005

Carboxylic Acids

Figure 2.12, showing the quantity of acetic acid in the effluent, provides contradicting data, which needs further investigation. Where there is a significant amount of ethanol in the effluent, there is no corresponding significant quantity of acetic acid. Whilst data for the 23rd of March (data highlighted in the red circle) shows a large quantity of acetic acid, the pH for this day is significantly higher than all the days preceding it, which implies that this is a rogue value. This also confirms the disagreement between the actual and calculated COD on this day.

It would be expected that the levels of acetic acid in the effluent would mirror those of ethanol, but they seem to be more sporadic and follow no clear pattern. Many samples had no appreciable levels of carboxylic acids at all.

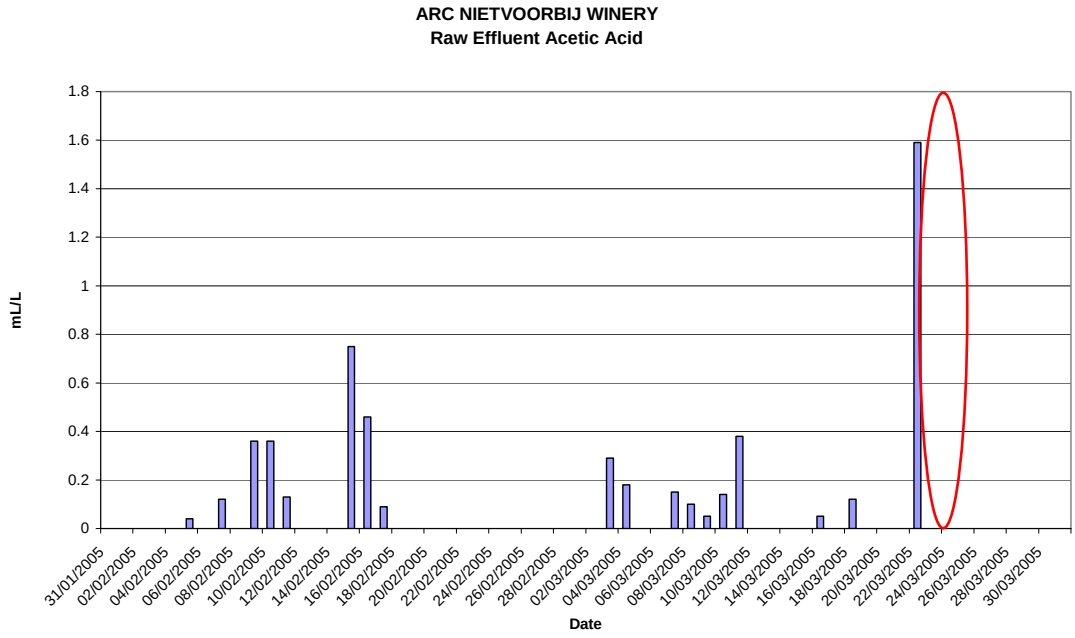

Figure 2.12 - Raw effluent acetic acid at Winery 2 as detected for the time period of January 2005 to March 2005

In Figure 2.13 the concentration of propionic acid and butyric acid are given. They are generally low (between 0 mℓ/ℓ and 0.2 mℓ/ℓ) with the exception of the 10th of March. This is a day with a recognised rogue point as shown in Figure 2.8. Whilst these acids are not largely significant mathematically, they are significant environmentally because they are the most noxious smelling of all the winery effluent constituents.

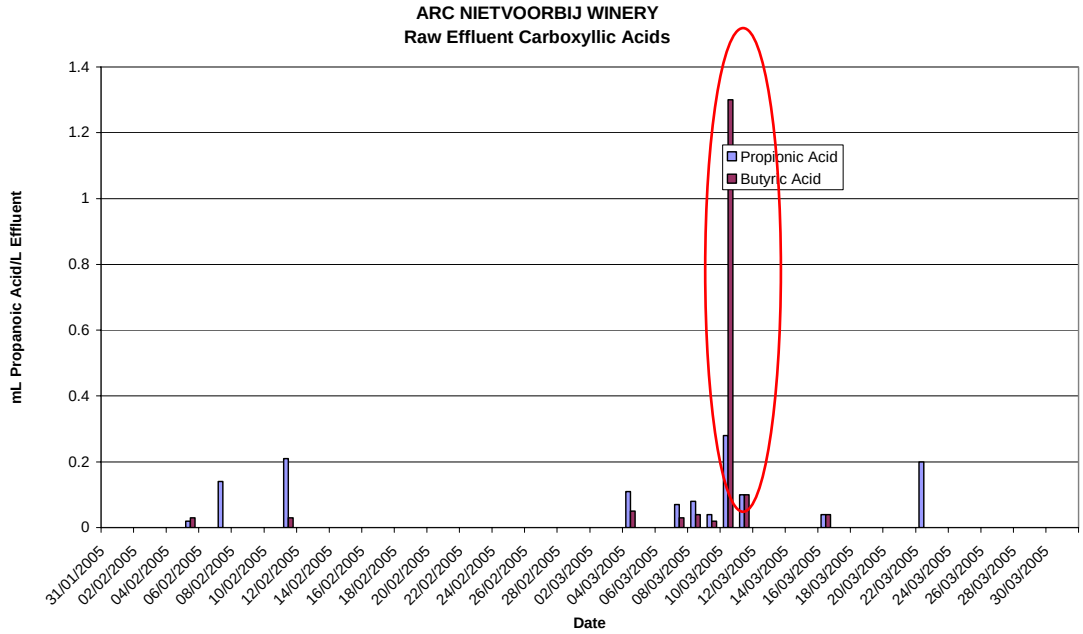

Figure 2.13 - Raw effluent carboxylic acids detected in Winery 2 over the time period of January 2005 to March 2005

Metallic species

The quantity of both sodium and potassium detected is given in Figure 2.14. Different conclusions can be drawn for differing constituents. Sodium appears to be less affected than potassium by the physical process of harvesting. Sodium is expected to arise from cellar cleaning operations (caustic soda and sodium carbonates are used to clean stained tanks). Sodium values rise from 32 mg/ℓ at the beginning of the harvest to 121 mg/ℓ at the peak of the harvest and drop towards the end (a rise of approximately 400%). The quantity of sodium in the effluent ranges from 8.91 mg/ℓ to 121 mg/ℓ. The overall potassium is strongly affected by the point of time within the harvest season. Potassium values rise strongly from 25 mg/ℓ to 225 mg/ℓ at the peak of the harvest and then decline to base levels at the end of the harvest. This is an increase of 900%. This is to be expected because potassium is the major inorganic constituent of grapes. Thus, as the harvest gets underway, the levels should rise, and as it comes to an end, it is expected that the levels of potassium would drop. The findings for both sodium and potassium for winery 2 are in agreement with those found in winery 1. Potassium was detected in a range of 22.9 to 215 mg/ℓ.

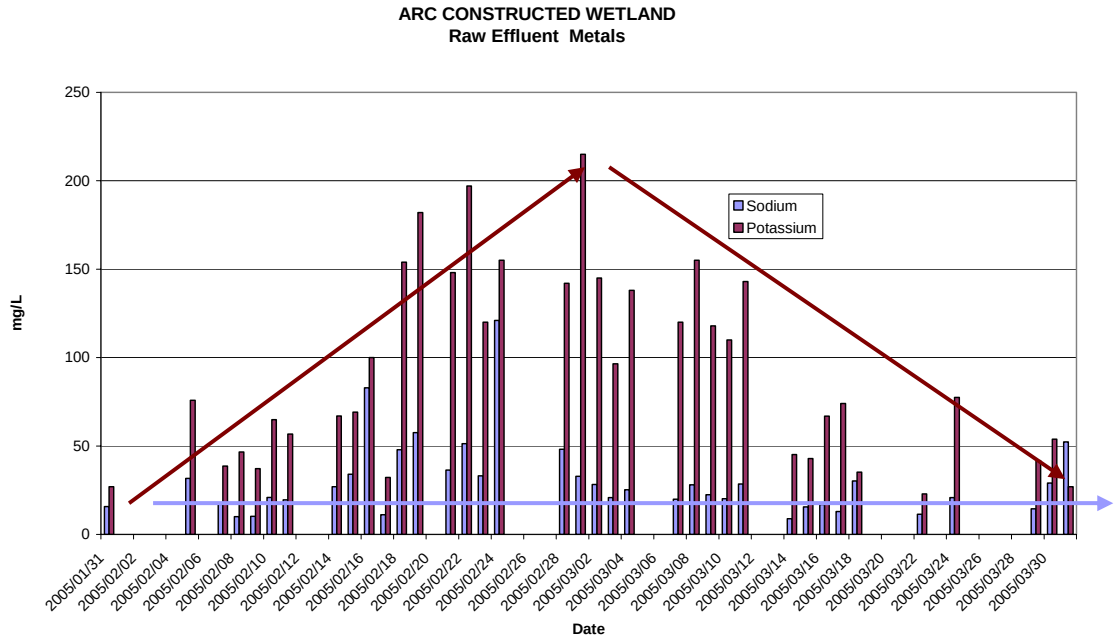

Figure 2.14 - Effluent metallic species levels detected at Winery 2 over the time period of January 2005 to March 2005

2.5 Conclusions on methods and trends observed from the data obtained

- Ethanol is the major constituent of winery effluent and contributes approximately 86% to 91% of the COD. Ethanol levels may rise to above 4 mℓ/ℓ.
- Acetic acid is the second organic component that contributes significantly to the COD of winery effluent. Acetic acid may occur at concentrations as high as 0.4 mℓ/ℓ.
- The pH of the effluent during the harvest period is roughly 4.5.
- The quantity of potassium is strongly dependant on the time of year and the intensity of the harvest season. The quantity of potassium in the effluent ranges from 25 mg/ℓ to 225 mg/ℓ.
- Sodium levels in the effluent are less dependent on the harvest than potassium. The sodium levels range between 25 mg/ℓ and 125 mg/ℓ.
- Whilst other components such as longer (C_3, C_4, C_5) carboxylic acids, other organic acids and phenolic components are present in winery effluent, they are not significant as modelling parameters.

2.6 References

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CHAPTER 3
MODELLING OF CHEMICAL REMOVAL PROCESSES IN WETLANDS

3.1 Introduction

The design of a wastewater treatment system requires a mathematical model. The purpose of the model should be twofold, *viz*. to provide an engineer with a size to build the treatment system, and to provide an engineer with a final effluent specification. The model has to incorporate aspects of time (temporal aspects), space (spatial aspects) and the degradation kinetics of the chemical species present in the effluent. As such, the model should attempt (within acceptable limits) to describe the surface plots for different chemical species within the effluent. As discussed in earlier reports, most models used for designing and describing constructed wetlands seek to predict the breakdown of the COD, which is a lumped parameter, using either 1st order kinetics or regression correlations. What follows is a description of the model we are developing.

3.2 Model development

The fitting of a model becomes critical for the design of any constructed wetlands. A preliminary model has been developed and the workings therein will be explained in this section.

The tracer study provides enough information to calculate the degree of dispersion, the residence time of each tank, as well as the number of tanks needed to be modelled.

For Component I, the mass balance for a single CSTR is as follows:

$$Q \cdot C_{i,1} = Q \cdot C_{i,0} - r \cdot V \qquad [1]$$

$$V/Q = \tau \qquad [2]$$

$$r = -k \cdot C \qquad [3]$$

$$C_{i,1} = C_{i,0} - C_{i,1} \cdot k \cdot \tau$$ Substituting [2] and [3] into [1]

$$C_{i,1} = \frac{C_{i,0}}{1 + k \cdot \tau} \qquad [4]$$

$$C_{i,2} = \frac{C_{i,1}}{1 + k \cdot \tau}$$ If the outlet from CSTR1 was fed to CSTR2, equation 5 would be [5]

Thus

$$C_{i,2} = \frac{C_{i,0}}{1 + k \cdot \tau} \cdot \frac{1}{1 + k \cdot \tau}$$ Substituting [4] into [5] gives equation [6]:

$$C_{i,2} = \frac{C_{i,0}}{(1 + k \cdot \tau)^2}$$ ised form of the equation which is given by equation [7].

$$C_{i,N} = \frac{C_{i,0}}{(1 + k_i \cdot \tau)^N} \qquad [7]$$

where:

superscript numbers notate the number (in sequence) of the tank;

Q is the flow rate;

V is the volume of the CSTR;

r is the rate of reaction;

$C_{i,N}$ is the outlet concentration of component i after N tanks;

$C_{i,0}$ is the initial concentration of component i;

N is the Number of CSTR's in series;

k_i is the rate constant for component i;

τ is the mean residence time of each CSTR.

This derivation is based on the assumption that the reactions are first order, and that each CSTR has the same residence time, and that the flow into the system is equal to the flow out of the system. Since we are dealing with biological systems, the kinetics are most likely to follow the form of the Monod equation (4) and this has been indicated by (3). This CW was operated far from maximum loading rate, which means that it is safe to assume that the kinetics are linear (and that we are operating in the first order regime).

Because of the physical constraints of the CW, it isn't possible to directly measure the kinetics (the k-values). These have to be calculated from the tanks-in-series model. This is done in the following manner:

- A week of data from the 2005 harvest was selected for parameter fitting. This week was the 7th to the 11th of February 2005. This week was selected on the basis of significant flow as well as significant effluent strength.
- Each sample port has its own Residence Time Distribution (RTD). This means that from the beginning of the CW to *that port* may be modelled as a reactor with a given residence time based on the flow for that period of time. Each port is modelled as a series of tanks (determined by N) and these tanks have no relation to sample ports above the port in question. An attempt to show this graphically is given in Figure 3.1. What it doesn't imply is that since sample port 1a and b is halfway from the raw effluent and sample port 1, that

sample port 1a and b represents the outflow from reactor 1 in a 2 tanks in series model. These boundaries are theoretical.

- Sample ports 1a and b and 1 are used to find the rate constants. These rate constants are then tested on ports 2a and b and 2.
- No further ports (after port 2) can be assessed because all ethanol has been fully decomposed by port 2.
- The interpretation of N is interesting. For the sake of modelling, N will be used as calculated – i.e. if N is 1.33, then that value will be used. For design purposes, $N \in \mathbb{N}$, that is $N \in \{1, 2, 3, \ldots N\}$, such that if the model generated a value of N = 1.33, N would be rounded up to 2.

3.2.1 Determination of hydraulic parameters

A flawed assumption of earlier work in this project (and in many others) was that the constructed wetland could be modelled as an Ideal Plug Flow reactor. This was, however, recognised as such and to this end a tracer study was undertaken to determine the true hydraulic nature of the reactor. The tracer study provided detailed information on how to model the system.

The tracer chosen for the study was KBr, and samples were analysed for Br-. Bromide was chosen on the basis of it being:

- Highly soluble and therefore unlikely to precipitate
- Non-existent in the system being studied

5 kg of KBr was injected as a pulse into the inlet of the CW and samples were taken at each of the 15 ports shown in Figure 3.2. This experiment was run just before the 2006 harvest, as this is the period where least rainfall was expected. Each port was sampled every hour for the first 6 hours, every second hour for the next 18 hours, and then every four hours thereafter.

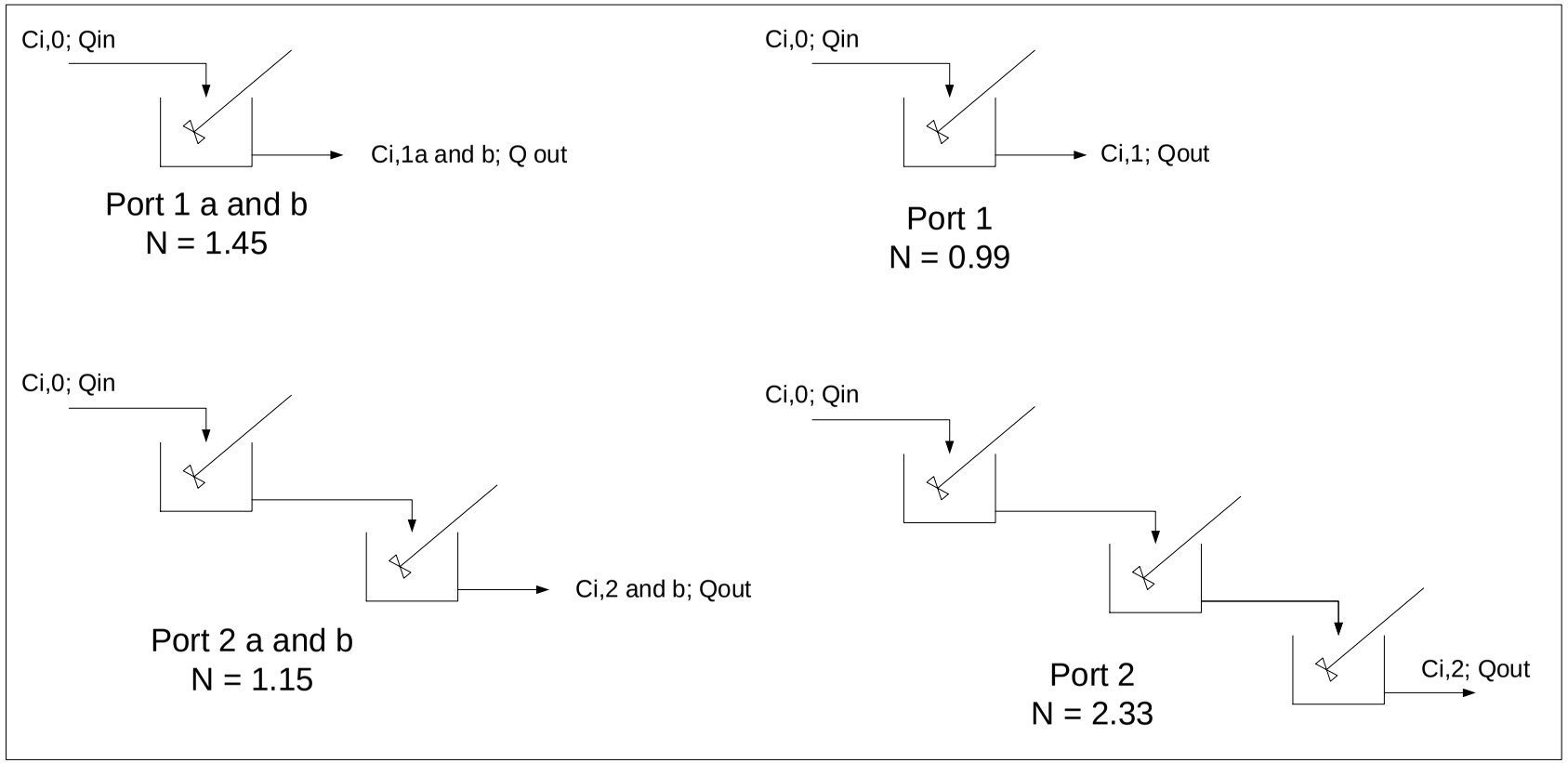

Figure 3.1 – Graphical description of the model

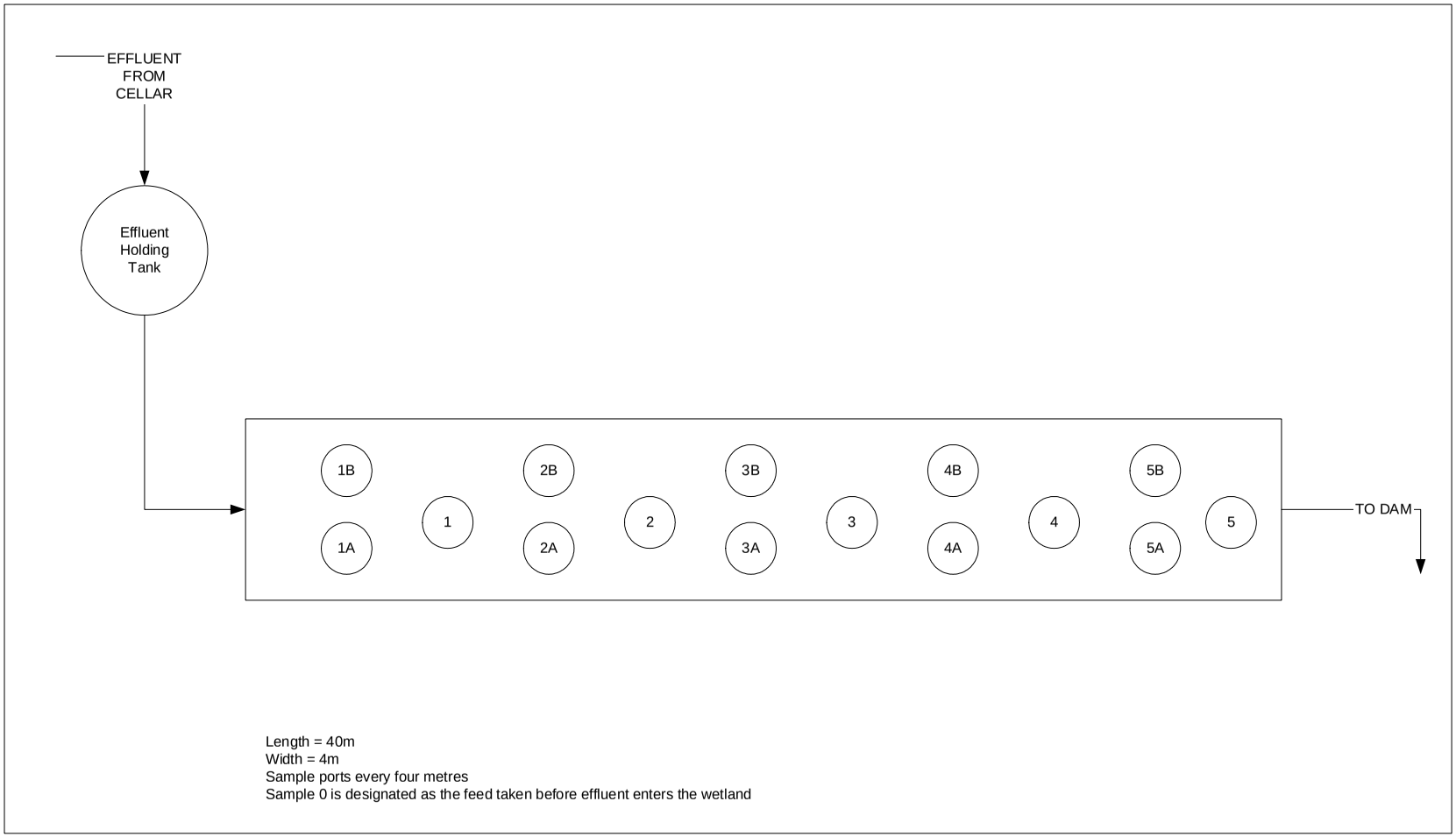

Figure 3.2 - Sample port location

The concentration profiles for each of the sample ports are given in Table 3.1. This data has been fully analysed and the residence time distributions have been calculated according to the methods described by Fogler (1) and Levenspiel (2). Two variables are calculated from the residence time distributions:

- The first moment of the distribution is τ which can be interpreted as the mean residence time of the system
- The Peclet number (a dimensionless number) can also be calculated. The inverse of the Peclet number is called the dispersion number, and provides information about the degree of dispersion. The Dispersion number can also be used to calculate the number of theoretical tanks in a tanks-in-series model.

The residence time function is generally defined as E (t) and has not been normalised for this system primarily because we are not comparing this system to others. All ports a and b have been averaged, to get an equivalent value for the centre of the constructed wetland (essentially reducing the surface curves shown in the December 2005 report to a line down the centre of the CW). All the E (t) vs. t curves and data tables are given in Appendix 2 of this report. In each case, the tail of the concentration curve was estimated by fitting exponential decay functions and extrapolating to infinity. These decay graphs are given in Appendix 3 of this report.

Table 3.1 – Bromide concentration profiles for the various ports indicated

Date and Time	Meter Reading	Flow / m^3	Port	Concentration /mg/L														
03/02/2006 17:10	125.37		1a	1b	1.00	2a	2b	2.00	3a	3b	3.00	4a	4b	4.00	5a	5b	5.00
03/02/2006 18:00	125.86	0.49	0.83	6.41	0.58	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
03/02/2006 19:04	126.41	0.55	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
03/02/2006 20:04	126.89	0.48	0.00	0.74	0.00	0.00	0.74	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.71	0.00	1.78
03/02/2006 21:10	128.44	1.55	183.42	145.07	12.78	0.00	11.63	0.00	0.00	0.00	0.00	0.00	0.00	1.09	0.00	0.00	132.48
03/02/2006 22:12	129.46	1.02	128.29	109.50	134.83	0.00	14.72	0.00	0.00	0.00	0.00	0.00	0.00	3.41	0.00	0.00	181.81
03/02/2006 22:52	129.96	0.50	95.96	95.47	167.69	0.00	25.29	1.36	0.00	0.00	0.00	0.00	0.00	2.73	0.00	0.00	122.43
03/02/2006 23:50	130.71	0.75	73.26	91.12	151.17	0.00	74.63	5.55	0.00	0.00	0.00	0.00	0.00	2.12	0.00	0.00	133.98
04/02/2006 02:06	132.52	1.81	51.65	105.17	67.78	5.24	160.56	25.24	2.37	3.65	0.00	0.00	0.00	8.55	0.00	0.00	134.14
04/02/2006 04:05	134.03	1.51	47.54	84.01	28.11	47.07	115.91	64.34	1.41	14.01	0.79	0.00	0.00	26.69	0.00	0.35	126.98
04/02/2006 06:03	135.52	1.49	44.96	52.84	17.77	62.30	77.07	141.84	1.17	24.00	3.39	0.00	0.31	41.97	0.32	3.26	106.69
04/02/2006 08:10	136.92	1.40	43.46	32.60	12.34	55.28	59.59	148.43	1.74	58.81	9.05	0.29	0.56	48.01	1.01	4.64	88.18
04/02/2006 10:12	138.16	1.24	39.34	20.47	8.9	37.31	47.44	121.81	3.25	70.45	13.47	0.59	0.8	41.24	1.4	7.64	67.16
04/02/2006 11:55	139.14	0.98	33.23	15.21	7.68	33.27	36.83	106.91	10.40	79.38	20.15	1.01	1.81	36.17	5.35	7.89	55.84
04/02/2006 13:30	140.26	1.12	28.76	14.19	6.52	30.63	29.71	96.07	24.49	74.07	47.77	1.09	3.73	32.33	3.08	12.81	55.58
04/02/2006 16:20	141.71	1.45	10.92	5.70	7.11	28.16	22.34	76.43	68.71	69.63	31.50	38.84	10.50	30.48	3.54	13.33	47.41
04/02/2006 19:30	143.18	1.47	3.91	5.48	10.77	22.24	17.19	56.02	78.88	62.29	55.27	60.57	29.94	35.53	5.06	38.27	46.16
05/02/2006 00:06	148.24	5.06	10.78	16.05	5.18	9.59	9.15	22.05	30.53	38.29	59.88	24.66	81.68	62.99	10.59	4.86	26.50
05/02/2006 05:06	150.53	2.29	7.72	8.74	4.01	8.50	9.26	13.58	17.11	24.10	42.48	48.00	62.92	48.03	42.67	68.56	27.86
05/02/2006 09:15	151.00	0.47	6.09	7.70	4.42	7.75	9.39	12.09	14.35	23.24	36.25	44.41	55.30	46.40	45.65	79.61	36.56
05/02/2006 11:53	154.95	3.95	5.37	7.55	2.49	5.89	7.48	10.39	9.24	16.49	21.58	33.67	38.77	49.75	55.99	84.54	21.77
05/02/2006 15:56	156.21	1.26	3.52	7.55	5.03	4.46	6.23	7.16	8.14	14.86	14.30	17.97	29.70	34.09	44.90	66.42	29.65
06/02/2006 09:30	159.24	3.03	1.89	2.27	2.21	3.49	4.75	6.00	7.09	12.51	11.47	16.71	20.24	28.58	43.08	54.73	14.29
17/02/2006 13:35	195.20	35.96	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	3.31	2.37	3.23	0.00	4.22	3.34	0.00
24/02/2006 12:00	208.81	13.61	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
03/03/2006 12:00	212.52	3.71	0.00	0.00	0.00	0.00	0.00	0.00	0.72	1.53	1.79	2.30	3.43	1.82	6.18	5.30	3.80

3.2.2 Residence time distribution study

Since each sample port was sampled, a residence time distribution of each port has been obtained. This provides a mean residence time (τ) as well as the number of tanks that would be needed if one were to model that system as a series of reactors. These values are given in Table 3.2.

Table 3.2 - Tau and N values

Port Number	Length / m	Residence Time (τ) / hours	Dispersion Number (D)	Number of Tanks (N)
1a and b	4	15.49	0.81	1.45
1	8	14.62	32.0	0.99
2a and b	12	20.28	1.040	1.15
2	16	22.62	0.300	2.33
3a and b	20	31.55	0.125	4.46
3	24	36.13	0.080	6.86
4a and b	28	41.47	0.060	9.36
4	32	37.89	0.120	4.7
5a and b	36	49.7	0.040	12.75
5	40	22.36	0.620	1.63

This implies that as the effluent progresses down the reactor, the flow changes from stirred tank behaviour towards plug flow behaviour. At no point does it behave as a plug flow reactor though. Ports 4 and 5 show anomalies in the number of the tanks needed in the model, but this can be explained by assuming there is short-circuiting through the system. This is a consequence of poor design of the CW itself, whereby short-circuiting wasn't prevented (usually this is done through the use of baffles).

3.2.3 K-value determination

Table 3.3 shows the calculations whereby the values of k are determined for each of the days of sampling from Port 0 to Port 1a and b. This is calculated for each of the parameters that were deemed important. Note that the concentration of all organic species is mℓ/ℓ, the concentration of Na and K is mg/ℓ (ppm) and that pH has no dimensions.

This was repeated for the reactor from Port 0 to Port 1, and the k-values for each of the components were then determined and averaged. These average k-values are given in Table 3.4. Under the conventions used in this model, a negative value for k means that with time, component *i* will decrease, and for a positive value of k, with time the concentration of component *i* will increase.

Table 3.3 - K-value calculations

Sample Port 1a and b - N=1								
07 February 2005	Ethanol	Propanol	Acetic Acid	Propionic Acid	Butyric Acid	pH	Na	K
Feed Concentration	0.32	0.00	0.12	0.04	0.00	4.39	17.03	38.70
Concentration at the port	0.06	0.00	0.03	0.02		6.66	32.00	47.00
Flow/m^3/hr	0.07	0.07	0.07	0.07	0.07	0.07	0.07	0.07
Tau / hrs	166.07	166.07	166.07	166.07	166.07	166.07	166.07	166.07
K	-0.03	#DIV/0!	-0.02	-0.01	#DIV/0!	0.00	0.00	0.00
Sample Port 1a and b - N=1								
08 February 2005	Ethanol	Propanol	Acetic Acid	Propionic Acid	Butyric Acid	pH	Na	K
Feed Concentration	0.41	0.00	0.00	0.00	0.00	4.31	10.03	46.60
Concentration at the port	0.07	0.01	0.00	0.00	0.00	6.60	15.00	46.98
Flow/m^3/day	0.06	0.06	0.06	0.06	0.06	0.06	0.06	0.06
Tau / hrs	196.48	196.48	196.48	196.48	196.48	196.48	196.48	196.48
K	-0.02	0.01	#DIV/0!	#DIV/0!	#DIV/0!	0.00	0.00	0.00
Sample Port 1a and b - N=1								
09 February 2005	Ethanol	Propanol	Acetic Acid	Propionic Acid	Butyric Acid	pH	Na	K
Feed Concentration	0.34	0.00	0.36	0.00	0.00	4.37	10.03	37.31
Concentration at the port	0.00	0.00	0.00	0.00	0.00	6.57	14.60	38.50
Flow/m^3/day	0.08	0.08	0.08	0.08	0.08	0.08	0.08	0.08
Tau / hrs	148.40	148.40	148.40	148.40	148.40	148.40	148.40	148.40
K	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	0.00	0.00	0.00
Sample Port 1a and b - N=1								
10 February 2005	Ethanol	Propanol	Acetic Acid	Propionic Acid	Butyric Acid	pH	Na	K
Feed Concentration	0.92	0.07	0.36	0.00	0.00	4.37	20.98	64.89
Concentration at the port	0.36	0.08	0.30	0.00	0.00	6.56	14.50	39.00
Flow/m^3/day	0.07	0.07	0.07	0.07	0.07	0.07	0.07	0.07
Tau / hrs	169.09	169.09	169.09	169.09	169.09	169.09	169.09	169.09
K	-0.01	0.00	0.00	#DIV/0!	#DIV/0!	0.00	0.00	0.00
Sample Port 1a and b - N=1								
11 February 2005	Ethanol	Propanol	Acetic Acid	Propionic Acid	Butyric Acid	pH	Na	K
Feed Concentration	1.03	0.07	0.36	0.00	0.00	4.37	20.98	64.89
Concentration at the port	0.26	0.08	0.30	0.00	0.00	6.56	14.50	39.00
Flow/m^3/day	0.12	0.12	0.12	0.12	0.12	0.12	0.12	0.12
Tau / hrs	100.72	100.72	100.72	100.72	100.72	100.72	100.72	100.72
K	-0.03	0.00	0.00	#DIV/0!	#DIV/0!	0.00	0.00	-0.01

Table 3.4 - Average k-values

Component	k value / hr	Std Deviation	k value / day
Ethanol	-0.020	0.008	-0.482
Propanol	0.003	0.003	0.083
Acetic acid	-0.010	0.008	-0.24
Propionic acid	-0.006	<0.001	-0.149
PH	0.003	0.002	0.066
Na^+	0.0001	0.0026	0.0034
K^+	-0.001	0.003	-0.026
COD	-0.032	0.06	-0.759

The CW clearly works (given that after sample port 2 there is no ethanol left in the effluent) and the k-values calculated for each component make sense with the exception of propanol and sodium.

The model predicts that the concentration of propanol will rise, which is not true, but since the quantity of propanol is so small in relation to the quantity of ethanol, this is not a critical modelling parameter. The standard deviation of this rate constant is also of the same order of magnitude which makes this a meaningless value.

The concentration of sodium is also predicted to rise, which may be true given that the CW is exposed to the effects of evapo-transpiration which may concentrate the effluent. However, because the rate constant is very small and has a large degree of uncertainty, it is not possible to say what happens with any degree of statistical certainty.

The rate constant indicates that there would be a decrease in potassium in solution, and this makes sense as potassium is a primary plant metabolite.

pH was consistently observed to rise, and stabilise at approximately 7.5 to 8. However, modelling this is difficult because the pH is very dependant on many factors, from the concentration of organic acids such as acetic acid, to the concentration of conjugate acids such as Na^+ and K^+ and there is a very strong buffering system at work because the dolomitic matrix of the CW is composed primarily of calcium and magnesium carbonates. This combined with the CO_3^{2-} buffering system and trying to do a charge balance simultaneously would involve far too many variables that haven't and cannot be practicably measured and are not needed. The valuable information is that the pH rises from and average of 4.9 to approximately 7.5.

It has been postulated that the ethanol is first degraded to acetic acid, and this is then further metabolised by the biofilm and associated microorganisms to CO_2 and CH_4. Thus acetic acid should have a rate of formation and a rate of consumption. Because it is not possible to measure both these parameters simultaneously in the field, an overall rate degradation constant has been calculated. Thus, some of the uncertainty in this data may come from the production kinetics.

The most significant of all the k-values is for that of ethanol, and the model predicts this value with the least amount of uncertainty (the lowest Std Deviation as a percent – 40%) and the value of k to be -0.482 per day is similar to those values found for the COD in industry [Shepherd *et al.* (5) give a COD rate constant of 4.5/day]. It can be shown that their calculations are out by a factor of 10, which implies that the rate constant found on the same effluent is 0.45/day for the COD. The difference with the approach in this study is that the when the COD was modelled, the k-value was nearly twice that of the k-value for ethanol, and the standard deviation of the value was 1.875 time the value. This means that the value of 0.759/day for COD is not statistically significant.

3.3 Testing of the model

The model was tested from port 0 (the feed) to port 2a and b as well as from port 0 to port 2 and the results are presented in Table 3.5 and Table 3.6 respectively.

Table 3.5 – Model test – Port 2a and b

Port 2a and b		k/day	K Ethanol	K Propanol	K Acetic Acid	K Priopionate	K pH	K Na	K K	K COD
Tau Test	20.280		-0.020	0.003	-0.010	-0.006	0.003	0.000	-0.001	-0.032
Q Test	0.750									
N (1.15)	2.000									
	K Ethanol	K Propanol	K Acetic Acid	K Priopionate	K pH	K Na	K K	K COD		
Port 2a and b										
07 February 2005	Ethanol	Propanol	Acetic Acid	Propionic Acid	Butyric Acid	pH	Na	K	COD	
Feed Concentration	0.320	0.000	0.120	0.040	0.000	4.390	17.030	38.700	570.000	
Flow/m^3/day	0.070	0.070	0.070	0.070	0.070	0.070	0.070	0.070	0.070	
Tau / days	217.286	9.054	9.054	9.054	9.054	9.054	9.054	9.054	9.054	
Concentration at the port	0.011	0.000	0.101	0.036		4.639	17.074	37.940	342.679	
Measured Concentration	0.000	0.000	0.000	0.000	0.000	6.780	25.000	28.000		
Port 2a and b										
08 February 2005	Ethanol	Propanol	Acetic Acid	Propionic Acid	Butyric Acid	pH	Na	K	COD	
Feed Concentration	0.410	0.000	0.000	0.000	0.000	4.310	10.030	46.600	1230.000	
Flow/m^3/day	0.059	0.059	0.059	0.059	0.059	0.059	0.059	0.059	0.059	
Tau / hrs	185.704	185.704	185.704	185.704	185.704	185.704	185.704	185.704	185.704	
Concentration at the port	0.018	0.000	0.000	0.000		21.973	10.578	32.119	25.519	
Measured Concentration	0.000	0.000	0.000	0.000	0.000	6.380	12.220	34.430	645.000	
Port 2a and b										
09 February 2005	Ethanol	Propanol	Acetic Acid	Propionic Acid	Butyric Acid	pH	Na	K	COD	
Feed Concentration	0.340	0.000	0.360	0.000	0.000	4.370	10.030	37.310	1680.000	
Flow/m^3/day	0.078	0.078	0.078	0.078	0.078	0.078	0.078	0.078	0.078	
Tau / hrs	140.266	140.266	140.266	140.266	140.266	140.266	140.266	140.266	140.266	
Concentration at the port	0.023	0.000	0.062	0.000		13.026	10.440	27.994	55.770	
Measured Concentration	0.000	0.000	0.000	0.000	0.000	6.530	19.770	52.550	920.000	
Port 2a and b										
10 February 2005	Ethanol	Propanol	Acetic Acid	Propionic Acid	Butyric Acid	pH	Na	K	COD	
Feed Concentration	0.920	0.070	0.360	0.000	0.000	4.370	20.980	64.890	2400.000	
Flow/m^3/day	0.069	0.069	0.069	0.069	0.069	0.069	0.069	0.069	0.069	
Tau / hrs	159.818	159.818	159.818	159.818	159.818	159.818	159.818	159.818	159.818	
Concentration at the port	0.052	0.258	0.053	0.000		16.127	21.961	46.920	64.200	
Measured Concentration	0.290	0.130	0.140	0.000	0.000	6.240	18.810	55.810	1630.000	
Port 2a and b										
11 February 2005	Ethanol	Propanol	Acetic Acid	Propionic Acid	Butyric Acid	pH	Na	K	COD	
Feed Concentration	1.030	0.070	0.360	0.000	0.000	4.370	20.980	64.890	2400.000	
Flow/m^3/day	0.115	0.115	0.115	0.115	0.115	0.115	0.115	0.115	0.115	
Tau / hrs	95.199	95.199	95.199	95.199	95.199	95.199	95.199	95.199	95.199	
Concentration at the port	0.122	0.137	0.094	0.000		8.562	21.556	53.159	146.583	
Measured Concentration	0.070	0.230	0.000	0.000	0.000	6.290	17.160	50.150	1630.000	

Table 3.6 - Model test – Port 2

Port 2			K Ethanol	K Propanol	K Acetic Acid	K Priopionate	K pH	K Na	K K	K COD
Tau Test	22.620		-0.020	0.003	-0.010	-0.006	0.003	0.000	-0.001	-0.032
Q Test	0.750									
N (2.33)	3.000									
	K Ethanol	K Propanol	K Acetic Acid	K Priopionate	K pH	K Na	K K	K COD		
Sample Port 2										
07 February 2005	Ethanol	Propanol	Acetic Acid	Propionic Acid	Butyric Acid	pH	Na	K	COD	
Feed Concentration	0.320	0.000	0.120	0.040	0.000	4.390	17.030	38.700	570.000	
Flow/m^3/day	1.680	1.680	1.680	1.680	1.680	1.680	1.680	1.680	1.680	
Tau / hrs	242.357	242.357	242.357	242.357	242.357	242.357	242.357	242.357	242.357	
Concentration at the port	0.002	0.000	0.003	0.003		120.026	18.907	19.032	0.877	
Measured Concentration	0.000	0.000	0.000	0.000	0.000	7.130	47.190	35.700	25.000	
Sample Port 2										
08 February 2005	Ethanol	Propanol	Acetic Acid	Propionic Acid	Butyric Acid	pH	Na	K	COD	
Feed Concentration	0.410	0.000	0.000	0.000	0.000	4.310	10.030	46.600	1230.000	
Flow/m^3/day	1.420	1.420	1.420	1.420	1.420	1.420	1.420	1.420	1.420	
Tau / hrs	185.704	185.704	185.704	185.704	185.704	185.704	185.704	185.704	185.704	
Concentration at the port	0.004	0.000	0.000	0.000		37.062	10.863	26.666	3.793	
Measured Concentration	0.000	0.000	0.000	0.000	0.000	7.090	35.810	35.480	175.000	
Sample Port 2										
09 February 2005	Ethanol	Propanol	Acetic Acid	Propionic Acid	Butyric Acid	pH	Na	K	COD	
Feed Concentration	0.340	0.000	0.360	0.000	0.000	4.370	10.030	37.310	1680.000	
Flow/m^3/day	1.880	1.880	1.880	1.880	1.880	1.880	1.880	1.880	1.880	
Tau / hrs	140.266	140.266	140.266	140.266	140.266	140.266	140.266	140.266	140.266	
Concentration at the port	0.006	0.000	0.026	0.000		18.938	10.651	24.248	10.471	
Measured Concentration	0.000	0.000	0.000	0.000	0.000	6.780	49.120	51.540	430.000	
Sample Port 2										
10 February 2005	Ethanol	Propanol	Acetic Acid	Propionic Acid	Butyric Acid	pH	Na	K	COD	
Feed Concentration	0.920	0.070	0.360	0.000	0.000	4.370	20.980	64.890	2400.000	
Flow/m^3/day	1.650	1.650	1.650	1.650	1.650	1.650	1.650	1.650	1.650	
Tau / hrs	159.818	159.818	159.818	159.818	159.818	159.818	159.818	159.818	159.818	
Concentration at the port	0.012	0.778	0.021	0.000		24.956	22.469	39.898	10.828	
Measured Concentration	0.000	0.000	0.000	0.000	0.000	6.650	48.390	59.910	705.000	
Sample Port 2										
11 February 2005	Ethanol	Propanol	Acetic Acid	Propionic Acid	Butyric Acid	pH	Na	K	COD	
Feed Concentration	1.030	0.070	0.360	0.000	0.000	4.370	20.980	64.890	2400.000	
Flow/m^3/day	2.770	2.770	2.770	2.770	2.770	2.770	2.770	2.770	2.770	
Tau / hrs	95.199	95.199	95.199	95.199	95.199	95.199	95.199	95.199	95.199	
Concentration at the port	0.042	0.231	0.048	0.000		10.890	21.850	48.115	37.242	
Measured Concentration	0.000	0.000	0.000	0.000	0.000	6.850	27.730	63.060	1200.000	

It is most fortunate that the model (with the exception of 1 data point) under-predicts the performance of the CW for the most important parameter. That is, for a given initial concentration, the final concentration predicted is almost always higher than the actual concentration. A measure of this error can be defined as $E = \frac{C_{calculated_i,N} - C_{measured_i,N}}{C_{i,0}}$, and the Errors (E) are given in Table 3.7.

The interpretation of the error function is such that the lower the magnitude of E, the more accurate the model. Positive value of E implies that the model is predicting more than the actual value (for example, ethanol has a positive E value) whilst negative values of E mean that the model is under-predicting (for example, there is more sodium in the final port than predicted by the model). The data is presented in the form of the E ± standard deviations as a percentage. Original E calculations are given in Appendix 2.

Table 3.7 – Error% ± Standard Deviation%

Component i	Port 2a and b True N (1.15)	Port 2a and b Rounded N (2)	Port 2 True N (2.33)	Port 2 Rounded N (3)
Ethanol	18.52 ± 3.36%	4.71 ± 1.27%	6.89 ± 5.33%	1.75 ± 1.37%
Propanol	71.43 ± 116.14%	142.86 ± 240.%2	200.29 ± 320.67%	331.71 ± 565.51%
Acetic acid	22.97 ± 14.34%	7.11 ± 4.54%	6.59 ± 5.51%	3.47 ± 3.2%
Sodium	-67.26 ± 91.57%	-62.72 ± 91.50%	-186.48 ± 131.24%	-182.59 ± 131.01%
Potassium	-7.80 ± 27.43%	-17.61 ± 26.51%	-31.40 ± 20.62%	-38.01 ± 20.35%
COD	N/A	N/A	10.02 ± 7.64%	1.23 ± 8.06%

This data shows that the model becomes more accurate with increasing length, but this is to be expected as we are dealing with exponential decay in most cases. The model also becomes more accurate when dealing with Natural numbers but this cannot be given any meaning. Data for COD is not available for the a and b ports.

The model is most accurate for the degradation of ethanol (the worst difference in conversion is 6%) and acetic acid (similarly, the worst difference in conversion is 6%). The model is not useful in prediction the concentration of sodium, although it can predict that the concentration of potassium decreases. The model can predict the decay of COD albeit with less certainty than it predicts the decay of ethanol. This difference in conversion for COD decay is as high as 10%.

3.4 Generalisation of model

It is also possible to investigate the effect of the length of the wetland on the mixing regimes. For this purpose a plot of N (number of tanks-in-series) vs. Length can be drawn. Based on the theory presented by Fogler (1) and Levenspiel (2), it is possible to show that the residence time is linearly related to the length of the wetland, and that the Number of Tanks is proportional to the square of the length. A correlation of these relationships is shown in Figure 3.3.

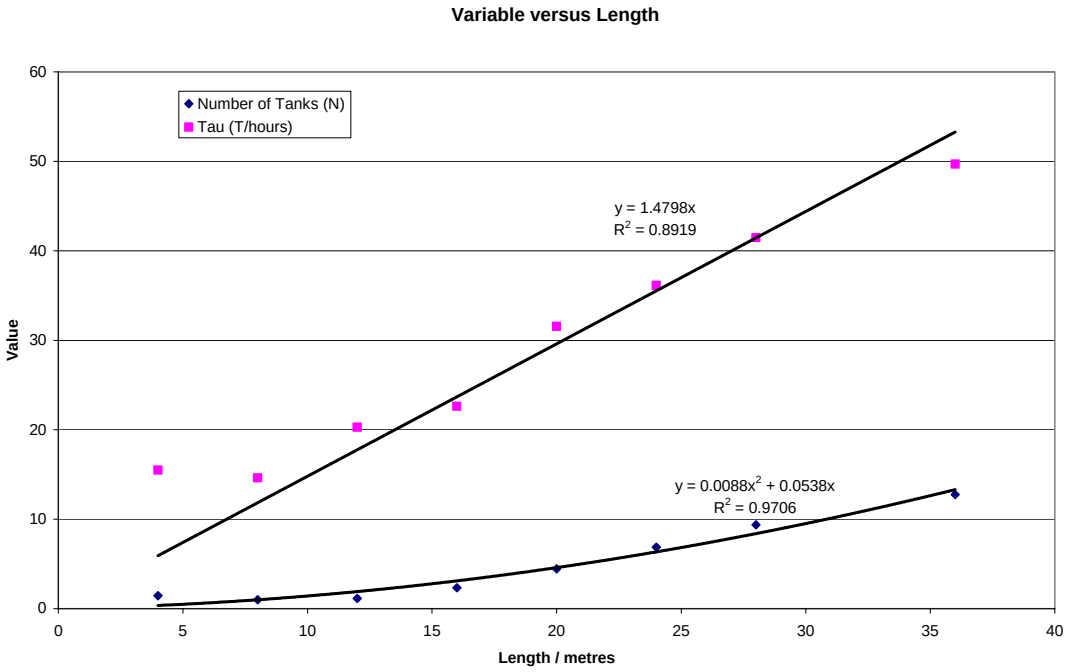

Figure 3.3 – Different Variables as a function of Length

It was found that the residence time is indeed linearly related to the length, and the number of tanks is clearly related to the square of the length. The sample ports at 32 and at 40m have been excluded from this analysis because there was a significant bypass. This bypass is caused by channelling within the constructed wetland and is a consequence of insufficient baffling during the construction of this CW.

3.5 Conclusions

Based on the information presented earlier, the following model is derived below:

- From the tracer study, a residence time (Tau) was measured for a given flow rate Q. An effective Volume (V_{eff}) can thus be defined as:

$$V_{Eff} = Q \cdot \tau \qquad [8]$$

- For our real wetland we have the equation for component i:

$$C_{i,N} = \frac{C_{i,0}}{(1 + k_i \cdot \tau)^N} \qquad [9]$$

- This can be solved (see Appendix 4 for the derivation) to yield the following equation

$$C_{i,l} = \frac{C_{i,0}}{\left(1 + k_i \cdot \frac{5}{3} \frac{\varepsilon \cdot l \cdot d \cdot w}{Q \cdot (0.0088 \cdot l^2 + 0.0538 \cdot l)}\right)^{0.0088 \cdot l^2 + 0.0538 \cdot l}} \qquad [10]$$

Where:

$C_{i,l}$ is the concentration of Component i at length l,

$C_{i,0}$ is the concentration of Component i initially

k_i is the rate constant of component i

ε is the void fraction of the gravel in the bed

l is the length of the bed

d is the depth of the bed (should be 1m for the hydraulic modelling to be accurate)

w is the width of the bed (should be 4m for the hydraulic modelling to be accurate)

Q is the flow rate of effluent into the CW

This is the model that has been used to generate the data in Table 3.8 below. This spreadsheet is included in Appendix 5 on the CD attached to this report. The inputs for the model are all in green cells and the outputs of the model are given in orange text and include the concentration and the number of tanks that the model is using. The reaction rate constants are given in blue font and design variables are given in purple cells. The values shown in the green cells are those given found at the CW on the 1st of March 2005.

In general, the smaller the error function, the more accurate the model. For the 11th of February, the Error for ethanol is less than the error for COD predictions. This is, however, one of the days that the model was calibrated with, so this data may not be used for model validation. For this reason, two other random days were chosen during the harvest, with these days being the 18th of March 2005 and the 1st of March 2005. In almost all instances, the Error for ethanol is less than the Error for COD. However, the model is conservative, both at high flow rates (the 18th of March) and at high effluent strengths (the 1st of March). Due to the large degree of uncertainty over the metallic species in the wetland it is not possible to predict with any degree of certainty how the metallic constituents will change.

The following parameters are included in the model:

- Ethanol Concentration
- Acetic Acid Concentration
- Sodium concentration
- Potassium concentration
- COD

Table 3.8 – Model Predictions at each Sample Port

For the 11th of Febrary				For the 18th of March				For the 1st of March			
4 m	C predicted	C Actual	Error Function (E)	4 m	C predicted	C Actual	Error Function (E)	4 m	C predicted	C Actual	Error Function (E)
Ethanol / mL/L	0.54		N/A	Ethanol / mL/L	0.54		N/A	Ethanol / mL/L	1.82		N/A
Acetic Acid / mL/L	0.08		N/A	Acetic Acid / mL/L	0.09		N/A	Acetic Acid / mL/L	0		N/A
Sodium / mg/L	20.14		N/A	Sodium / mg/L	30.49		N/A	Sodium / mg/L	34.04		N/A
Potassium / mg/L	52.48		N/A	Potassium / mg/L	34.34		N/A	Potassium / mg/L	194.5		N/A
COD / mg/L	1890		N/A	COD / mg/L	2555		N/A	COD / mg/L	5240		N/A
8 m	C predicted	C Actual	Error Function (E)	8 m	C predicted	C Actual	Error Function (E)	8 m	C predicted	C Actual	Error Function (E)
Ethanol / mL/L	0.22	0.32	-9.71%	Ethanol / mL/L	0.63	0.4	17.56%	Ethanol / mL/L	0.65	0.4	6.61%
Acetic Acid / mL/L	0.04	0	30.77%	Acetic Acid / mL/L	0.07	0	58.33%	Acetic Acid / mL/L	0	0	#DIV/0!
Sodium / mg/L	20.67	17.16	1979.54%	Sodium / mg/L	30.72	19.12	3008.81%	Sodium / mg/L	35.21	47.68	3375.94%
Potassium / mg/L	48.26	50.15	-3.33%	Potassium / mg/L	33.47	23.63	27.94%	Potassium / mg/L	174.61	168	3.07%
COD / mg/L	1425	1630	-8.54%	COD / mg/L	2325	2755	-15.38%	COD / mg/L	3695	4315	-8.86%
12 m	C predicted	C Actual	Error Function (E)	12 m	C predicted	C Actual	Error Function (E)	12 m	C predicted	C Actual	Error Function (E)
Ethanol / mL/L	0.08		N/A	Ethanol / mL/L	0.4		N/A	Ethanol / mL/L	0.19		
Acetic Acid / mL/L	0.02		N/A	Acetic Acid / mL/L	0.05		N/A	Acetic Acid / mL/L	0		
Sodium / mg/L	21.21		N/A	Sodium / mg/L	30.95		N/A	Sodium / mg/L	36.41		
Potassium / mg/L	44.29		N/A	Potassium / mg/L	32.61		N/A	Potassium / mg/L	156.27		
COD / mg/L	1055		N/A	COD / mg/L	2105		N/A	COD / mg/L	2530		
16 m	C predicted	C Actual	Error Function (E)	16 m	C predicted	C Actual	Error Function (E)	16 m	C predicted	C Actual	Error Function (E)
Ethanol / mL/L	0.02	0.06	-3.88%	Ethanol / mL/L	0.25	0.03	16.79%	Ethanol / mL/L	0.05	0	1.32%
Acetic Acid / mL/L	0.01	0	7.69%	Acetic Acid / mL/L	0.04	1.53	-1241.67%	Acetic Acid / mL/L	0	0.41	#DIV/0!
Sodium / mg/L	21.76	18	2084.26%	Sodium / mg/L	31.19	23.42	3041.60%	Sodium / mg/L	37.65	81.33	3517.57%
Potassium / mg/L	10.61	54	-76.39%	Potassium / mg/L	31.77	24.81	19.76%	Potassium / mg/L	139.64	116	11.00%
COD / mg/L	770	1200	-17.92%	COD / mg/L	1910	1985	-2.68%	COD / mg/L	1700	620	15.43%
24 m	C predicted	C Actual	Error Function (E)	24 m	C predicted	C Actual	Error Function (E)	24 m	C predicted	C Actual	Error Function (E)
Ethanol / mL/L	0	0	0.00%	Ethanol / mL/L	0.09	0	6.87%	Ethanol / mL/L	0	0	0.00%
Acetic Acid / mL/L	0	0	0.00%	Acetic Acid / mL/L	0.02	0	16.67%	Acetic Acid / mL/L	0	0.38	#DIV/0!
Sodium / mg/L	22.89	46.36	2052.71%	Sodium / mg/L	31.66	46.36	3012.79%	Sodium / mg/L	40.25	53.8	3861.32%
Potassium / mg/L	34.1	65.96	-56.09%	Potassium / mg/L	30.15	65.96	-101.68%	Potassium / mg/L	111.24	120	-4.07%
COD / mg/L	405	530	-5.21%	COD / mg/L	1563	1175	13.88%	COD / mg/L	745	530	3.07%
32 m	C predicted	C Actual	Error Function (E)	32 m	C predicted	C Actual	Error Function (E)	32 m	C predicted	C Actual	Error Function (E)
Ethanol / mL/L	0	0	0.00%	Ethanol / mL/L	0.03	0	2.29%	Ethanol / mL/L	0	0	0.00%
Acetic Acid / mL/L	0	0	0.00%	Acetic Acid / mL/L	0.01	0	8.33%	Acetic Acid / mL/L	0	0.44	#DIV/0!
Sodium / mg/L	24.09	29.3	2259.66%	Sodium / mg/L	32.14	26.83	3125.34%	Sodium / mg/L	43.02	32.1	4204.34%
Potassium / mg/L	28.6	29.6	-1.76%	Potassium / mg/L	28.62	20.16	24.02%	Potassium / mg/L	88.47	71.65	7.82%
COD / mg/L	210	0	8.75%	COD / mg/L	1280	470	28.98%	COD / mg/L	320	25	4.21%
40 m	C predicted	C Actual	Error Function (E)	40 m	C predicted	C Actual	Error Function (E)	40 m	C predicted	C Actual	Error Function (E)
Ethanol / mL/L	0	0	0.00%	Ethanol / mL/L	0.01	0	0.76%	Ethanol / mL/L	0	0	0.00%
Acetic Acid / mL/L	0	0	0.00%	Acetic Acid / mL/L	0	0	0.00%	Acetic Acid / mL/L	0	0	#DIV/0!
Sodium / mg/L	25.35	29.3	2385.66%	Sodium / mg/L	32.63	27.09	3173.48%	Sodium / mg/L	45.97	26.38	4516.74%
Potassium / mg/L	23.98	29.6	-9.89%	Potassium / mg/L	27.16	22.52	13.17%	Potassium / mg/L	70.3	52.05	8.49%
COD / mg/L	105	0	4.38%	COD / mg/L	1045	780	9.48%	COD / mg/L	15	25	-0.14%

It is possible to say that potassium is reduced over the course of the wetland and that sodium is not significantly affected. This is caused by the absorption of potassium by the plants, but not sodium. This may change if halophytic plants are used for the design. Alternatively, it is probably more sensible to avoid using caustic soda when operating the cellar. Uncertainty may also be caused by absorption, desorption and re-absorption down the wetland. This is typical behaviour in biological systems and is typically displayed in many drug trials (absorption, metabolism and excretion).

The data generated combined with the model may generate the following information:

- pH – pH rises rapidly and stabilises at approximately 7.5 to 8. This is a useful value in that effluent may be released into a river at this pH.
- Na – Sodium is not significantly affected.
- K – Potassium is reduced, although the model is not able to accurately predict the extent.
- Propanol, propionic acid, and butyric acid – these minor components of the organic constituents of the effluent are removed fully well before ethanol and acetic acid and can thus be neglected from modelling.
- Ethanol – this is the critical parameter – the model is reasonably accurate in determining the rate of removal – typical to a conversion of within approximately 15 percent of the real conversion. Ethanol conversion is under predicted – this is good for design purposes because it implies that the model is conservative. This means any resulting CW designed will be oversized.
- Acetic acid – this is a significant yet non-critical parameter. The overall rate of decay is accurate to within approximately 50%. On the 18th of March there was a significant value of acetic acid in sample port 2 (16m) which may be an inaccurate reading, or may reflect the degradation of ethanol to acetic acid. This rate of formation has not been included in this model and only an overall rate of degradation has been calculated. Further down the wetland, the acetic acid was fully consumed.
- COD – the accuracy of the COD degradation is less than that of the ethanol, but this is to be expected and has been explained earlier in this report. The conversion of COD is accurate to within 30%.

3.6 References

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CHAPTER 4
GENERAL SURVEY OF BOTANICAL FEATURES OF A WINERY WETLAND

4.1 Introduction

The plant population and distribution is an important aspect of any wetland because the plants contribute to the ecosystem, providing nutrients and surfaces, and modifying the water and soil composition (discussed below and in previous progress report, July 2004). In this project, the identification of the plants present in the natural wetlands that receives winery effluent is important because of the contribution they may make in the bioremediation of winery effluent.

There are a number of ways in which the plant communities affect wetland dynamics (2). The wetlands plants prevent blockage of the soil by root death; as roots grow, they create channels in the gravel matrix, and when the roots die, and decompose, create flow channels. Over the whole wetland, this should reduce or eliminate the blocking in the long term. Root death is also an important aspect for wetland microbiology in that the decomposition of roots also provides a source of carbon, nitrogen and micronutrients for the microbial communities. Plants also provide the matrix and associated microorganisms with oxygen – either through parenchyma cells, or through active transport. *Phragmites australis* has been shown to be able to transport up to 10 mℓ/min of oxygen anywhere in the plant. Plants are responsible for the absorption of inorganic elements in the effluent. These inorganic components include nitrogen (in all its associated forms), phosphorous and ionic species including, in some cases, salt. Plants have also been shown to consume organic components of the effluent; phenols are degraded via catechol and further meta-ring cleavage by microorganisms, producing low molecular weight organic aliphatic compounds, which then become available to the plants. Plants may be responsible for inhibition of some pathogen growth. It remains unclear as to how this occurs (2), since certain microbes are encouraged, and others are discouraged, simultaneously.

4.2 Methods

In the summer season survey, plant material was collected on 16 February 2005. Representative samples were selected over the whole area of the natural wetland. Plants were selected for sampling on the basis of visible assessment of the most prolific species. Leaves and roots were collected where possible, and for those plants with flowering material, flowers were also collected to aid identification. The plant specimens were refrigerated and transported within hours of collection, maintained at 4^{o}C and sent for identification the following day. Plant specimens were pressed, and voucher specimens will be obtained to

authenticate the results for the purpose of data publication. Specimens were identified at the Bolus Herbarium at the Department of Botany, University of Cape Town.

This information has been collated into a GPS mapping system showing the physical location of the sampling points in the marsh and the areas where the plants occur.

4.3 Results

4.3.1 Plant distribution

The location and distribution of plants in the wetland is given in Figure 4.1.

- Region 1: *Carex* (species unknown, more flowering material required for accurate identification)
- Region 2: *Epibolium hirstutum* L. (Great Willowherb: Cape to West Asia)
- Region 3: *Pesicaria decipiens* (R. Br.) Wilson – Widespread
- Region 4: *Typha capensis* (Rohrb.) N.E. Br. (Bullrush: South and Tropical Africa)
- Region 5: *Phytolacca octandra* L. (Inkberry)
- Region 6: *Juncu effuse hochstra* (Rush, Biesie: Cape to Mozambique)
- Region 7: *Pennisetum macrourum trin*. (Beddingras: Bokkeveld to Tropical Africa)
- Region 8: *Zantedeschia aethiopica* (Arum Lilies: Western Cape through the Eastern Cape, KwaZulu-Natal, Mpumalanga and into the Northern Province)

4.4 Discussion – implications for wetland design

Of the plant species catalogued, *Typha capensis* and *Zantedeschia aethiopica* are most commonly found in wetlands, based on visual observation of many natural wetlands in the Western Cape. *Zantedeschia aethiopica* has been used in a lab scale Constructed Wetland (CW) by others (1) with varying degrees of success in terms of bioremediation; *viz*. a significant reduction in nitrogenous chemicals was observed in this study, but the COD of the effluent was not decreased. However, this report was not based on remediation of winery effluent. In our wine wetland study, we may expect a reduction in the COD since the winery effluent has a larger proportion of organic components. To our knowledge, there are no other wetlands studies using South African plants and thus direct comparison is not possible.

In the natural wetland under investigation in this project, a fluid flow channel (depicted with a red arrow line shown in Figure 4.2) is predominated by *Zantedeschia aethiopica* (Arum Lilies). It is apparent that this plant is well suited to winery effluent remediation because the plant growth is visibly healthy, which suggests that these plants are utilising nutrients from the effluent and that the nutrients are beneficial to arum lilies.

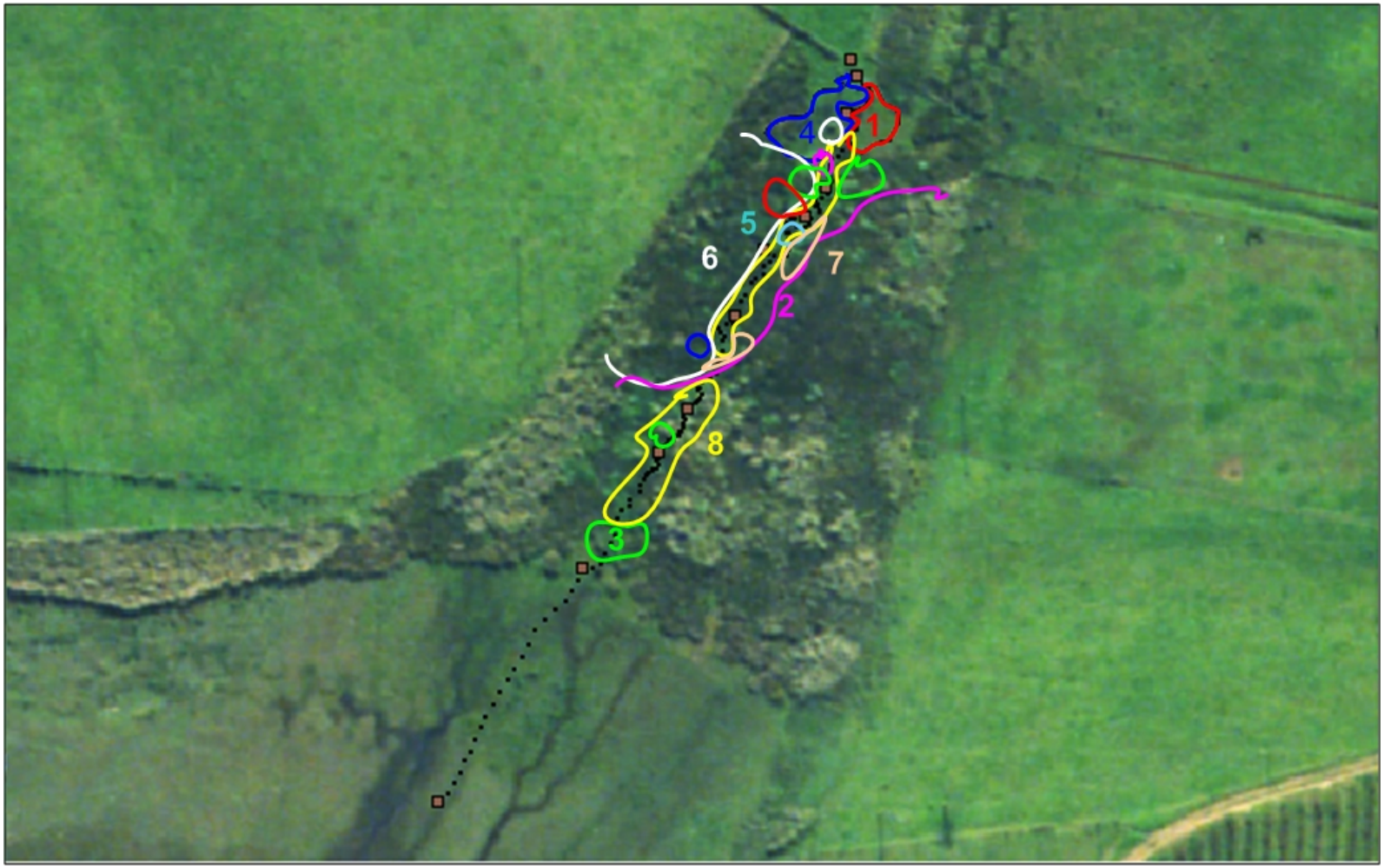


Figure 4.1 - Predominant plant species observed in natural wetlands

Figure 4.2 – Arum lilies growing along the nutrient path/flow channel

All the plant species identified are indigenous to South Africa. This indicates that the use of indigenous plants in constructed wetlands is feasible, and also that some of these plants may be harvested and sold as ornamental plants e.g. arum lilies and some restios.

The variation of plant species distribution along the length of the wetland is likely be related to the composition of the effluent. For example, at the inflow, the effluent has a high COD and a high concentration of organic components, and these gradually decrease down the length of the wetland. Similarly, the dissolved inorganic components may increase down the wetland due to evapo-transpiration.

4.5 Conclusions

Based on the findings of the botanical survey, it is recommended that the constructed wetland be sub-divided into regions. The length of the wetland should be divided into four equal regions. The first region should be planted with a combination of *Carex* (common sedge) and *Zantedeschia aethiopica* (Arum Lily). The second region should be planted with *Zantedeschia aethiopica* (Arum Lily) and *Typha capensis* (Bullrush). Region three should be planted with Arum lilies and Region 4 should be planted with *Pesicaria decipiens*. This would provide for a greater degree of plant biodiversity, which would in turn provide increased bio-remediation. These plants are known to be resistant to winery effluent and

are indigenous. Any other indigenous wetland plants may also be interspersed within this system as an experiment during the construction of CWs especially for winery effluent remediation.

4.6 References

1. **Belmont, M. & Metcalfe, C.** (2003). Feasibility of using ornamental plants (*Zantedischia aetiopica*) in subsurface flow treatment wetlands to remove nitrogen, COD and nonylphenol ethoxylate surfactants – a laboratory scale study. *Ecological Engineering* **21**: 233 – 247.
2. **Stottmeister, U., Kuschk, A., Kappelmeyer, U., Kästener, M., Müller, R. & Moorman, H.** (2003). Effects of plants and microorganisms in constructed wetlands for wastewater treatment. *Biotechnology Advances* **22**: 93 – 117.

CHAPTER 5
INVESTIGATION OF BIOCHEMICAL ACTIVITY DETECTION IN WETLANDS

5.1 Introduction

Wetlands, as in all ecological systems, show a high diversity in their microbiota. In cases where wetlands, natural or constructed, are used for the disposal of wastewater generated from industrial and agricultural processes, an understanding of the microbiota and enzyme processes found is crucial. Monitoring the changes in microbial diversity and enzyme processes over time in such wetlands allows for a better understanding of the mechanisms of bioremediation of such wastewater.

Monitoring enzyme activity in natural systems provides insight into the degradative processes taking place. However, often the levels of such enzymes are extremely low and may be beyond the detection limits of published methods. In such cases these methods need to be manipulated in order to optimise them for the detection of low levels of enzyme activity. This may be done by changing the ratio of enzyme to substrate or assay reaction mixture. These ratios may be changed by increasing the sample volume, decreasing the assay reaction mixture volume, or a combination of the two. Enzyme assays may also be manipulated by concentrating the proteins found in samples in order to make the enzyme activity easier to detect. This may be done by means of freeze-drying, dialysis and filtering the sample through a selectively permeable membrane. However, methods such as freeze-drying may denature the enzymes so that, although protein recovery is high, the enzyme activity observed is lower than expected.

Water and soil samples were collected from a local winery where there is a natural wetland (NW), and from a constructed wetland (CW) at ARC, Nietvoorbij. Samples of gravel and water were also collected from the model-constructed wetland maintained at the Bioprocess Engineering Research Unit, Chemical Engineering, University of Cape Town. Enrichment cultures were produced from these latter samples and the intracellular enzymes assayed for activity. In the case of the wetland samples, the activities found were correlated with the bacterial species identified in Section 6. The ratio of sample to assay reaction mixture was manipulated during these assays in order to determine which ratio produced the best enzyme activity. Enrichment samples from the constructed wetland were used in the manipulations of assay reaction mix ratios. A portion of these samples was also concentrated (using freeze-drying, dialysis and filtration) and assays were carried out using the optimal ratio observed. A further laccase assay was evaluated as a comparison with the ABTS laccase assay, which showed anomalous bleaching previously. A new method of directly assaying the soil samples was also tested.

5.2 Methods

5.2.1 Methods for sampling, sample preparation and culture

Water samples were collected from CW from enclosed ports within the constructed wetland. Water samples from NW were collected from demarcated areas (sample ports) in the natural wetland. These were collected from below surface level. It was previously discovered that bacterial growth in enrichment cultures, and therefore enzyme activity, is not significantly affected by the depth from which the samples are collected. In both cases the water samples were collected in sterile 15 mℓ plastic tubes and transported in a cooler box. Upon reaching UCT, the samples were placed in a 2°C refrigerator until processing.

Soil samples and soil/gravel samples were also collected from NW and CW respectively. Samples were collected from previously designated areas and stored in Zip-lock bags at 2°C until processed. A total of 8 samples and 6 samples were collected from CW and NW respectively over the time period of 10 February 2006 to 03 April 2006. All samples were collected at a depth of 5 – 10 cm.

A model-constructed wetland is maintained in the Bioprocess Engineering Research Unit, Chemical Engineering, University of Cape Town. Samples of gravel and water were collected from this model wetland was used in the analysis of the use of enrichment cultures**,** a 1 mℓ of 0.1% Tween 80 was added to each sample and the samples were vortexed for five minutes. 10 mℓ of each sample was added to 100 mℓ of enrichment broth.

Enrichment cultures were carried out in 100 mℓ of nutrient broth (Biolab) and 100 mℓ of malt extract broth (2% malt extract, 2% glucose, 0.1% bacteriological peptone). These volumes were dispensed into Ehrlenmeyer flasks and 10 mℓ of inoculums was added. The cultures were shaken at 150 rpm and 37ºC for 48 hours. The culture was centrifuged and both the supernatant and the cellular pellet retained. The pellet was resuspended in 5 mℓ 0.05M potassium phosphate buffer (pH 6.8) and sonicated. This sonicate was then microfuged and the supernatant retained. The pellet was resuspended in 250µℓ 0.05M potassium phosphate buffer (pH 6.8). This was placed at -20°C overnight.

In standard McCartney containers (approximately 25 mℓ total volume capacity), approximately 2 – 3 mℓ of soil/gravel was mixed with 600 µℓ of 1% Tween 80 and 5.4 mℓ of reagents required for specific enzyme assays. An additional container per sample and assay contained all the components except soil – to be used as a background control. Initially a total volume of 3 mℓ of reagents was tested, but particulate matter in the samples made it difficult to collect samples at different intervals, therefore the final volume was increased to 6 mℓ. Samples were also incubated with 5.4 mℓ sterile water and 600 µℓ of 1% Tween 80 to determine the total protein concentration in the samples.

Each soil sample was vortexed for 5 minutes to ensure release of bacterial cells from soil particles and incubated at room temperature (approximately 22 - 23°C) on a rocking shaker. Containers were

covered with foil (incubation in the dark) to prevent light oxidation of the substrates used. 1 mℓ of each sample was removed directly after the samples were vortexed and the supernatant fluid collected by centrifugation at 10 000 rpm for 5 minutes (these samples were designated t = 0). Further samples were collected after 3 hours (t = 3) and 24 hours (t = 24) of incubation. Samples collected were stored at -20°C until analysed.

Enzyme activity tested for, included laccase (using ABTS and guaiacol as substrates), tyrosinase, peroxidase, alcohol dehydrogenase, glucose oxidase, glucose-6-phosphate dehydrogenase, esterase, lipase, catechol 1,2-dioxygenase and catechol 2,3-dioxygenase.

The absorbance of the samples was determined in duplicate at the specified wavelengths indicated for the different assays. Samples were not concentrated prior to determination of absorbance. Absorbance readings were determined using a Unicam, Helios α UV/visible spectrophotometer. The change in absorbance per minute was determined for each sample. Activity in U/mℓ could not be determined due to the unknown volume of the soil/gravel samples used.

Due to the low levels of enzyme activity previously detected it was decided to manipulate the assay reaction mixture volumes of the assay. In most assays the ratio of assay reaction mixture to enzyme is 30:1. The assay reaction mixture was altered, retaining the ratios of components as in the original assays, to 9:1, 4:1, 3.5:1.5, 3:2 and 1:1 (Table 5.1). All other assay conditions were maintained.

Table 5.1 - Lists the samples used in the assay manipulations. The code shows which enrichment medium was used and the amount of sample and assay reaction mixture.

Sample	Code
N1	100 µℓ nutrient broth sonication pellet + 900 µℓ assay reaction mixture
N2	200 µℓ nutrient broth sonication pellet + 800 µℓ assay reaction mixture
N3	300 µℓ nutrient broth sonication pellet + 700 µℓ assay reaction mixture
N4	400 µℓ nutrient broth sonication pellet + 600 µℓ assay reaction mixture
N5	500 µℓ nutrient broth sonication pellet + 500 µℓ assay reaction mixture
M1	100 µℓ malt extract sonication pellet + 900 µℓ assay reaction mixture
M2	200 µℓ malt extract sonication pellet + 800 µℓ assay reaction mixture
M3	300 µℓ malt extract sonication pellet + 700 µℓ assay reaction mixture
M4	400 µℓ malt extract sonication pellet + 600 µℓ assay reaction mixture
M5	500 µℓ malt extract sonication pellet + 500 µℓ assay reaction mixture

A single port 0 sample, from the constructed wetland, was divided equally into volumes of 170 mℓ. These portions were concentrated by means of freeze-drying, dialysis and Amicon Ultra Centrifugal Filter Devices (1) (hereafter referred to as centrifugation). Dialysis was done using 20% polyethylene glycol (PEG). All samples were resuspended in 2 mℓ 50mM potassium phosphate buffer. Assays were done using a ratio of 9:1 assay reaction mixture and sample.

Enzyme activity observed was correlated with data on bacterial and archaeal genera represented in soil samples (data obtained from Section 6).

5.2.2 Methods of enzyme activity measurement

Laccase Assay: 2.5 mℓ of a 0.1M sodium acetate buffer (pH 5.0) was added to 0.33 mℓ of a 5mM ABTS solution. To this, 0.17 mℓ of enzyme solution was added and the reaction was read at 420nm for 80 seconds. This assay was compared with laccase oxidation of guaiacol (at the same concentration) measured at 470nm for three minutes (9).

Polyphenol Oxidase (Tyrosinase) Assay: A solution of 10mM DOPA was made up in 50mM potassium phosphate buffer (pH 6.0). 0.1 mℓ of enzyme solution was added to 2.9 mℓ of the DOPA buffer and the reaction was read 475nm for 10 minutes (6).

Peroxidase Assay: 1.4 mℓ of 0.0017M hydrogen peroxide solution, was prepared in 0.2M potassium phosphate buffer (pH 7.0), was added to 1.5 mℓ of a 0.0025M 4-aminoantipyridine / 0.17M phenol solution. 0.1 mℓ of enzyme solution was added to this and the reaction was read at 510nm for 5 minutes (48).

Glucose oxidase assay: 50mg *o*-Dianisidine was dissolved in 7.55 mℓ distilled water. 1 mℓ of this solution was added to 100 mℓ 0.1M potassium phosphate buffer (pH 7.0) which was sparged with compressed air for 10 minutes prior to use. 2.5 mℓ of this solution was added to 0.5 mℓ 0.55M glucose solution and 0.01 mℓ 50U.mℓ$^{-1}$ peroxidase solution. 0.05 mℓ of the enzyme solution was added to this and the reaction was read at 436nm for 5 minutes (40).

Esterase: Esterase was assayed for by means of the method of Gupta *et al.* (16).

Lipase assay: Solution A - 0.02g *p*-Nitrophenyl butyrate in 1 mℓ isopropanol; Solution B - 0.01g sodium cholate and 0.01g Gum Arabic dissolved in 8 mℓ phosphate buffer (5mM, pH 8.0). Mix by vortexing and add 0.2 mℓ Trixton X100. Make the volume to 9 mℓ with buffer - if not fully dissolved, microwave for 5 seconds. The substrate solution consists of 1 mℓ Solution A + 9 mℓ solution B (stable for a week at 4^{o}C). For the assay, 0.1 mℓ enzyme solution was added to 2.4 mℓ substrate solution and the reaction was read at 410 nm for three minutes.

Alcohol dehydrogenase: 1.5 mℓ of 0.1M sodium pyrophosphate buffer (pH 9.2) was added to 0.5 mℓ 2M ethanol, 0.1 mℓ 0.0015M NAD, 0.1 mℓ 1% BSA and 0.1 mℓ of the enzyme solution. The reaction was read at 340 nm for 4 minutes.

Glucose-6-phosphate dehydrogenase: 2.7 mℓ of 0.055 M Tris-HCl buffer (pH 7.8), to which 0.0033 M $MgCl_2$ had been added, was mixed with 0.1 mℓ 0.006M NADP, 0.1 mℓ 0.1 M glucose-6-phosphate and 0.1 mℓ of the enzyme solution. The reaction was read at 340 nm for 5 minutes (48).

Catechol 1,2-dioxygenase and catechol 2,3-dioxygenase assay: A solution of 0.5 mM catechol was made up in 50 mM potassium phosphate buffer (pH 7.2). 0.1 mℓ of enzyme solution was added to 2.9 mℓ of the catechol buffer.

For catechol 1,2-dioxygenase: Increase in absorbance at 260 nm monitored for the formation of *cis*-muconic acid ($\varepsilon_M = 1.75 \times 10^3$ mol^{-1} 1 cm)

For catechol 2,3-dioxygenase: Increase in absorbance at 375 nm monitored for the production of 2-hydroxymuconic semialdehyde ($\varepsilon_M = 4.4 \times 10^4$ mol^{-1} 1 cm)

Bradford's (protein) Assay: 0.9 mℓ of Coomasie Brilliant Blue solution was added 0.1 mℓ of enzyme solution. This was mixed and allowed to stand for 5 minutes before being read at 595 nm (7).

5.3 Results of measurements over two seasons

Table 5.2 shows the samples represented in the graphs later in the document, with all of the sample codes. The codes may be divided up into:

- the wetland from which the samples were collected (C or N)
- the port from which the sample was collected (1-6)
- the enrichment medium used (nutrient broth or malt extract)

Table 5.2 - Lists the samples produced from the enrichments. The code shows which wetland the sample was collected from, which port and which enrichment medium was used.

Sample	Code
C1NB	Constructed, port 1, nutrient
C1ME	Constructed, port 1, malt extract
C2NB	Constructed, port 2, nutrient
C2ME	Constructed, port 2, malt extract
C3NB	Constructed, port 3, nutrient
C3ME	Constructed, port 3, malt extract
C4NB	Constructed, port 4, nutrient
C4ME	Constructed, port 4, malt extract
C5NB	Constructed, port 5, nutrient
C5ME	Constructed, port 5, malt extract
N1NB	Natural, port 1, nutrient broth
N1ME	Natural, port 1, malt extract
N2NB	Natural, port 2, nutrient broth
N2ME	Natural, port 2, malt extract
N5NB	Natural, port 5, nutrient broth
N5ME	Natural, port 5, malt extract
N6NB	Natural, port 6, nutrient broth
N6ME	Natural, port 6, malt extract

5.3.1 Laccase

Due to the bleaching of ABTS observed during the laccase assays carried out previously another substrate needed to be tested. This was done to determine whether or not the bleaching was due to the ABTS or a component found in the samples. Guaiacol was selected and tested with crudely purified laccase extract from a *Trametes pubescens* strain. The reaction of laccase with guaiacol was slower than with ABTS and the activity observed was lower at the same enzyme concentration (Figure 5.1). Activity increased up to three minutes, after which the rate of activity levelled off. Therefore the reaction was only run for 3 minutes. Guaiacol was tested at concentrations of 5mM, 2.5mM and 1mM to determine the effect of substrate concentration on activity. No difference in activity was observed at the different substrate concentrations. Due to the fact that the activity observed using guaiacol as a substrate was lower than that of ABTS, and that the bleaching observed did not appear to be due to the ABTS, it was decided to continue using ABTS as a substrate for the laccase assay.

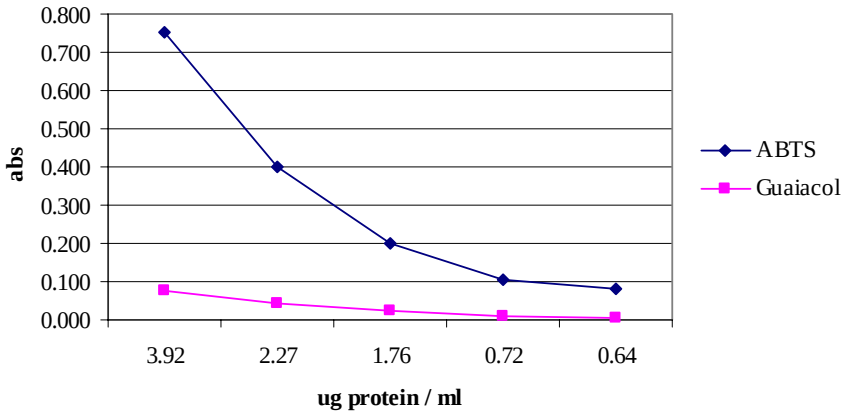
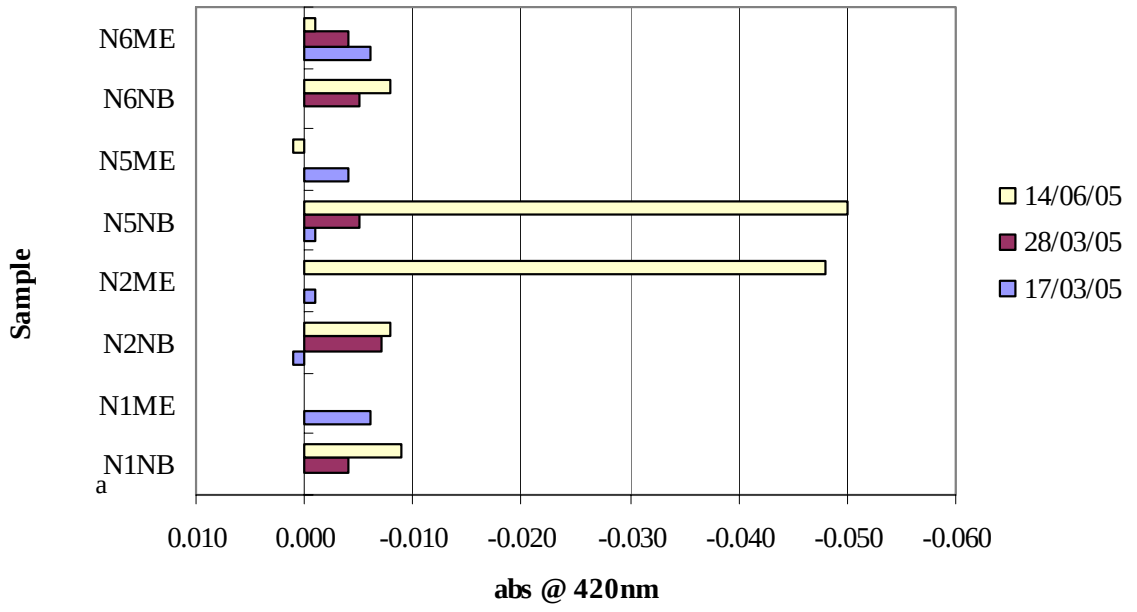


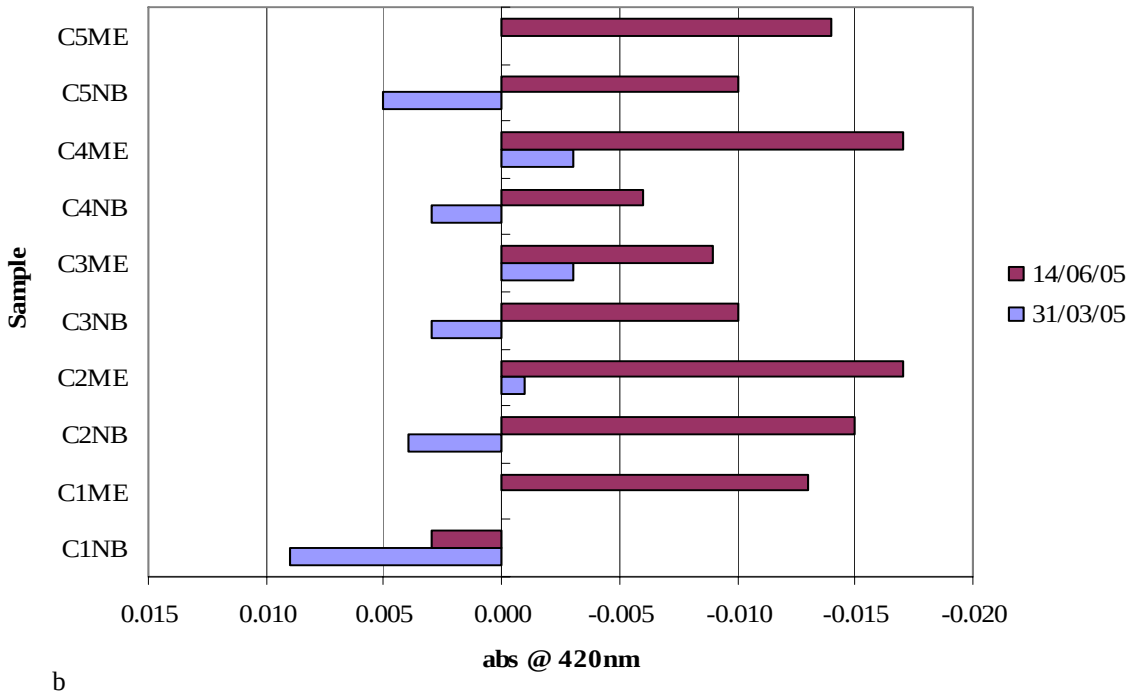
Figure 5.1 - The change in laccase activity, with changing enzyme concentration, shown by ABTS and guaiacol.

Samples collected from CW and NW on 14/06/2005 were enriched using nutrient broth and malt extract broth. The laccase assay was performed on the intracellular enzymes extracted from these enrichment cultures. This was done to determine whether or not any significant microbial changes had occurred in the extended period since the last samples were collected. Figure 5.2 represents a comparison in activity for laccase between the samples collected on 14/06/2005 and those collected on 31/03/2005 (CW) and 17/03/2005 and 28/03/2005 (NW). As in the previous report, no trends may be observed with regard to the changes in enzyme activity over time and through the two wetlands.

Once again, bleaching of ABTS was noted during the laccase assay. This bleaching was more marked, from the samples of both wetlands, than in previous samples. This is clearly demonstrated in Figure 5.2. The assay was repeated using guaiacol as a substrate, but almost no enzyme activity was detected (Table 5.3). No bleaching of the guaiacol was observed.



a



b

Figure 5.2 - Comparison of the change in absorbance for the laccase assay between samples collected on 14/06/2005 and those collected on a) 17/03/2005 and 28/03/2005 (NW) and b) 31/03/2005 (CW). The substrate used was ABTS.

Table 5.3 - The rates of activity observed for different concentrations of guaiacol and enzyme. No difference in activity was observed between the different substrate and enzyme concentrations.

		Sample 1		Sample 2	
[guaiacol]	**[enzyme]**	**Abs:**	**Activity:**	**Abs:**	**Activity:**
5mM	0.080	0.059	0.039	0.054	0.035
	0.062	0.034	0.022	0.034	0.022
2.5mM	0.080	0.059	0.039	0.035	0.022
	0.062	0.037	0.024	0.037	0.024
1mM	0.080	0.060	0.039	0.060	0.039
	0.062	0.034	0.022	0.034	0.022

Due to the low protein concentration and enzyme activity observed thus far, it was decided to manipulate the volume of assay reaction mixture in order to decrease the ratio of sample / enzyme to substrate. Figure 5.3 shows the changes in absorbance for the laccase assay. The change in absorbance increases with a decrease in ratio between assay reaction mixture and sample.

Concentrated samples (freeze-dried, dialysised and centrifuged samples) were analysed for activity. From Figure 5.4, it is clear that centrifugation resulted in the highest laccase activity when ABTS was used as the substrate.

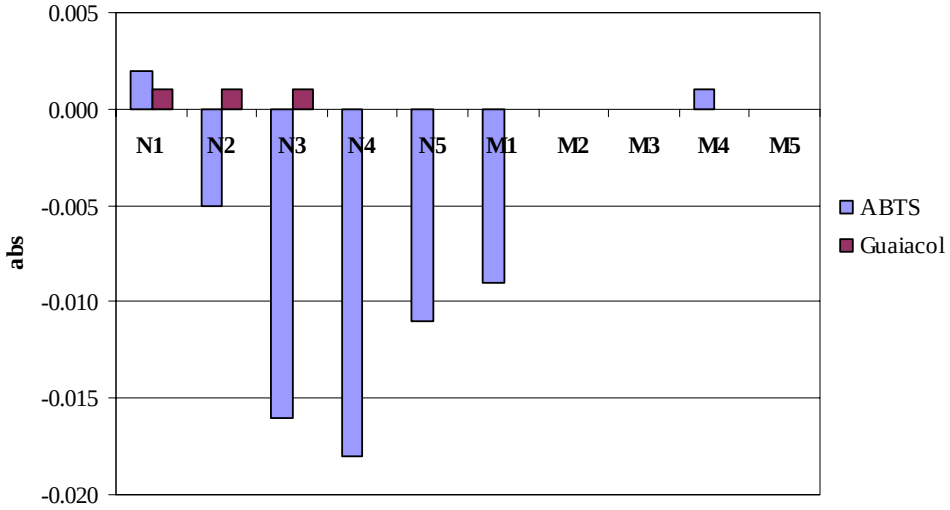


Figure 5.3 - The changes in absorbance observed for ABTS and guaiacol in the laccase assay using various ratios of assay reaction mixture and sample.

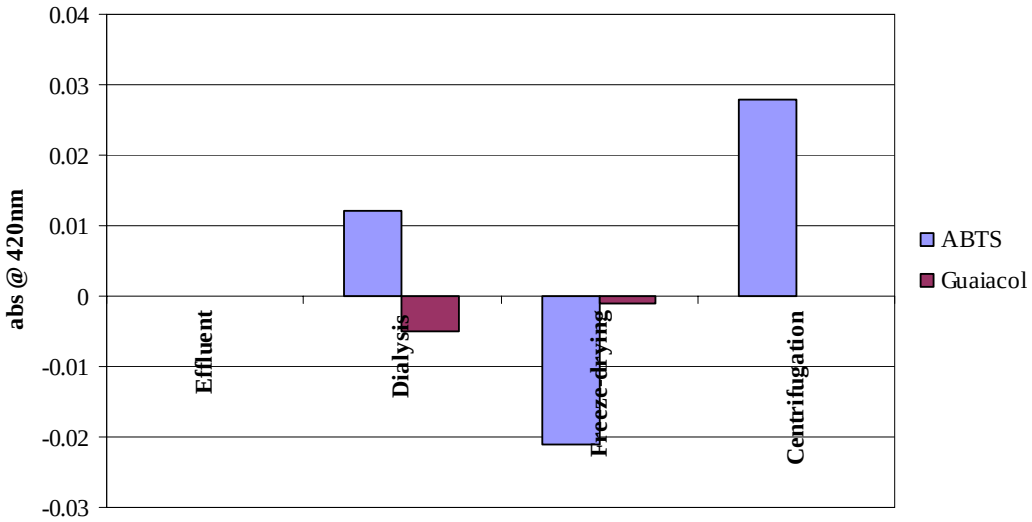


Figure 5.4 - The change in absorbance observed for the laccase assay, using ABTS and guaiacol, before and after concentration of the effluent sample.

Soil samples collected from CW and NW over the time period of 10 February 2006 to 03 April 2006, was tested for the same enzyme activity as all the previous samples, with the exception that the substrate solutions were added directly to the soil and absorbance readings taken directly at time = 0, time = 3hrs and time = 24hrs. Direct processing of the soil samples did not show any bleaching when ABTS was used as the substrate for the laccase assay. Both ABTS and guaiacol was therefore used in the direct analysis of the soil samples analysed (Figure 5.5 and Figure 5.6).

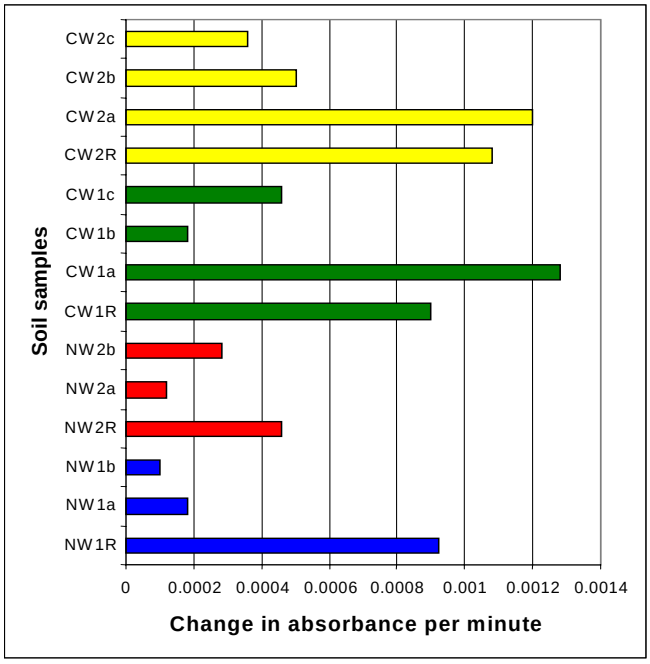


Figure 5.5 - Comparison of the change in absorbance per minute for the laccase assay (ABTS as substrate) between samples collected from CW and NW over the time period of 10 February 2006 to 03 April 2006, where R = reference sample collected on 10 February 2006, a = collected on 17 February, b = collected on 25 February 2006 (except for NW1b and NW2b which was collected on 03 April 2006), c = collected on 03 March 2006.

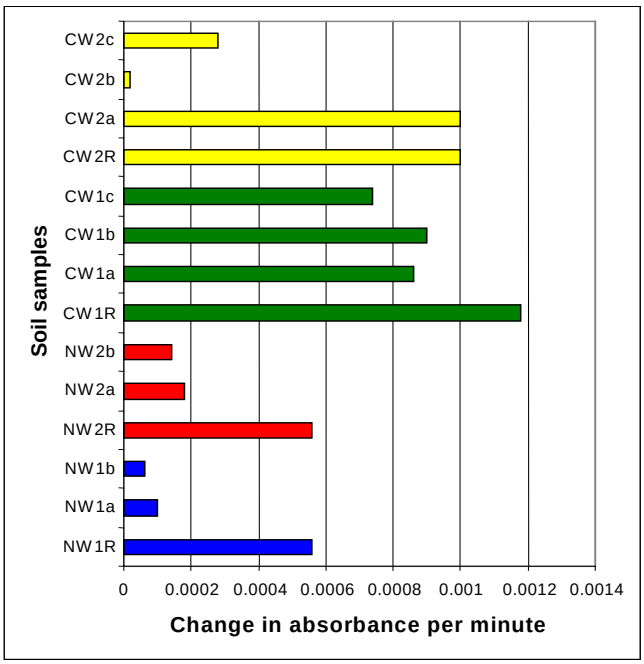


Figure 5.6 - Comparison of the change in absorbance per minute for the laccase assay (guaiacol as substrate) between samples collected from CW and NW over the time period of 10 February 2006 to 03 April 2006, where R = reference sample collected on 10 February 2006, a = collected on 17 February, b = collected on 25 February 2006 (except for NW1b and NW2b which was collected on 03 April 2006), c = collected on 03 March 2006.

5.3.2 Alcohol dehydrogenase

Intracellular enzymes extracted from enrichment cultures were screened for alcohol dehydrogenase, polyphenol oxidase, peroxidase, esterase, glucose-6-phosphate dehydrogenase and glucose oxidase activities. This was done to determine whether or not any significant microbial changes had occurred in the extended period since the last samples were collected. Once again, no polyphenol oxidase (PPO), peroxidase (Perox) or glucose oxidase (GluOx) activity was observed; laccase activity and total protein concentration are also included (Table 5.4).

Table 5.4 - The activities observed for the various assays, using the intracellular enzyme extract from the enrichment cultures of the water samples from CW and NW. Due to the bleaching of ABTS during the laccase assay, the assay was repeated using guaiacol.

	[protein]	ABTS*	Guaiacol	PPO	ADH	Esterase	Perox	G-6-PD	GluOx
C1NB	37.458	0.003	0.001	0.000	0.002	0.026	0.000	0.005	0.000
C1ME	31.390	-0.013	0.000	0.000	0.000	0.038	0.000	0.045	0.000
C2NB	36.587	-0.015	0.000	0.000	0.000	0.014	0.000	0.005	0.000
C2ME	30.211	-0.017	0.000	0.000	0.004	0.003	0.000	0.040	0.000
C3NB	36.278	-0.010	0.000	0.000	0.000	0.017	0.000	0.006	0.000
C3ME	28.188	-0.009	0.000	0.000	0.001	0.005	0.000	0.016	0.000
C4NB	36.812	-0.006	0.000	0.000	0.000	0.018	0.000	0.003	0.000
C4ME	24.480	-0.017	0.000	0.000	0.000	0.002	0.000	0.008	0.000
C5NB	35.520	-0.010	0.000	0.000	0.000	0.029	0.000	0.000	0.000
C5ME	27.486	-0.014	0.000	0.000	0.003	0.003	0.000	0.033	0.000
N1NB	36.840	-0.009	0.000	0.000	0.001	0.010	0.000	0.007	0.000
N1ME	28.722	0.000	0.000	0.000	0.004	0.005	0.000	0.034	0.000
N2NB	36.110	-0.008	0.000	0.000	0.000	0.010	0.000	0.006	0.000
N2ME	24.649	-0.048	0.000	0.000	0.002	0.036	0.000	0.011	0.000
N5NB	34.817	-0.050	0.000	0.000	0.000	0.015	0.000	0.010	0.000
N5ME	35.660	0.001	0.000	0.000	0.000	0.016	0.000	0.003	0.000
N6NB	36.840	-0.008	0.000	0.000	0.000	0.009	0.000	0.000	0.000
N6ME	32.992	-0.001	0.000	0.000	0.011	0.017	0.000	0.020	0.000

* change in absorbance not activity. ADH = alcohol dehydrogenase; G-6-PD = glucose-6-phosphate dehydrogenase.

Figure 5.7 represents a comparison in activity for alcohol dehydrogenase between the samples collected on 14/06/2005 and those collected on 31/03/2005 (CW) and 17/03/2005 and 28/03/2005 (NW). As in the previous report, no trends may be observed with regard to the changes in enzyme activity over time and through the two wetlands.

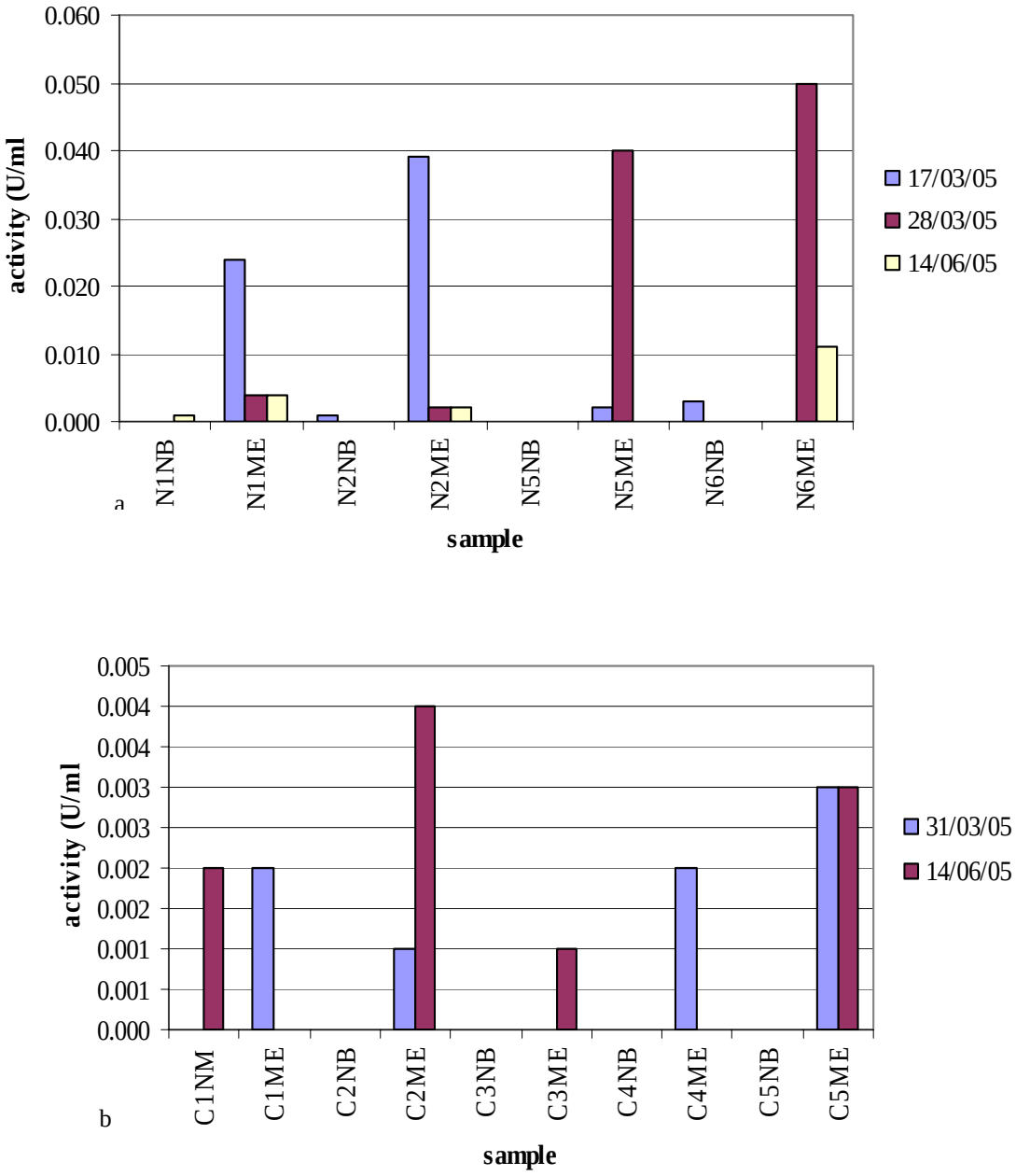


Figure 5.7 - Comparison of the change in activity for the alcohol dehydrogenase assay between samples collected on 14/06/2005 and those collected on a) 17/03/2005 and 28/03/2005 (NW) and b) 31/03/2005 (CW).

No alcohol dehydrogenase activity was observed when the ratio of assay reaction mix to sample was manipulated. Activity was however detected when effluent samples were concentrated using dialysis, but not when the samples were freeze-dried or concentrated by centrifugation. The direct soil assay technique showed a clear trend of increased alcohol dehydrogenase activity, especially in samples taken after an influx of winery wastewater (the 1a and 2a samples – Figure 5.8).

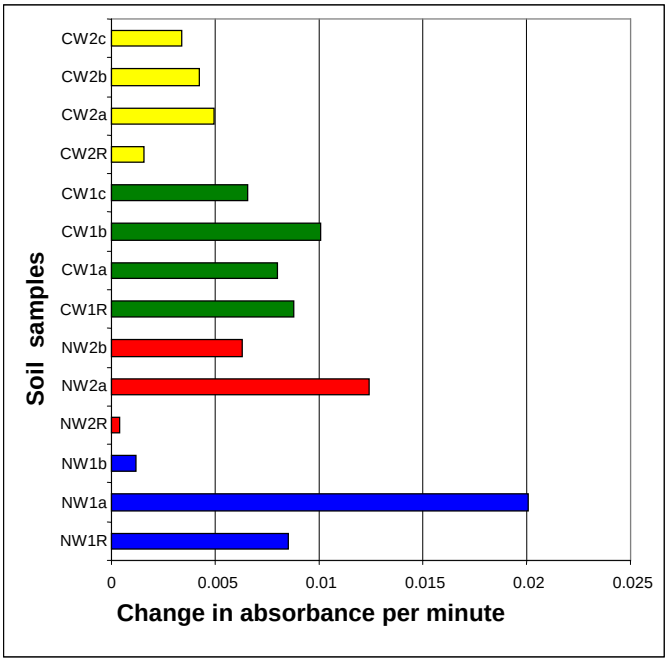


Figure 5.8 - Comparison of the change in absorbance per minute for the alcohol dehydrogenase assay between samples collected from CW and NW over the time period of 10 February 2006 to 03 April 2006, where R = reference sample collected on 10 February 2006, a = collected on 17 February, b = collected on 25 February 2006 (except for NW1b and NW2b which was collected on 03 April 2006), c = collected on 03 March 2006.

5.3.3 Esterase

Esterase activity was detected in the intracellular enzyme extraction from enrichment cultures (Figure 5.9). Esterase activity was also clearly affected by changes in the ratios of assay reaction mixture to sample, with the best activity detected for the 9:1 ratio (Figure 5.10). No esterase activity was detected with the direct assay technique. An increase in colour intensity was observed, but the background control sample showed a similar increase in intensity – possibly due to other components in the soil samples that were affecting the substrate used in this assay.

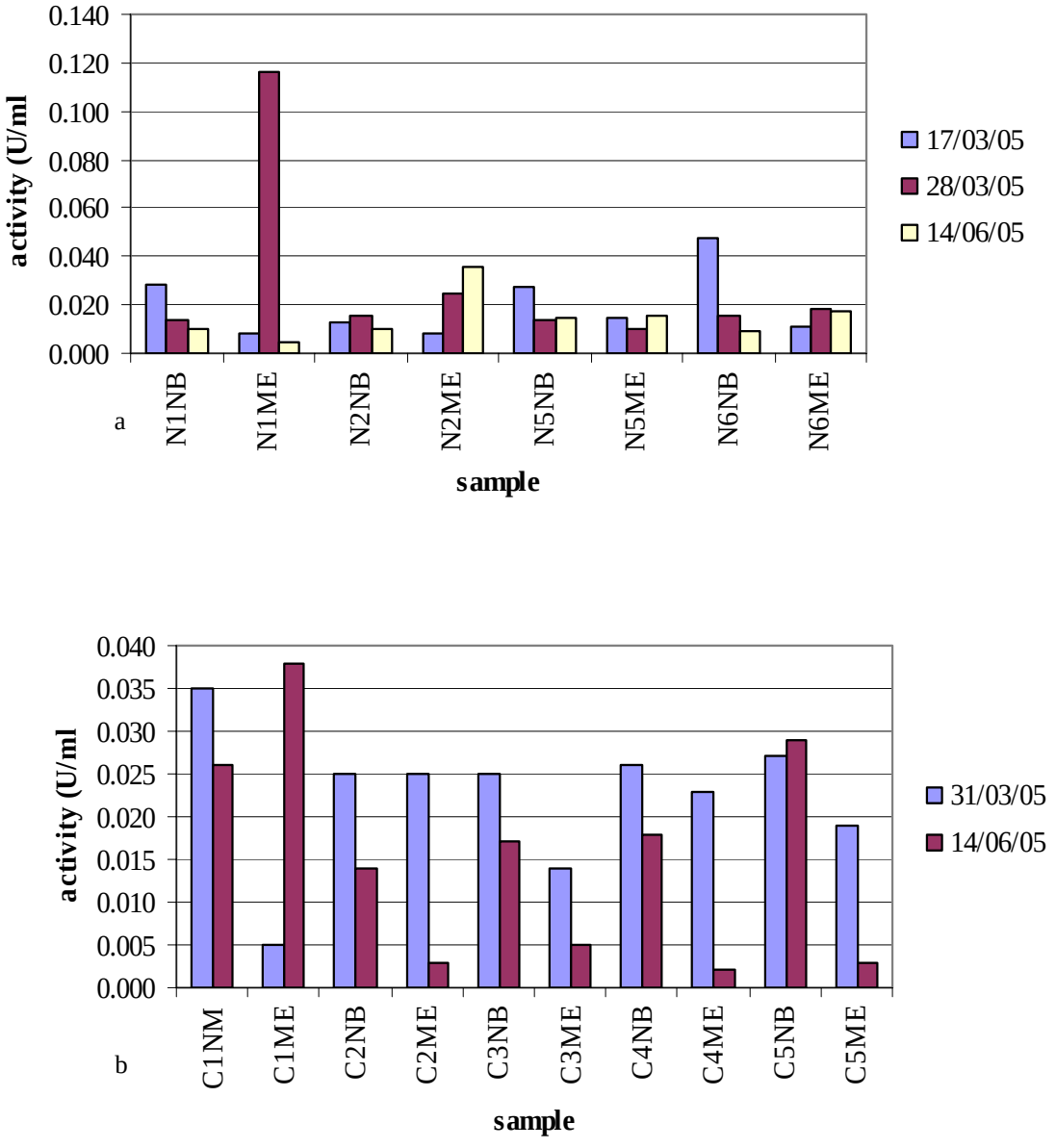


Figure 5.9 - Comparison of the change in activity for the esterase assay between samples collected on 14/06/2005 and those collected on a) 17/03/2005 and 28/03/2005 (NW) and b) 31/03/2005 (CW).

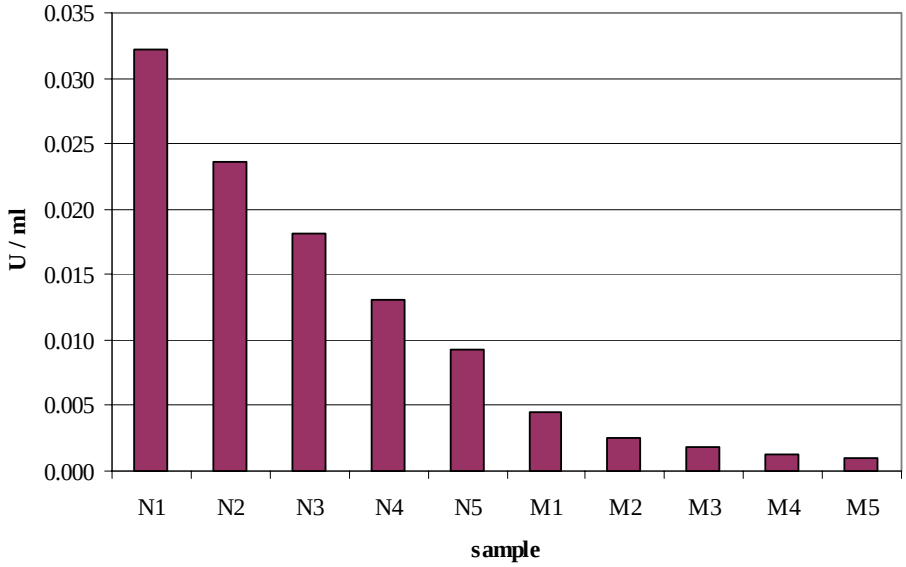
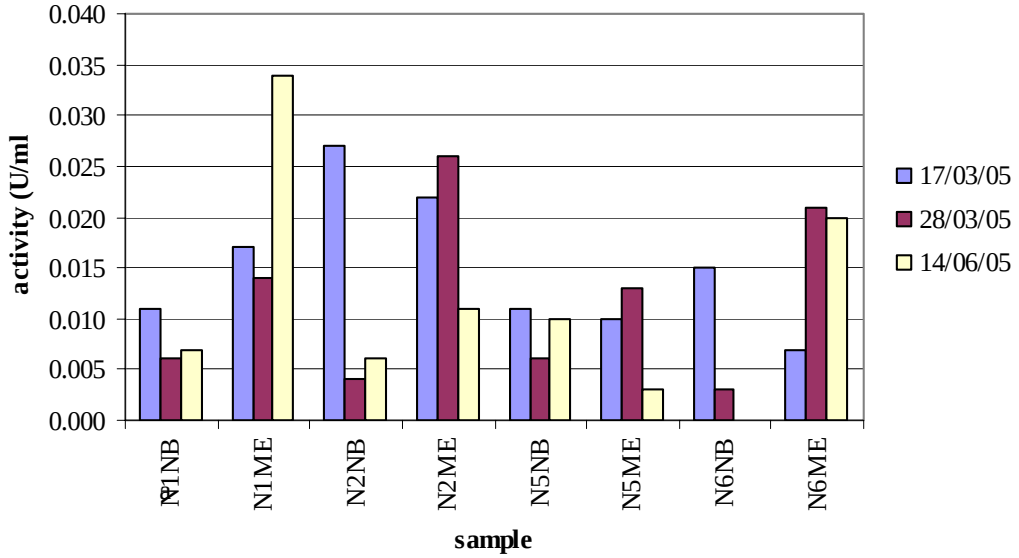


Figure 5.10 - The changes in activity observed for the esterase assay using various ratios of assay reaction mixture and sample.

5.3.4 Glucose-6-phosphate dehydrogenase

Intracellular glucose-6-phosphate dehydrogenase activity was detected in the sonicated enrichment cultures (Figure 5.11) and activity is clearly affected by the manipulation of the assay reaction mix to sample ratio as can be seen in Figure 5.12. The direct soil assay confirmed the presence of glucose-6-phosphate dehydrogenase, but no clear trends were observed (Figure 5.13). It was determined that concentration by centrifugation would increase the chances for glucose-6-phosphate dehydrogenase activity in effluent samples.



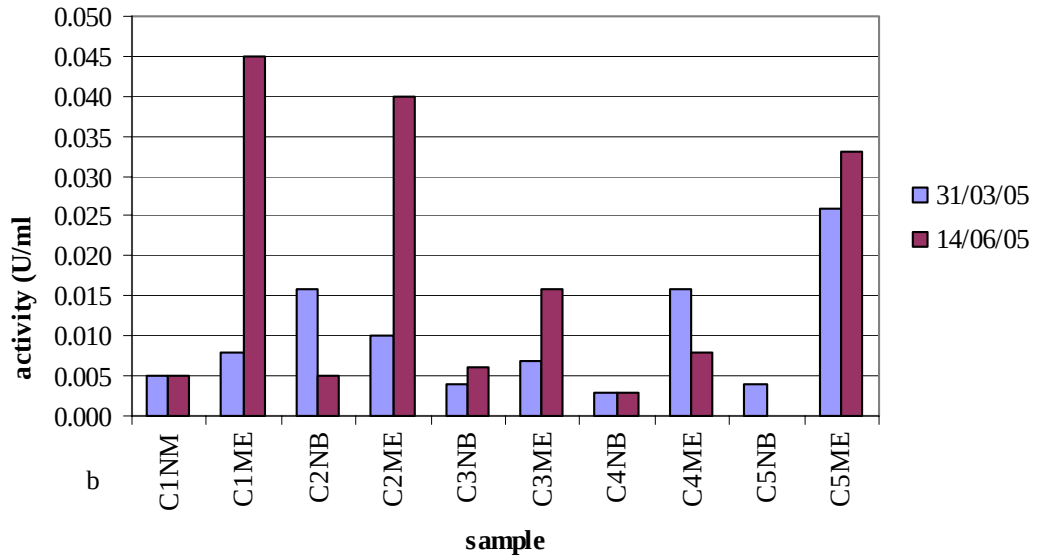


Figure 5.11 Comparison of the change in activity for the glucose-6-phosphate dehydrogenase assay between samples collected on 14/06/2005 and those collected on a) 17/03/2005 and 28/03/2005 (NW) and b) 31/03/2005 (CW).

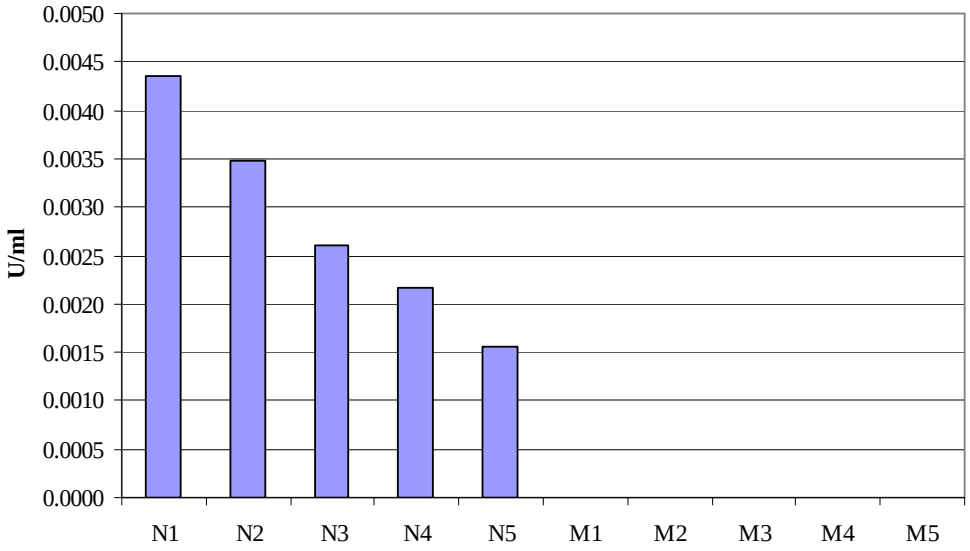


Figure 5.12 - The changes in activity observed in the glucose-6-phosphate dehydrogenase assay using various ratios of assay reaction mixture and sample.

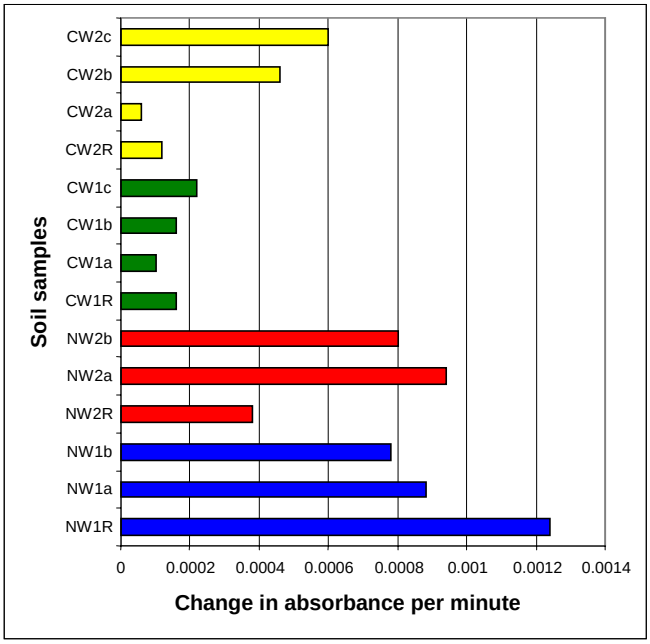


Figure 5.13 - Comparison of the change in absorbance per minute for the glucose-6-phosphate dehydrogenase assay between samples collected from CW and NW over the time period of 10 February 2006 to 03 April 2006, where R = reference sample collected on 10 February 2006, a = collected on 17 February, b = collected on 25 February 2006 (except for NW1b and NW2b which was collected on 03 April 2006), c = collected on 03 March 2006.

5.3.5 Peroxidase

No peroxidase activity was detected in the enrichment cultures, even when the ratio of assay reaction mix to sample was manipulated. Concentration of the effluent sample via dialysis showed an increased level of activity, stronger than the activity observed when the effluent was concentrated by freeze-drying and centrifugation. Peroxidase was consistently detected when the direct soil assay was employed (Figure 5.14). No clear trends were observed.

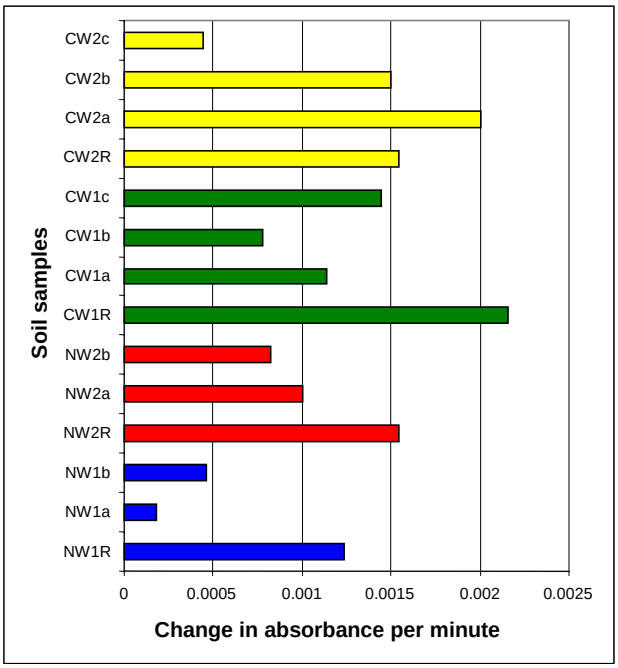


Figure 5.14 - Comparison of the change in absorbance per minute for the peroxidase assay between samples collected from CW and NW over the time period of 10 February 2006 to 03 April 2006, where R = reference sample collected on 10 February 2006, a = collected on 17 February, b = collected on 25 February 2006 (except for NW1b and NW2b which was collected on 03 April 2006), c = collected on 03 March 2006.

5.3.6 Tyrosinase

Tyrosinase activity was not detected in any of the effluent samples, even when the samples were concentrated, or enrichment cultures employed. Activity was however detected when the direct soil assay was employed (Figure 5.15).

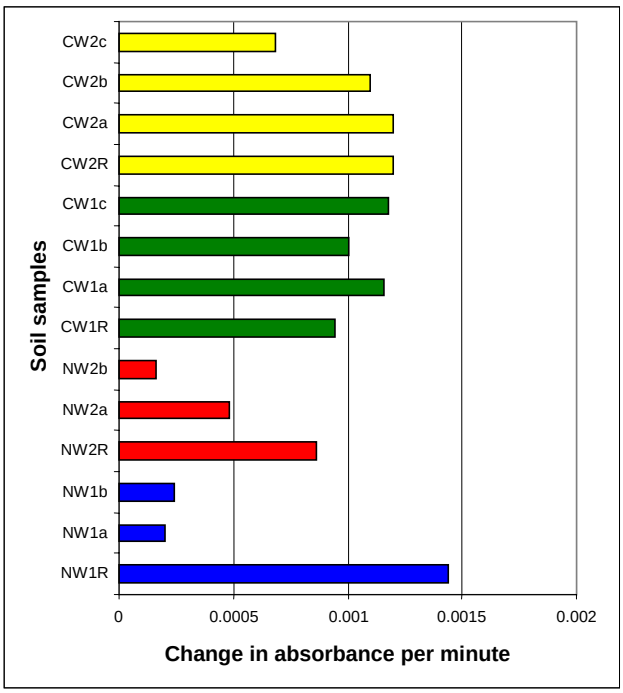


Figure 5.15 - Comparison of the change in absorbance per minute for the tyrosinase assay between samples collected from CW and NW over the time period of 10 February 2006 to 03 April 2006, where R = reference sample collected on 10 February 2006, a = collected on 17 February, b = collected on 25 February 2006 (except for NW1b and NW2b which was collected on 03 April 2006), c = collected on 03 March 2006.

5.3.7 Glucose oxidase

Glucose oxidase activity was only detected in the effluent after the sample was concentrated by centrifugation. The use of enrichment cultures and assay ratio manipulation did not allow for glucose oxidase detection. The use of the direct soil assay, allowed for detection of activity (Figure 5.16).

5.3.8 Catechol 1,2-dioxygenase and catechol 2,3-dioxygenase

Activity of these enzymes were not previously tested for, but were detected when the direct soil assay technique was employed (Figure 5.17 and Figure 5.18 for the activity of catechol 1,2-dioxygenase and catechol 2,3-dioxygenase respectively).

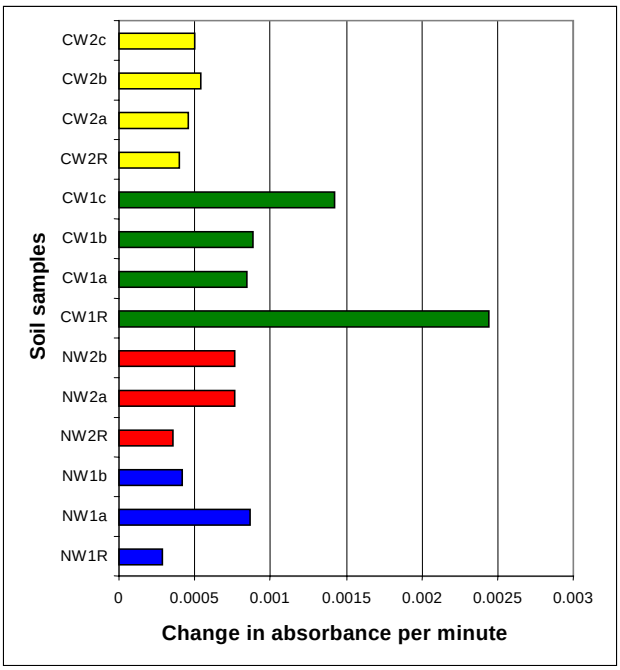


Figure 5.16 - Comparison of the change in absorbance per minute for the glucose oxidase assay between samples collected from CW and NW over the time period of 10 February 2006 to 03 April 2006, where R = reference sample collected on 10 February 2006, a = collected on 17 February, b = collected on 25 February 2006 (except for NW1b and NW2b which was collected on 03 April 2006), c = collected on 03 March 2006.

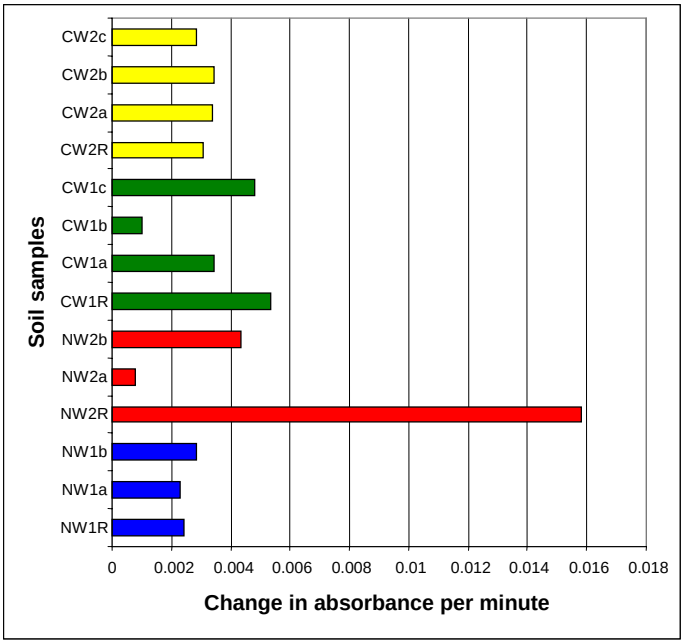


Figure 5.17 - Comparison of the change in absorbance per minute for the catechol 1,2-dioxygenase assay between samples collected from CW and NW over the time period of 10 February 2006 to 03 April 2006, where R = reference sample collected on 10 February 2006, a = collected on 17 February, b = collected on 25 February 2006 (except for NW1b and NW2b which was collected on 03 April 2006), c = collected on 03 March 2006.

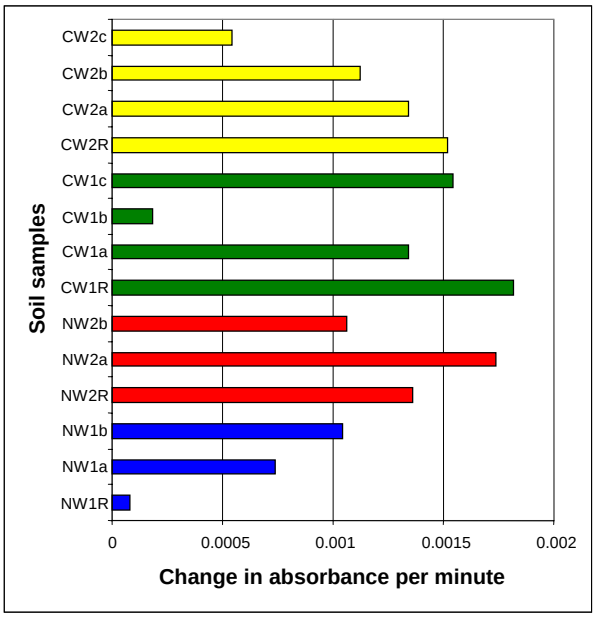


Figure 5.18 - Comparison of the change in absorbance per minute for the catechol 2,3-dioxygenase assay between samples collected from CW and NW over the time period of 10 February 2006 to 03 April 2006, where R = reference sample collected on 10 February 2006, a = collected on 17 February, b = collected on 25 February 2006 (except for NW1b and NW2b which was collected on 03 April 2006), c = collected on 03 March 2006.

5.4 Conclusions

5.4.1 Trends observed

The testing of ABTS with crude laccase extract showed definitively that the bleaching observed was not due to an inconsistency in the ABTS. It also showed that laccase showed a higher activity with ABTS than with guaiacol. The bleaching of ABTS by the samples of 14/06/2005 was higher than in previous samples and this can still not be explained. Further investigation will need to be carried out on this phenomenon. No bleaching of ABTS was observed with the direct soil sample analysis, which suggests that the bleaching effect could be due to any of the processes applied, e.g. due to enrichment culture technique or any of the sample concentration techniques.

Once again, no oxidase activity was observed in the samples and no particular trends were observed for laccase, esterase, alcohol dehydrogenase and glucose-6-phosphate dehydrogenase through the length of the wetlands. Comparison of the samples of 14/06/2005 with previous samples showed no trends over time either. In contrast to this, enzyme activity was detected in all of the soil samples when the direct assay technique was applied. Trends observed for laccase activity was different from one substrate to another, suggesting that one substrate should be chosen (ABTS or guaiacol) to ensure continuity of results observed. In the NW samples, a clear decrease in tyrosinase activity was observed over time, whereas the levels of tyrosinase in the CW samples did not seem to vary too much. No particular trends were observed for the other enzyme activities, although it was interesting to note the increase of alcohol dehydrogenase activity in some of the samples. There is, however, a clear difference in enzyme activities observed in the NW samples to that observed in the CW samples. It is recommended that in future, direct soil analysis should be performed, but that samples should be

taken over shorter time periods (bi-weekly) for the duration of a year. Furthermore, soil sample absorbances should also be taken over shorter time periods - a similar change in absorbance could occur over 3 hrs as over 30 minutes – and should be investigated in future studies.

The results obtained confirm that the biodegradation of winery waste taking place in the wetland can be effectively characterised. The degradation of the main constituents of winery effluents (glucose, fructose, ethanol, and phenolic substances) has been characterised in the detailed analysis (see Report 1) and this information has been used as the basis for the model of the wetland biodegradation. The microbial community present in the wetlands can be effectively characterised using phylogenetic techniques (see Report 2). The anaerobic metabolic (enzymatic) activities occurring have been detailed and to some extent are now correlated with the microbial population analysis (Report 2). The direct detection of enzyme activities has proved to be more difficult than anticipated. However, much has been learned. The last part of the project involved a new round of sampling, as new effluent entered the wetlands in January 2006, and modified enzyme assays were completed on fresh samples. These same samples were also used for confirmatory phylogenetic analysis to provide the correlation in the way that we now know is necessary.

5.4.2 Correlation with chemical and microbial data

Preliminary genera identification results from the natural wetland are presented in Table 5.5. The tentative correlation of enzyme activity observed to genera detected in the NW samples (Table 5.5), are preliminary. It is possible that when further results are available, and a time scale of species changes has been developed, the apparent lack of enzymatic trends may be more easily explained.

The growth of *Acinetobacter* sp. is stimulated by the presence of *Sacchromyces cerevisiae* due to the ethanol production of that yeast (42). This does not correlate with the lowered alcohol dehydrogenase activity found in the samples of 14/06/2005, in which the fermentation smell associated with yeast was noted.

Something that is interesting to note, is the presence of bacteria that may be causative agents of periodontal/endodontal diseases (Table 5.5). This could be explained if the winery is disposing of the contents of spittoons used during wine tasting into the natural wetlands as well. The total lack of detection of any actinomycetes was also interesting, since actinomycetes are well known for their predominance in various types of soils. All of the phylogenetic information given in this report came from samples taken from the natural wetlands area. It would be interesting to determine whether a similar type of bacterial/archaeal populations would be observed in the constructed wetlands, which would give answers to the questions of whether we would need to seed artificial wetlands with specific bacteria or whether other populations have the ability to perform a similar role in the constructed wetlands where existing populations have adapted to the changing environment. The differences observed in enzyme activity in itself would suggest that the two environments would have different

predominant bacterial/archaeal populations a fact which would also be influenced by the type of soil, fauna, flora and other external environmental factors at the two sites of interest.

5.4.3 Key findings and recommendations for further research

- Standard enzyme assays can be manipulated to allow for the detection of specific enzyme activities in soil samples
- Key enzymes that may be involved in wine wastewater degradation can be detected by using these modified assays
- Enzyme activity detected, even though precautions were taken, may be due to plant/fungal enzyme activity and not due to the bacteria/archaea detected in the soil
- Components in the soil may prevent actual detection of enzymes due to their strong oxidative properties. For example, it was difficult to determine esterase and lipase activity when direct soil sample analysis was performed, even though it was clear that a change in colour intensity occurred, which in itself was suggestive of esterase and lipase activity

Further studies using the laboratory scale model wetland would be very useful. This is a controlled environment that lends itself to testing the effect of microbial action on effluent, without extraneous factors, which might be found in either the natural or constructed wetlands. Studies of changes in bacterial community and enzyme activity before, during and after the addition of effluent may be carried out using this small-scale wetland. Various concentrations of effluent may be tested, as well as various components of the effluent. Once the changes in bacterial community and enzyme activity have been correlated it would be possible to seed the wetland with various strains of bacteria in order to determine whether or not it is feasible to develop a starter culture, which would increase the efficiency with which a constructed wetland degrades winery effluent

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CHAPTER 6
INVESTIGATION OF TRENDS IN MICROBIAL POPULATIONS IN WETLANDS

6.1 Introduction

The Breede, Berg and Olifants Rivers receive the bulk of winery effluent in South Africa. This wastewater mainly originates from various washing operations during the crushing and pressing of grapes, as well as from rinsing of fermentation tanks, barrels and other equipment (25). It typically has a pH of 3-5 and is highly concentrated in organic acids, sugars, alcohols, and phenolic substances (18). The levels of Chemical Oxygen Demand (COD) vary considerably from season to season, with the highest levels occurring in the harvest period from January to April, with subsequent lesser peaks later in the year when the wine is racked (11,16). In order to minimise the environmental pollution and to comply with legal requirements, there is a need for an efficient and sustainable treatment method for winery effluents (28). Conventional treatments, such as anaerobic or aerobic digesters, are often not cost effective and difficult to control, due to the seasonal fluctuations (7,11).

Constructed wetlands have been identified as a suitable means for treating winery effluent (11), providing a cheap alternative treatment method with significant additional marketing potential for wineries, e.g., conservation and recreational areas and benefits from demonstrating commitment to environmental causes. However, there are a number of problems associated with constructing wetlands, such as sensitivity of many plants to high COD levels and phenolic compounds, and poor understanding of the principles of wetland effluent treatment and of the contribution of plants and microorganisms to biodegradation. In research on wetlands for effluent treatment, there has been only limited focus on the microbial populations, though they are most probably the key factors in the bioremediation process.

In this local study, selected taxonomic groups of the microbial community in a winery wetland soil were identified and temporal changes that occurred within a year characterised using molecular phylogenetic techniques. The main objectives of this study were the following:

- in general, to contribute to the understanding and finally to the construction of successful and sustainable wetland systems for winery effluent treatment
- to identify key microorganisms present in the wetland
- to get an overview on the complexity and diversity of the microbial population in winery wetlands
- to detect shifts within this population due to the seasonal fluctuations of winery effluents

6.2 Methods

6.2.1 Sampling site and sample collection

Soil samples were collected from a selected winery wetland in Stellenbosch. The main inflow area (ca. 5 m^2) was chosen as the sampling site since it is assumed that the effects of the winery effluents are most intense there (Figure 6.1). While surface mud samples (up to approx. 5 cm depth) were obtained with sterile specimen containers, soil from depths ≥20 cm was recovered using decontaminated plastic corers (diameter, 25 mm). Between 5 and 20 cm depth there is a layer mainly consisting of plant material and water, so that soil samples are hardly retrievable. The collected samples (ca. 70-100 g) were cooled on ice immediately, homogenised by hand in plastic bags and stored at -80°C within two hours. Table 6.1 gives an overview on the samplings in the period from 15 February 2005 to 16 February 2006.

Figure 6.1 - Surroundings of the selected wetland in Stellenbosch (including dam, left) and close-up of the sampled main inflow, photographed during the harvest period in March (right).

Table 6.1 - Soil samples collected from a winery wetland in Stellenbosch.

Sample no.	Date	Depth (cm)
A[a]	23.11.2004	0-5
B[a]		20-28
11	15.02.2005	0-5
12		0-5
13		0-5
14		0-5
15		0-5
16		20-28
17		20-28
18		20-28
19		20-28
20		20-28
31	23.02.2005	0-5
32		0-5
33		0-5
34		0-5
35		0-5
36		20-28
37		20-28
38		20-28
39		20-28
40		20-28
41	02.03.2005	0-5
42		0-5
43		0-5
44		0-5
45		0-5
46		20-28
47		20-28
48		20-28
49		20-28
50		20-28
51	18.03.2005	0-5
52		0-5
53		0-5
54		0-5
55		20-28
56		20-28
57		20-28
58		20-28
60	02.04.2005	0-5
61		0-5
62		0-5
63a		20-28
63b		20-28
71	29.04.2005	0-5
72		0-5
73		0-5
74		20-28
75		20-28
76		20-28

Table 6.1 (continued) - Soil samples collected from a winery wetland in Stellenbosch.

Sample no.	Date	Depth (cm)
81	25.05.2005	0-5
82		0-5
83		0-5
84		20-28
85		20-28
86		20-28
91	29.06.2005	0-5
92		0-5
93		0-5
94		20-28
95		20-28
96		20-28
101	03.08.2005	0-5
102		0-5
103		20-28
104		20-28
111	08.09.2005	0-5
112		0-5
113		0-5
114		20-28
115		20-28
116		20-28
121	06.10.2005	0-5
122		0-5
123		0-5
124		20-28
125		20-28
126		20-28
141	16.11.2005	0-5
142		0-5
143		0-5
144		20-28
145		20-28
146		20-28
151	22.12.2005	0-5
152		0-5
153		0-5
154		20-28
155		20-28
156		20-28
161	02.02.2006	0-5
162		0-5
163		0-5
164		20-28
165		20-28
166		20-28
171	16.02.2006	0-5
172		0-5
173		0-5
174		20-28
175		20-28
176		20-28

[a] DNA isolated from samples A and B was used for the construction of the mini clone library and the identification of methanogenic *Archaea* and syntrophic *Bacteria*.

6.2.2 DNA extraction from soil samples and purification

A modified protocol of the method described by Miller *et al.* (21) was applied. Aliquots of the soil samples were thawed and – if necessary – centrifuged in a 2-mℓ tube (15000 x g, 1 min) to remove surplus water. Then, 500±10 mg of the well-mixed pellet was weighed in a 2-mℓ screw-capped tube containing 500±10 mg of quartz sand (Sigma S-9887). Amounts of 300 µL of each 100 mM sodium phosphate (pH 8) and lysis buffer (100 mM NaCl, 500 mM Tris [pH 8], 100 g/ℓ SDS) were added, and the tube was inverted to mix. After the addition of 300 µL of chloroform/isoamyl alcohol (24:1, v/v) the mixture was vortexed at maximum level for 120 s, and the cell debris were pelleted by centrifugation (15000 x g, 5 min). Ammonium acetate (7 M) was added to the supernatant to a final concentration of 2.5 M. The tube was shaken by hand to mix and centrifuged at 15000 x g for 10 min. The DNA was precipitated from the supernatant by addition of 0.6 volumes of isopropanol, incubation at room temperature for 15 min and centrifugation (15000 x g, 10 min). The pellet was desalted with 1 mℓ of 70% EtOH, air-dried and dissolved in 100 µL of TE (10 mM Tris, 1 mM EDTA, pH 8).

The DNA extracted using the modified Miller method was further treated by the following 2-step purification procedure:

Step 1: PVPP purification

Self-constructed minicolumns packed with polyvinylpolypyrrolidone (PVPP) were used for the removal of humic substances (3). Briefly, 400 µL of a 100 g/ℓ PVPP suspension (in TE) were loaded on a 20-µL pipette filter tip (centrifugation, 200 x g, 2 min). The minicolumn was washed twice with 150 µL of TE (600 x g, 10 min), then dried. The DNA sample (33 µL) was applied and, after a 2-min incubation, eluted by centrifugation (600 x g, 5 min, followed by 1700 x g, 10 min).

Step 2: Sephacryl purification

A gel filtration chromatography matrix, also packed in minicolumns, was applied to remove small fragments of nucleic acids (32). An amount of 400 µL of Sephacryl S-500 HR suspension (Amersham Biosciences 17-0613-10) was loaded on a 20-µL pipette filter tip (centrifugation, 600 x g, 2 min), then the column was washed four times with 120 µL of TE (centrifugation after fourth time, 800 x g, 3 min). The DNA eluate from PVPP column, ca. 35 µL, was applied and eluted by centrifugation (600 x g, 2 min).

The concentration (calculated as $OD_{260\ nm}$ x 50 ng/µL) and purity (ratio $OD_{260\ nm}/OD_{280\ nm}$) of the processed DNA were determined using the NanoDrop ND-1000 spectrophotometer.

6.2.3 Polymerase chain reaction (PCR)

PCR amplifications were performed in 0.2-mℓ thin-walled tubes using a thermocycler equipped with a heated lid (e.g., PCR Sprint Temperature Cycling System, Thermo Hybaid, Ashford, GB). A standard 50-µL reaction contained 1x PCR buffer [10x being 200 mM Tris pH 8.8, 100 mM KCl, 100 mM $(NH_4)_2SO_4$, 20 mM $MgSO_4$, 1% (w/v) Triton X-100], 0.2 mM each of dATP, dCTP, dGTP and dTTP,

0.5 µM of each primer (1.25 µM for A340F-GC/A533R), an appropriate amount of *Taq* DNA polymerase, and 25 ng of metagenomic DNA as the template. For DNA amplifications from whole cells, small amounts of freshly grown colonies were transferred to the reaction tubes using a 10-µL pipette. The primers used as well as the cycling conditions applied are given in Table 6.2.

Table 6.2 - PCR primers and cycling conditions.

Primer	Sequence (5'-3')	Ref.	Specificity	Cycling conditions
E9F U1510R	GAGTTTGATCCTGGCTCAG GGTTACCTTGTTACGACTT	(13) (27)	most *Bacteria*	94°C/4 min 30x (94°C/30 s - 52°C/30 s - 72°C/105 s) 72°C/10 min
341F[b] 534R	CCTACGGGAGGCAGCAG ATTACCGCGGCTGCTGG	(22) (22)	most *Bacteria*	94°C/4 min 20x (94°C/45 s - 65°C/45 s - 72°C/60 s) 20x (94°C/30 s - 55°C/30 s - 72°C/60 s) 72°C/10 min
A3Fa Ab927R	TCCGGTTGATCCYGCCGG CCCGCCAATTCCTTTAAGTTTC	(9,20) (mod.) (15)	most *Archaea*	94°C/4 min 20x (94°C/45 s - 55°C/45 s - 72°C/75 s) 10x (94°C/30 s - 55°C/30 s - 72°C/75 s) 72°C/10 min
A340F[b] A533R	CCCTACGGGGYGCASCAG TTACCGCGGCKGCTG	(23) (23)	most *Archaea*	94°C/4 min 30x (94°C/30 s - 53.5°C/30 s - 72°C/60 s) 72°C/10 min
M340F[b] M707R	CCCTACGGGGCGCAGCAG GGATTACARGATTTCAC	(37) (37)	most metha-nogenic *Archaea*	94°C/4 min 35x (94°C/30 s - 53°C/45 s - 72°C/60 s) 72°C/10 min
Syn682F Syn1196R	GGTGTAGAGGTGAAATTCGT CATAAAGGCCATGAGGACTT	(19) (19)	some syntrophic bacteria	94°C/4 min 30x (94°C/30 s - 65°C/45 s - 72°C/60 s) 72°C/10 min
M13fw M13rev	CCCAGTCACGACGTTGTAAAACG AGCGGATAACAATTTCACACAGG		cloning vector pTZ57R	94°C/4 min 35x (94°C/30 s – 64°C/30 s - 72°C/[var]) 72°C/10 min

[a] Corresponds to the numbering of the *E. coli* 16S rRNA gene.
[b] The following GC clamp was added at the 5' ends to form primers 341F-GC, A340F-GC, and M340F-GC, respectively: CGCCCGCCGCGCGCGGCGGGCGGGGCGGGGGCACGGGGGG

6.2.4 Cloning of PCR-amplified DNA fragments into *Escherichia coli*

PCR amplicons were ligated into the vector pTZ57R using the "InsT/Aclone PCR Product Cloning Kit" (Fermentas K1214) according to the manufacturer's instructions. $CaCl_2$-competent *E. coli* DH5α cells were prepared and transformed according to the Inoue protocol described in Sambrook and Russell (29). Alternatively, electrocompetent *E. coli* XL1-Blue were prepared and transformed using standard methods (29).

6.2.5 Amplified rDNA restriction analysis (ARDRA)

Amplified rDNA restriction analysis (ARDRA) of PCR amplicons was done in 20-µL reaction mixtures containing 100 ng of DNA, 1 U of restriction endonuclease (Fermentas) and the appropriate 10x buffer. The reactions were incubated overnight at 37°C, and the restriction digests were separated using agarose gel electrophoresis (2.5% gels). The appropriate restriction enzymes were selected using WatCut, an on-line tool available on http://watcut.uwaterloo.ca/.

6.2.6 Denaturing gradient gel electrophoresis (DGGE)

PCR amplicons obtained with either the bacterial (341F-GC and 534R) or the archaeal primer pair (A340F-GC and A533R) were separated on 16.5/16.5 cm, 1 mm thick 9-% (w/v) polyacrylamide (37.5:1 acrylamide:bisacrylamide) gels with varying denaturing gradients [Biorad gradient former; 100% being 7 M urea and 40% (v/v) formamide] using a Scie-Plas V20-HCDC apparatus (Scie-Plas, Warwickshire, GB). Electrophoresis was carried out at 100 V for 16 h at 60°C in 1x TAE buffer. Gels were stained (0.5 µg/mℓ ethidium bromide in 1x TAE for 20 min), destained (1x TAE for 15 min) and finally visualised using the AlphaImager 3400 imaging system equipped with the AlphaEase FC image processing and analysis software (Alpha Innotech, San Leandro, CA).

In order to retrieve DNA fragments from the gel, specific bands were cut out, the gel slices washed with 1 mℓ of ddH_2O and then soaked over night in 40 µL of TE. The eluted DNA (3.5 µL) was then used as a template to reamplify the fragment in a PCR reaction with the DGGE primers. The products were rerun on a DGGE gel to ensure the amplification of the correct fragment and then cloned in *E. coli* as described above. Screening for the correct clone was done by reamplifying the DGGE fragment through colony PCR.

6.2.7 Nucleotide sequence determination and analysis

DNA sequencing was done by the Sequencing Service of the Department of Molecular and Cell Biology at the University of Cape Town or by Inqaba Biotechnical Industries (Pretoria). BLAST analysis of the edited sequences was performed using the online tools provided by the National Center for Biotechnology Information (NCBI, http://www.ncbi.nlm.nih.gov). Putative chimeric 16S rDNA sequences were detected using the software of the Ribosomal Database Project II website (6).

6.3 Results

6.3.1 DNA extraction and purification from wetland soil samples

The development of a suitable and straightforward procedure for the direct isolation of total microbial DNA from wetland soil samples was described in detail in the November 2004 progress report. Further extractions showed that this procedure could be simplified without loss of DNA quality. Firstly, the sample pretreatment was reduced to a short centrifugation to remove surplus water. Secondly, since no RNA could be detected on agarose gels after the Sephacryl purification of crude DNA, the RNase digestion was omitted. Thirdly, the phenol extraction/ethanol precipitation step was found to result in a big loss of DNA without improving its quality – and was therefore omitted as well.

Using the protocol described in Materials and Methods, DNA was successfully extracted from 107 of the collected soil samples, with an average yield of 15 µg of purified DNA per g of soil (wet weight). More DNA was obtained from surface samples (range, 5.6-38.6 µg/g) than from soil collected at about 25 cm depth (range, 4.8-16.6 µg/g), which could indicate a higher microbial activity in the surface layer. For all samples, the $OD_{260\ nm}/OD_{280\ nm}$ ratio was between 1.70 and 1.88, values suggesting the absence of major contaminants. The DNA concentration was standardised to 25 ng/µL, and an aliquot was analysed by agarose gel electrophoresis. As Figure 6.2 shows, the samples mainly consist of high molecular weight (>5 kb) DNA fragments.

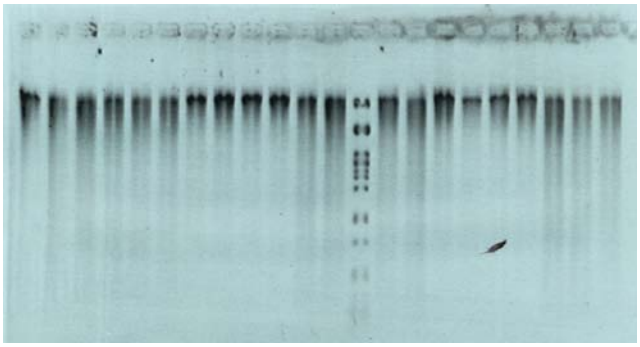

Figure 6.2 - Agarose gel (1%) of total DNA (125 ng) extracted from soil samples (selection). Marker: *Pst*I-restricted *λ*-DNA.

6.3.2 Construction of a "mini" 16S rDNA clone library

16S rDNA clone libraries constructed from metagenomic DNA are a useful means to determine the composition of microbial communities since there is no selection by culturing methods. However, for highly diverse environments like soil (containing several hundred to several thousand species), this involves a huge amount of sequencing, which is financially not feasible within this study. Therefore, we focus much more on changes in community composition than on the species composition itself. So the aim of the "mini" 16S rDNA clone library presented here could not be to get a representative picture of the microorganisms present in the wetland. However, it was done to establish and test our methods and to get a very rough idea of which taxonomic groups of bacteria there are. In addition, the obtained clones were very useful to prepare a marker for DGGE, which enables the comparison of samples run on different gels (see below).

DNA extracted from samples A and B (Table 6.1) was used as a template in PCR reactions with the primer pair E9F/U1510R, which amplifies the nearly complete 16S rRNA gene from the majority of bacterial organisms (1). The products obtained had the expected size of ca. 1.5 kb. To eliminate non-specific amplicons, the main bands were cut out and extracted from the agarose gel slices (QIAquick PCR Purification Kit, Qiagen 28106). The mixture of 16S rRNA gene fragments was then cloned into *Escherichia coli* as described in Materials and Methods. The 16S rDNA fragments of 10 clones of each sample were partially sequenced (ca. 600 bp), and the most similar sequences stored in public

databases were identified. As Table 6.3 shows, the majority of these 'best hits' are 16S rDNA genes of so far uncultured bacteria and provide little taxonomic information. Therefore, the names of the most similar cultured and identified species were also included in the table; however, most of them share less than 97% with the wetland clone sequences, which means that the latter originate from un-described species (33). Having a look at the sources from which the 'best hit' organisms were isolated, it can be stated that most of them are likely to be typical water or soil bacteria.

To get a better overview on the distribution of the taxonomic groups to which the retrieved sequences belong, a multiple alignment was compiled, which was used to construct a phylogenetic tree (Figure 6.3). The size of the library is not big enough to see any differences between the two depths sampled. However, the tree shows that half of the sequences group within the phylum *Bacteroidetes*, which includes the *Bacteroidales*, *Flavobacteria*, and *Sphingobacteria*. For the latter group (Mid-3c and 3q), the assignment is based on very low confidence percentages (as obtained from the Classifier software of the Ribosomal Database Project II, http://rdp.cme.msu.edu), and therefore any discussion of properties would be pure speculation. The family *Flavobacteriaceae*, which Mid-2a, 2b and 2l were attributed to with a confidence of over 90%, is a very diverse group of Gram-negative bacteria (aerobic to anaerobic, mesophilic to psychrophilic) that can be found in a wide range of habitats, including soil and freshwater environments (2). The four *Bacteroidales* sequences were all assigned to the family *Porphyromonadaceae*, of which most representatives are involved in the pathogenesis of periodontal disease (e.g., *Porphyromonas* and *Tannerella*) or other infections (*Dysgonomonas*). A very recent publication, however, describes the novel species *Proteiniphilum acetatigenes*, which belongs to the same family (5). *P. acetatigenes* was isolated from an upflow anaerobic sludge blanket (UASB) reactor treating brewery wastewater and is characterised as a Gram-negative, mesophilic, obligate anaerobic, chemoorganotrophic bacterium. It was not able to ferment carbohydrates, but produced acetic and propionic acids with yeast extract or peptone as energy sources. Added to a methanogenic consortium, it accelerated the acetic acid and methane formation. The occurrence of a related bacterium in the methanogenic winery wetland, where lysed yeast cells are likely to be present, seems possible. It has to be mentioned, though, that the sequence similarity (to the wetland clones' 16S rDNA) of *P. acetatigenes* is not higher than of those of the oral pathogens and with less than 90%, is quite low.

Of the seven representatives of the huge phylum *Proteobacteria* (the classical Gram-negative bacteria), only the three belonging to the γ-subdivision can be assigned to specific genera with sufficient certainty. Bacteria belonging to the genus *Acinetobacter* (Mid-3d) are common, free-living saprophytes found e.g. in soil, water, sewage, and foods. They are metabolically very versatile, which means that they may play an important role in a variety of commercially important industrial processes, as well as in the biodegradation of a wide range of environmental pollutants (35). *Citrobacter freundii* (Mid-2c) is regarded as an opportunistic pathogen. The members of this genus are believed to be commensal inhabitants of the intestine of humans and animals and, most likely as a consequence of fecal shedding, are present in the environment and can be recovered from water, sewage and soil (4).

The saprophytic pseudomonads (Mid-2i) are found in nature together with very diverse organisms and in some instances they constitute a substantial fraction of the microflora. They can be considered ubiquitous (24). In addition, the clone Mid-3h shall be mentioned here because of its considerable sequence similarity to the genus *Syntrophus*, a strictly anaerobic bacteria which ferments aromatic compounds to acetate, CO_2 and hydrogen syntrophically in co-culture with hydrogen-consuming methanogens (17). The presence of a related microorganism in the wetland could be interesting in the context of the biodegradation of phenolic substances occurring in winery effluents.

Finally, the phylum *Verrucomicrobia* (Mid-3p and 3g) counts only a small number of cultivated representatives, but is dominated by environmental rRNA gene clones that are ubiquitous in natural freshwater and soil microbial communities (26).

Table 6.3 - BLAST results of 20 partial 16S rDNA sequences obtained from wetland soil samples. Clone IDs containing the number "2" refer to sample A (retrieved from wetland surface), whereas "3" refers to sample B (from ca. 25 cm depth).

Clone ID	Best hit	% Identity (no. of bp)	Source	Acc. no.	Closest cultured and identified bacterial species	% Identity (no. of bp)	Acc. no.
Mid-2a	Aquatic bacterium R1-C1	93 (563)	Lake Inba, Japan	AB195777	*Cytophaga* sp. An36	95 (489)	AJ551174
Mid-2b	Unc. *Bacteroidetes* clone TAF-A87	99 (675)	River Taff epilithon	AY038761	*Riemerella* sp. Lo3	98 (621)	AB192398
Mid-2l	Unc. *Bacteroidetes* clone ODP1176A22X_12	95 (547)	Nankai Trough sediments	AY191341	*Riemerella* sp. Lo3	95 (547)	AB192398
Mid-3c	Unc. *Bacteroidetes* clone GWS-Kdna25	95 (711)	German Wadden Sea	AY515486	*Ornithobacterium rhinotracheale*	88 (505)	ORU87101
Mid-3q	Unc. bacterium clone TSBZ10	94 (757)	Polychlorinated dioxin-dechlorinating community	AB186818	*Flavobacillus indica* str. GPTSA100-9	89 (397)	AY904351
Mid-3e	Unc. bacterium clone TANB60	93 (579)	Trichloroethene-dechlorinating groundwater	AY667262	*Bacteroides merdae* ATCC 43184T	88 (557)	X83954
Mid-2d	Unc. *Bacteroides* sp. clone KB-1 2	92 (729)	Chlorinated ethene-dechlorinating enrichment culture	AY780553	*Bacteroidales* bacterium str. WB4	93 (549)	AB078842
Mid-2q		92 (742)				93 (562)	
Mid-2n		92 (764)				95 (584)	
Mid-2e	Unc. bacterium clone 118ds10	93 (701)	Water downstream manure	AY212569		93 (700)	
Mid-2o	most probably chimera						
Mid-3n	Uncultured bacterium clone HTA1	96 (594)	Freshwater reservoir	AF418941	*Oxalobacter formigenes* str. OXCR	89 (594)	U49754
Mid-2c	*Citrobacter freundii* str. SSCT56	99 (585)	Sewage sludge compost	AB210978	*Citrobacter freundii* str. SSCT56	99 (585)	AB210978
Mid-2i	Unc. bacterium clone 187up	98 (727)	Water upstream manure	AY212639	*Pseudomonas migulae*	96 (702)	AF321026
Mid-3d	*Acinetobacter junii* str. S33	99 (742)	Sewage activated sludge	AB101444	*Acinetobacter junii* str. S33	99 (742)	AB101444
Mid-3o	Unc. bacterium clone 1700b-41	96 (741)	Hawaiian volcanic deposits (anno 1700)	AY917466	*Acidobacteria* bacterium str. Ellin7184	91 (741)	AY673350
Mid-3g	Unc. soil bacterium clone PBS-III-20	92 (515)	Soil of flooded rice cultures	AJ390453	*Cytophaga* sp. Dex80-64	90 (188)	AJ431235
Mid-3p	Unc. *Verrucomicrobium* clone YNPRH34A	88 (633)	Yellowstone NP, soil near geyser	AF465651	*Verrucomicrobia* subd. 3 bacterium str. Ellin518	88 (433)	AY960781
Mid-3h	Unc. bacterium clone SHA-42	92 (759)	Dichloropropane-dechlorinating community	AJ306771	*Syntrophus gentianae*	91 (755)	X85132
Mid-3l	Unc. soil bacterium clone S124	93 (607)	Agricultural soil	AY037635	*Fibrobacter intestinalis* str. JG1	88 (347)	M62690

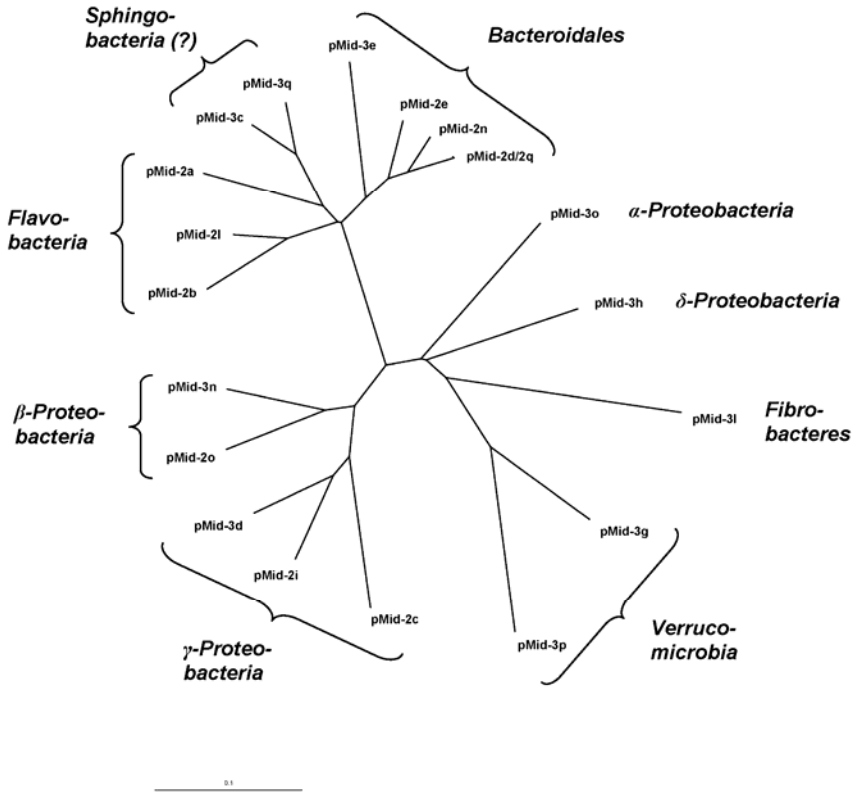

Figure 6.3 - Unrooted neighbour-joining tree based on 488-bp 16S rDNA fragments amplified from wetland soil samples. The alignment was done with the ClustalX software (34) using the standard parameters as proposed by Hall (12). Regions containing mainly gaps were excluded from the analysis. The scale bar refers to 10 substitutions per 100 bp. The sequences were attributed to the different taxonomic groups using the Classifier software of the Ribosomal Database Project II (6).

6.3.3 Identification of key microorganisms of the methanotrophic consortium in the winery wetland soil

Wetlands are typical environments in which organic matter is decomposed anaerobically. Thereby polymers are gradually degraded and finally converted into methane and CO_2 (Figure 6.4). A wide variety of microorganisms participate in this process, many of them relying on the activity of others. Most prominent is the interdependence of the acetogenic and/or hydrogen producing *Bacteria* ("secondary fermenters" or "syntrophs") and the methanogenic *Archaea*, which together form the core of all methane producing communities. The identification of members of these two microbial groups was therefore used to show the presence of an active methanogenic community in the winery wetland (19).

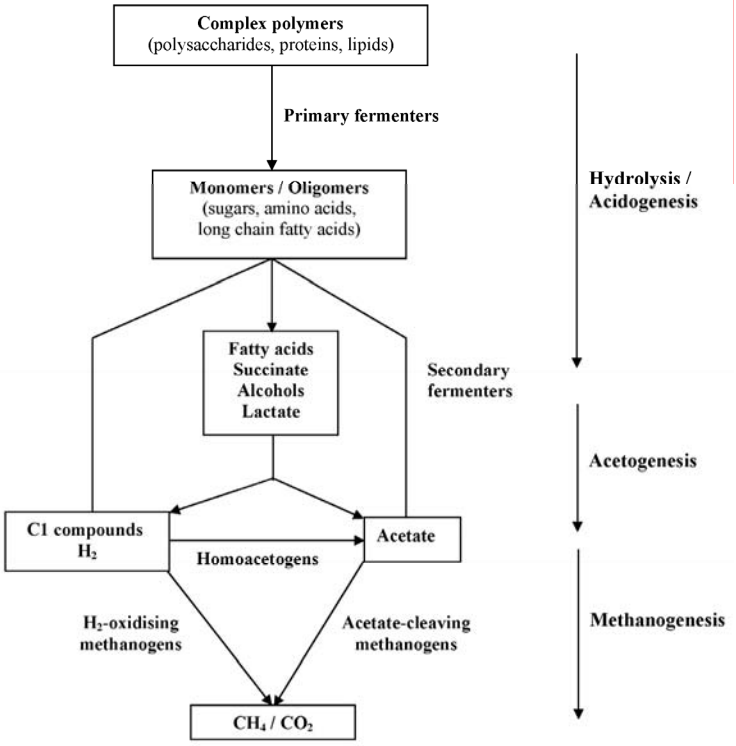

Figure 6.4 - Overview on processes and microbial groups involved in the anaerobic digestion of organic matter [adapted from (31)].

Metagenomic DNA extracted from samples A and B (see Table 6.1) was used in PCR reactions with primer set M340F/M707R (specific for methanogenic *Archaea*) for the amplification of partial 16S rDNA. After cloning the mixed products in *E. coli*, 45 clones containing the correct insert size were subjected to ARDRA (*Alu*I, *Hae*III and *Rsa*I) in order to assess the variety of DNA sequences of the small library (Figure 6.5). Up to 7 distinct patterns were obtained, whereby one was dominating in both the surface (A) and lower core (B) samples, comprising 70 to 80% of the clones. One representative of each pattern (per sample) was sequenced. The BLAST results and an overview on the phylogenetic origin of the analysed sequences are given in Table 6.4 and Figure 6.6, respectively.

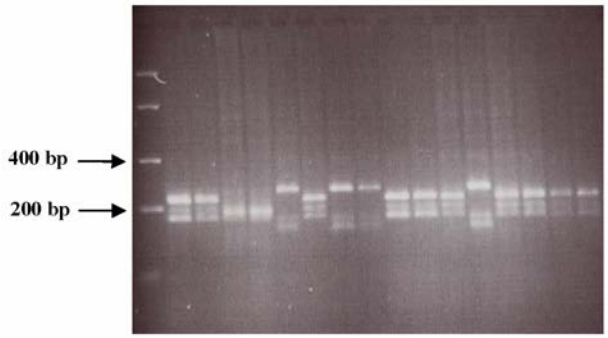

Figure 6.5 - ARDRA patterns (using restriction enzyme *Alu*I) of 16S rDNA PCR products of methanogenic *Archaea* (selection).

Table 6.4 - BLAST results of 22 partial 16S rDNA sequences obtained from wetland soil samples, using primer set M340F/M707R. Clone IDs containing the letter "T" were retrieved from the wetland surface, whereas "B" refers to a depth of ca. 25 cm.

Clone ID	Best hit	% Identity	Accession no.
T1	Uncultured *Methanomicrobiaceae* archaeon	93	AY133922
T2	methanogenic endosymbiont of *Caenomorpha* sp. 2	98	AJ132652
T14	Uncultured *Methanosaeta* sp.	98	AY780569
T15	Uncultured *Methanomicrobiaceae* archaeon	98	AY133901
T17	*Methanobacterium* sp. IM1	99	AY274451
T22	Uncultured *Methanosaetaceae* archaeon	97	AY125710
T24	Uncultured *Methanosaetaceae* archaeon	98	AY133916
T26	Uncultured bacterium	97	AY134895
T28	*Methanobacterium* sp. IM1	100	AY274451
B1	Uncultured *Methanomicrobiaceae* archaeon	99	AY133896
B4	Uncultured *Methanomicrobiaceae* archaeon	97	AY133896
B6	Uncultured *Methanosaeta* sp.	98	AY780569
B7	Uncultured *Methanomicrobiaceae* archaeon	99	AY125717
B9	Uncultured *Methanosaetaceae* archaeon	98	AY125710
B11	methanogenic endosymbiont of *Caenomorpha* sp. 2	98	AJ132652
B14	Uncultured *Methanosaetaceae* archaeon	100	AY133932
B17	Uncultured *Methanogenium* sp.	97	AY177809
B23	Uncultured *Methanosaeta* sp.	99	AY780569
B24	Uncultured *Methanomicrobiaceae* archaeon	96	AY133924
B26	Uncultured *Methanomicrobiaceae* archaeon	99	AY133896
B27	Uncultured *Methanomicrobiaceae* archaeon	98	AY133896
B30	Uncultured *Methanosaetaceae* archaeon	99	AY133932

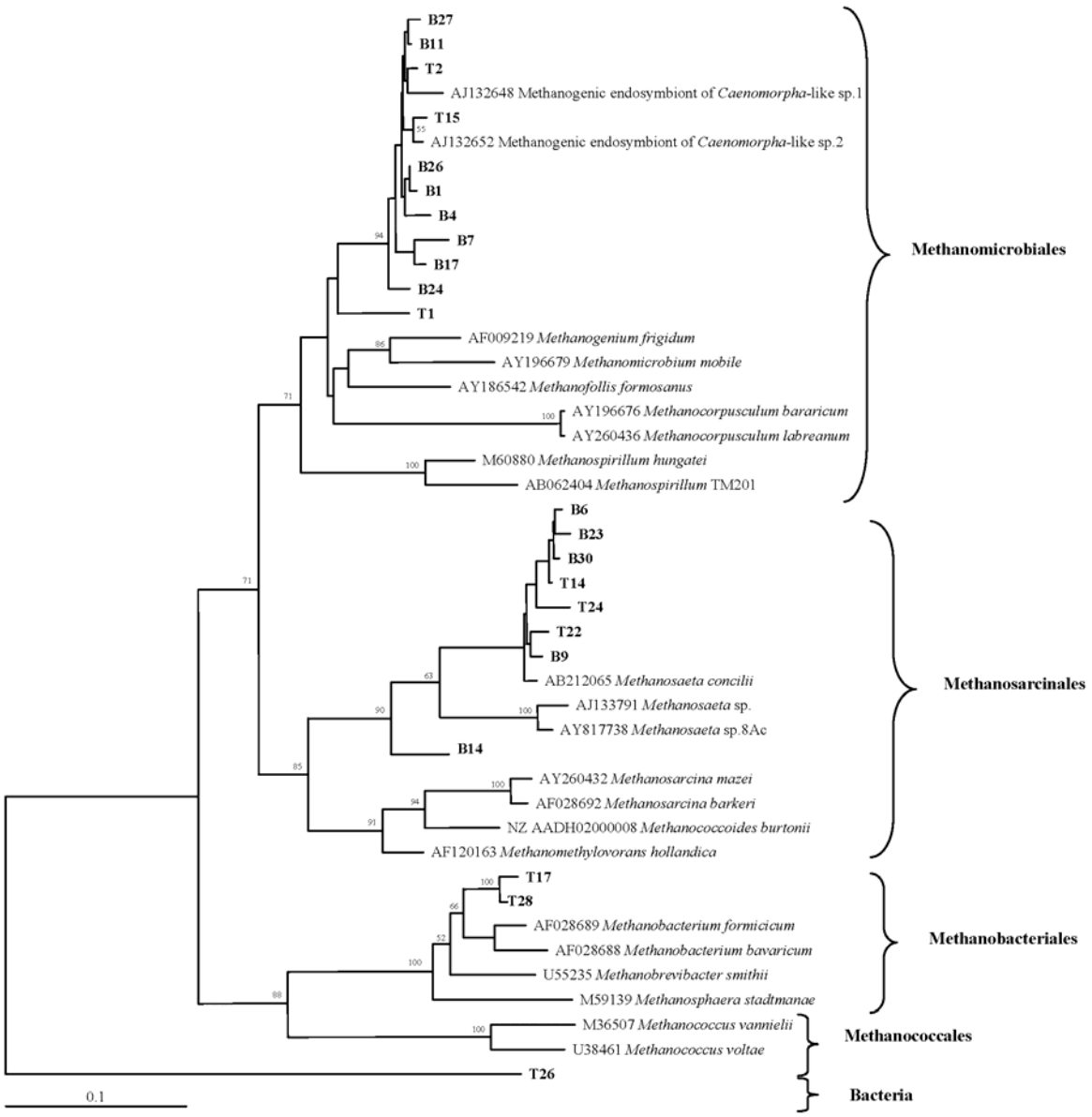

Figure 6.6 - Phylogenetic tree of partial 16S rRNA gene sequences of methanogenic *Archaea* amplified from wetland soil. The tree was constructed with the software Phylo_win 2.0 using the neighbour-joining algorithm. The scale bar represents a 10% difference in nucleotide sequences. The sequences obtained in the present study are shown in bold, accession numbers of the remaining sequences are indicated. Numbers at nodes represent the bootstrap values (from 1000 replicates; only values >50% are shown). T26, a bacterial sequence, was used as an outgroup.

Half of all phylotypes sequenced, including the two representatives of the dominant ARDRA pattern, belong to the order *Methanomicrobiales* and are positioned near the family *Methanomicrobiaceae*. They are most closely related to yet unnamed endosymbiontic methanogens isolated from *Caenomorpha* sp., a free-living anaerobic ciliated protozoan (36). *Methanomicrobiales* are hydrogenotrophic, and hydrogenotrophy has been shown to be the dominant nutrition route in peatlands and other wetlands [e.g. (8)]. In this study, more *Methanomicrobiales* sequences were recovered from the surface than from the deeper layer. However, the number of analysed clones is certainly too small to draw statistically significant conclusions in this regard. About a third of the sequences analysed cluster with the family *Methanosaetaceae*. These strictly acetoclastic methanogens typically thrive in low acetate concentrations (14) and are often outcompeted by other methanogens at higher acetate levels. It will therefore be interesting to investigate if their population size decreases with the beginning of the harvest season. First DGGE experiments that have already been done to approach this question (19) looks promising, however, the method still needs to be optimised. *Methanobacteriales* are very strict anaerobes; this might explain why they were not found in the surface sample. Representatives of the two other orders of the methanogenic *Archaea* were not expected to occur in the wetland since they are either hyperthermophilic (*Methanopyrales*) or have so far only been detected in marine environments (*Methanococcales*) (38).

The syntrophic *Bacteria* are crucial in every methanogenic community since they oxidise primary fermentation products and thereby generate acetic acid, hydrogen and/or carbon dioxide, which are the substrates for methanogens. Syntrophs are found within two main groups of the taxonomic system, namely, the *Firmicutes* (low G+C gram-positives) and the *δ-Proteobacteria* (30). This makes it impossible to target this functional group with a single primer pair. We decided to focus on two genera within the *Syntrophobacterales* (*δ-Proteobacteria*) by designing primers Syn682F and Syn1196R that target *Syntrophobacter*, known to oxidise propionate, pyruvate and fumarate, and *Syntrophus*, degrading benzoate and other aromatic compounds (30). A library of 69 clones was constructed and subjected to ARDRA (*Mva*I, *Hpy*F10VI, *Sdu*I). With all three enzymes, different patterns were found to be dominant in the surface and deeper layer samples. This is an indication that different taxonomic groups could be dominant at different depths, e.g. due to oxygen or temperature sensitivity or concentration gradients of specific nutrients. From each unique ARDRA pattern of each sample, one or two clones were sequenced. The BLAST results and an overview on the phylogenetic origin of the analysed partial 16S rRNA genes are given in Table 6.5 and Figure 6.7, respectively.

Table 6.5 - BLAST results of 29 partial 16S rDNA sequences obtained from wetland soil samples, using the primer pair Syn682F/Syn1196R, which targets syntrophic bacteria. Clone IDs containing the letter "T" were retrieved from the wetland surface, whereas "B" refers to a depth of ca. 25 cm.

Clone ID	Best hit	% Identity	Accession no.
T2	*Rhodobacter* sp. R-8	97	AY914074
T3	*Sinorhizobium* sp. c37	96	AB167207
T5	Uncultured sulfate-reducing bacterium	98	AB069774
T8	Uncultured sulfate-reducing bacterium	98	AB069774
T15	*Stella humosa*	90	AJ535710
T18	*Syntrophus gentianae*	99	X85132
T21	Uncultured sulfate-reducing bacterium	88	AB069773
T27	Uncultured *δ-Proteobacterium*	91	AY164375
T28	*Catellibacterium nectariphilum*	96	AB101543
T30	*Sphingomonas* sp. CS101	98	AY522503
T41	Uncultured sulfate-reducing bacterium	98	AB069773
T42	*Roseomonas lacus*	98	AJ78600
T45	Uncultured sulfate-reducing bacterium	98	AB069773
B2	Uncultured *Syntrophus* sp.	96	AY261813
B3	Uncultured δ-*Proteobacterium*	96	AY499729
B4	Uncultured *Syntrophus* sp.	95	AY780562
B5	Uncultured sulfate-reducing bacterium	98	AB069773
B6	*Syntrophus* sp.	95	AJ133794
B7	*Labrys* sp. CC-BB4	99	DQ062742
B11	Uncultured *δ-Proteobacterium*	93	AY940124
B13	Uncultured *δ-Proteobacterium*	95	AM071378
B15	Uncultured *δ-Proteobacterium*	99	AB074950
B17	*Pelobacter carbinolicus* DSM 2380	90	CP000142
B20	*Syntrophus* sp.	97	AJ133794
B23	*Sphingomonas* sp.	97	AJ011505
B25	Uncultured *Syntrophobacteraceae* bacterium	96	AY167444
B31	Uncultured α-*Proteobacterium* clone	99	AY921929
B40	*Smithella propionica*	98	AF126282
B42	*δ-Proteobacterium* UI	98	AB212873

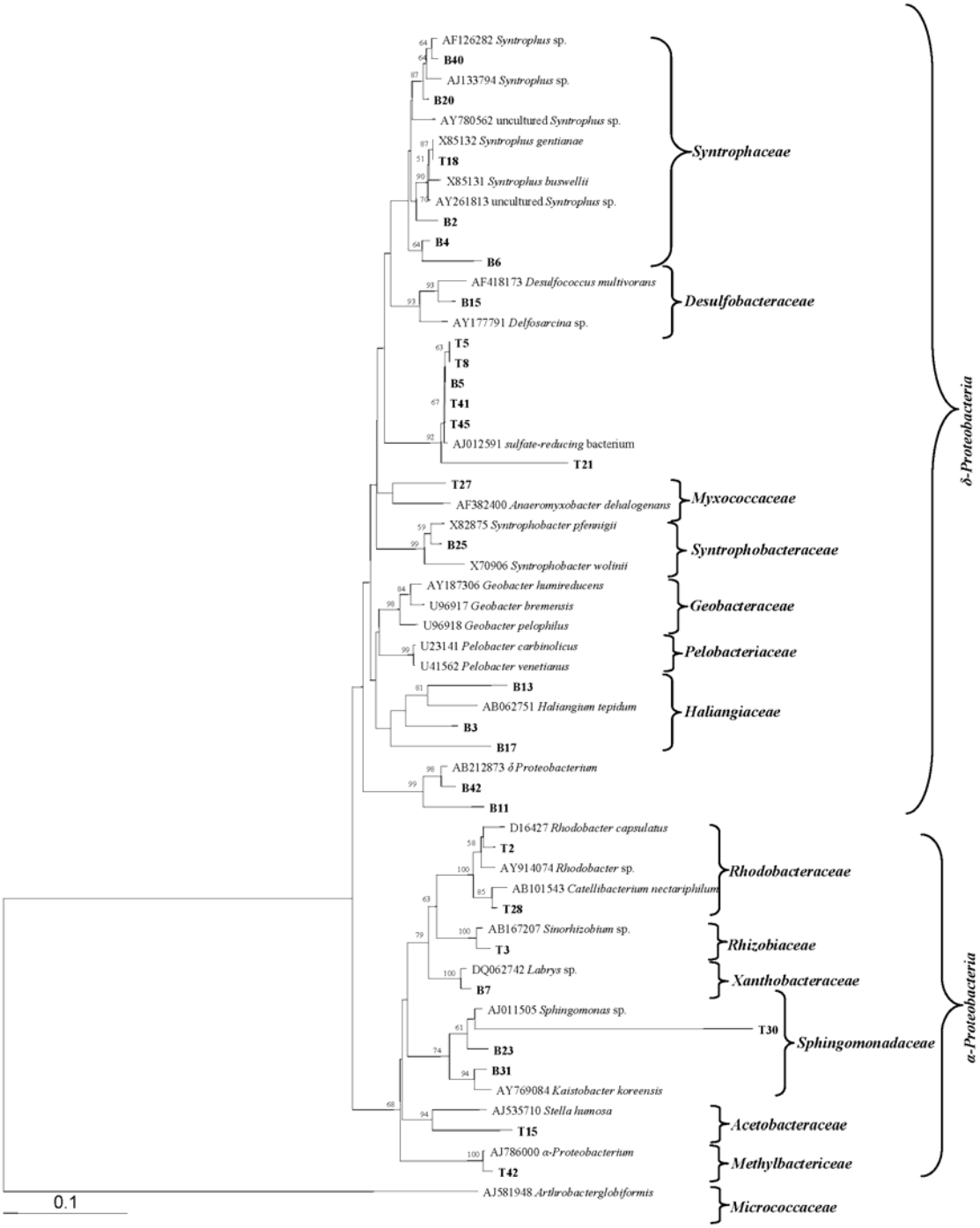

Figure 6.7 - Phylogenetic tree of partial 16S rRNA gene sequences from obtained with the primer pair Syn682F/Syn1196R. The tree was constructed with Phylo_win 2.0 using the neighbour-joining algorithm. The bar indicates 10% sequence divergence. The sequences obtained in the present study are shown in bold, accession numbers of the remaining sequences are indicated. Numbers at nodes represent the bootstrap values (from 1000 replicates; only values >50% are shown). *Arthrobacter globiformis* was used as an outgroup.

Of the 29 sequences, 20 (69%) belonged to *δ-Proteobacteria*, of which six and one clustered with the targeted families *Syntrophaceae* and *Syntrophobacteraceae*, and six others are closely related to an uncultured sulfate-reducing bacterium, with which they might form a so far unnamed family. The presence of the targeted syntrophic groups (and the identified methanogens) shows that the sampled wetland soil has the potential of converting substrates like propionate, pyruvate and aromatic compounds into methane. It has to be mentioned, however, that the newly designed primers are not as specific as expected after database comparisons, even with optimised PCR conditions. In order to prevent the amplification of α-proteobacterial 16S rDNA with primers Syn682F and Syn1196R (31% of the sequences), a recently published method called SuPER (Suicide Polymerase Endonuclease Restriction)-PCR could be applied in future experiments (10).

6.3.4 Comparative bacterial phylogenetics from 16S rDNA libraries

In order to detect possible striking differences in the bacterial composition of the wetland soil between harvest and off-season, 16S rDNA libraries from samples taken in Spring (i.e., non-effluent impacted) and in Autumn (winery effluent impacted) were constructed as described above (section 6.3.2), using the universal bacterial primer set E9F/U1510R. In total, 43 clones were sequenced and phylogenetically characterised (Tables 6.6a and 6.6b, Figure 6.8).

Table 6.6a - BLAST results of 21 partial 16S rDNA sequences obtained from wetland soil samples (surface mud) collected in spring (6 October; non effluent-impacted), using universal bacterial PCR primers E9F/U1510R.

Clone ID	Best hit	% Identity (no. of bp)	Source	Accession no.	Closest cultured and identified species	% Identity (no. of bp)	Accession no.
Mid-S1	Unc. soil bacterium clone L1A.6E11	98 (659)	Soil (Alaska)	AY988982	*Thiobacillus denitrificans* ATCC 25259	95 (659)	CP000116
Mid-S3	*Methylobacter tundripaludum* SV96	98 (701)	Arctic wetland soil (Spitsbergen, Norway)	AJ414655	*Methylobacter tundripaludum* SV96	98 (701)	AJ414655
Mid-S4	Unc. bacterium clone RA13C8	96 (548)	Reactor treating monochlorobenzene contaminated groundwater	AF407407	*Clostridium* sp. P6	94 (550)	AY949857
Mid-S5	Unc. beta proteobacterium clone 44a-B2-3	97 (704)	Biofilm in subsurface acid mine drainage system	AY082472	*Rhodocyclus tenuis* DSM 110	96 (704)	D16209
Mid-S7	Unc. bacterium clone WCHB1-03	95 (728)	Hydrocarbon- and chlorinated-solvent-contaminated aquifer	AF050594	no related isolates described	---	---
Mid-S8	Unc. bacterium clone pLW-101	93 (700)	Lake Washington sediment	DQ066981	no related isolates described	---	---
Mid-S9	Unc. bacterium clone rRNA381	99 (711)	Human vaginal epithelium	AY959154	*Acinetobacter* sp. phenon 8 LUH 4713	99 (711)	AJ633634
Mid-S10	Unc. soil bacterium clone PAH-Bio-12	98 (710)	PAH-contaminated soil	DQ123682	*Nitrosococcus oceani* ATCC 19707	89 (355)	CP000127
Mid-S13	*Spirochaeta* sp. TM3	91 (702)	???	X97096	*Spirochaeta* sp. TM3	91 (702)	X97096
Mid-S14	Arsenic resistant soil bacterium 7-03	99 (710)	Arsenic contaminated smelter wastes	AF308875	*Frateuria* sp. WJ64	96 (687)	AY495957
Mid-S16	Unc. bacterium clone W-Btb7_16	99 (710)	European upland stream	DQ017922	*Xanthomonas oryzae* LMG 5047-T	92 (712)	X95921
Mid-S17	Unc. bacterium clone W4-B31	97 (646)	Lake Waiau sediment (Hawaii)	AY345499	no related isolates described	---	---
Mid-S19	Unc. gamma proteobacterium	94 (682)	Symbiont of a Laxus sp. (nematode) found in Belize	U14727	*Thiococcus* sp. AT2204	93 (683)	AJ401211
Mid-S20	Unc. bacterium clone JH12_C51	95 (715)	Intertidal flat of Ganghwa island (South Korea)	AY568890	no related isolates described	---	---
Mid-S21	Unc. bacterium clone ELB25-087	92 (497)	Lake Bonney (Antarctica), at 25 m depth	DQ015772	no related isolates described	---	---
Mid-S22	Unc. bacterium clone QLS30-B10	97 (707)	Saline Qinghai Lake sediments (China)	AY940544	*Syntrophobac-ter* sp. TsuA1	88 (541)	AJ237605
Mid-S24	Unc. cyanobacterium clone PI_4e4h	98 (728)	Coastal bacterioplankton of Plum Island Sound Estuary (USA)	AY580403	*Synechocystis trididemni*	85 (688)	AB011380
Mid-S28	Unc. bacterium clone FE2MidBac41	92 (677)	Sulfide- and methane-rich cold seep	AY769017	*Flavobacte-rium mizutaii* ATCC 33299T	87 (545)	X67853
Mid-S29	*Roseomonas lacus* TH-G33	99 (683)	Lake Taihu sediments (China)	AJ786000	*Roseomonas lacus* TH-G33	99 (683)	AJ786000
Mid-S30	Unc. Clostridium sp. clone U3A9	90 (506)	Acetate utilizing anaerobic microbial community (Florida)	DQ201601	*Geobacillus gargensis* Ga	90 (192)	AY193888
Mid-S31	Unc. bacterium clone W4-B56	98 (686)	Lake Waiau sediment (Hawaii)	AY345493	*Rubrivivax gelatinosus* OK3O3	97 (679)	AF487435

Table 6.6b - BLAST results of 22 partial 16S rDNA sequences obtained from wetland soil samples (surface mud) collected in autumn (2 March 2005; effluent-impacted), using universal bacterial PCR primers E9F/U1510R.

Clone ID	Best hit	% Identity (no. of bp)	Source	Acc. no.	Closest cultured and identified species	% Identity (no. of bp)	Acc. no.
Mid-A3	*Desulfovibrio* sp. RPf35E1	98 (701)	Reactor treating acidic, metal-containing wastewater	AY548772	*Desulfovibrio desulfuricans* CSN	98 (701)	AF354664
Mid-A4	Unc. bacterium clone EUB8-2	95 (708)	Granular sludge treating cassava starch wastewater	AY693826	*Capnocytophaga* sp. ChDC OS43	86 (486)	AF543293
Mid-A5	Unc. bacterium clone N52	99 (677)	Anaerobic sludge	AB195904	*Propionibacterium lymphophilum* DSM 4903	93 (666)	AJ003056
Mid-A6	Unc. *Olsenella* sp. clone J27	99 (681)	Anaerobic sludge	DQ168843	*Olsenella* sp. S13-10	94 (659)	AY880047
Mid-A7	*Sphingomonas* sp. IW3	100 (655)	???	AB076396	*Sphingomonas* sp. IW3	100 (655)	AB076396
Mid-A8	Unc. bacterium clone 054D07_B_DI_P58	95 (665)	Municipal anaerobic sludge digester	CR933223	*Clostridium viride* T2-7 (DSM 6836)	91 (547)	X81125
Mid-A9	Unc. bacterium clone 20up	99 (711)	Water 20 m upstream of manure	AY212656	*Pseudomonas mendocina* PC1	96 (710)	DQ178219
Mid-A12	Unc. gamma proteobacterium clone MTBII46	100 (685)	Biofilm from brewery bottling plant	AJ715501	*Pseudomonas boreopolis* ATCC 33662T	97 (710)	AB021391
Mid-A13	*Chthoniobacter flavus* Ellin428	91 (684)	Rye grass and clover pasture soil	AY388649	*Chthoniobacter flavus* Ellin428	91 (684)	AY388649
Mid-A14	most probably chimeric sequence						
Mid-A16	Unc. soil bacterium clone PBS-III-16	96 (657)	Flooded rice field	AJ390451	no related isolates described	---	---
Mid-A17	Unc. soil bacterium clone MFC-EB19	98 (673)	Electricity-generating microbial fuel cell	AJ630287	no related isolates described	---	---
Mid-A18	*Pelobacter propionicus* DSM2379	98 (721)	???	X70954	*Pelobacter propionicus* DSM2379	98 (721)	X70954
Mid-A20	Unc. bacterium clone N52	99 (677)	Anaerobic sludge	AB195904	*Propionibact. lymphophilum* DSM 4903	93 (666)	AJ003056
Mid-A21	Unc. clostridia bacterium clone X9Ba77	96 (693)	Anoxic rice field soil	AY607207	*Lachnospiraceae* bacterium 19gly4	87 (359)	AF550610
Mid-A23	Unc. bacterium clone W4-B56	98 (696)	Lake Waiau sediment (Hawaii)	AY345493	*Ideonella* sp. 0-0013	96 (696)	AB211233
Mid-A24	Unc. bacterium clone nsc126	94 (564)	Simanto river system (Japan)	DQ211476	*Tannerella forsythensis* TR6	89 (544)	AB053947
Mid-A25	*Prevotella* sp. HY-36-2	94 (536)	???	AY581270	*Prevotella* sp. HY-36-2	94 (536)	AY581270
Mid-A27	Unc. low G+C Gram-positive bacterium	93 (194)	Coal tar waste-contaminated groundwater	AF351218	*Desulfito-bacterium metallireducens*	93 (192)	AF297871
Mid-A28	Unc. bacterium clone 137	99 (698)	Upper Mystic Lake sediment (USA)	DQ165101	*Anaeromyxobac-ter dehalogenans* 2CP-3	93 (698)	AF382400
Mid-A30	Unc. bacterium clone A1-84	96 (477)	Reactor for nitrogen and phosphous removal	AM180053	no related isolates described	---	---
Mid-A31	Unc. bacterium clone LS4-227	94 (564)	Sediment and soil	AB234247	*Salegentibacter holothuriorum* KMM 3524	86 (487)	AB116148

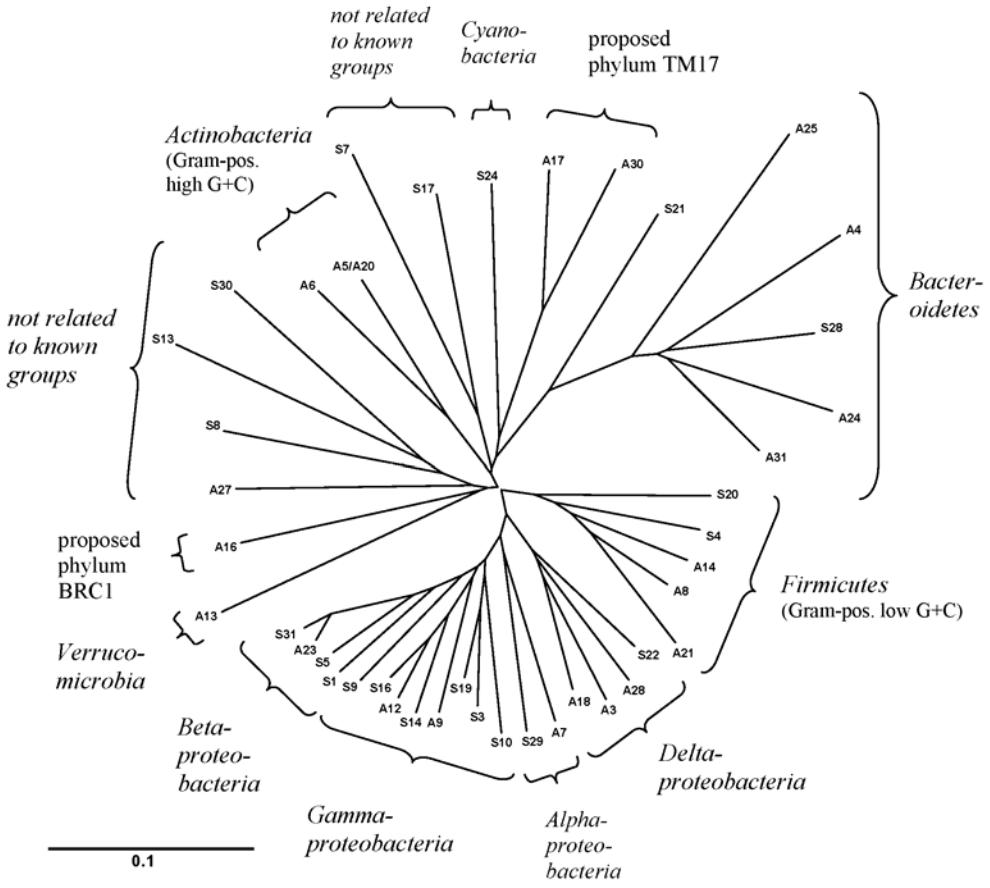

Figure 6.8 - Unrooted phylogenetic neighbour-joining tree based on 43 sequences of a comparative 16S rDNA library from wetland samples (main inflow area, surface). A, sequences retrieved from Autumn samples (2 March 2005, winery effluent-impacted); S, sequences from Spring samples (6 October 2005, non effluent-impacted). The alignment was done with the ClustalX software (34) using the standard parameters as proposed by Hall (12). Regions containing mainly gaps were excluded from the analysis. The scale bar refers to 10 substitutions per 100 bp. The sequences were attributed to the different taxonomic groups using the Classifier software of the Ribosomal Database Project II (6).

As expected, the majority of phylotypes found are closely related to strains recovered from similar environments (like fresh water sediments or anaerobic sludge) from all around the world, showing that the geographical location only has a minor influence on microbial growth compared to the micro-environmental conditions. 70% of the 16S rDNA sequences show less than 95% identity to previously cultured and identified bacteria. They are therefore likely to belong to yet un-described genera, which makes it virtually impossible to speculate on their metabolic properties. As in the previous library (section 6.3.2), members of the phyla *Bacteroidetes* and *Proteobacteria* were also dominant in this comparative analysis. Interestingly, gram-positive bacteria (*Firmicutes* and *Actinobacteria*) were

detected in considerable numbers in this experiment (17%), but not in the first library. They contain species that might be crucial for the function of the wetland: Propionibacteria ferment lactic acid (generated by glucose or ethanol fermentation) to propionic and acetic acid, and different groups of the class *Clostridia* accept a wide variety of substrates (including glucose, fructose, pectin, ethanol and amino acids) to produce hydrogen, CO_2, and butyric and acetic acids. Another example of a potentially important strain in the wetland is Mid-A18: *Pelobacter propionicus* is known to ferment ethanol and related substrates to propionic acid.

Significant differences between harvest and non-harvest season however could not be detected. Because of the extremely high bacterial diversity, far more phylogenetic data would have to be compiled. Nonetheless, this experiment gives us an idea about the bacterial groups present in the functionally active layer of the wetland. Figure 6.9 shows the relative representation of the different taxonomic groups (combined data of all phylogenetic data from the surface layer).

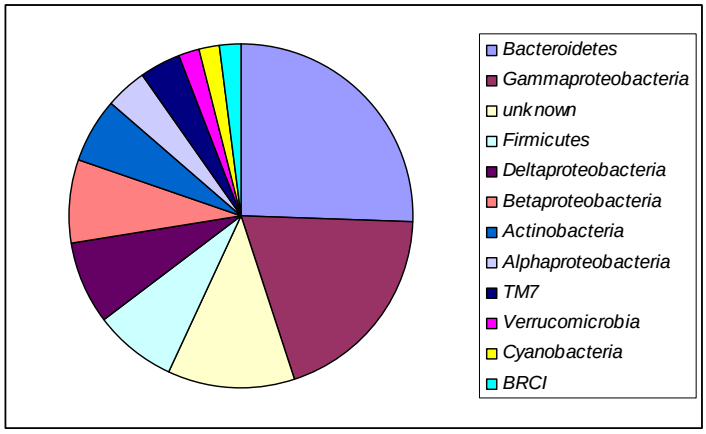

Figure 6.9 - Representation of phylogenetic groups in the surface mud of the winery wetland, as inferred from a total of 51 partially sequenced rRNA genes amplified with universal bacterial primers.

6.3.5 Microbial diversity and (micro-) heterogeneity in the wetland soil

In order to ensure reproducibility and to compare patterns of environmental samples loaded on different DGGE gels, it is necessary to add a standard marker to every set of samples. To prepare such a marker, plasmid DNA was extracted from the wetland 16S rDNA library clones and used as a template in PCR reactions with the primers 341F-GC and 534R. The amplicons were run on a DGGE gel, and as Figure 6.10 shows, one major signal was obtained from each clone (the very weak secondary bands could originate from non-specific fragments amplified during the PCR). The final 13-band marker (see e.g. Figure 6.11) was prepared by mixing the PCR products of the following clones [approximate retardation factors (R_f) relative to *E. coli* in brackets]: Mid-2q (0.31), 3e (0.54), 2a (0.72), 2b (0.74), 3d (0.77), *E. coli* (1.00, stronger band), 3l (1.06), 2c (1.16), 3q (1.18), 2d (1.35), 3n (1.53), 3o (1.64), 2o (1.69).

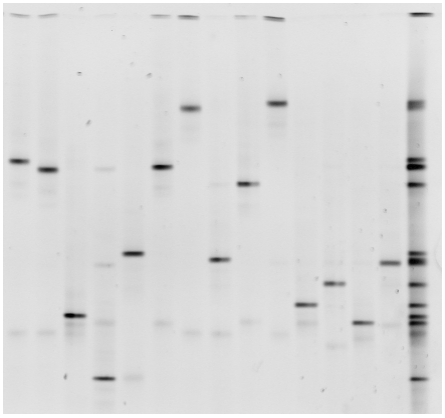

Figure 6.10 - DGGE gel (gradient, 40-60%) of PCR products amplified from wetland clones using primer pair 341F-GC/534R. Lanes, 1-13, clones Mid-2a, 2b, 2c, 2d, 2h, 2i, 2l, 3c, 3d, 3e, 3h, 3l, 3q; 14, *E. coli*; 15, mixture of samples loaded in lanes 1-14.

After the optimal amount of template for the PCR reaction (25 ng) and the ideal range of denaturant concentration in the DGGE gel (40-60% for *Bacteria*, 45-65% for *Archaea*) had been determined for the wetland samples, an experiment was carried out to test the reproducibility of the method. Four replicate DNA extractions were performed from two wetland soil samples (namely 32 and 75, collected on different days and different depths). PCR reactions were done and the amplicons separated by DGGE. No differences could be identified between the replicate patterns, as seen in Figure 6.11. This indicates that the soil samples as well as the metagenomic DNA preparations used are highly homogenous and therefore suitable for further DGGE analyses.

The micro-heterogeneity refers to the differences between replicate soil samples collected on the same day and at the same depth, within the specified sampling area. For the subsequent detection of community composition shifts occurring within weeks or months, it is crucial to first ensure that the differences between such replicates are negligible in comparison to the expected temporal variations.

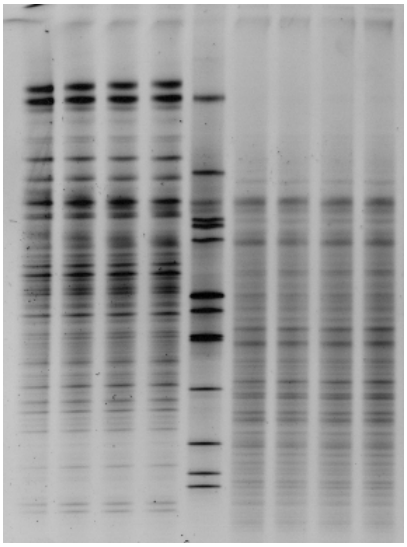

Figure 6.11 - DGGE gel (gradient, 30-70%) of partial 16S rRNA genes amplified from DNA extracted from wetland samples using primer pair 341F-GC/534R. Lanes: 1-4, replicate DNA extractions from sample 32 (wetland surface); 5, marker; 6-9, replicate DNA extractions from sample 75 (ca. 25 cm depth).

For this purpose, two sets of five replicate samples were chosen, collected on different days, one from the wetland surface, one at a depth of ca. 25 cm. The result of the respective PCR-DGGE analysis is depicted in Figure 6.12. There are hardly any qualitative differences within the two sets of banding patterns only the intensities of some signals vary from one sample to another. This indicates that although there is not a complete homogeneity (which can not be expected in a natural environment), the micro-heterogeneity in the sampling area is small enough to allow the subsequent temporal analyses. From Figure 6.12, it can also be concluded that the combination of sampling parameters (soil sample size, homogenisation, amount of soil used for DNA extraction, amount of template in the PCR etc.) is appropriate for the planned experiments.

Despite the very high diversity in both communities (many bands or “operational taxonomic units”, OTUs), Figure 6.11 and Figure 6.12 show clearly the large differences in the composition between the wetland surface and the lower region. There seem to be several common taxons in both layers, but the average G+C content of the 16S rDNA is considerably lower for the surface community (denaturing at lower denaturants concentrations). In addition, there are several bands of high intensity present in the surface samples, while the distribution seems to be much more even around 25 cm below.

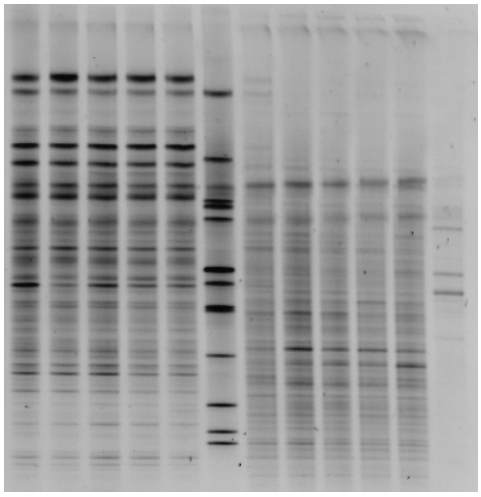

Figure 6.12 – Micro-heterogeneity analysis. DGGE gel (gradient, 30-70%) of partial 16S rRNA genes amplified from DNA extracted from wetland samples using primer pair 341F-GC/534R. Lanes: 1-5, samples 11-15 (wetland surface, 15 Feb 2005); 6, marker; 7-11, samples 36-40 (ca. 25 cm depth, 23 Feb 2005); 12, negative control.

6.3.6 Temporal changes in wetland soil community composition

In order to identify variations in the bacterial community composition from season to season, DNA from soil samples collected during the period of 15 February 2005 to 16 February 2006 was extracted, and the bacterial 16S rDNA gene fragments were PCR-amplified using the universal bacterial primers 341F-GC/534R. An agarose gel with the respective PCR products is shown in Figure 6.13, and the separation of the PCR products by DGGE are depicted in Figure 6.14 (wetland surface samples) and Figure 6.15 (samples from ca. 25 cm depth). The dendogram derived from the DGGE gel shown in Figure 6.14, are depicted in Figure 6.16.

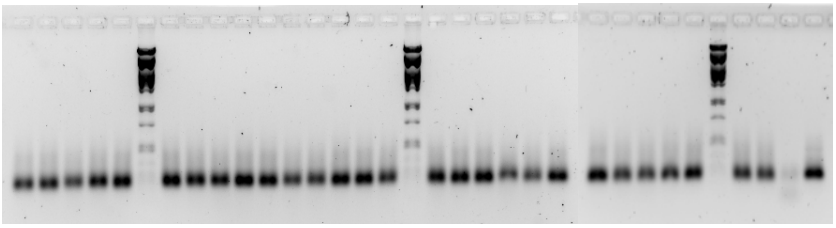

Figure 6.13 - Agarose gel (1%) of partial 16S rRNA genes amplified from DNA extracted from wetland samples using primer pair 341F-GC/534R (selection). Lanes: 1-5, samples 12, 14, 17, 19, and 34; 7-16, samples 35, 37, 40, 41, 43, 46, 48, 53, 54, and 55; 18-28, samples, 57, 61, 62, 63a, 63b, 72, 73, 74, 76, 81, and 83; 30-31, samples 85b and 86; 32, neg. control; 33, pos. control (*E. coli*); 6, 17 and 29, *Pst*I-restricted *λ*-DNA.

Within the sampled period of one year, major changes can be detected in the surface samples: Several initially strong bands disappear, while weak bands become dominant. The relation of strong bands on a gel to dominant species in an ecosystem has to be done with caution, since biases during DNA extraction and PCR amplification can be introduced. In addition, numerically dominant species are not necessarily the metabolically most active. But the fact that most changes in Figure 6.14 occur continuously could indicate that band intensities are directly related to the number of templates and therefore to the population size of the respective species. The comparison of the deeper layer samples gives a much more homogenous picture. Very slight differences in the patterns or intensities of single bands are visible, but no significant systematic changes can be detected. This is a strong indication that major bacterial community changes occur at the surface of the winery wetland, whereas much more stable conditions are found at 25 cm below the surface. This is not unexpected since the effluent flow is virtually restricted to the surface at the sampled wetland entry. Furthermore, environmental factors, e.g. temperature, oxygen and water content, influence from animals and birds, are likely to be much less variable at lower depths.

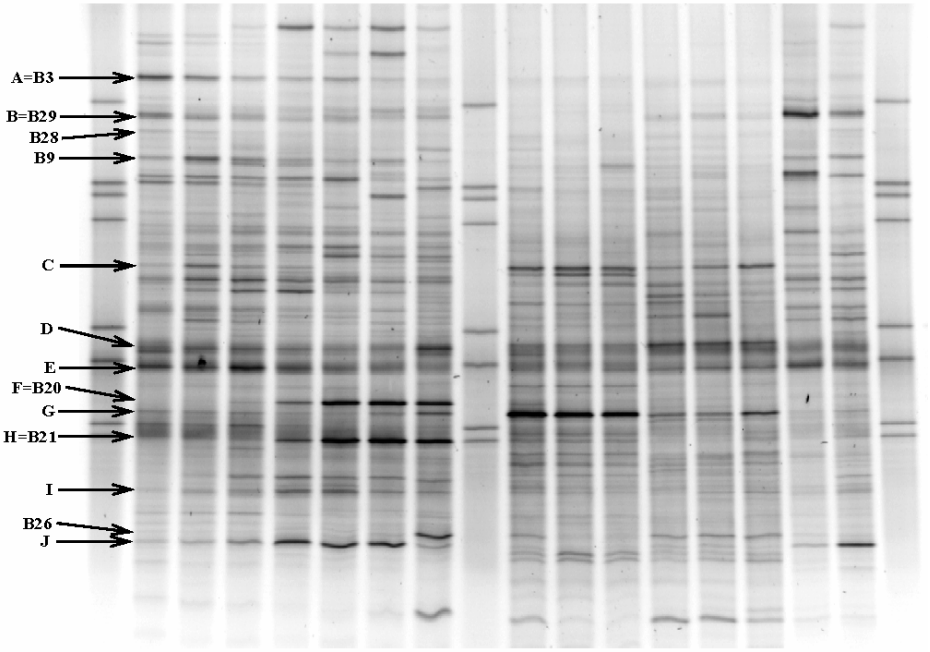

Figure 6.14 - Temporal changes in bacterial community composition (wetland surface). DGGE gel (gradient, 40-60%) of partial 16S rRNA genes amplified from DNA extracted from wetland samples using universal bacterial primers. Lanes: 2, 15 Feb 2005; 3, 23 Feb; 4, 2 Mar; 5, 18 Mar; 6, 2 Apr; 7, 29 Apr; 8, 25 May; 10, 29 Jun; 11, 3 Aug; 12, 8 Sep; 13, 6 Oct; 14, 16 Nov; 15, 22 Dec; 16, 2 Feb 2006; 17, 16 Feb; 1, 9 and 18, marker. The band labels correspond to those used in Figure 6.17 and Figure 6.18.

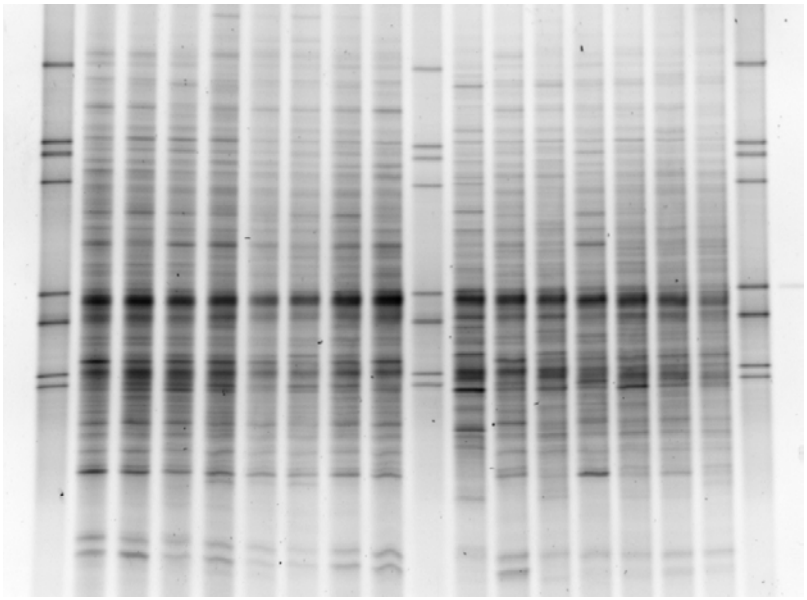

Figure 6.15 - Temporal changes in bacterial community composition (at 20-28 cm depth). DGGE gel (gradient, 40-60%) of partial 16S rRNA genes amplified from DNA extracted from wetland samples using universal bacterial primers. Lanes: 2, 15 Feb 2005; 3, 23 Feb; 4, 2 Mar; 5, 18 Mar; 6, 2 Apr; 7, 29 Apr; 8, 25 May; 9, 29 Jun; 11, 3 Aug; 12, 8 Sep; 13, 6 Oct; 14, 16 Nov; 15, 22 Dec; 16, 2 Feb 2006; 17, 16 Feb; 19, negative control; 1, 10 and 18, marker.

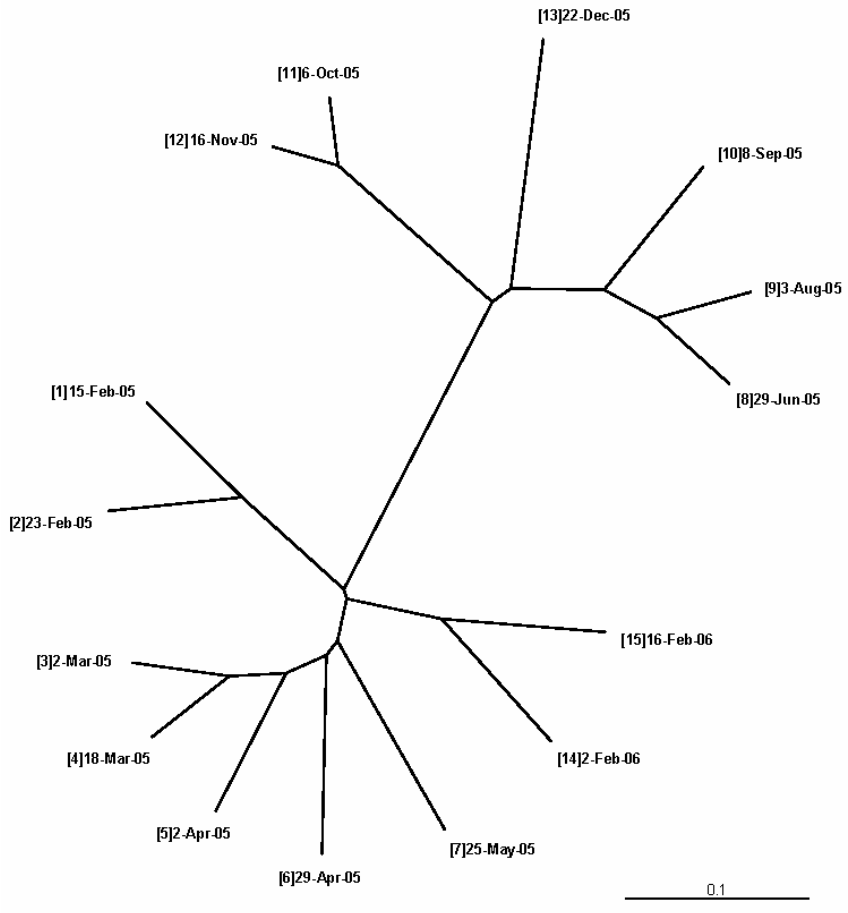

Figure 6.16 - Dendrogram derived from the DGGE gel shown in Figure 6.14 (temporal profile of the bacterial community in the wetland surface). The dendrogram based on the presence or absence of corresponding bands was obtained using the DGGEstat software (Erik van Hannen; available from http://www.sb-roscoff.fr/marine_microbes). The distance matrix was calculated using the Dice coefficient, and the clustering was done with the UPGMA (Unweighted Pair Group Method with Arithmetic Mean) method.

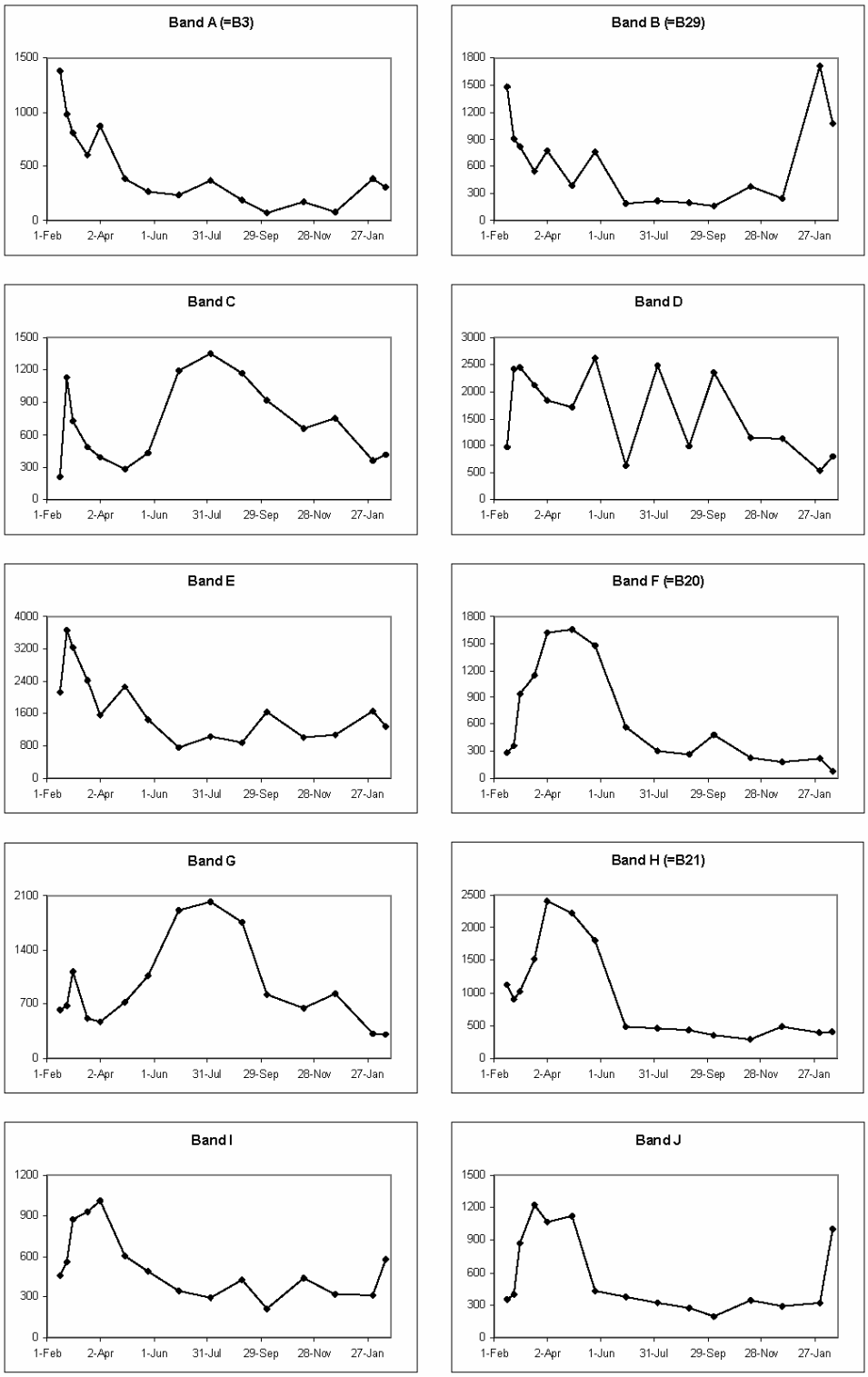

Figure 6.17 - Intensity changes of selected DGGE bands of Figure 6.14, which were obtained by amplification of bacterial 16S rDNA from the wetland surface between February 2005 and February 2006. The analysis is based on the peak area (Y axis) calculated from the digitalised gel image.

Since the community shifts observed in the surface mud seem to occur continuously and do not clearly correlate with the harvest period, it cannot be maintained that they are related to the amount of winery effluents flowing into the wetland. There are of course many other seasonal factors that can influence the microbial composition, such as temperature, plant growth, animals etc. It was hoped, however, that by excising and sequencing the most 'interesting' bands from the gel, it would be possible to assign them to specific taxonomic groups and therefore to estimate their putative metabolic role in the wetland soil. Twenty bands were cut out from the DGGE gel (Figure 6.18) and the eluted DNA cloned in *E. coli*. This was found to be very challenging due to the enormous number and density of bands. The procedure is definitely only suited in cases of low diversity, where the fragments are well separated, for example when specific taxonomic groups are being investigated. Of the 20 bands, 11 could finally be cloned and sequenced successfully. The respective BLAST results are listed in Table 6.7.

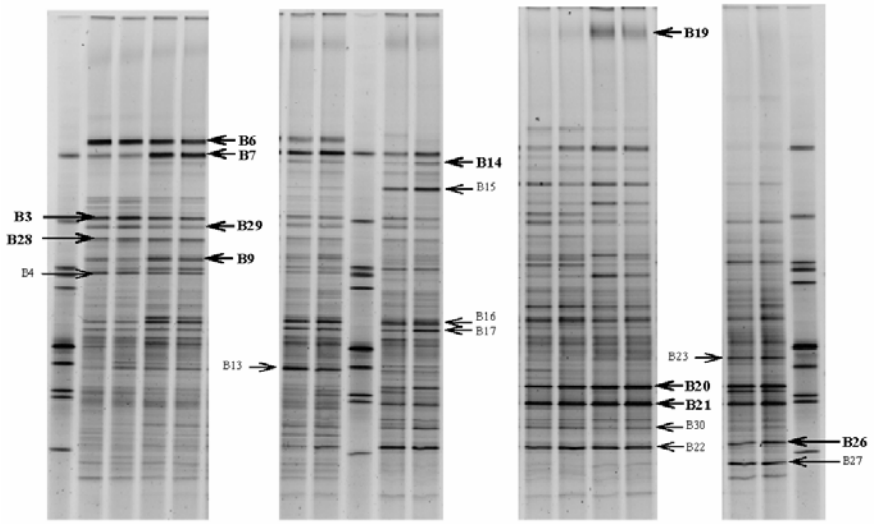

Figure 6.18 - DGGE gel (gradient, 30-60%) reflecting the bacterial community in the wetland surface at different time points (selection of samples depicted in Figure 6.14). All labelled bands were cut out from the gel and cloned. Sequences were obtained from the bands labelled in bold. N.B.: More bands are visible in this gel compared to Figure 6.14 because of the wider denaturing gradient range applied.

Table 6.7 - BLAST results of the sequences of 16S rDNA fragments recovered from a DGGE gel. The numbers correspond to those used in Figure 6.18.

Clone ID	Best hit	% Identity (no. of bp)	Source	Accession no.	Closest cultured and identified species	% Identity (no. of bp)	Accession no.
Mid-B3	Unc. bacterium, DGGE band UTFS-OF09-02 and Unc. bacterium, clone UTFS-OF09-d22-03	100 (135)	Anaerobic aerobic sequencing batch reactor, fed with acetate Activated sludge from lab-scale anaerobic aerobic sequencing batch reactor, fed with glucose	AB106408 AB200301	Wolinella succino-genes cp02.17	87 (132)	AY827911
Mid-B6	Unc. Bacteroidaceae bacterium, clone:Rs-N41	92 (146)	Gut homogenate from termite (Reticulitermes speratus)	AB088947	Bacteroides goldsteinii WAL 12034	91 (146)	AY974070
Mid-B7	Unc. bacterium, clone nsc126	90 (155)	Shimanto River system (Japan)	DQ211476	Tannerella forsythensis 338	89 (155)	L16495
Mid-B9	Sulfurospirillum sp. EK7 and many unc. bacteria, isolated from river or lake sediments, groundwater, anaerobic digester or mangrove plancton	99 (135)	River sediment (Germany)	AJ535704 and AB237694 AY756183 AY756596 AY780560 DQ191241 DQ227583 DQ228219 DQ234114 DQ234133 DQ234203 DQ234216 DQ234236 DQ234237 L14632	Sulfuro-spirillum sp. EK7 and strain JPD-1 and Campyloba-cter sp. DSM 806	99 (135)	AJ535704 AY189928 AF144694
Mid-B14	Prevotella sp. N12-20, N19-22, and N19-31 and many unc. bacteria isolated from human lung	95 (155)	Non-tumourous mucosal tissue from a patient with oral squamous cell carcinoma	AY880052 AY880053 AY880054 and AF385551 AY9577 AY806234 AY806417 AY806424 AY806453 AY807021 AY807028 AY807074 AY807628 AY807654 DQ054709	Prevotella sp. N19-31	95 (155)	AY880054
Mid-B19	Unc. bacterium, clone 118ds10	98 (155)	Water 10 m downstream of manure	AY212569	Paludibacter propionici-genes (Bacteroida-les sp. WB4)	97 (155)	AB078842
Mid-B20	Human oral bacterium C65	98 (155)	Human	AF201972	Prevotella oris ATCC 33573	96 (155)	L16474

Table 6.7 (continued) **-** BLAST results of the sequences of 16S rDNA fragments recovered from a DGGE gel. The numbers correspond to those used in Figure 6.18.

Clone ID	Best hit	% Identity (no. of bp)	Source	Accession no.	Closest cultured and identified species	% Identity (no. of bp)	Accession no.
Mid-B21	Human oral bacterium C65	98 (155)	Human	AF201972	Prevotella oris ATCC 33573	96 (155)	L16474
Mid-B26	Unc. bacterium, clone FOTU9(2-6) and unc. bacterium, clone Qui2P2-58 and unc. bacterium, clone P1CN57	100 (160)	Bioreactor pretreating potable water Sediment Sewage sludge	DQ066681 AJ518511 AJ504518	Dechloromo-nas sp. MissR	99 (160)	AF170357
Mid-B28	Unc. bacterium, DGGE_band_B09	100 (135)	Lagoon (Japan)	AB158693	no related bacteria described	---	---
Mid-B29	Unc. bacterium, clone nsc128	99 (153)	Shimanto River system (Japan)	DQ211478	Bacteroides sp. ECP-C1	93 (155)	AF529225

The fragments were found to originate from only three different major bacterial groups. Since only about 150 bp can be used for database comparisons, accurate phylogenetic allocations are not possible. The sequences of most bands (B6, B7, B14, B19, B20, B21, and B29) were found to be similar to 16S rDNA genes from members of the order *Bacteroidales*, which was well represented in the analysed clone libraries. Species of the genera *Bacteroides* and the closely related *Prevotella* and *Tannerella* are mainly known to be human-associated. However, as mentioned in section 6.3.2, a similar bacterium has recently been isolated from a reactor treating brewery wastewater. Bands B3 and B9 are most closely related to *ε-Proteobacteria*. Interestingly, members of this group were not found in previous experiments of this study. Band B28 can be affiliated with the proposed phyla TM7, of which no representatives have been cultured so far; their metabolic activities are therefore unknown.

Archaeal microorganisms are less likely to be the primary degraders of winery waste. However, as shown above, the methanogenic *Archaea* play a key role in the last step of anaerobic decomposition of organic matter, and different groups of methanogens dominate at different concentrations of substrates such as acetate. The experiment was done in analogy to the bacterial analysis. A nested PCR approach was applied, using primers A3Fa/Ab927R for the first round and the DGGE primers A340F-GC/A533R for the second round. The results obtained are shown in Figure 6.19 (wetland surface samples) and Figure 6.20 (samples from ca. 25 cm depth). The dendogram derived from the DGGE gel shown in Figure 6.19 is depicted in Figure 6.21, while the intensity changes of selected DGGE bands of Figure 6.19 are depicted in Figure 6.22.

With between 30 (surface samples) and 50 (deeper layer) distinguishable bands, the archaeal diversity in the wetland soil is much lower than the bacterial. This was expected, since *Archaea* normally form a minority among the soil microorganisms. The banding patterns of the two gels (surface and deeper

layer) look considerably different, which reflects the different community compositions at different depths, probably due to the different growth conditions (oxygen, substrates, irradiation etc.).

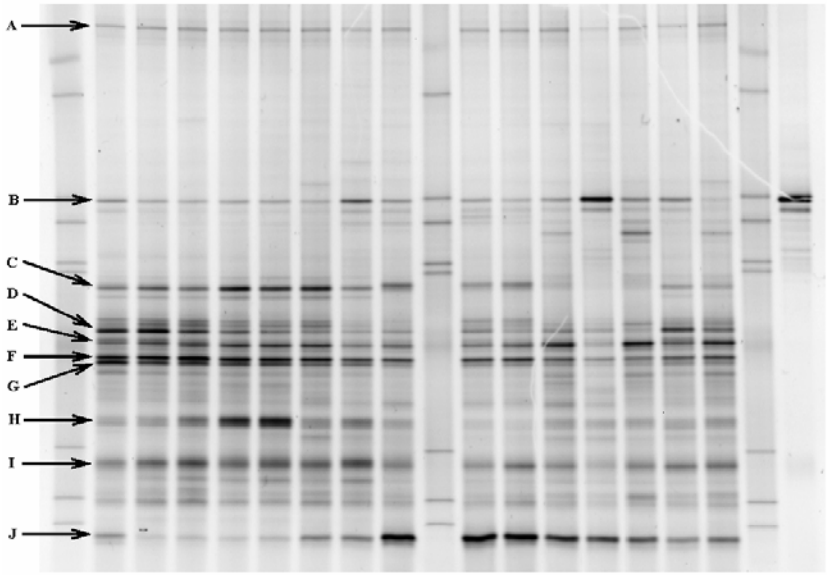

Figure 6.19 - Temporal changes in archaeal community composition (wetland surface). DGGE gel (gradient, 45-65%) of partial 16S rRNA genes amplified from DNA extracted from wetland samples using universal archaeal primers. Lanes: 2, 15 Feb 2005; 3, 23 Feb; 4, 2 Mar; 5, 18 Mar; 6, 2 Apr; 7, 29 Apr; 8, 25 May; 9, 29 Jun; 11, 3 Aug; 12, 8 Sep; 13, 6 Oct; 14, 16 Nov; 15, 22 Dec; 16, 2 Feb 2006; 17, 16 Feb; 19, negative control; 1, 10 and 18, marker. The band labels correspond to those used in Figure 6.22.

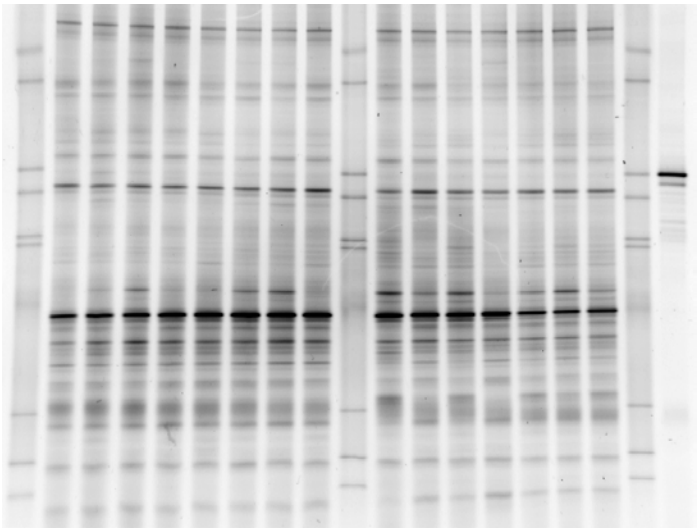

Figure 6.20 - Temporal changes in archaeal community composition (at 20-28 cm depth). DGGE gel (gradient, 45-65%) of partial 16S rRNA genes amplified from DNA extracted from wetland samples using universal archaeal primers. Lanes: 2, 15 Feb 2005; 3, 23 Feb; 4, 2 Mar; 5, 18 Mar; 6, 2 Apr; 7, 29 Apr; 8, 25 May; 9, 29 Jun; 11, 3 Aug; 12, 8 Sep; 13, 6 Oct; 14, 16 Nov; 15, 22 Dec; 16, 2 Feb 2006; 17, 16 Feb; 19, negative control; 1, 10 and 18, marker.

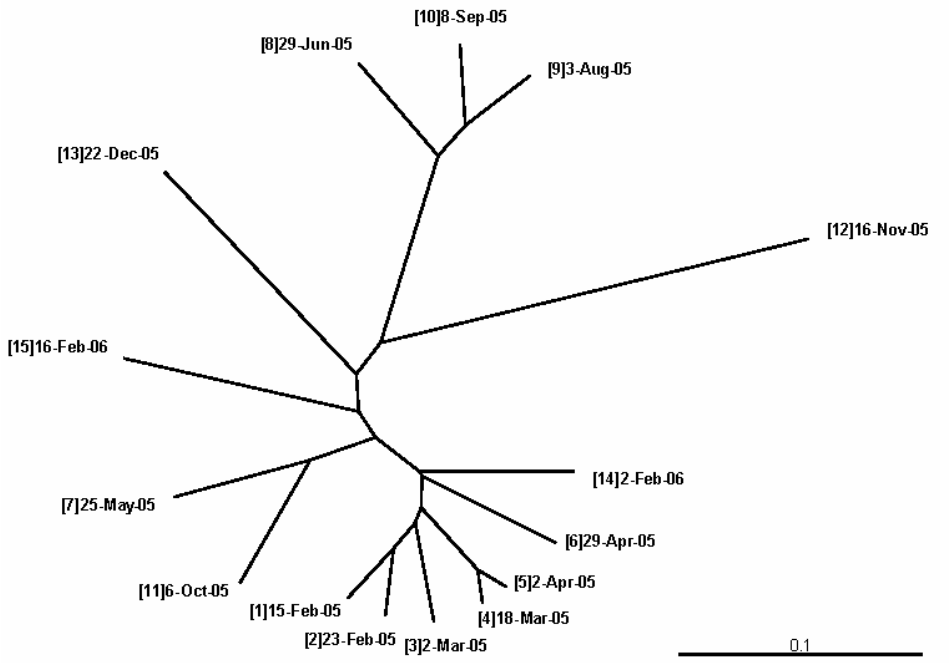

Figure 6.21 - Dendrogram derived from the DGGE gel shown in Figure 6.19 (temporal profile of the archaeal community in the wetland surface). The dendrogram based on the presence or absence of corresponding bands was obtained using the DGGEstat software (Erik van Hannen; available from http://www.sb-roscoff.fr/marine_microbes). The distance matrix was calculated using the Dice coefficient, and the clustering was done with the UPGMA (Unweighted Pair Group Method with Arithmetic Mean) method.

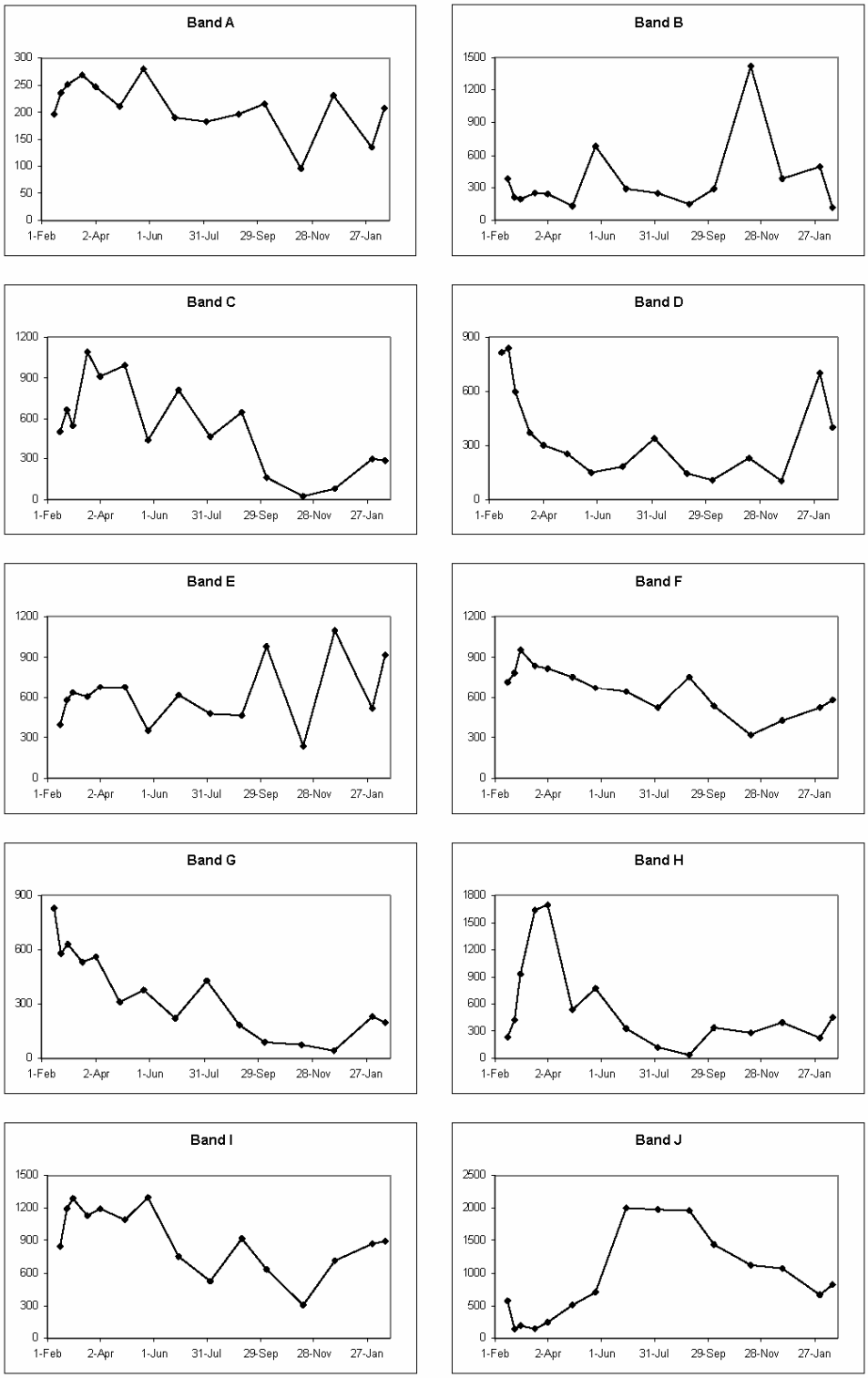

Figure 6.22 - Intensity changes of selected DGGE bands of Figure 6.19, which were obtained by amplification of archaeal 16S rDNA from the wetland surface between February 2005 and February 2006. The analysis is based on the peak area (Y axis) calculated from the digitalised gel image.

6.3.7 Overview of trophic interactions in the winery wetland

The phylogenetic data so far collected from the wetland show and confirm the potential of the present microbial community to degrade the main constituents of winery effluents, including glucose, fructose, ethanol, and phenolic substances. Figure 6.23 gives an overview of the trophic interactions that are likely to occur in this anaerobic environment, together with examples of microorganisms possibly involved and found in this study. Not included in the figure are the aerobic degradative processes that can take place at the very surface of the wetland, by which a wide variety of substrates are completely oxidised to carbon dioxide (e.g. by the ubiquitous pseudomonads).

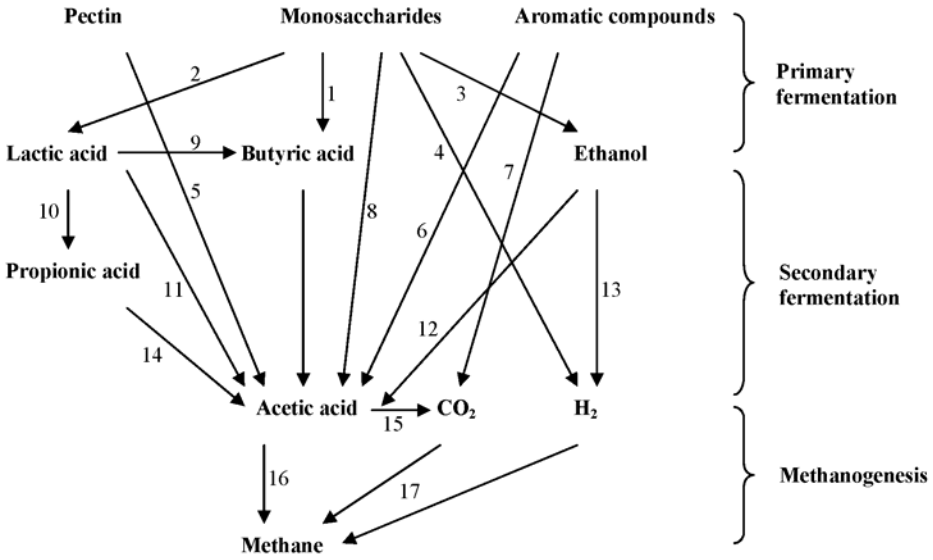

Figure 6.23 - Overview on the trophic interactions likely to contribute to the anaerobic degradation of winery wastewater constituents in the wetland. The following microorganisms that have already been identified in this study might be involved in the specific steps: 1, *Clostridium*; 2, *Citrobacter*, *Acinetobacter*; 3, *Citrobacter*; 4 and 5, *Clostridium*; 6, *Syntrophus*, *Acinetobacter*, *Rhodocyclus*; 7, *Syntrophus*; 8, *Clostridium*, *Frateuria*; 9, *Clostridium*; 10, *Propionibacterium*; 11, *Citrobacter*; 12, *Pelobacter*, *Frateuria*; 13, *Clostridium*, *Pelobacter*; 14, *Syntrophobacter*; 15 and 16, *Methanosarcinales*; 17, *Methanomicrobiales*.

6.4 Conclusions and trends observed

- Community DNA was successfully extracted from winery wetland sediment samples taken at regular intervals throughout a full yearly cycle.
- Analysis of bacterial molecular phylogenetic markers (16S rRNA gene sequences) identified a wider range of aerobic and anaerobic microbial phylotypes.
- Amplification with archaeal-specific 16S primers indicated that the anaerobic sediments (all but the upper few mm of the sediment cores) contained a wide variety of methanogenic archaea.

- Organisms known to have a major role in degrading ethanol (e.g., *Pelobacter propionicus*) were identified in core samples.
- 16S libraries did not provide sufficient data for significant differences in species distribution to be detected between samples recovered during harvest, and in the 'off-season'.
- Analysis of microbial species distribution using DGGE proved to be a reliable and effective means of comparing seasonal changes.
- While most phylotypes were visible through the annual sampling cycle, significant changes in a number of phylotypes, associated with the seasonal harvest, were identified.
- Changes in archaeal populations suggest that these anaerobic organisms play a major role in secondary degradation of winery wastes, and may be a major contributor to carbon loss (as methane) from the system.
- Many phylotypes showing major quantitative seasonally-related changes belonged to uncultured microbial groups.

The use of molecular phylogenetics to assess changes in microbial population structure in relation to significant environmental impacts (seasonal release of winery waste) demonstrates the strengths and weaknesses of this technology.

Positive: Molecular methods are capable of identifying a vastly greater range of microorganisms than can be identified by culture-dependent methods.

Dominant organisms identified by molecular phylogenetics are frequently not those identified by culturing.

Key organisms may be identified, providing avenues for further characterization of their *in situ* function.

Weakness: Many of the key phylotypes identified are assignable only as 'uncultured organisms'. In consequence, little to nothing can be said about their physiology.

An open wetland is a multi-variable system, and population changes are suggestive, rather than direct proof, of a role in degradation of winery waste products.

6.5 References

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CHAPTER 7
INTEGRATION OF RESULTS FOR DESIGN OF WETLANDS EFFECTIVE IN TREATING WINERY WASTEWATER

7.1 Correlation of chemical, biochemical and microbial information

Enzyme activity measurement and correlation with the microbial populations being detected was considered in two phases in the course of the project. In the early approach, various methods were employed to maximise enzyme detection, including the manipulation of enzyme to reaction mixture ratio, concentration of the proteins detected in the samples, and the analysis of enrichment cultures. A limited degree of success was obtained, with the detection of alcohol dehydrogenase, esterase, glucose-6-phosphate dehydrogenase, laccase and peroxidase activities in the various samples. The second phase, based on data collected in the early 2006 season, was more successful. A new approach was developed in the analysis of the new soil samples collected from the two sites of interest. Soil samples were used directly in the detection of enzyme activity through the incubation of the soil samples with the substrate/reaction mix used in the detection of specific enzymes. Although the levels of activity detected were low, the following enzyme activities were positively demonstrated: alcohol dehydrogenase, catechol 1,2-dioxygenase, catechol 2,3-dioxygenase, glucose-6-phosphate dehydrogenase, laccase, peroxidase and polyphenol oxidase (tyrosinase).

Further, an attempt was made to correlate the bacterial/archaea genera detected within the natural wetlands soil samples with the enzyme activities detected. Twenty-three of the fifty-five genera identified, are known for their ability to produce some of the enzymes assayed for. The results obtained therefore suggest that the organisms detected are involved in the biodegradation of the winery waste that flows into the natural wetlands. Preliminary genera identification results from the natural wetland were presented in Table 7.1 below and tentative correlation of enzyme activity observed to genera detected in the NW samples is proposed. Certain specific examples can be considered: The growth of *Acinetobacter* sp. is stimulated by the presence of *Sacchromyces cerevisiae* due to the ethanol production of that yeast (42). This does not correlate with the lowered alcohol dehydrogenase activity found in the samples of 14/06/2005, in which the fermentation smell associated with yeast was noted. It is interesting to note, is the presence of bacteria that may be causative agents of periodontal/endodontal diseases (Table 7.1). This could be explained if the winery is disposing of the contents of spittoons used during wine tasting into the natural wetlands as well. The total lack of detection of any actinomycetes was also interesting, since actinomycetes are well known for their predominance in various types of soils. All of the phylogenetic information given in this report came from samples taken from the natural wetlands area. It would be interesting to determine whether a

similar type of bacterial/archaeal populations would be observed in the constructed wetlands, which would give answers to the questions of whether we would need to seed artificial wetlands with specific bacteria or whether other populations have the ability to perform a similar role in the constructed wetlands where existing populations have adapted to the changing environment. The differences observed in enzyme activity in itself would suggest that the two environments would have different predominant bacterial/archaeal populations a fact which would also be influenced by the type of soil, fauna, flora and other external environmental factors at the two sites of interest.

Table 7.1 - Correlation of bacterial/archaeal genera tentatively identified to be present in soil samples analysed by DGGE and enzyme activity detected in samples

Bacterial and archaeal genera represented in soil samples	Enzymes produced by identified genus	Enzyme activity detected	Comments
Acidobacteria strain			Class of bacteria which include the genera *Acidobacterium*, *Geothrix* and *Holophaga* * *Acidobacterium* spp. produces various hydrolases[a] * *Geothrix* spp. are involved in Fe (III) reduction[b] **Holophaga* spp. perform *o*-demethylation reactions[c]
Acinetobacter	Esterase[1], alcohol dehydrogenase[11], glucose oxidase, lipase, catechol 1,2-dioxygenase	Esterase, alcohol dehydrogenase, glucose oxidase, catechol 1,2-dioxygenase	
Anaeromyxobacter	G-6-PD	G-6-PD	
Bacteroides	Esterase[4], G-6-PD[9]	Esterase, G-6-PD	
Campylobacter	Glucose oxidase, alcohol dehydrogenase	Glucose oxidase, alcohol dehydrogenase	
Capnocytophaga			Causes periodontal diseases[d]
Catellibacterium			
Chthoniobacter			Not a validly published genus*
Citrobacter	Esterase[5]	Esterase	
Clostridium	Lipase, glucose oxidase, G-6-PD, esterase, alcohol dehydrogenase	Glucose oxidase, G-6-PD, esterase, alcohol dehydrogenase	
Cytophaga	Esterase[3], Alcohol dehydrogenase[7]	Esterase, Alcohol dehydrogenase	
Dechloromonas			Perchlorate reducing bacteria[e]
Desulfitobacterium			Performs reductive dechlorination[f]
Desulfovibrio	Alcohol dehydrogenase	Alcohol dehydrogenase	
Fibrobacter	Esterase[6]	Esterase	
Flavobacillus			Not a validly published genus*
Flavobacterium			Produces laccase-like nucleoside oxidases[g]
Frateuria	Catechol 1,2-dioxygenase	Catechol 1,2-dioxygenase	
Geobacillus	Lipase, esterase, catechol 2,3-dioxygenase, alcohol dehydrogenase	Esterase, catechol 2,3-dioxygenase, alcohol dehydrogenase	
Ideonella			Performs degradation of chlorate and perchlorate[h]
Labrys			Facultative methylotroph[i]
Lachnospira			Pectin-degrading rumen bacteria[j]

Methanobacterium	Alcohol dehydrogenase	Alcohol dehydrogenase	
Methanogenium	Alcohol dehydrogenase	Alcohol dehydrogenase	
Methanosaeta			Methanogen[k]
Methylobacter	Alcohol dehydrogenase	Alcohol dehydrogenase	
Nitrosococcus	G-6-PD, esterase, catechol 1,2-dioxygenase	G-6-PD, esterase, catechol 1,2-dioxygenase	
Olsenella			Associated with endodontic diseases[l]
Ornithobacterium	Laccase[8]	Laccase	Causes diseases in ducks and other birds[m]
Oxalobacter			Enteric bacteria involved in oxalate metabolism[n]
Paludibacter			Propionate-producing bacteria[o]
Pelobacter	G-6-PD	G-6-PD	
Prevotella			Opportunistic human pathogens[p]
Propionibacterium	G-6-PD, esterase, alcohol dehydrogenase	G-6-PD, esterase, alcohol dehydrogenase	
Pseudomonas	Esterase[1], laccase[2], alcohol dehydrogenase[10], glucose oxidase, lipase, G-6-PD, catechol 1,2- and 2,3-dioxygenase	Esterase, laccase, alcohol dehydrogenase, glucose oxidase, G-6-PD, catechol 1,2- and 2,3-dioxygenase	
Rhodobacter	G-6-PD, catechol 2,3-dioxygenase, alcohol dehydrogenase	G-6-PD, catechol 2,3-dioxygenase, alcohol dehydrogenase	
Rhodocyclus			Perform the degradation of anthaquinone dyes[q]
Riemerella			Causes diseases in ducks and other birds[r]
Roseomonas			Opportunistic human pathogens[s]
Rubrivivax			Photosynthetic bacteria[t]
Salegentibacter			Marine bacteria[u]
Sinorhizobium	Laccase, G-6-PD	Laccase, G-6-PD	Renamed to the genus *Ensifer**
Smithella			Propionate-degrading bacteria[v]
Sphingomonas	Alcohol dehydrogenase	Alcohol dehydrogenase	
Spirochaeta			Associated with plants and algae[w]
Stella			-
Sulfurospirillum			Arsenate-reducing bacteria[x]
Synechocystis	G-6-PD	G-6-PD	
Syntrophus			Members of this genus has the ability to degrade long-chain fatty acids, e.g. decanoic acids[y]
Tannerella			Periodontal pathogens[z]
Thiobacillus			Perform H_2S oxidation[A]
Thiococcus			Perform sulphur oxidation[B]
Verrucomicrobium			-
Wolinella			Non-pathogenic host-associated oragnisms[C]
Xanthomonas	Laccase, G-6-PD, esterase, alcohol dehydrogenase	Laccase, G-6-PD, esterase, alcohol dehydrogenase	

* Information obtained from: Euzéby (14)

- Wording in red = information obtained from "The Comprehensive Enzyme Information System" (http://www.brenda.uni-koeln.de)

[1]: Bornscheuer, (5); [2]: Solana *et al.* (43); [3]: Jager *et al.* (23); [4]: Rudek & Haque (39); [5]: Kumar *et al.* (29); [6]: McDermid *et al.* (35); [7]: Kazuoka *et al.* (26); [8]: Jansen *et al.* (24); [9]: Shah & Williams (41); [10]: Gorisch (15); [11]: Smith *et al.* (42); [a]: Inagaki *et al.* (19); [b]: Coates *et al.* (10); [c]: Kreft & Schink (28); [d]: Douvier *et al.* (12); [e]: Bender *et al.* (3); [f]: Lee *et al.* (30); [g]: Koga *et al.* (27); [h]: Karlsson & Nilsson (25); [i]: Miller *et al.* (36); [j]: Duskova & Marounek (13); [k]: Ma *et al.* (32); [l]: Rocas & Siqueria (38); [m]: Canal *et al.* (8); [n]: Stewart *et al.* (44); [o]: Ueki *et al.* (47); [p]: Berger *et al.* (4); [q]: Dong *et al.* (11); [r]: Higgins *et al.* (17); [s]: Nolan & Waites (37); [t]: Tanskul

et al. (46); [u]: Ivanova *et al.* (21); [v]: Liu *et al.* (31); [w]: Magot *et al.* (34); [x]: Stolz *et al.* (45); [y]: Jackson *et al.* (22); [z]: Inagaki *et al.* (20); [A]: Ma *et al.* (33); [B]: Imhoff *et al.* (18); [C]: Baar *et al.* (2).

Based on the enzyme activities detected and the microbial phylogenetics results, it is also possible to propose a tentative correlation as illustrated in Figure 7.1 below.

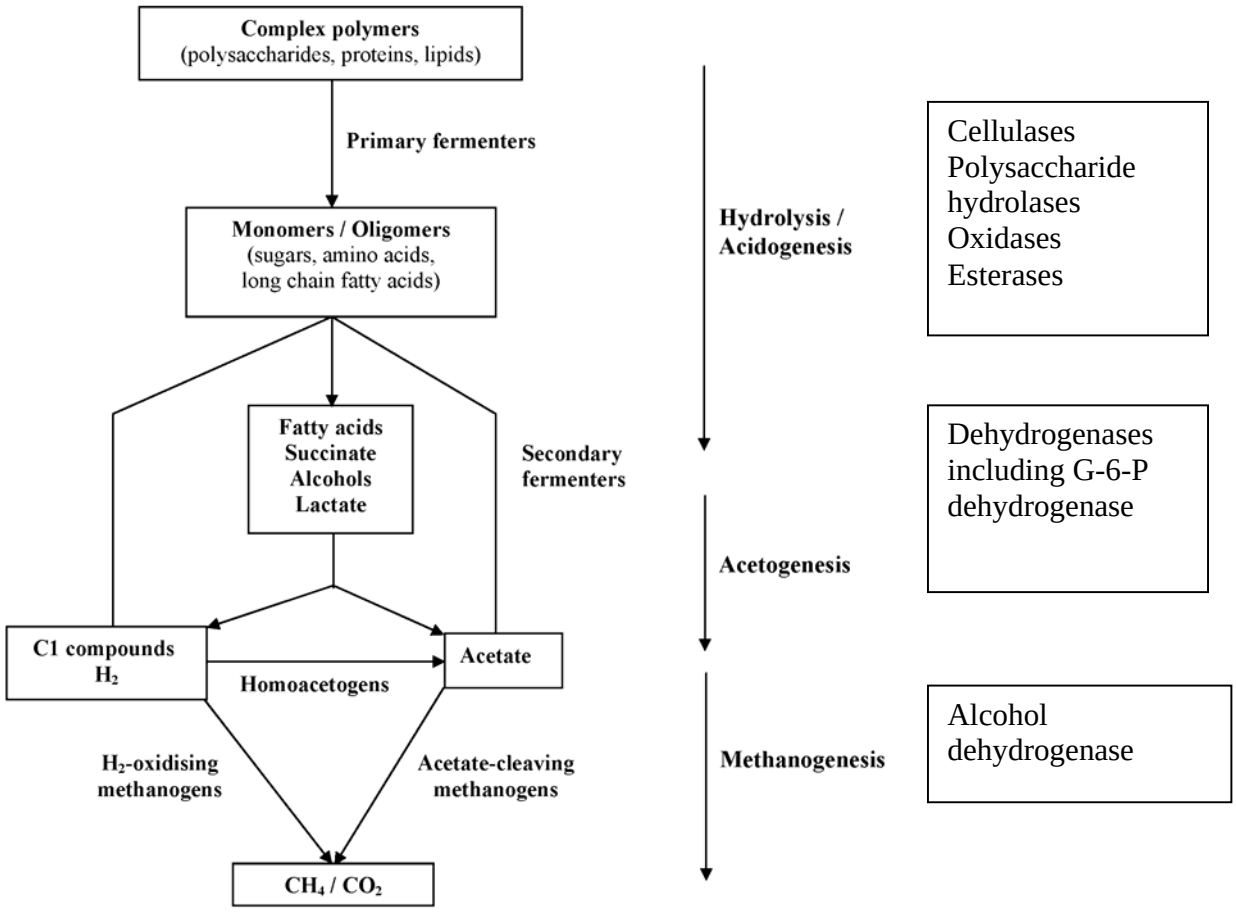


Figure 7.1 - Correlation of enzyme activities and microbial groups

7.2 Conclusions on relationships between chemical, biochemical and microbial data and implications for wetland design

This feasibility study has demonstrated an approach to monitoring wetlands used to reduce the pollutant load of wastewaters in wetlands. Chemical and biochemical analyses and molecular phylogenetics were used in preliminary characterization of the active microbial population, and in the development of a predictive model of degradation rates in a wetland exposed to winery wastewater. It is clear from this study that the impact of pollutant addition on natural microbial populations can be demonstrated by molecular methods. These methods allow us to monitor the survival, and more importantly, the health of the microbial population responsible for the biodegradation of the impacting pollutant. However, natural microbial communities are complex, containing hundreds or thousands of individual species, in multiple trophic layers of primary, secondary and tertiary degraders. Different microorganisms degrade different classes of compounds, and not all the species are necessarily directly involved in the primary degradation process. It is recognised that in degradative niches (such as wetland sediments) relatively few species are directly involved in primary degradation of pollutants and a very limited number of species may dominate and this study supports this. Thus, definitive correlation of chemical composition with microbial and/or enzymatic activity is extremely challenging.

We conclude that while this project has established useful methodologies and the approaches are useful more in-depth study is required in order to effectively design wetlands for optimal microbial activity, as follows:

(1) The effect of pollutants on wetland microbial population distributions must be better understood in order to manage wetlands' effectiveness in reducing pollution of local water resources.

(2) The effect of pollutants on the metabolism of microbial populations in wetlands must be better understood in order to ensure that the requisite enzymes are expressed and active when xenobiotics are present.

(3) Constructed wetlands provide suitable cheap, low-tech, environmentally sustainable systems for reducing the impact of the pollutants on surface and ground water but we need to know how more about how they can be adapted, to effectively remediate the pollutants impacting them

CHAPTER 8
CONCLUSIONS AND RECOMMENDATIONS

8.1 Key findings

This project has been conducted as a feasibility study, to determine whether it is feasible to use the type of data generated in this study, to facilitate the modelling and effective design of wetlands. This study has demonstrated that the approach is successful, but there is a great deal more that can now be achieved using the technology and understanding that have been developed.

The results obtained confirm that the biodegradation of winery waste taking place in the wetland can be effectively characterised. The degradation of the main constituents of winery effluents (glucose, fructose, ethanol, and phenolic substances) has been characterised in the detailed analysis and this information has been used as the basis for the model of the wetland biodegradation.

The microbial community present in the wetlands can be effectively characterised using phylogenetic techniques. The anaerobic metabolic (enzymatic) activities occurring have been detailed and to some extent are now correlated with the microbial population analysis.

The direct detection of enzyme activities has proved to be more difficult than anticipated. However, much has been learned. The last part of the project involved a new round of sampling, as new effluent entered the wetlands in January 2006, and modified enzyme assays were completed on fresh samples. These same samples were also used for confirmatory phylogenetic analysis to provide the correlation in the way that we now know is necessary.

By January 2006 the monthly triplicate soil samples collected from the wetland inflow area (at two different depths), and the respective extracted DNA samples, covered a full year. Using this sample set, the methods developed were applied to study the microbiological changes occurring in the wetland, which may be caused by the seasonal load with winery effluents. DGGE was used to detect qualitative or semi-quantitative changes in the composition of the bacterial community.

The ideal method to identify the most abundant microbial species in the wetland would be to sequence a large number (>500) of amplified 16S rDNA. Within the current feasibility study, clone libraries containing ca. 40 clones each were prepared from February and October samples, of which at least half are being sequenced. This would yield more data to use for correlations.

A major problem, of course, is that many other factors apart from the winery effluents may induce changes in the bacterial composition and activity in the wetland soil during the year (temperature, rain, sun, changing vegetation, birds etc.). It would therefore be very interesting and highly valuable for future research, to determine whether at least some of the data obtained from the wetlands could be reproduced in experiments with the lab-scale wetland. Soil from the wetlands could be applied to the model, from which samples would then be taken before, during and after the application of winery effluents. We do have a model wetland set up and samples could be taken for analysis in a future project.

Constructed wetlands have been demonstrated to be both feasible and practical method of treating winery effluent. A conservative semi-empirical model has been developed that predicts the effluent treatment efficiency of the constructed wetlands. The correct plants have been identified for use in constructed wetlands for use in the Western Cape Province and include primarily sedge species and Arum Lilies. This model, in conjunction with the botanical data, can be used for the design of new constructed wetlands for the wine industry

8.2 Results arising from phylogenetics studies

The primary objective of the molecular phylogenetic analyses was to assess the feasibility of different systems as a means of determining microbial diversity, and in particular the fraction of the population with a functional role in degradation of winery waste stream components. This objective has been effectively satisfied.

Trials using both 16S library analyses and 16S DGGE analyses demonstrated clearly that the latter method can be applied effectively to determine the dominant members of the microbial community. In addition, this method showed considerable promise as a technique for monitoring temporal changes in microbial populations. The effectiveness of this method thus allowed us to identify specific microorganisms which responded (both positively and negatively) to the seasonal changes in winery waste releases. We have thus been able to identify certain genera (target organisms) which are implicated in the direct metabolic degradation of winery wastewater chemical constituents, and which together constitute trophic components in the mineralization process.

8.3 Recommendations for further research

The success of this trial study has many implications for further work. Using DGGE analysis of amplified 16S rRNA marker genes as a basic identification tool, the following are possible lines of investigation aimed at further understanding the details of the degradative process, the specific enzymes involved and the process kinetics.

- Identified organisms can be isolated and cultured, allowing the opportunity to investigate their specific enzymology, degradation capacity, reaction kinetics, response to concentration changes, etc.
- Quantitative changes in target organism populations can be more accurately monitored using qPCR (quantitative PCR).
- A knowledge of specific key organisms leads directly to the identification of key enzymes. In some instances, detailed information on these enzymes may be available in the scientific literature or through sequenced genomes. In either case, it is possible to specifically track the expression and activity of such organism-specific enzymes, using either specific assays of enzyme gene-specific qPCR.
- An understanding of which organisms play a key role in degradative processes permits a higher level of monitoring and control of the system. For example, the impact of potentially deleterious processes (such as intermittent releases of high or low pH water streams) could be monitored and understood.
- These techniques can potentially be applied to any wastewater system, particularly wastewaters containing where high concentrations of known chemical constituents.
- Further studies using laboratory scale model wetland would be very useful. This is a controlled environment which lends itself to testing the effect of microbial action on effluent, without extraneous factors which might be found in either the natural or constructed wetlands. Studies of changes in bacterial community and enzyme activity before, during and after the addition of effluent may be carried out using this small-scale wetland. Various concentrations of effluent may be tested, as well as various components of the effluent. Once the changes in bacterial community and enzyme activity have been correlated it would be possible to seed the wetland with various strains of bacteria in order to determine whether or not it is feasible to develop a starter culture which would increase the efficiency with which a constructed wetland degrades winery effluent.

Appendix 1
COD Calculations

A theoretical COD value can be calculated if one knows the chemical composition of the effluent. This is done by working out the combustion reactions for each of the specific components.

Thus, for an effluent containing 1 mℓ/ℓ of ethanol, 0.2 mℓ/ℓ of acetic acid and 0.1 mℓ/ℓ of propionic acid the following procedure can be followed:

a. Convert the liquid concentrations into molar concentrations (multiply the concentration by the density and divide by the molar mass). This means that the above concentrations become 0.0197M ethanol, 0.00349 M acetic acid and 0.00134 M of propionic acid.

b. Combust each of the species with O_2.

$$C_2H_6O + 3O_2 \rightarrow 2CO_2 + 3H_2O$$

$$C_2H_4O + 2O_2 \rightarrow 2CO_2 + 2H_2O$$

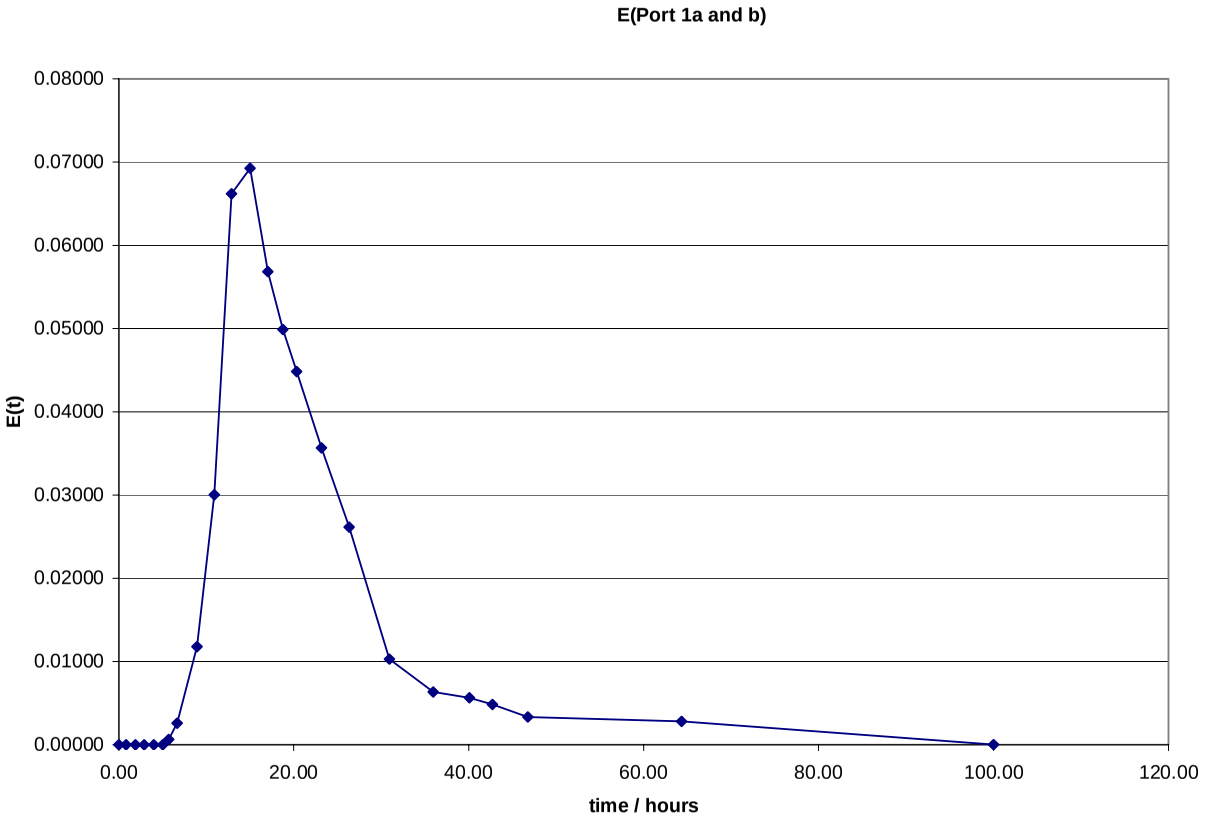
$$C_3H_6O_2 + \frac{7}{2}O_2 \rightarrow 3CO_2 + 3H_2O$$

c. Work out the stoichiometric O_2 requirement – in this case it is $0.0197 \cdot 3 + 0.00349 \cdot 2 + 0.00134 \cdot 7/2$ which is 0.07077 Mol/ℓ .

d. Convert back to mass (mg/ℓ) which is the normal value – in this case 0.07077Mol/ℓ*31.999g/mol which is 2.264g/ℓ or approximately 2265 mg/ℓ.

Appendix 2
Age Distribution graphs and data tables

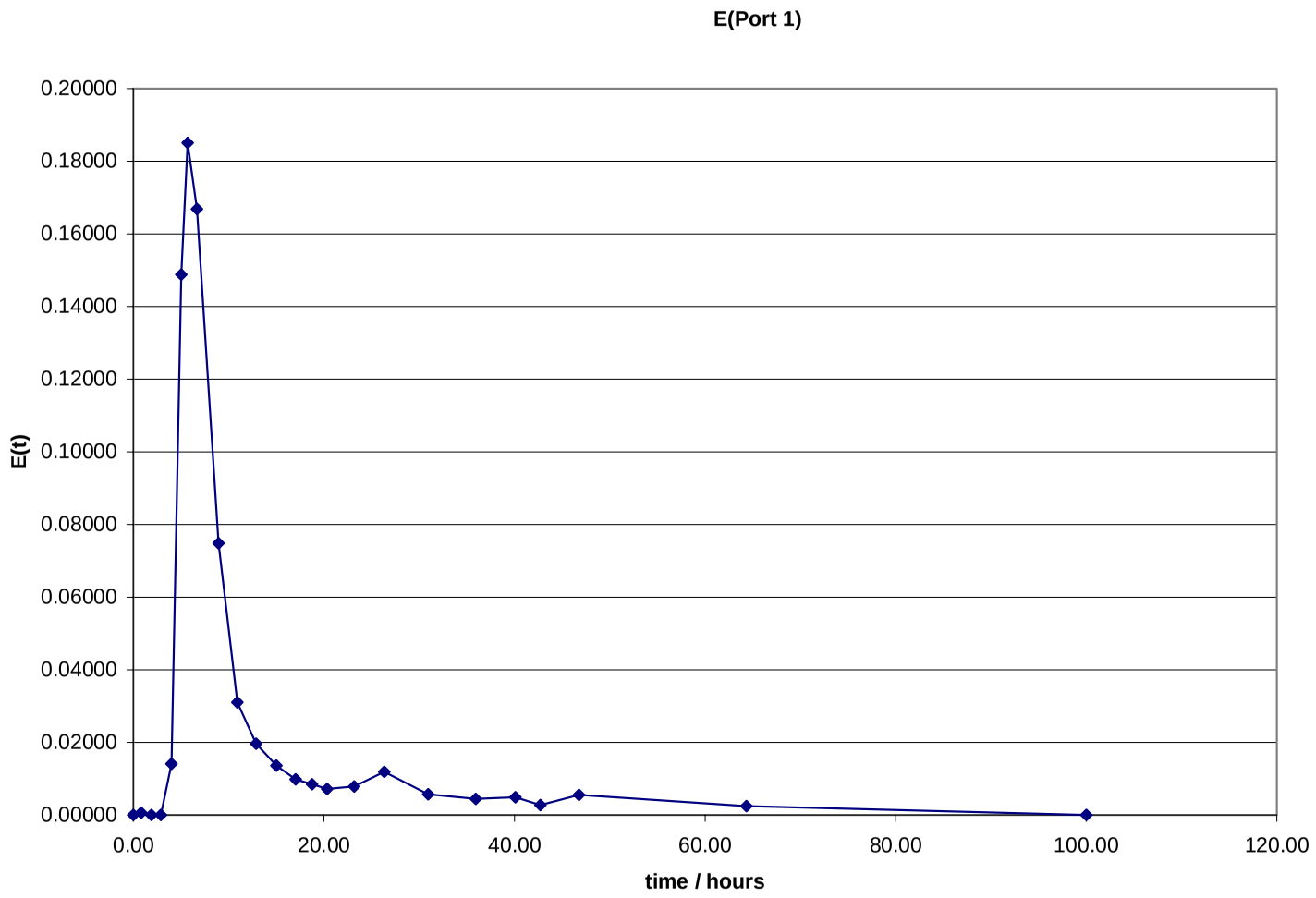
A2.1 - Sample port 1a and 1b graph



A2.2 - Sample port 1a and 1b data table

Date and Time	Time / hours	Meter Reading	Flow / m^3	Port	Concentration /mg/L			Q (sigC.Dt)	E(C/Q)	Integral of E	ti.ei.dt	ti^2.Ei.dt
2006/02/03 17:10	0.00	125.37		1a	1b	1 a/b Average	1 a/b Average	1317.26	0.00000			
2006/02/03 18:00	0.83	125.86	0.49	0.83	6.41	3.62	3.62	3.02	0.00275	0.00	0.00	0.00
2006/02/03 19:04	1.90	126.41	0.55	0.00	0.00	0.00	0.00	0.00	0.00000	0.00	0.00	0.00
2006/02/03 20:04	2.90	126.89	0.48	0.00	0.74	0.37	0.37	0.37	0.00028	0.00	0.00	0.00
2006/02/03 21:10	4.00	128.44	1.55	183.42	145.07	164.25	164.25	180.67	0.12469	0.14	0.55	2.19
2006/02/03 22:12	5.03	129.46	1.02	128.29	109.50	118.90	118.90	122.86	0.09026	0.09	0.47	2.36
2006/02/03 22:52	5.70	129.96	0.50	95.96	95.47	95.72	95.72	63.81	0.07266	0.05	0.28	1.57
2006/02/03 23:50	6.67	130.71	0.75	73.26	91.12	82.19	82.19	79.45	0.06239	0.06	0.40	2.68
2006/02/04 02:06	8.93	132.52	1.81	51.65	105.17	78.41	78.41	177.73	0.05952	0.13	1.21	10.77
2006/02/04 04:05	10.92	134.03	1.51	47.54	84.01	65.78	65.78	130.45	0.04993	0.10	1.08	11.80
2006/02/04 06:03	12.88	135.52	1.49	44.96	52.84	48.90	48.90	96.17	0.03712	0.07	0.94	12.12
2006/02/04 08:10	15.00	136.92	1.40	43.46	32.60	38.03	38.03	80.50	0.02887	0.06	0.92	13.75
2006/02/04 10:12	17.03	138.16	1.24	39.34	20.47	29.91	29.91	60.81	0.02270	0.05	0.79	13.39
2006/02/04 11:55	18.75	139.14	0.98	33.23	15.21	24.22	24.22	41.58	0.01839	0.03	0.59	11.10
2006/02/04 13:30	20.33	140.26	1.12	28.76	14.19	21.48	21.48	34.00	0.01630	0.03	0.52	10.67
2006/02/04 16:20	23.17	141.71	1.45	10.92	5.70	8.31	8.31	23.55	0.00631	0.02	0.41	9.59
2006/02/04 19:30	26.33	143.18	1.47	3.91	5.48	4.70	4.70	14.87	0.00356	0.01	0.30	7.83
2006/02/05 00:06	30.93	148.24	5.06	10.78	16.05	13.42	13.42	61.71	0.01018	0.05	1.45	44.83
2006/02/05 05:06	35.93	150.53	2.29	7.72	8.74	8.23	8.23	41.15	0.00625	0.03	1.12	40.34
2006/02/05 09:15	40.08	151.00	0.47	6.09	7.70	6.90	6.90	28.61	0.00523	0.02	0.87	34.90
2006/02/05 11:53	42.72	154.95	3.95	5.37	7.55	6.46	6.46	17.01	0.00490	0.01	0.55	23.56
2006/02/05 15:56	46.77	156.21	1.26	3.52	7.55	5.54	5.54	22.42	0.00420	0.02	0.80	37.22
2006/02/06 09:30	64.33	159.24	3.03	1.89	2.27	2.08	2.08	36.54	0.00158	0.03	1.78	114.80
2006/02/06 15:00	100.00	212.52	53.28			0.16855515	0.17	6.01	0.00013	0.00	0.46	
										1.00		
										Tau	15.49	
										Sigma^2	165.62	
										N	1.45	
										Sigma Theta^2	0.69	
										Disp	0.81	
										Sigma Theta^2	0.69	
										N	1.45	

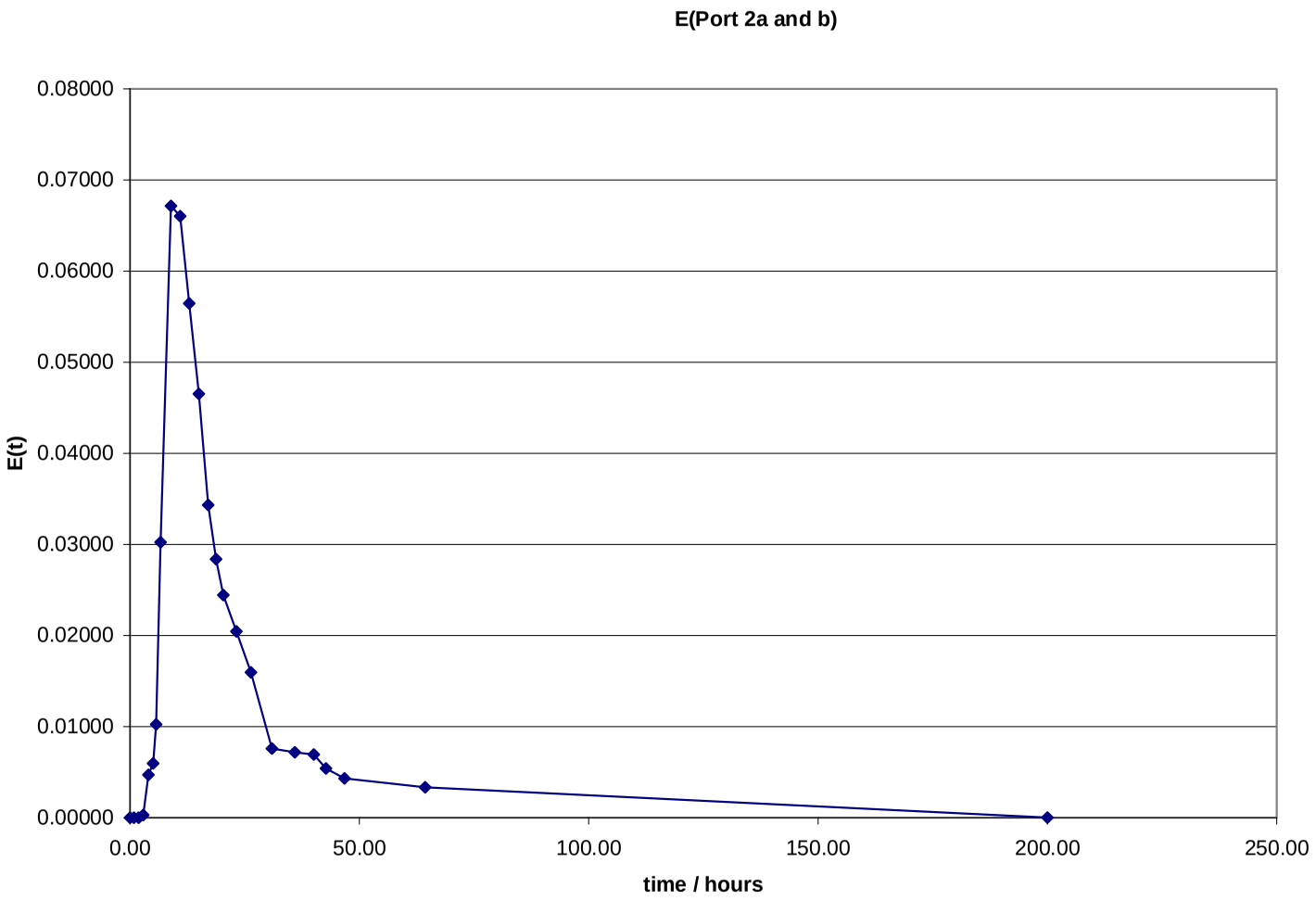
A2.3 - Sample port 1 graph



A2.4 - Sample port 1 data table

Date and Time	Time / hours	Meter Reading	Flow / m^3	Concentration /mg/L		Q (sigC.Dt)	E(C/Q)	Integral of E	ti.ei.dt	ti^2.Ei.dt
2006/02/03 17:10	0.00	125.37		1.00	1.00	906.07	0.00000			
2006/02/03 18:00	0.83	125.86	0.49	0.58	0.58	0.48	0.00064	0.00	0.00	0.00
2006/02/03 19:04	1.90	126.41	0.55	0.00	0.00	0.00	0.00000	0.00	0.00	0.00
2006/02/03 20:04	2.90	126.89	0.48	0.00	0.00	0.00	0.00000	0.00	0.00	0.00
2006/02/03 21:10	4.00	128.44	1.55	12.78	12.78	14.06	0.01410	0.02	0.06	0.25
2006/02/03 22:12	5.03	129.46	1.02	134.83	134.83	139.32	0.14881	0.15	0.77	3.90
2006/02/03 22:52	5.70	129.96	0.50	167.69	167.69	111.79	0.18507	0.12	0.70	4.01
2006/02/03 23:50	6.67	130.71	0.75	151.17	151.17	146.13	0.16684	0.16	1.08	7.17
2006/02/04 02:06	8.93	132.52	1.81	67.78	67.78	153.63	0.07481	0.17	1.51	13.53
2006/02/04 04:05	10.92	134.03	1.51	28.11	28.11	55.75	0.03102	0.06	0.67	7.33
2006/02/04 06:03	12.88	135.52	1.49	17.77	17.77	34.95	0.01961	0.04	0.50	6.40
2006/02/04 08:10	15.00	136.92	1.40	12.34	12.34	26.12	0.01362	0.03	0.43	6.49
2006/02/04 10:12	17.03	138.16	1.24	8.9	8.90	18.10	0.00982	0.02	0.34	5.79
2006/02/04 11:55	18.75	139.14	0.98	7.68	7.68	13.18	0.00848	0.01	0.27	5.12
2006/02/04 13:30	20.33	140.26	1.12	6.52	6.52	10.32	0.00720	0.01	0.23	4.71
2006/02/04 16:20	23.17	141.71	1.45	7.11	7.11	20.15	0.00785	0.02	0.52	11.93
2006/02/04 19:30	26.33	143.18	1.47	10.77	10.77	34.11	0.01189	0.04	0.99	26.10
2006/02/05 00:06	30.93	148.24	5.06	5.18	5.18	23.83	0.00572	0.03	0.81	25.16
2006/02/05 05:06	35.93	150.53	2.29	4.01	4.01	20.05	0.00443	0.02	0.80	28.57
2006/02/05 09:15	40.08	151.00	0.47	4.42	4.42	18.34	0.00488	0.02	0.81	32.53
2006/02/05 11:53	42.72	154.95	3.95	2.49	2.49	6.56	0.00275	0.01	0.31	13.20
2006/02/05 15:56	46.77	156.21	1.26	5.03	5.03	20.37	0.00555	0.02	1.05	49.17
2006/02/06 09:30	64.33	159.24	3.03	2.21	2.21	38.82	0.00244	0.04	2.76	177.33
2006/02/06 15:00	100.00	212.52	53.28	0.00	0.00	0.01	0.00000	0.00	0.00	0.06
								1.00		
								Tau	14.62	
								Sigma^2	215.04	
								N	0.99	
								Sigma Theta^2	1.01	
								Disp	10000000.00	
								Sigma Theta^2	1.01	
								N	0.99	

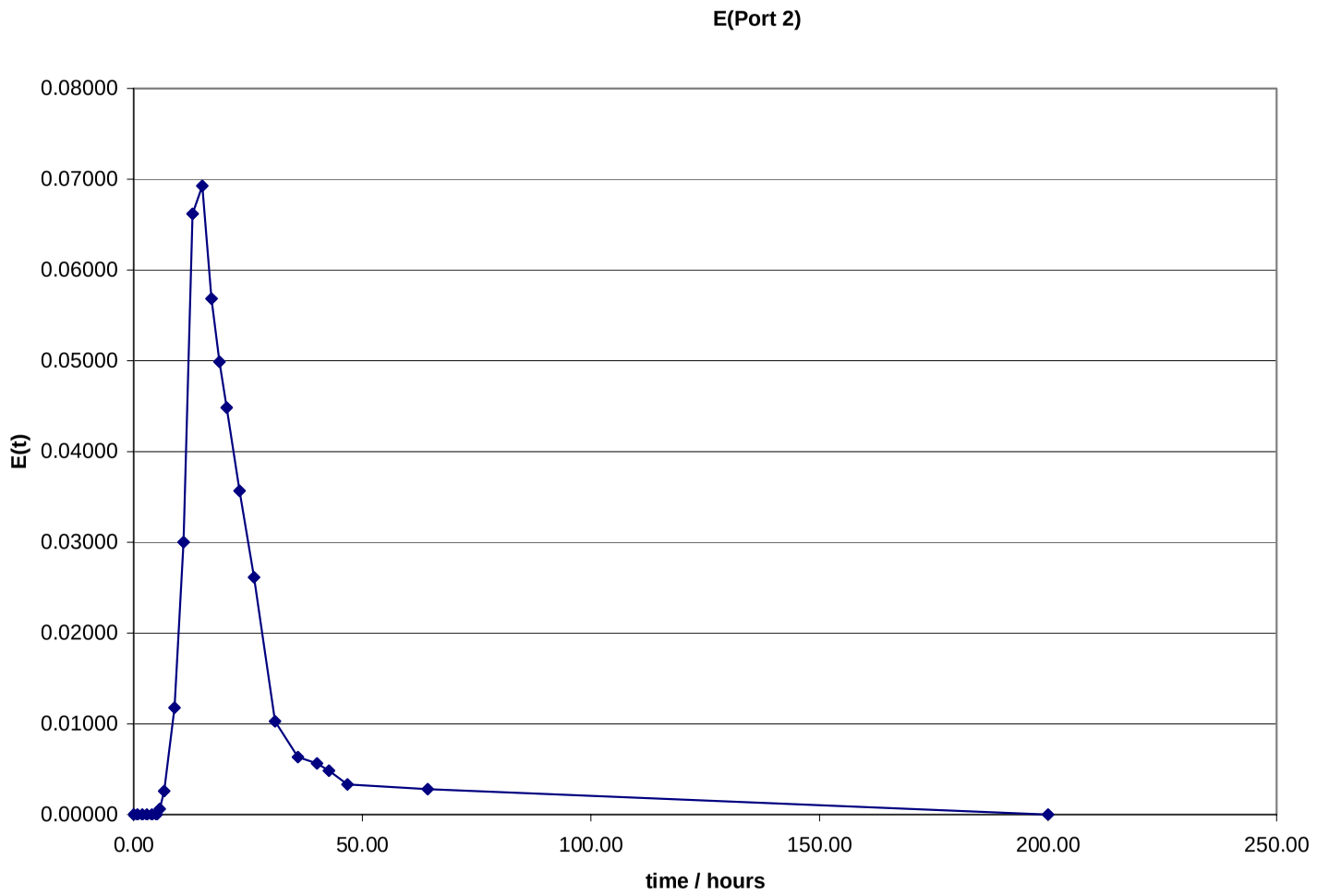
A2.5 - Sample port 2a and 2b graph



A2.6 - Sample port 2a and 2b data table

Date and Time	Time / hours	Meter Reading	Flow / m^3			Concentration /mg/L	Q (sigC.Dt)	E(C/Q)	Integral of E	ti.ei.dt	ti^2.Ei.dt
2006/02/03 17:10	0.00	125.37		2a	2b	Average 2a and 2b	1234.32	0.00000			
2006/02/03 18:00	0.83	125.86	0.49	0.00	0.00	0.00	0.00	0.00000	0.00	0.00	0.00
2006/02/03 19:04	1.90	126.41	0.55	0.00	0.00	0.00	0.00	0.00000	0.00	0.00	0.00
2006/02/03 20:04	2.90	126.89	0.48	0.00	0.74	0.37	0.37	0.00030	0.00	0.00	0.00
2006/02/03 21:10	4.00	128.44	1.55	0.00	11.63	5.82	6.40	0.00471	0.01	0.02	0.08
2006/02/03 22:12	5.03	129.46	1.02	0.00	14.72	7.36	7.61	0.00596	0.01	0.03	0.16
2006/02/03 22:52	5.70	129.96	0.50	0.00	25.29	12.65	8.43	0.01024	0.01	0.04	0.22
2006/02/03 23:50	6.67	130.71	0.75	0.00	74.63	37.32	36.07	0.03023	0.03	0.19	1.30
2006/02/04 02:06	8.93	132.52	1.81	5.24	160.56	82.90	187.91	0.06716	0.15	1.36	12.15
2006/02/04 04:05	10.92	134.03	1.51	47.07	115.91	81.49	161.62	0.06602	0.13	1.43	15.60
2006/02/04 06:03	12.88	135.52	1.49	62.30	77.07	69.69	137.05	0.05646	0.11	1.43	18.43
2006/02/04 08:10	15.00	136.92	1.40	55.28	59.59	57.44	121.57	0.04653	0.10	1.48	22.16
2006/02/04 10:12	17.03	138.16	1.24	37.31	47.44	42.38	86.16	0.03433	0.07	1.19	20.25
2006/02/04 11:55	18.75	139.14	0.98	33.27	36.83	35.05	60.17	0.02840	0.05	0.91	17.14
2006/02/04 13:30	20.33	140.26	1.12	30.63	29.71	30.17	47.77	0.02444	0.04	0.79	16.00
2006/02/04 16:20	23.17	141.71	1.45	28.16	22.34	25.25	71.54	0.02046	0.06	1.34	31.11
2006/02/04 19:30	26.33	143.18	1.47	22.24	17.19	19.72	62.43	0.01597	0.05	1.33	35.07
2006/02/05 00:06	30.93	148.24	5.06	9.59	9.15	9.37	43.10	0.00759	0.03	1.08	33.41
2006/02/05 05:06	35.93	150.53	2.29	8.50	9.26	8.88	44.40	0.00719	0.04	1.29	46.45
2006/02/05 09:15	40.08	151.00	0.47	7.75	9.39	8.57	35.57	0.00694	0.03	1.15	46.29
2006/02/05 11:53	42.72	154.95	3.95	5.89	7.48	6.69	17.60	0.00542	0.01	0.61	26.02
2006/02/05 15:56	46.77	156.21	1.26	4.46	6.23	5.35	21.65	0.00433	0.02	0.82	38.36
2006/02/06 09:30	64.33	159.24	3.03	3.49	4.75	4.12	72.37	0.00334	0.06	3.77	242.68
2006/02/06 15:00	200.00	212.52	53.28			0.03	4.53	0.00003	0.00	0.73	146.90
									1.00		
									Tau	21.01	
									Sigma^2	328.29	
									N	1.34	
									Sigma Theta^2	0.74	
									Disp	1.04	
									Sigma Theta^2	0.74	
									N	1.34	

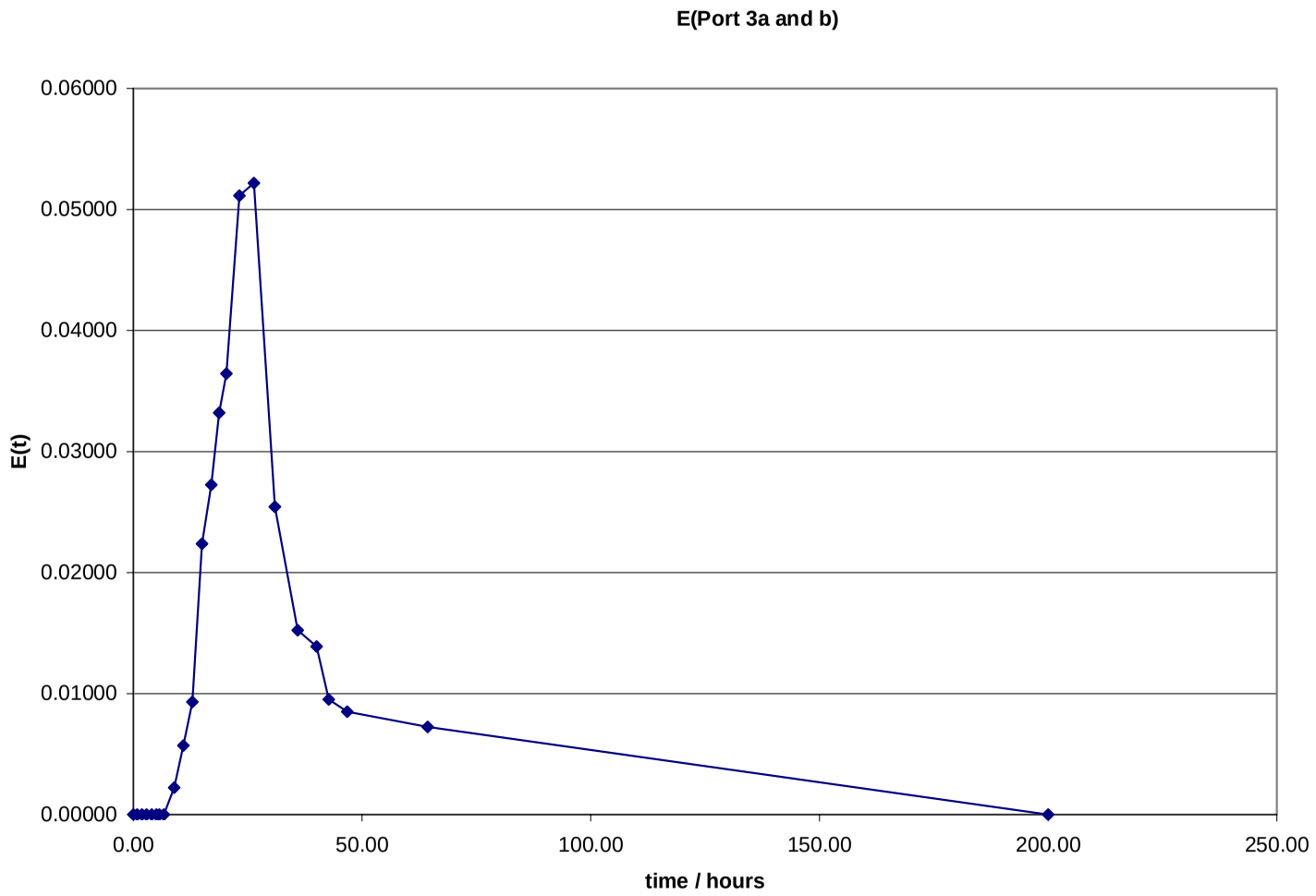
A2.7 - Sample port 2



A2.8 - Sample port 2 data table

Date and Time	Time / hours	Meter Reading	Flow / m^3	Conc	Q (sigC.Dt)	E(C/Q)	Integral of E	ti.ei.dt	ti^2.Ei.dt
2006/02/03 17:10	0.00	125.37		2.00	2142.75	0.00000			
2006/02/03 18:00	0.83	125.86	0.49	0.00	0.00	0.00000	0.00	0.00	0.00
2006/02/03 19:04	1.90	126.41	0.55	0.00	0.00	0.00000	0.00	0.00	0.00
2006/02/03 20:04	2.90	126.89	0.48	0.00	0.00	0.00000	0.00	0.00	0.00
2006/02/03 21:10	4.00	128.44	1.55	0.00	0.00	0.00000	0.00	0.00	0.00
2006/02/03 22:12	5.03	129.46	1.02	0.00	0.00	0.00000	0.00	0.00	0.00
2006/02/03 22:52	5.70	129.96	0.50	1.36	0.91	0.00063	0.00	0.00	0.01
2006/02/03 23:50	6.67	130.71	0.75	5.55	5.37	0.00259	0.00	0.02	0.11
2006/02/04 02:06	8.93	132.52	1.81	25.24	57.21	0.01178	0.03	0.24	2.13
2006/02/04 04:05	10.92	134.03	1.51	64.34	127.61	0.03003	0.06	0.65	7.10
2006/02/04 06:03	12.88	135.52	1.49	141.84	278.95	0.06620	0.13	1.68	21.61
2006/02/04 08:10	15.00	136.92	1.40	148.43	314.18	0.06927	0.15	2.20	32.99
2006/02/04 10:12	17.03	138.16	1.24	121.81	247.68	0.05685	0.12	1.97	33.54
2006/02/04 11:55	18.75	139.14	0.98	106.91	183.53	0.04989	0.09	1.61	30.11
2006/02/04 13:30	20.33	140.26	1.12	96.07	152.11	0.04483	0.07	1.44	29.35
2006/02/04 16:20	23.17	141.71	1.45	76.43	216.55	0.03567	0.10	2.34	54.24
2006/02/04 19:30	26.33	143.18	1.47	56.02	177.40	0.02614	0.08	2.18	57.41
2006/02/05 00:06	30.93	148.24	5.06	22.05	101.43	0.01029	0.05	1.46	45.29
2006/02/05 05:06	35.93	150.53	2.29	13.58	67.90	0.00634	0.03	1.14	40.92
2006/02/05 09:15	40.08	151.00	0.47	12.09	50.17	0.00564	0.02	0.94	37.62
2006/02/05 11:53	42.72	154.95	3.95	10.39	27.36	0.00485	0.01	0.55	23.30
2006/02/05 15:56	46.77	156.21	1.26	7.16	29.00	0.00334	0.01	0.63	29.60
2006/02/06 09:30	64.33	159.24	3.03	6.00	105.40	0.00280	0.05	3.16	203.58
2006/02/06 15:00	200.00	212.52	53.28	0.03	4.40	0.00002	0.00	0.41	82.05
							1.00		
							Tau	22.62	
							Sigma^2	219.36	
							N	2.33	
							Sigma Theta^2	0.43	
							Disp	0.30	
							Sigma Theta^2	0.43	
							N	2.33	

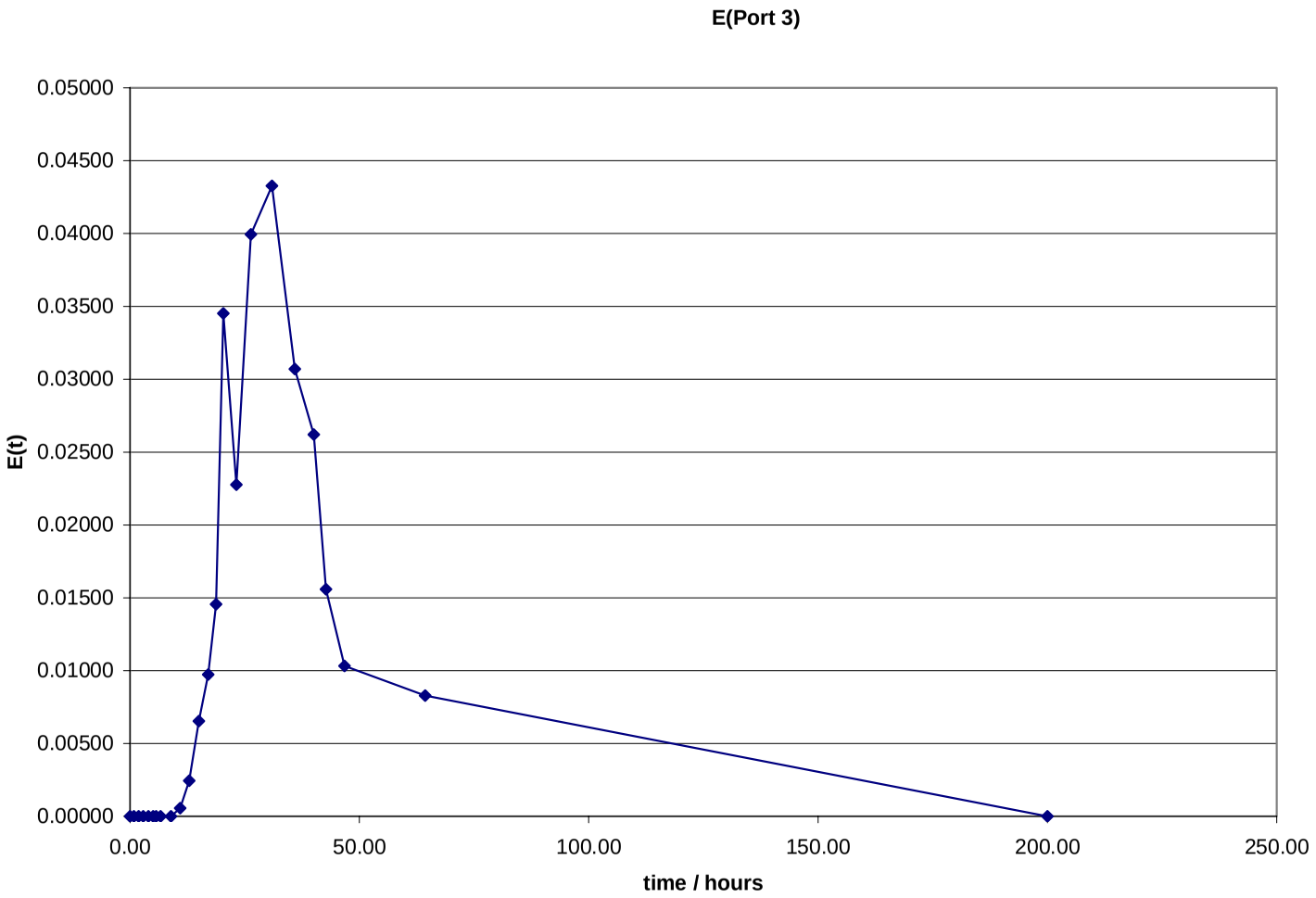
A2.9 - Sample port 3a and 3b



A2.10 - Sample port 3a and 3b data table

Date and Time	Time / hours	Meter Reading	Flow / m^3			Concentration /mg/L	Q (sigC.Dt)	E(C/Q)	Integral of E	ti.ei.dt	ti^2.Ei.dt
2006/02/03 17:10	0.00	125.37		3a	3b	Average 2a and 2b	1352.38	0.00000			
2006/02/03 18:00	0.83	125.86	0.49	0.00	0.00	0.00	0.00	0.00000	0.00	0.00	0.00
2006/02/03 19:04	1.90	126.41	0.55	0.00	0.00	0.00	0.00	0.00000	0.00	0.00	0.00
2006/02/03 20:04	2.90	126.89	0.48	0.00	0.00	0.00	0.00	0.00000	0.00	0.00	0.00
2006/02/03 21:10	4.00	128.44	1.55	0.00	0.00	0.00	0.00	0.00000	0.00	0.00	0.00
2006/02/03 22:12	5.03	129.46	1.02	0.00	0.00	0.00	0.00	0.00000	0.00	0.00	0.00
2006/02/03 22:52	5.70	129.96	0.50	0.00	0.00	0.00	0.00	0.00000	0.00	0.00	0.00
2006/02/03 23:50	6.67	130.71	0.75	0.00	0.00	0.00	0.00	0.00000	0.00	0.00	0.00
2006/02/04 02:06	8.93	132.52	1.81	2.37	3.65	3.01	6.82	0.00223	0.01	0.05	0.40
2006/02/04 04:05	10.92	134.03	1.51	1.41	14.01	7.71	15.29	0.00570	0.01	0.12	1.35
2006/02/04 06:03	12.88	135.52	1.49	1.17	24.00	12.59	24.75	0.00931	0.02	0.24	3.04
2006/02/04 08:10	15.00	136.92	1.40	1.74	58.81	30.28	64.08	0.02239	0.05	0.71	10.66
2006/02/04 10:12	17.03	138.16	1.24	3.25	70.45	36.85	74.93	0.02725	0.06	0.94	16.07
2006/02/04 11:55	18.75	139.14	0.98	10.40	79.38	44.89	77.06	0.03319	0.06	1.07	20.03
2006/02/04 13:30	20.33	140.26	1.12	24.49	74.07	49.28	78.03	0.03644	0.06	1.17	23.85
2006/02/04 16:20	23.17	141.71	1.45	68.71	69.63	69.17	195.98	0.05115	0.14	3.36	77.78
2006/02/04 19:30	26.33	143.18	1.47	78.88	62.29	70.59	223.52	0.05219	0.17	4.35	114.61
2006/02/05 00:06	30.93	148.24	5.06	30.53	38.29	34.41	158.29	0.02544	0.12	3.62	111.99
2006/02/05 05:06	35.93	150.53	2.29	17.11	24.10	20.61	103.03	0.01524	0.08	2.74	98.36
2006/02/05 09:15	40.08	151.00	0.47	14.35	23.24	18.80	78.00	0.01390	0.06	2.31	92.67
2006/02/05 11:53	42.72	154.95	3.95	9.24	16.49	12.87	33.88	0.00951	0.03	1.07	45.71
2006/02/05 15:56	46.77	156.21	1.26	8.14	14.86	11.50	46.58	0.00850	0.03	1.61	75.32
2006/02/06 09:30	64.33	159.24	3.03	7.09	12.51	9.80	172.15	0.00725	0.13	8.19	526.85
2006/02/06 15:00	200.00	212.52	53.28	0.00	0.00	0.00	0.00	0.00000	0.00	0.00	0.00
									1.00		
									Tau	31.55	
									Sigma^2	223.32	
									N	4.46	
									Sigma Theta^2	0.22	
									Disp	0.13	
									Sigma Theta^2	0.22	
									N	4.46	

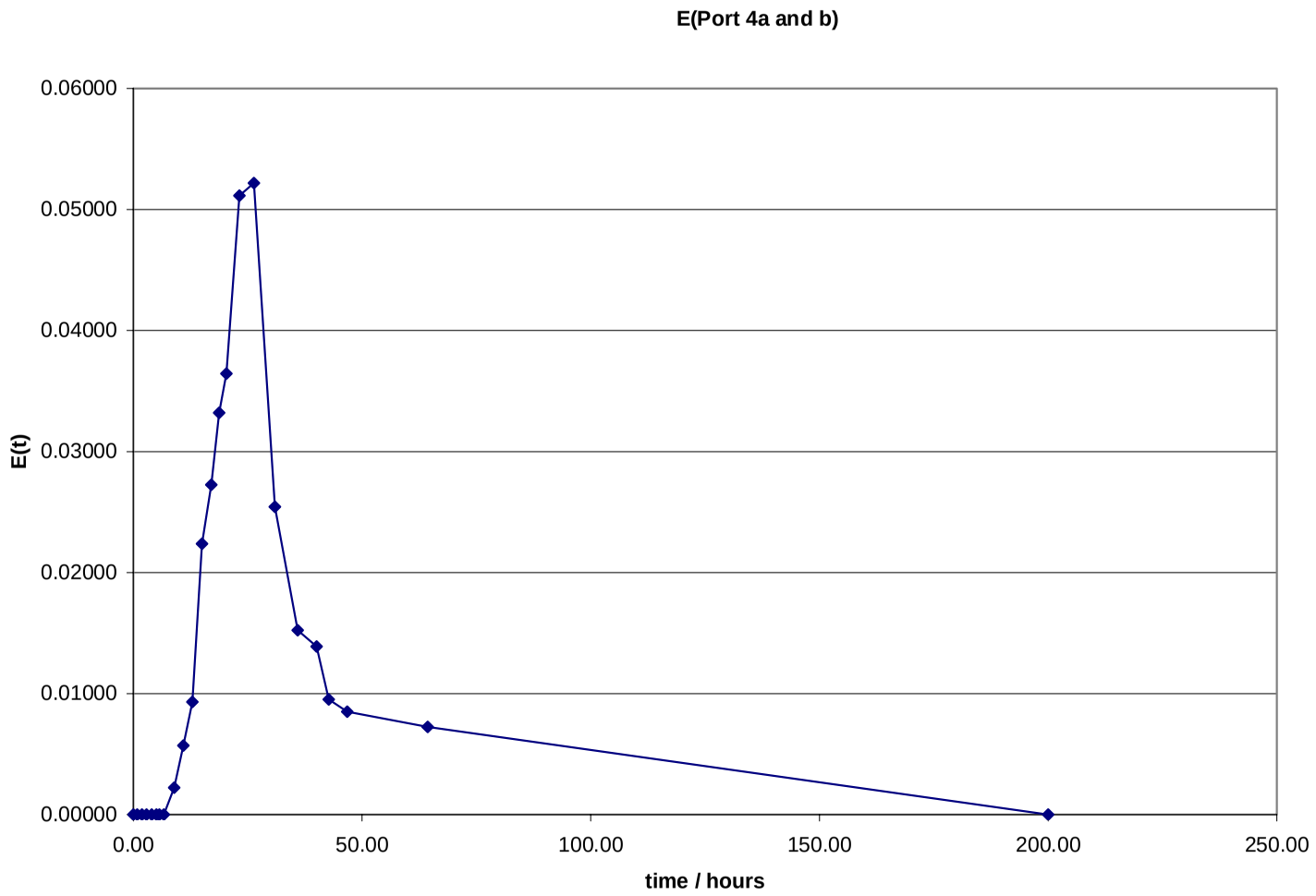
A2.11 - Sample port 3



A2.12 - Sample port 3 data table

Date and Time	Time / hours	Meter Reading	Flow / m^3	Conc	Q (sigC.Dt)	E(C/Q)	Integral of E	ti.ei.dt	ti^2.Ei.dt
2006/02/03 17:10	0.00	125.37		3.00	1383.79	0.00000			
2006/02/03 18:00	0.83	125.86	0.49	0.00	0.00	0.00000	0.00	0.00	0.00
2006/02/03 19:04	1.90	126.41	0.55	0.00	0.00	0.00000	0.00	0.00	0.00
2006/02/03 20:04	2.90	126.89	0.48	0.00	0.00	0.00000	0.00	0.00	0.00
2006/02/03 21:10	4.00	128.44	1.55	0.00	0.00	0.00000	0.00	0.00	0.00
2006/02/03 22:12	5.03	129.46	1.02	0.00	0.00	0.00000	0.00	0.00	0.00
2006/02/03 22:52	5.70	129.96	0.50	0.00	0.00	0.00000	0.00	0.00	0.00
2006/02/03 23:50	6.67	130.71	0.75	0.00	0.00	0.00000	0.00	0.00	0.00
2006/02/04 02:06	8.93	132.52	1.81	0.00	0.00	0.00000	0.00	0.00	0.00
2006/02/04 04:05	10.92	134.03	1.51	0.79	1.57	0.00057	0.00	0.01	0.13
2006/02/04 06:03	12.88	135.52	1.49	3.39	6.67	0.00245	0.00	0.06	0.80
2006/02/04 08:10	15.00	136.92	1.40	9.05	19.16	0.00654	0.01	0.21	3.11
2006/02/04 10:12	17.03	138.16	1.24	13.47	27.39	0.00973	0.02	0.34	5.74
2006/02/04 11:55	18.75	139.14	0.98	20.15	34.59	0.01456	0.02	0.47	8.79
2006/02/04 13:30	20.33	140.26	1.12	47.77	75.64	0.03452	0.05	1.11	22.60
2006/02/04 16:20	23.17	141.71	1.45	31.50	89.25	0.02276	0.06	1.49	34.61
2006/02/04 19:30	26.33	143.18	1.47	55.27	175.02	0.03994	0.13	3.33	87.71
2006/02/05 00:06	30.93	148.24	5.06	59.88	275.45	0.04327	0.20	6.16	190.47
2006/02/05 05:06	35.93	150.53	2.29	42.48	212.40	0.03070	0.15	5.52	198.19
2006/02/05 09:15	40.08	151.00	0.47	36.25	150.44	0.02620	0.11	4.36	174.67
2006/02/05 11:53	42.72	154.95	3.95	21.58	56.83	0.01559	0.04	1.75	74.93
2006/02/05 15:56	46.77	156.21	1.26	14.30	57.92	0.01033	0.04	1.96	91.54
2006/02/06 09:30	64.33	159.24	3.03	11.47	201.49	0.00829	0.15	9.37	602.63
2006/02/06 15:00	200.00	212.52	53.28	0.00	0.00	0.00000	0.00	0.00	0.00
							1.00		
							Tau	36.13	
							Sigma^2	190.31	
							N	6.86	
							Sigma Theta^2	0.15	
							Disp	0.08	
							Sigma Theta^2	0.15	
							N	6.86	

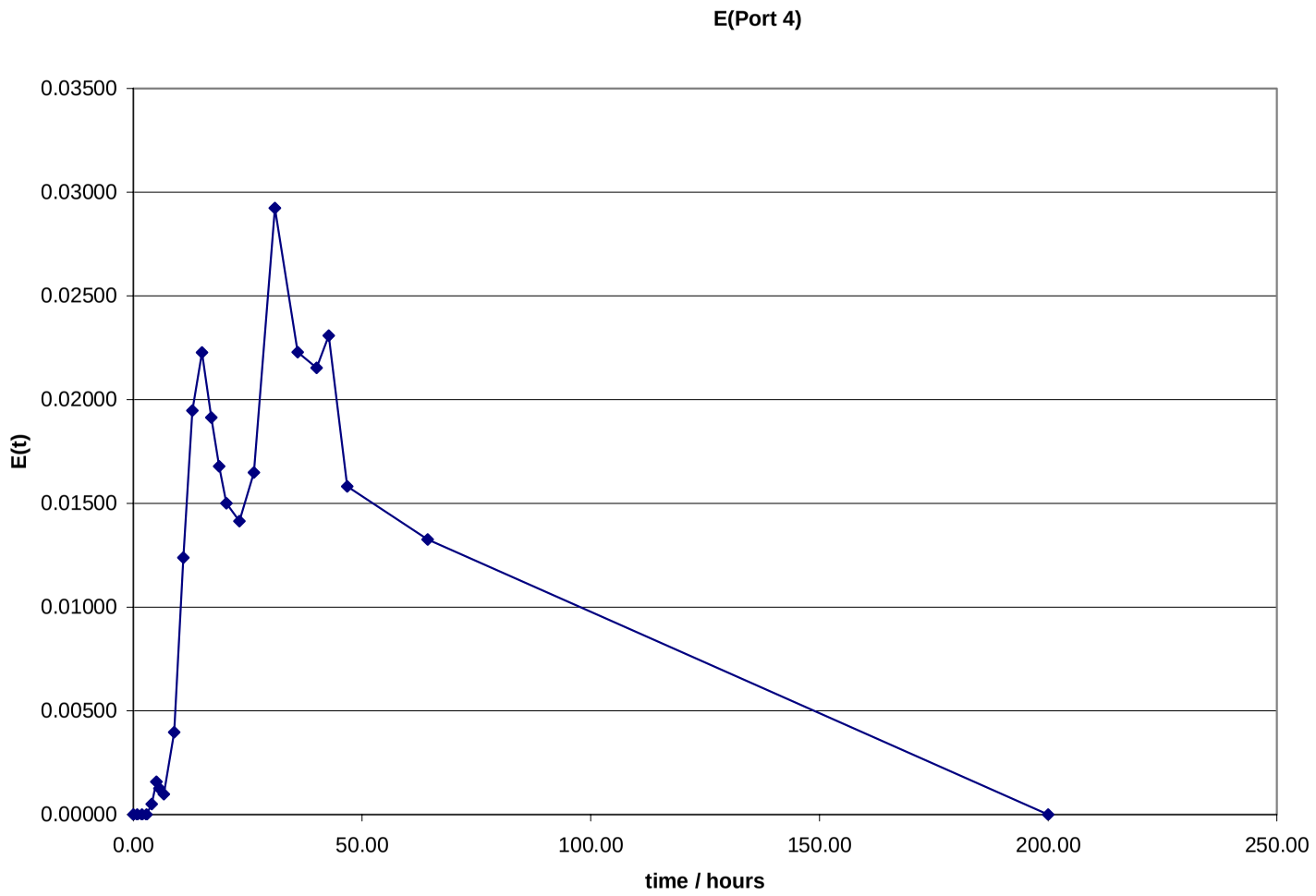
A2.13 - Sample port 4a and 4b



A2.14 - Sample port 4a and 4b data table

Date and Time	Time / hours	Meter Reading	Flow / m^3			Concentration /mg/L	Q (sigC.Dt)	E(C/Q)	Integral of E	ti.ei.dt	ti^2.Ei.dt
2006/02/03 17:10	0.00	125.37		4a	4b	Average 4a and 4b	1467.30	0.00000			
2006/02/03 18:00	0.83	125.86	0.49	0.00	0.00	0.00	0.00	0.00000	0.00	0.00	0.00
2006/02/03 19:04	1.90	126.41	0.55	0.00	0.00	0.00	0.00	0.00000	0.00	0.00	0.00
2006/02/03 20:04	2.90	126.89	0.48	0.00	0.00	0.00	0.00	0.00000	0.00	0.00	0.00
2006/02/03 21:10	4.00	128.44	1.55	0.00	0.00	0.00	0.00	0.00000	0.00	0.00	0.00
2006/02/03 22:12	5.03	129.46	1.02	0.00	0.00	0.00	0.00	0.00000	0.00	0.00	0.00
2006/02/03 22:52	5.70	129.96	0.50	0.00	0.00	0.00	0.00	0.00000	0.00	0.00	0.00
2006/02/03 23:50	6.67	130.71	0.75	0.00	0.00	0.00	0.00	0.00000	0.00	0.00	0.00
2006/02/04 02:06	8.93	132.52	1.81	0.00	0.00	0.00	0.00	0.00000	0.00	0.00	0.00
2006/02/04 04:05	10.92	134.03	1.51	0.00	0.00	0.00	0.00	0.00000	0.00	0.00	0.00
2006/02/04 06:03	12.88	135.52	1.49	0.00	0.31	0.16	0.30	0.00011	0.00	0.00	0.03
2006/02/04 08:10	15.00	136.92	1.40	0.29	0.56	0.43	0.90	0.00029	0.00	0.01	0.14
2006/02/04 10:12	17.03	138.16	1.24	0.59	0.8	0.70	1.41	0.00047	0.00	0.02	0.28
2006/02/04 11:55	18.75	139.14	0.98	1.01	1.81	1.41	2.42	0.00096	0.00	0.03	0.58
2006/02/04 13:30	20.33	140.26	1.12	1.09	3.73	2.41	3.82	0.00164	0.00	0.05	1.08
2006/02/04 16:20	23.17	141.71	1.45	38.84	10.50	24.67	69.90	0.01681	0.05	1.10	25.57
2006/02/04 19:30	26.33	143.18	1.47	60.57	29.94	45.26	143.31	0.03084	0.10	2.57	67.73
2006/02/05 00:06	30.93	148.24	5.06	24.66	81.68	53.17	244.58	0.03624	0.17	5.16	159.50
2006/02/05 05:06	35.93	150.53	2.29	48.00	62.92	55.46	277.30	0.03780	0.19	6.79	244.02
2006/02/05 09:15	40.08	151.00	0.47	44.41	55.30	49.86	206.90	0.03398	0.14	5.65	226.55
2006/02/05 11:53	42.72	154.95	3.95	33.67	38.77	36.22	95.38	0.02468	0.07	2.78	118.61
2006/02/05 15:56	46.77	156.21	1.26	17.97	29.70	23.84	96.53	0.01624	0.07	3.08	143.89
2006/02/06 09:30	64.33	159.24	3.03	16.71	20.24	18.48	324.54	0.01259	0.22	14.23	915.44
2006/02/06 15:00	200.00	212.52	53.28			0.00	0.00	0.00000	0.00	0.00	0.00
									1.00		
									Tau	41.47	
									Sigma^2	183.66	
									N	9.36	
									Sigma Theta^2	0.11	
									Disp	0.06	
									Sigma Theta^2	0.11	
									N	9.36	

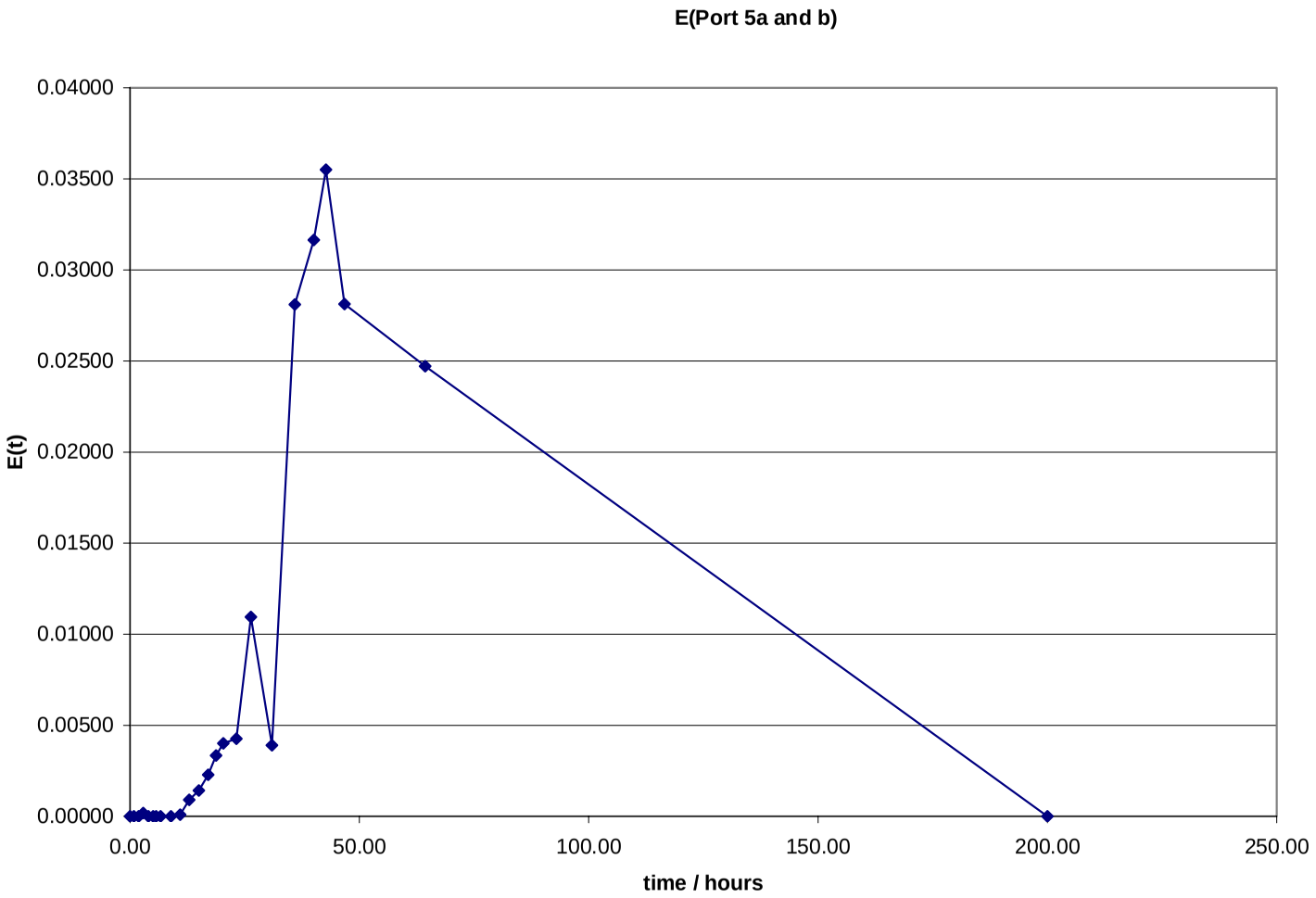
A2.15 - Sample port 4



A2.16 - Sample port 4 data table

Date and Time	Time / hours	Meter Reading	Flow / m^3	Conc	Q (sigC.Dt)	E(C/Q)	Integral of E	ti.ei.dt	ti^2.Ei.dt
2006/02/03 17:10	0.00	125.37		4.00	2154.67	0.00000			
2006/02/03 18:00	0.83	125.86	0.49	0.00	0.00	0.00000	0.00	0.00	0.00
2006/02/03 19:04	1.90	126.41	0.55	0.00	0.00	0.00000	0.00	0.00	0.00
2006/02/03 20:04	2.90	126.89	0.48	0.00	0.00	0.00000	0.00	0.00	0.00
2006/02/03 21:10	4.00	128.44	1.55	1.09	1.20	0.00051	0.00	0.00	0.01
2006/02/03 22:12	5.03	129.46	1.02	3.41	3.52	0.00158	0.00	0.01	0.04
2006/02/03 22:52	5.70	129.96	0.50	2.73	1.82	0.00127	0.00	0.00	0.03
2006/02/03 23:50	6.67	130.71	0.75	2.12	2.05	0.00098	0.00	0.01	0.04
2006/02/04 02:06	8.93	132.52	1.81	8.55	19.38	0.00397	0.01	0.08	0.72
2006/02/04 04:05	10.92	134.03	1.51	26.69	52.94	0.01239	0.02	0.27	2.93
2006/02/04 06:03	12.88	135.52	1.49	41.97	82.54	0.01948	0.04	0.49	6.36
2006/02/04 08:10	15.00	136.92	1.40	48.01	101.62	0.02228	0.05	0.71	10.61
2006/02/04 10:12	17.03	138.16	1.24	41.24	83.85	0.01914	0.04	0.66	11.29
2006/02/04 11:55	18.75	139.14	0.98	36.17	62.09	0.01679	0.03	0.54	10.13
2006/02/04 13:30	20.33	140.26	1.12	32.33	51.19	0.01500	0.02	0.48	9.82
2006/02/04 16:20	23.17	141.71	1.45	30.48	86.36	0.01415	0.04	0.93	21.51
2006/02/04 19:30	26.33	143.18	1.47	35.53	112.51	0.01649	0.05	1.38	36.21
2006/02/05 00:06	30.93	148.24	5.06	62.99	289.75	0.02923	0.13	4.16	128.68
2006/02/05 05:06	35.93	150.53	2.29	48.03	240.15	0.02229	0.11	4.00	143.91
2006/02/05 09:15	40.08	151.00	0.47	46.40	192.56	0.02153	0.09	3.58	143.59
2006/02/05 11:53	42.72	154.95	3.95	49.75	131.01	0.02309	0.06	2.60	110.95
2006/02/05 15:56	46.77	156.21	1.26	34.09	138.06	0.01582	0.06	3.00	140.14
2006/02/06 09:30	64.33	159.24	3.03	28.58	502.06	0.01326	0.23	14.99	964.37
2006/02/06 15:00	200.00	212.52	53.28	0.00	0.00	0.00000	0.00	0.00	0.00
							1.00		
							Tau	37.89	
							Sigma^2	305.52	
							N	4.70	
							Sigma Theta^2	0.21	
							Disp	0.12	
							Sigma Theta^2	0.21	
							N	4.70	

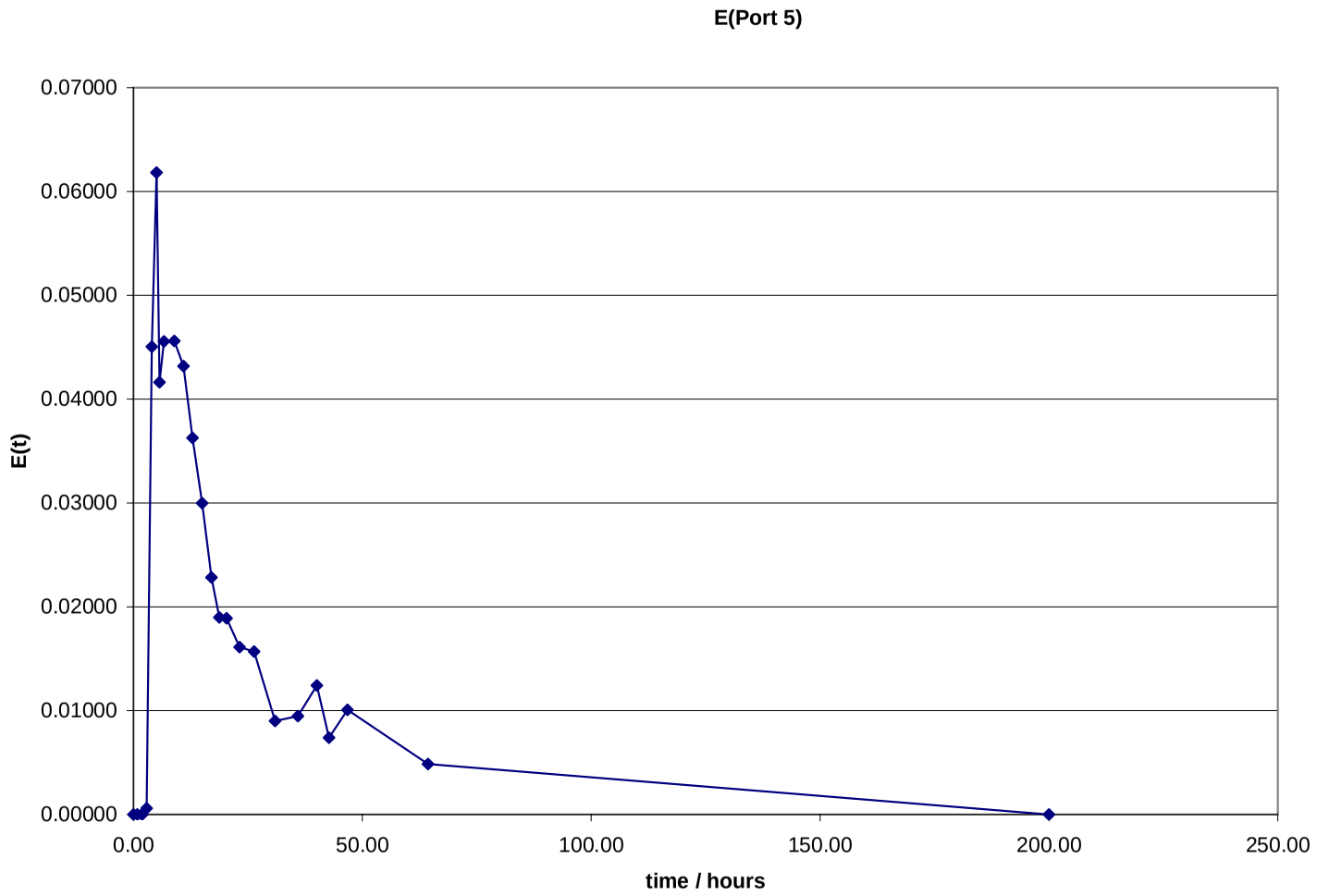
A2.17 - Sample port 5a and 5b



A2.18 - Sample port 5a and 5b data table

Date and Time	Time / hours	Meter Reading	Flow / m^3			Concentration /mg/L	Q (sigC.Dt)	E(C/Q)	Integral of E	ti.ei.dt	ti^2.Ei.dt
2006/02/03 17:10	0.00	125.37		5a	5b	Average 4a and 4b	1978.92	0.00000			
2006/02/03 18:00	0.83	125.86	0.49	0.00	0.00	0.00	0.00	0.00000	0.00	0.00	0.00
2006/02/03 19:04	1.90	126.41	0.55	0.00	0.00	0.00	0.00	0.00000	0.00	0.00	0.00
2006/02/03 20:04	2.90	126.89	0.48	0.71	0.00	0.36	0.36	0.00018	0.00	0.00	0.00
2006/02/03 21:10	4.00	128.44	1.55	0.00	0.00	0.00	0.00	0.00000	0.00	0.00	0.00
2006/02/03 22:12	5.03	129.46	1.02	0.00	0.00	0.00	0.00	0.00000	0.00	0.00	0.00
2006/02/03 22:52	5.70	129.96	0.50	0.00	0.00	0.00	0.00	0.00000	0.00	0.00	0.00
2006/02/03 23:50	6.67	130.71	0.75	0.00	0.00	0.00	0.00	0.00000	0.00	0.00	0.00
2006/02/04 02:06	8.93	132.52	1.81	0.00	0.00	0.00	0.00	0.00000	0.00	0.00	0.00
2006/02/04 04:05	10.92	134.03	1.51	0.00	0.35	0.18	0.35	0.00009	0.00	0.00	0.02
2006/02/04 06:03	12.88	135.52	1.49	0.32	3.26	1.79	3.52	0.00090	0.00	0.02	0.30
2006/02/04 08:10	15.00	136.92	1.40	1.01	4.64	2.82	5.98	0.00143	0.00	0.05	0.68
2006/02/04 10:12	17.03	138.16	1.24	1.4	7.64	4.52	9.19	0.00228	0.00	0.08	1.35
2006/02/04 11:55	18.75	139.14	0.98	5.35	7.89	6.62	11.36	0.00335	0.01	0.11	2.02
2006/02/04 13:30	20.33	140.26	1.12	3.08	12.81	7.95	12.58	0.00401	0.01	0.13	2.63
2006/02/04 16:20	23.17	141.71	1.45	3.54	13.33	8.44	23.90	0.00426	0.01	0.28	6.48
2006/02/04 19:30	26.33	143.18	1.47	5.06	38.27	21.67	68.61	0.01095	0.03	0.91	24.04
2006/02/05 00:06	30.93	148.24	5.06	10.59	4.86	7.73	35.54	0.00390	0.02	0.56	17.18
2006/02/05 05:06	35.93	150.53	2.29	42.67	68.56	55.62	278.08	0.02810	0.14	5.05	181.44
2006/02/05 09:15	40.08	151.00	0.47	45.65	79.61	62.63	259.91	0.03165	0.13	5.26	211.02
2006/02/05 11:53	42.72	154.95	3.95	55.99	84.54	70.27	185.03	0.03551	0.09	3.99	170.61
2006/02/05 15:56	46.77	156.21	1.26	44.90	66.42	55.66	225.42	0.02813	0.11	5.33	249.14
2006/02/06 09:30	64.33	159.24	3.03	43.08	54.73	48.91	859.10	0.02471	0.43	27.93	1796.75
2006/02/06 15:00	200.00	212.52	53.28			0.00	0.00	0.00000	0.00	0.00	0.00
									1.00		
									Tau	49.70	
									Sigma^2	193.68	
									N	12.75	
									Sigma Theta^2	0.08	
									Disp	0.04	
									Sigma Theta^2	0.08	
									N	12.75	

A2.19 - Sample port 5

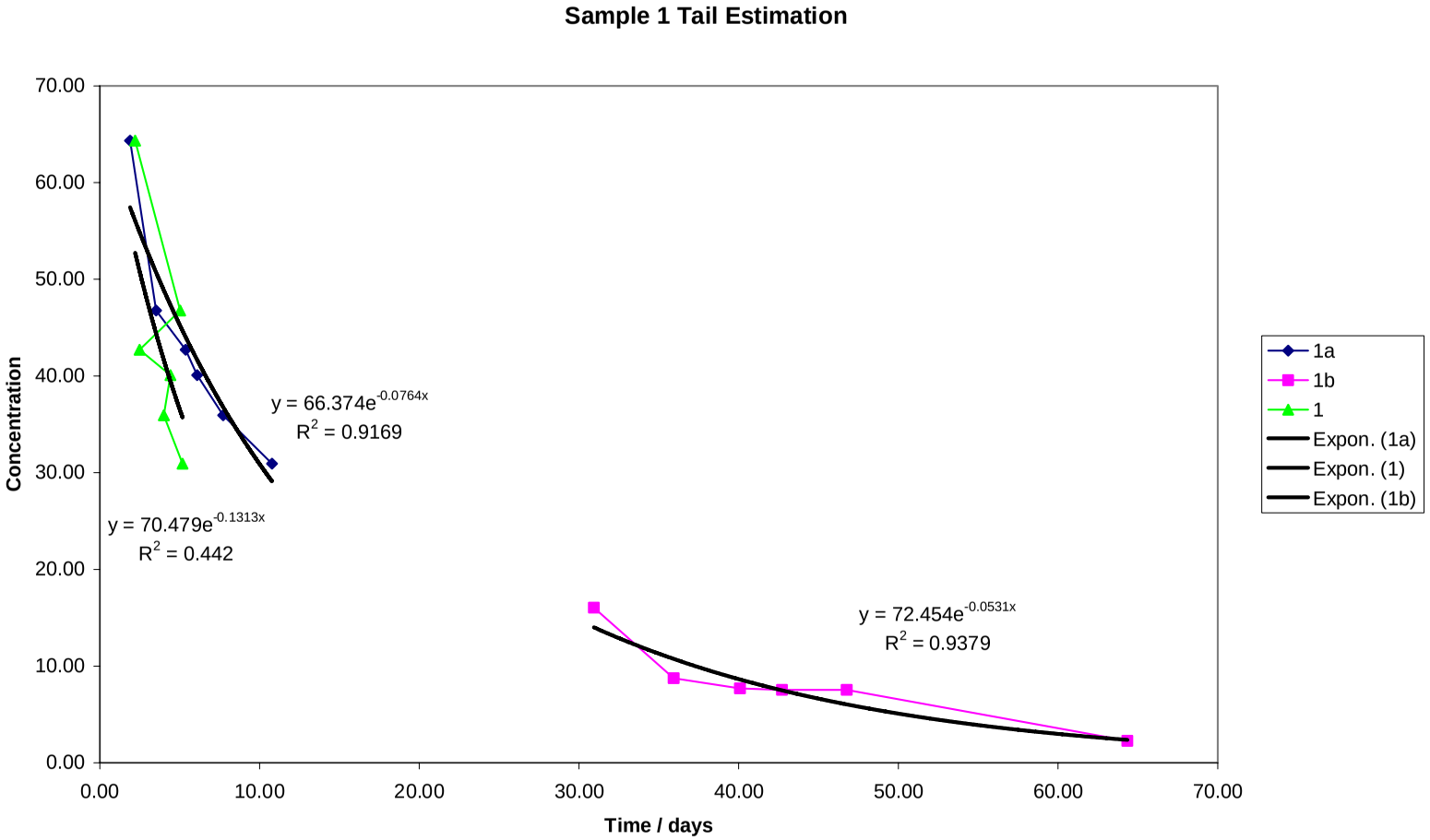


A2.20 - Sample port 5 data table

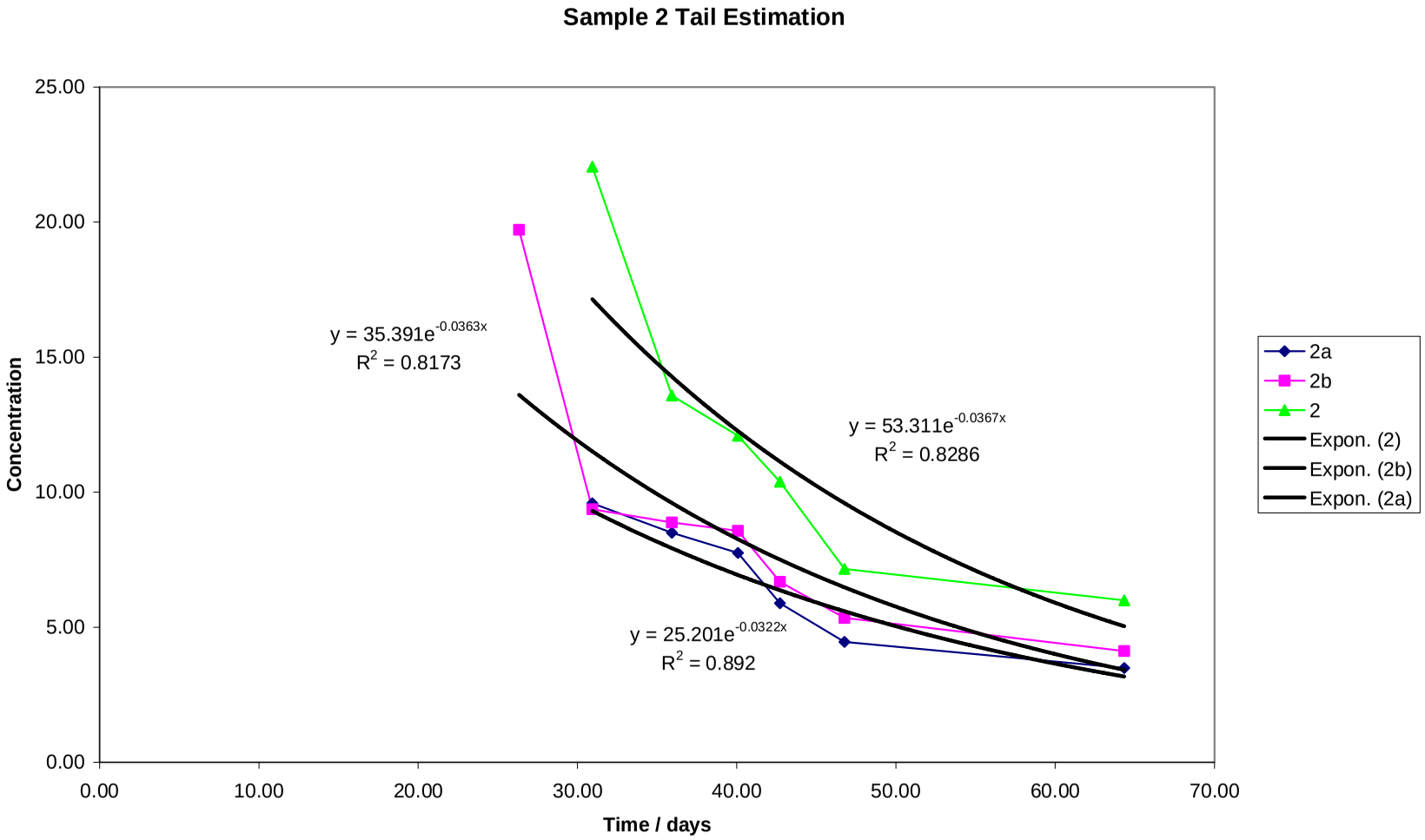
Date and Time	Time / hours	Meter Reading	Flow / m^3	Conc	Q (sigC.Dt)	E(C/Q)	Integral of E	ti.ei.dt	ti^2.Ei.dt
2006/02/03 17:10	0.00	125.37		5.00	2941.16	0.00000			
2006/02/03 18:00	0.83	125.86	0.49	0.00	0.00	0.00000	0.00	0.00	0.00
2006/02/03 19:04	1.90	126.41	0.55	0.00	0.00	0.00000	0.00	0.00	0.00
2006/02/03 20:04	2.90	126.89	0.48	1.78	1.78	0.00061	0.00	0.00	0.01
2006/02/03 21:10	4.00	128.44	1.55	132.48	145.73	0.04504	0.05	0.20	0.79
2006/02/03 22:12	5.03	129.46	1.02	181.81	187.87	0.06182	0.06	0.32	1.62
2006/02/03 22:52	5.70	129.96	0.50	122.43	81.62	0.04163	0.03	0.16	0.90
2006/02/03 23:50	6.67	130.71	0.75	133.98	129.51	0.04555	0.04	0.29	1.96
2006/02/04 02:06	8.93	132.52	1.81	134.14	304.05	0.04561	0.10	0.92	8.25
2006/02/04 04:05	10.92	134.03	1.51	126.98	251.84	0.04317	0.09	0.93	10.20
2006/02/04 06:03	12.88	135.52	1.49	106.69	209.82	0.03627	0.07	0.92	11.84
2006/02/04 08:10	15.00	136.92	1.40	88.18	186.65	0.02998	0.06	0.95	14.28
2006/02/04 10:12	17.03	138.16	1.24	67.16	136.56	0.02283	0.05	0.79	13.47
2006/02/04 11:55	18.75	139.14	0.98	55.84	95.86	0.01899	0.03	0.61	11.46
2006/02/04 13:30	20.33	140.26	1.12	55.58	88.00	0.01890	0.03	0.61	12.37
2006/02/04 16:20	23.17	141.71	1.45	47.41	134.33	0.01612	0.05	1.06	24.51
2006/02/04 19:30	26.33	143.18	1.47	46.16	146.17	0.01569	0.05	1.31	34.46
2006/02/05 00:06	30.93	148.24	5.06	26.50	121.90	0.00901	0.04	1.28	39.66
2006/02/05 05:06	35.93	150.53	2.29	27.86	139.30	0.00947	0.05	1.70	61.15
2006/02/05 09:15	40.08	151.00	0.47	36.56	151.72	0.01243	0.05	2.07	82.88
2006/02/05 11:53	42.72	154.95	3.95	21.77	57.33	0.00740	0.02	0.83	35.57
2006/02/05 15:56	46.77	156.21	1.26	29.65	120.08	0.01008	0.04	1.91	89.30
2006/02/06 09:30	64.33	159.24	3.03	14.29	251.03	0.00486	0.09	5.49	353.24
2006/02/06 15:00	200.00	212.52	53.28	0.00	0.00	0.00000	0.00	0.00	0.00
							1.00		
							Tau	22.36	
							Sigma^2	307.77	
							N	1.63	
							Sigma Theta^2	0.62	
							Disp	0.62	
							Sigma Theta^2	0.62	
							N	1.63	

Appendix 3
Decay Curves for RTD Tail Estimation

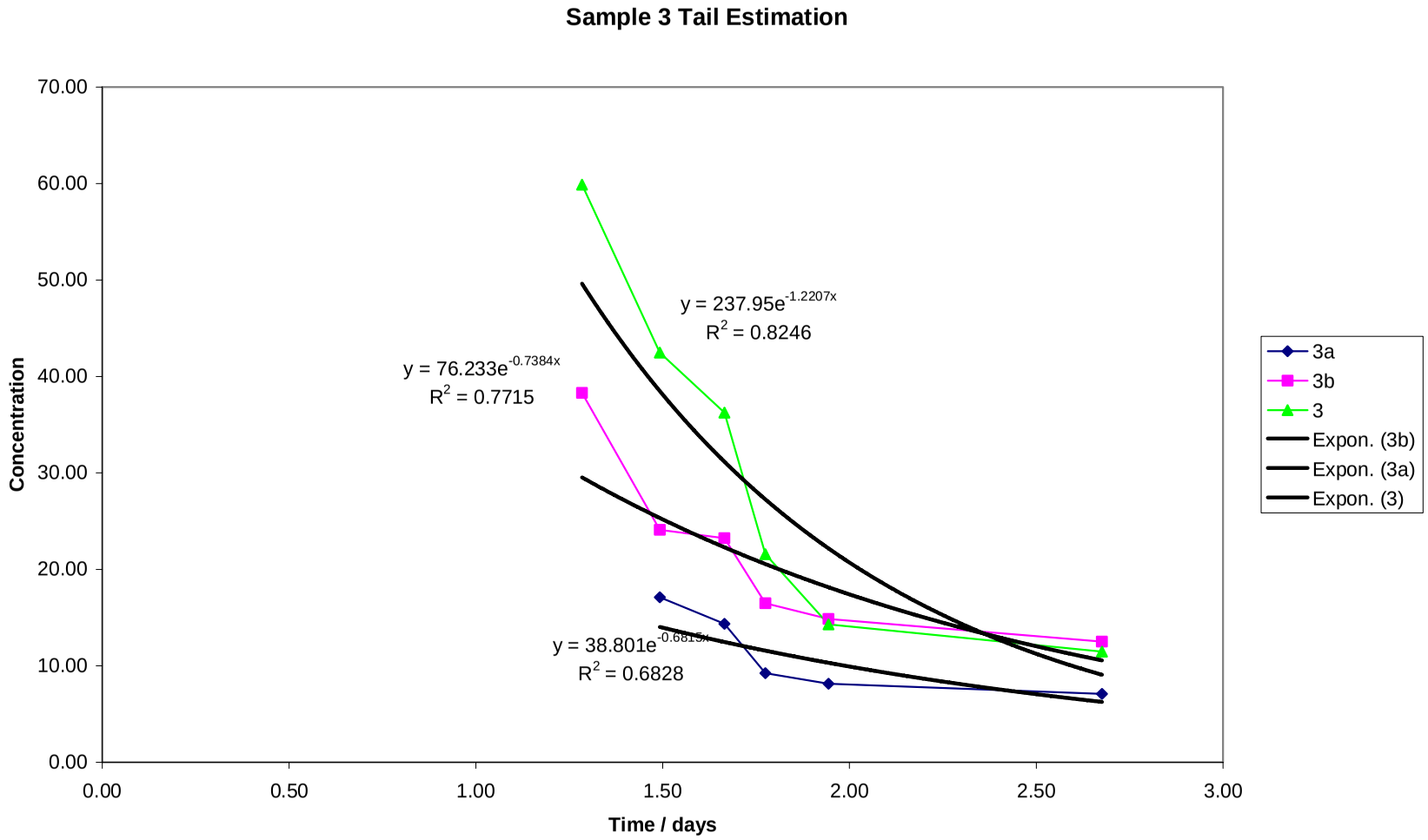
A3.1 - Sample Ports – i = 1



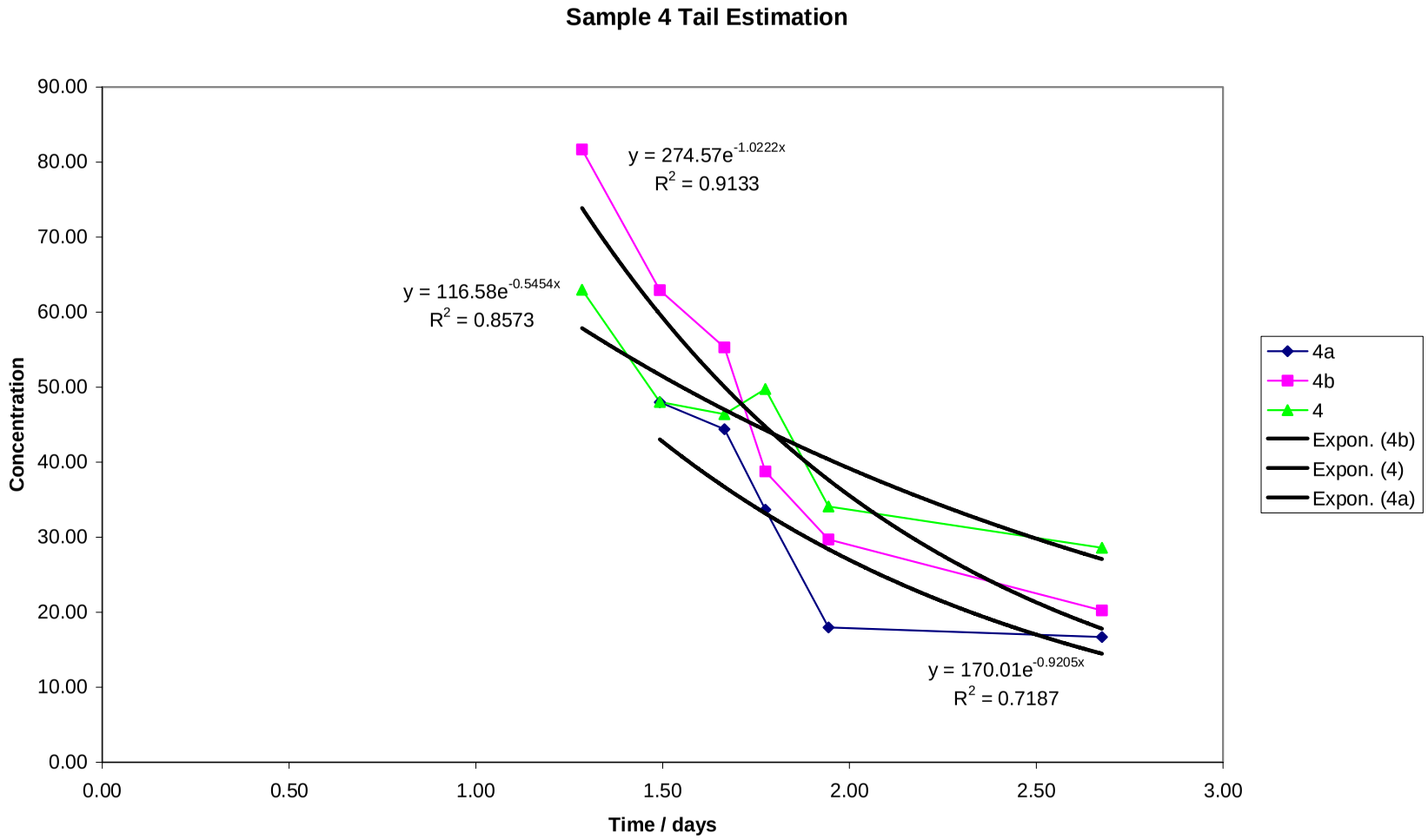
A3.2 - Sample Ports – i = 2



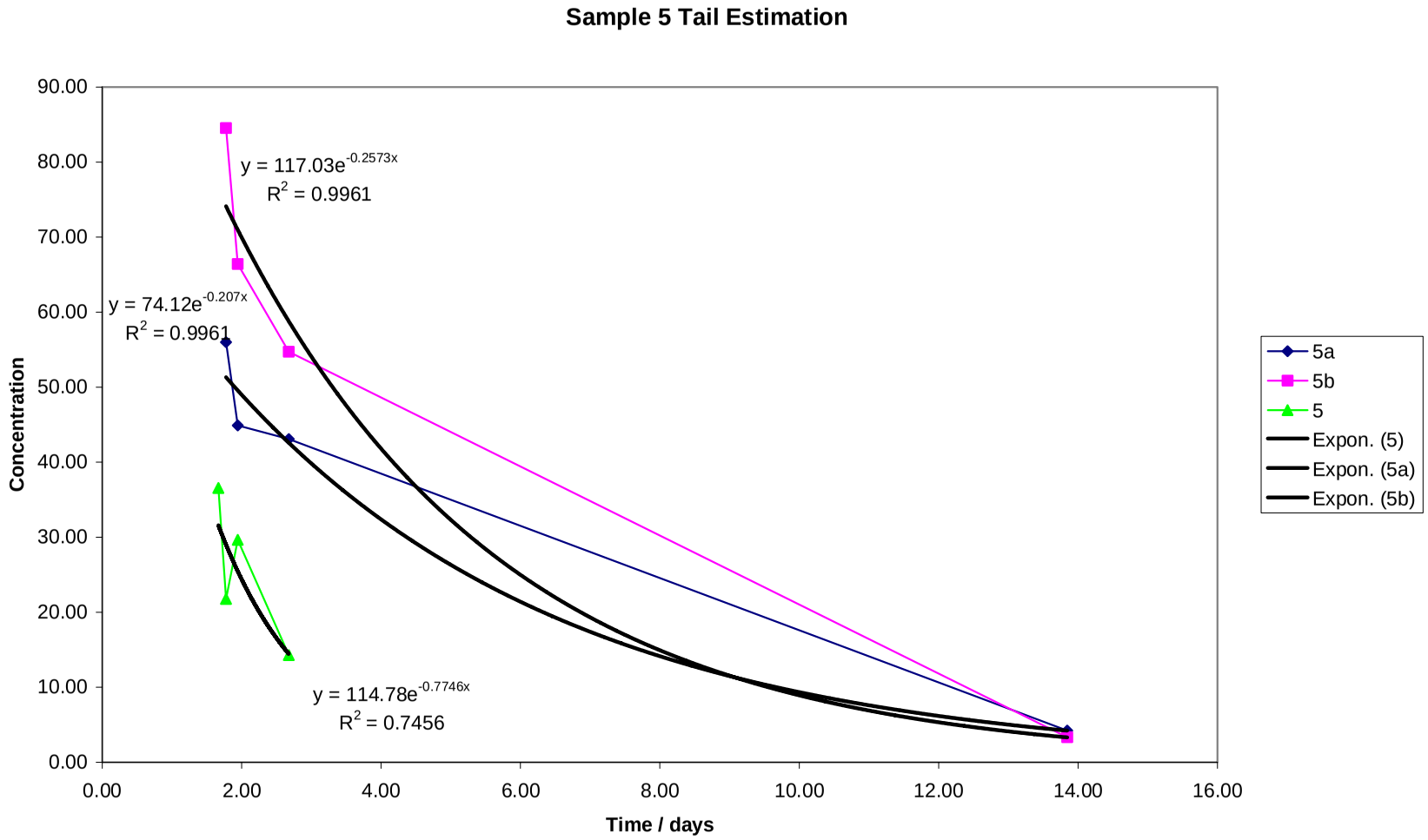
A3.3 - Sample Ports – i = 3



A3.4 - Sample Ports – i = 4



A3.5 - Sample Ports – i = 5



Appendix 4
Derivation of Equation of the General Model

Given equations [1] and [2]:

$$C_{i,N} = \frac{C_{i,0}}{\left(1 + k_i \cdot \tau_{Overall}\right)^N} \quad [2]$$

$$V_{Eff} = Q \cdot \tau_{Overall} \quad [1]$$

and

$$\tau_{Overall} = N \cdot \tau \quad [4]$$

We can substitute equations [1] and [4] into [2], which will yield:

$$C_{i,N} = \frac{C_{i,0}}{\left(1 + k_i \cdot \frac{V_{Eff}}{Q \cdot N}\right)^N} \quad [5]$$

From the tracer study, at sample port 2:

$$V_{Eff} = Q_{Test} \cdot \tau_{Test}$$

$$V_{Eff} = \frac{0.75 m^3}{hr} \cdot 22.62 hrs$$

$$V_{Eff} = 16.96 m^3$$

The real volume is calculated using the following formula:

$$V = \varepsilon \cdot l \cdot d \cdot w \quad [6]$$

where ε is the void fraction (calculated at 0.4) of the gravel bed, l is the length (16m at port 2), d is the depth (1m) and w is the width (4m).

Thus:

$$V = 0.4 \cdot 16 \cdot 1 \cdot 4$$

$$V = 25.6 m^3$$

Thus:

$$\frac{V_{Eff}}{V} = \frac{16.96 m^3}{25.6 m^3} = 0.6625 \approx \frac{2}{3}$$

$$V_{Eff} = \frac{2}{3} \cdot V \quad [7]$$

Substituting [7] into [5],

$$C_{i,N} = \frac{C_{i,0}}{\left(1 + k_i \cdot \frac{2}{3} \frac{V}{Q \cdot N}\right)^N} \quad [8]$$

If [6] is substituted into [8], equation [9] follows:

$$C_{i,N} = \frac{C_{i,0}}{\left(1 + k_i \cdot \frac{2}{3} \frac{l \cdot d \cdot w}{Q \cdot N}\right)^N} \quad [9]$$

A relationship can be found between N and l – i.e.

$$N = 0.0088 \cdot l^2 + 0.0538 \cdot l \quad [10]$$

Substituting [10] into [9] yields:

$$C_{i,N} = \frac{C_{i,0}}{\left(1 + k_i \cdot \frac{2}{3} \frac{l \cdot d \cdot w}{Q \cdot (0.0088 \cdot l^2 + 0.0538 \cdot l)}\right)^{0.0088 \cdot l^2 + 0.0538 \cdot l}} \quad [11]$$

Where:

$C_{i,N}$ is the concentration of Component i at length l,

$C_{i,0}$ is the concentration of Component i initially

k_i is the rate constant of component i

l is the length of the bed

d is the depth of the bed (should be 1m for the hydraulic modelling to be accurate)

w is the width of the bed (should be 4m for the hydraulic modelling to be accurate)

Q is the flow rate of effluent into the CW

The void fraction can be included in this model by adjusting the k-values – the k-values were calculated for reactors that had zero void fraction – thus we need a modification of the effective volume ratio modify to include the void fraction.

This can be calculated as follows:

$$newratio \cdot \varepsilon = oldratio \quad [12]$$

$$x \cdot 0.4 = \frac{2}{3}$$

$$x = \frac{5}{3}$$

Such that the general form of the equation can be given as:

$$C_{i,l} = \frac{C_{i,0}}{\left(1 + k_i \cdot \frac{5}{3} \frac{\varepsilon \cdot l \cdot d \cdot w}{Q \cdot (0.0088 \cdot l^2 + 0.0538 \cdot l)}\right)^{0.0088 \cdot l^2 + 0.0538 \cdot l}} \quad [13]$$