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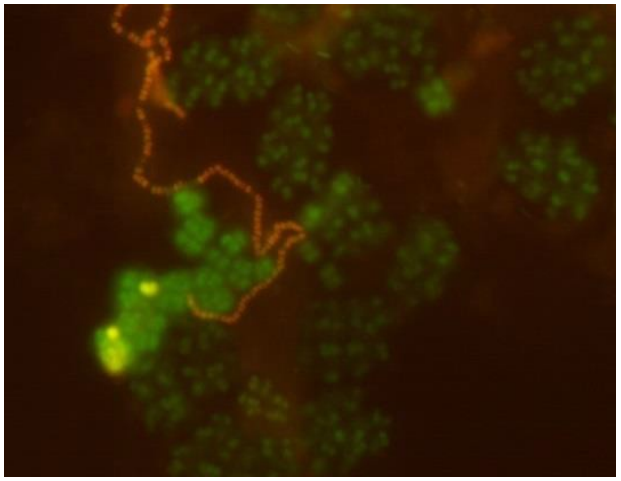
Developing a fundamental understanding of the microbiological treatment of winery wastewater



FINAL REPORT to

AUSTRALIAN GRAPE AND WINE AUTHORITY

Project Number: UA 1301

Principal Investigator: **Paul R Grbin**

Research Organisation: **The University of Adelaide**

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Developing a fundamental understanding of the microbiological treatment of winery wastewater

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16 December 2016

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Table of Contents

Contents

Abbreviations:

CAL	Covered Anaerobic Lagoon
CCA	Canonical Correlation Analysis
COD	Chemical Oxygen Demand
DNA	Deoxyribonucleic Acid
EBPR	Enhanced Biological Phosphorus Removal
Ec	Electrical conductivity
Eff	Effluent
FISH	Fluorescent in situ Hybridisation
F:M	Food to Microbe ratio
GALO	Gordonia Amarae-Like Organism
GAO	Glycogen Accumulating Organism
GC	Gas Chromatography
gDNA	Genomic Deoxyribonucleic Acid
HPLC	High Performance Liquid Chromatography
HRT	Hydraulic Retention Time
ICP-MS	Inductively Coupled Plasma Mass Spectrometry
Inf	Influent
MLSS	Mixed Liquor Suspended Solids
PCA	Principal Component Analysis
PG	Pinot Gris/Grigio
PHA	Polyhydroxyalkanoate
rRNA	Ribosomal Ribonucleic Acid
SBR	Sequencing Batch Reactor
SRT	Sludge Retention Time
SV	Sludge Volume
WWTP	Winery Wastewater Treatment Plant
WWW	Winery Wastewater

1. Abstract

The objective of this project was to develop a fundamental understanding of the microbial populations that exist in the treatment of winery wastewater. Winery wastewater treatment plants (WWTP) were surveyed across Australia, at different stages of wine processing, and from anaerobic and aerobic treatment and storage lagoons. Conventional and molecular microbiology data identified microbial communities and relationships with effluent chemistry and plant operations were extrapolated. Pre-vintage preparation, solids management in the winery, surge water management and nutrient dosing were found to be key to promoting a healthy microbial community, essential for efficient and effective wastewater treatment.

2. Executive summary

Biological treatment of winery wastewater is common, but it has been poorly understood and difficult to manage. This project aimed to provide industry with strategies to reduce these difficulties, making the process more efficient and effective. It was a collaborative project between the University of Adelaide Wine Microbiology and Biotechnology Laboratory, CSIRO Land and Water and JJC Engineering.

We surveyed over 30 plants for three consecutive vintages, providing each winery a snapshot of what their treatment plant looked like from a microbiological point of view, improving the understanding of their treatment systems. So-called 'G-bacteria' dominated many plants and were associated with poor settling and cloudy supernatant, which prompted a PhD project titled, 'Identity and Ecophysiology of Glycogen Accumulating Organisms (GAO) in winery wastewater treatment plants' to be established to specifically target their dominance. *Alysiosphaera europea*, *Gordonia amarae*-like organisms, *Zoogloeae* and Type 0041/0675 are all commonly observed in WWTP. Yeast also dominated WWW in many plants and were associated with poor solids management in the winery.

Additionally, four WWTP were frequently sampled and analysed over three years to develop an understanding of trends that occur at the start of vintage, during peak vintage and at quiescent periods, both in terms of the microbiology of wastewater treatment plants, and the chemistry of influents and effluents. Each plant displayed a unique microbial community and while the communities at three plants were very stable, one plant was very dynamic and regularly changed in response to environmental changes and contained many novel organisms. Each of the plants was treating wastes from wineries with different operations however they shared similar characteristics. Influents were characterised by high Ec, low pH, low nitrogen and phosphorus levels and very high COD, which was attributed to large concentrations of phenols, ethanol and tartaric acid.

The application of anaerobic digesters to treat winery wastewater is growing internationally however they are still rare in Australia. Anaerobic treatment offers significant environmental and economic benefits and we believe this to be the direction in which wineries should be moving. We investigated two plants to gain a snapshot of the organisms present and the environmental factors that influence

the community structure. It was found that temperature, pH and phosphorus have the greatest impact on the community structure and microbiology revealed low methanogen populations, so there is scope to improve performance significantly.

In response to consultation with wineries, two additional studies were undertaken. One to assess any potential health hazard associated with working with lagoon water and a second to investigate the impact of common additives (charcoal, perlite, skim milk and bentonite) used in the winemaking process on an SBR.

Potentially toxic cyanobacteria were measured in storage lagoons. Levels were found to be very low, posing no health threat to plant operators. Toxin levels were well within the World Health Organization guidelines for irrigation water.

Scientific data confirmed empirical observations by plant operators that charcoal has a significant negative effect on SBRs. Charcoal released the phenolics, ethanol, sugars and organic acids it had stripped from wine and juice back into the SBR water, significantly increasing the COD load on plants and lowering the pH.

Acknowledgements

The research and activities covered in this project were financially supported by the Australian Grape and Wine Authority (trading as Wine Australia).

This project was a joint venture between the University of Adelaide, JJC Engineering and CSIRO Land and Water. The team at the University of Adelaide was led by Paul Grbin and Kathryn Eales, with Research Assistant Patrick Rea and PhD candidate Cristobal Onetto, and all the members of Wine Microbiology and Biotechnology Group at the University of Adelaide. The team at CSIRO was led by Dr. Anu Kumar, and special thanks go to Debra Gonzago for her technical skills and organisation. Mike Carson and John Constable of JJC Engineering provided engineering and operational expertise.

The project would not have been possible without the support of over 30 wineries Australia wide. Berri Estates, Brown Brothers, Campbells, Casella Family Wines, Coldstream Hills, d'Arenberg, De Bortoli, Domaine Chandon, Grant Burge, Houghton Wines, Hunter Valley Wine Group, Josef Chromy Wines, Karadoc Lindemanns, Littore, Margaret River Wine Production, Medhurst, NPEC, Oakridge, Pernod Ricard, Pfeiffer Wines, Rosemount, Taylors, The Wine Group, Southern Estate Wines, Wickham Hills, Winemaking Tasmania, Wirra Wirra, Wolf Blass, Wynns, Yabby Lake and Yalumba Oxford Landing,

Finally, we would like to acknowledge a few individuals at our four main winery sites. Frank Zirilli and Jon Jefferson at Southern Estate Wines, Griffith, NSW. Bart Challacombe and Ross Webster from Casella Family Wines, Yenda, NSW. Darryl Grear and Grant Kohlhagen from Pernod Ricard Wines, Rowland Flat, SA and Luke Wilson, Alana Seabrook and Robby Mercuri of Yalumba Wines. Each person was incredibly generous with their time and readily shared all their data. Without their efforts and commitment this project could not have been successful.

3. Background

From 2008-2012 we microscopically examined a large number of sludge samples from WWTP. *Zoogloeae*, Glycogen Accumulating Organisms (GAO), *Gordonia amarae*-like organisms (GALO), *Nostocoida limicola* II and cyanobacteria were all commonly observed and frequently caused problems. *Zoogloeae*, GAO and *Nostocoida limicola* II often cause bulking and poor sludge dewatering. The filamentous bacteria GALO produces stable foams and cyanobacteria (blue green algae) putrefy and cause off-odours. Many of the problematic bacteria observed are due to nutrient deficiency and can potentially be avoided if there is a better understanding of the microbial community dynamics and WWW chemistry. Additionally, unidentified filamentous bacteria had also been observed. The function of these filaments, advantageous or not, remains unknown.

We have presented in several WWW workshops and there is always enthusiasm for more microbiology knowledge. Microbiological analysis was considered the critical missing component for better understanding WWW systems. Currently, the biodiversity and ecophysiology of microbial communities in WWW treatment plants is largely unknown, this was easily established by undertaking a literature survey. Similarly, the effects of fluctuating flow volume and chemical composition of WWW on plant microbiota has not been determined.

The unique operational parameters of WWW treatment do not apply in other industrial or domestic wastewater treatment plants. Many of the standard values that are used by plant operators such as Sludge Volume Index (SVI), retention times and Carbon:Nitrogen:Phosphorus ratios are unachievable for WWW, and specific guidelines for WWW are required to help direct process operations to control microbial populations.

Winery wastewater treatment is unique. There are large temporal fluctuations in flow volume, with more than 40 % occurring during vintage, along with variation in COD, pH and electrical conductivity (Fernandez et al. 2007; Quayle et al. 2009). Despite this unique ecophysiology, characterisation of the microbiology of WWW treatment plants has been limited. A few studies have focused on plant design but not microbial community analysis (Eusebio et al. 2004; Malandra et al. 2003). The dominance of G-bacteria in WWW sludge has only been recently reported (Kiss et al. 2011; S. McIlroy et al. 2011). Kiss et al. (2011), reported them to be beneficial, however that has not been our experience or that of

McIlroy (2011). Further, other problems relating to the microbiology of WWW have been reported, for example the production of volatile fatty acids (Bories et al. 2007) and lack of available nutrients (Rodriguez-Caballero et al. 2012). Therefore, microbiological characterisation of WWW treatment plants will provide a greater understanding of the treatment process and drive the development of tools to manage them more effectively.

The highly variable nature of WWW impacts on microbiological plant health, as evidenced by events of foaming, poor settling, overloading and process failure. When this occurs, wineries incur additional costs from increased aeration to replacing equipment. Furthermore, EPA or local government fines may be issued and if problems persist, then forced plant closure can occur. This has cascading negative economic (particularly if during vintage), environmental and social effects.

As intensification of winemaking in Australia continues, wineries will be under increased pressure to control odour, recycle wastewater and reduce environmental footprint, therefore all will benefit from improved understanding, design and operation of biological treatment processes.

4. Project Aims and Performance Targets

The overall aim of this project was to develop a better understanding of the unique microbiology of WWW treatment to improve plant operation.

To achieve this, a number of sub aims and outputs were addressed.

Aim 1. Investigate the normal microbial communities of winery activated sludge plants at three key stages throughout the year.

Output 1: *The microbiology of Australian winery wastewater treatment plants during peak vintage.* Approximately 30 wineries were sampled and microbiologically investigated during peak vintage in 2014, 2015 and 2016.

Output 2: *Anaerobic processing*. Samples were taken routinely over 2.5 years from two anaerobic digesters; one in the Barossa Valley and one in Griffith. These samples were chemically and microbiologically examined.

Output 3: *Seasonal changes in microbial communities.* Molecular community profiles were analysed and changes through winery operations during a yearly cycle were followed to develop a fundamental understanding of biological WWW treatment.

Output 4: *Novel isolates.* Identify (e.g. novel filaments) and assess viability of microorganisms isolated to enable optimised treatment operations for the maintenance of a healthy system.

Output 5: *Chemistry of winery wastewater* and *operational parameters.* Chemical, production and processing data was collected over the period of the project.

Aim 2. Determine factors affecting growth and nutrition of the microbial consortia, to eliminate/prevent the development of problematic microbes.

Output 6: *The dominant organisms and factors affecting their growth.* Observations and statistics were used to identify relations between microbial populations and environmental parameters.

Output 7: *Additives study.* The effect of charcoal, skim milk, perlite and bentonite on SBR chemistry was determined.

Output 8: *Full scale trials.* Full scale trials were proposed for each of the four main sites.

Aim 3. Develop practical methods for the examination and characterisation of WWW.
Develop guidelines for plant operators to provide tools to maximise plant microbial efficiency and reduce the likelihood of microbiologically related plant failures. These guidelines were produced in a web-based format and/or manual and that easily identify the microbe and relate it to operational parameters that assist in control.

Output 6 and Appendix 1: Communication

5. Methods

5.1 Winery wastewater treatment plants examined in this study

This research was made possible by the 32 wineries across Australia that supported the project and provided wastewater samples during peak vintage over three years; 2014, 2015 and 2016 (Figure 5.1). Winery wastewater treatment plants were described based on location, crush size, winery operations (grape varieties and solids management), bottling, plant design (pretreatments, anaerobic/aerobic processing, type of aeration) and level of process control (monitoring, maintenance and management).

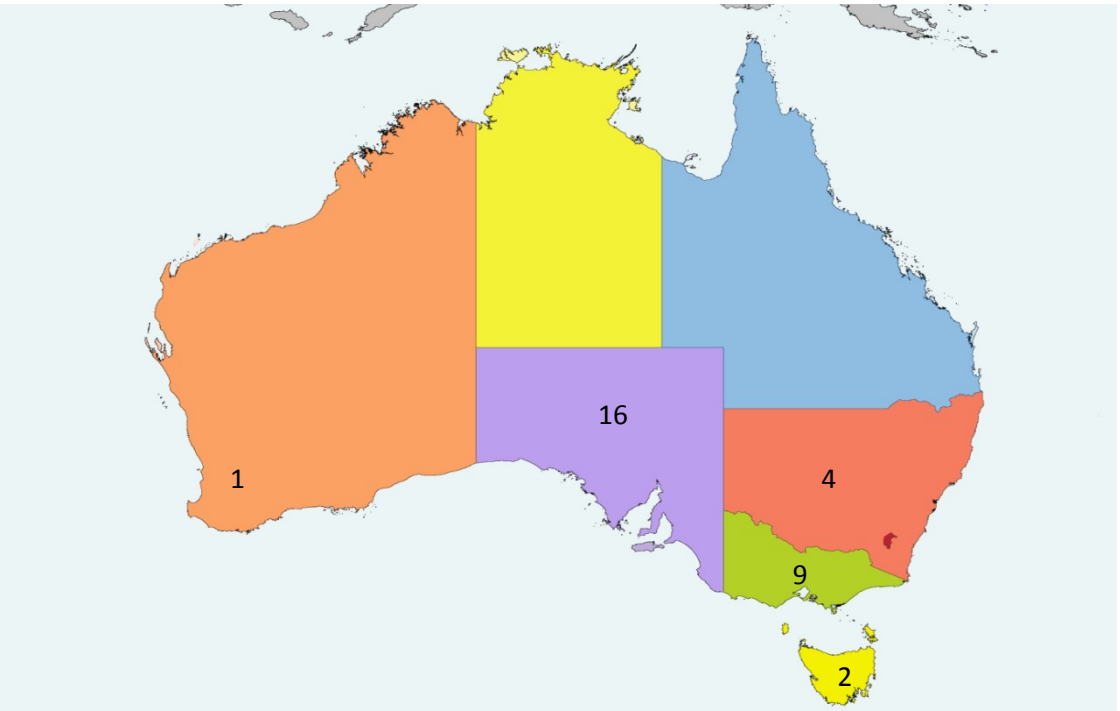


Figure 5.1: Location of wineries sampled for the microbiology survey.

There was a significant focus on four sites, each of these plants was designed by John Constable at JJC Engineering and their ongoing management is assisted by Mike Carson (JJC Operations) and John Constable. Briefly, each plant has a pretreatment zone where influent is screened and settled before commencing biological treatment (Table 5.1). COD removal across the plants was effective with removal all above 90%.

Grab samples of raw influent and mixed liquor were requested from WWTP and couriered to the laboratory. All samples were stored at 4^{o}C for no more than 48h before analysis. Each

sample was microscopically examined after Gram, Neisser, Sudan and Nigrosin staining. Microscopy was carried out with a Nikon Eclipse microscope.

Table 5.1 Description of four main sites investigated in this project.

	Plant A	Plant B	Plant C	Plant D
Location	Griffith	Barossa	Griffith	Barossa
Crush size (t)	180,000	22,000	18,000	25,000
Bottling	Yes	Yes	No	No
CAL (ML)	30	N/A	N/A	5
SBR (ML)	6	5	0.6	1.5
Aeration	Coarse/surface	surface	Fine	Coarse/surface

Fluorescence in situ hybridisation (FISH) analyses of samples and biovolume estimates

Environmental samples were fixed and the FISH protocol conducted as described by Amann et al. (1995). Oligonucleotide probes used in FISH identification studies are listed in Appendix 5.1. Biovolume fractions were estimated using imaging analyses software DAIME v2.1 using specific probe and EUB338mix probe for total biovolume.

Cyanobacteria identification

Samples were collected from storage lagoons and transported on ice and tested within 3 days. Samples were examined by ELISA assay for the presence of microcystin and nodularin toxins using Abraxis Microcystins –DM ELISA microtitre plates, according the manufacturer's instructions.

Wastewater analysis

pH, Ec, SV and MLSS were assessed in accordance with standard procedures (Rice Bridgewater and Association, 2012). Sludge volume was recorded at 30min (SV30) and 60min (SV60). COD was determined using Hach mercury free COD 2 high range kits (Hach). Turbidity was determined using a turbidity meter and the results reported in Nephelometric Turbidity Units (NTU). Clarity was determined by measuring the absorbance at 650nm using a UV-Vis spectrophotometer.

Organic acids (acetic, tartaric, malic, lactic, succinic), sugars (glucose and fructose), ethanol and glycerol were measured using an HPLC fitted with an ion-exchange Aminex HPX-87H column (Bio-Rad, USA), coupled to a refractive index detector (Agilent Technologies).

Total phenolics were measured by UV-Vis absorbance at 280nm.

Descriptive statistics and graph analyses showing most common statistical parameters, such as means, medians and correlations etc. illustrating changes and shifts in the chemical composition of the plants were determined.

DNA extraction, PCR amplification and pyrosequencing.

DNA extraction was conducted using the FastDNA spin kit for soil (MP biomedical) with optimised modifications for activated sludge (Albertsen et al. 2015). 16S rRNA amplicons were generated targeting the V3-V4 region using primers 341F and 806R (Table 5.2) (Muyzer et al. 1993) and sequenced on a MiSeq (Illumina) at the Australian Centre for Ecogenomics (Brisbane).

Table 5.2 Primers and sequences applied

	Primer	Sequence specific region
341F	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCCTACGGGNGGCWGCAG	CCTACGGGNGGCWGCAG
806R	GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGACTACHVGGGTWTCTAATCC	GACTACHVGGGTWTCTAATCC

Sequencing analysis

All data analysis and visualisations were conducted using R. The raw sequence data from Illumina analysis were quality checked and trimmed with Trimmomatic (Bolger et al. 2014), and forward and reverse reads merged in FLASH (Magoč and Salzberg 2011). The merged reads were dereplicated and formatted for use in the UPARSE workflow (Edgar 2013). These reads were clustered and OTU abundance was estimated using USearch. Taxonomy was assigned using the MiDAS database (S. J. McIlroy et al. 2015). Samples were rarefied and cut to 12000 for comparison. Amplicon data was analysed and visualised using the 'ampvis' package which builds on the R packages 'phyloseq' and 'vegan'.

Bioinformatic analysis

To analyse the any potential statistical correlations between OTU species defined by MiDAS and environmental variables of the WWTPs a number of different statistical methods were applied. Twenty six environmental variables were analysed for their significance SV60, MLSS, CODf, NPOC, TN, P, F:M, COD:N, COD:P, citric, tartaric, malic, succinic, lactic, acetic, glucose, fructose, glycerol, ethanol, phenols, calcium, potassium, magnesium, sodium, sulfur and Effluent SS. The metadata file is available in Appendix 5.4.

Principal component analysis (PCA) was conducted, using vegan with square root transformed OTU counts. Significance of treatments was tested, using the envfit parametric test on the first two principal components and on the Bray-Curtis dissimilarity matrix.

Canonical Correlation Analysis was applied to determine relationships between the two sets of multivariate data (16S rRNA gene sequence and the chemistry and operational data). Analysis was performed using Vegan and permutation tests for significance performed.

Two additional outputs were included in the project:

1. There was some concern and interest regarding potential health impacts on staff and use with irrigation. Therefore cyanobacteria testing was carried out on storage lagoons.
2. Plant operators were reporting increased difficulties in managing wastewater treatment systems when Pinot Gris/Grigio grapes and wines were being

processed, which led to an investigation on the impact of charcoal on these treatment plants. We expanded the investigation to include other common additives in wineries i.e. perlite, bentonite and PPVP.

6. Results/Discussion

6.1 Output 1: Annual survey of the microbiology of WWTP

Aerobic biological treatment is common in winery wastewater treatment plants both in Australia and internationally, yet there are very few reports of the bacteria associated with these systems. Understanding the microbial community is helpful in optimising the efficiency of these plants and surveys of this kind have been common in industrial wastewater treatment plants. This is the first report of an extensive survey looking particularly at winery wastewater treatment plants and aimed to characterise both the problematic and common organisms in WWTP.

After three years of surveying, we now have a very clear idea of the common microbes associated with WWTP and these are summarised in Table 6.1. G-bacteria were present in over 50% of all plants surveyed. *Nostocoida limicola* II, *Gordonia amarae*-like organisms, *Zoogloeae*, Type 0041/0675 and Type 0092 should also be considered common populations in WWTP.

Nostocoida limicola II, GALO and *Zoogloeae* are all very common. Type 0041/0675 G-bacteria are the most commonly observed organism in WWTP. They can be beneficial as they remove excessive carbon beyond their growth requirements, assisting to reduce the high COD levels typical of WWW. However, they can also be problematic as they disassociate from the floc and cause cloudy supernatant and poor settling. Furthermore, the G-bacteria can proliferate during quiescent periods when food is low and they can use their internal carbon reserves and continue to thrive, leading to poor diversity, an unbalanced community and problems in the WWTP. At the four main sites, we now have detailed ecogenomic profiles of the populations present in these systems and are relating population shifts over the key stages of winery operations.

Table 6.1: Common microbes associated with WWTPs over a three year period.

	2014				2015				2016			
ORGANISM	Dominant	Secondary	Observed	**Total**	Dominant	Secondary	Observed	**Total**	Dominant	Secondary	Observed	**Total**
G-BACTERIA	7	7	5	**19**	5	6	4	**15**	9	5	2	**16**
N.LIMICOLA II	2	2	10	**14**	2	3	7	**12**	0	5	6	**11**
YEAST	5	0	2	**7**	6	0	5	**11**	4	0	3	**7**
GALO	3	1	3	**7**	3	3	3	**9**	1	2	4	**7**
TYPE 0041/0675	4	1	1	**6**	3	1	2	**6**	4	0	3	**7**
GRAM +VE SINGLE CELLS	3	1	2	**6**	1	2	1	**4**	0	0	2	**3**
TYPE 0092	0	3	3	**6**	0	2	4	**6**	0	0	2	**2**
ZOOGLOEA	0	2	4	**6**	1	1	4	**6**	2	1	4	**7**
TYPE 0803/0914	2	0	2	**4**	1	1	1	**3**	1	0	1	**2**

UNKNOWN	3	0	0	**3**	3	0	0	**3**	1	0	0	**1**
THIOTHRIX	1	0	2	**3**	1	0	1	**2**	3	0	1	**4**
TYPE 0411	0	1	1	**2**	0	0	2	**2**	0	0	0	**0**
TYPE 0961	1	0	0	**1**	1	0	0	**1**	0	0	0	**0**

6.1.1 G-bacteria

The most commonly observed organisms were the G-bacteria. G-bacteria are a phenotypically related group of bacteria with many phylotypes (Seviour and Nielsen 2010). FISH analysis showed that G-bacteria belonging to the *Alphaproteobacteria*, *Actinobacteria* and *Gammaproteobacteria* are all present in WWW, however the most commonly observed were the Alphaproteobacterial, *Defluviicoccus vanus* related G-bacteria (Figure 6.1.1b). Large populations are often present and have accounted for 60% of the entire bacterial community as determined by biovolume and 16S rRNA gene profiling.

G-bacteria are widely distributed, appearing in over 50% of all plants surveyed, with a range of configurations and crushing between 200-200,000 tonne. G-bacteria have been observed in WWTP before (Kiss et al. 2011; S. McIlroy et al. 2011) and should be considered a common component of WWTP. In a balanced population when they are forming part of the floc, G-bacteria can be beneficial in removing large concentrations of carbon from the influent. However, in plants where they proliferate, they can become troublesome causing poor settling in SBRs and a decrease in effluent quality with high turbidity and hence COD.

Identification: Cells are spherical and 2µm in diameter, they are arranged in tetrads or grape like clusters. They can have various Gram and Neisser staining depending on the phylotype present. They stain positively for Sudan Black.

Physiology: G-bacteria have been well studied in domestic wastewater treatment plants. They assimilate simple organic acids, thriving on acetate and are associated with nutrient deficient conditions.

Control: Direct feeding and nutrient dosing, particularly nitrogen.

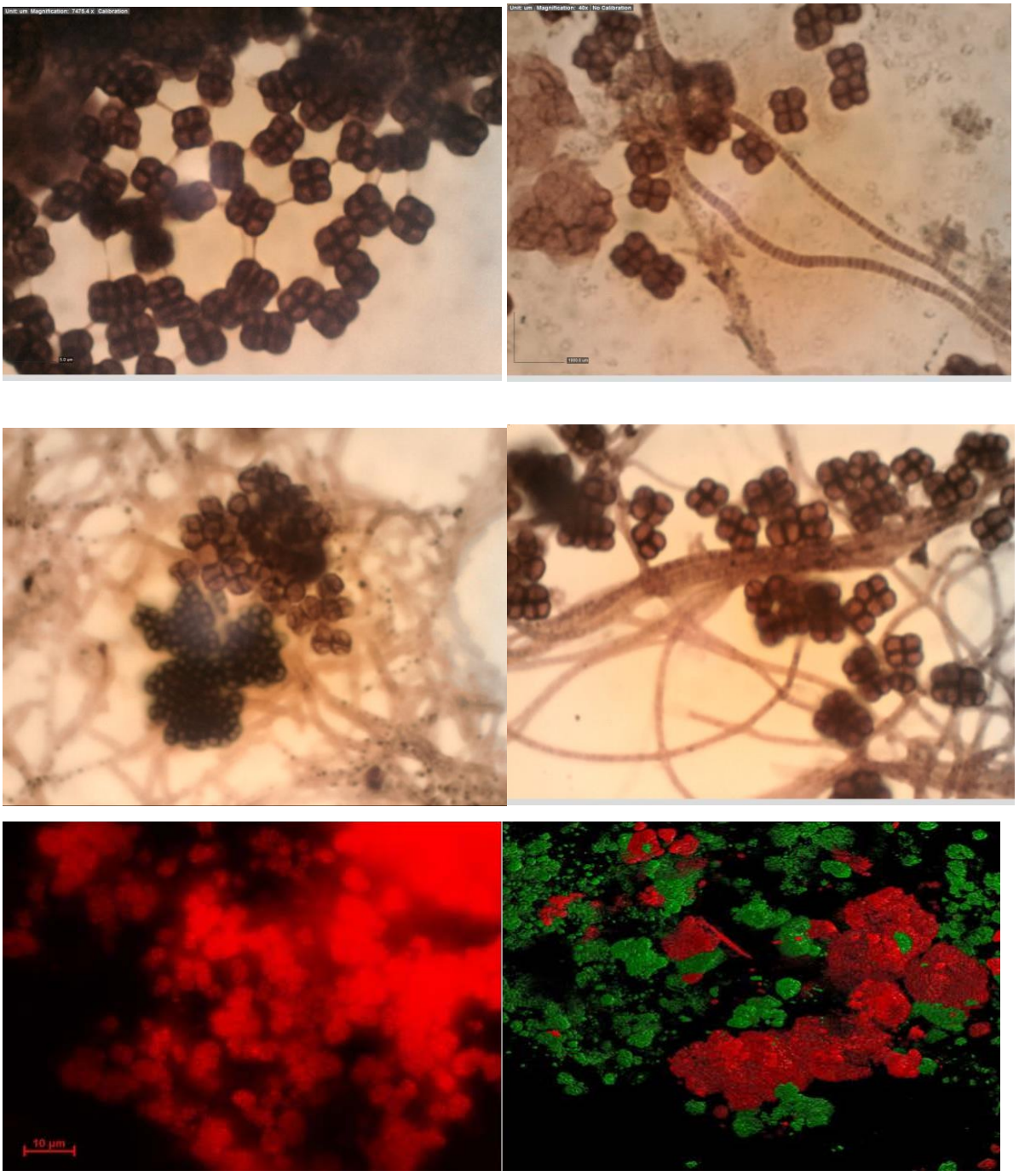


Figure 6.1.1 (a-d) Different phylotypes of G-bacteria with different secondary filaments.(e) DF2 probe confirming these cells as *Defluviicoccus vanus*. (f) Biovolume of DF2 (CY3, red) and EUB338mix (Fluos, green).

6.1.2 Nostocoida limicola II

Nostocoida limicola II was commonly observed as a secondary filament, especially when G- bacteria are present. Although *Nostocoida limicola* is often associated with bulking in other activated sludge systems (Seviour and Nielsen, 2009) it rarely proliferates to troublesome levels in winery systems. Again, *Nostocoida limicola* II is a phenotypic characterisation, FISH confirmed that this Gram negative, Neisser positive morphotype routinely observed in these systems belongs to *Alysiosphaera europea.*

Identification: Oval shaped cells with a diameter of 1-1.4µm, in coiled filaments. Gram and Neisser variable depending on pylotype, Sudan black positive.

Physiology: They assimilate sugars and ethanol, but not acetate.

Control: Increase aeration and mixing. Reduce sludge age.

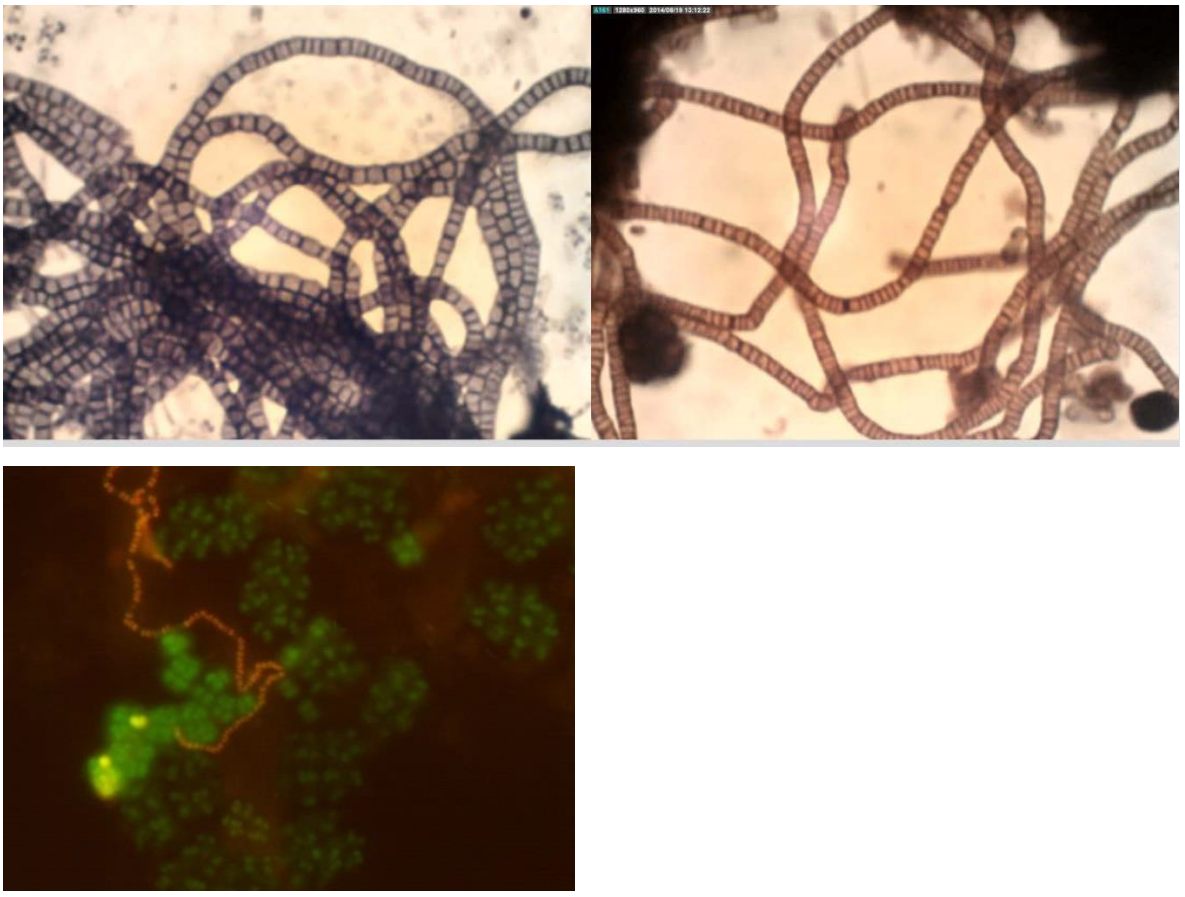


Figure 6.1.2 Neisser positive Nostocoida limicola II and positive reaction to Noli664 probe (CY3, orange) targeting *Alysioshpaera europea* with EUB338mix (Fluos, green) a universal probe, many G- bacteria morphology cells present.

6.1.3 Yeast and Gram positive bacteria

The common occurrence of yeast and Gram positive bacteria (identified as lactic acid bacteria) are due to the nature of the winery waste, as these organisms perform wine fermentation. It was found that they are usually present in plants with low process control and that could be characterised as overloaded, with a low pH and under aerated. These treatment plants were not removing the required COD.

Identification: *Saccharomyces cerevisiae* cells are oval shaped with a diameter of 4µm.

Physiology: normal component of wine fermentation. In a wastewater treatment plant, they indicate low process control and overloading, low pH and under aeration and failing. These plants are not removing the required COD.

Control: Cleaner production strategies and improved solids separation in the winery.

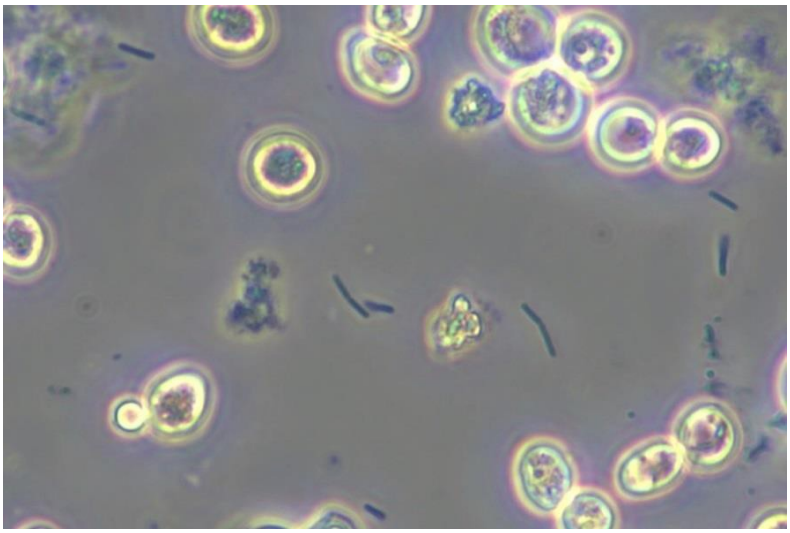


Figure 6.1.3 WWW sample examined under phase microscopy, yeast cells are clearly visible.

6.1.4 Type 0041/0675

Type 0041/0675 was only observed as the dominant species in aerobic zones that were preceded by anaerobic digestion. Type 0041/0675 have been associated with the *Chloroflexi* and their physiology has been tied to protein degradation.

Identification: Cells are squares or rectangles, 0.5-1.5µm X 0.7-2.5µm, that grow in long straight filaments. Type 0675 is usually regarded as smaller than Type 0041, however cell properties are often difficult to observe due to the presence of large populations of attached growth. Easily identified by the large numbers of cells attached to filaments. Gram, Neisser and Sudan variable.

Physiology: Feeds on N-acetylglucosamine, a major component of bacterial cells that is released in wastewater systems as cells degrade. Type 0041/0675 is associated with long sludge age, low F:M ratios and nutrient deficiencies.

Control: Sludge wasting, direct feeding and nutrient dosing.

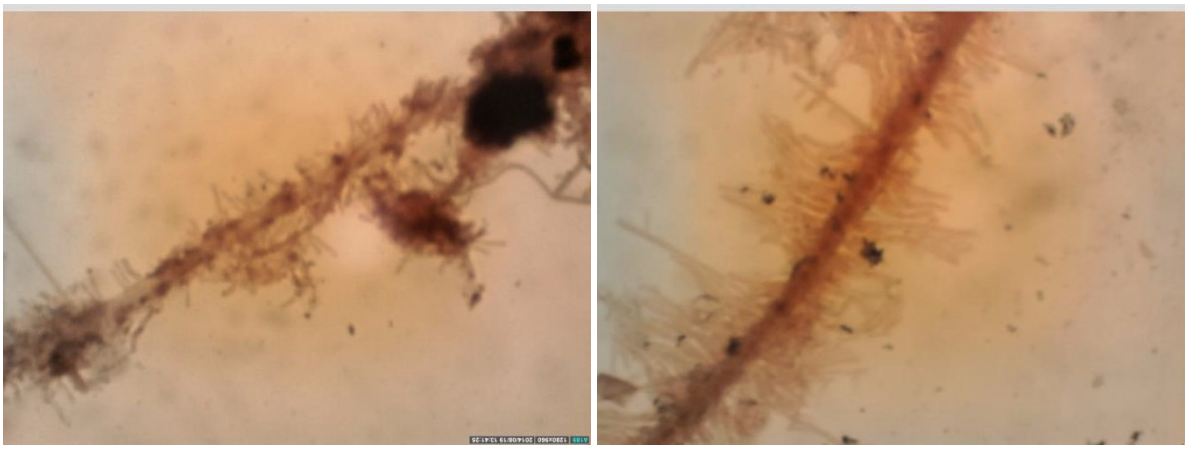


Figure 6.1.4 Type 0041/0675 showing both long and short attached growth.

6.1.5 *Gordonia amarae*-like organisms (GALO)

Plants experiencing foaming episodes were dominated by GALO. Empirical observations indicated a relationship between high temperatures and GALO. From this study, it is not clear if the GALO foam layer acted as insulation causing the temperature of the SBR to rise, or if in fact the increase in temperature of the SBR favoured the growth and proliferation of GALO.

Identification: Gram, Neisser and Sudan Black positive cells that are arranged in filaments with right angle branching.

Physiology: GALO are physiologically very diverse, assimilating a wide range of organic acids, sugars and amino acids under anoxic and aerobic conditions GALO are capable of producing their own surfactants and have hydrophobic cell surfaces, therefore when they are present in WWTP they may often cause stable foams. Production of surfactants has been associated with nutrient deficient environments. GALO also prefer warmer temperatures.

Control: Water sprays, minimise aeration, nutrient dosing, reduce sludge age.

Figure 6.1.5 GALO with typical right angle branching and thick viscous foam.

6.1.6 Zoogloea

Zoogloea were also common but rarely dominant, they are essential to good floc structure, producing large concentrations of extracellular polysaccharide material. This material causes the sludge to become slimy, causing poor settling and difficulties with dewatering.

Identification: Gram negative rods, 0.5-1um X 1.0 – 3.6um. Gram and Neisser negative, Sudan black positive. They cluster together either in an amorphous or finger-like morphology.

Physiology: *Zoogloea* are denitrifiers and can utilise a wide range of substrates. Excessive production of extracellular polysaccharide is associated with low nutrients.

Control: Sludge wasting and nutrient dosing.

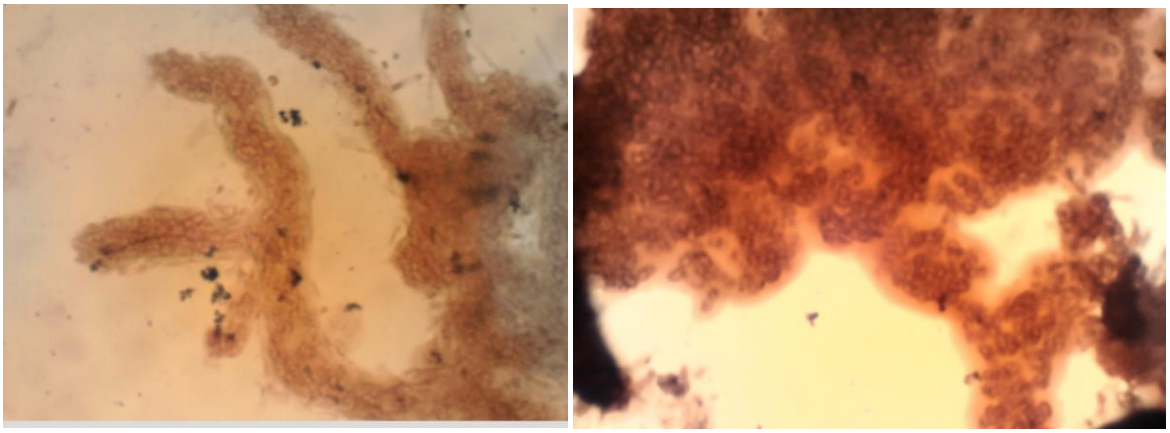


Figure 6.1.6 Zoogloea showing typical finger-like microcolonies and producing excessive amounts of polysaccharide material.

6.1.7 Type 0803/0914

Type 0803 and Type 0914 are nearly indistinguishable visually, the difference being that Type 0914 is believed to contain sulfur granules. These filaments are morphotypes with several phylotypes, 16SrRNA gene sequencing identified these filaments as belonging to *Defluviifilum* species.

Identification: Square to rectangular cells 0.7 X 1.5µm. Slightly bent filaments that when present in high numbers align parallel to each other forming bundles. Gram and Neisser negative, Sudan black positive.

Physiology: Assimilates mono- and poly-saccharides under aerobic, anoxic and anaerobic conditions. Type 0914 is associated with wastes containing sulfur compounds. They have hydrophobic cell membranes and can be associated with foaming.

Control: Nutrient dosing, increase sludge wasting, increase aeration.

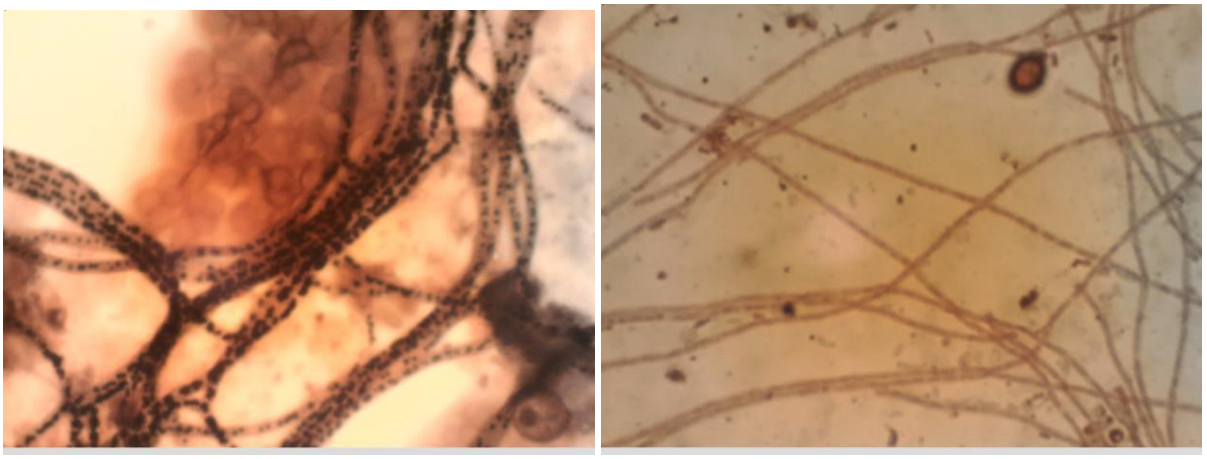


Figure 6.1.7 Images showing filaments forming parallel bundles and traverse each other

Conclusions

In a move towards sustainable management of the microbiology of wastewater treatment plants, three key factors arise; nutrients, monitoring and good cellar management.

Nutrients: All organisms commonly observed are associated with nutrient deficiency, which is not surprising given the very high organic carbon load in winery wastewater and the lack of nitrogen in the winemaking process. Wine yeast and bacteria need nutrient supplementation in order to complete fermentation and prevent sluggish performance, the same applies to the bacteria in the wastewater treatment system. Provide these organisms with the nutrients they need for efficient growth and the plant will perform better.

Monitor: Excessive carbon load, poor solids management and under aeration can lead to unbalanced populations. The proliferation of one organism is problematic as diversity creates a robust and resilient system.

Good cellar management: Essential for a healthy, efficient WWW process. Yeast and lactic acid bacteria are routinely observed and should be considered problematic and are avoidable in wastewater treatment through the application of cleaner production techniques.

6.2 Output 2: Anaerobic populations

Samples were taken from two anaerobic digesters 16 times during peak vintage and non-vintage over the three year period (2014-2016). All samples were taken at the outfall into the SBR. Samples were examined by community profiling.

Anaerobic treatment is an alternative to the traditional approach of aerobic treatment and has several potential benefits including, less area demand, low energy consumption, lower sludge production, energy generation from biogas and no noxious odours.

Anaerobic treatment is the use of microorganisms to hydrolyse and consume waste substrates in the absence of oxygen. Anaerobic organisms thrive in the high organic acid and low nutrient environment that is typical of winery wastewaters. Anaerobic treatment of waste is a three phase biological process.

1. Acidogenic bacteria convert various waste components into simpler organic acids.
2. Acetogenic bacteria convert these acids into acetic acid
3. Methanogenic bacteria convert acetic acid into methane

The two covered anaerobic lagoons (CALs) investigated in this study have very similar configuration, both designed by JJC Engineering. Plant A is 30ML and Plant D is 5ML lagoon both with HRT of >20days and they receive wastewater of similar characteristics (Table 6.2.1). They are operated differently, as Plant A is managed to optimise COD removal with regular inflow and discharge volumes and achieves a COD removal of approximately 55-60% during peak vintage and around 30% during quiescent season. Plant D is operated to act as a storage lagoon to protect the downstream SBR from being overloaded but still achieves good COD removal when fed and discharged regularly.

Table 6.2.1 Covered anaerobic lagoon

		pH	EC	COD	CODf	SS	NPOC	TN	temp
Plant A	Mean	5.07	1595.3	3956.6	2853.7	1428.4	854.5	45.8	23.9
	Max	6.6	1994	11000	5900	5660	1383	97.8	30.1
	Min	4.4	1273	1370	1200	120	393	0.89	13.7
Plant D	Mean	5.59	2406.7	9414.4	4240.2	8778.7	1067.5	100.4	21.5
	Max	7.4	5240	37200	14900	34720	2040	640.6	28.2
	Min	4.1	1610	60	100	220	33.3	0.89	15.5

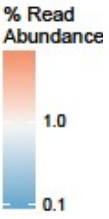
The microbial population must be in balance and environmental factors must be maintained for favourable growth conditions, to achieve a high reaction rate and minimum residence time. Growth rates of anaerobic bacteria are particularly sensitive to abiotic factors like pH and temperature. If too much acid is produced, pH will drop and the system will fail. If the pH is too low, methanogens will cease to grow, but acetogens and acidogens will continue to produce biomass, but produce no methane.

Microbial populations a metagenomics approach.

The microbial populations of Plant A and D were distinct, however they did share some of the most abundant OTUs (Table 6.2.2). Overall the population was heavily dominated by acidogens.

Table 6.2.2 OTU read abundance of Plant A and D

	A	D
Saccharibacteria; c__Saccharibacteria_OTU_6; OTU_6	7.1	4.7
Proteobacteria; Pseudomonas; OTU_8	5.1	4.3
Actinobacteria; Atopobium; OTU_3	7.9	1
Acidobacteria; f__Acidobacteriaceae (Subgroup 1)_OTU_2; OTU_2	6.8	2
Bacteroidetes; Rikenellaceae RC9 gut group; OTU_5	0.7	7
Euryarchaeota; Methanobrevibacter; OTU_1	4.1	3.6
Firmicutes; Pseudoramibacter; OTU_7	4.4	2.3
Firmicutes; Erysipelotrichaceae UCG−004; OTU_23	2.8	3.1
Firmicutes; f__Acidaminococcaceae_OTU_10; OTU_10	5.6	0
Firmicutes; Ruminococcaceae UCG−014; OTU_4	5.1	0.1
Bacteroidetes; f__Prevotellaceae_OTU_13; OTU_13	1.8	3.2
Caldiserica; Caldisericum; OTU_24	1.3	3.2
Bacteroidetes; Prevotella 7; OTU_11	0.2	4.2
Firmicutes; Megasphaera; OTU_19	0.1	3.8
Caldiserica; Caldisericum; OTU_22	2.3	1.5
Firmicutes; Erysipelotrichaceae UCG−004; OTU_16	0.9	2.3
Firmicutes; Acetitomaculum; OTU_9	2.3	1
Spirochaetae; f__Spirochaetaceae_OTU_21; OTU_21	1.2	1.7
Firmicutes; o__Lactobacillales_OTU_12; OTU_12	0.1	2.7
Bacteroidetes; f__Prevotellaceae_OTU_20; OTU_20	2	0.8
Bacteroidetes; o__Bacteroidales_OTU_25; OTU_25	1.2	1.5
Bacteroidetes; f__ratAN060301C_OTU_18; OTU_18	2.5	0
Bacteroidetes; U29−B03; OTU_15	2.4	0
Bacteroidetes; f__Prevotellaceae_OTU_27; OTU_27	1.1	1
Proteobacteria; Acetobacter; OTU_17	0.1	2
Proteobacteria; Acidocella; OTU_14	1.9	0
Proteobacteria; Pseudomonas; OTU_41	0.7	1.1
Caldiserica; Caldisericum; OTU_51	1	0.7
Firmicutes; Catenibacterium; OTU_29	0	1.3
Firmicutes; Megasphaera; OTU_96	0	1.3
Bacteroidetes; f__Prevotellaceae_OTU_66; OTU_66	0	1.3
Bacteroidetes; Parabacteroides; OTU_45	1.1	0
Proteobacteria; Thiothrix; OTU_26	1.1	0
Proteobacteria; o__Rhizobiales_OTU_28; OTU_28	1	0.1
Caldiserica; Caldisericum; OTU_684	0.3	0.7
Firmicutes; Megasphaera; OTU_30	0	1
Firmicutes; Clostridium sensu stricto 12; OTU_44	0	0.9
Actinobacteria; f__Coriobacteriaceae_OTU_43; OTU_43	0	0.9
Proteobacteria; Defluviicoccus; OTU_60	0	0.8
Bacteroidetes; f__ratAN060301C_OTU_55; OTU_55	0.7	0.2
Bacteroidetes; f__Prevotellaceae_OTU_38; OTU_38	0.2	0.6
Firmicutes; Succiniclasticum; OTU_58	0.7	0.1
Bacteroidetes; Prevotella 7; OTU_67	0	0.7
Actinobacteria; Atopobium; OTU_34	0.5	0.2
Bacteroidetes; U29−B03; OTU_50	0.3	0.4
Firmicutes; Ruminiclostridium 5; OTU_49	0.1	0.6
Parcubacteria; c__Parcubacteria_OTU_40; OTU_40	0.6	0.1
Bacteroidetes; Prevotella 7; OTU_42	0	0.7
Spirochaetae; f__Spirochaetaceae_OTU_75; OTU_75	0.5	0.2
Firmicutes; Ruminiclostridium 5; OTU_32	0	0.7



A diverse range of acidogens were revealed belonging to the Proteobacteria, TM7, Firmicutes and Bacteriodetes. OTU_6 has the highest abundance; this sequence belongs to the Candidatus phylum *Saccharibacteria* formerly known as TM7, but has very low similarity to anything in the 16S databases. OTU_8 dominates both plants in August. This OTU can be identified as Proteobacterial species, *Pseudomonas fluorescens*. This organism has been studied extensively due to its potential benefit in bioremediation against several strains of plant pathogens. It has been reported in anaerobic wastewater samples and can assimilate aromatic compounds. Firmicutes were represented by the genus *Pseudoramibacter* and *Erysipelotriaceae*.

There was not one particular acetogen population that dominated the samples, but many OTUs belonged to the Class *Costridia* that that contain known acetogens in the genera *Acetitomaculum*, *Syntrophomonas* and *Gelria*. Members of the Deltaproteobacterial family *Synergistaceae* were also present that are acetogens.

Methanogens identified in profiling belong to the genera *Methanobrevibacter*, *Methanosarcina*, *Methanoregula*, *Methanocorpusculum* and *Methanosaeta*. OTU_1 is present in both plants, and at times in high abundance. BLASTn searches reveal that OTU_1 belongs to the genus *Methanobrevibacter* and has only 96% similarity to cultured organisms *M. acididurans* and *M. ruminantium* and hence is most likely a novel species of bacteria.

Relatively slow growing methanogens must be present in high numbers to ensure the final step of methanogenesis is complete and biomass production is low and methane is produced. In these systems methanogen populations were relatively small, therefore there is scope to improve the performance of these plants by changing parameters to favour their growth conditions.

Canonical Correspondence Analysis (CCA) illustrates the greatest impact on the populations are temperature, pH and phosphorus (Figure 6.2.1). The pH of Plant A ranged from 4.4-6.6 and Plant D 4.1-7.4 over the three years. OTU_6 only dominated at pH>5, *Saccharibacteria* have been associated with low pH before (ref). The temperature range for the plants was 15.3-30.1 ^{o}C and 13.6-28.1 ^{o}C for Plant A and D respectively. *Methanobrevibacter* was most

abundant at temperatures around 20°C. OTU_20, a Bacteriodes belonging to the Prevotellaceaea, was associated with high phosphorus.

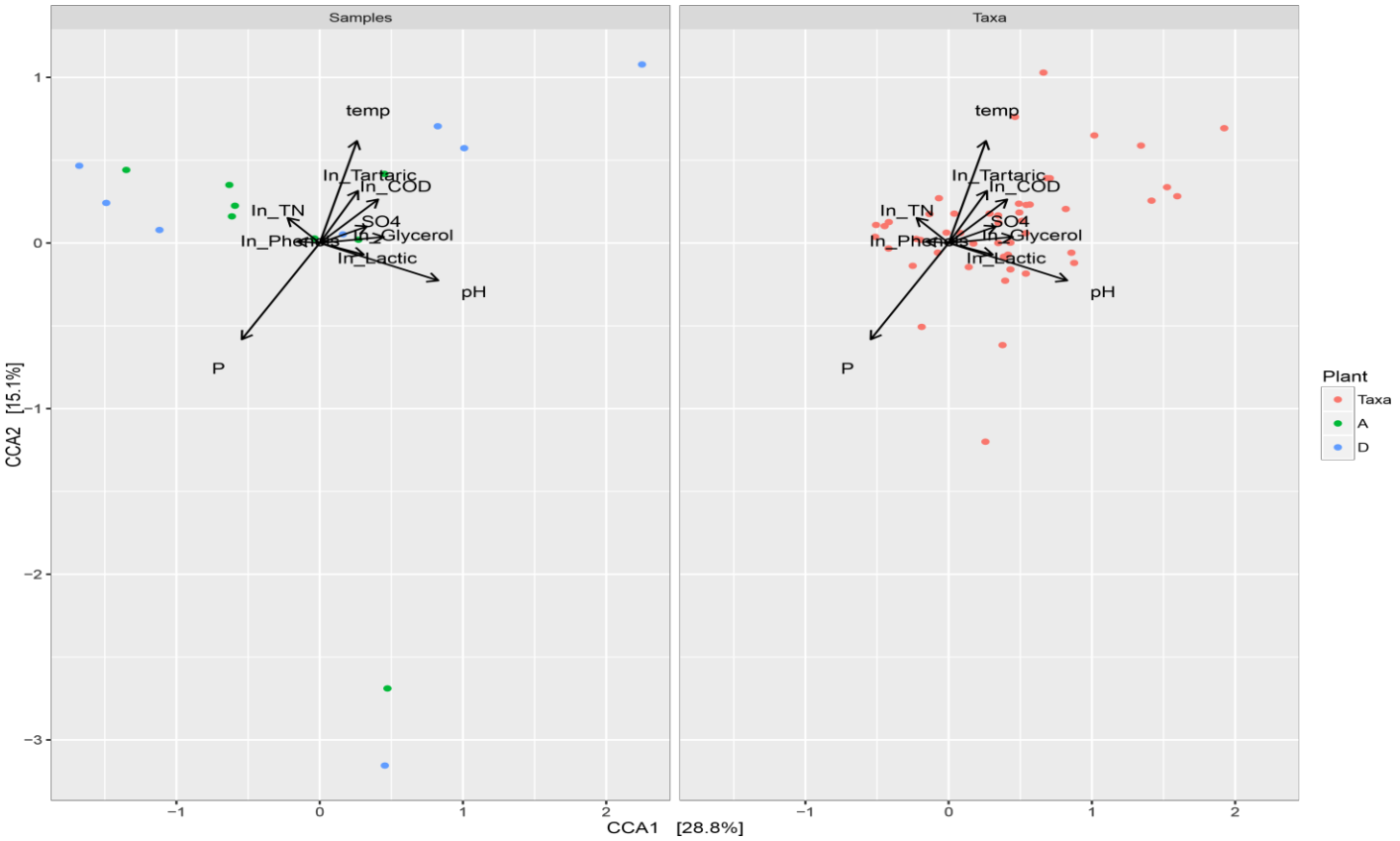


Figure 6.2.1 CCA analysis of the Covered Anaerobic Lagoons.

These samples were taken from the outfall of the CAL that is feeding into the SBR. It is highly probable that microenvironments exist within these lagoons and that both the microbiology and the water chemistry varies considerably. These plants are designed so that sludge can build up on the base over time, relying on gas production from microbial metabolism for mixing. This project aimed to take a snap shot of the microbial organisms present to observe any commonality between plants and observe influential parameters. Genomic fingerprinting suggests that novel organisms are in high abundance in anaerobic WWTP. Maintaining a healthy pH and temperature are key for optimal operation and are the most influential parameters in determining the microbial community.

Further studies into the microenvironments within lagoons are required to truly understand the microbiology of these systems and to optimise their performance by increasing methanogen populations.

6.3 Output 3: Profiling of the four plants over the three years

Bacterial community compositions of four treatment plants were compared using conventional microscopy techniques and molecular community profiling (Figure 6.3.1). Overall, microscopy was very good at identifying the dominant populations present and good correlation existed between microscopic observations and 16S rRNA gene profiling. Each community displayed a unique community profile. Plants A, B and D are stable over time under normal operating conditions. Plant C however, is highly variable. Due to the uniqueness of each plant as shown by PCA, each plant will be assessed individually.

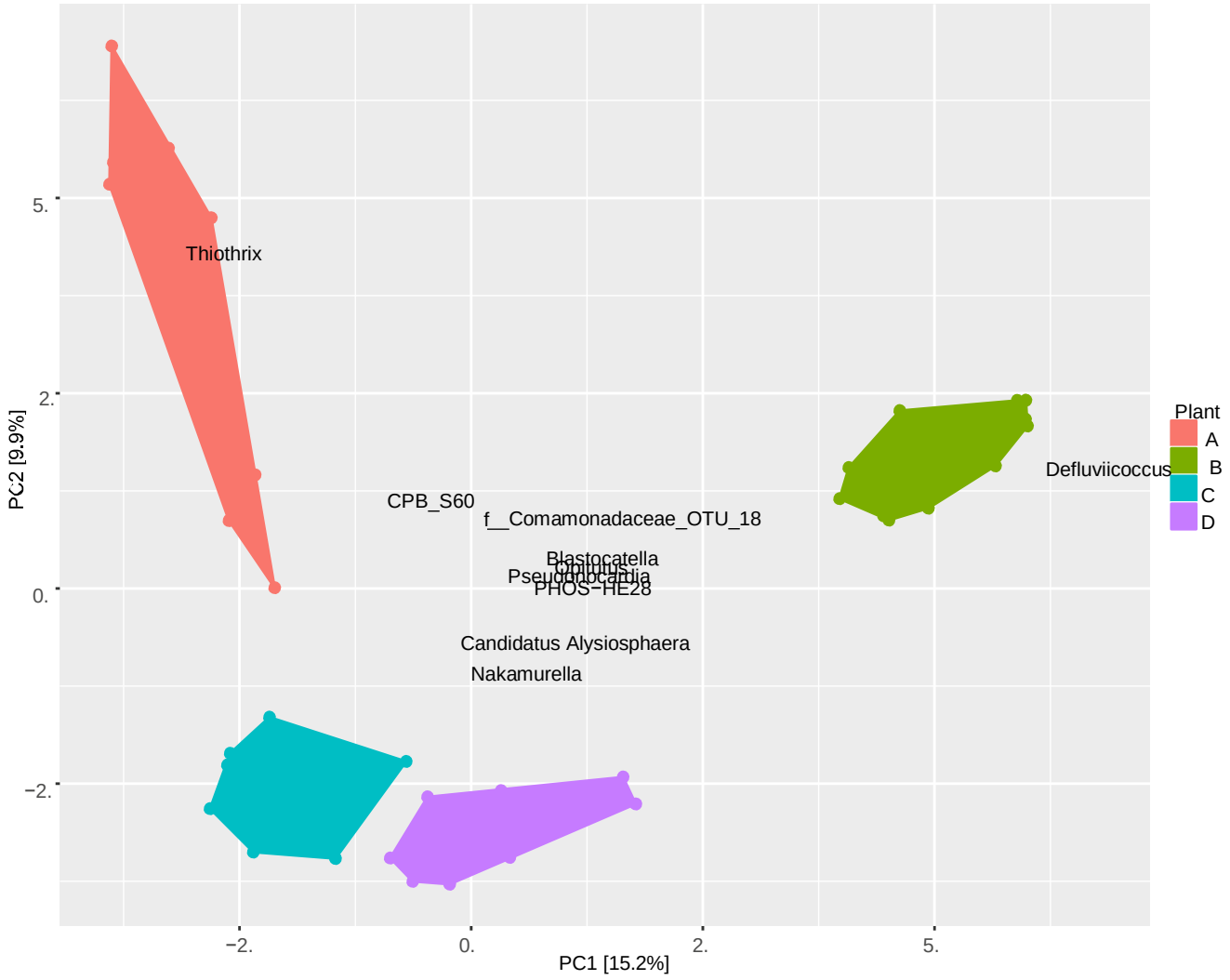
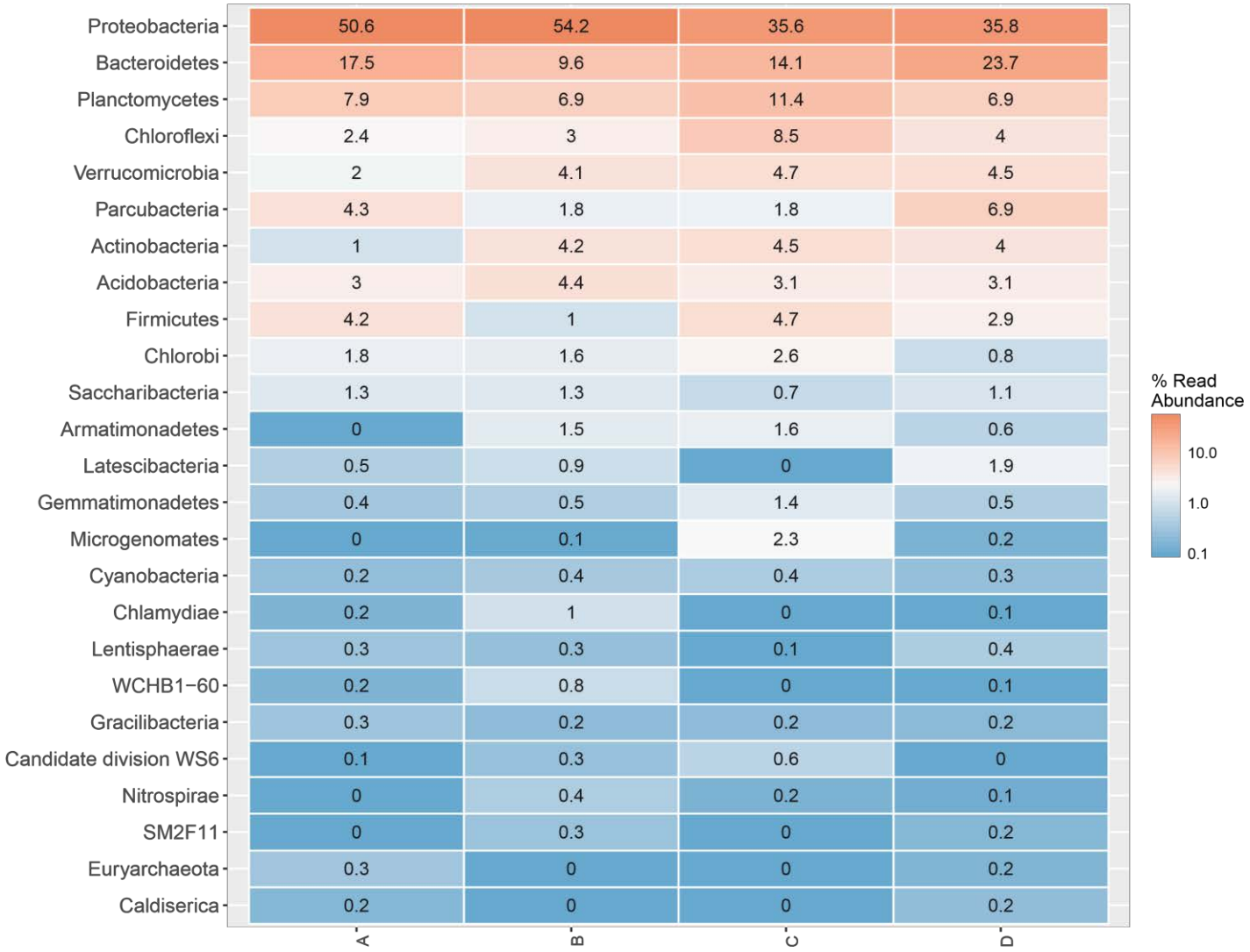


Figure 6.3.1 Illustrates how well the populations from each of the four plants cluster and how distinct the microbiology of the plants is. Plant B is characterised by *Defluviicoccus* and Plant A by *Thiothrix*, this is consistent with microscopic observations.

Trends at the phyla level can be seen (Table 6.3.1) with *Proteobacteria*, *Bacteriodes*, *Planctomycetes, Chloroflexi, Verrucomicrobia, Parcubacteria, Actinobacteria, Acidobacteria, Firmicutes* and *Chlorobi*. Analysis of activated sludge systems through high throughput sequencing has shown the domination of *Proteobacteria* in these four plants (35-54%), *Proteobacteria* commonly dominate activated sludge treating both domestic and industrial wastewaters (Saunders et al. 2016). *Bacteroidetes* also have a high importance in all samples (9.3-23.7%) of the total community.

Table 6.3.1 Heatmap of relative abundance of bacterial phyla in the activated sludge of the SBRs from each of the four plants.



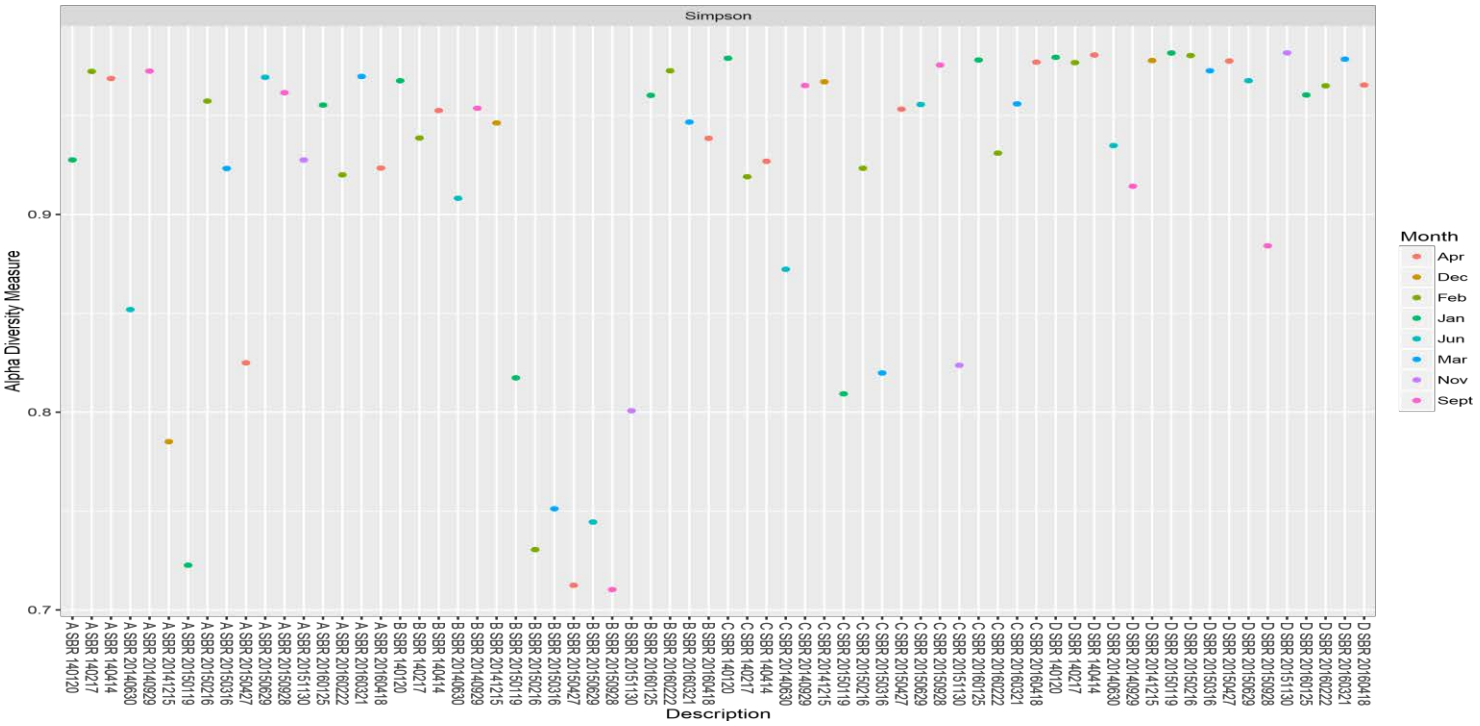


Figure 6.3.2 Simpson's alpha diversity of each sample

Species richness and diversity for each sample were assessed using Simpson's alpha diversity (Figure 6.3.2). All samples measured 0.7 or greater indicating low diversity. Bray-Curtis dissimilarity matrices were used to assess differences between samples. Plants A, B and D were generally very stable over the three years. Plant C however varied greatly, generating very high dissimilarity values. Plant C is the smallest plant investigated in detail in this project. It is has less buffering capacity and experiences peaks and dramatic changes in influent chemistry and load more than the other plants; it is discussed in detail later.

From 251827 successful reads, the MiDAS database defined 3663 OTUs. Only about 1200 of the OTUs could be classified to the genus level indicating the uniqueness of this environment.

At the genus level, *Planctomycete* genus SM1A02 and Proteobacterial genus *Defluviicoccus* feature in several plants and in high abundance. Phylogenetic trees were constructed based on the V3-V4 16S rRNA gene sequences retrieved to observe diversity within the genera.

The MiDAS database classified 35 OTUs belonging to the SM1A02 genus. Currently there are no cultured species, only clones. The 16S rRNA gene phylogenetic tree revealed deep branching and some distinct clustering suggesting several novel species are present (Figure

6.3.3). Little is known about this genus and the role it plays in activated sludge; its common occurrence and distribution in WWTP is interesting.

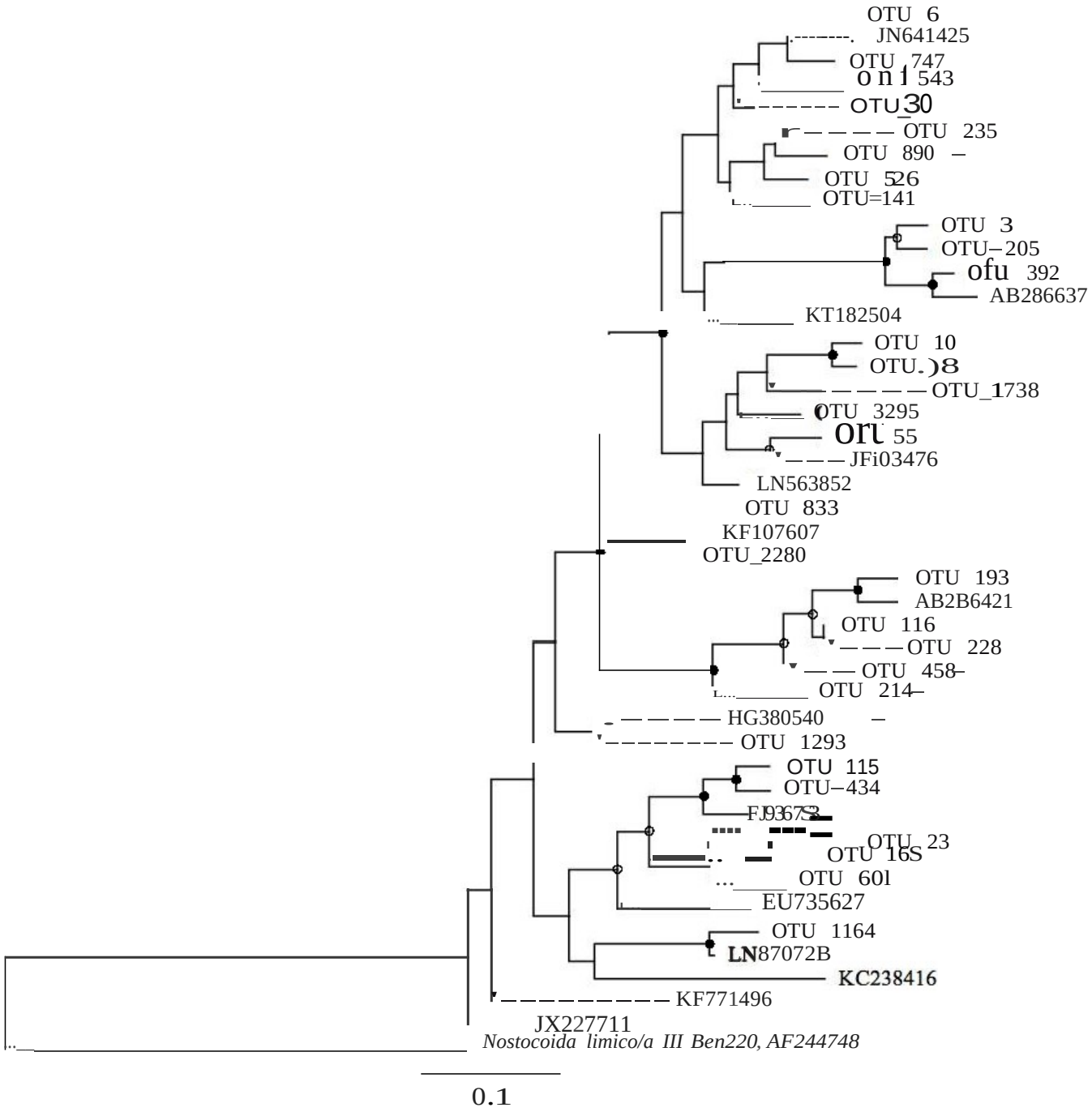


Figure 6.3.3.Phylogenetic tree of >440bp from the V3-V416S RNA gene of the Genus SM1A02. Sequences from this study are highlighted in bold. Bootstrap values were calculated as percentages of 1000 analysis, open circles indicate values >500, closed circles >75%.The scale bar corresponds to 0.1 substitutions per nucleotide.

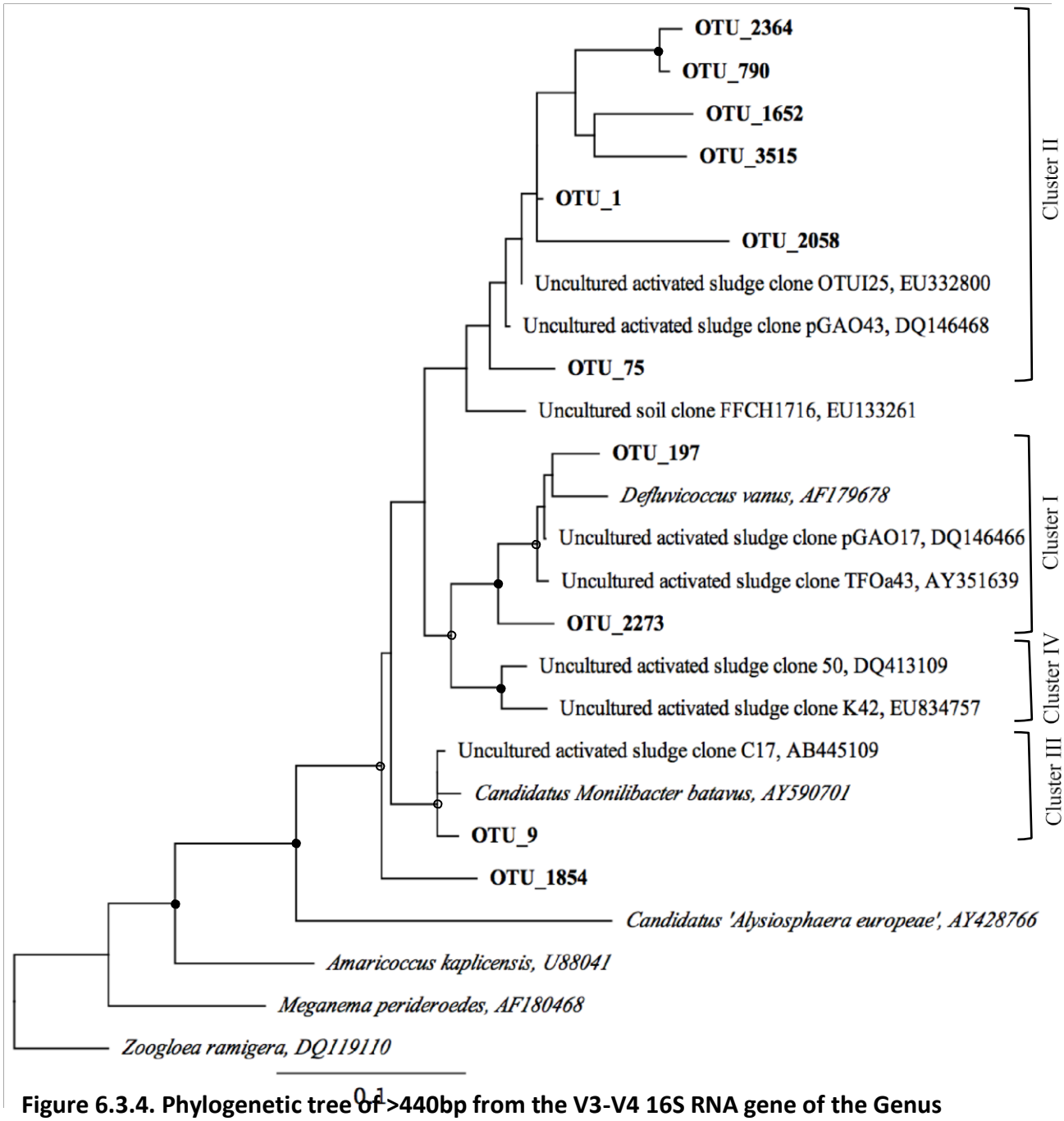


Figure 6.3.4. Phylogenetic tree of >440bp from the V3-V4 16S RNA gene of the Genus Defluviicoccus. Sequences from this study are highlighted in bold. Bootstrap values were calculated as percentages of 1000 analysis, open circles indicate values >50%, closed circles >75%. The scale bar corresponds to 0.1 substitutions per nucleotide.

Unlike SM1A02, the *Defluviicoccus* are well studied and their role as glycogen assimilating organisms is well defined. To date, four distinct clusters exist within the genus. The phylogenetic tree indicates that organisms belonging to each are present in WWTP (Figure 6.3.4). The most abundant OTU_1 falls within *Defluviicoccus* group II; this has the conventional tetrad or grape bunch morphology associated with GAO. The second most abundant is OTU_9 belonging to group III. Most closely related to *Monilibacter batavus*, this organism is filamentous and has *Nostocoida limicola* morphology; with light microscopy, this organism could easily be misidentified.

The microbiology of Plant A over three years.

Plant A was dominated by *Thiothrix* for the first two years. While microscopy identified the filament as a *Thiothrix* species (Figure 6.3.5), 16S rRNA gene sequencing is required to identify the filament to a species level, as 99% similar to *Thiothrix disciformis* (DSM 14473). OTU_53 was present at all times but peaked in February in 2014 and 2015. OTU_53 belongs to the genus *Competibacter* and is consistent with the G-bacteria phenotype observed microscopically. *Zoogloea* appear during non-vintage periods. The abundant OTU_40 appears to be a novel organism related to the family *Hyphomicrobiaceae*.

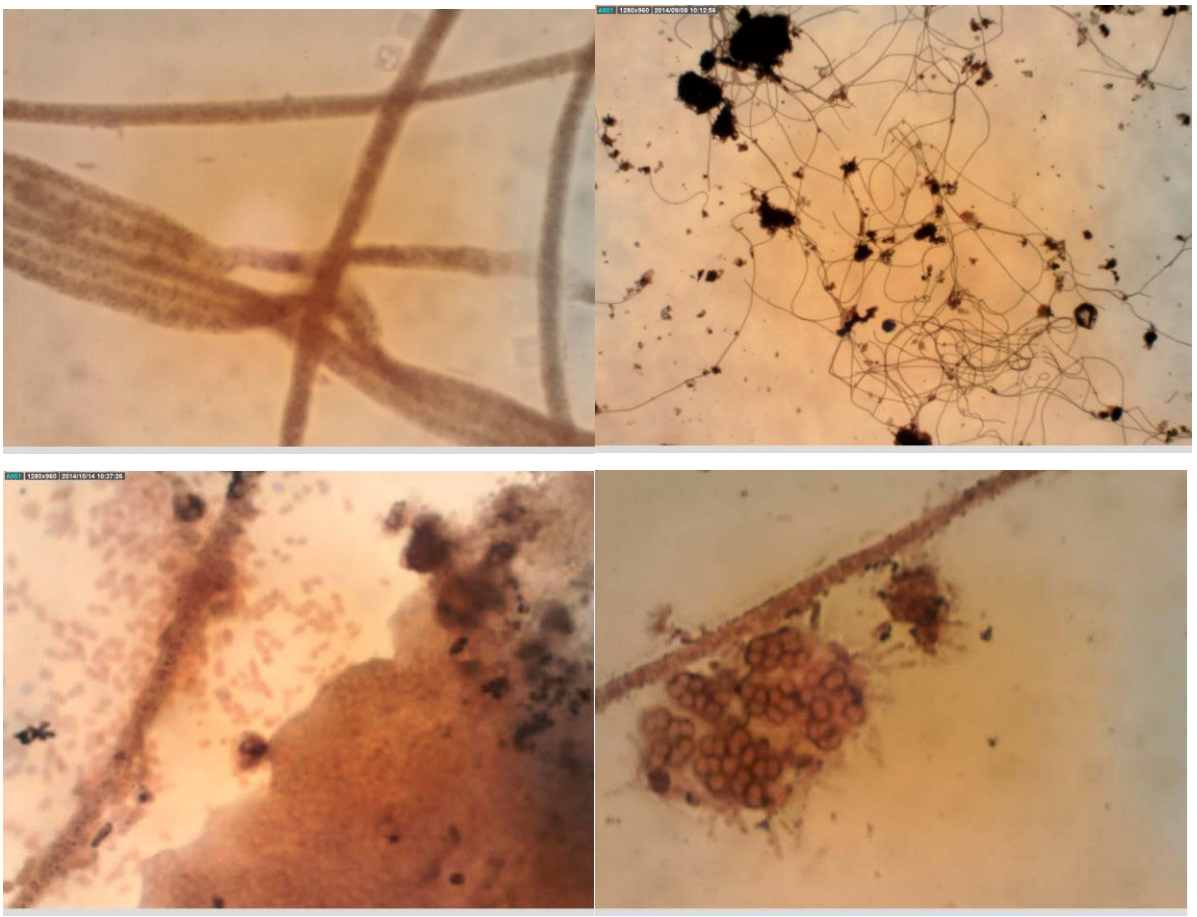
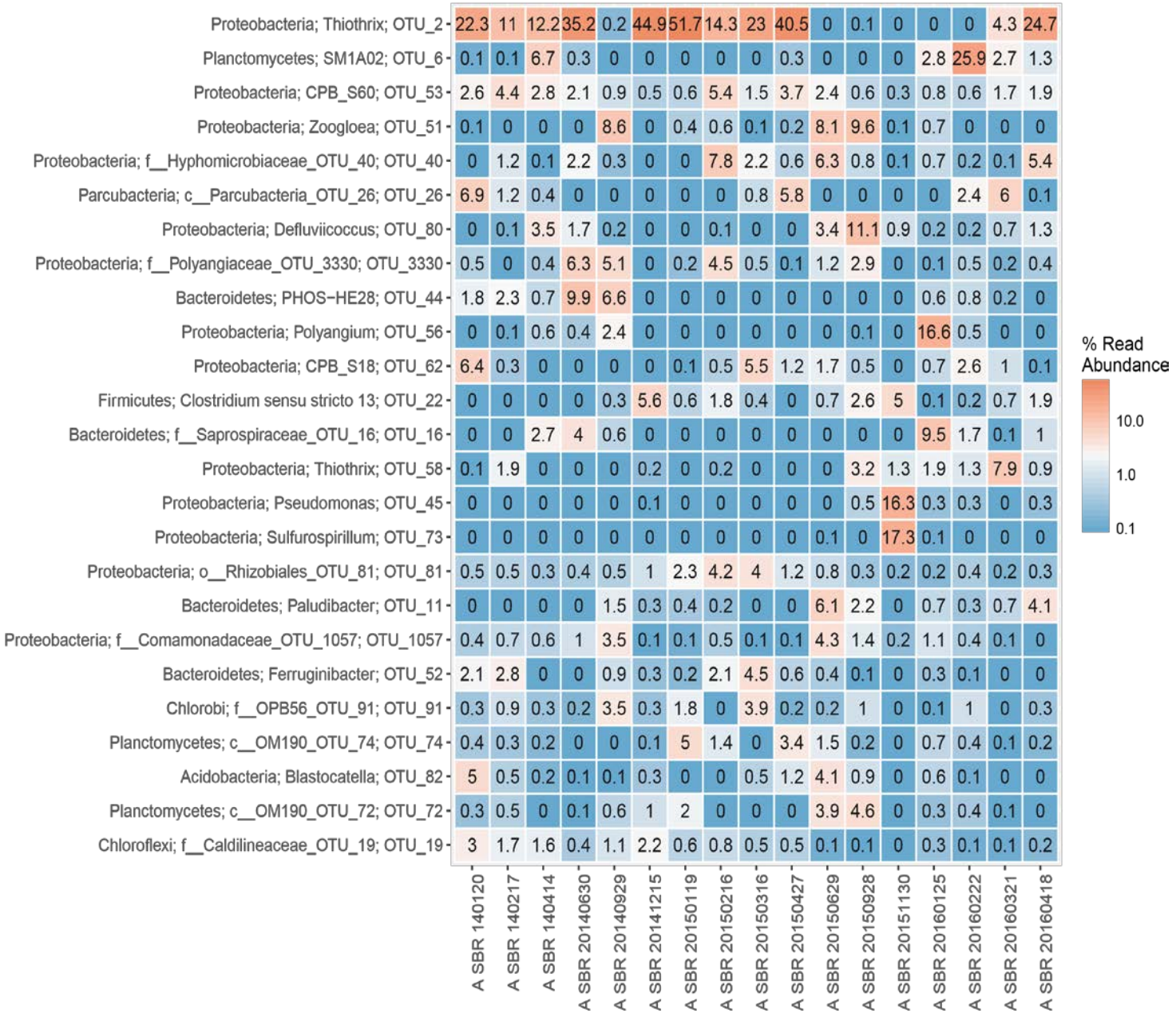


Figure 6.3.5. Light microscopy images typical of Plant A. Showing an abundance of filaments, *Thiothrix*, *Zoogloea* and G-bacteria identified by genomics as *Competibacter*.

Table 6.3.2 Heatmap of relative abundance of bacterial OTUs in the activated sludge of the SBRs from plant A.



The microbiology of Plant B.

Plant B was very stable over the three years, with G-bacteria, *Defluviicoccus* II dominating the sample (Figure 6.3.6). The *Nostocoida limicola* morphotype that was regularly observed under microscopy was *Alysiosphaera europeae*, but OTU_9 is also present, the *Defluviicoccus* that also exhibits *N. limicola* morphology. Genomic analysis determined that the GALO present in large numbers are *Millisia brevis,* a relatively rare member of the Mycolata.

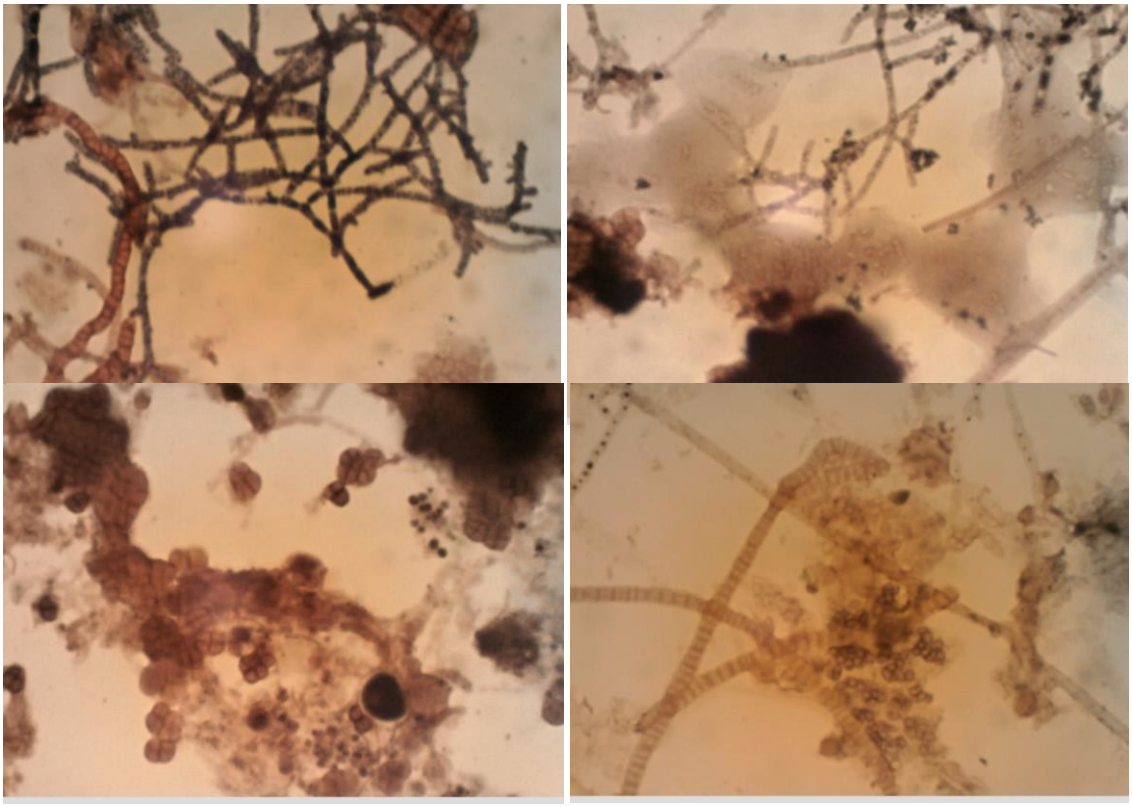


Figure 6.3.6 Light microscopy typical images from Plant B illustrating GALO, Zoogloea, G-bacteria and N.limicola.

The microbiology of Plant C.

The microbiology of Plant C was constantly evolving, but generated interesting seasonal changes. Plant C is discussed in more detail in section 6.5.

The microbiology of Plant D.

Plant D was very stable. Microscopically it was dominated by a filamentous bacteria Type 0041/0675 (Figure 6.3.7). It also had large populations of G-bacteria present. Genomics analysis revealed that *Defluviicoccus* II and III are present at different times. OTUs classified as genus SM1A02 are also abundant in this plant (Table 6.3.3). *Zoogloea* are always present at populations ranging 1-12% and represented by several OTUs. OTU_5 is the most abundant belonging to the Bacteriodetes family Saprospiraceae. Members of this family are often reported in activated sludge and are thought to be involved in the breakdown of complex organic compounds. OTU_63 belonging to the Gammaproteobacteria is present in high abundance in several samples. It has 97% similarity to *Competibacter* but given the low similarity may represent a new species of the genus. Seasonal fluctuations are not observed.

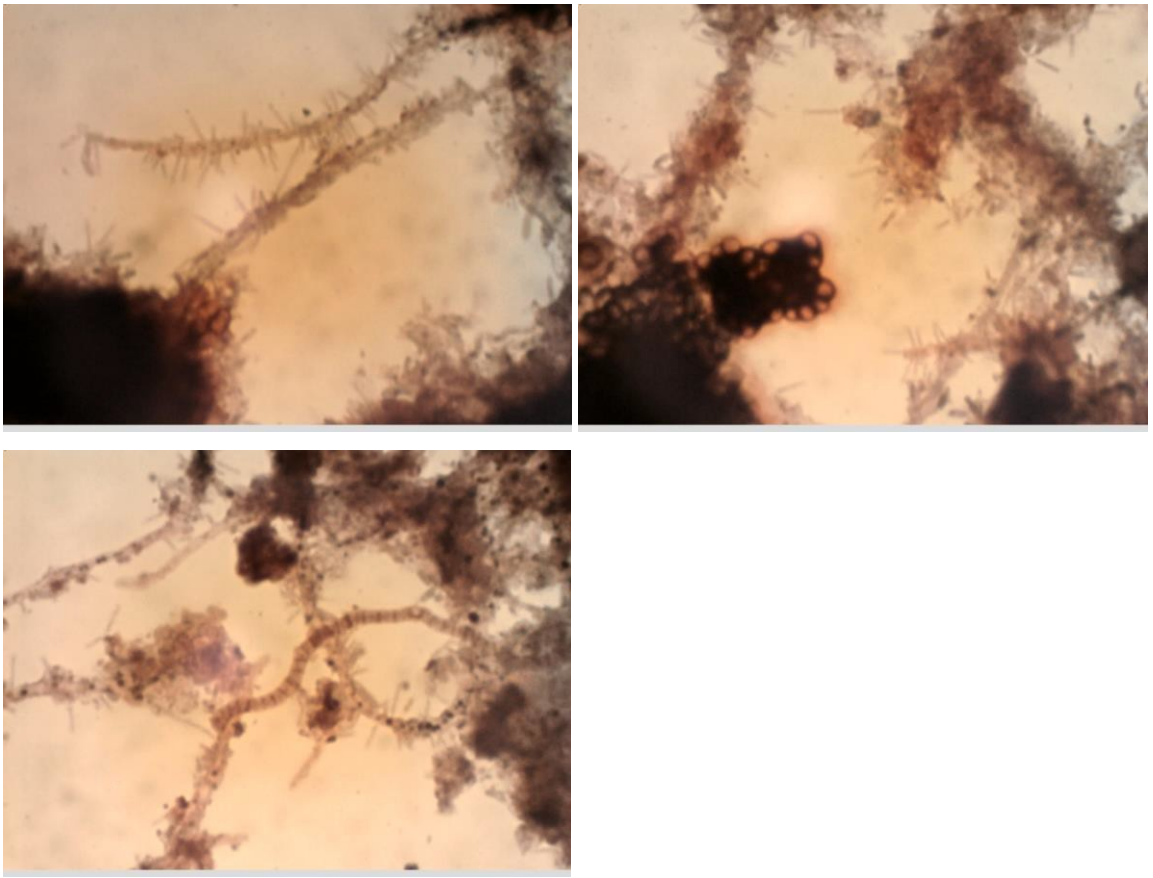
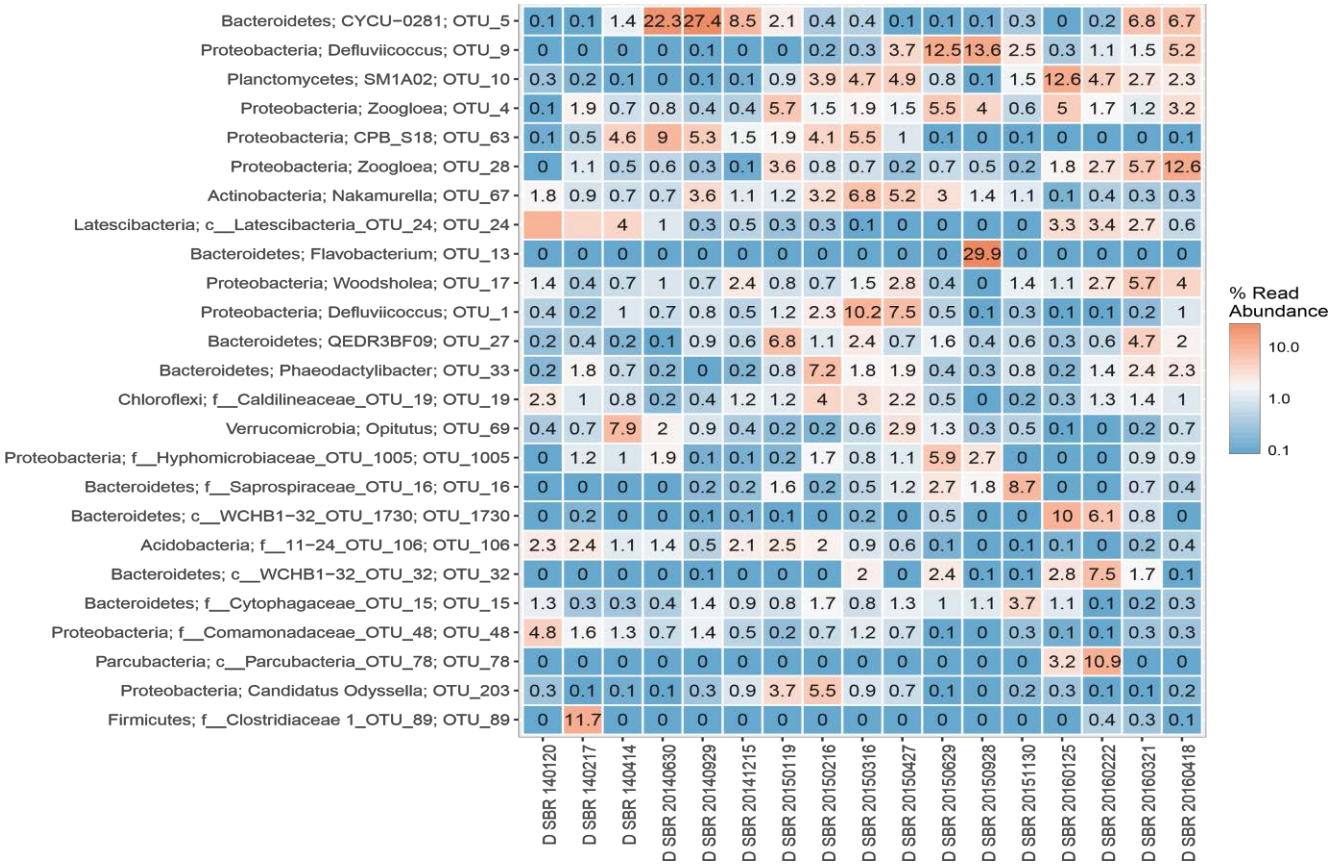


Figure 6.3.6. Light microscopy typical images of Plant D.

Table 6.3.3. Heatmap showing the relative abundance of OTUs in Plant D.



6.4 Output 4: Novel organisms

Several novel organisms were observed during microscopic examination of the WWTPs (Figure 6.4.1-3). Attempts were made to culture these organisms into pure cultures with limited success using R2A and GYE (Appendix 5.2). Identification of some organisms was made possible through the application of 16S rRNA gene sequencing.

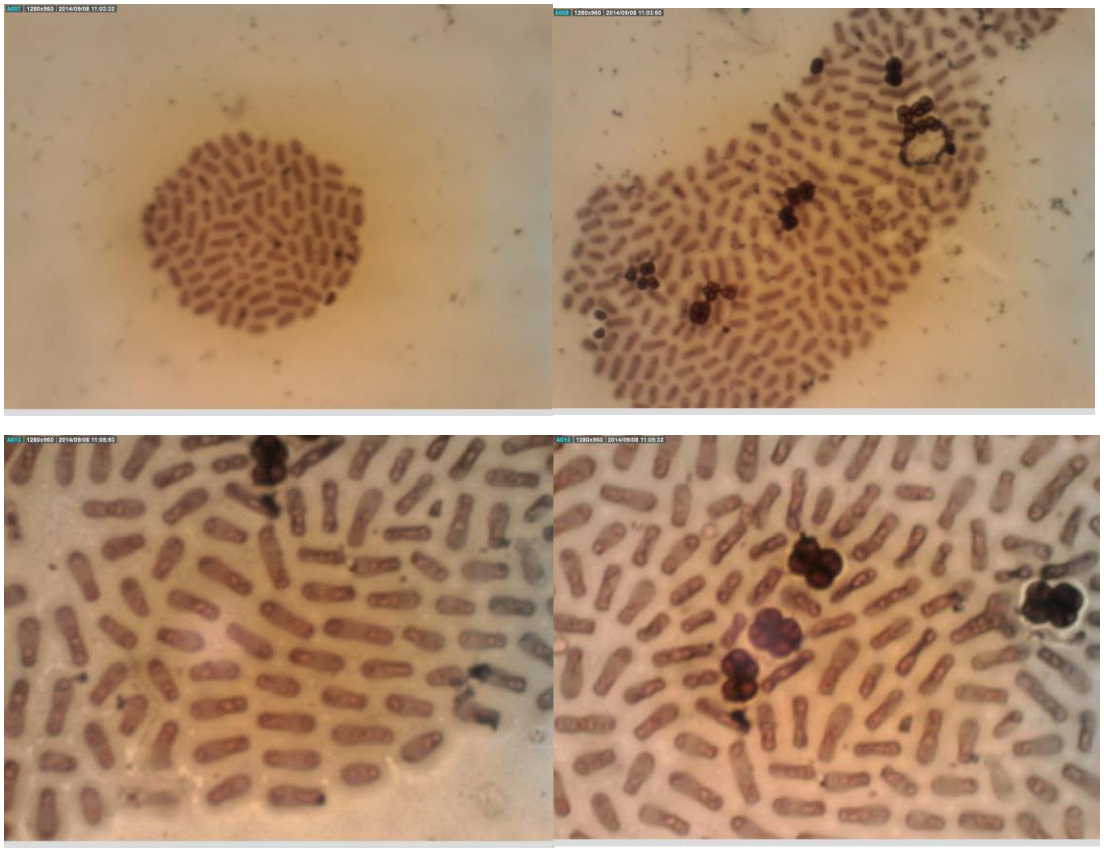


Figure 6.4.1 Unidentified 1 (a,b) 400X magnification illustrating the microcolony shapes. (c,d) 1000X magnification showing the cells in more detail.

This organism created very turbid waters, which carried over from SBRs into treated water tanks. These Gram negative rods produced polysaccharide material and formed microcolonies with rounded edges, at times making near perfect circles.
Ecogenomics data based on an effluent sample that was heavily dominated by this organism generated an OTU table with a single, unique OTU accounting for 36.5% of the population. The V3-V4 16S rRNA gene sequence of OTU_1 is in Appendix 5.3. RDP and SILVA classify this as a novel bacteria belonging to the Alphaproteobacterial class

Rhizobiales. Attempts were made to isolate the organism into pure culture but were unsuccessful. BLASTtn searches reveal the closest relative is an unidentified clone retrieved from brewery wastewater (JQ072482) at 97% identity.

Figure 6.4.12 describes a second unidentified organism. This organism was identified as Eikelboom's Type 0803 belonging to the *Chloroflexi* Candidate genus *Defluviifilum*. The filaments observed in WWTP exceeded 150µm in length and the filaments rarely bundled together therefore not displaying the normal distinguishing features of Type 0803. This filament has been well studied in activated sludge. It cannot consume acetate, but is involved in macromolecule conversion and assimilates glucose.

Figure 6.4.2. Unidentified 2

A third unidentified organism is described in Figure 6.4.3. Unidentified 3 is readily visible under low magnification. It is a tightly coiled filament with large cells approximately 3µm in diameter.

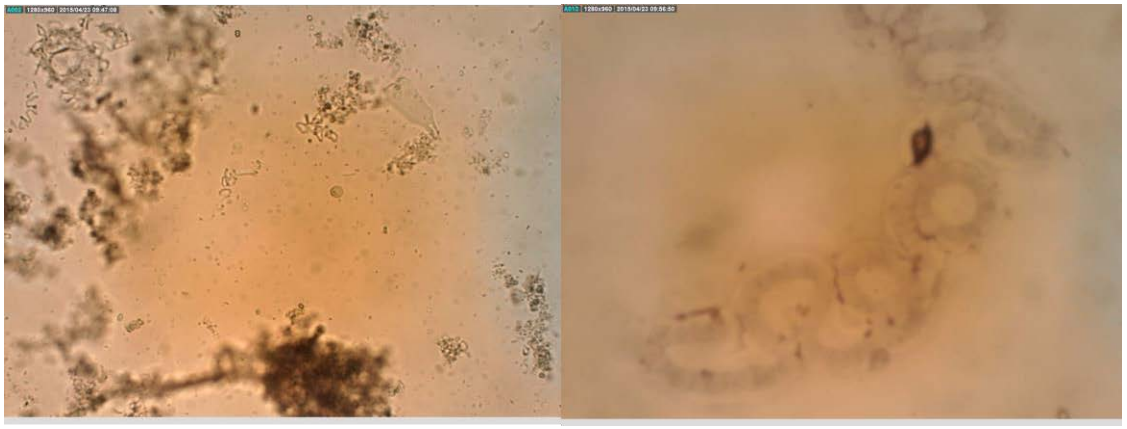


Figure 6.4.3. Unidentified 3

The inability to culture these organisms is not surprising as it is estimated that only 2% of all bacteria can be cultured in the laboratory. This is one of the driving factors for genomics studies in microbiology.

6.5 Output 5: Chemistry of winery effluent

Studies have shown that influent chemistry was an overriding factor shaping the microbial composition of activated sludge. The chemistry of WWW has been characterised as having a low pH and nutrient status, with high Ec and COD. Overall this statement is supported by the analyses undertaken, but huge variation and fluctuations exist as documented in Table 6.5.1, showing the range of these values over the three years of the project.

Table 6.5.1. Characteristics of winery wastewater, lowest and highest values recorded, with the median in brackets.

	PLANT A	PLANT B	PLANT C	PLANT D
pH	3.9-11 (5.3)	4.1-6.6 (4.8)	3.3-11.7 (4.8)	3.2-10.2 (4.7)
Ec	422-1618 (1106)	844-1995 (1401)	354-4150 (1631)	873-2290 (1513)
Total Nitrogen	18.2-5484 (7.934)	0.9-70 (8.6)	5.01-113.8 (16.54)	0.89-28.15 (9.553)
Total Phosphorus	1.41-43.4 (14)	3.4-21.8 (7.67)	1.05-29.60 (10.08)	1.44-68.0 (6.78)
COD	460-19700 (4500)	3800-13100 (5905)	450-36100 (7950)	890-30000 (5960)

Kumar et al. (2009) stated that it is not the COD of wastewater but the ion content that is important to assess for its treatment. Therefore in this study, particular attention was given to cation concentrations (Table 6.5.2). A major impact on cation concentrations is the choice and management of winery cleaning products. Another significant impact is the quality of the process water. Ion concentrations can impact on microbial growth.

Table 6.5.2 Mean ion concentration (mg/L) of the plants over the three years.

	PLANT A	PLANT B	PLANT C	PLANT D
Sodium	272.05	254.9	223.65	61.23
Potassium	126.86	245.3	468.53	571.72
Calcium	20.23	25.3	29.64	46.84
Magnesium	8.21	14.7	16.60	13.97
Aluminium	0.81	0.4	1.10	0.66
Iron	1.25	1.1	6.37	1.68

An imbalance in divalent and monovalent cations has been linked to poor settling, specifically that monovalent ions cause dispersion in flocs, and conversely divalent cations improve floc stability. Figure 6.5.1 suggests that monovalent ions may contribute to high SV. It does not account for all variability, but a trend is evident.

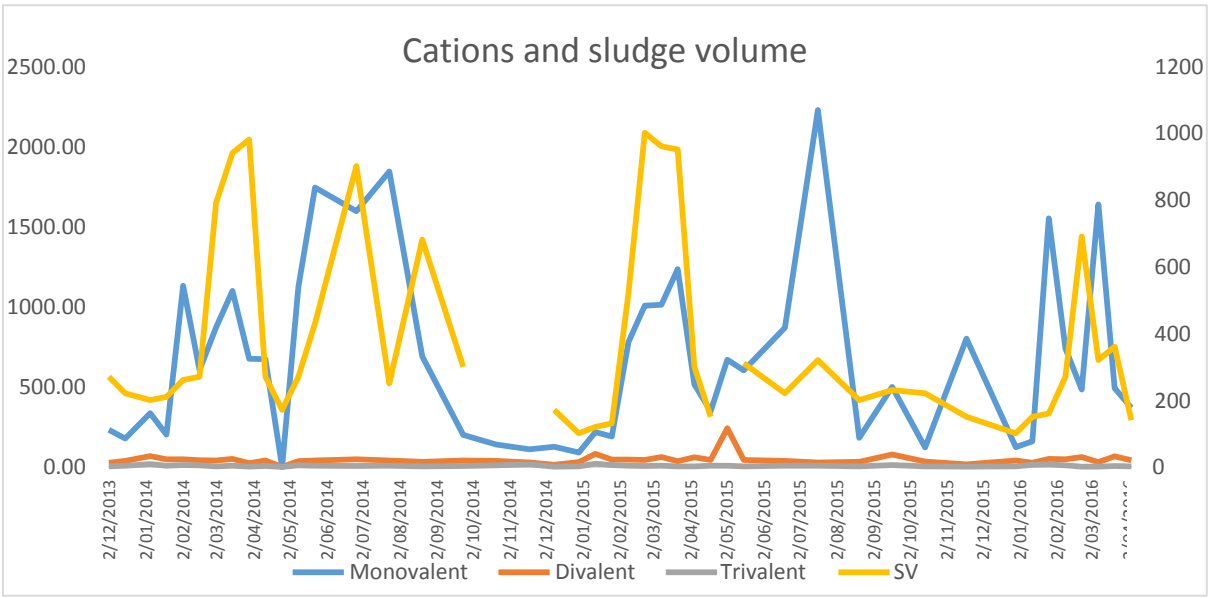


Figure 6.5.1 Cation concentrations (mg/L) and SV (mL).

The COD of the plants can vary considerably. Understanding the carbon composition of influent can help to appreciate why certain populations are dominating, as some species have distinct carbon requirements for growth. HPLC and total phenols analysis revealed that the

plants shared the same three principal components; ethanol, tartaric acid and phenolics (Figure 6.5.2). This influent composition is unique to the wine industry. Wineries have been reported to have high concentrations of readily biodegradable organic compounds, but the high concentrations of phenols are noteworthy. Phenolics can add colour, have a particularly high COD and are often resilient to biological breakdown. Malandra et al. (2003) reported that sugars were a major component, however in this study they were only present in very low concentration.

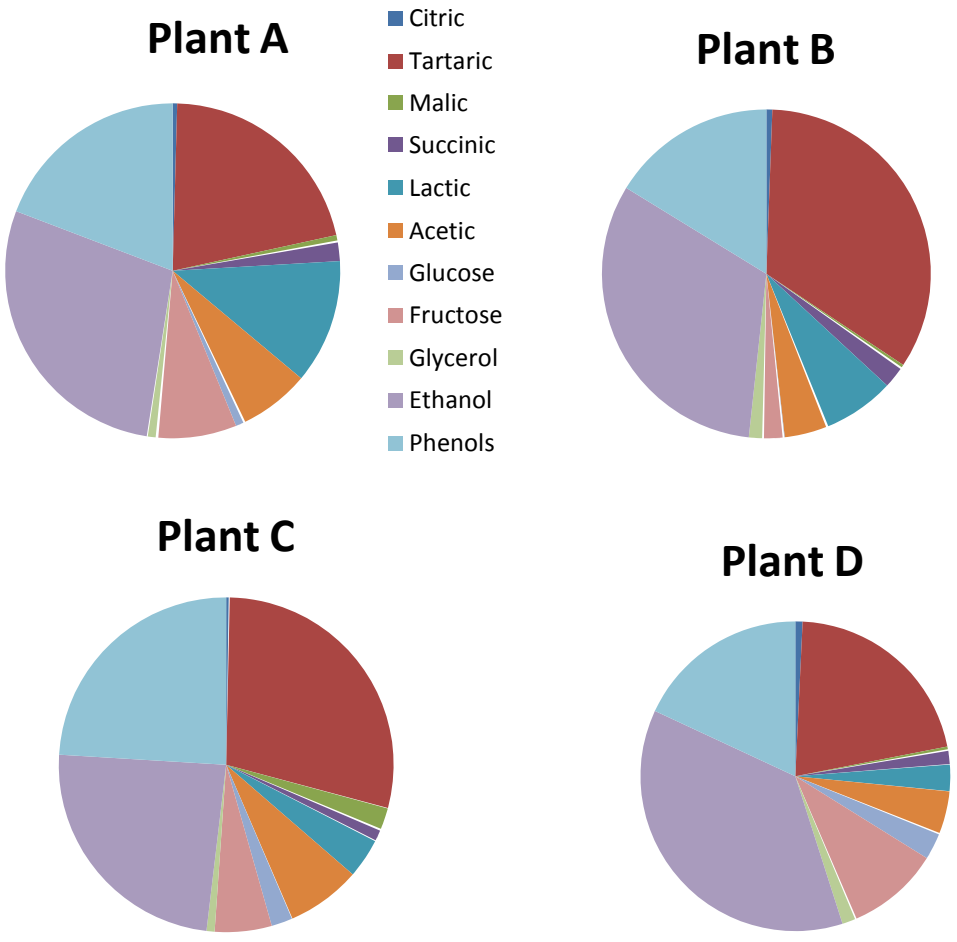


Figure 6.5.2 Pie charts showing the typical carbon content of each plant.

Interestingly, the short residence time in surge storage dramatically changes the carbon composition (Figure 6.5.3). The tartaric acid and other organic acids are reduced and the concentration of acetic acid increases markedly.

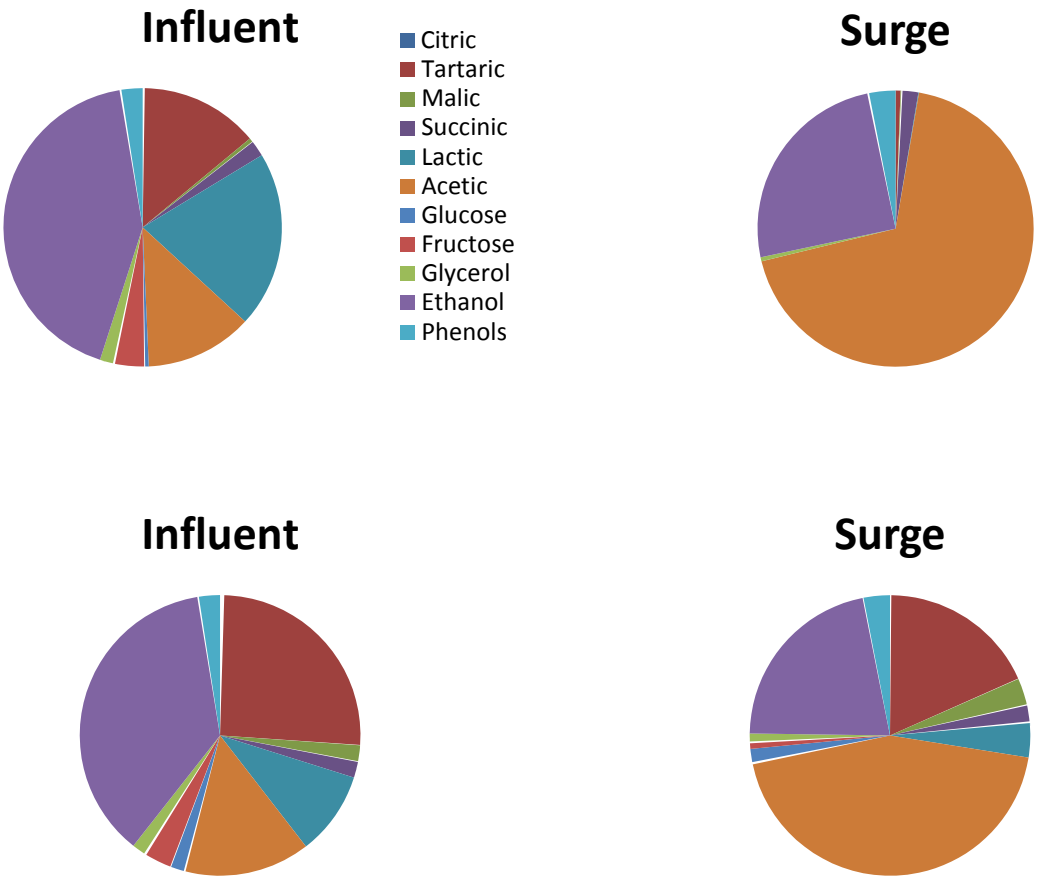


Figure 6.5.3 Changes in influent and surge storage carbon composition.

Winery wastewater can now be described as having high phenolics, ethanol, tartaric and acetic acid and are low in sugars.

Although operations within wineries can vary dramatically, there is high similarity in the chemical composition of winery wastewater.

Turbidity and Clarity

Turbidity and clarity are key indicators of wastewater quality (Figure 6.5.4). Winery wastewater treatment plant operators need a rapid and reliable turbidity measure to tell them if effluent falls within the allowable thresholds for discharge to sewer, reuse in irrigation or storage. Turbidity is the cloudiness of water caused by suspended solids. Suspended solids in winery wastewater treatment plants can come from:

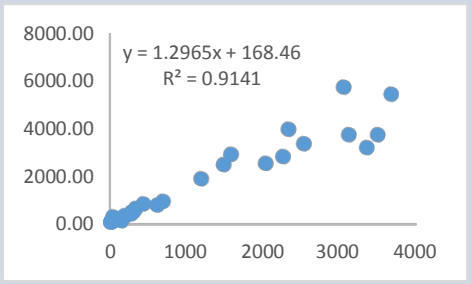
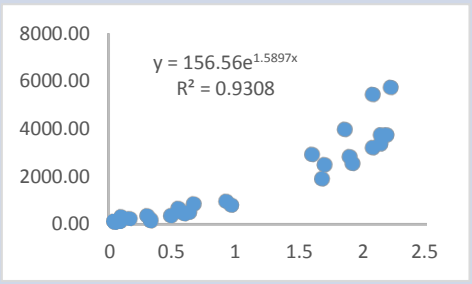
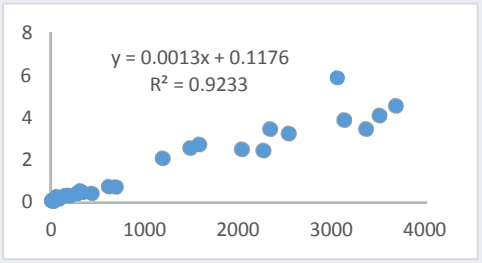
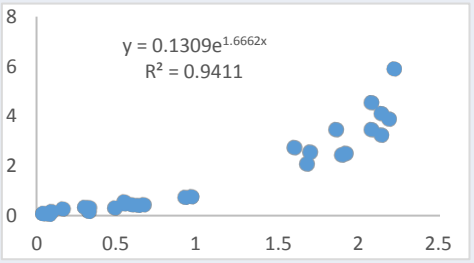
1. Grape and wine solids
2. Solids matter used in wine processing e.g. diatomaceous earth, perlite
3. Biosolids from the water treatment process
4. Chemical precipitates

Figure 6.5.4. Differences in sludge volume (SV30) observed in WWTP.

Turbid waters are problematic because of their physical and chemical properties. Physically the solids block filters and clog pipes. Chemically, many solids are carbon based and require management as they can cause odours, deplete environments of oxygen and create algal blooms and biofilms.

The plotted data of turbidity and clarity against suspended solids and COD generate high coefficients of determination suggesting strong relationships (Figure 6.5.5). Measuring turbidity and clarity is faster and cheaper than measuring COD or SS and provides a rapid way to assess the quality of wastewater effluent. Whilst COD and SS should be measured on a weekly basis, turbidity or Absorbance can be measured on a daily basis.

Table 6.5.5 Chemical Oxygen Demand and Suspended Solids against absorbance and turbidity.

	NTU	Abs @ 650nm
COD (mg/L)		
SS (g/L)		

These data can be useful tools in water management decisions (store, blend, discharge or irrigate) and how to regulate the wastewater treatment plant operations. We have developed a ranking of turbidity/Abs, so the results can be useful for winery wastewater management. This is detailed below:

Rank 1 or 2. Safe. Adequate for reuse as irrigation water and poses a low risk of blockage and has a COD and SS below all council regulations.
Rank 3. Caution. SS and COD may begin to exceed council, Environmental Protection Authority and irrigation thresholds. Preventative action advisable.
Rank 4 or 5. Contingency plan required. Such contingency plans could include extending settling time, reducing solids in reactor or blending with other water supplies.

	1	2	3	4	5
Abs (650nm)	<0.1	0.11-0.49	0.5-0.7	0.7-1.0	>1
Turbidity (NTU)	<50	51-200	201-500	500-700	>700
COD (mg/L)	<100	100-400	400-700	701-1000	>1000
SS (g/L)	<0.1	0.1-0.4	0.4-0.6	0.6-0.8	>0.8

6.6 Output 6: Relationships between microbiology and chemistry and plant operations

Some relationships between microbiology and chemistry or plant operations are well documented. G-bacteria are associated with very high levels of readily degradable organic acids (particularly acetate), and proliferate when nitrogen is limited. *Nostocoida limicola* II, thrives in high sugar and ethanol environments. Type 0041/0675 is a sign of long sludge age and macromolecule breakdown. GALO prefer warmer weather, produce a biosurfactant causing stable foam formation and are physiologically very diverse. Type 0803/0914 occurs when there is insufficient air or mixing.

These relationships have been documented over time and are the result of empirical evidence and astute observation. Our attempt to build on our understanding of microbial relationships with chemistry and plant operations consists of two approaches. The first is the traditional, observation based approach. The second is a statistical approach, based purely on numerical data.

Observations we have made as a team of scientists, engineers and plant operators that add to the existing knowledge of the above organisms and are particularly relevant to the wine industry:

- *Nostcoida limicola* is often present as a secondary organism to G-bacteria.
- Type 0041/0675 is only present in large numbers after a covered anaerobic lagoon. This is consistent with its role in metabolising the breakdown of cells.
- GALO foams often appear when the treatment plant is running hot e.g. >40^{o}C. The foam layer acts as insulation and the temperature will continue to rise unless removed.
- Yeast and Gram-positive bacteria are present in plants with poor solids management in the winery.

Other observations without numerical data include the effect of pre-dosing a plant with carbon before the onset of vintage to improve plant performance. In vintage 2015, three of

the sites added a carbon load to their treatment plants in early January to prepare the bacteria in the WWTP for the start of vintage. Plant operators at two of the sites believed the plants performed better for a longer period of time as they were easier to manage. These plants were not overloaded until nearly the end of vintage, whereas in the previous years, they had appeared overloaded and were struggling by mid vintage. COD data indicate that the plant pre-dosed with wine coped better than those pre-dosed with waste RTDs and molasses.

During the past 12 months, two of the four plants have been working to carbon, nitrogen and phosphorus levels. Phosphorus has not been observed to be limiting in these systems. Nitrogen levels are innately very low in WWW and this can cause limitations in the growth of many organisms in the WWTP. Several problematic organisms are associated with nitrogen limitation and these were commonly observed in many of WWTP surveyed in this study.

During discussions with the 30+ wineries in 2014 we described the benefit of nitrogen dosing. Several wineries dosed with urea during peak vintage of 2015 and reported better settling and effluent quality in terms of clarity and COD. It was observed that those systems with anaerobic digestion prior to aerobic treatment did not have excessive growth of the organisms associated with nitrogen limitation. Further investigation showed that the waste from the anaerobic digesters is high in nitrogen, usually in the form of ammonia, reducing the need for urea addition.

The second approach is based on statistical analysis of two numerical data sets, microbiological data in the form of an OTU table and chemical analysis. The metadata files used for this statistical analysis are available in Appendix 5.4. Twenty six variables were tested for their significance on the microbial population. ANOVA analysis was performed on the Bray- Curtis distance matrix of OTU abundance, to find the best set of environmental variables that describe the community structure. The Adonis function from the vegan library was applied based on linear distance matrices and uses permutation test with Pseudo F-ratio. Eighteen environmental variables were found to be significant (Table 6.6.1) with SV60, MLSS, TN and F:M ratio the most significance.

Table 6.6.1 Significance testing of environmental variables to describe the microbial community structure.

	Df	SumsOfSqs	MeanSqs	F.Model	R2	Pr(>F)	
SV60	1	1.2139	1.21387	4.0509	0.04602	0.001	***
MLSS	1	0.9155	0.91550	3.0552	0.03471	0.001	***
CODf	1	0.5653	0.56526	1.8864	0.02143	0.011	*
NPOC	1	0.4661	0.46613	1.5556	0.01767	0.031	*
TN	1	1.0061	1.00605	3.3574	0.03815	0.001	***
P	1	0.5124	0.51241	1.7100	0.01943	0.023	*
FM	1	1.1017	1.10166	3.6765	0.04177	0.001	***
CODN	1	0.5798	0.57983	1.9350	0.02198	0.009	**
CODP	1	0.2414	0.24140	0.8056	0.00915	0.774	
Citric	1	0.3842	0.38419	1.2821	0.01457	0.130	
Tartaric	1	0.3653	0.36531	1.2191	0.01385	0.187	
Malic	1	0.4592	0.45923	1.5325	0.01741	0.035	*
Succinic	1	0.2724	0.27237	0.9089	0.01033	0.594	
Lactic	1	0.6172	0.61723	2.0598	0.02340	0.008	**
Acetic	1	0.4727	0.47266	1.5774	0.01792	0.049	*
Glucose	1	0.2525	0.25250	0.8426	0.00957	0.710	
Fructose	1	0.4475	0.44755	1.4935	0.01697	0.046	*
Glycerol	1	0.4858	0.48581	1.6212	0.01842	0.024	*
Ethanol	1	0.4784	0.47838	1.5965	0.01814	0.046	*
Phenols	1	0.5336	0.53357	1.7806	0.02023	0.018	*
Ca	1	0.4996	0.49958	1.6672	0.01894	0.025	*
K	1	0.3624	0.36238	1.2093	0.01374	0.204	
Mg	1	0.3835	0.38345	1.2797	0.01454	0.132	
Na	1	0.5453	0.54533	1.8199	0.02068	0.010	**
S	1	0.5833	0.58334	1.9467	0.02212	0.008	**
EffSS	1	0.3436	0.34358	1.1466	0.01303	0.216	
Residuals	41	12.2858	0.29965		0.46582		
Total	67	26.3744			1.00000		

Signif. codes: 0 ‘***’ 0.001 ‘**’ 0.01 ‘*’ 0.05 ‘.’ 0.1 ‘ ’ 1

A CCA plot with only these 18 environmental variables that were found to be significant was constructed (Figure 6.6.2). The biplot shows clustering of activated sludge bacterial populations according to the plant. Samples from WWTP are represented by coloured circles, where red circles represent OTUs. Correlations between environmental variables and CCA axes are represented by the length and angle of arrows. Plant CCA analysis revealed that Plants B, C and D had a positive correlation with ethanol and sulfur, while Plant A was positively correlated with phosphorus. Relationships between environmental factors and microbiology were not strong when analysing all four plants together.

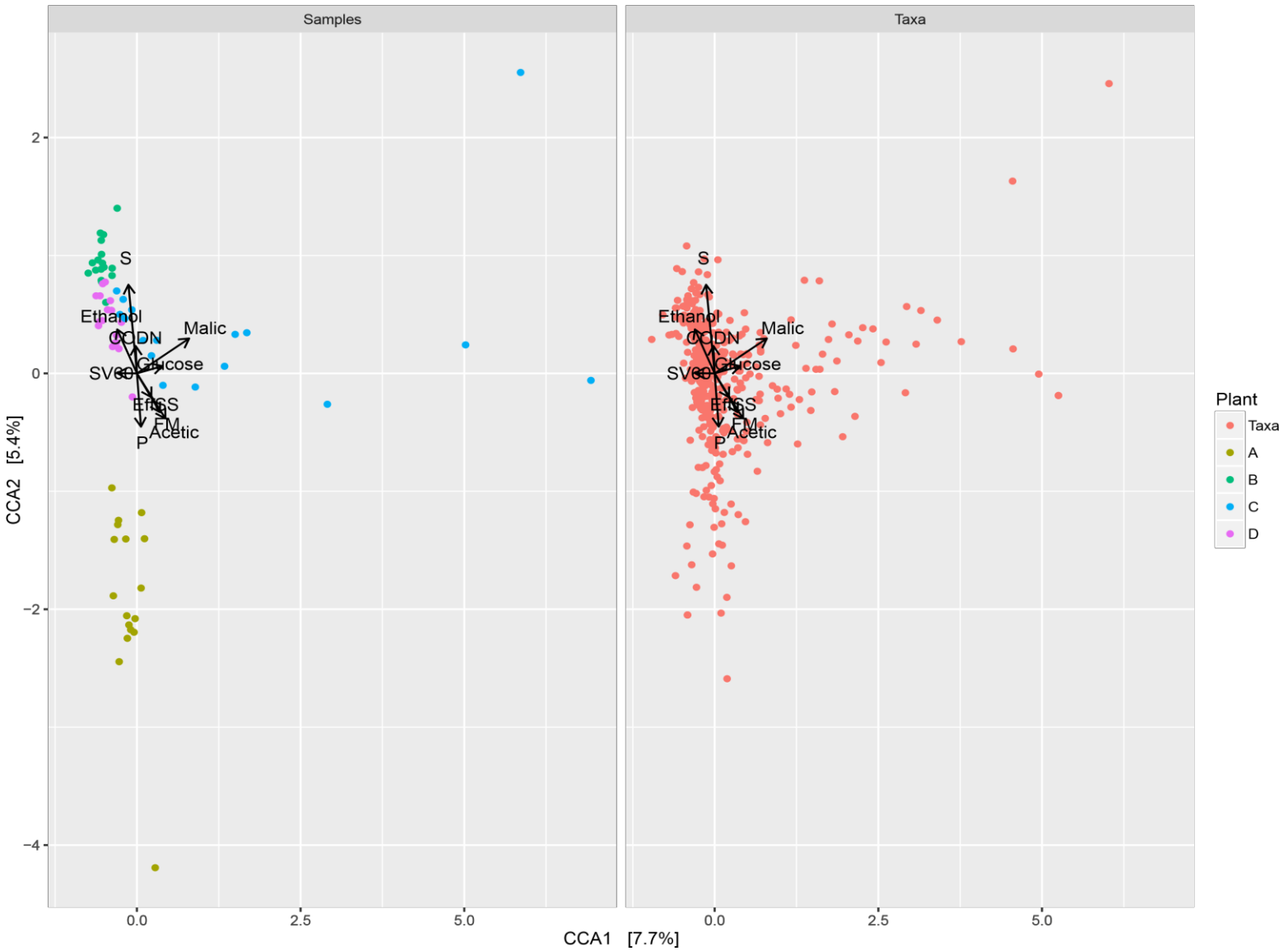
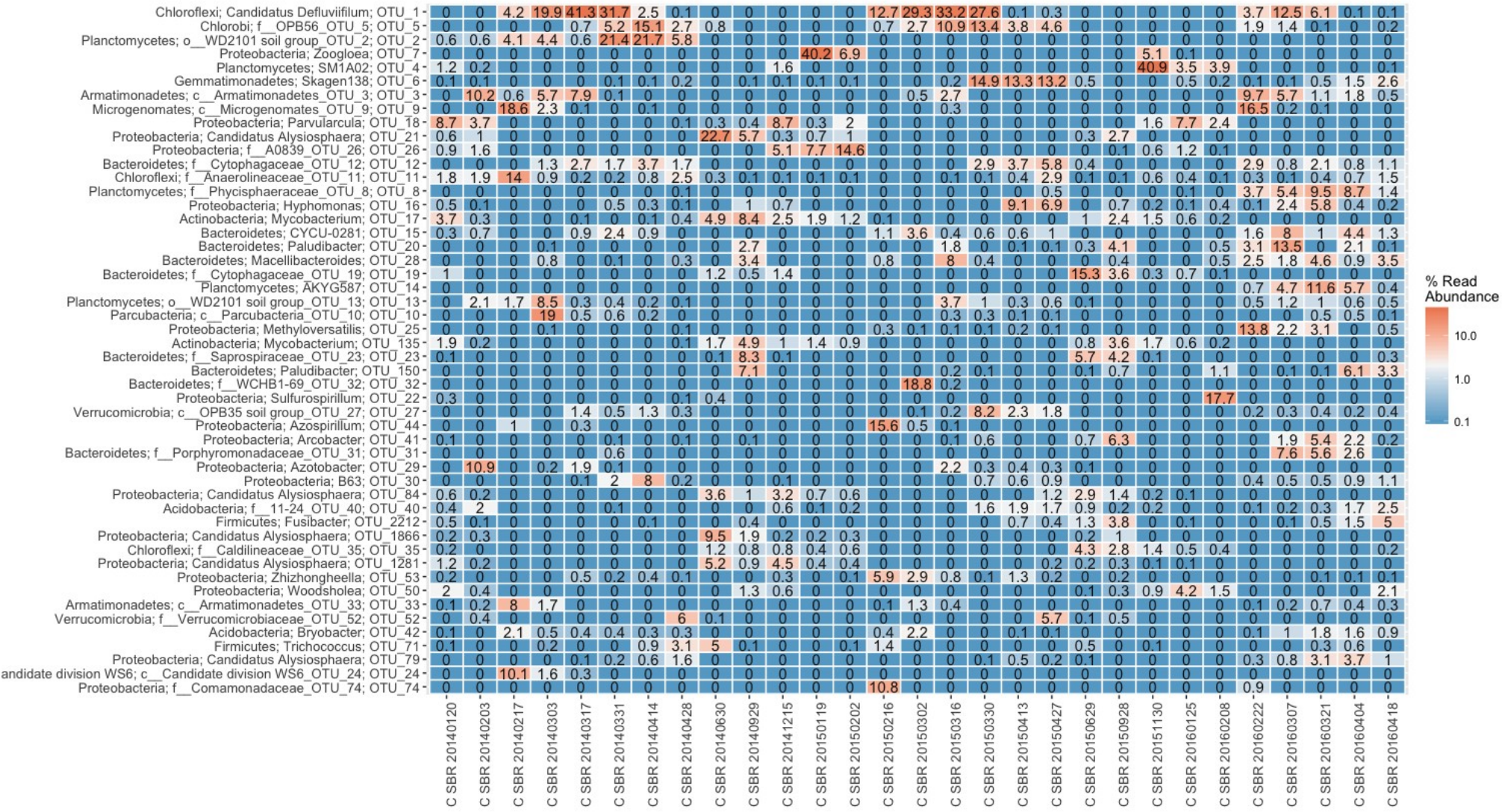


Figure 6.6.2 Canonical Correspondence Analysis by A) Plant samples and environmental variables. B) Taxa and environmental variables.

Plant C in blue, does not cluster well, from microbiology (section 6.3) and chemistry (section 6.5). We know that Plant C fluctuates significantly. When examining the heatmap generated from the abundance of OTUs, seasonal trends are obvious (Table 6.6.2). During peak Vintage, OTU_1, *Defluviifilum* (Type 0803) appears, as the population of OTU_1 reduces, OTU_5 emerges, belongs to a newly described, deeply branched lineage of Chlorbi, the OPB56. There are no cultured organisms belonging to this branch and they are poorly understood. OTU_3 is also present during the intense weeks of vintage, BLASTn searches

reveal that this read is also highly unique, with only 90% similarity to anything in the current database.


Chloroflexi; Candidatus Defluviifilum; OTU_1
Chlorobi; f__OPB56_OTU_5; OTU_5
Planctomycetes; o__WD2101 soil group_OTU_2; OTU_2
Proteobacteria; Zoogloea; OTU_7
Planctomycetes; SM1A02; OTU_4
Gemmatimonadetes; Skagen138; OTU_6
Armatimonadetes; c__Armatimonadetes_OTU_3; OTU_3
Microgenomates; c__Microgenomates_OTU_9; OTU_9
Proteobacteria; Parvularcula; OTU_18
Proteobacteria; Candidatus Alysiosphaera; OTU_21
Proteobacteria; f__A0839_OTU_26; OTU_26
Bacteroidetes; f__Cytophagaceae_OTU_12; OTU_12
Chloroflexi; f__Anaerolineaceae_OTU_11; OTU_11
Planctomycetes; f__Phycisphaeraceae_OTU_8; OTU_8
Proteobacteria; Hyphomonas; OTU_16
Actinobacteria; Mycobacterium; OTU_17
Bacteroidetes; CYCU-0281; OTU_15
Bacteroidetes; Paludibacter; OTU_20
Bacteroidetes; Macellibacteroides; OTU_28
Bacteroidetes; f__Cytophagaceae_OTU_19; OTU_19
Planctomycetes; AKYG587; OTU_14
Planctomycetes; o__WD2101 soil group_OTU_13; OTU_13
Parcubacteria; c__Parcubacteria_OTU_10; OTU_10
Proteobacteria; Methyloversatilis; OTU_25
Actinobacteria; Mycobacterium; OTU_135
Bacteroidetes; f__Saprospiraceae_OTU_23; OTU_23
Bacteroidetes; Paludibacter; OTU_150
Bacteroidetes; f__WCHB1-69_OTU_32; OTU_32
Proteobacteria; Sulfurospirillum; OTU_22
Verrucomicrobia; c__OPB35 soil group_OTU_27; OTU_27
Proteobacteria; Azospirillum; OTU_44
Proteobacteria; Arcobacter; OTU_41
Bacteroidetes; f__Porphyromonadaceae_OTU_31; OTU_31
Proteobacteria; Azotobacter; OTU_29
Proteobacteria; B63; OTU_30
Proteobacteria; Candidatus Alysiosphaera; OTU_84
Acidobacteria; f__11-24_OTU_40; OTU_40
Firmicutes; Fusibacter; OTU_2212
Proteobacteria; Candidatus Alysiosphaera; OTU_1866
Chloroflexi; f__Caldilineaceae_OTU_35; OTU_35
Proteobacteria; Candidatus Alysiosphaera; OTU_1281
Proteobacteria; Zhizhongheella; OTU_53
Proteobacteria; Woodsholea; OTU_50
Armatimonadetes; c__Armatimonadetes_OTU_33; OTU_33
Verrucomicrobia; f__Verrucomicrobiaceae_OTU_52; OTU_52
Acidobacteria; Bryobacter; OTU_42
Firmicutes; Trichococcus; OTU_71
Proteobacteria; Candidatus Alysiosphaera; OTU_79
andidate division WS6; c__Candidate division WS6_OTU_24; OTU_24
Proteobacteria; f__Comamonadaceae_OTU_74; OTU_74
C SBR 20140120
C SBR 20140203
C SBR 20140217
C SBR 20140303
C SBR 20140317
C SBR 20140331
C SBR 20140414
C SBR 20140428
C SBR 20140630
C SBR 20140929
C SBR 20141215
C SBR 20150119
C SBR 20150202
C SBR 20150216
C SBR 20150302
C SBR 20150316
C SBR 20150330
C SBR 20150413
C SBR 20150427
C SBR 20150629
C SBR 20150928
C SBR 20151130
C SBR 20160125
C SBR 20160208
C SBR 20160222
C SBR 20160307
C SBR 20160321
C SBR 20160404
C SBR 20160418
% Read Abundance
10.0
1.0
0.1

During non-vintage the microbial populations shift dramatically with *Nostocoida limicola* phylotype *Alysiosphaera europea* and GALO identified as *Mycobacterium* sp in abundance. At the end of December until start-up, *Mycobacterium* continue their presence but *A. europea* disappears. OTU_18 belonging to the Alphaproteobacterial genus Parvularcula emerges in high abundance. The PCA illustrates the relationships between OTUs and microbial communities defined by month (Figure 6.6.3).

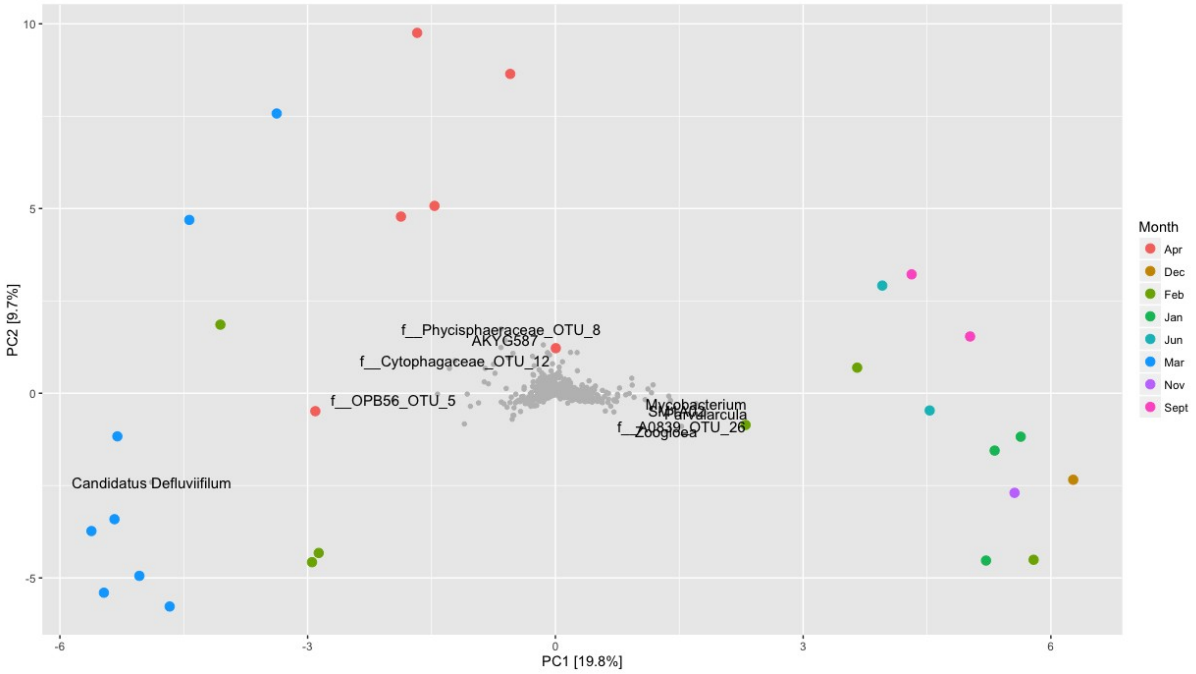


Figure 6.6.3 PCA of Plant C microbial communities defined by month.

6.7 Output 7: Additives

It has been observed by winery staff that each year during Pinot Gris/Grigio (PG) processing wastewater treatment plants are adversely affected. They often become overloaded and fail. Attempts have been made to understand this by studying the effect of common PG processing additives on an SBR.

Pinot Gris wine and juice, obtained from the University of Adelaide winery, was treated with common additives used in wine processing and added to wastewater.

250mL aliquots of juice and wine were treated with charcoal (2g/L), casein (0.25g/L), perlite (1g/L) and bentonite (1g/L) on a shaker for 2h, then filtered through 0.2µm filter. The filtrate was measured.

The solids were added to SBR water and mixed on a shaker for 2h, then filtered through a 0.2µm filter. The SBR filtrate was measured to assess the impact of these additives on pH, Ec, COD, total phenols, HPLC, ICP and suspended solids.

Figure 6.7.1 Pinot Gris juice and wine used in the experiment

The common components of winery wastewater are considered to be red wine, white wine and yeast. Additionally the main cleaning products are caustic soda and citric acid. We assessed the impact of these components in Table .6.7.1.

Table 6.7.1 Common components of winery wastewater

Sample	*COD (mg/L)*	*EC mS/cm*	*pH*	*Ca (mg/L)*	*Mg (mg/L)*	*K (mg/L)*	*Na (mg/L)*
Red Wine	*128800*	*2.03*	*3.50*	*59.2*	*112*	*953*	*27.4*
White Wine	*188600*	*1.4*	*3.24*	*50.4*	*156*	*478*	*32*
Soda Ash (2% w/v)	*18.0*	*23.3*	*11.32*	*0.2*	*0.1*	*0.2*	*9540*
Citric Acid (2% 2/v)	*13.5*	*3.05*	*2.35*	*2.7*	*0.2*	*0.3*	*19.6*
Tap Water	*0*	*0.35*	*7.1*	*29*	*9.5*	*3.7*	*47*
1g Yeast	*474213*			*0.6*	*1.56*	*28*	*0.54*

The biggest impact on COD is yeast, and as described in Section 6.1, there are often large concentrations of lees in the waste stream. Wines are high in COD and potassium, with a low pH, potassium is higher in red wines than white wines. Caustic soda increases the pH and adds large concentrations of sodium, while citric acid will reduce the pH.

The amount of solids entering wastewater treatment plants is significant, especially at small wineries (Figure 6.7.1). The solids can consist of grape skins and lees from the raw products as mentioned above. But also solids from the additives used during juice and wine processing such as bentonite, perlite, casein and charcoal. Perlite had minimal impact but contributes to suspended solids. The other additives investigated all impacted differently; charcoal increased the Ec, casein increased the COD and bentonite increased the pH.

Table 6.7.2 Additives

	pH	**EC (mS/cm)**	**COD (mg/L)**
Uni tap water	4.770	9.000	230.954
Charcoal (2g/L)	4.220	53.000	283.013
Casein (0.25g/L)	5.840	23.000	364.415
Perlite (1g/L)	5.067	9.667	286.800
Bentonite (1g/L)	6.620	17.667	254.617

When these solids have been used to treat wine and juice they carry some components with them that can be released into the SBR water. The treatment of juice and wine reduces pH and increases COD load (Table 6.7.3). The composition of the COD load varies though and may significantly impact the microbial populations that can grow.

Table 6.7.3 Solids from treated wine and juice

WINE	pH	Ec	COD mg/L	**JUICE**	pH	Ec	COD mg/L
SBR	9.0	879	85	SBR 3	9.1	907	30
Wine + SBR	8.9	872	678	Juice + SBR 3	8.8	877	505
Wine + SBR + Charcoal	8.3	778	1749	Juice + Charcoal + SBR 3	8.1	783	757
Wine + SBR + Casein	8.5	860	868	Juice + Casein + SBR 3	8.2	856	451
Wine + SBR + Perlite	8.7	867	1366	Juice + Perlite + SBR 3	8.6	865	506
Wine + SBR + Bentonite	8.4	839	1001	Juice + Bentonite + SBR 3	8.4	854	393

Additives that had been used to treat wine were added to SBR water and shaken for 2h, the solids were then filtered out and the carbon released into the water measured (Table 6.7.4). Charcoal released ethanol and phenols with some tartaric and acetic acids, reducing pH. Phenols have a high COD and would play a major role in the significant COD increase observed. Perlite released ethanol, citric acid and glycerol, while bentonite released ethanol and phenolics.

Table 6.7.4 Carbon components (g/L) measured in filtered SBR water after being incubated with additives used to treat wine.

	citric	*tartaric*	*glucose*	*malic*	*fructose*	*succinic*	*lactic*	*glycerol*	*acetic*	*ethanol*	*phenols*
Control	0.038	0.000	0.000	0.000	0.001	0.000	0.008	0.020	0.011	0.296	0.006
Charcoal	0.000	0.013	0.000	0.000	0.000	0.000	0.003	0.004	0.026	0.766	0.045
Casein	0.000	0.003	0.000	0.000	0.000	0.000	0.000	0.002	0.000	0.412	0.009
Perlite	0.076	0.000	0.000	0.000	0.000	0.001	0.017	0.039	0.015	0.665	0.007
Bentonite	0.000	0.002	0.008	0.000	0.000	0.000	0.000	0.000	0.010	0.451	0.011

Additives that had been used to treat juice were measured in the same way (Table 6.7.5), casein, perlite and bentonite did not impact the SBR water significantly. Charcoal released a considerable amount of fructose and succinic acid and some citric acid and phenols.

Table 6.7.5 Carbon compounds (g/L) measured in filtered SBR water after being incubated with additives used to treat juice.

	citric	*tartaric*	*glucose*	*malic*	*fructose*	*succinic*	*lactic*	*glycerol*	*acetic*	*ethanol*	*phenols*
Control	0.087	0.000	0.178	0.000	0.266	0.001	0.000	0.000	0.000	0.000	0.006
Charcoal	0.134	0.000	0.005	0.000	0.487	0.131	0.000	0.000	0.003	0.000	0.023
Casein	0.049	0.000	0.041	0.000	0.260	0.000	0.000	0.000	0.000	0.000	0.009
Perlite	0.081	0.000	0.156	0.000	0.268	0.003	0.000	0.000	0.000	0.000	0.006
Bentonite	0.059	0.000	0.000	0.011	0.252	0.000	0.000	0.000	0.004	0.000	0.011

From this study, perlite, skim milk and bentonite have minimal impact on SBR chemistry. However, charcoal releases the phenols, ethanol, sugars and organic acids it has stripped from wine and juice into the SBR water. This release of carbon significantly increases the COD load on the system and can cause a rapid drop in pH, destabilising and overloading the microbial population.

The use of charcoal in the wine industry has been increasing recently due to the increased production and processing of Pinot Gris/Grigio grapes. It is essential to keep charcoal out of the wastewater system in order to minimise shock in the SBRs during vintage.

6.8 Output 8: Full scale trials

Changes have been implemented at wineries in an attempt to improve plant performance. Nutrient dosing was undertaken at many plants using urea and/or DAP. Operational parameters that have been changed include:

Feeding regimes (Plant A). Small volumes of raw wastewater have been directed to aerobic lagoons, by-passing the anaerobic digester. Chemical analysis of the wastewater shows that a range of organic acids, phenols, sugars and ethanol are present in the raw wastewater. After anaerobic digestion most of these carbon forms are converted into acetate. This was our most successful and complete trial and is detailed in below.

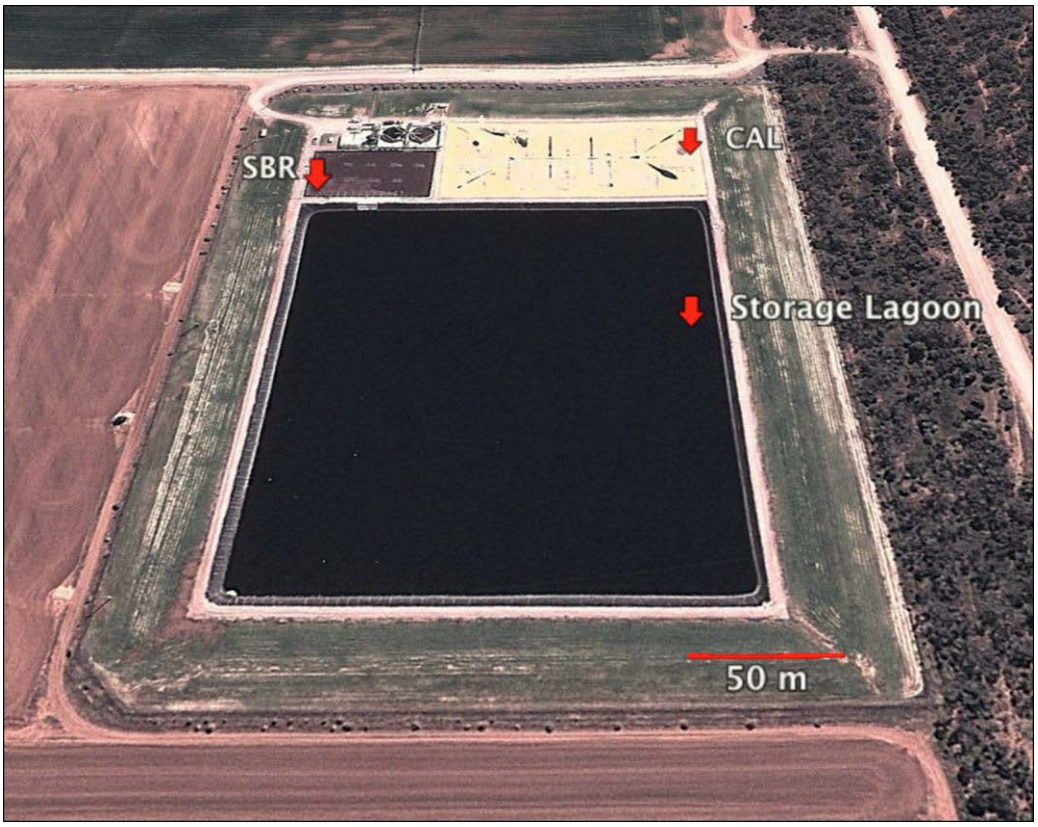
Aeration (Plant B). Changes in the aeration patterns of a cycle can trigger population and floc structure changes. This is achieved by manipulating dissolved oxygen set points in the program. Such changes can also save energy/money by reducing over-aeration. Unfortunately, due to maintenance issues with the aerators this trial was abandon after a few weeks.

Configuration (Plant C): At one site, a trial is underway to see if the surge tank can be used as an additional SBR during peak vintage to cope with the peak load. This plant is configured with a surge tank similar in size to the SBR and is fitted with a fine air diffuser. It was observed during 2014 and 2015 that the surge tank removed a large proportion of the COD during early vintage, taking the pressure off the SBR unit. However, approximately 4-6 weeks into vintage the pH dropped considerably to pH 4 and then no further COD removal could be achieved and the SBR soon became overloaded and failed. Our proposal was to buffer the surge tank using calcium carbonate (lime) to extend the time in which the surge tank could continue to act as an SBR, potentially getting the SBR through the peak period. While the operators were keen, there was no money in the budget for the additional lime and the project did not go ahead.

Biomass management (Plant D): Changing the suspended solids content of the aeration system changes the amount of food available per microbe (F:M), different organisms favour different F:M ratios and will thrive under the changed conditions. Another scheme has been to use anaerobic biomass as a potential carbon and nitrogen source during quiescent periods.

Plant A: Raw feeding regime.

Samples were taken from a full-scale beverages treatment plant in NSW, Australia during the period of April – August 2015. Influent samples were taken post-screening. The plant is continuously fed into a 30ML covered anaerobic lagoon with a HRT averaging 26 days. Over the six month trial, the SBR (6ML) processed three batches/day of 450kL. From 1 June, one batch/day contained 100kL of raw influent bypassing the CAL. Influent, CAL, SBR and Effluent samples were assessed by pH, Ec, temperature, MLSS, SV, COD, HPLC and total phenols (Rice and Bridgewater 2012) where appropriate.



FISH was performed for the identification of *Thiothrix* spp. (G123T and competitor) (Kanagawa et al. 2000) and biovolume fractions were estimated using a G123T probe as specific target and EUB338mix probe for total biovolume.

DNA was extracted, sequenced and processed as above.

This plant has long suffered from slow settling sludge, reducing efficiency and effluent quality. The WWTP treats 1500 tonnes of COD annually and consists of a Covered Anaerobic

Lagoon (CAL) followed by an SBR. Microscopic examination indicates that *Thiothrix sp.* are the cause of poor settling (Figure 6.8.1).

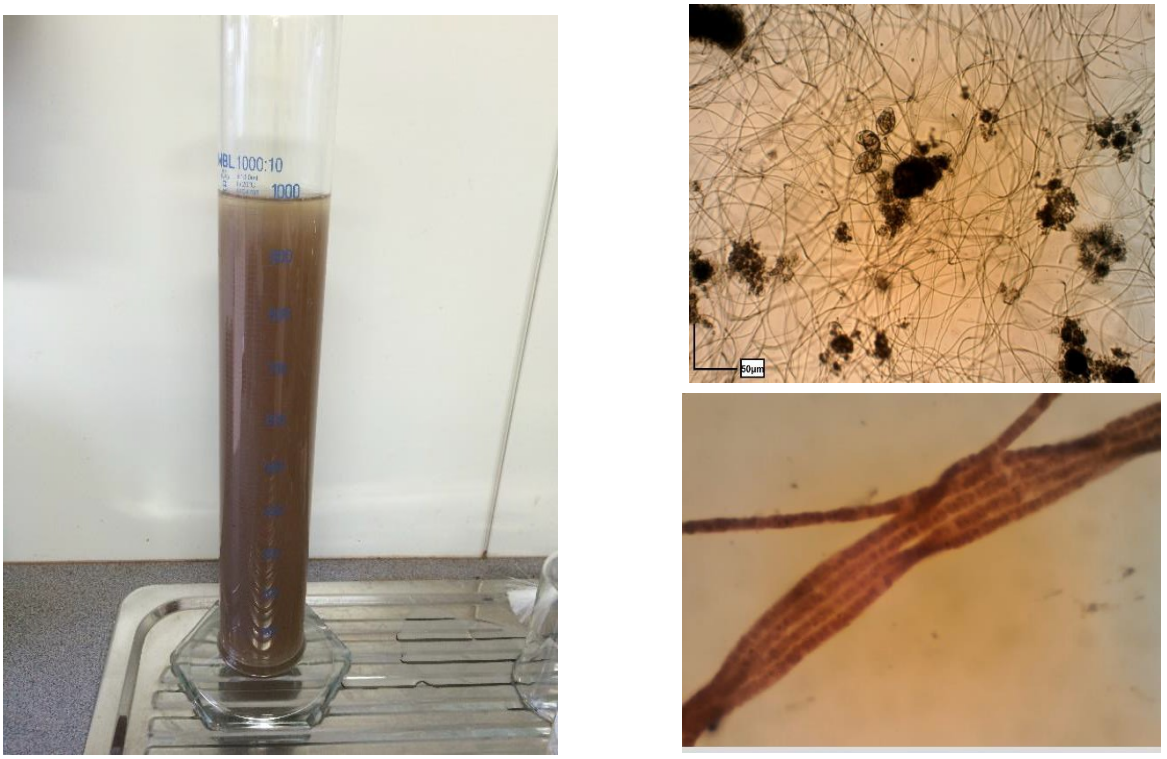


Figure 6.8.1 SBR sample and microscopic analysis on 26 May 2016 a) SV30= 970mL b) Brightfield microscopy at 100X showing excessive filament growth c) Gram stain (X1000 magnification) Typical *Thiothrix* morphology.

Thiothrix is chemolithotrophic, oxidising hydrogen sulfide as an inorganic energy source and can store sulfur internally. Their dominance in wastewater is associated with septic wastewater, simple organic acids, nutrient deficiency and high sulfur. Common remedies include increased aeration and supplementation of nutrients.

Chemical analysis of the raw influent shows a cocktail of organic acids, phenols and alcohols while the feed from the CAL is high in acetic acid and phenols (**Table 6.8.1**).

Table 6.8.1. Average composition of raw feed and covered anaerobic lagoon during the period of April-August 2015. (Values are in g/L unless otherwise stated).

	Tartaric acid	Succinic acid	Lactic acid	Acetic acid	Glycerol	Ethanol	Phenols	COD (mg/L)	pH	EC (uS/cm)
Raw Feed	0.486 ± 0.27	0.073 ± 0.07	0.561± 0.29	0.214 ± 0.08	0.033 ± 0.01	1.132 ± 0.10	1.076 ± 0.10	3970 ± 200	6.1 ± 0.50	1081 ± 207
CAL	-	-	-	0.781 ± 0.61	-	0.402 ± 0.30	0.940 ± 0.57	3435 ± 666	5.1 ± 0.70	1485 ±193

From an understanding of the microbiology of the treatment plant and the chemical composition of the wastewater, an attempt was made to manage the microbial populations to improve settling in this full scale system. Nutrients and aeration were not limited, so a small volume of raw influent was fed directly into the SBR to increase the carbon compound range that the SBR was receiving to reduce septicity. Therefore a small volume (100kL/day) of raw influent was fed directly into the SBR, bypassing the CAL.

After direct feeding, a shift in microbial populations could be observed. Microscopic examination and application of 16S rRNA targeted probes showed the community was dominated by *Thiothrix sp.* (Figure 6.8.2). The biovolume of *Thiothrix sp.* reduced from 31% to 1%.

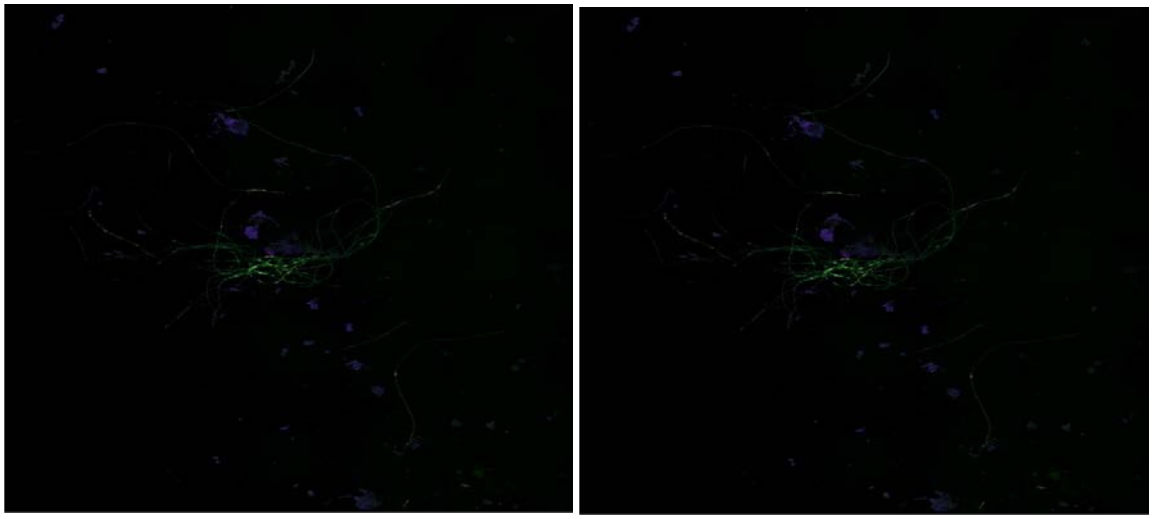
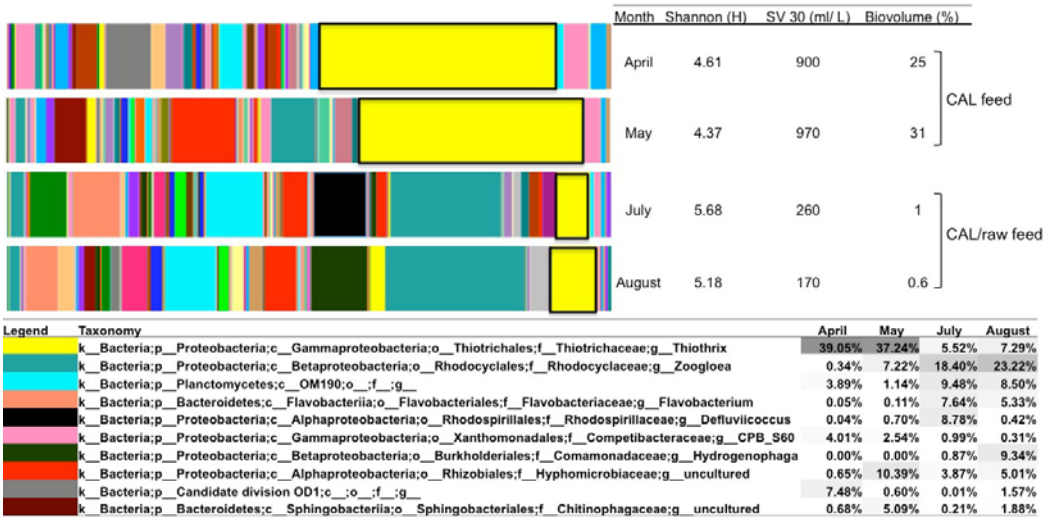


Figure 6.8.2. FISH application of G123T (Green) and EUB338mix (Blue) to estimate biovolume.

Metagenomics analysis also showed an increased biodiversity with the Shannon index increasing from 4.37 to 5.68, and the appearance of species belonging to *Zoogloea* (Figure 6.8.3).



Legend	Taxonomy	April	May	July	August
	k__Bacteria;p__Proteobacteria;c__Gammaproteobacteria;o__Thiotrichales;f__Thiotrichaceae;g__Thiothrix	39.05%	37.24%	5.52%	7.29%
	k__Bacteria;p__Proteobacteria;c__Betaproteobacteria;o__Rhodocyclales;f__Rhodocyclaceae;g__Zoogloea	0.34%	7.22%	18.40%	23.22%
	k__Bacteria;p__Planctomycetes;c__OM190;o__;f__;g__	3.89%	1.14%	9.48%	8.50%
	k__Bacteria;p__Bacteroidetes;c__Flavobacteriia;o__Flavobacteriales;f__Flavobacteriaceae;g__Flavobacterium	0.05%	0.11%	7.64%	5.33%
	k__Bacteria;p__Proteobacteria;c__Alphaproteobacteria;o__Rhodospirillales;f__Rhodospirillaceae;g__Defluviicoccus	0.04%	0.70%	8.78%	0.42%
	k__Bacteria;p__Proteobacteria;c__Gammaproteobacteria;o__Xanthomonadales;f__Competibacteraceae;g__CPB_S60	4.01%	2.54%	0.99%	0.31%
	k__Bacteria;p__Proteobacteria;c__Betaproteobacteria;o__Burkholderiales;f__Comamonadaceae;g__Hydrogenophaga	0.00%	0.00%	0.87%	9.34%
	k__Bacteria;p__Proteobacteria;c__Alphaproteobacteria;o__Rhizobiales;f__Hyphomicrobiaceae;g__uncultured	0.65%	10.39%	3.87%	5.01%
	k__Bacteria;p__Candidate division OD1;c__;o__;f__;g__	7.48%	0.60%	0.01%	1.57%
	k__Bacteria;p__Bacteroidetes;c__Sphingobacteriia;o__Sphingobacteriales;f__Chitinophagaceae;g__uncultured	0.68%	5.09%	0.21%	1.88%

Figure 6.8.3. SBR microbial community composition, Shannon's diversity index, sludge volume 30 and *Thiothrix sp.* biovolume fraction before and after direct raw feeding.

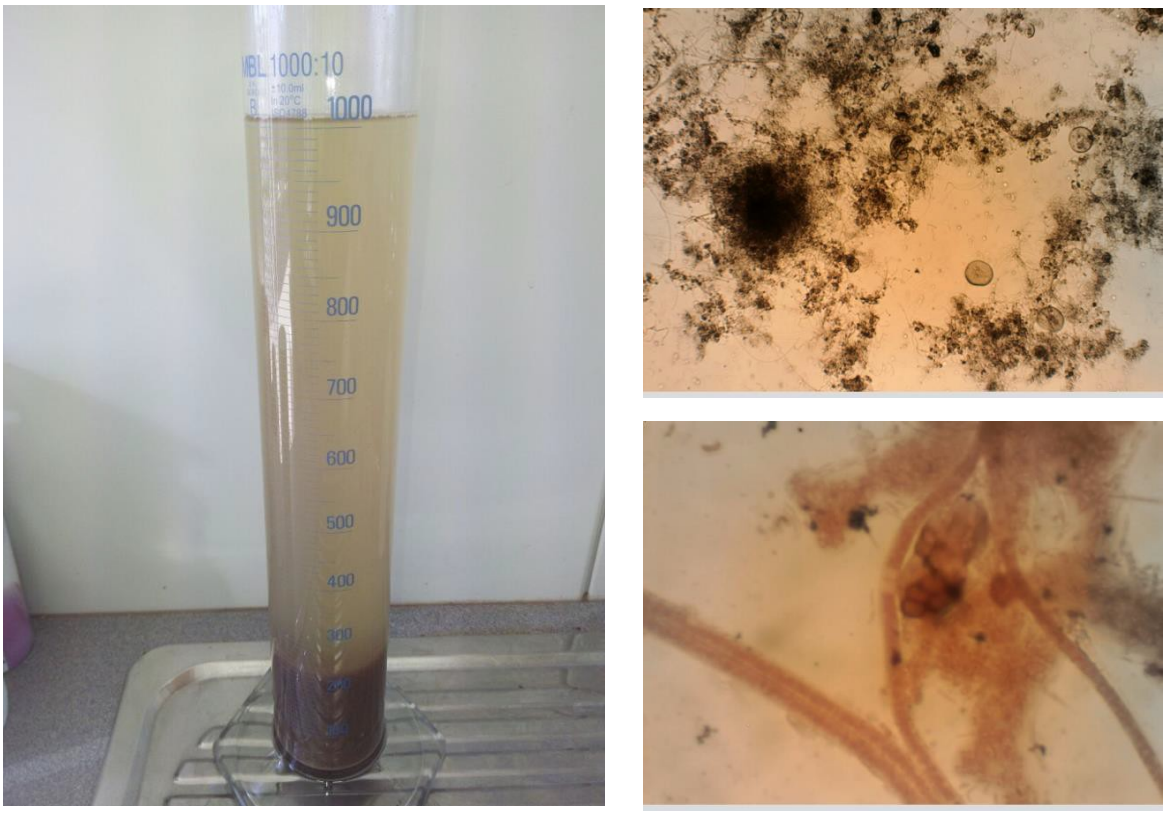


Figure 6.8.4. SBR sample taken 27 July 2016. a) SV30= 260 b) Light microscopy reveals better floc structure and few filaments c) Gram staining illustrating the presence of both Thiothrix and Zoogloea.

Sludge volume decreased from SV30=970 to SV 260 after direct feeding. Microscopy revealed a notable reduction in filaments and the appearance of Zoogloea.

Whilst there is a need to send most of the raw influent into the CAL, introducing a relatively small volume of raw influent with a cocktail of organic acids, phenols and alcohols improved settling, efficiency and quality of the effluent.

Cyanobacteria in storage lagoons

Cyanobacteria, also known as blue green algae, often proliferate in storage lagoons and maturation ponds. Many cyanobacterial species can produce toxins of potential concern, including neurotoxins, hepatotoxins and cytotoxins. These toxins can infect plants, animals and humans and a common question we receive from plant operators is about their health when dealing with wastewater on a daily basis. Recently there has been increasing concern about the impact of using storage water as irrigation water due to the presence of cyanobacteria and their associated toxins. The most commonly reported species in lagoons is *Microcystis aeruginosa* which produces a range of microcystins, many of which are hepatotoxic. In this study we measured microcystins as they are the most common and important in wastewater management.

In Australia there are no federal or state standards for cyanobacteria in water, however the NHMRC Australian Drinking Water Guidelines (2011) specify a guideline value of 1.3µg/L for total microcystins (expressed as microcystin-LR toxicity equivalents). The World Health Organization (WHO) has set a provisional limit of 1µg/L for microcystin-LR in drinking water and 10ppb for irrigation and recreational use.

	Plant A	Plant B	Plant D	Plant N	Plant N2	Plant K
25/08/2014	0.88	0.02		0.55	0.52	
27-Oct	0.76	0.23	0.53			
24/11/2014	1	0.06	0.83			
5/01/2015	1.65		8.07			
19/01/2015	1.6	0.35	4.91			
5/02/2015	1.47	0.27	4.75			
18/02/2015	1.12	0.29	2.78			
5/03/2015	2.25	0.24	1.91			
16/03/2015	1.63	0.35	1.66			0.61
30/03/2015	2.78	0.25	1.21			
13/04/2015	2.49	0.26	0.87			
27/04/2015	1.45	0.18	0.67			
11/04/2015	1.27	0.11	0.73			
25/05/2015	1.36	0.23	0.69			

All plants were well below recommended levels for irrigation and reuse.

7. Outcomes/Conclusions

All outcomes for the project were achieved, plus an additional two outputs investigating cyanobacteria risk and the effect of winery additives on wastewater chemistry. Key conclusions from the detailed microbiological analyses are:

- G-bacteria and *Nostocoida limicola* II are routinely observed in WWTP. The importance of G-bacteria, are now being investigated in a PhD student project.
- Microbial communities were very similar from one vintage to the next, with individual plants tending to maintain a similar community over time (e.g. Plants A, B and D), unless significant upstream changes occur (e.g. Plant C, high levels of activated carbon in the WWW).
- Novel organisms were frequently observed in the study. Further investigation is required to determine their role in WWTP.
- Microbiology is not directly related to the size of the winery, but the configuration and level of process control can determine which populations will flourish.

Practical implications of the research results have already been implemented, with a successful full-scale trial undertaken, the presentation of project outcomes at a workshop for industry practitioners (see Appendix 1.1) and the development of posters outlining key WWW microbes and standardised methods for turbidity measurements.

Several other practical solutions for improvement of plant performance have been determined including:

- Diversity in bacterial populations improves plant performance, therefore, steps taken to enhance diversity, such as direct feeding are simple, practical solutions.
- Improve solids management to reduce the burden on wastewater treatment plants, reducing risk of plant failure, particularly during vintage.
- Prepare plants for vintage by adding carbon (molasses, wine, grape juice).
- Dosing with nitrogen (urea and or DAP). We have provided a clear understanding that nutrients are needed for bacterial growth, so nutrient dosing can be critical.

- Winery additives, such as activated carbon, can potentially cause plant failure, as they can release compounds back into the WWW, which can affect bacterial growth. Separation of these additives from the WWW stream will reduce the risk of failure.

The economic benefits relating to the outcomes from this project are mainly indirect. Improved plant performance means less time, therefore costs associated with running the plant e.g. labour and energy. The cost of plant failure can be significant, both in labour cost, plus potentially the transport of WWW to an alternative site (tens of thousands of dollars), fines and cessation of winery operations, particularly during vintage. The majority of practical solutions developed from this project are relatively inexpensive, not requiring major capital investment. However, the opportunity provided by the low operational cost of anaerobic treatment warrants further investigation, particularly in association with co-generation of energy. That the treated water can be reused for irrigation (low cyanobacterial risk) can be of direct economic benefit to grape and wine producers.

While more efficient and effective biological treatment does not produce a direct improvement in the quality of grapes or wine for grape growers or winemakers, the potential reuse of water can be part of an irrigation program (without clogging pipes) to produce high quality grapes, which is a direct environmental benefit to both producers and to the broader community. More efficient treatment also reduces the carbon footprint in the wine sector through reduced energy usage.

8. Recommendations

Extension

From the workshop, there was great enthusiasm from plant operators to have annual get-togethers. These workers rarely have the opportunity to gather and discuss problems and experiences. The observations of operators of WWTP were the drivers of this research. They know their plants well, monitor them and maintain them carefully, but they are looking for more support.

Practical advice for industry

The top five tips for industry for a successful biological wastewater system are:

- Solids management/cleaner production
- Maintenance and preparation
- Monitor your system
- Provide a healthy microbial environment
- Contingency Plan

Future research

Key areas for further research and development are focused around anaerobic digestion; improving existing systems and designing a small flexible version. Anaerobic waste treatment in the wine industry is expanding rapidly internationally due to its favourable economic and environmental qualities. Its potential to treat other wastes such as lees and marc is advantageous but needs to be explored further.

9. Appendix 1: Communication

Site visits

Site visits were a major aspect of our project. Each year during peak vintage trips were made to the Barossa, McLaren Vale, Yarra Valley, Rutherglen and Griffith so we could meet with WWW operators, discuss their issues and collect samples. Thirty samples were collected and a microbiological report including photographs was given to the operators after examination of their sludge.

Our four major sites, two in the Barossa and two in Griffith, were visited three times each year;

- Pre-vintage to discuss preparation for the onslaught of vintage,
- Peak vintage, to observe how things were going, identify any problems and provide advice/direction where wanted.
- Post vintage, to review all the data and assess what changes would need to be implemented for a more successful project and WWW treatment.

Written reports to wineries

In peak vintage 2014 (32), 2015 (28) and 2016 (22), wineries submitted wastewater samples, all received written reports with photos describing the microbiology of their samples.

Presentations

WIC Crush conference (Sept 2014): The microbiology of Australian Winery Wastewater Treatment Plants. Presenter: Kathryn Eales

7th Australian Wine Industry Environment Conference (Sept 2014): The microbiology of Australian Winery Wastewater Treatment Plants. Presenter: Paul Grbin.
2014.

International Water Association: Specialised Conference on Sustainable Viticulture, Winery Wastes and Agri-industrial Wastewater Management. Stellenbosch, South Africa. 4/11/2015

‘Microbial populations of winery wastewater treatment plants in Australia’. Presenter: Kathryn Eales

Interwinery Analysis Group Seminar on 10/12/2015 in Hahndorf SA. The presentation was entitled ‘What makes your wastewater system tick?’ Presenter: Paul Grbin

Posters

Australian Wine Industry Technical Conference (2016):

Winery Wastewater: Microbiological Lessons Learnt. Authors: Kathryn Eales, Patrick Rea, Cristobal Onetto and Paul Grbin.

Winery Wastewater: Standardising methods for turbidity. Authors: Kathryn Eales, Patrick Rea, Cristobal Onetto and Paul Grbin.

International Water Association: MEWE Conference:

Winery Wastewater: Serving Cocktails improves diversity. Authors: Cristobal Onetto, Kathryn Eales and Paul Grbin.

Webinars

edX MOOC a video summary of the project was prepared and included in the online course titled ‘World of Wine: from Grape to Glass’ (see www.Wine101x). This course has had more than 20,000 learners enrolled, from more than 150 countries. Presenter: Paul Grbin.

AWRI webinar (10/12/2015) entitled ‘Microbial populations of Winery Wastewater Treatment Plants’. Presented by Kathryn Eales

Workshop

Winery Wastewater Workshop. Barossa Valley

Radio

Paul Grbin interviewed by ABC Pt Pirie

Appendix 1.1

Survey Monkey Results:

Winery Wastewater Workshop

Q1 **Overall, how would you rate this workshops usefulness for your workplace?**

- Answered: 8
- Skipped: 0

	NOT USEFUL		OK		VERY USEFUL	TOTAL	WEIGHTED AVERAGE
	0.00%	0.00%	0.00%	62.50%	37.50%		
RESPONSES	0	0	0	5	3	8	4.38

Q2 **Did you learn what you wanted to about winery wastewater at this workshop? Did it meet your expectations?**

- Answered: 8
- Skipped: 0

ANSWER CHOICES	RESPONSES
YES	100.00%
	8
NO, PLEASE GIVE DETAILS OF WHAT WE MISSED.	0.00%
	0
TOTAL	8

Q3 **How useful was the information in each part of the workshop?**

- Answered: 8
- Skipped: 0

	NOT USEFUL		**MODERATE**		**VERY USEFUL**	**TOTAL**	WEIGHTED AVERAGE
MICROBIAL POPULATIONS IN WINERY WASTEWATER TREATMENT PLANTS	0.00% 0	0.00% 0	12.50% 1	25.00% 2	62.50% 5	8	4.50
MAXIMISING THE POTENTIAL OF GRAPE MARC	0.00% 0	25.00% 2	50.00% 4	12.50% 1	12.50% 1	8	3.13
GYCOGEN ACCUMULATING ORGANISMS; UREA DOSING	0.00% 0	0.00% 0	25.00% 2	50.00% 4	25.00% 2	8	4.00
SERVING COCKTAILS IMPROVES DIVERSITY; DIRECT FEEDING	0.00% 0	0.00% 0	25.00% 2	50.00% 4	25.00% 2	8	4.00
IMPACT OF ADDITIVES ON WASTEWATER TREATMENT	0.00% 0	0.00% 0	12.50% 1	50.00% 4	37.50% 3	8	4.25
GLOBAL PERSPECTIVE OF WATER IN THE WINE INDUSTRY	0.00% 0	25.00% 2	12.50% 1	37.50% 3	25.00% 2	8	3.63
WINERY WASTEWATER ANAEROBIC PROCESSING	0.00% 0	0.00% 0	12.50% 1	37.50% 3	50.00% 4	8	4.38
SITE VISITS TO YALUMBA AND NPEC	0.00% 0	14.29% 1	0.00% 0	42.86% 3	42.86% 3	7	4.14

Q4 **Can we improve the information delivered in the workshop?**

- Answered: 3
- Skipped: 5

Showing 3 responses

- Thorough and great detail.
- Overall good workshop
- The information was all very interesting, but in hindsight, while I felt I took a lot of notes, I'm now not clear what the really important points were - it is now just a bunch of points. Anything I gained during the workshop I have subsequently lost.

Q5 **What additional information would assist you in managing wastewater in your workplace?**

- Answered: 6
- Skipped: 2

Showing 6 responses

- Detailed pictures of organisms.
- Weather effects on CAL and SBR, i.e. temp changes
- Stabilising waste streams pre treatment plant
- More site visits, Annual workshops to get together with other plant operators in the industry
- The development of standard reference sheets regarding types of microbial populations, nutrient specs etc
- Not more information, but really simple, clear summaries of what are good operational rules. Statements like 'feeding microbes improves diversity' are interesting but not helpful for a plant operator. They need to know X is the optimal rate for chemical Y, or when X falls below ... then dose with ..... One of the presentation finished with future directions and where to from here, which isn't the right end point for this audience

Q6 **What information from today's workshop will you use in your workplace?**

- Answered: 7
- Skipped: 1

Showing 7 responses

- The importance of educating cellar staff of what goes down the drain will affect your waste water.
- Pre vintage preparation.
- Acclimatisation of bio- reactors to sugars in December to prevent indigestion during beginning vintage.
- Serving cocktails, Direct feeding, Microbial populations,
- Looking at microbial populations and the impact of additives on wastewater treatment
- I really only have broad principles now. I feel a bit disappointed this is the case
- additives info was useful, also feeding strategies

Q7 **Will you change what you do in your workplace after this workshop?**

- Answered: 8
- Skipped: 0

ANSWER CHOICES	RESPONSES
YES	87.50%
	7
NO	12.50%
	1
TOTAL	8

- **SETTLING TIMES, AERATION TIMES AND EDUCATION OF WASTE WATER.**
- **AS PER 6**
- **ALREADY HAVE SOME OF THESE PROCESS IN PLACE, POTENTIALLY EXPERIMENT MORE WITH DIRECT FEEDING**
- **REDUCING AND RECAPTURING WINERY WASTE BEFORE IT ENTERS THE TREATMENT PLANT, MORE FOCUS ON MICROBIAL AND NUTRIENT LEVELS**
- **I'M NOT SURE THAT I CAN**
- **POSSIBLY, WE COULD LOOK AT DIFFERENT NUTRIENT STRATEGIES**

Q8 **If you answered no to question 7, please tell us why?**

- Answered: 2
- Skipped: 6

ANSWER CHOICES	RESPONSES
TOO COMPLICATED	50.00%
	1
TOO TIME CONSUMING	0.00%
	0
DON'T SEE ANY BENEFIT	0.00%
	0
NEED MORE TRAINING	0.00%
	0
TOO MUCH EFFORT	0.00%
	0
ALREADY TOO BUSY	0.00%
	0
COLLEAGUES WONT EMBRACE CHANGES REQUIRED	0.00%
	0
WORKPLACE ALREADY USES EXCELLENT PRACTICES	50.00%
	1
OTHER (PLEASE SPECIFY)	50.00%
	1

TOTAL RESPONDENTS: 2

- Not sure what is required

Q9 **Any other comments?**

- Answered: 7
- Skipped: 1

Showing 7 responses

- Excellent workshop and much appreciated for the invite.
- It was a good day. Like to compare how plants work
- Well done - look forward to the next one. Would like to have access to power point presentation as display and printouts where difficult to read.
- Great day, Useful information.
- Would be good to see continued research and shared experience within this topic
- Facts and numbers are helpful. The posters of microbes are good. The rest is just interesting
- great initiative and well presented

10. Appendix 2: Intellectual Property:

No intellectual property arose from this project.

11. Appendix 3: References

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12. Appendix 4: Staff

The staff engaged on this project were:

Associate Professor Paul Grbin – Project supervisor

Dr Kathryn Eales – Chief Investigator

Mr Patrick Rea – Research Assistant

Mr Cristobal Onetto – PhD Student

Mr John Constable – Collaborator

Mr Mike Carson – Collaborator

Dr Anu Kumar – Collaborator

Ms Debra Gonzago – Research Assistant

13.

Appendix 5:

Appendix 5.1 FISH Probes

Probe name	Target	Sequence 5' to 3'	Reference
EUB338I	Most bacteria	GCT GCC TCC CGT AGG AGT	(Amann et al. 1990)
EUB338II	Planctomycetes	GCA GCC ACC CGT AGG TGT	(Daims et al. 1999)
EUB338III	Verrucomicrobiales	GCT GCC ACC CGT AGG TGT	(Daims et al. 1999)
NonEUB	Control	ACT CCT ACG GGA GGC AGC	(Wallner et al. 1993)
Alf968	Alphaproteobacteria	GGT AAG GTT CTG CGC GTT	(Neef et al. 1999)
Beta42a	Betaproteobacteria	GCC TTC CCA CAT CGT TT	(Manz et al. 1992)
Gam42a	Gammaproteobacteria	GCC TTC CCA CAT CGT TT	(Manz et al. 1992)
HGC69a	Actiobacteria	TAT AGT TAC CAC CGC CGT	(Roller et al. 1994)
LGC354	Firmicutes	TGG AAG ATT CCC TAC TGC	(Meier et al. 1999)
DF988	Defluviicoccus group II		(Wong et al. 2004)
DF1020	Defluviicoccus group II		(Wong et al. 2004)
Noli644	Alysiosphaera europeae	TCC GGT CTC CAG CCA CA	(Snaidr et al. 2002)

Appendix 5.2 Culture media

R2A

Formula	***g/L***
Yeast extract	0.5
Proteose peptone	0.5

Casein hydrolysate	0.5
Glucose	0.5
Starch	0.5
Di-potassium phosphate	0.3
Magnesium sulfate	0.024
Sodium pyruvate	0.3
Agar	15.0
pH 7.2 ± 0.2 @ 25°C	

Glucose Yeast Extract

Formula	***g/L***
Glucose	20
Yeast extract	10
$CaCO_3$	10
Agar	17

Appendix 5.3

V3-V4 16S rRNA gene sequence of OTU_1 from effluent

CCTACGGGTGGCTGCAGTGGGGAATATTGGACAATGGGCGCAAGCCTGATCCAGCCATGCCGC

GTGTGTGATGAAGGCCTTAGGGTTGTAAAGCACTTTCGCACGTGACGATAATGACGGTAGCGT

GAGAAGAAGCCCCGGCTAACTTCGTGCCAGCAGCCGCGGTAATACGAAGGGGGCTAGCGTTGT

TCGGAATCACTGGGCGTAAAGCGCACGTAGGCGGATGCTTAAGTCAGGGGTGAAATCCCGGG

GCTCAACCTCGGAACTGCCCTTGATACTGGGTTTCTTGAGTTCGGGAGAGGTTGGTGGAACTGC

GAGTGTAGAGGTGAAATTCGTAGATATTCGCAAGAACACCAGTGGCGAAGGCGGCCAACTGGC

CCGATACTGACGCTGAGGTGCGAAAGCGTGGGGAGCAAACAGGATTAGAAACCCTAGTAGTC

Appendix 5.4 Metadata

Four plants, Covered Anaerobic Lagoons, Plant C.

SeqID	Sample ID	Description	Plant	Month	Raey	SV60	MLSS	CODf	NPOC	TN	P	F:M	COD:N	COD:P	Citric	Tartaric	Malic	Succinic	Lactic	Acetic	Glucose	Fructose	Glycerol	Ethanol	Phenols	Ca	K	Mg	Na	S	EffSS
J44_S1	S0625	A SBR 140120	A	Jan	2014	640	3120	1720	670	28	13.8	0.110256	61.42857	124.6377	0	0.0029	0	0	0.015	0.6095	0	0	0.0111	1.4172	0.977	30.8	108	8.66	311	7.08	40
J44_S5	S0629	A SBR 140217	A	Feb	2014	470	5240	3850	1319	44	8.91	0.146947	87.5	432.0988	0	0.0065	0	0	0	1.9521	0	0	0	1.0054	0.234	17.5	154	8.28	307	6.31	300
J44_S9	S0633	A SBR 140414	A	Apr	2014	840	5580	2990	1011	34	12.2	0.107168	87.94118	245.082	0	0.0081	0	0	0	1.824	0	0	0	0.661	0.668	14.3	120	6.97	260	4.55	80
J210_S15	S3903	A SBR 20140630	A	Jun	2014	340	3440	3210	1094	25.47	10.9	0.147747	126.0306	294.4954	0	0.0054	0	0	0	1.7722	0	0	0	0.7407	0.808	17.9	133	8.4	319	6.36	40
J210_S16	S3904	A SBR 20140929	A	Sept	2014	370	3840	2620	861.9	33.59	15.5	0.113715	77.9994	169.0323	0	0	0	0	0	0.6651	0	0	0	0.3232	0.482	12.4	51.2	8.07	249	5.47	80
J210_S17	S3905	A SBR 20141215	A	Dec	2014	850	1540	1840	618.5	38.38	13.8	0.199134	47.94164	133.3333	0	0.0447	0	0	0	1.3917	0	0	0	0.298	0.607	6.85	31.2	3.57	377	3.48	160
J210_S18	S3906	A SBR 20150119	A	Jan	2015	720	1940	2040	582.1	41.11	16.2	0.227835	49.62296	125.9259	0	0	0	0	0	1.3285	0	0	0	0.3619	0.767	14.8	55.3	6.42	307	4.2	40
J210_S19	S3907	A SBR 20150216	A	Feb	2015	300	4240	3420	993.6	97.75	9.73	0.174764	34.98721	351.4902	0	0	0	0.3683	0	1.757	0	0	0.0469	0.8755	0.874	29.1	194	11.5	164	9.07	200
J210_S20	S3908	A SBR 20150316	A	Mar	2015	880	6560	3060	882	53.51	11.2	0.085518	57.18557	273.2143	0	0	0	0	0.0203	1.6598	0	0	0	0.6034	1.197	23.6	173	12	146	7.5	580
J210_S21	S3909	A SBR 20150427	A	Apr	2015	780	2560	2270	668.5	64.65	26.8	0.192122	35.11214	84.70149	0	0	0	0	0	1.2888	0	0	0	0.258	1.197	13.3	139	5.85	178	3.5	720
J531_S146	SA3762	A SBR 20150629	A	Jun	2015	200	4680	3490	1025	9.957	14.9	0.149145	350.5072	234.2282	0	0.0039	0	0	0	0.7845	0	0	0	0.7407	1.806	13.8	114	6.53	245	4.23	160
J531_S150	SA3766	A SBR 20150928	A	Sept	2015	540	2380	2950	849.6	48.75	24.05	0.227241	60.51282	122.6611	0	0.0129	0	0	0	0.2137	0	0	0	0.0448	1.324	14.175	36.515	10.195	361.065	7.41	180
J531_S154	SA3770	ASBR 20151130	A	Nov	2015	870	5380	2970	525.7	47.01	30	0.101208	63.17805	99	0	0	0	0	0	1.12045	0	0	0	0.205082	1.324	14.5	68	6.66	385	4.55	120
J531_S158	SA3774	A SBR 20160125	A	Jan	2016	480	6020	2000	551.6	63.04	23	0.069214	31.72589	86.95652	0	0	0	0	0	0.647324	0	0	0	0.250924	1.324	14.5	68	6.66	385	4.55	160
J531_S161	SA3777	A SBR 20160222	A	Feb	2016	740	7560	3300	861.7	88.2	9.21	0.087302	37.41497	358.3062	0	0	0	0	0	1.248037	0	0	0	0.48506	1.03	19	167	9.47	203	5.77	160
J531_S164	SA3780	A SBR 20160321	A	Mar	2016	820	5680	5000	934.8	71.9	18	0.190728	69.54103	277.7778	0	0	0	0	0	1.984434	0	0	0.002981	0.423227	1.11	20.6	198	9.42	273	5.94	700
J531_S167	SA3783	A SBR 20160418	A	Apr	2016	900	2100	5900	982.2	64.22	5.31	0.632143	91.87169	1111.111	0.003644	0	0	0	0	2.536876	0	0	0.004939	0.453723	0.463	16	187	6.66	295	1	660
J44_S2	S0626	B SBR 140120	B	Jan	2014	220	2560	5220	1530	69	6.62	0.101953	75.65217	788.5196	0	0.0815	0	0.0272	1.3063	0.4079	0	0	0.0912	1.8478	1.199	31.4	131	11.7	398	33.7	260
J44_S6	S0630	B SBR 140217	B	Feb	2014	260	3280	2870	844	30	5.04	0.14875	95.66667	569.4444	0	0.0965	0	0	0.0643	0.454	0	0	0.0011	0.2539	0.138	24.2	256	9.43	270	13.9	450
J44_S10	S0634	B SBR 140414	B	Apr	2014	600	4380	4880	1389	47	4.2	0.089132	103.8298	1161.905	0	0	0	0	0	0.561	0	0	0	0.6583	0.393	21	151	8.05	262	12.2	360
J210_S22	S3910	B SBR 20140630	B	Jun	2014	400	4240	2500	715	45	12.8	0.04717	55.55556	195.3125	0	0	0	0	0.1163	1.1923	0	0.0015	0.0009	0.1675	0.412	24	215	8.78	208	13.2	80
J210_S23	S3911	B SBR 20140929	B	Sept	2014	970	5060	7590	2384	87.58	10.7	0.075	86.66362	709.3458	0	0.969	0	0.0683	0.7195	0.4029	0	0	0.0353	2.7047	0.72	32.8	267	8.87	219	15.2	100
J210_S24	S3912	B SBR 20141215	B	Dec	2014	850	3520	3570	1003	202	6.04	0.040568	17.67327	591.0596	0	0	0	0	0.1142	1.3044	0	0	0	0.7333	0.418	26.4	79.5	9.57	184	16.8	120
J210_S25	S3913	B SBR 20150119	B	Jan	2015	890	4120	8510	2108	760.8	5.29	0.247864	11.18559	1608.696	0.050332	0	0	0.1097	0.2638	0.8687	0	0	0	3.0663	1.04	27.3	233	8.73	285	13.9	40
J210_S26	S3914	B SBR 20150216	B	Feb	2015	930	6000	4870	1403	52.94	8.99	0.113633	91.99033	541.7133	0	0.5883	0	0.041	2.0841	0.4515	0	0.098	0.0477	1.0983	1.521	21.5	239	9.39	337	14.4	120
J210_S27	S3915	B SBR 20150316	B	Mar	2015	940	7900	7590	2132	74.55	10.6	0.096076	101.8109	716.0377	0	1.553	0.0644	0.2961	1.4452	0.8735	0	0	0.0868	1.7239	0.819	22.7	361	10.3	231	16.4	160
J210_S28	S3916	B SBR 20150427	B	Apr	2015	820	6780	5540	1489	189.7	5.33	0.032684	29.20401	1039.4	0.005907	0	0.0088	0	0	0.3319	0	0	0	1.0747	1.365	25.5	288	9.02	348	12	60
J531_S147	SA3763	B SBR 20150629	B	Jun	2015	910	10000	7390	2042	102.7	11.1	0.02956	71.95716	665.7658	0	0	0	0	0	1.0948	0	0	0	2.1897	1.324	23.8	317	10.7	217	16.8	40
J531_S151	SA3767	B SBR 20150928	B	Sept	2015	900	7740	5980	1718	85.53	10.695	0.030904	69.91699	559.1398	0	0	0	0	0	0.7271	0	0	0	1.1156	1.342	18.845	178.295	10.885	266.21	18.565	160
J531_S155	SA3771	B SBR 20151130	B	Nov	2015	490	3860	6020	1302	83.99	8.82	0.031192	71.67522	682.5397	0	0	0	0.02502	0.610714	0.51265	0	0	0.014909	1.829995	1.191	30.5	318	10.7	291	18.1	60
J531_S159	SA3775	B SBR 20160125	B	Jan	2016	520	3820	4940	1227	281.2	9.29	0.103455	17.56757	531.7546	0	0	0	0	0	1.175599	0	0	0	1.696261	0.79	27.4	149	9.97	460	16.9	60
J531_S162	SA3778	B SBR 20160222	B	Feb	2016	870	5980	5210	1632	83.15	4.97	0.121973	62.65785	1048.29	0.002613	1.235643	0.011445	0.140443	1.184376	0.213158	0	0.244236	0.265433	2.786569	0.874	23.8	296	13.1	233	28.9	100
J531_S165	SA3781	B SBR 20160321	B	Mar	2016	920	7500	16200	2722	184.3	7.67	0.2592	87.90016	2112.125	0.166822	0.679147	0.055326	0.104687	0.145334	0.769928	1.093953	1.52058	0.139235	1.914088	0.897	23.2	357	10.2	187	13.9	60

J531_S168	SA3784	B SBR 20160418	B	Apr	2016	910	9560	11300	1513	82.81	14.3	0.04728	136.4569	790.2098	0	0	0.002447	0.047464	0.102511	1.864773	0	0	0.002719	1.922325	1.11	23.5	443	11.1	279	44.7	140
J44_S3	S0627	C SBR 140120	C	Jan	2014	210	6340	1680	410	6.2	5.41	0.044164	270.9677	310.536	0	0	0	0	0	0.2882	0	0	0	0.5862	2.228	30.6	159	16.2	42.6	14.2	10
J44_S7	S0631	C SBR 140217	C	Feb	2014	270	8840	5250	2086	21	8.1	0.148473	250	648.1481	0.1462	3.295	0.7916	0.2471	0.1312	0.4079	0	0	0.0094	0.344	0.558	30.8	483	10.5	121	9.17	400
J44_S11	S0635	C SBR 140414	C	Apr	2014	270	7960	4920	1284	16	10.5	0.061809	307.5	468.5714	0	0	0	0	0	0.3927	0	0	0.0007	0.5914	0.908	28.2	226	10.7	446	7.01	200
J210_S30	S3918	C SBR 20140630	C	Jun	2014	900	2740	11310	2812	11.35	27.1	0.206387	996.4758	417.3432	0	0	0	0	0.1345	1.9299	0	0	0	3.3088	3.482	28.6	1110	18.1	485	19.3	1000
J210_S31	S3919	C SBR 20140929	C	Sept	2014	300	5200	9180	2473	11.53	18.8	0.058846	796.1839	488.2979	0	0	0	0	0	0.9793	0	0	0.0279	3.2072	0.76	25.7	134	14.7	65.4	21.5	420
J210_S32	S3920	C SBR 20141215	C	Dec	2014	170	11480	90	19.08	28.27	4.76	0.000261	3.183587	18.90756	0	0	0	0	0	0	0	0	0	0.1278	0.254	9.46	69.4	4.74	56.3	4.79	60
J210_S33	S3921	C SBR 20150119	C	Jan	2015	120	4920	300	18.14	7.574	8.85	0.007114	39.60919	33.89831	0	0	0	0	0	0.05813	0	0	0	0.0625	2.183	57.1	126	23.6	91.6	18.7	120
J210_S34	S3922	C SBR 20150216	C	Feb	2015	520	8100	13480	5072	106.4	5.56	0.832099	126.6917	2424.46	0	2.2283	0.3105	0.5502	1.7128	8.0736	1.3041	0.2756	0.4446	0.0309	1.643	31	405	14.5	372	11.9	260
J210_S35	S3923	C SBR 20150316	C	Mar	2015	960	8440	3580	1284	24.82	14.5	0.113112	144.2385	246.8966	0	1.7411	0.3106	0.1341	0.7159	0.7688	0	0	0.0425	0.0202	2.235	45.2	509	17	503	7.46	380
J210_S36	S3924	C SBR 20150427	C	Apr	2015	150	4700	6120	1605	18.85	12.3	0.217021	324.6684	497.561	0	0	0	0.0173	0	3.9896	0	0	0.0011	0.1776	0.918	33.4	281	9.42	66.1	20	180
J531_S148	SA3764	C SBR 20150629	C	Jun	2015	220	4100	3090	1073	2.693	4.67	0.025122	1147.419	661.6702	0	1.8255	0.006	0.006	0	0.0769	0	0	0	0.5639	0.984	30.2	834	6.83	34.3	6.76	160
J531_S152	SA3768	C SBR 20150928	C	Sept	2015	230	4260	1830	662.3	3.911	28.78	0.014319	467.911	63.58582	0	0	0	0.002	0	1.6134	0	0	0	0	2.746	53.67	332.805	24.54	165.56	26.835	280
J531_S156	SA3772	C SBR 20151130	C	Nov	2015	150	5440	180	30.73	6.382	5.39	0.001103	28.20432	33.39518	0	0	0	0	0	0	0	0	0	0.684805	1.44	12	126	3.74	673	4.19	40
J531_S139	SA3755	C SBR 20160125	C	Jan	2016	150	5420	2040	531.1	12.47	4.62	0.031365	163.5926	441.5584	0	0	0	0.003712	0	0.421723	0.001738	0	0	0.300008	0.854	17.6	132	7.73	27.9	5.95	40
J531_S141	SA3757	C SBR 20160222	C	Feb	2016	270	6380	4830	1482	122.8	15.7	0.151411	39.33225	307.6433	0	0	0	0	0	1.182446	0.000376	0	0.013179	1.686133	1.365	30.2	373	17.7	365	11.5	280
J531_S143	SA3759	C SBR 20160321	C	Mar	2016	320	3960	6900	1343	5.752	6.27	0.203283	1199.583	1100.478	0.005022	0	0	0	0.015384	1.69261	0	0	0	1.178946	1.91	25	297	4.7	1340	21.1	400
J531_S145	SA3761	C SBR 20160418	C	Apr	2016	140	4180	16100	2168	58.44	10.3	0.160486	275.4962	1563.107	0	0	0	0	0	1.629942	0	0	0.011953	2.334538	1.446	27.4	80.2	13.4	289	16.2	260
J44_S4	S0628	D SBR 140120	D	Jan	2014	790	7260	15010	4340	28.15	17.6	0.206749	533.2149	852.8409	0	0.0191	0.0034	0.1909	0.5923	0.2204	0	0	0.5121	7.3904	2.279	52	361	17.4	58.5	26.5	80
J44_S8	S0632	D SBR 140217	D	Feb	2014	990	10060	9350	2730	5.735	3.23	0.185885	1630.34	2894.737	0	1.4341	0.0187	0.1031	0.198	0.0994	0.099	0.8072	0.0693	1.2051	0.274	40.2	623	13.6	57.3	19.3	1160
J44_S12	S0636	D SBR 140414	D	Apr	2014	900	7580	5730	1675	12.85	25.3	0.080633	445.9144	226.4822	0.0329	1.5869	0.0366	0.0425	0.012	0.1087	0	0.0215	0.0194	0.7127	0.804	34	559	14.3	62.7	13.7	120
J210_S43	S3931	D SBR 20140630	D	Jun	2014	590	7820	2670	654.1	0.8986	5.68	0.022762	2971.289	470.0704	0	0.0148	0	0.0153	0.0555	0.3362	0	0	0.0198	0.8425	0.526	40.3	495	8.69	55.4	10	140
J210_S44	S3932	D SBR 20140929	D	Sept	2014	700	6740	2190	713.4	8.137	2.36	0.021662	269.141	927.9661	0	0.0747	0	0	0	0.4027	0	0	0.0217	0.6997	0.5	44.2	583	7.64	58.5	13.4	180
J210_S45	S3933	D SBR 20141215	D	Dec	2014	790	8740	13900	3206	20.39	12.9	0.106026	681.7067	1077.519	0	0.5254	0	0.0791	0.4726	0.33	0	0	0.1979	4.8161	2.461	64.8	569	12.6	47.7	18	280
J210_S46	S3934	D SBR 20150119	D	Jan	2015	960	10620	890	166.4	9.441	4.84	0.00838	94.26967	183.8843	0	0	0	0	0	0.3254	0	0	0	0.0807	0.732	25.9	662	10.4	77.8	17	140
J210_S47	S3935	D SBR 20150216	D	Feb	2015	960	8180	14300	3473	5.18	4.74	0.233089	2760.618	3016.878	0	1.2392	0	0.1348	1.5477	0.2697	0	0.3008	0.5019	4.4135	0.874	50.4	490	13	57.8	20.1	300
J210_S48	S3936	D SBR 20150316	D	Mar	2015	910	7900	9710	2814	11.49	14.8	0.245823	845.0827	656.0811	0.1567	1.5215	0.0408	0.1127	0.7269	1.7482	0	0.0914	0.218	2.6786	1.701	46.3	542	14.2	51.9	14.8	160
J210_S49	S3937	D SBR 20150427	D	Apr	2015	760	4900	5520	1638	18.7	6.66	0.120163	295.1872	828.8288	0.4419	2.1697	0	0.1128	0.0666	2.5935	0	0	0.0848	0.0682	1.202	40.9	717	9.75	48.6	23.4	200
J531_S149	SA3765	D SBR 20150629	D	Jun	2015	970	7360	7580	2237	14.16	8.39	0.068659	535.3107	903.4565	0.1901	1.5383	0.0115	0.0905	0.1924	0.1171	0	0	0.165	2.7815	3.192	37.7	686	11	52.9	15	160
J531_S153	SA3769	D SBR 20150928	D	Sept	2015	960	3420	5960	1745	24.23	6.555	0.05809	245.9761	909.2296	0	0.7464	0	0.0404	0.1071	0.4309	0	0	0.0372	2.1058	1.643	38.265	511.4	10.46	56.755	14.96	520
J531_S157	SA3773	D SBR 20151130	D	Nov	2015	960	9660	2350	541.9	6.305	6.02	0.016218	372.7201	390.3654	0	0	0	0	0	0.590965	0	0	0	0.953032	0.999	59.8	486	12	82.8	13.2	180
J531_S160	SA3776	D SBR 20160125	D	Jan	2016	910	5940	7360	1860	17.5	43.2	0.123906	420.5714	170.3704	0	0.48318	0.011868	0.074794	2.696445	0.301288	0	0.234932	0.30968	2.008232	1.046	57.5	582	15.7	82.2	21.6	520
J531_S163	SA3779	D SBR 20160222	D	Feb	2016	940	6300	9610	2885	14.62	5.1	0.254233	657.3187	1884.314	0	1.061026	0.002006	0.082439	1.587682	0.332078	0.551658	1.895332	0.263377	2.399858	0.88	45.3	549	13.4	55.6	17.6	360
J531_S166	SA3782	D SBR 20160321	D	Mar	2016	940	6660	15300	2375	3.82	6.71	0.306306	4005.236	2280.179	0.022428	0.515027	0.016627	0	0.290034	1.163234	0	0.005906	0.054727	3.052167	1.26	62.6	531	14.5	52.8	14.2	140
J531_S169	SA3785	D SBR 20160418	D	Apr	2016	670	4340	30000	3582	22.42	14.8	0.691244	1338.091	2027.027	0.058816	0.277528	0.040278	0.040723	0.494317	1.058747	0	0	0.304599	5.141416	2.334	40.8	581	14.7	59.6	19.7	160

SeqID	Sample ID	Description	Plant	Month	Raey	SV60	MLSS	CODf	NPOC	TN	P	F:M	COD:N	COD:P	Citric	Tartaric	Malic	Succinic	Lactic	Acetic	Glucose	Fructose	Glycerol	Ethanol	Phenols	Ca	K	Mg	Na	S	EffSS
J210_S30	S3918	C SBR 20140630	C	Jun	4	900	0	10	2	5	27.1	6	996	417	0.00 0	0.000	0.00 0	0.000	0.13 5	1.93 0	0.000	0.000	0.000	3.309	3.482	28.6 0	1110.00	18.1 0	485.00	19.3 0	100 0
J210_S31	S3919	C SBR 20140929	C	Sept	4	300	0	0	3	3	18.8	9	796	488	0.00 0	0.000	0.00 0	0.000	0.00 0	0.97 9	0.000	0.000	0.028	3.207	0.760	25.7 0	134.00	14.7 0	65.40	21.5 0	420
J210_S32	S3920	C SBR 20141215	C	Dec	4	170	80	90	19.0 8	28.2 7	4.76	0.00 0	3	19	0.00 0	0.000	0.00 0	0.000	0.00 0	0.00 0	0.000	0.000	0.000	0.128	0.254	9.46	69.40	4.74	56.30	4.79	60
J531_S123	SA373	C SBR 20140120	C	Jan	4	210	634 0	168 0	410	6.2	5.41	0.04 4	271	311	0.00 0	0.000	0.00 0	0.000	0.00 0	0.28 8	0.000	0.000	0.000	0.586	2.228	30.6 0	159.00	16.2 0	42.60	14.2 0	10
J531_S124	SA374	C SBR 20140203	C	Feb	4	260	720 0	107 10	381 8	20	10.3 0	0.24 8	536	1040	0.00 0	2.250	0.21 8	0.086	0.05 1	3.52 1	0.000	0.000	0.019	0.782	1.324	32.2 0	836.00	14.5 0	293.0 0	18.9 0	10
J531_S125	SA374	C SBR 20140217	C	Feb	4	270	884 0	525 0	208 6	21	8.1	0.14 8	250	648	0.14 6	3.295	0.79 2	0.247	0.13 1	0.40 8	0.000	0.000	0.009	0.344	0.558	30.8 0	483.00	10.5 0	121.0 0	9.17	400
J531_S126	SA374	C SBR 20140303	C	Mar	4	790	970 0	207 0	828. 3	35.1 4	13.9 0	0.06 4	59	149	0.00 0	1.241	0.00 0	0.018	0.05 5	0.22 6	0.000	0.000	0.000	0.138	1.620	30.2 0	553.00	10.2 0	317.0 0	7.24	320
J531_S127	SA374	C SBR 20140317	C	Mar	4	940	936 0	690	197 4	6.89	24.4 0	0.01 5	100	28	0.00 0	0.131	0.01 5	0.019	0.01 7	0.03 2	0.000	0.010	0.000	0.000	1.828	32.5 0	949.00	16.6 0	149.0 0	12.1 0	640
J531_S128	SA374	C SBR 20140331	C	Mar	4	980	988 0	470	211. 1	7.9	8.51	0.01 3	59	55	0.00 0	0.029	0.00 0	0.000	0.00 0	0.04 4	0.000	0.000	0.001	0.000	0.870	17.2 0	399.00	275.0 5.70	0	7.82	160
J531_S129	SA374	C SBR 20140414	C	Apr	4	270	796 0	492 0	128 4	16	10.5	0.06 2	308	469	0.00 0	0.000	0.00 0	0.000	0.00 0	0.39 3	0.000	0.000	0.001	0.591	0.908	28.2 0	226.00	10.7 0	446.0 0	7.01	200
J531_S130	SA374	C SBR 20140428	C	Apr	4	170	528 0	347 0	907	4.7	13.0 0	0.06 6	738	267	0.00 0	0.000	0.00 0	0.000	0.00 0	0.54 2	0.000	0.000	0.000	0.407	1.592	26.9 0	1000.0 0	7.57	122.0 0	11.7 0	100
J531_S131	SA374	C SBR 20150119	C	Jan	5	120	492 0	300	18.1 4	7.57 4	8.85	0.00 7	40	34	0.00 0	0.000	0.00 0	0.000	0.00 0	0.05 8	0.000	0.000	0.000	0.063	2.183	57.1 0	126.00	23.6 0	91.60	18.7 0	120
J531_S132	SA374	C SBR 20150202	C	Feb	5	130	484 0	280	42	6	5.64	0.00 6	47	50	0.00 0	0.000	0.00 0	0.000	0.00 0	0.00 0	0.000	0.000	0.000	0.069	1.498	32.4 0	136.00	12.1 0	53.60	7.64	40
J531_S133	SA375	C SBR 20150216	C	Feb	5	520	810 0	134 80	507 2	106. 4	5.56	0.83 2	127	2424	0.00 0	2.228	0.31 1	0.550	1.71 3	8.07 4	1.304	0.276	0.445	0.031	1.643	31.0 0	405.00	14.5 0	372.0 0	11.9 0	260
J531_S134	SA375	C SBR 20150302	C	Mar	5	0	201 0	100 0	808 0	127 00	491 0	0.26 57	7.7	2	0.00 224	1643	1.15 0	2.915	0.73 8	9.09 5	0.565	0.312	0.314	0.408	0.244	29.7 0	537.00	12.8 0	468.0 0	9.33	220
J531_S135	SA375	C SBR 20150316	C	Mar	5	960	844 0	358 0	128 4	24.8 2	14.5	0.11 3	144	247	0.00 0	1.741	0.31 1	0.134	0.71 6	0.76 9	0.000	0.000	0.043	0.020	2.235	45.2 0	509.00	17.0 0	503.0 0	7.46	380
J531_S136	SA375	C SBR 20150330	C	Mar	5	950	660 0	458 0	298 6	20	18	0.13 9	227	252	0.00 0	0.000	0.00 0	0.000	0.00 0	0.64 0	0.000	0.000	0.000	0.047	2.020	24.0 0	699.00	12.1 0	534.0 0	10.1 0	180
J531_S137	SA375	C SBR 20150413	C	Apr	5	300	646 0	963 0	279 0	13.8	6.91	0.29 8	698	1394	0.00 0	0.000	0.00 0	0.000	0.00 0	4.37 8	0.000	0.000	0.007	2.063	0.961	47.5 0	441.00	10.9 0	73.00	7.78	280
J531_S138	SA375	C SBR 20150427	C	Apr	201 5	150	470 0	612 0	160 5	18.8 5	12.3	0.21 7	325	498	0.00 0	0.000	0.00 0	0.017	0.00 0	3.99 0	0.000	0.000	0.001	0.178	0.918	33.4 0	281.00	9.42	66.10	20.0 0	180
J531_S139	SA375	C SBR 20160125	C	Jan	6	150	542 0	204 0	531. 1	12.4 7	4.62	0.03 1	164	442	0.00 0	0.000	0.00 0	0.004	0.00 0	0.42 2	0.002	0.000	0.000	0.300	0.854	17.6 0	132.00	7.73	27.90	5.95	40
J531_S140	SA375	C SBR 20160208	C	Feb	6	160	574 0	941 0	372 6	11	12	0.21 9	895	771	0.00 0	2.512	0.00 0	0.163	1.01 6	2.81 9	0.546	2.149	0.200	0.068	1.417	35.9 0	1400. 00	13.2 0	151.0 0	12.5 0	100
J531_S141	SA375	C SBR 20160222	C	Feb	6	270	638 0	483 0	148 2	122. 8	15.7	0.15 1	39	308	0.00 0	0.000	0.00 0	0.000	0.00 0	1.18 2	0.000	0.000	0.013	1.686	1.365	30.2 0	373.00	17.7 0	365.0 0	11.5 0	280
J531_S142	SA375	C SBR 20160307	C	Mar	6	690	612 0	220 0	608	3	12	0.55 6	773	186	0.00 0	0.349	0.04 9	0.073	0.30 7	1.21 7	0.000	0.000	0.002	0.150	1.585	39.8 0	298.0 0	21.2 0	185.0 0	5.09	340
J531_S143	SA375	C SBR 20160321	C	Mar	6	320	396 0	690 0	134 3	5.75 2	6.27	0.20 3	1200	1100	0.00 5	0.000	0.00 0	0.000	0.01 5	1.69 3	0.000	0.000	0.000	1.179	1.910	25.0 0	297.00	1340. 4.70	00	21.1 0	400
J531_S144	SA376	C SBR 20160404	C	Apr	6	360	400 0	989 0	283 2	26	30	0.24 7	382	334	0.00 0	0.000	0.00 0	0.000	0.00 0	0.82 2	0.000	0.000	0.000	3.158	2.328	50.8 0	394.00	14.6 0	96.00	23.5 0	760
J531_S145	SA376	C SBR 20160418	C	Apr	6	140	418 0	161 00	216 8	58.4 4	10.3	0.16 0	275	1563	0.00 0	0.000	0.00 0	0.000	0.00 0	1.63 0	0.000	0.000	0.012	2.335	1.446	27.4 0	80.20	13.4 0	289.0 0	16.2 0	260
J531_S146	SA376	C SBR 20150629	C	Jun	5	220	410 0	309 0	107 3	2.69 3	4.67	0.02 5	1147	662	0.00 0	1.826	0.00 6	0.006	0.00 0	0.07 7	0.000	0.000	0.000	0.564	0.984	30.2 0	834.00	6.83	34.30	6.76	160
J531_S147	SA376	C SBR 20150928	C	Sept	5	230	426 0	183 0	662. 3	3.91 1	28.7 8	0.01 4	468	64	0.00 0	0.000	0.00 0	0.002	0.00 0	1.61 3	0.000	0.000	0.000	0.000	2.746	53.6 7	332.81	24.5 4	165.5 6	26.8 4	280
J531_S148	SA377	C SBR 20151130	C	Nov	5	150	544 0	180	30.7 3	6.38 2	5.39	0.00 1	28	33	0.00 0	0.000	0.00 0	0.000	0.00 0	0.00 0	0.000	0.000	0.000	0.685	1.440	12.0 0	126.00	673.0 3.74	0	4.19	40

SeqID	SampleID	Description	Month	Year	pH	EC	temp	SS	In_COD	In_COD (f)	Ex_COD	Ex_CODf	NH_4-N	NO_x-N	NO_2-N	Cl^-	NO_3^-	$SO_4^{=}$	Ca	K	Mg	Na	S	P
J44_S15	S0639	A CAL 140203	Feb	2014	5.2	1731	30.1	980	7080	6450	2600	1880	0.06005	0.01	0.05624	21	0.16	16	17.6	137	7.97	211	6.74	4.96
J44_S17	S0641	A CAL 140414	April	2014	4.6	1570	22.4	1140	5670	5030	4050	2990	0.07393	0.1114	0.06581	21	0.14	12	14.3	120	6.97	260	4.55	12.2
J210_S9	S3897	A CAL 20140512	May	2014	4.6	1456	19.8	1140	4250	4230	4400	2520	0.3483	0.096	0.04203	26.995	0.64937	19.867	17.3	145	7.7	190	7.41	7.93
J210_S10	S3898	A CAL 20140825	August	2014	5.9	1707	15.3	1220	3640	3450	3100	2850	0.1228	0.1032	0.02852	36.124	0.118	13.595	17.1	92.8	9.31	320	5.84	18.1
J210_S11	S3899	A CAL 20150119	Jan	2015	5	1448	25.7	260	2750	2440	2240	2040	0.06613	0.07049	0.0166	23.45	0.05	13.56	14.8	55.3	6.42	307	4.2	16.2
J210_S12	S3900	A CAL 20150216	Feb	2015	4.9	1994	28	5660	8880	8360	7370	3420	0.07794	0.009399	0.005	29.441	0.072	21.625	29.1	194	11.5	164	9.07	9.73
J210_S13	S3901	A CAL 20150316	March	2015	4.6	1717	24.6	1880	8040	7400	4460	3060	0.1224	0.08255	0.07019	32.699	0.05	20.049	23.6	173	12	146	7.5	11.2
J210_S14	S3902	A CAL 20150427	April	2015	4.7	1392	19.1	520	4210	3880	2720	2270	5.941	0.1326	0.0779	15.235	0.05	8.356	13.3	139	5.85	178	3.5	26.8
J44_S16	S0640	D CAL 140203	Feb	2014	7.1	3990	28.2	34720	9660	8610	2990	100	0.06023	0.1139	0.05595	70	1.1	50	57.5	431	15.9	64.2	23.7	3.23
J44_S18	S0642	D CAL 140414	April	2014	4.6	2390	19.2	360	6400	5730	6660	6250	0.2806	0.2249	0.1063	76	0.52	36	34	559	14.3	62.7	13.7	25.3
J210_S37	S3925	D CAL 20140512	May	2014	4.7	1970	18	260	3560	2540	3980	3950	0.3157	0.03069	0.01892	74.045	0.73196	34.7	39.9	609	13.3	66.6	13.5	6.42
J210_S38	S3926	D CAL 20140825	August	2014	6.1	2210	13.6	500	5360	5340	4360	3940	2.057	0.6526	0.1965	95.18	1.242	39.457	49.1	550	12.1	76.1	15.1	24.5
J210_S39	S3927	D CAL 20150119	Jan	2015	5.1	2430	24.2	240	980	890	5600	5180	3.673	0.09613	0.01656	106.61	0.29	41.6	25.9	662	10.4	77.8	17	4.84
J210_S40	S3928	D CAL 20150216	Feb	2015	5.5	1747	26.2	22260	15500	14300	28500	4000	0.09027	0.007497	0.005	78.175	0.085	46.168	50.4	490	13	57.8	20.1	4.74
J210_S41	S3929	D CAL 20150316	March	2015	4.1	1830	21.9	540	10480	9710	7190	6680	0.1741	0.2694	0.1844	71.18	0.058	41.06	46.3	542	14.2	51.9	14.8	14.8
J210_S42	S3930	D CAL 20150427	April	2015	5.1	1940	15.5	29020	5560	5520	37200	4400	1.243	0.1882	0.0748	59.087	0.569	19.202	40.9	717	9.75	48.6	23.4	6.66

In_NPOC	In_TN	In_Phenols	In_Citric	In_Tartaric	In_Malic	In_Succinic	In_Lactic	In_Acetic	In_Glucose	In_Fructose	In_Glycerol	In_Ethanol	Ex_NPOC	Ex_TN	Ex_Citric	Ex_Tartaric	Ex_Malic	Ex_Succinic	Ex_Lactic	Ex_Acetic	Ex_Glucose	Ex_Fructose	Ex_Glycerol	Ex_Ethanol
1456	4.282	482.000	0.009	0.407	0.012	0.021	0.119	0.117	0.000	0.101	0.027	0.580	654.000	46.000	0.000	0.000	0.000	0.000	0.000	1.152	0.000	0.000	0.000	0.794
1455	3.951	668.000	0.005	0.812	0.000	0.052	0.069	0.182	0.000	0.027	0.012	0.661	1011.000	34.000	0.000	0.000	0.000	0.000	0.000	1.984	0.000	0.000	0.000	0.423
947	7.555	532.000	0.000	0.464	0.008	0.074	0.019	0.182	0.000	0.000	0.001	0.524	811.900	38.550	0.000	0.000	0.000	0.000	0.000	2.048	0.000	0.002	0.000	0.373
983.4	0.8986	416.000	0.000	0.003	0.000	0.020	0.651	0.338	0.000	0.000	0.013	0.908	831.000	21.000	0.000	0.000	0.000	0.000	0.000	0.577	0.000	0.000	0.000	0.256
603.4	4.325	767.000	0.000	0.081	0.000	0.000	0.414	0.382	0.000	0.000	0.000	0.660	582.100	41.110	0.000	0.000	0.000	0.000	0.000	1.329	0.000	0.000	0.000	0.362
2326	2.64	874.000	0.000	0.781	0.000	0.075	1.235	0.937	0.000	0.253	0.298	2.616	993.600	97.750	0.000	0.000	0.000	0.368	0.000	1.757	0.000	0.000	0.047	0.876
2098	6.174	1197.000	0.000	0.893	0.034	0.128	0.595	2.154	0.000	0.000	0.069	1.454	882.000	53.510	0.000	0.000	0.000	0.000	0.020	1.660	0.000	0.000	0.000	0.603
958.4	20.33	1197.000	0.000	0.000	0.000	0.004	0.000	0.287	0.000	0.000	0.000	1.005	668.500	64.650	0.000	0.000	0.000	0.000	0.000	1.289	0.000	0.000	0.000	0.258
2556	4.666	430.000	0.386	1.391	0.024	0.318	0.264	0.068	1.163	2.874	0.067	1.032	72.000	343.000	0.000	0.000	0.000	0.000	0.000	0.501	0.000	0.000	0.000	0.318
1675	12.85	804.000	0.033	1.587	0.037	0.043	0.012	0.109	0.000	0.022	0.019	0.713	1855.000	7.000	0.000	0.000	0.000	0.000	0.000	3.658	0.000	0.000	0.000	1.991
710.5	8.277	332.000	0.000	0.379	0.000	0.031	0.025	0.322	0.028	0.034	0.000	0.341	1108.000	5.700	0.000	0.000	0.000	0.000	0.000	0.377	0.000	0.000	0.000	0.388
1522	13.06	1005.000	0.117	0.980	0.000	0.050	0.095	0.182	0.000	0.000	0.105	1.588	1112.000	20.590	0.045	0.000	0.000	0.000	0.000	0.584	0.000	0.000	0.000	0.419
166.4	9.441	732.000	0.000	0.000	0.000	0.000	0.000	0.325	0.000	0.000	0.000	0.081	1369.000	55.470	0.000	0.090	0.000	0.000	0.000	1.013	0.000	0.000	0.000	1.036
3473	5.18	874.000	0.000	1.239	0.000	0.135	1.548	0.270	0.000	0.301	0.502	4.414	3473.000	5.180	0.000	0.046	0.000	0.000	0.000	0.512	0.000	0.000	0.000	0.373
2814	11.49	1701.000	0.157	1.522	0.041	0.113	0.727	1.748	0.000	0.091	0.218	2.679	1913.000	3.286	0.000	0.000	0.000	0.000	0.000	2.253	0.000	0.000	0.001	2.371
1638	18.7	1202.000	0.442	2.170	0.000	0.113	0.067	2.594	0.000	0.000	0.085	0.068	1053.000	80.400	0.000	0.067	0.000	0.000	0.063	0.598	0.000	0.000	0.754	0.156