THE UNIVERSITY of ADELAIDE

Australian Government

Australian Grape and Wine Authority

Travel to 8th Specialized Conference on Sustainable Viticulture, Winery Wastes & Agri-industrial Wastewater Management, Chile, November 2017

8 th IWA Specialized Conference on Sustainable Viticulture, Winery Wastes and Agri-industrial Wastewater Management

November 12-15th 2017 / Viña del Mar

FINAL REPORT to

AUSTRALIAN GRAPE AND WINE AUTHORITY

Project Number: **AGT-1619**

Principal Investigator: **PAUL GRBIN**

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AGT 1619 Project Title: Travel to 8th Specialized Conference on Sustainable Viticulture, Winery Wastes & Agri-industrial Wastewater Management, Chile, November 2017

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Executive Summary:

- Attended The 8th Specialized Conference on Sustainable Viticulture, Winery Wastes & Agri-industrial Wastewater Management, 12-15 November, 2017, Viña del Mar, Chile

- Invited to present plenary lecture titled "An overview of the Australian wine industry: insights into history, regions, varieties and wastewater treatment". This lecture included a study undertaken as part of Wine Australia project UA1301

- Co-authored two other oral presentations given (by Dr Kathryn Eales, Cristobal Onetto) presenting work from Wine Australia projects UA1301 and GWR Ph 1316. Cristobal Onetto's presentation wins best talk of the conference.

- Invited to be a session Chair and elected to the Scientific Committee for 9th Specialized Conference in 2019, University of Mons, Belgium

- Undertook a range of technical visits to vineyards, wineries and a research facilities.

- Invited to present at Viña Concha y Toro Centro de Investigación e Innovación. This presentation highlighted research from Wine Australia projects UA1302, GWR Ph 1308 and GWR Ph 0901.

- Established various linkages with researchers in Chile and South Africa, and exploring opportunities for new PhD projects and visiting researchers.

Key activities and outcomes:

The 8th Specialized Conference on Sustainable Viticulture, Winery Wastes & Agri-industrial Wastewater Management is a special interest group under the auspices of the International Water Association (IWA). The IWA is the peak water association and has more than 7000 members and works across water challenges throughout the world. Attendance at this conference was beneficial in gaining an understanding into international trends in sustainability and treatment issues in the wine industry. A variety of topics where addressed (Appendix 1), including establishment of regional and national approaches to sustainability via codes of practice, and guidelines for development of sustainable wineries. Alternative approaches to the treatment of winery wastes was presented by several authors. Of particular focus was the use of anaerobic treatment, generating biogas for potential energy production, trickle filter technology and mathematical modelling to improve process control of anaerobic treatment systems.

Wine Australia funded research was presented in two sessions. Dr Kathryn Eales (formerly University of Adelaide, (UoA)) presented a paper titled "*Tackling filamentous bulking in winery wastewater treatment plants; a full scale experiment*" (see Appendix 2), this was work undertaken as part of project UA1301 Developing a fundamental understanding of the microbiological treatment of winery wastewater. Cristobal Onetto (UoA PhD student, GWR Ph 1316) presented a paper titled "*Excessive proliferation of GAOs in winery wastewater: causes, consequence and potential solutions*" (see Appendix 3). This paper was judged (by the Scientific Committee) as the best presentation of the conference. In another session Assoc Prof Paul Grbin, was invited to present a plenary lecture. This presentation was titled "*An overview of the Australian wine industry: insights into history, regions, varieties and wastewater treatment*". This lecture included a study undertaken as part of UA1301 on the effect of activated carbon and other common winemaking processing aids in the waste stream on wastewater treatment plants (Appendix 4). Paul Grbin was also invited to participate as a session chair and was elected to the Scientific Committee for the 9th Specialized Conference. This conference will take place in 2019 at the University of Mons, Belgium. New themes will be included, such as brewery and distillery waste treatment. Opportunity for a potential PhD project was discussed with Prof Rolando Chamy, Pontificia Universidad Católica de Valparaíso, with a potential student identifed. Futher, the possiblity of a visiting researher from ARC Infruitec-Nietvoorbij, Stellenbosch, South Africa was discussed with Dr Reckson Mulidzi (Research Team Manager). Contact has been made and arrangements discussed.

Figure 1: Cristobal Onetto wins best presentation, with Prof Rolando Chamy., Conference

The conference concluded with technical visits to two wineries (including vineyards) in the Casablanca Valley region in Chile. The first visit was to Viña Veramonte, here the organic practices in the vineyard where discussed, by the Vineyard Manager. This included detailed on preparation of mulch and compost. All of which occurred on site. This was followed by a tour of the winery facilities, conducted by a member of the winemaking team. The technical visits concluded with a tour of Matetic Vineyards, in the San Antonio appellation. This family owned company is certified organic and biodynamic. The winery is set up for gravity flow production. Matetic flagship wine is cool climate Syrah. The tour of the production facilities was followed by a structured tasting including Sauvignon Blanc and a red blend (Cab Franc, Malbec, Syrah and Petit Verdot).

Figure 2: Casablanca Valley, Viña Veramonte

Further technical visit where undertaken in the Colchagua Valley. A site visit was carried out at large export focused company, Viña Luis Felipe Edwards. With production facilities for more than 25,0000 tonnes. Viña Luis Felipe Edwards exports to more than 100 countries. The company owns more than 2000 Ha in multiple regions in Chile. Luis Nicolas Bizzarri- Director of Winemaking (UoA Wine Alumnus) hosted the visit. Discussion focused on winery wastewater treatment, and contact was established with Australian based consultants to assist with upgrading the wastewater treatment system. A visit to vineyards, winery and wastewater treatment facility was also undertaken at Los Vascos (Domaine's Baron de Rothschild). The visit was hosted by Marcelo Gallardo (Chief Winemaker) and Sebastian Cruz (Production Manager). Finally, an extensive tour and tasting was undertaken at Viu Manent. The tasting included production samples and packaged wines. Discussion occurred with Patricio Celedōn (Chief Winemaker) regarding a range of issues including smoke taint,

Figure 3: Winery wastewater treatment facilities at Los Vascos, Colchagua Valley

recently highlighted in Chile, after extensive forest fires early in 2017.

A presentation was also given at the Viña Concha y Toro Centro de Investigación e Innovación, Pencahuie, (Maule region). This invited presentation titled "*Fit-for-Purpose Bacteria via Directed Evolution*". Wine Australia funded research from UA1302, GWR Ph 1308 and GWR Ph 0901 was presented to an audience of researchers and winemaking staff from Concha y Toro. An in-depth discussion of Concha y Toro research agenda (with Gerard Casaubon, Director; Álaro González Assistant Manager Research & Development and Sebastián Vargas, Project Engineer) was undertaken and a tour of the research laboratories and winery. Potential for future collaboration was discussed and continue.

Figure 4: Viña Concha y Toro Centro de Investigación e Innovación Research winery

Outputs as outlined in original application

Originally proposed with 1 oral presentation and 2 posters to describe the Wine Australia funded project UA1301/GWR Ph 1316. Instead three oral presentations where given at the 8th Specialized Conference on Sustainable Viticulture, Winery Wastes & Agri-industrial Wastewater Management, including an invited plenary lecture. Further, one presentation (Cristobal Onetto) was awarded the best talk overall. A further oral presentation was also made at Viña Concha y Toro Centro de Investigación e Innovación. This presentation highlighted Wine Australia funded research (UA1302, GWR Ph 1308 and GWR Ph 0901). Linkages has been established with Pontificia Universidad Católica de Valparaíso, ARC Infruitec-Nietvoorbij, Stellenbosch and Viña Concha y Toro Centro de Investigación e Innovación, all with potential to develop into new research projects.

Appendix I

13 November, 2017 (Monday)

13 November, 2017 (Monday)

Room 1

Time	Country	Programme
8:55-9:00		**Welcome**
9:00-9:45		**PLENARY** **JÖEL ROCHARD** **L'Institut Français de la Vigne et du Vin** **Paris, France**
9:45-11:15		**"CHALLENGES OF VITICULTURE AND WATER MANAGEMENT", Chair: German Buitrón;** **Expositors: Juan Sutil (Corporación Reguemos Chile, Chile)** **Jöel Rochard (L'Institut Français de la Vigne et du Vin, France) and Rolando Chamy (Núcleo Biotecnología Curauma PUCV, Chile)**
11:15-11:30		**Coffee & Break**
Time	**Country**	**Block 1, Chair: Rolando Chamy**
11:30-11:50	Australia	The Effect Of Activated Carbon And Other Winemaking Processing Aids In The Waste Stream On Wastewater Treatment Plants, **Author:** Cristobal Onetto, Kathryn L Eales and **Paul R Grbin**
11:50-12:10	Australia	Tackling Filamentous Bulking In Winery Wastewater Treatment Plants; A Full Scale Experience, **Author:** Cristobal A Onetto, Kathryn L Eales and **Paul R Grbin**
12:10-12:30	Australia	Excessive Proliferation Of GAOs In Winery Wastewater:Causes, Consequences And Potential Solutions. **Author:** Cristobal A Onetto, Kathryn L Eales and **Paul R Grbin**
12:30-12:50	Mexico	Evaluation Of The Biochemical Methane Potential From Winery Vinasses, **Author:** Felipe Ojeda**, Germán Buitrón**
12:50-13:10	Mexico	Treating Craft Brewing Wastewater: Up-flow Anaerobic Sludge Blanket -- Hybrid Membrane Bioreactor. **Author: M. F. Ortiz-Sánchez,** G. Cuevas-Rodríguez
13:10-13:30	Portugal	Biological Treatment Of Wine--making Effluent Of SOGRAPE, Portugal, Author: **C-Y. Cheng** and F. Braga
13:30-15:00		**Lunch**
15:00-16:30		**"CLIMATE CHANGE AND CARBON FOOTPRINT"** **Chair: Rolando Chamy; Expositors: Jörgen Villy Fenhann (Universidad Técnica de Dinamarca, Dinamarca)** **Sara González (Universidad de Santiago de Compostela, España)** **Elba Vivanco (Núcleo Biotecnología Curauma PUCV, Chile)**
16:30-16:45		**Coffee & Break**
Time	**Country**	**Block 2, Chair: Paul R Grbin**
16:45-17:05	Chile	Reducing The Carbon Footprint Of Wine Fermentation. The Role Of Modeling In Energy Management Optimization. **Author: Andrés Donoso-Bravo,** Constanza Sadino-Riquelme, Cristian Paris, David Jeison
17:05-17:25	France	Wine Sustainable Development: Inventory Of Fixtures International Strategic And Regulatory, **Author:** Pierot Isabelle and **Rochard Joël.**
17:25-17:45	Spain	Bio Refinery Of Cabernet Grape Pomace For Extraction Of Polyphenols: Process Intensification, Optimisation And Study Of, **Author**: **Arunima Nayak**, Brij Bhushan
17:45-18:05	South Africa	Effect Of Irrigation With Winery Wastewater Containing High Levels Of Potassium And Low Sodium On Poorly Drained Soils. **Author: A.R. Mulidzi***, C.E. Clarke, P.A. Myburgh1
18:05-18:25	Chile	Importancia del desarrollo del conocimiento tecnologico en el mercado laboral Chileno. **Author**: **Drago Radovic**
18:25-18:45	Portugal	WineWATERFootprint Project-Water Footprint Assessment In Portuguese Wine Chain, **Margarida Correia de Oliveira**

14 November, 2017 (Tuesday)

14 November, 2017 (Tuesday)

Room 1

Time		Programme
8:55-9:00		**Welcome**
9:00-9:45		**School of Agriculture, Food and Wine from University of Adelaide, Australia**
9:45-11:15		**"SUSTAINABLE ENOLOGY"** **Chair: Sara González;** **Expositors: Eduardo Gratacós (Centro Regional de Innovación Hortofrutícola PUCV, Chile)** **Alain Vande Wouwer (Université de Mons, Belgium),** **ASCC agent**
11:15-11:30		**Coffee & Break**
Time	**Country**	**Block 1, Chair: Juan Lema**
11:30-11:50	France	Ecological Engineering Applied To The Liquid Waste Processing Of Cellar: Example Of The Blueset Device Of Buzet, **Author: Joel Rochard,** Julie Nehmtow, Runying Wang and Arnaud Alary
11:50-12:10	France	Processes For Wineries Wastewater Treatment Adapted Organic And Sustainable Wines: Prospect And Inventory Of Fixtures, **Author: J. Rochard**
12:10-12:30	Mexico	Two Stage Biohydrogen And Methane Production From Wine Vinasses: Effect Of Substrate Concentration. **Author:** JB. A. Albarrán-Contreras, J. Carrillo-Reyes and **G. Buitrón**
12:30-12:50	Canada	Electricity generation from winery wastewater using an air cathode microbial fuel cell: effect of buffering. **Author: Tianlong Liu,** Anupama Pillai, and Deborah J. Roberts
12:50-13:10	Spain	Porous Carbons From Exhausted Grape Pomace For Recovery Of Polyphenols From Wine Wastewaters: A Sustainable Approach, **Author: Arunima Nayak**, Brij Bhushan
13:10-13:30	Portugal	Challenges For Modern Wine Production In Dry Areas: Wastewater As A Viable Resource?, **Author: M. Oliveira.** J. M.Costa. R. Fragoso, R. Egipto, C. M. Lopes, E. Duarte
13:30-15:00		**Lunch**
15:00-16:30		**"INNOVATION AND VITICULTURE"** **Chair: Joël Rochard;** **Expositors: Franco Cecchi (Università degli Studi di Verona, Italia)** **Juan Lema (Universidad Santiago de Compostela, España)** **Lorena Wilson (Pontificia Universidad Católica de Valparaíso, Chile)**
16:30-16:45		**Coffee & Break**
Time	**Country**	**Block 2, Chair: Andres Donoso**
16:45-17:05	Mexico	A Low-cost Automated Alkalinity Measurement Device For On-line Asessement Of Winery Vinasses Anaerobic Treatment, **Author: A. Vargas***, A.N. López-Moreno*, D.A. Patlán Zarazúa* and J. Carrillo-Reyes*
17:05-17:25	Chile	Model-Based Control Analysis Of Anaerobic Digestion Of Industrial Winery Wastewater. **Author: Gustavo Vargas-Morales**, Gonzalo Ruíz-Filippi, Santiago García Gen, Rolando Chamy
17:25-17:45	Belgium-Chile	Derivation Of A Reduced-order Model For Anaerobic Digestion Control, **Author:** G. Giovannini, M. Sbarciog, R. Chamy, **A. Vande Wouwer**
17:45-18:05	Belgium	Estimation And Control Of Winery Wastewater Treatment By Anaerobic Digestion, Author: M. Sbarciog, G. Giovannini and **A. Vande Wouwer**
18:05-18:30		**Closing Ceremony**

Appendix 2

Tackling filamentous bulking in winery wastewater treatment plants; a full scale experience

Cristobal A Onetto *, Kathryn L Eales * and Paul R Grbin *

* Department of Wine & Food Science, University of Adelaide, Waite Campus, South Australia

Keywords: Full scale wastewater treatment plant, raw feeding, Thiothrix, Type 021N.

Abstract

A winery wastewater treatment plant (WWTP) in NSW Australia has long suffered from bulking problems associated with the proliferation of filamentous *Thiothrix* spp.. The WWTP consists of a large covered anaerobic lagoon (CAL) followed by a sequencing batch reactor (SBR). The CAL functions as both an anaerobic digester and surge lagoon for the irregular flow of wastewater generated from the production of seasonal products. Chemical analyses of the raw influent was composed of a complex mixture of organic acids, phenols and alcohol. The CAL effluent was characterised by high acetic acid and phenolic concentrations. An attempt was made to manipulate the SBR microbial community to improve settling by direct feeding small volumes (100kL/day) of raw influent bypassing the CAL into the SBR. After raw feeding, the plant ceased bulking, as indicated by the SV60 reducing from 930 to 200 mL/L. 16S rRNA community profiling and qFISH biovolumes of SBR samples revealed major changes in the microbial community structure. *Thiothrix* spp. population decreased from 36.8% to 0.2%. *Zoogloea* spp. dominated in all samples after raw feeding. In this study we propose direct feeding as a control method for industrial plants with large surge/anaerobic lagoons to manage the bulking problems caused by *Thiothrix* spp. in downstream SBRs.

Introduction

Bulking sludge is considered a frequent cause of operational problems in activated sludge systems often attributed to filamentous bacteria (Seviour & Nielsen 2010). Type 021N is a well-documented and globally distributed filamentous bacteria comprising several species of the *Thiothrix* genus that can cause severe bulking and high sludge volume index (Dumonceaux et al. 2006; Kanagawa et al. 2000). The literature suggests that high concentrations of readily biodegradable substrates will favour the growth of Type 021N *Thiothrix* (Seviour & Nielsen 2010). It is therefore plausible that changes in wastewater composition from mainly low molecular weight readily biodegradable substrates to a more diverse range of substrates will have an influence on the abundance of Type 021N/*Thiothrix*.

In this study we investigated a plant that has long suffered from bulking, reducing efficiency and quality of the effluent. Periodic microscopic examinations indicated that *Thiothrix spp.* are the cause of poor settling. From an understanding of the microbiology of the treatment plant and the chemical composition of the wastewater, an attempt was made to manipulate the microbial populations to improve settling in this full-scale system by direct feeding small volumes of raw influent bypassing the CAL into the SBR.

Material and Methods

Sampling of the Wastewater Treatment:

Samples were taken from a full-scale beverages treatment plant in NSW, Australia during the period of April 2015 – August 2015. Influent samples were taken post screening via a composite sampler. Wastewater was continuously feed into a 30ML CAL with a HRT averaging 26 days, CAL samples were taken at the point of discharge into the SBR. Over the 6 month trial, the SBR (6ML) processed 3 batches/day of 450kL with an average SRT of 33 days. From the 1st of June, 1 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 & Bridgewater 2012) where appropriate. The SBR was regularly dosed with urea to prevent nutrient deficiency.

*Fluorescence in Situ Hybridization (FISH)***:**
FISH was performed using formaldehyde fixed cells for the identification of *Thiothrix* spp. (G123T and competitor) (Kanagawa et al. 2000). Biovolumes fractions were estimated using imaging analyses software DAIME v2.1 using G123T probe as specific target and EUB338mix probe for total biovolume.

DNA extraction, sequencing and bioinformatics processing:
Biomass DNA was extracted using a FastDNA SPIN Kit (MP Biomedicals, Santa Ana, CA) following the manufacturer's instructions with modifications in the homogenization step. Amplicon libraries and sequencing were performed by the Australian Centre for Ecogenomics using primers targeting the V3-4 16S rRNA region and the Miseq (Illumina) platform. Trimming and quality filtering was performed using Trimmomatic v. 0.32. Reads were merged using Pandaseq then dereplicated and formatted for the UPARSE workflow (Edgar 2013). Taxonomy assignation and diversity analyses was performed using QIIME 1.9.1, and the MiDAS v1.20 database (McIlroy et al. 2015).

Results and Conclusions
Microscopic examination and application of 16S rRNA targeted probes to SBR samples before direct feeding showed the community was dominated by *Thiothrix spp.,* after direct feeding SBR samples were dominated by *Zoogloea* spp. (Figure 1). Chemical analysis of the raw influent shows a cocktail of organic acids, phenols, alcohols and low sulfur concentrations, while the fed from the CAL is high in acetic acid and phenols (Table 1).

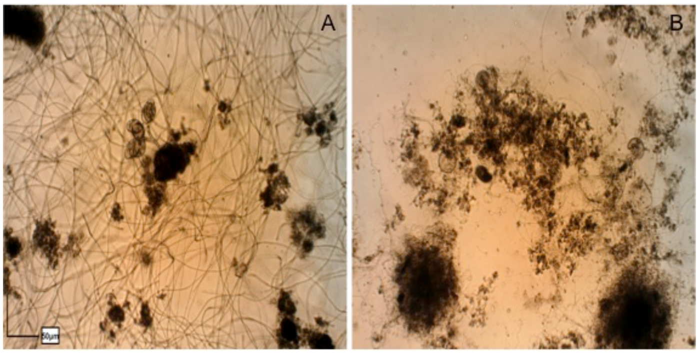

Figure 1. Light micrographs showing floc structure of samples taken before the feeding trial, May 2015 (A) and after July 2015 (B). Scale bar represents 50 µm.

Table 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

Sludge volume decreased from SV30=970 to SV=260 after direct feeding and a shift in microbial populations could be observed. Biovolumes of *Thiothrix sp.* reduced from 31% to 1%. Metagenomics analysis also showed an increased biodiversity with Shannon index increasing from 4.37 to 5.68, and the appearance of species belonging to the *Zoogloea, Planctomycetes and Flavobacterium* (Figure 2).

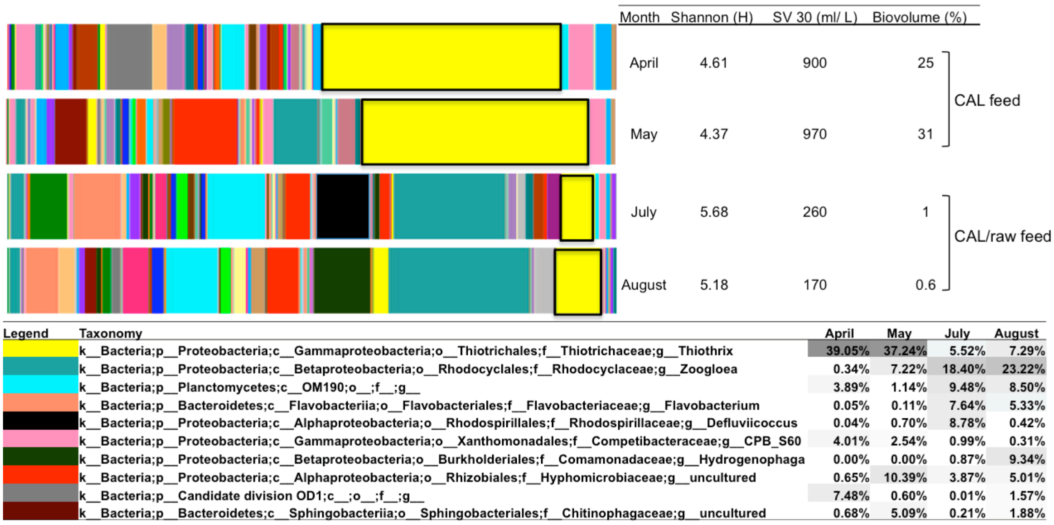

Figure 2. SBR microbial community composition, Shannon's diversity index, sludge volume 30 and *Thiothrix sp.* biovolume fraction before and after direct raw feeding.

In this study, knowingly changing the chemical characteristics of the SBR feed altered the metabolic selection of organisms and improved biodiversity and settling. 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 carbon substrates may be an easy and economical strategy for industrial plants with large surge lagoons to manage their microbial communities and control filamentous bulking caused by *Thiothrix spp.*.

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Excessive proliferation of glycogen accumulating organisms in winery wastewater: Causes, consequences and potential solutions.

Cristobal A Onetto *, Kathryn L Eales * and Paul R Grbin *

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Abstract
Glycogen accumulating organisms (GAO) regularly proliferate in Australian winery wastewater treatment plants handling waste high in carbon and nutrient deficient. When GAO dominate, they are often associated with poor performance; slow settling biomass and turbid effluents. A 3-year survey of an Australian winery wastewater (WWW) treatment plant revealed the presence GAOs belonging to the Alphaproteobacteria, Actinobacteria and Gammaproteobacteria, however the most commonly observed were the Alphaproteobacterial, *Defluviicoccus vanus* related GAO with observed abundances as high as 50% of the entire bacterial community. High COD:N ratios were a common trend and therefore thought to be the cause of GAO high abundance. Activated sludge samples dominated by *Defluviicoccus vanus* related GAO were incubated under different carbon to nitrogen (COD:N) ratios (100:1, 60:1 and 20:1) using ^{13}C acetate and ^{15}N urea and GAO cell assimilation quantified using Fluorescence in situ hybridisation in conjunction with Nanoscale secondary ion mass spectrometry (FISH-NanoSIMS), a novel technique with no previous report in this field. NanoSIMS revealed that low (COD:N of 100:1) or null nitrogen concentrations enhanced the carbon uptake of GAO cells. COD:N ratios of 60:1 and 20:1 reduced the carbon uptake of GAO cells. This data shows that nitrogen dosing at COD:N ratios of 60:1 or higher is a feasible strategy for controlling the excessive growth of these microorganisms in WWW treatment plants.

Keywords
Glycogen accumulating organisms; activated sludge; winery wastewater; nitrogen dosing

INTRODUCTION

Wastewater generated by the wine industry differs considerably from that of domestic wastewater, as it contains high organic loads, low nutrient levels and low pH. For successful aerobic biological treatment of wastewater an accepted rule of thumb is a Carbon:Nitrogen:Phosphorus (C:N:P) ratio of 100:5:1. WWW carbon oxygen demand (COD) values are very high (approximately 1000-16000 mg/L), N very low and P adequate. This results in influent COD:N ratios as high as 1000:1 with pH values below 4.0, making biological treatment challenging. The high COD levels primarily explained by high concentrations of sugars, organic acids, ethanol and polyphenolic compounds reflect the need for nutrient supplementation, tackled by some wineries with the addition of urea and/or diammonium phosphate to facilitate its biological treatment.

Few studies have been conducted in recent years associated with WWW microorganisms [1-3]. A large survey of Australian WWW treatment plants showed glycogen accumulating organisms (GAO) identified as Alphaproteobacterial, *Defluviicoccus* species often dominated, and were suggested as a possible cause of non-filamentous bulking.

It has been shown that GAO have the ability to assimilate carbon sources stored as both polyhydroxyalkanoates (PHA) and glycogen. Although not investigated before, these unique metabolic characteristics may favour their growth under nutrient limiting conditions and enhance their proliferation in WWW environments. In this study the effect of nitrogen dosing over the metabolism of the GAO community in WWW was assessed by FISH-NanoSIMS, and the production of PHA, glycogen and DNA quantified.

MATERIALS AND METHODS

Sampling Grab samples of raw influent and mixed liquor were taken from an Australian WWW treatment plant over a 3-year period (2014, 2015 and 2016).

Chemical Analyses Samples were assessed by SV, COD, total nitrogen and phosphorus.

DNA extraction and Bioinformatics Biomass DNA was extracted using a FastDNA SPIN Kit for soil (MP Biomedicals, Santa Ana, CA) following the standard protocol except for four times increased bead beating duration. Amplicon libraries and sequencing were performed by the Australian Centre for Ecogenomics. Bioinformatics processing was performed as previously described by Albertsen et al. (2015) [4].

Fluorescence in situ hybridization (FISH) Cells where fixed using standard procedures [5]. Fluorescence *in situ* hybridization was performed as previously described by Li et al. (2008) [6] and re-suspended in 50 µl of sterile MilliQ water.

Incubation conditions and nanoscale secondary ion mass spectrometry (NanoSIMS) analyses. Six treatments (control, acetate control, nitrogen control, COD:N 100:1, 60:1 and 20:1) in duplicate were carried out using ^{13}C- acetate and ^{15}N-urea during an 8 h aeration cycle. Isotopic mapping was carried out using the Cameca NanoSIMS 50 at the Centre for Microscopy, Characterisation and Analysis (University of Western Australia). Target cells were re-allocated in the NanoSIMS by using previously obtained positive FISH images of the samples.

RESULTS AND DISCUSSION

Microscopic analyses of mixed liquor samples showed GAO were routinely the most abundant organism present. FISH analyses showed that GAO belonging to the Alphaproteobacteria, Actinobacteria and Gammaproteobacteria are all present, however the most commonly observed were the Alphaproteobacterial, *Defluviicoccus* cluster II related GAO (Figure 1), exhibiting typical tetrad or grape bunch morphology associated with GAO.

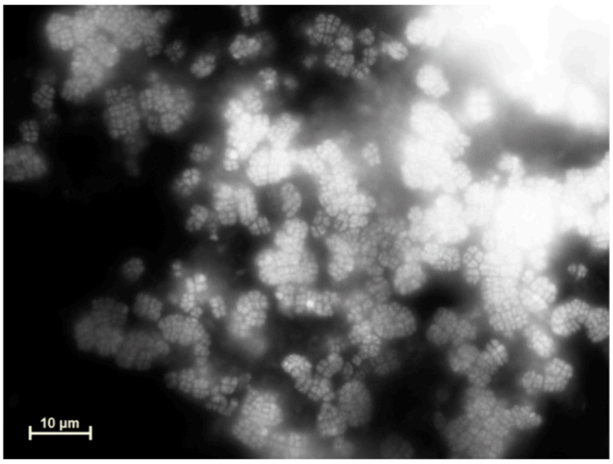

Figure 1. FISH micrograph of *Defluviicoccus* Cluster II cells using probes DF2 mix (DF988 and DF1020).

The presence of GAO is not rare for plants treating high COD industrial waste [7] and has previously been reported specifically for WWW treatment plants [1] and should be considered a common component of WWW activated sludge. The lack of nutrient supplementation in the presence of high COD loads appears as a common trend between previous reports, suggesting that these conditions support the growth of GAO. Furthermore, the ability of high COD waste to support the growth of GAO has been investigated for their high PHA production potential for use in bioplastics manufacture. In a balanced activated sludge microbial population when they are forming part of the floc, GAO can be beneficial at removing large concentrations of carbon from the influent wastewater. However, our observations indicate that when they proliferate they can become troublesome causing poor settling in SBRs and a decrease in effluent quality with high turbidity and hence COD.

Metagenomic analyses confirmed the common occurrence of *Defluviicoccus* related GAO belonging to the 4 distinct clusters discovered to date (Figure 2B) with abundances up to 50% of the whole bacterial community (Figure 2A). The most abundant OTU_1 falls within *Defluviicoccus* cluster II (DF2)(Figure 2B), consistent with FISH analysis.

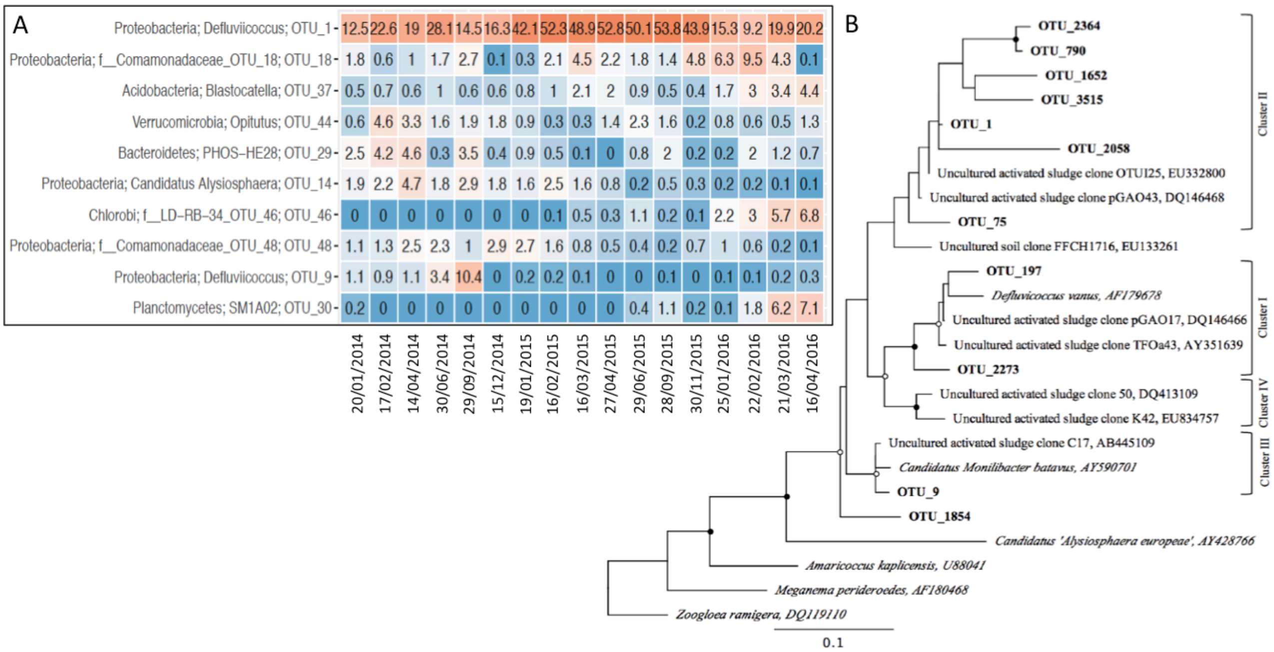

Figure 2. (A) Heatmap representing the percentage read abundance of the 10 most abundant genera in SBR samples. If no genus name could be assigned the best classification is provided. (B) Phylogenetic tree of >440bp from the V3-V4 16S rRNA gene of the Genus *Defluviicoccus*. Sequences from this study 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.

The metabolism of DF2 has been well studied. Their metabolic storage characteristics increase their competitiveness during famine stages and possibly under WWW nutrient limiting conditions. As DF2 is the dominant and, probably, the most metabolically active population, they will assimilate nutrients and carbon quickly, leading to their proliferation.
In the wastewater industry, dosing with urea is common practise to eliminate nutrient deficiency, however dosing at a COD:N ratio of 20:1 is not economically viable or practical in the wine industry. For this reason it is beneficial to determine if lower levels of nitrogen supplementation, that are workable and feasible, will allow non-GAO populations to compete for carbon substrates.
To answer these questions, samples were incubated with stable isotope labelled substrates under different COD:N. Changes in the DF2 cells uptake of carbon (^{13}C-Acetate) and nitrogen (^{15}N-Urea) were assessed by FISH-NanoSIMS. NanoSIMS provided single cell quantification and visualization of multiple stable isotopes, enabling the precise quantification of substrate uptake by DF2 cells. Areas of interest were predefined using DF2 positive FISH confocal microscopy images (Figure 3A). The bottom right panel in Figure 3A shows a $^{13}C/^{12}C$ ratio image, with the colour scale indicating the degree of ^{13}C enrichment; in this case blue represents natural abundance. This allows the direct visualisation of the uptake of the label by the DF2 cells. NanoSIMS data showed a marked increase in the ^{13}C abundance of DF2 cells in the acetate control (5.2 at%) and 100:1 (6.3 at%) (Figure 3B). The results for treatments 60:1 and 20:1 showed that DF2 cells are assimilating

comparatively less carbon, and was therefore assimilated by other microorganisms in the biomass. Under these conditions, more homogenous ^{13}C enrichment was observed throughout the microbial community with a clear lower enrichment in DF2 cells. This information combined with previous reports of GAO excessive abundance in WWW activated sludge supports the view that high COD feeds with low nutrient levels enhance the carbon assimilation of DF2. COD:N ratios of 60:1 and 20:1 reduced carbon assimilation by DF2 and maximised their nitrogen uptake and should help control the growth of these organisms. Whilst a 20:1 ratio may theoretically increase biomass production in industrial plants, the quantity of urea required is often excessive and not economically viable for industrial plants. Our results indicate that dosing at a more economically viable 60:1 ratio will lead to a more balanced microbial community in the activated sludge, and reduce the problems associated with GAO proliferation.

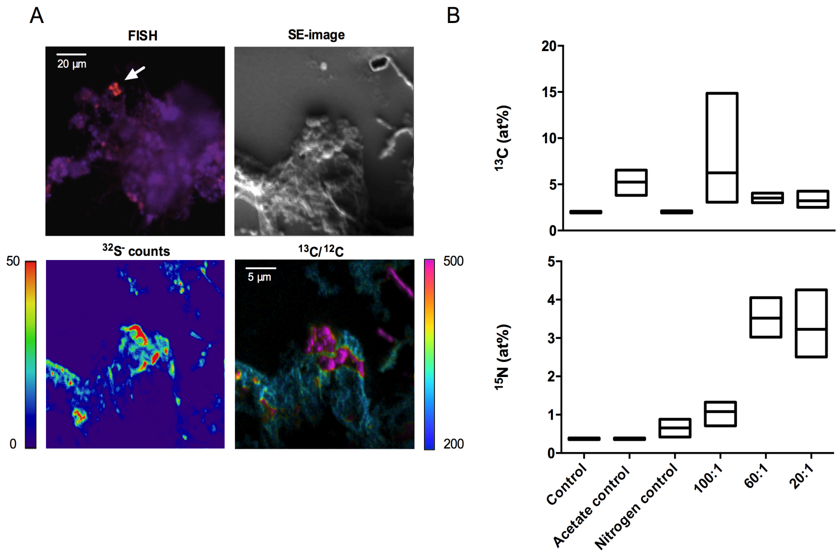

Figure 3. (A) Representative FISH and NanoSIMS images, showing DF2 cells in the same field of view after 8 h of incubation. Cells were located using FISH with the DF2mix (DF988 and DF1020). The $^{13}C/^{12}C$ ratio image demonstrates high ^{13}C uptake by DF2 cells, the colour scale indicates the degree of ^{13}C enrichment; in this case blue represents natural abundance. The $^{32}S^-$ ion counts image shows the biomass arrangement. (B) ^{13}C and ^{15}N uptake of *Defluviicoccus* cluster II (DF2) cells quantified by NanoSIMS after the 8 h incubation. The isotope content is presented as the isotope fraction $^{13}C/(^{12}C + ^{13}C)$ given in atomic %. The box plots summarize the mean and the minimum and maximum value.

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Appendix 4

The effect of activated carbon and other common winemaking processing aids in the waste stream on wastewater treatment plants.

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Abstract

Pinot Gris/Grigio (PG) popularity is increasing rapidly in Australia. Wastewater treatment plant operators have observed that during PG processing wastewater treatment plants can be adversely affected. As PG production intensives the burden on the plants can become difficult to manage. Plants reportedly become overloaded and COD removal is reduced in SBRs. This has been investigated by studying the affect common winemaking processing aids used in PG production may have on a wastewater treatment. The processing aids included, activated carbon, perlite, casein and bentonite. Changes in chemistry of SBR water exposed to solids from PG processing were assessed and highlighted the burden activated carbon can have on a wastewater treatment plant. Activated carbon releases 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. Emphasizing the need for strict solids management in winery, limiting their presence in the waste streams generated.

Keywords

Wastewater treatment; Pinot Gris; activated carbon;

Introduction

Winery wastewater is characterised as low in pH and nutrients, and high in salt, COD and solids. The common components of winery wastewater are considered to be juice, wine and cleaning products, particularly caustic soda and the impact of these on wastewater are well understood and managed accordingly.

The amount of solids entering wastewater treatment plants is significant, especially at small wineries. The solids can consist of grape skins and lees from the raw products, but also solids from the additives used during juice and wine processing such as bentonite, perlite, casein and activated carbon.

Pinot Gris popularity has increased significantly in Australia in the past few years (Anderson and Aryal, 2014). The main point of difference in the processing of Pinot Gris (PG) is there is a decolorisation step commonly applied, where activated carbon is applied to reduce the slightly pick colour naturally found in this grape variety to a colorless wine. Empirical observations suggests that PG processing carries an increased burden on wastewater treatment plants. In this study we investigated the impact of various processing aid solids on waste streams.

Materials and Methods

Figure 1 Pinot Gris juice and wine used in the experiment

Pinot Gris wine and juice (Adelaide Hills) was obtained from the University of Adelaide winery (Figure 1). Wine and juice of Pinot Gris was treated with activated carbon (2g/L), casein (0.25g/L), perlite (1g/L) and bentonite (1g/L) and then filtered through a 0.2um membrane. The solids were added to SBR water and mixed on a shaker for 2h, then filtered through a 0.2um filter. The SBR filtrate was measured to assess the impact these additives had in relation to pH, EC, COD, total phenols, HPLC, ICP-MS and suspended solids.

Results and discussion

The impact processing aids from PG production have on SBR water was assessed in terms of pH, EC and COD. All additives treating both juice and wine reduced pH and increased COD load (Table 1). Activated carbon had the most marked impact on all 3 parameters, lowering pH and EC and increasing COD.

Table 1: 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 + AC*	8.3	778	1749	Juice + AC +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

*Activated carbon

The composition of the COD load was characterised by HPLC and absorbance (Table 2 and 3). From the treatment of wine, activated carbon released ethanol and phenolic compounds with some tartaric and acetic acids, the acids accounting for the reduction in pH observed. Phenols have a high COD and would play a major role in the significant COD increase observed. Perlite released ethanol, citric and glycerol, while bentonite and casein released ethanol.

Table 2: Carbon components (g/L) measured in filtered SBR water after being incubated with additives treating 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
Activated carbon	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

Processing aids that had treated juice were measured in the same way (Table 3), casein, perlite and bentonite did not impact the SBR water significantly. Activated carbon released a considerable amount of fructose and succinic and some citric acid and phenols.

Table 3. Carbon compounds (g/L) measured in filtered SBR water after being incubated with additives treating 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
Activated carbon	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

Conclusions

From this study, perlite, skim milk and bentonite have minimal impact on SBR chemistry. However, activated carbon releases the phenols, ethanol, sugars and organic acids it has stripped from wine and juice into the SBR water. This release of organic materials 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 activated carbon in the wine industry has been increasing recently due to the increased production and processing of Pinot gris/grigio grapes. It is essential to exclude activated carbon out of the wastewater system in order to minimise shock in the SBRs during grape processing.

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