

# **A high rate biofilm contact reactor for winery wastewater treatment**

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## ABSTRACT

Winemaking produces variable volumes wastewater rich in biodegradable organic material, with fluctuating chemical composition and pH values according to the seasonal activities of the cellar. Releasing untreated winery wastewater into the environment can cause eutrophication and toxicity in surface water and has detrimental effects on soil condition and ground water quality. Rising costs of effluent disposal, limited availability of freshwater resources and increasingly stringent water use regulations imposed on wineries are enthralling interest in low cost, sustainable, and robust wastewater treatment solutions for wineries. The objective of this study was to design, construct and implement an easily pre-assembled, energy efficient pilot scale biofilm reactor with a small footprint for winery wastewater treatment. A commercial cooling tower as a trickling filter reactor unit was central to the design. The system was tested at a winery in Stellenbosch and after proving to be effective, was up-scaled by adding a second cooling tower to the system as a secondary reactor, treating the effluent from the first subunit, contributing to the overall waste removal efficiency of the system. The double-unit pilot system was tested in six trials over three years. The system showed effective, robust treatment of winery wastewater of varying strengths with minimal solid waste production, consistently reducing chemical oxygen demand (COD) (average 93% reduction), total nitrogen, sulfate, phosphate and suspended solids (average 90% reduction) to meet prescribed regulations for irrigation. The system performed at its peak when treating highly concentrated wastewater during harvest season. The pH of treated wastewater was consistently buffered from highly acidic and basic values to close to neutral. To understand how the biofilm worked to remove contaminants within the system, and how the additional cooling tower unit expanded the treatment scope of the system, a three-tiered investigation of the microbial community structure, distribution of microorganisms and collective metabolic capabilities of biofilm samples from each cooling tower subunit was investigated. Next generation sequencing revealed that the biofilm populations of the two reactor subunits were phylogenetically distinct, with only 12% of operational taxonomic units (OTUs) overlapping between the two biofilms. Taxonomic data indicated that carbohydrate reducing bacteria dominated the population of the first cooling tower, while nitrifying and denitrifying bacteria dominated the second. Fluorescent in situ hybridization coupled with confocal laser

scanning microscopy (FISH-CLSM) revealed the stratified distribution of aerobic *Gammaproteobacteria* across the depth of the biofilm from the first cooling tower unit, and showed distinct distribution patterns of *Nitrosomonas* and *Nitrospirae* in biofilm samples from the first and second cooling tower units. Substrate utilization analyses using the Biolog system revealed that the majority of the carbon substrates that were tested were utilized in the biofilm samples from both cooling towers, but that important metabolic utilization capabilities fell exclusively either within the consortium of the biofilm from tower 1 or tower 2. Collectively, the data from each of the three analytical approaches indicated that by adding a second subunit to the bioreactor, the treatment capacity of the system was not merely expanded, but that the second reactor subunit added to the microbial and metabolic diversity of the system.

## OPSOMMING

Die wynmaakproses produseer veranderlike volumes afvalwater wat ryk is aan bioafbreekbare organiese materiaal, met wisselende chemiese samestelling en pH-waardes volgens seisoenale aktiwiteite van die kelder. Die vrystelling van onbehandelde kelderafvalwater in die omgewing kan nadelige voedingstofverryking en toksisiteit in oppervlakwater veroorsaak en kan die grondsamenstelling en grondwatergehalte negatief beïnvloed. Stygende kostes van uitvloeiselbeskikking, beperkte beskikbaarheid van varswaterbronne en toenemend strengere regulasies met betrekking tot waterverbruik by wynkelders kweek belangstelling in lae koste, volhoubare en robuuste afvalwateroplossings vir wynkelders. Hierdie studie was daarop gemik om 'n biofilmreaktor vir kelderafvalwaterbehandeling op proefskaal te ontwerp, bou en implementeer wat maklik voor installasie in 'n fabriek saamgestel kan word, wat op 'n energie-doeltreffende en volhoubare wyse werk en 'n kompakte ontwerp beslaan. Die basiese ontwerp was 'n sypelfilterreaktoreenheid bestaande uit 'n kommersiële koeltoring. Die stelsel is getoets by 'n kelder in Stellenbosch, en nadat die stelsel se doeltreffendheid bewys is, is dit opgeskaleer deur 'n tweede koeltoring as sekondêre reaktor by te voeg. Die behandelde uitvloeisel van die eerste koeltoring-subeenheid is derhalwe in die tweede koeltoringeenheid behandel, wat die doeltreffendheid van die stelsel as geheel uitgebrei het. Die dubbeleenheid-proefreaktorstelsel is in ses afsonderlike toetse oor drie jaar getoets. Die stelsel het kelderafvalwater doeltreffend behandel met minimale vaste afvalproduksie. Die

stelsel het kontaminante in die afvalwater konsekwent verminder. Chemiese suurstofbehoefte (COD) is gemiddeld met 93% verminder, stikstof, sulfate en fosfate het afgeneem en vaste stowwe in suspensie het met gemiddeld 90% afgeneem; trouens, die behandelde water se gehalte het binne die voorgeskrewe besproeiingsregulasies geval. Die stelsel was op sy doeltreffendste tydens die behandeling van hoogs gekonsentreerde afvalwater tydens oestyd. Die pH van behandelde afvalwater is deurgaans gebuffer van baie suur en basiese waardes na naastenby neutraal. Ten einde die biofilm se verwydering van besoedeling te verstaan en hoe die bykomende koeltoringeenheid die stelsel se behandelingsomvang uitbrei, is 'n drieledige ondersoek van stapel gestuur om die mikrobiële gemeenskapstruktuur, die verspreiding van mikro-organismes en kollektiewe metaboliese vermoëns van biofilmmonsters van elke koeltoringeenheid te ondersoek. Nuutste generasie volgordebepaling het gewys dat die biofilmbevolkings van die twee koeltoring-subeenhede filogeneties verskil, met slegs 12% oorvleueling van operasionele taksonomiese eenhede tussen die twee biofilms. Volgens taksonomiese data het koolhidraat-metaboliserende bakterieë die bevolking van die eerste koeltoring oorheers, terwyl nitrifiserende en denitrifiserende bakterieë die gemeenskap van die tweede toring oorheers het. Fluoreserende *in situ*-hibridisasie met konfokaleskandeerderlasermikroskopie het gestratifiseerde verspreiding van aërobiese Gammaproteobacteria regoor die diepte van die biofilm van die eerste koeltoringeenheid geopenbaar, en het die verskillende verspreidingspatrone van Nitrosomonas en Nitrospirae in die biofilmmonsters van die eerste en tweede koeltoringeenhede uitgewys. Biolog-substraatbenuttingsontledings het getoon dat die meerderheid getoetste koolstofs substrate deur die biofilmmonsters van beide koeltorings verbruik is, maar dat belangrike metaboliese benuttingsvermoëns uitsluitlik óf binne die konsortium van die biofilm uit toring 1 óf toring 2 geval het. Gesamentlik het die data van elk van die drie analitiese benaderings aangedui dat die toevoeging van 'n tweede koeltoringeenheid by die bioreaktor die stelsel se behandelingskapasiteit uitgebrei het, en dat die tweede subeenheid bydra tot die mikrobiële en metaboliese diversiteit van die stelsel en die uitbreiding van die behandelingsomvang van die stelsel as geheel.

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## CHAPTER 1

### Introduction

Responsible management of freshwater resources is central to the 2030 sustainable development goals (UN 2015). The growing world population, which is expected to reach 9.1 billion by 2050, climate change, increasing levels of urban development and pollution all contribute to the fact that freshwater is now a threatened and overexploited resource in many regions of the world (WHO 2004). To help protect and remediate already compromised ecosystems and the communities that depend on them, water consumption and wastewater management should be carefully controlled.

Globally, agriculture consumes approximately 70% of “blue water” i.e. groundwater, and surface water such as rivers and lakes. Furthermore, approximately 40% of the world’s food crops are cultivated on irrigated land. This figure is expected to increase by another 19% by 2050 (FAOSTAT 2015). The wine industry is considered a major global agricultural activity (FAOSTAT 2015), with grapes ranking among the top 40 produced crops worldwide. Wine production is a water-intensive process, with the water footprint for one glass of wine estimated at 109 L. This includes all agricultural, vineyard and cellar activities contributing to the production of wine (Hoekstra *et al.* 2011; Mekonnen and Hoekstra 2011). The implementation of water re-use strategies in the wine industry can therefore make an important contribution to sustainable management of water as a resource by minimising the volumes of spent water that needs to be disposed of or fed into overstressed municipal wastewater treatment plants. Recycled wastewater can be used for any purpose, providing that it is of sufficient quality for the intended use. If the level of wastewater treatment required is achievable at a lower cost than freshwater consumption, running costs of a process may be decreased by using treated or recycled wastewater (Alves *et al.* 2006).

Winemaking produces wastewater that is rich in biodegradable organic material, varies in concentration and volumes over seasons, and fluctuates between highly acidic and basic pH values (Bustamante *et al.* 2008). Releasing untreated winery wastewater into the environment can cause eutrophication and toxicity in surface water and has detrimental effects on soil condition and ground water quality (EPA 2004; Serrano *et al.* 2011). Rising costs of effluent disposal, limited availability of



freshwater resources and increasingly stringent water use regulations imposed on wineries globally are enthralling interest in wastewater reuse in this industry (Van Schoor 2005; Gabzdylova *et al.* 2009; Mosse *et al.* 2011b; Preston-Wilsey 2015).

When considering wastewater treatment solutions for a specific winery, factors that are taken into account include the intended end-use of the treated wastewater, which will specify the required level of purification. Furthermore, the size of the winery, the amount of wastewater produced, the availability and expertise of staff required to operate a treatment system, financial resources, infrastructure and land available to accommodate such a system all serve as deciding factors in choosing a suitable wastewater solution for a specific winery (Van Schoor 2005; Mosse *et al.* 2011a; Litaor *et al.* 2015).

Current technologies available in winery wastewater treatment include physico-chemical treatment processes, biological treatment, membrane filtration, advanced oxidation processes and various combinations of biological and chemical processes (Ioannou *et al.* 2015). Due to the high amount of readily biodegradable matter in winery wastewater, biological wastewater treatment is considered the most economical treatment method (Van Loosdrecht and Heijnen 1993).

Biological wastewater treatment utilises the metabolic activity of microorganisms, which oxidise and assimilate contaminants in wastewater as a means to generate energy and biomass. The biochemical reactions that are responsible for the removal of pollutants in biological wastewater treatment systems are influenced by the configuration of the bioreactor, as this determines the phases (solid, liquid and gaseous phases, or a combination of these) included in the system, which ultimately determines which redox environments (aerobic, anaerobic, anoxic or a combination of these) exist within a reactor. Biofilm reactors retain biomass within a biofilm that is attached to a substrate exposed to wastewater in the presence or absence of aeration. Soluble organic micro- and macromolecules diffuse through the extracellular polymeric substances (EPS) of the biofilm, allowing microbes in the biofilm to oxidise readily biodegradable chemical oxygen demand (COD) at a high rate (Andreottola *et al.* 2005). In a mature biofilm in an aerated system, an oxygen concentration gradient exists across the biomass, providing different biochemical

redox environments within the biofilm (Lewandowski and Boltz 2011). This creates a multi-niche microenvironment in which microorganisms with distinct metabolic profiles can flourish and contribute to multi-contaminant waste removal, making an aerobic biofilm reactor ideal for application in winery wastewater treatment.

The objectives of this study were to design, build and test a pilot scale biofilm reactor for winery wastewater treatment on site at a winery. Furthermore, an in-depth study into the biofilm within this system was planned to shed light on how the microbial community structure, distribution of microorganisms and collective metabolic capabilities of a biofilm contribute to winery wastewater treatment.

The design of the pilot reactor was based on pre-fabricated cooling tower units used as naturally ventilated high rate trickling filters. The simple design was easily pre-assembled off site for quick and easy installation, and was easily expandable to upscale the capacity and treatment scope of the system. Furthermore, simple and energy efficient operation with low maintenance and minimal waste production was achieved. Once the system was fully operational as a high rate biological contact reactor and producing improved quality treated effluent, compliant with legal requirements for disposal via beneficial irrigation or release into the environment, an in-depth characterisation of the microbial consortium of the biofilm within the reactor was proposed.

Until recently, bioreactors were considered “black boxes” with all knowledge on their functionality based on observations such as effluent quality, making these systems hard to predict and control (Herrero and Stuckey 2015). However, bioreactor performance depends on good bioreactor design, which provides the optimal environment for selective development of microorganisms with the most desirable set of metabolic capabilities for the treatment requirements at hand (Herrero and Stuckey 2015).

In the case of the pilot system designed for this study, it was necessary to understand how the biofilm worked within the system, and how up-scaling the system by adding an additional cooling tower unit to the reactor might influence the treatment scope of the system.

To gain maximal insight into the biofilm composition and functionality in this system, a three-tiered approach to microbial community analyses was suggested, with metagenomics for characterising the microbial community and defining dominant organisms, followed by fluorescent *in situ* hybridisation coupled with confocal laser scanning microscopy (*FISH-CLSM*) for visual analysis, which made confirmation of the presence, and analyses of the spatial distribution of specific groups with defined metabolic capabilities within the biofilm possible. Stratified distribution and microcolony formation of organisms in the biomass could indicate the presence of multiple biochemical redox niches, and thus an oxygen gradient across the biofilm. Finally phenotypic, culture based substrate utilisation tests were chosen to investigate the metabolic capabilities of the biofilm to shed further light on how the biofilms function within this system to treat winery wastewater.

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## CHAPTER 2

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## Introduction to winery wastewater

Wine production is a major global agricultural activity, with wine grapes being among the top 40 produced crops worldwide (FAOSTAT 2015). The main wine producing countries are France, Italy, Spain, the United States, Argentina, Australia, South Africa and China (OIV 2015). The production of wine has a large water footprint estimated globally at 109L of water per glass of wine produced (Mekonnen and Hoekstra 2011). This takes into account the total agricultural, vineyard and cellar activities contributing to water consumption such as evaporation and incorporation during production as well as the water polluted in the life cycle of the product (Mekonnen and Hoekstra 2011; Hoekstra and Mekonnen 2012). The volume and characteristics of wastewater produced varies greatly between wineries in relation to the size of the winery, the types of wine produced (Artiga *et al.* 2005a; Bustamante *et al.* 2008), the winemaking style, techniques and equipment used at a particular winery, and the season's working period (i.e. vintage, racking and bottling). An estimated 1.3-1.5 kg of waste is produced for each liter of wine produced, of which 75% is wastewater, amounting to up to 4 m<sup>3</sup> wastewater per m<sup>3</sup> of wine that is produced (Lucas *et al.* 2010). Major processes that contribute to wastewater generation is the washing of floors and equipment, especially during vintage, rinsing of transfer pipelines and filtration units, barrel cleaning, transfer and bottling wastage, and rainwater captured in the wastewater management system (Vlyssides *et al.* 2005; Bustamante *et al.* 2008).

Organic waste contributes up to 85% of the contaminants in winery wastewater (Ruggieri *et al.* 2009). Vinification introduces grape pulp, skins, seeds and dead yeast into the wastewater stream (Moldes *et al.* 2008). Organic contamination is quantified as chemical oxygen demand (COD), biological oxygen demand (BOD) and total suspended solids (TSS). During vintage, COD can reach concentrations of up to 45 000 mg L<sup>-1</sup>, BOD, 20 000 mg L<sup>-1</sup>, and TSS, 30 000 mg L<sup>-1</sup> (Mosse *et al.* 2011). Soluble organic acids, alcohols, esters, sugars, tannins, lignins and polyphenols are also present (Mosse *et al.* 2011). The inorganic component of winery wastewater consists of N derived from clarification proteins, inorganic P from cleaning agents and potassium, which is prevalent in grape skins, contributing to the high salinity (>3

dS m<sup>-1</sup>) and sodicity with a sodium absorption of >9 (meq L<sup>-1</sup>)<sup>0.5</sup> (Bustamante *et al.* 2005). The characteristics of winery wastewater are summarised in table 1.

Table 1. Reported characteristics of winery wastewater. (Adapted from Ioannou *et al.* 2015)

<b>Parameter</b>	<b>Unit</b>	<b>Min</b>	<b>Max</b>	<b>Mean</b>
Chemical Oxygen Demand (COD)	mg L <sup>-1</sup>	320	49105	11886
Biochemical Oxygen Demand (BOD5)	mg L <sup>-1</sup>	203	22418	6570
Total Organic Carbon (TOC)	mg L <sup>-1</sup>	41	7363	1876
pH	–	2.5	12.9	5.3
Electrical Conductivity (EC)	mS cm <sup>-1</sup>	1.1	5.6	3.46
Total Solids (TS)	mg L <sup>-1</sup>	748	18332	8660
Total Volatile Solids (TVS)	mg L <sup>-1</sup>	661	12385	5625
Suspended Solids (SS)	mg L <sup>-1</sup>	66	8600	1700
Total Phosphorous (TP)	mg L <sup>-1</sup>	2.1	280	53
Total Nitrogen (TN)	mg L <sup>-1</sup>	10	415	118
Total Phenolic Compounds (TPh)	mg L <sup>-1</sup>	0.51	1450	205

Due to high transport costs and the large fluctuations in both volume and chemical composition of winery wastewater produced over time, disposal into municipal wastewater treatment systems is not a feasible solution (Shepherd *et al.* 2001). A robust wastewater treatment regime must be in place to cope with fluctuations in both volume and constituents of wastewater corresponding to the commencement and termination of different work periods and intervals of low or no activity in the cellar (Melamane *et al.* 2007).

Untreated winery effluent, which ends up in the environment, can cause serious damage to ecosystems (Serrano *et al.* 2011) and is summarised in Table 2. The application of untreated winery effluent as irrigation causes contamination of soil with organic and inorganic material. Leaching from these contaminants affect colour, pH, dissolved oxygen concentration and electrical conductivity of surface and ground water. This will damage the physicochemical characteristics of the soil and can cause odour problems. The low pH of winery wastewater and the phytochemicals present in vinification lees and grape must are toxic when applied to crops (Moldes *et al.* 2008) and wetlands (Shepherd *et al.* 2001; Arienzo *et al.* 2009a). Certain phenolic compounds present in winery waste are resistant to degradation and are toxic to humans, animals and some microorganisms, even at low concentrations (Nair *et al.* 2008). These factors outline the importance of winery wastewater treatment. Increased international wine production, and stricter wastewater management strategies (Table 3) being enforced in the major wine producing regions of the world are promoting responsible wastewater management in the wine industry (EPA 2004; Van Schoor 2005; Gabzdylova *et al.* 2009; Mosse *et al.* 2011; Preston-Wilsey 2015).

The first research on winery wastewater treatment was published in 1940 by the Australian engineering and water supply department when concerns were raised about the fate of wastewater from the rapidly expanding wine industry in Southern Australia. It was noted that introducing winery wastewater into the municipal activated sludge wastewater treatment system made the treatment of domestic sewage difficult and at times impossible. Separate treatment was suggested. Pre-treatment of the winery effluent was achieved by mixing it in a ratio of 1 to 5 with raw sewage, treating it chemically with lime, followed by pH adjustment and dilution, after which



it could be applied to a two-stage biological trickling filter. Resulting effluent was of such a quality that it could be discharged into the municipal wastewater treatment plant (Hodgson and Johnston 1940). The literature shows that the expanding knowledge base of winery wastewater and the treatment thereof has grown rapidly during the last 20 years. Figure 1 illustrates the number of publications addressing winery wastewater treatment per annum from 1995 to 2015. Studies fall into two broad themes, firstly, the treatment efficiency of various processes by evaluating the efficacy of newly developed processes and combined or up-scaled existing processes and secondly, the characterisation of winery effluent and determining its environmental impacts (Ioannou *et al.* 2015).

Table 2. Environmental impacts of winery wastewater (Adapted from EPA 2004).

Winery wastewater components	Indicators	Effects
Organic matter	BOD, TOC, COD	Reduces oxygen levels - death of fish and other aquatic organisms. Odours generated by anaerobic decomposition.
Alkalinity/acidity	pH	Death of aquatic organisms at extreme pH. Affects the solubility of heavy metals in the soil and availability and/or toxicity in waters affects crop growth.
Nutrients	N, P, K	Eutrophication or algal bloom. N as nitrate and nitrite in drinking water supply can be toxic to infants.
Salinity	EC, TDS	Impacts undesirable taste to water, toxic to aquatic organisms, affects water uptake by crops.
Sodicity	SAR, ESP	Affects soil structure resulting in surface crusting. Low infiltration and hydraulic conductivity.
Heavy metals	Cu, Ni, Pb, Zn, Hg	Toxic to plants and animals
Solids	TSS	Can reduce light transmission in water, thus, compromising ecosystem health, smothers habitats, odour generated from anaerobic decomposition.

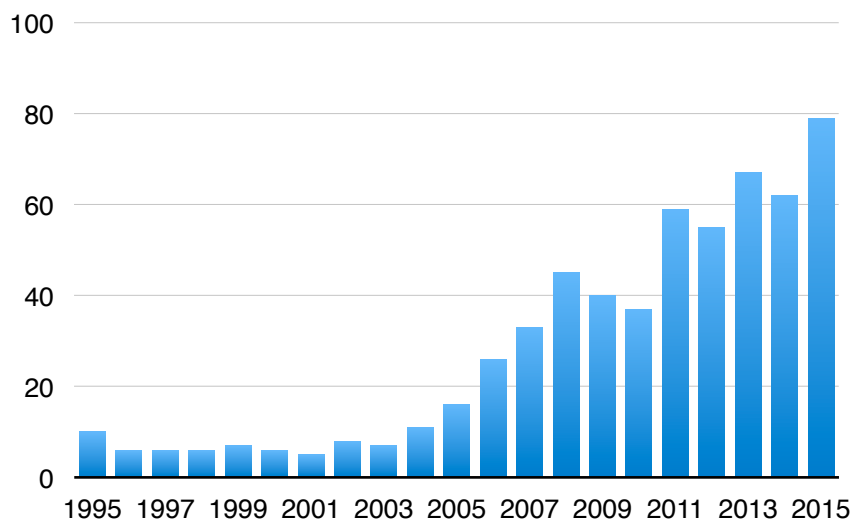


Figure 1. Publications on winery wastewater treatment from 1995 to 2015.

Table 3. Required standards of treated winery wastewater for irrigation purposes in South Africa (DWAF 2004).

Irrigation volume (m <sup>3</sup> )	Faecal coliforms/ 100 ml	COD (mg.L <sup>-1</sup> )	pH	SS (mg.L <sup>-1</sup> )
< 2000	< 1000	75	>5.5 or <9	<25
< 500	< 100 000	400	>6 or <9	
< 50	< 100 000	5000	>6 or <9	

All existing methods of winery wastewater treatment that have been successfully applied in practice and described in the literature over the last 20 years have recently been reviewed and compared (Ioannou *et al.* 2015). The review discusses the available technologies in five categories, namely physico-chemical treatment, biological treatment, membrane filtration, advanced oxidation processes and combinations of biological and chemical processes. Choosing a suitable technique for a specific winery depends on the required level of purification, which is determined by the intended end-use for the treated water, whether it will be re-used, irrigated or released into a river. Furthermore, the size of the winery, the amount of wastewater produced, the availability and expertise of the staff, the financial resources and

infrastructure or land available to accommodate such a system are taken into account (Van Schoor 2005; Mosse *et al.* 2011; Litaor *et al.* 2015).

This review discusses a brief overview of current methods in winery wastewater treatment, with special focus on biofilm bioreactors.

### **Non-biological winery wastewater treatment**

Physicochemical methods of winery wastewater treatment involve the physical separation of contaminants from the wastewater stream by the addition of chemicals or the application of physical processes that cause precipitation or flocculation of the suspended contaminants in wastewater. These methods aim to improve biodegradability before using biological treatment methods (Lucas *et al.* 2009), and are also used to reduce colour, remove metal contamination, and remove residual non-biodegradable COD after biological treatment (Andreottola *et al.* 2007; Ioannou *et al.* 2013). The efficacy of such processes are strongly influenced by the concentration and the properties of the chemical coagulant or flocculent that is used and the mixing speed, reaction time, current density and pH in the system (Ioannou *et al.* 2015). Treatment efficiency is measured in the reduction of COD, TSS and turbidity. Chemical precipitation with the chelating agent trimercaptotriazine was effective for removing copper and zinc contamination from winery wastewater, but only a 9% COD reduction was achieved (Andreottola *et al.* 2007). In a study by Rytwo *et al.* (2011), a two-step sedimentation that uses sepiolite to change the surface charge properties of the dispersed particles in winery wastewater is very effective at TSS removal (98%), and COD removal (20-40%), and they presumed that the remaining COD was dissolved organic matter for which this process is ineffective. Coagulation and flocculation with  $\text{Ca}(\text{OH})_2$  and  $\text{Al}_2(\text{SO}_4)_3$  as coagulant tested by Braz *et al.* (2010) was effective at removing TSS (95%) and turbidity (92%) but less than 68% of COD was removed. Electrocoagulation was effective at removing colour and turbidity, with an Fe anode removing 46.6% COD and an Al anode removing 48.5% COD (Kara *et al.* 2013). The most effective physicochemical method for COD reduction is coagulation with the natural coagulant chitosan, achieving up to 73% COD reduction (Rizzo *et al.* 2010). Other examples of non-biological methods used in winery wastewater treatment include electrodialysis and reverse osmosis which are very

effective at removing salinity from winery wastewater, but are not widely applied due to high operation costs and energy requirements (Lucas *et al.* 2009; Mosse *et al.* 2011). Advanced oxidation processes (AOP) generate highly reactive radical species such as hydroxyl radicals from reagents such as ozone, hydrogen peroxide and energy light sources (Ioannou *et al.* 2013; Agustina *et al.* 2008). Ozone oxidation has proven effective at pilot scale COD reduction (Lucas *et al.* 2009) and photo-fenton oxidation has been used to mineralise biological resistant COD after biological treatment (Ioannou *et al.* 2013) as well as a primary winery wastewater treatment step with up to 80% COD removal during vinification (Velegraki and Mantzavinos 2015). These processes are expensive to maintain and perform, and required highly trained personnel, making these suitable for application in large wineries (Mosse *et al.* 2011). Furthermore, these treatment methods applied in isolation will not be sufficient for winery wastewater treatment, but are excellent as supplementary treatment to biological treatment methods (Ioannou *et al.* 2013).

### **Biological winery wastewater treatment**

High concentrations of readily biodegradable organic matter make biological processes the most economical treatment option for winery wastewater (Van Loosdrecht and Heijnen 1993). The biochemical reactions that are responsible for the removal of pollutants in biological wastewater are influenced by the configuration of the bioreactor, as this determines the phases included in the system, which ultimately determines which redox environments exist within a reactor.

As microorganisms oxidise chemicals in wastewater to obtain energy for growth, electrons are released. The type of electron acceptor that is available determines the biochemical environment in a reactor. Aerobic environments are defined by the presence of oxygen as electron acceptor in non-rate limiting quantities. Aerobic systems support high amounts of biomass production per unit waste removed from wastewater, which will require proper management (Metcalf and Eddy 2003). Aerobic systems favour nitrification, converting ammonium ions to nitrite and then to nitrate ions (Sharma and Ahlert 1977). With nitrate or nitrite as electron acceptor, the environment is defined as anoxic, and the production of biomass is less efficient than in aerobic systems. When inorganic compounds such as carbon dioxide or sulfur serve

as electron acceptors, the environment is defined as anaerobic, and such an environment is the least effective at biomass production. Furthermore, such an environment also allows denitrification from wastewater with high organic load (Metcalf and Eddy 2003). Anaerobic wastewater treatment has the advantage of low energy requirements, as no aeration is required, and a lower production of biomass occurs due to the slower growing organisms favoured by these conditions. Furthermore, methane gas is a byproduct, which can be harvested and used as an energy source (Lettinga 1995). A disadvantage is the production of volatile fatty acids as a by-product, which causes malodour (Bories *et al.* 2005a), but this can be controlled by the addition of nitrate salts (Bories *et al.* 2007), however, this is a high cost solution, also impacting the final quality of the treated wastewater (Burgin and Hamilton 2007). The biochemical environment in a bioreactor thus has a major impact on the resulting wastewater treatment efficacy, and controlling that environment ensures conditions that are conducive to optimal waste removal.

The biochemical redox environment (aerobic, anaerobic, anoxic or a combination of these) within a bioreactor is determined by the phases it consists of. A reactor can consist of a single phase only, such as suspended growth bioreactors or it can consist of a combination of any or all of the following phases, namely a solid phase, a liquid phase and a gaseous phase. Biofilm bioreactors always consist of more than one phase (Leslie Grady *et al.* 2011).

The most common parameters that are used to monitor biological treatment efficiency in winery wastewater are COD, BOD, total Nitrogen and total Phosphate. The efficacy of the process is sensitive to the initial BOD concentration and TSS, temperature, DO concentration of the water in treatment, the ratio of nutrients to microorganisms within the system, growth kinetics and the parameters under which the system is operated (Ioannou *et al.* 2015).

### *Carbon removal*

Microbial metabolism comprises of catabolic reactions which supply energy for cellular activities, and anabolic reactions, in which the energy is consumed to synthesise biomass from carbon sources. Carbon sources for anabolism can be

organic or inorganic. Energy sources for catabolism can be organic carbon in organotrophs, inorganic carbon in lithotrophs or light in phototrophs. Glycolysis followed by the tricarboxylic acid cycle (TCA) is the main biochemical means of microbial energy generation. Glucose is broken down to pyruvate and acetyl-coA, which cycles into the TCA, where chemical energy is transferred to ATP, and electrons reduce the coenzyme nicotinamide dinucleotide (NAD<sup>+</sup>) to NADH. In aerobic or anoxic conditions, O<sub>2</sub>, NO<sub>3</sub><sup>-</sup> or NO<sub>2</sub><sup>-</sup> serve as electron acceptors for the transport of electrons from NADH via the electron transport chain over the cell membrane. A pH and charge gradient is generated over the cell membrane, creating a proton motive force which drives membrane transport of compounds into the cell and the generation of ATP from ADP by ATP-ase, transporting protons back into the cell. Under anaerobic conditions, no electron acceptor is available outside the cell membrane, therefore, NADH cannot be produced in the TCA, and pyruvate is reduced to acetate and propionate by the NADH generated in glycolysis via fermentation (Fig. 2 and 3) (Henze 2008).

Aerobic heterotrophs oxidise organic matter by the following reaction:



Fermenting organisms convert glucose to acetic acid by the following reaction:

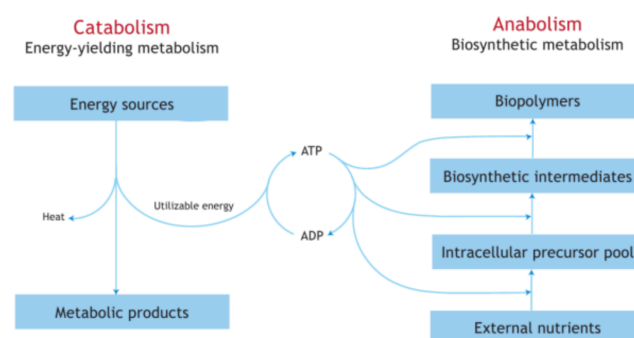


Figure 2. Anabolism and catabolism in microbial metabolism (Henze 2008).

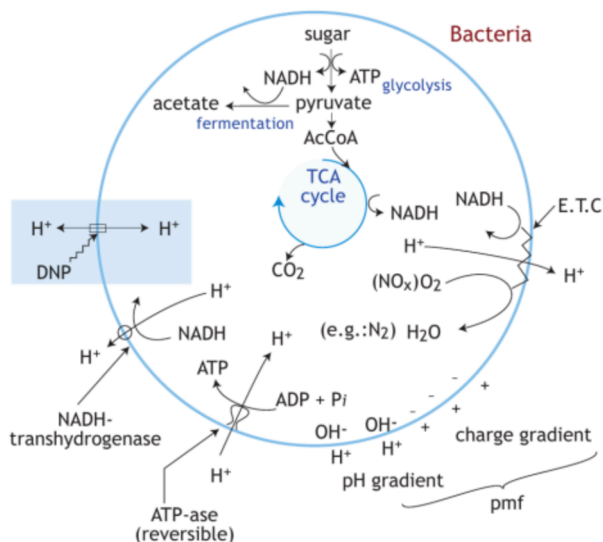


Figure 3. Microbial carbon metabolism for energy generation (Henze 2008).

### *Nitrogen removal*

Ammonium ( $\text{NH}_4^+$ ) and nitrate ( $\text{NO}_3^-$ ) are the main sources of nitrogen in wastewater (Tchobanoglous and Burton 1991). Nitrogen contamination of environmental water sources is toxic to aquatic life; it causes eutrophication of surface water (Ghafari *et al.* 2008) and renders water less responsive to chlorine disinfection. This makes nitrogen removal from wastewater one of the main goals of wastewater treatment. Nitrogen removal is possible with various physicochemical methods, but due to its high efficacy and relatively low cost, biological nitrogen removal is preferred (EPA 1993). It is removed by aerobic and anaerobic conversion to gaseous nitrogen, which is released back into the environment. Ammonium is converted to nitrate via nitrite by aerobic nitrification, and ammonia and nitrite oxidising bacteria are responsible for this process. The nitrate that is produced by nitrification is in turn converted anaerobically to gaseous nitrogen by denitrifying bacteria (Fig. 4) (Ahn 2006).

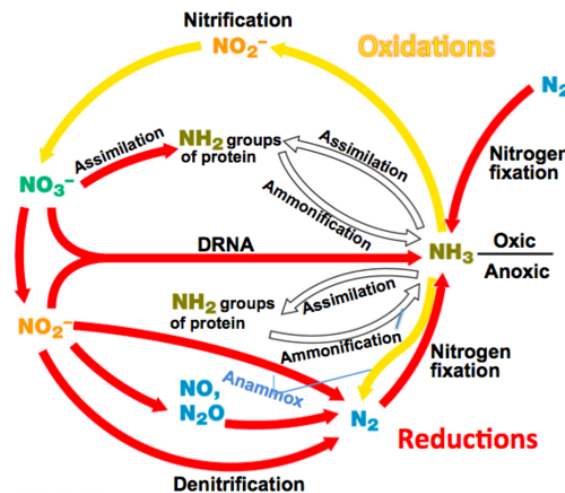
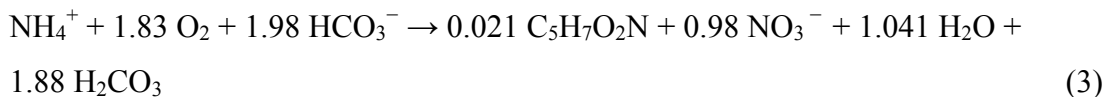
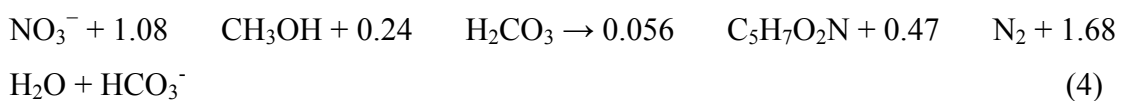


Figure 4. Nitrogen removal in oxic and anoxic conditions (Martinko and Madigan, 2005).

Nitrification is carried out by chemoautotrophs under strict aerobic conditions in two steps, namely the oxidation of ammonia to nitrite followed by nitrite to nitrate. Bacteria that utilise ammonia and nitrate as energy sources, with oxygen as electron acceptor are involved, and common examples include *Nitrosomonas*, *Nitrosopira*, *Nitrosovibrio*, *Nitrosococcus* and *Nitrosolobus*. An equation for nitrification was derived from the experimental determination of oxygen consumption during nitrification by *Nitrosomonas* and *Nitrobacter* (equation 3) (EPA 1993).

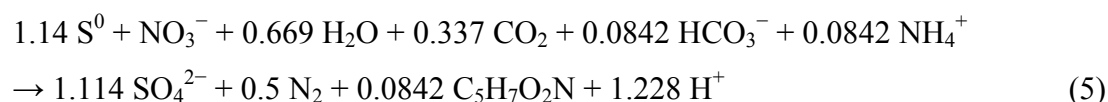


Denitrification is carried out by heterotrophs under anoxic (facultative anaerobic) conditions. These organisms utilise nitrite and nitrate as electron acceptors and carbon as energy source, yielding gaseous nitrogen. Gram-negative alpha and beta *Proteobacteria* such as *Pseudomonas* and some gram-positive bacteria such as *Bacillus* are denitrifiers. In a study using methanol as a carbon source, the following equation was derived for denitrification (equation 4) (EPA 1993):





Nitrification is, in some exceptional instances, possible under microaerophilic conditions, such as with *Magnetospirillum*, which consume oxygen while denitrifying (Bazyliniski and Blakemore 1983). Nitrification can also be performed by autotrophs such as sulfur and hydrogen-oxidising *Thiobacillus denitrificans* (equation 5) with reduced sulfur compounds as electron donor (Robertson and Kuenen 1984).



Finally, anaerobic ammonium oxidation (Anammox) (equation 6) is performed by lithoautotrophs that consume ammonia in the absence of oxygen, where nitrite is the electron acceptor, and belong to the *Planctomycete* bacteria. Anammox can also use CO<sub>2</sub> as a carbon source, utilising nitrite as an electron acceptor (equation 7) (Jetten *et al.* 2001).



Conventional nitrification and denitrification, albeit effective, are energy consuming processes in most wastewater treatment applications that are currently used, and for this reason, there has been a sharp increase in Anammox related research as a more economical method of nitrogen removal from wastewater (Ahn 2006).

### *Phosphate removal*

Phosphate removal from wastewater is essential to prevent eutrophication of surface water bodies (EPA 2004). Biological phosphate removal is a highly effective solution, used widely in municipal and industrial wastewater treatment (Lewandowski *et al.* 2011). In biological phosphate removal, phosphate accumulating microorganisms (PAO) are exploited for their ability to assimilate and store more phosphate than metabolically required (Mino *et al.* 1998). Assimilated phosphate is stored intracellularly as polyphosphate (poly-P) under aerobic or anoxic conditions, with oxygen or nitrate as electron acceptors. Under anaerobic conditions, PAO's assimilate volatile fatty acids and store them intracellularly as polyhydroalkanoates (PHA) by

hydrolysing internal poly-P to provide ATP for the PHA assimilation process, and phosphate is released into the surrounding environment. PAO's are exposed to alternating aerobic and anaerobic conditions, known as enhanced biological phosphate removal, in order to optimise phosphate assimilation. Wasting portions of biomass (poly-P sludge) then allows net removal of phosphate from the system. Dominant PAO's identified in wastewater treatment systems are *Betaproteobacteria* named *Candidatus Accumilobacter phosphatis* (Sudiana *et al.* 1999; Ginige, Keller & Blackall 2005).

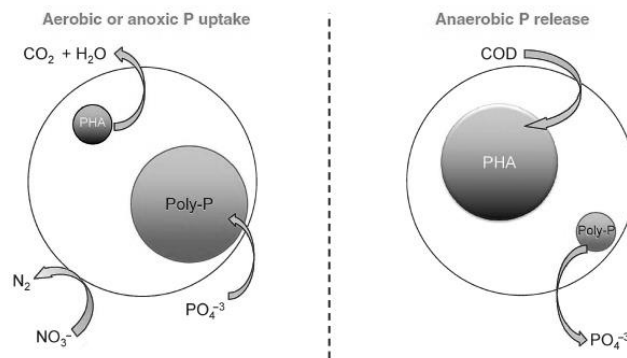


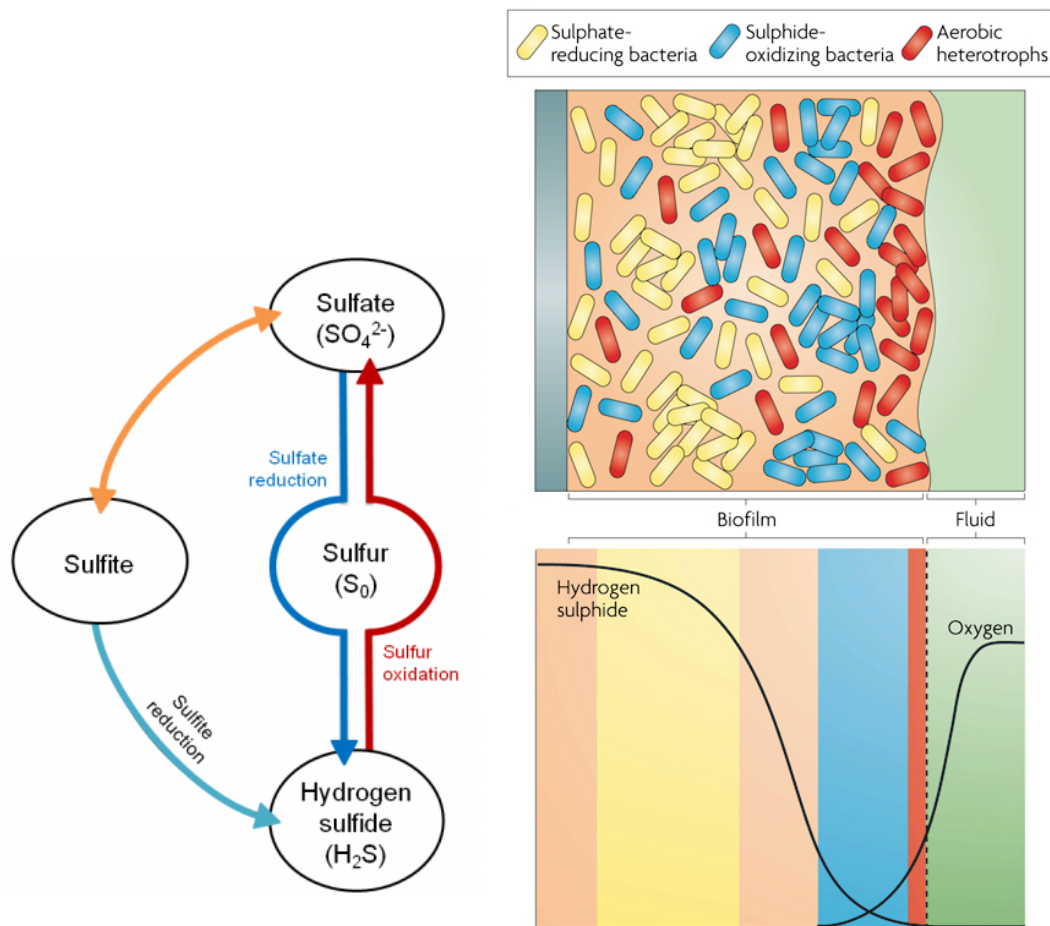
Figure 5. The balance between phosphate assimilation and phosphate release in PAO in aerobic and anaerobic conditions, adapted from Ginige *et al.* (2005).

### *Sulfate removal*

Wastewater rich in sulfate is produced by sources such as the pharmaceutical, paper, pulp and molasses industries (Mohan *et al.* 2005), and does occur in winery wastewater at times (Stevenson *et al.* 2007). Physicochemical removal of sulfate from wastewater is effective, but disadvantages such as solid disposal and high operation costs are encouraging use of biological sulfate removal methods (Silva *et al.* 2002).

The sulfur cycle consists of mineralisation of organic sulfur to the inorganic form of hydrogen sulfide gas, the oxidation of sulfide to elemental sulfur and sulfate, and the reduction of sulfate to sulfide (Wang *et al.* 2005). Microbes then assimilate sulfur

compounds. Sulfur assimilation is carried out by plants, fungi and prokaryotes where sulfate is reduced to organic sulfhydryl groups (R-SH) which are assimilated into biomass. Biological sulfate removal involves aerobic and anaerobic reactions by a range of microorganisms. Anaerobic wastewater treatment is detrimentally influenced by the anaerobic reduction of sulfate, as the H<sub>2</sub>S that is produced is toxic to microorganisms. Sulfate reducing bacteria (SRB) are obligate anaerobes, and in biofilm reactors, they carry out dissimilatory desulfurilation at the anaerobic layer of the biofilm closest to the substrate surface. SRB use low molecular weight molecules as electron donors to reduce organic sulfur molecules to H<sub>2</sub>S. H<sub>2</sub>S gas can diffuse through biomass to where oxygen is present. Here, sulfide oxidising bacteria (SOB), which are colourless sulfide-oxidising bacteria, green and purple sulfur bacteria and some chemolithotrophs such as *Theobacillus denitrificans*, oxidise H<sub>2</sub>S as electron donor to elemental sulfur with oxygen as the electron acceptor and CO<sub>2</sub> as a carbon source (Janssen *et al.* 1997). Elemental sulfur can then in turn be oxidised further to sulfate or H<sub>2</sub>S, thereby resulting in net removal of sulfate from the system (Krishnakumar *et al.* 2005).



(a) (b)  
 Figure 6. (a) The balance between anaerobic sulfate reduction and aerobic sulfur oxidation. (b) A biofilm with SRB in the anaerobic zone close to the substrate surface of the biofilm, SOB in the aerobic or anoxic zone and heterotrophs in the aerobic zones (Stewart and Franklin 2008).

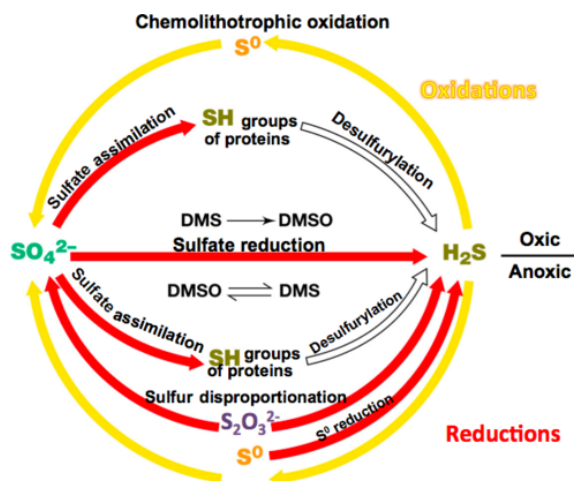


Figure 7. A schematic representation of the sulfate cycle and its oxic and anoxic reactions (Martinko and Madigan, 2005).

## Suspended growth biological reactors applied in winery wastewater treatment

In suspended growth bioreactors, the microorganisms are suspended in the water being treated, and require agitation or stirring to keep the microbes in suspension and the pollutants in the water uniformly mixed and in contact with the biomass for optimal nutrient removal (Leslie Grady *et al.* 2011). The biomass is often recycled within the system, and operation should include a surplus biomass disposal plan. Suspended growth reactors often consist of more than one treatment stage, with varying biochemical conditions between them, allowing multiple biological nutrient removal objectives to be met (Leslie Grady *et al.* 2011).

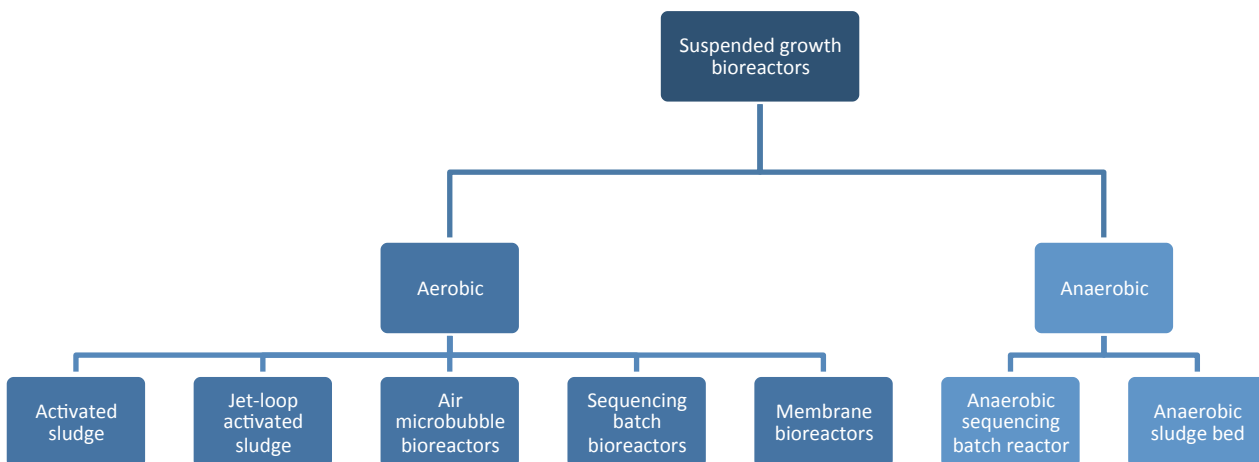


Figure 8. Suspended growth biological reactors tested for winery wastewater treatment.

### *Aerobic suspended growth reactors in winery wastewater treatment*

#### *1. Activated sludge*

The conventional activated sludge (CAS) process is used widely throughout the wine industry for wastewater treatment due to its easy management and efficacy. The major drawback of this method is that it requires nutrient supplementation with N and P to support sufficient microbial growth in winery wastewater treatment (Fumi *et al.* 1995). To address this problem, Beck *et al.* (2005) suggested and optimised the

treatment of winery effluent along with domestic wastewater in a municipal wastewater treatment plant by treating the effluent in two aerated reactors separated by a secondary clarifier, which achieved a 50% reduction in BOD and COD. Bolzonella *et al.* (2010) monitored the performance of a municipal activated sludge treatment plant which also received winery effluent of varying strengths and observed a COD removal of up to 75%. Activated sludge treatment systems can adjust to the variation in volume and wastewater quality associated with winery wastewater, and can achieve COD removal of as high as 98% (Fumi *et al.* 1995), 95% Phosphate removal (Petruccioli *et al.* 2000) and 50% BOD removal (Beck *et al.* 2005). Brucculeri *et al.* (2005) observed COD reduction of 90% and a total nitrogen reduction of 60%. High efficiency in nutrient removal aside, these systems produce small amounts of sludge, do not require pH adjustment, and do not require intensive or highly skilled management. Furthermore, a vertical design results in a system with a small footprint. These qualities make the activated sludge process suitable for medium to large sized wineries (Fumi *et al.* 1995). The process of activated sludge treatment in winery wastewater treatment is thus effective and suitable for optimisation, but these systems remain vulnerable to toxic polyphenols in winery wastewater (Lucas *et al.* 2009). Furthermore, variations in seasonal winery wastewater production can disrupt sludge settleability, cause flock disintegration and increase the amount of solids in the effluent (Brito *et al.* 2007; Lofrano and Meric 2015).

## 2. *Jet-loop activated sludge*

Jet-loop activated sludge reactors (JLR) introduce a jet of fluid delivered by a nozzle into the wastewater sludge mixture, creating a turbulent, well aerated environment which provides optimal conditions for high rate mass transfer and bioconversion of contaminants in wastewater without the need for large oxidation tanks. Initial studies on the application of JLR to high organic load wastewater involved pilot scale systems treating high strength brewery wastewater (Dilek *et al.* 1996; Bloor *et al.* 1995). Such a system removed 97% of the COD, but it was established that only 60-80% of the COD was biodegradable, and that further treatment would be required to remove remaining BOD as well as the high concentrations of suspended bacteria in the effluent (Bloor *et al.* 1995). Petruccioli *et al.* (2000) and Eusebio *et al.* (2004)

applied JLR to winery wastewater, and achieved COD removal of over 90%, even during fluctuations in organic load throughout the vintage season. The major organisms detected suspended in the effluent and in the biofilm that formed on the walls of the system were *Pseudomonas* and *Saccharomyces cerevisiae*, and, as reported in other JLR, there were no filamentous organisms and protozoa present, which was ascribed to the sheer forces present in a JLR, which negatively impacts settleability (Bloor *et al.* 1995; Petruccioli *et al.* 2000), which is the likely reason why JLR has not extensively been applied to winery wastewater treatment aside from pilot scale projects, despite its advantageous compact footprint.

### 3. *Air microbubble bioreactors*

Air microbubble bioreactors are similar to JLR, but in order to optimise mass transfer, a venturi injector, coupled with multiple nozzles introduces a stream of air into the suspended biomass. This yields a system that is compact, and is highly effective, with a COD removal of 93-99% with a retention time of 14-15 days (Oliveira *et al.* 2009a; Silva *et al.* 2012; Silva 2012).

### 4. *Sequencing batch reactors*

Sequencing batch reactors (SBR) are activated sludge processes which operate in stages, with different biochemical environments and agitation present in each stage. SBR was first used for winery wastewater treatment by Torrijos and Moletta (1997), with a 95% removal of soluble COD and 97.5% removal of BOD during the harvest period. Aerobic granular SBR stably removes up to 95% of organic load at fluctuating influent concentrations and allows reactions and settling to occur in one tank, without the need for additional clarifiers, lowering maintenance and operation costs (López-Palau *et al.* 2012). Compared to wetlands and ponds, SBR have a smaller footprint and higher tolerance to seasonal fluctuations in organic load and flow volumes, and are therefore widely implemented for winery wastewater management (Lopez-Palau *et al.* 2009; McIlroy *et al.* 2011).

## 5. Membrane bioreactors

Membrane bioreactors (MBR) provide a smaller footprint solution for winery wastewater treatment than activated sludge processes, with no need for additional clarifiers, reduced sludge production and constant efficacy throughout fluctuations in organic load and flow volume of winery effluent (Valderrama *et al.* 2012a). They remove organics, suspended solids, nitrogen and phosphorous whilst also removing microbial contamination (Artiga *et al.* 2005b; Guglielmi *et al.* 2009). In a comparison of full scale applications of MBR and CAS to winery wastewater following harvest, the two systems delivered similar COD removal, with 97% for the MBR and 95% for the CAS. The MBR delivered effluent that met international guidelines for re-use in terms of disinfection based on total and fecal coliforms, whilst CAS effluent required further treatment. An estimate of operational cost for CAS and MBR showed that the systems have similar operational costs, but that MBR was less labour intensive (Valderrama *et al.* 2012a).

### *Anaerobic suspended growth reactors in winery wastewater treatment*

Anaerobic digestion is the biological transformation of organic matter to biogas by anaerobic microorganisms. Biogas consists of methane, carbon dioxide, and to a lesser extent di-hydrogen, carbon monoxide, and di-hydrogen sulfide (Moletta 2002). This reaction occurs in two steps, firstly the fermentation of organic matter by acidogenic bacteria to volatile fatty acids, alcohol, acetic acid, di-hydrogen and carbon dioxide. Secondly, acetogenic bacteria convert volatile fatty acids (VFA) and alcohol into acetic acid, hydrogen and carbon dioxide. Finally, methane is produced by acetoclastic methanogens from acetic acid, and hydrogenophilic methanogens from hydrogen and carbon dioxide (Moletta 2005). The major advantages of anaerobic digestion are lower energy requirements in the absence of aeration, and that more than 50% of COD can be converted to biogas, which is a renewable fuel, whilst producing less sludge than aerobic processes (España-Gamboa *et al.* 2011).

Anaerobic suspended growth reactors are generally applied to winery wastewater that contain high concentrations of suspended solids, and can be completely mixed reactors with settlers and sludge recirculation, such as an anaerobic sequencing batch



reactor or anaerobic sludge beds, where the reaction and settling occurs inside one digester, such as anaerobic bed reactors and anaerobic lagoons (Moletta 2005).

#### 6. *Anaerobic sequencing batch reactors*

Anaerobic sequencing batch reactors (ASBR) are used widely for the treatment of glucose rich vinasse and high organic wastewater from the food industry, achieving effective COD removal and hydrogen production (Venkata *et al.* 2007; Sreethawong *et al.* 2010; España-Gamboa *et al.* 2011). ASBR is a fill and draw process, where wastewater is added to a digester containing anaerobic sludge, which is mixed. Ruíz *et al.* (2002) showed that ASBR effectively removes 98% of COD in winery wastewater with an influent of 8600mg/L at a retention time of two days, with a break in gas production during the transition from fermentation of organic matter to the breakdown of VFA. ASBR is not frequently used at wineries. In a study investigating the co-digestion of winery wastewater with swine manure in a continuously stirred reactor, winery wastewater was added to the manure in increasing increments, showing effective COD reduction and hydrogen production improving with each added increment of winery wastewater.

#### 7. *Anaerobic sludge beds*

Non-aerated evaporative ponds and lagoons function as anaerobic sludge beds, and are used at many wineries to store and treat winery wastewater due to their low running cost, technical simplicity and adaptability to seasonal fluctuations in effluent. Quiescent winery wastewater, however, quickly becomes anaerobic, causing VFA formation. Furthermore, sulphate-reducing bacteria will produce hydrogen sulphide in winery wastewater that is rich in sulphates. Both hydrogen sulphide and VFA's have high olfactory indexes (Qatibi *et al.* 1990; Le Cloirec *et al.* 1991), causing malodour, a common complaint where ponds and lagoons are used (Guillot *et al.* 2000). Successful remedies for the malodour at ponds include acidification as a pre-treatment step, which reduced odour intensity by a factor of ten (Desauziers *et al.* 2006), the addition of nitrate to odorous ponds, which assisted microorganisms in VFA degradation (Bories *et al.* 2005b). In a pilot scale system, aerated lagoons were operated in a sequential, fed-batch mode, and achieved 91% COD removal after 21

days of operation (Montalvo *et al.* 2010).

### **Biofilm reactors applied in winery wastewater treatment**

Fixed growth biological contact reactors retain biomass within a biofilm that is attached to a substrate exposed to wastewater in the presence or absence of aeration. Soluble organic micro and macromolecules diffuse through the EPS, allowing the biofilm to oxidise readily biodegradable COD at a high rate (Andreottola *et al.* 2005).

Biofilm reactors can achieve similar results in wastewater treatment as suspended growth bioreactors in terms of carbon removal, nitrification, denitrification and sulfur removal. The biochemical reactions involved are dependent on the diffusion of substrates into the EPS, therefore mass transport limitations should be taken into consideration (Lewandowski *et al.* 2011). A drawback of biofilm reactors is a slightly longer start-up period, allowing time for the biofilm to develop (Andreottola *et al.* 2005).

Biofilm reactors can be grouped into packed bed reactors, which could be stationary, or moving, rotating contact reactors and biologically active filters (Leslie Grady *et al.* 2011; Lewandowski *et al.* 2011).

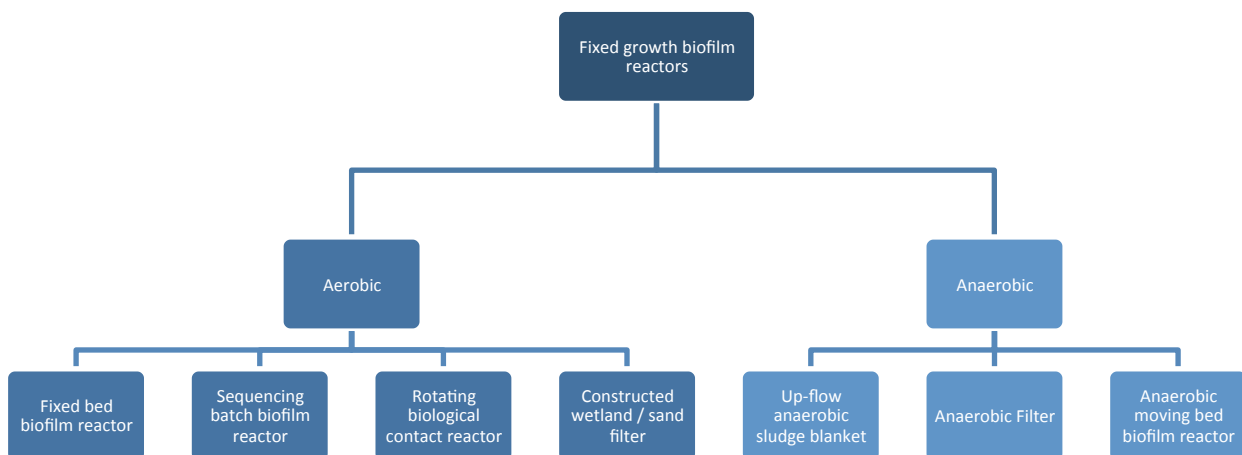


Figure 9. Fixed growth biological reactors tested for winery wastewater treatment.

## *Aerobic Biofilm reactors*

### *1. Fixed bed biofilm reactors*

Fixed bed biofilm reactors (FBBR) are aerobic three-phase biofilm reactors consisting of ventilated packed towers of media. Wastewater trickles over the media with or without circulation, and a biofilm develops (Lewandowski *et al.* 2011). Wastewater is introduced to a packed tower of media within a retaining structure by a distribution system that disperses the wastewater over the media, allowing it to trickle through the system by gravity. The porous fill media can be rock, slag, plastic cross- or vertical flow fill media blocks or randomly shaped plastic biofilm carrier structures with a large void space for proper aeration and prevention of clogging (Metcalf and Eddy 2003). When compared to CAS, FBBR systems have the advantage of a smaller volume and a higher specific biomass contact area, resulting in a smaller footprint; reduced bulking problems, as only detached biomass reaches the settler; no need for backflow and backwashing, due to the high void volume, and easier process management overall.

FBBR are effective at removing soluble organic matter from winery wastewater and the oxidation of ammonia to nitrate (Kim *et al.* 2014), but are ineffective at removing insoluble organics from wastewater, and are not widely applied to winery wastewater. Liquid-solid separation is required as there is a net production of total suspended solids originating from the breakdown of biomass. Andreottola *et al.* (2005) achieved a 91% reduction in COD with a two-stage trickling filter, and found that the remaining COD was resistant to bio deterioration. Kim *et al.* (2014) combined a trickling filter with a partially saturated vertical constructed wetland (VFCW) for winery wastewater treatment. The trickling filter served as the first round of treatment, followed by two rounds of partially saturated VFCW. The bulk of the dissolved carbon and nitrification took place in the trickling filter and the first step of VFCW with a COD and nitrogen reduction of over 94%.

## 2. Sequencing batch biofilm reactors

Sequencing batch biofilm reactors (SBBR) combine MBR with SBR. The presence of the plastic media, however, means that settlement cannot take place in the oxidation tank as with SBR, requiring a separate settling tank, compromising the compactness of the system. In a pilot scale application to winery wastewater, a COD reduction of between 86% and 99% was achieved (Andreottola *et al.* 2002).

## 3. Rotating biological contact reactors

Rotating biological contact reactors (RBC) consist of corrugated media on a rotating axis, forming shafts of media, which rotate through a reaction area by air or motor drive (Patwardhan 2003). The axis rotates slowly, exposing biofilm that develops on the media to wastewater and air alternately. The rotating reactor is covered to keep light from entering, preventing algal growth. Reactors can be arranged in stages in series to increase treatment efficiency, and each stage can have multiple shafts operating in parallel, increasing the treatment capacity of the system. The density of the media used influences the application. Low density media has a lower specific surface area available for biofilm growth, and is typically used at the initial treatment stages in a RBC to remove the bulk of BOD and preventing solid accumulation further downstream. Higher density media, with higher biofilm supporting capacity, is typically used for nitrification (Leslie Grady *et al.* 2011; Lewandowski *et al.* 2011). In a recent review of all current research of RBC applications in wastewater treatment, Hassard *et al.* (2015) highlights how operational adjustments such as gaseous headspace, submergence (Courstens *et al.* 2014), rotation speed and biofilm age control (Yun *et al.* 2004) can be manipulated to achieve optimal conditions for specific waste removal by creating different biochemical environments (Wuertz *et al.* 2004). A small scale evaluation of a RBC applied to winery wastewater showed only a 43% reduction in COD, but at a retention time of only 1h. The study also investigated the microbial community within the biofilm and revealed that yeast also play a major role in COD removal. When compared to CAS and other suspended growth systems, the compact size, versatility, low maintenance and cost effective system was worth considering as a treatment option for winery wastewater, as higher

efficacy was expected with longer retention time (Malandra *et al.* 2003). The future of RBC reactors for high organic load wastewater will likely be in combination with suspended growth as a part of novel hybrid activated sludge processes, which deliver improved organic removal rates and denitrification (Hoyland *et al.* 2010).

#### 4. *Constructed wetlands and biological sand filters*

Constructed wetlands consist of contained basins filled with sand, gravel or earth, and are categorised according to the type of water flow, namely surface or subsurface flow, whether macrophytic growth is present, the nature of the growth, and the flow path of sub-surface flow wetlands, which can be vertical or horizontal (Vymazal 2014). When compared to other biological treatment methods, constructed wetlands offer cost effective treatment of winery wastewater with low energy consumption and operational costs, providing the winery has the land available to accommodate the system (Christen *et al.* 2010). A wide variety of constructed wetlands are successfully implemented at wineries for the reduction of organic load (Grismer *et al.* 2003; Arienzo *et al.* 2009b; Zingelwa *et al.* 2009; Serrano *et al.* 2011). A pre-treatment step of coarse filtration and settlement (Christen *et al.* 2010) or anaerobic digestion (De la Varga *et al.* 2013) will avoid clogging of the wetland drainage. Christen *et al.* (2010) used a planted wetland in a bed of porous soil, draining vertically, which was flood-irrigated with winery wastewater to achieve a 87% reduction in BOD. (Serrano *et al.* 2011) achieved up to 93% COD and 95% BOD removal in a series of three parallel subsurface horizontal flow constructed wetlands coupled with a hydrolytic up-flow sludge bed as pre-treatment, and established that temperature and surface loading rate were the main parameters that influenced the efficacy of the system. In the hybrid VFCW investigated by (Kim *et al.* 2014), the bulk of the COD removal and nitrification occurred in the trickling filter pre-treatment step, but denitrification occurred in the flooded zone of the partially saturated VFCW.

A major drawback of planted constructed wetlands for winery wastewater treatment is the fact that the macrophytic wetland species are sensitive to the high concentrations of phytotoxic compounds in winery wastewater (Arienzo *et al.* 2009b). Sand bioreactors are biological sand filters that serve as unplanted constructed wetlands. A research group in South Africa have investigated the performance and microbial

ecology of pilot scale biological sand filters applied to winery wastewater (Welz *et al.* 2011; Welz *et al.* 2012; Rodriguez-Caballero *et al.* 2012; Ramond *et al.* 2013; Welz and Le Roes-Hill 2014; Welz *et al.* 2014a). The performance and microbial community structure of a biological sand filter treating simulated winery wastewater of two different compositions was assessed, which showed that the redox status has a major influence on the selection of bacterial consortia, with different communities detected at superficial depths than at deeper levels (Welz *et al.* 2014b). Organics were effectively removed and glucose, ethanol and phenolic compounds were degraded more effectively at deeper levels, where VFA accumulation is also favoured (Welz *et al.* 2014b). A COD removal of 98% and a removal of 99,2% phenolics was reported (Ramond *et al.* 2013). Acetate accumulation over time was also identified as a problem in pilot scale investigations (Welz and Le Roes-Hill 2014). Therefore, alternating redox environments were recommended for effective organics removal, which can be achieved by including intermittent aeration, or by configuring multiple reactors in series. The issues of operational cost and availability of surplus land were addressed by Litaor *et al.* (2015) who designed a mobile hybrid biological sand filter system consisting of a coagu-flocculation module with nanocomposites to reduce TSS, followed by a series of containers filled with volcanic tuff of progressively decreasing particle size. Each container is equipped with forced-air circulation, increasing treatment capacity and efficacy. The system was mounted on two flat lorry beds, which enabled easy transportation before and after vintage season. 95% of TSS was reduced by the nanocomposite module, while the COD removal efficiency was between 90 and 95%. Clogging of the volcanic tuff beds was addressed by lifting the nylon sacks containing the tuff material, and hosing off the layer of fouling which accumulated on the outside, which restored impaired drainage. This was maintained by removing, washing and drying the tuff at the end of the vintage season. Alternatively, the application of a H<sub>2</sub>O<sub>2</sub> solution was found to be effective in prevention of clogging. With COD removal of as high as 95-98% (Welz *et al.* 2014b; Litaor *et al.* 2015), unplanted constructed wetlands or biological sand filters can be considered the most effective aerobic methods for reducing organic load in winery wastewater.

### *Anaerobic biofilm reactors*

Anaerobic biofilm reactors have the advantage of producing lower volumes of sludge, and requiring less energy input than aerobic systems, and can generate profit by producing high quality biogas. They are however, sensitive to spikes in organic concentrations and low pH, which are both characteristic of winery effluent, which can cause disintegration of granulated biofilms and failure of the process. Furthermore, VFA accumulation can be problematic, and treatments by these systems alone are not sufficient to achieve low COD concentrations that comply with standards for discharge (DWAF, 2004).

#### *5. Up-flow anaerobic sludge blanket*

In an up-flow anaerobic sludge blanket (UASB) reactor, wastewater enters an anaerobic reactor from the bottom, and flows upward through a blanket of sludge, which is suspended by means of the combined effects of the upward flow and gravity. Flow conditions select for organisms that have the capability to aggregate and form sludge biofilm granules (Leslie Grady *et al.* 2011). UASB are effective at reducing organics in winery wastewater, with COD reductions of 70% achieved (Kalyuzhnyi *et al.* 2001; Keyser *et al.* 2003). Efficacy of the process is, however, strongly influenced by the organisms used in the seeding sludge, and a conditioning step that acclimatises the sludge to the low carbohydrate conditions in winery wastewater will assist granulation, which will reduce the start-up time and increase the COD removal efficiency to 90% (Keyser *et al.* 2003). Furthermore, temperature and pH influences the granulation process, with no granulation detected at submesophylic conditions (18-21C), but good granule development at mesophylic conditions (35C), which is imperative to adequate sludge retention (Kalyuzhnyi *et al.* 2001). The organic load applied to a UASB after start-up should be low and increased gradually once COD removal efficiency is above 90%. This will ensure a successful start-up within one day once the plant has been out of operation (Wolmarans and De Villiers 2004). In their review, Ioannou *et al.* (2015) noted that in all studies applying UASB to winery wastewater, the COD was only reduced sufficiently to below 800 mg/L in two applications (Ruíz *et al.* 2002; Keyser *et al.* 2003). In all other cases, the COD of the

treated effluent was above 800 mg/L, which means a polishing step is required before the effluent will be suitable for environmental discharge or re-use.

## 6. *Anaerobic filters*

Anaerobic filters (AF) are up-flow bioreactors filled with high void volume, high specific area packed media, which is the same as the packed media used in aerobic trickling filters. Effluent is recirculated to maintain uniform hydraulic loading, and treatment is carried out by suspended and attached biomass. The majority of the active biomass is retained, while effluent is released from the top of the system, and biogas is collected from the base of the reactor (Leslie Grady *et al.* 2011). In one example of successful application to winery wastewater, a system has shown 96-98% COD reduction at a winery over a four year period with negligible production of VFA's and producing biogas with a methane concentration of up to 70%, making it a viable energy source. Furthermore, this system recovered fast from destabilisation due to organic overload, and had a short start-up time after a long period of being out of use (Molina *et al.* 2007). In another study investigating the effect of reactor size and specific surface area on performance, it was found that the smallest reactor with the highest specific surface area had the best performance in terms of COD reduction and excess sludge production. The system consumed less energy and produced less sludge than high rate aerobic systems, but COD remained above 3 000 mg/L, which means further treatment is required prior to discharge (Ganesh *et al.* 2009). In a recent application to rice winery wastewater treatment, the effect of hydraulic retention time (HRT) on system performance in terms of COD reduction and methane production was investigated. With an initial HRT of 10 days, COD reduction was 91%, but a shorter HRT led to more methane production. HRT was therefore reduced as far as possible to promote methane production without a major loss in COD removal efficiency. The optimal HRT was found at 2.2 days with >84% COD removal (Jo *et al.* 2015a). It should be noted that the 84% COD removal still yielded an effluent with a COD of >2 500 mg/L, thus further treatment is required prior to discharge.



## 7. *Anaerobic moving bed bioreactors*

Anaerobic moving bed bioreactors (AMBB) first showed promise in winery wastewater treatment in a bench-scale treatment, which achieved up to 89% COD removal at a HRT of 1.55 days (Sheli and Moletta 2007). The same group achieved 86% COD removal at a HRT of 2.5 days in a full-scale reactor using small, low density polyethylene support structures as biofilm carriers. Furthermore, the group investigated the effect of packing material structure and supplementation with trace elements on the performance of the system, and found that short term dosing of essential trace metals and using fill media with higher specific surface area increases the COD removal efficiency of the system (Chai *et al.* 2014).

### **Concluding remarks**

Table 4 summarises all the biological wastewater treatment methods that have been applied to winery wastewater at a full scale to date. Figure 10 indicates the most frequent methods applied to winery wastewater in general (Mosse *et al.* 2011). Wineries can implement different strategies to minimise wastewater production (Conradie 2015), but winemaking will always produce some effluent that requires responsible and efficient treatment and disposal. Non-biological treatment methods have been found to be effective as pre-or post-treatment for biological treatment methods. In terms of residual COD after treatment, aerobic and anaerobic biological wastewater treatment are the most effective methods of winery wastewater treatment (Fig. 11) (Ioannou *et al.* 2015). Small wineries with available land will likely benefit sufficiently from well managed wetlands and aerated ponds, while large wineries will benefit from activated sludge and anaerobic reactors, and very large wineries may find that investing in large anaerobic systems with biogas capture for meeting energy requirements a viable option. Although promising, limited data is available for MBR and membrane filtration and separation processes, and more full scale studies are required to confirm the efficacy and performance of these systems in practice (Mosse *et al.* 2011). Biofilm bioreactors offer resistance to fluctuations in organic load and wastewater volumes, have low sludge production, have a high-specific area to volume ratio, thus a small footprint and offer efficient mass transfer across the nutrient and biochemical environment gradient that exists across the layers of the biofilm, making

fixed growth biological reactors ideal for winery wastewater treatment (Andreottola *et al.* 2002; Sheli and Moletta 2007; Serrano *et al.* 2011). Literature suggests that AOP are promising as post-treatment for biological processes, and that in future, the most effective systems are likely to be hybrid systems, combining more than one treatment method (Ioannou *et al.* 2015). From an economical point of view, research around proper cost analyses of investment and operational costs of new and implemented treatment systems is lacking. There is no standardised set of parameters on which running and installation costs of new and implemented systems can be compared, making it difficult to determine which is the best available technology for a specific winery, and future studies should be done to establish such a standard set of costing parameters (Ioannou *et al.* 2015).

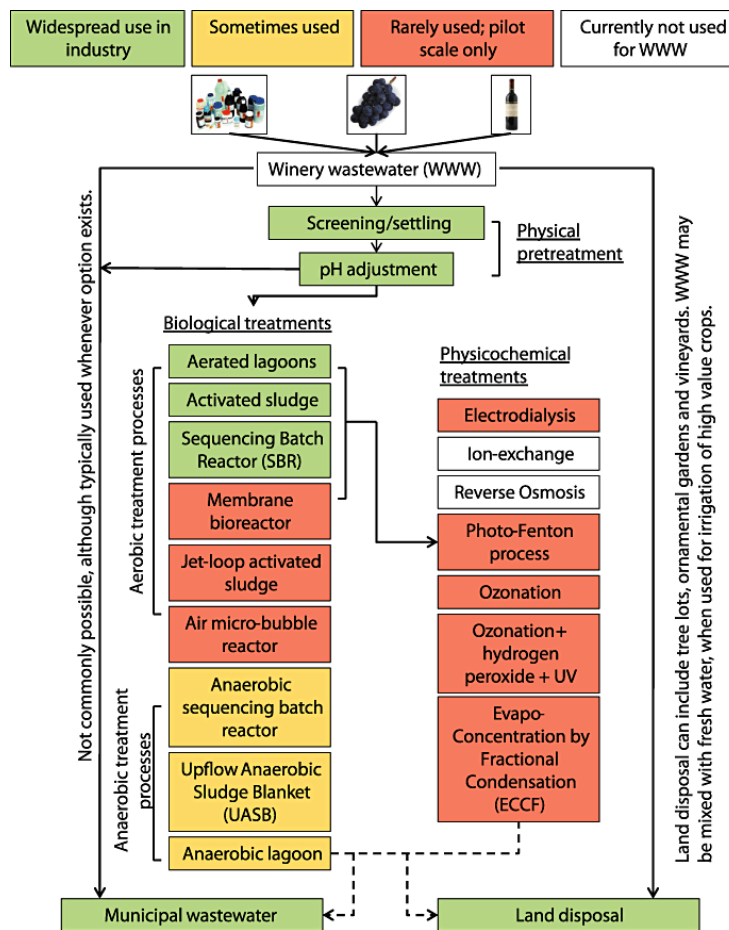
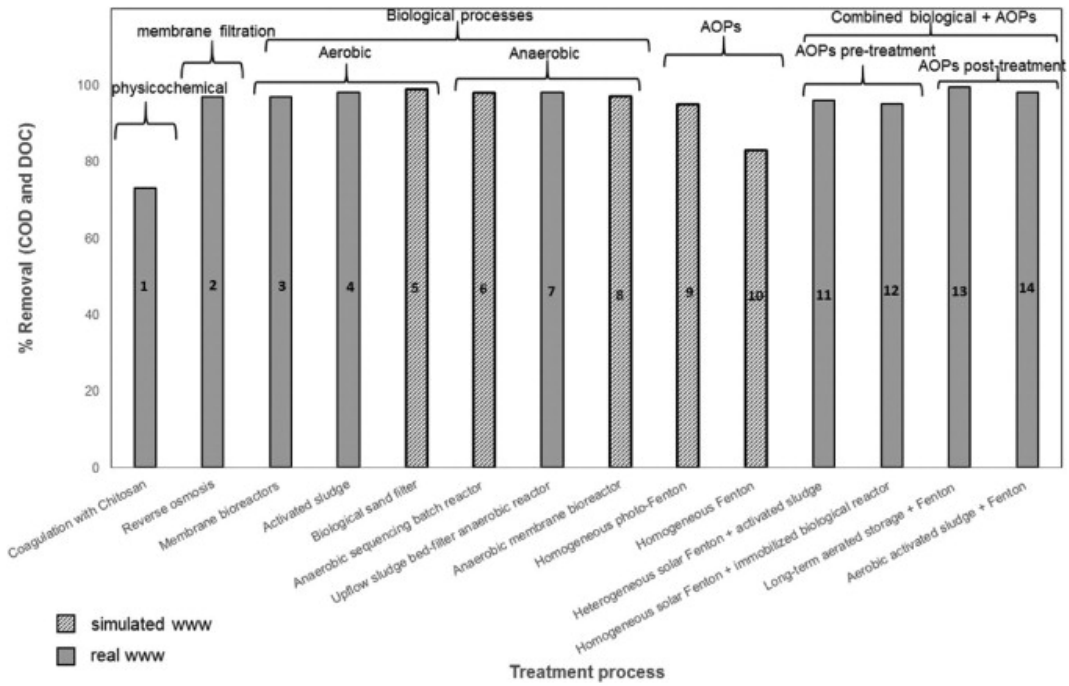


Figure 10. Current and potential treatment methods for winery wastewater and the fate of treated effluent (Mosse *et al.* 2011).



Bar N <sup>o</sup>	Influent COD (mg L <sup>-1</sup> )	Residual COD (mg L <sup>-1</sup> )	Bar N <sup>o</sup>	Influent COD (mg L <sup>-1</sup> )	Residual COD (mg L <sup>-1</sup> )
1	1550	418	8	4400	132
2	5353	140	9	2250 *(DOC)	112 *(DOC)
3	2500	75	10	970	164
4	5500	110	11	3300	132
5	7587	75	12	2958	147
6	8600	172	13	20000	100
7	8000	160	14	18500	370

Figure 11. A summary of the most effective non-biological, biological, advanced oxidation and combined methods of winery wastewater treatment based on COD removal (Ioannou *et al.* 2015).

Table 4. A summary of biological treatment methods for winery wastewater

	Treatment	Advantages	Disadvantages	Applicability	COD reduction %	residual COD	reference
Suspended growth biological reactors	Aerobic	Activated sludge	Easy management.	Energy intensive. Requires nutrient addition (N, P).	Medium to large wineries	98%	130 mg/L (Fumi <i>et al.</i> 1995)
		Jet-loop reactor	High efficiency with low energy requirement.	Limited applications in practice	Potentially all wineries	98%	500 mg/L (Petruccioli <i>et al.</i> 2002)
		Air microbubble reactor	High efficiency.	Limited applications in practice	Potentially all wineries	93%	200 mg/L (Oliveira, <i>et al.</i> 2009b)
		Sequencing batch reactor	Simple automation, low running costs, applied widely in the industry.	Requires multiple tanks, large footprint.	Medium to large wineries	95%	90 mg/L (Lopez-Palau, <i>et al.</i> 2009)
		Membrane bioreactor	High efficiency, small footprint, low sludge production.	High installation cost, membrane fouling	Large wineries requiring high quality effluent	97%	900mg/L (Valderrama <i>et al.</i> 2012b)
	Anaerobic	Anaerobic Sequencing batch bioreactor	Low sludge production, biogas production.	Requires multiple tanks, large footprint.	Medium to large wineries	98%	172mg/L (Ruiz <i>et al.</i> 2002)
		Anaerobic sludge bed reactor	Low cost, biogas production.	Long start-up time, malodour.	All wineries	91%	1683mg/L (Montalvo <i>et al.</i> 2010)
Biofilm biological reactors	Aerobic	Fixed bed biofilm reactor	Resistant to fluctuations in COD concentration and flow.	Requires sludge separation	All wineries	91%	642 mg/L (Andreottola <i>et al.</i> 2002)
		Sequencing batch biofilm reactor	High efficiency, no bulking, simple management	Requires pH control, separate sludge settling tank, limited application in practice, nutrient supplementation.	Medium to large wineries	99%	88mg/L (Andreottola <i>et al.</i> 2002)
		Rotating biological contact reactor	Short start-up, low maintenance, no bulking, compact footprint.	Low efficacy, requires pH control, low treatment rate, nutrient supplementation, limited application in practice.	Small to medium wineries	43%	3475 mg/L (Malandra <i>et al.</i> 2003)
		Constructed wetlands/Biological sand filters	High efficiency, capacity for high organic load, low energy use.	High retention time, large footprint, sensitive to TSS and pH fluctuations, malodour.	Medium to large wineries	73%	400 mg/L (Serrano <i>et al.</i> 2011)
	Anaerobic	Up flow anaerobic sludge blanket	High efficiency, low sludge production.	High installation cost.	Medium to large wineries	90%	260mg/L (Keyser <i>et al.</i> 2003)
		Anaerobic filters	High efficiency at low HRT, Low sludge production, biogas production.	Requires low TSS, clogging	Medium to large wineries	84%	2500mg/L (Jo <i>et al.</i> 2015b)
	Anaerobic moving bed bioreactors	Easy management, high biomass retention, biogas production.	Requires trace element supplementation.	Medium to large wineries	89%	5011mg/L (Sheli and Moletta 2007)	

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## CHAPTER 3

### Understanding biofilms in wastewater treatment: biofilm processes and a modern three tiered approach to investigating biofilm communities in wastewater treatment systems

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## Introduction

The functionality of a biofilm wastewater treatment system is dependent on the collective metabolism of the microbial consortium within the biofilm, and is governed by a complicated assembled community within the biofilm structure, in which a multitude of microorganisms interact via quorum sensing and horizontal gene transfer (Verstraete 2007).

Bioreactor performance depends on good bioreactor design, which provides the optimal environment for selective development of microorganisms with the most desirable set of metabolic capabilities for the treatment requirements at hand. Until recently, bioreactors were considered “black boxes” with all knowledge on their functionality based on observations such as effluent quality, making these systems hard to predict and control (Herrero and Stuckey 2015). It is possible that poor bioreactor performance can be ascribed to a shortage of specific microorganisms with key metabolic capabilities for degradation of the target contaminants (Herrero and Stuckey 2015) and that performance can be improved by changing the operational parameters within the system to favour the development of more microorganisms with desirable metabolic capabilities, as demonstrated in constructed wetlands with different oxygen conditions (Adrados *et al.* 2014).

A better understanding of the development and processes of the biofilm and the composition of the microbial community under specific conditions will be a major advantage in effective wastewater treatment system design (Lazarova and Manem 1995).

To date, a range of methods have been used to study community structures within biofilms (Steele *et al.* 2005). Culture dependent methods, such as the Biolog system can supply information on metabolic activities of a sample (Almstrand *et al.* 2013b), but is limited due to the exclusion of unculturable organisms. It is estimated that only 1% of a microbial community can be isolated from an environmental sample by culture-dependent methods (Amann *et al.* 1995; Hugenholtz *et al.* 1998; Torsvik *et al.* 1998). Molecular approaches each have their own limitations, but are more robust and inclusive (Amann *et al.* 1995).



Sequences gained through next generation sequencing (NGS) can be used to synthesise Fluorescent in situ hybridisation (FISH) probes targeting microorganisms of interest. FISH coupled with confocal laser scanning microscopy (CLSM) can be a powerful tool to investigate biofilms at a visual level, especially the distribution of specific microorganisms in a biofilm (Almstrand *et al.* 2013b). Fingerprinting techniques such as denaturing gradient gel electrophoresis (DGGE) separate amplified DNA markers based on sequence heterogeneity (Muyzer *et al.* 1993). Terminal restriction fragment length polymorphism (TRFLP) determines community structure based on heterogeneity in restriction digest sites on conserved genes between taxa (Fisher and Triplett 1999). DNA microarrays (Dubois *et al.* 2004) and automated ribosomal RNA intergenic spacer analysis (ARISA) distinguish members of a community based on 16S-23S intergenic space length (Brown *et al.* 2005). These techniques are cost-effective, and provide high-resolution data of entire microbial communities, providing a means of studying community changes (Borneman and Triplett 1997). Clone libraries and PCR-based molecular fingerprinting techniques can provide information on different taxa present within a microbial community, however, many clones must be sequenced and analysed to determine species distribution and differences between communities, resulting in time, labour and cost intensive methods. Furthermore, traditional PCR based fingerprinting techniques tend to provide data that underestimates the abundance of the most prevalent members of a community and overestimates the least abundant members of a community (Lueders and Friedrich 2003; Brown *et al.* 2005). Recent developments in NGS have made novel techniques accessible for the characterisation of complex microbial consortia through parallel sequencing of short, hyper variable regions of small subunit RNA (Huse *et al.* 2008), making it possible to detect organisms of low relative abundance in a microbial consortium (Petrosino *et al.* 2009; Shokralla *et al.* 2012).

At a phenotypic level, community level physiological profiling (CLPP) can be used to gain insight into the metabolic capabilities of substrate utilisation of a microbial community (Garland *et al.* 2003a).

This review discusses the fundamentals of biofilm formation and processes in

wastewater treatment applications. An overview of the latest available techniques for studying microbial communities within wastewater treatment systems suggests a three-tiered approach to microbial community analyses, with metagenomics for characterising the microbial community, followed by FISH-CLSM for visual confirmation of the presence, and analyses of the spatial distribution of specific groups within the community. Finally a phenotypic investigation into the metabolic capabilities of such a microbial consortium sheds further light on how biofilms function in wastewater treatment applications.

## **The fundamentals of biofilms for wastewater treatment**

### *Biofilm formation*

Communities of microorganisms growing on surfaces as biofilms are the most abundant form of life on earth (Römling 2013). Biofilm formation is a highly organised, step-wise process. In the broad spectrum of biofilm studies, these steps are defined as (1) adsorption, (2) consolidation and (3) colonisation (Notermans *et al.* 1991). It was accepted that the first step in biofilm formation is the adsorption or attachment of planktonic cells to a surface, which then proliferate into microcolonies and produce extra polymeric substances (EPS). Recently, investigations into the mechanism of microbial adhesion to surfaces for biofilm formation has shed light on how exactly planktonic cells adhere to substrate surfaces to form biofilms. It is now recognised that a surface primed with organic matter is a prerequisite for cell adhesion. Wang *et al.* (2012) demonstrated how natural organic matter (NOM) forms a conditioning layer on surfaces in aquatic environments, and that this is a requirement before the attachment of bacterial cells can take place to form biofilms.

The conditioning film consists of dissolved organic matter or EPS secreted by planktonic bacteria. It has been proven that the presence of NOM conditioning films improve the initial adhesion of microorganisms to a surface in freshwater (Fig. 1) (Berman *et al.* 2011; Wang *et al.* 2012).

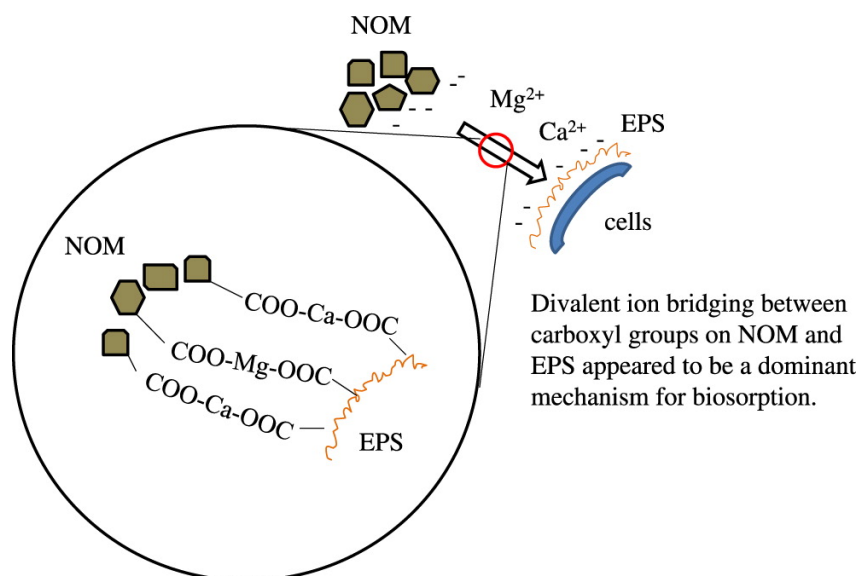


Figure 1. NOM as a priming layer, to which EPS can adhere by forming divalent ion bridges between carboxyl groups on NOM and EPS (Wang *et al.* 2012).

The mechanism of cell attachment and arrangement into microcolonies in young biofilms is not fully understood. An investigation by Zhao *et al.* (2013) into the process of attachment to the substrate and organisation of individual cells into microcolonies as the first step of biofilm formation, demonstrated how exopolysaccharides not only act as a molecular glue between cells and the substrate, but also to enable the attached cells to form self-organised microcolonies. *Pseudomonas aeruginosa* cells secrete the exopolysaccharide PsI, leaving a trail of PsI on the substrate surface. Type IV pili in these organisms play a pivotal role in surface motility, extending from the cell and exploring the region around the cell. It is proposed that the pili interact with PsI, drawing cells towards PsI. Cells attracted towards PsI depositions, will in turn deposit more PsI, attracting more cells to the region, resulting in a positive feedback for the formation of a microcolony at that location (Fig. 2) (Zhao *et al.* 2013).

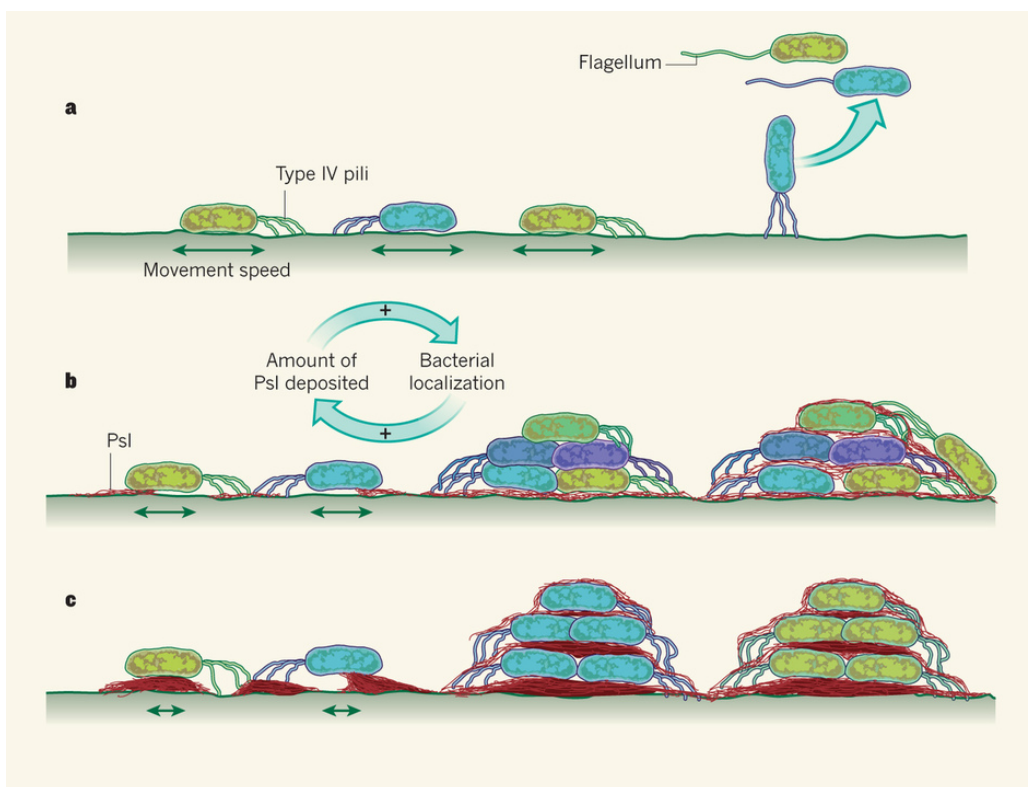


Figure 2. *P.aeruginosa* cells explore surfaces for biofilm formation with type IV pili. Biofilm formation is initiated and directed by the secretion of an adhesion molecule Psl. (a) Mutant *P.aeruginosa* cells produce no Psl, and thus explore large surface areas, without adhering and forming microcolonies. (b) Wild-type *P.aeruginosa* leave a Psl trail on the surface (indicated in red), attracting more Psl secreting cells to the site. The presence of Psl causes the cells to be attracted to the region, and remain there, thus initiating self-organised microcolony formation through a positive feedback loop. (c) Because Psl encourages cells to stay in one place, cells that produce high amounts of Psl do not take part in surface exploration, meaning that daughter cells are retained at the site of cell division, leading to exponential growth and microcolony maturation (Zhao *et al.* 2013).

Attachment of cells to the substrate is also influenced by the physical surface characteristics of the cells and the substrate (Renner and Weibel 2011). Cells with hydrophobic characteristics and rough surfaces will form biofilms more easily than hydrophilic, smooth cells (Mazumder *et al.* 2010). Biofilms form more readily on hydrophobic surfaces due to adhesion through hydrophobic interactions, although adhesion through adsorption to hydrophilic surfaces is also possible (Gómez-Suárez *et al.* 2002).

Microcolonies secrete EPS, providing a protective environment for biofilm growth and development, changing the attachment to the substrate from reversible to

irreversible. These cells are difficult to disperse due to the EPS and attachment to the surface and one another with extracellular adhesive organelles such as curli and pili (Karatan and Watnick 2009).

EPS secretion is initiated by communication between cells within the microcolonies and between microcolonies by quorum sensing, a cell-to-cell communication system which regulates motility and secondary metabolite production (Renner and Weibel 2011). The EPS is an amphiphilic mixture of proteins, nucleic acids, polysaccharides, humic substances, lipids and inorganic complexes (Characklis 1990; Nielsen *et al.* 1992; Frølund *et al.* 1996; Dignac *et al.* 1998; Wagner *et al.* 2009; D'Abzac *et al.* 2010). Carbohydrates can occur freely suspended in the EPS matrix, or as exopolysaccharides on the bacterial cell surface. They can form complexes with proteins, lipids and surface substrates. Polysaccharides can have linear, branched or cyclic structures, most frequently being in the  $\beta$  configuration with 1,3 or 1,4 linkages in the polymer backbone. Proteins in the EPS are derived from living and dead cells, and are responsible for the hydrophobic properties of EPS, assisting attachment of the biofilm to hydrophobic and negatively charged surfaces (Characklis 1990; Allison 2000).

Lecitins are the most abundant proteins in the EPS, and they enable the adherence of pathogenic cells to their hosts, and in biofilms, enable cell-to-cell adhesion. Polysaccharases are also abundant, degrading EPS and components in the surrounding environment, providing nutrients to the cells within the biofilm.

Microcolony structure depends on the conditions in the biofilm system such as nutrient distribution and hydrodynamic forces, as well as the organisms present in the biofilm. Microcolonies, developing and thriving within the microenvironment of the EPS then gradually propagate by rippling, streaming, rolling, detaching and seed dispersal to cover the entire surface, forming a mature biofilm (Fig. 3 and 4) (Lewandowski *et al.* 2011; Stewart 2012).

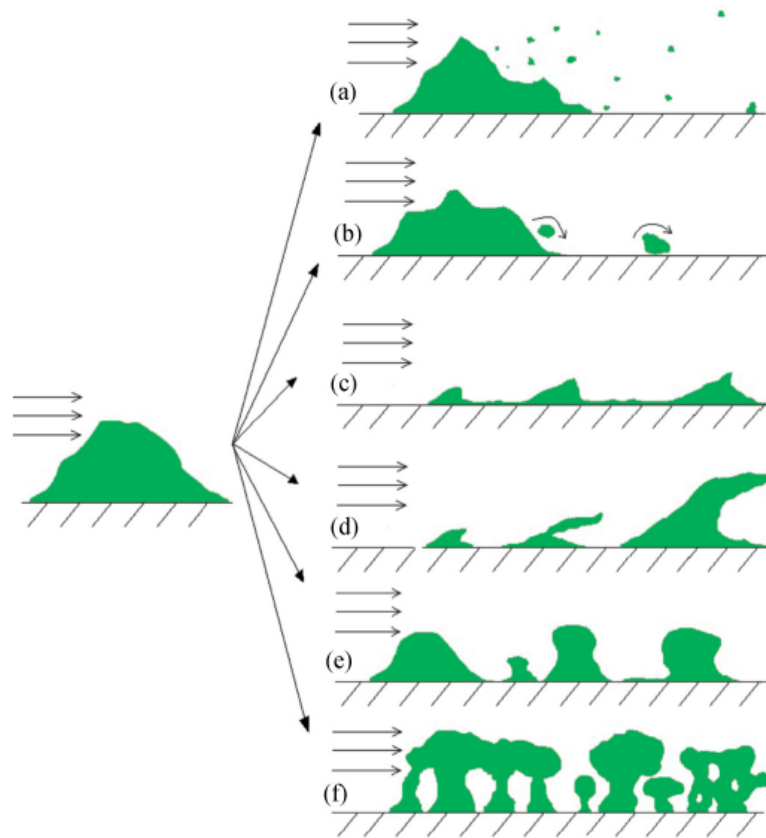


Figure 3. Different patterns of biomass development and distribution in submerged biofilms, variations depending on the organisms within the biofilm, the flow velocity of fluid over the biofilm surface and nutrient distribution in the liquid around the biofilm. (a) Darting, where planktonic cells are expelled from microcolonies, (b) rolling, where bacteria detach in clusters and roll over the substrate surface, (c) ridges form on the biofilm surface (d) streaming fingers of biomass in the direction of flow, (e) mounds in low flow velocity systems and (f) mushroom structures with pores and channels within the biomass (Lewandowski *et al.* 2011).

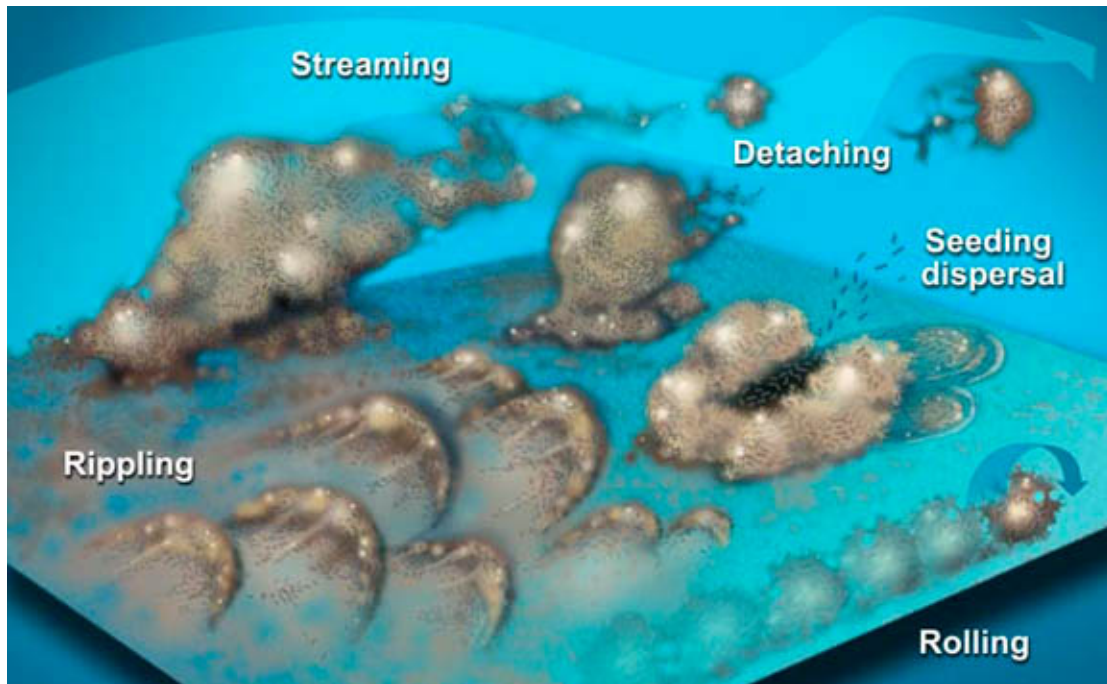


Figure 4. Biofilm propagation on the substrate by rippling, rolling, streaming, detachment and dispersal (Center for Biofilm Engineering at MSU-Bozeman, P Dirckx, 2003).

### *Biofilm systems*

Biofilm systems refer to biofilms along with all the processes that they carry out within their environment. Biofilm systems can exist spontaneously, where they develop in an ideal niche, or due to human intervention. If biofilm development is encouraged, suppressed or controlled in some way, and the activity of the biofilm is measured, monitored and applied for a defined purpose, the biofilm system is known as a biofilm reactor (Lewandowski *et al.* 2011).

### *Processes of a biofilm system*

Biofilm processes include all the biological, physical and chemical processes that affect a biofilm or are affected by the rate of the biofilm development and the microbial processes within the biofilm. These processes are exploited when using biofilm reactors. Broadly speaking, biofilm systems consist of four components, namely the surface to which the biofilm is attached, the biofilm within its EPS matrix, the nutrient containing liquid phase, and the gas phase surrounding the biofilm (Lewandowski *et al.* 2011).

*Biofilm activity*

Average biofilm activity is a useful indicator of the efficacy of a biofilm reactor, and biofilm reactors are optimised in terms of biofilm activity.

Biofilm activity indicates the rate of the utilisation of the growth-limiting nutrient within a bioreactor, and it can be calculated from the mass balance of the growth-limiting nutrient.

$$\text{Biofilm activity} = \frac{(C_{\text{influent}} - C_{\text{effluent}}) \times Q}{A} \quad (6)$$

Where C is the concentration of the growth limiting nutrient and Q is the volumetric flow rate ( $\text{m}^3\text{s}^{-1}$ ) in the reactor, and A is the surface covered by the biofilm. In this instance, very sensitive and specific probes are used to monitor nutrient concentrations.

*Biomass quantification as a measure of biofilm activity*

The rate of biofilm biomass production is also an indicator of biofilm activity, and can be measured in various ways. Simpler and more sensitive methods than nutrient concentration measurements exist, and are particularly relevant in systems where biofilm formation or inhibition is of importance. Examples are various techniques in microscopy, such as scanning and transmission electron microscopy (Bakke *et al.* 2001; Sommerfeld *et al.* 2014), and confocal microscopy for the quantitative three dimensional analyses of biofilm structure with specifically targeted fluorescent dyes (Daims *et al.* 2011; Bar-Zeev *et al.* 2014). Other methods include measuring changes in a surface's ability to reflect light due to biofilm formation and thickening, such as with the rotoscope (Cloete *et al.* 2005) and the quartz crystal microbalance (Reipa *et al.* 2006), image acquisition of biofilm growth over time (Milferstedt *et al.* 2006), piezoelectric sensor devices, which measures the changes in resonance frequency of a tuning fork as a quantitative indicator of biofilm growth (Waszczuk *et al.* 2012) and electrochemical sensors that change their electrochemical behaviour due to biofilm deposition (Pires *et al.* 2013; Zheng *et al.* 2013). Finally, there is also a novel thermal sensor, which measures changes in the thermal excitation of a biofilm to quantify it by



monitoring its thermophysical properties in real time (Reyes-Romero *et al.* 2014).

### *Hydrodynamic forces influencing mass transfer, biofilm propagation and detachment*

In wastewater treatment settings, mass transfer is the biofilm process of greatest importance, as this is the process by which the biofilm will remove pollutants from polluted water. Hydrodynamics at the biofilm-liquid phase interface depend on the flow velocity of the liquid phase over the biofilm, and affects mass transfer of nutrients to and from the biofilm, and thus biofilm activity (Beyenal and Lewandowski 2002). In a model proposed by Lewandowski *et al.* (2011), the effect of hydrodynamics on mass transfer was explained (Fig. 5).

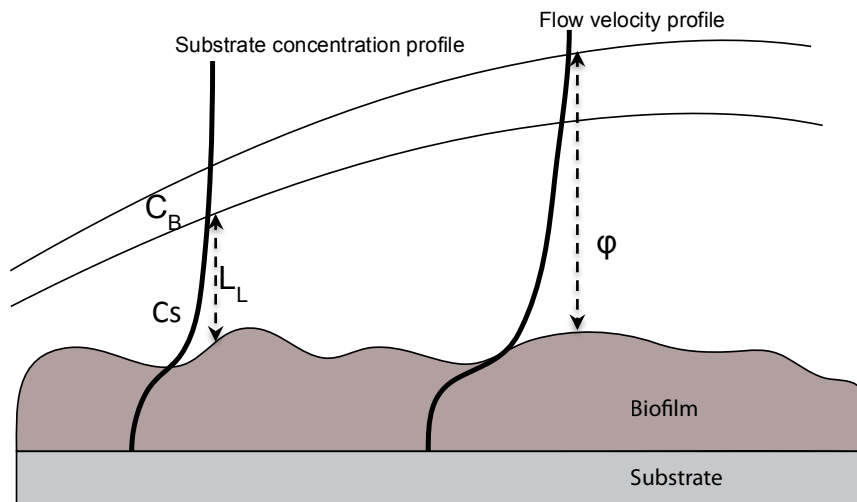


Figure 5. Substrate concentration and flow velocity profiles with growth-limiting nutrient concentration (Lewandowski *et al.* 2011).

In an ideal biofilm model, the average flow velocity of the stream is considered, denoted  $C_B$ . The flow velocity decreases towards the biofilm surface in accordance with hydrodynamics, denoted  $C_S$ . The layer of liquid that is just above the biofilm layer, where flow velocity is decreased due to hydrodynamics, is denoted  $\phi$ . Further away from the surface of the biofilm, where the flow velocity is higher, mass transport is driven by convection currents carrying nutrients away, whereas closer to the surface of the biofilm, in the hydrodynamic boundary layer  $\phi$ , mass transport is due to diffusion. The diffusion is driven by a nutrient concentration gradient which is created by the microorganisms within the biofilm, using the nutrients in the hydrodynamic boundary layer, thus leaving the layer of liquid just above the biofilm,

denoted  $L_L$ , with a lower nutrient concentration. This is the mass transport boundary layer.

When a specific substance is considered, in the case of biofilm activity evaluation, the growth-limiting nutrient, the rate of mass transfer and the amount of mass transfer that is possible at a certain flow rate can be calculated.

The flux of mass into the biofilm, denoted  $k$ , is dependent on the diffusivity of the substance of interest in water, denoted  $D_W$  ( $\text{m}^2 \text{s}^{-1}$ ) and the depth of the boundary layer  $L_L$  (equation 6). The amount of mass that is transferred is the product of the flow velocity and the mass transfer rate (equation 7 and 8) (Lewandowski *et al.* 2011).

$$k = \frac{D_W}{L_L} = \frac{1}{R_L} \quad (7)$$

$$N = k (C_B - C_S) \quad (8)$$

The mass transfer boundary layer decreases in diameter exponentially with increased flow velocity, thus, a slower flow velocity and larger boundary layer will offer more resistance to mass transfer,  $R_L$ , and a slower mass transfer flux. Modelling experiments predict that biofilm reactors with rapid flow velocities will be more effective in terms of mass transfer (De Beer *et al.* 1994; Taherzadeh *et al.* 2012) and this has been confirmed by practical experiments using probes to measure concentration profiles. Examples demonstrating the mass transfer resistance in the boundary layer in studies on oxygen flux into biofilms demonstrated oxygen concentration profiles resembling those predicted theoretically (Ramsing *et al.* 1993; De Beer *et al.* 1994; Rasmussen and Lewandowski 1998). Figure 6 demonstrates how the concentration of oxygen is decreased in the fluid layer just above the biofilm surface.

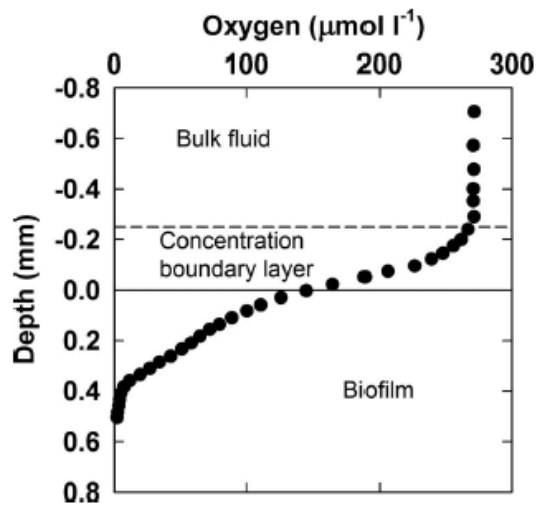


Figure 6. Oxygen concentration profile, showing how the concentration of oxygen decreases close to the surface of the biofilm in the concentration boundary layer (Ramsing *et al.* 1993; Stewart 2012).

The effect of the lower nutrient concentrations occurring in the mass transfer boundary layer is that biofilms start growing in finger-like protrusions from the biofilm surface to access areas of higher nutrient concentrations beyond the mass transfer boundary layer (Fig. 7). This was predicted in theoretical models (Picioreanu *et al.* 2000), and demonstrated in biofilm reactors with low flow velocities (Groenenboom 2000; Risse-Buhl and Küsel 2009; Graba *et al.* 2013).

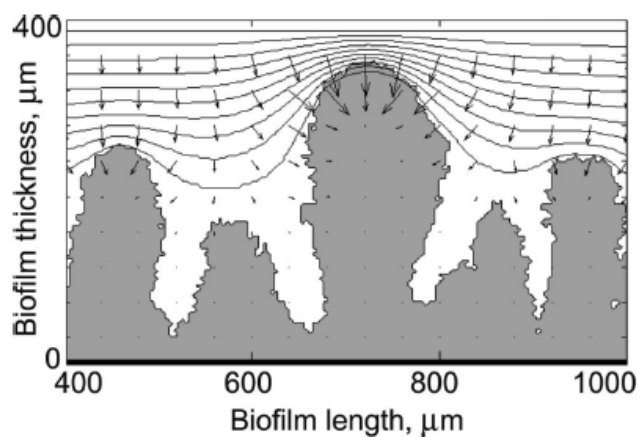


Figure 7. Finger-like protrusions of biofilm growth develop as the organisms in the biofilm try to reach an area of higher nutrient concentration beyond the mass transfer boundary layer. The lines indicate higher nutrient concentration levels, and the arrows show the direction and amount of nutrient flux into the biofilm (Picioreanu *et al.* 1998).

Biofilm detachment is an important process in bioreactor settings, as detachment controls the dissemination of bacteria into the process system keeping the amount of biofilm in balance. The performance and stability of the bioreactor depends on the microbial residence time in the system, and detachment frequency determines the functional cycle length of a bioreactor. Biofilm detachment can be divided into three

phases, namely sloughing, which is the loss of large pieces of biomass from the biofilm, erosion, which is a gradual loss of single cells or small pieces of biomass from the system, and finally, abrasion. Abrasion affects the entire biofilm and is caused by sheer stress on the biofilm surface due to liquid flow or collisions on the surface of the biofilm (Derlon *et al.* 2008). It has been shown that an increase in sheer stress on a biofilm is associated with a sharp increase in the size of sloughed particles, and increased erosion and frequency of sloughing events (Walter *et al.* 2013).

### **Metagenomic characterisation of microbial communities in wastewater treatment applications.**

#### *A brief history*

DNA sequencing dates back to the 1960s, when Frederic Sanger first sequenced the ribosomal RNA of *E. coli*, followed by the entire genome of a bacterial phage. In 1977, he developed a chain termination method of sequencing (Sanger *et al.* 1977), which became known as Sanger sequencing. Other DNA sequencing methods of the time were laborious and required the use of radioactive materials. Due to its efficiency and low radioactivity, Sanger sequencing became the preferred technology, and was used in first generation DNA sequencing laboratories. Applied Biosystems announced the first automated sequencing machine in 1987.

This allowed the extraction of nucleic acids, amplification of rRNA with suitable primers, and the construction of clone libraries using sequencing vectors (Rondon *et al.* 2000). Selected clones were then sequenced and the nucleotide sequence of the rRNA obtained is then compared to sequences available in databases such as Silva and GenBank. This allowed an estimation of the microbial diversity within a sample, and has been used widely in the characterisation of microbial populations in water (Martiny *et al.* 2003; Lipponen *et al.* 2004). Improved Sanger sequencing methods was pivotal to the completion of the human genome project in 2001 (Liu *et al.* 2012).

When the entire genome of an environmental sample is sampled to taxonomically characterise the microbial community, the approach is known as metagenomics. Shotgun sequencing is an improved sequencing technique previously used in metagenomic

studies, and was used in 2004 by Venter *et al.* to characterise the microbial diversity and relative organism abundance in an ocean water sample (Venter *et al.* 2004). Taxonomic assignments of such data are typically executed by using database search and alignment algorithms such as BLAST (Altschul *et al.* 1990). Although these approaches were successful, they were extremely expensive and time consuming.

The demand for increased accuracy, lowered cost and higher throughput caused a movement away from Sanger sequencing, and next generation sequencing (NGS) methods started to emerge. The company 454 introduced 454 sequencing in 2005. In 2006, Solexa released the Genome Analyzer and Agencourt introduced the Sequencing by Oligo Ligation Detection (SOLiD) platform. Applied Biosystems purchased Agencourt and in 2007, 454 was purchased by Roche and Solexa was purchased by Illumina. Vigorous competition between manufacturers sustains continuous improvement of technology, with sequencing capabilities currently doubling every 6-9 months (Loman *et al.* 2012). Today, the Roche 454, AB SOLiD, and Illumina platforms are the market leaders in high throughput NGS Technologies (Liu *et al.* 2012).

NGS technologies are characterised according to the template that is used for sequencing reactions. The most common type of sequencing uses immobilised libraries of clone amplified templates, made from a single DNA molecule. The DNA templates are required to be short (200-1000 bp) and that each template contains a binding site for both a reverse and forward primer (Glenn 2011). More recent technologies sequence single DNA molecules without amplification.

### *Next generation sequencing platforms*

#### Roche/454

The 454 was the first commercially available NGS platform, and has been used widely for genomic sequencing and microbial ecology metagenomic studies (Sogin *et al.* 2006). The pyrosequencing process involves the denaturation of template DNA library with 454-specific adaptors into single strands. These strands are then

immobilised onto individual beads. Each bead is amplified by emulsion PCR. Millions of beads are loaded onto a picotiter plate with wells only big enough to accommodate a single bead. All the beads are sequenced in parallel by flowing specific dNTP's over the plate surface. For each complementary base that binds, ATP is converted to pyrophosphate (PPi), which converts present luciferin to oxyluciferin, emitting detectable visible light. Light emissions for each bead with each dNTP are recorded, and the complement nucleotide sequence is recorded. In 2008, the system was combined with GS FLX Titanium software, which improved the accuracy, and is responsible for signal conversion into sequence data, yielding standard flowgram format (SFF) files, which contain the quality scores for each read, and can be applied to further data analyses. Currently, the massive advantage of using Roche 454 is the speed of the analysis, at 10 hours from start to completion, and has a relatively high read length. The major drawback is the high reagent cost and high error rate in poly bases longer than 6 bp.

#### Life technologies SOLiD

SOLiD uses ligation to determine sequences. A library of DNA fragments is covalently bound to a glass slide. Fragments contain a ligation site at the first base, a cleavage site at the fifth base. A set of four fluorescent di-base probes compete for ligation to the primer. Fluorescence is detected when probes complementary to the template strand binds. Multiple cycles of ligation, detection, and cleavage reveal the sequence of the template fragment. SOLiD reads have more reads at lower cost than other platforms, but can take up to 7 days to complete, and produces up to 4TB of data, both of which are major drawbacks to the system (<http://www.lifetechnologies.com>; <http://allseq.com/knowledgebank>; (Van Dijk *et al.* 2014).

#### Illumina

This system sequences by synthesis. The library is denatured to single strands and then grafted to a flow cell. Bridge amplification forms clusters, and the four fluorescent dye-labelled terminator dNTP's are added to the clusters. The sequence at that position is then determined for all the clusters. The dye is then cleaved and another round of dye-labelled terminator is added, and the process is repeated. After

multiple cycles, the complement sequence is recorded. The platform was improved to the HiSeq in 2010, with increased output of 200G per run and a much lower error rate. Runs take 8 days to complete, but runs are much cheaper than with 454 and SOLiD platforms. The HiSeq requires an additional HiSeq control system and a real time analyser to generate data from detected signals. A compact desktop version of this platform, namely MiSeq was launched in 2011, with on-board data generating software, completing runs in as little as 8 hours (<http://www.illumina.com>; (Van Dijk *et al.* 2014).

### Ion torrent

The ion personal genome machine (Ion PGM) was launched as a compact desktop sequencing platform that is based on semiconductor sequencing. A template strand of DNA is attached to a chip. The chip is flooded by one of the four nucleotides at a time. When a complementary nucleotide is incorporated by DNA polymerase, a proton is released, which can be detected by the system as a pH change. The absence of a charge indicates a non-complementary nucleotide, and double the charge indicates two nucleotides added in succession. The PGM platform has made affordable, fast and scalable high throughput sequencing accessible to small and medium scale laboratories. Increased primary sequence generation is possible, making it the preferred platform for sequence generation in microbial ecology studies (Whiteley *et al.* 2012).

Microbial ecologists use the sequences of 16S rRNA genes for phylogenetic analyses and for designing specific PCR primers and FISH probes. Initially, sequences were obtained from Sanger sequencing methods, which gave rise to only a few hundred full length sequences from several samples that were deposited into databases as reference sequences. With NGS technologies, it became possible to generate thousands of sequences from several hundreds of samples. The higher throughput came at the cost of read length, resulting in a deterioration of the length of sequences deposited into reference databases (Schloss *et al.* 2015). A high demand therefore exists for a technology that can generate full length sequences at high throughput to overcome the current limitations in taxonomic classifications using short sequence databases.

## Single molecule sequencing.

Until 2013, sequencing platforms have targeted DNA from mixed chromosomes isolated from a bacterial population. Only recently, platforms have become available that will sequence a single DNA molecule isolated from a single cell without using PCR amplification. This allows the analysis of the genome of a single cell. The Heliscope, (Helicos Biosciences Corp) (Thompson and Steinmann 2010) single molecule real time (SMRT) sequencer from Pacific Biosciences (PacBio) (Chin *et al.* 2013) and the Oxford nanopore DNA sequencer (Maitra *et al.* 2012) are newly developed single molecule sequencing platforms. The advantage of these technologies is speed with 200-400 bases read per second, minimal sample preparation and long read lengths. With single cell genomic capabilities, one of the major challenges in metagenomics, namely the accurate analysis of genetic variations of microorganisms at strain level, is overcome.

The PacBio SMRT sequencer was tested by (Schloss *et al.* 2015) for application in microbial ecology. This platform was compared to the 454 and MiSeq platforms for sequencing the 16S rRNA gene. Both the 454 and MiSeq platforms yielded a moderate number of high quality 200-250nt sequences at error rates below 0.02% (Schloss *et al.* 2011). The PacBio system generated a small number of near full length sequences, but at an error rate of nearly 5-fold higher than the other platforms. Further development of such platforms is required, as high error rate currently limits the application of this technology to microbial ecology (Schloss *et al.* 2015).

### *Current NGS platform technology compared*

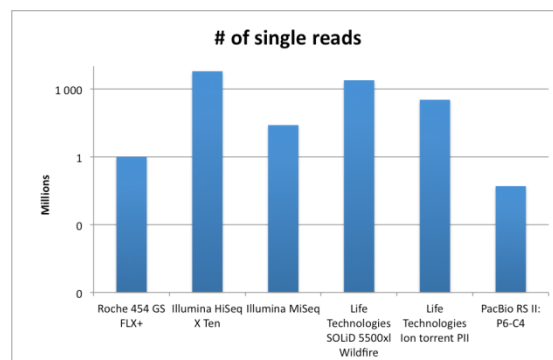
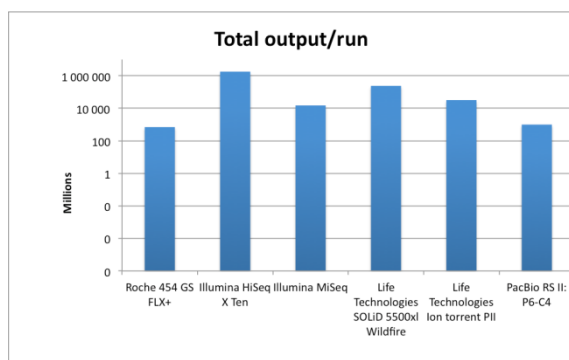
The specifications and running costs of the most widely applied NGS platforms are evolving each year. A snapshot comparing the specifications of the current market leaders was compiled using the most up to date data available from the manufacturers for 2015 (Fig. 8 and table 1). Illumina HiSeq offers the highest throughput at the lowest cost per base, and is therefore considered the leading NGS platform (Van Dijk *et al.* 2014). Data was collected from Roche (<http://www.454.com>), Illumina (<http://www.illumina.com>), Life technologies (<http://www.lifetechnologies.com>) and



the Allseq independent knowledge bank of current NGS specifications (<http://allseq.com/knowledgebank>).

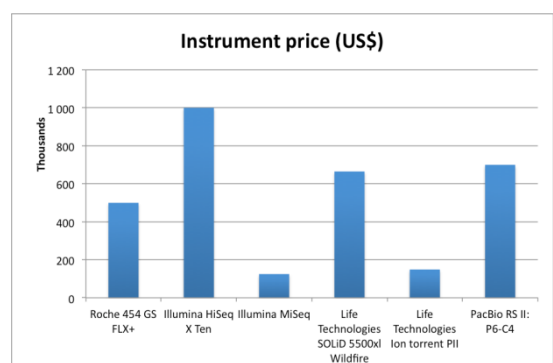
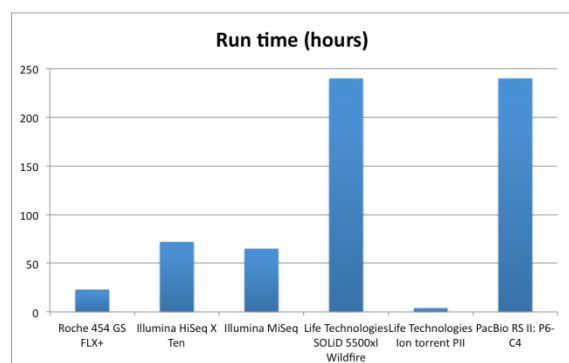
Table 1. A summary of the specifications of the market leading NGS platforms mid 2015.

	Roche 454 GS FLX+	Illumina HiSeq X Ten	Illumina MiSeq	Life Technologies SOLiD 5500xl Wildfire	Life Technologies Ion torrent PII	PacBio RS II: P6-C4
<b>Total output/run</b>	700 Mb	1.8 Tb	15 Gb	240 Gb	~32Gb (at launch)	~500 Mb - 1 Gb
<b>Run time</b>	23 hrs	3 days	~65 hrs	10 days	2-4 hrs	~2 Gb
<b>Output/day</b>	700 Mb	600 Gb	~5.5 Gb	24 Gb	~64Gb	up to 240 min
<b>Read length</b>	up to 1kb	2 x 150	2 x 300	2 x 50	100b	10-15 kb
<b># of single reads</b>	1M	6B	25M	2.4B	up to 330M	~50k
<b>Instrument price</b>	~\$500k	\$1M*	\$125k	\$665k	\$149k	~\$700k
<b>Run price</b>	~\$6k	~\$12k	1400	5000	1000	~\$400



(a)

(b)



(c)

(d)

Figure 8. (a) Illumina HiSeq generates by far the largest output with nearly 2T bases per run. (b) HiSeq also produces the largest number of single reads, with SOLiD and Ion torrent platforms as close seconds. (c) Ion torrent provides the fastest run time, which along with its relatively high output per run

and number of single reads and instrument affordability (d) makes it the most accessible and practical platform for bench top research application.

### **Visual characterisation using FISH**

FISH has become the method of choice for detection, identification and characterisation of microorganisms due to the combined reliability of molecular methods with direct visualisation (Amann and Ludwig 2000). FISH combined with flow cytometry (FCM) is a rapid cell enumeration process with high throughput, allowing rapid quantification. FISH–FCM can also be used to identify metabolic activity, physiological characteristics, classification according to metabolic activity and functional genes in biofilm samples (Wagner *et al.* 2009). The use of FISH to characterise nitrogen-, phosphate- and sulfate removing bacteria, and yeast in wastewater treatment will be discussed in the following sections.

#### *Characterisation of nitrogen metabolising bacteria in wastewater treatment biofilms*

Nitrogen removal is a two-step biological oxidation-reduction process consisting of nitrification, carried out by autotrophic nitrobacteria and denitrification, carried out by heterotrophic denitrifiers (Menoud *et al.* 1999; He *et al.* 2009; He *et al.* 2011).

Nitrifiers consist of relatively few groups of organisms while denitrifiers are organisms with heterotrophic and autotrophic metabolism, from a diverse range of genera (Wagner and Loy 2002; Thomsen *et al.* 2004).

To identify the population of a denitrifying community is challenging when using molecular methods due to the high taxonomic diversity, but many successful community studies have been achieved using FISH.

Denitrifiers most frequently found in waste water treatment plants (WWTPs) are *Pseudomonas*, *Cytophaga-Flavobacter-Bacteriodes* (CFB), *Hyphomicroba* (Wagner and Loy 2002; Ginige *et al.* 2004; Ginige *et al.* 2005) and in activated sludge systems,

the most common denitrifiers are *Aquaspirillum*, *Azoarcus*, *Thauera*, *Rhodocyclus*, *Thiobacillus denitrificans* and *Thiomicrospira denitrificans* (Ginige *et al.* 2004).

The most abundant filamentous organisms in WWTPs with N and P removal are *Microthrix*, and *Chloroflexi* type 0092, 0803 and TM7 (Nielsen *et al.* 2010; Mielczarek *et al.* 2012). *Microthrix* is responsible for complicating solids-liquid separation, contributing to foam bulking in wastewater treatment plants and cooling water (Rossetti *et al.* 2005), while *Chloroflexi* are common in activated sludge floc biomass (Björnsson *et al.* 2002).

When biofilms develop in environments with relatively low concentrations of organic substrate, nitrifying organisms thrive, whilst only a limited denitrifying community will develop due to insufficient electron donors present. If concentrated organic carbon is present, a limited nitrifying community will develop, as heterotrophs will compete for oxygen as electron acceptor (LaPara *et al.* 2006).

In a study investigating the development of both nitrifying and denitrifying communities within an aerated biofilm reactor, FISH probes were used to determine how varying levels of COD and nitrogen ratios affect the development, spatial distribution and microbial population of nitrifying and denitrifying organisms within a biofilm (Huijun Liu *et al.* 2010). Table 2 summarises the FISH probes used most commonly and successfully in municipal and industrial wastewater treatment applications.

Table 2. 16S rRNA probes used for the detection of nitrogen metabolising bacteria in wastewater applications.

Probes	Sequence (5'–3')	Target organisms (position)	References
EUB338	GCTGCCTCCCGTAGGAGT	Domain bacteria (338–355)	Amann 1995
EUB338 II	GCAGCCACCCGTAGGTGT	Planctomycetales (338–355)	Daims <i>et al.</i> 1999
EUB338 III	GCTGCCACCCGTAGGTGT	Verrucomicrobia (338–355)	Daims <i>et al.</i> 1999
Non 338	ACTCCTACGGGAGGCAGC	Competitor to EUB338	Coskuner <i>et al.</i> 2005
ALF1b	CGTTCG(C/T)TCTGAGCCAG	Alphaproteobacteria (19–35)	Daims <i>et al.</i> 1999
BET42a	GCCTTCCCACCTTCGTTT	Betaproteobacteria (1027–1043)	Daims <i>et al.</i> 1999
GAM42a	GCCTTCCCACATCGTTT	Gammaproteobacteria (1027–1043)	Daims <i>et al.</i> 1999
Nso190	CGATCCCCTGCTTTTCTCC	Ammonia-oxidising Betaproteobacteria (190–208)	Mobarry <i>et al.</i> 1996
Nsv443	CCGTGACCGTTTCGTTCCG	Nitrosospira (444–462)	Mobarry <i>et al.</i> 1996
Nsm156	TATTAGCACATCTTTCGAT	Nitrosomonas (156–174)	Mobarry <i>et al.</i> 1996
NIT3	CCTGTGCTCCATGCTCCG	Nitrobacter (1035–1048)	Mobarry <i>et al.</i> 1996
CNIT3	CCTGTGCTCCAGGCTCCG	Competitor to Nit3	Wagner <i>et al.</i> 1996
NSR1156	CCCGTTCTCCTGGGCAGT	Nitrospira (1156–1173)	Mobarry <i>et al.</i> 1996
HYP1241	GCTGCSCATTGTCACCGCC	Hyphomicrobia (1241–1260)	Layton <i>et al.</i> , 2000
CF319a	TGGTCCGTGTCTCAGTAC	CFB groups (319–336)	Manz <i>et al.</i> 1996
PM	GATCCGGACTACGACGGTTT	Pseudomonas (1284–1304)	Gunasekera <i>et al.</i> 2003

Arch915	GTGCTCCCCGCCAATTCCT	Archaea (915–934)	Alm <i>et al.</i> 1996
Ntspa 662	GGAATTCCGCGCTCCTCT	Nitrospira spp (662-679)	Daims <i>et al.</i> 2000
Cntspa 662	CGCCTTCGCCACCGGTGTTCC	competitor Cntspa 662	Daims <i>et al.</i> 2000
CFXMIX (GNSB941, <b>CFX1223</b> )	CCATTGTAGCGTGTGTGTMG	Chloroflexi	Bjornsson <i>et al.</i> 2002
CFXMIX ( <b>GNSB941</b> , CFX1223)	AAACCACACGCTCCGCT	Chloroflexi	Gich <i>et al.</i> 2001
CFXMIX (mixture of <b>MPA60</b> + MPA223 + MPA645)	GGATGGCCGCGTTCGACT	Microthrix parvicella (60-77) , M. calida	Erhart <i>et al.</i> 1997
CFXMIX (mixture of MPA60 + <b>MPA223</b> + MPA645)	GCCGCGAGACCCTCCTAG	Microthrix parvicella (223-240), M. calida	Erhart <i>et al.</i> 1997
CFXMIX (mixture of MPA60 + MPA223 + <b>MPA645</b> )	CCGGACTCTAGTCAGAGC	Microthrix parvicella (645-661) , M. calida	Erhart <i>et al.</i> 1997
Nso 1225	CGCCATTGTATTACGTGTGA	Most betaproteobacterial AOB (1224-1243)	Mobarry <i>et al.</i> 1996; 1997

### *Characterisation of phosphate removing bacteria in wastewater treatment applications*

Phosphate accumulating organisms (PAO's) take up more phosphorous than what is required for normal cellular metabolism, and this is encouraged by exposure to environments with anaerobic conditions with no oxygen or nitrate, alternating with anoxic or aerobic conditions. This process is known as enhanced bio-phosphate removal (EBPR) (Mino *et al.* 1998).

The organisms involved with phosphate removal are relatively poorly described, mostly due to uncultureability, but the application of FISH has made it easier.

In municipal wastewater treatment plants, alpha and beta subclass *Proteobacteria* dominate municipal wastewater sludge. Other common bacterial genera in plants with EBPR were *Rhodocyclus*, *Micrococcus*, *Staphylococcus* and *Acidovorax* (Hiraishi *et al.* 1998; Sudiana *et al.* 1999). Table 3 summarises the FISH probes used most commonly and successfully in municipal and industrial wastewater treatment applications.

Table 3. 16S rRNA FISH probes for the detection of phosphate metabolising bacteria in wastewater treatment applications.

Probes	Sequence (5'-3')	Target organisms (position)	References
EUB338	GCTGCCTCCCGTAGGAGT	Bacteria (10-900)	Amann <i>et al.</i> 1990
Alf1b	CGTTCGYTCTGAGCCAG	Alpha subclass of the Proteobacteria (225)	Amann <i>et al.</i> 1990
Bet42a	GCCTTCCCACCTTCGTTT	Beta subclass of the Proteobacteria (80)	Amann <i>et al.</i> 1990
GAM42a	GCCTTCCCACATCGTTT	Gamma subclass of the Proteobacteria (80)	Amann <i>et al.</i> 1990
CF319	TGGTCCGTGTCTCAGTAC	Cytophaga-Flavobacteria group (80)	(Wagner <i>et al.</i> 1994)
ACA23a	ATCCTCTCCCATACTCTA	Acinetobacter spp. (80)	Amann <i>et al.</i> 1990
HGC69a	TATAGTTACCACCGCCGT	Gram-positive bacteria with high DNA G+C content (GPBHGC) (225)	(Roller <i>et al.</i> 1994)
MNP1	TTAGACCCAGTTTCCCAGGCT	Nocardioforme actinomycetes (10)	(Schuppler <i>et al.</i> 1998)
Miclun1	CACCTCAGCAGATGTCGGC	Microlunatus (183-201)	Kyselkova <i>et al.</i> , 2008

### *Characterisation of sulfate removing bacteria in wastewater treatment*

Sulfate reducing bacteria (SRB) are responsible for the biological removal of sulfates from wastewater. Under anaerobic conditions, sulfate is the terminal electron acceptor, and is reduced to sulfide (Sinbuathong *et al.* 2007).

An anaerobic sludge reactor was tested for sulfate removal under conditions of varied COD to sulfate ratios. The microbial population was studied and characterised with FISH probes EUB338 (*Bacteria* domain), SRB385 (*beta-Proteobacteria*) and ARC915 (Archean domain) (Hirasawa *et al.* 2008). These probes were also used in a study on the microbial community composition of a sulfate removing bioreactor (Li *et al.* 2011).

In a study into the relationship between the composition of feed water for an anaerobic sulfate removing bioreactor and the corresponding SRB population, FISH probes were utilised to identify *Desulfonema*, *Desulfobacterium*, *Desulfobacteriaceae* and *Desulfobacter* (Icgen and Harrison 2006). Table 4 summarises the FISH probes used most commonly and successfully in municipal and industrial wastewater treatment applications to study sulfate removing populations.



Table 4. 16S rRNA FISH probes for the detection of sulfur metabolising bacteria in wastewater treatment applications.

<b>Probes</b>	<b>Sequence (5'–3')</b>	<b>Target organisms (position)</b>	<b>References</b>
EUB338	GCTGCCTCCCGTAGGAGT	Most bacteria	Amann <i>et al.</i> 1990
SRB385	CGGCGTCGCTGCGTCAGG	Sulfate reducing bacteria	Sandaa <i>et al.</i> 1999
ARC915	GTGCTCCCCCGCCAATTCCT	Archaea	Stahl 1991
SRB129	CAGGCTTGAAGGCAGATT	Desulfobacter	Devereux <i>et al.</i> 1992
SRB221	TGCGCGGACTCATCTTCAA	Desulfobacterium	Devereux <i>et al.</i> 1992
SRB657	TTCCGCTTCCTCTCCATA	Desulfonema	Fukui <i>et al.</i> 1999
SRB687	TACGGATTTCACTCCT	Desulfobacteriaceae	Devereux <i>et al.</i> 1992

### *Characterisation of yeasts in wastewater treatment applications*

Yeast is unicellular fungi that belong to phyla Ascomycota and Basidiomycota. They are acidophilic and occur in wastewater produced by industries that require fermentation or produce acidic, high organic load wastewater (Yang and Zheng 2014).

Considerable diversity exists among the yeast species that occur in industrial wastewater treatment systems, with 48 taxa belonging to 21 different genera that have been identified. Populations from the *Rhodotorula*, *Candida*, *Trichosporon*, *Pichia* species and some unidentified *Ascomycetes* occurred most frequently in industrial wastewater treatment applications (Yang *et al.* 2011).

The wine industry produces large amounts of acidic, concentrated organic wastewater. A study conducted on the yeast population on a biofilm contact reactor used to treat winery wastewater showed the following yeast species were present: *Williopsis saturnus*, *Picia membranaefaciens*, *Candida intermedia*, *Eremothecium gossyohi*, *Saccharomyces cerevisiae* and *Hanseniaspora uvarum* (Malandra *et al.* 2003).

FISH has successfully been utilised in wine industry related studies to detect yeast species (Stender *et al.* 2002; Xufre *et al.* 2006; Andorra *et al.* 2011; Yang *et al.* 2011).

The D1/D2 domains at the 5' end of 26S rRNA has high interspecies sequence variation, giving an ideal foundation for the development of specie specific FISH probes in yeasts. Using this principle, Xufre *et al.* developed a set of FISH probes specific for the detection of yeast species associated with the wine industry (Xufre *et al.* 2006). Table 5 summarises the FISH probes used to study yeast populations successfully in industrial wastewater treatment applications.

Table 5. 18S and 23S rRNA FISH probes for the detection of yeast in organic wastewater treatment applications.

Probes	Sequence (5'-3')	Target organisms (position)	References
PF2	CTCTGGCTTCACCCTATT C	All yeasts (18S rRNA) (618-636)	Kempf 2000
Cint	TTATCCACCCCTAGCA	<i>Candida intermedia</i> (26S rRNA) (1415-1430)	Mounier <i>et al.</i> 2009
Cst	CTCTATGGCGTTTCTTTC	<i>C. stellata</i> (D133)	Xufre <i>et al.</i> 2006
Hgu	CAATCCCAGCTAGCAGTAT	<i>H. guilliermondii</i> (D506)	Xufre <i>et al.</i> 2006
Huv	TCAATCCCGGCTAACAGTA	<i>H. uvarum</i> (D507)	Xufre <i>et al.</i> 2006
Kma	AGCTACAAAGTCGCCTTC	<i>K. marxianus</i> (D94)	Xufre <i>et al.</i> 2006
Kth	ATAGGACTAGACTCCTCG	<i>K. thermotolerans</i> (D196)	Xufre <i>et al.</i> 2006
Pan	GACAGGCAATATCAGCAGA	<i>P. anomala</i> (D499)	Xufre <i>et al.</i> 2006
Pme	AGAGCTTCGCACGGCACC	<i>P. membranifaciens</i> (D167)	Xufre <i>et al.</i> 2006
Sce	TGACTTACGTCGCAGTCC	<i>S. cerevisiae</i> (D527)	Xufre <i>et al.</i> 2006
Tde	GCAGTATTTCTACAGGAT	<i>T. delbrueckii</i> (D495)	Xufre <i>et al.</i> 2006

### **FISH-CLSM to investigate the distribution of specific groups of microorganisms within a biofilm community**

FISH together with high-resolution CLSM imaging elucidates visually how specific groups of organisms function within biofilms. Computer software has been developed that translates high resolution images into qualitative and quantitative data about specific groups of organisms within biofilms (Gantner *et al.* 2006; Daims 2009; Daims *et al.* 2011). The spatial distribution of a group amongst the substrate- and biochemical redox gradients that exist within a biofilm can shed light on how that group functions metabolically (Almstrand *et al.* 2013a). Furthermore, when multiple probes are used, insight is gained into the stratified growth patterns of specific groups, and how they orientate themselves towards groups of different metabolic orientations, shedding light on the collective metabolic activities within a consortium (Almstrand *et al.* 2013a).

In an investigation into the impact of operational parameters and carbon source in a lab-scale phosphate removing biofilm system, FISH-CLSM was used to investigate a possible correlation between increased and decreased phosphate removing efficacy with shifts in the microbial population of the biofilm with gene probes targeting denitrifying and phosphate removing organisms (Fig. 9). By studying the microbial population over time, changes in efficiency could be attributed to changes in the operational environment (Falkentoft *et al.* 2002).

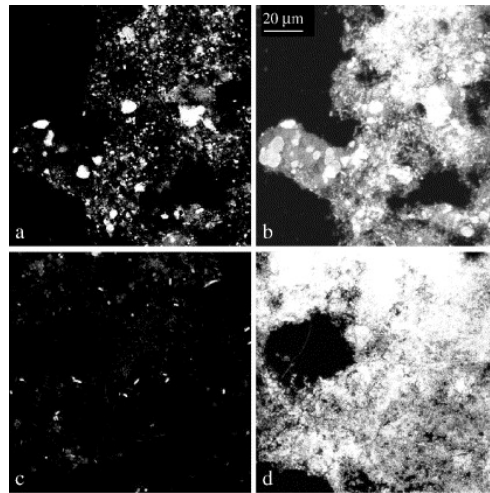


Figure 9. FISH using probes Cy3-labeled ALF1b (alpha subclass of *Proteobacteria*) and a Cy5-labeled EUB338 (*Bacteria* domain). Images a and b are of the aerobic biofilm 2 weeks into the sampling period, and b and c show images of samples taken two weeks after start-up of the denitrifying biofilm in the lab-scale reactor. Almost all of the alpha bacteria disappeared within the first two weeks following transfer of the biomass to the denitrifying setup. (a) ALF1b, aerobic sample. (b) EUB338, aerobic sample. (c) ALF1b, denitrifying sample. (d) EUB338, denitrifying sample (Falkentoft *et al.* 2002).

An example of the application of FISH-CLSM to investigate the distribution of specific organisms in an anisotropic biofilm is the study by Almstrand *et al.* (2013) where sequential rounds of FISH with different probes were performed on one biofilm sample, allowing the detection of multiple populations (Fig. 10). Software with an automated tool for vertical-distribution analysis, along with a newly developed algorithm for co-aggregation analysis of microbial populations was used to show distinctly different stratification patterns of ammonia oxidisers and nitrite oxidisers in different types of wastewater biofilms, shedding light on the ecological interactions within these biofilms as *Nitrospira* co-aggregated more closely with *Nitrosomonas* in pilot scale nitrifying trickling filters (NTF) and moving bed bioreactors than in a full scale NTF (Almstrand *et al.* 2013a).

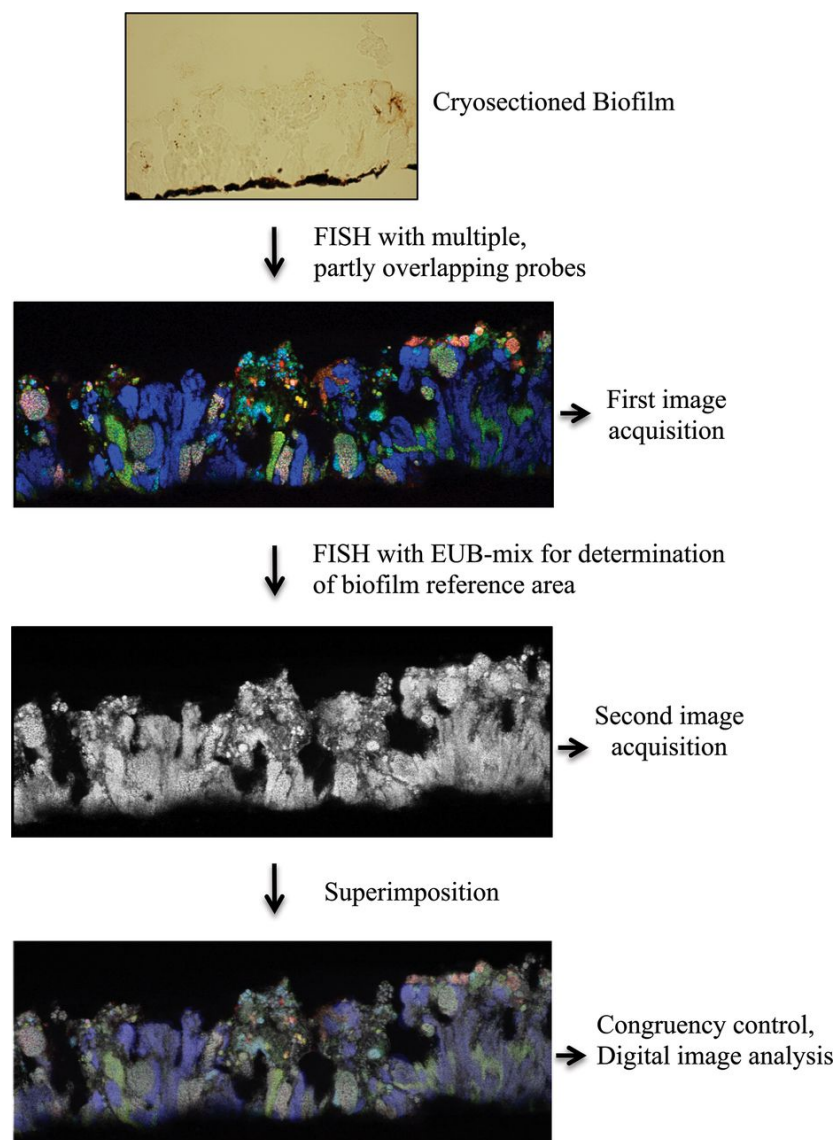


Figure 10. The sequential FISH procedure, with a first round performed with multiple partly overlapping probes targeting NOB, AOB, or Betaproteobacteria, and a second round with the EUB228 probe mixture, for detection of all bacteria. The images were superimposed in silico (Almstrand *et al.* 2013a).

Ricardo *et al.* (2012), was investigating the kinetics of simultaneous nitrate and perchlorate removal in an ion exchange membrane biofilm reactor. FISH was performed to gain insight into the stratified development of denitrifying and perchlorate reducing bacteria within the biofilm. From the FISH analyses it was established why both denitrification and perchlorate removal could happen simultaneously. The nitrifiers and perchlorate removers developed at opposite interfaces of the biofilm (Fig. 10), thus outperforming suspended growth reactors, as nitrate is an inhibitor to perchlorate removal, preventing simultaneous removal in the absence of a concentration gradient.

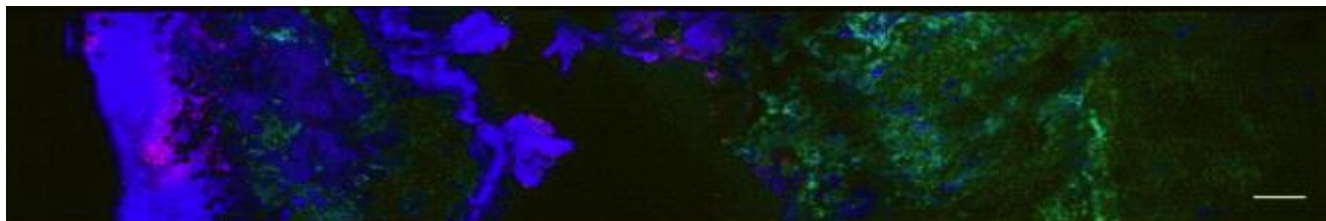


Figure 10. FISH micrograph of a longitudinal cut of the biofilm hybridised with Cy3-labeled Dechl2 (in magenta, resulting from the overlap of red and blue) and FitC-labeled THAU832 (in cyan, resulting from the overlap of green and blue), with Cy5-labeled EUBmix (blue). The biofilm was supported on a membrane (not visible) located on the right side of the image. Bar = 50  $\mu\text{m}$  (Ricardo *et al.* 2012).

### **Community level physiological profiling (CLPP) for the metabolic characterisation of biofilms**

The rapid classification of heterotrophic microbial communities based on metabolic traits was first developed as a screen of substrate utilisation by Garland *et al.* in 1991, and the term community-level physiological profiling was devised by Lehman *et al.* (1995). CLPP exposes a microbial sample to an array of substrates, and can be used to monitor changes in substrate utilisation in different conditions, over time or between samples, allowing assessment of relative change in microbial communities, or can be used to define the metabolic capabilities of a microbial consortium (Garland and Lehman 1999). CLPP can be direct detection of metabolic activity via measurement of oxygen consumption or carbon dioxide production, or indirect measurement of respiration via measurement of tetrazolium dye reduction (Garland *et al.* 2003b). Garland *et al.* (2003) proposed using the oxygen biosensor system by BD Biosciences (BDOB) along with defined substrates for CLPP in an environmental microbial sample. The BDOB microplate system is based on detecting fluorescence as a measurement of respiration from an oxygen sensitive fluorophore. The fluorophore is strongly quenched by oxygen, thus emission increases with oxygen consumption as an indicator of metabolic activity. Oxygen biosensors require short incubations and are sensitive to low substrate concentrations of 50-100 mg/L. BD Biosciences BDOB is no longer commercially available, however oxygen sensitive fluorophores are available as free probes for mix-and-measure procedures, for example MitoXpress from Luxcel Biosciences, but aside from the costs involved with the specialist instruments required to monitor results, no standard protocol has yet been developed for the application in environmental microbial samples (Stefanowicz 2006). Measuring  $\text{CO}_2$  as a direct metabolic response in the presence of a substrate in

environmental microbial consortia has been performed in environmental samples. Gas chromatography (Degens and Harris 1997), CO<sub>2</sub> responsive colorimetric assays and monitoring the utilisation of radioactive carbon sources by scintillation counting (Campbell *et al.* 2003) were tested in soil samples. The major advantages of these methods were rapid detection of changes in substrate utilisation, and not excluding unculturable strains as no culturing steps are required (Stefanowicz 2006), however, these techniques are expensive, and, according to the literature, have not been applied to biofilms.

The Biolog system, has, on the other hand, been used to study substrate utilisation in biofilms (Rusznayák *et al.* 2008; Gu *et al.* 2014; Takabe *et al.* 2014). Microbial samples are incubated in 96-well plates with arrays of substrates, with one substrate apportioned to each well on every plate, allowing multiple parallel assays. A range of Biolog plates are available for testing different substrates in different sample types, with specific supplementation and incubation protocols for specific cultures, for example gram positive bacteria (Biolog GP plates), gram negative bacteria (Biolog GN plates), mixed cultures (Biolog Eco Plates) and fungi (Biolog FF plates). Samples are cultured and cells are plated into the appropriate 96-well Biolog plates at defined concentrations. Samples can be supplemented if necessary, and incubated along with the substrates in the presence of a colorimetric redox dye. When a substrate is oxidised, the colourless tetrazolium redox dye in a well is reduced to form violet formazan, which is detected and quantified as optical density (OD) with a spectrophotometric plate reader, assigning OD's to each well, corresponding to each substrate tested, resulting in a metabolic fingerprint or CLPP for that sample (Stefanowicz 2006). The majority of Biolog plates are designed to be used for CLPP in defined, pure cultures, for which spectrophotometric data can be collected over time and used to define growth kinetics for each substrate tested (Paulsen and Holmes 2014a). Biolog ECO plates contain 31 carbon sources in triplicate on each plate, making them ideal to perform reproducible CLPP in environmental samples (Classen *et al.* 2003).

An example of how ECO plates have been applied to optimise the functionality of a wastewater treatment system is a study investigating the effect of carrier filling ratios on the performance of a moving bed biofilm reactor treating industrial wastewater. ECO plates were used to determine which carrier filling ratio supports a biofilm community with optimal functionality by calculating the functional diversity index from the CLPP data (Gu *et al.* 2014). ECO plates have also been used to study the phases of development of microbial



consortia in constructed wetlands for wastewater treatment (Weber and Legge 2011) and to investigate how the metabolic capabilities of microbial communities differ in constructed wetlands with different designs and operational parameters (Button *et al.* 2015). ECO plates are limited to carbon sources. Biolog's phenotypic microarray (PM) plates are a range of 20 plates with approximately 200 carbon sources, 400 nitrogen sources, 100 phosphorous and sulfur sources, 240 antibiotics and various pH and osmolarity ranges to test sensitivity, amounting to more than 1000 phenotypic traits that can be screened simultaneously. These tests were designed for pure cultures, but protocols can be adjusted to suit environmental samples (Paulsen and Holmes 2014b). Biolog PM Plates have been applied to study the chemical sensitivity of a *Pseudomonas alcaliphila* biofilm to 22 anti-microbial compounds, and to compare sensitivity of the biofilm to that of planktonic cells (Santopolo *et al.* 2012). In a clinical study, the carbon utilisation patterns of both planktonic and biofilm samples of human pathogen *Salmonella Typhi* (Chelvam *et al.* 2015). The Biolog system has numerous drawbacks, such as the exclusion of unculturable organisms and loss of diversity within wells with extended incubation times, however, it provides a high throughput, rapid, relatively cost effective method of establishing substrate utilisation patterns in microbial samples (Santopolo *et al.* 2012). Due to the complex nature of environmental microbial communities such as multiple growth requirements and different responses to stresses, PM plates are difficult to apply to multiple strain samples, but it is possible to tailor the protocols to suit studies of microbial communities (Paulsen and Holmes 2014b).

### **Concluding remarks**

A thorough understanding of the development, mass transfer processes, microbial make-up and metabolic capabilities of a biofilm is instrumental in designing and maintaining a biofilm wastewater reactor that functions optimally (Lazarova and Manem 1995; Verstraete 2007; Herrero and Stuckey 2015). This is a review of current insights into biofilms in wastewater treatment, discussing the formation of biofilms, the processes that make biofilm wastewater treatment possible, and the characterisation of biofilms with the latest available techniques. NGS, FISH-CLSM and CLPP are discussed as possible characterisation techniques, summarising all the resources available for biofilm characterisation within each of these techniques. Since each technique focuses only on a specific aspect of the biofilm under investigation, it is suggested that multiple characterisation techniques are applied that address biofilm characterisation at different levels, such as metagenomic, visual and metabolic

analyses as a three-tiered investigation, producing complementary data sets by which a biofilm can be characterised.

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## CHAPTER 4

### A high rate biofilm contact reactor for the treatment of winery wastewater

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## Introduction

The growing global wine industry (OIV 2015) is facing challenges such as water scarcity, rising energy costs, growing concerns about chemical pollution and climate change (Guthey and Whiteman 2009), motivating implementation of more sustainable winemaking practices. Stakeholders and increasingly strict legal requirements further motivate sustainable practice, resulting in pollution prevention efforts, natural resource protection and the development and application of product innovations that help wineries achieve environmentally responsible and legally compliant water use and waste disposal. International legal requirements for winery wastewater as stated by the South African National Water Act (1998) General Authorizations published in the Government notice Nr. 399 (26 March 2004), the guidelines of the Southern Australian Environmental Protection Agency (EPA 2004) and the General waste discharge requirements for discharges of wine, beverage and food processor waste to land of the California Environmental Protection Agency (U.S. EPA 2016) deem untreated winery wastewater unsuitable for release into the environment and disposal through beneficial irrigation due to the environmental risks posed by the high biodegradable organic load, large amount of suspended solids and acidic pH. Biological wastewater treatment systems that have been tested in the wine industry often pose challenges to wineries that are aiming to deal with wastewater in an affordable and sustainable manner. Conventional activated sludge techniques (Fumi *et al.* 1995; Brucculeri *et al.* 2005), sequencing batch reactors (Andreottola *et al.* 2002; Ruiz *et al.* 2002), rotating biological contact reactors (Malandra *et al.* 2003), and highly aerated systems (Oliveira *et al.* 2009; Litaor *et al.* 2015) are all energy intensive processes. Furthermore, many wineries do not have space available to accommodate large systems. Constructed wetlands (Arienzo *et al.* 2009; Serrano *et al.* 2011) require the permanent allocation of land to wastewater treatment. Some processes such as membrane bioreactor systems require intensive maintenance (Valderrama *et al.* 2012). Therefore, a high demand exists for a compact, energy efficient, cost effective biological wastewater treatment solution which is simple in operation and maintenance. The objective of this study was to design, build and test a pilot scale biofilm reactor that can be pre-assembled off site, is easy to install and operate, has a low running cost and can improve winery wastewater quality to meet legal standards for disposal via beneficial irrigation or release into the environment. A previous study by Cloete, Smith & Saayman (1999) showed that a commercial water cooling tower can be used as a trickling filter for municipal

wastewater treatment, resulting in a cooling tower based design in the present study. A commercial cooling tower, consisting of a fiberglass hollow tower, filled with highly porous polypropylene fill media with a collection basin at the base is used as the filtration structure. A cooling tower is designed to have maximal ventilation and a very fine water distribution system, thus yielding a much higher aeration rate than a conventional trickling filter and fine droplet distribution, aiding the rapid degradation of organic pollutants in the wastewater. Setting this system apart from a trickling filter is the addition of a centrifugal separator, which removes any detached biomass from the system based on relative density. The centrifugal separator removed any detached biomass in the wastewater stream by centrifugal force into a separation chamber, from which the solids are expelled. This resulted in the production of small volumes of sludge during operation and purging of spent biofilm at the end of each biofilm cycle, eliminating the need for a sedimentation tank. Wastewater treatment efficacy was tested by monitoring chemical oxygen demand (COD) as the main parameter for organic contamination in the tests, as well as pH, alkalinity, total sulphates, total phosphates, total nitrogen, and the total suspended solids in the input wastewater and treated water streams. The feasibility of upscaling of the system was tested by adding an additional cooling tower in series, and the possible reuse of the wasted biofilm sludge from the system was investigated. The goal of the treatment process is yielding treated winery wastewater that meets the legal standards for river disposal or beneficial irrigation. The guidelines are described in the scheme for integrated production of wine (IPW) of the South African wine and spirit board, and comply with the general authorization of the Department of Water and Sanitation (DWS) of South Africa. The IPW complies with international sustainability criteria in the wine industry including the International Federation of Wine and Spirits (FIVS) 'Global Wine Sector Environmental Sustainability Principles' and the International Organisation of Vine and Wine (OIV) 'Guidelines for sustainable Viticulture: Production, processing and packaging of products'. Wineries belonging to the scheme are audited by the IPW to obtain an integrity and sustainability certification of their wines. The guidelines are summarised in table 1.

Table 1. Summarised guidelines for winery wastewater disposal and irrigation in South Africa. (Adapted from IPW 2015).

Parameter	River disposal	Beneficial irrigation	Beneficial irrigation
	General limit	(up to 500 m <sup>3</sup> /day)	(up to 2 000 m <sup>3</sup> /day)
Chemical oxygen demand (mg/L)	75	400	75
pH	5.5-9.5	6.0-9.0	5.5-9.5
Nitrogen (mg/L)	6		3
Nitrite (mg/L)	15		15
Phosphate (mg/L)	10		10
Suspended solids (mg/L)	25		25

Table 2. Standards for treated effluent water applied to vineyards in South Africa (Van Schoor 2005).

Parameter	Vineyard irrigation	
	Optimum value	Maximum value
Chemical oxygen demand (mg/L)	<60	<100
pH (KCl)	6.5-8.4	6.0-9.0
Nitrogen (mg/L)	<5	<10
Phosphate (mg/L)	<5	<10
Sulphate (mg/L)	<150	<250
Total dissolved solids (mg/L)	<500	<1 000

## Materials and methods

### 1. Pilot reactor set-up, operation and experimental process.

The pilot reactor was installed on site at a winery in Stellenbosch, with easy access to the collection tank for pooled wastewater directly from the cellar. The initial pilot reactor consisted of a small cooling tower with a sump volume of 80L (ict 650 small 1500, Industrial Cooling Towers, Alberton, South Africa) connected to a BADU<sup>TM</sup>45/11 0.45kW circulation pump (Speck Pumps, Cape Town, South Africa) and a Jumag SCV 0716D vertical centrifugal separator equipped with a self-priming pressure circulation pump and an electronic valve (Prozone Systems, Cape Town, South Africa). The pilot plant was connected to an input of wastewater directly from the cellar. Electronic valve controlled flow through the system allowed the automation of wastewater intake, the operational cycle length and timing of output from the system by a programmable logic controller. The system was



operated by filling the 80L sump of the cooling tower, and allowing this volume to recirculate in the system for a set amount of time. After one season of testing, the system was expanded by including a second, larger cooling tower into the system with a sump volume of 950L (ict 1250, Industrial Cooling Towers, Alberton, South Africa) (Fig. 1 and 2).

For all subsequent tests, the system was operated by filling the 80L sump of the first cooling tower unit. This volume was then circulated through the first unit for a set amount of time, before being dumped into the second cooling tower subunit, after which the 80L sump was refilled and another cycle commenced. This process filled the 950L sump of the second cooling tower, and when it reached capacity, its circulating pump switched on, and circulated the 950L continuously. The second cooling tower unit was kept at capacity by the time controlled addition of water from the first cooling tower unit, coordinated with the release of an equal volume of water from the outlet, creating a time controlled bleed on bleed off system in the second cooling tower unit.

The system was operated intermittently over the course of three years spanning from September 2012 to September 2015, with optimisation starting in September 2012. The first full trial with the initial single reactor system was during harvest season in March 2013. The system was subsequently expanded, and the double-reactor system was tested in three rounds of trials in the early summer (November 2013 and November/December 2014), the late winter/early spring (August 2014 and September 2015) and harvest season (February/March 2014 and 2015) respectively. The early summer months are mild to warm and dry, and water consumption in the cellar is low. Cellar activities in this season include cleaning bottling equipment with caustic and bottling wine, yielding wastewater with COD concentrations between 1 000 and 4 000 mg/L (EPA 2004). Late winter and early spring are cold and wet, and cellar activities during this season include putting red wine into barrels and filtering the previous year's red wine (EPA 2004), which can elevate organic contents of the wastewater produced to between 4 000 and 10 000 mg/L COD. During harvest season, cellar activities are at their height, and highly concentrated wastewater is produced with COD values in excess of 10 000 mg/L.

Each trial spanned the lifetime of the biofilm that developed within the first subunit of the system, varying between 10 and 16 days. Once the biofilm reached a thickness of approximately 2 cm, it detached from the fill media, and was expelled via the centrifugal separator. The biofilm was consistently sloughed after reaching a thickness of 2cm,

irrespective of the age of the biofilm, and it was therefore deduced that detachment events are linked to the weight of the biofilm as opposed to the age. When the system was operated continuously, biofilm development and detachment was sustained in uninterrupted cycles. The biofilm in the first subunit started developing within 24 h of the previous detachment event, and treatment continued. The biofilm in the second subunit never exceeded 0.5 cm in thickness, and remained attached to the fill media for as long as the system was in operation.

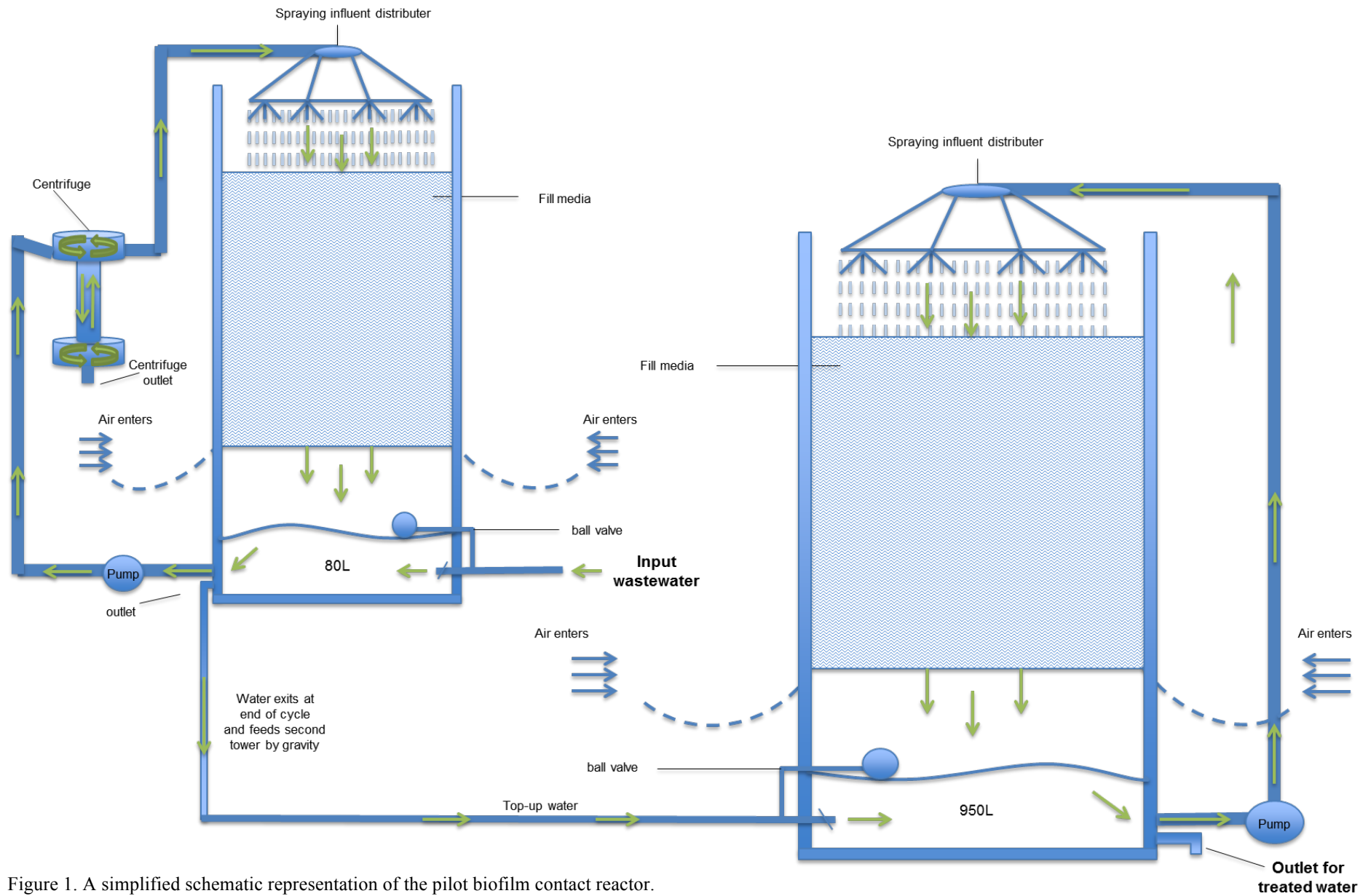


Figure 1. A simplified schematic representation of the pilot biofilm contact reactor.



(a)



(b)



(c)

Figure 2. Pilot scale biofilm reactor (a) showing the small cooling tower unit with the centrifugal separator, (b) wastewater in circulation, trickling into the sump and (c) the expanded pilot reactor, including a larger cooling tower unit (right).

## 2. *Optimisation of operational parameters*

The harvest season trial with the initial single subunit system was conducted at a 4-h cycle length. In order to establish the optimal operational parameters for the expanded system, especially when the demands on the system were high during harvest season, both the effect of the duration of the recirculation period, as well as the biofilm maturity (time from start-up) were investigated. In an attempt to improve on the treatment results obtained with the single tower reactor system, two experiments were conducted, the first with a cycle length of 4 h,

with sampling on day 7 and day 10. The system was subsequently cleared of biomass with a high pressure hose, and the experiment was repeated at a cycle length of 6 h.

### 3. *Water analyses*

Samples were collected at the input of the system, and from the sump of each tower. Samples were kept cool during transportation, and were analysed on the day of sampling. pH was measured using the PCTestr35 (Eutech, USA). Suspended solids values were determined using the pre-programmed turbidity test for total suspended solids on the Spectroquant™Pharo 300 (Merck Pty Ltd, South Africa). All other tests were conducted using standardised test kits for the Merck Spectroquant™ system, with spectrophotometric and turbidity measurements taken with the Spectroquant™Pharo 300 (Merck Pty Ltd, South Africa).

#### 3.1. *COD*

The COD Cell test kit 114541 (Merck Pty Ltd, South Africa) was used. Shortly, the water sample is oxidised with a hot sulfuric solution of potassium dichromate with silver sulfate as the catalyst. Chloride is masked with mercury sulfate. The concentration of green  $\text{Cr}^{3+}$  ions is then determined photometrically.

#### 3.2. *Acid capacity*

The acid capacity measures the ability of the water sample to bind to  $\text{H}^+$  ions, exerting a buffering effect. The acid capacity test kit 101756 (Merck Pty Ltd, South Africa) was used to measure the reaction of protonatable substances in the water samples with the reagent AC-1, resulting in a change of the pH and the colour of an indicator in direct proportion to the acid capacity. The resultant colour is evaluated photometrically.

#### 3.3. *Total sulfates*

Total sulfates were measured using cell test kit 114548 (Merck Pty Ltd, South Africa) in which sulfate ions react with barium ions to form slightly soluble barium sulfate. The resulting turbidity is measured in the photometer.

### *3.4. Total phosphates*

Total Phosphates were measured using the 114842 cell test kit (Merck Pty Ltd, South Africa). Orthophosphate ions react in a sulfuric solution with ammonium vanadate and ammonium heptamolybdate to form orange-yellow molybdovanadophosphoric acid that is determined photometrically.

### *3.5. Total nitrogen*

The total nitrogen cell test kit 114537 (Merck Pty Ltd, South Africa) was used to determine total nitrogen by measuring the transformation of organic and inorganic nitrogen compounds into nitrate according to Koroleff's method by treatment with an oxidizing agent in a thermoreactor. The nitrate reacts with a benzoic acid derivative in concentrated sulfuric acid to form a red nitro compound that is determined photometrically.

## *4. Sludge analyses*

Spent biofilm sludge was released from the system after a mature biofilm sloughed off after a 14 day treatment cycle during the harvest season of 2015. The sludge was expelled from the system via the centrifugal separator and left in the sun to dry completely, before submitting the sample to Bemlab (Somerset-West, South Africa) for a total composition analysis applicable to compost and soil.

## **Results and discussion**

### *5. Trial 1: Single subunit pilot system during harvest, March 2013*

This trial was conducted at a 4h cycle length, and COD values in the cellar effluent varied between 2 800 and 10 000 mg/L. The treatment cycle spanned 10 days, as the biofilm detached after 10 days of operation. The data collected in this trial served as the foundation for the optimisation of the system and further trials with the up-scaled, two subunit pilot reactor.

### 5.1. COD

The average percentage reduction in COD achieved over this period was 80%, with lower COD removal efficiency for the first two days of the trial as the biofilm was developing. A maximum removal efficiency of 92% was achieved on day 5 when the effluent from the cellar had a high COD of 7929 mg/L. On day 10 the cellar effluent had a COD concentration of above 9900 mg/L, but the removal efficiency was only 80% (Fig. 3). The total suspended solids increased sharply on day 10, suggesting that the biofilm was detaching, and making less biomass available to reduce COD. When considering the COD data alone, the first trial suggested that the biofilm starts removing organics from the wastewater within the first 24h since start-up, and that system performance improves with increased COD levels. Furthermore, the treatment efficiency starts to decline with the first signs of biomass detachment at the end of the biofilm lifetime. The reduction of COD was not sufficient to comply with required reduction to below 400 mg/L, indicated on the graph (Table 1, Table 2), indicating that a longer cycle length may be required.

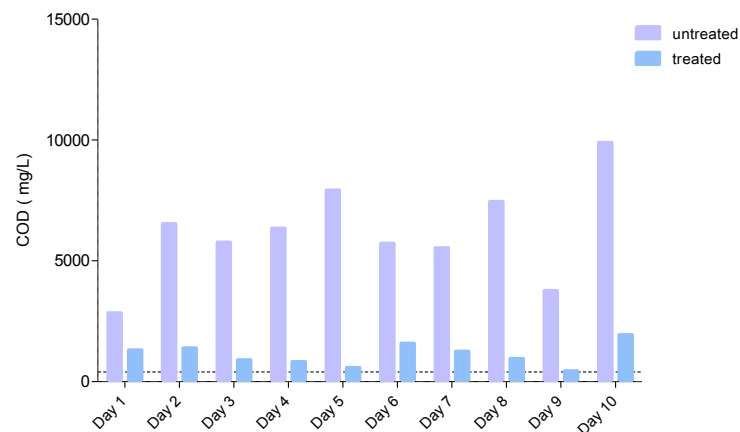


Figure 3. COD removal in the single subunit pilot reactor. The line is at 400 mg/L.

### 5.2. pH

The pH of the cellar effluent varied between 10.5 and 4, and the pH of the treated water was consistently improved to between 6.9 and 7.8, which complied with the required parameters for irrigation (Fig. 4) (Table 1, Table 2).

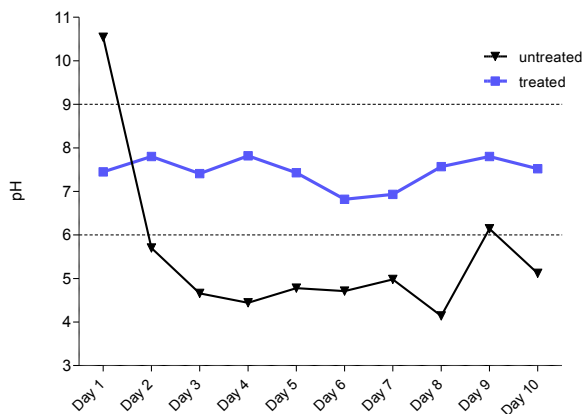


Figure 4. pH of the winery wastewater before and after treatment in the single subunit pilot reactor.

### 5.3. Total nitrogen

Nitrogen levels in the cellar effluent fluctuated between 0.11 and 3 mg/L, which was below the required limit, but, the system was effective at removing nitrogen on all days except for day 4 and day 9 of the cycle (Fig. 5). The elevated concentration of nitrogen in the treated water stream on day 9 was ascribed to nitrogen in the biomass which was detaching and entering the sump at that stage.

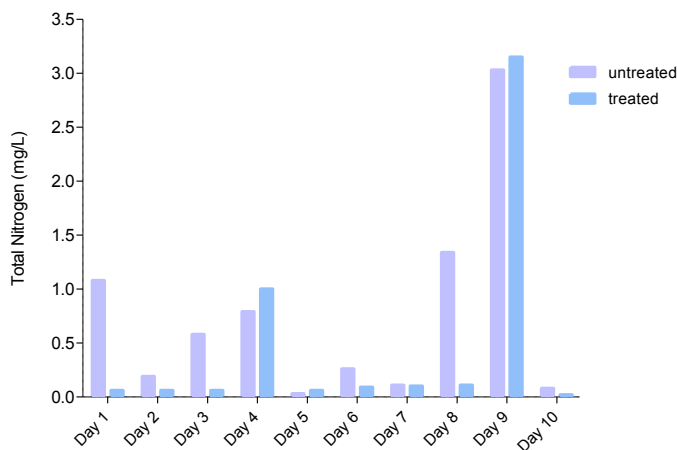


Figure 5. Total nitrogen removal in the single subunit pilot reactor.



#### 5.4. Total sulfate

No sulfate was detected in the winery wastewater on day 1 and 2. On days 2 and 3, sulfate levels were above the limits for irrigation (Table 2) and were reduced to within limits. On subsequent days, sulfate removal was inconsistent, with increased sulfate concentrations detected in the treated water on days 6 and 8 (Fig. 6). These inconsistent results suggested that biofilm age could influence sulfate removal.

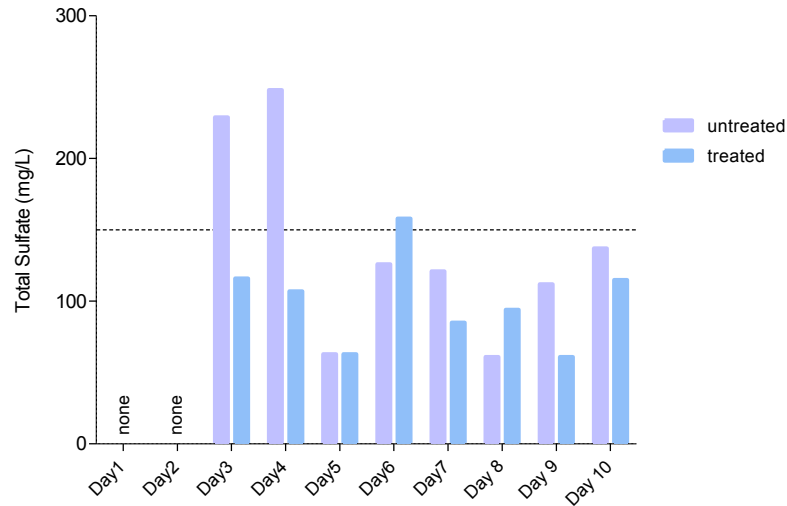


Figure 6. Total sulfate removal in the single subunit pilot reactor.

#### 5.5. Total phosphate

Phosphate levels were consistently reduced from between 5 and 20 mg/L to within prescribed limits (Table 1, Table 2) for irrigation purposes on all days except day 10, when the biofilm detached (Fig. 7).

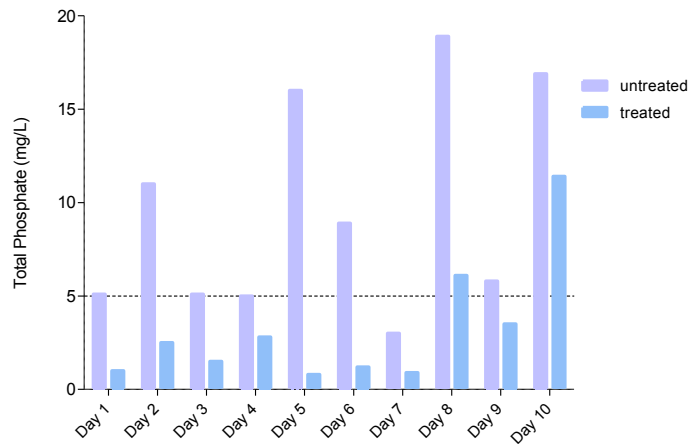


Figure 7. Total phosphate removal in the single subunit pilot reactor.

### 5.6. Suspended solids

The concentration of suspended solids in the untreated winery effluent was low for the first 4 days of the trial. Suspended solids were consistently reduced on all days, but reductions were not sufficient to comply with required limits for irrigation, namely 25mg/L (Table 1), indicated by the line on figure 8. The suspended solids in the pooled centrifuge effluent for each day is more concentrated than the untreated wastewater on days 5, 6, 9, suggesting that the centrifuge was removing detached biomass from the system, which was confirmed on day 10, when the centrifuge eliminated the bulk of the detached biomass at the end of the biofilm lifetime.

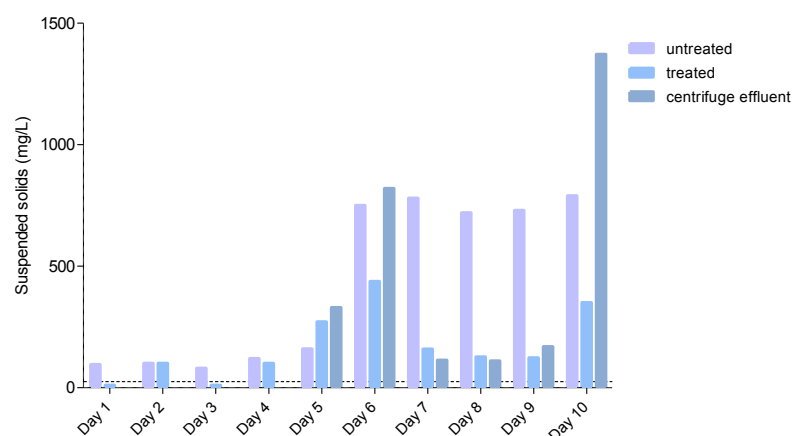


Figure 8. Total suspended solids removal in the single subunit pilot reactor.

Collectively, the data from the first trial indicated that the system was effective at reducing COD and nutrient concentrations, as well as buffering high and low pH values, although not all parameters were sufficiently improved to meet the required limits for disposal through irrigation with the system operating with a cycle length of 4 h. Longer cycle periods were proposed, as well as the addition of a second cooling tower unit to the pilot reactor system to investigate how two cooling tower subunits operated in series within the system might affect the treatment capacity and the overall performance of the system.

## 6. Optimisation of operational parameters for the up-scaled, two subunit pilot system

The two trials conducted during harvest season of 2014 helped to determine that the two-subunit pilot system achieves similar efficacy in COD removal at a biofilm age of 7 and 10 days, and that COD removal is improved by approximately 10% by increasing the cycle period from 4 to 6 h. All further trials were conducted using a 6-h cycle duration.

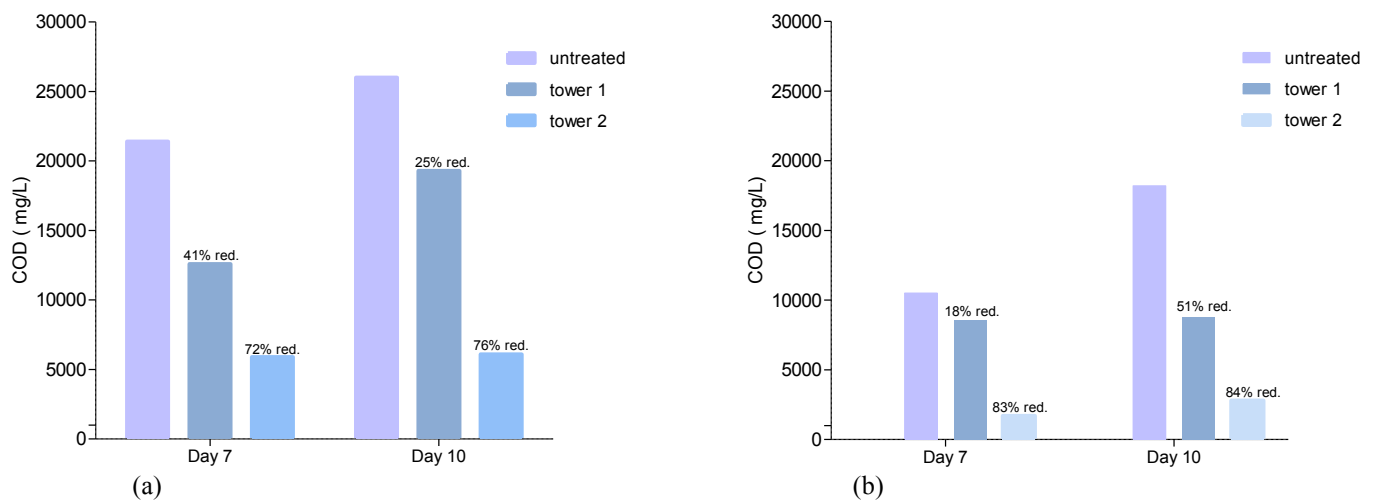


Figure 9. COD removal performance of the two subunit pilot reactor during harvest conditions, using a 4h and a 6h cycle length.

## 7. Trials 2,3 and 4: Up-scaled double subunit pilot system performance during late winter/early spring (August 2014 and September 2015), early summer November/December 2013 and 2014, and harvest season (February/March 2014 and 2015).

To evaluate the performance of the double reactor pilot system under different environmental and wastewater strength conditions, the data of trials from different seasons were compared for each parameter.

### 7.1. COD

During the late winter and early spring of 2014 and 2015, the COD of the wastewater from the cellar varied between 3 000 and 10 000 mg/L. The biofilm in both late winter/ early spring trials detached from the reactor after 16 days of operation (Fig. 10a and 10b). The slightly longer cycle length can be ascribed to cold temperatures causing slower biofilm growth. In these trials, the COD was consistently reduced by treatment in the first tower, except for day 10 of the 2014 trial (Fig. 10a), and reduced further by the second tower at an average of 87%. The COD concentration of the treated water for each of the late winter/ early spring trials was  $1013 \pm 223$  mg/L and  $298 \pm 28$  mg/L respectively, amounting to an average COD reduction of 87%. Unusually high amounts of rainfall occurred on day 10 and 11 of the 2014 trial, and water in the sump of both towers were diluted, affecting the accuracy of the measurements on day 10. For the summer trials, biofilm age only reached 14 days. The COD concentration of the effluent from the cellar was lower than in winter, ranging between 1 000 and 4 000 mg/L COD (Fig. 10c and 10d). In both summer trials, the first tower reduced the COD, but not as effectively as in the winter trials which had a higher input COD concentration. COD concentration was reduced, on average by 50% in the first tower, and further reduced to a total of 88% after treatment in the second tower. The average COD of the treated water in the summer trials was 239 mg/L, which is well within the prescribed limits for irrigation purposes (Table 1). During harvest season, when the wastewater from the cellar had COD concentrations varying between 5 000 and 35 000 mg/L COD, the system was most effective at removing COD (Fig. 10e and 10f). On average, the first tower removed 86% of COD. The overall COD reduction was 91% in the first harvest trial, and the treated water had a COD concentration of  $1\,229 \pm 139$  mg/L. In the second harvest trial, there was a 98% reduction in COD, yielding treated water with a COD of  $307 \pm 37$  mg/L COD (Table 3). The evaluation of the system in the harvest season of 2014 was plagued by grape material clogging a drainage valve between tower 1 and tower 2 and from the data it is clear that the COD removal in tower 1 was not as effective as was expected from previous trials. Biofilm development was also slower, with detachment only occurring after day 18. This indicates that the overall COD removing abilities of the system depends on effective COD removal in the first cooling tower subunit when treating highly concentrated wastewater, and that the second subunit is only able to reduce COD concentrations to below 400 mg/L (line indicated on the graphs, Fig. 10) if the first subunit removed at least 50% of the initial COD. From Table 3, it is clear that the system is more effective at COD removal when treating high

organic load wastewater, but that the COD concentration in treated water was, on average, improved to within range of the required limits for irrigation when treating wastewater with low, mid-range and high concentrations across seasons.

Table 3. The mean COD concentrations of the untreated and treated wastewater, as well as % reduction of COD.

COD (mg/L)	late winter/ early spring		early summer		harvest season		average across seasons (n=6)
	2014 (n=8)	2015 (n=8)	2013 (n=7)	2014 (n=7)	2014 (n=18)	2015 (n=14)	
<b>Untreated</b>	5400 ± 969	4250 ± 719	1983 ± 450	2128 ± 137	14011 ± 3136	20539 ± 2711	8052 ± 3081
<b>Treated</b>	1013 ± 233	298 ± 28	259.6 ± 70	182 ± 34	1229 ± 139	307.9 ± 37	548 ± 184
<b>% Reduction</b>	81.24	92.99	86.94	91.45	91.23	98.53	93.19

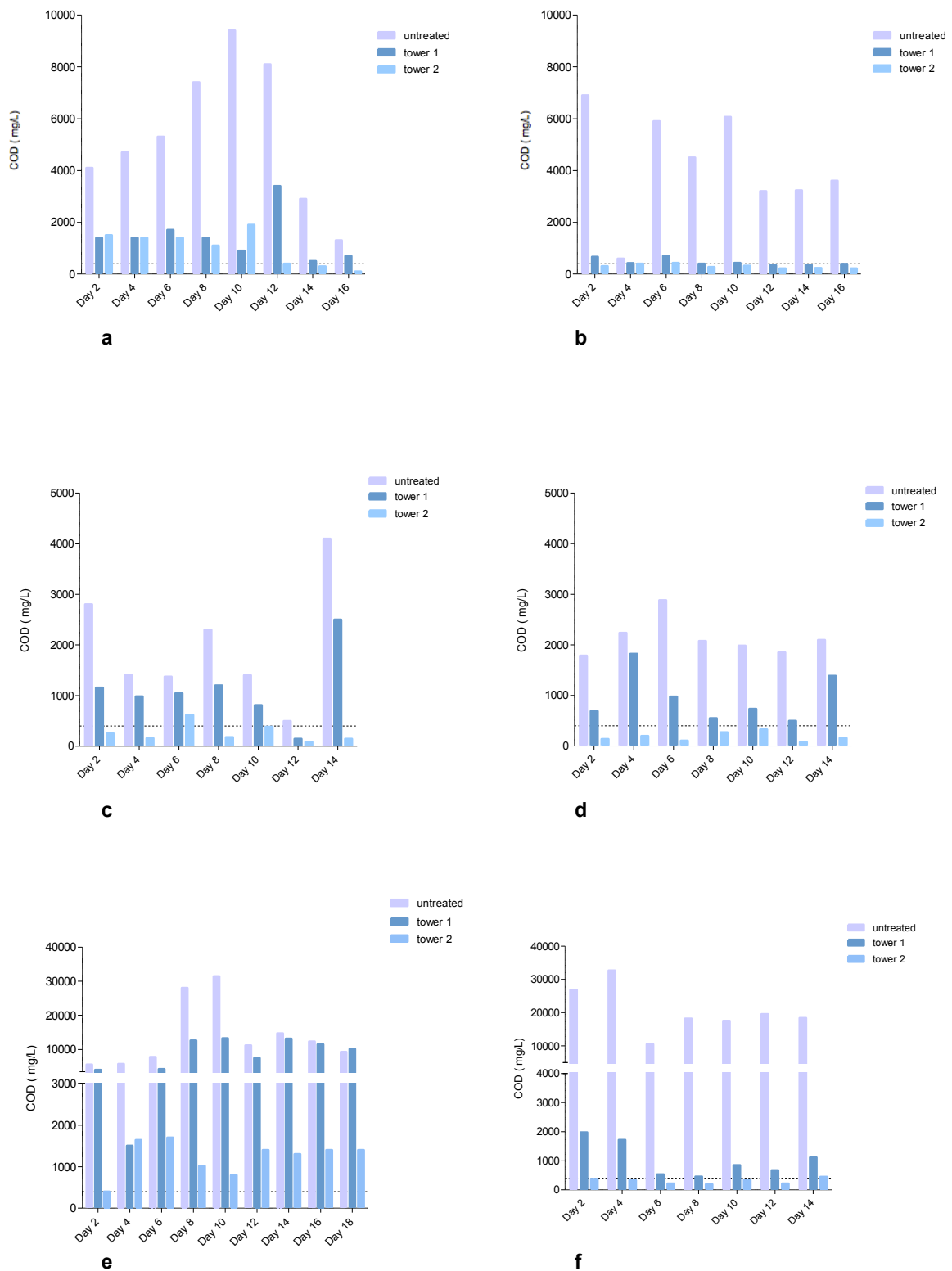


Figure 10. COD reduction over three seasons. (a) August/September 2014, (b) September 2015, (c) November/December 2013, (d) November/December 2014, (e) February 2014 and (f) February 2015.

## 7.2. pH

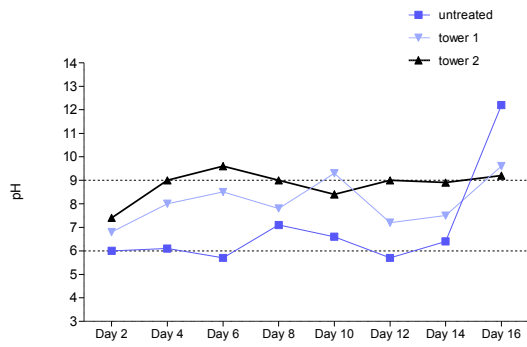
The system had a consistent buffering effect on the pH of the treated water across all six trials. In the late winter/early spring and the summer trials, the pH of the wastewater fluctuated between 4.2 and 11 (Fig. 11a-d). Varying cellar activities, including cleaning processes, resulted in high pH values and bottling and wasting resulted in low pH values in these seasons. Across all four these trials, treatment in the first subunit was sufficient to either reduce or increase the pH to within the required range of 6-9. Subsequent treatment in the second unit increased the pH by roughly another unit. Alkalinity (Table 4) was measured as the acid capacity (mg/L CaCO<sub>3</sub>) in the treated and untreated water. Acid capacity measures the ability of a water sample to bind to the anions of carbonic acids, exerting a buffering effect. At pH 4.3, such anions are completely protonated, and the water has no buffering capacity. A low acid capacity value thus indicates poor buffering capacity. The average acid capacity of the untreated wastewater outside of harvest time was  $12.63 \pm 3.9$  mg/L CaCO<sub>3</sub>. This indicated that buffering ions were present such as carbonates and hydroxides, likely from cleaning activities in the cellar as well as ammonia, phosphate and sulfate ions, exerting a buffering effect on the acidity of the water (Shanahan and Semmens 2015). There is a strong correlation between pH, buffering capacity and nitrogen removal, which can explain the buffering effect that was observed (Shammas 1986; Wild *et al.* 1971; Chen *et al.* 2006; Shanahan and Semmens 2015,). Nitrification releases two hydrogen ions for each mole of ammonium that is oxidised, which causes a reduction in pH. The amount of pH reduction, is, however, dependant on the alkalinity and ammonium concentrations of the water being treated (Shanahan and Semmens 2015). Optimum nitrification takes place at a pH of 7-9 (Chen *et al.* 2006). Nitrification can thus provide the hydrogen ions required to reduce the pH when wastewater with a high pH enters the system. Denitrification takes place optimally between pH 5 and 7, and releases one mole of hydroxide for each mole of nitrate or nitrite reduced, which can provide sufficient buffering capacity in a system when there is a lack of alkalinity (Shanahan and Semmens 2015). During the two harvest season trials, the average pH of the untreated water was 4.6, and alkalinity was extremely low at around 2 mg/L CaCO<sub>3</sub>, meaning that any acidity present in the water at that stage would contribute strongly to an acidic pH. At such high COD concentrations, the biofilm in tower 1 was likely breaking down large amounts of carbohydrates to organic acids, keeping the pH low. The second cooling tower then provided sufficient buffering capacity, likely through nitrification and

denitrification to increase the pH, as was reflected by the higher concentrations of alkalinity after treatment.

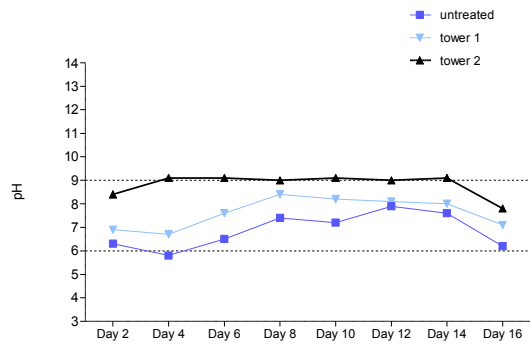
Table 4. The mean acid capacity concentrations as mg/L CaCO<sub>3</sub> of the untreated and treated wastewater.

Acid capacity (mg/L CaCO <sub>3</sub> )	late winter/ early spring		early summer		harvest season		average across seasons (n=6)
	2014 (n=8)	2015 (n=8)	2013 (n=7)	2014 (n=7)	2014 (n=18)	2015 (n=14)	
<b>Untreated</b>	9.14 ± 1.6	24.53 ± 1.71	9.6 ± 4.24	7.31 ± 1.81	2.11 ± .97	2.04 ± 0.27	9.12 ± 3.36
<b>Treated</b>	16.45 ± 3.13	71.35 ± 5.18	12.55 ± 4.47	14.3 ± 3.08	19.09 ± 2.24	30.84 ± 2.92	27.43 ± 9.17

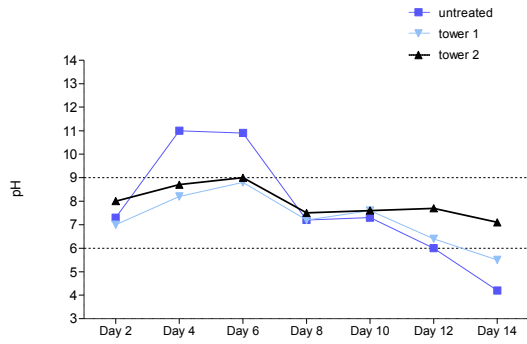




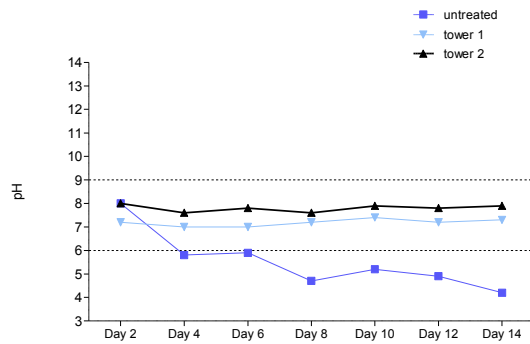
**a**



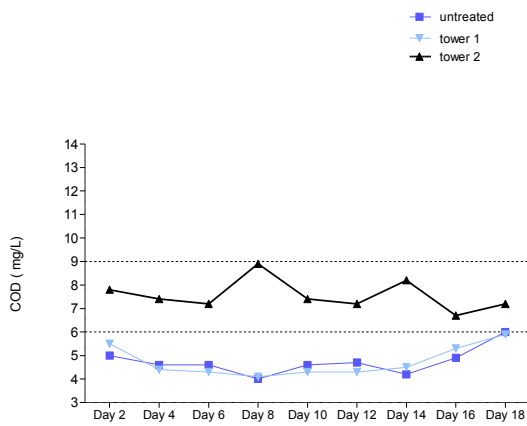
**b**



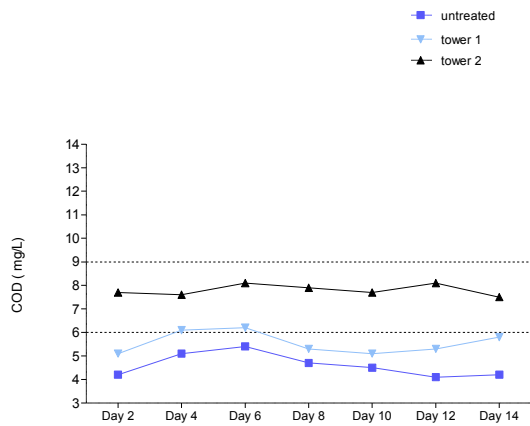
**c**



**d**



**e**



**f**

Figure 11. The pH of untreated and treated wastewater in (a) August/September 2014, (b) September 2015, (c) November/December 2013, (d) November/December 2014, (e) February 2014 and (f) February 2015.

### 7.3. Total Nitrogen

Nitrogen concentrations in the untreated wastewater rarely exceeded 5mg/L (line indicated on graphs, Fig. 12) outside of harvest season, and varied from high ( $17 \pm 4.06$  mg/L) during the 2014 harvest and moderate ( $8.4 \pm 0.13$  mg/L) during the 2015 harvest. At low concentrations, there was only between 5 and 50% reductions in total Nitrogen levels and results were inconsistent. When nitrogen concentrations exceeded 5mg/L, the system was consistently effective at removing 78–93% of nitrogen concentration, meeting prescribed guidelines, except in the last days of the trials. Nitrogen concentrations in treated wastewater exceeding the concentration of the untreated wastewater was observed after treatment after day 10 of both the 2014 and 2015 late winter/ early spring trials (Fig. 12a and 12b), the 2013 summer trial (Fig. 12c) and the 2014 harvest trial (Fig. 12d). The rising nitrogen concentrations in the system towards the end of each trial were likely the first indicators of detaching biomass at the end of the biofilm lifetimes. Furthermore, high levels of Nitrogen in the effluent towards the end of the biofilm maturity can be an indication of increased ammonia levels, with aerobic ammonia oxidation decreasing as the biofilm becomes dominated by anaerobic and anoxic zones, with reduced oxygen diffusion into the biofilm as it increases in thickness and age.

Table 5. The mean total nitrogen concentrations of the untreated and treated wastewater, and percentage reduction.

Total Nitrogen (mg/L)	late winter/ early spring		early summer		harvest season		average across seasons (n=6)
	2014 (n=8)	2015 (n=8)	2013 (n=7)	2014 (n=7)	2014 (n=18)	2015 (n=14)	
<b>Untreated</b>	$3.5 \pm 0.89$	$4.8 \pm .91$	$3.2 \pm 0.66$	$3.3 \pm 0.65$	$17.0 \pm 4.06$	$8.4 \pm 0.13$	$6.7 \pm 2.21$
<b>Treated</b>	$3.3 \pm 0.97$	$3.05 \pm 0.77$	$2.3 \pm 0.62$	$1.7 \pm 0.67$	$3.8 \pm 1.78$	$0.61 \pm 0.21$	$2.4 \pm .47$
<b>% Reduction</b>	5.71	36.46	28.13	48.48	77.65	92.74	64.18

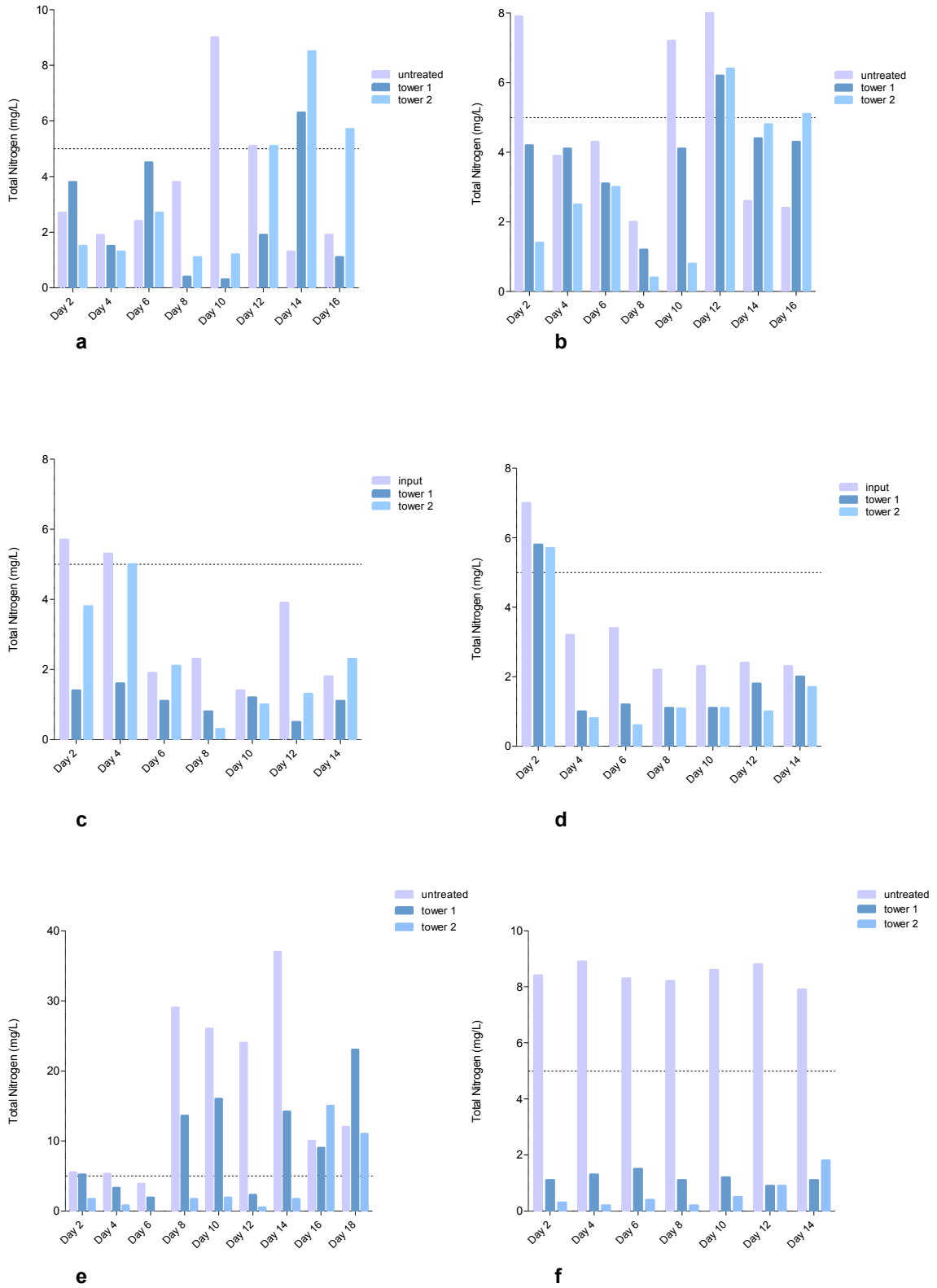


Figure 12. Total nitrogen reduction over three seasons. (a) August/September 2014, (b) September 2015, (c) November/December 2013, (d) November/December 2014, (e) February 2014 and (f) February 2015.

#### 7.4. Total sulfate

Sulfate levels in the winery wastewater very rarely exceeded 150 mg/L (Fig. 13), and was reduced, on average, by 63% to  $21.4 \pm 5.22$  mg/L. No correlations could be drawn between biofilm age and sulfate removal efficacy, but, due to the sulfate reduction observed, it was suspected that the biofilm in this system is stratified, with distinct biochemical redox layers, including an anoxic or anaerobic layer in which sulphate reduction can occur. In all of the trials, the bulk of the sulphate removal happened in the second cooling tower unit, confirming that the biofilm in the second cooling tower must be stratified.

Table 6. The mean total sulfate concentrations of the untreated and treated wastewater, and percentage reduction.

Total Sulphate (mg/L)	late winter/ early spring		early summer		harvest season		average across seasons (n=6)
	2014 (n=8)	2015 (n=8)	2013 (n=7)	2014 (n=7)	2014 (n=18)	2015 (n=14)	
<b>Untreated</b>	67.7 ± 22.13	63.8 ± 4.49	41.3 ± 9.83	46.0 ± 9.67	92.3 ± 17.23	32.4 ± 4.56	57.13 ± 8.9
<b>Treated</b>	42.8 ± 18.14	25.8 ± 2.08	14.0 ± 3.42	18.3 ± 3.37	22.89 ± 5.13	4.86 ± 0.77	21.4 ± 5.22
<b>% Reduction</b>	37.31	60.32	65.85	60.22	75.30	85.19	62.52

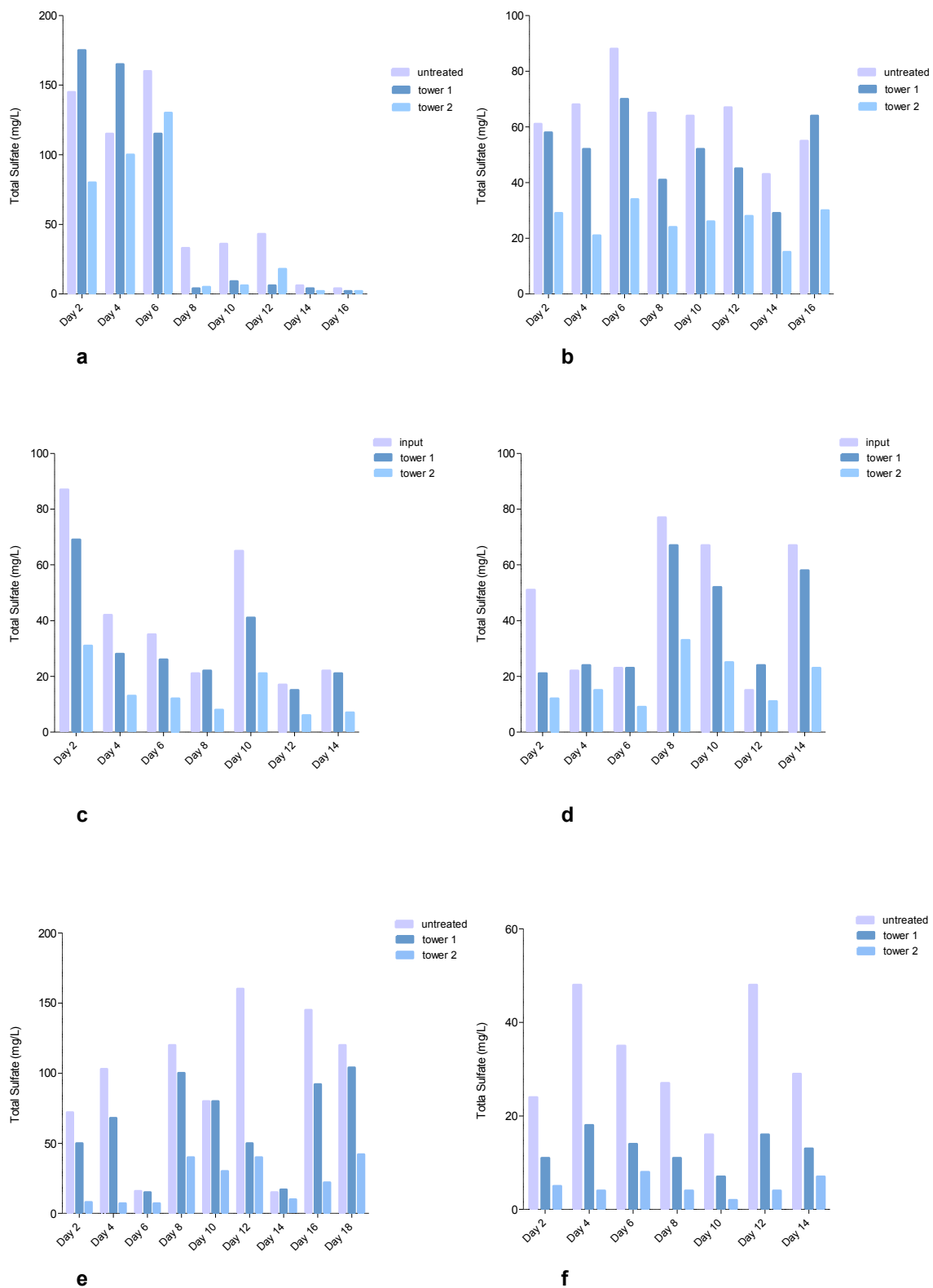


Figure 13. Total sulfate reduction over three seasons: (a) August/September 2014, (b) September 2015, (c) November/December 2013, (d) November/December 2014, (e) February 2014 and (f) February 2015.

### 7.5. Total phosphate

Outside of harvest season, the phosphate concentration in the untreated wastewater rarely exceeded 5 mg/L (lines indicated on Fig. 14). On average, a 66% phosphate removal efficacy was achieved, bringing the average phosphate concentration in treated wastewater to  $3.1 \pm 0.82$  mg/L (Table 5). Phosphate removal is carried out by phosphate accumulating organisms that assimilate and store phosphate as poly-phosphate under aerobic and anoxic conditions. Under anaerobic conditions, these organisms hydrolyse their internal polyphosphate stores, releasing phosphate into the surrounding environment. The fact that both phosphate uptake and phosphate release into the treated water are observed, serves as a further indication that the biofilms of this system are stratified, and contain distinct biochemical redox zones.

Table 5. The mean total phosphate concentrations of the untreated and treated wastewater, and percentage reduction.

Total Phosphate (mg/L)	late winter/ early spring		early summer		harvest season		average across seasons (n=6)
	2014 (n=8)	2015 (n=8)	2013 (n=7)	2014 (n=7)	2014 (n=18)	2015 (n=14)	
<b>Untreated</b>	$2.4 \pm 0.57$	$2.6 \pm 0.18$	$10.67 \pm 3.05$	$6.3 \pm 0.73$	$15.29 \pm 2.75$	$17.08 \pm 3.27$	$9.0 \pm 2.5$
<b>Treated</b>	$1.1 \pm 0.31$	$1.4 \pm 0.17$	$4.4 \pm .56$	$1.7 \pm 0.4$	$4.0 \pm 0.64$	$6.1 \pm 0.88$	$3.1 \pm 0.82$
<b>% Reduction</b>	54.17	46.15	58.76	73.02	73.33	64.71	66.67

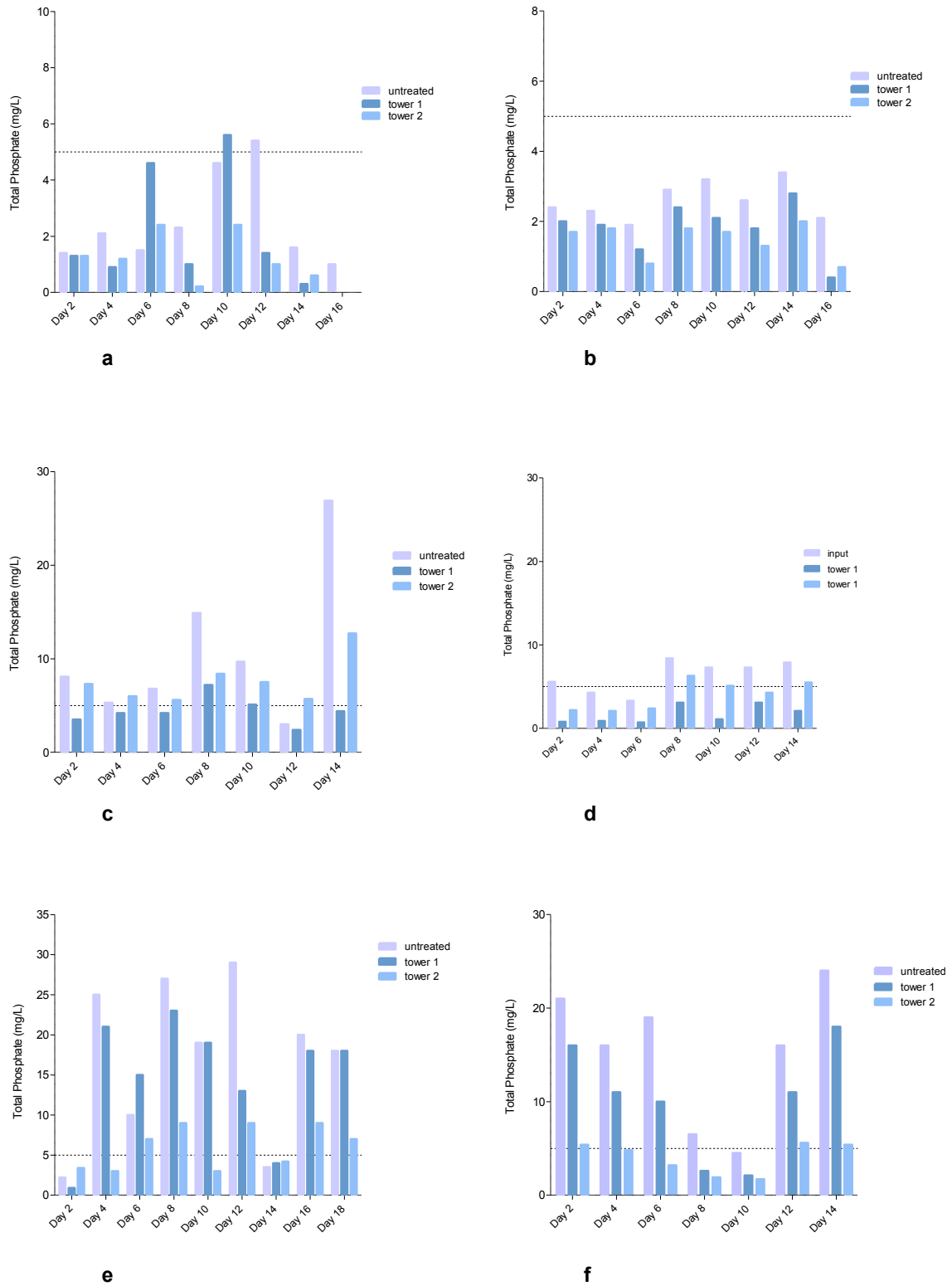


Figure 14. Total phosphate reduction over three seasons. (a) August/September 2014, (b) September 2015, (c) November/December 2013, (d) November/December 2014, (e) February 2014 and (f) February 2015.

### 7.6. Total suspended solid

The removal of total suspended solids was consistently effective, with 90.7% average removal, with an average concentration of  $74.8 \pm 6.5$  mg/L. Again, the second subunit was responsible for the bulk of the solids removal (Fig. 15). The required limit for irrigation of a volume of  $500\text{m}^3$  /day is 25mg/L. A concentration of 75 mg/L will be suitable for irrigation of lower volumes than  $500\text{m}^3$  of treated water per day.

Table 6. The mean total suspended solids concentrations of the untreated and treated wastewater, and percentage reduction.

Total suspended solids (mg/L)	late winter/ early spring		early summer		harvest season		average across seasons (n=6)
	2014 (n=8)	2015 (n=8)	2013 (n=7)	2014 (n=7)	2014 (n=18)	2015 (n=14)	
<b>Untreated</b>	1199 ± 116.1	761.1 ± 65.6	414.7 ± 98.13	370 ± 84.63	1207 ± 501.3	814.7 ± 131.8	794.2 ± 148.4
<b>Treated</b>	86.7 ± 21.0	48.1 ± 12.4	69.4 ± 30.33	69.14 ± 8.67	86.67 ± 25.74	91.86 ± 14.69	74.83 ± 6.5
<b>% Reduction</b>	92.83	93.69	83.33	81.35	92.83	88.82	90.68



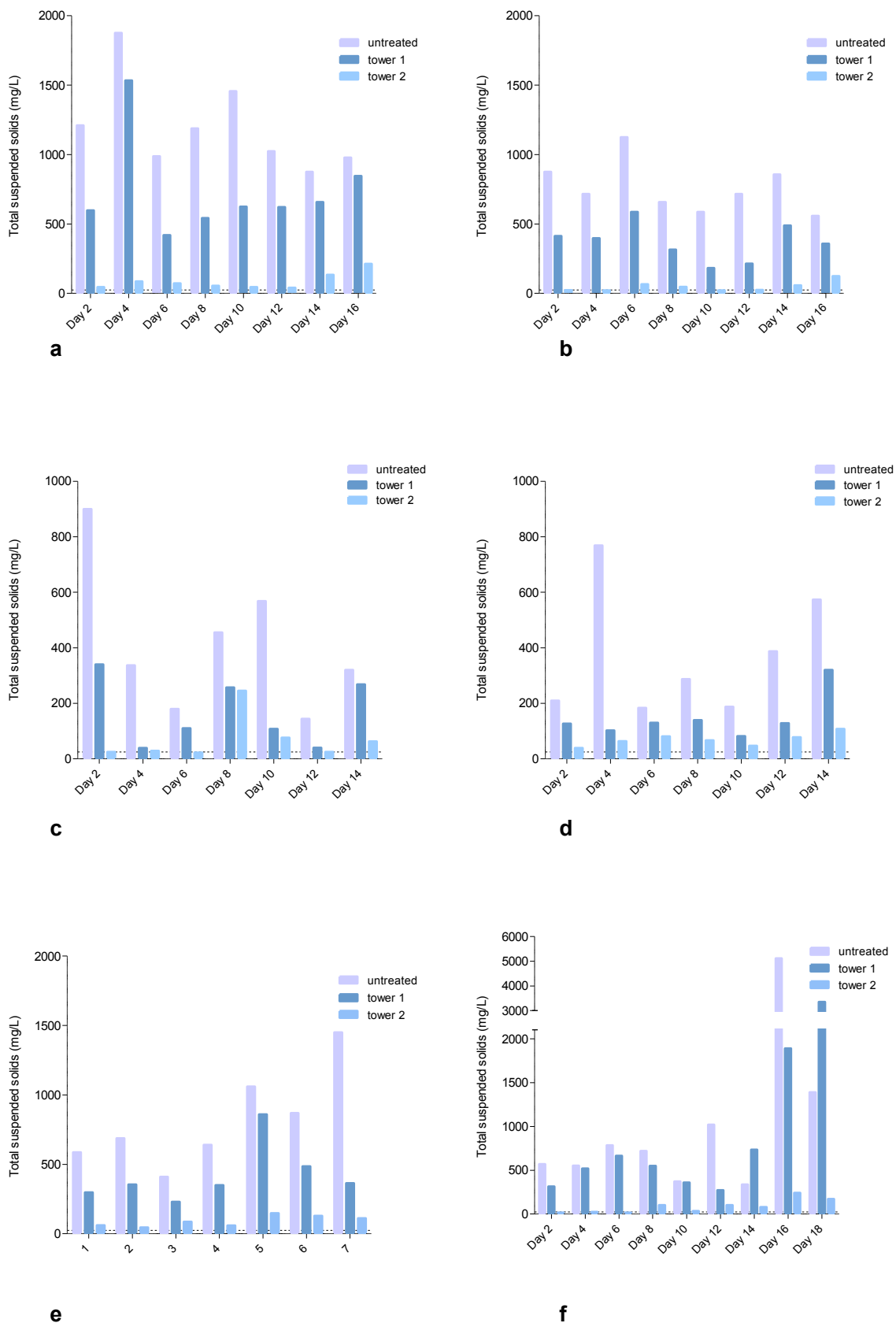


Figure 15. Total suspended solids reduction over three seasons. (a) August/September 2014, (b) September 2015, (c) November/December 2013, (d) November/December 2014, (e) February 2014 and (f) February 2015.

## 8. Sludge analysis

To determine whether the solid waste from the system could be re-used as a soil amendment medium, the spent biofilm which was expelled from the system was dried and chemically analysed. Saviozzi *et al.* (1994) have previously shown that sludge from the aerobic treatment of winery wastewater can be applied as soil amendment, and noted that, over a 180 day trial, the bioavailable N, P, K, S and carbon in the soil was improved, and the pH, COD and microbial activity in the soil remained unchanged. The pH of the sludge from this study was close to neutral, and the electrical conductivity was well within the prescribed limit of 70 mS/m for irrigation and the general limit for water disposal (IPW 2015). Furthermore, the sodium adsorption ratio (SAR) of 0.05 was also below the allowed limit of 5 for irrigation purposes (IPW 2015) (table 7). It should be kept in mind that Saviozzi *et al.* (1994) observed a dangerous build-up of salt in the soil during their 180 day trial, which was detrimental to soil condition and crops. The application of sludge from this system should be monitored over time to establish if it causes a similar build-up of salt, and the potential phytotoxicity of the sludge needs to be established before it can be deemed a suitable amendment for crop-bearing soil.

Table 7. The chemical composition of the solid waste of the winery wastewater treatment process.

pH	Conductivity (ohm)	Sodium Adsorption Ratio	% Moisture	Density (kg/m <sup>3</sup> )	N %	P %	K %	Ca %	Mg %	Na (g/kg)	Carbon %	Ash %
7.9	100	0.05	14.2	419	1.95	0.34	1.19	3.46	0.27	1.11	16.5	77.6

## Conclusion

A pilot scale biofilm contact reactor was built and tested for winery wastewater treatment efficacy over the course of three years. An initial, single unit pilot scale reactor successfully treated winery wastewater by reducing COD, neutralising high and low pH values and removing nutrients and suspended solids from winery wastewater during harvest season, when the winery was producing high-strength wastewater. The system was up-scaled by adding a second cooling tower to the system, operating as a secondary reactor, treating the effluent from the first subunit. The second unit contributed to the overall waste removal efficiency. The double-unit pilot system was tested in six trials, two per season over three

years. On average, the system was consistently effective at reducing COD, total nitrogen, sulfate, phosphate and suspended solids to meet prescribed regulations for irrigation. The system performed at its peak when treating highly concentrated wastewater during harvest season. The waste removal capabilities of the system indicated the presence of anaerobic microbial metabolic activities in the biofilms based on the sulphate and phosphate removal data. This implied that the biofilm was possibly stratified, providing more than one biochemical redox niche for waste removal. Overall, the system and its performance met the objectives of this study, namely providing an easily constructible, compact and highly effective wastewater treatment solution for wineries. The system proved to be easily up-scalable by adding an additional cooling tower unit, was simple to operate, did not produce malodours, and required only enough electricity to power three small pumps. The only operational difficulties arose from grape material in the wastewater stream during harvest season, which clogged and hampered the operation of solenoid valves in the system. By replacing all the valves with industrial grade electronic ball valves, which will be incorporated in future designs, this problem will be avoided. The system showed effective, robust treatment of winery wastewater of varying strengths with minimal solid waste production. The quality of the solid waste was analysed and deemed potentially suitable for re-use by application as a soil amendment medium.

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## CHAPTER 5

### The microbial community of a biofilm contact reactor for the treatment of winery wastewater

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## Introduction

Wineries produce highly concentrated organic wastewater, which can vary in composition and volume seasonally (Moldes *et al.* 2008; Lucas *et al.* 2010). Organic waste contributes up to 85% of the contaminants in winery wastewater, and is introduced at various stages of the winemaking process as grape material, yeasts, alcohols, esters, sugars, soluble organic acids, tannins, lignins and polyphenols (Mosse *et al.* 2011). Consequently, winery wastewater can have chemical oxygen demand (COD) values of up to 45 000 mg L<sup>-1</sup>, biological oxygen demand (BOD) values of up to 20 000 mg L<sup>-1</sup> and total suspended solids (TSS) of up to 30 000 mg L<sup>-1</sup> during vintage. The high concentration of readily biodegradable organic matter makes biological wastewater treatment the most economical option for winery wastewater (Van Loosdrecht and Heijnen, 1993).

Biological wastewater treatment relies on the metabolic activities of microorganisms, which oxidise contaminants in wastewater to obtain energy for growth. The biochemical redox environment (aerobic, anaerobic, anoxic or any combination of these) in a wastewater treatment system is determined by the phases included in the design of the system, and defines which electron acceptor is available for microbial metabolism. A single phase reactor, such as a suspended growth bioreactor can be aerobic or anaerobic. Reactors can also consist of a combination of any or all of the following phases, namely a solid phase, a liquid phase and a gaseous phase (Leslie Grady *et al.* 2011). Fixed growth biological contact reactors retain biomass within a biofilm that is attached to a substrate exposed to wastewater in the presence or absence of aeration. Soluble organic micro and macromolecules diffuse through the extracellular polymeric substances (EPS), allowing the biofilm to oxidise readily biodegradable COD at a high rate (Andreottola *et al.* 2005). In a mature biofilm in an aerated system, an oxygen concentration gradient exists across the biomass, providing different biochemical redox environments within the biofilm (Lewandowski and Boltz, 2011). This creates a multi-niche microenvironment in which microorganisms with distinct metabolic profiles can flourish and contribute to waste removal. Biofilm reactors can achieve similar

results in wastewater treatment as suspended growth bioreactors in terms of carbon removal, nitrification, denitrification and sulfur removal (Lewandowski and Boltz 2011). The design and operational parameters of a biofilm reactor can have a major impact on the microbial consortium and thus the treatment capabilities of a biofilm (Adrados *et al.* 2014). To optimise the design and functionality of a biofilm wastewater reactor, it is important to understand how the biofilm works within the system to remove contaminants. In this study, a three tiered approach provides insight into the microbial community structure, the spatial distribution and the metabolic capabilities of organisms of a biofilm in a high rate biological contact reactor treating winery wastewater. The system (Chapter 4 of this manuscript), consisting of two reactor subunits operating in series, was effective at reducing COD, N, P, S, suspended solids and neutralising pH in winery wastewater with the first reactor subunit (tower 1), and showed increased treatment efficacy after treatment with a second reactor subunit (tower 2). The treated water from tower 1, which had a reduced organic load and a pH restored to neutral, served as the input water for tower 2, meaning that the biofilm in tower 2 developed under chemically distinct conditions from the biofilm in tower 1, which had high organic load, low pH wastewater as input. The question therefore arose whether the second reactor subunit contributed to the overall performance of the system by simply increasing the treatment capacity of the system, or whether the microbial community of the biofilm in tower 2 expanded the treatment scope of the system, rendering the second reactor a polishing unit, removing contaminants that fell outside of the metabolic scope of the consortium in the first reactor. Investigating the differences in metagenomic profiles, organism distribution patterns and metabolic profiles of the biofilms in tower 1 and tower 2 will shed light on how the inclusion of separate subunits in the design of a high rate biological contact reactor for the treatment of winery wastewater, will influence reactor performance. The results will provide pivotal information on how such bioreactors should be up-scaled.

The three tiered approach to investigating the system at hand involved studying the complete metagenomes of the biofilms by next generation sequencing (NGS), followed by analyses of the distribution of specific organisms within the biofilms using fluorescent in situ hybridisation (FISH) coupled with confocal microscopy using organism-specific probes, and finally an investigation into the metabolic activities of the biofilms from the reactor using the Biolog system. NGS, which is culture-independent, was used to study the bacterial and fungal metagenomes for towers 1 and 2 and is considered the most inclusive technique for analysing environmental communities in microbial ecology (Shokralla *et al.* 2012). NGS has been



widely used as a rapid, cost effective, high throughput tool to study complex environmental microbial communities in wastewater treatment applications (Ducey and Hunt, 2013; Li *et al.* 2013; Kim *et al.* 2014), and has to our knowledge not yet been applied to study the microbial community of a winery wastewater treatment system. FISH targeting microorganisms with specific metabolic properties was used to investigate the spatial distribution of different organisms within the biofilm. FISH is a specific, culture independent technique used widely in biofilm and wastewater treatment studies to identify microbes and study their distribution and growth patterns (Wagner *et al.* 1995, Daims *et al.* 2001a, Guo *et al.* 2013, Basset *et al.* 2016). Finally, the metabolic capabilities of biofilm samples from each reactor subunit, tower 1 and tower 2, were compared based on substrate utilisation patterns using the Biolog system, as previously also applied to heterotrophic environmental microbial samples (Garland and Mills 1991, Kelly and Tate 1998, Preston-Mafham *et al.* 2002, Grayston *et al.* 2004). Incubation of biofilm samples in Biolog 96 well microtiter plates containing single substrates and a colourless redox dye can, through oxidative substrate utilisation and a subsequent colour reaction, produce patterns of substrate utilisation or a community-level physiological profile (CLPP) for each biofilm community. This allows comparative multivariate analyses of the substrate utilisation profiles of the two biofilm communities (Garland, 1996). Although the Biolog system is culture-based, it has been shown that non-culturable cells do respond to Biolog assays (Garland, 1999) and are not as biased as traditional culture based techniques (Preston-Mafham *et al.* 2002).

## **Materials and methods**

### ***1. Biofilm community analysis in a high rate biological contact reactor treating winery wastewater with NGS.***

#### *1.1. Sampling of biomass*

A 5 g sample of biomass was collected from the base of the fill media, from a spot towards the center of the tower for each subunit of the biofilm reactor during the harvest season of 2014, when high strength effluent with a COD of above 20 000 mg/L was being treated.

## 1.2. NGS of the bacterial metagenome

For each subunit, 0,25 g of the biomass sample was applied to the ZR™ Soil microbe DNA Miniprep Kit (Zymo Research Corporation, U.S.A.) for isolation of PCR-quality DNA, which was used to prepare an amplicon library for next generation sequencing (NGS) at the Central Analytical Facility (CAF, Stellenbosch). Extraction yield was evaluated by electrophoresis in 0.8% (w/v) agarose gels stained with ethidium bromide and bands were compared to a 1 000 bp DNA ladder (GeneRuler, Fermentas). The variable region V3 to V4 of the bacterial 16S rRNA genes were targeted for PCR amplification using Fusion primers (Huse *et al.* 2008) to create amplicon libraries (IDT, USA). Multiplex sequencing was achieved using barcodes (Ion Xpress barcodes, IDT). The amplicons were enriched using the Ion PGM™ Template OT2 400 Kit on the Ion OneTouch™ 2 System. The samples were loaded onto an Ion 318 Chip for directional multiplex sequencing using the personal genome machine PGM (Thermo Fisher, USA). The sequencing data was processed using MOTHRUR version 1.33.3 (Schloss *et al.* 2009). The BAM file format was converted to a standard flowgram format (.sff) followed by trimming of the barcodes and adaptors. Low-quality sequences were removed by eliminating sequences that did not have an exact match to the forward primer, contained any ambiguous bases and were shorter than 200 bp. PCR chimeras were identified and removed using Uchime (Edgar *et al.* 2011; Schloss *et al.* 2011). The ion torrent sequencing process produced 2 462 (tower 1) and 3 004 (tower 2) high quality V3-V4 tags of the 16S rRNA gene, with an average length of 384 bp. Taxonomic classification was done via BLAST alignment against the ARB-SLIVA database down to phylum, class and genus level using a confidence threshold of 80%. Sequence clusters were classified using the RDP v14 reference taxonomy. Sequences were clustered into operational taxonomic units (OTUs) 97% and 95% sequence similarity (a 0.03 or 0.05 distance limit) using MOTHRUR at a uniform length of 300 bp, avoiding possible miss-assignments due to differences in sequence length. According to Kunin *et al.* (2010), clustering should not be done at a threshold of higher than 97% sequence similarity, as this leads to overestimation of diversity. Rarefaction curves were generated for each sample at 0.03 and 0.05 distance levels. The Chao1 richness estimator, which calculates the minimal number of actual OTUs present in a sample (Chao, 1984), the Shannon diversity index (Ludwig and Reynolds, 1988) and Good's coverage, measuring the percentage of individuals sampled in a microbial community (Good, 1953), were calculated for each sample.

### *1.3. NGS of the fungal metagenome*

The Internal Transcribed Spacer (ITS1 and ITS2) regions of the fungal 18S rRNA genes were amplified using eukaryotic primers using a set of 6 uniquely barcoded forward ITS1f primers and one reverse primer. PCR product quality and size were determined by Agarose gel electrophoreses. Equimolar pooling of amplicons and library templating were followed by sequencing on a 318 chip using the OT2 and Ion Torrent PGM systems (Thermo Fisher, USA). The quality of raw sequencing reads were consecutively checked using the open source bioinformatics programs QIIME (Caporaso *et al.* 2010) and PIPITS, an automated pipeline for analysis of fungal ITS sequences (Gweon *et al.* 2015). Unique, non-chimeric sequences greater than 160 bp were aligned to the UNITE (Abarenkov *et al.* 2010) reference database and clustered into OTUs at a 97% similarity. Taxonomic identification was performed at an 80% similarity cut-off value. The 18S rRNA data from individual samples were rarefied to equal sample size based on the sample with lowest number of sequences.

## ***2. CLSM and Fluorescent in-situ hybridisation for the characterisation of biofilm composition and structure.***

Biofilm sample preparation and FISH were performed using the guidelines described in the FISH Handbook for Biological Wastewater Treatment (Nielsen *et al.* 2009) and for the application of multiple probes per sample, an adjusted protocol from Suarez *et al.* (2015) and Thurnheer (2004) was followed. The process is summarised in Figure 1.

### *2.1. Biofilm sample collection and preparation*

Biofilm samples were taken from the reactor (chapter 4) post-harvest season in April 2015 when cellular activities yielded high-strength wastewater with COD values above 20 000 mg/L. Sections of fill media supporting biofilm growth from tower 1 and tower 2 were harvested carefully with in-tact, undisturbed biofilm still attached to the surface. The samples were gently submerged in a 4% paraformaldehyde PBS solution for fixation at 4 °C for 1 h.

The screening of biofilm samples with each individual FISH probe was performed on slides prepared with biofilm suspensions. For this purpose, fixed biofilm material from each tower was removed from the fill media substrate, and homogenised in PBS. Suspended samples

were stored in 1:1 PBS:Ethanol at -20 °C. Suspended biofilm samples were prepared for microscopy by spotting 10µl aliquots onto pretreated microscope slides, and left to air dry. Subsequently, the samples were permeabilised with lysozyme (Sigma-Aldrich), 70 000 U/ml in Tris-HCL pH7.5 for 10 min at 37 °C. For multiplex FISH, cryosections of biofilms from tower 1 and tower 2 were prepared. The substrate with the attached biofilm was embedded in tissue freezing medium (Leica Biosystems GmbH, Wetzlar, Germany) overnight at 4 °C. The samples were then placed in liquid nitrogen until the freezing medium was frozen. Using a scalpel and forceps, the pliable fill media substrate was carefully separated from the intact frozen biofilm sample to expose the substrate interface of the biofilm. The orientation of the sample was labelled to indicate the substrate and water interfaces, and after embedding the entire sample in tissue freezing medium, it was stored at -80 °C until use. Cryosectioning was carried out on a Leica CM1860 UV Cryostat (Leica Biosystems GmbH, Wetzlar, Germany) at -20 °C with 15-20 µm thick slices collected on pre-treated microscope slides. The samples were dehydrated in an ethanol series (50, 80 96% v/v) and permeabilised with lysozyme (Sigma-Aldrich), 70 000 U/ml in Tris-HCL, pH7.5 for 10 min at 37 °C. Samples were stored at -20 °C until use.

All microscope slides were pre-treated to enhance the attachment of biofilm material and minimize the loss of biomass during FISH. Slides were etched with 1M HCl at 60 °C for 8 h, followed by a dH<sub>2</sub>O and ethanol rinse, and left to air dry. Subsequently, etched slides were coated with 0.01% poly-L-lysine solution (Sigma-Aldrich, St.Louis, USA) by soaking for 5 min at room temperature and leaving in a vertical position to air dry in a dust-free environment.

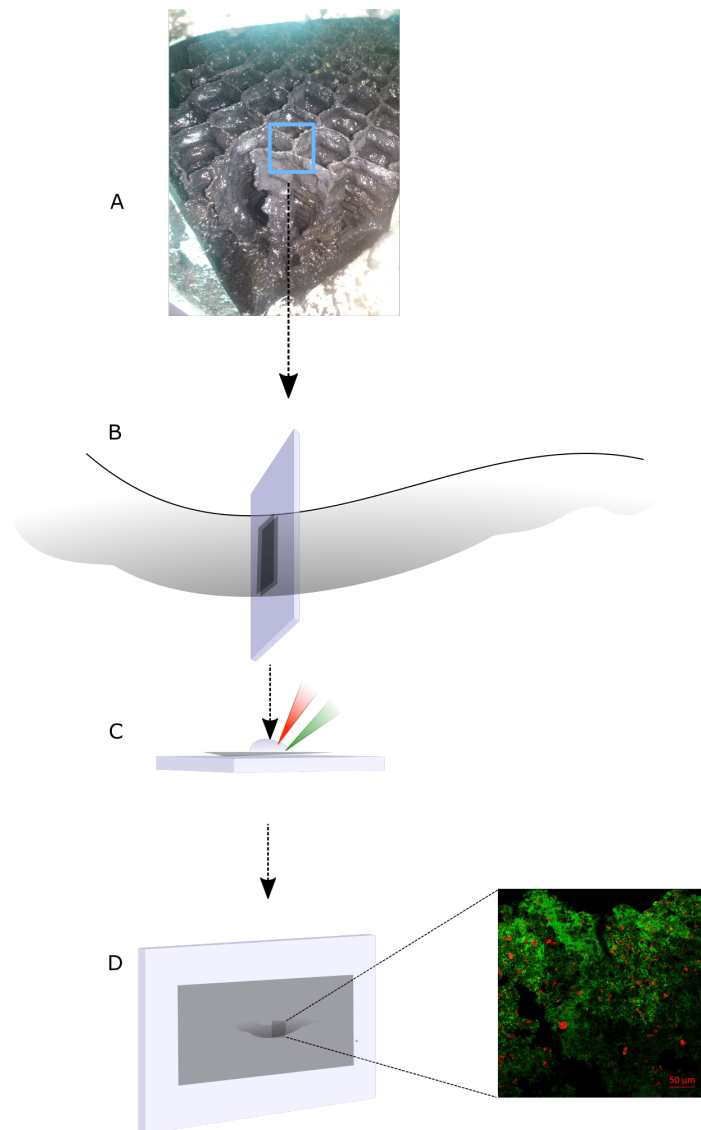


Figure 1. A graphical representation of the FISH process. (a) The biofilm growing on fill media in tower 1 of the bioreactor, from where it was harvested. (b) Biofilm intact on fill media substrate, encapsulated in tissue freezing medium, cryosectioned perpendicular to the substrate interface of the biofilm through to the water interface. (c) Cryosection on a microscope slide with FISH performed by adding hybridisation cocktails. (d) The biofilm section is imaged with multiple tiled scans producing an image traversing the breadth of the sample.

## 2.2. FISH

FISH probes were obtained from Biomers.net GmbH (Ulm, Germany). Probe sequences for the detection of the relevant target organisms were retrieved from the literature. The probes were labeled at the 5' end with Atto fluorescent dyes from ATTO-TEC GmbH (Siegen, Germany). All buffers were made up to the specifications of Nielsen *et al.* (2009). Slides with prepared biofilm samples were pre-incubated with hybridisation buffer at 46 °C for 15 min. Table 1 lists the FISH probes and the hybridisation conditions that were used. Samples were incubated with 2ml of hybridisation cocktail at 46° C for 3 h according to the protocol described by Nielsen *et al.* (2009). Multiple probe analyses were carried out by combining probes with similar hybridisation stringencies in the hybridisation cocktail. When probes with different hybridisation stringencies were used on one sample, sequential hybridisations were performed, starting with probes requiring the most stringent conditions and ending with hybridisations with probes requiring less stringent conditions. Hybridisation was followed by a 15 min incubation with washing buffer at 46 °C after which samples were dipped in cold dH<sub>2</sub>O and left to air dry. Samples were embedded in a small drop of Dako fluorescence mounting agent (Dako, Denmark) containing an anti-fading agent to preserve fluorescence, and covered with a cover slip. Slides were stored in the dark until microscopy.

Table 1. A summary of the FISH probes applied in this study.

	function in wastewater treatment	% FA	Probe	Probe sequence (5'-3')	Reference
<u>Organism (Eukarya)</u>					
Eukarya	universal probe for yeast	30	Euk 1195	GGG CAT CAC AGA CCT GTT	(Giovannoni <i>et al.</i> 1988)
<i>Candida intermedia</i>	COD reduction	20	Cint	TTA TCC ACC CCT AGC A	(Mounier <i>et al.</i> 2009)
<i>Saccharomyces cerevisiae</i>	present in the winemaking process	10	Sce	TGACTTACGTCGCAGTCC	(Xufre <i>et al.</i> 2006)
<u>Organism (Prokarya)</u>					
EUB338	universal probe for bacteria	30	EUB338 pair	GCTGCCTCCCGTAGGAGT	(Amann <i>et al.</i> 1990)
<i>Alphaproteobacteria</i>	Denitrifiers/PAO	20	ALF1b	CGTTCG(C/T)TCTGAGCCAG	(Amann <i>et al.</i> 1990)
<i>Betaproteobacteria</i>	Denitrifiers/PAO	35	BET42a	GCCTTCCCACTTCGTTT	(Amann <i>et al.</i> 1990)
<i>Gammaproteobacteria</i>	NOB/PAO	35	GAM42a	GCC TTC CCA CAT CGT TT	(Manz <i>et al.</i> 1992)
AOB	AOB	55	Nso190	CGATCCCCTGCTTTTCTCC	(Mobarry <i>et al.</i> 1996)
Most betaproteobacterial AOB	AOB	35	Nso 1225	CGCCATTGTATTACGTGTGA	(Mobarry <i>et al.</i> 1996)
<i>Nitrospirae</i>	NOB	50	Ntspa712	CGCCTTCGCCACCGCCTTCC	(Daims <i>et al.</i> 2001b)
<i>Nitrobacter</i>	NOB	40	NIT3	CCTGTGCTCCAGGCTCCG	(Wagner <i>et al.</i> 1996)
<i>Azoarcus</i>	NOB	20	AZA645	GCCGTA CTCTAGCCGTGC	(Hess <i>et al.</i> 1997)
<i>Actinobacteria</i>	PAO	30	Actino221 pair	CGCAGGTCCATCCCAGAC	(Kong <i>et al.</i> 2006)
Most <i>Accumilobacter</i>	PAO	35	PAO462	CCGTCATCTACWCAGGGTATTAAC	(Crocetti <i>et al.</i> 2000)
<i>Deltaproteobacteria</i>	Sulfate reducers	35	DELTA495a pair	AGTTAGCCGGTGCTTCCT	(Loy <i>et al.</i> 2002)

### 2.3. Microscopy and image analyses

Confocal microscopy was performed using a Zeiss LSM780 (Carl Zeiss, Germany) equipped with Zen 2011 imaging software (Carl Zeiss, Germany) a GaAsP detector 32+2PMT for fluorescence and a transmitted light detector T-PMT. The lasers that were used were the Argon multiline laser 25 mW at 458 nm, 488 nm and 514 nm; the DPSS 561 nm laser and the HeNe 633 nm laser. The beam splitters were MBS 405 for ATTO 390 labelled probes, MBS488 for ATTO 488 labelled probes, MBS 458/514 for ATTO 514 labelled probes and MBS 488/561/633 for ATTO 550 and ATTO 590 labelled probes. Single high resolution images were acquired using a LCI plan-apochromat 63x/1.4 oil immersion DIC M27 objective. The composite image to investigate the distribution of eukaryotic and bacterial cells across the biofilm was composed of nine 135  $\mu\text{m}$  x 135  $\mu\text{m}$  images taken with the plan-apochromat 63x/1.4 oil immersion DIC M27 objective using the tile scan image collection function of the ZEN software. The abundance of eukaryotic cells and bacterial cells were calculated using the biovolume fraction analysis function in Daime digital image analysis software version 2.0 (Daims *et al.* 2006). Twenty five random, non-overlapping high resolution images were imported to Daime, segmented, and biomass was detected by using modified robust automated threshold selection (RATS-L thresholding) setting before the biovolume fraction was calculated for signal from each probe. The distribution of Gammaproteobacteria was investigated using a composite tile image of a cryosection through the depth of a biofilm sample taken from tower 1 (Fig. 11a). The stratified distribution of Gammaproteobacteria was studied and calculated in Daime software by segmenting the image with edge detection of biomass with a dark threshold parameter of 20. A slicer template (Fig. 11b) (Almstrand *et al.* 2003) was generated which divided the biomass in the image into stacked slices of 116  $\mu\text{m}$  each between the substrate interface and the water interface of the biofilm. The relative abundance of Gammaproteobacteria in each layer was calculated as percentage biomass, giving an indication of the stratification of Gammaproteobacteria across the depth of the biofilm. The 3D projection showing the distribution of *S.cerevisiae* cells among *Nitrobacter* clusters was composed using multiple high resolution images taken with the Z-stack function of Zen 2011 software.



### **3. Community level physiological profiling of biofilm substrate utilisation using Biolog ECO and Biolog Phenotypic Microarray analyses.**

Biolog ECO plates were used as a general screening tool to determine whether the substrate utilisation profiles of the biofilms from tower 1 and tower 2 differed. ECO plates are designed for analyses of heterogeneous environmental samples, and have been applied in biofilm studies. More in-depth analyses of substrate utilisation were done using Biolog phenotype microarray (PM) plates. PM plates are intended for use with single, defined cultures, and are paired with growth media to support specific groups of organisms such as gram-negative or gram-positive bacteria. This study also served as an evaluation of the feasibility of applying the PM plate system to heterogeneous environmental samples. The growth media and protocols were adjusted according to suggestions by the manufacturer.

#### *3.1. Sampling*

Biofilm samples were collected from tower 1 and tower 2 in September 2015, during a period when cellular activities yield wastewater of a moderate strength of a COD between 3 000 and 7 000 mg/L, which is the strength that was typically recorded throughout the most of the year excluding vintage periods, when high COD values are recorded. The rationale was that monitoring metabolic activity of biofilms formed when treating moderate strength wastewater would give insight into the basal metabolism of the organisms within the biofilms. A sample of 1 g of biofilm was removed from each tower, and suspended in 10 ml PBS. A suspension of each biofilm was formed by vortex mixing at top speed for 10 min at room temperature.

#### *3.2. Inoculation*

For carbon utilisation profiles, Biolog ECO microplates (Biolog Inc., Hayward CA, USA) were inoculated with 150 µl of a  $10^{-3}$  dilution of biofilm suspensions from each tower. For a broader investigation into substrate utilisation, phenotype microarrays, Biolog PM microplates (Biolog Inc., Hayward CA, USA) were used: PM1 and PM2 for carbon metabolism, PM3 for nitrogen metabolism, PM4 for phosphorous and sulfur metabolism, PM5 for growth factor requirements, PM6,7 and 8 for peptide nitrogen metabolism, PM9 for osmotic stress response and PM10 for optimum growth pH. Plates were inoculated with biofilm suspensions from each tower, and the inoculation protocol from Biolog designed for

pure *Bacillus* cultures was adjusted to suit the growth requirements of heterogenic biofilms growing in a winery wastewater environment. Plates PM1-PM8 were inoculated with 100  $\mu\text{l}$  of a  $10^{-3}$  dilution of biofilm suspension in inoculating medium IF-0a GN/GP (Biolog Inc., Hayward CA, USA) supplemented according the manufacturer's protocol. PM9 and PM10 were inoculated with 100  $\mu\text{l}$  of a  $10^{-3}$  dilution of biofilm suspension in inoculating medium IF-10b GN/GP (Biolog Inc., Hayward CA, USA) supplemented according the manufacturer's protocol. For plates PM3-PM8, the inoculating fluid was supplemented with 0.08 M Acetic acid (Sigma-Aldrich), to provide an organic carbon source native to the winery wastewater environment (Malandra *et al.* 2003). Furthermore, each inoculum was adjusted to pH 4.5 to simulate winery wastewater conditions, and included a tetrazolium dye "Redox dye mix H" for monitoring substrate utilisation of medium to slow growing organisms. All plates were incubated without agitation at 21 °C and the absorbance of each well was measured at 590 every 24 h for 144 h using a BioTek PowerWave microplate spectrophotometer (BioTek, VT, USA).

### 3.3. Analysis of microplate data

The spectrophotometric data of each plate was corrected by subtracting the water blank as well as the absorbance value at  $t=0$  from each well, which eliminates interference from the density of the inoculum with the results. The colour score in each well was expressed as average well colour development (AWCD) (equation 1 and 2). The absorbance value at 590 nm in each well was normalised by dividing the corrected absorbance values by the AWCD for that specific plate (equation 3), as recommended by Garland *et al.* (1996).

$$AWCD = \frac{1}{31} \sum_{i=31}^{31} \textit{absorbance} \text{ for ECO plates} \quad (1)$$

$$AWCD = \frac{1}{95} \sum_{i=95}^{95} \textit{absorbance} \text{ for PM plates} \quad (2)$$

$$\textit{Absorbance}_{\text{normalised}} = \frac{\textit{absorbance}}{AWCD} \quad (3)$$

The normalised absorbance was then used as a colour score for substrate utilisation. The extent of substrate utilisation in each well was defined by comparing the colour development to the control well as also implemented by Nai *et al.* (2013) when studying substrate

utilisation in PM 1 and 2 by an environmental fungal sample. Wells with less than 0.75 absorbance units were defined as having weak substrate utilisation. Wells with between 0.75 and 1.5 absorbance units were considered positive for substrate utilisation and wells with absorbance units above 1.5 were considered to have strong substrate utilisation. All experiments were done in triplicate.

## Results and discussion

### 4. *Biofilm community analysis in a high rate biological contact reactor treating winery wastewater with NGS.*

#### 4.1. *Biofilm bacterial community richness and diversity*

The complexity of the bacterial communities in the biofilm samples were assessed by determining the number of OTUs present, the Shannon diversity index, the Chao species richness estimator and Good's coverage. Alignment at a uniform length of 300 bp clustered the highest number of OTUs for the biofilm sample taken from tower 2 with 922 OTUs at a distance limit of 0.03 and 592 OTUs at a 0.05 distance limit (Table 2). Shannon diversity indices showed similar bacterial diversities in the biofilms of tower 1 and tower 2, and that the biofilm from tower 2 had slightly higher bacterial diversity than the biofilm from tower 1 (Table 2). The rarefaction curve of both samples at 0.03 and 0.05 distance limits (Fig. 2) indicates only slightly higher phylotype richness in the biofilm from tower 2. The curves do not level out, indicating that sequencing depth was not sufficient to detect all the species present in the sample, and that further sequencing is required to accurately calculate bacterial richness in these samples. This is confirmed by Chao1 bacterial richness estimations, which were much higher than the OTUs observed in each sample, indicating underestimated bacterial richness when only considering the observed OTUs. When considering a Venn diagram (Fig. 3) of the overlap in OTUs between the bacterial populations of the biofilms from tower 1 and tower 2, only 203 OTUs out of 1699 occur in both towers. In a study by Lemos *et al.* (2011) it was determined that more than 10 000 sequences are required to achieve at least 80% coverage, thus to accurately represent a data set in taxon based approaches, coverage of  $\geq 90\%$  is required, and they found that they could achieve that coverage with 20 000 sequences. Although we have an indication of diversity within the

samples, further sequencing is required to achieve a sufficient sequencing depth to accurately characterize populations based on OTUs.

Table 2. Richness and diversity estimations of the bacterial phylotypes of the biofilms in tower 1 and tower 2.

	Number of Sequences	3% distance				5% distance			
		OTU	Chao1	Shannon	Coverage	OTU	Chao1	Shannon	Coverage
Tower 1	2462	777	2942.06	5.07	75.63%	439	1220.39	4.29	88.18%
Tower 2	3004	922	4314.67	5.11	75.77%	529	1700.84	4.37	87.82%

*Chao1 richness estimator: the total number of OTU's estimated by infinite sampling. (A higher number=higher richness)*

*Shannon diversity index: characterizes species diversity. (A higher number = more diversity)*

*Good's coverage: estimation of the probability that the next read is part of an OTU that has already been found.*

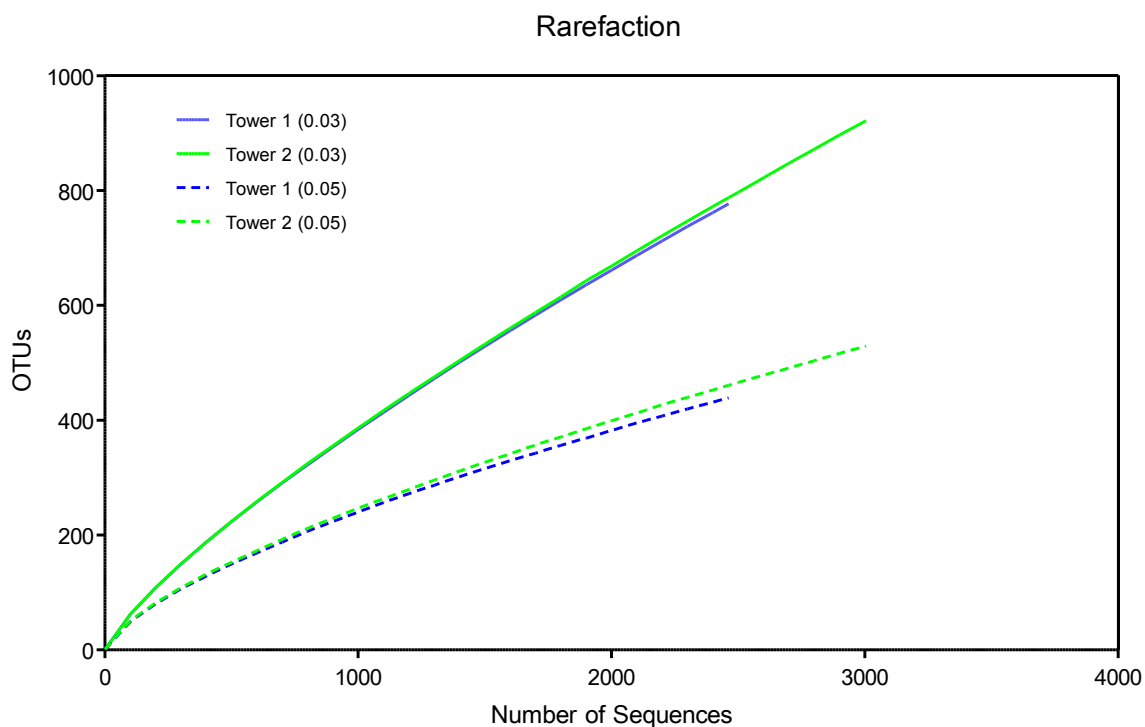


Figure 2. Rarefaction curves of the bacterial communities of biofilm samples from tower 1 and tower 2, with OTUs assigned at distance cutoff levels of 0.03 (solid lines) and 0.05 (dashed lines).

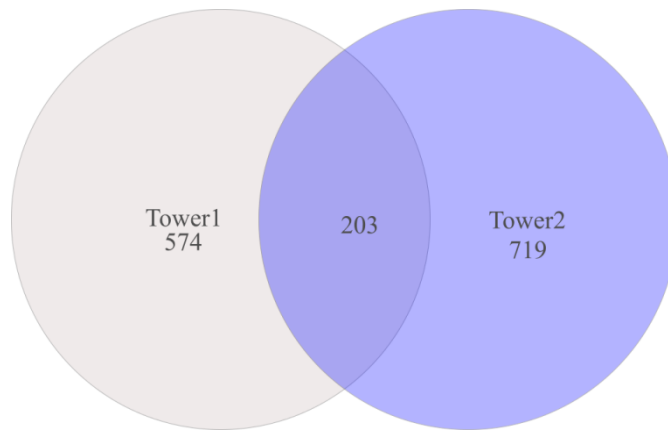


Figure 3. A Venn diagram of the bacterial communities in biofilms from tower 1 and tower 2 based on OTUs at 0.03 cutoff level, indicating that only 203 OTUs overlap between the two samples.

#### 4.2. Taxonomic composition of the bacterial community of biofilms from tower 1 and tower 2.

The bacterial communities of the biofilm samples were characterized according to their taxonomic groups. Fig. 4(a) shows the relative abundance of the bacterial community at class level. In total, 11 classes were identified, and all of them were observed in both biofilm samples. In both towers, 45-50% of sequences were assigned to *Actinobacteria*, followed by *Alphaproteobacteria* with 13-14% abundance and *Flavobacteria* with 10-12% abundance. The only major difference in the classifications in the two samples is that tower 1 showed a higher abundance of *Bacilli* than tower 2. Between 6 and 7% of sequences could not be classified. At order level (Fig. 4b), it is apparent that *Lactobacilliales* are more abundant in tower 1 than tower 2, and at family level (Fig. 4c) the 50% of sequences that could be classified were similar in abundance in both towers. Organisms that had a relative abundance below 1% were grouped together as “other”. At genus level (Fig. 5), 19 groups were identified with similar relative abundance in both towers. They were *Acetobacterium*, *Actinomyces*, *Aeromicrobium*, *Amaricoccus*, *Arcobacter*, *Bdellovibrio*, *Bosea*, *Devosia*, *Dysgonomonas*, *Fluviicola*, *Kaistia*, *Lactobacillus*, *Leadbetterella*, *Mezorhizobium*, *Myroides*, *Paracocci*, *Patulibacter*, *Pseudoxanthomonas* and *Trichococcus*. *Trichococcus* was defined as the most abundant group, being more abundant in tower 1 than tower 2.

The majority of the organisms that were classified at genus level have previously been associated with wastewater treatment systems. Of these 19 organisms, those that were

previously found to fulfill carbohydrate removing or biodegradation roles in wastewater treatment were *Acetobacterium*, *Lactobacillus*, *Leadbetterella*, *Kaistia*, *Actinomyces*, *Arcobacter*, *Fluviicola* and *Trichococcus*. All of these organisms, except for *Arcobacter*, were more prevalent in tower 1, which can be ascribed to the fact that tower 1 treats water with a much higher organic load than tower 2. *Acetobacterium* are anaerobic fermenters, converting lactate or ethanol to acetate, and are common in wastewater from food industries (Hassan and Nelson 2012). Also common in wastewater from food industries are *Lactobacillus*, which are facultative anaerobes that utilise carbohydrates to form lactate, and are used for decolourisation in food industry wastewater (Lamia and Moktar, 2003; Tondee and Sirianuntapiboon 2008; Aouidi *et al.* 2009). *Leadbetterella* are carbohydrate utilizing organisms isolated from activated sludge (Ryu *et al.* 2006). *Kaistia* is an aerobic chemo-organotroph which is involved in biodegradation of organic matter, and was first isolated from an industrial wastewater stream (Im *et al.* 2004) and subsequently in anaerobic granules of an upflow anaerobic sludge blanket reactor treating wastewater from brewing factories in Korea (Lee *et al.* 2007). *Actinomyces* are facultative anaerobes that produce enzymes which can oxidize organic material and have been detected in low pH distillery wastewater (Kim *et al.* 2014). *Arcobacter* are microaerophilic pathogens that have been isolated from a wide range of environments, including wastewater treatment systems (Zhao *et al.* 2015, Lee *et al.* 2016). *Fluviicola* is a strict aerobe, which has been isolated from industrial wastewater (Yang *et al.* 2014) and was proven to remove a range of organic nutrients rapidly in a lab scale sequencing batch reaction kettle treating municipal wastewater, and nutrient removal by *Fluviicola* improved with increased aeration intensity (Xin *et al.* 2016). *Trichococcus* are aerobic lactic acid bacteria that have been identified widely in wastewater treatment systems, including sulfur reducing systems, where they degrade organics to short-chain volatile fatty acids, which serve as substrates for sulfur reducing bacteria (Stams *et al.* 2009; Qian *et al.* 2015), high strength organic wastewater (Lee *et al.* 2008) and in activated sludge sewage treatment plants (Shchegolkova *et al.* 2016).

*Mesorhizobium*, *Devosia*, *Pseudoxanthomonas*, *Bosea* and *Paracocci* have been identified in systems that utilise nitrogen metabolism for wastewater treatment. All of these organisms had a higher relative abundance in tower 2, except for *Paracoccus*, which were equally abundant in both towers. It can be argued that the conditions in tower 2 are more favourable for nitrification and denitrification, as these processes stop below pH 5. The input water for tower 2 is above pH 6.5. *Mesorhizobium* are aerobic heterotrophs that are capable of nitrogen

fixation and denitrification, and have been detected in a mature biofilm that was fouling a membrane in a membrane nitrification reactor (Lu *et al.* 2016) and in the activated sludge of an intermittently aerated nitrifying-denitrifying plant treating nitrogen rich industrial wastewater (Juretschko *et al.* 2002). *Devosia* was detected as a dominant strain in granule forming biomass of aerobic granular sludge in a system treating beer wastewater (Song *et al.* 2010) and was identified as a halophilic nitrifier in aerobic granules of a continuous flow nitrification reactor treating high salinity wastewater (Wan *et al.* 2014). *Pseudoxanthomonas* are strictly aerobic denitrifiers used in denitrification biofilters (Meng *et al.* 2015) and was identified in a packed-bed bioreactor removing dye from textile industry wastewater (Chen *et al.* 2016). *Bosea* are denitrifying biofilm-forming organisms, which have been detected in biofilms in membrane wastewater treatment systems (Ivnitsky *et al.* 2007), an anaerobic digester (Ouattara *et al.* 2003) and a hospital water supply (La Scola *et al.* 2003). *Paracocci* are a metabolically versatile group of organisms that can include chemoorganoheterotrophs, utilizing organic substrates, facultative chemolithoautotrophs, utilising sulfides and hydrogen as energy sources, methylotrophs utilising methyl compounds, and under anaerobic conditions, denitrification (Bartosik *et al.* 2003). They have been isolated from a range of biological wastewater treatment systems such as a fluidized bed reactor where sulfide oxidation and denitrification took place (Robertson and Kuenen 1983), sewage, sludge (Kelly *et al.* 2006), in a denitrifying dimethoate wastewater treatment plant (Li *et al.* 2010) and denitrifying sand filter treating municipal wastewater (Neef *et al.* 1996).

*Dysgonomonas* are facultative anaerobes detected in aerobic microbial granules treating wastewater (Ivanov and Tay, 2006) and are associated with wastewater treatment systems treating sulfate rich wastewater (Sarti *et al.* 2010; Gao *et al.* 2011). *Amaricoccus* are phosphate and glycogen accumulating organisms that occur in tetrad formation or in regular packets, and have been isolated in wastewater treatment systems from activated sludge biomass (Maszenan *et al.* 1997), as well as in an abattoir wastewater treatment system (Ge *et al.* 2015) and winery wastewater treatment systems (Kiss *et al.* 2011; McIlroy *et al.* 2011). *Patulibacter* has not been described widely in wastewater systems. It was first isolated from soil (Takahashi *et al.* 2006) and finally isolated from the sludge of a wastewater plant, and was found to degrade ibuprofen (Almeida *et al.* 2013).

*Myroides*, *Aeromicrobium* and *Bdellovibrio* are common in aquatic environments. *Myroides* are mesophilic aeromonads that are common in a wide range of aquatic systems (Popovic *et*

*al.* 2015). *Aeromicrobium* are aerobic *Actinomycetes* that occur widely in fresh and marine aquatic environments (Zaitlin and Watson 2006) and *Bdellovibrio* is an obligate aerobic predator, common in aquatic environments which targets gram-negative organisms in mixed species biofilms. Its lytic action on biofilms has been investigated as a means to combat membrane biofouling (Kim *et al.* 2013; Özkan *et al.* 2014).



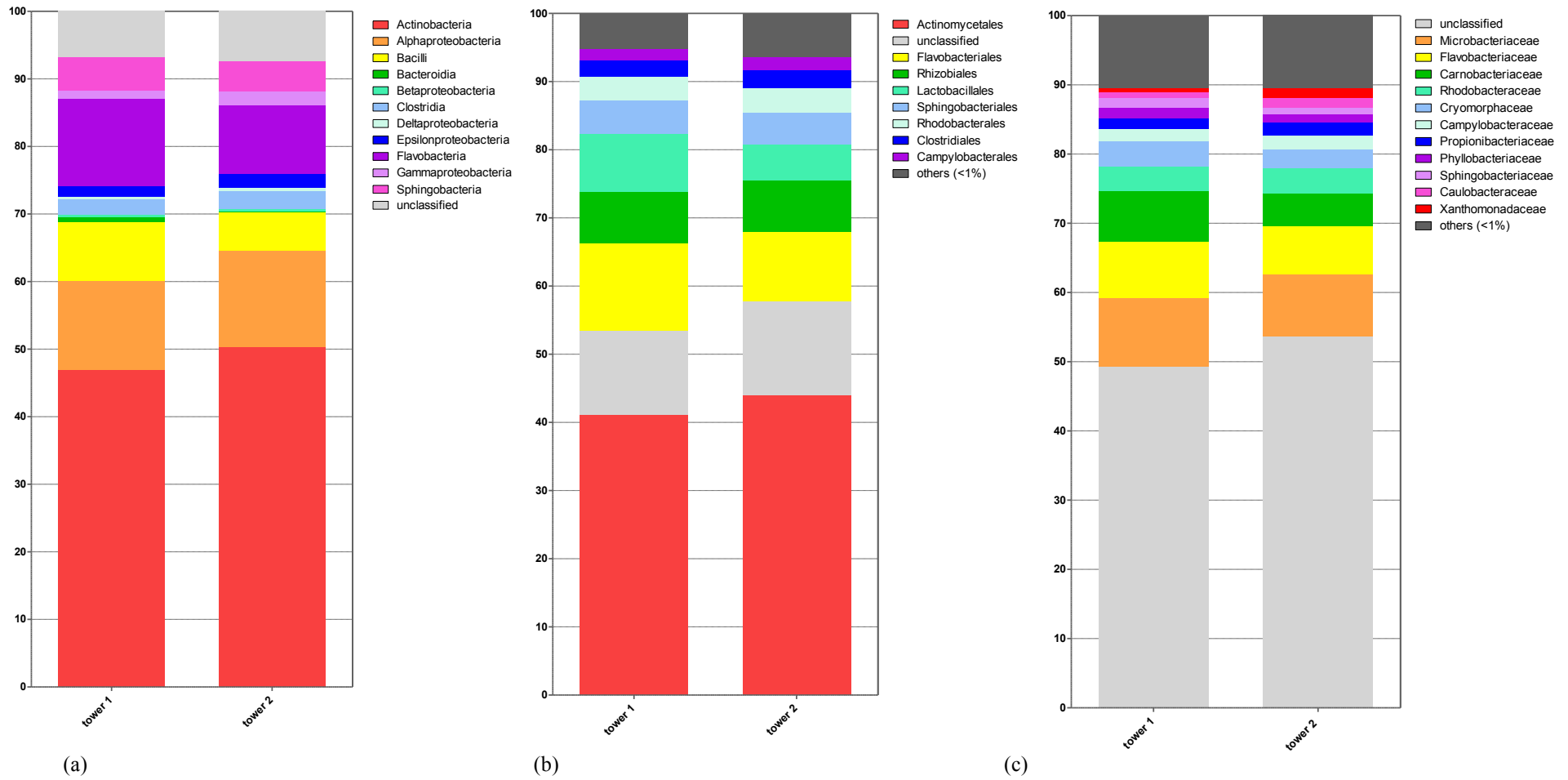


Figure 4. Relative abundance of bacteria at (a) class level, (b) order level and (c) family level in the biofilm samples from tower 1 and tower 2.

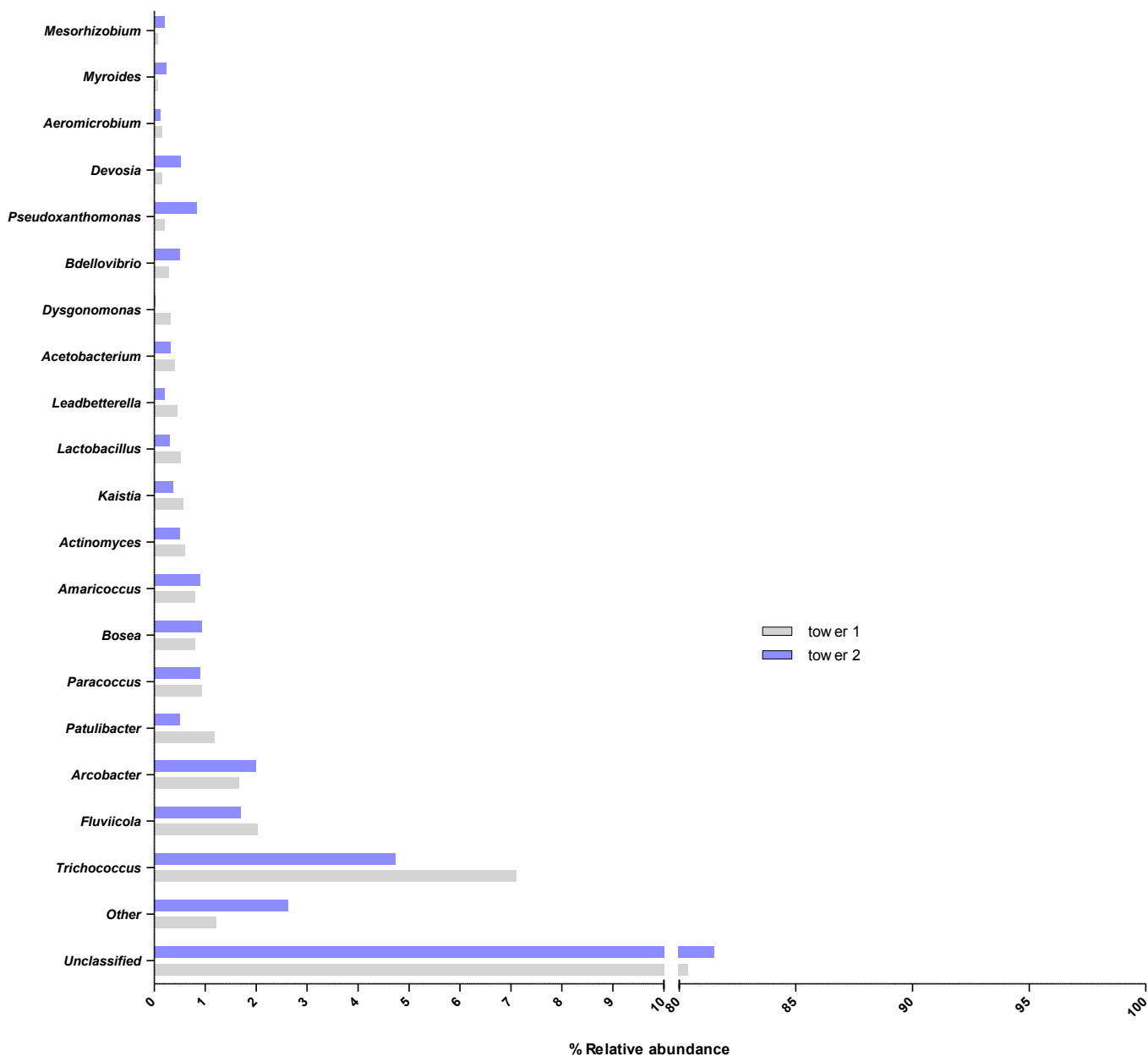


Figure 5. Relative abundance of the most prevalent organisms identified at genus level for the biofilm samples from tower 1 and tower 2.

#### 4.3. *The structure of the fungal community of the biofilms in sequential subunits in a high rate biological contact reactor treating winery wastewater*

Taxonomic classification of the fungal community of biofilms from tower 1 and tower 2 revealed that unlike the bacterial communities, the fungal communities in each tower differed. *Candida* was the most abundant among the identified genera in the population of tower 1, followed by *Trichosporon* and *Fusarium*, and in tower 2, *Trichosporon* was the most abundant genus, followed by *Candida* and an unidentified genus of *Nectriaceae* (Fig. 6). Table 3 summarizes the most abundant species in the populations of tower 1 and tower 2.

*Candida intermedia* has been identified in high strength organic wastewater systems (Chigusa *et al.* 1996) and was reported, along with *Saccharomyces cerevisiae* as the dominant yeasts isolated from the biofilm of a rotating biological contact reactor used to treat winery wastewater (Malandra *et al.* 2003). *Candida sojae* was first isolated from defatted soybean flakes (Nakase *et al.* 1994), and has rarely been described in a wastewater context except for one study in high organic pulp mill wastewater (Trosok *et al.* 2002). *Candida sake* was originally isolated from apples (Vinas *et al.* 1998) and is not widely reported in wastewater environments, but have been isolated from grapes where it is reported to form a coating protecting grapes from gray mold (Marín *et al.* 2008; Calvo-Garrido *et al.* 2013). *Trichosporon laibachii* and *T.monoliforme* have been isolated from industrial wastewater sources treating chemical effluent (Badia-Fabregat *et al.* 2015; Gargouri *et al.* 2015) and *T. laibachii* from a fungal system treating effluent from a veterinary hospital (Badia-Fabregat *et al.* 2015). Both *T. laibachii* and *T. vадense* have been isolated from industrial wastewater treatment systems that were used for dye decolourisation (Pajot *et al.* 2008; Dias *et al.* 2010) and *T. jirovecii* was detected in wastewater treatment system sludge (Quan *et al.* 2013). *Fusarium* sp. are usually found in the air, soil, or associated with plants (Gupta *et al.* 2000) and *F.merismoides* has been isolated from a constructed wetland treating polycyclic aromatic hydrocarbons (Giraud *et al.* 2001). *Magnusiomyces capitatus* is a lipase producing yeast isolated from the wastewater of an olive mill (Salgado, 2015). *Cosmospora butyri* belong to the family *Netriaceae*, were originally isolated from butter, and have not been associated with wastewater (Summerbell *et al.* 2011). *Dipodascaceae* is known to be involved in biodegradation (Falkiewicz-Dulik *et al.* 2015) but has not previously been associated with wastewater. *Ascomycota* sp. have been widely associated with municipal (Matsunaga *et al.* 2014) and pharmaceutical wastewater treatment plants (Deng *et al.* 2012; Zhang *et al.* 2013)

as well as industrial wastewater treatment plants removing pesticides from wastewater (Saad *et al.* 2000; XiaoXing *et al.* 2010), and has been identified as a main organism in a biofilm fouling a membrane in a desalinating reverse osmosis system (Al Ashhab *et al.* 2014).

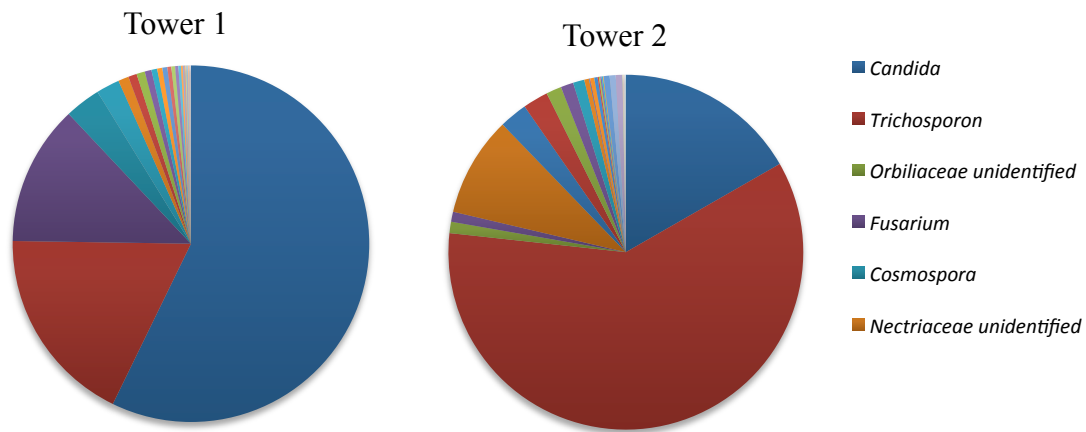


Figure 6. Relative abundance of the classified fungal genera for the biofilm samples from tower 1 and tower 2.

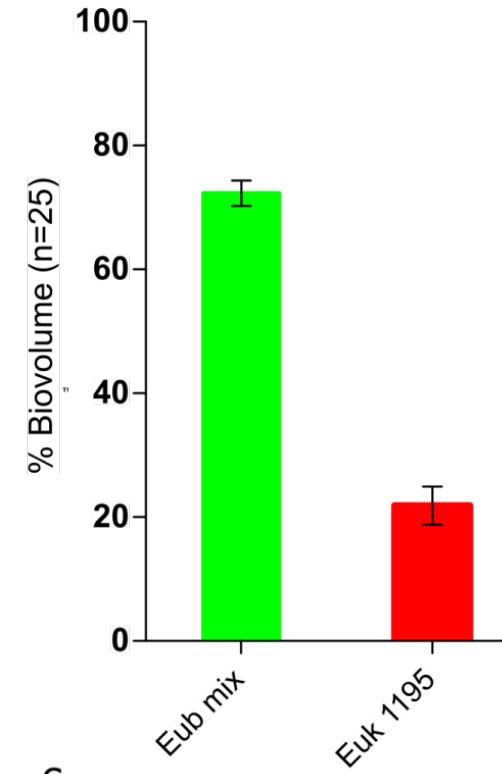
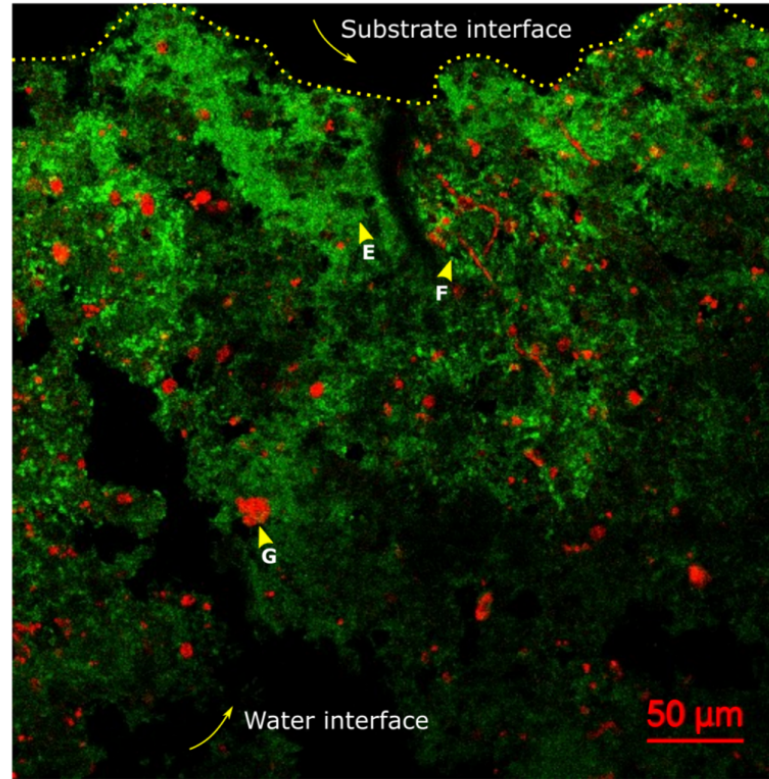
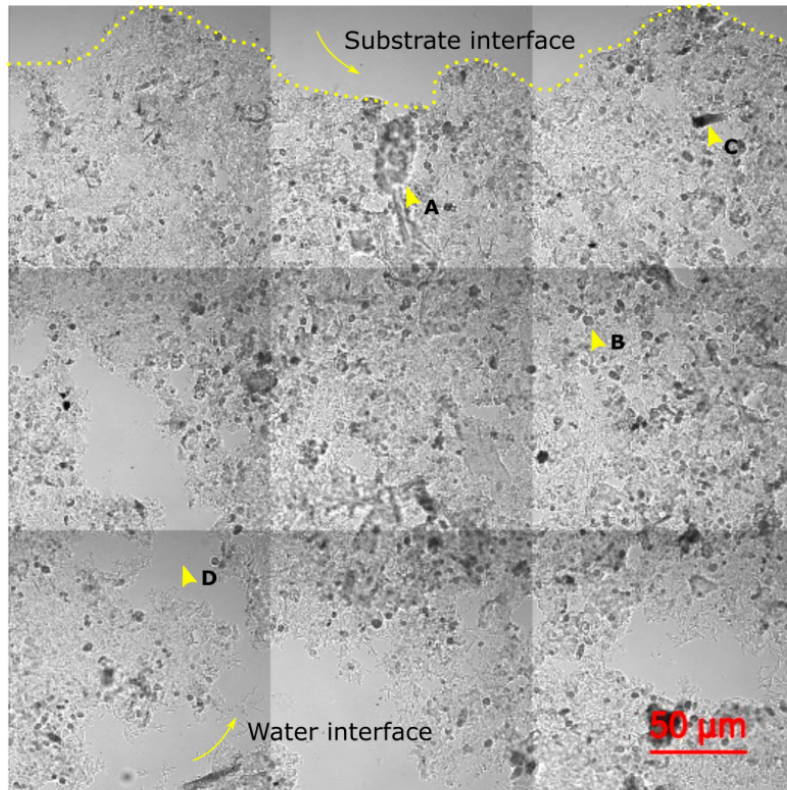
Table 3. The relative abundance of fungal species characterized for the biofilm samples from tower 1 and tower 2.

<i>Tower 1</i>		<i>Tower 2</i>	
<i>Candida intermedia</i>	3.40%	<i>Trichosporon laibachii</i>	9.16%
<i>Candida sojae</i>	1.33%	<i>Candida intermedia</i>	5.30%
<i>Candida sake</i>	0.40%	<i>Trichosporon vadense</i>	4.34%
<i>Trichosporon jirovecii</i>	0.37%	<i>Nectriaceae sp</i>	3.24%
<i>Fusarium merismoides</i>	0.32%	<i>Magnusiomyces capitatus</i>	1.01%
<i>Cosmospora butyri</i>	0.22%	<i>Trichosporon moniliiforme</i>	0.77%
<i>Phoma radicina</i>	0.20%	<i>Nectriaceae sp</i>	0.66%
<i>Trichosporon laibachii</i>	0.16%	<i>Dipodascaceae sp</i>	0.46%
<i>Trichosporon moniliiforme</i>	0.15%	<i>Ascomycota sp</i>	0.43%

### 5. CLSM and Fluorescent in-situ hybridisation for the characterisation of biofilm composition and structure.

Confocal bright field images of a 15µm cryosection of the biofilm taken from tower 1 (Fig. 7a) revealed dense biomatter at the substratum interface, and channels and voids (D) permeating the water interface of the biofilm. Also observed were possible algal cell fragments (A and C) and microalgae cells (B). FISH images of the same section revealed that

72.31%  $\pm$  2.03 of the biovolume hybridised with the general bacterial probe mix EUB 338 (green), with yeast cells hybridised with EUK1195 (red), making up 21.89%  $\pm$  3.07 of the biovolume distributed evenly within the biofilm as single oval cells and filaments (Fig. 7b and c). This was consistent with results obtained in biomass taken from an aerobic sequencing batch reactor treating winery wastewater in Australia (McIlroy *et al.* 2011). The microalgae which were observed in Fig. 7a did not auto-fluoresce, thus are not photosynthetic, nor did they hybridise with the eukaryotic probe, as the permeabilisation steps that were followed were not stringent enough to permeabilise algal cells (Palacios and Marín, 2008). A screening of biofilm samples from both towers with FISH probes (Table 1) revealed the presence of *Alpha*, *Delta* and *Gammaproteobacteria* at class level, with inconclusive detection of *Betaproteobacteria* due to non-specific signal, discussed in the troubleshooting section added to the end of this chapter.



a

b

c

Figure 7. (a) bright field and (b) FISH-CLSM composite images of a lengthwise (substrate to water interface) 15μm cryosection of a biofilm taken from tower 1. Green: Bacteria hybridised with EUB338 ; Red: Yeast cells hybridised with EUK1195 (c) the percentage of the total biovolume that hybridised with the EUB probe mix and the EUK 1195 probe.

*Alphaproteobacteria* in winery wastewater treatment systems can be nitrifiers such as *Nitrobacter* (Beltran *et al.* 2000). Glycogen accumulating organisms (GAO) such as *Amaricoccus* was detected with ALF1b in biofilm samples from both towers (Fig. 8). The cells were observed in tetrad and clustered tetrad formation, which resemble the *Alphaproteobacteria* tetrad forming organisms (TFO), which were detected by FISH in activated sludge from the anaerobic phase of a membrane bioreactor treating wastewater in an enhanced biological phosphorous removal process (Ge *et al.* 2015).

*Deltaproteobacteria* were detected by the DELTA495a probe pair in tower 1 only (Fig. 9).

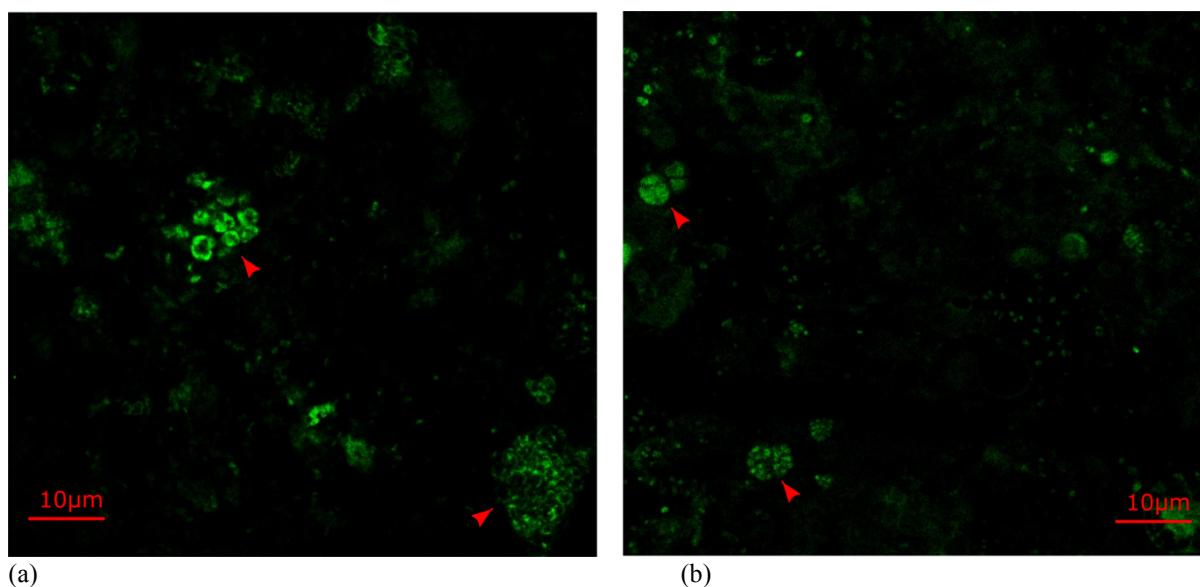


Figure 8. FISH-CLSM images of *Alphaproteobacteria* hybridised with ALF1b, observed in tetrad formation tower 1 (a) and tower 2 (b).

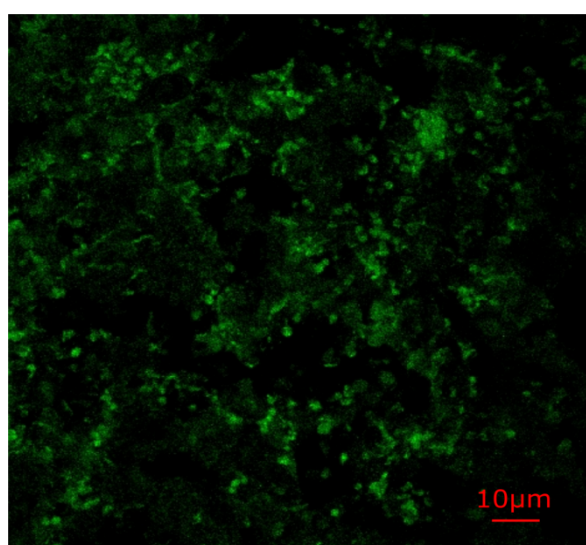


Figure 9. FISH-CLSM image of *Deltaproteobacteria* hybridised with DELT495a observed dispersed evenly among biomass in a cryosection of the biofilm from tower 1.

*Gammaproteobacteria*, probed for using GAM42a, were observed as dispersed single cells in a biomass sample from tower 2. A lengthwise (substrate to water interface) cryosection across the biofilm revealed a visible stratified hybridisation with GAM42a, with *Gammaproteobacteria* comprising  $30\% \pm 3.11$  of the biovolume closest to the substrate interface and  $63\% \pm 3.67$  of the biovolume closest to the water interface (Fig. 10).

AOB were screened using probes Nso190 and Nso1225, which probe for most *Betaproteobacterial* AOB. NOB were screened with Ntspa712, which probes for most members of the phylum *Nitrospirae* and NIT3, which probes for *Nitrobacter* species. No hybridisations with NIT3 were observed, however, Nso190, Nso1225 and Ntspa712 hybridised to dispersed cells in biomass from tower 1, and all hybridised exclusively with clusters of cells in the biomass of tower 2 (Fig. 11).



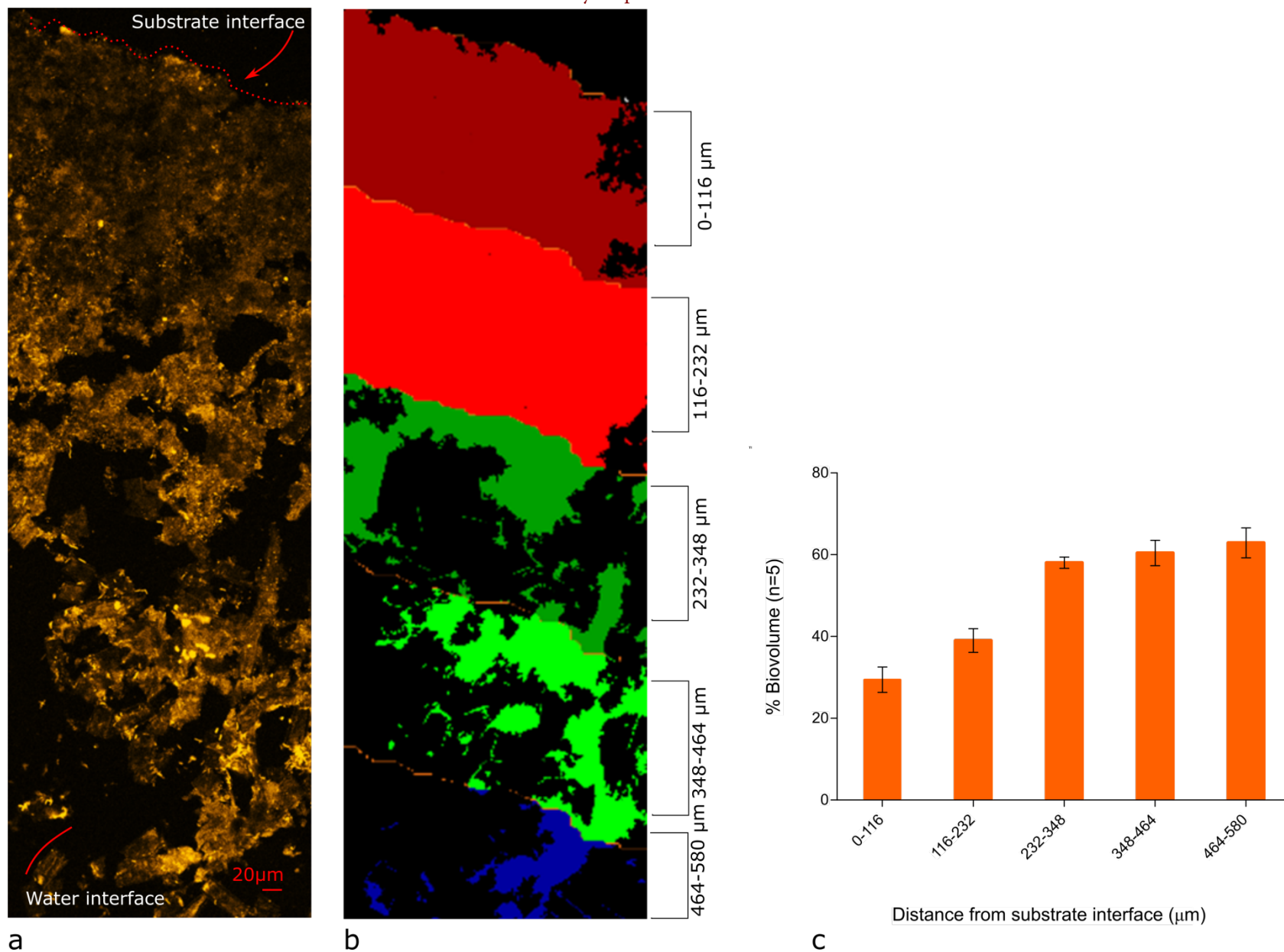


Figure 10. (a) FISH-CLSM image of *Gammaproteobacteria* hybridised with GAM42a, concentrated at the water interface, (b) projected sections by DAIME software for sectional biovolume calculations (c) the % biovolume per section distance from the water interface comprised of biomass hybridized with GAM42a.

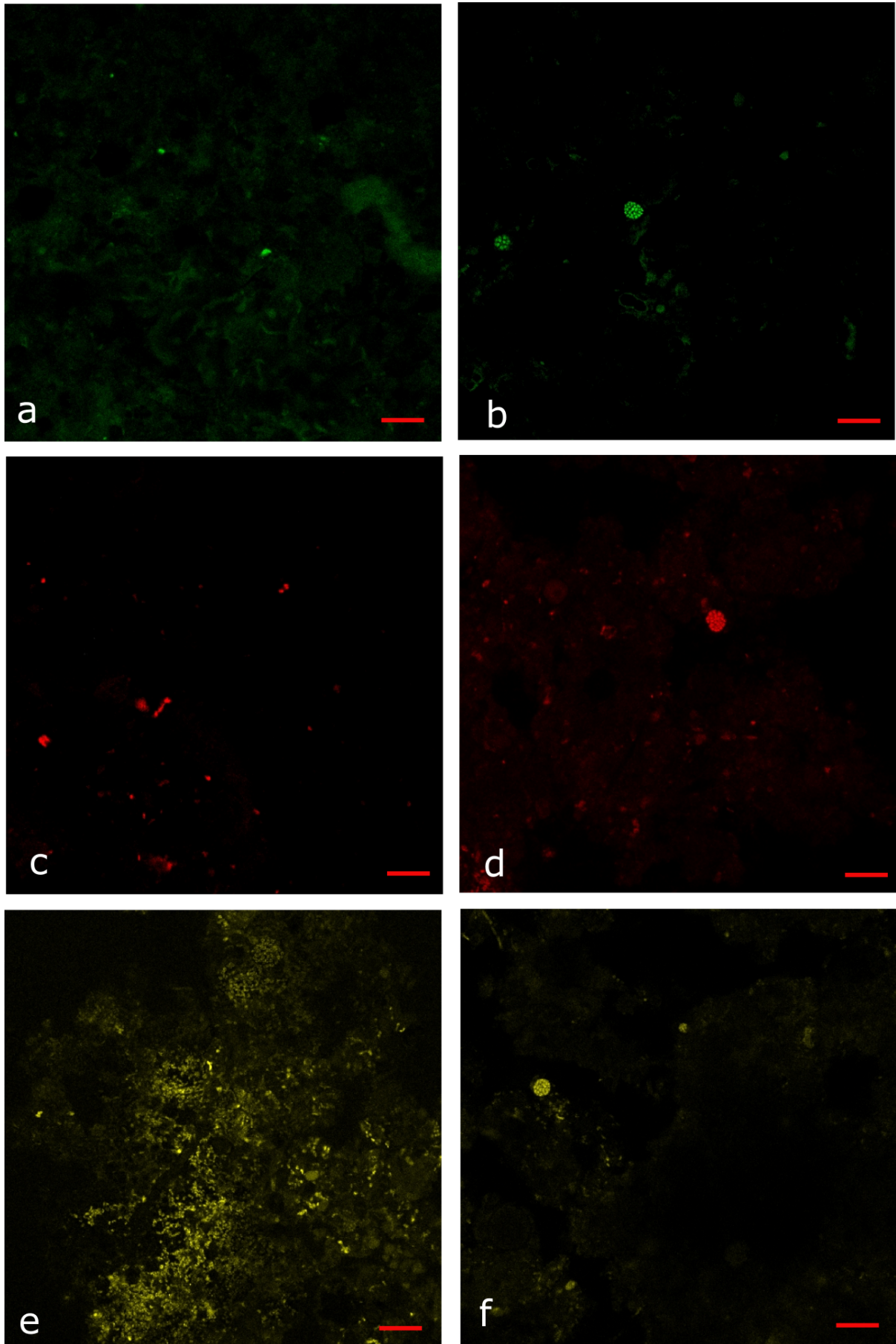


Figure 11. FISH-CLSM images of biofilm samples probed with Nso 190 (red) and 1225 (green), and Ntsps 712 (yellow), showing that these organisms are distributed as dispersed cells in tower 1 (a,c and e) and occur in cluster formation in tower 2 (b,d and f).

PAO were screened using Actino221 and PAO462, and hybridisations were only observed with PAO462 hybridising with biomass from tower 2, dispersed among AOB probed by Nso 190 (Fig. 12).

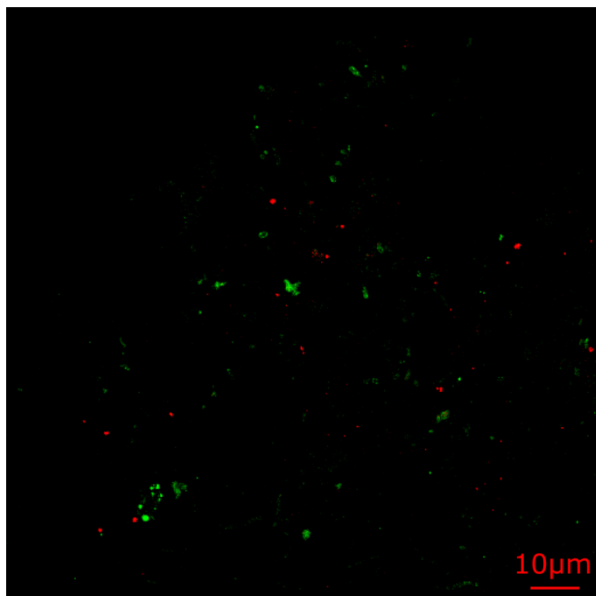


Figure 12. FISH-CLSM image of sparse individual AOB cells (red, Nso190) and PAO cells (green, PAO462) in a biofilm sample taken from tower 2.

The pseudohyphal structures that hybridised with EUK 1195 (Fig. 7b) resemble that of *Candida intermedia* (Lachance *et al.* 2011) and its presence was detected in biomass from both towers when hybridised with its specific probe Cint. Investigation of its distribution among other organisms by multiplex FISH was hampered by overlap in the green range from blue range non-specific signal from with *Betaproteobacteria* probe. *Saccharomyces cerevisiae* was observed dispersed as single cells in biomass samples and biofilm cryosections from both towers and was prevalent among clusters of *Nitrobacter* cells (Fig. 13).

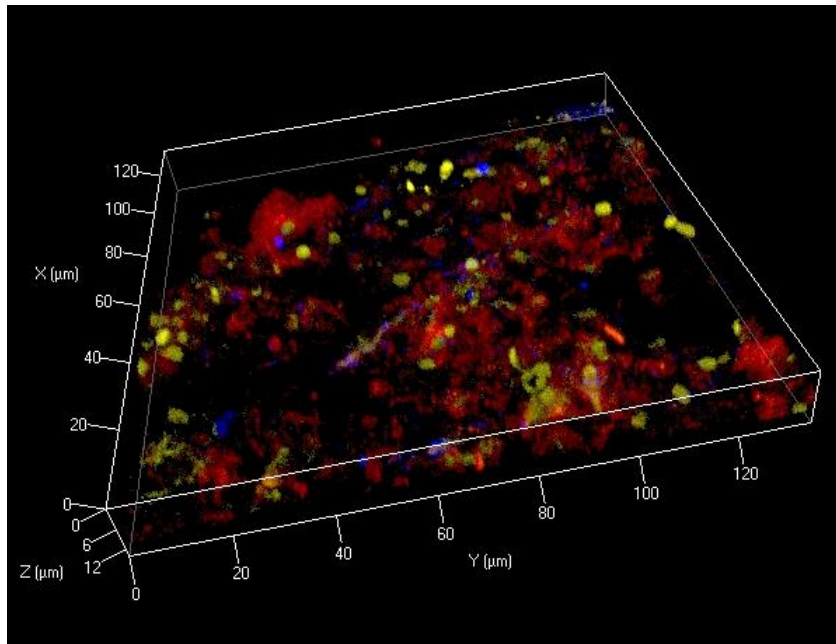


Figure 13. 3D projection of a Z-stack analysis of a 15µm cryosection of biofilm from tower 1, showing the distribution of individual *Saccharomyces cerevisiae* cells (yellow) among clusters of *Nitrobacter* cells (red). Also visible is non-specific signal (blue) due to the presence of the Aza645 probe labelled with ATTO390, which caused signal emission from the EPS around the cells.

#### **6. Community level physiological profiling of biofilm substrate utilisation using Biolog ECO and Biolog Phenotypic Microarray (PM) analyses.**

From the absorbance data and visual inspection for all the ECO and PM plates, it was clear that the incubation time required for a colour signal to develop in each plate varied, which can be ascribed to variations in the biofilm samples tested, as well as substrates present on each plate. Therefore Garland *et al.* (1996) suggested calculating the average well colour development (AWCD) at every time point recorded for each plate, then choosing plate readings that show approximately the same AWCD to use for further analyses, irrespective of the incubation time that was required to achieve the chosen AWCD. Smalla *et al.* (1998) showed that incubation time also has an influence on the community structure within a well, with diversity being lost with long incubation times. Therefore, choosing an AWCD that does not require extended incubation times to achieve in the plates that are tested, provide a more accurate representation of the substrate utilisation profiles of the entire community present in the inoculum. For environmental samples, an AWCD of 0.75 is regarded as optimal, as at this

value, colour responses are seen in most wells, and wells with most active substrate utilisation reach their colour development asymptote (Garland and Mills 1994).

### 6.1. Biolog ECO plates

The incubation time that was required to reach an AWCD of 0.75 absorbance units in ECO plates were 120 h for the biofilm sample from tower 1 and 192 h for the biofilm sample from tower 2 (Fig. 14).

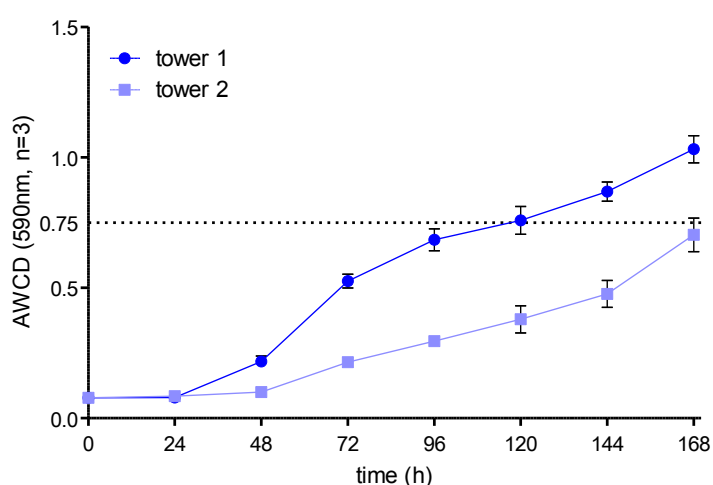


Figure 14. AWCD at 590 nm over 7 days for ECO plates inoculated with biofilm suspensions from bioreactor subunits tower 1 and tower 2.

Analyses of corrected and normalised data from these time points revealed that the biofilm suspension from tower 1 utilised 11 of the 31 carbon substrates, four of them strongly, while the biofilm suspension from tower 2 utilised 10 carbon sources, and three of them strongly. Only  $\alpha$ -D-Lactose and N-Acetyl-D-Glucosamine were utilised by both samples, and none of the strongly utilised substrates were utilised by both biofilm samples. The most notable discrepancy between utilisation of carbon source categories was that no polymers were metabolised by the sample from tower 1, while Tween 40, Tween 80 and  $\alpha$ -Cyclodextrin were metabolised by the sample from tower 2. Screening of the CLPP of the two biofilm samples with ECO plates were reproducible, and showed that the metabolic profiles of the two biofilms are dissimilar (table 4).

Table 4. Substrate utilisation by biofilm suspensions from winery wastewater treatment reactors subunits tower 1 and tower 2 in ECO plates, with moderate substrate utilisation (colour score  $\geq 0.75$ ) in pink and strong substrate utilisation (colour score  $\geq 1.5$ ) in red. These are average colour scores from triplicate experiments.

	Carbon substrate	Nutrient	utilisation by biofilm	
			tower 1	tower 2
Polymers	Tween 40	C		1.228
	Tween 80	C		2.556
	Glycogen	C		
	$\alpha$ -Cyclodextrin	C		1.968
Carbohydrates	D-Cellobiose	C	1.319	
	$\alpha$ -D-Lactose	C	1.725	0.754
	$\beta$ -Methyl-D-Glucoside	C	1.167	
	D-Xylose	C		
	i-Erythritol	C		
	D-Mannitol	C		
	N-Acetyl-D-Glucosamine	C + N	2.104	0.849
Sugar Phosphates	Glucose-1-Phosphate	C + P	1.404	
	D, L -glycerol Phosphate	C + P		
Carboxylic acids	Pyruvic acid methyl ester	C	1.358	
	D-Galactonic acid- $\gamma$ -Lactone	C	1.524	
	D-Glucosaminic acid	C + N	1.001	
	D-Galacturonic acid	C	1.794	
	Itaconic acid	C		
	$\alpha$ -Ketobutyric acid	C		1.082
	$\gamma$ -Hydroxybutyric acid	C		0.938
	D-Malic acid	C		
Amino acids	L-Arginine	C + N		
	L-Asparagine	C + N	0.796	
	L-Phenylalanine	C + N		1.836
	L-Serine	C + N	0.753	
	L-Threonine	C + N		1.129
	Glycyl-L-Glutamic acid	C + N		
Phenolic compounds	2-Hydroxybenzoic acid	C		1.429
	4-Hydroxybenzoic acid	C		

## 6.2. Biolog PM plates

Biolog PM plates 1 and 2 contain 190 carbon sources, offering more in-depth analyses of carbon metabolism than ECO plates. The incubation time that was required to reach an AWCD of 0.75 absorbance units in PM 1 plates were 48 h for the biofilm sample from tower 1 and 72 h for the biofilm sample from tower 2. For the PM 2 plates, required incubation times were 96 h for the biofilm sample from tower 1 and 144 h for the biofilm sample from tower 2 (Fig. 15).

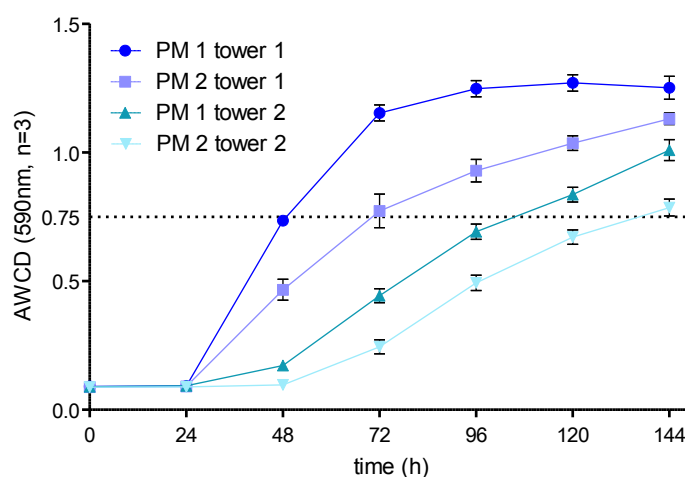


Figure 15. AWCD at 590 nm over 6 days for Biolog PM 1 and PM 2 plates inoculated with biofilm suspensions from bioreactor subunits tower 1 and tower 2.

The CLPP of the two biofilm samples for the 190 carbon sources revealed that collectively, biofilms from the system positively metabolised 129 of the carbon substrates (absorbance  $\geq 0.75$ , table 5). Of these substrates, 63% were metabolised in both tower 1 and tower 2, however, the biofilm in tower 1 was exclusively responsible for metabolising 18% of the total substrates, and the remaining 19% were exclusively metabolised in tower 2 (Fig. 16). The utilised metabolites were grouped according to their related metabolic pathways in microbes, or at least assigned to a biochemical category. The majority of the groups were dominated by metabolites which were utilised in both tower subunits, save for the polymer metabolic substrates and the majority of the pentose catabolic pathway substrates. The substrates which were strongly utilised (absorbance  $\geq 1.5$ , table 5), were mostly distributed among the substrates that were utilised in both towers. In agreement with the ECO plate results, utilisation of polymers occurred exclusively in tower 2. The polymers Tween 20, 40 and 80 are nonionic polysorbate surfactants that serve as carbon sources to organisms that can utilise the oleic acid moiety of long chain fatty acids (Slijkhuis 1983, Yeh and Pavlostathis, 2001).

Non-ionic wetting agents such as these polysorbate surfactants are found in detergents used in winery cleaning processes (Zoecklein *et al.* 1995; Fugelsang 1997) and microbial surfactant degradation contributes to an important waste removal trait of the system. Furthermore, inulin, a fructooligosaccharide found in grapes (Apolinário *et al.* 2014), was utilised only by the biofilm from tower 2. Inulin can be degraded by a range of microbes including *Candida* and *Fusarium* (Kango and Jain, 2011), which were detected by NGS in the biofilm samples from both tower 1 and 2 of the reactor, and *Saccharomyces cerevisiae*, which was detected by FISH in biofilm samples from both towers, suggesting that the *Candida* and *Fusarium* populations vary at species level between the two towers. Glycogen utilisation, which is associated with glycogen accumulating organisms (GAO) was also exclusively observed in tower 2. GAO *Amaricoccus*, an *Alphaprotobacterial* tetrad forming organism that has been observed in aerobic and anaerobic winery wastewater treatment systems (Kiss *et al.* 2011; McIlroy *et al.* 2011) was classified as more abundant in tower 2 than in tower 1 with the metagenomic study of this system (Fig. 5), which likely contributes to the fact that glycogen utilisation is only detected in tower 2 by Biolog analyses. When considering the organic acid metabolism, it is noted that acetic acid, propionic acid and citric acid, the most abundant organic acids detected in winery wastewater (Malandra *et al.* 2003), are metabolised exclusively in tower 2. The CLPP analysis of carbon substrates thus demonstrated that the bulk of the substrates are metabolised in both towers, but that tower 2 expanded the scope of the carbon substrate utilisation capabilities of the system.

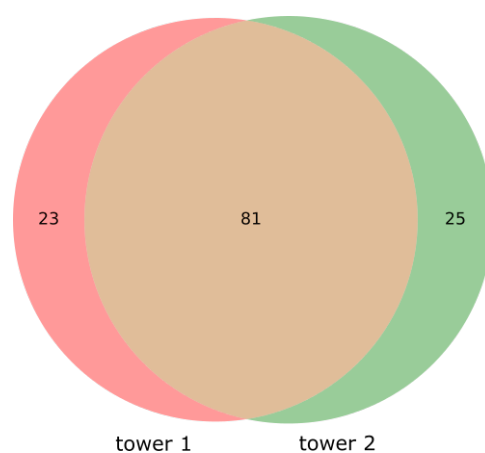


Figure 16. A Venn diagram of the utilisation of PM carbon sources in tower 1 and tower 2, showing the number of substrates that are utilised in each tower exclusively, as well as the number of substrates utilised in both towers.



Table 5. A summary of the carbon substrates utilised (colour development absorbance  $\geq 0.75$ ) by the biofilms of tower 1 and tower 2 on PM 1 and PM2. Substrates in yellow were utilised by biofilms from both tower 1 and tower 2. Substrates in red were utilised exclusively in tower 1, whilst substrates in green were utilised exclusively in tower 2. The substrates marked with ■ were utilised strongly (colour development absorbance  $\geq 1.5$ ) in tower 1, ♦ were utilised strongly in tower 2 and ● were utilised strongly in both tower 1 and 2.

Metabolism	substrate	Metabolism	substrate
Pentose catabolic pathway	L-Arabinose	Pyruvate metabolism	Methyl Pyruvate
	D-Xylose		Pyruvic Acid
	D-Ribose	Cell wall and aromatic pathways	D-Glucosaminic Acid
	D-Arabitol		Uridine
	D-Arabinose		Inosine
Galactose pathway	3-O-β-D-Galacto-pyranosyl-D-Arabinose	4-Hydroxy benzoic acid	
	Stachyose	β-Hydroxy Butyric Acid	
	D-Sorbitol	D,L-Octopamine	
	D-Melibiose	M-Hydroxy Phenyl Acetic Acid	
	α-D-Lactose	Amino acid and protein metabolism	L-Proline
	L-Galactonic Acid-g-Lactone		D-Serine
	N-Acetyl-D-Galactosamine		D-Aspartic Acid
	α-Methyl-D-Galactoside		N-Acetyl-D-Mannosamine
	β-Methyl-D-Galactoside		L-Leucine
	D-Raffinose		D,L-Carnitine
D-Galactose	Putrescine		
D-Tagatose	L-Aspartic Acid		
Starch and sugar metabolism	Lactulose		D-Alanine
	Maltose		L-Glutamic Acid
	Sucrose	L-Rhamnose	
	Maltotriose	L-Asparagine	
	D-Cellulose	L-Glutamine	
	α-Cyclodextrin	L-Serine	
	β-Cyclodextrin	L-Malic Acid	
	γ-Cyclodextrin	Gelatin	
	Dextrin	N-Acetyl-Neuraminic Acid	
	Maltitol	D-Fucose	
	Palatinose	D-Glucosamine	
	Turanose	L-Arginine	
	Glycogen	L-Alanyl-Glycine	
	Inulin	L-Histidine	
Organic acid metabolism	Malonic Acid	Hydroxy-L-Proline	
	Meliibionic Acid	L-Ornithine	
	γ-Amino Butyric Acid	L-Phenylalanine	
	δ-Amino Valeric Acid	Glycerol	
	Butyric Acid	Glycolic Acid	
	Caproic Acid	L-Alaninamide	
	Quinic Acid	N-Acetyl-L-Glutamic Acid	
	Succinamic Acid	L-Alanine	
	D-Tartaric Acid	Acetoacetic Acid	
	L-Tartaric Acid	L-Isoleucine	
	Acetic Acid	L-Lysine	
	Propionic Acid	L-Valine	
	Citraconic Acid	2,3-Butanediol	
	α-Keto-valeric acid	Glycoside	Amygdalin
	D-Lactic Acid Methyl Ester		Glycolysis pathways
	Oxalomalic Acid	D-Glucose-1-Phosphate	
	Polymer metabolism	Tween 20	
Tween 40		b-Methyl-D-Glucoside	
Tween 80		N-Acetyl-D-Glucosamine	
Tricarboxylic acid cycle	Succinic Acid	D-Mannose	
	D,L-Malic Acid	D-Gluconic Acid	
	α-Keto-Glutaric Acid	L-Lactic Acid	
	Fumaric Acid	D-Fructose	
	Oxalic Acid	α-D-Glucose	
	Succinic Acid	β-D-Allose	
	α-Keto-butyric Acid	Arbutin	
	Citric Acid	Gentiobiose	
Uronic acid metabolism	Glucuronamide	L-Glucose	
	D-Glucuronic Acid	3-Methyl Glucose	
	Mucic Acid	Salicin	
	Pectin	L-Sorbose	
	D-Galacturonic Acid	5-Keto-D-Gluconic Acid	
Sugar alcohols	D-Saccharic Acid	D-Trehalose	
		D-Mannitol	
		i-Erythritol	

Analyses of biofilm samples using Biolog PM 3-PM 8 plates only showed colour development after 5 days of incubation, and yielded non-reproducible colour development data between repeat experiments. Plates PM 9 and PM 10 showed colour development after 24 h of incubation, but the absorbance values recorded were not reproducible between repeat experiments. This could be ascribed to the possibility that the prescribed defined growth medium which is supplemented to support the growth of a defined single culture is not sufficiently supplemented to support the growth of a heterogeneous environmental sample. Furthermore, the variations in colour development in wells of repeat experiments can be ascribed to loss of diversity in the well during incubation. Therefore, the CLPP of the nitrogen, phosphorous and sulfur metabolisms, growth factor requirements, peptide nitrogen metabolism, osmotic stress response and optimum growth pH for the biofilm samples could not be determined, deeming the Biolog PM system unsuitable for CLPP determinations other than carbon metabolism in these heterogeneous environmental biofilm samples.

## **Conclusion**

Each characterisation method used in this study was effective at describing the biofilms at hand. Collectively, the results provided insight into the composition and the functionality of the biofilms in the reactor. The three-tiered approach yielded complementary data, allowing more conclusive deductions about the biofilm communities and functionality than would be possible with each characterisation method in isolation. Although sequencing depth was insufficient to conclusively report on the phylotype richness of the bacteria in the biofilm samples, the analyses did indicate a somewhat higher phylotype richness and phylogenetic diversity in the bacterial consortium of tower 2. Furthermore, only 12% of the OTUs overlapped between the bacterial samples, indicating that the two samples are phylogenetically distinct. This was confirmed by taxonomic data that indicated that the dominant bacteria in the two towers differed. Bacteria that are primarily responsible for the removal of carbohydrates, sugars and alcohol from wastewater were more abundant in tower 1 than tower 2 due to higher organic load input water and bacteria that typically fulfil nitrifying and denitrifying roles in wastewater treatment were more abundant in tower 2 due to the higher, more favourable pH conditions. Furthermore, the yeast populations differed considerably in terms of the dominant organisms in each tower. FISH provided insight into the distribution of organisms within the biofilms and confirmed the suspected presence of an

oxygen gradient across the biofilm depth. FISH was also effective at identifying differences in how certain organisms arrange themselves within the two biofilms, such as the *Nitrosomonas* and the *Nitrospirae* that were probed for, and detected *Accumilobacter*, which was not classified in the taxonomic analyses, with PAO462 only in tower 2. With the Biolog system, ECO plates revealed that the carbon metabolizing profiles of the two biofilms were dissimilar for 6 groups of carbon sources. Analyses using the Biolog PM system were only successful for the carbon source tests. It was revealed that the majority of the substrates were utilised in biofilm samples from both cooling towers, but that important metabolic utilisation capabilities fell exclusively either within the consortium of the biofilm from tower 1 or tower 2. This concludes that ECO plates are a rapid, effective means to determine metabolic profiles of biofilm samples, and can detect differences in metabolic profiles of two biofilm samples, while PM plates 1 and 2 can provide in-depth analyses of carbon substrate utilisation in environmental heterogeneous biofilm samples, and that these tests can provide extensive insight into the carbon metabolism of a sample, allowing deeper understanding of the metabolic consortium. Each approach used in this study revealed some shortcomings in characterizing the biofilm samples. NGS fell short on sequencing depth, and a great deal of the OTUs were left taxonomically unassigned, which will remain a problem for as long as environmental databases are still being established. FISH was very effective at detecting and characterizing the growth and distributional patterns of specific organisms. The drawbacks were that multiple probe experiments were laborious and optimisation of the experiments and equipment was time consuming. The study was hampered by losing two of the selected probes to unexpected non-specific binding reactions in the biofilm material, and data loss through the overlap with other probes by non-specific signal. The Biolog system provided valuable insight into the carbon metabolic profiles of the biofilm samples, but it should be kept in mind that these tests are affected by cultureability of organisms as well as longer incubation times. Collectively, the data indicated that by adding a second subunit to the bioreactor, the treatment capacity was not merely expanded, but that the second reactor subunit added to the microbial and metabolic diversity of the system, expanding the treatment scope of the system. A three-tiered biofilm analysis can therefore provide the data required to optimise the design of a bioreactor to provide favourable conditions for the development of a microbial consortium which has optimal waste removal properties for the treatment requirements at hand.

## Addendum

### *Troubleshooting note on FISH*

Hybridisation with *Betaproteobacteria* and members of the *Azoarcus* cluster could not be investigated, as the fluorescent label ATTO 390, which was conjugated to the BET42a and the AZA645 probes yielded non-specific signal emission on the blue range, appearing to originate from the EPS surrounding the cells. Furthermore, where ATTO 390 and ATTO 488 were used together in multiple-stained samples, bright, non-specific signal was observed in the green range. This occurrence in FISH-CLSM with biofilms has not been reported in the literature. ATTO 390 is, however a slightly hydrophilic coumarin-derived fluorescent label, which is well known to conjugate with lectins and proteins, both which are abundant components of the EPS (Flemming 2011). A coumarin derived blue fluorescent dye has even been used as a labeling tool for glycoproteins in biological imaging (Rong *et al.* 2013). Coumarin analogues were also evaluated for intrinsic fluorescence resonance energy transfer (iFRET) of tryptophan residues in proteins, and, upon excitation of tryptophan containing proteins as FRET donors at 280 nm, resonance energy was carried over to the coumarin derivatives as FRET acceptors at 360 nm, which in turn caused strong emission over 460 nm. The EPS of an environmental biofilm is too biochemically complex and ill-defined to exclude the possibility of similar reactions taking place, which could explain the non-specific signal observed in the green range around 550 nm upon excitation at 300 nm (ATTO). This should be considered when choosing fluorescent labels for application in FISH of environmental biofilm samples.

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## CHAPTER 6

### Conclusion

Wineries can minimise wastewater production through the implementation of various water saving and pollution minimising strategies (Conradie 2015), but winemaking will always produce some effluent requiring treatment. Biological wastewater treatment has proven to be an efficient and cost effective solution for winery wastewater treatment (Ioannou *et al.* 2015). Biofilm reactors in particular offer resistance to fluctuations in organic load and wastewater volumes, have low sludge production and have high-specific area to volume ratios. These characteristics result in small footprints and offer efficient mass transfer across the nutrient and biochemical environment gradient that exists within a stratified biofilm, making fixed growth biological reactors ideal for winery wastewater treatment (Andreottola *et al.* 2002; López-Serrano *et al.* 2002; Sheli and Moletta 2007).

The objectives of this study were met by the successful construction and implementation of a pilot scale biofilm contact reactor for winery wastewater, which was analysed for treatment efficacy through different seasons and wastewater quality conditions. An initial, single unit pilot scale reactor successfully treated winery wastewater by reducing COD, neutralising high and low pH values and removing nutrients and suspended solids from winery wastewater during harvest season, when the winery was producing high-strength wastewater. The system was up-scaled by adding a second cooling tower to the system, operating as a secondary reactor, treating the effluent from the first subunit. The second unit contributed to the overall waste removal efficiency of the system. The double-unit pilot system was tested in six trials over three years. The system showed effective, robust treatment of winery wastewater of varying strengths with minimal solid waste production. The quality of the solid waste was analysed and deemed potentially suitable for re-use by application as a soil amendment medium. On average, the system was consistently effective at reducing COD, total nitrogen, sulfate, phosphate and suspended solids to meet prescribed regulations for irrigation. The system performed at its peak when treating highly concentrated wastewater during harvest season.

The waste removal capabilities of the system indicated the presence of anaerobic microbial metabolic activities in the biofilms based on the sulphate and phosphate removal data. This implied that the biofilm was possibly stratified, providing more than one biochemical redox niche for waste. An investigation into the microbial community composition through next-

generation sequencing as well as the analysis of the distribution patterns of specific organisms with defined metabolic profiles by means of FISH confirmed that the biofilm was indeed stratified, with an oxygen gradient existing across its depth, giving rise to more than one biochemical redox niche. The three-tiered investigation into the biofilms in the reactor proved effective at providing an overview of the composition and the functionality of the biofilms and collectively provided insight into how the addition of a second cooling tower unit affects the microbial population and the treatment scope of the entire system. Metagenomic analyses revealed a higher phylotype richness and phylogenetic diversity in the bacterial consortium of the second subunit in the reactor and only a 12% OTU overlap between the bacterial samples from the two subunits, indicating that the biofilm populations of the two reactor subunits were phylogenetically distinct. This was confirmed by taxonomic data that indicated that the dominant bacteria in the two subunits differed, with the bacterial population in the first tower dominated by carbohydrate reducing bacteria, and the second tower dominated by nitrifying and denitrifying bacteria. Furthermore, the yeast populations differed considerably in terms of the dominant organisms in each tower. The distinct nature of the microbial populations in each tower was ascribed to the fact that biochemically distinct niches exist within each, with the first subunit mostly treating acidic, carbohydrate rich water, and the second subunit treating water which was less concentrated in organics and had a pH which was closer to neutral. FISH provided insight into the distribution of organisms within the biofilms and confirmed the suspected presence of an oxygen gradient across the biofilm depth. FISH was also effective at identifying differences in how certain organisms such as the *Nitrosomonas* and the *Nitrospirae* arrange themselves distinctively within the two biofilms. In the substrate utilisation analyses using the Biolog system, it was revealed that the majority of the carbon substrates that were tested were utilised in biofilm samples from both cooling towers, but that important metabolic utilisation capabilities fell exclusively within the consortium of the biofilm from either tower 1 or tower 2. The Biolog system proved to be a rapid, effective means to detect differences in metabolic profiles of two biofilm samples. Collectively, the data from each of the three analytical approaches indicated that by adding a second subunit to the bioreactor, the treatment capacity of the system was not merely expanded, but that the second reactor subunit added to the microbial and metabolic diversity of the system, expanding the treatment scope of the system.

From an economical point of view, research around proper cost analyses of investment and operational costs of new and implemented treatment systems is lacking. There is no



standardised set of parameters on which running and installation costs of new and implemented systems can be compared, making it difficult to determine which is the best available technology for a specific winery, and future studies should be done to establish such a standard set of costing parameters (Ioannou *et al.* 2015).

The three-tiered biofilm analysis approach used in this study provided both culture dependent and culture independent approaches to understanding the functionality of a biofilm in a bioreactor at the level of community composition through genomics, physical distribution of organisms through hybridisation and microscopy and metabolic abilities through phenotypic profiling. Data from such a multi-level approach can prove to be an invaluable tool in bioreactor design and optimisation strategies, especially when used to complement system performance data, shedding light on how adjustments to the design and operation of a bioreactor might improve or expand the waste removal capabilities of a system.

This system proved highly effective at treating winery wastewater with high organic waste content and a low pH. Current developments in the application of this technology includes the full scale commercial application of this system at wineries as well as a broader application to other kinds of organically polluted, low pH industrial wastewater, with tests that will be conducted on effluent from dairies, abattoirs, fruit canning and fruit juice factories.

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