Microbial community structure and dynamics in wastewater treatment over a year

A thesis

submitted in partial fulfilment

of the requirements for the degree

of

Masters of Science (Research)

at

The University of Waikato

by

Shaun Sanders

THE UNIVERSITY OF
WAIKATO
Te Whare Wānanga o Waikato

2019

Abstract

Wastewater treatment plants are necessary for the release of effluent into the natural environment. The wastewater treatment plant functions as a bioreactor cultivating a diverse microbial community for the removal of nutrients such as carbon, nitrogen, and phosphorous from the effluent. The Tahuna treatment plant was the focus of this study because there was simultaneous removal of nitrogen and phosphorous, although the treatment plant was not designed for both these processes. Biochemical parameters were taken, specifically; dissolved oxygen, pH, oxidation-reduction potential, total suspended solids, total phosphorous, dissolved reactive phosphorous, nitrogen levels, and temperature. Through the use of Ion Torrent sequencing of the 16S rRNA gene, and analysis of sequences using the RDP and MIDAS databases, this study was able to elucidate the diversity of microbes within the Tahuna wastewater treatment plant. The analysis of the biochemical parameters and sequencing results revealed the presence and activity of functional groups of organisms important in wastewater. These organisms were involved in ammonium oxidation, nitrite oxidation, denitrification and phosphorous removal. Ammonium was metabolised by the ammonium oxidising bacteria *Nitrosomonas* producing nitrite. The nitrite was subsequently converted to nitrate by the nitrite oxidising bacteria *Nitrospir*a. Finally, *Dechloromonas* functioned as a denitrifying phosphorous accumulating organism in the treatment plant. As a denitrifying phosphorous accumulating organism, *Dechloromonas* was able to metabolise nitrate into dinitrogen gas as well as accumulate phosphorous for removal. The community remained relatively stable over the course of the study, with CAP and CAA plots as well as ANCOM analysis revealing that the greatest driver of the microbial community was temporal change.

Acknowledgements

I would like to thank my supervisors Ian McDonald, Hugh Ratsey, and Graeme Glasgow for the support they provided. I would like to acknowledge the Kamai Valley Services for the grant they provided to help with my research. I would also like to thank the lab technicians in the thermophile research unit, specifically Roanna for all the help she provided in the lab. Thanks to the Dr Alexis Richards for all the great advice and help with R studio and Dr Huw Marshall for reading and giving comments on my thesis. Thank you to my family who has always supported me. Lastly, thanks to all the past and present TRU students who I have spent so much time with and have not only become close friends but helped me retain my sanity over the course of my thesis.

Table of Contents

Table of Figures

Chapter 1:

Literature review

Wastewater treatment plants (WWTP) are bioreactors which cultivate microorganisms to reduce the nutrient load in wastewater before it is released back into the environment. The efficiency of wastewater treatment plants is reliant on the microorganism's present, so microbial community structure and dynamics are of utmost importance. The microorganisms in WWTP can be arranged simply by taxonomy, but it is useful to place them into functional groups (Figure 1) as this gives an insight into the processes occurring during treatment.

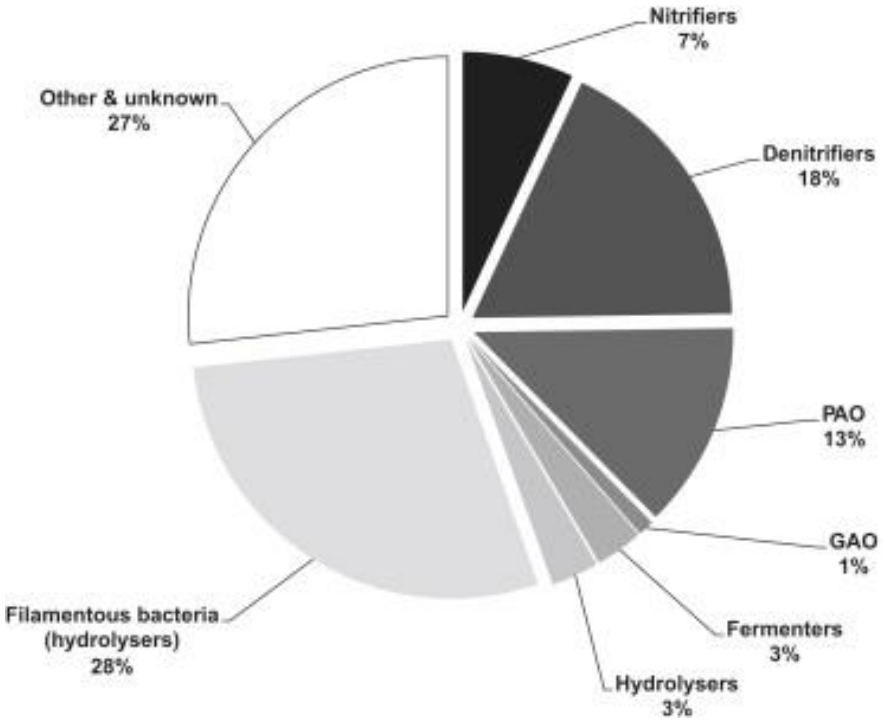

Figure 1. The microbial community in a WWTP organised by functional groups (Nielsen et al., 2010).

In wastewater treatment, carbon, nitrogen, and phosphorous removal are necessary; without removal by microorganisms the release of these nutrients can have detrimental environmental impacts such as eutrophication in lakes and streams (Álvarez et al., 2017; Conley et al., 2009; Mainstone & Parr, 2002;

Rodríguez-Gallego et al., 2017; Smith et al., 1999). A range of processes, such as denitrification, nitrification, anaerobic ammonia oxidation, glycogen accumulation, and phosphorous uptake occurs in WWTP. The presence of the organisms required to undertake all the nutrient removal processes listed above means WWTPs have an impressive and diverse microbial community.

Microbial diversity in wastewater communities

The diversity of microorganisms in WWTPs differs between systems due to factors such as plant configuration, nutrient load, and the oxygenation system. During plant operation both a transient and core microbiome exist, an understanding of which can help elucidate what processes are occurring within the system. The core microbiome is the members of a microbial community that are commonly present in that environment, while the transient microbiome consists of the microbial members present only during specific times or conditions (Shade & Handelsman, 2012).

The most common form of the wastewater treatment process is the activated sludge process, which in its most basic form consists of an aeration tank and a settling tank. In this aeration influent is combined with a stable microbial biomass, and aeration allows aerobic microorganisms to utilise nutrients in the influent and form flocs which are then transferred to a settling tank, so the flocculant can be removed (Haandel, 2015; Metcalf & Eddy, 2014). In activated sludge, Proteobacteria is the dominant phylum (Chen et al., 2016; Fan et al., 2017; Xu et al., 2018; Ye & Zhang, 2013; B. Zhang et al., 2017; Zhang et al., 2018). *Dechloromonas, Thauera, Nitrosomonas* and *Propionivibrio* are present in the core microbiome, these belong to the β-Proteobacteria (Fan et al., 2017; McIlroy et al., 2016). The α-Proteobacteria present in conventional WWTPs include *Reyranella*, which is a micro-aerophilic gram negative heterotrophic bacillus (Fan et al., 2017; Inaba et al., 2018; Pagnier et al., 2011). Another important member in WWTP is *Arcobacter* from ε-Proteobacteria (Faust et al., 2015; Fernández et al., 2008; Ma et al., 2015), while a major microbe in the y-Proteobacteria is *Pseudomonas* (Lu et al., 2014).

Other bacteria which also play a role include Actinobacteria, Firmicutes, Bacteroidetes, Acidobacteria, *Nitrospira*e, and Planctomycetes (Chen et al., 2016; Fan et al., 2017; Xu et al., 2018; Ye & Zhang, 2013; Zhang et al., 2017; Zhang et al., 2018). Actinobacteria present in wastewater include filamentous bacteria such as 'Candidatus *Microthrix'*, while the Firmicutes include bacteria such as *Trichococcus* and *Enterococcus* (Fan et al., 2017). Bacteriodetes in wastewater treatment includes a range of microorganisms, such as *Flexibacter* and *Ferruginibacter*. Another important dominant organism is *Nitrospira* from the order *Nitrospira*e (Fan et al., 2017).

As the microbial life in WWTP determines what nutrients are removed during treatment, knowledge of the microbial life within the WWTP community can be beneficial for the running of WWTPs. The diversity and community structure of the wastewater can be determined by a range of techniques. Historically, these techniques involved the use of cultivation, while more recently advanced techniques such as sequencing have been implemented (Kampfer et al., 1996).

Cultivation techniques of nitrifying bacteria allow physiological, genetic, and biochemical properties to be analysed in detail. However, cultivation is biased to the bacteria that can be grown on the media used and cultivation of pure strains of many bacteria is difficult. Early cultivation studies indicated that it was *Nitrobacter* which was the dominant nitrifier in wastewater. Eventually, molecular methods identified that it was *Nitrosomonas* that was dominant. More recent studies using advanced cultivation techniques, including selective enrichment used in conjunction with cell sorting, has allowed other Ammonia Oxidising Bacteria (AOB) such as *Nitrosospira* to be grown in pure culture (Fujitani et al., 2015; Ushiki et al., 2013).

Molecular techniques used to study AOB, such as Denaturing Gradient Gel Electrophoresis (DGGE) , Fluorescent In Situ Hybridisation (FISH)(Araujo et al., 2000), and sequencing usually involve the use of 16SrRNA sequences (Eschenhagen et al., 2003), or DNA sequences relating to specific enzymes involved in the oxidation of ammonium, specifically the enzyme ammonia

monooxygenase (AmoA) (Purkhold et al., 2000). The use of these techniques has shown that different AOB populations change abundance depending on the environmental conditions (Purkhold et al., 2000). Therefore, cultivation methods which rely on synthetic wastewater, as opposed to domestic wastewater, may show the incorrect dominant AOB. The use of FISH allows for the visualisation of defined microorganisms in a sample, this can also for the identification of organisms potentially working in consortia.

Nitrogen removal

Nitrification and nitrifying organisms

The importance of bacteria in wastewater treatment is derived from their ability to utilize nitrogen, phosphorous, and carbon. Conventional nitrogen removal in WWTPs involves organisms undertaking denitrification and nitrification. Nitrification is the biological process in which oxygen is used to oxidise ammonium or nitrite to nitrate. Nitrification is usually a two-step process involving two phylogenetically distinct groups of organisms, AOB and Nitrite Oxidising Bacteria (NOB), which can interact as a consortia (Haandel, 2015; Lam & Kuypers, 2011). In aerobic tanks, oxygen is supplied, via aeration and mixing, which allows for both the degradation of organic material and the process of nitrification to occur by aerobic microorganisms (Haandel, 2015; Lam & Kuypers, 2011).

The first step of nitrification begins with AOB. In the literature, these bacteria are often identified using 16S rRNA and amoA genes (Head et al., 1993; Purkhold et al., 2000; Purkhold et al., 2003). Known nitrifying bacteria in wastewater include *Nitrosomonas* and *Nitrosospira,* which belong in the β-Proteobacteria, and *Nitrosococcus*, which belongs in the y-Proteobacteria (Head et al., 1993). *Nitrosomonas* are the main ammonium oxidising bacteria in WWTPs, these organisms utilise the enzyme ammonia monooxygenase to oxidise ammonia to hydroxylamine ($NH_3 + O_2 \rightarrow NH_2OH$)(Lam & Kuypers, 2011; Ma et al., 2013). The AOB then use hydroxylamine oxidoreductase to oxidise the hydroxylamine to nitrite ($NH_2OH + O_2 \rightarrow NO_2^- + H_2O + 2H^+$). The second group of

organisms involved in nitrification are NOB. NOB in wastewater include the *Nitrospira*, of the phylum *Nitrospira*e, and *Nitrobacter* (Fan et al., 2017; Xu et al., 2018). NOB can oxidise nitrite to nitrate using oxygen and the enzyme nitrite oxidoreductase ($NO_2^- + 0.5O_2 \rightarrow NO_3^-$)(Lam & Kuypers, 2011).

Studies examining the nitrifying communities in WWTP often observe different nitrifiers dominating the sludge. Differences in the WWTP community are most likely caused by a variety of factors such as Dissolved Oxygen (DO), pH, sludge retention time, and temperature.

As nitrifying organisms are beneficial in WWTP for the removal of ammonium, the operating conditions, and environmental parameters which impact them, are also of importance. As nitrifiers need oxygen to oxidise ammonium the aeration and consequent DO levels in the wastewater tank can affect the growth rate and function of the AOB. The oxygen level, represented by the DO, increases nitrification rates when above 3 mg/L. As AOB and NOB are aerobic microorganisms, low oxygen impedes the growth of these organisms, with concentrations below 2 mg/L resulting in inhibition (Metcalf & Eddy, 2014; Noda et al., 2003; Park & Noguera, 2004). Heterotrophic bacteria can more effectively utilize oxygen in a system, which means that excessive oxygen concentrations can cause nitrifiers to be outcompeted (Metcalf & Eddy, 2014). However, there is evidence that prolonged exposure to low DO (less than 0.5 mg/L), facilitated by a Sludge Retention Time (SRT) of 40 days, can result in a change to a stable dominant nitrifier community able to undertake full nitrification at low oxygen concentrations (Liu & Wang, 2013). Nitrification occurring at different levels of DO can be attributed to shifts in *Nitrosomonas* dominating the nitrifier communities, depending on their affinity for oxygen (Park & Noguera, 2004).

pH is an important parameter for wastewater treatment, for although pH varies greatly between WWTPs it also appears to be a major determining factor for the community structure (Gao et al., 2016). Additionally, AOB are more resistant to changes in pH than NOB. NOB are inhibited when an excess of ammonium or free nitrous acid is added, due to change in the pH (Anthonisen et

al., 1976). There are also other factors which can affect nitrification such as SRT, with a low SRT reducing nitrification efficiency (Noda et al., 2003).

Denitrification and denitrifying organisms

Denitrification plays an important role in conventional wastewater treatment, reducing the overall amount of nitrogen compounds in the effluent. Specifically, denitrification is the reduction of nitrite or nitrate to nitrogen oxides, which are then eventually converted to dinitrogen gas (Knowles, 1982). In both natural and wastewater systems, denitrification is mainly undertaken by bacteria. In wastewater, facultative anaerobic heterotrophic bacteria dominate the denitrification community (Knowles, 1982). The importance of denitrification in wastewater treatment is that during nitrification nitrate is produced which, when released, can act as a strong greenhouse gas. Denitrification allows this nitrite to be transformed into the less polluting dinitrogen gas.

The process of denitrification involves the uptake of nitrite or nitrate which are then used as electron acceptors in the absence of oxygen to oxidise carbon compounds, this process reduces nitrite/nitrate to dinitrogen gas ($NO_3^- \rightarrow NO_2^- \rightarrow NO \rightarrow N_2O \rightarrow N_2$) (Figure 2) (Haandel, 2015; Knowles, 1996; Lam & Kuypers, 2011).

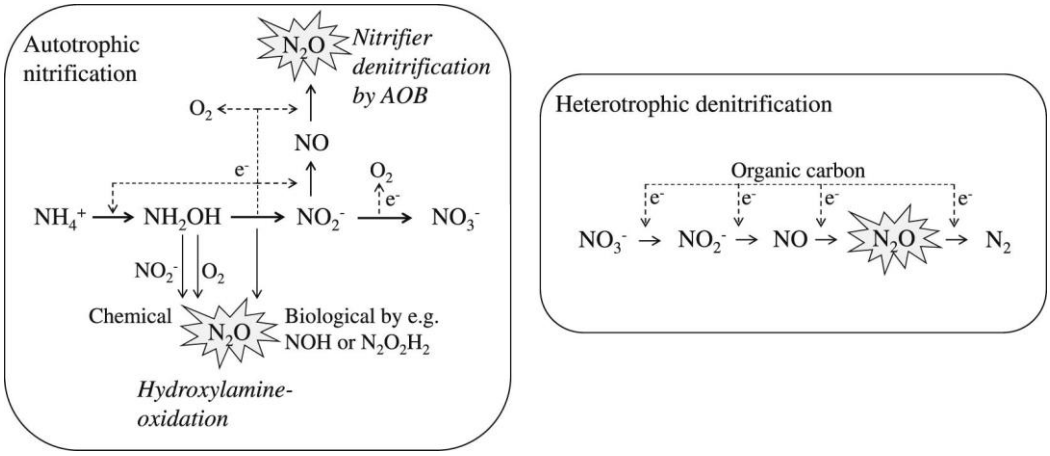

Figure 2. The biochemical pathway of nitrification/denitrification (Wunderlin et al., 2012).

A series of nitrate reductases are required for the reduction of NO_3^- to N_2, these proteins are nitrate reductase (Nar), nitrite reductase (Nir), nitric oxidoreductase (Nor), and nitrous oxide reductase (Nos) (Zumft, 1997). For denitrification to function, there needs to be nitrification occurring, nitrification provides the necessary nitrate for the facultative anaerobic bacteria (Haandel, 2015). As the bacteria involved in denitrification are heterotrophs they also require an adequate supply of organic matter as an electron donor (Kampschreur et al., 2009; Lam & Kuypers, 2011; Zumft, 1997). There is a diverse range of bacteria which undertake denitrification in wastewater, but the majority of denitrifying bacteria belong to the β-proteobacteria (Thomsen et al., 2007). These β-proteobacteria denitrifiers include *Azoarcus*, *Thauera*, *Curvibacter*, and *Dechloromonas* (Ginige et al., 2004; Thomsen et al., 2007). In the core microbiome of wastewater treatment, the main denitrifying bacteria are *Dechloromonas* and *Thauera* (Fan et al., 2017).

Factors which control the functioning of denitrifying bacteria in wastewater are primarily the amount of nitrogen present, pH, temperature, the carbon source, and DO. Nitrogen in the form of nitrite is a major factor which limits the growth of nitrifiers, as it is used as an electron acceptor. Generally, the internal recycle provides the nitrate which has been generated in an aerobic tank by nitrification (Metcalf & Eddy, 2014). The carbon source for denitrification can be supplied by the incoming influent or from dead cell mass. However, the addition of external carbon compounds such as ethanol, methanol, and acetate have been shown to increase denitrification rates (Lew et al., 2012; Peng et al., 2007). The level of nitrite and nitrous oxide, the intermediates in denitrification can also affect the efficiency of denitrification, with excessive levels inhibiting the process. The effect of these intermediates is usually linked to pH, with treatment out of the range of pH 7-9 experiencing intermediate build-up and inhibition. Temperature also has a strong effect on denitrification with temperatures outside of 20 - 30°C having an inhibitory effect (Lu et al., 2014).

Carbon source has a strong impact on the denitrifiers community structure. As microorganisms tend to specialise in the use of specific carbon compounds, the use of single or multi-carbon compounds encourages or limits the organisms present in the community. Methylotrophic denitrifiers such as *Methyloversatilis* spp. and *Hyphomicrobium* spp. dominate the denitrifier community when the external carbon is methanol (Baytshtok et al., 2009; Osaka et al., 2006). When acetate is used as the carbon source the denitrifiers are microbes closely related to the bacterial families Comamonadaceae, which include *Comamonas* and *Acidovorax,* and Rhodocyclaceae, which include *Thauera* and *Dechloromonas* (Ginige et al., 2005; Osaka et al., 2006). Ethanol was shown to be more easily utilised by denitrifiers than other carbon compounds and was dominated by *Thauera* and *Dechloromonas* (Christensson et al., 1994; Peng et al., 2007; Sun et al., 2016).

The DO in wastewater treatment plants has a significant effect on the efficiency of denitrification. The proteins associated with denitrification, Nar, Nir, Nor, and Nos are suppressed by the addition of oxygen. Furthermore, as oxygen can be used as a superior electron acceptor than nitrite, a high level of oxygen can cause the denitrifying bacteria to be outcompeted by other, aerobic bacteria (Lu et al., 2014).

Anammox

In unconventional nitrogen removal, the wastewater treatment systems utilize anaerobic ammonium oxidation (Anammox) (Du et al., 2015). Anammox bacteria are autotrophic lithotrophs which can oxidise ammonium in anaerobic conditions by utilising nitrite or nitrate to produce dinitrogen gas (Cao et al., 2016; Mulder et al., 1995). The presence of microorganisms capable of oxidising ammonium to dinitrogen without oxygen was first hypothesised in 1977 (Broda, 1977), before the first Anammox bacteria was described in a bioreactor in the Netherlands in 1995 (Mulder et al., 1995). A range of Anammox bacteria have since been discovered and enriched from activated sludge, these include; Candidatus *Kuenenia* (van der Star et al., 2008), Candidatus *Brocadia* (Oshiki et

al., 2011), Candidatus *Anammoxoglobus* (Kartal et al., 2007), Candidatus *Scalindua* (Awata et al., 2013) and Candidatus *Jettenia* (Ali et al., 2015).

Anammox bacteria are a monophyletic order of Brocadiales within the Planctomycetes phylum (Strous et al., 2006; van Niftrik & Jetten, 2012). Planctomycetes are a major phylum of the bacterial domain although 16S rRNA has demonstrated that they are distinct from other members of eubacteria (Strous et al., 2006; van Niftrik & Jetten, 2012). The Planctomycetes are also unique in the eubacteria domain as they do not possess peptidoglycan on the outside of their cell wall but do possess intracytoplasmic membranes which produce cell compartmentation (Figure 3).

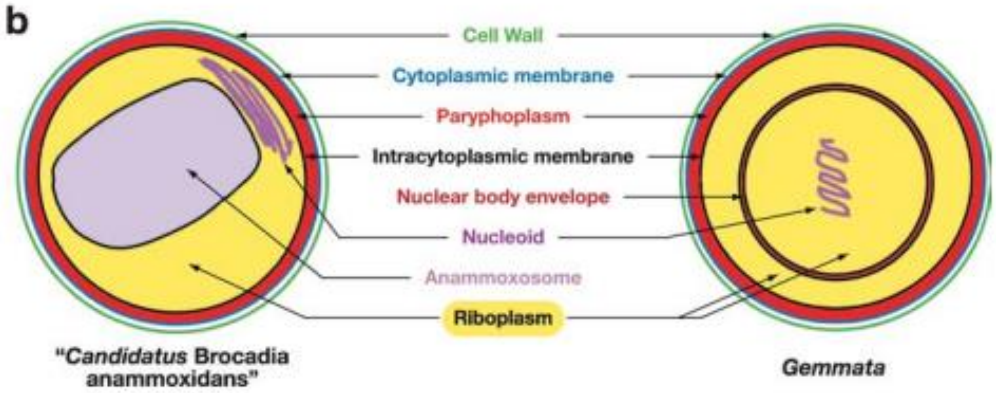

Figure 3. Cell organisation and unique compartmentation of "Candidatus Brocadia Anammoxidans", and Gemmata (Fuerst, 2005).

The intracytoplasmic membranes play an important role in Anammox bacteria, forming three organelles; the Anammoxosome, riboplasm, and the paryphoplasm, each possessing their own bilayer of intracytoplasmic membranes (Lindsay et al., 2001; van Niftrik et al., 2004). The Anammoxosome is a ribosome-free organelle unique to Anammox bacteria and is the location of Anammox catabolism (Lindsay et al., 2001; van Niftrik et al., 2004). In the bacterial cell, the Anammoxosome is surrounded by a single membrane, and the outside of that single membrane is surrounded by the riboplasm. The riboplasm contains many electron dense ribosome-like particles and the cell fibrillar nucleoid and is also surrounded by an intracytoplasmic membrane which contains the paryphoplasm. The paryphoplasm is a region in the cell where the cytoplasm does not contain ribosome-like particles. The paryphoplasm is surrounded by two membranes, the

outer side bound by the cytoplasmic membrane and the inner by the intracytoplasmic membrane (Lindsay et al., 2001).

The Anammoxosome allows for an important aspect of Anammox bacteria, which is their ability to oxidise ammonia in anaerobic conditions. This ability is unique in the Planctomycetes phylum with the majority of Planctomycetes being aerobic chemoorganotrophs, while Anammox bacteria are anaerobic lithoautotrophs (van Niftrik et al., 2004). The Anammox process utilises the Anammoxosome, and the enzymatic machinery within, to oxidise ammonium using nitrite or nitrate to produce dinitrogen gas (Figure 4).

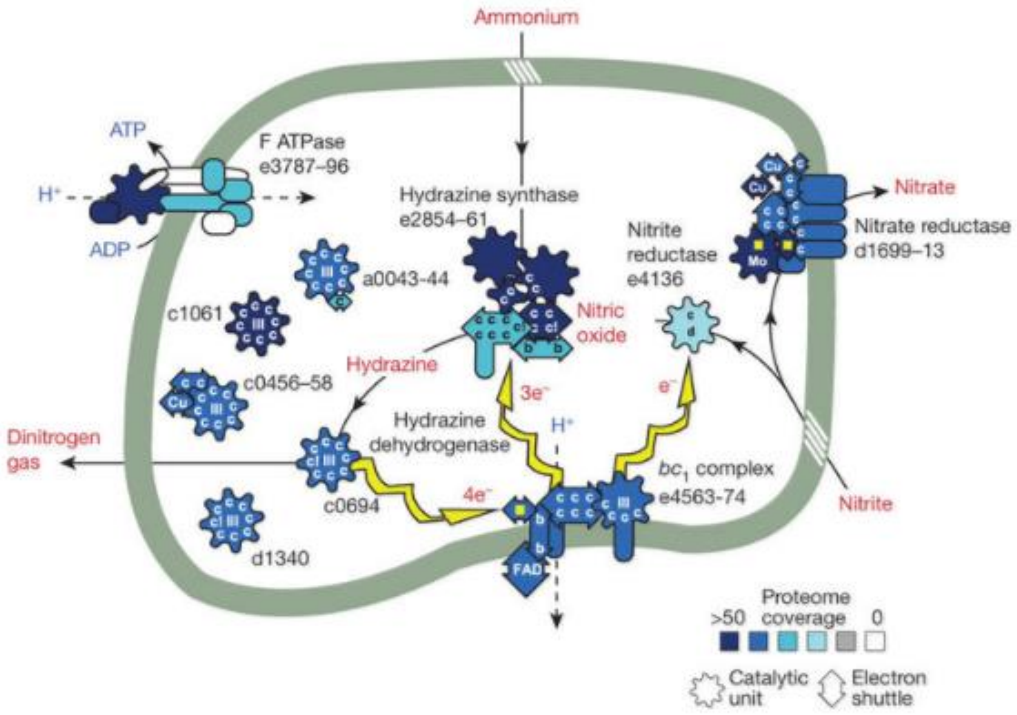

Figure 4. The biochemical reactions which occur in the Anammoxosome to utilise ammonium and produce dinitrogen gas (Kartal et al., 2011).

The mechanism for Anammox involves three redox reactions (Figure 4). It begins with nitrite entering the Anammoxosome where it is reduced to nitric oxide by nitrite reductase ($NO_2^- + 2H^+ + e^- = NO + H_2O$). An N-N bond is then formed when the nitric oxide is further reduced and simultaneously condensed with incoming ammonium by hydrazine synthase, resulting in the generation of hydrazine ($NO + NH_4^+ + 2H^+ + 3e^- = N_2H_4 + H_2O$) (Kartal et al., 2011). Hydrazine is a toxic compound and as such needs to be contained within the Anammoxosome

(Strous et al., 2006) so that it does not damage the cell. All known Anammox bacteria contain hydrazine within the Anammoxosome using ladderane lipids (Rattray et al., 2008) (Figure 5). These lipids are particularly dense and impermeable, due to their unusual linearly concatenated cyclobutene moieties, protecting the cell by restricting diffusion of hydrazine (Sinninghe Damsté et al., 2002). Lastly, the hydrazine is oxidised to dinitrogen gas by hydrazine dehydrogenase ($NH_4^+ + NO_2^- = N_2 + 2H_2O$) (Kartal et al., 2011). This Anammox cycle generates a proton motive force enabling the bacteria to produce ATP (Strous et al., 2006).

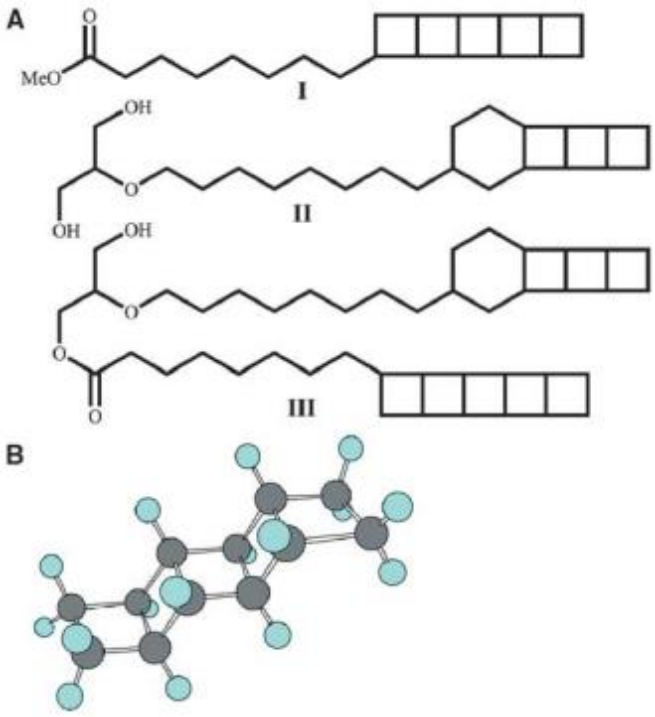

Figure 5. Structures of various ladderane lipids of "Candidatus Brocadia. (Jetten et al., 2003).

Anammox bacteria have been of interest for wastewater treatment due to their ability to function without the addition of extra carbon, their lower sludge production and their low oxygen requirements, all of which could significantly reduce the cost of treatment (Jetten et al., 2001). Anammox rates have been shown to be optimal in WWTPs with high ammonium content and low carbon input (Ganigué et al., 2009). However, Anammox bacteria have a range of requirements and limitations for their use in treatment plants. One of the main

limitations of Anammox bacteria is their slow generation time, which can be around ten to eleven days in optimum conditions (Jetten et al., 2001; Schmidt et al., 2003; Strous et al., 1998; van der Star et al., 2007). Other limitations include their inhibition by substrates such as ammonium and nitrite, and inhibition due to environmental conditions, including oxygen (Corbalá-Robles et al., 2016), pH (Jaroszynski et al., 2011), temperature (Lotti et al., 2015), organic matter (Ni et al., 2012), salts (Zhang et al., 2016), heavy metals, phosphate, and sulfide (van der Star et al., 2008).

Factors controlling Anammox

Nitrite is the substrate used by Anammox bacteria in the catabolism of ammonium, and a ratio of ammonium to nitrate at 1:1.32 is required for Anammox to occur (Kartal et al., 2011; Strous et al., 1998). However, nitrite is a toxic compound, which in sufficient quantities can inhibit a wide range of organisms (Zhou et al., 2011). Previous studies have differed on the amount of nitrite needed for inhibition of Anammox activity (Bettazzi et al., 2010; Carvajal-Arroyo et al., 2014; Cho et al., 2010; Dapena-Mora et al., 2007; Fernández et al., 2012; Isaka et al., 2007; Jaroszynski et al., 2011; Jin et al., 2013; Kimura et al., 2010; Lotti et al., 2012; Torà et al., 2010; Van Hulle et al., 2010; Zhou et al., 2011). Strous originally demonstrated that nitrite would inhibit Anammox at 100 mg L^{-1} (Strous et al., 1999). However, further studies showed a wide range of tolerance to nitrite, some studies showing inhibition occurring with concentrations from 400 mg L^{-1} to as high as 700 mg L^{-1}(Cho et al., 2010; Lotti et al., 2012; Tang, Zheng, Hu, et al., 2010).

Studies of nitrite inhibition show that Anammox tolerance depends on a variety of parameters, such as the type of Anammox bacteria, pH, plant configuration, temperature, pH, and the presence of other substrates such as ammonium (Cho et al., 2010). In the presence of ammonium the nitrite tolerance of Anammox bacteria increases (Lotti et al., 2012). One study, by Carvajal-Arroys, demonstrated an increase in tolerance from 53 mg L^{-1} to 384 mg L^{-1} in the presence of ammonium (Carvajal-Arroyo et al., 2014). The effect of nitrite is also altered by the morphology of the biomass, with homogenized biomass having a

significantly lower nitrite inhibition threshold than granular biomass (Cho et al., 2010). Studies show that different treatment plants have different rates of inhibition, but that 280 mg L^{-1} of influent nitrite or 100 mg L^{-1} of effluent nitrite should be considered warning levels and precautions should be taken to lower nitrite levels. Although there has been debate over whether it was free nitrous acid or nitrite which was the main inhibitor (Zhang et al., 2016; Zhou et al., 2011), further evidence has demonstrated nitrite to be the main inhibitor, with free nitrous acid still effecting Anammox but to a lesser degree (Lotti et al., 2012; Puyol et al., 2014).

Ammonium is a major substrate for Anammox bacteria and its breakdown is of importance in wastewater treatment. Several studies have shown that extremely high levels of ammonium can have detrimental effects on the Anammox process but have differed on the level of ammonium tolerance and the form of ammonium which has the greatest inhibitory effect. However, the tolerance of Anammox bacteria to ammonium appears to be greater than tolerance for nitrite, its other substrate (Dapena-Mora et al., 2007; Strous et al., 1999).

As Anammox bacteria operate in anaerobic environments one obvious inhibitor of Anammox is oxygen. In WWTPs this is measured as DO. The correlation between oxygen and reversible/irreversible inhibition of Anammox has been well studied and recognised (Egli et al., 2001; Strous et al., 1997). pH and temperature are important parameters which control the rate of Anammox, with statistical analysis showing that pH had greater importance for the inhibition of Anammox (Daverey et al., 2015). The pH range recommended for wastewater differs between studies, with the optimum pH ranging between 6.5 and 8.5, while the temperature is recommended to be between 30 to 40°C (Egli et al., 2001; Strous et al., 1999; Van Hulle et al., 2010; Yin et al., 2016). The pH appears to buffer the effect temperature has on Anammox bacteria, with lower temperatures being tolerated with higher pH (Daverey et al., 2015). Another aspect of importance is the tolerance for changes in pH, with studies showing that Anammox functions significantly better in stable pH conditions (Jaroszynski

et al., 2011). Anammox appears tolerant of lower temperatures, until around 15˚C where the biomass becomes unstable. An increase in temperature causes an irreversible failure of Anammox once it reaches 45˚C (Dosta et al., 2008).

Anammox bacteria are autolithotrophs and do not require the addition of organic matter as a substrate (Jetten et al., 2001). The addition of organic matter can therefore adversely affect the growth of Anammox bacteria as they are outcompeted by faster-growing organisms, such as heterotrophic denitrifiers that utilize the organic matter (Ni et al., 2012; Tang, Zheng, Wang, et al., 2010). Addition of greater than 300 mg Chemical Oxygen Demand (COD)/L would inactivate the Anammox process (Chamchoi et al., 2008). However, in some studies, Anammox bacteria were shown to be able to utilize organic matter such as propionate, but large amounts would still affect the process negatively, while alcohols such as methanol were shown to have a strong detrimental effect on the system (Guven et al., 2005; Isaka et al., 2008). The ability of Anammox bacteria to be able to utilize some organic matter means that they can be useful in wastewater treatment where organic matter may be high (Kartal et al., 2008).

Phosphorous accumulating organisms

The biological removal of phosphorous is undertaken by microorganisms for either direct microbial growth or for storage as polyphosphate, the organisms doing the latter are referred to as Polyphosphate Accumulating Organisms (PAOs) (Wagner et al., 2002). Treatment plants that are designed for the organic removal of phosphorous rely on PAOs and refer to the process as luxury phosphorous uptake or Enhanced Biological Phosphorous Removal (EBPR) (Haandel, 2015; Wagner et al., 2002) (Figure 6). Polyphosphate is stored in the biomass of PAOs to be used as an energy supply in the uptake of carbon sources when the microbes are in anaerobic environments (Mino et al., 1998).

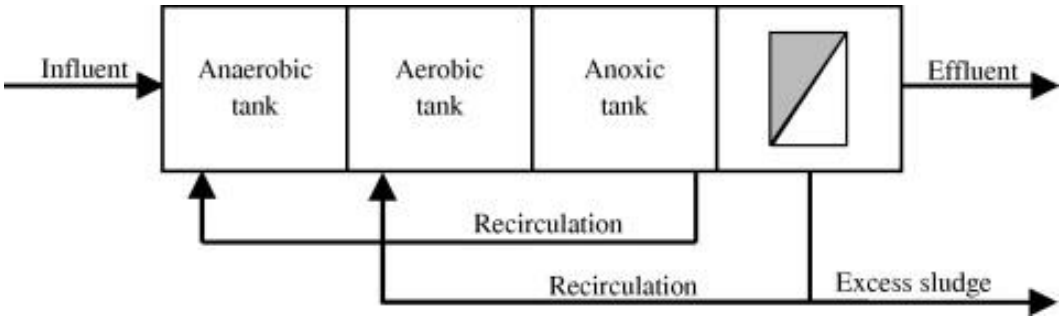

Figure 6. Schematic drawing showing the configuration of a treatment plant designed for luxury phosphorous uptake (Zuthi, Guo, Ngo, Nghiem, & Hai, 2013).

The main PAO identified in the wastewater phosphorous removal treatment process is 'Candidatus *Accumulibacter phosphatis*' a coccoid-rod shaped bacteria from the β-Proteobacteria (Hesselmann et al., 1999). The other known PAOs include *Tetrasphaera* sp. from the family Intrasporangiaceae and the genus Actinobacteria (Kong et al., 2005). *Tetrasphaera* forms short rods or cocci in tetrads and differs from *Accumulibacter* as it does not form polyhydroxyalkanoates and also uses different forms of carbon (Kong et al., 2005). *Tetrasphaera* also does not appear to compete with *Accumulibacter* and may be beneficial to *Accumlibacter* as they can ferment glucose, providing acetate for *Accumlibacter* growth (Nielsen et al., 2010).

Factors controlling PAO communities

The inhibition of PAOs can come from a variety of sources, such as pH, temperature, COD/P ratio, level of oxygenation, nitrite level, as well as competition from other bacteria.

The main competitors of carbon sources for PAOs in WWTPs come from Glycogen Accumulating Organisms (GAO), such as Candidatus *Competibacter phosphatis* and bacteria related to *Defluviicoccus vanus* (Mielczarek et al., 2013). GAO's are able to compete with PAOs more effectively at warmer temperatures causing a decrease in phosphorous removal (Oehmen et al., 2007). The efficiency of PAOs is affected when the temperature is above 30°C, possibly due to the ability of GAOs to uptake more acetate at warmer temperatures than their PAO counterparts (Lopez-Vazquez et al., 2007; Oehmen et al., 2007). PAOs have also

been shown to increase in numbers in wastewater when the pH is higher than 7.25 (Haandel, 2015).

Filamentous bacteria

Non-floc forming filamentous bacteria are often found during the wastewater treatment process. Filamentous bacteria can have both beneficial and detrimental roles in treatment depending on their abundance (Sezgin et al., 1978). When filamentous bacteria are at low abundance, they can function as scaffolding to help floc formation. However, an overabundance of filamentous bacteria can result in bulking or foaming, which prevents flocculation and settling of the wastewater biomass (Gnida et al., 2018). In the past, the identification of these filaments was achieved by examining morphological features (Eikelboom, 1975; Li et al., 2008). However, there is evidence that morphological features do not reliably distinguish the identity of the filamentous organisms, with one morphotype often belonging in more than one genus. The use of techniques such as FISH probes targeting 16rRNA has provided evidence of filamentous bacteria in Proteobacteria, Bacteriodetes, Chloroflexi, Firmicutes, Actinobacteria, Mycolata, Planctomycetes, and Tetrasphaera (Speirs et al., 2009).

Wastewater treatment plants

Wastewater treatment plants associated with conventional nitrogen removal

The first WWTPs removed nutrients by separating solids and liquids from wastewaters in a primary treatment (Haandel, 2015). Since large amounts of organic material remained after primary treatment a secondary treatment was developed for the biological removal of organic matter (Bitton, 2010; Haandel, 2015). Further advancements led to the use of activated sludge, where aerated sludge would be settled and returned to the start of the process before effluent was discharged. There are a variety of WWTP configurations, although, conventional activated sludge involves single or multiple aeration tanks (Haandel, 2015; Metcalf & Eddy, 2014). The modern WWTP involves variations of single or multiple aerated tanks using either complete-mixed activated sludge, sequencing batch reactors, or the staged activated sludge process (Haandel,

2015; Metcalf & Eddy, 2014). The complete-mixed reactor uses an aeration tank where complete aeration takes place, enabling the oxygen and substrates in the wastewater to homogenise via thorough mixing in the system. The defining feature of sequencing batch reactors is the activation and deactivation of aeration in five steps; fill, react, settle, discharge, and pause/idle (Haandel, 2015; Metcalf & Eddy, 2014). The sequencing batch process can be altered to a continuous flow system with the addition of tanks which undergo the alternating steps (Haandel, 2015). The staged activated sludge process involves a series of plug flow tanks, which are narrow tanks separated by baffle walls. The separation of tanks allows different aeration conditions to occur in different tanks (Haandel, 2015; Metcalf & Eddy, 2014).

Conventional biological wastewater treatment generally uses nitrification and denitrification to remove nitrogen. Treatment plants with nitrogen removal capabilities all require an aerobic and an anoxic zone. There are three major configurations relating to nitrogen removal; pre-anoxic denitrification, post-anoxic denitrification, and single reactor nitrification/denitrification processes (Figure 7).

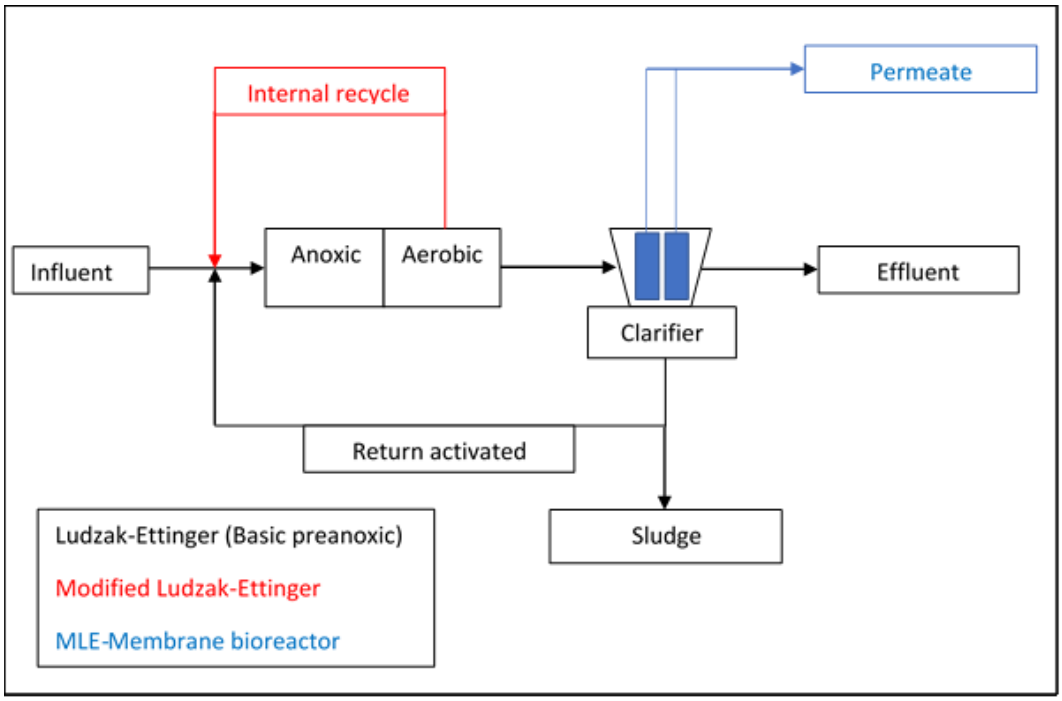

Figure 7. Examples of post-anoxic denitrification configurations- influent enters the anoxic tank supplying the denitrifying bacteria with carbon after denitrification occurs the wastewater is transferred to the aerobic tank where nitrification occurs. The internal

recycling returns nitrate produced from the oxidation of ammonium in the aerobic tank, to the anoxic tank. The Ludzack-Ettinger process in black is the base configuration. The modified Ludzack-Ettinger process in red adds in the internal recycling. The modified Ludzack-Ettinger process-membrane bioreactor in blue includes a membrane into the final clarifier. Modified from (Metcalf & Eddy, 2014).

The pre-anoxic denitrification process includes plant types such as the Ludzak-Ettinger, the modified Ludzak-Ettinger (MLE), the MLE-membrane bioreactor (MBR), the step feed biological nitrogen removal system, the step fed MBR, and the sequencing batch reactor (SBR). Apart from the SBR, all of these have a sequential series of tanks beginning with an anoxic tank leading to an aerobic tank and finishing in a secondary clarifier. Nitrification occurs in the aerobic tank which produces nitrate, this nitrate is then returned to the wastewater before it enters the anoxic zone. The addition of nitrate encourages denitrification to occur in the anoxic tank where the nitrate is converted to dinitrogen gas using carbon compounds from the incoming influent (Metcalf & Eddy, 2014). As the nitrification process causes a decrease in pH, the pre-anoxic denitrification process has the benefit of buffering the system with alkalinity before the wastewater enters the aerobic tank (Metcalf & Eddy, 2014; Zhang & Bishop, 1996).

The postanoxic denitrification system has various designs, ranging from the two-reactor system, the two-sludge nitrification-denitrification, the four-stage bardenpho system, the four-stage bardenpho MBR, the dual sludge system, and the MLE-packed bed postanoxic. Two-reactor postanoxic denitrification systems are rarely used as it is more beneficial to use a pre-anoxic system as the two-reactor postanoxic denitrification process leaves a remaining amount of nitrite that needs to be removed chemical addition. The Bardenpho systems essentially has an additional anoxic and aerobic step to a pre-anoxic denitrification system as shown in Figure 8, this allows the system to remove more than the 75% of nitrate remaining after the initial anoxic/aerobic step (Metcalf & Eddy, 2014).

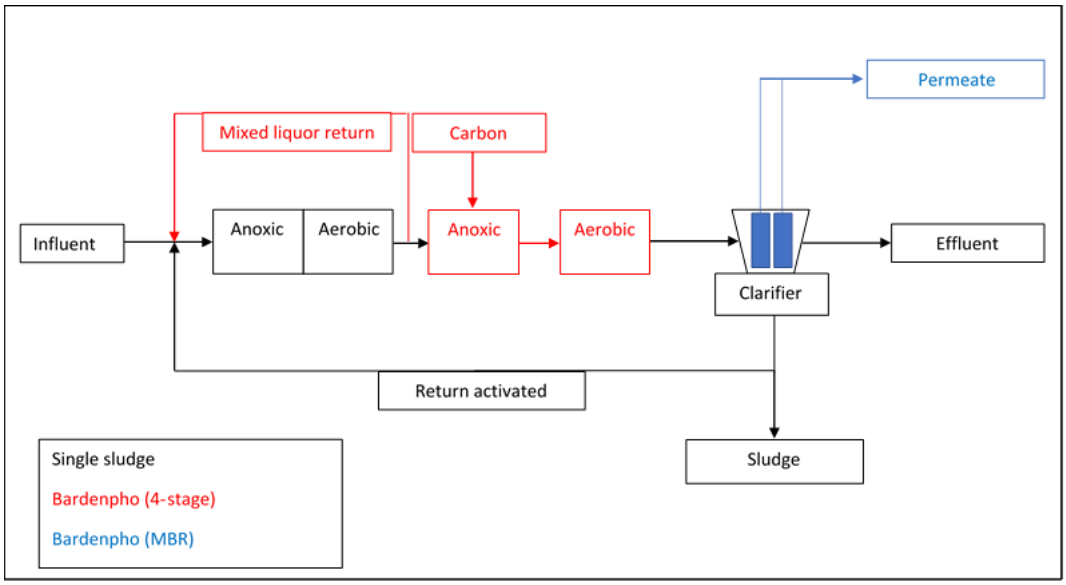

Figure 8. Diagram showing the different forms of the Bardenpho treatment, the basic sludge system in black, the 4-stage Bardenpho system in red and black, and the Barendpho (MBR) in black, red, and blue. Modified from (Metcalf & Eddy, 2014).

WWTP relying on a single reactor for nitrification and denitrification rely on either one of two systems, simultaneous nitrification/denitrification or cyclic nitrification-denitrification. The simultaneous nitrification/denitrification system utilises low DO and includes, low DO oxidation ditches, the Orbal process, and the low DO MBR. In simultaneous nitrification/denitrification systems, the bacteria on the outside of the flocs have oxygen available, but there is not enough oxygen to permeate the floc, this leaves the internal bacteria to be in an anoxic environment. The cyclic nitrification-denitrification system differs from the simultaneous nitrification/denitrification system in that although it can function in a single reactor the system functions by changing aeration either temporally or spatially (Metcalf & Eddy, 2014). This system includes the oxidation ditch and dNOX systems.

Wastewater treatment plants associated with Anammox

Three types of WWTPs have been designed to utilize Anammox; the two reactor nitritation-Anammox process, a one reactor nitritation-Anammox process, and the one reactor denitrification-Anammox process (van der Star et al., 2007). In the two reactor nitritation-Anammox process, the first reactor allows for some ammonium to be oxidised into nitrite before the wastewater

moves into another anoxic tank (van der Star et al., 2007). The one-reactor denitrification-Anammox process was the first system in which Anammox was discovered and involves the use of one tank in which both nitritation, specifically the oxidation of ammonium to nitrite, and Anammox occur (Mulder et al., 1995). This configuration is called DEAMOX (Kalyuzhnyi et al., 2006), DENAMMOX, or simply Anammox in the literature (Mulder et al., 1995; van der Star et al., 2007). In the one reactor nitritation-Anammox process, the entire process can be undertaken in one tank but requires strict control and changes of aeration (Innerebner et al., 2007). The type of treatment plants that involve the one reactor nitritation-Anammox process includes; CANON (Completely Autotrophic Nitrogen-removal Over Nitrite) (Third et al., 2001), DEMON (Deammonification system) (Innerebner et al., 2007), and OLAND (Oxygen-Limited Autotrophic Nitrification Denitrification) (Philips et al., 2002; Pynaert et al., 2002).

The CANON process involves the use of one tank containing both aerobic ammonia oxidisers and Anammox bacteria which form consortia of granular biomass. The tank is kept under constant microaerophilic conditions which allows the aerobic ammonia oxidisers to oxidise some of the ammonium to nitrite. The Anammox bacteria are in a low oxygen environment, inside the granular biomass, and so are able to use the nitrite to oxidise the remaining ammonium to dinitrogen gas (Third et al., 2001).

The DEMON process is a one reactor plant design that has intermittent periods of aeration controlled by monitoring time, DO levels, and the pH of the system. The time parameter involves three cycles running over an eight-hour period, these cycles; fill, settle, and decant, determine when aeration occurs. The monitoring of DO levels allows for the inhibition of the second step of nitrification which would reduce the amount of nitrite available to the Anammox bacteria. During partial nitrification, the pH decreases causing an increase in aeration, then during Anammox the pH increases which in turn decreases aeration (Gonzalez-Martinez et al., 2015; Innerebner et al., 2007). The DEMON process has also been attached to other wastewater treatment configurations as a sidestream addition (Wett et al., 2015).

The OLAND process was thought to be primarily driven by nitrifiers. The hypothesis was that the nitrifiers possessed the ability to switch their metabolism from nitrification to oxidation of ammonia by using nitrite when oxygen was limited (Philips et al., 2002). Later, evidence showed that the OLAND process was similar to other systems, and involved both aerobic AOB and Anammox bacteria (Third et al., 2001). This process works by the partial nitrification of ammonium in aerobic zones of the biofilm by AOB and conversion of the remaining ammonium by Anammox bacteria (Courtens et al., 2014).

Wastewater treatment plants associated with Enhanced Biological Phosphorous Removal (EBPR)

There are a variety of plant configurations designed for EBPR. WWTPs undertaking EBPR requires contact between activated sludge and the incoming influent in an anaerobic environment, usually accomplished by alternating anaerobic and aerobic tanks (Metcalf & Eddy, 2014). There are three general types of configurations that WWTP fall under when designed for EBPR; Phoredox, high phosphorous/BOD removal with nitrification, and low phosphorous/BOD removal with nitrification (Metcalf & Eddy, 2014). The phoredox, which is sometimes referred to as the anaerobic/aerobic (A/O) process, involves the use of two reactors. In the anaerobic tank at the beginning of the A/O process which receives the influent and return sludge and an aerobic reactor.

In the anaerobic tank the PAOs uptake available carbon to generate polyhydroxyalkanoates (PHA), a form of volatile fatty acids (Kerrn-Jespersen & Henze, 1993; Wagner et al., 2002). To form PHAs the PAOs need reducing power and some energy, the reducing power is generated through the conversion of glycogen to PHA, while the energy is gained from hydrolysis of stored polyphosphate into orthophosphate (Hesselmann et al., 1999; Wagner et al., 2002). In aerobic tanks, the PAOs oxidise the stored PHAs for energy while at the same time restoring their reserves of phosphorous (Ghehi et al., 2014). Consequently, the intracellular phosphates are then extracted once the sludge is removed (Wagner et al., 2002). A wide range of configurations have been

designed for phosphorous removal (Figure 9), these include; Phoredox, the anaerobic-anoxic-aerobic (A^2O) process which is a modified Ludzack-Ettinger process altered to include an anaerobic contact zone, the University of Capetown (UCT) process, the Virginia initiative plant (VIP) process, the Modified Bardenpho, the Johannesburg (JHB) process, Westbank, and the Phostrip – sidestream removal process (Metcalf & Eddy, 2014).

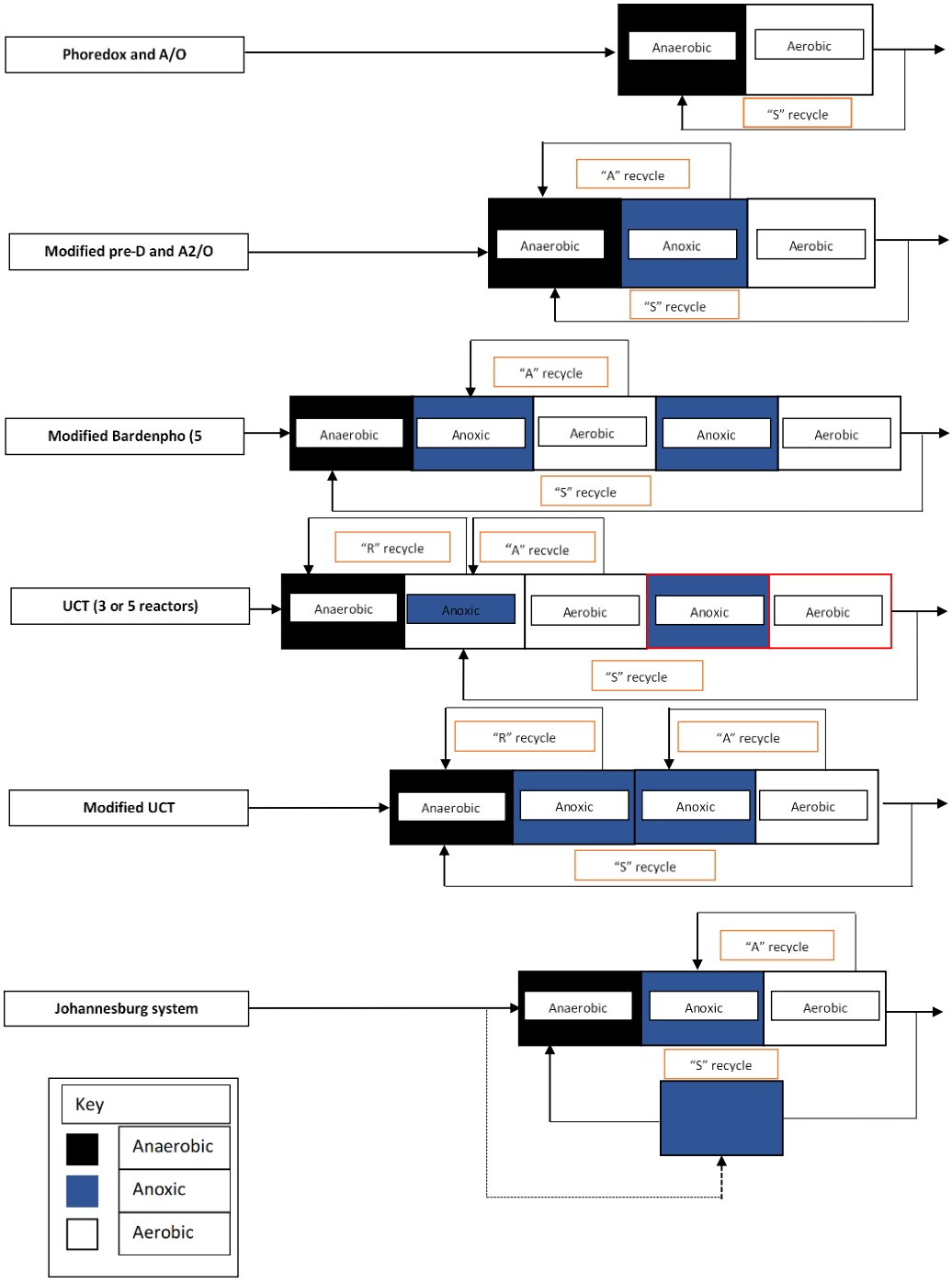

Figure 9. Layout of treatment processes involved in EBPR. Modified from (Metcalf & Eddy, 2014).

A range of parameters determines the microbial life which resides in an activated sludge treatment process. By examining a treatment plant over an extended amount of time with differing biochemical parameters the microbial community structure and dynamics can be analysed.

Study aims

- **How is a four-stage Bardenpho treatment plant capable of nitrogen and phosphorous removal without additional treatments?**
- **How does the diversity and dynamics in the microbial community affect the removal of nutrients from a wastewater treatment plant?**
- **How do temporal and biochemical changes affect a microbial community in a wastewater treatment plant?**

Chapter 2:

Methodology

Sampling

The wastewater treatment plant in this study was designed as a four-stage process (Figure 10 & Figure 11). The plant receives soda ash if the pH lowers too much, additional carbon input in the form of acetic acid, and occasionally alum if the level of phosphorous in the final effluent is deemed too high (Figure 12). The WWTP was designed with two points for acetate dosing, these were in the anoxic tanks where denitrification was planned to occur. However, acetate dosing only occurred in the first anoxic tank during this study. The aerobic tank in the WWTP had similar DO content to the anoxic tanks. During the year the WWTP was successful in removing carbon, ammonium, and phosphorous. Biochemical data and sequencing results were analysed to determine how the microbial community changed over a year, and what functional groups were the reason for the successful operation of the plant. Five samples were collected from the Tahuna wastewater treatment plant from each of the four tanks and one sample from the influent approximately once a month from October 2017 to October 2018.

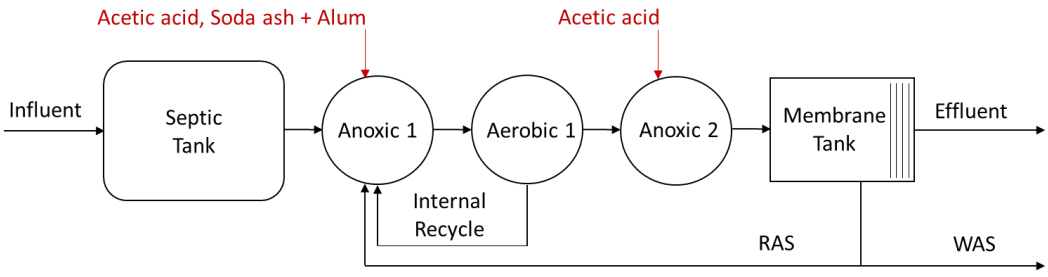

Figure 10. Diagrammatic layout of Tahuna wastewater treatment plant

The treatment plant differs from most bardenpho plants as it has an additional septic tank at the beginning of the process. During the sampling biochemical parameters were taken, specifically; DO, pH, oxidation-reduction potential (ORP), total suspended solids (TSS), total phosphorous (TP), dissolved reactive phosphorous (DRP), nitrogen levels (ammonium, nitrogen dioxide, and

nitrate), and temperature (Figure 13**)**. Grab samples retrieved from the plant were labelled with the tanks they were retrieved from; anoxic 1, MBR, anoxic 2, post-septic, and aerobic. Samples were then frozen at -80°C until the DNA could be extracted.

Figure 11. Photos were taken at Tahuna treatment plant showing the treatment tanks

Figure 12. Photos showing the acetate, alum, and soda ash which was added to the treatment plant at different times throughout the year.

Figure 13. Photos of the equipment used to take samples and measurements of pH, ORP, and temperature in the Tahuna wastewater treatment plant.

Extraction

Prior to extraction samples were thawed, and a 1000 µL aliquot removed, spun down, and the supernatant removed. Extractions were undertaken using the CTAB extraction protocol (Ni et al., 2010). Involving a bead beating step which was shown to be beneficial in DNA extractions involving wastewater (Albertsen et al., 2015).

The sample was then placed in UV treated 2.0 mL screw-capped conical bottomed polypropylene tube containing 0.5 g of both 0.1 mm and 2.5 mm silica-zirconia beads. Samples were then suspended in 300 mL of phosphate buffer saline (100 mM NaH_2PO_4), and 300 mL SDS lysis buffer was added. The samples were then shaken using a bead beater for 10 seconds before being placed on a Vortex-Genie 2 (MO BIO Laboratories Inc, Carlsbad, CA, USA) to shake horizontally for 10 minutes. Samples were then centrifuged at 10,000rpm for 30 seconds to compact samples. 180 µL of cetyltrimethylammonium bromide-polyvinylpyrrolidone (CTAB) extraction buffer (100 mM Tris-HCl, 1.4 M NaCl, 20 mM EDTA, 2% CTAB, 1% polyvinylpyrrolidone and 0.4% β-mercaptoethanol) was added to the supernatant.

Samples were then vortexed for 10 seconds and incubated at 60°C and 300 rpm for 30 minutes on a rocking bed. Samples were then centrifuged at 10,000 rpm for 30 seconds and 350 µL of chloroform/isoamyl alcohol (24:1) was

added to the supernatant. Samples were then vortexed for 15 seconds, placed on a rocking bed for 30 minutes, and then centrifuged at 10,000rpm for 5 minutes. The aqueous phase was then transferred to a new UV treated microfuge tube. 10 M of Ammonium acetate was added to reach a final concentration of 2.5 M, the samples were vortexed for 10 seconds, and then centrifuged for 5 minutes at 12,500 rpm. The aqueous layer was then removed to a new UV treated microfuge tube and 0.54 volumes of isopropanol were added and mixed by inversion.

Samples were then left overnight at −20°C before being centrifuged at 13,200 rpm for 20 min at 4°C. The supernatant was then removed, the pellet washed with 1 mL of 70% AR grade ethanol and centrifuged for 1 minute at 13,200 rpm. The ethanol was then removed, sample dried on a speed vacuum, and the DNA re-suspended in 50 µL sterile LO-TE. The Samples were quantified with Nanodrop 1000 using $A_{260/230nm}$ and $A_{260/280nm}$. The samples were then re-frozen at -80◦C until further use (Archer et al., 2015).

PCR and Sequencing

All extracted DNA underwent PCR with the EU A/B primers as a check for competency as well as to determine if there was any inhibition of the samples. Dilution of the samples before PCR was necessary as the DNA concentration was extremely high. Any samples above 500 ng/µL were diluted to approximately 100 ng/µL, the samples were then re-quantified with the Nanodrop 1000 using $A_{260/230nm}$ and $A_{260/280nm}$.

The diluted samples were then further diluted to 10 ng/µL. The EUB B/A PCR protocol used duplicate samples and positive and negative controls. In a PCR cabinet, the required 0.2 µL PCR tubes, 1.5 mL centrifuge tubes, and MiliQ H_20 was UV treated for 15 minutes. The required 1.2 µL 50 mM MgCl2, 2 µL 2 mM dNTPs, 2 µL 10x buffer, and 1.2 µL 0.4 mg/mL BSA per sample were defrosted, vortexed, and spun down in a centrifuge. In the PCR cabinet these reagents, including the MiliQ H_20, were added to an Eppendorf tube labelled as a master mix. The empty tubes and mastermix were then moved to a biosafety cabinet

which had previously been sprayed with 70% ethanol and undergone UV. Once in the biosafety cabinet, the primers and the Taq polymerase were added to the mastermix, which was then vortexed. 19 µL of the mastermix was then aliquoted out to each of the PCR reaction tubes. Water was added to be a negative control and the vortexed and spun DNA samples were added to the remaining tubes, except for the positive control which used an *E. coli* sample. The PCR tubes were then spun using a microfuge and placed in a thermocycler. The program for the thermocycler is as follows; 94°C for 2 minutes, 94°C for 45 seconds, 55°C for 30 seconds, 72°C for 2 minutes, the thermocycler then cycled back to 94°C for 45 seconds 29 times and the run finished with 72°C for 7 min. The Eu A/B amplicons were then run on a 1% agarose gel at 70 V for 20 mins, using *E. coli* as a positive control. If clear bands were seen the diluted samples were frozen for later use in PCR of the 16S rRNA gene in preparation for ion torrent sequencing.

Ten samples (two months) were sequenced first as a test to be assured the following fifty samples (10 months) would sequence correctly. The Ion torrent sequencing protocol relied on the amplification of the 16S rRNA gene by PCR, specifically an approximately 500 bp amplicon of the V4 region using barcoded fusion primers 515f and 926R. The samples were run in triplicate and then pooled at the end of the process. The program for the thermocycler is as follows; 94°C for 3 minutes, 94°C for 45 seconds, 50°C for 1 minute, 72°C for 1.5 minutes, the thermocycler then cycled back to 94°C for 45 seconds 30 times and the run finished with 72°C for 10 minutes. 5 µL of each of the pooled amplicons were run on a 1% agarose gel with a 1 kb ladder for 30 minutes at 75 volts to check the PCR was successful and that inhibition did not occur.

Once the amplicons were shown to be reliable they were pooled and normalised using a 96 well sequel prep normalisation plate, according to manufacturer's instructions (Life Technologies, Auckland). Working in a biosafety cabinet 25 µL of each sample of PCR amplicons were added to each well. 25 µL of Normalisation DNA binding buffer was then added to the wells, the buffer and amplicons mixed by repeated pipetting, with care taken not to touch the sides or bottom of the wells. Once all samples were added to the plate a sterile PCR plate

seal was placed to cover the plate. The plate is then left at room temperature for an hour. The binding buffer was then removed to a sterile labelled tube and placed in a freezer at -20°C as a precautionary measure, in case the amplicons do not become bound. 50 µL of wash buffer was then added to each well, with repeated pipetting to mix. The wash buffer was then pipetted off with care taken to remove all residual wash buffer. Normalization Elution buffer was then added to each well at a volume of 20 µL and the samples thoroughly mixed. The plate then sat for five minutes at room temperature. The solution was then transferred to a labelled sterile tube and stored at 4°C until sequenced.

Bioinformatics

The normalised amplicons were sequenced at the Waikato DNA Sequencing Facility at the University of Waikato using an Ion Torrent PGM DNA sequencer with an Ion 318v2 chip (Life Technologies). Raw sequences in the FASTQ format were first filtered in mothur to remove short reads, long reads, and reads with excessive homopolymers. The sequences were then run through USEARCH (ver 9) for filtering.

Two separate databases were used to assign taxonomy to the raw fasta file. First, the Ribosomal Database Project (RDP) classifier from Michigan State University (Wang et al., 2007) was used to convert the generated sequence FASTA file to assign taxonomy to the generated sequences (Wang et al., 2007). The QIIME software was then used to combine and convert the newly generated taxonomy and the OTU table to a BIOM file to be used in R studio. The second database was the MIDAS taxonomy (Saunders et al., 2015), which also correlated wastewater specific taxonomy to function.

The RDP database (Wang et al., 2007) was used to create a taxonomic table which was then run in R studio (Team, 2016). The relative abundance of the phylum with any taxa with less than 80% confidence classed as unknown. The OTU table and taxonomy were then imported into R studio and into an object using the phyloseq package (McMurdie & Holmes, 2013). The phyloseq object was then used with ggplot2 (Kassambara, 2018) to create graphs with colours

provided from RColorbrewer (Neuwirth, 2014) that showed the taxonomy at the phylum and class level. The RDP database was also used to create a canonical analysis of principal coordinates plot (CAP)(Anderson & Willis, 2003) using the ggplot2 function (Kassambara, 2018). The MIDAS database (Saunders et al., 2015) was used to create functional group heatmaps and a core community Venn diagram using Ampvis2 (Andersen K.S. et al., 2018).

Chapter 3:

Results and discussion

Results

Biochemical results

Biochemical parameters were measured over a 12-month period. The dissolved oxygen (DO) of the WWTP was directly controlled by aerators in the tanks and the level of aeration differed in the individual tanks over the study period (Figure 14). It is of note that the aerobic tank had a similar level of DO to the anoxic tanks, it was only during the months of June and July that the tanks were noticeably more aerobic reaching between 2.7 and 4.5 mg/L of DO. The MBR tank maintained an aerobic environment averaging 4.8 mg/L DO over the study period.

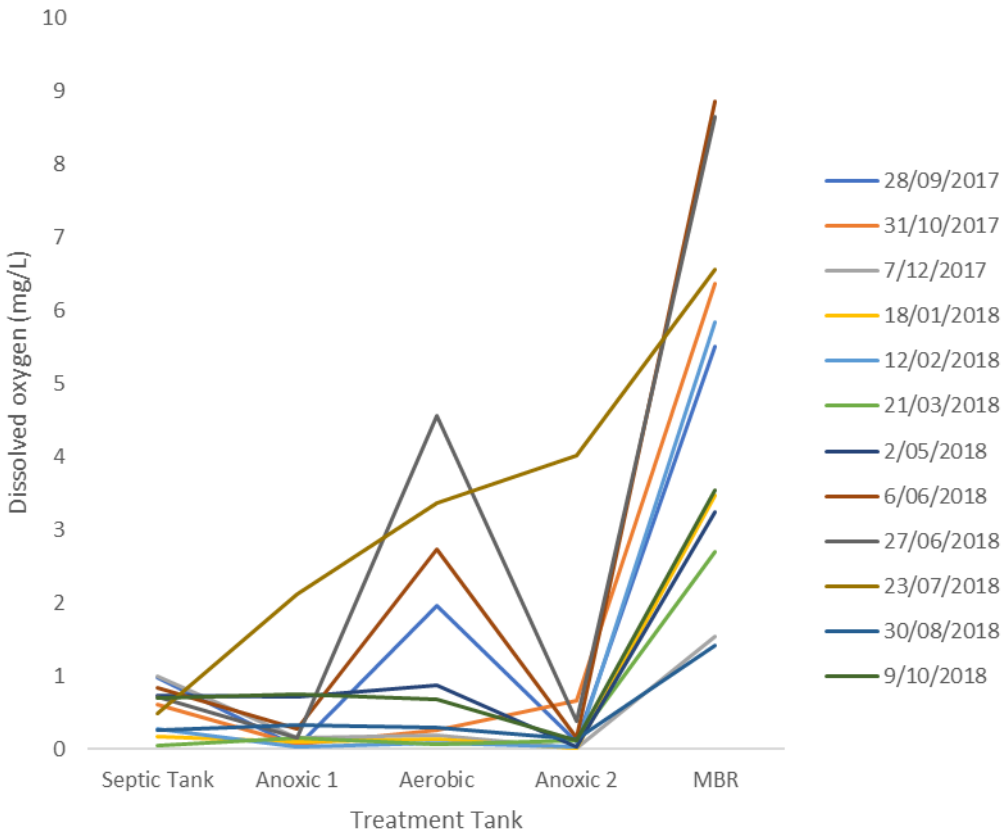

Figure 14. Dissolved oxygen across treatment tanks between September 2017 and October 2018.

ORP is the oxidation-reduction potential measured (in millivolts) in the tanks of a wastewater treatment plant. This measurement is important as a representation of the microbial community's ability to undertake a range of biological processes, such as nitrification, denitrification, luxury phosphorous uptake, and carbon removal. The ORP in the treatment plant tended to follow a trend where the ORP increased from an average -211 mV in the septic tank to an average of -101.5 mV upon entering the first anoxic tank (Figure 15). The ORP continues to increase reaching its highest in the MBR tank where it reaches an average of 82.9 mV. ORP has a strong correlation with dissolved oxygen, therefore it follows a similar pattern to the treatment plant aeration.

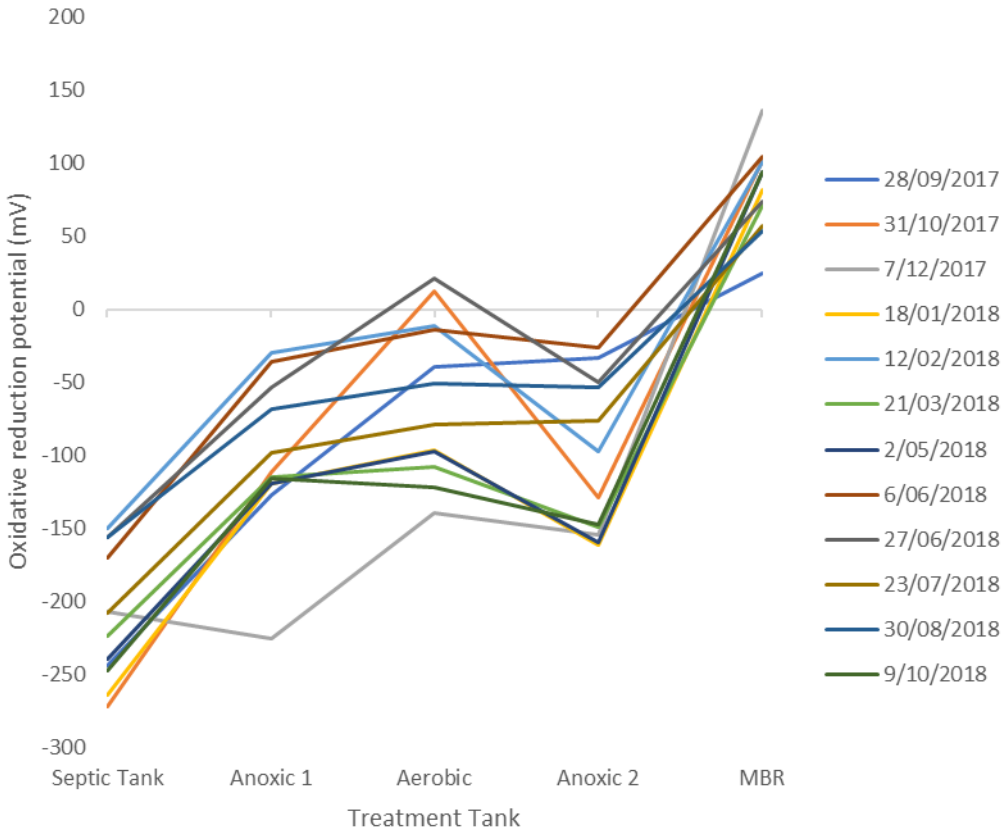

Figure 15. ORP concentration between September 2017 and October 2018.

The pH in the WWTP remained relatively stable (Figure 16). pH was lowest in the septic tank before entering the treatment tanks (6.75). Although the pH of the treatment tanks was relatively stable there was slight variation in

the aerobic and anoxic tanks. The pH generally increased from the septic tank to the permeate by 0.84.

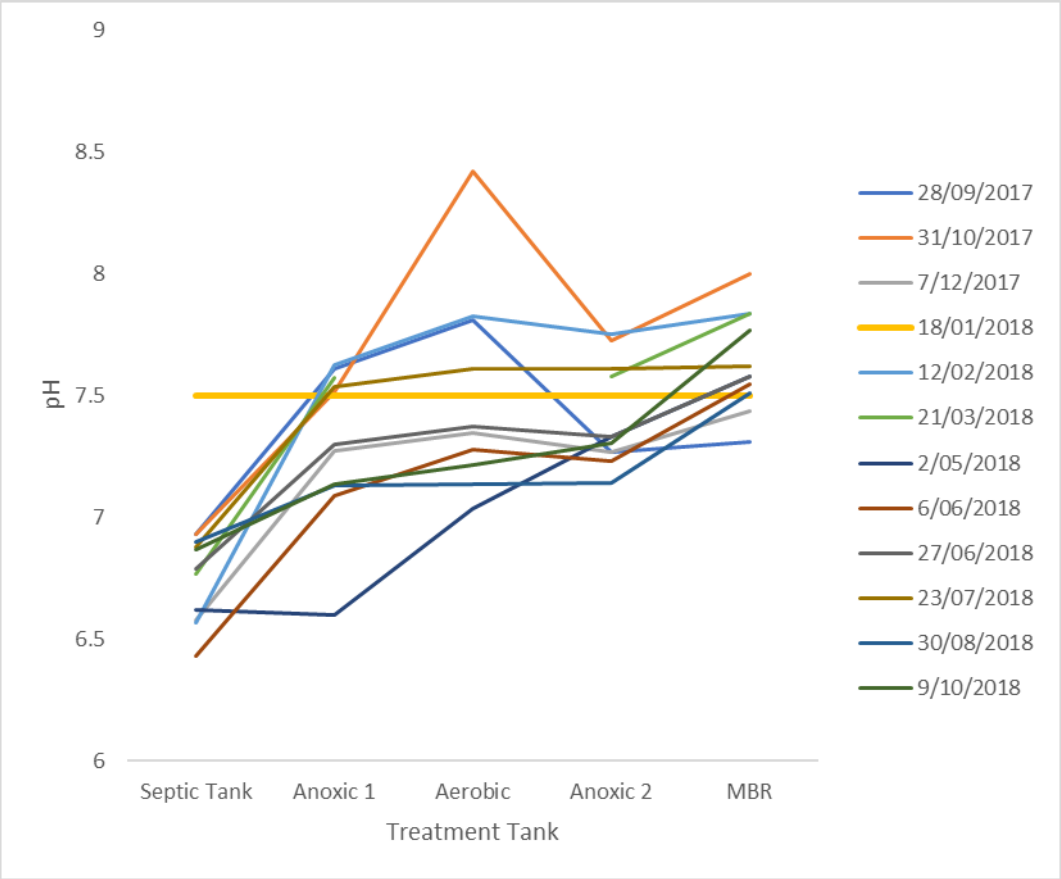

Figure 16. pH levels over the course of the year.

Phosphorous is measured during the process as it is necessary to be removed during the treatment process. There are two types of phosphorous measured in WWTP, Total phosphorous (TP) and dissolved reactive phosphorous (DRP) (Figure 17), which is the biologically available phosphorous, both generally follow a pattern of releasing phosphorous in the anoxic tanks and absorption of phosphorous in the aerobic tanks. However, when the EBPR was deemed inefficient at removing phosphorous, alum was added to the WWTP to lower the

phosphorous in the final effluent.

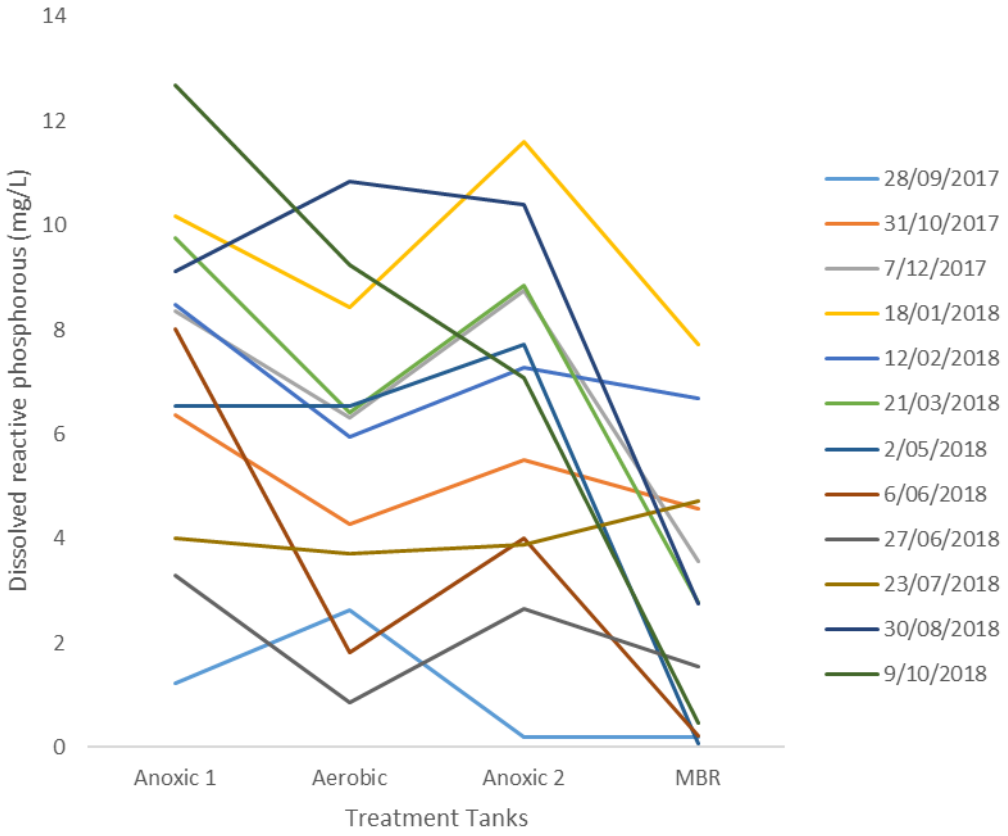

Figure 17. DRP across treatment tank between September 2017 and October 2018.

The different types of nitrogen that were measured in this study were ammonium, nitrite, and nitrate. The majority of nitrogen in the septic tank was ammonium, with the highest concentration being 57.2 mg/L and the average concentration being 26.38 mg/L. Ammonium shows a drop as the effluent enters the aerobic tanks but has peaks in the second anoxic tank (Figure 18).

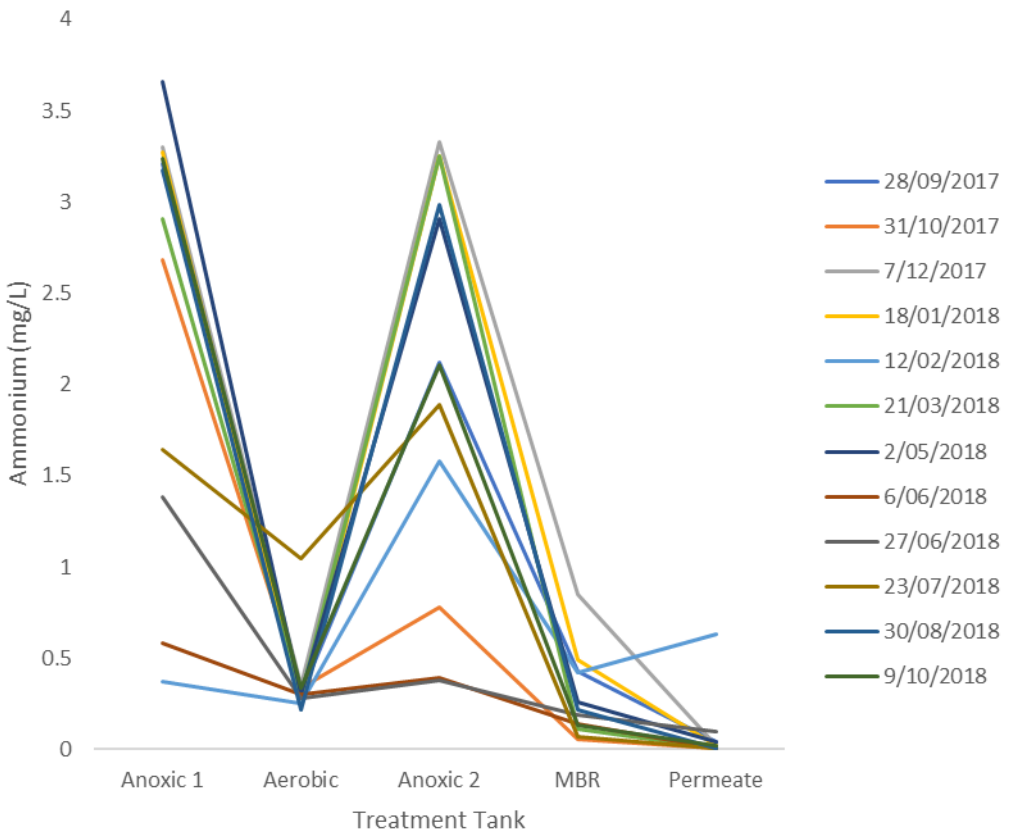

Figure 18. Ammonium concentration across the treatment tanks between September 2017 and October 2018. The Post-septic tank was removed, due to being before treatment and as such skewed the data.

The lower ammonium in the aerobic MBR tank was correlated with an increase in nitrite in the same tank (Figure 19). The resulting final effluent was significantly reduced in ammonium with an average concentration of 0.07 mg/L. The nitrite is highest before entering the treatment plant, with an average of 0.17 mg/L. The nitrite lowers to 0.02 mg/mL as it enters the treatment tanks, due to dilution from the RAS internally recycled wastewater and remains low in all of the treatment tanks. There is a peak of in the MBR tank during August and September where it reached 0.45 and 0.23 mg/L respectively.

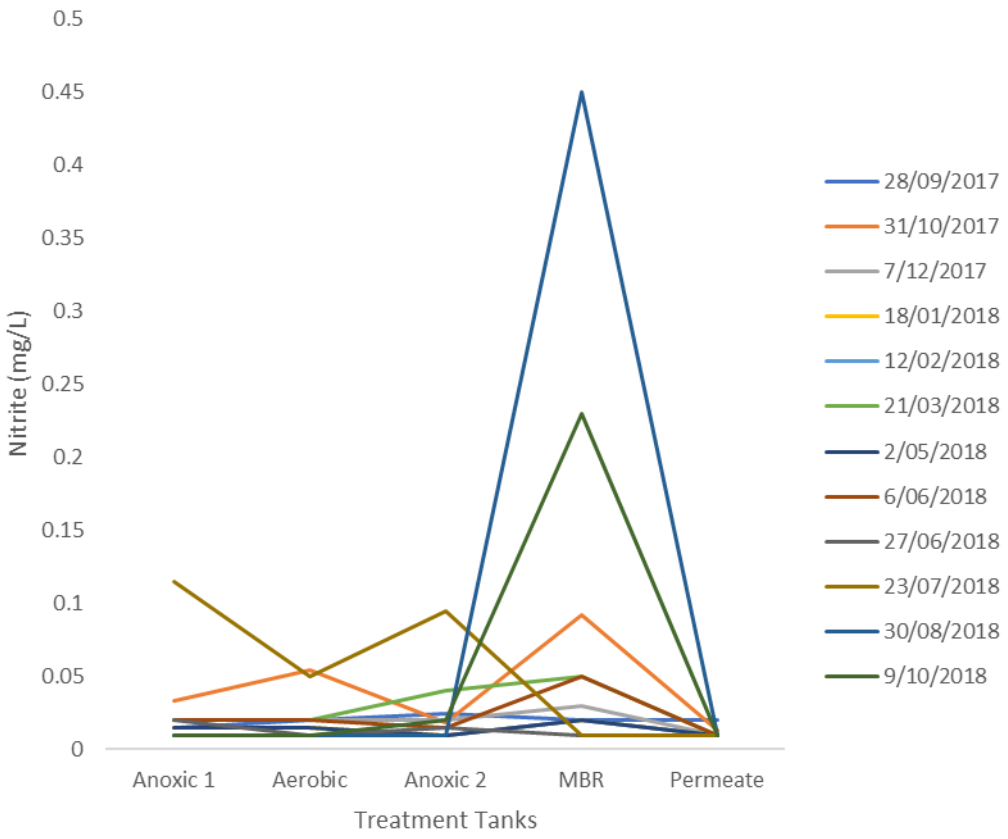

Figure 19. Nitrite concentration across the treatment tanks between September 2017 and October 2018.

The nitrate levels entering from the septic tank varied between months with the minimum concentration being 0.26 mg/L in June and the highest being a concentration of 3.55 mg/L during August. Unlike the nitrite and ammonium, the concentration of nitrate in the RAS were similar to the influent nitrate concentration. The nitrate was seen to increase in the MBR tank where it was most aerobic (Figure 20), there was little decrease after the MBR tank, with

nitrate levels being higher in the final effluent than the starting influent.

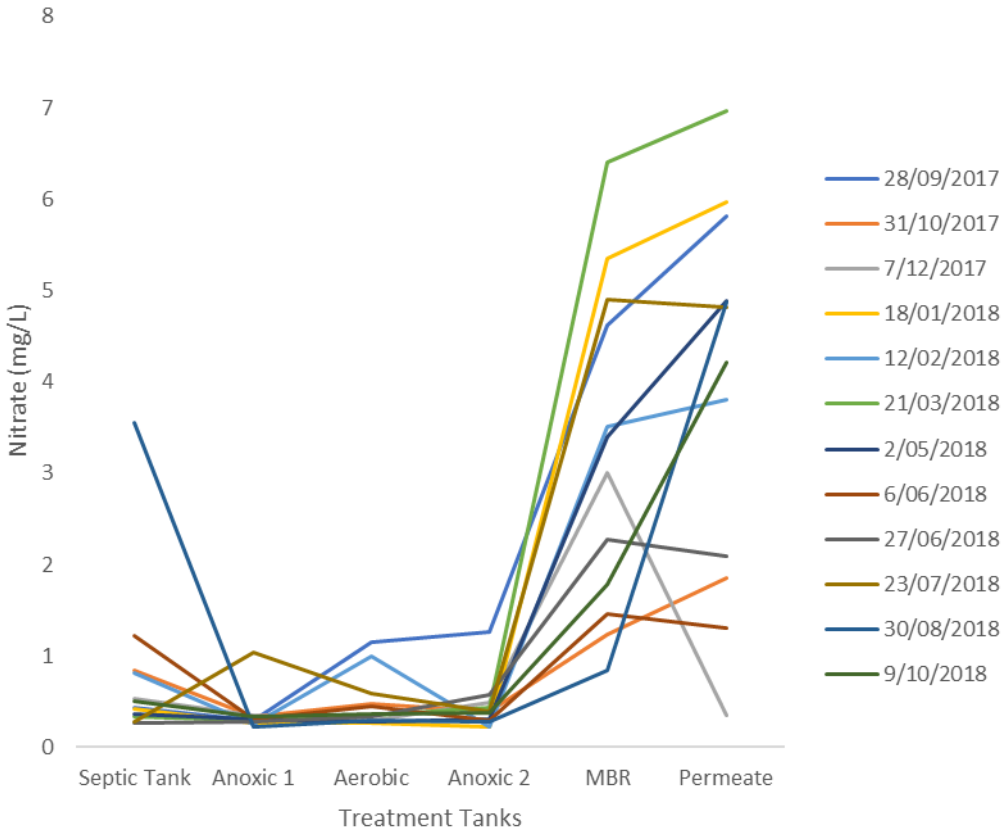

Figure 20. Nitrate concentration across the treatment tanks between September 2017 and October 2018.

Temperature changed across the tanks over the year (Figure 21) with the lowest temperature been 14.7°C and the highest 24°C. The septic tank showed less variation in temperature than the treatment tanks with a standard deviation of 2.47 degrees and a low temperature of 14.7°C in June and a high of 21.2°C in February. The lowest temperatures were between June and August, while the highest temperatures were between December and March. However, the difference in temperature across tanks each month was low.

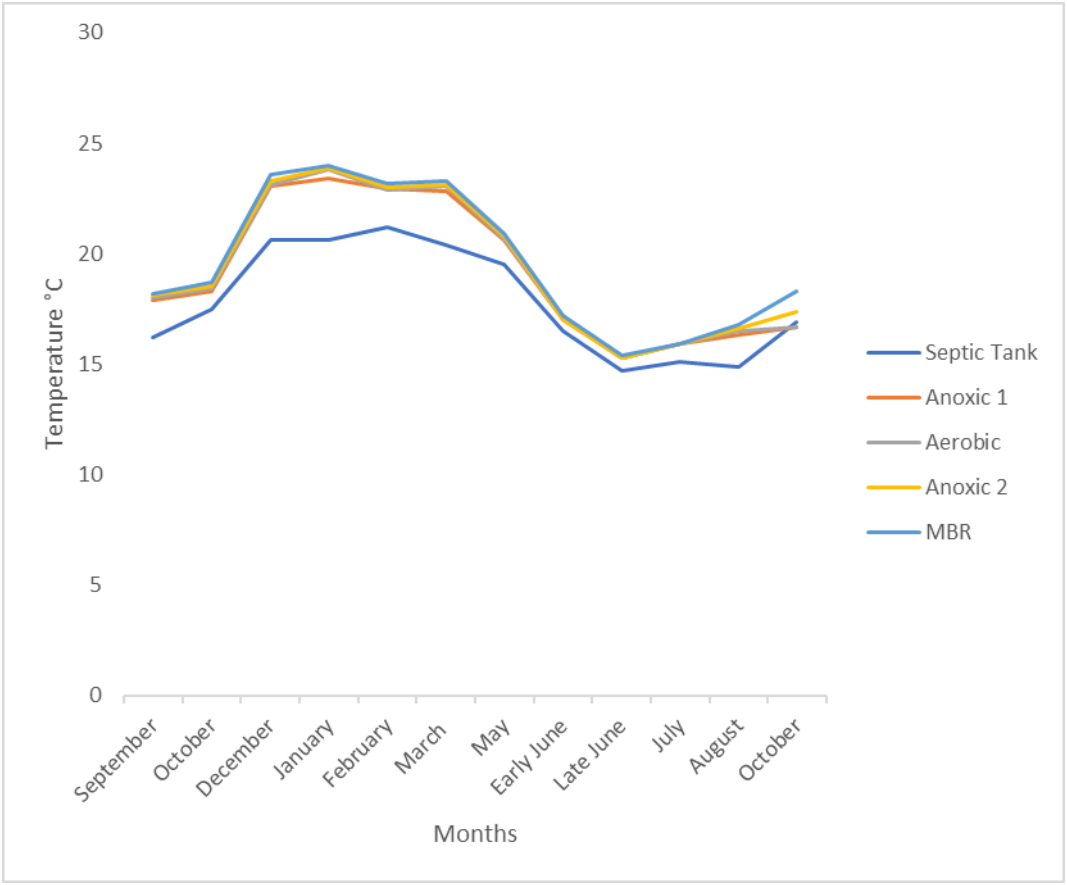

Figure 21. Temperature variation across the months during the study period.

Sequencing results

Sequencing resulted in a minimum read count per sample of 10979, and a maximum count of 64894 reads. A total of 33 phyla were observed from the 48 samples and annotated through the RDP database (Wang et al., 2007) (Figure 24), with approximately 30% of the OTUs classed as unknown. This data also showed that over the course of the year there were two phyla which showed the highest relative abundance in the WWTP. Analysis using a rarefied OTU matrix rarefraction curve shows that that almost all the diversity in the WWTP plant was observed (Appendix: Figure 41).

Graphs analysing Phylum level abundance were created using the RDP database (Figure 22)**.** In this study, Proteobacteria was shown to be the most abundant phylum in all samples with an average abundance of 27% correlating with 831 OTUs. Bacteroidetes was the other dominant phylum in this study with an average abundance of 25% and correlating with 535 OTUs. In the treatment tanks (The anoxic 1, aerobic, anoxic 2, and MBR tank), Proteobacteria and Bacteroidetes were the two major phyla with a relative abundance of approximately 25% for each phylum. While, the post-septic samples had three major phyla; Proteobacteria, Bacteroidetes, and Firmicutes at a relative abundance of 34%, 29%, and 28% respectively. Specifically, in the aerobic tank (the second tank in the treatment), the two major phyla were of similar abundance with Proteobacteria between 21 and 27%, with an average abundance of 25%. While, the abundance of Bacteroidetes was between 18 and 29%, with an average of 24%. The samples, when grouped by similarity, showed that all but the septic tanks had a strong correlation to both the sequencing run and time of their PCR (Figure 28).

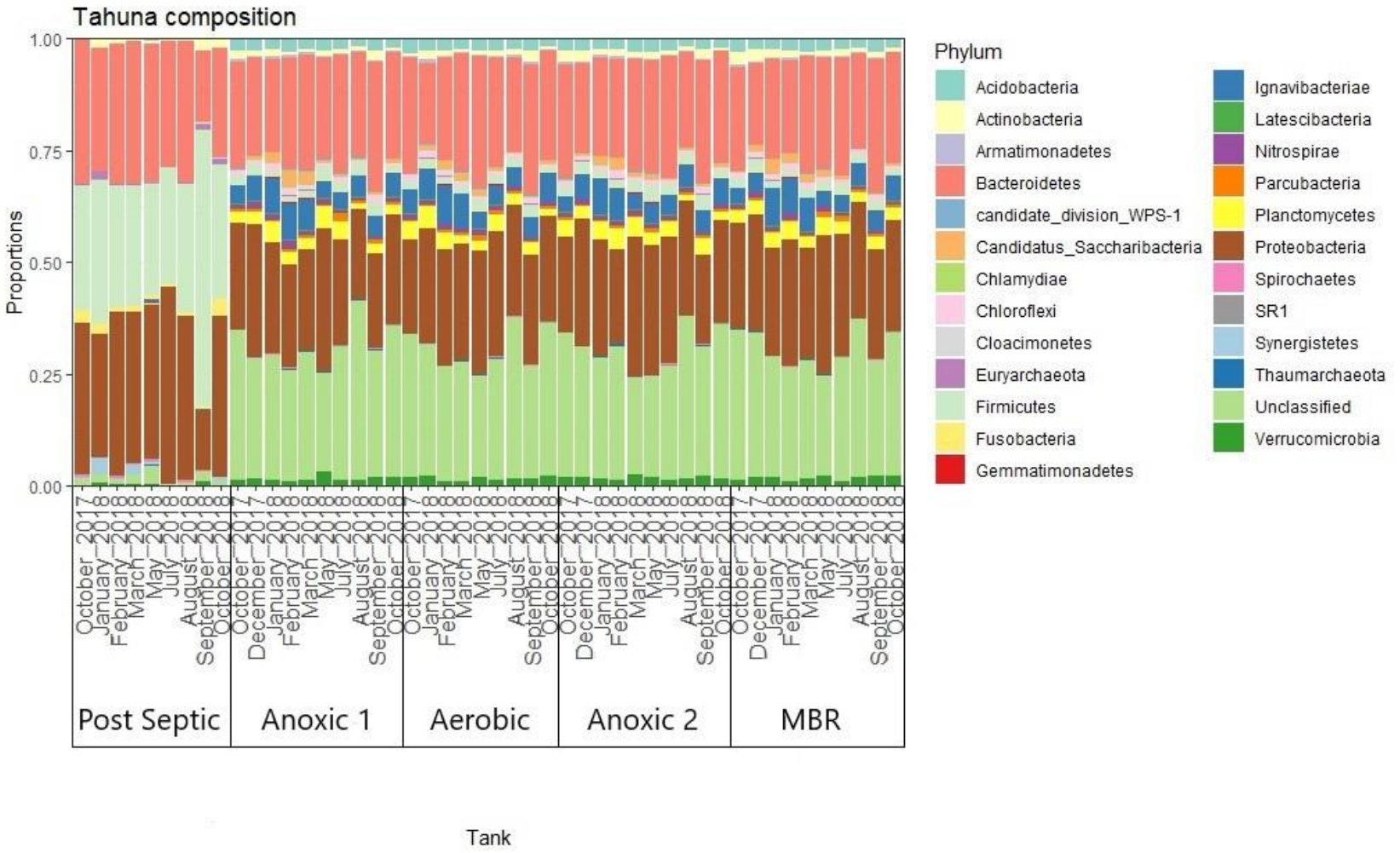

Figure 22. Phylum level relative abundance of the Tahuna wastewater treatment plant. Taxonomy was classified using the RDP database and graphed using phyloseq and ggplot2 in R studio.

At the class level sequencing revealed the microorganism with the most relative abundance on average in the treatment tanks was Sphingobacteriia (Figure 23**)**, the only representative of the Bacteroidetes above 1% at the class level. Sphingobacteriia had a relative abundance between 7 and 19% and had its lowest relative abundance in December (7-8%) and its highest in September (18-19%).

Proteobacteria diversity was correlated with five classes; Betaproteobacteria, Alphaproteobacteria, Deltaproteobacteria, Gammaproteobacteria, and Epsilonproteobacteria. Betaproteobacteria was the most abundant class after Sphingobacteriia with a relative abundance between 7-13%. The lowest abundance being recorded during the months of September and October where the abundance was 7-10%, while the highest abundance was seen during March and May where it was 10-13%. Gammaproteobacteria had a relative abundance of 5-9%, Alphaproteobacteria (4-8%), Deltaproteobacteria (1-2%), and Epsilonproteobacteria (0-1%).

In the treatment tanks there were four subgroups of Acidobacteria, group 3, 4, 7, and 17. In order from lowest abundance to highest the average abundance was, Acidobacteria_Gp7 (0.17- 0.67%), Acidobacteria_Gp17 (0.15- 0.51%), Acidobacteria_Gp3 (0.28- 0.82%), and Acidobacteria_Gp4 (0.53- 1.23%). The remaining classes were as follows Bacilli (0.05% to 0.24%), Fusobacteriia (0.01- 0.27%), Erysipelotrichia (0.06- 0.35%), Subdivision3 (0.09- 0.44%), Negativicutes (0.03- 0.57%), Caldilineae (0.14- 0.67%), Flavobacteriia (0.07- 0.87%), Anaerolineae (0.33- 1.31%), Nitrospira (0.43- 1.79%), Clostridia (0.57- 2.38%), Verrucomicrobiae (0.32-2.61%), Bacteroidia (0.09-2.69%), Actinobacteria (0.57 -2.97%), Planctomycetia (1.27 -5.07%), Cytophagia (3.06 -7.11%), and Ignavibacteria (3.43-8.72%).

At the class level, the relative abundance changed across the months with an average of 3.63%. The greatest relative change across the months was 14.07% with the Epsilonproteobacteria changing from 0. 79% in July to 0.056% in

September 2018. Meanwhile, the lowest relative change across the months was 1.41% with Betaproteobacteria, which changed from 8.4% in October 2017 to 11.8% in May 2018. The largest change across the months was from Sphingobacteria rising from 7.2% in December 2017 to 18.6% in September, an increase of 11.6%. The smallest change was from Bacilli a change from 0.081% in January to 0.19% in September 2018, a change of 0.11%.

The change across the months was greater than the change across the individual tanks each month. The individual tank average relative change was 1.77%. However, the largest relative change across the tanks was larger than the change across the months was Epsilonproteobacteria in May with a low of 0.085% and a high of 1.938%, an increase of 22.71%. The smallest relative change across the tanks was from Acidobacteria_Gp3 during December 2017 at 1.046%.

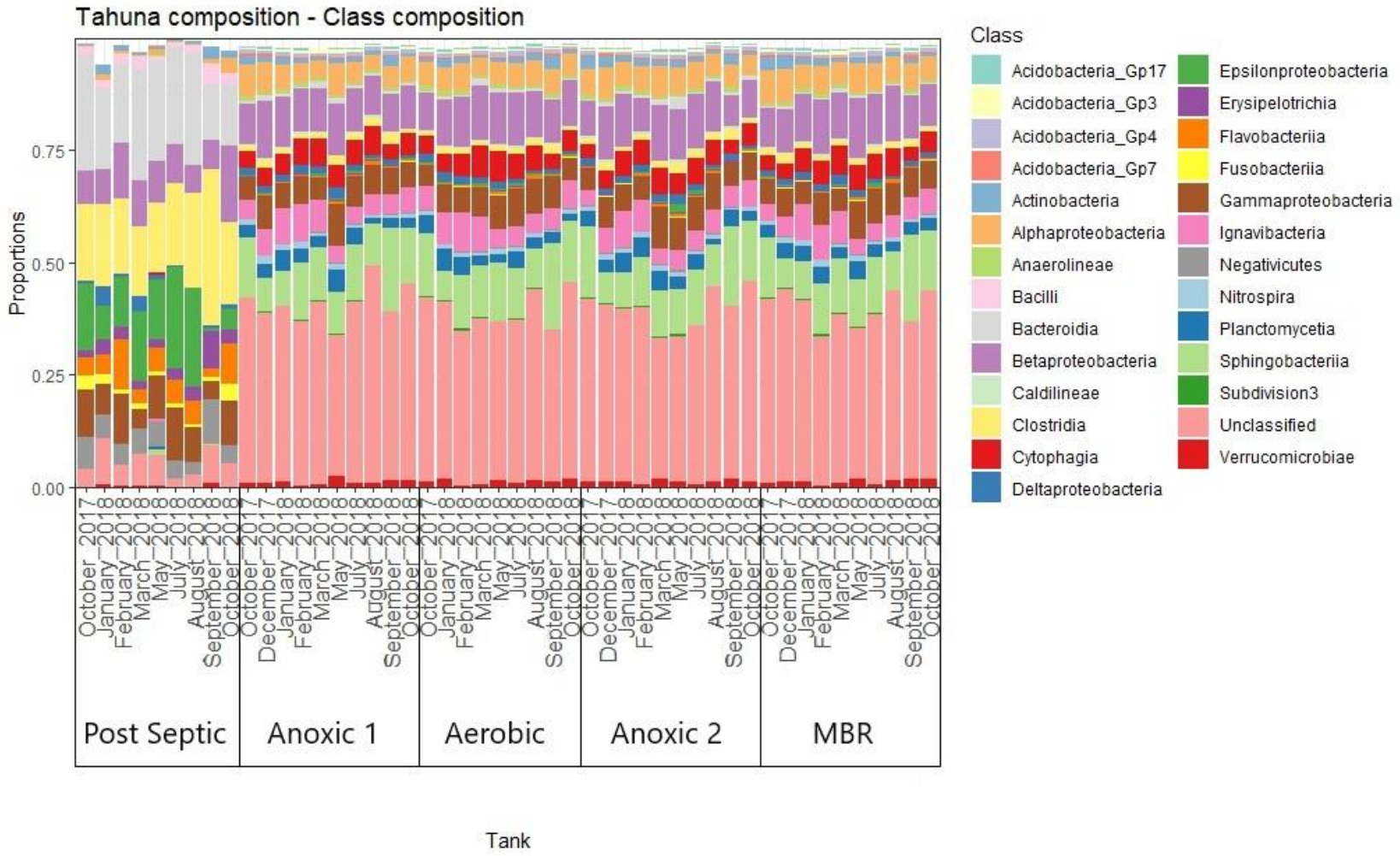

Figure 23. Class level relative abundance of the Tahuna wastewater treatment plant. Taxonomy was classified using the RDP database and graphed using phyloseq and ggplot2 in R studio.

Taxonomy utilising the RDP database (Wang et al., 2007) was analysed using heatmaps (Figure 24 and Figure 25) and boxplots (Figure 26) showed that there was a strong difference between the post-septic tanks and the treatment tanks. Although, all the tanks contained the same microorganisms the difference in relative abundances was vastly different between the treatment and post-septic tanks. In the post-septic samples, only 4 to 7.6% of the relative abundance of microorganisms could not be identified, this was vastly different from the treatment tanks where the unclassified microorganisms were 19.3 to 23.3%.

At the phylum level, all the tanks both shared a high abundance of Bacteroidetes and Proteobacteria. Proteobacteria had an average relative abundance of 25 to 26% in the treatment tanks and 34% in the post-septic tanks. Bacteroidetes had an average relative abundance of 23 to 24% in the treatment tanks and 29% in the post-septic. The most abundant Proteobacteria in the treatment tanks were an unclassified betaproteobacteria (3.3 – 6%), Rhizobiales from Alphaproteobacteria (3.2 – 4.8%), and an unclassified Gammaproteobacteria (2.7 – 4%). These abundances differed from the septic tanks which were unclassified betaproteobacteria (0.7 – 2.6%), Rhizobiales (0.3 – 0.7%), and unclassified Gammaproteobacteria (0.2 – 0.4%). The most abundant Proteobacteria in the post-septic samples were Burkholderiales from Betaproteobacteria (2.6 – 5%).

The most abundant class of Bacteroidetes in the post-septic samples was Bacteroidea with an abundance of 14 to 21.9%, in the treatment tanks Bacteroidea had less abundance at 6.9 to 10.9%. In the treatment tanks, Sphingobacteria was the most abundant class in Bacteroidetes with an abundance of 6.9 to 9.8% compared to 0.4 to 2.5% in the post-septic tanks.

There were two microorganisms which showed the highest relative abundance in the post-septic tanks was Clostridia and Bacteroidea. The highest relative abundance in the post-septic treatment tanks was the class Clostridia from the phylum Firmicutes at 19 to 28.5%.

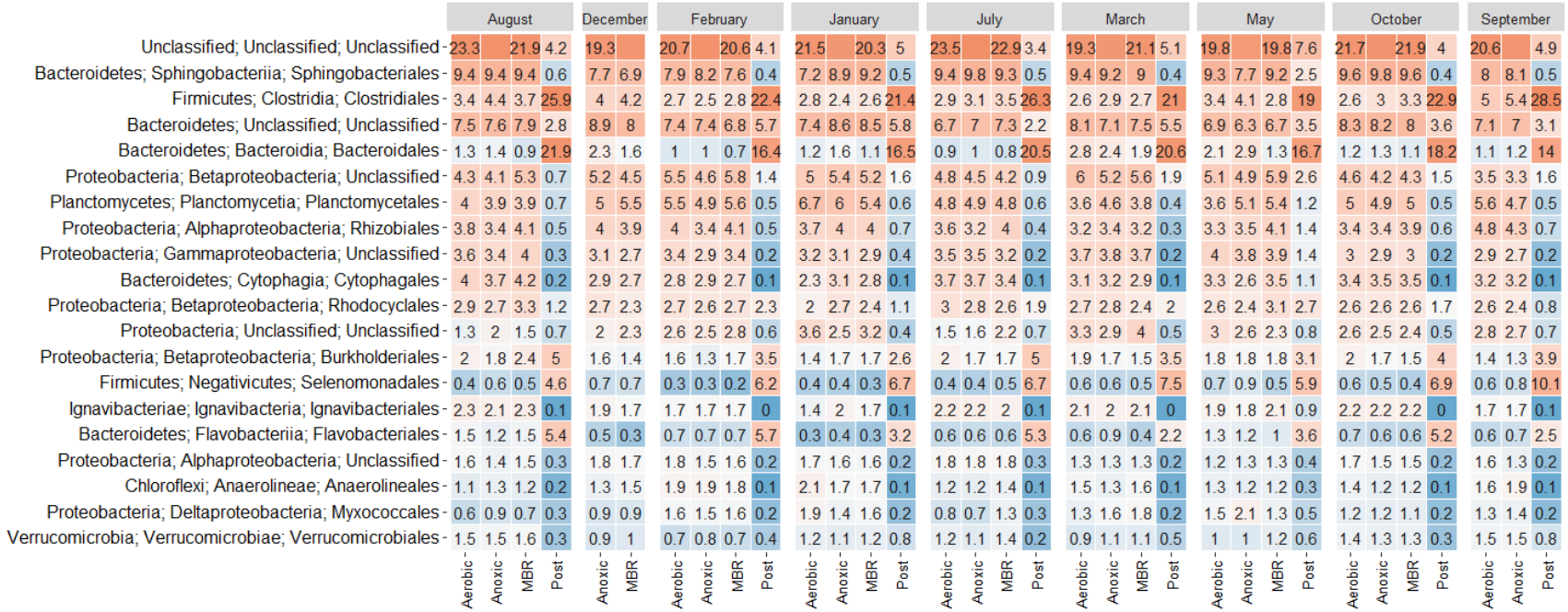

Figure 24. Heatmap of the 20 most abundant microorganisms ordered by tank and month.

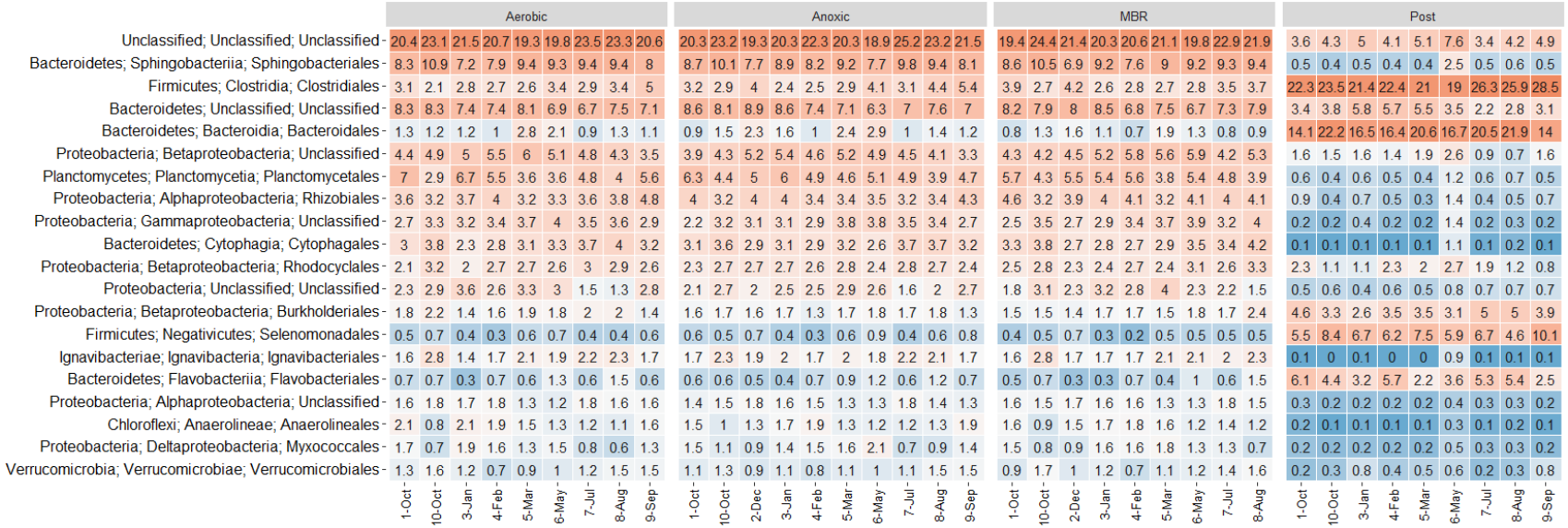

Figure 25. Heatmap of the 20 most abundant class ordered by date and tank.

Figure 26. Boxplot of the relative abundance classes in all tanks .

Using the MIDAS database, a heatmap with the treatment tanks but without the post-septic samples was created with the 40 most abundant organisms (Figure 27). It is of note that the MIDAS database occasionally used slightly different nomenclature to the RDP database. Disregarding the unclassified organisms, the highest relative abundance was Candidatus CU923752. The next abundant organism was from the phylum Chlorobi and the class Chlorobia, named under the MIDAS database as K2-30-37 was between 5.2 and 5.7%. The next abundant organism was from the phylum Bacteroidetes and from the class Sphingobacteriia, referred to as QEDR3BF09 and had between 3.4 and 4% relative abundance. Candidatus 'competibacter' from Class Gammaproteobacteria was the next abundant with 3.2 to 3.4%, its alternative name being CPB_S60, this was also positively correlated with being a GAO. *Dechloromonas* had an abundance between 2.6 and 2.9%, this genus belongs to the class Betaproteobacteria and order Rhodocyclales and has been shown to function as a PAO. The last of the most relative abundant organisms belong to *Terrinmonas* at 2.3 to 2.6% abundance, and an organism from the family Cytophagaceae which had an abundance between 2.2 and 2.5%.

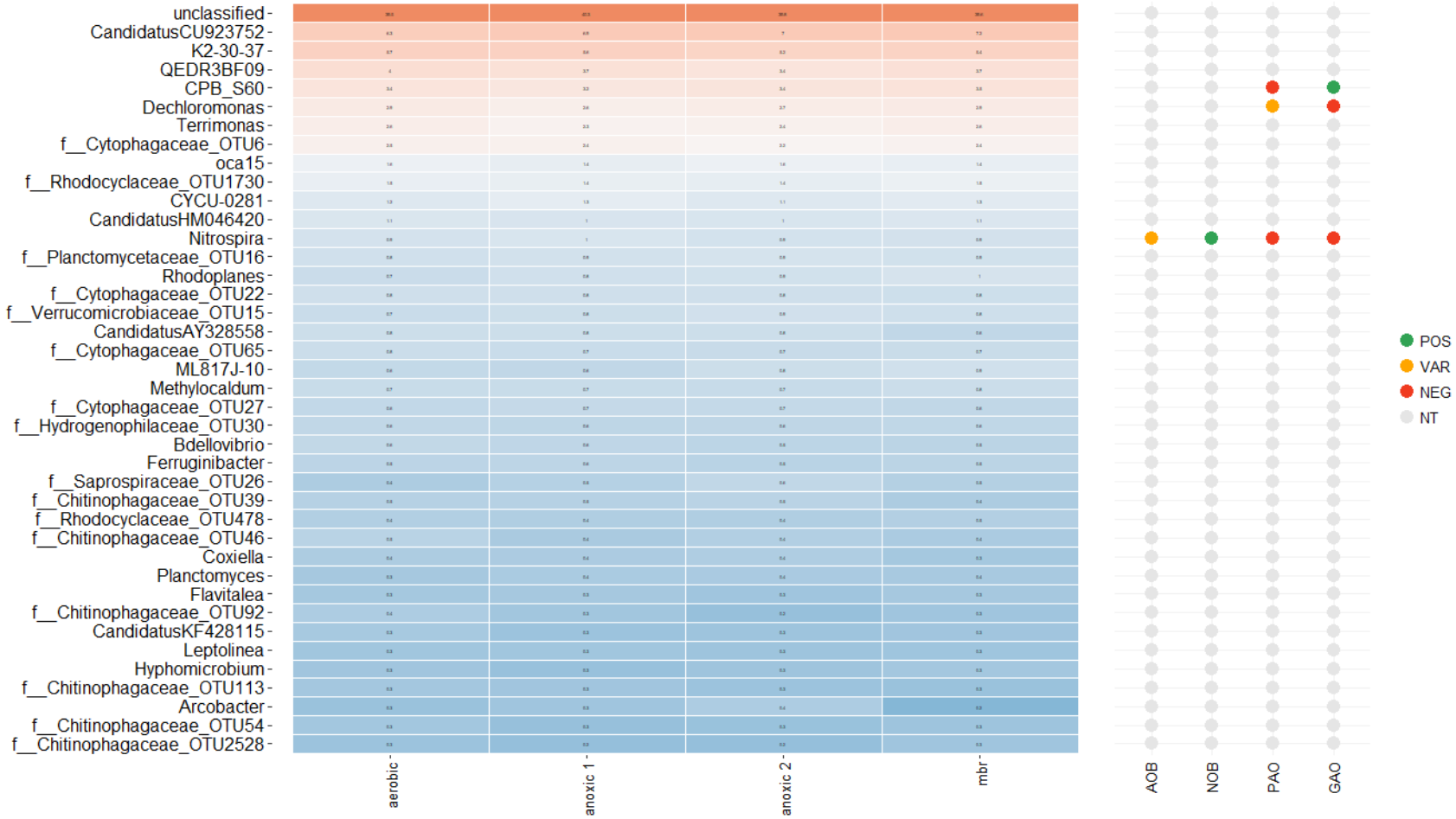

Figure 27. Heatmap using MIDAS database showing the 40 highest relative abundance without the post-septic tank The heatmap shows dark orange for higher abundance and light blue for low abundance.

Graphs analysing differences across the samples were used to find any useful correlation. Hierarchal clustering showed four clusters (Figure 28), the first cluster is by post-septic tanks. The next cluster was grouped by the month of July and August. The smallest cluster consisted of two samples, one from anoxic tank 1 during May and the second was an aerobic tank during January. The final cluster was all the remaining samples.

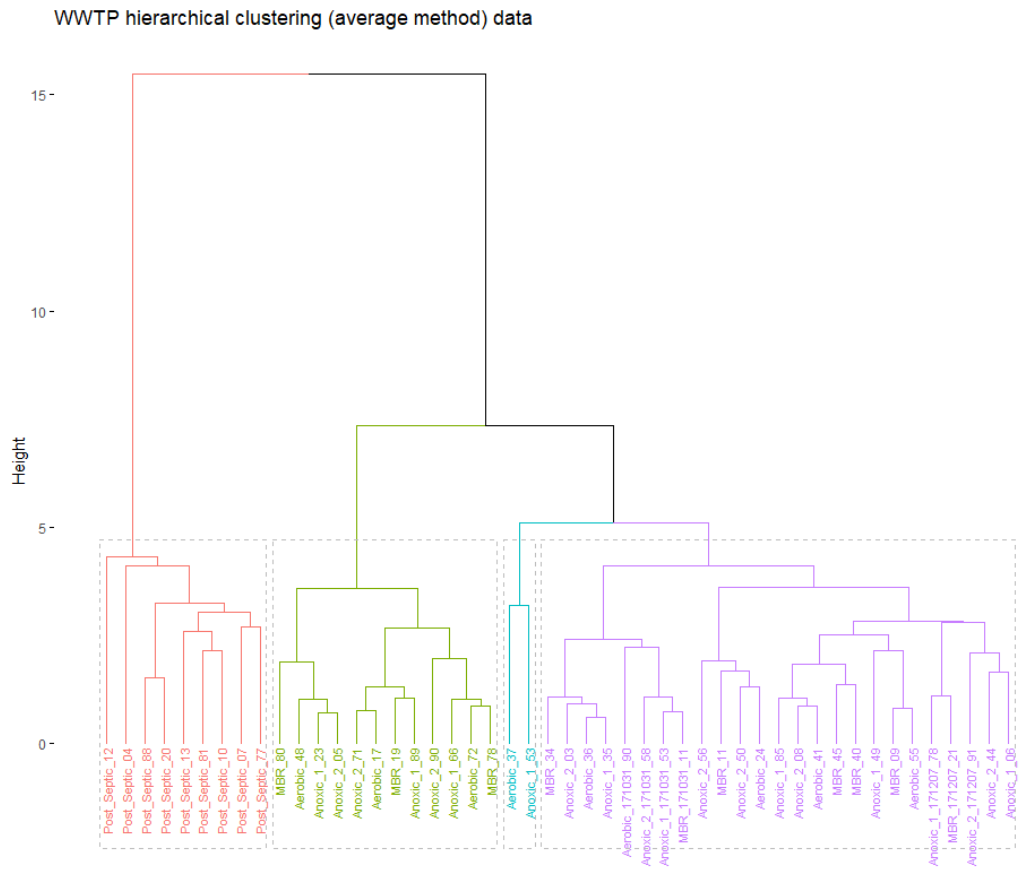

Figure 28. Hierarchical clustering graphing showing four separate groups clustered together.

Graphs were created that compared the differences in the tanks to the parameters of interest. PCR date and the month the samples were collected were compared to the differences in the tanks (Figure 29, Appendix: Figure 38, Figure 39, & Figure 40). There is some similarity between PCR date and a strong similarity across sequential months. However, the different tanks in either month

or PCR date showed little difference to each other (Figure 30), with no strong correlation with the sequencing run, but there was a correlation with date.

The graph does show that there is a strong correlation to PCR date and month of sample collection. The second MDS/PcoA graph (Figure **31**) shows correlation of differences in the samples to tank type. However, there is a correlation to the date of the samples. The last two principal component analysis graphs both show the differences between tanks when compared to the date they were sampled. However, one graph has the addition of the post-septic samples (Figure 31) while the other shows the differences only between treatment tanks (Figure 32).

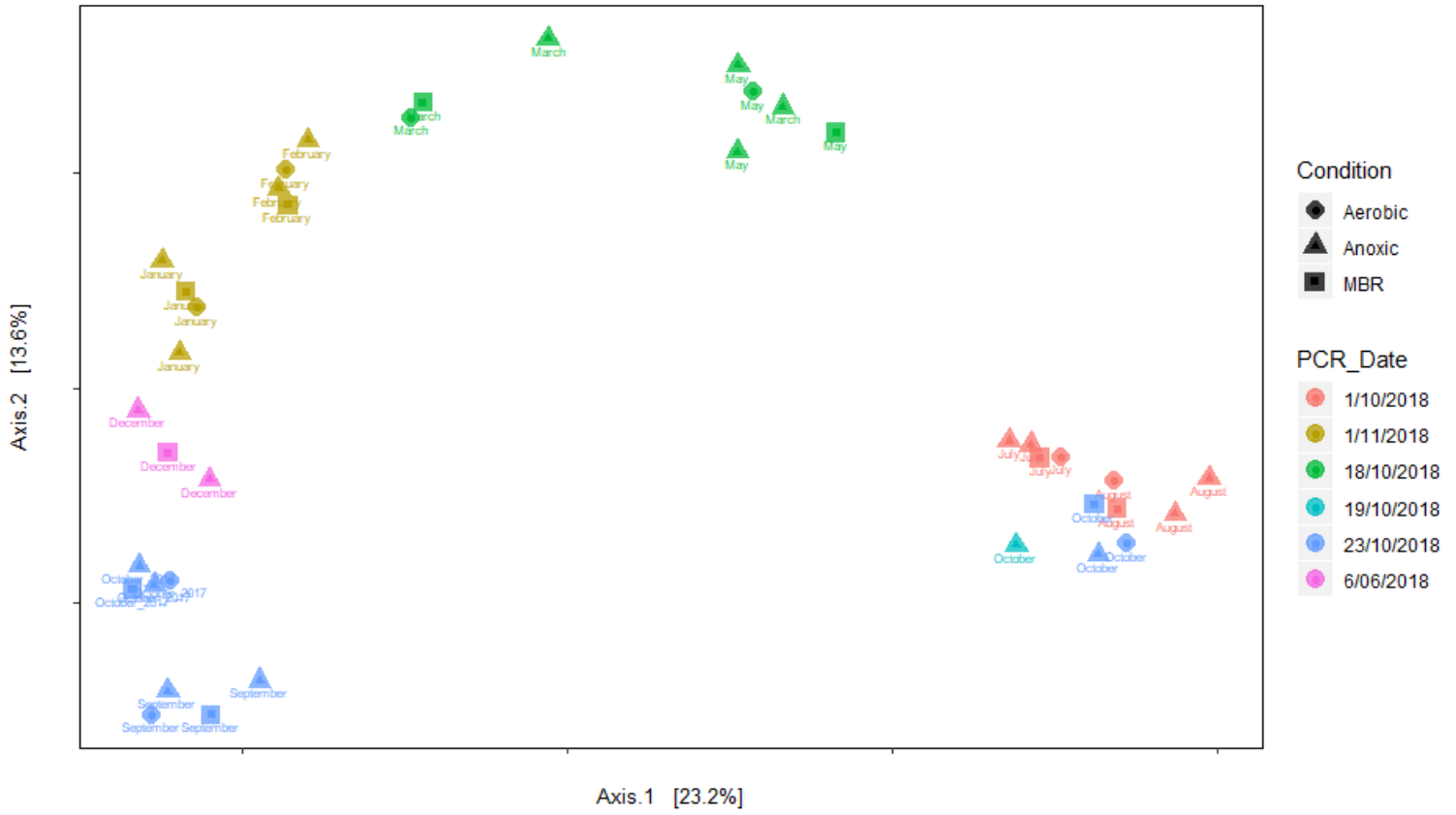

Figure 29. MDS/PCoA graph correlating difference in treatment tanks alongside the date of PCR.

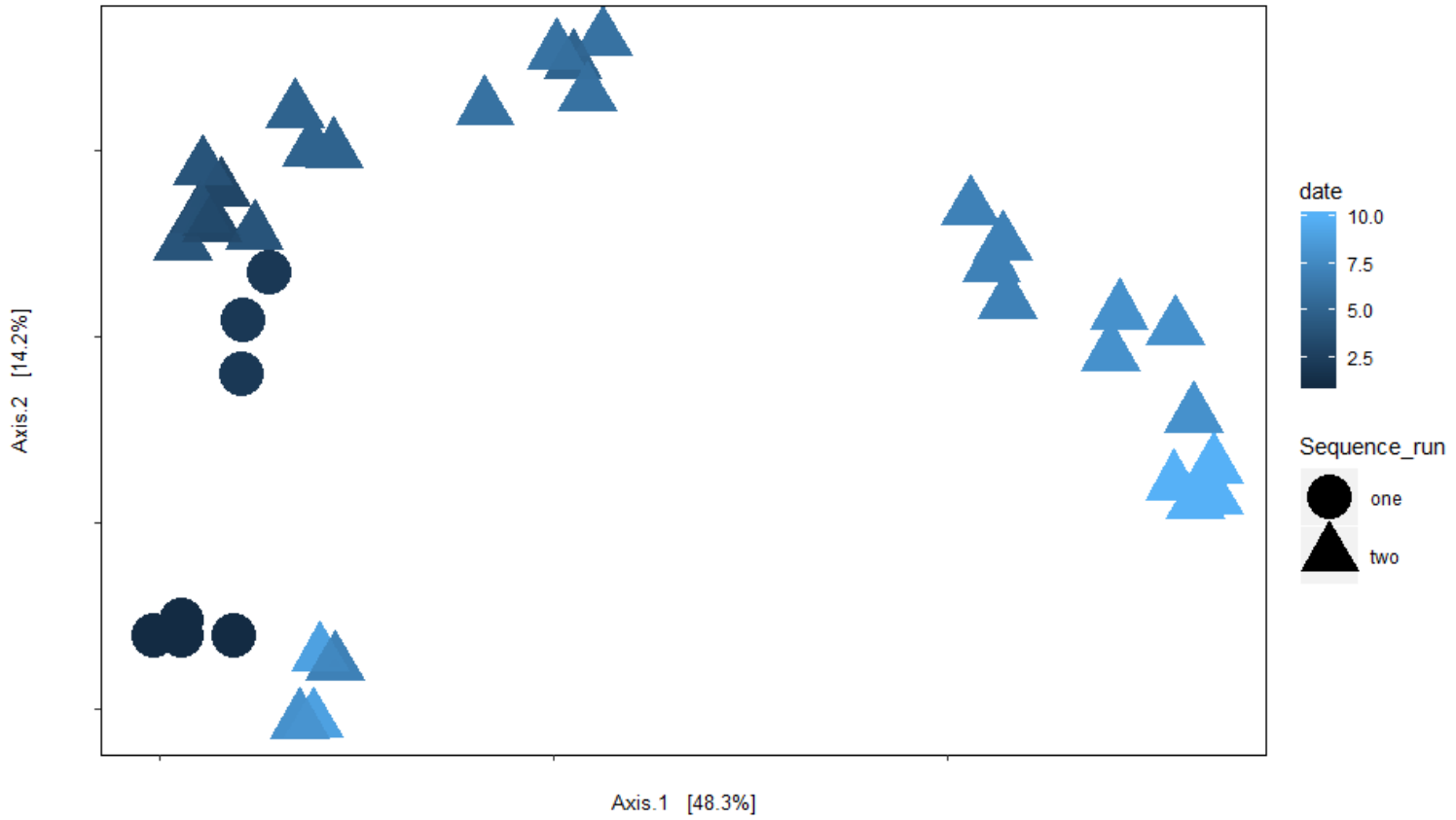

Figure 30. MDS/PCoA on Weighted-UniFrac distance, rarefied without replacement without condition Post-septic.

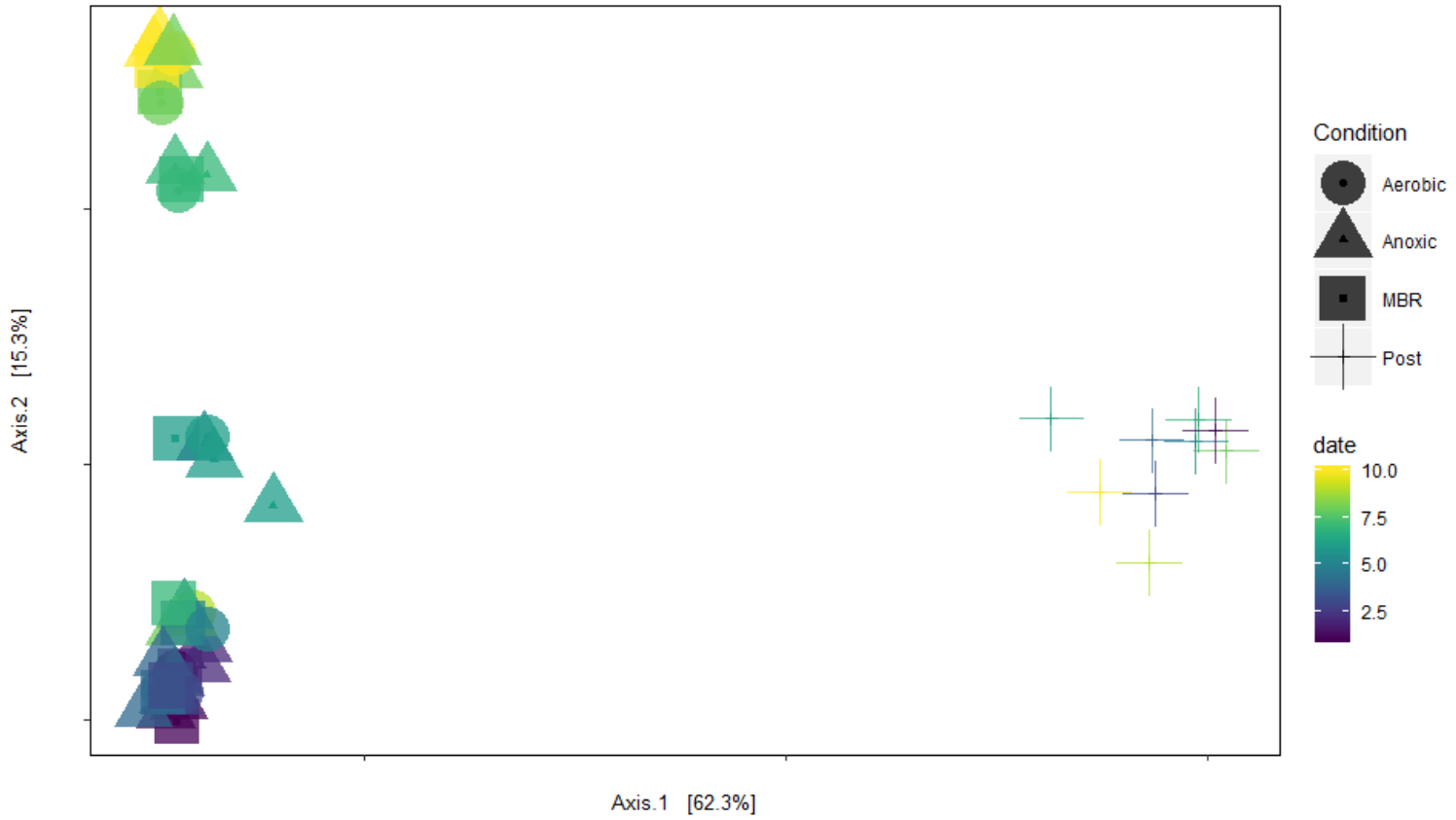

Figure 31. MDS/PCoA graph correlating difference in treatment tanks alongside sample date.

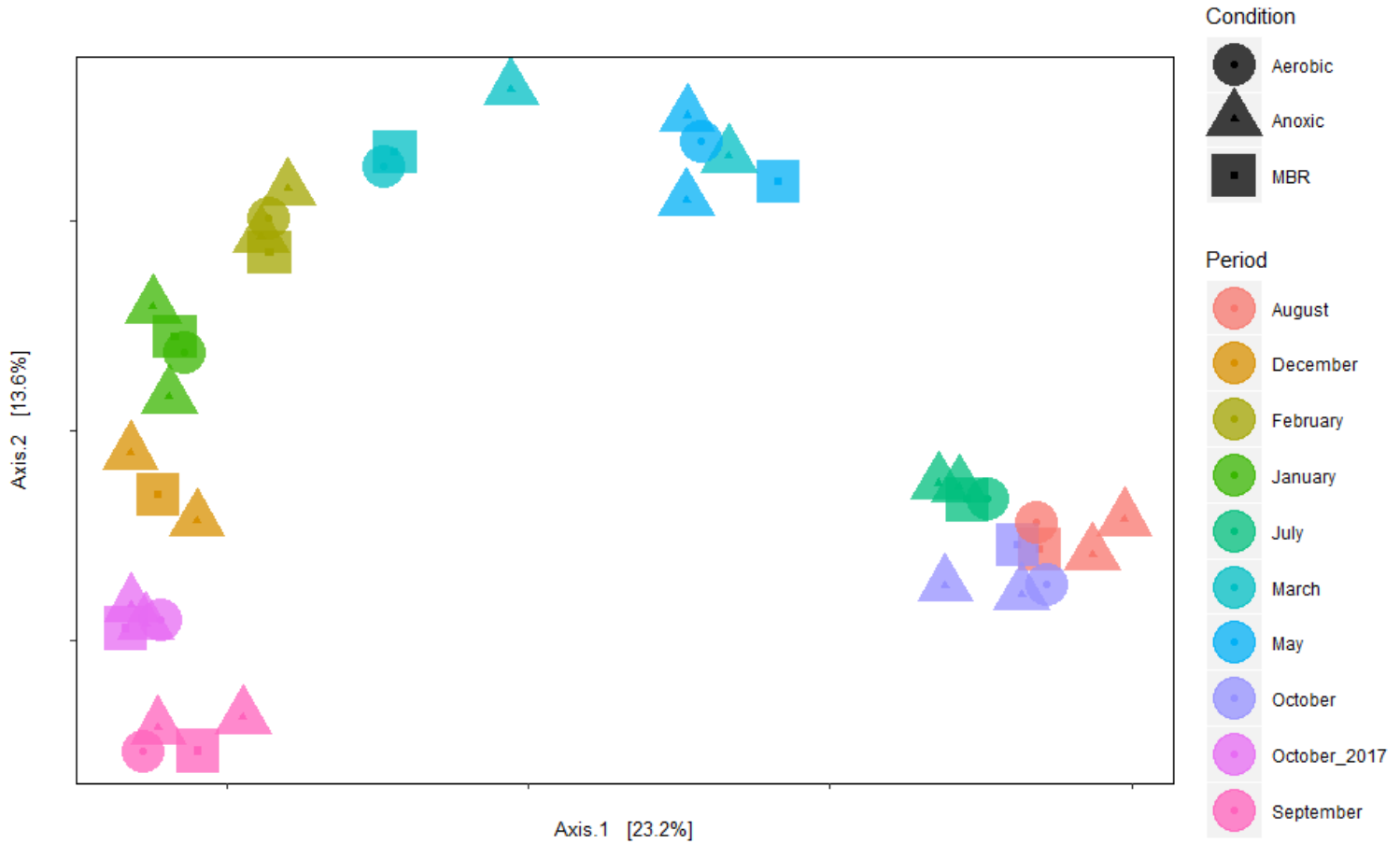

Figure 32. Principal component analysis plot which displays months of samples as different colours and tank conditions as shapes.

The MIDAS database is dedicated to wastewater treatment systems and has microbial functional information specific to wastewater treatment. This database was used to form a taxonomic table which also contained functional information. This data revealed 17 genera that correlated with functional groups related to wastewater, either ammonia oxidation, nitrite oxidation, phosphorous accumulation, and glycogen accumulation, as well as information about filamentous bacteria (shown in Figure 33).

The genera were CPB_S60, *Nitrospira*, *Leptolinea*, *Defluviicoccus*, *Nitrosomonas*, CCM19a, *Anaerolinea*, *Turicibacter*, *Catenibacterium*, *Fodinicola*, *Propionivibrio*, *Tetrasphaera*, spb280, *Thiothrix*, *Gordonia*, *Skermanina*, and *Micropruina* (Figure 33). These genera were not all linked to individual OTUs, instead, the 17 genera were comprised of 54 OTUs.

CPB_S60 was the most abundant of the bacteria with a known function of interest. CPB_S60 along with *Defluviicoccus,* CCM19a, *Propionivibrio,* spb280, and *Micropruina* are all known GAOs. *Nitrospira* was the only NOB discovered in the study. *Nitrospira* is also indicated as a possible AOB, this is due to some studies showing strains of *Nitrospira* were also capable of COMMAMOX, thus also functioning as an AOB.

Filamentous bacteria are linked to bulking and foaming in WWTP. In the study there were multiple filamentous bacteria, *Leptolinea* showed the most relative abundance. However, there was also *Anaerolinea*, *Turicibacter*, *Catenibacterium*, *Fodinicola, Thiothrix*, *Gordonia*, and *Skermanina* which were shown to be filamentous. *Tetrasphaera* and *Dechloromonas* were the only genera related to PAOs discovered in the WWTP.

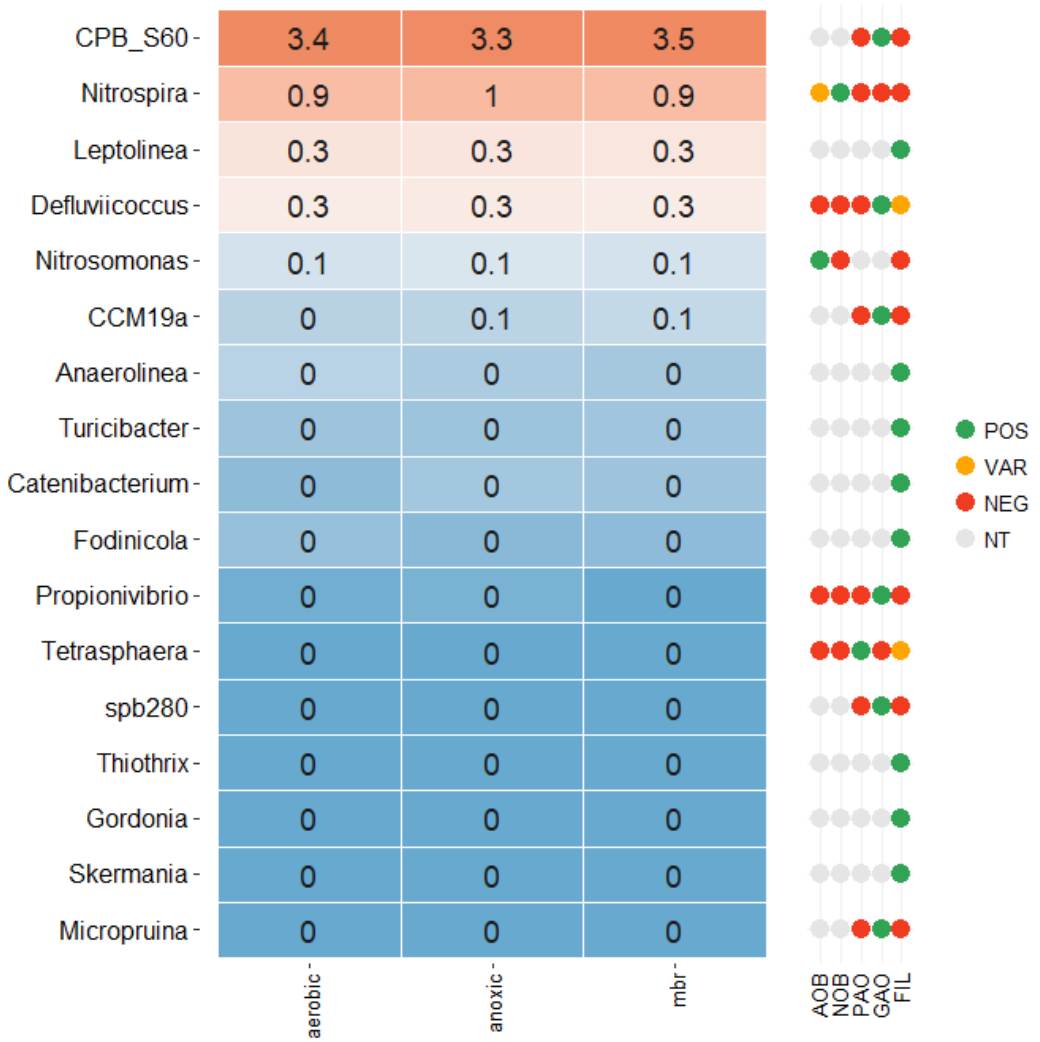

Figure 33. Relative abundance of important functional groups in the Tahuna wastewater treatment plant. POS = positive, VAR = variable, NEG = negative, NT = not assessed.

The core microbiome of the wastewater treatment tanks was analysed using R studio (amp_vis was used with the amp_core function) and the MIDAS database. The analysis revealed that 76.3% of the OTUs comprised the core microbiome of all the treatment tanks (Figure 34).

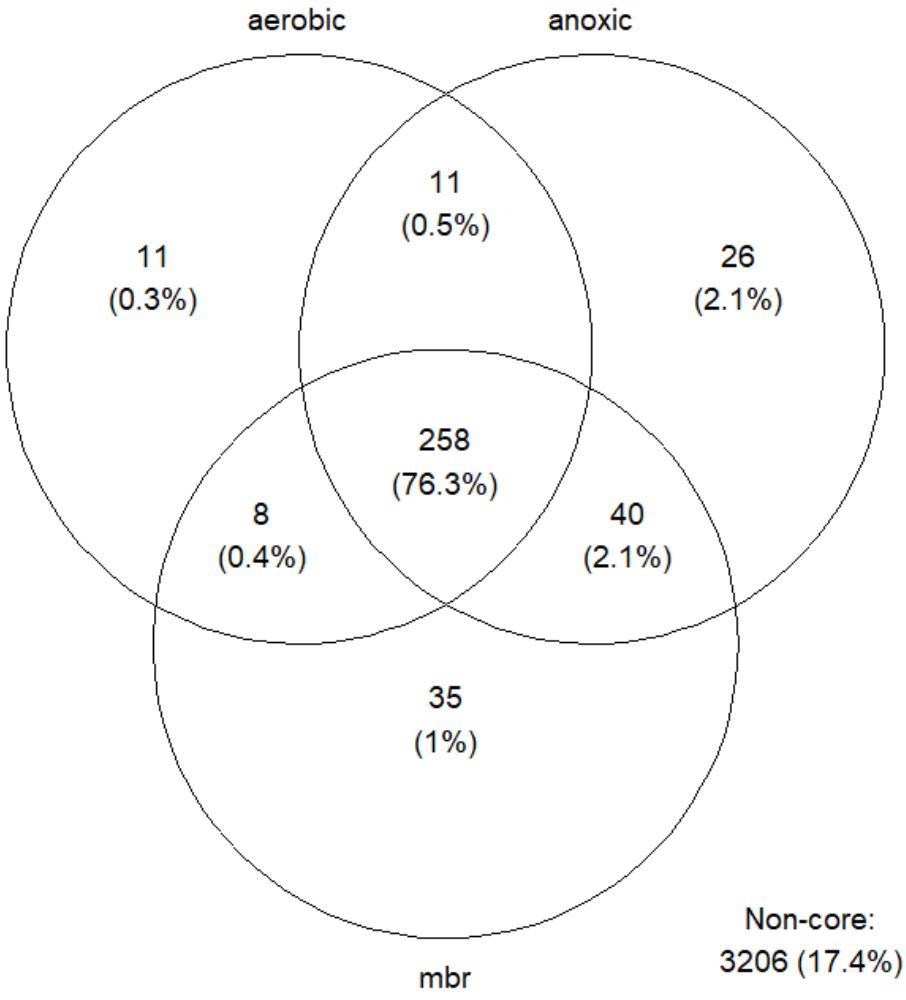

Figure 34. Venn diagram showing the percentage of OTUs in the core microbiome.

Using analysis of composition of microbiomes (ANCOM) (Mandal et al., 2015), and examining only the tanks in the treatment process (excluding the post-septic tank) only one OTU was identified as significantly different, OTU 1089 (not shown). OTU 1089 was assigned to the Chitinophagaceae family using the RDP database (Wang et al., 2007). The Chitinophagaceae family are a group of microorganisms which in wastewater treatment have been shown to be a low

DO nitrifier. Using ANCOM with both anoxic tanks being classed as one variable ANCOM presented two OTUs, OTU 1177 and OTU 3697 as significantly different (Figure 35). OTU 1177 was classified as Parcubacterium group, while OTU 3697 was classified as *Prevotella* from the phylum *Bacteroidales*.

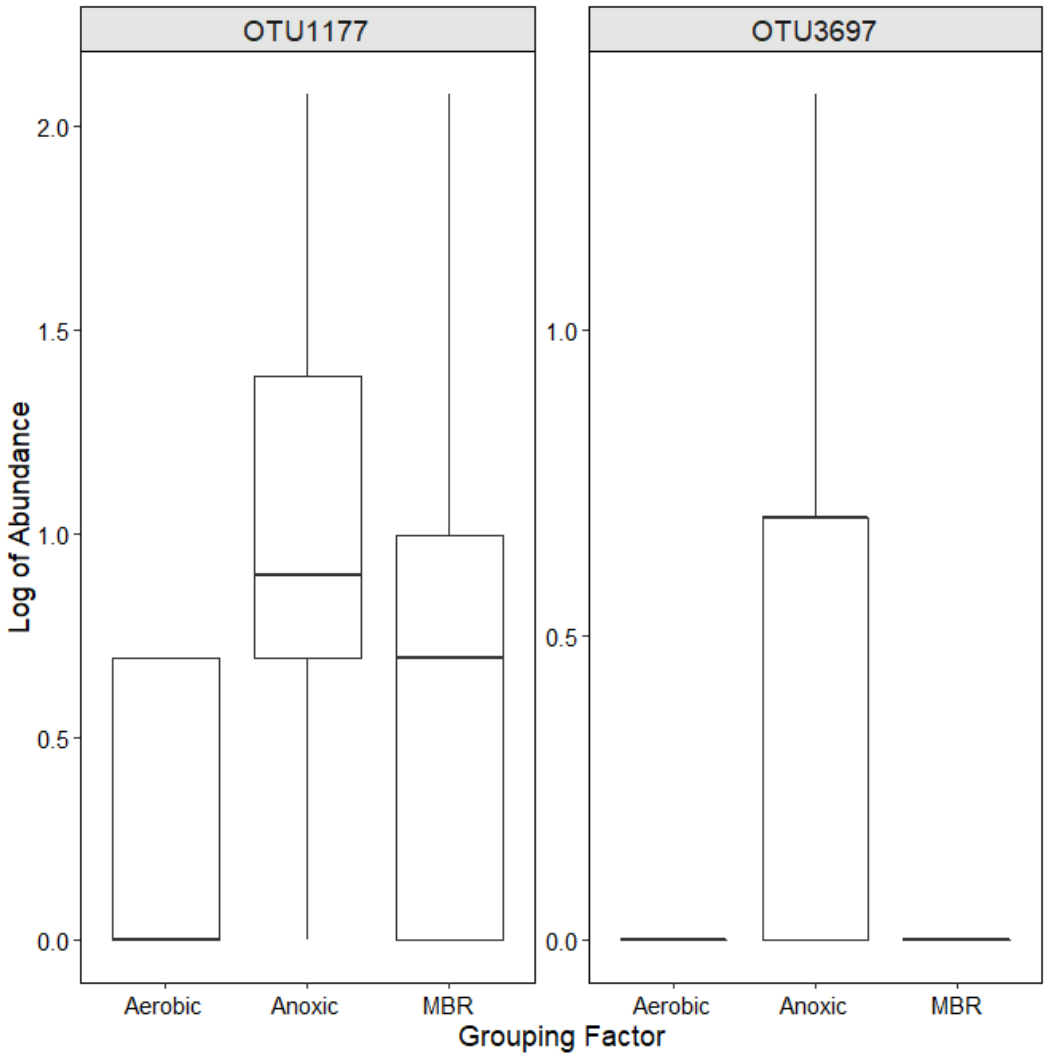

Figure 35. ANCOM analysis of the treatment tanks W/O post-septic tanks.

Microscopic analysis

Traditional microscopy analysis is commonly used to analyse the condition of WWTP. The microscopic analysis of the Tahuna WWTP was done so that it could be compared to the taxonomic information produced by sequencing. Using phase contrast and bright field microscopy with Neisser staining the Tahuna WWTP were analysed. The appearance of monocolonies was

seen using phase contrast at 400X magnification (Figure 36). These monocolonies, when examined in bright field with neisser staining (Figure 37), showed the presence of PHA granules. A filamentous bacteria was also observed in high abundance in the WWTP, although did not appear to be causing any problem for the plant. According to traditional methods of identification, this was identified as Eikelboom type 0092 (Eikelboom, 1975).

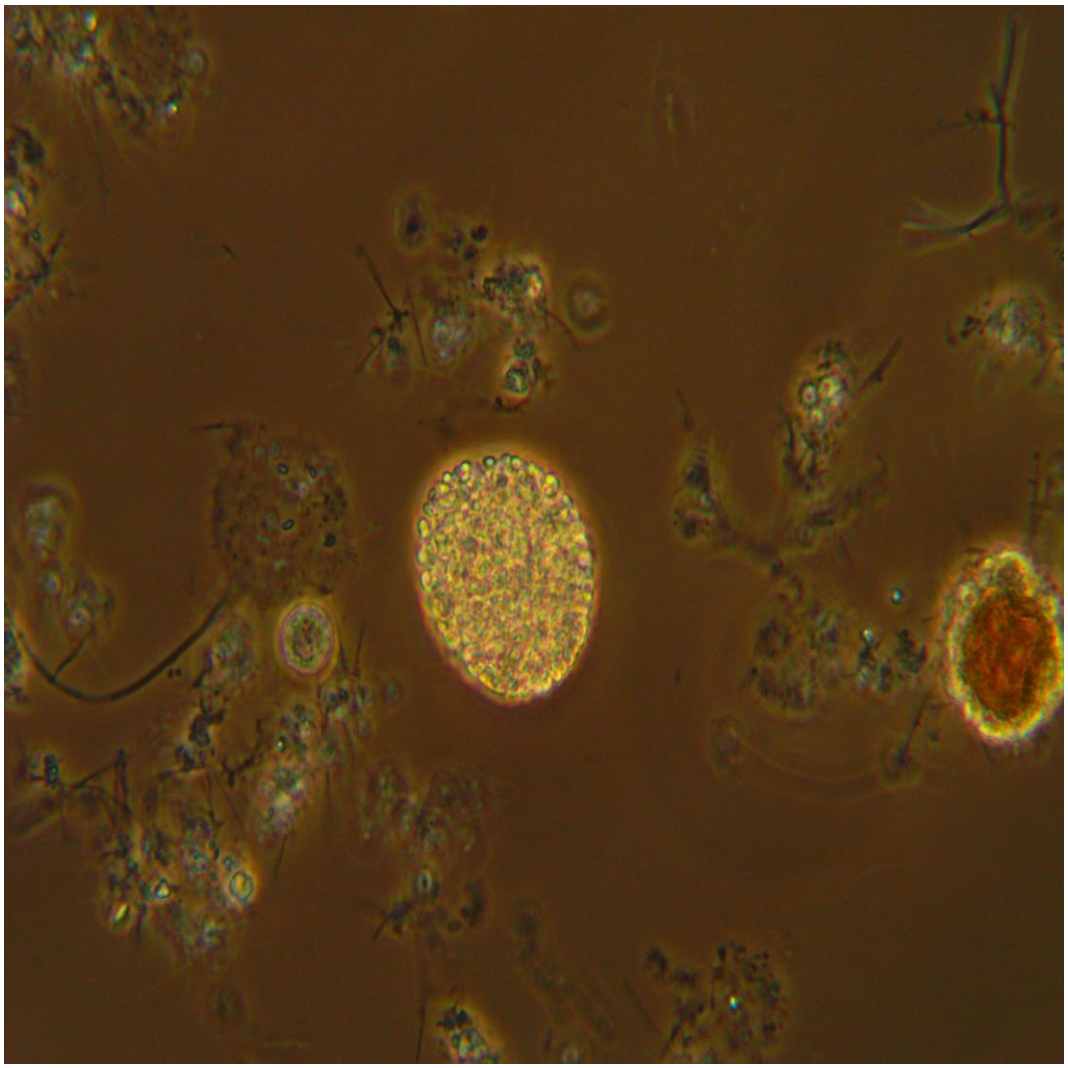

Figure 36. Photo of monocolonies at 400X magnification using phase contrast microscopy, taken on the 10th of January 2018.

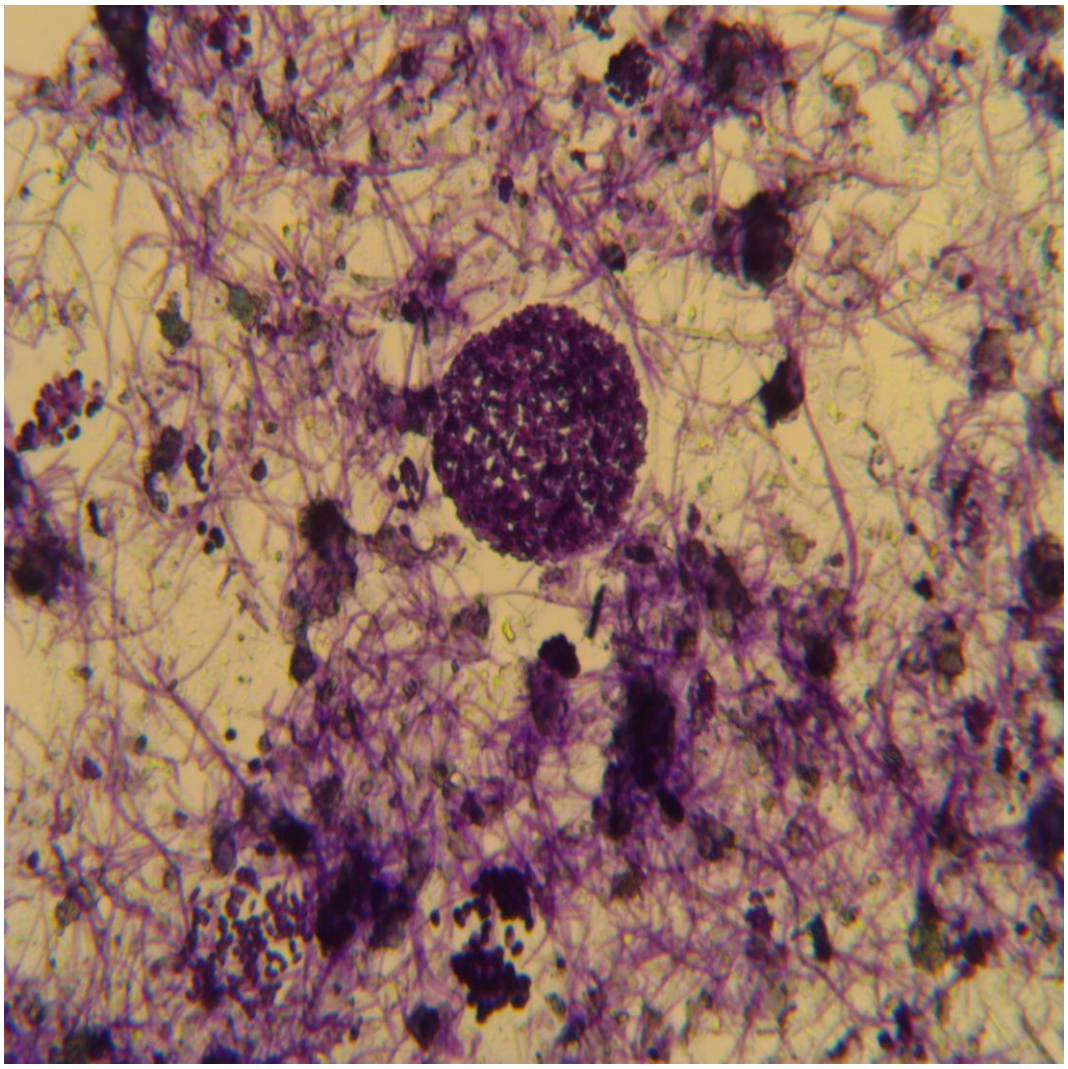

Figure 37. Photo of monocolonies and filamentous bacteria taken at 400X magnification using bright field and Neisser stain, taken in the 10th of January 2018.

Discussion

The diversity of microorganisms at the phylum and class level were very similar between treatment tanks (the anoxic, aerobic, and MBR tanks) and a large proportion of the community (76.3%) comprised the core microbiome (Figure 34). A lack of change in diversity between tanks has been shown previously in other studies (Xia et al., 2010; Zhang et al., 2018). In WWTPs there are several phyla which have often been shown to be highly abundant. These phyla include Proteobacteria, Bacteroidetes, Chloroflexi and Acidobacteria (Chen et al., 2016; Wang et al., 2012). Proteobacteria is the most abundant phylum in the study WWTP, this has also been shown to be the dominant phylum in previous studies (Xia et al., 2010; Xu et al., 2018; Zhang et al., 2018). Bacteroidetes was also highly abundant, which has also been the case in other studies (Chen et al., 2016). However, although they were present, the phyla Chloroflexi and Acidobacteria differed from the literature showing low abundance in the study WWTP (Chen et al., 2016; Wang et al., 2012).

A range of biochemical parameters was measured during the study including; DO, pH, ORP, TP, DRP, NH_4-N, NO_2-N, NO_3-N, and temperature. The correlation between the DO (Figure 14) and the phosphorous (Figure 17) in the sequential tanks, as well as the low concentration of TP in the final effluent, implies the existence of functioning phosphorous accumulating organisms (PAOs).

The decrease in ammonium concentration in the treatment tanks (Figure 18) demonstrates that ammonium oxidation is occurring in the WWTP. There was a decreased amount of ammonium and an increase in the nitrite concentration in the MBR tank. The decrease of ammonium and increase in nitrite in the aerobic tanks implies that nitrification, as opposed to Anammox, is the process which is breaking down ammonium (Park & Noguera, 2004; van Niftrik & Jetten, 2012).

pH is known to influence microbial communities and can change during the nitrification and denitrification process (Glass & Silverstein, 1998; Villaverde

et al., 1997). Removal of ammonium could conceivably be due to conventional nitrogen removal as if in a two-sludge process. The ammonium decreased during the treatment process, except for a temporary increase in the second anoxic tank, most likely caused by heterotrophic activity and release of complex nitrogen species being converted into ammonium. The decrease of ammonium, as well as the increase in both nitrite and nitrate in the aerobic MBR tank, would indicate that this is where the Ammonia Oxidising Bacteria (AOB) begin the nitrification process. The sequencing results revealed five OTUs identified as *Nitrosomonas* from the Proteobacteria phylum. *Nitrosomonas* was found to be in the 40 most relative abundant organisms and has been previously shown to be one of the core bacteria involved in nitrification in WWTPs. *Nitrospira* was the only other microorganism identified that has the possibility of functioning as an AOB. If *Nitrospira* was the organism undertaking ammonia oxidation it would be likely that it was functioning as a COMAMMOX organism (Daims et al., 2015). However, the abundance of Denitrifying organisms means that it is unlikely that COMAMMOX would be occurring. *Nitrospira* was the only organism found through sequencing that was known to function as a Nitrifying Oxidising Bacteria (NOB). The lack of any other NOB, as well as the abundance of Nitrospira, would indicate that this genus was the main NOB in the system. Six OTUs were correlated with *Nitrospira* with two OTUs, OTU91 and OTU4160, being the most dominant with a relative abundance of 0.48% and 0.34% respectively. The dominant OTU was analysed using the Blast function through the NCBI database, showing a sequence correlation with '*Nitrospira cf. moscoviensis'.*

Denitrification should be observable in the WWTP by monitoring nitrite concentrations in the treatment tanks. There is an increased concentration of nitrite in the MBR tank due to nitrification. The process of denitrification is known to increase pH (Cao et al., 2013). However, changes in pH in this study were too small to indicate denitrification or nitrification (Figure 16). If denitrification was occurring is most likely through *Dechloromonas*, which was found in the WWTP as three separate OTUs at relatively high abundance. This microorganism has been shown in the literature to be one of the main core

denitrifying bacteria in WWTP (Fan et al., 2017; McIlroy et al., 2016). *Dechloromonas* is known to reduce nitrate in anoxic conditions, where it utilises acetate as an electron donor (Ginige et al., 2005).

DO has a major effect on the community structure of the WWTP as well as the effectiveness of various microbial functions such as nitrification, denitrification, and heterotrophic carbon removal (Liu & Wang, 2013; Park & Noguera, 2004; Wang et al., 2012; Wilén & Balmér, 1999). Although the treatment plant was designed as a four-stage Bardenpho system, the DO concentrations of each tank means that the treatment plant was essentially functioning as a two-stage treatment system. If this is the case, then the MBR tank would be functioning as a single aerobic tank while the aerobic tank functioned as an anoxic tank. The treatment plant could then be removing phosphorous in a similar system to the phoredox process (Metcalf & Eddy, 2014).

There were periods during the year when phosphorous removal was deemed not satisfactory, and the removal of phosphorous was accomplished by the addition of alum. The time periods where it was necessary to use alum should have then correlated with a lower abundance of PAO. However, the abundance of PAOs did not differ much during the year. The sequencing found two organisms which were known PAOs. The first OTU recognised as a PAO was *Tetrasphaera*, however, the relative abundance of *Tetrasphaera* was low in all samples. *Tetrasphaera* is an organism suggested to be able to accumulate phosphate, although in a different way (Kristiansen et al., 2013). The second organism correlated with two OTUs and was identified as *Dechloromonas* which had a higher abundance than *Tetrasphaera* and has been shown in some studies to be capable of both denitrification and phosphorous accumulation (Lv et al., 2014; Y. Y. Zhang et al., 2017). Further investigation of the *Dechloromonas* sequence using BLAST on the NCBI database allowed the putative placement as Candidatus '*Accumulibacter phosphatis*' with an indent and query cover of 100%. Candidatus '*Accumulibacter phosphatis*' is thought to be the main PAO in wastewater communities (Hesselmann et al., 1999).

There is evidence that Glycogen Accumulating Organisms (GAOs) impede the efficiency of PAOs as they compete for similar resources (Mielczarek et al., 2013; Oehmen et al., 2006). In the activated sludge plant in this study, six genera were recognised as GAOs. The most abundant GAO was CPB_S60, also known as 'Candidatus Competibacter', a non-filamentous aerobic heterotroph with some strains capable of using nitrate as an electron donor (Kong et al., 2006). Although 'Candidatus Competibacter' was seen in high abundance (~3.4%), it was also comprised of only a single OTU. The next abundant GAO was *Defluviicoccus* with a relative abundance of 0.3%. *Defluviicoccus* is from the Proteobacteria phylum, family Rhodospirillaceae (Wong & Liu, 2007) and has previously been known as Candidatus 'Monolibacter batavus' and 'Nostocoida limicola' (McIlroy et al., 2010; Nittami et al., 2009). In wastewater, this organism is known to form tetrads and filaments (Nittami et al., 2009; Wong & Liu, 2007). The filamentous morphotype may cause bulking in WWTP (Nittami et al., 2009), while the tetrads which stain positive for PHAs in anaerobic conditions are apparent in deteriorating EBPR plants (Nittami et al., 2009). *Defluviicoccus* has been shown to be able to use not only the carbon compounds that *Competibacter* uses but also glucose (Burow et al., 2007). The remaining GAOs had a relative abundance of less than 0.1% and were CCM19a, *Propionivibrio*, SPB280, *Micropruina*, in order of highest to lowest abundance.

The filamentous bacteria are often a concern for WWTP, the microscopic analysis of the Tahuna treatment plant revealed one filamentous bacteria that was particularly abundant (Figure 37). Traditional microscopic methods of identification through morphology of the filament would identify it as Eikelboom type 0092 (Eikelboom, 1975). However, analysis of the sequencing results as well as past studies into the taxonomy of Eikelboom type 0092 would reveal this organism from the phylum Chloroflexi, class Anaerolinea and the genus *Leptolinea* (Lachlan Speirs et al., 2009; Yamada & Sekiguchi, 2009).

At the phylum level, there is some similarity between the post-septic and the treatment tanks, with both Proteobacteria and Bacteroidea being in high abundance (Figure 22). However, at closer taxonomic ranks the similarity

becomes less and there is a clear separation between the post-septic tanks and the treatment tanks (Figure 23), showing that the treatment process significantly alters the microbial community.

The PCoA graphs which correlate the samples with PCR bias and date show that there may be some PCR bias in the samples (Figure 29). However, the correlation with month shows each month is closely related to its following month (Figure 32), showing that there is a temporal variation occurring over the year.

Chapter 4:

Conclusions and future perspectives

The Tahuna wastewater treatment plant was capable of nitrogen and phosphorous removal due to the microbiological life. The nitrogen removal was due to a group of microorganisms, the AOB, NOB, and denitrifiers. In regards to nitrification, it is most likely that *Nitrosomonas* is the microorganism that is functioning as an AOB. The only NOB discovered in the treatment plant was *Nitrospira*. There was an abundance of *Dechloromonas* in the treatment, which has the capability of acting as a denitrifier. The concentration of nitrate released in the final effluent while higher than in the tanks is still substantially lower than the incoming ammonium and remaining nitrite, this implies that denitrification is occurring. The microscopic analysis of the WWTP showed monocolonies which stained Neisser positive and thought to be indicative of PAOs such as 'Candidatus *Accumulibacter phosphatis*'. However, 'Candidatus *Competibacter*' was in high relative abundance in the WWTP and are known to be GAOs. This organism is known to appear as Neisser positive, although they are not PAOs and can detrimentally affect phosphorous removal. Phosphorous removal was most likely caused by *Dechloromonas* which had some abundance in the WWTP and functions as a PAO, although with a different metabolism and morphology to other PAOs.

Studies of this type could be further enhanced using transcriptomics. Using transcriptomics and community analysis, the organisms could be identified as well as whether they were functionally active. A high amount of OTUs in the study were unclassified. If metagenomics was applied to the analysis of wastewater treatment it would allow for some of the functional attributes of the unknown OTUs to be identified.

References

Albertsen, M., Karst, S. M., Ziegler, A. S., Kirkegaard, R. H., & Nielsen, P. H. (2015). Back to Basics - The Influence of DNA Extraction and Primer Choice on Phylogenetic Analysis of Activated Sludge Communities. *Plos One, 10*(7). doi:10.1371/journal.pone.0132783

Ali, M., Oshiki, M., Awata, T., Isobe, K., Kimura, Z., Yoshikawa, H., Hira, D., Kindaichi, T., Satoh, H., Fujii, T., & Okabe, S. (2015). Physiological characterization of anaerobic ammonium oxidizing bacterium 'Candidatus Jettenia caeni'. *Environmental Microbiology, 17*(6), 2172-2189. doi:10.1111/1462-2920.12674

Álvarez, X., Valero, E., Santos, R. M. B., Varandas, S. G. P., Sanches Fernandes, L. F., & Pacheco, F. A. L. (2017). Anthropogenic nutrients and eutrophication in multiple land use watersheds: Best management practices and policies for the protection of water resources. *Land Use Policy, 69*, 1-11. doi:https://doi.org/10.1016/j.landusepol.2017.08.028

Andersen K.S., Kirkegaard R.H., Karst S.M., & M., A. (2018). ampvis2: an R package to analyse and visualise 16S rRNA amplicon data. *bioRxiv*. doi:http://dx.doi.org/10.1101/299537

Anderson, M. J., & Willis, T. J. (2003). Canonical analysis of principal coordinates: A useful method of constrained ordination for ecology. *Ecology, 84*(2), 511-525.

Anthonisen, A. C., Loehr, R. C., Prakasam, T. B. S., & Srinath, E. G. (1976). Inhibition of nitrification by ammonia and nitrous acid. *Journal of the Water Pollution Control Federation, 48*(5), 835-852.

Araujo, J. C., Brucha, G., Campos, J. R., & Vazoller, R. F. (2000) Monitoring the development of anaerobic biofilms using fluorescent in situ hybridization and confocal laser scanning microscopy. In*: Vol. 41. Water Science and Technology* (pp. 69-77).

Archer, S. D. J., McDonald, I. R., Herbold, C. W., Lee, C. K., & Cary, C. S. (2015). Benthic microbial communities of coastal terrestrial and ice shelf Antarctic meltwater ponds. *Frontiers in Microbiology, 6*(485). doi:10.3389/fmicb.2015.00485

Awata, T., Oshiki, M., Kindaichi, T., Ozaki, N., Ohashi, A., & Okabe, S. (2013). Physiological characterization of an anaerobic ammonium-oxidizing bacterium belonging to the "Candidatus scalindua" group. *Appl Environ Microbiol, 79*(13), 4145-4148. doi:10.1128/aem.00056-13

Baytshtok, V., Lu, H., Park, H., Kim, S., Yu, R., & Chandran, K. (2009). Impact of varying electron donors on the molecular microbial ecology and

biokinetics of methylotrophic denitrifying bacteria. *Biotechnology and Bioengineering, 102*(6), 1527-1536. doi:doi:10.1002/bit.22213

Bettazzi, E., Caffaz, S., Vannini, C., & Lubello, C. (2010). Nitrite inhibition and intermediates effects on Anammox bacteria: A batch-scale experimental study. *Process Biochemistry, 45*(4), 573-580. doi:https://doi.org/10.1016/j.procbio.2009.12.003

Bitton, G. (2010). *Wastewater Microbiology*. Hoboken, UNITED STATES: Wiley.

Broda, E. (1977). Two kinds of lithotrophs missing in nature. *Z Allg Mikrobiol, 17*(6), 491-493.

Burow, L. C., Kong, Y., Nielsen, J. L., Blackall, L. L., & Nielsen, P. H. (2007). Abundance and ecophysiology of Defluviicoccus spp., glycogen-accumulating organisms in full-scale wastewater treatment processes. *Microbiology-Sgm, 153*, 178-185. doi:10.1099/mic.0.2006/001032-0

Cao, S., Du, R., Niu, M., Li, B., Ren, N., & Peng, Y. (2016). Integrated anaerobic ammonium oxidization with partial denitrification process for advanced nitrogen removal from high-strength wastewater. *Bioresource Technology, 221*(Supplement C), 37-46. doi:https://doi.org/10.1016/j.biortech.2016.08.082

Cao, X. S., Qian, D., & Meng, X. Z. (2013). Effects of pH on nitrite accumulation during wastewater denitrification. *Environmental Technology, 34*(1), 45-51. doi:10.1080/09593330.2012.679700

Carvajal-Arroyo, J. M., Puyol, D., Li, G., Lucero-Acuña, A., Sierra-Álvarez, R., & Field, J. A. (2014). Pre-exposure to nitrite in the absence of ammonium strongly inhibits anammox. *Water Research, 48*, 52-60. doi:https://doi.org/10.1016/j.watres.2013.09.015

Chamchoi, N., Nitisoravut, S., & Schmidt, J. E. (2008). Inactivation of ANAMMOX communities under concurrent operation of anaerobic ammonium oxidation (ANAMMOX) and denitrification. *Bioresource Technology, 99*(9), 3331-3336. doi:https://doi.org/10.1016/j.biortech.2007.08.029

Chen, Y., Zhao, Z., Peng, Y., Li, J., Xiao, L., & Yang, L. (2016). Performance of a full-scale modified anaerobic/anoxic/oxic process: High-throughput sequence analysis of its microbial structures and their community functions. *Bioresource Technology, 220*, 225-232. doi:https://doi.org/10.1016/j.biortech.2016.07.095

Cho, S., Takahashi, Y., Fujii, N., Yamada, Y., Satoh, H., & Okabe, S. (2010). Nitrogen removal performance and microbial community analysis of an anaerobic up-flow granular bed anammox reactor. *Chemosphere, 78*(9), 1129-1135. doi:https://doi.org/10.1016/j.chemosphere.2009.12.034

Christensson, M., Lie, E., & Welander, T. (1994). A comparison between ethanol and methanol as carbon sources for denitrification. *Water Science and Technology, 30*(6), 83-90. doi:10.2166/wst.1994.0255

Conley, D. J., Paerl, H. W., Howarth, R. W., Boesch, D. F., Seitzinger, S. P., Havens, K. E., Lancelot, C., & Likens, G. E. (2009). Controlling Eutrophication: Nitrogen and Phosphorus. *Science, 323*(5917), 1014-1015. doi:10.1126/science.1167755

Corbalá-Robles, L., Picioreanu, C., van Loosdrecht, M. C. M., & Pérez, J. (2016). Analysing the effects of the aeration pattern and residual ammonium concentration in a partial nitritation-anammox process. *Environmental Technology, 37*(6), 694-702. doi:10.1080/09593330.2015.1077895.

Courtens, E. N. P., Boon, N., De Clippeleir, H., Berckmoes, K., Mosquera, M., Seuntjens, D., & Vlaeminck, S. E. (2014). Control of nitratation in an oxygen-limited autotrophic nitrification/denitrification rotating biological contactor through disc immersion level variation. *Bioresource Technology, 155*, 182-188. doi:https://doi.org/10.1016/j.biortech.2013.12.108

Daims, H., Lebedeva, E. V., Pjevac, P., Han, P., Herbold, C., Albertsen, M., Jehmlich, N., Palatinszky, M., Vierheilig, J., Bulaev, A., Kirkegaard, R. H., von Bergen, M., Rattei, T., Bendinger, B., Nielsen, P. H., & Wagner, M. (2015). Complete nitrification by Nitrospira bacteria. *Nature, 528*(7583), 504-509. doi:10.1038/nature16461 http://www.nature.com/nature/journal/v528/n7583/abs/nature16461.html#supplementary-information

Dapena-Mora, A., Fernández, I., Campos, J. L., Mosquera-Corral, A., Méndez, R., & Jetten, M. S. M. (2007). Evaluation of activity and inhibition effects on Anammox process by batch tests based on the nitrogen gas production. *Enzyme and Microbial Technology, 40*(4), 859-865. doi:https://doi.org/10.1016/j.enzmictec.2006.06.018

Daverey, A., Chei, P. C., Dutta, K., & Lin, J. G. (2015). Statistical analysis to evaluate the effects of temperature and pH on anammox activity. *International Biodeterioration & Biodegradation, 102*, 89-93. doi:10.1016/j.ibiod.2015.03.006

Dosta, J., Fernández, I., Vázquez-Padín, J. R., Mosquera-Corral, A., Campos, J. L., Mata-Álvarez, J., & Méndez, R. (2008). Short- and long-term effects of temperature on the Anammox process. *Journal of Hazardous Materials, 154*(1), 688-693. doi:https://doi.org/10.1016/j.jhazmat.2007.10.082

Du, R., Peng, Y., Cao, S., Wang, S., & Wu, C. (2015). Advanced nitrogen removal from wastewater by combining anammox with partial denitrification. *Bioresource Technology, 179*(Supplement C), 497-504. doi:https://doi.org/10.1016/j.biortech.2014.12.043

Egli, K., Fanger, U., Alvarez, P. J. J., Siegrist, H., van der Meer, J. R., & Zehnder, A. J. B. (2001). Enrichment and characterization of an anammox bacterium from a rotating biological contactor treating ammonium-rich leachate. *Archives of Microbiology, 175*(3), 198-207. doi:10.1007/s002030100255

Eikelboom, D. H. (1975). Filamentous organisms observed in activated sludge. *Water Research, 9*(4), 365-388. doi:https://doi.org/10.1016/0043-1354(75)90182-7

Eschenhagen, M., Schuppler, M., & Röske, I. (2003). Molecular characterization of the microbial community structure in two activated sludge systems for the advanced treatment of domestic effluents. *Water Research, 37*(13), 3224-3232. doi:https://doi.org/10.1016/S0043-1354(03)00136-2

Fan, X. Y., Gao, J. F., Pan, K. L., Li, D. C., & Dai, H. H. (2017). Temporal dynamics of bacterial communities and predicted nitrogen metabolism genes in a full-scale wastewater treatment plant. *Rsc Advances, 7*(89), 56317-56327. doi:10.1039/c7ra10704h

Faust, L., Szendy, M., Plugge, C. M., van den Brink, P. F. H., Temmink, H., & Rijnaarts, H. H. M. (2015). Characterization of the bacterial community involved in the bioflocculation process of wastewater organic matter in high-loaded MBRs. *Applied Microbiology and Biotechnology, 99*(12), 5327-5337. doi:10.1007/s00253-015-6402-y

Fernández, I., Dosta, J., Fajardo, C., Campos, J. L., Mosquera-Corral, A., & Méndez, R. (2012). Short- and long-term effects of ammonium and nitrite on the Anammox process. *Journal of Environmental Management, 95*, S170-S174. doi:https://doi.org/10.1016/j.jenvman.2010.10.044

Fernández, N., Díaz, E. E., Amils, R., & Sanz, J. L. (2008). Analysis of Microbial Community during Biofilm Development in an Anaerobic Wastewater Treatment Reactor. *Microbial Ecology, 56*(1), 121-132. doi:10.1007/s00248-007-9330-2

Fuerst, J. A. (2005). INTRACELLULAR COMPARTMENTATION IN PLANCTOMYCETES. *Annual Review of Microbiology, 59*(1), 299-328. doi:10.1146/annurev.micro.59.030804.121258

Fujitani, H., Kumagai, A., Ushiki, N., Momiuchi, K., & Tsuneda, S. (2015). Selective isolation of ammonia-oxidizing bacteria from autotrophic nitrifying granules by applying cell-sorting and sub-culturing of microcolonies. *Frontiers in Microbiology, 6*(1159). doi:10.3389/fmicb.2015.01159

Ganigué, R., Gabarró, J., Sànchez-Melsió, A., Ruscalleda, M., López, H., Vila, X., Colprim, J., & Balaguer, M. D. (2009). Long-term operation of a partial nitritation pilot plant treating leachate with extremely high ammonium concentration prior to an anammox process. *Bioresource Technology, 100*(23), 5624-5632. doi:https://doi.org/10.1016/j.biortech.2009.06.023

Gao, P., Xu, W., Sontag, P., Li, X., Xue, G., Liu, T., & Sun, W. (2016). Correlating microbial community compositions with environmental factors in activated sludge from four full-scale municipal wastewater treatment plants in Shanghai, China. *Applied Microbiology and Biotechnology, 100*(10), 4663-4673. doi:10.1007/s00253-016-7307-0

Ghehi, T. J., Mortezaeifar, S., Gholami, M., Kalantary, R. R., & Mahvi, A. H. (2014). Performance evaluation of enhanced SBR in simultaneous removal of nitrogen and phosphorous. *Journal of Environmental Health Science and Engineering, 12*. doi:10.1186/s40201-014-0134-2

Ginige, M. P., Hugenholtz, P., Daims, H., Wagner, M., Keller, J., & Blackall, L. L. (2004). Use of Stable-Isotope Probing, Full-Cycle rRNA Analysis, and Fluorescence In Situ Hybridization-Microautoradiography To Study a Methanol-Fed Denitrifying Microbial Community. *Applied and Environmental Microbiology, 70*(1), 588-596. doi:10.1128/aem.70.1.588-596.2004

Ginige, M. P., Keller, J., & Blackall, L. L. (2005). Investigation of an acetate-fed denitrifying microbial community by stable isotope probing, full-cycle rRNA analysis, and fluorescent in situ hybridization-microautoradiography. *Applied and Environmental Microbiology, 71*(12), 8683-8691. doi:10.1128/aem.71.12.8683-8691.2005

Glass, C., & Silverstein, J. (1998). Denitrification kinetics of high nitrate concentration water: pH effect on inhibition and nitrite accumulation. *Water Research, 32*(3), 831-839. doi:https://doi.org/10.1016/S0043-1354(97)00260-1

Gnida, A., Zabczynski, S., & Surmacz-Gorska, J. (2018). Filamentous bacteria in the nitrifying activated sludge. *Water Science and Technology, 77*(11), 2709-2713. doi:10.2166/wst.2018.215

Gonzalez-Martinez, A., Rodriguez-Sanchez, A., Muñoz-Palazon, B., Garcia-Ruiz, M.-J., Osorio, F., van Loosdrecht, M. C. M., & Gonzalez–Lopez, J. (2015). Microbial community analysis of a full-scale DEMON bioreactor. *Bioprocess and Biosystems Engineering, 38*(3), 499-508. doi:10.1007/s00449-014-1289-z

Guven, D., Dapena, A., Kartal, B., Schmid, M. C., Maas, B., van de Pas-Schoonen, K., Sozen, S., Mendez, R., Op den Camp, H. J. M., Jetten, M. S. M., Strous, M., & Schmidt, I. (2005). Propionate oxidation by and methanol inhibition of anaerobic ammonium-oxidizing bacteria. *Applied and Environmental Microbiology, 71*(2), 1066-1071. doi:10.1128/aem.71.2.1066-1071.2005

Haandel, A. C. v. (2015). *Handbook of biological wastewater treatment : design and optimisation of activated sludge systems* (Second edition.. ed.): London : IWA Publishing.

Head, I. M., Hiorns, W. D., Embley, T. M., McCarthy, A. J., & Saunders, J. R. (1993). THE PHYLOGENY OF AUTOTROPHIC AMMONIA-OXIDIZING BACTERIA AS DETERMINED BY ANALYSIS OF 16S RIBOSOMAL-RNA GENE-SEQUENCES. *Journal of General Microbiology, 139*, 1147-1153. doi:10.1099/00221287-139-6-1147

Hesselmann, R. P. X., Werlen, C., Hahn, D., van der Meer, J. R., & Zehnder, A. J. B. (1999). Enrichment, Phylogenetic Analysis and Detection of a Bacterium That Performs Enhanced Biological Phosphate Removal in Activated Sludge. *Systematic and Applied Microbiology, 22*(3), 454-465. doi:https://doi.org/10.1016/S0723-2020(99)80055-1

Inaba, T., Hori, T., Navarro, R. R., Ogata, A., Hanajima, D., & Habe, H. (2018). Revealing sludge and biofilm microbiomes in membrane bioreactor treating piggery wastewater by non-destructive microscopy and 16S rRNA gene sequencing. *Chemical Engineering Journal, 331*, 75-83. doi:https://doi.org/10.1016/j.cej.2017.08.095

Innerebner, G., Insam, H., Franke-Whittle, I. H., & Wett, B. (2007). Identification of anammox bacteria in a full-scale deammonification plant making use of anaerobic ammonia oxidation. *Systematic and Applied Microbiology, 30*(5), 408-412. doi:https://doi.org/10.1016/j.syapm.2007.02.001

Isaka, K., Sumino, T., & Tsuneda, S. (2007). High nitrogen removal performance at moderately low temperature utilizing anaerobic ammonium oxidation reactions. *Journal of Bioscience and Bioengineering, 103*(5), 486-490. doi:https://doi.org/10.1263/jbb.103.486

Isaka, K., Suwa, Y., Kimura, Y., Yamagishi, T., Sumino, T., & Tsuneda, S. (2008). Anaerobic ammonium oxidation (anammox) irreversibly inhibited by methanol. *Applied Microbiology and Biotechnology, 81*(2), 379-385. doi:10.1007/s00253-008-1739-0

Jaroszynski, L. W., Cicek, N., Sparling, R., & Oleszkiewicz, J. A. (2011). Importance of the operating pH in maintaining the stability of anoxic ammonium oxidation (anammox) activity in moving bed biofilm reactors. *Bioresource Technology, 102*(14), 7051-7056. doi:10.1016/j.biortech.2011.04.069

Jetten, M. S. M., Sliekers, O., Kuypers, M., Dalsgaard, T., van Niftrik, L., Cirpus, I., van de Pas-Schoonen, K., Lavik, G., Thamdrup, B., Le Paslier, D., Op den Camp, H. J. M., Hulth, S., Nielsen, L. P., Abma, W., Third, K., Engström, P., Kuenen, J. G., Jørgensen, B. B., Canfield, D. E., Sinninghe Damsté, J. S., Revsbech, N. P., Fuerst, J., Weissenbach, J., Wagner, M., Schmidt, I., Schmid, M., & Strous, M. (2003). Anaerobic ammonium oxidation by marine and freshwater planctomycete-like bacteria. *Applied Microbiology and Biotechnology, 63*(2), 107-114. doi:10.1007/s00253-003-1422-4

Jetten, M. S. M., Wagner, M., Fuerst, J., van Loosdrecht, M., Kuenen, G., & Strous, M. (2001). Microbiology and application of the anaerobic

ammonium oxidation (‘anammox’) process. *Current Opinion in Biotechnology, 12*(3), 283-288. doi:https://doi.org/10.1016/S0958-1669(00)00211-1

Jin, R.-C., Xing, B.-S., Yu, J.-J., Qin, T.-Y., & Chen, S.-X. (2013). The importance of the substrate ratio in the operation of the Anammox process in upflow biofilter. *Ecological Engineering, 53*, 130-137. doi:https://doi.org/10.1016/j.ecoleng.2012.12.027

Kalyuzhnyi, S., Gladchenko, M., Mulder, A., & Versprille, B. (2006). DEAMOX—New biological nitrogen removal process based on anaerobic ammonia oxidation coupled to sulphide-driven conversion of nitrate into nitrite. *Water Research, 40*(19), 3637-3645. doi:https://doi.org/10.1016/j.watres.2006.06.010

Kampfer, P., Erhart, R., Beimfohr, C., Bohringer, J., Wagner, M., & Amann, R. (1996). Characterization of bacterial communities from activated sludge: Culture-dependent numerical identification versus in situ identification using group- and genus-specific rRNA-targeted oligonucleotide probes. *Microbial Ecology, 32*(2), 101-121.

Kampschreur, M. J., Temmink, H., Kleerebezem, R., Jetten, M. S. M., & van Loosdrecht, M. C. M. (2009). Nitrous oxide emission during wastewater treatment. *Water Research, 43*(17), 4093-4103. doi:http://dx.doi.org/10.1016/j.watres.2009.03.001

Kartal, B., Maalcke, W. J., de Almeida, N. M., Cirpus, I., Gloerich, J., Geerts, W., Op den Camp, H. J. M., Harhangi, H. R., Janssen-Megens, E. M., Francoijs, K.-J., Stunnenberg, H. G., Keltjens, J. T., Jetten, M. S. M., & Strous, M. (2011). Molecular mechanism of anaerobic ammonium oxidation. *Nature, 479*, 127. doi:10.1038/nature10453 https://www.nature.com/articles/nature10453#supplementary-information

Kartal, B., Rattray, J., van Niftrik, L. A., van de Vossenberg, J., Schmid, M. C., Webb, R. I., Schouten, S., Fuerst, J. A., Damsté, J. S., Jetten, M. S. M., & Strous, M. (2007). Candidatus “Anammoxoglobus propionicus” a new propionate oxidizing species of anaerobic ammonium oxidizing bacteria. *Systematic and Applied Microbiology, 30*(1), 39-49. doi:https://doi.org/10.1016/j.syapm.2006.03.004

Kartal, B., van Niftrik, L., Rattray, J., de Vossenberg, J., Schmid, M. C., Damste, J. S. S., Jetten, M. S. M., & Strous, M. (2008). Candidatus 'Brocadia fulgida': an autofluorescent anaerobic ammonium oxidizing bacterium. *FEMS Microbiology Ecology, 63*(1), 46-55. doi:10.1111/j.1574-6941.2007.00408.x

Kassambara, A. (2018). ggpubr: 'ggplot2' Based Publication Ready Plots. *R package version 0.2.*

Kerrn-Jespersen, J. P., & Henze, M. (1993). Biological phosphorus uptake under anoxic and aerobic conditions. *Water Research, 27*(4), 617-624. doi:https://doi.org/10.1016/0043-1354(93)90171-D

Kimura, Y., Isaka, K., Kazama, F., & Sumino, T. (2010). Effects of nitrite inhibition on anaerobic ammonium oxidation. *Applied Microbiology and Biotechnology, 86*(1), 359-365. doi:10.1007/s00253-009-2359-z

Knowles, R. (1982). Denitrification. *Microbiological Reviews, 46*(1), 43-70.

Knowles, R. (1996). Denitrification: microbiology and ecology. *Life support & biosphere science : international journal of earth space, 3*(1-2), 31-34.

Kong, Y., Xia, Y., Nielsen, J. L., & Nielsen, P. H. (2006). Ecophysiology of a group of uncultured Gammaproteobacterial glycogen-accumulating organisms in full-scale enhanced biological phosphorus removal wastewater treatment plants. *Environmental Microbiology, 8*(3), 479-489. doi:doi:10.1111/j.1462-2920.2005.00914.x

Kong, Y. H., Nielsen, J. L., & Nielsen, P. H. (2005). Identity and ecophysiology of uncultured actinobacterial polyphosphate-accumulating organisms in full-scale enhanced biological phosphorus removal plants. *Applied and Environmental Microbiology, 71*(7), 4076-4085. doi:10.1128/aem.71.7.4076-4085.2005

Kristiansen, R., Nguyen, H. T. T., Saunders, A. M., Nielsen, J. L., Wimmer, R., Le, V. Q., McIlroy, S. J., Petrovski, S., Seviour, R. J., Calteau, A., Nielsen, K. L., & Nielsen, P. H. (2013). A metabolic model for members of the genus Tetrasphaera involved in enhanced biological phosphorus removal. *Isme Journal, 7*(3), 543-554. doi:10.1038/ismej.2012.136

Lam, P., & Kuypers, M. M. M. (2011). Microbial Nitrogen Cycling Processes in Oxygen Minimum Zones. *Annual Review of Marine Science, 3*(1), 317-345. doi:10.1146/annurev-marine-120709-142814

Lew, B., Stief, P., Beliavski, M., Ashkenazi, A., Svitlica, O., Khan, A., Tarre, S., de Beer, D., & Green, M. (2012). Characterization of denitrifying granular sludge with and without the addition of external carbon source. *Bioresource Technology, 124*, 413-420. doi:https://doi.org/10.1016/j.biortech.2012.08.049

Li, J., Li, Y., Ohandja, D.-G., Yang, F., Wong, F.-S., & Chua, H.-C. (2008). Impact of filamentous bacteria on properties of activated sludge and membrane-fouling rate in a submerged MBR. *Separation and Purification Technology, 59*(3), 238-243. doi:https://doi.org/10.1016/j.seppur.2007.06.011

Lindsay, M. R., Webb, R. I., Strous, M., Jetten, M. S., Butler, M. K., Forde, R. J., & Fuerst, J. A. (2001). Cell compartmentalisation in planctomycetes: novel

types of structural organisation for the bacterial cell. *Archives of Microbiology, 175*(6), 413-429. doi:10.1007/s002030100280

Liu, G., & Wang, J. (2013). Long-Term Low DO Enriches and Shifts Nitrifier Community in Activated Sludge. *Environmental Science & Technology, 47*(10), 5109-5117. doi:10.1021/es304647y

Lopez-Vazquez, C. M., Song, Y. I., Hooijmans, C. M., Brdjanovic, D., Moussa, M. S., Gijzen, H. J., & Loosdrecht, M. M. C. v. (2007). Short-term temperature effects on the anaerobic metabolism of glycogen accumulating organisms. *Biotechnology and Bioengineering, 97*(3), 483-495. doi:doi:10.1002/bit.21302

Lotti, T., Kleerebezem, R., & van Loosdrecht, M. C. M. (2015). Effect of Temperature Change on Anammox Activity. *Biotechnology and Bioengineering, 112*(1), 98-103. doi:10.1002/bit.25333

Lotti, T., van der Star, W. R. L., Kleerebezem, R., Lubello, C., & van Loosdrecht, M. C. M. (2012). The effect of nitrite inhibition on the anammox process. *Water Research, 46*(8), 2559-2569. doi:https://doi.org/10.1016/j.watres.2012.02.011

Lu, H., Chandran, K., & Stensel, D. (2014). Microbial ecology of denitrification in biological wastewater treatment. *Water Research, 64*, 237-254. doi:http://dx.doi.org/10.1016/j.watres.2014.06.042

Lv, X. M., Shao, M. F., Li, C. L., Li, J., Gao, X. L., & Sun, F. Y. (2014). A Comparative Study of the Bacterial Community in Denitrifying and Traditional Enhanced Biological Phosphorus Removal Processes. *Microbes and Environments, 29*(3), 261-268. doi:10.1264/jsme2.ME13132

Ma, J., Wang, Z., Yang, Y., Mei, X., & Wu, Z. (2013). Correlating microbial community structure and composition with aeration intensity in submerged membrane bioreactors by 454 high-throughput pyrosequencing. *Water Research, 47*(2), 859-869. doi:http://dx.doi.org/10.1016/j.watres.2012.11.013

Ma, J., Wang, Z., Zang, L., Huang, J., & Wu, Z. (2015). Occurrence and fate of potential pathogenic bacteria as revealed by pyrosequencing in a full-scale membrane bioreactor treating restaurant wastewater. *Rsc Advances, 5*(31), 24469-24478. doi:10.1039/C4RA10220G

Mainstone, C. P., & Parr, W. (2002). Phosphorus in rivers — ecology and management. *Science of The Total Environment, 282-283*, 25-47. doi:https://doi.org/10.1016/S0048-9697(01)00937-8

Mandal, S., Van Treuren, W., White, R. A., Eggesbo, M., Knight, R., & Peddada, S. D. (2015). Analysis of composition of microbiomes: a novel method for

studying microbial composition. *Microb Ecol Health Dis, 26*, 27663. doi:10.3402/mehd.v26.27663

McIlroy, S. J., Nittami, T., Seviour, E. M., & Seviour, R. J. (2010). Filamentous members of cluster III Defluviicoccus have the in situ phenotype expected of a glycogen-accumulating organism in activated sludge. *FEMS Microbiology Ecology, 74*(1), 248-256. doi:10.1111/j.1574-6941.2010.00934.x

McIlroy, S. J., Starnawska, A., Starnawski, P., Saunders, A. M., Nierychlo, M., Nielsen, P. H., & Nielsen, J. L. (2016). Identification of active denitrifiers in full-scale nutrient removal wastewater treatment systems. *Environmental Microbiology, 18*(1), 50-64. doi:10.1111/1462-2920.12614

McMurdie, P. J., & Holmes, S. (2013). phyloseq: An R package for reproducible interactive analysis and graphics of microbiome census data. *Plos One, 4*(8).

Metcalf, & Eddy, a. (2014). *Wastewater engineering : treatment and resource recovery* (Fifth edition / revised by George Tchobanoglous, Professor Emeritus of Civil and Environmental Engineering, University of California at Davis, H. David Stensel, Professor of Civil and Environmental Engineering, University of Washington, Seattle, Ryujiro Tsuchihashi, Wastwater Technical Leader, AECOM, Franklin Burton, Consulting Engineer, Los Altos, CA

contributing authors, Mohammad Abu-Orf, North American Biosolids Practice Leader, AECOM, Gregory Bowden, Wastewater Technical Leader, AECOM, William Pfrang, Wastewater Treatement Technology Leader, AECOM.. ed.): New York, NY : McGraw-Hill Education.

Mielczarek, A. T., Nguyen, H. T. T., Nielsen, J. L., & Nielsen, P. H. (2013). Population dynamics of bacteria involved in enhanced biological phosphorus removal in Danish wastewater treatment plants. *Water Research, 47*(4), 1529-1544. doi:https://doi.org/10.1016/j.watres.2012.12.003

Mino, T., van Loosdrecht, M. C. M., & Heijnen, J. J. (1998). Microbiology and biochemistry of the enhanced biological phosphate removal process. *Water Research, 32*(11), 3193-3207. doi:https://doi.org/10.1016/S0043-1354(98)00129-8

Mulder, A., van de Graaf, A. A., Robertson, L. A., & Kuenen, J. G. (1995). Anaerobic ammonium oxidation discovered in a denitrifying fluidized bed reactor. *FEMS Microbiology Ecology, 16*(3), 177-183. doi:https://doi.org/10.1016/0168-6496(94)00081-7

Neuwirth, E. (2014). RColorBrewer: ColorBrewer Palettes. . *R package version 1.1-2.*

Ni, S.-Q., Lee, P.-H., Fessehaie, A., Gao, B.-Y., & Sung, S. (2010). Enrichment and biofilm formation of Anammox bacteria in a non-woven membrane reactor. *Bioresource Technology, 101*(6), 1792-1799. doi:https://doi.org/10.1016/j.biortech.2009.10.050

Ni, S.-Q., Ni, J.-Y., Hu, D.-L., & Sung, S. (2012). Effect of organic matter on the performance of granular anammox process. *Bioresource Technology, 110*, 701-705. doi:https://doi.org/10.1016/j.biortech.2012.01.066

Nielsen, P. H., Mielczarek, A. T., Kragelund, C., Nielsen, J. L., Saunders, A. M., Kong, Y., Hansen, A. A., & Vollertsen, J. (2010). A conceptual ecosystem model of microbial communities in enhanced biological phosphorus removal plants. *Water Research, 44*(17), 5070-5088. doi:https://doi.org/10.1016/j.watres.2010.07.036

Nittami, T., McIlroy, S., Seviour, E. M., Schroeder, S., & Seviour, R. J. (2009). Candidatus Monilibacter spp., common bulking filaments in activated sludge, are members of Cluster III Defluviicoccus. *Systematic and Applied Microbiology, 32*(7), 480-489. doi:https://doi.org/10.1016/j.syapm.2009.07.003

Noda, N., Kaneko, N., Milkami, M., Kimochi, Y., Tsuneda, S., Hirata, A., Mizuochi, M., & Inamori, Y. (2003). Effects of SRT and DO on N2O reductase activity in an anoxic-oxic activated sludge system. *Water Science and Technology, 48*(11-12), 363-370.

Oehmen, A., Lemos, P. C., Carvalho, G., Yuan, Z., Keller, J., Blackall, L. L., & Reis, M. A. M. (2007). Advances in enhanced biological phosphorus removal: From micro to macro scale. *Water Research, 41*(11), 2271-2300. doi:https://doi.org/10.1016/j.watres.2007.02.030

Oehmen, A., Saunders, A. M., Vives, M. T., Yuan, Z., & Keller, J. (2006). Competition between polyphosphate and glycogen accumulating organisms in enhanced biological phosphorus removal systems with acetate and propionate as carbon sources. *Journal of Biotechnology, 123*(1), 22-32. doi:https://doi.org/10.1016/j.jbiotec.2005.10.009

Osaka, T., Yoshie, S., Tsuneda, S., Hirata, A., Iwami, N., & Inamori, Y. (2006). Identification of Acetate- or Methanol-Assimilating Bacteria under Nitrate-Reducing Conditions by Stable-Isotope Probing. *Microbial Ecology, 52*(2), 253-266. doi:10.1007/s00248-006-9071-7

Oshiki, M., Shimokawa, M., Fujii, N., Satoh, H., & Okabe, S. (2011). Physiological characteristics of the anaerobic ammonium-oxidizing bacterium 'Candidatus Brocadia sinica'. *Microbiology, 157*(6), 1706-1713. doi:doi:10.1099/mic.0.048595-0

Pagnier, I., Raoult, D., & La Scola, B. (2011). Isolation and characterization of Reyranella massiliensis gen. nov., sp nov from freshwater samples by

using an amoeba co-culture procedure. *International Journal of Systematic and Evolutionary Microbiology, 61*, 2151-2154. doi:10.1099/ijs.0.025775-0

Park, H.-D., & Noguera, D. R. (2004). Evaluating the effect of dissolved oxygen on ammonia-oxidizing bacterial communities in activated sludge. *Water Research, 38*(14), 3275-3286. doi:https://doi.org/10.1016/j.watres.2004.04.047

Peng, Y.-z., Ma, Y., & Wang, S.-y. (2007). Denitrification potential enhancement by addition of external carbon sources in a pre-denitrification process. *Journal of Environmental Sciences, 19*(3), 284-289. doi:https://doi.org/10.1016/S1001-0742(07)60046-1

Philips, S., Wyffels, S., Sprengers, R., & Verstraete, W. (2002). Oxygen-limited autotrophic nitrification/denitrification by ammonia oxidisers enables upward motion towards more favourable conditions. *Applied Microbiology and Biotechnology, 59*(4), 557-566. doi:10.1007/s00253-002-1059-8

Purkhold, U., Pommerening-Roser, A., Juretschko, S., Schmid, M. C., Koops, H. P., & Wagner, M. (2000). Phylogeny of all recognized species of ammonia oxidizers based on comparative 16S rRNA and amoA sequence analysis: Implications for molecular diversity surveys. *Applied and Environmental Microbiology, 66*(12), 5368-5382. doi:10.1128/aem.66.12.5368-5382.2000

Purkhold, U., Wagner, M., Timmermann, G., Pommerening-Roser, A., & Koops, H. P. (2003). 16S rRNA and amoA-based phylogeny of 12 novel betaproteobacterial ammonia-oxidizing isolates: extension of the dataset and proposal of a new lineage within the nitrosomonads. *International Journal of Systematic and Evolutionary Microbiology, 53*, 1485-1494. doi:10.1099/ijs.0.02638-0

Puyol, D., Carvajal-Arroyo, J. M., Sierra-Alvarez, R., & Field, J. A. (2014). Nitrite (not free nitrous acid) is the main inhibitor of the anammox process at common pH conditions. *Biotechnology Letters, 36*(3), 547-551. doi:10.1007/s10529-013-1397-x

Pynaert, K., Sprengers, R., Laenen, J., & Verstraete, W. (2002). Oxygen-Limited Nitrification and Denitrification in a Lab-Scale Rotating Biological Contactor. *Environmental Technology, 23*(3), 353-362. doi:10.1080/09593332508618419

Rattray, J. E., van de Vossenberg, J., Hopmans, E. C., Kartal, B., van Niftrik, L., Rijpstra, W. I. C., Strous, M., Jetten, M. S. M., Schouten, S., & Damste, J. S. S. (2008). Ladderane lipid distribution in four genera of anammox bacteria. *Archives of Microbiology, 190*(1), 51-66. doi:10.1007/s00203-008-0364-8

Rodríguez-Gallego, L., Achkar, M., Defeo, O., Vidal, L., Meerhoff, E., & Conde, D. (2017). Effects of land use changes on eutrophication indicators in five coastal lagoons of the Southwestern Atlantic Ocean. *Estuarine, Coastal and Shelf Science, 188*, 116-126. doi:https://doi.org/10.1016/j.ecss.2017.02.010

Saunders, A. M., Hansen, A. A., McIlroy, B., Nielsen, J. L., Albertsen, M., Nierychlo, M., McIlroy, S. J., Karst, S. M., & Nielsen, P. H. (2015). MiDAS: the field guide to the microbes of activated sludge. *Database, 2015*. doi:10.1093/database/bav062

Schmidt, I., Sliekers, O., Schmid, M., Bock, E., Fuerst, J., Kuenen, J. G., Jetten, M. S. M., & Strous, M. (2003). New concepts of microbial treatment processes for the nitrogen removal in wastewater. *FEMS Microbiology Reviews, 27*(4), 481-492. doi:https://doi.org/10.1016/S0168-6445(03)00039-1

Sezgin, M., Jenkins, D., & Parker, D. S. (1978). A Unified Theory of Filamentous Activated Sludge Bulking. *Journal (Water Pollution Control Federation), 50*(2), 362-381.

Shade, A., & Handelsman, J. (2012). Beyond the Venn diagram: the hunt for a core microbiome. *Environmental Microbiology, 14*(1), 4-12. doi:10.1111/j.1462-2920.2011.02585.x

Sinninghe Damsté, J. S., Strous, M., Rijpstra, W. I. C., Hopmans, E. C., Geenevasen, J. A. J., van Duin, A. C. T., van Niftrik, L. A., & Jetten, M. S. M. (2002). Linearly concatenated cyclobutane lipids form a dense bacterial membrane. *Nature, 419*, 708. doi:10.1038/nature01128 https://www.nature.com/articles/nature01128#supplementary-information

Smith, V. H., Tilman, G. D., & Nekola, J. C. (1999). Eutrophication: impacts of excess nutrient inputs on freshwater, marine, and terrestrial ecosystems. *Environmental Pollution, 100*(1), 179-196. doi:https://doi.org/10.1016/S0269-7491(99)00091-3

Speirs, L., Nittami, T., McIlroy, S., Schroeder, S., & Seviour, R. (2009). *Filamentous Bacterium Eikelboom Type 0092 in Activated Sludge Plants in Australia Is a Member of the Phylum Chloroflexi* (Vol. 75).

Speirs, L., Nittami, T., McIlroy, S., Schroeder, S., & Seviour, R. J. (2009). Filamentous Bacterium Eikelboom Type 0092 in Activated Sludge Plants in Australia Is a Member of the Phylum Chloroflexi. *Applied and Environmental Microbiology, 75*(8), 2446-2452. doi:10.1128/aem.02310-08

Strous, M., Heijnen, J. J., Kuenen, J. G., & Jetten, M. S. M. (1998). The sequencing batch reactor as a powerful tool for the study of slowly growing anaerobic

ammonium-oxidizing microorganisms. *Applied Microbiology and Biotechnology, 50*(5), 589-596. doi:10.1007/s002530051340

Strous, M., Kuenen, J. G., & Jetten, M. S. M. (1999). Key physiology of anaerobic ammonium oxidation. *Applied and Environmental Microbiology, 65*(7), 3248-3250.

Strous, M., Pelletier, E., Mangenot, S., Rattei, T., Lehner, A., Taylor, M. W., Horn, M., Daims, H., Bartol-Mavel, D., Wincker, P., Barbe, V., Fonknechten, N., Vallenet, D., Segurens, B., Schenowitz-Truong, C., Médigue, C., Collingro, A., Snel, B., Dutilh, B. E., Op den Camp, H. J. M., van der Drift, C., Cirpus, I., van de Pas-Schoonen, K. T., Harhangi, H. R., van Niftrik, L., Schmid, M., Keltjens, J., van de Vossenberg, J., Kartal, B., Meier, H., Frishman, D., Huynen, M. A., Mewes, H.-W., Weissenbach, J., Jetten, M. S. M., Wagner, M., & Le Paslier, D. (2006). Deciphering the evolution and metabolism of an anammox bacterium from a community genome. *Nature, 440*, 790. doi:10.1038/nature04647 https://www.nature.com/articles/nature04647#supplementary-information

Strous, M., Van Gerven, E., Kuenen, J. G., & Jetten, M. (1997). Effects of aerobic and microaerobic conditions on anaerobic ammonium-oxidizing (anammox) sludge. *Applied and Environmental Microbiology, 63*(6), 2446-2448.

Sun, Y. X., Shen, D. D., Zhou, X. L., Shi, N., & Tian, Y. (2016). Microbial diversity and community structure of denitrifying biological filters operated with different carbon sources. *Springerplus, 5*. doi:10.1186/s40064-016-3451-3

Tang, C.-J., Zheng, P., Hu, B.-L., Chen, J.-W., & Wang, C.-H. (2010). Influence of substrates on nitrogen removal performance and microbiology of anaerobic ammonium oxidation by operating two UASB reactors fed with different substrate levels. *Journal of Hazardous Materials, 181*(1), 19-26. doi:https://doi.org/10.1016/j.jhazmat.2010.04.015

Tang, C.-j., Zheng, P., Wang, C.-h., & Mahmood, Q. (2010). Suppression of anaerobic ammonium oxidizers under high organic content in high-rate Anammox UASB reactor. *Bioresource Technology, 101*(6), 1762-1768. doi:https://doi.org/10.1016/j.biortech.2009.10.032

Team, R. (2016). RStudio: Integrated Development for R. Retrieved from http://www.rstudio.com/

Third, K. A., Sliekers, A. O., Kuenen, J. G., & Jetten, M. S. M. (2001). The CANON System (Completely Autotrophic Nitrogen-removal Over Nitrite) under Ammonium Limitation: Interaction and Competition between Three Groups of Bacteria. *Systematic and Applied Microbiology, 24*(4), 588-596. doi:https://doi.org/10.1078/0723-2020-00077

Thomsen, T. R., Kong, Y., & Nielsen, P. H. (2007). Ecophysiology of abundant denitrifying bacteria in activated sludge. *FEMS Microbiology Ecology, 60*(3), 370-382. doi:10.1111/j.1574-6941.2007.00309.x

Torà, J. A., Lafuente, J., Baeza, J. A., & Carrera, J. (2010). Combined effect of inorganic carbon limitation and inhibition by free ammonia and free nitrous acid on ammonia oxidizing bacteria. *Bioresource Technology, 101*(15), 6051-6058. doi:https://doi.org/10.1016/j.biortech.2010.03.005

Ushiki, N., Fujitani, H., Aoi, Y., & Tsuneda, S. (2013). Isolation of Nitrospira belonging to Sublineage II from a Wastewater Treatment Plant. *Microbes and Environments, 28*(3), 346-353. doi:10.1264/jsme2.ME13042

van der Star, W. R. L., Abma, W. R., Blommers, D., Mulder, J.-W., Tokutomi, T., Strous, M., Picioreanu, C., & van Loosdrecht, M. C. M. (2007). Startup of reactors for anoxic ammonium oxidation: Experiences from the first full-scale anammox reactor in Rotterdam. *Water Research, 41*(18), 4149-4163. doi:https://doi.org/10.1016/j.watres.2007.03.044

van der Star, W. R. L., Miclea, A. I., van Dongen, U., Muyzer, G., Picioreanu, C., & van Loosdrecht, M. C. M. (2008). The membrane bioreactor: A novel tool to grow anammox bacteria as free cells. *Biotechnology and Bioengineering, 101*(2), 286-294. doi:10.1002/bit.21891

Van Hulle, S. W. H., Vandeweyer, H. J. P., Meesschaert, B. D., Vanrolleghem, P. A., Dejans, P., & Dumoulin, A. (2010). Engineering aspects and practical application of autotrophic nitrogen removal from nitrogen rich streams. *Chemical Engineering Journal, 162*(1), 1-20. doi:https://doi.org/10.1016/j.cej.2010.05.037

van Niftrik, L., & Jetten, M. S. M. (2012). Anaerobic Ammonium-Oxidizing Bacteria: Unique Microorganisms with Exceptional Properties. *Microbiology and Molecular Biology Reviews, 76*(3), 585-+. doi:10.1128/mmbr.05025-11

van Niftrik, L. A., Fuerst, J. A., Damste, J. S. S., Kuenen, J. G., Jetten, M. S. M., & Strous, M. (2004). The anammoxosome: an intracytoplasmic compartment in anammox bacteria. *Fems Microbiology Letters, 233*(1), 7-13. doi:10.1016/j.femsle.2004.01.044

Villaverde, S., García-Encina, P. A., & Fdz-Polanco, F. (1997). Influence of pH over nitrifying biofilm activity in submerged biofilters. *Water Research, 31*(5), 1180-1186. doi:https://doi.org/10.1016/S0043-1354(96)00376-4

Wagner, M., Loy, A., Nogueira, R., Purkhold, U., Lee, N., & Daims, H. (2002). Microbial community composition and function in wastewater treatment plants. *Antonie Van Leeuwenhoek International Journal of General and Molecular Microbiology, 81*(1-4), 665-680. doi:10.1023/a:1020586312170

Wang, Q., Garrity, G. M., Tiedje, J. M., & Cole, J. R. (2007). Naive Bayesian classifier for rapid assignment of rRNA sequences into the new bacterial taxonomy. *Appl Environ Microbiol, 73*(16), 5261-5267. doi:10.1128/aem.00062-07

Wang, X., Hu, M., Xia, Y., Wen, X., & Ding, K. (2012). Pyrosequencing Analysis of Bacterial Diversity in 14 Wastewater Treatment Systems in China. *Applied and Environmental Microbiology, 78*(19), 7042-7047. doi:10.1128/aem.01617-12

Wett, B., Podmirseg, S. M., Gomez-Brandon, M., Hell, M., Nyhuis, G., Bott, C., & Murthy, S. (2015). Expanding DEMON Sidestream Deammonification Technology Towards Mainstream Application. *Water Environment Research, 87*(12), 2084-2089. doi:10.2175/106143015x14362865227319

Wilén, B.-M., & Balmér, P. (1999). The effect of dissolved oxygen concentration on the structure, size and size distribution of activated sludge flocs. *Water Research, 33*(2), 391-400. doi:https://doi.org/10.1016/S0043-1354(98)00208-5

Wong, M. T., & Liu, W. T. (2007). Ecophysiology of Defluviicoccus-related tetrad-forming organisms in an anaerobic-aerobic activated sludge process. *Environmental Microbiology, 9*(6), 1485-1496. doi:10.1111/j.1462-2920.2007.01267.x

Wunderlin, P., Mohn, J., Joss, A., Emmenegger, L., & Siegrist, H. (2012). Mechanisms of N2O production in biological wastewater treatment under nitrifying and denitrifying conditions. *Water Research, 46*(4), 1027-1037. doi:https://doi.org/10.1016/j.watres.2011.11.080

Xia, S. Q., Duan, L. A., Song, Y. H., Li, J. X., Piceno, Y. M., Andersen, G. L., Alvarez-Cohen, L., Moreno-Andrade, I., Huang, C. L., & Hermanowicz, S. W. (2010). Bacterial Community Structure in Geographically Distributed Biological Wastewater Treatment Reactors. *Environmental Science & Technology, 44*(19), 7391-7396. doi:10.1021/es101554m

Xu, S., Yao, J., Ainiwaer, M., Hong, Y., & Zhang, Y. (2018). Analysis of Bacterial Community Structure of Activated Sludge from Wastewater Treatment Plants in Winter. *BioMed Research International, 2018*, 8. doi:10.1155/2018/8278970

Yamada, T., & Sekiguchi, Y. (2009). Cultivation of Uncultured Chloroflexi Subphyla: Significance and Ecophysiology of Formerly Uncultured Chloroflexi 'Subphylum I' with Natural and Biotechnological Relevance. *Microbes and Environments, 24*(3), 205-216. doi:10.1264/jsme2.ME09151S

Ye, L., & Zhang, T. (2013). Bacterial communities in different sections of a municipal wastewater treatment plant revealed by 16S rDNA 454

pyrosequencing. *Applied Microbiology and Biotechnology, 97*(6), 2681-2690. doi:10.1007/s00253-012-4082-4

Yin, Z. X., dos Santos, C. E. D., Vilaplana, J. G., Sobotka, D., Czerwionka, K., Damianovic, M., Xie, L., Morales, F. J. F., & Makinia, J. (2016). Importance of the combined effects of dissolved oxygen and pH on optimization of nitrogen removal in anammox-enriched granular sludge. *Process Biochemistry, 51*(9), 1274-1282. doi:10.1016/j.procbio.2016.05.025

Zhang, B., Xu, X. Y., & Zhu, L. (2017). Structure and function of the microbial consortia of activated sludge in typical municipal wastewater treatment plants in winter. *Scientific Reports, 7*. doi:10.1038/s41598-017-17743-x

Zhang, B., Yu, Q. W., Yan, G. Q., Zhu, H. B., Xu, X. Y., & Zhu, L. (2018). Seasonal bacterial community succession in four typical wastewater treatment plants: correlations between core microbes and process performance. *Scientific Reports, 8*. doi:10.1038/s41598-018-22683-1

Zhang, T. C., & Bishop, P. L. (1996). Evaluation of substrate and pH effects in a nitrifying biofilm. *Water Environment Research, 68*(7), 1107-1115. doi:10.2175/106143096x128504

Zhang, Y., He, S., Niu, Q., Qi, W., & Li, Y.-Y. (2016). Characterization of three types of inhibition and their recovery processes in an anammox UASB reactor. *Biochemical Engineering Journal, 109*, 212-221. doi:https://doi.org/10.1016/j.bej.2016.01.022

Zhang, Y. Y., Islam, M. S., McPhedran, K. N., Dong, S. M., Rashed, E. M., El-Shafei, M. M., Noureldin, A. M., & El-Din, M. G. (2017). A comparative study of microbial dynamics and phosphorus removal for a two side-stream wastewater treatment processes. *Rsc Advances, 7*(73), 45938-45948. doi:10.1039/c7ra07610j

Zhou, Y., Oehmen, A., Lim, M., Vadivelu, V., & Ng, W. J. (2011). The role of nitrite and free nitrous acid (FNA) in wastewater treatment plants. *Water Research, 45*(15), 4672-4682. doi:https://doi.org/10.1016/j.watres.2011.06.025

Zumft, W. G. (1997). Cell biology and molecular basis of denitrification. *Microbiology and Molecular Biology Reviews, 61*(4), 533-+.

Appendix

Table 1. Raw Tahuna WWTP biochemical data.

Sample_ID	Date	DO	pH	ORP	TP	DRP	NH4_N	NO2_N	NO3_N	Temp
Post_Septic_04	28/09/2017	0.99	6.93	-244	8.44	4.64	43.5	NA	0.44	16.2
Post_Septic_77	31/10/2017	0.61	6.93	-272	7.3	2.91	24.2	0.119	0.848	17.5
Post_Septic_07	18/01/2018	0.17	NA	-264	7.29	5.99	50.4	0.06	0.42	20.6
Post_Septic_81	12/02/2018	0.28	6.57	-150	2.05	1.32	9.47	0.2	0.82	21.2
Post_Septic_10	22/03/2018	0.06	6.77	-224	12.7	4.48	5.3	0.05	0.33	20.4
Post_Septic_12	2/05/2018	0.73	6.62	-239	4.86	3.62	31.7	0.02	0.36	19.5
Post_Septic_20	28/07/2018	0.5	6.88	-208	3.71	2.32	2.56	0.03	0.28	15.1
Post_Septic_88	30/08/2018	0.26	6.9	-156	4.44	3.51	29.3	0.13	3.55	14.9
Post_Septic_13	9/10/2018	0.7	6.87	-247	6.16	4.79	40.5	0.05	0.5	16.9
Anoxic_1_35	28/09/2017	0.035	7.61	-127	15.6	1.235	3.21	0.015	0.3	17.9
Anoxic_1_06	18/01/2018	0.1	NA	-119.5	15.18	10.175	3.27	0.015	0.26	23.4
Anoxic_1_85	12/02/2018	0.04	7.63	-29.5	31.6	8.485	0.375	0.01	0.255	22.95
Anoxic_1_49	22/03/2018	0.16	7.575	-115	18.85	9.755	2.905	0.02	0.295	22.85
Anoxic_1_53	2/05/2018	0.725	6.6	-119	13	6.535	3.66	0.015	0.305	20.6
Anoxic_1_66	28/07/2018	2.12	7.54	-98.5	11.225	3.995	1.64	0.115	1.045	15.9
Anoxic_1_89	30/08/2018	0.325	7.135	-68	15.1	9.12	3.175	0.01	0.22	16.35
Anoxic_1_23	9/10/2018	0.755	7.14	-116	30.7	12.675	3.24	0.01	0.335	16.7
Anoxic_1_171031_53	31/10/2017	0.075	7.515	-111.5	27.9	6.37	2.685	0.0335	0.3355	18.3
Anoxic_1_171207_78	7/12/2017	0.165	7.275	-225.5	15.1	8.36	3.3	0.02	0.35	23.05
Aerobic_36	28/09/2017	1.965	7.81	-39	14.7	2.62	2.185	0.02	1.145	18
Aerobic_37	18/01/2018	0.145	NA	-96	20.7	8.435	3.255	0.015	0.265	23.8
Aerobic_41	12/02/2018	0.095	7.83	-11	29.85	5.96	0.585	0.01	1	22.9
Aerobic_55	22/03/2018	0.065	NA	-108	15.35	6.415	1.615	0.02	0.345	23.05

Aerobic_24	2/05/2018	0.875	7.04	-97.5	13.95	6.53	2.8	0.015	0.285	20.7
Aerobic_72	28/07/2018	3.37	7.61	-78.5	9.405	3.7	1.95	0.05	0.585	15.9
Aerobic_17	30/08/2018	0.295	7.14	-50.5	22.2	10.845	3.15	0.01	0.3	16.5
Aerobic_48	9/10/2018	0.685	7.215	-122	30.8	9.245	2.2	0.01	0.36	16.65
Aerobic_171031_90	31/10/2017	0.265	8.425	12.5	27.4	4.265	1.06	0.054	0.476	18.4
Anoxic_2_03	28/09/2017	0.11	7.27	-33.5	13	0.185	2.12	0.025	1.26	18.1
Anoxic_2_44	18/01/2018	0.025	NA	-161	21.15	11.6	3.255	0.01	0.23	23.85
Anoxic_2_08	12/02/2018	0.03	7.755	-97.5	33.1	7.27	1.58	0.01	0.23	23
Anoxic_2_50	22/03/2018	0.115	7.58	-149	18.55	8.84	3.25	0.04	0.44	23.1
Anoxic_2_56	2/05/2018	0.03	7.335	-159.5	15.35	7.73	2.905	0.01	0.305	20.8
Anoxic_2_90	28/07/2018	4.015	7.61	-76	10.07	3.885	1.89	0.095	0.4	15.9
Anoxic_2_71	30/08/2018	0.135	7.145	-53	22.6	10.4	2.985	0.01	0.28	16.6
Anoxic_2_05	9/10/2018	0.13	7.305	-147	32.9	7.085	2.11	0.02	0.375	17.35
Anoxic_2_171031_58	31/10/2017	0.66	7.73	-128.5	22.1	5.5	0.7805	0.0185	0.3975	18.55
anoxic_2_171207_91	17/12/2017	0.02	7.27	-154	15.25	8.76	3.33	0.02	0.49	23.3
MBR_34	28/07/2018	5.52	7.31	25	14.7	0.19	0.43	0.02	4.62	18.2
MBR_40	18/01/2018	3.48	NA	82	9.4	7.72	0.49	0.02	5.35	24
MBR_45	12/02/2018	5.84	7.84	101	36	6.69	0.42	0.02	3.51	23.2
MBR_09	22/03/2018	2.7	7.84	71	11.4	2.78	0.11	0.05	6.41	23.3
MBR_11	2/05/2018	3.25	7.58	94	16.4	0.07	0.26	0.02	3.4	20.9
MBR_78	28/07/2018	6.56	7.62	57	7.65	4.71	0.07	0.01	4.9	15.9
MBR_19	30/08/2018	1.43	7.51	54	14.4	2.76	0.22	0.45	0.84	16.8
MBR_80	9/10/2018	3.55	7.77	94	32.8	0.47	0.13	0.23	1.78	18.3
MBR_171207_21	7/12/2017	6.37	8	102	29.2	4.58	0.056	0.092	1.24	18.7
MBR_171031_11	31/10/2017	1.54	7.44	136	17.5	3.56	0.85	0.03	3.01	23.6

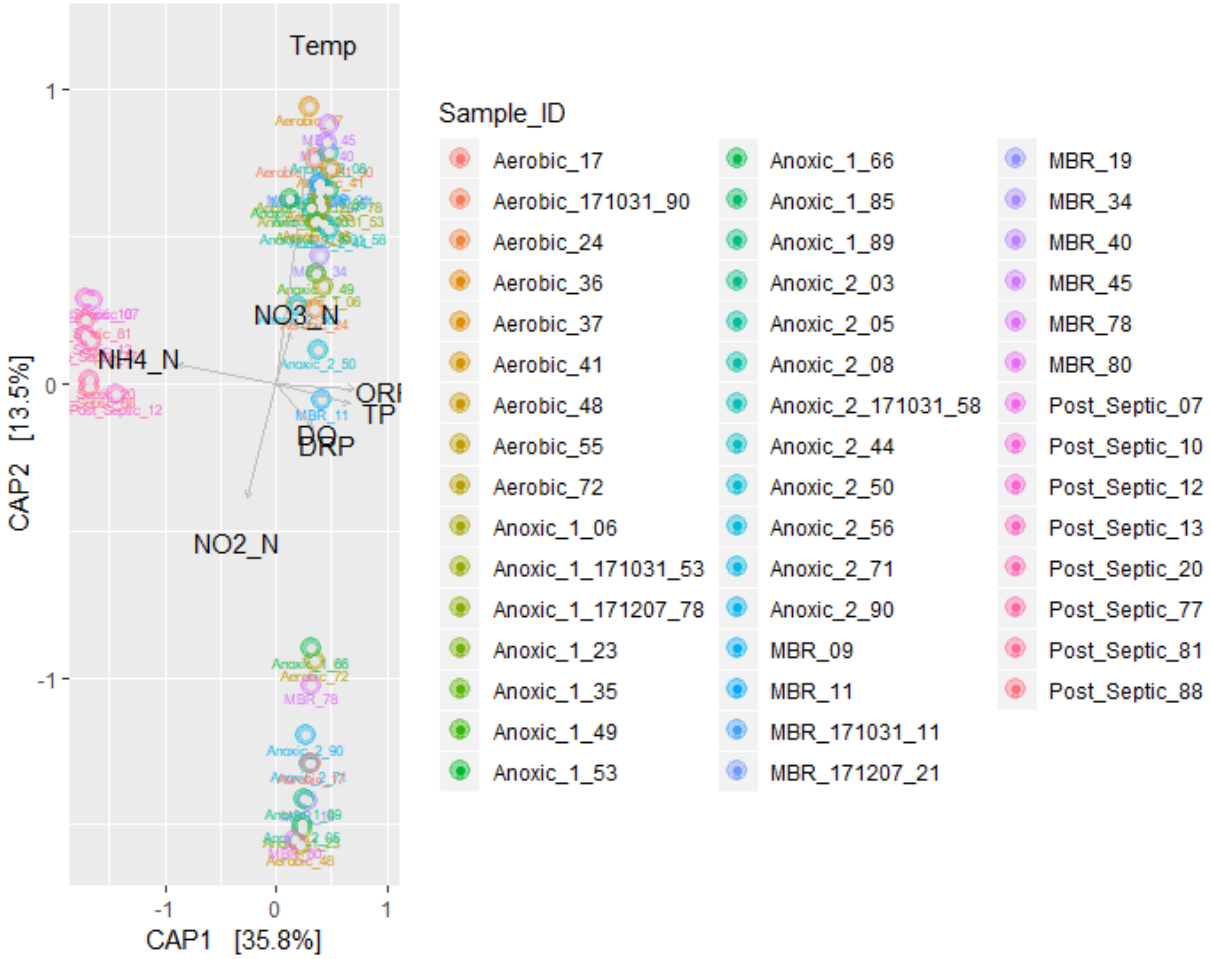

Figure 38. CAP plot showing correlation with biochemical parameters.

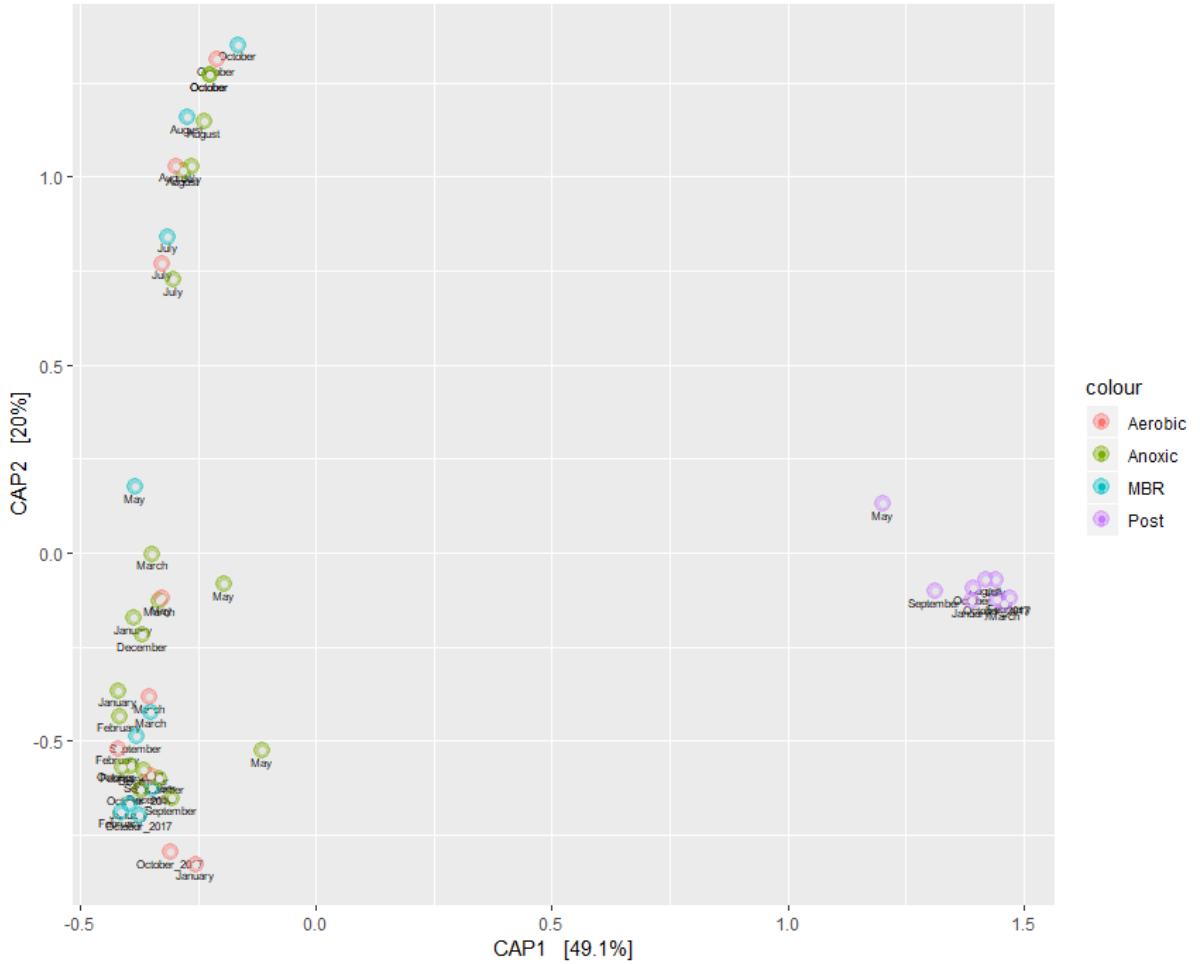

Figure 39. CAP plot showing the similarity between tanks

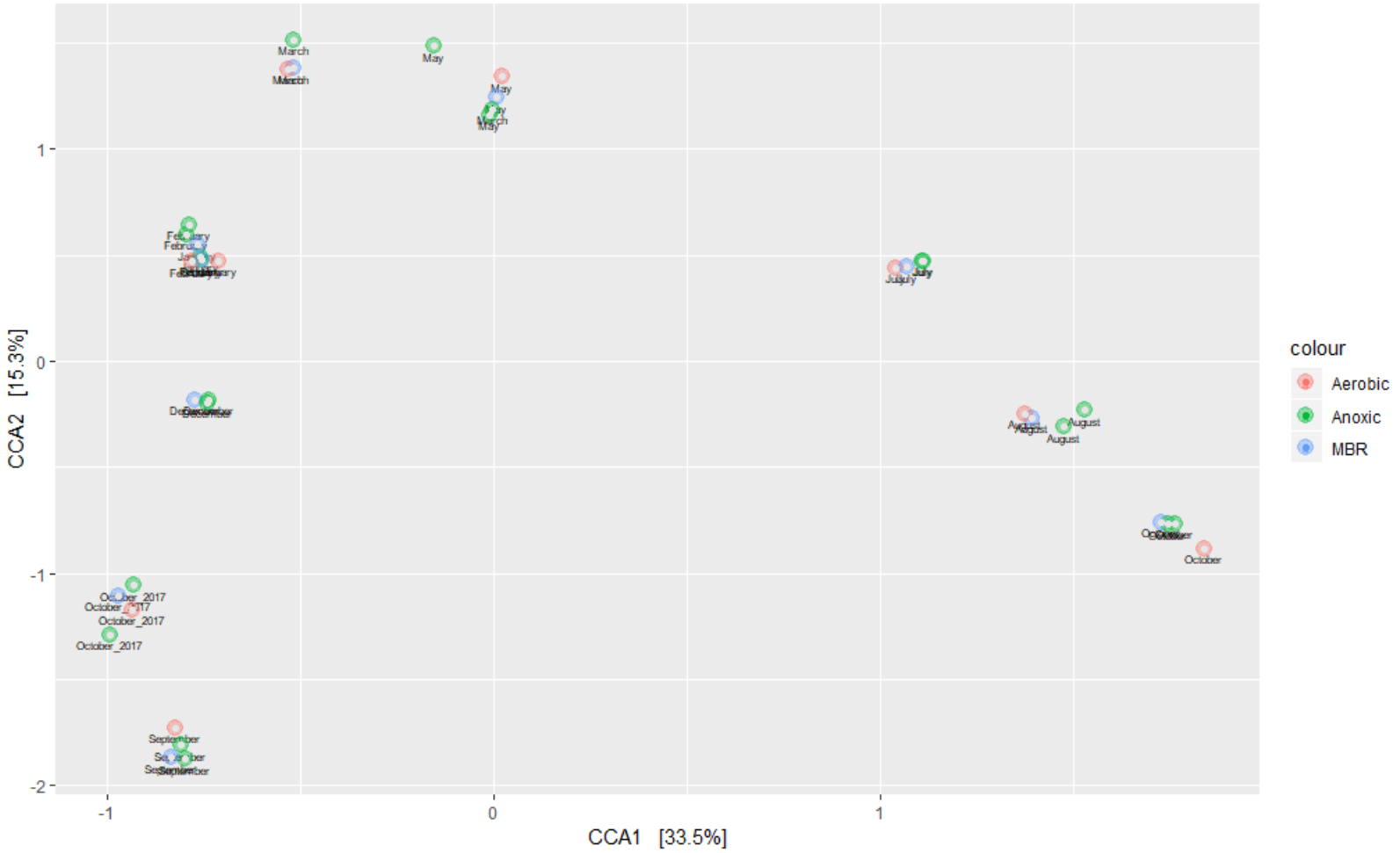

Figure 40. CCA plot showing the differences between tanks, excluding the post-septic tanks

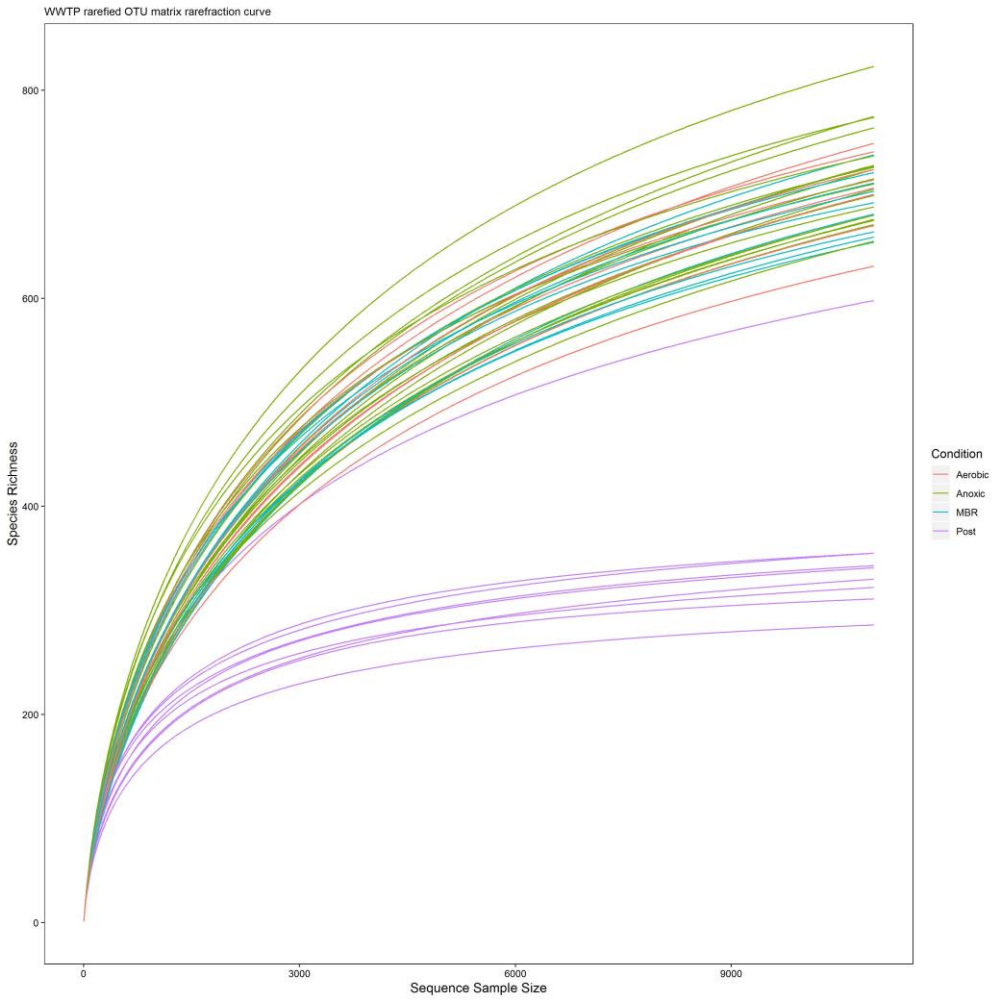

Figure 41. WWTP rarefied OTU matrix rarefraction curve, shows that that almost all the diversity in the WWTP plant was observed.