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IRVINE

DISSERTATION

Submitted in Partial Satisfaction of the Requirements for the Degree of

DOCTOR OF PHILOSOPHY

in Engineering

by

Tongzhou Wang

Dissertation Committee

Professor Betty H. Olson, Chair

Professor Diego Rosso

Professor Sunny C. Jiang

2014

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To

My Family

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ACKNOWLEDGEMENTS

I want to thank God for all of the gifts that he has given in my life: my wonderful parents, my beautiful and supportive wife, my son, and the strength and belief to overcome difficult times in life.

I am extremely grateful for Professor Betty H. Olson's support, including TAships and guidance over the past five years. She has taught me a lot about patience and how to deal with difficult situations in life. She always encouraged me with passion and dignity: "We will worry about it when we get there." Even though I had to leave school to work abroad about a year and half, she still emailed me to come back and finish doctoral degree. Without such support, I would not be able to achieve this true accomplishment.

Professors Diego Rosso and Sunny Jiang also have given me great insights about the field of wastewater engineering and microbiology. Dr. Rosso was always willing to sacrifice his time and set up meetings with me to discuss specific matters in my research. Moreover, I want to thank Dr. Jiang and the department that provided me two quarters of departmental scholarship/funding when I was second year graduate student at UC Irvine. I am extremely thankful that they were able to give several opportunities throughout my oral exam and final PhD defense so my work can be of top quality.

I would also like to thank the operators and laboratory personnel at Michelson Water Reclamation Plant for their support in providing data and assistance in collecting samples. This research is funded by National Water Research Institute (Award number no. 08-TM.004).

Completion of this PhD degree has helped me come to realize that pursuing a career in research is not suitable for me nor will it bring me long term happiness. Therefore, I will continue my career as an educator, since I have been extremely passionate about teaching for past ten years. I want to thank everyone who helped me come to this self-realization: Pitiporn Asvapathanagul, Chengyao Tsai, Philip Gedalanga, and Zachary Scott.

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ABSTRACT OF THE DISSERTATION

Understanding of Nitrifying and Denitrifying Bacterial Population Dynamics in an Activated Sludge Process

By

Tongzhou Wang

Doctor of Philosophy in Engineering

University of California, Irvine, 2014

Professor Betty H. Olson, Chair

Nitrogen removal from wastewater has been an important objective in treatment since the 1960s and is one of the most important biological processes used. The progression of knowledge has evolved in stages moving from simple stoichiometric equations into the modern activated sludge models of today. These models use surrogates such as volatile solids for biomass and outcome parameters such as nitrate and nitrite in the secondary effluent to simulate biological activity. Thus, even the most complex models fail to capture the cyclical

nature of bacterial abundance and the operating parameters which drive these cycles in full-scale plants. Better understanding of microbial communities has been attempted through the application of florescent in situ hybridization (FISH), which has determined the presence of specific organisms and the distributions of nitrifying and denitrifying populations within a single grab sample. New techniques such as quantitative polymerase chain reaction (qPCR) allowed the identification and quantification of nitrifying and denitrifying bacterial populations over time in full- scale plants. This has permitted the determination of relationships between organisms and operating parameters, which is missing from the majority of earlier microbial studies of wastewater treatment processes.

Intense monitoring of bacterial populations involved in nitrification and denitrification was used in this dissertation to identify and illustrate how application of these molecular tools can be used improve plant performance. The overall findings of this study showed that plant performance should be optimized seasonally for maximum nitrification and to maximize denitrification anoxic dissolved oxygen needs to be carefully monitored during the winter and spring to prevent excess oxygen from inhibiting denitrification activity. Furthermore, this study suggests that consortia of bacterial groups carried out denitrification and no one single group could be identified which represented more than 50% of the population. This latter finding suggests that interactions, of what might otherwise be considered as minor groups, become important in understanding overall influences on the denitrification process. This

was shown by the inhibition of the abundance of denitrifying bacteria through the production of nitrite by a bulking organism (*Thiothrix eikelboomii*).

In the first study, we determined the nitrifying populations (ammonia oxidizing bacteria, *Nitrobacter* spp. and *Nitrospira* spp.) and the total bacterial population were most affected by five of the major physicochemical parameters. Water temperature, nitrite produced, nitrate produced, solids retention time, and pH were found to be the major physicochemical parameters controlling these bacterial dynamics. Two clusters in Principal Component 1(PC1) reflected a seasonality shift at 26.9^{o}C. Temperature was found to be the parameter most directly affecting all bacterial populations in the warmer seasons (July-December), while nitrite produced and pH showed direct negative impacts on the bacterial populations in the cooler seasons (January-June) in the principal component analysis plot. PC1 and PC2 together accounted for 59.8% of the total variance, and the first six Principal Components accounted for 90.2% of total variance. Nitrifying and total bacterial abundance were strictly dependent on temperature in the summer time and inhibited by pH and nitrite in the winter season. This study found SRT needs to be extended by approximately 3.6 days to achieve optimum nitrification and the reduction of the ammonia-oxidizing bacteria: nitrite-oxidizing bacteria ratio of 9.5:1 to 2:1, because the SRT is too short for the *Nitrobacter* spp. and *Nitrospira* spp. growth rates.

In the second study, two major denitrifying microbial groups, *Thauera*-like bacteria and *Zoogloea-Methyloversatilis*-like bacteria, which accounted for 34% on average of the total bacterial community measured using quantitative PCR (qPCR), were investigated in relation to the denitrification ability in a full scale plant. In this study of 11-months in warm wastewater (23-28.6 ^{o}C), dissolved oxygen (DO) in the anoxic zone was the most important parameter that determined denitrification efficiency when the temperature was below 27°C. *Zoogloea-Methyloversatilis*-like bacteria correlated significantly with denitrification (r= 0.52, $p < 0.05$) under hypoxic conditions (0.2 mg l^{-1} < DO < 0.6 mg l^{-1}), while *Thauera*-like bacteria abundance negatively correlated (r= -0.55, $p < 0.05$) with DO and significantly correlated with denitrification (r= 0.55, $p < 0.05$) under strict anoxic condition (DO < 0.2 mg l^{-1}). Methanol dosing only played an important role in the *Zoogloea-Methyloversatilis*-like bacterial abundance (r=0.45, $p < 0.05$) when temperature was above 27°C, and led to no correlation with *Thauera*-like bacteria. *Thauera*-like bacteria abundance correlated positively with COD removal when temperature was less than 27°C (r= 0.52, p< 0.05). Operating the anoxic zone of the plant in the higher DO range (0.2 mg l^{-1} < DO <0.6 mg l^{-1}) when the temperature was below 27°C reduced the overall denitrification and corresponded to a reduction in *Thauera*-like bacteria bacterial abundance. At temperatures above 27°C, the denitrification efficiency was independent of the DO level, with the same number of events of denitrification exceeding 70% for both hypoxic and strict anoxic conditions.

In the third study, we found that *Burkholderia*-like bacterial populations and *Paracoccus*-like bacterial populations were out grown by *Thiothrix Eikelboomii* (bulking organisms) during three different time periods (March, May, July). Anoxic DO was the most important factor creating negative impacts on the bacterial abundances of *Burkholderia*-like bacteria (r = -0.45, p $<$ 0.05) and *Paracoccus*-like bacteria (r = -0.46, $p < 0.05$). However, hypoxic DO positively correlated with *Thiothrix*-like bacteria (r =0.38, p <0.05). Nitrite accumulations also imposed a negative impact on *Paracoccus*-like bacteria (r = -0.33, p<0.05) but correlated positively with *Thiothrix Eikelboomii* (r = 0.44, $p < 0.05$), primarily due to fluctuations in the build-up of nitrite accumulation (up to 0.6 mg l^{-1}). Elevated anoxic dissolved oxygen (DO), nitrite buildup, and increasing temperature have significantly influenced the increasing population of bulking oraganisms and the suppressed population dynamics of denitrifying bacterial populations.

Chapter 1. Introduction

1.1 Background

Nitrogen (N_2) constitutes 78% of the atmosphere, making the nitrogen cycle one of the most important processes on the planet. The dynamics of nitrification and denitrification are subjects of continuing interest in the field of science and engineering. Historically, nitrogen removal in wastewater engineering under anoxic and aeration conditions was studied from the perspective of plant design and operation (Anthonisen et al., 1976, Mulder et al., 1995, Wild et al., 1995).

Recent microbial studies have been used to examine population abundance and diversity using a variety of molecular tools (Lee et al., 1999; MacGregor et al., 1999; Lee et al., 2008). Engineering studies have taken a number of approaches examining optimization of nitrogen removal including stoichiometric mass balances, bench scale studies on kinetics using pure and mixed cultures; and respirometric studies describing substrate utilization, oxygen uptake rates, and growth rates (Spanjers et al., 1995; Garcia-Ochoa, 2010).

Few studies have been conducted on full scale wastewater removal plants due to inability to control external factors that affect ammonia oxidation and carbon removal. Researchers usually extrapolate from laboratory studies to predict rates of removal in biological processes of pilot plants. However, Rittman et al. (1999) and Henze et al. (2000) developed activated sludge models to predict bacterial components such as heterotrophic and ammonia oxidizing

bacteria using fluorescent RNA oligonucleotide probes and then calculated the fraction of volatile suspended solids representing these organisms based on stoichiometric mass balances. With these findings, novel avenues of biomass representation remain to be explored. Direct measurements of bacterial populations associated with nutrient removal related to concurrent measurements of physicochemical parameters can provide an increased understanding of plant operation, since these groups actually drive the biological processes for carbon and nitrogen removal. However, ability to use the biomass to control biological processes becomes more complex, because these groups can use a variety of diverse substrates as is the case with autotrophic and heterotrophic bacteria.

Physicochemical parameters in wastewater treatment facilities are important to optimize efficiency of nitrogen removal and to determine types and abundances of bacterial populations. The abundance of bacterial populations determined in numerous bench, or pilot scale studies were highly dependent on various environmental and operational parameters such as temperature, pH, dissolved oxygen (DO), mixed liquor suspended solids (MLSS), hydraulic retention time (HRT), and solids retention time (SRT) (Prinčič et al., 1998; Randall et al., 1992; Grady & Lim, 1980; Narkis et al, 1978; Watson et al, 1981; Yoo et al., 1999).

For autotrophic bacteria, two main bacterial groupings, responsible for the oxidation of ammonia to nitrate are ammonia oxidizing bacteria (AOB) and nitrite oxidizing bacteria (NOB). *Nitrosomonas*-like populations are found to be the predominant genus among AOB

populations in the activated sludge process (Dionisi et al., 2002; Egli et al., 2003; Park and Noguera, 2004; Hallin et al., 2005; LaPara and Ghosh, 2006). Additionally, the effects of physicochemical parameters on nitrite oxidizing bacteria (NOB), specifically *Nitrospira spp*. and *Nitrobacter spp.* have also been studied in bench-scale reactors and full-scale wastewater treatment processes (Nogueira and Melo, 2006; Siripong and Rittmann, 2007; Huang et al., 2010). Several studies suggested that competition between *Nitrobacter spp*. and *Nitrospira spp*. were primarily dependent on dissolved oxygen (DO) (Schramm et al., 2000; Downing and Nerenberg et al., 2008), and nitrite concentrations (Balmelle et al., 1992; Kim and Kim, 2006; Blackburne et al., 2008; Dytczak et al., 2008). Changes in the communities of AOB and NOB populations were related to free ammonia concentrations (Hawkins et al., 2005; Lydmark, 2007) and temperature (Balmelle 1992; Hunik, 1993; Mauret et al., 1996; Yoo et al., 1999). AOB to NOB ratios ranged from greater than 85:1 in a bench scale study (Gieseke et al.; 2001) to a ratio of 2-3.5:1 in plants with optimized nitrification processes (Copp and Murphy, 1995; You et al. 2003; Dytezek et al., 2008).

Among bacterial populations of dentrifiers that convert nitrate to nitrogen gas, the genera of *Curvibacter*, *Thauera*, *Azoarcus* and *Zoogloea spp.* have been identified as the dominant genera in several industrial and municipal wastewater treatment plants in Northern Europe (Juretschko *et al*., 2002; Wagner and Loy, 2002; Thompsen et al, 2007). However, parameters that control dominant denitrifiers in each treatment process are still unclear. There has not

been any long term study on the distribution of these denitrifying populations, nor on the interrelationship among nitrifying and denitrifying populations as their abundances relate to a wide range of physicochemical parameters in a complete nitrification wastewater treatment plant.

The proposed study will use quantitative polymerase chain reaction (qPCR) due to the relative ease of sample preparation and analysis, low detection limits, and high levels of specificity (Graham et al., 2007). These qPCR results are compared to florescent in situ hybridization (FISH) (Tsushima et al., 2006) for nitrifying bacteria. Such a methodology can be applied to quantifying nitrifying and denitrifying bacteria to determine: 1) the optimum operating conditions in the nitrification and denitrification process for a specific plant; 2) important physicochemical factors that impact the abundance of these populations' biomass in wastewater treatment, 3) kinetic parameters in a dynamic system that can be adjusted resulting in better plant operation, especially if biomass of different groups can be measured directly and substituted for MLSS by operators for daily process optimization.

1.2 Research Motivation

Despite the problems in the initial design of the biological nitrogen removal process in activated sludge systems, research and development in the field of wastewater engineering combined anoxic biological denitrification with nitrification to carry out complete nitrogen removal. The bacterial biomass carrying out these functions was quantified using mixed

liquor suspended solids (MLSS) since the actual abundance could not be determined due to the lack of microbial tools. Wastewater treatment processes were mainly operated based on trial and error and long term experience of the engineers and operators.

With more stringent water quality control by the National Pollutant Discharge Elimination System on the nitrogen pollutants, such as nitrate and ammonia, being discharged into surface water. Optimization of wastewater treatment plant operations through control of the dynamics of nitrification and denitrification processes have become and remain an important topic within the scientific community. To date, estimated averages of bacterial abundance based on pure culture studies helped to determine optimum parameters such as half-saturation coefficient for substrates (K_s), half-saturation coefficient for oxygen (K_o), and growth rate (μ), while molecular techniques allowed engineers to determine optimum μ that are matched to HRT and SRT for ammonium oxidation. These methodologies have improved the optimization of treatment process over the past decades (Rittmann, 2001). However, population dynamics of denitrifying populations have still not been studied in any of the past research. Utilizing newly-developed microbial techniques to obtain nitrifying and denitrifying microbial indicators of nitrogen removal are important to understand their population dynamics throughout different seasons of the year. This will lead to adjustments to optimize nitrogen removal and will improve the efficiencies in long term operations of the activated sludge process.

1.3 Objectives and Hypothesis

The main purpose of this research is to understand the dynamics of major bacterial populations which correspond with nitrification and denitrification in a wastewater treatment plant. This research is also to identify the important physicochemical parameters that are driven by or impact the dynamics of various nitrifying and denitrifying populations within the activated sludge process using an 11-month study.

By examining the effect of various physicochemical variables in relation to nitrifying populations through different seasons, we identified key operational parameters including nitrite accumulation, nitrate accumulation, and pH that have significant negative impacts on the nitrifying populations (ammonia-oxidizing bacteria, *Nitrospira*, and *Nitrobacter*). The abundance ratios between AOB and NOB are strongly influenced by temperature. Since a greater AOB to NOB ratio can result in greater nitrite accumulation, we hypothesized that increasing SRT decreases the AOB to NOB ratios, which can lead to low nitrite accumulation. From on our results, we are able to determine the solution to develop an adequate AOB to NOB ratio in relation to SRT and temperature. This minimizes the nitrite accumulation and obtains optimization of nitrification process in the Southern California warm climate zone.

In our second research study on denitrifying populations, we examined the population dynamics of denitrifying organisms to understand the shift from anaerobic denitrification to aerobic denitrification and to compare efficiencies of each. By looking at the impact of DO

concentrations in the range of 0 mg l^{-1} < DO < 0.6 mg l^{-1} in the anoxic zone and bacterial abundance of predominant denitrifiers significant insights into improving denitrification efficiency can be achieved. Based on this interest, we hypothesized that

: a) lower temperature (22 ^{o}C to 26°C) and hypoxic DO above 0.1 mg l^{-1} have inhibited the growth of two denitrifying bacterial populations, *Thauera*-like bacteria and *Zoogloea-Methyloversatilis* which represented 34% of total bacterial population, b) *Thauera*-like bacteria are a better indicator of denitrification than *Zoogloea-Methyloversatilis*-like bacteria under warm temperature and strict anoxic DO condition, c) anoxic DO conditions favored the abundance of one of the major denitrifiers. Our study utilized quantitative polymerase chain reaction (qPCR) over an 11-month period to quantify two predominant and related genera *Zoogloea-Methyloversatilis*-like bacteria and *Thauera*-like bacteria in the denitrifying community of wastewater to test these hypotheses.

The last focal point of the research studying the population dynamics of bulking organism and denitrifying populations, enabled us to determine the key operational parameters that denitrifying bacterial populations in this treatment process with the optimum DO conditions in the anoxic zone. This information allows us to hypothesize that a nitrite producing bulking organisms (*Thiothrix eikelboomi*) with partial denitrification ability will out-compete denitrifiers under hypoxic DO conditions in a warm climate. By conducting the

qPCR experiment to quantify the abundance of a bulking organism (*Thiothrix eikelboomi*) and two groups of denitrifiers (*Paracoccus*-like bacteria and *Burkholderia*-like bacteria) and utilizing pearson correlational analyses of the bacterial abundance and environmental parameters, we are able to determine: a) physicochemical parameters causing bulking organisms to outgrow denitrifiers which are different than those that promote bulking , b) the solutions to prevent the growth of a bulking organism, and c) which of the bacterial groups of denitrifiers (aerobic or anaerobic) represent a better indicator for denitrification.

Utilizing abundance of bacterial populations through an application of a fine-scale qPCR technique as a surrogate for biomass in nitrification and denitrification processes provides an ealier indication of ongoing problems within the wastewater treatment process than the MLSS, which will be a continue refinement in wastewater modeling and operation.

1.4 Research Methodology

The research methodology involved the study of bacterial abundances and environmental and operational parameters in relation to nitrification and denitrification. This study took place over an 11-month period at a full-scale methanol-fed nitrification-denitrification activated sludge process and involved 42 DNA extractions in triplicate from environmental samples, primer development for four denitrifiers, and qPCR analysis on seven bacterial populations. Environmental and physicochemical parameters results were measured and provided by the wastewater treatment plant personnel at the Michelson Water Reclamation

Plant (MWRP), and the microbial populations of ammonia-oxidizing bacteria, nitrite-oxidizing bacteria (*Nitrobacter* spp. and *Nitrospira* spp.), and total bacteria were determined by quantitative polymerase chain reaction (qPCR) over the course of the period.

Pearson moment correlation coefficient analysis was used to calculate the interrelationships between nitrifying organisms, total bacterial populations, and different physicochemical parameters. Pearson moment correlation analysis also determined the relationships between bacterial abundance, methanol dose, and denitrification to identify if the major organisms are utilizing methanol to carry out denitrification.

Various denitrifying bacteria are also quantified using qPCR. Since denitrifying bacteria have been reported to represent 80% of the total bacterial population in the type of treatment in this study (Ferris et al., 1996), total bacteria are quantified using a universal primer and probe set that incorporated autotrophic and heterotrophic bacteria (Weisburg et al., 1991; Ferris et al., 1996). Four other denitrifying organisms were quantified and represented the genera of *Burkholderia*, *Paracoccus*, *Thauera*, and *Zoogloea-Methyloversatilis*. All of the primers and probes for these these denitrifying populations were developed by Chengyao Tsai (Tsai, 2012) and Tongzhou Wang (in this dissertation).

1.5 Organization of the dissertation

The dissertation is divided into five chapters. Chapter 2 reviews the literature and prior research concerning nitrification and denitrification. Description of specific pathways in

nitrification and denitrification is followed by a summary of predominant bacterial populations that have been previously identified across different geographic locations and found to be responsible for nitrification and denitrification in wastewater treatment plants.

Chapter 3 discusses the study of ammonia oxidizing bacteria (AOB), nitrite oxidizing bacteria (NOB) populations (*Nitrobacter* spp., *Nitrospira* spp.), and total bacterial populations at MWRP using qPCR. The objective of the study is to determine: (1) key physicochemical parameters that control AOB and NOB and improve bacterial growth in a complete nitrifying system, (2) the examination of key denitrifiers as indicators for the process, and (3) further investigation of specific denitrifiers.

Chapter 4 discusses population dynamics of two predominant denitrifying populations, *Thauera*-like bacteria and *Zoogloea*-*Methyloversatilis*-like bacteria. Physicochemical parameters of denitrifying bacteria such as anoxic DO, methanol dosage, and level of denitrification are discussed to investigate interrelationships of these genera over an 11-month period in a full-scale wastewater treatment system. The study examines how denitrification and denitrifying populations are affected by hypoxic denitrification and anoxic denitrification.

Chapter 5 examines the population dynamics of three bacterial populations which explain the competition between two denitrifying bacteria (*Paracoccus*-like bacteria and *Burkholderia*-like bacteria) and a bulking partial denitrifier (*Thiothrix Eikelboomi*).

Physicochemical parameters studied include temperature, nitrite accumulation, DO, and nitrate removal. Understanding the physicochemical variable which controls the population dynamics of these organisms can lead to better optimization of efficiency of denitrification and prevent bulking events in the activated sludge process.

Chapter 6 draws conclusions from the results of the studies presented in the dissertation. The conclusion section contains additional recommendations and suggestions for future research.

Chapter 2. Literature Review

2.1 Nitrogen cycle

Nitrogen (N) is a major element of all organisms and it accounts for 6.25% of their dry mass on average (Bothe, 2006). Biochemically, N undergoes a variety of oxidation and reduction states that produce compounds with oxidation with valences ranging from +5 (as in nitrate, NO_3^-) to -3 (as in ammonia, NH_3). Reactions steps within the nitrogen cycle are carried out by Bacteria, Archaea and specialized eukaryotic fungi. Nitrogen fixation, the conversions of N_2 to NH_4^+, is primarily performed by microorganisms known as *diazotrophs* under both aerobic and anaerobic conditions. Ammonium ions in water can undergo three different biochemical reactions: 1) assimilation by bacterial cells via the glutamine synthetase and the glutamate-oxoglutarate aminotransferase pathways to form proteins for cell growth

(Bothe, 2006), 2) reaction with nitrite to form the end product nitrogen by bacteria belonging to the Phylum *Planctomycetes*, 3) and oxidation to nitrate (nitrification) in the presence of oxygen by ammonia oxidizing bacteria (AOB) and Archaea (AOA) as well as nitrite oxidizing bacteria (NOB). Part of the assimilated nitrate denitrified (denitrification) by denitrifying bacteria forms nitrogen gas and is recirculated to the beginning of the nitrogen cycle (Bothe, 2006).

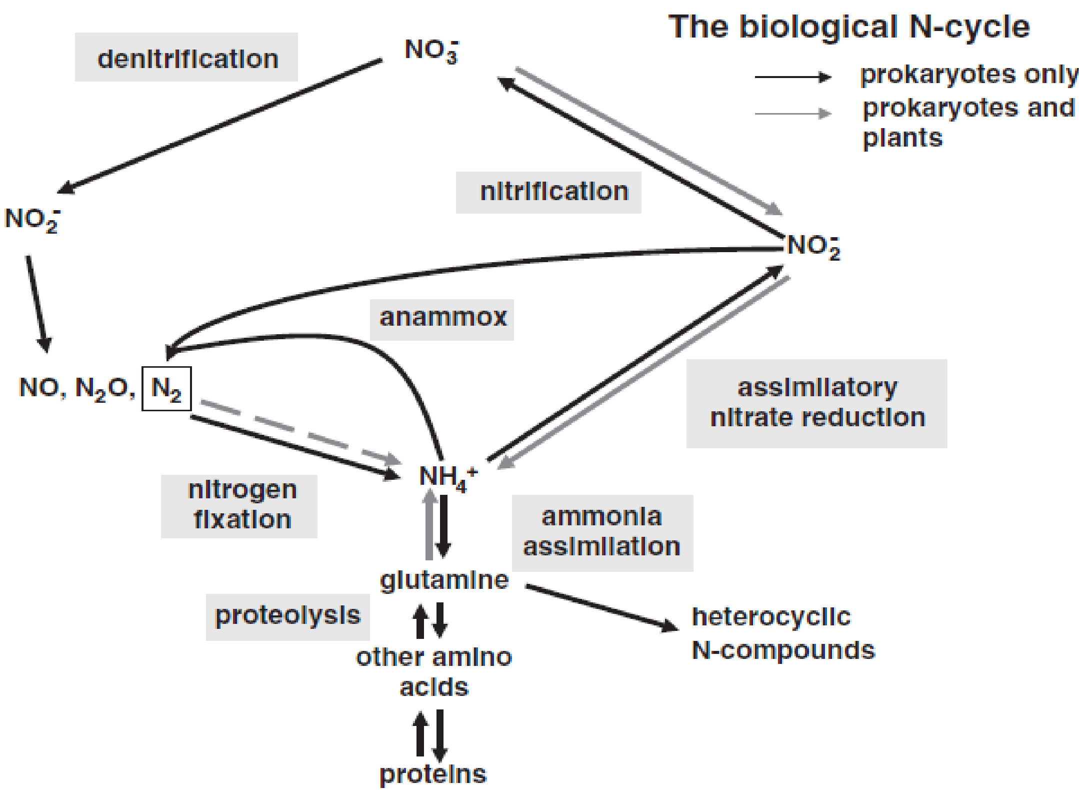


Figure 2-1 Biological nitrogen cycle with biochemical pathways carried out by different organisms, adapted from Bothe, (2006).

Nitrification and denitrification are the most important steps in wastewater engineering for the purpose of nitrogen removal. During the last few decades, regulations of nutrient discharges from domestic wastewater into streams and water bodies have become more stringent to prevent eutrophication and avoid toxic effects of ammonia release to organisms living in receiving water bodies (Tchobanoglous et al, 2003). Nitrogen compounds such as ammonia and nitrate have been hazardous to the environment and are monitored and regulated in various forms depending on the sensitivity of the receiving waters. Ammonia discharge into coastal waters is under scrutiny currently because of its potential role in dinoflagellate blooms which are hazardous to humans and fish, therefore discharging permits are required for each wastewater treatment plant (Russo and Thurston, 1991). The United States Environmental Protection Agency currently limits the concentrations of nitrate to 10 mg l^{-1} in discharges water bodies in the US (EPA National Pollutant Discharge Elimination System (NPDES)). Many states have more stringent discharge limits such as California where the Regional Water Quality Control Boards set standards for respective watershed (Porter Colonge Water Quality Control Act, 1969). Therefore, from a regulatory standpoint the steps involved in nitrification and denitrification are important reactions in biological nitrogen removal and control wastewater effluent quality to meet increasing standard stringency.

2.2 Nitrification in Activated Sludge Process

Nitrification consists of a two step process, and the two bacterial groupings responsible for the oxidation of ammonia to nitrate are ammonia oxidizing bacteria (AOB) and nitrite oxidizing bacteria (NOB). Equation 1 (Ward and O'Mullan, 2002) shows the transformation of ammonia to nitrite mediated by AOB which utilize two operons which encoded for enzyme in this process ammonia monooxygenase (*amo*) and hydroxylamine oxidoreductase (*hao*). The conversion of nitrite to nitrate by NOB is described in equation 2 (Wolfe and Lieu, 2002). It was generally assumed that there are one copy of 16S rDNA per cell for *Nitrospira* (Dionisi, 2003) and two copies per cell of *amo* gene (Yapsakli, 2010) and three copies of the gene (hao) gene for the ammonia-oxidizing bacterial cell (DJ Arp et al., 2002).

$$NH_3 + \frac{3}{2}O_2 \xrightarrow[\text{Amonia monooxygenase and Hydroxylamine oxidoreductase}]{} NO_2^- + H^+ + H_2O \quad (1)$$

$$NO_2^- + \frac{1}{2}O_2 \xrightarrow{\text{Nitrite oxidoreductase}} NO_3^- \quad (2)$$

2.2.1 Nitrifiers

It has been found that numerous types of organisms including Archaea and Proteobacteria can carry out ammonia oxidation in nature and are referred to as ammonia oxidizing bacteria

(AOB). Moreover, most of the chemolithoautotrophic AOB responsible for the rate-limiting step of nitrification that converts ammonia to nitrite are found within the group of *Betaproteoteobacteria* (Seviour et al., 2010). As shown in Figure 2.2, with this group, *Nitrosomonas*-like populations related to *N. europaea* and *N. eutropha* were found to be the predominant species among AOB populations in the activated sludge process (Painter,1986; Purkhold et al., 2000).

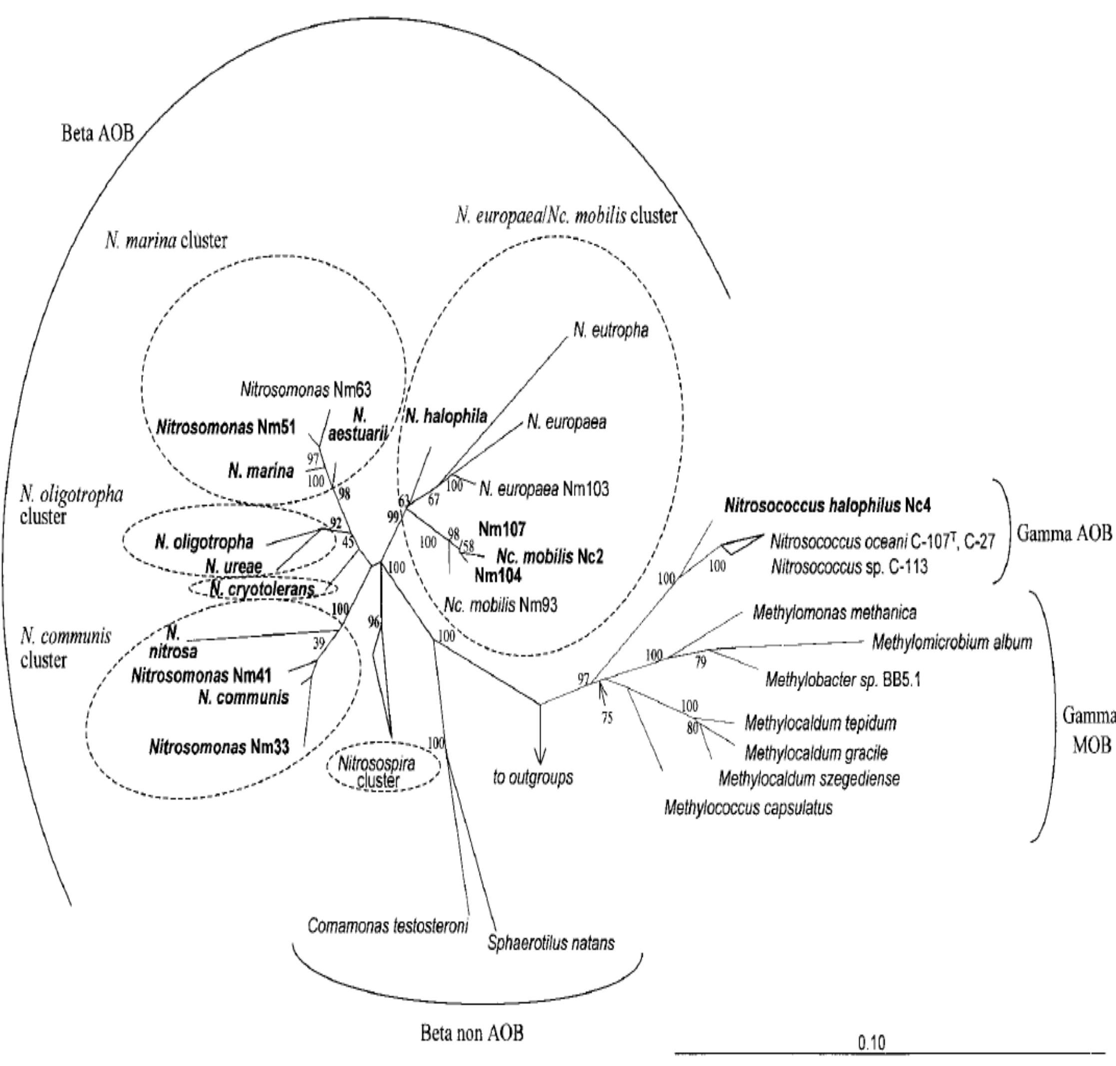


Figure 2-2 Phlogenetic tree of ammonia oxidizers taken from Purkhold et al, 2000

For nitrite oxidizing bateria (NOB), there are four genera known as NOB that control the second step of nitrification: *Nitrobacter, Nitrospira, Nitrococcus*, and *Nitrospina*, and of these genera *Nitrobacter* and *Nitrospira* have been shown to be the dominant groups in activated sludge. *Nitrospira* (NIS) are k-strategists and are better adapted to low substrate concentrations of nitrite and oxygen. On the other hand, *Nitrobacter*（NB）are r-strategists, and can out compete *Nitrospira* when more nitrite is available and DO is in excess (Anthonisen et al., 1976). It has been shown by Blackburn (2007) that NIS and NB have relatively similar oxygen half-saturation constants (K_O) values, with 0.54±0.14 and 0.43±0.08 mg•L^{-1} respectively, but the maximum oxygen uptake for NB is 289±15 mg g $VSS^{-1}h^{-1}$ or about 9 times of the maximum oxygen uptake for NIS (32±2 mg g $VSS^{-1}h^{-1}$). This suggests that oxygen affinity may play an important role since it has been shown in a previous study that NIS was dominant over NB in oxygen limited processes (Huang et al., 2010)

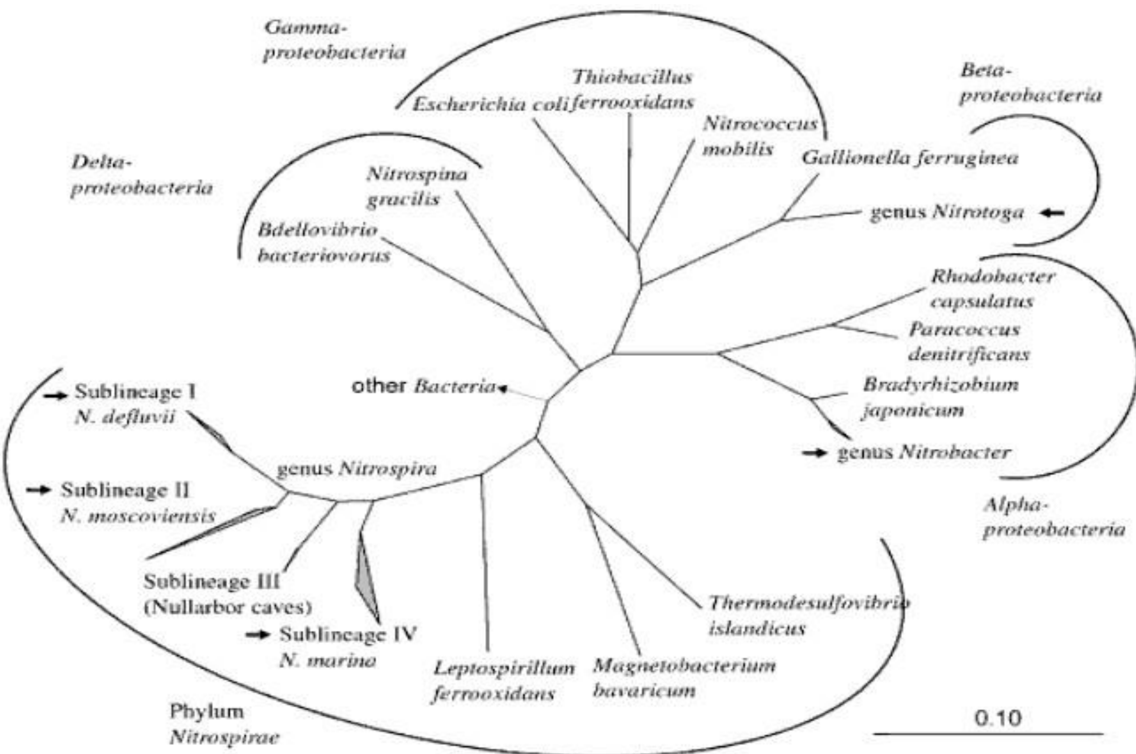


Figure 2-3 The Phylogenetic tree of nitrite oxidizers taken from Daims and Wagner

2.3 Denitrification

2.3.1 Denitrifying Enzyme

In general, denitrification is defined as the dissimilatory reduction of NO_3^- or NO_2^- to N_2O and N_2 (Hayatsu et al., 2008) and is encoded by different genes. The enzymes transcribed from these genes are shown in figure 2.4. The denitrification pathway consists of four steps. The first step is the conversion of nitrate (NO_3^-) to nitrite (NO_2^-) is encoded by either the *narG/napA* gene. Nitrite is further reduced to nitric oxide (NO) reductase encoded by *nirS/nirK* gene, which is followed by nitric oxide reductase which contains the genetic information (*cnorB/qnorB)* to convert to nitrous oxide (N_2O). During the final step of

denitrification, the majority of the N_2O will be converted to nitrogen gas by nitrous oxide reducase (*NosZ*).

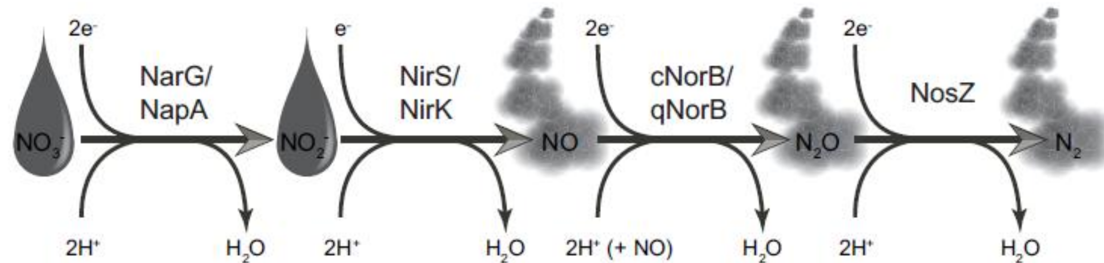


Figure 2-4 Four step enzymatic reduction of nitrate to nitrogen gas by different enzyme, Zumft, 2005

2.3.2 Dominant denitrifying populations in nitrifying-denitrifying activated sludge processes

These bacteria belong to a variety of bacterial groups: *Curvibacter, Azoarcus* and *Theaura* and *Zoogloea spp.* are the dominant groups in numerous treatment processes located in Europe, Australia, and Japan based on 16s rRNA FISH probe data and other 16s rRNA based microbial methods. Table 2-1 is constructed based on the different denitrifiers, microbial methods, process type, bacterial abundances, the location of the plant, and denotes the major the major denitrifiers identified in each study. All of the plants included in the table carried out complete nitrification and denitrification, and the heterotrophs utilized external carbon sources such as methanol and acetate as electron donors.

Table 2-1 Denitrifying Bacteria in Waste Water Treatment Processes.

Dominant Denitrifiers	Method	Type of Process	Bacterial Abundance	Location	Reference

Azoarcus and *Thauera, Zoogloea*	16S rRNA analysis, FISH-MAR	Activated Sludge in Industrial Plant	34%	Germany	Juretschko, 2002
Curvibacter delicatus and *C. lanceolatus*	Micro-manipulation 16S rRNA analysis, FISH-MAR	Municipal and industrial plants	30%	Denmark	Thompsen et al, 2004
Methylophilales and *Hyphomicrobium, Paracoccus*	16 rRNA analysis, FISH-MAR	Methanol-fed lab-scale SBR	Main denitrifiers but relative low concentration	Australia	Ginige et al, 2004
Rhodocylus and *Phosphate accumulating organisms*	FISH-MAR	Municipal and industrial EBPR Plant	17-22%	Denmark	Kong et al, 2004
Acidovorax, Dechloromonas and *Thauera*	16S rRNA analysis, FISH-MAR	RAS in full-scale plant and lab scale SBR	16%	Australia	Ginige et al, 2005
Meganema perideroedes	FISH-MAR	Industrial Plant	N/A	Denmark	Kragelund et al, 2005
Phylogenetically Heterogeneous Filaments	FISH-MAR	Activated Sludge from 21 plants	low quantity	Denmark	Thomsen et al, 2006b
Alphaproteoacterial Filaments	FISH-MAR	Industrial Plant	low quantity	Denmark	Kragelund et al, 2006
Hyphomicrobium	FISH and micro-electrodes	Biofilm	N/A	Japan	Satoh et al, 2006
Cruvibacter, Azoarcus, and Thauera.	FISH-MAR	Municipal Plant	20-49%	Denmark	Thompsen et al, 2007
Azoarcus, Zoogloea spp. and Alpha and	FISH-MAR	Municipal Plant	53% of all Eubacteria	Sweden	Hagman et al, 2008

Gamma-proteobacteria					
Curvibacter, Azoarcus, Thauera, Accumulibacter	FISH-MAR	Domestic Plant	30%	Denmark	Morgan-Sagastume et al, 2008

However, none of the above studies have looked at the effect of seasonal variation on organisms within plants. Interestingly studies have revealed that dominant denitrifying bacteria are different depending on the design and location of the denitirification tank as well as the substrate used. For example, adaptation to methanol takes longer than adaptation to substrates such as acetate or ethanol and different organisms predominant. In plants using methanol, *Paracoccus*, *Hyphomicrobium,* and *Methylophiles* are found to be highly associated with one another (Hallin et. al, 2006), while Thauera and Zoogloea communities were also found in relatively high abundances (Osaka et. al, 2006). On the other hand, ethanol and acetate selected for *Azoarcus, Dechloromonas, Thauera*, and *Acidovorax*-like bacteria. It has also been shown that plants such as the one in the proposed research which has the anoxic zone prior to the aeration tank with inputs of primary effluent, RAS and recycled mixed liquor are far more complex in their species diversity. Process did respond differently due to change in operational parameters, and it appeared that the dominant organism within each wastewater treatment plant may vary due to changes of influent and physicochemical conditions (Hallin et al., 2006). In addition, inability to find unique 16s rRNA-based phylogeny to identify denitrifying organisms suggested using functional genes may be a more accurate way to detect and quantify total denitrifiers.

2.4 Quantitative polymerase chain reaction (qPCR)

Quantitative polymerase chain reaction (qPCR) is a molecular technique that uses small and highly specific DNA primers (oligonucleotides) that attach onto the corresponding 5' to 3' and 3' to 5' strand of a predetermined DNA fragment to amplify a new DNA strand through the use of an enzyme of Taq polymerase. Additional primers will then attach onto the new strand and undergo the same cyclical processes to achieve exponential synthesis. The amount of DNA theoretically doubles with every cycle of PCR with 2^N number of DNA strands after N number of cycles. TaqMan oligoprobe containing a reporter and non-fluorescent quencher attaches onto the consensus site of the designated species. Once the primer hybridizes across the DNA polymerase, reporter is detached from the quencher of the oligoprobe, and it releases excitation energy at a wavelength that can be detected by the monitoring instrument. (Pelt-Verkuil, 2008)

Temperature and magnesium gradient experiments should be performed to optimize the annealing efficiency of the DNA hybridization. A temperature gradient is used to determine the best temperature to carry out the annealing process. Additional magnesium concentration stabilizes and prevents denaturation of the DNA strand. The annealing temperature of the forward and reverse primer should be within the range of $\pm 5^oC$, and the guanine and cytosine nucleotide content in the primers should be about 50% to improve the efficiency rate of the PCR reaction. A calculation of PCR annealing temperature for T_m and

salt adjusted T_m, which accounts for the nucleotide composition including A, T, G, C can be estimated by the equation below. (Pelt-Verkuil, 2008)

$$T_m= 64.9+41([yG+zC-16.4]/[xT+wA+zC])$$

Where y, z , w, z represent the numbers of A, T, G, C neucleotides in the molecule.

Chapter 3. Understanding the role of Nitrifying Populations, Total Bacterial Populations and their effect on ammonia removal and denitrification in an Activated Sludge Process under Warm-Climate Condition

3.1 Abstract

This study determined the nitrifying populations that were most affected by four of the major operating parameters over an 11 month period at a wastewater treatment plant located in Southern California. Quantitative polymerase chain reaction (qPCR) was used to determine the bacterial abundances of ammonia oxidizing bacteria, *Nitrobacter* spp., *Nitrospira* spp., and the total bacterial population. Water temperature, nitrite produced, nitrate produced, solids retention time, and pH were found to be the major physicochemical parameters controlling bacterial dynamics. Temperature was strongly and positively correlated with ammonia oxidizing bacteria (AOB) (r=0.48, p< 0.01), *Nitrobacter* spp. (NB) (r=0.47, p<0.01) and *Nitrospira* spp. (NS) (r=0.64, p<0.0001) and did not correlate with total bacteria (TB). Nitrite produced was negatively correlated with ammonia oxidizing bacteria (r=-0.50, p< 0.001), total bacteria (r=-0.33, p<0.05), *Nitrobacter* spp. (r=-0.41, p< 0.001), and *Nitrospira* spp. (r=-0.57, p< 0.001). Nitrate produced was negatively correlated with ammonia oxidizing bacteria (r=-0.38, p< 0.001), *Nitrobacter* spp. (r=-0.45, p< 0.001), and

Nitrospira spp. (r=-0.45, p< 0.001). The pH of wastewater was also found to be negatively correlated with all bacterial abundances: AOB (r=-0.58, p<0.0001), TB (r=-0.32, p<0.05), NB (r=-0.52, p<0.001), and NS (r=-0.51, p<0.001).

Two clusters in Principal Component 1(PC1) reflected a seasonality change at 26.9^{o}C. Temperature was found to be the parameter most directly affecting all bacterial populations in the warmer seasons (July-December), while nitrite produced and pH showed direct negative impact on the bacterial populations in the cold seasons (January-June) in the PCA plot. PC1 and Principal Component 2 (PC2) together accounted for 59.8% of the total variance. Nitrifying and total bacterial populations were strictly dependent on temperature in the summer time and inhibited by pH and nitrite in the winter season. The SRT needs to be extended by approximately 3.6 days to reduce AOB to NOB ratio of 9.5:1 to 2:1 to achieve optimum nitrification. We recommend examining and identifying the denitrifying organisms as indicator for denitrification since total bacterial population did not represent a strong indicator our study.

3.2 Introduction

Two biochemical steps are involved in the process of nitrification in the wastewater treatment process: 1) converting ammonia to nitrite by ammonia oxidizing bacteria (AOB), and 2) forming nitrite to nitrate by nitrite-oxidizing bacteria (NOB). However, physicochemical factors that control AOB and NOB populations in full-scale wastewater

treatment plant (WWTP) are not well understood.

For nitrite-oxidizing bacteria, effects of physicochemical parameters on *Nitrospira spp*. and *Nitrobacter spp.* were studied in bench-scale and pilot-scale wastewater treatment plants (Nogueira and Melo, 2006; Siripong and Rittmann, 2007; Huang et al., 2010). Several studies suggested that the competition of *Nitrobacter spp*. and *Nitrospira spp*. were primarily dependent on dissolved oxygen (DO) (Schramm, 1999; Blackburne et al., 2007; Downing et al., 2008) and nitrite concentrations, (Balmelle 1992; Kim and Kim, 2006; Dytczak et al., 2008; Ruiz et al., 2003; Ruiz et al., 2006). Changes in the community of AOB and NOB populations were found to be related to the ammonium concentration (Lydmark et al., 2007) and temperature (Balmelle 1992; Hunik 1993; Kim et. al, 2006). However, there is no discussion on the interrelationships among AOB, NOB, and total bacterial communities in a complete nitrification process based on the wide ranges in physicochemical parameters in a full-scale process. Determination of important physicochemical factors that impacted the abundance of bacterial populations in wastewater treatment is beneficial in enhancing plant optimization. To detect and quantify AOB and NOB, the quantitative polymerase chain reaction (qPCR) was the preferred technique to determine AOB and NOB populations in both lab-scale and full-scale wastewater treatment plants due to its low detection limit and high sensitivity (Graham et al., 2007).

Major parts of the Southern California are located in a warm climate region, where the wastewater annual temperatures range between 20-30 ^{o}C and are frequently higher than seasonal temperatures in the Eastern United States (10-25 ^{o}C), Europe (5-25 ^{o}C), and Australia (10-25 ^{o}C), where nitrification studies were first conducted (Harms et al., 2003; Limpiyakorn et al., 2006; Zhang et al., 2006; Blackburn et al., 2007; **Figuerola et al., 2010**). Variations in temperature dictate changes in operational parameters due to changes in the population dynamics of nitrifying bacteria according to the activated sludge models (Henze et. al, 2000).

Utilizing abundance of bacterial populations as a surrogate for biomass in nitrification processes provides us continued refinement of wastewater treatment modeling and operation compared to the mixed liquor suspended solids (MLSS). In this study the Michelson Wastewater reclamation plant (Southern California) was operated for complete ammonia (NH_3-N), biochemical oxygen demand (BOD) and chemical oxygen demand (COD) removal under relatively high DO (3.2-4.2 mg l^{-1}) and high temperature conditions (23.0 ^{o}C to 28.6 ^{o}C). This suggested that oxygen and BOD and COD are not limiting factors for the growth of bacterial population, while ammonia concentration is a limiting substrate for the growth of AOB populations in this treatment process.

Improved understanding of the growth of both AOB and NOB are needed to be understood in order to achieve optimum nitrification at the lowest cost, and it has been

reported by Blackburn et. al., (2007) that NOB bacterial populations have a higher maximum growth rate than AOB. This infers that given shorter solid retention time (SRT), conditions will favor the growth of AOB populations over NOB populations under ammonia limiting conditions.

Higher AOB to NOB ratios can result in high nitrite accumulation (Oh et. al., 1999), which has been shown to inhibit the nitrification process. Hence, we hypothesized that increasing SRT decreases the AOB to NOB ratios in order to prevent the accumulation of nitrite concentrations. Utilizing a fine-scale qPCR monitoring technique to improve the nitrification process, we are able to determine the adequate AOB to NOB ratio for nitrification optimization in the Southern California warm climate zone.

3.3 Materials and Methods

3.3.1 Plant Description and Sample Collection

Michelson Water Reclamation Plant (MWRP) located in Irvine, CA operated a preanoxic process with an average flow rate of 68.1 million L/day million (18 MGD), methanol was added into the anoxic zone daily to promote denitrification. Primary effluent underwent biological treatment for biochemical oxygen demand (BOD), chemical oxygen demand (COD), and ammonia (NH_3-N) removal in the activated sludge process. Ammonia removal in the activated sludge process consisted of both anoxic and aerobic zones designed for denitrification and nitrification, respectively. Samples were taken at the primary clarifier

effluent, aeration basin, and secondary clarifier effluent on a weekly basis. Approximately 250 mls of samples were collected in sterile bottles and placed on ice transported to the laboratory and analyzed for or stored at 4 ^{o}C until DNA extraction. All physical and chemical measurements, including temperature, DO, pH, ammonia, nitrite and nitrate concentrations, HRT, SRT, total BOD, total COD, alkalinity (Alk), methanol dosages were laboratory data which followed the standard methods (APHA et al., 1998). The following table shows the means and ranges of the operational parameters.

Table 3-1 Operational parameters and average concentrations at MWRP.

Parameter	Mean	Range
Flow (MGD)	18.1	14.3 - 19.9
BOD Removal (%)	94.7	79.0 - 97.4
NH_3-N Removal (%)	98.4	94.4 - 99.9
Influent NH3-N (mg l^{-1})	28.4	21.7 - 36
SRT (days)	8.3	5.9 - 9.5
HRT (hours)	6.0	5.4 - 7.5
MLSS (mg l^{-1})	2440	1980 - 2800
Temperature (℃)	26.7	23.7 - 28.6
Methanol (mg l^{-1})	9.14	6.53-13.1

pH	6.89	6.5-7.2

3.3.2 DNA Extraction

DNA extraction of the environmental samples followed the protocol (Yu and Mohn 1999; Gedalanga and Olson 2009).

3.3.3 Quantitative Polymerase Chain Reaction

DNA extracted samples were diluted (1:10, 1:100 or 1:000) with sterile HPLC water depending on activated sludge concentration and expected concentration of target organism and then stored at -80 C until time of analysis. Primers for *Nitrospira spp.*, *Nitrobacter spp.*, *β*-proteobacteria AOB subclass, and total bacteria (TB) using 16S rDNA genes were amplified by qPCR and are listed in Table 3-2.

Table 3-2 Nucleotide sequence of primers and dual-labeled probes for TB, AOB and NOB

Target organism	Primers or probes	Sequence (5'→3')	T_a (^{o}C)[a]	Reference
Total Bacteria	Forward primer	ATGGCTGTCGTCAGCT	50	Weisburg and Lane 1991
	Reverse primer	ACGGGCGGTGTGTAC		Ferris et al. 1996
	Probe	FAM-CAACGAGCGCAACCC-TAM		Harms et al. 2003
*Beta*proteo-bacteria AOB subclass	Forward	GGAGGAAAGCAGGGGATCG	60	Hermansson and Lindgren 2001
	Reverse	CGTCCTCTCAGACCARCTACTG		
	Probe	FAM-CAACTAGCTAATCAGRCATCRGCCGCTC-BHQ1		
Nitrospira	Forward	GCGGTGAAATGCGTAGAK	60	Graham et al.

spp.	primer	ATCG		(2007).
	Reverse primer	TCAGCGTCAGRWAYGTTCCAGAG		
	Probe	FAM-CGCCGCCTTCGCCACCG-TAMRA		
Nitrobacter spp.	Forward primer	ACCCCTAGCAAATCTCAAAAAACCG	68	Graham et al. (2007).
	Reverse primer	CTTCACCCCAGTCGCTGACC		
	Probe	FAM-AACCCGCAAGGAGGCAGCCGACC-TAMRA		
a. : denotes for annealing temperature was modified				

All qPCR reactions analysis were carry out on a Rotorgene 3000 (Qiagen, Valencia, California) or an Eppendorf RealPlex EP (Eppendorf, Hauppauge, New York). It was generally assumed that there had one copy of 16S rDNA per cell for *Nitrospira* and *Nitrobacter* (Dionisi, 2003) and three copies for ammonia-oxidizing bacterial cell (Hommes, 2002) for calculating the average cell numbers in the activated sludge sample.

Every qPCR run was accompanied with a negative control and a standard curve consisting of *AOB spp.*, *Nitrospira spp.* and *Nitrobacter spp.* DNA fragments of known concentrations, and NOB. The minimum detection limit of the assay was $5x10^2$ copies per reaction for *AOB spp.*, *Nitrospira spp.* and *Nitrobacter spp*. The average values and standard deviations of efficiency, coefficient of determination, y-intercept, and slope of all qPCR runs are shown in table S1 below (standard curves are shown in the supplemental figures in Appendix A).

3.3.4 Positive control for TB, AOB, NIS, and NB

The positive control for AOB, *Nitrospira* spp. and *Nitrobacter* spp. followed the methodology carried out by Harms et al (2009) and Huang et al (2010).

3.3.5 Pearson's correlation and principal component analysis

Pearson's correlation analysis was used to determine the relationship between percent removal of BOD, COD, NH_3-N, and Alkalinity consumed (Alk) with bacterial populations. Removal of BOD, COD, NH_3-N, and Alk consumed were normalized by influent concentrations in terms of percentage due to large fluctuations in influent concentration for each of the parameters. Normalization of the data simplifies the fluctuations in influent and provides a clearer understanding of trends between changes in bacterial populations and the product formation by these organisms. Nitrate and Nitrite produced/accumulation are obtained from the difference between primary and secondary clarifier effluents. Moreover, the estimate of nitrogen denitrified (%D) was calculated based upon the difference between nitrogen concentrations (NO^-_2-N, NO^-_3-N and NH_4^+-N) in the primary clarifier effluent and the remaining nitrogen measured in the secondary clarifier effluent. Calculations are shown in equation 1 and 2 assuming that no ammonia is assimilated in the anoxic zone.

$$\%\ \text{Denitrified} = \frac{N_{in} - NO^-_{3\,out} - NO^-_{2\,out}}{N_{in}} \times 100\% \qquad \text{(eq.1)}$$

$$N_{in} = NH^+_{4\,in} + NO^-_{3\,in} + NO^-_{2\,in} \qquad \text{(eq. 2)}$$

3.4 Results

3.4.1 Biological and physicochemical parameters on overall ammonia removal and denitrification

The physicochemical and biological data obtained from Michelson Water Reclamation Plant (MWRP) throughout eleven months are shown in Figure 3-1 and Figure 3-2.Figure 3-1 (a, b, e) and present the weekly bacterial population concentrations, DO, and temperature in the aeration basin. All bacterial concentrations tend to follow an upward trend with increasing temperature in the aeration basin. The water temperature in the aeration basin ranged from 23.7-28.6 ^{o}C, increasing from January (minimum) to October (maximum) and then decreasing from October to December. A similar result was also reflected in the correlations of bacterial populations with temperature. Temperature strongly and positively correlates with AOB (r=0.48, $p<$ 0.01), NB (r=0.47, $p<$0.01) and NS (r=0.64, $p<$0.0001) but does not correlate with TB. These findings suggested the total bacteria populations behave significantly differently from the nitrifying population with respect to temperature. It has been generally believed that heterotrophic denitrifying bacteria plays a more important role than nitrifying populations in the activated sludge process in activated sludge microbial ecology (Seviour et. al, 2010). Table 3-3 summarizes the minimum, mean, and maximum concentration of all bacterial populations.

Table 3-3-Range of bacterial population Concentrations (cells l^{-1})

Concentration	AOB	TB	NB	NIS
Minimum	4.51×10^{11}	7.11×10^{12}	1.05×10^{9}	1.21×10^{11}
Mean	1.56×10^{12} $\pm8.51\times10^{11}$	1.79×10^{13} $\pm6.18\times10^{12}$	3.65×10^{10} $\pm3.85\times10^{10}$	3.57×10^{11} $\pm2.12\times10^{11}$
Maximum	3.40×10^{12}	3.44×10^{13}	1.56×10^{11}	1.10×10^{12}

Total bacterial abundance is an order of magnitude higher than other studies by Graham et al., (2007) and Limpiyakorn et al., (2006) in pilot and full-scale studies and remained at $1.79\times10^{13}\pm6.18\times10^{12}$ cells l^{-1}, with a MLSS concentration remained relatively stable at 2440 ± 180 mg l^{-1} throughout the 11 month period. AOB had a mean concentration of $1.56\times10^{12}\pm8.51\times10^{11}$ cells l^{-1}, represented $5.99\pm0.13\%$ of total bacterial population. Once again we reported a higher concentration than reported for other nitrifying facilities (Harms et al., 2003; Limpiyakorn et al., 2006; Zhang et al., 2006; Figuerola et al., 2010). NB concentration and NIS concentration averaged at $3.65\times10^{10}\pm3.85\times10^{10}$ cells l^{-1} (0.19 ± 0.18 %) and $3.57\times10^{11}\pm2.12\times10^{11}$ cells l^{-1} ($2.07\pm1.08\%$), respectively. Total NOB abundance, which the sum of *Nitrobacter* spp. and *Nitrospira* spp., was greater than reported in the experiment by Harm et al., (2003). The causes of these variations could be due to changes in environmental and operational conditions as well as quantitative methodology and DNA extraction approach but are certainly related to shorter mean cell residence times and warmer climates.

DO ranges from 2.15 to 4.94 mg l^{-1} with an average DO concentration of 2.83±0.53 mg l^{-1}, with a DO set point of 3.0 mg l^{-1}. Hence DO is not limiting in this process. At this plant *Nitrobacter* spp. and *Nitrospira* spp. were positively correlated (r=0.78, p< 0.0001), and *Nitrospira* spp. was dominant over *Nitrobacter* spp. with an average NIS to NB ratio of 9.8 to 1. Comparing these data to a previous study conducted by Huang et al. (2010) on an incomplete nitrifying plant where oxygen was a limiting factor (average DO = 0.87±0.56 mg l^{-1}) and the water temperature (23 ^{o}C-29 ^{o}C) was similar, *Nitrobacter* spp. and *Nitrospira* spp. were negatively correlated and while *Nitrospira* was determined to be the dominant NOB, the average ratio NIS to NB of 3.2:1 was much lower. This outcome suggested the predominance of NIS over NB becomes more significant when DO is a limiting factor since the activity of heterotrophic bacteria is higher in the summer time and allows a faster growthrate for NIS. Blackburne et al. (2007) showed that NIS and NB had relatively similar oxygen half-saturation constants (K_o) values, with 0.54±0.14 and 0.43±0.08 mg l^{-1} respectively. However, the maximum oxygen uptake rate (OUR) for NB is 289±15 mg $VSS^{-1}h^{-1}$ or about nine times the maximum oxygen uptake for NIS (32±2 mg $VSS^{-1}h^{-1}$). While NIS has a higher K_o, its lower OUR may provide NIS a selective advantage over NB even when oxygen is not limiting. The maximum specific growth rate for NB and NIS were reported to be 0.57 ±0.09 day^{-1} and 0.63 ±0.03 day^{-1} (Blackburne et al., 2007).

The dominance of *Nitrospira* spp. over *Nitrobacter* spp. can also be explained by previous studies which suggested that *Nitrospira* spp. are K-strategists and are better adapted to low substrate concentrations of nitrite and oxygen, and it helps explain why it is negatively correlated with nitrite build up (Table 4, r= -0.57, $p<0.001$) . It has been shown that nitrite accumulation of 0.2 mg l^{-1} can have an adverse impact on the *Nitrospira* and *Nitrobacter* bacterial populations (Odell et al., 1996; Zhou et al., 2011). Thus, the difference in dominance ratios between the two plants reflects differences in the amounts of biomass between these genera and is related primarily to DO (3 mg l^{-1} compared to 1 mg l^{-1}) and SRT (8.3 days compared to 4.2 days), as influent ammonia concentrations are similar between the two plants. On the other hand, *Nitrobacter* spp. is disadvantaged under low nitrite and oxygen concentrations, which resulted in lower biomass (Anthonisen et al., 1976; Ruiz et al., 2003).

In addition, K_o for AOB was reported to be 0.33±0.003 mg l^{-1} by Blackburne et al (2008) in another study, which compared the maximum oxygen uptake rate (OUR) and maximum growth rate of AOB and NOB. The OURs for AOB and NOB were approximately 245 mg $VSS^{-1}h^{-1}$ and 260 $VSS^{-1}h^{-1}$, respectively based on interpolation of the data in that study. This also implies the presence of excess nitrite since there is greater abundance of AOB than NOB. Therefore, NOB populations had greater advantage over AOB, while the maximum growth rate of AOB (0.54 ±0.09 day^{-1}) was lower than NOB (0.67 ±0.03 day^{-1}) (Blackburne et al., 2008). This suggests one solution to the high AOB to NOB ratio would be to increase the

SRT so NOB biomass is increased, which will improve long term stability of operational performance.

Although it has been found that optimum pH for the growth of *Nitrosomonas* (AOB) and *Nitrobacter* in batch studies were between 7.5 and 8.6 (Yoo et. al., 1999), full-scale studies reported that maximum nitrification were achieved when pH was in range of 6.5-7.0 (Antoniou et. al., 1990; Shammas et. al., 1986). The pH levels (6.7-7.2) in this study also fall close to the category of full-scale processes that have achieved complete nitrification. However, our study revealed that nitrification was not fully optimized based on the *in-situ* measurement of nitrifying bacterial populations, where pH concentrations showed significant negative impacts on all nitrifying bacterial groups in this study. With pH having an overall effect on total bacteria (r= -0.32, p<0.05) and a more significant and negatively correlated impact with AOB (r=-0.58, p<0.01), NB (r=-0.52, p< 0.0001), and NS (r=-0.51, p<0.0001) (Table 4), this implied that nitrification can be further optimized by maintaining at pH the level of 6.5-6.7.

SRT and HRT averaged at 8.3±0.8 days and 6.0±0.6 hr, respectively, which was also seen in a fully nitrifying plant in northern California (Lee et. al 2000). BOD removal (94.7±3.1%) and COD removal (90.9±2.9%) remained relatively stable with the exception of two significant drops in BOD and COD removal on March 16 (86.6% for BOD and 84.1% for COD) and June 15 (79% for BOD and 77.2% for COD) as a result of chlorination in the return activated sludge (RAS) to reduce bulking. The mean concentrations of NH_4-N, BOD, and COD removal (Figure 3-1) all leveled off (>90%), so the correlations with bacteria were

insignificant. Importantly, the chlorination even in March had a lesser effect on AOB and TB than the events in June, which was accompanied by decreasing SRT.

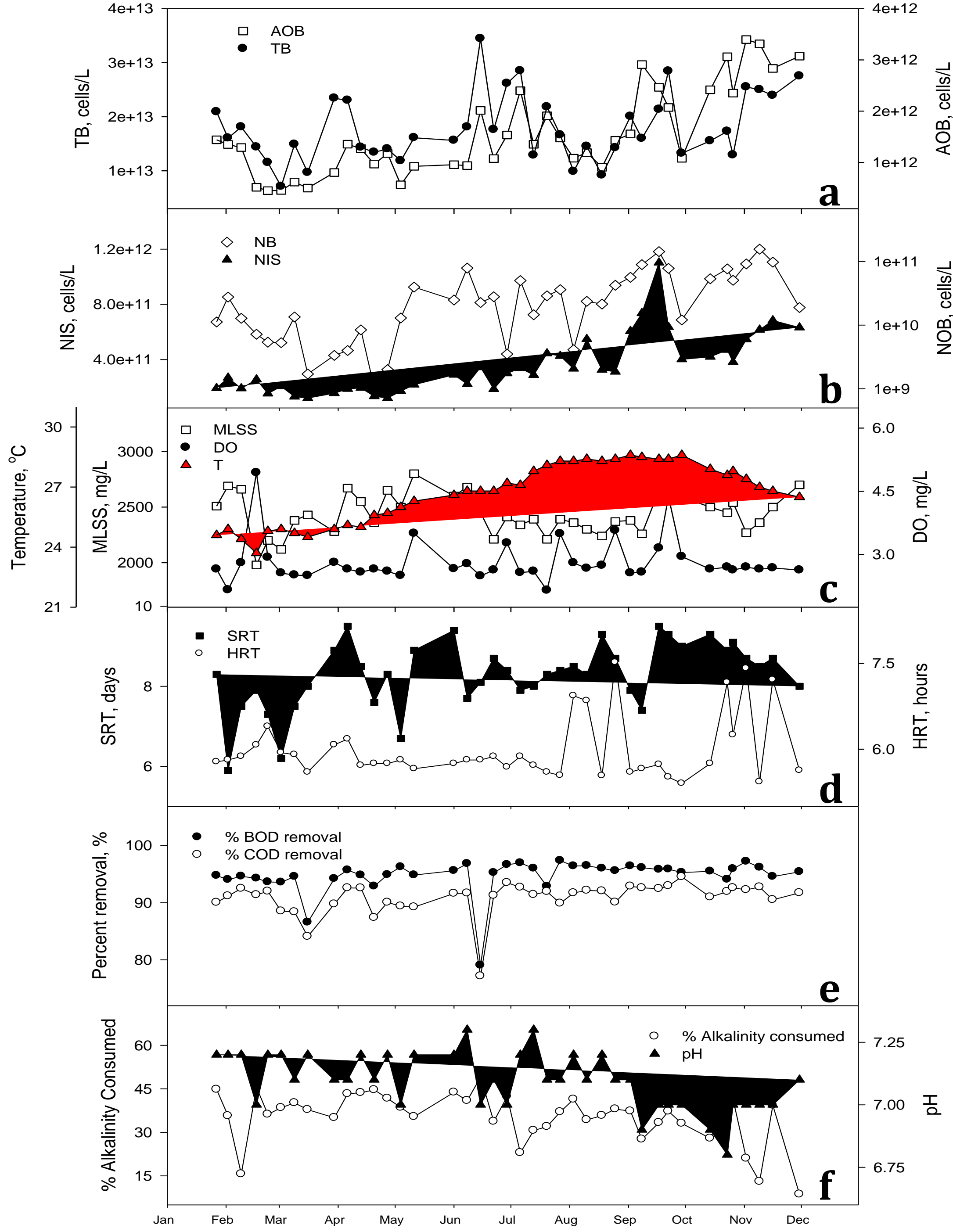


Figure 3-1 Comparison of weekly physicochemical parameters with bacterial populations over a year of period. (a) TB, AOB, NB and NIS abundances in the aeration basin. (b) Temperature, DO and MLSS in the aeration basin. (c) SRT and HRT. (d) BOD and COD

removal (e) % Alkalinity consumed between primary and secondary clarifier effluent and secondary clarifier effluent and pH in the aeration tank.

Figure 3-2a shows ammonium concentrations averaged 28.5±3.2 mg l^{-1} in the primary clarifier effluent and 0.16±0.25 mg l^{-1} in the secondary clarifier effluent with a peak of 1.6 mg l^{-1} on July 2009. Percent NH_4^+-N removal between the primary and secondary clarifier was stable at 98.4±1.54 %. Nitrate was below 3 mg l^{-1} in the primary clarifier effluent for the entire period with an average concentration of 0.80±0.6 mg l^{-1} and remained stable at 8.2±1.1 mg l^{-1} in the secondary clarifier effluent, which also showed significant negative correlation with the nitrifying populations (Table 4). Nitrite concentrations averaged 0.34±0.2 mg l^{-1} in the primary clarifier effluent, and 0.24±0.2 mg l^{-1} in the secondary clarifier. Temperature and nitrite produced/accumulation appeared to be a dominant factor affecting bacterial populations in this plant. This is shown by the strong positive correlation between temperature and amount of nitrite accumulation/produced (r=0.76, $p < 0.01$), which agreed with and further refined the theoretical study conducted by Randall et. al (1984) at a lower temperature range of (15-25 ^{o}C). It was also reported a nitrite concentration of 0.34 mg l^{-1} can result in the slowdown of denitrification and nitrification activity in a batch study (Zhou et. al., 2011). Nitrate was also reported to have negative effect on the growth of ammonia-oxidizing bacteria when nitrate exceeded 25 mg l^{-1} (Odell et al., 1996).

Significant build-up of nitrite accumulation has also been proven to have adverse effect on nitrification (Odell et al., 1996; Zhou et al., 2011) and can result from a high AOB

to NOB ratio. This range of AOB to NOB ratio has been found to be greater than 85:1 in a bench study (Gieseke et al., 2001). Optimum AOB to total NOB ratios of 2-3.5 were found in good nitrification processes (Copp and Murphy, 1995; You et al., 2003), which reported no nitrite build up. The AOB to NOB ratio and temperature produced a negative correlation, which may account for nitrite buildup in the cooler months ($r = -0.33$, $p < 0.05$). The maximum AOB to NOB ratio of 9.5 was on April 20 (25.7^{o}C) and a minimum AOB to NOB ratio of 2.0 on September 17 (28.4 ^{o}C). Based on the maximum growth rate of AOB and NOB by Blackburn et al (2008), we calculated that the SRT needs to be extended by approximately 3.6 days for an AOB ratio of 9.5:1 down to 2:1. This also can be achieved by online monitoring the AOB to NOB ratio while increasing the SRT.

Percentage nitrogen denitrified fluctuated throughout the whole sampling period (62% to 77%) with maximum denitrification (77%) occurring in September. This variability may be due to changes in the denitrifying bacterial populations related to temperature and substrate or either time in the anoxic zone, or the presence of DO in the anoxic zone.

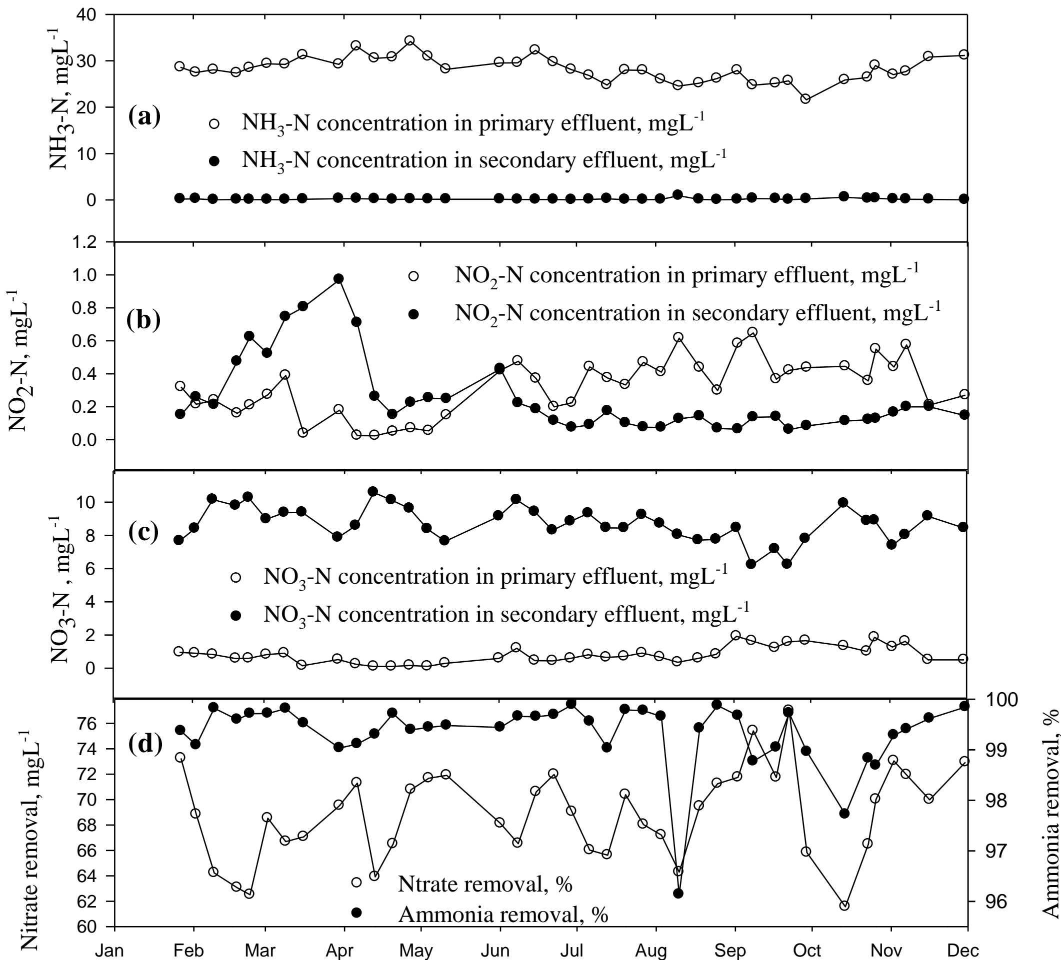


Figure 3-2 Comparisons of weekly nitrogen parameters with bacterial populations over 11 month of period: (a) Ammonia concentrations in primary and secondary effluent, (b) Nitrite concentrations in primary and secondary effluent (c) Nitrate concentrations in primary and secondary effluent, (d) Total nitrate removal and ammonia removal (%).

3.4.2 Determination of the most important physicochemical parameters affecting nitrifying bacteria using Pearson's correlation

Temperature has the most important factor at the treatment process on nitrifying bacterial populations, which ranged between 23.7-28.6 ^{o}C, with the maximum temperature of 28.6 ^{o}C on Sep 29, 2009. Temperature was strongly correlated with bacterial population abundance throughout the 11 month sampling period (See section 3.1). For temperatures

above 20 °C, the growth rate of AOB is greater than NOB (Brouwer et al. 1996) which also was reflected by the greater cell abundance of AOB. Since the results showed strong correlations between AOB and NOB with temperature, this implied that other bacterial groups in the total bacteria were negatively affected or less sensitive to the temperature range of 23.7-28.6 °C which contributed to the lack of correlation between total bacteria and temperature (*r*=0.15).

Table 3-4 Pearson Moment Correlation Coefficients between Physicochemical parameters and bacterial abundances

	Correlation Coefficient			
Type	AOB	TB	*Nitrobacter*	*Nitrospira*
AOB	-	**0.60**[d]	**0.73**[d]	**0.73**[d]
TB	**-**	-	**0.37**[a]	**0.40**[a]
Nitrobacter	**-**	**-**	-	**0.78**[d]
Nitrospira	**-**	**-**	**-**	-
MLSS	0.10	0.11	0.08	0.05
% BOD removed	0.12	-0.19	0.23	0.21
% COD removed	0.21	-0.09	0.25	0.29
SRT	0.30	0.25	0.27	**0.32**[a]
HRT	0.21	-0.03	0.12	0.08
DO	-0.13	0.06	0.06	0.10
Temperature	**0.48**[b]	0.15	**0.47**[b]	**0.64**[d]
% NH_4^+-N removed	0.25	0.15	0.19	0.19
NO^-_3 produced, secondary clarifier	**-0.38**[a]	-0.16	**-0.45**[b]	**-0.45**[b]
NO^-_2 produced, secondary clarifier	**-0.50**[b]	**-0.33**[a]	**-0.41**[b]	**-0.57**[c]
% Alk consumed	**-0.53**[c]	-0.27	**-0.38**[b]	**-0.37**[b]
% denitrification	**0.36**[a]	**0.32**[a]	0.26	0.24

pH	**-0.58[d]**	**-0.32[a]**	**-0.52[c]**	**-0.51[c]**
Methanol	0.19	0.08	0.14	0.01
Bolded values indicates a significant correlation, (r> 0.3 a: P < 0.05; b: P < 0.01; c: P < 0.001; d: P < 0.0001).				

According to the correlation analysis in Table 4, HRT was not correlated with any bacterial populations since organisms were more likely to form flocs but only *Nitrospira* spp. were significantly correlated with SRT. This was largely due to the fact that *Nitrospira* spp. lies with the center of the floc, while *Nitrobacter* spp. and AOB lies at the outer surface of the sludge floc (Wagner et. al, 1996), which makes the growth rate of *Nitrospira* spp. to be slower than the other nitrifying populations and is therefore dictated the SRT.

3.4.3 Principal Component Analysis

Based on the correlational analyses in Table 4, the environmental factors that showed significant associations (in bold) with bacterial abundances are included in the principal component analysis (PCA) for further investigations: temperature, SRT, nitrate produced, nitrite produced, alkalinity consumed, percent of nitrate denitrified, and pH (Table 4). The PCA analysis has visually allowed us to determine the key parameters that need to be controlled during different parts of the season for optimum growth of TB, AOB and NOB populations.

The PCA included seven environmental parameters involved SRT, temperature (T), NO_3-N produced, NO_2-N produced percent $Alk_{consumed}$, percent of nitrate denitrified and pH, as well as four groups of bacterial populations. Principal component analysis indicated a

strong seasonality as shown by the clustering of individual data points with specific physicochemical and biological parameters (Figure 3-3). Two main overlapping clusters are presented relating to strong seasonal patterns of the observations. Cluster A consisted of observations dated from January to the mid August. Cluster B consisted of observations dated from June to November, and the overlaping of the observations cluster A and B between June and August. The separation of these two clusters was primarily driven by temperature. Principal component 1(PC1) and principal component 2 (PC2) explained 59.8% of the total variance, and the following nine components explained 40.2 % of the total variance.

The PCA containing PC1 and PC2 loadings and score combined into a bi-plot are shown in Figure 3-3. The negative portions of the axis were scores of the observations that fell on the left of the x-axis and lower half of the y-axis, and the top of y-axis and and the right hand side of the x-axis represented positive portions in the bi-plot consisting of loadings for the observations. Loadings greater than 0.5 and less than -0.5 are significant because they are the major influencing factors in the system. Results reflected significant loading in both PC1 and PC2. PC1, accounted for 44.2% of the total variance and had positive loadings (>0.5) on AOB, NIS and NB. It also had negative loadings on NO_3-N produced and NO_2-N (<-0.5). PC2 which accounted for 15.6 % of the total variance, containing high positive loadings on SRT (>0.5), NO_3-N produced (>0.5), and % Alk consumed (>0.25), as well as negative loadings on percent denitrified (<0.5) which accounted for lesser variances.

The three most important physicochemical chemical parameters that yield the greater eigenvectors from the principal component analysis were denitrification (percent denitrified), SRT and temperature that were positively correlated with the bacterial populations, while nitrate produced, Alk, nitrite produced and pH range negatively were correlated with the bacterial populations. With this analysis, we were able to observe that nitrifying and total bacterial populations were strictly dependent on temperature in the summer time (in cluster A) and inhibited directly by pH and nitrite in the winter season (cluster B).

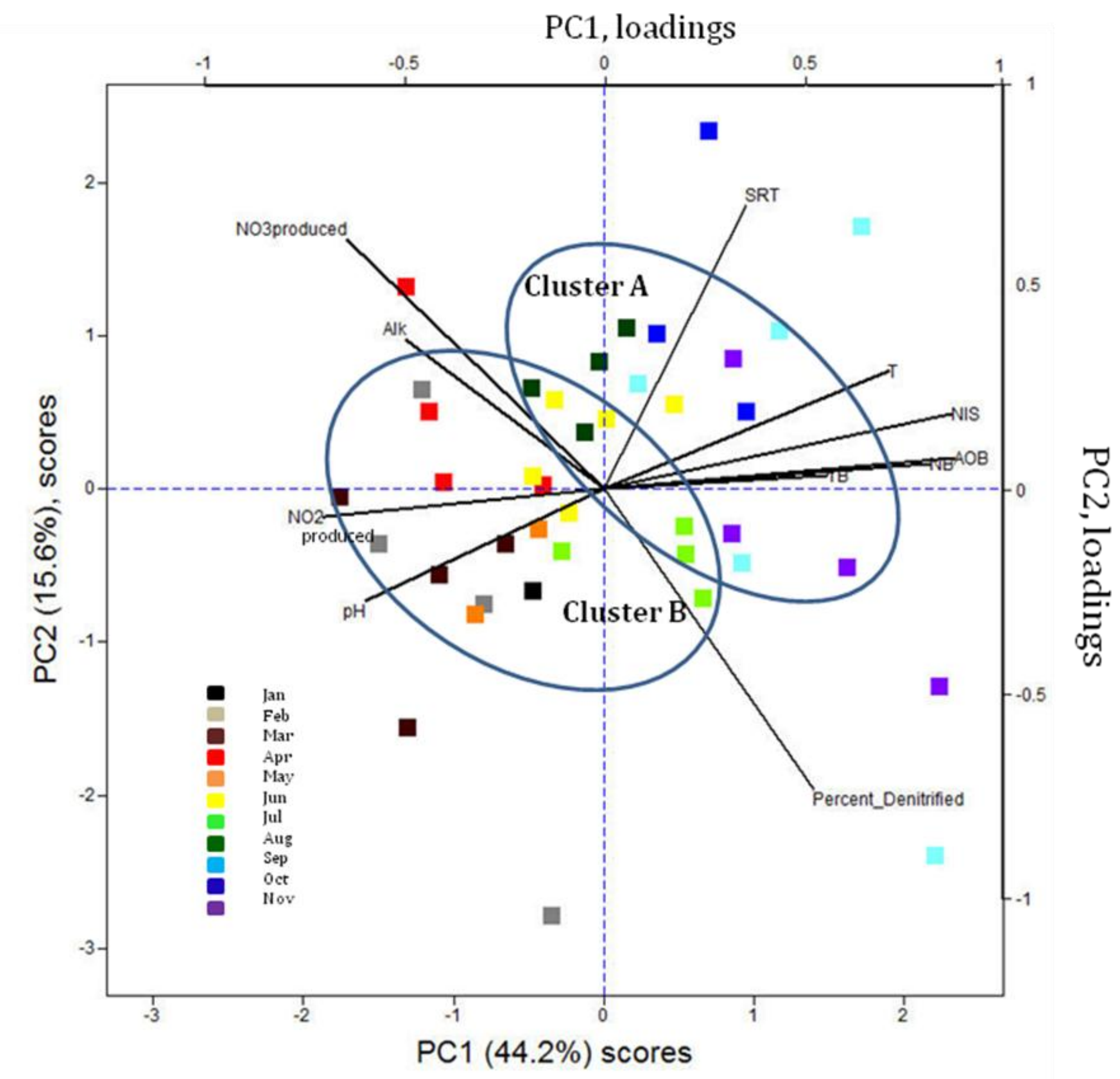


Figure 3-3 PCA bi-plot and scores plot for 11 physicochemical and biological parameters with 40 observations

The significance of seasonality in controlling plant operation can be further understood by dividing the two overlapping clusters in two temperature ranges. Figure 3-4 shows the dividing temperature that separates the two clusters. From the middle of January to the beginning of June, average monthly temperatures were below 26.9 ^{o}C. From middle of June to November, temperature was above 26.9 ^{o}C. This result is illustrated by the PCA study, temperature was the main parameter that drove the pattern of the biological process when DO

in the aeration zone was not a limiting factor, which is the case in this study. While observations scattered around cluster B did not fall in the warm season (>26.9°C) category, therefore they were more likely driven by other physical parameters such as: NO_3-N produced, percent Alk consumed, NO_2-N produced, pH, and percent denitrified. The total bacterial population showed no significant relationship to temperature and therefore loaded closely to the x-axis. This finding suggested that the data set should be further divided into two separate seasons where T<26.9 and T>26.9 (Fig. 3.4) in order to determine the seasonal behaviors of other predominant bacterial populations (denitrifying bacteria) in later studies for a better process control of denitrification.

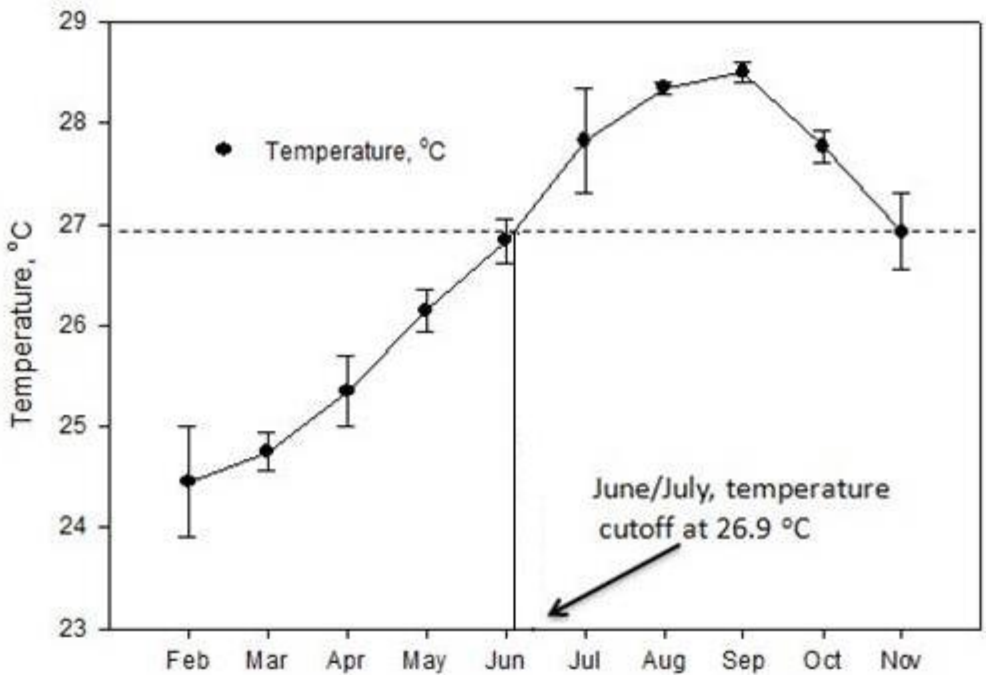


Figure 3-4**-** Monthly average temperature showing cutoff temperature at 26.9 °C

3.4.4 The need for further research in finding denitrification indicators

Although there have been many studies on the total abundance of dominant organisms across the different treatment processes (Juretschko et al., 2002; Wagner et al., 2002; Thomsen et. al., 2004; Ginige et. al., 2004; Kong et. al., 2004; Osaka et al., 2006; Thomsen et. al., 2007; Hagman et. al., 2008; Morgan-Sagastume et. al., 2008), these studies have only been done on a single grab sample scale. Therefore, no analysis of physicochemical data related to denitrifiers can actually be associated with overall denitrification in the process. Our study is the first to relate environmental and operational parameters with bacterial abundance over a long period. The lack of a direct and strong relationship in the PCA analysis between the total bacterial populations and denitrification indicate the population dynamics in in denitrifying population in activated sludge processes (Seviour et. al., 2010) should be better described with a practical end of identifying the denitrifying organisms as an indicator of denitrification.

3.5 Conclusions

PCA analysis revealed that the nitrifying bacteria and total bacteria are mainly driven by temperature in the warm seasons (June – November) and negatively impacted by nitrite accumulations and pH in the colder seasons (January – June). Hence, operational paraters can be adjusted according optimum performance even with temperature influences. Nitrite accumulation was largely due to the high AOB to NOB ratios, and inhibition by nitrite on all

of the nitrifying bacteria. Controlling the AOB to NOB ratio to the proper level of 2:1 can be the most important step to optimize the nitrification process. Since pH is also another operational factor, nitrifying populations can also be promoted by maintaining pH level close to 6.5-6.7 to improve overall activity of nitrification. Finally, total bacteria did not represent a strong indicator of overall denitrification, illustrating the need to find better biomarkers for denitrification.

Chapter 4. Investigation on the effect of physicochemical parameters on denitrifying bacteria and denitrification process in a full-scale water reclamation plant

4.1 Abstract

Two major denitrifying microbial groups, *Thauera*-like bacteria and *Zoogloea*-*Methyloversatilis*-like bacteria, which accounted for 34% on average of the total bacterial community using quantitative PCR (qPCR), were investigated in relation to the denitrification ability in a full scale plant. In this study of 11-months in warm wastewater (23-28.6 ^{o}C), dissolved oxygen (DO) in the anoxic zone was the most important parameter that determined denitrification efficiency when the temperature was below 27°C. *Zoogloea*-*Methyloversatilis*-like bacteria correlated significantly with denitrification (r=0.52, $p < 0.05$) under hypoxic conditions (0.2 mg L^{-1} < DO <0.6 mg L^{-1}), while *Thauera*-like bacteria abundance negatively correlated (r=-0.55, $p < 0.05$) with DO and significantly correlated with denitrification (r=0.55, $p < 0.05$) under strict anoxic condition (DO < 0.2 mg L^{-1}). Methanol dosing only played an important role in the *Zoogloea*-*Methyloversatilis*-like bacterial abundance (r=0.45, $p < 0.05$) when temperature was above 27°C, and led to no correlation with *Thauera*-like bacteria. *Thauera*-like bacteria abundance correlated positively with COD removal when temperature was less than 27°C (r=0.52, p<0.05). Operating the anoxic zone of the plant in the higher DO range (0.2 mg L^{-1} < DO <0.6 mg L^{-1}) when the temperature was below 27°C reduced the overall denitrification

because of a reduction in *Thauera*-like bacterial abundance. At temperature above 27°C, the denitrification efficiency was independent of the DO level, with denitrification exceeding 70% for both hypoxic and strict conditions.

4.2 Introduction

Removal of nitrogen compounds from municipal wastewater has been of continued interest in water engineering research due to regulatory policies on nitrogen discharge (Tchobanoglous et al., 2011). These policies are driven by increasing environmental concerns on accumulative nutrient loadings (Nyenje et al., 2010; Karydis et al., 2012; Kitsiou and Karydis, 2011). Nitrogen entering *in sewage* is mainly present in the form of organic *nitrogen* and is broken down during transport to ammonium (NH_4^+). The oxidation of ammonia has become a main processes in water reclamation plants and is achieved by biological nitrification. Nitrification can be modeled as a two-step process as it is carried out by ammonia-oxidizing bacteria (AOB) and nitrite-oxidizing bacteria (NOB) under aerobic conditions (Henze et al, 2000). NH_4^+ is oxidized into nitrite (NO_2^-) and subsequently to nitrate (NO_3^-) as end products (see equation 1). Properly, the first step is referred to as nitritation, and the second as nitrification. In practice, it is common to refer to the two-step process as nitrification, aiming at the final product (i.e., nitrate). Denitrification completes the removal of nitrogen, is primarily achieved by heterotrophic bacteria under anoxic conditions,

and can be modeled as a four-step biochemical process converting NO_3^- into nitrogen gas (N_2) (see equation 2).

$$NH_4^+ \rightarrow NO_2^- \rightarrow NO_3^- \qquad (1)$$

$$NO_3^- \rightarrow NO_3^- \rightarrow NO^- \rightarrow N_2O \rightarrow N_2 \qquad (2)$$

Historically, there have been significantly more studies on nitrifying bacteria than denitrifying bacteria (Seviour, 2010). The predominant nitrifiers are proteobacteria and belong to the genera of *Nitrosomonas*, *Nitrobacter* and *Nitrospira* and have been studied intensively in various wastewater treatment processes across the globe (*inter alia*, Juretschko et al, 1998; Blackburne et al, 2007; Whang et al, 2009). Importantly, a few studies have monitored AOB and NOB abundance in plants to address plant performance so that plant operations can be further improved (Harms et al., 2003; Kim et al, 2011). The quantification of total nitrifiers (i.e., total AOB + total NOB) have shed light on the relationship between nitrifying populations and the various physicochemical parameters in full-scale processes (Harms et al., 2003; Huang et al., 2010; Blackburne et al., 2007).

On the other hand, there is a limited amount of studies on denitrifying populations. Denitrifiers are mostly heterotrophs with higher phylogenetic diversity, and a number of denitrifying bacterial genomes have yet to be sequenced. Since there is not a single group that can be followed as with AOB and NOB, other methods have been used to investigate denitrifiers. Conventional culture-dependent methods have been utilized since the 1960s to

determine the bacterial diversity of denitrifying populations in wastewater (Dias et al., 1964). Hence, most denitrifying bacteria were initially isolated by culture and quantified in the laboratory. However, it is difficult to extrapolate these findings to denitrifying bacteria in full-scale processes, and culture methods are considered biased by Wagner et al (1993). The culture-dependent methods later combined with an in-situ physiological method by Nielsen et al (1999) lead to fluorescence in-situ hybridization-microautoradioactivity (FISH-MAR). FISH-MAR enables quantitative determination of the microbial diversity in wastewater treatment processes, and its limitations are only been able to examine small volumes, and relies on short probes (≈ 35 mer) for identification. Previous studies with the FISH-MAR method have shown the predominant denitrifying populations to composed of the genera of *Curvibacter*, *Azoarcus*, *Thauera*, *Zoogloea* and *Acculibacter* (betaproteobacteria), *Methyophilus* and *Hyphomicrobiu* (alphaproteobacteria) (Seviour et al., 2010). Important physicochemical parameters which control the activity and growth of denitrifying bacteria are temperature, dissolved oxygen (DO) concentration, nutrient (e.g., N, P) and organic carbon availability.

Temperature is one of most important factors that promotes the growth of bacterial populations and is difficult to control under field conditions. The optimum temperature for a bacterial population is primarily determined from laboratory or pure culture studies. The activated sludge model (ASM; Henze et al., 2000) was developed in regions with colder

climates than Southern California. Hence, the effect of water temperature which ranges of 20 ^{o}C to 30 ^{o}C on specific denitrifying populations in full-scale processes is not well documented for warmer climates.

Another important parameter controlling the growth of denitrifying populations in the activated sludge process is DO in the anoxic reactors. We can define hypoxic conditions as those where nitrate is present but the DO is not strictly in the anoxic region (i.e., $0.2 < DO < 0.6$ mg L^{-1}). Denitrification is optimized when the DO concentration is kept below 0.2 mg L^{-1}, which also corresponds to oxidation-reduction potentials below 0 mV (Tchobanoglous et al., 2003). Although denitrifying populations are purported to be dominated by facultative anaerobic heterotrophic bacteria, our understanding of aerobic denitrification is less well documented (Baumann et al., 1996).

Supplemental carbon sources may be required to enhance denitrification especially in colder climates; methanol has been used widely for its availability and cost-effectiveness. Other carbon compounds, such as acetate and ethanol, have also been added at different wastewater systems (Hallin et al, 1998; Osaka et al, 2006). The reason for the addition of different carbon sources to complement the influent wastewater composition is attributable to the differences in bacterial nutritional diversity. However, it is still unclear if populations of denitrifiers whose activity and growth are selectively promoted by supplemental carbon are the best competitors in wastewater.

We examined the dynamic abundance and interrelationships of the two populations under strict anoxic or hypoxic conditions with physicochemical parameters including DO, methanol dosage, and level of denitrification to insights into the optimization of full-scale wastewater treatment processes for denitrification process using fine-scale qPCR monitoring technique. Our hypotheses in this study are: a) decreasing temperature and hypoxic DO have inhibited the growth of two denitrifying bacterial populations which represented 34% of total bacterial population, b) *Thauera*-like bacteria is a better indicator of denitrification than *Zoogloea-Methyloversatilis*-like bacteria under warm temperature and anoxic DO condition, c) anoxic favored the abundance of the two major denitrifiers.

This study utilized quantitative polymerase chain reaction (qPCR) over an 11-month period to quantify two predominant and related genera *Zoogloea* *Methyloversatilis*-like bacteria and *Thauera*-like bacteria in the denitrifying community of wastewater. These organisms had previously been reported to be predominant bacterial populations within the *B*etaproteobacteria in some wastewater processes (Dias et al, 1964; Thomsen et al, 2004; Thomsen et al, 2007; Mao et al, 2012).

4.3 Materials and Methods

4.3.1 Plant Description and Sampling

The Michelson Water Reclamation Plant (MWRP) located in Irvine, CA operated at an average flow rate of 68.1 million L day^{-1} or 18.1 million gallons per day (MGD). The plant was designed and is operated as a Modified Ludzak Ettinger (MLE) process with complete nitrification and incomplete denitrification. All physical and chemical measurements were performed in accordance with standard methods (APHA, 1998) and were obtained from laboratory personnel and plant operators.

4.3.2 Sample Collection

Samples of 250 ml were collected sterilely from the primary clarifier effluent, anoxic zone mix liquor, aeration basin mixed liquor, and secondary clarifier effluent on a weekly basis from January 27, 2009 to November 30, 2009. Samples were placed on ice and transported to the laboratory. Samples were stored at 4°C and DNA was extracted within 24 h.

4.3.3 DNA extraction

Genomic DNA was extracted from triplicate samples using a modified bead beating protocol (Yu and Mohn 1999; Gedalanga and Olson 2009). DNA concentration and purity were measured using DU®7400 spectrophotometer (BECKMAN, USA) at260/280 nm, while the purity(A_{260}/A_{280}) ranged between 1.60-1.80. DNA extract samples were diluted (1:10, 1:100

or 1:000) with sterile HPLC water to account for the concentration of target organisms and stored at -80°C until time of analysis.

4.3.4 *Zoogloea* bacteria Positive Control

Zoogloea spp. strain (B-3640) obtained from the Agricultural Research Service (ARS) culture collection was grown according to USDA protocol. The growth medium was Tryptone Glucose Yeast (TYG) Broth containing 0.5% tryptone, 0.1% glucose, and 0.1% K_2HPO_4. Cultures were incubated in an orbital shaker (Forma Scientific, Marietta, OH) at 28°C at 60 rpm for 24 hr until significant growth occurred. Colony isolation was achieved by streaking the culture onto 2% TGY agar and incubating as stated above. An individual colony was then transferred from the agar medium to a tube containing TGY broth and incubated as before. The culture was then subjected to the same DNA extraction protocol as described below and stored at -80°C as a pure extract and in 1:10 dilutions.

4.3.5 *Thauera*- like bacteria Positive Control

The positive control for *Thauera* was obtained from the amplified target amplicon, which was the PCR product using16S rRNA gene primers (Table 4-1) of DNA extract from activated sludge. Details of the DNA extraition process are contained in Section 4.3.3

4.3.6 qPCR *Thauera*-like bacteria and *Zoogloea- Methyloversatilis*-like bacteria Primers and Probes development

The *Thauera*-like bacteria primer and probe set was designed in three sequential steps: (1) The NCBI/BLAST/blastn suite alignment search was utilized to identify the regions of

homology and heterogeneity between the *Thauera* MZ1T (NR074711.1) and 16S rRNA genes (5'-3' sequences) of other *Thauera* species. (2) Uncultured and environmental Genbank entries that showed high dissimilarity with the reference sequence were excluded from the alignment process. (3) Highly specific regions that carried no homology with other bacterial genera, while sharing homology with the *Thauera* MZ1T, were selected for use in the design of the primer and probe set. The primers were screened against all bacterial taxids other than the target using *in silico* analyses (Moss et al. 2007). If homology was found in either the forward and reverse primer such that an amplicon could be generated, the size was ascertained. The same steps were performed in the design *Zoogloea* spp. primer and probe set using the reference sequence of *Zoogloea ramigera* [X74913]. The primer and probe set were later determined to capture both *Zoogloea* spp. and *Methyloversatilis* spp. (see results section 3.1).

The *Thauera*-like bacteria spp. primers yielded a 400 basepair (bp) amplicon; the *Zoogloea-Methyloversatilis*-like bacteria primers yielded a 121(bp) basepair amplicon.

4.3.7 Quantitative Polymerase Chain Reaction (qPCR)

Primers and probes for *Thauera*-like bacteria spp. and *Zoogloea-Methyloversatilis*-like bacteria spp. for the 16S rRNA genes used in the qPCR analyses are listed in Table 2. All qPCR reactions were performed on an Eppendorf RealPlex EP (Eppendorf, Hauppauge, New York). Each qPCR reaction contained a total volume of a 25 **μl** solution with the following

reagents: 5 µl DNA extract/template, and 20 µl containing 2.5 mM $MgCl_2$, 1 X PCR buffer, 200 nM dNTP, 1 U AmpliTaq DNA polymerase, 100 nM dual-labeled probe, 150 nM forward primer, and 150 nM reverse primer. The qPCR protocol for environmental DNA extracts included 35 cycles of 2 minutes holding at 94°C followed by denature at 94°C of the DNA strands for 40s followed by 20 s at their respective annealing temperatures (listed in Table 2).

Table 4-1 -Primer and Probe sets for *Thauera*-like bacteria and *Zoogloea-Methyloversatilis*-like bacteria

Target Group	Name	Sequence (5'to 3')	Annealing Temperature* (°C)
Thauera-like bacteria	Forward Primer	CGTGGTCTCTAACATAGGCC	66.9
	Probe	Fam-TACCGGACTAAGAAGCACCGGCTAACTACGT-BHQ1	
	Reverse Primer	CGGAGCAGCAATGCACTGAG	
Zoogloea-Methyloversatilis-like bacteria	Forward Primer	TCCTGGGCCAGTACTGA	59.2
	Probe	Fam-CGAAAGCGTGGGGAGCAAACAGGATTAGATA-BHQ1	
	Reverse Primer	GTGAGGAGACTCATTGAGTAA	

The 16S rRNA gene copy number per cell was based on the *Thauera MZ1T* genome (NR074711.1), which has four copies per genome. Four copies of 16S rRNA gene per cell was assumed for all *Thauera*-like bacteria in calculating the average cell numbers in activated sludge samples. There is no genome sequence available for *Zoogloea* spp., while *Zoogloea*

spp. and *Thauera*-like bacteria spp. are within the same family, so we assumed four copies per cell for calculating the average *Zoogloea-Methyloversatilis*-like bacteria cell numbers in the activated sludge sample.

The standard curves ranged from $1.75 \times 10^3 - 1.75 \times 10^6$ copies per reaction and $9.5 \times 10^2 - 9.5 \times 10^6$ copies per qPCR reaction for *Thauera*-like bacteria spp. *and Zoogloea*-like bacteria spp., respectively (see standard curves of *Zoogloea-Methyloversatilis*-like spp. and *Thauera*-like spp. in Appendix B1 and Appendix B2 of the supplemental material). A standard curve was generated for each set of samples analyzed such that the target in the samples fell within the standard curve range. A negative control was incorporated into each of qPCR run. The minimum detection level per rxn was 5.0×10^3 copies for *Thauera*-like bacteria spp. and 1.75×10^3 copies *Zoogloea-Methyloversatilis*-like *bacteria*, respectively. The mean and standard deviation of efficiency for all qPCR runs were 1.033 ± 0.012 for 0.955 ± 0.033 for *Zoogloea*-like bacteria spp. and *Thauera*-like *bacteria*. The coefficients of determination (R^2) were 0.983 ± 0.01 and 0.968 ± 0.04, respectively. The slopes of standard curves were -3.29 ± 0.07 for *Zoogloea*-like bacteria spp. and -3.47 ± 0.11 for *Thauera*-like bacteria, and the y-intercept of the standard curve was 46.04 ± 1.61 for *Zoogloea-Methyloversatilis* spp. and for 40.39 ± 1.68 for *Thauera*-like bacteria.

4.3.8 Melt Curve

For melt curve analyses 32X Sybr green I (Invitrogen, Carlsbad, CA) was added to the master mix to yield a final concentration of 0.05X for both bacteria. Sybrgreen Reactions were conducted using Eppendorf RealPlex EP instrument (Eppendorf, Hauppauge, NY) with the temperature ramped in 1°C increments from 50 to 99°C.

4.3.9 PCR product purification, and DNA sequencing and Optimization of primer annealing temperature

Gel electrophoresis was conducted after PCR to confirm the size of the amplicon Briefly the protocol for electrophoresis was 5 µl of PCR product was mixed with 3 µl of 6X gel loading buffer type IV (Sambrook and Russell, 2001) which was then loaded into a 2% (w/v) agarose gel (Fisher Scientific, Fair Lawn, NJ) soaked with 0.375 µg/ml ethidium bromide (Fisher Scientific, Fair Lawn, NJ). The gel was run at 85V or 90V for 90 min with 50 or 100 bp DNA mini ladders (2.5 µl) (*ex*ACTG*ene*, Fisher Scientific Interational, Cannada) in the first and last lanes of the gel wells. Additionally positive and negative controls were run with each gel electrophoresis. Product bands were photographed using FluorChem^{TM} (Alpha Innotech Corporation, San Leandro, CA) or using AlphaEaseFC^{TM} software (Alpha Innotech Corporation, San Leandro, CA). The optimizations of annealing temperature were for each organism is shown in Appendix B3 and Appendix B4.

Positive bands were excised from the gel and purified by using QIAquick gel extraction kits (ZYMO, Irvine, CA) according to the manufacturer's instructions. The 4 ng μl^{-1} of 119 (16S rRNA genes for *Zoogloea*) basepair amplicons and 10 ng $\mu l^{-1}$of 400 basepair amplicons for 16S rRNA genes for *Thauera* were sequenced at UC Davis sequencing facility (Davis, CA). Both 3'to 5' and 5' to 3' strands of all samples were sequenced. The sequencing results were visualized using Sequence Scanner V1.0 software (Applied biosystems, Foster City, CA). The forward sequence and reverse compliment of the 3'-5'strand were utilized to generate a final consensus sequence using CLUSTALW software (Subramaniam, 1998) or basic local alignment search tool such as NCBI/ BLAST/ BLASTN suite (Altschul et al., 1990). Degeneracy replacement was done manually using the sequence chromatogram (Sequence scanner V1.0 software (Applied Biosystems, Foster City, Calif.) to check potential errors or remove degeneracies carried in only 1 strand. Fragment alignment was performed using CLUSTALW software or Basic Alignment Search Tool and submitted to NCBI for sequence match (Benson et al., 2008).

4.3.10 Statistical calculation

For interpreting the relationship between bacterial abundance and physicochemical parameters, Pearson correlation analyses were used. Pearson correlation coefficients (r) were obtained using Microsoft Excel with a sample size (n) of 40, and results yielding a level of

significance ($p<0.05$) were considered to be statistically significant using the two tail T-distribution function.

4.4 Results

4.4.1 Sequencing Results for *Zoogloea-Methyloversatilis* like bacteria and *Thauera*-like bacteria.

Although the design of *Zoogloea* primer and probe set was genus specific, sequence results from sample DNA extracts of MLSS amplicon indicated a 98% coverage over the amplicon length to *Methyloversatilis universalis* (Acession No. DQ442273) with a 97% maximum identity to the target sequence (The sequencing results are presented in the supplemental data Appendix B-Table1, page 121). The sequence from this study shared 100% homology with four *Zoogloea* type species over the 16S rRNA gene amplicon length with a 100% maximum identity to: *Zoogloea ramigera* (X74913), *Zoogloea resiniphila* (AJ011506), *Zoogloea oryzae* (AB201043), *Zoogloea caneni* (DQ413148) (see phylogenic analysis of *Zoogloea* spp. related sequences, Appendix B5 in the supplemental material). The construction of the primer and probe set and the completion of the work predated the publication of the sequence for *Methyloversatilis universalis* in August of 2011. Hence, the target organism is referred to as *Zoogloea*-*Methyloversatilis*-like bacteria. The rapid increase in 16S rRNA gene sequences added to Genbank and other data bases emphasized the difficultly in assuring that a primer and probe set was unique from newly sequenced organisms as was the case in this study and identifies a potential pitfall existing for probes used in both qPCR and *in situ* hybridization.

qPCR would not distinguish between *Zoogloea* spp. and *Methyloversatilis* spp. based on the two mismatches in the forward and the one mismatch in the reverse primers (Appendix B5). No differences would be obtained by qPCR even if the mismatches were in the region of the probe as five mismatches out of twenty were required for differentiation based on the work of Xiao et al (2010) for *Nocardia*. This finding pointed out the need to continuously refine primer and probe sets to insure specificity with the target organisms in activated sludge. On the other hand, the *Thauera*-like bacteria sequence results from sample DNA extracts of MLSS was significantly different from its neighboring species in Appendix B6, The amplicons in this study shared 100% coverage with two *Thauera* spp. type species over the 16SrRNA gene amplicon length with a 100% maximum identity to *Thauera* sp. MZ1T (NR074711) and *Thauera aminoaromatica* (AJ315677).

The melting temperature (Tm) of the amplicon for the control *Zoogloea* (3640) and 26 MWRP mixed liquor amplicons ranged between 85.0 ^{o} C and 87.5 ^{o} C (see Appendix B7 in the supplemental material). Two outlier samples from August 18, 2009 reflected a different Tm. This suggests the presence of *Methyloversatilis* spp. in this specific sample which has a theoretical range of 1 ^{o}C higher than the *Zoogloea* spp. A melt curve analysis was also conducted for *Thauera*-like bacteria. The melting temperature for *Thauera*-like bacteria based on the 26 MWRP mixed liquor amplicons contained one group of temperature peaks ranging between 79.5 ^{o} C and 80.5 ^{o} C, which were nearly identical to the Tm of 80 ^{o} C for the known

400bps *Thauera* spp. amplicon and is in the acceptable range of variation for a given genera (see Appendix B8 in the supplemental material).

4.4.2 Effect of Temperature on *Zoogloea-Methyloversatilis*-like bacteria and *Thauera spp.* bacteria communities

Zoogloea-Methyloversatilis-like bacterial and *Thauera*-like bacterial abundances showed different overall relationships to the temperature of the mixed liquor activated sludge over the 11-month study (Figure 4-1). *Zoogloea-Methyloversatilis*-like *bacteria* remained in pseudo-steady state fluctuating around a concentration 10^{12} cells l^{-1} from January through June 2009 except for a drop of approximately one log as a result of a chlorination event to reduce bulking organisms on April 12, 2009. In the overall correlation between *Zoogloea-Methyloversatilis*-like bacteria concentrations and temperature (Figure 4-2a) we see a negative relationship ($r = -0.33$ $p < 0.05$). Breaking the year into two temperature segments produced a clearer picture of the response of *Zoogloea-Methyloversatilis*-like bacteria to temperature (Figure 4-2b and 4-2c) showing a negative correlation (r=-0.59, $p < 0.01$) when the temperature grouping was > 27 ^{o}C and no relationship when the temperature was ≤ 27° C. In pure culture the optimum temperature for several *Zoogloea* species was higher (Joyce and Dugan, 1972), which pointed out the importance of understanding how optimal conditions were altered for an organism in complex environments in a full scale wastewater treatment plant.

Thauera-like bacteria concentrations closely followed the temperature pattern over the 11-month period (Figure 4-1). Again analyzing the overall correlation between *Thauera*-like bacteria concentrations and temperature, showed a positive relationship of $r= 0.46$, $p<0.005$ (Figure 4-3a). Interestingly, breaking the data into the two temperature segments addressed above (Figure 4-3b and 4-3c), showed the major relationship between *Thauera*-like bacteria and temperature was derived in the range of 23-26.9 °C, and not from the relationship when the temperatures were above 27 °C (Figure 4-1). These findings helped explain why *Zoogloea-Methyloversatilis*-like *bacteria* did not dominate over *Thauera*-like bacteria in the summer months. Moreover, the abundance of *Thauera*-like bacteria was nearly one log lower when the temperature was in the 23-27°C range compared to its abundance at 27-29°C (Figure 4-1).

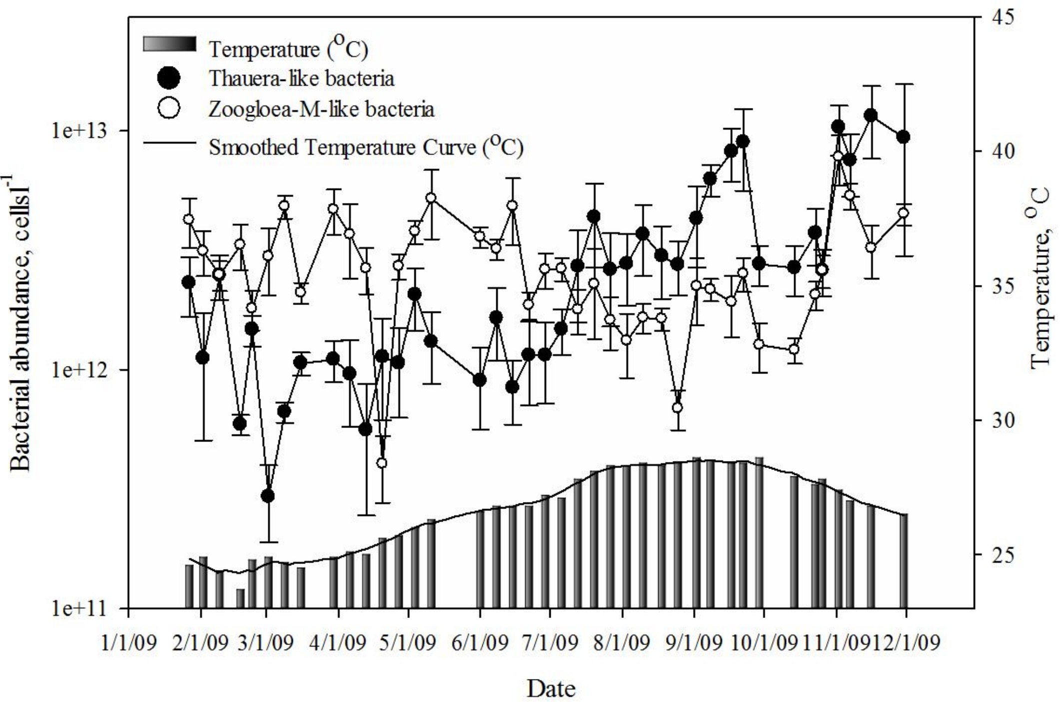


Figure 4-1 Relationship between bacterial abundance of *Zoogloea-Methyloversatilis*-like bacteria, *Thauera*-like bacteria and temperature over the study period. The error bars stands for standard deviation of the triplicate samples.

The effect of temperature has strong impacts on these denitrifying genera. Studies using pure cultures of *Zoogloea* spp. and *Thauera MTZ1* spp. showed the optimum temperature for growth to be 28°C (Joyce and Dugan., 1972) and 30°C (Duldhardt et al., 2007), respectively. Our data concurred with these pure culture studies in that, *Zoogloea-Methyloversatilis* like bacteria had a lower optimum growth temperature than *Thauera*-like bacteria but also clearly points out how population dynamics in the MLSS is modulated by operating parameters, such that *Zoogloea-Methyloversatilis*-like bacteria could not maintain optimum growth at 28 °C as seen in pure culture studies (Isaka et al., 2007).

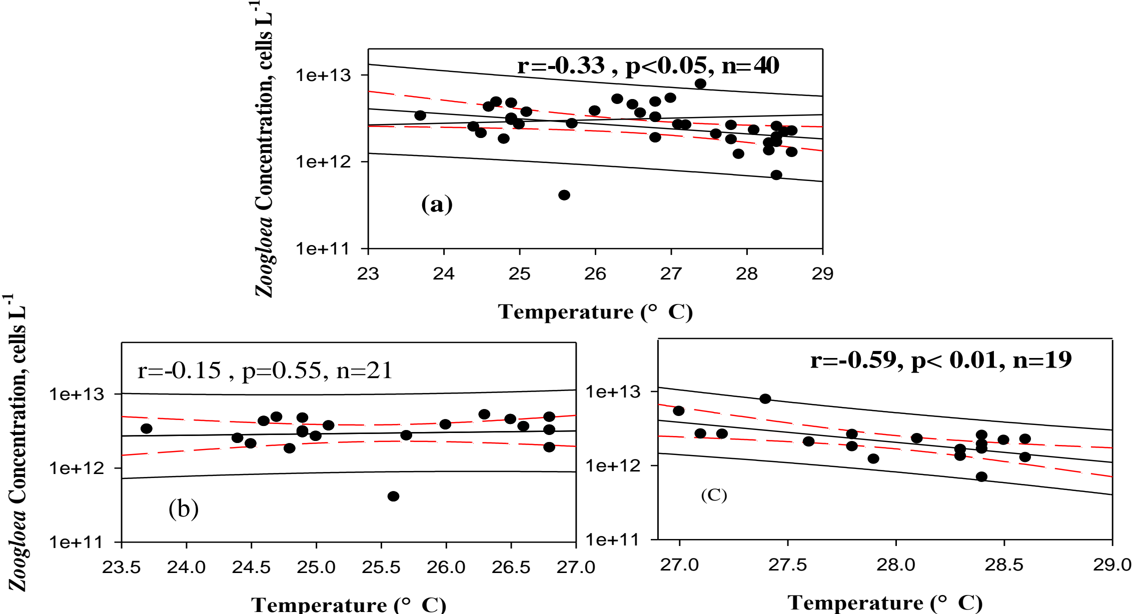
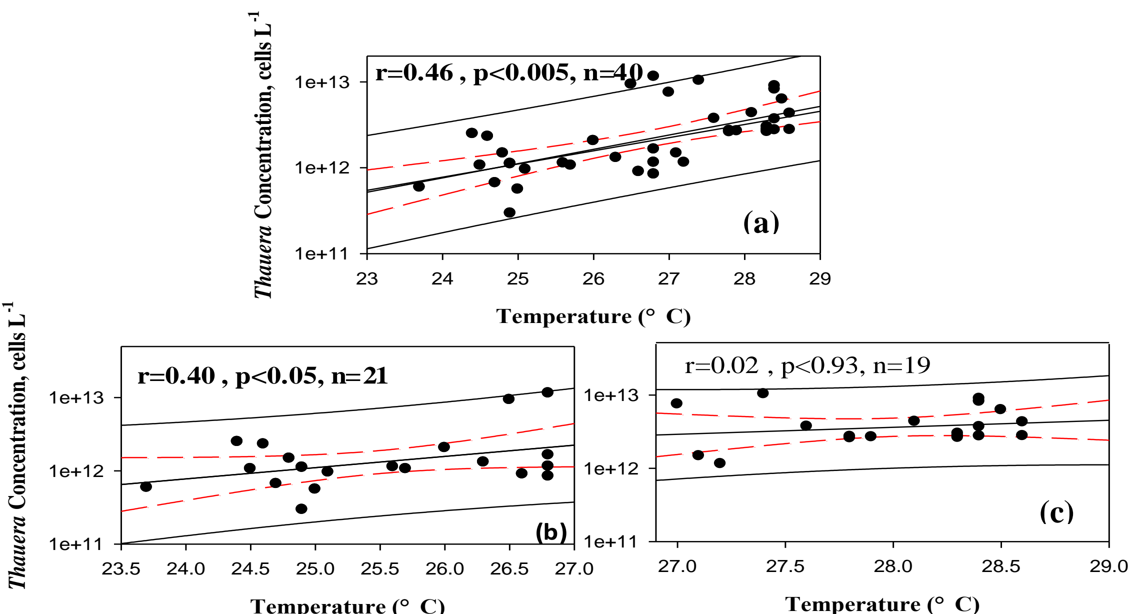


Figure 4-2-Relationship between temperature and *Zoogloea-Methyloversatilis* like bacterial abundance (*Zoogloea* Concentration) in activated sludge tank of a complete nitrifying plant. (a) all temperatures; (b) temperature 23° C to 26.9° C; (c) tempeatures >27 °C, where n=sample size. (The double dashed line -------- indicate 95% confidence interval. The double solid line — indicate 95% prediction interval).



4-3-Relationship between temperature and *Thauera*-like bacteria abundance (*Thauera* Concentration) in an activated sludge tank of MWRP. (a) all temperatures; (b) temperature range 23°C to 26.9^{o}C; (c) temperatures >27°C; where n=sample size. (The double dashed line -------- indicate 95% confidence interval. The double solid line — indicate 95% prediction interval).

4.4.3 Relationship between substrate and *Zoogloea- Methyloversatilis*-like bacterial and *Thauera-like* bacterial populations in activated sludge

The role of substrate utilization in denitrification was measured by removal of COD and nitrate as well as methanol addition. Each of these parameters was compared to the abundance of each bacterial grouping over the two temperature ranges (Figure 4-4 and Figure 4-5). For temperatures below 27°C a positive correlation coefficient was found for *Zoogloea-Methyloversatilis*-like bacterial abundance and nitrate removal (Figure 4-4 a) (r=0.54, p<0.05), and no correlation was found between *Zoogloea-Methyloversatilis*-like bacteria abundance with COD removal and methanol dose. At the higher temperature range (Figure 4-4 d, e, f) methanol dosage (r= 0.45, p<0.05), showed a positive correlation with

Zoogloea-Methyloversatilis like bacteria and also a significant positive correlation with percent denitrification (r=0.43, p =0.05) but no significant relationship with COD removal in the both cold and warm seasons (Figure 4-4c). Examining Figure 4-1, it is clear that this organism's abundance was primarily driven by temperature as it increased when temperature approached to 28°C.

Thauera-like bacteria only showed a positive correlation (r=0.53, p<0.05) with COD removal below 27°C (Figure 4-5c). Above 27°C only denitrification and *Thauera*-like bacterial abundance were significantly correlated (r= 0.70, p<0.05) (Fig 4-5d) but not correlated with COD removal or methanol (Figure 4-5e and 4-5f). Our data agreed with a previous study that showed three *Thauera* species were unable to utilize methanol in pure culture for growth (Cantafio et al., 1996; Scholten et al., 1999). Acetate, propionate, butyrate and ethanol have been reported to be carbon sources utilized by *Thauera* bacteria *(*Macy et al, 1993; Lukow and Diekmann, 1997), and *Methylophilius* and *Methylobacillus* of Betaproteobacteria along with *Hyphomicrobium* spp. and other Alphaproteobacteria have been identified as the predominant methanol utilizers in methanol-fed denitrification plants. (Ginige et al., 2004; Ruiz et al., 2009). This result showed at this plant the methanol utilization by *Zoogloea-Methyloversatilis* like bacteria which were present at a greater abundance than non-methanol utilizing *Thauera*-like bacteria which used COD during the colder months at this plant.

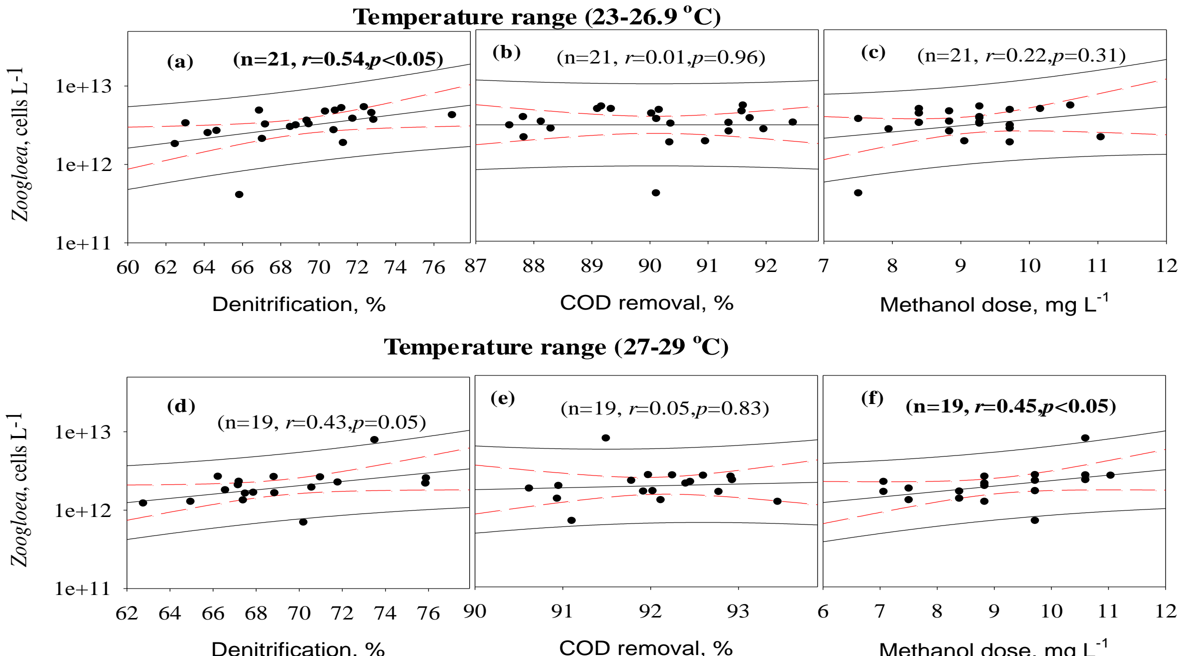


Figure 4-4-Relationship between nitrate removal, COD removal, methanol dosage, and the *Zoogloea- Methyloversatilis*-like bacteria populations in MLSS of MWRP over two temperature ranges. (a, b, c) temperatures 23°C to 26.9°C; (d, e, f) temperatures >27°C, n=sample size, , and bold =statistically significant (The double dashed line -------- indicate 95% confidence interval. The double solid line — indicate 95% prediction interval).

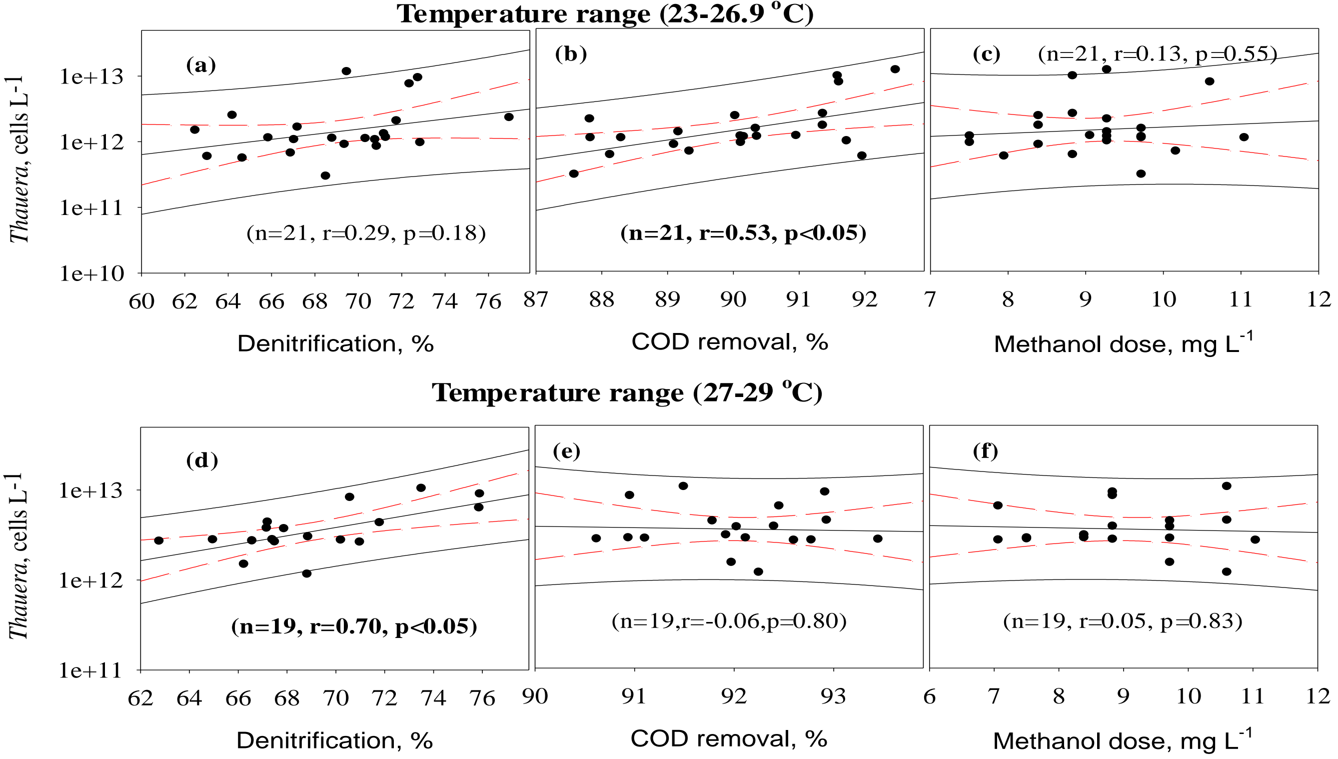


Figure 4-5-Relationship between nitrate removal, COD removal, methanol dosage and the *Thauera*-like bacteria populations in MLSS in MWRP over two temperature ranges. (a, b, c) temperatures 23°C to 26.9°C; (d, e, f) temperatures >27°C, n =sample size, and bold =statistically significant (The double dashed line -------- indicate 95% confidence interval. The double solid line — indicate 95% prediction interval).

These data showed that *Zoogloea-Methyloversatilis*-like bacteria were a good indicator of nitrate removal over the entire temperature range. However, *Thauera*-like *bacteria* were a better indicator than *Zoogloea-Methyloversatilis*-like bacteria when the temperature was greater than 27°C. In the higher temperature range, *Zoogloea-Methyloversatilis*-like bacteria and *Thauera* bacteria abundances were increased with increasing methanol dosage, and COD removal, respectively.

4.4.4 Aerobic versus Anoxic Denitrification

Apart from temperature, dissolved oxygen in the anoxic tank is another important variable to both the growth of denitrifiers and the denitrification process. General practice is to keep the DO below 0.2 mg l^{-1} in the anoxic tank (Anderson et al., 1986; Kester et al., 1997). Seventy percent denitrification represents what is considered the plant optimum. However, other factors during plant operation result in greater fluctuations of anoxic DO and the percentage of denitrification.

To further examine these data, temperature and DO were stratified by efficiencies in denitrification, and the results are shown in Figure 4-6. Approximately 27.5% of total events with greater than 70% denitrification were at low DO concentrations compared to 22.5% denitrification when DO was greater than 0.2 mg l^{-1}. At temperatures greater than 27°C, anoxic conditions produced approximately six times more events with greater than 70%

denitrification compared to those conditions with DO > 0.2 mg l^{-1}. At greater than 27 ^{o}C the percent of events producing less than 70% removal were equal between the two DO conditions. These data suggested that denitrification will significantly benefit from strict anoxic conditions (DO< 0.2 mg l^{-1}) and will greatly benefit over hypoxic conditions (DO>0.2 mg l^{-1}) when temperature less than 27°C. Since majorities of denitrifying organisms have been reported in batch studies to have greater denitrification activity and bacterial abundance at the higher temperature range (Holt et al., 1994), the need to keep DO below 0.2 mg l^{-1} is more important in the late fall-winter period than the summer-early fall period (Figure 4-6).

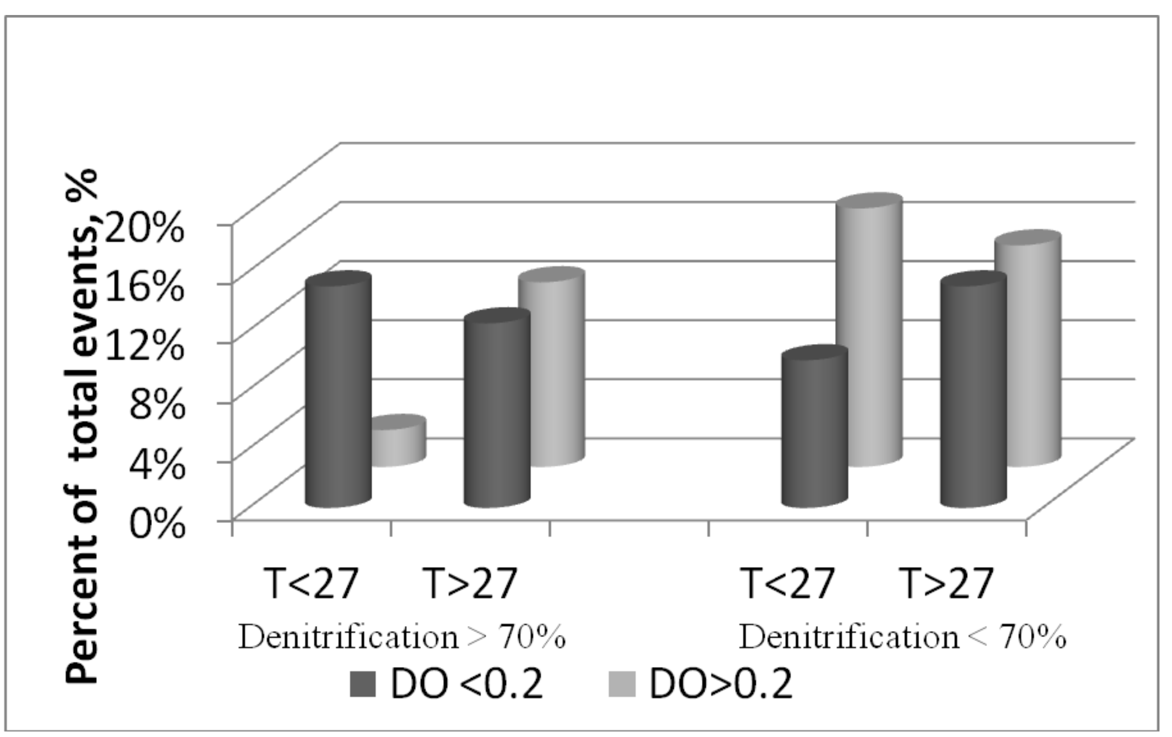


Figure 4-6-Effect of DO (mg l^{-1}) concentration in the Anoxic Zone and Temperature on Denitrification (expressed as percent of samples falling into each category)

Furthermore, we examined the amount of nitrogen removed when DO > 0.2 mg l^{-1} but < 0.6 mg l^{-1} and when it was less than 0.2 mg l^{-1} (Figure 4-7) with nitrate removal from 62% to

76%. Again using denitrification of 70% under anoxic conditions slightly more values (n= 10) fell into this category compared to (n=7) when DO was greater than 0.2 mg l^{-1} shown in Figure 4-7 This reinforced the concept that two denitrifying populations of bacteria predominated in this plant (aerobic and anoxic).

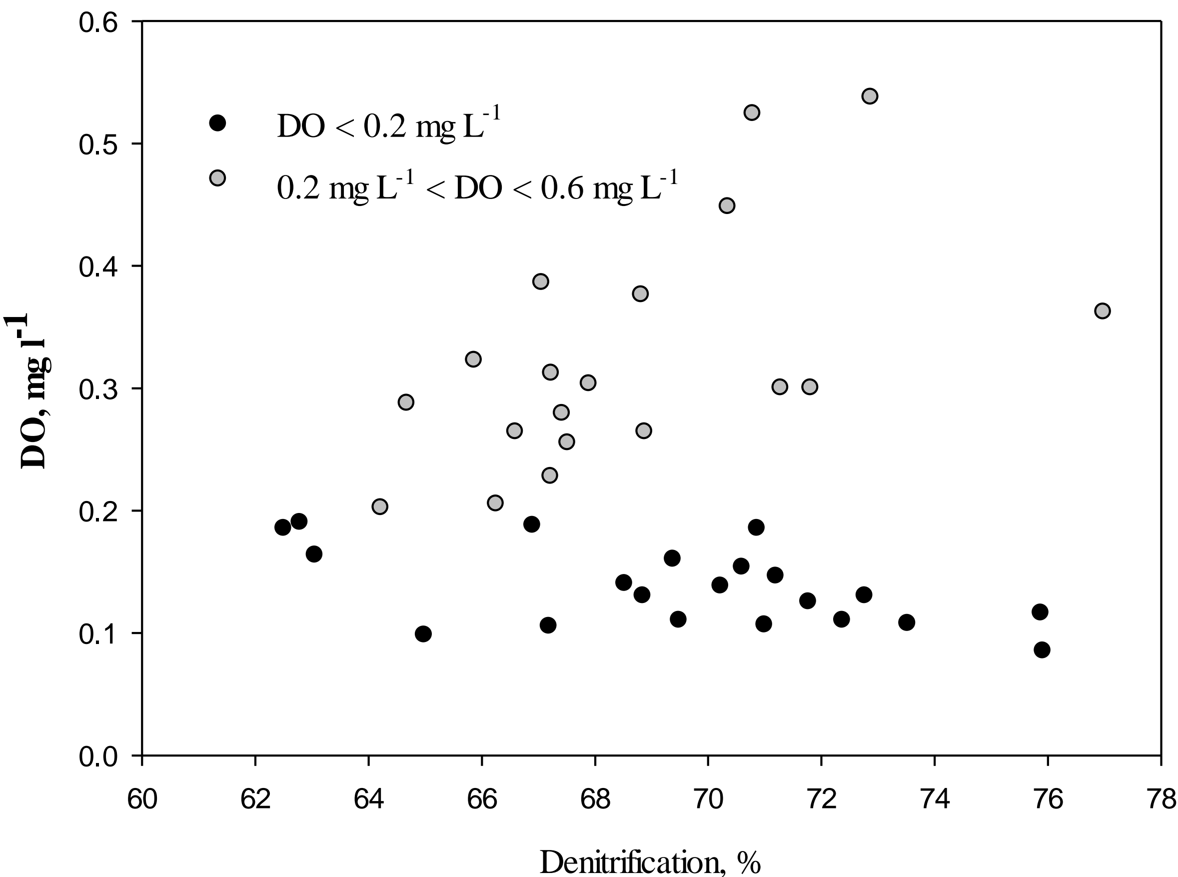


Figure 4-7--Dissolved oxygen and denitrification at MWRP stratified by DO< 0.2mg l^{-1} ●, and 0.2<DO<0.6 mg l^{-1} ○ in the anoxic zone.

Since we had two bacterial genera which represented 34% of the total bacterial community and reacted differently to temperature, we examined their response to DO and denitrification based on population abundance and the results are shown in Figure 4-8. For

Zoogloea-Methyloversatilis-like bacteria denitrification trends upward with both DO conditions (Figure 4-8a, b), with a significant relationship when denitrification ($r = 0.52$, $p < 0.05$) when DO was greater than 0.2mg l^{-1}. On the other hand, *Thauera*-like bacterial abundance increased when denitrification increased when DO was less than 0.2 mg l^{-1}(Figure 4-8c), and no correlation existed when DO was greater than 0.2mg l^{-1}(Fig. 4-8d). In fact, the concentration of *Thauera*-like bacteria decreased as the DO concentration increased from 0.2 mg l^{-1} to 0.6 mg l^{-1} (Figure 4-8d and Figure 4-9). The findings that *Zoogloea-Methyloversatilis*-like bacteria carried out denitrification under anoxic and hypoxic wastewater conditions have also been shown in a study (Patureau et al, 2000). Our findings indicate that *Zoogloea-Methyloversatilis*-like bacteria were far less impacted by fluctuations in DO concentrations than *Thauera*-like bacteria. Hence, higher concentrations of oxygen negatively impact the growth of *Thauera*-like bacteria. In the plant studied, the DO did not decrease overall denitrification efficiency in the summer but did reduce the abundance of one of major groups (*Thauera*-like bacteria) involved in denitrification, which had the greatest impact during colder months, when bacterial activities are low.

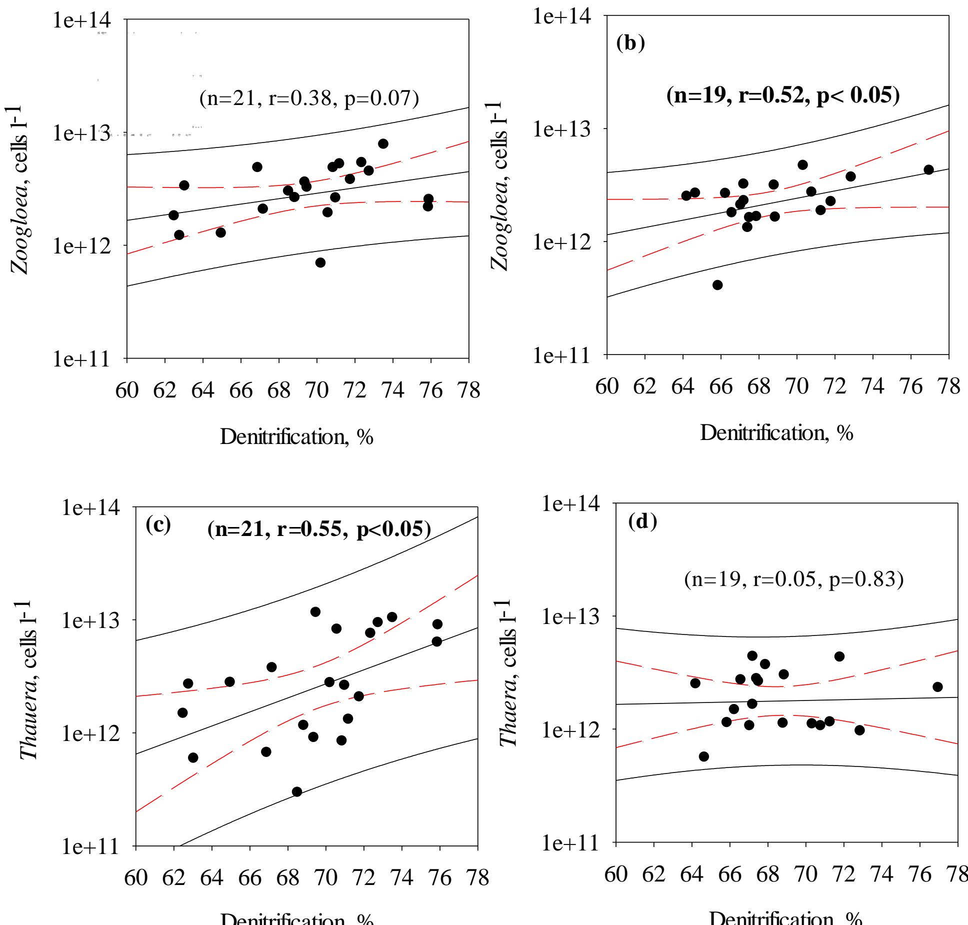


Figure 4-8-Comparison of *Zoogloea-Methyloversatilis*-like bacteria spp. and *Thauera*-like bacteria abundance to denitrification at DO less than 0.2 mg l^{-1} (a, c) and greater than 0.2 mg l^{-1} (b, d) in the anoxic zone (The double dashed line -------- indicate 95% confidence interval. The double solid line —— indicate 95% prediction interval).

Furthermore, this finding extends our understanding of denitrification by *Zoogloea-Methyloversatilis*-like bacteria, between the DO range of (0 mg l^{-1} - 0.2 mg l^{-1} and 0.2 - 0.6 mg l^{-1}), showing *Zoogloea-Methyloversatilis*-like bacteria can carry out denitrification regardless of these changes in DO concentrations.

Thauera-like bacterial concentrations related to DO in Figure 4-9c, reaffirms the negative correlation observed between the two variables. Since *Thauera*-like bacteria abundance was

negatively related to oxygen even below 0.2 mg l^{-1}, *Thauera*-like bacteria appeared from these data to be a strictly anaerobic denitrifier. Reexamining Figure 4-8c it is clear that the higher abundance was observed at the lowest oxygen concentration. Both findings suggest that the growth of *Thauera*-like bacteria is most favorable under strict anoxic conditions and also suggest best nitrate removal when DO is less than 0.1 mg l^{-1}. This evidence concurs with a study by Oh and Silverstein which suggested that inhibition of denitrification occurred when DO was as low as 0.09 mg l^{-1}, (Oh and Silverstein, 1999a). Therefore a set point of < 0.1 mg l^{-1} is more favorable to *Thauera*-like bacteria, since a nearly one log decrease in *Thauera*-like bacterial abundance (Figure 4-9d) was seen when DO was greater than 0.2 mg l^{-1}.

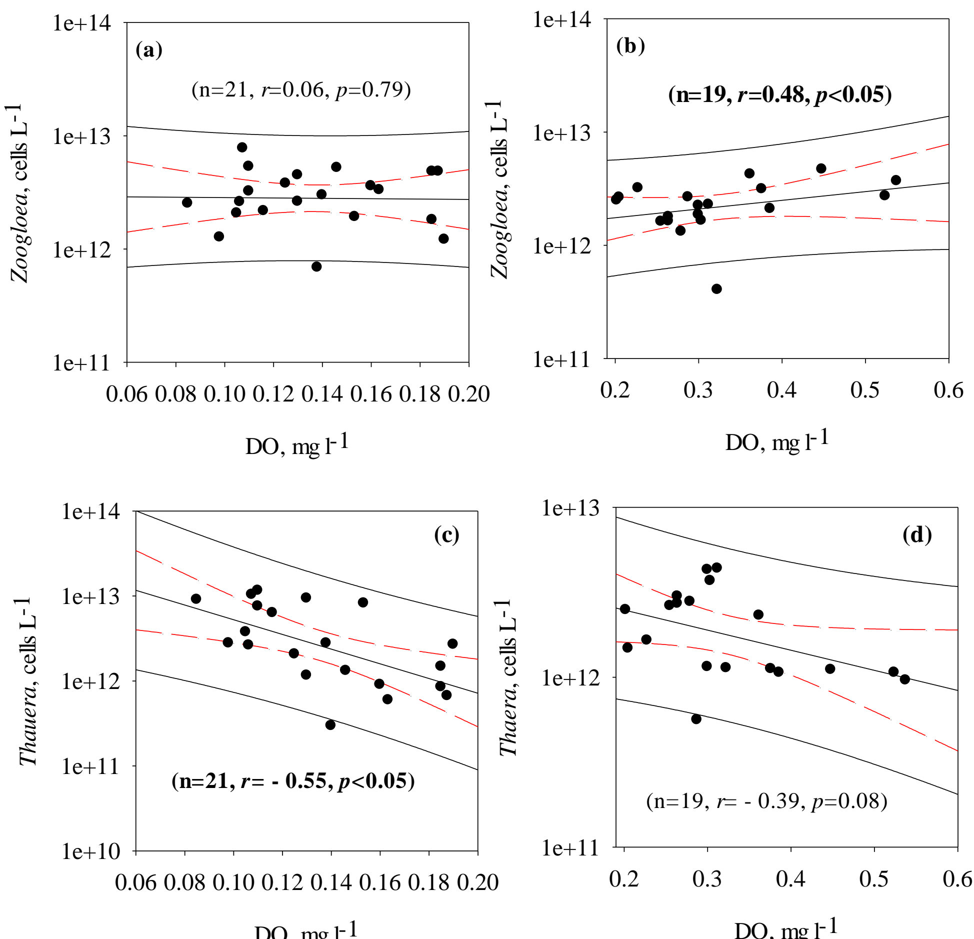


Figure 4-9 Comparison of *Zoogloea-Methyloversatilis*-like bacteria spp. and *Thauera*-like bacteria abundance to DO at DO less than 0.2 mg l^{-1} (a, c) and greater than 0.2 mg l^{-1} (b, d) in the anoxic zone (The double dashed line -------- indicate 95% confidence interval. The double solid line —— indicate 95% prediction interval).

4.6 Conclusions

Overall, temperature dependency also affected different substrate utilization. *Zoogloea-Methyloversatilis*-like bacteria used methanol at a higher temperature range, while *Thauera*-like bacteria abundance was enhanced with increasing COD removal when temperature was in the lower temperature range. Understanding the temperature dependence of species as well as their response to DO in this study showed that higher temperature

retarded the abundance of *Zoogloea-Methyloversatilis*-like bacteria; while higher anoxic DO concentrations during the warm season has retarded *Thauera*-like bacteria growth.

In this treatment plant, considerable amounts of aerobic denitrification occurred when temperatures were less than 27°C and at higher temperatures (>27°C). Strict anoxic conditions were the most important factors since the number of events resulting in greater 70% of NO_3-N removed fell dramatically when DO was greater than 0.2 mg l^{-1} when temperature was less than 27°C.

Chapter 5. Competitions among Heterotrophic Denitrifiers and Bulking Organisms in an Activated Sludge Process under Warm-Climate Condition

5.1 Abstract

In this study, population dynamics of two denitrifiers (*Paracoccus*-like bacteria and *Burkholderia*-like bacteria) and one bulking organism (*Thiothrix eikelboomi*) were investigated to examine the interactions of denitrification versus nitrate reduction to nitrite. The *Burkholderia*-like bacterial population and the *Paracoccus*-like bacterial population were out grown by *Thiothrix*-like bacteria (bulking organisms) during three different time periods (March, May, July), which correlated significantly with the nitrite accumulation ($r=0.44$, $p< 0.05$), high anoxic dissolved oxygen (DO)($r=0.38$, $p<0.05$). DO was the most important factor creating negative impacts on the bacterial abundances of *Burkholderia*-like bacteria ($r = -0.45$, $p < 0.05$) and *Paracoccus*-like bacteria ($r = -0.46$, $p < 0.05$). However, hypoxic DO positively correlated with *Thiothrix eikelboomi* ($r = 0.38$, $p < 0.05$). Nitrite accumulations negatively impacted *Paracoccus*-like bacteria ($r = -0.33$, $p<0.05$) but correlated positively with *Thiothrix eikelboomi* ($r = 0.44$, $p < 0.05$). Temperature correlated positively with Paracoccus-like bacteria ($r = 0.36$, $p < 0.05$) and *Burkholderia*-like bacteria ($r = 0.34$, $p < 0.05$), while negatively correlated *Thiothrix eikelboomi* ($r = -0.41$, $p < 0.05$).

5.2 Introduction

Increasing difficulties in achieving higher efficiencies of denitrification have received greater attention due to the occurrence of a large number of inhibitors effecting denitrifying bacterial populations in activated sludge systems (Glass et al., 1997; Oh et al., 1999b; O'Connor and Hondzo, 2007). Some of the parameters inhibiting the growth of the myriad of populations involved in denitrification may include: significant buildup of nitrite accumulation from 0.5 to 20 mg l^{-1} *(Glass et al., 1998; Wilderer et al., 1987; Pan et. al., 2012)*, moderate changes in dissolved oxygen (DO) concentration in the anoxic zone (DO exceeds 1 mg l^{-1}) (Liu et al., 2010), significant temperature variations (Ilies., 2001; Carrera., 2004; Saleh-Lakha et al., 2009), and high abundances of bulking organisms and foaming organisms, which all can disrupt the continuous dynamics of bacterial growth and its ability to denitrify (Asvapathanagul, 2011; Tian et. al., 2011; Chevakidagarn et al., 2012; Noutsopoulos et. al., 2012).

Since denitrifying bacteria are so heterogenous it maybe that consortia of minor populations contribute in a major way to denitrification (Jeretschko et al., 2002; Gomge et al., 2004; Thomsen et. al., 2007; Hagman et. al., 2008). This observation is also based on (the study in Chapter 4) Wang's (2014) work that showed the two major groups to represent approximately 34% of the denitrifying population at the Michelson Water Reclamation Plant,

and subsequent 454 sequencing data which showed a consortia of denitrifiers (data not shown).

We have investigated *Burkholderia* and *Paracoccus*-like bacteria in this study, two minor bacterial populations, representing together about 1.0 % of the total bacteria. *Burkholderia* species are among the most common heterotrophic bacteria found in wastewater treatment processes that are associated with removal of chemical oxygen demand (COD) (Ren et. al, 2005; Wang et. al, 2002; Salmerón-Alcocer et. al., 2007). Although several species of *Burkholderia* (*B.*), such as *B. cepacia*, *B. pseudomallei*, and *B. mallei*, have been reported with the ability to denitrify in batch and pilot studies (Brenner et. al., 2005), there is no subsequent study that monitors *Burkholderia* bacterial populations to understand their relationships to denitrification in a full-scale activated sludge process under varying anoxic conditions over yearly temperature changes. On the other hand, *Paracoccus* species are commonly found in methanol-fed municipal wastewater treatment plants, where the genus of *Paracoccus denitrificans* has been used as a bacterial model for the study denitrification (Glass et. al., 1998). Among all the denitrifiers within the genus of *Paracoccus* (*P.*), several species including *P. pantotrophus*, *P. denitrifican*, *P. alcaliphilus*, *P. kocurii*, *P. marcusii*, *P. solventivorans*, *P. thiocyanatus*, *P. versutus* are denitrifying organisms that reduce nitrate to nitrogen gas (N_2). *P. alkenifer*, *P. aminophilus*, *P. aminovorans* can only convert nitrate to nitrite while *P. denitrificans* is reported to be capable of utilizing methanol as the carbon

source for growth under both aerobic and anaerobic conditions (Robertson et., al., 1989; Barak et. al., 2000).

Investigations of how *Paracoccus*-like bacterial and *Burkholderia*-like bacterial populations contribute to the overall denitrification in the wastewater treatment process under both anoxic and hypoxic conditions were conducted in this study. Moreover, a group of bulking organisms, *Thiothrix eikelboomi* was also found at the same treatment plant (Asvapatanagul, 2011) during our study. It is also identified as one of the nitrate removing based genera because of its ability to convert nitrate to nitrite (Kanagawa et al., 2000; Dumonceaux et al., 2006; Trubitsyn et al., 2013). We have incorporated this bacterial group into the analysis to understand the possible interactions amongst these three organisms in light of inhibition of denitrification by nitrite and the matched population size of organisms. This study relates the abundance of these bacterial populations with physicochemical parameters to understand the population dynamics the *Burkholderia*-like and *Paracoccus*-like bacterial populations in a full-scale process, when bulking is occurring.

The hypothesis is that a nitrite producing bulking (*Thiothrix eikelboomi*) with partial denitrification ability will out-compete denitrifiers under hypoxic DO conditions in warm climate. By conducting the qPCR experiment to quantify the abundance of a bulking organism (*Thiothrix eikelboomi*) and two groups of denitrifiers (*Paracoccus*-like bacteria and *Burkholderia*-like bacteria) and utilizing pearson's correlational analysis of the bacterial

abundance and environmental parameters, we are able to determine: a) physicochemical parameters causing bulking organisms to outgrow denitrifiers, b) the solutions to prevent the growth of a bulking organism, c) which of the bacterial group of denitrifiers (aerobic or anaerobic) represent a better indicator for denitrification.

5.3 Materials and Methods

5.3.1 Plant Description

See section 4.3.1.

5.3.2 Sample Collection

See section 4.3.2.

5.3.3 *Burkholderia* spp. and *Paracoccus* spp. Positive Control

Paracoccus denitrificans (B-3785) and *Burkholderia cepacia* (NRRLB-14809) were obtained from the Agricultural Research Service (ARS) culture collection was grown according to USDA protocol. The growth medium was Tryptone Glucose Yeast (TGY) Broth containing 0.5% tryptone, 0.1% glucose, and 0.1% K_2HPO_4. Cultures were incubated in an orbital shaker (Forma Scientific, Marietta, OH) at 28°C at 60 rpm for 24 hr until significant growth occurred. Colony isolation was achieved by streaking the culture onto 2% TGY agar and incubating as stated above. An individual colony was then transferred from the agar medium to a tube containing TGY broth and incubated as before. The culture was then

subjected to the same DNA extraction protocol as described below and stored at -80°C as a pure extract and in 1:10 dilution.

5.3.4 DNA Extraction

The qPCR and SYBR Green qPCR methods were described in section 3.3.9 and 3.3.10.

5.3.5 qPCR *Paraccocous spp.* and *Burkholderia* spp. Primers and Probes development

The *Paracoccus*-like bacteria primer and probe set was designed in three sequential steps: (1) The NCBI/BLAST/blastn suite alignment search was utilized to identify the regions of homology and heterogeneity between the *Paracoccus denitrificans* (NR074711.1) and 16S rRNA genes (5'-3' sequences) of other *Paracoccus* species. (2) Uncultured and environmental Genbank entries that showed high dissimilarity with the reference sequence were excluded from the alignment process. (3) Highly specific regions that carried no homology with other bacterial genera, while sharing homology with the *Paracoccus denitrificans*, were selected for use in the design of the primer and probe set. The primers were screened against all bacterial taxids other than the target using *in silico* analyses (Murray et al. 2007). If homology was found in either the forward and reverse primer such that an amplicon could be generated, the size was ascertained. The same steps were performed in the design *Burkholderia*-like bacteria primer and probe set using the reference sequence of *Burkholderia cepacia*. The *Paracoccus*-like bacteria spp. primers yielded a 165 basepair (bp) amplicon, and the *Burkholderia*-like bacteria spp. primers yielded a 183(bp)

basepair amplicon. The details of sequencing results and the discussions on the specificity of primer and design can be found in the work conducted by Chengyao Tsai (2012).

5.3.6 Quantitative Polymerase Chain Reaction (qPCR)

Primers and probes for *Burkholderia spp. and Paracoccus spp.* for the 16S rRNA genes used in the qPCR analyses are listed in Table 2. All qPCR reactions were performed on an Eppendorf RealPlex EP (Eppendorf, Hauppauge, New York). Each qPCR reaction contained a total volume of a 25 **µl** solution with the following reagents: 5 µl DNA extract/template, and 20 µl containing 2.5 mM $MgCl_2$, 1 X PCR buffer, 200 nM dNTP, 1 U AmpliTaq DNA polymerase, 100 nM dual-labeled probe, 150 nM forward primer, and 150 nM reverse primer. The qPCR protocol for environmental DNA extracts included 35 cycles of 2 minutes holding at 94°C followed by denature at 94°C of the DNA strands for 40s followed by 20 s at their respective annealing temperatures (listed in Table 2).

Table 5-1 Primer and Probes set for *Burkholderia*-like bacteria and *Paracoccus*-like bacteria

Target Group	Name	Sequence (5'to 3')	Annealing Temperature (^{o}C)
Burkholderia –like bacteria	Forward Primer	AATACAGTCGGGGGATGACGGTA	66.2
	Probe	Fam-CGCAGGCGGTTTGCTAAGACCGATGTGAAATCCCC-BHQ1	
	Reverse Primer	GGGAACTGCATTGGTGACTGGC	

Paracoccus –like bacteria	Forward Primer	GGGCAGCATGCTGTTCGGTG	60.0
	Probe	Fam-ACACCTAACGGATTAAGCATT CCGCCTGGG-BHQ1	
	Reverse Primer	TTACCAACCCTTGACATCGC	

The 16S *rRNA* gene copy number per cell was based on the *Paracoccus* genome which has 3 copies per genome (Klappenbach et al., 2001). While 5.3 copies of 16S *rRNA* per cell was obtained by calculating the *Burkholderia* spp. average cell numbers from the available *Burkholderia* spp. genome data in Genebank for activated sludge samples. The calculated average copy per cell to calculate *Burkholderia* spp. is shown in Table Apendix C1.The standard curves ranged from 3.75×10^3 – 1.75×10^6 copies per rxn and 3.75×10^2 – 3.75×10^6 copies per qPCR rxn for *Burkholderia cepacia* (NRRLB-14809) *and Paracoccus denitrificans* (B-3785), respectively. A standard curve was generated for each set of samples analyzed such that the target in samples fell within the standard curve range. A negative control was incorporated into each of qPCR run. The minimum detection level per rxn was 5.0×10^3 copies for the reaction. The mean and standard deviation of efficiency for all qPCR runs were 0.99±0.02 for *Paracoccus denitrificans* (B-3785) and 0.98±0.03 for *Burkholderia cepacia* (NRRLB-14809), while the coefficient of variation (R^2) were 0.99±0.02 and 0.99±0.03, respectively. The slope of standard curves was -3.31±0.14 for *Paracoccus*

denitrificans (B-3785) and -3.368±0.11 for *Burkholderia cepacia* (NRRLB-14809), and the y-intercept of the standard curve was 38.54±0.7 for *Paracoccus denitrificans* (B-3785) and for 40.0±1.2 for *Burkholderia cepacia* (NRRLB-14809) (Appendix C1 and Appendix C2). The same experimental procedure was conducted for *Thiothix*-like bacteria and the experimental data are shown by Asvapathanagul, et al., (2011).

5.3.7 PCR product purification, and DNA sequencing and Optimization of primer annealing temperature

Gel electrophoresis was conducted after PCR to confirm the size of the amplicon Briefly the protocol for electrophoresis was 5 µl of PCR product was mixed with 3 µl of 6X gel loading buffer type IV (Sambrook and Russell, 2001) which was then loaded into a 2% (w/v) agarose gel (Fisher Scientific, Fair Lawn, NJ) soaked with 0.375 µg ml^{-1} ethidium bromide (Fisher Scientific, Fair Lawn, NJ). The gel was run at 85V or 90V for 90 min with 50 or 100 bp DNA mini ladders (2.5 µl) (*ex*ACTG*ene*, Fisher Scientific Interational, Cannada) in the first and last lanes of the gel wells. Additionally positive and negative controls were run with each gel electrophoresis. Product bands were photographed using FluorChem™ (Alpha Innotech Corporation, San Leandro, CA) or using AlphaEaseFC™ software (Alpha Innotech Corporation, San Leandro, CA). The optimizations of annealing temperature for each organism is shown in Appendix C3 and Appendix C4 (see supplementary data). Positive bands were excised from the gel and purified by using QIAquick gel extraction kits (ZYMO, Irvine, CA) according to the manufacturer's instructions. 6 ng ·µl^{-1} of 183 (16S

rRNA genes for *Burkholderia*-like bacteria) basepair amplicons and 7 ng $\cdot\mu l^{-1}$ of 165 base pair amplicons for 16S rRNA genes for *Paracoccus*-like bacteria were sequenced at UC Davis sequencing facility (Davis, CA). Both 3'to 5' and 5' to 3' strands of all samples were sequenced. The sequencing results were visualized using Sequence Scanner V1.0 software (Applied biosystems, Foster City, CA). The forward sequence and reverse compliment of the 3'-5'strand were utilized to generate a final consensus sequence using CLUSTALW software (Subramaniam, 1998) or basic local alignment search tool such as NCBI/ BLAST/ BLASTN suite, (Altschul et al., 1990). Degeneracy replacement was done manually using the sequence chromatogram (Sequence scanner V1.0 software Applied Biosystems, Foster City, CA) to check potential errors or remove degeneracies carried in only 1 strand. Fragment alignment was performed using CLUSTALW software or Basic Alignment Search Too on NCBI for sequence match (Benson et al., 2008). The results are analyzed using phylogenetic analysis to show specificity of the sequence results for MWRP samples containing *Burkholderia*-like bacteria and *Paracoccus*-like bacteria and are shown in Appendix C5 and Appendix C6.

5.3.8 Melt Curve for *Paracoccus*-like bacteria and *Burkholderia*-like bacteria

For melt curve analyses was conducted to confirm specificity of the primer and probe sets, where 32X Sybr green I (Invitrogen, Carlsbad, CA) was added to the master mix to yield a final concentration of 0.05X for both bacteria. Sybr green Reactions were conducted using Eppendorf RealPlex EP instrument (Eppendorf, Hauppauge, NY) with the temperature

ramped in increments from 50 to 99°C (Appendix C7 and Appendix C8 in supplemental data).

5.3.9 Statistical Analysis

For interpreting the relationship between bacterial abundance and physicochemical parameters, Pearson correlation analyses were used. Pearson correlation coefficients (r) were obtained using Microsoft Excel with a sample size (n) of 40, and results yielding a level of significance ($p < 0.05$) were considered to be statistically significant using the two tail T-distribution function.

5.4 Results and Discussion

5.4.1 Physicochemical and Biological Parameters

As shown in Figure 5-1, the water temperature in the aeration basin ranged from 23.7-28.6 °C, increasing from January (minimum) to October (maximum) and decreasing from October to November. DO in the anoxic zone ranged from 0.06 to 0.60 mg l^{-1} with an overall decreasing trend from the January to November (Figure 5-1a). Total nitrate removals ranged between 15.8 mg l^{-1} to 24 mg l^{-1} (Figure 5-1c), while nitrite concentrations (Figure 5-1c) appeared to accumulate from February through May, with no accumulation during the rest of the sampling dates. The concentrations of nitrite in primary effluent samples were less than those in the secondary effluent (Figure 5-1c). All bacterial groups' abundance significantly dropped from the month of September into October which was followed by a

significant reduction in the total nitrogen influent 5.8 mg l^{-1} or 12.5% overall nitrogen removal.

Burkholderia-like bacteria, *Paracoccus*-like bacteria, and a previously determined group of *Thiothrix eikelboomi* bacteria were monitored in the full-scale, incomplete denitrifying plant, throughout this the same period (Figure 5-1b). *Burkholderia*-like bacteria have an overall decreasing trend from 1.1×10^{10} to 6.62×10^{8} mg l^{-1} for January through August, and increase from of 6.62×10^{8} to 1.61×10^{10} mg l^{-1} for the period of August through November. *Paracoccus*-like bacteria followed closely with trends of *Burkholderia*-like bacteria abundances throughout the 11 month period with a significant Pearson Moment correlation of ($r = 0.78$, $p < 0.0001$). This suggests similar growth behavior among these denitrifying bacteria in activated sludge process. Abundance of *Paracoccus*-like bacteria appeared to be steady from January through April ($8.5\times10^{9} \pm 3\times10^{9}$ cells l^{-1}) peaking in May and decreasing through August (4.0×10^{10} to 6.8×10^{9} cells l^{-1} cells l^{-1}). From August to November this group increased from 6.8×10^{9} to 4.8×10^{10} cells l^{-1}. The changes in bacterial abundances and the existence of competitions between these populations were resulted from changes in environmental conditions, such as fluctuations of nitrite accumulation, anoxic dissolved oxygen (DO), and temperature (Figure 5-1b).

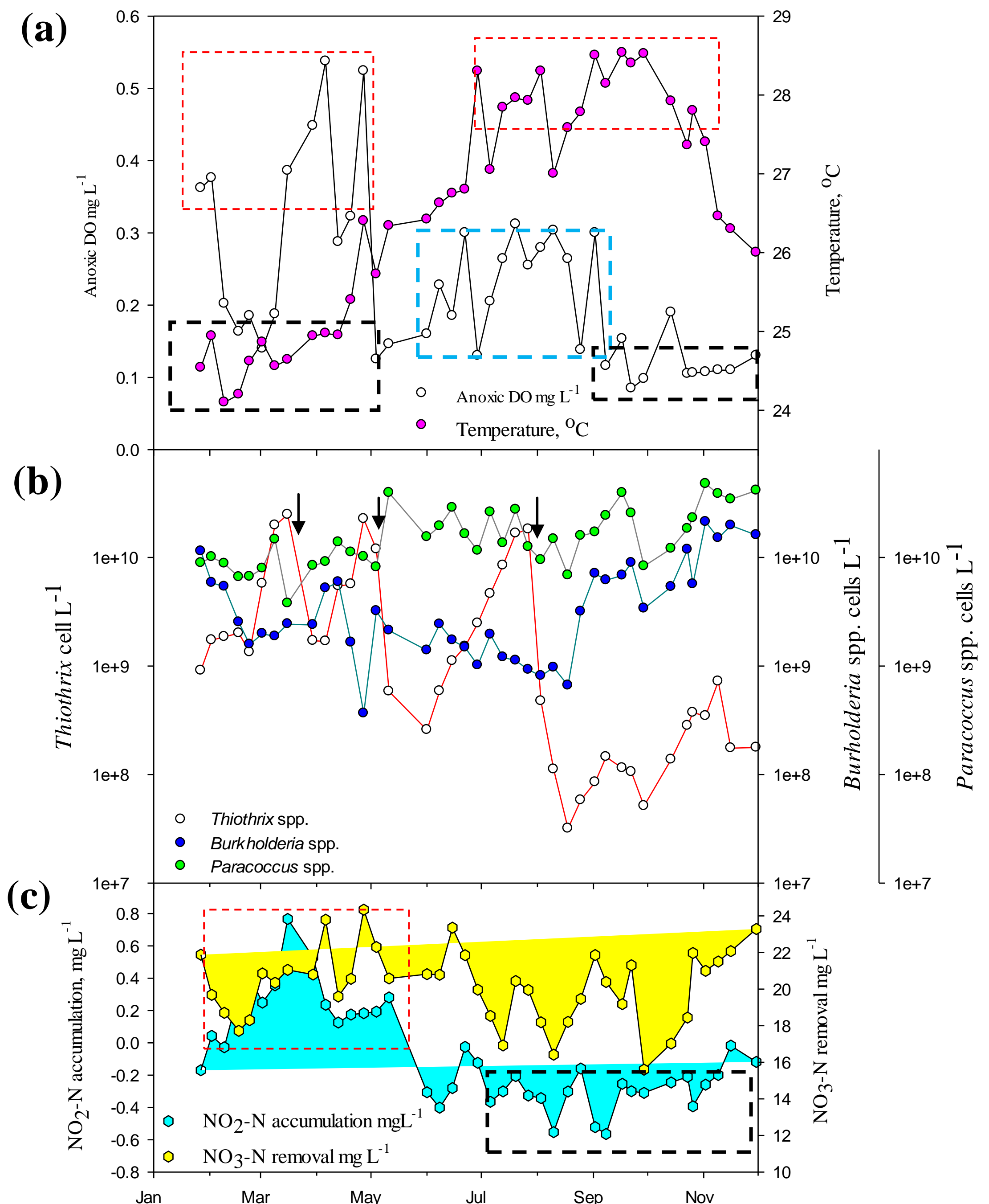


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Figure 5-1 (a) Anoxic DO concentration and temperature (b) Bacterial abundances of *Thiothrix eikelboomi* bacteria, Burkholderia-like bacteria, and Paracoccus-like bacteria (c) Nitrite (NO2-N) accumulations and total nitrate removals. Where [red dashed box] represents high operational range, [blue dashed box] represents moderate operational range, and [black dashed box] represents low operational range. ↓ represents the time of chlorination event took place.

5.4.2 Effect of DO and Temperature on Bacterial Abundance

Three different trends in DO concentrations in the anoxic zone, which are categorized as high, moderate, and low operational ranges, were observed during three different periods (Figure 5-1a): January-May, May-August, and August-November, respectively. From January until the end of May, a significant number of events with hypoxic DO concentrations (DO > 0.2 mg l^{-1}) occurred in the anoxic tank.

Under hypoxic anoxic DO conditions (0.15 mg l^{-1}< DO< 0.54 mg l^{-1}) from January to May (Fig 5-1a and 5-1b). *Thiothrix eikelboomi* bacteria concentrations quickly increased from 9.09×10^{8} cells l^{-1} to 2.26×10^{10} cells l^{-1} with increasing temperature from 23.7 °C to 26.5 °C (r=0.91, p< 0.0001). Although chlorination of returned activated sludge (RAS) occurred after each of the *Thiothrix eikelboomi* bacterial abundance peaks during the first two bulking events, the increase in *Thiothrix eikelboomi* bacterial abundance from June to July (third peak) occurred during moderate hypoxic DO concentrations (0.2 mg l^{-1} < DO < 0.35 mg l^{-1}, see Figure 5-1b) and increased in temperature (26.5 °C to 28.5 °C). However, temperature correlated positively with Paracoccus-like bacteria ($r = 0.36$, $p < 0.05$) and *Burkholderia*-like bacteria ($r = 0.34$, $p < 0.05$), while negatively correlated *Thiothrix eikelboomi* ($r = -0.41$, $p < 0.05$) for the overall period. This suggested that *Paracoccus*-like bacteria and *Burkholderia*-like bacteria *Thiothrix eikelboomi* are more likely to be outgrown by *Thiothrix eikelboomi* in the colder season.

In contrast, after the third chlorination event, all bacterial populations closely followed the trend of nitrate removal. Anoxic DO concentrations kept at a stabilized level (less than 0.2 mg l^{-1}) had favored *Burkholderia*-like bacteria and *Paracoccus*-like bacteria, while temperature decreased from September to November (28.6 °C to 26.5 °C). *Paracoccus*-like bacteria and *Burkholderia*-like bacteria significantly increased from August to November under anoxic DO conditions (DO < 0.2 mg l^{-1}), where the populations of *Thiothrix eikelboomi* bacteria stayed at the low levels (cells number $< 1 \times 10^9$) , with no occurrence of bulking events. During this period there was no nitrite accumulation (nitrite in the secondary effluent is less than primary effluent) and temperature increase (Fig. 5-1b, c), which optimized the conditions for the growth of *Paracoccus*-like and *Burkholderia*-like bacterial populations.

Moreover, *Paracoccus*-like bacteria correlated positively with denitrification under strict anoxic conditions (DO < 0.2 mg l^{-1}, $r = 0.50$, $p < 0.05$). However, *Burkholderia*-like bacteria, correlated significantly with denitrification when anoxic tank was under hypoxic condition (0.2 mg l^{-1}< DO < 0.6 mg l^{-1}, $r = 0.50$, $p < 0.05$) (Fig 5-1). This implies that *Paracoccus*-like bacterial abundances were found to be strict anoxic denitrifiers, while *Burkholderia*-like bacterial abundances were found to be hypoxic denitrifying bacteria. These results agreed with literature that was studied in batch process and pure culture systems (Robertson et., al., 1989; Barak et. al., 2000). Interestingly, these hypoxic DO concentrations in the anoxic zone

were found to have significant negative impacts on the bacterial abundances of *Burkholderia*-like bacteria (r = -0.45, $p < 0.05$) and *Paracoccus*-like bacteria (r = -0.46, $p <$ 0.05). However, the overall DO concentrations in the anoxic tank correlated positively with *Thiothrix eikelboomi* bacterial abundance (r = 0.38, $p <0.05$). This suggested both *Burkholderia*-like bacteria and *Paracoccus*-like bacteria may have higher growth rate under anoxic condition. Our results agreed with the trend of overall denitrification in our previous study where shows the denitrification $\geqq$ 0.2 mg l^{-1} is minimized (Wang et al., 2014).

The result for *Paracoccus*-like bacteria in this study contradicts a pilot-scale study that had found *Paracoccus*-like bacteria undergo denitrification under both aerobic and anoxic conditions (Barak et. al., 2000). The effect of an anoxic DO range of 0.2 mg $l^{-1} <$ DO$<$ 0.6 mg l^{-1} on *Paracoccus*-like bacteria in a full-scale treatment process has been reported in the literature.

5.4.3 Effect of Nitrite Accumulation on Bacterial Abundance

Nitrite accumulation calculated based on the difference between secondary effluent and primary effluent was also found to be related to bacterial abundance. Nitrite accumulation has a negative impact on the bacterial abundance of *Paracoccus*-like bacteria (r = -0.33, $p < 0.05$) and has no significant correlation with *Burkholderia*-like bacteria (r = -0.17, p = 0.42). Our results agreed with other studies showing nitrite accumulation can be problematic to the denitrification (Betlach et. al., 1983; Glass et. al., 1998). It has also been

reported that a nitrite concentration of 0.34 mg l^{-1} can result in the slowdown of denitrification and nitrification activity in a batch study (Zhou et. al., 2011). Interestingly, we also took this opportunity to look at the effect of nitrite accumulations on the two predominant bacterial populations (*Thauera*-like bacteria and *Zoogloea*-like bacteria Table 5-2), and we were able to see that only the abundance of the strictly anoxic denitrifier *Thauera*-like bacteria was significantly impacted by nitrite accumulation (r= -0.41, p<0.05). The correlation analysis of nitrite accumulations on all bacterial populations are shown in Table 5-2. Additionally, the accumulation of nitrite may result from *Thiothrix eikelboomi* related bulking organism's inability to fully convert nitrate to nitrogen gas since *Thiothrix eikelboomi* bacteria have been reported to relate to elevated nitrite concentrations (Hong et al., 2013), while nitrite is positively associated with *Thiothrix eikelboomi* bacterial abundance ($r = 0.44$, $p < 0.05$) in our study. We hypothesized that nitrite may have more negative impacts on strict anoxic denitrifiers. Due to our limited data at one plant, further investigations on the effect of nitrite on a variety of bacteria as well as overall denitrification are required in order to draw such a conclusion. Although it is clear that the abundance of *Thiothrix eikelboomi* was positively associated with nitrite accumulation in the anoxic zone, these organisms can have indirect impacts on two of the strict anoxic denitrifiers.

In additions, the main controlling factor in changes in bacterial abundances can be associated with solid retention time (SRT) (Tchobanoglous et. al., 2004). Our study (Table 5-2) showed

that SRT only had a significant negative correlation with the abundance of *Thiothrix eikelboomi*, and had no impact on the other bacterial populations. Therefore it is clear that SRT was not the controlling factor of denitrifying populations in this process.

Table 5-2. Correlation analysis of nitrite to bacterial populations

Organisms to nitrite	Strict anoxic denitrifiers		Aerobic denitrifiers		Bulking/ denitrifiers
	Paracoccus-like bacteria	*Thauera*-like bacteria	*Zoogloea*-like bacteria	*Burkholderia*-like bacteria	*Thiothrix eikelboomi*
Nitrite	**-0.33**	**-0.41**	0.19	-0.17	**0.44**
SRT	0.27	0.26	0.16	0.13	**-0.37**

Bolded values are statistically significant, where $p < 0.05$

5.4.4 Effect of seasonality on bacterial abundance

We also generalized the conditions, which favored (+) and disfavored (-) the three bacterial populations in Table 4, with time frames based on the different ranges of anoxic DO and temperature. Both of the moderate and high anoxic DO months have negative impact on the bacterial abundances of *Paracoccus*-like bacteria and *Burkholderia*-like bacteria, the optimum condition for these denitrifiers occurred during the fall season (Fig.5-1).

The chlorination of RAS (Fig. 5-1b) caused *Thiothrix eikelboomi* bacterial abundance to reduce significantly (over one log), while chlorination of the RAS created less of an impact on the *Paracoccus*-like than on *Burkholderia*-like bacteria which experienced reduced population size. *Paracoccus*-like bacteria increased after each chlorination event. Burkholderia showed no significant response to the first chlorination event, while a 0.5 log decrease in bacterial abundance occurred during the second event and less than 0.25 log decrease in bacterial abundance in the third event, which is with methodological variation. Thus, chlorination imposed a greater effect on the bulking organism *Thiothrix eikelboomi* than *Paracoccus*-like and *Burkholderia*-like bacteria. This type of comparison on the effect of chlorination to different bacterial abundance has not been reported in previous literature

Table 4. Effect of temperature, DO , and nitrite concentrations on bacterial growth

Type	Time and Environmental Conditions		
	January-May	May-August	August-November
Organism	23.7< T < 26.5 (^{o}C, increasing T) 0.15< DO< 0.54 (mg l^{-1})	26.5 <T < 28.6 (^{o}C, increasing T) 0.15<DO < 0.33 (mg l^{-1})	26.5 <T < 28.6 (^{o}C, decreasing T) 0.05 < DO < 0.2 (mg l^{-1})

	NO_2-N >0 (mg l^{-1})	No Nitrite accumulation	No Nitrite accumulation
Thiothrix eikelboomi bacteria	(+)	(+)	(-)
Paracoccus-like bacteria	(-)	(-)	(+)
Burkholderia-like bacteria	(-)	(-)	(+)

‘

5.6 Conclusions

In this study, high anoxic DO in plant operation can and lower temperatures to significant increases in the abundance of certain bulking organisms, which resulted in significant nitrite accumulations and negatively impacted the abundance of strict denitrifying bacteria in the treatment process. Improvement of DO control by strict anoxic condition will promote optimum growth for denitrifying populations (*Paracoccus*-like bacteria and *Burkholderia*-like bacteria) and simultaneously reduces the abundance of bulking organisms (*Thiothrix eikelboomi* bacteria). Such approaches will disfavor nitrite producers such as *Thiothrix eikelboomi* bacteria and improved the overall denitrification activity.

Chapter 6. Conclusions

This research showed that fine scale monitoring of bacterial populations involved in nitrification and denitrification in a full-scale plant would allow operations to respond in real time to keep processes optimized. Currently, bacterial biomass in wastewater treatment is assessed by a surrogate measurement, volatile suspended solids. This material contains a considerable volume of nonviable substances such polysaccharides, extracellular DNA, cell wall fragments, etc. Hence, changes in VSS are far slower than those actually occurring in the active bacterial population. This dissertation provides a framework of how full scale plants can be monitored for bacterial abundance of key populations and how the changes in population abundance can be related to operating and environmental parameters. This research has provided interesting and new information on three fronts.

The first is the nitrification process and seasonal changes that retard the bacterial activity of ammonia oxidizers and nitrite oxidizers. Specifically, the research showed that although ammonia, BOD, and COD removal were close to complete removal, total bacteria, ammonia oxidizing bacteria, *Nitrospira*, and *Nitrobacter* were found to be inhibited by nitrite accumulation and pH in the winter while enhanced by SRT and temperature in the summer. SRT is recommended to increase by 3.6 days in the colder season to adjust AOB to NOB ratio and minimize nitrite accumulation and maximize nitrification process. Nitrification can be

further improved by maintaining the pH level in the range of 6.5-6.7 to enhance the growth of nitrifying bacteria, which is lower than optimum in pure cultures studies. The abundance of the total bacterial population failed to show a direct relationship with denitrification efficiencies based our PCA analysis and has led to an additional study identifying a strong denitrification bioindicator for the improvement of denitrification efficiencies.

Development of primer and probe sets for two predominant denitrifying bacterial populations (*Zoogloea-Metholovasatlis*-like bacteria and Thauera-like bacteria) was conducted to understand population dynamics and the contribution to the denitrification process of these two microbial groups. We observed that the *Zoogloea-Metholovasatalis*-like bacteria dominated over *Thauera*-like bacteria during colder season, while *Thauera*-like bacteria dominated over *Zoogloea-Metholovasatalis*-like bacteria during the warmer season due to increase in water temperature and strictly anoxic DO conditions.

Zoogloea-*Metholovasatalis*-like bacteria were unaffected by both hypoxic and anoxic DO conditions, and *Thauera*-like bacteria were favored in anoxic conditions. Anoxic conditions tend to have greater denitrification efficiencies (more events over 70% denitrification) than hypoxic conditions under in the temperature season. This result implied the importantance of monitoring different denitrifying populations since only consortia within the total bacterial population contribute in a major way to denitrification during different seasons of the year.

Greater control of the anoxic DO system is needed to sustain a low DO range (DO < 0.1 mg

l^{-1}), which will increase denitrification efficiencies by producing a greater time period where denitrification is greater than 70% and by shifting the efficiencies from 63% to over 70%. Lastly, the abundance of two denitrifying populations *Burkholderia*-like bacteria, and *Paracoccus*-like bacteria were quantified to investigate the inhibitory factors: (excess anoxic DO, nitrite accumulation, and bulking organisms (*Thiothrix eikelboomii*). *Burkholderia*-like bacteria and *Paracoccus*-like bacteria were over competed by *Thiothrix eikelboomii* bacteria during three different periods of the year, which led to higher nitrite accumulations and subsequently reduced the abundance of *Burkholderia*-like bacteria and *Paracoccus*-like bacteria. Thus chlorination of return activated sludge is required to prevent occurrence of *Thiothrix eikelboomii* and bulking events. Maintaining anoxic DO under strict anoxic conditions can also reduce the risk of significant nitrite accumulation in the wastewater treatment process and reduce the abundance of bulking organisms which in turn reduces competition with nitrite denitrifying populations. This finding has helped us gain insights into understanding the population dynamics of denitrifiers.

Overall, this study advances the field by utilizing a newly developed microbial application to monitor different microbial populations for the improvement in efficiencies of biological nitrogen removal in the wastewater process. As this molecular technique become more popular in the industry and is developed into maturity, wastewater treatment will no longer have to be treated as a black box as it has done for the past century. Subsequently, treatment

performance can then be maiximized based on the actual bacterial distributions for any biological wastewater treatment processes.

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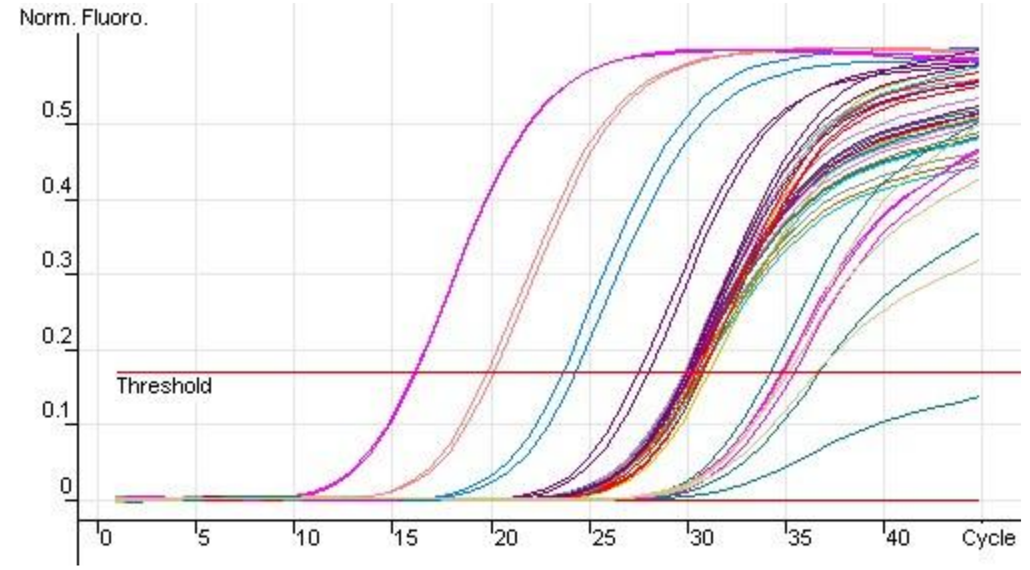
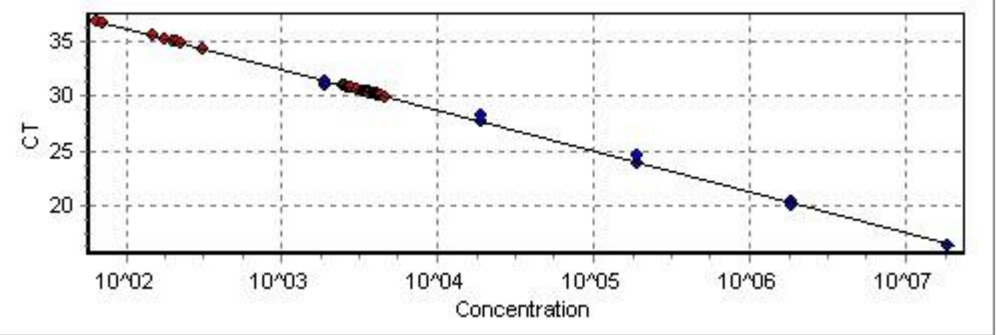
Appendix A Table-1. Reaction efficiency, Slope, R^2 Value, and Intercept for Standard Curves

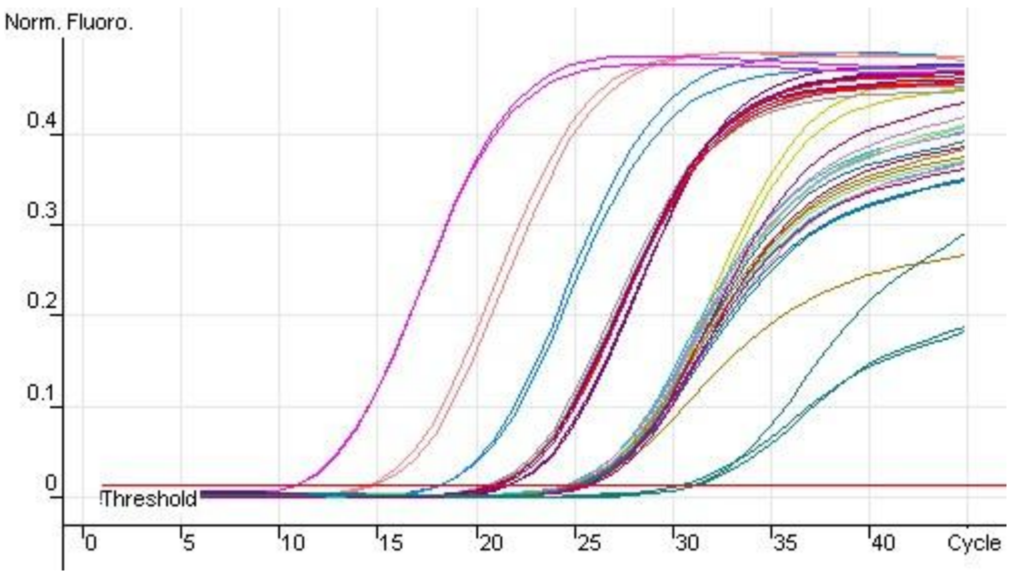
Organisms	Reaction efficiency	Slope	R^2 Value	Intercept
AOB	0.98±01	-3.74±21	0.97±02	43.7±0.8
Nitrospira spp.	1.00±02	-3.37±17	0.95±03	41.2±1.4
Nitrobacter spp.	0.99±02	-3.63±10	0.99±01	37.5±0.4

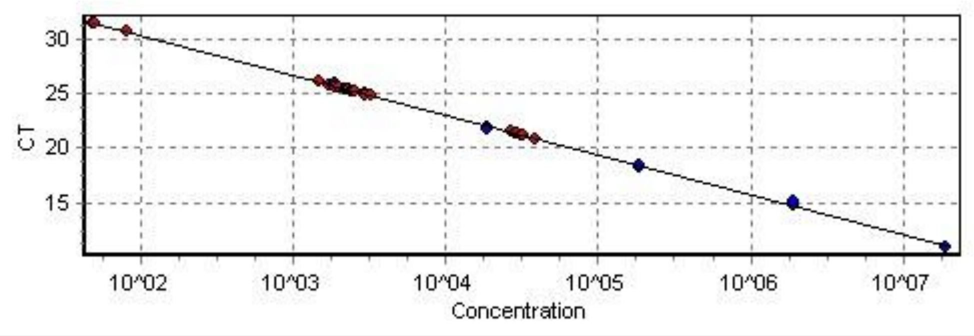
Appendix A1. Standard curve of AOB qPCR



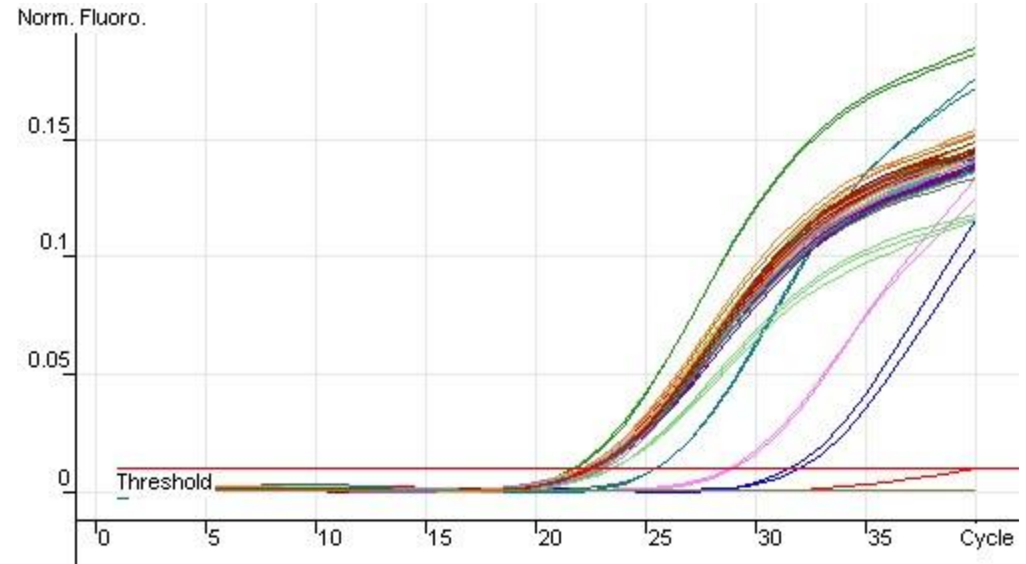


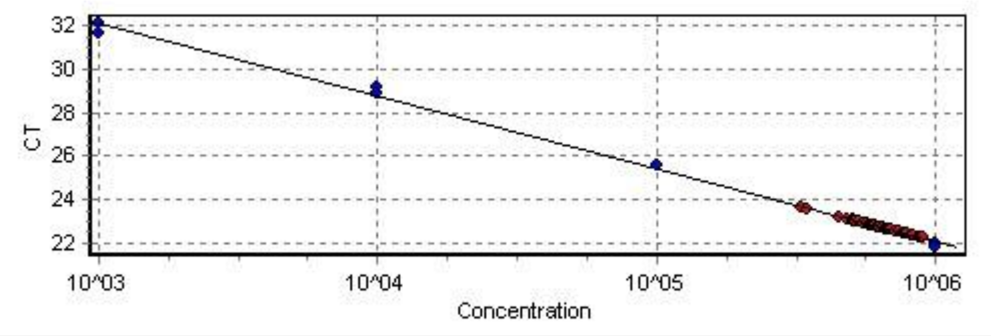
Appendix A2. Standard Curve of *Nitrospira* spp.



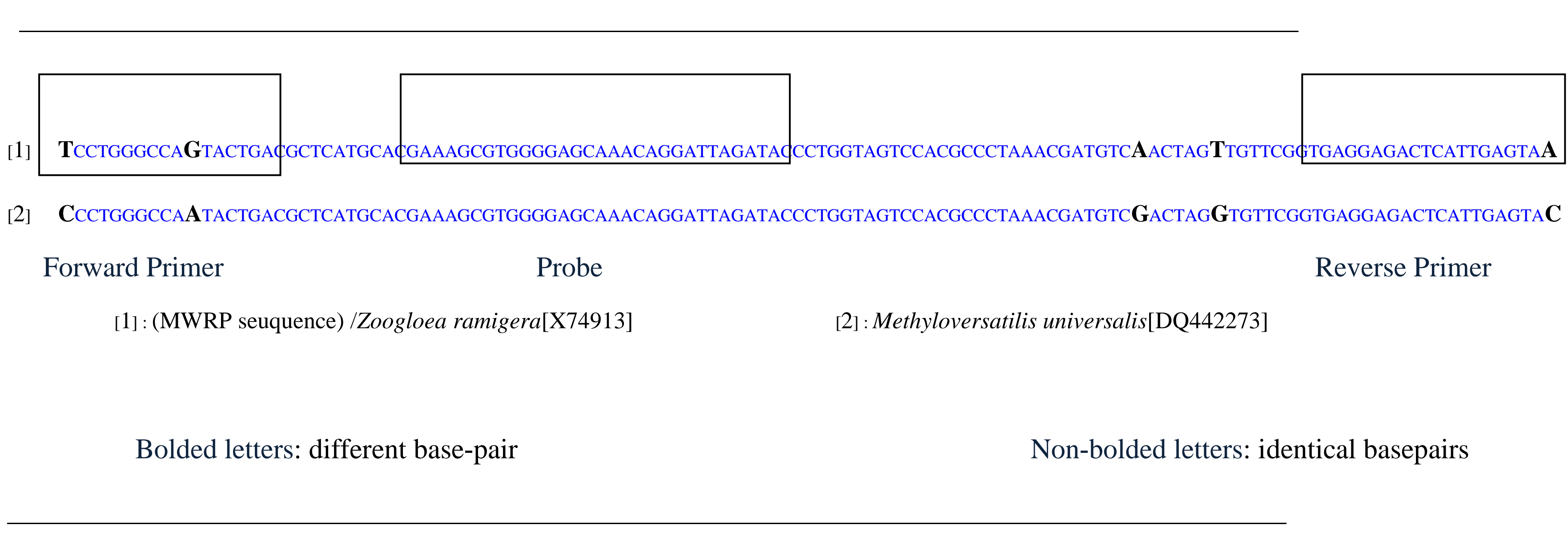


Appendix A3. Standard Curve of *Nitrobacter* spp.

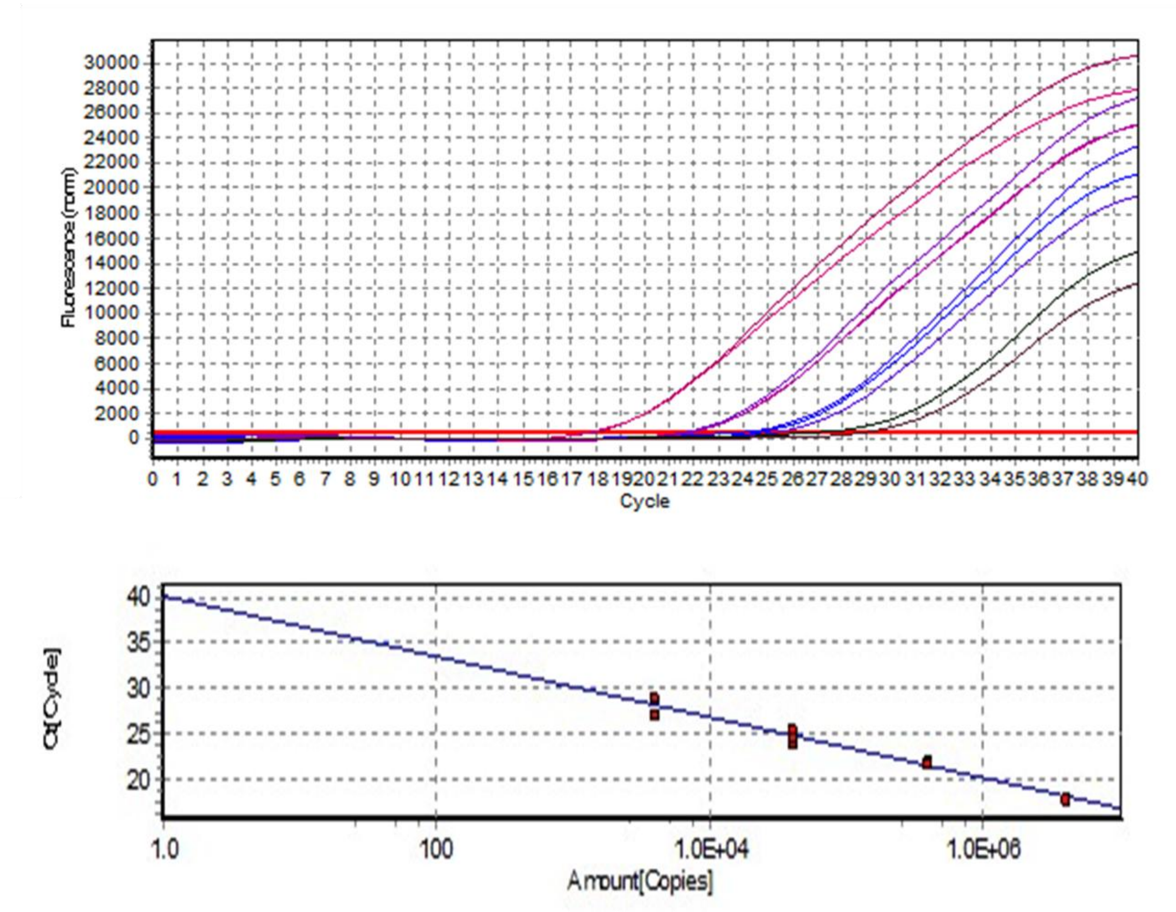




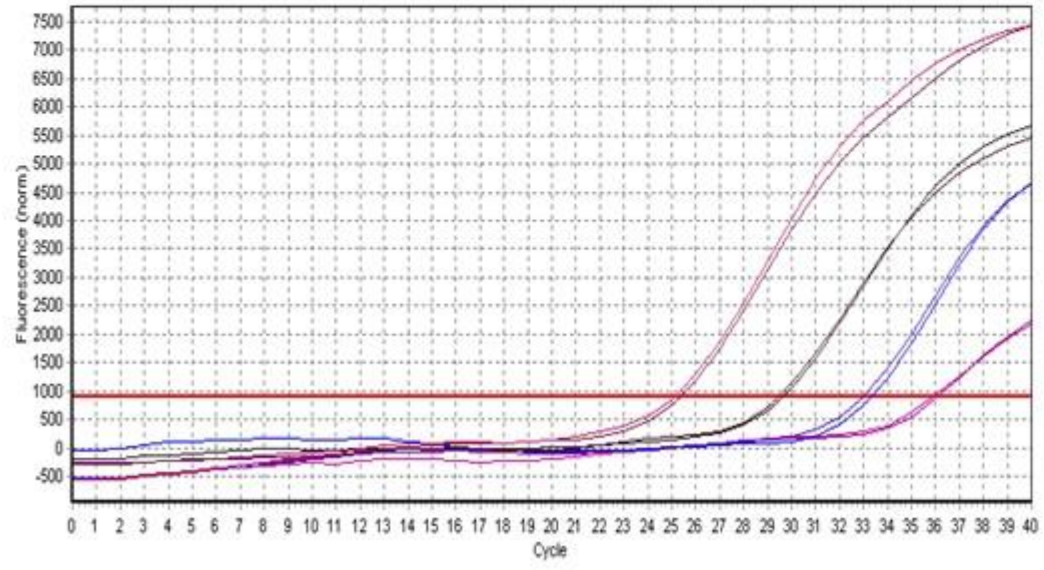
Comparison of *Methyloversatil* spp. and Zoogloea spp. sequences

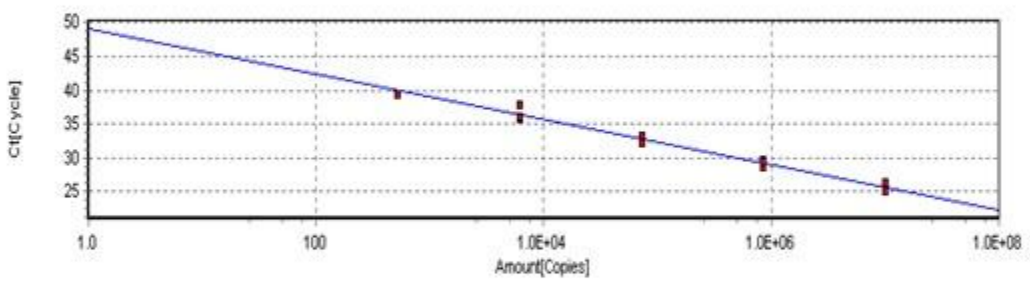


Appendix B1. Stand Curve for *Thauera* spp. qPCR

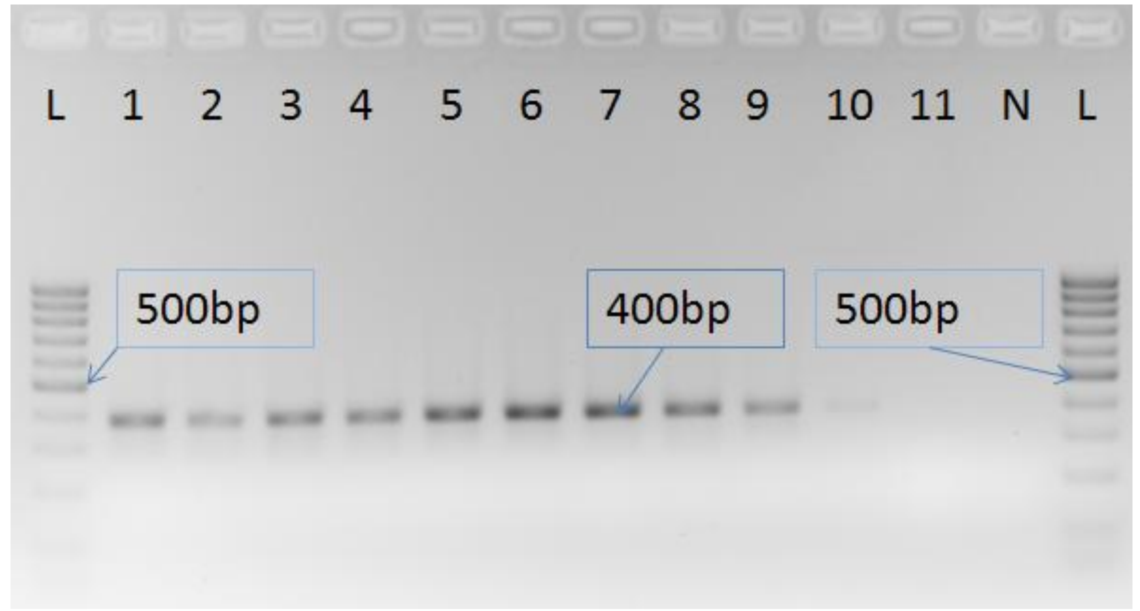


Appendix B2. Stand Curve for *Zoogloea* spp. qPCR



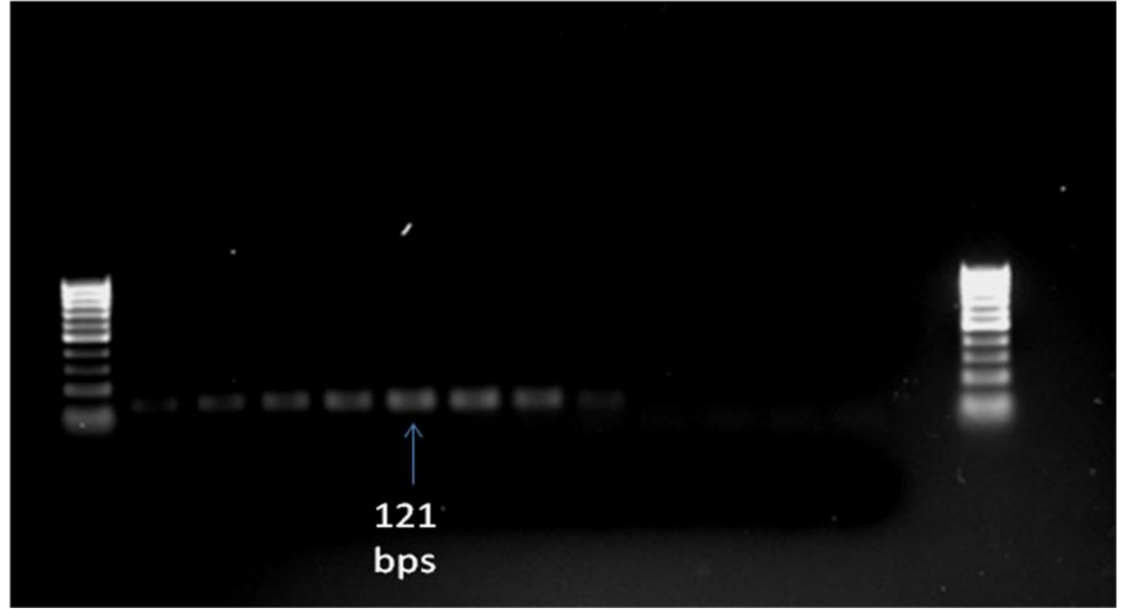


Appendix B3. Gel electrophoresis of temperature optimization gradient for Thauera sp.



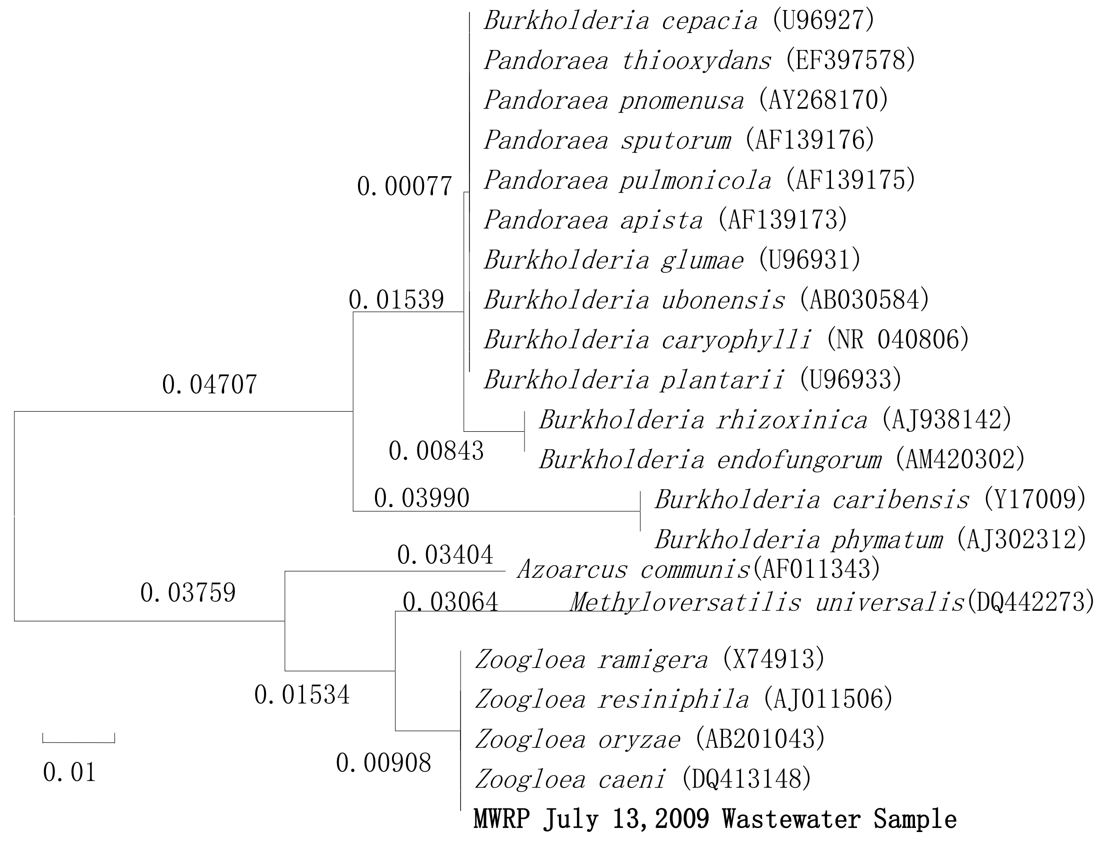
Lane number () followed by temperature in ^{o}C: Lane (1) 56.8, Lane (2) 57.1, Lane (3) 58.3, Lane (4) 60.0, Lane (5) 62.1, Lane (6) 64.4, Lane (7) 66.9, Lane (8) 69.3, Lane (9) 71.4, Lane (10) 73.2 and Lane (11) 74.4^{o}C, respectively. where 66.9 ^{o}C isis the optimal annealing temperature. 400 bp was found to be the size of the PCR amplicon. The well labeled Lane N was negative control and L was 100 bp ladders.

Appendix B4. Process flow diagram at Michelson Water Reclamation Plant Gel electrophoresis of temperature optimization gradient for Zoogloea sp.

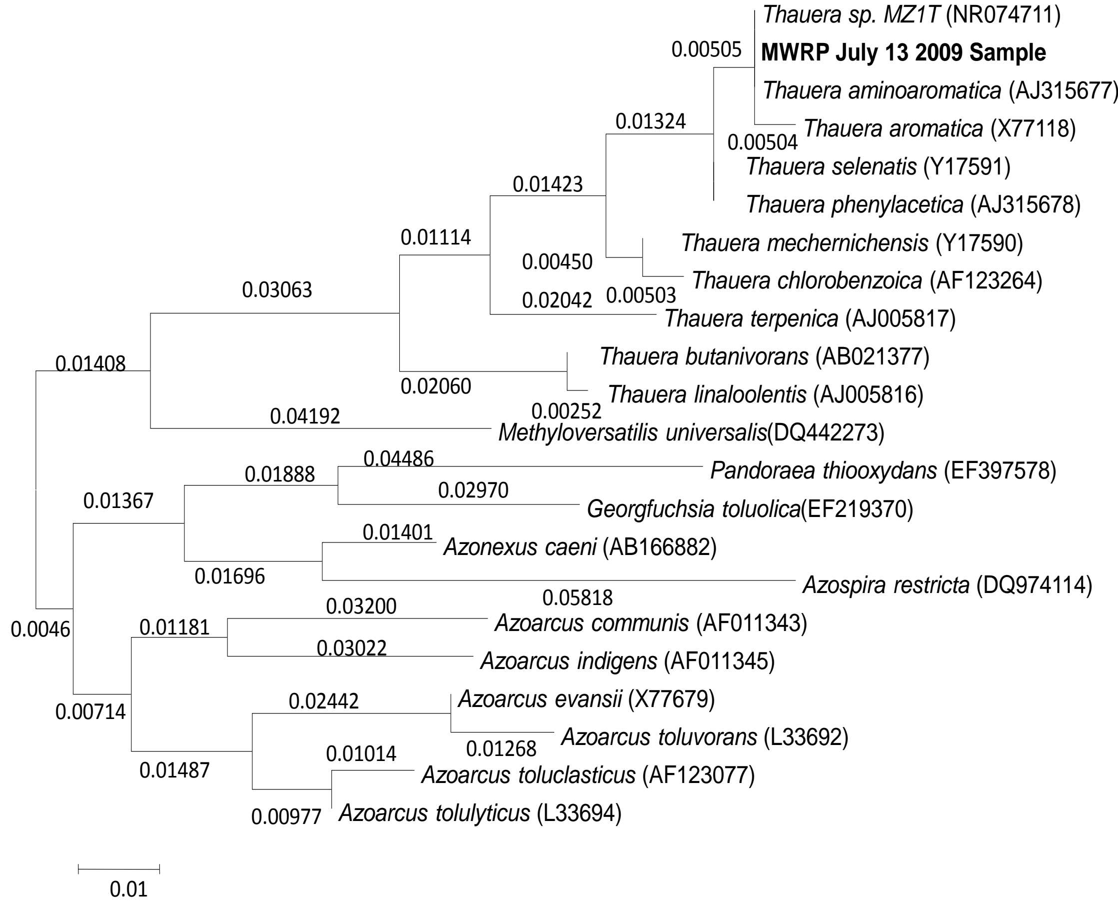


Lane number () followed by temperature in °C: Lane (1) 54.8, Lane (2) 55.1, Lane (3) 56.1, Lane (4) 57.5, Lane (5) 58.5, Lane (6) 61.2, Lane (7) 63.2, Lane (8) 65.2, Lane (9) 67.0, Lane (10) 68.5, Lane (11) 69.6 °C, where 58.5 is the optimal annealing temperature. 121 bp was found to be the size of the PCR amplicon. The well labeled Lane N was negative control and L was 100 bp ladders

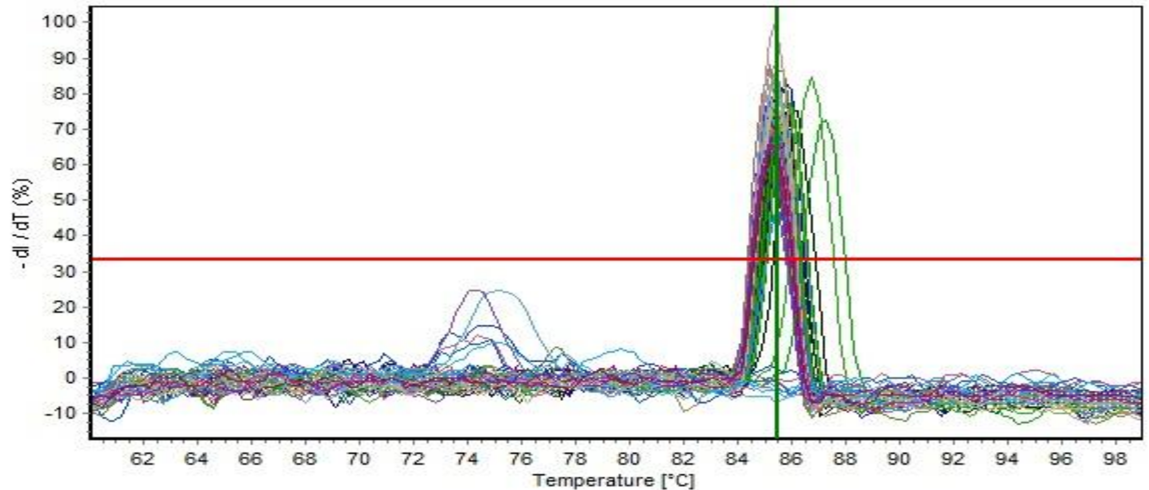
Appendix B5. Phylogenetic analysis of the 16S rRNA gene sequences of the 121-bps *Zoogleoa* spp. sequences



Appendix B6. Phylogenetic analysis of the 16S rRNA gene sequences of the 400-bps *Thauera* spp.

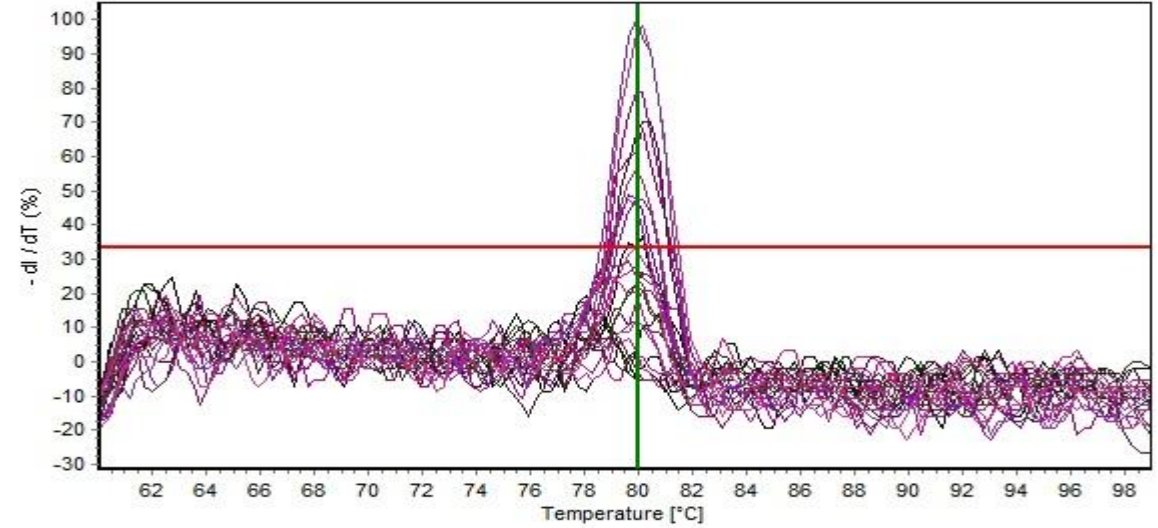


Appendix B7. Melt curve analysis of *Zoogloea-Methyloversatilis*-like bacteria. in MWRP samples



Threshold: 33%

Appendix B8. Melt curve analysis of positive amplicon control, *Thauera* spp.



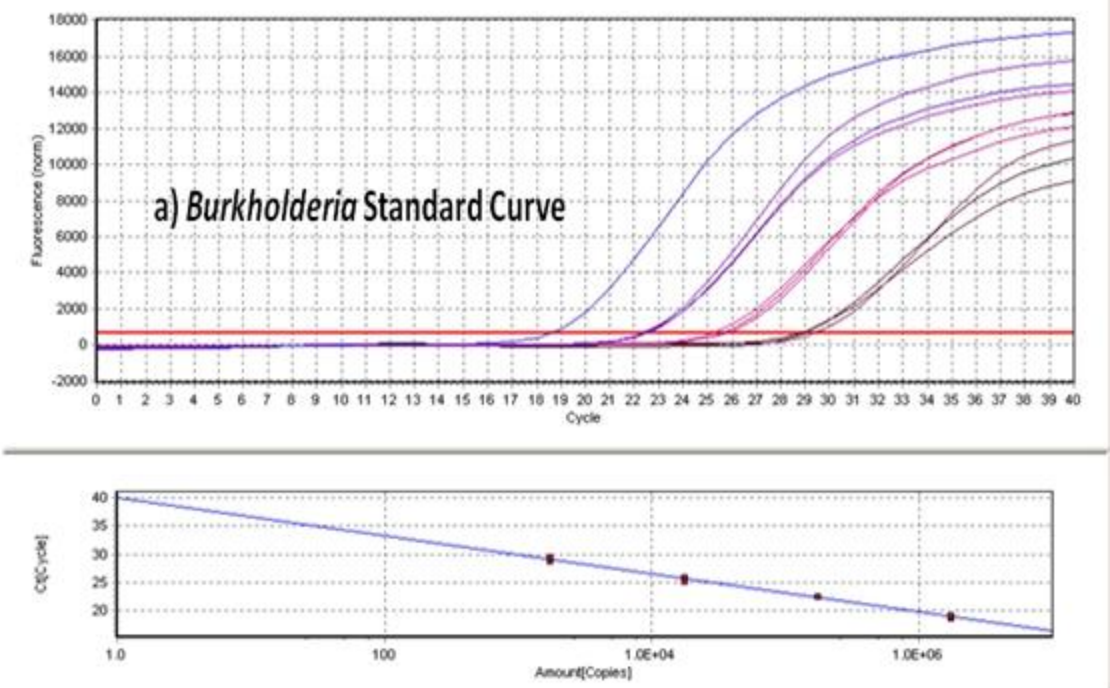
Threshold: 33%

Appendix C-Table1 *Burkholderia* species with copies per cell based on the complete genome

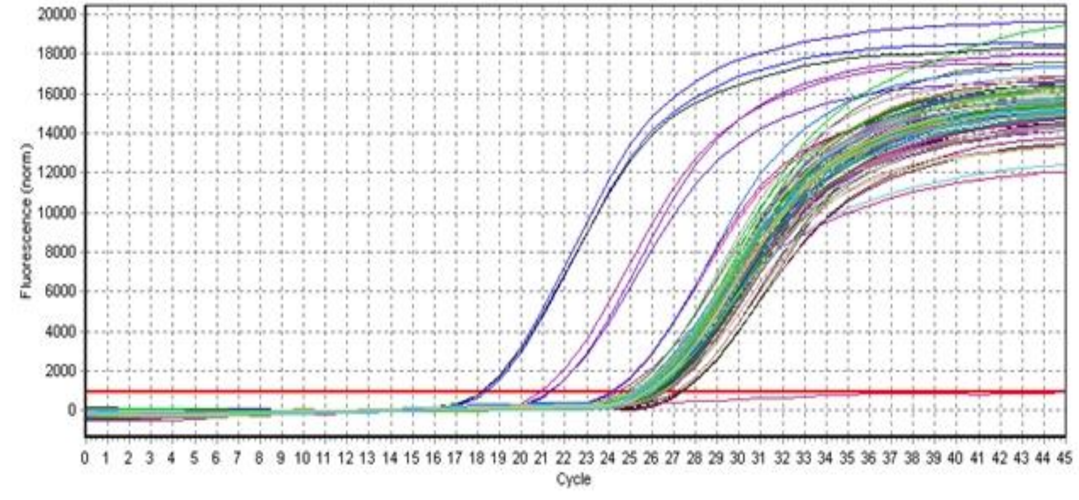
Burkholderia species	Copies/cell
Burkholderia ambifaria AMMD	6
Burkholderia ambifaria MC40-6	6
Burkholderia cenocepacia AU 1054	6
Burkholderia cenocepacia HI2424	6
Burkholderia cenocepacia J2315	6
Burkholderia cenocepacia MC0-3	6
Burkholderia glumae BGR1	5
Burkholderia mallei ATCC 23344	4
Burkholderia mallei NCTC 10229	4
Burkholderia mallei NCTC 10247	4
Burkholderia mallei SAVP1	5
Burkholderia multivorans ATCC 17616	5
Burkholderia phymatum STM815	6
Burkholderia phytofirmans PsJN	6
Burkholderia pseudomallei 1106a	4
Burkholderia pseudomallei 1710b	4
Burkholderia pseudomallei 668	4

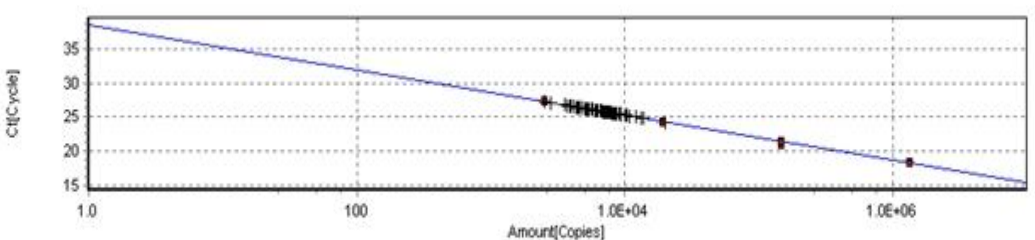
Burkholderia pseudomallei K96243	4
Burkholderia pseudomallei MSHR346	4
Burkholderia rhizoxinica HKI 454	3
Burkholderia sp. 383	6
Burkholderia sp. CCGE1001	6
Burkholderia sp. CCGE1002	6
Burkholderia sp. CCGE1003	6
Burkholderia thailandensis E264	4
Burkholderia vietnamiensis G4	6
Burkholderia xenovorans LB400	6
Calculated average copies/cell for *Burkholderia* species	5.11

Appendix C1. Standard curve of *Burkholderia cepacia* (NRRLB-14809) qPCR

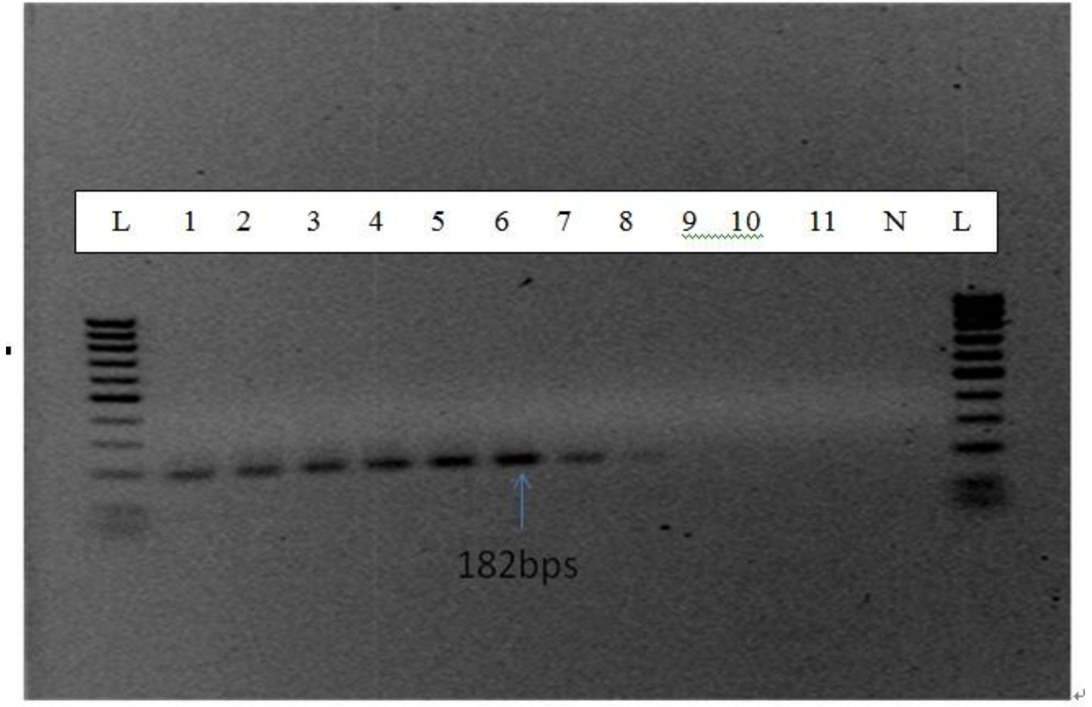


Appendix C2. Stand Curve for Paracoccus denitrificans (B-3785) qPCR



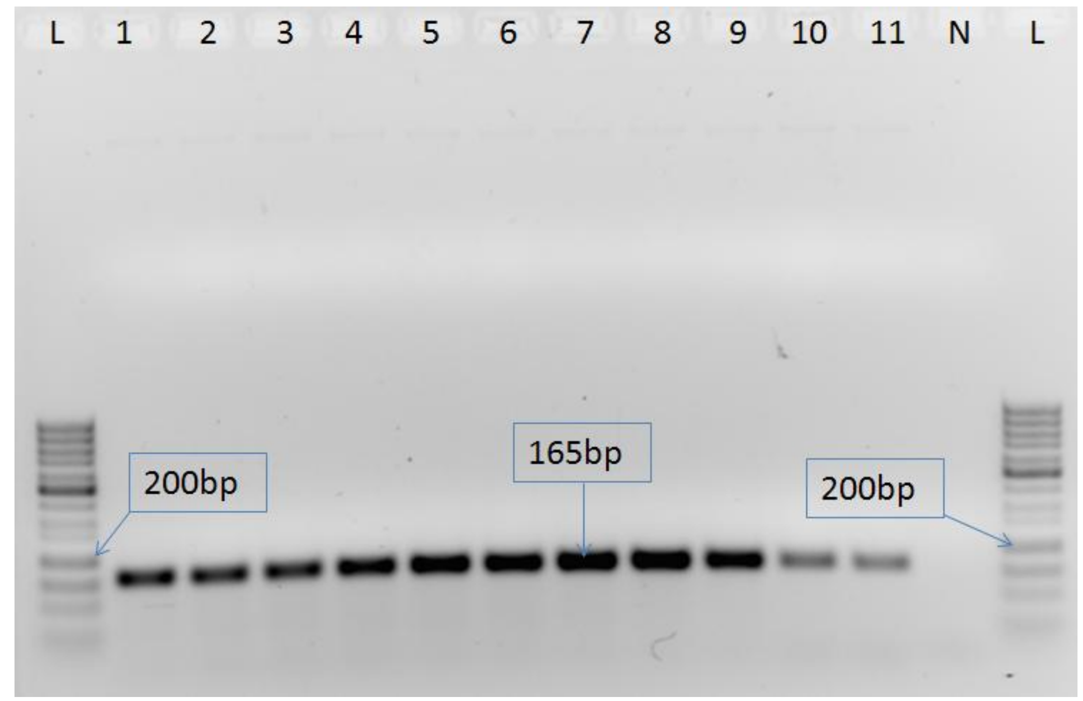


Appendix C3. Gel electrophoresis of temperature optimization gradient for *Burkholderia cepacia* (NRRLB-14809)



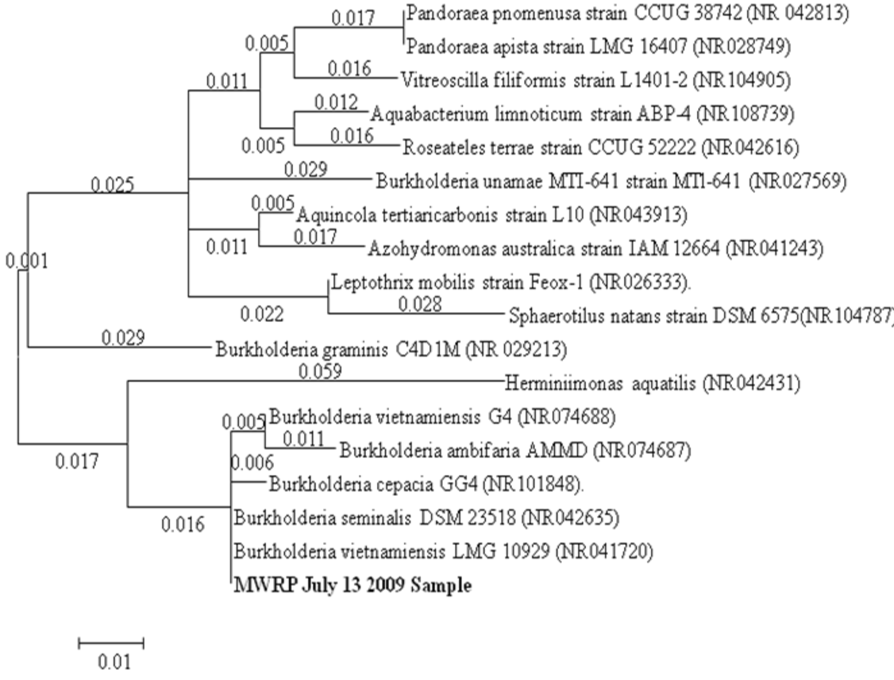
Lane number () followed by temperature in ^{o}C: Lane (1) 59.7, Lane (2) 60.1, Lane (3) 61, Lane (4) 62.4, Lane (5) 64.2, Lane (6) **66.2**, Lane (7) 68.2, Lane (8) 70.2, Lane (9)72.0, Lane (10) 74.6, Lane (11) 75.1 ^{o}C, where 66.2 is the optimal annealing temperature. 182 bp was found to be the size of the PCR amplicon. The well labeled Lane N was negative control and L was 100 bp ladders

Appendix C4 Gel electrophoresis of temperature optimization gradient for *Paracoccus denitrificans* (B-3785)



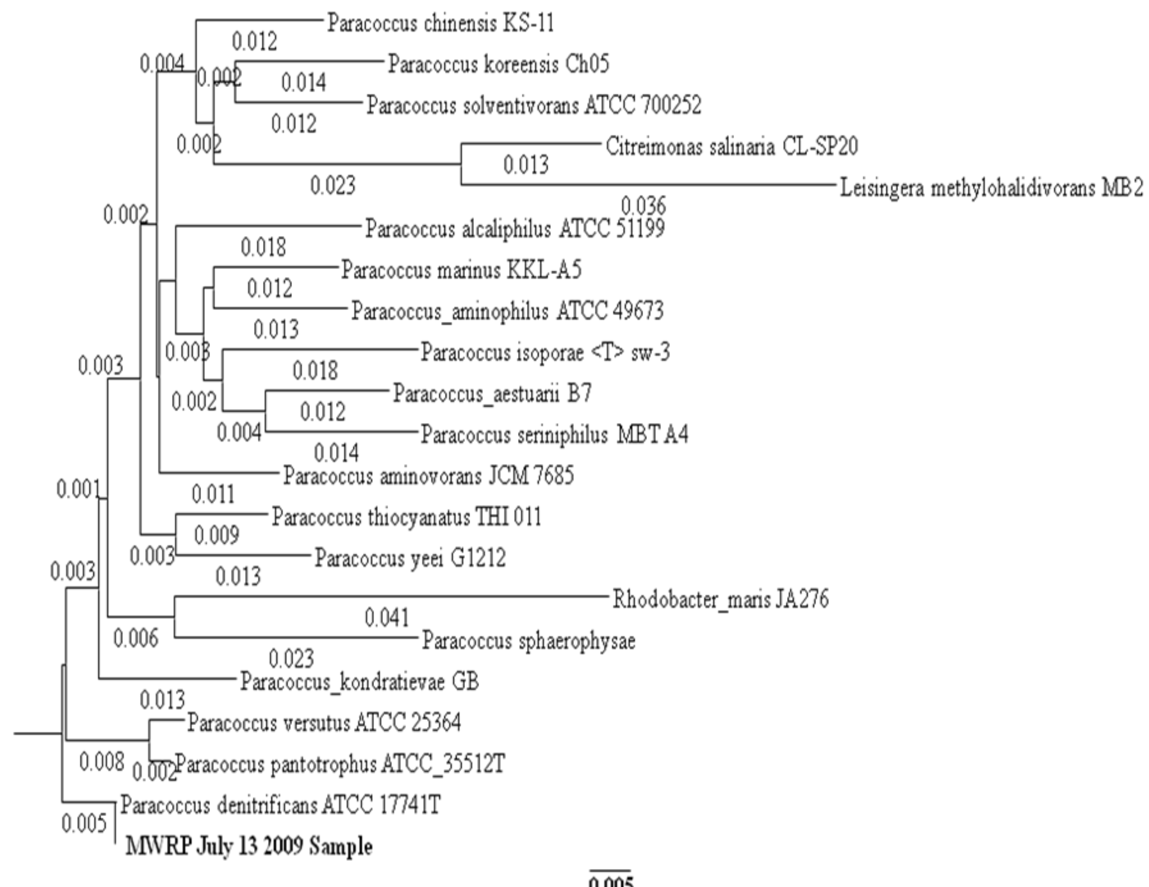
Lane number () followed by temperature in ^{o}C: Lane (1) 49.8, Lane (2) 50.3, Lane (3) 51.5, Lane (4) 53.4, Lane (5) 55.7, Lane (6) 58.3, Lane (7) 60, Lane (8) 63.7, Lane (9)66.1, Lane (10) 68.0, Lane (11) 69.4 ^{o}C, where 60 is the optimal annealing temperature. 165 bp was found to be the size of the PCR amplicon. The well labeled Lane N was negative control and L was 50 bp ladders.

Appendix C5 Phylogenetic analysis of the 16S rRNA gene sequences of the 183-bps *Burkholderia*-like bacteria sequences



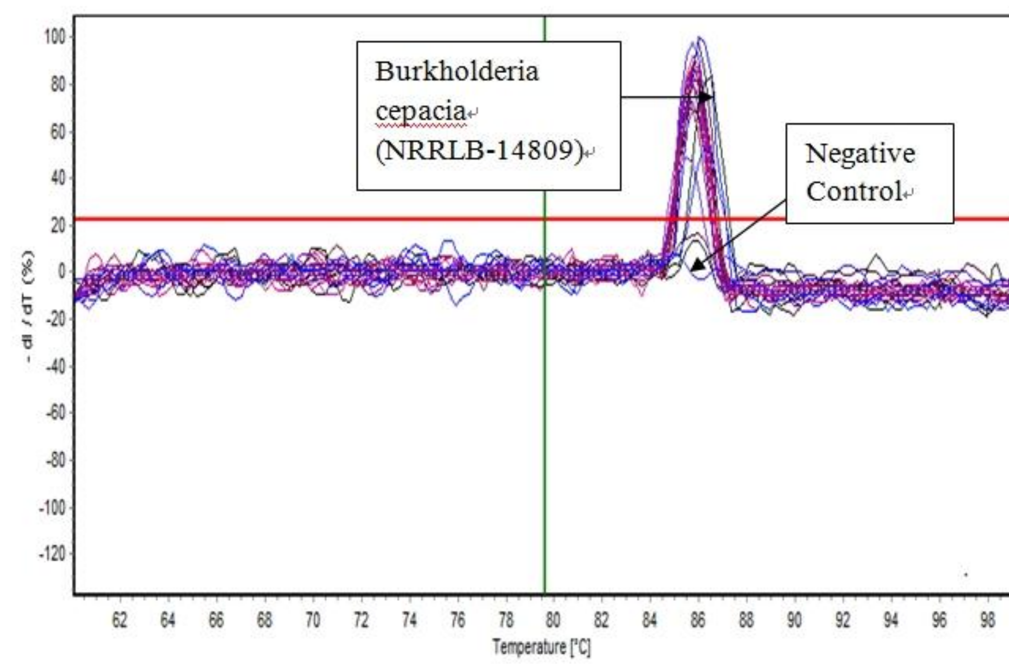
Closely related genera from the GenBank database and MWRP July 13 2009 sample. The numbers above the branches indicate the dissimilarity among the sequences. GenBank accession numbers are in parentheses. The scale bar indicates 0.01 inferred (1-nucleic-acid differences in 100 nucleic acid base pairs).

Appendix C6 Phylogenetic analysis of the 16S rRNA gene sequences of the 165-bps *Paracoccus*-like bacteria sequences



Closely related genera from the GenBank database and MWRP July 13 2009 sample. The numbers above the branches indicate the dissimilarity among the sequences. GenBank accession numbers are in parentheses. The scale bar indicates 0.005 inferred (5-nucleic-acid differences in 1000 nucleic acid base pairs)

Appendix C7 Melt curve analysis of *Burkholderia* spp. positive amplicon control



Appendix C8 Melt curve analysis of *Paracoccus* spp. positive amplicon control