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COMMUNITY ANALYSIS OF A UNIQUE FULL-SCALE WASTEWATER TREATMENT PLANT AS REVEALED BY 454-PYROSEQUENCING

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by

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ABSTRACT

Activated sludge is one of the most commonly applied biological treatment processes for wastewater treatment. The microorganisms grown in activated sludge systems are responsible for the removal and transformation of wastewater contaminants. These bacterial populations are sensitive to wastewater characteristics, plant design and operational parameters. Because the effectiveness of activated sludge relies on microbial activity, a better understanding of the microbial communities and how they respond to constraints may be useful for improving plant performance.

Environmental conditions in the activated sludge process are complex and the identification and classification of the communities present has proven a challenge for engineers as well as microbiologists and ecologists. Many bacteria in activated sludge cannot be cultured and conventional culture dependent techniques are not adequate in defining these populations in depth. Recent molecular methods have advanced the understanding of this complicated ecosystem, yet much remains unclear due to plant

specific dynamics that influence community structure. Sequencing techniques are a novel approach to the identification of the microbial populations of activated sludge. This new technology allows researchers to ask exploratory questions about specific and distinct wastewater treatment processes that could not be efficiently completed with other methods.

One such unique wastewater treatment plant that may benefit from the use of pyrosequencing techniques is the Los Alamos National Laboratory Sanitary Wastewater System (LANL SWWS) located in Los Alamos, New Mexico. This plant experiences highly variable flow and utilizes glycerin as a supplemental carbon source due to low-strength carbon influent. In addition, the plant experienced an upset in 2012 with respect to excessive tardigrade growth. In response introduced sludge from another wastewater treatment plant (seed event) to help improve the microbial populations. 454-pyrosequencing was used as a method to identify and analyze the microbial populations of this glycerin enriched activated sludge wastewater treatment plant.

The objectives of this research were to 1) determine the differences between LANL SWWS activated sludge population and that in other plants, 2) identify and evaluate the effects of glycerin as a supplemental carbon source on the microbial population, 3) identify any adaptive characteristics or population changes due to the seeding event, 4) determine if there were certain operational conditions or environmental factors that influence specific microbial populations.

In order to accomplish these objectives, samples were taken from LANL SWWS and other nearby wastewater treatment plants. These samples were pyrosequenced and the

results were analyzed with various bioinformatics tools. The microbial communities were compared between LANL SWWS and other plants that did not utilize an external carbon source. Analysis of the activated sludge population before and after the seed event was performed and contrasted with the microbial structure and distribution of the activated sludge used to inoculate LANL SWWS. Wastewater characteristics and plant parameters were investigated to determine bacterial responses to environmental and operational conditions.

There was a distinction between the microbial populations of LANL SWWS and conventionally fed wastewater treatment plants. The relative abundance of the dominant taxa found in the samples exposed to glycerin as an external carbon source exhibited a different distribution compared to the samples obtained from conventional treatment facilities. It was also apparent that the introduction of foreign biomass into the LANL SWWS activated sludge via the seed event had no direct influence on the microbial community. The main contributing parameter that affected the microbial population of LANL SWWS appeared to be the solids residence time, which was likely the cause of the tardigrade proliferation and could also justify the distribution of the consortia within the activated sludge.

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3-CA	chloroanaline
AOB	ammonia oxidizing bacteria
BOD_5	five day biological oxygen demand
bsCOD	biodegradable soluble COD
COD	chemical oxygen demand
CTAB	hexadecyltrimethylammonium
CTAB S	hexadecyltrimethylammonium with sucrose lysis buffer
DGGE	denaturing gradient gel electrophoresis
DO	dissolved oxygen
EBPR	enhanced biological phosphorous removal
EDTA	Ethylenediaminetetraacetic acid
EGSB	expanded granular sludge bed
EQ	equalization basin
FISH	fluorescence in situ hybridization
GAO	glycogen accumulating organism
IFAS	integrated fixed-film activated sludge
LAC WWTP	Los Alamos County wastewater treatment plant
LANL SWWS	Los Alamos National Laboratory Sanitary Wastewater System
MBR	membrane bioreactor
MGD	million gallons per day
MLSS	mixed liquor suspended solids
MLVSS	mixed liquor volatile suspended solids
NOB	nitrite oxidizing bacteria
OTU	operational taxonomic unit
PAO	polyphosphate accumulating organisms
PBS	phosphate buffer saline
PCR	polymerase chain reaction
QIIME	Qualitative Insights Into Microbial Ecology
RDP	Ribosomal Database Project
RPM	revolutions per minute
RTL	Research Testing Labs, Lubbock Texas
SLB	sucrose lysis buffer
SMP	soluble microbial products
SRT	solids residence time
SVI	sludge volume index
Tris	Tris(Hydroxymethyl)aminomethane
TSS	total suspended solids
VSS	volatile suspended solids
WWTP	wastewater treatment plant

CHAPTER 1 INTRODUCTION

Activated sludge is one of the most commonly used biological treatment processes for wastewater treatment in the U.S. The microorganisms in activated sludge systems are responsible for the removal and transformation of wastewater contaminants. These microbial populations are sensitive to wastewater characteristics, plant design and operational parameters. Because the effectiveness of activated sludge relies on microbial activity, a better understanding of the microbial communities and how they respond to conditions within the activated sludge process may be useful for improving plant performance.

There have been many attempts to identify and classify microbes in activated sludge, including culture and isolation based techniques, and various molecular approaches. However, activated sludge is a complex environment and most bacteria cannot be isolated with culture-dependent techniques (Snaidr, Amann et al. 1997). Culture-independent molecular methods such as denaturing gradient gel electrophoresis (DGGE), fluorescence in situ hybridization (FISH) and 16S rDNA gene clone libraries may provide a more accurate representation of microbial populations (Sanz and Köchling 2007). However these techniques are time consuming, costly, and labor intensive, and they typically only identify a small subset of an activated sludge microbial community. Next-generation high-throughput pyrosequencing is a cost effective, expedient method that offers a more complete picture of the consortia present in complex environments (Roesch, Fulthorpe et al. 2007).

Next generation DNA sequencing technologies, such as 454-pyrosequencing, have been recently used to study wastewater treatment microbial populations including describing the diversity of a single full-scale plant (Sanapareddy, Hamp et al. 2009), specific nutrient removal processes (Ye, Shao et al. 2011; Tu and Schuler 2013) and comparing the communities between different treatment plants (Hu, Wang et al. 2012; Wang, Hu et al. 2012; Zhang, Shao et al. 2012). This sequencing technique allows researchers to quantify relative abundance, identify shared communities, and assess population dynamics within activated sludge systems.

Influent characteristics impact microbial growth and population distributions and therefore affect plant performance. Just as overloaded plants can exhibit instability, so can those treating wastewater with low organic content. Adequate sustenance is required for microbial metabolism and the addition of a supplemental carbon source is a remedy for low influent strength conditions. The majority of the research on supplemental carbon sources has focused on compounds such as methanol and acetate (Hallin, Rothman et al. 1996; Ginige, Keller et al. 2005; Osaka, Yoshie et al. 2006). Glycerol, a byproduct of biodiesel production, has become an attractive alternative due to increased availability and decreased cost. Studies on glycerol as an external carbon source are limited, but have confirmed that the addition was beneficial to certain bacteria (Lu and Chandran 2010). The effects of glycerol addition on the microbial communities of activated sludge have been little studied, largely because conventional identification methods in microbiology have limitations.

The design and operation of a wastewater treatment plant is dependent on the influent characteristics and effluent quality requirements. The application of pyrosequencing to

full-scale activated sludge systems may offer insights into plant specific populations. Unique parameters of an individual plant may enrich for distinct microbial communities which in turn impact plant performance. The identification of these populations could be useful for operational optimization. In the event of a plant upset, 454-pyrosequencing as a method to detect microbial indications of instability might also be used to mitigate the likelihood of failure or to monitor progress during plant recovery.

One such unique wastewater treatment plant that may benefit from the use of pyrosequencing techniques is the Los Alamos National Laboratory Sanitary Wastewater System (LANL SWWS) located in Los Alamos, New Mexico. This plant utilizes glycerin as a supplemental carbon source due to low influent five day biological oxygen demand (BOD_5) concentration (70-80 mg/L) and experiences highly variable flow. In addition, the plant experienced an upset in 2012 with respect to excessive tardigrade growth, and in response introduced sludge (re-seeded) from another wastewater treatment plant to help improve the microbial populations.

The objectives of the research described in this thesis were:

1) Determine the differences between LANL SWWS activated sludge population and other plants.
2) Identify and evaluate the effects of glycerol as a supplemental carbon source on the microbial population.
3) Define any adaptive characteristics or population changes due to the seeding event.

4) Determine if there were identifiable operational conditions or environmental factors that influence specific microbial populations.

The research approach was to use 454-pyrosequencing to examine the microbial populations of the activated sludge. The relative abundance and diversity of the communities were compared with those from other treatment plants. The dynamics of the consortia surrounding the upset event and the effects of the foreign biomass were investigated. Other environmental factors that have been attributed to impacting the microbiota in activated sludge were also explored. Research into the microbial populations as revealed by 454-pyrosequencing will improve our understanding of the complex nature of activated sludge.

CHAPTER 2 PERTINANT LITERATURE REVIEW

2.1 Methods of Identifying Microbial Populations in Activated Sludge

Activated sludge is commonly used in biological wastewater treatment and is the result of combining wastewater with biomass, primarily bacteria, which metabolize the waste to remove soluble compounds. It is a complex microbial environment. The bacteria in this mixed liquor are responsible for a myriad of different transformations, including the removal of organics and nutrients. The ability of this secondary treatment process to operate effectively and produce high quality effluent relies on the microbial community within the sludge to interact and function in a stable manner. While advancements towards a greater understanding of microbial populations within activated sludge have increased dramatically over the last few decades, a comprehensive definition of the dynamics and composition of these communities continue to evolve.

There are many methods used to identify and characterize microbial populations. Conventional techniques such as pure culture isolation and microscopy require prior knowledge of the bacteria and their physiological properties and therefore have limitations associated with studying these communities as a whole within complex ecosystems such as activated sludge (Sanz and Köchling 2007). There are many bacteria in activated sludge that cannot be cultured and this method of investigation which limits the effectiveness of classical culture techniques for studying microbial dynamics in the activated sludge process (Wagner, Amann et al. 1993). With the advent of molecular techniques such as fluorescence *in situ* hybridization (FISH), denaturant gradient gel electrophoresis (DGGE) and 16S rDNA gene cloning and sequencing methods (Amann,

Lemmer et al. 1998; Nogales, Moore et al. 2001; Wagner, Loy et al. 2002), the understanding of the microbial populations in wastewater treatment have increased substantially, yet these communities remain unclear due to plant specific dynamics that influence community structure.

Recently, 454-pyrosequencing has gained popularity due to its performance and relatively low cost. With an average read length of 400 base pairs, and the ability to process over 1 million reads per run in ten hours (Roche Diagnostics Corporation n.d.), research using this technique as a molecular method for identifying the microbial communities in activated sludge has increased (Ye, Shao et al. 2011; Hu, Wang et al. 2012; Zhang, Shao et al. 2012; Ye and Zhang 2013). This technology does not require prior information about the consortia and questions about entire communities can be answered more efficiently than with conventional molecular methods (Wagner, Loy et al. 2002; Calderón, González-Martínez et al. 2012). This method has enabled researchers to study microbial community structures in full-scale wastewater treatment plants (WWTPs) under different operating configurations including anoxic/oxic, anaerobic/anoxic/oxic, conventional activated sludge, membrane bioreactors (MBRs) and enhanced biological phosphorous removal (EBPR) reactors (Yang, Zhang et al. 2011; Ye, Shao et al. 2011; Hu, Wang et al. 2012; Zhang, Shao et al. 2012; Ibarbalz, Figuerola et al. 2013; Ma, Wang et al. 2013; Mielczarek, Saunders et al. 2013). They have observed changes in populations over different geographic locations, temporal distributions, aeration characteristics, influent parameters and nutrient conditions. While each investigation identified varying distributions of microbial communities, there was evidence that certain microbes were ubiquitous in activated sludge. *Proteobacteria, Bacteriodetes, Firmicutes,*

Acidobacteria, Nitrospira, Planctomycetes, Verrucomicrobia, and *Chloroflexi* were phyla found in nearly all the literature. The theme from the multiple conclusions of each study was that the population dynamics were specific to each facility; however a consensus of common phyla present in multiple activated sludges could not have been expediently performed without pyrosequencing.

Research on the interactions between microbes in activated sludge to better understand the ecological relationships within these communities are often described in terms of certain functional groups. From an engineering perspective, the study of the microbial populations in wastewater treatment must yield applicable information in order to be implemented (Daims, Taylor et al. 2006) and so much engineering research is process specific. Among the groups of interest are ammonia oxidizing bacteria (AOB), nitrite oxidizing bacteria (NOB), polyphosphate-accumulating organisms (PAOs), glycogen-accumulating organisms (GAOs). Pyrosequencing has allowed comparisons to be made between different functional groups such as AOB and NOB under attached and suspended growth conditions (Kim and Kwon 2011), or PAO and GAO competition with various acetate concentrations (Tu and Schuler 2013). The ease of investigating the functionality of certain groups of bacteria involved in nutrient removal has increased due to pyrosequencing.

The various methods for identifying and classifying bacteria in wastewater treatment have resulted in a general agreement that several core taxa are present in most sludge and the study of these populations *in situ* could not be performed using culture dependent techniques. Advances in molecular methods have resulted in numerous applicable findings, however, to capture the complex ecosystem of activated sludge as a whole,

next-generation sequencing techniques such as 454-pyrosequencing have proven robust. With the advent of this technique, an influx of information about microbial populations in activated sludge has occurred with the central paradigm being that the community abundance and distribution is highly variable, An all-inclusive, generalized definition of the microbial consortia of activated sludge may not be possible due to its complexities, yet within the boundaries of each study the biodiversity can now be readily investigated with pyrosequencing.

2.2 Microbial Responses to Supplemental Carbon

One of the central challenges that face microbial ecologists and wastewater engineers revolves around the dynamic, adaptive nature of microbial populations within activated sludge. The structure and functionality of any microbial community is dependent upon numerous environmental factors that not only affect specific organisms within the community but their interactions, and consequently the biodegradation properties of the entire process (Forney, Liu et al. 2001). An engineer may seek to capitalize on these relationships to meet certain wastewater treatment goals. In order for biological wastewater treatment to be successful, sufficient nutrients must be present to support the growth and energy requirements of the microbes responsible for biodegredation. Carbon is one of these nutrients and is typically present in influent as some form of organic compound that can be metabolized by heterotrophic bacteria. If there is not enough carbon in the system, common practice is to add an external carbon source (Temudo, Muyzer et al. 2008; da Silva, Mack et al. 2009; Swinarski, Makinia et al. 2009; Yin, Wang et al. 2009; Guerrero, Taya et al. 2012).

Influent characteristics are one of many parameters that affect the microbial consortia in activated sludge, and they adapt in order to maximize the benefits from that substrate. The various pathways of glycerol metabolism is an example of the ability of microbes to tailor their functionality to satisfy their needs (Metsoviti, Paramithiotis et al. 2012). For example, if an influent is high in organic carbon, it could be assumed that heterotrophic bacteria will dominate, whereas autotrophs should be more prolific where inorganic carbon in the form of carbon dioxide is plentiful. Nitrogen and phosphorous removal are examples of processes in wastewater treatment that exploit chemoautotrophic organisms (Tchobanoglous, Burton et al. 2003). An analysis of microbial phospholipid fatty acid profiles of activated sludge in response to the reactor feed composition reflected the alterations in microbial community structure within activated sludge(Forney, Liu et al. 2001). Samples that were fed glucose-peptone for at least 30 days were distinct from samples that were collected from conventionally fed wastewater treatment facilities. Other research has investigated the influence of glucose, glycerol, and xylose on culture grown microbial communities (Temudo, Muyzer et al. 2008). While the type of substrate varied, the total amount of carbon was maintained at a constant 4 g/L. The communities were different in each case with *Klebsiella oxytoca* and species related to *Clostridium* dominating the glycerol fed community. The by-products ethanol, acetate and butyrate also changed in response to increased substrate concentrations. When the substrate was changed to a combination of glucose, glycerol or xylose, the communities changed depending on how they were initially cultivated, and mixotrophic organisms became dominant due to their ability to process multiple sources of carbon.

These studies illustrate that cultured microbial populations within laboratory conditions are dynamic and responsive according to carbon sources. The functionality of the system may indicate shifts in the microbial community as a result of different organisms becoming dominant due to specialized functions. However, the application of this research to full scale treatment plants remains unclear due to the greater microbial diversity in activated sludge than in lab scale reactors and the limitations of defining the functionality of each microbe in a mixed setting. The investigation of these relationships via pyrosequencing is warranted.

2.3 Treating Low Strength Wastewater with Glycerol and the Impacts on the Microbial Populations

The composition of carbon compounds entering a WWTP can vary and it is usually measured in terms of how much oxygen is required by the microbes to degrade the waste, termed as biological oxygen demand (BOD) or chemical oxygen demand (COD). A domestic WWTP treating influent with less than 200mg/L BOD or 400mg/L COD is often characterized as treating low strength wastewater (Tchobanoglous, Burton et al. 2003). Low strength conditions are detrimental to plant stability and combating this situation with an external, or supplemental carbon source has been well researched.

The addition of supplemental carbon has proven effective in increasing BOD in experimental systems(Carrera, Baeza et al. 2003). Methanol addition was a common practice in several studies due to its low cost and high accessibility (Cherchi, Onnis-Hayden et al. 2009), as well as its association with low sludge production (Hallin, Rothman et al. 1996). Methanol was found to be an effective supplemental carbon source

to enhance denitrification(Nyberg, Aspegren et al. 1992; Nyberg, Andersson et al. 1996), yet as a combustive compound, along with complications in low temperatures (Yin, Wang et al. 2009), alternatives were also considered such as acetate, ethanol, and glycerol (Osaka, Shirotani et al. 2008; Ginige, Bowyer et al. 2009; Swinarski, Makinia et al. 2009; Lu and Chandran 2010; Peng, Guo et al. 2012). Until recently, it was thought that glycerol was too costly to use as a supplemental carbon source, however as a byproduct of biodiesel production, the price of crude glycerol has dropped dramatically due to a resurgence in alternative fuel sources (Bodík, Blšťáková et al. 2009). Research comparing external carbon sources on biofilm reactors demonstrated that glycerol had a higher nitrification rate than ethanol or methanol yet exhibited a greater biofilm biomass accumulation (Bill, Bott et al. 2009). The addition of glycerol has also been studied in conjunction with phosphorous removal (Guerrero, Taya et al. 2012) and once the anaerobic phase of the EBPR was lengthened, it was found that phosphorous removal was enhanced by the addition of glycerol.

While studies have been performed analyzing the effects of glycerol as a supplemental carbon source on the processes in biological nutrient removal, few have been done on the impacts to the microbial populations. Many investigations of glycerol metabolism were implemented under culture dependent conditions and as discussed in section 2.1, this can be a limited approach in the study of the microbial ecology of activated sludge. Glycerol can be metabolized through a variety of pathways, both oxidative or reductive and can result in various useful by-products (da Silva, Mack et al. 2009) which has motivated much of the research. The pure cultures *Citrobacter freundii*, *Klebsiella pneumoniae*, *Enterobacter agglomerans*, *Enterobacter aerogenes*, belonging to the phylum

Proteobacteria, class *Gammaproteobacteria* (Homann, Tag et al. 1990; Barbirato, Soucaille et al. 1996; Biebl, Zeng et al. 1998; Ito, Nakashimada et al. 2005), and *Clostridium pasteurianum, Clostridium butyricum, and Lactobacillus reuteri* of the Firmicutes phylum (Talarico, Axelsson et al. 1990; Biebl, Marten et al. 1992; Biebl 2001) were found to grow anaerobically with glycerol as a sole carbon source. Under aerobic as well as anaerobic conditions in batch reactors, strains cultivated from environmental consortia closely related to *Klebsiella pneumoniae* were found to convert raw glycerol into 1,3-propanediol (Rossi, da Costa et al. 2012). Stable isotope probing has been used to identify glycerol assimilating bacteria in mixed cultures in batch reactors. Research comparing the microbial communities of glycerol assimilating bacteria in a denitrifying integrated fixed-film activated sludge (IFAS), demonstrated that the communities were different in the biofilm phase versus the suspended phase of the process (Lu and Chandran 2010). They identified species closely related to *Comamonas badia* and *Diaphorobacter* of the *Betaproteobacteria* class in the suspended phase. In the biofilm phase *Comamonas badia* were again identified as well as *Bradyrhizobium*, and *Tessaracoccus bendigoensis* from the *Alphaproteobacteria* class and *Propionibacteriaceae* from *Actinobacteria*. Each of these species relative abundance within the microbial community dramatically increased over the year of exposure to glycerol as a carbon source to enhance denitrification.

Glycerol as a supplemental carbon source for low strength wastewater is becoming a popular process manipulation. It has been shown to be an effective denitrification enhancer as well as fostering biological phosphorous removal. The effects of glycerol on

pure cultures have been studied, yet more research is needed to identify the changes in the communities as a whole in activated sludge.

2.4 Microbial Responses to Seeding Events

There are circumstances in which a wastewater treatment plant may need to add a supplemental microbial culture to the system; this process is known as seeding. A plant may choose to add alternate biota to their system in order to expedite the startup process and decrease the acclimation time for the biological treatment to become effective (Dabert, Delgenès et al. 2005). A WWTP may need to alter or manipulate the microbial population within the system in order to meet new or more stringent requirements (Stephenson and Stephenson 1992). This process is also utilized in the event of a system failure. Seeding can be accomplished by the addition of customized bacterial cultures (bioaugmentation), or addition of mixed liquor suspended solids from another facility. The survival rate and adaptation of these microbes within a full-scale system have not been studied extensively nor has their impact on the indigenous community been investigated.

In the event of an abnormal inflow of pollutants, also known as a shock, a WWTP may need to take precautionary measures to ensure that the resulting disturbance to the microbial population is minimal. Buffer tanks may be used to prevent these loads from entering the system all together, but the use of genetically engineered microorganisms comparable to the pre-shocked population has been documented to (Eichner, Erb et al. 1999). With the advances in molecular techniques some research has focused on the recovery of reactors after bioaugmentation and found that recovery time is less for

bioaugmented sludge versus a culture that was allowed to recuperate without biosolids addition (Bokhamy, Deront et al. 1997; Boon, Goris et al. 2000; Boon, Top et al. 2003). The functionality of reactors with and without bioaugmented inoculate as measured through nitrification, carbon removal and sludge compaction after a shock of chloroaniline (3-CA) were compared (Boon, Top et al. 2003). It was reported that the reactors that were inoculated exhibited similar carbon removal efficiency to a reactor than did not receive a shock. Of the reactors that were exposed to 3-CA, it took 48 hours for the inoculum to degrade the 3-CA compared to six days for the 3-CA to washout of the system on its own. After 4 days, the bioaugmented reactors recovered the ability to perform nitrification which the researchers attributed to an increase in AOB. Because 3-CA inhibits denitrification, there was a buildup of ammonia in the system and once the inoculum degraded this compound, AOBs dominated.

Other research has investigated the effects of bioaugmentation to enhance microbial acclimatization in EBPRs. The anaerobic-aerobic cycling to foster PAO growth within sludge typically takes more than 40 days (Korstee, Appeldoorn et al. 1994) and the introduction of specialized biomass for phosphorous removal to hasten the process was studied (Dabert, Delgenès et al. 2005). It was found to be effective in decreasing the acclimatization time by two weeks. Moreover, it was found that the 3% inoculation biomass resulted in overall increased phosphorous removing capability. In analyzing the microbial populations of the reactor that was inoculated to a control reactor, it was reported that 14 days into the study the two reactors had very different microbial populations, however after 105 days, the communities appeared nearly identical, suggesting that the organisms responsible for phosphorous removal were not specific to

the inoculate. It is possible that this convergence of diversity was due to other interactions between the microbes, and a similar investigation with next-gen sequencing techniques may offer clarification.

One study utilized 454-pyrosequencing to investigate the microbial communities of a dry anaerobic digester (Cho, Im et al. 2013) in comparison to that of an inoculum sludge. This study focused on methanogenic genus diversity and reported that the diversity of the seed sludge communities decreased once the population acclimated to dry conditions at 200 days. The diversity of the dry sludge was less than that of the original inoculum sludge, from 18 different genera to over 90% *Methanosarcina* which has been shown to be shock tolerant and utilize multiple pathways for methanogenesis. This research illustrated that microbial communities may shift after a seed event or inoculation, but as there was no preliminary comparison to the original dry sludge, further investigation is needed.

Through the advancements in next generation high-throughput sequencing, such as 454-pyrosequencing, a more complete understanding of the microbial populations of activated sludge is being accomplished (Sanapareddy, Hamp et al. 2009; Ye, Shao et al. 2011; Hu, Wang et al. 2012; Ma, Wang et al. 2013; Ye and Zhang 2013). The microbial responses of the inoculum and the receiving sludge can be identified in detail with this technology, and perhaps the effects of a seed event or bioaugmentation can be optimized.

2.5 Environmental Conditions and Operational Characteristics Affecting Microbial Populations

Biological wastewater treatment revolves around the bacteria present to remove or treat the constituents present in the waste to regulatory standards. As previous sections of this work have suggested, many microbes are ubiquitous in WWTPS, and serve as core microbial populations, yet vary in their communal distribution based on many factors including geographic location, influent characteristics, and plant operation. With the advent of molecular methods and next generation sequencing techniques, studies of the microbial populations within full-scale treatment plants under various conditions have increased the understanding of how certain organisms are affected by their environment.

It is well known that temperature has a large impact on microbial metabolism and that their growth rates are temperature dependent and with an optimal range for most bacteria is 25-35°C (Tchobanoglous, Burton et al. 2003). Pyrosequencing studies have found that temperature explained the most variation between the microbial population of multiple WWTPs (Wang, Hu et al. 2012). Another study illustrated that as temperature increase to 45°C, the number of DGGE bands also increase, but decreased as the temperature exceeded 62° C (LaPara, Konopka et al. 2000). Other research has shown that microbial populations cluster according to season and have attributed temperature as the main contributor to this pattern (Moura, Tacao et al. 2009). Most research has investigated the impact of temperature on specific categories of microorganisms focusing on those involved in nutrient removal or sludge bulking. Optimal phosphorous removal occurs at temperatures between 10-30°C (Mamais and Jenkins 1992). Low temperatures have been shown to inhibit aerobic phosphorous removal (Helmer and Kunst 1998). GAOs out-compete PAOs when the temperature exceeds 20°C (Panswad, Doungchai et al. 2003; Lopez-Vazquez, Song et al. 2007; López-Vázquez, Hooijmans et al. 2008). The

temperature for optimal growth rate of nitrifiers (both AOBs and NOBs) has been shown to be 15-35°C (Antoniou, Hamilton et al. 1990). Low temperatures enhanced the growth of *Nitrospira* over AOBs such as *Nitrosomonas* due to nitrite availability (Siripong and Rittmann 2007). Temperature has also been studied in correlation with filamentous bacteria and has it has been suggested that as temperature increases, dissolved oxygen decreases and this condition favors the growth of filamentous bacteria (Liu and Liu 2006). A positive correlation between SVI and temperature has also been demonstrated (Krishna and Van Loosdrecht 1999).

Most bacteria prefer a pH 6.5-7.5 (Tchobanoglous, Burton et al. 2003). Research has shown that pH levels significantly impact the EBPR process evident in the absence of *Betaproteobacteria*, *Actinobacteria*, *Bacteriodetes* and *Chlorobi* when the pH declined from 7.0 to 6.5 (Zhang, Liu et al. 2005). The ability of an expanded granular sludge bed (EGSB) reactor to remove nitrogen was drastically impaired when the pH of the influent dropped below 6.2 (Liao, Shen et al. 2013). Nitrifying bacteria required a pH range of 6.45-8.95 outside of which, it was found that nitrification was inhibited (Ruiz, Jeison et al. 2003).

Dissolved oxygen (DO) is a parameter of wastewater treatment. Temperature, pH and the SRT can affect the DO concentrations in a system, and these levels have been correlated with microbial population variance (Moura, Tacao et al. 2009; Wang, Hu et al. 2012). Under low DO concentrations, the growth rates of aerobic microbes will slow, allowing for the slower growing bacteria such as nitrifiers and filamentous bacteria to proliferate. An AOB similar to *Nitrosomonas* was found only in a full scale plant once the DO had dropped below 1mg/L (Park and Noguera 2004). Low DO concentrations

(<2.0 mg/L) have been linked to filamentous bacteria (Wilén and Balmér 1999) and poor settleability (Martins, Heijnen et al. 2003).

The solids residence time (SRT) is one of the most important operating parameters of the activated sludge process. The time that the sludge remains in the system can dictate the extent to which wastewater is treated. SRT is the inverse of the specific growth rate. Furthermore, it is directly correlated to the substrate concentration in the system. Influent characteristics, desired nutrient removal, effluent requirements and temperature play a role in how long the sludge should remain in the system and so depending on the goals of the plant, the SRT can vary from three to five days, to more than fifty days (Tchobanoglous, Burton et al. 2003). The length of time that biosolids are in a system effects many aspects of the activated-sludge process. Studies show that in order for nitrification to take place, longer SRTs were necessary to allow slow growing organisms such as ammonia-oxidizing bacteria to reproduce as well as ensure oxygen depletion to the level that nitrifiers will proliferate (Chen, Xu et al. 2004). Low-loaded plants run at high SRT values to help maintain high biomass concentrations in the biological reactors (Yoochatchaval, Ohashi et al. 2008). In systems with variable flow, longer SRTs were recommended to enhance system stability especially under low-strength influent conditions and at low temperatures (Angenent, Banik et al. 2001). MBRs utilize longer SRTs and this has been shown to lead to more biodiversity within studied reactors (Duan, Moreno-Andrade et al. 2009), and results from MBR research has indicated that *Delta* and *Epsilonproteobacteria* growth rates increase with SRT (Ahmed, Cho et al. 2007). Conversely, it has also been suggested that reactors with longer residence times have less bacterial diversity (Saikaly, Stroot et al. 2005).

Environmental parameters often have cumulative effects on the microbial population in activated sludge. These factors also impact various treatment processes in different ways and so it has proven difficult for researchers to confirm the effects of any one variable on the microbial community of full-scale treatment plants using conventional techniques of microbial identification. Environmental influences and operational parameters coupled with pyrosequencing results may offer a more in depth view of the deterministic responses of the activated sludge microbial populations.

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CHAPTER 3 RESEARCH OBJECTIVES

The unique operation of the LANL SWWS offered the opportunity to study the microbial community of activated sludge in response to long term supplemental carbon addition. There was also an upset incident to the activated sludge population caused by an overabundance of tardigrades thought to be the result of a lengthy solids residence time within the aeration basins of LANL SWWS. Plant personnel introduced mixed liquor from another treatment facility to aid in the plant recovery and this inoculation allowed for the evaluation of the microbial reaction to an inoculation of foreign biomass. It was hypothesized that: 1) the microbial population of LANL SWWS would be different from the population of plants treating domestic wastewater, 2) that there would be less diversity in a plant that receives a more homogeneous substrate such as glycerin, 3) the population would adapt, yet revert to the original composition following the exposure to the foreign biomass, 4) the presence and proliferation of certain microbes could indicate plant performance.

To demonstrate that 454-pyrosequencing is a robust and applicable method for studying the dynamic microbial community within activated sludge, the objectives of this research were to: 1) determine the differences between LANL SWWS activated sludge population and other WWTPs, 2) identify and evaluate the effects of glycerol as a supplemental carbon source on the microbial population, 3) define any adaptive characteristics or population changes due to the seeding event, 4) determine if there are certain operational or environmental conditions that influence specific microbial populations.

The methodology of this research was to use 454-pyrosequencing and bioinformatics tools to analyze the populations of several activated sludge samples from LANL SWWS as well as other WWTPs. Twenty two samples were collected over a seven month time frame, fourteen from LANL SWWS, and eight from other full-scale WWTPs in central New Mexico in an effort to identify any community variation due alternative carbon sources, operational characteristics and parameters.

CHAPTER 4 METHODS AND MATERIALS

4.1 Los Alamos National Laboratories Sanitary Wastewater System Description

Los Alamos National Labs Sanitary Wastewater System (LANL SWWS) utilizes suspended growth activated sludge as secondary treatment. It is a relatively small wastewater treatment plant with a design capacity of 0.6 MGD and an average monthly influent flow of 0.333 MGD. Daily measured operating parameters and wastewater characteristics are included in Appendix B. The facility only treats wastewater from the Los Alamos National Laboratory and a small trailer park and hence experiences highly variable inflows that correspond to operations at the laboratory, with peak flows Monday through Thursday and minimal flows Friday through Sunday. It also receives wastewaters with very low organic content such as wash down from rainfall or snowmelt and return flow from the drain pump station.

A schematic of the facility (Appendix A) is provided and briefly discussed here. Influent wastewater enters the LANL SWWS at the entrance works (labeled). A splitter box with a pump is located at the entrance works and glycerin is added to the system at this point. The flow then proceeds to the equalization and aeration basins. In order to dampen the effects of influent flow variability, equalization basins (EQ basins) are utilized to stabilize the flow based on operational set points maintained by plant personnel. The EQ basins are aerated for an eight minute cycle, with two minutes on and six minutes off to promote biomass stability before the flow is released to the aeration basins. These two basins have a combined holding capacity of 400,000 gallons (1514 m^3). There are four aeration basins, with an allowable 250,000 gallon (946 m^3) volume each, however only

three operate, the fourth is used for emergency overflow (total aerated volume of 750,000 gallons or 2839 m^3). The plant has two clarifiers, though both are not used for their designed purpose. One was converted to an aerobic digester in which the sludge thickening process takes place before the clarified water is decanted into the effluent holding pond and subject to MiOx chlorine disinfection. The thickened sludge is sent to drying beds.

4.1.2 Aeration Basins

There are three aeration basins for biological treatment. These sequential aeration basins perform internal nitrification/denitrification. The flow through this portion of the treatment train begins in the southwest basin (SW), proceeding to the southeast (SE), the northeast (NE) and finally out to the clarifiers. Cyclic aeration occurs in each basin at different intervals and durations as described in table 4.1. The length of air on and off is dictated by ammonia and nitrate levels and runs on a forty five minute cycle for each basin.

Table 4.1: Aeration duration of each LANL SWWS basin

	Programmed durations (minutes)		**Actual durations during time of project (minutes)**	
	Air on	**Air off**	**Average on**	**Average off**
SW basin	7	38	6	39
SE basin	4	41	3.6	41.4
NE basin	1	44	1.5	43.5

The SW basin is exposed to the longest air on cycle and ammonia degradation takes primarily place here. As the flow proceeds through the basins, the oxygen concentration decreases, and the anaerobic conditions result in denitrification. This is a successful operation as the effluent ammonia and nitrate levels were consistently below regulatory requirements.

4.1.3 LANL SWWS Glycerin Addition

Prior to July, 2010, the five day biological oxygen demand (BOD_5) concentration within the LANL SWWS system was approximately 70-80 mg/L, and ranged from as low as 25 mg/L to as high as 150 mg/L. Even at the highest BOD_5 concentrations, this is well below the typical domestic characteristics of 190 mg/L (Tchobanoglous, Burton et al. 2003). This low concentration resulted in an unstable system and the plant was vulnerable to upsets. Plant personnel began adding crude glycerin from Southwest Biofuels LLC to the system July, 2010. This crude glycerin was the result of biodiesel production and "contained ~30% glycerin, with the balance being saponification products, fatty acids, etc." (S. Trujillo personal communication, June 22, 2012). In October 2011, personnel noticed inconsistencies in the crude material and theorized the product to be less than 5% glycerin. In mid-November 2011, they began supplementing with a 99.7% purified United States Pharmacopeia (USP) grade glycerin from Vitusa Products Inc. (http://www.vitusaproducts.com/) and have continued using this product. They used the pure glycerin in full concentration through February 2012, however due to viscosity related mechanical issues, such as pump clogging, tube rupture and other malfunctions, have since diluted this product with 50% water as of February 29, 2012. Glycerin was added as a pulse with duration and rate varying depending on the plant

dynamics. A handwritten log was provided by LANL SWWS personnel from January, 2012 through May, 2012. Due to intermittent recordings, missing values were assumed equal to adjacent values. Based on this data, the daily dose rates were calculated and averaged into monthly doses reported in table 4.2. Pumping typically began between in the late morning (10am) and the pump duration ranged from thirty minutes to four hours. The drastic increases in pump duration and glycerin flow after February reflects additional pumping time required to feed the 50% diluted glycerin solution.

Table 4.2: Monthly average glycerin dose rates and pump durations

	Glycerin dose rate	Length of time pump on	Glycerin volume added per day
Month	**mL/min**	**min**	**mL**
January	219.7	36.3	7972.2
February	244.9	71.9	17607.2
March	471.0	186.8	87964.6*
April	395.0	177.5	70112.5*
May	451.0	142.8	64402.8*

* Larger volume reflects longer pump duration due to 50% dilution

Comment [BMT1]: What is the daily mass of glycerin in kg glycerin/d as well as kg theoretical BOD/d. How does this compare to kg BOD/d from wastewater?

According to plant personnel, the addition of the supplemental carbon source has resulted in an increase in BOD_5 concentration to at least 200 mg/L. They attributed an increase in biomass production to an observed decrease in effluent nitrates as well as an increase in both effluent total suspended solids (TSS) and sludge volume index (SVI).

4.2 Other Wastewater Treatment Plant Descriptions

Los Alamos County WWTP (LAC WWTP) was selected for comparison to LANL SWWS because it is near LANL SWWS and experiences similar temporal effects and precipitation events. This plant also provided suspended solids to LANL SWWS as a

seed inoculate due to experienced upsets previously discussed. Four other treatment facilities were also sampled for comparison. These plants were chosen because they were similar in size, configuration or geographical location to LANL SWWS and offered the best possible consistencies for full-scale comparison. Table 4.3 illustrates relevant design and operating parameters of these plants.

Table 4.3: Plant operation and characteristics

Plant Name and Location	Plant Capacity (MGD)	Biological treatment system	SRT (days)
City of Santa Fe Paseo Real WWTP Santa Fe, NM	13	Activated sludge Continuous flow	15+
Bernalillo Treatment Plant Town of Bernalillo, NM	1.3	Activated sludge Continuous flow	18+
Rio Del Oro WWTP Village of Los Lunas, NM	1	Activated sludge Membrane Bioreactor (2 separate systems)	6-8 25+
Belen WWTP Belen, NM	1.6	Activated sludge SBR	18-30
Wastewater Treatment Plant in Pueblo Canyon County of Los Alamos, NM	1.4	Activated sludge	8-18
LANL SWWS Los Alamos, NM	0.6	Activated sludge	70+

4.3 Sampling Methods and Preservation

Samples were collected by plant personnel from the LANL SWWS southeast aeration basin at approximately 2 pm each collection day during the period November 14, 2011 through June 11, 2012. The mixed liquor was immediately transported to an on-site laboratory where it was centrifuged and immediately frozen. Two samples from the Los Alamos County Wastewater Treatment Plant in Pueblo Canyon on February 15, 2012 and March 30, 2012 were also provided by personnel. These samples were handled identically to the samples collected from LANL SWWS. The samples were transported to UNM on ice and stored at -23°C until DNA extraction.

Five other samples were collected by hand from Santa Fe, Los Lunas, Bernalillo, and Belen, New Mexico from May 22-24, 2012. 250 mL of mixed liquor were collected on site from the aeration basins of each plant and immediately stored on ice in 500 mL bottles. These samples were transported directly to UNM. Upon arrival, 1 mL of well-mixed activated sludge was removed from each bottle and put into microtubes. These samples were centrifuged at 6000 revolutions per minute (rpm) for 4 minutes. The supernatant was removed and the solids were washed with 1 mL of phosphate buffered saline (PBS). They were again centrifuged for 4 minutes after which the buffer was removed, and were immediately frozen at -23°C until DNA extraction.

4.4 DNA extraction

A modified preservation and extraction method referred to as CTAB S was used to extract the DNA from each sample. A study performed in 2008 by Kendra R. Mitchell and Cristina D. Takacs-Vesbach observed that greater DNA yield and diversity was obtained using sucrose lysis buffer (SLB) as a preservative and

hexadecyltrimethylammonium (CTAB) as the extraction method (Mitchell and Takacs-Vesbach 2008). This method is described below and the procedure is thoroughly outlined with solution composition in Appendix C.

Samples were thawed to room temperature and approximately 100 µL of the biomass were taken from each test tube. The biomass was inserted into a microtube and 300 µL of Tris pH 9 was added to each sample tube. These were then sonicated for 8 seconds at 10% to thoroughly mix and break up any possible flocs that might have formed. 300 µL per microtube was then pulled off for a remaining volume of 100 µL. An equal volume of SLB was added for a volume of 200 µL of sample solution per microtube. The samples were frozen again at -23°C overnight.

The samples were thawed to room temperature and 400 µL of CTAB Buffer was added to each totaling 600 µL in each microtube. A proteinase K solution with a concentration of 100 µg/mL was added, (in this case 3µL of 200x stock solution). Each sample was once again sonicated for 8 seconds at 10%. All samples were then inserted into a water bath for one hour at 60°C, inverting every 20 minutes for mixing. 120 µL of 10% SDS was added to each sample tube. The samples were again incubated in a 60°C water bath for another hour with periodic inversions. Equal volumes of Phenol:chloroform:isoamyl alcohol was used once for extraction (720 µL) extracting the top portion of the 2 phase sample followed twice more by equal volumes of chloroform, (again retaining only the top phase of the sample). Finally, 0.1 volumes of 3M NaOAc and two volumes of ethanol were added to each sample tube and allowed to precipitate overnight at -23°C. The thawed samples were centrifuged for 45 minutes at 11,000 rpm and the supernatant from each precipitated sample was drawn off. Each sample was washed with 500µL of

70% ethanol, spun again at 11,000 rpm for 30 minutes, the ethanol was extracted, and the sample was allowed to dry completely. The resulting DNA pellet was suspended in 50 µL 10mM Tris pH8 and frozen at -23°C until shipment for pyrosequencing.

4.5 PCR Amplification and 454-Pyrosequencing

The extracted DNA samples were shipped frozen to Research and Testing Laboratory (RTL) in Lubbock, Texas for 16S rDNA 454-pyrosequencing, the methods for this process briefly described were provided by RTL. Initial generation of the sequencing library utilized a one-step polymerase chain reaction (PCR) with a total of 30 cycles, a mixture of Hot Start and HotStar high fidelity taq polymerases. Given that a typical read length from 454-pyrosequencing is approximately 400 base pairs (bp), the 16S-amplicons were PCR-amplified over the hypervariable regions V1-V3 with the forward primer Gray28F (5'-TTTGATCNTGGCTCAG) and reverse primer Gray519r (5'-GTNTTACNGCGGCKGCTG) (Handl, Dowd et al. 2011). This region was found to have the fewest sequence errors associated with it while still capturing bacterial diversity down to the genus level (Wang, Garrity et al. 2007). Bacterial tag-encoded FLX amplicon pyrosequencing (bTEFAP) utilized the Roche 454 FLX instrument with Titanium reagents and procedures based upon RTL protocols and previous literature (Dowd, Callaway et al. 2008) (RTL, Lubbock, TX).

4.6 Post-run Data Preparation with QIIME

The open source software Qualitative Insights Into Microbial Ecology 1.80 (QIIME) (Caporaso, Kuczynski et al. 2010) was used for processing and analyzing the data received from RTL. The entire workflow for this process is provided in Appendix C

The original, "raw" data received from RTL resulted in 175,426 unique sequences for all 22 samples. Filtering and denoising was employed in order to remove erroneous sequences pertaining to the pyrosequencing process (Huse, Welch et al. 2010). The pyrosequencing results from RTL were provided in .sff format. These were then converted to text files. In QIIME, .sff files were processed into flowgrams and converted into readable .txt files that could then be opened and the barcodes extracted. This text file was used in the denoising process in order to remove possible sequencing errors due to imprecise signals from the 454-pyrosequencing. QIIME has a built in collection of programs for denoising entitled AmpliconNoise, developed by Chris Quince et. al in which the code written allows for noise removal from the 454-pyrosequencing process and the PCR point errors (Quince, Lanzen et al. 2009). In order to run AmpliconNoise on the new text files, 22 mapping files were created. A tab delimited file containing the sample I.D., the Barcode sequence, the Linker Primer sequence, and a description of the sample was created. Each individual sample was denoised using its respective mapping file. The number of processors was specified based on availability and the platform Titanium was used because RTL performed 454-pyrosequencing with Titanium Series Reagents.

Once the files were denoised, they were concatenated into one large file containing all samples for comparison. This step resulted in 104,046 effective reads (21,422 sequences were removed due to noise) that have been cleaned and the chimeras removed and therefore allowed for a more reliable analysis further downstream.

In order to extract useful information about the microbial population of each sample, an operational taxonomic unit (OTU) table was created. Based on a specified sequence 97%

similarity, the sequences were clustered into OTUs using the default method uclust (Edgar 2010). One representative sequence was then chosen for each OTU. The algorithm Ribosomal Database Project's Naïve Bayesian Classifier (RDP 2.2) (Wang, Garrity et al. 2007) compared these representative sequences to an outside curated environmental database's (Greengenes 12_10 (McDonald, Price et al. 2012; Werner, Koren et al. 2012)) sequences and assigned a taxonomic name to the OTU. Finally, the abundance of each OTU within each sample was tabulated and an OTU.biom table was formed with the taxonomic identities assigned to each OTU. A total of 22 samples were processed in this way, resulting in 9579 OTUs from the representative sequences. The sample with the minimum amount of effective sequences was LANL.2.7.12 (1343) and LL.6.18.12 contained the most effective sequences (19,703). The standard deviation among all 22 samples was 3834 reads per sample. This OTU table was used for all subsequent analyses.

In order to make a phylogenetic comparison, a phylogenetic tree was created. The sequences were aligned with templates sequences from the Greengenes database using the algorithm PyNAST (Caporaso, Bittinger et al. 2010) and filtered. By default QIIME utilized FastTree 2.1.3 (Price, Dehal et al. 2010) to produce a phylogenetic tree as a result of approximately-maximum-likelihood from multiple sequence alignment.

4.7 Taxonomic Summary

Various levels of taxonomic assignment were used throughout this project. To gain insight into the taxonomic grouping within each sample, the taxonomic summaries from the phylum to the genus level were created from the OTU.biom table in QIIME. These summaries were imported into a spreadsheet program (Microsoft Excel® 2010) to make

observations and comparisons. Those taxa occurring in a least one sample at greater than 1% relative abundance were considered to be the impacting biota on the plant performance. All other microbes were grouped into the "Minor taxa" category.

4.8 Diversity Analysis

In order to analyze and compare the microbial communities of the activated sludge, the diversity of the systems was investigated. The microbial diversity within each sample, known as alpha diversity, was utilized to compare how many taxa were in each sample. Beta diversity was used to make comparisons between the samples. A quantitative approach was taken in an effort to distinguish variations among the samples resulting from changes in the composition and distribution of the microbial communities (Lozupone, Hamady et al. 2007).

4.8.1 Alpha Diversity

Alpha diversity was used to describe the diversity within each sample, or groups of samples. The Shannon diversity index for diversity accounts for both the taxonomic richness and evenness within a sample. This index was used to characterize the total amount of OTUs found in each sample as well as the number of times each OTU was observed.

$$H' = -\sum_{i=1}^{R} p_i \ln(p_i)$$

Where:

H' = the Shannon's diversity index

R= the total number of OTUs in a sample

p_i = the proportion of a specific OTU relative to R

If there is only one type of microbe in the sample, $p_i = 1$, $\ln(1) = 0$ and H'= 0, there is no diversity. A larger H' reflects greater microbial diversity within the sample.

This alpha diversity measure was calculated using the QIIME 1.80 software. The minimum number of sequences in any of the samples analyzed was 1,343. To avoid skewing the diversity measures due to greater sample depth, the OTU tables were rarefied to the minimum number of sequences. The alpha diversity script was run on the rarefied OTU tables and the Shannon index was specified as one of the metrics. The results were then plotted with the diversity index (H') on the y-axis and the sequences per sample on the x-axis.

4.6.2 Beta Diversity

Beta diversity was used to compare the diversity between each sample. A quantitative approach to beta diversity accounted for the relative abundance of each OTU. There are different ways to approach the beta diversity. From a taxonomic perspective, the taxa within a sample are equally related; either they belong to one community or another. From a phylogenetic perspective, the taxa within a sample are related based on their lineage which account for varying degrees of divergence (Lozupone and Knight 2005). Beta diversity metrics utilize pairwise distance matrices for comparison and depending on the approach used, different matrices for analysis are necessary. These distances can then be visualized using different ordination methods for multivariate analysis.

There are several ordination methods that aid in the visualization of multidimensional data. Given multiple samples, each sample has its own set of variables (in this case, the microbial populations) and multivariate analysis is often necessary to elucidate any latent patterns in the data set. Principal component analysis (PCA) and principal coordinate analysis (PCoA) were two ordination techniques used to visualize the beta diversity of the samples.

Principal Component Analysis (PCA) is a linear exploratory method for visualizing patterns in high dimensional data. PCA is a popular multivariate method for reducing the original number of variables into a lower number of synthetic variables, and projecting them in such a way that can be interpreted with the maximum variation explained by the components (Ramette 2007). In other words, large data sets are transformed into a distance matrix that keep the variables within the dimensions stable, the "new" data points were plotted, and rotated so that two perpendicular lines bisect largest portion of the data and explained the maximum variation.

PCA was used to analyze the microbial populations between the various activated sludge samples from a taxonomic perspective. The statistical computing and graphics software R (R Core Team 2013) was used to carry out this procedure with the function "prcomp". The data was first standardized by mean centering. The mean of each dimension was subtracted from each set of data points and resulted in a matrix where each data set was expressed as a distance from the mean. Because the data used was of the same units of measure (microbial abundance), the covariance of each data set was calculated. A covariance matrix was used to iteratively calculate the eigenvalues and unit eigenvectors. The eigenvectors of the covariance matrix are the principal components and account for

the variation in a data set. The data was then rotated so that the x-axis is the principal component with the highest eigenvalue or most variation explained (PC1) and the y-axis is the orthogonal component that has the next highest eigenvalue (PC2).

A biplot was then generated, allowing the samples and variables to be plotted on the same graph. The samples were displayed as points and the microbial populations were displayed as vectors. The direction of these vectors equates to the greatest change among the variable, while the magnitude of the vector represents the rate of that change. So if there are more of a certain bacteria in a certain sample, the vector will point towards that sample and if that sample has a lot more of that certain bacteria compared to all other samples, the vector will be long.

Principal coordinate analysis (PCoA) is a separate multivariate statistical ordination technique that allows for exploring beta diversity from a phylogenetic approach. The components are not linearly derived from the original data set like in PCA, but are representations based on the maximum distance between the samples (Ramette 2007). The weighted unique factor (UniFrac) metric was used to create a distance matrix. Weighted UniFrac is best used if attempting to describe changes in the most abundant lineages (Chen, Bittinger et al. 2012). It was believed that within activated sludge, the rare microbes would not impact plant performance, and quantitative or weighted UniFrac was specified as the distance measure for the matrix. This measure approaches diversity from a phylogeny-based perspective in which taxa may be more similar to one community than another based on their evolutionary history. Using the QIIME software, a phylogenetic tree was created from the OTU table. Each branch on the tree corresponded to a taxa that was present in any of the samples. The weighted UniFrac

distance measure accounted for the abundance of taxa within each sample by weighing the phylogenetic branch with more abundantly occurring microbes more heavily than those with less abundance (Lozupone and Knight 2008).

$$u = \sum_{i}^{n} b_i \times \left| \frac{A_i}{A_T} - \frac{B_i}{B_T} \right|$$

Where:

u = the weighted UniFrac value

n = the total branches in the phylogenetic tree

b_i = the length of a branch

A_i = the number of sequences than descend from a branch in community A

A_T = the total number of sequences in community A

B_i = the number of sequences than descend from a branch in community B

B_T = the total number of sequences in community B

This algorithm was performed in QIIME for every OTU in the samples and resulted in square distance matrices based on the number of samples. This distance matrix was transformed into a multidimensional space and the principal coordinates were generated to maximize the variation between the reproduced distances. It is important to note that due to the transformation and projection of the distances between each sample, the original data set is no longer correlated with the principle coordinates.

4.7 Statistical Analysis

Multiple statistical analyses were used in this project. In order to make reliable comparisons across each sample, the effective reads were rarefied or normalized to the minimum number of sequences (1,343). Two-tailed, unequal variance t-tests were used for significance testing between different groups of bacteria and operational parameters. Correlations were considered significant at $p<0.05$ (95% confidence interval).

To elucidate any linear trends, univariate linear correlations were carried in out in Microsoft Excel 2010 between the most abundant phyla and full-scale treatment plant parameters. The least squares method was used to correlate microbial populations with specific plant parameters. The function (LINEST) was used for linear regression analysis. The F probability distribution (FDIST) was used to determine if the correlations were significant. With alpha specified as 0.05, correlations were considered significant if the F critical was greater than the F value.

4.8 C/N Ratio Calculation

LANL SWWS did not measure influent BOD or COD during the study; however, given the chemical equation for glycerin ($C_3H_8O_3$ with a molecular weight of 92.1 mg/mmol) the theoretical BOD of glycerin was calculated:

Comment [BMT2]: Theoretical BOD or COD = 1.22 mg BOD/mg glycerin. Assuming .68 mg BOD5/mg UBOD, calculate added BOD load to pland.

$$C_3H_8O_3 + 3.5O_2 + \rightarrow 3CO_2 + 4H_2O$$

$$\frac{\Delta O_2}{\Delta\, C_3H_8O_3} = \frac{3.5\left(32\frac{mg}{mmol}\right)}{\left(92.1\frac{mg}{mmol}\right)} = 1.22\ \left(\frac{mg\ BOD}{mg\ glycerin}\right)$$

Assuming that the BOD_5 is 68% of the ultimate BOD (Tchobanoglous, Burton et al. 2003), the added BOD load to the plant due to glycerin was calculated:

$$1.22\left(\frac{mg\ BOD}{mg\ glycerin}\right) * 0.68 = 0.83\left(\frac{mg\ added\ BOD}{mg\ glycerin}\right)$$

This calculated value was sufficient to perform further calculations with glycerin as a contributing organic load given the lack of measured BOD data. In order to estimate the carbon to nitrogen ratio (C/N), the carbon due to the glycerin addition was calculated. The average volume of glycerin was calculated based on data provided by plant personnel. The concentration of the pure glycerin was altered during this project (Table 4.2) and the calculation to accommodate the dilution was:

$$\left[flow_{Jan-Feb}\left(\frac{mL}{min}\right) * pump\ on(min)\right] + \left[\frac{flow_{Mar-May}}{2}\left(\frac{mL}{min}\right) * pump\ on(min)\right]$$

$$= avg\ glycerin\ flow\ (\frac{mL}{day})$$

The manufacturer supplied the specific gravity of the glycerin (1.2626). The density of the 99.7% glycerin was calculated as:

$$SG * \rho_{H2O\ 4℃}\left(\frac{mg}{mL}\right) = \rho_{glycerin}\left(\frac{mg}{mL}\right)$$

The average mass of bulk glycerin added per day was calculated as:

$$\rho_{glycerin}\left(\frac{mg}{mL}\right) * avg\ glycerin\ flow\ \left(\frac{mL}{d}\right) = avg\ mass\ of\ glycerin\ \left(\frac{mg}{d}\right)$$

The molecular weight (MW) of glycerin is 92.1 g/mol and the mols per day of glycerin added were calculated as:

$$\frac{avg\ mass\ of\ glycerin\left(\frac{g}{day}\right)}{MW_{glycerin}\left(\frac{g}{mol}\right)} = Mol\ of\ glycerin\ \left(\frac{mol}{day}\right)$$

The influent flow was reported in MGD and was converted to L/day multiplying the flow by 3.785 L/gal. The mmol of glycerin per liter of influent was calculated as follows:

$$\frac{mmol\ of\ glycerin\ \left(\frac{mmol}{day}\right)}{influent\ flow\ \left(\frac{L}{day}\right)} = \frac{glycerin\ (mmol)}{influent\ (L)}$$

The chemical equation for glycerin is $C_3H_8O_3$ therefore the molar ratio of carbon to glycerin was calculated as 3:1 and the molar concentration of carbon due to glycerin was estimated:

$$\frac{glycerin\ (mmol)}{influent\ (L)} * \frac{3\ carbon\ (mmol)}{1\ glycerin\ (mmol)} = Carbon\ \left(\frac{mmol}{L}\right)$$

The average influent ammonia as nitrogen was calculated from data provided by plant personnel. The MW of ammonia is 14 g/mol as nitrogen. The molar concentration of the ammonia as nitrogen entering the system was calculated:

$$\frac{avg\ NH_3 - N\left(\frac{mg}{L}\right)}{MW_{NH_3-N}\left(\frac{mg}{mmol}\right)} = NH_3 - N\ \left(\frac{mmol}{L}\right)$$

The C/N ratio of due to glycerin was estimated as:

$$\frac{Carbon\ \left(\frac{mmol}{L}\right)}{NH_3 - N\ \left(\frac{mmol}{L}\right)} = \frac{Carbon}{Nitrogen}\ ratio$$

This ratio was then compared to the C/N ratio necessary for bacterial cell growth. The assumed chemical equation for biomass in the system is $C_5H_7O_2N$ (MW= 113 g/mol),

and the COD of cell tissue was assumed to be 1.42 g O_2/ g cells (Tchobanoglous, Burton et al. 2003) calculated by the following stoichiometric equation and calculation:

$$C_5H_7O_2N + 5O_2 + \rightarrow 5CO_2 + NH_3 + 2H_2O$$

$$\frac{\Delta O_2}{\Delta\, C_5H_7O_2\mathrm{N}} = \frac{5(32\ g/mol)}{(113\ g/mol)} = 1.42\ COD\ (\frac{O_2}{g\ cells})$$

Given the chemical equation for biomass above, a C/N of 5:1 would be needed for only growth. The synthesis yield coefficient, assuming the maximum biomass per unit of biodegradable soluble COD, 0.6 mg VSS/mg bsCOD (Tchobanoglous, Burton et al. 2003) was used was used to account for a portion of the carbon going towards maintenance and energy.

The C/N ratio needed for anabolism and catabolism was calculated:

$$\frac{\frac{5\ mol\ C}{1\ mol\ N}}{1.42\frac{g\ COD}{g\ VSS} * 0.6\ \frac{g\ VSS}{g\ bsCOD}} = \frac{mol\ C}{1\ mol\ N}\ needed\ for\ growth\ and\ energy$$

This value was compared to the C/N ratio provided by the glycerin addition.

4.9 Solids residence time calculations

The solids residence time (SRT), or the length of time that sludge remained in the system, was calculated assuming steady state conditions. The SRT for LANL SWWS was calculated based on data provided by plant personnel (Appendix B) and the following equation (Tchobanoglous, Burton et al. 2003):

$$SRT = \frac{Mass\ in\ the\ system}{Mass\ leaving\ the\ sytem} = \frac{VX}{(Q - Q_W)X_e + (Q_W X_R)}$$

V = aeration basin volume (L)

X = biomass concentration in the aeration basin (mg/L)

Q = equalization basin flowrate (L/day)

Q_w = waste sludge flowrate (L/day)

X_e = concentration of biomass in effluent (mg/L)

X_R = concentration of biomass recycled (mg/L)

Volumes and flows were reported in million gallons (MG) and mg per day (MGD). These were converted to liters (L) and L/day prior to calculations. As previously stated, the plant did not run at full capacity over the weekends and so many values were not reported. These missing values (such as the MLSS and TSS) were averaged based on the proximate recorded values. Typical activated sludge operations maintain continuous flow through the treatment train however, LANL SWWS did not. During this project, the reported waste activated sludge flow rates at LANL SWWS were intermittent due to sludge storage issues. Rather than continuous or daily wasting, they wasted only a few times per month. Due to no waste, the majority wasted solids leaving the system resulted in zero values. For this reason the effective SRT was calculated by taking a 45 day moving average of the above mentioned masses to allow the sporadic wasting rates to have less severe effects on the calculations.

The expected biomass concentration was also calculated and compared to the measured MLVSS concentration in the reactor (Tchobanoglous, Burton et al. 2003). Average constituent concentrations and flows were used, and typical kinetic coefficients were specified (Table 7.3 and 7.4). The SRT was designated as the minimum from the previous calculation (70 days).

$$X = \left(\frac{SRT}{\tau}\right) * \left(\frac{Y(S_o - S)}{1 + (k_d SRT)}\right)$$

Where:

X= heterotrophic biomass concentration (mg VSS/L)

SRT = solids residence time (days)

τ = hydraulic detention time (days)

Y = synthesis yield coefficient (mg VSS/mg bsCOD)

S_o = influent soluble substrate concentration (mg bsCOD/L)

S = effluent soluble substrate concentration (mg bsCOD/L)

k_d = endogenous decay coefficient (mg VSS/ mgVSS d)

In order to calculate the biomass, several preliminary calculations had to be performed (Tchobanoglous, Burton et al. 2003):

$$\tau = \left(\frac{reactor\ volume\ (L)}{influent\ flow\ \left(\frac{L}{d}\right)}\right) = hydraulic\ detention\ time\ (days)$$

$$S = \left(\frac{K_s(1 + k_d * SRT)}{SRT(Yk - k_d) - 1}\right)$$

Where:

S = effluent soluble substrate concentration (mg bsCOD/L)

SRT = solids residence time (days)

k = maximum specific substrate utilization rate (g bsCOD/g VSS day)

Y = synthesis yield coefficient (mg VSS/mg bsCOD)

K_s = half velocity constant (mg/L)

k_d = endogenous decay coefficient (mg VSS/ mgVSS d)

The influent soluble substrate concentration (S_o) was calculated from the given average influent TSS converted VSS using the biomass conversion of 0.85 VSS/TSS (Tchobanoglous, Burton et al. 2003) to COD :

$$\frac{inf\ TSS\ \left(\frac{mg\ TSS}{L}\right) * biomass\ conversion\ \left(\frac{VSS}{TSS}\right)}{Y\ \left(\frac{mg\ VSS}{mg\ bs\ COD}\right)} = inf\ TSS\ \left(\frac{mg\ bsCOD}{L}\right)$$

The average influent glycerin COD was calculated from the balance stoichiometric equation:

$$C_3H_8O_3 + 3.5O_2 \rightarrow 3CO_2 + 4H_2O$$

$$\frac{\Delta O_2}{\Delta\ C_3H_8O_3} = \frac{3.5(32\ g/mol)}{(92.1\ g/mol)} = 1.22\ \left(\frac{g\ bsCOD}{g\ glycerin}\right)$$

$$\frac{inf\ mass\ C_3H_8O_3\ \left(\frac{mg\ glyc}{d}\right) * COD_{C_3H_8O_3}\left(\frac{mg\ bsCOD}{mg\ glyc}\right)}{influent\ flow\ \left(\frac{L}{d}\right)} = \inf C_3H_8O_3\left(\frac{mg\ bsCOD}{L}\right)$$

The influent soluble substrate was:

$$S_o = inf\ TSS\ \left(\frac{mg\ bsCOD}{L}\right) + inf\ C_3H_8O_3\left(\frac{mg\ bsCOD}{L}\right)$$

The heterotrophic biomass concentration (X) was compared to the measured MLVSS. The total MLVSS was also calculated to include inert substrates in the system (Tchobanoglous, Burton et al. 2003):

Comment [BMT3]: Fine. Now compare the BOD due to glycerine to that for the influent wastewater, recognizing all of the inaccuracies due to lack to data. The point is to estimate the relative increase in BOD loading of the glycerine addition.

$$X_T = \left(\frac{SRT}{\tau}\right) * \left(\frac{Y(S_o - S)}{1 + (k_d SRT)}\right) + f_d k_d (X) SRT + \left(\frac{X_{oi} SRT}{\tau}\right)$$

Where:

f_d = fraction of biomass as cell debris (g VSS/g VSS)

X_{oi} = the nonbiodegradable VSS concentration (mg/L)

CHAPTER 5 THE EFFECTS OF GLYCERIN AS A SUPPLEMENTAL CARBON SOURCE ON THE MICROBIAL POPULATIONS OF ACTIVATED SLUDGE

5.1 Introduction

Activated sludge is a commonly used biological process for removal of organic and inorganic constituents in wastewater treatment plants (WWTPs). The microbes involved in this process comprise a diverse and dynamically reactive ecosystem. The complexities of activated sludge consortia make it difficult to fully understand these communities. Conventional culture-dependent research techniques are limited to studying isolates and require prior knowledge in order to research a microbe. Many molecular methods (such as FISH, DGGE, and 16s rDNA cloning) are too time consuming, too expensive, and not sensitive enough for investigating large samples (Sanz and Köchling 2007). More recently-developed next-generation sequencing techniques, such as 454-pyrosequencing, developed by Roche 454 Life Science, are useful tools in studying these populations in their entirety (Quince, Lanzen et al. 2009), and have allowed researchers to view biological systems in full-scale WWTPs in a revolutionary way. Comparisons of the microbial population in activated sludge have been made between different WWTPs (Hu, Wang et al. 2012; Zhang, Shao et al. 2012), within the same treatment plant (Sanapareddy, Hamp et al. 2009; Ye and Zhang 2013), and studies of the communities involved in nutrient removal (Ye, Shao et al. 2011; Ma, Wang et al. 2013; Tu and Schuler 2013) have been performed with 454-pyrosequening. They have resulted in a better understanding of this complex ecosystem however the consensus is that microbial distributions are plant specific.

The research presented here is focused on gaining a better understanding of the microbial community at a WWTP utilizing glycerin as a supplemental carbon source. Los Alamos National Laboratory Sanitary Wastewater System (LANL SWWS) has had operational difficulties due to a low-strength influent. In order to address this problem, plant personnel have been adding glycerin as an external carbon source to the system upstream from the aeration tanks and have since seen an increase in plant effectiveness. According to communications with plant personnel, the effluent quality has greatly improved and toxic upsets are less frequent. Studies have shown that the addition of external carbon increases nitrification rates (Nyberg, Aspegren et al. 1992; Nyberg, Andersson et al. 1996; Park, Chung et al. 2005; Mokhayeri, Riffat et al. 2008; Osaka, Shirotani et al. 2008; Yin, Wang et al. 2009), and enhances phosphorous removal (Oehmen, Saunders et al. 2006), yet research on the impacts of supplemental carbon to the entire microbial consortia is limited.

It was hypothesized that the addition of this compound to the LANL SWWS would impact the microbial population of the activated sludge. The objectives of this body of work were to 1) compare the communities of activated sludge samples that utilized glycerin as a supplemental carbon source to those that did not use any supplement (referred to as Other or conventionally fed plants), 2) identify significant bacterial differentiations between LANL SWWS samples and the Other samples, 3) determine and contrast the biodiversity of the samples. Samples from LANL SWWS and eight other treatment plants were pyrosequenced and analyzed in order to achieve these objectives.

5.2 Methods and Materials

Six full-scale WWTPs were sampled over the course of seven months (from November, 14 2011 through June, 18 2012). Fourteen samples were taken from LANL SWWS, and eight samples from conventionally fed WWTPs, hence referred to as other plants (See table 4.1 for details on other plants). The samples were collected from the aeration basins of each plant, DNA extraction was performed via the CTAB S method (detailed in Section 4.2) (Mitchell and Takacs-Vesbach 2008), and the samples were sent to RTL (Lubbock, TX) for 454-pyrosequencing as discussed in Section 4.3. A Roche 454FLX instrument (Roche Inc., Indianapolis, IN) with Titanium reagents and the universal eubacterial pyrosequencing primers forward primer 28F (5'GAGTTTGATCNTGGCTCAG3') and the reverse primer 519R (5' GTNTTACNGCGGCKGCTG'3) were used (Dowd, Callaway et al. 2008).
Raw sequencing data was received from the pyrosequencing lab, the data was then manually filtered, trimmed and denoised using AmpliconNoise (Quince, Lanzen et al. 2011) and the effective reads were then processed with the bioinformatics software QIIME 1.80 (Caporaso, Kuczynski et al. 2010) in order for the sequences to be classified and identified as operational taxonomic units (OTUs). A detailed description of this process is provided in Section 4.4. A taxonomic summary was performed on these OTUs from the phylum to the genus level. In order to reduce the dataset and to prioritize more common organisms, rare taxa were not investigated. Taxa that were included were present in at least one sample at a relative abundance greater or equal to 1% of the total OTUs. All less abundant microbes were combined into the category "Minor" phyla, class, etc.

Two tailed unpaired t-tests of the most abundant phyla (assuming unequal variance in order to be conservative) were used to identify any significant differences at the phylum level with respect to the microbial populations present in the samples collected from the glycerin fed plant (LANL SWWS) and the Other WWTPs. Those phyla that were found to be significantly different ($p<0.05$) between the two treatments were further analyzed at the family level and significant differences were tested as well. The Shannon index for diversity was used to determine the diversity within each group of samples, (the LANL SWWS samples and Other WWTPs). To demonstrate dissimilarities between samples, Principal Coordinate Analysis (PCoA) was implemented on the OTU table of all twenty two samples (specifying whether the sample was glycerin fed or conventionally fed) to examine possible patterns across the microbial communities (see Section 4.4 for details).

5.3 Results and Discussions

The raw sequences resulting from the pyrosequencing, as provided by RTL, totaled 175,426 unique sequences. After denoising them with AmpliconNoise, 104,046 effective sequences with a maximum length of 400 base pairs remained, 58% of the original raw sequences. These sequences were clustered, assigned OTUs and taxonomic classification was performed as described in Section 4.6. Eighteen unique phyla occurred in at least one sample at greater than 1% abundance, and all others were grouped into a "Minor phyla" category (Figure 5.1). The taxa labelled as "Unassigned" denoted a group of 2248 OTUs that could not be confidently assigned with the Greengenes database and were not considered in this study. It should be noted however, that the LANL SWWS samples contained a larger relative abundance of this category compared to the other WWTP

samples however investigation into the identity of each OTU was beyond the scope of this thesis.

5.3.1 Taxonomic summary

Of the eighteen unique phyla, nine classifiable phyla were found in every sample, albeit at varying distributions (Figure 5.1). The figure depicts the relative abundance of classified bacteria, where every identified OTU was grouped into a taxon and represented in the graph as a percentage of the total number of phyla identified within each sample. The nine phyla observed in every sample were *Proteobacteria*, *Chloroflexi*, *Firmicutes*, *Bacteroidetes*, *Planctomycetes*, *Acidobacteria*, *Nitrospirae*, and *TM7* and have been commonly found in previous research on microbial populations in activated sludge (Chen, Xu et al. 2004; Yang, Zhang et al. 2011; Zhang, Shao et al. 2012; Ibarbalz, Figuerola et al. 2013; Mielczarek, Saunders et al. 2013; Winkler, Kleerebezem et al. 2013). The eighteen unique phyla were found in all of the other WWTP samples with the exception of the LAC.3.30.12 sample from the Los Alamos County WWTP. This sample had no occurrence of *Chlorobi*, *GN02*, *Armatimonadetes*, *Spirochaetes*, *Elusimicrobia*, or *Verrucomicrobia*. Excluding the "Unassigned" phyla, *Proteobacteria* were the most abundant in the other plants whereas *Chloroflexi* were the most abundant phyla in the LANL SWWS samples. These two phyla are discussed in detail in the following sections.

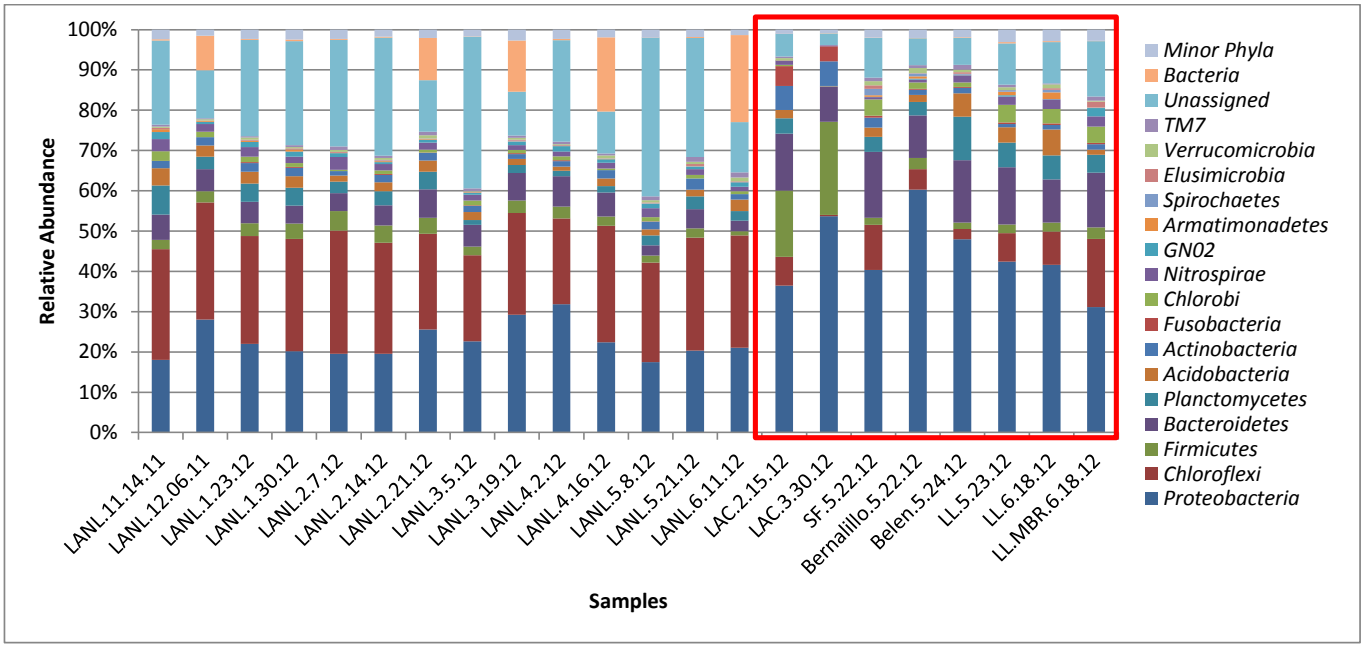

Figure 5.1: Taxonomic summary of all 22 samples at the Phylum level. LANL SWWS samples are on the left and all conventionally fed, other WWTP samples are on the right boxed in red

5.3.1.1: Chloroflexi

Chloroflexi is a phylum commonly found in suspended culture wastewater treatment plants. Groups within this phylum have been proven capable of degrading carbohydrates (Yamada, Sekiguchi et al. 2005; Kragelund, Levantesi et al. 2007) and have therefore been deemed beneficial to mitigating membrane fouling in membrane bioreactors (MBRs) (Miura, Watanabe et al. 2007). Filamentous *Chloroflexi* have been identified in activated sludge which performed nitrification (Bjornsson, Hugenholtz et al. 2002; Kragelund, Levantesi et al. 2007) and have been associated with sludge bulking (Bjornsson, Hugenholtz et al. 2002). 454-pyrosequencing studies have found them to be present in several different treatment configurations from anaerobic/anoxic/oxic (A/A/O) reactors, to MBRs and oxidation ditches (Yang, Zhang et al. 2011; Hu, Wang et al. 2012; Zhang, Shao et al. 2012), yet *Proteobacteria* were observed dominant over *Chloroflexi* in these studies. *Chloroflexi* abundance was high (26% relative abundance) in MBRs with high mixed liquor suspended solids (MLSS = 10000 mg/L), lengthy solids residence times (SRT = 49 and 95 days), and high soluble microbial products (SMP) due to cell decay, therefore it was suggested that *Chloroflexi* prefer high MLSS, long SRTs and may degrade SMPs (Miura, Hiraiwa et al. 2007; Miura, Watanabe et al. 2007), however *Proteobacteria* still dominated community composition over *Chloroflexi* in the researched study.

Chloroflexi were more prevalent in the LANL SWWS samples than in any of the conventional treatment samples with a relative abundance ranging from 21.3% to 30.6%. Contrary to the previously cited research, *Chloroflexi* generally dominated these samples over *Proteobacteria*. On average, LANL SWWS operated under and SRT over 70 days

(detailed discussion of the SRT is presented in Section 7.3.3). LANL SWWS also successfully nitrogen removal via microbial nitrification/denitrification as indicated by very low nitrogen effluent concentrations (typical effluent NO_3 < 1mg/L and NH_3 < 0.1mg/L), (detailed description of the nitrification/denitrification are provided in Section 4.1 and daily values can be found in Appendix B), and a high relative abundance of *Chloroflexi* was expected. Of the other conventionally fed plant samples, the Los Lunas MBR (LL.MBR.6.18.12) had the most *Chloroflexi* (17.0%). The Los Lunas systems has parallel biological treatment systems (an MBR and an anoxic/oxic (A/O) process), which allowed assessment of the effect of process configuration independent of influent quality. The LL.6.18.12 sample which was taken from the aeration basin of the parallel A/O process, had only 8.3% Chloroflexi, which was less than half that in the MBR, suggesting that the MBR configuration tended to increase the Chloroflexi content. These findings suggest that *Chloroflexi* may be enriched in systems with longer SRTs as in the LANL SWWS (SRT ~ 70 d) and the Los Lunas MBR (SRT > 25 days). It may also be inferred that the elevated *Chloroflexi* populations in the LANL SWWS may be related to this phyla's preference for simple carbohydrates such as glycerin, but more research on the metabolic function of this microbe is needed to confirm this possibility.

To further analyze the most abundant phylum in the LANL SWWS samples, the composition of *Chloroflexi* was examined at the class level. The majority of *Chloroflexi* in most samples belonged to the class *Anaerolineae* (Figure 5.2),and have been identified as the most abundant class within *Chloroflexi* found in activated sludge (Yoon, Park et al. 2010). This class was analyzed to the order level in figure 5.3 which illustrated dissimilarities between the LANL SWWS samples and the conventionally fed plant

samples within the class *Anaerolineae*. Specifically, order *DRC31* was more abundant in the LANL SWWS samples, as well as the Los Lunas MBR sample (LL.MBR.6.18.12). Like most of the identified groups, the specific functions of order DRC31 are not fully known and further research is necessary to elucidate this phenomenon.

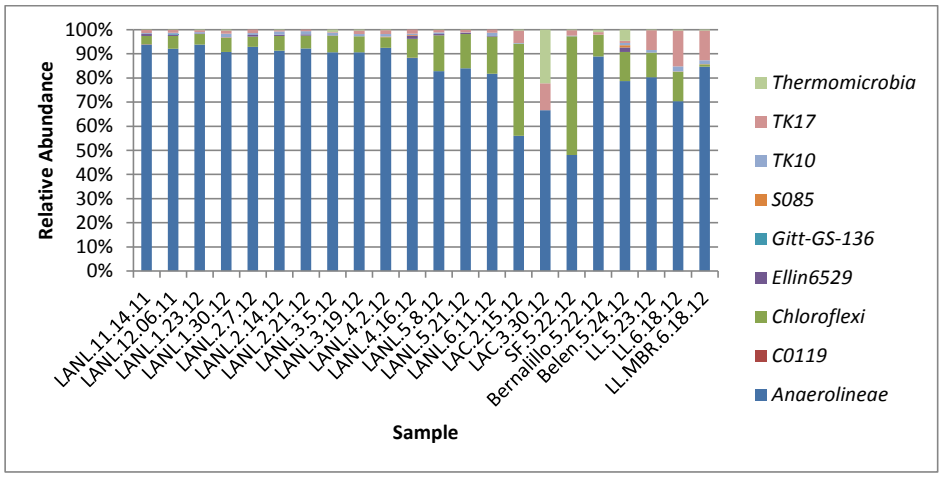

Figure 5.2: The relative abundance of the classes within Chloroflexi. The abundance is shown as the percent contribution to the phyla Chloroflexi

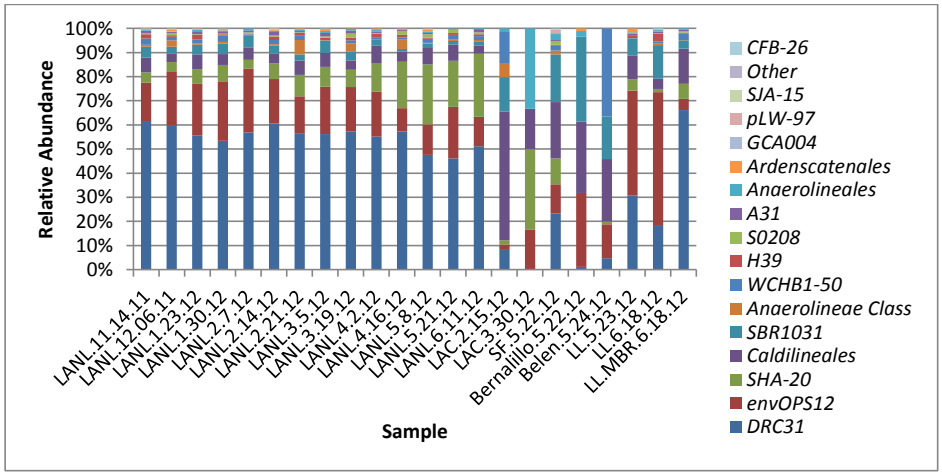

Figure 5.3: The relative abundance of the orders within the class of Anaerolineae, phylum Chloroflexi. The abundance is shown as the percent contribution to the class Anaerolineae

The phylum *Chloroflexi* has been extensively researched. It has been proposed that groups within this phylum are capable of degrading carbohydrates such as glucose (Miura, Watanabe et al. 2007). Both glycerin and glucose are easily biodegradable, although their chemical structures are quite different, and so a competitive advantage of some Chloroflexi when growing on readily biodegradable compounds could explain the large Chloroflexi populations in the LANL system samples. Yet as a diverse phyla and slow growing microbe (Miura, Watanabe et al. 2007),consensus on their phylogenetic development remains unclear and further research is needed to elucidate functionality and metabolic activity at deeper taxonomic levels.

5.3.1.2: Proteobacteria

Proteobacteria is one of the most common and studied phyla of bacteria with over 2000 recognized species and it is no surprise that they are dominant in many wastewater

treatment systems. *Proteobacteria* had the largest relative abundance with an average of 30.5% of all OTUs across all 22 samples, ranging from a maximum of 60.3% (Bernalillo.5.23.12) to a minimum of 17.4% (LANL.5.8.12) (Figure 5.1). The *Proteobacteria* were generally more abundant in the other plants without carbon addition than in the LANL samples.

Other studies have reported that *Proteobacteria* were the most prolific microbe in wastewater treatment systems and discuss them at the class level (Sanapareddy, Hamp et al. 2009; Hu, Wang et al. 2012; Ma, Wang et al. 2013; Ye and Zhang 2013). The distribution of the classes within the phyla *Proteobacteria* expressed as percent contribution to that phylum is illustrated in Figure 5.3. The most dominant class of *Proteobacteria* in each of the 22 samples was *Betaproteobacteria* with an average of 14.7% relative abundance, occurring with a maximum of 32.8% in LAC.3.30.12 and a minimum 7.4% in the LANL.11.14.11 sample. *Alphaproteobacteria* was the next most abundant in most samples followed by *Gamma*, *Delta* and finally *Epsilon*. This distribution was consistent with the literature (Yang, Zhang et al. 2011; Hu, Wang et al. 2012).

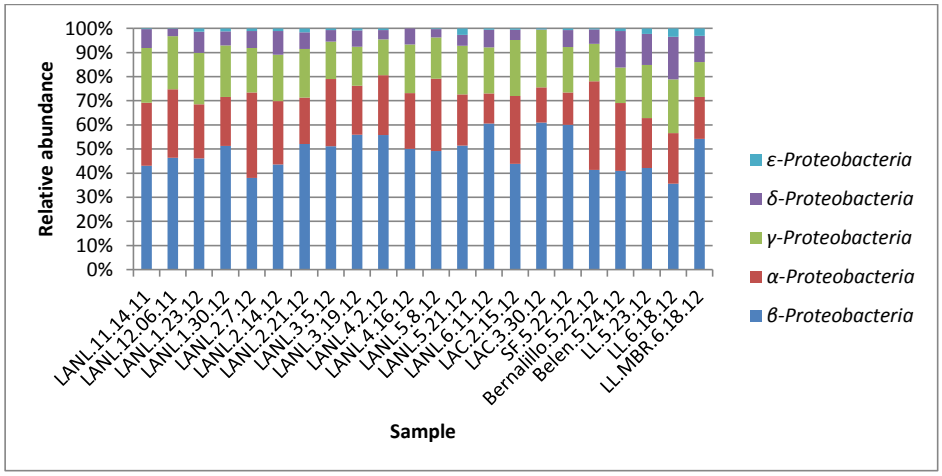

Figure 5.4: The relative abundance of the classes within the phylum Proteobacteria. The abundance is shown as the percent contribution to the phylum Proteobacteria

Betaproteobacteria were the most prevalent *Proteobacteria* in all samples and, as seen in figure 5.4, the LAC.3.30.12 sample's *Betaproteobacteria* population was dominated by the order *Burkholderiales*. This order was generally dominant in all other samples as well followed by *Rhodocyclales*. Other studies of full-scale MBRs have also found that within the class of *Betaproteobacteria*, *Burkholderiales* and *Rhodocyclales* were the dominant *Betaproteobacteria* (Hu, Wang et al. 2012).

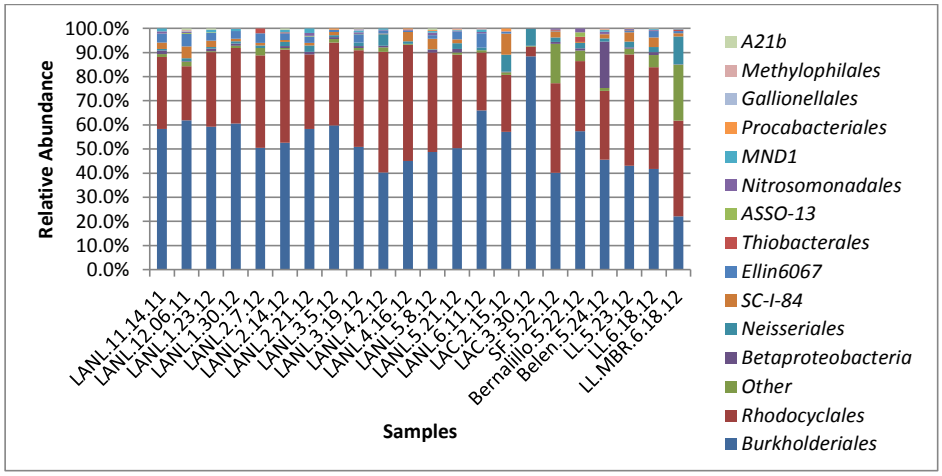

Figure 5.5: The relative abundance of the orders within the class of Betaproteobacteria. The abundance is shown as the percent contribution to the class Betaproteobacteria

Figure 5.6 depicts the *Alphaproteobacteria* distribution. *Rhizobiales* had the largest contribution to the *Alphaproteobacteria* population, and this is in agreement with the distribution of studied MBRs (Hu, Wang et al. 2012).

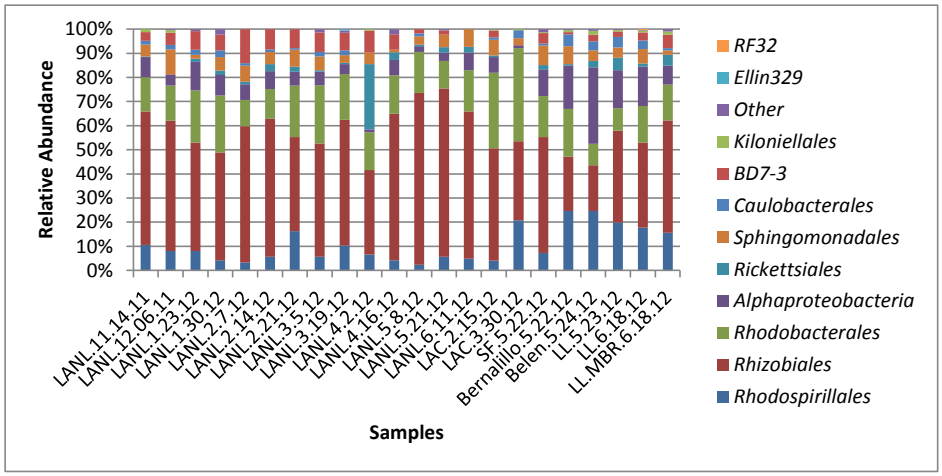

Figure 5.6: The relative abundance of the orders within the class of Alphaproteobacteria. The abundance is shown as the percent contribution to the class Alphaproteobacteria

From figure 5.4, the sample LAC.3.30.12, had a very small *Deltaproteobacteria* contribution to the phyla *Proteobacteria*. Figure 5.7 illustrates the variation in *Deltaproteobacteria* abundance between each sample, and the LAC.3.30.12 sample was populated by only two orders of *Deltaproteobacteria*. *Myxococcales* generally the dominant δ-*Proteobacteria*, as reported in other studies (Zhang, Shao et al. 2012).

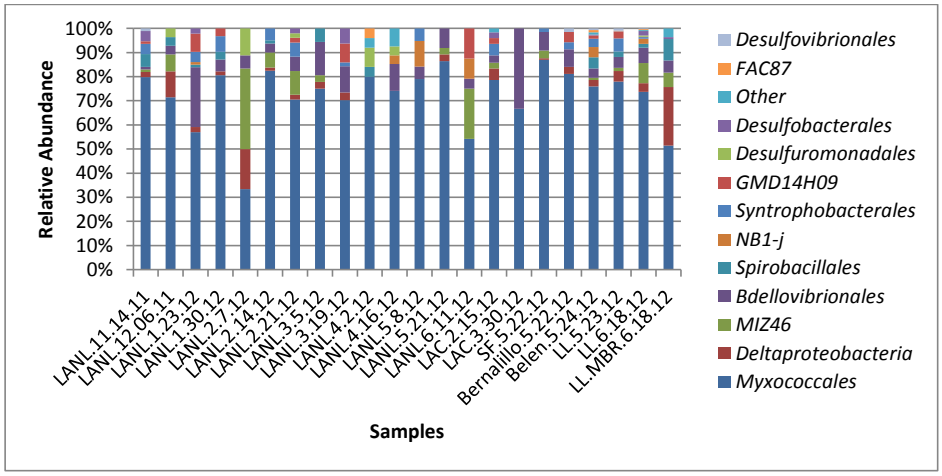

Figure 5.7: The relative abundance of the orders within the class of Deltaproteobacteria. The abundance is shown as the percent contribution to the class Deltaproteobacteria

Xanthomonadales were the dominant *Gammaproteobacteria* in nearly every MBR from the research(Hu, Wang et al. 2012), and this order also largely contributed to the *Gammaproteobacteria* population in this study as well (Figure 5.8), however it was also prevalent in non MBR samples such as the Santa Fe mixed liquor solids sample. *Pseudomonadales* was the main contributor in both Los Alamos County samples (LAC2.15.12 and LAC.3.30.12). *Thiotrichales* was more abundant in all of the LANL SWWS samples over the other WWTP samples, and this order of *Gammaproteobacteria* was not present in the literature (Hu, Wang et al. 2012)

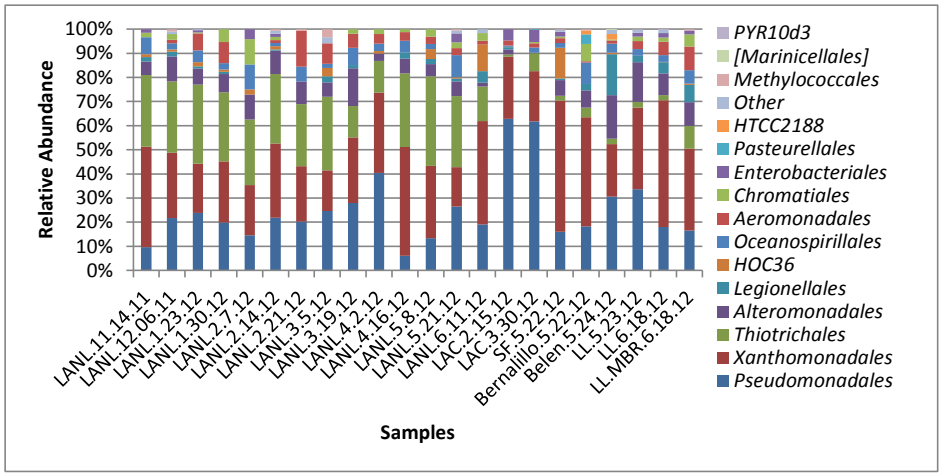

Figure 5.8: The relative abundance of the orders within the class of Gammaproteobacteria. The abundance is shown as the percent contribution to the class Gammaproteobacteria

The *Epsilonproteobacteria* occurred at very low levels (typically less than 1% of the total OTUs) in all samples. This is a common occurrence in the microbial populations of activated sludge (Hu, Wang et al. 2012; Wang, Hu et al. 2012; Zhang, Shao et al. 2012).

5.3.1.3: Other Phyla of Interest

Bacteroidetes was another phylum common to all samples, with a maximum relative abundance of 16.4% (SF.5.22.12) to a minimum of 2.6% (LANL.6.11.12). They were generally more abundant at LANL than in the other treatment plants compared to LANL SWWS (Figure 5.1). These microbes have been associated with sludge bulking (Kragelund, Levantesi et al. 2008) and have typically been reported as the second most abundant phyla behind *Proteobacteria* in most of the literature (Hu, Wang et al. 2012; Zhang, Shao et al. 2012; Ma, Wang et al. 2013).

Both of the Los Alamos County (LAC) samples had more *Firmicutes* than any other sample at 16.4% and 23.1% relative abundance (Figure 5.1). This phylum is common in wastewater under variable operational conditions (Yang, Zhang et al. 2011; Hu, Wang et al. 2012; Zhang, Shao et al. 2012) yet more information on the LAC WWTP operations is needed to explain their elevated presence.

The "Unassigned" phylum indicates the OTUs for this taxonomy were not assignable, and the taxonomic resolution could not be determined. There were 2248 OTUs in this category. The "Unassigned" bacterium was present at much higher quantities in the LANL SWWS samples than that of the conventional plants, with the caveat that this assignment comprised more than one OTU and possibly multiple taxa. Yet this abundance of "Unassigned" bacteria could indicate that the LANL SWWS samples have more lineages that have yet to be formally identified. Of the 9579 OTUs, there were 2248 OTUs that were designated as "Unassigned" and formidable research would need to be performed on these sequences in order to elucidate their identities.

The taxonomic summary of the 22 samples acquired from various activated sludge demonstrates a clear difference between the microbial population utilizing glycerin as a supplemental carbon source and those that do not. The three phyla that were consistently dominant in the LANL SWWS samples were *Proteobacteria*, "Unassigned" and *Chloroflexi*. The relative abundance of each of these phyla varied from one sample to the other, however generally the LANL SWWS samples had a similar microbial distribution with *Chloroflexi* as the dominant phyla. The plants without carbon addition have a dissimilar microbial community compared to LANL SWWS in that *Proteobacteria* was

dominant. Further statistical analysis and diversity measurements were performed to confirm the dissimilarities between the samples.

5.3.2 Two tailed Un-paired T-test

Two tailed un-paired t-test with unequal variance were performed on the phyla occurring in at least one of the 22 samples, at greater than 1% relative abundance. The 14 samples from LANL SWWS were designated as one group (Glycerin fed plant) and the 8 samples from the other conventional treatment plants as a second group (Other plants). Table 5.1 lists the phyla and whether there was significant difference between each phylum present in the glycerin fed plant versus the conventionally fed plants. The phyla considered were only those present in at least one sample at greater than 1% abundance. Those that yielded statistically different values between the two data sets ($p < 0.05$) are shown in bold font.

Table 5.1: Results of unpaired t-test assuming unequal variance on the most abundant phyla

	Mean relative abundance		
Phylum	**Glycerin fed plant**	**Other plants**	**p-value**
Proteobacteria	22.70%	44.23%	**1.79E-04**
Chloroflexi	26.47%	7.35%	**4.69E-06**
Firmicutes	2.90%	6.58%	2.53E-01
Bacteroidetes	5.28%	13.00%	**1.94E-05**
Planctomycetes	3.14%	4.78%	9.76E-02
Acidobacteria	2.29%	2.92%	2.33E-01
Actinobacteria	1.78%	2.55%	1.83E-01
Fusobacteria	0.10%	1.35%	5.16E-02
Chlorobi	1.00%	2.40%	6.39E-02
Nitrospirae	1.81%	1.38%	2.73E-01
GN02	0.97%	0.36%	**4.47E-02**
Armatimonadetes	0.19%	0.48%	2.16E-01
Spirochaetes	0.05%	0.45%	8.17E-02
Elusimicrobia	0.20%	0.40%	3.04E-01
Verrucomicrobia	0.50%	0.68%	2.73E-01
TM7	0.68%	0.68%	9.71E-01
Unassigned	22.62%	8.24%	**9.27E-05**

Proteobacteria, *Chloroflexi*, *Bacteroidetes*, *GN02*, and "Unassigned" were found to be statistically significantly different between the glycerin fed plant and the other plants. From the taxonomic summary in the previous section, *Chloroflexi*, and "Unassigned" were found to be more abundant in the LANL plant than the other plants, and the t-test analyses confirmed those observations. *GN02* was also statistically more prevalent in the LANL SWWS samples; however at a low relative abundance (less than 1% average over all 14 LANL SWWS samples) it was unlikely that these bacteria had a large impact on plant function. *Proteobacteria* and *Bacteroidetes* were more abundant in the conventionally fed treatment plants. The other phyla exhibited no significant difference between each variable. Based on these results, it is possible that glycerin as a supplemental carbon source may have had an effect on the distribution of microbial

communities within activated sludge, however further investigation into the effects of plant parameters and wastewater characteristics to confirm this speculation

5.3.3 Alpha Diversity

In order to describe the diversity within each sample and to evaluate further whether the microbial population of activated sludge utilizing glycerin as a supplemental carbon source was dissimilar from those using conventional substrate, the Shannon's index of diversity was used (see Section 4.8 for a detailed description of the analysis). This index accounts for both richness and evenness of the microbes present in each sample and a higher value represents a higher diversity. In order to avoid any diversity inflation errors due to sequencing depth, the samples were rarefied or normalized to 1340 random sequences per sample because the least amount of sequences in any sample was 1343. The results of this analysis are plotted below in the rarefaction curve (Figure 5.9). It was confirmed from this curve that the samples from the other treatment plants (red boxes and line) were more diverse than the LANL SWWS samples because they plot above the LANL SWWS (blue diamonds and line).

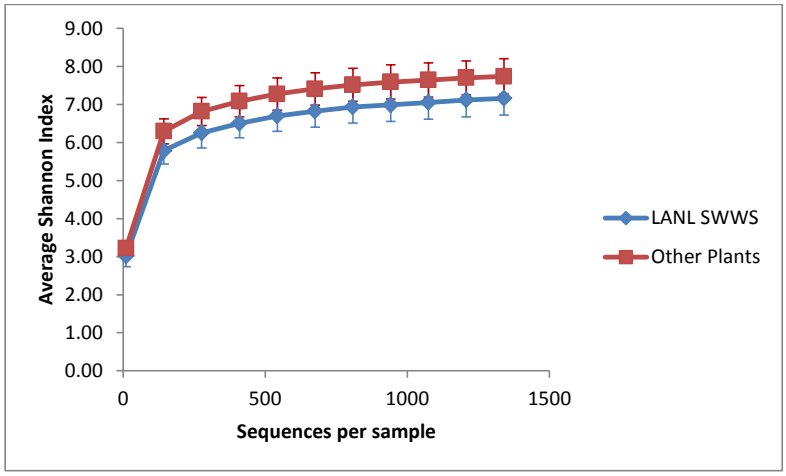

Figure 5.9: Rarefaction curve of average Shannon Diversity Index of glycerin fed plant (LANL SWWS in blue) and conventionally fed plants (Other Plants in red)

The actual values from the index are tabulated below to further clarify the differences (Table 5.2). The least diverse sample was LANL.5.8.12 while the sample from the Los Lunas MBR (LL.6.18.12) was the most diverse. Good's coverage ranged from 80% to 96% with an average of 88%.

Table 5.2: Shannon Diversity Index comparison between LANL SWWS and Other Plants

Sample	Shannon Diversity Index	Good's Coverage
LANL.11.14.11	7.52	92%
LANL.12.06.11	7.54	82%
LANL.1.23.12	7.39	90%
LANL.1.30.12	7.37	91%
LANL.2.7.12	7.10	80%
LANL.2.14.12	7.22	87%
LANL.2.21.12	7.66	86%
LANL.3.5.12	6.91	84%
LANL.3.19.12	7.64	83%
LANL.4.2.12	7.43	83%
LANL.4.16.12	6.83	82%
LANL.5.8.12	6.13	87%
LANL.5.21.12	6.99	90%
LANL.6.11.12	6.50	85%
LAC.2.15.12	7.59	95%
LAC.3.30.12	6.75	90%
SF.5.22.12	7.79	91%
Bernalillo.5.22.12	8.08	83%
Belen.5.24.12	7.68	89%
LL.5.23.12	8.11	94%
LL.6.18.12	8.29	96%
LL.MBR.6.18.12	7.48	90%

This diversity metric illustrates that the other plant's microbial populations were more generally diverse than the LANL SWWS populations. The reason for this may be due to the diverse nature of substrates in municipal wastewater in comparison to that treated by the LANL SWWS. The lower BOD_5 concentration of the influent entering the system coupled with the more homogenous substrate such as glycerin may enrich for the growth of certain organisms in the activated sludge and a decreased variation of the microbial community.

5.3.4 Beta Diversity

Beta diversity can be used to evaluate differences between communities. A matrix of the distance between each pair of samples is generated. Principal coordinate analysis (PCoA) is a technique for visualizing these distances. It allows for the samples to be grouped based on their phylogenetic differences. It should be noted that with this method the axes have no meaning, this technique is a graphical tool for visualizing the distance matrix that compares each pair of OTUs (Section 4.8 provides more detail). As in the previous diversity analysis, the samples were rarefied to the lowest sequencing depth. The weighted, discrete PCoA (Figures 5.10) depict a clear clustering between the two types of WWTPs given the different carbon source. There was a distinct dichotomy between the sample groups along principal coordinate one (PC1), with 39.03% of the variation between the samples explained by PC1. 17.83% of the sample variability was explained by PC2. The LANL SWWS (green triangles) samples clustered closely together along both PC1 and PC2, suggesting they were more similar to each other rather than samples from the other conventionally fed WWTPs. The other samples grouped closely along PC1, and there was no clear clustering along PC2. There was also a general separation among the other treatment plants themselves and a slight shift of the Los Lunas MBR (yellow square) towards the LANL SWWS samples along PC1 which may have been related to the relatively long SRT of this MBR system as previously described in section 5.3.

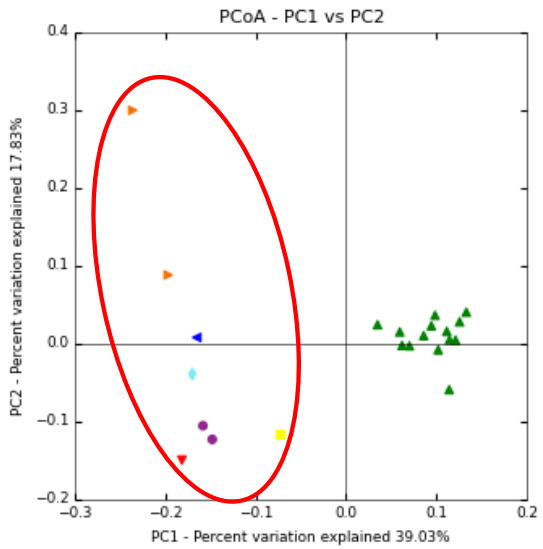

Figure 5.10: PCoA of LANL SWWS samples (green triangles) and other conventionally fed plants (circled in red). Color coded by plant location (2 orange triangles are LAC, 2 purple circles are Los Lunas, cerulean diamond is SF, red triangle is Belen, yellow square is Los Lunas MBR and blue triangle is Bernalillo)

Analysis of only the other samples illustrated a clustering of the Los Lunas samples (purple circles and yellow square) cluster together along PC2 (near -0.1 along the y-axis), with a divergence along PC1 towards the LANL SWWS samples by the LL MBR (yellow square). LAC samples clustered (orange triangles) along PC1 but diverged greatly along PC2. Overall, the other samples clustered closely along PC1 (near -0.2 along the x-axis) with the exception of the LL.MBR sample.

5.4 Conclusions

This chapter addresses the findings of utilizing 454-pyosequencing as a technique for analyzing microbial populations in activated sludge. It investigates the differences in the

distribution and abundance of the microbial populations of LANL SWWS activated sludge and that of other WWTPs. It is apparent that the microbial communities of LANL SWWS and other conventionally fed treatment plants were different based on observation of the taxonomic summary, two-sample un-paired t-tests, and alpha and beta diversity analyses. Glycerin as a supplemental carbon source may be a contributing factor to the community dissimilarities, however, other factors such as unique influent characteristics and solids residence time may also influence the biodiversity and further research is needed. The LANL SWWS samples were less diverse than conventionally fed treatment plants and differed phylogenetically from these plants as well. Glycerin as a supplemental carbon source may have altered the microbial population of the activated sludge in LANL SWWS, but unfortunately cannot be definitively confirmed because no samples were taken from LANL SWWS prior to the glycerin addition.

Due to the complex ecological nature of the microbial population within this environment, other contributions besides glycerin could have influenced the activated sludge communities. The possibility of environmental impacts, operating parameters or a combination of these conditions effecting the population is discussed in more detail in Chapter 7 of this work. While every WWTP is unique and comparing the microbial consortia of different plants directly cannot necessarily be generalized, utilizing 454-pyrosequencing as a method to identify, classify and subsequently analyze and compare these communities as a whole can be accomplished.

CHAPTER 6 THE EFFECTS OF A SEEDING EVENT ON THE MICROBIAL COMMUNITY OF LANL SWWS ACTIVATED SLUDGE

6.1 Introduction

During system startup and in response to system upsets wastewater treatment plants may inoculate, or "seed" their activated sludge reactors by importing biomass from another wastewater treatment system or other sources. Adding such biomass can expedite the initial startup process and decrease the acclimation time for microbial cultures (Dabert, Delgenès et al. 2005; Bartrolí, Carrera et al. 2011). In order to meet new or more stringent regulatory requirements or treat a certain constituent, a WWTP may need to alter or manipulate the microbial population within the system by seeding (Stephenson and Stephenson 1992). This process is also utilized in the event of a system failure or inefficiency. Seeding can be accomplished by the addition of customized bacterial cultures (bioaugmentation), or a sludge culture from other facilities. The survival rate and adaptation of these novel microbes within a system has been studied, yet most research has focused on seeding with bioaugmented biota (Bokhamy, Deront et al. 1997; Boon, Goris et al. 2000; Boon, Top et al. 2003). The effects of seeding activated sludge on the original populations have not been studied extensively due to the complex and dynamic nature of the wastewater environment, and the previous unavailability of suitable methods for microbial population characterization. With the advent of high throughput nucleic acid sequencing, the study of microbial population changes over time has become feasible.

In February 2012, LANL SWWS experienced an upset due to elevated levels of tardigrades, commonly referred to as water bears (Figure 6.1). Tardigrades exist in most

wastewaters, yet as predators of bacteria responsible for removal of wastewater contaminants, these eukaryotic organisms can degrade plant performance when present in very large numbers by reducing bacterial concentrations. Tardigrades are polyextremophiles, meaning they can survive under many different types of stress, including low temperatures and pH, high and low pressures, and anoxic conditions. They produce a sort of cocoon called a tun, made from sugars, and under extreme conditions they can revert to this dormant phase and remain viable for years (Miller 2011). Like many other higher organisms, tardigrades are slow growing relative to bacteria, and so they are often found in wastewater plants with long SRTs. In one laboratory-scale study of activated sludge reactors that also included a biofilm phase, tardigrades were present at 10 day and 30 day SRTs, but their quantity greatly increased at an SRT of 75 days (Moghaddam, Guan et al. 2003). This may be why they are often detected in nitrifying systems as well, which tend to have relatively long SRTs (Chen, Xu et al. 2004).

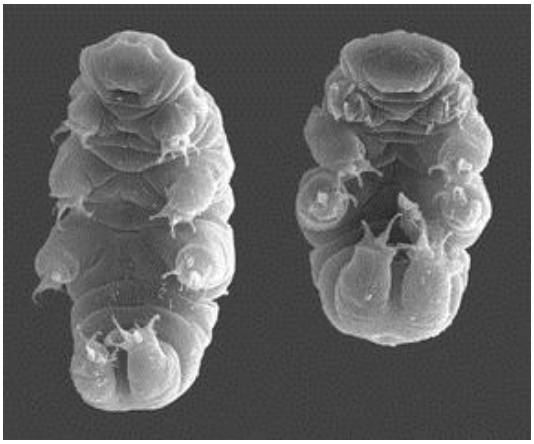

Figure 6.1: Two individual adult Tardigrades, Hypsibius dujardini species imaged by scanning electron microscopy (Gabriel, McNuff et al. 2007)

The cause of the preponderance of these organisms was unclear to plant personnel and they began purging small volumes of activated sludge and replacing it with sludge from a similar system, the nearby Los Alamos County Wastewater Treatment Plant in Pueblo Canyon (LAC WWTP). This seeding event occurred four times over the course of this project (2/15/2012, 2/17/2012, 2/25/2012 and 3/5/2012) and offered the opportunity to study the effects of seeding a full-scale wastewater treatment plant through 454-pyrosequencing.

It was hypothesized that the seeding event would alter the microbial community of the LANL SWWS activated-sludge, and that the consortia would eventually revert to pre-seed conditions once the inoculation ceased. The intentions of this research were to

1) classify the most abundant consortia within LANL SWWS activated sludge and the LAC WWTP inoculum,

2) compare the microbial communities throughout the pre-seed, during inoculation, and post-seed conditions of LANL SWWS activated sludge,

3) identify any adaptive or divergent patterns during the seed event.

Activated sludge samples collected over time were pyrosequenced, analyzed and compared in order to accomplish these goals.

6.2 Methods and Materials

6.2.1 Sample collection, DNA extraction and Pyrosequencing

Samples from the LANL SWWS activated sludge system were collected to examine the microbial populations. Samples were collected from the LANL SWWS southeast aeration basin (the middle basin in the aeration basin treatment train), at approximately 2 pm each collection day from November 14, 2011 through June 11, 2012. The mixed liquor was stored as described in Chapter 4, section 4.1 and DNA extraction was performed via the CTAB S method on all samples as described in section 4.2. The inoculation sludge (in the form of RAS) was provided by LAC WWTP, a nearby anoxic/oxic treatment plant with a design capacity of 1.4 MGD, and an SRT ranging 8-18 days. Various volumes (Table 6.1) were added to the LANL SWWS reactor (reactor volume = 750,000 gal or 2839 m^3) until the tardigrade population diminished.

Table 6.1: Los Alamos County biomass inoculation dose

Dates of dose	Volume of inoculate added (gal)	Volume of inoculate added (m3)	% of LANL SWWS aeration basin (v/v)
2/15/2012	6000	22.71	0.8%
2/17/2012	9000	34.065	1.2%
2/25/2012	21000	79.485	2.8%
3/5/2012	4500	17.0325	0.6%

Comment [BMT4]: Do you know the MLSS concentration or any other information about the sludge samples?

Can you estimate the fraction of total biomass in the system that was due to seed?

Two samples of the mixed liquor from LAC WWTP (LAC.2.15.12 and LAC.3.30.12) were collected by LANL plant personnel and were processed in the same way as the LANL SWWS samples. A total of 16 samples (14 from the LANL system and 2 samples from LAC WWTP) were collected and sent to Research and Testing Laboratory (RTL) in Lubbock, Texas for 454-pyrosequencing, as discussed in Section 4.3.

6.2.2 Analytical Methods

The pyrosequencing results were denoised, classified, and analyzed using QIIME 1.80 software as described in Section 4.4. The taxonomic analysis was performed from the phylum to the genus level, and the operational taxonomic units (OTUs) present in at least one sample at greater than 1% abundance were used for comparison. Less common OTUs were omitted from further analysis to reduce the number of taxa in the analysis. Statistical analysis was performed at the phylum level of classification to determine if there was a significant difference in microbial populations. Two-tailed t-tests assuming unequal variance were performed comparing the populations between the pre-seed and post-seed populations as fractions of the total populations (Table 6.2). Statistical significance was not evaluated for comparisons with the "during seeding" samples or the LAC samples as there were only two samples taken for each of those categories.

Table 6.2: List of samples pyrosequenced according to seed event

Pre-seed	During seed	Post-seed	LAC Inoculum	LAC non-inoculum
LANL.11.14.11	LANL.2.21.12	LANL.3.19.12	LAC.2.15.12	LAC.3.30.12
LANL.12.06.11	LANL.3.5.12	LANL.4.2.12		
LANL.1.23.12		LANL.4.16.12		
LANL.1.30.12		LANL.5.8.12		
LANL.2.7.12		LANL.5.21.12		
LANL.2.14.12		LANL.6.11.12		

Alpha and beta diversity analysis (as specified in Section 4.6), including rarefaction curves, the Shannon Index, Principal Component Analysis (PCA), and Principal Coordinate Analysis (PCoA) were used to further evaluate the community differences around the seed event.

6.3 Results and Discussion

6.3.1 Taxonomic Summary

The taxonomic summary of the sixteen samples at the phylum taxa level is shown in Figure 6.2. The LAC WWTP samples are the columns furthest to the right and the LANL SWWS are the boxed in samples on the left. When considering only the samples taken from LANL SWWS and LAC WWTP, fourteen unique phyla (excluding minor phyla) at the greater than 1% abundance cutoff were studied. It is important to note, that of the two samples from LAC WWTP, only LAC.2.15.12 (indicated by the red arrow below) was used during the seed event. LAC.3.30.12 served only as a comparison sample.

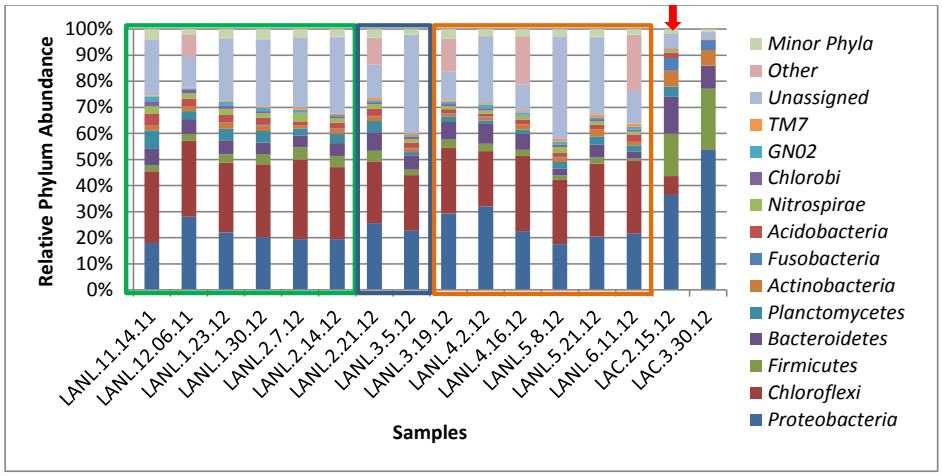

Figure 6.2: The taxonomic summary of the 1% most abundant phyla identified from LANL SWWS samples (14 on the right, green box is pre-seed, blue box is during seed, and orange box is post-seed) and LAC WWTP samples (2 left most columns). Red arrow indicates sample that was used for inoculation (LAC2.15.12).

Figure 6.2 shows that the microbial communities in the LANL and LAC wastewater treatment plants were dissimilar. *Chloroflexi* was more abundant in the LANL SWWS, as discussed in Chapter 5.3. *Proteobacteria* and *Firmicutes* were more prevalent in the LAC samples than in the LANL samples. There were large variations between the two samples LAC WWTP samples, (taken six weeks apart). *Proteobacteria* and *Firmicutes* were more common in the LAC.3.30.12 sample than in the LAC.2.15.12 sample. *Bacteriodetes, Planctomycetes* and *Chloroflexi* were more abundant in the LAC.2.15.12 sample than in the LAC.3.30.12 sample. The plant operated under the same conditions over this time period, and so possible reasons for the variation in microbial populations at the LAC plant between 2/15 and 3/30 are not known.

The phyla *Proteobacteria* was found to be abundant and was further divided to the class levels (*Alphaproteobacteria, Betaproteobacteria, Deltaproteobacteria, Gammaproteobacteria,* and *Epsilonproteobacteria).* Figure 6.3 illustrated that the most abundant class relative to the *Proteobacteria* within all samples was *Betaproteobacteria*.

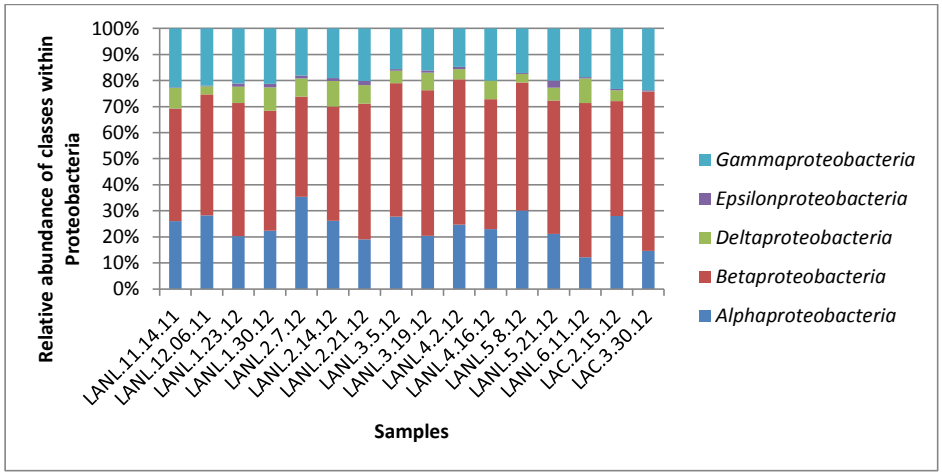

Figure 6.3: Relative abundance of the classes within phylum Proteobacteria found in LANL SWWS samples (14 on the right) and LAC samples (2 on the left).

6.3.2 Significance tests

In order to determine if the samples were significantly different from one another, un-paired t-tests, (assuming unequal variance) were performed on the LANL SWWS phyla. It was hypothesized that those phyla found in greater abundance in the LAC.2.15.12 sample would have an effect on the microbial populations of the LANL SWWS samples. Unfortunately, only two samples were taken from the LANL SWWS aeration basin during the seed event and could not be reliably statistically compared to samples taken pre or post-seed. The samples occurring within pre-seed and post-seed condition sample groups (Table 6.2) were the two groups that were analyzed with the un-paired t-test. From figure 6.2 (excluding the Unassigned phylum), *Chloroflexi* was more abundant in the LANL samples than the LAC samples, while *Firmicutes* and *Proteobacteria* were more abundant in the LAC.2.15.12 sample than in any of the LANL SWWS samples. It

was expected that these three phyla would be among those found to be significantly different between the pre-seed and post-seed samples. *Firmicutes* and *Planctomycetes* (highlighted in bold) were the only two phyla found to be significantly different ($p<0.05$) between these groups. Table 6.3 lists the mean relative abundance and p-values of the abundant phyla. The mean relative abundance demonstrated that *Firmicutes* and *Planctomycetes* decreased from pre to post-seed conditions. *Proteobacteria*, and *Chloroflexi*, were not significantly dissimilar before and after the seed event.

Table 6.3: Significantly different phyla comparing pre-seed and post-seed conditions

	Mean relative abundance		
Phylum	**Pre-seed**	**Post-seed**	**p-value**
Proteobacteria	21.9%	22.8%	0.77
Chloroflexi	28.4%	26.2%	0.20
Firmicutes	**3.7%**	**2.0%**	**0.01**
Bacteroidetes	5.0%	4.8%	0.85
Planctomycetes	**4.2%**	**2.2%**	**0.03**
Actinobacteria	1.9%	1.9%	0.95
Fusobacteria	0.1%	0.1%	0.35
Acidobacteria	2.5%	1.8%	0.13
Nitrospirae	2.1%	1.5%	0.13
Chlorobi	0.9%	0.8%	0.54
GN02	0.9%	1.0%	0.75
TM7	0.5%	0.9%	0.09

It was expected that *Firmicutes* would be comparatively higher in the during seed samples than in the pre-seed samples, however, as stated previously, inadequate sample depth prevented statistical comparisons. The mean relative abundance of the significantly different phyla was plotted and included the samples taken during the inoculation as well as the inoculum for a general comparison (Figure 6.4). The error bars

are standard deviations and were high in the during seed samples because there were only two samples taken. *Firmicutes* were more abundant in the Inoculum samples, yet the pattern from the LANL SWWS samples demonstrated that the mean relative abundance decreased throughout the inoculation from pre-seed to post-seed. *Planctomycetes* was slightly less abundant in the Inoculum sample than the mean of the pre-seed samples, yet higher than the during and post-seed samples. Still, there was no increase in the LANL samples that would indicate a contribution from the inoculation.

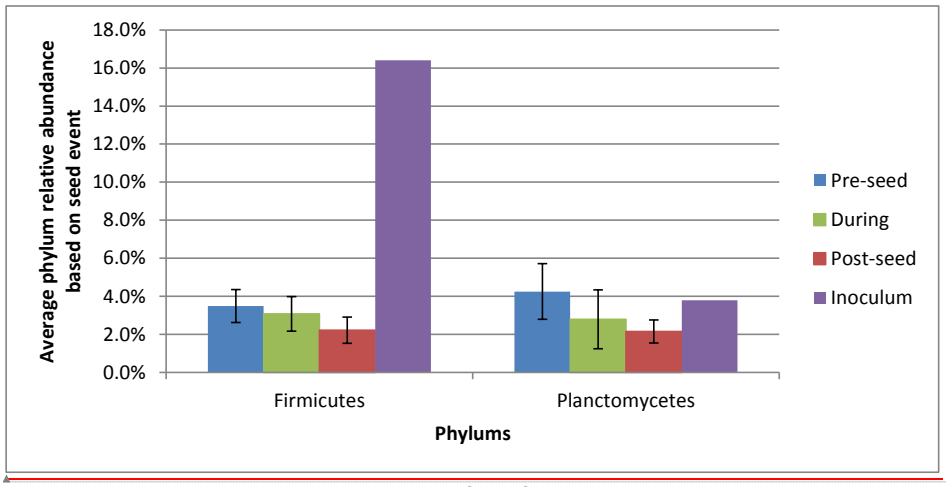

Figure 6.4: Average relative abundance of the four LANL SWWS sample phyla found to be significantly different from pre-seed to post-seed conditions. Error bars are the standard deviation of phyla within each group

The mean relative abundance of all of these groups decreased over the course of the treatment, and the changes per sample were investigated (Figures 6.5 and 6.6). *Firmicutes* (Figure 6.5), revealed an increasing trend (R^2=0.80) in relative abundance prior to the seed event, and decreased post-seed (R^2=0.82). As previously discussed, evidence of the abundance of *Firmicutes* in the LAC.2.15.12 (16.4%) sample was not apparent in the LANL SWWS samples after inoculation. Because the post-seed samples

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have a negative slope, the results show that there was a change in the distribution of this phylum, but it was not a result of the seed event.

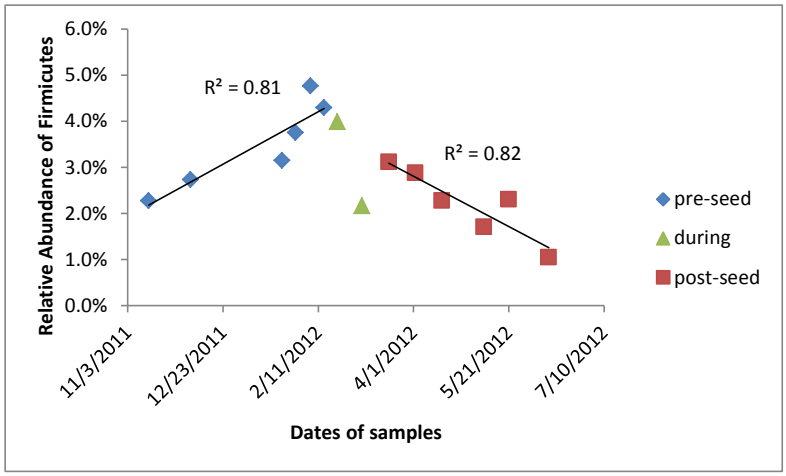

Figure 6.5: Relative abundance of Firmicutes within the LANL SWWS over the course of the seed event

A decreasing trend in the phylum *Planctomycetes* was noticed prior to the seed event, while relative abundance increased post-seed (Figure 6.6). The relative abundance within the inoculum (3.8%) may have contributed to the positive trend after the seed event, but this cannot be confirmed based on the number of samples taken.

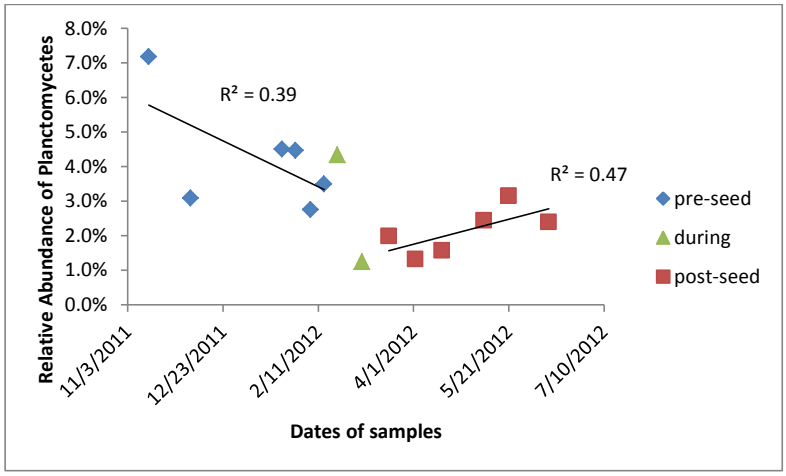

Figure 6.6: Relative abundance of Planctomycetes within the LANL SWWS over the course of the seed event

These results indicated that there were other factors than the simple addition of organisms through seeding that contributed to the shifts in microbial populations. Previous research has suggested that the microbial populations of activated sludge are an interactive ecosystem that may be affected by many changing conditions (Wang, Hu et al. 2012; Zhang, Shao et al. 2012; Ye and Zhang 2013). The method in which LANL SWWS inoculated their system may not result in identifiable changes to the microbial consortia that can be confidently linked to only the seed event.

Previous research has shown that successful bioaugmentation requires an adequate quantity of inoculum (Stephenson and Stephenson 1992) and while plant personnel noticed improvement in operation due to the seed events, it is possible that the inoculation constituted too small a fraction of the total amount of biomass in the activated sludge system to produce a measureable impact on the microbial communities. The inoculations occurred on 2/15, 2/17, 2/25, and 3/5 over the course of this project. The mass fractions of the added biomass (added biomass/system biomass) were calculated to

help evaluate whether the microbial mass added was likely to have affected the total populations. The three aeration basin volume totaled 2839 m^3 (ignoring biomass in the clarifiers), and the average biomass in the LANL SWWS was calculated using the aeration basin volume and the average MLSS concentration for February, 2012.

$$Volume * MLSS = (2.84 * 10^3 m^3) * \left(2.97 * 10^7 \frac{mg}{m^3}\right) = 8.43 * 10^3 kg\ biomass$$

It was assumed that the inoculation doses were from the return activated sludge (RAS) line of LAC WWTP, assuming a conservative RAS recycle ratio of 50%, the following calculation was performed to obtain an average RAS concentration (X_R) with a typical MLSS concentration of 2000 mg/L for the LAC WWTP.

$$X_R = \left(\frac{1.5Q}{0.5Q}\right) * MLSS = \left(\frac{1.5Q}{0.5Q}\right) * \left(2.00 * 10^3 \frac{mg}{L}\right) = 6000\ mg/L$$

The mass of biomass added per dose was then calculated:

$$Volume_{added\ biomass} * X_R = mass\ of\ added\ biomass$$

and as a fraction of the biomass in the system:

$$\left(\frac{biomass\ added\ by\ seeding}{biomass\ in\ the\ system}\right) * 100\% = mass\ fraction\ of\ biomass\ added$$

The results of the calculations are tabulated below (Table 6.4).

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Comment [M5]: Did I get this right?

Comment [BMT6]: Units problem . Q * MLSS gives mass/d.

Table 6.4: Mass fraction of added biomass to LANL SWWS from inoculation

Dates of dose	Volume of inoculate added (m3)	Biomass per dose (kg)	Mass fraction of biomass added
2/15/2012	22.7	136.3	1.6%
2/17/2012	34.1	204.4	2.4%
2/25/2012	79.5	476.9	5.7%
3/5/2012	17.0	102.2	1.2%

While according to plant personnel, the seed event helped to remediate the tardigrade outbreak, the volumes of inoculum were somewhat low compared to other studies which used 6.6% to 37.1% inoculated quantity represented as suspended solids. (Bouchez, Patureau et al. 2000; Bouchez, Patureau et al. 2000). Samples taken directly after the inoculation, along with variable dose frequencies and intensities could offer more insight and warrants future research.

6.3.3 Alpha Diversity Analysis

Comparisons of the microbial population changes over the course of the seed event based on taxonomic relative abundance were inconclusive. Because of the complexity of the activated sludge populations, a better description of the impact of the inoculation may be obtained by investigating changes in diversity. The Shannon diversity index (expanded on in Section 4.8) was used to study the diversity within the LANL samples, as well as the LAC.2.15.12 sample. This diversity index is useful for studying diversity within samples, or groups of samples (pre-seed, during seed, and post-seed). While all of the sample groups were diverse, the inoculum sample was the most diverse, followed by the pre-seed and during seed sample groups, and the post-seed sample group was the least diverse, according to the calculated rarefaction curves using the Shannon diversity index as an indication of richness and evenness of the microbial population (Figure 6.7).

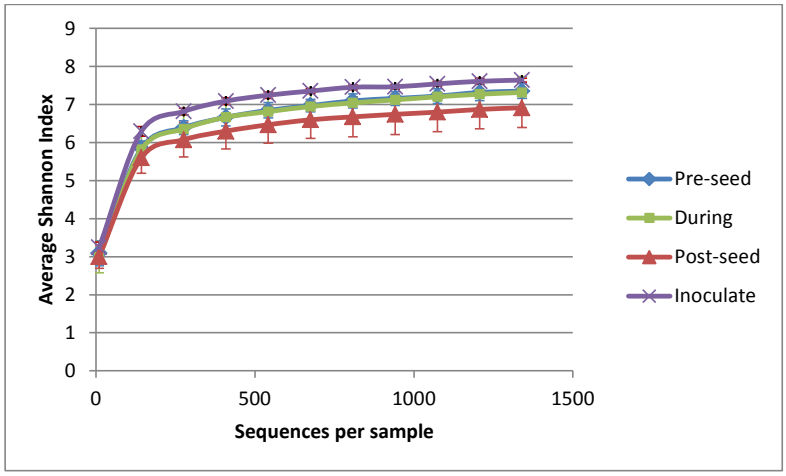


Figure 6.7: Rarefaction curves of LANL SWWS samples, (pre-seed, during and post-seed) and LAC.2.15.12 (Inoculum)

The rarefaction curves shown in Figure 6.5 illustrated that the diversity of the LANL sample microbial populations did not increase after the seed events, although the seeding events added new bacteria to the LANL system.

Comment [AS7]: It would be interesting to look for bugs that were not present in the pre-seed sample but were present in the seed - did any of these show up, even in very small amounts, and the post-seed and during-seed samples?

Comment [M8]: There are 5 phyla that are absent from pre-seed, but occur in in seed, show by % difference? (data in THESIS ch 6 percdiff)

6.3.4 Beta Diversity Analysis

Taxon-based Principal component analysis (PCA) was used to evaluate beta diversity (differences between sample groups) at the phylum level (*Proteobacteria* was analyzed at the class level: Alpha, Beta, Delta, Gamma and Epsilon) A matrix with the phylum relative abundance within each sample and whether the sample categorized as pre-seed, during, post-seed or inoculum was imported into the statistical software R. Using the "prcomp" function, as discussed in Section 4.8, PCA was performed (Figure 6.8). The arrows are vectors, representing the variables, in this case the phyla, and the points are the samples. They were grouped by color according to when they were taken during the seed event. PC1 explained 40.3% of the variation between the samples, PC2 explained

18.0%. There was a separation of the pre-seed (blue dots) and post-seed (red dots) samples along PC2, and a large variance between the inoculum sample (purple point) and all of the LANL SWWS samples along PC1. The during seed samples (green dots) were between the pre and post-seed samples along PC2. The vectors represented the phyla and the length of each reflects the overall relative abundance. For example, *Delta and Epsilonproteobacteria* were not prevalent in any samples and their vectors (nearly overlapping) were short, whereas *Chloroflexi* was abundant in the LANL SWWS samples and the vector representing that phylum was long and oriented away from the Inoculum sample. The vectors in the direction of the Inoculum sample were *Alphaproteobacteria, Gammaproteobacteria, Firmicutes, Bacteroidetes, Fusobacteria,* and *Actinobacteria*. *Firmicutes* were more abundant in the Inoculum sample (Figure 6.4) and the direction and magnitude of this vector was expected. The PCA also supported the statistical analysis that both *Firmicutes* and *Planctomycetes* were significantly more abundant (Table 6.3) in the pre-seed samples than in the post-seed samples. The phyla groups near the pre-seed samples were *Nitrospirae, Chlorobi, Acidobacteria,* and *Planctomycetes*. These phyla were found to have a larger mean relative abundance in the pre-seed samples versus the post-seed samples (Table 6.3). *TM7, GN02* and *Proteobacteria* were more abundant in the post-seed samples (Table 6.3). The vector representing *TM7* followed the expected orientation, pointing towards the post-seed. The *Proteobacteria* were dissected to the class level for the PCA, yet the most abundant *Proteobacteria*, *Betaproteobacteria* tends toward the post-seed samples. *GN02* points towards the pre-seed samples, and reasons for this were unknown.

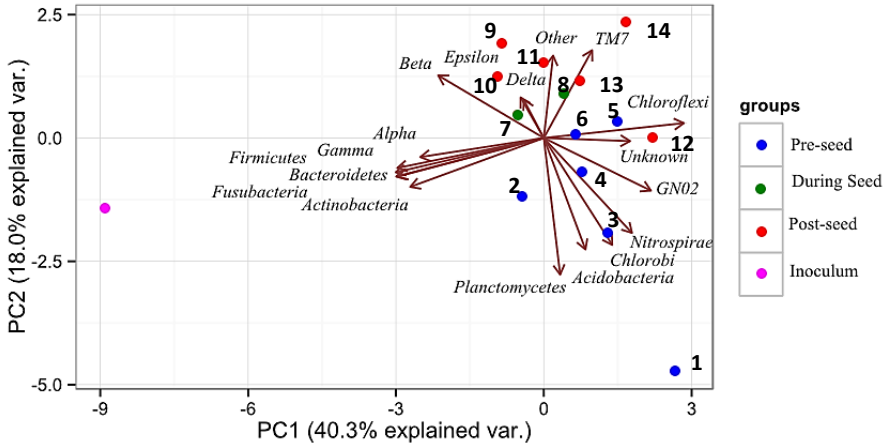


Figure 6.8: PCA of the most abundant phyla (Proteobacteria is divided into its respective classes). The vectors are the Phyla, the points are the samples grouped by time taken during the seed event and numbered by date sampled from earliest to latest

From this analysis, it was not clear if the Inoculum had an effect on the microbial population of the LANL SWWS samples. Along PC2, post-seed samples were less similar to the inoculum than the during-seed samples, suggesting that any long-term effects of the inoculum on the LANL SWWS system did not appear in this analysis. The samples were numbered to demonstrate that there appeared to be a temporal trend as well, from the earliest sample (1, taken 11/14/11) to the latest sample (14, from 6/11/12). The temperature in the aeration basin (Appendix B) throughout the study was mild, and did not fluctuate drastically, so seasonality was not likely the cause. The clustering of the pre-seed and post-seed samples may have been due to a constituent passing through the system over time or another event unrelated to the inoculation.

The aforementioned taxon-based approaches (the taxon summaries, Shannon diversity index, and PCA) to microbial diversity can be ambiguous. The threshold of similarity

that would classify a sequence into a specific OTU has not been historically consistent and this has resulted in various definitions of OTUs (Martin 2002). The taxon-based approach also assumes that taxa are equally related, and don't account for taxonomic similarity as a range (Martin 2002; Lozupone and Knight 2005). A phylogenetic (lineage based) perspective may offer alternative information about the dynamics of a population based on divergence. For example, a group of taxa may not be exactly alike, yet they are evolutionarily closely related. The weighted unique factor (UniFrac) measure was used to compute the phylogenetic beta diversity distance matrix accounting for relative abundance of each OTU within each sample. A detailed description of this algorithm is provided in Section 4.8. Principal coordinate analysis (PCoA) is an ordination method that allows for visualization of the distance matrix. Unlike PCA, this method is not linear and the variables from the original data cannot be correlated with the principal coordinates (axes on the plot). The axes represent the variation of the distances between the samples rather than the variation directly within the data set. It is important to note that due to this manipulation, the axes on the plot become irrelevant; they serve only as a reference for scale. The samples were categorized according to the seed event and the weighted UniFrac PCoA is shown in figure 6.9. The pre-seed samples (green squares) grouped together and the post-seed samples (orange triangles) grouped together. Because weighted UniFrac was used, these groupings suggested that the communities within the pre-seed samples were more phylogenetically similar to each other than they were to the communities in the post-seed samples. Not only did the OTUs share similar lineage, specific OTUS were also relatively abundant in these samples. The first and second principal coordinates (PC1 and PC2) accounted for 38.36% and 18.19% of the variation

within the distance matrix. The distance between the inoculum sample and the LANL SWWS samples along PC1 denoted that the phylogeny of the activated sludge of the two treatment plants was dissimilar. Along PC2, the inoculum sample lay between the pre and post-seed groupings, meaning that these samples had dissimilar phylogenetic trees along PC2. Perhaps this pattern was the result of the seed event and the inoculum sample facilitated the shift from pre-seed to post-seed microbial communities. However, this array along PC2 could also be attributed to a temporal aspect as the samples seem to follow a general trend from earliest sample taken at the top of the plot to latest sample taken at the bottom of the plot.

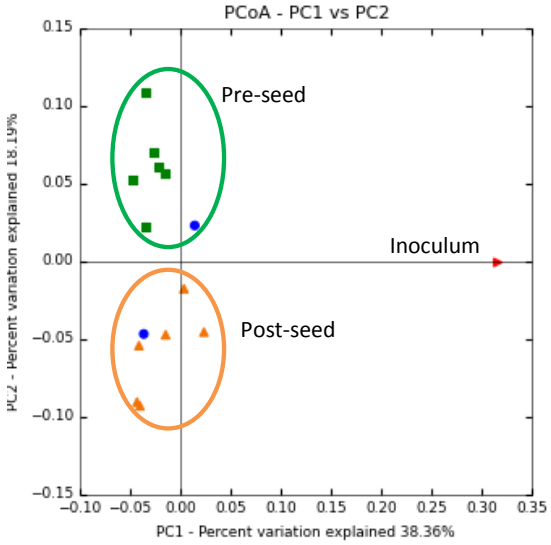


Figure 6.9: PCoA of seed event samples, pre-seed (green squares), during seed (blue circles), post-seed (orange triangles), and inoculum (red triangle).

Comment [M9]: Probably need to label samples

As previously stated, the variables within the original data set no longer correlate to the axes in the plot therefore the underlying causes for the sample distribution cannot be

definitively determined. In comparison to the PCA plot (Figure 6.8) there were similar groupings of the pre-seed and post-seed samples and it is probable that the most abundant phyla contributed to the distribution of the samples.

6.4 Conclusions

This chapter evaluated the responses of microbial communities in a full-scale treatment plant to a seed event utilizing 454-pyrosequencing as a method to detect reactions of the consortia in activated sludge to foreign biomass. Taxonomic classification of the microbial communities within the two treatment plants was achieved. *Chloroflexi* dominated the LANL SWWS samples, whereas *Proteobacteria* was most abundant in the LAC WWTP samples. The summary demonstrated that the two samples from the LAC WWTP were very different while the differences between the LANL SWWS samples were more subtle. Statistical analysis revealed that the microbial populations differed around the seed event. From a taxonomic perspective, two dominant phyla were significantly dissimilar before and after the inoculations. Interestingly, the most abundant phyla across all of the samples were not impacted by the treatment, leading to questions about the inoculation dose concentrations. The microbial changes were not correlated directly with the inoculum consortia. Taxonomically, there was not enough evidence to confirm any divergence or adaptation related to the addition of external biomass. However, it should be noted that only two samples were taken during the time of the seed event, and only one from the inoculum sludge itself, and statistically significant correlations between them were not possible. PCA demonstrated that the samples clustered based on whether they came from pre-seeded samples or post-seeded samples. PCoA suggested that the pre-seed, and post-seed samples were

phylogenetically different however, it remains unclear if the pattern was in direct response to the seed event.

Utilizing next generation pyrosequencing to identify population changes in activated sludge in response to a treatment is a relatively new technique. It allows for taxonomic as well as phylogenetic comparisons to be made efficiently. This work demonstrates that the effects of a seed event on the microbial populations of activated sludge could be accomplished with stringent sampling depth and frequency coupled with environmental and operational characteristics and measurements to enhance the explanations behind biological responses.

CHAPTER 7 THE EFFECTS OF ENVIRONMENTAL AND OPERATIONAL CHARACTERISTICS ON THE MICROBIAL POPULATIONS OF ACTIVATED SLUDGE

7.1 Introduction

Environmental factors directly affect microbial populations within activated sludge systems. Temperature, pH, dissolved oxygen, and nutrient loads are a few parameters that can impact the biodiversity within a system. The effectiveness of activated sludge relies on the optimization of these conditions to enrich for the survival of the biota needed to treat various constituents. Wastewater treatment plant (WWTP) operators manipulate process parameters such as sludge age to manage plant performance. Conversely deviation from certain environmental conditions can adversely affect microbial populations. WWTPs monitor many system characteristics in order to optimize performance and mitigate upsets. This approach is process driven and has proven effective. However indications of performance from this perspective are symptomatic and a more proactive approach may be to monitor the microbes themselves. With next-generation sequencing techniques becoming increasingly efficient and cost effective (Schuster 2007), it may soon be possible to utilize the microbial population as an indication of plant performance.

Temperature is an important environmental factor affecting microbial communities in activated sludge systems (Hu, Wang et al. 2012; Zhang, Shao et al. 2012; Ye and Zhang 2013). Most activated sludge bacteria are mesophilic and function well under moderate temperatures (25-35°C) (Tchobanoglous, Burton et al. 2003). Sludge volume index (SVI) is an indication of the settleability of the sludge and is a measure of sludge bulking,

with values greater than 150 mL/g considered to be “bulking”, or poorly-settling sludge (Tchobanoglous, Burton et al. 2003). Excessive growth of filamentous bacteria is a primary contributing factor to sludge bulking problems (Sezgin, Jenkins et al. 1978).

Carbon sources and availability also affect microbial dynamics. Many bacteria are heterotrophic, meaning they obtain carbon for growth from organic sources, while autotrophs such as nitrifiers utilize inorganic carbon for cell material. Nitrifiers consist of two groups of organisms, ammonia oxidizing bacteria (AOB), and nitrite oxidizing bacteria (NOB). AOB convert ammonia to nitrite, and NOB convert nitrite to nitrate. In activated sludge, *Nitrosomonas* has long been considered to be the dominant AOB and *Nitrobacter* is the dominant NOB (Coskuner and Curtis 2002), yet other studies have found AOB *Nitrosospira* and NOB *Nitrospira* in activated sludge (Siripong and Rittmann 2007). It has been demonstrated that the carbon to nitrogen ratio (C/N) greatly influenced these microbes (Chiu, Lee et al. 2007).

The solids residence time (SRT) is an important operating parameter of the activated-sludge process as it determines the rate of the growth of organisms in the culture. Influent characteristics, desired nutrient removal, effluent requirements and temperature play a role in establishing the desired SRT. SRTs can vary from three days to as high as fifty days, with typical values of 3-5 days for BOD removal and 18 days for nitrification (Tchobanoglous, Burton et al. 2003). Plants with low-strength influent are often run at high SRT values to help maintain high biomass concentrations in the biological reactors (Yoochatchaval, Ohashi et al. 2008). Research has found that activated sludge plants with low influent concentration should operate at SRTs between 5 and 15 days to avoid accumulation of slowly biodegradable microbial products due to cell decay and prevent

overgrowth of filamentous bacteria (Masse, Sperandio et al. 2006). In systems with variable flow, longer SRTs are recommended to enhance system stability, especially under low-strength influent conditions and at low temperatures (Angenent, Banik et al. 2001). Very long SRTs have been associated with some process phenomena, such as excessive growth of eukaryotic organisms such as tardigrades. The LANL system experienced such an upset in February 2012 due to tardigrades (discussed in Chapter 6.1) and evaluation of possible causes of this upset was of interest in this study.

It was hypothesized that environmental factors at LANL SWWS would influence the microbial population. The objectives of this portion of the research were to 1) determine if certain microbial communities were correlated to environmental conditions at LANL SWWS, 2) evaluate possible limiting conditions within the plant that may influence the consortia distribution, and 3) evaluate excessive SRT as a possible cause of the tardigrade related upset at the LANL plant. Pyrosequencing results were analyzed along with measured plant conditions in order to accomplish the objectives. While these findings may not be generalized to other WWTPs, they would serve as a base line or microbial blue print to better combat possible future plant upsets.

7.2 Methods and Materials

7.2.1 Sample Collection, DNA extraction, PCR amplification and Pyrosequencing

Fourteen activated sludge samples were collected from LANL SWWS south east (SE) aeration basin, as described in Section 4.1. The samples were preserved until DNA was extraction which was executed via the CTAB S method (Section 4.4). Adequate DNA

was verified by gel electrophoresis and the samples were cold shipped to Research and Testing Laboratory (RTL) in Lubbock, Texas for 454-pyrosequencing.

7.2.2 Analytical Methods

Bioinformatics software (QIIME 1.80) was used to analyze the results of the sequencing as detailed in Section 4.6. Phyla occurring in at least one sample at greater than 1% relative abundance were considered for the remainder of analysis, while others were considered to be unlikely to contribute significantly to system function are were not included in the analyses. The phyla *Proteobacteria* was divided into its respective classes for subsequent investigation. Measurements were taken by plant personnel throughout this project at various locations along the treatment train (Appendix B). As described in Section 4.1, there are three aeration basins at the LANL SWWS, and data pertaining to the aeration basin that the activated sludge samples were collected from (SE aeration basin) were considered in subsequent analysis, the other two aeration basins were not. These values were averaged one week prior and one week after each activated sludge sample collection date (Table 7.1). Univariate linear correlations were evaluated between each parameter and each phylum and those phyla found to be significantly correlated (using the F probability distribution with $\alpha<0.05$) with at least one process parameter were further investigated for linear correlations at the order level of taxonomy.

7.2.3 SRT and C/N Calculations

The carbon to nitrogen ratio (C/N) and SRT calculations were performed as described in Sections 4.8 and 4.9 respectively, based on measurements provided by LANL SWWS personnel. The average influent flow into the aeration basins was taken as the flow from

the EQ basins, the influent nitrogen was assumed equal to the influent ammonia as total kjeldahl nitrogen was not measured. With the chemical equations for glycerin and biomass (Section 4.9), the molar concentration into the system due to only glycerin was calculated. The molar concentration of the carbon due to glycerin divided by the molar concentration of the influent ammonia was the C/N ratio.

All values for the SRT were calculated from data provided by LANL SWWS. Missing values were estimated as the average between the most proximate recorded values. With the intermittent wasting employed at the LANL SWWS, the calculation of the SRT required using a moving average for the wasting rates, as described in Section 4.9. The kinetic coefficients for the activated sludge process for the removal of organic matter were taken from the literature (Tchobanoglous, Burton et al. 2003).

7.3 Results and Discussions

The parameters of interest that were measured by the LANL SWWS were averaged over two weeks around each sample date to provide representative indications of conditions and performance surrounding the sampling dates (Table 7.1) as the measurements were from grab samples. The temperature of the activated sludge in the SE aeration basin was fairly steady, however it was also low compared to what most microbes prefer, at an average of 18.1°C, peaking at 20.3°C and measuring 16.7°C minimum from November, 2011 through June 2012. The SVI was more than double (Table 7.1) that of recommended values (<100mL/g), which was an indication of poor settling possibly due to filamentous growth. The MLSS concentration and pH were within typical ranges. For carbonaceous removal the DO concentrations were slightly low, but above a detrimental limit for aerobic oxidation of 0.5 mg/L (Tchobanoglous, Burton et al. 2003). The SE

aeration basin is the second basin in the cyclic aeration basin train (See Appendix A for schematic). Every 45 minutes, the basin was aerated for a short duration to provide oxygen for ammonia oxidation (nitrification) followed by no aeration for the remaining 45 minutes to allow for denitrification (a more detailed explanation of this process is provided in Section 4.1). The effluent ammonia and nitrate concentrations support that this process was effective during the time of this work as they were well below the mandated requirements (Table 7.1).

Table 7.1: LANL SWWS parameters studied in relation to microbial populations

			Southeast aeration basin measurements						Effluent measurements	
Date	**MLSS mg/L**	**SVI mL/g**	**pH**	**Temp °C**	**DO mg/L**	**DO on* min**	**NH_3 mg/L**	**NO_3 mg/L**	**NH_3 mg/L**	**NO_3 mg/L**
L.11.14.11	2865.00	321.00	7.17	18.53	1.07	2.87	0.78	3.98	0.09	2.51
L.12.06.11	2882.22	321.78	7.00	16.65	1.12	3.27	1.07	4.65	0.05	3.89
L.1.23.12	2755.00	347.60	6.88	17.99	1.31	3.73	1.08	4.01	0.08	2.26
L.1.30.12	2748.00	348.56	6.85	17.98	1.15	3.93	1.41	3.18	0.10	1.80
L.2.7.12	2899.09	331.60	6.89	17.87	1.06	3.87	1.50	2.67	0.06	1.30
L.2.14.12	2946.00	331.90	6.93	17.54	1.08	3.57	1.08	2.56	0.04	1.03
L.2.21.12	2977.00	331.30	6.89	16.61	1.12	3.46	1.25	2.54	0.02	0.90
L.3.5.12	3151.54	319.64	6.95	17.03	0.99	3.53	1.58	0.81	0.04	0.43
L.3.19.12	3271.82	300.36	6.92	17.55	1.15	4.07	0.86	0.30	0.05	0.23
L.4.2.12	3337.27	290.55	7.08	19.13	1.00	3.79	1.07	0.01	0.03	0.01
L.4.16.12	3193.64	303.00	7.07	18.39	1.02	3.73	0.96	0.16	0.03	0.00
L.5.8.12	2987.00	326.25	7.04	18.34	1.35	3.57	0.89	1.21	0.05	0.41
L.5.21.12	2947.00	328.50	7.00	19.63	1.15	3.46	0.79	2.13	0.04	1.09
L.6.11.12	2681.67	351.25	7.04	19.80	1.26	3.73	1.05	3.07	0.02	1.80
Averages	2974.45	325.23	6.98	18.07	1.13	3.61	1.10	2.23	0.05	1.26

* The SE basin was aerated on a 45 minute cycle. The DO on indicates the minutes the basin was on per 45 minute cycle

7.3.1 Correlations of microbial populations with environmental factors and plant parameters

LANL SWWS is located in a canyon and experiences shaded conditions most of the year. It was observed that the temperature of the activated sludge basin averaged 18.1°C (±1°C) over the course of this work, ranging from 20.3°C in June to 16.7°C in March (possibly due to snowmelt runoff). These temperatures are relatively cold for most mesophilic bacteria and were lower than typical conventional activated sludge systems in temperate climates. No significant correlation was found among the most abundant microbes and the activated sludge temperature, which was contradictory to previous research (Wang, Hu et al. 2012), possibly because temperature variations at the LANL plant were relatively small.

The average SVI of LANL SWWS was well above 150 mL/g and it was hypothesized that filamentous bacteria may have been prevalent. It should be noted that the SVI value was determined without dilution and so this performance parameter may not be comparable to other studies due to empirical errors (Tchobanoglous, Burton et al. 2003). The SVI as a performance parameter was significantly (using the F probability distribution as described in Section 4.7with $\alpha < 0.05$) negatively correlated with the phylum *Bacteroidetes* and the class *Alphaproteobacteria*, R^2 values of 0.32 and 0.36 respectively.

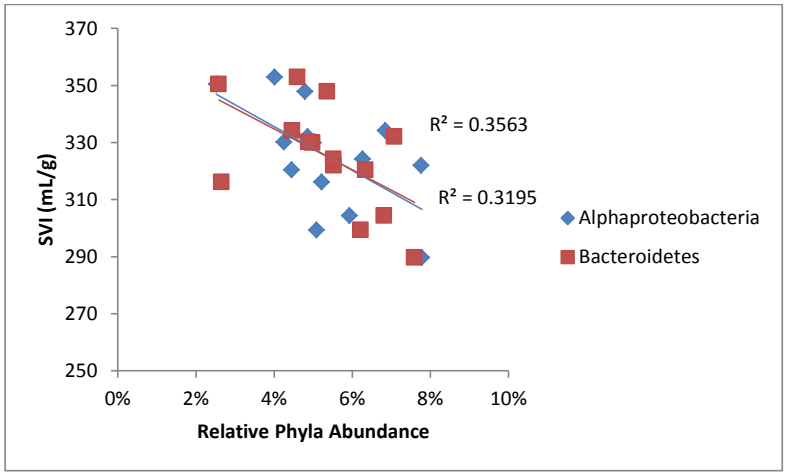


Figure 7.1: The relationship between SVI and significantly (α<0.05) correlated phyla

Filamentous *Bacteroidetes* have been associated with sludge bulking, specifically the genus *Haliscomenobacter* of the order *Saprospirales* (Kragelund, Levantesi et al. 2008). *Saprospirales* was the most relatively abundant order of *Bacteroidetes* in the samples (Figure 7.2).

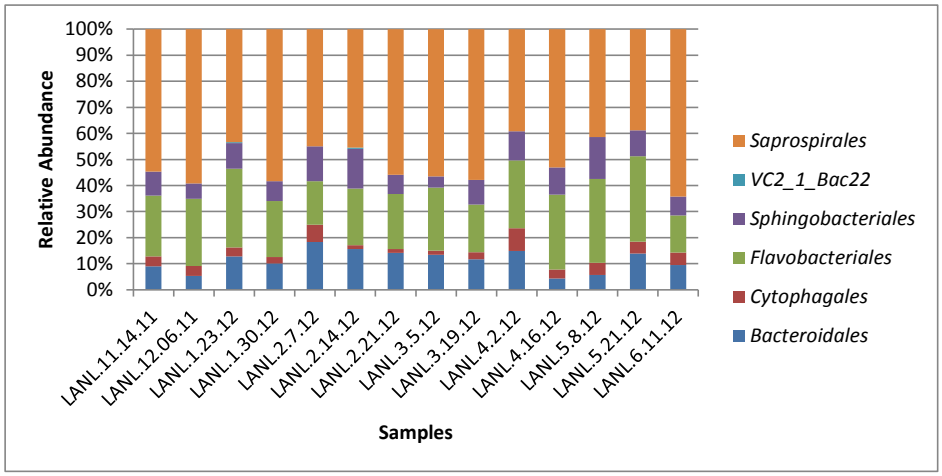


Figure 7.2: The relative abundance of the orders within the phylum *Bacteroidetes*

Upon further inspection down to the order level, three orders belonging to the phylum *Bacteroidetes* were significantly ($\alpha<0.05$) negatively correlated to the SVI (Figure7.3). *Saprospirales* was not one of them. It was determined that at the genus level (not shown), *Haliscomenobacter* was not abundant (<0.3% maximum relative abundance) and this may explain the lack of correlation. *Flavobacteriales* ($R^2 = 0.33$) were identified as significantly correlated to the SVI and their relative abundance in LANL SWWS was a maximum of 1.7%. They have been characterized as floc-forming organisms and may be responsible for breaking down carbohydrates (Manz, Amann et al. 1996). It could be inferred from the negative correlation between *Flavobacteriales* and the SVI that while these microbes may flocculate, they were not detrimental to sludge settling. *Cytophagales* ($R^2 =0.34$) has been isolated from activated sludge (Seviour, Blackall et al. 1997), and *Sphingobacteriales* ($R^2 =0.35$) has been identified as a common order within activated sludge samples from North America (Zhang, Shao et al. 2012) but neither have been well researched.

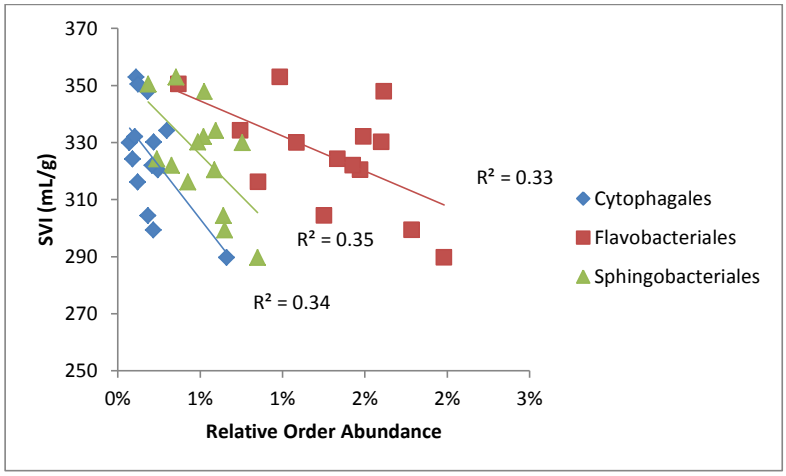


Figure 7.3: The relationship between SVI and significantly correlated (α<0.05) *Bacteroidetes* relative abundance

The order *Rhizobiales* were the most dominant within the class *Alphaproteobacteria* in the LANL SWWS samples and species from this order (*Meganema perideroedes)* have been linked with sludge bulking (Kragelund, Nielsen et al. 2005), yet the genus *Meganema* were not present in the samples. When significant orders within *Alphaproteobacteria* were identified, only *Rickettsiales* was significant (Figure 7.4), yet this was due to one sample (4.2.12) with a dramatically increased relative abundance for that sample only (2% versus <0.1%) therefore *α-Proteobacteria* was not considered accurately correlated to the SVI.

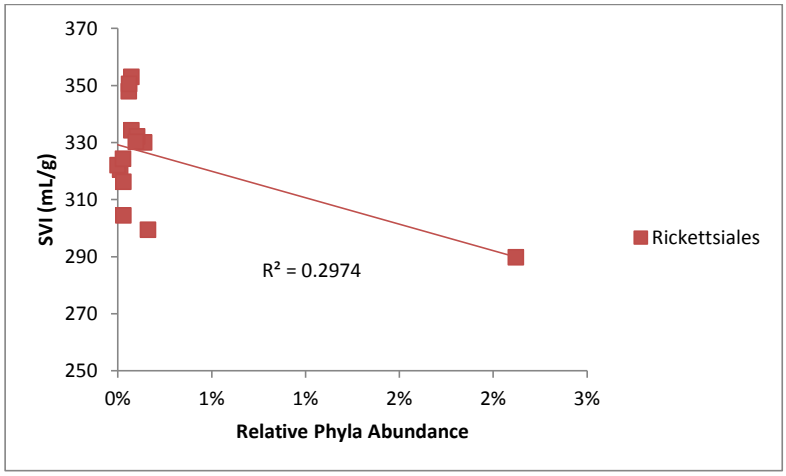


Figure 7.4: The relationship between SVI and significantly correlated (α<0.05) *Alphaproteobacteria* relative abundance

Many filamentous bacteria have been identified in the phylum *Chloroflexi* (Kragelund, Levantesi et al. 2007) and they were abundant in the LANL SWWS samples, but their abundance not significantly correlated to the SVI. The SVI of LANL SWWS measured high in comparison to typical values which could indicate settling problems such as sludge bulking and filamentous growth. The statistical analysis presented above illustrated that this parameter was not correlated with any known problematic bacteria and as evident by an average effluent TSS < 3 mg/L, effluent quality was not affected by the elevated SVI.

The average MLSS concentration of LANL SWWS (Table 7.1) was within normal ranges (Tchobanoglous, Burton et al. 2003). There were five phyla significantly correlated with MLSS (α<0.05) (Figure 7.5), two were positively correlated, *β-Proteobacteria* (R^2=0.30), and *Bacteroidetes* (R^2=0.30), and three were negatively correlated, *Chloroflexi* (R^2=0.33), *Planctomycetes* (R^2=0.36), and *Acidobacteria* (R^2=0.43).

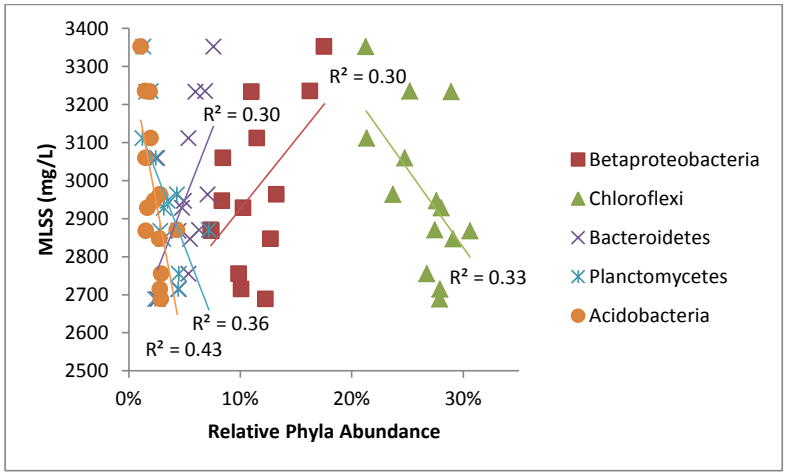


Figure 7.5: The relationship between MLSS and significantly (α<0.05) correlated phyla

Analysis at the order level, (Figure 7.6) included three orders from the class *Betaproteobacteria*: an order of *Betaproteobacteria* that could not be assigned a taxonomy beyond the class level and was hence classified as an order of *Betaproteobacteria* (R^2=0.41), *Neisseriales* (R^2=0.30), and *Rhodocyclales* (R^2=0.71). Little is known of the order *Neisseriales* other than they have been found in very small relative abundance in wastewater (Wang, Hu et al. 2012). *Rhodocyclales* have historically been categorized as a versatile group of organisms common to wastewater (Juretschko, Loy et al. 2002) with the ability to perform nitrification/denitrification and utilize an array of different carbon sources (Hesselsoe, Fuereder et al. 2009). Treatment plants that perform nitrogen transformation tend to have a longer SRT and therefore accumulate suspended solids. The LANL SWWS plant not only performs internal nitrification/denitrification, it operates at a very lengthy SRT and the strong positive

correlation between *Rhodocyclales* and the MLSS concentration could be an indication of the SRT.

The orders *Flavobacteriales* (R^2=0.29), and *Sphingobacteriales* (R^2=0.32), of the phylum *Bacteriodetes* were also positively correlated to the MLSS ($\alpha < 0.05$). These orders were negatively correlated to the SVI (Figure 7.3). The SVI is calculated by the settled sludge volume divided by the MLSS, and the fact that the same *Bacteroidetes* were found with significant inverse correlations as they were with the SVI was to be expected. This prompted the investigation into the correlation between the SVI and the MLSS concentration (Figure 7.6). There was a significant ($\alpha < 0.05$) negative correlation between the two measurements suggesting the SVI should be diluted in order to increase accuracy (Tchobanoglous, Burton et al. 2003).

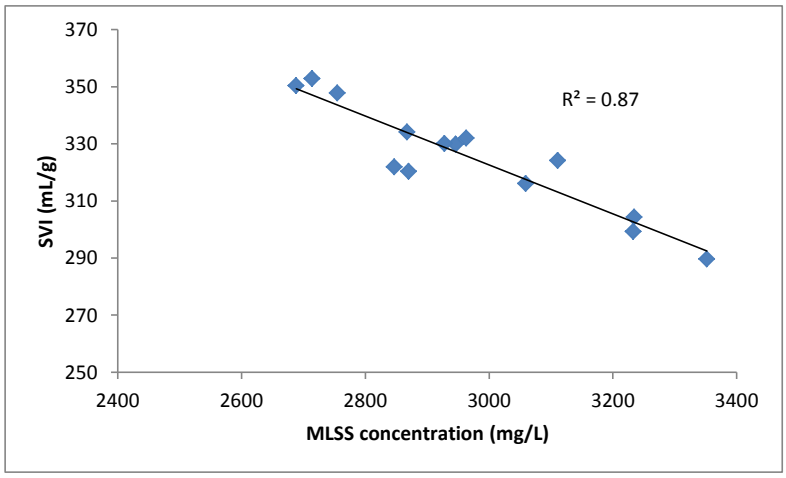


Figure 7.6: The relationship between SVI measurements and MLSS concentrations of LANL SWWS

Pirellulales (R^2=0.29) of the phylum *Planctomycetes* was negatively correlated. *Planctomycetes* have been identified in multiple wastewater systems, yet little has reported on them (Neef, Amann et al. 1998; Fuerst and Sagulenko 2011; Chiellini, Munz et al. 2013). From the phyla *Chloroflexi* and *Acidobacteria*, there were no orders that were statistically significant with $\alpha < 0.05$.

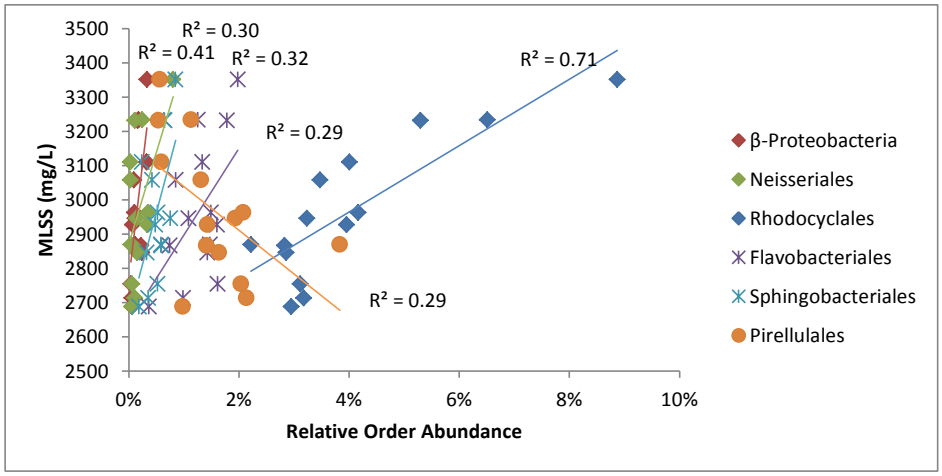


Figure 7.7: The relationship between MLSS and significantly correlated (α<0.05) *β-Proteobacteria* (diamonds), *Bacteroidetes* (asterisks), and *Planctomycetes* (circle) relative abundance

The pH of LANL SWWS was near neutral (average pH= 6.98 ±0.09) with little deviation through this study, even so phyla of bacteria responded significantly to changes in pH (Figure 7.8). *Epsilonproteobacteria* and *Firmicutes* decreased with increasing pH while *Chlorobi* was positively correlated.

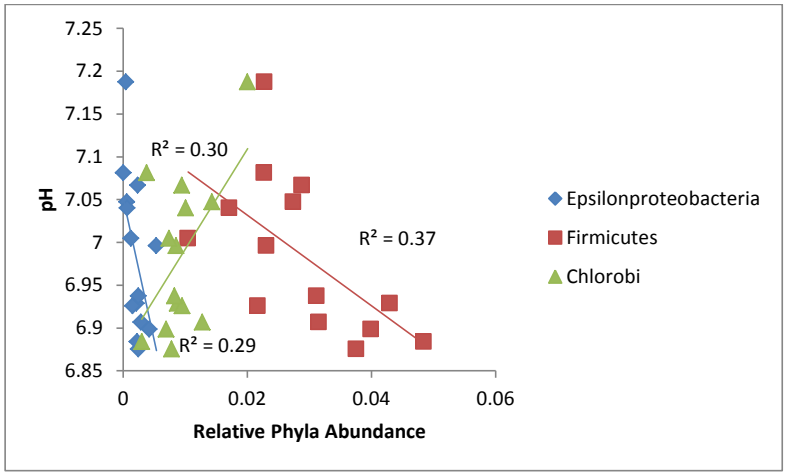


Figure 7.8: The relationship between pH and significantly (α<0.05) correlated phyla

The significantly correlated orders belonging to these phyla were *Campylobacterales* (R^2=0.29), *Clostridiales* (R^2=0.39), and *Ignavibacteriales* (R^2=0.39), respectively. As pH is a critical environmental variable affecting all bacteria, it is not surprising that some microbes correlated with pH changes. Because the observed changes in pH were small, correlations between abundance in pH in this study may have indicated a particular sensitivity to pH. *Campylobacterales* have been found in activated sludge treating influent from a slaughter house (Zhang, Shao et al. 2012), yet the pH of the waste was not specified in the study and reasons for this correlation remain unclear. They have been found to survive in bile (Okoli, Wadstrom et al. 2007), and the correlation shown in figure 7.9 agreed with the possibility they preferred slightly acidic conditions. *Clostridiales* have been found in several WWTPs and were considered to be one of many shared orders among various activated sludge with pH ranging from 6.41 to 7.29 (Wang,

Hu et al. 2012). Less is known about *Ignavibacteriales* response to small changes in pH, but they have been found in industrial wastewaters (Zhu, Tian et al. 2013).

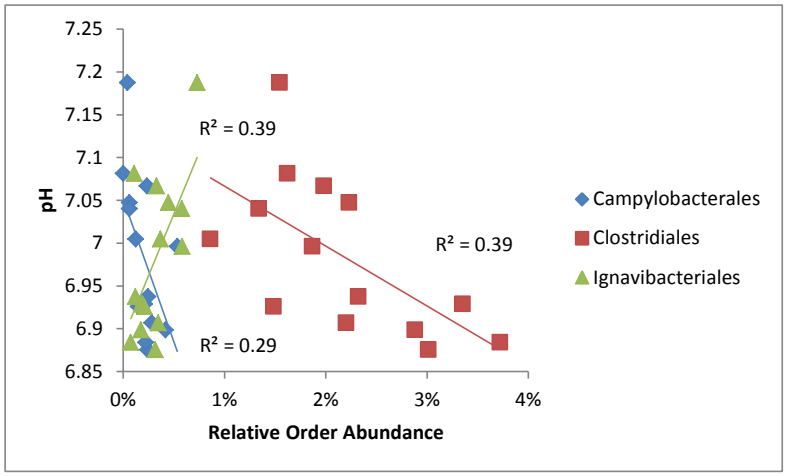


Figure 7.9: The relationship between pH and significantly correlated (α<0.05) ε-*Proteobacteria, Firmicutes* and *Chloroflexi* relative abundance

The average DO in the Southeast aeration basin was 1.13 mg/L and within all three aeration basins, did not exceed 2.1 mg/L. These values are considered low and poor settleability and filamentous growth have been connected with low DO concentrations (Martins, Heijnen et al. 2003; Park and Noguera 2004). *Bacteroidetes* were the only phylum that was correlated with the maximum DO concentration. Within the phylum *Bacteroidetes*, the orders *Bacteroidales*, *Flavobacteriales*, and *Sphingobacteriales* were negatively correlated with the maximum DO in the Southeast aeration basin (Figure 7.10). *Bacteroidales* are anaerobic and their negative correlation to oxygen availability was expected (Bae and Wuertz 2009). *Flavobacteriales*, and *Sphingobacteriales* have been shown to be facultative anaerobes (Bruns, Rohde et al. 2001).

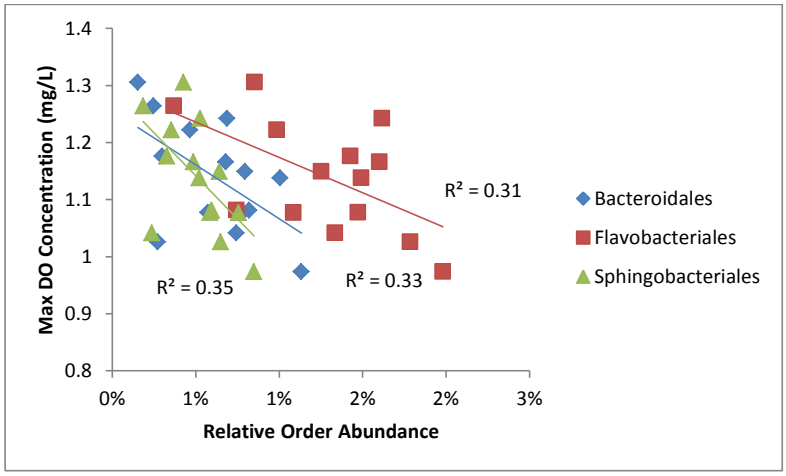


Figure 7.10: The relationship between maximum dissolved oxygen concentrations in SE aeration basin and significantly correlated (α <0.05) orders within the phylum *Bacteroidetes* relative abundance

The length of exposure to oxygen may impact the microbial population as well, specifically with respect to nitrifiers. When the correlations were performed on the length of time the basin was aerated per 45 minute aeration cycle, three phyla were negatively correlated (Figure 7.11). NOB and AOB would be expected to increase in abundance with increased aeration if oxygen was limiting their activity. However, AOB were very rare in all of the LANL samples and the only identifiable NOB was *Nitrospira* at very low abundance (average of 1.8%). Neither group of bacteria was significantly correlated to the dissolved oxygen concentration or the duration of aeration. It is possible that the oxygen availability in the aeration basin was too low to foster the growth of nitrifiers and that the success of the LANL SWWS nitrification/denitrification process was due to the length of the SRT rather than the manipulation of aerobic and anoxic zones for the enrichment of nitrogen removing bacteria.

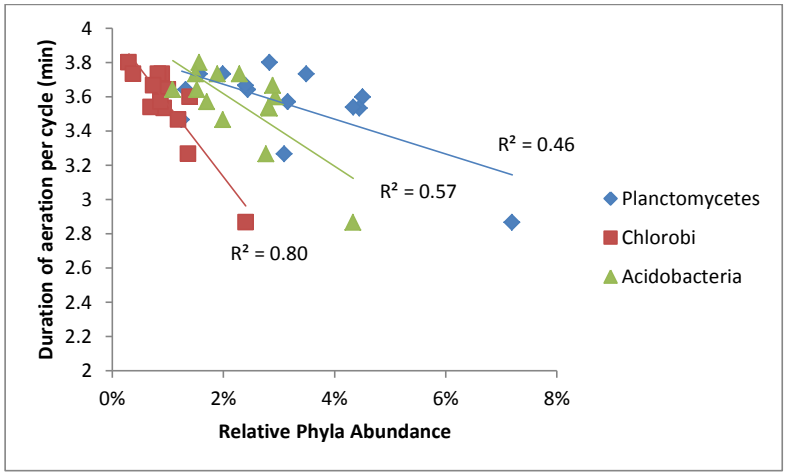


Figure 7.11: The relationship between aeration duration in the SE aeration basin and significantly correlated (α <0.05) phyla

Each order in the *Chlorobi* phylum (*Ignavibacteriales, OPB56 order*, and *SJA-28 order)* decreased with increased aeration time (Figure 7.12), suggesting these organisms may have competitive advantage under low oxygen conditions and research has shown that *Ignavibacteriales* are facultative anaerobes (Liu, Frigaard et al. 2012). *Acidobacteria* order *RB41*, and the *Planctomycetes* orders *Gemmatales* and *Pirellulales* also exhibited negative relationships and have been associated in small abundance with anaerobic sludge digestion (Riviere, Desvignes et al. 2009).

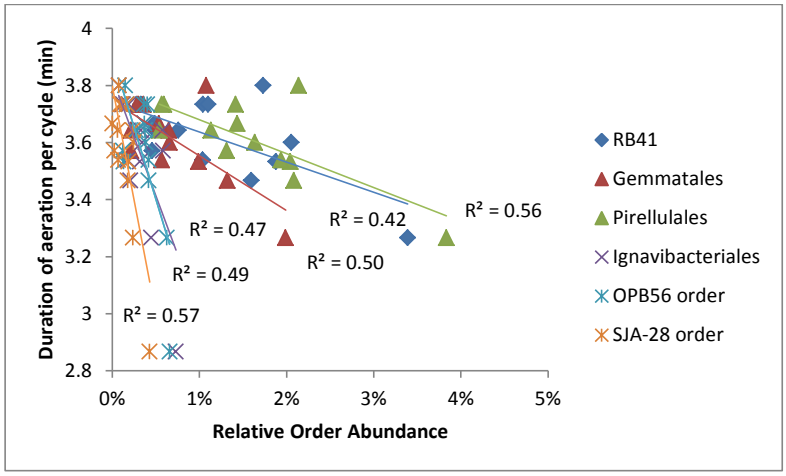


Figure 7.12: The relationship between aeration duration in the SE aeration basin and significantly correlated (α <0.05) *Chlorobi* (asterisks), *Acidobacteria* (diamond) and *Planctomycetes* (triangles) relative abundance

Ammonia and nitrate concentrations were monitored in the aeration basin as well (Table 7.1). These concentrations were low, indicating effective nitrification/denitrification. Interestingly, the NOB *Nitrospirae* did not correlate with nitrate. *Firmicutes* increased with ammonia concentration (Figure 7.13), specifically the order *Clostridiales* (R^2=0.34). *Clostridiales* bacteria are obligate anaerobes. An elevated level of ammonia would indicate lessened nitrification rates which could be a symptom of low oxygen supplies.

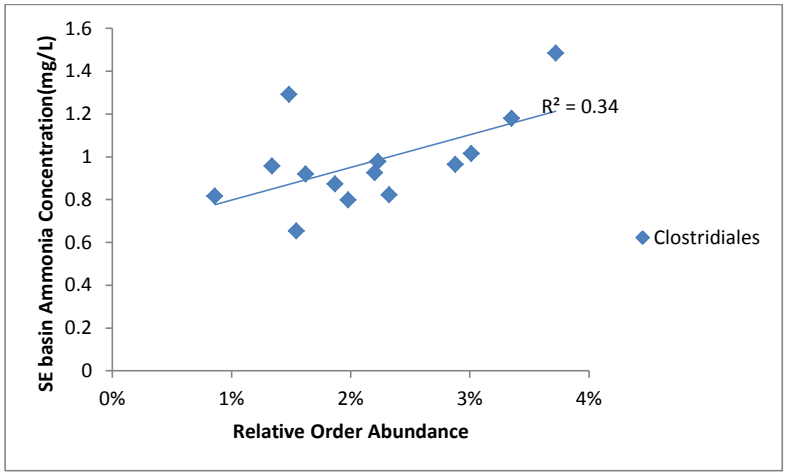


Figure 7.13: The relationship between ammonia concentrations in the SE aeration basin and significantly correlated (α<0.05) order *Clostridiales* of the phylum *Firmicutes* relative abundance

The phyla *Planctomycetes* and *Acidobacteria* were positively correlated with nitrate concentrations (Figure 7.14), but were not associated with its removal.

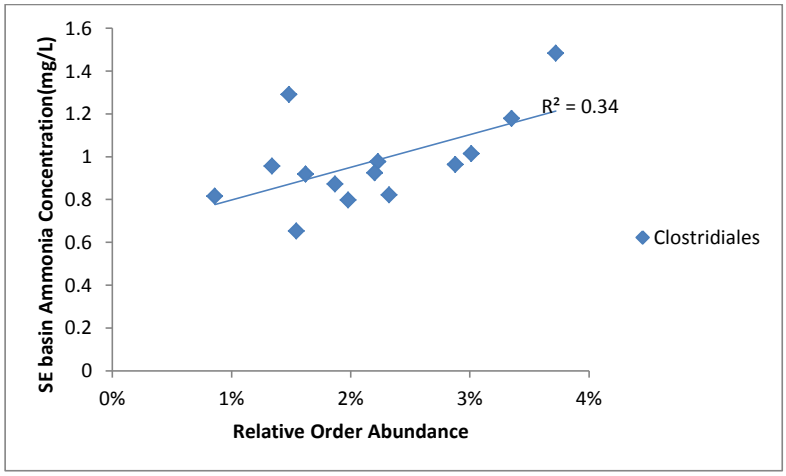


Figure 7.14: The relationship between nitrate concentrations in the SE aeration basin and significantly correlated (α<0.05) phyla relative abundance

The orders that correlated with nitrate concentrations in the SE aeration basin (Figure 7.15) were similar to those that correlated with the aeration duration (Figure 7.12), however rather than a negative correlation, they were positively correlated. This relationship could again be attributed to the oxygen availability rather that the nitrate concentration as none these bacteria are known to uptake nitrate.

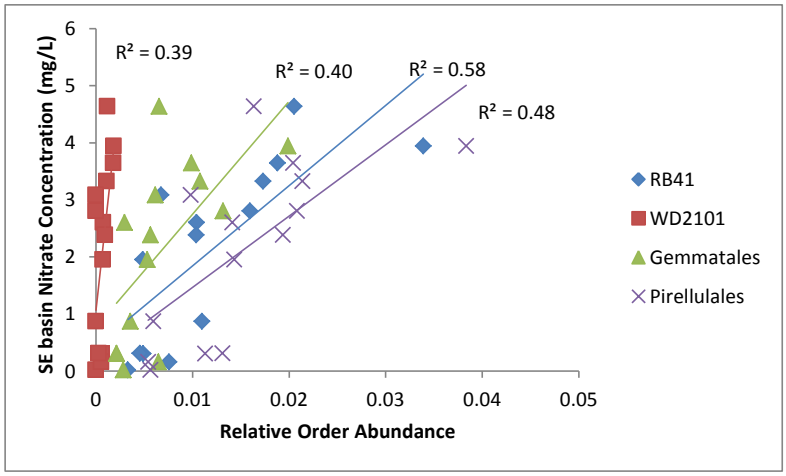


Figure 7.15: The relationship between nitrate concentrations in the SE aeration basin and significantly correlated (α<0.05) *Acidobacteria* and *Planctomycetes* relative abundance

A final correlation was found between the phylum *Nitrospirae* and the effluent ammonia concentration (Figure 7.16). *Nitrospirae* is a NOB and were overall part of the most abundant phyla, yet were generally sparse (1.8% maximum relative abundance). *Nitrosomonadales*, an AOB, was present in the SE aeration basin yet at an average relative abundance of less than 0.05%, they were scarce and it stands to reason that the ammonia passing through the treatment train would not be converted into nitrate and would therefore wash through the system.

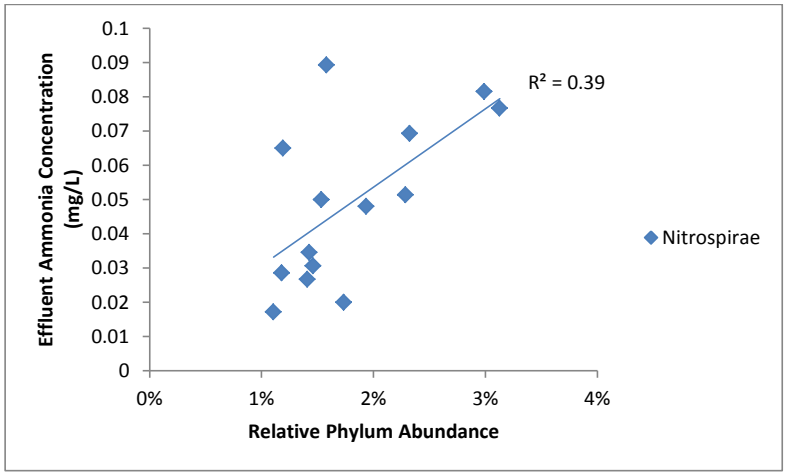


Figure 7.16: The relationship between effluent ammonia concentrations and significantly correlated (α<0.05) phyla relative abundance

This correlation was surprising because *Nitrospirae* should correlate with effluent nitrate. With very few AOB to produce nitrite, the reason for the presence of these NOB may be due to denitrification reduction from nitrate to nitrite. Nitrifiers are slow growers and an increased abundance could be the result of the lengthy SRT of LANL SWWS. The rarity of AOB should result in an accumulation of ammonia because the bacteria responsible for oxidizing ammonia were not present in large enough quantities and the figure above supports this conclusion. The nitrate concentrations through the LANL SWWS (Figure 7.17) demonstrate that the aeration basins contained greater nitrate concentrations than the influent, suggesting that nitrification was taking place. The presence of the nitrite oxidizing *Nitrospirae* in the SE aeration basin confirmed that the oxidation of nitrite to nitrate took place. Ammonia was being converted or consumed as the wastewater passed

through the system (Figure 7.18), with a drastic concentration decrease once exposed to the aeration basins.

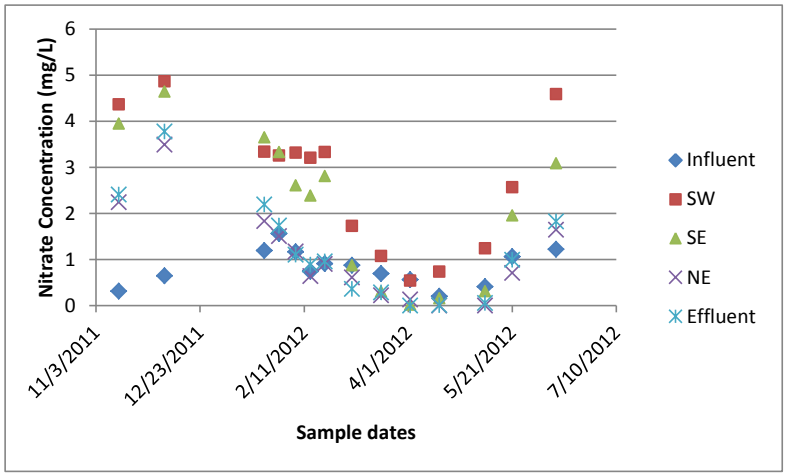


Figure 7.17: Nitrate concentrations through the LANL SWWS treatment train

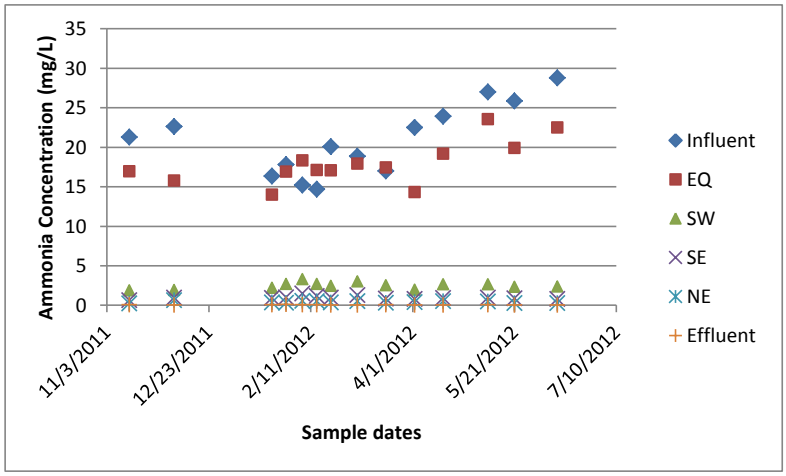


Figure 7.18: Ammonia concentrations through the LANL SWWS treatment train

The decrease in ammonia concentration coupled with the limited AOB abundance warranted an investigation into the carbon to nitrogen ratio as an alternative explanation for the diminution of ammonia.

7.3.2 The Influent Carbon to Nitrogen Ratio of LANL SWWS

Nitrifying bacteria are autotrophic, meaning they utilize CO_2 as a carbon source. They are chemolithotrophs, and obtain their energy from ammonia (AOB) or nitrite (NOB). Compared to heterotrophic bacteria, which use organic carbon, they are slow growing and produce a small amount of biomass for the amount of nitrogen they consume. Within the mixed population of activated sludge, competition occurs among the various types of bacteria. Heterotrophic bacteria have been known to outcompete autotrophs because they grow faster, deplete the available oxygen and occupy more space in a wastewater treatment system (Hanaki, Wantawin et al. 1990; Ohashi, deSilva et al. 1995; Fdz-Polanco, Mendez et al. 2000), particularly in biofilms where space is limited. When the carbon to nitrogen ratio (C/N) is high, organic carbon is readily available, and heterotrophs tend to dominate. One study illustrated that adding glucose as a supplemental carbon source increased the amount of heterotrophs in a suspended growth reactor, and observed that heterotrophic assimilation of ammonia occurred, resulting in the inhibition of ammonia oxidation. (Hanaki, Wantawin et al. 1990). Nitrifiers catabolize and anabolize ammonia, they utilize available nitrogen for cell growth as well as for energy (Tchobanoglous, Burton et al. 2003).

The scarcity of AOB and relatively low abundance of NOB prompted an examination into the carbon to ammonia as nitrogen (C/N) ratio at LANL SWWS. Within biofilm

reactors, ammonia oxidation was the rate limiting step for nitrification (Xia, Li et al. 2010). It has been reported that soluble COD limits nitritation, the process by which ammonia is oxidized to nitrite (Zeng, Zhang et al. 2009). A study of AOB within a denitrifying-nitrifying system under a low C/N ratio, (2:1), reported that influent ammonium was removed and that it was either oxidized in the aerobic reactor or assimilated for cell growth (Ballinger, Head et al. 2002).

The C/N ratio was calculated to test whether the LANL SWWS influent was carbon limited. The plant did not measure influent BOD concentrations and so the minimum C/N ratio due only to the addition of glycerin of was calculated with the average parameters shown in table 7.2.

Table 7.2: Average influent parameters used to calculate the C/N ratio

Influent flow (L/d)	$1.29*10^6$
Influent ammonia as N (mg/L)	20.9
Influent glycerin (mL/d)	$4.98*10^4$

The influent molar concentration of ammonia as nitrogen was:

$$\frac{20.9\left(\frac{mg\ NH_3-N}{L}\right)}{14\left(\frac{mgNH_3-N}{mmol\ N}\right)}=1.5\ \frac{mmol\ N}{L}$$

The influent molar concentration of 99.7% purified United States Pharmacopeia (USP) grade glycerin (ρ = 1.26 g/mL) (Vitusa Products Inc., Berkeley Heights, NJ, USA, http://www.vitusaproducts.com/) as carbon was calculated as:

$$4.98*10^4\left(\frac{mL\ C_3H_8O_3}{day}\right)*\ 1.26\left(\frac{g}{mL}\right)=62.8*10^6\left(\frac{mg\ C_3H_8O_3}{day}\right)$$

$$\frac{62.8 * 10^6 \left(\frac{mg C_3H_8O_3}{L}\right)}{92 \left(\frac{mg\ C_3H_8O_3}{mmol}\right)} / 1.29 * 10^6 \frac{L}{d} = 0.53 \left(\frac{mmol C_3H_8O_3}{L}\right)$$

There are 3 mols of carbon per mol of glycerin. The molar concentration in the system due to glycerin was:

$$0.53 \frac{mmol\ C_3H_8O_3}{L} * \frac{3\ mmol\ C}{1\ mmol\ C_3H_8O_3} = 1.59 \frac{mmol\ C}{L}$$

The minimum C/N in LANL SWWS due to glycerin was estimated:

$$C/N\ due\ to\ glycerin = \frac{1.59 \left(\frac{mmol\ C}{L}\right)}{1.5 \left(\frac{mmol\ N}{L}\right)} = 1.1$$

The C/N due to glycerin in the LANL SWWS was estimated as a minimum value. Limited measurements of the influent BOD were supplied by plant personnel for the month of March, 2012 (3/8 and 3/14-3/20) with an average 36 mg/L (±23 mg/L). The eight measurements were not from composite samples and the naturally occurring COD in the influent could not be reliably determined for the entire seven month study. Because glycerin was supplementing low influent organic concentrations, it was believed that the minimum estimated C/N (1.1:1) was a reasonable approximation.

The above value was compared to the C/N ratio needed for cell growth to verify if the system was carbon limited. The molar ratio for growth alone for biomass, $C_5H_7O_2N$ (Tchobanoglous, Burton et al. 2003) is 5:1. The oxygen equivalent of cell tissue was taken to be 1.42 g bsCOD/g VSS (Tchobanoglous, Burton et al. 2003). To account for the of amount of cell tissue produced from the influent carbon, the synthesis yield of 0.6

mg VSS/ mg bsCOD (Tchobanoglous, Burton et al. 2003) was used. Therefore, the approximate C/N for anabolism was calculated as:

$$C/N\ needed\ for\ cell\ growth = \left(\frac{g\ bsCOD}{1.42\ g\ VSS}\right) * \left(\frac{g\ VSS}{0.6\ g\ bsCOD}\right) * \frac{5\ mol\ C}{1\ mol\ N} = 5.9$$

From the above calculation, the heterotrophic bacteria would require 5.9 mols of carbon and 1 mol of nitrogen. The estimated C/N due to glycerin in the LANL SWWS was 1.1:1 and LANL SWWS appeared to be carbon limited. Once the influent carbon from glycerin was utilized for growth, there was excess ammonia for the nitrifiers to assimilate. This finding did not agree with the nitrifying bacterial population of the activated sludge, AOB (<0.17% maximum) and NOB (<3.1% maximum). It was possible that there was more influent carbon than was measured however this could not be verified.

An alternative reason for the lack of autotrophic nitrifiers was the low dissolved oxygen concentration in the SE aeration basin (average maximum DO = 1.1 mg/L) as DO <0.50mg/L has been reported to inhibit nitrification (Tchobanoglous, Burton et al. 2003). Yet figure 7.18 demonstrated that ammonia was being efficiently removed and studies have observed complete nitrification under low DO with various aeration rates due to AOB (Bellucci, Ofiţeru et al. 2011). Both AOB and NOB were found capable of nitrification under intermittent aeration at DO concentrations during aeration as low as 1.4 mg/L and were able to enrich for AOB by altering the aeration cycles (Mota, Head et al. 2005). LANL SWWS operated with intermittent aeration (Table 4.1), and despite the low DO of the system, the cyclic aeration could account for some presence of nitrifiers.

Yet with few AOB in the system, the high ammonia removal efficiency of LANL SWWS was perplexing. Heterotrophic nitrification could explain for the depletion of ammonia as strains of bacteria have been found to utilize ammonia including *Pseudomonas stutzeri* YZU-001(Zhang, Wu et al. 2011), *Comamonas* sp. GAD4 *(*Chen and Ni 2011) effectively. The species *Paracoccus denitrification* sp. (Blaszczyk 1993) and *Comamonas denitrificans* sp. (Gumaelius, Magnusson et al. 2001) have also illustrated heterotrophic nitrification capabilities. The 454-pyrosequencing results from this study were not performed to the strain or species level, but at the genus level (Figure 7.19) the heterotrophic nitrifiers were generally more abundant than the known AOB.

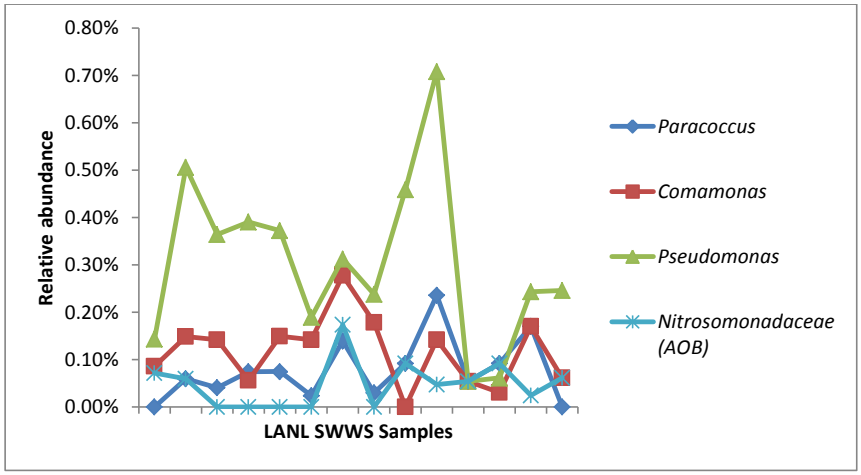


Figure 7.19: Comparison of relative abundance of ammonia oxidizing bacteria (AOB) to possible heterotrophic nitrifying bacteria (Paracoccus, Comamonas, Pseudomonas) at the genus level

Yet heterotrophic nitrifying bacteria still require an adequate C/N(Van Loosdrecht and Jetten 1998). Alternatively, anaerobic ammonia oxidation (anammox) could be responsible for the high ammonia removal. Anammox bacteria oxidize ammonia in the

absence of oxygen and are slow growing like autotrophic nitrifiers. In systems with low DO, and long SRTs, (Van Loosdrecht and Jetten 1998), much like LANL SWWS. Genera attributed with this capability under low COD concentrations have been identified as belonging to the order *Planctomycetales* (van Dongen, Jetten et al. 2001). This order of bacteria belongs to the phyla *Planctomycetes* and was found to be more abundant than the AOB in the LANL SWWS communities (Figure 7.20), although investigation past the genus level would need to be performed in order to confirm the anammox species presence.

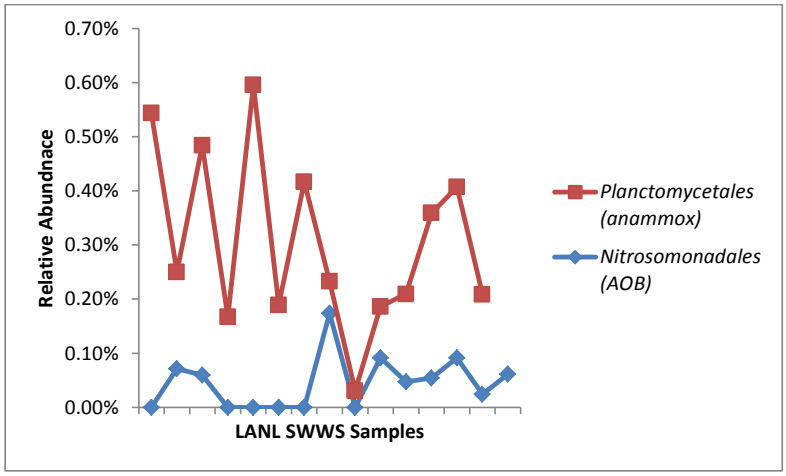


Figure 7.20: Comparison of relative abundance of possible anammox bacteria (*Planctomycetales*) to AOB at the order level

The nitrification/denitrification processes of the LANL SWWS should be further investigated and the microbial populations involved merit further study.

7.3.3 The Solids Residence Time of LANL SWWS

Tardigrades are ubiquitous (Miller 2011; Mobjerg, Halberg et al. 2011) yet within wastewater treatment environments, the literature suggested they were found most often in plants that operate at lengthy solids residence times (SRT) (Moghaddam, Guan et al. 2003). As discussed previously in this work, LANL SWWS experienced an upset in 2012 linked to excessive tardigrade growth. Research suggests that these organisms proliferate in systems with long SRTs and it was believed that LANL SWWS also ran at a longer SRT than most conventional activated sludge systems.

In an effort to identify the cause of the escalated tardigrade presence at LANL SWWS, the effective SRT was estimated. It was hypothesized that the increased abundance of tardigrades was due to high SRT values.

Unlike typical domestic wastewater treatment plants, the LANL SWWS wasted biomass from their systems sporadically over the course of this project. In the month of December, 2011, for example, they wasted biomass from the system once. A sample calculation of the instantaneous SRT calculated on a day when wasting took place is provided below:

$$SRT_{instantaneous} = \frac{VX}{(Q - Q_W)X_e + (Q_W X_R)}$$

$$\frac{(2.8 * 10^6 L) * (3.14 * 10^{-3} \frac{kg}{L})}{\left(9.0 * 10^5 \frac{L}{day}\right) * \left(1.6 * 10^{-6} \frac{kg}{L}\right) + \left(4.5 * 10^4 \frac{L}{day}\right) * \left(5.3 * 10^{-3} \frac{kg}{L}\right)} = 36.9\ days$$

The uneven rate of wasting at the LANL plant resulted in highly variable calculated instantaneous SRTs, with large wasting ($Q_W X_R$) amounts on some days, and zero wasting

amounts on others, therefore a moving average of 45 days was used and demonstrated below:

$$SRT_{45\ days} = \frac{45\ day\ average\ mass\ in\ the\ system}{45\ day\ average\ mass\ leaving\ the\ sytem}$$

$$\frac{9173\ kg}{122.6\ \frac{kg}{d}} = 75\ days$$

By this calculation, the average SRT of LANL was 142 days (±85) during the study period, with a minimum of 70 days. This indicated that the time that the activated sludge solids were in the system was much more than even an extended aeration treatment plant.

The SRT can also be related to the specific biomass growth rate:

$$\mu = \frac{1}{SRT}$$

$$\frac{1}{142\ days} = 0.007 d^{-1}$$

The average specific biomass growth rate calculated from the 45 day moving average SRT values was 0.0088 d^{-1} (±0.003) or . This is a very low value as a typical growth rate for even slow growing nitrifiers is 0.75 d^{-1} (Tchobanoglous, Burton et al. 2003).

The plant began wasting more consistently after the December holiday period (during which time wasting was decreased because the staff at LANL was greatly decreased, leading to decreased loadings to the plant). Figure 7.21 depicts the 45 moving average SRT along with the mass in the system after the holiday period. There was a peak in the SRT towards the end of January 2012 and the tardigrade outbreak began in early February. It is probable that the tardigrade proliferation was induced by the lengthy SRT

(148 days) at this time of the year and this finding was supported by previous research (Moghaddam, Guan et al. 2003).

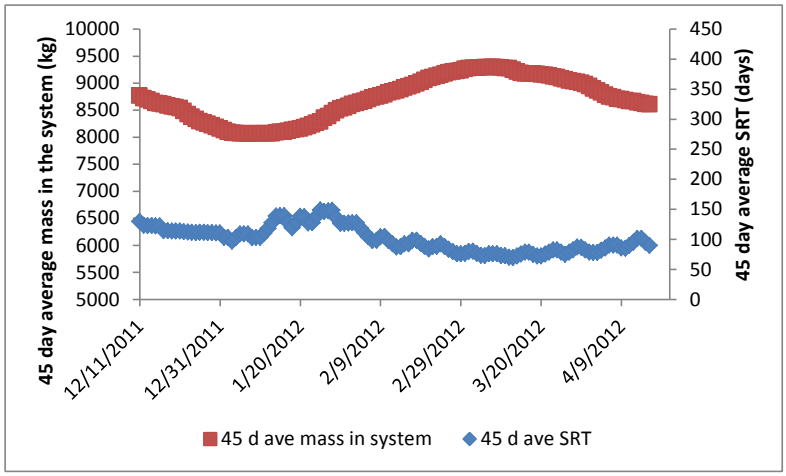


Figure 7.21: Calculated SRT results and the mass in LANL SWWS aeration basin with 45 day moving average

The SRT and the wasting rate are inversely related. A higher SRT results from less wasting, and tends to produce greater solids accumulation in the system (Tchobanoglous, Burton et al. 2003). This trend was reflected somewhat in figure 7.21, considering that variations in plant influent also affect biomass production. Furthermore, the 45 day moving average of the mass of solids in the system peaked towards the end of the seed event (Table 6.4) and declined once the seed event stopped.

The expected biomass concentration in the aeration tank was calculated to assess whether the measurements of influent concentrations and wasting rates and the biomass (MLSS) concentrations were in approximate agreement with the following equation (Tchobanoglous, Burton et al. 2003):

$$X = \left(\frac{SRT}{\tau}\right) * \left(\frac{Y(S_o - S)}{1 + (k_d SRT)}\right)$$

Several constituents and parameters were averaged (Table 7.3), and typical kinetic coefficients were assumed (Tchobanoglous, Burton et al. 2003) (Table 7.4) in order to perform the calculation. The minimum SRT of 70 days (Figure 7.21) was used.

Table 7.3: Average constituents and parameters used to calculate the biomass concentration within the aeration basins

Influent flow	$1.3 * 10^6$ (L/d)
Influent glycerin dose	$4.98 * 10^4$ (mL/d)
Influent TSS	221 (mg/L)
Reactor volume	$2.8 * 10^6$ (L)
Reactor MLSS	2969 (mg/L)
Reactor MLVSS	2394 (mg/L)

Table 7.4: Typical kinetic coefficients for the removal of organic matter from domestic wastewater*

k	5 (g bsCOD/g VSSd)
K_s	40 (mg bsCOD/L)
Y	0.4 (mg VSS/mg bsCOD)
k_d	0.1(g VSS/g VSSd)
VSS/TSS ratio	0.85 (g VSS/g TSS)
f_d	0.15 (g VSS/g TSS)

*(Tchobanoglous, Burton et al. 2003)

The following preliminary calculations were made:

$$\tau = \left(\frac{V}{Q}\right) = \left(\frac{2.8 * 10^6 L}{1.3 * 10^6 \frac{L}{d}}\right) = 2.2\ days$$

$$S = \left(\frac{K_s(1 + k_d * SRT)}{SRT(Yk - k_d) - 1}\right)$$

$$\frac{\left(40\ \frac{mg\ bsCOD}{L}\right)(1 + 0.1\frac{gVSS}{gVSS\ d} * 70\ days)}{70\ days\left(\left(0.4\ \frac{mg\ VSS}{mg\ bsCOD} * 5\frac{mg\ bsCOD}{mg\ VSSd}\right) - 0.1\frac{gVSS}{gVSS\ d}\right) - 1} = 2.42\ \frac{mg\ bsCOD}{L}$$

The influent suspended solids concentration (S_o), was calculated from the influent TSS, assuming 85% of TSS was biomass (Tchobanoglous, Burton et al. 2003):

$$\frac{221\left(\frac{mg\ TSS}{L}\right) * 0.85\left(\frac{VSS}{TSS}\right)}{0.4\ (\frac{mg\ VSS}{mg\ bs\ COD})} = 470\left(\frac{mg\ bsCOD}{L}\right)$$

The addition of the glycerin was also taken into account:

$$\frac{6.3 * 10^7\ \left(\frac{mg\ C_3H_8O_3}{d}\right) * 1.22\left(\frac{mg\ bsCOD}{mg\ C_3H_8O_3}\right)}{1.3 * 10^6\ (\frac{L}{d})} = 60\left(\frac{mg\ bsCOD}{L}\right)$$

The total influent soluble substrate as COD was:

$$S_o = 470\left(\frac{mg\ bsCOD}{L}\right) + 60\left(\frac{mg\ bsCOD}{L}\right) = 530\left(\frac{mg\ bsCOD}{L}\right)$$

The final concentration of heterotrophic biomass including growth on the influent COD and accounting for decay was calculated:

$$X = \left(\frac{70\ d}{2.2\ d}\right)\frac{0.4\ \frac{mg\ VSS}{mg\ bsCOD} * \left(530\ \frac{mg\ bsCOD}{L} - 2.42\frac{mg\ bsCOD}{L}\right)}{1 + \left(0.1\frac{gVSS}{gVSS\ d} * 70d\right)} = 837\ \left(\frac{mg\ VSS}{L}\right)$$

The synthetic yield coefficient and endogenous decay coefficient were responsible for a large decrease in the heterotrophic biomass concentration. The SRT of LANL SWWS was calculated to be high compared to typical SRTs, however due to the low influent COD concentration it was likely necessary in order to maintain a sufficient biomass concentration in the system. The measured average MLVSS (Table 7.3) of the LANL SWWS was 2394 mgVSS/L, which was much higher than the calculated value (837 mgVSS/L). An explanation for this discrepancy may be that much of the measured biomass in the reactors was in the form of cell debris.

The total MLVSS in the aeration basins includes biomass and inert materials in the form of cell debris and nonbiodegradable substrate (Tchobanoglous, Burton et al. 2003):

$$X_T = \left(\frac{SRT}{\tau}\right) * \left(\frac{Y(S_o - S)}{1 + (k_d SRT)}\right) + f_d k_d(X) SRT + \left(\frac{X_{oi} SRT}{\tau}\right)$$

The concentration of cell debris in the aeration basins was calculated:

$$f_d k_d(X) SRT = 0.15 \frac{g\ VSS}{g\ VSS} * 0.1 \frac{g\ VSS}{g\ VSS\ d} * 837 \frac{mg\ VSS}{L} * 70d = 879 \frac{mg\ VSS}{L}$$

This calculated value was higher than the heterotrophic biomass, most likely due to the lengthy SRT of LANL SWWS.

The nonbiodegradable VSS (nbVSS) in the influent was not measured and was calculated assuming that the remaining measured VSS in the aeration basins was nonbiodegradable:

$$\frac{X_{oi} SRT}{\tau} = (measured\ MLVSS - X - cell\ debris) = 678 \frac{mg\ VSS}{L}$$

The total MLVSS was calculated:

$$X_T = 837\frac{mg\ VSS}{L} + 879\ \frac{mg\ VSS}{L} + 678\ \frac{mg\ VSS}{L} = 2394\ \frac{mg\ VSS}{L}$$

It should be noted that the nonbiodegradable VSS was calculated using the measured concentrations of the MLVSS in the aeration basins. Unfortunately nbVSS was not measured by plant personnel and could not be verified. However, including heterotrophic biomass and cell debris at a 70 day SRT better compared with the measured MLVSS concentrations:

$$X_{biomass\ and\ debris} = 837\frac{mg\ VSS}{L} + 879\ \frac{mg\ VSS}{L} = 1715\ \frac{mg\ VSS}{L}$$

This calculated concentration accounted for 72% of the measured MLVSS in the aeration tanks. According to communications with plant personnel they have "significant side stream flow from runoff, sludge drying beds, and the dumping of honey wagons" which were not measured, and which would add additional influent solids and influent COD that were not accounted for in the above calculations, therefore it was assumed that the remaining VSS measured in the aeration basins was not nbVSS, but an unmeasured and likely biodegradable influent. The discrepancy between the calculated biomass and cell debris concentrations compared to the measured MLVSS supported this assumption.

The calculated heterotrophic biomass and cell debris concentrations were plotted as a function of increasing SRT (Figure 7.20). From this graph, it was concluded that while it was necessary for LANL SWWS to operate at high SRTs in order to retain enough biomass in the system, heterotrophic biomass accumulation plateaued at approximately 50 days. Operating at an SRT longer than 50 days likely had no benefit with respect to biomass. The figure also illustrated that at greater than a 65 day SRT, cell debris

accumulation exceeded biomass accumulation. This relationship between the increased length of time that biomass remained in a system to an increase in decay was expected.

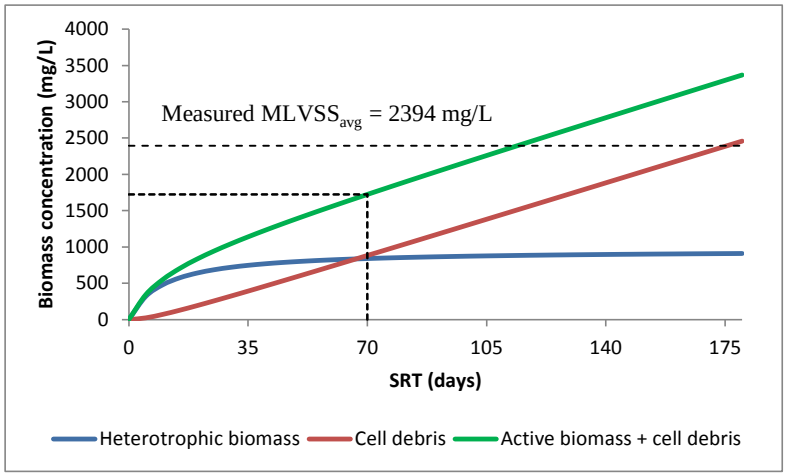


Figure 7.22: The effect of increased SRT on the heterotrophic biomass accumulation and cell debris accumulation of LANL SWWS

From the figure, it could also be observed that at a SRT of 114 days, the calculated total biomass in the system was equal to the average measured MLVSS of the plant.

7.4 Conclusions

Environmental parameters impact activated sludge microbial populations. This work demonstrated that 454-Pyrosequencing was useful to classify and identify the population responses to variations in plant conditions. Unfortunately due to the complexities of the consortia within activated sludge, little is known of each microbe's functionality and so few generalizations can be made presently. Furthermore, it is difficult to attribute one parameter to the proliferation of any microbe as some parameters are symptoms of others and that microorganisms often require a combination of various conditions.

Correlations were found between certain taxa and the operational characteristics and constituents of LANL SWWS. The SVI and the abundance of certain bacteria associated with flocculation were found to be negatively correlated. It could be inferred from this relationship that an increased abundance of these bacteria was beneficial for selling and compaction of the sludge. Yet further investigation into the measured SVI revealed that this parameter was correlated with the MLSS concentration and was likely not diluted and not an accurate indication of settleability. Other parameters that have causal interaction with bacteria such as the MLSS and DO were also correlated, however these relationships were likely symptomatic of operational characteristics of the plant (long SRT and nitrification/denitrification). Temperature variations had no effect on shifts in the microbial populations. The pH, and nitrogen availability within the SE aeration basin were correlated to certain taxa, yet were not found to be functionally connected to these wastewater characteristics. It should be noted that rather than sample once per week, an analysis of daily samples may provide more information on the impacts of environmental conditions.

A correlation was found between effluent ammonia and the relative abundance of *Nitrospirae* within the SE aeration basin. This NOB produces nitrate and the reason for the correlation requires further study. Nitrifiers were not prolific in the LANL SWWS, yet investigation into the effluent ammonia and nitrate concentrations suggested successful nitrification/denitrification. The C/N was calculated to clarify influent limitations that may affect these autotrophic bacteria. It appeared that the LANL SWWS was carbon limited based on calculations performed assuming a minimum C/N with glycerin as the sole carbon source. The C/N due to glycerin was calculated to be 1.1:1,

and was compared with the C/N necessary for balanced growth. The calculated C/N for both anabolism and catabolism was 5.9:1. This ratio was higher than the estimated ratio in the LANL SWWS and did not explain the scarcity of nitrifying bacteria. Alternative hypotheses for the effective nitrification/denitrification of the LANL SWWS were proposed and further research is needed. The absence of nitrifiers was observed because of the pyrosequencing technique, and may have not been investigated if other molecular methods were used.

The escalated presence of tardigrades in the LANL SWWS may be a symptom of the sludge remaining in the system for too long. An investigation into the SRT was performed with data provided by plant personnel. Wasting rates were sporadic during the time of this study and a 45 day moving average was used to calculate and effective SRT with a minimum of 70 days, and average of 142 days (± 85). This work proved that the SRT of LANL SWWS was lengthy for an activated sludge system and that while this was necessary to maintain biomass in the system due to the under loaded conditions, it may be excessive. The specific biomass growth rate was very low. Comparisons were made between the calculated biomass concentration in the system and the measured MLVSS in the system. The values did not correspond until and SRT of 114 days and it was believed that some influent biodegradable soluble substrate was entering the system without being measured. The inert material within the aeration basins was also calculated and a large proportion of cell debris within the aeration basins was indicative of a lengthy residence time.

454-pyrosequencing was robust enough for this analysis, yet the functionality of many of the microbes in activated sludge was unknown and reasons for their variation under

different parameters remains unclear. The coupling of 454-pyrosequencing with functional genetics may prove extremely useful from this perspective.

CHAPTER 8 CONCLUSIONS

The microbial communities of activated sludge were revealed by 454-pyrosequencing. This section summarizes the findings of this thesis presented in Chapter 5 through Chapter 7. The research objectives of this thesis were:

1) Determine the differences between LANL SWWS activated sludge population and other plants.
2) Identify and evaluate the effects of glycerin as a supplemental carbon source on the microbial population.
3) Define any adaptive characteristics or population changes due to the seeding event.
4) Determine if there were certain operational conditions or environmental factors that influence specific microbial populations.

In Chapter 5, comparison and analysis of the microbial communities of LANL SWWS, a unique WWTP utilizing glycerin as a supplemental carbon source, to Other WWTP activated sludge populations were performed. The principal conclusions from this chapter were:

1) The microbial consortia of the samples from LANL SWWS were taxonomically different compared to samples obtained from WWTPs that did not utilize and external carbon source. The principal differences between the LANL SWWS samples and the Other WWTPs was that the LANL SWWS samples had a higher

relative abundance of *Chloroflexi* versus the Other WWTP sample were abundant with *Proteobacteria*.

2) Unpaired two tailed T-tests of the glycerin fed plant (LANL SWWS) and the conventionally fed plants (Other WWTPs) samples confirmed that four phyla were significantly different between the two groups. This difference could be attributed to the glycerin as a supplemental carbon source, however the LANL SWWS operated at a lengthy SRT as well and further investigation is needed.
3) Alpha diversity analysis demonstrated that the microbial diversity within the Other WWTP samples was greater than the microbial diversity within the LANL SWWS samples.
4) Beta diversity analysis as visualized with PCoA concluded that the LANL SWWS samples were phylogenetically more similar to each other than they were to any of the Other WWTP samples.

Chapter 6 investigated the microbial community responses around a seed event. LANL SWWS experienced an upset and used biomass from LAC WWTP to recover. The findings from this chapter were:

1) Within the LANL SWWS samples, two phyla were found to be significantly different when compared before the seed event and after the seed event. This difference was not a reflection of the microbial population of the inoculum. Both phyla were found to be less abundant in the post-seed samples than the pre-seed samples, even though the sample taken from LAC WWTP exhibited greater relative abundance of the bacteria. This relationship may have been due to seasonal change rather than the effects of the seed event.

2) The amount of inoculum added to the system was not significantly large enough to induce a noticeable microbial shift.
3) Alpha diversity analysis revealed that the inoculum consortia were more diverse than all the LANL SWWS samples. The samples taken post-seed were the least diverse and the samples taken pre-seed and during the seed even were equally diverse.
4) Beta diversity from the taxonomic and phylogenetic perspective confirmed that the samples from the LANL SWWS and the inoculum were very dissimilar. There was a general clustering among the LANL SWWS samples according to pre-seed, during, and post-seed groupings. These clusters may be attributed to the microbial populations; however they also cluster based on seasonality.

Chapter 7 investigated to impacts of the operational and environmental characteristics on the microbial community in the LANL SWWS activated sludge. Primary conclusions were:

1) Microbial populations correlated with some environmental and operational parameters. The functions of most microbes are still unclear and diagnosing the implications behind the correlations was not straightforward.
2) Even though LANL SWWS added glycerin as a supplemental carbon source, the system was carbon limited. The C/N ratio was not favorable to autotrophic growth. Heterotrophs utilized influent ammonia to compensate for the carbon limitation and there was limited excess for the slower growing AOB and NOB. Nitrifiers were not very abundant in the aeration basin, confirming the competition between the two groups of bacteria.

The SRT was likely the reason behind the microbial dynamics within LANL SWWS. The SRT was calculated to be greater than 70 days and probably caused the proliferation of tardigrades, as it was greatest prior to the upset event. In order to retain biomass in the system, a lengthy SRT was beneficial, however over 50 days, it was no longer advantageous because biomass accumulation ceased, and cell debris accumulated.

CHAPTER 9 APPLICABILITY AND RECOMMENDATIONS FOR FUTURE RESEARCH

9.1 Applicability

This study demonstrated that utilizing 454-pyrosequencing as a method for identifying and classifying the microbial populations of activated sludge from a full-scale wastewater treatment plant is a useful technique. With the advancements in technology, sequencing methods will become more common as the efficiency and affordability continue to improve.

9.1.1 Full-scale wastewater treatment plant microbial identification

454-pyrosequencing and other sequencing technologies may be applied by individual facilities to gain a better understanding of the microbial communities present in the biological treatment train. While each treatment facility may have a unique distribution of consortia members, high throughout sequencing techniques may offer extensive insight that could not have been gleaned from traditional classification techniques. A baseline identification of the individual microbes that participate in each biological treatment facility would be useful. Rather than monitoring the effluent quality as a symptom of the effectiveness of the biological treatment, identifying changes in the microbial community as an indication of the health of the system may be more proactive and could mitigate any lengthy adverse effects. In the event of a plant upset, utilizing pyrosequencing to examine the recovery of the microbial population could be informative. A WWTP may also be required to treat constituents to more stringent standards, and to be able to identify microbes that are capable of degrading or treating certain components of their wastewater would aid in plant optimization.

9.1.2 Utilizing glycerin as a supplemental carbon source

LANL SWWS supplemented their low organic influent with glycerin. This study demonstrated that this external carbon source was useful in supporting adequate biomass for wastewater treatment. As a byproduct of biodiesel production, this compound is readily available as well as more cost effective and safer than other external carbon sources such as methanol or ethanol (Bodík, Blšťáková et al. 2009; da Silva, Mack et al. 2009; Guerrero, Taya et al. 2012).

9.2Recommendations

9.2.1 The microbial responses to the unique characteristics of LANL SWWS need further evaluation

Activated sludge is a complex environment and the pyrosequencing results from this study reflected the complicated nature of the microbial populations within this setting. This study confirmed that the community of organisms in a WWTP utilizing glycerin as a supplemental carbon source were different compared to those from conventional treatment plants. However, the dissimilarities may be due to operational parameters, namely the SRT. Further research is warranted to confirm if the variations were the result of glycerin enriching for certain organisms or that the lengthy SRT allowed for the proliferation of slow growing microbes and additional regression analysis may offer clarification. Results from this research would be useful as a reference point to evaluate the microbial community changes in response to various glycerin concentrations and increased wasting rates to decrease the SRT of the plant. Operating a lab scale reactor and manipulating the glycerin and SRT may also provide clarification.

9.2.2 The effects of the seeding event could be readdressed

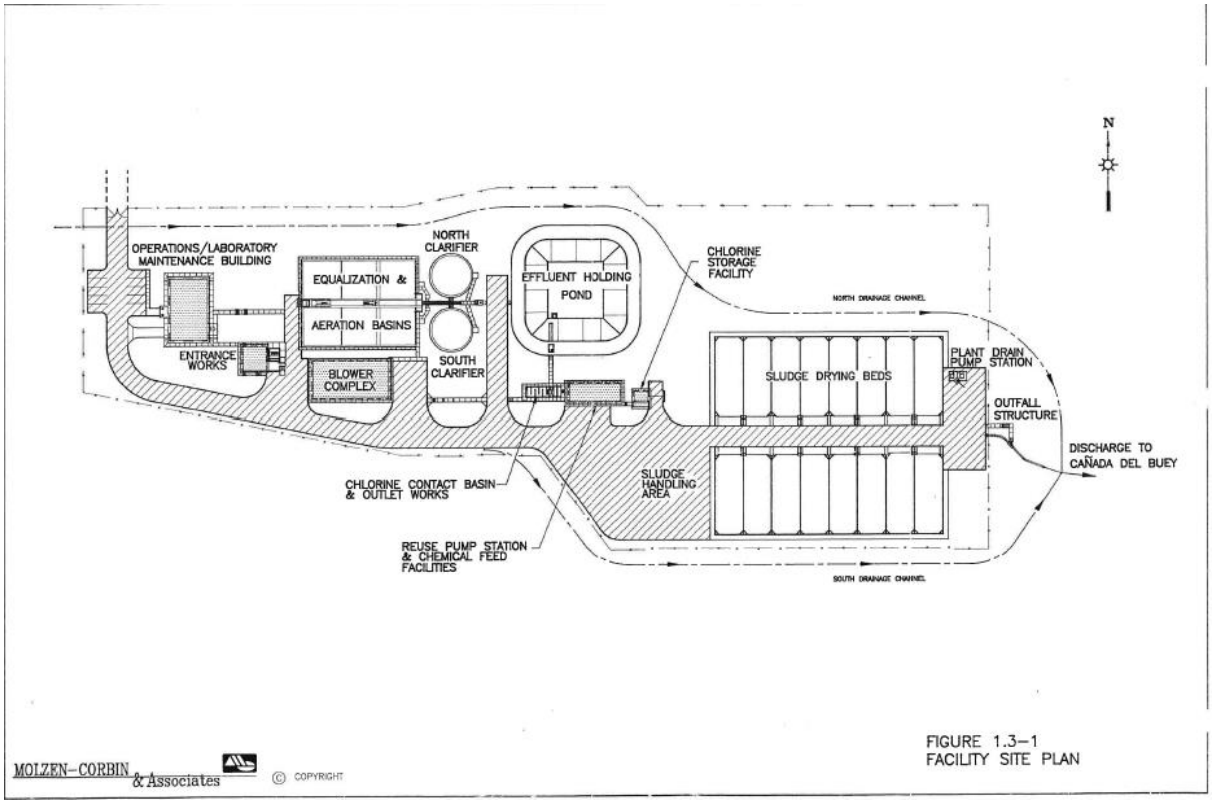
This study could not confirm a population response to the introduction of biomass from an outside facility. In the event that another upset requires LANL SWWS to reseed a portion of their activated sludge, samples should be taken directly surrounding the event, not only from LANL SWWS, but from the inoculation facility as well. Operational conditions and influent characteristics should also be monitored.

9.2.3 Plant operational characteristics and environmental conditions warrant further investigation on the microbial populations

This study demonstrated that there were certain parameters and conditions that were significantly correlated to a variety of microbes. Further investigation into these relationships is warranted as there was no definitive causality for many of the correlations. Additional measurements and stringent evaluation of the influent characteristics such as BOD and glycerin addition could yield more conclusive results. Additional analysis could also be performed on the plant parameters in conjunction with the pyrosequencing results. Multivariate data analysis methods such as analysis of similarity, and other nonparametric statistical methods may provide further insight. The results from this study indicate that LANL SWWS operates at an excessive SRT and an increased wasting rate is recommended as a possible solution to the preponderance of tardigrades.

APPENDICES

APPENDIX A: LANL SWWS SCHEMATIC



APPENDIX B: AVERAGE MEASURED PARAMETERS AND CHARACTERISTICS OF LANL SWWS

Date	Averages of week prior and post sample	Influent Flow MGD	EQ Flow MGD	Treated Water Flow MGD	Reuse Flow MGD	Total Volume of Basins Number	RAS Flow MGD	WAS Flow MGD	Influent pH SU	Influent Temperature Deg C
11/14/2011	11/7-11/21	0.27	0.35	0.30	0.29	0.75	0.30	0.01	7.90	19.26
12/6/2011	11/29-12/13	0.29	0.37	0.37	0.37	0.75	0.54	0.00	7.93	17.81
1/23/2012	1/16-1/30	0.34	0.38	0.36	0.37	0.75	0.31	0.01	7.46	18.68
1/30/2012	1/23-2/6	0.33	0.37	0.36	0.35	0.75	0.29	0.01	7.51	18.78
2/7/2012	1/31-2/14	0.33	0.36	0.35	0.35	0.75	0.29	0.01	7.61	18.58
2/14/2012	2/7-2/21	0.31	0.37	0.34	0.34	0.75	0.28	0.01	7.60	18.11
2/21/2012	2/14-2/28	0.29	0.35	0.33	0.32	0.75	0.28	0.01	7.56	17.65
3/5/2012	2/27-3/12	0.30	0.35	0.33	0.31	0.75	0.29	0.01	7.58	18.10
3/19/2012	3/12-3/26	0.30	0.36	0.34	0.34	0.75	0.25	0.01	7.55	18.29
4/2/2012	3/26-4/9	0.26	0.33	0.31	0.30	0.75	0.25	0.01	7.69	19.74
4/16/2012	4/9-4/23	0.24	0.31	0.28	0.27	0.75	0.27	0.01	7.55	18.69
5/8/2012	5/1-5/15	0.22	0.30	0.26	0.26	0.75	0.25	0.01	7.59	18.34
5/21/2012	5/14-5/28	0.19	0.25	0.23	0.23	0.75	0.28	0.01	7.62	19.66
6/11/2012	6/4-6/18	0.22	0.27	0.25	0.24	0.75	0.26	0.01	7.52	19.56
TOTAL AVERAGES		**0.28**	**0.34**	**0.32**	**0.31**	**0.75**	**0.30**	**0.01**	**7.62**	**18.66**

Date	Influent Alkalinity grab mg/L	Influent Alkalinity Composite mg/L	Influent TSS mg/l	Influent Ammonia mg/L	Influent Nitrate mg/L	EQ pH SU	EQ Temperature Deg C	EQ Alkalinity mg/L	EQ Ammonia mg/L	MLSS mg/L
11/14/2011	186.67	218.86	181.50	21.28	0.31	7.73	19.01	223.20	16.95	2870.00
12/6/2011	163.73	235.20	171.00	22.64	0.65	7.67	17.27	192.13	15.77	2846.67
1/23/2012	139.27	166.00	251.20	16.34	1.19	7.34	18.18	164.27	13.99	2754.55
1/30/2012	134.20	156.00	322.00	17.80	1.56	7.44	18.32	175.07	16.90	2714.00
2/7/2012	130.40	163.87	258.67	15.23	1.16	7.53	18.07	180.40	18.32	2867.27
2/14/2012	129.47	166.40	211.55	14.70	0.74	7.49	17.70	177.47	17.11	2946.00
2/21/2012	124.53	168.57	193.78	20.06	0.91	7.47	16.90	181.33	17.09	2963.00
3/5/2012	149.33	184.86	159.80	18.86	0.87	7.33	16.81	190.13	17.93	3110.77
3/19/2012	159.47	190.93	155.27	16.99	0.69	7.42	17.69	191.60	17.44	3234.55
4/2/2012	153.73	193.57	210.82	22.50	0.56	7.98	19.01	218.00	14.33	3351.82
4/16/2012	161.60	191.33	248.20	23.93	0.20	8.01	18.39	243.73	19.18	3232.73
5/8/2012	165.60	201.07	273.75	27.00	0.41	7.62	18.55	246.53	23.56	3059.00
5/21/2012	154.00	188.60	248.44	25.87	1.06	7.59	19.06	228.93	19.90	2928.00
6/11/2012	181.33	232.40	213.11	28.79	1.22	7.73	20.11	238.67	22.50	2688.33
TOTAL AVERAGES	**152.38**	**189.83**	**221.36**	**20.86**	**0.82**	**7.60**	**18.22**	**203.68**	**17.93**	**2969.05**

Date	MLVSS mg/L	MLSS Volatile Fraction %	Sludge Volume Index mL/g	Oxygen Uptake Rate mg/L-min	Respiration Rate mg/gm/hr	RAS Concentration mg/L	RAS Volatile Conc mg/L	RAS Volatile % %
11/14/2011	2280.00	79.71	320.43	0.08	2.14	5575.71	4404.29	79.00
12/6/2011	2267.78	79.67	322.00	0.07	1.86	4173.33	3311.11	79.44
1/23/2012	2184.00	79.60	347.89	0.07	1.91	5303.64	4130.00	78.70
1/30/2012	2169.00	79.90	352.89	0.08	2.22	5643.00	4506.00	79.70
2/7/2012	2311.82	80.64	334.20	0.09	2.35	6063.64	4870.00	80.27
2/14/2012	2385.00	81.10	329.90	0.09	2.20	5922.00	4750.00	80.30
2/21/2012	2392.00	80.80	332.10	No Data	No Data	5915.10	4760.00	80.50
3/5/2012	2517.27	81.18	324.18	No Data	No Data	6267.77	5060.91	81.18
3/19/2012	2629.09	81.27	304.36	No Data	No Data	6623.64	5490.91	83.00
4/2/2012	2713.64	81.09	289.64	No Data	No Data	6450.00	5223.64	81.00
4/16/2012	2614.55	81.00	299.27	0.14	3.18	5981.82	4818.18	80.55
5/8/2012	2488.00	81.34	316.13	0.17	4.03	5457.00	4423.40	81.06
5/21/2012	2386.00	81.47	330.11	0.16	4.10	5230.00	4211.40	80.59
6/11/2012	2184.55	81.59	350.44	No Data	No Data	4940.00	4031.82	81.08
TOTAL AVERAGES	**2394.48**	**80.74**	**325.25**	**0.11**	**2.67**	**5681.90**	**4570.83**	**80.45**

Date	SW AB pH su	SW AB Temp Deg C	SW AB Alkalinity mg/l	SW DO High mg/L	SW DO Minutes on min	SW DO Minutes off min	SW DO Minutes on Total min	SW DO Minutes off Total min	SW AB Ammonia mg/L	SW AB Nitrate mg/L
11/14/2011	7.16	18.65	156.80	1.94	5.33	39.67	170.67	1269.33	1.89	4.37
12/6/2011	7.09	16.80	134.80	2.10	5.20	39.80	166.40	1273.60	1.93	4.87
1/23/2012	6.88	17.76	129.07	1.73	5.40	39.60	172.80	1267.20	2.22	3.34
1/30/2012	6.89	18.07	133.87	1.66	5.73	39.27	183.47	1256.53	2.68	3.26
2/7/2012	6.90	17.93	139.20	1.53	6.60	38.40	211.20	1228.80	3.31	3.31
2/14/2012	6.91	17.57	143.47	1.45	6.47	38.53	206.93	1233.07	2.71	3.21
2/21/2012	6.90	16.81	144.40	1.42	5.77	39.23	184.62	1255.38	2.45	3.33
3/5/2012	6.91	16.61	166.80	1.44	5.80	39.20	185.60	1254.40	3.03	1.73
3/19/2012	6.91	17.38	174.27	1.42	6.40	38.60	204.80	1235.20	2.54	1.07
4/2/2012	7.13	18.80	197.47	1.21	6.43	38.57	205.71	1234.29	1.97	0.54
4/16/2012	7.16	18.47	193.60	1.54	6.53	38.47	209.07	1230.93	2.65	0.73
5/8/2012	7.08	18.45	190.40	1.64	6.50	38.57	207.60	1232.40	2.65	1.24
5/21/2012	7.06	19.35	163.60	1.39	5.71	38.93	185.14	1254.86	2.33	2.57
6/11/2012	7.04	20.19	163.73	1.65	6.13	38.87	196.27	1243.73	2.36	4.59
TOTAL AVERAGES	**7.00**	**18.06**	**159.39**	**1.58**	**6.00**	**38.98**	**192.16**	**1247.84**	**2.48**	**2.73**

Date	SE AB pH su	SE AB Temp Deg C	SE AB Alkalinity mg/l	SE DO High mg/L	SE DO Minutes on min	SE DO Minutes off min	SE DO Minutes on Total min	SE DO Minutes off Total min	SE AB Ammonia mg/L	SE AB Nitrate mg/L
11/14/2011	7.19	18.73	153.87	1.08	2.87	42.13	91.73	1348.27	0.65	3.95
12/6/2011	7.05	16.75	133.87	1.18	3.27	41.73	104.53	1335.47	0.98	4.64
1/23/2012	6.91	17.86	128.13	1.24	3.60	41.40	115.20	1324.80	0.92	3.65
1/30/2012	6.88	18.15	133.20	1.22	3.53	41.47	113.07	1326.93	1.01	3.33
2/7/2012	6.88	17.99	137.73	1.08	3.80	41.20	121.60	1318.40	1.48	2.61
2/14/2012	6.93	17.61	140.67	1.08	3.73	41.27	119.47	1320.53	1.18	2.39
2/21/2012	6.90	16.78	142.67	1.14	3.54	41.46	113.23	1326.77	0.96	2.81
3/5/2012	6.93	16.74	164.13	1.04	3.47	39.13	153.60	1286.40	1.29	0.87
3/19/2012	6.94	17.49	173.07	1.15	3.73	41.27	119.47	1320.53	0.82	0.31
4/2/2012	7.07	18.96	196.80	0.97	3.64	41.36	116.57	1323.43	0.80	0.02
4/16/2012	7.08	18.56	199.60	1.03	3.73	41.27	119.47	1320.53	0.92	0.16
5/8/2012	7.04	18.61	187.20	1.31	3.64	41.36	116.57	1323.43	0.96	0.31
5/21/2012	7.00	19.29	162.53	1.17	3.57	41.43	114.29	1325.71	0.87	1.95
6/11/2012	7.00	20.29	163.73	1.26	3.67	41.33	117.33	1322.67	0.82	3.09
TOTAL AVERAGES	**6.98**	**18.13**	**158.37**	**1.14**	**3.56**	**41.27**	**116.87**	**1323.13**	**0.98**	**2.15**

Date	NE AB pH su	NE AB Temp Deg C	NE AB Alkalinity mg/l	NE DO Minutes on min	NE DO Minutes off min	NE DO High mg/L	NE DO Minutes on Total min	NE DO Minutes off Total min	NE AB Ammonia mg/L	NE AB Nitrate mg/L
11/14/2011	7.22	18.63	161.07	1.07	43.93	0.65	34.13	1405.87	0.25	2.24
12/6/2011	7.10	16.57	138.13	1.27	43.73	0.73	40.53	1399.47	0.62	3.49
1/23/2012	6.97	17.83	131.87	1.53	43.47	0.82	49.07	1390.93	0.37	1.83
1/30/2012	6.94	18.03	136.40	1.80	43.20	0.96	57.60	1382.40	0.31	1.51
2/7/2012	6.96	17.93	141.20	2.00	43.00	0.83	64.00	1376.00	0.55	1.17
2/14/2012	6.95	17.60	147.33	1.67	43.33	0.65	53.33	1386.67	0.50	0.63
2/21/2012	6.91	16.79	149.33	1.31	43.69	0.61	41.85	1398.15	0.35	0.90
3/5/2012	6.90	16.56	168.53	1.47	43.53	0.74	46.93	1393.07	0.52	0.61
3/19/2012	6.89	17.41	172.80	1.40	43.60	0.76	44.80	1395.20	0.30	0.22
4/2/2012	7.05	18.79	198.93	1.50	43.50	0.73	48.00	1392.00	0.41	0.13
4/16/2012	7.10	18.41	199.60	1.53	43.47	0.57	49.07	1390.93	0.51	0.00
5/8/2012	7.11	18.49	190.93	1.57	43.43	0.74	50.29	1389.71	0.48	0.00
5/21/2012	6.96	19.21	168.67	1.50	43.50	0.73	48.00	1392.00	0.28	0.71
6/11/2012	7.01	20.23	167.47	1.60	43.40	0.85	51.20	1388.80	0.26	1.64
TOTAL AVERAGES	**7.01**	**18.04**	**162.30**	**1.52**	**43.48**	**0.74**	**48.49**	**1391.51**	**0.41**	**1.08**

Date	Treated Water pH SU	Treated Water Temperature Deg C	Treated Water Alkalinity Grab mg/L	Treated Water Alkalinity Composite mg/L	Treated Water Chlorine Residual Free mg/L	Treated Water Chlorine Residual Total mg/L	Treated Water TSS mg/L	Treated Water Ammonia mg/L	Treated Water Nitrate mg/L
11/14/2011	7.68	19.35	160.13	159.60	1.44	2.15	13.04	0.08	2.41
12/6/2011	7.61	16.94	134.13	132.43	1.30	2.16	8.11	0.05	3.77
1/23/2012	7.37	18.06	126.27	126.00	1.42	1.96	2.44	0.07	2.19
1/30/2012	7.28	18.21	132.80	129.71	1.44	1.87	2.20	0.09	1.74
2/7/2012	7.22	18.09	132.53	129.50	1.47	1.78	1.47	0.08	1.10
2/14/2012	7.29	17.75	134.00	131.67	1.55	1.99	1.24	0.05	0.89
2/21/2012	7.29	16.87	137.33	136.00	1.56	2.10	1.02	0.02	0.95
3/5/2012	7.19	16.24	151.07	151.71	1.79	2.29	1.30	0.03	0.36
3/19/2012	7.29	17.36	162.67	156.67	1.54	2.05	0.92	0.07	0.28
4/2/2012	7.41	18.76	182.40	188.00	1.75	2.30	1.37	0.03	0.00
4/16/2012	7.46	18.24	181.33	185.57	1.89	2.40	1.37	0.03	0.01
5/8/2012	7.49	18.46	171.73	174.40	1.27	1.89	1.72	0.05	0.05
5/21/2012	7.41	18.71	154.80	154.13	1.34	1.94	1.93	0.03	0.99
6/11/2012	7.49	20.14	251.71	157.60	1.25	1.77	3.44	0.02	1.83
TOTAL AVERAGES	**7.39**	**18.08**	**158.07**	**150.93**	**1.50**	**2.05**	**2.97**	**0.05**	**1.18**

APPENDIX C: PROTOCOLS

CTAB S DNA extraction by Microbial Observatory Method

Reference: (Mitchell and Takacs-Vesbach 2008)

Equipment

1. Microtubes
2. Pipettes
3. Waterbath
4. Freezer
5. Sonicator
6. Centrifuge
7. Fume hood

Solutions

1. Sucrose lysis buffer (SLB): 20 mM EDTA, pH 8.0; 400 mM NaCl; 0.75 M sucrose; 50 mM Tris-HCl, pH 9.0
2. CTAB Buffer: 1% CTAB, 0.75 M NaCl, 50 mM Tris pH8; 10 mM EDTA
3. Proteinase K
4. 10% SDS
5. Phenol:chloroform:isoamyl alcohol (24:24:1)
6. Chloroform
7. 3 M Na Acetate
8. Absolute ethanol
9. 70 % ethanol
10. 10 mM Tris pH 8.0, Filter sterilized

Procedure

1. Add equal volume (100 µL) of SLB to sample, freeze (-23°C) overnight or until ready to extract DNA
2. Add 400 µL of CTAB buffer to 200 µL thawed sample from above
3. Add proteinase K to a final concentration of 100 µg/mL (3 µL of 200x stock solution)
4. Incubate for 1 hour in a water bath at 60°C, inverting periodically to mix
5. Add SDS to a final concentration of 2% (200 µL sample with 10% SDS = 120 µL of SDS)
6. Incubate for 1 hour in a water bath at 60°C, inverting periodically to mix

7. Under a fume hood add an equal volume of Phenol:chloroform:isoamyl alcohol (720 µL)
8. Invert to mix, allow to precipitate and draw off top aqueous portion into new microtube, discard remainder
9. Add an equal volume of chloroform
10. Allow to precipitate and draw off top aqueous portion into new microtube, discard remainder
11. Add an equal volume of chloroform
12. Allow to precipitate and draw off top aqueous portion into new microtube, discard remainder
13. Add 0.1 volumes of 3 M NaOAc, invert gently,
14. Add 2 volumes of absolute ethanol
15. Precipitate for 1 hour or overnight at -23°C
16. Centrifuge thawed samples for 45 minutes at 11,000 rpm
17. Draw off supernatant
18. Add 500 µL of 70% ethanol
19. Centrifuge samples for 30 min at 11,000 rpm
20. Draw off most of the ethanol and allow the rest to volatize until completely dry
21. Add 50 µL of 10 mM Tris pH 8.0 and freeze until ready to sequence

QIIME Protocol

References: (Lozupone and Knight 2005; Wang, Garrity et al. 2007; Quince, Lanzen et al. 2009; Caporaso, Bittinger et al. 2010; Caporaso, Kuczynski et al. 2010; Edgar 2010; Price, Dehal et al. 2010; McDonald, Price et al. 2012; Werner, Koren et al. 2012)

Equipment:

1. QIIME software version 1.8.0
   Install QIIME:
   - Depending on the version of QIIME the user needs and the operating system he or she will be using will dictate how this is done.
   - www.qiime.org contains the most recent versions of QIIME for download. Follow the instructions under the heading "Getting started with QIIME Installing:" based on the necessary operating system.
2. .sff files from Research Testing Labs (RTL) Lubbock, Texas
   - When RTL has finished the 454-pyrosequencing, they will email 3 links, analysis, FASTA files, and .sff files.
   - The .sff files were the only files used in this protocol because filtering and denoising was performed by the user.

****general codes used throughout the procedure are in bold print
****all script in [ ] are suggestions the user can define such as file paths and names of out puts. Do not use [ ] in the actual script.
****when naming a file or directory, do not use special characters or spaces, as this will confuse QIIME

Procedure:

1. **print_qiime_config.py**
   This script ensured that QIIME was installed correctly. This will print out the versions and various dependencies that are being used in the pipeline.

Preparing the raw data:

1. Process the "raw" .sff files
   RTL will provide .sff files for all samples individually. In order to read these files, convert them to FASTA files, which contain 3 types of files, a .qual, a .txt, and a .fna (FASTA nucleic acid).

   process_sff.py -i [path to sff file directory] -f -o [path to new directory]

The –f converted individual .sff files into a readable text file (also known as a flowgram, hence the -f). This will result in a new directory with .fna, .qual. and .txt file.

2. Create mapping files
   Individual mapping files were created for each sample. The mapping files relay information about components of the sample that the researcher is interested in. There are a minimum of four tab delimited columns necessary for the mapping file. These files were created in Microsoft Excel 2010 for each sample and saved as tab delimited files. The first row (four columns) must be exactly in the format below:

#SampleID	BarcodeSequence	LinkerPrimerSequence	Description
LANL.11.14.11	AACTAACG	GAGTTTGATCNTGGCTCAG	LANL.11.14.11

- The #SampleID is how the sample is identified further down the pipeline.
- The BarcodeSequence is the barcodes used for each sample. They were obtained from the .txt files created from processing the .sff files from RTL. The .txt files created from step 1 contain many different flowgrams for the different sequences found in this sample. The first twelve bases (usually in lowercase text) of the sequences are descriptive bases used to identify the samples. The first four are the order in which the nucleotides were flown over each sequencing plate during pyrosequencing. The following eight are the barcodes.
- The LinkerPrimerSequence will vary depending which primer was requested, (in this case, 28-F). RTL provided this primer sequence, but it can be found in the original .txt file from the FASTA file provided by RTL as well.
- The Description is any descriptive terminology useful for analysis.

A total of 22 mapping files were created and imported into QIIME.

- Additional columns may be added to this mapping file in order to study the effects of different groupings or manipulations on the overall study distribution, such as pH levels, flow characteristics, or defined controls and variables like before and after a treatment or seasonality.

****Initially, one mapping file per sample must be created, however these can be combined later.

validate_mapping_file.py -m [path to mapping file.txt] -o [path to mapping output]
This will ensure that the mapping files are in the correct format and free of typos. In the newly created mapping output file, information about errors and warnings can be viewed as well as a corrected_mapping.txt. file which will show you where your errors might be.

3. Trimming/Filtering and Denoising the files
   The algorithm AmpliconNoise (Quince, Lanzen et al. 2009) was used in place of demultiplexing/quality filtering and manually denoising the sequences. AmpliconNoise removed the barcodes and forward primer, pyrosequencing noise, and PCR amplification point errors. Perseus removed the chimeras. Denoising must be run on each individual sample.

   ampliconnoise.py -i [path to sff.txt files from step 1] -o [path to denoised file directory] -m [path to mapping file for individual sample] -n [number of processors to use] –platform [type of chemistry used by sequencing lab]

   During this project, multiple processors were available and depending on the size of the sff.txt file, between 4 and 8 processors were used. RTL used Titanium chemistry during pyrosequencing and that was the specified platform. AmpliconNoise automatically truncated the sequences to 400bp for Titanium. Each sample took between 4 and 48 hours to denoise and resulted in fewer, yet more reliable sequences.

4. Concatenate the denoised files
   The samples were then concatenated into one larger .fna file based on which samples were to be tested further down the pipeline. The * command was used to group all file types specified.

   cat [path to where all denoised .fna files/*.fna] > [path to newly named large .fasta file]

Analysis:

1. Make an OTU table and build a phylogenetic tree

   pick_de_novo_otus.py -i [path to concatenated .fna files] -o [path to OTU directory]

- The sequences were clustered. The default method for clustering sequences was uclust. An initial sequence was designated as a seed, and the next sequence must be 97% (default) similar to be clustered with it. If it was not 97% similar, a new cluster was created with that sequence as a second independent seed.
- A representative sequence was then determined from each cluster to represent a specific OTU.
- Taxonomy was assigned to each OTU using the classifier algorithm RDP 2.2 (Ribosomal Database Project's Naïve Bayesian Classifier). These representative sequences were matched to an outside curated environmental database (Greengenes).
- The result of this algorithm was an OTU table in .biom format with numeric representations and taxonomic labels for each OTU. This OTU.biom table was used for subsequent taxonomic analysis.
- The sequences were also aligned and filtered in order to form a phylogenetic tree. The alignment tool Python Nearest Alignment Space Termination (PyNAST) was used to align the sequences with existing aligned sequences from the Greengenes data base. These sequences were then filtered again to remove any alignments with gaps or outside the V1-V3 hypervariable region.
- An approximately-maximum-likelihood phylogenetic tree was built from these aligned and filtered sequences with the software FastTree 2.1.3. This tree was used for subsequent phylogenetic analysis.

To view the statistics of this table, such as the sequences per sample, the minimum or maximum sequences per sample and the number of unique OTUs: **biom summarize-table -i [path to OTU_table.biom] -o [path to summary.txt]**

2. Taxonomic composition summary

   summarize_taxa_through_plots.py –i [path to OTU_table.biom] -o [path to taxa summary] -m [path to mapping file]

   This resulted in a new directory with .biom and .txt files for each level of taxonomy L2=phylum level, L6=genus level. There will also be taxa_summary_plots file that contains HTML connections to view these summaries in area or bar chart formats.

3. Alpha Diversity

The diversity within each sample or groups of samples, also known as alpha diversity was investigated. There are multiple diversity metrics that may be specified depending on the ecological approach of the researcher. The Shannon Diversity Index was used to investigate the diversity because it measure species richness and evenness and was designated in a parameter.txt file. The OTU table was first rarefied to the least amount of sequences per sample (1343 from sample LANL.2.7.12). This step was necessary because it randomly chooses the same number of sequences from each sample to mitigate influence caused by sequencing depth or heterogeneity.

alpha_diversity.py –i [path to OTU_table.biom] –m [path to mapping file] –o [path to alpha diversity directory] –t [path to rep_set.tre file] –e number of sequences per sample to rarefy to –p [path to parameter .txt file to specify metrics used]

This workflow resulted in rarefaction plots HTML links and text files that were imported into Microsoft Excel for further inspection.

4. Beta Diversity

Beta diversity was used to demonstrate the diversity between samples. The samples were again rarefied to the lowest amount of sequences per sample. Based on the composition of each community, a phylogenetic tree was created and square matrices were created in which the distance between each pair of samples was calculated via UniFrac. Principal Coordinates Analysis (PCoA) was used to visualize these matrices. PCoAs axes represent the principal coordinates and have no correlation to the original data set, they are rotated axis to show the maximum distances between samples. Samples that cluster together have similar phylogenetic trees.

beta_diversity_through_plots.py -i [path to OTU_table.biom] -o [path to directory for beta diversity files] -m [path to mapping files] -t [path to rep_set.tre] - e number of sequences per sample to rarefy to

This workflow resulted in several files, each labeled either weighted or unwieghted. The weighted files were used in this analysis because the aim was to evaluate the samples based on relative abundance rather than presence/absence. Distance matrices and principal coordinate matrices were generated as well as links to 3 dimensional emperor PCoA plots.

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