Analysis of the Continually Diversifying Microbial Community in a Wastewater Sequencing Batch Reactor

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Abstract:

Sequencing Batch Reactors (SBRs) increase the biofilm surface area in sewage treatment units that decrease the nitrogen content of wastewater. Decreased nitrate concentrations improve the effluent quality and therefore reduce the risk associated with eutrophication of sensitive aquatic habitats. My hypothesis is that the species composition of the SBR biofilm will change during the course of operation. Samples were collected from the first batch reactor at two separate time points in 2006, the influent that feeds the reactor in 2007 and the newest SBR biofilm in 2008.

SBR and influent samples were diluted and cultured on tryptic soy agar and other differential media at different temperatures. Cultured organisms were identified by amplifying the 16S rRNA genes of each culture and sequencing the 16S rRNA gene. Biochemical tests were also used to analyze some specific metabolic characteristics of the cultured organisms. Nitrate reduction, denitrification, and urease activity were the specific characteristics that were of interest in this study. 16S rRNA genes from uncultured organisms were amplified and cloned. These organisms were sequenced and analyzed at a later date.

The prevalent community members changed with time and this study showed that a shift was made from a predominantly γ- Proteobacteria (Enterobacteriaceae) community to one that was much richer in diversity. This study supported the basic ideas behind biofilm formation and showed that over time, the slow colonizers were able to become integral members of the community.

Introduction:

Sequencing Batch Reactors (SBRs) are a relatively new improvement to the world of wastewater treatment. They provide a smaller and more convenient method of wastewater treatment for home and business owners who are not connected to a town's water and sewer system. SBRs also eliminate the need for sand mounds associated with septic systems. In a SBR, there are physical separations between various processes, as well as biological conditions, such as oxygen levels, separating different processes. The SBR provides timed cycles of aerobic and anaerobic conditions to treat the wastewater. In the aerobic cycle, bacteria have oxygen available for respiration and are able to carry out various metabolic processes. In the anaerobic cycling phase, nitrate reduction and denitrification occur which reduces nitrate and nitrite loads in the effluent. Denitrification occurs when nitrate is reduced to nitrite and finally to nitrogen gas which is released into the atmosphere. This is important since high nitrate and nitrite levels in the river cause algal blooms and fish kills. Lower nitrate and nitrite levels are especially prevalent in the Williamsport area since the Susquehanna River is the largest waterway to feed into the extremely sensitive waters of the Chesapeake Bay. The overall goal of wastewater treatment is to reduce nutrient levels in the effluent. SBRs are beginning to provide a better way than fixed bed systems to accomplish this. In one study, a SBR with a denitrifying biofilm was able to reduce 80% more nitrate and nitrite than a comparable wastewater treatment plant with only a nitrifying biofilm (Daims et. al., 2001).

Prior Research

Many researchers have tried to determine ways that a SBR can have optimal conditions to grow the desired nitrifying and denitrifying microbes. Some groups have also examined the composition of the community to find ammonium oxidizing bacteria (Daims et. al., 2001). One group looked specifically at aerobic granulation and the effect this had on the reduction of nitrate. A granule is a small conglomeration of microbes. Daims found that the granules in their SBR designated SBR1 grew at a much faster rate than the granules in the SBR designated as SBR2. The difference between the two systems was a pulse environment in SBR2. Pulses are intentional physical disturbances in the reactor that contribute to the overall stability of the microbial ecosystem. The larger granules of SBR1 were initially beneficial because they removed more nitrogen. On the other hand, the larger granules started to fall apart when they reached a maximum size and the system had a significant decrease in nitrate reduction and denitrification. The granules that grew at a steadier, slower rate never seemed to reach a maximum size in the time frame examined in the study. These granules also continued to reduce more nitrate as time passed. This led the research group to conclude that the community was never exceptionally stable in SBR1 (larger granules) but was much more stable in SBR2. It was concluded that the pulse slowed the maturation of the organisms and therefore affected the stability of the granules (Xia, Zhang, Wang, 2006).

Zaiat's group also found that the agitation type and timing greatly affected the microbial community in the system. Again, it was concluded that too much agitation destroys the granules. This destruction is detrimental to the efficiency of the system to

remove nutrients. Temperature also affects the microbes that are present. Lower temperatures, especially around 5℃, seem to increase the diversity not only of microbes on the inside of the granules but in the overall population as well (Zaiat et. al., 2001).

It has also been found that a more alkaline pH increases the efficiency of the community in decreasing organic nutrients, especially the efficiency of denitrifying organisms (Wang et. al., 2007). This is relevant to this project because the microbial communities will be examined. The nitrate reducing and denitrifying portion of the overall communities are of specific interest to this project. This may also be helpful later when others are developing optimal conditions for the SBR; perhaps a pulse environment can be applied to develop and maintain a more stable community. Temperature, pH conditions, and timing of the cycles in the reactor could also be optimized for the desired microbial community.

Another recent development in the field of wastewater treatment is based on the discovery of “Anammox” bacteria. The term “Anammox” is applied to organisms that are capable of oxidizing ammonium in an anaerobic environment. The identity of organisms capable of this metabolic pathway in a sequencing batch reactor, or any wastewater treatment facility, would be a wonderful find since these organisms can oxidize ammonium to nitrogen gas. The reactants of this pathway are ammonium and nitrogen dioxide and the products are nitrogen gas and water. The nitrogen gas is released into the atmosphere and the effluent poses less of a threat to the surrounding waterways than effluent with higher nitrate and nitrite levels. Anammox is currently estimated to remove 50% of fixed nitrogen from the global ocean system (Strous et. al.,

2006). The removal of fixed nitrogen from a wastewater treatment reactor would be ideal but identifying organisms capable of Anammox is not an easy task. The organisms that are known to carry out Anammox are extremely slow growing, with division only occurring once every two weeks if the bacteria are dividing at maximum speed (Strous et. al., 2006). While there are media that select specifically for ammonia oxidizing bacteria, the rate of growth is still very slow. Therefore, since culturing organisms capable of Anammox is so time consuming and difficult, non-culture based methods are the best choice to identify these organisms.

Another group researched the differences in community richness in thermophilic (temperature range from 45℃ to 65℃) and mesophili c (temperature range from 25℃ to 35℃) reactors. Although there were organisms g rowing in the thermophilic reactor, there was much more diversity in the reactor with the mesophilic temperature. Their research also supported the hypothesis that longer retention times, 15 hours as opposed to 7 hours, in each stage of the treatment process increased the overall diversity of the community (LaPara et. al., 2000). Again, this study is important because possible optimizations could be made to the reactor based on these data.

Many methods were utilized to identify organisms from the above studies. Some groups believe that the newer molecular methods are the most useful in their research areas. Others have found that the best techniques are the more traditional ones. Microbial culturing on agar plates is still an excellent method to use, but one must account for the fact that less than 10% of microbes can be grown in the laboratory. Therefore it is important to keep this in mind when assessing, and making conclusions about, the microbial population (Amann, Ludwig, Schleifer, 1995). It is important to

realize that “even if the plate is called non-selective, one can not obtain a reasonable insight into the microbial community by only using one medium” (Amann, Lemmer, Wagner, 1998). Another problem with culturing organisms is that many of the organisms are part of granules. It is not an easy task to convert the granules full of organisms into single cells (Amann, Lemmer, Wagner, 1998).

To combat these problems, RNA specific probes have been developed, and some researchers have relied on fluorescent in-situ hybridization (FISH) to determine the composition of the microbial community. FISH uses specific probes to identify members of the microbial community. There are even specific probes for nitrate reducing bacteria but these have not been obtained yet for this study. All of the above methods will be taken into consideration in regards to this work.

Terahara et. al. believe that cloning and denaturing gradient gel electrophoresis (DGGE) are too time consuming for common usage. DGGE shows individual bands for the numerous members present in the community. It also gives an overall view of the diversity of the community. This group resorted to using terminal restriction fragment length polymorphism via 16S rRNA and 16S rDNA analysis (Terahara, et. al., 2004). While this molecular approach is very beneficial for identification of organisms in a SBR, cloning will be used in this project to identify members of the microbial community. The terminal restriction fragment length polymorphism and DGGE techniques will not be used in this study. While the advantages of DGGE are evident in obtaining a view of the microbial community as a whole, it does not allow one to identify individual organisms without further DNA sequencing.

Biofilm Formation

In a biofilm, the initial colonizers form the "base" for all of the later colonizers to build upon. The later colonizers that may not be able to adhere to the surface material may be able to adhere better to the initial colonizers and the extracellular polysaccharides that are being produced and secreted. Once enough microbes have integrated into the biofilm; quorum sensing (intercellular signaling) is used to indicate a need for growth through cell division or recruitment. Biofilms are highly competitive once they are established and organisms are able to secrete substances that are toxic to other organisms in the biofilm, thus freeing up space and nutrients (Watnick and Kolter, 2000). It is through this process that biofilms continually change and carry out different metabolic process as different organisms integrate into the biofilm. The organisms that integrate more slowly into the biofilm, such as those capable of nitrate reduction and denitrification, are of great interest to this research.

Cromaglass Technology

The Cromaglass sequencing batch reactors (Appendix Figure 1) operate on the basic premise of aerobic and anaerobic cycling. Since the environments in the reactor are separated by time and not space, Cromaglass has been able to develop reactors on a much smaller scale than a traditional fixed bed sewage treatment system. Suspended in the reactor are multiple PVC tubes that contain a specific form of "fixed film media". This media allows for biofilm formation in the reactor. The placement of the "fixed film media" is important because activated sludge is the most metabolically active portion of the sewage treatment process. Hanging the "fixed film media" in the chamber where

the activated sludge is present will allow for a more diverse and active community to establish the metabolically diverse biofilm.

This project will provide increased evidence that the microbial community in an SBR affects the overall performance. The knowledge gained about the nitrate reducing and denitrifying communities will also supplement current knowledge on better ways to use the natural metabolisms of microbes to treat wastewater. Data collected from sample analysis will hopefully provide more insight into possible methods for optimization of SBR conditions based on the desired community.

Materials/Methods:

Collection of samples

Samples from the SBR biofilm or the influent were collected at the Williamsport Sanitary Authority Central Plant, located just off of Interstate 180E, on four different occasions. The first samples were collected from the biofilm on September 28 and December 27, 2006 at 2 and 12 weeks after the commencement of operation of the SBR in 2006. The influent sample was collected from the influent wastewater that feeds the SBR on October 16, 2007. The most recent sample was taken from the SBR biofilm on January 22, 2008 at 2 week after the start-up of the new SBR.

All samples, excluding the influent, were collected directly from the SBR biofilm (Appendix Figure 2). An approximately 1”x1” sample of the biofilm was collected from the “fixed film media”, also known as a “coffee can” (Appendix Figure 3), which was located in a PVC tube and suspended in the SBR. The biofilm samples were inoculated into tubes with sterile water and were transported back to the lab for further purposes.

Methods for Identifying Cultured Organisms

Multiple samples were, and will continue to be, collected to determine whether the microbial community is changing and increasing over the desired time frame. When the sample was first collected from the biofilm of the SBR "coffee can" (Appendix Figure 3), it was diluted 10^{-2}, 10^{-4}, and 10^{-6} with vigorous but brief vortexing at maximum speed to separate the cells. 100 uL of each dilution was spread onto microbial growth plates which were incubated at room temperature, 37℃ and 45℃ for 2 days to 1 week to obtain a more diverse collection of organisms.

Multiple microbial growth media types were used in the most recent sample to obtain an even greater diversity of organisms. Tryptic Soy agar (BD Diagnostic Systems) was the basic media type that was used, since it is a general medium and many organisms will grow on it. Hektoen Enteric agar (BD Diagnostic Systems) was also used and is specific for enteric bacteria including *Salmonella* and *Shigella* species. Mannitol Salt agar (BD Diagnostic Systems) was also used; this agar selects specifically for *Staphylococcus* species but can grow other organisms, though not very well. After more than 120 well-isolated colonies were obtained, colonies were patched onto fresh plates containing the same media that they were originally grown on. The plates were incubated for 2 to 3 days.

After the colonies were patched, each patch that grew was inoculated into 500 uL microcentrifuge tubes with 100 uL of sterile water. This cell and water mixture was then frozen and thawed twice at -80° for rapid freezing and on a heating block a t 37° for rapid thawing. Subsequently, a PCR master mix was prepared and 19 uL of master mix were aliquoted into separate labeled tubes and to each tube was added 1 uL of the

frozen and thawed cells. The primers used in these reactions were 27f and 1492r (Sigma-Genosys). These primers are considered universal for performing a PCR of the 16S rRNA gene in bacteria because they amplify the 16S rRNA sequence in almost all bacteria. However, these primers do not work so well for organisms such as Archaea. The primer sequences are seen in Appendix Figure 4 with the PCR recipe and program. These PCR tubes were kept on ice until all were completed and amplified using the PCR program rRNA_fl. After the PCR amplification was complete, each 20 uL sample was loaded into a 1% agarose gel and electrophoresed. The gel was then examined by ultraviolet light exposure and a photo was taken. If the DNA fragment was at the expected location, approximately 3675 base pairs when compared with the λ-Bste molecular weight marker, then the gel slices were cut out and weighed in clean 1.5 uL microcentrifuge tubes. These gel slices were then taken through a gel purification process using the microcentrifuge procedure in the Qiagen manual. After gel purification, 5 uL of the purified DNA was run out on another 1% agarose gel to insure that the DNA was present after purification and also to assess the DNA concentration for sequencing purposes. The gel purification steps were only used with the 2006 and 2007 samples since the new sequencing company Agencourt does not require purified PCR products for sequencing.

PCR amplifications and gel purifications were done numerous times to insure the maximum number of usable cultures for sequencing and also a good DNA concentration from each culture. Eventually, usable DNA extracts were obtained and prepared for DNA sequencing. The samples were pipetted into a 96 well plate. A primer plate was also prepared which contained 27f or 1492r primer. These two plates

were sent to Agencourt for sequencing and the sequence files were received about five days later. When the data were received, the organisms were identified using the Ribosomal Database Project and the National Center for Biotechnology's Basic Local Alignment Search Tool (BLAST) using the nucleotide collection (nr/nt) megablast function for highly similar sequences. The BLAST2 function was used to compare the sequence of the organism in question with its closest RDP match. This was done to determine the match percent and the probable identity (Appendix Figure 5).

Methods for Culture Independent Analysis

Less than 10% of microbes are able to be grown in the laboratory. To obtain a more accurate survey of the community members, a cloning procedure was also used to identify community members. The 16S rRNA genes of the 10^{-0} biofilm or influent water samples were PCR amplified using 2x Premix Ex-Taq (TaKaRa) and then cloned into the TA vector pCR2.1 following the procedure that came with the TOPO TA cloning kit (Invitrogen). This procedure included the addition of the PCR products from the amplified water/ biofilm sample to a mixture of a salt solution and the TA vector pCR2.1. After a brief incubation at room temperature, the PCR product/ vector mixture was transformed into chemically competent *E. coli* cells (strain TOP10F'). These cells were spread onto LB plates containing Isopropyl β-D-1-thiogalactopyranoside (IPTG) and D-galactopyranoside (X-Gal) and incubated at 37° over night. The cells that took up the vector containing the insert were white and the organisms that did not take up the insert were blue in color. These plates were refrigerated and individual clones were used later in sequencing reactions. White colonies were inoculated into sterile medium and the

plasmid DNA was isolated. The sequencing reactions were prepared and sent to Agencourt.

These procedures were done with the biofilm samples collected in 2006 and the influent sample collected in the fall of 2007. Culture independent identification methods will also be used to identify organisms from the two week sample taken in January. This will be consistent with any samples collected at later time points. Approximately 100 clones and 100 cultured organisms were identified from the most recent influent time point since the 96 well plate was used for sequencing.

DNA Sequence Analysis

The raw data was analyzed using the Ribosomal Database Project and the BLAST2 alignment program. BLAST 2 aligns two sequences at areas where the nucleotide sequences are similar. A percent similarity can be calculated based on the total number of bases in the alignment divided by the bases that were identical versus those that had a gap inserted. Using the number for percent similarity, the organisms were identified and determined to be the same or different species. Any percent similarity number that was less than 97% indicated the possibility that the identified organism/ clone was a new species. The RDP and BLAST2 databases did reveal some questionable identities and percent identity matches, so these sequences were reexamined to determine if the sequence data was questionable or if the identity was accurate. Later sequence files, of the new 2006 clone data and the 2008 culture data, were analyzed with assistance from research students in Dr. Newman's lab. The tentative identifications were checked to insure accuracy.

Nitrate Reduction Assays

Nitrate reduction and denitrification assays were done with every cultured organism. Tubes containing nitrate broth were inoculated with the identified sample and then incubated for 2 days at 30℃. The broth conta ined potassium nitrate as a nitrate source. After the incubation time, nitrate reduction was tested by adding a few drops each of α-napthylamine and sulfanilic acid to the medium. If the medium turned red, then it was concluded that the nitrate had been reduced to nitrite and the test was positive for nitrate reduction. If the medium stayed the original color, zinc powder was added to the medium. The zinc reduced any free nitrate in the tube to nitrite. If the medium was still the original color after addition of the zinc, then it was concluded that denitrification had taken place and the nitrogen had been released into the atmosphere as N_2 gas. If the medium turned pink after addition of the zinc powder, then it was determined that there was still nitrate in the tube and the organism was not capable of nitrate reduction or denitrification (Newman, 2007) (Appendix Figure 6). The results of the nitrate reduction and denitrification tests were entered into an Excel spreadsheet based upon positive results for nitrate reduction and denitrification.

Urease Assays

The urease assay tested for the presence of the enzyme urease which breaks urea down into ammonia and carbon dioxide. This test was important since the organisms in this study were taken from a wastewater treatment plant and the ability to convert urea into its two products would be very useful in sewage. To do this test, each identified organism was inoculated into the 1x Urea broth medium. These tubes were incubated for two days at 30℃ and the results were recorded. If the urea broth was

close to the original color, then the organism was determined to be negative for the urease enzyme. If however, the medium had turned a bright pink to magenta color, the organism was determined to be positive for urease. The change in broth color from pale pink to magenta was due to a rise in pH when ammonia was produced (Newman, 2007) (Appendix Figure 7). The results were then entered into the Excel spreadsheet.

Glucose and Lactose Fermentation Assays

Both the glucose and lactose tests were used to determine if the identified organisms could use the respective sugar as a sole carbon source during fermentation. The method for this test was similar to the nitrate and urease tests. Each identified organism was inoculated into tubes with either glucose or lactose medium and the phenol red indicator to test for acid production. A Durham tube was also placed in the tube to test for gas production. The tubes were then incubated for two days at 30℃ and the results were recorded. The medium started out as an orange - red color; however, if the inoculum had turned yellow, the result was positive for acid production. If the sugar broth was close to the original color, then the organism was determined to be unable to ferment the respective sugar to produce acid byproducts. The next result was based on the production of gas during fermentation. If a bubble was present in the Durham tube, then the organism was recorded as positive for gas production and if there was not a bubble in the tube, then the organism was negative for gas production (Newman, 2007) (Appendix Figure 8). The results were then entered into Excel spreadsheets. A complete graphical overview of the methods can be seen in the Appendix Figure 9.

To obtain data on the metabolic properties of uncultured proteobacterial members of the community, Bergey's Manual of Systematic Bacteriology was consulted. Metabolic characteristics at not only the family and genus levels were examined, but also the species level, if the information was available. Results of nitrate reduction and denitrification assays from organisms that were grown in culture were also compared with the Bergey's Manual of Systematic Bacteriology.

Results:

Plate photos are shown in the appendix to illustrate the growth on various types of media. Each plate shows a different stage in the culturing process. Figure 10 in the appendix is a photo of the dilution plate made from the original sample that was incubated for 2 - 4 days. The individual colonies were selected and "patched" onto other plates, like those seen in Appendix Figures 11 – 13. The organisms were patched onto plates made with the original media on which they were grown. The patch plates were also incubated for 2 – 4 days.

Gel photos were taken of each set of electrophoresed samples. Though not all are included here, one representative gel for each step of the preparatory steps is included. Figure 14, in the appendix, is a gel photo of the PCR products obtained from cultured organisms that were grown in the methods previously described. Figure 15 is a photo of a gel containing the purified PCR products. These purified samples were electrophoresed to insure that DNA was present and to estimate the concentration for sequencing. Figure 16 shows a PCR of the 16S rRNA amplification of an entire sample. This PCR product was then prepared using the cloning and selection procedures

mentioned above for uncultured organisms. All of the organisms or clones that had definite bands on the gel were used for sequencing.

After the samples were sequenced by Agencourt, the files were received and analyzed by the freshmen, other members of Dr. Newman's lab, and myself. Many of the organisms were identified multiple times in the sequence analysis. A complete view of the community broken down by family can be seen in the Appendix Figure 17. The division of columns was based on the sampling time and the percentages of the families add up to 100%.

In the 2 week sample from 2006, the bulk of the community identified through culture based methods was made up of γ-Proteobacteria, especially members of the Enterobacteriaceae and Xanthomonadaceae families. There were a very small percentage of organisms identified from the γ-Proteobacteria in the Moraxellaceae and Pseudomonadaceae families. There were also some members identified in the Firmicutes phylum from the Bacillaceae and Streptococcaceae families (Appendix Figure 18). Nitrate reduction and denitrification assays showed that 17 of 34 organisms (50%) were able to reduce nitrate but none were shown to denitrify (Appendix Table 1). The community members that were identified through non-culture based methods were somewhat repetitious when compared with the cultured community members but were more diverse. Actinobacteria (Mycobacteriaceae and Microbacteriaceae) were identified in small numbers as well as Bacteroidetes (Flavobacteriaceae, Flexibacteraceae, and Sphingobacteriaceae). Firmicutes were slightly more predominant and were divided into three families (Clostridiaceae, Peptococcaceae, and Syntrophomonadaceae). The α-Proteobacteria (Methylocystaceae) made up a small

percentage while β-Proteobacteria (Burkholderiaceae, Comamonadaceae, Oxalobacteraceae, Neisseriaceae, Rhodocyclaceae) and γ-Proteobacteria (Pseudoalteromonadaceae, Enterobacteriaceae, Moraxellaceae, Pseudomonadaceae, Xanthomonadaceae) dominated the collection. δ-Proteobacteria (Geobacteraceae, and Haliangiaceae) and Verrucomicrobia (Verrucomicrobiaceae) were a marginal part of the identified community (Figure 19).

In the 12 week sample from 2006, the community was more diverse but γ-Proteobacteria, especially members of the Enterobacteriaceae and Aeromonadaceae families again made up a large percentage. Equal percentages of γ-Proteobacteria (Pseudomonadaceae), Actinobacteria, Firmicutes, and β-Proteobacteria were identified (Appendix Figure 20). Results of the nitrate reduction and denitrification assays showed that 13 of 21 organisms (62%) were able to reduce nitrate and 2 of 21 (10%) were shown to denitrify (Appendix Table 2). The community members that were identified through non-culture based methods were more diverse but some repetition was observed when compared with the cultured community. Actinobacteria (Mycobacteriaceae) made up a small percentage of the community while the Bacteroidetes made up a higher percentage of the community and were divided into 6 families (Flavobacteriaceae, Flexibacteraceae, Sphingobacteriaceae, Bacteroidaceae, Porphyromonadaceae, and Crenotrichaceae). Firmicutes were less dominant and divided into 5 families (Clostridiaceae, Peptococcaceae, and Syntrophomonadaceae, Acidaminococcaceae, Thermoanaerobacteriaceae). The β-Proteobacteria (Burkholderiaceae, Comamonadaceae, Oxalobacteraceae, Neisseriaceae, Rhodocyclaceae, Incertae sedis 5, Nitrosomonadaceae) dominated the collection and

the γ-Proteobacteria (Xanthomonadaceae) only made up a very small percentage. ε-Proteobacteria (Helicobacteraceae), Chlamydiae (Chlamydiaceae), Cyanobacteria (Family 1.1), and Planctomycetes (Planctomycetaceae) made up a small part of the identified community (Appendix Figure 21).

In the influent, sampled in October of 2007, the majority of the community was part of the phylum γ-Proteobacteria. There were also some organisms identified that belonged to both the α and β-Proteobacteria. The remaining organisms were identified to be members of the Firmicutes, Actinobacteria, and Bacteroidetes (Appendix Figure 22). While the species diversity was not very high in regards to the culture based identification methods, there was more diversity found in this sample than in the sample taken from the biofilm two weeks after SBR set-up last year. Nitrate reduction and denitrification results showed that every organism was capable of reducing nitrate except *Pseudomonas plecoglossicida* (F- 037). *Serratia liquefaciens* (F-041) tested positive for both nitrate reduction and denitrification. The urease, glucose and lactose tests gave some conflicting results between members identified as the same species and may need to be done a second time (Appendix Table 3). Similar to what was observed in both the 2 and 12 week samples from 2006, the community members that were identified through non-culture based methods were somewhat repetitious when compared with the cultured community members but were more diverse. Bacteroidetes (Bacteroidaceae, Porphyromonadaceae, Prevotellaceae, Rikenellaceae, Flavobacteriaceae, Flexibacteraceae, Acidaminococcaceae, Clostridiaceae, Eubacteriaceae, Lachnospiraceae, and Syntrophomonadaceae) made up a small portion of the community as well as Fusobacteria (Fusobacteriaceae), Spirochaetes

(Spirochaetaceae), α-Proteobacteria (Sphingomonadaceae), and δ-Proteobacteria (Desulfovibrionaceae). β-Proteobacteria (Comamonadaceae, Neisseriaceae, Rhodocyclaceae), ε-Proteobacteria (Campylobacteraceae), and γ-Proteobacteria(Aeromonadaceae, Moraxellaceae, Pseudomonadaceae, Xanthomonadaceae) made up larger percentages of the community members (Appendix Figure 23).

In the 2 week sample from 2008, there was a greater diversity seen in the community, when compared with the 2 and 12 week samples from 2006. Again, γ-Proteobacteria (Aeromonadaceae, Alteromonadaceae, Halothiobacillaceae, Enterobacteriaceae, Moraxellaceae, Pseudomonadaceae, and Xanthomonadaceae) made up a substantial portion of the collection but there were some organisms from the phyla Bacteroidetes (Bacteroidaceae, Flavobacteriaceae, and Crenotrichaceae), Firmicutes (Bacillaceae, Streptococcaceae, Clostridiaceae, and Syntrophomonadaceae), Cyanobacteria (Family 4.1) and Actinobacteria (Micrococcineae) identified. There was also some representation of the sub-phyla α (Acetobacteraceae), β (Comamonadaceae, Incertae sedis 5, and Rhodocyclaceae), and ε-Proteobacteria (Helicobacteraceae) (Appendix Figure 24). The urease, glucose, lactose, nitrate reduction, and denitrification tests gave some conflicting results and may be redone at a later time since there was such a great variation between some organisms identified as the same species (Appendix Table 4). The data from the uncultured samples has not been analyzed yet but the community diversity and members will be reported at a later time.

Discussion:

In wastewater treatment, one of the most recent innovations is sequencing batch reactors. They provide a smaller more convenient method for treating wastewater in residential and business settings. SBRs also provide the anaerobic – aerobic cycling of the larger fixed bed system on a much smaller scale. Optimally, nutrient levels would be reduced significantly when the effluent leaves an SBR, but some new methods are needed to improve this system. Decreasing the effluent nutrient load not only makes SBRs more marketable but also healthier for the environment. Microbes which are both nitrate reducing and denitrifying are one of the most desired groups of organisms in sequencing batch reactors and many researchers have tried to determine ways to optimize conditions for these microbes. This project was designed to examine the biofilm composition and possibly suggest methods to improve the SBR for these organisms that could improve the quality of the SBR effluent before it enters the Susquehanna River and Chesapeake Bay. Lowered nutrient levels are not only applicable in the waters that feed the Chesapeake Bay but anywhere that wastewater is flowing into a sensitive aquatic habitat.

Cromaglass units are the specific units being monitored in this project, but there are other units available from various companies. The main differences between reactors are the way that the sewage is processed. In the case of the Cromaglass unit, the sewage enters the first chamber and is aerated as it fills and after the filling stops. The second step is the optional denitrification process in which the pumps are turned off for a set time and the microbes can metabolize in an anaerobic environment. The treated wastewater is then transferred across a screen to another chamber and the

sludge settles to the bottom. Lastly, the treated water is discharged from the reactor. The set-up of the reactor is different in actual physical characteristics from other batch reactors, but the cycling process is the same.

In the Fall, reactor start-up was delayed so the influent was collected to establish a working baseline to be compared with the samples that were and will be obtained and analyzed from the biofilm in the SBR. The methods for sample analysis, PCR amplification and cloning of the 16S rRNA genes present in the waste water sample, were consistently used throughout the course of the project. Any knowledge gained from the comparison of influent to coffee can biofilm may be pertinent to improving the conditions for the aforementioned desired microbes.

While the methods of identification remained consistent, the scale of identified organisms increased dramatically in the most recent samples. The main reason for this is that a company (Agencourt) was found that has a less expensive price for sequencing reactions. The only stipulation for the lower price was that samples be prepared in 96 well plates. Due to the less expensive reactions, more were performed. At the prior sequencing company, 46 reactions would have been prepared in individual tubes and cost the same amount as 96 samples in one plate. Agencourt also does not require gel purification of PCR products so this was a time and money saver. The increase in scale was also beneficial in obtaining a better survey of the community due to a larger sampling size. Not only did a larger sample size increase the number of organisms identified, but it also showed that while the sampling parameters have gradually been improving, they are still not even close to measuring the full community of the biofilm and the SBR.

Another method that also saved time and money was the use of CTAB DNA plasmid preparations instead of the traditional Quiagen preps. The Quiagen method was used with the older samples but did not work particularly well with the new samples since DNA yields were not as high and inserts were not identified in a very high percentage of the clones. The CTAB protocol was used in place of Quiagen to prepare plasmids for sequencing. The plasmid DNA was sequenced after being prepared using the CTAB method.

One method that was problematic was the use of only TSA plates. This is the main reason why other differential plates were used. The diversity was originally limited because many of the organisms were morphologically similar on TSA and after sequence analysis, many identities were repeated. Using multiple media types did provide a more diverse survey of the most prominent culturable members of the microbial community. Another problem regarding these organisms was that many of the organisms chosen for patching did not grow very well after being patched onto the new plates. The reason for this is not quite clear since the organisms seemed to grow well on the first set of plates. The increase of diversity in identified cultured organisms in the most recent 2008 two week samples is attributed not only to the use of more media types, but also a larger sample size.

PCR amplification and gel purification also provided some problems such as low yields of DNA for sequencing. This limited the number of organisms that could be used for sequencing. Some organisms with very low yields were used in sequencing reactions and some of the sequence data reflected that with little or no sequence data.

While this was the case for a few organisms, those are organisms that could not be identified accurately and counted in the results.

DNA sequence analysis went relatively well, but again there were some organisms that did not have any sequence to be identified from or the sequence data was not able to be used due to possible contamination of the culture. One indication of this was overlapping peaks at the same spot in the trace file. This in turn resulted in very low identity percent matches. The data was examined and in the case of most organisms, the sequence traces showed that the sequence data could not be relied on. The data for those organisms was disregarded. On average in a 96 well plate of samples, between 4 and 6 samples were unable to be identified by their sequence. In the case of some organisms though, the percentage of match identity was legitimately low enough to consider these organisms as possible new species.

In all 4 samples, the numbers that corresponded to the percent match varied greatly between culture based and non-culture based identifications. The cultured organisms had a much higher percent identity number because most organisms had been cultured and characterized previously in other research. In this case, the RDP was a useful tool to find the probable identity of an organism based on its sequence. On the other hand, the percent match numbers were much lower for the non- culture based identifications since most of the identities made through the cloning method were for organisms that were identified through similar methods elsewhere. These organisms have probably never been cultured since so few microbes are able to be grown in typical laboratory conditions at this point in time. Since the match percent numbers were so low, usually in the high 80 – low 90% range, the identities assigned to

the clones were usually not type strain identities from the RDP but identities based on a BLAST search.

For the influent, and the 2 and 12 week samples from 2006, many of the organisms fell into the sub-phylum of γ-Proteobacteria; there were some organisms from the phyla Bacteroidetes, Firmicutes, and Actinobacteria. There was also some representation of the sub-phyla α and β-Proteobacteria. These phyla were not highly represented in this sample. Therefore, it is very possible that they make up more of the bacterial community than what was recorded in the study. In other research with SBRs, many Proteobacteria have been found, especially β- Proteobacteria. Some α, γ, and δ– Proteobacteria have also been found in smaller numbers, (LaPara et. al., 2000). Also, depending on the conditions of the reactor and the specific probes used for studies, nitrifying bacteria such as *Nitrosomonas* and *Nitrobacter* species have been identified, as well as ammonia oxidizing bacteria (Daims et. al., 2001).

In the 2 week sample from 2008, there was a greater diversity seen in the community. Again, γ-Proteobacteria made up a substantial portion of the sample but there were some organisms from the phyla Bacteroidetes, Firmicutes, Cyanobacteria and Actinobacteria identified. There was also some representation of the sub-phyla α, β, and ε-Proteobacteria. While many metabolic characteristics were examined, the ones most interesting to this project were metabolism of glucose, lactose, and Urease. Nitrate reduction and denitrification were also of interest. Of the organisms identified and metabolically characterized from all time points, 34% of the identified community members were able to denitrify. Urease was found to be metabolized by 12% of the community. Both of these metabolic properties are important for organisms living in a

wastewater environment since urea and nitrates are common components of wastewater. With the development of the right techniques, these metabolic characteristics could be exploited to improve the quality of treated wastewater.

Other organisms of interest such as Archaea were never identified but different primers could be used to amplify the rRNA gene for Archaea. This would also provide valuable knowledge about the SBR since methanogenic Archaea are capable of anaerobic digestion and are useful in the sewage treatment process. Archaea are especially good at removing ammonia from waste, and unlike bacteria, can remove it consistently even when the waste load fluctuates. Under typical conditions in a sewage treatment reactor, Archaea removed up to 50% of the ammonia present (Ambachtsheer and Childs, 2003). The use of different primers for PCR to identify organisms is a very plausible option that will be applied to the next phase of this project.

Like the Archaea, nitrifying bacteria are difficult to find with traditional methods. However, techniques already mentioned such as DGGE of a 16S rDNA library are plausible to identify nitrifying bacteria. Fluorescent In-Situ Hybridization with probes specific for nitrifying bacteria is also useful in identifying this specific group of organisms. These bacteria are crucial in the removal of ammonia, nitrate and nitrite (Schramm et. al.,1998). Since these organisms are so desired and are most likely present in the reactor, this is one means which may be used to identify them.

The community seen in the 2 week sample from 2006 was relatively young and had not had much time for the slower colonizers to integrate into the community. The identified organisms are probably the most prominent members of the community that were able to grow on the TSA and other differential plates in the given incubation

period. In the fourth set of data, the samples taken in January of 2008, the sequencing batch reactor had only been operating for 2 weeks before samples were collected. Again, this was probably not enough time for more than a small proportion of the community to establish itself in the biofilm, although more diversity was seen in the 2008 two week sample than in the one taken in 2006 after the same time of operation.

This project has provided a view of the changing diversity in the SBR at multiple time points in its operation. It also may provide pertinent knowledge for the betterment and optimization of the SBR when the entire project is finished and summarized. Nitrate Reduction and Denitrification will be analyzed and methods for improvement suggested based on accrued data.

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Appendix:

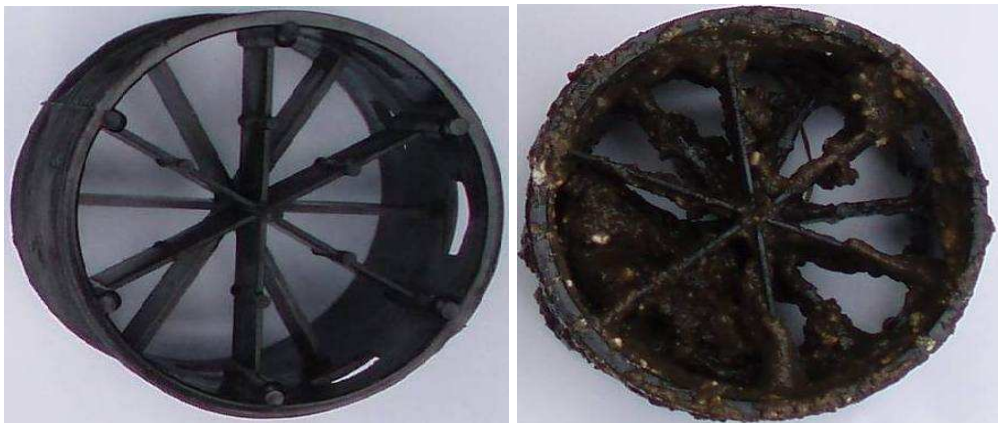
Figure 1: Cromaglass Unit

(http://www.cromaglass.com/products/index.html)
View of a Cromaglass unit during installation.

Figure 2: Collection of Samples from SBR Biofilm

On the left, Michael Gerardi (Cromaglass microbiologist) was removing the PVC tube from SBR. On the right, Michael and I were collecting biofilm scrapings from the PVC tube and the coffee can.

Figure 3: “Coffee Cans”



The coffee can on the left looks as it would before it went into the SBR. The coffee can on the right is what it would look like when the biofilm was scraped off for analysis.

Figure 4: Sequences of the Primers that were used and PCR Recipe and Program Used for all Polymerase Chain Reaction (PCRs)

Primer Sequences (Lane et. al., 1991)

- 27f - 5' – AGAGTTTGATCMTGGCTCAG
- 1492r - 5' – TACGGYTACCTTGTTACGACTT

Recipe for PCR (20uL reaction mixture)

Premix Taq (TaKaRa)	10.0uL
Primer 1 (27f) (5mM)	4.0uL
Primer 2 (1492r) (5mM)	4.0uL
dH2O	1.0uL
2x Frozen/Thawed Cells	1.0uL

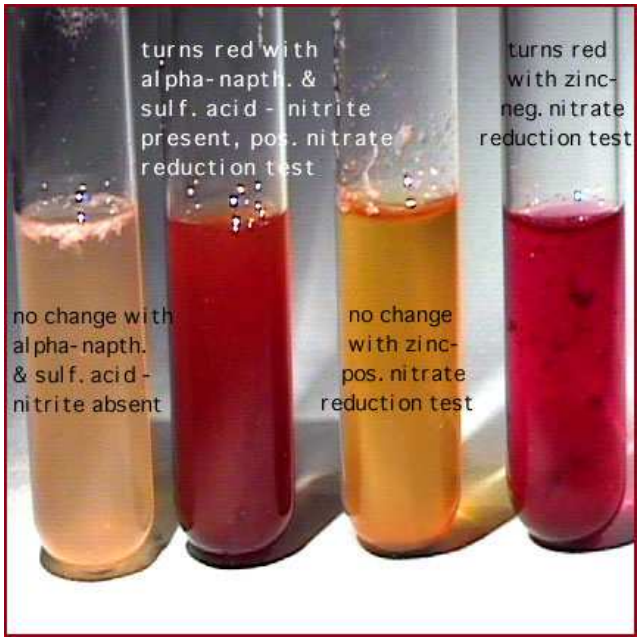
Program rRNA_fl

Phase 1	1 cycle	
Initial Denaturation		2 minutes at 94ºC
Phase 2	35 cycles	
Standard Denaturation		30 seconds at 94ºC
Primer Annealing		30 seconds at 50ºC
Primer Extension		90 seconds at 72ºC
Phase 3	1 cycle	
Primer Extension		10 minutes at 72ºC

Figure 5: BLAST 2 Alignment used to determine the Match Percent between a Known Organism for the Ribosomal Database Project and an Unknown Organism

```
 Score = 1150 bits (598),  Expect = 0.0
 Identities = 684/720 (95%), Gaps = 4/720 (0%)
 Strand=Plus/Minus

Query  8    CCCACCCTTTCGCTCCTCAGCGGTCAGTTACGGCCCAGAGATCTGCCTTCGCCATCGGTG  67
            ||||||||||||||||||||| ||||||||||||||||||||||||||||||||||||||
Sbjct  716  CCCACCCTTTCGCTCCTCAGCG-TCAGTTACGGCCCAGAGATCTGCCTTCGCCATCGGTG  658

Query  68   TTCCTCCTGATATCTGCGCATTCCACCGCTACACCAGGAATTCCAATCTCCCCTACCGCA  127
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  657  TTCCTCCTGATATCTGCGCATTCCACCGCTACACCAGGAATTCCAATCTCCCCTACCGCA  598

Query  128  CTCTAGTCTGCCCGTACCCACTGCAGGCCCGAGGTTGAGCCTCGGGATTTCACANCAGAC  187
            ||||||||||||||||||||||||| |||||||||||||||||| |||||||| |||||
Sbjct  597  CTCTAGTCTGCCCGTACCCACTGCAAGCCCGAGGTTGAGCCTCGGAATTTCACAGCAGAC  538

Query  188  GCGACAAACCGCCTACGAGCTCTTTACGCCCAATAATTCCGGATAACGCTTGCACCCTAC  247
            |||||| ||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  537  GCGACAGACCGCCTACGAGCTCTTTACGCCCAATAATTCCGGATAACGCTTGCACCCTAC  478

Query  248  GTATTACCGCGGCTGCTGGCACGTAGTTAGCCGGTGCTTTTTCTGCAGGTACCGTCACTT  307
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  477  GTATTACCGCGGCTGCTGGCACGTAGTTAGCCGGTGCTTTTTCTGCAGGTACCGTCACTT  418

Query  308  TCGCTTCTTCCCTGCTAAAAGAGGTTTACAACCCGAAGGCCGTCATCCCTCACGCGGCGT  367
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  417  TCGCTTCTTCCCTGCTAAAAGAGGTTTACAACCCGAAGGCCGTCATCCCTCACGCGGCGT  358

Query  368  TGCTGCATCAGGCTTCCGCCCATTGTGCAATATTCCCCACTGCTGCCTCCCGTAGGAGTC  427
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  357  TGCTGCATCAGGCTTCCGCCCATTGTGCAATATTCCCCACTGCTGCCTCCCGTAGGAGTC  298

Query  428  TGGGCCGTGTCTCAGTCCCAGTGTGGCCGGTCACCCTCTCAGGCCGGCTACCCGTCGACG  487
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  297  TGGGCCGTGTCTCAGTCCCAGTGTGGCCGGTCACCCTCTCAGGCCGGCTACCCGTCGACG  238
```

Figure 6: Graphic Showing a Nitrate Reduction/ Denitrification Assay

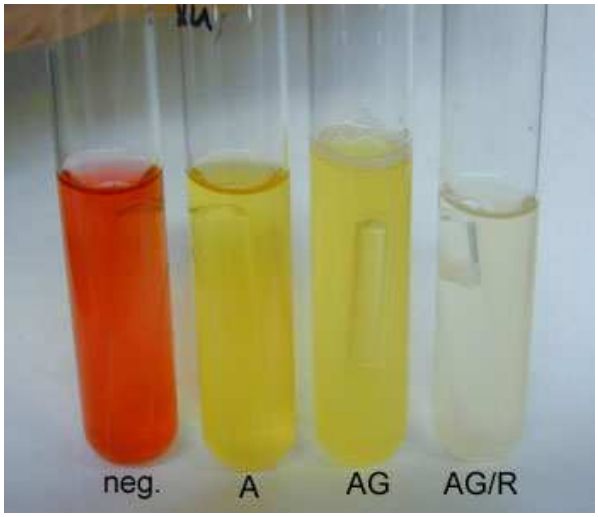


http://www.mc.maricopa.edu/~johnson/labtools/Dbiochem/nit1.jpg

Figure 7: Graphic Showing a Urease Assay

http://www.kcom.edu/faculty/chamberlain/Website/lab/idlab/urease.htm

Figure 8: Graphic Showing a Glucose Fermentation Assay



http://biology.uwsp.edu/faculty/TBarta/ferm.html

In this photo, neg. means that that organism was incapable of fermenting glucose to produce acid or gas. In the tube labeled A, this organism was able to ferment the glucose to produce acid. In the tube labeled AG, the organism not only fermented the sugar to make acid, but produced gas byproducts as well. In the tube labeled AG/R, this organism was capable of producing both acid and gas and reducing a dye present in the tube, I did not utilize any dye so the forth tube does not apply to this study.

Figure 9: Graphical Overview of Methods

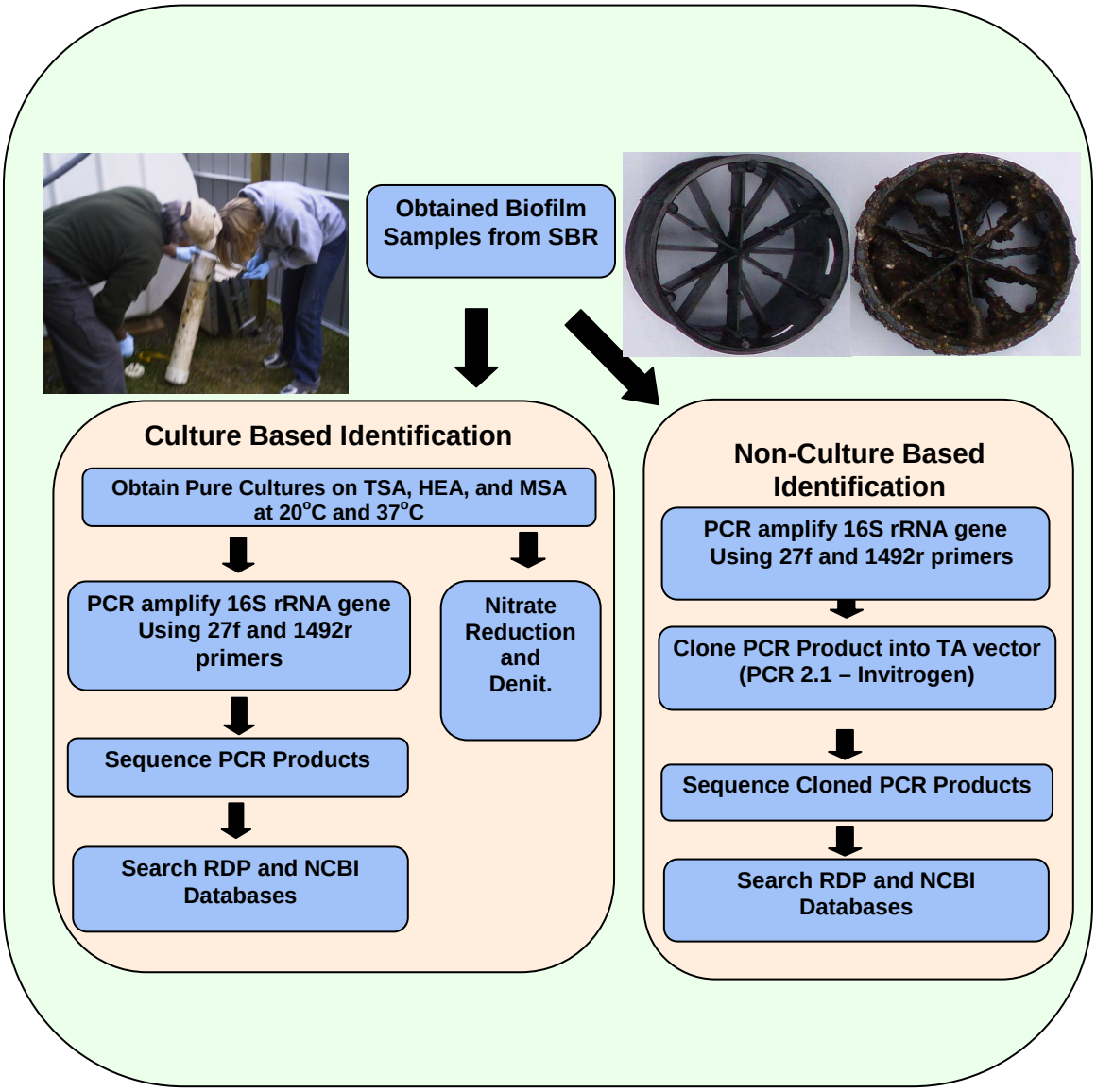
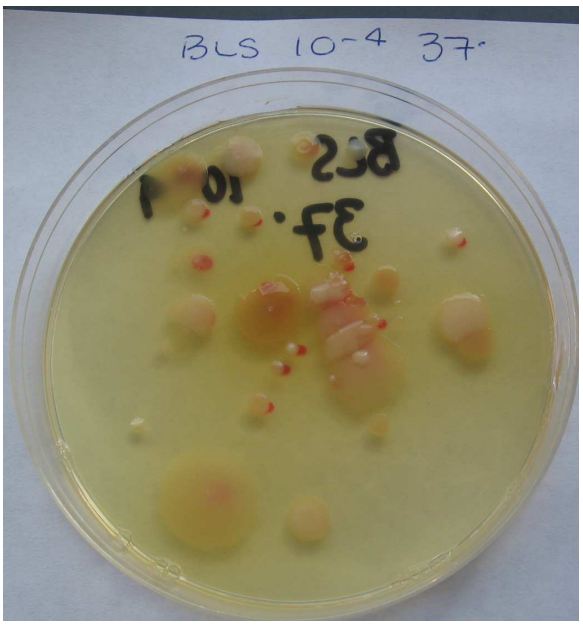
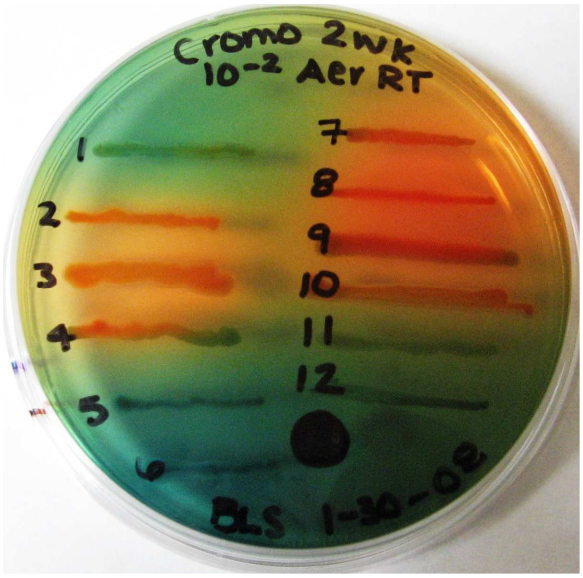


Figure 10: Dilution Plate (10^{-4} - 37°) – TSA



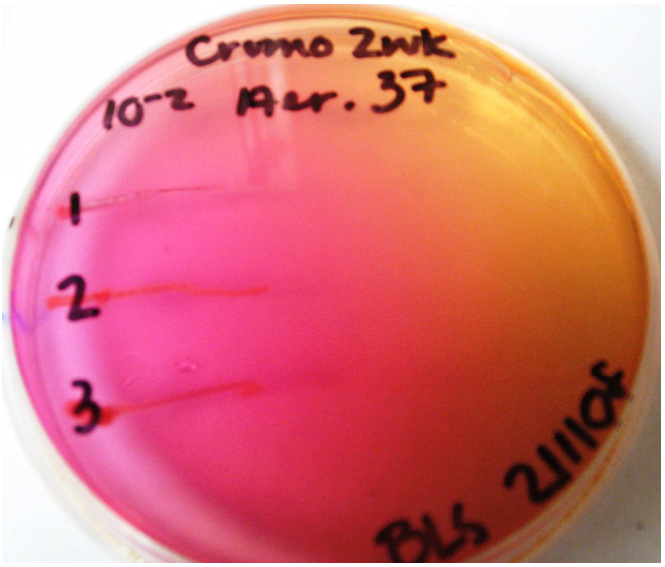
TSA plate grown at 37°. These organisms were selec ted for possible identification and patched onto new plates.

Figure 11: Hektoen Enteric Plate (10^{-2} – RT)



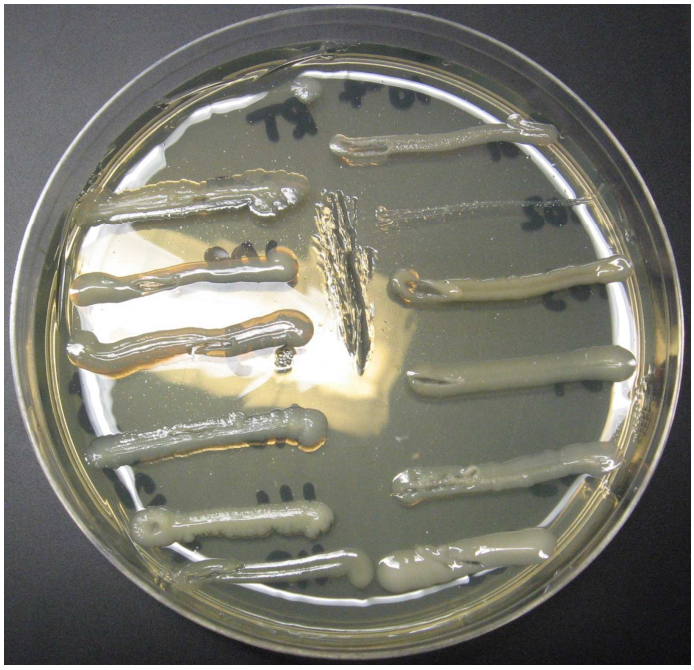
HEA plate grown at RT. The organisms were grown and identified using PCR and other methods mentioned above.

Figure 12: Mannitol Salt Plate (10^{-2} – 37º)



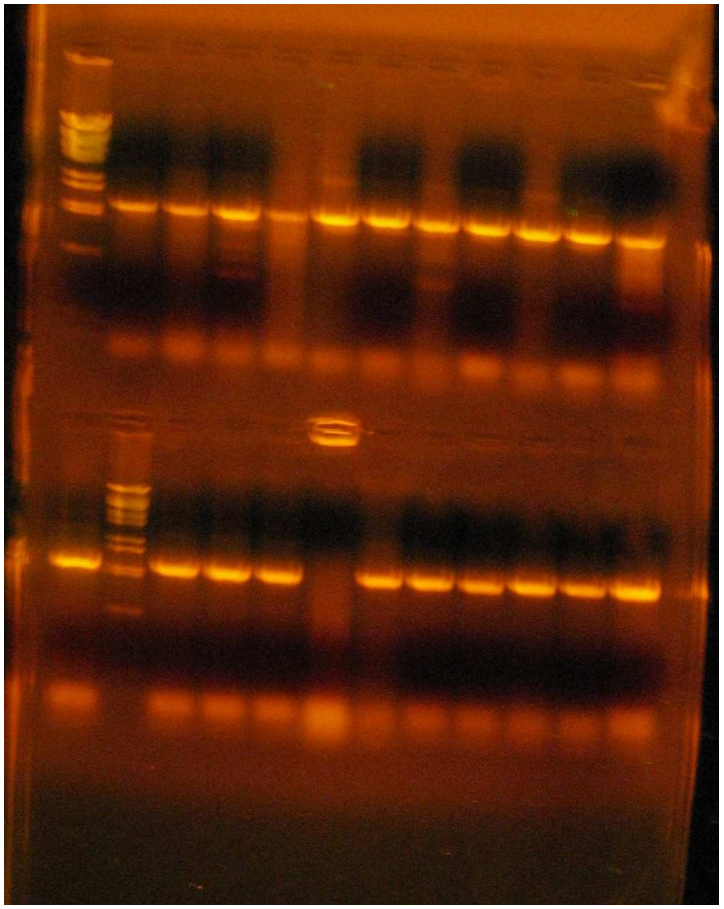
MSA plate grown at 37℃. The organisms were grown and identified using PCR and other methods mentioned above.

Figure 13: Patch Plate (10^{-4} – RT) - TSA



TSA plate grown at RT. The organisms were grown and identified using PCR and other methods mentioned above.

Figure 14: Gel Photo of PCR Products (Gel 3a)



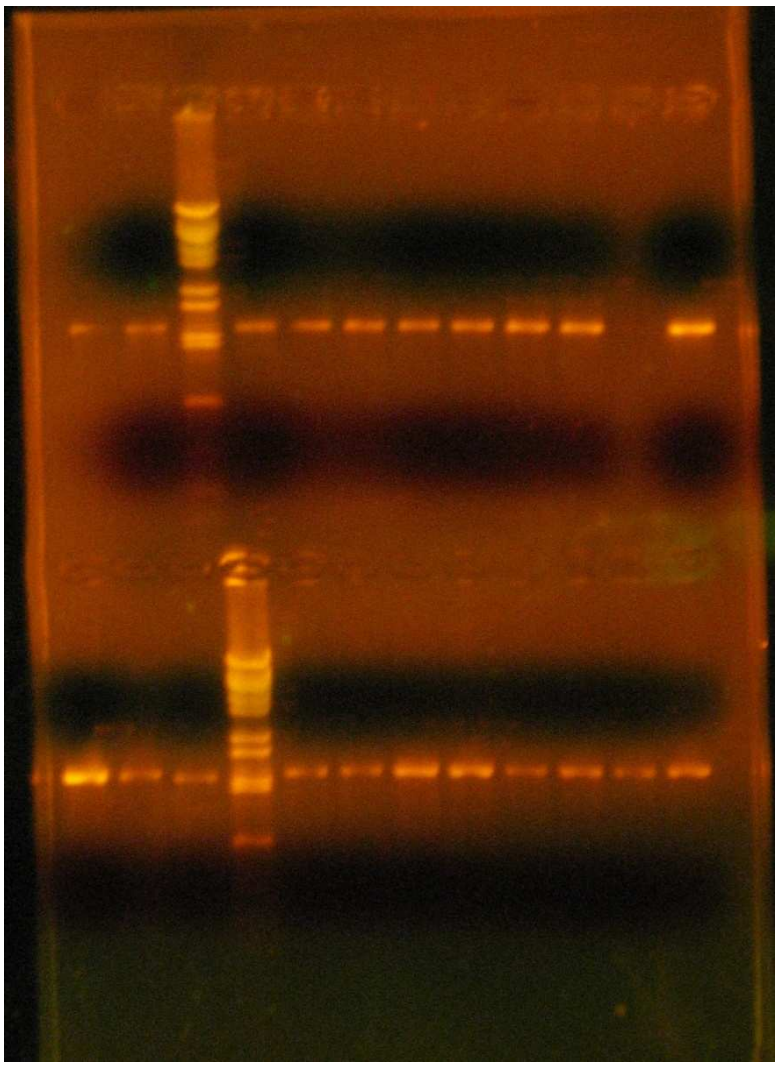
Lambda – Bste II Marker

http://www.sigmaaldrich.com/sigma/datasheet/d9793dat.pdf

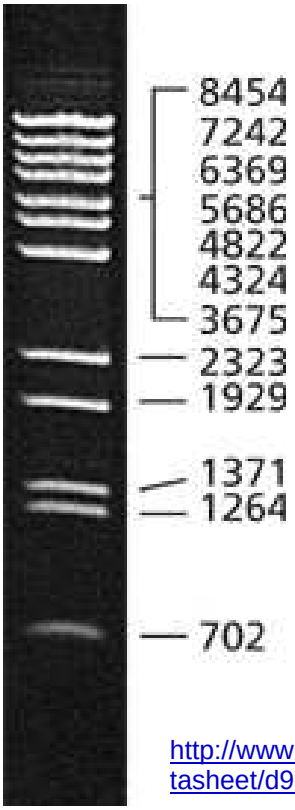
TOP:
Lane 1 – λ-Bste Marker
Lane 2 – BLS 3
Lane 3 – BLS 5
Lane 4 – BLS 8
Lane 5 - BLS 9
Lane 6 - BLS 11
Lane 7 - BLS 12
Lane 8 – BLS 16
Lane 9 – BLS 18
Lane 10 – BLS 19
Lane 11 –BLS 21
Lane 12 - BLS 24

BOTTOM:
Lane 1 – BLS 27
Lane 2 – λ-Bste Marker
Lane 3 – BLS 30
Lane 4 – BLS 32
Lane 5 - BLS 33
Lane 6 - BLS 37
Lane 7 - BLS 39
Lane 8 – BLS 40
Lane 9 – BLS 41
Lane 10 – BLS 42
Lane 11 –BLS 47
Lane 12 - BLS 48

Image 15: Gel Photo of Gel Purified PCR Products (Gel 4b)



Lambda – Bste II Marker

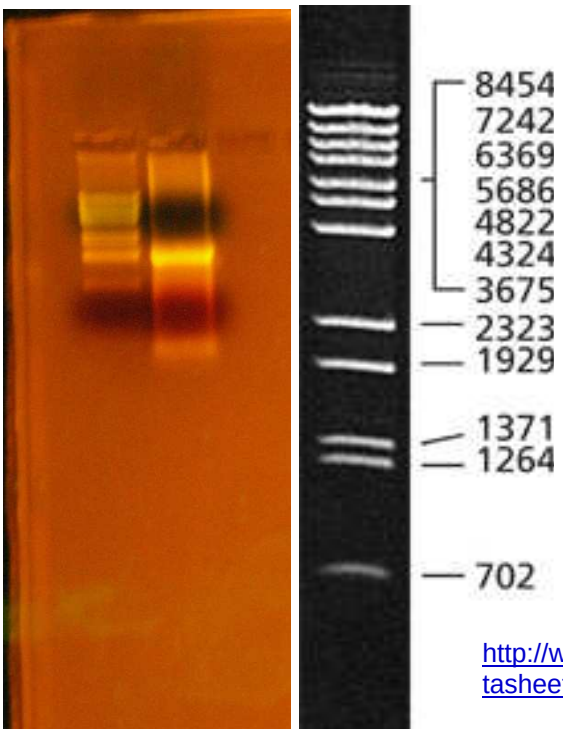


http://www.sigmaaldrich.com/sigma/datasheet/d9793dat.pdf

TOP:
Lane 1 – BLS 23
Lane 2 – BLS 24
Lane 3 – λ –Bste
Lane 4 – BLS 25
Lane 5 – BLS 27
Lane 6 – BLS 28
Lane 7 – BLS 29
Lane 8 – BLS 30
Lane 9 – BLS 31
Lane 10 – BLS 32
Lane 11 – BLS 33
Lane 12 – BLS 34

BOTTOM:
Lane 1 – BLS 38
Lane 2 – BLS 39
Lane 3 – BLS 40
Lane 4 – λ -Bste
Lane 5 – BLS 41
Lane 6 – BLS 42
Lane 7 – BLS 43
Lane 8 – BLS 44
Lane 9 – BLS 47
Lane 10 – BLS 48
Lane 11 – BLS 53
Lane 12 – BLS 54

Figure 16: Gel Photo of PCR Amplified Cromaglass Sample Used in Cloning Procedure

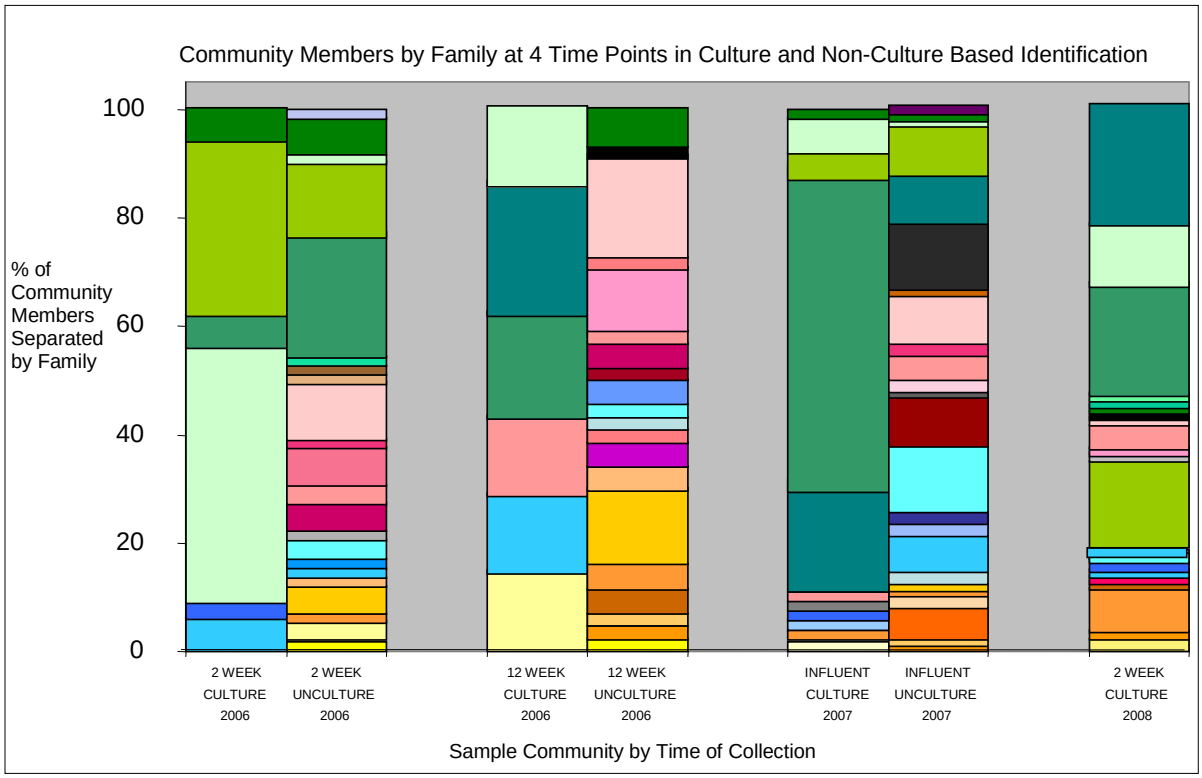


Lambda – Bste II Marker

http://www.sigmaaldrich.com/sigma/datasheet/d9793dat.pdf

Lane 1 - λ-Bste
Lane 2 – BLS PCR CE2
Lane 3 – EMPTY
Lane 4 – EMPTY
Lane 5 – EMPTY
Lane 6 – EMPTY
Lane 7 – EMPTY
Lane 8 – EMPTY

Figure 17: Complete View of Community Members by Family for each of 4 Samples.



Legend:

Actinobacteria
Microbacteriaceae
Micrococcineae
Corynebacteriaceae
Mycobacteriaceae

Bacteroidetes
Flavobacteriaceae
Crenotrichaceae
Sphingobacteriaceae
Prevotellaceae
Bacteroidaceae
Flexibacteraceae
Porphyromonadaceae
Rikenellaceae

Chlamydiae
Chlamydiaceae

Cyanobacteria
Family 1.1
Family 4.1

Firmicutes
Bacillaceae
Enterococcaceae
Syntrophomonadaceae
Acidaminococcaceae
Lachnospiraceae
Thermoanaerobacteriaceae
Streptococcaceae
Clostridiaceae
Peptococcaceae
Eubacteriaceae

Fusobacteria
Fusobacteriaceae

α Proteobacteria
Brucellaceae
Methylocystaceae
Acetobacteraceae
Sphingomonadaceae

β Proteobacteria
β Proteobacteria
Burkholderiaceae
Neisseriaceae
Comamonadaceae
Incertae sedis 5
Oxalobacteraceae
Nitrosomonadaceae
Rhodocyclaceae

γ Proteobacteria
Pseudomonadaceae
Enterobacteriaceae
Xanthomonadaceae
Halothiobacillaceae
Pseudoalteromonadaceae
Aeromonadaceae
Moraxellaceae
Alteromonadaceae

δ Proteobacteria
Geobacteraceae
Desulfovibrionaceae
Haliangiaceae

ε Proteobacteria
Helicobacteraceae
Campylobacteraceae

Planctomycetes
Planctomycetaceae

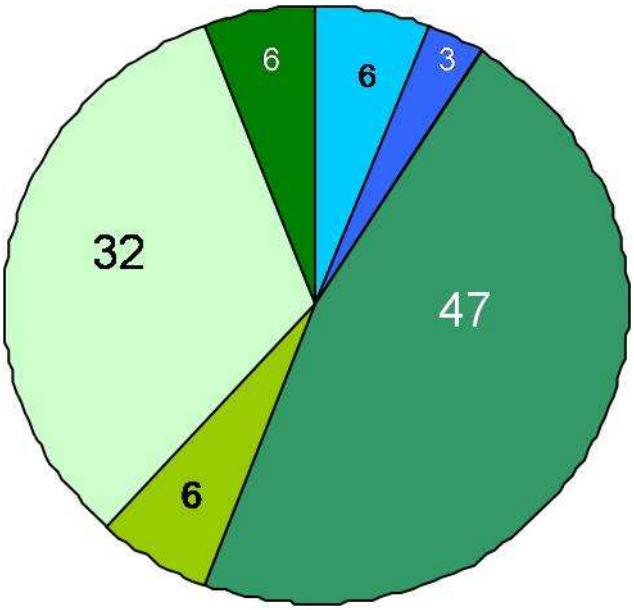
Spirochaetes
Spirochaetaceae

Verrucomicrobia
Verrucomicrobiaceae

Table 1: Breakdown of Cultured Organisms by Family and Genus. Results of Metabolic Tests for Identified Organisms. 2 Week Samples – 2006.

Division/Phylum	Family	Genus	NR	Denit
91.3% g-Proteobacteria	47.1% Enterobacteriacae	Citrobacter, Enterobacter, Klebsiella, Kluyvera, Raoultella	+ + +/- + +	- - - - -
	5.9% Moraxellaceae	Acinetobacter	-	-
	32.4% Pseudomonadaceae	Pseudomonas	+/-	-
	5.9% Xanthomonadaceae	Stenotrophomonas	+/-	-
8.8% Firmicutes	5.9% Bacillaceae	Exiguobacterium	-	-
	2.9% Streptococcaceae	Lactococcus	--	-

Figure 18: Percentage of 2 Week (2006) Community Members Separated by Family (Culture Based Methods)

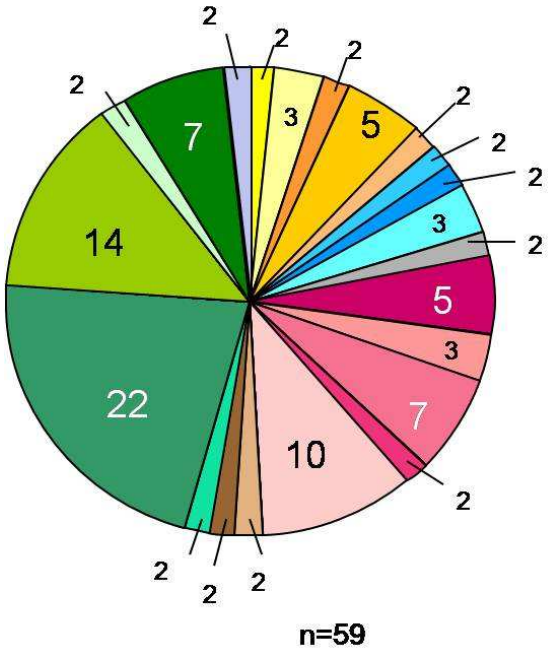


n=34

Legend:

Firmicutes; Bacillaceae
Firmicutes; Streptococcaceae
γ Proteobacteria; Pseudomonadaceae
γ Proteobacteria; Enterobacteriaceae
γ Proteobacteria; Moraxellaceae
γ Proteobacteria; Xanthomonadaceae

Figure 19: Percentage of 2 Week (2006) Community Members Separated by Family (Non-Culture Based Methods)



n=59

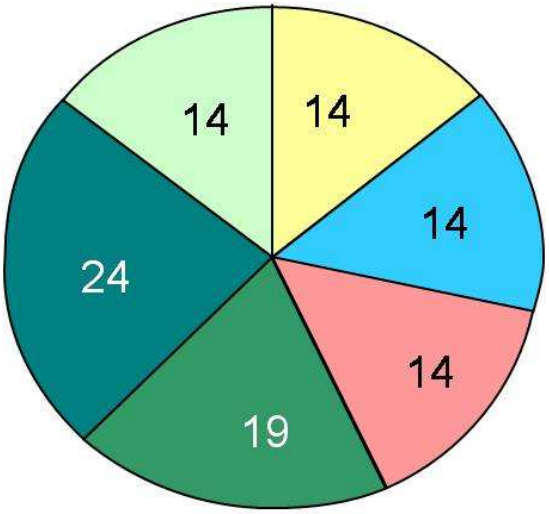
Legend:

Actinobacteria; Mycobacteriaceae
Actinobacteria; Microbacteriaceae
Bacteroidetes; Flavobacteriaceae
Bacteroidetes; Flexibacteraceae
Bacteroidetes; Sphingobacteriaceae
Firmicutes; Clostridiaceae
Firmicutes; Peptococcaceae
Firmicutes; Syntrophomonadaceae
α Proteobacteria; Methylocystaceae
β Proteobacteria; Burkholderiaceae
β Proteobacteria; Comamonadaceae
β Proteobacteria; Oxalobacteraceae
β Proteobacteria; Neisseriaceae
β Proteobacteria; Rhodocyclaceae
δ Proteobacteria; Geobacteraceae
δ Proteobacteria; Haliangiaceae
γ Proteobacteria; Pseudoalteromonadaceae
γ Proteobacteria; Enterobacteriaceae
γ Proteobacteria; Moraxellaceae
γ Proteobacteria; Pseudomonadaceae
γ Proteobacteria; Xanthomonadaceae
Verrucomicrobia; Verrucomicrobiaceae

Table 2: Breakdown of Identified Organisms by Family and Genus. Results of Metabolic Tests for Identified Organisms. 12 Week Samples – 2006.

Division/Phylum:	Family:	Genus:	NR	Denit
14.3 % Actinobacteria	Microbacteriaceae	Microbacterium	+/-	-
14.3% Firmicutes	Bacillaceae	Bacillus	+/-	-
14.3% b – Proteobacteria	Comamonadaceae	Acidovorax, Comomonas	+ -	+ -
57.1% g - Proteobacteria	19.0% Enterobacteriaceae	Enterobacter, Klebsiella, Serratia, Raoultella, Pantoea	- + + + +	- - - - -
	14.3% Pseudomonadaceae	Pseudomonas	+/-	-
	23.8% Aeromonadaceae	Aeromonas	+	-

Figure 20: Percentage of 12 Week (2006) Community Members Separated by Family (Culture Based Methods)

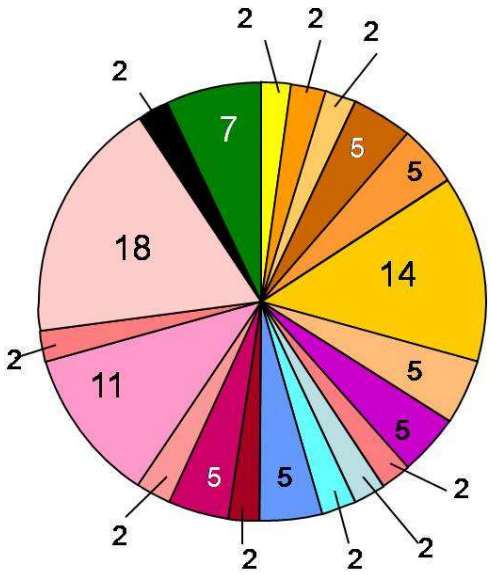


n=21

Legend:

Actinobacteria; Microbacteriaceae
Firmicutes; Bacillaceae
β Proteobacteria; Comamonadaceae
γ Proteobacteria; Pseudomonadaceae
γ Proteobacteria; Enterobacteriaceae
γ Proteobacteria; Aeromonadaceae

Figure 21: Percentage of 12 Week (2006) Community Members Separated by Family (Non-Culture Based Methods)



n=44

Legend:

Actinobacteria; Mycobacteriaceae
Bacteroidetes; Bacteroidaceae
Bacteroidetes; Porphyromonadaceae
Bacteroidetes; Crenotrichaceae
Bacteroidetes; Flavobacteriaceae
Bacteroidetes; Flexibacteraceae
Bacteroidetes; Sphingobacteriaceae
Chlamydiae; Chlamydiaceae
Cyanobacteria; Family 1.1
Firmicutes; Acidaminococcaceae
Firmicutes; Syntrophomonadaceae
Firmicutes; Thermoanaerobacteriaceae
Planctomycetes; Planctomycetaceae
β Proteobacteria; Burkholderiaceae
β Proteobacteria; Comamonadaceae
β Proteobacteria; Incertae sedis 5
β Proteobacteria; Nitrosomonadaceae
β Proteobacteria; Rhodocyclaceae
ε Proteobacteria; Helicobacteraceae
γ Proteobacteria; Xanthomonadaceae

Table 3: Breakdown of Identified Organisms by Family and Genus. Results of Metabolic Tests for Identified Organisms. Influent Samples – 2007.

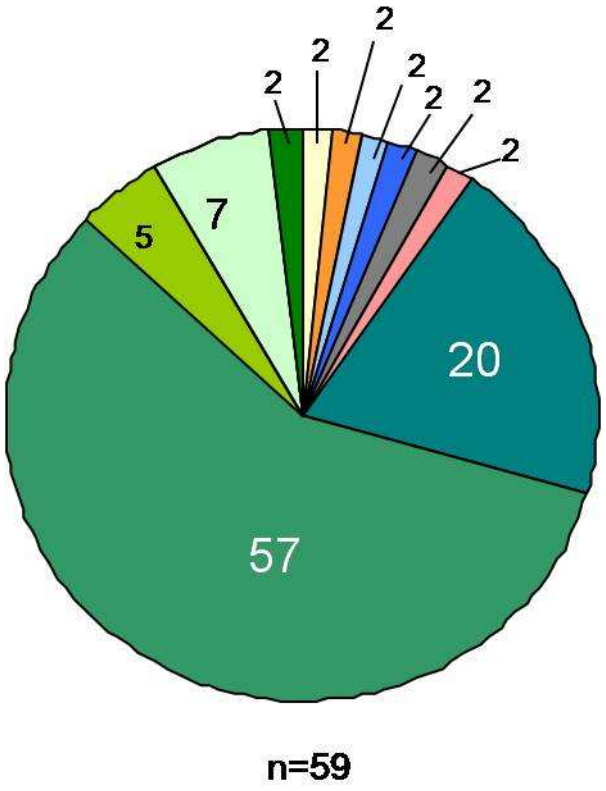
Division/Phylum:	Family:	Genus:	NO^{3-} Red.:	Urease:	Glucose:	Lactose
1.6% Actinobacteria	1.6% Corynebacteriaceae	Corynebacterium	+	+	+/+	+/+
1.6% Bacteroidetes	1.6% Flavobacteriaceae	Empedobacter	+	+	+/+	+/+
3.2% Firmicutes	1.6% Enterococcaceae	Enterococcus	+	+	+/+	+/-
	1.6% Streptococcaceae	Streptococcus	+	+	+/+	+/+
1.6% a - Proteobacteria	1.6% Brucellaceae	Brucella	N/A	N/A	N/A	N/A
1.6% b - Proteobacteria	1.6% Comamonadaceae	Acidovorax	+	+	+/+	+/-
90.2% g - Proteobacteria	19.7% Aeromonadaceae	Aeromonas	+	+	+/-	+/-
	57.4% Enterobacteriaceae	Citrobacter, Enterobacter, Klebsiella, Serratia, Kluyvera, Proteus, Raoultella, Shigella	+ + + + denit ,+ + + + +	+ + +/- +/- +/- + + +/-	+/+ +/+ +/+ +/+ +/+ +/+ +/+ +/+	+/+ +/V +/+ +/V +/+ +/-/+/- +/+ +/+
	4.9% Moraxellaceae	Acinetobacter	+	+	+/+	+/+
	6.6% Pseudomonadaceae	Pseudomonas	+/-	+/-	+/V	+/V
	1.6% Xanthomonadaceae	Pseudoxanthomonas	+	+	+/+	-/-

* A “V” designation in a category on the table stands for variable. In this case, organisms of the same genus had varying positive and negative results for a given test.

* A “UK” designation in a category means that the characteristic is currently undetermined. There are multiple reasons for this but the most prevalent is that the organism did not grow in the specific test media.

* For glucose and lactose fermentation tests, +/- = acid, but no gas, a +/+ = acid and gas, a -/+ = no acid, but gas, and a -/- = no acid and no gas.

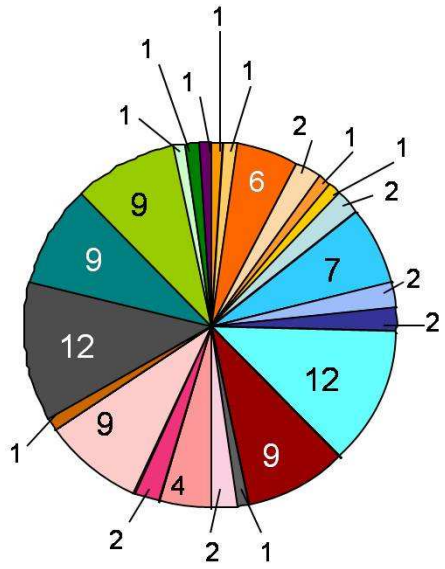
Figure 22: Percentage of 2007 Influent Community Members Separated by Family (Culture Based Methods)



Legend:

Actinobacteria; Corynebacteriaceae
Bacteroidetes; Flavobacteriaceae
Firmicutes; Enterococcaceae
Firmicutes; Streptococcaceae
α Proteobacteria; Brucellaceae
β Proteobacteria; Comamonadaceae
γ Proteobacteria; Aeromonadaceae
γ Proteobacteria; Pseudomonadaceae
γ Proteobacteria; Enterobacteriaceae
γ Proteobacteria; Moraxellaceae
γ Proteobacteria; Xanthomonadaceae

Figure 23: Percentage of 2007 Influent Community Members Separated by Family (Non-Culture Based Methods)



n=90

Legend:

Bacteroidetes; Bacteroidaceae
Bacteroidetes; Porphyromonadaceae
Bacteroidetes: Prevotellaceae
Bacteroidetes: Rikenellaceae
Bacteroidetes; Flavobacteriaceae
Bacteroidetes; Flexibacteraceae
Firmicutes; Acidaminococcaceae
Firmicutes; Clostridiaceae
Firmicutes; Eubacteriaceae
Firmicutes; Lachnospiraceae
Firmicutes; Syntrophomonadaceae
Fusobacteria: Fusobacteriaceae
α Proteobacteria; Sphingomonadaceae
β Proteobacteria
β Proteobacteria; Comamonadaceae
β Proteobacteria; Neisseriaceae
β Proteobacteria; Rhodocyclaceae
δ Proteobacteria; Desulfovibrionaceae
ε Proteobacteria: Campylobacteraceae
γ Proteobacteria; Aeromonadaceae
γ Proteobacteria; Moraxellaceae
γ Proteobacteria; Pseudomonadaceae
γ Proteobacteria; Xanthomonadaceae
Spirochaetes: Spirochaetaceae

Table 4: Breakdown of Identified Organisms by Family and Genus. Results of Metabolic Tests for Identified Organisms. 2 Weeks Samples – 2008.

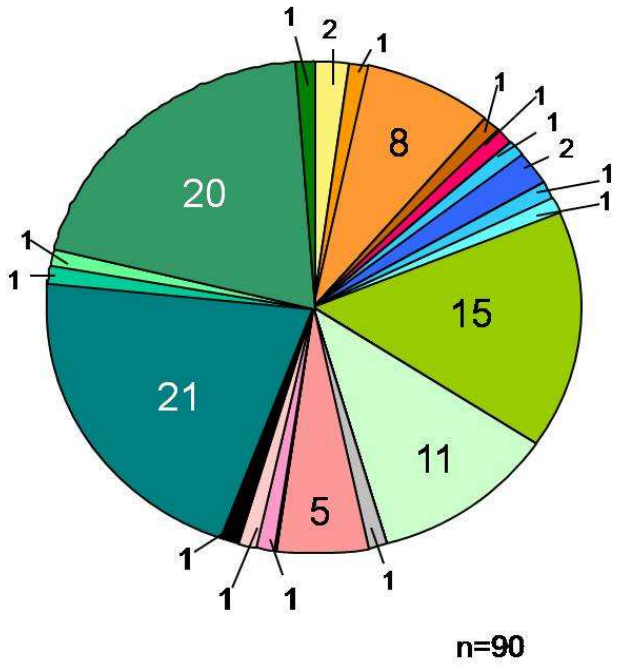
Division/Phylum:	Family:	Genus:	NO^{3-} Red.:	Urease:	Glucose:	Lactose
2.2% Actinobacteria	2.2% Micrococcineae	Micrococcus, Brevibacterium	-	-	-/-	-/-
10.0% Bacteroidetes	1.1% Bacteroidaceae					
	7.8% Flavobacteriaceae	Flavobacterium, Chryseobacterium, Sejongia	UK UK +	UK UK -	+/- -/- -/-	-/- -/- -/-
	1.1% Crenotrichaceae	Crenotrichaceae	UK	UK	UK	UK
1.1% Cyanobacteria	1.1% Family 4.1	Anabaena	UK	UK	UK	UK
5.5% Firmicutes	1.1% Bacillaceae	Bacillus	UK	UK	+/-	-/-
	2.2% Streptococcaceae	Streptococcus	UK	UK	+/V	+/+
	1.1% Clostridiaceae	Clostridium	UK	UK	UK	UK
	1.1% Syntrophomonadaceae	Anaerobaculum	UK	UK	UK	UK
1.1% a - Proteobacteria	1.1% Acetobacteraceae	Acetobacter	UK	UK	UK	UK
7.8% b - Proteobacteria	5.6% Comamonadaceae	Acidovorax, Malikia	+ denit, + +	- -	V/V -/-	+/+ -/-
	1.1% Incertae Sedis 5	Leptothrix	+	-	+/+	+/+
	1.1% Rhodocyclaceae	Zoogloea	UK	UK	UK	UK
1.1% e - Proteobacteria	1.1% Helicobacteraceae	Helicobacter	+	-	+/+	-/-
71.1% g - Proteobacteria	21.1% Aeromonadaceae	Aeromonas	+	-	V/-	V/V
	20.0% Enterobacteriaceae	Cedecea, Citrobacter, Enterobacter, Yersinia, Serratia, Kluyvera	UK + + + + +	UK - + V + +	UK +/+ +/+ +/V +/+ +/+	UK +/+ +/+ +/V +/+ +/+
	1.1% Alteromonadaceae	Alteromonas	+	-	+/+	+/+
	1.1% Halothiobacillacaeae	Thioalkalimicrobium	+	+	+/+	+/+
	1.1% Xanthomonadaceae	Ignatzschineria	UK	UK	+/+	+/+
	15.6% Moraxellaceae	Acinetobacter	V	-	V/V	V/V
	11.1 Pseudomonadaceae	Pseudomonas	V	-	V/V	V/V

*A “V” designation in a category on the table stands for variable. In this case, organisms of the same genus had varying positive and negative results for a given test.

* A “UK” designation in a category means that the characteristic is currently undetermined. There are multiple reasons for this but the most prevalent is that the organism did not grow in the specific test media.

* For glucose and lactose fermentation tests, +/- = acid, but no gas, a +/+ = acid and gas, a -/+ = no acid, but gas, and a -/- = no acid and no gas.

Figure 24: Percentage of 2 Week (2008) Community Members Separated by Family (Culture Based Methods)



Legend:

Actinobacteria; Micrococcineae
Bacteroidetes; Bacteroidaceae
Bacteroidetes; Flavobacteriaceae
Bacteroidetes; Crenotrichaceae
Cyanobacteria; Family 4.1
Firmicutes; Bacillaceae
Firmicutes; Streptococcaceae
Firmicutes; Clostridiaceae
Firmicutes; Syntrophomonadaceae
γ Proteobacteria; Moraxellaceae
γ Proteobacteria; Pseudomonadaceae
α Proteobacteria, Acetobacteraceae
β Proteobacteria; Comamonadaceae
β Proteobacteria; Incertae sedis 5
β Proteobacteria; Rhodocyclaceae
ε Proteobacteria; Helicobacteraceae
γ Proteobacteria; Aeromonadaceae
γ Proteobacteria; Alteromonadaceae
γ Proteobacteria; Halothiobacillaceae
γ Proteobacteria; Enterobacteriaceae
γ Proteobacteria; Xanthomonadaceae