

ABSTRACT

NGUYEN, VIVI LE. A Method to Quantify In-Situ Growth Rates of a Filament and a Floc-former Using Real-time Quantitative PCR. (Under the direction of Francis L. de los Reyes III).

Filamentous bulking is a common problem in activated sludge wastewater treatment plants caused by the excessive growth of filamentous bacteria. To date, engineering controls have not proven consistent in eliminating the problem; therefore focus has shifted in recent years to investigating the mechanisms of floc-filament competition. Two models which attempt to explain this competition are the kinetic selection theory and the diffusion limitation model. The kinetic selection theory cites differences in kinetic parameters as the cause of variation in filament/floc-former ratio. At high substrate concentrations, floc-formers out-compete filaments whereas filaments are favored at lower substrate concentrations. The diffusion limitation model assumes equal kinetic parameters and cites the difference in morphology as the mechanism for bulking.

The goal of this project is to develop a species-specific method to quantify the in-situ growth rate of one filament and one floc-former in activated sludge. Growth rate is an important parameter in modeling of activated sludge which has been, until now, estimated using non-species specific methods. The method proposed here will allow future researchers to determine the effect of different operating conditions on the growth rate of a given species, both experimentally and through modeling efforts.

It has been shown for a number of different organisms that RNA level increases as growth rate increases. Chemostats were used to study pure cultures of *Sphaerotilus natans*, as the representative filament, and *Arthrobacter globiformis*, as the representative flocc-former, to determine the relationship between growth rate and RNA:DNA ratio for each species. Real-time qPCR and reverse transcription real-time qPCR were used to measure DNA and RNA levels respectively. The relationship between RNA:DNA ratio and growth rate was found to be positive and linear, R^2 of 0.58 and 0.98 for *S. natans* and *A. globiformis* respectively. This relationship was used to determine in-situ growth rate of *S. natans* in activated sludge from the aeration basin of the North Cary WWTP and in samples collected from a previous study on the effect of different substrates on bulking. *A. globiformis* could not be detected in any of the WWTP samples. This study represents the first time qPCR and RT-qPCR have been used to quantify in-situ microbial growth rates in activated sludge.

A Method to Quantify In-Situ Growth Rates of a Filament and a Floc-Former Using
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BIOGRAPHY

Vivi Le Nguyen was born in Lincoln, Nebraska, USA to parents Thanh Ngoc Nguyen and Myle Nguyen in 1982. She happily spent the first 18 years of her life in Nebraska. In fall of 2001, she began her college career at the University of Pennsylvania in Philadelphia, PA as a chemical engineering major. It was during this time at UPENN that her passion for environmental work came to life. Professors Wen K. Shieh and Manaf Farhan inspired her to pursue a minor in Environmental Studies. In the spring of 2005, she received a Bachelor of Science in Engineering in Chemical Engineering. During the summer of 2005, she worked as an intern at the Environmental Protection Agency (EPA) office in Washington DC under the direction of Nhan Nguyen, Branch Chief of the Chemical Engineering Branch under the Office of Pollution Prevention and Toxics. In fall of 2005, she began work as a research assistant at North Carolina State University under the direction of Dr. Francis L. de los Reyes. At NCSU, she became heavily involved in Engineers Without Borders, a non-profit, humanitarian organization devoted improving the quality of life for people in developing countries through engineering solutions. Upon graduation she hopes to obtain a position as a consulting engineer in water, wastewater treatment and one day hopes to be able to apply this knowledge to humanitarian work in developing nations.

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Introduction

Activated sludge for wastewater treatment is the most common use of biotechnology in the world. Well settling, flocculated sludge is vital to the proper function of such systems. Filamentous bulking is a major problem in activated sludge wastewater treatment caused by excessive growth of filamentous organisms. Filaments prevent sludge from settling, compacting, or both by bridging flocs or causing loosely formed flocs, depending on the causative filament. Poorly settling sludge can lead to loss of solids in the aeration basin and in severe cases, can cause solids overflow in the secondary clarifier and hydraulic overload of solids separation equipment. Many studies have reported bulking problems in wastewater treatment plants (WWTPs) worldwide (30).

To date, most studies have focused on engineering methods to solve the bulking problems (19). Oxidation using chemicals such as chlorine and ozone is the most common method of treating bulking sludge. However, these non-specific solutions only treat the symptoms of the problem, and will therefore require continuous and consequently costly treatment.

More knowledge about the microorganisms (identification, growth requirements, and physiology) responsible for bulking, is needed in order to develop specific treatment methods. As a result, work has recently shifted to molecular methods to study these microorganisms. Methods such as fluorescence in-situ hybridization (FISH) and real-time PCR have opened the doors to new understanding of these organisms by allowing researchers to study them with species level specificity in-situ. FISH has been used extensively to study biofilms, activated sludge floc and even to relate species levels to bulking (27). Real-time PCR has

been used to correlate filament levels with bulking events (49) and to quantify *Microthrix parvicella* (21) but to date, no one has used molecular methods to measure the in-situ growth rate of filamentous bacteria in activated sludge.

The objective of this work was to develop and validate a method for measuring the growth rate of *Sphaerotilus natans* (SNA), a filament, and *Arthrobacter globiformis* (AGF), a floc-former, in activated sludge. The approach is to correlate growth rate with the 16S rRNA:DNA ratio. RNA has been shown to be positively correlated with growth rate for a number of microorganisms including *E. coli*, *S. typhimurium* (26), *A. aerogenes* (4), various marine bacteria isolates (24) and resin-acid-degrading bacteria (34). The amount of DNA within a cell does not change with growth rate and therefore serves as a measure of biomass to normalize RNA content. RNA and DNA were quantified with qPCR and RT-qPCR respectively. The thesis is divided into four sections: a literature review, qPCR and RT-qPCR assay development, chemostat reactor experiments to determine the 16S rRNA:DNA versus growth rate curve and using the assay to determine the in-situ growth rate of SNA and AGF in activated sludge.

1 Literature Review

1.1 Introduction

Filamentous bulking is a potentially serious and costly problem in activated sludge (AS) wastewater treatment plants (WWTPs). It has been estimated that bulking is a prevalent problem in as many as half of the AS WWTPs worldwide (50). The literature review is aimed at providing the reader with adequate background information to form a basis of understanding for the work presented. Topics to be discussed are: factors affecting growth rate of filamentous bacteria, techniques used to treat bulking, competition mechanisms between filaments and floc-formers and a review of methods used to measure bacterial growth rate.

1.2 Factors Affecting Growth Rate

The factors affecting growth of filamentous organisms are the same as those affecting all other types of organisms in activated sludge. These can be categorized as wastewater characteristics, process design parameters or treatment plant operation conditions. Wastewater characteristics which affect bulking are: nutrient balance (N and P concentrations) and the presence of certain substrates such as readily metabolizable soluble organics, dissolved sulfide, lipids, and particulate substrates such as starch. Treatment plant operating conditions which can affect bulking are dissolved oxygen (DO) concentration, pH and temperature.

Process design parameters affecting bulking include: net growth rate (MCRT, F/M), aeration basin configuration and redox conditions, wastewater feeding regime and upstream biological treatment units, sewer surfaces and in-plant surfaces (19).

1.3 Techniques to Control Bulking

Techniques for controlling bulking include non-specific methods which affect all microorganisms within the system, and specific methods targeting only filamentous organisms through metabolic and physiological control. Non-specific bulking control methods include: manipulation of return activated sludge (RAS) flow rates and aeration basin feed points, addition of chemicals and inert solids to enhance activated sludge settling rates and addition of various types of oxidants (chlorine, hydrogen peroxide, ozone, filamenticides) (19).

Specific methods of bulking control require knowledge of the causative organisms so that process design parameters and treatment plant operating conditions can be tailored to select for floc-forming over filamentous bacteria. Techniques that have been used include adjusting nutrient concentrations (nitrogen, phosphorous, sulfur), changing the aeration basin configuration or feeding regime, and the use of metabolic selectors. The term selector was devised by Chudoba et al. (7) to refer to an AS system configuration which contains compartmentalized regions of differing conditions which select for certain groups of organisms and against others. Selectors have been proven to successfully control bulking in many cases, however they are not always successful, and more research is needed about the causative organisms to devise more efficient ways to control the problem.

1.4 Competition mechanisms

The engineering approaches discussed previously have had variable success in eliminating bulking problems. A large body of literature explores competition between floc-forming and filamentous organisms. Understanding the competition mechanism may unlock the door to finding a cure for activated sludge bulking. The kinetic selection theory (KST) is the most widely cited theory for explaining floc-filament competition. This theory, based on Monod kinetics, presumes different growth constants (μ_{\max} and K_s) for different organisms. Applied to activated sludge, this means that filaments should prevail at conditions with low substrate concentration because they are low K_s organisms and vice versa for floc-forming organisms. This theory necessitates the concept that at long, steady state incubations competitive exclusion occurs and only one type of organism will eventually take over completely (7).

Diffusion limitation theory states that filaments are always present within sludge flocs, and that bulking occurs under low substrate conditions due to the ability of filaments to grow outside the floc. In diffusion-based selection (DBS), kinetic parameters are presumed to be equal for both types of organisms and bulking is explained by morphology. Under diffusion dominated conditions (low substrate concentration), bulking occurs because filaments gain an advantage by extending outside of the floc toward substrate. Under high substrate conditions, filaments gain no such advantage and so remain inside the floc (30).

1.5 Measuring Growth Rate

The terms metabolism, metabolic activity, activity and microbial growth have all been used, sometimes interchangeably, in reference to the growth rate of bacteria. Metabolism, metabolic activity, and activity are terms which refer to both anabolic and catabolic reactions within the cell. Growth however refers to the ability of cells to divide and create new biomass (40). Measuring metabolic activity is important for applications where the interest is in degradation of substrates or consumption of a reactant. For applications in which cell numbers or volume of biomass is the important factor, such as in filamentous bulking, knowing the in-situ growth rate of a specific organism is most advantageous.

Numerous methods have been used to characterize growth rates of bacteria. These methods can be categorized as either direct or indirect methods. Direct methods include microscopy and plate counts. Indirect methods require correlation of growth rate with some external factor which can be measured. This factor is quantified for environmental samples, and growth rate is then determined using the correlation. Factors that have been used include turbidity, biomass, total nitrogen or protein, incorporation of labeled nucleic acid precursors, and amount of nucleic acids themselves, in particular RNA. The following sections provide a review of direct and indirect methods for measuring growth rates of bacteria.

1.5.1 Direct counts

Microbial growth can be evaluated directly by counting cells under a microscope at two points in time. The hemocytometer is a device commonly used to count cells. It is

essentially a microscope slide with a chamber of known volume with a grid which has specific dimensions so that the number of cells counted in a sample can be used to calculate the concentration in the original culture. Though this method is used extensively to enumerate bacteria in culture, it has severe shortcomings limiting usefulness for enumerating cells in environmental samples. Considering the limited number of possible cell morphologies versus the number of species of bacteria currently known, it is logical to see that most cells are not different enough to be easily identifiable under a microscope. As such, measuring growth rate of a single species within mixed cultures under a microscope is practically impossible. In addition, microscopy cannot distinguish between live, dead and live but not growing cells. Including dead and non-growing cells in the count will underestimate the growth rate of the active population. Other limitations are that concentrations under 10^6 cells/mL are generally too dilute to be counted, small cells are difficult to count, and cells prone to forming flocs or filaments cannot be distinguished and therefore do not provide accurate numbers with this technique (29).

Fluorescent stains such as fluorescein diacetate (FDA), acridine orange (AO), 4,6-diamidino-2-phenylindole (DAPI) and fluorescein isothiocyanate (FITC) can enhance microscopy by making cells easier to see and also by allowing for discrimination between live and dead cells. Though using stains will increase accuracy of counting by making cells easier to visualize, the stains act indiscriminately and will stain all types of cells, therefore even with fluorescent staining, microscopy cannot be used for species specific measurements (29).

Plate counts are an alternative to microscopy which does differentiate between live and dead cells however, creates the great bias of only allowing enumeration of cultivable cells. It has been estimated that only 1-10% (28) of microorganisms can be cultured under laboratory conditions. Plate counts have been used for decades to enumerate bacteria, however, this technique is not capable of accurately representing mixed culture environmental samples.

1.5.2 Turbidity

Turbidity, a measure of the cloudiness of a liquid sample, is quantified with a spectrophotometer. A spectrophotometer measures optical density (OD), which is the degree to which a sample transmits light. Particles, or cells, within a liquid sample scatter light therefore preventing some amount from passing through the sample. A direct relationship (over a certain range of turbidities) exists between number of bacteria and amount of scattered light. Using turbidity to measure the growth rate of bacterial cells requires generation of a standard curve which relates some measure of cells (number or mass) to optical density (OD). This method, though relatively quick and easy, inherently has many of the same limitations as the direct methods. A spectrophotometer cannot differentiate between live cells, dead cells or other material present in the sample nor can it differentiate between different microorganism species. Turbidity is a useful measurement for enumerating cells in pure culture but cannot be used to measure species specific growth rate within mixed cultures (28).

1.5.3 Total protein

Total protein can be used to quantify changes in biomass due to cell growth. The three most common methods used for quantifying total protein are the Lowry, Bradford and bicinchoninic acid (BCA) assays. All three assays are based on the binding of different reagents with the protein in a sample. The reaction induces a color change which can be detected spectrophotometrically. Choosing the best assay depends on the type of sample being analyzed (pure or mixture of proteins) and the potential for interference by sample components. The major limitation for use of total protein measurements for quantitation of environmental samples is that none of the assays mentioned above can differentiate the source of protein. Therefore protein in environmental samples coming from plants and microscopic animals will also be detected. A more common method of determining growth rate is by measuring rate of protein synthesis by leucine incorporation, discussed in Section 1.5.4.4.

1.5.4 Incorporation of labeled nucleic acid precursors

Incorporation of labeled nucleic acid precursors is an established method for measuring the in-situ growth rate of microorganisms. Among the labeled precursors used are tritiated thymidine, tritiated adenine, tritiated leucine and bromodeoxyuridine.

Except for leucine, which is incorporated into protein, the general principle is that microorganisms favor incorporation of exogenous nucleic acid precursors over *de novo* synthesis and the rate of incorporation of these precursors is proportional to nucleic acid synthesis and therefore growth rate. If the nucleic acid precursor is labeled in a quantifiable

manner, the growth rate of the bacteria can be determined by measuring the rate of nucleic acid synthesis.

1.5.4.1 Tritiated thymidine

The tritiated thymidine growth assay is perhaps the most well known method of this type (2, 11, 32, 48). This method quantifies the amount of [³H]thymidine incorporated into DNA to determine the increase in new bacterial cells per unit time (growth). Cells are incubated with [³H]thymidine for a short period of time, then DNA is extracted, and the amount of [³H]thymidine is quantified with a scintillation counter.

Bacterial growth rates are calculated from the assumptions that thymine bases are approximately 25% of total DNA bases and that the average genome size of DNA of bacteria are 4×10^{-15} g DNA per cell (31). The factor converting [³H]thymidine incorporation to new cells has been determined both empirically and theoretically, the mean value of which is 2×10^9 cells nmol^{-1} (32, 40).

The tritiated thymidine method requires short incubation periods to reduce artifacts created by extended incubation of environmental samples. This requirement also makes it convenient for field studies. Thymidine kinase is the enzyme which converts thymidine to dTMP. It is required in order for the labeling of DNA to occur to significant extent. Fungi, microalgae and cyanobacteria and many eucaryotic organisms do not have thymidine kinase, therefore, unlike with protein measurements, this assay is specific for bacteria. Perhaps the most significant advantage of the tritiated thymidine assay is that it is culture and biomass

independent, which means it can be used to evaluate the growth rate of microorganisms in-situ.

Though the technique is well accepted, it is not without limitations which are discussed in great detail in *Measurements of Bacterial Growth Rates* (31) and will be summarized here.

Dilution of the labeled thymidine incorporated into DNA occurs because of *de novo* synthesis of dTTP precursors. The degree of dilution can be measured with dilution experiments in which different amounts of non-labeled thymidine are added to a constant amount of labeled thymidine, then the amount of label incorporated into DNA is measured. Pollard and Moriarty (41) found that adding labeled thymidine in a high enough concentration (usually 20 nM in water) suppresses *de novo* synthesis so that dilution experiments do not have to be performed for every sample. A method to determine the concentration at which *de novo* synthesis is insignificant is to add increasing amounts of tritiated thymidine at the same activity until no additional incorporation occurs.

It is possible for non-bacterial organisms to take up the radio-labeled substrate through degradation of thymidine to thymine then further breakdown of thymine. The tritium label enters the pool of general metabolites and can thus be incorporated into DNA. However, these reactions take time, and if the incubation period is kept short (usually under 1 hour), non-specific labeling of DNA is considered insignificant.

A few species of *Pseudomonas* have been shown to be unable to take up [³H]thymidine. This is hypothesized to be due to the lack of mechanisms for efficient transport of nucleosides or bases within the cell. It is proposed that bacteria with very limited nutrient requirements such as chemolithotrophic and sulfate-reducing bacteria also lack such mechanisms and explaining the reason for poor [³H]thymidine incorporation into their DNA. Bacteria which are unable to take up [³H]thymidine cannot be assessed using this method.

Disturbing the interactions between different microorganisms and their environment during experimental manipulation can affect rate of DNA synthesis. This limitation applies mostly to analysis of sediment samples because the sediment needs to be mixed to incorporate the [³H]thymidine so that all bacteria in the sample will have access. Moriarty explains that disturbances affect the rate of DNA synthesis less rapidly than either protein or phospholipid synthesis because the regulation of DNA is complex. It is dependent on many other factors such as energy generation and protein synthesis. Cells take up [³H]thymidine much faster than it can be used. As a result, DNA synthesis rate is independent of the amount of thymidine added and sediments can be disturbed without having a large impact on the rate of DNA synthesis (32).

One of the limitations of the tritiated thymidine method is that it is incapable of assessing species specific in-situ growth rates. This limitation was addressed by Pollard by combining the tritiated thymidine method with a reverse probe DNA hybridization technique (39). The in-situ growth rate of *Bacillus cereus* and *Zoogloea ramigera* in activated sludge

was quantified using this method. Though the method provides a way of measuring in-situ growth rates of microorganisms in mixed cultures, the limitations associated with the tritiated thymidine method still hold. In addition, a new host of issues introduced by the reverse DNA hybridization technique, including cross hybridization and hybridization efficiency, prevent the method from becoming widely adopted.

1.5.4.2 Bromodeoxyuridine incorporation

Bromodeoxyuridine (BrdU), a thymidine analogue, is detected via immunocytochemistry. Instead of calculating growth rate from a conversion factor, a standard curve correlating fluorescence intensity to specific growth rate is required.

BrdU offers several advantages over [³H]thymidine. It is non-radioisotopic and also offers greater specificity of detection because the antibodies will not detect chemicals formed from metabolism of BrdU. The assay is able to preserve microspatial growth information and can distinguish between closely located individual cells unlike microautoradiography (15).

Though the advantages over [³H]thymidine make the assay useful for certain types of studies, BrdU still has some of the same limitations as [³H]thymidine. It is not species specific, though it could be if combined with an identification method such as fluorescence in-situ hybridization (FISH) (38). Also, like thymidine, not all bacteria are capable of incorporating BrdU, and the assay is only able to assess those organisms which are capable.

Additional limitations of BrdU, not associated with the thymidine method are cell permeability, BrdU's mutagenic properties and metabolism of BrdU.

BrdU is a fixed cell method. Extensive optimization is required to treat cells so that cell walls and membranes are permeable enough for penetration of the enzyme to bind to labeled DNA. Pernthaler and Hamasaki demonstrate this for marine bacteria, but the process would need to be optimized specifically for each type of assay. Additionally, the permeabilization method often leads to high levels of species-specific cell loss (38).

BrdU is known to undergo tautomeric shifts that allow it to bind to guanine instead of adenine. Upon replication, DNA polymerase recognizes the mismatch and eliminates one of the two bases (38) potentially causing a mutation which can affect growth rate. BrdU also increases susceptibility of DNA to inactivation by UV or visible light, requiring incubations to take place in the dark which would make the technique not viable for phototrophic bacteria. The optimized amount of BrdU addition is 1 μM in natural seawater (15) and at this concentration, stimulation of growth by BrdU metabolism may become significant resulting in misleading growth rate estimates.

1.5.4.3 Adenine

Similar to thymidine incorporation into DNA, adenine incorporation into RNA has also been investigated as a measure of growth rate. Adenine nucleotide pool turnover is another radiolabeling technique used to measure growth rate of bacteria in-situ. The theoretical basis of this technique is that the flux of precursor into nucleotide triphosphate

pools (i.e., ATP, TTP, UTP, etc.), is in equilibrium with the removal of the triphosphate precursors required for cellular biosynthesis which is directly related to growth rate (μ). The adenine nucleotide (AN) pool does not systematically vary with changes in growth rate. Therefore the turnover rate of the intracellular AN pool must vary in direct proportion to the rates of nucleic acid synthesis. Therefore, a positive correlation is expected to exist between cellular AN flux and μ . AN pool turnover time can be calculated by monitoring the change in the specific activity of the ATP pool with incubation time. One turnover is the time required for the ATP pool to achieve a specific radioactivity that is equal to 50% of the value at isotopic equilibrium. The growth rate is calculated from AN pool turnover as follows (22):

- Specific activity of the ATP pool at any time (SA_t) = $1 - (2^{-N})$, where N = # of turnover cycles
- AN pool turnover time (T) = t/N , where t = incubation period
- T = 2.5% of generation time (T_d) irrespective of growth rate
- Therefore, the growth rate (μ) = $1/T_d \times \ln 2$

The limitations of this technique are similar to the tritiated thymidine assay. It is not species specific and even more so, is less specific than [^3H]thymidine because [^3H]adenine can be incorporated by eucaryotic organisms as well, though like [^3H]thymidine, not all organisms can incorporate [^3H]adenine and the assay can only assess those organisms which are capable of incorporating [^3H]adenine. Also, because the method provides an average growth rate of the entire community incorporating [^3H]adenine, the assay is most accurate when all microbes are growing at identical rates. Additionally it can be argued that a

weakness of the assay is that it requires a large number of independent assumptions; should any of these be proven not applicable, the method would be invalid for that particular system.

1.5.4.4 Leucine incorporation

[³H]leucine or [¹⁴C]leucine is used to measure the rate of protein synthesis similarly to [³H]adenine incorporation into RNA or [³H]thymidine into DNA. The rate of protein synthesis gives a more direct measure of cell biomass and can be correlated with growth rate (32). Riemann and Bell (42) found that the [³H]leucine and [³H]thymidine give equivalent results, but that the [³H]leucine had the added advantages of increased sensitivity (cells incorporate 10 times more leucine during growth than thymidine) and also increased specificity. Incorporation of leucine in molecules other than protein is less likely than thymidine being incorporated into non-DNA molecules. Though there is increased specificity as far as incorporation into only protein, there is less specificity in this method due to the fact that the leucine is incorporated into all protein, algae and bacteria alike. Size fractionations have been suggested as a method of differentiating the growth rates (42).

1.5.5 Frequency of dividing cells

The frequency of dividing cells (FDC) is a method of estimating bacterial growth by counting the number of dividing cells at a given point in time. Hagstrom et al. (14) used the method to determine bacterial growth rates in water samples from the Baltic Sea. Cells were preserved with formaldehyde and stained with acridine orange. Dividing bacteria were defined as those showing a visible invagination but not a clear intervening zone between cells.

Growth rate was determined from a graph by knowing temperature and percentage of dividing cells.

Riemann et al. (42) found good correlation between FDC and thymidine incorporation for bacteria in coastal environments. However, the FDC method assumes all bacteria are active. When FDC estimates of biomass were corrected for percent active bacteria determined from microautoradiography, production rates increased by 28%. If a significant portion of the population is not active, the FDC method will underestimate the production rate of the active portion.

The greatest advantage of this method is that it is relatively basic, requiring only a microscope. However, as a result, FDC has many of the same limitations as microscopy counting of cells. It is difficult to distinguish dividing cells using only epifluorescence microscopy, scanning electron microscopy (SEM) solves this problem, but is too tedious to employ for frequent measurements. Also, FDC curves have only been determined for a small number of environments and can only determine the average growth rate of the community being analyzed. Unless combined with an identification technique such as 16S rRNA FISH, FDC cannot be used for evaluation of species-specific growth rates.

1.5.6 Adenylate energy charge

ATP is a molecule synthesized as a means of short-term energy storage for cells. The level of ATP has been found to be proportional to growth rate. Relative abundance of ATP to its precursors ADP and AMP indicate how rapidly the highest energy state (ATP) is

formed. The adenylate energy charge (AEC) is the most commonly used expression of this ratio (52):

$$\text{AEC} = (\text{ATP} + \frac{1}{2} \text{ADP}) / (\text{ATP} + \text{ADP} + \text{AMP})$$

Chapman (6) measured AEC during different growth stages of *E. coli* and found that actively growing cells had an AEC of 0.8, while viability is maintained at AEC from 0.8-0.5 and dying cells have AEC lower than 0.5.

Wiebe and Bancroft (52) used AEC to measure the growth state of marine bacteria both in water and sediment. Jewson and Dokulil (20) measured AEC of freshwater bacteria and Webster et al. (51) measured AEC during the growth of *Bacillus stearothermophilus*. All three groups recognized the largest limitation of the method to be the difficulty of extracting ATP without changing the AEC. Filtration and especially centrifugation significantly lower AEC. Wiebe and Bancroft found that filtration lowered the AEC to the point of not being able to distinguish between bacteria in the stationary versus log phase. Additionally, AEC can only vary from 0 to 1, and since most active cells have an AEC > 0.5, this parameter does not have a wide enough range to accurately describe the possible range of growth rates of microorganisms. Therefore, the AEC can only be used to describe growth rate generally (9). Another limitation is that all organisms contain ATP, and therefore the method cannot be made to target specific organisms or even types of organisms. Though there is the benefit of no incubation or added substrate requirements, the limitations of this method prevent it from being widely used as a measure of in-situ growth rate of bacteria.

1.5.7 RNA-based techniques

RNA has long been thought to be positively correlated with growth in cells. Caldwell, Mackor and Hinshelwood (4) found this to be true for *Aerobacter aerogenes* in 1950. DNA per cell was found to be constant regardless of growth conditions. Schaechter, Maaloe and Kjeldgaard (44) also showed this relationship to be true for *Salmonella typhimurium* in 1958.

Dortch et al. (9) assembled a compilation graph of growth rate versus RNA:DNA ratio for a number of these early studies on *Aerobacter aerogenes*, *Salmonella typhimurium* and *Escherichia coli*. Applying a single linear correlation to all of the data, an R^2 value of 0.634 was obtained. The RNA:DNA ratios for these organisms all fall in the range of approximately 3-14, for a growth rate range of 0.2-2.4 h^{-1} .

More recently, Muttray and Mohn used modified orcinol and diphenylamine reactions to study the relationship between growth rate and RNA:DNA ratio of five isolates of resin acid-degrading bacteria in activated sludge treating pulp and paper mill wastewater. Four of the five isolates showed positive, linear correlation between growth rate and RNA:DNA ratio ($r^2 > 0.9$) (34). One strain was examined by slot-blot hybridization and also showed positive correlation between growth rate and RNA:DNA ratio (33).

Kemp, Lee and LaRoche (24) found high correlations for marine bacterial isolates from the Sargasso Sea and Georges Bank water columns and also Georges Bank sediment. RNA content per cell was also positively correlated ($r = 0.67-0.90$) but not as well as for

RNA:DNA ratio ($r = 0.93-0.99$). RNA content per cell vs. cell volume was not at all correlated.

Kerkhof and Ward (25) compared ethidium bromide (EtBr) fluorometry and membrane hybridization for determining the relationship for another marine bacteria, *Pseudomonas stutzeri*. The EtBr method measured only relative fluorescence of RNA and DNA; absolute quantities were not determined. Growth rates ranged from approximately 0.01-0.125 (h^{-1}) and RNA:DNA ratios ranged from approximately 1.98-2.7 for fluorometry and 0.5-1.5 for membrane hybridization. The suspected cause of the lower ratios for membrane hybridization was inefficient detection of rRNA versus the rRNA gene. This group also compiled historical data set using the same *A. aerogenes* and *S. typhimurium* but different *E. coli* data sets as Dortch et al. (9) and also including *P. stutzeri* data from their study and interestingly obtained an $r^2 = 0.922$ for a least squares fit of all the data points.

Ribosomal RNA has been targeted extensively in microbial ecology because of its characteristics. It is structurally conserved across all three domains: Eucarya, Bacteria and Archaea, but also contains regions which are highly specific allowing for species level identification. Of the three subunits of ribosomal RNA, the 16S is the most targeted. The 5S is too small to offer enough variability, and the 23S, due to its size, has not been sequenced much and the database of 23S sequences is not very extensive in comparison with the 16S database. Though the 16S database offers a lot more information, this gene may not be specific enough to differentiate between sub-species. Those looking for this level of specificity may have to turn to 23S rRNA. Pre 16S rRNA has also been investigated for its

relationship to growth rate (5, 37). Though pre 16S rRNA is much more specific, it is also much more labile, and the relationship to growth rate needs more investigation.

Many methods are available for quantitating RNA and DNA within the context of determining species-specific growth rate. EtBr fluorometry (25, 24), membrane hybridization (33), FISH (17), competitive PCR (35) and most recently real-time PCR are among the most popular techniques. Of these, real-time PCR offers the greatest sensitivity with a theoretical detection limit of one gene copy, the largest dynamic detection range of up to 10^8 fold and also the highest throughput, up to 384 samples simultaneously depending on the system, with results in a matter of hours.

1.6 Conclusions

Bulking is a serious problem in WWTP worldwide caused by overgrowth of filamentous bacteria. Determining the in-situ growth rate of these organisms would lead to vast opportunities in understanding more about these organisms to develop more effective ways of controlling the problem. An easy, accurate and straightforward method to measure in-situ growth rate of microorganisms with species level specificity has eluded microbiologists for decades. With the exception of the DNA hybridization/thymidine assay and RNA based techniques, the methods described are only capable of assessing the growth rate of pure cultures or the average growth rate of a community.

The method proposed here is to use real-time PCR to quantitate RNA and DNA to correlate the ratio to the growth rate of a specific microorganism. The technique offers the

ability to relatively simply and accurately determine the in-situ growth rate of a particular species within a complex mixed-culture environment.

2 Developing PCR Assays to Quantify DNA & RNA of *Arthrobacter globiformis* & *Sphaerotilus natans*

2.1 Competitive PCR

2.1.1 Introduction

The Polymerase Chain Reaction (PCR) is a powerful method used in biotechnology. At its core, PCR is essentially a method of making many copies of a given target sequence of DNA. A typical PCR reaction mix requires buffer, primers, dNTPs, DNA template and DNA polymerase, usually *Taq* polymerase. The reaction takes advantage of DNA's thermodynamic properties by defining the three steps of the reaction by different temperatures: denature (95°C), anneal (50-60°C) and elongate (72°). The value of the technique is in its versatility. Depending on the design of the primers, different parts of DNA can be manipulated and amplified. Some applications of PCR include species identification, cloning and site-directed mutagenesis. In combination with other molecular techniques, the questions to be answered using this technique are limitless.

Though conventional PCR is an invaluable technique in the biotechnology world, its use is limited to qualitative purposes. During the initial cycles of the PCR reaction, the efficiency is very near to 100% which means that the number of copies of the amplicon is doubled each cycle. As the reaction progresses, efficiency decreases due to a number of reasons which could include breakdown of enzyme and reactant limitations. The number of copies eventually plateaus. The cycle number at which the plateau phase occurs varies with

every reaction, even in replicates, and makes it impossible to quantify species specific DNA in the original sample based on final concentrations.

Competitive PCR (cPCR) is a method which allows for quantitation of DNA by using an internal standard. The standard, or competitor, is amplified by the same primers as the target sequence but differs from the target enough to be detected in end point analysis. Since the competitor and target DNA are amplified by the same set of primers, they compete for the primers during the PCR reaction. The target and competitor oligonucleotides are co-amplified in the same tube therefore the factors thought to affect PCR amplification efficiency such as salt concentration and presence of inhibitors affect both oligonucleotides equally.

The original amount of target in the sample can be back calculated from the formula:

$$\frac{T_f}{C_f} = \frac{T_i (1+e)^N}{C_i (1+e)^N} = \frac{T_i}{C_i} \quad (1)$$

where 'T' and 'C' are the concentrations of target and competitor DNA respectively, 'e' is the reaction efficiency, 'N' is the number of cycles, and the 'i' and 'f' subscripts refer to initial and final concentrations (18). The final quantities of target and competitor DNA are known as well as initial quantity of competitor. Since the efficiency for target and competitor DNA and number of cycles are equal for both target and competitor, the initial quantity of target DNA can be calculated from a simple ratio.

The objective of this work was to develop cPCR assays to accurately quantify RNA and DNA of *Sphaerotilus natans* and *Arthrobacter globiformis* in environmental samples.

2.1.2 Materials and methods

2.1.2.1 Bacterial strains and culture conditions

Sphaerotilus natans (SNA) was chosen as the representative filament for a number of reasons. It is historically the most studied bulking organism and has been shown to cause bulking in activated sludge wastewater treatment plants worldwide (50). Additionally, previous work has shown that wastewater treatment plants in Raleigh and Cary, North Carolina contain SNA and have the potential to bulk (23). The SNA strain, obtained from ATCC (15291), was isolated from paper-mill slime and was grown in CYGA medium (pH 8) on a shaker at 26°C. One liter of CYGA medium contains 5 g casitone (BD 225930), 10 g glycerol, 1 g yeast extract, 1 liter of distilled water (1). *Arthrobacter globiformis* (AGF) was chosen as the representative floc-former for this project. The AGF strain, also obtained from ATCC (8010), was grown on nutrient broth (Difco 234000) at 26°C. Glycerol stocks of both cultures were made for long-term storage. These stocks were made according to the following protocol:

1. Grow bacteria overnight in liquid media at 26°C
2. Centrifuge 15 mL of cell culture and decant liquid
3. Resuspend cell pellet in 0.85 mL of fresh, sterile media
4. Transfer mixture to a sterile 2 mL centrifuge tube
5. Add 150 µL of sterile glycerol, vortex to mix
6. Stored at -80°C.

2.1.2.2 cPCR Primer Design

PCR primers targeting the 16S rRNA gene were designed for both SNA and AGF by creating an alignment of all known sequences of the respective organism from the GenBank database (36). In addition to the sequences retrieved from GenBank, two sequences from MWG Biotech, Inc. were also included in the alignment. PCR product amplified from genomic DNA with bacterial primers 8F and 1492R were prepared and sent for sequencing as described in Section 2.1.2.6. Two aliquots of the same purified PCR product were sent, one to be sequenced with the 8F primer, the other with the 1492R primer. Two aliquots were sent because the maximum DNA length that can be analyzed is approximately 700 bp. The sequence from the 8F primer was inserted into the alignment as returned from MWG. The reverse complement of the 1492R sequence was used in the alignment.

Table 2.1 lists the accession number and description of all sequences used in the SNA alignment. Full sequences can be found in Appendix A. *Eikelboom 1701* and *Leptothrix mobilis* are close relatives of SNA; these sequences were included in the alignment as a negative check. Regions of the gene sequence that targeted all of the SNA sequences but not *Eikelboom* nor *Leptothrix* were chosen as potential priming sites. Potential priming sites were then screened using the Ribosomal Database Project (RDP). Sites that had the highest specificity in RDP were then Blasted against sequences in GenBank. Trial and error was used to find the most specific primers possible. All primers were ordered from Integrated DNA Technologies (IDT).

AGF primers were designed in a similar fashion. Table 2.2 shows the sequences used for the alignment, SNA along with *L. mobilis*, *M. luteus*, *A. rhombi* and *B. rhamnosum* sequences were added as negative checks. The results of the primer design are show in Table 2.3 and RDP search and Blast results of each primer as of July 11, 2007 are shown in Appendix C.

Table 2.1 - Sequences used for SNA primer design

Seq #	Accession	Description
00	Z18534	<i>S.natans</i> 16S ribosomal DNA
01	AB087568	<i>Sphaerotilus</i> sp. L19 gene for 16S rRNA, partial sequence
02	AB087567	<i>Sphaerotilus</i> sp. L13 gene for 16S rRNA, partial sequence
03	AB087566	<i>Sphaerotilus</i> sp. L12 gene for 16S rRNA, partial sequence
04	AB087564	<i>Sphaerotilus</i> sp. L7 gene for 16S rRNA, partial sequence
05	AB087563	<i>Sphaerotilus</i> sp. L6 gene for 16S rRNA, partial sequence
06	AB087560	<i>Sphaerotilus</i> sp. L1 gene for 16S rRNA, partial sequence
07	AB087565	<i>Sphaerotilus</i> sp. L8 gene for 16S rRNA, partial sequence
08	AB087561	<i>Sphaerotilus</i> sp. L2 gene for 16S rRNA, partial sequence
09	AB087562	<i>Sphaerotilus</i> sp. L3 gene for 16S rRNA, partial sequence
10	AF072915	<i>Sphaerotilus</i> sp. IF5 16S ribosomal RNA gene, partial sequence
11	AF072914	<i>Sphaerotilus</i> sp. IF4 16S ribosomal RNA gene, partial sequence
12	AF072916	<i>Sphaerotilus</i> sp. IF9 16S ribosomal RNA gene, partial sequence
13	AF072917	<i>Sphaerotilus</i> sp. IF14 16S ribosomal RNA gene, partial sequence
14	AB072236	<i>Sphaerotilus natans</i> gene for 16S rRNA, partial sequence
15	L33980	<i>Sphaerotilus natans</i> 16S ribosomal RNA (16S rRNA)
16	L33978	<i>Sphaerotilus natans</i> 16S ribosomal RNA (16S rRNA)
17	L33977	<i>Sphaerotilus natans</i> 16S ribosomal RNA (16S rRNA)
18	L33976	<i>Sphaerotilus natans</i> 16S ribosomal RNA (16S rRNA)
19	L79964	<i>Eikelbloom type 1701</i> ribosomal RNA gene, complete sequence
20	X97071	<i>L.mobilis</i> 16S rRNA gene
21	-----	<i>S.natans</i> MWG sequence from 8F primer
22	-----	<i>S.natans</i> MWG sequence from 1492 (reverse complement)
23	AJ534666	Uncultured beta proteobacterium partial 16S rRNA gene, clone S15A-MN107

Table 2.2 - Sequences used for AGF primer design

Seq #	Accession	Description
00	AB089841	<i>Arthrobacter globiformis</i> gene for 16S rRNA
01	AB098573	<i>Arthrobacter globiformis</i> gene for 16S rRNA, partial sequence
02	F321068	<i>Arthrobacter globiformis</i> strain U17 16S ribosomal RNA gene, partial sequence
03	X80736	<i>A.globiformis</i> 16S rDNA
04	AY561601	<i>Arthrobacter</i> sp. RG-39 16S ribosomal RNA gene, partial sequence
05	Z18534	<i>S.natans</i> 16S ribosomal DNA
06	X97071	<i>L.mobilis</i> 16S rRNA gene
07	AJ409095	<i>Micrococcus luteus</i> 16S rRNA gene, strain D7
08	AJ415376	<i>Brachybacterium rhamnosum</i> partial 16S rRNA gene, type strain LMG 19848T
09	AY509239	<i>Arthrobacter rhombi</i> strain S189 16S ribosomal RNA gene, partial sequence
10	-----	AGF sequence from MWG (8F)
11	-----	AGF sequence from MWG (1492R - reverse complement)

Table 2.3 - cPCR Primer sequences and specifications

primer name	Sequence	length (bp)	Tm ¹	GC%	location	Accession No.
SNA229F	CTACCAAGCCTGCGATCTGTAGCT	24	60.50	54.20	229-252	AB087568
SNA550R	GGGGATTTACATCTGTCTTATGGAA	26	56.70	42.30	550-575	AB087568
AGF946F	TGGACCGGACCGCCGCAGAAATGTG	25	67.20	64.00	946-970	AB089841
AGF1066R	CCCATGAGTCCCCGCCATTACGCG	24	66.00	66.70	1082-1105	AB089841

¹ Calculated using OligoAnalyzer 3.0 (47)

2.1.2.3 Creating competitor

The competitor for each species was created using conventional PCR with modified reverse primers using genomic DNA as template. For SNA, two modified reverse primers were designed, SNARloop and SNARlinear. The SNARloop reverse primer included a 41

base pair tail which was composed of a 24 base insertion sequence which included cutting sites for HindIII and BamHI restriction enzymes.

The loop primer was named such because the primer is designed so that the 24 base insertion forms a loop when the primer initially anneals to the target sequence (see Figure 2.1). The SNARlinear primer was designed for SNA such that the 17 bases were complementary to a region 24 bases upstream of the 24 bases that are complementary to the target DNA (see Figure 2.1). The forward primers used to create competitors are the same as those used for cPCR. The competitors designed in this way will have the same priming sites as the target DNA, but will be distinguishable on an agarose gel because they will be 24 bases longer. The size of the insertion for the competitor is somewhat arbitrary. It is assumed that 24 bases are large enough to differentiate on regular agarose gels, while still small enough to not greatly affect PCR efficiency. Sequences of primers for creation of competitors are shown in Table 2.4.

The 25 μ L PCR reaction contained 15 μ L of PCR buffer mix, 0.5 μ L of forward primer, 0.5 μ L of the modified reverse primer, 8.2 μ L of sterile water, 0.3 μ L *Taq* polymerase and 0.5 μ L of extracted genomic DNA as template. The PCR cycle had an initial denaturing step for 5 min at 94°C, then 30 cycles of denaturing for 1 min at 95°C, annealing for 50 s at 55°C and extension for 1 min at 72°C and a final extension step for 20 min at 72°C. The FailSafe PCR System from Epicentre Biotechnologies with PreMix E was used for all cPCR and conventional PCR runs.

Genomic DNA was obtained for PCR by growing SNA culture overnight, pelleting cells, then extracting with the Powersoil DNA Isolation Kit from MoBio (Cat. No 12888-50). The manufacturer's instructions were followed with no modifications.

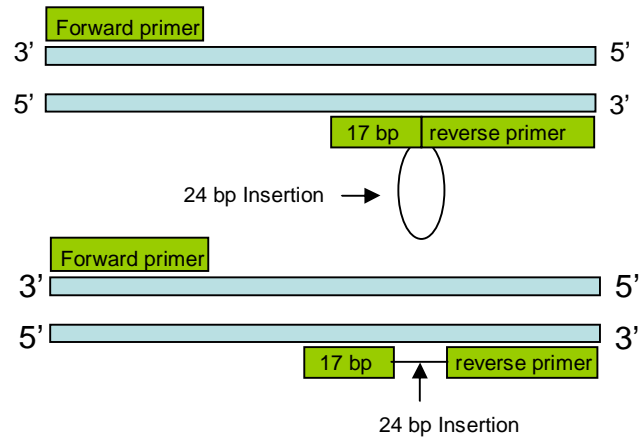


Figure 2.1 - Linear and loop primers for creating competitor

Table 2.4 - Modified reverse primer sequences used to create competitors

primer name	Sequence
SNARloop	GGGGATTTCACATCTGTCTTATGGAA AAGCTT AGTCATAGA ACT GGATCC CCGCCTGCGCACGCTTT
SNARlinear	GGGGATTTCACATCTGTCTTATGGAA AAGCTT AGTCATAGA ACT GGATCC GTAATTCCGATTAACGC
AGFloop	CCCATGAGTCCCCGCCATTACGCG AAGCTT AGTCATAGA ACTG GATCCC TGGCAACATGGAACGA

AAGCTT – Cutting site for Hind III

GGATCC – Cutting site for Bam HI

ATAGAA – 24 base insertion (not complementary to template DNA)

RNA competitors were created by in vitro transcription of the DNA competitor. SNA DNA competitors were linearized with EcoRV restriction enzyme (Promega) at 37°C for 1.5 hours. The enzyme was then inactivated by heating for 15 min at 80°C. Each 20 µL digest contained 13.3 µL of sterile water, 2 µL of 10X RE Buffer, 0.2 µL of Acetylated BSA (10 µg/ µL), 3 µL of DNA (1 µg/ µL) and 0.5 µL of EcoRV (10 U/ µL). The results were verified on a gel and quantified with a spectrophotometer. Next, the linearized competitor DNA was in vitro transcribed to produce RNA with the AmpliScribe™ T7 High Yield Transcription kit from Epicentre. The RNA from in vitro transcription was quantified using a spectrophotometer, then was reverse transcribed to produce cDNA using the RETROscript® kit from Ambion. PCR is then run on the cDNA.

2.1.2.4 Cloning

Copies of the competitor were made by cloning to reduce the chance of sequence errors introduced by *Taq* Polymerase. Cloning was carried out using an Invitrogen TA Cloning kit. Each 10 µL ligation mix contained 1 µL of competitor PCR product, 1 µL 10X ligation buffer, 2 µL of PCR vector 2.1, 5 µL of sterile water and 1 µL of T4 DNA Ligase. The mix was incubated at 14°C overnight, then stored at -20°C until the transformation reaction. The plasmid was transfected into chemically competent *E.coli* cells according to the following protocol (adapted from the Invitrogen TA Cloning Manual, (16)):

1. Thaw One Shot® cells on ice.
2. Pipette 1-2 µl of each ligation reaction into cells and stir gently with pipette tip to mix.

3. Incubate the vials on ice for 30 minutes.
4. Heat shock for 30 seconds at 42°C without shaking. Transfer vials to ice.
5. Add 250 µl of S.O.C. medium to each vial.
6. Shake the vials at 37°C for 1 hour at 225 rpm.
7. Plate 10 µl to 200 µl from each transformation vial on an LB plate containing 100-µg/ml ampicillin.
8. Incubate plates overnight at 37°C.

Thirteen and ten colonies were chosen for SNA and AGF respectively for PCR verification of presence of the competitor. The cells were diluted in sterile water and 0.5 µL of the dilution was used as template in whole-cell PCR. Each 12.5 µL reaction contained 6.25 µL of 2X buffer, 0.15 µL of Taq DNA polymerase, 4.975 µL of sterile water, 0.3125 µL of the forward and reverse primers, and 0.5 µL of template. The PCR products were verified on a 1% agarose gel run for 1 hour at 100V. After the gel, five clones were grown overnight at 37°C then pelleted, and plasmid was extracted using the Wizard Plus SV Minipreps DNA Purification System from Promega (Cat. No. A1330). Aliquots of the extracted plasmids were sequenced for confirmation of the presence of the correct competitor sequence. Once the sequence was confirmed, one clone was chosen and grown in a large amount of LB media overnight to obtain enough competitor plasmid for use during all future experiments. Plasmid was extracted from pelleted cells, quantified using a spectrophotometer and stored until use at -20°C.

2.1.2.5 cPCR

Each cPCR run consists of many individual PCR reactions containing a constant amount of environmental sample with serial dilutions of the competitor. Each 25 μL mix contained 12.5 μL of buffer, 0.5 μL of each primer (0.5 μM), 0.3 μL of *Taq* polymerase, 0.5 μL template, 0.5 μL of one part of the serial dilution of competitor plasmid.

The cycling parameters of the reaction are as follows: 94°C for 5 min, then 30 cycles of 94°C for 50 s, 60°C for 45 s, and 72°C for 1 min, then 72° for 7 min and a final hold at 4°C.

SNA PCR products were visualized on a 3% agarose gel stained with ethidium bromide for 4 hours at 80V. AGF PCR products were visualized on 2% agarose gels for 1.5 hours at 105 volts. Each cPCR run included a positive control of genomic DNA of SNA or AGF and a negative control with deionized water.

Gel-Pro Analyzer software from Media Cybernetics was available to quantify bands on gels.

2.1.2.6 Sequencing

Clones were sequenced by MWG Biotech, Inc. to verify the presence of the competitor. Additionally, SNA and AGF samples were sequenced occasionally to verify the purity of cultures. In order to sequence clones, transformed *E.coli* cells were grown to log-

phase then pelleted. Plasmid DNA was extracted from the pellet, quantified via spectrophotometry and diluted to MWG's specifications, then mailed and sequenced.

To verify purity of cultures, bacteria were grown to mid-log phase then pelleted. DNA was extracted from the pellet and PCR amplified with bacterial primers 8F and 1492R. The PCR product was purified with Qiagen's QIAquick PCR Purification kit (Cat. No. 28104), quantified with a spectrophotometer, diluted to MWG's specifications, then 2 aliquots were mailed and sequenced, one with the 8F primer and the other with 1492R. The sequences were Blasted to determine if they were from the correct species of bacteria.

2.1.3 Results and discussion

2.1.3.1 Creating competitors

Conventional PCR was run to create competitors. Figures 2.2 and 2.3 show the creation of SNA and AGF competitors respectively. Competitors were created with normal forward primers and modified reverse primers. Target DNA refers to genomic DNA of each species amplified with cPCR primers. Though the target and competitor DNA appear to be the same size in Figure 2.2, this is because the 1% gel was only run for 1 hour at 95 volts. Further experiments (Section 2.1.3.3) were performed to optimize gel conditions for separation of SNA competitor and target DNA. The SNA competitor created with the loop primer was used for all future experiments. The loop primer method was also employed for creating the AGF competitor.

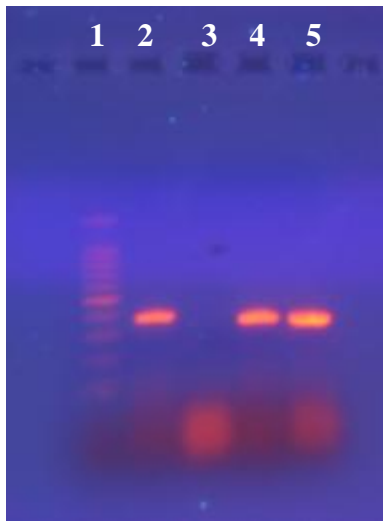


Figure 2.2 - Verification of creating SNA competitors

Lane 1 – Ladder; lane 2 – competitor created with loop primer; lane 3 – NTC; lane 4– competitor created with linear primer; lane 5 – SNA target DNA

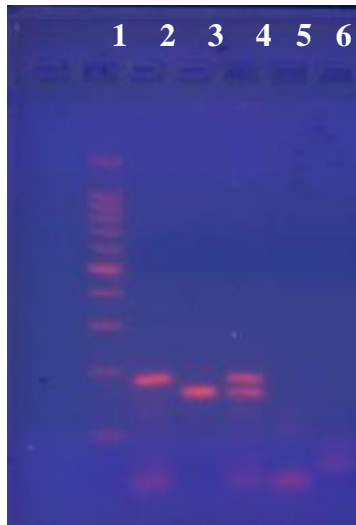


Figure 2.3 - Verification of creating AGF competitors
Lane 1 – ladder, lane 2 – AGF competitor; lane 3 – AGF target; lane 4 – mix of AGF competitor and target; lane 5 – NTC with competitor primers; lane 6 – NTC with target primers

2.1.3.2 Cloning competitors

Copies of competitors were made by cloning instead of additional PCR to avoid sequence errors introduced by *Taq* polymerase. The steps of cloning and verification of cloning products were:

1. Ligation of plasmid with PCR 2.1 vector
2. Transfection of ligation product into competent *E.coli* cells
3. Plate cells and grow overnight
4. Pick at least 10 colonies to analyze for plasmid
5. Perform whole cell PCR on cells from chosen colonies with cPCR primers
6. Visualize PCR products on an agarose gel

Clones containing competitor sequence should show a bright band, clones with no competitor should show no band. Figures 2.4 and 2.5 show the cloning results of SNA and

AGF respectively. Thirteen SNA and ten AGF clones were chosen for analysis, all of which contained competitor plasmid.

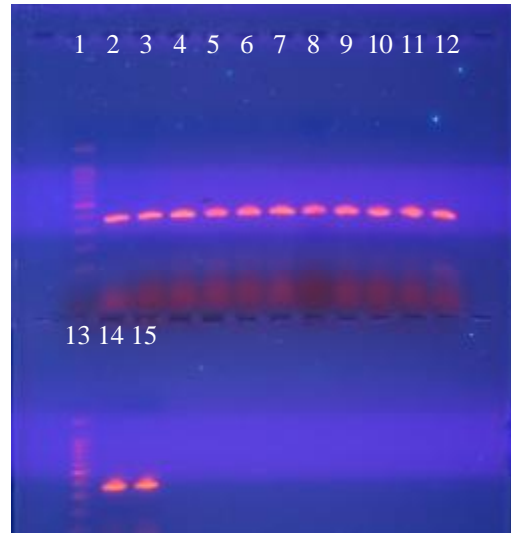


Figure 2.4 - Gel of SNA cloning results
Lanes 1 and 13 – 50 bp step ladder; lanes 2 – 12, 14 and 15 – PCR product of SNA clones showing presence of competitor

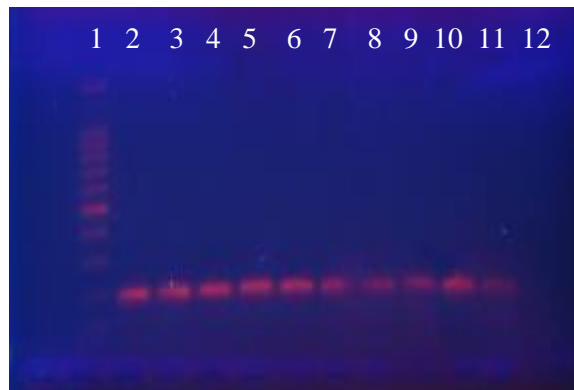


Figure 2.5 - Gel of AGF cloning results
Lane 1 – 50 bp step ladder; lanes 2-11 – PCR product of AGF clones showing presence of competitor; lane 12 – no template negative control

2.1.3.3 Optimizing gel conditions

Several experiments were run to optimize the gel conditions for separating SNA target DNA from SNA competitor DNA. Agarose concentration, time and amount of DNA

were tested. Figures 2.6-2.8 show three of these runs. Each run had one lane of target DNA, one lane of competitor DNA (PCR amplicon, not competitor plasmid), and a mix of target and competitor DNA. After many attempts, the conditions chosen for cPCR were 3% agarose run for 4 hours at 80V.

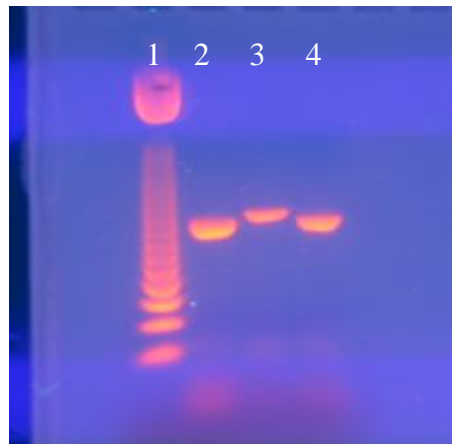


Figure 2.6 - Optimizing gel conditions for SNA cPCR 1
2% agarose, 2 hours, 80V; Lane 1 – 50 bp step ladder (5 mL); lane 2 – target SNA DNA (4 mL); lane 3 – SNA competitor DNA (4 mL); lane 4 – mix of target and competitor DNA (4 mL of each)

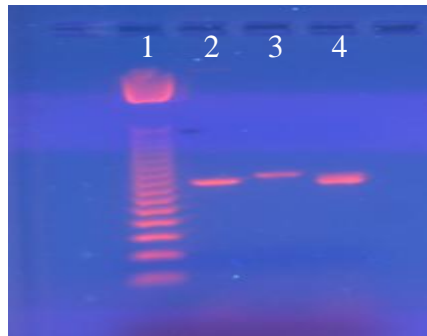


Figure 2.7 - Optimizing gel conditions for SNA cPCR 2
2% agarose, 2 hrs, 80V; Lane 1 – 50 bp step ladder (2.5 mL); lane 2 – target SNA DNA (1 mL); lane 3 – SNA competitor DNA (1 mL); lane 4 – mix of target and competitor DNA (1 mL of each)

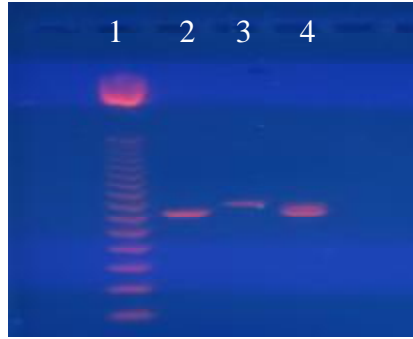


Figure 2.8 - Optimizing gel conditions for SNA cPCR 3
 2% agarose, 3 hours, 80 V; Lane 1 – 50 bp step ladder (2.5 mL); lane 2 – target SNA DNA (1 mL); lane 3 – SNA competitor DNA (1 mL); lane 4 – mix of target and competitor DNA (1 mL of each)

2.1.3.4 cPCR - DNA

The first cPCR run for SNA was done keeping competitor concentration constant with a 10X serial dilution of genomic DNA. The amount of SNA genomic DNA per PCR reaction ranged from 6.5 ng to 6.5×10^{-9} ng. The amount of competitor used per PCR reaction was 3.25 ng. Figure 2.9 shows the results of this cPCR run. The results show no clear separation of target and competitor DNA. After several attempts to adjust gel conditions, it was determined that because the competitor plasmid is so much smaller, in terms of base pairs, than genomic DNA, the copy number of amplicon in the competitor DNA was far greater than the copy number in genomic DNA for the same amount of nanograms. Therefore, a second cPCR run was done keeping genomic DNA amount constant at 6.5 ng and serially diluting competitor DNA (see Figure 2.10). Competitor DNA amount ranged from 0.445 ng to 4.45×10^{-5} ng. The results of the second cPCR run show two rows of bands, the bottom row corresponding to target DNA, the top row to competitor DNA. The competitor row appears to have the strongest band at lane 2 and successively gets

lighter in lanes 3-7, while the target is opposite. The target DNA band appears lightest in lane 2 and gets successively darker in lanes 3-7. This result is expected, as the amount of competitor decreases, more target DNA gets amplified.

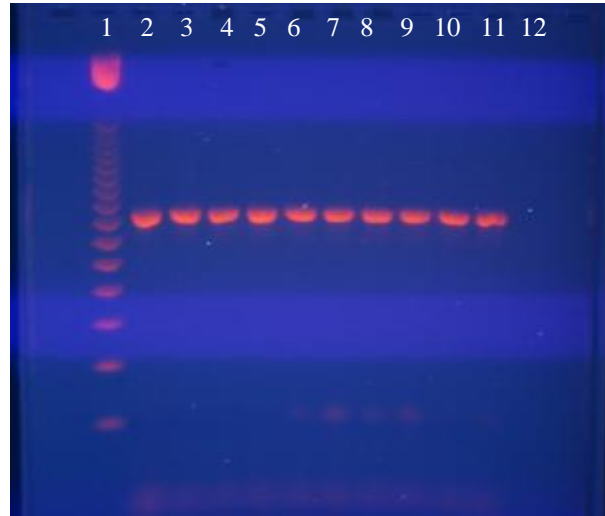


Figure 2.9 - SNA cPCR run #1
Serial dilution of target DNA, amount of competitor kept constant
2% agarose gel run for 3 hours at 80V; lane 1 – 50 bp step ladder; lanes 2-11 – successive cPCR reactions; lane 12 – no template control



Figure 2.10 - SNA cPCR run #2

**Serial dilution of competitor, amount of target DNA kept constant
3% agarose gel run for 4 hours 80V; lane 1 – 50 bp step ladder; lanes 2-6 – cPCR products; lane 7 – PCR product of target DNA; lane 8 – PCR product of competitor plasmid; lane 9 – mix SNA target and competitor PCR products; lane 10 – 50 bp step ladder**

Figure 2.11 shows the results of a successful AGF cPCR run. The competitor was serially diluted 6X, the amount of DNA ranged from 748 ng to 0.577 ng. The amount of AGF genomic DNA used per reaction was 200 ng. The results for AGF are clearer than for SNA, two rows of bands, the top row (competitor) getting fainter from left to right across the gel, and the bottom row (target) of bands getting brighter from left to right. The equivalence point (where amount of target and competitor are equal) appears to be between lanes 5 and 6.



Figure 2.11 - cPCR AGF cPCR run
2% agarose gels run for 1.5 hours at 105 volts; lane 1 – 50 bp step ladder; lanes 2-6 – cPCR products; lane 7 – PCR product of target DNA; lane 8 – no template control

2.1.3.5 RT-PCR preliminary results

Preliminary work to develop a RT-PCR assay for this project was performed but was not completed because the work was being shifted to real-time PCR instead of cPCR. A discussion of the process can be found in Appendix B.

2.1.4 Conclusions

Competitive PCR is a method of quantitative PCR. Competitive RT-PCR allows for quantification of RNA. The use of co-amplified standards takes away the concern of different amplification efficiencies, and is the technique's largest draw. However, the dynamic range is only about 1000 fold and therefore the technique requires extensive optimization to get the amount of competitor in the correct range as the template. Real-time PCR is quickly gaining force as the next quantitative PCR method. Though generally external standards are used, the dynamic range is up to 10^7 fold. Another advantage is that

the method is automated (45). There are no gels, and the quantitation is done by computer programs, which dramatically increases throughput. For these reasons, the quantitative PCR method for this project was switched to real-time PCR instead of continuing with cPCR.

2.2 Developing real-time qPCR and RT-qPCR Assays

2.2.1 Introduction

Real-time PCR is an adaptation of traditional PCR utilizing fluorescent chemistry to allow for monitoring of the reaction in real time. The efficiency of PCR is closest to 100% at the very beginning of the reaction. As the reaction progresses, the efficiency decreases, and eventually the number of copies of DNA will plateau. Even in replicate reactions, the plateau has been shown to result in differing final concentrations of amplicon. Real-time PCR is unique in its ability to quantify the amount of amplified product at the beginning of the reaction for more accurate quantitation of starting material.

A number of different detection chemistries exist for real-time assays. SYBR Green I is the only non-specific method. SYBR Green I is a dye which fluoresces upon binding to the minor groove of double stranded DNA (dsDNA). As the PCR reaction progresses and more amplicon is formed, the dye binds to the DNA, causing fluorescence. The intensity of fluorescence is then recorded. The dye binds non-specifically to dsDNA therefore careful primer design for the SYBR Green assay is of utmost importance.

Fluorescent probes are another type of detection chemistry which allows for multiplex assays (detecting multiple targets in one tube). The most popular types of fluorescent probes are TaqMan and Molecular Beacons. These probes have 2 fluorescent dye molecules, one reporter and one quencher, at each end. If these two molecules are in close proximity, the quencher absorbs the signal from the reporter and little fluorescence is

detected. TaqMan probes are activated by the nuclease activity of *Taq* polymerase. During PCR, the probe hybridizes with the template and the reporter molecule is cleaved by *Taq* polymerase thus separating the reporter from the quencher and allowing for measurable fluorescence. Molecular Beacons act similarly to TaqMan probes but instead of the reporter molecule being cleaved, the probe stays intact. To keep the reporter and quencher molecules in close proximity, the probe forms a stem-loop structure in free solution. The molecules are separated upon hybridization with the target molecule. Scorpions, hybridization probes, Eclipse, and Ampifluor are additional types of detection chemistries all of which use the quencher-reporter system (3).

The choice of reaction chemistry depends on the purpose of the experimental work. The use of fluorescent probes allows for multiplexing, but fluorescent probes are expensive, and designing probe-primer systems can get complicated. Detection with SYBR Green is less expensive for single gene detection assays and allows for generation of a melt-curve to verify the absence of non-specific products.

The most important concept in real-time PCR is the threshold cycle (C_t). The C_t value is the cycle number during a PCR reaction at which the fluorescence reaches a detectable level. Quantification at the threshold cycle ensures that the efficiency of the reaction is closest to 100%. An important consideration while designing an assay is whether the experimental design calls for absolute or relative quantification. Absolute quantification requires the generation of a standard curve from known amounts of DNA (or RNA for real-time RT-PCR), which is then used to extrapolate the concentration of DNA or RNA in

samples. Relative quantification normalizes gene expression to an endogenous housekeeping gene. No such gene is known to exist for bacteria.

The objective of this work was to develop real-time PCR assays to quantify 16S rRNA and the rRNA gene of *Sphaerotilus natans* and *Arthrobacter globiformis*. Absolute quantification with SYBR Green was chosen as the most appropriate type of assay. Development of the assays involved primer design and testing as well as generation of standard curves.

2.2.2 Material and Methods

2.2.2.1 Primer Design

Careful primer design for SYBR Green detection is of utmost importance because of the sensitivity of real-time detection. Typical design criteria include the following (3):

- Primer length should be kept relatively short, 20-30 bp
- Design amplicon length to be between 75-200 bp. Longer amplicons will cause reaction efficiency to decrease.
- Keep melting temperature in the range of 50°C and 65°C
- Maintain GC content between 50-60%
- Avoid secondary structure in primers and amplicon
- Avoid repeats of Gs or Cs longer than three bases in the primers and >4 bp repeat in the amplicon
- Avoid 3' complementarity of forward and reverse primers to prevent primer-dimer formation

For this assay, primers specific to SNA and AGF were designed adhering to as many of the criteria above as possible without sacrificing specificity. The method used to design the real-time primers was the same as described for cPCR, using the same consensus alignment, but were redesigned for increased species specificity.

In addition to primers for real-time PCR, primers were also needed to create RNA and DNA standards. The forward and reverse primers used to create the standards were designed to target regions upstream and downstream respectively of the real-time primers.

The forward primer contains a T7 promoter site at the 5' end to allow for in vitro transcription to create the RNA standard.

Initially, the same set of primers was used to create the standard as real-time PCR, but this resulted in overestimation of the standard when creating the standard curves. The details of this are discussed in Section 2.2.3.2. The suspected reason for this is that the PCR binding sites may not be amplified completely, or may be degraded somewhat during the initial PCR reaction so that during the real-time reaction, though the amplicon exists (and is therefore quantifiable by spectrophotometry), the primer binding sites may not be fully intact. In contrast, if the initial PCR created a standard which was larger and inclusive of the real-time binding sites, it is more likely that these sites will remain intact in the case of degradation or incomplete replication. The primers used for real-time PCR and for creation of the standard are listed in Table 2.5.

Table 2.5 - Primer specifications for real-time PCR

primer name	Sequence	length (bp)	Tm ¹	GC%	location	Accession No.	Purpose
SNA417F	TTCTGGGCTAATACCTCGGGAGGA	24	61.0	54.2	417-440	AB087568	real-time
SNA550R	GGGGATTCACATCTGTCTTATGGAA	26	56.7	42.3	550-575	AB087568	real-time
AGF965F	AATGTGGTTTCTCCTTTTGGGGCC	24	60.1	50.0	965-988	AB089841	real-time
AGF1066R	ATTACGCGCTGGCAACATGGAACG	24	62.4	54.2	1066-1089	AB089841	real-time
SNA243F T7	TAATACGACTCACTATAGGATCTGTA GCTGGTCTGAGAGGA	41	63.6	43.9	243-264	AB087568	standard
SNA595R	CTAGCTCCACAGTCACAAATG	21	53.3	47.6	595-615	AB087568	standard
AGF739F T7	TAATACGACTCACTATAGGATACCCT GGTAGTCCATGCCGTAAAC	45	64.8	44.4	739-764	AB089841	standard
AGF1435R	CCTTGTTACGACTTAGTCCCAATCGC	26	59.2	50.0	1435-1460	AB089841	standard

¹ Calculated using OligoAnalyzer 3.0 (47)

2.2.2.2 Real-time PCR and RT-PCR

The iQ5 Real-Time PCR Detection System (Bio-Rad Laboratories Inc.) was used for all real-time PCR and real-time RT-PCR reactions. Each 25 μL real-time PCR reaction mix contained: 12.5 μL of 2X iQ SYBR Green Supermix, 9.5 μL of nuclease-free H_2O water, 0.5 μL of each primer (0.2 μM) and 2 μL of template. The cycling conditions were as follows: initial denaturation for 5 min at 95°C; then 45 cycles of 50 s at 95°C, 25 s at 60°C, 45 s at 72°C; and a final elongation of 7 min at 72°C. Melt curves were also performed over the range of 55°C-95°C, increasing 0.5°C per 30 s. A no template control with sterile water instead of template was performed for each master mix.

One step RT-PCR was chosen over two step RT-PCR because only one gene was analyzed. Cycling parameters were as follows: reverse transcription at 50°C for 10 min, denaturation of reverse transcriptase at 95°C for 5 min, 45 cycles of denature at 95°C for 50 s, anneal at 60°C for 25 s, elongate for 72°C for 45 s, then a final elongation step for 7 min at 72° followed by a final denature and anneal for 1 min each at 95°C and 55°C respectively, then a final hold at 4°C. Each 25 μL real-time RT-PCR reaction mix contained: 12.5 μL of 2X SYBR Green RT-PCR reaction mix, 8.5 μL of nuclease-free H_2O water, 0.75 μL of each primer (0.2 μM), 2 μL of template and 0.5 μL of RT iScript MMLV Reverse Transcriptase. A no template control with sterile water instead of template was performed for each master mix. Additionally, a negative RT reaction, in which RT enzyme was not added, was performed for each template to control for DNA contamination.

2.2.2.3 Creating Real-time PCR standards

The DNA standard was created by amplifying genomic DNA of each species with the appropriate primers listed in Table 2.5 with conventional PCR. The expected sizes of the SNA and AGF amplicons are 392 and 741 bp respectively. Each 25 μL reaction contained 12.5 μL of 2X buffer E (Epicentre), 10.2 μL sterile water, 0.5 μL of each primer (10 pM), 0.3 μL of *Taq* DNA polymerase (2.5 U/ μL) and 2 μL of DNA template. The cycling conditions were as follows: one cycle of 95°C for 5 min, 30 cycles of 95°C for 50 s, 57°C for 50 s, 72°C for 45 s, one cycle of 72°C for 7 minutes and hold at 4°C. The PCR product was purified with the Qiagen PCR purification kit, and then verified on a 1% agarose gel run at 100V for one hour. The purified PCR product was quantified with a spectrophotometer and then diluted serially 10-fold. The serial dilutions were used as template for real-time PCR to generate a standard curve. The serial dilutions covered a range of 0.678 ng to 6.78 $\times 10^{-8}$ ng of SNA DNA which corresponds to 9.2 to 0.2 log copies and 10 ng to 10⁻⁸ ng of AGF DNA which corresponds to 9.1 to 0.1 log copies.

The RNA standard was created by starting with the DNA standard which contains a T7 binding site at the 5' end due to the specially designed forward primers. The DNA standard was used as template for in vitro transcription (Promega Riboprobe Cat. No. P1440). Each 50 μL reaction contained 10 μL buffer, 5 μL 100 mM DTT, 1.25 μL (50U) RNase inhibitor, 2.5 μL of each rNTP, 1 μL (20 U) T7 RNA polymerase, 3 μL template (1-2.5 μg) and 19.75 μL of sterile water. The reaction was incubated at 37°C for 1.5 hours. Following in vitro transcription, the sample was DNased (Qiagen Cat. No. 79254) at room temperature

(25°C) for 15 minutes, then purified with the Qiagen RNeasy Mini Kit (Cat. No. 74104). An additional DNase reaction was performed on-column during purification. After purification, samples were quantified with a spectrophotometer and serially diluted 10-fold. The serial dilutions were used as template for one-step RT-qPCR. The serial dilution covered a range of 6.84 ng to 6.84×10^{-9} ng of SNA RNA which corresponds to 10.51 to 1.51 log copies and 8.54 ng to 8.54×10^{-10} ng of AGF RNA which corresponds to 10.31 to 0.31 log copies for AGF.

2.2.2.4 Calculating copy number

The copy number of DNA and RNA standards for real-time qPCR was calculated with Equation 2 (43). To calculate RNA copy number, 340 g/mol bp was used instead of 660 g/mol bp.

$$\begin{aligned}
 \text{copy \#} = & \left(\text{DNA conc} \left[\frac{\text{ng}}{\text{ml}} \right] \right) X \left(\frac{1 \text{ g}}{10^9 \text{ ng}} \right) X \left(\frac{1 \text{ mol bp DNA}}{660 \text{ g DNA}} \right) X \left(\frac{6.023 \times 10^{23} \text{ bp}}{\text{mol bp}} \right) X \\
 & \left(\frac{1 \text{ copy}}{\text{size of gene [bp]} } \right) X (\text{vol of template [ml]})
 \end{aligned} \tag{2}$$

2.2.3 Results and Discussion

2.2.3.1 Primer Design Optimization

The final results of the primer design process are shown in Table 2.5 of Section 2.2.2.1. Extensive design troubleshooting took place before finding primers that worked. The cPCR primers fit most of the criteria for real-time PCR except for the fact that they were not specific enough. Both SNA primers and the forward AGF primer were redesigned for increased species specificity. The combined specificity was greater for the new SNA primers and AGF primers than for the cPCR primers as show by the number of hits on the Ribosomal Database Project (RDP), see Table 2.6. Specifications of the new sets of primers are listed in Table 2.7.

Table 2.6 - Comparing species specificity of cPCR and real-time primers

cPCR primers	RDP hits	new primers	RDP hits
SNA229F	168	SNA958F	22
SNA550R	33	SNA1068R	104
AGF946F	30	AGF 965F	8

Table 2.7 - SNA primer set #2 designed for real-time PCR

primer name	Sequence	length (bp)	Tm	GC%	location	Accession No.
SNA1068R	GTAGCAACTGATGACAA GGGTTGCGC	26	61.0	54.0	1068-1093	AB087568
SNA958F	GGCAGGAATCCCGCAGA GATGTGGGA	26	64.0	62.0	958-983	AB087568
AGF965F	AATGTGTTTTCTCCTTTT GGGGCC	24	60.1	50.0	965-988	AB089841
AGF1066R	ATTACGCGCTGGCAACAT GGAACG	24	62.4	54.2	1066-1089	AB089841

Table 2.8 - Description of real-time PCR runs testing various parameters

Run	Template	Annealing temperature (°C)	Template concentration range			Well Positions
1	Unpurified PCR Product from genomic DNA and SNA1068R/958F	60	75	7.50×10^{-6} ng	A01-A08	
2	Unpurified PCR Product from genomic DNA and SNA1068R/958F	60	75	7.50×10^{-9} ng	D01-D11	
3	Purified PCR Product from genomic DNA and SNA1068R/958F	60	78.5	7.85×10^{-5} ng	A01-A07	
4	Purified PCR Product from genomic DNA and bacterial primers 8F and 1495R	60	75	7.50×10^{-5} ng	A01-A07	
5	Dilution series from Run 4 re-diluted 10X and 20X	60	7.5 7.50×10^{-1}	7.50×10^{-6} 7.5×10^{-7} ng	A01-A07: SNA 10X B01-B07: SNA 20X	
6	Serial dilution made from dilution 6 of Run 4 dilution series	63.3	7.50×10^{-4}	7.50×10^{-11} ng	C05-C12	

Upon testing the new primers in a real-time PCR reaction, the AGF primers gave good preliminary results, however the more specific SNA primers gave unusual fluorescence patterns. These primers were not capable of amplifying properly in real-time PCR. They caused fluorescence to drop off sharply after a peak, instead of forming a plateau as expected. A number of different factors were investigated to try to resolve this problem: annealing temperature, template concentration, genomic DNA versus PCR product as template, purified versus unpurified PCR product as template, well position on the PCR plate and primer concentration. The amplification curves of real-time runs testing these parameters are shown in Figures 2.12-2.19. Table 2.8 summarizes some of the real-time PCR runs testing several

parameters. For each run, a serial 10-fold dilution was made of template DNA over the range of concentrations indicated in Table 2.8. The primers used for all reactions were the newly designed real-time primers.

As the figures show, none of the factors tested in these six real-time PCR runs seemed to resolve the issue of the decreasing fluorescence. Each run had multiple samples which did not amplify correctly.

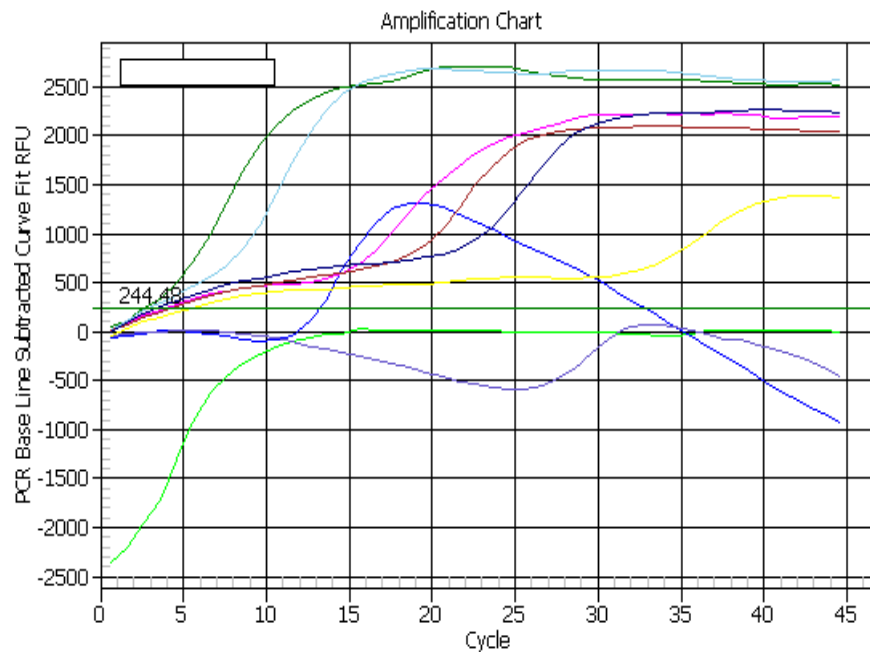


Figure 2.12 - Troubleshooting SNA primers for real-time PCR, PCR Run 1

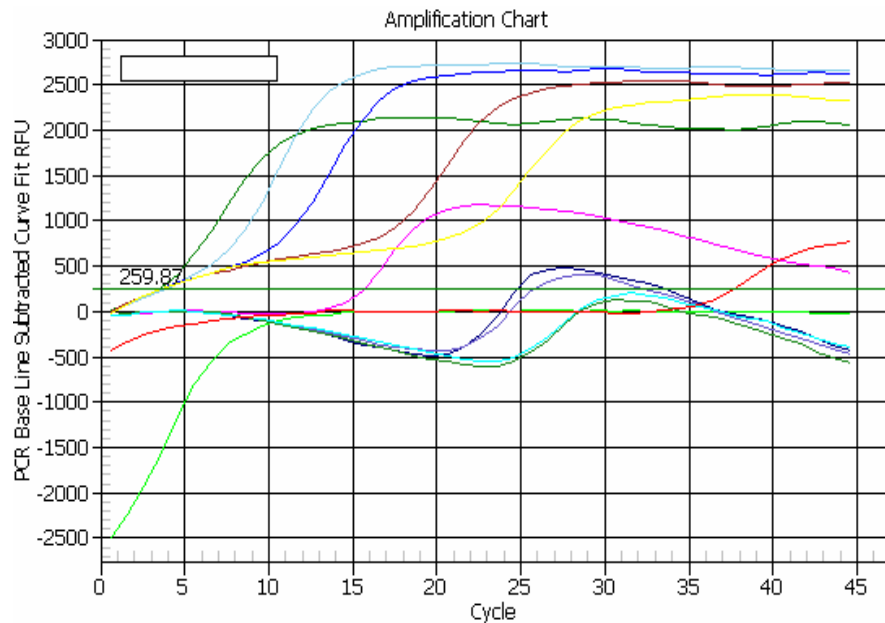


Figure 2.13 - Troubleshooting SNA primers for real-time PCR, PCR Run 2

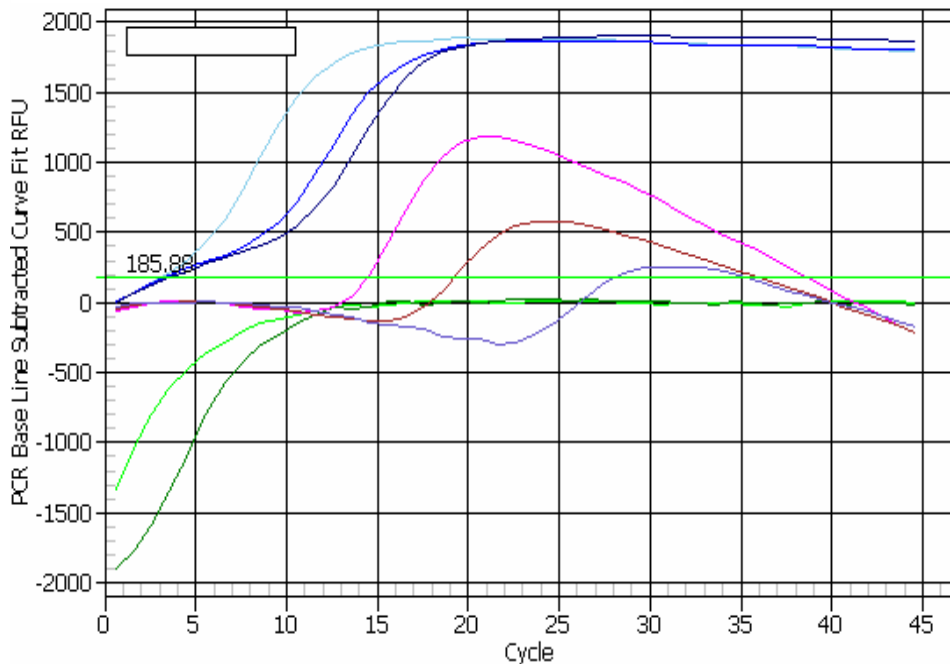


Figure 2.14 - Troubleshooting SNA primers for real-time PCR, PCR Run 3

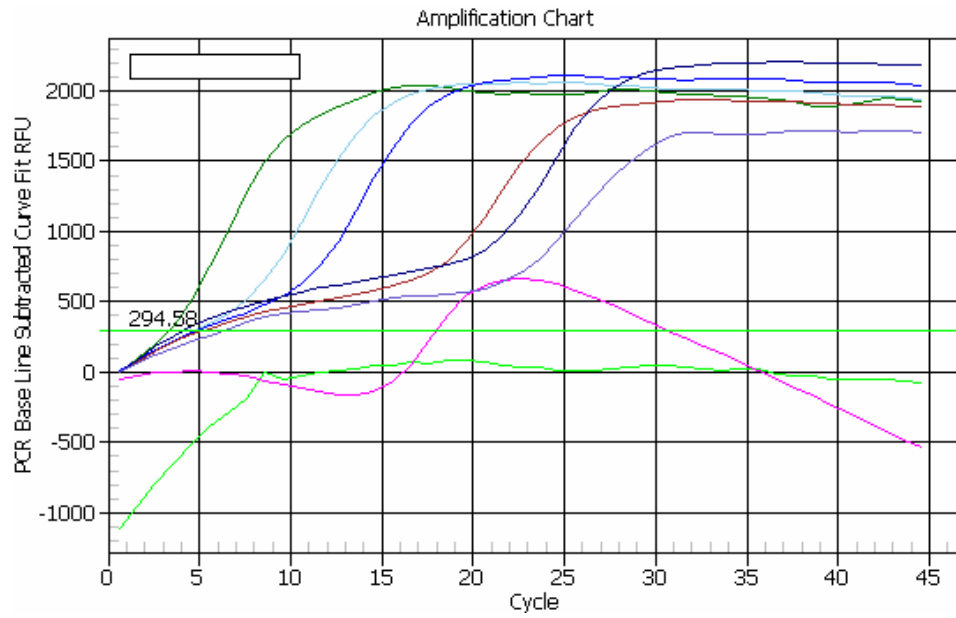


Figure 2.15 - Troubleshooting SNA primers for real-time PCR, PCR Run 4

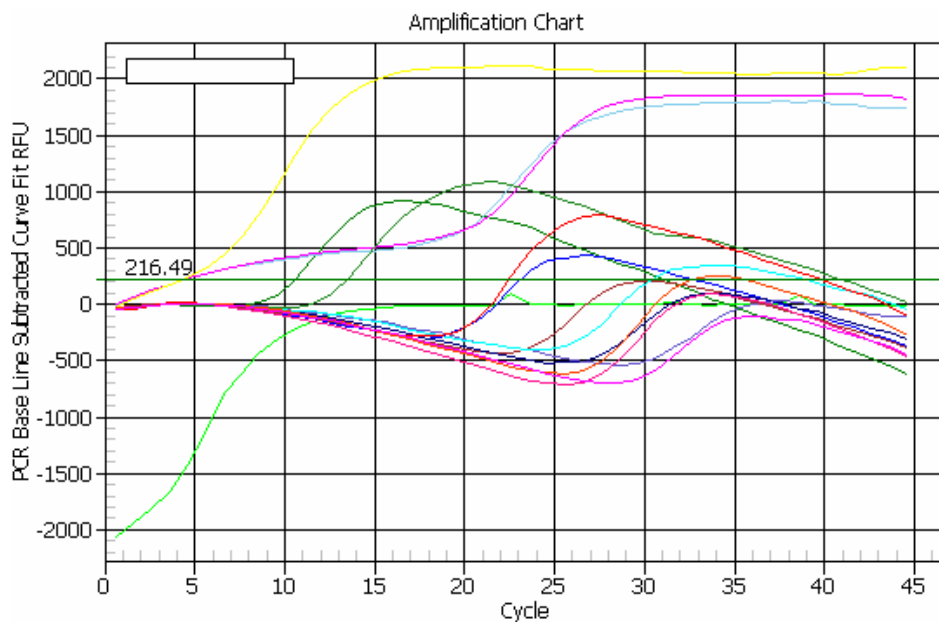


Figure 2.16 - Troubleshooting SNA primers for real-time PCR, PCR Run 5

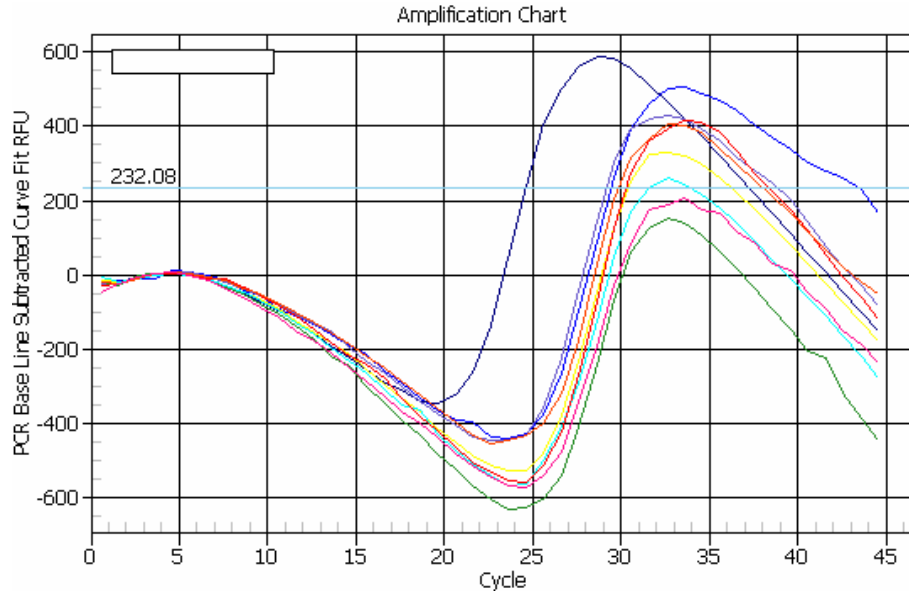


Figure 2.17 - Troubleshooting SNA primers for real-time PCR, PCR Run 6

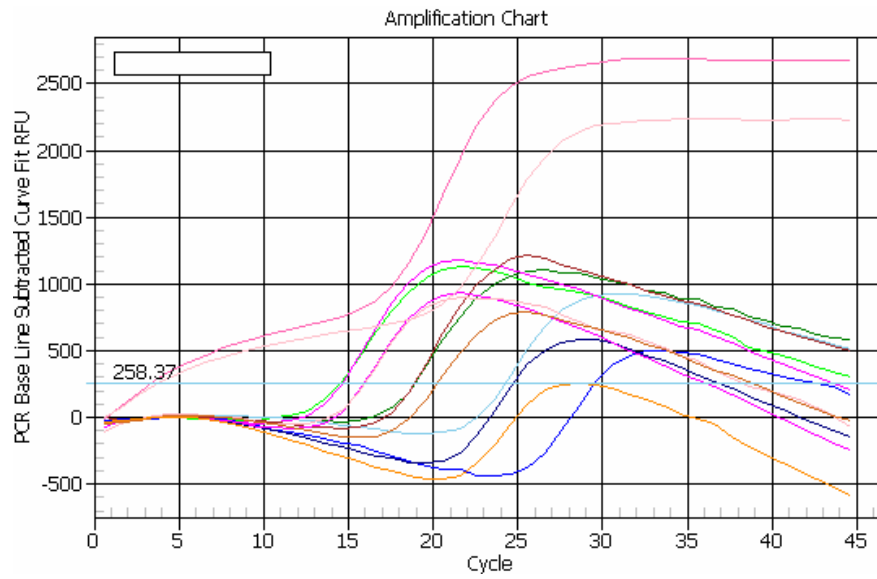
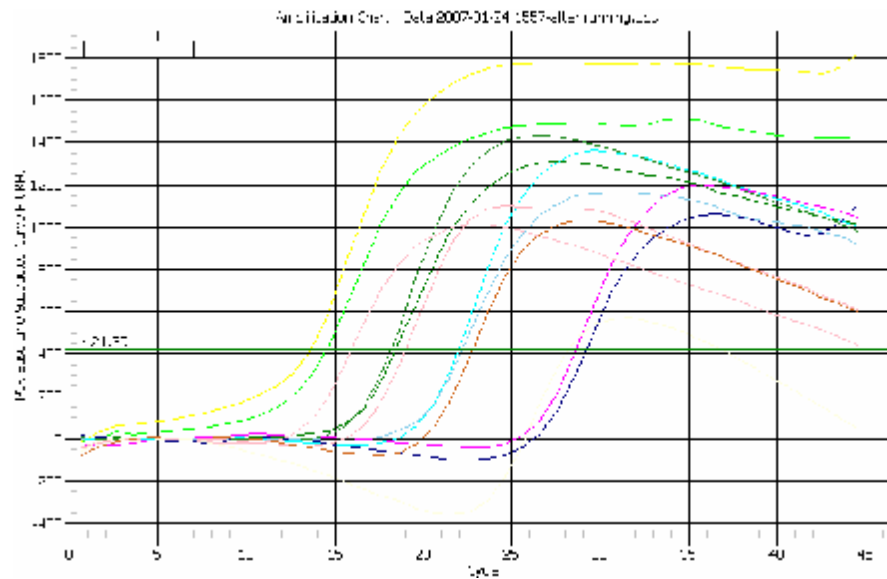


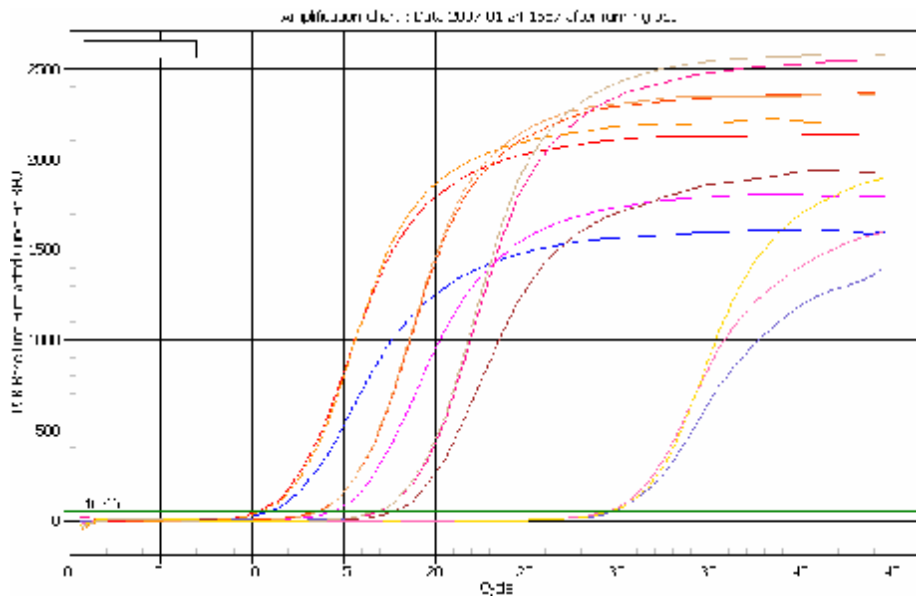
Figure 2.18 - Optimizing Annealing Temperature - SNA primers

After testing purified versus unpurified PCR product, SNA primers versus bacterial primers, template concentration and well position, the next factor tested was annealing temperature. Three template amounts (0.075, 0.0075, 0.00075 ng) were tested at four annealing temperatures (55.8, 58.9, 63.3, 65°C). As seen in Figure 2.18, most of the samples did not amplify properly. The next target of investigation was the template. Three different concentrations of genomic DNA (26, 2.6 and 0.26 ng) were used as target instead of PCR product, again the fluorescence decreased in most of the samples instead of forming a plateau as expected (Figure 2.19). Also tested in the same PCR run were the primers themselves. Genomic DNA with the same concentrations listed previously was amplified with the primers designed for cPCR. Finally, this run gave good results. The fluorescence curve is flat at the initial part of the run, increases during the exponential phase, and then forms a plateau at the end of the reaction (Figure 2.20).

Next, PCR product generated from genomic DNA and SNA cPCR primers, in a 10X dilution series ranging from 5×10^{-2} ng/ μ L to 5×10^{-8} ng/ μ L was used as template in a real-time reaction. The results showed excellent fluorescence and standard curves (Figures 2.21-2.22). The standard curve had an $R^2 = 0.999$ and an efficiency of 106.2% which indicates a high quality standard curve. However, since it was determined that the cPCR primers were not specific enough, the primers were redesigned once again, using the iterative procedure described previously. The final result of the primer design is listed in Table 2.5 of Section 2.2.2.1



**Figure 2.19 - Troubleshooting SNA primers for real-time PCR
Genomic DNA + primers SNA958F/SNA1068R**



**Figure 2.20 - Troubleshooting SNA primers for real-time PCR
Genomic DNA + primers SNAR2/SNAF2**

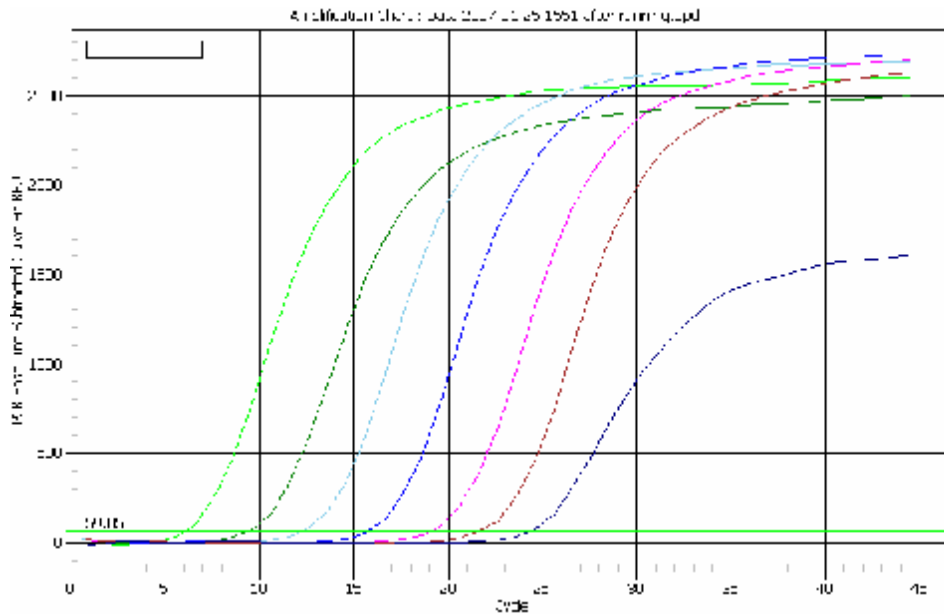


Figure 2.21 - Amplification curve
 PCR product of genomic DNA/SNAR2/SNAF2 as template + primers SNAR2/SNAF2

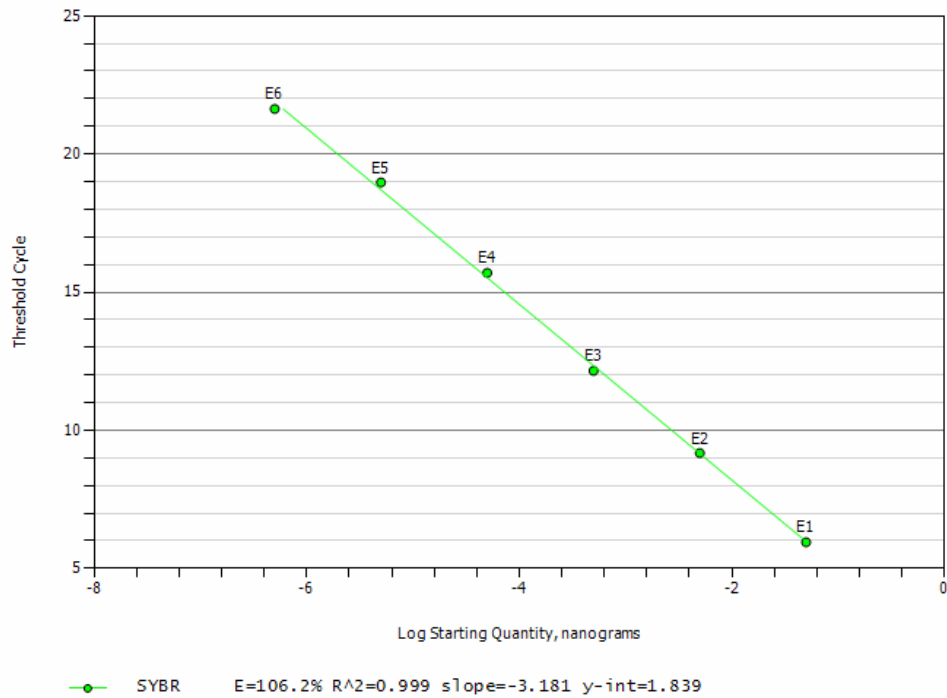


Figure 2.22 - Troubleshooting SNA primers - Standard curve
 PCR product of genomic DNA/SNAR2/SNAF2 as template + primers SNAR2/SNAF2

After deciding on the final sequences of the SNA and AGF primers, the annealing temperature for the primers was optimized. The temperature range tested was 55-65°C. The results for AGF reveal no real difference between the annealing temperatures. For SNA, the temperature that gave the lowest C_t value was 65°C, however, the rest of the temperatures had similar C_t values and all amplified well; therefore an annealing temperature of 60°C was chosen for both species (Figures 2.23-2.24).

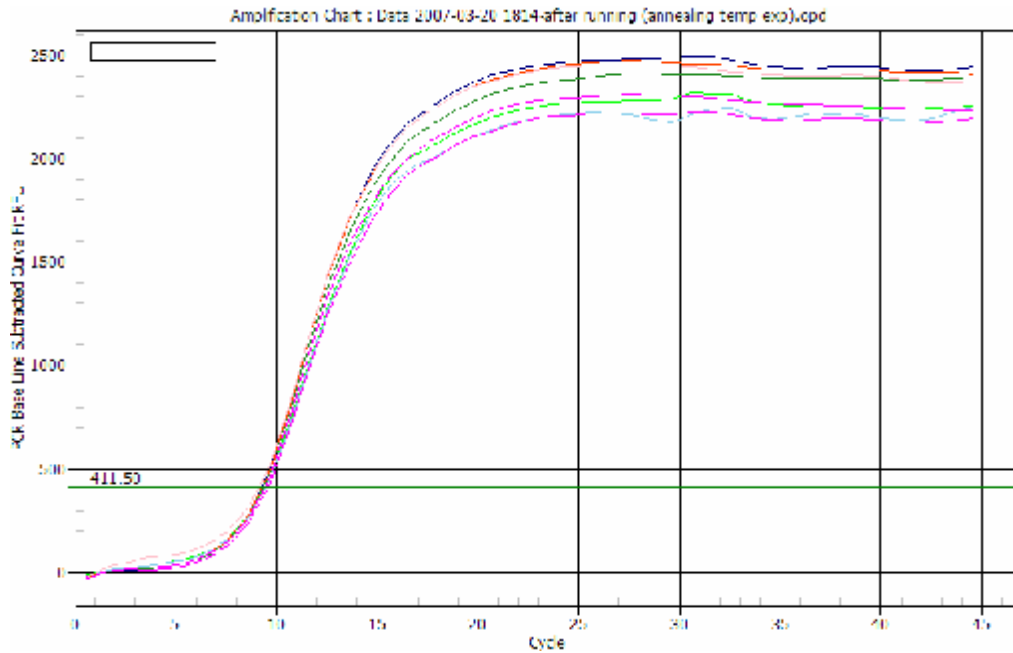


Figure 2.23 - Optimizing annealing temperature of AGF primers
Temperature range = 55-65°C

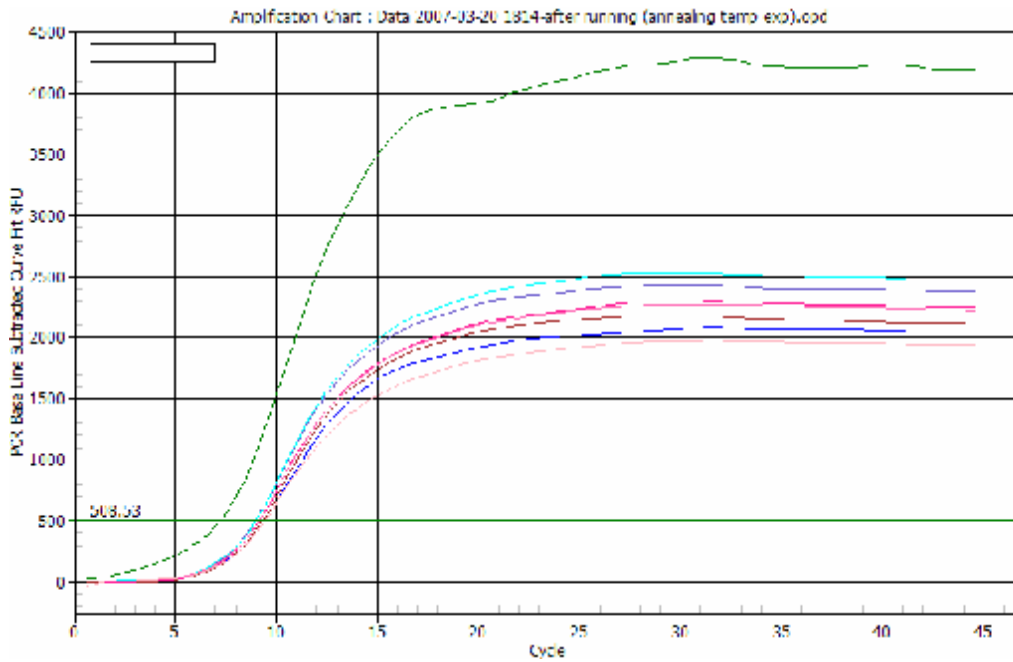


Figure 2.24 - Optimizing annealing temperature of SNA primers
Temperatures tested were 55-65°C, the one that gave the lowest Ct value was at 65°C, but since all of the rest of the products amplified similarly well, and similarly, the temperature chosen was 60°.

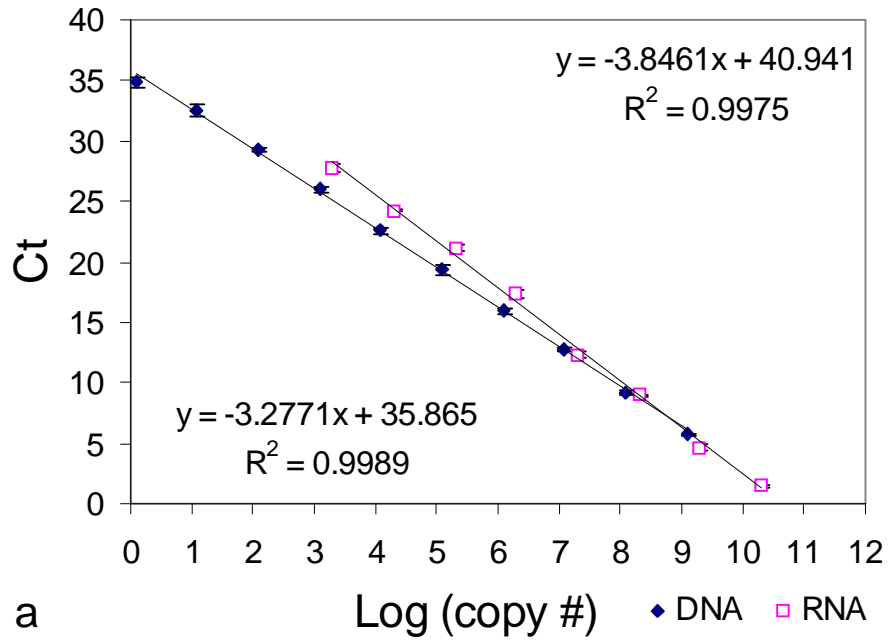
2.2.3.2 Real-time DNA and RNA Standards

After designing new primers for real-time PCR, the next step was to generate standard curves. Initially, the same primers used for real-time PCR to make the standard curve were used to generate the DNA standards. As stated earlier, this resulted in overestimation of the standard, meaning the spectrophotometer could quantify all of the product, but only some of it could be amplified in the reaction. The standard curves generated from these reactions are shown in Figures 2.26-2.29. For all of the graphs, the C_t value starts to tail off toward lower initial template concentrations. Since the assay is only able to detect concentrations in the linear range, this would result in too high detection limits. For SNA RNA, the detection limit would be 10^8 copies which means that the assay would not be able to detect the presence of SNA RNA in an RNA extraction sample unless there are more than 10^8 copies present. Fey et al. (12) had the same problem with using the same primers for generating standards as running PCR reactions. The solution was to use different primers to generate RNA and DNA standards that would create a larger amplicon encompassing the sites for the real-time primers.

Figure 2.25 shows the results of the standard curves for SNA and AGF respectively, after employing the same strategy adapted by Fey et al. Both RNA and DNA curves for SNA are linear over a range of 10^9 copies. AGF DNA and RNA have linear ranges of 10^{10} and 10^8 copies respectively. The detection limits of each assay are 954 and 2 copies for SNA RNA and DNA respectively and 3,591 and 16 copies for AGF RNA and DNA respectively. These limits were determined by taking the C_t value of the no template control (NTC),

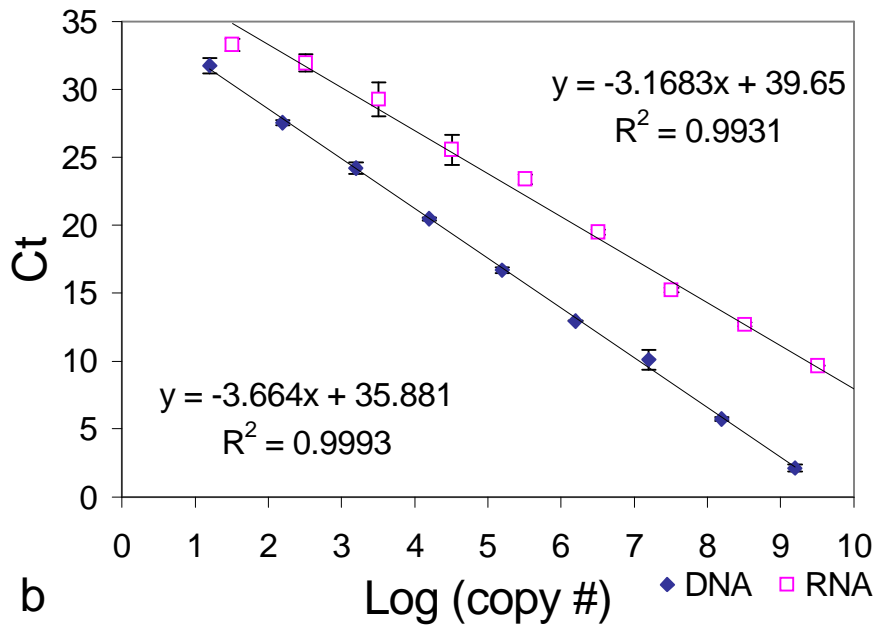
subtracting 3.3 (which corresponds to a 10-fold dilution) then calculating the corresponding copy number (46). The limit for SNA DNA was determined by taking the highest C_t value (instead of the NTC) minus 3.3 since there was no amplification in the NTC for this assay. Real-time PCR assays can be less accurate at very low copy numbers, therefore data outside this limit was considered to be under the detection limit of the assay. Each data point is an average of triplicate real-time reactions run from the same dilution set. The standard deviation for AGF ranged from 0.10 to 0.31 and 0.09 to 0.54 for RNA and DNA respectively. The ranges of standard deviations for SNA were 0.04-1.25 and 0.03-0.74 for RNA and DNA respectively. The error bars are shown on the graph.

AGF Standard Curves



a

SNA Standard Curves



b

Figure 2.25 - Real-time qPCR and real-time RT-qPCR standard curves
a) AGF and b) SNA

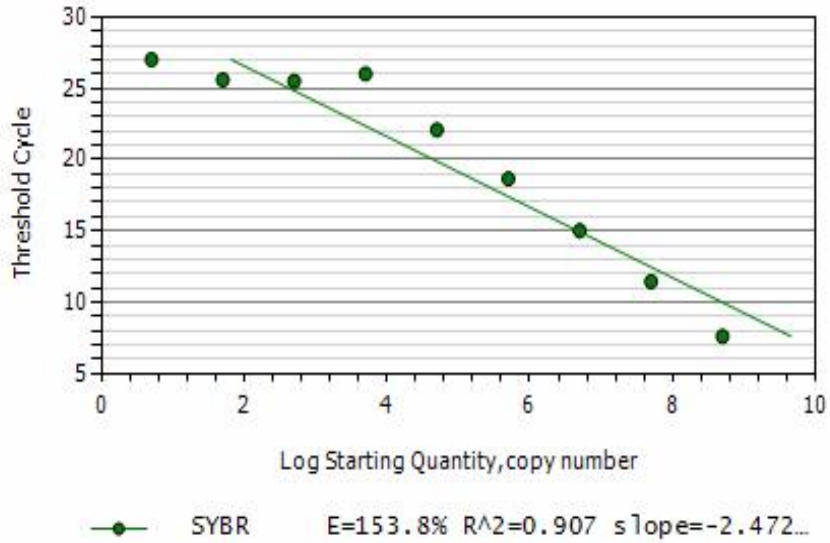


Figure 2.26 - AGF DNA Standard curve attempt
 AGF1066R/AGF965F primers were used to generate standards and run PCR.
 DNA dilution range = 8.7 - 0.7 log copies

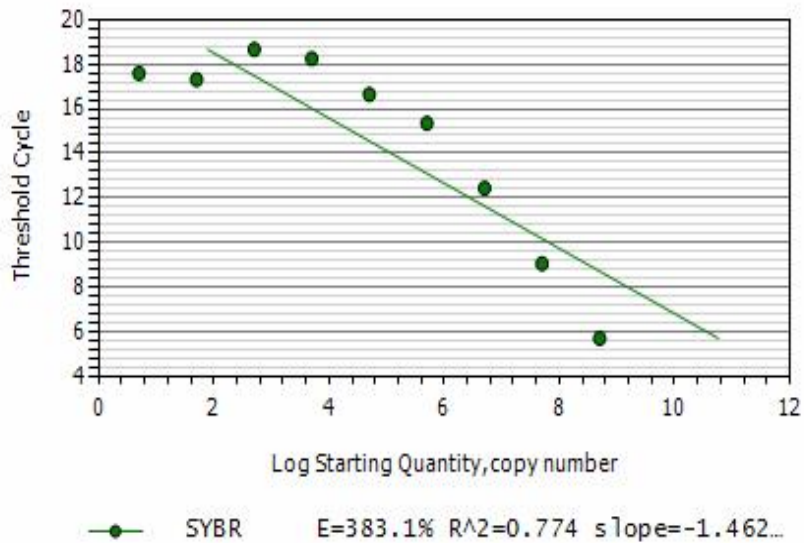


Figure 2.27 - SNA DNA Standard curve attempt
 SNA550R/SNA417F primers were used to generate standards and run PCR. DNA dilution range = 8.7 - 0.7 log copies

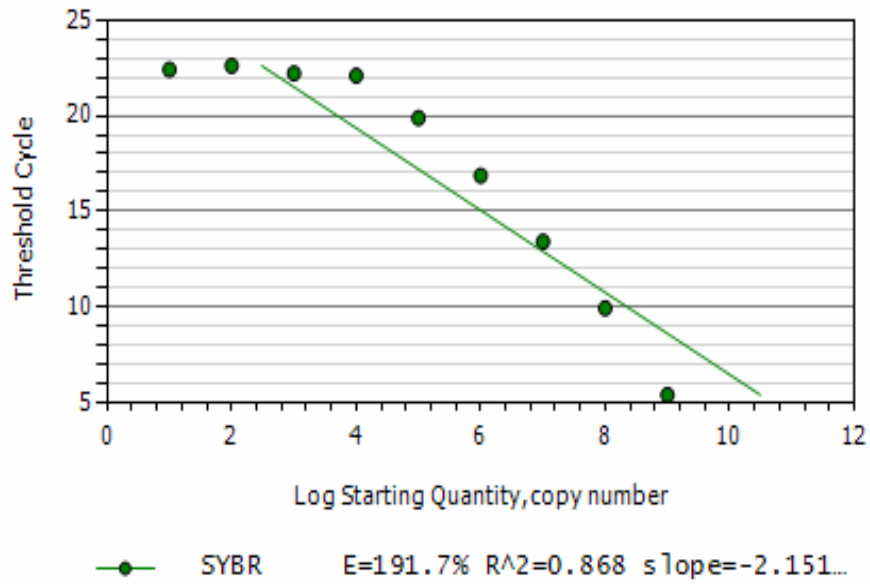


Figure 2.28 - AGF RNA Standard curve attempt AGF1066R/AGF965F primers were used to generate standards and run PCR. DNA dilution range = 10¹⁰ copies - 1 copy

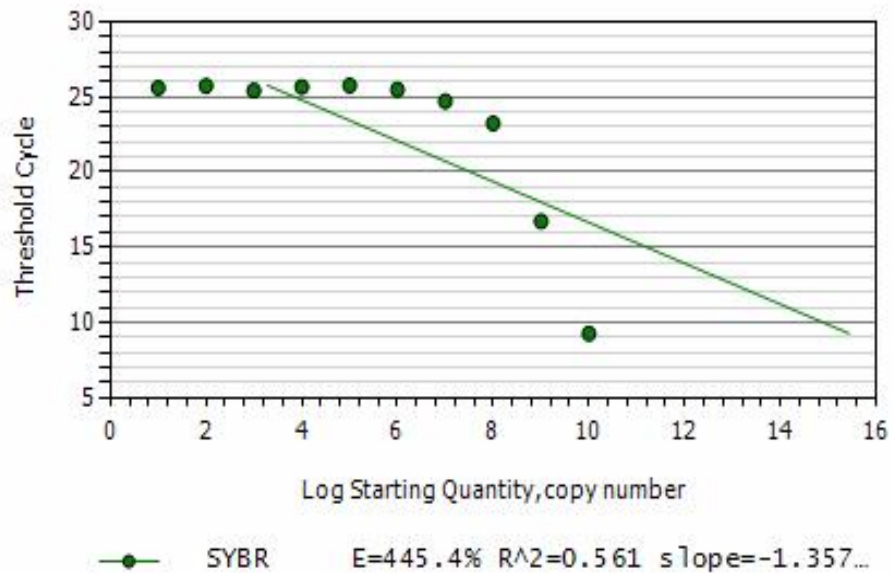


Figure 2.29 - SNA RNA Standard curve attempt SNA550R/SNA417F primers were used to generate standards and run PCR. DNA dilution range = 10¹⁰ copies - 1 copy

2.2.3.3 Specificity of SNA real-time primers

A Blast (Basic Local Alignment Search Tool) search was performed on all primers to test the specificity *in silico*. The Blast results of real-time primers are shown in Appendix C. Though the primers were designed for maximum specificity, the results of Blast show that both the SNA550R and SNA417F primers for SNA target a number of unclassified and uncultivated organisms. Sequences that were targeted by both the forward and reverse primers were chosen for a phylogenetic tree analysis to determine how similar these organisms are to *Sphaerotilus natans*. The unknown organisms along with several SNA species sequences and close relatives of SNA were aligned in the RDP phylogenetic tree analysis program (8). The phylogenetic tree generated by the program is shown in Figure 2.30. The caveat included in the instructions of the program state that it is not to be used quantitatively. Even so, the tree is still useful in providing basic information. This tree shows that the uncultivated and unclassified organisms are more similar to SNA than to *Leptothrix*, *Rubrivivax* or *Ideonella* species which are close relatives of SNA. Therefore, the primers are specific to SNA and unidentified “SNA-like” organisms. No tree was generated for AGF primers because the combined specificity of these primers showed no bacteria other than *Arthrobacter spp.* were targeted.



Figure 2.30 - Phylogenetic tree showing sequences targeted by SNA550R and SNA417F primers

The Blast searches and phylogenetic tree analysis determines specificity of the primers to organisms other than SNA and AGF by comparing sequences. Another way to test specificity is by running a melt curve after a real-time PCR run. Melt curves were generated by increasing the temperature 0.5°C every 30 seconds after the end of a PCR run. As the dsDNA denatures, the fluorescence decreases. The negative first derivative of the fluorescence curve is plotted as a function against temperature (3). Each peak of the melt curve represents one amplified product similar to bands on an electrophoresis gel. Some melt curves are shown in Figures 2.31-2.34. All of these melt curves show only one peak indicating the primers were specific. The primers did not target other organisms in the North Cary sludge. Also, they did not amplify other products within the SNA and AGF genomes, and no primer-dimers were formed.

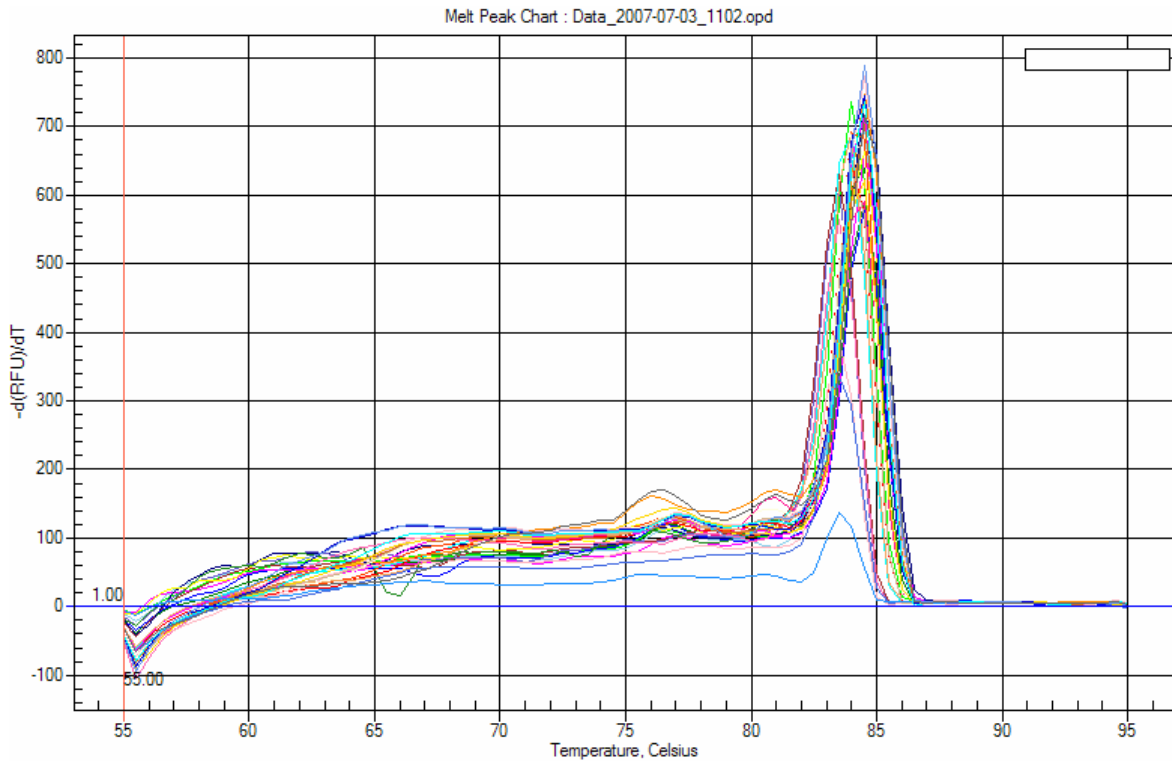


Figure 2.31 - Melt curve of RT-qPCR of N. Cary samples - AGF

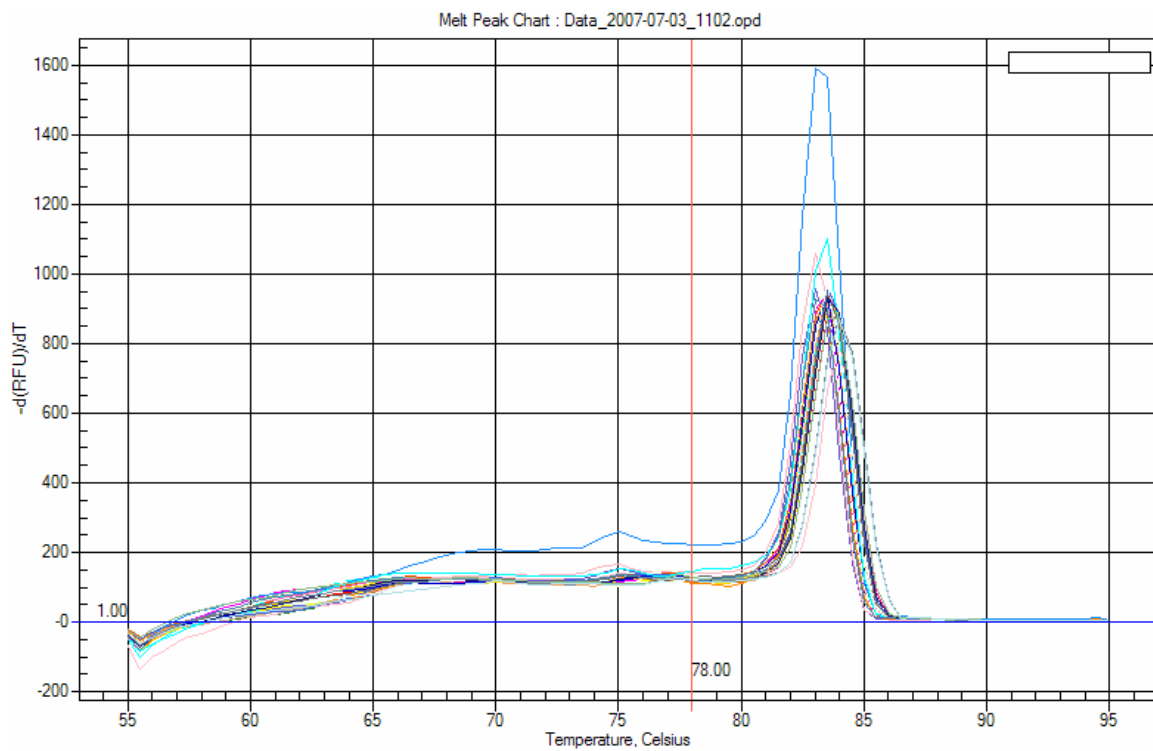


Figure 2.32 - Melt curve of RT-qPCR of N. Cary samples with SNA primers

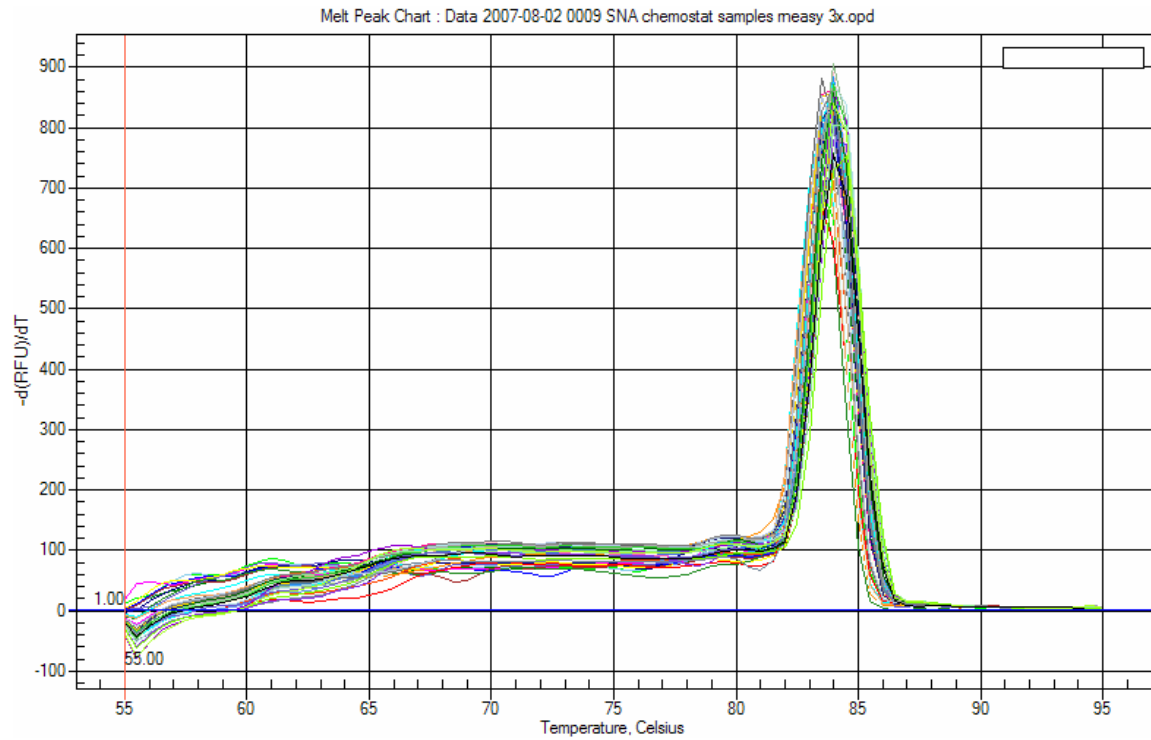


Figure 2.33 - Melt curve of RT-qPCR of SNA chemostat samples - SNA

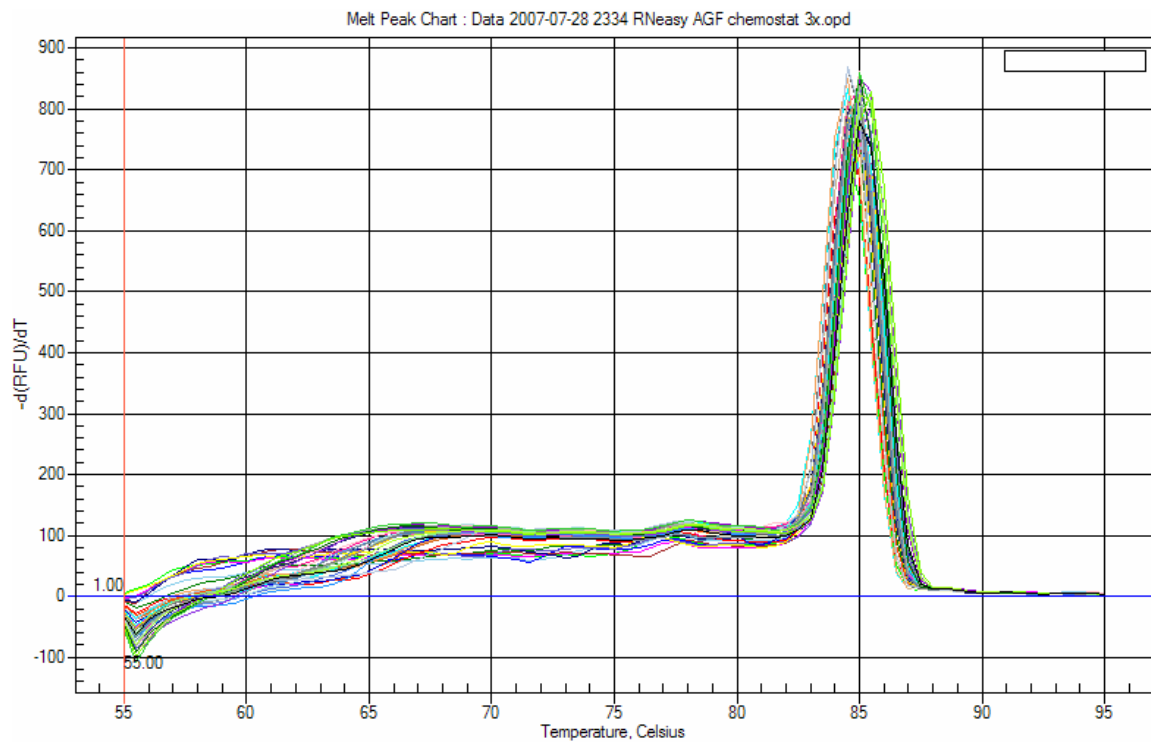


Figure 2.34 - Melt curve of RT-qPCR of AGF chemostat samples - AGF

2.2.4 Conclusions

Real-time qPCR and real-time RT-qPCR assays were developed to quantify DNA and RNA, respectively, for SNA and AGF. Development of the assay included primer design and optimization, and generation of standard curves.

A total of four standard curves of C_t versus log copy number of known amounts of nucleic acid were generated. The equations for these curves are as follows:

- SNA RNA log copy # = $12.515 * (C_t \text{ value}) - 0.316$
- SNA DNA log copy # = $9.793 * (C_t \text{ value}) - 0.273$
- AGF RNA log copy # = $10.645 * (C_t \text{ value}) - 0.260$
- AGF DNA log copy # = $10.944 * (C_t \text{ value}) - 0.305$

3 Determining RNA:DNA versus μ relationship for *Sphaerotilus natans* & *Arthrobacter globiformis*

3.1 Introduction

The 16S rRNA:DNA ratio has been correlated with growth rate (μ) for a number of organisms. However, the relationship has been found to vary across different species. In order to use the RNA:DNA ratio to determine in-situ growth rate of an organism, the relationship between this ratio and growth rate must first be established.

The objective of this study was to determine the relationship between RNA:DNA ratio and growth rate for bacterial species *Sphaerotilus natans* (SNA) and *Arthrobacter globiformis* (AGF). This relationship will be used to evaluate in-situ growth rates of these organisms in activated sludge. The competition between filamentous and floc-forming bacteria have long been studied, and the ability to determine in-situ growth rates of these organisms could potentially contribute to better methods for control of filamentous bulking in activated sludge.

The approach to determine the RNA:DNA versus μ relationship was to grow each organism in pure culture at various growth rates using chemostat reactors. The chemostat reactor is a continuously stirred tank reactor (CSTR) used for culturing microorganisms. In a CSTR, hydraulic residence time (HRT) is equal to the mean cell residence time (MCRT), or solids retention time (SRT), therefore the growth rate of organisms within the system can be controlled simply by adjusting the liquid flow rate through the system. Growth rate is calculated as the inverse of the HRT. Reactors were run in pure culture at five different

growth rates for each organism ranging from 5 hours to 5 days. RNA and DNA concentrations were analyzed for samples from each SRT using the assay described above. From this, the correlation curve was generated.

3.2 Materials and Methods

3.2.1 Chemostat setup

The reactor was a 1L jacketed glass spinner flask with a top and two side-arms on opposite sides of the vessel (Bellco 1965-51000). One side-arm was plugged with a BugStopperTM closure (Whatman 6713-3010) which allows air to pass but filters out bacteria and viruses. The other side arm was sealed with a rubber stopper with two glass tubes going through it connected to feed and effluent lines. The detailed set-up of chemostats is shown in Figure 3.1. Table 3.1 lists tubing and connector specifications. The effluent line began in the flask with 11.5 cm of tubing 1 (Cole-Parmer 06424-67) connected to the glass tubing inside the flask. On the other end of the glass tube, outside the flask, was 23 cm of tubing 1, then approximately 150 cm of tubing 2 connected with connector 1. Tubing 2 led directly into the effluent bottle, and was held in place with a foam stopper. The foam stopper and opening of the effluent bottle was covered with aluminum foil. Tubing 1 was connected to the glass tube by stretching it over the end of the glass tube.

The influent line began with approximately 10 cm of tubing 3 connected to the barbed spout at the bottom of the influent bottle. Tubing 3 was connected to 15 cm of tubing 1, which led to a one-way check valve (Bel-Art 197150000) to prevent backflow, to another 15 cm of tubing 1. Next was connector 1, then 90 cm of tubing 2, then another connector 1, 20 cm of tubing 1 which was connected to the glass tube in the rubber stopper. On the other end of the glass tube was about 5 cm of tubing 1.

Feed and effluent to each chemostat were pumped to and from the flask via a single cartridge pump (Masterflex cartridge pump model # 7519-15, cartridge model #7519-85). Two different pump motors were used, one with a range of 6-600 RPM (model # 7553-70) for faster flow rates, and one with a range of 1-100 RPM (model # 7553-80) for slower flow rates. Each pump required a speed controller: one pump was connected to a Masterflex speed controller (model # 7553-17) and the other to a Stir-pak speed controller (model # 4554-12). Changing the rotation speed of the pump by adjusting the knob on the speed controller allowed for coarse adjustment of flow rate. Fine tuning of flow rate was achieved by adjusting the knob on the cartridge. This had the effect of flattening or un-flattening the tubing.

Temperature was kept constant at 22°C via a water jacket on the flask. The water jacket had one barbed hose connector at the top and one at the bottom on opposite sides of the flask. An aquarium pump circulated water from a water bath through the water jacket. Tubing 3 was used to connect the lower barbed hose connector of the water jacket to connector 3 which was connected to approximately 1.5m of tubing 4 which was connected to the aquarium pump. On the effluent side of the water jacket, tubing 3 connected the higher barbed hose of the water jacket to 1.5m of tubing 4 which dropped directly in the water bath. The water bath was not capable of cooling water to 22° C, however it was capable of holding the temperature steady at 22°C if cooler water was pumped in, therefore the water in the water bath was circulated with water in a cooler filled with ice water (Figure 3.4). A Masterflex pump similar to the ones on the effluent and feed lines was used to circulate water, however Norprene tubing was used instead of tubing 2, because it was much more durable.

All glass containers were kept in a Plexiglas box during operation of the chemostat in an attempt to minimize contamination. Holes were drilled in the sides of the box to allow tubing to be connected to pumps and the water bath. The flasks were equipped with magnetic stir bars and were placed on top of mixers to maintain suspension of the cultures.

To reduce the possibility of contamination in the system, the entire reactor setup, including media, feed and effluent bottles and all lines were set up and autoclaved for 1.5 hours. The system was inoculated by removing the BugStopper™, pouring in 100 mL of culture grown overnight and quickly replacing the BugStopper™.

Table 3.1 - Chemostat parts and descriptions

Name	Description	Company	Cat. No.
Connector 1	Barbed fittings, Reducing Connector, Kynar, 1/8" x 1/16" ID, 1/32", 13/16", 7/32";	Cole-Parmer	30703-41
Connector 2	Barbed fittings, Reducing Connector, Kynar, 1/4" x 1/8" ID, 1/16", 1-15/32", 1/2"	Cole-Parmer	30703-50
Tubing 1	C-FLEX® tubing, 1/8"ID x 1/4"OD	Cole-Parmer	06424-67
Tubing 2	Masterflex® peroxide-cured silicone tubing, L/S®13 ID = 0.03 in	Cole-Parmer	96400-13
Tubing 3	Clear C-FLEX® tubing, 1/4"ID x 3/8"OD	Cole-Parmer	06422-10
vinyl tubing	ID = 3/8", OD = 1/2"	Fisher Sci.	14-169-7G

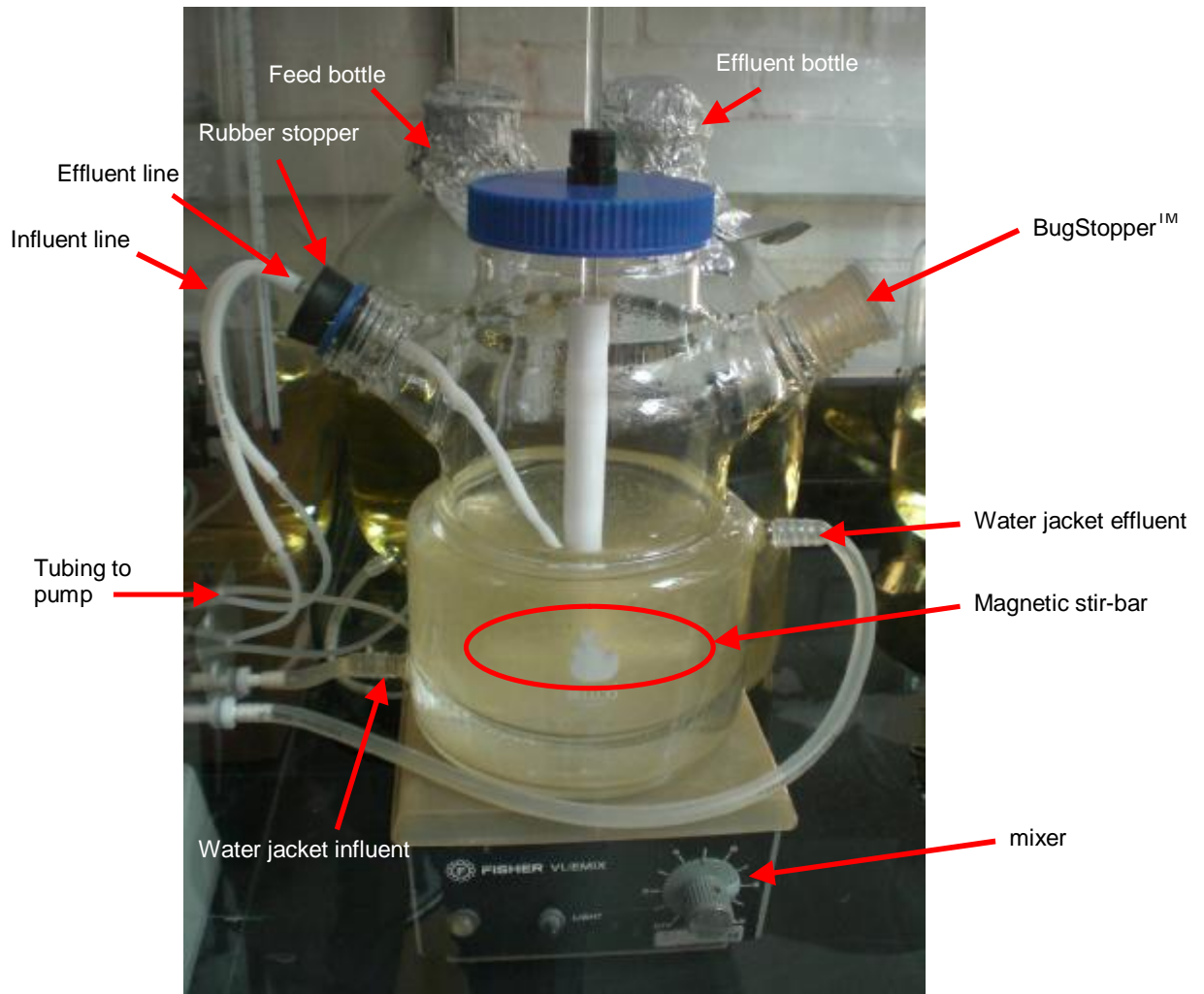


Figure 3.1 - Diagram of chemostat setup



Figure 3.2 - Overall setup of chemostat reactors
From L to R: cooler, water bath, pump and controller 1, Plexiglas box containing chemostat/feed/effluent systems 1&2, pump and controller 2.



Figure 3.3 - Close-up of pump (to show fine and coarse adjustments)



Figure 3.4 - Cooler and water bath setup

3.2.2 Chemostat operation

The volume in the chemostats was kept as close to 1L as possible. Tubing cut to length on the effluent line was used as constant level-control to fix the volume in the reactor. This was done by keeping flow rate in the effluent line slightly higher than in the feed line. Chemostats were operated for at least 3x SRT to obtain steady state in the system.

The media used was prepared as recommended by ATCC. For SNA the feed was diluted 2X, so that influent COD was at about 10,000 mg/L. The media for *Arthrobacter globiformis* was nutrient broth (Difco 234000).

3.2.3 Measuring flow rate

The flow rate of the system was measured at the end of each run. The feed tube was disconnected from the spinner flask and propped up at the same height so that the feed media

could flow into a graduated cylinder. The flow rate was determined by measuring the time needed to fill the cylinder up to a given volume.

3.2.4 Determining SRT

Solids retention time was calculated as the volume of liquid in the flask divided by the flow rate of the feed, or $SRT = V/F$.

3.2.5 Collecting Samples

After running the chemostats for at least 3X SRT, the reactors were taken down for sampling. The spinner flasks were placed on a mixer to maintain suspension of the culture within a laminar flow hood to prevent contamination. As many 50 mL samples as possible were taken from the well-mixed reactor. The volume of the reactor was measured by totaling the number of 50 mL samples plus any residual liquid in the reactor. The samples were pelleted, then the liquid portion was removed and the pellet stored immediately at -80°C .

3.2.6 Nucleic acid extraction

RNA and DNA extractions were performed using the RNeasy Mini Kit (Cat. No. 74104) for RNA and DNeasy Blood and Tissue Kit (Cat. No. 69504) for DNA from Qiagen. Cell pellets intended for DNA extraction were pre-treated with the protocol for gram-positive bacteria. The pre-treatment consisted of a 40 minute incubation at 37°C with enzymatic lysis buffer (20 mM Tris-Cl, pH 8.0, 2 mM sodium EDTA, 1.2% Triton® X-100, lysozyme) and a 30 minute incubation at 56°C with proteinase K and a buffer containing a chaotropic salt. After lysis, the mixture was purified with a spin-column protocol.

Cells for RNA extraction were also pre-treated with lysozyme and proteinase K. The protocol used was Protocol 4 from the RNAprotect Bacteria Reagent Handbook, however steps 2-6 were omitted because the RNAprotect reagent can decrease RNA yield. Twenty μL of proteinase K was used. All RNA extracts were DNased twice using Qiagen's RNase-Free DNase Set (Cat. No. 79254). The amount of DNase I was doubled for increased DNA removal. Doubling the amount of DNase, required doubling the amount of buffer RDD, Buffer RLT and ethanol during the purification protocol. In between the first and second DNase reactions, the RNA was purified with the Qiagen RNeasy Mini Kit.

3.2.7 Solids Measurements

Total suspended solids (TSS) for chemostat samples were measured according to Standard Methods (10). For TSS analysis of the chemostat samples, 45 μm filters (Whatman) were prepared by drying at 105°C for at least 20 minutes, cooled in a dessicator then the weight was recorded. Fifty mL of culture were used for analysis for each chemostat run. Filters were weighed immediately before use and the weight was recorded (A). Samples were heated at 105°C for 20 minutes, cooled in a dessicator, and then the weight was recorded (B). TSS was calculated using the formula:

$$\text{TSS} = \frac{B - A}{\text{sample volume}} \quad (3)$$

Since the chemostats contained pure cultures, it is assumed that TSS = VSS.

3.2.8 RNA:DNA versus μ relationship for SNA and AGF

The RNA:DNA versus μ relationship for each species was determined by running pure culture chemostats at 5 different SRTs. Triplicate samples from each SRT were collected for RNA and DNA extraction. Each extraction replicate was then run in triplicate PCR reactions. The C_t values from the PCR reactions were used to calculate the concentration of RNA and DNA in the original culture sample using the standard curves and Equation 4. Sample calculations are shown in Appendix G.

3.2.9 Calculating copy number

RNA and DNA concentrations from real-time PCR reactions were determined from the standard curves. These are expressed as copy # per μ L of original chemostat sample. The formula used to convert copy # per PCR reaction to copy # per μ L of sample is as follows:

$$\frac{\text{copy \#}}{\text{ml of sample}} = \frac{\left(\frac{\text{copy \#}}{\text{PCR reaction}} \right) \times \left(\frac{\text{volume of DNA eluate}}{\text{DNA extraction}} \right)}{\left(\frac{\text{vol. of DNA eluate}}{\text{PCR reaction}} \right) \times (\text{vol. of original culture sample})} \quad (4)$$

This copy number was converted to nanograms of RNA or DNA so that the RNA:DNA ratio can also be expressed as ng RNA/ng DNA as well. This was done by manipulating Equation 2.

3.3 Results and Discussion

3.3.1 RNA:DNA versus μ relationship for SNA and AGF

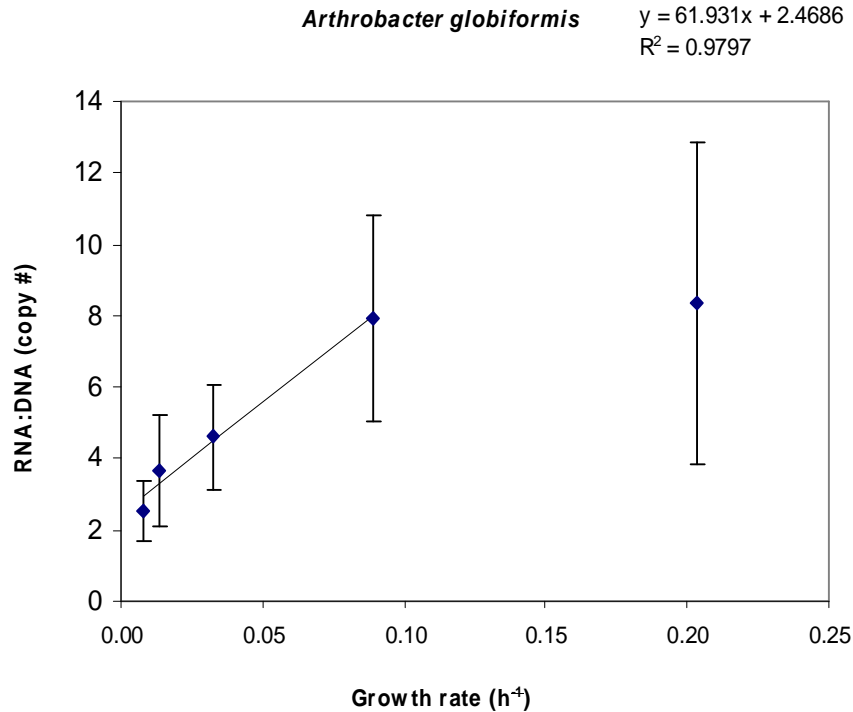


Figure 3.5 - RNA:DNA ratio vs. specific growth rate of AGF
Each point is an average of all replicates, $n=9$ (three extractions, triplicate PCR run for each extraction).

Figure 3.5 shows that the relationship between growth rate and RNA:DNA ratio for AGF is both positive and linear ($R^2 = 0.98$) for growth rates ranging from 0.008 to $0.090 h^{-1}$. The $0.203 h^{-1}$ data point was not included in the linear regression. Presumably at this point, the cells have reached maximum capacity and the RNA:DNA ratio versus growth rate relationship breaks down. The RNA:DNA ratios determined for AGF are in the range of those determined for several other species including marine isolates (24), and resin-acid degrading bacteria (34).

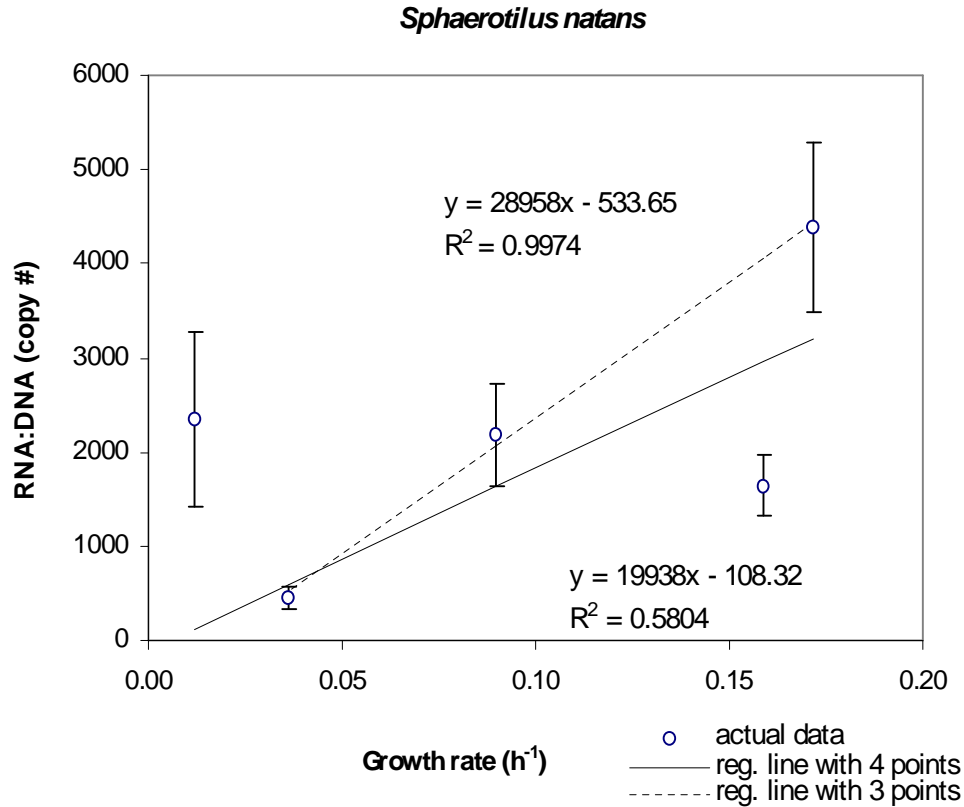


Figure 3.6 - RNA:DNA ratio vs. specific growth rate of SNA
 Each point is an average of all replicates, n=9 (three extractions, triplicate PCR run for each extraction).

Figure 3.6 shows the relationship between RNA:DNA ratio and the growth rate of SNA. The solid regression line is the one used to calculate the in-situ growth rate of SNA in WWTP samples. The 3.5 day SRT (growth rate = $0.012 h^{-1}$) was omitted from this regression line. Excessive biofilm formation inside the chemostat and on the walls of tubing may have affected the growth rate of the bacteria at such a long SRT. A second, dotted regression line is shown with a very high R^2 value of 0.99, which excludes both the 6 hour (growth rate = $0.15 h^{-1}$) and 3.5 day SRTs (growth rate = $0.012 h^{-1}$). It seems that this may be the more correct relationship, however, only more chemostat experiments could verify whether or not this is true.

3.3.2 Conclusions

The relationship between 16S rRNA:DNA ratio and growth rate for SNA and AGF was determined using chemostat reactors and the real-time PCR assays described previously. Each species was grown in pure culture chemostats at five different growth rates (SRTs). At each growth rate, samples were collected for RNA and DNA extraction and quantitation by real-time RT-qPCR and real-time qPCR respectively. The relationship was found to be linear and positive for both SNA ($R^2 = 0.58$) and AGF ($R^2 = 0.98$). In-situ growth rate can be determined using these equations:

$$\text{SNA} \rightarrow \text{RNA:DNA (copy \#)} = 19938 * (\text{growth rate, h}^{-1}) - 108.32,$$

$$\mu = 0.0363 - 0.1715 \text{ h}^{-1}$$

$$\text{AGF} \rightarrow \text{RNA:DNA (copy \#)} = 61.931 * (\text{growth rate, h}^{-1}) + 2.4686,$$

$$\mu = 0.008 - 0.0891 \text{ h}^{-1}$$

4 In-situ growth rate of SNA and AGF in wastewater

4.1 Introduction

The real-time PCR assay was used to quantify the amount of SNA and AGF DNA in activated sludge samples collected from the Neuse River WWTP in Raleigh, NC. During transportation from the WWTP to the lab, these samples were treated with three different methods to determine the effect on RNA and DNA concentration. The three different holding methods were: immediate freezing with ethanol and dry ice, holding on ice, and holding at room temperature.

Additionally, samples from two previous studies were also analyzed. One study determined the effect of readily metabolizable substrates on bulking (13). In this study by Gulez (13) sequencing batch reactors (SBR) were inoculated with sludge from the Neuse River WWTP in Raleigh, NC and fed different substrates to determine the effect on the community structure and bulking. Molecular methods, FISH and denaturing gradient gel electrophoresis (DGGE) were compared with traditional staining methods Gram and Neisser staining to analyze the samples. The other study used membrane hybridization to survey the filamentous population in different WWTPs across North Carolina (23).

4.2 Materials and Methods

4.2.1 Sample holding treatment study

Sludge from North Cary (NC) was collected from the mixed liquor of the aeration basin. The sludge was poured into a one gallon jug, and 15 mL aliquots were transferred into sterile 15 mL conical centrifuge tubes. These samples were handled during transport with three different holding treatments to determine if there was any significant effect on DNA and RNA concentrations. The three treatments were: immediate freezing in a mixture of ethanol and dry ice (E), holding on ice until processing (I) and holding at room temperature with no ice (N) until processing. Approximately five of the 15 mL centrifuge tubes were used for each holding treatment. Upon reaching the lab, approximately ten cell pellets were made for each treatment by spinning down 2 mL of resuspended MLSS in sterile 2 mL screw cap centrifuge tubes. The ethanol treatment samples were thawed at room temperature for approximately 30 minutes before processing. Two 10 mL samples from the no-ice treatment were used for solids analysis. The time between collection and start of processing for all samples was not greater than two hours.

4.2.2 Nucleic acid extraction

Samples from the North Cary WWTP were extracted with MoBio Powersoil kits for RNA (Cat. No. 12866-25) and DNA (Cat. No. 12888-50). RNA was extracted using the manufacturer's protocol with no modifications. After extraction with the Powersoil kit, RNA samples were DNased and purified twice as described in Section 3.2.6. The DNA protocol

was modified to prevent loss of DNA sample. Modifications are marked on the manufacturer's protocol (Appendix D). Samples from both the substrate study and survey of NC WWTPs were extracted with Qiagen kits as described in Section 3.2.6.

4.2.3 Solids analysis

For wastewater treatment plant (WWTP) samples, both TSS and VSS were measured. The filters were prepared by passing through (10 mL) of deionized water three times, dried for 20 min at 105°C, then heated at 550°C until the weight changed by no more than 0.05 mg. Filters were stored in a dessicator at room temperature until use. 10 mL of MLSS collected from the aeration basin of the WWTPs were used for solids analysis. After being filtered, the sample was washed by passing 10 mL of deionized water through the filter. The sample was dried at 105°C until the weight changed by no more than 0.05 mg and the weight was recorded. Next the sample was incinerated at 550°C until the weight changed by no more than 0.05 mg and the weight recorded.

4.3 Results and Discussion

4.3.1 Sample holding treatment study

RNA and DNA were extracted from wastewater treatment plant samples using MoBio Powersoil kits. Concentration and purity of the extracts were determined with the NanoDrop spectrophotometer (NanoDrop Technologies, Wilmington, Delaware). Real-time PCR was run on each sample in triplicate and the concentration of SNA and AGF DNA and RNA in the extract were calculated from standard curves. From this number, SNA DNA and RNA copy number in the original MLSS sample were calculated using Equation 4. AGF DNA and RNA concentrations were below the detection limit of the assay, therefore the data is not presented. Two-sided paired t-tests were performed on the SNA copy number/mL of sample. Significant differences were found in DNA concentration between all methods and in RNA concentrations for samples held on ice versus frozen immediately in ethanol and dry ice (Table 4.1).

Figure 4.1 shows SNA DNA decreasing with increasing temperature, but RNA increasing with increasing temperature. This indicates the possibility that some cells are dying at the higher temperature, but the cells that are living are more active. The objective of this study was to determine how the holding method for transporting samples from WWTPs to the lab would affect RNA and DNA concentrations in the samples, with the hope of identifying the best method. The criterion for best method was highest yield of both DNA and RNA. The no-ice treatment gave the highest concentration of SNA RNA but the lowest concentration of SNA DNA. The EtOH/dry ice treatment gave the highest concentration of

SNA DNA but the lowest concentration of SNA RNA. Since the ice method did not give the lowest values for either DNA or RNA concentration, it was determined that holding on ice is the most suitable method for transporting samples.

Table 4.1 - Results of t-tests – Effect of holding methods on SNA DNA & RNA

T-test	SNA RNA		SNA DNA	
	p-value	conclusion	p-value	conclusion
E and I	0.7935	not significantly different	0.0484	significantly different
N and I	0.0508	not significantly different	0.0444	significantly different
N and E	0.0191	significantly different	0.0002	significantly different

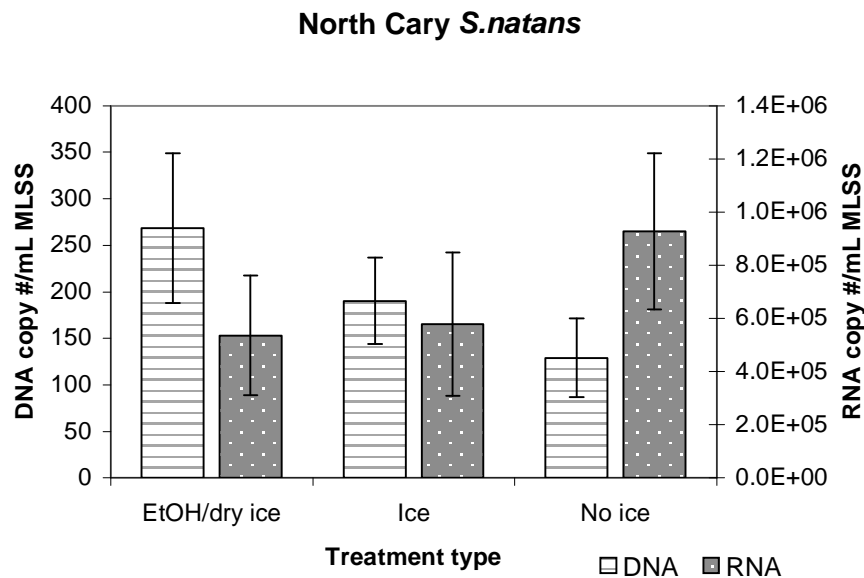


Figure 4.1 - SNA RNA and DNA in North Cary WWTP activated sludge

4.3.2 In-situ growth rate

In a previous study, sequencing batch reactors were inoculated with sludge from the Neuse River (NR) WWTP in Raleigh, NC and fed various substrates to determine the effect on community structure and bulking. Each reactor run lasted 10-12 days and the reactor was

fed the same substrate from beginning to end. Samples were collected approximately every 2 days for staining and FISH analysis. Additional samples were collected for RNA and DNA extraction (13). Three of these samples were chosen from each reactor run for growth rate analysis, one from the beginning of the reactor run, one from the middle and one from the end.

Results from this analysis are shown in Figure 4.2. Unfortunately, the in-situ growth rate of only a few points could be determined with accuracy from the correlation curve. Most of the data falls outside of the growth rate range determined with the chemostats. However, if the growth rates are extrapolated from the curve assuming the relationship holds for all possible growth rates, the data compares reasonably well with previously determined FISH results (Figures 4.4 - 41.7). For glucose, the first FISH picture shows minimal SNA while succeeding pictures show vastly increased levels of the filament. The growth rate data reflects this change, increasing from 0.01 to 0.16 to 35.10 (h^{-1}). The same trend is seen for propionate with the growth rate reflecting what the FISH pictures depict. Though FISH was not done on the methanol sample, the growth rate shows a similar trend, but on a smaller scale. Though FISH pictures show similar trends for acetate and pyruvate, this was not reflected in the growth rate determinations. Both sets of samples showed increasing RNA and DNA from beginning to end of the reactor run, however, the RNA did not increase proportionately high enough to show increasing RNA:DNA ratios. This could be attributed to possible overloading of the filter during RNA extraction. The optimal cell number for *B. subtilis* as determined by Qiagen is 1×10^8 - 2.5×10^8 cells. AGF was under the detection limit of the assay and therefore no data for AGF is presented. See Appendix F for raw data.

The in-situ growth rates of SNA in activated sludge at the North Cary WWTP from the different holding treatments are presented in Figure 4.3. Some data points were outside of the range of the growth rate standard curve as determined from the chemostats, however were included to show the trends of the data. Each bar presented is an average of nine data points (3 extractions, 3 PCR runs per extraction). AGF DNA and RNA were below the detection limit of the assay therefore no data is presented. See Appendix F for raw data.

Samples from another previous study (23), were also analyzed for in-situ growth rate. The study was a survey of several North Carolina WWTPs, and used membrane hybridization to determine the SNA abundance as a percentage of total RNA in the aeration basin, RAS and foam of each plant. The samples chosen for analysis were from the aeration basin of the following WWTPs: Town of St. Paul's WWTP, Eagle Road WWTP in Cramerton, Gunpowder Creek WWTP in Hudson, Town of Monroe WWTP and Long Creek WWTP in Gastonia. Membrane hybridization determined that SNA was present at abundances of 6.5%, 9%, 9%, 5.5%, and 10% of total rRNA respectively. However, with real-time PCR, SNA was barely detectable (≤ 100 copies / mL of sample) and AGF was not detectable in any of the samples therefore growth rate data is not presented. Raw data is presented in Appendix F. Inability to detect SNA with real-time PCR is most likely due to the fact that the samples were fairly old (collected in 2001) and the nucleic acids have probably degraded with repeated freeze-thaw cycles.

Growth rate of *S.natans* on various substrates

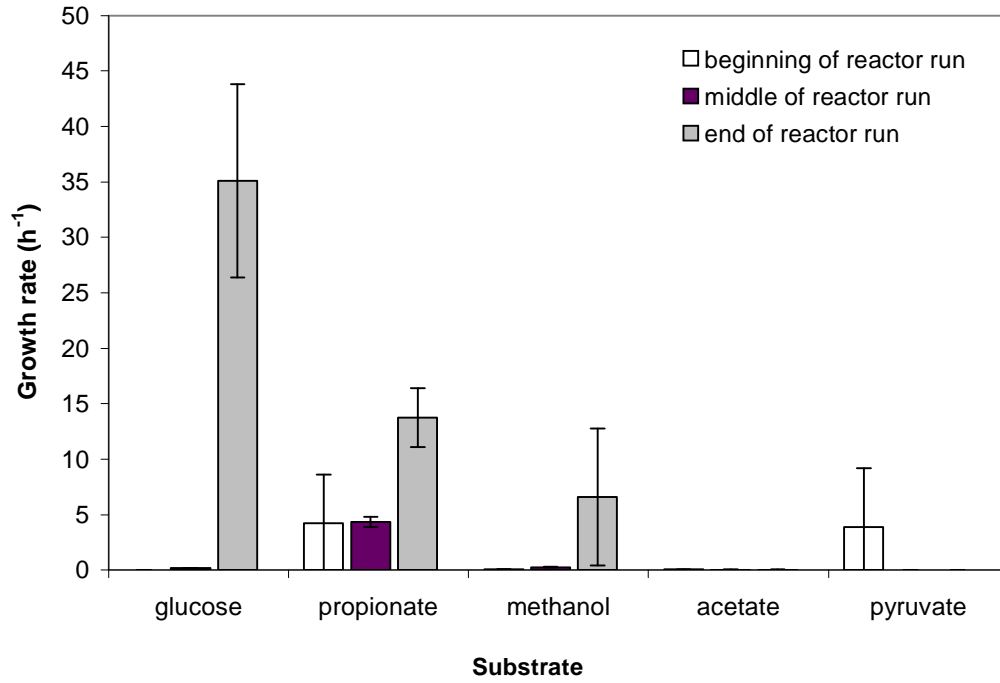


Figure 4.2 - Growth rate of SNA on different substrates

North Cary *S.natans*

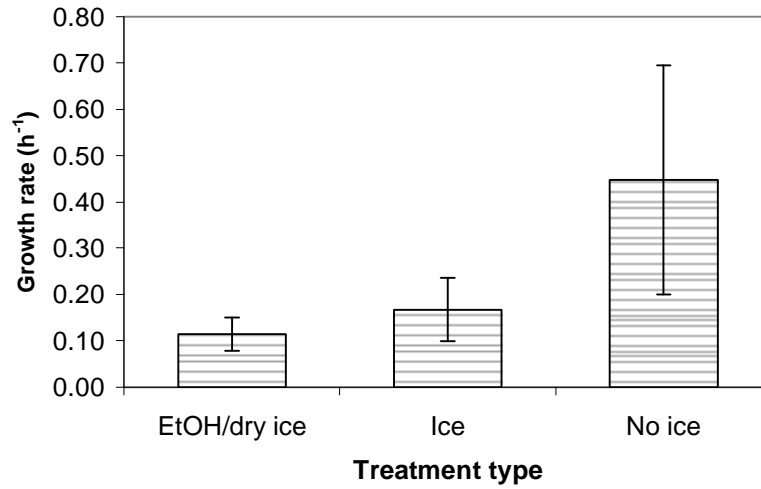
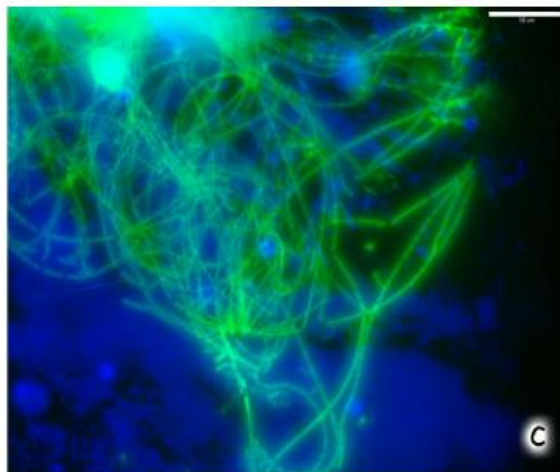
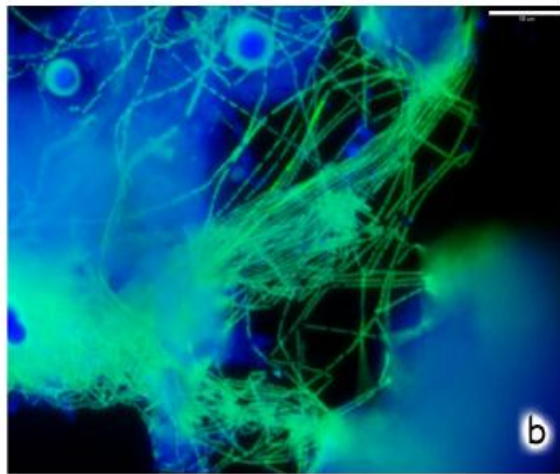
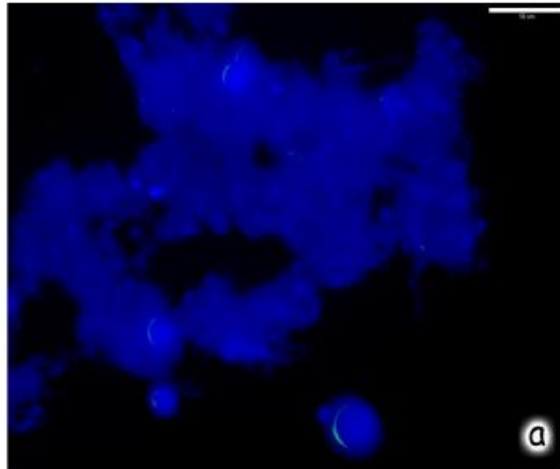
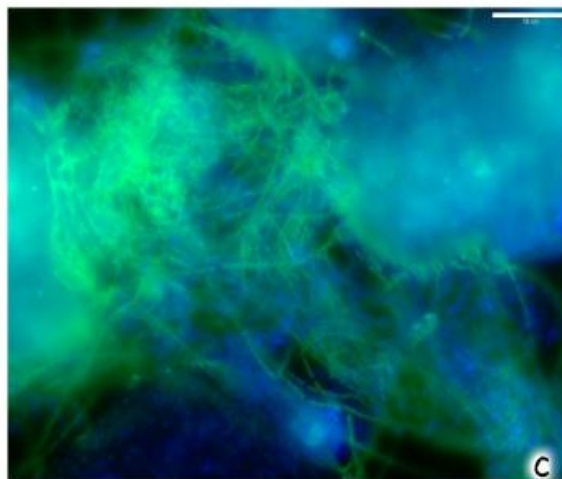
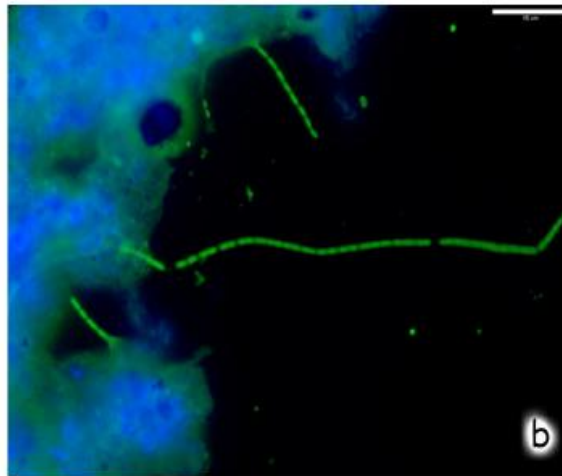
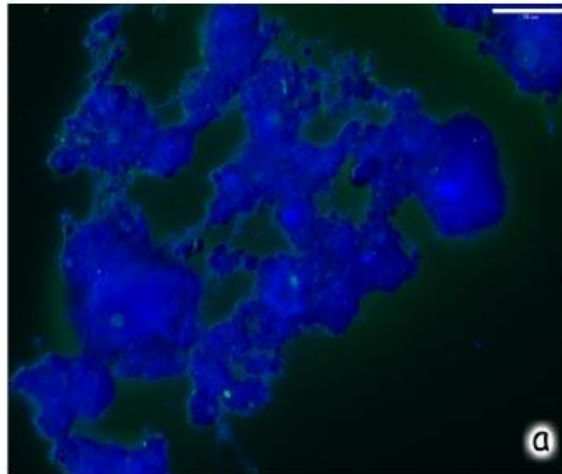


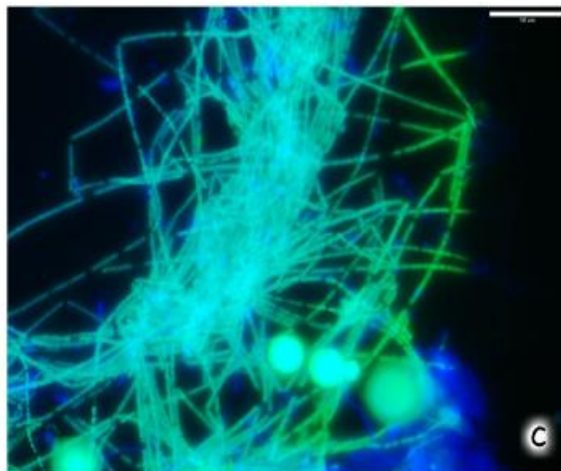
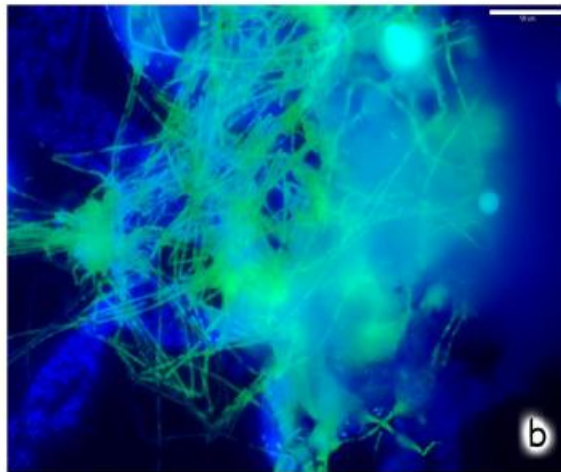
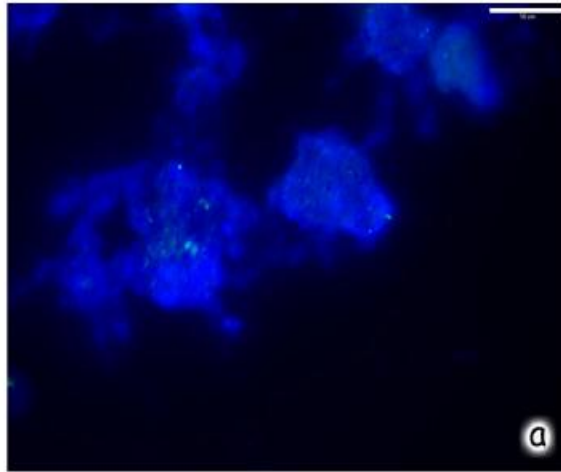
Figure 4.3 - In-situ growth rate of SNA - North Cary WWTP



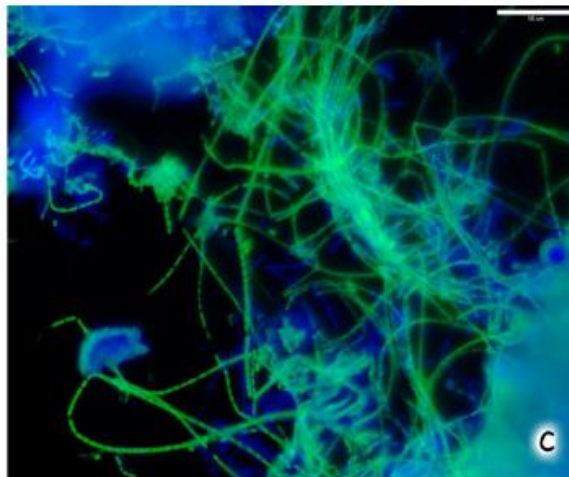
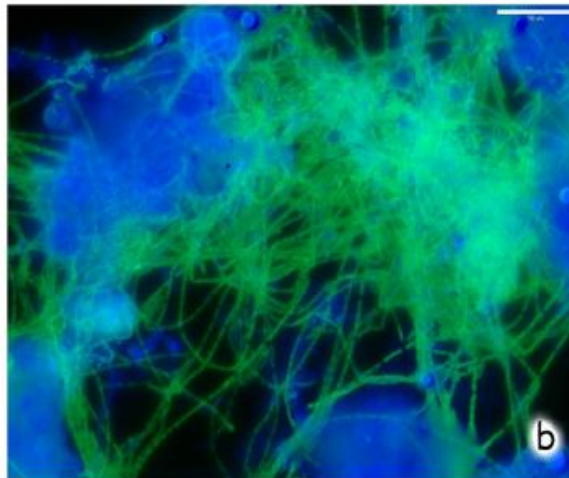
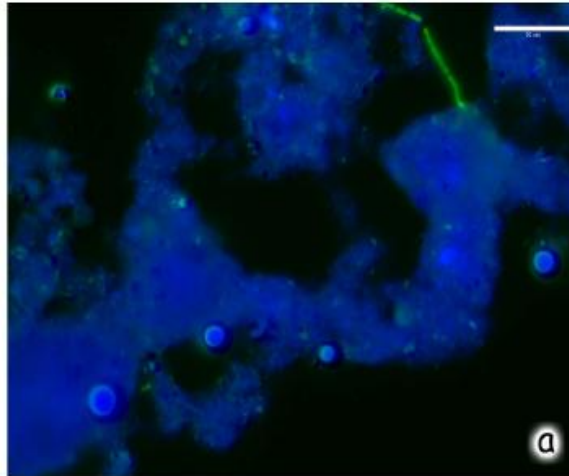
**Figure 4.4 - FISH image for SNA in glucose fed NR WWTP inoculated reactor
a) G1 b) G2 c) G3, bar=50 μ m (13)**



**Figure 4.5 - FISH image for SNA in propionate fed NR WWTP inoculated reactor
a) PR1 b) PR2 c) PR3, bar=50 µm (13)**



**Figure 4.6 - FISH image for SNA in acetate fed NR WWTP inoculated reactor
a) A1 b) A2 c) A3, bar=50 μm (13)**



**Figure 4.7 - FISH image for SNA in pyruvate fed NR WWTP inoculated reactor
PY1 b) PY2 c) PY3, bar=50 μ m (13)**

4.4 Conclusions

The in-situ growth rate of SNA in several different types of samples was determined by correlation to RNA:DNA using real-time PCR and RT-PCR to quantify DNA and RNA. In samples from the North Cary WWTP, it was determined that the holding method during transportation of the samples (ethanol/dry ice, ice, no ice) affects RNA and DNA concentration, which will in turn affect the growth rate measurement. DNA seemed to increase whereas RNA decreased with colder holding temperature. The most accurate measure would result from processing immediately after collection, however, since this was not feasible, holding the samples on ice would probably be best.

For samples from the substrate study, in-situ growth rate results for SNA were compared with previously generated FISH pictures. The results from glucose and propionate fed reactors seemed to agree well with the FISH pictures, while pyruvate and acetate growth rate calculations did not. This could be attributed to a number of possible issues including possible overloading of nucleic acid extraction filters or nucleic acid degradation.

The survey study samples were shown to contain some SNA by membrane hybridization but SNA could not be detected with real-time PCR. This is most likely due to the age of the samples, as they were collected in 2001. AGF could not be detected in any of the samples.

5 Summary of Conclusions

The objective of this project was to develop a relatively easy and fast method to measure specific in-situ growth rate of a floc-forming and a filamentous bacterial species in activated sludge. Being able to measure the in-situ growth rate of a particular species within a complex mixed culture environment opens the doors to studying these organisms in a way which has, until fairly recently, eluded microbiologists and engineers alike. This goal was achieved for *Sphaerotilus natans* (a filament) and *Arthrobacter globiformis* (a floc-former) using real-time quantitative PCR and correlating RNA:DNA ratio to growth rate.

The detection limits of each assay are 954 and 2 copies for SNA RNA and DNA respectively and 3591 and 16 copies for AGF RNA and DNA respectively. The range of growth rates which can be determined accurately from the correlation curves are 0.0363 – 0.1715 (h^{-1}) for SNA and 0.008 – 0.0891 (h^{-1}) for AGF. The assay was used to determine in-situ growth rates of AGF and SNA from three sources of WWTP samples, two of which are from previous studies and one was collected from the North Cary WWTP. AGF could not be detected in any of the samples. SNA was detected in the samples from the substrate effect study (13) and at the North Cary WWTP but not in the samples from the NC WWTP survey study (23). Unfortunately most of the SNA growth rates in the samples from the substrate effect study were outside of the range of growth rate from the correlation curve, however if the data is extrapolated as if the relationship holds for all growth rates, important information on growth trends is revealed. The real-time PCR assays were able to quantify change in

growth rates of SNA as bulking progressed. Qualitative changes in filament levels, as shown by FISH micrographs, corresponded with increasing growth rates of SNA.

6 Recommendations for Future Work

Some recommendations are included here for work which could provide a more complete picture of bulking caused by SNA using the information generated for this thesis.

1. The R^2 for the correlation curve for SNA RNA:DNA ratio versus growth rate could potentially be higher if more data points are gathered. Since many of the RNA:DNA ratios of SNA in samples from the substrate effect study were out of range on the high side of the correlation curve, more focus should especially be put on obtaining RNA:DNA ratios for the higher growth rates.
2. The in-situ growth rate data of the substrate effect study reveals that the RNA:DNA ratio can be used to track the growth rate of a certain population over time. Tracking the growth rate of AGF and SNA before, during and after a bulking event using sequencing batch reactors could reveal valuable information about floc and filament competition.
3. Sequencing batch reactors could also be used to investigate the effect of any number of parameters on SNA growth rate. Parameters of interest include flow conditions (completely mixed versus plug-flow) and particulate versus soluble substrate.
4. To complete the picture, a modeling component should be included such that the growth rate data would be an input parameter into a model of a WWTP system which would be able to accurately predict bulking events given certain operating parameters.

5. Since some problems were encountered obtaining primers which were specific enough, pre-cursor RNA and 23S rRNA could also be investigated as potential targets for correlation to growth rate.

6. Preliminary work was completed to optimize RNA and DNA extraction procedures (Appendix E). Additional work is needed as the methods are not fully optimized.

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APPENDICES

8 Appendix A - Sequences

8.1 SPHAEROTILUS ALIGNMENT SEQUENCES

zero

gi|47280|emb|Z18534.1|SN16SRDNA

S.natans 16S ribosomal DNA

```
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tacccttgacatgtctgaaatcctgcagagatgtgggagtgctcgaagagaatcagaacacaggtgctgcatg
gcegtcgtcagctcgtgctgtagatgttgggttaagtcccgcacagcgcgaacccttgtcattagttgctac
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gtagttagcctaaccgc
```

one

gi|28372140|dbj|AB087568.1|

Sphaerotilus sp. L19 gene for 16S rRNA, partial sequence

```
attgaacgctggcgggtatgccttacacatgcaagtcgaacggtagaggggcaacccttgagagtggcgaacggg
tgagtaatacatcggaacgtgccagtcgtgggggataacgtagcgaagctacgctaataaccgcatacagacct
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ggtacctgaagaataagcaccgggtaactacgtgccagcagccgcggttaatacgtaggggtgcaagcgttaactc
gaattactgggcgtaaagcgtgcgagggcgttccataagacagatgtgaaatccccgggctcaacctgggaac
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cggatcgtagctgcaactcgactgctggaagtgggaatcgctagtaatcgcggtatcagaatgtcncgggtgaat
acgttcccgggtcttgtagacacaccgcccgtcacaccatgggagcgggttctgcccagaagtagttagcctaaccg
caaggagggcgattaccacggcagggttctgtag
```

two

gi|28372139|dbj|AB087567.1|

Sphaerotilus sp. L13 gene for 16S rRNA, partial sequence

attgaacgctggcggatgccttacacatgcaagtcgaacggtagaggggcaacccttgagagtggcgaacggg
tgagtaatacatcggaacgtgccagtcgtgggggataacgtagcgaaagctacgctaataccgcatacgcct
gagggtgaaagcgggggatcgcaagacctcgcgcgattggagcggccgatggcagattaggtagttgggtgggg
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agactcctacgggaggcagcagtggggaatTTTggacaatgggcgaaagcctgatccagccataccgcgtgagg
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gaattactgggcgtaaaagcgtgcgagggcggTccataagacagatgtgaaatccccgggctcaacctgggaac
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aaacaggattagataccctggtagtccacgcctaaacgatgtcaactgggtggtgggaggggttctctcagt
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accgcacaagcgggtggatgatgtggtTTaattcgatgcaacgcgaaaaaccttacctacccttgacatggcag
gaatccccgagagatgtgggagtgctcgaaagagaacctgcacacaggtgctgcatggccgctcgtcagctcgt

three

gi|28372138|dbj|AB087566.1|

Sphaerotilus sp. L12 gene for 16S rRNA, partial sequence

attgaacgctggcggatgccttacacatgcaagtcgaacggtagaggggcaacccttgagagtggcgaacggg
tgagtaatacatcggaacgtgccagtcgtgggggataacgtagcgaaagctacgctaataccgcatacgcct
gagggtgaaagcgggggatcgcaagacctcgcgcgattggagcggccgatggcagattaggtagttgggtgggg
aaaggcctaccaagcctgcgatctgtagctggctctgagaggacgaccagccacactgggactgagacacggccc
agactcctacgggaggcagcagtggggaatTTTggacaatgggcgaaagcctgatccagccataccgcgtgagg
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naacaggattagataccctggtagtccacgcctantcgatgtcaactgggtggtgggaggggttctctcagt
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accgcacaagcgggtgnatgatgtggtTTaattcnatgcaacgcgaaaaaccttacctacccttgacatggcag
gaatccccgagagatgtgggagtgctcgaaagagaacctgcacacaggtgctgcatggccgctcgtcagctc

four

gi|28372136|dbj|AB087564.1|

Sphaerotilus sp. L7 gene for 16S rRNA, partial sequence

attgaacgctggcggatgccttacacatgcaagtcgaacggtagaggggcaacccttgagagtggcgaacggg
tgagtaatacatcggaacgtgccagtcgtgggggataacgtagcgaaagctacgctaataccgcatacgcct
gagggtgaaagcgggggatcgcaagacctcgcgcgattggagcggccgatggcagattaggtagttgggtgggg
aaaggcctaccaagcctgcgatctgtagctggctctgagaggacgaccagccacactgggactgagacacggccc
agactcctacgggaggcagcagtggggaatTTTggacaatgggcgaaagcctgatccagccataccgcgtgagg
gaagaaggccttcgggttgtaaaccgctTTTgtcagggaaagaaatcTTctgggctaatacctcgggaggatgac
ggtacctgaagaataagcaccggcctaactacgtgccagcagccgcggtaatacgtaggggtgcaagcgttaatcg
gaattactgggcgtaaaagcgtgcgagggcggTccataagacagatgtgaaatccccgggctcaacctgggaac
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aaacaggattagataccctggtagtccacgcctaaacgatgtcaactgggtggtgggaggggttctctcagt
aacgaagctaacgcgtgaagttgaccgcctggggagtagcggccgcaaggTTgaaactcaaaggaattgacgggg
accgcacaagcgggtggatgatgtggtTTaattcgatgcaacgcgaaaaaccttacctacccttgacatggcag
gaatccccgagagatgtgggagtgctcgaaagagaacctgcacacaggtgctgcatggccgctcgtcagctcgt
tcgta

five

gi|28372135|dbj|AB087563.1|

Sphaerotilus sp. L6 gene for 16S rRNA, partial sequence

attgaacgctggcggatgccttacacatgcaagtcgaacggtagaggggcaacccttgagagtggcgaacggg
tgagtaatacatcggaacgtgccagtcgtgggggataacgtagcgaaagctacgctaataccgcatacgcct
gagggtgaaagcgggggatcgcaagacctcgcgcgattggagcggccgatggcagattaggtagttggtgggg
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agactcctacgggaggcagcagtggggaatTTTggacaatgggcgaaagcctgatccagccataaccgctgctg
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gaattactgggcgtaaaagcgtgcgagggcggttccataagacagatgtgaaatccccgggctcaacctgggaac
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atgctggaggaacaccaatggcgaaggcaatccccggacctgactgacgctcatgctggggagcaaacaggat
tagataccctggtagtccacgcctaaacgatgtcaactggttggtgggagggTTTTctctcagtaacgaagct
aacgctgaagttgaccgctggggagtagcggccgcaaggttgaaactcaaaggaattgacggggaccgcaca
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agagatgtgggagtgctcgaaagagaacctgcacacaggtgctgcatggccgctcgtcagctcgtgct

six

gi|28372132|dbj|AB087560.1|

Sphaerotilus sp. L1 gene for 16S rRNA, partial sequence

attgaacgctggcggatgccttacacatgcaagtcgaacggtagaggggcaacccttgagagtggcgaacggg
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gagggtgaaagcgggggatcgcaagacctcgcgcgattggagcggccgatggcagattaggtagttggtgggg
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agactcctacgggaggcagcagtggggaatTTTggacaatgggcgaaagcctgatccagccataaccgctgctg
gaagaaggccttcgggttgtaaaccgctTTTgtcagggaaagaaatcttctgggctaatacctcgggaggatgac
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accgcacaagcggtagatgatgtggtTTAattcgatgcaacgcgaaaaaccttacctacccttgacatggcag
gaatccccgagagatgtgggagtgctcgaaagagaacctgcacacaggtgctgcatggccgctcgtcagctcgtg
tcgt

seven

gi|28372137|dbj|AB087565.1|

Sphaerotilus sp. L8 gene for 16S rRNA, partial sequence

attgaacgctggcggatgccttacacatgcaagtcgaccggtagaggggcaacccttgagagtgtcgaacggg
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gagggtgaaagcgggggatcgcaagacctcgcgcgattggagcgtccgatggcagattaggtagttggtgggg
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agactcctacgggaggcagcagtggggaatTTTggacaatgggcgaaagcctgatccagccataaccgctgctg
gaagaaggccttcgggttgtaaaccgctTTTgtcagggaaagaaatcttctgggctaatacctcgggaggatgac
ggtacctgaagaataagcaccggcctaactacgtgccagcagccgcggttaatacgtaggggtgcaagcgttaatcg
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acgttcccgggtcttgtacacaccgcccgtcacaccatgggagcgggttctgccagaagtagttagcctaaccg
caaggagggcgattaccacggcaggggttcgtgactgggggtgaagtcgtaaca

eight

gi|28372133|dbj|AB087561.1|

Sphaerotilus sp. L2 gene for 16S rRNA, partial sequence

attgaacgctggcgggtatgccttacacatgcaagtcgaacggtagaggggcaacccttgagagtggcgaacggg
tgagtaatacatcggaacgtgccagctcgtgggggataacgtagcgaagctacgctaataccgcatacgcact
gaggggtgaaagcgggggatcgcaagacctcgcgcgattggagcggccgatggcagattaggtagttgggtgggg
aaaggcctaccaagcctgcatctgtagctgggtctgagaggacgaccagccacactgggacttgagacacggcc
cagactcctacgggagggcagcagtggggaattttggacaatgggcgaaagcctgatccagccataccgcgtgcg
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cacgtcatacaatggccggtacagagggctgccaaaccgcgagggggagccaatcccagaaaaccgggtcgtagtc
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gcaaggagggcgattaccacggcaggggttcgtgactgggggtgaagtcgtaaca

nine

gi|28372134|dbj|AB087562.1|

Sphaerotilus sp. L3 gene for 16S rRNA, partial sequence

attgaacgctggcgggtatgccttacacatgcaagtcgaacggtagaggggcaacccttgagagtggcgaacggg
tgagtaatacatcggaacgtgccagctcgtgggggataacgtagcgaagctacgctaataccgcatacgcact
gaggggtgaaagcgggggatcgcaagacctcgcgcgattggagcggccgatggcagattaggtagttgggtgggg
aaaggcctaccaagcctgcatctgtagctgggtctgagaggacgaccagccacactgggactgagacacggccc
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gt

ten

gi|4165069|gb|AF072915.1|AF072915

Sphaerotilus sp. IF5 16S ribosomal RNA gene, partial sequence

agagtttgattatggctcagattgaacgctggcgggtatgccttacacatgcaagt cgaacggtagaggggcaac
ccttgagagtgaggcaacgggtgagtaatacatcggaacgtgccagtcgtgggggataacgtagcgaaagctac
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cccgggctcaacctgggaactgcatttgtgactgtggagctagagtagcggtagagggggatggaattccgctg
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agaagtagttagcctaaccgcaaggagggcgattaccacggcaggggttcgtgactgggggtgaagtcgtaacaag
gtagccgtatcggaaggtgcccgtggatcacctcctttctg

eleven

gi|4165068|gb|AF072914.1|AF072914

Sphaerotilus sp. IF4 16S ribosomal RNA gene, partial sequence

agagtttgattctggctcagattgaacgctggcgggtatgccttacacgtgcaagt cgaacggtagaggggcaac
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nctaataccgcatacagacctgaggggtgaaagcgggggatcgcaagacctcgcgcgattggagcggccgatggca
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cccttatgggtagggctacacacgtcatacaatggccgggtacagagggctgccaaaccgcgagggggagccaat
cccagaaaaccggctcgtagtcgggatcgtagctgcaactcgactgctggaagtcggaatcgctagtaatcgcg
gatcagaatgtcgcggtgaatacgttcccgggtcttgtacacaccgcccgtcacaccatgggagcgggttctgc
cagaagtagttagcctaaccgcaaggagggcgattaccacggcaggggttcgtgactgggggtgaagtcgtaaaa
ggtagccgtatcggaaggtgcccgtggatcacctcctttctg

twelve

gi|4165070|gb|AF072916.1|AF072916

Sphaerotilus sp. IF9 16S ribosomal RNA gene, partial sequence

gtgagtaatacatcggaacgtgccagtcgtgggggataacgtagcgaaagnnacnntaataaccgcatacagacc
tgaggggtgaaagcgggggatcgcaagacctcgcgcgattggagcggccgatggcagattaggtagttgggtgggg

taaaggcctaccaagcctgcgatctgtagctggtctgagaggacgaccagccacactgggactgagacacggcc
cagactcctacgggagggcagcagtggtgggaat tttggacaatgggcgcaancntgatccagccataccgctgcg
ggaagaaggccttcgggttgtaaaccgcttttgtcaggggaagaaatcttctgggctaatacctcgggaggatga
cggtagctgaagaataagcaccggctaactacgtgccagcagccgcggttaatacgtagggtgcaagcgttaatc
ggaattactgggctaaagcgtgcgagggcgggttctataagacagatgtgaaatccccgggctcaacctgggaa
ctgcatttgtgactgtggagctagagtagcgttagagggggatggaattccgctgttagcagtgaaatgctgtaga
tatgctggagggaacaccaatggcggaaggcaatccccctggacctgtagcgtcatgacgaaagcgtggggag
caaacaggattagataccctggtagtccacgcccctaaacgatgtcaactggttgttgggagggtttcttctcag
taacgaagctaacgctgaagttagaccgctggggagtagcggccgcaagggtgaaactcaaaggaattgacggg
gaccgcacaagcgggtggatgatgtggtttaattcgatgcaacgcaaaaaaccttacctacccttgacatggca
ggaatcccgcagagatgtgggagtgctcgaaagagaacctgcacacaggtgctgcatggccgtcgtcagctcgt
gtcgtgagatggttgggttaagtcccgcaacgagcgcgaaccttgtcatcagttgctacgaaagggcactctga
gagactgcccgtgacaaaccggaggaaggtggggatgacgtcaggtcctcatggcccttatgggtagggtaca
cacgtcatacaatggcgggtacagagggctgccaaccgagggggagccaatcccagaaaaacggctcgtagt
ccggatcgtagtctgcaactcactgctggaagtgcgaatcgctagtaatcgcggtcagaatgtcgcggtgaa
tatcgttcccgggtcttgtacacaccgcccgtcacaccatgggagcgggttctgccagaagtagttagcctaac
cgcaagga

thirteen

gi|4165071|gb|AF072917.1|AF072917

Sphaerotilus sp. IF14 16S ribosomal RNA gene, partial sequence

gtgagtaatacatcggaacgtgccagtcgtgggggataacgtagcgaagctnnnntaataaccgcatacagacc
tgaggggtgaaagcgggggatcgcaagacctcgcgcgattggagcggccgatggcagattaggtagttggtgggg
taaaggcctaccaagcctgcgatctgtagctggtctgagaggacgaccagccacactgggactgagacacggcc
cagactcctacgggagggcagcagtggtgggaat tttggacaatgggcgcaancctgatccagccataccgctgcg
ggaagaaggccttcgggttgtaaaccgcttttgtcaggggaagaaatcttctgggctaatacctcgggaggatga
cggtagctgaagaataagcaccggctaactacgtgccagcagccgcggttaatacgtagggtgcaagcgttaatt
cggattactgggctaaagcgtgcgagggcgggttccataagacagatgtgaaatccccgggctcaacctgggaa
tatgctggagggaacaccaatggcggaaggcaatccccctggacctgtagcgtcatgacgaaagcgtggggag
caaacaggattagataccctggtagtccacgcccctaaacgatgtcaactgggttgttgggagggtttcttctcag
taacgaagctaacgctgaagttagaccgctgggggagtagcggccgcaagggtgaaactcaaaggaattgacgg
ggaccgcacaagcgggtggatgatgtggtttaattcgatgcaacgcaaaaaaccttacctacccttgacatggc
aggaatcccgcagagatgtgggagtgctcgaaagagaacctgcacacaggtgctgcatggccgtcgtcagctcg
tgtcgtgagatgttgggttaagtcccgcaacgagcgcgaaccttgtcatcagttgctacgaaagggcactctga
tgagactgcccgtgacaaaccggaggaaggtggggatgacgtcaggtcctcatggcccttatgggtagggtac
acacgtcatacaatggcgggtacagagggctgccaaccgagggggagccaatcccagaaaaacggctcgtag
tccggatcgtagtctgcaactcactgctggaagtgcgaatcgctagtaatcgcggtcagaatgtcgcggtgaa
atacgttcccgggtcttgtacacaccgcccgtcacaccatgggagcgggttctgccagaagtagttagcctaac
cgcaaggaggggcgatta

fourteen

gi|19744162|dbj|AB072236.1|

Sphaerotilus natans gene for 16S rRNA, partial sequence

gggaccgcacaagcgtggatcgatgtggtttaattcgatgcaacgcaaaaaaccttacctacccttgacatgt
ctgaaatcctgcagagatgtgggagtgctcgaaagagaatcagaacacaggtgctgcatggccgtcgtcagctc
gtgtcgtgagatgttgggttaagtcccgcaacgagcgcgaaccttgtcattagttgctacgaaagggcactcta
atgagactgcccgtgacaaaccggaggaaggtggggatgacgtcaggtcctcatggcccttatgggtagggtac
acacgtcatacaatggcgggtacagagggctgccaaccgagggggagccaatcccagaaaaacggctcgtag
tccggatcgcagatgcaactcactgctggaagtgcgaatcgctagtaatcgcggtcagcttgcgcgggtgaa
atacgttcccgggtcttgtacacaccgcccgtcacaccatgggagcgggttctgccagaagtagttagcctaac
cgcaaggaggggcgattaccacggcagggtcgtgactgggggtgaaagtctacaaggtagcctatcggaagg

fifteen

gi|504539|gb|L33980.1|SHLRRDC

Sphaerotilus natans 16S ribosomal RNA (16S rRNA)

agagtttgatcatggctcagattgaacgctggcggatgccttacacatgcaagt cgaacggtagaggggcaac
ccttgagagtggcgaacgggtgagtaatacatcggaacgtgccagtcgtgggggataacgtagcgaaagctac
gctaataaccgcatacgcctgaggggtgaaagcgggggatcgcaagacctcgcgcgattggagcggccgatggca
gattaggtagttggtggggtaaaggcctaccaagcctgcatctgtagctggtctgagaggacgaccagccaca
ctgggactgagacacggcc

sixteen

gi|504538|gb|L33978.1|SHLRRDB

Sphaerotilus natans 16S ribosomal RNA (16S rRNA)

agagtttgatcatggctcagattgaacgctggcggatgccttacacatgcaagt cgaacggtagggggagcaa
tcccctgagagtggcgaacgggtgagtaatacatcggaacgtgccagtcgtgggggataacgtagcgaaagct
acgctaataaccgcatacgcctgaggggtgaaagcgggggaccgtaaggcctcgcgcgattggagcggccgatgg
cagattaggtagttggtggggtaaaggcccaccaagcctgcatctgtagctggtctgagaggacgaccagcca
cactgggactgagacacggcc

seventeen

gi|504537|gb|L33977.1|SHLRRDA

Sphaerotilus natans 16S ribosomal RNA (16S rRNA)

agagtttgatcatggctcagattgaacgctggcggatgccttacacatgcaagt cgaacggtagaggagcaat
cctcgagagtggcgaacgggtgagtaatacatcggaacgtgccagtcgtgggggataacgtagcgaaagctac
gctaataaccgcatacgcctgaggggtgaaagcgggggactcgcaagagacctcgcgcgattggagcggccgatgg
cagattaggtagttggtggggtaaaggcccaccaagcctgcatctgtagctggtctgagaggacgaccagcca
cactgggactgagacacggcc

eighteen

gi|504536|gb|L33976.1|SHLRRD

Sphaerotilus natans 16S ribosomal RNA (16S rRNA)

agagtttgatcatggctcagattgaacgctggcggatgccttacacatgcaagt cgaacggtagaggggcaac
ccttgagagtggcgaacgggtgagtaatacatcggaacgtgccagtcgtgggggataacgtagcgaaagctac
gctaataaccgcatacgcctgaggggtgaaagcgggggatcgcaagacctcgcgcgattggagcggccgatggca
gattaggtagttggtggggtaaaggcctaccaagcctgcatctgtagctggtctgagaggacgaccagccaca
ctgggactgagacacggcc

nineteen

gi|6667247|gb|L79964.1|L79964

Eikelbloom type 1701 ribosomal RNA gene, complete sequence

tttgatcctgcctcagattgaacgctgncgncatgccttacacatgcaagt cgaacggtagggggagcaatccc
ctgagagtggcgaacgggtgagtaatacatcggaacgtgccagtcgtgggggataacgtagcgaaagctacgc
taataaccgcatacgcctgaggggtgaaagcgggggacctgcaagggcctcgcgcgattggagcggccgatgtca
gattaggtagttggtggggtaaaggcctaccaancctgcatctgtagctggtctgagaggacgaccagccaca
ctgggactgagacacggcccagactcctacgggaggcagcagtggggaat tttggacaatgggcgcaagcctga
tccanccatgccgctgcggaagaaggccttcgggttgtaaaccgct tttgtcaggggaagaaatcctttgggc
taataccctggagggatgacggtaacctgaagaataagcaccggcctaactacgtgccagcagccgcggtaatcgc
tagggtgcaagcgttaactcggaattactgggcgtaaacgctgagcagggcgggttggtgtaagacagatgtgaaatc
cccggctcaacctgggaactgcattttgtgactgcacagctagagtagcggtagagggggatggaattccgctg
tagcagatgaaatggtatgatgagggaacaccgatggcgaaggcagtcacctggacctgtactgacgctca
tgcacgaaagcgtggggagcaaacaggattagataccctggtagtccacgccctaaacgatgtcaactgggtgt
tgggaggggtttctctcagtaacgaagctaacgcgtgaagttgaccgcatggggagtagcggccgcaaggttgaa
actcnnaggaattgacggggaccgcacaagcgggtggatgatgtggtttaattcgatgcaacgcgaaaaacctt
acctacccttgacatgtctgagatcctgacgtgttggtggngtgcctgaaagngaatacagaacacaggtgctgc
atggccgctcgtcagctcgtgctgagatggtgggttaagtcggcaacgagcggcaacccttctcattagttgc

tacgaaagggcactttaatgagactgcccgggtgacaaaccggaggaaggtggggatgacgtcaggtcctcatggc
ccttatgggtagggctacacacgtcatacaatggccgggtacagagggctgccaaccgcgagggggagccaatc
ccagaaaaccggtngtagtcgggatcgcagtttgcaantngactgctggaagtccgaatcgctagtaa

twenty

gi|1263136|emb|X97071.1|LM16SRR

L.mobilis 16S rRNA gene

cagagtttgatcctggctcagattgaacgctggcggcatgctttacacatgcaagtccaacggtagaggggcaa
cccctgagagtgccgaacgggtgagtaatgcatcggaaacgtgccagtagtgggggatagcccggcgaaagccg
gattaataccgcatgagacctgaggggtgaaagcgggggactcgcaagagcctcgcgctactggagcggccgatg
tcagattagctagttgggtggggtaaaggcctaccaaggcgacgatctgtagctggctgagaggacgaccagcc
acactgggactgagacacggccagactcctacgggagcagcagtggggaattttggacaatgggcgcaagcc
tgatccagccatgcccgcgtgcccgaagaaggccttcgggttgtaaacgcttttgtcaggggaagaaatcttctg
ggctaataaccctgggaggatgacggtagcctgaagaataagcaccggctaactacgtgcccagcagccggtaat
acgtaggggtgcaacgcttaactcggaaactactggcgtaaaagcgtgcccagggcgttatataagacagatgtgaa
atccccgggctcaacctgggaactgcattttgtgactgtatagctagagtagcggtagagggggatggaattccgc
gtgtagcagtgaaatgctgtagatatgcccggaggaacaccgatggcgaaggcagtcacctggacctgtactgacgc
tcatgacgaaagcgtggggagcaaacaggattagataccctggtagtccacgcccctaaacgatgtcaactgggt
tgttgggaggggtttcttctcagtaacgtagctaacgcgtgaagttgaccgctggggagtagcggccgcaaggtt
gaaactcaaaggaattgacggggaccgcacaaagcgggtggatgatgtgggttaattcgatgcaacgcgaaaaac
cttacctacccttgacatgctaggaatcctgcagagatgtgggagtgctcgaaagagaacctagacacaggtgc
tgcattggccgctcgtcagctcgtgctgtagatgttgggttaagtcgcccaacgagcgcgaacccttgtcattagt
tgctacgaaagggcactctaataagactgcccgggtgacaaaccggaggaaggtggggatgacgtcaggtcctcat
ggcccttatgggtagggctacacacgtcatacaatggccgggtacagagggctgccaaccgcgagggggagcca
atcccagaaaaccggctcgtagtcgggatcgcagctcgaactcgactgctggaagtccgaatcgctagtaatcg
cggatcagcttgcccgggtgaatacgttcccgggtcctgtacacaccgcccgtcacaccatgggagcgggttct
gccagaagtagttagcctaaccgcaaggagggcgattaccacggcaggggttcgtgactgggggtgaagtcgtaac
aaggtagccgtatcggaaaggtgcccgtggatcacctcctt

twenty-one

MWG sequence from 8F bacterial primer

tgagtgtagggtttaccacacatgcaagtcgaacggtagaggggcaacccttgagagtgccgaacgggtgagta
atacatcggaaacgtgccagctcgtgggggataacgtagcgaagctacgctaataccgcatacagacctgagggg
gaaagcgggggatcgcaagacctcgcgcgattggagcggccgatggcagattaggtagttgggtggggtaaaggc
ctaccaagcctgcatctgtagctggctgtagaggacgaccagccacactgggactgagacacggccagactc
ctacgggagggcagcagtggggaattttggacaatgggcgcaagcctgatccagccataaccgctgcccgaagaa
ggccttcgggttgtaaacgcttttgtcaggggaagaaatcttctgggctaataacctcgggaggatgacgggtacc
tgaagaataagcaccggctaaactacgtgccagcagccgcccgttaatacgtaggggtgcaacgcttaactcggaa
ctggggcgtaaagcgtgcccagggcgttccataagacagatgtgaaatccccgggctcaacctgggaactgcatt
tgtgactgtggagctagagtagcggtagagggggatggaattccgcgctgtagcagtgaaatgctgtagatatgccc
aggaacaccaatggcgaaggcaatcccctgggacctgtactgacgctcatgacgaaagcgtggggagcaaaaca
ggattagatacccctggtagtccacg

twenty-two

reverse complement-MWG sequence from 1492R bacterial primer

agatatgcccggaggaacaccaatggcgaaggcaatccccctggacctgtactgacgctcatgacgaaagcgtgg
ggagcaaacaggattagataccctggtagtccacgcccctaaacgatgtcaactgggtgttgggaggggtttcttc
tcagtaacgaagcctaaccgctgaagttgaccgctggggagtagcggccgcaaggttgaaactcaaaggaattga
cggggaccgcacaaagcgggtggatgatgtgggttaattcgatgcaacgcgaaaaaccttacctacccttgacat
ggcaggaatcccgcagagatgtgggagtgctcgaagagaaacctgcacacaggtgctgcatggccgctcgtcagc
tcgtgctcgtgagatgttgggttaagtcgcccaacgagcgcgaacccttgtcatcagttgctacgagagggcactc
tgatgagactgcccgggtgacaaaccggaggaaggtggggatgacgtcaggtcctcatggcccttatgggtagggc
tacacacgtcatacaatggccgggtacagagggctgccaaccgcgagggggagccaatcccagaaaaccggctcg
tagtccggatcgtagctcgaactcgactgctggaagtcggaaatcgctagtaatcgccggatcagaatgtcgcgg

tgaatacgttcccgggtcttgtacacaccgcccgtcacaccatgggagcgggttctgccagaagtagttagccta
atctcaccagtgacgactagagttatcctgcc

twenty-three

gi|26986476|emb|AJ534666.1|

Uncultured beta proteobacterium partial 16S rRNA gene, clone S15A-MN107

tgacgctggcggcatgccttacacatgcaagtcgaacggcagcgcgggagcaatcctggcggcgagtgccgaac
gggtgagtaatatatcggaacgtgcccagagtgggggataactagtcgaaagattggctaataaccgcatacga
tctatggatgaaagtgggggattcgcaaggacctcatgctcctggagcggccgatatctgattagctagttggt
ggggtaaaggcccaccaaggcttcgatcagtagctggtctgagaggacgaccagccacactgggactgagacac
ggcccagactcctacgggaggcagcagtggggaatgggacaatgggcgcaagcctgatccagcaatgccgcg
tgtgtgaagaaggccttcgggttgtaaagcactttgtcagggagaagaacgggttctggccaatacccggagcta
atgacggtaacctgaagaataagcaccggctaactacgtgccagcagcggtaatacgtaggggtgcaagcgtt
aatcgggaattactgggcgtaaaagcgtgcgagggcgggttatgtaagttagatgtgaaatccccggctcacctggg
aattgcatttgagactgcatggctagagtgatcagaggggggtagaattccacgtgtagcagtgaaatgcgta
aagatgtggaagaataaccgatggcgaaggcagcccctggatacactgacgctcatgcacgaaagcgtggggagc
aacaggattagatccctggtagtccacgccttaaacgatgtctactagtgctcgggtcttaattgacttggtaac
gcagctaacgcggtgaagtagaccgctgggagtagcggtcgcaagattaaaactcaaaggaattgacggggacc
gcacaagcgggtggatgatgtggattaattcgatgcaacgcgaaaaaccttacctacccttgacatggcaggaat
cccgcagagatgcgggagtgctcgaagagaacctgcacacaggtgctgcatggctgtcgtcagctcgtgtcgt
gagatgttgggttaagtcccgcacagcgcgaacccttgtcattagttgctacatttgggtgggcactctaattg
agactgccgggtgacaaaccggaggaaggtggggatgacgtcaagtcctcatggcccttatgggtagggcttcac
acgtcatacaatggtacatacagagggccgccaaccgcgagggggagctaataccagaaagtgtatcgtagtc
cggattggagctcgtcaactcgactccatgaagttggaatcgctagtaatcgcggatcagcatgtcgcgggtgaat
acgttcccgggtcttgtacacaccgcccgtcacaccatgggagcgggtttcaccagaagtaggtagcctaaccg
caaggagggcgcttaccacgggtgggattcgtgactgggg

8.2

ARTHROBACTER ALIGNMENT SEQUENCES

00

gi|35210320|dbj|AB089841.1|

Arthrobacter globiformis gene for 16S rRNA

tgaacgctggcggcgctgcttaacacatgcaagtcgaacgatgatccgggtgcttgaccggggattagtgggcgaacgggtgagtaaacacgtgagtaaacctgcccttgactctgggataagcctgggaaactgggtctaataaccggatatgactcctcatcgcatggtgggggggtggaaagcttttgggttttggatggactcgccggcctatcagcttgttggtgaggtaatggctcaccaaggcgacgacgggtagccggcctgagaggggtgaccggccacactgggactgagacacggcccagactcctacgggaggcagcagtggggaatatgacacaatgggcgaaagcctgatgcagcgacgccgcgtgaggggatgacggccttcgggttgtaaacctcttccagtagggaagaagcgaaagtgacgggtacctgcagaagaagcgccggctaactacgtgccagcagccgcggtaatacgtagggcgcaagcgttatccggaattattgggcgtaaagagctcgtaggcgggttctgcgctctgccgtgaaagtcggggctcaactccggatctgccgtgggtacgggcagactagagtgatgtaggggagactggaattcctgggtgtagcgggtgaaatgcccagatatcaggaggaacaccgatggcgaaggcaggtctctgggcattaactgacgctgaggagcgaaagcatggggagcgaacaggattagatccctggtagtccatgccgtaaacgttgggcactaggtgtgggggacattccacgttttccgcgccgtagctaacgattaagtgccccgcctggggagtagcggccgcaaggctaaaactcaaaggaattgacggggggcccgcacaaagcgggagcatgcccgttaattcgatgcaacgcgaagaaccttaccaggcttgacatggaccggaccgcgcgaagaatggtttctccttttggggccgggttcacaggtggtgcatggttctcgtcagctcgtgctcgtgagatggtgggttaagtcgccgaacgagcgaaccctcgttccatggtgccagcgcgtaatggcggggactcatgggagactgccccgggtcaactcggaggaaggtggggacgacgtcaaatcatcatgcccttatgtcttgggcttcacgcatgctacaatggccgggtacaaaggggttgcgatactgtgaggtggagctaatccccaaaagccgggtctcagttcggatgggggtctgcaactcgaccccatgaagtggagtcgctagtaatcgagatcagcaacgctgcccgtgaatacgttccccgggccttgtacacaccgcccgtcaagtcacgaaagtggtaacaccggaagccgggtggcctaacccttggggagggagccgtcgaaggtgggactggcgattgggactaagtcgtaacaaggtagccgtaccggaagg

aatgt = real-time priming sites

atacc = priming sites for creating standards

01

gi|27530892|dbj|AB098573.1|

Arthrobacter globiformis gene for 16S rRNA, partial sequence

agagtttgatcctggctcaggatgaacgctggcggcgctgcttaacacatgcaagtcgaacgatgatccgggtgcttgaccggggattagtgggcgaacgggtgagtaaacacgtgagtaaacctgcccttgactctgggataagcctgggaactgggtctaataaccggatatgactcctcatcgcatggtgggggggtggaaagcttttgggttttggatggactcgccggcctatcagcttgttgggtgaggtaatggctcaccaaggcgacgacgggtagccggcctgagaggggtgacggccacactgggactgagacacggcccagactcctacgggaggcagcagtggggaatatgacaaatggggcgaagcctgatgcagcagccgcgctgagggatgacggccttcgggttgtaaacctcttccagtagggaagaagcgaaagtacgggtacctgcagaagaagcggcggtaactacgtgcccagcagccgcggtaatacgtagggcgcaagcgttatccggaattattgggcgtaaaagagctcgtaggcgggttctgcgctctgccgtgaaagtcggggctcaactccggatctgcccgtgggtacgggcagactagagtgatgtaggggagactggaattcctgggtgtagcggtgaaatgcccagatatcaggaggaacaccgatggcgaaggcaggtctctgggcattaactgacgctgaggagcgaagcatggggagcgaacaggattagataccctggtagtccatgccgtaaacgttgggcactaggtgtgggggacattccacgttttccgcgccgtagctaacgcattaagtccccgcctggggagtagcggccgcaaggctaaaactcaaaggaattgacggggggcccgcacaaagcggcggagcatgcccgttaattcgatgcaacgcgaagaaccttaccaggcttgacatggaccggaccgcccagaaatgtgggtttctccttttggggccgggttcacaggtggtgcatggttctcgtcagctcgtgctcgtgagatgttgggttaagtcgccgaacgagcgaaccctcgttccatgttgccagcgcgtaatggcggggactcatgggagactgcccgggtcaactcggaggaaggtggggacgacgtcaaatcatcatgcccccttatgtcttgggcttcacgcatgctacaatggccgggtacaaaggggttgcgatactgtgaggtggagctaatccccaaaagccgggtctcagttcggattggggctctgcaactcgaccccatgaagtcggagtcgctagtaatcgagatcagcaacgctgcccgtgaatacgttccccgggccttgtacacaccgcccgtcaagtcacgaaagtggtaacaccggaagccgggtggcctaacccttctggtgggagggagccgtcgaaggtgggactggcgattgggactaagtcgtaacaagtagccgtaccggaaggtgcccgtggatcacctcc

02

gi|15384500|gb|AF321068.1|AF321068

Arthrobacter globiformis strain U17 16S ribosomal RNA gene, partial sequence

gcagcgcgcccgcgtgagggatgacggccttcggggttgtaaacytctttcagtagggaagaagcgaagtgacg
gtacctgcagaagaagcgcggcctaactacgtgccagcagccgcggtaatacgtagggcgcgagcgttatccgg
aattattgggcgtaaagagctcgtagggcgggttctgctgcgctctgccgtgaaagtcgggggcttaaccccgatct
gcgggtgggtacgggcagactagagtgacgtaggggagactggaattcctggtgtagcgggtgaaatgcccagata
tcaggaggaacaccgatggcgaaggcaggtctctgggctgtaactgacgctgaggagcgaagcatggggagcg
aacaggattagataccctggtagtcctatgccgtaaacggttgggcactaggtgtgggggacattccacggtttcc
gcgccgtagctaacgcattaagtgcgccgccc

03

gi|639803|emb|X80736.1|

A.globiformis 16S rDNA

cctggctcaggatgaacgctggcggcgtgcttaacacatgcaagtcgaacgatgatccgggtgcttgaccgggg
attagtggcgaacgggtgagtaaacacgtgagtaacctgcccttgactctgggataagcctgggaaactgggtct
aataccggatatgactcctcatcgcattgggtgggggggtggaaagcttttgggttttggatggactcgcggccta
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gggactgagacacggcccagactcctacgggaggcagcagtggggaatattgcacaatgggcgaaagcctgatg
cagcgcgcccgcgtgagggatgacggccttcggggttgtaaacctctttcagtagggaagaagcgaagtgacgg
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ggcccgcacaagcggcgggagcatgcccattaatctgactgcaacgcgaagaaccttaccaggcttgacatggac
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tcgtgagatgttgggttaagtcccgcacagcgcgaacctcgttccatgttgccagcgcgtaattggcgggggac
tcattgggagactgcccgggtcaactcggaggaagggtggggacgacgctcaaatcatcatgcccttatgtcttgg
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ctcagttcggattggggctcgaactcgaccccatgaagtcggagtcgctagtaatcgcagatcagcaacgctg
cgggtgaatacgttcccgggcttgtacacaccgcccgtcaagtcacgaaagtgggtaacaccggaagcgggtgg
cctaacccttctgtgggagggagccgtcgaagggtgggactggcgattgggactaagtgc

04

gi|45504978|gb|AY561601.1|

Arthrobacter sp. RG-39 16S ribosomal RNA gene, partial sequence

agatgaacgctggcggcgtgcttaacacatgcaagtcgaacgatgatccgggtgcttgaccgggggattagtggc
gaacgggtgagtaaacacgtgagtaacctgcccttgactctgggataagcctgggaaactgggtctaataccgga
tatgactcctcatcgcattgggtgggggggtggaaagcttttgggttttggatggactcgcggcctatcagcttgt
tggtgaggtaatggctcaccaaggcgcagcagcgggtagccggcctgagaggggtgaccggccacactgggactgag
acacggcccagactcctacgggaggcagcagtggggaatattgcacaatgggcgaaagcctgatgcagcgcgc
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taacgcattaagtgcgccgctggggagtagcggccgcaaggctaaaactcaaaggaattgacggggggcccgcac
aagcggcgggagcatgcccattaatctgatgcaacgcgaagaaccttaccaggcttgacatggaccggaccgccc
gcagagatg

05

gi|47280|emb|Z18534.1|

S.natans 16S ribosomal DNA

caatcctcgagagtggcgaacgggtgagtaatacatcggaacgtgccagtcgtgggggataacgtagcgaaac
tacgctaataaccgcatacagaccgaggggtgaaagcgggggactcgcaagagcctcgcgcgattggagcggccga
tggcagattaggtagttggtggggtaaaggcccaccaagcctgcgatctgtagctggtctgagaggcgaccagc
cacactgggactgagacacggcccagactctacgggagggcagcagtggggaattttggacaatgggcgaaagcc
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gtagcagtgaaatgctgtagatatgctgggaggaacaccgatggcgaaggcaatcccctggacctgactgacgctc
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gcttgccgcgggtgaatacgttcccgggtcttgtacacaccgcccgtcacaccatgggagcgggtctcgccagaa
gtagttagcctaaccgc

06

gi|1263136|emb|X97071.1|

L.mobilis 16S rRNA gene

cagagtttgatcctggctcagattgaacgctggcggcatgctttacacatgcaagtgcaacggtagagggggcaa
cccctgagagtgccgaacgggtgagtaatgcatcggaacgtgccagtagtgggggatagcccgccgaaagccg
gattaataaccgcatgagacctgaggggtgaaagcgggggactcgcaagagcctcgcgctactggagcggccgatg
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atcccagaaaaccggctcgtagtcgggatcgcagctcgtcaactcgactgcgtgaagtcggaatcgctagtaatcg
cggatcagcttgccgcgggtgaatacgttcccgggtcttgtacacaccgcccgtcacaccatgggagcgggttct
gccagaagtagttagcctaaccgcaaggagggcgattaccacggcagggttcgtgactgggggtgaagtcgtaac
aaggtagccgtatcgggaagggtgcccgtggatcacctcctt

07 (close relative of *A.globiformis*)

gi|13276764|emb|AJ409095.1|

Micrococcus luteus 16S rRNA gene, strain D7

catgcaagtcgaacgatgaagcccagnntgctgggtgattaatggcgaacgggtgagtaaacacgtgagtnacct
gcccttaactctgggataagcctgggaaactgggtctaataccggataggagcgtccaccgcatgggtgggtgtt

ggaaagatttatcggttttggatggactcgcggcctatcagcttgttgggtgaggtaatggctaccaagggcag
gacgggtagccggcctgagagggtgaccggccacactgggactgagacacggcccagactcctacgggagggcag
cagtggggaatattgcacaatgggcgaaagcctgatgcagcgcgcgcgtgagggatgacggccttcgggttg
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agccgcggtaatacgtaggggtgcgagcgttatccggaattattgggctaaagagctcgtagggcggtttgcgc
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cggagtcgctagtaatcgcagatcagcaacgctgcgggtgaatacgttcccgggccttgtacacaccgcccgtca
agtcacgaaagtggtaacaccggaagccgggtggcctaacccttgtggggggagccgtcgaaggtgggaccagc
gattgggactaagtngtacaagg

08 (another close relative)

gi|19069510|emb|AJ415376.1|

Brachy bacterium rhamnosum partial 16S rRNA gene, type strain LMG 19848T

gacgaacgctggcggcgtgcttaacacatgcaagtcgaacgatgacgaccgagcttgctcgggtctgattagtg
cgaacgggtgagtaaacacgtgagcaacctgcccttcactctgggataacctcgggaaatcggggctaataccgg
atatgagctcctgtcgcacatggcgggtgttggaaagtttttcgggtgaaggatgggctcgcggcctatcagttgt
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cgcgtgagggatgacggccttcgggttgtaaacctctttcagcaggggaagaagcgaagtgacggtacctgcag
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cgtaaagagcttgtaggtggcttgtcgcgtctgcccgtgaaaacccgaggctcaacctcgggcgtgcgggtgggta
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caccgatggcgaagggcaggtctctgggcccattactgacactgagaagcgaagcatggggagcgaacaggatta
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taacgcattaagtgccccgcctggggagtagcggccgcaaggctaaaactcaaaggaattgacggggggcccgcac
aagcggcggagcatgaggatataatcgatgcaacgcgaagaaccttaccaggcttgacatgacccggacgact
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ccgggggtcaactcggaggaaaggtggggacgacgtcaaatcatcatgcccttatgtcttgggcttcacgcatgc
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ggggctcgcgaactcgaccccatgaagtggagtcgctagtaatcgcagatcagcaacgctgcgggtgaatacgtt
cccgggccttgtacacaccgcccgtcaagtcacgaaagtcggtaacaccggaagccagtgggccatcctcgtga
gggagctgtcgaaggtgggatcgggtgattgggactaagtcgtaacaaggtagccgtaccggaagg

09

gi|40456396|gb|AY509239.1|

Arthrobacter rhombi strain S189 16S ribosomal RNA gene, partial sequence

cgtgcttaacacatgcaagtcgaacgatgatcccggtgcttgcaccgggtgattagtgggcgaacgggtgagtaa
cacgtgagtaacctgccccgactctgggataagcccgggaaaccgggtctaataccggatattcacttccttc
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ggcccaccaagggcagcagggtagccggcctgagaggggtgaccggccacactgggactgagacacggcccaga
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tcccccttggggccggttcacaggtggtgcatggttgcgctcagctcgtgctcgtgagatggtgggttaagtccc
gcaacgagcgcgaaccctcgttccatggtgccagcagctgatggtggggactcatgggagactgccggggtcaac
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gtacaatgggttgcgatactgtgaggtggagctaatccccaaaagccggctcagttcggattgggggtctgcaa
ctcgacccccatgaagtggagtcgctagtaatcgagatcagcaacgctgcggtgaatacgttccccggccttg
tacacaccgcccgtcaagtacgaaagtggtaaacaccgcaagccgggtggcaaccctttgtgggagggagccg
tcgaaggtgggaccgctgattgggactaagtcgtaaca

10

>AGF_4-8F 84..809 of trace file (MWG Sequence with 8F primer)

Gtaacctgcccttgactctgggataagcctgggaaactgggtctaataccggatatgactcctcatcgcatggt
gggggggtggaaagcttttgggttttggatggactcgccgctatcagcttggtggtgaggtaatggctacca
agggcagcagcgggtagccggcctgagaggggtgaccggccacactgggactgagacacggcccagactcctacgg
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cgggttgtaaacctctttcagtagggaagaagcgaagtgacgggtacctgcagaagaagcgcgggctaactacg
tgccagcagccgcggttaatacgtagggcgcaagcgttatccggaattattgggctaaagagctcgtagggcgt
ttgtcgcgctctgccgtgaaagtccggggctcaactccggatctgcggtgggtacgggcagactagagtgatgta
ggggagactggaattcctggtgtagcgggtgaaatgcgagatcaggaggaacaccgatggcgaaggcaggtc
tctgggcattaactgacgctgaagagcgaagcatggggagcgaacaggattagataccctggtagtccatgcc
gtaaacgttgggactaggtgtgggggacattccacgttttccgcccgtagctaacgc

11

>AGF4R-1492R 74..798 of trace file (MWG Sequence with 1492R primer)

gggcgggtgtgtacaaggccccgggaacgtattcaccgcagcgttgctgatctgagattactagcactccgactt
catgggggtcgagttgcagaccccccaatccgaactgagaccggcttttgggattagctccacctcacagtatcgc
aacctttgtaccggccattgtagcatgcgtgaagcccaagacataaggggcatgatgattgacgctcgtcccc
accttctccgagttgaccccgagctctccatgagctcccgcattacgcgctggcaacatggaacgaggggt
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cgaattaatccgcatgctccgcccgttggtgcccggcccccgctcaattcctttgagttttagccttgccggcgtac
tccccagggcggggcacttaatgcgttagctacggcgggaaaacgtgtaagtccccccacacctagtgcccaac
gtttacggcatggactaccaggggtatctaactcctgttcgctccccatgctttcgtcctcagcgtcagttaatg
cccagagacctgccttcgcatcggtgttctcctgatctgcccatttaccgcta

9 Appendix B – Process for creating RNA Standard for cPCR

1. PCR to make T7 PCR product (11/07/06)
2. In-Vitro transcription (11/13/06) of T7 PCR products
3. RT-PCR results (show gel 11/14/06) -> -RT product shows DNA contamination
4. 12/14/06-12/18/06: retry process (Dnase 11/13/07 product and did RT-PCR again, show gel 12/18/06) no products in any wells except + control
5. 12/20/06 – ran PCR of RT results again, diluted RT products to 20 ng/uL to use as template, products were positive but did not run + controls with AGF/SNA primers
6. 1/17/07 – repeated in vitro transcription (templates = 11/07/06 PCR product)
7. 02/09/07 – started with new SNAF3 primer (PCR with T7 primer, PCR clean up, in vitro with Epicentre, Dnase and purified)
8. 02/10/07 – Dnase and purified AGF in vitro product from 1/17/07, RT reaction with SNA & AGF
9. 2/12/07 – PCR of RT products
10. 2/13/07 – ran gel (-RT controls showed bright bands, + control did not show band at all)
11. 02/13/07 – repeated in-vitro
12. 02/14/07 – Dnased and purified in-vitro products
13. 02/17/07 – RT-PCR of in vitro products
14. 2/19/07 – Gel of RT-PCR results (-RT showed bright bands again)
15. 2/20/07 – tested Dnase (ambion and Epicentre) and RT kits (Ambion), both Dnases were working fine, RT kits were variable!
16. 03/06/07 – repeated invitro (Epicentre)
17. 03/08/07 –Dnased (Epicentre) & purified invitro products
18. 03/09/07 – Dnased again (Ambion), RT-PCR, comparing I-script vs. ambion RT kits
19. 03/13/07 – GEL of RT-PCR results
20. 03/21/07 – tested Iscript one step real-time RT-pcr (ran one sample of each RNA template & NTC, did not run -RT controls, real time results showed AGF was overloaded, but looks like SNA worked)
21. 03/27/07 – Riboprobe invitro transcription, Dnased invitro product with 2X with ambion kit
22. 03/29/07 – 1 step RT-PCR (show real time results)
23. 04/04/07 – RT-PCR to make RNA standard curves (results = very high detection limit!)
24. redesigned primers
25. 5/14/07 - PCR with new SNA standard primers, qiagen cleanup, riboprobe invitro, gel to check T7 PCR result
26. 5/28/07 - repeat above process for new AGF standard primers, PCR with T7, cleanup DNA with qiagen kit, riboprobe invitro, cleanup RNA with qiagen kit, RT-PCR (SNA and AGF together)
27. repeated experiments until we got the standard curves we wanted
28. present standard curve results

10 Appendix C – Blast results

SNA229F – cPCR

Sequences producing significant alignments:
 (bits) Score E-value Length

Accession	Description	Max score	Total score	Query coverage	E value	Max ident.
CP_50857.1	Uncultured bacterium F5601-106 ribosomal RNA gene, partial sequence	40.1	40.1	100%	2e-04	100%
F01199.1	Uncultured bacterium F0109-162 ribosomal RNA gene, partial sequence	38.1	38.1	100%	7e-03	100%
CP_49949.1	Uncultured bacterium K31-10-111 ribosomal RNA gene, partial sequence	40.1	40.1	100%	2e-04	100%
EF_49750.1	Uncultured bacterium F101-100 ribosomal RNA gene, partial sequence	48.1	48.1	100%	7e-04	100%
E010929.1	Uncultured bacterium F101-137 ribosomal RNA gene, partial sequence	38.1	38.1	100%	2e-03	100%
CP_49159.1	Uncultured bacterium J01-106 ribosomal RNA gene, partial sequence	40.1	40.1	100%	2e-04	100%
EF_10122.1	Uncultured bacterium F001-162 ribosomal RNA gene, partial sequence	38.1	38.1	100%	7e-03	100%
E0113224.1	Uncultured bacterium F101-100 ribosomal RNA gene, partial sequence	38.1	38.1	100%	2e-03	100%
CP_13296.1	Uncultured bacterium F01-162 ribosomal RNA gene, partial sequence	40.1	40.1	100%	7e-04	100%
DQ961047.1	Uncultured bacterium clone AY147_G03 16S ribosomal RNA gene, partial sequence	38.1	38.1	100%	7e-03	100%
CP_24950.1	Klebsiella qilinensis strain 187-115 ribosomal RNA gene, partial sequence	40.1	40.1	100%	2e-04	100%
EF_72068.1	Uncultured Clostridium sp. clone QADP-FA292_G10 16S ribosomal RNA gene, partial sequence	48.1	48.1	100%	7e-04	100%
E0142716.1	Uncultured bacterium F01-162 ribosomal RNA gene, partial sequence	38.1	38.1	100%	2e-03	100%
CP_25147.1	Uncultured Bacillus subtilis strain 100-194-1002-1 16S ribosomal RNA gene, partial sequence	40.1	40.1	100%	2e-04	100%
E0142717.1	Uncultured bacterium F01-162 ribosomal RNA gene, partial sequence	38.1	38.1	100%	7e-03	100%
E0522095.1	Uncultured bacterium clone E-01-162 ribosomal RNA gene, partial sequence	40.1	40.1	100%	2e-04	100%
E0112580.1	Uncultured Bacillus subtilis clone B-1166-1 16S ribosomal RNA gene, partial sequence	48.1	48.1	100%	7e-04	100%
DQ162805.1	Uncultured Bacillus subtilis clone B-1166-1 16S ribosomal RNA gene, partial sequence	38.1	38.1	100%	2e-03	100%
DQ951174.1	Klebsiella sp. 91-542-109 ribosomal RNA gene, partial sequence	40.1	40.1	100%	2e-04	100%
DQ877097.1	Uncultured bacterium clone P401_A01 16S ribosomal RNA gene, partial sequence	38.1	38.1	100%	7e-03	100%
DQ963084.1	Uncultured bacterium clone K31103 16S ribosomal RNA gene, partial sequence	40.1	40.1	100%	2e-04	100%
DQ963085.1	Uncultured bacterium clone Bab17 16S ribosomal RNA gene, partial sequence	40.1	40.1	100%	7e-04	100%
DQ963086.1	Uncultured bacterium clone FF-4 16S ribosomal RNA gene, partial sequence	38.1	38.1	100%	2e-03	100%
DQ963087.1	Uncultured bacterium clone B-1166-1 16S ribosomal RNA gene, partial sequence	40.1	40.1	100%	2e-04	100%
AY26817.1	Uncultured Aquaspirillum sp. partial 16S rRNA gene, clone GTU1	48.1	48.1	100%	7e-04	100%
AJ267418.1	Leishmania sp. 92 partial 16S rRNA gene, isolate 52	38.1	38.1	100%	2e-03	100%
AY159732.1	Uncultured bacterium partial 16S rRNA gene, clone Y9229	40.1	40.1	100%	2e-04	100%
DQ963088.1	Uncultured beta-proteobacterium clone P4010 16S ribosomal RNA gene, partial sequence	38.1	38.1	100%	7e-03	100%
DQ963089.1	Uncultured bacterium clone F0110-83 ribosomal RNA gene, partial sequence	40.1	40.1	100%	2e-04	100%
DQ963090.1	Bacterium CVC-J0210 16S ribosomal RNA gene, partial sequence	48.1	48.1	100%	7e-04	100%
DQ963125.1	Uncultured bacterium clone 04 16S ribosomal RNA gene, partial sequence	38.1	38.1	100%	2e-03	100%
AY251156.1	Uncultured bacterium clone P2q1-247 16S ribosomal RNA gene, partial sequence	40.1	40.1	100%	2e-04	100%
AY251157.1	Uncultured bacterium clone P2q1-226 16S ribosomal RNA gene, partial sequence	38.1	38.1	100%	7e-03	100%
AY251158.1	Uncultured bacterium clone 114-01 16S ribosomal RNA gene, partial sequence	40.1	40.1	100%	2e-04	100%
AY109770.1	Uncultured alpha-proteobacterium clone 5199-1573 16S ribosomal RNA gene, partial sequence	40.1	40.1	100%	7e-04	100%
AY18757.1	Uncultured alpha-proteobacterium clone T015 16S ribosomal RNA gene, partial sequence	38.1	38.1	100%	2e-03	100%
AY251159.1	Burkholderia thailandica strain 91-115 ribosomal RNA gene, partial sequence	40.1	40.1	100%	2e-04	100%
AY251160.1	Burkholderia thailandica strain 91-115 ribosomal RNA gene, partial sequence	48.1	48.1	100%	7e-04	100%
AY251161.1	Acetivibrio sp. F-108 16S ribosomal RNA gene, partial sequence	38.1	38.1	100%	2e-03	100%
AY251162.1	Uncultured bacterium clone SJB5 16S ribosomal RNA gene, partial sequence	40.1	40.1	100%	2e-04	100%
AY119283.1	Uncultured anaerobic bacterium clone B70-1 16S ribosomal RNA gene, partial sequence	38.1	38.1	100%	7e-03	100%
AY145072.1	Minimal medium isolate clone K11-111 ribosomal RNA gene, partial sequence	40.1	40.1	100%	2e-04	100%
AY178471.1	Grassland soil clone S12-624 16S ribosomal RNA gene, partial sequence	48.1	48.1	100%	7e-04	100%

SNA550R – cPCR and real-time PCR

Sequences producing significant alignments:
(10188 hits) (Accession number shown)

Accession	Description	Max score	Total score	Query coverage	E value	Max ident
AF021487.1	Jncultured Rhodospirillaceae bacterium clone 16C rRNA gene, clone A44	52.0	52.0	100%	4e-11	100%
F09500C.1	Jncultured bacterium clone 143-11 16S ribosomal RNA gene, partial sequence	52.0	52.0	100%	De-CE	100%
F09499C.1	Jncultured bacterium clone D76-12 16S ribosomal RNA gene, partial sequence	52.0	52.0	100%	De-LL	100%
AF021025.1	Jncultured beta proteobacterium SBK11-21 16S ribosomal RNA gene, partial sequence	52.0	52.0	100%	3e-11	100%
AF048544.1	Jncultured bacterium clone F19-6 16S ribosomal RNA gene, partial sequence	52.0	52.0	100%	De-CE	100%
AF047081.1	Jncultured bacterium clone m016hC2 16S ribosomal RNA gene, partial sequence	52.0	52.0	100%	4e-11	100%
AY03700C.1	Jncultured bacterium clone m016hC1 16S ribosomal RNA gene, partial sequence	52.0	52.0	100%	De-CE	100%
AF037025.1	Jncultured bacterium clone m016hC0 16S ribosomal RNA gene, partial sequence	52.0	52.0	100%	3e-11	100%
AY037011.1	Jncultured bacterium clone m016hA4 16S ribosomal RNA gene, partial sequence	52.0	52.0	100%	3e-11	100%
AY035974.1	Jncultured bacterium clone m015h11 16S ribosomal RNA gene, partial sequence	52.0	52.0	100%	De-CE	100%
AF037044.1	Jncultured bacterium clone m015h12 16S ribosomal RNA gene, partial sequence	52.0	52.0	100%	De-LL	100%
AY03797C.1	Jncultured bacterium clone m015h11 16S ribosomal RNA gene, partial sequence	52.0	52.0	100%	3e-11	100%
AF042481.1	Jncultured bacterium clone m012.1 16S ribosomal RNA gene, partial sequence	52.0	52.0	100%	3e-11	100%
AF042475.1	Jncultured bacterium clone m012.1 16S ribosomal RNA gene, partial sequence	52.0	52.0	100%	4e-11	100%
AF007550.1	Enhydrobia sp. L11 gene for 16S rRNA, partial sequence	52.0	52.0	100%	De-CE	100%
AF087797.1	Cohaerzilus sp. L10 gene for 16S rRNA, partial sequence	52.0	52.0	100%	De-LL	100%
AF087796.1	Cohaerzilus sp. L12 gene for 16S rRNA, partial sequence	52.0	52.0	100%	3e-11	100%
AF007555.1	Cohaerzilus sp. L8 gene for 16S rRNA, partial sequence	52.0	52.0	100%	De-CE	100%
AF087792.1	Cohaerzilus sp. L7 gene for 16S rRNA, partial sequence	52.0	52.0	100%	4e-11	100%
AF007553.1	Enhydrobia sp. L6 gene for 16S rRNA, partial sequence	52.0	52.0	100%	De-CE	100%
AF087798.1	Cohaerzilus sp. L3 gene for 16S rRNA, partial sequence	52.0	52.0	100%	De-LL	100%
AF087795.1	Cohaerzilus sp. L2 gene for 16S rRNA, partial sequence	52.0	52.0	100%	3e-11	100%
AF007550.1	Enhydrobia sp. L11 gene for 16S rRNA, partial sequence	52.0	52.0	100%	De-CE	100%
AF031112.1	Jncultured beta proteobacterium clone m015B 16S ribosomal RNA gene, partial sequence	52.0	52.0	100%	4e-11	100%
AY03909C.1	Jncultured Rhodospirillaceae bacterium clone 449A7 16S ribosomal RNA gene, partial sequence	52.0	52.0	100%	De-CE	100%
AF039085.1	Jncultured Rhodospirillaceae bacterium clone 449A4 16S ribosomal RNA gene, partial sequence	52.0	52.0	100%	De-LL	100%
AY039087.1	Jncultured Rhodospirillaceae bacterium clone 449A2 16S ribosomal RNA gene, partial sequence	52.0	52.0	100%	3e-11	100%
AF039424.2	Jncultured Bacillales bacterium clone 75157 16S ribosomal RNA gene, partial sequence	52.0	52.0	100%	De-CE	100%
AF039414.1	Cohaerzilus sp. L14 16S ribosomal RNA gene, partial sequence	52.0	52.0	100%	4e-11	100%
AF072915.1	Cohaerzilus sp. L13 16S ribosomal RNA gene, partial sequence	52.0	52.0	100%	De-CE	100%
AF072914.1	Cohaerzilus sp. L14 16S ribosomal RNA gene, partial sequence	52.0	52.0	100%	3e-11	100%
AY037014.1	Jncultured bacterium clone m016hA5 16S ribosomal RNA gene, partial sequence	50.1	50.1	76%	1e-11	100%
AY035902.1	Jncultured bacterium clone m015D7 16S ribosomal RNA gene, partial sequence	50.1	50.1	96%	De-C4	100%
F09498C.1	Jncultured bacterium clone D76-10 16S ribosomal RNA gene, partial sequence	48.1	48.1	92%	De-L4	100%
F09500C.1	Jncultured bacterium clone 143-11 16S ribosomal RNA gene, partial sequence	48.1	48.1	70%	5e-11	100%
AF034242.1	Jncultured beta proteobacterium C0152 16S ribosomal RNA gene, partial sequence	48.1	48.1	92%	De-C4	100%
AF021488.1	Jncultured Rhodospirillaceae bacterium clone 10S rRNA gene, clone 09	46.1	46.1	48%	1e-11	100%
AF042463.1	Jncultured bacterium clone m012.1 16S rRNA gene, clone A12.0r	46.1	46.1	70%	0.002	100%
AF042462.1	Jncultured beta proteobacterium clone 100F.L. 50 16S ribosomal RNA gene, partial sequence	46.1	46.1	88%	J.LJ2	100%
F09500C.1	Jncultured bacterium clone 143-11 16S ribosomal RNA gene, partial sequence	46.1	46.1	88%	1.77E	100%
F09500C.1	Jncultured bacterium clone 138-95 16S ribosomal RNA gene, partial sequence	46.1	46.1	70%	0.002	100%
AF039425.1	Jncultured beta proteobacterium clone C001 16S ribosomal RNA gene, partial sequence	46.1	46.1	48%	1e-11	100%

SNA417F – real-time primer

Accession	Description	Max score	Total score	Query coverage	E value	Max ident
EF149790.1	Uncultured bacterium F1Clone131 16S ribosomal RNA gene, partial sequence	48.1	48.1	100%	3e-04	100%
EF149789.1	Uncultured bacterium F1Clone130 16S ribosomal RNA gene, partial sequence	48.1	48.1	100%	3e-04	100%
EF149698.1	Uncultured bacterium F1Clone38 16S ribosomal RNA gene, partial sequence	48.1	48.1	100%	3e-04	100%
EF140095.1	Uncultured bacterium clone RNL1Clone41 16S ribosomal RNA gene, partial sequence	48.1	48.1	100%	3e-04	100%
AY758254.1	Uncultured bacterium clone 20K-9 16S ribosomal RNA gene, partial sequence	48.1	48.1	100%	3e-04	100%
AY758250.1	Uncultured bacterium clone 20K-1 16S ribosomal RNA gene, partial sequence	48.1	48.1	100%	3e-04	100%
AF175607.1	Uncultured eubacterium WJGRT-62 16S ribosomal RNA gene, partial sequence	48.1	48.1	100%	3e-04	100%
AF247775.1	Uncultured bacterium DF03 16S ribosomal RNA gene, partial sequence	48.1	48.1	100%	3e-04	100%
AY537080.1	Uncultured bacterium clone mdt16h02 16S ribosomal RNA gene, partial sequence	48.1	48.1	100%	3e-04	100%
AY537033.1	Uncultured bacterium clone mdt16c07 16S ribosomal RNA gene, partial sequence	48.1	48.1	100%	3e-04	100%
AY537029.1	Uncultured bacterium clone mdt16c03 16S ribosomal RNA gene, partial sequence	48.1	48.1	100%	3e-04	100%
AY537014.1	Uncultured bacterium clone mdt16a08 16S ribosomal RNA gene, partial sequence	48.1	48.1	100%	3e-04	100%
AY537011.1	Uncultured bacterium clone mdt16a04 16S ribosomal RNA gene, partial sequence	48.1	48.1	100%	3e-04	100%
AY536982.1	Uncultured bacterium clone mdt15f07 16S ribosomal RNA gene, partial sequence	48.1	48.1	100%	3e-04	100%
AY536974.1	Uncultured bacterium clone mdt15e11 16S ribosomal RNA gene, partial sequence	48.1	48.1	100%	3e-04	100%
AY536940.1	Uncultured bacterium clone mdt15b12 16S ribosomal RNA gene, partial sequence	48.1	48.1	100%	3e-04	100%
AY537370.1	Uncultured bacterium clone mek62d11 16S ribosomal RNA gene, partial sequence	48.1	48.1	100%	3e-04	100%
AY491590.1	Uncultured bacterium clone oc49 16S ribosomal RNA gene, partial sequence	48.1	48.1	100%	3e-04	100%
AY491569.1	Uncultured bacterium clone oc25 16S ribosomal RNA gene, partial sequence	48.1	48.1	100%	3e-04	100%
AY501613.1	Uncultured prokaryote isolate DGGGE band TDW-5(B) 16S ribosomal RNA gene, partial sequ	48.1	48.1	100%	3e-04	100%
AY212645.1	Uncultured bacterium clone 192up 16S ribosomal RNA gene, partial sequence	48.1	48.1	100%	3e-04	100%
DQ106968.1	Uncultured bacterium clone RABC-B67 16S ribosomal RNA gene, partial sequence	48.1	48.1	100%	3e-04	100%
AY337603.1	Beta proteobacterium HS5/S24542 16S ribosomal RNA gene, partial sequence	48.1	48.1	100%	3e-04	100%
AF523022.1	Uncultured Aquabacterium sp. clone C-7 16S ribosomal RNA gene, partial sequence	48.1	48.1	100%	3e-04	100%
AB205965.1	Uncultured bacterium gene for 16S rRNA, partial sequence, clone:OS-34	48.1	48.1	100%	3e-04	100%
AB205869.1	Uncultured bacterium gene for 16S rRNA, partial sequence, clone:12C-M31	48.1	48.1	100%	3e-04	100%
AB158681.1	Uncultured bacterium gene for 16S rRNA, partial sequence, isolate:37-05	48.1	48.1	100%	3e-04	100%
D84641.1	Beta proteobacterium S24542 gene for 16S ribosomal RNA, partial sequence	48.1	48.1	100%	3e-04	100%
AB211233.1	Ideonella sp. 0-0013 gene for 16S rRNA, partial sequence	48.1	48.1	100%	3e-04	100%
AY945923.1	Uncultured bacterium clone DR-13 16S ribosomal RNA gene, partial sequence	48.1	48.1	100%	3e-04	100%
AF525829.1	Uncultured soil bacterium clone G12-1236-5 small subunit ribosomal RNA gene, partial seq	48.1	48.1	100%	3e-04	100%
AJ318109.1	Uncultured beta proteobacterium 16S rRNA gene, clone B1ci13b	48.1	48.1	100%	3e-04	100%
AJ318108.1	Uncultured beta proteobacterium 16S rRNA gene, clone B1ci13	48.1	48.1	100%	3e-04	100%
AB087576.1	Leptothrix sp. L18 gene for 16S rRNA, partial sequence	48.1	48.1	100%	3e-04	100%
AB087575.1	Leptothrix sp. L17 gene for 16S rRNA, partial sequence	48.1	48.1	100%	3e-04	100%
AB087574.1	Leptothrix sp. L16 gene for 16S rRNA, partial sequence	48.1	48.1	100%	3e-04	100%
AB087572.1	Leptothrix sp. L11 gene for 16S rRNA, partial sequence	48.1	48.1	100%	3e-04	100%
AB087571.1	Leptothrix sp. L10 gene for 16S rRNA, partial sequence	48.1	48.1	100%	3e-04	100%
AB087570.1	Leptothrix sp. L5 gene for 16S rRNA, partial sequence	48.1	48.1	100%	3e-04	100%
AB087569.1	Leptothrix sp. L4 gene for 16S rRNA, partial sequence	48.1	48.1	100%	3e-04	100%
AB087568.1	Sphaerotilus sp. L19 gene for 16S rRNA, partial sequence	48.1	48.1	100%	3e-04	100%
AB087567.1	Sphaerotilus sp. L13 gene for 16S rRNA, partial sequence	48.1	48.1	100%	3e-04	100%

SNA416F (cont'd)

AB087569.1	Leptothrix sp. L4 gene for 16S rRNA, partial sequence	48.1	48.1	100%	3e-04	100%
AB087568.1	Sphaerotilus sp. L19 gene for 16S rRNA, partial sequence	48.1	48.1	100%	3e-04	100%
AB087567.1	Sphaerotilus sp. L13 gene for 16S rRNA, partial sequence	48.1	48.1	100%	3e-04	100%
AB087566.1	Sphaerotilus sp. L12 gene for 16S rRNA, partial sequence	48.1	48.1	100%	3e-04	100%
AB087565.1	Sphaerotilus sp. L8 gene for 16S rRNA, partial sequence	48.1	48.1	100%	3e-04	100%
AB087564.1	Sphaerotilus sp. L7 gene for 16S rRNA, partial sequence	48.1	48.1	100%	3e-04	100%
AB087563.1	Sphaerotilus sp. L6 gene for 16S rRNA, partial sequence	48.1	48.1	100%	3e-04	100%
AB087561.1	Sphaerotilus sp. L2 gene for 16S rRNA, partial sequence	48.1	48.1	100%	3e-04	100%
AB087560.1	Sphaerotilus sp. L1 gene for 16S rRNA, partial sequence	48.1	48.1	100%	3e-04	100%
DQ234243.2	Uncultured Burkholderiales bacterium clone DS161 16S ribosomal RNA gene gene, partial s	48.1	48.1	100%	3e-04	100%
AF072917.1	Sphaerotilus sp. IF14 16S ribosomal RNA gene, partial sequence	48.1	48.1	100%	3e-04	100%
AF072916.1	Sphaerotilus sp. IF9 16S ribosomal RNA gene, partial sequence	48.1	48.1	100%	3e-04	100%
AF072915.1	Sphaerotilus sp. IF5 16S ribosomal RNA gene, partial sequence	48.1	48.1	100%	3e-04	100%
AF072914.1	Sphaerotilus sp. IF4 16S ribosomal RNA gene, partial sequence	48.1	48.1	100%	3e-04	100%
AY540763.1	Uncultured bacterium clone PCF24-(HA4) 16S ribosomal RNA gene, partial sequence	48.1	48.1	100%	3e-04	100%
X72724.1	I.dechloratans gene for 16S ribosomal RNA	48.1	48.1	100%	3e-04	100%
D16214.1	R.gelatinosus gene for 16S ribosomal RNA	48.1	48.1	100%	3e-04	100%
EF150223.1	Uncultured bacterium F5Clone57 16S ribosomal RNA gene, partial sequence	46.1	46.1	95%	0.001	100%
EF140584.1	Uncultured bacterium clone BNL2Clone88 16S ribosomal RNA gene, partial sequence	46.1	46.1	95%	0.001	100%
EF140570.1	Uncultured bacterium clone BNL2Clone74 16S ribosomal RNA gene, partial sequence	46.1	46.1	95%	0.001	100%
EF140562.1	Uncultured bacterium clone BNL2Clone65 16S ribosomal RNA gene, partial sequence	46.1	46.1	95%	0.001	100%
EF140516.1	Uncultured bacterium clone BNL2Clone17 16S ribosomal RNA gene, partial sequence	46.1	46.1	95%	0.001	100%
EF140500.1	Uncultured bacterium clone BNL2Clone1 16S ribosomal RNA gene, partial sequence	46.1	46.1	95%	0.001	100%
AF175625.1	Uncultured eubacterium WJGRT-88 16S ribosomal RNA gene, partial sequence	46.1	46.1	95%	0.001	100%
AB087573.1	Leptothrix sp. L14 gene for 16S rRNA, partial sequence	42.1	42.1	87%	0.020	100%
AB291350.1	Uncultured bacterium gene for 16S rRNA, partial sequence, clone: N1B149	40.1	40.1	100%	0.081	95%
DQ342819.1	Uncultured bacterium clone ADPS1_08H 16S ribosomal RNA gene, partial sequence	40.1	40.1	100%	0.081	95%
DQ342812.1	Uncultured bacterium clone ADPS1_01F 16S ribosomal RNA gene, partial sequence	40.1	40.1	100%	0.081	95%
DQ342656.1	Uncultured bacterium clone PS1_01H 16S ribosomal RNA gene, partial sequence	40.1	40.1	100%	0.081	95%
DQ342554.1	Uncultured bacterium clone PSAD2_08B 16S ribosomal RNA gene, partial sequence	40.1	40.1	100%	0.081	95%

AGF1066R – cPCR and real time primer

Sequences producing significant alignments:

(Click headers to sort columns)

Accession	Description	Max score	Total score	Query coverage	E value	Max ident
AB284261.1	Actinosynnema violaceoruber gene for 16S rRNA, partial sequence	48.1	48.1	100%	3e-04	100%
EF195089.1	Arthrobacter sp. DiSca3 16S ribosomal RNA gene, partial sequence	48.1	48.1	100%	3e-04	100%
DQ442518.1	Streptomyces libani subsp. libani strain NRRL B-3446T 16S ribosomal	48.1	48.1	100%	3e-04	100%
EF110914.1	Arthrobacter sp. CN-1 16S ribosomal RNA gene, partial sequence	48.1	48.1	100%	3e-04	100%
AB279890.1	Arthrobacter sp. KV-653 gene for 16S rRNA, partial sequence	48.1	48.1	100%	3e-04	100%
AB279889.1	Arthrobacter sp. KV-651 gene for 16S rRNA, partial sequence	48.1	48.1	100%	3e-04	100%
DQ985281.1	Arthrobacter sp. EV4 16S ribosomal RNA gene, partial sequence	48.1	48.1	100%	3e-04	100%
DQ158005.1	Arthrobacter sp. J3.62 16S ribosomal RNA gene, partial sequence	48.1	48.1	100%	3e-04	100%
DQ158001.1	Arthrobacter sp. J3.46 16S ribosomal RNA gene, partial sequence	48.1	48.1	100%	3e-04	100%
DQ157998.1	Arthrobacter sp. J3.40 16S ribosomal RNA gene, partial sequence	48.1	48.1	100%	3e-04	100%
DQ157995.1	Arthrobacter sp. J3.33 16S ribosomal RNA gene, partial sequence	48.1	48.1	100%	3e-04	100%
DQ157992.1	Arthrobacter sp. FB24 16S ribosomal RNA gene, partial sequence	48.1	48.1	100%	3e-04	100%
DQ157991.1	Arthrobacter sp. 31.32 16S ribosomal RNA gene, partial sequence	48.1	48.1	100%	3e-04	100%
DQ157989.1	Arthrobacter sp. 25.32 16S ribosomal RNA gene, partial sequence	48.1	48.1	100%	3e-04	100%
DQ157988.1	Arthrobacter sp. 16.43 16S ribosomal RNA gene, partial sequence	48.1	48.1	100%	3e-04	100%
DQ157986.1	Arthrobacter sp. 16.18 16S ribosomal RNA gene, partial sequence	48.1	48.1	100%	3e-04	100%
DQ157985.1	Arthrobacter sp. 16.6 16S ribosomal RNA gene, partial sequence	48.1	48.1	100%	3e-04	100%
CP000454.1	Arthrobacter sp. FB24, complete genome	48.1	240	100%	3e-04	100%
AM403316.1	Uncultured actinobacterium partial 16S rRNA gene, clone JG35-K2-	48.1	48.1	100%	3e-04	100%
DQ345443.1	Phycosicoccus jejuensis strain KSW2-15 16S ribosomal RNA gene,	48.1	48.1	100%	3e-04	100%
DQ881476.1	Uncultured bacterium clone Ms-B19 16S ribosomal RNA gene, partial	48.1	48.1	100%	3e-04	100%
AB271054.1	Pseudonocardia bacterium Gsoil 857 gene for 16S rRNA, partial	48.1	48.1	100%	3e-04	100%
AM292606.1	Arthrobacter sp. JG37-Iso3 partial 16S rRNA gene, isolate JG37-Iso3	48.1	48.1	100%	3e-04	100%
DQ649438.1	Actinomyces sp. YACS-36 16S ribosomal RNA gene, partial sequence	48.1	48.1	100%	3e-04	100%
DQ490461.1	Micrococcaceae bacterium KVD-unk-27 16S ribosomal RNA gene,	48.1	48.1	100%	3e-04	100%
DQ490460.1	Micrococcaceae bacterium KVD-unk-31 16S ribosomal RNA gene,	48.1	48.1	100%	3e-04	100%
DQ490459.1	Micrococcaceae bacterium KVD-unk-30 16S ribosomal RNA gene,	48.1	48.1	100%	3e-04	100%
AM236151.1	Arthrobacter sp. IN13 partial 16S rRNA gene, strain IN13	48.1	48.1	100%	3e-04	100%
DQ102726.1	Arthrobacter sp. SR2-6a 16S ribosomal RNA gene, partial sequence	48.1	48.1	100%	3e-04	100%
DQ181633.1	Kutzneria sp. 744 16S ribosomal RNA gene, partial sequence	48.1	48.1	100%	3e-04	100%

AGF1066R (cont'd)

AB248288.1	Saccharothrix espanaensis gene for 16S rRNA, partial sequence,	48.1	48.1	100%	3e-04	100%
DQ124817.1	Uncultured Arthrobacter sp. clone cloET17 16S ribosomal RNA gene,	48.1	48.1	100%	3e-04	100%
DQ125934.1	Uncultured bacterium clone AKAU4222 16S ribosomal RNA gene,	48.1	48.1	100%	3e-04	100%
DQ125933.1	Uncultured bacterium clone AKAU4221 16S ribosomal RNA gene,	48.1	48.1	100%	3e-04	100%
DQ125932.1	Uncultured bacterium clone AKAU4220 16S ribosomal RNA gene,	48.1	48.1	100%	3e-04	100%
DQ125926.1	Uncultured bacterium clone AKAU4206 16S ribosomal RNA gene,	48.1	48.1	100%	3e-04	100%
DQ125924.1	Uncultured bacterium clone AKAU4199 16S ribosomal RNA gene,	48.1	48.1	100%	3e-04	100%
DQ125923.1	Uncultured bacterium clone AKAU4198 16S ribosomal RNA gene,	48.1	48.1	100%	3e-04	100%
DQ125921.1	Uncultured bacterium clone AKAU4195 16S ribosomal RNA gene,	48.1	48.1	100%	3e-04	100%
DQ125915.1	Uncultured bacterium clone AKAU4182 16S ribosomal RNA gene,	48.1	48.1	100%	3e-04	100%
DQ125899.1	Uncultured bacterium clone AKAU4160 16S ribosomal RNA gene,	48.1	48.1	100%	3e-04	100%
DQ125896.1	Uncultured bacterium clone AKAU4157 16S ribosomal RNA gene,	48.1	48.1	100%	3e-04	100%
DQ125890.1	Uncultured bacterium clone AKAU4143 16S ribosomal RNA gene,	48.1	48.1	100%	3e-04	100%
DQ125887.1	Uncultured bacterium clone AKAU4140 16S ribosomal RNA gene,	48.1	48.1	100%	3e-04	100%
DQ125885.1	Uncultured bacterium clone AKAU4138 16S ribosomal RNA gene,	48.1	48.1	100%	3e-04	100%
DQ125883.1	Uncultured bacterium clone AKAU4134 16S ribosomal RNA gene,	48.1	48.1	100%	3e-04	100%
DQ125881.1	Uncultured bacterium clone AKAU4131 16S ribosomal RNA gene,	48.1	48.1	100%	3e-04	100%
DQ125871.1	Uncultured bacterium clone AKAU4120 16S ribosomal RNA gene,	48.1	48.1	100%	3e-04	100%
DQ125870.1	Uncultured bacterium clone AKAU4119 16S ribosomal RNA gene,	48.1	48.1	100%	3e-04	100%
DQ125849.1	Uncultured bacterium clone AKAU4080 16S ribosomal RNA gene,	48.1	48.1	100%	3e-04	100%
DQ125848.1	Uncultured bacterium clone AKAU4077 16S ribosomal RNA gene,	48.1	48.1	100%	3e-04	100%
DQ125847.1	Uncultured bacterium clone AKAU4076 16S ribosomal RNA gene,	48.1	48.1	100%	3e-04	100%
DQ125844.1	Uncultured bacterium clone AKAU4073 16S ribosomal RNA gene,	48.1	48.1	100%	3e-04	100%
DQ125841.1	Uncultured bacterium clone AKAU4067 16S ribosomal RNA gene,	48.1	48.1	100%	3e-04	100%
DQ125833.1	Uncultured bacterium clone AKAU4059 16S ribosomal RNA gene,	48.1	48.1	100%	3e-04	100%
DQ125832.1	Uncultured bacterium clone AKAU4058 16S ribosomal RNA gene,	48.1	48.1	100%	3e-04	100%
DQ125831.1	Uncultured bacterium clone AKAU4057 16S ribosomal RNA gene,	48.1	48.1	100%	3e-04	100%
DQ125827.1	Uncultured bacterium clone AKAU4050 16S ribosomal RNA gene,	48.1	48.1	100%	3e-04	100%
DQ125822.1	Uncultured bacterium clone AKAU4039 16S ribosomal RNA gene,	48.1	48.1	100%	3e-04	100%
DQ125820.1	Uncultured bacterium clone AKAU4035 16S ribosomal RNA gene,	48.1	48.1	100%	3e-04	100%
DQ125819.1	Uncultured bacterium clone AKAU4034 16S ribosomal RNA gene,	48.1	48.1	100%	3e-04	100%

AGF1066R (cont'd)

DQ125818.1	Uncultured bacterium clone AKAU4033 16S ribosomal RNA gene,	48.1	48.1	100%	3e-04	100%
DQ125815.1	Uncultured bacterium clone AKAU3961 16S ribosomal RNA gene,	48.1	48.1	100%	3e-04	100%
DQ125799.1	Uncultured bacterium clone AKAU3940 16S ribosomal RNA gene,	48.1	48.1	100%	3e-04	100%
DQ125797.1	Uncultured bacterium clone AKAU3938 16S ribosomal RNA gene,	48.1	48.1	100%	3e-04	100%
DQ125796.1	Uncultured bacterium clone AKAU3937 16S ribosomal RNA gene,	48.1	48.1	100%	3e-04	100%
DQ125795.1	Uncultured bacterium clone AKAU3934 16S ribosomal RNA gene,	48.1	48.1	100%	3e-04	100%
DQ125794.1	Uncultured bacterium clone AKAU3933 16S ribosomal RNA gene,	48.1	48.1	100%	3e-04	100%
DQ125793.1	Uncultured bacterium clone AKAU3932 16S ribosomal RNA gene,	48.1	48.1	100%	3e-04	100%
DQ125792.1	Uncultured bacterium clone AKAU3930 16S ribosomal RNA gene,	48.1	48.1	100%	3e-04	100%
DQ125785.1	Uncultured bacterium clone AKAU3920 16S ribosomal RNA gene,	48.1	48.1	100%	3e-04	100%
DQ125776.1	Uncultured bacterium clone AKAU3908 16S ribosomal RNA gene,	48.1	48.1	100%	3e-04	100%
DQ125773.1	Uncultured bacterium clone AKAU3904 16S ribosomal RNA gene,	48.1	48.1	100%	3e-04	100%
DQ125772.1	Uncultured bacterium clone AKAU3903 16S ribosomal RNA gene,	48.1	48.1	100%	3e-04	100%
DQ125770.1	Uncultured bacterium clone AKAU3901 16S ribosomal RNA gene,	48.1	48.1	100%	3e-04	100%
DQ125769.1	Uncultured bacterium clone AKAU3900 16S ribosomal RNA gene,	48.1	48.1	100%	3e-04	100%
DQ125762.1	Uncultured bacterium clone AKAU3876 16S ribosomal RNA gene,	48.1	48.1	100%	3e-04	100%
DQ125751.1	Uncultured bacterium clone AKAU3864 16S ribosomal RNA gene,	48.1	48.1	100%	3e-04	100%
DQ125747.1	Uncultured bacterium clone AKAU3859 16S ribosomal RNA gene,	48.1	48.1	100%	3e-04	100%
DQ125741.1	Uncultured bacterium clone AKAU3852 16S ribosomal RNA gene,	48.1	48.1	100%	3e-04	100%
DQ125739.1	Uncultured bacterium clone AKAU3849 16S ribosomal RNA gene,	48.1	48.1	100%	3e-04	100%
DQ125730.1	Uncultured bacterium clone AKAU3832 16S ribosomal RNA gene,	48.1	48.1	100%	3e-04	100%
DQ125718.1	Uncultured bacterium clone AKAU3814 16S ribosomal RNA gene,	48.1	48.1	100%	3e-04	100%
DQ125714.1	Uncultured bacterium clone AKAU3810 16S ribosomal RNA gene,	48.1	48.1	100%	3e-04	100%
DQ125700.1	Uncultured bacterium clone AKAU3792 16S ribosomal RNA gene,	48.1	48.1	100%	3e-04	100%
DQ125692.1	Uncultured bacterium clone AKAU3779 16S ribosomal RNA gene,	48.1	48.1	100%	3e-04	100%
DQ125687.1	Uncultured bacterium clone AKAU3771 16S ribosomal RNA gene,	48.1	48.1	100%	3e-04	100%
DQ125678.1	Uncultured bacterium clone AKAU3752 16S ribosomal RNA gene,	48.1	48.1	100%	3e-04	100%
DQ125676.1	Uncultured bacterium clone AKAU3748 16S ribosomal RNA gene,	48.1	48.1	100%	3e-04	100%
DQ125674.1	Uncultured bacterium clone AKAU3746 16S ribosomal RNA gene,	48.1	48.1	100%	3e-04	100%
DQ125671.1	Uncultured bacterium clone AKAU3741 16S ribosomal RNA gene,	48.1	48.1	100%	3e-04	100%
DQ125670.1	Uncultured bacterium clone AKAU3739 16S ribosomal RNA gene,	48.1	48.1	100%	3e-04	100%
DQ125666.1	Uncultured bacterium clone AKAU3735 16S ribosomal RNA gene,	48.1	48.1	100%	3e-04	100%
DQ125664.1	Uncultured bacterium clone AKAU3730 16S ribosomal RNA gene,	48.1	48.1	100%	3e-04	100%
DQ125660.1	Uncultured bacterium clone AKAU3725 16S ribosomal RNA gene,	48.1	48.1	100%	3e-04	100%
DQ125659.1	Uncultured bacterium clone AKAU3724 16S ribosomal RNA gene,	48.1	48.1	100%	3e-04	100%
DQ125658.1	Uncultured bacterium clone AKAU3723 16S ribosomal RNA gene,	48.1	48.1	100%	3e-04	100%
DQ125648.1	Uncultured bacterium clone AKAU3697 16S ribosomal RNA gene,	48.1	48.1	100%	3e-04	100%
DQ125642.1	Uncultured bacterium clone AKAU3686 16S ribosomal RNA gene,	48.1	48.1	100%	3e-04	100%
DQ291145.1	Lentzea kentuckyensis strain NRRL B-24416 16S ribosomal RNA gene,	44.1	44.1	100%	0.005	95%

AGF965F – real-time primer

Sequences producing significant alignments:

(Click headers to sort columns)



Accession	Description	Max score	Total score	Query coverage	E value	Max ident
DQ985470.1	Arthrobacter sp. CMU6 16S ribosomal RNA gene, partial sequence	48.1	48.1	100%	3e-04	100%
DQ985279.1	Arthrobacter sp. EV2 16S ribosomal RNA gene, partial sequence	48.1	48.1	100%	3e-04	100%
AY853403.1	Arthrobacter sp. HF-2 16S ribosomal RNA gene, partial sequence	48.1	48.1	100%	3e-04	100%
AY177350.3	Arthrobacter sp. I3 16S ribosomal RNA gene, partial sequence	48.1	48.1	100%	3e-04	100%
AB089841.1	Arthrobacter globiformis gene for 16S rRNA	48.1	48.1	100%	3e-04	100%
AB098573.1	Arthrobacter globiformis gene for 16S rRNA, partial sequence	48.1	48.1	100%	3e-04	100%
X80736.1	A.globiformis 16S rDNA	48.1	48.1	100%	3e-04	100%
M23411.1	A.globiformis small subunit ribosomal RNA	48.1	48.1	100%	3e-04	100%
DQ298297.1	Uncultured bacterium clone SR44 16S ribosomal RNA gene, partial	46.1	46.1	95%	0.001	100%
AF423303.1	Uncultured soil bacterium clone 987-1 16S ribosomal RNA gene,	46.1	46.1	95%	0.001	100%
AF423301.1	Uncultured soil bacterium clone 960-2 16S ribosomal RNA gene,	46.1	46.1	95%	0.001	100%
AF423234.1	Uncultured soil bacterium clone 167-2 16S ribosomal RNA gene,	46.1	46.1	95%	0.001	100%
AJ785568.1	Arthrobacter sp. S/4 partial 16S rRNA gene	46.1	46.1	95%	0.001	100%
AF197053.1	Arthrobacter sp. 'SMCC ZAT200' 16S ribosomal RNA gene, partial	46.1	46.1	95%	0.001	100%
AF197040.1	Arthrobacter sp. 'SMCC G991' 16S ribosomal RNA gene, partial	46.1	46.1	95%	0.001	100%
AF197039.1	Arthrobacter sp. 'SMCC G986' 16S ribosomal RNA gene, partial	46.1	46.1	95%	0.001	100%
AF197023.1	Arthrobacter sp. 'SMCC G954' 16S ribosomal RNA gene, partial	46.1	46.1	95%	0.001	100%
AY391045.1	Uncultured bacterium clone 70 16S ribosomal RNA gene, partial	40.1	40.1	100%	0.082	95%
AE014134.5	Drosophila melanogaster chromosome 2L, complete sequence	36.2	36.2	75%	1.3	100%
CT737257.2	Pan troglodytes chromosome X clone CH251-43F04 map Xq28,	36.2	36.2	75%	1.3	100%
XM_539912.2	PREDICTED: Canis familiaris similar to GTPase, IMAP family member	36.2	36.2	75%	1.3	100%
NM_164817.1	Drosophila melanogaster CG31898-RA (CG31898), mRNA	36.2	36.2	75%	1.3	100%
AC016670.7	Homo sapiens BAC clone RP11-11K6 from 2, complete sequence	36.2	36.2	75%	1.3	100%
AY061364.1	Drosophila melanogaster LD28680 full length cDNA	36.2	36.2	75%	1.3	100%
AL022164.1	Human DNA sequence from clone RP4-581F7 on chromosome	36.2	36.2	75%	1.3	100%
AC092234.1	Drosophila melanogaster, chromosome 2L, region 29C-29D, BAC	36.2	36.2	75%	1.3	100%
AC092220.1	Drosophila melanogaster, chromosome 2L, region 28B-29X, BAC	36.2	36.2	75%	1.3	100%
AC008898.6	Homo sapiens chromosome 5 clone CTD-2236M5, complete sequence	36.2	36.2	75%	1.3	100%
AP006841.1	Bacteroides fragilis YCH46 DNA, complete genome	36.2	36.2	75%	1.3	100%
CT574563.2	Pan troglodytes chromosome X clone CH251-7J11 map Xq28,	36.2	36.2	75%	1.3	100%
AC004423.1	Drosophila melanogaster DNA sequence (P1 DS02110 (D147)),	36.2	36.2	75%	1.3	100%
AM471474.1	Vitis vinifera, whole genome shotgun sequence, contig	34.2	34.2	70%	5.1	100%

RDP Search Results

SNA229F

domain Bacteria (175/409692) (hits/total searched) [[list results for this node](#)]

- phylum Aquificae (0/1173)
- phylum Thermotogae (0/150)
- phylum Thermodesulfobacteria (0/118)
- phylum Deinococcus-Thermus (0/875)
- phylum Chrysiogenetes (0/4)
- phylum Chloroflexi (0/3542)
- phylum Thermomicrobia (0/23)
- phylum Nitrospira (0/1349)
- phylum Deferribacteres (0/307)
- phylum Cyanobacteria (0/12569)
- phylum Chlorobi (0/515)
- phylum **[Proteobacteria](#)** (175/153950)
- phylum Firmicutes (0/104178)
- phylum Actinobacteria (0/34747)
- phylum Planctomycetes (0/3832)
- phylum Chlamydiae (0/456)
- phylum Spirochaetes (0/3438)
- phylum Fibrobacteres (0/181)
- phylum Acidobacteria (0/13402)
- phylum Bacteroidetes (0/45622)
- phylum Fusobacteria (0/1203)
- phylum Verrucomicrobia (0/4270)
- phylum Dictyoglomi (0/14)
- phylum Gemmatimonadetes (0/949)
- phylum Lentisphaerae (0/113)
- phylum BRC1 (0/50)
- phylum OP10 (0/216)
- phylum OP11 (0/100)
- phylum TM7 (0/585)
- phylum WS3 (0/131)
- phylum Dehalococcoides (0/180)
- phylum SR1 (0/29)
- phylum OD1 (0/146)
- unclassified_Bacteria (0/21275)

SNA550R

domain Bacteria (33/409692) (hits/total searched) [[list results for this node](#)]

- phylum Aquificae (0/1173)
- phylum Thermotogae (0/150)
- phylum Thermodesulfobacteria (0/118)
- phylum Deinococcus-Thermus (0/875)
- phylum Chrysiogenetes (0/4)
- phylum Chloroflexi (0/3542)
- phylum Thermomicrobia (0/23)
- phylum Nitrospira (0/1349)
- phylum Deferribacteres (0/307)
- phylum Cyanobacteria (0/12569)
- phylum Chlorobi (0/515)
- phylum **Proteobacteria** (33/153950)
- phylum Firmicutes (0/104178)
- phylum Actinobacteria (0/34747)
- phylum Planctomycetes (0/3832)
- phylum Chlamydiae (0/456)
- phylum Spirochaetes (0/3438)
- phylum Fibrobacteres (0/181)
- phylum Acidobacteria (0/13402)
- phylum Bacteroidetes (0/45622)
- phylum Fusobacteria (0/1203)
- phylum Verrucomicrobia (0/4270)
- phylum Dictyoglomi (0/14)
- phylum Gemmatimonadetes (0/949)
- phylum Lentisphaerae (0/113)
- phylum BRC1 (0/50)
- phylum OP10 (0/216)
- phylum OP11 (0/100)
- phylum TM7 (0/585)
- phylum WS3 (0/131)
- phylum Dehalococcoides (0/180)
- phylum SR1 (0/29)
- phylum OD1 (0/146)
- unclassified_Bacteria (0/21275)

SNA958F

domain Bacteria (22/409692) (hits/total searched) [[list results for this node](#)]

- phylum Aquificae (0/1173)
- phylum Thermotogae (0/150)
- phylum Thermodesulfobacteria (0/118)
- phylum Deinococcus-Thermus (0/875)
- phylum Chrysiogenetes (0/4)
- phylum Chloroflexi (0/3542)
- phylum Thermomicrobia (0/23)
- phylum Nitrospira (0/1349)
- phylum Deferribacteres (0/307)
- phylum Cyanobacteria (0/12569)
- phylum Chlorobi (0/515)
- phylum **Proteobacteria** (22/153950)
- phylum Firmicutes (0/104178)
- phylum Actinobacteria (0/34747)
- phylum Planctomycetes (0/3832)
- phylum Chlamydiae (0/456)
- phylum Spirochaetes (0/3438)
- phylum Fibrobacteres (0/181)
- phylum Acidobacteria (0/13402)
- phylum Bacteroidetes (0/45622)
- phylum Fusobacteria (0/1203)
- phylum Verrucomicrobia (0/4270)
- phylum Dictyoglomi (0/14)
- phylum Gemmatimonadetes (0/949)
- phylum Lentisphaerae (0/113)
- phylum BRC1 (0/50)
- phylum OP10 (0/216)
- phylum OP11 (0/100)
- phylum TM7 (0/585)
- phylum WS3 (0/131)
- phylum Dehalococcoides (0/180)
- phylum SR1 (0/29)
- phylum OD1 (0/146)
- unclassified_Bacteria (0/21275)

SNA1068R

domain Bacteria (110/409692) (hits/total searched) [[list results for this node](#)]

- phylum Aquificae (0/1173)
- phylum Thermotogae (0/150)
- phylum Thermodesulfobacteria (0/118)
- phylum Deinococcus-Thermus (0/875)
- phylum Chrysiogenetes (0/4)
- phylum Chloroflexi (0/3542)
- phylum Thermomicrobia (0/23)
- phylum Nitrospira (0/1349)
- phylum Deferribacteres (0/307)
- phylum Cyanobacteria (0/12569)
- phylum Chlorobi (0/515)
- phylum **Proteobacteria** (108/153950)
- phylum **Firmicutes** (2/104178)
- phylum Actinobacteria (0/34747)
- phylum Planctomycetes (0/3832)
- phylum Chlamydiae (0/456)
- phylum Spirochaetes (0/3438)
- phylum Fibrobacteres (0/181)
- phylum Acidobacteria (0/13402)
- phylum Bacteroidetes (0/45622)
- phylum Fusobacteria (0/1203)
- phylum Verrucomicrobia (0/4270)
- phylum Dictyoglomi (0/14)
- phylum Gemmatimonadetes (0/949)
- phylum Lentisphaerae (0/113)
- phylum BRC1 (0/50)
- phylum OP10 (0/216)
- phylum OP11 (0/100)
- phylum TM7 (0/585)
- phylum WS3 (0/131)
- phylum Dehalococcoides (0/180)
- phylum SR1 (0/29)
- phylum OD1 (0/146)
- unclassified_Bacteria (0/21275)

AGF946F

domain Bacteria (32/409692) (hits/total searched) [[list results for this node](#)]

- phylum Aquificae (0/1173)
- phylum Thermotogae (0/150)
- phylum Thermodesulfobacteria (0/118)
- phylum Deinococcus-Thermus (0/875)
- phylum Chrysiogenetes (0/4)
- phylum Chloroflexi (0/3542)
- phylum Thermomicrobia (0/23)
- phylum Nitrospira (0/1349)
- phylum Deferribacteres (0/307)
- phylum Cyanobacteria (0/12569)
- phylum Chlorobi (0/515)
- phylum Proteobacteria (0/153950)
- phylum Firmicutes (0/104178)
- phylum [Actinobacteria](#) (32/34747)
- phylum Planctomycetes (0/3832)
- phylum Chlamydiae (0/456)
- phylum Spirochaetes (0/3438)
- phylum Fibrobacteres (0/181)
- phylum Acidobacteria (0/13402)
- phylum Bacteroidetes (0/45622)
- phylum Fusobacteria (0/1203)
- phylum Verrucomicrobia (0/4270)
- phylum Dictyoglomi (0/14)
- phylum Gemmatimonadetes (0/949)
- phylum Lentisphaerae (0/113)
- phylum BRC1 (0/50)
- phylum OP10 (0/216)
- phylum OP11 (0/100)
- phylum TM7 (0/585)
- phylum WS3 (0/131)
- phylum Dehalococcoides (0/180)
- phylum SR1 (0/29)
- phylum OD1 (0/146)
- unclassified_Bacteria (0/21275)

AGF 965F

domain Bacteria (8/409692) (hits/total searched) [[list results for this node](#)]

- phylum Aquificae (0/1173)
- phylum Thermotogae (0/150)
- phylum Thermodesulfobacteria (0/118)
- phylum Deinococcus-Thermus (0/875)
- phylum Chrysiogenetes (0/4)
- phylum Chloroflexi (0/3542)
- phylum Thermomicrobia (0/23)
- phylum Nitrospira (0/1349)
- phylum Deferribacteres (0/307)
- phylum Cyanobacteria (0/12569)
- phylum Chlorobi (0/515)
- phylum Proteobacteria (0/153950)
- phylum Firmicutes (0/104178)
- phylum [Actinobacteria](#) (8/34747)
- phylum Planctomycetes (0/3832)
- phylum Chlamydiae (0/456)
- phylum Spirochaetes (0/3438)
- phylum Fibrobacteres (0/181)
- phylum Acidobacteria (0/13402)
- phylum Bacteroidetes (0/45622)
- phylum Fusobacteria (0/1203)
- phylum Verrucomicrobia (0/4270)
- phylum Dictyoglomi (0/14)
- phylum Gemmatimonadetes (0/949)
- phylum Lentisphaerae (0/113)
- phylum BRC1 (0/50)
- phylum OP10 (0/216)
- phylum OP11 (0/100)
- phylum TM7 (0/585)
- phylum WS3 (0/131)
- phylum Dehalococcoides (0/180)
- phylum SR1 (0/29)
- phylum OD1 (0/146)
- unclassified_Bacteria (0/21275)

11 Appendix D – Protocols

11.1 PowerSoil™ DNA Isolation Kit Protocol from MoBio

1. To the PowerBead Tubes provided, add 0.25 gm of soil sample.
2. Gently vortex to mix.
3. **Check Solution C1.** If Solution C1 is precipitated, heat solution to 60°C until dissolved before use.
4. Add 60µl of Solution C1 and invert several times or vortex briefly.
5. Secure PowerBead Tubes horizontally using the MO BIO Vortex Adapter tube holder for the vortex (MO BIO Catalog No. 13000-V1) or secure tubes horizontally on a flat-bed vortex pad with tape. Vortex at maximum speed for 10 minutes.
6. Make sure the PowerBead Tubes rotate freely in your centrifuge without rubbing. Centrifuge tubes at 10,000 x g for 30 seconds at room temperature. **CAUTION:** Be sure not to exceed 10,000 x g or tubes may break.
7. Transfer the supernatant to a clean 2 ml Collection Tube (provided).
Note: Expect between 400 to 500µl of supernatant. Supernatant may still contain some soil particles.
8. Add 250µl of Solution C2 and vortex for 5 seconds. Incubate at 4°C for 5 minutes.
9. Centrifuge the tubes at room temperature for 1 minute at 10,000 x g.
10. Avoiding the pellet, transfer up to, but no more than, 600µl of supernatant to a clean 2 ml Collection Tube (provided). ****Transfer ALL of supernatant**
11. Add 200µl of Solution C3 and vortex briefly. Incubate at 4°C for 5 minutes. ****Add more Solution C3 in proportion to amount of supernatant transferred in Step 10.**
12. Centrifuge the tubes at room temperature for 1 minute at 10,000 x g.
13. Avoiding the pellet, transfer up to, but no more than, 750µl of supernatant into a clean 2 ml Collection Tube (provided). ****Transfer ALL of supernatant to 15 mL sterile centrifuge tube.**
14. Add 1200µl of Solution C4 to the supernatant and vortex for 5 seconds. ****Add more Solution C4 in proportion to amount of supernatant transferred in Step 13.**
15. Load approximately 675µl onto a Spin Filter and centrifuge at 10,000 x g for 1 minute at room temperature. Discard the flow through and add an additional 675µl of supernatant to the Spin Filter and centrifuge at 10,000 x g for 1 minute at room temperature. Load the remaining supernatant onto the Spin Filter and centrifuge at 10,000 x g for 1 minute at room temperature.
Note: A total of three loads for each sample processed are required. ****More loads will be required depending on amount of liquid transferred in step 13. Repeat until all liquid has been loaded and spun onto filter.**
16. Add 500µl of Solution C5 and centrifuge at room temperature for 30 seconds at 10,000 x g.
17. Discard the flow through.
18. Centrifuge again at room temperature for 1 minute at 10,000 x g.
19. Carefully place Spin Filter in a clean 2 ml Collection Tube (provided). Avoid splashing any Solution C5 onto the Spin Filter.
20. Add 100µl of Solution C6 to the center of the white filter membrane. Alternatively, sterile DNA-Free PCR Grade Water may be used for elution from the silica Spin Filter membrane at this step (MO BIO Catalog No. 17000-10).
21. Centrifuge at room temperature for 30 seconds at 10,000 x g.

22. Discard the Spin Filter. The DNA in the tube is now ready for any downstream application. No further steps are required. We recommend storing DNA frozen (-20° to -80°C). Solution C6 contains no EDTA. To concentrate the DNA see the Additional Information Section.

11.2

PowerSoil™ RNA Isolation Kit Protocol from MoBio

1. Add up to 2 g of soil to the 15 ml Bead Tube (provided). **Note:** Please refer to Additional Information Section for information regarding the amount of soil to process.
2. Add 2.5 ml of Bead Solution to the Bead Tube and vortex to mix.
3. Add 0.25 ml of Solution SR1 to the Bead Tube and vortex to mix.
4. Add 0.8 ml of Solution SR2 and place the Bead Tube on the Vortex Adapter (MO BIO Laboratories Catalog # 13000-V1-15 for Vortex Genie 2 or 13000-LV2-15 for Labnet Vortex) and vortex at maximum speed for 5 minutes.
5. Remove the Bead Tube from the Vortex Adapter and add 3.5 ml of phenol: chloroform:isoamyl alcohol (pH 6.5 – 8.0, [User supplied]) and vortex to mix until the biphasic layer disappears.
6. Place the Bead Tube on the Vortex Adapter and vortex at maximum speed for 10 minutes.
7. Remove the Bead Tube from the Vortex Adapter and centrifuge at 2500 x g for 10 minutes at room temperature.
8. Remove the Bead Tube from the centrifuge and carefully transfer the upper aqueous phase (avoiding the interphase and lower phenol layer) to a clean 15 ml Collection Tube (provided). The thickness of the interphase will vary depending on the type of soil used. Discard the phenol:chloroform:isoamyl alcohol in an approved waste receptacle. **NOTE:** The biphasic layer will be thick and firm in soils high in organic matter and may need to be pierced to remove the bottom phenol layer.
9. Add 1.5 ml of Solution SR3 to the aqueous phase and vortex to mix. Incubate at 4°C for 10 minutes.
10. Centrifuge at 2500 x g for 10 minutes at room temperature.
11. Transfer the supernatant, without disturbing the pellet, to a new 15 ml Collection Tube (provided).
12. Add 5 ml of Solution SR4 to the Collection Tube containing the supernatant, invert or vortex to mix, and incubate at -20°C for 30 minutes.
13. Centrifuge at 2500 x g for 30 minutes at room temperature.
14. Decant the supernatant and invert the 15 ml Collection Tube on a paper towel for 5 minutes. **NOTE:** Depending on soil type, the pellet may be large and/or dark in color.
15. Add 1 ml of Solution SR5 to the 15 ml Collection Tube and resuspend the pellet completely. (**NOTE:** Depending on the soil type, the pellet may be difficult to resuspend. Resuspension may be aided by placing the tubes in a heat block or water bath at 45°C for 10 minutes, followed by vortexing. Repeat until the pellet is resuspended.)
16. Prepare one RNA Capture Column (provided) for each RNA Isolation Sample:
 - a. Remove the cap of a 15 ml Collection Tube (provided) and place the RNA Capture Column inside the 15 ml Collection Tube. The column will hang in the 15ml tube.
 - b. Add 2 ml of Solution SR5 to the RNA Capture Column and allow it to gravity flow through the column and collect in the 15 ml Collection Tube. Allow Solution SR5 to completely flow through the column (**OPTIONAL:** The Collection Tube may be emptied after Solution SR5 has completely flowed through the column. **NOTE: DO NOT ALLOW THE COLUMN TO DRY OUT PRIOR TO LOADING THE RNA ISOLATION SAMPLE.**)
17. Add the RNA Isolation Sample from Step 15 onto the RNA Capture Column and allow it to gravity flow through the column. Collect the flow through in the 15 ml Collection Tube.
18. Wash the column with 1 ml of Solution SR5. Allow it to gravity flow and collect the flow through in the 15 ml Collection Tube.

19. Transfer the RNA Capture Column to a new 15 ml Collection Tube (provided) and add 1 ml of Solution SR6 to the RNA Capture Column to elute the bound RNA into the 15 ml Collection Tube. Allow Solution SR6 to gravity flow into the 15 ml Collection Tube.
20. Transfer the eluted RNA to a 2.2 ml Collection Tube (provided) and add 1 ml of Solution SR4. Invert at least once to mix and incubate at -20°C for 10 minutes.
21. Centrifuge the 2.2 ml Collection Tube at 13,000 x g for 15 minutes at room temperature to pellet the RNA.
22. Decant the supernatant and invert the 2.2 ml Collection Tube onto a paper towel for 10 minutes to air dry the pellet.
23. Resuspend the RNA pellet in 100 µl of Solution SR7. (NOTE: Although DNA carryover does not occur with the majority of soil types, certain soils high in organic matter may present unique carryover situations. In situations where the absence of DNA contamination is critical, the purified RNA should be tested for potential DNA carryover by performing PCR with qualified primers on the isolated RNA without performing prior reverse transcription amplification. The absence of a detectable amplification fragment by agarose electrophoresis indicates the absence of detectable carryover DNA. In the event DNA is detected, DNase treatment of the isolated RNA is recommended; see Additional Information Section for instruction).

12 Appendix E – RNA and DNA Extraction Optimization Experiments

12.1 Comparing MoBio and Qiagen DNA and RNA extraction kits

12.1.1 Introduction

The quality of the nucleic acid extraction process is vital to quantitation. The ideal extraction process is consistent across replicates and produces both high yield and high quality DNA or RNA. Therefore, some work was dedicated to optimizing the nucleic acid extraction process. Two types of kits were compared: the RNA and DNA Powersoil kits from MoBio, Inc. and the RNeasy and DNeasy kits from Qiagen.

The Powersoil kits are designed for extraction of nucleic acid from soils and are designed to remove high levels of humic acids. Both the RNA and DNA procedure use mechanical bead beating to lyse cells. The RNA protocol uses phenol:chloroform:isoamyl alcohol extraction, while the DNA protocol uses a spin filter procedure. The Qiagen DNeasy and RNeasy kits allow for more flexibility. The user can decide which protocol to use for cell lysis. Enzymatic lysis with lysozyme and Proteinase K digestion was chosen for both DNA and RNA extraction as recommended by Qiagen Technical Support for more efficient cell lysis.

12.1.2 Materials and Methods

DNA and RNA extractions of samples from the Neuse River WWTP in Raleigh and the North and South Cary Water Reclamation Facilities (WRF) were extracted for RNA and DNA using MoBio and Qiagen kits. Modifications to the protocols are listed below. These

modifications were used for all extractions. Any additional modifications for experiments are described within the discussion of the particular experiment.

12.1.2.1 Powersoil DNA

- Step 10: Transfer all supernatant instead of only 600 μ L
- Step 11: Scale up amount of Solution C3 proportionately to volume transferred in step 10
- Step 13: Transfer all supernatant to a sterile 15 mL collection tube
- Step 14: Scale up Solution C4 in proportion to volume transferred in step 13

12.1.2.2 Qiagen RNA

- Use Protocol 4 of RNA protect Bacteria Reagent Handbook from Qiagen: Enzymatic Lysis and Proteinase K Digestion of Bacteria
- DO NOT perform steps 2-6, these are for use of RNAprotect which can decrease RNA yields
- Step 7: Use 20 μ L of Proteinase K
- Step 8: Incubate for 20 minutes instead of 10 minutes
- Step 9: Use 700 μ L of Buffer RLT + β -mercaptoethanol
- Step 10: Use 500 μ L of 96-100% ethanol
- Step 11: Proceed to Protocol 7 – Purification of Total RNA from Bacterial Lysate Using the RNeasy Mini Kit
- Step 1: Perform this step 2 times to use all of cell lysis material
- Elute with 50 μ L of RNase-free water

12.1.2.3 Qiagen DNA

- Use the Pretreatment for Gram-Positive Bacteria
- Step 3: Incubate for 40 minutes instead of 30 minutes
- Elute with 200 μ L of Buffer AE

12.1.3 Results and Discussion

12.1.3.1 Experiment 1 - RNA extraction using the MoBio Powersoil kit

Samples collected from the North Cary Water Reclamation Facility were treated with three different holding methods during transportation from the plant to the laboratory. The three methods were: immediate freezing with ethanol and dry ice (E), keeping cold on ice (I), and holding at room temperature (N).

Triplicate samples from each treatment method were extracted for RNA using MoBio Powersoil kit two times. For the first extraction, one modification to the protocol was made. Instead of incubating the sample at -20°C for 30 minutes at step 12, the sample was incubated overnight per the suggestion of MoBio Technical Support to increase RNA yields. For the second extraction, three modifications were made: step 9 – samples were incubated for 15 minutes instead of 10 minutes, step 12 – samples were incubated for 40 minutes instead of 30, step 20 – samples were incubated for 15 minutes instead of 10 minutes.

After extraction, the RNA was quantified using a spectrophotometer. The results are shown in Table E.1.

Table E.1 – Comparing 2 RNA extractions using MoBio’s Powersoil kit of replicate WWTP samples

Sample ID	Extraction 1			Extraction 2		
	ng/mL	260/280	260/230	ng/mL	260/280	260/230
E1	2.46	1.61	0.97	133.41	2.18	2.37
E2	4.63	1.83	1.02	71.35	2.22	2.32
E3	6.7	1.56	1.26	86.49	2.19	2.3
I1	5.19	1.91	1.52	67.01	2.26	2.28
I2	6.13	1.92	1.15	138.1	2.15	2.41
I3	4.62	1.95	1.67	127.9	2.22	2.26
N1	6.13	1.61	0.68	133.86	2.18	2.38
N2	0.96	1.62	1.16	164.79	2.18	2.4
N3	1.72	2.5	0.54	167.36	2.18	2.42

The results show that the yield from Extraction 1 is much lower than the yield from Extraction 2. In addition, the purity ratios for Extraction 1 are lower than for Extraction 2,

which indicates protein contamination in the samples from Extraction 1. Though modifications were made to the protocol provided by the manufacturer, these modifications were relatively minor and should not have produced such a great difference in yield. These results show the inconsistency of the MoBio RNA Powersoil kit.

12.1.3.2 Experiment 2 – Comparing RNA and DNA yield for MoBio and Qiagen kits for replicate samples from WWTP

DNA and RNA were extracted using each kit from triplicate MLSS samples (2 mL) from the Neuse River WWTP and South Cary WRF. The results are shown in tables E.2 - E.3. Since the MoBio and Qiagen kits call for different final elution volumes, the total amount (ng) of nucleic acid extracted (concentration x elution volume) is reported rather than the concentration. The averages and standard deviations of the results are presented in Figure E.1. Based on the average values, the the Qiagen kit gave about 31% and 313% higher yield than the MoBio kit for Raleigh and S. Cary RNA, respectively. The Qiagen DNA extractions show an even greater difference between the two kits with 624% and 1809% yield improvements for Raleigh and S. Cary DNA, respectively. Though the DNA purity is lower for the Qiagen extraction, the preliminary real-time PCR results using these samples as template show no indication of PCR inhibition. For these reasons, the Qiagen RNeasy and DNeasy kits were chosen as the extraction method for all chemostat samples. Though data shows the Qiagen kits to be an improvement over the MoBio kits, further study is needed to confirm that the Qiagen kits can quantitatively extract RNA and DNA. Quantitative extraction is defined as obtaining a proportionately higher yield from a higher cell load.

Table E.3 – Extraction results using MoBio Powersoil extraction kits

Mobio	RNA		DNA	
	total ng	260/280	total ng	260/280
RAL 1	18255	2.2	618	2.37
RAL 2	19436	2.19	556	2.04
RAL 3	13648	2.2	7294	1.97
S.CARY 1	5524	2.23	1294	1.72
S.CARY 2	6213	2.2	740	1.35
S.CARY 3	3122	1.72	775	2.05

Table E.4 – Extraction results using RNeasy and DNeasy kits from Qiagen

Qiagen	RNA		DNA	
	total ng	260/280	total ng	260/280
RAL 1*	25894	2.09	85068	3.29
RAL 2	20461	2.03	20015	1.73
RAL 3	20810	2.03	20842	1.65
S.CARY 1	20968	2.05	17755	1.71
S.CARY 2	20373	2.05	17261	1.73
S.CARY 3	20086	2.07	18600	1.66

*Sample was mishandled and is therefore excluded from analysis

MoBio vs. Qiagen

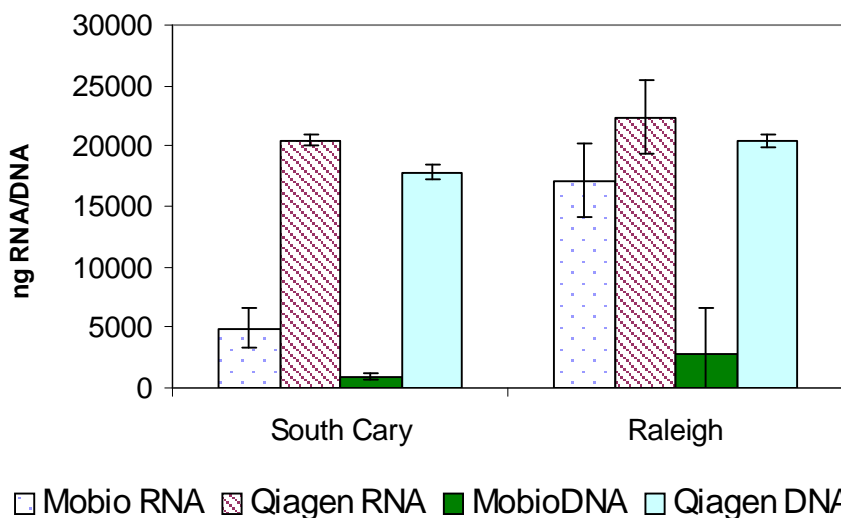


Figure E.1 - Comparison of RNA/DNA yield between MoBio and Qiagen extraction kits

13 Appendix F - Raw Data

13.1 Chemostat - SNA RNA Standard Curve

data - 06/04/2007

ID	Ct	Log Copy #	copy #
SNA -RT 1	34.05	N/A	0.0E+00
SNA -RT 2	32.57	N/A	0.0E+00
SNA -RT 3	34.53	N/A	0.0E+00
SNA NTC 1	33.69	N/A	0.0E+00
SNA NTC 2	33.33	N/A	0.0E+00
SNA NTC 3	N/A	N/A	0.0E+00
SNA1	5.73	10.236	3.2E+10
SNA1	9.70	9.236	3.2E+09
SNA1	12.56	8.236	3.2E+08
SNA1	15.20	7.236	3.2E+07
SNA1	19.70	6.236	3.2E+06
SNA1	23.27	5.236	3.2E+05
SNA1	26.33	4.236	3.2E+04
SNA1	29.90	3.236	3.2E+03
SNA1	32.44	2.236	3.2E+02
SNA1	33.34	1.236	3.2E+01
SNA1	33.35	0.236	3.2E+00
SNA2	5.61	10.236	3.2E+10
SNA2	9.64	9.236	3.2E+09
SNA2	12.68	8.236	3.2E+08
SNA2	15.39	7.236	3.2E+07
SNA2	19.39	6.236	3.2E+06
SNA2	23.81	5.236	3.2E+05
SNA2	N/A	4.236	3.2E+04
SNA2	30.09	3.236	3.2E+03
SNA2	32.17	2.236	3.2E+02
SNA2	33.73	1.236	3.2E+01
SNA2	33.59	0.236	3.2E+00
SNA3	5.54	10.236	3.2E+10
SNA3	9.63	9.236	3.2E+09
SNA3	12.85	8.236	3.2E+08
SNA3	15.07	7.236	3.2E+07
SNA3	19.37	6.236	3.2E+06

ng	copy #	Ct*	log copy #	log ng	Stdev
6.8E+00	3.2E+10	5.63	10.51	0.84	0.10
6.8E-01	3.2E+09	9.66	9.51	-0.16	0.04
6.8E-02	3.2E+08	12.70	8.51	-1.16	0.15
6.8E-03	3.2E+07	15.22	7.51	-2.16	0.16
6.8E-04	3.2E+06	19.49	6.51	-3.16	0.19
6.8E-05	3.2E+05	23.40	5.51	-4.16	0.36
6.8E-06	3.2E+04	25.56	4.51	-5.16	1.09
6.8E-07	3.2E+03	29.27	3.51	-6.16	1.25
6.8E-08	3.2E+02	31.94	2.51	-7.16	0.64
6.8E-09	3.2E+01	33.29	1.51	-8.16	0.47
6.8E-10	3.2E+00	33.40	0.51	-9.16	0.17
NTC	0.0E+00	33.51	0		
-RT	0.0E+00	33.72	0		

Detection limit (copies) = 953.91
 Detection limit (ng) = 2.03E-07

* average of triplicate PCR reactions except for 3.2 x 10⁴ copy # reaction, one of the replicates did not amplify properly

13.2

Chemostat - SNA DNA Standard Curve

Raw data - 6/10/2007

ID	Ct	Log copy #	Copy #
sna ntc 1	N/A	N/A	0.0E+00
sna ntc 2	N/A	N/A	0.0E+00
sna ntc 3	N/A	N/A	0.0E+00
SNA1	2.41	9.196	1.6E+09
SNA1	5.88	8.196	1.6E+08
SNA1	9.67	7.196	1.6E+07
SNA1	12.94	6.196	1.6E+06
SNA1	16.53	5.196	1.6E+05
SNA1	20.41	4.196	1.6E+04
SNA1	23.79	3.196	1.6E+03
SNA1	27.65	2.196	1.6E+02
SNA1	31.19	1.196	1.6E+01
SNA1	38.58	0.196	1.6E+00
SNA2	2.02	9.196	1.6E+09
SNA2	5.65	8.196	1.6E+08
SNA2	9.66	7.196	1.6E+07
SNA2	12.92	6.196	1.6E+06
SNA2	16.92	5.196	1.6E+05
SNA2	20.41	4.196	1.6E+04
SNA2	24.62	3.196	1.6E+03
SNA2	27.69	2.196	1.6E+02
SNA2	31.77	1.196	1.6E+01
SNA2	N/A	0.196	1.6E+00
SNA3	1.95	9.196	1.6E+09
SNA3	5.63	8.196	1.6E+08
SNA3	10.94	7.196	1.6E+07
SNA3	12.97	6.196	1.6E+06
SNA3	16.66	5.196	1.6E+05
SNA3	20.6	4.196	1.6E+04
SNA3	24.21	3.196	1.6E+03
SNA3	27.35	2.196	1.6E+02
SNA3	32.33	1.196	1.6E+01
SNA3	N/A	0.196	1.6E+00

ng	copy #	Ct*	log copy #	log ng	Stdev
6.8E-01	1.6E+09	2.13	9.20	-0.169	0.25
6.8E-02	1.6E+08	5.72	8.20	-1.169	0.14
6.8E-03	1.6E+07	10.09	7.20	-2.169	0.74
6.8E-04	1.6E+06	12.94	6.20	-3.169	0.03
6.8E-05	1.6E+05	16.70	5.20	-4.169	0.20
6.8E-06	1.6E+04	20.47	4.20	-5.169	0.11
6.8E-07	1.6E+03	24.21	3.20	-6.169	0.42
6.8E-08	1.6E+02	27.56	2.20	-7.169	0.19
6.8E-09	1.6E+01	31.76	1.20	-8.169	0.57
6.8E-10	1.6E+00	38.58	0.20	-9.169	N/A
NTC	0.0E+00	N/A	0.00	0.000	0.05

Detection limit (copies) = 1.46
 Detection limit (ng) = 6.30E-10

* average of triplicate PCR reactions except for lowest dilution which has only one replicate

13.3

Chemostat - AGF DNA Standard Curve

Raw data - 06/08/07

ID	Ct	Log Copy #	copy #
AGF1	5.83	10.09	1.2E+09
AGF1	9.11	9.09	1.2E+08
AGF1	13.02	8.09	1.2E+07
AGF1	16.2	7.09	1.2E+06
AGF1	19.81	6.09	1.2E+05
AGF1	22.73	5.09	1.2E+04
AGF1	26.17	4.09	1.2E+03
AGF1	29.48	3.09	1.2E+02
AGF1	33.08	2.09	1.2E+01
AGF1	34.49	1.09	1.2E+00
NTC	35.89	N/A	0.0E+00
AGF2	5.78	10.09	1.2E+09
AGF2	9.33	9.09	1.2E+08
AGF2	12.65	8.09	1.2E+07
AGF2	15.88	7.09	1.2E+06
AGF2	19.14	6.09	1.2E+05
AGF2	22.69	5.09	1.2E+04
AGF2	25.9	4.09	1.2E+03
AGF2	29.38	3.09	1.2E+02
AGF2	32.01	2.09	1.2E+01
AGF2	34.6	1.09	1.2E+00
NTC 2	34.46	N/A	0.0E+00
AGF3	5.65	10.09	1.2E+09
AGF3	9.2	9.09	1.2E+08
AGF3	12.67	8.09	1.2E+07
AGF3	15.7	7.09	1.2E+06
AGF3	19.1	6.09	1.2E+05
AGF3	22.35	5.09	1.2E+04
AGF3	25.81	4.09	1.2E+03
AGF3	29.17	3.09	1.2E+02
AGF3	32.42	2.09	1.2E+01
AGF3	35.35	1.09	1.2E+00
NTC 3	35.38	N/A	0.0E+00

ng	copy #*	Ct	log copy #	log ng	Stdev
1.0E+00	1.2E+09	5.75	9.09	0.00	0.09
1.0E-01	1.2E+08	9.21	8.09	-1.00	0.11
1.0E-02	1.2E+07	12.78	7.09	-2.00	0.21
1.0E-03	1.2E+06	15.93	6.09	-3.00	0.25
1.0E-04	1.2E+05	19.35	5.09	-4.00	0.40
1.0E-05	1.2E+04	22.59	4.09	-5.00	0.21
1.0E-06	1.2E+03	25.96	3.09	-6.00	0.19
1.0E-07	1.2E+02	29.34	2.09	-7.00	0.16
1.0E-08	1.2E+01	32.50	1.09	-8.00	0.54
1.0E-09	1.2E+00	34.81	0.09	-9.00	0.47
NTC	0.0E+00	35.24	0.00		0.72

Detection limit (copies) = 15.73
 Detection limit (ng) = 1.28E-08

*average of triplicate values

13.4

Chemostat - AGF RNA Standard Curve

Raw data - 06/18/2007

ID	Ct	Log Copy #	copy #
AGF1	1.62	1.0E+01	2.0E+10
AGF1	4.37	9.3E+00	2.0E+09
AGF1	9.11	8.3E+00	2.0E+08
AGF1	12.1	7.3E+00	2.0E+07
AGF1	17.67	6.3E+00	2.0E+06
AGF1	21.43	5.3E+00	2.0E+05
AGF1	24.09	4.3E+00	2.0E+04
AGF1	28.13	3.3E+00	2.0E+03
AGF1	28.77	2.3E+00	2.0E+02
AGF1	29.77	1.3E+00	2.0E+01
AGF1	28.96	3.1E-01	2.0E+00
AGF2	1.44	1.0E+01	2.0E+10
AGF2	4.8	9.3E+00	2.0E+09
AGF2	8.94	8.3E+00	2.0E+08
AGF2	12.52	7.3E+00	2.0E+07
AGF2	17.22	6.3E+00	2.0E+06
AGF2	21.03	5.3E+00	2.0E+05
AGF2	24.27	4.3E+00	2.0E+04
AGF2	27.53	3.3E+00	2.0E+03
AGF2	28.81	2.3E+00	2.0E+02
AGF2	28.7	1.3E+00	2.0E+01
AGF2	29.31	3.1E-01	2.0E+00
AGF3	1.47	1.0E+01	2.0E+10
AGF3	4.83	9.3E+00	2.0E+09
AGF3	8.98	8.3E+00	2.0E+08
AGF3	12.35	7.3E+00	2.0E+07
AGF3	17.19	6.3E+00	2.0E+06
AGF3	20.95	5.3E+00	2.0E+05
AGF3	24.3	4.3E+00	2.0E+04
AGF3	27.68	3.3E+00	2.0E+03
AGF3	29.29	2.3E+00	2.0E+02
AGF3	28.62	1.3E+00	2.0E+01
AGF3	29.01	3.1E-01	2.0E+00
NTC1	30.06	N/A	0.0E+00
NTC 2	29.75	N/A	0.0E+00
NTC 3	29.15	N/A	0.0E+00
-RT 1	28.2	N/A	0.0E+00
-RT 2	28.75	N/A	0.0E+00
-RT 3	28.15	N/A	0.0E+00

ng	copy #	Ct	log copy #	log ng	Stdev
8.5E+00	2.0E+10	1.51	10.31	0.93	0.10
8.5E-01	2.0E+09	4.67	9.31	-0.07	0.26
8.5E-02	2.0E+08	9.01	8.31	-1.07	0.09
8.5E-03	2.0E+07	12.32	7.31	-2.07	0.21
8.5E-04	2.0E+06	17.36	6.31	-3.07	0.27
8.5E-05	2.0E+05	21.14	5.31	-4.07	0.26
8.5E-06	2.0E+04	24.22	4.31	-5.07	0.11
8.5E-07	2.0E+03	27.78	3.31	-6.07	0.31
8.5E-08	2.0E+02	28.96	2.31	-7.07	0.29
8.5E-09	2.0E+01	29.03	1.31	-8.07	0.64
8.5E-10	2.0E+00	29.09	0.31	-9.07	0.19
NTC	0.0E+00	29.65	N/A		0.33
-RT	0.0E+00	28.37	N/A		

Detection limit (copies) = 3591.36

Detection limit (ng) = 1.50E-06

*average of triplate values

13.5 RNA:DNA vs growth rate curve AGF DNA

AGF DNA - Raw data - 07/28/2007

Volume of eluate used in PCR reaction 2.00E+00 ul
 Dilution factor of eluate for PCR reaction 1.00E+01
 Size of 16s rDNA 1.50E+03 bp

ID	Ct	volume	log copy #	copy #	Vol. of culture used (ul)	copy # / uL of culture	ng 16S rDNA/uL of culture
5h A	12.68	196	7.07	1.19E+07	25000	4.66E+05	7.66E-04
5h B	13.61	188	6.79	6.18E+06	25000	2.32E+05	3.82E-04
5h C	13.94	194	6.69	4.90E+06	25000	1.90E+05	3.13E-04
5h A	13	196	6.98	9.49E+06	25000	3.72E+05	6.11E-04
5h B	13.96	188	6.68	4.83E+06	25000	1.82E+05	2.99E-04
5h C	14.65	194	6.47	2.98E+06	25000	1.15E+05	1.90E-04
5h A	13.62	196	6.79	6.14E+06	25000	2.41E+05	3.95E-04
5h B	14.22	188	6.60	4.03E+06	25000	1.51E+05	2.49E-04
5h C	14.69	194	6.46	2.89E+06	25000	1.12E+05	1.85E-04
NTC	N/A						
11h A	13.53	184	6.82	6.54E+06	25000	2.41E+05	3.96E-04
11h B	12.55	190	7.11	1.30E+07	25000	4.95E+05	8.13E-04
11h C	13.61	190	6.79	6.18E+06	25000	2.35E+05	3.86E-04
11h A	13.29	184	6.89	7.74E+06	25000	2.85E+05	4.68E-04
11h B	12.55	190	7.11	1.30E+07	25000	4.95E+05	8.13E-04
11h C	13.53	190	6.82	6.54E+06	25000	2.48E+05	4.08E-04
11h A	13.49	184	6.83	6.73E+06	25000	2.47E+05	4.07E-04
11h B	12.71	190	7.07	1.16E+07	25000	4.42E+05	7.27E-04
11h C	13.71	190	6.76	5.76E+06	25000	2.19E+05	3.60E-04
NTC	N/A						

(continued)

ID	Ct	volume	log copy #	copy #	Vol. of culture used (ul)	copy # / uL of culture	ng 16S rDNA/uL of culture
1D A	16.37	194	5.95	8.89E+05	12500	6.90E+04	1.13E-04
1D B	15.71	184	6.15	1.41E+06	12500	1.04E+05	1.71E-04
1D C	15.97	186	6.07	1.18E+06	12500	8.76E+04	1.44E-04
1D A	16.17	194	6.01	1.02E+06	12500	7.94E+04	1.30E-04
1D B	15.95	184	6.08	1.19E+06	12500	8.79E+04	1.44E-04
1D C	16.4	186	5.94	8.70E+05	12500	6.48E+04	1.06E-04
1D A	16.37	194	5.95	8.89E+05	12500	6.90E+04	1.13E-04
1D B	16.15	184	6.02	1.04E+06	12500	7.64E+04	1.26E-04
1D C	16.16	186	6.01	1.03E+06	12500	7.67E+04	1.26E-04
NTC	N/A						
3D A	13.26	186	6.90	7.90E+06	12500	5.88E+05	9.67E-04
3D B	12.7	186	7.07	1.17E+07	12500	8.72E+05	1.43E-03
3D C	13.06	188	6.96	9.10E+06	12500	6.84E+05	1.12E-03
3D A	13.13	186	6.94	8.66E+06	12500	6.44E+05	1.06E-03
3D B	13.07	186	6.96	9.03E+06	12500	6.72E+05	1.10E-03
3D C	13.35	188	6.87	7.42E+06	12500	5.58E+05	9.17E-04
3D A	13.45	186	6.84	6.92E+06	12500	5.15E+05	8.46E-04
3D B	12.74	186	7.06	1.14E+07	12500	8.47E+05	1.39E-03
3D C	13.62	188	6.79	6.14E+06	12500	4.62E+05	7.59E-04
5D A	12.92	188	7.00	1.00E+07	12500	7.55E+05	1.24E-03
5D B	12.74	188	7.06	1.14E+07	12500	8.57E+05	1.41E-03
5D C	12.32	188	7.18	1.53E+07	12500	1.15E+06	1.89E-03
5D A	12.83	188	7.03	1.07E+07	12500	8.04E+05	1.32E-03
5D B	13.06	188	6.96	9.10E+06	12500	6.84E+05	1.12E-03
5D C	12.67	188	7.08	1.20E+07	12500	9.00E+05	1.48E-03
5D A	13.45	188	6.84	6.92E+06	12500	5.20E+05	8.55E-04
5D B	13.16	188	6.93	8.48E+06	12500	6.38E+05	1.05E-03

13.6

RNA:DNA vs growth rate curve AGF RNA

AGF RNA - Raw data - 07/28/2007

Volume of eluate used in PCR reaction

2 ul

Dilution factor of eluate for PCR reaction

10

Size of 16s rRNA

1500 bp

ID	Ct	-RT	vol	log copy #	copy #	Vol. of culture used (uL)	copy #/uL of culture	ng 16S rRNA/uL of culture	RNA:DNA (copy #)	RNA:DNA (ng)
5h A	9.73	34.69	49	8.12	1.33E+08	25000	1.30E+06	1.10E-03	2.79E+00	1.44E+00
5h B	9.56	34.68	49	8.17	1.48E+08	25000	1.45E+06	1.23E-03	6.23E+00	3.21E+00
5h C	9.65	32.66	49	8.14	1.40E+08	25000	1.37E+06	1.16E-03	7.19E+00	3.71E+00
5h A	9.64	33.92	49	8.15	1.40E+08	25000	1.38E+06	1.17E-03	3.70E+00	1.91E+00
5h B	9.6	35.45	49	8.16	1.44E+08	25000	1.41E+06	1.20E-03	7.77E+00	4.00E+00
5h C	9.53	33.15	49	8.18	1.51E+08	25000	1.48E+06	1.25E-03	1.28E+01	6.58E+00
5h A	9.46	34.29	49	8.20	1.57E+08	25000	1.54E+06	1.31E-03	6.41E+00	3.30E+00
5h B	9.27	33.79	49	8.25	1.78E+08	25000	1.74E+06	1.47E-03	1.15E+01	5.92E+00
5h C	9.15	32.79	49	8.28	1.92E+08	25000	1.88E+06	1.59E-03	1.67E+01	8.61E+00
NTC	34.62	38.3								
11h A	8.65	31.91	49	8.42	2.63E+08	25000	2.58E+06	2.18E-03	1.07E+01	5.51E+00
11h B	9.36	33.33	49	8.22	1.68E+08	25000	1.64E+06	1.39E-03	3.32E+00	1.71E+00
11h C	9.1	32.94	50	8.30	1.98E+08	25000	1.98E+06	1.67E-03	8.42E+00	4.34E+00
11h A	8.6	32.41	49	8.43	2.71E+08	25000	2.66E+06	2.25E-03	9.33E+00	4.81E+00
11h B	8.95	33.53	49	8.34	2.17E+08	25000	2.13E+06	1.80E-03	4.31E+00	2.22E+00
11h C	8.97	33.03	50	8.33	2.15E+08	25000	2.15E+06	1.82E-03	8.64E+00	4.45E+00
11h A	8.63	32.8	49	8.43	2.66E+08	25000	2.61E+06	2.21E-03	1.05E+01	5.43E+00
11h B	8.78	33.03	49	8.38	2.42E+08	25000	2.37E+06	2.01E-03	5.37E+00	2.76E+00
11h C	8.83	33.34	50	8.37	2.35E+08	25000	2.35E+06	1.99E-03	1.07E+01	5.52E+00
NTC	34.45	N/A								
1D A	12.35	31.89	50	7.40	2.53E+07	12500	5.06E+05	4.28E-04	7.33E+00	3.78E+00
1D B	12.7	32.96	50	7.31	2.03E+07	12500	4.05E+05	3.43E-04	3.90E+00	2.01E+00
1D C	13.47	28.64	50	7.10	1.25E+07	12500	2.49E+05	2.11E-04	2.84E+00	1.46E+00

(continued)

AGF RNA - Raw data - 07/28/2007 (continued)

ID	Ct	-RT	vol	log copy #	copy #	Vol. of culture used (uL)	copy #/uL of culture	ng 16S rRNA/uL of culture	RNA:DNA (copy #)	RNA:DNA (ng)
NTC	35.13	38.5								
3D A	9.93	31.83	50	8.07	1.17E+08	12500	2.34E+06	1.98E-03	3.98E+00	2.05E+00
3D B	10.8	30.91	48	7.83	6.74E+07	12500	1.29E+06	1.10E-03	1.49E+00	7.65E-01
3D C	9.74	32.62	50	8.12	1.32E+08	12500	2.64E+06	2.23E-03	3.86E+00	1.99E+00
3D A	10.1	32.07	50	8.02	1.05E+08	12500	2.10E+06	1.78E-03	3.26E+00	1.68E+00
3D B	10.76	30.72	48	7.84	6.92E+07	12500	1.33E+06	1.12E-03	1.98E+00	1.02E+00
3D C	9.68	32	50	8.14	1.37E+08	12500	2.74E+06	2.32E-03	4.91E+00	2.53E+00
3D A	9.92	31.65	50	8.07	1.18E+08	12500	2.35E+06	1.99E-03	4.57E+00	2.36E+00
3D B	10.03	30.73	48	8.04	1.10E+08	12500	2.11E+06	1.78E-03	2.49E+00	1.28E+00
3D C	9.55	32.84	50	8.17	1.49E+08	12500	2.97E+06	2.52E-03	6.44E+00	3.32E+00
5D A	10.49	26.81	48	7.91	8.20E+07	12500	1.58E+06	1.33E-03	2.09E+00	1.08E+00
5D B	10.19	25.95	49	8.00	9.92E+07	12500	1.94E+06	1.65E-03	2.27E+00	1.17E+00
5D C	10.17	26.69	48	8.00	1.00E+08	12500	1.93E+06	1.63E-03	1.68E+00	8.64E-01
5D A	10.44	27.72	48	7.93	8.47E+07	12500	1.63E+06	1.38E-03	2.02E+00	1.04E+00
5D B	10.1	26.44	49	8.02	1.05E+08	12500	2.06E+06	1.74E-03	3.01E+00	1.55E+00
5D C	10.32	26.71	48	7.96	9.14E+07	12500	1.75E+06	1.49E-03	1.95E+00	1.00E+00
5D A	10.16	27.6	48	8.00	1.01E+08	12500	1.94E+06	1.64E-03	3.73E+00	1.92E+00
5D B	9.76	26.33	49	8.11	1.30E+08	12500	2.55E+06	2.16E-03	4.00E+00	2.06E+00
5D C	10.24	26.76	48	7.98	9.61E+07	12500	1.85E+06	1.56E-03	2.15E+00	1.11E+00

13.7**RNA:DNA vs growth rate curve AGF**

SRT (hr)	m, h⁻¹	RNA:DNA* (copy number)	Stdev (RNA:DNA copy #)	RNA:DNA (ng)	Stdev (RNA:DNA ng)
4.92	0.2033	8.34	4.50	4.30	2.32
11.22	0.0891	7.93	2.87	4.08	1.48
30.83	0.0324	4.60	1.45	2.37	0.74
74.33	0.0135	3.66	1.56	1.89	0.80
124.34	0.0080	2.54	0.83	1.31	0.43

13.8 RNA:DNA vs growth rate curve SNA DNA

SNA DNA Raw data - 07/28/2007

Volume of eluate used in PCR reaction 2 ul

Dilution factor of eluate for PCR reaction 10

Size of 16s rDNA 1500 bp

ID	Ct	volume	log copy #	copy #	Vol. of culture used	copy #/uL of culture	ng 16S rDNA / uL of culture
5h A	15.45	200	5.58	3.77E+05	10000	37680.05	6.19E-05
5h B	15.74	198	5.50	3.14E+05	10000	31088.30	5.11E-05
5h C	16.04	200	5.42	2.60E+05	10000	26006.55	4.27E-05
5h A	15.31	200	5.61	4.11E+05	10000	41145.44	6.76E-05
5h B	15.75	198	5.49	3.12E+05	10000	30893.54	5.08E-05
5h C	15.31	200	5.61	4.11E+05	10000	41145.44	6.76E-05
5h A	15.42	200	5.58	3.84E+05	10000	38397.19	6.31E-05
5h B	15.76	198	5.49	3.10E+05	10000	30700.00	5.05E-05
5h C	15.71	200	5.51	3.20E+05	10000	31999.98	5.26E-05
NTC	N/A						
6h A	14.95	197	5.71	5.16E+05	10000	50817.43	8.35E-05
6h B	15.31	202	5.61	4.11E+05	10000	41556.89	6.83E-05
6h C	14.56	202	5.82	6.59E+05	10000	66579.51	1.09E-04
6h A	14.42	197	5.86	7.20E+05	10000	70903.17	1.17E-04
6h B	14.79	202	5.76	5.70E+05	10000	57619.13	9.47E-05
6h C	14.87	202	5.73	5.43E+05	10000	54793.91	9.01E-05
6h A	14.49	197	5.84	6.89E+05	10000	67851.67	1.12E-04
6h B	14.82	202	5.75	5.60E+05	10000	56542.99	9.29E-05
6h C	14.12	202	5.94	8.69E+05	10000	87786.91	1.44E-04
NTC	N/A						

(continued)

ID	Ct	volume	log copy #	copy #	Vol. of culture used	copy #/uL of culture	ng 16S rDNA / uL of culture
11h A	15.22	200	5.64	4.35E+05	10000	43539.70	7.16E-05
11h B	14.40	198	5.86	7.29E+05	10000	72164.43	1.19E-04
11h C	14.84	200	5.74	5.53E+05	10000	55283.92	9.09E-05
11h A	14.46	200	5.85	7.02E+05	10000	70195.97	1.15E-04
11h B	14.39	198	5.87	7.34E+05	10000	72619.37	1.19E-04
11h C	14.72	200	5.78	5.96E+05	10000	59614.29	9.80E-05
11h A	15.00	200	5.70	5.00E+05	10000	49995.39	8.22E-05
11h B	14.07	198	5.95	8.97E+05	10000	88795.32	1.46E-04
11h C	14.91	200	5.72	5.29E+05	10000	52904.63	8.70E-05
NTC	N/A						
1D A	14.94	204	5.72	5.19E+05	5000	105909.76	1.74E-04
1D B	14.81	200	5.75	5.63E+05	5000	112672.18	1.85E-04
1D C	14.49	204	5.84	6.89E+05	5000	140525.28	2.31E-04
1D A	14.16	204	5.93	8.48E+05	5000	172910.50	2.84E-04
1D B	14.32	200	5.88	7.67E+05	5000	153303.61	2.52E-04
1D C	14.57	204	5.82	6.55E+05	5000	133634.95	2.20E-04
1D A	14.75	204	5.77	5.85E+05	5000	119341.82	1.96E-04
1D B	14.45	200	5.85	7.06E+05	5000	141277.01	2.32E-04
1D C	14.21	204	5.91	8.21E+05	5000	167561.74	2.75E-04
3D A	14.53	199	5.83	6.72E+05	5000	133678.07	2.20E-04
3D B	14.34	202	5.88	7.57E+05	5000	152902.71	2.51E-04
3D C	15.23	198	5.64	4.33E+05	5000	85668.53	1.41E-04
3D A	14.33	199	5.88	7.62E+05	5000	151581.49	2.49E-04
3D B	14.02	202	5.97	9.26E+05	5000	186961.75	3.07E-04
3D C	15.10	198	5.67	4.70E+05	5000	92961.30	1.53E-04
3D A	14.78	199	5.76	5.74E+05	5000	114242.49	1.88E-04
3D B	14.23	202	5.91	8.11E+05	5000	163846.61	2.69E-04
3D C	14.87	198	5.73	5.43E+05	5000	107417.76	1.77E-04

13.9

RNA:DNA vs growth rate curve SNA RNA

SNA chemostat samples 2x Dnased, Raw data - 08/08/2007

Volume of eluate used in PCR reaction 2 ul

Dilution factor of eluate for PCR reaction 1

Size of 16s rDNA 1500 bp

ID	Ct	-RT	volume (ul)	log copy #	copy #	Vol. of culture used (uL)	copy #/uL of culture	ng 16S rDNA/uL of	RNA:DNA (copy #)	RNA:DNA (ng)
5h A	5.7	32.14	50	10.72	5.19E+10	10000	1.30E+08	1.10E-01	3446.49	1775.47
5h B	5.7	33.43	50	10.72	5.19E+10	10000	1.30E+08	1.10E-01	4177.26	2151.92
5h C	5.43	33.2	51	10.80	6.32E+10	10000	1.61E+08	1.36E-01	6197.64	3192.72
5h A	5.55	32.83	50	10.76	5.79E+10	10000	1.45E+08	1.23E-01	3519.75	1813.20
5h B	5.76	33.25	50	10.70	4.97E+10	10000	1.24E+08	1.05E-01	4024.24	2073.09
5h C	5.33	32.53	51	10.83	6.80E+10	10000	1.73E+08	1.47E-01	4212.60	2170.13
5h A	5.39	32.82	50	10.81	6.51E+10	10000	1.63E+08	1.38E-01	4236.77	2182.58
5h B	5.72	33.11	50	10.71	5.12E+10	10000	1.28E+08	1.08E-01	4169.06	2147.70
5h C	5.31	32.64	51	10.84	6.90E+10	10000	1.76E+08	1.49E-01	5495.85	2831.20
NTC	34.19									
6h A	5.98	34.83	50	10.63	4.24E+10	10000	1.06E+08	8.97E-02	2084.97	1074.08
6h B	6.24	36.26	50	10.55	3.51E+10	10000	8.77E+07	7.43E-02	2110.60	1087.28
6h C	5.98	33.75	51	10.63	4.24E+10	10000	1.08E+08	9.15E-02	1623.20	836.19
6h A	6.01	36.29	50	10.62	4.15E+10	10000	1.04E+08	8.78E-02	1462.10	753.20
6h B	6.53	36.43	50	10.45	2.84E+10	10000	7.10E+07	6.02E-02	1232.96	635.16
6h C	6.08	34.1	51	10.60	3.94E+10	10000	1.00E+08	8.51E-02	1834.08	944.83
6h A	6.05	35.19	50	10.61	4.03E+10	10000	1.01E+08	8.53E-02	1484.08	764.53
6h B	6.12	35.4	50	10.58	3.83E+10	10000	9.57E+07	8.10E-02	1692.57	871.93
6h C	5.94	33.66	51	10.64	4.36E+10	10000	1.11E+08	9.42E-02	1267.38	652.89
NTC	33.85									

(continued)

ID	Ct	-RT	volume (ul)	log copy #	copy #	Vol. of culture used (uL)	copy #/mL of culture	ng 16S rDNA/mL of	RNA:DNA (copy #)	RNA:DNA (ng)
11h A	5.59	32.81	48	10.75	5.63E+10	10000	1.35E+08	1.14E-01	3101.66	1597.82
11h B	5.71	33.81	48	10.71	5.16E+10	10000	1.24E+08	1.05E-01	1715.07	883.52
11h C	5.78	34.41	50	10.69	4.90E+10	10000	1.23E+08	1.04E-01	2216.36	1141.76
11h A	5.47	33.14	48	10.79	6.14E+10	10000	1.47E+08	1.25E-01	2099.15	1081.38
11h B	5.66	33.82	48	10.73	5.35E+10	10000	1.28E+08	1.09E-01	1767.39	910.47
11h C	5.88	34.37	50	10.66	4.56E+10	10000	1.14E+08	9.65E-02	1911.28	984.60
11h A	5.54	32.95	48	10.77	5.84E+10	10000	1.40E+08	1.19E-01	2801.12	1443.00
11h B	5.63	33.86	48	10.74	5.47E+10	10000	1.31E+08	1.11E-01	1477.28	761.03
11h C	5.64	34.51	50	10.73	5.43E+10	10000	1.36E+08	1.15E-01	2564.09	1320.89
NTC	33.82									
1D A	7.63	32.37	50	10.11	1.28E+10	5000	6.39E+07	5.41E-02	603.14	310.71
1D B	8.18	33.43	51	9.93	8.57E+09	5000	4.37E+07	3.70E-02	387.74	199.74
1D C	7.45	32.09	49	10.16	1.46E+10	5000	7.13E+07	6.04E-02	507.74	261.56
1D A	7.63	32.15	50	10.11	1.28E+10	5000	6.39E+07	5.41E-02	369.43	190.31
1D B	8.13	33.84	51	9.95	8.88E+09	5000	4.53E+07	3.84E-02	295.52	152.24
1D C	7.31	31.94	49	10.21	1.61E+10	5000	7.90E+07	6.69E-02	591.10	304.51
1D A	7.62	33.09	50	10.11	1.29E+10	5000	6.43E+07	5.45E-02	539.16	277.75
1D B	8.07	34.33	51	9.97	9.28E+09	5000	4.73E+07	4.01E-02	334.97	172.56
1D C	7.5	32.49	49	10.15	1.40E+10	5000	6.88E+07	5.83E-02	410.62	211.53
3D A	5.81	32.53	50	10.68	4.80E+10	5000	2.40E+08	2.03E-01	1793.66	924.01
3D B	5.69	32	50	10.72	5.23E+10	5000	2.62E+08	2.22E-01	1711.04	881.44
3D C	5.36	31.54	50	10.82	6.65E+10	5000	3.33E+08	2.82E-01	3881.61	1999.62
3D A	5.83	32.4	50	10.67	4.73E+10	5000	2.36E+08	2.00E-01	1558.98	803.11
3D B	5.54	32.16	50	10.77	5.84E+10	5000	2.92E+08	2.47E-01	1560.51	803.90
3D C	5.32	31.3	50	10.84	6.85E+10	5000	3.42E+08	2.90E-01	3682.61	1897.10
3D A	5.76	33.03	50	10.70	4.97E+10	5000	2.49E+08	2.11E-01	2176.47	1121.21
3D B	5.58	32.42	50	10.75	5.67E+10	5000	2.83E+08	2.40E-01	1729.65	891.03
3D C	5.41	31.66	50	10.81	6.41E+10	5000	3.21E+08	2.72E-01	2985.21	1537.84

13.10 RNA :DNA vs growth rate curve SNA

SRT (hr)	m, h ⁻¹	RNA:DNA (copy #)	stdev	RNA:DNA (ng)	stdev
5.83	0.1715	4386.63	895.87	2259.78	461.51
6.31	0.1585	1643.55	320.26	846.68	164.98
11.13	0.0899	2183.71	541.17	1124.94	278.78
27.53	0.0363	448.82	113.82	231.21	58.64
84.13	0.0119	2342.19	929.14	1206.58	478.65

13.11 Chemostat Raw Data – SNA

Date collected	Measuring flow rate			total volume, mL	SRT, d	SRT, h	m, h ⁻¹	Solids analysis		
	Volume collected	Time, sec	flow rate, mL/min					dish + filter (g)	dried at 104C (g)	TSS, mg/L
9/14/2006	8.6	180	2.8667	1003	0.24	5.83	0.1715	52.6704	52.6882	356
8/18/2006	8.2	196	2.5102	950	0.26	6.31	0.1585	61.9655	61.9748	186
8/11/2006	8	327	1.4679	980	0.46	11.13	0.0899	61.96	61.98	400
8/14/2006	14.1	1539	0.5497	908	1.15	27.53	0.0363	61.9655	61.9935	560
9/13/2006	3.8	1332	0.1712	864	3.51	84.13	0.0119	61.9671	62.0095	848

13.12 Chemostat Raw Data – AGF

Date collected	Measuring flow rate			total volume, mL	SRT, d	SRT, h	m, h ⁻¹	Solids analysis		
	Volume collected	Time, sec	flow rate, mL/min					dish + filter (g)	dried at 104C (g)	TSS, mg/L
10/17/2006	8	161	2.9814	880	0.20	4.92	0.2033	53.0188	53.0214	52
11/4/2006	8	405	1.1852	798	0.47	11.22	0.0891	52.6655	52.6671	32
10/9/2006	14.1	1539	0.5497	1017	1.28	30.83	0.0324	52.6646	52.6688	84
10/4/2006	4.22	1338	0.1892	844	3.10	74.33	0.0135	52.6642	52.6769	254
11/9/2006	4.5	1848	0.1461	1090	5.18	124.34	0.0080	52.6700	52.69	480

13.13 North Cary WWTP - SNA

Sample	Vol. RNA eluate (uL)*	Vol. DNA eluate (uL)	RNA			DNA		RNA:DNA (copies)	Growth rate (h ⁻¹)
			Ct	Ct (-RT)	copy # / ul sample	SNA Ct	copy # / ul sample		
E1	50	104.0	15.2	29.09	6.52E+05	20.68	366.15	1779.56	0.09469
E1-2	50	104.0	15.25	29.02	6.28E+05	20.64	375.47	1673.46	0.08937
E1-3	50	104.0	14.41	29.28	1.16E+06	21	299.45	3863.66	0.19922
E2	50	98.5	16.01	35.13	3.62E+05	21.7	182.67	1979.88	0.10473
E2-2	50	98.5	16.06	34.35	3.49E+05	21.59	195.75	1781.70	0.09479
E2-3	50	98.5	15.81	37.57	4.18E+05	22.02	149.40	2799.64	0.14585
E3	50	98.5	15.57	28.05	4.98E+05	21.25	242.38	2054.46	0.10848
E3-2	50	98.5	15.35	28.03	5.84E+05	20.89	303.91	1922.56	0.10186
E3-3	50	98.5	15.48	27.96	5.32E+05	20.91	300.12	1771.36	0.09428
I1	50	99.0	16.21	N/A	3.13E+05	22.29	126.72	2467.98	0.12922
I1-2	50	99.0	16.36	40.53	2.80E+05	21.82	170.26	1647.10	0.08804
I1-3	50	99.0	16.39	N/A	2.74E+05	22.2	134.09	2046.28	0.10807
I2	50	99.0	14.71	29.69	9.30E+05	21.81	171.34	5429.76	omitted
I2-2	50	99.0	14.71	30.04	9.30E+05	21.35	228.77	4066.61	0.20940
I2-3	50	99.0	14.7	29.72	9.37E+05	21.71	182.45	5136.23	omitted
I3	50	99.0	15.31	35.36	6.02E+05	21.62	193.07	3115.63	0.16170
I3-2	50	99.0	15.39	38.73	5.68E+05	21.21	249.81	2271.93	0.11938
I3-3	50	99.0	15.04	35.02	7.32E+05	21.18	254.56	2875.26	0.14964
N1	50	102.0	14.37	26.44	1.19E+06	22.14	143.47	8302.27	omitted
N1-2	50	102.0	14.45	26.41	1.12E+06	21.61	200.17	5614.27	omitted
N1-3	50	102.0	15.2	26.53	6.52E+05	21.73	185.63	3510.13	0.18148
N2	50	100.0	14.73	37.57	9.17E+05	22.86	89.46	10248.96	omitted
N2-2	50	100.0	14.82	37.1	8.59E+05	22.59	106.01	8101.81	omitted
N2-3	50	100.0	15.24	N/A	6.33E+05	22.22	133.76	4731.88	omitted
N3	50	101.0	14.09	34.15	1.46E+06	23.03	81.20	17978.76	omitted
N3-2	50	101.0	14.14	34.1	1.41E+06	22.83	92.08	15289.47	omitted
N3-3	50	101.0	15.09	36.8	7.06E+05	22.3	128.47	5494.03	omitted
NTC 1			37.25	N/A		N/A			
NTC 2			42.9	N/A		N/A			
NTC 3			35.53	38.13		N/A			

N/A = sample did not amplify, omitted = growth rate not applicable because it is outside of the range of standard curve determined from chemostats

13.14 North Cary WWTP - AGF

Sample	Vol. RNA eluate (uL)*	Vol. DNA eluate (uL)	RNA			DNA		RNA:DNA (copies)	Growth rate (h ⁻¹)
			Ct	Ct (-RT)	copy # / ul sample	AGF Ct	copy # / ul sample		
E1	50	104.0	23.38	31.76	4.60E+02	30.24	1.35	339.95	omitted
E1-2	50	104.0	24.24	24.12	2.75E+02	30.07	1.53	180.28	omitted
E1-3	50	104.0	23.45	N/A	4.41E+02	29.9	1.72	256.73	omitted
E2	50	98.5	25.47	27.75	1.32E+02	31.71	0.46	288.52	omitted
E2-2	50	98.5	25.91	N/A	1.01E+02	30.73	0.91	111.36	omitted
E2-3	50	98.5	26.16	41.24	8.71E+01	30.25	1.27	68.43	omitted
E3	50	98.5	25.6	28.24	1.22E+02	30.1	1.41	86.12	omitted
E3-2	50	98.5	26.03	34.32	9.42E+01	29.33	2.43	38.76	omitted
E3-3	50	98.5	25.97	31.95	9.76E+01	28.77	3.60	27.11	omitted
I1	50	99.0	28.23	33.34	2.52E+01	31.69	0.47	54.23	omitted
I1-2	50	99.0	28.04	32.59	2.83E+01	30.29	1.24	22.72	omitted
I1-3	50	99.0	28.22	40.75	2.54E+01	29.87	1.67	15.19	0.20539
I2	50	99.0	25.43	29.23	1.35E+02	32.45	0.27	494.51	omitted
I2-2	50	99.0	25.49	34.6	1.30E+02	30.13	1.39	93.47	omitted
I2-3	50	99.0	25.18	27.54	1.57E+02	29.77	1.79	87.38	omitted
I3	50	99.0	25.54	37.2	1.26E+02	31.9	0.40	314.59	omitted
I3-2**	50	99.0	27.11	N/A	4.93E+01	4.15	969849180.74	0.00	omitted
I3-3	50	99.0	37.29	N/A	1.11E-01	30.47	1.10	0.10	omitted
N1	50	102.0	23	27.33	5.78E+02	31.49	0.55	1047.32	omitted
N1-2	50	102.0	23.44	30.59	4.44E+02	30.12	1.44	307.35	omitted
N1-3	50	102.0	23.24	33.63	5.00E+02	30.05	1.52	329.82	omitted
N2	50	100.0	27.41	39.11	4.12E+01	31.08	0.72	57.14	omitted
N2-2	50	100.0	35.41	N/A	3.43E-01	28.7	3.84	0.09	omitted
N2-3	50	100.0	N/A	N/A	#VALUE!	29.88	1.68	#VALUE!	#VALUE!
N3	50	101.0	26.16	29.6	8.71E+01	29.04	3.06	28.52	omitted
N3-2	50	101.0	26.57	32.15	6.82E+01	29.02	3.10	22.00	omitted
N3-3	50	101.0	26.32	31.78	7.92E+01	28.64	4.05	19.57	omitted
NTC 1			29.08	34.3		34.22			
NTC 2			28.06	36.09		34.48			
NTC 3			28.64	33.76		35.01			

*volume used to elute, not actual volume of eluate, **sample did not amplify correctly and was therefore omitted

13.15

Raw Data – Samples from substrate study (13) – SNA growth rate

volume of RNA eluate used in PCR reaction 2 uL
 volume of initial sample from WWTP 2000 uL

Sample	Vol. RNA eluate (uL)	Vol. DNA eluate (uL)	RNA			DNA		RNA:DNA (copies)	Growth rate (h ⁻¹)	Avg growth rate	Stdev
			Ct	Ct (-RT)	copy # / ul sample	Ct	copy # / ul sample				
glu a	49	200.0	15.39	32.58	5.562E+05	13.14	8.045E+04	6.913	0.01	0.01	0.00
glu a	49	200.0	15.69	33.67	4.472E+05	10.77	3.568E+05	1.254	0.01		
glu a	49	200.0	15.5	33.1	5.135E+05	8.38	1.602E+06	0.320	0.01		
glu b	47	192.0	7.09	32.14	2.223E+08	13.13	7.772E+04	2859.636	0.15	0.16	0.04
glu b	47	192.0	7.12	33.83	2.175E+08	13.69	5.467E+04	3978.164	0.20		
glu b	47	192.0	7.01	32.17	2.356E+08	12.92	8.869E+04	2656.109	0.14		
glu c	49	195.0	7.6	32.62	1.600E+08	22.62	2.029E+02	788515.198	39.55	35.10	8.74
glu c	49	195.0	7.59	33.21	1.611E+08	21.88	3.230E+02	498882.384	25.03		
glu c	49	195.0	7.5	32.88	1.720E+08	22.55	2.120E+02	811461.592	40.70		
ac a	49	190.0	14.02	33.75	1.505E+06	19.93	1.072E+03	1404.639	0.08	0.05	0.02
ac a	49	190.0	13.97	33.59	1.561E+06	18.57	2.519E+03	619.671	0.04		
ac a	49	190.0	13.93	33.65	1.607E+06	18.51	2.616E+03	614.343	0.04		
ac b	47	192.0	9.72	30.18	3.287E+07	13.84	4.975E+04	660.669	0.04	0.04	0.01
ac b	47	192.0	9.31	30.89	4.428E+07	13.49	6.199E+04	714.278	0.04		
ac b	47	192.0	9.29	30.65	4.492E+07	13.79	5.134E+04	875.102	0.05		
ac c	48	196.0	7.7	32.11	1.457E+08	11.95	1.666E+05	874.798	0.05	0.03	0.03
ac c	48	196.0	7.79	31.82	1.365E+08	9.24	9.145E+05	149.232	0.01		
ac c	48	196.0	7.57	31.67	1.601E+08	N/A	#VALUE!	#VALUE!	#VALUE!		
pyr a	50	150.0	15.09	29.05	7.058E+05	N/A	#VALUE!	#VALUE!	#VALUE!	3.89	5.32
pyr a	50	150.0	14.91	29.33	8.045E+05	21.51	3.135E+02	2566.373	0.13		
pyr a	50	150.0	14.98	29.18	7.646E+05	28.09	5.015E+00	152442.143	7.65		
pyr b	50	194.0	12.26	28.74	5.520E+06	12.51	1.159E+05	47.606	0.01	0.01	0.00
pyr b	50	194.0	13.18	28.79	2.828E+06	12.52	1.152E+05	24.548	0.01		
pyr b	50	194.0	13.17	28.81	2.849E+06	11.64	2.003E+05	14.223	0.01		

(continued)

Sample	Vol. RNA eluate (uL)	Vol. DNA eluate (uL)	RNA			DNA		RNA:DNA (copies)	Growth rate (h ⁻¹)	Avg growth rate	Stdev
			Ct	Ct (-RT)	copy # / ul sample	Ct	copy # / ul sample				
pyr c*	49	194.0	12.23	30.54	5.529E+06	13.22	7.421E+04	74.496	0.01	0.01	0.00
pyr c*	49	194.0	12.28	30.02	5.331E+06	13.47	6.342E+04	84.059	0.01		
pyr c*	49	194.0	12.15	30.25	5.860E+06	12.23	1.383E+05	42.382	0.01		
pro a *	49	194	14.65	31.16	9.524E+05	26.48	1.784E+01	53377.871	2.68	4.21	4.40
pro a *	49	194	14.86	31.45	8.175E+05	28.68	4.477E+00	182610.166	9.16		
pro a *	49	194	14.8	31.33	8.540E+05	24.67	5.565E+01	15346.645	0.78		
pro b*	48	195	7.5	29.49	1.685E+08	19.2	1.740E+03	96827.256	4.86	4.36	0.47
pro b*	48	195	7.76	29.71	1.395E+08	19.3	1.634E+03	85354.579	4.29		
pro b*	48	195	7.55	29.35	1.625E+08	18.92	2.075E+03	78306.124	3.93		
pro c	48	218	6.53	29.04	3.410E+08	20.22	1.025E+03	332755.654	16.69	13.73	2.67
pro c	48	218	6.66	28.84	3.103E+08	19.78	1.351E+03	229617.161	11.52		
pro c	48	218	6.53	28.85	3.410E+08	19.82	1.318E+03	258793.547	12.99		
me a	48	195	17.42	30.39	1.246E+05	35.22	7.383E-02	1687666.485	84.65	0.05	0.01
me a	48	195	17.67	30.69	1.039E+05	23.44	1.212E+02	857.558	0.05		
me a	48	195	17.67	30.59	1.039E+05	23.8	9.663E+01	1075.272	0.06		
me b	48	194	16.4	34.24	2.615E+05	24.81	5.096E+01	5131.695	0.26	0.26	0.04
me b	48	194	16.64	33.8	2.197E+05	25.35	3.629E+01	6051.901	0.31		
me b	48	194	16.47	34.53	2.485E+05	24.61	5.778E+01	4301.112	0.22		
me c*	48	194	16.46	30.66	2.504E+05	27.17	1.156E+01	21648.998	1.09	6.60	6.18
me c*	48	194	16.54	30.66	2.362E+05	29.82	2.187E+00	108007.001	5.42		
me c*	48	194	16.44	30.81	2.540E+05	31.13	9.601E-01	264577.682	13.28		
NTC			34.07			29.34					
NTC			33.89			28.71					
NTC			31.92			28.63					
*Dnased 2x											
average of duplicate instead of triplicate											
within the limits of growth rate standard curve											

13.16

Raw Data – Samples from substrate study (13) – AGF

volume of RNA eluate used in PCR reaction	2	uL								
volume of initial sample from WWTP	2000	uL								
			AGF							
			RNA			DNA				
Sample	Vol. RNA eluate (uL)	Vol. DNA eluate (uL)	Ct	Ct (-RT)	copy # / ul sample	Ct	copy # / ul sample	RNA:DNA (copies)	Growth rate (h ⁻¹)	
glu a	49	200.0	28.06	29.24	14.94	35.37	0.0708	210.92	3.37	
glu b	47	192.0	29.74	30.84	4.95	34.84	0.0987	50.16	0.77	
glu c	49	195.0	27.84	30.54	17.17	34.47	0.1300	132.11	2.09	
ac a	49	190.0	28.05	29.08	15.03	34.33	0.1397	107.59	1.70	
ac b	47	192.0	27.65	29.16	18.57	33.05	0.3471	53.52	0.82	
ac c	48	196.0	28.89	30.98	8.66	35.33	0.0714	121.24	1.92	
pyr a	50	150.0	33.01	30.26	0.67	N/A	#VALUE!	#VALUE!	#VALUE!	
pyr b	50	194.0	29.24	30.91	7.23	31.37	1.1417	6.33	0.06	
pyr c*	49	194.0	27.64	28.67	19.49	33.84	0.2013	96.80	1.52	
pro a *	49	194	28.23	29.43	13.42	35.64	0.0568	236.06	3.77	
pro b*	48	195	27.29	28.66	23.82	34.95	0.0928	256.79	4.11	
pro c	48	218	28.81	N/A	9.10	41.60	0.0010	9390.03	151.58	
me a	48	195	28.87	29.3	8.77	33.27	0.3020	29.03	0.43	
me b	48	194	29.28	30.91	6.76	38.47	0.0078	869.13	13.99	
me c*	48	194	28.23	29.52	13.14	N/A	#VALUE!	#VALUE!	#VALUE!	
NTC			29.61							
NTC			28.71							
NTC			28.46							
*Dnased 2x										

13.17

Raw Data – Samples from survey study (23) – SNA

volume of DNA eluate used in PCR reaction	2	uL								
volume of initial sample from WWTP	2000	uL								
			SNA							
			RNA			DNA				
Sample	Vol. RNA eluate (uL)	Vol. DNA eluate (uL)	Ct	Ct (-RT)	copy # / ul sample	SNA Ct**	copy # / ul sample	RNA:DNA (copies)	Growth rate (h ⁻¹)	
STP A	47	192.0	25.5	N/A	343.65	26.86	13.91	24.71	0.00667	
STP A	47	192.0	25.99	N/A	240.69	27.21	11.16	21.57	0.00651	
STP A	47	192.0	26.34	N/A	186.63	26.8	14.44	12.92	0.00608	
STP B	49	203.0	26.69	N/A	150.87	27.49	9.90	15.25	0.00620	
STP B	49	203.0	26.25	N/A	207.72	27.66	8.89	23.36	0.00660	
STP B	49	203.0	26.41	N/A	184.92	27.73	8.51	21.73	0.00652	
STP C	46	193.0	N/A	N/A	0.00	25.75	28.08	0.00	0.00543	
STP C	46	193.0	N/A	N/A	0.00	25.46	33.70	0.00	0.00543	
STP C	46	193.0	N/A	N/A	0.00	25.88	25.88	0.00	0.00543	
CRAM A	49	192.0	N/A	N/A	0.00	24.49	61.67	0.00	0.00543	
CRAM A	49	192.0	N/A	N/A	0.00	24.38	66.08	0.00	0.00543	
CRAM A	49	192.0	N/A	N/A	0.00	24.5	61.28	0.00	0.00543	
CRAM B	50	192.0	N/A	N/A	0.00	24.93	46.77	0.00	0.00543	
CRAM B	50	192.0	N/A	N/A	0.00	25.42	34.37	0.00	0.00543	
CRAM B	50	192.0	N/A	N/A	0.00	24.65	55.77	0.00	0.00543	
CRAM C	50	192.0	N/A	N/A	0.00	23.91	88.79	0.00	0.00543	
CRAM C	50	192.0	N/A	N/A	0.00	23.84	92.78	0.00	0.00543	
CRAM C	50	192.0	34.6	N/A	0.49	23.66	103.90	0.00	0.00543	
GP A	48	190.0	28.01	N/A	56.63	25.2	39.06	1.45	0.00551	
GP A	48	190.0	28.28	N/A	46.54	25.22	38.57	1.21	0.00549	
GP A	48	190.0	28.67	N/A	35.05	25.22	38.57	0.91	0.00548	
GP B*	49	191.0	23.47	N/A	1566.53	26.87	13.75	113.95	0.01115	

(continued)

Sample	Vol. RNA eluate (uL)	Vol. DNA eluate (uL)	SNA						
			RNA			DNA		RNA:DNA (copies)	Growth rate (h ⁻¹)
			Ct	Ct (-RT)	copy # / ul sample	SNA Ct**	copy # / ul sample		
GP B*	49	191.0	22.96	N/A	2269.40	26.71	15.20	149.29	0.01292
GP B*	49	191.0	23.03	N/A	2156.84	26.78	14.55	148.26	0.01287
GP C	49	193.0	28.36	N/A	44.82	26.99	12.88	3.48	0.00561
GP C	49	193.0	28.84	N/A	31.62	26.81	14.43	2.19	0.00554
GP C	49	193.0	28.56	N/A	38.76	26.84	14.16	2.74	0.00557
GAS A	52	182	24.38	N/A	858.07	25.31	34.92	24.57	0.00667
GAS A	52	182	27.96	N/A	63.62	25.63	28.56	2.23	0.00554
GAS A	52	182	26.83	32.36	144.62	26.05	21.93	6.59	0.00576
GAS B	53	188	26.41	N/A	200.02	25.7	28.23	7.09	0.00579
GAS B	53	188	26.29	N/A	218.24	25.11	40.90	5.34	0.00570
GAS B	53	188	26.78	31.14	152.86	25.91	24.74	6.18	0.00574
GAS C	50	188	24.14	N/A	982.29	25.65	29.13	33.72	0.00712
GAS C	50	188	24.98	N/A	533.47	25.03	43.01	12.40	0.00605
GAS C	50	188	25.47	31.6	373.64	25.19	38.89	9.61	0.00591
MON A	53	195	31.46	N/A	5.09	24.56	59.94	0.09	0.00544
MON A	53	195	28.91	N/A	32.51	24.3	70.58	0.46	0.00546
MON A	53	195	29.25	N/A	25.39	24.37	67.54	0.38	0.00545
MON B	49	190	N/A	N/A	0.00	23.78	95.35	0.00	0.00543
MON B	49	190	N/A	N/A	0.00	23.61	106.10	0.00	0.00543
MON B	49	190	N/A	N/A	0.00	23.71	99.63	0.00	0.00543
MON C	49	190	28.24	N/A	48.91	24.73	52.48	0.93	0.00548
MON C	49	190	28.43	N/A	42.60	24.75	51.83	0.82	0.00547
MON C	49	190	28.5	31.21	40.49	24.88	47.76	0.85	0.00548
NTC			N/A	N/A		N/A			
NTC			36.29	36.29		34.61			
NTC			N/A	N/A		35.81			
*Dnased 2x									

13.18

Raw Data – Samples from survey study (23) – AGF

Sample	Vol. RNA eluate (uL)	Vol. DNA eluate (uL)	AGF						
			RNA			DNA		RNA:DNA (copies)	Growth rate (h ⁻¹)
			Ct	Ct (-RT)	copy # / ul sample	Ct	copy # / ul sample		
STP A	47	192.0	27.89	30.72	15.92	33.14	0.33	48.88	0.75
STP B	49	203.0	27.26	28.52	24.73	33.62	0.25	100.59	1.58
STP C	46	193.0	25.64	27.11	64.71	33.24	0.31	211.97	3.38
CRAM A	49	192.0	N/A	N/A	0.00	34.31	0.14	0.00	-0.04
CRAM B	50	192.0	N/A	N/A	0.00	30.72	1.78	0.00	-0.04
CRAM C	50	192.0	N/A	N/A	0.00	35.11	0.08	0.00	-0.04
GP A	48	190.0	29.14	29.53	7.37	33.74	0.21	34.87	0.52
GP B*	49	191.0	26.87	27.74	31.65	32.2	0.63	50.45	0.77
GP C	49	193.0	28.61	29.13	10.53	32.08	0.69	15.26	0.21
GAS A	52	182	29.41	30.74	6.73	33.33	0.27	24.92	0.36
GAS B	53	188	28.8	30.51	10.10	35.04	0.08	120.26	1.90
GAS C	50	188	27.96	29.16	16.21	33.8	0.20	80.77	1.26
MON A	53	195	29	29.89	8.90	33.93	0.19	46.84	0.72
MON B	49	190	28.74	29.93	9.70	33.11	0.33	29.45	0.44
MON C	49	190	28.02	29.78	15.29	32.67	0.45	34.09	0.51
NTC			35.56			34.19			
NTC			31.8						
NTC			30.06						

*Dnased 2x

14 Appendix G – Sample Calculations

Sample Calculations	AGF RNA:DNA ratio	VLN 08-20-2007
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$C_t \text{ value} = -3.2771 (\text{Log copy \#}) + 35.865$
 $\text{Log copy \#} = \frac{C_t \text{ value} - 35.85}{3.2771} = -0.30514(C_t) + 10.94404$

for 5h - A sample :

$\text{Log copy \#} = -0.30514(12.68) + 10.94404$
 $= 7.07$
 $\text{copy \#} = 10^{(7.07)}$
 $= 1.19 \times 10^7 \text{ copies}$

Since template was diluted 10x for PCR reactions,

$\frac{\text{copy \#}}{\text{PCR rxn}} = 1.19 \times 10^7 \text{ copies}$

$\frac{\text{copy \#}}{\text{mL original culture}} = \frac{\left[\frac{\text{copy \#}}{\text{PCR rxn}} \right] \times \left[\frac{\text{volume of DNA}}{\text{extraction}} \right]}{\left[\frac{\text{volume of DNA eluate}}{\text{PCR rxn}} \right] \times \left[\text{volume of original culture used for extraction} \right]}$

↑ eluate

$= \frac{(1.19 \times 10^7) \times (196 \text{ ul})}{(2 \text{ ul}) \times (25000 \text{ ml})}$
 $= 4.66 \times 10^5 \text{ copies/mL original culture}$

to convert to ng :

$\frac{\text{copy \#}}{\text{mL original culture}} \left[\frac{10^9 \text{ ng}}{1} \right] \left[\frac{1500 \text{ bp}}{\text{copy}} \right] \left[\frac{660^* \text{ ng}}{\text{mol bp}} \right] \left[\frac{\text{mol bp}}{6.023 \times 10^{23} \text{ bp}} \right]$
 $= [4.66 \times 10^5] \left[\frac{10^9 \text{ ng}}{1} \right] \left[\frac{1500 \text{ bp}}{\text{copy}} \right] \left[\frac{660 \text{ ng}}{\text{mol bp}} \right] \left[\frac{\text{mol bp}}{6.023 \times 10^{23} \text{ bp}} \right]$
 $= 7.66 \times 10^{-4} \frac{\text{ng 16s rDNA}}{\text{mL of culture}}$

* for RNA, use 340 instead of 660.

$\frac{\text{RNA copy \#}}{\text{mL culture}} = 1.30 \times 10^6$

$\text{RNA:DNA (copy \#)} = \frac{1.30 \times 10^6}{4.66 \times 10^5} \approx 2.8$

Substrate effect study, glucose-A sample:

RNA:DNA ratio calculated same as previously described.

$$\text{RNA:DNA (copy \#)} = 210.92$$

$$\text{RNA:DNA} = 61.931(\text{growth rate}) + 2.4686$$

$$\text{Growth rate} = \frac{\text{RNA:DNA} - 2.4686}{61.931}$$

$$= \frac{210.92 - 2.4686}{61.931}$$

$$= 3.37 \text{ h}^{-1}$$

* the RNA and DNA concentrations were under the detection limit of the real-time assays \therefore this growth rate is not valid. The calculations were shown for demonstration purposes only.