Abstract

Keith, Julia Elizabeth. Molecular Characterization of Microbial Populations in Full-Scale Activated Sludge Plants and Bioaugmentation Sites. (Under the direction of Francis de los Reyes.)

The application of molecular biology techniques to the study of wastewater treatment and bioaugmentation was demonstrated in three studies. In the first study, full scale activated sludge treatment plants in North Carolina were surveyed to (1) determine the extent of filamentous bulking and foaming, and (2) relate these problems to the microbial community structures in the activated sludge reactors. Oligonucleotide probes targeting the rRNA of the major sublasses of the Proteobacteria, the mycolic acid containing actinomycetes (mycolata) and *Sphaerotilus natans* were used in quantitative hybridizations with samples from sixteen full scale plants. The survey results showed that 88% and 63% of plants in North Carolina have experienced bulking and foaming, respectively. No statistically significant correlations between the frequency and severity of foaming and the levels of any of the microbial groups were observed. However, several of the plants that had the most severe foaming problems had high levels of mycolata and alpha Proteobacteria. Bulking in the sampled plants is probably not caused by *Sphaerotilus natans* but by other filaments, or other floc characteristics. The sampling protocol raised questions about possible change between collection and analysis, and a second study was designed to determine optimal storage conditions. Quantitative hybridizations with the same set of probes used in the first study showed that treatment with chloramphenicol proved best in minimizing change over time in wastewater samples. However, storage at room temperature was also a viable storage option. In the third study, molecular methods were used to detect the bacterium *Bacillus* DA33 in augmented wastewater and soil samples. A probe targeting the 16S rRNA of this organism was designed and characterized for use in quantitative membrane hybridizations. Because a unique target sequence was not available within the 16S rRNA, another probe, this time targeting the 16S-23S intergenic spacer region, was designed and characterized. Hybridization results showed that levels of *Bacillus* DA33 were higher in soil than in wastewater. In several bioaugmented soil and wastewater samples, *Bacillus* DA33 had higher levels than in non-bioaugmented samples, but the results were mixed in other cases. Molecular methods allow us to analyze environmental samples in more detail. Integrating these methods in future studies of wastewater treatment and bioaugmentation should provide more information that will help improve design and operation.

Molecular Characterization of Microbial Populations in Full-Scale Activated Sludge Plants and Bioaugmentation Sites

by

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Dedication

This document is dedicated to my parents for their love and support, to Molly Puente for coping with the crazy dog, and of course to Corey Cavalier.

Biography

Julia Keith received her B.S. in Biology from Duke University in May 2000. During her undergraduate career, she was fascinated by biology and environmental science. Upon completing the degree, she decided to apply the knowledge and skills gained to environmental problems. As a graduate student, she worked at the interface between environmental engineering and biology, applying molecular biology techniques to the study of problems in wastewater treatment and bioremediation. Upon completion of the Master of Science degree in Environmental Engineering, she will work in environmental science and engineering.

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Introduction

The purpose of this study is to quantify specific microbial populations in wastewater treatment plants and bioaugmentation sites using oligonucleotide probe hybridizations. Molecular methods, particularly those targeting the ribosomal ribonucleic acid (rRNA), have become important techniques in microbial ecology, and are increasingly being used to investigate engineered systems such as wastewater treatment plants. These techniques allow the identification and quantification of microbial populations, without the limitations of traditional microbiological approaches.

Three separate projects were performed as part of the thesis research. Chapter 1, “Bulking and Foaming in North Carolina Wastewater Treatment Plants and their Relationship to Microbial Community Structure”, describes a survey designed to identify problems in wastewater treatment plants in North Carolina and the analysis of microbial community structure at those plants. Chapter 2, “The Effects of Sample Storage on the Microbial Community Structure in Wastewater Treatment Plants” is an in-depth analysis of samples collected from two of the plants studied in Chapter 1. The project analyzed the effect of sampling procedures on group-specific changes in RNA levels in wastewater samples and possible protocols that could be used to prevent change. Chapter 3, “Detection and Quantification of a *Bacillus* Strain Used for Bioaugmentation”, examines the design and characterization of oligonucleotide hybridization probes targeting *Bacillus sp.* DA33 and their application to soil and activated sludge samples.

Chapter 1, Bulking and foaming in North Carolina wastewater treatment plants and their relationship to microbial community structure

Abstract

A study was conducted to determine the prevalence of filamentous foaming and bulking in wastewater treatment plants in North Carolina, and to relate these problems to microbial community structure. Of the 47 plants surveyed, 88% had experienced foaming. Plants that contained high levels of the alpha-subclass of the Proteobacteria and relatively high levels of mycolata tended to experience the most severe foaming. Bulking was not as common a problem as foaming and affected 63% of the plants surveyed. There was no correlation between bulking and *Sphaerotilus natans* RNA levels, indicating that some other filament or other floc characteristics are responsible for bulking in these plants.

Introduction

Foaming and bulking problems in activated sludge wastewater treatment plants can adversely affect environmental quality and in some cases cause a wastewater treatment plant to violate its permit. Foaming refers to a thick brown scum that forms on the surface of aeration basins and secondary clarifiers. This scum interferes with equipment and can become a health and safety hazard (de los Reyes *et al.*, 1997). Filamentous bulking occurs when filamentous microorganisms push flocs apart, interfering with activated sludge settling (Jenkins *et al.*, 1993). These problems have been connected to the massive growth of specific filamentous microorganisms: foaming has been related to *Gordonia amarae* and "*Microthrix parvicella*" among others (de los Reyes and Raskin, 2002), and bulking can be caused by species including *Sphaerotilus natans* and Eikelboom Type 1851 (Jenkins *et al.*, 1993; de los Reyes *et al.*, 1997).

The objectives of this study were 1) to determine the extent of foaming and bulking, and 2) to perform an initial analysis of the microbial community structures full-scale wastewater treatment plants in North Carolina. This analysis will generate baseline data, and is the first step in forming recommendations to solve microbiologically-based problems such as foaming and bulking.

A two-pronged approach was used to analyze the problems, the microorganisms that cause them, and the conditions that allow the causative organisms to proliferate. The first step in this approach was a written survey that asked wastewater operators questions about problems that occur in their plants. This allowed the identification of plants that have experienced bulking or foaming problems. The second step was collecting activated sludge samples from several plants that have experienced problems, and quantifying the microbial populations in the samples. By relating the levels of specific microorganisms to plant processes, we can ultimately identify the niche of the specific microbial populations. The challenge is to identify the problem-causing microorganisms, quantify their levels, and determine the conditions that promote their growth. Activated sludge systems can then be designed and operated to avoid conditions that lead to excessive filamentous growth.

When applied to wastewater, many traditional methods of identification and quantification give misleading results. Culture techniques are inherently flawed: it has been shown that greater than 99% of microorganisms present in wastewater and other environments cannot be cultivated, and analyses based on cultivation will overlook the vast majority of organisms present (Wagner *et al.*, 1993; Kampfer *et al.*, 1996). Standard light microscopy allows more reliable observation of microorganisms within a sample, but many different bacteria have similar shapes, and it can be very difficult to distinguish between the many rod-shaped or filamentous microorganisms present (Blackall, 1994). The use of molecular methods circumvents these problems. Molecular methods involving nucleic acid analysis allow us to analyze environmental samples without cultivation. Because they are very specific, they also allow us to distinguish between unrelated organisms, even those that are morphologically similar.

These molecular techniques include ribosomal RNA (rRNA) analysis, which can be used to quantify the presence and the activity of different microorganisms within an environmental sample. In this study, we used oligonucleotide probe hybridization techniques targeting rRNA. This molecule, which includes a large subunit and small subunit, is involved in protein synthesis and is therefore present in all organisms. Some sequences within the molecule are highly conserved, while other sequences are variable. Closely related organisms have high sequence similarities in their 16S rRNA. Because of these properties, probes can be designed to target different phylogenetic levels. For example, hybridizations may use a universal probe that targets all organisms, while group-specific probes target specific genera or species. Using these probes for quantitative membrane hybridization then allows a description of the microbial community structure within a sample.

A few studies have attempted to describe microbial community structure using rRNA-targeted probes and correlate that structure to problems in wastewater treatment (Juretschko *et al.*, 2002; de los Reyes *et al.*, in press). This study is only the second to survey a large number of plants, and the only study that has evaluated plants in North Carolina. The state contains an unusually high number of plants that perform biological nutrient removal, and the associated long solids retention times (SRTs) may promote filamentous foaming and bulking (Pitt and Jenkins, 1990; Jenkins *et al.*, 1993).

Materials and Methods

Survey of North Carolina Activated Sludge Plants

A survey was developed to determine the extent of foaming and bulking in North Carolina (Appendix). Approximately 200 surveys were sent to plants around the state; 47 of these were completed and returned. Contact information was obtained from the EPA Envirofacts Warehouse Database (http://www.epa.gov/enviro/index_java.html).

The survey asked plant operators to describe their plants in terms of type of activated sludge process (i.e. conventional activated sludge, oxidation ditch, etc) and the design and operational conditions (hydraulic retention time [HRT], solids retention time [SRT], flow rate, etc). The survey asked wastewater operators their opinions on the cause of bulking or foaming at their plants and the success of control methods applied. Questions on bulking and foaming asked whether plants had experienced these problems and their frequency, and in the case of foaming, operators were also asked to estimate the average foam depth.

Sample Collection

Based on survey results, the several plants with bulking or foaming problems were selected. The descriptions of the wastewater treatment plants are summarized in Table 1.2. Sampling packages consisting of a cooler, ice packs, and 50-mL centrifuge tubes, were sent to these plants. Wastewater operators were asked to collect four grab samples from each plant: one each from the aeration basin, secondary clarifier effluent, return activated sludge line, and from any foam on the surface of the aeration basin. Clarifier effluent samples contained too little biomass to analyze and were used to visually examine effluent quality. Effluent quality was adequate in all cases.

RNA Extraction

RNA was extracted using a modified low pH, hot phenol method. (Stahl *et al.*, 1988; Raskin *et al.*, 1994) Cell pellets were obtained in 2 mL screwcap tubes to which silica zirconium beads (BioSpec, Bartlesville, OK), pH 5.1 buffer (2 mM ethalenediaminetetraacetic acid [EDTA] and 10 mM sodium acetate), and phenol (pH 5.1) were added. The tubes were beaten on a BioSpec mini BeadBeater (BioSpec, Bartlesville, OK) for 2 minutes, incubated at 60°C for ten minutes and then returned to the bead beater for two minutes. The beads were separated from the remaining material by centrifuging at 2300 x g. 200 uL of buffer was added to the beads and the beads were beaten for another minute. Aqueous material was separated from the beads and combined with the mixture of organic and aqueous material. The samples were centrifuged for 10 minutes at 9300 x g, and the aqueous material was transferred to clean tubes. The aqueous material was re-extracted once with phenol, twice with 4:1 phenol: chloroform, and once with chloroform. RNA was precipitated overnight by adding ½ volume of 10 M NH_4Ac and 2 volumes of absolute ethanol to the aqueous material. After overnight storage in a -20°C freezer, the RNA was collected by centrifuging for 30 minutes at 16000 x g and resuspended in 250 uL of sterile water. The quality of extracted RNA was evaluated using poylacrylamide gel electrophoresis and quantified using the Gel-Pro Image Analysis software v. 3.1 (Media Cybernetics, Silver Spring, MD) using *E. coli* 16S rRNA standards (Roche Diagnostics, Indianapolis, IN).

Temperature Dissociation (T_d) Study

The dissociation temperature (T_d) of the probe S-S-S.nat-656-A-a-18 was experimentally determined by performing a T_d study. The T_d, which occurs at 50% probe washoff, was used as the wash temperature in subsequent hybridizations.

Membrane Hybridizations

Quantitative membrane hybridizations were used to determine the rRNA fractions of three major Bacterial groups in wastewater (the Alpha, Beta and Gamma subclasses of the Proteobacteria (Kampfer *et al.*, 1996)). We also tested individual genera and species of filamentous microorganisms that have been connected to foaming and bulking. These include the mycolic-acid containing actinomycetes (Mycolata), *Gordonia*, and *Gordonia amarae*, which have been shown to cause foaming in wastewater treatment plants (de los Reyes *et al.*, 1997), and the filament *Sphaerotilus natans* which can cause bulking (Wagner *et al.*, 1994d). Probe names, sequences, target groups, and was temperatures are shown in Table 1.1. RNA at an initial concentration of 100 ng/uL was diluted 1:3 in 2% glutaraldehyde and denatured for 10 minutes at room temperature. Samples were then diluted in sterile water containing RNase free bromophenol blue to a final concentration of 1 ng/uL. Standards were denatured in the same manner, but a dilution series was prepared following denaturation. Concentrations of the dilution series ranged from 0.025-1.28 ng/uL. Samples and standards were applied in triplicate to a MagnaCharge nylon membrane (Osmonics; Minnetonka, MN).

Oligonucleotide probes were obtained from Sigma Genosys (The Woodlands, TX), labeled with ^{32}P, and membranes were prehybridized for 30 minutes to 2 hours in 18 mL PerfectHyb Plus hybridization buffer (Sigma-Aldrich Chemicals; St. Louis, MO). The probe labeling reaction was performed using 1-5 µL probe, 1-3 µL ^{32}P-γATP (ICN Biomedicals; Costa Mesa, CA), 1 µL T_4 polynucleotide kinase (Promega Corp.; Madison, WI), 3 µL 10x polynucleotide kinase buffer (Promega Corp.; Madison, WI), 1.5 µL 1% Igepal type NP40 (Sigma-Aldrich Chemicals; St. Louis, MO) and water to a final volume of 30 µL. The reaction proceeded for 30-45 minutes at 37^{o}C. Labeled probe was purified using mini-Quick Spin Oligo Columns (Roche Diagnostics Corp.; Indianapolis, IN), following the manufacturer's instructions. Purified probe was added to membranes, and hybridized overnight at 40^{o}C. Membranes were then washed twice for 30 minutes in 100 mL wash buffer (1% sodium citrate, 1% sodium dodecyl sulfate) at 40^{o}C, and washed once for 30 minutes in 350 mL wash buffer at the probe-specific wash temperature. The membranes were dried and exposed for 1-3 days on a Phosphorscreen. The screen was scanned on a Phosphorimager (Amersham Biosciences; Piscataway, NJ) and the resulting image was analyzed using the ImageQuant software package (Amersham Biosciences; Piscataway, NJ).

Each set of membranes was tested against a group-specific probe as well as Bacterial and Universal probes. The standards series on each membrane was analyzed and linear regression was used to determine the relationship between signal intensity and ng RNA. This relationship was used to determine the ng of group, Bacterial and total rRNA for each sample. RNA quantities of group specific probes were then normalized using the formula:

$$\% \text{ RNA} = [\text{ ng group RNA} / (\text{ng Bacterial or Universal})] * 100\%.$$

Bacterial RNA values were occasionally inconsistent, so all analysis was performed on a basis of total (Universal probe) values. RNA of standards and selected samples were examined on a non-denaturing polyacrylamide gel.

Statistical analysis

Possible correlations between RNA levels within the samples to bulking or foaming were compared using a z-correlation test. The data was analyzed in the StatView software package (SAS Institute; Cary, NC).

Results and Discussion

Survey Results

The average SRT of the activated sludge processes reported by survey respondents was 22 days. This number is high when compared to those found in other studies. This is unsurprising, as many plants in North Carolina maintain high solids retention times (SRT), which have been connected to foaming (Pitt and Jenkins, 1990). Although only 9 of the plants surveyed indicated that they performed biological nutrient removal, several plant operators expressed plans to upgrade. Six of the plants sampled performed biological nutrient removal. Survey results for the plants sampled are shown in Figure 1.2.

Of the survey respondents, 88% and 63% reported having experienced episodes of foaming and bulking, respectively. Table 1.3 shows how these results compare to those obtained in other studies. The levels of foaming and bulking observed in this study are high relative to the European studies and comparable to those observed in the Australian study. Various studies have observed a higher frequency of foaming and bulking in plants that perform biological nutrient removal (BNR) than in other wastewater treatment plants (Krhutkova *et al.*, 2002). Several researchers believe that this can be attributed to the higher solids retention times used in biological nutrient removal plants (refs). In this study, BNR plants had higher average foam depths (6.3 inches) than those that did not perform BNR (3.6 inches), while both groups had similar foaming frequencies (3.6 vs. 4.0 out of 8). In this study, BNR plants had an average SRT of 361 hours, while non-BNR plants had an average SRT of 510 hours. The higher average foam depths in BNR vs. non-BNR plants is probably related to some factor other than SRT.

The frequency and severity of foaming at the different plants sampled are shown in Table 1. Most survey respondents that reported bulking observed it infrequently, and foaming was more common. Frequency of foaming and bulking were rated on a scale of 1 = never to 8 = continually. On this scale, the average frequency of foaming was 4 and the average frequency of bulking was only 2.

Table 1 shows that three plants sampled were bulking, and almost all were foaming at the time of sampling. The thickness of foaming (in inches) was used as a measure of the severity of foam. The severity of bulking was measured using the sludge volume index (SVI). SVI is calculated based on the formula:

$$\mathrm{SVI} = \frac{\mathrm{SV}_{30}\ (\mathrm{mL} / \mathrm{L})}{\mathrm{SS}\ (\mathrm{g} / \mathrm{L})}$$

Where: SV30 is the volume of sludge obtained by allowing 1 L of wastewater to settle for 30 minutes;and SS is the suspended solids concentration (Jenkins *et al.*, 1993).

An SVI of 150 or greater indicates that bulking is occurring. Samples 1, 2, 5 and 11 (Raleigh, St. Paul's, North Cary, and Blowing Rock) were bulking. The Raleigh and Cary plants had SVI values just over 150, but the St. Paul's plant had an SVI of over 2000. Even if this value resulted from a misplaced decimal point, and the actual SVI was approximately 200, the plant still has a bulking problem. The plant operator indicated that bulking does not affect operation because the plant functioned with excess clarifier capacity.

All but one of the plants sampled had experienced foaming. Foam occurred relatively frequently at most of the plants, and several plants had foam at least two inches thick. Foaming was a very serious problem at the Greenville Utilities Commission WWTP, with an average depth of 27 inches. The North Cary plant presents an interesting case: the plant operator insisted that foaming never occurred, but others' observations indicated that foaming was always present, with a typical depth of at least 5 inches. Therefore we considered this plant to have foaming frequency of 8.

When analyzing the data, it is important to be cognizant of the subjective nature of the survey and the fact that plant operators were the primary source of information. Operators' estimations of the severity of problems may be affected by their personal experience. In plants near capacity, foam can spill onto walkways and create a safety hazard but in a plant well below capacity, the same amount of foam may not interfere with operation. The operator of the first plant would be very likely to report it, but the operator of the second might deemphasize foaming. In most cases, the researchers cannot determine the accuracy of values written on the survey. One questionable value was the SVI of over 2000 obtained from the Saint Paul's plant. The value written down on the survey may be correct, or it may involve a misplaced decimal point. The researchers felt that the additional data obtainable through surveys far outweighs the error introduced by the occasional error in responses.

The personal opinion of wastewater operators can also be important in understanding problems like foaming and bulking. Operators view the plant constantly, and are personally familiar with any problems that occur in the treatment process. As part of the survey, we asked operators to list what they perceived as the causes of foaming and bulking and the most effective techniques to control these problems. Causes and controls of foaming are reported in Table 1.4, and those of bulking are reported in Table 1.5. The most common cause cited for foaming was temperature change in fall or spring. Other commonly listed causes were industry discharge and surfactants. The most effective control mechanisms for foaming were to increase wasting and decrease aeration. Fewer plants speculated about the causes of bulking; some of those reported included hydraulic overload, low F/M ratio, and old sludge. One operator's bulking control method was an increase in MLSS, while another found a decrease in MLSS effective. The causes of bulking may be plant-specific, but there is not enough data to draw definite conclusions.

To determine what operational conditions lead to foaming and bulking, it was beneficial to analyze the commonalities between plants that experienced these problems but also how these plants differed from those that did not observe foaming or bulking. Z-tests for correlation were used to compare depth of foaming and SVI to solids retention time. The relationship was not statistically significant, but when depth of foaming was grouped about appropriate values, it was found that plants with foam up to 1" deep had average SRTs of 407 hrs. Plants with foam greater than 1" deep and up to 6" deep had average SRTs of 470 hours, and plants with foam greater than 6" deep had average SRTs of 519 hours. Non-bulking sludges had average SRTs of 424 hours, while bulking sludges had average SRTs of 496 hours.

Based on operators' suggestions for causes of bulking and foaming, data were analyzed to determine whether foaming or bulking sludges had higher levels of industrial waste or surfactant inflow. There was no significant difference between foaming and non-foaming or bulking and non-bulking sludges. Sludges that did not suffer filamentous problems had as much industrial inflow as sludges that did suffer foaming and bulking.

Microbial Community Structure

Before hybridization, selected RNA of standards and samples was analyzed on an acrylamide gel (data not shown). Clear bands showed for the 16S and 23S regions of RNA. No obvious degradation was present in samples and standards tested.

Figures 1.1-1.3 show the levels of alpha, beta, and gamma Proteobacteria in collected samples. All microbial groups were compared to SVI using a Z-correlation test. There were no correlations between microbial group and SVI. For each plant tested, the levels of a particular group RNA are approximately the same in the RAS line as in the aeration basin, with a few exceptions. At plant 4, levels of beta and gamma Proteobacteria rRNA are higher in the RAS line than in the aeration basin. At plant 16, levels of beta Proteobacteria are significantly higher in the RAS line than in the aeration basin. Plants 9 and 11 show higher rRNA levels of alpha Proteobacteria in the RAS line than in the aeration basin. Plant 10 shows higher rRNA levels of beta Proteobacteria in the aeration basin than in the RAS line.

The alpha-subgroup of the Proteobacteria is clearly the dominant phylogenetic group at plants 1, 2, 5, 6, 7 and 8. With the exception of plant 2, foaming tended to be a more severe problem at these plants than at other plants. Figure 1.4 compares foam thickness to levels of alpha Proteobacteria found in the samples. Although there appears to be some relationship between high levels of alpha Proteobacteria and higher values of foam thickness, linear regression was not a good fit for the data. Z-tests for correlation significance were used to compare levels of alpha Proteobacteria to foam thickness in all samples tested and in the subset of samples where alpha Proteobacteria was dominant, but the relationship was not statistically significant in either case. Beta Proteobacteria, which has been the dominant phylogenetic group in many previous studies on wastewater, (de los Reyes, 2002; Juretschko *et al.*, 2002; Wagner *et al.*, 2002) was dominant only at plant 10 in the current study. This may be because of climate differences, and the relatively high SRTs in North Carolina wastewater treatment plants. Figure 1.5 compares the levels of beta Proteobacteria to foam thickness. As with the alpha Proteobacteria, there appears to be some increase in foam height with increasing levels of this phylogenetic group. This trend appears to be clearer than that seen in the alpha Proteobacteria, but is also not statistically significant. There was no relationship between levels of gamma Proteobacteria and foam height (data not shown).

Samples from 14 of the plants were analyzed for Mycolata, *Gordonia*, and *Gordonia amarae*. Mycolata results are shown in Figure 1.6, but due to hybridization errors the *Gordonia* and *G. amarae* results are not shown.

The mycolata is a bacterial group that contains many filamentous microorganisms. *Gordonia* (formerly *Nocardia*) *amarae* is one of the best studied of these species, but other members of the Mycolata group can cause foaming. Levels of mycolata RNA are typically higher in the foam samples than in other samples taken. Foaming members of this bacterial group can partition to foam and in some cases can remain even if their levels decline in the MLSS (de los Reyes *et al.*, 1997). Figure 1.7 compares foam height to levels of mycolata in the samples, and there was no clear relationship between the two. Z-tests compared foam height to levels of mycolata in all samples and in the subset of sample with elevated mycolata levels in foam, but failed to show any correlations. This indicates that mycolata is not causing foam in all cases, but the cases discussed below indicate that mycolata may be one factor that contributes to foaming. The apparent relationships between alpha and beta Proteobacteria and foaming was discussed earlier; perhaps this indicates that mycolata and some member(s) of the alpha or beta Proteobacteria are both contributors to foaming in particular wastewater treatment plants.

Plants 4 and 5 are likely candidates for mycolata foaming. In the foam sample from plant, the Mycolata contribute 14% of total rRNA while they contribute less than 1% of total rRNA in the mixed liquor sample from the same plant. The foam at this plant was estimated to be 5 inches thick. Although it does not cause problems in plant operation, the researchers have observed that it appears on the aeration basin almost continually. Plant 4 experiences very severe foaming, ranging from 18-36 inches deep. This plant has elevated levels of mycolata in the foam sample, and mycolata levels in the MLSS are also higher than average. It is likely that the mycolata contribute significantly to foaming at these plants.

Levels of *G. amarae* and *Gordonia* in the samples were extremely low (data not shown). This indicates that members of the Mycolata other than *Gordonia* are present, and that non-*Gordonia* mycolata may cause foam.

To examine bulking, we compared levels of microbial groups in the samples to sludge volume index (SVI). There was no relationship between alpha, beta, or gamma Proteobacteria and sludge volume index. Assays were also performed on *Sphaerotilus natans*. This filament contributes to bulking in wastewater treatment plants and lab-scale reactors and is probably the best researched bulking filament. Levels of *S. natans* in the samples are shown in Figure 1.8. Levels of *S. natans* range from 2.4 % to 13.1 % of total RNA, as determined using the Universal probe. Figure 1.9 compares SVI to levels of *S.*

natans in the samples. There does not appear to be a correlation between the presence of this species and bulking in the wastewater treatment plants. SVI and levels of the filament were compared using Z-correlation tests. There was no significant correlation between SVI and *S. natans* either when all samples or when the subset of samples that were bulking were analyzed. Resarch has also shown that *Sphaerotilus natans* does not grow as well in environmental samples as in lab reactors; and that it may not be the most common bulking-causing filament in wastewater treatment plants ((Blackbeard *et al.*, 1986)). This may indicate that a different filamentous bacterium might be causing the bulking. In a recent study, our laboratory group has determined that EikleboomType 1851 was more prevalent in several plants (unpublished data).

Relationship of observed microbial community structure to average conditions

In this study, we collected only one set of samples from each of the plants. While this was the only feasible way to collect samples from many different plants, many of the responses to survey questions gave average values of operational parameters (SVI, MLSS) or problems (bulking, depth of foam). The microbial community structure in individual samples may not represent the average microbial community structure for the plant, and comparing it to the average values obtained through the survey may make it more difficult to demonstrate statistically significant trends in the data. For example: 63% of surveyed plants reported experiencing bulking, but only 4 of the 16 (25%) plants sampled were bulking at the time the sample was collected. This may indicate that there were fewer bulking filaments than average in collected sanples and our results could be an underestimate of the total amount of bulking filaments present. This does not invalidate the research, but should be kept in mind when performing analysis.

Conclusions

Foaming appears to be a more severe problem in North Carolina wastewater treatment plants than in studies conducted in Europe. Although more than half of plants observed bulking, its occurrence was generally infrequent; the average frequency value of bulking was 2 out of a possible 8. There was no correlation between bulking and the levels of *S. natans*. Foaming was a widespread problem, occurring in a majority of wastewater plants surveyed. 71% of plants reported at least moderate foaming, and 17% of plants reported heavy foaming. In most cases, those plants that observed more severe foaming had high levels of Mycolata or Alpha Proteobacteria.

This project is a preliminary analysis of bulking and foaming in North Carolina wastewater treatment plants. More analyses need to be performed on the samples to draw more definite conclusions. Because the usual suspects (i.e. *Gordonia amarae* and *Sphaerotilus natans*) did not seem to be causing foaming or bulking in all of the plants, it could prove worthwhile to probe for other filaments within the samples. Probing for several different filaments should help us to determine which filaments are present in the samples and also which filaments are the most important causes of foaming and bulking. It is also important to remember that the experiments described in this section were performed on grab samples. Conditions at the time of sampling may not reflect average severity of bulking and foaming at every plant tested. A very important area of future research is the collection of many samples over a long period of time. By analyzing many samples taken from a few wastewater treatment plants over several months or years, we can better understand the relationship between filament levels and operating conditions at those plants. By combining these in-depth analyses with survey data, we can extrapolate to other survey respondents.

The relatively high foam depths in BNR plants also bears further study. BNR plants are often connected to foaming and bulking because the associated long solids retention times are thought to promote growth of filaments. Surprisingly, although North Carolina BNR plants suffered greater foaming than non-BNR plants, the non-BNR plants had far higher average SRTs. This indicates that other operational conditions are more likely to lead to foaming. One possibility would be lower oxygen levels, which favor the growth of various filament types (Jenkins, 1992), in plants that perform biological nitrogen or phosphorous removal vs. continuously aerated plants. Further research in this area should help us better understand filamentous growth in the North Carolina plants.

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Table 1.1. Probes and standards used for each group of Bacteria.

Target group	**Probes used**	**Probe sequence**	**Standard**	**Wash Temperature (T_d), °C**	**Reference**
α Proteobacteria	S-Sc-aProt-0019-a-A-17[c]	CGTTCG C/T TCTGAGCCAG	*Gluconacetobacter hansenii*	53	(Manz *et al.*, 1992)
β Proteobacteria	L-Sc-bProt-0042-a-A-17[d]	GCCTTCCCACTTCGTT	*Sphaerotilus natans*	58	(Manz *et al.*, 1992)
γ Proteobacteria	L-Sc-gProt-0042-a-A-17[d]	GCCTTCCCACATGGTTT	*Leucothrix mucor*	58	(Manz *et al.*, 1992)
Mycolata	S-*-Myb-0736-b-A-22	CAGCGTCAGTTACT (Nit 1) CCCACAC	*Gordonia amarae*	51	(de los Reyes *et al.*, 1997)
Sphaerotilus natans	S-Sp-S.nat-656-a-A-18	CTGCCGTACTCTAGTTAT	*Sphaerotilus natans*		(Wagner *et al.*, 1994a)

Table 1.2. Survey Results for the Plants Sampled.

Sample Number	Town	Plant Name	Flow Rate (10^6 L/day)	SRT (hrs)	F/M	SVI at time of sampling	Frequency of foaming	Foam Thickness (in)
1	Raleigh	Neuse River WWTP	227.1	240-360	n/r**	157 *	6	2.5
2	St. Paul's	Town of St. Paul's WWTP	1.9	n/r	0.003	2414 *	7	0.5
3	Cramerton	Eagle Road WWTP	15.1	> 720	n/r	120	7	0.5
4	Greenville	Greenville Utilities Commission WWTP	66.2	264-480	0.1/.06	96	4	27
5	North Cary	North Cary WRF	37.8	696	0.07	150 *	Reported = 1, Observed = 8	5
6	Boone	Town of Boone WWTP	18.2	288	0.15	n/r	5	4
7	Monroe	Town of Monroe WWTP	34.1	396	< 0.1	14	6	6
8	Gastonia	Long Creek WWTP	60.6	408	.08	95	4	7
9	Lenoir	Lower Creek WWTP	22.7	sum.: 360 win.: 480	s: 0.12 w: 0.09	135	3	< 1
10	Hudson	Gunpowder Creek WWTP	7.6	s: 336 w: 672	0.085	124	4	1.5
11	Blowing Rock	Blowing Rock WWTP	3.0	n/r	n/r	275 *	5	n/r
12	Charlotte	Mallard Creek WWTP	30.3	240	n/r	117	8	4
13	Charlotte	McAlpine Creek WWTP	n/r	n/r	n/r	n/r	No surveys	
14	Charlotte	McDowell Creek WWTP	22.7	n/r	n/r	92	No surveys	
15	Charlotte	Sugar Creek WWTP	50.9	13.9	.14	96	4	1
16	Charlotte	Irwin Creek WWTP	43.3	11.47	n/r	82	No surveys	

* Plant was bulking.

** n/r: Value was not reported in the survey

Table 1.3. Percentage of plants in different studies reporting foaming and bulking (when reported).

	Reference:	No. of plants surveyed	% of plants reporting foaming	% of plants reporting bulking
North Carolina	This study	47	88	63
Australia, 1990	(Blackall *et al.*, 1991)	46	92	60
Australia, 1994	(Seviour *et al.*, 1994)	65	68	82
California	(Pitt and Jenkins, 1990)	134	66	n/r*
Czech Rep., 1996	(Wanner *et al.*, 2000)	78	28	n/r
Czech Rep., 1997	(Wanner *et al.*, 2000)	72	46	n/r
Denmark	(Wanner, 1994)		50	
Italy	(Rossetti *et al.*, 1994)	39	50	49
Illinois	(de los Reyes, 2002)	33	97	
Massachusetts	(Schwitzenbaum *et al.*, 1992)	50	n/r	60
Netherlands	(Eikelboom, 1994)	70	48	70
South Africa	(Blackbeard *et al.*, 1986)	111	40	32

*n/r indicates that the data were not reported

Table 1.4. Most common causes of and methods used to control foaming, as reported by survey respondents.

Most common causes of foaming	**Number of plants reporting each cause**	**Most common foaming controls (most to least effective)**	**Average effectiveness? (1 = most effective to 6 = ineffective)**
Seasonal change	11*	1. Increase F/M or MLSS	2.5**
Oil and grease	8	2. Decrease RAS	2**
Industry discharges	7	3. Increase wasting	2.2
Imbalance F/M	5	4. Decrease aeration	2.3
Surfactants/detergents	5	5. Chlorinate	3.2
Old sludge	3	6. Water spray	3.4
Dead bugs	1	7. Reduce SRT	3.6
Very young sludge	1	8. Remove manually	4

*For 4 respondents, foaming increased in spring-summer. For 4, foaming increased in both spring and fall. The remaining 3 plants reported seasonal change but did not specify when it occurred.

**Few plants reported these control methods, and the average effectiveness reported cannot be considered significant.

Table 1.5. Most common causes of and methods used to control bulking, as reported by survey respondents.

Most common causes of bulking	**Number of plants reporting each cause**	**Most common bulking controls (most to least effective)**	**Average effectiveness? (1=most effective to 6=ineffective)***
Hydraulic overload	2	1. Decrease F/M or MLSS	1
Low F/M	2	2. Increase MLSS	1.5
Low DO (due to industrial waste)	1	3. Increase wasting	2
Oil and grease	1	4. Decrease aeration	2
Very young sludge	1	5. Reduce SRT	2.5
Low return and low wasting	1	6. Chlorinate	3
Old sludge	1	7. Increase aeration	4.25
Denitrification	1		

*Because few plants reported their methods used to control bulking, the average effectiveness reported cannot be considered significant.

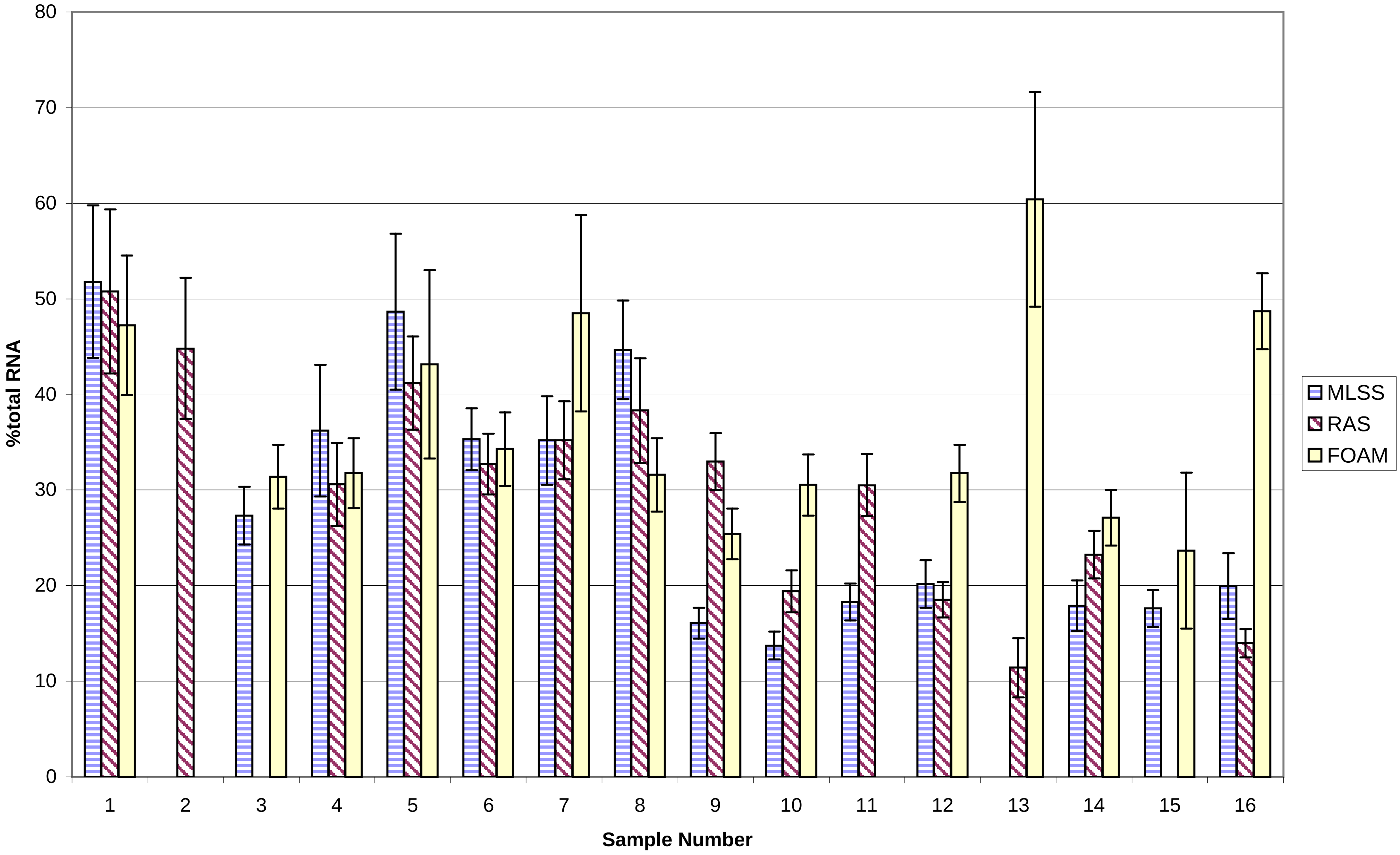

Figure 1.1. Levels of Alpha Proteobacteria in North Carolina Wastewater Treament Plants

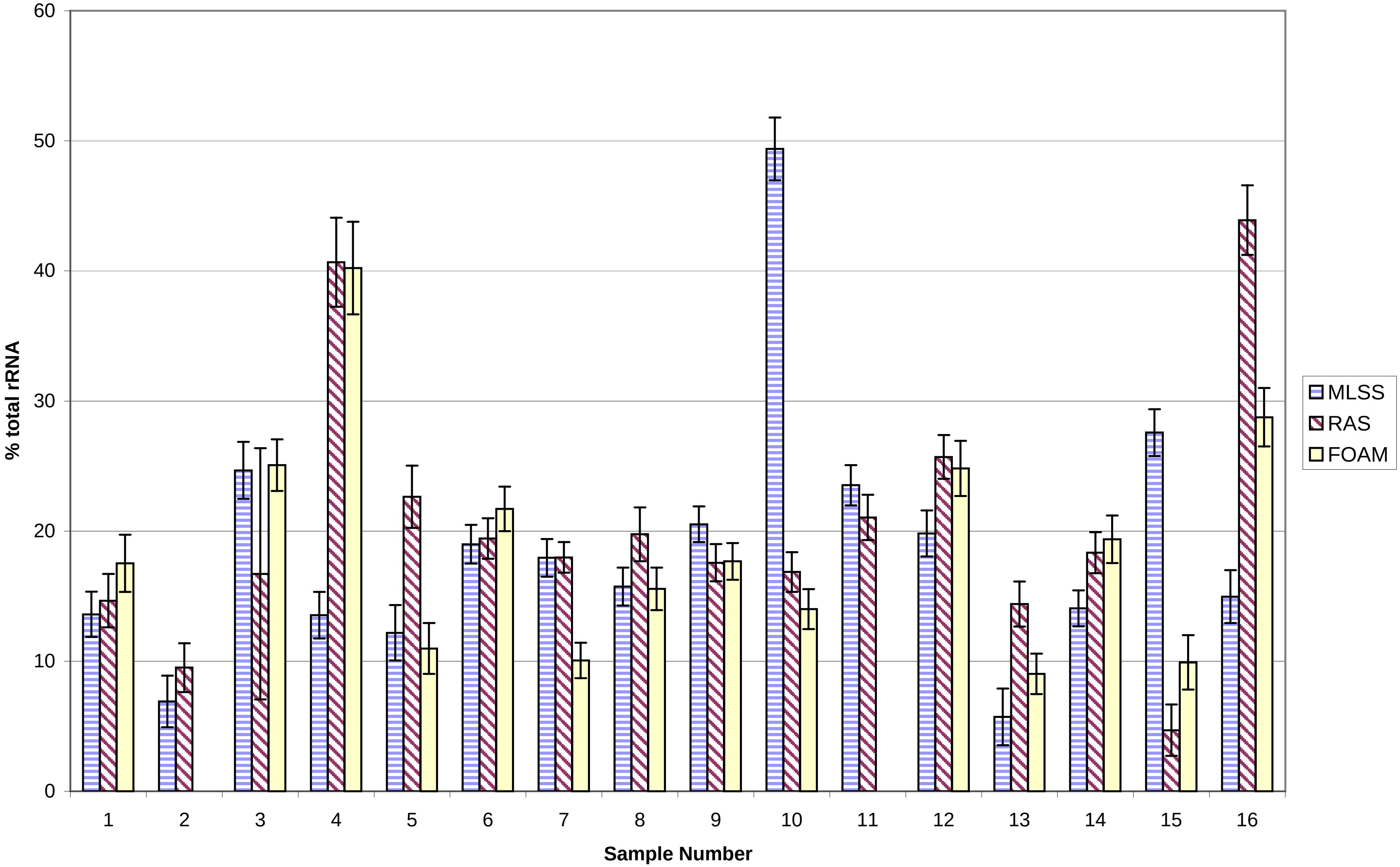

Figure 1.2. Levels of Beta Proteobacteria in North Carolina Wastewater Treatment Plants

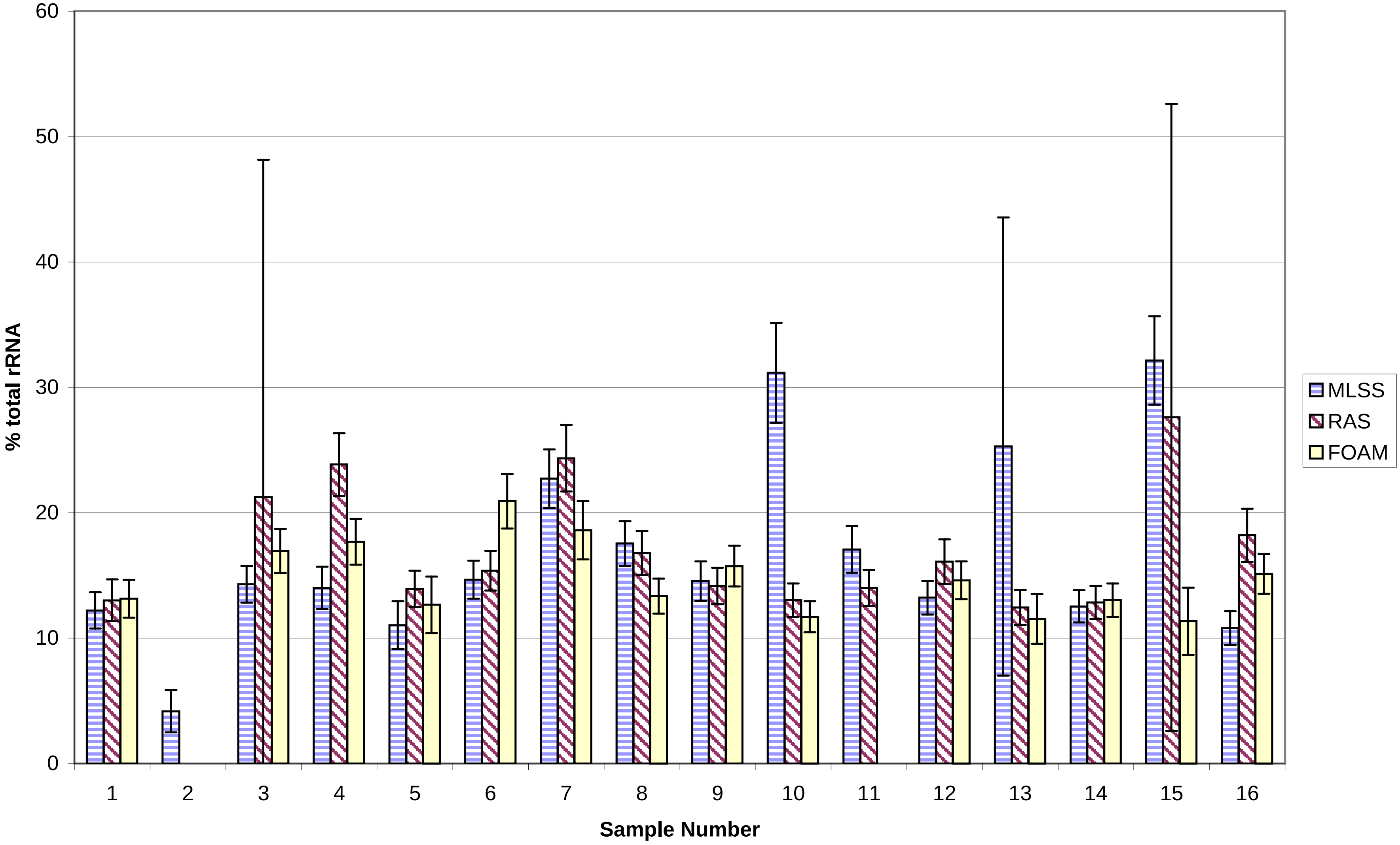

Figure 1.3 Levels of Gamma Proteobacteria in North Carolina Wastewater Treatment Plants

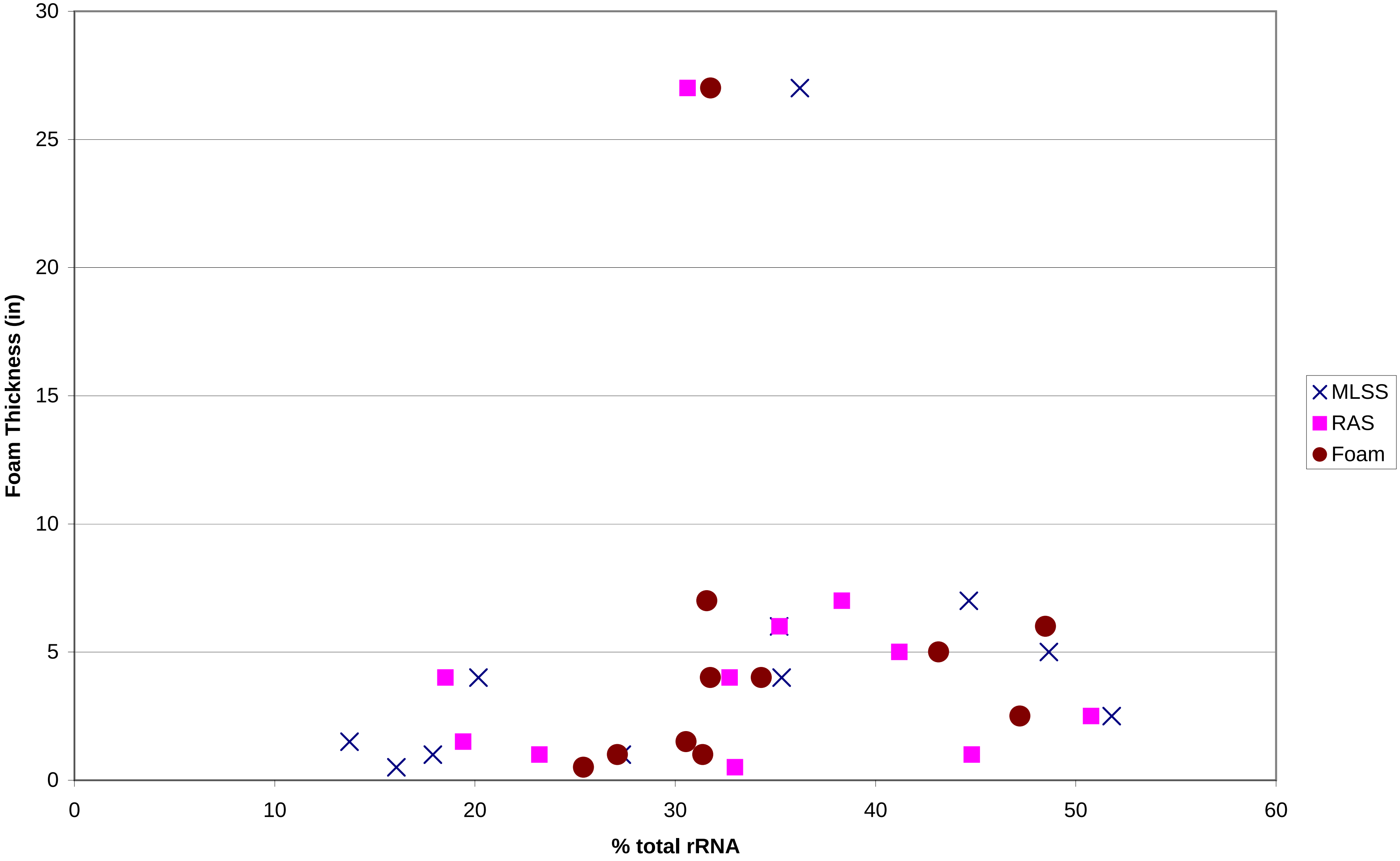

Figure 1.4. Comparison of Foam Thickness to Levels of Alpha Proteobacteria

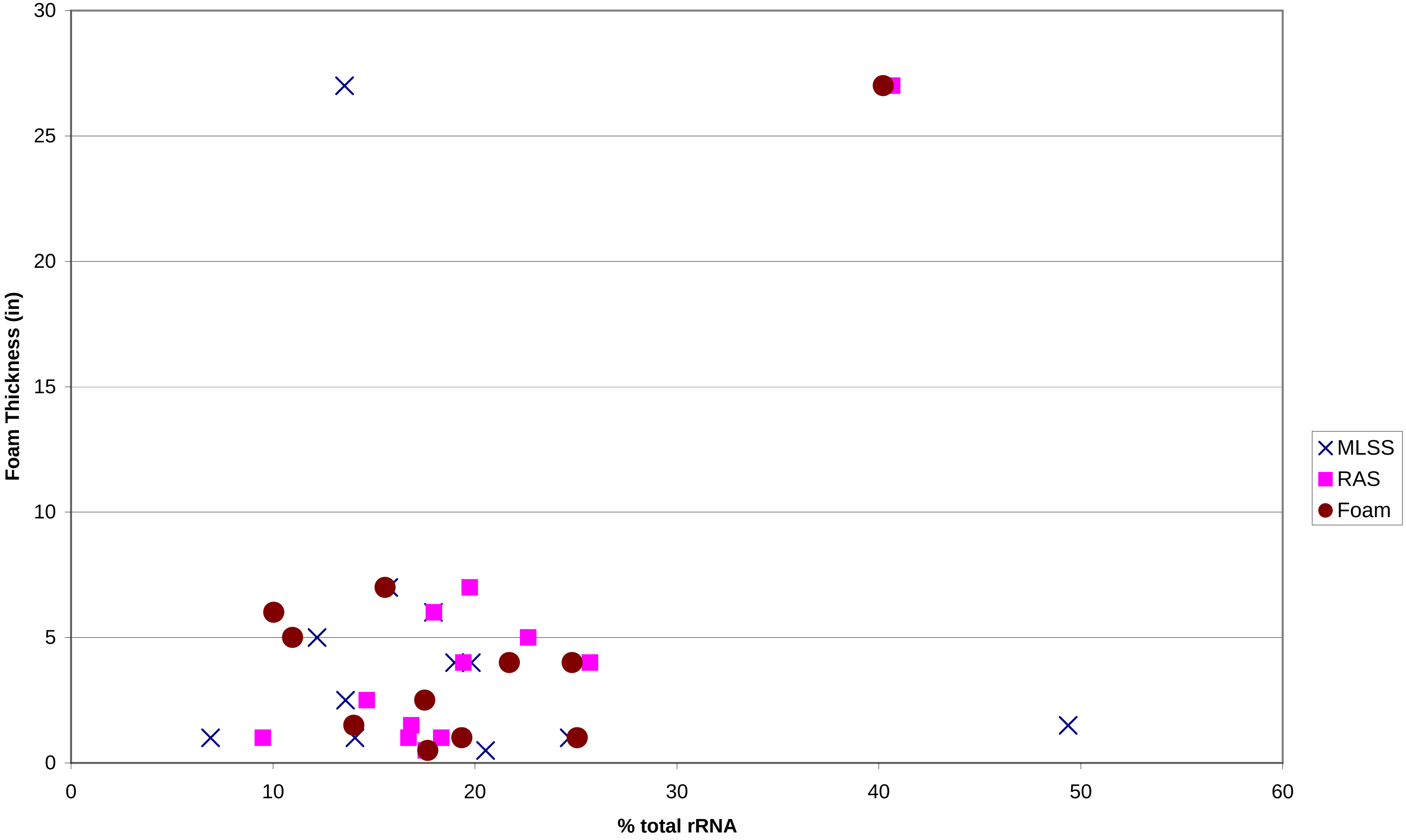

Figure 1.5. Comparison of Foam Thickness to Levels of Beta Proteobacteria in Collected Sample

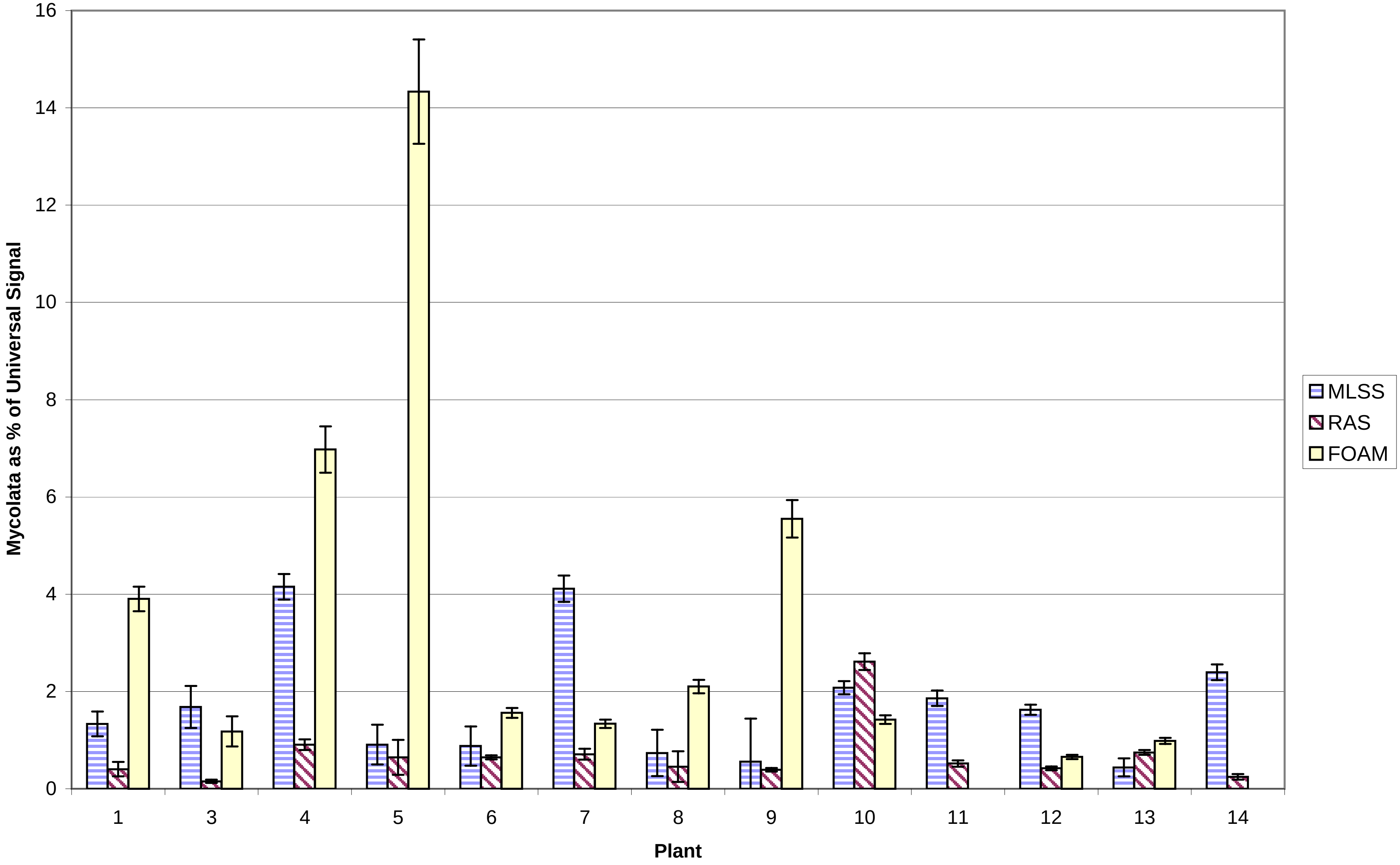

Figure 1.6. Levels of Mycolata in North Carolina Wastewater Treament Plants

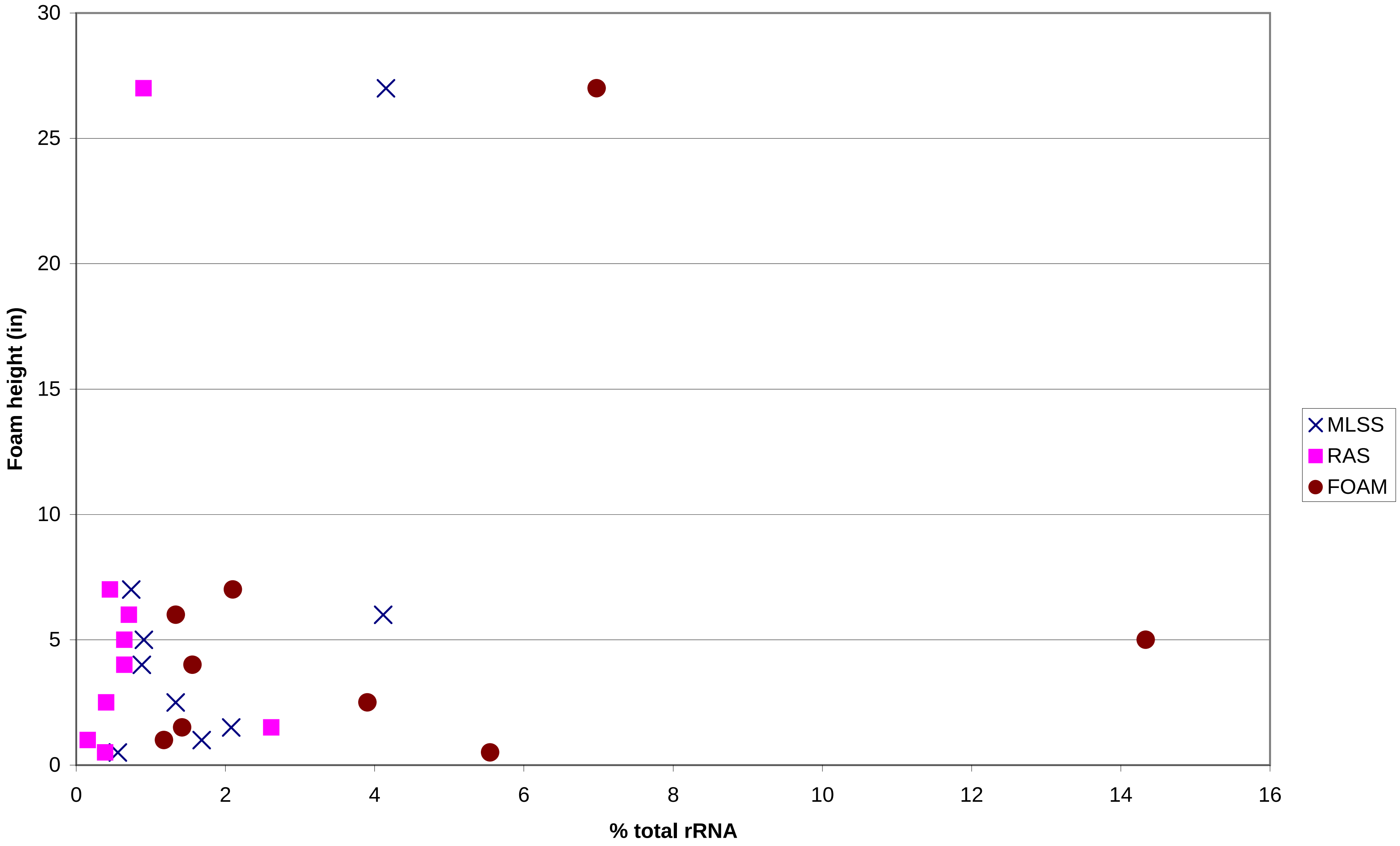

Figure1.7. Relationship Between Foam Height and Levels of Mycolata rRNA in the Samples.

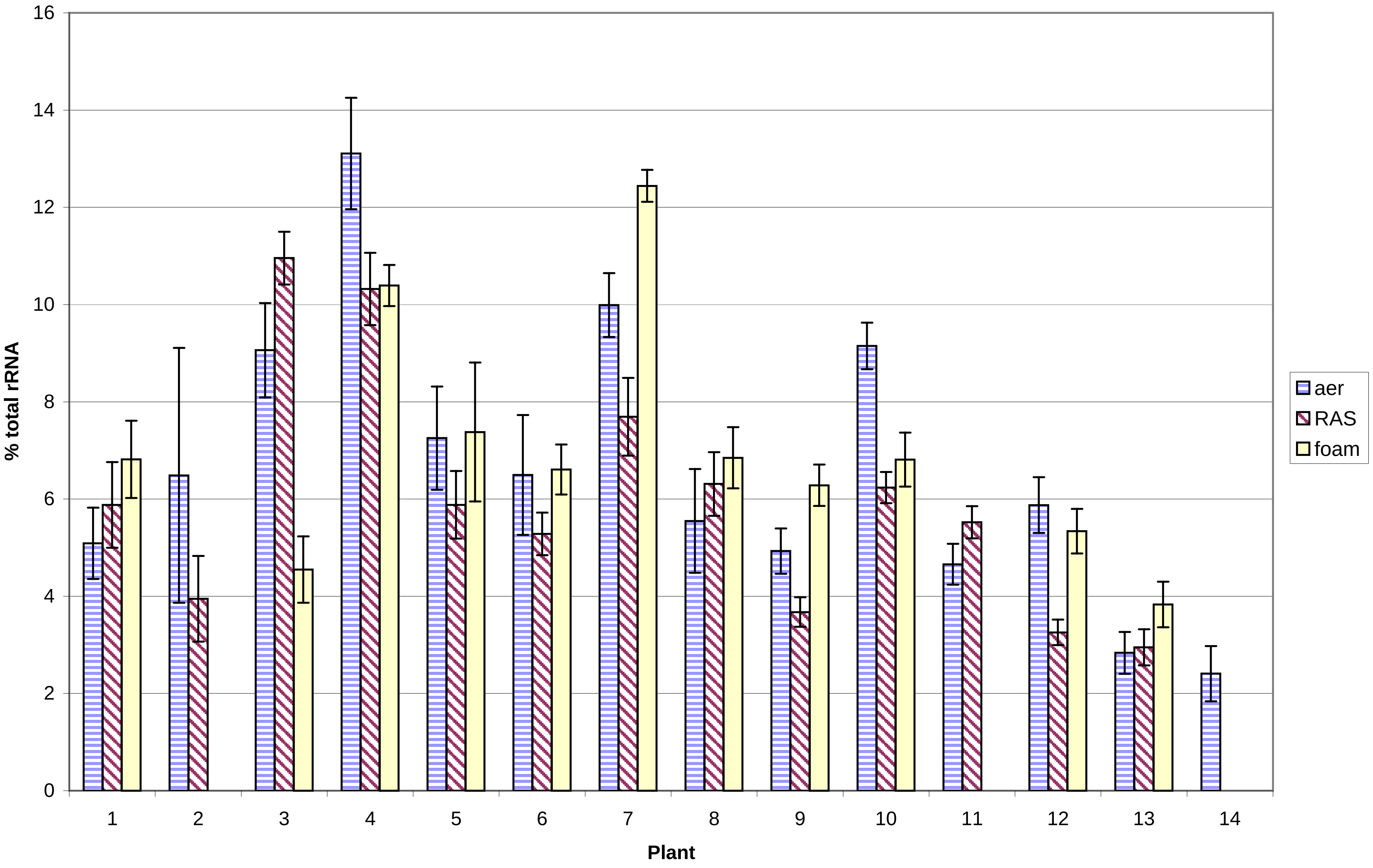

Figure 1.8. Levels of *Sphaerotilus natans* in North Carolina Wastewater Treament Plants

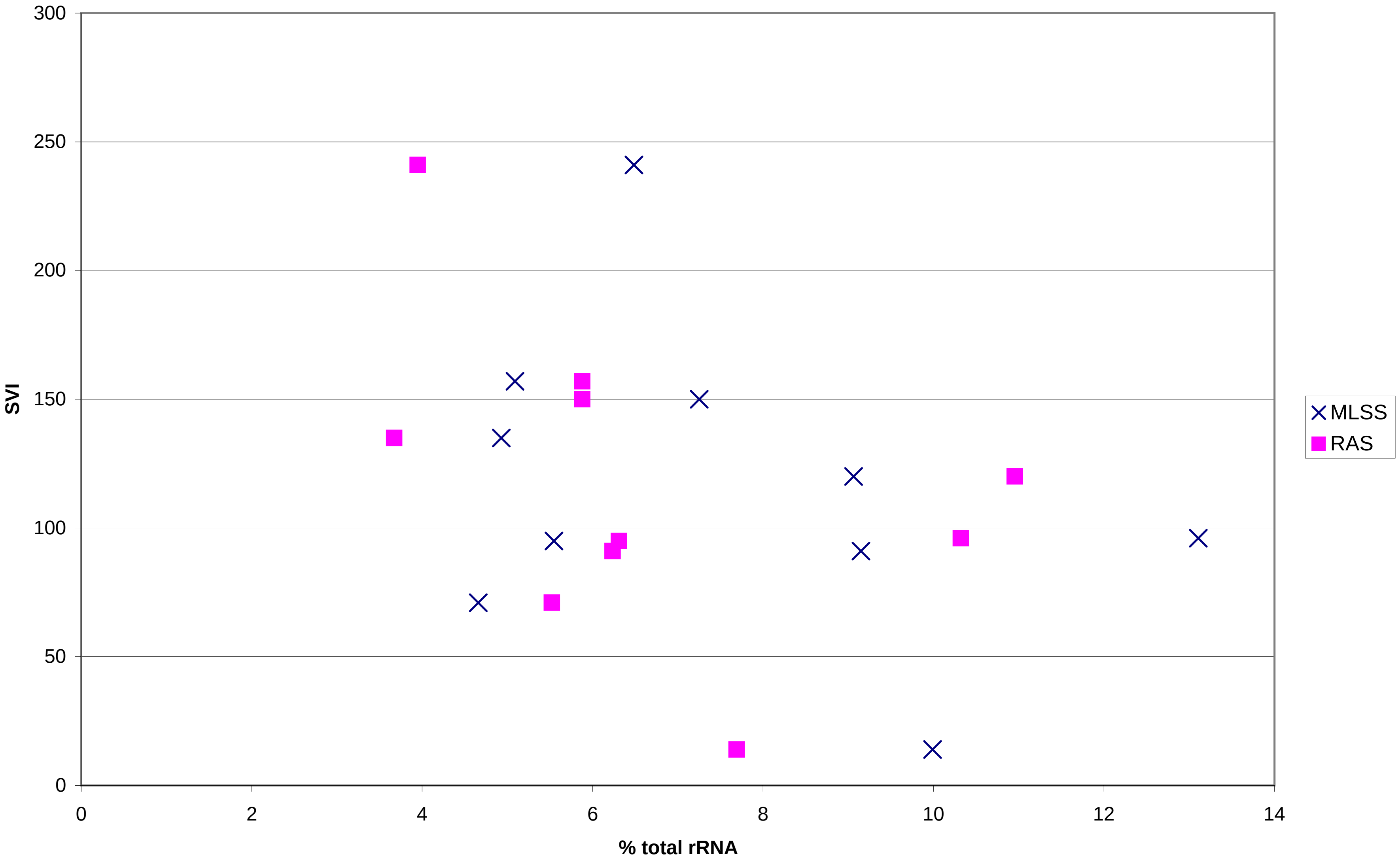

Figure 1.9. Relationship of SVI to levels of *Sphaerotilus natans* in samples

Chapter 2, Effects of sample storage on the microbial community structure in wastewater treatment plant samples

Abstract

Oligonucleotide probe hybridizations targeting rRNA are sensitive tools for the analysis of microbial community structure in wastewater treatment plant samples. This sensitivity raises concerns for many researchers, because when collecting environmental samples it is often not possible to analyze the samples immediately. If RNA levels change in response to storage conditions, quantitative membrane hybridizations may not accurately reflect *in situ* levels of microbial populations. We analyzed the effect of different sample storage conditions on the relative RNA levels of major Bacterial groups in wastewater. Results showed that RNA levels in samples treated with chloramphenicol will remain approximately constant for at least 48 hours, while RNA levels in samples stored at room temperature will remain constant for at least 24 hours. Either might be a viable storage option for sample collection, but storage at room temperature is more practical.

Introduction

Engineers and operators of wastewater treatment plants are interested in identifying and quantifying microorganisms in bioreactors to promote the growth of desired organisms (e.g., phosphate accumulating organisms and nitrifiers) and to control the growth of problem organisms, (e.g., filaments that promote bulking or foaming). Molecular techniques have been used since the early 1990's to identify and quantify microorganisms in wastewater treatment systems. Using ribosomal ribonucleic acid (rRNA) sequence analysis, engineers and operators can begin to optimize wastewater treatment plant design and operation to favor microbial communities that effectively treat wastewater while avoiding the occurrence of problems.

Membrane hybridizations (also known as Northern blots or hybridization after extraction) are one method of rRNA analysis. Ribosomal RNA samples and standards series are blotted onto a membrane in triplicate, and then labeled probe is added to the membrane. The probe binds to the RNA of interest, and the levels of RNA in the sample can be determined through quantification of the resulting signal. This method is powerful because it is quantitative and allows analysis of many samples simultaneously. Statistical analysis on the data shows that the results of this method are more quantitative and precise than those obtained with many of the PCR-based methods common in environmental microbiology (Reue, 1998).

When collecting samples for RNA- or DNA-based molecular characterization, immediate analysis or immediate freezing for later analysis should show the microbial community structure at the time of collection. When samples are not analyzed immediately, changes in dissolved oxygen levels, biochemical oxygen demand (BOD), temperature and other factors may begin to affect the microbial community structure. RNA levels may shift in response to these changes, and thus affect the results of molecular biology analyses.

A variety of studies have examined the stability of RNA in animal tissues (Barton *et al.*, 1993; Leonard *et al.*, 1993; Marchuk *et al.*, 1998; Fitzpatrick *et al.*, 2002). These studies have found that the stability of rRNA varies depending on the tissue of origin. In one study, rRNA extracted from bovine reproductive tissues degraded between 24 and 96 hours (Fitzpatrick *et al.*, 2002). In another study, rRNA extracted from connective tissue remained stable but rRNA extracted from the liver was degraded in a sample stored for 48 hours. The authors believed that greater rRNA degradation in the liver was related to the relatively high levels of RNases present in that organ (Marchuk *et al.*, 1998). Although these studies concluded that RNA degradation did not present significant problems for further molecular analyses, this may not be the case for wastewater samples, where Bacteria form a large fraction of the biomass. Because bacterial cells contain approximately twice as much RNAse as eukaryotic cells (Sambrook *et al.*, 1989), rRNA levels in a bacterial sample might be expected to change more over time. This change may also be group-specific or species-specific, with rRNA from some groups or species more sensitive to degradation than others.

A few studies have examined the effect of sampling on the microbial community structure of soil samples. One study examined the effect of varying substrate loading rates and storage on a soil core (Griffiths, 1998). This study used %G+C analysis and community DNA hybridization to examine microbial community structure, and does not demonstrate the effects of storage on RNA. A recent study examines the effects of freezing and storage at room temperature on soil samples (Sessitch, 2002). The authors found that the quality of RNA was equivalent in frozen and room temperature samples, but that frozen samples gave higher RNA yields. This article analyzed total RNA, leaving open the question of group-specific degradation or production. It should also be noted that soil samples contain far lower biomass levels than do activated sludge samples, so the results of a storage experiment in wastewater treatment plants may not correspond to the results seen in soil.

Such changes after sampling can present many problems when trying to analyze different wastewater treatment plants. It is unlikely that any wastewater treatment plant could justify investing in the technology to perform molecular techniques, and treatment facilities would most likely send samples to an outside lab for analysis. The sample may spend several days in transit, during which time rRNA composition may change significantly. We conducted a study of wastewater treatment plants in North Carolina (Chapter 1) and examined several options for storage and shipment of samples. Dry ice for immediate freezing was unavailable in most of the plants. Thus the collected samples were shipped overnight on wet ice. Despite these precautions, some samples had reached room temperature by the time they arrived at the laboratory.

Previous research into changes in microbial community structure in environmental samples have used broad measurements (% G+C, community DNA hybridization) (Griffiths, 1997) or more specific but less quantitative methods such as restriction fragment length polymorphism (RFLP) (Tiedje *et al.*, 1998). Although RFLP and other similar signature technologies can be semi-quantitative, PCR-based methods always raise concerns of PCR bias (Altwegg, 1995; Amann and Ludwig, 2000). We wanted a reliable quantitative analysis to use in the current study that would lend itself to statistical analysis and that could also be refined to show small-scale phylogenetic differences within the samples. We chose the technique of quantitative membrane hybridization because it allowed us to reliably quantify rRNA present in stored samples and to analyze samples on a number of different phylogenetic levels. The current study was designed to determine: 1) how the microbial communities in activated sludge samples changed after collection as measured by rRNA hybridization and 2) how samples can be handled to increase the reliability of rRNA hybridizations. In this study we concentrated on group-specific rRNA, but if more specific identification were required the analysis could be expanded using genus- or species-specific probes.

Four treatment conditions were identified to explore these questions. Storage at 4^{o}C simulated shipment on ice, while storage at room temperature (20-25 ^{o}C) simulated a sample allowed to warm during shipment. Samples were also treated with chloramphenicol (CM), a broad spectrum antibiotic that inhibits RNA production, and with phenol to prevent RNA degradation (Sambrook *et al.*, 1989). Studies have shown that treatment with CM can improve results obtained by fluorescence in situ hybridization and by stopping processing of rRNA in cells, preserving RNA for analysis (Ouverney and Fuhrman, 1997; Oerther *et al.*, 2000). Any change in rRNA levels in CM-treated samples would therefore be due to RNA degradation, while change in rRNA levels in phenol-treated samples would be due to from production of new RNA. The use of these two chemicals should allow us to identify instances of group-specific RNA degradation or production in the samples. By testing samples stored for different time periods, we attempted to quantify how the microbial community structure changed over time under different storage conditions.

Materials and Methods

Sample Collection

Activated sludge samples were collected from the North Cary Water Reclamation Facility (Cary, NC) on July 24, 2001 and from the Neuse River Wastewater Treatment Plant (Raleigh, NC) on July 25, 2001. The North Cary wastewater treatment plant is permitted to treat 20 million gallons of wastewater per day, and uses a Kruger A2O process to perform nitrogen and phosphorous removal. The Raleigh wastewater treatment plant is an activated sludge plant performing nitrogen and phosphorous removal, and is permitted to treat 64 million gallons of wastewater per day.

One liter of activated sludge mixed liquor was taken from the aeration basin of each plant. The master sample was taken back to the laboratory and 96 14-mL aliquots were prepared from a well-mixed sample. These aliquots were centrifuged at 3220 x g for 5 minutes. After decanting the liquid, RNase free water was added to the samples to a final volume of 2 mL and the samples were transferred to 2.0 mL screwcap tubes. The samples were centrifuged at 3300 x g for 5 minutes and remaining liquid was decanted. Samples were then stored under one of 16 different regimens. All samples were processed within one hour after collection.

Storage of Samples

Storage conditions were designed as a randomized complete block. To test the effect of storage over time, samples were stored for 0, 24, 48, or 72 hours before being frozen in -80^{o}C ethanol. There were four different groups of samples at each time period:

“phenol”, “CM”, “4^{o}”, and “RT” (or room temperature). There were 2-3 replicate samples within each treatment-time combination. A schematic representation of the treatment regimens is shown in Figure 2.1.

Room Temperature samples. Room temperature (RT) samples were stored at room temperature for 0, 24, 48 or 72 hours before being frozen.

Refrigerated samples. Refrigerated samples were stored in a 4^{o}C refrigerator for 0, 24, 48 or 72 hours before being frozen.

Phenol samples. Phenol-treated samples were suspended in 1 mL phenol and stored in a 4^{o}C refrigerator for 0, 24, 48 or 72 hours until frozen.

Chloramphenicol samples. In the CM-treated samples, CM was added to a final concentration of 200 µg/µL based on the original 14 mL sample (500 uL of 5.6 g/L CM was added to the samples following centrifugation). Treated samples were stored in a 4^{o}C refrigerator for 0, 24, 48 or 72 hours until frozen.

RNA extractions

RNA extractions were performed as described in Chapter 1.

Membrane Hybridizations

Quantitative membrane hybridizations were performed as described in Chapter 1. Hybridizations were used to determine the rRNA fractions of the major Bacterial groups in wastewater (the Alpha, Beta and Gamma Proteobacteria, the Cytophaga-Flavobacteria-Bacteriodes [CFB] group, and the mycolic acid-containing actinomycetes [Mycolata])

(Manz *et al.*, 1992; de los Reyes *et al.*, 1997). Initial experiments also tested some individual species—*Gordonia amarae, Leucothrix mucor*—but because levels of these species were extremely low when compared with the larger groups, they are not reported here and further experiments only tested the group-level probes. Table 2.1 shows the probes used for each group of bacteria and the corresponding species that provided reference RNA.

Statistical Analysis

Analyses were performed separately on each microbial group. The results were compared using Analysis of Variance (ANOVA) and Tukey's test. The analysis was performed using SAS (SAS Institute, Cary, NC). For the ANOVA, each combination of treatment and time was treated as a separate category and plant (i.e Raleigh or Cary) was considered to be a random factor. If the ANOVA determined that change across the samples was significant, individual results were compared using Tukey's test.

Results

Figures 2.2-2.9 show the results of the quantitative membrane hybridizations. The trends seen in the membrane hybridizations are summarized in Table 2.2. In all cases, controls are the average of all 0 hr 4^{o}C and RT samples. These samples were frozen immediately, and the results obtained from these samples should be representative of the original microbial community. Because this effectively removed two of the sample blocks, the degrees of freedom in the final analysis decreased. Two samples (Raleigh /

4^{o}C / 0 hr / replicate 3 and Raleigh / 4^{o}C / 24 hr / replicate 1) were accidentally combined during the extraction process and were not analyzed. This left two replicates in the Raleigh / 4^{o}C / 0 hr and /24 hr sample blocks, and three in all other blocks.

Table 2.2 shows that the levels of the bacterial groups tested vary between the two plants. At both plants, Alpha Proteobacteria was the dominant phylogenetic group, followed by Beta Proteobacteria. The trends of change in rRNA fractions of the major groups are quite different in the samples taken from each plant. Over time, all microbial groups tested increased in samples taken from the Raleigh plant and treated with phenol. This is not the case for the corresponding samples from the Cary wastewater treatment plant.

Because the samples taken from the two plants behaved differently, the samples from each plant are discussed separately.

Cary, Alpha Proteobacteria

The results of the alpha Proteobacteria analysis on Cary samples are shown in Figure 2.2. Levels of alpha Proteobacteria in CM-treated samples remain statistically constant up to 48 hours and decrease between 48 and 72 hours. Alpha Proteobacteria in phenol-treated samples are lower than control at all time periods tested. Refrigerated samples show increased levels of alpha Proteobacteria RNA (relative to control) by 72 hours, and the levels of this group do not change significantly in samples stored at room temperature.

Raleigh, Alpha Proteobacteria

The results of the alpha Proteobacteria analysis on Raleigh samples are shown in Figure 2.3. Samples treated with phenol and chloramphenicol and samples stored at room temperature do not differ significantly from control. Refrigerated samples contained significantly higher levels of alpha Proteobacteria RNA than control by 24 hours. Those levels then declined between 24 and 48 hours.

Cary, Beta Proteobacteria

The results of the Cary beta Proteobacteria experiment are shown in Figure 2.4. Levels of beta Proteobacteria RNA remain approximately constant in CM-treated, phenol-treated, and refrigerated samples. By 48 hours, levels of beta Proteobacteria RNA in room temperature samples are significantly higher than control.

Raleigh, Beta Proteobacteria

The results of the beta Proteobacteria analysis on Raleigh samples are shown in Figure 2.5. CM-treated, Refrigerated, and room temperature samples do not differ significantly from control throughout the 72-hour period. The levels of beta Proteobacteria RNA in phenol-treated samples increased by approximately 50% between 48 and 72 hours.

Cary, Gamma Proteobacteria

The results of this experiment are shown in Figure 2.6. Phenol-treated, refrigerated, and room temperature samples do not differ significantly from control. Levels of gamma Proteobacteria RNA in CM-treated samples decreased by 72 hours.

Raleigh, Gamma Proteobacteria

The results of this experiment are shown in Figure 2.7. Refrigerated and room temperature samples do not differ from control. CM-treated samples show an increase by 72 hours. The levels of this group in phenol-treated samples had increased by approximately 50% by 72 hours.

Cary, Mycolata

Levels of mycolata RNA in Cary samples are shown in Figure 2.8. The standard deviations of these measurements were relatively large. No sample shows statistically significant change.

Raleigh, Mycolata

Levels of mycolata RNA in Raleigh samples are shown in Figure 2.9. CM-treated and room temperature samples do not differ significantly from control. Refrigerated samples contain significantly lower levels of mycolata RNA that control at 48 and 72 hours. The mycolata RNA increased dramatically in phenol samples: the levels at 72 hours are over 100% greater than the levels in control.

Discussion

The experiment was designed as a randomized complete block. We formulated two questions to test the data: 1) for each treatment, what is the change over time, and 2) for each time step, what is the difference between treatments? Through statistical tests, we hoped to answer a third, overriding question: what is the “best” treatment? This is a qualitative measurement determined from the results of the statistical analyses. The ideal treatment would be one that showed no change over all tests, treatments, and time periods. If change was observed for every treatment, we could then define the best treatment in one of two ways. The best treatment could be that which shows change in the fewest number of samples or it could be the treatment for which change takes the longest to occur. We chose the second criterion, believing that it was a more valid measurement. A treatment that led to change over many samples only after they had been stored for over 48 hours would give consistent results in a sample stored for less than 48 hours. In contrast, treatment that led to change in a limited number of samples by 24 hours would give results that were not always reliable. Thus we might see change in the 72-hour sample of the “best” treatment, but not before that time step.

No treatment completely inhibited change over time. When samples across all phylogenetic groups are compared, the samples treated with chloramphenicol show change, but only after at least 48 hours of storage. Room temperature samples also show less change than expected. Only one sample stored at room temperature showed statistically significant change by 48 hours. Although fewer tests revealed change in refrigerated samples, in one case that change occurred before 24 hours. Samples treated with phenol changed in several cases. The change was not always consistent and samples from the two different plants showed different trends.

As with anything related to microbial community structure, the mechanisms underlying change in these samples are complex. Each plant tested contained its own characteristic mix of microorganisms, and these reacted differently to a specific treatment. All Proteobacteria groups tested tended to decrease in samples taken from the Cary plant and treated with CM, while those from the Raleigh plant either increased (alpha and gamma Proteobacteria) or decreased (beta Proteobacteria). This indicates that the members of the Proteobacteria groups present in the Cary wastewater treatment plant were more susceptible to RNA degradation than other species. At Raleigh, it is likely that members of the alpha Proteobacteria were less susceptible to degradation than other species present.

Samples treated with phenol also showed change, allowing us to deduce the mechanism of change in RNA levels. When tested for beta and gamma Proteobacteria, phenol samples tended to increase while corresponding samples treated with CM either decreased or remained constant. This indicates that RNA production is the most important mechanism of change within the beta and gamma Proteobacteria.

In other cases, the change was not always consistent with those seen in CM treated samples. This suggests that more than one mechanism affected RNA levels within a phylogenetic group. When tested for the mycolata rRNA, Raleigh samples treated with phenol increased sharply. This indicates that RNA production can favor the mycolata relative to members of other phylogenetic groups in the Raleigh plant, and the dramatic increase leads us to hypothesize that perhaps members of this phylogenetic group are metabolizing phenol. For example, the genus *Rhodococcus*, many species of which can degrade phenol, is targeted by the mycolata probe (Ambujom, 2001; Prieto *et al.*, 2002). It should also be noted that in Raleigh samples, the mycolata was the only phylogenetic group tested where the 4^{o}C samples were significantly lower than control. This indicates that without phenol present as a substrate, RNA production in other phylogenetic groups is substantially greater than the mycolata RNA production. Because the mycolata levels in Cary samples were very low and had high variability, no statistically significant change was observed within the samples.

When tested for alpha Proteobacteria, Cary samples treated with phenol decreased while Raleigh samples showed no significant change (there was an insignificant increase). Combined with the observation that CM-treated samples from Cary decreased after 48 hours and those from Raleigh increased (it should be noted that the increase was not statistically significant), this indicates that both RNA production and RNA degradation may be affecting the levels of alpha Proteobacteria. In the Cary samples, RNA degradation affected the alpha Proteobacteria rRNA, while RNA production may have favored other phylogenetic groups more than the alpha Proteobacteria. Members of the alpha Proteobacteria at Raleigh were not as susceptible to RNA degradation, and were not out-competed by RNA production in other groups.

Conclusions and Recommendations

Over the course of 72 hours, some change did occur within CM –treated samples as well as the phenol-treated samples. This indicates that both RNA production and RNA degradation are important mechanisms of group-specific change. At least on a group level, it appears that in most cases these changes cancel each other out. Although CM might be the best treatment in terms of preventing change for a longer period of time, samples held up to 24 hours can also be stored at room temperature with little change in the group-level microbial community structure.

CM and room temperature storage appear to be the most effective treatments. CM is effective at delaying change in a samples stored up to 48 hours, while room temperature is effective for 24 hours of storage. However, these are by no means final results. Although these treatments delayed group-specific change in most cases, we cannot yet conclude that it would have the same effect on other microbial groups. The microbial groups tested here were chosen because they are the largest components of biomass present in wastewater samples. Further research should examine the effect of storage on smaller groups and individual species of interest. One approach could involve a lab-scale experiment wherein a limited number of bacterial species are cultivated in a reactor. By collecting samples over time and storing them, it would be possible to examine the evolution of the microbial community over time and how storage under different conditions affected rRNA levels.

Another important experiment would include samples treated with both phenol and CM. The combined use of these chemicals should prevent both RNA degradation and RNA production. Comparing the results with those obtained from room temperature samples should show whether CM or room temperature are truly viable storage options.

As their use increases, molecular methods may become important tools to engineers and operators of wastewater treatment plants. They will be able to take advantage of these methods by shipping samples to an outside laboratory. The results of molecular analyses will be far more reliable if the sample can be stored in such a way to inhibit change in RNA levels. We have demonstrated that both treatment with chloramphenicol and room temperature storage can help limit change in samples collected. As we look to the future, we must consider the practicality of a storage technique as well as its effectiveness. Chloramphenicol is the more effective treatment, but CM is a powerful antibiotic and subject to regulation. A sample stored at room temperature would present fewer complications, and would also ensure that the researcher could buy one less chemical. Based on the information currently available, we recommend that researchers use room temperature storage for samples that cannot be frozen immediately.

Acknowledgements

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Tables

Table 2.1. Probes and standards used for each group of Bacteria.

Target group	**Probes used**	**Probe sequence**	**Standard**	**Wash Temperature (T_d), ^oC**	**Reference**
α Proteobacteria	S-Sc-aProt-0019-a-A-17[c]	CGTTCG C/T TCTGAGCCAG	*Gluconacetobacter hansenii*	53	(Manz *et al.*, 1992)
β Proteobacteria	L-Sc-bProt-0042-a-A-17[d]	GCCTTCCCACTTCGTT	*Sphaerotilus natans*	58	(Manz *et al.*, 1992)
γ Proteobacteria	L-Sc-gProt-0042-a-A-17[d]	GCCTTCCCACATGGTTT	*Leucothrix mucor*	58	(Manz *et al.*, 1992)
Mycolata	S-*-Myb-0736-b-A-22	CAGCGTCAGTTACT (Nit 1) CCCACAC	*Gordonia amarae*	51	(de los Reyes *et al.*, 1997)

Table 2.2. Summary of levels of groups tested over different sample treatments. Percentage values of the control groups are shown, as is the trend of statistically significant changes over time. Individual graphs from which this data is drawn appear in Figures 1-9.

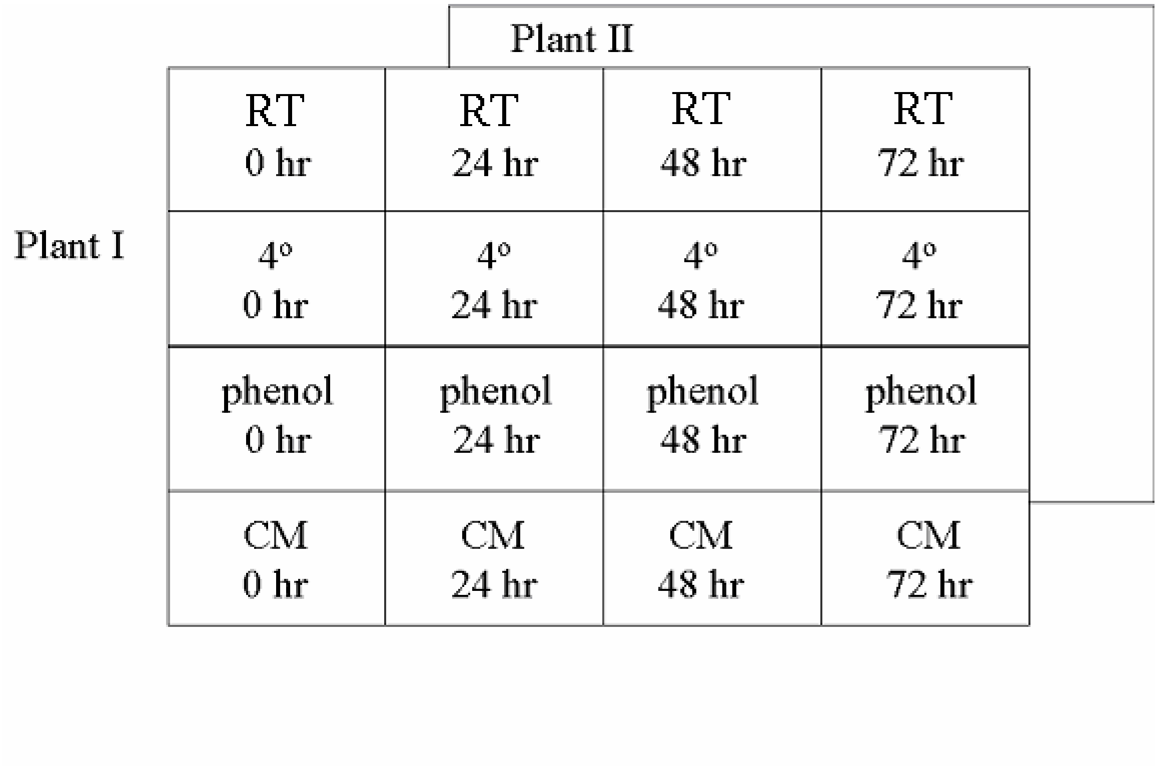
Group	**Value of control sample, %Universal**									
	Cary	Raleigh	Cary				Raleigh			
			CM	Ph	4o	RT	CM	Ph	4^{o}	RT
α Proteobacteria	36%	32%	↓	↓	↑		↑		↓**	
β Proteobacteria	18%	24%				↑		↑↑***		
γ Proteobacteria	5%	8%	↓				↑	↑↑***		
Mycolata	0.5%	1%					↑	↑↑***	↓	
G. amarae*	0.3%									

* The levels of this sample were extremely low and the associated error was almost as large as the sample value. This species was not analyzed.

** In these samples, levels of Alpha Proteobacteria were lower than control at 24 hours, but by 48 hours they had increased approximately to control levels. The overall trend is considered to be a decrease.

*** Three of the groups tested showed dramatic increases when treated with phenol.

Figures



RT 0 hr	RT 24 hr	RT 48 hr	RT 72 hr
4° 0 hr	4° 24 hr	4° 48 hr	4° 72 hr
phenol 0 hr	phenol 24 hr	phenol 48 hr	phenol 72 hr
CM 0 hr	CM 24 hr	CM 48 hr	CM 72 hr

Figure 2.1. Schematic representation of sample block design. There are 32 total sampling blocks; 16 for each wastewater treatment plant.

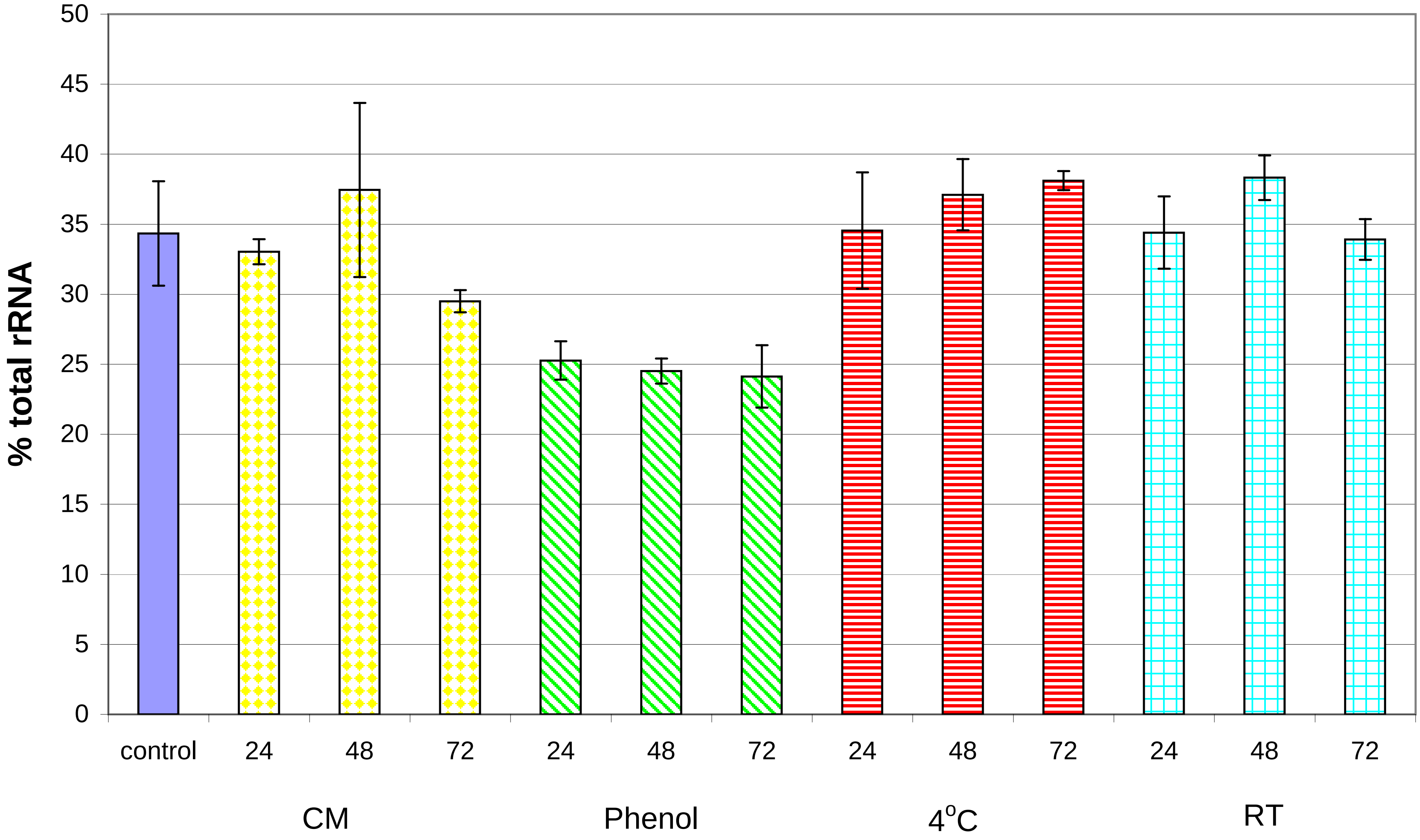


Figure 2.2. Results of the membrane hybridization for Cary alpha Proteobacteria

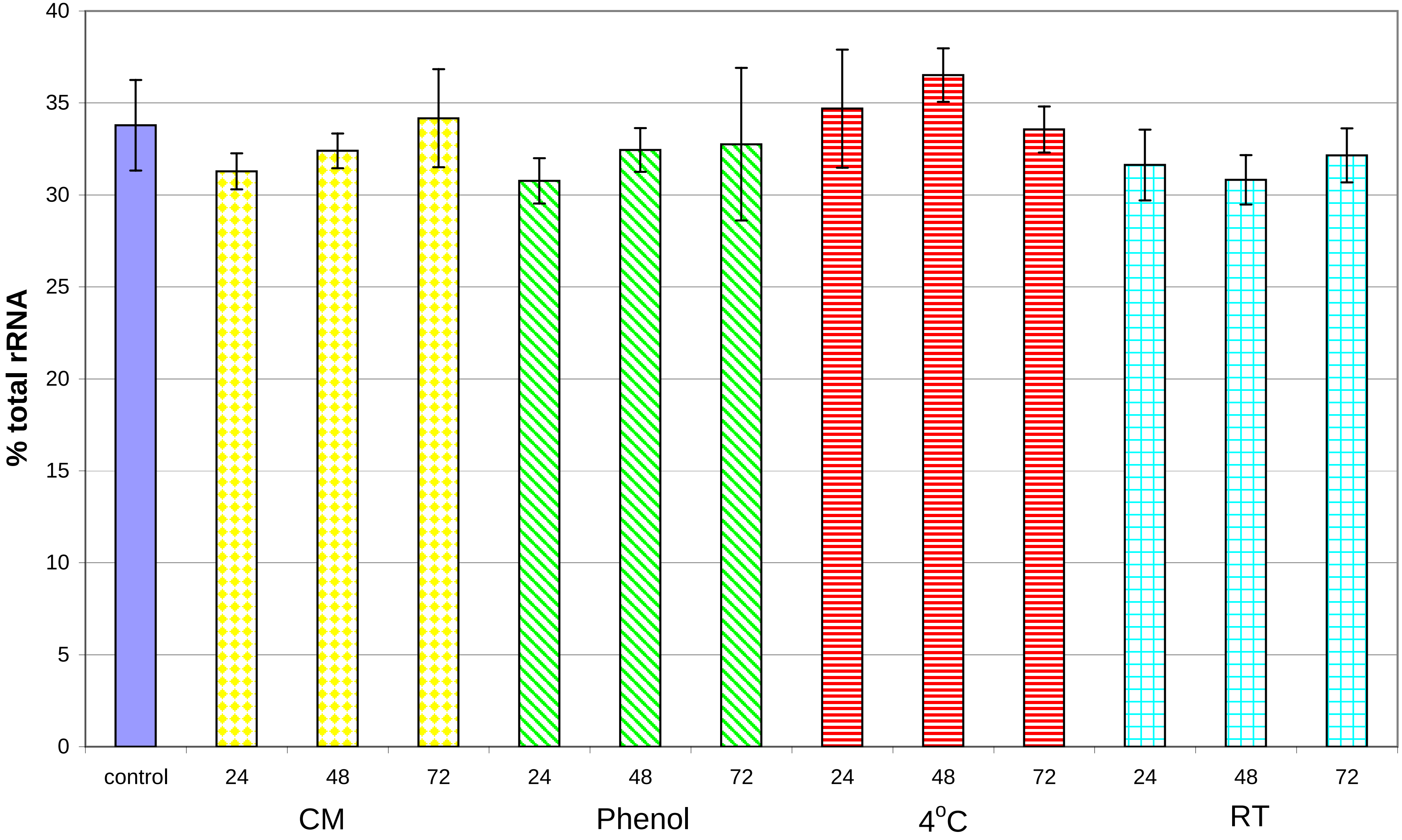


Figure 2.3. Results of the membrane hybridization for Raleigh alpha Proteobacteria

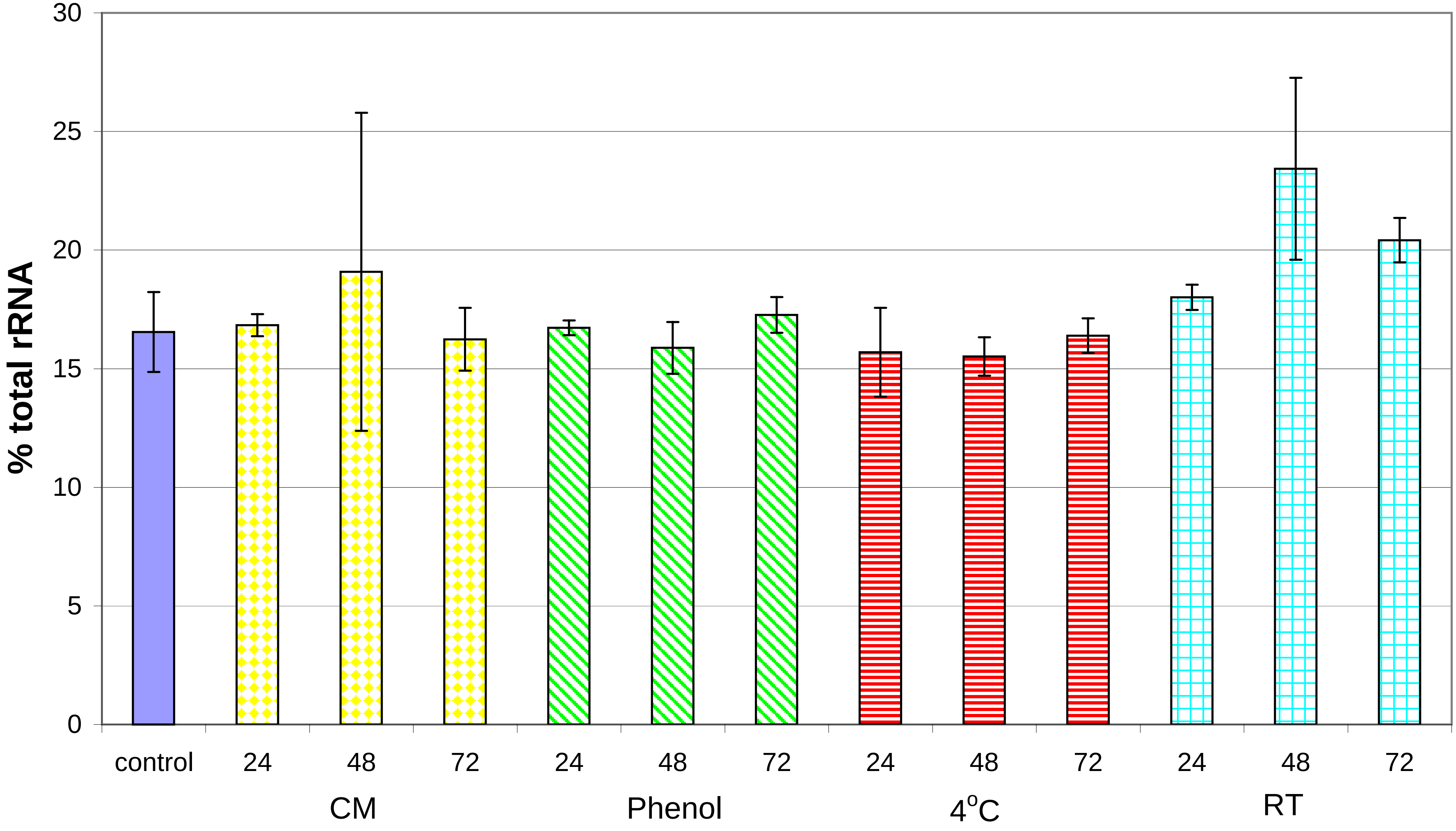


Figure 2.4. Results of the membrane hybridization for Cary beta Proteobacteria

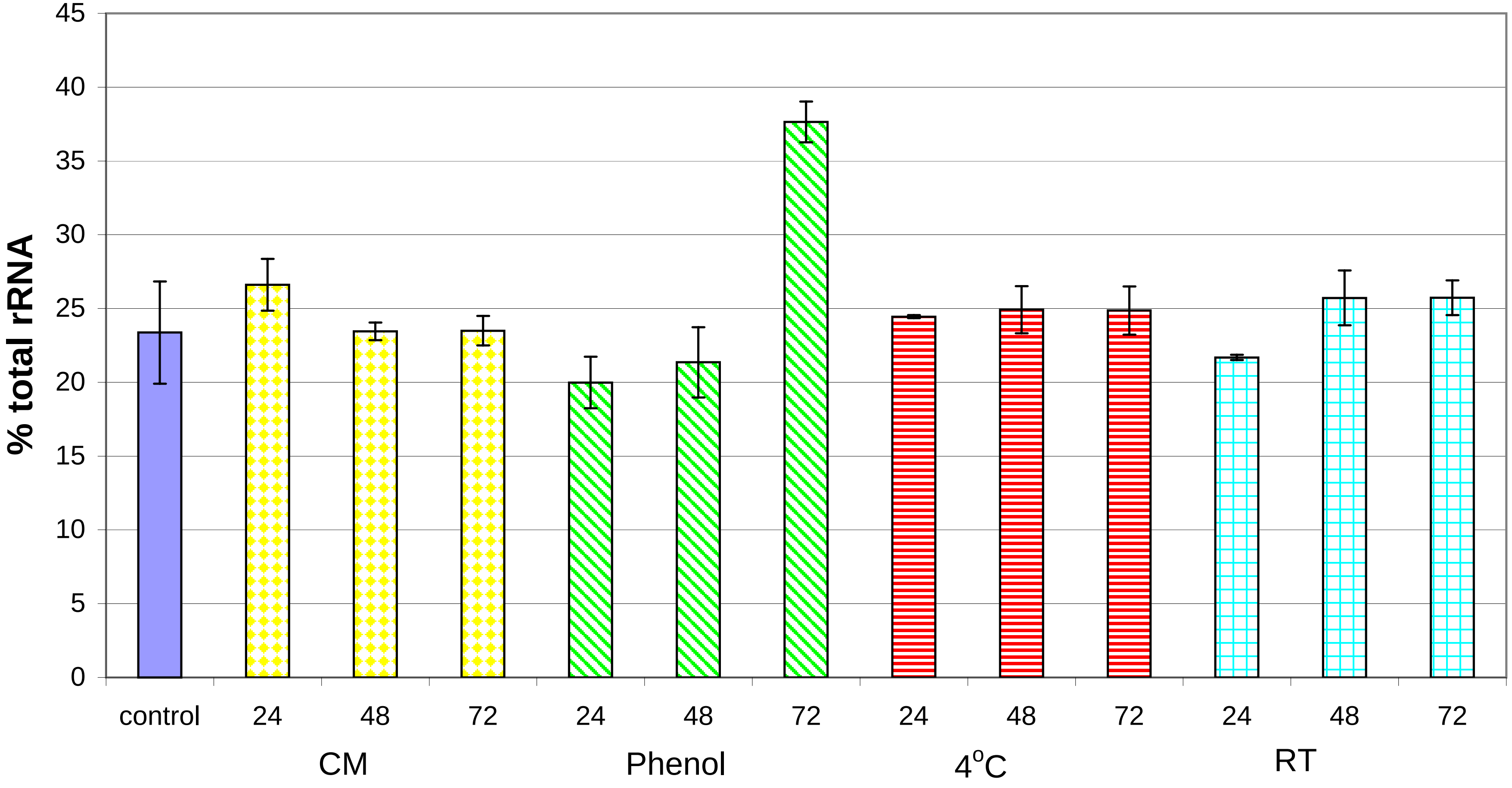


Figure 2.5. Results of the membrane hybridization for Raleigh beta Proteobacteria

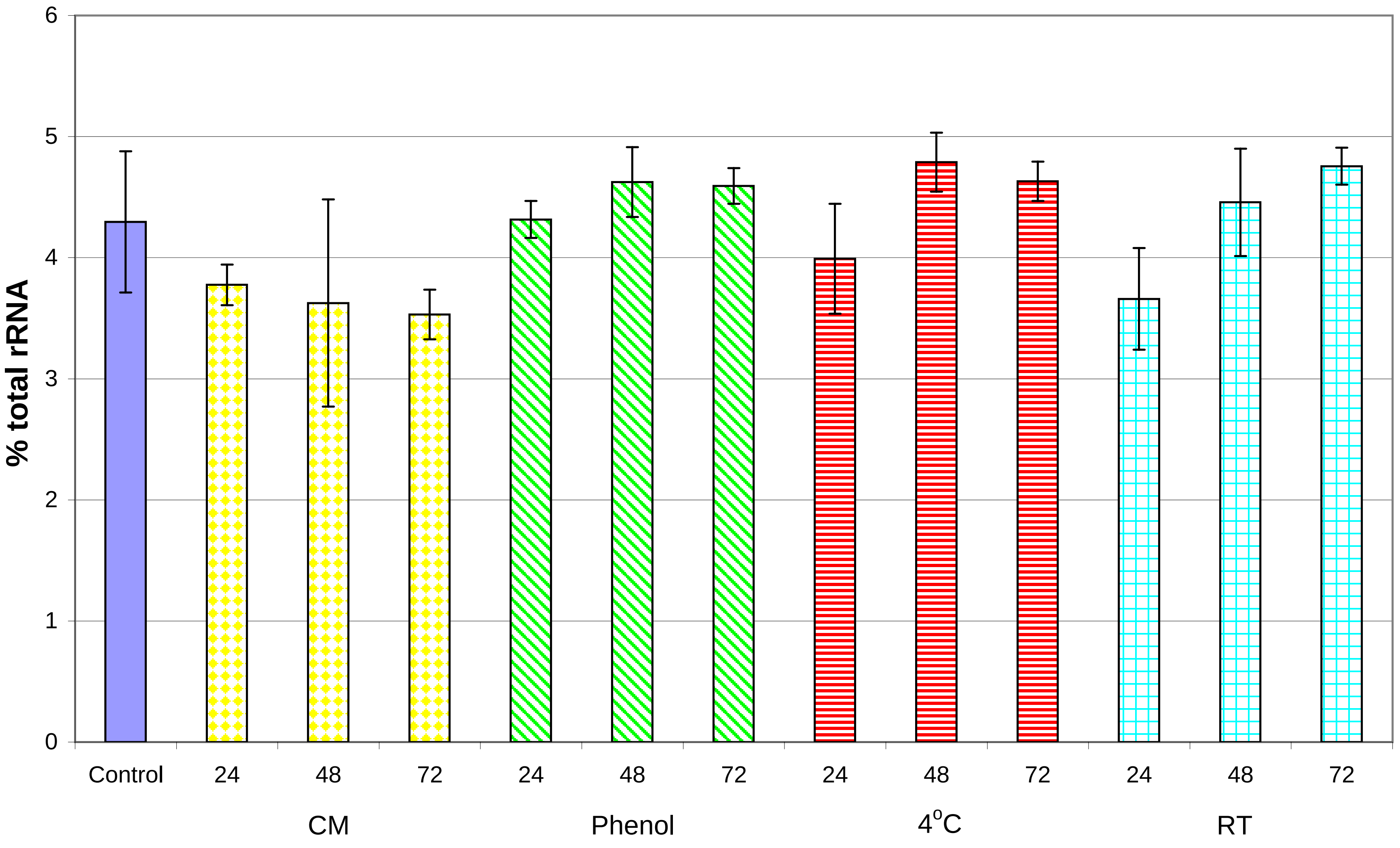


Figure 2.6. Results of the membrane hybridization for Cary gamma Proteobacteria

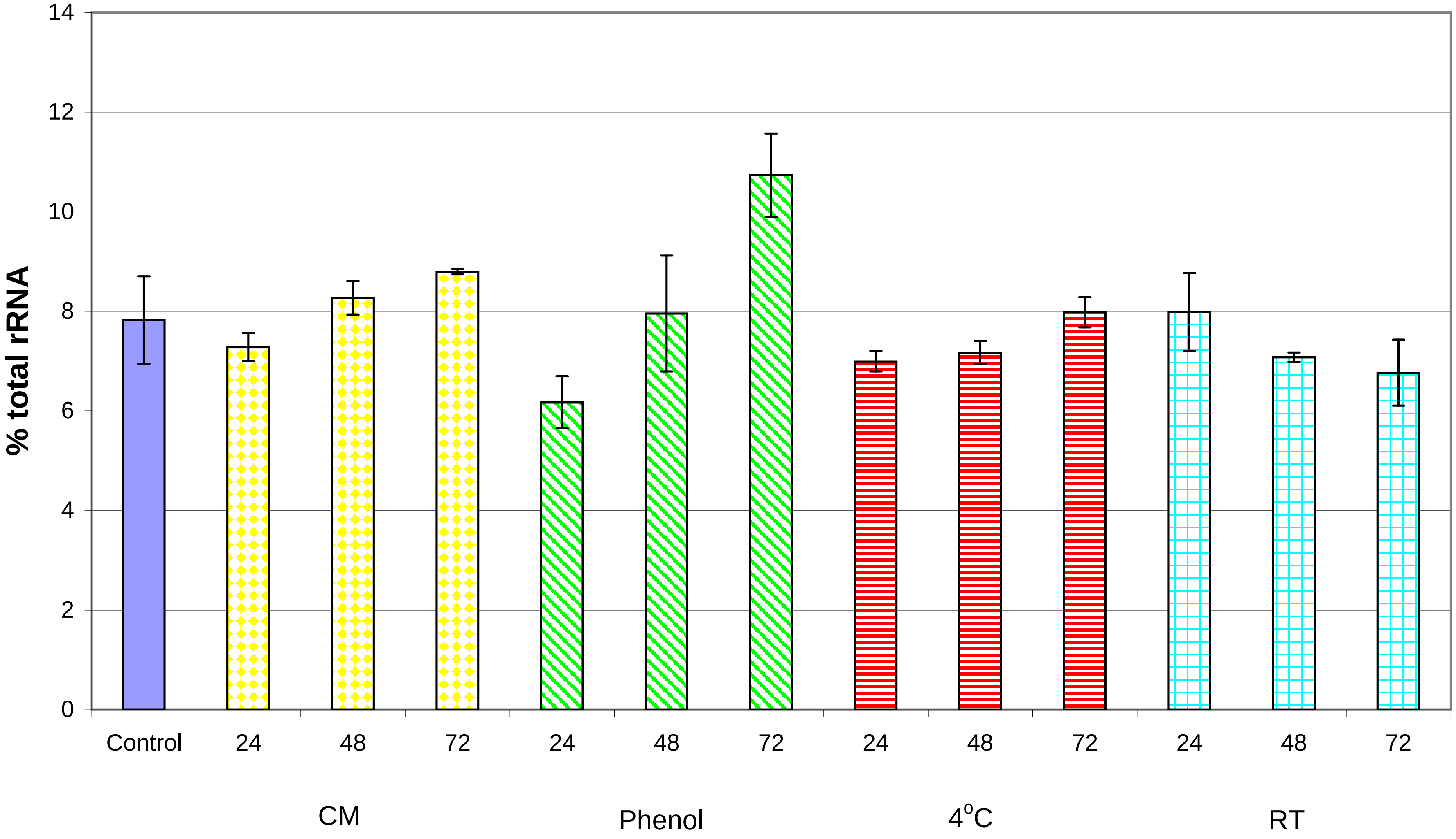


Figure 2.7. Results of the membrane hybridization for Raleigh gamma Proteobacteria

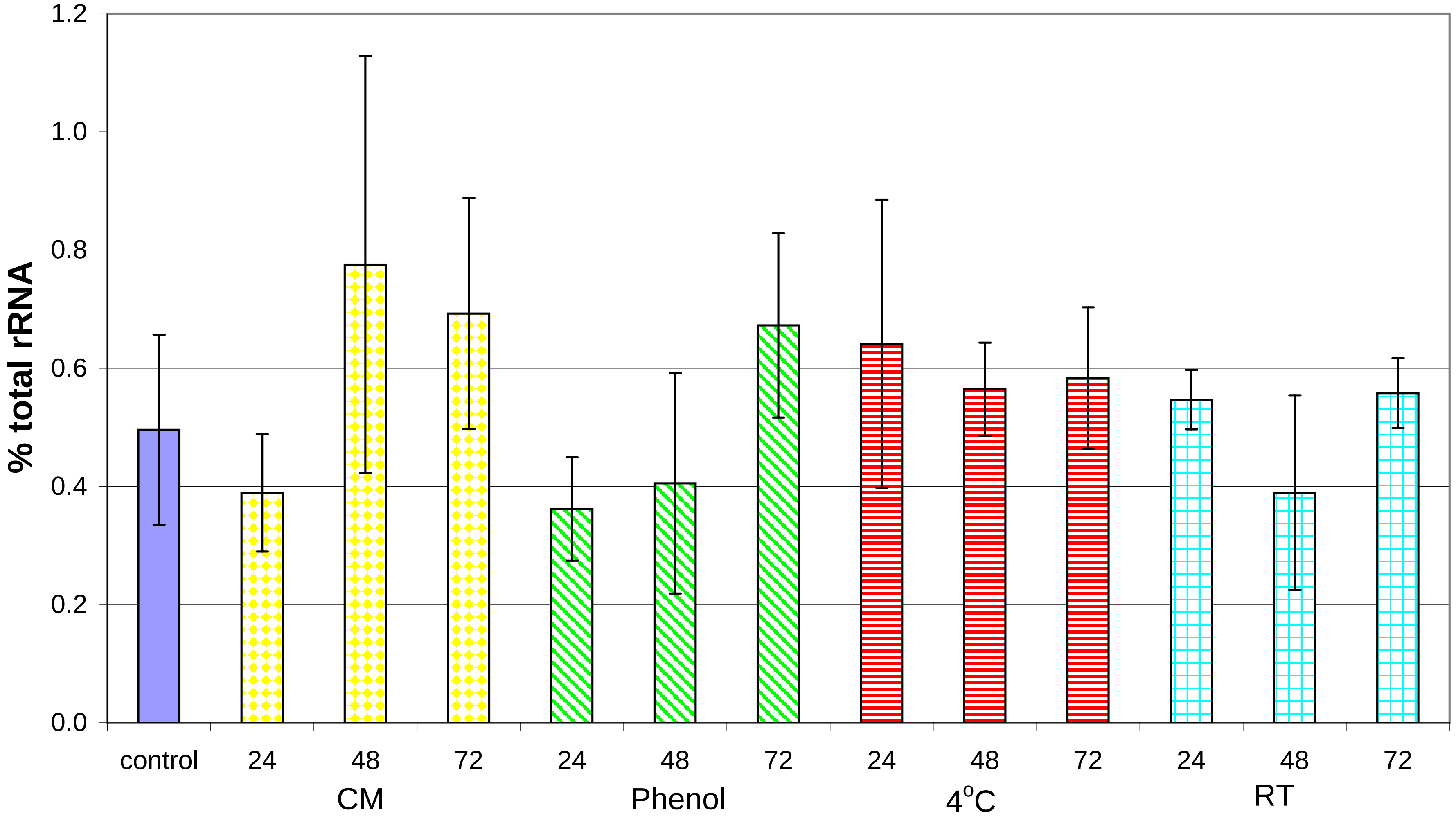


Figure 2.8. Results of the membrane hybridization for Cary mycolata

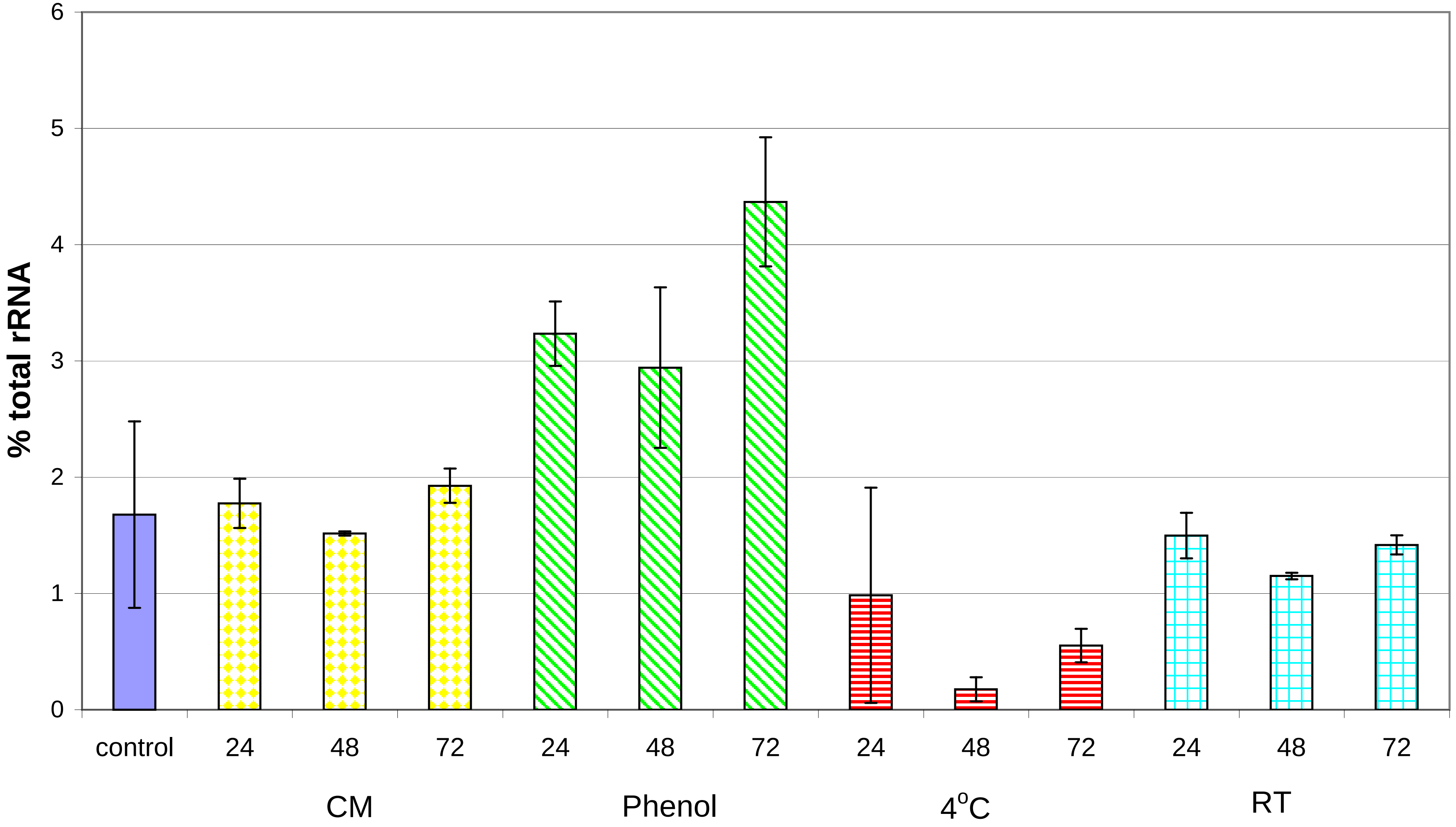


Figure 2.9. Results of the membrane hybridization for Raleigh mycolata

Chapter 3, Detection and quantification of a Bacillus strain used for bioaugmentation

Abstract

Bacillus sp. DA33 is a *Bacillus* isolate that produces an endogenous fungicide and hydrolyzes grease. This isolate has been incorporated in a commercial formulation that is used as an additive to soil (for turfgrass management) and wastewater (for increasing grease biodegradation). To track the fate of this organism in bioaugmented turfgrass plots and wastewater treatment plants, we designed an oligonucleotide hybridization probe targeting the 16S rRNA of *Bacillus* DA33. The probe was thoroughly characterized for quantitative membrane hybridizations and used to quantify *Bacillus* rRNA in treated and control samples. Because this probe was not uniquely specific to DA33, a probe targeting the 16S-23S rRNA intergenic spacer region of *Bacillus* DA 33 was also designed and characterized. It was hypothesized that the abundance of the intergenic spacer region would provide a measure of growth activity of the strain. The hybridization results showed that the amended soil samples had higher levels of *Bacillus* DA33 rRNA than in wastewater treatment plant samples, although the levels of DA33 rRNA were not directly correlated to application of the specific commercial product. In wastewater treatment plants, several samples that were bioaugmented had high DA33 rRNA, suggesting that *Bacillus* DA33 was retained in these treatment plants. However, low hybridization signals were obtained with the intergenic spacer region probe. This may be due to the combined effects of high specificity and low growth activity in unmodified environmental samples.

Introduction

The genus *Bacillus* has frequently been used in bioaugmentation and bioremediation. Some strains of *Bacillus subtilis* form symbiotic relationships with plants and promote the plant's growth (Ferguson *et al.*, 2000). Other *Bacillus* species, notably *B. thurigensis* and *B. atrophaeus*, have been shown to be toxic to mosquitoes, and humans have benefited from this trait (Vidyarthi *et al.*, 2001, other ref). Recent reports also indicate that surfactants derived from *Bacillus thurigensis* and *Bacillus subtilis* aid in the remediation of hydrocarbons and are easily broken down in the environment once their task is complete (Moran *et al.*, 2000; De Lourdes Tirado Montiel *et al.*, 2001).

The isolate *Bacillus* DA33 was isolated from the environment by Novozymes Biologicals, Inc (Salem, VA) for use in commercial formulations. The strain has been shown to improve treatment of fats oils, and grease in wastewater. When applied to turf grass, it acts as a biocontrol agent by limiting fungal growth, reducing dependence on fertilizers and pesticides. These properties make the isolate a useful addition to wastewater or soil. While the effect of bioaugmenting *Bacillus* DA33 formulations in wastewater treatment plants and turfgrass has been documented in Novozymes' field trials, the fate of *Bacillus* DA33 has not been monitored.

Recent research has suggested that no matter how effective bioaugmentation appears in a controlled laboratory environment, a bioagent will only perform its function if it is well-adapted to its environment (Bouchez *et al.*, 2000). The augmented bacterium must persist following application long enough to perform its function. To fully evaluate the effectiveness of *Bacillus* DA33 in bioaugmentation, we need to be able to identify the organism, and also to assess its activity, within a sample.

Bioremediation has been used in the degradation of hazardous wastes in wastewater, the removal of metals from the environment, and the breakdown of persistent hydrocarbons contaminating soils, among other uses. Bioremediation can involve genetic manipulation of plants or bacteria to remove a specific contaminant from the environment, or in a more general form as bioaugmentation, where microorganisms already possessing desired traits are cultured to very high levels and then returned to the environment (Pieper and Reineke, 2000; Dua *et al.*, 2002).

The challenge with any bioremediation project is to ensure that the microorganism will perform its function in the environment. Although laboratory bioremediation projects are often very successful, when the same microorganism is added to an environmental system, selection pressures and lack of access to its substrate can cause the augmentation effort to fail (Dua *et al.*, 2002). It has been suggested that selection of an organism that is well adapted to the environment is a very important component of bioaugmentation projects (Bouchez *et al.*, 2000). *Bacillus* is a very common soil bacterium, and one might expect bioaugmented *Bacillus* DA33 to survive well in soil. Recent research has shown that members of the *Bacillus subtilis* subgroup survive well in wastewater (Wattiau *et al.*, 2001).

Ribosomal RNA (rRNA) analysis provides a very effective tool to identify bioaugmented species. This molecule contains variable regions as well as highly conserved regions. Ribosomal RNA levels also vary with activity: actively growing cells continually synthesize proteins and have high levels of the molecule. We therefore designed a probe to target the rRNA of *Bacillus* DA33. The first probe designed targeted the 16S (small subunit) region of the organism's rRNA.

We also designed a probe targeting the intergenic spacer region (ISR), the region between the 16S and 23S rRNA. It is removed during processing of the mature rRNA molecule, but in very rapidly growing cells, production outpaces processing (Cangelosi and Brabant, 1997). Very rapidly growing cells will thus have relatively high levels of ISR RNA. Growth activity has a far greater effect on ISR RNA than on 16S rRNA. Because of this, 16S rRNA-targeted probes can be used primarily to measure the abundance of particular microorganisms in the environment, while ISR probes primarily measure the activity of these microorganisms. Combining a 16S and ISR probe can yeild a great deal of information about the levels of the organism in the environment, its activity, and also its potential effectiveness as a bioagent (Oerther *et al.*, 2000).

Materials and Methods

Bacterial cultures

Samples of *Bacillus DA33* were obtained from Novozymes Biologicals (Salem, VA). Petri dishes of Plate Count Agar or 100 mL of plate count broth (BD; Franklin Lakes, NJ) were inoculated with colonies of the isolate and maintained at 30°C for 10-18 hours. Other closely related *Bacillus* species (*Bacillus subtilis* ATTC 21332, *Bacillus subtilis* 6051a, *Bacillus licheniformis* 12713 and *Bacillus licheniformis* 14580) were obtained from Novozymes Biologicals or the American Type Culture Collection (ATCC; Alexandria, Virginia) and cultured under ATCC-recommended conditions.

RNA extraction

RNA was extracted as described in Chapter 1.

Polymerase Chain Reaction and Sequencing

DNA was extracted according to the microwave protocol (Bollet *et al.*, 1991). Cultures were centrifuged at 32000 x g for 5 minutes to obtain cell pellets. The pellets were washed in 1 mL tris-EDTA (TE, pH approximately 9.5) and resuspended in 100 μl TE with 50 μl of 10% SDS added. The pellets were incubated at 65°C for 30 minutes, then centrifuged and the lysate was decanted. The pellets were microwaved three times for one minute at a time. 200 μl TE and 200 μl phenol-chloroform, pH 7, were added, and the pellets were shaken for 15 minutes, then centrifuged for 5 minutes at 2300 x g. The aqueous layer was transferred to a clean tube and DNA was precipitated overnight in absolute ethanol.

One μl of the resulting DNA solution was used in a 50 μl PCR reaction. The concentrations of the PCR products were measured using a spectrophotometer (Spectronic Uvicam UV1) to determine appropriate dilutions before sequencing. The sequencing procedure required 10 ng of DNA per 100 base pairs in length.

Polymerase chain reaction was performed on an Eppendorf Mastercycler Gradient thermal cycler (Eppendorf AG, Hamburg, Germany). Reagents were obtained as a PCR kit (consisting of Taq polymerase, 10x PCR buffer and dNTP mix) manufactured by TaKaRa Shuzo (Kyoto, Japan, distributed by PanVera Corp., Madison, WI). One μl of template was added to 49 μl reaction mixture (10 mM Tris-HCl, 50 mM KCl, 1.5 mM

$MgCL_2$, 100 pmol each forward and reverse primers, 1.6 mM dNTP mix and 2.5 units TaKaRa Taq™). The initial PCR reaction amplified the 16S ribosomal DNA using the bacterial primers S-D-Bact-0011-A-S17 and S-D-Bact-1492-B-A16 (de los Reyes *et al.*, 1998), at an annealing temperature of 53.5-56.5^{o}C. Thirty cycles of denaturation, annealing, and extension were used. The PCR products were run on a 0.7% agarose gel in 1x Tris-borate EDTA (TBE) for 1.75-2.5 hours (75 V, approximately 80 mA), with the molecular weight marker pGEM (Promega Corp., Madison, WI). Products were then sequenced at the Duke University DNA analysis facility (Durham, NC). Once the partial sequence of the 16S rDNA was obtained, we designed the nested primers Bac-f (5'-AACGCCGCGTGAGTGATG-3') and Bac-r (5'-ATGCACCACCTGTCACTCTG-3'). The PCR and sequencing were repeated to obtain the full 16S rDNA sequence.

PCR and sequencing were also performed on the intergenic spacer region (ISR) that lies between the 16S and 23S rDNA. Primers KF-5 (5'-GAAGTCGTAACAAGG-3') and KF-17 (5'-CGGGTACTTAGATGTTTCA-3') (Johnson *et al.*, 2000) were used to amplify this region of DNA. The products were run on a 1% agarose gel for 1-1.5 hours (100 V, approximately 110 mA), and sequenced at the Duke University facility.

Alignment and probe design

Sequences were aligned using the the SeqLab and SeqWeb analysis programs (Accelerys Corp., Madison, Wisc.), and the closest relatives to the isolate were determined using the Basic Local Alignment Search Technique (BLAST) program of the National Institutes of Health (Bethesda, MD) (Altschul *et al.*, 1990). The 16S rDNA sequence was then manually aligned with representatives of ten of the most closely related *Bacillus* species. The alignments were used to identify possible probe candidates. The alignment and probe design steps were repeated with the ISR region sequence.

Enrichment of intergenic spacer region DNA

Before extraction of ISR region RNA, samples were cultured and treated with chloramphenicol (CM). This broad-spectrum antibiotic inhibits RNA processing and has been shown to increase the relative levels of ISR RNA within Bacteria (Cangelosi and Brabant, 1997; Oerther *et al.*, 2000). *Bacillus* cultures were grown overnight at 30°C. The isolate was diluted 1:20 and other *Bacillus* species were diluted 1:10 in new media. After an additional hour of growth at 30°C, CM was added to a final concentration of 20 mg/L. The cultures were maintained for an additional hour at 30°C, and then centrifuged and transferred to screwcap tubes and stored at –80°C until extraction.

Oligonucleotide probes and determination of optimal wash temperatures

Oligonucleotide probes were obtained from Sigma-Genosys (The Woodlands, TX). Dissociation temperature (T_d) studies were performed as described in Chapter 1. *Bacillus* DA33 as well as *Bacillus subtilis* ATTC 21332, *Bacillus subtilis* 6051a, *Bacillus licheniformis* 12713 and *Bacillus licheniformis* 14580 were used in dissociation temperature studies (Zheng, 1995).

Membrane hybridizations

Membrane hybridizations were performed as described in Chapter 1.

Probe specificity studies

The specificity of the oligonucleotide probes was examined by membrane hybridizations with nucleic acids from *Bacillus subtilis* ATTC 21332, *B. subtilis* 6051a, *B. licheniformis* 12713 and *B. licheniformis* 14580, representing species closely related to the target *Bacillus* strain. The previously determined T_d for each probe was used as the final wash temperature for the membranes. Hybridization signals were quantified by storage phosphor analysis using a 400 series PhosphorImager and ImageQuant software package (Molecular Dynamics, Sunnyvale, CA).

Bioaugmentation experiments.

Two sets of samples were analyzed for the presence of *Bacillus* DA33. The first group of samples consisted of grab samples taken from wastewater and soil cores that had been bioaugmented with a commercial product containing *Bacillus* DA33. The activated sludge samples—Dairy-1 and -2, Yogurt plant, Citgo aer, Cifcon, Kenyon Industries, Kolb-Lena-1 and 2—and soil samples—MJ1, MJ2, MJ3, and MJ4—were received during October 2001. Samples Dairy-1 and Dairy-2 were misnomers: the samples were collected from fixed-growth bioreactor treating wastewater (and not a dairy plant). The other wastewater samples represented industrial wastewater that had been amended with *Bacillus* DA33. The soil samples in this experiment had all been treated with the isolate. Samples MJ-2 through -4 had been treated with increasing concentrations of an additive designed to promote the growth of *Bacillus* DA33, while the three replicates of sample MJ-1 served as a control.

The second set of samples consisted of soil samples taken from plots treated with DA33 and control plots; they were received in September, 2002. Eight plots were planted with Penncross bentgrass, as in the diagram shown in Figure 3.1. The plots sloped from left to right (i.e. from plot 1 to plot 8). Even-numbered samples were treated with a formulation including *Bacillus* DA33, and odd numbered samples were treated with a competitor's product. The cells had open irrigation and were fertilized as described in previous Novozymes trials. Samples were collected four days after bioaugmentation. Levels of *Bacillus* DA33 in the samples were measured in a blind experiment. Soil cores were collected from the plots and sent to the laboratory. Three subsamples were taken from each of the eight soil cores. Levels of *Bacillus* DA33 RNA were quantified using membrane hybridization.

Quantitative membrane hybridization

Membrane hybridizations were performed as described in Chapter 1. All samples were tested against probes S-Sp-B.DA33-1449-a-A-18 (16S probe) and S-S-B.DA33-1737-a-A-27 (ISR probe) as well as Bacterial and Universal probes.

Results and Discussion

Probe Design and Characterization

To design a probe targeting the 16S region of *Bacillus* DA33 rRNA, the 16S rDNA sequence had to be determined. PCR with Bacterial primers successfully amplified DNA extracted from the *Bacillus* DA33 culture. The gel used to test amplification products of 16S rDNA is shown in Figure 3.2. The PCR products were approximately 1500 bases in length, and the full 16S rDNA sequence is shown in Table 3.1. BLAST results showed that the strain was most closely related to *Bacillus subtilis* strain OG-01-center (Genbank accession no: AB018484) (Suzuki, 1999). Comparison of the sequence with its closest relatives showed that there was not a unique probe site within the 16S rDNA region. Through manual alignment of the DA33 isolate with its closest relatives, we identified a possible probe sequence that bound to the fewest number of organisms possible. This probe, S-Sp-B.33-1449-A-a-18 (5'—TCC ACC TTC GGC GGC TGG—3', with a predicted T_d of 55 °C) bound to strains of several different bacterial species. Table 3.2 shows the BLAST results from the DA33 16S probe, which also binds to the p47-phox gene of *Mus. Musculus* as well as to the 16S rDNA of *Bacillus subtilis*, *Bacillus fusiformis*, *Bacillus infernus*, *Bacillus sp.* FE1, and *Listeria monocytogenes*.

Hybridization to the mouse gene was probably not problematic, since we expect low levels of this RNA in environments such as activated sludge or soil. *Bacillus infernus* was an anaerobic thermophile that reduces Fe (III) and Mn (IV) in deep terrestrial subsurfaces, so again, this organism would not interfere with the use of the DA33 probe. *B. sphaericus* and *B. fusiformis* are both members of the mesophilic roundspored bacteria that are typically pathogenic to mosquitoes. Even though this probe binds to non-target species, we proceeded to characterize this probe for membrane hybridization. Use of this probe for quantitative studies should take these limitations in consideration. For example, there is a published probe for *B. sphaericus*, and, if this is used in conjunction with the DA33 probe, the effect of *B. sphaericus* can be subtracted from the DA33 probe results.

Because the 16S probe was not uniquely specific, we searched for an additional candidate probe in the ISR of RNA. The ISR region lies between the 16S and 23S regions of RNA; it contains non-functional sequence and can also contain sequences encoding transfer RNAs. It is transcribed with these regions, but is removed during processing of the rRNA. Consequently, ISR sequences have high variability and can be used to distinguish very closely related microorganisms. To develop a probe in this region, we sequenced the 16S-23S spacer region of rDNA using previously published primers. PCR results are shown in Figure 3.3, and the ISR rDNA sequence (which also contained portions of the 16S and 23S rDNA sequences) is shown in Table 3.3. By manual alignment and visual inspection, we were able to identify a unique probe binding site. Two probes were designed: S-S-B.DA33-1737-a-A-24 (5'—CTA CGT GAT ATC TTG TCT TAC TAA—3', with a predicted T_d of 50.4^{o}C) and S-S-B.DA33-1737-a-A-27 (5'—TAC CTA CGT GAT ATC TTG TCT TAC TAA—3', with a predicted T_d of 54 ^{o}C). The latter probe is uniquely specific to *Bacillus sp.* DA33, with at least two mismatches to the closest nontarget sequences.

To determine its dissociation temperature, a T_d study was performed with probe S-Sp-B.33-1449-A-a-18. The probe was tested with rRNA from *Bacillus sp.* DA33 and two close relatives, *B. subtilis* ATCC 21332 and *B. subtilis* ATCC 6051a. The T_d curve is shown in Figure 3.4. The T_d of probe S-Sp-B.33-1449-A-a-18 was determined to be 60°C. At this wash temperature, the probe also binds to the 16S rRNA of the other *B. subtilis* strains. At the wash temperature, probe S-Sp-B.33-1449-A-a-18 binds (with varying specificity) to other *Bacillus* strains, but that it does not bind to other Gram-positive or to Gram-negative microorganisms.

The results of the T_d study for the two ISR probes are shown in Figure 3.5. The 24-base probe had a T_d of 51°C, while the 27-base probe had a Td of 54°C. We used the 27-base probe in all further analyses because it had a slightly higher T_d, indicating more stable binding to its target. The probe does not bind to any other DNA sequence in the database.

Sample Analysis

Soil and wastewater samples that had been bioaugmented with *Bacillus* DA33 were tested with both the 16S and ISR probes. The levels of ISR in the first group of samples were extremely low and very difficult to quantify (results not shown). The samples were collected some time after inoculation, and the low levels of ISR indicate that *Bacillus* DA33 in the samples had already passed through its time of peak growth. The results from the 16S probe are shown in Figures 3.6 (wastewater samples) and 3.7 (soil samples). The levels of DA33 were higher in soil than in wastewater. This was expected, as *Bacillus* is a common genus of soil bacteria.

Wastewater samples Dairy-1 and Dairy-2 were collected from a membrane bioreactor. Levels of *Bacillus* DA33 in these samples were collected from the same wastewater treatment plant but differ substantially; this may be due to differences across the biofilm. In contrast, the 16S rRNA results from samples Kolb-Lena-1 and Kolb-Lena-2 are almost identical. Levels of *Bacillus* DA33 rRNA in Dairy-1 are significantly higher than those in any other wastewater samples.

All soil samples in Figure 3.7 were augmented with *Bacillus* DA33. Samples MJ-2, MJ-3, and MJ-4 were treated with increasing concentrations of an additive designed to favor growth of *Bacillus* DA33, with samples MJ-1a, b, and c as control. The replicates of sample MJ-1 show higher levels of *Bacillus* DA33 rRNA than do MJ-2, -3, and -4. This indicates that the additive does not provide any benefit to *Bacillus* DA33 and may even inhibit its growth.

Figures 3.8 and 3.9 compare soil samples treated with *Bacillus* DA33 to control soil samples. Figure 3.8 shows the results of 16S probe hybridizations to compare the two. The average levels of *Bacillus* DA33 rRNA are equivalent in treated and untreated samples. The levels of *Bacillus* DA33 rRNA as measured with the 16S probe are significantly higher in lower-numbered (i.e. samples 1-3) than higher numbered samples (sample 6-8). In other words, the samples at the top of the slope had greater response to the *Bacillus* DA33 16S probe than did the samples at the bottom of the slope. ISR data does not show any difference between treated and untreated samples (Figure 3.9). This is the opposite of expected values. If spores had remained in place, the levels of *Bacillus* DA33 should have been higher in the even-numbered plots. If instead they had been carried by water, more *Bacillus* spores should have been deposited at the bottom of the slope (samples 6-8) than the top. The presence of higher levels of *Bacillus* DA33 at the top of the hill than the bottom could possibly be explained by spore transfer. The system sampled was also open, with nothing to prevent transfer of *Bacillus* spores from one plot to another. In the future, more separated and controlled experiments would permit better analysis of *Bacillus* DA33.

Conclusions

Both the 16S and the ISR probe target *Bacillus sp.* DA33 effectively. When using the 16S probe we must remember that it also targets other close relatives of the organism. On the other hand, the ISR probe is uniquely specific to B. DA33. Although levels of the ISR probe are extremely low in many samples, we would expect them to be highest in samples that are growing rapidly. Combined use of the 16S and ISR probe should yield information about the presence and activity of DA33 in a sample.

Results to date have shown that *Bacillus* DA33 maintains relatively high levels in soil following bioaugmentation. Less of the isolate remains in wastewater than in soil. This does not necessarily indicate that *Bacillus* DA33 does not perform well in wastewater; if it persists in wastewater even at low levels, the isolate may be capable of performing its function and hydrolyzing grease. This also indicates that more frequent amendments are required to wastewater than to soil.

Future Research

In the current study, we have concentrated primarily on the presence of *Bacillus* DA33 in a sample, and have only examined its *in situ* activity in a limited way. The ISR probe in relation to the 16S probe should make it possible to quantify the activity of

Bacillus DA33 in bioaugmented samples. Before it can be used reliably in environmental samples, we need to develop a relationship between growth of *Bacillus* DA33 and ISR levels in a lab system. One way of doing this would be to test samples from a laboratory reactor. A time series of samples could be drawn following inoculation: samples would be taken at regular time intervals based on the expected doubling time of the organism. Because *Bacillus* grows so quickly, hourly samples taken over the course of two or three days could span a wide rage of growth rates. By comparing ISR levels to growth rate, we can determine the relationship between growth rate and ISR levels in samples treated with *Bacillus* DA33.

To truly quantify the isolate's effectiveness in bioaugmentation experiments, *Bacillus* DA33 needs to be examined with molecular probes following bioaugmentation. The relationship between growth rate and ISR levels described above should allow us to identify the point after augmentation when *Bacillus* DA33 is growing most rapidly. The combination of that probe and the 16S probe should allow us to track *Bacillus* DA33 levels in the sample. If this organism is truly effective in bioaugmentation, fungicidal activity and hydrolysis of grease should correlate to its activity within the sample.

We also intend to develop chemiluminescent quantification using the *Bacillus* DA33 probes. The current study used ^{32}P for membrane hybridizations. It would be difficult for our industry partner to obtain a license for ^{32}P, so we are developing non-isotopic quantification of the probes. This effort should result in a reliable quantification method that does not demand radioactive material.

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<u>Tables</u>

Table 3.1. 16S rDNA sequence of *Bacillus* DA33. The highlighted region is the probe binding site.

```
   1   GCTGGCGGCG TGCCTAATAC ATGCAAGTCG AGCGGACAGA TGGGAGCTTG
  51   CTCCCTGATG TTAGCGGCGG ACGGGTGAGT AACACGTGGG TAACCTGCCT
 101   GTAAGACTGG GATAACTCCG GGAAACCGGG GCTAATACCG GATGCTTGTT
 151   TGAACCGCAT GGTTCAAACA TAAAAGGTGG CTTCGGCTAC CACTTACAGA
 201   TGGACCCGCG GCGCATTAGC TAGTTGGTGA GGTAACGGCT CACCAAGGCA
 251   ACGATGCGTA GCCGACCTGA GAGGGTGATC GGCCACACTG GGACTGAGAC
 301   ACGGCCCAGA CTCCTACGGG AGGCAGCAGT AGGGAATCTT CCGCAATGGA
 351   CGAAAGTCTG ACGGAGCAAC GCCGCGTGAG TGATGAAGGT TTTCGGATCG
 401   TAAAGCTCTG TTGTTAGGGA AGAACAAGTA CCGTTCGAAT AGGGCGGTAC
 451   CTTGACGGTA CCTAACCAGA AAGCCACGGC TAACTACGTG CCAGCAGCCG
 501   CGGTAATACG TAGGTGGCAA GCGTTGTCCG GAATTATTGG GCGTAAAGGG
 551   CTCGCAGGCG GTTTCTTAAG TCTGATGTGA AAGCCCCCGG CTCAACCGGG
 601   GAGGGTCATT GGAAACTGGG GAACTTGAGT GCAGAAGAGG AGAGTGGAAT
 651   TCCACGTGTA GCGGTGAAAT GCGTAGAGAT GTGGAGGAAC ACCAGTGGCG
 701   AAGGCGACTC TCTGGTCTGT AACTGACGCT GAGGAGCGAA AGCGTGGGGA
 751   GCGAACAGGA TTAGATACCC TGGTAGTCCA CGCCGTAAAC GATGAGTGCT
 801   AAGTGTTAGG GGGTTTCCGC CCCTTAGTGC TGCAGCTAAC GCATTAAGCA
 851   CTCCGCCTGG GGAGTACGGT CGCAAGACTG AAACTCAAAG GAATTGACGG
 901   GGGCCCGCAC AAGCGGTGGA GCATGTGGTT TAATTCGAAG CAACGCGAAG
 951   AACCTTACCA GGTCTTGACA TCCTCTGACA ATCCTAGAGA TAGGACGTCC
1001   CCTTCGGGGG CAGAGTGACA GGTGGTGCAT GGTTGTCGTC AGCTCGTGTC
1051   GTGAGATGTT GGGTTAAGTC CCGCAACGAG CGCAACCCTT GATCTTAGTT
1101   GCCAGCATTC AGTTGGGCAC TCTAAGGTGA CTGCCGGTGA CAAACCGGAG
1151   GAAGGTGGGG ATGACGTCAA ATCATCATGC CCCTTATGAC CTGGGCTACA
1201   CACGTGCTAC AATGGACAGA ACAAAGGGCA GCGAAACCGC GAGGTTAAGC
1251   CAATCCCACA AATCTGTTCT CAGTTCGGAT CGCAGTCTGC AACTCGACTG
1301   CGTGAAGCTG GAATCGCTAG TAATCGCGGA TCAGCATGCC GCGGTGAATA
1351   CGTTCCCGGG CCTTGTACAC ACCGCCCGTC ACACCACGAG AGTTTGTAAC
1401   ACCCGAAGTC GGTGAGGTAA CCTTTTAGGA GCCAGCCGCC GAAGGTGGAC
1451   AGA
```

Table 3.2. Alignment of probe S-Sp-B.DA33-1449-a-A-18 with other closely related species.

Probe Target	1	....	CCAGCCGCCGAAGGTG-GA	....	18
B. sp. DA33	1427	GGAG	CCAGCCGCCGAAGGTG-GA	-CAGATGAT	1457
B. sp. FE1	1435	GGAG	CCAGCCGCCGAAGGTG-GA	-TAGATTTG	1461
B. subtilis	1435	GGAG	CCAGCCGCCGAAGGTG-GA	-CAGATGAT	1465
B. sphaericus	1441	GGAG	CCAGCCGCCGAAGGTG-GA	-TAGATGAC	1471
B. fusiformis	1447	GGAG	CCAGCCGCCGAAGGTG-GA	-TAGATGAT	1477
B. infernus	1448	GGAG	CCAGCCGCCGAAGGTG-GA	ACAGATGAT	1479
Pae. chinjuen	1418	GGAG	CCAGCCGCCGAAGGTGGGG	-TAGATGAT	1439
E. coli	1454	GGAG	GGCGCTTACCACTTTGTGA	-TTCATGAC	1485

Table 3.3. Intergenic spacer region sequence and portions of the 16S and 23S rDNA sequence of *Bacillus* DA33. The highlighted region is the probe binding site. The underlined regions represent the portion of the 16S (positions 1-55) and 23S (positions 214-409) sequences also obtained through sequencing.

```
  1  AAGGTGCGGC TGGATCCCTC CTTTCTAAGG ATATTTACGG AATATAAGAC
 51  CTTGGGTCTT ATAAACAGAA CGTTCCCTGT CTTGTTTAGT TTTGAAGGAT
101  CATTCCTTCG AAACGTGTTC TTTGAAAACT AGATAACAGT AGACATCACA
151  TTCAATTAGT AAGACAAGAT ATCACGTAGT GATTCTTTTT AACGGTTAAG
201  TTAGAAAGGG CGCACGGTGG ATGCCTTGGC ACTAGGAGCC GATGAAGGAC
251  GGGACGAACA CCGATATGCT TCGGGGAGCT GTAAGCAAGC TTTGATCCGG
301  AGATTTCCGA ATGGGGAAAC CCACCACTCG TAATGGAGTG GTATCCATAT
351  CTGAATTCAT AGGATATGAG AAGGCAGACC CGGGGAACTG AAACATCTAA
401  GTACCCGAG
```

Figures

Figure 3.1. Diagram of the plots used in the second soil sampling experiment. Even-numbered cells were treated with a formulation including *Bacillus* DA33 and odd-numbered cells were treated with a competitor’s product. The plots sloped from left to right.

x	1	2	3	4	5	6	7	8	x

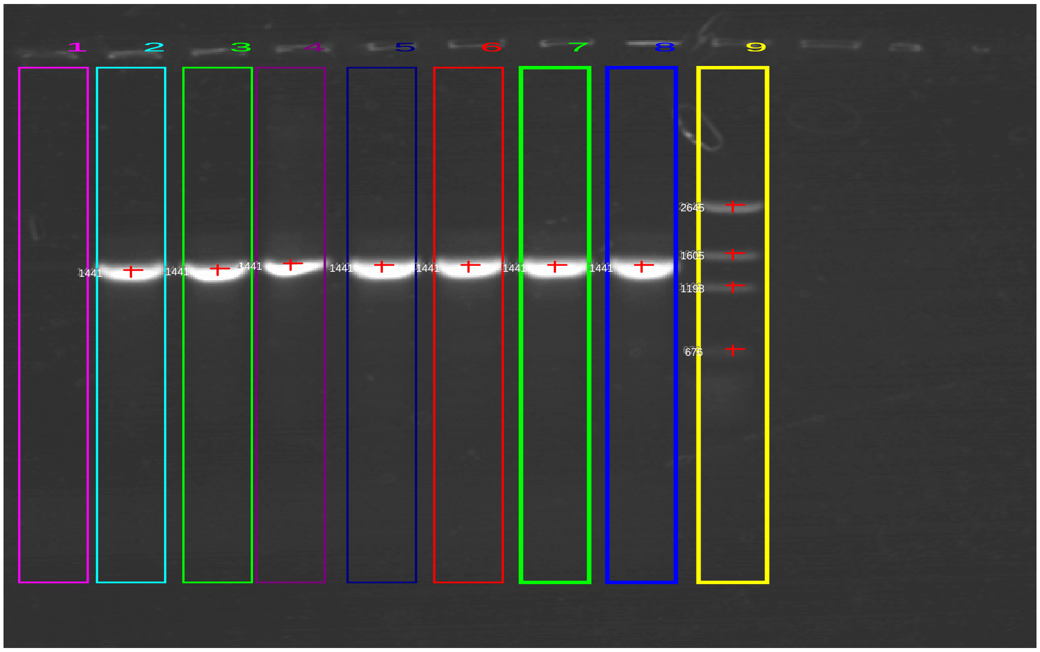


Figure 3.2. 16S rDNA amplified with the primers 11f and 1492r. Lane 1 is the negative control, conducted at 55°C. Lanes 2-8 are PCR products obtained across a gradient of 53.5-56.5°C (0.5°C/well), and lane 9 is the molecular weight marker pGEM. The migration of the bands shows that the PCR product obtained is approximately 1500 bases in length.

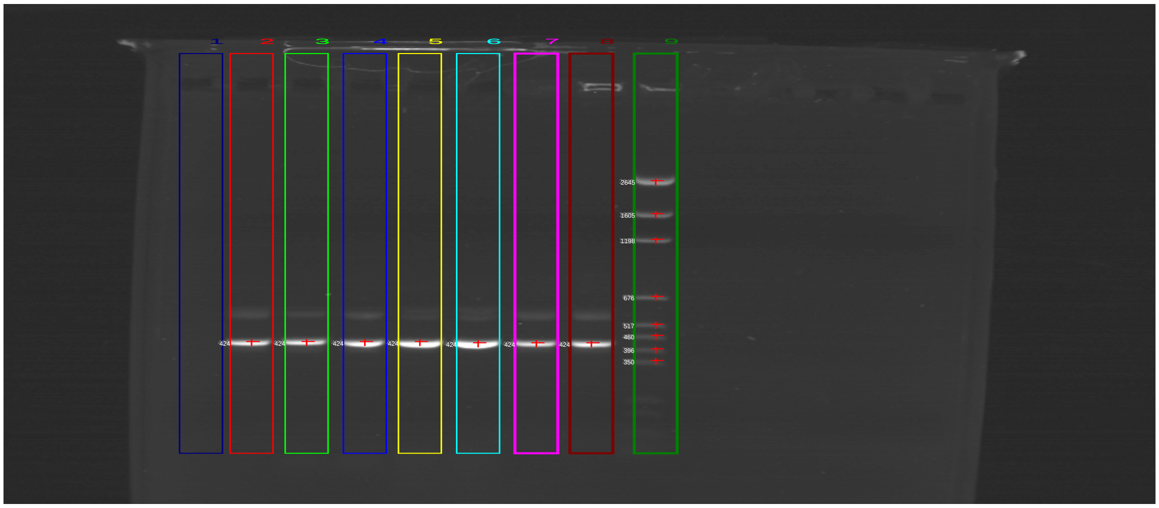
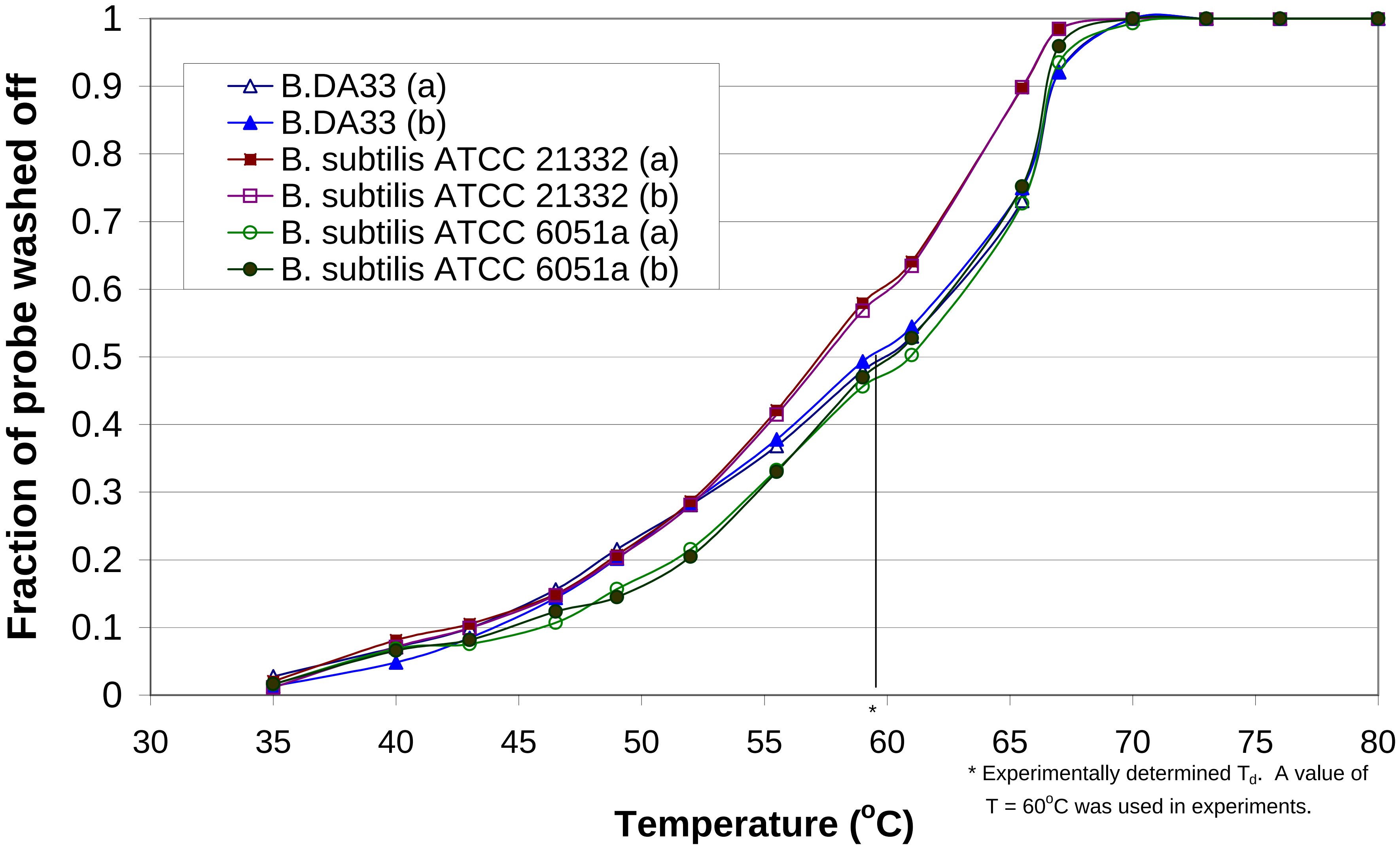
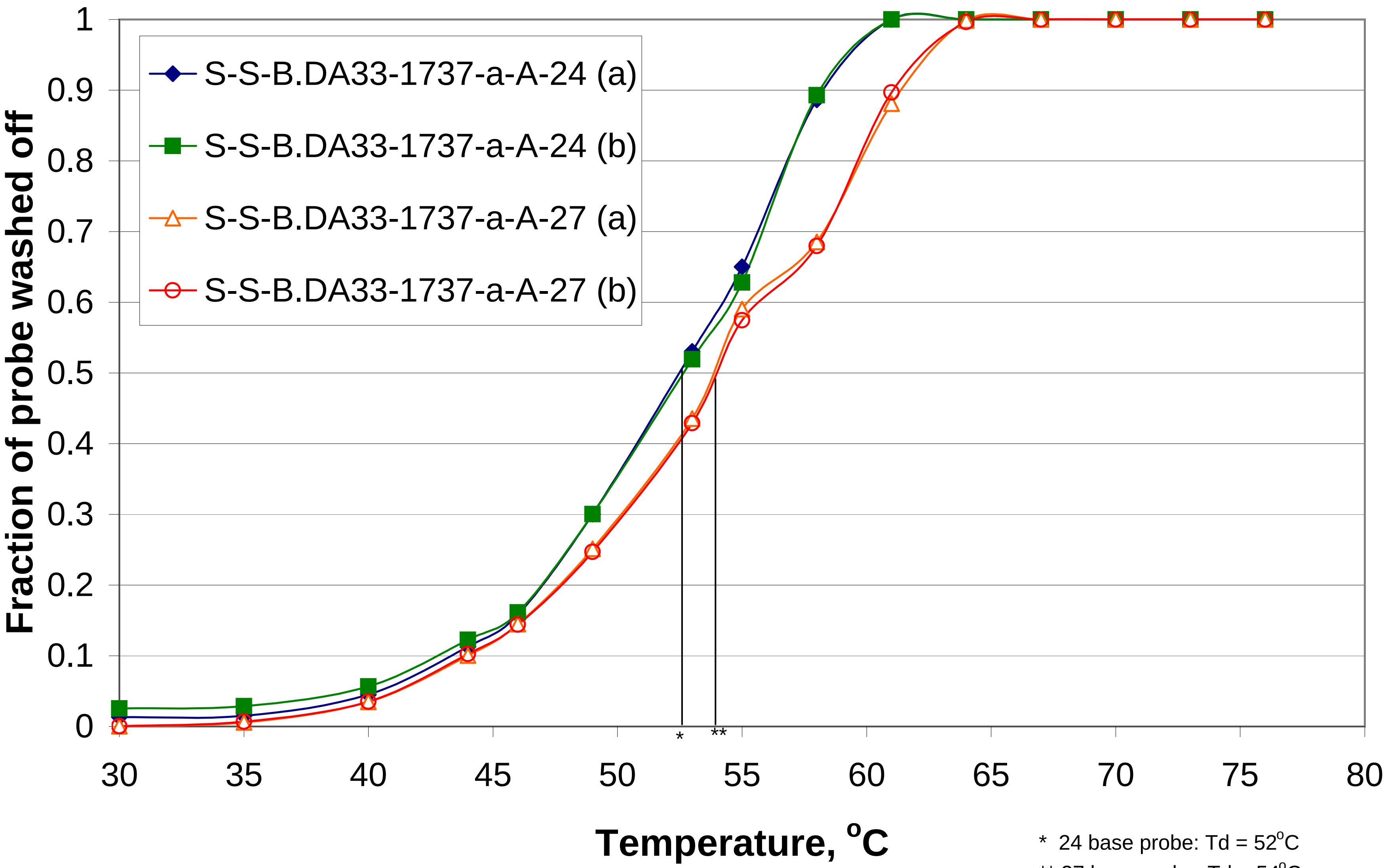


Figure 3.3. Intergenic spacer region (ISR) DNA amplified with products KF17 and KF5. Molecular weight markers are shown in lane 8. The bright bands in the image represent ITS products. These were approximately 424 base pairs in length.



* Experimentally determined T_d. A value of T = 60°C was used in experiments.

Figure 3.4. Td of the 16S probe. Probe washoff is expressed in terms of the fraction of total



* 24 base probe: Td = 52°C

** 27 base probe: Td = 54°C

Figure 3.5. Results of the T_d study performed on the ISR probes.

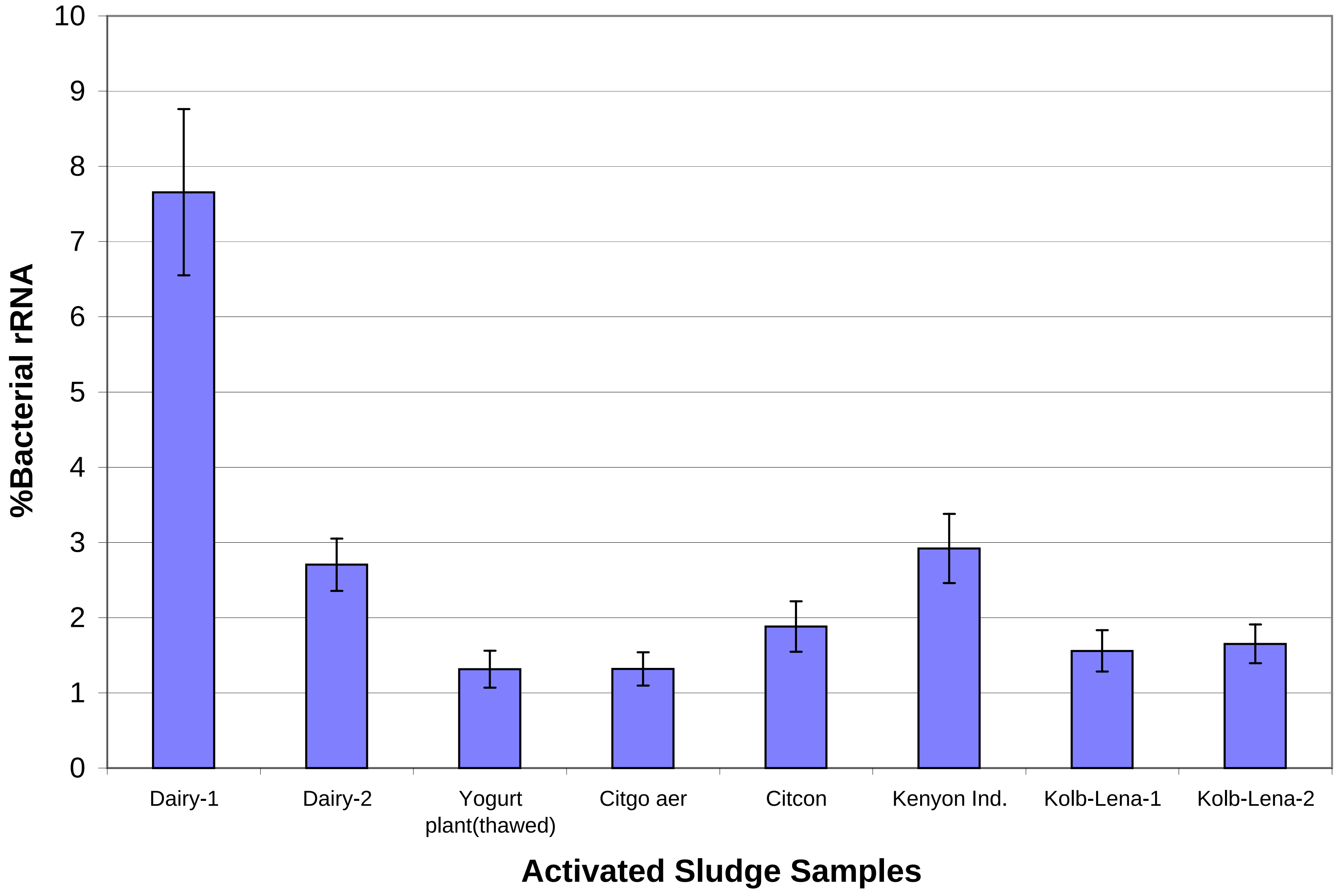


Figure 3.6. Wastewater samples tested with the *Bacillus* DA33 16S probe

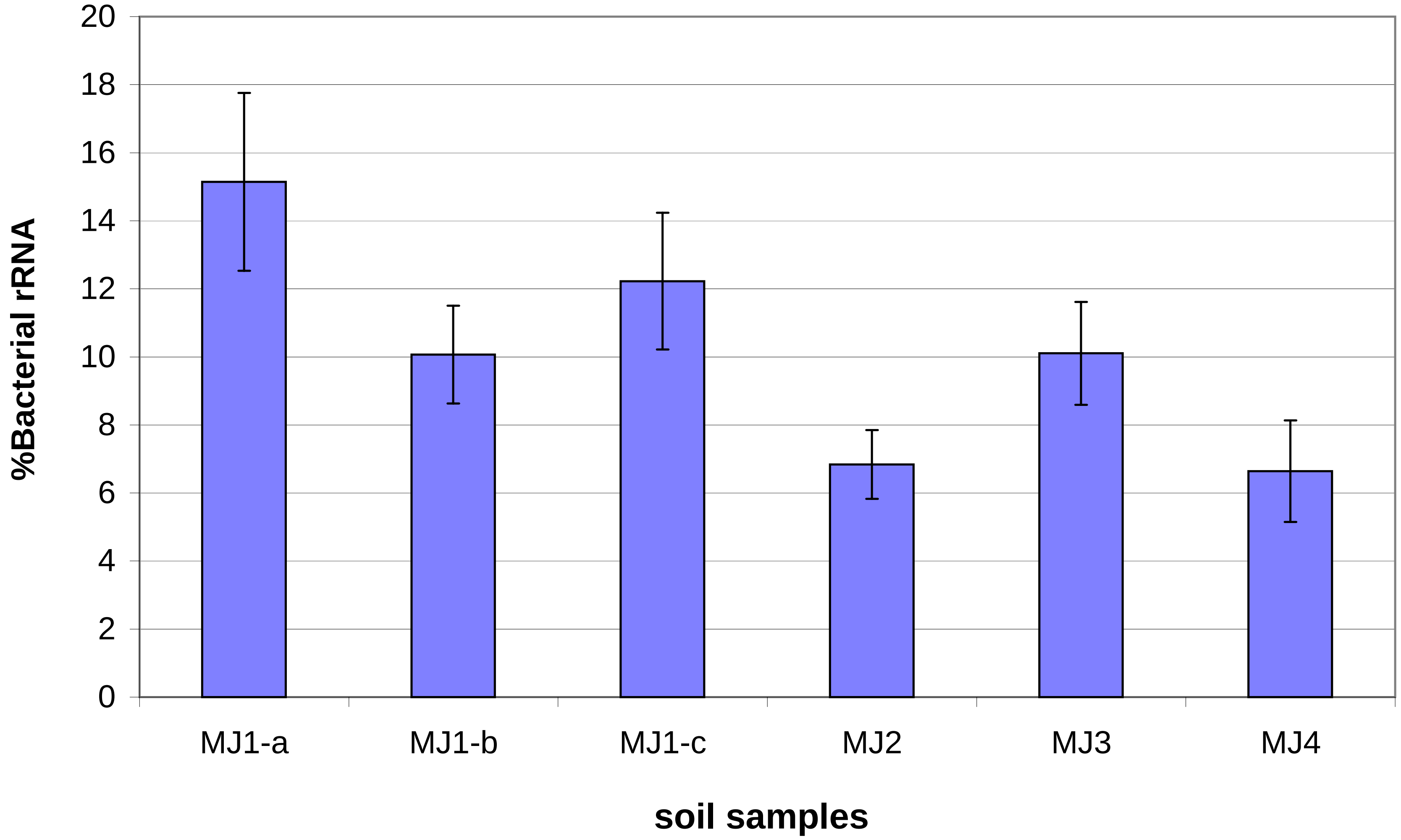


Figure 3.7. Soil samples tested with the *Bacillus* DA33 16S probe

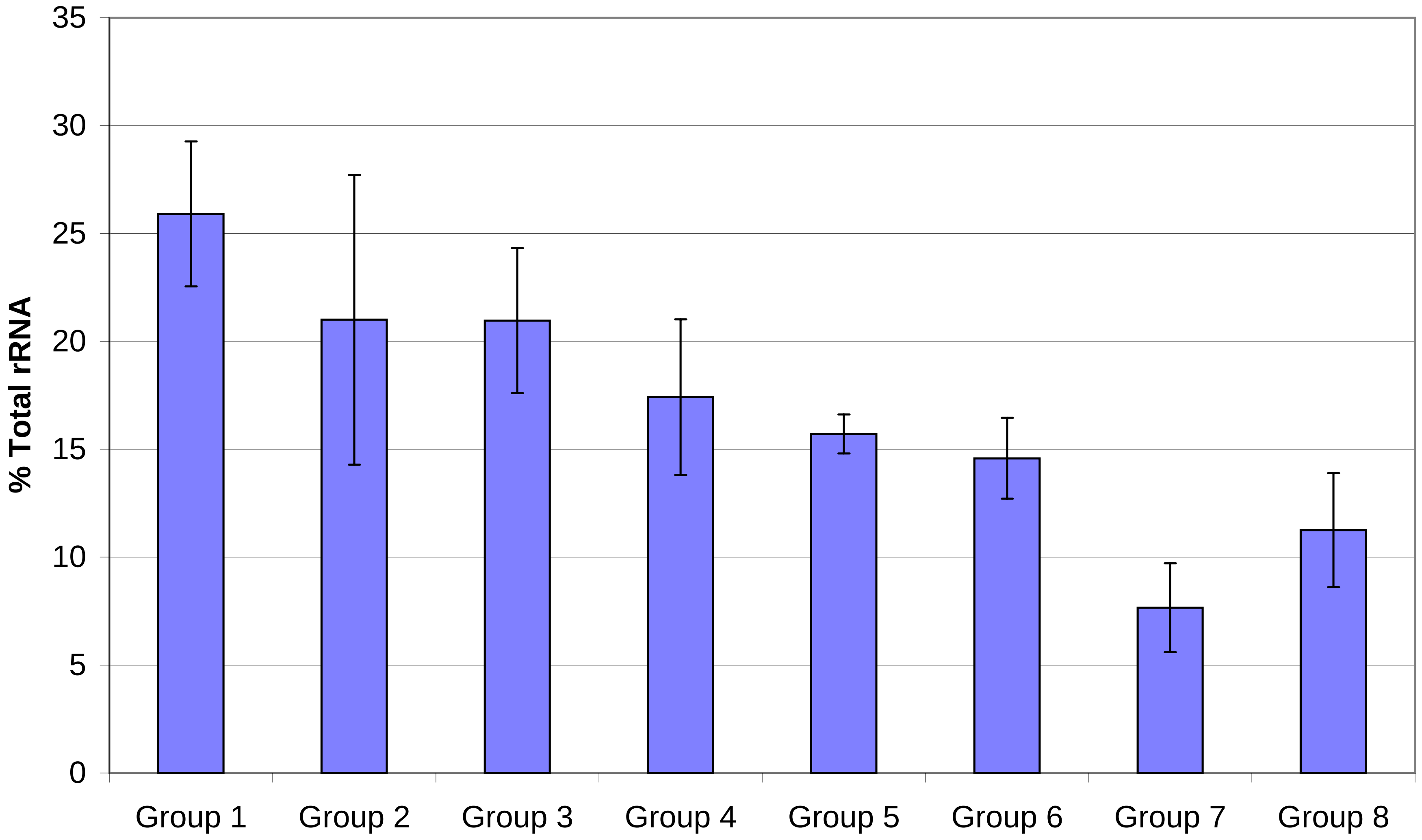


Figure 3.8. Soil samples representing treated and control plots tested with the *Bacillus* DA33 16S probe. the treated plots have been bioaugmented with B. DA33

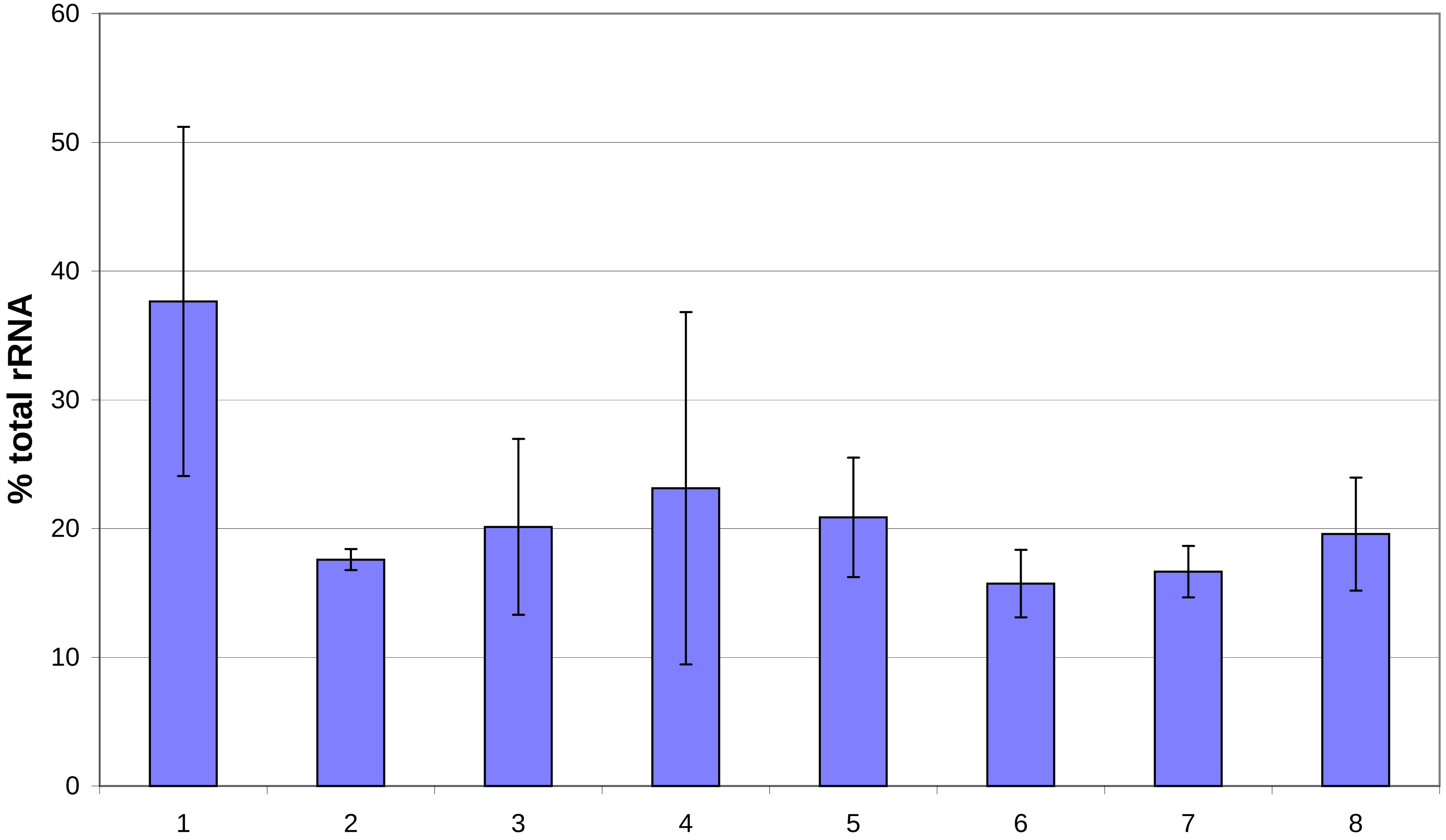


Figure 3.9. Soil samples representing treated and control plots tested with the Bacillus DA33 ISR probe. The treated plots (even numbered samples) have been bioaugmented with B. DA33.

Appendix: Survey sent to North Carolina Wastewater Treatment Plants

BACKGROUND INFORMATION

Name of plant: ______________________________

Plant Location: ______________________________

Contact Person: ______________________________

Address: ______________________________

Phone: ______________

Fax: ______________

Email: ______________

BASIC PLANT INFORMATION

Is the plant mostly treating domestic or industrial wastewater? ______________

If the plant treats industrial wastewater, please describe the type of wastewater and flow percentage.

____

Design flow (cite units, e.g., MGD) ______________________________

Is actual flow higher or lower than design flow? ______________________________

Flow range? ______________When is it high (season?) ______________

PLANT PROCESSES

If available, please include a layout of the plant's configuration showing all liquid and solid streams, including all recycle flows (e.g., supernatant from anaerobic digester, liquid fraction from thickeners, etc.). This may be important in the analysis of activated sludge problems.

(See sample layout)

DESCRIPTION OF UNIT PROCESSES

Please fill out any categories that apply. If you think some detail or special characteristics are relevant, please do not hesitate to include them.

ACTIVATED SLUDGE

What type of activated sludge system do you use (contact stabilization, oxidation ditch?)?

Number of individual units	Average HRT	Average SRT	MLSS	MLVSS	F/M ratio (units?)	Mixing device
Type of aeration/mixing	DO (range? Average?)	Average RAS concentration	Average settleability			

Do you currently have any problems with the operation of this system (bulking, foaming?)?

Is there any foam trapping between the activated sludge basin and the secondary clarifier? Where?

Secondary Clarifier:

Are there scum baffles in the secondary clarifier?

Where does the scum go?

Please describe any problems (such as foaming or bulking) in the secondary clarifier.

Digesters:

	HRT	SRT	MLSS	% VS destruction
Anaerobic digester				
Aerobic digester				

What is the shape of the anaerobic digester?

What is the gas production rate of the anaerobic digester?

Are there currently any problems with operation

Of the anaerobic digester?

Of the aerobic digester?

Are there any design problems

In the anaerobic digester?

In the aerobic digester?

Other solids treatment:

Please describe additional treatments used (i.e. gravity belt thickener, belt press)

Do any problems arise at this stage of treatment?

How do you dispose of the sludge?

SPECIFIC PROBLEMS:

Please use the scale below for questions a, b and c:

never		occasionally		requently		continually	
1	2	3	4	5	6	7	8

1. Foaming (brown scum layer on surface of aeration basin)

a. How often has this plant experienced foaming in aeration basin (scale of 1-8) _________

b: In secondary clarifier? _____________________

c. In anaerobic digester? ______________________

Is this plant currently experiencing foaming? ____________________

Has this plant ever experienced foaming? ______________________

What problems if any, have been/are being caused by foaming?

____

____

Describe the foam (please be as descriptive as possible)

____

____

2. During Foaming

How thick is foam (Average, maximum)? _____________________

Estimate percentage of foam coverage (e.g., 10%, 25%, 50%)

In aeration basin surface (average, maximum) ___________

In secondary clarifier surface? (average, maximum) ___________

What do you think is the cause of foam? (OK to guess)

____

Please describe methodology used (e.g., microscopy, observation of correlation with F/M, etc.)
____

____

Is foaming seasonal (once or twice a year), intermittent (comes and goes at random), or always present?
____

Any observed patterns to foaming? (e.g., every start of spring or fall, when industry discharges, etc.) Please describe.

____

____

Current Control Procedures (e.g., chlorine sprays, water sprays, chlorinate RAS, reduce sludge age, increase/decrease aeration, etc.)

____

____

On a scale of 1-6 (see below), rate the control procedure(s) used in the past.

Very successful		Moderately successful		Not successful	
1	2	3	4	5	6

Control procedure	Rating
_____________	_________
_____________	_________
_____________	_________

3. Bulking (sludge does not settle in secondary clarifier)

How often has this plant experienced bulking? (scale of 1-8, as above) __________

Has this plant ever experienced bulking? _______________

Range of SVI during bulking incidents: ________________

What were/are the causes of bulking?_______________________________________

Please describe methodology used (e.g., microscopy revealed filaments, if so, which filaments; changes in loading, etc.)

____

____

____

Control Procedures (e.g., chlorinate RAS, reduce sludge age, increase/decrease aeration, etc.)

____

____

On a scale of 1-6 (see below), rate the control procedure(s) used.

Very successful		Moderately successful		Not successful	
1	2	3	4	5	6

Control procedure	Rating
_____________	_________
_____________	_________
_____________	_________

Have you had any other solids separation problems (e.g., pinpoint floc, viscous bulking, etc.)? Please specify.
____

How did the plant control/manage the problem? ____

____

Has the plant ever had problems meeting standards?
-If so, please describe which standards are not met, and if you wish please estimate the fee that results from noncompliance:

____

-Are there plans to upgrade the plant in the future? If so, please list planned upgrades and time frame.

____

If you have any recommendations, suggestions, or other tips that you would like to share with other WWTPs about specific plant problems (foaming, bulking, other solids separation problems, solids treatment problems, flow problems), please feel free to write them on this space. One of the purposes of the survey is to disseminate some good advice/ share experiences with other WWTPs in North Carolina.

____

____

____

____

____

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____

____

____

Will you be interested in the results of the survey (solutions to problems, no names of specific WWTPs)?_________

If the results of this survey (solutions to problems, no names of specific WWTPs) were made available to all participating plants, do you think you will find the information useful? _____________________________

If the results of this survey (solutions to problems, no names of specific WWTPs) were made available via the World Wide Web (NC State website), will you be able to access them? _____________________________

Would you prefer the results (solutions to problems, no names of specific WWTPs) to be made available on the World Wide Web (on an NC State website) or as a printed report? _________________________________

Will your plant be interested in sending activated sludge and/or anaerobic digester samples for analysis of the microorganisms (this will be free of charge, the results will show the microbial populations in your sample)? The data will be used solely for research purposes, and you will receive a copy of the data for your plant. (YES/NO) ___

Thank you very much for your participation!