

Abstract

Liao, Jiangying. Quantifying Activated Sludge Bulking-Causative Filamentous Bacteria Using Molecular Methods (Under the direction of Francis de los Reyes.)

Filamentous bulking is a widespread problem in activated sludge wastewater treatment plants. In North Carolina, 63% of wastewater treatment plants (WWTPs) surveyed have experienced bulking. Determining the dominant bulking-causative bacteria and their level of proliferation is a necessary step in bulking control. This study used molecular techniques, i.e. quantitative Fluorescent *in situ* Hybridization (FISH) and membrane hybridization to identify and quantify the specific filamentous microorganisms and their threshold values for causing bulking in both lab scale reactors and full scale treatment plants. Filament length of a specific filamentous organism, Eikelboom Type 1851, correlated strongly with the sludge volume index (SVI) and was identified to be the major bulking-causative microorganism in lab scale reactors and a full scale activated sludge plant. The full scale plant is a biological nutrient removal (BNR) plant, a common operational mode in North Carolina, suggesting that this organism may be prevalent in North Carolina WWTPs. The threshold value for Eikelboom Type 1851-caused-bulking was determined. This threshold value will allow the monitoring of incremental improvements in control methods and the delineation of the niche of Eikelboom Type 1851 in activated sludge. Furthermore, the lab scale experiments verified the kinetic selection theory and the filamentous backbone theory for Type 1851.

Quantifying Activated Sludge Bulking-Causative Filamentous
Bacteria Using Molecular Methods

By

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A thesis submitted to the Graduate Faculty of
North Carolina State University
in partial fulfillment of the
requirements for the Degree of
Master of Science

DEPARTMENT OF CIVIL ENGINEERING
Environmental Engineering and Water Resources

Raleigh
2003

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Dedicated to my father, to whom I am so deeply indebted; my mother, who has done so much for the family; my sister, whom I hope will find her own happiness and my nephew, whom I hope will be somebody someday.

Biography

Jiangying Liao, also known as Joanne, got her Bachelor of Science degree from College of Environmental Science, Engineering and Management of Nankai University, Tianjin, China. There she was exposed to all facets of knowledge concerning environmental problems. She then came to the United States to pursue her Master of Science degree in Department of Civil Engineering of North Carolina State University. As a graduate student, she focused on the research of activated sludge filamentous bulking problem using molecular techniques under the direction of the very nice Dr. Francis de los Reyes. She is now eager to see the big big world out there and her wish is to be a better person everyday.

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OVERVIEW

The activated sludge process, the most widespread technology for wastewater treatment at present, is an engineered, enhanced self-purification process of a water body. The engineering intensifies the treatment, but basic understanding of the microorganisms and their activity under different conditions are keys for successful operation. This study is focused on the microorganisms causing one of the most common activated sludge problems - filamentous bulking. According to the filamentous backbone theory proposed by Sezgin *et al.* (1978), bulking is due to the excessive growth of filamentous bacteria that interfere with the compaction and settling of the activated sludge either by producing a very diffuse floc structure or by growing in profusion beyond the confines of the floc into the bulk medium and bridging between flocs (Jenkins *et al.*, 1993). Despite the attention that researchers and practitioners have focused on this problem, it is still not clear how and why certain filaments cause bulking.

There have been suggestions for controlling bulking. Remedial methods, e.g., addition of oxidants or talc have been investigated (Saayman *et al.*, 1996; Eikelboom and Grovenstein, 1997) but these only treat the consequences or symptoms and do not “cut the weeds and remove the roots”. Biological control methods, (including kinetic selection and metabolic selection), most thoroughly studied by Chudoba and colleagues (Chudoba *et al.*, 1973a; Chudoba *et al.*, 1973b; Chudoba *et al.*, 1973c; Chudoba, 1989; Chudoba, 1985), and Blackall *et al.* (1991) are intended to suppress the growth of filamentous bacteria. These control methods are designed to either impose a high process loading factor on the biomass to provide a selective advantage for those microorganisms with the ability to take up readily biodegradable substrate at high rates, or control the

terminal electron acceptor to eliminate certain filaments. However, traditional biological control measures do not always work because of problems with identifying the major bulking-causative bacteria and the lack of knowledge of the filaments' environmental niches.

In 1975, Eikelboom published a breakthrough paper, defining “Keys” using morphology, physiology and staining characteristics to identify filamentous organisms in activated sludge (Eikelboom 1975). The Eikelboom keys have been invaluable to wastewater professionals. However, this identification tool has serious limitations. It was found that one Eikelboom “Type” might not consist of one single phenotype, and that morphological characters are not reliable indicators for distinguishing phylogenetic groups because the morphology of some filaments is known to change (Howarth *et al.*, 1999).

To gain insight into activated sludge bulking, we asked the following research questions: For a given activated sludge sample, which filamentous bacteria are dominant? Under what conditions do certain filaments cause bulking, and to what extent? How does operation affect the bulking problem? To attempt to answer these questions, we conducted batch, lab and full scale studies. Model organisms were selected and studied in pure culture experiments, full-scale wastewater treatment plant performance was monitored over time and related to filamentous bacteria levels, and lab scale reactors were operated to simulate various operating environments. This work uses molecular techniques in addition to classical chemical and biological tests to give a more complete and detailed picture of what is occurring inside the activated sludge black box. The

accurate identification and quantification of bulking-causative organisms may guide future activated sludge modeling and the development of rational control measures.

Molecular Methods:

Although it has been widely accepted since the late 1980's that the massive growth of around 30 different filamentous "Types" is responsible for the decreased settleability of activated sludge, the effects and contributions of specific filament "Types" or species on bulking is not known. Filamentous bulking studies have been limited primarily by the inadequacy and inaccuracy of traditional identification methods that use morphology, physiology and staining characteristics and the lack of quantitative methods for determining filamentous biomass *in situ*. The growth of 16S and 23S ribosomal RNA sequence databases has enabled researchers to use rRNA-targeted hybridization for studying activated sludge biomass. Oligonucleotide probes targeting specific domains, genera, species, or even strains have been developed. These probes can be hybridized either to isolated nucleic acids immobilized on membrane filters (membrane hybridization or Northern blots) or directly to the rRNA in whole, fixed cells (whole-cell hybridization or Fluorescent *In Situ* Hybridization). These phylogenetic methods have the following advantages compared to the traditional identification methods. (1) Molecular methods are accurate: unlike morphology, physiology and staining characteristics, conserved phylogenetic identity does not change over time or under different conditions. (2) The methods can yield target-specific, quantitative data. This study is based mainly on rRNA-targeted oligonucleotide hybridizations, with occasional use of traditional methods for comparison.

Fluorescence *In Situ* Hybridization (FISH)

FISH originated in cytogenetics (Vandekken *et al.*, 1990) and clinical research (Heitz *et al.*, 1991) and is now applied intensively in environmental microbiology. The method combines the specificity of nucleic acid sequences with the sensitivity of detection systems based on fluorochromes. FISH combined with digital image analysis (use of a digital camera, image analysis software and computer) makes it possible to obtain not only qualitative information from the samples but also quantitative data. FISH uses fluorescent dyes for detection. The rRNA probes are conjugated with fluorochromes and are excited by incandescent light (in epifluorescence microscopy) or a laser (in confocal laser scanning microscopy). The probe, whose own sequence is complementary to the target rRNA sequence, will only bind to specific rRNA in target cells, and the rest of the fluorescent probe will be washed off. The samples are fixed onto microscopic slides and only the cells containing the target sequence will give a sufficient signal above background when viewed under a proper light source.

Membrane hybridization

In membrane hybridization, the method of signal indication is radioactivity instead of fluorescence. Ribosomal RNA samples and standards series are blotted onto a membrane, and radiolabeled 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 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).

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Chapter One: Development of a Quantitative FISH Method for Determining the Biomass of Bulking-Causative Filaments in Activated Sludge

Introduction:

Filamentous bulking is caused by the excessive growth of filamentous organisms that extend from flocs into the bulk solution and interfere with compaction, settling, thickening and concentration of activated sludge (Jenkins, *et al.*, 1993). Several researchers have related filamentous organism level (both cell counts and filament length) to activated sludge settling properties. Finstein and Heukelekian (1967) first showed that the SVI of activated sludge could be related to the total filament length per floc. The total extended filament length (TEFL) in activated sludge was discovered to correlate with sludge volume index (SVI), diluted SVI (DSVI) and zone settling velocity (which are measures of activated sludge settling) (Jenkins *et al.*, 1993). However, these efforts identified the lumped effects of all filaments in the sludge, and the individual contribution of particular filaments remains unknown.

The objective of this study was to develop a method to quantify the specific contribution of individual filament species to bulking. This is important because (1) given any condition, only one or two filaments may dominate the sludge; and (2) it may require different levels of filaments to cause bulking so the total filament threshold may not be accurate and useful to direct the control of specific filament-caused bulking. This information can be used in activated sludge modeling and serve as a guide for WWTP

operators. Using quantitative Fluorescent *in situ* Hybridization (FISH), species-specific organisms can be identified from the original sludge sample without extraction of RNA, and their levels can be measured by the combined use of a microscope, digital camera, computer, and image analysis software. We assumed that the ratio of filament length to volatile suspended solids level of an organism in pure culture was similar to that in mixed liquor of activated sludge, allowing the use of the correlations established for the pure culture experiment in activated sludge samples. By measuring the filament length in the sludge, the specific biomass of that organism can be obtained.

Previous attempts to conduct quantitative image analysis of activated sludge used area coverage, percent coverage, cell number or cell size distribution (Li *et al.*, 1997). Other workers used different shape parameters to classify the objects as floc or filaments. Five shape parameters were evaluated and the reduced radius of gyration (RG) were identified as the most suitable parameter for determining filament to floc ratio (Cenens *et al.*, 2002). RG is based on the moments of an object (Pons *et al.*, 2000) and quantifies how dispersed the pixels in an object are from their centroid (Cenens *et al.*, 2002). In our experiment, we used manual count for filament length measurement.

In contrast to quantitative FISH, membrane hybridization can quantify the rRNA level of specific filaments in activated sludge samples. Both approaches were used in this study and the results were compared.

Sphaerotilus natans has been historically acknowledged as a bulking-causing microorganism in wastewater. It is a sheath forming bacterium with rod-shaped cells and belongs to the beta-subclass of Proteobacteria. The cells are about 2 μm in width and the filament can be 1000 μm in length (Jenkins *et al.*, 1993). *Sphaerotilus natans*

has been the focus of research in the 1970's (Yoshikawa and Takiguchi, 1979; Vanveen *et al.*, 1978; Takiguchi *et al.*, 1978; Dondero, 1975; Curtis *et al.*, 1971; Bott *et al.*, 1970) and is still detected around the world (Lacko *et al.*, 1999; Liu *et al.*, 2002; Pellegrin *et al.*, 1999). *Leucothrix mucor* has always been compared to *Thiothrix* spp. and Eikelboom 021N due to their similar morphology. Though it is often argued that *Leucothrix* is a marine organism, many non-marine *Leucothrix*-like bacteria have been discovered (Williams and Unz, 1985; 1989). It was also discovered to cause bulking for a petrochemical wastewater treatment facility (Poffe *et al.* 1979). Since there is still unresolved debate on *L. mucor*, it is important to investigate its significance in activated sludge. For these reasons, we initially chose *S. natans* and *L. mucor* as our model organisms for investigating the contribution of specific filamentous bacteria to bulking.

Materials And Methods:

Sampling

Sampling packages consisting of a cooler, ice packs, and 50-mL centrifuge tubes were sent to the Greenville Wastewater Treatment Plant (WWTP). Grab samples were taken by wastewater operators from the activated sludge mixed liquor in 50-mL tubes, and sent overnight to the North Carolina State University Environmental Engineering laboratory. For membrane hybridizations, 14-mL samples were centrifuged at 2,000 x g and cell pellets were stored at -80°C until nucleic acid extraction.

Organisms Used And Growth Conditions

Pure cultures of *S. natans* (ATCC 13395) and *L. mucor* (ATCC 25107) were grown in CYGA medium (5 g/l casitone, 10 g/l glycerol, 1 g/l yeast autolysate) and ATCC medium 429 (11.7 g/l NaCl, 5.35 g/l MgCl₂, 2 g/l Na₂SO₄, 0.75 g/l CaCl₂·2H₂O, 0.35 g/l KCl, 0.5 g/l Tris buffer, 0.05 g/l Na₂HPO₄, 10 g/l sodium glutamate, pH 7.6) respectively at 26°C in a shaker at 100 rpm.

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 65°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 µL 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 NH₄Ac 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 µL of sterile water. The quality of

extracted RNA was evaluated using polyacrylamide 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).

Oligonucleotide Probes

The oligonucleotide probes used in quantitative membrane hybridizations and FISH are listed below.

Table 1.1. Probe names, sequences, target groups and hybridization conditions used in this study

Probe name ^a	Target group	Probe use	T _w (°C)	Formamide %	Reference
S-*-Univ-1390-a-A-18	Almost all organisms	Membrane	44	N/A	Zheng <i>et al.</i> , 1996
S-D-Bact-0338-a-A-18	domain Bacteria	Membrane	56	N/A	Amann <i>et al.</i> , 1990
S-S-S.nat-0656-a-A-18	<i>Sphaerotilus natans</i>	Membrane/ FISH	46	45	Wagner <i>et al.</i> , 1994, this study
S-S-L.muc-0652-a-A-18	<i>Leucothrix mucor</i>	Membrane/ FISH	65	35	Wagner <i>et al.</i> , 1994a this study

^a Probe names have been standardized according to the Oligonucleotide Probe Database (Alm *et al.*, 1996).

For FISH, the *S. natans* and *L. mucor* probes were obtained from Sigma-Genosys (The Woodlands, Texas) and Integrated DNA Technologies, Inc. (Coralville, IA). The probes were labeled with Cy3 or Oregon Green (fluorescent dyes with red or green signals respectively). For membrane hybridization, oligonucleotides were obtained from Sigma-Genosys (The Woodlands, Texas). Probes were 5' end labeled with [γ^{32} -P]ATP (ICN Radiochemicals, Irvine, California). The probe labeling reaction was performed using 1-5 μ L probe, 1-3 μ L [γ^{32} -P]- ATP (ICN Biomedicals; Costa Mesa, CA), 1 μ L T₄

polynucleotide kinase (Promega Corp.; Madison, WI), 3 μ L 10x polynucleotide kinase buffer (Promega Corp.; Madison, WI), 1.5 μ L 1% Igepal type NP40 (Sigma-Aldrich Chemicals; St. Louis, MO) and water to a final volume of 30 μ L. The reaction proceeded for 30-45 minutes at 37 °C. Labeled probe was purified using mini-Quick Spin Oligo Columns (Roche Diagnostics Corp.; Indianapolis, IN), following the manufacturer's instructions.

Membrane Hybridizations and Dissociation Temperature Study

RNA at an initial concentration of 100 ng/ μ L was diluted 1:3 in 2% glutaraldehyde and denatured for 10 minutes at room temperature. rRNA samples were then diluted in sterile water containing RNase free bromophenol blue to a final concentration of 1 ng/ μ L. 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/ μ L. Samples and standards were applied in triplicate to a MagnaCharge nylon membrane (Osmonics; Minnetonka, MN).

Purified probe was added to membranes, and hybridized overnight at 40 °C. Membranes were then washed twice for 30 minutes in 100 mL wash buffer (1% sodium citrate, 1% sodium dodecyl sulfate) at 40 °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 standard 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 mass of group, Bacterial and total rRNA for each sample. RNA quantities for group specific probing 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 analyses were performed on the basis of total (Universal probe) values. RNA of standards and selected samples were examined on a non-denaturing polyacrylamide gel.

Since the *S. natans*-specific and *L. mucor*-specific probes were originally designed for FISH, optimal wash conditions after membrane hybridization were determined by performing dissociation temperature (T_d) studies using an elution method (Zheng *et al.*, 1996). The temperature at which 50% of the hybridized probe was washed off was determined to be the T_d of the probe. Nucleic acid samples from *S. natans* and *L. mucor* cultures (100 ng) were slot-blotted in duplicate to nylon membranes (Magna Charge, Micron Separation Inc., Westboro, MA). Baked membranes were prehybridized for 2 h at 40°C and hybridized overnight at 40°C. Subsequently, the membranes were washed in 100 ml of 1% SDS-1X SSC (0.15 M NaCl, 0.015 M sodium citrate) twice for 1 h at 40°C. The membranes were then cut into separate individual hybridized blots. Each blot was washed in 3 ml 1% SDS-1X SSC for 10 min at the first temperature (30°C). The blot was then removed and transferred to a new scintillation vial with a

wash solution at the next higher temperature, and washes were continued at increasing temperatures until a temperature of 80°C was reached (total of sixteen temperatures). The amount of probe released at each wash temperature was quantified by liquid scintillation counting using a Model 1600CA Liquid Scintillation Analyzer (Packard Instruments, Downers Grove, IL).

FISH and Filament Length Quantification

To develop the relationship of filament length (quantified by image analysis after FISH) to biomass, 100 ml batch cultures of *S. natans* and *L. mucor* were grown and fixed using 4% (w:v) paraformaldehyde for 2 h at 4°C, and stored in phosphate buffered saline/ethanol (1:1, v:v) at -20°C until FISH. 3 µl of sample was applied to each microscopic slide (Supercured Heavy Teflon-coated slides, Cel-Line Associates Inc., Newfield, NJ) well and air dried before serial immersion into 50%, 80% and 96% ethanol for 3 min each. Then slides were incubated at 46 °C until hybridization. Hybridization buffer consisted of sodium chloride, Tris-HCl, 45% formamide for *S. natans* and 35% for *L. mucor* and a final concentration of 1% sodium dodecyl sulfate (SDS). 8 µl of buffer at 46 °C was put on each well and 1 µl of specific probe was applied to each buffered well. The slides were incubated in moisture chambers at 46 °C for 2 hours before washing. The wash buffer contained sodium chloride, EDTA and Tris-HCl, with a final concentration of 1% SDS. The slides were washed at 48 °C for 15 min. The slides were dipped in cold 4'6-diamidino-2-phenylindole-2HCl (DAPI; Sigma, St. Louis, MO.) solution followed by ice-cold water briefly and air dried quickly. The slides were mounted in Citifluor (Citifluor Ltd., London, United Kingdom) to prevent

deterioration of fluorescence signal. Images were captured with a Photometrics CCD camera mounted on a Nikon Optiphot II fluorescence microscope. Images were analyzed for filament length using Metamorph software (Universal Imaging Corp., Silver Spring, MD).

FISH was conducted to dilutions of pure cultures of *S. natans* and *L. mucor*. Total and volatile suspended solids were determined in triplicate by Standard Methods (1994). At each dilution, three wells on the microscope slide were used. Ten random fields were captured from each well. The total filament length from each image field was then determined manually, and the means and average standard deviation were computed. When the length measurement for a field gave a standard deviation/mean ratio of greater than 0.4, the filament dispersion was judged to be non-uniform, and the result was discarded (Hernandez *et al.* 1994). Filament length per volume (L_v , $\mu\text{m}/\text{ml}$) was calculated using the following equation (Hernandez *et al.*, 1994)

$$L_v = [(L_f \times A_w)/(V \times a)] \times \text{DF} \quad (1)$$

where:

L_f = average total filament length per field (μm)

A_w = area of well (μm^2)

V = volume of sample applied (ml)

a = area of microscope field (μm^2)

DF = dilution factor

The same sample size was used in determining the levels of filament length in activated sludge samples. For activated sludge samples, two independent observers performed the measurements and the average of the measurements were obtained.

Statistical analysis

Data was log-transformed and the probability of rejecting the null hypothesis of no difference between two dilutions was determined by the F-statistics using the StatView software package (SAS Institute; Cary, NC).

Results And Discussion:

Dissociation Temperature Study

The *S. natans*-specific and *L. mucor*-specific probes were originally designed for FISH, and therefore optimum wash conditions in membrane hybridizations have not been previously determined. An accurate determination of the post-hybridization wash temperature is crucial in order to assure probe specificity. Wash temperatures of the *S. natans* and *L. mucor* probes are shown in Figures 1.1 and 1.2, respectively. The results of the T_d study showed that wash temperatures of 46 °C and 65 °C corresponded to 50% elution of hybridized probes, and represented specific probe binding to *S. natans* and *L. mucor* rRNA. These temperatures were adopted in subsequent hybridizations.

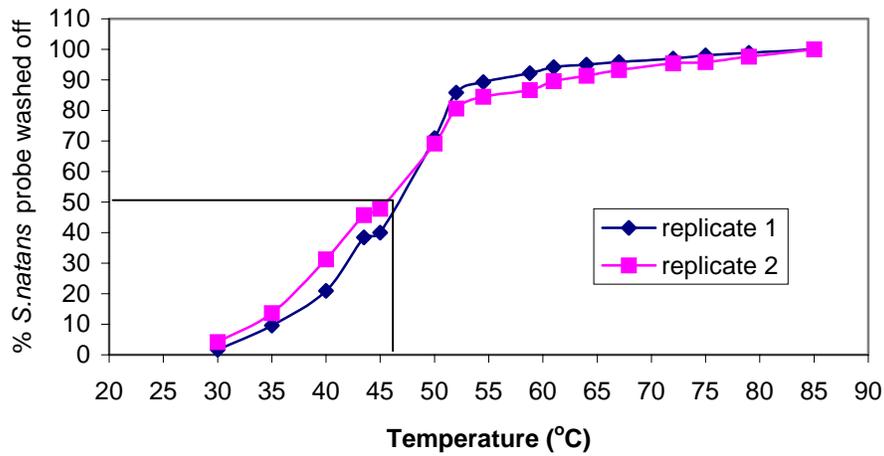


Figure 1.1. Optimum wash temperature for probe S-S-S.nat-0656-a-A-18.

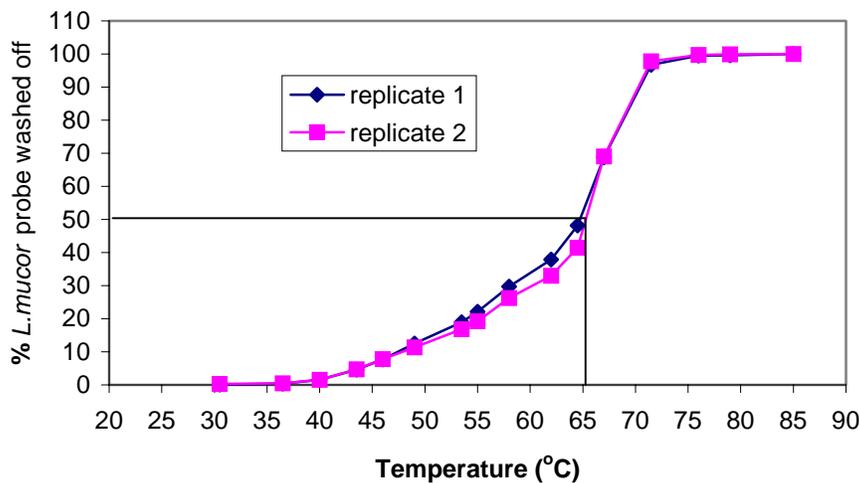


Figure 1.2. Optimum wash temperature for S-S-L.muc-0652-a-A-18.

Quantitative FISH

FISH has been used extensively to identify bacterial populations *in situ* in activated sludge. However, the use of FISH for generating quantitative data is still limited. A novel approach for quantifying filamentous biomass *in situ* was developed for analyzing filamentous foaming (Hernandez *et al.*, 1994; de los Reyes *et al.*, 1998).

The same approach was used in this study for *S. natans* and *L. mucor*. A comparison of

manual measurement of filament length and automatic measurement with image analysis software (data not shown) showed that manual measurement is more accurate. The filaments often go in and out of the floc, and the auto-thresholding function of the software could not distinguish the filament from background adequately. Manual counting involves some human judgment, but was deemed more accurate than the systematic and random error of auto-thresholding. Thus, manual measurement was used in all FISH experiments.

The correlation between filament length and VSS for *S. natans* is shown in Figure 1.3. The correlation for *L. mucor* is shown in Figure 1.4.

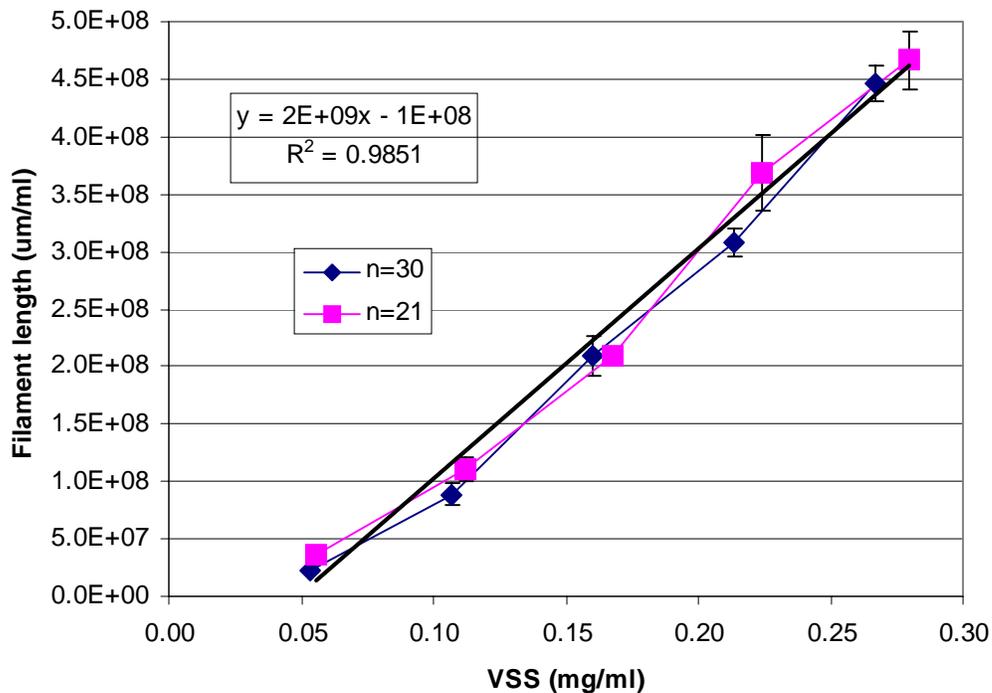


Figure 1.3. Correlation between filament length determined by FISH and *S. natans* biomass.

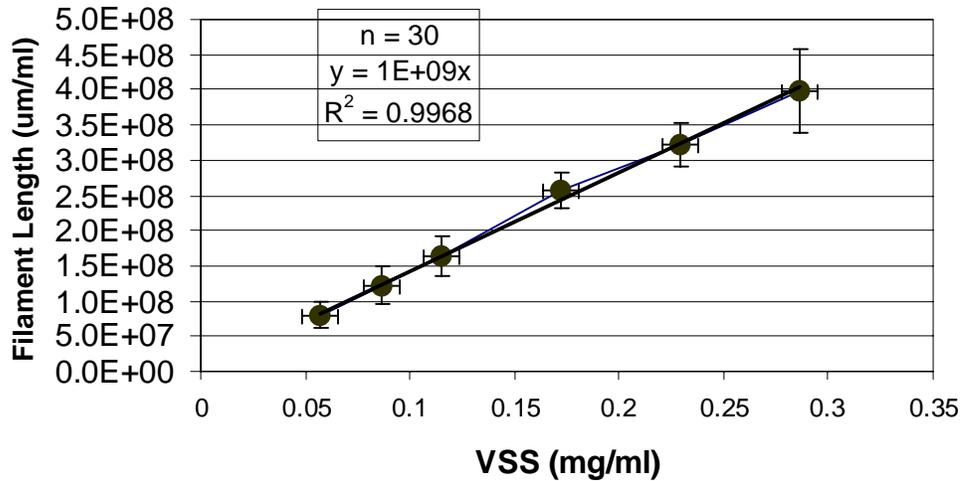


Figure 1.4. Correlation between filament length determined by FISH and *L. mucor* biomass.

Two independent cultures of *S. natans* were grown, and the regression curve for both cultures resulted in the same regression equation, showing the reproducibility of the data. The regression curve did not go to zero, which is caused by the difficulty for detecting *S. natans* cells under the level of 0.05 mg VSS/ml sample. This curve thus should not be used when the cell concentration is below this level. According to the table that shows power as a function of sample size determined by the F-statistics, three wells and ten pictures per well results in a power of 0.5 for random data, which is acceptable for detecting the true difference between dilutions. Since we already discarded data sets that were considered non-uniform, we consider this sample size adequate for the purpose of this study. The fraction of *S. natans* in environmental samples could be estimated using the following equation (Hernandez *et al.*, 1994):

$$\% (g \text{ } S. \text{ natans}/g \text{ VSS}) = \{ [R_c \times (L_v + 1 \times 10^8)] / \text{VSS} \} * 100\% \quad (2)$$

where:

R_c = regression constant from Figure 1.3 (4.93×10^{-10} mg/ μm)

L_v = average length per volume from (1)

VSS = volatile suspended solids concentration (mg/ml)

The biomass of concentration of *L. mucor* can be determined using a similar equation, with the respective regression coefficient (7×10^{-10} mg/ μm). In general, the biomass of any filament can be determined using this method.

The regression coefficient for *L. mucor* is slightly larger than that for *S. natans* and they are both in the same order of magnitude as the regression coefficient for *Gordonia amarae* (4.14×10^{-10}) determined earlier (de los Reyes, *et al.* 1998). Since *L. mucor* is normally 2-4 μm in diameter while the other two have 1.5 μm and 1 μm diameters respectively, these data indicate, that the different species should have comparable density.

Samples from Greenville WWTP were obtained and analyzed using quantitative FISH and membrane hybridization. Since it is impossible to detect the difference of the organisms in pure culture and in wastewater samples, and we do not expect too much change in cell properties under these two conditions, we used the regression equation to determine the biomass contribution of *S. natans* to the total biomass was determined. *Leucothrix* was not detected in the Greenville WWTP sample so the comparison between the two methods was not carried out.

Based on FISH, the biomass contribution of *S. natans* in the Greenville plant mixed liquor was 5.13% of the total VSS, which is slightly lower than the contribution of *S. natans* to the total rRNA (7.2%) determined by membrane hybridization (Figure 1.5).

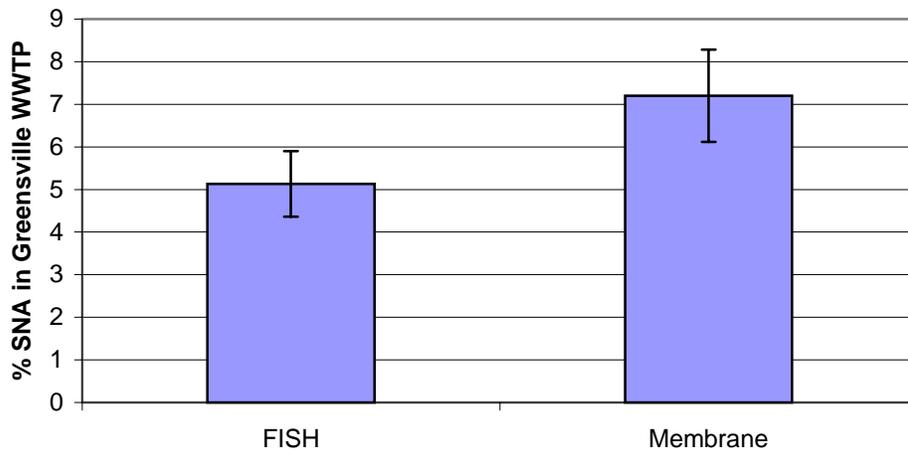


Figure 1.5. Comparison of *S. natans* levels obtained by quantitative FISH and membrane hybridization in the Greenville WWTP

While the results of the two assays are comparable, it should be remembered that the two methods measure different parameters. Hybridization to extracted rRNA measures the lumped effects of cell abundance and cell activity, since rRNA content is related to growth activity. Therefore, it may be hypothesized that *S. natans* has an equal, or slightly higher contribution to the total growth activity, compared to its contribution to the total biomass. In comparison, very slow growers such as *Gordonia* have the opposite effect. *Gordonia* cells apparently have lower intrinsic rRNA content, and have a higher fraction of inactive cells (de los Reyes *et al.*, 1998).

The limit of detection of the quantitative FISH method can be greatly improved by optimizing the dispersion of activated sludge samples during the FISH procedure. Bright signals from filaments were observed inside flocs; however, portions of the filaments disappeared from the image plane, and therefore were not measured with the FISH method. A representative FISH micrograph is shown in Figure 1.6. Here, portions

of the filament decrease in fluorescence intensity as part of the filament disappear from the image plane into the floc. Optimizing floc break-up (e.g., by sonication), or the use of confocal laser microscopy to reduce out-of-focus fluorescence may help in addressing these problems.

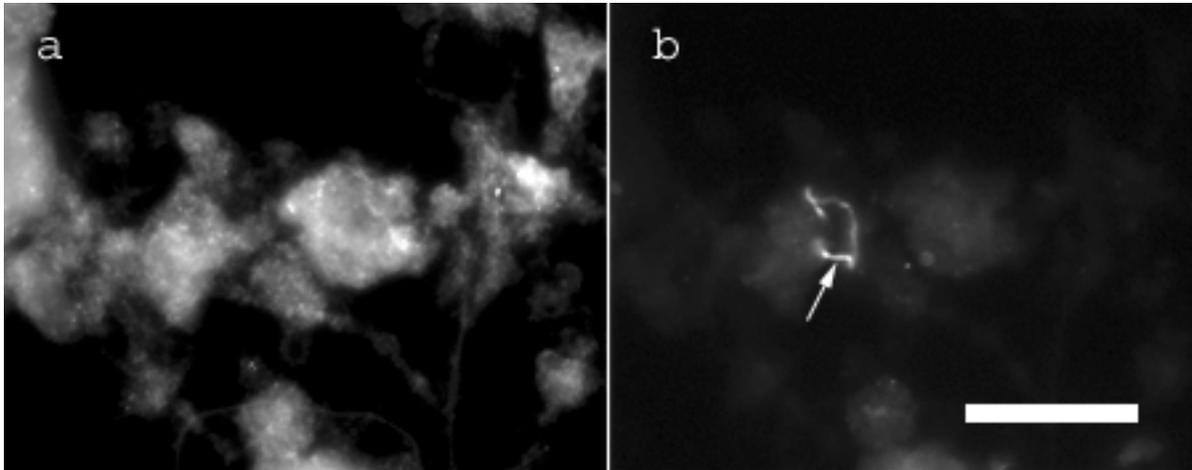


Figure 1.6. *Sphaerotilus natans* in activated sludge (Greenville plant) detected by FISH. a. DAPI micrograph showing all cells with DNA. b. Epifluorescence micrograph of the same image field. The arrow shows a bright portion of the filament. Bar = 90 μm .

Because bulking is more logically related to cell biomass than cell rRNA contribution, FISH appears to be a better method to study bulking. However, due to the detection limit of FISH, combining the two hybridization formats is a better approach than FISH or membrane hybridization alone. By increasing the number of image fields analyzed, the detection limits of the quantitative FISH method should be improved. The quantitative assessment of specific filaments in activated sludge will allow the tracking of filamentous organisms during bulking, and will be key tools in relating the growth and proliferation of specific filaments to specific operating conditions.

Conclusions:

A quantitative method for estimating *S. natans* and *L. mucor* biomass in activated sludge was developed. Before this study, it was impossible to quantify specific bulking-causing filamentous biomass in an activated sludge system. This study related the measurable filament length to the species-specific biomass and reveals the specific contribution to bulking of individual filaments. The results of quantitative FISH and membrane hybridization for *S. natans* were comparable for the Greenville WWTP sample. The combined use of these two hybridization assays represents a new approach of studying this filament, and will be a useful tool in studies of filamentous bulking.

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Chapter Two: Determining Species-Specific Filamentous Bulking Thresholds Using Molecular and Reactor Studies

Introduction:

Bulking is normally considered to occur when the sludge volume index (SVI) reaches 150 ml/g. Previous work showed that above a certain level of total extended filament length (TEFL), the SVI increases rapidly above 100 ml/g. The TEFL level corresponding to SVI 150 can thus be considered the threshold for bulking to occur due to all the filamentous bacteria present (Jenkins *et al.*, 1993). Recent research correlated the relative levels of rRNA and volatile suspended solids (VSS) of *Gordonia spp.* to another activated sludge problem – foaming (de los Reyes and Raskin, 2002), but no research has related the specific rRNA level or VSS of specific filamentous bacteria to bulking. With the quantitative FISH method developed in Chapter One, the correlation curves between filament length and VSS for *Sphaerotilus natans* and *Leucothrix mucor* can be used to determine the threshold value of biomass for *Sphaerotilus* and *Leucothrix*-caused bulking, while the specific rRNA contribution of the filaments can be determined using membrane hybridization.

Because a full-scale wastewater treatment plant (WWTP) sample with *Sphaerotilus* or *Leucothrix*-caused bulking was not obtained, lab-scale reactors were set up to simulate bulking. The approach was to quantify the increase in filament length of specific organisms before and during bulking. This would allow the estimation, for the first time, of species-specific bulking thresholds. These thresholds can then be used to

monitor incremental improvements in control measures and can thus serve to direct rational control methods.

The laboratory scale reactors also allowed the direct testing of the kinetic selection theory. Kinetic selection is achieved by imposing a high process loading factor on the biomass, thereby providing a selective advantage for those microorganisms with the ability to take up readily biodegradable substrate at high rates (Grady *et al.*, 1999). The goal is to produce a substrate concentration at the inlet to the bioreactor that favors the growth of floc-forming bacteria at a faster rate than that of the filamentous bacteria. This can be accomplished by providing plug-flow-like conditions within the bioreactor. The three lab scale reactors we set up are one completely stirred tank reactor (CSTR), one fast feeding sequencing batch reactor (SBR) and one slow feeding SBR. The fast feeding SBR is the closest set-up to a plug-flow reactor (PFR). Therefore, it is supposed to provide the substrate gradient that favors the floc-forming bacteria, and be the best reactor configuration for controlling bulking. On the other hand, the CSTR has the smallest substrate concentration in the reactor and favors the high substrate affinity filamentous bacteria, and is thus expected to be the easiest configuration to achieve bulking. The slow feeding SBR is an intermediate process and is expected to have a performance in between the PFR and the CSTR.

Material and methods:

Flask Experiment

The first experiment was performed similar to one described by Seka *et al.* (2001). Three 2L-flasks were set up as CSTRs by shaking at 100 rpm. The CSTRs were

fed with nonfat dry milk powder at a concentration of 1 g/L (approximate COD content: 1g COD/g milk). The milk was prepared daily to avoid degradation and the feed solution was continuously mixed by a magnetic stir bar. Polyethylene tubing connected the milk jar and the flasks, with three pump heads driving the milk solution into the reactors. The tubings were kept as short as possible to minimize their effect on flow rate. The configuration is as shown in Figure 2.1.

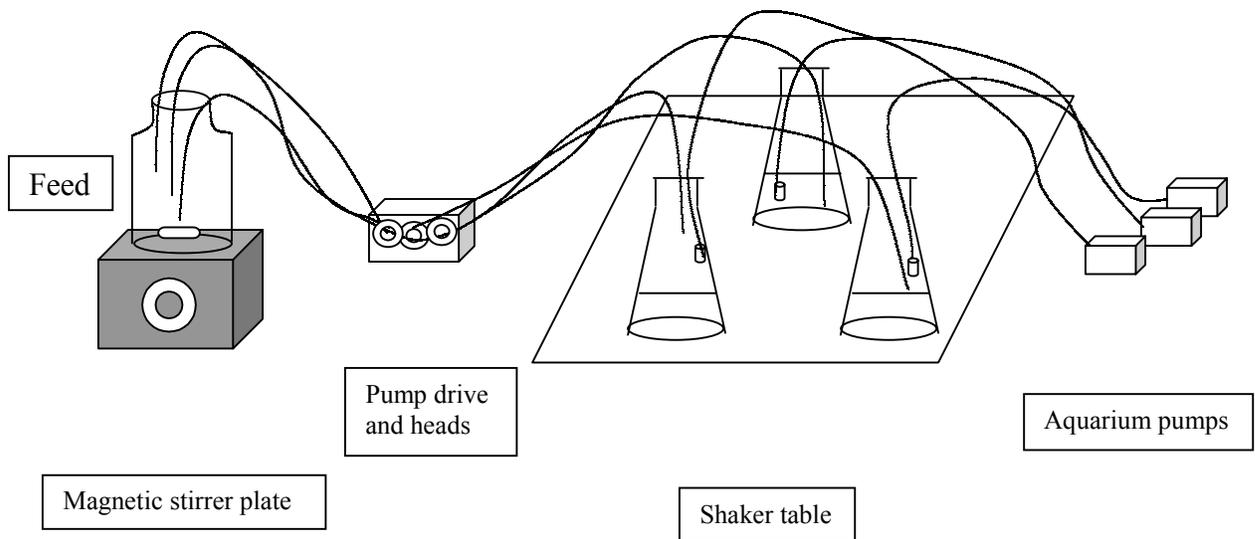


Figure 2.1. Configuration of CSTR for first bioaugmentation experiment

Activated sludge was taken from the return activated sludge tank of the Neuse River Wastewater Treatment Plant, Raleigh, NC. One liter of sludge was settled to 100 ml before being added to the flasks. Different volumes of *S. natans* and *L. mucor* cells were fed to the flask and after one day and two days of growth, the diluted sludge volume indexes (DSVIs) of the sludge were measured in a one-liter graduated cylinder.

The initial experiments were improved in subsequent runs with the following changes: aeration as well as mixing was provided by aquarium pumps because both *S.*

natans and *L. mucor* are aerobic bulking organisms and because the shaker in the first experiment did not provide enough mixing to keep the sludge in suspension.

Lab Scale Reactors

Three batch scale reactors were set up to simulate different treatment conditions. One was operated as a CSTR and the other two were operated as sequencing batch reactors (SBR). The three reactors each consisted of a 12 cm diameter plastic column with an effective reaction volume of 8 L. The CSTR was operated with an additional conical glass clarifier with a 10 cm diameter opening. Configurations are shown in Figure 2.2.

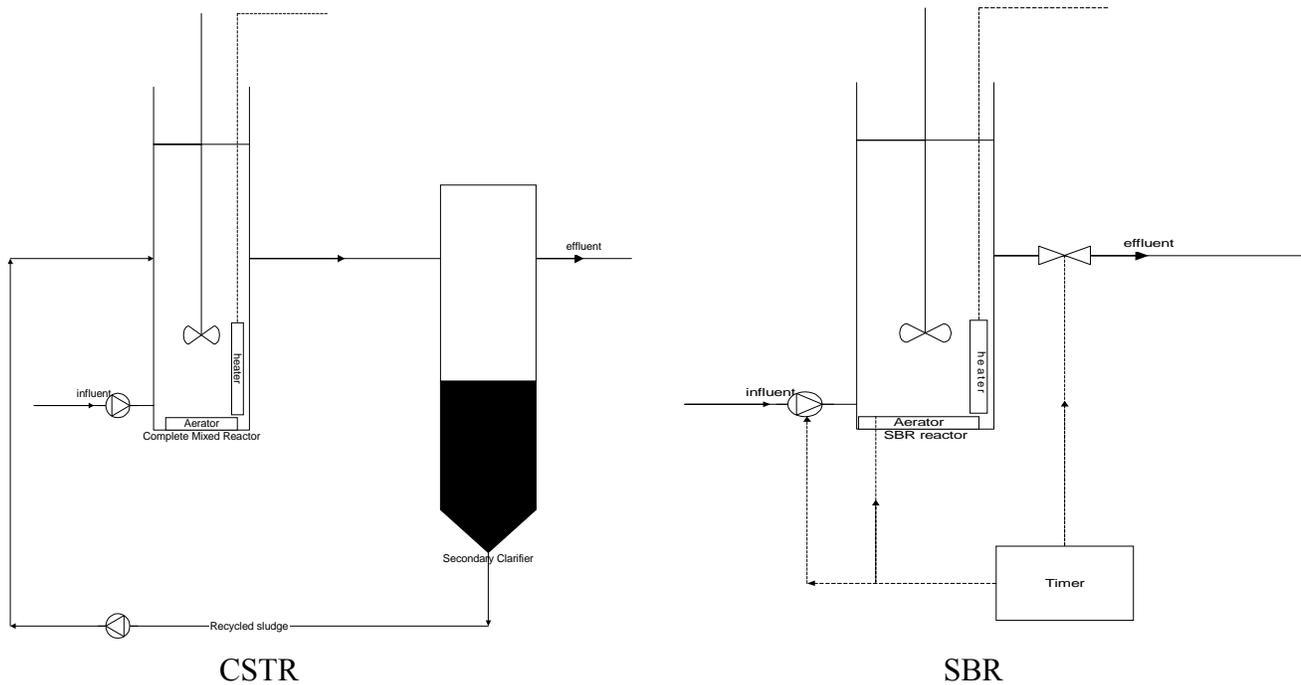


Figure 2.2. Configuration of lab scale reactors

The inflow rates and sludge recycle ratio of the CSTR and the influent and effluent flow rates of the two SBRs were controlled by two Masterflex peristaltic pumps (Cole-Parmer, Vernon Hills, IL). The operational conditions are specified in the Operational Stages section. The operation of feeding, mixing, aerating, settling, discharging, and the cycle of periods for the SBRs were set by time controllers (ChronTrol Corporation, San Diego, CA). Mixing was achieved using Stir-Pak Laboratory mixer (Cole-Parmer, Vernon Hills, IL). Dissolved oxygen was measured using YSI 5100 Dissolved Oxygen Meter (Yellow Spring Instruments, OH). All three reactors were aerated by aquarium pumps. The temperature was maintained at 22 ± 1 °C.

Sludge volume index (SVI), dissolved oxygen (DO), chemical oxygen demand (COD), mixed liquor suspended solids (MLSS), mixed liquor volatile suspended solids (MLVSS), total nitrogen (TN) and total phosphorous (TP) were measured periodically by Standard Methods (1998).

The reactors were fed by synthetic wastewater, with a COD, TN and TP of approximately 550 mg/L, 80 mg/L and 60 mg/L respectively. The components are shown in Table 2.1.

Table 2.1. Composition of synthetic wastewater (mg/L)

Glucose	250	KCl	7
Yeast extract	150	NH ₄ Cl	150
MgSO ₄ ·7H ₂ O	50	KH ₂ PO ₄	196.4
MnSO ₄ ·7H ₂ O	5	NaHCO ₃	555.6
FeSO ₄ ·7H ₂ O	2.2	CaCl ₂	3.8

The reactors were seeded with activated sludge mixed liquor from the North Cary Water Reclamation Facility (NCWRF). The SVI of the seed sludge (1/17/2002) was 253 ml/g.

Operational Stages

Depending on experimental needs and requirements, the operational conditions were modified. The experimental run was divided into three stages:

Stage I: Since the SVI of the seed sludge exceeded the standard value viewed as the indication of bulking (150 ml/g), the first task was to reduce the filament level in this sludge and start the experiment from a non-bulking status. In this stage, two SBRs were operated simultaneously under alternating anaerobic and aerobic conditions. One cycle period was 8 hr, which consisted of 2 hr non-aeration, 4 hr aeration, 2 hr settling and discharge. In each cycle, two-thirds of the effective volume ($16/3$ L) was replaced by fresh influent, setting the Hydraulic Retention Time (HRT) to 12 hr, and 500 ml of mixed liquor was removed from the reactors, setting the Solids Resident Time (SRT) to 16 days. The DO was controlled to 0.03 mg/l by adjusting the aeration.

Stage II: The objective of this stage was to investigate the effect of addition of the filamentous organism *Sphaerotilus natans* on sludge bulking in the reactors. Pure cultures of *S. natans* were grown to a total mass of 0.4 grams and harvested. *S. natans* cells were added to the mixture of the sludge from both reactors to a final concentration of 0.017g cells/L, which is 0.5 % of the total sludge. The new sludge was evenly

distributed into the three reactors described above. The operational conditions were the same as Stage I, except that aeration was kept on during the whole reaction phase in Stage II. The influent volumes for the three reactors were the same, but the feeding times were different: 12 min for SBR (A); 3 hr for SBR (B); 24 hr for CSTR (C), respectively. Using these feeding times effectively made SBR A simulate a plug flow reactor, while SBR B simulated a reactor with flow characteristics between a PFR and a CSTR.

Stage III: The objective of the third stage was to suppress the filamentous growth and return the sludge in the reactors back to non-bulking status. In this stage, all three reactors were operated as SBRs with the same operational conditions as in Stage I.

Quantitative FISH

FISH samples were taken almost every other day. Bead-beating as pretreatment was performed for Stage I samples to ensure probe accessibility because of the granular, compacted status of the sludge. FISH was performed before and after inoculation of *S. natans*. Quantification of filament lengths was performed as described in Chapter One. With Stage II sludge, two sets of measurements were carried out: (1) the total filament length determined from the signal of the species-specific probe illustrated and (2) the portion of the filaments that is outside the floc, or the extended filament length, where the floc boundary was defined by 4',6-diamidino-2-phenylindole-2HCl (DAPI) staining and the autofluorescence of the floc structure. DAPI is a fluorescent probe which binds to DNA and is excited by UV light. The DAPI signal shows all the cells containing DNA.

Results and Discussion:

To quantify the threshold value of filament length for *Sphaerotilus*- and *Leucothrix*-caused bulking, we needed to find a sample plant with bulking problem caused mainly by *Sphaerotilus* or *Leucothrix*. As *Sphaerotilus* thrives with high amount of soluble substrate (Contreras *et al.*, 2000; Donkin and Russell, 1997; Gaudy and Wolfe, 1961), and has been observed in paper mill plants (Pellegrin *et al.*, 1999), several paper mill plants were contacted for samples. FISH was performed on these samples but very few *Sphaerotilus* cells were detected. Similarly we could not find a bulking WWTP with *L. mucor* as the dominant species. We therefore decided on another experimental approach - enrich augmented *S. natans* or *L. mucor* in an environment favoring its growth to the level that will cause bulking.

Flask Experiments

In the first attempt, the diluted SVI (DSVI) of a mixture of 900 ml milk and 100 ml sludge was measured. Since the DSVI did not change, the milk had no effect on the sludge settleability so any change in DSVI should be due to the bioaugmentation of microorganisms. *Leucothrix mucor* was used as the test organism because the growth in pure culture was in filament form. A total mass of 0.2 g cells was added to one of the flasks. Twenty-four hours after inoculation, the DSVI of the milk-sludge mixture decreased from 175 L/g to 135 L/g. After 38 hrs, the DSVI was 140 L/g. We postulated that heterotrophs in the sludge were consuming the milk substrate and growing faster,

making the VSS increase while having no effect on the settled sludge volume, causing the SVI to decrease.

In a second experiment, new sludge from Neuse River WWTP with a DSVI of 280 L/g was used. 100, 200 and 400ml of *L. mucor* with a VSS concentration of 0.28 g/L was added to the three flasks. After 80 hrs, the DSVIs were 280 L/g (no change), 260 L/g (decrease, with a more compact, tiny-flaked sludge) and 290 L/g (minor increase), respectively.

FISH was conducted and the predominant filaments in the sludge did not hybridize with the *L. mucor* probes, indicating that *Leucothrix mucor* had not incorporated into the indigenous sludge successfully.

400 ml of *Sphaerotilus natans* at a concentration of 0.3 g/L was then added in a similar experiment to the Neuse River WWTP sludge and the SVI changed from 250 to 235 ml/g. The negative result is easier to explain: the cells were growing as rods in pure culture and were added to the reactors in this form. A micrograph of reactor sludge after one day of *S. natans* inoculation is shown below.

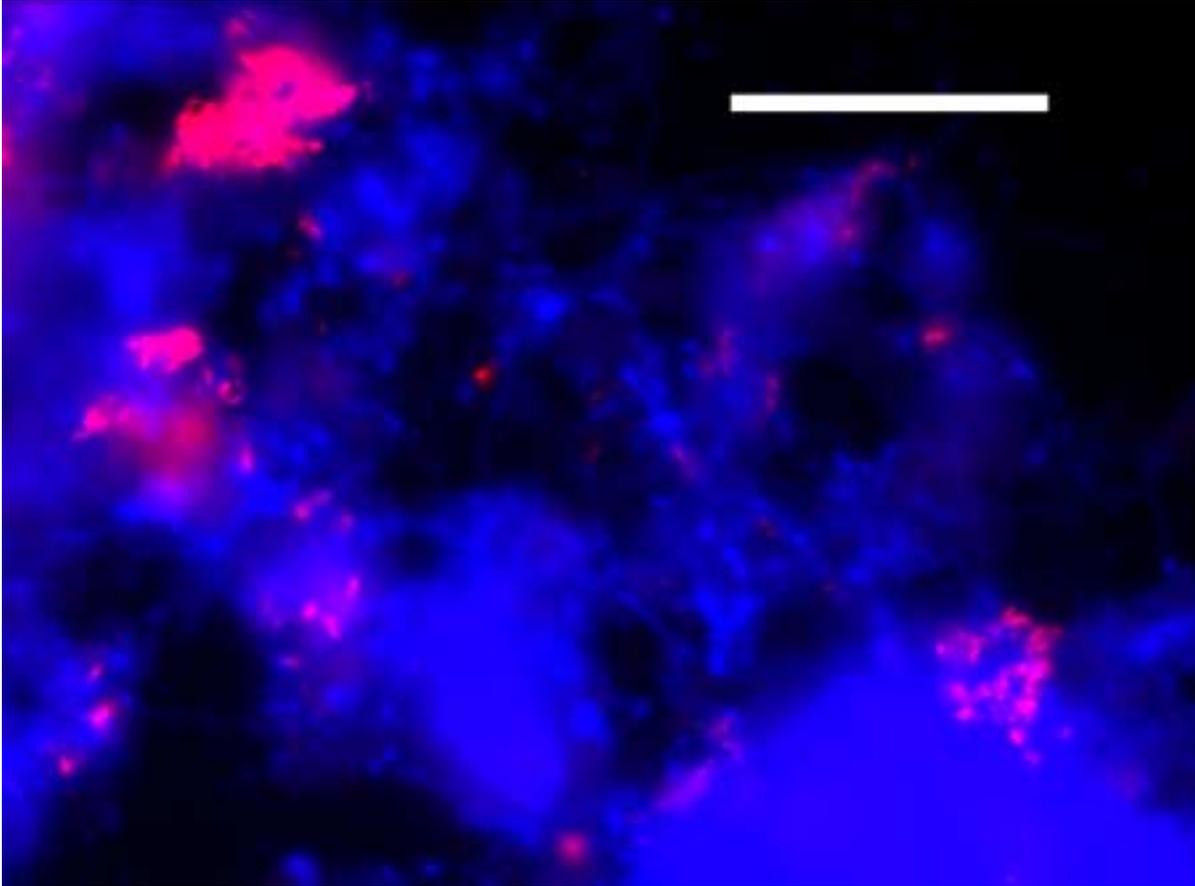


Figure 2.3. *S. natans* after one day inoculation. Bar=50 μm . Red signals are *S. natans* cells.

After the failure of the first attempt, we concluded that the reactors (2L flasks) were too small and the run time was not long enough for bulking to occur. We then decided to conduct a new set of experiments with laboratory reactors.

Laboratory scale Reactors

An important factor affecting sludge settlability is floc formation. For a good floc to form there must be a balance between floc-forming and filamentous bacteria. Too many filaments push the floc apart and keep the sludge from flocculating and settling.

Floc formers are generally thought to have low substrate affinity and high substrate utilization rate, and thus are favored at higher substrate concentrations. On the other hand, filaments have higher substrate affinities and low substrate utilization rate. In a CSTR, filaments are more likely to form because of the lower substrate concentration in the reactor. On the contrary, a selective pressure for floc formers is present in a SBR, where substrate gradients occur over time. Also, most filamentous bacteria only use oxygen as terminal electron acceptor while many of floc formers can take up readily biodegradable organic matter under either anoxic or anaerobic conditions. Anaerobic conditions can thus provide a powerful selective pressure against filamentous bacteria. This is the concept of metabolic selection (Grady and Daigger, 1999).

In Stage I, the objective was to reduce the SVI to less than 150 ml/g. By transferring the sludge from NCWRF to the lab scale reactors, and running the reactors as SBRs with an anaerobic stage followed by an aerobic stage, a substrate gradient that favored the floc forming bacteria was created.

At the beginning of Stage I, a portion of the activated sludge was wasted through the exit port (1/3 of effective height from the bottom). After several days, the MLSS started to increase and after one-month's operation reached 4 g/L. MLSS of both SBRs was maintained at 3.5-4.0 g/L. The SVI of both reactors decreased from 270 ml/g at the beginning of this stage to around 50 ml/g. Microscopic observation showed that most filamentous bacteria were washed out or decayed. One month after setup, the SVIs of both the reactors decreased to around 40ml/g. This stage lasted for more than 4 months

(1/17/02-5/27/02) until the sludge stabilized to a yellowish, granular appearance with good settleability. At this point the bioaugmentation experiment was begun.

In Stage II, *S. natans* pure cultures were added to stimulate bulking. One week after the addition of *S. natans* and change of the operational condition, reactor C (CSTR) began bulking suddenly, and the SVI increased from 92.9 ml/g (June 5) to 198.2 ml/g (June 6). At that time the SVI in reactor B (SBR) also increased from the original 31 ml/g to 90.2 ml/g, while the SVI for reactor A (SBR) remained constant at about 40.3 ml/g. Starting on June 10, SBR B started to experience bulking, with SVI of 217.6 ml/g. The sludge bulking in SBR B and CSTR continued, with the SVI reaching the maximum of 558 ml/g in SBR B and 1283 ml/g in CSTR. Since the SVI was too high to be an accurate measurement (Jenkins *et al.* , 1993), DSVI was used to measure the sludge settleability. During the entire Stage II, the SVI in SBR A did not change much, and the values remained within the 40.3-83.8 ml/g range.

In Stage III, all of three reactors were changed into SBRs and operated as in Stage I. The SVIs of the three reactors decreased sharply, and within one SRT returned to non-bulking status with a final concentration of 52.4 ml/g in SBR A, 129 ml/g in SBR B and 72.5 ml/g in CSTR. This stage lasted for 10 days (7/02/02 to 7/12/02).

Since SBR A had the highest substrate gradient, it was expected to be best for filamentous bulking control; the CSTR had the lowest substrate gradient and was

expected to experience bulking; and the slowly fed SBR B was expected to behave in between the two extreme conditions. The SVI data confirmed the above hypothesis.

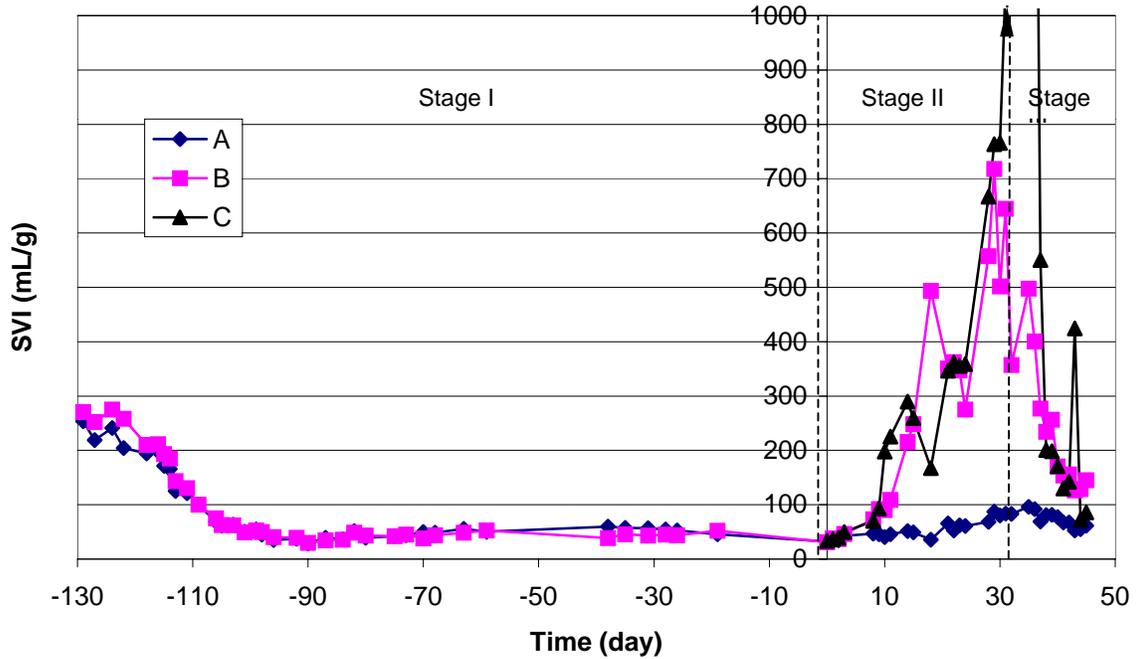


Figure 2.4. SVI changes over time

The level of *Sphaerotilus natans* increased dramatically the day after inoculation from 1.0×10^8 to 7.2×10^8 $\mu\text{m}/\text{ml}$, then slightly went down and stabilized at the level of 6.5×10^8 $\mu\text{m}/\text{ml}$ (Figure 2.5). FISH showed that instead of *S. natans*, Eikelboom Type 1851 was the dominant filament in the sludge during the whole course of bulking. Stage II micrographs showing the increasing abundance of this filament is shown in Figure 2.6.

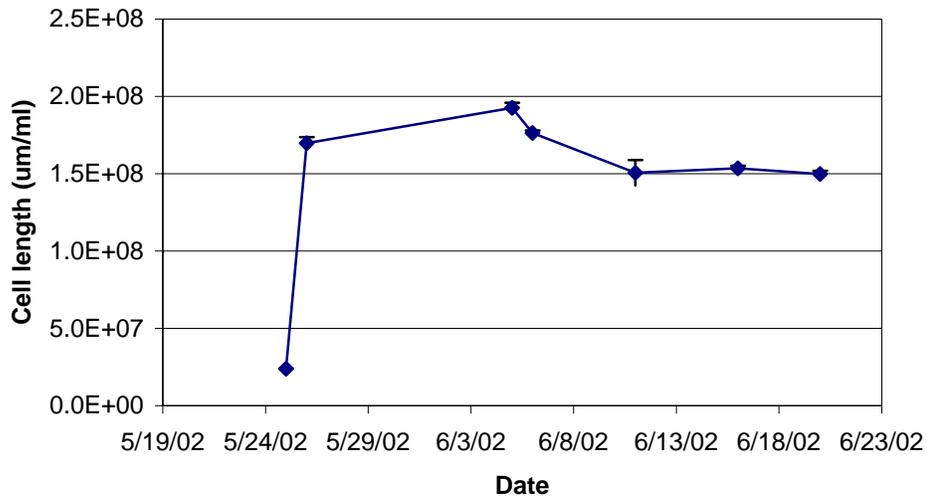


Figure 2.5. Change in length of *S.natans* in reactor C over time

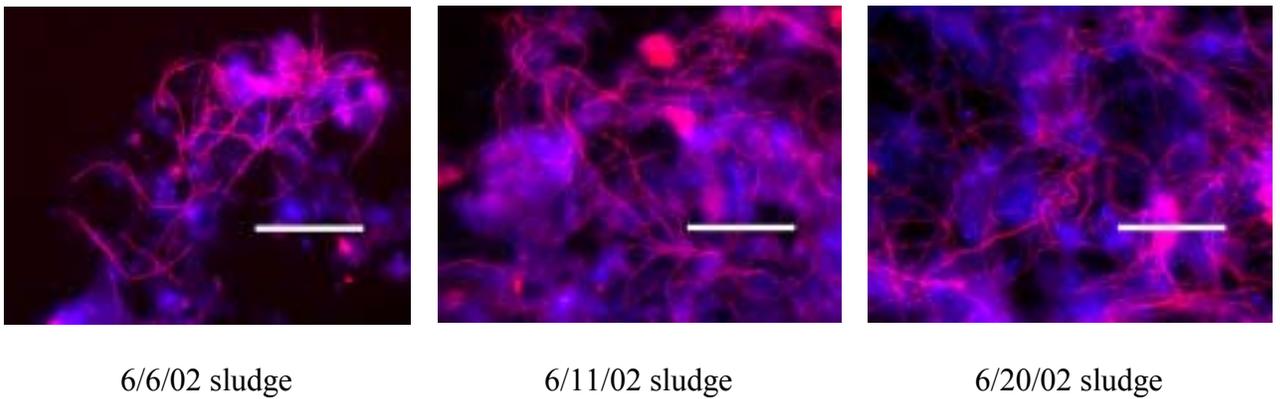


Figure 2.6. Increase of Type 1851 levels over time in reactor C

In reactor A (SBR), high levels of Type1851 were measured, but there was no correlation between Type 1851 filament length and reactor SVI (Figure 2.7). When reactor A had the highest level of total filament length, the ratio between the extended filament length (measured as filament outside the floc) to the total filament length (inside + outside the floc) was around 40%.

In reactor C (CSTR), quantitative FISH showed that SVI and filament length of Type 1851 strongly correlated with each other ($R=0.96$), which suggests that Type 1851 proliferation may be the cause of bulking in reactor C. The result is shown in Figure 2.8. Compared to reactor A, the filaments were mostly outside the floc, and the extended to total ratio for reactor C is around 70%. Therefore, what is important in bulking-causing incidence is not the total filament length but the extended filament length.

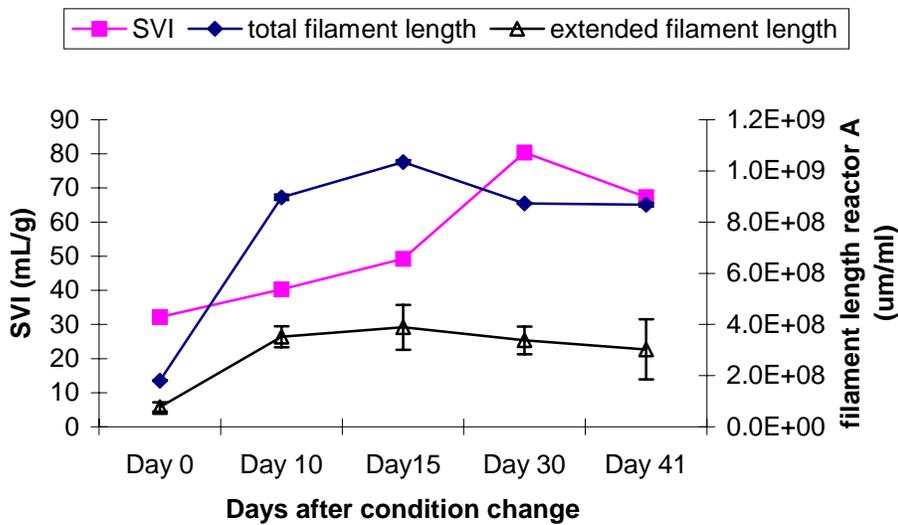


Figure 2.7. SVI vs. filament length curve for reactor A

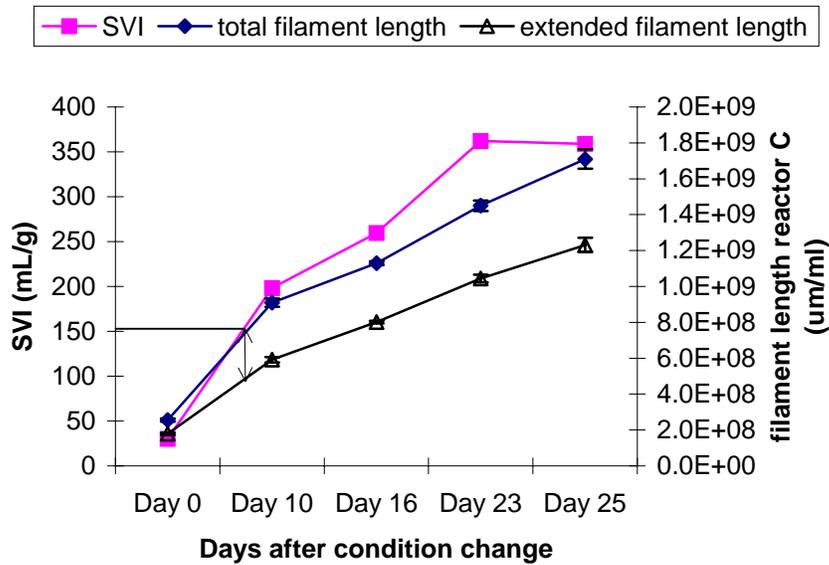


Figure 2.8. SVI vs. filament length curve for reactor C

According to SVI 150, the species-specific bulking threshold for Type 1851 is estimated to be $8 \times 10^8 \mu\text{m}$ total filament length/ml or around $6 \times 10^8 \mu\text{m}$ extended filament length/ml. The highest total filament length level in reactor A exceeded the threshold value determined by reactor C data without causing bulking because the extended portion was only 40% that value. This observation confirms the filamentous backbone theory, proving that extended filaments cause bulking. Meanwhile, substrate diffusion seems to be another important factor affecting filamentous bulking since filamentous bacteria have to extend outside of floc to get the substrate in bulk solution when substrate does not diffuse into the floc efficiently.

However, this result is a little surprising because previous studies have shown that in general, the SVI increases rapidly above 100 ml/g when total extended filament length values increased above $10^7 \mu\text{m/ml}$ (Jenkins *et al.*, 1993). The specific threshold value for

Type 1851 is one order of magnitude greater than the general one, which suggests different filaments contribute differently to bulking.

Thiothrix spp. was discovered to also increase in number during the time of this experiment. When bulking initially happened, Type1851 was definitely dominant, indicating that the bulking causing bacteria was Type1851; however as the experiment proceeded, *Thiothrix spp.* co-dominated in reactor C with Type1851. Figure 2.9 shows *Thiothrix spp.* in the CSTR. Because reactor C was bulking, the reactor had severe loss of solids, which made it difficult to quantify the filaments after June 21. Only qualitative observations of this phenomenon were thus performed.

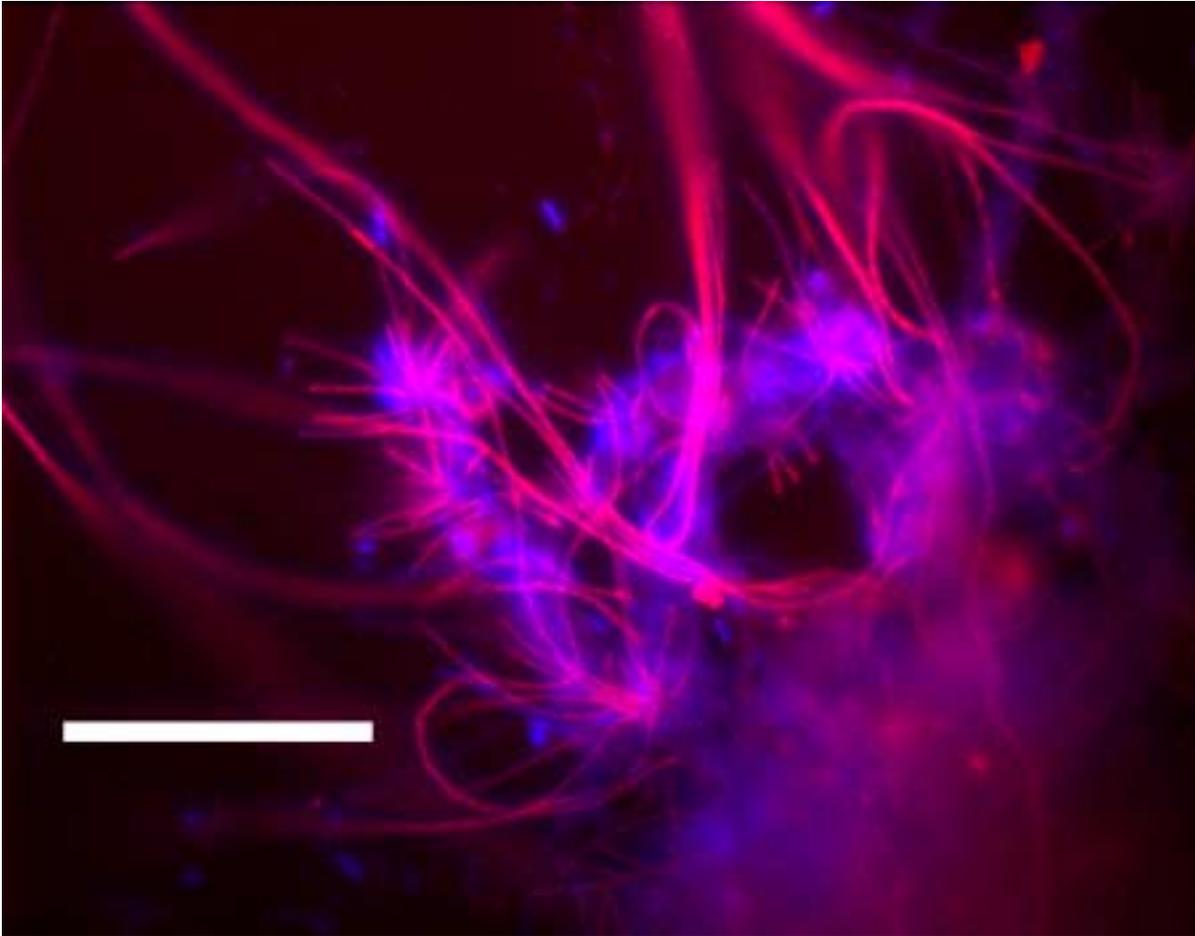


Figure 2.9. *Thiothrix* spp. on Day 26. Bar = 50 μ m. Red signal are *Thiothrix* cells.

According to Jenkins *et al.* (1993), the growth of many filamentous organisms, including Eikelboom Type 1851 and *Thiothrix* spp. is generally encouraged by the use of uniformly aerated, completely mixed, continuously fed aeration basins. These two filamentous bacteria are also both favored by readily metabolizable substrates and moderate to high SRT and they are both organisms with attached growth. Type 1851 is associated with quite high SRTs (> 10 days) and low F/M (as kg BOD₅ / kg MLVSS) feed (< 0.2) and is often seen when simple sugars are present in the wastewater. Previous studies have shown that Eikelboom Type 1851 is an extremely slow grower in pure

culture (Beer *et al.*, 2002; Kohno *et al.*, 2002). Since we have an SRT of 16 days, this seems to be long enough to initiate Eikelboom Type 1851-caused bulking. *Thiothrix* is always considered a sign for nutrient (nitrogen and/or phosphorus) deficiency in the activated sludge system. It was found to grow best with low levels of lactate, ammonium and phosphate (Williams and Unz, 1989). These two filaments were both observed in the seed sludge from NCWRF. Reactor C was operated as a CSTR and was continuously fed the readily biodegradable glucose as carbon source and had a relatively long SRT. All these factors combined stimulated the proliferation of Eikelboom Type 1851 and *Thiothrix* spp.

Since *Sphaerotilus* was proved to be not the bulking-causing microorganism in the bioaugmentation experiment (see Figure 2.5), an experimental run was carried out without its addition. The repeat experiment began on July 16 using new seed sludge from the NCWRF. Since the new sludge had a high SVI of 344 ml/g, the reactors were operated as in Stage I to lower the SVI. After all three reactors reached SVIs of around 80, the reactors were operated as one CSTR(C), one quick feeding SBR (A) and one slow feeding SBR (B) as in Stage II of the first round. At first the SVI kept going down in reactors B and C, till in mid August, reactor B started to bulk first, while the SVI of C was still decreasing. A week later, C started bulking too and in another week, its SVI surpassed B. The dominant filament was again Eikelboom Type 1851. This experiment shows that Type1851-caused bulking need not be initiated by the addition of *S. natans*.

Since in the first round reactor C lost a lot of sludge and large amount of the filamentous bacteria was washed out, it took longer for reactor C to start bulking on the

second round. But once it started bulking, the SVI increased very quickly, indicating it is still the easiest set-up to get bulking.

Conclusions:

Instead of the initial test organism, *Sphaerotilus natans*, Eikelboom Type 1851 was found to be the dominant and causative filamentous bacteria in the bulking sludge in laboratory-scale reactors fed with synthetic wastewater. The correlation coefficient between the reactor SVI and Type 1851 filament length was 0.96, which suggests cause and effect of Type 1851 proliferation and bulking. The threshold value was estimated to be 8×10^8 μm total filament or 6×10^8 μm extended filament /ml sludge. One interesting phenomenon we observed in the CSTR is the growth of *Thiothrix* after Type 1851-caused bulking, and the co-dominance of the two filaments. One recent research concluded that if another filament began to proliferate it would cause the regression of the one which was formerly dominant (Gaval *et al.*, 2002). Our observation in the CSTR differed from this conclusion. It appears that two filaments can co-dominate in a given sludge.

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Chapter Three: Spatial and Temporal-Scale Study of North Carolina Wastewater Treatment Plants

Introduction:

Filamentous bulking is a complex problem because different types of filamentous bacteria have different environmental niches so changes in temperature and operational conditions can cause changes in the dominant filaments.

The effect of temperature on biological nutrient removal processes has been studied by Mamais and Jenkins (1992), McClintock *et al.* (1993) and Brdjanovic *et al.* (1997, 1998). The growth of filamentous bacteria and sludge settlability in activated sludge systems have been found to be affected by temperature (Knoop and Kunst, 1998; Krishnam *et al.*, 1999). Donkin (1999) has shown that operation conditions have a big effect on filament dominance.

The objective of this study was to delineate the environmental and operational conditions that affect the growth of specific filaments in North Carolina activated sludge plants. This study consists of two parts: a spatial study and a temporal study. For the spatial study, samples were collected from different WWTPs across North Carolina. Due to the different operational conditions and configurations of the treatment plants responded, we expected different rRNA levels of our model organism, *Sphaerotilus natans* as determined by membrane hybridization. The North Cary Water Reclamation Facility (NCWRF) cooperated with us for the temporal study. To link bulking to levels of bulking-causing organisms, the microbial community change of the major microbial groups over a one-year period and the dominant filamentous bacteria for a time period

covering the coldest to the hottest weather in North Carolina were studied. Group-specific rRNA probes were used to detect major groups of bacteria in the activated sludge system, i.e., alpha, beta, gamma subclass of *Proteobacteria* (Manz *et al.*, 1992; Wagner *et al.*, 1994), and species-specific probes were used to determine the dominant filaments. Correlations of plant SVI with all the major operational parameters were established. One study showed that for samples from Nancy-Mxaeville, France, WWTP, there was no relationship between floc characteristics and the SVI over a period of one year while filamentous bulking events associated with variations of the SVI were detected from changes of total filament length, filament number/image and the proportion of filamentous bacteria with respect to the total activated sludge (da Motta *et al.*, 2001). Another study focusing on yearly operational changes (especially DO and F:M ratio) defined the optimum conditions for the proliferation of six filaments (Scruggs and Randall, 1998). In this present study, we combined quantitative FISH with membrane hybridizations, which, along with the operational data, allowed us to draw conclusions on the effect of these factors to bulking in a full-scale WWTP.

Materials and methods:

Sampling

Grab samples were obtained from the activated sludge mixed liquor and return activated sludge tank of eleven full-scale wastewater treatment plants as described in Chapter One.

Grab samples for membrane hybridization were obtained for a period of one year (9/4/01-8/30/02) from the North Cary Water Reclamation Facility for the temporal study.

For the quantitative FISH study, 3 ml of sample was taken from the activated sludge mixed liquor of NCWRF, collected in 15-mL tubes, fixed using 9ml of 4% (w:v) paraformaldehyde for 2 h at 4°C, and stored in phosphate buffered saline/ethanol (1:1, v:v) at -20°C until FISH. Sampling began in Feb. 2002 and ended in Sept. 2002. Samples were taken every week for the first half-year and once in August and September.

Probes

Membrane hybridization probes used in this study are listed in Table 3.1.

A series of FISH probes were used to determine which filaments were dominant in the activated sludge. Group specific probes were first tried, including probes targeting the alpha-, beta- and gamma- subclasses of the *Proteobacteria*. FISH probes used in this study are listed in Table 3.2.

Operational data, including temperature, effluent flow rate, reactor sludge volume index (SVI), solid retention time (SRT), food to microorganism (F/M) ratio, dissolved oxygen (DO), and others were obtained from NCWRF since these factors have been identified to affect bulking (Jenkins *et al.*, 1993).

Table 3.1. Probes and standards used in membrane hybridization

Probe name	Target group	RNA organism	reference	Tw (°C)	Sequence	Reference
S-*-Univ-1390-a-A-18	Almost all organisms	/		44	GACGGGCGGTGTGTACAA	Zheng et al., 1996
S-D-Bact-0338-a-A-18	domain Bacteria	/		56	GCTGCCTCCCGTAGGAGT	Amann et al., 1990
S-D-Bact-0338-b-A-18		/		56	GCAGCCACCCGTAGGTGT	Daims et al, 1999
S-D-Bact-0338-b-A-18		/		56	GCTGCCACCCGTAGGTGT	Daims et al, 1999
L-Sc-aProt-0019-a-A-17	α -Proteobacteria	<i>Gluconacetobacter hansenii</i>		53	CGTTCG(C/T)TCTGAGCCAG	Manz et al, 1992 de los Reyes et al. 2002
L-Sc-bProt-0042-a-A-17	β -Proteobacteria	<i>Sphaerotilus natans</i>		52	GCCTTCCCACTTCGTTT	Wagner et al, 1994b de los Reyes et al. 2002
L-Sc-gProt-0042-a-A-17	γ -Proteobacteria	<i>Leucothrix mucor</i>		58	GCCTTCCCACATCGTTT	Wagner et al, 1994b de los Reyes et al. 2002
S-Sp-S.nat-0656-a-A-18	<i>Sphaerotilus natans</i>	<i>Sphaerotilus natans</i>		46	CATCCCCCTCTACCGTAC	Wagner et al, 1994a this study (Chapter 1)

Table 3.2. FISH probe used in this study

Probe name	Target group	Formamide%	Reference
S-*-1851-0592-a-A-21	Eikelboom Type 1851	35	Beer et al, 2002
S-Sp-L.muc-0652-a-A-18	<i>Leucothrix mucor</i>	35	Wagner et al, 1994a
S-Sp-H.hyd-0655-a-A-18	<i>Haliscomenobacter hydrossis</i>	20	Wagner et al, 1994a
S-*-021N-0652-a-A-18	<i>Eikelboom type 021N</i>	35	Wagner et al, 1994a
S-Sp-S.nat-0656-a-A-18	<i>Sphaerotilus natans</i>	45	Wagner et al, 1994a
S-Sp-Thio-0652-a-A-17	<i>Thiothrix</i>	45	Wagner et al, 1994a
S-Sp-A.eur-0644-a-A-17	<i>Alisphaera europea</i>	30	Snaidr et al 2001
L-Sc-aProt-0019-a-A-17	α -Proteobacteria	20	Manz et al, 1992
L-Sc-bProt-0042-a-A-17	β -Proteobacteria	35	Wagner et al, 1994b
L-Sc-gProt-0042-a-A-17	γ -Proteobacteria	35	Wagner et al, 1994b

Results and discussion:

Spatial Study

The SVIs, MLSS (mixed liquor suspended solids) concentrations, and configurations for the sampled plants and the plant code are shown in Tables 3.3 and 3.4. Of these eleven plants, 5 were bulking at the time of sampling (i.e., had SVI values above 150 ml/g).

Table 3.3. Summary of relevant information from full-scale wastewater treatment plants

Plant Code	Design Flow Rate (m³ day⁻¹ x 10⁻³)	Activated sludge configuration	SVI (ml/g)	MLSS (mg/l)
B	18.2	Oxidation ditch with nitrification	132	3340
C	15.1	Extended aeration	156	1150
NC	37.8	Phased oxidation ditch	230	2820
Gas	60.6	A ² O	170	2865
G	66.2	Oxidation ditch with BNR	133	2847
LC	22.7	A ² O	278	3500
LG	7.6	Sequencing batch reactor	124	2300
MC	30.3	Conventional	117	3342
MCD	22.7	Conventional	92	2557
M	34.1	Conventional	77	4200
R	227.1	Conventional with nitrification	170	4009

Table 3.4. Names of plants sampled

Plant Code	Plant Name
B	Town of Boone WWTP
C	Eagle Road WWTP
NC	North Cary WRF (Water Reclamation Facility)
Gas	Long Creek WWTP
G	Greenville Utilities Commission WWTP
LC	Lower Creek WWTP
LG	Gunpowder Creek WWTP
MC	Mallard Creek WWTP
MCD	McDowell Creek WWTP
M	Town of Monroe WWTP
R	Neuse River WWTP

The results of quantitative hybridization with the *S.natans* specific probe to extracted RNA are shown in Figure 3.1.

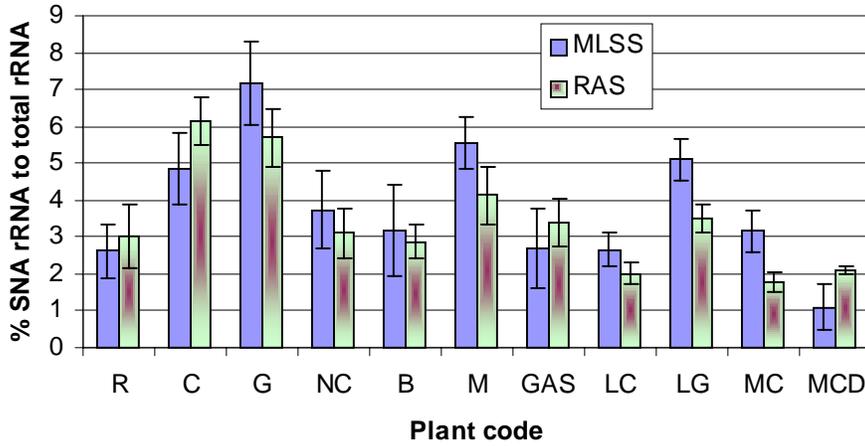


Figure 3.1. Results of quantitative membrane hybridizations with probe S-S-S.nat-0656-a-A-18.

The average *S. natans* rRNA levels (as percentages of the total 16S rRNA) in the mixed liquor and return activated sludge (RAS) of all plants were 3.8% and 3.4%, respectively. However, there appears to be no correlation between settling ability (as measured by the SVI) and *S. natans* rRNA levels in the mixed liquor and RAS. Bulking sludges did not necessarily have higher *S. natans* levels (Figure 3.2). In particular, the plants with the three highest *S. natans* rRNA levels in the mixed liquor (plants G, LG, and M) had relatively low SVIs. Of the two plants with the next highest *S. natans* rRNA percentages in the mixed liquor, one (plant C) had an SVI just slightly above 150 ml/g, while the other (plant NC) was clearly in the bulking region, with an SVI of 230 ml/g. It was therefore apparent that the settling ability in these full-scale plants was not directly related to *S. natans* biomass alone. This is to be expected: a variety of factors, such as extracellular polymeric substances, surface chemistry, floc size, etc., affect sludge

settling. Other filamentous species or types are also likely to be present, and may affect sludge settling.

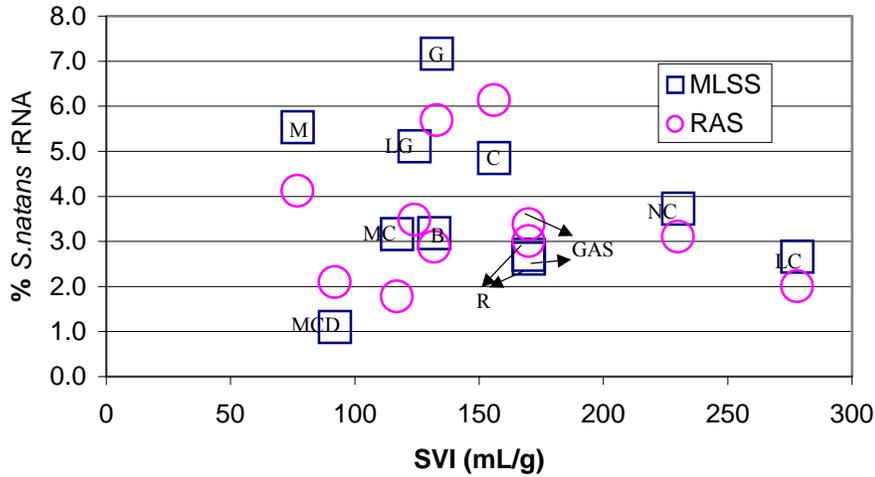


Figure 3.2. Plot of SVI versus *S. natans* rRNA percentages (of total 16S rRNA).

As stated in Chapter One, the two methods (FISH and membrane hybridization) gave comparable results for the Greenville WWTP (sample G) and only this plant out of the eleven plants samples had sufficient filament lengths above the limit of detection (~0.05 mg *S. natans* VSS/ml) for FISH. Positive signals were also observed for the sample from NCWRF. However, problems with excessive floc interference prevented the reliable quantification of *S. natans* biomass. The floc structure lowered probe accessibility to the target organism. Interestingly, *S. natans* was not observed by FISH in samples with relatively high *S. natans* rRNA levels (e.g., plants C, M, and LG). Again, this may be due to the inherent limitations of the FISH method. The limits of detection with FISH are dependent on the number of image fields and volumes of sample used. In practice, sample volumes in the microliter range are examined. On the other

hand, membrane hybridizations extract RNA from volumes in the milliliter range. Because sample heterogeneity (i.e., cell distribution, floc dispersal, etc.) is greater in FISH than in membrane hybridization, it is very possible that cells originally present in the samples may be overlooked. This phenomenon has been called ‘no cells, no signals’ (Amann, 1990).

Temporal Study

To determine the dominant filamentous bacteria in NCWRF, Gram staining was performed occasionally on the samples. The dominant filament was Gram-negative. The alpha Proteobacteria probe bound to the majority of the cells and resulted in very strong signals while the gamma and beta probes only bound to a small portion of the cell population stained with 4'6-diamidino-2-phenylindole-2HCl (DAPI). From a literature review of the alpha-subclass of Proteobacteria, several candidate filaments were identified. *Acetobacter spp.* have the morphology of rods in chain, but microscopic observation eliminated this possibility. *Alisphaera spp.* was also believed to be filamentous (Bergey's Manual, 1994). A probe targeting *Alisphaera europea* (EU24) (Snaidr 2001) was then used, but the result was negative. Among the six species-specific probes available, the probe targeting Eikelboom Type1851 bound to the greatest amount of filamentous cells.

Ten samples were selected over the six-month period (Feb. to Aug., 2002) and FISH was performed to determine the change in filament length level of Type1851. The quantitative FISH data was correlated with the SVI value provided by NCWRF. The

result is shown in Figure 3.3 and the correlation of Type 1851 filament length and the SVI of NCWRF is shown in Figure 3.4.

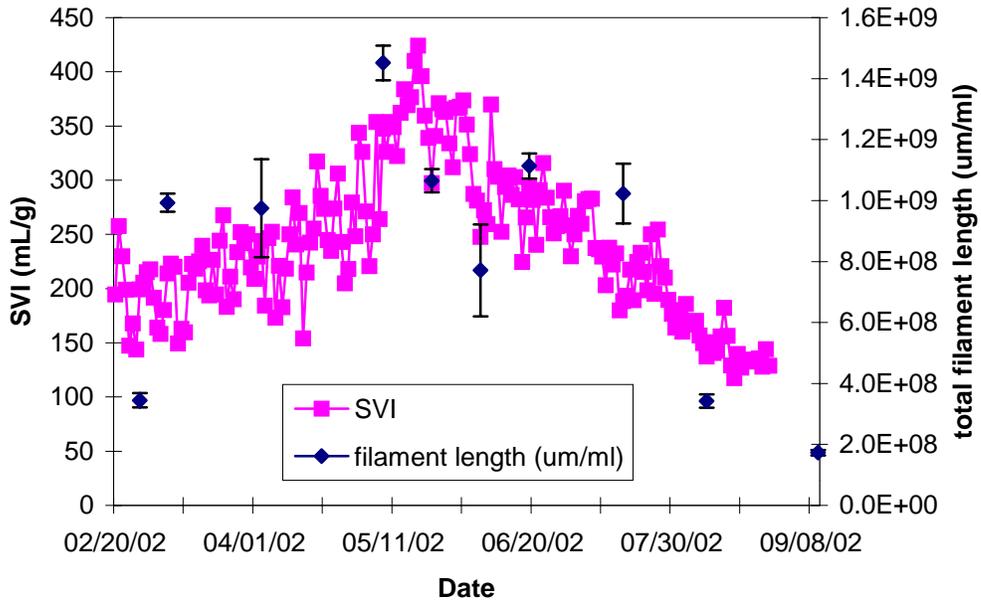


Figure 3.3. Quantitative FISH over time vs. SVI of NCWRF

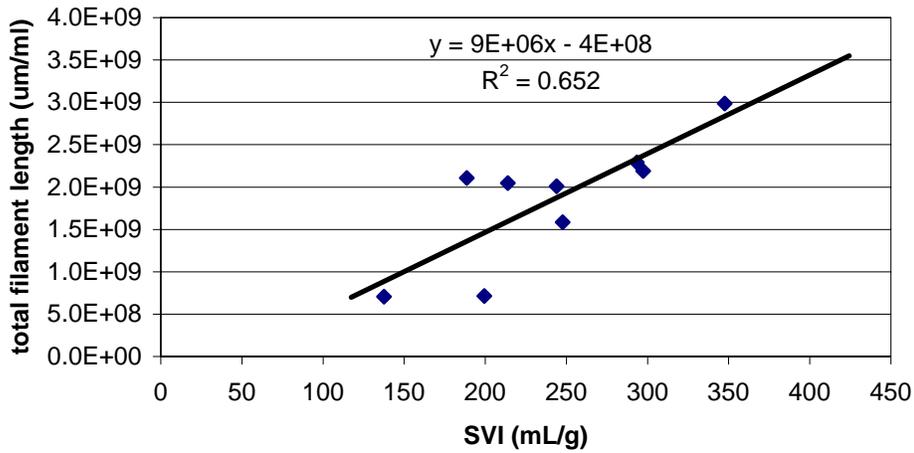


Figure 3.4. Correlation of Type 1851 filament length with the SVI of NCWRF

Figure 3.4 shows that the SVI of NCWRF is positively correlated with the filament length of Eikelboom Type 1851, with a correlation coefficient of 0.81. In Figure 3.3, SVI 150 corresponds to Type 1851 total length of $6 \times 10^8 \mu\text{m/ml}$. This number is very similar to that determined from the reactor experiment in Chapter Two ($8 \times 10^8 \mu\text{m/ml}$). This indicates (1) the robustness of the quantitative FISH method, and (2) that this range of filament length corresponds to the threshold level for bulking due to Type 1851.

Twenty samples of NCWRF mixed liquor from Sept. 4, 2001 to Aug. 30, 2002 were chosen and quantitative membrane hybridizations with the alpha, beta, gamma Proteobacteria probes to extracted RNA over the one-year period were conducted. The results are shown in Figure 3.5.

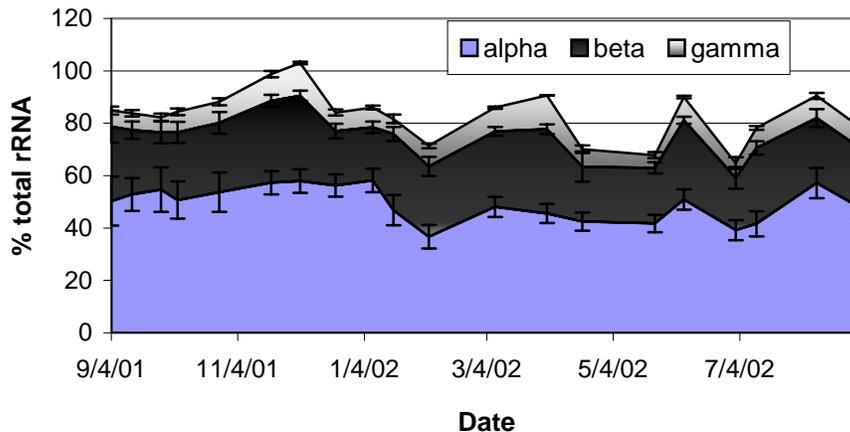
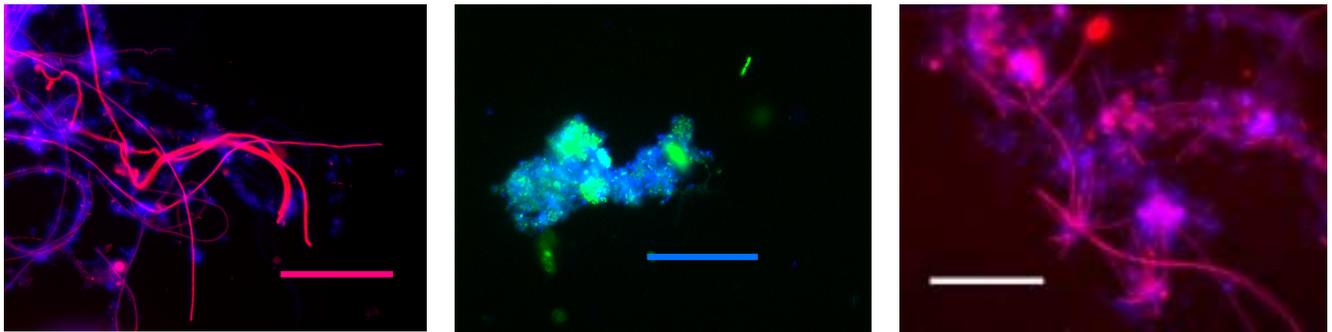


Figure 3.5. Quantitative membrane hybridization with α , β , γ Proteobacteria probes on NCWRF samples over time

As shown in Figure 3.5, the Alpha Proteobacteria make up the major group of bacteria in mixed liquor of NCWRF throughout the year. They constitute around 50% of the total population rRNA. The second largest group is comprised of Beta Proteobacteria, which contributes around 30% of the total rRNA. Gamma Proteobacteria contribute the least to the total rRNA among the groups tested in this particular activated sludge with around 10% of the whole population rRNA. FISH observation agrees with this result. FISH pictures for representative alpha-, beta-, gamma-subclass Proteobacteria in NCWRF are shown in Figure 3.6. Among the commonly recognized bulking-causing bacteria, *Sphaerotilus spp.* belong to the beta-subclass of *Proteobacteria* and *Thiothrix spp.* belong to the gamma-subclass. No significant seasonal change in the major population was apparent in NCWRF.



Alpha-Proteobacteria in NCWRF

Beta-Proteobacteria in NCWRF

Gamma-Proteobacteria in NCWRF

Figure 3.6. Alpha-, Beta-, Gamma-subclass Proteobacteria in NCWRF

To determine the main factors affecting bulking, operational data were related to the plant SVI and their respective correlations are shown (Figures 3.7 to 3.16)

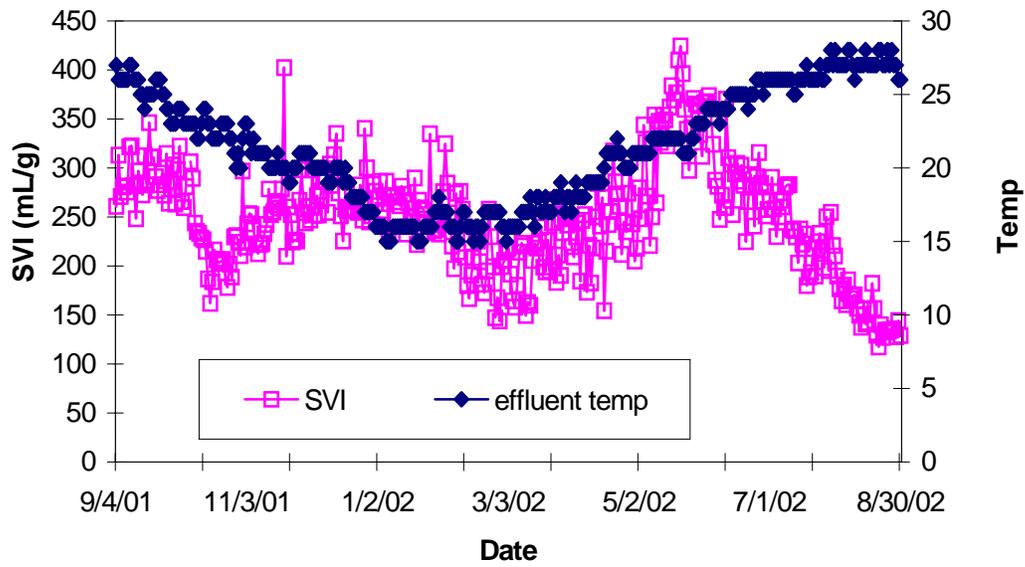


Figure 3.7. Temperature and SVI of NCWRF over time

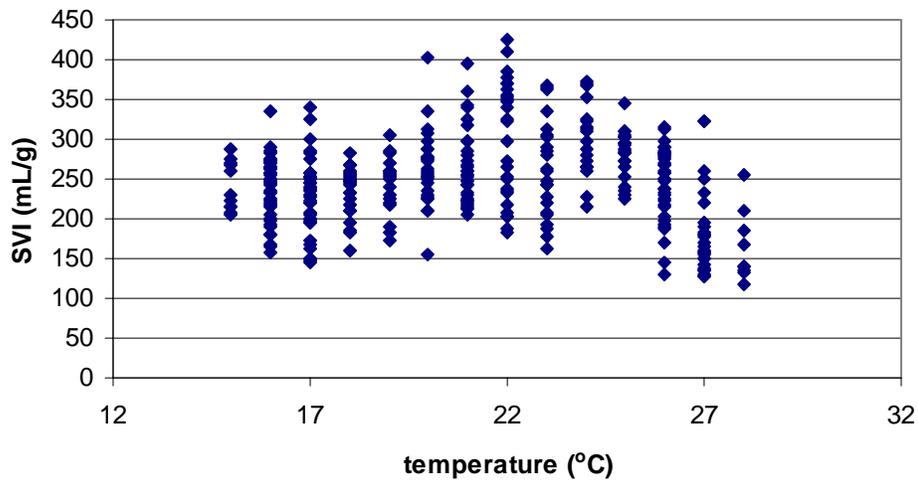


Figure 3.8. Correlation of temperature and SVI of NCWRF

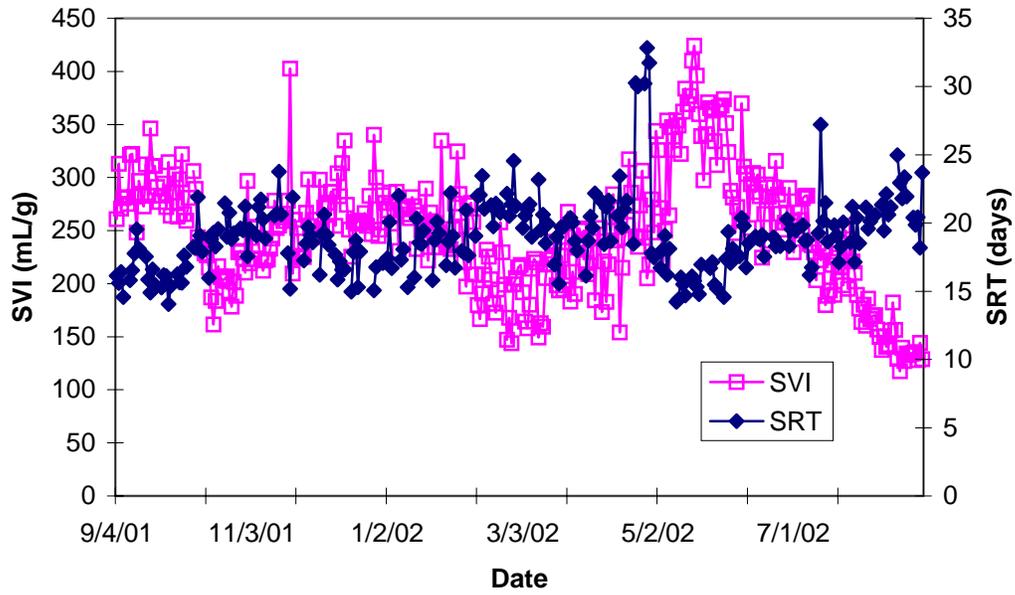


Figure 3.9. SRT and SVI of NCWRF over time

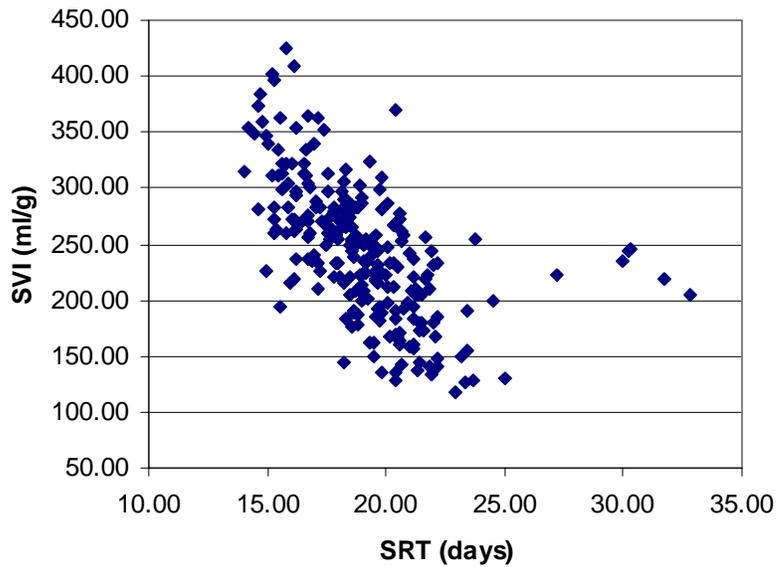


Figure 3.10. Correlation of SRT and SVI of NCWRF

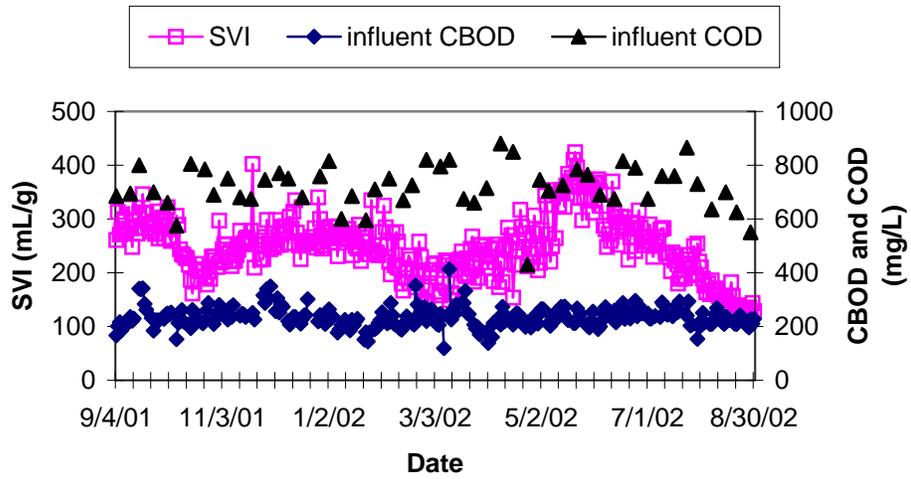


Figure 3.11. Influent COD, CBOD level and SVI of NCWRF over time

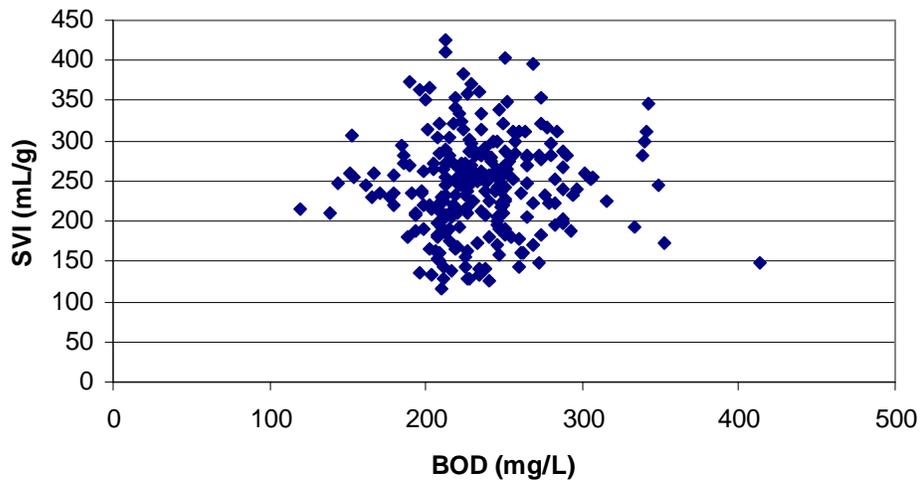


Figure 3.12. Correlation of influent BOD level and SVI of NCWRF

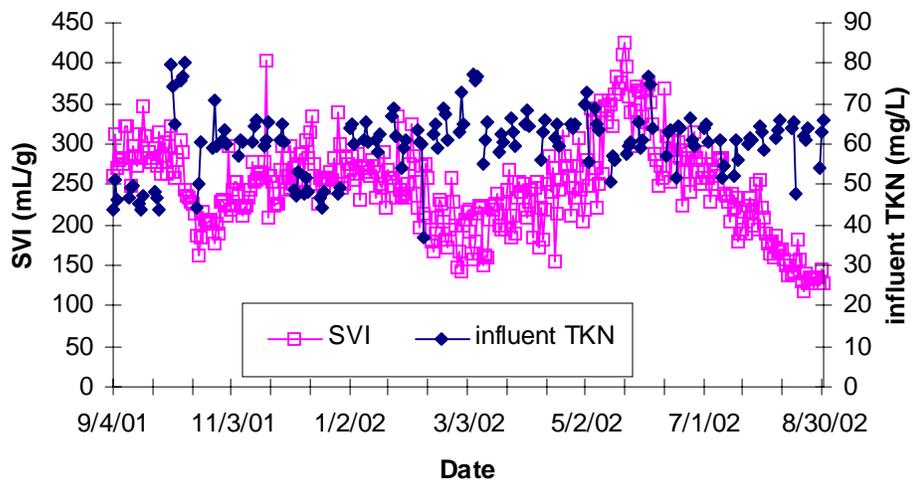
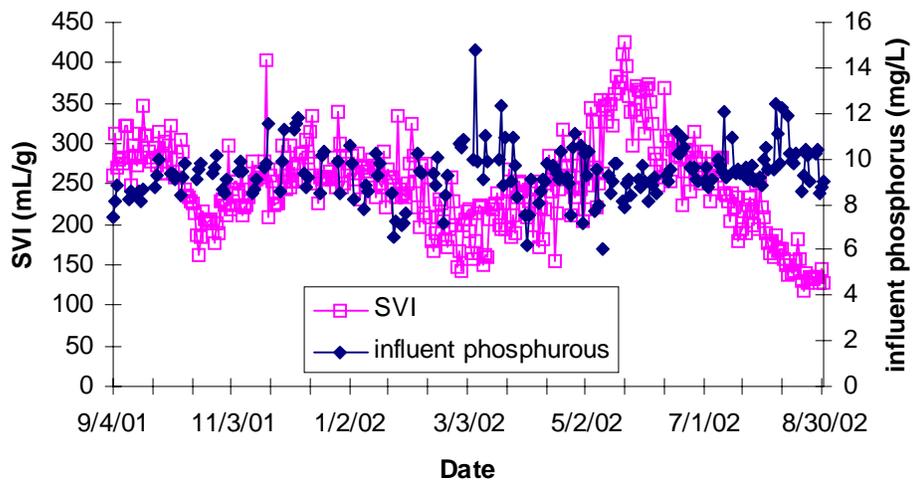


Figure 3.13. Influent P, N level and SVI

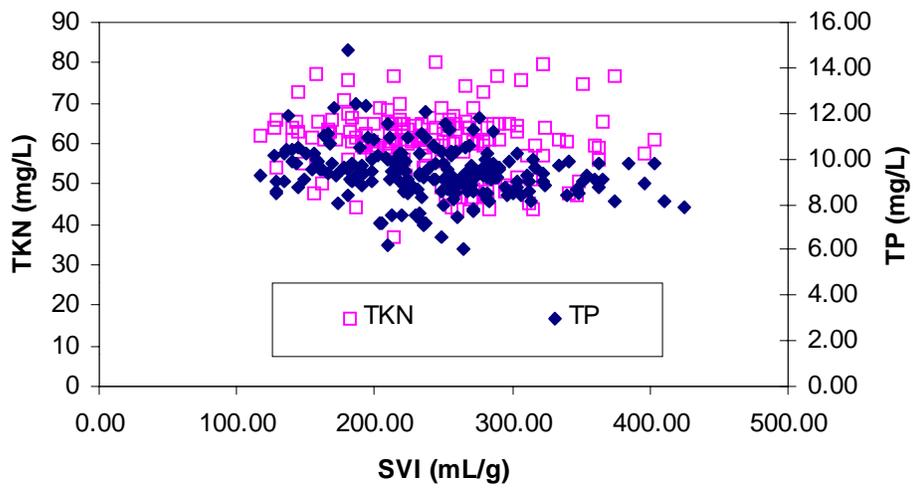


Figure 3.14. Correlation of influent P, N level and SVI of NCWRF

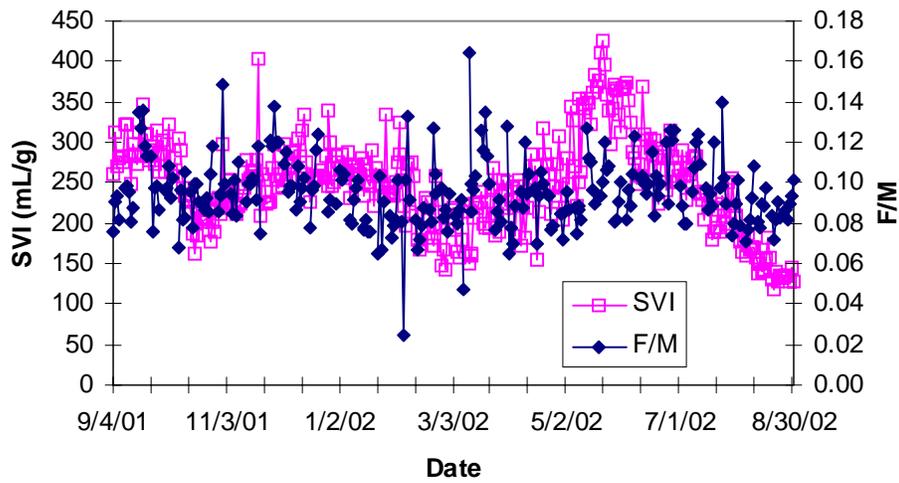


Figure 3.15. F/M ratio and SVI of NCWRF

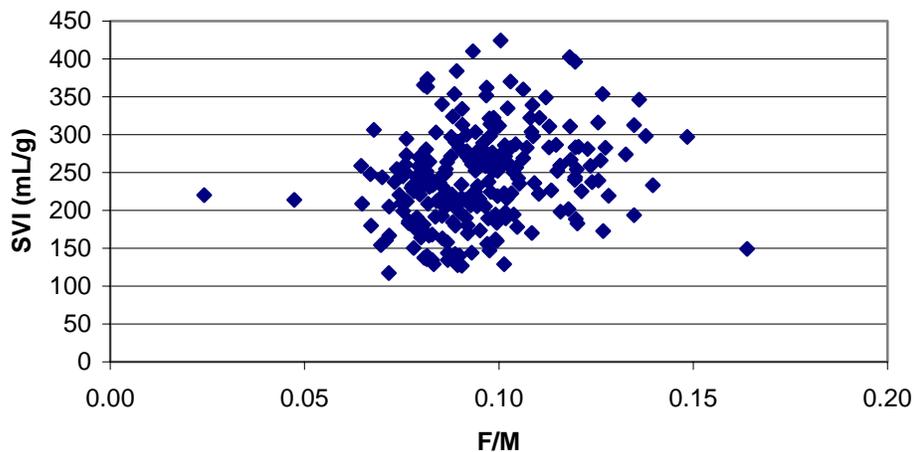


Figure 3.16. Correlation of F/M ratio and SVI of NCWRF

As shown in Figure 3.7, temperature did not affect filamentous bulking at NCWRF directly. Temperature was not the highest when the highest SVI (June 2002) occurred. When the temperature was highest (August and September for both years), the SVI values were not at the same level, indicating that other factors have a greater effect on the occurrence of bulking. Similar conclusions can be made for influent TKN, P, COD, BCOD and F/M ratio. However, SRT did correlate with SVI negatively (correlation coefficient of -0.56). Especially when SRTs are between 14 and 25 days, the correlation coefficient is 0.7. Since Type 1851 length correlates with SVI positively, and if this plant has to operate at high SRTs (> 10 days), increasing the SRT might be a way to reduce the amount of Type 1851, and controlling filamentous bulking.

In another study on foaming we conducted concurrently with this one, high temperature correlates well with foaming (data not shown). In foaming plants, high temperature stimulates the growth of mycolic acid containing actinomycetes, such as *Gordonia amarae* (de los Reyes *et al.*, 1997), which produces biosurfactant and stabilizes

air bubbles. On the other hand, the growth of Type 1851 is dependent on other factors, in this case, mainly SRT. In the threshold value experiment in Chapter Two, we controlled the SRT of the three reactors to be 16 days. Compared to the NCWRF one-year data, this is towards the lower end of the full scale SRT range, which explains the bulking occurrence in the lab-scale experiment.

Type 1851 has been discovered in both industrial wastewater (including cattle slaughtering, fruit juice processing, miso manufacturing, chicken slaughtering industries) and domestic sewage (Kohno *et al.*, 2002); in Australia (Beer *et al.*, 2002) and in Japan (Kohno *et al.*, 2002). According to Beer *et al.* (2002), Eikelboom Type 1851 belongs to the *Chloroflexi* group. Bjornsson L. *et al.* (2002) concluded that filamentous *Chloroflexi* (green non-sulfur bacteria) are abundant in BNR WWTPs. NCWRF is a nitrogen removal plant, as are many WWTPs in North Carolina. Therefore the Type 1851 problem may not be confined to this one plant. According to Jenkins *et al.* (1993), Type 1851 ranks 13th on causing bulking and foaming problem in the US before 1993. In a survey conducted in 1989 to 1991 on Danish BNR plants, Type 1851 was the sixth dominant filamentous microorganism (Kristensen *et al.*, 1994). Another survey on nutrient removal activated sludge systems of South Africa revealed that Type 1851 was the fourth in frequency (Lacko *et al.*, 1999). These studies show that Type 1851 is a very common filament in BNR plants. Since more and more WWTPs are required to upgrade to perform BNR, the extent of Type 1851 caused bulking may be on the rise.

Conclusions:

Membrane hybridization detected *S. natans* in plants where FISH detected none, which shows the limitation of the FISH method, and confirmed the necessity of combining the two methods. The results of membrane hybridizations showed no correlation between sludge settling ability (as measured by the SVI) and *S. natans* rRNA content with the 11 plants sampled, indicating that other filamentous organisms, or other physical and chemical factors may have a greater effect on the sludge settling in these plants. Using FISH, we determined that Eikelboom Type 1851 is the major bulking-causing filament in the NCWRF. A similar approach can be used to determine the dominant filament in other plants.

Both FISH and membrane hybridizations show that alpha Proteobacteria make up the major group of bacteria in mixed liquor of the NCWRF. The bacterial community structure in NCWRF appeared to be stable through the one-year period.

SRT seems to be the major factor affecting Eikelboom Type 1851 growth. The filament length of Type 1851 was positively correlated with the plant SVI (correlation coefficient of 0.81), while the SRT (within the range of 14 to 25 days) correlated negatively to the plant SVI, suggesting that increasing the SRT of this plant may result in slower Type 1851 growth, decreasing the chance for bulking to occur in this plant. Jenkins *et al.* (1993) concluded that high SRT favors Type 1851 growth and defined the lower limit of SRT for Type 1851-caused bulking, which is 10 days. Since the SRTs of the North Cary WRF are all above 14 days, the reverse correlation of SVI and SRT might exist for SRTs exceeding 14 days. There are not enough data points to reach a conclusion about SRTs exceeding 25 days on bulking. Beer *et al.* (2002) and Kohno *et al.* (2002) both claimed that this organism grows very slowly in axenic culture.

However, pure culture experiments are not adequate and *in situ* data are important in the study of bulking phenomena. Since the number of BNR plants is increasing, the Type 1851-caused bulking problem may be more and more significant and additional studies on Type 1851 occurrence and environmental requirements are needed.

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SUMMARY AND FUTURE STUDIES

A quantitative method for estimating the biomass of the filamentous organisms *S. natans* and *L. mucor* in activated sludge was developed. Curves showing the correlation between species-specific filament length and cell biomass were established, which revealed the specific contribution of individual filaments to bulking. The results of quantitative FISH and membrane hybridization for *S. natans* were comparable for the Greenville WWTP sample. Membrane hybridization detected *S. natans* in plants where FISH detected none, showing the limitation of the FISH method, and confirmed the necessity of combining the two methods. The results of membrane hybridizations showed no correlation between sludge settling ability (as measured by the SVI) and *S. natans* rRNA content in the 11 plants sampled, indicating that other filamentous organisms, or other physical and chemical factors may have a greater effect on sludge settling in these plants. A combined FISH/membrane hybridization approach can be used to determine the dominant filament in other plants. In the case of the North Cary WRF, Eikelboom Type 1851 was determined to be the major bulking-causing filament. Both FISH and membrane hybridizations show that alpha Proteobacteria make up the major group of bacteria in mixed liquor of the NCWRF. The bacterial community structure in NCWRF appeared to be stable through the one-year period. The combined use of these two hybridization assays represents a new approach of studying the filaments, and will be a useful tool in studies of filamentous bulking.

Instead of the initial test organism (*Sphaerotilus natans*), Eikelboom Type 1851 was found to be the dominant and causative filamentous bacterium in the bulking sludge in laboratory-scale reactors fed with synthetic wastewater. The correlation between the

reactor SVI and Type 1851 filament length was 0.96, suggesting Type 1851 proliferation was the cause of bulking. The threshold value in the lab scale study was estimated to be 8×10^8 μm total filament length/ml sludge. This volume is similar to the threshold determined from NCWRF, showing the validity of the quantitative FISH method. *Thiothrix* later co-dominated the CSTR with Eikelboom Type 1851. Similar control measures can be taken against the two filaments.

SRT seems to be the major factor affecting Eikelboom Type 1851 growth in NCWRF. The filament length of Type 1851 was positively correlated with the plant SVI while the SRT correlated negatively to the plant SVI, suggesting that increasing the SRT of this plant may result in slower Type 1851 growth, decreasing the chance for bulking to occur in this plant. Since NC WRF is a BNR plant and more and more WWTPs are required to do BNR in North Carolina, Eikelboom Type 1851 is a filament whose environmental niche should be further studied.

In the future, pure culture of Type 1851 should be obtained and the filament length of this organism should be correlated to its VSS as we did for *S. natans* and *L. mucor* so that its specific biomass contribution to bulking can be determined by FISH. Also, its rRNA contribution to bulking can be determined by membrane hybridization. Lab scale reactors should be operated under different SRTs (especially higher than 25-day SRTs), F/M ratios and others to determine its optimum growth condition. Once its environmental niche is defined, we can expect better control over Type 1851-caused bulking.