QUANTIFYING FILAMENTOUS MICROORGANISMS IN ACTIVATED SLUDGE BEFORE, DURING, AND AFTER AN INCIDENT OF FOAMING BY OLIGONUCLEOTIDE PROBE HYBRIDIZATIONS AND ANTIBODY STAINING

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Abstract—Quantitative oligonucleotide probe hybridizations, immunostaining, and a simple foaming potential test were used to follow an incident of seasonal filamentous foaming at the Urbana-Champaign Sanitary District, Northeast Wastewater Treatment Plant. A positive correlation was observed between an increase in foaming potential and the appearance of foam on the surfaces of aeration basins and secondary clarifiers. In addition, during the occurrence of foaming, the mass and activity of *Gordonia* spp. increased as measured by fluorescence in situ hybridization, antibody staining, and quantitative membrane hybridization of RNA extracts. An increase in *Gordonia* spp. rRNA levels from 0.25 to 1.4% of total rRNA was observed using quantitative membrane hybridizations, whereas during the same period, the fraction of mixed liquor volatile suspended solids attributed to *Gordonia* spp. increased from 4% to more than 32% of the total mixed liquor volatile suspended solids. These results indicate that both the activity and biomass level of *Gordonia* spp. in activated sludge increased relative to the activity and the biomass level of the complete microbial community during a seasonal occurrence of filamentous foaming. Thus, *Gordonia* spp. may represent a numerically dominant but metabolically limited fraction of the total biomass, and the role of *Gordonia* spp. in filamentous foaming may be linked more tightly to the physical presence of filamentous microorganisms than to the metabolic activity of the cells. © 2001 Elsevier Science Ltd. All rights reserved

Key words—contact stabilization activated sludge, filamentous foaming, wastewater treatment, oligonucleotide hybridization probes, ribosomal RNA, antibody stain

NOMENCLATURE

ANOVA analysis of variance
BOD<sub>5</sub> five-day biochemical oxygen demand, mg l<sup>-1</sup>
DO dissolved oxygen, mg l<sup>-1</sup>
FISH fluorescence in situ hybridization
FITC fluorescein isothiocyanate
F/M food to microorganism ratio, g BOD<sub>5</sub>[g suspended solids day]<sup>-1</sup>
MLSS mixed liquor suspended solids, mg l<sup>-1</sup>
RAS return activated sludge
RNA ribonucleic acid
rRNA ribosomal ribonucleic acid
SI scum index
SRT solids retention time, day
SVI<sub>30</sub> thirty-min sludge volume index, ml g<sup>-1</sup>
TRITC tetramethyl rhodamine isothiocyanate
UCSD, Urbana-Champaign Sanitary District, North
NEWWTW east Wastewater Treatment Plant
WAS waste activated sludge

INTRODUCTION

Filamentous foaming, the formation of a “stable, viscous, chocolate-colored scum layer” (Soddell and Seviour, 1990) on the surfaces of activated sludge aeration basins and secondary clarifiers, is a common problem in wastewater treatment plants. Research using classical microbiological techniques, such as microscopic observation and culture-based enumeration, has demonstrated a link between the predominance of filamentous microorganisms and instances of foaming in laboratory-, pilot-, and full-scale wastewater treatment plants (Goddard and Forster, 1986; Pitt and Jenkins, 1990; Jenkins et al., 1993). The current understanding of filamentous foaming suggests that certain conditions experienced by the activated sludge (e.g., low dissolved oxygen concentrations [DO], low food to microorganism ratios [F/M], or high solids retention times [SRTs]) create a selective advantage for the growth of filamentous microorganisms, which are subsequently captured in the wastewater treatment plant through physical configurations (e.g., scum baffles in the secondary...
clarifiers and subsurface hydraulic withdrawal) (Jenkins et al., 1993; Wanner, 1994). Thus, the shift from floe formers to filaments as predominant microbial populations, combined with specific hardware configurations, provide conditions suitable for the initiation and persistence of filamentous foaming in activated sludge systems.

Historically, studies of filamentous foaming have examined three topics: (i) causes and conditions for the initiation of foaming, (ii) stability and persistence of foam, and (iii) potential control measures. Many studies focusing on the causes and conditions of foaming initiation have correlated results from classical microbiological techniques with a quantification of foaming. The methods described for quantifying foaming primarily rely on direct measurements of foam. Compressed gas purging (Khan and Forster, 1990; Pitt and Jenkins, 1990; Blackall et al., 1991; Ho and Jenkins, 1991) and the formation of gaseous CO$_2$ from Alka-Seltzer™ tablets (solid pellets of sodium bicarbonate) (Ho and Jenkins, 1991) have been used to generate foam from samples in a graduated vessel. The height (or volume) of foam formed when bubbles of gas pass through a sample is recorded, and the stability of the foam during aeration and after the stream of bubbles has subsided is monitored over time. Foaming was quantified for samples from different full-scale activated sludge systems with or without filamentous foam (Khan and Forster, 1990; Blackall et al., 1991) or for samples from laboratory-scale systems (Pitt and Jenkins, 1990; Ho and Jenkins, 1991). In one study, foaming potential was measured in a full-scale wastewater treatment plant incorporating an anaerobic selector (Pitt and Jenkins, 1990).

Despite these research efforts, ambiguous results and conflicting anecdotal recommendations from wastewater treatment plant personnel have confounded efforts to understand and systematically eliminate filamentous foaming. These difficulties are due in part to the general inability of classical microbiological techniques to accurately quantify the number and activity of suspect microbial populations (e.g., Gordonia [formerly Nocardia] amarae) in activated sludge. Culture-based enumeration assays of activated sludge have been shown to be biased towards microorganisms that grow rapidly on standard nutrient media (Soddell and Seviour, 1990; Wagner et al., 1993). Similarly, identification keys based on phenotypic characteristics such as morphology and staining properties also suffer from limitations. Morphology and staining characteristics can vary greatly for the same organism depending on environmental conditions (Soddell and Seviour, 1990; Kochianova et al., 1992). In addition, similarities in morphology between microorganisms with different metabolic properties or growth kinetics often lead to ambiguous identification (Soddell and Seviour, 1990; Seviour et al., 1997). To avoid many of these problems, molecular signature methods can be employed. Primarily, two families of molecular signature methods, oligonucleotide probe hybridization and antibody staining, have been used to measure the levels of microorganisms in activated sludge (Amann et al., 1998).

Hybridizations to whole cells or membrane immobilized nucleic acid extracts with oligonucleotide probes targeting ribosomal RNA (rRNA) provide a reliable means to quantify the number and activity of specific microbial populations (for recent reviews see, Raskin et al., 1997; Stahl, 1997; Amann et al., 1998). These methods have been used to characterize microbial population structure in a variety of activated sludge systems (Manz et al., 1992; Wagner et al., 1993, 1994a,b; de los Reyes et al., 1997, 1998a). Oligonucleotide probes targeting the rRNA of several filamentous microorganisms have been developed and applied to characterize activated sludge (Wagner et al., 1994a,b; de los Reyes et al., 1997, 1998a; Erhart et al., 1997; Schuppeler et al., 1998).

Staining of whole cells with mono- and polyclonal antibodies also has been used to identify and enumerate microorganisms in a variety of environments (Bohlool and Schmidt, 1980; McDermott, 1997; Oerther et al., 1999). The target specificity of antibody stains cannot be as easily adjusted as with oligonucleotide probes. However, antibody stains can be used to detect microorganisms that exhibit low levels of metabolic activity (i.e., low levels of rRNA) or even nonviable cells (McDermott, 1997; Oerther et al., 1999). A polyclonal antibody serum developed using a pure culture of G. amarae strain ASF-3 was characterized previously for the detection of whole cells of G. amarae in anaerobic digester (Hernandez and Jenkins, 1994) and activated sludge foams (Hernandez et al., 1994; de los Reyes et al., 1998; Oerther et al., 1999).

In this study, we extended this previous work and used oligonucleotide probe hybridizations targeting rRNA and whole cell antibody staining to quantify changes in Gordonia spp. population levels during the initiation of seasonal filamentous foaming in a full-scale wastewater treatment plant. In addition, we examined the foaming potential of the activated sludge before, during, and after a seasonal occurrence of filamentous foaming.

**MATERIALS AND METHODS**

**Description of the full scale wastewater treatment plant**

The Urbana-Champaign Sanitary District, Northeast Wastewater Treatment Plant (UCSD, NEWWTP) provides treatment to a municipal waste stream with an average influent flow rate of 5.7 x 10$^6$ m$^3$ day$^{-1}$ (15 million gallons day$^{-1}$) and an average influent BOD$_5$ concentration of 150 mg l$^{-1}$ (1.8 x 10$^4$ lbs day$^{-1}$) (Bachman, personal communication). As shown in Fig. 1, the UCSD, NEWWTP consists of primary treatment units and two secondary treatment schemes, contact-stabilization activated sludge and a rock medium trickling filter. During most of the year, the trickling filter serves as a roughing filter operated in
Foaming potential test

Mixed liquor samples (7 l) were collected from the head of the contact basin (Fig. 1, cell B2) and from the end of the re-aeration basin (Fig. 1, cell A2). Samples of RAS (7 l) were taken from the RAS line (Fig. 1). Well-mixed samples from cell B2 were distributed into seven, 1-l graduated cylinders to allow quiescent settling of solids for 30 min. The supernatant was collected, the settled solids were pooled, and the volume of settled solids was measured. The samples from cell A2 and RAS were used directly without quiescent settling. Subsamples were prepared by diluting (settled) samples with secondary effluent according to the following dilution series: volume (settled) sample (ml): volume effluent (ml); 250:0; 225:25; 200:50; 175:75; 150:100; 125:125; 100:150; 75:175; 50:200; 25:225; and 0:250. Well mixed samples (250 ml) were placed into 500-ml graduated cylinders and two tablets of Alka-Seltzer™ (two tablets contain 650 mg sodium acetylsalicylate, 3832 mg heat-treated sodium bicarbonate, and 2000 mg citric acid; Bayer Corp., Elkhart, IN) were added. The volume of foam generated from the evolution of CO₂ gas was measured as the total maximum volume of sample in the cylinder minus the initial 250 ml.

Rapid determination of suspended solids levels

Suspended solids concentrations were determined by Standard Methods (Greenberg et al., 1992) or with a modified microwave drying procedure. Mixed liquor or RAS samples were collected as described above, stored on ice, and transported to the laboratory for analysis. Different volumes (2, 4, 6, 8, and 10 ml) of the samples were vacuum filtered through glass micro-fiber filter paper (47 mm, Whatman 934-AH, Clifton, NJ) prepared according to Standard Methods (Greenberg et al., 1992) (12 samples for each volume). To provide rapid drying after filtration, three samples were placed together in a microwave oven (Speedwave, 2000, Tappan, Dublin, OH) along with a
250-ml beaker with 25 ml of deionized H2O. The samples were heated at 900 W for various times (5, 10, 15, or 20 min). After cooling in a desiccator, suspended solids were determined by weight difference.

Quantification of extracted rRNA with membrane hybridization

Grab samples (15 ml) of the mixed liquor and RAS of the UCSD, NEWWTP were pelleted at 5000 × g, the supernatant was decanted, and the samples were frozen in an ethanol/dry ice bath and stored at −80 °C. Total RNA was extracted using a low-pH, hot-phenol bead-beating protocol (Stahl et al., 1988; Raskin et al., 1995). RNA extracts were quantified by spectrophotometry, examined for degradation by polyacrylamide gel electrophoresis (Zheng et al., 1996), and stored at −80 °C.

Dilutions of RNA extracted from pure cultures and approximately 300 ng of each environmental extract were denatured in 1.5% (vv⁻¹) glutaraldehyde and blotted in triplicate (100 ng for each slot) to Magna Charge membranes (Micron Separation Inc., Westboro, MA) (Zheng et al., 1996). Oligonucleotide hybridization probes (S-G-16S-Gor-0596-a-A-18, 5' to 3' nucleotide sequence GACGGGCGGTGTGTTCAAC, Zheng et al., 1996; and S-G-Gor-0596-a-A-22, 5' to 3' nucleotide sequence TGCGAATTTSCACAGACGACGC, de los Reyes et al., 1997) were labeled with 32P (ICN Radiochemicals, Irvine, CA) (Raskin et al., 1994). Probe nomenclature is based on the oligonucleotide probe database (Alm et al., 1996). Hybridizations and two initial washes were conducted at 40 °C for 12–16 h and 30 min each, respectively (Raskin et al., 1994). Final washes were conducted for 30 min at 44 °C for probe S-A-Univ-1390-a-A-18 (Zheng et al., 1996) and at 54 °C for probe S-G-Gor-0596-a-A-22 (de los Reyes et al., 1997). Radioactive signal was quantified with an Instant Imager (Packard Instruments, Downers Grove, IL). The results of the membrane hybridizations with the specific probe S-G-Gor-0596-a-A-22 were expressed as the percentage of 16S rRNA measured with the universal probe S-A-Univ-1390-a-A-18.

Quantification of Gordonia mass using antibody staining and FISH

Grab samples of activated sludge from the UCSD, NEWWTP were fixed in 4% paraformaldehyde (4 g paraformaldehyde in 100 ml 1× phosphate-buffered saline [PBS] pH 7.2 [1× PBS = 130 mM NaCl and 10 mM sodium phosphate]) for 1 min at room temperature, washed twice with 1× PBS, and stored in 50% (vv⁻¹) ethanol in 1× PBS at −20 °C. Fixed cells were treated with a 1:100 dilution of Rabbit anti G. amarae serum and a 1:200 dilution of Cy3 conjugated Sheep anti-Rabbit IgG as previously described (Oerther et al., 1999). The stained cells were applied to a microscope slide, air-dried, and treated as described below. For FISH, fixed cells (1 μl) were applied in a sample well on a washed super cured, heavy teflon coated microscope slide (Cel-Line Associates Inc., Newfield, NJ), air dried, and dehydrated using an ethanol series and hybridized with the oligonucleotide probe S-G-Gor-0596-a-A-22 labeled with tetramethyl rhodamine isothiocyanate (TRITC) or fluorescein isothiocyanate (FITC) (Genosys Corp., The Woodlands, TX) as previously described (de los Reyes et al., 1997). For both antibody staining and FISH, the slides were placed in 100 ml of fresh 4.6-diamidino-2-phenylindole dihydrochloride (DAPI) staining solution (100 mM Tris HCl [pH 7.2], 0.9 M NaCl, and 25 [g DAPI] 1⁻¹ H2O) for 2 min, then rinsed with ice-cold water (de los Reyes et al., 1997). Finally, stained or hybridized cells were mounted with Citifluor (UKC Chemical Laboratory, Canterbury, UK) and a cover slip. Visualization of the fluorescence signal was conducted with an epifluorescence microscope (Axioskop, Zeiss, Germany) and dichromatic filter sets 41001-fluorescein, 41002-rodamine, and 31000-DAPI (Chroma Tech. Corp., Brattleboro, VT). Digital images were captured with a KAF 1400 charge coupled device (Photometrics, Tucson, AZ), and data were analyzed with IP Laboratory Spectrum IO software version 3.0 (Signal Analytics, Vienna, VA).

To determine the percentage of Gordonia spp. in the biomass, 24 random image fields were captured (eight images each from three microscope slides), and the total filament length from each image field was determined (de los Reyes et al., 1998b). The mass of Gordonia spp. was estimated using equation (1) (de los Reyes et al., 1998b)

\[
\text{mg Gordonia spp.-VSS} = \frac{R_c 	imes (L_w 	imes A_w)/(V 	imes a)}{c_R}
\]

where \( R = 4.14 \times 10^{-10} \text{ mg Gordonia spp.-VSS} \mu m^{-1} \), \( L_w \) the average total filament length per field for the 24 random images (μm), \( A_w \) the area of microscope slide sample well (μm²), \( V \) the volume of sample applied to the microscope slide sample well (ml), \( A \) the area of microscope field (μm²).

The total filament length per field for the eight random image fields were averaged. Thus, three averages were generated from the three microscope slides. These three averages were used to calculate the standard deviation of the average total filament length per field.

RESULTS AND DISCUSSION

The UCSD, NEWWTP has a history of seasonal filamentous foaming anecdotally attributed to loading changes caused by the arrival and departure of students at the University of Illinois at Urbana-Champaign (Bachman, personal communication). Approximately 43% of the municipal population served by this facility consists of students (33,950 full time students in Spring 1997/79,864 individuals served by the UCSD, NEWWTP). To examine the link between the operation of the UCSD, NEWWTP, the abundance and activity of Gordonia spp., and the formation and persistence of filamentous foaming, we monitored the foaming potential and microbiological population composition of the UCSD, NEWWTP throughout 1997.

UCSD, NEWWTP operational data

Figures 2 and 3 present data collected by the on-site laboratory at the UCSD, NEWWTP during 1997. The aeration basin was configured for contact-stabilization operation from days 1 to 149 and from days 197 to 365. From days 150 to 196, the aeration basin was configured for operation as a conventional activated sludge system (indicated by the shaded areas in Figs 2 and 3). Various events in 1997 are indicated by diamonds in Figs 2 and 3. The University of Illinois Spring semester started on day 20 (diamond 1) and ended on day 137 (diamond 3). The Fall semester began on day 238 (diamond 4) and ended on day 353 (diamond 5). The one-week Spring Holiday started on day 80 (diamond 2), while the four-day Thanksgiving break began on day 332 (diamond 5). From days 190 to 294, filamentous foaming was observed on the surfaces of the aeration basin and secondary clarifiers.
Fig. 2. Operational data for the 1997 calendar year for the UCSD, NEWWTP. (a) Influent BOD$_5$ and daily solids removal (WAS + effluent suspended solids [SS]); (b) average solids concentration of composite sample from the contact basins (B, C, and D) and suspended solids concentration in the re-aeration basin (basin A); (c) $F/M$ and SRT. The shaded area indicates operation in conventional activated sludge mode. Diamonds one through six represent the first day of instruction for the Spring semester at the University of Illinois, the first day of the Spring Vacation, the last day of exams for the Spring semester, the first day of instruction for the Fall semester, the first day of the Thanksgiving break, and the last day of exams for the Fall semester, respectively.
Figure 2(a) shows changes in the BOD$_5$ treated in the activated sludge system as well as the suspended solids removed from the system. As suggested by personnel at the UCSD, NEWWTP, some changes in the influent BOD$_5$ corresponded to changes in the student population. For instance, when the Spring and Fall semesters began, the influent BOD$_5$ increased approximately 71% and 52%, respectively (diamonds 1 and 4, Fig. 2(a)). Similarly, at the start of the Spring Holiday (diamond 2, Fig. 2(a)), at the start of the Thanksgiving break (diamond 5, Fig. 2(a)), and at the end of the Fall semester (diamond 6, Fig. 2(a)), the influent BOD$_5$ decreased 52%, 44%, and 44%, respectively. In contrast, the end of the Spring semester did not correspond to a change in the influent BOD$_5$ (diamond 3, Fig. 2(a)).

Also, the increase in influent BOD$_5$ in mid-February and the decrease in influent BOD$_5$ at the end of October did not correspond to changes in the student population. Thus, although some changes in the influent BOD$_5$ were associated with changes in the student population, additional unidentified factors affected the influent BOD$_5$.

Figure 2(a) also shows changes in the suspended solids removed from the activated sludge system. A comparison of these data with the influent BOD$_5$ data suggests that changes in the rate of solids removal corresponded to changes in the influent BOD$_5$. For example, in mid-January, the increase in the suspended solids removed coincided with an increase in influent BOD$_5$. The increase in the suspended solids removed in early July corresponded to an attempt by the UCSD, NEWWTP personnel to control the initiation of filamentous foaming by increasing the solids removal rate.

In Fig. 2(b), the MLSS concentration in the re-aeration basin (A) and the MLSS concentration of the composite sample from the contact basins (B, C, and D) are shown. When the plant was operated in contact-stabilization mode, the MLSS level in the contact basin was relatively stable with an average value of approximately 1100 mg l$^{-1}$ (Fig. 2(b)). In contrast, the MLSS concentration in the re-aeration basin was highly variable ranging from approximately 6200 mg l$^{-1}$ in mid-March to 3500 mg l$^{-1}$ in early November (Fig. 2(b)). Changes in the removal of suspended solids (Fig. 2(a)) corresponded to changes in the MLSS levels in the re-aeration basin (Fig. 2(b)).

From days 150 to 196, when the plant was operated in conventional activated sludge mode (shaded areas in Fig. 2), the removal of suspended solids was decreased (Fig. 2(a)) and the MLSS concentration in the aeration basins increased (Fig. 2(b)). Coincidently, when the MLSS level in the aeration basins reached 1900 mg l$^{-1}$ during the first week of July, foam appeared on the surfaces of aeration basins and secondary clarifiers (Fig. 2(a)–(c)). Despite the return to contact-stabilization mode on day 197, foaming persisted for approximately 12 weeks until late October (Fig. 2(a)–(c)). The return to contact-stabilization mode diverted solids from the contact basin to the re-aeration basin (Fig. 2(b), early July). The changes in operation associated with the switch from conventional to contact-stabilization mode may have been partly responsible for providing an environment suitable for the persistence of foaming.

The F/M ratio (calculated as the daily mass of BOD$_5$ present in the primary effluent divided by the mass of MLSS in the aeration basins [basins A, B, C, and D]) and the SRT (calculated as the mass of MLSS in the aeration basins divided by the mass of solids removed per day [mass of WAS plus effluent suspended solids removed per day]) are shown in Fig. 2(c). When the plant was operated in contact-
stabilization mode (from days 1 to 149 and from days 197 to 365), the $F/M$ ratio varied from 0.18 to 0.5 (g BOD$_5$/[g suspended solids day]$^{-1}$) and the SRT was maintained between 2.3 and 5 days. From days 150 to 196, the change in operation from contact-stabilization to conventional mode resulted in considerable variability in the $F/M$ ratio and the SRT (Fig. 2(c)). Although this variability suggests that the system was unstable, foam did not appear until the first week of July. This observation suggests that conditions in addition to the change in operation from contact-stabilization to conventional mode were required for the initiation of filamentous foaming.

Figure 3 shows the SVI$_{30}$ and the temperature of the mixed liquor in aeration basin D as well as the daily high and low ambient temperatures. The temperature of the mixed liquor showed the same general trend as the ambient temperature, and reached a maximum of approximately 23°C from late-June to mid-August (Fig. 3). The initiation of filamentous foaming coincided with the beginning of this period, suggesting that high temperatures may be one trigger to encourage the growth of filamentous microorganisms involved in the initiation of foaming.

The SVI$_{30}$ values in the aeration basin were low (100–150 ml g$^{-1}$) during the initiation of filamentous foaming. Thus, the foaming event coincided with well settling biomass (Fig. 3) and was not associated with sludge bulking. In contrast, during the winter season (January, February, November, December), the SVI$_{30}$ values were often above 200 ml g$^{-1}$, suggesting moderate bulking.

### Rapid solids determination

We developed a protocol for the rapid determination of suspended solids levels using microwave drying. One-way Analysis of Variance (ANOVA) was conducted on the means of suspended solids concentrations determined by standard methods and the alternative microwave drying procedure (Table 1). An ANOVA $P$-value greater than an $x$ of 0.05 (i.e., 95% confidence interval) was used to indicate that the means of replicate suspended solids concentrations determined by standard methods and the alternative microwave drying method were not significantly different from each other (Table 1). Sample volumes that met this comparability criterion (i.e., sample A, 2-, 4-, and 6-ml volumes; sample B, 6- and 8-ml volumes; and sample RAS, 2- and 6-ml volumes) were further examined with repeated $t$-tests to isolate the treatment method that produced the least deviation from standard methods (underlined values in Table 1, e.g., sample A, 2-ml volume heated for 20 min).

The ANOVA analysis showed that the 6-ml samples produced results consistently comparable with standard methods. In addition, the $t$-test comparisons for all 6-ml samples demonstrated the lowest average value deviation for samples microwaved for 15 min. Together, these results suggest that 15 min of microwave drying of a filter containing solids from 6 ml of sample produced results that were statistically comparable to the concentration of suspended solids determined with standard methods. The advantage of the alternative microwave drying protocol is the shorter time necessary to dry a sample in the microwave compared to a conventional oven at 105°C. Therefore, this protocol can be used to determine the concentration of suspended solids in a sample while performing the 30-min quiescent settling for the foaming potential test.

### Foaming potential

To measure the foaming potential of the UCSD, NEWWTP and monitor changes in microbial population levels, multiple samples were taken from three locations in the plant before, during, and after the

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**Table 1.** Analysis of variance (ANOVA) and $t$-test comparisons of the microwave solids drying protocol compared to the determination of suspended solids using standard methods.

<table>
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<tr>
<th>Sample</th>
<th>Volume (ml)</th>
<th>$P$-value</th>
<th>5 min</th>
<th>10 min</th>
<th>15 min</th>
<th>20 min</th>
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incident of filamentous foaming (Fig. 4(a)). The RAS line was sampled just before returning the activated sludge to re-aeration basin A (before cell A6), the re-aeration basin was sampled in cell A2 near the location where mixed liquor was mixed with primary effluent, and the contact basin was sampled in cell B2 close to the place where primary effluent was introduced (Fig. 1). Thus, the biomass samples should represent the highest solids concentrations (i.e., RAS line), least active/most highly endogenously respired biomass (i.e., end of the re-aeration basin), and most active biomass (i.e., start of the contact basin). During the occurrence of foaming, the highest surface coverage and the zone of thickest, darkest foam was found around the sampling point for the re-aeration basin, while the surface of the contact basin was typically free from filamentous foam but occasionally exhibited a slight frothy white foam.

In contrast to previous work, which examined measures of foaming potential for a single MLSS concentration (e.g., Pitt and Jenkins, 1990; Ho and

Fig. 4. Results of the application of the foaming potential test to the UCSD, NEWTTP. (a) Sampling schedule; (b) foam volume for different suspended solids levels before (April and May), during (July, August, and September), and after (December) the appearance of foam; (c) plot of the power functions (equations (2), (3) and (4) that were fitted to the results of the foaming potential test.
Jenkins, 1991), we measured foaming potential over a range of MLSS levels (Fig. 4(b)). Each sample obtained from the RAS line and cells A2 and B2 (Fig. 4(a)) was used to prepare 11 subsamples with different suspended solids concentrations. The results of the foaming potentials for all subsamples are presented in Fig. 4(b). These data were fitted using power functions (of the form $y = ax^b$, where $a$ and $b$ are constants) and linear regressions (of the form $y = c + dx$, where $c$ and $d$ are constants). The results were similar, but the power functions exhibited better fits as indicated by their higher $R^2$ values (data not shown). Thus, the results from the foaming potential test before, during, and after foaming can be described by the following three linearized power functions in which $x$ and $y$ represent MLSS (mg suspended solids l$^{-1}$) and foam volume (ml), respectively

$$\log y = (\log 46.196) + [0.1914] \log x; \quad R^2 = 0.7707$$

(2)

$$\log y = (\log 83.479) + [0.1621] \log x; \quad R^2 = 0.7713$$

(3)

$$\log y = (\log 24.988) + [0.2487] \log x; \quad R^2 = 0.8725$$

(4)

These equations are shown graphically in Fig. 4(c). One-way ANOVA of the values of the slopes (in square brackets in equations (2), (3) and (4)) showed these were not significantly different from each other at an $x$ of 0.05 (95% confidence interval). In contrast, ANOVA demonstrated that the intercept terms (in parentheses in equations (2), (3) and (4)) were significantly different. These results suggest that the concentration of MLSS affects the foaming potential test in a constant manner (i.e., the results of the foaming potential tests from before, during, and after foaming shared a common slope). Additionally, the results of the foaming potential test appeared to depend upon the levels of surfactants or other soluble components present in the mixed liquor (i.e., the results of the foaming potential tests from before, during, and after foaming demonstrated unique intercepts). The results demonstrate that this simple test can be used to compare the foaming potential of activated sludge at different time points in a single wastewater treatment plant. Furthermore, the results indicate that the levels of suspended solids in the samples affect the foaming potential, making it important to adjust the solids levels before each foaming potential test or to perform the test for a range of solids levels.

**Membrane hybridizations**

If the cause of foaming is the presence of a specific microbial population, then a correlation should be observed between the occurrence of foaming and the abundance or physiology of this microbial population in a wastewater treatment plant. Since our previous results suggested that *Gordonia* spp. were responsible for foaming in the UCSD, NEWWTP (de los Reyes *et al.*, 1997, 1998a,b), quantitative membrane hybridizations were performed with an oligonucleotide probe targeting the 16S rRNA of *Gordonia* spp. (probe S-G-Gor-0596-a-A-22). Figure 5(a) shows the relative abundance of *Gordonia* rRNA in samples taken from re-aeration basin A (cell A2), contact basin B (cell B2), and RAS. Before the initiation of foaming, the levels of *Gordonia* rRNA were approximately 0.25% of the total rRNA for all three sampling sites (Fig. 5(a)). During foaming, the levels of *Gordonia* rRNA increased to approximately 1.4% of total rRNA, and after the disappearance of foaming, the levels of *Gordonia* rRNA decreased to levels comparable to those found before the initiation of foaming (Fig. 5(a)).

To examine the effects of sample site selection on the hybridization results, samples were removed from various locations in the activated sludge system on August 26, 1997 (day 238) (Fig. 5(b)). The surface foam on re-aeration basin A contained approximately 2.5 times higher levels of *Gordonia* rRNA than the mixed liquor (Fig. 5(b)). In contrast, the rRNA levels were higher in the mixed liquor than in the surface foam for the contact basins (with the exception of cell D2). The hybridization results are consistent with visual observations of the foam in the plant (data not shown). For instance, the foam in re-aeration basin A was darker in color, thicker, and covered a larger fraction of the surface than the foam in contact basin B. These results suggest that sample selection is important when attempting to quantify changes in the levels of *Gordonia* spp. in wastewater treatment plants.

**FISH and antibody staining**

To complement the measurement of rRNA abundance quantified with membrane hybridizations, we conducted whole cell antibody staining and FISH to determine the mass of *Gordonia* using a previously established relationship between mass and total extended filament length developed for a pure culture of *G. amarae* SE-102 (de los Reyes *et al.*, 1998b). The mass of *Gordonia* spp., reported as the % *Gordonia* VSS of the total VSS, was measured in a limited number of samples removed before (two samples from cell B2), during (two samples from RAS and one sample from cell B2), and after (two samples from cell B2) the seasonal occurrence of filamentous foaming. The results from whole cell antibody staining and FISH for the seven samples are shown in Fig. 6. The results show that the % *Gordonia* VSS of the total VSS measured with antibody staining was similar to the result obtained with FISH for three of
the seven samples (i.e., samples from cell B2 on days 107, 113, and 266). In contrast, the % *Gordonia* VSS of the total VSS measured with antibody staining was higher than the results obtained with FISH for the other four samples (i.e., samples from RAS on days 197 and 266 and samples from cell B2 on days 304 and 343). The discrepancy between the levels of *Gordonia* spp. measured with antibody staining and FISH is likely the result of low levels of rRNA in individual cells of *Gordonia* spp. Similar observations have been discussed previously (Oerther et al., 1999). We do not believe that the lower FISH response was due to insufficient cell fixation since we optimized cell fixation methods for *Gordonia* spp. to ensure the cells were permeable to the fluorescently labeled oligonucleotide probes (de los Reyes et al., 1997). Furthermore, the fact that *Gordonia* spp. contribute only a small fraction to the total rRNA despite their numerical abundance, as suggested by combining FISH and membrane hybridization results, supports the notion that the intracellular rRNA levels of *Gordonia* spp. are low and that a fraction of the *Gordonia* biomass is metabolically less active. Concerns about incomplete recovery of rRNA during the RNA extraction step performed before membrane hybridization have been addressed previously (Raskin et al., 1997).

The results of whole cell antibody staining and FISH show a five-fold increase in the % *Gordonia* VSS during foaming in cell B2 (from less than 4% of day 113 to almost 20% on day 266, Fig. 6). This result is consistent with the approximately five-fold increase in the percentage of total rRNA attributed to the *Gordonia* genus (Fig. 5(a)). Thus, the increase in activity attributed to *Gordonia* (as measured by quantitative membrane hybridizations) is similar to
the increase in mass of *Gordonia* (as measured with whole cell antibody staining). Ten days after the disappearance of all traces of surface foam (day 304), the % *Gordonia* VSS remained high (24%) in cell B2 (Fig. 6). This result demonstrates that filamentous foaming can subside even in the presence of elevated mass fractions of *Gordonia* spp. in the mixed liquor, and therefore suggests that additional factors are necessary for the persistence of filamentous foaming. The antibody staining and FISH results for day 343 show that the mass of *Gordonia* spp. eventually returned to levels comparable to those in samples taken before the appearance of surface foam.

**CONCLUSIONS**

In this study, results from a foaming potential test, oligonucleotide probe hybridizations, and antibody staining correlated with a seasonal occurrence of filamentous foaming at the UCSD, NEWWTP. We demonstrated that a simple Alka-Seltzer™ based test successfully quantified filamentous foaming at different time points within a single wastewater treatment plant. Additionally, our results indicate that the levels of suspended solids affect the foaming potential test, but that the relationship between foaming potential and suspended solids can be determined. The differences in foaming potential observed at various time points are hypothesized to be the result of variable levels of surfactants or other soluble components. To allow a broader application of the foaming potential test, future work should evaluate if this test can be used to predict the occurrence of filamentous foaming before foam appears on the surfaces of aeration basins and clarifiers, and should establish if results of this test can be compared for different plants.

Results obtained with oligonucleotide probe hybridizations and antibody staining indicate that the abundance of *Gordonia* rRNA as well as the % *Gordonia* VSS transiently increased approximately five-fold during the appearance of foaming. Thus, *Gordonia* spp. can represent an important fraction of the total biomass of a wastewater treatment plant suffering from filamentous foaming (> 20% of total VSS). However, a comparison of the results obtained with antibody staining and FISH suggest the presence of significant levels of metabolically less active *Gordonia* spp. Similarly, a comparison of the results obtained with membrane hybridizations and those obtained with antibody staining and FISH suggest that on average for every 24% *Gordonia* VSS of the total VSS, *Gordonia* spp. contributed only 1% of the total rRNA. Thus, a numerically dominant microbial population may contribute only a small fraction of the total rRNA pool. Based on these results, future studies of microbial populations in activated sludge should rely upon quantification of both the relative abundance of rRNA (e.g., by membrane hybridizations) and the fractional mass contributed by target populations (e.g., by whole cell antibody staining and FISH).

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