An Inexpensive, Reproducible Method to Quantify Activated Sludge Foaming Potential: Validation Through Lab-Scale Studies and Year-Long Full-Scale Sampling Campaign

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Abstract

Activated sludge is a conventional treatment process for biochemical oxygen demand (BOD) and total suspended solids (TSS) removal at water resource recovery facilities (WRRFs). Foaming events are a common operational issue in activated sludge and can lead to decreased treatment efficiency, maintenance issues, and potential environmental health risks. Stable foaming events are caused by biological and chemical drivers (i.e., microbes and surfactants) during the aeration process. However, foaming events are difficult to predict and quantify. We present an inexpensive and easy-to-use method that can be applied at WRRFs to quantify foaming potential. Subsequently, the method was applied over a year-long full-scale study while data on microbial community composition and functional parameters associated with foaming potential were collected from activated sludge samples at South Shore Water Reclamation Facility (WRF) (Oak Creek, WI). Results from the development of the foaming potential method using linear alkylbenzene sulfonate (LAS) showed that the method was reproducible (relative standard deviation <20%) and able to capture changes in foam-inducing constituents. Using full-scale activated sludge samples, higher relative abundance values for the following genera were associated with foaming events: *Zoogloea*, *Flavobacterium*, *Variovorax*, and *Bdellovibrio*. This is the first report that *Variovorx* and *Bdellovibrio* relative abundance was correlated with foaming events in activated sludge. Furthermore, the foaming potential positively correlated (ρ = 0.24) with soluble total nitrogen. Characterizing foaming events through frequent sampling and monitoring of specific genera and functional parameters may allow for predictions and preemptive mitigation efforts to avoid negative consequences in the future.

Practitioner Points

- A reproducible method to measure foaming potential in activated sludge is available.
- Genera *Zoogloea*, *Flavobacterium*, *Variovorax*, and *Bdellovibrio* correlated with foaming events.
- A year-long sampling campaign of activated sludge measuring foaming potential and microbial community composition was conducted at South Shore Water Reclamation Facility in Oak Creek, WI.
- More research at other facilities with this method is needed to understand links between microbes and foaming

Introduction

Water resource recovery facilities (WRRFs) commonly apply the activated sludge treatment process to remove biochemical oxygen demand (BOD) and total suspended solids (TSS). Foaming events are an operational issue that sporadically affect WRRFs (Jenkins et al., 2004; Metcalf & Eddy, 2014). Foam produced in activated sludge basins causes safety, efficiency, and maintenance issues for plants. Increased effluent concentrations of BOD and TSS, possible pathogen and antibiotic resistance exposure to plant operators, pipe blockages, and interference with monitoring equipment are all potential problems (Jenkins et al., 2004; Vardar-Sukan, 1998; Zhang et al., 2021). Foaming is a particular challenge because it is both difficult to predict and to quantify. Developing methods to quantify and to predict foaming events in activated sludge would allow operators to mitigate events before they occur and prevent subsequent, negative consequences.

Foaming is caused by the dispersion of gas through the mixed liquor (Vardar-Sukan, 1998). One theory, known as flotation theory, suggests that within activated sludge basins, three mechanisms can contribute to a stable foaming event: air bubbles, surfactants (synthetic or biological), and hydrophobic cells (Petrovski et al., 2011). Foaming characterization and quantification is difficult for WRRFs because there is not currently a standard method. This has led to several characterization methods, such as visual observation, quantified observations, and measured foaming (Dunkel et al., 2018; Frigon et al., 2006; Fryer et al., 2011). Measurements through observation are based on whether the activated sludge basin has had a history of foaming: yes or no (Dunkel et al., 2018; Jiang et al., 2016; Rosso et al., 2019; Zhang et al., 2021). Quantified observations typically adopt the Frigon et al. (2006) method where operators describe the level of foaming in the activated sludge basin on a scale of 0–4 (0, *being no foaming* and 4, *being foam in the aeration basin and secondary clarifiers*) (Li et al., 2020; Wang & Yu, 2016). Quantified foaming is often measured using one of the following tools: Alka-Seltzer™ tablets, diffusers, or sintered discs (De Los Reyes & Raskin, 2002; Fryer et al., 2011; Oerther et al., 2001). While the quantified measurement is the most useful measure to capture slight changes and remove bias, a current problem is that there is no standardized quantifiable measurement that is easy-to-use and relatively inexpensive for implementation at WRRFs. Thus, there is a need for a standard method to measure activated sludge foaming potential that provides reproducible, quantitative results. Such a method would help WRRFs to not only track foaming events but also understand what causes foaming events through further research.

Both biological and chemical drivers have been linked to foaming. Biological drivers, specifically, are foam-forming microbes. Mycolata, which are mycolic acid producing *Actinobacteria*, have been reported typically as the common foam formers in activated sludge (Dunkel et al., 2018; Jenkins et al., 2004; Petrovski et al., 2011; Pitt & Jenkins, 1990). Furthermore, *Gordonia* spp. and *Gordonia amarae*, which are Mycolata (and historically misidentified as *Nocardia*), have been identified historically as foam-forming bacteria in mixed liquor from foaming activated sludge (De Los Reyes & Raskin, 2002; Dunkel et al., 2018; Frigon et al., 2006; Oerther et al., 2001; Pitt & Jenkins, 1990). Additionally, *Microthrix parvicella* (*M. parvicella*), of the *Actinobacteria* phylum, has been associated with activated sludge foaming (Jenkins et al., 2004). While common foam-forming microbes have been reported for inducing activated sludge foaming events, research has shown, with the use of next-generation sequencing (NGS) tools, that not all foaming events can be attributed to these microbes (Guo et al., 2015). Therefore, there may be novel populations of foam-forming microbes in activated sludge outside the previously classified microbes.

Biotechnologies, such as activated sludge, rely on microbial communities to drive function. The biological drivers of foaming events in activated sludge are associated with functional parameters (e.g. BOD_5 removal). By creating an optimal environment for these microbes, the functional parameters may induce a foaming event in the activated sludge. Functions such as settleability, measured as sludge volume index (SVI), nitrogen and phosphorus concentrations, surfactant concentrations, and seasonal temperature have previously been associated with foaming or the presence of common foaming microbes (Dunkel et al., 2018; Jiang et al., 2016; Li et al., 2020; Rosso et al., 2019; Wang & Yu, 2016). However, if there are novel foam-forming populations, then there may be dynamic relationships between foaming events and functional parameters beyond those commonly reported. Therefore, the

relationship between activated sludge functional parameters and foaming potential needs to be investigated for novel foam formers.

The goal of this research was to develop a method to quantify foaming potential so that a better understanding of activated sludge foaming drivers could be established. The research objective was to apply the foaming potential measurement to activated sludge to identify relationships between foam formation and the occurrence of particular microorganisms and water quality parameters. Weekly samples over a 1-year period at South Shore Water Reclamation Facility (WRF) in Oak Creek, WI, USA, were collected to identify and characterize foaming events at the facility. 16S rRNA gene sequencing was conducted to observe full-scale microbial community composition changes in relation to foaming potential over the course of 1 year. Additionally, the influence of activated sludge functional parameters was investigated to understand these drivers.

Materials And Methods

Development of foaming potential method

The foaming potential method
The potential for foaming in activated sludge was measured using the foaming potential apparatus (Figure 1). A 0.2–2.5-LPM Flow Meter (VWR International, Radnor, PA) and an Aquatic Eco-Systems Sweetwater Air Diffuser (Model: AS4 1.5 × 1.5 in.) (Pentair, Minneapolis, MN) with a bubble size of 1–3 mm were used to adjust the flow of air and diffuse air into the sample and generate bubbles, respectively. Up to 10 L of mixed liquor were collected and from that sample, 200 mL of mixed liquor were added to a 1-L graduated cylinder. The mixed liquor was well mixed prior to subsampling to minimize subsampling error. The diffuser was placed in the sample, and the flowrate of air was adjusted to 1.0 LPM. The air was directed into the sample for 1 min at room temperature. The active foam volume (foam formed on the surface when air is diffusing) was recorded by reading the graduated cylinder lines after aeration. The air was then turned off, and the resting foam volume was recorded after 1 min of no air flow. An active foam volume less than 5 mL per 200 mL of mixed liquor was not distinguishable by eye. Therefore, foaming potential values, measured as active foam volume per 200 mL of mixed liquor, less than 0.025 mL/mL were not discernable and were considered the limit of detection. Values below this were reported as <0.025 mL/mL. This method uses a similar approach of diffusing air into a sludge sample as employed by Novak et al. (1990) and used recently by Kersch et al. (2020). Here, we provide details on supplies, manufacturers, and reproducibility in surfactant and mixed liquor solutions to validate the method.

FIGURE 1 Apparatus to measure foaming potential of activated sludge. The apparatus was connected to controlled air flow in a fume hood. The diffuser was submerged in the activated sludge sample. Foaming potential was measured by observing the foam volume in the graduated cylinder.

Linear alkylbenzene sulfonate (LAS) and the foaming potential method

The foaming potential method was developed with the goal of being inexpensive, easy-to-use, and reproducible. Before the method was used for activated sludge measurements, the foaming potential method was tested under controlled conditions using a LAS solution (Fisher Scientific, Hampton, NH) in deionized water. LAS has been shown to cause foaming, and this was a controlled approach to test method reproducibility (Cohen et al., 1993). The active and resting foam volumes for increasing LAS concentrations in deionized water were determined following the foaming potential method described above.

Wastewater samples and the foaming potential method

Primary effluent (PE) and recycled activated sludge (RAS) samples were mixed at varying ratios to test the reproducibility of the foaming potential method in various wastewater samples. PE and RAS samples (sample volume = 10 L) were collected from an aeration basin at South Shore WRF on Friday, July 23, 2021. The PE sample was taken from the overflow weir of the primary clarification basin. The RAS sample was taken from the recirculation pipe. The PE and RAS were transported to the Water Quality Center Lab at Marquette University (~30 min) and mixed as indicated by Table 1 to generate five samples with ranging percentages of RAS compared to PE.

TABLE 1. Primary effluent (PE) to recycled activated sludge (RAS) ratio experimental setup #1 (determined by the percentage of RAS on a volumetric basis) for foaming potential.

Percent of recycled activated sludge (%)	Primary effluent (mL)	Recycled activated sludge (mL)
100	0	200

75	50	150
50	100	100
25	150	50
0	200	0

The foaming potential method was later tested again using a wider variety of PE to RAS ratios. PE and RAS samples (sample volume = 10 L) were manually pulled at South Shore WRF on Wednesday, April 27, 2022. The samples were mixed as indicated by Table 2 to generate eight mixes of RAS to PE. The foaming potential of each sample was measured as described in The foaming potential method section.

TABLE 2. Primary effluent (PE) to recycled activated sludge (RAS) ratio experimental setup #2 (determined by the percentage of RAS on a volumetric basis) for foaming potential.

Percent of recycled activated sludge (%)	Primary effluent (mL)	Recycled activated sludge (mL)
100	0	200
90	20	180
80	40	160
75	50	150
60	80	120
50	100	100
25	150	50
0	200	0

One-year sampling campaign to observe relationships of microbial community composition and functional parameters to foaming potential

Activated sludge samples (sample volume = 10 L) from the West Plant at South Shore WRF were obtained approximately once a week over the course of 54 weeks (n = 53; one sampling week missed due to COVID-19 quarantine protocol). The South Shore WRF employs aerobic tanks for BOD_5 removal (no anoxic zones). The plant receives influent from separated sewers (i.e., no combined sewers). The aeration basins are classified as "west" or "east" basins. The set of west basins is adjacent to the set of east basins and receives the same influent flow. All samples were collected from west basins unless noted (east basins were only sampled if west were undergoing maintenance). The first of the 53-week sampling days was Wednesday, June 10, 2020, at 9:00 a.m. The samples were collected at the midpoint of the basin approximately 2 ft beneath the surface. Additional samples over the course of 5 weeks (Week 54–Week 57) were taken in the front 1/3 and back 1/3 of the basin, which resulted in two samples every week except for Week 55 (n = 7). The samples were transported to the Water Quality Center Lab (~30 min), and 10 mL of the 10-L sample was saved and stored at −20°C until the DNA was extracted.

Analytical methods

Total solids (TS), TSS, volatile solids (VS), and volatile suspended solids (VSS) analyses were performed according to Standard Methods 2540 B, 2540 D, and 2540E, respectively (APHA-AWWA-WEF, 1998). Sludge settleability and SVI were measured in accordance with Standard Methods 2710 C and D (APHA-

AWWA-WEF, 1998). Sludge settleability was measured unstirred. Soluble chemical oxygen demand (sCOD) was measured by filtering the mixed liquor sample through a 0.45-µm cellulose nitrate membrane filter (Whatman™, Marlborough, MA), and the filtrate was analyzed following Standard Method 5220 D (APHA-AWWA-WEF, 1998). Total dissolved phosphorus and dissolved reactive phosphorus concentrations were measured by filtering the mixed liquor sample through a 0.45-µm cellulose nitrate membrane filter (Whatman™, Marlborough, MA), and the filtrate was measured using the ascorbic acid method, Standard Method 4500-P E. (APHA-AWWA-WEF, 1998). For the ascorbic acid method, the appropriate pH was reached with the addition of 6 N NaOH using a phenolphthalein indicator and further adjusted using one drop of 50% HCl.

Conductivity was measured using ExStik® II pH/Conductivity/TDS Meter (Extech Instruments, Nashua, NH). The pH was measured using an Orion™ 4-Star Plus pH/DO Benchtop Meter (Thermo Fisher Scientific, Waltham, MA). Soluble anionic surfactant and soluble total nitrogen concentrations were measured by filtering the mixed liquor sample through a 0.45-µm cellulose nitrate membrane filter (Whatman™, Marlborough, MA) and following the supplier instructions for the TNT 874 and TNT 826 vial tests, respectively (Hach®, Loveland, CO). Capillary suction time (CST) of the mixed liquor was measured using the Triton Type 319 Multi-purpose CST (Triton Electronics Limited, Essex, England) by placing 3 mL of sample into the 1.8 cm stainless steel funnel attached to the single radius test head with cellulose chromatography paper (Whatman™, Marlborough, MA).

The sCOD and soluble total nitrogen measurements were performed on Week 20 to Week 33 using a 0.7-µm glass fiber filter (MilliporeSigma™, Burlington, MA) instead of the 0.45-µm cellulose nitrate membrane filter (Whatman™, Marlborough, MA). It was determined that the difference between the two filters yielded concentrations that were not statistically significant (n = 5 samples) ($p \leq 0.05$) using a Student's t-test. There was no soluble anionic surfactant, total dissolved phosphorus, and dissolved reactive phosphorus data for Week 20–Week 33.

Molecular methods

DNA extractions
The FastDNA™ SPIN Kit (MP Biomedicals, Santa Ana, CA) was used to extract DNA. Lysis buffer (CLS-TC) was added to the activated sludge samples (volume = 0.5 mL), and then the samples were subjected to three freeze–thaw cycles utilizing liquid nitrogen to maximize lysis of bacteria (Kimbell et al., 2021). DNA purification followed the manufacturer instructions with the following modifications: (1) The samples were centrifuged for 30 min at 14,000 ×g to pellet debris; (2) gentle agitation occurred for 20 min at room temperature on a variable speed rocker (VWR International, Radnor, PA); (3) the sample/binding matrix suspension was centrifuged for 15 min at 14,000 ×g to pellet binding matrix; (4) an additional 250 µL of SEWS-M was added and centrifuged until no liquid contents remained; and (5) a double elution of 50 µL of DES was performed. Samples were quantified using NanoDrop™ One/OneC Microvolume UV–Vis Spectrophotometer (Thermo Fisher Scientific, Waltham, MA). DNA extractions were stored at −20°C until further downstream analysis. The DNA extractions for Weeks 1, 32, and 47 failed and were not carried on to downstream analyses.

Illumina DNA sequencing
The V4 region of bacterial 16S rRNA gene was PCR amplified from purified activated sludge sample DNA using the primers 515F (Parada et al., 2016) and 806R (Apprill et al., 2015) containing Nextera

adapter sequences (Ilumina, Inc., San Diego, CA). The following PCR setup was used: 12.5-μL 2X KAPA HiFi HotStart ReadyMix PCR (Roche, Pleasanton, CA); 1.5 μL each of a 5-μM forward and 5-μM reverse primer solution; 7.5-μL HyClone molecular grade water; and 2-μL 10×-diluted DNA template (dilution to minimize inhibition). PCR was run on a vapo-protect Mastercycler pro S (Eppendorf) under the following conditions: 95°C for 5 min; 25 cycles of 95°C for 30 s, 50°C for 30 s, 72°C for 1 min; 72°C for 1 min; and 4°C hold. One extraction blank and one mock community (ZymBOIOMICS Microbial Community DNA standard, Zymo Research, Irvine, CA) were sequenced to assess contamination and sequencing error rate. Triplicate PCRs were run from a single DNA extraction for each sample. PCR products were then screened by electrophoresis on 1.5% agarose gel to determine unsuccessful amplification or a lack of specificity. Following PCR and screening, the triplicate remaining PCRs were pooled (~75 μL) and cleaned with 60 μL of Agencourt AMPure XP beads (Beckman Coulter, Brea, CA), following the manufacturer's protocol. The final purified DNA was resuspended in 40-μL 10-mM Tris-EDTA. Then, individual sample libraries were prepared according to the Illumina MiSeq protocol in the Nextera XT Index kit (Illumina, Inc., San Diego, CA). Indexed PCR amplicons were cleaned with AMPure XP beads, and the resultant sample DNA quality and concentration were assessed using the BroadRange Qubit 2.0 spectrophotometric assay (Thermo Fisher Scientific Inc., Waltham, MA, USA). Libraries were then normalized for DNA concentration, pooled, and prepared for sequencing with the SequalPrep Kit (ThermoFisher Scientific). Sequencing was carried out on an Illumina MiSeq using a 2 × 250-cycle paired-end protocol at the University of Wisconsin-Milwaukee Great Lakes Genomics Center (Milwaukee, WI USA). Primer and barcode sequences from resulting reads were removed using Cutadapt (Martin, 2011).

Data processing and statistical analysis

RStudio (version 3.12; [R Core Team, 2018]) package "dada2" was used to process the DNA sequences, create amplicon sequence variants (ASVs), and assign taxonomy to the ASVs (Callahan et al., 2016). The packages "phyloseq" (McMurdie & Holmes, 2013), "ggplot," "ggplot2" (Wickham, 2016), and "vegan" were used to transform, compare, and visualize the microbial community composition (ASV) data. Alpha diversity was measured using the Shannon Index. The "aov" function was used for ANOVA analysis of alpha diversity values. Principal coordinate analysis (PCoA) was run with the "ape" package and was calculated from whole community relative-abundance based Bray–Curtis dissimilarity values comparing all samples. The "corrplot" package was used to calculate a Spearman's rank sum correlation between each of the functional parameters and the ASVs. The ASV was labeled in figures as the genus to which it belonged because not all ASVs were identified at the species level.

The relative abundance differential rankings were calculated using the relative abundances from the most significant foaming event (Week 28, foaming potential = 0.57 mL/mL) and the average relative abundances from low foaming events (foaming potential ≤0.18 mL/mL). The low foaming events had a similar Bray–Curtis measurement (Weeks 25, 27, and 29) to the significant foaming event. We conducted this procedure as a means of shortening the time period being compared, which reduced the influence of the large-scale seasonal community change on the observed relationships. ASVs identified as having high relative abundance change associated with foaming were then compared with the ASVs identified by the Spearman's rank correlation as being most associated with the foaming potential parameter across the entire sampling campaign.

This was done as a means of cross-referencing the highly associated ASVs from the Spearman's rank correlation while accounting for seasonal changes that occurred in the microbial community over the course of the 57-week sampling campaign.

Relative standard deviation is the standard deviation multiplied by 100 and then divided by the mean; the units are in percent.

Results & Discussion

Validation of the foaming potential method using LAS

The foaming potential method was used to characterize changes in foaming induced by a surfactant (Figure 2a). The data were log-transformed because of heteroscedasticity (increasing deviations with increasing surfactant concentrations) (Figure 2b). There was a positive relationship between the log LAS concentration and the log active foaming potential (R^2 = 0.92; 95% confidence intervals on slope of 1.027 to 1.341), an indication that the foaming potential method captures changes in foaming potential induced by changes in the sample matrix.
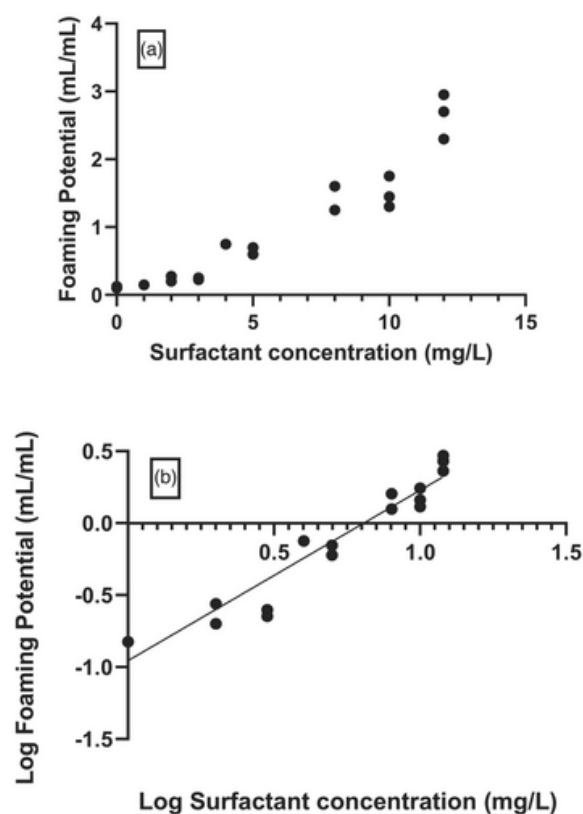

FIGURE 2 (a) The impact of linear alkylbenzene sulfonate solution on foaming potential. Concentrations were tested in triplicate. (b) Log–log plot of same data set. The R^2 of the log–log plot was 0.92, and the 95% confidence intervals for the slope were 1.027 to 1.341. The foaming potential method captured a positive trend between surfactant concentration and foaming potential.

The foaming potential method was reproducible using LAS as the foam inducer; the relative standard deviation (RSD) of the foaming potential was quantified to understand reproducibility of the foaming potential method. The average RSD was 9.8%. The higher surfactant concentrations yielded higher RSD

values indicating that substantial foaming events will likely have more variability when measured using this method.

Validation of the foaming potential method using wastewater samples

The foaming potential method captured changes in foaming potential at varying percentages of RAS, compared to PE, of the wastewater samples from both sampling dates. For the first ratio test with PE and RAS samples from July 23, 2021, the largest mean foaming potential occurred at 100% of RAS, which was over 10 times greater than the other samples (Figure 3a). The 75% (the second highest average) and 100% (the highest average) RAS testing parameters were significantly different (Mann–Whitney U test [$p \leq 0.05$]). The 0% and 75% RAS testing parameters were not significantly different (Mann–Whitney U test, $p > 0.05$).
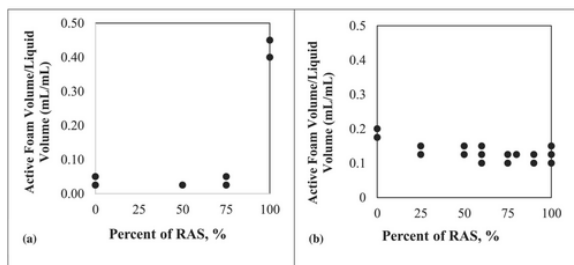

FIGURE 3 (a) Foaming potential at different percentages of recycled activated sludge (RAS). No discernable foaming was observed at the RAS percentage of 25%, and only one of three triplicates yielded a value above detection limit (0.025 mL/mL) at a RAS percentage of 50%. Primary effluent (PE) and RAS were sampled on July 23, 2021, from South Shore Water Reclamation Facility (WRF). (b) Foaming potential at different percentages of RAS; PE and RAS were sampled on April 27, 2022, from South Shore WRF. Some dots overlap. All conditions tested had readings above detection limit.

Results from a second round of active foam production testing in RAS to PE ratios contradicted the first test, as foam production was not related to RAS percentage (Figure 3b). Overall, these data implicate the dynamic nature of sludge systems, which change chemically and biologically over time based on influent characteristics and recycled material.

These data also indicate that sludge foaming should be measured over time to better understand dynamics at a treatment plant and how RAS to PE ratios impact foaming within changing plant dynamics. Despite the differences in the results from the RAS to PE ratio experiments, the reproducibility for individual samples was high among all samples. The standard deviation of foaming potential measurements was <5% for all RAS to PE mixtures. This is further evidence that this method is reproducible and applicable to full-scale wastewater samples.

The foaming potential method is simple to employ and cost effective. The entire test takes approximately 10 min from start to finish for triplicate technical replicates. The majority of the testing components consist of common lab equipment, such as a graduated cylinder, rubber tubing, and a clamp stand. The more specialized equipment, the 0.2–2.5-LPM Flow Meter (VWR International, Radnor, PA) and an Aquatic Eco-Systems Sweetwater Air Diffuser (Model: AS4 1.5 × 1.5 in.) (Pentair, Minneapolis, MN), as described in Development of foaming potential method section, are readily available and cost less than $150 (at the time of purchasing and not including labor costs).

Reproducibility of the method can be further defined as the method is used across more full-scale studies.

Microbial community composition and foaming potential
The definition of a stable foaming event using the new method presented herein was when the resting foaming volumes were greater than zero; there were only two stable foaming events over the course of 1 year at South Shore WRF (Figure 4). The most notable foaming event occurred on Week 28. This event produced foaming potential values that were over three times greater than the weekly average active foaming potential. The five highest foaming events occurred during Weeks 28, 53, 52, 26, and 4, and were 3.3, 2.6, 2.0, 1.8 and 1.7 times greater, respectively, than the average active foaming potential. Overall, the activated sludge basins at South Shore WRF were healthy in terms of foaming potential during the 57-week sampling period.
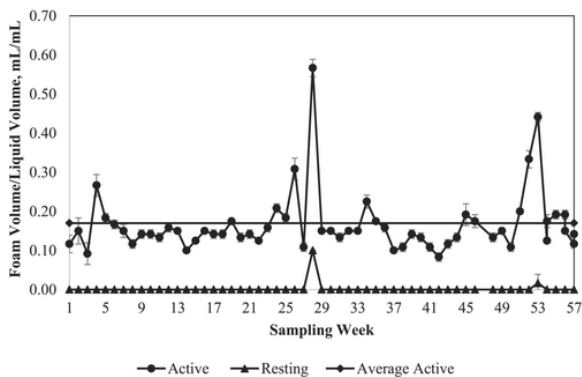

FIGURE 4 The foaming potential of the activated sludge over the course of 57 weeks from the West Activated Sludge Plant at South Shore Water Reclamation Facility (WRF) (n = 3 samples). The error bars are the average deviation. The start date (Week 1) was June 10, 2020. Active foam values are measured while air is actively diffusing through the sludge, whereas resting foam values are measured after discontinuing air diffusion for 1 min.

The Week 28 foaming event consisted of white, billowy foam, whereas the Week 53 foaming event produced a brown, denser foam. Both events were considered stable because the resting foaming potential values were greater than the minimum detection value. Foaming events have been previously characterized by foam type with possible foam-inducing variables and control methods (Gerardi, 2002; Jenkins et al., 2004). While Petrovski et al. (2011) found that air bubbles, hydrophobic cells, and surfactants are all required for stable foaming, the changing foam types observed in this study suggest that either different components in the sludge or different ratios of those components (e.g., hydrophobic cells, surfactants) are driving each given foaming event. With this noted variability in contributing foaming factors, it is critical to have standardized methods for measuring foaming intensity and duration. Without that standardization, it will remain difficult to establish meaningful relationships between foaming and its driving sources.

An annual, cyclical microbial community composition turnover was observed over the course of the 57-week sampling period (Figure 5). The microbial community compositions at the beginning and ending of the 57-week sampling campaign were very similar, whereas the communities from opposite seasons (e.g., summer vs. winter) were most dissimilar. Griffin and Wells (2017) also noticed seasonal, cyclical changes in the microbial community composition in activated sludge reactors in Illinois, USA, which

was driven by seasonal temperature differences in the activated sludge. Peces et al. (2022), in a multi-plant multi-year study, showed that these seasonal changes were repeatable across several years and consistent across treatment plants in the same region. It is clear climatic seasonality in temperate climates plays a strong role in structuring activated sludge microbial communities. Activated sludge basin temperature was not recorded in this study; but given the proximity to the plants from the Griffin and Wells (2017) study, it is most likely that basin temperature is playing a key role in driving community change. The relationship between whole community composition change and foaming was not clear in our study. We did not observe large-scale changes in the microbial community composition during any of our measured foaming events, as can be seen by the lack of overall community change between Week 28, the sample with the highest foaming potential, and the samples from the proceeding and following weeks (Figure 6). This has not been the case in other studies, as Wang and Yu (2016) found "extreme differences" in the foaming versus non-foaming activated sludge microbial communities. More work is needed to understand which foaming events cause large community change and why this may be occurring.
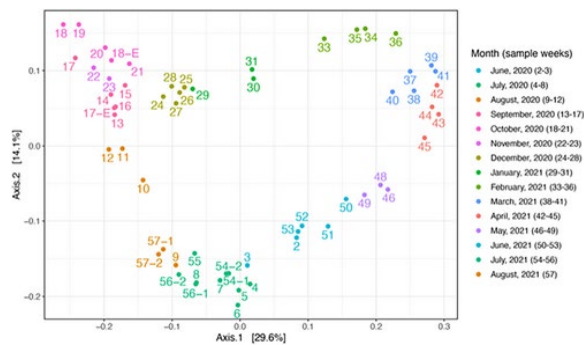

FIGURE 5 The principal coordinates analysis (PCoA) plot of the 57-week samples measured by Bray–Curtis dissimilarity and distinguished by month of the year sampled. Point labels refer to the sampling week. Point color indicates the month in which the sample was taken starting in June of 2020 and ending in August of 2021 (21). The "E" indication represents samples from the East Activated Sludge basins at South Shore Water Reclamation Facility (WRF). All other samples were from the West Activated Sludge basins at South Shore WRF. The circle indicates sample weeks with a similar microbial community composition as Week 28 (the most substantial foaming event). Additional samples over the course of 5 weeks (Week 54–Week 57) were taken in the front 1/3 and back 1/3 of the basin, which made for two samples every week except for Week 55 ($n = 7$). These additional samples are indicated with a "-1" and "-2 =," respectively. Sample weeks as numbered on the plot are indicated next to the sample month in the legend.
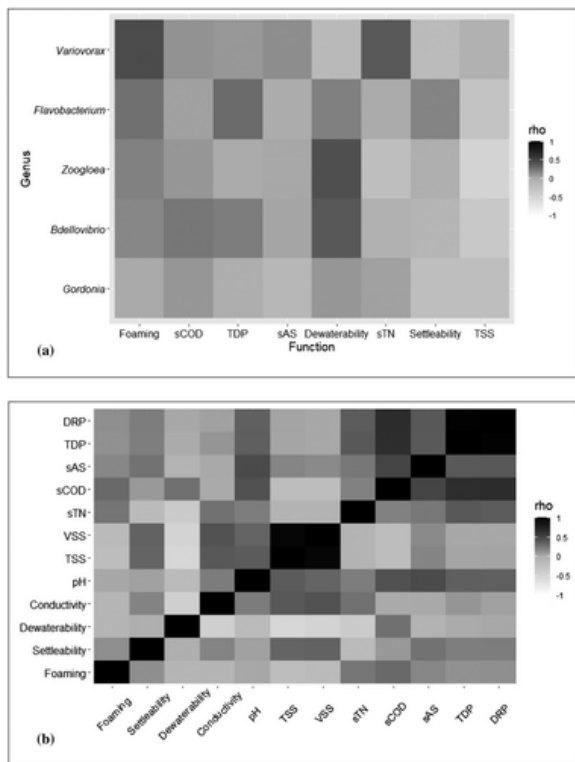
FIGURE 6 (a) Spearman rank correlation matrix for the functional parameters (function) of the activated sludge over the 57-week sampling period in relation to the foam-forming microbes at South Shore Water Reclamation Facility (WRF) (individual amplicon sequence variants [ASVs] that were identified as *Variovorax* [ASV105], *Flavobacterium* [ASV16], *Zoogloea* [ASV1], and *Bdellovibrio* [ASV176]) and the commonly reported foam former *Gordonia*. The functional relationships are as defined as follows: "Foaming" = active foaming potential of activated sludge (mL/mL), "sCOD" = soluble chemical oxygen demand concentration of activated sludge (mg/L), "TDP" = total dissolved phosphorus concentration of activated sludge (mg/L), "sAS" = soluble anionic surfactant concentration of activated sludge (mg/L), "Dewaterability" = capillary suction time (CST) of activated sludge (seconds), "sTN" = soluble total nitrogen concentration of activated sludge (mg/L), "Settleability" = sludge volume index (SVI) (mL/g TSS), and "TSS" = total suspended solids of activated sludge (g/L). (b) Spearman rank correlation matrix for the functional parameters of the activated sludge over the 57-week sampling period. The functional relationships are as defined as follows: "foaming" = active foaming potential of activated sludge (mL/mL), "Settleability" = SVI (mL/g TSS), "Dewaterability" = CST of activated sludge (seconds), "Conductivity" = conductivity of activated sludge (mS/cm), "pH" = pH of activated sludge, "TSS" = total suspended solids of activated sludge (g/L), "VSS" = volatile suspended solids (g/L), "sTN" = soluble total nitrogen concentration of activated sludge (mg/L), "sCOD" = soluble chemical oxygen demand concentration of activated sludge (mg/L), "sAS" = soluble anionic surfactant concentration of activated sludge (mg/L), "TDP" = total dissolved phosphorus concentration of activated sludge (mg/L), and "DRP" = dissolved reactive phosphorus concentration of activated sludge (mg/L). The darkness of the square corresponds to the magnitude (i.e., ρ) of the correlation.

Indicator microbes of foaming potential at South Shore WRF

Given the low number of foaming events that occurred during the sampling campaign and the strong seasonal effect on community composition, we focused on Weeks 25–29, a period of rapid foaming potential change, to identify microorganisms whose shift in relative abundance (relative abundance differential ranking, as outlined in Data processing and statistical analysis section) correlated with increased foaming potential in the basin. Due to limited foaming events, we did not expect to observe strong correlations. Still, we were interested to see which microbes had the strongest correlation to

foaming, even if the correlation was weak. Future research studies that capture more foaming events could follow up on these associations between microbes and foaming.

Although the overall microbial community composition did not change significantly between the foaming and non-foaming periods, there were some large changes in the relative abundances of individual ASVs between these periods. ASVs associated with genera that have previously been associated with foaming, classified to *Zoogloea* and *Flavobacterium*, were the most enriched during the significant foaming event when compared to the low foaming samples (Table 3). In previous studies, *Zoogloea* has been associated with activated sludge foaming in part due to the high amounts of extracellular polymeric substances (EPSs) produced by the microbe (Gerardi, 2002). *Zoogloea* is associated particularly with "billowy white foam," which is an accurate description of the substantial foaming event on Week 28 (Gerardi, 2002). *Zoogloea* is influenced by the following functional parameters: high or low food to microbe ratio, high mean cell residence time (MCRT), long hydraulic retention time (HRT), nutrient deficiency, and presence of readily degradable carbonaceous biochemical oxygen demand (Gerardi, 2002).

TABLE 3. Microbes with greatest enrichment during the Week 28 foaming event. See ASV table in Data S1 for full list of ASVs.

Genus	Relative abundance differential ranking
Zoogloea (ASV1 & ASV2)	1 & 2
Flavobacterium (ASV16 & ASV22)	3 & 6
Variovorax (ASV105)	24
Bdellovibrio (ASV176)	36

Flavobacterium, of the *Bacteroidetes* phylum, had a Spearman rank value of $\rho = 0.27$. *Flavobacterium* also has been found previously to be enriched in activated sludge during foaming events (Guo et al., 2015). Additionally, *Flavobacterium* has been shown to produce biosurfactants, which may be a contributing mechanism in activated sludge foaming (Bodour et al., 2003). The presence of *Flavobacterium* in the activated sludge foaming samples indicates that the microbe likely plays a role in the mechanism of activated sludge foaming.

Variovorax, of the *Proteobacteria* phylum, had the highest Spearman rank value of $\rho = 0.46$ across the 57-week sampling campaign (Spearman rank ρ values found in Data S2 and S3). While there is no previous association between *Variovorax* and activated sludge foaming reported in the literature to the authors' knowledge, *Variovorax paradoxus* is a known bio-emulsifying agent, which may be contributing to the foam formation (Franzetti et al., 2012). This is the first report that *Variovorax* relative abundance is correlated to activated sludge foaming events.

Bdellovibrio had a Spearman rank value of $\rho = 0.16$. There is no known association between *Bdellovibrio* and activated sludge foaming. However, *Bdellovibrio* is a predatory bacterium within activated sludge that preys on gram-negative microbes, particularly from *Proteobacteria* and *Bacteroidetes* (Feng et al., 2017). *Proteobacteria* and *Bacteroidetes* are the phylum of the other microbes in this study associated with foaming potential: *Zoogloea*, *Flavobacterium*, and *Variovorax*. The use of parasitic bacteria has been suggested

as a means of biocontrol of activated sludge foaming (Batinovic et al., 2021). Therefore, the association of *Bdellovibrio* with foaming may be a naturally occurring response in the activated sludge basin from the growth of the foam-inducing microbes. Further investigation is required to determine if *Bdellovibrio* is a biocontrol for foam-forming microbes. This is the first report that *Bdellovibrio* correlates to activated sludge foaming events.

Notably, *Gordonia* and *M. parvicella* were not foam inducers in this study, as indicated by the 57-week sampling period at South Shore WRF. These microbes have been widely reported as classic foam-inducing microbes in activated sludge (Jenkins et al., 2004). *Gordonia* was not highly abundant in any of the activated sludge samples across the 57-week sampling campaign and was not correlated to foaming potential (Spearman rank value of $\rho = 0.02$). *M. parvicella* was not identified in these activated sludge samples.

The microbes found to be associated with foaming potential at South Shore WRF, *Variovorax*, *Flavobacterium*, *Zoogloea*, and *Bdellovibrio*, did not always have the same positive or negative association with activated sludge functional parameters (Figure 6a). Dewaterability (measured as CST), soluble total nitrogen, and settleability (measured as SVI) had varying relationships among the indicator microbes, whereas soluble anionic surfactants, soluble COD, total dissolved phosphorus, and TSS had the same relationship with all indicator microbes, but the relationship direction varied (positive or negative). Due to that the reported functional parameters are not always correlated in the same direction (positively or negatively) with the foaming microbes, it suggests that the microbes do not grow or decay as a single mechanism with changes in the activated sludge basin.

The study from Dunkel et al. (2018) concluded that the noted foam-forming microbes (*Chryseobacterium*, *M. parvicella*, and *Gordonia* sp.) in the activated sludge had the same relationships to the measured functional parameters. There was no crossover between the foam-forming microbes identified in this study and the Dunkel et al. (2018) study; however, the microbes in this study did not experience the same overall preference in relation to the functional parameters of the activated sludge.

Activated sludge functional parameters and foaming potential
Foaming potential was correlated to soluble total nitrogen concentration ($\rho = 0.24$) (Figure 6b). A study of mesophilic anaerobic digesters, although citing other foam-forming microbes, also found a positive correlation between total nitrogen concentration and foaming events (Jiang et al., 2021). Previous findings showed that nitrogen-oxidizing microbes (ammonia-oxidizing bacteria [AOBs] and nitrite-oxidizing bacteria [NOBs]) were not enriched in the activated sludge foam (Guo et al., 2015; Wang & Yu, 2016). The microbial community composition of the foam was not analyzed in this study; however, assuming the same principles apply, the positive relationship between active foaming potential and soluble total nitrogen concentration may be occurring because of the possible occurrence of cationic surfactants or biosurfactants. Cationic surfactants are sometimes known to be quaternary ammonium-based compounds (QACs) (Games et al., 1982; Ying, 2004). The concentration of cationic surfactants was not measured during this study; however, if there was an increase in the QAC-based cationic surfactant concentrations due to the pandemic Hora et al. (2020), the surface tension of the activated sludge basin would decrease, and the soluble total nitrogen concentration would increase. Also, another possible explanation between the relationship between foaming potential and soluble total

nitrogen may be because the organic nitrogen-containing chemicals could be acting as surfactants themselves. Further investigation is required to understand the reason that soluble total nitrogen is positively correlated to foaming potential.

TSS/VSS concentration had a negative relationship with the active foaming potential (Figure 6b). This finding is contradictory to the observations in other published literature. Frigon et al. (2006) and Li et al. (2020) found that mixed liquor suspended solids (MLSS) concentration is positively correlated to observed foaming quantifications and foam-forming microbes (*Gordonia* and *M. parvicella*), respectively. Therefore, the relationship of TSS/VSS to foaming potential may be dependent on the foam-forming microbes in the activated sludge basin.

There was a positive relationship ($\rho = 0.16$) between active foaming potential and soluble anionic surfactants (Figure 6b). The low rho value indicates that soluble anionic surfactants are not the driving surfactant mechanism. Biosurfactants and cationic and nonionic surfactants should also be considered in future research. *Flavobacterium* and *Variovorax* are biosurfactant and bio-emulsifying producers, respectively, and also foam-forming indicators at South Shore WRF (Bodour et al., 2003; Franzetti et al., 2012). Therefore, the biosurfactant produced by these microbes may be providing the surfactant mechanism, as indicated by flotation theory (Petrovski et al., 2011).

Conclusions

A method was developed to quantify the foaming potential of an activated sludge sample in a manner that is relatively quick, inexpensive, easy-to-use, and reproducible. The foaming potential method was subsequently applied to correlate foaming events to microbial indicators and functional parameters. This study was limited by only having two stable foaming events throughout the studied period. Interpretation of correlations needs to consider this limitation. Certainly, correlation is not causation. This research presents an early step in better understanding the causes of foaming through short-term and long-term studies. To understand mechanisms of foaming, a reproducible method to quantify foaming is first needed. By developing a way to quantify foaming, there is a framework in which WRRFs could eventually predict and mitigate foaming events in activated sludge. The goal would be to provide these identified foaming indicators to treatment facilities as a measurable way to predict such events. Therefore, mitigation efforts could be implemented before treatment disruption. Additionally, this method could be used by WRRFs to demonstrate reduction of the occurrence of foaming problems. The following conclusions are based on the study of South Shore WRF samples:

1. The foaming potential measurements were correlated to increased LAS concentration and yielded reproducible results (RSD < 20%). Additionally, the foaming potential method was reproducible in the wastewater samples. This foaming potential method yields quantifiable measurements of foaming that can be implemented in future foaming studies to allow for easy comprehension and comparison of the extent of the foaming event.

2. The following genera were identified as taxa with relative abundance values that increased with the occurrence of foaming events in activated sludge at South Shore WRF: *Zoogloea*, *Flavobacterium*, *Variovorax*, and *Bdellovibrio*. The relative abundance of *Gordonia* was not identified as being related to foaming, even though it is commonly reported as a foam-inducing microbe. This is the first report

that *Variovorax* and *Bdellovibrio* might be related to foaming events in activated sludge. More full-scale studies on foaming events and microbes are required to determine if there is a correlation between these microbes and foaming.

3. The foaming potential positively correlated with soluble total nitrogen (Figure 6b).

The conclusions of this study have led to questions to consider for future research. Answering these questions will better inform prediction and mitigation efforts at full-scale facilities experiencing activated sludge foaming. The following considerations should be included in activated sludge foaming work moving forward from this study:

1. The correlation of *Variovorax* and *Bdellovibrio* relative abundance with foaming events in activated sludge should continue to be studied to corroborate results reported herein and to determine fundamental mechanisms of the relationship between taxon presence and foam formation. In order to understand these mechanisms, bacterial cultures of the identified microbes may be introduced into a controlled foaming assay to observe the effect on foaming potential.

2. The potential relationship between biologically produced organic nitrogen in activated sludge RAS and foaming events and their intensity should be studied.

3. The foaming potential method can be further validated using more complicated and controlled matrices including activated sludge from a variety of full-scale WRRFs.

4. The relative role of chemical drivers (i.e., surfactants) compared with biological drivers (i.e., microbes) of activated sludge should be measured and compared to understand how it affects foaming potential and foam type.

5. *Bdellovibrio* should be studied as a possible biocontrol for the biological drivers of the *Proteobacteria* and *Bacteroidetes* phylum in activated sludge foaming events.

6. The variability and inconsistency of results between studies highlights the ill-understood nature of this problem. Larger-scale studies that employ the same method across multiple facilities of varying characteristics would help alleviate this problem.

Author Contributions

Grace Scarim: Investigation; writing—original draft; methodology; validation; writing—review and editing; formal analysis; data curation. **Emily Lou LaMartina:** Visualization; writing—review and editing; formal analysis. **Kaushik Venkiteshwaran:** Investigation; writing—review and editing; methodology; formal analysis. **Daniel H. Zitomer:** Conceptualization; funding acquisition; writing—review and editing; resources. **Ryan J. Newton:** Funding acquisition; writing—review and editing; formal analysis. **Patrick J. McNamara:** Conceptualization; funding acquisition; writing—review and editing.

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Data Availability Statement

The data that supports the findings of this study are available in the supplementary material of this article.

Supporting Information

Filename	Description
wer10856-sup-0001-Data S1_ASV Table.csvCSV document, 1.7 MB	**Data S1.** Supporting information.
wer10856-sup-0002-Data S2_Rho Functional.csvCSV document, 3.7 KB	**Data S2.** Supporting information.
wer10856-sup-0003-Data S3_Rho Indicator.csvCSV document, 4.5 KB	**Data S3.** Supporting information.

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