A NEW FRAMEWORK FOR ANALYZING FILAMENTOUS BULKING IN ACTIVATED SLUDGE: ROLES FOR KINETICS AND DIFFUSION

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ABSTRACT

Filamentous bulking is caused by the excessive growth of filaments over floc formers, and their competition was previously described using kinetic selection. However, recent studies reported that instead of kinetic selection, diffusion limitation inside the flocs may be the crucial factor in microbial selection. To clarify the roles of these factors in explaining filamentous bulking, a new conceptual qualitative framework was developed in this study. We hypothesize that the growth rates of filaments and floc formers are affected by the combination of kinetic selection and substrate diffusion limitation. Three different regions (bulking, transitional, and non-bulking region) based on substrate concentration are suggested. In the bulking region, kinetic selection controls the growth rate process and favors filaments. In the non-bulking region, kinetic selection also is controlling, and favors the growth of floc formers. However, in the transitional region, substrate diffusion limitation, determined by the floc size, plays an important role in causing bulking. To test this framework, sequencing batch reactors (SBRs) were operated with influent COD of 100, 300, 600 and 1000 mg/L, and the sludge settleability was measured at various floc size distributions developed using different mixing strengths. The experimental data in the bulking and transitional regions supported the proposed framework.

KEYWORDS

Activated sludge, filamentous bulking, diffusion limitation, floc size

INTRODUCTION

Filamentous bulking is caused by the excessive growth of filamentous organisms outside the activated sludge flocs (Jenkins et al. 2003). The difference in growth kinetics between filaments and floc formers was widely used for explaining their competition (Chudoba et al. 1973c; Chudoba et al. 1973b; Chudoba et al. 1973a). Floc formers are thought to have high values of $\mu_{\text{max}}$ (maximum growth rate) and are favored at high substrate concentration, while filaments are thought to have low $K_s$ and thus have high values of growth rate at low substrate concentration (Figure 1). Based on the substrate concentration, the bulking and non-bulking regions can be determined. In the non-bulking region, the growth rate of filaments is higher than that of formers.
However, a recent study (Martins et al. 2003a) suggests that instead of kinetic selection, substrate diffusion limitation inside the flocs may be the important factor in microbial selection. It was hypothesized that filaments and floc formers have the same kinetics. At low substrate concentration in bulk solution, because the diffusion of substrate inside the floc is very low, floc formers cannot access substrate for growth, while filaments, due to their morphology, can grow faster than floc formers and easily reach substrate outside the flocs. Extended filaments then cause bulking. At high substrate concentration, filaments are postulated to have no such diffusion limitation advantage to be predominant, and most grow inside floc, and bulking does not occur. If this mechanism is correct, there is no need to invoke differences in growth kinetics, \( \mu_{\text{max}} \) and \( K_s \) of the two types of organisms for explaining bulking. The implication of this hypothesis is that under the same substrate concentration, bulking tends to occur at large floc sizes due to higher substrate diffusion resistance.

To clarify the roles and to what extent diffusion and kinetics influence filamentous bulking, we used quantitative fluorescence in situ hybridization (FISH) to measure the filament length inside and outside the activated sludge flocs in bulking and non-bulking sludge. It was found that both types of sludge have high levels of total filament length. The difference is that in non-bulking sludge, most filaments grew inside the flocs, which was consistent with the diffusion limitation hypothesis (Liao, et al., 2004). However, our later study (Lou and de los Reyes III, 2005b) using substrate uptake tests and metabolic modeling showed that non-bulking sludge has a higher maximum growth rate than bulking sludge. This result agreed with the kinetic selection theory. Considering our previous experiments, we hypothesized in the current study that the growth rates of filaments and floc formers are affected by the combination of kinetic selection and substrate diffusion limitation, and the dominant effect depends on the substrate concentrations and the floc sizes in the activated sludge system.

The concept of combining substrate diffusion limitation and microbial kinetics has been previously reported for predicting the performance of biological film reactors (Atkinson et al. 1968), and a series of mathematical models based on the Monod maximum utilization rate and the substrate diffusion coefficients was developed (Williams and McCarty 1976). Since the biomass in activated sludge is composed of many bacterial floc particles (microbial suspension...
aggregate, it is reasonable to speculate that substrate diffusion limitation as well as kinetic selection affect the organisms’ growth. Within the floc matrix, the metabolic reactions occur simultaneously with mass transfer and a concentration gradient would be established, which limits the metabolic rates of the cells inside the flocs. Such conditions may lead to endogenous respiration and cell lysis near the center of flocs if the substrate is exhausted or a change from aerobic respiration to anaerobic fermentation if oxygen is exhausted (Benefield and Molz 1983). Oxygen diffusion limitation inside the activated sludge floc has also been mentioned to induce filamentous bulking (Sezgin et al. 1978). However, research integrating substrate-diffusion-limitation degradation and microbial growth for explaining filamentous bulking is very limited. In addition, most research has been conceptual, and not accompanied by experimental data.

In the present study, a new conceptual qualitative framework integrating kinetics and diffusion inside the activated sludge flocs for explaining filamentous bulking was developed. Three different regions (bulking, transitional and non-bulking region) based on substrate concentration are suggested. In the bulking and non-bulking regions, kinetic selection controls the growth rate process and favors filaments and floc formers, respectively. However, in the transitional region, substrate diffusion limitation, determined by the floc size, plays an important role in causing bulking. To test this framework, sequencing batch reactors (SBRs) were operated at various substrate concentrations and sludge settleability was measured at different floc size distributions.

**MATERIALS AND METHODS**

**Sequencing batch reactor for floc size control**
The SBRs, each with active volumes of 8 L, were operated to produce bulking and non-bulking activated sludge. The reactors were seeded with sludge from the North Cary Wastewater Reclamation Facility (Cary, NC), and treated synthetic wastewater [50 mg/L MgSO₄·7H₂O, 7 mg/L KCl, 150 mg/L NH₄Cl, 141.8 mg/L, KH₂PO₄, 54.91 mg/L K₂HPO₄, 555.6 mg/L NaHCO₃, glucose and yeast extract depending on the COD concentrations]. The pH and temperature of the reactors were maintained at 7.0 ± 0.1 and 22 ± 1 °C respectively. Four SBRs with glucose concentrations expressed as COD of 100, 300, 600 and 1000 mg/L respectively, were operated with a 2-hour fill time and 1-hour settling time. The cycle period of operation was 8 hours. Hydraulic retention time (HRT) and sludge retention time (SRT) were 12 hours and 16 hours, respectively. Two-thirds of active volume (5.33 L) of effluent was replaced by the new feed at the end of the period. The mixing strength or the shear rate, expressed as $G = (\rho N_p N^3 D^5 / \mu V)^{1/2}$ ($\rho$: density of fluid; $N_p$: dimensionless quantities power number; $N$: impeller speed, $D$: impeller diameter, $\mu$: fluid viscosity; $V$: active volume of reactor), was controlled by the impeller speed, keeping other parameters constant. Two impellers speeds N, 50 rpm (revolutions per minute) and 126 rpm were used in the 100 mg/L and 300 mg/L influent COD reactors, and 50 rpm and 200 rpm were applied to 600 mg/L and 1000 mg/L influent COD reactors. A higher rpm (200 rpm), instead of 126 rpm, was used in the high influent COD reactor to keep a similar range of floc size distribution, since at the higher COD concentrations, larger flocs were formed. Based on the equation for $G$, after inputting the referenced parameters (Oldshue, 1983), we obtain $G_{200}=110.4 \text{ s}^{-1}$; $G_{126}=55.2 \text{ s}^{-1}$; and $G_{50}=13.8 \text{ s}^{-1}$, i.e., $G_{200}=2G_{126}=8G_{50}$ (subscripts represent the values of the rpm) for the reactor operation. Thus the substrate diffusion can be compared, as the larger the floc size, the more difficult the substrate can diffuse into the flocs. The dissolved
oxygen (DO) concentration was maintained at 6 to 7 mg/L to avoid oxygen limitation inside the activated sludge flocs (Lau et al. 1984).

MLSS (mixed liquor suspended solids concentration), MLVSS (mixed liquor volatile suspended solids) and SVI (30 minute sludge volume index) were periodically measured using Standard Methods (Greenberg et al. 1998). DO was measured using a YSI5300 DO probe meter (YSI, Yellow Springs, OH).

**Floc size measurement**

Depending on the biomass concentration in activated sludge, floc particles were stained with an appropriate amount of methylene blue solution [methylene blue: 0.5 g; KOH (1% solution): 1 mL; ethanol (95%): 30 mL; distilled water: 100 mL], and were immobilized in agarose gel for imaging. The images were captured with a Photometrics Sensys CCD camera mounted on a Nikon Optiphot II fluorescence microscope for size analysis using Metamorph™ (Universal Imaging Corp., Silver Spring, MD). More than 500 floc particles (10 shots) were imaged and analyzed to obtain mean particle size values for each test. The average radius of floc size (R) was determined using \( R = \left( \frac{3V}{4\pi} \right)^{1/3} \), where \( V \) is the average volume of particles was defined as the total volume of flocs divided by number of flocs.

The procedure of floc size analysis using Metamorph was performed in three stages. The measured distance was first calibrated, the floc sizes were then thresholded, and the size distribution was measured using the internal program of integrated morphometry. Floc size thresholding was performed manually and based on personal judgment. Three types of thresholding (normal, overestimated and underestimated) were used to test the effect of personal judgment on the sensitivity of floc size measurement. The average floc size radii for the overestimated case (137.4 \( \mu \)m) and the underestimated case (110.0 \( \mu \)m) were within about 10% of the value of normal thresholding (123.4 \( \mu \)m).

**RESULTS**

**a. Qualitative framework (hypothesis)**

The hypothesis integrating kinetic selection and diffusion limitation effects on filamentous bulking is summarized in Figure 1, which describes the growth rates of filaments and floc formers versus the substrate concentrations with or without substrate diffusion limitation. When the organisms are free-living, and no floc effects exist, the growth rates for filaments and floc formers follow Monod kinetics, i.e., only kinetic selection affects the competition. If the substrate concentration is lower than \( C_{SA} \) (bulking region), filaments win the competition and bulking occurs. However, when the activated sludge flocs composed of filaments and floc formers are formed, diffusion resistance is introduced to the system which results in differences in concentration in the bulk solution and in the floc interior. The larger and denser the flocs, the higher the diffusion resistance, and the measured apparent \( K_s \) value would be larger. The apparent \( K_s \) (in the dash growth rates of Figure 1) is the \( K_s \) combining the true value (in the solid growth rates of Figure 1) and the diffusion limitation effect. This causes the growth rate curves to shift to the right, while keeping the \( \mu_{max} \) the same. If the substrate concentration is higher than \( C_{SB} \) (non-bulking region), bulking does not occur. Thus, in both the bulking and non-bulking
region, diffusion limitation is insignificant and has no effect on sludge bulking. In the transitional region, bulking may or may not occur, depending on the degree of substrate diffusion limitation. Figure 1 illustrates that due to the effect of substrate diffusion limitation, the bulking region will be increased by the shaded area \( (C_{SA}-C_{SB}) \) that previously was in the non-bulking region in the absence of diffusion. The shaded region is reduced when the diffusion resistance is decreased, e.g., by decreasing floc sizes using higher shear. This framework may be used to explain the contradiction in our previous experimental results (Liao, et al., 2004; Lou and de los Reyes III, 2005b) that showed different percentages of filament levels inside and outside the flocs using the same substrate concentration and operational conditions. The hypothetical framework also predicts that decreasing floc size in the transition region would shift bulking sludge to non-bulking sludge. In addition, it should be noticed that in the framework, the values of the critical region-determined substrate concentrations, \( C_{SA} \) and \( C_{SB} \), are not fixed, and can be varied depending on many factors, e.g., the definition of bulking threshold (SVI=150 mL/g); operational conditions, such as types of reactors, substrate concentration and fill time; and floc properties, such as diffusion coefficient, and mass density. The significance of this framework is that it may explain the contradictory experimental data in the literature by showing how kinetic selection alone does not determine bulking occurrence, and how substrate diffusion may be a crucial factor under certain operational conditions.

b. SBR floc size-controlled experiments

Using different mixing strength in SBRs was successful for controlling the floc sizes in this experiment, and the measurements were repeatable. The large particles, around 230 μm average radius, were formed when the impeller speed 50 rpm was applied, while the small particles, 130-140 μm average radius were obtained when the impeller speed was 126 or 200 rpm. A lower average radius of 192 μm, measured using 50 rpm in the 100 mg/L COD reactor, was possibly because of the low influent substrate concentration that decreased the organisms’ growth. Much smaller particles determined in the 600 mg/L and 1000 mg/L influent COD reactors were due to the high mixing strength (200 rpm), for which the growth of flocs at higher substrate concentrations could not compensate. The volume distribution of the floc size is shown in Figure 3 -6. In the 100 mg/L influent COD reactor, a high volume percentage (74.5%) of flocs was in the range of 500-800 μm at the low impeller speed, while only 16.9% of flocs in this range at the high speed. Approximately 66 to 74% of flocs were found in the 300 influent COD mg/L reactor ranging 900-1300 μm at the low speed, and 64% of flocs was from 500 to 800 μm at the high speed. For 600 mg/L and 1000 mg/L influent COD reactors, a high volume percentage (68%-76%) of flocs was found greater than 1000 μm at 50 rpm. However using the high mixing strength at 200 rpm, the floc sizes distributed homogenously in the range of 25 to 450 μm in the 600 mg/L COD reactor, and about 70% of flocs were 375 to 700 μm in 1000 mg/L COD reactor. Thus the various impeller speeds produced different shear rate to control the floc sizes.
Figure 3. Floc size distribution in COD 100 mg/L SBRs with (a) 50 rpm and (b) 126 rpm

Figure 4. Floc size distribution in COD 300 mg/L SBRs with (a) 50 rpm; (b) 126 rpm and (c) 50 rpm (return from 126 rpm)

Figure 5. Floc size distribution in COD 600 mg/L SBRs with (a) 200 rpm, (b) 50 rpm and (c) 200 rpm (return from 50 rpm)
The experiment was performed in two stages (for the 100 mg/L influent COD reactor) or three stages (for the 300 mg/L influent COD, the 600 mg/L influent COD, and the 1000 mg/L influent COD reactors). In the 100 mg/L and 300 mg/L influent COD reactors, the mixing impeller speed of 50 rpm was applied in stage 1 to form large floc particles. After inoculating with activated sludge, both reactors started to bulk with SVI (Figure 7) increasing from 122 mL/g to 1278 mL/g in the 100 mg/L influent COD reactor, and from 101 mL/g to 353 mL/g in the 300 mg/L influent COD reactor. In stage 2, the impeller speed was changed from 50 rpm to 126 rpm. The reactors showed different behaviors. The SVI in the 100 mg/L COD reactor decreased from 1278 mL/g to 612 mL/g, and bulking still occurred. However, in the 300 mg/L COD reactor, the sludge became non-bulking with SVI decreasing from 353 mL/g to 97 mL/g, and corresponding small floc sizes (Figure 4) were measured. In stage 3, the impeller speed was changed back to 50 rpm, and the 300 mg/L COD reactor started to bulk again with SVI increasing from 94 mL/g to 205 mL/g at the end of the stage.

In the 600 mg/L and 1000 mg/L influent COD reactors, 200 rpm was used in stage 1 to reduce the floc sizes. A higher rpm was used in the first stage to maintain similar floc sizes as in the 300 mg/L influent COD reactor. Approximately one month after seeding activated sludge, non-bulking sludge was obtained for both reactors with SVI stabilizing at about 100 mL/g. In stage 2, the SVI for both reactors increased to more than 150 mL/g in about 4 days after the mixing strength was decreased from 200 rpm to 50 rpm. SVI was 200 mL/g at the end of the stage. In stage 3, the high mixing strength (200 rpm) was applied again in both reactors, and the SVI decreased to 120 mL/g in the 600 mg/L reactor and 150 mL/g in the 1000 mg/L reactor in one week.
**DISCUSSION**

An important point of the proposed framework is that the values of substrate concentrations in Figure 1 do not need to be fixed for each time period, e.g., the constant substrate concentrations in CMR at steady state, but that these can represent the initial substrate concentrations in reactors, allowing different shapes of the Monod growth rate curves of filaments and floc formers at different conditions. Thus the operational conditions should be stated clearly when describing the region-determined substrate concentrations. In addition, this framework is a general framework that can be applied in all the operational conditions for explaining filamentous bulking when kinetic selection and substrate diffusion limitation are the dominant mechanisms.

Our experimental data supported the hypothesis that filamentous bulking is caused by the combination of kinetic selection and substrate diffusion limitation. Kinetic selection is a dominant factor in controlling the bulking process at low substrate concentrations, where diffusion limitation is insignificant to the competition between filaments and floc formers. The filaments, because of their low $K_s$, have high growth rates at low substrate concentration, and thus win the competition. At the same substrate concentration, the decrease in SVI values with decreasing floc sizes indicated the substrate diffusion effect. However, it cannot compensate for the effect of kinetic selection and bulking occurred. As the influent COD was increased from 100 mg/L to the higher substrate concentration, kinetic selection favored the floc formers and counteracted the substrate diffusion limitation effect on the excessive growth of filaments. The SVI profiles (Figure 7) show that the bulking occurred when particle sizes were large and non-bulking occurred when particle sizes were small. These phenomena were repeated in stage 3 for the 300, 600 and 1000 mg/L COD influent reactors. The results indicate that both kinetic selection and the substrate diffusion may occur in municipal wastewater treatment plants since...
the influent substrate concentration is around 250-300 mg/L, and thus diffusion limitation may be a critical factor in filamentous bulking in full-scale WWTPs.

To test the validity of the conceptual qualitative framework, we initially used the 100 mg/L influent COD reactor to test the bulking region, the 300 mg/L COD reactor to test the transitional region, and the 600 mg/L & 1000 mg/L reactors to test the non-bulking region in our floc size-controlled experiment. The experimental data supported the hypothesis in the bulking and transitional region, using SVI greater than or equal to 150 mL/g as bulking threshold. However, the non-bulking region has not been verified by our results. An influent substrate concentration of 1000 mg/L using 2-hour feeding seems to be too low to test the non-bulking region, and apparently was still in the transitional region (Figure 7d). The SVI data indicated that bulking occurred at 240.9 μm average floc size radius and non-bulking occurred at 127.0 μm.

Our study used different shear rates, expressed as G values, to control the floc size and consequently to test the effect of diffusion on bulking. The data seem to agree with our hypothesis that the occurrence of bulking is related to a combination of kinetics and substrate diffusion limitation. However, it is also possible that instead of diffusion limitation as the main factor that causes bulking, another mechanism may be present. One mechanism is physical shear that mechanically suppresses filamentous growth outside the floc directly, e.g., by cutting the filament length. Zahradka (1966) has previously reported that filaments were suppressed in the recycled activated sludge with a high energy pump. This phenomenon was also observed by other researchers (Galil, et al., 1991; Shin, et al., 1992). However, small floc sizes were also obtained in their experiments, implying that the effect of diffusion limitation was low. Thus it is not clear which mechanism led to the suppression of filamentous growth in their studies. Our framework only considers the effect of substrate diffusion limitation on filamentous growth, and does not include the mechanical shear effect directly on the filaments.

Activated sludge is a complicated system and many factors may also be involved in the competition between filaments and floc formers. Bacterial storage may be one of the important factors (Lou and de los Reyes III 2005a; Lou and de los Reyes III, 2005b), since the dynamic nature of substrate flows into wastewater treatment plants elicit transient responses from microorganism that affect the growth rates of filaments and floc formers. Floc formers have higher storage capacities and thus outcompete the filaments in famine condition. We do not include the storage phenomenon in our framework. In the SBR floc size-controlled experiments, we did not need to consider the storage effect on bulking, since our previous experimental data (Lou and de los Reyes III, 2005b) have shown that at a 2 hr fill time and influent COD lower than 1000 mg/L, the concentration of the storage product (glycogen when glucose was used as carbon source) is very low. However, it may be an important factor if a higher influent substrate concentration is used.

CONCLUSIONS

Our experimental results are consistent with the predictions of the suggested conceptual framework. At low substrate concentration (the bulking region), bulking occurred and was independent of floc size (kinetic selection control). At a higher substrate concentration
corresponding to the transitional region, the occurrence of the filamentous bulking is determined by the effect of substrate diffusion limitation. Bulking occurs at large floc sizes and non-bulking occurs at smaller floc sizes. The framework explains the paradox of our experimental observations. The results demonstrate that using either kinetics or diffusion exclusively to explain microbial competition is incorrect. We realize that the substrate concentration boundaries between bulking, transition, and non-bulking regions may be dependent on substrate type, bioreactor configuration, bulking threshold SVI and other factors. Nevertheless, we are confident that the underlying concepts in the proposed framework are intrinsically correct. Further studies demonstrating the non-bulking region at higher substrate concentrations should consider bacterial storage. The new framework provides a more integrated understanding of the competition between filaments and floc formers under different operational conditions. Based on this understanding, conditions promoting filamentous bulking in activated sludge may be avoided by controlling floc sizes using different mixing strengths.

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