Microbial community change of sulfate reduction and sulfur oxidation bacteria in the activated sludge cultivated with acetate and peptone

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Abstract The growth of sulfate reducing bacteria (SRB) and filamentous sulfur bacteria was monitored on a laboratory scale in activated sludge reactors using acetate and peptone as the artificial wastewater. When the artificial wastewater contained acetate and peptone, filamentous bacteria increased in the sludge and the SVI values increased. There was a good correlation between sulfate reducing activity and sulfur oxidation activity in the produced sludge. The microbial community change of filamentous sulfur bacteria and sulfate reducing bacteria was analyzed using the fluorescent *in situ* hybridization (FISH) method. The tendency for the growth of filamentous sulfur bacteria *Thiothrix eikelboomii* following the growth of SRB was observed. The percentage of SRB385- hybridized cells and DNMA657-hybridized cells found in the total cell area increased from 2–3% to 7–10% when the filamentous bulking occurred.

Keywords Activated sludge; filamentous sulfur bacteria; sulfate reducing bacteria; *Thiothrix eikelboomii*

Water Science & Technology **Vol 54 No 8 pp 111–119** © IWA Publishing 2006

Introduction

Sulfur oxidation and reduction bacteria are widely detected in the environment (Odom and Singleton, 1993). These bacteria play an important role in carbon, sulfur and nitrogen cycles. About 10 to 1000 mg/l of sulfate are contained in sewage. Sulfate reduction causes concrete corrosion and unpleasant odors in the sewerage system. It is known that sulfate reduction affects methane production in the anaerobic digestion process (Isa *et al.*, 1986). It has been reported that sulfate reduction was a main trigger of filamentous bulking due to sulfur bacteria type 021N (Yamamoto-Ikemoto *et al.*, 1988, 1994). Sulfate reduction in the anaerobic zone also affects phosphate removal (Yamamoto-Ikemoto *et al.*, 1991, 1996, 1998).

Microbial community and the role of sulfate reducing bacteria (SRB) in the wastewater biofilm (Okabe *et al.*, 1999, 2003; Ito *et al.*, 2002) and UASB granules (Araki *et al.*, 1999) were reported. It was reported that the numbers of SRB ranged from 0.5 to 8% of the total cells in the activated sludge (Manz *et al.*, 1998). However, the microbial community of SRB in the activated sludge is mostly unknown. The authors examined the municipal plant and found that the bulking sludge contained large amounts of filamentous sulfur bacteria *Thiothrix eikelboomii*, the abundance of probe DNMA657-hybridized cells (estimated to *Desulfonema* spp.) was high (Miyazato *et al.*, 2005). These results suggest that the monitoring and control of SRB especially *Desulfonema* spp. are important to control filamentous bulking. It is necessary to observe the real time changes of filamentous bacteria and the SRB community.

In this study, the abundance of filamentous sulfur bacteria and SRB was monitored in a laboratory scale reactor cultivated with peptone and acetate. Then the community changes of SRB and sulfur oxidizing bacteria were examined by *in situ* hybridization with 16S rRNA targeted origonucleotide probes.

doi: 10.2166/wst.2006.856

Materials and methods

Operational conditions

The laboratory scale activated sludge reactor shown in Figure 1 consisting of two aeration tanks (3 l) and a sedimentation tank (3.5 l) was used for the experiments at 20 °C. In Run 1, the unit was seeded with activated sludge from the municipal plant A, in which filamentous bacteria type 021N was rarely recognized, and was fed with the artificial wastewater 1 (AW1) consisting of peptone and acetate as shown in Table 1. In Runs 2 and 3, the seeding activated sludge was collected from the municipal plant S, in which a small amount of type 021N is usually observed and filamentous bulking sometimes occurs. Runs 2 and 3 were conducted simultaneously. While AW1 was used for influent wastewater in Run 2 as well as Run 1, AW2 without acetate was used in Run 3 for 48 days (Period 1). After 48 days, the influent wastewater was changed to AW1 (Period 2). In all runs, the hydraulic retention time (HRT) of the aeration tanks and the sedimentation tank were regulated to 6 and 3 hrs, respectively. Once a day, 300 ml of activated sludge mixture was withdrawn from the aeration tank to regulate the sludge age as 20 days. The DO concentrations in the aeration tanks were regulated at over 3 mg/l.

Analytical method

Influent, effluent, and mixed liquor in the aeration tanks were sampled occasionally. Sulfate, nitrite, and nitrate in the filtered samples were analyzed by an ion chromatograph (Shimadzu Corp., IC-10AD). Phosphate and organic acids were analyzed by a HPLC using electric conductive detection with post-column pH buffer (Shimadzu Corp., HIC-10AD). TOC and IC were analyzed by a TOC analyzer (Shimadzu Corp., TOC-5000).

Sulfur oxidation and reduction activities

MLSS and SVI at Phase I (diluted SVI when SV_{30} was below 30%) (Matsui and Yamamoto, 1984) were measured twice a week. Filament length (microscope-video monitor system) (Matsui and Yamamoto, 1984) and the number of SRB (MPN method with m-ISA medium) (Mara and Williams, 1970) were measured as the indicators of the presence of filamentous sulfur bacteria and SRB, respectively. The activities of sulfur oxidation and sulfate reduction were measured using batch experiments at 20 °C according to the following procedures. In the sulfur oxidation activity experiments, the centrifuged activated sludge and the substrate (127 mg/l of $Na_2S{\cdot}12H_2O$, 71 mg/l of $NaHCO_3$, 158 mg/l of $MgSO_4{\cdot}7H_2O$, 52 mg/l of $CaCl_2$ and 44 mg/l of KH_2PO_4) were mixed in a 300 ml conical flask and the final MLSS concentration was regulated to 2 g/l. The mixture in the flask was aerated and 20 ml of the mixture were sampled after being cultivated at

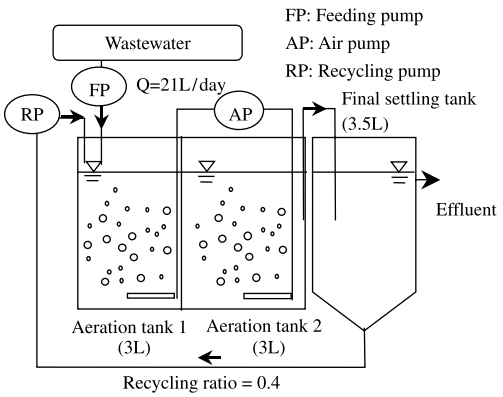

Figure 1 Experimental set up

Table 1 Composition of artificial wastewater

	Concentration (mol/l)	
	AW1	AW2
CH_3COOK	100	0
Polypeptone	200	200
Yeast extract	20	20
$NaHCO_3$	71	71
$MgSO_4{\cdot}7H_2O$	158	158
$CaCl_2$	39	39
KH_2PO_4	44	44

0, 1, 3, and 6 hr intervals. The concentrations of sulfate in the filtered samples were analyzed and the increasing rate of sulfate was calculated as the sulfur oxidation activity. In the sulfate reducing experiments, a plastic syringe was used to extract 50 ml of substrate and sludge. The centrifuged sludge and the substrate (100 mg/l of CH_3COOK, 200 mg/l of Polypeptone, 20 mg/l of yeast extract, 71 mg/l of $NaHCO_3$, 158 mg/l of $MgSO_4{\cdot}7H_2O$, 52 mg/l of $CaCl_2$ and 44 mg/l of KH_2PO_4), purged with nitrogen gas, were added to the syringe and mixed using a magnetic stirrer. The mixture was sampled at 24 hrs intervals, and the sulfate concentrations in the filtered samples were measured. The decreasing rate of sulfate was obtained as a result of the sulfate reducing activities.

Community analysis

Microbial community change was analyzed by the FISH method. The following 16S rRNA-targeted oligonucleotide probes were used; SRB385 (Amann *et al.*, 1992), five SRB group specific probes (SRB 687, 660, 221, 129 (Devereux *et al.*, 1992) and DNMA657 (Fukui *et al.*, 1999)) and four kinds of *Thiothrix* group specific probes (G1B, G2M, G3M and G123T) (Kanagawa *et al.*, 2000; Aruga *et al.*, 2002). The sequence, specification, hybridization conditions and references of each oligonucleotide probe are shown in Table 2. All probes were synthesized and labeled with indocarbocyanine (Cy-3), x-rhodamine isothiocyanate (XRITC) or fluorescein-5-isothiocyanate (FITC) by TAKARA Shuzo Co. Ltd. The activated sludge samples were taken from the aeration tank at several different time intervals during the experiment. The centrifuged sludge was fixed by paraformaldehyde. When the SRB group specific probes were used for hybridization, the fixed samples were dispersed with ultrasonic treatment for 5 min before the hybridization occurred. All *in situ* hybridizations were performed according to the procedure described by Amman (1995). The activated sludge samples were simultaneously stained with DAPI. The total percentages of SRB group specific probe-hybridized cells and DAPI-stained cells were analyzed and the ratio of probe-hybridized cells to DAPI-stained cells was calculated using the software WinRoof ver.5.02 by Mitani corp. The total percentages of *Thiothrix* group specific probe-hybridized filaments and DAPI-stained filaments were also analyzed and the individual filament lengths were estimated from the total filament length.

Results and discussion

Course of MLSS and settling characteristics

Figure 2 shows the courses of SVI at Phase I and filament length. In all runs, filamentous bacteria type 021N grew and the settling characteristics became poor. The SVI values and filament length of inoculated sludge of all runs were almost the same. However the amount of filamentous bacteria type 021N in the inoculated sludge of Run 2 was higher than that in Run 1. While SVI at Phase I increased after 40 days cultivation in Run 1,

Table 2 16S rRNA-targeted oligonucleotide probes used in this study

Probe name	Specificity	Sequence of probe (5′ → 3′)	Target site[a]	FA[b] concentration (%)	Reference
SRB385	SRB of the δ subdivision of the proteobacteria plus several Gram-positive bacteria (e.g., *Clostridium* spp.)	CGGCGTCGCTGCGTCAGG	385-402	30	Amann *et al.* (1992)
SRB687	*Desulfovibrio* spp. plus members of the genera *Geobacter*, *Desulfomonas*, *Desulfuromonas*, *Desulfomicrobium*, *Bilophila*, and *Pelobacter*	TACGGATTTCACTCCT	687-702	10	Devereux *et al.* (1992)
660	*Desulfobulbus* spp.	GAATTCCACTTTCCCCTCTG	660-679	30	Devereux *et al.* (1992)
221	*Desulfobacterium* spp.	TGCGCGGACTCATCTTCAAA	221-240	10	Devereux *et al.* (1992)
129	*Desulfobactor* spp.	CAGGCTTGAAGGCAGATT	129-146	20	Devereux *et al.* (1992)
DNMA657	*Desulfonema* spp., (*Desulfococcus* spp., *Desulfosarcina* spp.)	TTCCGCTTCCCTCTCCCATA	657-676	20	Fukui *et al.* (1999)
G1B	*Thiothrix disciformis*	TGTGTTCGATTCCTTGC	1029-1046	30	Kanagawa *et al.* (2000) Aruga *et al.* (2002)
G2M	*Thiothrix eikelboomii*	GCACCACCGACCCCTTAG	842-859	35	Kanagawa *et al.* (2000) Aruga *et al.* (2002)
G3M	*Thiothrix flexilis*	CTCAGGGATTCCTGCCAT	996-1013	30	Kanagawa *et al.* (2000) Aruga *et al.* (2002)
G123T	*Thiothrix* nivea group, *Thiothrix disciformis*, *Thiothrix eikelboomii* and *Thiothrix flexilis*	CCTTCCGATCTCTATGCA	697-714	40	Kanagawa *et al.* (2000) Aruga *et al.* (2002)

[a]16S rRNA position according to *E. coli* numbering
[b]Formamide concentration in the hybridization buffer

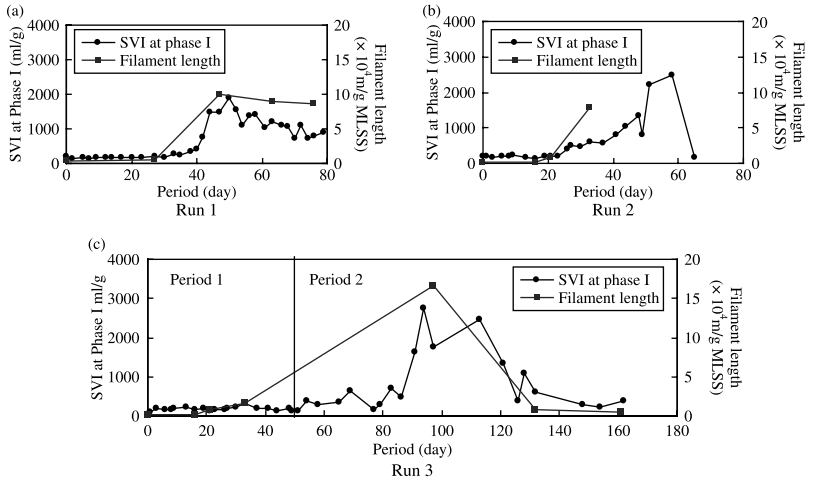

Figure 2 Courses of MLSS, SVI at Phase I and filament length of the activated sludge during the operation

bulking started after 20 days cultivation in Run 2. A similar result was reported in the lower organic loading conditions (Miyazato and Yamamoto-Ikemoto, 2003). These results suggested that the timelag before bulking occurrence depended on the initial amount of type 021N. In Period 1 of Run 3, in which the artificial wastewater without acetate was used for cultivation, filamentous bulking did not occur. After 30 days cultivation in Period 2 of Run 3, type 021N grew predominantly. The authors examined the municipal plant activated sludge, and reported that when the acetate concentration in influent to the aeration tank was high, filamentous bulking sometimes occurred (Miyazato *et al.*, 2005). The results suggested that the addition of acetate might be influential on the growth of filamentous bacteria. In all runs, when SVI at Phase I increased to over 1000 ml/g, MLSS decreased due to overflow from the sedimentation tank. In Run 3, after long term operation, the floc size increased enormously to 1,000 μm–10,000 μm due to growth of filamentous bacteria, and settling characteristics were improved. Figure 3 shows the relationship between filament length and SVI at Phase I in the produced sludge. It was reported that when the total extended length of filamentous bacteria increased to over 10^4 m/gMLSS, the settling characteristics became poor (Matsui and Yamamoto, 1984). In this experiment, the linear relation was obtained in filamentous length versus SVI at Phase I. It is clear that the growth of filamentous bacteria is a main cause of bulking in this experiment. Since filamentous bacteria grew logarithmically, filamentous bulking occurred sharply as shown in Figure 2. Therefore, the monitoring of filamentous bacteria is very important.

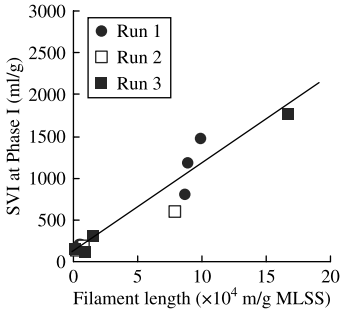

Figure 3 Relationship between filament length and SVI at Phase I in the activated sludge

Water characteristics of influent, effluent and aeration tanks

Figure 4 shows the concentrations of TOC, acetate, nitrate and sulfate in Run 3. Most of the TOC and acetate were removed in the first aeration tank. Nitrate was increased in the aeration tanks, indicating that nitrification had occurred. Nitrite was not detected and phosphate concentrations did not change in the aeration tanks. The efficiency of TOC removal and nitrification after 96 days of operation in which bulking occurred was almost the same as those after 6 days of operation. On the other hand, sulfate concentrations in the aeration tanks slightly decreased after bulking. In the 167 days of experimenting with the sludge, the enormous size of flocs which coexisted with the filamentous bacteria were observed in the aeration tanks. These results suggest that sulfate reduction occurred inside the enormous flocs.

Sulfate reduction and sulfur oxidation activities

Figure 5 shows the relationship between filament length and the sulfur oxidizing activity. There was a good correlation of filament length with the sulfur oxidizing activity, meaning that most of the filamentous bacteria which grew in these experiments had the ability to oxidize sulfur. Figure 6 shows the relationship between the sulfate reducing activity and the sulfur oxidizing activity after 20 day of cultivations. It was shown that when the activity of SRB in the activated sludge increased, the sulfur oxidizing activity also increased. It was confirmed that the growth of SRB was a main trigger of filamentous bulking caused by filamentous sulfur bacteria type 021N (Yamamoto-Ikemoto *et al.*, 1991, 1994, 1996). Similar results were obtained in the municipal sludge experiments (Miyazato *et al.*, 2005). However, the rate was lower in this experiment, than in the municipal sludge experiments. Since SRB were detected in the influent wastewater of the municipal plant, it was estimated that the sulfate reducing activity of the influent wastewater was included in the sludge activity.

Microbial Community analysis by in situ hybridization

Figure 7 shows the community change of filamentous bacteria and SRB in the activated sludge. In the initial stage with the activated sludge of Run 1, *Thiothrix* group specific probe-hybridized filamentous bacteria were not detected. The amount of probe SRB385-hybridized cells was low. A small amount of SRB group specific probe hybridized cells

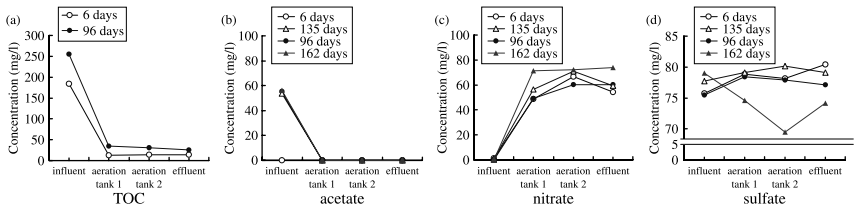

Figure 4 TOC, acetate, nitrate and sulfate concentrations in the activated sludge reactor of Run 3

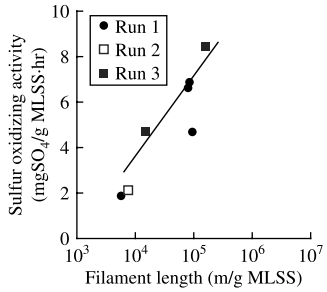

Figure 5 Relationship between filament length and sulfur oxidizing activity in the activated sludge

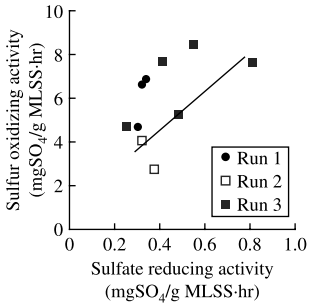

Figure 6 Relationship between sulfate reducing activity and sulfur oxidizing activity in the activated sludge

was detected. In the sludge cultivated after 27 days, an increase in *Thiothrix* spp. was not detected. However, in the sludge cultivated after 35 days, a small amount of G2M probe hybridized cells (estimated to *Thiothrix eikelboomii*) was detected. The SRB-group specific probe hybridized cells slightly increased. In the sludge cultivated after 47 days, a large amount of *Thiothrix eikelboomii* was detected. The probe SRB385-hybridized cells and the probe DNMA657-hybridized cells (estimated to *Desulfonema* spp.) increased in the sludge. In the sludge cultivated after 72 days, *Thiothrix eikelbomii* slightly decreased and the SRB group specific probe hybridized cells also decreased. These results suggested that the growth of *Thiothrix eikelboomii* was related to the growth of SRB. The results of Run 2, in which the operational conditions were almost the same as Run 1, are shown in Figure 7(b). Initial stage sludge contained a small amount of *Thiothrix eikelboomii*. The amount of SRB group specific probes-hybridized cells was greater than those in the inoculated sludge of Run 1. In the sludge cultivated after 30 days, an increase in *Thiothrix eikelboomii* was found. The probe SRB385-hybridized cells and probe DNMA657-hybridyzed cells also increased. However, in the sludge cultivated after 57 days, although a large amount of *Thiothrix eikelboomii* was detected, the number of SRB group cells decreased. Since the settling characteristics improved in the final stage of Run 2 (see Figure 2), it was estimated that the decrease in *Thiothrix eikelboomii* lagged behind decrease in SRB. The results of the community analysis of Run 3 are shown in Figure 7(c). *Thiothrix eikelboomii* and SRB did not increase during Period 1 fed with AW2 without acetate (see in the results of the 30 day operated sludge). In Period 2, both *Thiothrix eikelboomii* and SRB increased. These results indicate that the growth of

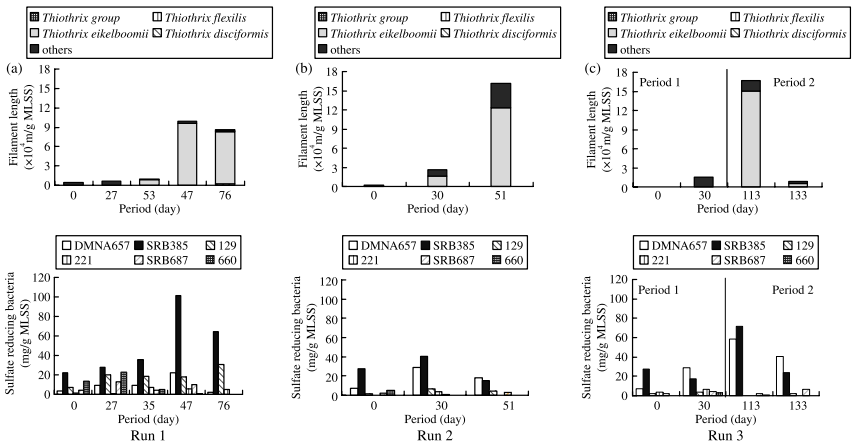

Figure 7 Community change of filamentous bacteria and sulfate reducing bacteria in the activated sludge estimated by fluorescence *in situ* hybridisation

Thiothrix eikelboomii was followed by the increase in SRB hybridized with probe SRB385 and DNMA657. After 132 days of operation, *Thiothrix eikelboomii* and SRB decreased. A large amount of enormous flocs containing filamentous bacteria were suspended and attached to the walls in the aeration tanks in this Period. Since the enormous flocs could not be dispersed by ultrasonication, the community analysis was carried out on the enormous flocs. It was estimated that SRB grew in the enormous flocs.

From the above results, it is considered that the growth of SRB, especially *Desulfonema* spp. is a trigger of the growth of *Thiothrix eikelboomii*. It was reported that *Thiothrix* sp. grew when the sulfide was contained in swage (Nielson and Keiding, 1998). Since the sulfide was also produced in the anaerobic selector, the anaerobic selector was not suitable for bulking control due to *Thiothrix* sp. (Tomei *et al.*, 1999). In this study, the artificial swage did not contain sulfide and the anaerobic selector was not introduced. DO concentration in the aerobic basin was over 3 mg/l, and the reactor was in the aerobic condition. However, SRB grew in the activated sludge flocs in this experiment. It was estimated that anaerobic conditions might be produced just around the sulfate reducing bacteria since *Thiothrix eikelboomii* oxdized sulfide and consumed oxgen.

In this experiment, acetate in the wastewater influenced the growth of both bacteria. Fatty acids and other organic acids are used for *Desulfonema* spp. as electron donors and carbon sources; oxidation of electron donors is complete and results in carbon dioxide. *Thiothrix eikelboomii* also grow on acetate. Acetate might affect the growth of both bacteria.

Conclusions

The community changes of filamentous sulfur bacteria and SRB were examined in the laboratory scale activated sludge reactors. The results are summarized as follows.

1. Filamentous bulking due to sulfur oxidizing bacteria occurred in the experimental conditions in this study. The length of filament sulfur bacteria in the inoculated sludge influenced the lag time of bulking occurrence.
2. There is a tendency that when the sulfate reduction activity increased, filamentous bacteria grew and sulfur oxidation activity increased.
3. When probe SRB385-hybridized cells (SRB) and probe DNMA657-hybridized cells (estimated to *Desulfonema* spp.) increased in the sludge, filamentous sulfur bacteria *Thiothrix eikelboomii* grew.

These results indicate that the monitoring of filament length and sulfate reduction is important to estimate the bulking occurrence.

Acknowledgements

The authors wish to express their thanks to Mr M. Ito and Ms N. Jouho, graduate students at Kanazawa University for their technical assistance.

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