Original Article

Effects of Sulfates on Enhanced Biological Phosphorus Removal in Three Waste Water Treatment Plants

Sui Kanazawa ^a, Ryoko Yamamoto-Ikemoto ^b, Norihisa Matsuura ^b

^a Graduate School of Natural Science & Technology, Kanazawa University, Kanazawa, Japan

^b Institute of Science and Engineering, Kanazawa University, Kanazawa, Japan

ABSTRACT

The effects of sulfates on enhanced biological phosphorus removal (EBPR) were investigated in three municipal wastewater treatment plants (WWTPs), which treat sewage containing several concentrations of sulfates. The behaviour of sulfates and phosphates in the anaerobic oxic process was analysed by considering the daily inflow rate and water quality. Although phosphorus release and uptake were recognized in all plants, phosphorus release from the WWTPs that were treating high-sulfate wastewater (high-sulfate WWTP), was lower than that of the WWTP treating low-sulfate wastewater (low-sulfate WWTP). In the high-sulfate WWTPs, sulfate decreased in the anaerobic zone and increased in the oxic zone. Analysis of the sulfate reduction and sulfur oxidation activities of the sludge showed that both activities were dependent on the influent sulfate concentration. The relative abundance of SRB which oxidize organic matters completely in high-sulfate WWTPs tends to be higher than that in low-sulfate WWTPs. On the other hand, a lower relative abundance of polyphosphate accumulating organisms (PAOs) was recognized in the high-sulfate WWTPs.

Keywords: enhanced biological phosphorus removal process, sulfate reduction, full-scale activated sludge plant, PAOs

INTRODUCTION

The anaerobic-oxic (AO) activated sludge process is widely used to remove phosphorus in sewage treatment plants. This process is performed by polyphosphate accumulating organisms (PAOs) in activated sludge. However, it was reported that biological phosphorus removal in this process is not stable because of several factors. The factors, such as pH, temperature, and volatile fatty acids (VFAs), enhance glycogen accumulating organisms (GAOs), which compete with PAOs for uptake of the substrate [1–4]. Other factors, such as nitrites, have a serious impact on PAOs because of toxicity [5].

Approximately 10–100 mg/L of sulfate is contained in sewage, and sulfate-reducing bacteria (SRB) are sometimes detected in activated sludge. Hydrogen sulfide produced by SRB is the cause of corrosion of sewer pipes and the odour from pump pits concentrated in downtown areas. SRB are divided into two major metabolic groups: those that incom-

pletely oxidize organic compounds to acetate and those that completely oxidize organic compounds to carbon dioxide [6]. Recently, Meyer *et al.* [7] investigated microbial communities of sulfur oxidizing bacteria (SOB) and SRB in six wastewater treatment plants (WWTPs) and reported that sulfur contributed to the structure of the main families related to the sulfur cycle. Because there are many hot springs and fishery harbours in Japan, the sulfate concentration in wastewater is thought to differ. Hence, it is considered that there are differences in the microbial community related to the sulfur cycle in WWTPs.

It was reported that sulfate reduction is the main trigger for the growth of filamentous sulfur bacteria in the AO process [8,9]. Moreover, Yamamoto-Ikemoto *et al.* conducted batch experiments using activated sludge from a municipal WWTP and reported that phosphorus release from the sludge, which had high sulfate reduction activity, tended to be lower than that of low-activity sludge [10]. Recently, a short-sludge age enhanced biological phosphorus removal (EBPR) process

Corresponding author: Ryoko Yamamoto-Ikemoto, E-mail: rikemoto@se.kanazawa-u.ac.jp

Received: June 29, 2018, Accepted: October 24, 2018, Published online: February 10, 2019

Copyright © 2019 Japan Society on Water Environment

Fig. 1 Sewer service area of the WWTPs.

Table 1 Outline of the WWTPs.

		D-WWTP	M-WWTP	S-WWTP
Collection area	km ²	6.0	23.7	25.8
Population		19,111	67,632	108,330
Daily inflow	m ³ /day	7,969	22,560	37,447
Start of the operation		December 1995	July 1995	March 1997

was investigated by Valverde-Perez *et al.* [11]. In this process, SRB outcompeted PAOs because of the combination of substrate competition and inhibition because of sulfides, and *Thiothrix* grew when sulfate was reduced. Rubio-Rincon *et al.* indicated the potential failure of EBPR receiving high-sulfate or sulfide-rich wastewater by conducting a series of batch activity tests [12]. There are some relationships between SRB, SOB, and PAOs in the EBPR process. However, the previous research was based on lab-scale experiments. To date, no information on the sulfur cycle for full-scale WWTPs is available.

To better understand the sulfur cycle in EBPR in operating WWTPs, we investigated three operating WWTPs receiving sewage containing different concentrations of sulfates. First, operational data for the three WWTPs, which treated several concentrations of sulfates were analysed. Next, the behaviour of sulfates and phosphates in each zone of the anaerobic oxic reactor were analysed by considering of time variation of the influent. Furthermore, microbial activities and the microbial community of the activated sludge were analysed.

MATERIALS AND METHODS

Wastewater treatment plants

Three municipal wastewater treatment plants (S, M, and D) in Ishikawa Prefecture, Japan were examined in this study. Figure 1 shows the service area and location of each plant.

Large hot spring areas were included in the service area of the smallest D-WWTP. The service area of M-WWTP was from the mountainside to seaside area and included a fishery harbor and some hot springs. The largest S-WWTP primarily covered the city area. Outlines of the WWTPs are shown in Table 1. All WWTPs were operated in AO conditions, except during the early summer in M-WWTP. We analyzed the annual operational reports of the WWTPs from 2007 to 2016 [13].

Sampling in WWTPs

Water and sludge samples were collected in each season at 10:00–11:00 AM from November 2015 to June 2017. The collected water samples were influent wastewater (IN), the effluent of a primary sedimentation tank (PS), and effluent of a final sedimentation tank (FS). The activated sludge samples were collected in the middle and the end of the anaerobic zone of a reactor (AN1 and AN2, respectively), and the middle and the end of the oxic zone of the reactor (OX1 and OX2, respectively). Temperature and pH of the collected samples were measured, and the samples were filtered (Omnipore™ 0.2 µm PTFE Membrane, Merk KGaA, Darmstadt, Germany) on site. Water quality was analyzed within a day. Samples from IN and FS were collected every 2 h beginning 24 h prior to regular sampling by using an auto sampler from October 2016 to June 2017.

The sulfate, nitrate, and nitrite concentrations in the super-

nant were measured by a high-performance ion chromatography (HIC-SP/HIC-NS, Shimadzu, Kyoto, Japan) equipped with a conductivity detector (CDD-10ADsp, Shimadzu) and Shim pack anion column (IC-SA2, Shimadzu, Kyoto, Japan). The phosphate concentration was measured by the molybdenum blue method. Mixed liquor suspended solids (MLSS) and sludge volume index (SVI) of the activated sludge collected from AN2 were measured.

Batch experiments were conducted to examine the sulfur oxidizing rate and sulfate reducing rate according to Miyazato *et al.* [9]. In the sulfur oxidizing batch test, the activated sludge was centrifuged, and we adjusted the MLSS concentration by adding substrate. The sludge was transferred to a conical flask and incubated at 20°C with aeration. We took the samples after 1, 3, and 6 h and analyzed the sulfate concentration by using a high-performance ion chromatography. We assumed that the sulfate increase until 6 h was a zero order reaction, and determined the slope as the sulfur oxidizing rate. In the sulfate reducing batch test, the sludge was centrifuged and mixed with the substrate. The adjusted sludge was transferred to 100 mL biochemical oxygen demand (BOD) bottles after nitrogen purge. We prepared four bottles each for every batch test, and one was immediately opened (zero hour). The other bottles were cultivated at 20°C and opened after 12, 24, and 48 h. The sulfate concentration of the samples was analyzed. We assumed that the rate of decrease in sulfates from 12 until 48 h was a zero order reaction, and determined the slope as the sulfate reducing rate.

Microbial community analysis

The microbial community in the activated sludge was examined using amplicon sequencing of the partial 16S rRNA gene with Illumina high throughput sequencing analysis. DNA was extracted using a Powersoil DNA Isolation Kit (MO Bio, Carlsbad, USA) according to manufacturer's protocol. The 16S rRNA gene amplicon was prepared according to Illumina's dual indexing strategy using a two-step PCR protocol. The first polymerase chain reaction (PCR) amplification was conducted with Uni515F and Uni806R primer sets [14]. The components of the first PCR reaction mixture (25 µL) were: DNA (1 ng/µL), 0.5 µM each of forward and reverse primer, 5 µL of Prime STAR GXL Buffer, 200 nM of dNTP mixture, and 0.625 units of PrimeSTAR GXL DNA Polymerase (TaKaRa Bio Inc., Kusatsu, Japan). Amplification was performed under the following conditions: 25 cycles at 95°C for 10 s, 55°C for 10 s, and 68°C 45 s. The first PCR amplicons were purified using Agencourt AM-Pure XP (Beckman Coulter, Pasadena, USA) following the

manufacturer's instructions. The second PCR amplification to attach Nextera (XT) indices and sequencing adapters (Illumina, San Diego, USA) were conducted according to the manufacturer's protocol, and then purified. The amplicons were mixed for sequencing on Illumina Miseq platform at Kanazawa University. The libraries were then sequenced on the instrument using V2 nano chemistry (Illumina), producing 2×250 bp paired reads.

Raw sequence data were analyzed according to Hewawasam *et al.* [15]. Clustering of operational taxonomic units (OTUs) was conducted at the 97% similarity level. The OTUs were then assigned to taxa using greengenes [16], with a taxonomy assignment tool PyNAST [17]. A heat map was generated using the heatmap3 R package [18].

RESULTS AND DISCUSSION

Analysis of the operational data

Influent concentration, effluent concentration, and removal efficiency of BOD, chemical oxygen demand (COD), suspended solids (SS), total nitrogen (TN), total phosphorus (TP), sulfates, MLSS, mixed liquid dissolved oxygen (MLDO), and sludge volume index (SVI) in each WWTP from 1997 to 2015 are summarized in **Table 2**. The influent concentration of BOD, COD, TN, and TP in S-WWTP was highest, followed by M-WWTP. The concentrations in D-WWTP was 50–60% of the concentrations in S-WWTP, indicating that sewage was diluted by hot spring water. However, the BOD/COD, BOD/N, and BOD/P ratios were almost the same in each plant, suggesting that the composition of organic substances were similar in all WWTPs. On the other hand, the sulfate concentration in D-WWTP was highest, and that in M-WWTP was also high. The effects of hot springs and sea water were relatively high. The SS, BOD, and COD removal efficiencies were consistently over 97%, 95%, and 85%, despite high variation in temperature. Nitrogen removal efficiencies were relatively low, although the efficiencies depended on temperature. Effluent concentration of phosphorus in S-WWTP and D-WWTP were always below 1.2 mg/L. In M-WWTP, in which the plant operation was sometimes switched from AO to conventional, the average effluent phosphorus concentration was higher than that of D-WWTP and S-WWTP.

Variation of water quality in each sampling point

Fig. 2 shows the results of the regular sampling. Most of DOC was removed in the middle of the aeration tank (**Fig. 2b**). Phosphates increased in the anaerobic zone and

Table 2 Operational data in the WWTPs from 2007 to 2016.

			D-WWTP	M-WWTP	S-WWTP
pH	Influent		7.2 ± 0.1 (7.0 ~ 7.2)	7.4 ± 0.1 (7.3 ~ 7.5)	7.8 ± 0.1 (7.6 ~ 7.9)
	Effluent		7.0 ± 0.1 (6.8 ~ 7.1)	7.0 ± 0.1 (6.9 ~ 7.1)	7.1 ± 0.1 (6.9 ~ 7.2)
Temperature	Influent	°C	20.6 ± 4.1 (11.5 ~ 28.0)	20.0 ± 3.6 (13.9 ~ 29.5)	19.9 ± 4.0 (12.1 ~ 27.0)
	Effluent	°C	21.0 ± 4.3 (13.0 ~ 28.8)	20.6 ± 3.8 (11.2 ~ 27.5)	21.0 ± 4.0 (13.7 ~ 28.0)
SS	Influent	mg/L	105.3 ± 7.7 (93.0 ~ 117.0)	126.0 ± 7.4 (115.9 ~ 138.1)	189.8 ± 5.0 (1.6 ~ 197.0)
	Effluent	mg/L	1.3 ± 0.4 (1.0 ~ 2.0)	2.8 ± 0.6 (2.0 ~ 4.0)	2.6 ± 0.9 (1.6 ~ 4.0)
	Removal efficiency	%	98.8 ± 0.4 (98.1 ~ 99.1)	97.8 ± 0.5 (96.8 ~ 98.4)	98.6 ± 0.5 (97.8 ~ 99.2)
BOD	Influent	mg/L	121.5 ± 19.1 (91.2 ~ 159.0)	145.8 ± 9.1 (130.3 ~ 157.0)	255.5 ± 33.2 (214.0 ~ 299.0)
	Effluent	mg/L	1.7 ± 0.4 (1.2 ~ 2.3)	3.1 ± 1.1 (2.1 ~ 5.9)	3.1 ± 1.6 (1.4 ~ 5.6)
	Removal efficiency	%	98.6 ± 0.4 (97.9 ~ 99.2)	97.8 ± 0.9 (95.5 ~ 98.7)	98.7 ± 0.8 (97.4 ~ 99.5)
COD _{Mn}	Influent	mg/L	71.1 ± 5.1 (61.6 ~ 80.2)	91.8 ± 16.5 (79.1 ~ 138.1)	157.2 ± 11.4 (142.0 ~ 174.7)
	Effluent	mg/L	8.6 ± 0.6 (7.3 ~ 9.5)	10.1 ± 1.1 (8.4 ~ 11.5)	12.5 ± 11.4 (10.3 ~ 14.1)
	Removal efficiency	%	87.8 ± 1.1 (86.0 ~ 89.5)	88.7 ± 1.9 (86.8 ~ 93.9)	92.0 ± 1.0 (90.2 ~ 93.3)
TN	Influent	mg/L	25.8 ± 2.7 (22.3 ~ 31.1)	35.4 ± 1.8 (32.1 ~ 37.9)	40.5 ± 0.6 (39.4 ~ 41.4)
	BOD/TN		4.8 ± 1.0 (3.5 ~ 6.5)	4.1 ± 0.3 (3.7 ~ 4.7)	6.3 ± 0.8 (5.3 ~ 7.4)
	Effluent	mg/L	19.3 ± 1.5 (16.3 ~ 21.1)	22.0 ± 3.0 (17.9 ~ 26.6)	19.6 ± 2.8 (15.2 ~ 23.2)
	Removal efficiency	%	25.0 ± 6.3 (16.9 ~ 36.7)	38.0 ± 5.8 (29.1 ~ 47.3)	51.6 ± 6.9 (42.3 ~ 61.8)
TP	Influent	mg/L	2.9 ± 0.1 (2.6 ~ 3.1)	4.1 ± 0.5 (3.2 ~ 5.0)	5.8 ± 0.2 (5.4 ~ 6.1)
	BOD/TP		42.0 ± 6.2 (34.8 ~ 53.5)	35.7 ± 3.9 (27.5 ~ 41.9)	44.2 ± 4.9 (36.8 ~ 50.1)
	Effluent	mg/L	0.7 ± 0.1 (0.5 ~ 0.9)	1.5 ± 0.3 (1.0 ~ 2.0)	0.7 ± 0.2 (0.5 ~ 1.2)
	Removal efficiency	%	76.6 ± 4.1 (69.4 ~ 82.3)	62.8 ± 7.8 (50.9 ~ 80.0)	87.2 ± 3.7 (78.9 ~ 91.7)
Sulfate	Influent	mg/L	79.9 ± 18.6 (60.8 ~ 125.5)	43.6 ± 5.8 (35.1 ~ 57.5)	26.8 ± 2.9 (23.5 ~ 32.4)
	BOD/Sulfate		1.6 ± 0.3 (0.9 ~ 2.0)	3.4 ± 0.4 (2.7 ~ 4.0)	9.7 ± 2.2 (6.6 ~ 12.5)
	Effluent	mg/L	66.9 ± 9.0 (59.4 ~ 84.9)	42.8 ± 3.5 (37.3 ~ 49.4)	31.9 ± 1.3 (29.9 ~ 34.6)
	Removal efficiency	%	14.6 ± 8.0 (0.7 ~ 33.4)	1.1 ± 7.3 (-11.0 ~ 14.1)	-19.8 ± 9.0 (-34.9 ~ -6.8)
MLSS		mg/L	1,413.9 ± 249.2 (249.2 ~ 1,960.0)	1,411.1 ± 225.7 (730.0 ~ 2,265.0)	1,640.8 ± 195.6 (1,190.0 ~ 2,260.0)
MLDO ^a		mg/L	0.8 ± 3.6 (0.3 ~ 3.6)	1.0 ± 0.7 (0.1 ~ 3.7)	1.5 ± 0.8 (0.1 ~ 4.4)
SVI		mL/g	228.0 ± 58.5 (136.4 ~ 420.0)	288.2 ± 122.9 (68.0 ~ 1,110.0)	169.0 ± 61.8 (80.0 ~ 400.0)

The values are the averages and standard deviations of each water-quality parameter.

The values within parentheses are the minimum and maximum values.

^a MLDO data were instantaneous values in OX2, and measured by using mounted DO meter.

Fig. 2 The course of average sulfate and phosphate concentrations in reaction tanks ($n = 6$). Bars indicate the minimum and maximum values. (a pH, b DOC, c DTN, d sulfate, e phosphate)

decreased in the oxic zone in all plants during the anaerobic-oxic operation as shown in **Fig. 2e**. In D-WWTP, which contained high concentrations of sulfates in the influent, sulfate concentration decreased significantly in the anaerobic zone from PS (**Fig. 2d**). On the other hand, sulfate concentration increased through the anaerobic and oxic zone in S-WWTP, which received low sulfate containing wastewater.

Daily variation of influent and effluent water quality

The daily variation of influent water quality is shown in **Fig. 3**. Similar time trends were recognised in flow rate, DOC, and DTN in all plants. Minimum flow rate appeared in the early morning and two peaks appeared during day and night time (**Fig. 3a**). Variation in flow rate in S-WWTP was greater than that of other plants. DOC and DTN concentrations of influent increased in the morning and that of effluent increased during mid-night, indicating a time lag between influent and effluent (**Fig. 3b** and **3c**). Although trends for influent phosphorus at S-WWTP was similar to that of DOC

and DTN (**Fig. 3e**), those in M-WWTP and D-WWTP were quite different, with DOC and DTN exhibiting very large variation. Influent sulfate in S-WWTP were almost constant, although the trends for sulfates in M-WWTP and D-WWTP were similar to that of TOC and DTN. Variation of the sulfate concentration in D-WWTP was large (**Fig. 3d**). These results indicated that influent wastewater variation needed to be considered to evaluate the behaviour of sulfates and phosphates in the plant.

Behaviour of sulfates and phosphates considering the influent variation

The changes in phosphate and sulfate per unit sludge were calculated from 2 h sampling data prior to regular sampling by considering the inflow rate. The results are shown in **Fig. 4**. Phosphorus release in the anaerobic zone and uptake in the aerobic zone were demonstrated well in all plants. In S-WWTP, significant phosphorus release was recognized in all seasons (**Fig. 4c**). Released phosphorus in S-WWTP was ap-

Fig. 3 Daily inflow variation ($n = 3$). Bars indicate the minimum and maximum values. **a** Flow rate, **b** DOC, **c** DTN, **d** Sulfate, **e** Phosphate.

proximately 1.5 times as much as that in the other two plants. On the other hand, sulfates were reduced in the anaerobic zone in M-WWTP (**Fig. 4b**). In D-WWTP, reduced sulfates in the middle of the anaerobic zone were higher than those at the end of the anaerobic zone because the sampling point for the end of the anaerobic zone was near the aerobic zone (**Fig. 4a**). However, reduced sulfates were higher than that in M-WWTP, and phosphorus release was lower. Yamamoto-Ikemoto *et al.* reported that phosphorus release of the sludge having high sulfate-reducing activity tended to be lower than that of low-activity sludge, and suggested SRB compete and/or inhibit PAOs [10]. The results of this study support this tendency, and that the sulfate reduction was accelerated, and phosphorus release was inhibited when the influent sulfate concentration was high.

Relationship between sulfate reduction rate, sulfur oxidation rate and released phosphate

Fig. 5a shows the results of the batch test under anaerobic conditions and the relationship between sulfate reduction and sulfur oxidation rates obtained by the batch tests. There was a tendency for the activated sludge with high-sulfate reduction activity to have high-sulfur oxidation activity. Both activities in high-sulfate WWTP (*i.e.*, D- and M-WWTPs) were highest, hence those in S-WWTP were lowest. These results indicated that sulfate reducing and sulfur oxidizing activities were dependent on the influent sulfate concentrations. The relationship between the sulfate reducing rate and maximum released phosphate is shown in **Fig 5b**. There was a tendency for the released phosphorus from the activated sludge, which had the high sulfate reducing rate to be smaller than that of low-rate sludge. It is inferred that there is some

Fig. 4 Behavior of sulfates and phosphates in the reactor. **a** D-WWTP, **b** M-WWTP, and **c** S-WWTP.

Fig. 5 Results of the batch test. a) the relationship between sulfate reducing and oxidizing rate; b) the relationship between sulfate reducing rate and maximum released phosphate. The symbols in orange, green, and blue show D-WWTP, M-WWTP, and S-WWTP, respectively. A symbol of M-WWTP on May 2016 is not shown in the figure because the plant was operating under the conventional method in this month.

relationship between SRB and PAOs, such as competition based on the organic matter, and accumulation of phosphorus was decreased because of sulfate reduction.

Microbial community in the activated sludge

Fig. 6 shows the heat map of the relative content of the

microbiome at the genus level across the samples. The heat map includes the top 48 genera of all bacterial 16S rRNA genes read. Cluster analysis showed differences between S-WWTP and the other two plants. In the cluster of S-WWTP, the microbial community of May 2016 and June 2017 were similar. *Leptospira*, *Xanthomonadaceae*, *Tolomonas*, and

Fig. 6 Heat map analysis based on the major bacterial genera. Cluster analysis was performed on the 48 most represented genera (with abundance > 0.1%).

“*Candidatus Symbiobacter*” were more abundant in the sample taken on September 2017, and four genera (*i.e.*, “*Candidatus Saccharimonas*”, *Lentimicrobiaseae*, *Niabella*, and *Filimonas*) were more abundant in December 2015. Conversely, the community from D-WWTP and M-WWTP had two clusters. Most of the communities clustered depending on the WWTP. The communities of D- and M-WWTP in September 2017 were clustered in the same group. *Dehalomonas*, *Ferruginibacter*, and *Cloacibacterium* were dominant in September 2017 in D-WWTP, and PHOS-HE51 was dominant in September 2017 in M-WWTP.

Table 3 shows the relative abundance of PAOs, GAOs, SRB, and SOB relative to total detected prokaryotes. Approximately 2.0 to 3.6% of the overall population were classified as PAOs. The dominant PAOs in the WWTPs were “*Candidatus Accumulibacter*”. The abundance of genus *Tetrasphaera*, which was also reported as an important PAO in full-scale WWTPs [19,20], was low. The relative abundance of PAOs in S-WWTP was highest, and that of D-WWTP was lowest. The relative abundance of GAOs (“*Candidatus Competibacter*” and *Defluviicoccus*) were 0.1 to 0.3%, respectively. Kim *et al.* analyzed the bacterial communities in a

Table 3 Relative abundance of genera of PAOs, GAOs, SRB, and SOB in activated sludge.

	S-WWTP						M-WWTP						D-WWTP					
	2015DEC	2016MAY	2016NOV	2017JUN	2017SEP	Average	2015DEC	2016OCT	2017JUN	2017SEP	Average	2015DEC	2016JUN	2016NOV	2017JUN	2017SEP	Average	
PAOs	3.5	3.5	3.3	4.4	3.1	3.6	1.8	3.3	3.4	3.0	2.9	2.4	2.4	2.3	1.8	1.2	2.0	
Candidatus Accumulibacter	3.4	3.4	3.2	4.4	3.1	1.8	1.8	3.4	3.0	0.0	2.0	2.3	2.4	2.3	1.8	1.2	2.0	
Tetrasphaera	0.1	0.1	0.1	0.1	0.0	0.1	0.1	0.1	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	
GAOs	0.6	0.2	0.4	0.2	0.2	0.3	0.1	0.1	0.0	0.0	0.1	0.2	0.1	0.1	0.1	0.2	0.1	
Candidatus Competibacter	0.6	0.2	0.4	0.2	0.2	0.0	0.1	0.1	0.0	0.0	0.1	0.2	0.1	0.1	0.1	0.2	0.1	
Defluviococcus	0.0	0.1	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	
Incompletely oxidizing SRB*	0.1	0.1	0.1	0.2	0.2	0.1	0.1	0.1	0.1	0.2	0.1	0.2	0.1	0.0	0.1	0.1	0.1	
Desulfomicrobium	0.0	0.1	0.0	0.1	0.1	0.1	0.0	0.0	0.0	0.1	0.0	0.2	0.1	0.0	0.1	0.0	0.1	
Desulfovibrio	0.1	0.0	0.0	0.0	0.1	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.1	0.1	0.0	
Desulfobulbus	0.0	0.0	0.1	0.1	0.0	0.0	0.0	0.1	0.1	0.1	0.1	0.2	0.1	0.1	0.1	0.0	0.1	
Completely oxidizing SRB*	0.0	0.0	0.1	0.1	0.1	0.0	0.1	0.1	0.2	0.2	0.1	0.5	0.6	0.3	0.3	0.3	0.4	
Desulfobacter	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.2	0.2	0.2	0.2	0.1	0.2	
Desulfonema	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.1	0.0	0.1	0.0	0.0	0.0	0.0	0.0	0.0	0.0	
Desulfomonile	0.0	0.0	0.0	0.1	0.0	0.0	0.0	0.0	0.1	0.1	0.1	0.0	0.2	0.1	0.1	0.1	0.1	
Desulfuromonas	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.3	0.2	0.0	0.0	0.1	0.1	
SOB	0.3	0.3	0.5	0.7	0.4	0.4	0.7	0.7	1.2	1.5	1.0	0.8	4.5	0.6	1.1	1.0	1.6	
Paracoccus	0.1	0.2	0.1	0.2	0.1	0.1	0.1	0.1	0.2	0.1	0.2	0.1	0.1	0.1	0.1	0.1	0.1	
Thiobacillus	0.1	0.0	0.0	0.0	0.0	0.0	0.2	0.4	0.1	0.5	0.2	0.5	3.1	0.4	0.2	0.0	0.8	
Pseudomonas	0.0	0.0	0.1	0.0	0.1	0.0	0.0	0.1	0.1	0.0	0.0	0.0	0.0	0.1	0.1	0.1	0.0	
Thiothrix	0.1	0.1	0.3	0.4	0.1	0.2	0.3	0.1	0.7	0.8	0.4	0.0	1.2	0.1	0.8	0.7	0.6	
Thioalkalipsira	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.1	0.0	0.1	0.0	0.0	0.0	0.1	0.0	

* Incompletely oxidized SRB means the group that oxidized organic substances incompletely to acetate, and completely SRB indicates another group that oxidized organic substrates completely to CO₂.

pilot scale anaerobic-anoxic-oxic process treating municipal sewage plant using pyrosequencing of partial 16S rRNA gene sequences and reported that “*Ca. Accumulibacter*” accounted for 3.1% of the total reads [21]. Law *et al.* revealed the microbial community for full-scale EBPR and showed that the abundance of the main *Accumulibacter*-annotated OTUs was 3.26%, and overall PAO relative abundance was less than 5% [22]. The relative abundance of *Competibacter* was always lower than that of *Accumulibacter*. Our results regarding relative abundances of PAOs and GAOs were similar with those of the above reports.

Conversely, incompletely oxidizing SRB were detected at approximately 0.1% and there were no differences among the plants. The relative abundances of completely oxidizing SRB in D-WWTP tended to be higher than that in the other plants, and *Desulfobacter* belonging to completely oxidizing SRB was only detected in D-WWTP. SOB was approximately 1.0% in M- and D-WWTPs and the abundance of SOB was lower in S-WWTP. *Thiothrix* belonging to the filamentous SOB was detected in all plants. In M- and D-WWTP, *Thiothrix* tended to be higher in June and September, and *Thiobacillus* was also observed in these WWTPs. Meyer *et al.* revealed the SRB and SOB communities of activated sludge in Brazil [7]. They observed *Syntrophobacteraceae* and *Nitrospiraceae* as the major SRB and *Rhodospirillaceae* and *Hyphomicrobiaceae* as the dominant SOB. The detected SRB and SOB in this study were different than that of their study. On the other hand, Brand *et al.* analysed the SRB population of activated sludge in nine WWTPs in the Netherlands by

T-RFLP and reported that *Desulfobacter* and *Desulfovibrio* were the most common and dominant SRB observed in the samples [23]. Although they suggested that sulfate variation was not a key parameter for the detected SRB population in the WWTPs, the SRB populations in this study depended on influent sulfates. Although both the sulfate reducing activity and sulfur oxidizing activity were observed in all WWTPs, the relative abundance of SRB was below 1.0%. It is suggested that the existence of other SRB was estimated.

The relative abundance of PAOs, SRB, and SOB was different in the WWTPs. Coats *et al.* characterized EBPR performance and associated mixed microbial consortium in lab- and full-scale EBPR systems [24]. They reported that the PAOs population in full-scale EBPR, which achieved excellent phosphorus removal, was quantitatively comparable to that of the lab-scale reactor achieving high phosphorus removal performance, although no correlation between the relative abundance of PAOs and the performance of phosphorus removal could be inferred. In our study, the WWTPs achieved phosphorus removal well, however, the released phosphates in S-WWTPs (low-sulfate WWTP) was higher than that in other plants. In this plant, a higher relative abundance of PAOs was detected. On the other hand, the relative abundance of SRB and SOB were related to reduced sulfates in the anaerobic zone and activity of the sulfur cycle.

These results suggested the following relationship of SRB, SOB, and PAOs in the activated sludge in full-scale plants receiving sewage containing high-sulfate concentrations. The activity of sulfate reduction and sulfur oxidation were high,

and a high relative abundance of SRB and SOB was observed, indicating that SRB and SOB might consist of the sulfur cycle in the activated sludge. Furthermore, the released phosphorus from the activated sludge, which had high sulfate-reducing activity, tended to be low. It is considered that the phosphorus accumulation of the sludge was decreased because of the inhibition of hydrogen sulfide and/or competition with SRB. Hydrogen sulfide produced by SRB is well known as a toxic compound to organisms. Rubio-Rincón *et al.* reported the findings: PAO could be inhibited when sulfide concentrations are more than 8 mg H₂S-S/L, and it leads to the potential failure of EBPR treating sulfate- or sulfide-rich wastewaters [12]. However, considering the concentrations of reduced sulfates, the concentration of hydrogen sulfide in D-WWTP was much lower than the reported level [10]. The influence of sulfide is small. The percentages of completely oxidizing SRB in D-WWTP was highest. They completely oxidized organic matter, such as propionate and acetate, which PAOs also utilize [25,26]. Valverde-Pérez *et al.* referred to the specific uptake rate by SRB, and mentioned that SRB could outcompete PAO for the uptake of volatile fatty acids [11]. Hence, it is inferred that phosphorus release by PAOs might be inhibited because of competition with the organic matter. It is possible to provide the substrates to PAOs by subjecting incompletely oxidizing SRB to dominate the activated sludge. Yamamoto-Ikemoto *et al.* carried out batch experiments using sludge from a municipal plant operated under AO conditions and reported that phosphate release and sulfate reduction occurred simultaneously, and PAOs utilized acetate produced by SRB under anaerobic conditions [27]. Although the plant they examined was operated under the AO method, the aeration used a small amount of the air in the anaerobic zone. Hence, the inhibition because of completely oxidizing SRB might be small because of decreasing DO concentrations, and the occurrence of filamentous bulking will be a concern. The measurement to control sulfate reduction for stable EBPR operation remains for a future study.

CONCLUSIONS

Sulfur cycle and phosphorus removal in three municipal WWTPs operating in anaerobic-oxic conditions were examined. The results are summarized as follows.

1. In the all investigated WWTPs, phosphorus uptake and release were recognized in the anaerobic-oxic conditions. In the high sulfate-WWTPs, sulfate reduction and sulfur oxidation occurred, and the phosphorus release was lower than those in the low sulfate WWTP. On the other hand,

significant phosphorus release and uptake were observed in the low-sulfate WWTP.

2. Both sulfate reduction and sulfur oxidation activities depended on the influent sulfate concentration.

3. The relative abundance of PAOs was higher in the low-sulfate WWTP, which observed high phosphate released in the anaerobic zone. On the other hand, the relative abundance of SRB and SOB were related reduced sulfate in the anaerobic zone and activity of the sulfur cycle. Completely oxidizing SRB was detected high sulfate WWTP, suggesting SRB might compete with PAOs in full scale plant.

ACKNOWLEDGEMENTS

We would like to thank the Ishikawa sewage systems agency for cooperation of the sampling and providing the annual reports. This work was supported by The Obayashi Foundation.

REFERENCES

- [1] Filipe CDM, Daigger GT, Grady CPL: A metabolic model for acetate uptake under anaerobic conditions by glycogen accumulating organisms: Stoichiometry, kinetics, and the effect of pH. *Biotechnol. Bioeng.*, **76**(1), 17–31, 2001. PMID:11400103 doi:10.1002/bit.1022
- [2] Panswad T, Doungchai A, Anotai J: Temperature effect on microbial community of enhanced biological phosphorus removal system. *Water Res.*, **37**(2), 409–415, 2003. PMID:12502069 doi:10.1016/S0043-1354(02)00286-5
- [3] Lopez-Vazquez CM, Oehmen A, Hooijmans CM, Brdjanovic D, Gijzen HJ, Yuan Z, van Loosdrecht MCM: Modeling the PAO–GAO competition: Effects of carbon source, pH and temperature. *Water Res.*, **43**(2), 450–462, 2009. PMID:19022471 doi:10.1016/j.watres.2008.10.032
- [4] Carvalheira M, Oehmen A, Carvalho G, Reis MAM: The effect of substrate competition on the metabolism of polyphosphate accumulating organisms (PAOs). *Water Res.*, **64**, 149–159, 2014. PMID:25051162 doi:10.1016/j.watres.2014.07.004
- [5] Jens M, Eva A, Steven I: Effect of nitrite on anoxic phosphate uptake in biological phosphorus removal activated sludge. *Water Res.*, **33**(8), 1781–1883, 1999.

- [6] Muyzer G, Stams AJM: The ecology and biotechnology of sulphate-reducing bacteria. *Nat. Rev. Microbiol.*, **6**(6), 441–454, 2008. PMID:18461075 doi:10.1038/nrmicro1892
- [7] Meyer DD, de Andrade PAM, Durrer A, Andreote FD, Corção G, Brandelli A: Bacterial communities involved in sulfur transformations in wastewater treatment plants. *Appl. Microbiol. Biotechnol.*, **100**(23), 10125–10135, 2016. PMID:27683212 doi:10.1007/s00253-016-7839-3
- [8] Yamamoto-Ikemoto R, Matsui S, Komori T: Ecological interactions among denitrification, poly-P accumulation, sulfate reduction, and filamentous sulfur bacteria in activated sludge. *Water Sci. Technol.*, **30**(11), 201–210, 1994. doi:10.2166/wst.1994.0560
- [9] Miyazato N, Yamamoto-Ikemoto R, Takamatsu S: Microbial community change of sulfate reduction and sulfur oxidation bacteria in the activated sludge cultivated with acetate and peptone. *Water Sci. Technol.*, **54**(8), 111–119, 2006. PMID:17163019 doi:10.2166/wst.2006.856
- [10] Yamamoto-Ikemoto R, Matsui S, Komori T: A study on filamentous bulking and sulfate reduction in anaerobic-oxic activated sludge system of the municipal plant. *Environ. Eng. Res.*, **31**, 295–303, 1994.
- [11] Valverde-Pérez B, Wágner DS, Lóránt B, Gülay A, Smets BF, Plósz BG: Short-sludge age EBPR process – Microbial and biochemical process characterisation during reactor start-up and operation. *Water Res.*, **104**(1), 320–329, 2016. PMID:27570133 doi:10.1016/j.watres.2016.08.026
- [12] Rubio-Rincón FJ, Lopez-Vazquez CM, Welles L, van Loosdrecht MCM, Brdjanovic D: Sulphide effects on the physiology of *Candidatus Accumulibacter phosphatis* type I. *Appl. Microbiol. Biotechnol.*, **101**(4), 1661–1672, 2017. PMID:27830293 doi:10.1007/s00253-016-7946-1
- [13] Ishikawa sewage public corporation: Izhi kanri nenpo (Operation and maintenance annual report) -2007 to 2016-. Ishikawa sewage public corporation, Kanazawa, Japan, 2007 to 2016. [in Japanese]
- [14] Caporaso JG, Lauber CL, Walters WA, Berg-Lyons D, Huntley J, Fierer N, Owens SM, Betley J, Fraser L, Bauer M, Gormley N, Gilbert JA, Smith G, Knight R: Ultra-high-throughput microbial community analysis on the Illumina HiSeq and MiSeq platforms. *ISME J.*, **6**(8), 1621–1624, 2012. PMID:22402401 doi:10.1038/ismej.2012.8
- [15] Hewawasam C, Matsuura N, Maharjan N, Hatamoto M, Yamaguchi T: Oxygen transfer dynamics and nitrification in a novel rotational sponge reactor. *Biochem. Eng. J.*, **128**(15), 162–167, 2017. doi:10.1016/j.bej.2017.09.021
- [16] DeSantis TZ, Hugenholtz P, Larsen N, Rojas E, Brodie EL, Keller K, Huber T, Dalevi D, Hu P, Andersen GL: Greengenes, a chimera-checked 16S rRNA gene database and workbench compatible with ARB. *Appl. Microbiol. Biotechnol.*, **72**(7), 5069–5072, 2006. PMID:16820507 doi:10.1128/AEM.03006-05
- [17] Caporaso JG, Bittinger K, Bushman FD, DeSantis TZ, Andersen GL, Knight R: PyNAST: a flexible tool for aligning sequences to a template alignment. *Bioinformatics*, **26**(2), 266–267, 2010. PMID:19914921 doi:10.1093/bioinformatics/btp636
- [18] Zhao S, Guo Y, Sheng Q, Shyr Y: Advanced heat map and clustering analysis using heatmap3. *BioMed Res. Int.*, **2014**, Article ID 986048, 6 pages, 2014.
- [19] Kong Y, Nielsen JL, Nielsen PH: Identity and eco-physiology of uncultured actinobacterial polyphosphate-accumulating organisms in full-scale enhanced biological phosphorus removal plants. *Appl. Environ. Microbiol.*, **71**(7), 4076–4085, 2005. PMID:16000823 doi:10.1128/AEM.71.7.4076-4085.2005
- [20] Nguyen HTT, Le VQ, Hansen AA, Nielsen JL, Nielsen PH: High diversity and abundance of putative polyphosphate-accumulating Tetrasphaera-related bacteria in activated sludge systems. *FEMS Microbiol. Ecol.*, **76**(2), 256–267, 2011. PMID:21231938 doi:10.1111/j.1574-6941.2011.01049.x
- [21] Kim BC, Kim S, Shin T, Kim H, Sang BI: Comparison of the bacterial communities in anaerobic, anoxic, and oxic chambers of a pilot A₂O process using pyrosequencing analysis. *Curr. Microbiol.*, **66**(6), 555–565, 2013. PMID:23358667 doi:10.1007/s00284-013-0311-z
- [22] Law Y, Kirkegaard RH, Cokro AA, Liu X, Arumugam K, Xie C, Stokholm-Bjerregaard M, Drautz-Moses DI, Nielsen PH, Wuertz S, Williams RBH: Integrative microbial community analysis reveals full-scale enhanced biological phosphorus removal under tropical conditions. *Sci. Rep.*, **6**(1), 25719, 2016. PMID:27193869 doi:10.1038/srep25719
- [23] van den Brand TPH, Roest K, Chen GH, Brdjanovic D, van Loosdrecht MCM: Occurrence and activity of sulphate reducing bacteria in aerobic activated sludge systems. *World J. Microbiol. Biotechnol.*, **31**(3), 507–516, 2015. PMID:25649202 doi:10.1007/s11274-015-1807-4

- [24] Coats ER, Brinkman CK, Lee S: Characterizing and contrasting the microbial ecology of laboratory and full-scale EBPR systems cultured on synthetic and real wastewaters. *Water Res.*, **108**(1), 124–136, 2017. PMID:27814897 doi:10.1016/j.watres.2016.10.069
- [25] Arun V, Mino T, Matsuo T: Biological mechanism of acetate uptake mediated by carbohydrate consumption in excess phosphorus removal systems. *Water Res.*, **22**(5), 565–570, 1988. doi:10.1016/0043-1354(88)90056-5
- [26] Pijuan M, Guisasola A, Baeza JA, Carrera J, Casas C, Lafuente J: Aerobic phosphorus release linked to acetate uptake: Influence of PAO intracellular storage compounds. *Biochem. Eng. J.*, **26**(2-3), 184–190, 2005. doi:10.1016/j.bej.2005.04.014
- [27] Yamamoto-Ikemoto R, Matsui S, Komori T, Bosque-Hamilton EJ: Symbiosis and competition among sulfate reduction, filamentous sulfur, denitrification, and poly-P accumulation bacteria in the anaerobic-oxic activated sludge of a municipal plant. *Water Sci. Technol.*, **34**(5-6), 119–128, 1996. doi:10.2166/wst.1996.0543