Short Communication

Activated sludge microbial communities of a chemical plant wastewater treatment facility with high-strength bromide ions and aromatic substances

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The prokaryotic and eukaryotic microbial communities of activated sludge in a chemical plant wastewater treatment facility, processing relatively oligotrophic wastewater containing aromatic compounds and high-strength bromide ions, were characterized by high-throughput sequencing of rRNA genes based on DNA and RNA extracts. The microbial community structure was distinct from those previously reported from domestic wastewater treatment plants. Several abundant OTUs in the RNA-based prokaryotic community were related to aromatic compound-degrading bacteria, which most likely contributed to the removal of recalcitrant chemicals from the wastewater. Furthermore, both prokaryotic and eukaryotic predators were highly abundant. These might promote stabilization of the microbial food chain and affect biomass in the activated sludge, maintaining the waste-removal function of the microbial community.

Key Words: activated sludge; chemical plant wastewater; eukaryotic microbial community; high-throughput sequencing; prokaryotic microbial community

Activated sludge composed of numerous kinds of microorganisms, including both prokaryotes and eukaryotes, e.g., fungi, protozoa, and metazoa, has been used for wastewater treatment for over a century (Pinto and Love, 2012; Vuono et al., 2015). The activities and composition of constituent microorganisms affect the performance of activated sludge processes, while the community struc-

ture is sensitive to environmental changes (Sato et al., 2016a, c). Especially, wastewater composition is a decisive factor shaping community structure; i.e., the microbial community develops uniquely depending on the wastewater composition (Inaba et al., 2018a; Navarro et al., 2016). Although the biological treatment of domestic wastewater has been intensely investigated, sludge microbial communities used in chemical industrial wastewater treatment remain largely unclarified. This is partly because wastewater from chemical plants often contains characteristic recalcitrant substances, e.g., halogenated and/or aromatic compounds, and information on such sludge features is often considered a matter of corporate confidentiality. However, knowledge of the constituent microorganisms of the activated sludge used to treat chemical industrial wastewater is valuable to environmental studies.

In this study, we have focused on the sludge microbial community of a wastewater treatment plant processing brominated functional materials (e.g., refractory materials) from a manufacturing factory using a combination of activated sludge and trickling-filter processes (Fig. S1). As most of the functional materials are composed of brominated aromatic substances, wastewater from the plant usually contains high-strength bromide ions (Br^-) and aromatic substances. To clarify the specialized microbial community of the chemical plant wastewater, both prokaryotic and eukaryotic microbial community structures in the activated sludge were analyzed using high-throughput sequencing based on 16S and 18S rRNA genes (Inaba et al., 2018b). Microbial analyses were performed using both genomic DNA and expressed rRNA as templates of amplicon sequences to evaluate the total and metabolically active populations of constituent microorganisms, respectively.

A sludge sample collected from the upper section of the

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Fig. 1. The prokaryotic microbial community structures revealed by 16S rRNA gene amplicon sequencing at class level.

RNA and DNA denote the community structures of the RNA- and DNA-based prokaryotic community, respectively. The extraction of total DNA and RNA was performed as reported previously (Aoyagi et al., 2015). From the extracted DNA and RNA, the 16S rRNA gene was amplified by primer sets of 515F/806R (Sato et al., 2016a). High-throughput Illumina sequencing of 16S rRNA amplicons and data processing by QIIME with the Green Gene database were performed as described previously (Caporaso et al., 2010; Sato et al., 2016a). The closest relatives of the identified OTUs were further determined based on the results of BLAST searches (<https://blast.ncbi.nlm.nih.gov/Blast.cgi>) using the National Center for Biotechnology Information database. All raw sequence data from this study were deposited in the DDBJ database under accession number DRA005907.

first (No. 1) trickling filter tower of a wastewater treatment plant (Fig. S1) was provided by Manac, Incorporated (Tokyo, Japan). Three replicates of the sample were centrifuged; the supernatants were used for chemical analyses and the pellets for DNA/RNA extractions. The chemical oxygen demand (COD) and total organic carbon (TOC) concentrations were analyzed as described previously (Sato et al., 2016b). The concentration of Br^- was determined using capillary electrophoresis (CE; Agilent, Santa Clara, CA, USA). The COD, TOC, and Br^- values are represented as the mean values of triplicate measurements. Aromatic substances were extracted from 1 ml of the supernatant sample with an equal amount of ethyl acetate by vortex for 2 min followed by centrifugation, and the extract was analyzed using a GC-mass spectrometer (GCMS-QP 2010; Shimadzu, Tokyo, Japan) equipped with a mass spectrometric detector and auto-injector (AOC-20i; Shimadzu). The temperature program for the GC oven was as follows; 50°C for 1.5 min, increase to 180°C at 8°C min⁻¹, then to 300°C at 12°C min⁻¹ with a final holding time of 10 min. Mass data in a range of 60.00 m z⁻¹ to 650.00 m z⁻¹ was analyzed.

The TOC and COD values of the supernatant were 633 and 357 mg/L, respectively (TOC/COD = 1.77). In our previous studies, the TOC and COD values of synthetic wastewater mimicking domestic wastewater were 1,130

and 450 mg/L, respectively (TOC/COD = 2.5) (Sato et al., 2016b), suggesting that the activated sludge sample contained relatively low amounts of organic substances that could easily be assimilated by heterotrophic microorganisms. The concentration of Br^- in the supernatant was 2,157 mg/L, which is approximately 30 times higher than that of seawater, possibly reflecting the condition of the raw wastewater, as the sludge sample was collected from the first (No. 1) trickling filter tower (Fig. S1). GC-MS analysis detected three dominant peaks from the ethyl acetate extract of the supernatant sample, whose mass spectra exhibited fragment ions at m/z 94 and 66, 107 and 77, and m/z 117 and 90, with retention times of 6.5, 8.3, and 12.2 min, respectively. The molecular mass and fragmentation patterns of these chemicals were identical to those of phenol, *p*-methyl phenol, and indole from a NIST database (data not shown). The peak-area ratio of the three chemicals was 1.0:2.0:1.1, respectively. The concentration of phenol was determined to be 5.9 mg/L using a standard curve. Results of the chemical analyses showed that the activated sludge was relatively oligotrophic and contained toxic aromatic compounds and high concentrations of Br^- .

The total number of 16S rRNA amplicon sequences obtained from six libraries (three RNA based; three DNA based) was approximately 0.23 million, corresponding to

Table 1. The 10 most abundant OTUs in the RNA-based prokaryotic library.

OTU	Related species*	Accession	Identity	Abundance**		Class
				RNA	DNA	
I	<i>Bdellovibrio bacteriovorus</i>	KU973531	100%	24.9%	1.9%	<i>Deltaproteobacteria</i>
II	<i>Methyloversatilis discipulorum</i>	NR_136517	100%	15.6%	3.9%	<i>Betaproteobacteria</i>
III	<i>Methylobacillus</i> sp. LF-1	EU780432	100%	7.4%	2.1%	<i>Betaproteobacteria</i>
IV	<i>Thauera</i> sp. HW-37	KP152655	100%	6.5%	0.30%	<i>Betaproteobacteria</i>
V	<i>Candidatus Gortzia infectiva</i>	HE797908	97%	4.6%	0.05%	<i>Alphaproteobacteria</i>
VI	<i>Paracoccus</i> sp. OTB16	KX022807	100%	4.6%	1.9%	<i>Alphaproteobacteria</i>
VII	<i>Myxobacterium</i> sp. AT1-01	AB246771	97%	4.0%	3.4%	<i>Deltaproteobacteria</i>
VIII	<i>Solitalea canadensis</i>	KF528160	87%	2.8%	19.8%	<i>Sphingobacteriia</i>
IX	Unclassified <i>Cryomorphaceae</i>	KR611617	88%	2.3%	7.5%	<i>Flavobacteriia</i>
X	Unclassified <i>Bacteroidetes</i>	HQ663407	96%	2.0%	2.7%	Unclassified

*The closest relatives of the identified OTUs were further determined based on the results of BLAST searches (<https://blast.ncbi.nlm.nih.gov/Blast.cgi>) using the National Center for Biotechnology Information database.

**RNA and DNA denote the relative abundance of the OTUs in RNA- and DNA-based prokaryotic libraries, respectively.

Table 2. The five most abundant OTUs in the RNA-based eukaryotic libraries.

OTU	Related species*	Accession	Identity	Abundance**		Phylum (or Class)
				RNA	DNA	
XI	<i>Opisthonecta henneguyi</i>	JN120201	99%	50.8%	54.5%	<i>Ciliophora</i>
XII	<i>Breviata anathema</i>	AF153206	99%	22.5%	0.37%	<i>Breviatea</i> (Class)
XIII	<i>Spumella</i> sp. TGKH6	LC000676	98%	4.4%	2.8%	<i>Chrysophyceae</i> (Class)
XIV	<i>Mononchoides</i> sp. FDL-2015	LN827618	99%	3.9%	12.0%	<i>Nematoda</i>
XV	<i>Anoetus</i> sp. AD664	JQ000062	96%	3.8%	14.2%	<i>Arthropoda</i>

*From the extracted DNA and RNA, the 18S rRNA gene was amplified by primer sets of TAREuk454FWD1/TAREukREV3 as described previously (Inaba et al., 2018b), and then sequenced using the Illumina MiSeq platform. Obtained 18S rRNA amplicon sequences were *de novo* assembled at the OUT level, and then the closest relatives were determined by homology search using the blastn program with the Silva rRNA database ver108 (Inaba et al., 2018b).

**RNA and DNA denote the relative abundances of the OTUs in the RNA- and DNA-based eukaryotic community, respectively.

an average of 38,122 sequences from each library. Class level phylogenetic analysis classified 91.5% (in RNA-based libraries) and 93.6% (in DNA-based libraries) of the obtained sequences and revealed apparent differences in composition between the DNA- and RNA-based libraries (Fig. 1). The DNA-based microbial community structure showed a predominance of *Sphingobacteriia* (relative abundance: 23.45%), uncultured *Bacteroidetes* group (VC2_1_Bac22) (20.05%), *Betaproteobacteria* (14.63%), *Flavobacteriia* (8.68%), and *Deltaproteobacteria* (6.41%), whereas the RNA-based structure showed a predominance of *Betaproteobacteria* (34.49%), *Deltaproteobacteria* (30.30%), *Alphaproteobacteria* (7.97%), and *Sphingobacteriia* (6.69%). As the organism abundances of DNA- and RNA-based microbial community structures were correlated with the total and metabolically active populations of constituent microorganisms, respectively, we hypothesized that dominant members of the RNA-based library played key roles in chemical plant wastewater treatment. Table 1 summarizes the relative abundances of the 10 most abundant bacterial operational taxonomic units (OTUs) I-X in the RNA-based library. The deltaproteobacterial OTU I related to *Bdellovibrio bacteriovorus* was found to be the most abundant, accounting for 24.9% of the total. *B. bacteriovorus* is a bacterial predator that preys on Gram-negative bacteria (Rendulic

et al., 2004). Another deltaproteobacterial OTU VII (*Myxobacterium* sp. AT1-01) belongs to the order *Myxococcales*. Several bacterial predators are known to be affiliated with this taxonomic group. For instance, *Myxococcus xanthus* can grow as a saprophyte or prey on a variety of both Gram-negative and -positive bacteria, as well as fungi (Muñoz-Dorado et al., 2016). The betaproteobacterial OTUs related to *Methyloversatilis discipulorum* (OTU II), *Methylobacillus* sp. LF-1 (OTU III), and *Thauera* sp. HW-37 (OTU IV) were the next most abundant, with relative abundances of 15.6%, 7.4%, and 6.5%, respectively. The genera *Methyloversatilis* and *Methylobacillus* can degrade aromatic compounds and utilize C1 compounds (e.g., methanol and methylamine) as their sole carbon source (Kumar and Maitra, 2016; Rochman et al., 2017). Several *Thauera* species are commonly found in activated sludge and are also able to degrade aromatic compounds (Mao et al., 2010). The next most abundant OTUs V and VI were affiliated with the class *Alphaproteobacteria*. The genus *Paracoccus*, which includes the OTU VI, is also commonly found in activated sludge and is reported to utilize polycyclic aromatic hydrocarbons (Teng et al., 2010). The other three OTUs, i.e., VIII, IX and X, were affiliated with the classes *Flavobacteriia* or *Sphingobacteriia*, commonly found in the activated sludge of various wastewater treatment

Fig. 2. Epifluorescence microscopic images of the activated sludge sample.

For microscopic analyses, the activated sludge sample was treated for 15 minutes with SYTO9 staining reagent (Molecular Probes, OR, US) at a final concentration of 5 μ M; SYTO9 stains both live and dead bacterial cells. Fluorescent and differentiated interference contrast (DIC) microscopy was performed using an Axio Observer. Z1 (Carl Zeiss, Jena, Germany) equipped with a 10 $\times$ objective (Plan-Apochromat 10 $\times$ /0.45 numerical aperture, Carl Zeiss) and CCD camera (AxioCam MR3, Carl Zeiss). Obtained images were annotated using AxioVision software (Carl Zeiss). A, B, C, E, DIC images of the activated sludge, where *Anoetus*-like microorganisms were frequently observed; D, F, superimposed DIC images (C and E) with fluorescent images. Bacteria (green) were abundantly present in and around the bodies of *Anoetus*-like microorganisms.

plants.

The eukaryotic microbial community structure of the activated sludge was also evaluated using both DNA- and RNA-based libraries of 18S rRNA genes. The total number of sequences obtained from six libraries (three RNA based; three DNA based) was approximately 0.21 million, corresponding to an average of 34,523 sequences from each library. Table 2 summarizes the relative abundances of the five most abundant eukaryotic OTUs, XI–XV, in the RNA-based library. The most abundant OTU, XI, which accounted for >50% of the total in both RNA- and DNA-based libraries, was related to a protozoan, *Opisthionecta henneguyi*, belonging to the phylum *Ciliophora*. Like other protozoa, *Opisthionecta* feeds on bacteria (Martín-Cereceda

et al., 1999). The second most abundant OTU, XII, was closely related with the ameboid flagellate, *Breviata anathema*, which has been found to dominate eukaryotic communities by preying on *Deltaproteobacteria* cells at a bioremediation site (Holmes et al., 2013). OTU XIII was related to a protozoan, *Spumella* sp. TGKH6, which has been detected in various environments, including the bioremediation site described above, and also feeds on bacteria as a growth substrate (Böhme et al., 2009). OTUs XIV and XV were related to a nematode *Mononchoides* sp. FDL-2015 and a mite *Anoetus* sp. AD664, respectively. A mite-like organism was frequently found in the activated sludge under microscopic observation (Fig. 2), and this was possibly the *Anoetus* species identified by 18S rRNA

sequencing. Notably, bacterial cells were found in abundance in and around the bodies of *Anoetus*-like organisms (Figs. 2D and 2F), suggesting predator-prey or symbiotic interactions.

In conclusion, high-resolution phylogenetic analysis of both prokaryotic and eukaryotic microbial communities in activated sludge used for chemical plant wastewater treatment indicated that the community structure was obviously distinct from those previously reported from domestic wastewater treatments. The identified microbial communities potentially reflect the chemical features of the activated sludge, i.e., the presence of low concentrations of organic substances, high-strength Br^- , and aromatic substances, which seems to be disadvantageous to the heterotrophic bacteria commonly found in domestic wastewater treatment plants. The high abundances of *Thauera*-, *Paracoccus*-, *Methyloversatilis*-, and *Methylobacillus*-related OTUs imply the importance of the ability to utilize aromatic compounds for survival in the chemical wastewater treatment plant, where these persistent chemicals were most likely degraded by these bacteria. Furthermore, high abundances of prokaryotic predators (*Bdellovibrio*- and *Myxobacterium*-related OTUs) as well as eukaryotic predators (the three protozoan OTUs, XI, XII and XIII, and the two metazoan OTUs, XIV and XV) suggested that predation activities could promote stabilization of the microbial food chain and affect the sludge biomass. Further investigations will be necessary for revealing a detailed relationship between the predatory process and the waste-removal function of the sludge microbial community.

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Supplementary Materials

Supplementary figure is available in our J-STAGE site (<http://www.jstage.jst.go.jp/browse/jgam>).

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