Original Article

Diversity of octylphenol polyethoxylate-degrading bacteria in river sediments and enrichment cultures, was revealed by 16S rRNA and *adh1*-gene molecular analysis

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To understand the extent of natural adaptation to chemical exposure within a microbial consortium, we scrutinized the dynamic relationship between the microbial diversity and biodegradation capacity of octylphenol polyethoxylates (OPEO_n), as representative alkylphenol polyethoxylates (APEO_n), using enrichment cultures of various sediments from the Iwata River system. To address the potential of microbes in river sediments to transform a surfactant into endocrine-disrupting chemicals, the ability of the microbes to degrade OPEO_n in six different sediment samples was assessed by enrichment culture. In addition, 16S rRNA gene-based denaturing gradient gel electrophoresis (DGGE) analysis was conducted to elucidate the microbial communities before and after enrichment of OPEO_n-degrading microbes. Functional gene (*adh1*) copies were also enumerated to estimate the potential of OPEO_n degradation in the six sediment samples. Moreover, microbial communities in three of the six sediment samples were characterized by 16S rRNA-based clone analysis. OPEO_n-degrading activity was determined to be present in five of the six enrichment cultures; however, no predominant species could be found by clone analysis. DGGE analysis revealed that the genus *Pseudomonas*, which contributes to the degradation of OPEO_n, was one of the major populations in the enrichment cultures. The *adh1* gene enumeration was shown to be relatively high when river flow was relatively slow. These results suggest that potential OPEO_n degraders are widely distributed in Iwata River with differences in their history of chemical exposure. © Pesticide Science Society of Japan

Keywords: alkylphenol polyethoxylates (APEO_n), octylphenol polyethoxylates (OPEO_n), endocrine disruptor, biodegradation, natural adaptation.

Introduction

Alkylphenol polyethoxylates (APEO_n) are one of the most widely used groups of surfactants. Their use includes detergents, adjuvants to pesticides, textiles, petroleum recovery chemicals, metalworking fluids, and personal care products. Although APEO_n with longer ethoxylate chains have no significant adverse effects, their breakdown products are frequently detected in the environment and are well-known estrogen agonists and androgen antagonists. These can interfere with nuclear receptor function, causing reproductive and de-

velopmental disorders in mammals, animals inhabiting aquatic environments, and birds.^{1–3)} The increasing chemical exposure to these surfactants, particularly in worldwide aquatic environments, has caused considerable public anxiety because of their toxicity. The Ministry of the Environment, Japan has publicly stated that octylphenols act as endocrine disruptors, especially in fish (<http://www.env.go.jp/chemi/end/kento1202/ref10.pdf>). Countries in North America and Europe, and organizations such as the Organization for Economic Cooperation and Development are currently evaluating the human health or ecological risks of these surfactants and their breakdown compounds⁴⁾; however, large amounts of APEO_n have been discharged into the environment as soil cleaners during the recent remediation of polyaromatic hydrocarbon contamination in soils and perchloroethylene contamination in water,^{5–11)} and they are used as additives in the for-

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mulation of pesticides.^{12,13)} Growing public concern has led to efforts to identify and characterize environmental pollution mechanisms in rivers and groundwater caused by the excessive use of APEO_n.^{14–16)} Because it is important to understand the mechanism underlying the biodegradation of APEO_n in farmland and the hydrosphere, we recently demonstrated the biodegradation of APEO_n in a pure culture of *Pseudomonas putida* strain S5 isolated from Japanese rice paddy fields.¹⁷⁾ According to the study of this strain, APEO_n were biodegraded under aerobic conditions by successive exocission of the ethylene oxide chain, accompanied by oxidation of the terminal hydroxyl group through carboxylic intermediates.^{17–20)} Moreover, the biodegradation of APEO_n is significantly dependent on environmental conditions.^{21,22)}

Consequently, the well-characterized microbial degradation of APEO_n highlights the importance of understanding and predicting microbial behavior for bioremediation or prevention of APEO_n pollution.^{17,20,21)} Although it is difficult to confirm changes in microbial diversity caused by human activity without supporting data, we notice these changes whenever we are faced with their adverse effects.

In this study, we used octylphenol polyethoxylates (OPEO_n) as a representative APEO_n and six freshwater sediment samples from a river that were enriched for aerobic OPEO_n-degrading activity. The bacterial communities in six of the sediments were then subjected to phylogenetic analysis by denaturing gradient gel electrophoresis (DGGE) and a clone library. The functional gene involved in OPEO_n degradation was also enumerated by quantitative PCR to determine the diversity of OPEO_n-degrading bacteria.

Materials and Methods

1. Sampling

All the river sediment samples used in this study were collected from Iwata River in Tsu City, Japan, in June 2006. The locations of the sampling sites were as follows: Site-1 (N34°42'22.0" E136°25'51.5"); Site-2 (N34°42'24.8" E136°26'09.2"); Site-3 (N34°42'59.5" E136°26'25.6"); Site-4 (N34°43'44.0" E136°26'14.3"); Site-5 (N34°43'02.2" E136°27'18.8"); and Site-6 (N34°43'43.6" E136°30'59.1"). The atmospheric temperature was around 30°C. The pH values at sampling sites were measured using a portable soil pH meter PRN-41 (Fujiwara Science Company). In addition of pH values, 0.5 g sediment samples were used for the determination of total organic carbon by a CN coder JM1000CN (J-SCIENCE LAB. Co. Ltd.). The sediment samples were immediately stored at 4°C until DNA extraction and bacterial enrichment.

2. Reagents

The surfactant α -[4-(1,1,3,3-tetramethylbutyl) phenyl]- ω -hydroxypoly-1, 2-ethanediyl, abbreviated to TX-100 (Aldrich Chemicals, WI, USA), was used as the substrate for bacterial OPEO_n biodegradation. Because this compound is character-

ized with an average of 9.5 ethoxy segments per molecule, we calculated its molecular weight to be 624.32. Surfactants with shorter ethylene oxide chains and the carboxylic acid derivatives used as standards were synthesized and purified by the method described by Shibata *et al.*²¹⁾

3. Enrichment culture of OPEO_n-degrading bacteria

To investigate the potential of OPEO_n degradation in sediments, we tried to enrich OPEO_n-degrading bacteria; 1 g sediment was inoculated in triplicate into basal salt liquid medium containing 1.6 mM TX-100,¹⁷⁾ and incubated at 27°C for 14 days with shaking (100 rpm). The basal salts contained in this medium were (grams/liter) Na₂HPO₄ (6.8), KH₂PO₄ (3.0), NaCl (0.5), (NH₄)₂SO₄ (2.0), MgSO₄ (0.24), CaCl₂ (0.01), together with 1.6 mM TX-100. After OPEO_n biodegradation was observed, 100 μ l culture was transferred to fresh media and incubated for 7 days. Several transfers were then made to facilitate enrichment. The bacterial growth of enrichment cultures was monitored by measurement of the optical density of the medium at 660 nm every 24 hr.

4. OPEO_n biodegradation assay by gas chromatography (GC) analysis

After enrichment of the OPEO_n-biodegrading bacteria, a 1 ml aliquot was collected from each culture and immediately extracted by the addition of an equal volume of ethyl acetate (containing *n*-eicosane as the internal standard: IS). GC analysis was conducted using methods described previously.²¹⁾ Enrichment cultures inoculated with each sediment sample were sterilized by serial autoclaving (twice, at 121°C for 30 min) and analyzed to investigate the abiotic degradation of OPEO_n as a control.

5. DNA extraction from the samples

DNA was extracted from sediment samples (0.25 g) using the PowerSoil™ DNA Isolation Kit (MO BIO Laboratories, Inc., CA, USA), according to the manufacturer's instructions. The purity and quantity of the DNA preparation was examined by measuring the UV absorption spectrum.²²⁾ DNA was extracted from 0.25 ml subsamples of enrichment cultures using the same method.

6. Clone analysis

The 16S rRNA genes from the purified DNA preparations were amplified by PCR with Premix Taq (Ex version) polymerase (TaKaRa Bio, Inc., Shiga, Japan), according to the manufacturer's instructions (100 ng template DNA, 25 pmol of each primer in a 50 μ l reaction volume). The PCR primers used in the amplification were universal primer 530F (5'-GT-GCCAGCMGCCGCGG-3', where M represents A or C; *Escherichia coli* positions 514–529^{23,24)}), and prokaryote-specific primer 1492R (5'-GGYTACCTTGTACGACTT-3', where Y represents C or T; *E. coli* positions 1492–1510²⁴⁾). The reaction conditions were as follows: initial denaturation

at 94°C for 2 min, followed by 20 cycles of 94°C for 30 sec, 55°C for 30 sec, 72°C for 1 min. The Dice Model TP600 thermal cycler (TaKaRa Bio, Inc.) was used. PCR amplicons corresponding to the expected size of 1.0 kb were purified with a QIAquick PCR Purification Kit (Qiagen, CA, USA). The rRNA gene fragments were cloned into plasmids using the pGEM-T Easy vector system (Promega, WI, USA). Cloned amplicons were prepared from randomly selected recombinants and used as templates for sequencing. Each clone was sequenced with the T7W and SP6W primers,²⁵⁾ the Dynamic ET Terminator Cycle Sequencing Kit (GE Healthcare, WI, USA), and an automated sequence analyzer (ABI PRISM 310 Genetic Analyzer; Applied Biosystems, CA, USA). rRNA gene sequences of about 950 bp were obtained for all clones. All sequences were checked for chimeric artifacts with the Check_Chimera program in the Ribosomal Database Project II (RDP II)²⁶⁾ and compared with similar sequences for reference organisms with a BLAST search.²⁷⁾

7. Phylogenetic and statistical analyses

Sequence data were used to construct a neighbor-joining phylogenetic tree using the MEGA package (version 3.1).²⁸⁾ Bootstrap analysis was used to estimate the reliability of the phylogenetic reconstruction (100 replicates). Operational taxonomic units (OTUs) were defined for this analysis as 16S rRNA gene sequence groups in which the sequences differed by <5%. Analysis of molecular variance²⁹⁾ was used to examine the partitioning of variation within sampling sites vs. among sites in the entire data set. One hundred permutations with random assemblages of these sequences were compared with observed distributions to test the statistical significance of nonrandom distributions.

8. PCR–denaturing gradient gel electrophoresis (DGGE) analysis

To provide a brief overview of the sediment and enrichment samples, we performed 16S rRNA gene-based PCR-DGGE analysis. PCR primers 341F-GC and 534R were used to amplify the variable V3 region of the bacterial 16S rRNA genes (corresponding to position 341–534 in the *E. coli* 16S rRNA gene sequence) connected to a GC clamp.³⁰⁾ PCR was performed using Premix Taq (Ex version) polymerase (TaKaRa), according to the manufacturer's instructions (100 ng template DNA, 25 pmol of each primer in a 50 µl reaction volume). The reaction conditions were as follows: initial denaturation at 94°C for 2 min, followed by 10 cycles each of denaturation at 94°C for 30 sec, annealing at variable temperatures for 30 sec, and extension at 72°C for 1 min. In the first cycle, the annealing temperature was set to 60°C, and for each of the 10 subsequent cycles, the annealing temperature was reduced by 1°C. This was followed by 20 cycles, each of 94°C for 30 sec, 50°C for 30 sec, and 72°C for 1 min.

DGGE analysis was performed with a DCode™ instrument (Bio-Rad, CA, USA), according to the manufacturer's instruc-

Fig. 1. Description of the sampling sites. Six sediment samples were collected along Iwata River in Tsu City, Japan. Structural formulae of (A) OPEO₂, (B) OPEO₃, (C) OPEC₂, (D) OP, which are potential pollutants formed by microbial metabolic toxicity.

tions. Gels were made using a gradient of denaturants between 30% (containing 2.10 M urea and 18.3% formamide) and 60% (containing 4.20 M urea and 24.0% formamide). Next, 500 ng of each PCR amplicon was subjected to electrophoresis on a 10% (w/v) polyacrylamide gel at 160 V for 5 hr at a running temperature of 58°C. After electrophoresis, the gel was stained with SYBR Gold (Invitrogen, CA, USA) for 30 min, according to the manufacturer's instructions. The DGGE profiles corresponding to both the sediments and enrichments are representative of triplicate samples because there were no significant differences between the results shown in Fig. 2 and other result replicates.

A gel slice containing a DNA band was excised and its

Fig. 2. 16S rRNA gene-based PCR–denaturing gradient gel electrophoresis (DGGE) profiles of bacterial populations in the enrichment cultures and isolate samples. (A) Sediment from Site-1 (Lane 1), Site-2 (Lane 2), Site-3 (Lane 3), Site-4 (Lane 4), Site-5 (Lane 5), Site-6 (Lane 6), Lane 2, (B) the second enrichment culture from Site-1 (Lane 7), Site-2 (Lane 8), Site-3 (Lane 9), Site-4 (Lane 10), Site-5 (Lane 11), Site-6 (Lane 12). The eighteen bands excised for DNA sequencing analysis are designated by a letter and number, e.g., E1.

DNA sequence was determined by the method of Watanabe *et al.*²⁵⁾ The resulting sequences were compared with the compilation of 16S rRNA genes available in the GenBank nucleotide library with a BLAST search²⁷⁾ through the DNA Data Bank of Japan (DDBJ).³¹⁾

9. Quantitative PCR

P. putida strain S5-alcohol dehydrogenase, *adh1* coding alcohol dehydrogenase 1 (ADH1), was selected to investigate the distribution of microbes capable of OPEO_n degradation in river sediments because this enzyme catalyzes the oxidation of the OPEO_n terminal alcohol group, which is considered one of the key enzymes involved in OPEO_n metabolism. qPCR for enumerating the *adh1* gene copies coding this enzyme was conducted with a StepOne plus™ Real-Time PCR system (Applied Biosystems, USA). Reaction mixtures volumes of 20 μ l were used, with each reaction mixture containing 10 μ l FAST SYBR green Master Mix (Applied Biosystems, USA), 7.6 μ l sterile water, 1.6 μ l DNA template and 0.4 μ M each of the forward and reverse primers. *adh1*-specific qPCR was performed using primers dg4F (5'-TCGGAGGCAGCAGCTC-GATCAAC-3') and RTdg4R (5'-CGGCAGVACRTCGT-CRTAGC-3')²³⁾ at an annealing temperature of 60°C. Both Uni16SF (5'-CTTACGACCAGGGCTACACAC-3') and Uni16SR (5'-CGGACTACGACGCACTTTAT-3')²⁰⁾ primers

were used for universal bacterial qPCR, at the same annealing temperature, as well as *adh1*-specific qPCR. The fragment sizes of both dg4F-RTdg4R and Uni16SF-Uni16SR primer sets were 113 and 95 bp, respectively. The amplification efficiency of both targets was around 100%. The specificity of amplicons was also confirmed by DNA sequencing. Calibration of both genes was performed with serial dilutions of a known quantity of genomic DNA obtained from *P. putida* S5. DNA copy numbers were calculated from the number of base pairs of genomic DNA using the average molecular weight of a base pair in double-stranded DNA (660 Da).³²⁾

10. Nucleotide sequence accession numbers

The sequences reported in this paper have been deposited in the GenBank, EMBL, and DDBJ nucleotide sequence databases under GenBank/EMBL/DDBJ accession Numbers AB374648–AB374913 (cloned 16S rRNA genes), AB374914 to AB374922 (DGGE bands from enrichment culture).

Results

1. Bacterial community composition in the river system

To investigate the potential for biodegradation of OPEO_n in the environment, we collected six sediment samples at the sites of different types of human activity along Iwata River as follows: Site-1, samples were collected from the head of the

Table 1. Distribution of 16S rRNA gene clones detected in Site-1, Site-4 and Site-6 soils

Group	Site-1 library			Site-4 library			Site-6 library		
	No. of OTUs	No. of Site-1 clones	Total clones (%)	No. of OTUs	No. of Site-4 clones	Total clones (%)	No. of OTUs	No. of Site-6 clones	Total clones (%)
Bacteria									
α -Proteobacteria	3	7	9	5	9	10	2	5	6
β -Proteobacteria	3	15	18	2	15	16	2	7	8
γ -Proteobacteria	3	7	8	2	6	7	1	17	19
δ -Proteobacteria	4	15	18	6	19	21	3	20	22
ε -Proteobacteria	1	1	1	1	1	1	1	3	3
Firmicutes	0	0	0	1	1	1	1	1	1
Actinobacteria	0	0	0	1	2	2	1	2	2
Planctomyces and relatives	3	6	7	0	0	0	2	4	4
CFB	3	8	10	1	12	13	4	9	10
Acidobacteria	3	5	6	2	5	5	0	0	0
Nitrospira	1	2	2	2	4	4	1	2	2
Green non-sulfur bacteria	1	1	1	0	0	0	0	0	0
Cyanobacteria	2	3	4	1	2	2	1	6	7
Uncultured bacteria	3	3	4	5	5	5	6	6	7
Archaea	0	0	0	0	0	0	1	7	8
Eukaryota	1	10	12	1	10	11	0	0	0

river beside a paddy field; Site-2, samples were collected from the river flowing through a golf course with a history of large amounts of pesticide application; Site-3, samples were collected from near a waterworks discharge point; Site-4, samples were collected upstream from where household wastewater would be discharged; and Site-5 and Site-6, samples were collected in the lower reaches of the river where household and/or industrial wastewater would be discharged (Fig. 1). The pH values of Site-1, Site-2, Site-3, Site-4, Site-5, and Site-6 were 7.31, 6.95, 6.71, 7.21, 6.75, and 7.39, respectively. The total organic carbon in Site-1, Site-2, Site-3, Site-4, Site-5, and Site-6 sediments was 65.1, 593.6, 211.9, 225.9, 1062.2, and 391.7 (mg/g dry soil), respectively.

To determine precise microbial diversity, DGGE profiles of 16S rRNA genes amplified from the enrichment cultures were generated (Fig. 2); however it was difficult to sequence the DNA excised from DGGE gels for sediments (Fig. 2A). Therefore, clone libraries of 16S rRNA genes were constructed from three of the six samples (Site-1, Site-4 and Site-6) considering the location. About 90 clones from each of the three libraries were sequenced as a representative survey of the microflora of the sediment. About 30 distinct operational taxonomic units (OTUs) were obtained from each library. The results are summarized in Table 1. This analysis indicated that the difference in genetic diversity at Site-1, Site-4, and Site-6 was not significant (Fig. 3); however, it was revealed that major bacterial populations consisted of five subclasses of Proteobacteria at the analyzed sites. Large-scale phylogenetic analysis of these OTUs was also performed to affiliate clonal sequences using the MEGA 3.1 program²⁸⁾ (Supplemental Fig. S1). It was revealed that the sequences related to the specific species involved in OPEO_n degradation were not predominant.

2. Enrichment for OPEO_n-biodegradation activity in sediment samples

Sediment samples were concentrated 100-fold and analyzed for contamination with OPEO_n and/or their breakdown products. Based on GC analyses, no OPEO_n or their metabolites were detected in the concentrated samples (data not shown). We then surveyed the potential for surfactant biodegradation in each sediment sample by enriching the OPEO_n-degrading microorganisms. OPEO_n were degraded to OPEO₂, OPEO₃, and OPEO₄ in Site-1, Site-2, Site-4, Site-5 and Site-6 enrichments. In these samples, OPEO₂ was the main OPEO_n degradation product that accumulated in the enrichment cultures (Fig. 4). Another intermediate, octylphenol diethoxy carboxylate (OPEC₂), which is produced by oxidation of the terminal hydroxyl group of OPEO₂, also accumulated in the enrichment cultures. In the second enrichment at Site-1, Site-2, and Site-4, the amount of OPEO_n intermediates increased 1.5- to 6-fold more than that of the first enrichment (Fig. 4). In contrast, the amounts of OPEO_n metabolites at Site-5 and Site-6 were not increased, although the concentration of metabolites

Fig. 3. Clone distribution from (A) Site-1 sample, (B) Site-4 sample, and (C) Site-6 sample.

in Site-6 was greater than that in other samples. During OPEO_n degradation, bacterial growth was observed in five active enrichment cultures, except for Site-3, with optical densities at 660 nm (OD_{660}) > 0.5 (data not shown). In addition, we investigated the change in microbial diversity by DGGE in each enrichment culture (Fig. 2B). DGGE bands were excised and a partial 16S rRNA gene fragment (about 200 bp) was amplified for sequence analysis. Supplemental Table S1 lists the database sequences with the highest nucleotide similarity for each DGGE band. According to sequence analysis of the DGGE bands from the enrichment cultures, the most prominent bands (E1, E2, E3, E7, E8, and E9) were affiliated with the genus *Pseudomonas*.

3. Enumeration of functional genes involved in OPEO_n degradation

Analysis of the bacterial community by DGGE and active enrichment cultures showed that OPEO_n-degrading bacteria

Fig. 4. Concentration of OPEO_n metabolites in the enrichment culture of (A) Site-1, (B) Site-2, (C) Site-3, (D) Site-4, (E) Site-5, and (F) Site-6. Gray bars indicate first enrichment culture, and right gray bars indicate second enrichment. ND: not detected.

would become dominant by exposing them to OPEO_n in the sediment (Figs. 2 and 4). We attempted to enumerate the bacteria that could be involved in the degradation of OPEO_n by molecular detection of the *adh1* gene. DNA fragments of the *adh1* and 16S rRNA genes amplified with primer sets from genomic DNA of *P. putida* strain S5 were sequenced and the corresponding sequences were confirmed (data not shown). Considering that the extraction efficiency of environmental DNA would have influenced the amplification of the target gene, we investigated the proportion of the *adh1* gene that oc-

curred compared to the 16S rRNA gene. The *adh1* gene copy numbers determined in the collected sediments are presented in Fig. 5A. Among the sediments, the copy number of the *adh1* gene was observed at 1.67×10^{10} to 8.01×10^{10} (copies/ng DNA) (Fig. 5A). The copy number of the 16S rRNA gene ranged from 4.64×10^{11} to 3.17×10^{12} (copies/ng DNA) (Fig. 5B).

Discussion

Pesticides and formulation components that are discharged

Fig. 5. Changes in (A) *adh1* gene and (B) 16S rRNA gene copy number in the sediment as estimated by qPCR. Error bars represent the standard deviation of copy numbers measured in triplicate samples.

into the non-target environment through surface water and/or groundwater transform into their biodegraded products (metabolic toxicants), and may exert certain chemical selective pressure on wildlife, including animals, plants, and microorganisms. In fact, toxic products can be generated by the microbial biodegradation of some non-toxic synthetic compounds, such as APEO_n^{14,15,20,21,33,34}); however, the extent to which discharged human-generated chemicals alter microbial ecology remains unclear, although such alterations may affect the biodegradation properties of microbial ecosystems.

To understand the in situ microbial communities, we performed 16S rRNA gene-based DGGE and clone analysis of representative bacteria from the river system. These analyses helped us to demonstrate the microbial diversity along the river before the treatment of sediment samples with enrichment cultures (Figs. 2A and 3). A previous report of OPEO_n-degrading bacteria indicated that they belong to α - and γ -Proteobacteria.^{17,35,36} Clone analysis demonstrated that clones corresponding to these two phyla were detected ranging from 17 to 25% at the three sites. These clones were affiliated with *Hyphomonas* sp. (Site-1) and *Sphingomonas* sp. (Site-4) belonging to α -Proteobacteria. The genus *Sphingomonas* has been reported in OPEO_n-degrading bacteria,³⁵ but not in *Hyphomonas* sp. Clone analysis also showed that clones assigned to the *Pseudomonas* group were found in Site-4 (96% similarity) and Site-6 (97% similarity) samples (Supplemental Fig. S1). Major band sequences from DGGE analysis also revealed that bacterial species related to the *Pseudomonas* group were dominant in the enrichment cultures. Considering that some strains of the genus *Pseudomonas* have been reported as OPEO_n degraders,^{17,20,36} these clones can be regarded as an OPEO_n degrader. Although other bacterial genera also degraded OPEO_n as a carbon source³⁷ and isolated as OPEO_n-degrading bacteria,³⁵ further isolation of OPEO_n-degrading bacteria is needed. In this study, 16S rRNA gene-based clone analysis also demonstrated that some clones are similar to a eukaryote. We consider that these clones are derived from plant DNA in the sediments, since plant mitochondria have a close evolutionary relationship to the bacterial 16S rRNA gene.³⁸

Because the predominant species could not be found among sampling sites (Table 1, Figs. 2A and 3), we enriched each sediment sample for bacteria capable of OPEO_n biodegradation to investigate whether there was potential surfactant biodegradation. Active enrichment cultures facilitated the proliferation of bacteria belonging to OPEO_n-degrading bacteria, as evidenced by DGGE and culturing (Fig. 2B, Supplemental Table S1, and Fig. 4). The sequences of these clones (E1 or E7) had more than 97% similarity with *P. putida* strain S5. Considering that no OPEO_n degradation was observed in the control, OPEO_n degradation was biological in the enrichment cultures. When we used an average of 5.5 copies of the 16S rRNA gene in one cell,³⁹ the bacterial cell numbers calculated by 16S rRNA gene copy numbers were

approximately 10¹¹ cells. Considering that microbial cells in sediment environments are generally enumerated as billions of cells, the cells calculated from 16S rRNA gene copy numbers among sampling sites were considered to be 100-fold more. Since this inconsistency may be due to unsuitable amplification efficiency, further primer modification for amplification of the 16S rRNA gene is needed. In addition to the enumeration of 16S rRNA gene copy numbers, we attempt to measure a number of functional gene (*adh1*) copies for the detection of OPEO_n-degrading bacteria by quantitative PCR. The *adh1* gene copy number was nearly parallel to the production of OPEO_n metabolic toxicants in each enrichment culture, except for Site-3 (Figs. 4 and 5A); however, a lower copy number of the *adh1* gene at Site-4 suggested that OPEO_n degradation occurred by other strains harboring a novel OPEO_n-degrading enzyme, since the metabolic toxicants produced from OPEO_n were generated as well as at Site-1 or Site-6 (Figs. 4 and 5).

All sediments, except for Site-3, showed the potential for metabolic toxicity derived from OPEO_n (i.e., metabolic toxicant production from OPEO_n). Considering that the results of pH values were not significantly different among sampling sediments, but that the amount of total organic carbon was different, this may be explained by the history of human-generated chemical exposure from a paddy field, golf course, household wastewater and the lower reaches of the river. We observed comparably high levels of metabolic toxicants from OPEO_n at Site-1 and Site-6 (Fig. 4A and F). Moreover, DGGE analysis demonstrated that related strains of OPEO_n degradation, *Pseudomonas* spp. and *Sphingomonas* sp., might exist at both sites (Fig. 2B and Supplemental Table S1). Since the genus *Sphingomonas* has been reported as an OPEO_n-degrading bacterium harboring an *adh1*-like gene,³⁵ *adh1* gene detection also revealed that bacteria capable of OPEO_n degradation would exist in both sediments (Fig. 5A).

The enrichment culture from Site-4, which showed lower levels of metabolic toxicants than both Site-1 and Site-6, demonstrated that the concentration of OPEO₂ increased 6-fold through subculturing (Fig. 4D). DGGE analysis of the enrichment culture showed that the predominant species might be related to *Methylobacterium* sp. and *Alcaligenes* sp. (Fig. 2B and Supplemental Table S1). Since *Methylobacterium* sp. and *Alcaligenes* sp. have not yet been reported as OPEO_n-degrading bacteria, these strains would be novel strains of OPEO_n degradation. The relatively low enumeration of the *adh1* gene at Site-4 would be due to the presence of bacteria with lower ADH1 activity or a novel *adh1*-like gene. Thus, it is reasonable that an inconsistency between OPEO_n degradation and *adh1* gene detection appears in the Site-4 sample.

At Site-2, low levels of metabolic toxicants produced from OPEO_n were observed in the enrichment culture (Fig. 4B). DGGE analysis of the enrichment culture showed that *Pseudoxanthomonas* sp., *Stenotrophomonas* sp. and

Rhodopseudomonas sp. were predominant (Fig. 2B and Supplemental Table S1). Since *Pseudoxanthomonas* sp. and *Stenotrophomonas* sp. have been reported as surfactant-degrading bacteria,⁴⁰⁾ these strains are involved in OPEO_n metabolic toxicity. Furthermore, the genus *Pseudomonas* was not detected and the *adh1* gene number was quite low (Fig. 5 and Supplemental Table S1). This could be explained by the OPEO_n-degrading bacteria and the human-generated chemicals being washed out at Site-2 because of the rate of river flow.

The metabolic toxicants produced from OPEO_n at Site-5 were different to those found at Site-6 (Fig. 4E and F). This result was supported by the microbial diversity found in enrichment cultures from both sites, demonstrated by DGGE profiles. Although *Rhodanobacter* sp. and *Rhodopseudomonas* sp. were predominantly detected in Site-5 enrichment culture, these strains were not OPEO_n-degrading bacteria (Fig. 2B and Supplemental Table S1). Considering that the genus *Pseudomonas*, which can efficiently degrade OPEO_n,¹⁷⁾ was not detected in the enrichment culture from Site-5, the discrepancy between the *adh1* gene copy number and the concentration of OPEO_n metabolites was due to the presence of bacteria that have lower ADH1 activity at Site-5 (Fig. 5).

No metabolic toxicant produced from OPEO_n was observed at Site-3 (Fig. 4C), although enumeration of the *adh1* gene indicated the presence of bacteria capable of producing OPEO_n (Fig. 5). However, since the concentration of OPEO_n was equivalent to that of the control in the enrichment of the Site-3 sediment, and the value of OD₆₆₀ was not increased in the enrichment culture (data not shown), we considered that no bacterial growth with OPEO_n degradation occurred, and that the lack of generation of metabolic toxicants of OPEO_n at Site-3 was due to the presence of OPEO_n-degrading microbes that have lower ADH1 activity. Further analysis of the lack of metabolic toxicity at Site-3 is needed. DGGE analysis of the Site-3 enrichment culture showed that *Thermomonas* sp., *Lysobacter* sp. and *Erythrobacter* sp. were present in the culture. Considering that no generation of metabolic toxicants of OPEO_n or bacterial growth was observed, these clones would be amplified from the sediment inoculated into the culture; however, since these genera have not yet been reported as OPEO_n-degrading bacteria, these strains would not be involved in OPEO_n degradation.

The rate of river flow at Site-2, Site-4, and Site-5 (460 m³/sec) was much faster than at Site-1 (10 m³/sec) and Site-6 (50 m³/sec).⁴¹⁾ DGGE analysis showed that enrichment culture from Site-1 and Site-6 sediments correlated with known OPEO_n-degrading bacteria, but that Site-2, Site-4, and Site-5 did not (Fig. 4B and Supplemental Table S1). Since Confer and Logan demonstrated that fluid mixing can substantially increase the uptake rate of environmental substrates,⁴²⁾ diverse bacterial species where high river flow would be dominant and could assimilate OPEO_n. Although the 16S rRNA gene

copy number demonstrated that bacterial cells were nearly equal in all sediments (Fig. 5B), OPEO_n-degrading bacteria harboring the *adh1* gene were relatively lower in high river flow than in low river flow (Fig. 5A). The detection of the *adh1* gene in sediment communities of Iwata River suggests that *adh1* genes are possibly responsible for the transformation of OPEO_n metabolic toxicants. This is the first report of the detection of *adh1*-like genes involved in OPEO_n degradation in a freshwater environment. In general, PCR analysis of functional genes complemented the culture-dependent measurements of specific strains,⁴³⁾ although it failed to detect their presence in sediments Site-3 and Site-4; therefore, modification of the primer set for *adh1* gene detection would be necessary to support the accurate evaluation of OPEO_n-degrading bacteria. It may also be concluded that quantitative PCR is supported by the culture-dependent assay in the evaluation of in situ microbial ecology. Considering that OPEO_n-degrading bacteria might exist in the sediment at these sites, river flow was considered to be a major factor for the bacterial uptake of OPEO_n in the river; therefore, the history of human-generated chemical exposure may differ due to the hydrology at each site, and the potential of metabolic toxicity by OPEO_n was considered to differ from Site-1 to Site-6.

In this study, we found that there was no predominant species in 16S rRNA gene-based molecular microbial diversity among sample sites; however, active OPEO_n-degrading enrichment cultures were generated. Moreover, quantification of the *adh1* gene also revealed that the bacteria involved in OPEO_n degradation were common among sampling sites. Our results suggest that OPEO_n-degrading bacteria commonly exist in Iwata River, regardless of human activity. Moreover, it has been reported that OPEO_n was toxic to some bacterial strains;⁴⁴⁾ therefore, if excess OPEO_n were applied as a detergent, these degrading bacteria would be enriched and produce endocrine-active substances because of changes in microbial diversity with increasing OPEO_n-degrading bacteria. Given the abundance of OPEO_n-degrading bacteria in the environment,^{17,34–37,40,45)} these bacteria might contribute to produce metabolic toxicants by OPEO_n degradation; therefore, preventing the release of surfactants might be best addressed by inhibiting bacteria that produce metabolic toxicants of OPEO_n. Furthermore, much attention has been directed toward detecting changes in the specific bacteria that harbor the *adh1*-like gene in the environment to prevent contamination from metabolic toxicants.

Moreover, large amounts of APEO_n are used as cleaning surfactants to remediate soil and/or groundwater with hydrophobic organic compounds.^{5–11)} If countries have no regulation on APEO_n use, APEO_n-degrading bacteria might produce an excess amount of metabolic toxicants. Consequently, the adjuvant has a significant influence on the natural adaptation of microbial consortia, so it is important to monitor and/or regulate the environmental fate of adjuvants as well as active ingredients.

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Supplemental Fig. S1 and Table S1 are available in the online publication at <http://www.jstage.go.jp/browse/jpestics/>.

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