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Identification of Final Biodegradation Product of Nonylphenol Ethoxylate (NPE) by River Microbial Consortia

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Both the production and consumption of non-ionic surfactants have been steadily increasing year by year. In Japan, the former reached 422,119 metric tons in 1993. Alkylphenol ethoxylate (APE) is the representative of polyoxyethylene-type nonionic surfactants along with alcohol ethoxylate (AE), and mainly consumed as industrial applications rather than household use. Most of the alkyl part constituting hydrophobes of APE is composed of the highly branched nonyl-group and is designated as nonylphenol ethoxylate (NPE). NPE has been recognized as a considerably refractory compound among chemicals released into surface waters (1981). In fact, no complete degradation of it has thus far been reported, although partial degradation was found to occur exclusively on their hydrophiles (i.e., ethylene oxide (EO) chains). Intermediates resulting from NPE biodegradation have been found in various environmental sources (1994; 1994; 1984). Through detailed investigations, these metabolites have been shown to be alkylphenol, NPE with one or two moles of EO chains, and their analogous forms with a carboxylated end of the EO chain instead of a hydroxyl end (1982). The above findings were also obtained through the NPE biodegradation by isolated bacterial strain (1994). It has also been revealed that the shorter the EO chain of an NPE becomes due to biodegradation, the stronger its toxicity (1986). Moreover, bioaccumulation of APE-degradation products has been found in some organisms (1990).

The effluent from the sewage treatment plant (STP) is mainly discharged to the river, the resource for city water in Japan. Therefore, much attention is paid to the toxicity potential, such as total organic halides (TOX) potential, trihalomethanes (THM) potential, mutagenicity and so on, of river water receiving the effluent from STP. These toxicity potentials are guessed to be caused by various organic chemicals including fumic acids. It is, therefore, expected that complete degradation or mineralization of chemicals included in wastewater should be achieved at STP. But some of them, which is supposed to be less than 10% of influent because of the removal efficiency, are discharged to the river without any degradation even if STP is operated normally.

In this paper, it is investigated that the biodegradabilities of NPE by the TOC-Handai method which was developed to evaluate the complete biodegradability of organic chemicals by microorganisms in natural aquatic environments via the loss of total organic carbon (TOC) (1993). Then, the structural analyses of the remaining intermediate of NPE (Triton N-101) degraded by river microbial consortia was carried out. It was found to be the uniformed form in contrast to the other so far reported NPE-

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biodegradation products. Finally we identified the final biodegradation product of NPE.

Water samples were collected at 4 stations as indicated in Table 1. All samples were kept at 4°C except during transport and used for biodegradation testing within 1 week. Dissolved oxygen was measured by a DO meter (Central Kagaku) equipped with an oxygen electrode. Water temperature was obtained by a thermometer fitted in the DO meter. pH was measured by a pH electrode (Sensonix Japan). Ethyl violet active substance (1982), which reflects anionic surfactants, was assayed instead of using the methyleneblue active substance (MBAS) assay. TOC was determined by a TOC analyzer (Shimadzu). Conductivities of water samples were measured using a conductivity meter (Horiba). Sodium, potassium, magnesium and calcium cations were determined using an atomic adsorption spectrophotometer (Hitachi). Bacterial counting was performed using a low-nutrient agar plate (0.5 g peptone, 0.25 g yeast extract, 0.1 g glucose in 1.0 L sampled river waters, solidified with 1.5% agar). Agar plates were incubated for 7 days, at 28°C. All other routine analyses followed the Japanese Industrial Standards (JIS) K0102 (1978).

Triton N-101, a nonylphenol ethoxylate in which the average number of ethylene oxide units is 9.5, was obtained from Rohm & Haas Corp. Trimethylsilyldiazomethane (0.01% [vol/vol] in *n*-hexane) was obtained from GL sciences Inc., Japan.

Five hundred milliliters of each river water was filtrated through a Millipore GV filter (Type GVWP04700; pore size 0.22 mm), and each filter was dipped into 50 ml of a dilution solution for BOD5 assay (21.75 mg K_2HPO_4, 8.5 mg KH_2PO_4, 44.6 mg $NaHPO_4 \cdot 12H_2O$, 1.7 mg NH_4Cl, 22.5 mg $MgSO_4 \cdot 7H_2O$, 27.5 mg $CaCl_2$, and 0.25 mg $FeCl_2 \cdot 6H_2O$ in 1.0 L deionized water) in a 100-ml beaker. The microorganisms collected on the Millipore filter were subsequently resuspended by sonication for 1 minute at a frequency of 20 kHz (Tosho Denki). Five milliliters of each microorganisms suspension was then inoculated into 45 ml of artificial river water in a 70-ml screw vial, supplemented with the compound to be tested at a concentration adjusted to approx. 40 $mg \cdot L^{-1}$(corresponding to 20 mg-TOC$\cdot L^{-1}$) in advance. A vial without chemical supplementation was prepared as a blank for each inoculum, and without inoculum as a control for the tested chemical. The vials were incubated at 28°C in the dark on a rotary shaker at 120 rpm, and 1-ml samples withdrawn from the culture media in the vials were subjected to TOC measurement periodically. All the above operations were carried out aseptically.

After the TOC values reached half of those at the start of test, each culture medium containing intermediates derived from NPE (Triton N-101) was extracted with 5 ml methylene chloride. The methylene chloride extracts were dehydrated with sodium sulfate, transferred into pear-shaped flasks, and concentrated by a rotary evaporator. The concentrated extracts were filtered through Pasteur pipettes filled with cotton plugs into small glass tubes, and dried by a nitrogen stream. The dried samples were redissolved with a small portion of chloroform, and injected into high-performance liquid chromatography (HPLC). The HPLC apparatus consisted of a solvent programmer, solvent delivery pump, solvent mixer and spectrophotometric detector (Tosoh) connected to an Advanced Computer Interface (Dionex) with chromatogram analyses performed by a chromatography work station (Dionex). Normal-phase HPLC, in which gradient elution was employed, was performed according to (1985) with slight modification. A prepacked amide chromatographic column, 250 X 4.6 mm (TSK-GEL Amide-80, Tosoh), was used.

Mobile phase A comprised *n*-hexane and mobile phase B in the ratio 9:1. Mobile phase B consisted of 2-propanol and methanol in the ratio 7:3. A linear program from 100% A to 100% B in 37.5 min. was carried out. The flow rate was 1 $ml \cdot min^{-1}$ and the detection wavelength 277 nm. A gas chromatography/mass spectrometry (GC/MS) instrument (Shimadzu) equipped with fused-silica capillary column (CBP1-M25-025, 25 m X 0.25 mm i.d., liquid phase thickness 0.25 mm; Shimadzu) was used. Helium was employed as the carrier gas. A l-µl sample was injected splittless for 30 sec. The gas chromatograph temperature programs were as follows: 70°C isothermal for 2 min, increasing to 320°C at $10°C \cdot mid^{-1}$, and then maintained at 320 °C for 5 min. The electron impact conditions were: ionization energy 70 eV; ionizer temperature 230°C; mass ranges 50-400 m/z; scantime 1.5 sec. Samples extracted as described above were allowed to stand at ambient temperature to evaporate the solvent, and then further dried in a desiccator under a vacuum. To derive samples to methyl esters, 0.9 ml of ethylacetate, 50 µl methanol and 50 µl trimethylsilyldiazomethane (0.01% [vol/vol] in *n*-hexane) solution was added to the dried samples and they were allowed to stand for 30 min at ambient temperature. Ethylacetate samples were injected directly to GC/MS.

RESULTS AND DISCUSSION

The water qualities of the 4 inoculum sampling stations are given in Table 1. To define the characteristics of each inoculum, we selected unpolluted extreme upper streams and lower parts of the same rivers into which household effluents are discharged. In the Hino and Wada/Joganji rivers, the water qualities of the upper streams were found to be very clean, and even the lower parts of the rivers were quite clean with respect to BOD, total nitrogen, anionic surfactants and concentrations of various ions. The degradation profiles of NPE are shown in Fig. 1. It was found that the trends in degradation of NPE varied according to the sampling station. On the whole, the degradations of NPE by the inocula of upper streams of each river were faster than those by inocula of their middle reaches, which indicates that the microbial consortia of middle reaches may be preacclimatized with discharged NPE. No apparent correlation between the degradation of NPE and the bacterial counts of the inoculum sampling stations was observed. For example, the degradations of NPE with inocula from the upper stream of the Hino river were superior to those from the Wada river, despite the fact that the number of colony forming units (CFU) of the former station was about one-fourth that of the latter.

Culture medium containing the degradation products of NPE was extracted with methylene chloride, and subjected to normal-phase HPLC. In this system, NPEs were separated according to the number of ethylene oxide (EO) units. As shown in Fig. 2, a single peak was basically observed for each sample, which corresponded to NPE bearing 2 moles of EO units. To try and identify the compound corresponding to this peak, fractionated samples were subjected to GC/MS. However, no significant peaks were observed. We therefore attempted to esterify the degradation samples, because it was apparent that the degradation products would give no signals on mass spectrometry if they were negatively charged molecules such as carboxylic acids. The mass chromatograms (MCs) of methyl esterified samples were shown in Fig. 3. Distinct peaks were observed on the MCs at m/z 207 corresponding to the methyl ester of NP1EC and at m/z 251 corresponding to that of NP2EC (see the mass spectrum of each molecule in Fig. 3). However, no peaks on the MCs corresponding to NP1EO or NP2EO (at m/z values of 179 and 223, respectively (1981)). All of the NPE degradation products were shown to transform to the nonylphenol ethoxylates bearing a carboxylated end in their hydrophilic

Table 1. Inoculum sampling stations and their water qualities

	Hino river, upper stream[a] (Fukui pref.)	Hino river, middle reach (Fukui pref.)	Wada river, upper stream[a, b] (Toyama pref.)	Joganji river, middle reach (Toyama pref.)
Temperature (°C)	9.4	15.5	11	15
DO (mg/L)	11.2	8.9	N. M.	N. M.
pH	6.78	6.76	7.69	7.53
Alkalinity (mg/L)	20.4	20.4	15.3	25.5
SS (mg/L)	1.14	2	0.143	1.43
TOC (mg/L)	N. D.	N. D.	N. D.	N. D.
BOD (mg/L)	0.082	0.98	0.29	0.33
T-N (mg/L)	0.329	0.517	0.481	0.372
T-P (mg/L)	0.432	0.404	0.164	0.596
EVAS[c] (mg/L)	0.00247	N. D.	0.0129	0.0238
Conductivity (μS/m)	65.6	70.8	33.4	75.3
Na^+ (mg/L)	0.2	3.4	N. D.	N. D.
K^+ (mg/L)	N. D.	N. D.	N. D.	0.1
Mg^{2+} (mg/L)	1.6	1.4	0.7	1.2
Ca^{2+} (mg/L)	1.6	1.3	N. D.	5.5
Bacterial count (CFU/ml)	8.5×10^2	7.6×10^3	3.3×10^3	6.9×10^3

N. D., not detected; N. M., not measured; a, tumbling mountain streams; b, the upper stream of a tributary of the Joganji river; c, ethyl violet active substance.

groups. The degradation intermediates derived from NPE were revealed to be NP1EC and very small amount of NP2EC and did not include NP1EO and NP2EO. Although both NPnEOs and NPnECs are the established biodegradation intermediates of NPE, the above finding is somewhat novel, since NPnEOs were previously found to be concomitant with NPnECs as degradation products of NPE (1994). In river waters, NPnEOs are reported to be further oxidized to NPnECs (1994). Moreover, octylphenol ECs were reported to be more resistant to biodegradation than octylphenol EOs (1989). Thus, the final form of NPE-biodegradation products generated in natural aquatic environments might possess a carboxylated end in the polyethoxylate hydrophilic group instead of a hydroxyl end. More detailed, NP2EC could be further degraded and/or oxidized to NP1EO and/or NP1EC, as well as NP1EO could also be oxidized to NP1EC. An NPE degradable pseudomonad bacterium is also capable of degrading only the half portion of TOC of NPE, and of degrading NPE to NP2EO and NP2EC but neither NP1EO nor NP1EC (Maki et al., 1994). It suggests that river microbial consortia contain bacteria which are capable of transforming metabolites of NPE further to NP1EC. Since nonylphenol (NP), the simplest form among a series of biodegradation products of NPE is produced preferentially in anaerobic process (Giger et al., 1984), such as anaerobic digestion of activated sludge, it seems improbable that NPE is degraded to NP under aerobic conditions. Hence, NP1EC is thought to be the most probable ultimate biodegradation product of NPE generated under aerobic conditions.

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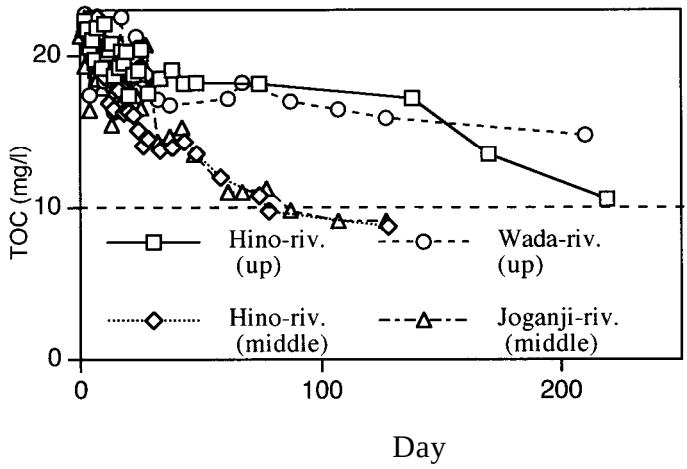

Figure 1. Comparison of the effects of each inoculum in the degradation

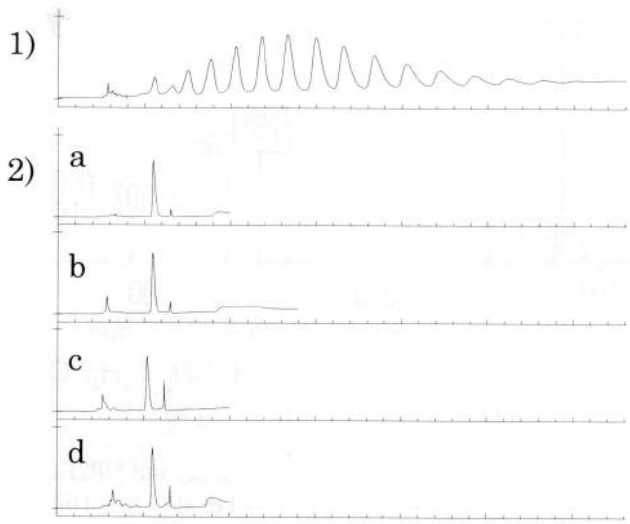

Figure 2. Chromatograms of degradation products of NPE on normal-phase HPLC. 1) Standard NPE (Triton N- 101). 2) Degradation products of NPE by iocula from a, Hino river, upper stream; b, Hino river, middle reach; c, Wada upper stream; d, Joganji river middle reach.

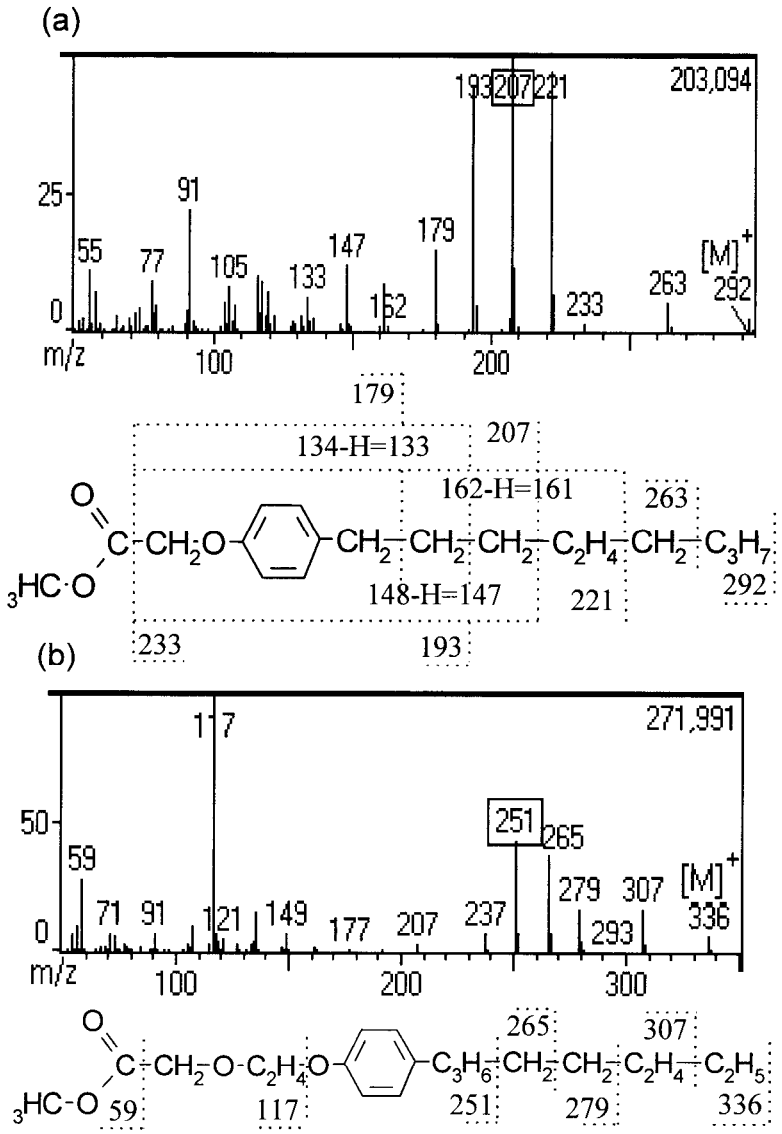

Figure 3. Mass spectra of methyl ester of NP1EC (a) and NP2EC (b). Mass spectra were interpreted according to Ahel et al (Ahel, 1987).

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