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Biodegradation of synthetic surfactants by river microorganisms

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Abstract—Four kinds of widely used synthetic surfactants and polyethylene glycol (PEG) which corresponds to the hydrophilic moieties of polyoxyethylene-type nonionic surfactants were subjected to a complete biodegradation test using microorganisms from relatively unpolluted river waters (the TOC-Handai method). Sodium dodecyl sulfate (SDS), alcohol ethoxylate (AE) and PEG were mineralized by most of the microorganisms used as inocula, irrespective of differences in their degradation rates, which is in agreement with their biodegradabilities confirmed so far by other tests. These degradable surfactants were, however, not completely degraded by some river microorganisms. Among the surfactants tested, SDS was degraded most rapidly by microorganisms of each river in the same manner but the degradation rates of AE and PEG differed considerably according to the inoculum sampling station. Sodium linear alkylbenzensulfonate (LAS) and nonylphenol ethoxylate (NPE), which are known to be recalcitrant molecules, were not degraded completely by microorganisms of any of the river waters within the testing period; degradation of the former rarely proceeded, while that of the latter ceased leaving the well-known intermediates. The residual metabolites of NPE generated by biodegradation, which were analyzed by high-performance liquid chromatography and gas chromatography/mass spectrometry, revealed that this surfactant was transformed to carboxylated metabolites, nonylphenoxy acetic acid (NP1EC) and [(nonylphenoxy) ethoxy] acetic acid (NP2EC) exclusively.

Keywords: biodegradation; surfactants; river microorganisms; TOC.

1 Introduction

Both the production and consumption of synthetic surfactants have been steadily increasing. In Japan, the former reached 1142136 metric tons in 1993. Linear alkylbenzensulfonate (LAS) and sodium dodecyl sulfate (SDS) are representatives of anionic surfactants, and alcohol ethoxylate (AE) and alkylphenol ethoxylate (APE) of nonionic surfactants. Together, these four synthetic surfactants can be regarded as exemplifying a series of pollutants which are being released rather abundantly into surface waters. A great deal of research has already been carried out on the biodegradabilities of synthetic surfactants, resulting in the establishment of basic data on the biodegradabilities of individual surfactant molecules (Swisher,

1987). In early work, indication of the degradation of a surfactant was restricted to a loss of the characteristics unique to the compound of interest as a surface active substance, with the remaining intermediates caused by the process of biodegradation not being considered (Huddleston, 1965; Manusner, 1969; Greff, 1965). With increased concern ones environmental aspects, the standard for the degradation of organic chemicals released into surface waters has shifted to complete degradation, in which the chemicals should be mineralized to be their primitive constituents, i. e., carbon dioxide and water. Several methods of evaluating the complete biodegradability of organic chemicals have been enveloped, for example, the Sturm, OECD (OECD, 1981), and MITI methods and derivatives of these (OECD, 1991). These methods, however, employ acclimatized activated sludges as inocula, which seem to simulate the treatability of organic pollutants by sewage treatment plants. In Japan, sewerage systems largely confined to urban areas, significant amount of household effluents containing synthetic surfactants are still discharged directly into natural surface water environments. Consequently, surfactant molecules should posses adequate degradability also in natural aquatic environments. The TOC-Handai method was developed to evaluate the complete biodegradability of organic chemicals by microorganisms in natural aquatic environments via the loss of total organic carbon (TOC; Nasu, 1993). The results obtained by this method differ significantly from those acquired by conventional means, such as the MITI method, in that the former method reflects differences in the biodegradability of each compound more distinctly than the latter. Here, we investigate the complete biodegradability of the four synthetic surfactants mentioned above by means of the TOC-Handai method, specifically by using microorganisms from relatively unpolluted river waters, and discuss the differences between readily biodegradable and recalcitrant surfactants from the viewpoint of their biodegrading abilities which are inherent in aquatic environments.

2 Materials and methods

2.1 Sampling of inocula for biodegradation tests and water quality analyses

Water samples were collected at 5 stations as indicated in Table 1. All samples were kept at 4℃ except during transport and used for biodegradation testing within 1 week. Dissolved oxygen was measured by a DO meter (Model of UC-12; Central Kagaku Co., Ltd.) equipped with an oxygen electrode. Water temperature was obtained by a thermometer fitted in the DO meter. pH was measured by a pH electrode (Model PE-2CN; Sensonix Japan Co., Ltd.). Ethyl violet active substance (Motomizu, 1982), which reflects anionic surfactants, was assayed instead of using the methyleneblue active substance (MBAS) assay. TOC was determined by a TOC analyzer (Model TOC-500; Shimadzu Ltd.). Conductivities of water samples was measured by conductivity meter (Model DC-12; Horiba Ltd.). Sodium, potassium, magnesium and calcium cations was determined by an atomic adsorption spectrophotometer (Model 208; Hitachi). Bacterial counting was performed using a low-nutrient medium (Yoshikura, 1981). All other routine analyses followed the Japanese Industrial Standards (JIS) K0102 (JIS, 1978).

2.2 Chemicals

Triton N-101, a nonylphenol ethoxylate in which the average number of ethylene oxide units is 9.5, was from Rohm & Haas Corp. AE was obtained from Sigma Chemicals, and LAS from Tokyo Chemical Industry Co., Ltd. SDS and polythylene glycol (PEG) were purchased from Kishida Chemical Co., Ltd. Trimethylsilyldiazomethane (0.01% [v/v] in n-hexane) was from GL sciences Inc., Japan.

2.3 Biodegradation testing by the TOC-Handai method

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 artificial river water (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 ("Biorupture" Model UCD-200U; Tosho Denki Co., Ltd.). Five milliliters of each microorganism 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 in advance to approx. 20 mg TOC/L. A vials without chemical supplementation was prepared as a blank for each inoculum, and without inoculum as a control for each tested chemical. The vials were incubated at 28℃ in the dark on a rotary shaker at 120 r/min, 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.

2.4 High-performance liquid chromatography (HPLC) and gas chromatography/mass spectrometry (GC/MS)

The culture media containing intermediates derived from NPE (Triton N-101) were 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 HPLC. The HPLC apparatus consisted of a PX-8010 solvent programmer, CCPD solvent delivery pump, MX-8010 solvent mixer and UV-8010 spectrophotometric detector (all from Tosoh Co., Ltd.) connected to a Chromatocorder 12 (System Instruments), or to an Advanced Computer Interface Dionex Corp.) with chromatogram analyses performed by a chromatography work station (AI-450, Ver. 3.320J; of Dionex Corp.). Normal-phase HPLC, in which gradient elution was employed, was performed according to Ahel and Giger (Ahel, 1985) with slight modification. A prepacked amide chromatographic column, 250×4.6 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/min and the detection wavelength 277 nm. A GC/MS instrument (Model GCMS-QP5000; Shimadzu) equipped

with fused-silica capillary column (CBP1-M25-025, 25m×0.25 mm i.d., liquid phase thickness 0.25 mm; Shimadzu) was used. Helium was employed as the carrier gas. A 1 ml sample was injected splittless for 30 sec. The gas chromatograph temperature programs were as follows: 70℃ isothermal for 2 min, increasing to 320℃ at 10℃/min, and then maintained at 320℃ for 5 min. The electron impact conditions were: ionization energy 70 eV; ionizer temperature 230℃; 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 trimethylsilyldiazo-methane (0.01% [v/v] 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.

3 Results

3.1 Comparison of surfactant degradation

The water qualities of the 5 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 practice, there

Table 1 Inoculum sampling stations and their water qualities

	Hino River, upper streama (Fukui prefecture)	Hino River, middle reach (Fukui prefecture)	Wada River, upper stream[a,b] (Toyama prefecture)	Joganji River, middle reach (Toyama prefecture)	Ai River, middle reach (Osaka prefecture)
Temperature, ℃	9.4	15.5	11	15	30.5
DO, mg/L	11.2	8.9	N.M.	N.M.	11.6
pH	6.78	6.76	7.69	7.53	8.54
Alkalinity, mg/L	20.4	20.4	15.3	25.5	66.3
SS, mg/L	1.14	2	0.143	1.43	2
TOC, mg/L	N.D.	N.D.	N.D.	N.D.	2.41
BOD, mg/L	0.082	0.98	0.29	0.33	1.5
T-N, mg/L	0.329	0.517	0.481	0.372	1.55
T-P, mg/L	0.432	0.404	0.164	0.596	0.32
EVAS[c], mg/L	0.00247	N.D.	0.0129	0.0238	0.115
Conductivity, μs/m	65.6	70.8	33.4	75.3	227.7
Na^+, mg/L	0.2	3.4	N.D.	N.D.	12.6
K^+, mg/L	N.D.	N.D.	N.D.	0.21	2.7
Mg^{2+}, mg/L	1.6	1.4	0.7	1.2	3.3
Ca^{2+}, mg/L	1.6	1.3	N.D.	5.5	18.6
Bacterial count, CFU/ml	8.5×10^2	7.6×10^3	3.3×10^3	6.9×10^3	7.7×10^3

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

were no significant differences in the biodegradation rates for each surfactant using inocula from 6 sampling stations of the Ai River). 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 water quality of the middle reach of the Ai River was not comparable to those of the other stations. The degradation profiles of the surfactants (Fig. 1) were similar, irrespective of the inoculum sampling station: SDS was degraded most rapidly, while AE and PEG were degraded similarly, but more slowly than SDS. PEG, which corresponds to the hydrophilic moieties of polyoxyethylene-type nonionic surfactants, was degraded faster than AE. Although in the case of the inoculum from the upper stream of the Hino River, the degradation of PEG was relatively slow and was exceeded by that of AE. The chemicals were found to be mineralized in the most cases. However, with the inoculum from the Wada River SDS was not completely degraded, while the degradation of AE by the same inoculum scarcely proceeded, even though it was mineralized by other inocula. LAS degradation was found to make little progress in all cases. NPE was apparently degraded more slow-

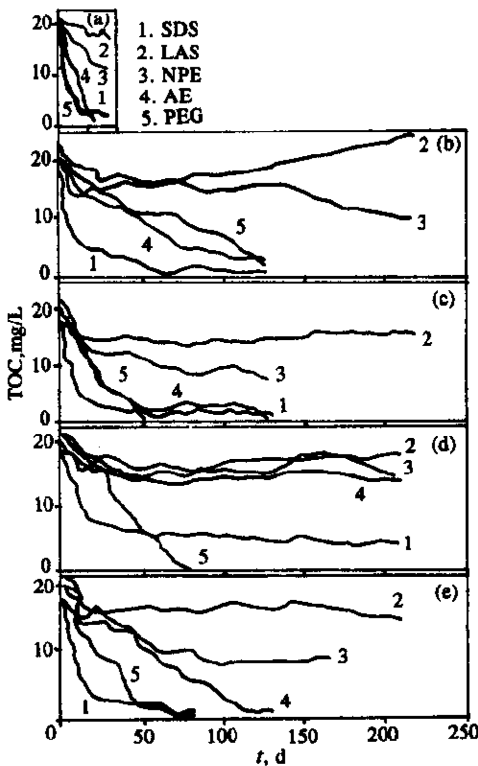

Fig. 1 Degradation of SDS, LAS, NPE, AE and PEG by inocula from the Ai River, middle reach (a); Hino River, upper stream (b); Hino River, middle reach (c); Wada River, upper stream (d); and Joganji River, middle reach (e)

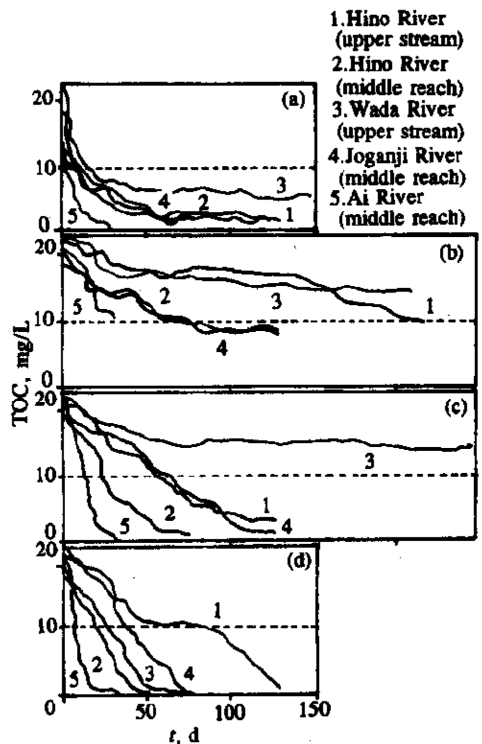

Fig. 2 Comparison of the effects of each inoculum in the degradation of SDS (a), NPE (b), AE (c) and PEG (d)

ly than AE, and ceased when the TOC values reached half of those at the start of test. In Fig. 2, the degradation data for eachsurfactant are combined so that the differences in the effects of each inoculum can be compared. SDS was shown to be degraded in the same manner by every inoculum, but the rates of degradation of polyoxyethylene-type compounds varied according to the sampling station. The average number of days for the TOC value to reach 10 mg/L (approximately equivalent to half of the initial concentrations) in the degradation of each chemical and their variance are shown in Table 2. SDS required the fewest days for the TOC to be reduced by half and showed the smallest variance (approx. 7.6 days and 8.64,

Table 2 Average number of days for the TOC of the tested chemicals to be reduced by half and their variances

	SDS	AE[a]	PEG	NPE[a]
Average days[b], $\bar{x}$	7.6	35.8	39.4	101.3
Variances, $\frac{\sum(\bar{x}-x_i^c)^2}{n^d-1}$	8.64	370.7	1020.6	5059.7

a. the numbers of inocula (n) for AE and NPE degradation is four since the TOC was not reduced to half by the inoculum from the upper stream of the Wada River within the testing period; b. calculated from Fig. 2; c. days required for TOC reduction by half by each inoculum for each chemical, d. number of inocula

respectively). The results for the AE and PEG degradation were similar to each others (35.8 days and 370.4 for AE, and 39.4 days and 1020.6 for PEG, respectively), while NPE needed 101.3 days and had a variance of 5059.7. It can be seen that the greater the average number of days needed for TOC reduction by half was, the larger the variances became. The chemicals also tended to degrade faster the more polluted the inoculum sampling station was. Thus, for example, the inoculum from Ai River, which was the only station where the TOC, BOD and total nitrogen concentrations exceed 2.0, 1.0 and 1.0 mg/L (Table 1), respectively, degraded all of the tested chemicals most rapidly, while degradations with inocula from the middle reaches were faster than those from the upper streams of the same rivers (The Wada River is the upper stream of a tributary of the Joganji River). However, no apparent correlation between the degradation of each chemical and the bacterial counts of the inoculum sampling stations was observed. For example, the degradations of all the chemicals 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.

3.2 HPLC and GC/MS analyses of degradation products of NPE

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. 3, 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 chromatorgrams (MCs) of methylesterified samples are shown in Fig. 4. 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. 4). However, no peaks on the MCs corresponding to NP1EO or NP2EO (at m/z values of 179 and 223, respectively (Giger, 1981)). All of the NPE degradation products were shown to transform to the nonylphenol ethoxylates bearing a carboxylated end in their hydrophilic groups.

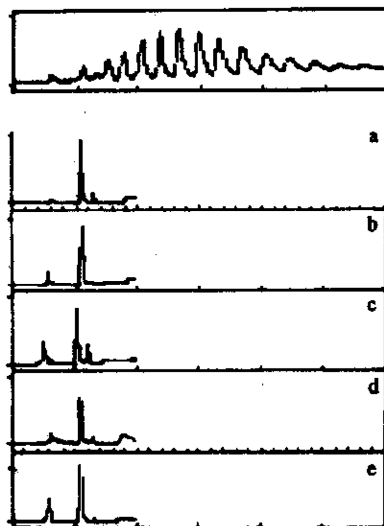

Fig. 3 Chromatogram of degradation products of NPE on normal-phase HPLC (1) Standard NPE (Triton N-101) (2) degradation products of NPE by inocula from a. Hino River, upper stream; b. Hino River, middle reach; c. Wada River upper stream; d. Joganji River middle reach; e. Ai River middle reach

4 Discussion

The overall results of the present study are agreement with findings obtained thus far in other biodegradation tests on restricted surfactant molecular species, i. e., readily biodegradable surfactants (chemicals), SDS, AE and PEG were found to be mineralized by microorganisms from most of the sampling stations, while NPE was only partially degraded leaving intermediates. The degradation intermediates derived from NPE were revealed to be NP1EC or NP2EC and did not to 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 (Corcia, 1994; Ahel, 1994a). In river waters, NPnEOs were further oxidized to NPnECs (Ahel, 1994b). Moreover, octylphenol ECs were reported to be more resistant to biodegradation than octylphenol EOs (Ball, 1989). Thus, the final form of NPE-biodegradation products generated in natural aquatic environments might posses a carboxylated end in the polyethoxylate hydrophilic group instead of a hydroxyl end.

LAS degradation was shown to proceed hardly at all, which is a novel finding because in other tests this synthetic surfactant has been reported to be degraded (Swisher, 1987; Itoh, 1988; Schöerl, 1988; Fischer, 1975). A likely explanation is inhibition to the degraders due to a high concentration of LAS supplementation (Swisher, 1987; Urano, 1985). In the case of this compound, 20 mg TOC/L (the initial concentration of the tested compounds) corresponds to approx. 30 mg LAS/L, which is rather high but a limited value for TOC determination. In some tests, however, the opposite result has been reported, i. e., LAS could be considerably degraded even at a high concentration (Swisher, 1981; Pitter, 1979). More-

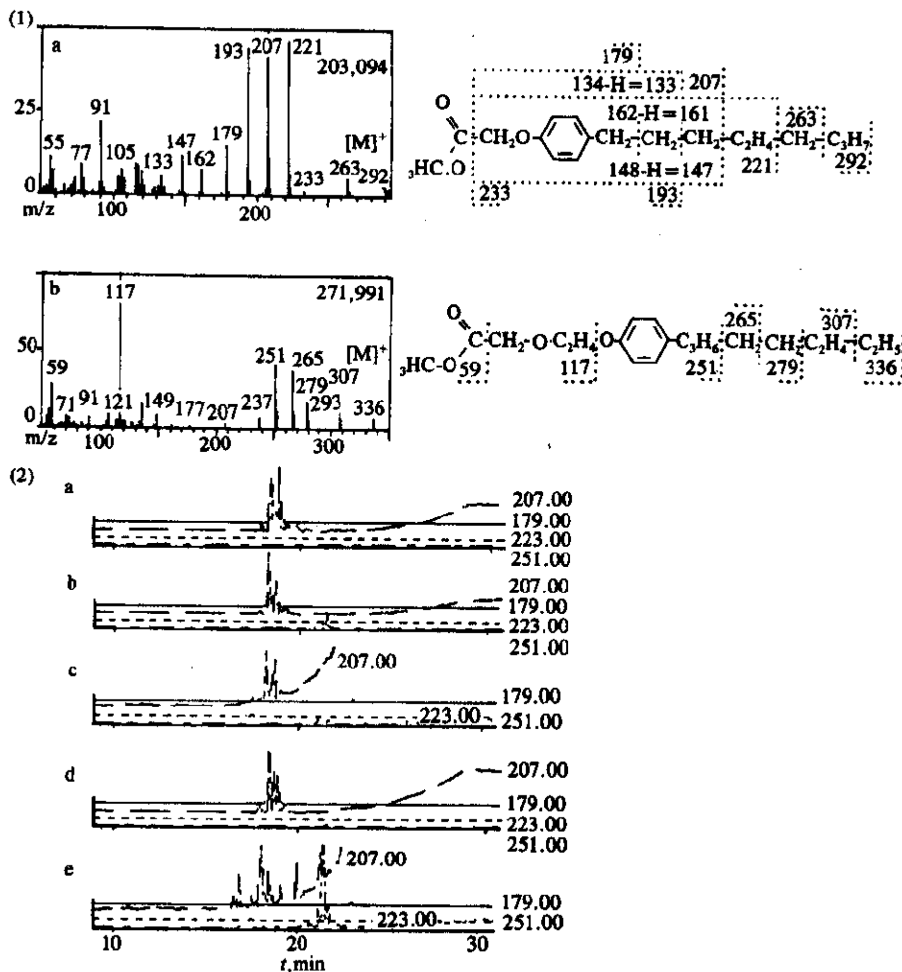

Fig. 4 (1) Mass spectra of methyl ester of NP1EC (a) and NP2EC (b) Mass spectra were interpreted according to Ahel *et al* (Ahel, 1987)
(2) Mass chromatogram (MCs) of methyl-esterified NPE- biodegradation products by inocula from the Hino River, upper stream (a); Hino River, middle reach (b); Wada River, upper stream (c); Joganji River, middle reach (d); and Ai River, middle reach (e). Numbers on the right of each MC represents characteristic m/z of a series of NPE-biodegradation products

over, some river die-away tests have also shown that high concentration of LAS could be degraded significantly (Maurer, 1974; de Oude, 1977). In contrast, a realistic concentration of LAS (10 μg/L) was hardly degraded by a particular river water (Palmisano, 1991). From the above contradictory results, it might be concluded that in aquatic environments microorganisms which are capable of degrading LAS are relatively localized as to their number and species, and that the conditions necessary for the adequate degradation of LAS to be achieved are also limited (marked inhibition against biodegradation due to a high concentration, etc.), although this surfactant is an unambiguously biodegradable molecule. In practice, complete LAS biodegradation has been reported to require the synergistic metabolism of the

defined bacterial group (Sigoillot, 1992). The following results obtained in our laboratory are also of interest: LAS was not degraded, even by the modified TOC-Handai method, where authentic river water from which microorganisms had been removed was used in place of artificial river water as the culture medium, although most of the chemicals tested were degraded more quickly by this modified procedure than by the standard one and 2, 4-dichlorophenol, which as well as LAS, was found not to be degraded by the standard TOC-Handai method was degraded by the modified procedure (data not shown).

It seems difficult to generalize what are readily degradable or recalcitrant chemicals. A primitive statistical analysis of the present degradation tests conducted in the present study showed that the shorter the time required for the completion of biodegradation, the smaller was the difference in the effect of different inocula. Although the molecular targets of biodegradation overlap, the degradation modes of PEG, AE and NPE differed; e.g., the degradations of AE and PEG by inocula from the upper stream and middle reach of the Hino River were relatively uniform whereas those from the Wada/Joganji River system were uneven, and with every inoculum NPE degradation was always initiated after some delay compared to AE and PEG. It has been suggested that the bacterial assimilabilities of AE and PEG overlap but that these bacteria are unable to degrade NPE (Maki, 1994), indicating that the bacterial composition differed among inoculum as to degraders of respective chemicals. Furthermore, there was no correlation between the number of bacteria in present inocula and their biodegradation ability. Therefore, it is likely that degraders of readily degradable surfactants and their derivatives are widely distributed indigenously in significant numbers whereas the opposite is the case with the recalcitrant ones, irrespective of the water quality of the aquatic environment.

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References

Ahel M, Giger W. Anal Chem, 1985; 57:1577

Ahel M, Conrad T, Giger W. Environ Sci Technol, 1987; 21:697

Ahel M, Giger W, Koch M. Wat Res, 1994a; 28:1131

Ahel M, Giger W, Schaffner C. Wat Res, 1994b; 28:1143

Ball HA, Reinhard M, MaCarty PL. Environ Sci Technol, 1989; 23:951

Corcia AD, Samperi R, Marcomini A. Environ Sci Technol, 1994; 28:850

de Oude Von NT. Tenside Deter, 1977; 14:189

Fischer WK, Gerike P. Wat Res, 1975; 9:1137

Giger W, Stephanou E, Schaffner C. Chemosphere, 1981; 10:1253

Greff RA, Setzkorn EA, Leslie WD. J of Am Oil Chem Soc, 1965;42:180

Huddleston RL, Allred RC. J of Am Oil Chem Soc, 1965; 42:983

Itoh S, Naito S, Unemoto T. Eisei Kagaku, 1988; 34:414

Japanese Industrial Standards Committee (ed.), Japanese industrial standard-testing methods for industrial waste water-JIS K 1012, Tokyo: Japanese Standards Association. 1978

Maki H, Masuda N, Fujiwara Y, Ike M, Fujita M. Appl Environ Microbiol, 1994; 60:2265

Manusner M, Benedict JH, Booman KA, Brenner TE, Conway RA, Duthie JR, Garrison LJ, Hendrix CD, Shewmaker JE. J of Am Oil Chem Soc, 1969; 46:432

Maurer EW, Cordon TC, Weil JK, Linfield WM. J of Am Oil Chem Soc, 1974; 51:287

Motomizu S, Fujiwara S, Fujiwara A Toei K. Anal Chem, 1982; 54:392

Nasu M, Song SN, Yamaguchi N, Shimazu A, Kondo M. Fresenius Environ Bull, 1993; 2:7

OECD. OECD Guidlines for Testing of Chemicals, OECD, Paris: 1981

OECD, Modified OECD 301E Biodegradation Screening Test, OECD Guidelines for Testing of Chemicals, Paris: 1991

Palmisano AC, Schwab BS, Maruscik DA. Can J of Microbiol, 1991; 37:939

Pitter P, Fuka T. Tenside Deter, 1979; 16:298

Schöerl P, Bock KJ, Huber L. Tenside Surfact Deter, 1988; 25:86

Sigoillot JC, Nguyen MH. Appl Environ Microbiol, 1992; 58:1308

Swisher RD. Tenside Deter, 1981; 18:57

Swisher RD. Surfactant biodegradation, 2nd ed. New York: Marcel Dekker Inc, 1987

Urano K, Saito M. Chemosphere, 1985; 14:1333

Yoshikura T, Oda K, Iida S. Bull Japan Soc Sci Fish, 1981; 47:183

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