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Uptake and Degradation of Arsenobetaine by the Microorganisms Occurring in Sediments

Ken'ichi Hanaoka,* Kenji Uchida,* Shoji Tagawa* and Toshikazu Kaiset
*Department of Food Science and Technology, Shimonoseki University of Fisheries, Nagata-honmachi 2-7-1, Shimonoseki 759-65, Japan, and †School of Life Science, Tokyo University of Pharmacy and Life Science, 1432-1 Horinouchi, Hachioji City, Tokyo 192-03, Japan

We have reported the degradation of arsenobetaine [$(CH_3)_3As^+CH_2COO^-$] to inorganic arsenic by microorganisms from various marine origins such as sediments. However, there was no information as to the fate of the ingested arsenobetaine within the body of the microorganisms before excretion.

In this study, arsenobetaine and sediments were added to two culture media (1/5 Zobell 2216E and a solution of inorganic salts) and aerobically incubated at 25 °C in the dark. Despite the degradation and complete disappearance of arsenobetaine from the filtrates of the incubation mixtures, the major arsenic compound from the microorganisms harvested from the mixtures was identified by HPLC as arsenobetaine throughout the incubation period. The presence of arsenobetaine was further confirmed by TLC and fast atom bombardment mass spectrometry (FAB MS). A minor arsenical also present in the incubated microorganisms, dimethylarsinic acid, was detected.

Keywords: arsenic; arsenobetaine; microorganisms; uptake; degradation; conversion; trimethylarsine oxide; dimethylarsinic acid; inorganic arsenic

INTRODUCTION

Arsenobetaine [$(CH_3)_3As^+CH_2COO^-$] was isolated for the first time as a naturally occurring organoarsenic compound from the western rock lobster by Edmonds, Francesconi and co-workers.[1] At present, it is known as a compound occuring ubiquitously in marine animals:[2–4] it has been detected in tissues or organs from marine animals not only belonging to higher trophic levels, such as Crustacea, Chondrichthyes and Osteichthyes (for example, the western rock lobster mentioned above, blue shark[5] and school whiting,[6] respectively) but also belonging to lower ones such as Echinodermata (for example, sea cucumber[7]). Especially in marine animals belonging to the highest trophic levels, almost all the arsenic is accumulated as arsenobetaine.

In order to clarify arsenic circulation in marine ecosystems, we have recently studied its microbial degradation in culture media amended with various sources of microorganisms, i.e. sediments,[4,8–11] the surface of marine algae,[12] the intestine of a mollusc[13] and suspended substances.[14] Arsenobetaine was degraded by microorganisms from all the sources: some arsenical degradation products were detected in the filtrates of the media, high microbial activity being observed with sediments and suspended substances in which arsenobetaine was degraded to inorganic arsenic. However, there was no information on the structure of the metabolite(s) accumulated in the bodies of the microorganisms themselves nor even on whether they had taken it up in their bodies. In this work, we studied the fate of arsenobetaine degraded by microorganisms: we investigated whether it is taken into their bodies and, if this occurs, the structure(s) as which it occurs in them. Sediments were used as the origin of the microorganisms, because of the high activity of microorganisms occurring in them for degradation of arsenobetaine.

MATERIALS AND METHODS

Sediments

Sediments were collected from the coastal waters of Yoshimi, Japan in October 1992, July 1993 and January 1994 in front of the National University of Fisheries, Shimonoseki, Japan, using an Eckman–Bardge grab sampler.

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Cultivation

Two culture media used in our previous experiments on degradation of organoarsenic compounds by microorganisms occurring in sediments or from other origins were used also in this study: namely, 1/5 ZoBell 2216E (g dm^{-3} filtered seawater: peptone 1.0; yeast extract 0.2, pH 7.5) and an aqueous solution of inorganic salts at pH 7.5 [g dm^{-3}: sodium chloride (NaCl) 30.0; calcium chloride ($CaCl_2 \cdot 2H_2O$) 0.2; potassium chloride (KCl) 0.3; iron (II) chloride ($FeCl_2 \cdot nH_2O$) 0.01; phosphates, KH_2PO_4 0.5 and K_2HPO_4 1.0; magnesium sulphate ($MgSO_4 \cdot 7H_2O$) 0.5; and ammonium chloride (NH_4Cl) 1.0]. The latter has been used as a medium without a carbon source: the microorganisms have to use the added arsenobetaine as the only carbon source except for trace amounts of organic matter introduced by the addition of the sediments. Sediments were added to each medium containing arsenobetaine and incubated aerobically at 25 °C in the dark under an atmosphere of air. At intervals of several days of incubation, 0.1 cm^3 portions of the mixtures were withdrawn, mixed with 2.0 cm^3 of water and filtered for analysis by high-performance liquid chromatography (HPLC).

Extraction and purification of arsenic compounds from microorganisms

After centrifugation (4000 *g*, 15 min) of each incubated mixture, harvested microorganisms were repeatedly suspended in an aliquot of the ZoBell medium and centrifuged to wash out the arsenicals present in the medium. Water-soluble arsenic compounds accumulated in the microorganisms were extracted with chloroform–methanol (2:1) as described previously.[4]

Extracted compounds were chromatographed with a cation-exchange resin, Dowex 50W-X8 (50–100 mesh, H^+ form) column (2.2 cm × 18.5 cm), and eluted with 400 cm^3 of water, 400 cm^3 of 2.0 mol dm^{-3} pyridine and 400 cm^3 of 1.0 mol dm^{-3} HCl, successively. The fraction eluted with the pyridine solution was further chromatographed with an anion-exchange resin, Dowex 1-X8 (50–100 mesh, OH^- form) column (2.2 cm × 18.5 cm) and eluted with 400 cm^3 of water (labelled [50W/pyridine → 1/water]) and 400 cm^3 of 2.0 mol dm^{-3} acetic acid ([50W/pyridine → 1/AcOH]), successively. The fraction [50W/pyridine → 1/water] was further applied to a Dowex 50W-X8 (200–400 mesh, pyridinium form) column (1 × 50 cm) equilibrated with 0.1 mol dm^{-3} pyridine–formic acid buffer (pH 3.1) and eluted with the same buffer (200 cm^3) and 0.1 mol dm^{-3} pyridine (200 cm^3).

High-performance liquid chromatography (HPLC)

Each diluted medium or arsenic-containing fraction was analysed by HPLC (Tosoh Co. Ltd, CCP 8000 series) using an ODS 120T column (4.6 mm × 250 mm; Tosoh Co. Ltd) with a mobile phase of 11.2 mmol dm^{-3} sodium heptanesulphonate solution in water–acetonitrile–acetic acid (95:5:6, by vol.; flow rate, 0.8 cm^3 min^{-1}; sample size, 10–20 mm^3).[15] A 20 mm^3 volume of each eluate collected every 25 s was injected into a graphite furnace atomic absorption spectrometer (GF AA) and analysed for arsenic as described previously.[8] The mixture of the authentic arsenic compounds (all with 100 mg as As per kg of water) which had been detected in the previous *in vitro* degradation experiments of arsenobetaine was also fractionated [retention times, s: inorganic arsenic(V) 150–225; inorganic arsenic(III) 225–300; methanearsonic acid 225–300; dimethylarsinic acid 325–400; arsenobetaine 525–625; trimethylarsine oxide 725–850].

Confirmation of the metabolites

The purified metabolites were chromatographed on a cellulose thin-layer (TLC) (Funakoshi Yakuhin Co. Ltd; Avicel SF, 0.1 mm thickness). Dragendorff reagent, $SnCl_2$–KI reagent[16] and iodine vapour were used for the detection of spots.

Mass spectra were recorded with a JEOL JMS DX-300 double-focusing mass spectrometer equipped with fast atom bombardment (FAB) (xenon atoms at 6 keV).

RESULTS

Arsenic compounds in the filtrates of the medium and within the bodies of the microorganisms

In order to investigate the arsenic compounds occurring in the bodies of the microorganisms, 800 cm^3 of each mixture of arsenobetaine (460 mg) and the sediments (October, 1992) was incubated. Figure 1 shows the time-course pat-

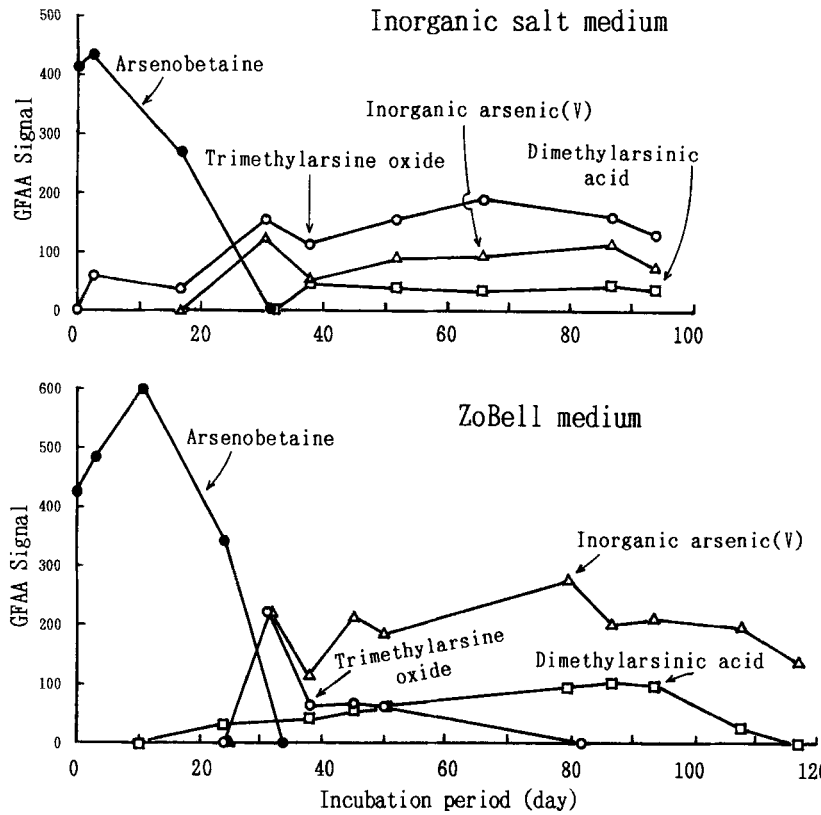

Figure 1 The degradation of arsenobetaine to three metabolites by sedimentary microorganisms during aerobic incubation at 25 °C in an inorganic salt medium (800 cm^3) and ZoBell medium (800 cm^3). The ordinate represents the observed GF AA signal of the diluted filtrates at 193.7 nm, and the abscissa represents the relative abundance of arsenic species in the eluate.

terns of arsenobetaine and its metabolites in the filtrates of the media exposed to the microorganisms introduced by addition of the sediments. Three metabolites were detected by HPLC in both media. Their retention times agreed with those of trimethylarsine oxide, dimethylarsinic acid and inorganic arsenic(V), respectively. In both media, arsenobetaine disappeared within about 30 days of incubation, trimethylarsine oxide, dimethylarsinic acid and inorganic arsenic(V) appearing with the disappearance of arsenobetaine.

On the other hand, when the arsenic compounds extracted from the harvested microorganisms from about half the medium incubated for 80 days were also analysed by HPLC, a single peak was detected in each extract and its retention time agreed with that of arsenobetaine.

Purification of arsenic compound from the microorganisms and the medium

To obtain a sufficient amount of arsenic species from the microorganisms for TLC and FAB mass spectrometry, a further experiment was carried out using 2000 cm^3 of ZoBell medium containing 1000 mg of arsenobetaine and the sediments (July, 1993). This medium was adopted here because of a higher degradation rate of arsenobetaine to inorganic arsenic(V) than in the inorganic salt medium in preliminary experiments (Fig. 1). Although the conversion rate was slightly higher with this sediment, the pattern of degradation was analogous to the former one (Fig. 2).

After 50 days of incubation where the only arsenic compound present in the filtrate was inorganic arsenic(V), microorganisms were harvested

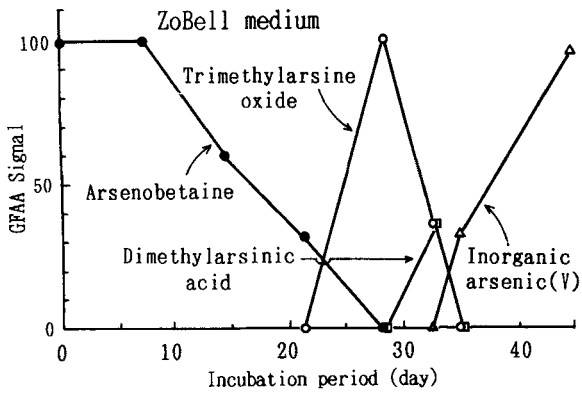

Figure 2 The degradation of arsenobetaine to three metabolites by sedimentary microorganisms during aerobic incubation at 25 °C in 2000 cm^3 of ZoBell medium. The ordinate represents the observed GF AA signal of the diluted filtrates at 193.7 nm.

from the medium by centrifugation to extract arsenic compounds from them. These extracted arsenic compounds were analysed by HPLC, showing a single peak whose retention time agreed with that of arsenobetaine.

In order to purify the extracted arsenic species, it was fractionated with Dowex 50W-X8 and Dowex 1-X8. Arsenic was detected in two fractions, [50W/pyridine → 1/water] and [50W/pyridine → 1/AcOH]. The relative distribution rates of arsenic were 87% in the former and 13% in the latter. A single arsenic peak was detected in each fraction with HPLC, showing a retention time agreeing with that of arsenobetaine ([50W/pyridine → 1/water]) or dimethylarsinic acid [50W/pyridine → 1/AcOH]. The arsenic compound in [50W/pyridine → 1/water] was further purified with a Dowex 50W-X8 (pyridinium form) column. Arsenic was detected in the fraction eluted with the buffer [50W/pyridine → 1/water → 50W/buffer].

On the other hand, when the supernatant of the centrifuged medium was subjected to the same chromatography, arsenic was detected in [50W/water → 1/water] as a single peak using HPLC, showing the same retention time as inorganic arsenic(V). This arsenic was eluted with buffer also in the chromatography with Dowex 50W-X8 (pyridinium form) colum [50W/water → 1/water → 50W/buffer].

TLC and FAB mass spectrometry of the purified metabolites

After lyophilization, the two fractions, [50W/pyridine → 1/water → 50W/buffer] from the microorganisms and [50W/water → 1/water → 50W/buffer] from the supernatant of the medium, were subjected to TLC. As shown in Table 1, a single spot was detected in each fraction and R_f value agreed with that of arsenobetaine (microorganism fraction) and inorganic arsenic(V) (supernatant fraction), in five solvent systems. The other arsenicals in the Table, which had the possibility of being derived from arsenobetaine, were not detected.

Figure 3 shows the FAB mass spectra of the arsenic compound from the microorganisms, and of synthetic arsenobetaine. The molecular ion peak of arsenobetaine, m/z 179 and some other ion peaks were shown in the spectrum of arsenobetaine. These were at m/z 120 $[(CH_3)_3As^+]$, 135 $[(CH_3)_4As^+]$, 201 $[(M+Na)]^+$, 357 $[(2M+1)^+]$ and 379 $[(2M+Na)^+]$. These ion peaks and a further peak at m/z 217 $[(M+K)^+]$ were shown in the spectrum of the pure arsenic compound.

We concluded that the major arsenic compound occurring in the microorganisms was arsenobetaine from results of HPLC, TLC and FAB mass spectrometry, the compound in the medium being inorganic arsenic(V) from HPLC and TLC.

Accumulation of arsenobetaine in microorganisms throughout the incubation period

As the final experiment in the study, the arsenic species in the microorganisms were successively investigated to confirm that arsenobetaine was consistently the major arsenical in the microorganisms from the early stages to the final stage of conversion of arsenobetaine in the medium.

Sixteen test tubes containing ZoBell medium (5 cm^3 each) containing arsenobetaine (8 mg) and the sediments (January 1994) were incubated at the same time, incubation in two tubes being stopped at intervals of several days. In each of these tubes, both arsenic compounds occurring in the microorganisms harvested by centrifugation and occurring in the filtrate of the medium were analysed by HPLC (Fig. 4).

Whilst after 21 days of incubation arsenobetaine disappeared from the filtrate, arsenobetaine was the only arsenical detected in the microorganisms by HPLC throughout the incubation period.

Table 1 R_f values from TLC of the arsenic compounds isolated from the medium and the microorganisms

Sample	R_f				
	Solvent system[a]				
	1	2	3	4	5
Isolated arsenic compound					
From medium	0.52	0.00	0.23	0.00	0.21
From microorganisms	0.79	0.75	0.60	0.37	0.53
Inorganic arsenic(V)	0.53	0.00	0.24	0.00	0.21
Arsenobetaine	0.78	0.76	0.59	0.37	0.54
Inorganic arsenic(III)	0.22	0.30	0.40	0.07	0.35
Methanearsonic acid	0.55	0.22	0.50	0.02	0.50
Dimethylarsinic acid	0.80	0.78	0.61	0.23	0.71
Arsenocholine	0.53	0.87	0.54	0.56	0.62

[a] Solvent systems: 1, ethyl acetate–acetic acid–water (3:2:1); 2, chloroform–methanol–25% aq. ammonia (3:2:1); 3, 1-butanol–acetone–formic acid–water (10:10:2:5); 4, 1-butanol–acetone–25% aq. ammonia–water (10:10:2:5); 5, 1-butanol–acetic acid–water (4:2:1).

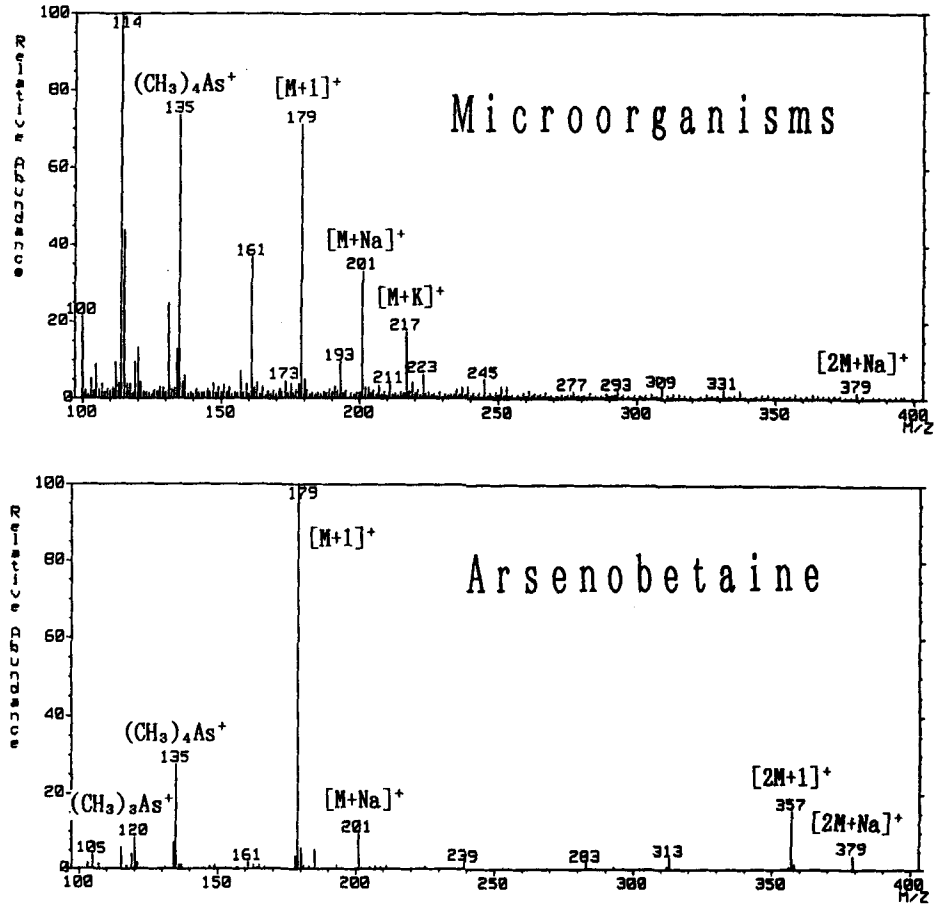

Figure 3 FAB mass spectra of the major arsenic compound accumulated in the microorganisms, and synthetic arsenobetaine.

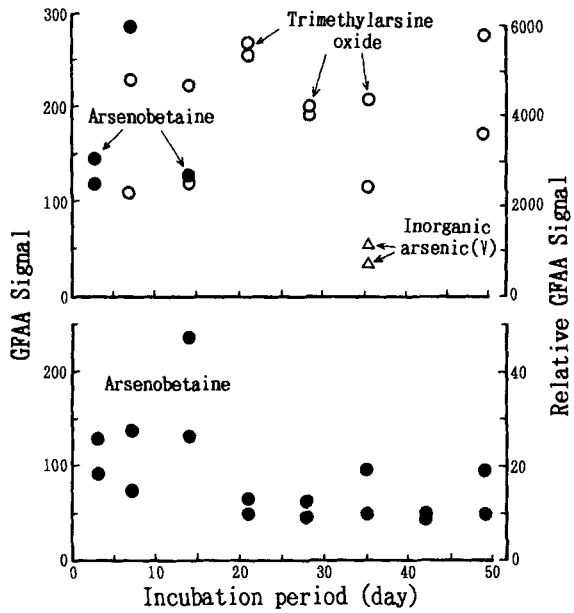

Figure 4 Arsenicals detected by HPLC in the filtrates of the media (upper) and in the extracts from the microorganisms harvested from the media (lower). The filtrates were diluted following the procedure in the text; the extracts from the microorganisms were dried and dissolved in 2 cm^3 of water. Each solution (a 50 mm^3 portion) was fractionated by HPLC. The left ordinate represents the observed GF AA signal of the solutions at 193.7 nm. The right ordinate represents the calculated GF AA signal on the basis of intact media.

DISCUSSION

In this study, it was clarified that the major arsenic compound occurring in the microorganisms was arsenobetaine, not only in the early stages of the degradation but also after its completion when inorganic arsenic (V) is the only arsenical in the filtrate. This may mean that when marine microorganisms degrade arsenobetaine, they take arsenobetaine in, cleave some useful groups such as the carboxymethyl moiety (which is a possible starting material for the synthesis of fatty acids) and discard the dangerous arsenic-containing residue; the result is that the major arsenic compound remaining there is arsenobetaine. There was, however, the possibility that the degradation had occurred extracellularly; other experiments are being conducted to clarify this. Besides arsenobetaine, dimethylarsinic acid was also detected in the microorganisms as a minor arsenical accumulated in there. This, however, does not mean that dimethylarsinic acid always accumulates with arsenobetaine during the degradation, because inorganic arsenic(V) has been detected in the extract from the microorganisms in place of dimethylarsinic acid in a similar preliminary experiment (unpublished). In the preliminary experiment, it was unfortunately not clear whether this inorganic arsenic(V) had really accumulated in the bodies of the microorganisms or merely adhered to their surface, because inorganic arsenic(V) was also present in the filtrate of the medium from which the microorganisms were harvested. On the other hand, we can conclude in this study that the dimethylarsinic acid accumulated with arsenobetaine in the microorganisms because no dimethylarsinic acid was detected in the filtrate of the medium when they were harvested from it after 49 days of incubation.

In every stage of conversion from arsenobetaine to inorganic arsenic(V), arsenobetaine only was detected by HPLC in the microorganisms harvested from the medium (Fig. 4): there was no doubt that arsenobetaine was the major arsenical in them during the incubation. This, however, may not indicate that arsenobetaine was the sole arsenical present. Taking account of the fact that 13% of the arsenic in the microorganisms was accumulated as dimethylarsinic acid, there could be other arsenic compounds in them, below the detection limit of the GF AA used in this study. Although important information on the conversion would result from investigation of these minor compound, their behaviour is not known, and is now under study.

CONCLUSIONS

The major arsenic compound accumulated in the body of microorganisms harvested from arsenobetaine-containing media is arsenobetaine, even after the arsenobetaine in the filtrate has been completely degraded to inorganic arsenic.

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REFERENCES

1. J. S. Edmonds, K. A. Francesconi, J. R. Cannon, C. L. Raston, B. W. Skelton and A. H. White, *Tetrahedron Lett.* **18**, 1543 (1977).

2. J. S. Edmonds and K. A. Francesconi, *Appl. Organomet. Chem.* **2**, 297 (1988).
3. W. Maher and E. Butler, *Appl. Organomet. Chem.* **2**, 191 (1988).
4. K. Hanaoka, H. Yamamoto, K. Kawashima, S. Tagawa and T. Kaise, *Appl. Organomet. Chem.* **2**, 371 (1988).
5. S. Kurosawa, K. Yasuda, M. Taguchi, S. Yamazaki, S. Toda, M. Morita, T. Uehiro and K. Fuwa, *Agric. Biol. Chem.* **44**, 1933 (1980).
6. J. S. Edmonds and K. A. Francesconi, *Mar. Pollut Bull.* **12**, 92 (1981).
7. K. Shiomi, M. Orii, H. Yamanaka and T. Kikuchi, *Nippon Suisan Gakkaishi* **53**, 103 (1987).
8. K. Hanaoka, T. Matsumoto, S. Tagawa and T. Kaise, *Chemosphere* **16**, 2545 (1987).
9. T. Kaise, K. Hanaoka S. Tagawa, *Chemosphere* **16**, 2551 (1987).
10. K. Hanaoka, S. Hasegawa, N. Kawabe, S. Tagawa and T. Kaise, *Appl. Organomet. Chem.* **4**, 239 (1990).
11. K. Hanaoka, S. Tagawa and T. Kaise, *Hydrobiologia* **256/257**, 623 (1992).
12. K. Hanaoka, K. Ueno, S. Tagawa and T. Kaise, *Comp. Biochem. Physiol.* **94B**, 379 (1989).
13. K. Hanaoka, T. Motoya, S. Tagawa and T. Kaise, *Appl. Organomet. Chem.* **5**, 427 (1991).
14. K. Hanaoka, H. Koga, S. Tagawa and T. Kaise, *Comp. Biochem. Physiol.* **101B**, 595 (1992).
15. R. A. Stockton and K. J. Irgolic, *J. Environ. Anal. Chem.* **6**, 313 (1979).
16. S. Tagawa, *Nippon Suisan Gakkaishi* **46**, 1257 (1980).