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Arsenic Compounds in Zoo- and Phyto-plankton of Marine Origin

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Major water-soluble arsenic compounds accumulated in some zoo- and phyto-plankton were identified. Zooplankton were collected at sampling stations in the Sea of Japan by a Norpac net towed from 600 m depth to the surface. Phytoplankton were cultivated under axenic conditions. Water-soluble arsenic compounds were extracted repeatedly from plankton tissues by aqueous methanol. The arsenic compounds in the extracts were analyzed by HPLC–ICP/MS.

Among zooplankton analyzed in the present study, two carnivorous species, i.e. Amphipoda (*Themisto* sp.) and Sagittoidea (*Sagitta* sp.), contained arsenobetaine as the dominant arsenic species. Arsenobetaine was the major species in Euphausiacea (*Euphausia* sp.), also. The most abundant arsenic compound in the herbivorous Copepoda species (*Calanus* sp.), on the other hand, was an arsenic-containing ribofuranoside with a sulfate ester group, and arsenobetaine was only a minor component. Phytoplankton contained arsenic-containing ribofuranosides apparently in a species-speific manner. The arsenic compounds in zooplankton seem to reflect their feeding habit; i.e. carnivorous species eating zooplankton or other small animals accumulate arsenobetaine, while herbivorous ones eating phytoplankton accumulate arsenic-containing ribofuranosides as major arsenic compounds.

Keywords: arsenic species; marine plankton; arsenobetaine; arsenic-containing ribofuranosides; arsenosugars; HPLC–ICP/MS

INTRODUCTION

Marine organisms, in general, are known to contain amounts of arsenic ranging typically from around 1 to more than 10 mg As kg^{-1} (wet basis), and much attention has been paid to the chemical form of accumulated arsenic for clarification of both toxicological implications and the environmental cycling of the element. After the purification and rigorous identification of arsenobetaine (**VIII** in Fig. 1)[1] and arsenic-containing ribofuranosides (arsenosugars: **X** and **XII**)[2] by Edmonds and co-workers, many reports have been published on the identification of arsenobetaine, and some of the arsenosugars; there seems to emerge a general consensus that marine animals, including fish, crustaceans and mollusks, contain arsenobetaine as a major, ubiquitous, water-soluble arsenic, while marine macroalgae (seaweeds) contain a series of arsenosugar derivatives (**X–XV**) in a species-specific manner.[3–6] Furthermore, many of the bivalve mollusks have been shown to contain some arsenosugars in addition to arsenobetaine.[7,8] Other organoarsenic compounds, including arsenocholine (**VII**), tetramethylarsonium ion (**VI**) and trimethylarsine oxide (**V**), are reported in some species while not in others,[3–6] and there remain several other arsenic compounds yet to be identified.

The origin of these organoarsenic compounds, especially arsenobetaine, is still a matter of debate. Edmonds *et al.* showed that arsenosugars in a brown alga, *Eckronia radiata*, were easily and efficiently decomposed to dimethylarsinoylethanol (**IX**) under anaerobic conditions.[9] They postulated that the arsenosugars produced in macroalgae were the origin of arsenobetaine in fish and crustaceans via dimethylarsinoylethanol which, either directly or indirectly converted further to other compound(s), was absorbed into the animals and converted finally to arsenobetaine.[10] Dimethylarsinoylethanol, however, was later shown to be not absorbed efficiently by a fish,[11] and there is no evidence to support further conversion of dimethylarsinoylethanol to arsenocholine or other arsenic compounds in the natural environment. Shibata and Morita, on the other

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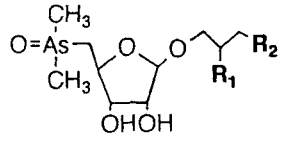
(I) Arsenate AsO_4^{3-} (II) Arsenite AsO_3^{3-} (III) Methane arsonate $CH_3AsO_3^{2-}$

(IV) Dimethyl arsinate $(CH_3)_2AsO_2^-$ (V) Trimethylarsine oxide $(CH_3)_3AsO$

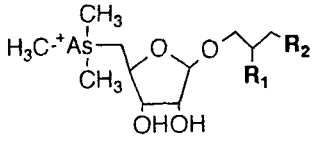
(VI) Tetramethylarsonium ion $(CH_3)_4As^+$ (VII) Arsenocholine $(CH_3)_3As^+CH_2CH_2OH$

(VIII) Arsenobetaine $(CH_3)_3As^+CH_2COO^-$ (IX) Dimethylarsinoylethanol $(CH_3)_2As(O)CH_2CH_2OH$

[Arsenosugars]



	R_1	R_2
(X)	OH	OH
(XI)	OH	$OPOCH_2CH(OH)CH_2OH$ (O, O⁻)
(XII)	OH	SO_3^-
(XIII)	NH_3^+	SO_3^-
(XIV)	OH	SO_4^-



(XV) OH SO_4^-

Figure 1 Arsenic standards used in the present study.

hand, reported the presence of a trimethylated arsenosugar derivative in an alga;[12] later this was shown to be decomposed directly to arsenocholine,[13] which was absorbed efficiently and accumulated as arsenobetaine in fish.[11] This trimethylated arsenosugar, however, has so far been found only of a very low level in a limited number of species.[12,14] and the significance of this compound is still not clear. Both fish[11] and bivalves[15] were shown to absorb and accumulate arsenobetaine efficiently from food and ambient seawater, respectively. These data strongly support the idea that arsenobetaine, a major constituent in marine animals, is absorbed and accumulated in these organisms via the food chain (or from seawater) intead of being synthesized *de novo* within their bodies.[11,15]

Zoo- and phyto-plankton are in lower trophic levels in the marine food web, and information about their arsenic compounds is important to reveal the cycling of arsenic in the marine environment, especially in the marine food web. So far, information on arsenic compounds in marine plankton is limited and contradictory.[6,16] Here, we report data on the speciation of arsenic in some selected zoo- and phyto-plankton by an HPLC–ICP/MS system developed in our laboratory.

MATERIALS AND METHODS

Zooplankton were collected at several stations in the Sea of Japan during the 1994 cruise in the Japan–Russia joint study of the marine environment of the Sea of Japan conducted by the Environmental Agency, Japan, and the Pacific Oceanological Institute, Russia, using the research vessel *Akademik M. A. Lavrentyev*. The sampling stations are summarized in Table 1. A Norpac net (mesh size 0.35 mm) was towed vertically from 600 m depth to the surface at each point. The sampling was conducted twice at each point, and the zooplankton collected in each sampling were used for counting the number of each plankton species, and for pollutant analysis (including arsenic speciation), respectively. Collected samples were separated by hand into several major species. The zooplankton samples

Table 1 Sampling stations for zooplankton in the Sea of Japan

Station	Location	Sampling date
5	N 40° 29′ E 134° 52′	27–28 Sept. 1994, midnight
6	N 39° 42′ E 135° 43′	27 Sept. 1994, morning
7	N 38° 58′ E 136° 34′	26 Sept. 1994, noon

were kept frozen at −20 °C until analysis. The samples were weighed, freeze-dried (weighed again; from 0.038 to 0.24 g), and were extracted three times with 5 ml aqueous methanol (1 : 1, v/v). The extracts were combined, evaporated to dryness and dissolved in 1 or 2 ml of water. The solution was filtered through a 0.5 μm disposable filter unit (Millipore Columngard-LCR13), and an aliquot of the solution was injected into the HPLC–ICP/MS system.

Culture strains of marine phytoplankton, a bacillariophyceae (diatom) *Skeletonema costatum*, and a raphidophyceae *Heterosigma akashiwo*, were obtained from the Microbial Culture Collection at the National Institute for Environmental Studies, Japan, and were cultivated in our laboratory under axenic conditions (10 000 lux, 24 h, 20 °C). The culture media was composed of natural, clean seawater obtained near Hachijo island, south of Tokyo, supplemented with several nutrients based on the f/2 formula.[17] After two weeks of cultivation in 500 ml medi (supposed to be near the plateau of growth), the phytoplankton were collected by gentle centrifugation (700–1000 rpm, 10–15 min), resuspended in clean seawater and centrifuged again, put on a dry paper towel for half a minute to remove excess water, and the pellets were weighed. Then water-soluble arsenic compounds in each pellet were extracted three times with aqueous methanol (1 : 1, v/v) solution by ultrasonication for 10 min, and the extracts were combined, evaporated, and dissolved in water. After filtration, with a 0.5 μm disposable filter unit, each solution was injected into the HPLC–ICP/MS system.

HPLC–ICP/MS analysis was conducted as reported previously[18] except that an Inertsil ODS column (4.6 mm × 250 mm; GL Science, Japan) was used instead of an Inertsil ODS-2 in the two ion-pair chromatographies.[19] Fourteen arsenic standards so far purified or synthesized in our laboratory and dimethylarsinoylethanol (**IX**) from Dr J. S. Edmonds, Western Australian Marine Research Laboratories, were used as authentic standards for identification of each arsenic species (Fig. 1).[18] Quantitation was performed by comparing the peak area of each compound with that of a known concentration of dimethylarsinate standard. Interference from Cl [$^{40}Ar^{35}Cl^+$ shows the same m/z (=75) as $^{75}As^+$] was detected by monitoring ion counts at m/z=77 (corresponding to $^{40}Ar^{37}Cl^+$) simultaneously.

RESULTS AND DISCUSSION

Chromatograms of the HPLC–ICP/MS analysis of several zooplankton samples are shown in Fig. 2. Non-fractionated mixtures of zooplanktons [large ctenophora (*Beroe* sp.) were removed] collected at Sta. 5 [Fig. 2(a)] clearly show the presence of one dominant arsenic compound and several other minor compounds in the extract. The major one was identified as arsenobetaine,

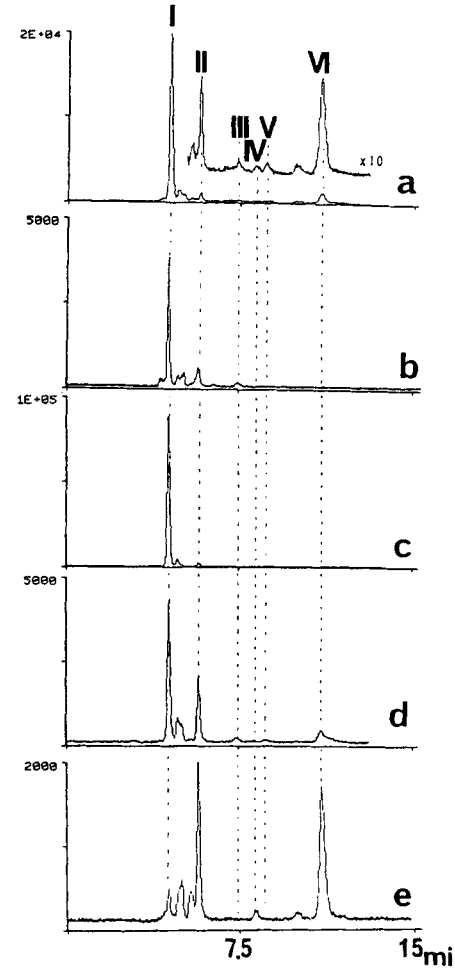


Figure 2 HPLC–ICP/MS chromatograms (m/z=75) of the zooplankton extracts. Column, Inertsil ODS; buffer, 10 mM tetraethylammonium hydroxide–4.5 mM malonic acid–0.05% methanol (pH 6.8); flow rate, 0.75 ml/min. (a) Non-fractionated mixture; (b) Sagittoidea; (c) Amphipoda; (d) Euphausiacea; (e) Copepoda. The peaks are labelled as follows: I, Arsenobetaine (**VIII**); II, unknown compound (r.t. 5.2 min); III, arsenosugar (**X**); IV, arsenosugar (**XII**); V, arsenosugar (**XI**); VI, arsenosugar (**XIV**).

and the second largest one as the arsenosugar containing sulfate ester at the end of a glycerol group (**XIV**). The third one, eluted at 5.2 min, did not correspond to any of the authentic standards currently in our hands. No obvious diference was observed between the chromatograms of the extracts of mixture samples collected in the three sampling stations (Sta. 5–7), suggesting that the pattern reflects general arsenic constituents in bulk zooplankton samples in the Sea of Japan at this season, i.e. late September.

The major zooplankton in the mixture were:

(1) *Sagitta* sp. in the phylum Chaetognatha, class Sigittoidea;
(2) Copepoda (Calanoida), Amphipoda and Euphausiacea, all in the phylum Arthropoda, class Crustacea;
(3) Siphonophora in the phylum Coelenterata, class Hydrozoa.

The number of each species in the net was counted separately by the Environmental Agency, Japan,[20] but no attempt was made to quantitate the amount of each species in the mixture on a weight basis. The result indicates that the most dominant species in Sagittoidea, Amphipoda, Euphausiacea and Copepoda were *Sagita elegans*, *Themisto* sp., *Euphausia pacifica* (Sta. 5) or *Thysanoessa longipes* (Sta. 6, 7), and *Calanus plumchrus* (Sta. 5, 6) or *Pseudocalanus minutus* (Sta. 7), respectively.[20]

Relatively large specimens (more than 5 mm in length) of Sagittoidea, Amphipoda, Euphausiacea and Copepoda were picked up from the bulk zooplankton with tweezers, and were put into separate vessels. Tentative assignments of the species are listed in Table 2. As the procedure was conducted on board ship without the help of a taxonomist, each fraction might contain a few related species in addition to the species assigned in the Table. The HPLC-ICP/MS chromatograms of the extracts from these samples are shown in Figs 2(b)–2(e), and the results of arsenic speciation are summarized in Table 3. We did not attempt to analyze the total arsenic content of each sample because of the limited availability. As shown in the Figure, Sagittoidea, Amphipoda and Euphausiacea species contain arsenobetaine as the dominant, water-soluble, arsenic compound. The arsenic content of the Amphipoda was especially high, and more than 90% of water-soluble arsenic in the organism was in the form of arsenobetaine. In all three samples, however, small amounts of some arsenosugar derivatives were detected (Table 3), and the relative amount of arsenosugars versus arsenobetaine was largest in Euphausiacea. A Copepoda, *Calanus* sp., on the other hand, contained an arsenosugar with a sulfate ester (**XIV**) as the most abundant species, and the arsenobetaine content was less than 10%. The second largest peak showed the same retention time (5.2 min) with an unknown compound in the mixture. The former three zooplankton also contained this as a minor arsenic compound.

As in the case of marine macroalgae, on the other hand, marine phytoplankton did not contain arsenobetaine at detectable levels. Their HPLC–

Table 2 Zooplankton species selected for arsenic speciation (Station 5)

Class/Order	Phylum	Tentative assignment
Sagittoidea	Chaetognatha	*Sagitta elegans*
Amphipoda	Arthropoda	*Themisto* sp.
Euphausiacea	Arthropoda	*Euphausia pacifica* + *Thysanoessa longipes*
Copepoda	Arthropoda	*Calanus* sp.

Table 3 Concentrations of arsenic species in the extracts of zooplankton (mg As kg^{-1}, dry wt)

		Arsenosugar				
Class/Order[a]	AB	**X**	**XI**	**XII**	**XIV**	Unknown at 5.2 min
Sagittoidea	2.66	0.16	—	—	—	0.65
Amphipoda	17.0	0.08	0.04	—	0.08	0.51
Euphausiacea	1.04	0.04	0.02	—	0.28	0.51
Copepoda	0.21	—	—	0.10	1.90	1.37
Mixture (Station 5)	3.91	0.03	0.04	0.03	0.46	0.18

[a] See Table 2 for more information.
[b] —, Not detected.

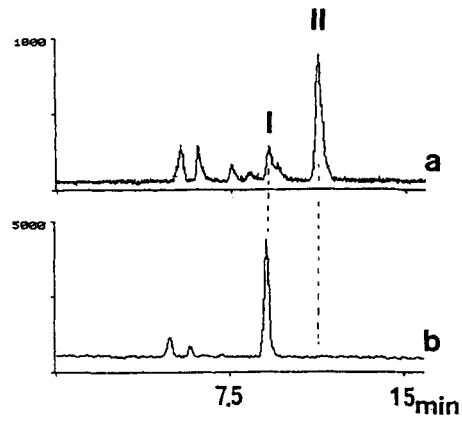


Figure 3 HPLC–ICP/MS chromatograms (m/z=75) of the phytoplankton extracts. The conditions were the same as in Fig. 2. (a) *Skeletonema costatum*; (b) *Heterosigma akashiwo*. Peak labels. I, arsenosugar (**XI**); II, arsenosugar (**XIV**).

ICP/MS chromatograms are shown in Fig. 3. The two phytoplankton species, i.e. *Heterosigma akashiwo* and *Skeletonema costatum*, contained arsenosugars apparently in a species-specific manner; i.e. *Heterosigma* contained (**XI**), while *Skeletonema* contained **XIV** in addition to **X** and **XI**.

We have experienced occasional decomposition of **XI** to **X** during the process of storage/extraction of water-soluble arsenic in marine macroalgae.[3] A similar experience was reported by Edmonds and co-workers.[21] Therefore, it seems appropriate, at this stage, to consider the amount of **X** in the extract to be a sum of **X** and a part of **XI**, rather than the amount of **X** only, within the organisms. The arsenosugar **XIV**, on the other hand, is stable, and its presence/absence will not reflect an artifact during sample preparation/storage.

The major arsenic compound in a Copepoda, *Calanus* sp., was the arsenosugar with a sulfate group (**XIV**), i.e. the major arsenic compound in the diatom; the concentration of arsenobetaine was low. On the other hand, the other three zooplankton species contained arsenobetaine as the major arsenic compound. These differences may reflect the differences in their feeding habits, i.e *Calanus* sp. is principally herbivorous, while the other three show more or less carnivorous characters. Among the three zooplanktons having arsenobetaine as the major compound. *Euphausia* sp. is reported to be dependent on both zooplankton and phytoplankton, i.e. it is omnivorous.[22] The ratio between arsenosugars and arsenobetaine is higher in Euphausiacea than in Amphipoda or Sagittoidea, again suggesting that their arsenic species reflect their feeding habits. On the other hand, the concentration of arsenobetaine in Amphipoda (*Themisto* sp.) was much higher than in the other zooplankton. The meaning of this specific character is not clear at this stage. The analysis of the unknown species in all of the zooplankton samples analyzed in the present study remains to be studied in the future. From the standpoint of metabolism and cycling of arsenic in the marine food web, it is especialy interesting that a fairly large amount of the unknown compound was detected in *Calanus* sp, which feeds on phytoplankton containing arsenosugar derivatives.

The arsenosugar **XIV** was first reported from the extract of kidney of a giant clam, *Tridacna maxima*,[7] and was attributed to a symbiotic alga, zooxanthellae, within the tissue of the clam. Later, it was found in brown algae in the family Sargassaseae, i.e. *Hizikia fusiforme*,[23] *Sargassum thunbergii*[12] and other *Sargassum* species,[24] as the most abundant organic arsenic compound. It was also found in some of the red algae[24] without showing any apparent relationship with phylogenetic speciation (the compound **XIV** in the present study was designated as **XIII** in Ref. 24). In the present study, it was found in a diatom, *S. costatum*, and a Copepoda, *Calanus* sp., as the major water-soluble arsenic compound. Cooney *et al.* analyzed arsenic compounds in a marine diatom, *Chaetoceros concavicornis*, by feeding radioactive arsenate to the medium.[25] Later their first assignment[25] was retracted, and possible structures were speculated, based on their chemical properties.[26] Three lipid-soluble arsenic (**I, II** and **III**) and four water-soluble ones (**A**, **B**, **C** and **D**) were recognized by TLC.[25] Lipid **II** was apparently a lyso-form of lipid **I**, and alkaline hydrolysis of lipid **I** produced a spot indistinguishable from **B**. Treatment of **B** with glycerophosphorylcholine diesterase, or **I** with phospholipase D, produced **C**. These data could be explained consistently[6, 10, 26] by suggesting that compound **B** and compound **C** are in fact **XI** and **X**, respectively, and that lipid **I** is a diacylated form of **XI**, which was rigorously identified as the lipid-soluble arsenic in a brown alga, *Undaria pinnatifida*.[27] The remaining two water-soluble compounds (**A** and **D**) were also assigned tentatively, based on other information;[26] i.e. the major one, **A**, was assigned to one of the

arsenosugars isolated from a brown alga, *Ecklonia radiata*, i.e. **XII**, while a minor one, **D**, was assigned to (**XV**). Erroneously or intentionally, however, the compound isolated from *E. radiata*, or compound **A**, was indicated as the sulfate form, **XIV**,[26] instead of the real structure having a sulfonate group (**XII**).[2] Our data clearly indicate that another diatom, *S. costatum*, contains **XIV** as the dominant arsenic compound, while **XII** was not detected in the plankton. Based on the present result, it seems that the major compound, **A**, in *Chaetoceros* species is also **XIV**. In fact, in our experience, **XII** was found only in brown algae, Pheophyta, among various seaweeds, and it was present as a ubiquitous and major arsenic compound in them.[24] The detection of **XII** in *Calanus* and zooplankton mixtures at low levels in the present study, however, suggests that some phytoplankton or marine microbes may contain this compound, too.

In repeated experiments to grow and analyze *S. costatum*, a small peak having an identical retention time with the authentic **XV** was also detected occasionally. Cooney *et al.* reported that the radioactivity of **D** (or **XV**) was 1/20 of that of **A** (**XIV**).[25] Our data also support the view that **XV**, even if present in the diatom, is only a minor constituent, and that **XV** in the alga may not be the major source of arsenobetaine in zooplankton or other marine animals.

Cullen *et al.* recently reported the analysis of arsenic species in a chlorophyta, *Polyphysa peniculus*, using hydride generation (HG)–gas chromatography (GC)–atomic absorption spectrometry (AA) and flow injection–microwave digestion–HG–AA.[16] They did not detect significant amounts of complex arsenic compounds, including arsenosugars, in the alga. Our findings, on the contrary, show that a diatom, *S. costatum*, and a Raphidophyceae, *H. akashiwo*, contain some of the arsenosugars as major water-soluble arsenic compounds. Furthermore, it has been shown that the result of a radioisotope study in another diatom, *Chaetoceros* sp., is consistent with the assumption that the major arsenic compounds of both diatom species are identical. The arsenic compounds in marine macroalgae, however, were found to vary considerably from one species to another,[24] and a similar variation may be present in phytoplankton species, too. Clearly more information is necessary to obtain a general view of the arsenic compounds in marine phytoplankton.

In conclusion, it has been shown in the present study that some zooplankton and phytoplankton contain arsenosugar derivatives as major water-soluble arsenic compounds. The arsenic species in zooplankton seem to reflect their feeding habit; i.e. herbivorous species, feeding mainly on phytoplankton contain a fair amount of arsenosugars, while carnivorous species contain arsenobetaine as the major arsenic. The present study clearly indicates that both arsenobetaine and arsenosugar derivatives should be considered as the major source of arsenic for marine organisms at higher trophic levels.

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