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Biomethylation of Arsenic in an Arsenic-rich Freshwater Environment

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Arsenic circulation in an arsenic-rich freshwater ecosystem was elucidated to detect arsenic species in the river water and in biological samples living in the freshwater environment. Water-soluble arsenic compounds in biological samples were extracted with 70% methanol. Samples containing arsenic compounds in the extracts were treated with 2 mol dm^{-3} of sodium hydroxide and reduced with sodium borohydride. The detection of arsenic species was accomplished using a hydride generation/cold trap/cryofocus/gas chromatography–mass spectrometry (HG/CT/CF/GC–MS) system. The major arsenic species in the river water, freshwater algae and fish are inorganic arsenic, dimethylarsenic and trimethylarsenic compounds, respectively. Trimethylarsenic compounds are also detected in aquatic macro-invertebrates. The freshwater unicellular alga *Chlorella vulgaris*, in a growth medium containing arsenate, accumulated arsenic and converted it to a dimethylarsenic compound. The water flea *Daphnia magna*, which was fed on arsenic-containing algae, converted it to a trimethylarsenic species. © 1997 by John Wiley & Sons, Ltd.

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INTRODUCTION

There are many volcanoes in Japan and hot springs are abundant in the volcanic areas. The volcanic hot springs, which flow into rivers, contain high amounts of arsenic. Therefore, the river water contains a higher level of arsenic than freshwater or groundwater. Many kinds of organisms live in the freshwater environments. From the point of view of environmental assessment, it is important that the influence of arsenic on freshwater biota and the circulation of arsenic in arsenic-rich environments is elucidated.

In marine ecosystems, it is thought that inorganic arsenic in the seawater is taken into marine algae or phytoplankton, and then converted to dimethylarsenic compounds in their tissues. Dimethylarsenic compounds are consumed by zooplankton, fish or other marine animals via the food chain. Dimethylarsenic compounds in the marine algae or phytoplankton are then transformed to trimethylarsenic compounds in the marine animals. These phenomena are explained as a circulation of arsenic in the marine environment and by biomagnification of arsenic in marine ecosystems.[1–18] The major dimethylarsenic compounds in marine algae are generally arsenosugars and the major trimethylarsenic compound in marine animals is arsenobetaine. The organoarsenic compounds present in marine organisms (arsenobetaine and arsenosugar) have very low toxicity for mammalia.[19–22]

On the other hand, experimental models of the biomethylation of arsenic in the aquatic environment have been developed by some groups. Cullen *et al*. reported the bioaccumulation and biomethylation of inorganic arsenic by a marine unicellular alga in artificial seawater.[23, 24] Arse-

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nate was converted to arsenite and arsenite was then converted to dimethylarsinate. Maeda *et al*. demonstrated experimentally the biomethylation and biotransformation of arsenic in a freshwater food chain.[25–30] We investigated the chemical species of arsenic present in arsenic-rich river water which was polluted from volcanic springs, and the freshwater biota in the Haya-kawa river at hot springs in Hakone, Kanagawa, Japan, using a hydride-generation/cold trap/cryofucus/gas-chromatographic mass spectrometry technique (HG/CT/CF/GC–MS). Also, to elucidate arsenic circulation in aquatic experimental organisms, a freshwater alga, *Chlorella vulgaris*, was grown with a culture medium containing inorganic arsenic, and the arsenic-containing alga was fed to the water flea *Daphnia magna*. The chemical species in their tissues were analyzed using the HG/CT/CF/GC–MS system. *C. vulgaris* converted arsenate to dimethylarsenic species and the water flea transformed a portion of the dimethylarsenic to a trimethylarsenic compound.

MATERIAL AND METHODS

Chemicals

Trimethylarsine oxide, $(CH_3)_3AsO$, was prepared according to our previous paper.[31] Methylarsonic acid, $CH_3AsO(OH)_2$, and dimethylarsinic acid, $(CH_3)_2AsO(OH)$, were purchased from Trichemical Co., Yamanashi, Japan. Arsenic standards of methyl, dimethyl and trimethyl species were prepared by serial dilutions from stock solutions (100 μg As cm^{-3}). Inorganic arsenic standard solution (1000 μg As(v) cm^{-3}), nitric acid (61% w/w), sulfuric acid (97% w/w) and perchloric acid (60% w/w) were obtained as analytical grade from Wako Pure Chemical Co., Tokyo, Japan. Sodium arsenate, $Na_2HAsO_4 \cdot 7H_2O$ and other reagents were of reagent grade.

Biological samples and water sample

Wild specimens of freshwater fish, sweet fish (*Plecoglossus altivelis*), the masu salmon (*Oncorhynchus masou masou*), the Japanese dace (*Tribolodon hakonensis*), other freshwater fish (*Rhinogobius* sp., *Sicyopterus japonicus*, *Phoxinus steindachneri*), freshwater macroinvertebrates, freshwater prawn (*Macrobranchiura nipponense*), a freshwater snail (*Semisulcospira libertina*), the larvae of a dobsonfly (*Plotohermes grandis*), the larvae and pupae of a caddisfly (*Stenopsyche marmorata*), green algae (*Clodophora glomerata*), diatoms on the stones and river water were collected from the Haya-kawa river at hot springs in Hakone, Kanagawa Prefecture, Japan. The species to be used for the biotransformation of arsenic in an experimental aquatic environment were a freshwater unicellular green alga (*Chlorella vulgaris*) and water flea (*Daphnia magna*), which were provided by Dr T. Hanasato at the National Institute for Environmental Studies of Japan.

Arsenic analysis

Total arsenic

The 1–2 g of biological samples (river water 10–20 cm^3) were digested with 5 cm^3 of nitric acid and 2 cm^3 of sulfuric acid on a hotplate below 100°C until the evolution of brown fumes ceased. After cooling, 5 cm^3 of nitric acid and 0.5 cm^3 of perchloric acid were added and then heated until dense fumes of sulfur trioxide appeared. The degraded acid solution was transferred to a 25 cm^3 volumetric flask, and 2.5 cm^3 of hydrochloric acid, 2.0 cm^3 of 10% (w/w) potassium iodide and 2 cm^3 of 20% (w/w) ascorbic acid were added. Water was then added to bring the solution to 25 cm^3. The total amount of arsenic was measured by reduction of the arsenic to arsine using a fully automated continuous arsine generation system (VGA 76) with 1% sodium borohydride (w/w in 0.5 mol dm^{-3} sodium hydroxide) and an atomic absorption spectrometer operated at 193.7 nm (Spectr AA400, Varian) equipped with a heated quartz tube. The flow rates in the arsine generation system were as follows; sample solution, 8 cm^3 min^{-1}; 6 mol dm^{-3} hydrochloric acid, 1 cm^3 min^{-1}; 1% sodium borohydride (w/w in 0.5 mol dm^{-3} sodium hydroxide) 1 cm^3 min^{-1}; argon, 100 cm^3 min^{-1}.

Chemical species of arsenic in biological samples

Each tissue (1–2 g) was homogenized with 70% methanol (30–50 cm^3) and then centrifuged. In the case of the freshwater micro-alga, *C. vulgaris*, and the water flea, *D. magna*, these samples were collected by centrifugation or filtration, and a small amount of sample

(0.05–0.1 g wet weight) was homogenized with 70% methanol (5 cm^3). The supernatants (4–5 cm^3) were digested with 2 mol dm^{-3} sodium hydroxide (5 cm^3) at 85°C for 3 h in a water bath. The arsenic sample solution was neutralized with dilute hydrochloric acid.

The river water (1000 cm^3) was concentrated to 18 cm^3 using a rotary evaporator, and 2 cm^3 of hydrochloric acid (2 mol dm^{-3}) was added to the concentrate which was filtered through a 0.45 μm membrane filter and appropriately diluted with deionized water. A 3 cm^3 portion of each sample solution was reduced with 2% sodium borohydride in 0.2 mol cm^{-3} sodium hydroxide and 0.6 mol cm^{-3} hydrochloric acid in a fully automated continuous arsine generation system (HFS-2, Hitachi). The water vapor was removed by passage of the gas stream through a vapor trap cooled with ice–sodium chloride. The arsines generated were collected in a U-tube trap in a liquid-nitrogen bath. The liquid nitrogen was then removed and the U-tube was heated. Arsines were introduced to the injection port of a gas chromatograph (HP 5890) and collected in the coiled head point (15 cm) of a capillary column (Urtla ALLOY 1 on a stainless capillary column, 30 m; Frontier Lab. Fukushima, Japan) precooled with liquid nitrogen for cryofocus condensation as shown in Fig. 1. The liquid nitrogen was then removed and the oven temperature was rapidly increased to 40°C. Arsines were separated by gas chromatography and analyzed using a mass spectrometer (HP 5972A mass selective detector) in the single-ion monitoring mode at *m/z* 76, 90, 103, 106 and 120. Inorganic arsenic and methylated, dimethylated and trimethylated arsenic compounds were identified and quantified as arsine (AsH_3), methylarsine (CH_3AsH_2), dimethylarsine [$(CH_3)_2AsH$] and trimethylarsine [$(CH_3)_3As$], respectively. These procedures are modified from the systems described in our previous paper.[32]

Culture of *Chlorella vulgaris* and incubation of *Daphnia magna*

Chlorella vulgaris, a freshwater unicellular green alga, was cultured with a culture medium ($NaNO_3$ 100 mg, K_2HPO_4 10 mg, $MgSO_4 \cdot 7H_2O$ 75 mg, $CaCl_2 \cdot 2H_2O$ 40 mg, Na_2CO_3 20 mg, ferrous citrate 6 mg, Na_2EDTA 6 mg and water 1000 cm^3 at pH 8.0)[33] containing sodium arsenate at a concentration of 100 μg As cm^{-3} at 27 °C with aeration and fluorescent lighting for 1 week. *C. vulgaris* was harvested by centrifugation and rinsed with an arsenic-free culture medium twice before analysis of the arsenic species and feeding to the water flea. The *Daphnia magna* was fed with arsenic-containing alga for one week at room temperature. The water flea was collected using a membrane cloth and washed with arsenic-free water. The chemical species of arsenic in both organisms were measured using the HG/CT/CF/GC–MS system.

RESULTS AND DISCUSSION

The determination of inorganic and methylated arsenic in biological samples was accomplished using the hydride generation coupled with atomic absorption spectrometry[23, 34] or mass spectrometry.[24, 31, 35] In previous work concerning the biomethylation of arsenic, Cullen *et al.* measured arsine or methylated arsines using an HG–GC–MS system operating in the wide-scan mode to investigate the biomethylation of arsenic by marine algae.[24, 35] In our study, the arsines were separated by gas chromatography and

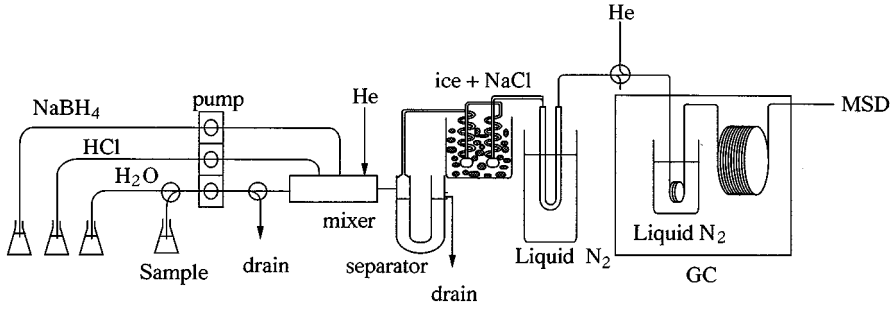

Figure 1 The hydride generation/cold trap/cryofocus/gas chromatography–mass spectrometry system for the collection of arsines, as described in the text. MSD, mass selective detector of a mass spectrometer.

Table 1 Arsenic content in biological samples living in a freshwater environment ($\mu g\ g^{-1}$)

	Total arsenic	Inorganic arsenic	Methylarsenic	Dimethylarsenic	Trimethylarsenic
River water	0.030	0.028 (93.2)[a]	ND	ND	0.002 (6.8)
Green alga					
Clodophora glomerata	0.453	0.044 (9.7)	ND	0.385 (85.0)	0.015 (3.3)
Diatom	0.124	0.010 (8.1)	ND	0.101 (81.0)	0.003 (2.4)
Freshwater fish					
Plecoglossus altivelis	0.051	ND	ND	0.005 (.8)	0.040 (78.4)
Oncorhynchus masou masou	0.146	ND	ND	0.063 (43.2)	0.081 (55.5)
Rhinogobius sp.	0.333	ND	ND	0.077 (18.3)	0.238 (71.5)
Phoxinus steindachneri	0.267	ND	ND	0.061 (22.8)	0.197 (73.8)
Tribolodon hakonensis	0.100	ND	ND	0.076 (76.0)	0.020 (20.0)
Sicyopterus japonicus	0.370	ND	ND	0.089 (24.1)	0.269 (72.7)
Prawn					
Macrobranchiura nipponense	0.817	ND	ND	0.614(75.2)	0.187 (22.9)
Marsh snail					
Semisulcospira libertina	0.186	ND	ND	0.050 (26.9)	0.116 (62.4)
Larva of dobsonfly					
Plotohermes grandis	2.875	ND	ND	2.762 (96.1)	0.043 (1.5)
Larva of caddisfly	0.236	ND	ND	0.202 (85.6)	0.022 (9.3)
Pupa of caddisfly	2.050	ND	ND	1.180 (57.6)	0.839 (40.9)
Stenopsyche marmorata					

[a] The ratio (%) of each species of arsenic to total arsenic is shown in parentheses.

identified and quantified by the selected-ion monitoring mode. Each arsine gave a sharp peak on the chromatogram. The HG/CT/CF/GC–MS system was useful for the determination of inorganic and methyl arsenic species in biological samples from the aquatic environment at trace levels.

Arsenic in river water

River water contained 0.030 $\mu g\ As\ cm^{-3}$ of total arsenic, as shown in Table 1. The content of arsenic was extremely high and the presence of these arsenicals was due to the influx of volcanic springs to the river. In this river water sample, inorganic arsenic was detected at 0.028 $\mu g\ As\ cm^{-3}$ and the ratio of inorganic arsenic to total arsenic was 93.2%. Almost all of the arsenic species in the river water were inorganic. Trimethylated arsenic was also detected at 0.002 $\mu g\ As\ cm^{-3}$ with a ratio to total arsenic of 6.8%. It is speculated that the trimethylated arsenic is likely to be trimethylarsine oxide, because trimethylarsine was detected in the river water after reduction with sodium borohydride without alkaline digestion, but arsenobetaine did not give trimethylarsine after reduction with sodium borohydride without alkaline digestion.[32]

Arsenic in algae and diatoms

Most of the arsenic compounds in the freshwater algae were water-soluble. The arsenic content in freshwaer algae was lower than in marine algae.[36, 37] The content of water-soluble dimethylated arsenic in algae was specifically higher than those of the other species of arsenic. The dimethylated arsenic compound was mainly detected in the algae at 0.385 $\mu g\ As\ g^{-1}$ wet wt and at 0.101 $\mu g\ As\ g^{-1}$ wet wt in the diatoms, while a small amount of inorganic arsenic was also detected at 0.044 $\mu g\ As\ g^{-1}$ and a 0.010 $\mu g\ As\ g^{-1}$, respectively. These contents are shown in Table 1. The ratio of dimethylated arsenic to total arsenic was 81–85%. The algae and diatoms accumulated inorganic arsenic from the freshwater, and almost all of the inorganics were converted to dimethylarsenic compounds in their tissue. Inorganic arsenic detected in the algae and diatoms was a precursor of the dimethylarsenic compounds. These tendencies are similar to those of marine algae, i.e. algae in marine or freshwater contain chiefly dimethylarsenic compounds. Dimethylarsenic compounds in marine algae have been found to be arsenosugars[38–43] but the chemical structures of the dimethylarsenic compounds in freshwater algae were not determined.

Arsenic in freshwater fish and aquatic macro-invertebrates

Freshwater fish and marsh snails contained 0.051–0.370 μg As g^{-1} of total arsenic in their tissues. Several fish analyzed in the experiment are edible. In freshwater fish and the marsh snail, the content of the trimethylarsenic compound was higher than that of the dimethylarsenic compounds except for *Tribolodon hakoensis*. Marine fish and marsh snails contained more trimethylarsenic compounds than dimethylarsenic ones, but the content of the dimethylarsenic compounds in the marine fish was low. Saito and Takahashi reported that arsenic was not detected in sweet fish in a river polluted with arsenic from

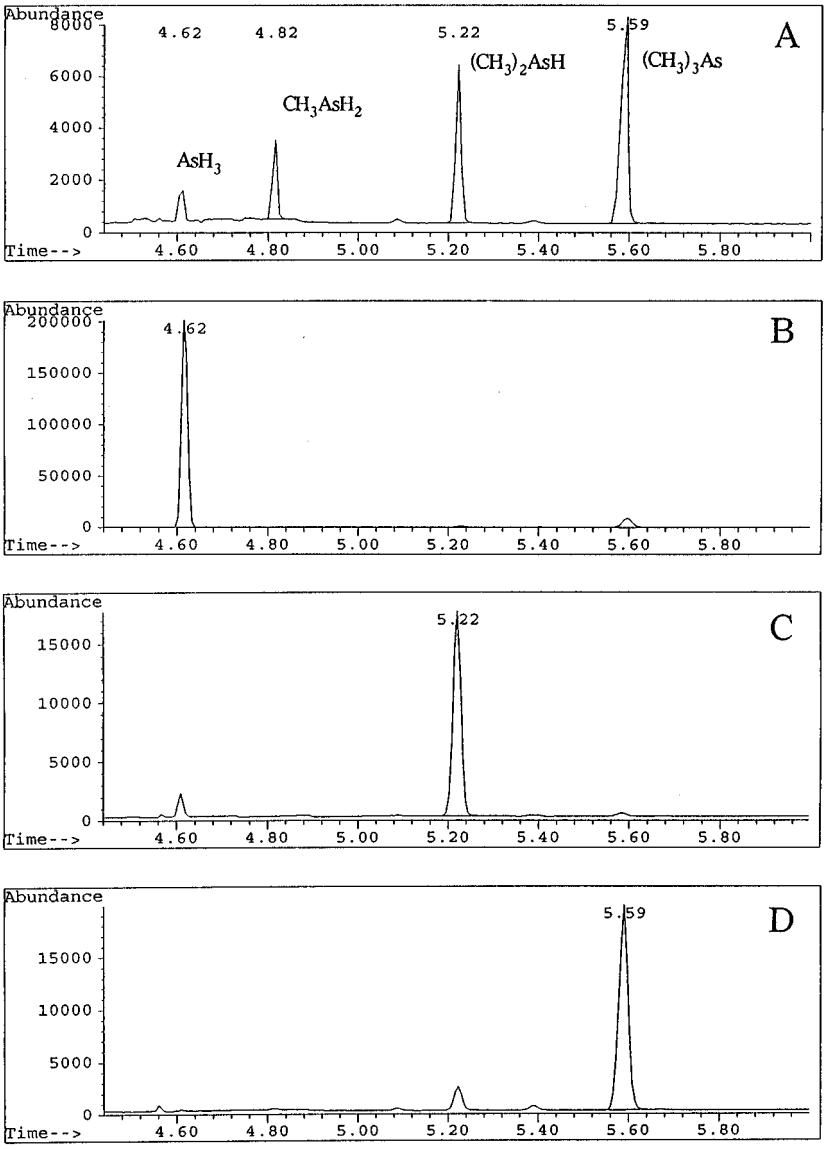

Figure 2 The total ion chromatograms of arsines in freshwater and wild specimens of freshwater biota. Ions of arsines were monitored at *m/z* 76, 90, 103, 106 and 120. (A) Authentic arsines: AsH_3 (arsine), CH_3AsH_2 (methylarsine), $(CH_3)_2AsH$ (dimethylarsine), $(CH_3)_3As$ (trimethylarsine) after introduction of a solution (3 cm^3) containing arsenite, methylarsonic acid, dimethylarsinic acid and trimethylarsine oxide into the hydride generation system, each at 10 ng As cm^{-3}. (B) Arsenic compounds in freshwater of the Haya-kawa river. (C) Arsenic compounds in freshwater green macro-algae *Clodophora glomerata* in the Haya-kawa river. (D) Arsenic compounds in the sweet fish *Plecoglossus altivelis*.

a hot spring,[44] but arsenobetaine in cultured specimens of rainbow trout (*Salmo gairdneri*) and wild specimens of Japanese smelt (*Hypomeus nipponensis*) was identified by Shiomi[45] *et al*. using an HPLC–ICP. The distribution of dimethylarsenic in freshwater fish and the marsh snail was different from that in marine fish. Arsenobetaine, which is a trimethylarsenic compound found in marine crustaceans is the major water-soluble arsenic and is found at very high levels;[36] on the other hand, the content of dimethylarsenic in a freshwater prawn was higher than that of the trimethylarsenic species. Also, the content of arsenic in the freshwater prawn was higher than in freshwater fish and the arsenic species consisted of 75.2% of a dimethylarsenic compound and 22.9% of a trimethylarsenic compound. It is thought that the disparity in the ratio of dimethylarsenic and trimethylarsenic in the freshwater prawn and marine crustacean was derived from the difference in the diets of these creatures.

Aquatic insects contain high levels of arsenic, except for the larva of the caddisfly. The chemical species of arsenic in their tissues are mostly dimethylarsenic compounds. Also, their diets account for the chemical species of arsenic in these aquatic insects.

Biotransformation of arsenic in wild specimens and experimental organisms

The chromatograms of arsine, methylarsine, dimethylarsine and trimethylarsine in typical samples are shown in Fig. 2. Based on these results of arsenic species from freshwater and freshwater biota, it is considered that the arsenic species in river water consisted of inorganic arsenic, and that inorganic arsenic is converted into dimethylated arsenic in freshwater algae and dimethylated arsenic is biotransformed into tri-

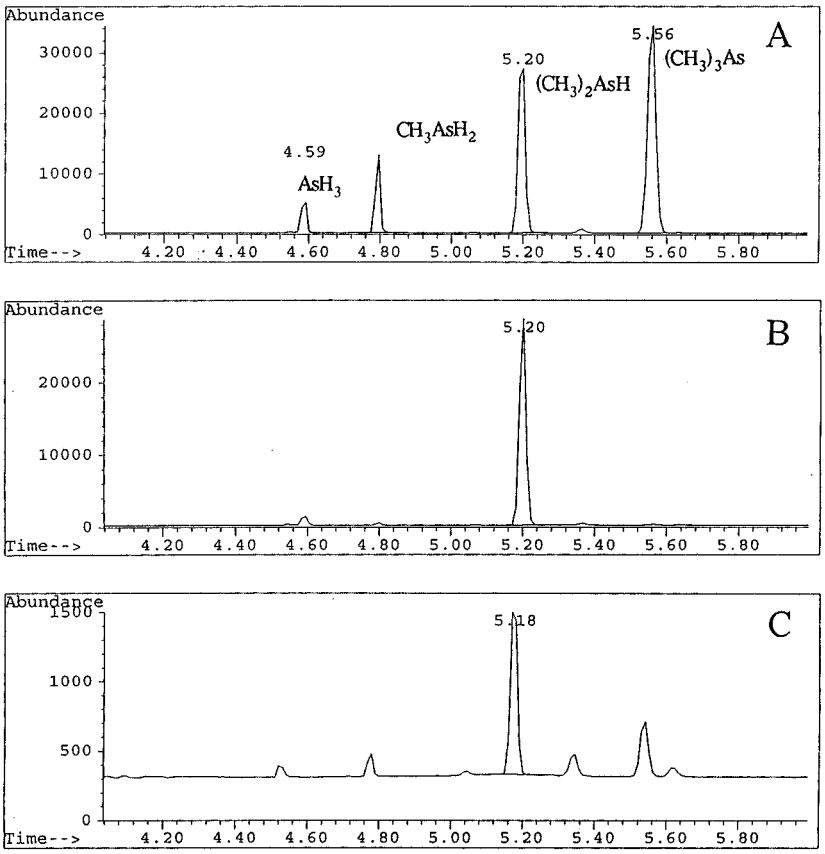

Figure 3 The total ion chromatograms of arsines in experimental organisms. Ions of arsines were monitored at *m/z* 76, 90, 103, 106 and 120. (A) Authentic arsines, as in Fig. 2 but each at 50 ng As cm^{-3}. (B) Arsenic compounds in micro-algae *Chlorella vulgaris*. (C) Arsenic compounds in water fleas *Daphnia magna*.

methylated arsenic in freshwater animals. Experimental organisms were studied in terms of transformation of the chemical form of arsenic, using the HG/CT/CF/GC–MS system. Inorganic arsenic added to the culture medium was converted to a dimethylarsenic compound with *C. vulgaris* and some of the dimethylarsenic compound in *C. vulgaris* was converted to a trimethylarsenic compound in the freshwater flea. The circulation of arsenic in the freshwater environment was ascertained based on the results of these experiments and likewise in wild specimens as shown in Fig. 3. Thus, the experimental results demonstrated biotransformation of arsenic in the freshwater environment via the food chain using the HG/CT/CF/GC–MS system.

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