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Metabolism of Arsenic Compounds by the Blue Mussel *Mytilus edulis* After Accumulation from Seawater Spiked with Arsenic Compounds

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Blue mussels (*Mytilus edulis*) were exposed to 100 μg As dm^{-3} in the form of arsenite, arsenate, methylarsonic acid, dimethylarsinic acid, arsenobetaine, arsenocholine, trimethylarsine oxide, tetramethylarsonium iodide or dimethyl(2-hydroxyethyl)arsine oxide in seawater for 10 days. The seawater was renewed and spiked with the arsenic compounds daily. Analyses of water samples taken 24 h after spiking showed that arsenobetaine and arsenocholine had been converted to trimethylarsine oxide, whereas trimethylarsine oxide and tetramethylarsonium iodide were unchanged. Arsenobetaine was accumulated by mussels most efficiently, followed in efficiency by arsenocholine and tetramethylarsonium iodide. None of the other arsenic compounds was significantly accumulated by the mussels. Extraction of mussel tissues with methanol revealed that control mussels contained arsenobetaine, a dimethyl(5-ribosyl)arsine oxide and an additional arsenic compound, possibly dimethylarsinic acid. Mussels exposed to arsenobetaine contained almost all their experimentally accumulated arsenic as arsenobetaine, and mussels exposed to tetramethylarsonium iodide contained it as the tetramethylarsonium compound. Mussels exposed to arsenocholine had arsenobetaine as the major arsenic compound and glycerylphosphorylarsenocholine as a minor arsenic compound in their tissues. The results show that arsenobetaine and arsenocholine are efficiently accumulated from seawater by blue mussels and that in both cases the accumulated arsenic is present in the tissues as arsenobetaine. Consequently arsenobetaine and/or arsenocholine present at very low concentrations in seawater may be responsible for the presence of arsenobetaine in *M. edulis* and probably also among other marine animals. The quantity of arsenobetaine accumulated by the mussels decreases with increasing concentrations of betaine. HPLC–ICP–MS was found to be very powerful for the investigation of the metabolism of arsenic compounds in biological systems.

Keywords: *Mytilus edulis*; arsenite; arsenate; methylarsonic acid; dimethylarsinic acid; arsenobetaine; arsenocholine; trimethylarsine oxide; tetramethylarsonium iodide; dimethyl(2-hydroxyethyl)arsine oxide; accumulation of arsenic; arsenic compounds in tissues

INTRODUCTION

The conversions of arsenic compounds in the marine environment are an integral part of the global arsenic cycle.[1] Many marine animals and marine algae used as human food contain arsenic compounds such as arsenobetaine, arsenocholine, tetramethylarsonium salts, trimethylarsine oxide, arsenosugars and arsenolipids.[2] Most of these arsenic compounds can now be separated and identified by liquid chromatography with arsenic-specific detectors such as GFAA, ICP–AE, or ICP–MS.[3,4] However, the arsenic cycle in the marine environment is still not completely understood.[2] One of the most challenging questions remaining is the elucidation of the origin of arsenobetaine in marine animals, such as crustaceans, fish, molluscs and echinoderms.

Arsenobetaine—widely distributed in marine animals independently of their feeding habits and the trophic level[5]—could be obtained by marine animals via ingestion of arsenic-containing food. However, several experiments have shown that marine animals are unable to convert dimethyl-

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(5-ribosyl)arsine oxide, the major arsenic compounds in algae, directly to arsenobetaine.[6–8]

Experiments with mussels (*Mytilus edulis*) exposed to seawater spiked with arsenobetaine led to accumulation of this arsenic compound in the tissues, whereas rock lobsters (*Panulirus cygnus*) accumulated arsenobetaine much less efficiently under the same conditions.[2] Because the natural levels of arsenobetaine are low in mussels and high in lobsters, arsenobetaine in seawater seems to be an unlikely source of arsenobetaine. Possibly, seawater contains a precursor of arsenobetaine at concentrations too low to be detectable by current analytical techniques. The origin of this unidentified precursor may be a product of the anaerobic decomposition of algal dimethyl-(5-ribosyl)arsine oxides and/or trimethyl-(5-ribosyl)arsonium cations in sediments.[9] The presence of unidentified arsenic species in coastal waters has been reported.[10–12] Uptake studies of organic arsenic compounds from water by marine organisms appear most promising to identify potential precursors of arsenobetaine in ocean water.

In this paper the results of experiments with the blue mussel *M. edulis* exposed in aquaria to 100 μg dm^{-3} arsenic in the form of arsenite, arsenate, methylarsonic acid, dimethylarsinic acid, trimethylarsine oxide, arsenobetaine, arsenocholine, tetramethylarsonium iodide and dimethyl-(2-hydroxyethyl)arsine oxide are reported.

EXPERIMENTAL

Chemicals

Arsenobetaine bromide was synthesized by the method described by McShane.[13] Purification by rectrystallization from ethanol yielded white crystals with a melting point of 225 °C (lit.[13] 227 °C). Arsenocholine bromide was synthesized according to a published procedure.[14] Tetramethylarsonium iodide prepared from trimethylarsine and methyl iodide was recrystallized from methanol. Disodium hydrogen arsenate heptahydrate was reagent-grade sodium arsenite. ($NaAsO_2$), dimethylarsinic acid and betaine hydrochloride, GPR grade (>97%), were purchased from BDH Chemicals. Trimethylarsine oxide prepared by oxidation of trimethylarsine with hydrogen peroxide was sublimed before use. Dimethyl(2-hydroxyethyl)arsine oxide was synthesized according to a published procedure.[15] Methylarsonic acid was recrystallized from ethanol/methanol (1:1). Pronalys 70% nitric acid (Rhone–Poulenc), Univar Analytical Reagent $HClO_4$ 70%, and AnalaR conc. H_2SO_4(BDH) were used as digestion acids. Stock solutions were prepared with deionized, doubly distilled water. Methanol was distilled before being employed as extracting solvent.

Stock solutions

Stock aqueous solutions containing 1000 mg As dm^{-3} were prepared for each of the nine arsenic compounds. The solutions were checked by HG–AA before spiking the experimental tanks. Aqueous betaine [trimethy(2-carboxymethyl)ammonium chloride] solutions contained, per 50 cm^3, 0.1029 g (0.013 mol dm^{-3}), 1.026 g (0.133 mol dm^{-3}), or 10.252 g (1.335 mol dm^{-3}) of the compound.

Exposure to arsenic compounds

Fresh *M. edulis* were obtained from a mussel farm situated near Garden Island, Perth, Western Australia. They were maintained in flowing seawater for two days prior to the exposure experiments. Groups of 25 mussels each were kept in four separate polypropylene tanks (48 cm × 54 cm, 33 cm high) filled with 50 dm^3 seawater (salinity 36.02 ± 0.16‰; seawater was 'sand-filtered' through quartz sand, particle size 0.3–1.0 mm). The size distribution of the mussels used in the exposure experiments is given in Table 1. One tank served as the control. Each one of the other three tanks was spiked with 5 cm^3 of one of the solutions of arsenic compounds (1000 mg dm^{-3} arsenic) to give a final concentration of 100 μg dm^{-3} arsenic. Each day the seawater in the tanks was replaced and a new arsenic spike (5 cm^3, 1000 mg As dm^{-3}) was added. This experiment was repeated two more times with four tanks each to provide exposure to all nine arsenic compounds (Table 1). The tanks were equipped with air stones. The water temperature was maintained at 22 °C. Water samples (50 cm^3) were taken immediately after addition of the arsenic compound and after 24 h, before the first change of seawater. The water samples were evaporated to dryness on a rotovap under an aspirator vacuum at temperatures not exceeding 50 °C. Before analysis, the residues were dissolved in distilled water (100 cm^3) to obtain 1:1

Table 1 Size distribution of mussels used in exposure experiments

Expt no.	Arsenic compound	Mean length (cm)[a]	No. of mussels
1	Control	7.4 (0.5)	25
	Arsenobetaine	7.4 (0.3)	25
	Arsenate	7.1 (0.3)	25
	Tetramethylarsonium	7.3 (0.4)	25
2	Control	6.3 (0.5)	25
	Dimethylarsinic acid	6.5 (0.4)	25
	Dimethyl(2-hydroxyethyl)arsine oxide	6.3 (0.4)	25
	Trimethylarsine oxide	6.4 (0.4)	25
3	Control	7.1 (0.3)	25
	Arsenocholine	6.9 (0.3)	25
	Arsenite	6.9 (0.2)	25
	Methylarsonic acid	7.0 (0.3)	25
4	Control	5.8 (0.2)	5
	Arsenobetaine	6.1 (0.5)	12
	Arsenobetaine : betaine (1 : 1)[b]	6.0 (0.4)	12
	Arsenobetaine : betaine (1 : 10)[b]	6.2 (0.4)	12
	Arsenobetaine : betaine (1 : 100)[b]	6.2 (0.4)	12

[a] SD in parentheses.
[b] Molar ratio.

diluted seawater. After 10 days 10 animals were shelled and the whole wet tissues from each mussel were separately weighed (wet weight) and stored frozen. The tissues were then freeze-fried (dry weight), and the dry material ground to a powder in a porcelain mortar. The mass of tissue obtained from a mussel was approximately 5 g wet weight and 1 g dry weight. A gill sample weighed 1.2 g wet and 0.15 g dry. From two animals the gills were excised and kept separately. The gills, and the tissues minus the gills, were also frozen, freeze-dried, and ground to a powder. The 13 remaining mussels were placed in a flow-through tank and seawater without added arsenic

Table 2 ICP-MS parameters used for HPLC–ICP–MS measurements

Plasma	Radio-frequency power	Forward 1.40 kW Reflected <5 W
Argon gas flows	Cool gas	13.5 $dm^3 min^{-1}$
	Auxiliary gas	1.1 $dm^3 min^{-1}$
	Nebulizer gas	0.73 $dm^3 min^{-1}$
Nebulization	Nebulizer	Meinhard concentric glass nebulizer type SB-30-A3
	Spray chamber	Double-pass Scott type, water-cooled (0 °C)
Ion sampling	Sample cone	Nickel orifice 1.00 mm diameter
	Skimmer cone	Nickel orifice 0.75 mm diameter
Vacuum	Expansion	1.6 mbar
	Intermediate	1.0×10^{-4} mbar
	Analyzer	2.1×10^{-6} mbar
Measuring parameters	Channels/amu	20
	Dwell time	320 μs
	Mass region	*m/z* 74.5–75.5
	Data acquisition	Scan mode
	Time/sweep	1.24 s

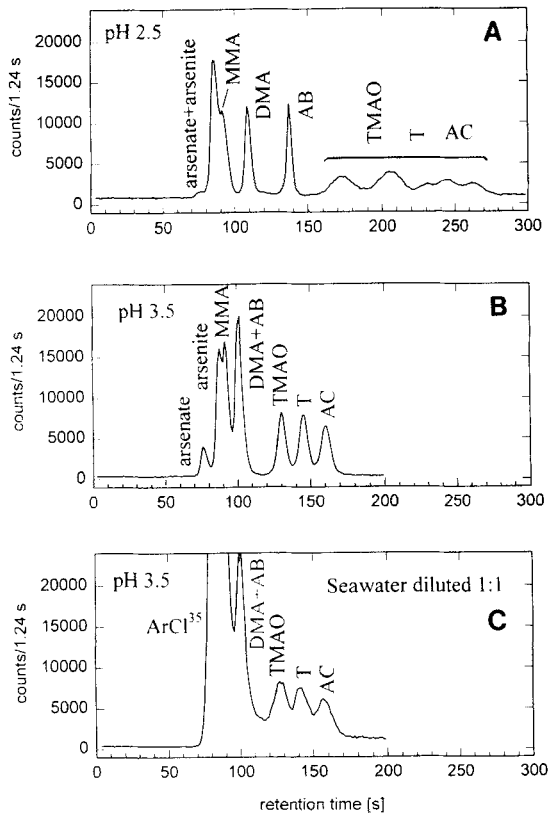

Figure 1 Separation of arsenate, arsenite, methylarsonic acid (MMA), dimethylarsinic acid (DMA), arsenobetaine (AB), trimethylarsine oxide (TMAO), tetramethylarsonium iodide (T) and arsenocholine (AC) [5 ng As of each compound in distilled water (**A**, **B**) and in seawater diluted 1:1 (**C**)] on a Hamilton PRP-1 column (25 cm × 4.1 mm i.d., 10 μm spherical particles). Injection volume, 100 μL; mobile phase 0.01 mol dm^{-3} 2-hydroxy-5-sulphobenzoic acid of pH 2.5 (**A**) or 3.5 (**B**, **C**); flow 1.5 cm^3 min^{-1}; ICP–MS detector.

compounds was pumped through for 14 days. These depurated mussels were shelled and treated as described above. Mussels that died during the experiments were removed from the tank.

Combined exposure to arsenobetaine and betaine

The four tanks containing 12 mussels were each filled with 50 dm^3 seawater and spiked with arsenobetaine to a concentration of 100 μg As dm^{-3}. Three tanks were treated with betaine solutions (5 cm^3 per 50 dm^3) to obtain final molar ratios of betaine/arsenobetaine of 1:1, 10:1 or 100:1. Water was changed and spiked with fresh arsenobetaine and betain solutions every day. After nine days the mussels were shelled and whole wet tissues from individuals were frozen, freeze-dried, ground to a fine powder and analysed separately for total arsenic by HG–AA. Arsenic concentrations were back-calculated to wet weight using the experimental wet weight/dry weight ratios.

Total arsenic analysis

Determinations of total arsenic were generally performed with a VGA-76 Hydride Generation Accessory connected to a Varian AA 875 flame atomic absorption spectrophotometer equipped with a flame-heated quartz tube. The Varian hollow-cathode lamp was operated at 7 mA. The arsenic signal was monitored at 193.7 nm. The

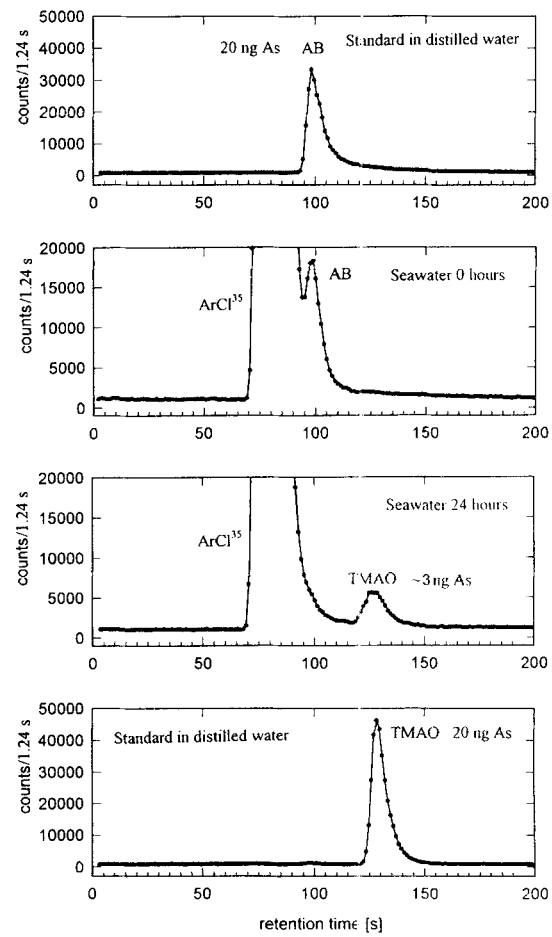

Figure 2 Chromatograms of the 1:1 diluted seawater samples from the tank spiked with trimethylarsine oxide (TMAO). Analytical details as for Fig. 1. Mobile phase pH 3.5.

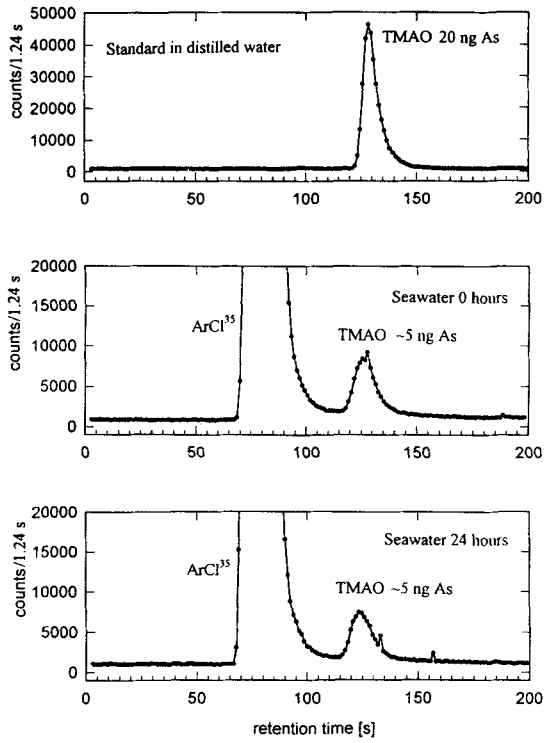

Figure 3 Chromatograms of the 1:1 diluted seawater samples from the tank spiked with tetramethylarsonium iodide (T). Analytical details as for Fig. 1. Mobile phase pH 3.5.

freeze-dried samples (*ca* 100 mg) were decomposed by heating in 10 cm^3 $HNO_3/HClO_4/H_2SO_4$ (7:2:1, by vol.) on a hotplate overnight (*ca* 100 °C). Arsenic concentrations are reported on a wet weight basis following back-calculation from the wet weight/dry weight ratios. NIES reference material No. 6 (mussels) certified at 9.2 ± 0.5 μg As g^{-1} was used to check the accuracy of the method. The mean value of nine determinations was 9.5 ± 0.3 μg As g^{-1}.

Total arsenic analyses to determine the extraction efficiency were carried out with a Varian AA 875 atomic absorption spectrophotometer in the GFAA mode using coated Varian plateau tubes and nickel nitrate as co-analyte. The methanol extracts were evaporated to dryness and dissolved in appropriate amounts of distilled water to give arsenic signals in the linear range of the calibration curve.

Extraction of arsenic compounds

Tissue samples from freeze-dried mussels from both the exposure and the depuration experiment were extracted with methanol. Only tissues from mussels from one control group and from those exposed to arsenobetaine, tetramethylarsonium iodide or arsenocholine were extracted. The finely powdered tissue samples were stirred with 20 cm^3 methanol for 10 min at room temperature on a magnetic stirring plate. The mixtures were filtered through a Whatman glass microfibre filter GF/A. The filter cake with the filter paper was extracted once more under the same conditions. The combined extracts were evaporated to dryness on a rotovap under an aspirator vacuum at temperatures not exceeding 50 °C. Before chromatography, the gums were shaken with a mixture of water (5 cm^3) and diethyl ether (5 cm^3) for 1 min. The aqueous phase was transferred to a volumetric flask (10–500 cm^3, depending on the amount of arsenic in the gums) and filled to the mark to provide concentrations of arsenic <1 μg cm^{-3}. The residue on the filter was dried at 100 °C and analysed for total arsenic by HG–AA. This procedure was carried out with approximately 500 mg of freeze-dried tissue, to which each of the 10 mussels in a group contributed 50 mg. From each of two gill samples, 50 mg was taken to obtain a total sample of 100 mg. The same mass of

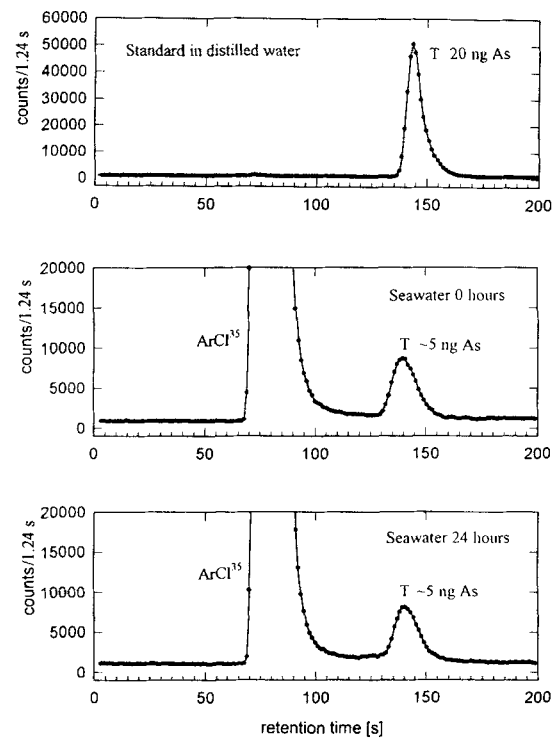

Figure 4 Chromatograms of the 1:1 diluted seawater samples from the tank spiked with arsenobetaine (AB). Analytical details as for Fig. 1. Mobile phase pH 3.5.

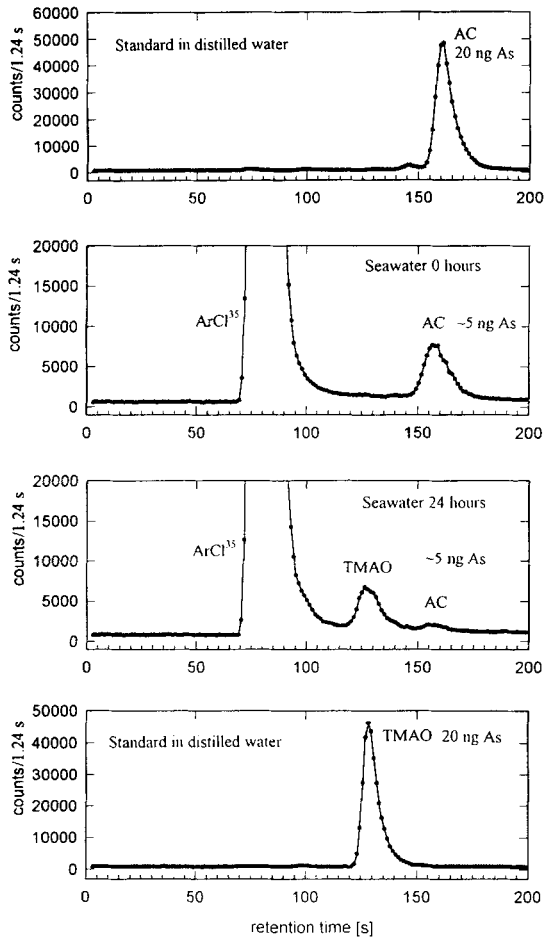

Figure 5 Chromatograms of the 1:1 diluted seawater samples from the tank spiked with arsenocholine (AC). Analytical details as for Fig. 1. Mobile phase pH 3.5.

tissue was taken from each of the two mussel tissues from which the gills had been removed.

HPLC–ICP–MS

The HPLC system consisted of a Milton Roy CM 4000 multiple solvent delivery unit and a PRP-1 reversed-phase column (Hamilton, Reno, Nevada, USA; 25 cm × 4.1 mm i.d.; spherical 10-μm particles of a styrene–divinylbenzene copolymer; stable between pH 1 and 13). A 100 μl loop was used in conjunction with a Rheodyne six-port injection valve. A guard cartridge (Hamilton, Reno, Nevada, USA) filled with the same stationary phase protected the analytical column. The HPLC column exit was connected to the ICP nebulizer with a steel capillary 75 cm long, 0.23 mm i.d. A VG Plasma Quad 2 Turbo Plus ICP–MS served to detect arsenic in the column effluent. The ICP–MS conditions are given in Table 2. An aqueous 0.01 mol dm^{-3} solution of 2-hydroxy-5-sulphobenzoic acid at pH 2.5 or 3.5 served as mobile phase at a flow rate of 1.5 cm^3 min^{-1}. The column was equilibrated with at least 100 cm^3 mobile phase before the injection of the extracts. Whereas water samples were analysed using only the pH 3.5 mobile phase, mussel extracts were investigated employing mobile phases at pH 2.5 and 3.5.

Statistics

For each experiment, analysis of covariance (ANCOVA) was used to determine significant differences in mean arsenic concentrations between arsenic-treated groups and controls. The analyses were carried out on log-transformed concentration with the log transformed dry mass of whole animals as the covariant and treatment group as a factor. The arsenic concentrations from the exposure and the depuration experiments were also compared.

RESULTS AND DISCUSSION

Although the forms of arsenic in marine biota are now well known, the origin of these arsenic compounds and therefore the arsenic cycle in the sea is still not completely elucidated. The study of biochemical transformations of arsenic compounds in marine organisms involving the administration and subsequent examination of metabolites in aquarium experiments on plants and animals may lead to a better understanding of the role of these organisms in the arsenic cycle in the ocean. We therefore exposed *M. edluis* in tanks (stationary system) for 10 days to 100 μg dm^{-3} arsenic concentrations of nine arsenic compounds in seawater.

Separation of arsenic compounds

To detect metabolites of the arsenic compounds added to the tanks and the arsenic compounds in mussel tissues, a chromatographic method was developed for the separation of arsenite, arsenate, methylarsonic acid, dimethylarsinic acid, arsenobetaine, trimethylarsine oxide, tetramethylarsonium iodide and arsenocholine (Fig. 1).[16] On the Hamilton PRP-1 reversed-phase

column with a 0.01 mol dm^{-3} aqueous solution of 2-hydroxy-5-sulphobenzoic acid at pH 3.5 as the mobile phase, arsenite and methylarsonic acid are not clearly separated and arsenobetaine and dimethylarsinic acid have the same retention time. All other arsenic compounds are well separated from each other. With the same mobile phase but at pH 2.5, arsenobetaine and dimethylarsinic acid are baseline-separated; the separation of arsenite from methylarsonic acid is not improved, and trimethylarsine oxide, tetramethylarsonium iodide and arsenocholine produce analytically unusable broad signals (Fig. 1).

Arsenic compounds in seawater in contact with mussels

Water samples were taken from the tanks immediately after spiking with arsenobetaine, trimethylarsine oxide, arsenocholine or tetramethylarsonium iodide and 24 h after addition of the spikes. These samples, after dilution with equal volumes of distilled water, were analysed chromatographically with the mobile phase at pH 3.5 and ICP–MS as detector. The chromatograms of the seawater samples contain a strong signal at the solvent front caused by $(Ar^{35}Cl)^+$ with the same m/z as arsenic. The signal obscures peaks associated with arsenite, arsenate and methylarsonic acid. The arsenobetaine signal sits on the long-retention side of the $ArCl^+$ signal and, therefore, can be used only for qualitative purposes (Fig. 2). Conversions of any of the arsenic compounds in the tanks to arsenite, arsenate and/or methylarsonic acid cannot be monitored under these conditions. The seawater spiked with trimethylarsine oxide or tetramethylarsonium iodide produced signals only for the compounds that were added (Figs 3 and 4). A conversion to dimethylarsinic acid would have been detected.

Arsenobetaine and arsenocholine were both transformed to trimethylarsine oxide. In the arsenobetaine-treated tank, trimethylarsine oxide was the only arsenic compound detected after 24 h (Fig. 2). This result is in accord with the observations of the conversion of arsenobetaine to trimethylarsine oxide by microorganisms associated with marine macroalgae[17] and by intestinal bacteria of a mollusc.[18] If all of the arsenobetaine had been converted to trimethylarsine oxide, approx. 5 ng of trimethylarsine oxide should have been present in seawater. Only 3 ng of trimethylarsine oxide was detected. Therefore, some unconverted arsenobetaine and perhaps dimethylarsinic acid that has the same retention time as arsenobetaine could have been present and could be responsible for the tailing $Ar^{35}Cl$

Table 3 Concentrations of arsenic in tissues of *M. edulis* after a 10-day exposure to arsenic compounds (100 µg dm^{-3} arsenic) in seawater

	As concentration (mg kg^{-1} wet wt)					
	Whole animal		Gills[b]		Whole animal – gills[b]	
Arsenic compound	Range	Mean	Range	Mean	Range	Mean
Control	3.5–5.0	4.2	2.7–2.9	2.8	3.6–4.3	3.9
Arsenobetaine	76.5–237	139	165–187	176	70–100	85
Arsenate	3.3–4.8	3.9	2.1–2.4	2.3	3.9–4.4	4.2
Tetramethylarsoniumiodide	10.5–19.8	15.1	25.7–32.5	29.1	14.6–15.9	15.2
Control	4.2–5.7	4.9	2.0–2.4	2.2	5.4–5.7	5.5
Dimethylarsinic acid	4.1–6.5	5.4	3.1–5.2	4.1	5.8–6.9	6.5
Dimethyl(2-hydroxyethyl)arsine oxide	4.5–6.3	5.3	1.8–2.2	2.0	5.7–6.1	5.9
Trimethylarsine oxide	4.2–5.4	4.9	2.5–3.1	2.8	5.7–5.8	5.8
Control	3.9–6.0	5.1	2.4–3.8	3.1	5.0–6.6	5.8
Arsenocholine	19.9–78.1	45.4	120–140	130	42.9–43.4	43.1
Arsenite	5.1–6.4	5.8	3.9–4.2	4.1	6.7–6.9	6.8
Methylarsonic acid	4.2–6.6	5.4	2.8–3.6	3.2	5.5–7.5	6.5

[a] Concentrations from 12 mussels including the two dissected animals.
[b] Concentrations from two mussels.

Table 4 Mass balance of arsenic in the exposure experiments with arsenobetaine, arsenocholine, and tetramethylarsonium iodide

Compound	Wt of As added[a] to tank (mg)	Wt of As in 25 mussels (mg)	$\frac{\text{As in 25 mussles}}{\text{Total As added}} \times 100$	As compound in mussels
Arsenobetaine	50	17.5	35	Arsenobetaine
Arsenocholine	50	4.75	9.5	Arsenobetaine
Tetramethylarsonium iodide	50	1.9	3.8	Tetramethylarsonium iodide

[a] Added in 10 instalments of 5 mg per day for 10 days.

signal. However, lack of evidence for the conversion of trimethylarsine oxide to dimethylarsinic acid under these conditions (Fig. 3) makes dimethylarsinic acid an unlikely decomposition product of arsenobetaine. Arsenocholine was not completely converted to trimethylarsine oxide within 24 h. A trace of unchanged arsenocholine is still present (Fig. 5). Arsenocholine could first be oxidized microbially to arsenobetaine,[19] which is then degraded to trimethylarsine oxide.

Accumulation of arsenic by the mussels

The concentrations of arsenic in the whole animals (12 mussels), in the gills (two mussels) and in mussels minus the gills (two mussels) after 10 days of exposure to 100 μg dm^{-3} arsenic in the form of nine arsenic compounds are summarized in Table 3. The concentrations of arsenic in whole mussels (approx. 5 mg As kg^{-1} wet weight) exposed to arsenite, arsenate, methylarsonic acid, dimethylarsinic acid, dimethyl(2-hydroxyethyl)arsine oxide or trimethylarsine oxide were not significantly higher than the arsenic concentrations of the controls ($P > 0.05$). That trimethylarsine oxide was not accumulated by *M. edulis* is in agreement with earlier experiments.[2] Dimethyl(2-hydroxyethyl)arsine oxide was not retained by fish.[20] The concentration of arsenic in the gills is approximately half the concentration in the whole animal and is also not significantly affected by exposure to arsenite, arsenate, methylarsonic acid, dimethylarsinic acid, dimethyl(2-hydroxyethyl)arsine oxide or trimethylarsine oxide.

Mussels exposed to arsenobetaine, arsenocholine or tetramethylarsonium iodide had arsenic concentrations significantly higher ($P < 0.001$) than the controls. The concentrations in the gills are even higher (Table 3). The average concen-

Table 5 Concentration of arsenic (mg kg^{-1} wet weight) in tissues of *M. edulis* following a 14-day depuration after a 10-day exposure to arsenic compounds (100 μg As dm^{-3}) in seawater

Arsenic compound	Whole animal		Gills[b]		Whole animal − gills[b]	
	Range	Mean	Range	Meann	Range	Mean
Control	3.3–4.7	3.9 (4.2)[c]	3.3, 3.4	3.3 (2.8)	4.2, 5.2	4.7 (3.9)
Arsenobetaine	46.4–198	106 (139)	132, 139	135 (176)	54, 63	58 (85)
Tetramethylarsonium iodide	6.3–16.8	11.2 (15.1)	7.8	7.8[d] (29.1)	4.5, 7.2	5.9 (15.2)
Control	3.3–4.5	3.9 (5.1)	1.7, 1.7	1.7 (3.1)	3.6, 4.1	3.8 (5.8)
Arsenocholine[e]	11.1–58.3	29.7 (45.4)	7.2, 25.0	16 (130)	15.3, 29.3	22.3 (43.1)

[a] Concentrations from 12 mussels including the two dissected ones.
[b] Concentrations from two mussels.
[c] Values in parentheses are average total arsenic concentrations in the mussel tissue after a 10-day exposure.
[d] Concentration from one gill only.
[e] Present as arsenobetaine in the tissues.

Table 6 Total arsenic (μg) in 12 mussels at the end of the accumulation and the depuration period

Arsenic compound	After accumulation in whole animal		After depuration in whole animal	
	Range	Mean	Range	Mean
Control	15.4–38	24.5	14.3–31	20.2
Arsenobetaine	456–1201	727	248–879	508
Tetramethylarsonium iodide	49–113	82	28.2–68	50
Control	13–41	24	11.2–20.8	14.5
Arsenocholine	99–317	197	39.4–234	130

tration (mg As kg^{-1} wet weight) of arsenic in *M. edulis* after an exposure of 10 days was highest in the arsenobetaine group (139 mg As kg^{-1}), lowest in the tetramethylarsonium group (15.1 mg kg^{-1}) and between these values in the arsenocholine group (45.4 mg As kg^{-1}). The arsenic concentration of the control animals was approx. 4.7 mg kg^{-1}.

When the complete conversion of arsenobetaine to trimethylarsine oxide during 24 h in the tanks is neglected, the 25 mussels in one tank incorporated into their tissues 35% of the total arsenobetaine added during 10 days (Table 4). This percentage would certainly be higher (perhaps by a factor of two) with consideration of the decrease of the arsenobetaine concentration with time. Kinetic data for the arsenobetaine→ trimethylarsine oxide conversion under the conditions used in the experiment are not available.

From the total arsenic added as arsenocholine to the tanks, approx. 10% was retained by the mussels, but not as arsenocholine. Because arsenocholine is also converted to trimethylarsine oxide (perhaps via arsenobetaine), uncertainty exists about the arsenic compound that is actually taken up by the mussels kept in the tank that was spiked with arsenocholine.

Tetramethylarsonium iodide remained unchanged in the water (Fig. 4) and was taken up by the mussels much less efficiently than arsenobetaine and arsenocholine. Only about 4% of the offered arsonium iodide was found unchanged in the tissues (Table 4).

Table 7 Concentration of arsenic (mg kg^{-1} wet weight) in whole animals of *M. edulis* following a 9-day exposure to 100 μg dm^{-3} arsenic as arsenobetaine and betaine/arsenobetaine molar ratios of 1:1, 10:1, 100:1 in seawater

Experiment	Whole animal[a]	
	Range	Mean
Control	3.3–4.9	4.1
Arsenobetaine	285–587	406
Arsenobetaine:betaine, 1:1	227–449	332
Arsenobetaine:betaine, 1:10	84–157	122
Arsenobetaine:betaine, 1:100	33.5–66	49

[a] Concentrates from 10 mussels.

Depuration of arsenic compounds

Thirteen of the 25 mussels from the control group (experiment 1, Table 1) and from the groups exposed to arsenobetaine, arsenocholine or tetramethylarsonium iodide for 10 days were removed to flow-through tanks and maintained in conti-

Table 8 Extraction of arsenic from freeze-dried tissues of mussels exposed to arsenobetaine and arsenocholine

	Extract no.	Total arsenic by GFAA (μg)	Arsenic (%)
Arsenobetaine	1	340	90.4
	2	26	6.9
	3	7.5	2.0
	4	2.5	0.7
	5	0.7	0.2
	Total	376[a]	
Arsenocholine	1	89	87.2
	2	9	8.8
	3	3.4	3.3
	4	0.7	0.7
	5	0	0
	Total	102[b]	

[a] Total arsenic in the mussel tissue: 392 μg, extractable 96%.

[b] Total arsenic in the mussel tissue: 120 μg, extractable 85%.

nuously flowing seawater for 14 days. During this depuration period the mussels lost approximately 10% of their tissue mass. This loss calculated on a dry weight basis was statistically significant ($P<0.01$) for each of the four groups.

The concentrations of total arsenic in the 'depurated' mussels previously exposed to arsenic compounds are summarized in Table 5. These concentrations in whole mussels are 24–35% lower than at the beginning of depuration. Depuration from the gills of the arsenobetaine-treated mussels is 24% within the range for the whole animals. However, the gills of the tetramethylarsonium- and arsenocholine-treated mussels lost approximately 80% of the arsenic during the depuration period. Gills consist of biologically very active tissue, and such thorough depuration is not unreasonable. Because only two mussels were dissected, statistically valid comparisons are not possible. Table 6 presents the ranges and averages of total arsenic in 12 mussels at the beginning and the end of the depuration period. The depurated animals contained approximately 35% less arsenic. The depuration of arsenic was significant in the arsenobetaine and the tetramethylarsonium group ($P<0.05$) and not signifi-

Table 9 Amounts of freeze-dried tissues extracted with methanol and amounts of arsenic in the extracts and in the tissues before and after extraction[a]

Tissue type		Wt of tissue (mg)			Wt of arsenic in tissue (μg)		
		Before extraction	After extraction	Dissolved[b]	Before extraction	After extraction	Wt. of arsenic in extract[b] (μg)
Control							
Accu:	WA	507.3	367.1	140.2	12.48	2.12	10.35
	Gills	91.3	71.8	19.5	2.18	0.28	1.90
	Rest	101.2	67.3	33.9	2.40	0.37	2.03
Depu:	WA	514.1	380	134.1	11.69	1.82	9.87
	Gills	99.2	63.9	35.3	2.60	0.32	2.27
	Rest	107.3	78.8	28.5	2.55	0.51	2.04
AB							
Accu:	WA	498.8	364.5	134.3	407.6	19.10	388.5
	Gills	101.8	63.4	38.4	141.5	0.94	140.4
	Rest	100.5	67.2	33.3	49.6	0.60	49.0
Depu:	WA	498.3	364.7	133.6	333.6	25.13	308.4
	Gills	101.4	65.5	35.9	110.5	0.97	109.5
	Rest	111.3	75	36.3	35.9	2.17	33.73
Tetra							
Accu:	WA	498.1	368.9	129.2	40.1	3.98	36.12
	Gills	115.6	72.7	42.9	26.91	0.49	26.42
	Rest	99.9	65.2	34.7	8.65	0.49	8.16
Depu	WA[c]	454.4	330.3	124.1	31.27	2.57	28.7
	Gills	101.3	74.0	27.3	—	—	—
	Rest	100.4	65.9	34.5	3.43	0.26	3.17
AC							
Accu:	WA	503.7	365.4	138.3	111.8	8.48	103.3
	Gills	99.4	61.3	38.1	102.7	1.60	101.1
	Rest	102.6	67.4	35.2	23.9	1.44	22.46
Depu:	WA[d]	353	244	109	64.9	4.34	60.6
	Gills	113.8	80.8	33	13.5	0.55	12.95
	Rest	101.8	75.4	26.4	11.54	1.17	10.37

[a] Abbreviations: AB, arsenobetaine; Tetra, tetramethylarsonium cation; AC, arsenocholine; Accu, after accumulation; Depu. after depurination; WA, whole animal; Rest, mussels minus gills.
[b] Calculated as the difference (wt before extraction—wt after extraction).
[c] Nine mussels.
[d] Seven mussels.

Table 10 *Identification and quantification of arsenic compounds in mussel tissues using HPLC–ICP–MS*[a]

Tissue type		Wt of arsenic[b] (μg) in form of							
		Total As	AB[c]	AC[d]	Tetra[d]	GPAC[c]	I[c]	II[c]	III[c]
Control									
Accu:	WA	10.4	6.7	—	—	—	—	6.8	0.5
	Gills	1.90	1.2	—	—	—	—	2.5	0.1
	Rest	2.03	1.2	—	—	—	—	1.9	0.2
Depu:	WA	9.9	6.4	—	—	—	—	2.0	0.4
	Gills	2.30	1.3	—	—	—	—	2.8	0.2
	Rest	2.04	1.5	—	—	—	—	1.4	0.2
Arsenobetaine									
Accu:	WA	388.5	338	—	—	—	8.5	—	—
	Gills	140.5	126.5	—	—	—	4.0	—	—
	Rest	49.0	48.6	—	—	—	1.6	—	—
Depu:	WA	308.4	305	—	—	—	9.5	—	—
	Gills	109.5	127	—	—	—	5.5	—	—
	Rest	33.7	41.3	—	—	—	2.5	—	—
Tetra									
Accu:	WA	36.1	5.7	—	23.2	—	—	5.4	—
	Gills	26.5	1.3	—	21	—	—	2.7	—
	Rest	8.16	1.7	—	5.5	—	—	1.5	—
Depu:	WA	28.7	7.4	—	16.5	—	—	7.1	—
	Gills	—	0.6	—	8	—	—	2.5	—
	Rest	3.17	1.3	—	2.1	—	—	0.8	—
Arsenocholine									
Accu:	WA	103.3	94	—	—	5	8.0	—	—
	Gills	101.1	90	—	—	1.5	4.0	—	—
	Rest	22.5	16.6	—	—	2.0	2.3	—	—
Depu:	WA	60.6	50.4	—	—	1.5	4.5	—	—
	Gills	12.9	15.7	—	—	0.3	2.6	—	—
	Rest	10.4	7.2	—	—	0.4	1.1	—	—

[a] Abbreviations: as in Table 9; GPAC, glycerylphosphorylarsenocholine.
[b] These amounts of arsenic are present in the weights of tissues given in Table 9.
[c] Identification via retention times on PRP-1 column with 0.01 mol dm^{-3} 2-hydroxy-5-sulphobenzoic acid adjusted to pH 2.5 with sodium hydroxide; flow, 1.5 cm^3 min^{-1}. Retention times: arsenobetaine, 131 s; glycerylphosphorylarsenocholine, 89 s; **I**, 71 s; **II**, 77 s; **III**, 104 s.
[d] Identification via retention times on PRP-1 column with 0.01 mol dm^{-3} 2-hydroxy-5-sulphobenzoic acid adjusted to pH 3.5 with sodium hydroxide; flow, 1.5 cm^3 min^{-1}. Retention times: tetramethylarsonium, 145 s; arsenocholine, 160 s

cant in the control ($P > 0.05$). For the arsenocholine group, depuration was just not significant at the 5% level ($P = 0.08$).

The animals lost arsenic. However, this loss was very likely to have been caused by two factors: the reduction of tissue mass and the absence of arsenic compounds in the flowing seawater. An allocation of the arsenic loss between these two factors is not possible with the available data.

Accumulation of arsenobetaine in the presence of betaine

The uptake of arsenobetaine from seawater by the mussel *M. edulis* could be influenced by betaine. Therefore, mussels were exposed for nine days to mixtures of arsenobetaine and betaine with molar ratios of betaine/arsenobetaine of 1:1, 10:1 or 100:1. The presence of betaine clearly reduces the uptake of

arsenobetaine by the mussels (Table 7). At a 100-fold molar excess of betaine over arsenobetaine, the concentration of arsenic in the whole animals is only 49 mg kg^{-1} (wet weight), approximately one-tenth of the concentration found in the absence of betaine.

Extraction of arsenic compounds from mussel tissues

Freeze-dried tissues (510.7 mg) from one mussel exposed to arsenobetaine for 10 days contained 392 μg As. Similarly, tissue from one mussel (510.2 mg) exposed to arsenocholine contained 120 μg As. These samples were extracted with five 20 cm^3 portions of methanol (10 min stirring

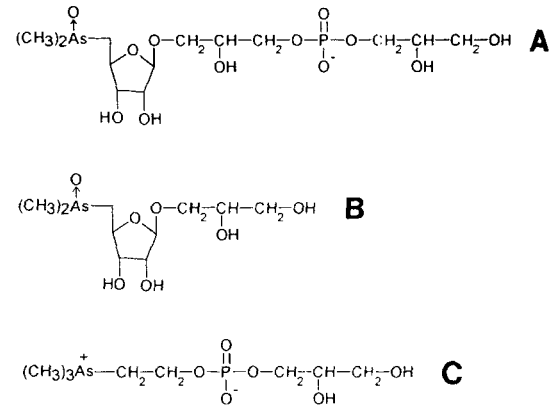

Figure 7 Chemical formulae of arsenic compounds **A**, **B**, **C** (see text).

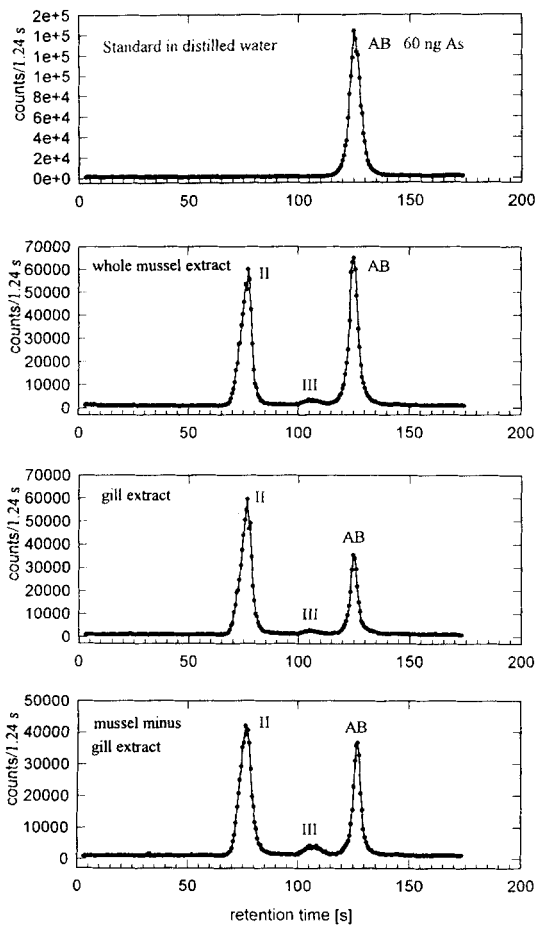

Figure 6 Chromatograms of the whole mussel and gill extracts of the control mussels after the accumulation period. Analytical details as for Fig. 1. Mobile phase pH 2.5.

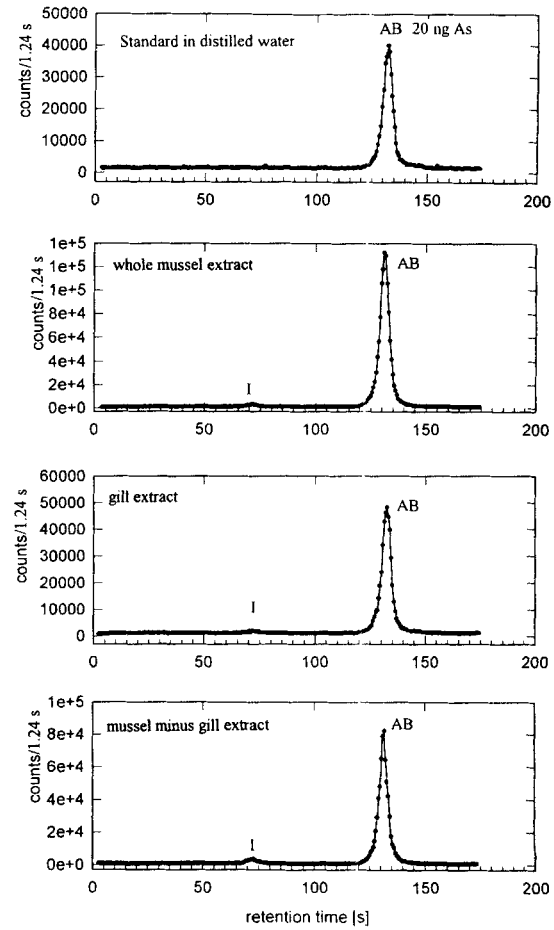

Figure 8 Chromatograms of whole mussel and gill extracts from mussels exposed to arsenobetaine (AB) for 10 days. Analytical details as for Fig. 1. Mobile phase pH 2.5.

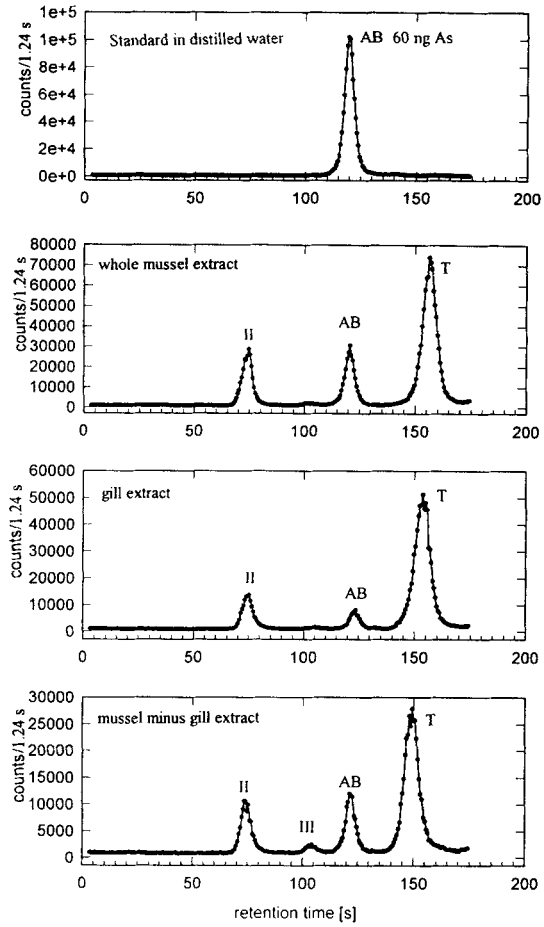

Figure 9 Chromatograms of whole mussel and gill extracts from mussels exposed to tetramethylarsonium iodide (T) for 10 days. Analytical details as for Fig. 1. Mobile phase pH 2.5.

with each portion). Total arsenic present in each portion was determined by GFAA to check on the extraction efficiency. Approximately 90% of the total arsenic was present in the first extract. The first two extractions removed 96% of the arsenic from the tissue (Table 8). The last three extractions accounted for only 3–4% of the total arsenic. Therefore, two extractions were used in the experiments to identify the arsenic compounds in the mussel tissues.

Identification and quantification of arsenic compounds in mussel tissues

Tissues from mussels were extracted with methanol. The amounts of tissues used and the weights of arsenic in the various samples are listed in Table 9. The extracts were evaporated to remove all methanol. The residue was dissolved in water (10–500 cm^3) and an aliquot of these resulting solutions (100 µl) was injected onto the column. Aqueous solutions of 2-hydroxy-5-sulphobenzoic acid (0.01 mol dm^{-3}) of pH 2.5 (mobile phase 1) or of pH 3.5 (mobile phase 2) were used as mobile phases at a flow rate of 1.5 cm^3 min^{-1}. ICP–MS was employed as the arsenic-specific detector with detection limits of 0.5 ng As absolute and 5 ng As cm^{-3}. The results are summarized in Table 10.

Control group

Three organoarsenic compounds were identified in the control mussels applying mobile phase 1 (Fig. 6). Arsenobetaine and compound **II** are the major arsenic compounds in all control tissues and compound **III** is a minor component.

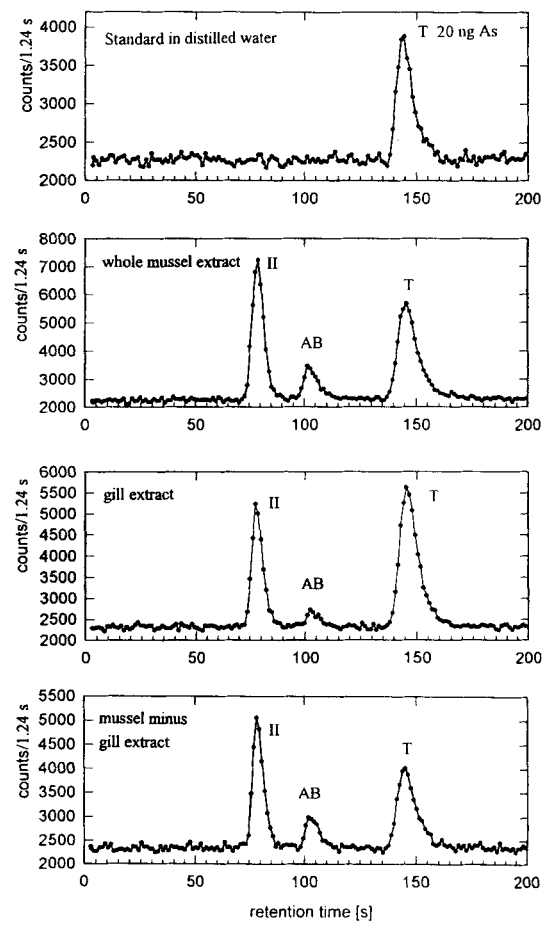

Figure 10 Chromatograms of whole mussel extracts from mussels exposed to tetramethylarsonium iodide (T) for 10 days. Analytical details as for Fig. 1. Mobile phase pH 3.5.

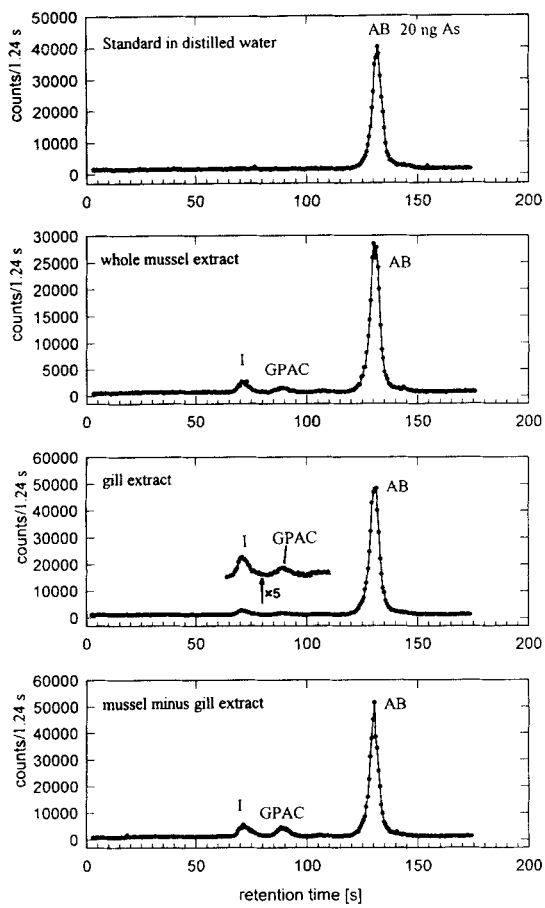

Figure 11 Chromatograms of whole mussel and gill extracts from mussels exposed to arsenocholine for 10 days. Analytical details as for Fig. 1. Mobile phase pH 2.5.

Compound **II** could not be identified with the available arsenic standards and may be an arsenic-containing ribofuranoside. Shibata and Morita[21] reported the presence of dimethyl-(5-ribosyl)arsine oxide (**A**) or arsine oxides **A** and **B** (Fig. 7) in *M. coruscum*, *M. edulis*, *Crassostrea gigas*, *Anadara broughtonii* and *Corbicula japonica*. Compound **A** was always present at higher concentrations than compound **B** . In *M. coruscum* dimethyl(5-ribosyl)arsine oxide **A** is a minor compound (7% of total water-soluble arsenic) whereas unidentified compound **II** is a major arsenic constituent of *M. edulis* (approx. 50% of total water-soluble arsenic). In the bivalve *Tresus keenae*[22] the concentrations of dimethyl-(5-ribosyl)arsine oxides **A** and **B** are of the same magnitude as the concentration of arsenobetaine. Dimethyl(5-ribosyl)arsine oxides **A** and **B** are considered to be the major arsenic species in bivalves.[21] Dimethyl(5-ribosyl)arsine oxides could be the precursors of arsenobetaine in the animal tissues.[22]

Arsenic compound **III**, a very minor constituent, had the same retention time as dimethylarsinic acid (Fig. 1). Shibata and Morita[21] reported the presence of dimethylarsinic acid in NIES No. 6 reference material made from *M. edulis*. Neither arsenocholine nor tetramethylarsonium was detected in the column effluent with mobile phase 2. Under these conditions these two compounds would elute after arsenobetaine (Fig. 1).

Comparing the amounts of the three arsenic compounds from the accumulation and the depuration experiments, only the amount of unidentified compound **II** was reduced by two-thirds in the whole animals; all other arsenic compounds in the depurated mussels were present in amounts of the same magnitude as those from the accumulation experiment (Table 10). The reduced amount of compound **II** (structure **A** in Fig. 7) in the depurated mussels may have been caused by its conversion to arsenobetaine as proposed by Edmonds *et al.*[23]

Arsenobetaine group

Because mussels in the arsenobetaine group had a very high arsenic concentration, the extracts had to be highly diluted with distilled water. Therefore compounds **II** and **III** found in the control animals were not detectable. Practically all the arsenic was present as arsenobetaine (AB) in the mussels before and after depuration (Fig.

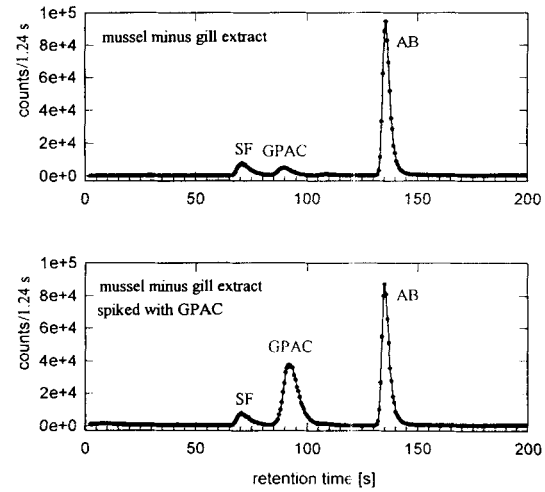

Figure 12 Identification of glycerylphosphorylarsenocholine (GPAC) in extracts of mussel tissue exposed to arsenocholine for 10 days. Analytical details as for Fig. 1. Mobile phase pH 2.5.

8). The very low-intensity signal at the solvent front (retention time 71 s) could be indicative of an arsenic compound, but may come from $^{40}Ar^{35}Cl$. Applying mobile phase 2, no arsenocholine and no tetramethylarsonium cation was detectable.

Tetramethylarsonium group

Because the dilution of the extracts was equal to the dilution of the control mussels, compound **II** was detected with mobile phase **I** in all tisses at a concentration comparable with that in the control mussels. Compound **III** was present in traces (Fig. 9). The amount of arsenobetaine in the various tissues was of the same magnitude as in the control mussels. Mobile phase 2 revealed that most of the accumulated arsenic is present as tetramethylarsonium cation. No arsenocholine was detectable (Fig. 10).

Arsenocholine group

Because the dilution of the extracts was again very high, compounds **II** and **III** were not detected with mobile phase 1 (Fig. 11). Arsenobetaine accounted for the majority (approx. 83%) of arsenic found in the whole mussel tissues. Moreover, glycerylphosphorylarsenocholine (structure **C** in Fig. 7) was identified in all tissues (Figs 11, 12) by spiking the diluted extract with a synthetic sample.[24] Compound **C** accounted for *ca* 5% of total arsenic. Using mobile phase 2, no arsenocholine was detected.

That most of the arsenic in mussels exposed to arsenocholine in seawater is present as arsenobetaine in the tissues, and that glycerylphosphorylarsenocholine is found in the mussel tissues, are in accord with past feeding experiments carried out with experimental fish. Yelloweye mullet (*Aldrichetta forsteri*) fed arsenocholine-treated beef readily converted arsenocholine to arsenobetaine (>90%) and two minor metabolites, glycerylphosphorylarsenocholine (*ca* 2%) and phosphatidylarsenocholine (*ca* 2%).[20, 24]

Arsenocholine could have been oxidized to arsenobetaine in the mussel tissue or in the water column. Because glycerylphosphorylarsenocholine was identified in all tissues of mussels exposed to arsenocholine, but not in mussels exposed to other arsenic componds, some arsenocholine might have been taken up by the mussels.

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