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Implications of Inorganic/Organic Interconversion on Fluxes of Arsenic in Marine Food Webs

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An organic form of arsenic is commonly encountered in marine organisms; in greysole and shrimp, it accounted for all arsenic found in muscle tissue. It has been isolated from flounder tissue by two independent procedures; it was hydrophilic, cationic, and was not decomposed to inorganic arsenic by hot nitric and sulfuric acids. NMR spectroscopy indicated all nonexchangeable protons to be equivalent; they behaved more like N-methyl protons than As-methyl protons. High-resolution mass spectroscopy from a heated probe yielded a spectrum corresponding to tetramethylarsonium ($AsMe_4^+$); the authentic ion, however, had TLC and ion-exchange behavior different from that of the natural product. Infrared spectrometry likewise produced conflicting or uninterpretable data. Decomposition of the compound for analytical purposes was accomplished by dry ashing under oxidizing conditions.

Sea urchins, like trout, converted arsenic to an organic form, but to a more limited degree.

Arsenic found naturally in sea urchins and in a species of macroalga was also organic. In individual containers, sea urchins were fed on the alga for 7 weeks. During this time they consumed 0.203 ± 0.075 mg total As and excreted only 0.036 ± 0.015 mg as feces. Measurement of inorganic As in the seawater did not account for the discrepancy, but measurements of total As did (0.202 ± 0.095 mg). Sea urchins, like humans, appear to be able to rapidly excrete these organic forms of arsenic.

Although arsenic is a moderately common anthropogenic pollutant, it can occur in high enough concentration under natural conditions to be of concern. The chemical handbooks list the concentration of arsenic in the lithosphere at 5 parts per million (ppm), sufficient, if it were evenly distributed, to bring about toxic reactions under most circumstances. In the terrestrial environment, geological disproportionation of the element has made natural instances of poisoning fairly rare; but in the more homogeneous marine environment, contact with arsenic is unavoidable. It occurs in seawater at 1–8 parts per billion (ppb) and in the sediments at 2–20 ppm (*1*). That these levels are biologically significant is indicated by the generally higher levels of arsenic in marine foods compared with terrestrial foods (*2*).

The levels of arsenic found in marine animals exceed those found in their surroundings: pelagic fish range from 0.3 to 3 ppm (*2–4*) and bottom-feeders from 1.4 to 55 ppm, higher than the averages of seawater and sediments, respectively. Therefore, bioaccumulation of arsenic must occur; biomagnification through the food chain, however, has been observed not to occur as a general phenomenon (*1, 5, 6*).

The work reported here is intended to supplement a growing body of evidence (*7–10*) that arsenic is converted by organisms to an organic storage form which is then excreted by the organism and also by predators, thus preventing food chain accumulation. Some characteristics of this detoxified

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form of arsenic have been studied in an attempt to discern its chemical structure.

Methods and Materials

Analytical Methods for Arsenic

Arsenic was measured by different methods at the two laboratories involved in this investigation. Most of the measurements on organisms, water and sediments were done in St. John's by using a variety of sample concentration or decomposition methods, followed by arsine evolution and absorption in silver diethyldithiocarbamate solution. These methods have already been thoroughly described (*11*). In Ottawa, a Philips model PW 1410 x-ray spectrograph was available. Samples dissolved in methanol were placed in a liquid sample holder, covered with Mylar film, and scanned at 1°20′/min. The x-rays were produced at a tungsten target at 50 kV and 50 ma; x-ray fluorescence was detected with a lithium fluoride analyzing crystal (2*d* spacing of 0.40276 nm) with the scintillation detector operated at 910 V.

Chemicals

Tetramethylarsonium iodide was prepared by slow addition of trimethylarsine (CAUTION: volatile, flammable poison) to excess cold methyl iodide; the white precipitate was recrystallized from methanol (J. I. Price, personal communication). Trimethylarsine was purchased from Alfa Inorganics or synthesized by the method of Ayscough and Emeléus (*12*).

^{74}As-arsenic acid was obtained from Amersham-Searle (Canada) Ltd.

All other chemicals were obtained from standard suppliers and were reagent grade.

Marine Organisms

Fish tissue containing high levels of arsenic was obtained from the Fish Inspection Laboratory, Fisheries and Marine Service, St. John's, Newfoundland through the courtesy of Mr. Keith Spencer. Samples consisted of frozen muscle from witch flounder (greysole, *Glyptocephalus cynoglossus*), "flounder" (a category including all commercial flatfish except greysole and halibut) and shrimp.

Sea urchins (*Strongylocentrotus droebachiensis*) were collected in localities near St. John's and kept in clean flowing seawater for several weeks prior to the experiments. A macroalga, *Fucus vesiculosus*, containing high levels of arsenic was collected near a stibnite mine at Moreton's Harbour, Newfoundland (*11*).

Purification of Arsenic Compounds from Marine Animals

The two laboratories involved in this study developed independent procedures for the purification of organoarsenic compounds.

In Ottawa, homogenized witch flounder muscle was first freeze-dried; 45 g dry muscle was extracted in a Soxhlet apparatus, first with $CHCl_3$, then with methanol. A portion of the methanol extract was taken to dryness on a rotary evaporator; the residue (1.3 g; 3 mg As) was then extracted sequentially with two 5-ml portions of chloroform containing 0%, 1%, 3%, 5%, 7%, 10%, 15%, 20%, 30%, 50%, and 100% methanol. The extracts containing the highest ratios of arsenic to dry weight were pooled and evaporated. The extraction procedure was repeated using 0.5% increments of methanol in chloroform. The procedure was repeated until the arsenic/dry weight ratio no longer increased. Chromatography on alumina, cation-exchange resins and gel-permeation substrates failed to increase the purity of the compound.

The procedure used in St. John's has already been described in part (*11*). This partial purification consisted of homogenization in trichloroacetic acid solution, removal of Cl_3CCOOH and lipids with benzene, and chromatography on a strong cation exchanger. The arsenic-containing fraction is eluted with 3% NH_4OH. Further purification of the greysole material was carried out as follows. The residue from the ion-exchange step was dissolved in 20 ml methanol and refluxed 24 hr. Large amounts of a white solid separated and were filtered off; all the arsenic remained in solution. Thin-layer chromatography revealed that the arsenic compound was unchanged in mobility. The filtrate was evaporated, redissolved in a minimum quantity of methanol, and deposited at the origin of a thick-layer alumina plate (Macherey-Nagel aluminum oxide G; 1 × 200 × 200 mm). The plate was developed in 95% methanol: 5% acetic acid. The arsenic band was located by removing and ashing small areas of adsorbent; the entire band was then removed and the compound eluted from the alumina with methanol. The evaporated eluate was dissolved in a minimum amount of warm methanol. The arsenic-containing material could then be precipitated with a few drops of chloroform.

Thin-Layer Chromatography

Macherey-Nagel SIL G-25 prepared silica gel plates (0.25 × 200 × 200 mm) were obtained from Brinkmann Instruments Ltd. Samples of substances to be compared were streaked on adjacent 7 cm segments of the origin line. Arsenite and arsenate were detected by spraying with ammoniacal

1% silver nitrate; tetramethylarsonium ion was detected with the Dragendorff spray reagent (*13*).

After development and spraying, the 7 cm tracks were scored into 1 cm segments. Each 1 × 7 cm segment of adsorbent was scraped into a 50 ml beaker for arsenic analysis. After analysis, a histogram of arsenic concentration versus distance was used to estimate an R_f value.

Conversion of Inorganic to Organic Arsenic in Sea Urchins

Arsenic acid-^{74}As (200 μCi; 0.2 μg) was added to 1500 ml seawater containing eight sea urchins (30–50 g). The seawater was aerated and kept at 14°C for 24 hr. The urchins were washed free of the radioactive seawater and kept in fresh seawater. At intervals, urchins were weighed. The gonad tissue was entirely removed and weighed, then 5.0 g gonad tissue was homogenized with 5.0 ml 10% sulfosalicylic acid. Total and nonanionic (organic) radio-arsenic were measured as previously described (*8*). Inorganic arsenic (+3 and +5) was separated by coprecipitation: 100μl of extract was diluted into 3.0 ml water, and 200 μl 0.45*M* ferric ammonium sulfate in 0.6*N* HCl was added; 1.0 ml 2*N* NH_4OH was mixed in slowly and the precipitate was centrifuged. The precipitate was washed with 2 ml 2*N* NH_4OH and dissolved in 3.0 ml 1*N* HCl. The entire amount was counted in 10 ml Aquasol. Quenching was not significant in the carbon-14 channel, and these figures were used in calculating recoveries. Standard addition controls revealed losses up to 25% of inorganic As in some homogenates.

Arsenic Budget in Sea Urchin–Algal System

Twelve one-liter jars were set up in a 4–6°C incubator with individual air stones. To each was added 500 ml fresh seawater and a weighed amount of algae containing 20 ± 6.2 ppm As. One sea urchin (from a group containing an average of 1.9 ± 0.37 ppm As in gonad tissue) was placed in each of eight jars. Each week, the water was changed and saved for analysis; the alga was weighed and replaced; and the feces were drained, weighed, and saved for analysis. Four jars were kept as controls, containing algae but no animals. After 7 weeks, the urchins were dissected into gonad, intestinal tissue, and blood, and analyzed for arsenic. Pooled feces and pooled water samples were analyzed.

Results and Discussion

Purification of Arsenic Compounds

The isolated arsenic compounds were white, waxy solids which appear to absorb some moisture in humid weather. The product of the Ottawa procedure varied in arsenic content from preparation to preparation, from 4% As by weight to as high as 14%. In spite of this, the NMR and mass spectra remained unchanged. This may have been a hydration phenomenon: arsine oxides and some related organoarsenic compounds are so hygroscopic they must be synthesized under very dry conditions; once hydrated, they cannot be dehydrated (*14*).

Both procedures yielded products with identical mass and NMR spectra. Thin-layer chromatography in the solvent systems listed in Table 1 and treatment with iodine vapor, sulfuric acid charring, ammoniacal silver nitrate, ninhydrin, ascorbate–molybdate and Dragendorff reagents and inspection under ultraviolet light revealed no contaminating substances. They were also identical in R_f to the arsenic in the original trichloroacetic acid homogenate.

Table 1. Thin-layer chromatography of arsenic compounds from shrimp and witch flounder.

	R_f values			
Solvent system	Greysole	Shrimp	Arsenite	Arsenate
n-BuOH:HOAc:H_2O (4:1:1.8)	0.20-0.21	0.20–0.21	0.39	0.00
n-PrOH:H_2O:HCOOH (50:45:5)	0.31	0.31		
n-PrOH:H_2O:NH_3 (80:20:1)	0.04	0.04		
MeOH:H_2O:HOAc (50:45:5)	0.44	0.45		
MeOH:HOAc (95:5)	0.61		0.1–0.2	0.8

The St. John's method was essentially quantitative to the end of the ion-exchange step. Recovery of arsenic from witch flounder was 100% (Table 2); since inorganic arsenite or arsenate are not retained on cation exchange resins, this means that essentially all the arsenic is in the organic form.

The same result was obtained in the case of shrimp muscle. All the arsenic was recovered from the ion-exchange step. The shrimp compound had the same R_f as the greysole compound in several solvent systems (Table 1) and was probably identical.

Table 2. Recovery of arsenic compound from witch flounder.

	Total As, μg	Recovery, %
Muscle (24 ppm)	2280	—
TCA extract after benzene extraction	2250	99
Portion of TCA extract	450	—
Cation-exchange eluate	462	103

Structure of the Arsenic Compounds

The properties manifested in the purification procedures indicated that the compound was hydrophilic and cationic.

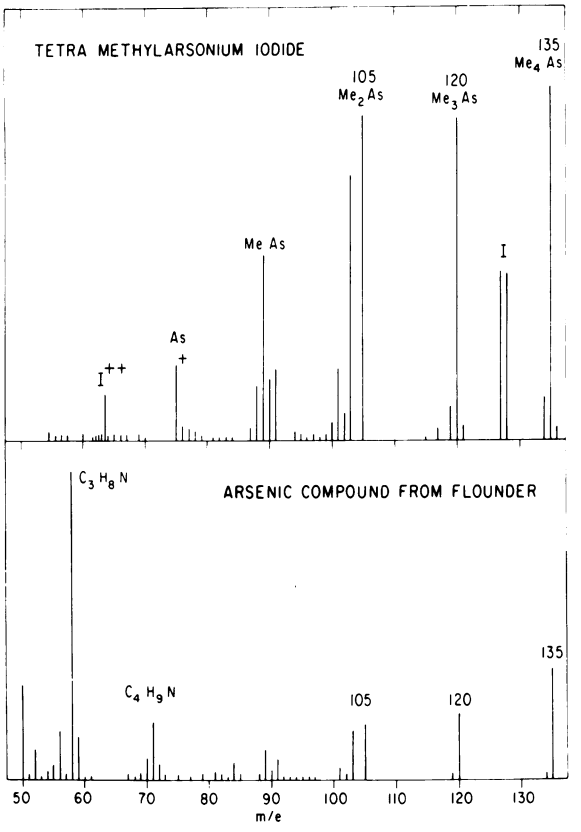

FIGURE 1. Low-resolution mass spectra of tetramethylammonium iodide and the arsenic compound isolated from witch flounder. In both cases, spectra appeared as the direct-introduction probe temperature passed 170-180 C. Empirical formulae were obtained by high-resolution mass spectroscopy.

The compound would not yield a mass spectrum at room temperature. On a heated probe, a spectrum began to appear above 170°C (Fig. 1). The high-resolution mass spectrum identified three of the fragments as $(CH_3)_nAs^+$ (n = 2, 3, and 4). There were also consistently two fragments at nominal masses 58 and 71 with empirical formulae C_3H_8N and C_4H_9N, respectively; these appeared and disappeared with the arsenic-containing ions as probe temperature increased. They, therefore, may originate from the compound.

We were prompted by the series of arsenic-containing ions to examine tetramethylarsonium ion; this is also an obvious candidate if well-established biological methylation mechanisms are carried to a conclusion. The synthetic material, although it gave the same series of ions (Fig. 1), behaved differently than the natural compound in thin-layer chromatography, and adsorbed so tightly to cation exchange resins that it could not be eluted (unlike the natural compound).

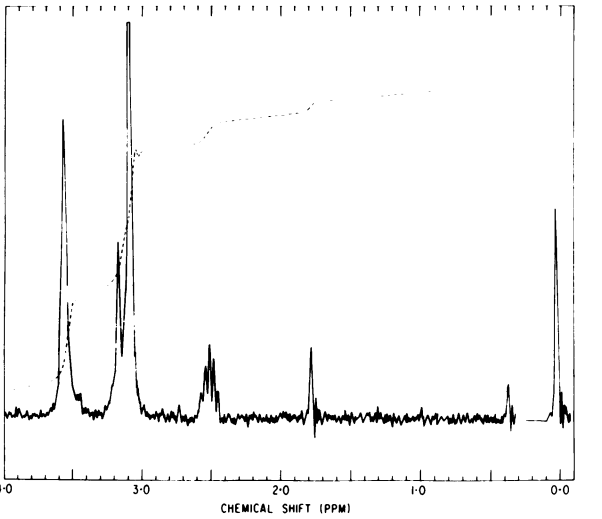

FIGURE 2. Nuclear magnetic resonance spectrum of the witch flounder arsenic compound dissolved in dimethyl sulfoxide-d_6.

The NMR spectrometer revealed a large, unsymmetrically split peak at 3.1 ppm (Fig. 2); this was in the region where *N*-methyl and *N*-methylene as well as some As-methyl protons would be expected (Fig. 3). A second, smaller peak at 1.8 ppm corresponds to As-methyl protons as seen in dimethylarsinic acid and tetramethylarsonium iodide. This peak has about 1/14 the combined area of the peaks at 3.1 ppm, but was present in the same proportions in two separate preparations; a downfield scan to 4.4 ppm assured that this smaller peak was not a sideband of the 3.1 ppm peak.

The infrared spectrum was difficult to interpret. The arsenic-methyl rock and stretch vibrations at 960 and 2940 cm^{-1}, respectively, were present, and the vibrations due to acidic arsenic (R_2AsO_2H) at 2600 and 2200 cm^{-1} are missing (*15*). We could draw

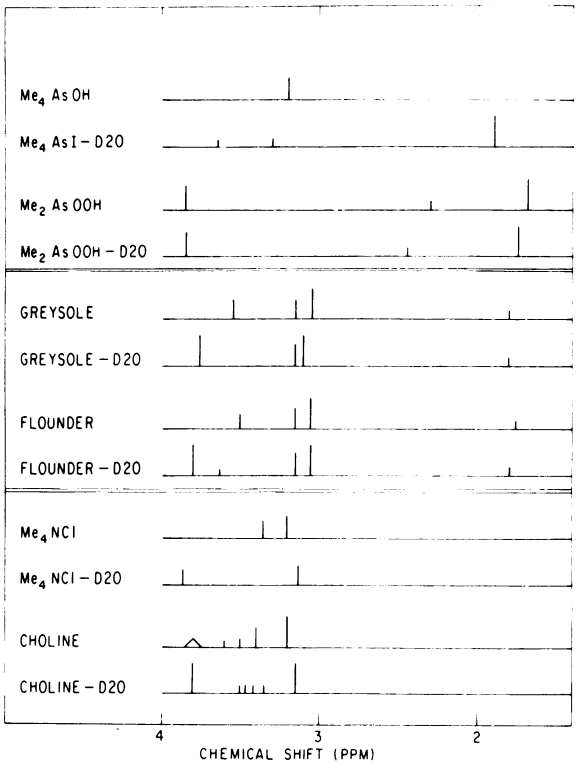

FIGURE 3. NMR resonances of the arsenic compounds from witch flounder and "flounder," and of selected reference compounds. The height of the line is approximately proportional to the peak height. The triangle symbol for choline indicates a very broad, indistinct peak. Dimethyl sulfoxide-d_6 was the solvent in all cases, and deuterium oxide (D_2O) was added where indicated.

no further information from the spectrum.

The structure of the organic arsenic compound has so far evaded elucidation. We believe it to be most important that this problem be solved, as research on the significance of arsenic in marine animals and its toxicology cannot proceed much farther otherwise.

Conversion of Inorganic to Organic Arsenic

Lunde (*9*) and Penrose (*8*) have demonstrated that inorganic arsenic is converted to an organic form by fish, provided that it is introduced by way of the digestive tract. Injecting the arsenic (*8*) or adding it to the ambient water (*9*) resulted in poor or negligible conversion. The implication is that intestinal microorganisms carry out at least the first stage of arsenic conversion. It is also known that algae are capable of carrying out this conversion (*10*). We examined sea urchins, an intermediate in littoral food chains, for this capability.

Force-feeding of urchins was not found to be feasible, so radioarsenic was administered by way of the seawater medium. After 24 hr, arsenic had been accumulated into gonad tissue to a concentration about 40% of that in the external medium. Over the next 7 days, little if any of the arsenic was lost from the animals (Table 3). Although some organic (nonanionic) arsenic was present and appeared to increase slightly over the 7 days, this increase was not statistically significant.

Table 3. Metabolism of ^{74}As-arsenious acid by sea urchins.[a]

Urchin no.	Time after end of exposure, hr	Accumulation factor[b]	Nonanionic arsenic, %	Inorganic arsenic, %
1	0.25	0.47	7.7	60
2	0.25	0.37	8.7	77
3	24	0.34	12.9	63
4	24	0.30	13.4	100
5	72	0.34	16.9	46
6	72	0.18	23.1	85
7	168	0.18	20.3	48
8	168	0.48	8.4	56
Significance of slope[c]		N.S.	N.S.	N.S.

[a]Urchins were exposed to a trace concentration of arsenic for 24 hr, then placed in a large volume of fresh seawater.

[b]Counts min per gram of gonad tissue divided by counts min/per ml seawater at the end of the exposure period.

[c]Linear regression of data on time, tested against slope = 0; N.S. = not significant at $p < 0.1$ level.

The data on inorganic arsenic must be viewed with some reservation, since the coprecipitation of inorganic arsenic in the presence of large amounts of dissolved organic material can result in underestimates of 0–25% (unpublished data). Clearly, however, inorganic arsenic persists in the urchin gonad for seven days without major excretion or metabolic conversion taking place.

The test for nonanionic arsenic is not subject to interference under these conditions and suitable controls have shown that nonanionic and inorganic (anionic) arsenic are cleanly separated (*8*). Hence, some conversion of arsenic does take place, although it does not exceed 23% of the absorbed dose. Furthermore, this organoarsenic compound is not rapidly excreted as is the compound in fish.

In a set of experiments done in another context (*11*), urchins collected very near a source of arsenic-bearing minerals appeared at first to contain very high levels of arsenic. This was traced to sand particles in the guts; when gonad tissue alone was measured, only slightly increased levels of arsenic were measurable. The same study demonstrated that arsenic was very easily leached from the min-

eral by seawater. Hence, sea urchins and presumably many marine animals have a ready source of arsenic in the sediments. The next question is, can the arsenic also be derived from their food?

Arsenic Budget in a Sea Urchin–Alga System

The urchins all survived to the end of the experiment, maintaining normal motility and adhesion to the walls of the containers, and feeding well. At the end of the 7-week experiment, they had consumed an average of 10.4 ± 3.82 g of alga each and produced 7.5 ± 3.14 of feces. At the time of this experiment, the nature of the arsenic compound was poorly understood, and only inorganic arsenic was measured in the seawater. There was an apparent disappearance of 77% of the consumed arsenic (Table 4, items A–D). Some months afterward, a better grasp of the chemistry involved prompted us to attempt the measurement of organic arsenic in the seawater samples. Seawater was mixed with the

Table 4. Arsenic budget for eight sea urchins fed 7 weeks on a macroalga high in arsenic.

	Arsenic, μg per animal
A. Arsenic consumed	203 ± 74.5
B. Fecal arsenic	36 ± 15.0
C. Arsenic lost (A minus B)	167 ± 61.3
D. Inorganic arsenic in seawater	38 ± 14.5
E. Total arsenic in seawater	202 ± 95.1
F. Organic arsenic in seawater (E minus D)	164 ± 96.2

$Mg(NO_3)_2$–MgO ashing aid, evaporated to dryness, and ashed by using the same temperature regime employed for tissue samples (*11*). The missing arsenic was found in these samples (Table 4, item E). Furthermore, the amount of total arsenic found in the seawater correlated well with the weight of alga consumed (r = 0.994; Fig. 4). The urchins themselves showed no difference in arsenic concentrations, about 2 ± 1.0 ppm in gonad tissue, and controls which contained alga but no urchins showed no increase in arsenic in the seawater above the 1.7 ppb basal level. The route of organic arsenic excretion is not known at this time, nor do we know whether consumed organic arsenic mingles in the same metabolic compartment with organic arsenic derived from ingested inorganic arsenic.

We can, however, propose a hypothesis that (1) organisms at each trophic level have a greater or lesser capacity to convert inorganic arsenic to a (presumably) detoxified organic form, and (2) organisms at the next higher trophic level can take advantage of this detoxification to rapidly excrete

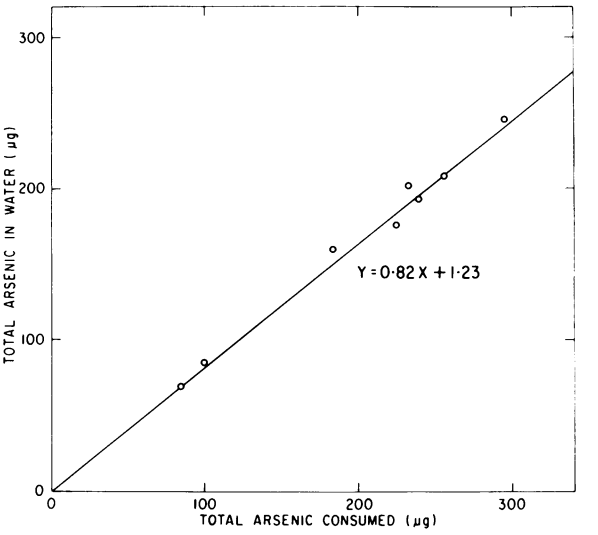

FIGURE 4. Relationship of total arsenic recovered from seawater to total arsenic consumed by individual sea urchins.

ingested organic arsenic. Rapid excretion of the organic arsenic compound has been demonstrated in fish (*8*) and man (*16*) as well as other mammals (S. Charbonneau, personal communication). Generally, there is no bioaccumulation of arsenic in food chains, probably as a result of this. Organisms containing high levels of arsenic in their tissues tend to be those that are prone to incidental ingestion of sediment particles while feeding (see Introduction). Certainly, considerably more work is needed on the converting ability and organic arsenic content of other organisms of importance in marine food webs in order to prove or disprove this generalization.

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