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The Synthesis of Fat and Water Soluble Arseno Organic Compounds in Marine and Limnetic Algae

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Two green algae (*Chlorella ovalis* and *Chlorella pyrenoidosa*), one blue green (*Oscillatoria rubescence*) and two diatoms (*Phaeodactylum tricornutum* and *Skeletonema costatum*) were cultivated in fresh and/or salt water media containing radioactive arsenic ions. The incorporation of arsenic into various lipid and water soluble fractions was studied by chromatographic separation and radioactivity measurements. All the algae were found to synthesise both lipid and water soluble arseno organic compounds. Acid treatment converted different arsenolipids into a water soluble product which seemed to be identical to an arseno organic compound isolated from fat free algal material under mild conditions.

Growth of the two algae, *Phaeodactylum tricornutum* and *Chlorella ovalis*, was not influenced by the presence in the medium of 10 – 30 ppm and 1 – 3 ppm of arsenic salts having a 1:1 ratio of tri to pentavalent arsenic ions. Enrichment of arsenic (as arseno organic compounds) in the algae corresponded to 200 – 3000 times the concentration in the medium. Algae seem to be an important source of the arseno organic compounds found in higher marine organisms.

Earlier work has shown that there is a significant difference in the level of arsenic in marine plants and animals as compared with that found in terrestrial plants and animals.[1,2] It has also been established that in marine animals the arsenic both in the lipid and the non lipid phase is present as arseno organic compounds.[3–5] Other results indicate that at least lipid soluble arseno organic compounds are present in marine algae.[6] Arsenic has not been detected in the lipid phase in either plants or animals of terrestrial origin, indicating that these do not synthesise arseno organic compounds corresponding to those found in marine organisms.[3]

Little is known about the structure and chemistry of the arseno organic compounds present in marine organisms. Earlier results indicate that they are biologically very stable, as they are not broken down to inorganic arsenic when they are taken in orally by mammals.[7] These results also imply that the arseno organic compounds which are found in marine samples are less toxic than inorganic arsenic, especially arsenite.

The presence of both lipid soluble and water soluble arseno organic compounds in fish and other marine animals suggest [6] that marine algae may serve as a source of the arseno organic compounds found in organisms higher in the food chain. Previous results indicate that fish (*Salmo gardneri*) is able to synthesise lipid and water soluble arseno organic compounds from arsenite/arsenate mixed into the feed. The amount of arseno organic compounds present in the fish from this source is, however, shown to be small compared to that derived from lower stages in the marine food chain.[8]

In view of these findings it is of interest to study the uptake of arsenic in algae and to characterise the lipid soluble (and eventually the water soluble) arseno organic compounds synthesised by the algae in more detail. An investigation of this type may be carried out under laboratory conditions by adding radioactive arsenic to algal cultures and subsequently analysing for the radioactive compounds synthesised.

EXPERIMENTAL

Growth experiments. The algae were grown in 2 l spherical flasks at the Norwegian Institute for Water Research as previously described [6,9] in nutrient enriched media [10] based on uncontaminated sea water for *Chlorella ovalis* Butcher, *Phaeodactylum tricornutum* Bohlin and *Skeletonema costatum* (Grev.) Cleve. The green algae *Chlorella pyrenoidosa* Chick as well as *Phaeodactylum tricornutum* and *Oscillatoria rubescens* (D. C.) were grown in a fresh water medium based on distilled water with nutrients added. When the growth of the algae was well under way (approx. one week), half of the algal solution was filtered off each day, and new culture medium was added from the stock solution. The filters were stored in chloroform prior to analysis.

For each culture experiment 10 l of medium were prepared and between 0.01 and 0.1 mCi of arsenic tracer (As-74, AJS.1, Amersham) was added. At the time of addition the radioactive arsenic tracer consisted of a mixture of arsenite and arsenate (3/2).

In a second series of experiments *Chlorella ovalis* and *Phaeodactylum tricornutum* were grown with inactive arsenite/arsenate (1/1) added to the radioactive arsenic in the following amounts: 3, 30, 3×10^2, 10^3, 3×10^3, 10^4, and 3×10^4 μg/l.

To compare arsenite and arsenate in the absorption process, two identical cultures of the alga *Phaeodactylum tricornutum* were started. To one culture was added 30 ppb As^{3+} together with 0.04 mCi As^{3+} tracer, and to the other 30 ppb As^{5+} with the corresponding amount of As^{5+} tracer. Half a liter of the culture was filtered after 1 d, 2 d, 4 d, and 8 d, respectively. The amount of As^{3+} and As^{5+} in the filtered medium, after the experiments were finished, was determined by molecular gel filtration.* Samples were stored at -20°C prior to analysis.

Separation of organic and inorganic arsenic. Lipids were extracted from the algal material with chloroform/methanol/water (4/2/1). Following separation from the aqueous-methanolic phase, the chloroform phase was washed twice with distilled water to which inactive arsenite-arsenate had been added in order to dilute any inorganic radioactive arsenic ions present in the chloroform. The algal material was subsequently boiled in water for 20 min to produce an aqueous extract. The pH of this was adjusted to 7 – 8 by NH_3, and the solution was run through a column of anion exchange resin (Dowex 2 × 8, 200 – 400 mesh) previously equilibrated with 0.2 N HCl and washed with water. Inorganic arsenic ions are adsorbed on the resin under these conditions, while organic bound arsenic is eluted.[5]

Inorganic radioactive arsenic was also removed as volatile $AsCl_3$ by distilling it off from a solution adjusted to 6.6 N HCl. During this treatment the As^{5+} present will be reduced to As^{3+}. $AsCl_3$ will distil at 100°C; to prevent decomposition of arseno organic

* The two valencies of arsenic will be separated on a 1 m column loaded with Sephadex G 25 Fine (Pharmacia Fine Chemicals, Sweden), using 0.05 M NH_3 as eluting agent (unpublished results).

compounds, the temperature should be kept at 100 – 105°C, particularly towards the end of the process. The degree of separation obtained was estimated on thin layer chromatography by autoradiography.

All measurements of radioactivity were performed with a 2 × 2″ NaI "welltype" scintillation detector.

Thin layer chromatography (TLC). Adsorbent layers of about 1 mm thickness were used for the analyses performed by TLC, in order to obtain sufficient amounts of the synthesised radioactive arsenic compounds to allow the autoradiographic measurements. The use of 1 mm adsorbent layers resulted, however, in a reduction of both the separation and reproducibility usually obtained with TLC.

A portion from the aqueous extracts produced by boiling of defatted algal material was chromatographed in a system of chloroform/methanol/ammonia (2/2/1) (system 1) with cellulose powder (MN 300, Macherey, Nagel & Co., Düsen, GFR) as adsorbent.[11] The plate dimension was 20 by 20 cm. Some fractions from separation of the water extracts by molecular gel-filtration were characterised in a two-dimensional TLC procedure, in which the solvent system described above was used in the first direction, and methanol/water/pyridine (10/5/1) in the second (system 2).

The lipids extracted from the algal material were investigated using two different techniques. A sample of 10 – 20 mg of oil was dissolved in chloroform and polar lipids separated from the non-polar ones on a silica gel column (SiO_2, 0.2 – 0.5 mm, for chromatography, E. Merck AG, Darmstadt, West Germany). The non-polar lipids were eluted with chloroform, and the polar lipids subsequently with 90 % methanol in chloroform. As more than 98 % of the radioactivity followed the polar fraction, the latter was characterised further by "Kieselgel" (7731, E. Merck AG, Darmstadt, West Germany) using chloroform/methanol/water (65/25/4) for the development [12] (system 3).

The algal lipids were also analysed directly by TLC in the system used for the water extract (system 1, described above). The lipid soluble arseno organic compounds followed the solvent front. They were detected by autoradiography, scraped out and refluxed in hydrochloric acid (6.6 N) for 2 h. The hydrolysate was concentrated by evaporation and the residue extracted with ethanol. Samples of the ethanol solution were subsequently analysed by TLC on system 1. All plates were sprayed with ninhydrin reagent in order to detect amino acids or other compounds containing amino groups.

To see whether inorganic arsenic ions could be complexed by algal material and move in the TLC systems employed, radioactive arsenite-arsenate was added to inactive aqueous algal extracts and subjected to analysis by TLC in system 1.

Molecular gel-filtration. Aqueous algal extracts were subjected to molecular gel filtration on a dextrane resin (Sephadex G 25 Fine, Pharmacia Fine Chemicals, Sweden). A column with a diameter of 15 mm and a height of 25 cm (K 15/25, Pharmacia Fine Chemicals, Sweden) was used, and absorbance at 254 nm in the eluate monitered by a Uvicord spectrophotometer (LKB, Sweden). A 0.005 N (pH 7 – 8) ammonia solution was used as eluting agent. The eluate was collected in 10 ml fractions and the activity of each was measured. The fraction with the highest radioactivity was analysed by TLC in system 1. Aliquots from each fraction were tested for ninhydrin-positive compounds by application to filter paper, drying and spraying with ninhydrin reagent in the conventional manner.

Autoradiography. Radioactive arsenic compounds separated on TLC were detected by autoradiography. The TLC plates were dried thoroughly and placed in contact with sensitive X-ray emulsion (Ilford Industry G film). Exposure time was 1 – 14 days, depending on the amount of activity present. The film was subsequently developed as ordinary X-ray emulsion.

RESULTS AND DISCUSSION

TLC analyses of the extracts from the defatted algae all show a basic spot with an R_F value of about 0.30 (A, Figs. 1 and 2). The thickness of the adsorbent layer and poor reproducibility in preparing the plates resulted in considerable variations of this R_F value. This spot (A) is present in all the aqueous extracts produced from different algal species investigated. Despite the

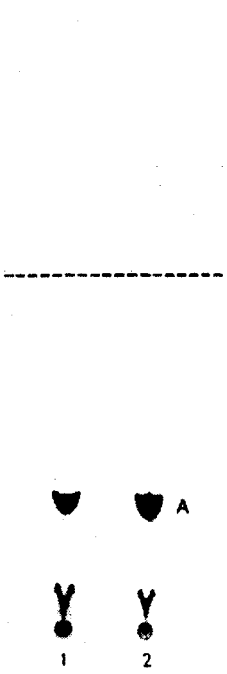

Fig. 1. Autoradiograph of TLC plate of aqueous extracts produced from *Phaeodactylum tricornutum* (1) and *Chlorella ovalis* (2). The exposed area (dark) shows the presence of radioactive arseno organic compounds. The main compound is marked A. More detail is shown on the original film. Analytical condition: Cellulose substrate (1 mm); developing solvent, chloroform/methanol/ammonia, 2/2/1 (system 1).

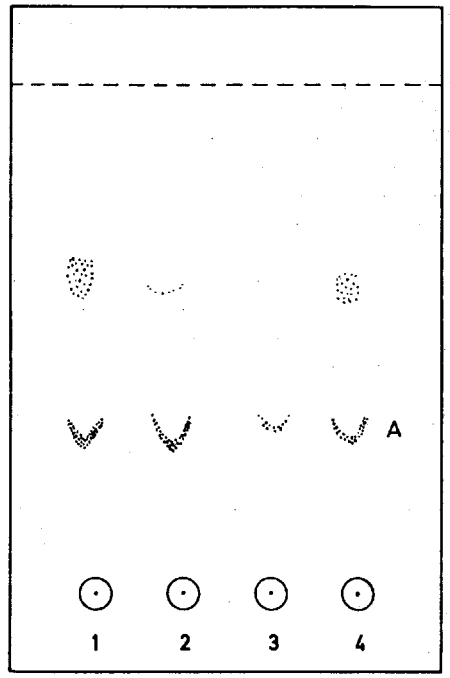

Fig. 2. Reproduction of an autoradiograph of TLC plate of aqueous extracts from *Phaeodactylum tricornutum* cultured in salt water (1) and in fresh water (2), and *Chlorella ovalis* in salt water (3) and *Chlorella pyrenoidosa* in fresh water (4). The main radioactive arseno organic compound present is compound A. Analytical conditions as described in Fig. 1.

lack of a rigid proof, the spot is supposedly caused by a single compound, namely compound A. Its presence is independent of the means used to remove the inorganic radioactive arsenic ions. There are also some weaker and more diffusely exposed areas corresponding to compounds with higher and lower R_F values. This is particularly so for the chromatograms where the aqueous extracts were treated by the ion exchange process. The amount of these compounds, in particular those with an R_F value greater than 0.30, are reduced in extracts subjected to treatment by 6.6 N hydrochloric acid. Compounds with R_F values less than 0.30, which are more polar than A, are present in all the water extracts.

A slight exposure at the spots of application on TLC plates is seen mainly for the extracts treated with hydrochloric acid. This should be compared

with results obtained when radioactive arsenite-arsenate was added to an inactive algal solution, demonstrating that the inorganic arsenic ions are stationary or moving only slightly under the TLC conditions used. Stationary radioactive arsenic compounds in the aqueous algal extract thus probably consist of arsenite-arsenate or other types of arsenic ions.

The ion exchange process is the preferred method for removal of inorganic radioactive arsenic from the aqueous algal extracts. The method is less drastic than the treatment with hydrochloric acid and seems not to cause changes in the chemical status of the arsenic. Some of the arseno organic compounds may, however, be irreversibly absorbed to the ion exchange resin. Changes, or decomposition of arseno organic compounds, may also take place in the preparation of the aqueous extracts, particularly during the boiling step. Formation of compound A is the most likely reaction.

TLC of the aqueous extracts from the algal clones of *Phaeodactylum tricornutum*, cultured both in fresh and salt water, and the two species, *Chlorella ovalis* and *Chlorella pyrenoidosa*, cultured in salt and fresh water, respectively, indicates that the concentration of compounds A is somewhat higher in the algae grown in salt water (Fig. 2).

The radioactivity in the non-polar and in the polar fractions of the algal oil, after these had been separated on the silica gel column, shows that nearly all the radioactivity (> 98 %), *i.e.* all the lipid soluble arseno organic compounds synthesised by the algae, follow the polar fraction. These results are in accordance with previous results reported for the lipid soluble arseno organic compounds present in fish oils.[13,14]

TLC-separation of the polar lipids made visible by autoradiography (Fig. 3) indicates the presence of lipid soluble arseno organic compounds different from those detected in the aqueous extracts. Moreover, the pattern of arsenic containing compounds varies with the various algae studied (*Chlor-*

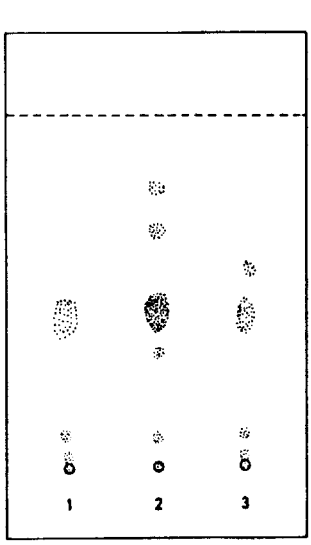

Fig. 3. Reproduction of an autoradiograph of TLC plate of the polar lipids from *Sceletonema costatum* (1). *Phaeodactylum tricornutum* (2) *and Chlorella ovalis* (3). Analytical conditions: Kieselgel G substrate (1 mm); chloroform/methanol/water, 65/25/4 (system 3).

ella ovalis, *Oscillatoria rubescens*, and *Phaeodactylum tricornutum*). A similar difference has been reported for lipid soluble arseno organic compounds in invertebrates.[15] This observation could be of significance, but new lipid soluble arseno organic compounds may be created as a result of the experimental conditions when extracting and fractionating the algal lipids.

TLC of algal lipids in system 1 shows the lipid soluble arseno organic compounds to move with the solvent front, as do the other algal lipids. In some of the samples, however, a well defined spot with an R_F value of *ca.* 0.30 was found. Presumably this is traces of compound A found in the aqueous extract.

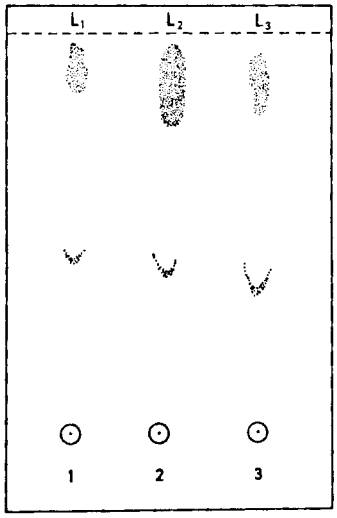

Fig. 4a. Reproduction of autoradiographs of TLC plates of the total amount of lipids extracted from *Oscillatoria rubescens* (1), *Phaeodactylum tricornutum* (2), and *Chlorella ovalis* (3). The lipids (L^{1-3}) will have R_F values close to 1 in system 1.

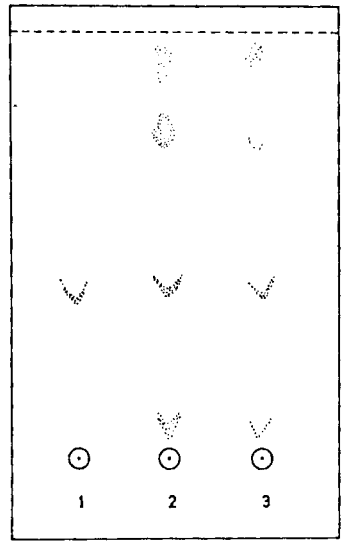

Fig. 4b. The lipids (L^{1-3}) shown in Fig. 4a were scraped out, treated with HCl and extracted with ethanol (see text). These extracts were analysed under the same conditions as described in Fig. 4a (see text).

Scraping out the lipid soluble compounds as detected by autoradiography and refluxing with hydrochloric acid (6.6 N) again produced a well defined spot at R_F 0.30 on repeated TLC-separation in system 1. Apparently compound A from the aqueous extract is also produced from the lipid soluble compounds on treatment with hydrochloric acid. A positive ninhydrin reaction is always associated with this compound. This fact becomes especially relevant when the positive reaction was obtained with compound A prepared from lipid soluble arseno organic compounds isolated by TLC, since this operation will remove most foreign ninhydrin positive material. Two-dimensional TLC of

the most radioactive fraction from the molecular gel-filtration gave coincidence between radioactivity and ninhydrin reaction.

Figs. 5 a-b show how the production of lipid and water soluble arseno organic compounds varies, as the concentration of inorganic arsenic ions is

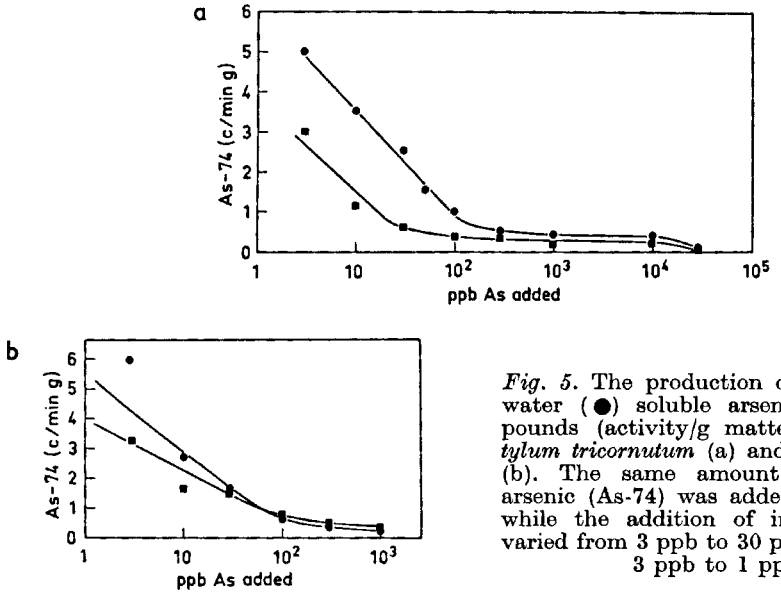

Fig. 5. The production of lipid (■) and water (●) soluble arseno organic compounds (activity/g matter) in *Phaeodactylum tricornutum* (a) and *Chlorella ovalis* (b). The same amount of radioactive arsenic (As-74) was added in each batch while the addition of inorganic arsenic varied from 3 ppb to 30 ppm (a) and from 3 ppb to 1 ppm (b).

increased in the medium under otherwise identical conditions. The ordinate is activity (counts/min) divided by weight of lipids and of dehydrated aqueous extracts, respectively. Both *Phaeodactylum tricornutum* and *Chlorella ovalis* show the same behaviour, which indicates two different patterns of uptake. One applies to media containing up to about 100 ppb of inactive arsenic ions added, and represent an active absorption of the arsenic. The percentage amount of arsenic converted to arseno organic compounds depends on the concentration of arsenic ions in the medium. The other applies to concentrations of arsenic from 100 ppb up to 10 – 30 ppm As for *Phaeodactylum tricornutum* and at least to 1 ppm As for *Chlorella ovalis*, and shows that an approximately constant fraction of the arsenic in the medium is absorbed. In the latter case an equilibrium between the arsenic absorbed and the arsenic present in solution seems to be established up to the concentration where the toxic effect of the arsenic begins to limit the alga's growth (and finally causes its death at around 30 ppm arsenic added (*Phaeodactylum tricornutum*)).

The results of the experiments where arsenite and arsenate were added independently and with radioactive arsenic tracer of the same valency show that more lipid soluble arseno organic compounds are synthesised by the culture with As^{5+} added. This is especially so in the first samples taken after one day. Gel filtration of the media shows that the arsenic in the two solutions

of As^{5+} and As^{3+}, respectively, will tend to reach an equilibrium between As^{3+} and As^{5+}. Although preliminary and not conclusive, the results indicate that the arsenate is used or preferred for the synthesis of arseno organic compounds in the algae.

Some results for the accumulation of arsenic in the algae compared to the concentration of arsenic in the culture media were obtained. The measurement of the radioactivity of the lipid and aqueous phase (counts As/g), produced from the various algae, was compared with corresponding measurements of the radioactivity of the medium. These results are shown in Table 1. They indicate

Table 1. Accumulation of arsenic [a] in algae as arseno organic compounds in the lipid phase and in the aqueous phase, respectively.

	Culturing media			
	Salt water		Fresh water	
	Lipid phase	Aqueous phase	Lipid phase	Aqueous phase
Phaeodactylum tricornutum	2900	2000	2800	1800
Chlorella ovalis	1600	1300		
Chlorella pyrenoidosa			400	190
Oscillatoria rubescens			540	240
Skeletonema costatum	1100	710		

[a] The calculation is based on the ratio between organic bound As-74 in the lipid phase and inorganic As-74 in the medium, and correspondingly in the aqueous phase and in the medium.

an accumulation factor of 250 – 3000 in the algae. It should be noted that the arsenic is somewhat more enriched in the lipid phase. From Figs. 5 a – b it must also be assumed that the accumulation is dependent upon the amount of arsenic present in the culture medium. Here the degree of accumulation will decrease with increasing arsenic concentration in the culture solution up to about 100 ppb (*Phaeodlactylum tricornutum* and *Chlorella ovalis*),

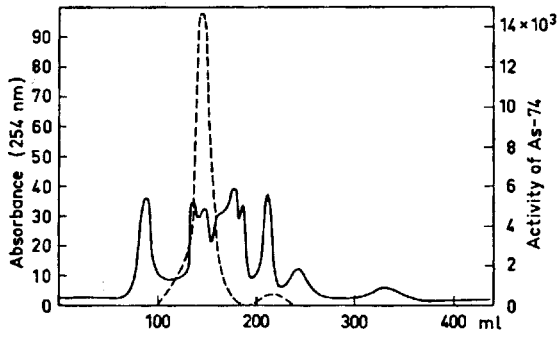

Fig. 6. UV absorbance (–) at 254 nm of molecular gel-filtrated aqueous extract from *Phaeodactylum tricornutum*. The radioactivity (– – –) of the eluate was measured in fractions of 10 ml.

and then be constant until at least a concentration of about 1 – 10 ppm arsenic in the solution is reached. The accumulation in this region is a factor 15 – 20 below the values found with no inactive arsenic added to the medium.

The results of the molecular gel filtration analysis of the water extracts are presented in Fig. 6. They show that the radioactive arseno organic compounds are eluted in two regions, one of smaller and one of larger molecular weight. The larger lies in the region where the majority of the water soluble arseno organic compounds from fish glue water are eluted. [4] This indicate that the main water soluble organic arsenic compounds in algae and fish are of similar molecular weight. By testing the fractions with ninhydrin reagent it is shown that these compounds are eluted before the amino acids. The two-dimensional TLC analyses of this fraction and subsequent autoradiography of the plate showed that no other radioactive arseno organic compounds could be detected and furthermore that the fraction shows a positive ninhydrin reaction.

CONCLUSIONS

On the basis of the results obtained in this work it is concluded that unicellular algae of limnetic as well as of marine origin are able to synthesise both fat soluble and water soluble arseno organic compounds from inorganic arsenic ions. The lipid soluble compounds are relatively unstable and may by a suitable treatment be converted to a water soluble arseno organic compound which cannot be distinguished from that which is most abundant in aqueous extracts. This main compound is present in all the algal species studied regardless of whether they are cultivated in salt or in fresh water. The results suggest that the arseno organic compounds present in the algae may be one important source for corresponding compounds found in marine organisms at higher stages in the marine food chain.

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