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Evidence Supporting the Presence of Dissolved Dimethylarsinate in the Marine Environment

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Experiments have been carried out to test the commonly held belief that the dissolved dimethylarsenic species present in marine waters is dimethylarsinate. By employing a novel combination of HPLC and cryogenic-trap hydride generation atomic absorption spectroscopy (HG AA), the sensitivity limitations of conventional in-line HPLC instrumentation can be overcome, permitting dimethylarsinate to be measured by HPLC at levels below 200 pg cm^{-3}. A comparison of results obtained by this procedure with those obtained by direct cryogenic-trap HG AA demonstrated that much of the dissolved dimethylarsenic present in the mouth of the Beaulieu River estuary (Hampshire, UK) had HPLC retention characteristics consistent with the presence of dimethylarsinate. Copyright © 1999 John Wiley & Sons, Ltd.

Keywords: dimethylarsenic; dimethylarsinate; seawater; arsenic; hydride; atomic absorption spectroscopy; methylation; HPLC; speciation; water analysis

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INTRODUCTION

Investigations carried out in the 1940s into the nature of Gosio gas[1] led Challenger to write his now-classic review of the biomethylation processes involved in the formation of methylated arsines[2]. In that review he outlined the steps which would be necessary to take arsenate through to a polymethylated arsenic oxy-anion, a process which would involve the sequential reduction and methylation of arsenic (Scheme 1), eventually producing the dimethylarsinate ion.

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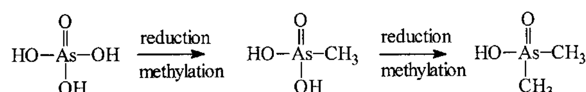
This has often been presented as a proven reaction mechanism. It has frequently been incorporated in modern arsenic biogeochemical cycles without identification of intermediate steps or sound evidence having been gathered that some of the species are present.

A very specific problem is that confusion has arisen in the literature as to whether the presence of the dissolved dimethylarsinate ion in the sea has ever been proven. In 1973 Braman and Foreback published the first report of dissolved methylated arsenic species being present in natural waters.[3] This information was obtained using newly developed cryogenic-trap procedures for the measurement of inorganic arsenic species. Unfortunately the fact that their technique would not distinguish between dimethylarsinate and any other chemical producing dimethylarsine was not generally appreciated and it was stated that dimethylarsinate had been found. Since that time the belief has been perpetuated that dissolved dimethylarsinate had been identified as being present in marine waters.

Dimethylarsenic has since been found in a variety of natural systems, ranging from seawater and marine algae[4] to urine.[5] In 1974 it was suggested that the interstitial waters of anoxic sediments might be the source of the methylated arsenic,[6] but no evidence could be found for its presence in the interstitial waters of sediments from the Santa Barbara Basin or from the deep Northeast Pacific. Dimethylarsenic was found in the photic zone, however, and its presence was correlated with indicators of primary productivity such as the chlorophyll concentration and ^{14}C uptake.[7] Only later were methylated arsenic species found to be



Scheme 1 Direct methylation of arsenate according to Challenger.

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present in the interstitial waters of estuarine sediments.[8–10]

The exceptional sensitivity of the cryogenic-trap hydride generation technique for arsenic speciation means that it is often the only suitable method. This is particularly the case in studies of the dissolved arsenic species in marine waters. The underlying principle is that sodium borohydride converts methylated arsenic species to their corresponding methylated arsines. There is currently no evidence to suggest that the borohydride reagent is capable of cleaving the As–C bond (except in some quaternary arsonium compounds) and the number of methyl groups attached to arsenic in these circumstances is believed to be conserved. Compounds such as dimethylarsinate, for example, yield dimethylarsine. This is not the case with the dimethylarsinosugars,[11,12] however, as in these compounds the dimethylarsenic moiety is attached to the sugar by an additional As–C bond. Evidence for the presence in the water column of dimethylarsenic species other than dimethylarsinate is to be found in the discovery of two operationally defined forms of dissolved dimethylarsenic known as 'hidden arsenic'[13] and 'refractory arsenic'.[14] Whether it is correct to deduce that dimethylarsinate is present from the generation of dimethylarsine rests on whether other compounds might be present which react with sodium tetrahydroborate to yield dimethylarsine. These might be, for example, reduced dimethylarsenic(III) compounds such as dimethylarsinous acid[15,16] or dimethylated arsenic(III) thiols.[17] Both of these have been found in anoxic systems and might be expected in oxic systems which are out of thermodynamic equilibrium.

Since the original discovery of dimethylarsenic in marine waters, the presumption has been perpetuated that this had been shown to be dimethylarsinate. There are still few, if any, reports of experiments to test the hypothesis that this dissolved dimethylarsenic is in fact dimethylarsinate.

In systems containing higher levels of arsenic, ion-exchange HPLC has become an accepted means of identifying the presence of dimethylarsenic acid (DMAA). Unfortunately current HPLC systems cannot detect the low level of dimethylarsinate which is present in an unpolluted seawater sample as it elutes. This is the case for both conventional HPLC instruments and for those which are capable of arsenic-specific detection (by atomic absorption spectroscopy, inductively coupled plasma mass spectrometry-MS etc). To overcome the problem, this paper reports a new approach which combines the identification of dimethylarsinate by HPLC retention behaviour with the sensitivity which can be achieved by using cryogenic-trap hydride generation atomic absorption spectroscopy (HG AA). Additional confirmation of the presence of a dimethylarsenic compound eluting in the dimethylarsinate retention time window is provided by the generation of dimethylarsine from the fraction.

EXPERIMENTAL

Analytical methods

This paper brings together two analytical procedures for the identification of dimethylarsinate in water. In the first stage of the analysis the arsenic species in the sample are separated by anion-exchange high-performance liquid chromatography (HPLC). By employing dimethylarsenic acid (DMAA) standard solutions and on-line arsenic-specific detection, the eluent fraction is identified which contains the eluting DMAA. This permits the DMAA fraction to be collected blind during the HPLC separation of samples containing such low arsenic levels that the in-line atomic absorption (AA) detection system is incapable of detecting them. Eluent fractions are collected from the HPLC separation which can then be analysed by cryogenic-trap hydride AA. Compound identification is provided by HPLC retention behaviour and supported by the generation of dimethylarsine in the subsequent cryogenic-trap (HG) AA procedure. Quantification of the DMAA is possible using the cryogenic-trap AA procedure.

Reagents and glassware

Standard solutions (1000 mg As dm^{-3}) of arsenite, arsenate, monomethylarsenic acid (MMAA) and DMAA were prepared from arsenic trioxide (As_2O_3), disodium hydrogen arsenate heptahydrate (Na_2HAsO_4), the disodium salt of monomethylarsonic acid ($Na_2CH_3AsO_3$) (reagent grade, Pfaltz and Bauer) and the sodium salt of dimethylarsinic acid [$Na(CH_3)_2AsO_2 \cdot 7H_2O$] (reagent grade, Pfaltz and Bauer), respectively. Arsenic concentrations were checked by flame AA. Secondary stock solutions, containing 10 mg As dm^{-3}, were then prepared by dilution of the 1000 mg As dm^{-3} solutions.

Sodium tetrahydroborate(III) ($NaBH_4$) (Aldrich) solutions were prepared daily in deionized water to

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give a concentration of 2 or 4% (w/v). Hydrochloric acid (Fluka, 'arsenic free') was diluted with deionized water to give final concentrations of 2% (v/v) or 0.05 mol dm^{-3}. L-Cysteine (reagent grade) was obtained from Sigma.

Potassium dihydrogen orthophosphate (KH_2PO_4) (BDH) solutions were prepared in deionized water, their pH was adjusted to the required value by the addition of sodium hydroxide, then they were degassed by ultrasonic treatment under vacuum.

HPLC separation of arsenic species

The HPLC separation of anionic arsenic species was carried out on a Partisil SAX10 column (25 cm × 4.6 mm i.d., 10 μm particle size) eluted with a potassium dihydrogen orthophosphate buffer.[18] In order to identify the DMAA retention time, the HPLC was fitted with an on-line HG AA detection system[8] derived from an ion chromatography system as reported by Ricci *et al.*[19] but omitting the persulphate oxidant. This was constructed by the direct coupling of the outlet tube from the HPLC column to a continuous-flow hydride generation AA system reported previously.[20] Solution eluting from the HPLC column was mixed with hydrochloric acid solution (3 mol dm^{-3}, 2.5 cm^3 min^{-1}) and then sodium tetrahydroborate solution (2% w/v, 2.5 cm^3 min^{-1}). After passing through a reaction coil, the liquid was mixed with a flow of nitrogen (100 cm^3 min^{-1}) and the gas phase was then separated from the liquid in a custom-built gas–liquid separator. The gas was dried by passage over sodium hydroxide pellets and the arsenic was detected by atomization in an electrical furnace (*ca* 1000 °C) aligned in the light path of an atomic absorption spectrometer (Varian AA5, Varian AA175 or Baird A5100).

The retention time window covering the elution of dimethylarsinate from the column was identified by the separation of arsenic species which had been added to seawater and by detecting the eluted arsenic compounds by on-line HG AA.

This on-line HG AA detection procedure cannot be employed, however, to determine directly the naturally occurring DMAA as such instrumentation has insufficient sensitivity (the naturally occurring dimethylarsenic species in coastal seawater samples are normally present at concentrations below *ca* 400 ng As dm^{-3}). Once the elution time of the DMAA had been determined by HG AA detection, the HG AA module was removed from the HPLC system and in subsequent experiments discrete eluate fractions were collected for analysis using the more sensitive cryogenic-trap system.

Cryogenic-trap HG AA of arsenic species

Two variants of the cryogenic-trap HG AA speciation procedure were employed. The first hydride procedure was a variation of our original procedure.[21,22] In subsequent work an alternative version of the technique was employed in which the sample was pre-treated with cysteine (20 mg cm^{-3}).[23] In both procedures the sample was acidified and reacted with sodium tetrahydroborate, producing arsines which were then swept by a flow of nitrogen into a cryogenic trap. This trap contained Chromosorb W-HP coated with 3% w/w silicone OV-3, and was cooled by liquid nitrogen. When all the arsines had been collected, the trap was gently warmed by electrical heating, sequentially releasing the arsines into a quartz-T tube held at *ca* 900 °C in an electrically heated furnace. Arsenic atoms generated in the furnace were monitored by an atomic absorption spectrometer (Varian AA5, Varian AA175 or Baird A5100).

Sampling

Samples for study were collected from the estuary of the River Beaulieu, which is an essentially non-polluted river in southern England, approximately 15 km long and draining the heathland and forest areas of the New Forest into the Solent. It is a well characterized estuary with dissolved methylated arsenic levels at their highest in the estuary mouth.[24]

Water was collected from Lepe Beach, just outside the Beaulieu Estuary mouth, and from three points just within the mouth of the estuary. All samples were taken close to high tide. The samples were collected directly into acid-washed polyethylene bottles and returned to the laboratory for immediate filtration (acid-washed Whatman GF/C).

Identification of dimethylarsinate

The cryogenic-trap HG AA procedure was employed as a screening procedure to identify samples containing dimethylarsenic species. Samples identified as containing dimethylarsenic were then subjected to an ion-exchange HPLC separation: 200 μl aliquots of the filtered sample were injected onto the HPLC column and the HPLC eluate was collected over the time window covering the elution of dimethylarsinate. This fraction was then analysed for the presence of dimethylarsenic by the cryogenic-trap HG AA procedure.

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RESULTS

The chromatographic behaviour of DMAA

Anion-exchange chromatography of seawater is affected by the ionic strength, pH and buffering capacity of the sample. Care must therefore be taken to ensure that the nature of the sample matrix does not significantly alter the retention behaviour of the DMAA, as in this work HPLC eluent fractions were to be collected 'blind' for subsequent cryogenic-trap HG AA analysis.

The pH and ionic strength of the solvent employed for the HPLC separation stage was adjusted to identify optimal analysis conditions and to ensure that the chromatographic behaviour of dimethylarsinate was understood when the DMAA was present in a seawater matrix. Preliminary experiments were therefore carried out using a directly coupled HPLC–HG AA system and employing standard arsenic species in solution at concentrations of *ca* 50 ng cm^{-3}.

Effect of eluent pH

Initial experiments were carried out to investigate the dependence of retention behaviour on pH. For a 30 mmol dm^{-3} phosphate eluent with pH values covering the range 4.5 to 7.0 the results are shown in Fig. 1.

Arsenite is present throughout this pH range as its fully protonated form [$As(OH)_3$] and is not retained by this chromatographic system, leading to a constant apparent retention time of *ca* 3.0 min. As the pH is increased the retention times of arsenate and MMAA decrease whilst that of DMAA increases until a maximum is reached at pH 6.2. The retention times of the methylated arsenic species converge *ca* pH 5.3, resulting in an inversion of their retention order at this pH. At pH 6.5 the retention of MMAA has decreased to the point where it ceases to be retained and it co-elutes with arsenite. A pH of 6.2 was chosen for further work as it permits the resolution of DMAA whilst limiting the overall length of the chromatogram, which is governed by the arsenate retention. At pH 6.2 the phosphate eluent also acts as a buffer which assists in controlling the column pH conditions when they are perturbed by the injection of the seawater matrix, which itself has a significant buffering capacity tending to shift the eluent pH towards 8.2.

Eluent concentration

Retention times of arsenic species on anion-exchange columns depend upon the concentration of the eluent. The eluent pH was therefore fixed at 6.2 and the phosphate strength was varied from 5 to 200 mmol dm^{-3} (Fig. 2).

Under these conditions the arsenite remains unretained. At low eluent concentrations the arsenate retention time is long; it elutes after more than 16 min with an eluent concentration of 5 mmol dm^{-3}. As the concentration is increased the retention times of all components fall, but the effect is most marked for arsenate. The MMAA retention time decreases and the anion is not retained above an eluent concentration of 100 mmol dm^{-3}. The DMAA retention is affected only little by the eluent strength, remaining effectively constant above 100 mmol dm^{-3}. An eluent concentration of 30 mmol dm^{-3} was chosen as at this concentration DMAA is well separated from the other species without giving rise to an excessively long arsenate retention time.

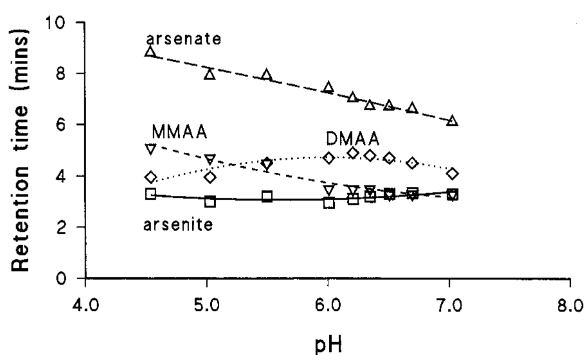

Figure 1 Effect of eluent pH on apparent retention time.

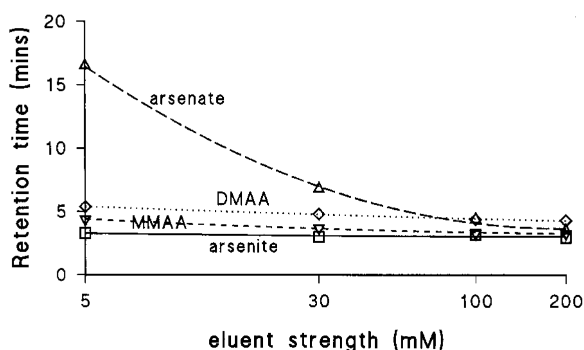

Figure 2 Effect of eluent concentration on the apparent retention time of four arsenic species.

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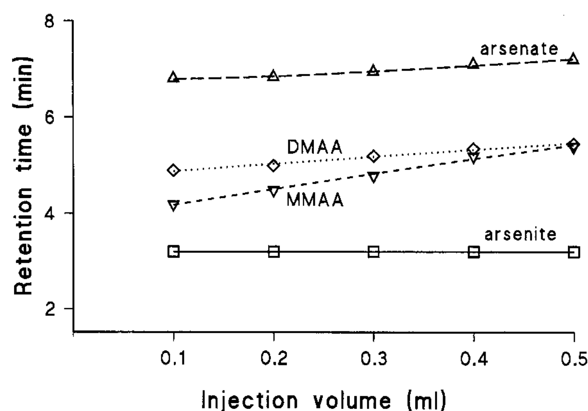

Figure 3 Alteration of apparent retention times of arsenic species with varying injection volumes.

Tolerance to injection volume

In order to achieve sufficiently low detection limits for seawater analysis, large-volume injections of the high-ionic-strength saline seawater solution are required. The effect of sample injection volume was therefore investigated for both standard solutions made up in distilled water and in seawater. For these experiments a 30 mmol dm^{-3} phosphate eluent was employed (pH 6.2) at a flow rate of 1.5 cm^3 min^{-1}. The effect of changing the volume of the seawater sample injected is shown in Fig. 3.

Arsenite is unretained on the column and its retention behaviour is therefore unaffected by the sample volume. Whilst there is a small increase in the retention times of arsenate and DMAA, the most significant effect on retention time is observed for MMAA. This of MMAA is increased as the injected volume increases, resulting in convergence of the MMAA and DMAA elution times when 500-μl injections are used. Injections larger than 500 μl result in significant peak broadening and they were therefore not considered. Although the convergence of the MMAA and DMAA peaks was not considered to be ideal, these two species were completely resolved in the subsequent cryogenic-trap analysis step.

Dimethylarsenic in estuarine waters

Water samples were taken regularly from the estuary of the River Beaulieu to monitor the presence of hydride-reducible dimethylarsenic species. Once a water sample had been identified as containing dimethylarsenic by cryogenic-trap HG AA, an aliquot of it was separated by HPLC and the eluate was collected during a tightly constrained time window covering the elution of the dimethylarsinate. This eluate fraction was then analysed using the cryogenic-trap HG AA procedure. The presence of hydride-reducible dimethylarsenic in this HPLC fraction was evidence supporting the presence of dimethylarsinate in the sample.

Table 1 Arsenic species (ng As cm^{-3}) in estuarine and coastal waters

Sample	Inorganic As(III) + As(V)	MMA	DMA	DMAA
Lepe	ND	ND	0.34, 0.34	0.33,0.36,0.33
Beaulieu 1	0.77	0.15	0.09	0.09
Beaulieu 2	0.79	0.13	0.14	<0.03
Beaulieu 3	0.87	0.02	0.09	0.10

In a preliminary experiment a single water sample was collected in June 1993 from Lepe Beach in Hampshire. This sample was filtered (Whatman GF/C) and then analysed by both cryogenic-trap HG AA[21] and using an early development of the HPLC procedure. In this sample the dimethylarsenic (DMA) concentration was not significantly different from the level of dimethylarsinate (DMAA) measured by HPLC (Table 1).

The second sample set was analysed using the HPLC conditions developed in this paper together with the cysteine-enhanced cryogenic-trap HG AA system. In these experiments three separate water samples were taken from the mouth of the River Beaulieu estuary. Whilst all three were found to contain some dimethylarsenic, only in two of the cases was this found to be fully explicable by the presence of dimethylarsinate. The reason for the absence of DMAA from Beaulieu sample 2 is currently unclear, but it may be due to the presence of currently unknown hydride-reducible dimethylarsenic species. All other analyses demonstrate that in these samples the dimethylarsenic content was consistent with the presence of dimethylarsinate.

DISCUSSION AND CONCLUSIONS

HPLC and cryogenic-trap HG AA, when used in combination, provide a powerful approach to the identification of DMAA in unpolluted natural waters. The HPLC separation permits the isolation of DMAA within a retention time window which

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might be expected to exclude most other dimethylarsenic species. The sensitivity of the cryogenic-trap technique, offering detection limits in the picogram range, is capable of detecting the small quantity of arsenic eluted from the HPLC column. At the same time, the trap AA procedure confirms the presence of a dimethylarsenic compound due to the identification of $(CH_3)_2AsH$ generated from the HPLC fraction. The improved detection limits attainable by this combination approach arises from the focusing of arsenic within the cryogenic trap. If a water sample were to contain, for example, 0.2 ng As cm^{-3} as DMAA, the injection of such a sample would lead to the elution of 100 pg of arsenic from the HPLC column. Spread over the width of a typical HPLC peak this would not be detectable by any direct HPLC–HG AA system. When focused by cryogenic trapping, however, 100 pg of arsenic moves into the AA system within a few seconds and is readily detected. In conventional HPLC systems the co-elution of monomethylarsonate with dimethylarsinate, when high-ionic-strength seawater samples are injected, would be a problem. When cryogenic-trap HG AA detection is employed, however, the dimethylarsine is resolved from monomethylarsine and the HPLC peak overlap is inconsequential.

Water samples have been analysed from the mouth of the River Beaulieu estuary, an area which has been shown in the past to contain significant levels of dimethylarsenic, but which is not significantly contaminated from geological, industrial or urban run-off. By comparing the levels of dimethylarsenic measured by the conventional cryogenic-trap HG AA system with the levels of dimethylarsenic measured within the dimethylarsinate HPLC retention window, it has been possible to illustrate both the probable presence of dimethylarsinate and that the majority of the hydride-reducible dimethylarsenic present in these water samples appeared to be present as dimethylarsinate. It should be pointed out that the correspondence is only valid for the hydride-reducible species (of which dimethylarsinate is one) and does not apply to the 'hidden' arsenic species which would not be detected in the cryogenic-trap procedure and that would be likely to elute outside the dimethylarsinate retention window.

How arsenate is converted to DMAA and 'hidden' dimethylarsenic has still to be firmly established. There is surprisingly little evidence to support the direct route to the formation of DMAA involving the sequential biomethylation of the arsenate anion through MMAA to DMAA, following the reaction sequence put forward by Challenger.[2]

The favoured explanation for the presence of dissolved DMAA is that it results from the degradation of larger biomolecules and not from the direct methylation of arsenate. It is now well established that most of the arsenic in marine algae is present as arsenosugars[11,25] and four dimethylarsenoribosides have been shown to account for 75% of arsenic in extracts of the edible brown seaweed, *Sphaerotrichia divaricata*.[26] The breakdown of dimethylarsenosugars, with conservation of the two CH_3As arsenic–carbon bonds during chemical and biological degradation (through 'hidden' arsenic species such as dimethyloxarsylethanol?)[27] would lead to the formation of DMAA, which would be very chemically stable under the oxidizing conditions of the aerobic marine environment. In this route arsenate is taken in by macroalgae (and phytoplankton?), converted to arsenosugars and released back into the water column as a result of excretion, grazing and decomposition of dead cells. Marine microbes would be expected to play a major role in changes to these compounds resulting in the generation of the metastable dimethylarsinate.

The arsenic compounds stored by marine fauna differ significantly from those found in marine plants and at present this area appears to be dominated by arsenobetaine.[28–40] Arsenobetaine is reported to be broken down to dimethylarsinate by bacteria isolated from coastal sediments[41] and by bacteria from the intestines of the chiton (mollusc) *Liolophura japonica*.[42] In culture studies arsenobetaine was broken down to trimethylarsine oxide[43] and then to arsenate.[44] From similar experiments the additional presence of dimethylarsinate was reported.[45] Arsenobetaine was degraded by bacteria in seawater to trimethylarsine oxide and inorganic arsenic(V).[46,47] The subsequent hydrolysis of trimethylarsine oxide would be expected to result in the formation of dimethylarsinate.

Arsenocholine is much less common than arsenobetaine in marine organisms[48] but it is reported to result from the anaerobic decomposition of arsenosugars.[49] Metabolism of arsenocholine by sediment micro-organisms has also been reported to result in the formation of arsenobetaine.[50]

It is not yet fully clear where 'hidden' dimethylarsenic fits into the story. The large pool of dimethylarsenic, held in organisms in the form of larger molecules, is released both actively during life and rapidly after death due to cell lysis and bacterial breakdown processes. This results in the

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release of compounds such as the arsenosugars and their breakdown products into the water column. Whilst there is evidence to suggest that active excretion and decomposition routes both contribute towards the presence of dimethylarsenic in the water column, the relative contributions of these two routes have yet to be established. In a previous study we have attempted, without success, to link the appearance of hydride-reducible DMA with the presence of particular phytoplankton or bacteria in the water column.[51] Despite a marked successional development of planktonic species, the appearance of dissolved DMA could not be linked to the presence of any particular species and its concentration slowly built up over the summer to dissipate eventually with deteriorating climatic conditions. Such observations would not be inconsistent with the appearance of dissolved DMA being related, not to active excretion of hydride-reducible DMA from living plankton, but to the release of 'hidden' DMA species through excretion, decompositional pathways and grazing. Only once the 'hidden' DMA decomposition pathways had reached a hydride-reducible species, such as DMAA, would the presence of hydride-reducible DMA be evident.

Most of the dimethylarsenic compounds which are not hydride-reducible have complex structures which make them useful carbon sources; their lifetimes in a biologically active system are likely to be relatively short, yet significant compared with the few days separating the development and demise of a phytoplankton population. Further microbial/chemical breakdown of these compounds will eventually lead to the presence of the metastable dimethylarsinate which is hydride-reducible. Subsequent demethylation of the DMAA would result in the eventual return of the arsenic to its arsenate form.[52]

This paper, in presenting evidence for the presence of dimethyl*arsinate*, has we believe gone further than previous work, which only produced evidence for dimethyl*arsenic*.

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