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Evaluation of extraction procedures for arsenolipids in algae & growing the unicellular alga *Dunaliella tertiolecta* in batch cultures

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Danksagung

Ich danke meiner Familie und meinen Freunden. Ihr seid es, an die ich denke, wenn ich zu Bett gehe. Ich bin froh, dass es euch gibt. Zudem danke ich Prof. Francesconi für seine Unterstützung und die vielen anregenden Gespräche während meiner Masterarbeit; Prof. Maher und Elliott Duncan für die Zeit in Canberra.

Ever tried.

Ever failed.

No matter.

Try again.

Fail again.

Fail better.

(Samuel Beckett)

Summary

Arsenic occurs in many different chemical forms (inorganic and organic) in the marine environment. Some of these arsenic compounds are soluble in low polarity organic solvents and are referred to generally as arsenolipids. Even though the existence of arsenolipids has been known since the late 1960s, the structures of most of these compounds have been identified only recently. Hence no work can be found in the literature which evaluates the extraction and purification of arsenolipids. We assessed and compared three solvent mixtures (chloroform/methanol, 2+1 v/v; methyl tertiary-butyl ether/methanol, 10+3 v/v; and methyl tertiary-butyl ether/methanol, 10+6 v/v) on their capability to extract arsenolipids from commercially available edible brown alga *Hizikia fusiforme* (Hijiki), and used the chloroform/methanol (2+1 v/v) mixture to extract arsenolipids from a certified reference material prepared from Hijiki. Further, we evaluated the influence of a silica column purification step on the composition of the arsenolipids and their behavior in the electrospray mass spectrometer. Inductively coupled plasma mass spectrometry after microwave-assisted acid digestion was used for total analysis of arsenic in the examined extracts and in the dried algal material. Speciation of the arsenolipids was carried out by high performance liquid chromatography with parallel detection by an inductively coupled plasma mass spectrometer and an electrospray mass spectrometer (HPLC/ICPMS/ESMS) which provided both elemental and molecular information.

The factors influencing the biosynthesis of arsenolipids and their role in the metabolism of marine organisms is unknown. Therefore the unicellular alga *Dunaliella tertiolecta* was grown in batch cultures under four sets of phosphorous and arsenic conditions. The effect of the various P/As regimes on cell growth and biomass were evaluated; further investigations on the composition of arsenolipids of the algal tissue will follow.

Contents

1 Introduction

1.1 Arsenic in the marine environment

Arsenic occurs in seawater, sediments and many marine organisms in various forms, which can be classified as inorganic arsenic (**1-2**) and organic arsenic (**3-11**) (Figure 1). Although the occurrence of arsenic in marine animals had been known since the early 1900s, it was another 70 years before the first organoarsenical, arsenobetaine (**7**), was identified in lobster by Edmonds et al. [1977]. Until now, more than 50 additional water-soluble arsenic compounds have been reported in marine organism since the identification of arsenobetaine [Francesconi 2010]; more common arsenicals (**1-9**) or arsenicals of particular interest for algae (**9-11**) are shown in Figure 1.

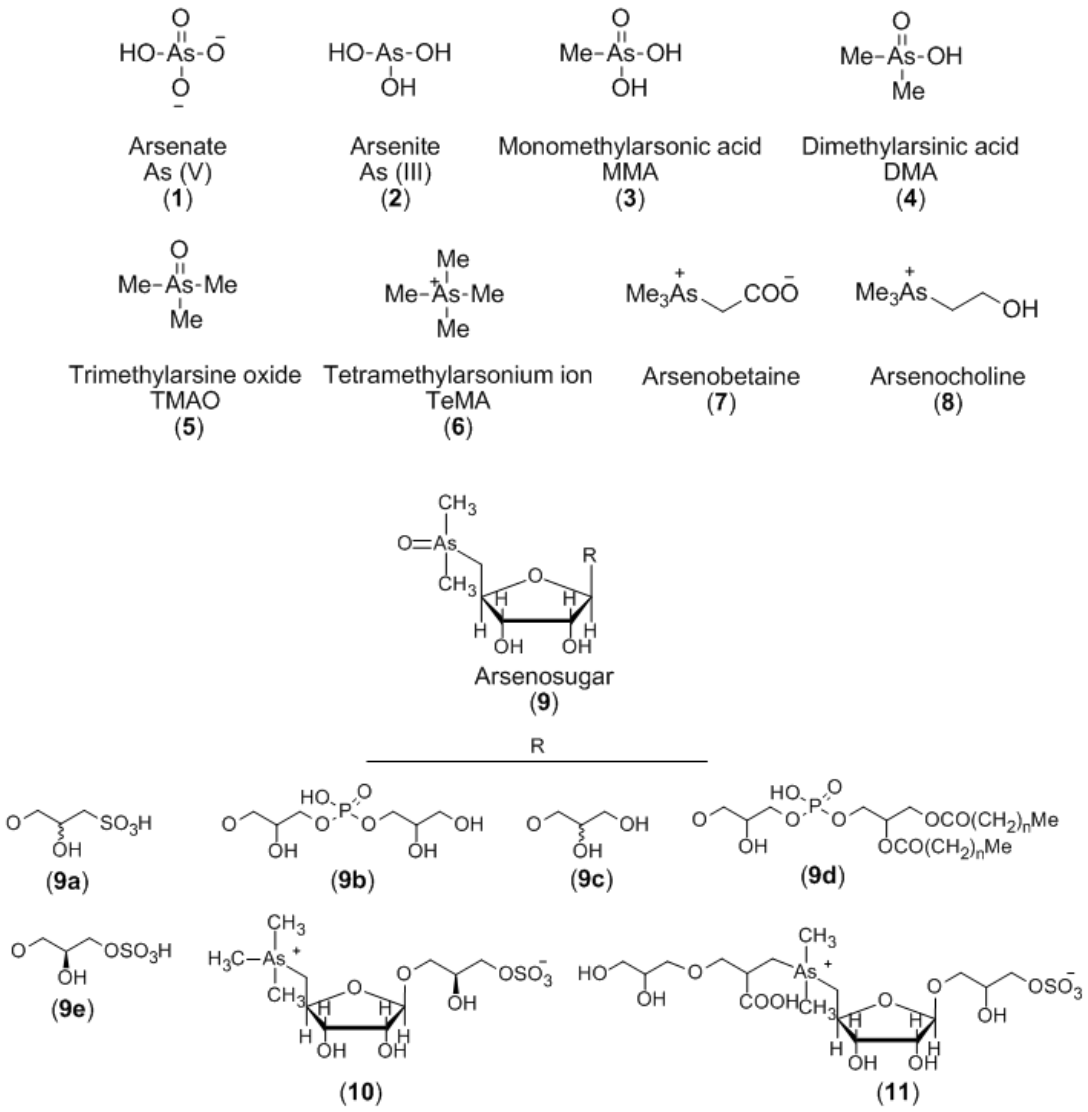


Figure 1 Names, abbreviations and structures of some marine arsenic-containing compounds.

This section gives an overview of water-soluble arsenicals in different marine samples; lipid soluble arsenicals will be discussed in Section 1.2. Although arsenic species can exist in both +3 and +5 oxidation states, As-(V) compounds are usually the predominant form. The various compounds occur as neutral or charged species depending on their chemical structure/properties (e.g. pK_a value) and the naturally occurring pH value. The pK_a values for some simple arsenicals are given in Table 1. The toxicity of arsenic depends as well on its chemical structure. Arsenate and arsenite are the most toxic compounds; the methylated species monomethylarsonic acid, dimethylarsinic acid and trimethylarsine oxide are considered to be less toxic; arsenobetaine, arsenocholine and the arsenosugars are considered to be non-toxic [Kaise and Fukui 1992; Oya-Ohta et al. 1996; Ellwood and Maher 2003]. The toxicity of the tetramethylarsonium ion is ambivalent. Tetramethylarsonium iodide showed acute toxicity when fed to mice [Kaise and Fukui 1992], but the toxicity on cultured human fibroblasts was low [Oya-Ohta et al. 1996].

Table 1 pK_a values for various arsenic species at 25°C adapted from Lafferty and Loeppert [2005] and for H_3PO_4 [Kohn and Dunlap 1998].

Arsenic species	pK_{a1}	pK_{a2}	pK_{a3}
H_3AsO_4	2.2	7.0	11.5
$H_2AsO_3(CH_3)$	4.2	8.8	–
$HAsO_2(CH_3)_2$	6.1	–	–
H_3AsO_3	9.2	12.1	13.4
H_3PO_4	2.1	7.3	12.3

1.1.1 Arsenic in seawater and sediments

The arsenic levels in the world oceans are uniform at about 0.5-2 µg/L [Andreae 1978; Santosa et al. 1994] with inorganic arsenic as the predominant form. The major As-(V) species in normal seawater (pH = 8.1) is $HAsO_4^{2-}$, calculated to be 98 % of total As-(V) [Turner et al. 1981]. The major As-(III) compound in seawater is the neutral H_3AsO_3. The ratio of As (III) to As-(V) in seawater is influenced by marine bacteria and marine phytoplankton [Johnson 1972; Johnson and Burke 1978]. Minor constituents in seawater are the methylated arsenic compounds MMA (**3**) and DMA (**4**). Andreae [1979] suggested that these methylated arsenic compounds and As-(III) could be released from phytoplankton after uptake and conversion of As-(V) from seawater. More complex arsenicals such as arsenobetaine (**7**) and arsenosugars (**9-11**) have not been detected so far in seawater. A reason for that might be that the standard analytical method for the detection of arsenic species in seawater converts arsenic compounds into volatile arsines, which were usually detected by atomic absorption spectrometry; arsenobetaine and arsenosugars remain undetected by this method because they do not form volatile arsines under these conditions [Francesconi and Edmonds 1997].

The arsenic concentrations in unpolluted near-shore marine sediments are normally between 0.1 and 50 µg/g, but could exceed 1000 µg/g through anthropogenic input [Maher and Butler 1988]. Only little information has so far been obtained on the arsenic species in sediments. Most of the work has focused on the arsenic species in porewater (interstitial waters) of sediments. It was found that As-(III) and As-(V) are major constituents of porewater of sediments beside minor amounts of DMA and MMA (<10 % of the total arsenic) and trace levels of trimethylarsine oxide (**5**) [Andreae 1979; Reimer and Thompson 1988]. Ellwood and Maher [2003] reported the existence of As-(III), As-(V) and arsenosugars (**9a** and **9e**) in sediments from a marine lake.

1.1.2 Arsenic in marine algae

The arsenic concentrations in marine algae are normally between 1 and 100 µg/g with large variations between algal types and locations (Table 2). Higher arsenic concentrations are found in brown algae compared to red- or green algae. Marine algae contain mainly arsenosugars, which were first identified from a methanol extract of *Ecklonia radiata*, a brown macroalga, by nuclear magnetic resonance (NMR) analysis after several chromatographic purification steps [Edmonds and Francesconi 1981]. Most of the arsenosugars are dimethylarsinylribosides (**9**) and differ in their aglycone (R), although two quaternary arsonio compounds (**10**) [Shibata and Morita 1988] and (**11**) [Francesconi et al. 1991] have also been reported in algae (Figure 1). The significant arsenic compounds for some brown-, green- and red algal species are given in Table 3.

Table 2 Arsenic concentrations in marine algae [Francesconi and Edmonds 1997].

		Arsenic concentration (mg/kg dry wt.)	
Type	**Location (no. of species)**	**Range**	**Mean**
Brown	Australia (14)	21 – 179	62
	USA (24)	1 – 32	10
	Japan (13)	2 – 72	21
Red	Australia (10)	12 – 31	19
	USA (15)	0.4 – 3.2	1.4
	Japan (25)	6 – 45	17
Green	Australia (9)	6 – 16	11
	USA (16)	0.2 – 23	1.5
	Japan (5)	12 – 19	16
Phytoplankton	Australia	Mixed samples	9

Arsenate is a minor constituent in algae except for the edible brown alga *Hizikia fusiforme* (Hijiki), where it accounts for 38 to 61 % of the total arsenic concentration [Yasui et al. 1978; Whyte and Englar 1983; Shinagawa et al. 1983]. These high amounts of toxic arsenate in Hijiki caused the British Food Standard Agency to advise people not to eat Hijiki [Ichikawa et al. 2006]. This advice initiated Ichikawa et al. [2006] to demonstrate that up to 90 % of the total arsenic can be removed from Hijiki by soaking and cooking the seaweed in water. Other arsenic constituents in algae are MMA and DMA [Francesconi and Edmonds 1997] at trace levels. Arsenobetaine, which was thought not to be present in algae for a long time

[Francesconi et al. 1999], was finally detected in brown, green and red algae by high performance liquid chromatography coupled online with an electrospray tandem mass spectrometer [Nischwitz and Pergantis 2005]. Arsenocholine, TMAO and TeMA have not been detected so far in marine algae. Algae also contain arsenolipids — these recently discovered compounds are currently being intensively investigated.

Table 3 Significant arsenic compounds in marine algae [Francesconi and Edmonds 1997].

Type	Species	Arsenic concentration (mg/kg wet or dry wt.)	% Water soluble	Significant arsenic compounds[a]
Brown	*Ecklonia radiata*	10 wet	>80	9a, 9b, 9c
	Hizikia fusiforme	10 wet	>80	1, 9e
	Undaria pinnatifida	2.8 wet	71	9d
	Sargassum thunbergii	4 wet	51	9e
Green	*Ulva pertusa*	17.1 dry	40	9c
	Bryopsis maxima	19.4 dry	20	9b
	Caulerpa brachypus	11.6 dry	32	u
Red	*Cyrtymenia sparsa*	44.8 dry	69	9b
	Coeloseira pacifica	23.1 dry	35	9b, u
	Laurencia okamurai	19.2 dry	47	9c, 9e

[a]Significant, ≥20 % of total water-soluble arsenic; u, unknown.

The occurrence of arsenic in marine algae is probably because of similarities with its Periodic Table group 15 member phosphorus. Arsenate and phosphate have similar atomic radii (0.248 nm; 0.238 nm) [Francesconi 2010] and pK_a values (Table 1) and therefore phosphate, a major nutrient for algae, exists in seawater in a chemical form similar to arsenate. The membrane transport system of algae is insufficiently selective to discriminate between these similar oxyanions, which leads to the uptake of arsenate [Francesconi 2010]. This infidelity does not have to be a disadvantage for all algal species. Maybe in a habitat lacking phosphate, algae can utilize arsenate for life-sustaining functions. It is already known that nature has different phosphate transport systems, which differ in phosphate affinity and selectivity [Willsky and Malamy 1980; Chung et al. 2003; Elias et al. 2012], but more work is necessary to characterize these transport systems in marine algae, and study their influence at different phosphate and arsenate levels on the algal metabolism.

1.1.3 Arsenic in marine animals

The arsenic concentrations in marine animals can vary widely, but are normally between 5 and 100 µg/g (Table 4). The major arsenic compound in most marine animals — e.g. fish, molluscs, crustaceans and marine mammals — is arsenobetaine, which can account for 95 % or even more of the total arsenic [Francesconi and Edmonds 1997]. Arsenobetaine was first isolated and identified in the tail muscle of the western rock lobster by Edmonds et al. [1977]. Arsenosugars, TeMA and trace levels of TMAO, arsenocholine, As-(V), As-(III) and DMA have also been reported in various marine animals [Francesconi and Edmonds 1997]. It is proposed that arsenosugars occur in some marine animals as ingested algal metabolites [Francesconi and Edmonds 1997]. Recent studies revealed the existence of lipid-soluble arsenic, so called arsenolipids, in fish (see Section *1.2*).

Table 4 Arsenic concentrations in marine animals [Francesconi and Edmonds 1997].

		Arsenic concentration (mg/kg wet or dry wt.)		
Type	**Location (no. of species)**	**Range**	**Mean**	
Finfish	Arabian Gulf (13)	0.3 – 32	4.9	wet
	Australia (9)	0.8 – 14	6.5	dry
	Greenland (5)	8 – 307	60	dry
	Northern Europe (14)	1.3 – 37	7.7	wet
	Norway (8)	0.6 – 8	2.7	wet
	USA (many)	Range of means from 1 to 7		wet
Crustaceans	Arabian Gulf (3)	6 – 19	12	wet
	Australia (5)	7 – 91	27	dry
	Scotland (3)	3 – 38	12	wet
	USA (16)	Range of means from 3 to 50		wet
Bivalve molluscs	Japan (5)	1 – 10	3.6	wet
	U. K. (5)	2.6 – 15	7.8	dry
	USA (12)	Range of means from 2 to 20		wet
Gastropod molluscs	Japan (10)	1.6 – 107	26	wet
	U. K. (6)	8.1 – 38	16	dry
	USA (2)	3.0 – 27	14	wet

1.2 Arsenolipids and their discovery

The term arsenolipids refers to those organoarsenic compounds that are soluble in less polar organic solvents. Lunde laid the foundation for this definition in 1967 with the discovery of lipid-soluble arsenic in fish oil and in chloroform/methanol 2+1 extracts of marine fishes — whole fish, tissue and liver extracts — and he speculated: *"It is difficult to say whether the arsenic is present as an arsenorganic compound or if the element is a substitute for phosphorus in one or some of the phospholipids* [Lunde 1967]". Lunde determined the arsenic content in his samples by neutron activation analysis, a method now rarely used. In 1972 he found evidence for the existence of arsenic in the lipid phase of various cultivated marine and limnetic unicellular algae after chloroform/methanol/water extraction [Lunde et al. 1972].

Later, Lunde grew unicellular algae in ^{74}As (radioactive) containing nutrient-enriched media and suggested that algae can synthesize water- and fat-soluble organoarsenic compounds from inorganic arsenic ions [Lunde 1973]. He extracted lipids from the algal tissue with a mixture of chloroform/methanol/water 4+2+1, re-dissolved them in chloroform and separated non-polar from polar lipids on a silica column. The non-polar lipids were eluted with chloroform and the polar lipids with 90 % methanol in chloroform. He showed that most of the radioactivity followed the polar lipid fraction. He also separated the lipid fraction by thin layer chromatography (TLC) with detection of ^{74}As by autoradiography and demonstrated that the lipid-soluble organoarsenic compounds could be converted into a water-soluble organoarsenic compound under acidic conditions indistinguishable from the original water-soluble organoarsenic compound. He found, in addition, that more of the lipid-soluble organoarsenic compounds were synthesized by the cultures if the arsenic source was arsenate compared to arsenite.

Further cultivation experiments with *Tetraselmis chuii* (a marine alga) and *Daphnia magna* (a small freshwater crustacean) in the presence of inorganic radioactive labeled ^{74}As led to speculations as to whether arsenic-containing phospholipids exist in nature and could be synthesized by these organisms [Irgolic et al. 1977]. Samples obtained from these culture experiments were extracted in chloroform/methanol 2+1 (v/v) and half of ^{74}As radioactivity was present in the lipid-containing chloroform layer. Silica gel chromatography followed by TLC and two dimensional TLC revealed the existence of six spots, five of which resulted from

one compound through the storage conditions. In two dimensional TLC, the original arsenic compound chromatographed at the same R_f-values as phosphatidylethanolamine. This original arsenic compound was treated with *phospholipases A, C* and *D,* and the generated products were partitioned between water and ether. After *phospholipase A* usage most of the ^{74}As radioactivity was found in the water phase, whereas the arsenic radioactivity after *phospholipase C* treatment was found in the ether phase. *Phospholipase D* led to an even distribution of radioactivity in both phases. Based on these experiments, Irgolic et al. [1977] suggested that the arsenic was incorporated into phospholipids, possibly containing an arsenocholine moiety.

In 1978, Benson and co-workers postulated a structure of an arsoniumphospholipid (O-phosphatidyltrimethyl-arsoniumlactic acid, Figure 2) in a marine unicellular alga (*Chaetoceros concavicornis*) as a detoxification product of arsenate [Cooney et al. 1978]. The marine alga was cultured in a medium containing ^{74}As labeled arsenate. The algal cells were harvested after four days and extracted with chloroform/methanol 2+1 (v/v) or ether/ethanol 1+3 (v/v). The alga had transformed the arsenate into three arsenolipids (I, II, III) and four water-soluble compounds (A, B, C and D). *Phospholipase* A_2 converted arsenolipid I into II (lyso form of lipid I) and *phospholipase D* converted I into C. Alkaline treatment of arsenolipids I and II transformed them into compound B. *Glycerophosphorylcholine diesterase* converted compound B into C, and C was transformed into D when heated at 100°C in 1 M KOH in ethanol. Compound D was identified as dimethylarsinic acid and C was claimed to be trimethylarsonium-lactate due to exact co-chromatography of the natural compound with a synthetic standard. Based on the presumed identity of C, arsenolipid I was claimed to be the trimethylarsoniumlactate phospholipid. Arsenolipid III and compound A stayed unidentified. Later, it was shown that compound C was not trimethylarsoniumlactate [Summons et al. 1982], and that compound A had been the dimethylarsinylriboside (**9a**) and compound B had been dimethylarsinylriboside (**9c**), and therefore the interpretation of the arsoniumphospholipid had been wrong [Benson 1989].

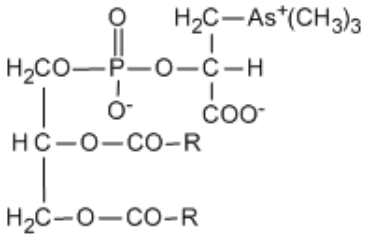


Figure 2 Arsoniumphospholipid postulated by Cooney et al. [1978].

Also other algae (diatom *Skeletonema costatum*, green alga *Platymonas cf. suecica*, dinoflagellate *Gonyaulax polyedra* and a coccolithophorid *Cricosphaera cateri*) were shown to biosynthesize water- and lipid-soluble organoarsenic compounds from arsenate in sea water [Andreae and Klumpp 1979]. Klumpp and Peterson [1981] showed that the macroalga *Fucus spiralis* biosynthesized twelve water- and one major lipid-soluble organoarsenic compound from ^{74}As labeled arsenate in seawater. Arsenate was first accumulated in the algal tissue in a water-soluble form and then converted into the lipid-soluble compound. The amount of the lipid-soluble compound increased over time and accounted for approx. 60 % of the total arsenic after 100 hours of exposure. Silica column chromatography revealed the polar nature of the arsenolipid and saponification transformed the arsenolipid into the main water-soluble organoarsenic compound.

The unicellular marine phytoplankton *Dunaliella tertiolecta* was grown in a nutrient-enriched medium containing ^{74}As labeled arsenate [Wrench and Addison 1981]. The harvested algal cells were extracted with a mixture of chloroform/methanol 2+1 (v/v). The profile of ^{74}As in the chloroform layer after silica gel TLC revealed the existence of three arsenolipids (1, 2 and 3). *Phospholipase D* completely hydrolyzed arsenolipid 1 while 2 and 3 were unaffected. Wrench and Addison treated $^{74}AsO_2^-$ with phosphatidyl inositol producing a compound that chromatographed in the same position as arsenolipid 1. Arsenolipid 2 and 3 eluted off a diethylaminoethyl cellulose anion-exchange column with neutral and zwitterionic lipids while arsenolipid 1 was more strongly retained on the column and was eluted with the anionic lipids (consistent with its being a phospholipid).

In 1981, the structures of two novel water-soluble organoarsenic compounds named dimethylarsinylribosides or arsenosugars (Figure 1; **9a** and **9c**) were identified from a methanol extract of *Ecklonia radiata*, a brown macroalga, by ^{1}H- and ^{13}C- NMR spectroscopic data [Edmonds and Francesconi 1981]. These two compounds accounted together for 81 % of the total arsenic in *Ecklonia radiata*. Strongly acidic or basic conditions were avoided

during the sample preparation to prevent decomposition of these arsenosugars to dimethylarsinic acid. The methanol extract was purified by gel permeation- and buffered ion-exchange chromatography followed by preparative layer chromatography and high performance liquid chromatography (HPLC). In a follow up study, examination of the methanol extract from *Ecklonia radiata* revealed the existence of arsenosugar **9b** a phosphoric acid diester [Edmonds and Francesconi 1983]. Acylation by long-chain fatty acids of the two free hydroxy groups of the terminal glycerol residue would produce an arsenosugar phospholipid which could account for at least one of the arsenolipids from former studies (Irgolic et al. 1977; Cooney et al. 1978; Klumpp and Peterson 1981; Wrench and Addison 1981). Thus the likely structural backbone of the first arsenolipids had been discovered.

Five years later, Morita and Shibata [1988] identified the arsenosugar phospholipid, dipalmitoylglycerophospho-2-hydroxypropyl-5-deoxy-5-(dimethylarsinoyl)-β-ribofuranoside, (Figure 3) in *Undaria pinnatifida* (Wakame), a brown macroalga. The wet macroalga was extracted in a mixture of chloroform/methanol 1+1 for two days. The evaporated chloroform layer was partitioned between hexane/acetonitrile and the arsenosugar phospholipid (recovered in hexane layer) was isolated by gel permeation chromatography and normal phase HPLC. Structural determination of the arsenosugar phospholipid was carried out by NMR analysis assisted by gas chromatography mass spectrometry (GC-MS) of the cleaved and esterified fatty acid side chains and arsenic and phosphorus detection by inductively coupled plasma atomic emission spectroscopy (ICPAES). The substitution of palmitic acid with other fatty acids - myristic acid, stearic acid and arachic acid – was predicted.

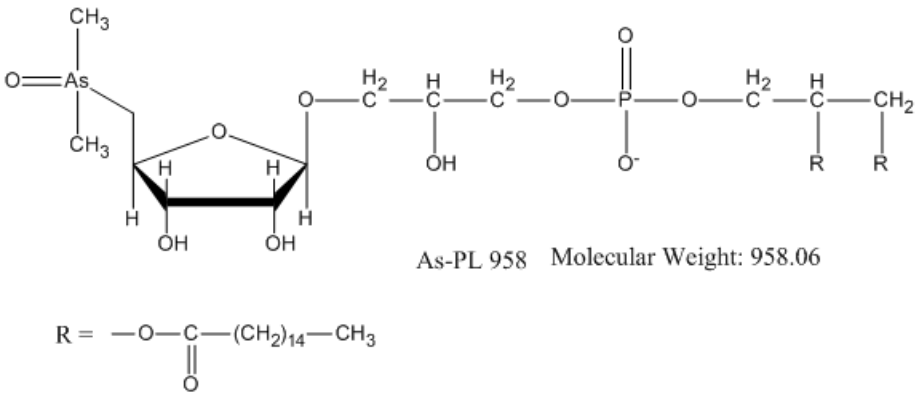


Figure 3 Arsenosugar phospholipid identified by Morita and Shibata [1988].

It took a long time from the first report of lipid-soluble arsenic in fish oil by Lunde to its identification. In 2008, two novel groups of arsenolipids were discovered in two studies on fish oils. First, six arsenic-containing fatty acids (AsFA) were identified in cod-liver oil (Figure 4) [Rumpler et al. 2008]. Crude cod liver oil was partitioned between hexane and aqueous methanol, and the polar aqueous methanol phase (containing ca. 30 % of the total arsenic) was taken for preparative size exclusion- and anion-exchange chromatography. About 50 % of the arsenic eluted off the anion-exchange column in the neutral/basic fraction, whereas the remaining 50 % eluted later as a single peak in the acidic fraction. Examination of the acidic fraction by reversed-phase HPLC- inductively coupled plasma mass spectrometry (ICPMS) demonstrated the existence of at least 15 arsenolipids. Further analysis of these acidic arsenolipids by HPLC-electrospray ionization MS (ESMS) revealed the molecular masses of six AsFAs; accurate mass determination by high resolution MS (HRMS) provide unequivocal molecular formulas for these compounds. The six AsFAs accounted for approx. 20 % of the total arsenolipid content of cod liver oil. The arsenic-containing fatty acid with molecular mass 362 (AsFA 362) was synthesized and the chromatographic and mass spectral properties were shown to be identical with those of the natural product [Rumpler et al. 2008].

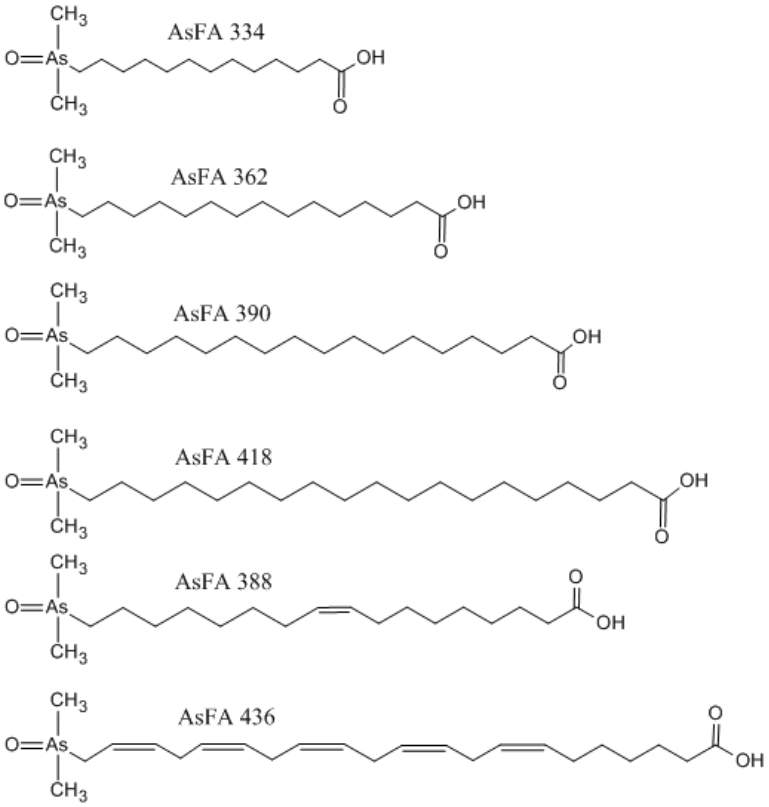


Figure 4 Arsenic-containing long-chain fatty acids identified in cod liver oil [Rumpler et al. 2008].

In the second study, capelin fish oil was analyzed in a manner similar to that used for cod liver oil [Taleshi et al. 2008]. On this occasion, however, most of the lipid-soluble arsenic compounds eluted at the void volume in the non-acidic fraction of the preparative anion-exchange column, and hence were not AsFAs. Reversed-phase HPLC-ESMS measurements of the non-acidic fraction revealed the existence of three arsenic compounds. A portion of the non-acidic material was purified by preparative reversed-phase HPLC. The collected fractions were analyzed for their arsenic content by ICPMS, and high resolution accurate mass spectrometry revealed the molecular formulas of three arsenolipids. The molecular formulas, together with the chromatographic properties indicated the presence of three arsenic-containing hydrocarbons (AsHC, Figure 5). AsHC 332 was also synthesized and the chromatographic and mass spectral properties were identical with those of the natural product. These three arsenic-containing hydrocarbons accounted for at least 70 % of the total arsenolipid content in capelin fish oil. The remaining arsenic consisted of less polar compounds which were not identified.

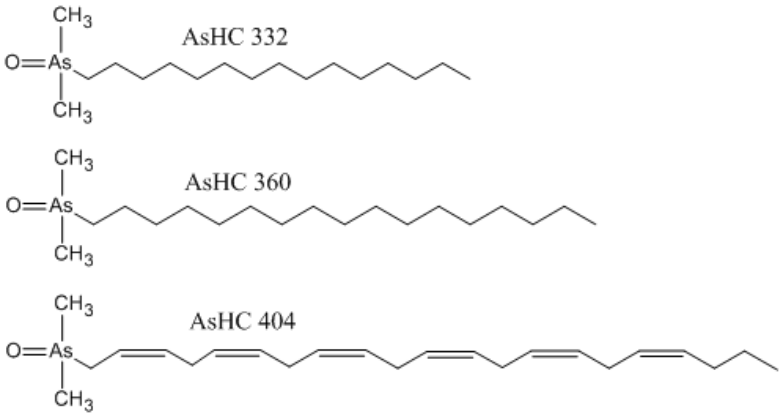


Figure 5 Arsenic- containing hydrocarbons identified in capelin fish oil [Taleshi et al. 2008].

One year later, Raber et al. [2009] developed a GCMS method to detect arsenic-containing hydrocarbons and confirmed with this method the existence of the three arsenic-containing hydrocarbons in capelin fish oil. Two years later, AsHC 332 and AsHC 404 were detected in sashimi-grade tuna fish [Taleshi et al. 2010]. Sashimi-grade tuna fish was extracted with a mixture of chloroform/methanol 2+1 (v/v), the extract was washed with water, and the resulting fat-soluble fraction was partitioned between hexane and water/methanol. The aqueous-methanol layer contained the polar arsenolipids which were further purified by a preparative anion-exchange column. Additional purification on a small preparative silica gel column was necessary to remove matrix constituents that would otherwise interfere with

the ESMS analysis. Identification of the compounds was carried out by HPLC-ICPMS, HPLC-ESMS and high resolution ESMS.

All three arsenic-containing hydrocarbons previously found in capelin fish oil were also detected in cod liver tissue [Arroyo-Abad et al. 2010]. The cod liver was extracted with methanol and purified by preparative column chromatography on silica gel. The three arsenolipids were identified by combining the elemental arsenic information of GC-ICPMS or GC-microwave induced plasma-AES (GC-MIPAES) with the molecular information of GC-MS. The finding was confirmed by accurate mass measurements of the compounds by time of flight MS (TOFMS) analysis. A new arsenic-containing long-chain fatty (AsFA 448) was discovered in fish meal from capelin (Figure 6) in addition to the known arsenolipids AsFA 362, AsFA 436, AsHC 332, AsHC 360 and AsHC 404 [Amayo et al. 2011]. These arsenolipids made up 12 % of the total arsenic content. Hexane was used to extract the arsenolipids from the fish meal, and the compounds were fractionated by vacuum liquid chromatography with a silica gel column. Identification and quantification of the arsenolipids was carried out by HPLC-ICPHRMS-ESHRMS.

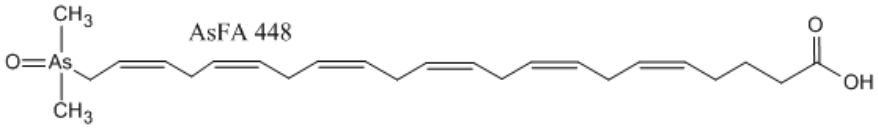


Figure 6 Arsenic-containing long-chain fatty acid identified in fish meal from capelin [Amayo et al. 2011].

One unknown arsenic-containing hydrocarbon (AsHC 388) in addition to the known AsHC 332 and AsHC 360 and eleven arsenosugar phospholipids (AsPL) with different side chains were identified in two species of brown macroalgae Hiziki (*Hizikia fusiform*) and Wakame (*Undaria pinnatifida*) [Garcia-Salgado et al. 2012] (Figure 7; Table 5). The macroalgae were extracted in chloroform/methanol (2+1, v/v), the extract washed with water and the resulting lipid-soluble fraction was purified on a preparative silica gel column. Identification and quantification of the arsenolipids was carried out by HPLC/ICPMS/ESMS and HRMS.

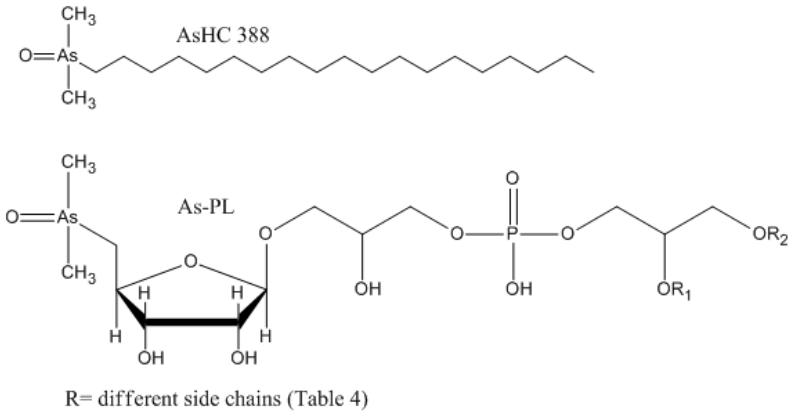


Figure 7 Arsenic-containing hydrocarbon and arsenosugar phospholipids identified in macro algae [Garcia-Salgado et al. 2012].

Table 5 Arsenosugar phospholipid side chains adapted from Garcia-Salgado et al. [2012].

Compound code	R1,R2**	
AsPL 930	$-C(O)(CH_2)_{12}CH_3$	$-C(O)(CH_2)_{14}CH_3$
AsPL 944	$-C(O)(CH_2)_{12}CH_3$	$-C(O)(CH_2)_{15}CH_3$
AsPL 956	$-C(O)(CH_2)_{14}CH_3$	$-C(O)(CH_2)_7CH=CH(CH_2)_5CH_3$
AsPL 958*	$-C(O)(CH_2)_{14}CH_3$	$-C(O)(CH_2)_{14}CH_3$
AsPL 982	$-C(O)(CH_2)_7CH=CH(CH_2)_5CH_3$	$-C(O)(CH_2)_7CH=CH(CH_2)_7CH_3$
AsPL 984	$-C(O)(CH_2)_7CH=CH(CH_2)_5CH_3$	$-C(O)(CH_2)_{16}CH_3$
AsPL 986	$-C(O)(CH_2)_{14}CH_3$	$-C(O)(CH_2)_{16}CH_3$
AsPL 1012	$-C(O)(CH_2)_{16}CH_3$	$-C(O)(CH_2)_7CH=CH(CH_2)_7CH_3$
AsPL 1014	$-C(O)(CH_2)_{16}CH_3$	$-C(O)(CH_2)_{16}CH_3$
AsPL 1042	$-C(O)(CH_2)_{16}CH_3$	$-C(O)(CH_2)_{18}CH_3$
AsPL 1070	$-C(O)(CH_2)_{18}CH_3$	$-C(O)(CH_2)_{18}CH_3$

*discovered by M. Morita and Y. Shibata; **see Figure 7.

The arsenic-containing fatty acids and arsenic-containing hydrocarbons are possibly the unknown arsenolipids reported in the earlier studies (Cooney et al. 1978; Wrench and Addison 1981). In contrast to the arsenosugar phospholipids, the arsenolipids based on fatty acids and hydrocarbons would not be cleaved to water-soluble products by a phospholipase. These three groups of arsenolipids are all polar arsenolipids.

Even though recent work has provided much information about the polar arsenolipids, the nature of the non-polar lipids is still unknown and awaits discovery.

1.3 Methods for determining total arsenic content and arsenolipids in marine samples

Arsenic occurs in many forms and matrices in marine biota which could influence the result of a total arsenic determination. Therefore dry ashing or wet digestion procedures are commonly used to oxidize the organic matter and to convert the different arsenic species into the same analyte, namely arsenate. Closed vessel systems are preferable over open vessel systems as they minimize the loss of volatile analytes. After conversion of the different arsenic species into arsenate, the determination of total arsenic is usually carried out by hydride generation atomic absorption spectrometry (HG-AAS), graphite furnace atomic absorption spectrometry (GFAAS), hydride generation atomic fluorescence spectrometry (HG-AFS), inductive coupled plasma optical emission spectroscopy (ICPOES) or ICPMS.

Arsenolipids are usually identified by coupled techniques after extraction and sample preparation as mentioned above. The first method for the determination of arsenolipids was based on the separation of the partially purified compounds in two independent HPLC runs (C_{18}-reversed-phase column) with detection by ICPMS or by ESMS. Atomic information of the $^{75}As^{+}$-ion was obtained by ICPMS and molecular masses were recorded by ESMS. The structures were postulated according to the molecular masses, preparative HPLC separation with fraction collection was then carried out and fractions were analyzed offline by HRMS which confirmed the molecular masses and provided molecular formulas [Rumpler et al. 2008; Taleshi et al. 2008; Taleshi et al. 2010].

Raber et al. [2009] showed that arsenic-containing hydrocarbons can be detected as arsines by GC-MS. Most likely the arsine oxide group of the arsenic-containing hydrocarbon is converted to an arsine group in the injection port of the GC. A further development of this approach identifies arsenic-containing hydrocarbons by GC-MIPAES or GC-ICPMS data in addition to GC-MS analysis [Arroyo-Abad et al. 2010]. The combination of atomic and molecular information and retention times reduces the possibility of misidentification of arsenolipids. The newest approach for identification of arsenolipids worked in a similar way. The arsenolipids were separated on a HPLC-column (C_{18}- or C_8-reverse phase column) by gradient elution and the effluent was split post-column to ICPHRMS and ESHRMS with simultaneous acquisition of atomic and molecular information [Amayo et al. 2011].

Quantification of the arsenolipids has been described for two simultaneous methods [Amayo et al. 2011; Garcia-Salgado et al. 2012] but with different approaches to compensate the different carbon content of the mobile phase during gradient elution. Compensation was necessary because a change in the organic solvent content of the mobile phase during gradient elution HPLC greatly influences the As^+ signals in ICPMS due to the C-enhancement effect [Allain et al. 1991]. One approach to compensate for the C-enhancement effect was to record the intensity of a constant concentration of DMA over the whole chromatogram and to calculate time dependent correction factors, which were then applied to the arsenolipids [Amayo et al. 2011]. The other approach was based upon the saturation of the ICPMS sample introduction system with a volatile organic solvent, which is introduced directly into the spray chamber of the ICPMS by a peristaltic pump, and therefore no change in sensitivity is observed [Raber et al. 2010]; this method was used by Garcia-Salgado et al. [2012].

1.4 Culture experiments of unicellular algae in arsenolipid research

To obtain sufficient quantities of algal biomass for experiments, it is usually necessary to use larger volumes of nutrient-enriched culturing media to enhance the growth of unicellular algae. Physical properties like temperature or amount of light are also important and influence the cell growth and hence the biochemical processes in the algal cell. In addition, the age and physiological state of the cultures at the time of inoculation, the presence of other microorganisms (e.g. bacteria) in the media, or the reactor type — batch and continuous flow reactor — can influence the algal cell biochemistry [Foster et al. 2008; Duncan et al. 2010].

The first study on arsenic uptake by unicellular algae was performed by Lunde et al. [1972]. He cultured marine and limnetic algae in nutrient-enriched media, based on seawater or distilled water [Lunde et al. 1972]. The flasks stood on a "vibrating table" and were illuminated by fluorescent lamps at 6000 lux. The concentration of arsenic in the culture media was in the order of 1 – 3 µg/L. After the algae had reached a certain point of growth, half of the culture was removed daily and replaced by fresh media. It took fourteen days until enough biomass was obtained for the analysis. Later on, he grew unicellular algae under similar conditions but added radioactive ^{74}As in the form of arsenite, arsenate or a mixture of both to the cultures [Lunde 1973]. Bottino et al. [1978] used a similar approach for the determination of the effect of arsenate and arsenite on the growth and morphology of unicellular algae. Algae were cultured in a temperature-controlled room at 20°C in modified seawater or artificial seawater at light intensities of 7000 lux or 12500 lux. The salinity of the media was adjusted to 20 ‰.

Recent culturing experiments describe the use of environmental chambers [Foster et al. 2008; Duncan et al. 2010]. In those experiments, axenic cultures of *Dunaliella tertiolecta* were cultured in sterile 2 L flasks in a nutrient-enriched media at 21°C in an environmental chamber at approx. 4000 lux on a 12:12 h light to dark cycle. Usually the algal cells were harvested from their growth media by filtration or centrifugation and processed according to the chosen analysis aim. So far studies of the arsenolipid metabolism with characterization of the intact arsenolipids have been restricted because of the lack of suitable analytical methods.

Work from Foster et al. [2008] showed, in accordance with former observations (Lunde 1973; Irgolic et al. 1977; Cooney et al. 1978; Andreae and Klumpp 1979; Wrench and Addison 1981), that the unicellular alga *Dunaliella tertiolecta* incorporated approx. 30 % of the total arsenic into the lipid fraction. The main arsenic compound in the hydrolyzed lipid extracts was arsenosugar **9c**, which could be a hydrolysis side product of the arsenosugar phospholipids.

1.5 Purpose of this work

A common method for the extraction of lipids uses a mixture of chloroform/methanol at a ratio of 2+1 (v/v) as first reported by Folch et al. [1957]. It is regarded as the "gold standard" for lipid extraction. In 2008, Matyash et al. [2008] reported an alternative extraction procedure for lipids, using methyl tertiary-butyl ether/methanol at a ratio of 10+3 (v/v) as extraction solvents.

The first part of this work compares and evaluates extraction systems based on mixtures of chloroform/methanol and of methyl tertiary-butyl ether/methanol for the extraction of arsenolipids, and describes sample preparation steps necessary for the ESMS determination of arsenolipids. The second part investigates the reference material NMIJ CRM 7405-a Trace Elements and Arsenic Compounds in Seaweed (Hijiki) for the existence of arsenolipids with the aim of providing a readily accessible source of these compounds. In the final part an experimental protocol for growing *Dunaliella tertiolecta* in batch cultures under various phosphorous to arsenic ratios is described.

2 Experimental

2.1 Chemicals

2.1.1 Standards and reagents

Milli Q water (18.2 MΩ cm; Millipore GmbH, Vienna Austria), referred to hereafter as water, was used throughout these experiments. Nitric acid (≥ 65 %, p.a.), aqueous ammonia (≥ 25 %, p.a.), methyl tertiary-butyl ether (MTBE, ≥ 95.5 %), formic acid (≥ 98 %, p.a.) and silica gel 60 (0.06-0.2 mm) were obtained from Carl Roth GmbH (Karlsruhe Germany); chloroform (99.0 – 99.4 %, p.a.) was obtained from Sigma Aldrich (Vienna, Austria), methanol from J.T.Baker (Deventer, Netherlands), ammonium bicarbonate (≥ 99.0 %, p.a.) from Fluka (Buchs, Switzerland), and acetic acid (96 %, p.a.) and acetone (≥ 99.8 %) from Merck (Darmstadt, Germany). Except for nitric acid, which was further purified in a quartz sub-boiling distillation unit, the chemicals were used as supplied.

Arsenic, germanium, indium and lutetium (1000 ± 3 µg/L in 2 % HNO_3) stock standard solutions for total measurements by ICPMS were purchased from CPI International (Santa Rosa, USA). Dimethylarsinate prepared from sodium dimethylarsinate (Fluka, Buchs, Switzerland) served as a standard for HPLC/ICPMS/ESMS measurements.

2.1.2 Certified reference materials

Standard Reference Material® 1643e Trace Elements in Water from NIST (Gaithersburg, USA) was used for quality control in total ICPMS measurements. Also certified reference material (CRM) from the National Metrology Institute of Japan, NMIJ CRM 7405-a Trace Elements and Arsenic compounds in seaweed (Hijiki) from AIST (Tsukuba, Japan), was used as a CRM for total arsenic determinations.

2.2 Samples

Commercially available Japanese seaweed (Hijiki), purchased from Mimasa (Barcelona, Spain), and the certified reference material NMIJ CRM 7405-a Trace Elements and Arsenic Compounds in Seaweed (Hijiki) from the National Metrology Institute of Japan (Tsukuba, Japan), kindly provided by Professor Narukawa, were used as samples. Axenic

cultures of *Dunaliella tertiolecta* were obtained from the Centre for Analytical Chemistry (CSIRO, Lucas Heights Science and Technology Centre, NSW, Australia).

2.3 Instrumentation

ICPMS measurements were carried out on an Agilent 7500ce ICPMS (Agilent Technologies, Waldbronn, Germany), equipped with a Burgener Ari MIST HP nebulizer (Burgener Research Inc., Mississauga, Canada), a Scott double-pass spray chamber, for total arsenic measurements, or a cyclonic spray chamber (Elemental Scientific Inc., Omaha, USA) for HPLC/ICPMS/ESMS measurements, an Integrated Sample Introduction System, and an ASX-500 auto-sampler (Agilent Technologies, Waldbronn, Germany). An Agilent Series 1100 LC/MSD single quadrupole mass spectrometer system (Agilent Technologies, Waldbronn, Germany) equipped with an atmospheric pressure ionization (API) LC-MS interface and an electrospray ionization source (ESI) was used for ESMS measurements. The Agilent 1100 HPLC-system used for the chromatographic separations was equipped with a binary pump, a vacuum degasser, column oven and an autosampler. A second Agilent 1100 HPLC system was used to mix the post-column effluent with an additional make-up mobile phase. The separating HPLC-system was connected with polyether ether ketone tubing to an adjustable flow splitter from ASI (Richmond, USA). Polyether ether ketone capillaries were also used for the connection of the flow splitter to the second HPLC system, the ICPMS and the ESMS.

GFAAS measurements were carried out on a Zeeman atomic absorption spectrometer (AA 240Z) with a GTA-120 Series graphite tube atomizer and PSD 120 auto sampler all from Varian Australia Pty Ltd (Mulgrave, Australia). Microwave digestion was performed with an UltraCLAVE IV (MLS GmbH, Leutkirch, Germany) in 12 mL quartz tubes with polytetrafluoroethylene caps. For centrifugation a Hettich Rotina 420 R or a Hettich 2043 Mikroliter centrifuge (Hettich, Tuttlingen, Germany) was used. Solvents were evaporated under reduced atmospheric pressure in a Maxi-Dry Plus (Heto Holten, Allerød, Denmark) equipped with a vacuum pump (Vakuumbrand, Wertheim, Germany). Samples were sonicated with a Transsonic T 700/H sonication bath (Elma Hans Schmidbauer GmbH & Co. KG., Singen, Germany). Samples were weighed with a SI-234 balance d=0.1 mg (Denver Instruments, Colorado, USA). Socorex Acura® pipettes (Ecublens, Switzerland) were used for preparing standards and sample solutions. Environmental chambers (3504 process controller, Eurotherm, Australia) were used to culture *Dunaliella tertiolecta*. Preparations

under aseptic conditions were carried out in a biological safety cabinet (Gelaire, BSB-12, Sydney, Australia).

2.4 Sample Preparation

Samples were extracted in triplicate in the various solvent mixtures for one hour on a rotating cross, unless otherwise specified. Afterwards, samples were centrifuged (3270 G, 10 minutes) and the supernatants were transferred to polypropylene tubes (Greiner, Bio-one, Frickenhausen, Germany). Three replicates of each solution were taken to determine the total content of arsenic by ICPMS. The remaining extractant was either washed with water or not — three replicates of each organic or aqueous layer were taken to determine the total content of arsenic by ICPMS — and evaporated overnight at room temperature under reduced pressure (approx. 10 mbar Maxi Dry Plus). The residue was re-extracted in a smaller volume of organic solvent (extraction was assisted by brief immersion in an ultrasonic bath). The extract was applied to a small silica column (Pasteur pipette packed to a height of 5 cm with silica gel, mesh size 0.06 – 0.2 mm) and fractions (1 mL) were collected. After the silica column purification, aliquots of the collected fractions were taken to determine the total content of arsenic by ICPMS or GFAAS. The fractions rich in arsenic were combined and evaporated. The residue was re-extracted with a small volume (usually 200 µL) of organic solvent (methanol or methanol/acetone 1+1 v/v). The mixtures were transferred to micro-centrifuge tubes (Sarstedt, Nümbrecht, Germany), centrifuged and the supernatant transferred to 250 µL vials for HPLC/ICPMS/ESMS measurements.

2.4.1 Extraction of a commercially available dried alga (Hijiki, *Hizikia fusiforme*) with various extraction solvents

2.4.1.1 Evaluation of extraction solvents (Experiment I)

A portion (0.8 g) of freeze-dried powdered alga was extracted either in methyl tertiary-butyl ether/methanol (MTBE/MeOH 10+3 v/v, 26 mL) or MTBE/MeOH (10+6 v/v, 26 mL) or chloroform/methanol ($CHCl_3$/MeOH 2+1 v/v, 18 mL). After the supernatant was transferred to a new tube, half of the MTBE/MeOH (10+6 v/v, 13 mL) solution was diluted with MTBE (8 mL). Water (5 mL) was added to all MTBE/MeOH solutions. The mixtures were shaken, centrifuged (3270 G, 10 min) and the upper organic layer was transferred to a new tube by transfer pipette. The $CHCl_3$/MeOH (2+1 v/v) solution was transferred to a separatory funnel and water (5 mL) containing 1 % ammonium bicarbonate (w/v) was added. After shaking the

solution, the organic and aqueous layers were separately collected. Solvents were evaporated and all residues were re-extracted with MeOH (200 µL).

2.4.1.2 Influence of the silica column pre-purification step (Experiment II)

A portion (0.8 g) of freeze-dried powdered alga was extracted either in MTBE/MeOH (10+3 v/v, 26 mL) or $CHCl_3$/MeOH (2+1 (v/v, 18 mL). The extractants were evaporated and the residues were re-extracted in MeOH/acetone (1+1 v/v, 2 mL). A Pasteur pipette was packed with silica and conditioned with MeOH/acetone (1+1 v/v) containing 1 % formic acid. A portion (0.5 mL) of the re-extracted residue was applied to the column and washed with MeOH/acetone (1+1 v/v, 5 mL) containing 1 % formic acid, MeOH (3 mL) and MeOH containing 1 % aqueous ammonia (10 mL). Fractions (1 mL) were collected and arsenic was located in the fractions by using GF-AAS (GF-AAS settings, Appendix A1). The main arsenic-containing fractions were combined and the solvent was evaporated. The residues were re-extracted with MeOH (200 µL).

2.4.1.3 Downscaling of the extraction procedure (Experiment III)

A portion (0.1 g) of freeze-dried powdered alga was extracted either in $CHCl_3$/MeOH (2+1 v/v, 2.5 mL) or MTBE/MeOH (10+6 v/v, 3.2 mL). After the supernatant was transferred to a new tube, MTBE (2 mL) was added to the MTBE/MeOH extract. Afterwards, water (1 mL) containing 1 % ammonium bicarbonate (w/v) was added to the MTBE/MeOH extracts as well. The mixtures were shaken and centrifuged (3270 G, 10 min) and the upper organic layer was transferred to a new tube by pipette. The aqueous layer was re-partitioned with MTBE (2 mL) and the organic phases were combined. Water (0.7 mL) containing 1 % ammonium bicarbonate (w/v) was added to the $CHCl_3$/MeOH extracts, and the mixture was transferred to a separatory funnel and shaken. The organic layer was collected; $CHCl_3$ (3 mL) was added to the aqueous layer and the mixture was again shaken. The organic layers were combined. Solvents were evaporated and all residues were re-extracted with MeOH/acetone (1+1 v/v, 200 µL).

2.4.2 Extraction of reference material NMIJ CRM 7405-a Trace Elements and Arsenic Compounds in Seaweed (Hijiki)

2.4.2.1 Chloroform/methanol extraction with water partitioning and silica column pre-purification (Experiment A)

A portion (1.6 g, n=1) of freeze-dried powdered alga was extracted in $CHCl_3$/MeOH (2+1 v/v, 36 mL). A portion (5 mL) of the extract was evaporated and the residue stored for later arsenolipid speciation. The rest of the algal extract (31 mL) was transferred to a separatory funnel and water (7.5 mL) containing 1 % ammonium bicarbonate was added and the mixture was shaken. After phase separation, the organic and the aqueous layers were separately collected. The solvents were evaporated. The residue of the organic phase was re-extracted in $CHCl_3$/acetone (1+1 v/v, 4 mL) and the residue of the aqueous layer was re-extracted in MeOH/acetone (1+1 v/v, 4 mL). A portion of the organic and of the aqueous layer was used for later arsenolipid speciation. A Pasteur pipette was packed with silica and conditioned with $CHCl_3$/acetone (1+1 v/v) containing 1 % formic acid. A portion (1 mL) of the re-extracted organic phase was applied to the column and washed with conditioning solvent (5 mL), MeOH (3 mL) and MeOH containing 1 % aqueous ammonia (20 mL). Fractions of 5 mL or 10 mL were collected and portions were used to determine the total arsenic content by ICPMS. For the two fractions that contained the most arsenic, the solvent was evaporated and the residue re-extracted with MeOH/acetone (1+1 v/v, 200 µL).

2.4.2.2 Chloroform/methanol extraction with silica column pre-purification, but without water partitioning (Experiment B)

A portion (0.1 g) of freeze-dried powdered alga was extracted in $CHCl_3$/MeOH (2+1 v/v, 10 mL or 5 mL). The extractant was evaporated and the residue was re-extracted in $CHCl_3$/acetone (1+1 v/v, 2 mL). A Pasteur pipette was packed and conditioned as described in Section 2.4.2.1, and a portion (1 mL) of the re-extracted residue was applied to the column. Fractions of 1 mL were collected and portions were used to determine the total arsenic content by ICPMS. Those fractions containing most of the arsenic were combined, the solvent was evaporated and the residue re-extracted in MeOH/acetone (1+1 v/v, 100 µL).

2.5 Determination of total arsenic by ICPMS

The solvent was evaporated from the samples, water (2 mL) and concentrated nitric acid (2 mL) were added to the residue, and a microwave-assisted acid digestion was carried out (microwave program, Appendix A2). Afterwards, the samples were diluted with water up to 9.8 g. 1 mL of internal standard (50 % methanol, 100 µg/L Ge, 100 µg/L In in water) was added to the solution before ICPMS measurements (ICPMS settings, Appendix A3). The recorded m/z ratios were 74 (Ge^+), 75 (As^+), 77($^{40}Ar^{37}Cl^+$) and 115 (In^+). For internal standardization, m/z ratios 74 and 115 were used. Quantification of arsenic was carried out by external calibration with As-standard solutions in the range of 0.1 µg/L to 500 µg/L. For additional quality control of the measurements, a drift control solution was measured every 15-30 samples within a batch run. SRM 1643e Trace Elements in Water was measured to evaluate the external calibration. The SRM contained a certified amount of 60.45 ± 0.72 µg arsenic per litre. The measured value by ICPMS was 61.83 ± 2.91 µg arsenic per litre (n=6). The algal reference material NMIJ CRM 7405-a was digested and measured to evaluate the microwave-assisted acid digestion method. The CRM contained a certified amount of 35.8 ± 0.9 µg arsenic per gram. the measured value by ICPMS was 35.5 ± 0.7 µg arsenic per gram (n=6).

2.6. Speciation analysis of arsenic by HPLC/ICPMS/ESMS

For the HPLC separation, 20 µL or 40 µL of sample were injected. The separation was carried out on a C_8-reversed-phase column (Agilent ZORBAX XDB-C8 4.6 mm x 150 mm; 5 µm; Agilent Technologies, Waldbronn, Germany) with gradient elution (10 mmol acetic acid in water with ammonia at pH 6.0 and methanol) at a flow rate of 1 mL/min. The gradient elution conditions were: 0-25 min, 50-95 % methanol; 25-40 min, 95 % methanol. The HPLC-effluent was split post-column by an adjustable flow splitter. A portion of the total flow (2.2 % or 10 %) went to the ICPMS and the rest went to the ESMS. For ICPMS measurements, the split flow was mixed with another flow (10 % or 5 % methanol, 0.1 % formic acid in water, 10 µg/L Ge; flow rate 0.2 mL/min or 1.0 mL/min) from a second HPLC pump. Carbon compensation [Raber et al. 2010] due to the gradient elution was carried out by directly pumping an aqueous solution of a volatile organic solvent (2 % acetone or 15 % methanol in water) over the integrated sample introduction system at 0.05 rounds per minute into the spray chamber of the ICPMS. The m/z ratios recorded by ICPMS were 53 ($^{40}Ar^{13}C^+$), 74 (Ge^+),

75 (As^+) and 77 ($^{40}Ar^{37}Cl^+$). Quantification of arsenic was carried out by ICPMS using external calibration with DMA-standard solutions in the range of 1 µg/L to 1000 µg/L. With ESMS, selected ion monitoring (SIM) was used to record the following m/z values: 333, 361, 389, 931, 945, 957, 959, 983, 985, 987, 1013, 1015, 1043, 1071; scan from m/z 200 to 1100 was also performed (ICPMS & ESMS settings, Appendix A3-A4).

2.7 Growing of *Dunaliella tertiolecta* in a batch reactor under various phosphorous to arsenic ratios

Axenic cultures of *Dunaliella tertiolecta* were grown at four ratios of phosphorous to arsenic: 1100 nM phosphorous to 67 nM arsenic; 1100 nM phosphorous to 200 nM arsenic; 8900 nM phosphorous to 67 nM arsenic; 8900 nM phosphorous to 200 nM arsenic.

2.7.1 Stock cultures

Axenic cultures of *Dunaliella tertiolecta* were transferred under aseptic conditions into sterile 50 mL polypropylene tubes containing 30 mL of sterile 0.3 µm filtered, f/10 seawater medium [Guillard and Ryther 1962]. The seawater media were prepared according to a modified recipe of Guillard and Ryther [1962], but in 10-fold dilution of the original instructions and at a phosphate concentration of approx. 0.85 mg per liter (8900 nM phosphorus). Cultures were incubated in environmental chambers under a light intensity of approximately 110 µmol photons m^{-2} s^{-1} (ca. 6000 lux), a 12:12 hour light to dark cycle and a temperature regime of 20°C during dark periods and 25°C during light periods. Stock cultures were tested for contamination with other culturable micro-organisms by swiping peptone yeast extract agar (PYEA) plates with the culture solution. Plates were incubated under the same conditions as the stock cultures for one week, after which they were examined for the presence of bacterial growth. The stock culture (1 mL) was transferred every week to fresh, sterilized f/10 seawater medium as described above to maintain exponential growth of the algal cells.

2.7.2 Batch cultures

Stock culture (30 mL) was transferred to sterilized 5 L flasks, containing 3 L of sterile 0.3 µm filtered f/10 seawater medium. The seawater media were prepared as described above but at varying phosphate levels. We started our experiments with three different phosphate treatments. The first treatment contained no added phosphate, the second contained

approx. 0.1 mg/L phosphate (approx. 1100 nM phosphorus) and the third contained approx. 0.85 mg/L phosphate (approx. 8900 nM phosphorus). A portion of the batch culture (20 mL) was transferred to a sterile polypropylene tube (50 mL) to test for contamination as described in paragraph 2.6.1 and to determine the cell growth. Batches were incubated for 7 days under the same conditions as mentioned above and were daily shaken and randomly placed in the environmental chambers. After 72 hours of incubation, arsenate stock solution (0.5 ml of either 0.4 mM or 1.2 mM), previously filtered (0.2 µm), was added to those batches which already contained additional phosphorus. A portion of the batch culture (20 mL) was transferred to a sterile polypropylene tube to test for the presence of bacteria and to determine the cell growth. In total, five batch cultures were prepared — four with different ratios of phosphorous to arsenic and one control sample, which contained no added phosphorous or added arsenic. At day seven, a portion of the batch culture (20 mL) was transferred to test for the presence of bacteria and to determine the cell growth. The cells of the batch cultures were harvested by centrifugation (4500 G, 10 min) and the algal tissue was frozen, freeze dried and stored for analysis. In total, 40 batches were cultured.

2.7.3 Growth determination

A portion of the sample (10 µL) was transferred to a haemocytometer slide. Cells within the four 4x4 grids at each of the corners of the slide were counted using a phase contrast microscope (Olympus model CHS, Tokyo, Japan) at a magnification of x 400 and the total number of cells per mL was calculated.

3 Results and discussion

3.1 Extraction of commercially available dried Hijiki

Three solvent mixtures (Experiment I) were tested for their ability to extract arsenic from algal tissue. After the extraction, water was added to induce phase separation, and the organic and aqueous layers were analysed. Next, a cleanup step by using a small silica column (Experiment II) was introduced after the extraction to purify the samples for improved ESMS measurements due to less matrix interference. Downscaling of the extraction (Experiment III) was then performed to test the handling of smaller amounts of sample.

3.1.1 Total arsenic extracted

The concentration of arsenic in commercial Hijiki was 141.7 ± 0.7 µg/g (dry mass; n=3) but only a small portion (ca 2-4 %) of that was extractable with organic solvents. $CHCl_3$/MeOH extracted about twice as much arsenic as did MTBE/MeOH 10+3 and about 30 % more arsenic than MTBE/MeOH 10+6. The downscaled procedure extracted approx. 10 % less arsenic compared to the larger scale procedures (Table 6). The mass of the residues after evaporation of the extractants were approx. 40 mg for $CHCl_3$/MeOH 2+1, approx. 40 mg MTBE/MeOH 10+6 and approx. 20 mg for MTBE/MeOH 10+3. The extraction mixtures that extracted more arsenic out of the algal tissue also extracted more matrix constituents.

Table 6 Extracted arsenic per gram dried alga (initial total [As] in alga was 141.7 µg/g).

Extractant	µg As/g dried alga	n	Experiment
MTBE/MeOH 10+3	2.44 ± 0.17	6	I;II
MTBE/MeOH 10+6	4.41 ± 0.10	3	I
$CHCl_3$/MeOH 2+1	5.69 ± 0.13	6	I;II
$CHCl_3$/MeOH 2+1	5.12 ± 0.27	3	III

The arsenic concentrations of the organic and of the aqueous layers after the water partitioning are shown in Table 7. The organic phases contained about 45 % to 70 % of the extracted arsenic in form of arsenolipids, whereas polar arsenic compounds were removed with the aqueous phase. The organic phase of $CHCl_3$/MeOH contained approximately 60 %

more arsenic than MTBE/MeOH 10+3. More arsenic (ca 50 %) was found in the organic phase of MTBE/MeOH 10+6 compared to MTBE/MeOH 10+3 (Table 7). Therefore, $CHCl_3$/MeOH was more efficient for the extraction of arsenolipids than MTBE/MeOH. Possibly, the polarity of $CHCl_3$/MeOH (2+1, v/v) was more suitable for the extraction of arsenolipids than the polarity of MTBE/MeOH (10+3 or 10+6, v/v).

Table 7 Extracted arsenic (expressed as µg As/g dry alga) of the organic phase after water partitioning and of the water phase.

Extractant	µg As/g dried alga (n=3)	Experiment
Organic phase		
MTBE/MeOH 10+3	1.68 ± 0.12	I
MTBE/MeOH 10+6	2.51 ± 0.46	I
$CHCl_3$/MeOH 2+1	2.78 ± 0.06	I
MTBE/MeOH 10+6	1.94 ± 0.03	III
$CHCl_3$/MeOH 2+1	2.51 ± 0.13	III
Aqueous phase		
MTBE/MeOH 10+3	1.02 ± 0.16	I
MTBE/MeOH 10+6	1.92 ± 0.30	I
$CHCl_3$/MeOH 2+1	2.98 ± 0.22	I
MTBE/MeOH 10+6	1.37 ± 0.06	III
$CHCl_3$/MeOH 2+1	2.25 ± 0.12	III

3.1.2 Arsenic species determined by HPLC/ICPMS/ESMS

3.1.2.1 Evaluation of extraction solvents (Experiment I)

The HPLC/ICPMS chromatograms of the extracts of Hijiki were similar to that previously reported [Garcia-Salgado et al. 2012] (Figure 8). The two predominant arsenolipids were AsHC 332 (peak C) and AsPL 958 (peak F). For MTBE/MeOH 10+3 the intensity of peak F relative to peak C was reduced compared to that for the other solvent mixtures suggesting that it was less efficient at extracting arsenosugar phospholipids (Table 8; Appendix A5-A9). In accordance with the total arsenic data (Table 7), the quantification of arsenolipids in the solvent mixtures suggested that $CHCl_3$/MeOH was more efficient for the extraction of arsenolipids than MTBE/MeOH. $CHCl_3$/MeOH 2+1 extracted more than twice as much of AsPL 958 than MTBE/MeOH 10+3 (Table 8). The amount of AsPL 958 in the MTBE/MeOH 10+6 extract was approx. 20 % lower than in the $CHCl_3$/MeOH 2+1 extract but extracted about twice as much of it as the MTBE/MeOH 10+3 extract. The MTBE/MeOH 10+6 extract showed a higher amount of the unknown compounds A and B.

Table 8 Quantification of arsenolipids for different extraction solvents.

	Unknown A	Unknown B	AsHC 332	AsPL 958
	ng As per g dried alga			
MTBE/MeOH 10+3 (n=2)	11; 10	43; 33	422; 435	391; 368
MTBE/MeOH 10+6 (n=3)*	35 ± 4	206 ± 23	488 ± 15	861 ± 75
MTBE/MeOH 10+6 (n=2)**	20; 20	104; 92	467; 416	720; 686
$CHCl_3$/MeOH 2+1 (n=3)	20 ± 8	24 ± 8	511 ± 12	1074 ± 40

*before water partitioning; ** after water partitioning.

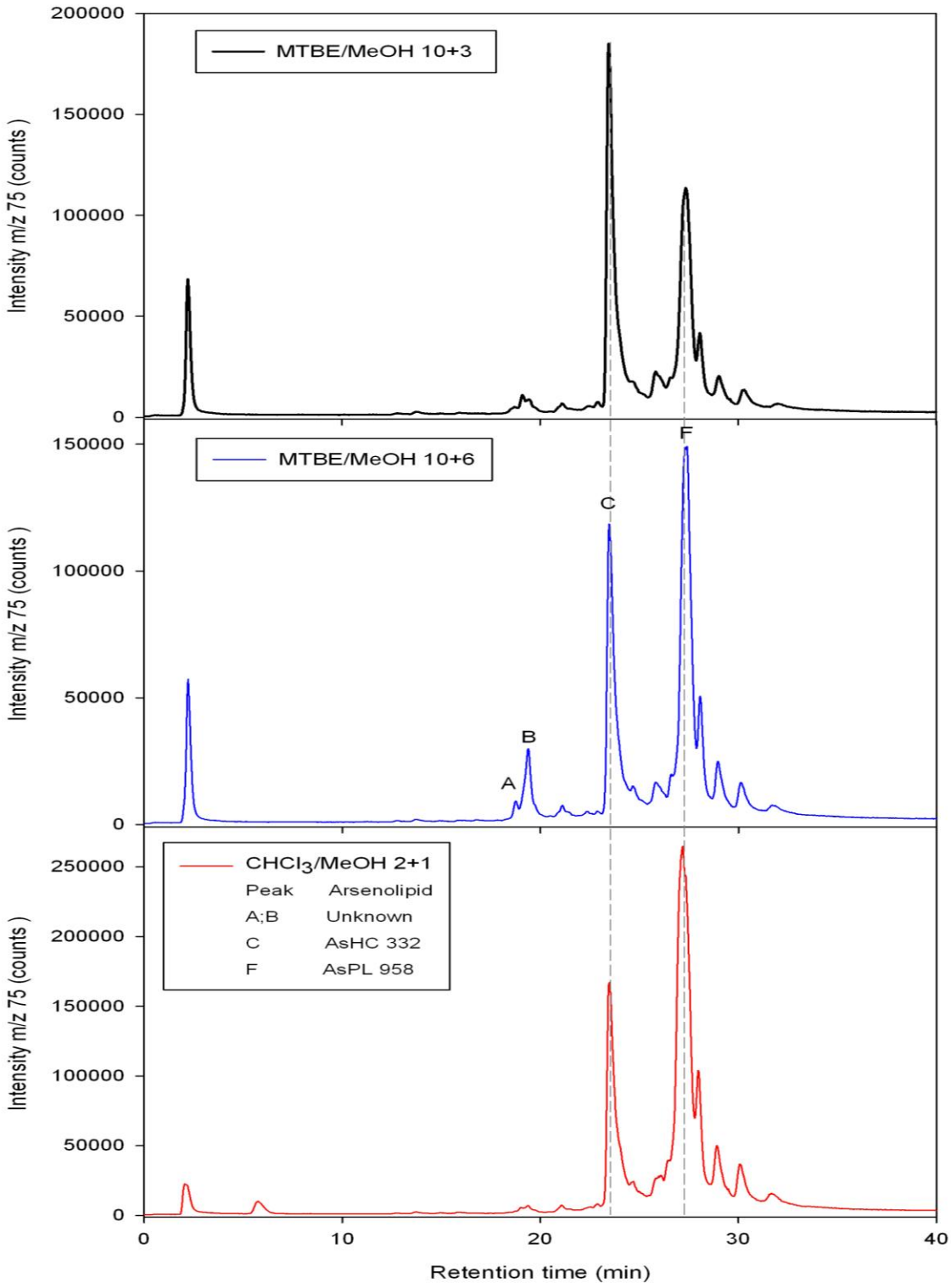


Figure 8 HPLC/ICPMS chromatograms for arsenolipids from different extraction solvents; stationary phase: ZORBAX XDB-C8 4.6 mm x 150 mm; 5 µm; mobile phase: 10 mmol acetic acid in water with NH_3 at pH 6.0 and methanol, 1 mL/min, gradient elution; gradient profile: 0-25 min, 50-95 % methanol; 25-40 min, 95 % methanol; post column split, 2.2 % to ICPMS; additional flow: 10 % methanol, 0.1 % formic acid in water, 0.2 mL/min; carbon compensation 2 % acetone; 20 µL sample injected.

3.1.2.2 Influence of the silica column pre-purification step (Experiment II)

$CHCl_3$/MeOH 2+1 and MTBE/MeOH 10+3 extracts were directly applied — without adding water — to a silica column for purification of the crude extracts. The quantitative data (Table 9; Appendix A10-A15) of the crude $CHCl_3$/MeOH and MTBE/MeOH 10+3 extracts showed the same pattern as described in Section 3.1.2.1 with one exception. The $CHCl_3$/MeOH extract contained a high amount of the unknown compounds A and B. These high amounts of A and B indicated that they were possibly removed during the water partitioning step in our first experiment. Most of the applied arsenic — 91 ± 9 % (n=3) for $CHCl_3$/MeOH and 102 ± 10 % (n=3) for the MTBE/MeOH — eluted off the silica column and nearly all within fraction 11 to 17 (Figure 9). The crude extract (before SiO_2-column) was dark green, and the residue obtained on evaporation of the extract had a mass of approx. 20 mg for MTBE/MeOH and of approx. 40 mg for $CHCl_3$/MeOH. The purified extracts of the combined fractions 11-17 for both procedures were colourless, and the residues after evaporation had masses of approx. 1-2 mg. Thus the silica column removed about 90 % of the total mass, while returning about 90 % of the total arsenic, and thereby provided much cleaner samples and sharper peaks in the chromatograms (Figure 10; Appendix A10-A11).

Table 9 Quantification of arsenolipids before the silica column.

	Unknown A+B	AsHC 332	AsPL 958
	ng As per g dried alga (n=1)		
Before silica column			
MTBE/MeOH 10+3	39	548	360
$CHCl_3$/MeOH 2+1	462	675	1054

The arsenolipid concentrations for the main arsenolipids after purification are shown in Table 10. One purified $CHCl_3$/MeOH extract out of three was stored for 10 days in its elution solvent, which contained 1 % ammonia; the elution solvents of the other two samples were removed within 1 day after storage. The amount of arsenosugar phospholipids was lower (approx. 30 %; Appendix A16) in the longer stored sample and was therefore removed as a replicate in Table 10. Possibly, base catalyzed cleavage of the ester bonds of the arsenosugar phospholipids occurred during the 10-day storage in 1 % ammonia. This bond cleavage might also occur during the silica column purification process or with short time storage, which

could be the explanation for the observed losses (approx. 30 %) of arsenosugar phospholipids during the silica clean-up step.

Table 10 Quantification of arsenolipids after the silica column.

	Unknown A	Unknown B	AsHC 332	AsPL 958
	ng As per g dried alga			
After silica column				
MTBE/MeOH 10+3 (n=3)	15 ± 1	62 ± 6	483 ± 20	265 ± 34
$CHCl_3$/MeOH 2+1 (n=2)	80; 70	340; 330	590; 540	710; 720

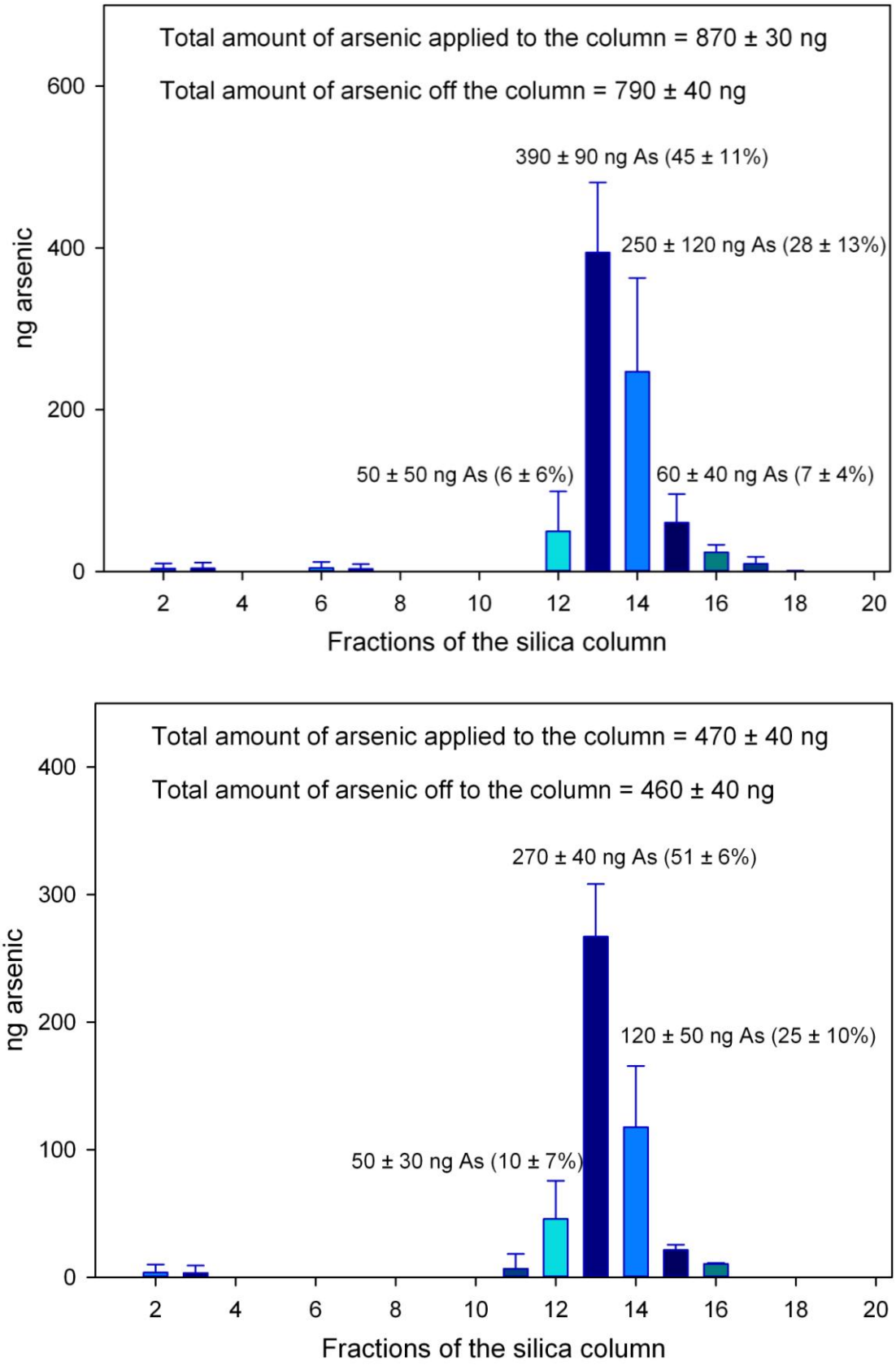


Figure 9 Distribution of arsenic in the silica column fractions for $CHCl_3$/MeOH (above) and MTBE/MeOH (beneath).

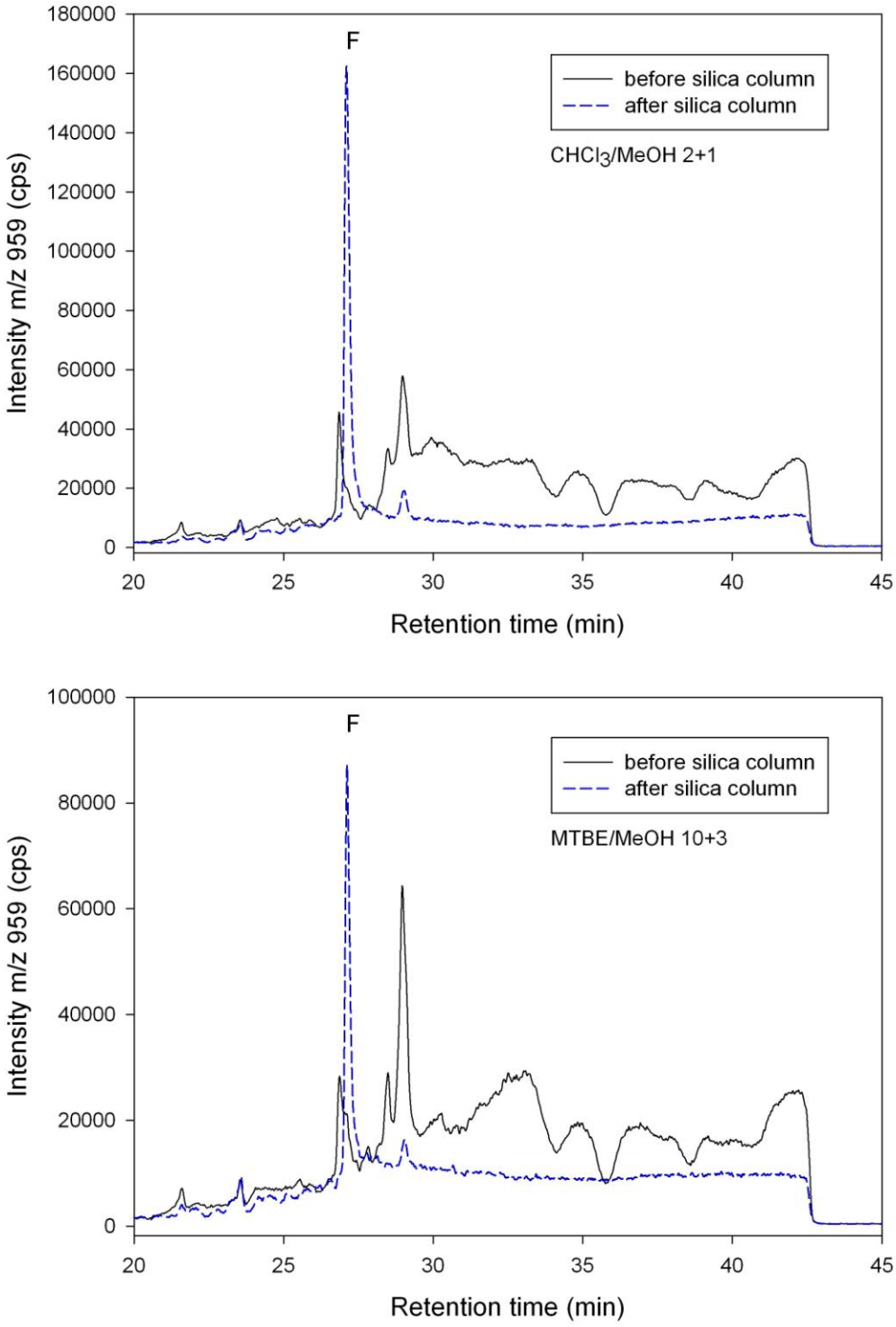


Figure 10 HPLC/ESMS chromatograms at m/z 959 of the silica column procedure, above: chloroform/methanol 2+1, beneath: methyl tertiary-butyl ether/methanol 10+3; stationary phase: ZORBAX XDB-C8 4.6 mm x 150 mm; 5 µm; mobile phase: 10 mmol acetic acid in water with NH_3 at pH 6.0 and methanol, 1 mL/min, gradient elution; gradient profile: 0-25 min, 50-95 % methanol; 25-40 min, 95 % methanol; post column split, 10 % to ICPMS; additional flow: 5 % methanol, 0.1 % formic acid in water, 1 mL/min; carbon compensation 15 % methanol; 40 µL sample injected.

3.1.1.3 Downscaling of the extraction procedure (Experiment III)

Downscaling of the extraction procedure was performed to test the handling of small extraction volumes. Experiment III is the downscaled form of experiment I for $CHCl_3$/MeOH 2+1 and MTBE/MeOH 10+6 with back-washing of the aqueous layer with organic solvent. The extractant to algal tissue ratio was the same for both experiments. The chromatograms for the downscaled procedure showed the same peaks for $CHCl_3$/MeOH 2+1 and MTBE/MeOH 10+6 (Figure 11). In accordance with experiment I, $CHCl_3$/MeOH 2+1 extracted more arsenosugar phospholipids (approx. 30 %) as compared to MTBE/MeOH 10+6 (Table 11). The water phase of the MTBE/MeOH extract was checked for losses of arsenolipids, but nearly all of the arsenic in the water phase eluted in the void volume besides small amounts of compounds **A** and **B**. The handling of the downscaled procedure during phase separation was more difficult as compared to the larger procedure. More care must be taken to avoid losses of solvent, e.g. during transferring the solvent or on the glass surface of the separatory funnel. To overcome this problem, more extraction solvent should be used next time.

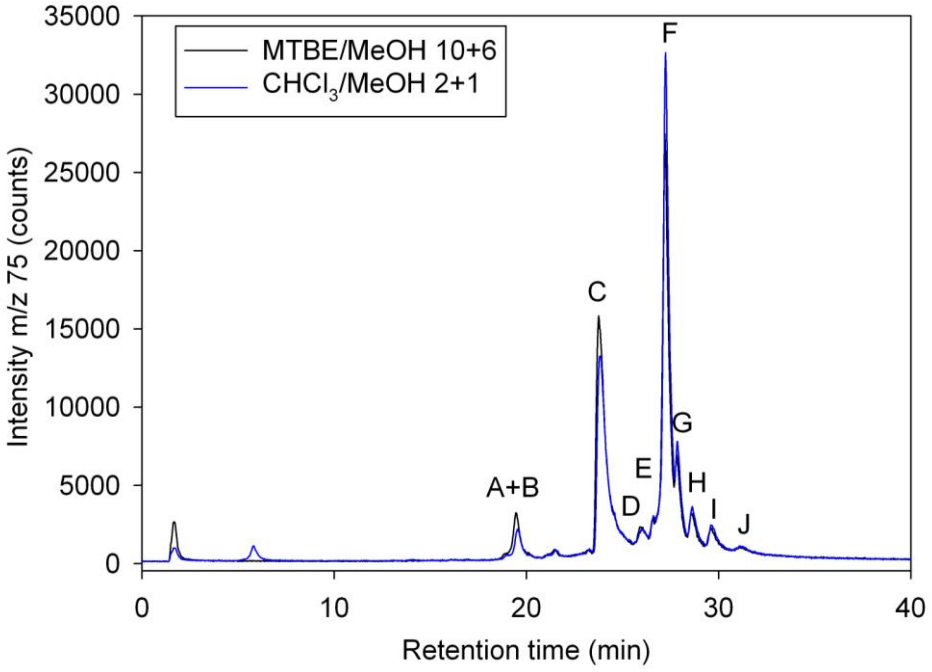


Figure 11 Comparison of HPLC/ICPMS chromatograms of the downscaled extraction procedure; ZORBAX XDB-C8 4.6 mm x 150 mm; 5 µm; mobile phase: 10 mmol acetic acid in water with NH_3 at pH 6.0 and methanol, 1 mL/min, gradient elution; gradient profile: 0-25 min, 50-95 % methanol; 25-40 min, 95 % methanol; post column split, 10 % to ICPMS; additional flow: 5 % methanol, 0.1 % formic acid in water, 1 mL/min; carbon compensation 15 % methanol; 20 µL sample injected.

Table 11 Quantification of arsenolipids for the downscaled procedure.

	A+B	AsHC 332	AsPL 958
		ng As per g dried alga	
MTBE/MeOH 10+6 (n=3)	81 ± 9	508 ± 30	553 ± 13
$CHCl_3$/MeOH 2+1 (n=3)	77 ± 19	555 ± 23	765 ± 46

MTBE/MeOH extraction had the advantage that it was not as time consuming as the $CHCl_3$/MeOH extraction because no additional cleaning steps during sample preparation were necessary, e.g. cleaning the separatory funnel to avoid contamination. Therefore, I would use the MTBE/MeOH extraction procedure if many samples were to be screened for the presence of arsenolipids. If quantification of the arsenolipids, especially arsenosugar phospholipids, is necessary, the $CHCl_3$/MeOH extraction is preferred because of the higher extraction efficiencies (Table 11).

3.1.1.4 HPLC-column recoveries

The HPLC-column recoveries (Table 12) for all experiments were calculated by the following equation:

$$HPLC\ column\ recovery\ \% = \frac{Integration\ of\ the\ chromatogram\ between\ 0-45\ min}{amount\ of\ arsenic\ injected\ onto\ the\ column} * 100 \quad (eq.1)$$

The amount of arsenic injected onto the column was calculated from the total arsenic ICPMS data, except for MTBE/MeOH 10+3 after SiO_2 and $CHCl_3$/MeOH 2+1 after SiO_2 where the GFAAS concentrations were used. A supplied arsenolipid solution prepared from a synthesised AsHC 360 compound and containing approx. 160 µg As/mL (concentration determined by ICPMS), was also analyzed by GFAAS and gave approx. 190 ± 20 µg As/mL. The mean value of the column recovery varied about 10 % depending on the baseline in the chromatogram (systematic error). The column recoveries for the extracts were within 70 — 110 %.

Table 12 HPLC-column recoveries for extracts obtained from the different extraction procedures.

Extractant	Column recovery %	n	Experiment
Before SiO_2			
MTBE/MeOH 10+3	83-99	2	I
MTBE/MeOH 10+6	90-102	2	I
$CHCl_3$/MeOH 2+1	95-105	3	I
MTBE/MeOH 10+3	100-110	1	II
$CHCl_3$/MeOH 2+1	90-100	1	II
MTBE/MeOH 10+6	87-97	3	III
$CHCl_3$/MeOH 2+1	82-92	3	III
After SiO_2			
MTBE/MeOH 10+3 (n=3)	69-79	3	II
$CHCl_3$/MeOH 2+1 (n=3)	65-75	3	II

3.2 Extraction of algal reference material NMIJ CRM 7405-a (Hijiki) with chloroform/ methanol

Because of higher extraction efficiency, the $CHCl_3$/MeOH (2+1, v/v) mixture was chosen to investigate the existence of arsenolipids in the CRM with the goal of providing a readily accessible source of these compounds. The reference material contained a certified amount of 35.8 ± 0.9 µg arsenic per gram and an certified amount of 10.1 ± 0.5 µg arsenate per gram, the measured value by ICPMS was 35.5 ± 0.7 µg arsenic per gram (n=6).

3.2.1 Total arsenic extracted

3.2.1.1 Chloroform/methanol extraction with water partitioning and silica column pre-purification (Experiment A)

Extraction of the reference material (1.6 g ≡ 56.8 µg As) with chloroform/methanol (2+1 v/v, 36 mL) yielded 16.2 ± 0.1 µg As (n=3). After addition of water to a portion (31 mL ≡ 14.0 ± 0.1 µg As) of the extract and separation of the phases, the organic layer contained 13.1 ± 0.1 µg As and the aqueous layer 0.9 ± <0.1 µg As. The sum of the organic and of the water phase gave on average ca 99 % of the extractable amount of arsenic. The mass of the residue of the organic phase was approx. 70 mg and approx. 100 mg of the water phase. Figure 12 shows a flow chart of the extraction. A portion of the organic phase (2.8 ± <0.1 µg As) was applied to the silica column. The crude extract (before SiO_2-column) was dark green and fraction 1 as well. Fraction 2 was light green and fractions 3 to 5 were clear solutions. Most of the arsenic (ca 95 %) eluted within fraction 2 and 3 (Figure 13). Fraction 2 contained approx. 0.7 µg As (25 %) and the mass of the residue after evaporation of the eluent was approx. 10 mg. Fraction 3 contained approx. 2.0 µg As (70 %) and the mass of the residue was approx. 4 mg. The silica column removed for the main arsenic fraction about 90 % of the total mass, while returning about 70 % of the total arsenic.

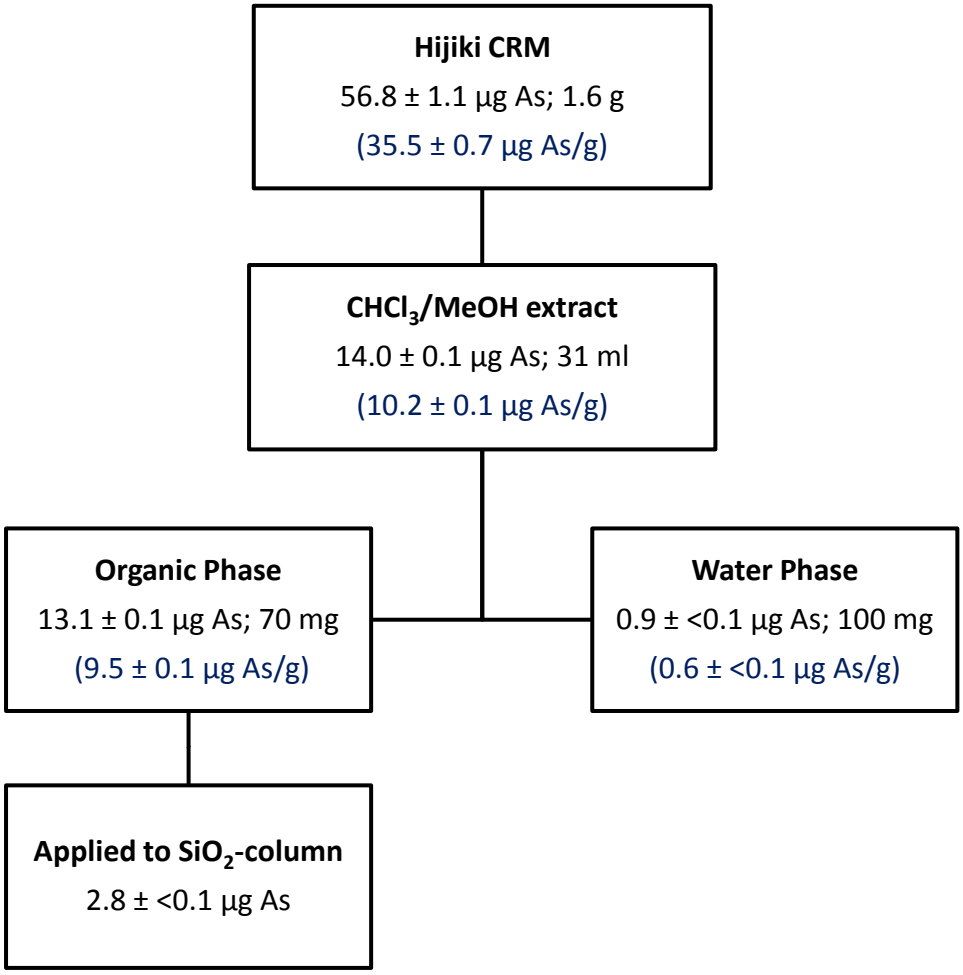


Figure 12 Flow chart of the arsenic distribution for experiment A.

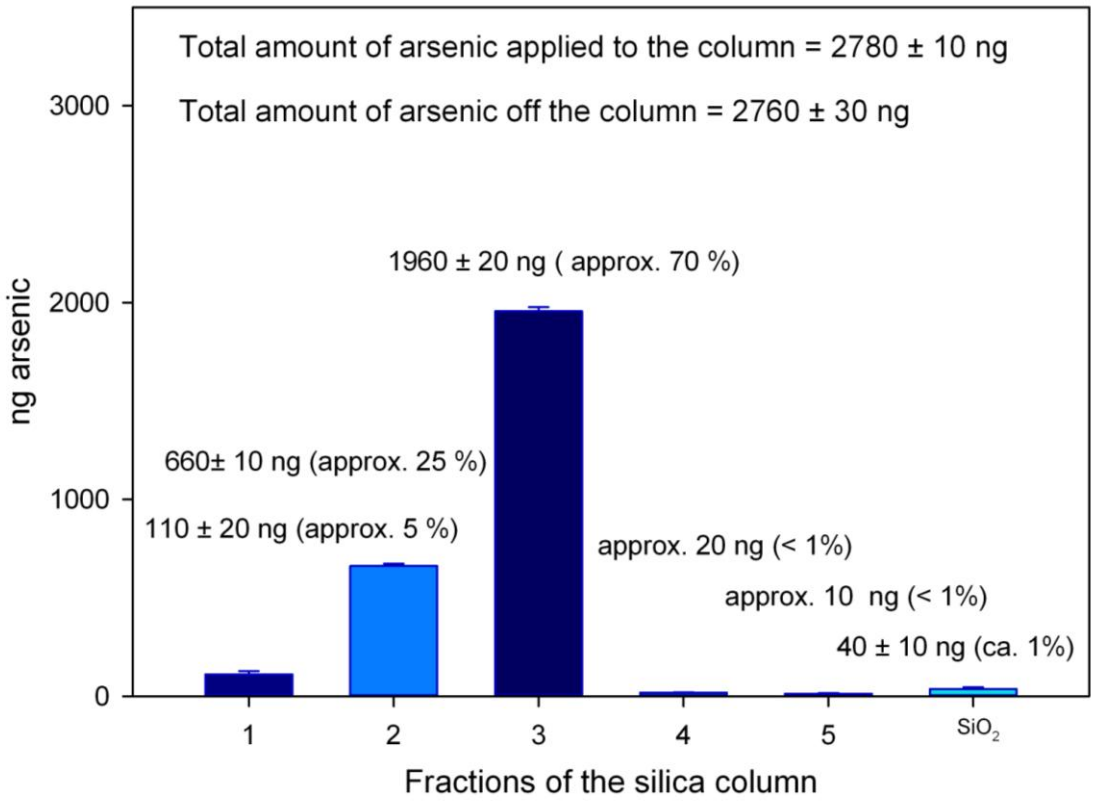


Figure 13 Distribution of arsenic in the silica column fractions for experiment A; fraction volume 5 mL or 10 mL.

3.2.1.2 Chloroform/methanol extraction with silica column pre-purification, but without water partitioning (Experiment B)

Extraction of the reference material (0.1 g ≡ 3.6 µg As) with chloroform/methanol (2+1 v/v) yielded 1.1 ± 0.1 µg As (n=6) with 10 mL and 5 mL extraction solvent (Figure 14). Therefore, the influence of different solvent volumes was negligible. The mass of the residue was approx. 50 mg (0.1 g alga) and compared to the residue of experiment A (170 mg; 1.6 g alga) it seems that more matrix constituents were extracted or the residue was not dry enough. The extractant to algal tissue ratio was a factor 2 or 5 higher for experiment B as compared to experiment A. A portion of the extract (50 %, 25 mg ≡ 0.50 µg As) was applied to the silica column. Most arsenic (approx. 90 %) eluted off the silica column and approx. 60 % was contained within fractions 9 to 11 (Figure 15). The mass of the residue for the combined fractions 9 to 11 was approx. 1 mg. Most of the extracted mass (ca. 15 mg) eluted within the first 5 mL in fraction 1. The sum of all masses of the residues gave a mass of approx. 20 mg.

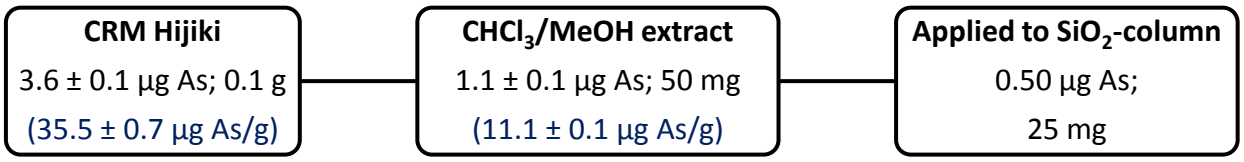


Figure 14 Flow chart of the arsenic distribution for experiment B.

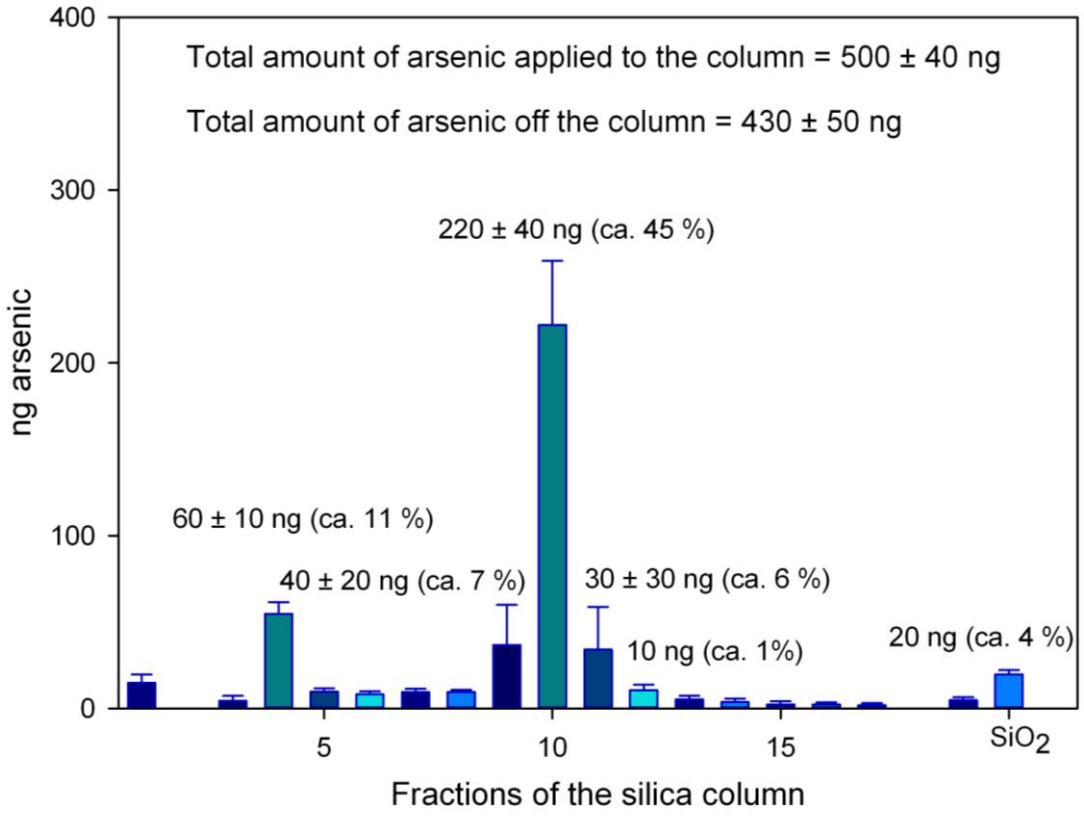


Figure 15 Distribution of arsenic in the silica column fractions for experiment B; fraction volume 1mL, except for fraction 1 (5 ml).

3.2.2 Arsenic species determined by HPLC/ICPMS/ESMS

The CRM-Hijiki contained a higher amount of arsenolipids (e.g. about twice as much AsPL 958) in the algal tissue compared to the commercial Hijiki (Tables 8 & 13). In addition, about twice as much arsenic (ca. 10 µg As/g) was extracted out of the CRM as compared to the commercial Hijiki (ca. 5 µg As/g), even though the total arsenic concentration for the commercial Hijiki (ca. 140 µg As/g) was about four times higher than in the CRM-Hijiki (ca. 36 µg As/g). A reason for that could be the natural variability of arsenic and possibly of arsenolipids in algae as described in Section 1.1.2. The CRM-Hijiki was collected from the east coast of Japan, whereas the commercial Hijki was from the south coast of Japan. Another reason for the arsenic variability could be different sample treatment during the production process. The CRM-Hijiki was washed, freeze-dried, freeze-pulverized, sieved and mixed for homogenization. The commercial Hijiki was, according to the provider, just dried in the sun. Most likely, the washing step of the CRM removed water-soluble arsenic and therefore the total arsenic concentrations are lower. Nevertheless, the washing step does not explain the differences for the arsenolipids, and possibly this observation indicated location dependent differences in the accumulation of arsenolipids. Further studies to confirm this observation are necessary.

The retention times for the identified arsenolipids in the commercial Hijiki match exactly with the retention times of the arsenolipids in the CRM (Figure 16). A comparison of a HPLC/ICPMS chromatogram of a purified CRM extract (fraction 3, experiment A) with its corresponding HPLC/ESMS chromatograms for the main compounds AsHC 332 (peak C) and As-PL 958 (peak F) showed the exact accordance of the ^{75}As-signal with their corresponding protonated molecular masses at m/z 333 and m/z 959 (Figure 17). AsHC 332 and As-PL 958 are the predominant arsenolipids in the CRM-Hijiki, in accordance with the finding of arsenolipids in Hijiki from Garcia-Salgado et al. [2012].

Quantification of the arsenolipids for the CRM (Table 13) showed that the peak at the void volume, the unknown compound A and unknown compound B occurred in higher quantities in the purified extract (fraction 3, experiment A; Table 13, Appendix A20) compared to its crude extract (extract before SiO_2-column; Table 13). Either A and B are degradation products of arsenolipids or they became detectable only after purification. The amount of AsPL 958 was lower (approx. 20 %) in the purified extract as compared to its crude extract.

Nevertheless, the identity of the main arsenolipids in the reference material NMIJ CRM 7405-a Trace Elements and Arsenic Compounds in Seaweed (Hijiki) was shown, and this CRM can now be used as an arsenolipid standard for HPLC/ICPMS analyses.

Table 13 Quantification of arsenolipids in an extract of the CRM obtained with chloroform/ methanol (2+1 v/v; Experiment A).

	Void Vol.	A	B	AsHC 332	AsPL 958
	ng As per g dried CRM				
Extract before water partitioning (n=1)	217	31	138	663	1771
Extract before SiO_2-column (n=1, organic layer)	26	12	32	653	1772
Extract before SiO_2-column (n=1, aqueous layer)	187	21	124	-	90
Purified extract (n=3)	241 ± 49	101 ± 5	495 ± 43	770 ± 21	1422 ± 76

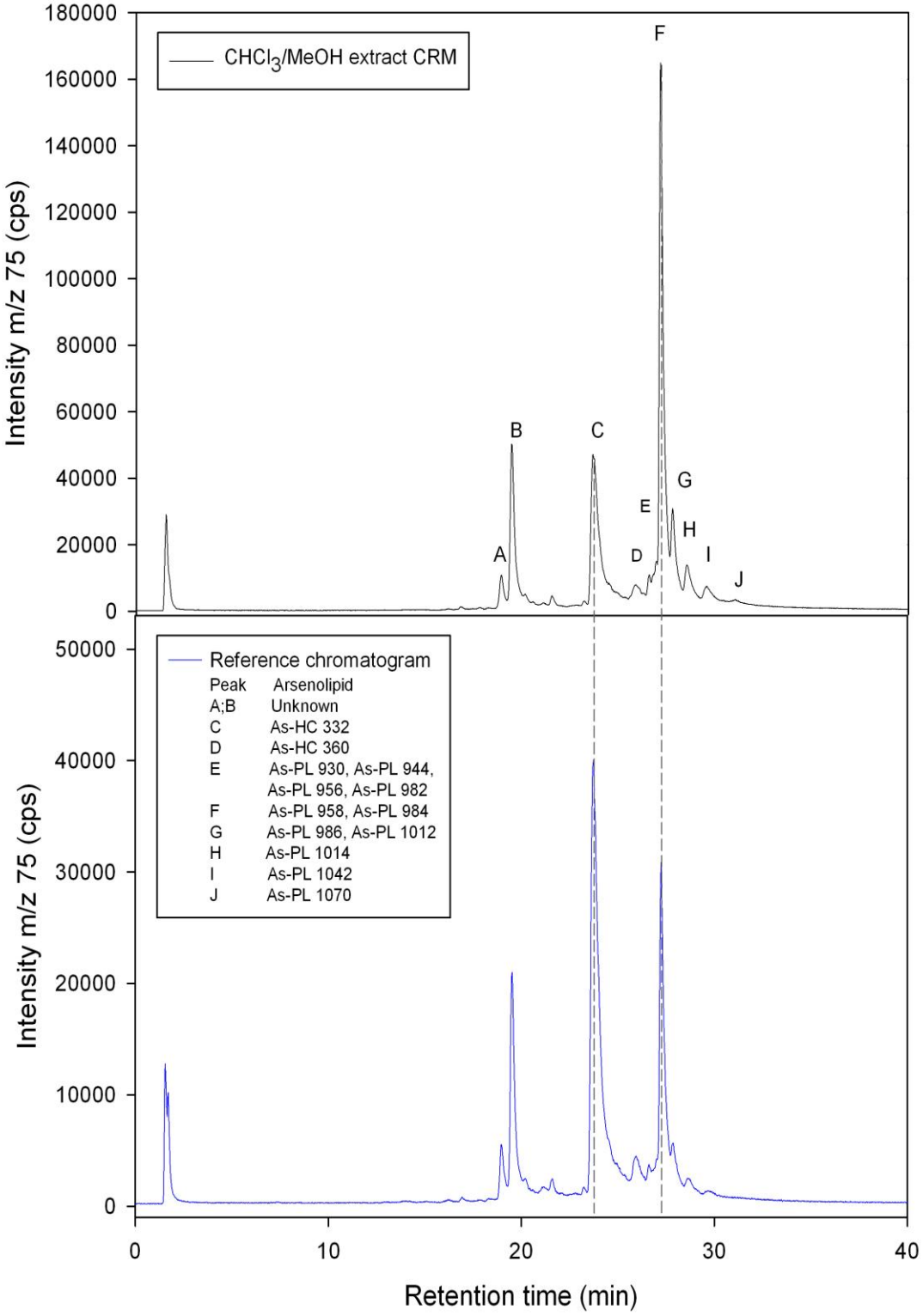


Figure 16 HPLC/ICPMS chromatograms for the CRM (above) against a reference (beneath) [Garcia-Salgado et al. 2012]; stationary phase: ZORBAX XDB-C8 4.6 mm x 150 mm; 5 µm; mobile phase: 10 mmol acetic acid in water with NH3 at pH 6.0 and methanol, 1 mL/min, gradient elution; gradient profile: 0-25 min, 50-95 % methanol; 25-40 min, 95 % methanol; post column split, 10 % to ICPMS; additional flow: 5 % methanol, 0.1 % formic acid in water, 1 mL/min; carbon compensation 15 % methanol; 20 µL sample injected.

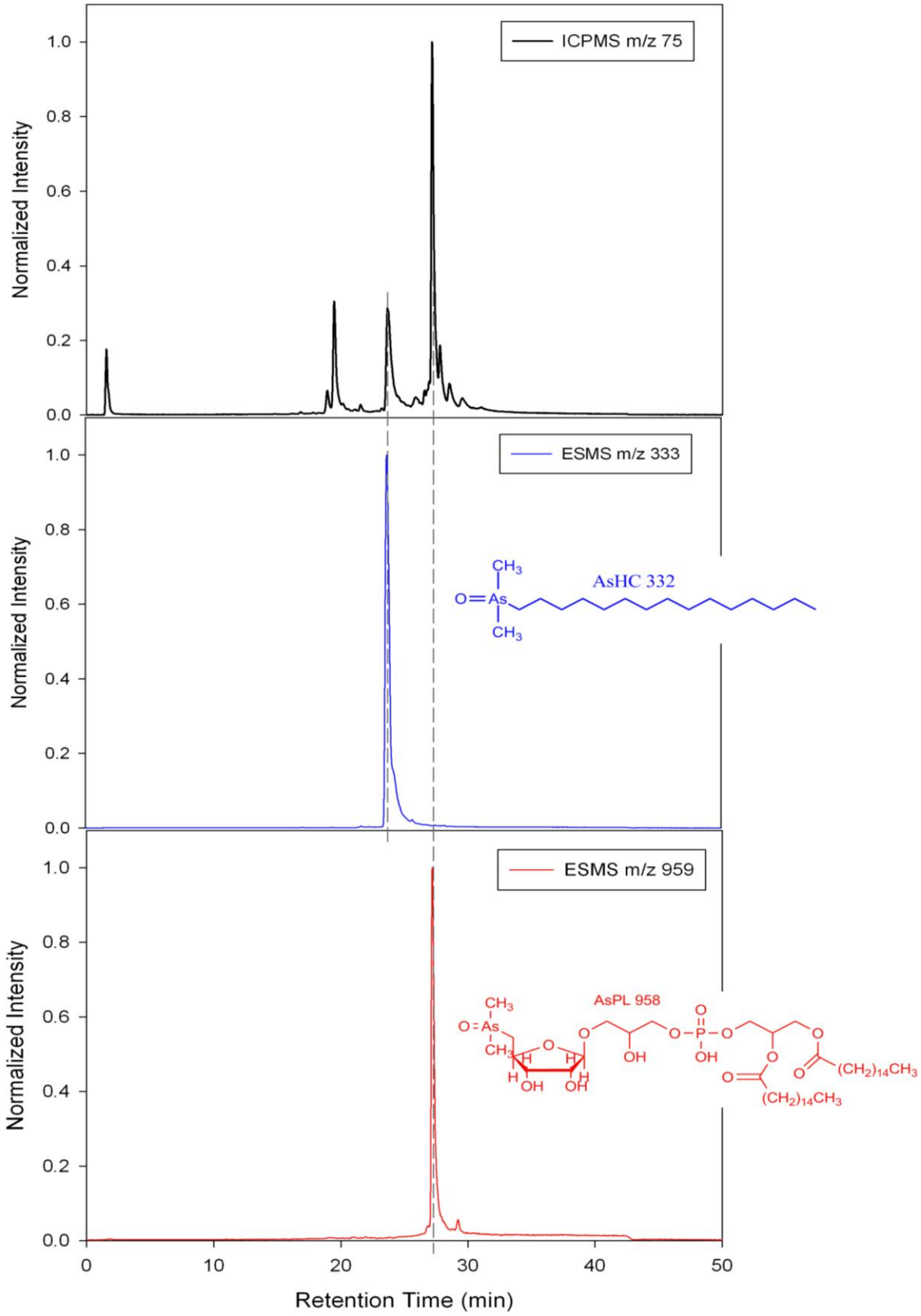


Figure 17 Comparison of a HPLC/ICPMS chromatogram (purified CRM extract, fraction 3, experiment A) with its corresponding HPLC/ESMS chromatogram; chromatographic conditions mentioned in Figure 16.

3.3 Growing Dunaliella tertiolecta in a batch reactor

Higher growth of *Dunaliella tertiolecta* was observed for the medium containing phosphorus at higher concentration levels (Figure 18). This result is in accordance with former work of Foster et al [2008]. Different arsenic concentrations at equivalent phosphorus concentrations showed no significant influence on cell growth.

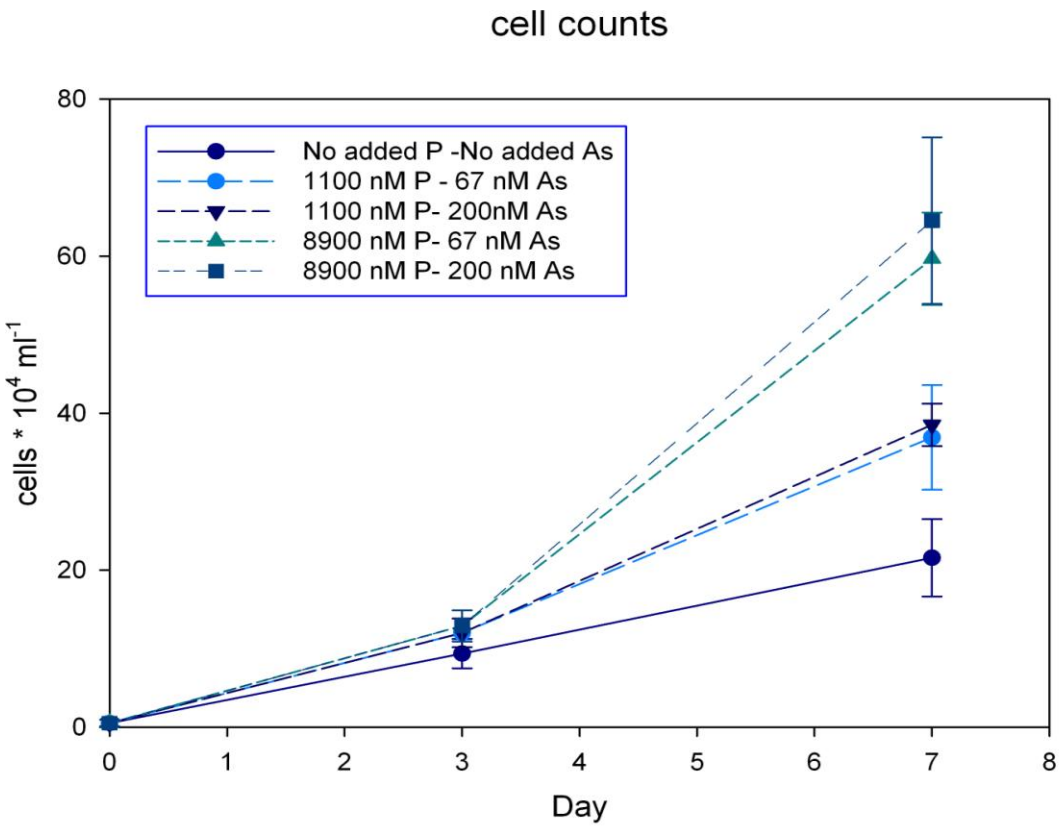


Figure 18 Cell growth of different phosphorus to arsenic treatments (n=4).

After 7 days of culturing, a biomass of approx. 60-80 mg (n=4) dry weight was obtained from a 3 L batch culture of the low phosphorous/low arsenic (1100 nM phosphorous to 67 nM arsenic) treatment. In comparison, a batch culture with the high phosphorous/low arsenic (8900 nM phosphorous to 67 nM arsenic) treatment yielded approx. 90-100 mg. The biomass obtained for all treatments is shown in Table 14. In total, approx. 300-500 mg (5-6 batches per treatment) from each treatment were obtained. More biomass was obtained for the treatments with the higher phosphorous concentration.

Investigations on arsenolipids in the stored algal tissue are necessary to determine if the different treatments had an influence on the composition of arsenolipids in *Dunaliella tertiolecta*. Foster et al. [2008] already showed that the uptake of arsenic for *Dunaliella tertiolecta* was not affected by various phosphorous concentrations in the range of 0.6 mg/L (ca. 6300 nM) to 3.0 mg/L (ca. 31500 nM) phosphate.

Table 14 Biomass and cell counts for the different batch culture treatments.

Treatment	Biomass	Cells $*10^4$/mL
Control sample	35-45 mg	22 ± 5
1100 nM P/ 67 nM As	60-80 mg	37 ± 7
1100 nM P/ 200 nM As	60-80 mg	39 ± 3
8900 nM P/ 67 nM As	90-100 mg	60 ± 6
8900 nM P/ 200 nM As	90-100 mg	65 ± 11

3.4 Conclusion

MTBE/methanol was able to extract arsenic hydrocarbons and arsenosugar phospholipids, but less efficiently than did $CHCl_3$/MeOH. The MTBE/methanol extraction has the advantage of the organic layer being on top compared to the $CHCl_3$/MeOH protocol. This feature allows easy separation of the organic phase without using a separatory funnel and without contaminating the organic phase with the water phase. Passage of the organic extracts through a small SiO_2 column removed about 90 % of the total mass, while returning about 70 % of the major arsenolipids. This clean-up step was essential for identification of the arsenolipids by ESMS. In summary, the MTBE/MeOH extraction was not as time consuming as the $CHCl_3$/MeOH extraction, but $CHCl_3$/MeOH was more efficient for the extraction of arsenolipids.

Extraction of the reference material NMIJ CRM 7405-a Trace Elements and Arsenic Compounds in Seaweed (Hijiki) revealed the existence of arsenolipids in this sample. Thus, this CRM is an accessible source of arsenolipids for laboratories which want to investigate arsenolipids and need an arsenolipid "standard" to test their analytical method or for comparison of their results.

3.5 An outline for future work

The results of the reference material NMIJ CRM 7405-a (Hijiki) need verification by additional independent measurements. Furthermore, the mixture of MTBE/methanol/water should be tested with the CRM and compared with the $CHCl_3$/MeOH method. The influence of acid and basic eluents on the stability arsenolipids should be studied. Moreover, investigations of the unknown compounds A and B by high resolution mass spectrometry would possibly reveal their structure. The total amount of arsenic should be determined for the *Dunaliella tertiolecta* samples of the different treatments, and arsenolipid speciation should be carried out on purified extracts from these samples. Depending on these results, further algal culturing experiments could be performed under various P/As regimes.

4 Appendix

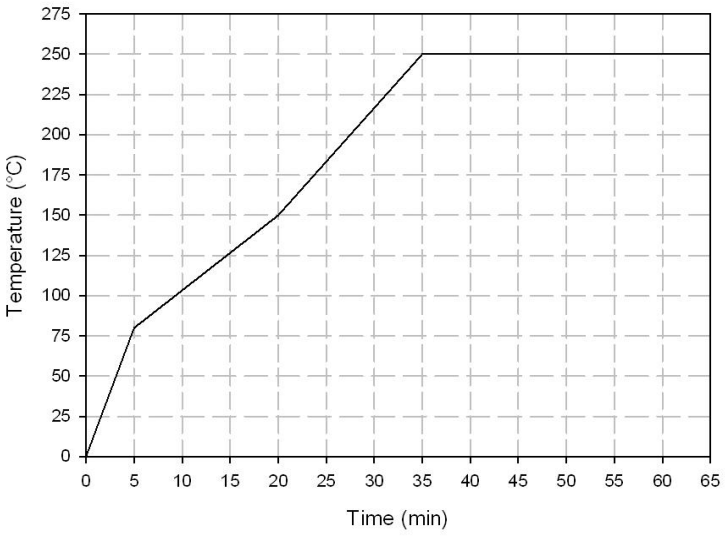
Wavelength: 193.7 nm Slit with: 0.5 R nm

Matrix modifier: 2000 mg/L nickel in water (5 µL co-injection)

Injection volume: 10 µL

A1, Graphite furnace settings.

Temperature (°C)	Time (s)	Gas flow (L/min)
85	5.0	0.3
95	40.0	0.3
150	20.0	0.3
150	20.0	0.3
1400	5.0	0.3
1400	1.0	0.3
1400	2.0	0.0
2600	0.6	0.0
2600	2.0	0.0
2600	2.0	0.3



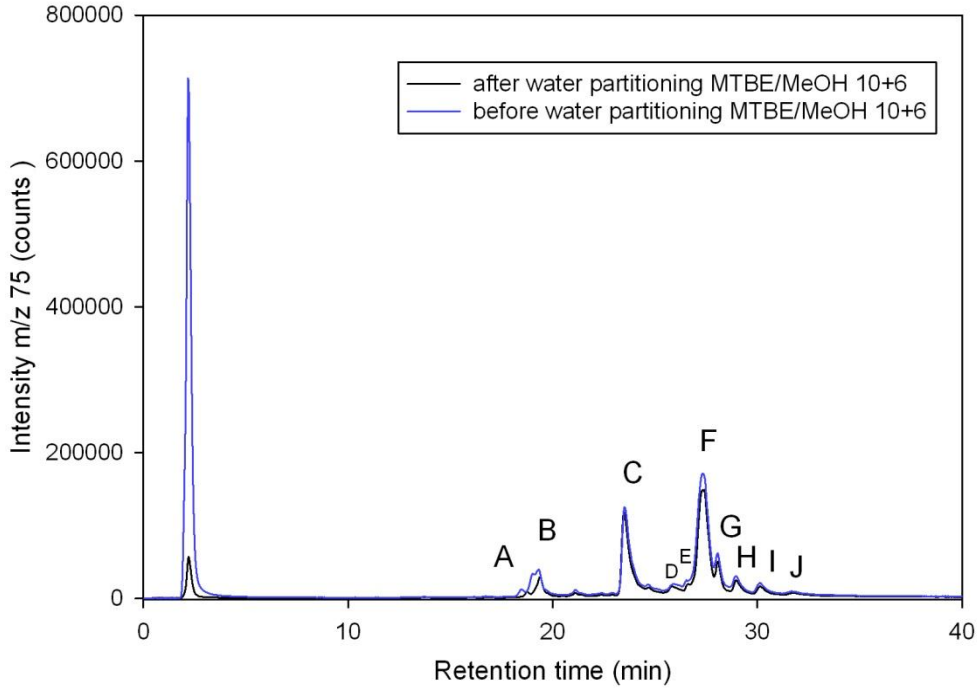
A2, Temperature profile of microwave-assisted acid digestion (UltraCLAVE IV).

A3, ICPMS settings.

ICPMS settings	Total arsenic	Speciation of arsenic
Nebulizer type	Burgener Ari Mist HP	Burgener Ari Mist HP
Spray chamber type	Peletier cooled Scott Type, double pass	Peletier cooled cyclonic
Spray chamber temperature	+2°C	+2°C
Torch	2.5 mm ID	1.5 mm ID
RF power	1550 W	1550 W
Plasma gas flow	15 L min^{-1}	15 L min^{-1}
Carrier gas flow	0.85 L min^{-1}	0.72 L min^{-1}
Auxiliary gas flow	0.22 L min^{-1}	-
Optional gas (20 % oxygen in argon)	-	5 %
Collision gas (He)	5.7 mL min^{-1}	-
Sample cone	Nickel	Platinum
Skimmer cone	Nickel	Nickel
Extract lense 1	0 V	1 V
Extract lense 2	-135 V	120 V
Omega Bias ce	-14 V	- 16 V
Omega lens ce	-0.4 V	- 0.4
Cell entrance	-30 V	- 26 V
QP focus	-11 V	3 V
Cell exit	-40 V	- 40 V

A4, ESMS settings.

ESMS settings	
Drying gas flow	10 L min^{-1}
Nebulizer pressure	60 psig
Drying gas temperature	350°C
Capillary voltage	+4000 V
Cycle time	0.78 s/cycle (70 % SIM, 30 % Scan)
Fragmentation voltage	100 or 150 V



A5 HPLC/ICPMS chromatograms experiment I; comparison of MTBE/MeOH 10+6 extraction before and after water partitioning; stationary phase: ZORBAX XDB-C8 4.6 mm x 150 mm; 5 µm; mobile phase: 10 mmol acetic acid in water with NH_3 at pH 6.0 and methanol, 1 mL/min, gradient elution; gradient profile: 0-25 min, 50-95 % methanol; 25-40 min, 95 % methanol; post column split, 2.2 % to ICPMS; additional flow: 10 % methanol, 0.1 % formic acid in water, 0.2 mL/min; carbon compensation 2 % acetone; 20 µL sample injected.

A6 Experiment I, MTBE/MeOH 10+3 (v/v).

Peak	Retention time [min] (n=2)	SD	ng As/g sample (n=2)	SD
Total	0-45		1566	41
Void volume	2.21	0.01	140	49
A	18.63	0.11	11	1
B	19.11	0.01	38	7
C	23.48	0.02	429	10
D	25.85	0.02	71	3
E	26.58	0.01	25	1
F	27.31	0.07	380	16
G	28.05	0.02	86	6
H	29.02	0.02	66	2
I	30.28	0.04	46	5
J	31.95	0.07	29	1

A7 Experiment I MTBE/MeOH 10+6 (v/v).

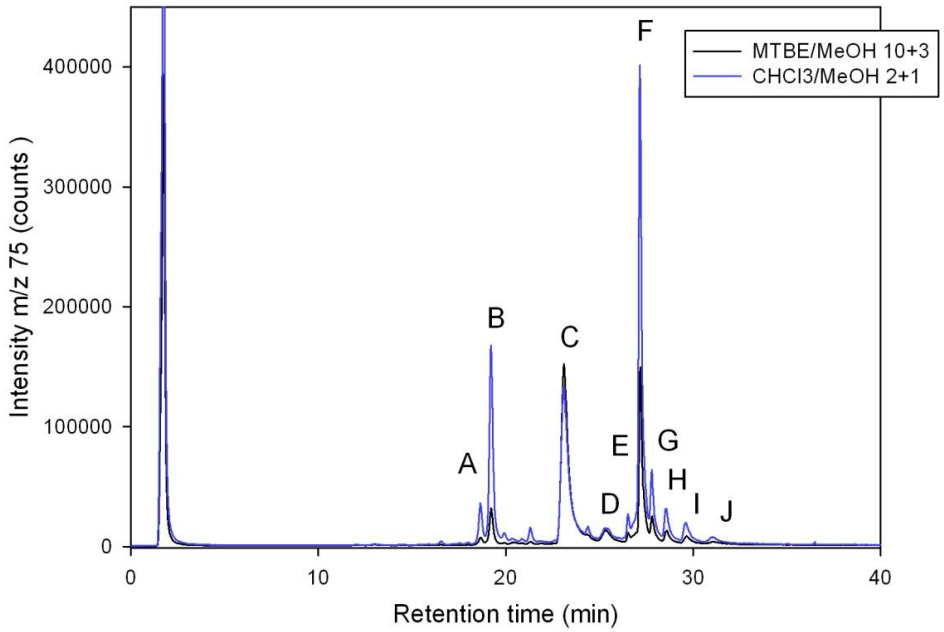
Peak	Retention time [min] (n=2)	SD	ng As/g sample (n=2)	SD
Total	0-45		2280	103
Void volume	2.21	0.01	135	7
A	18.77	0.03	20	1
B	19.40	0.02	98	9
C	23.51	0.04	442	36
D	25.85	0.04	90	1
E	26.65	0.04	44	4
F	27.42	0.01	703	24
G	28.09	0.02	135	6
H	28.99	0.02	105	6
I	30.14	0.02	78	1
J	31.77	0.06	56	4

A8 Experiment I, MTBE/MeOH 10+6 (v/v) before water partitioning.

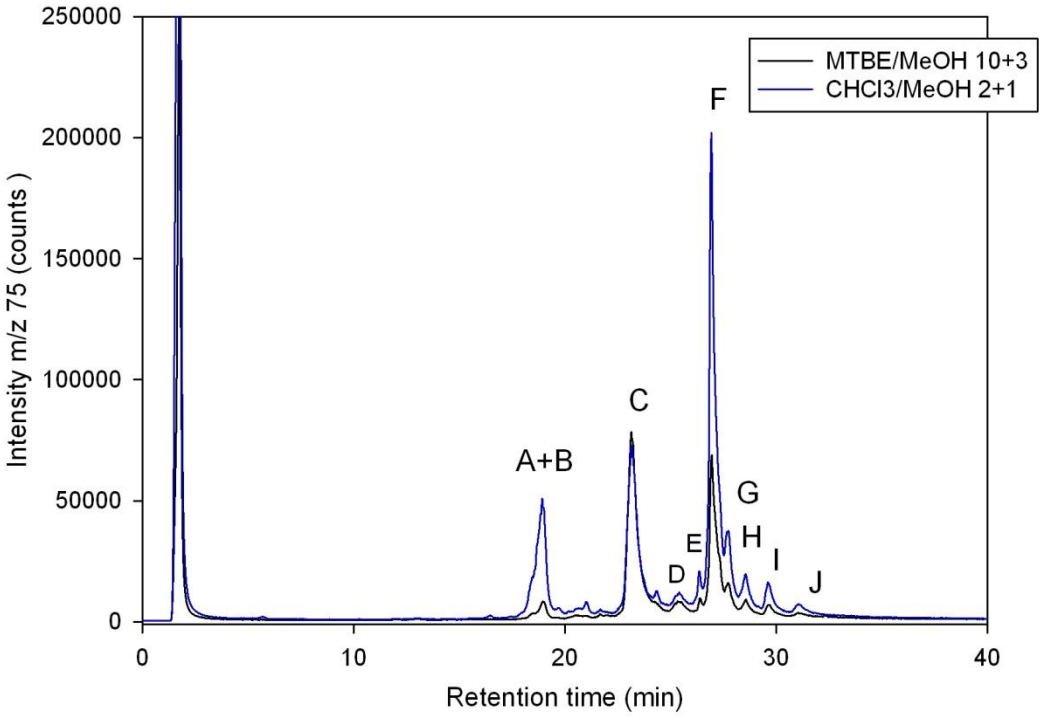
Peak	Retention time [min] (n=3)	SD	ng As/g sample (n=3)	SD
Total	0-45		4479	314
Void volume	2.19	0.02	1670	95
A	18.46	0.05	35	4
B	19.32	0.02	206	23
C	23.52	0.02	488	15
D	25.99	0.05	106	7
E	26.59	0.05	52	3
F	27.34	0.02	861	75
G	28.05	0.02	181	13
H	28.94	0.01	126	12
I	30.11	0.02	103	8
J	31.69	0.02	76	8

A9 Experiment I, chloroform/methanol 2+1 (v/v).

Peak	Retention time [min] (n=3)	SD	ng As/g sample (n=3)	SD
Total	0-45		2929	41
Void volume	2.13	0.06	95	41
A	19.05	0.03	20	8
B	19.37	0.02	24	8
C	23.50	0.01	511	12
D	26.09	0.02	100	1
E	26.47	0.05	59	2
F	27.18	0.02	1074	40
G	27.99	0.02	223	13
H	28.92	0.02	151	6
I	30.08	0.02	131	3
J	31.67	0.02	87	4



A10 HPLC/ICPMS chromatograms after silica column experiment II; stationary phase: ZORBAX XDB-C8 4.6 mm x 150 mm; 5 µm; mobile phase: 10 mmol acetic acid in water with NH_3 at pH 6.0 and methanol, 1 mL/min, gradient elution; gradient profile: 0-25 min, 50-95 % methanol; 25-40 min, 95 % methanol; post column split, 10 % to ICPMS; additional flow: 5 % methanol, 0.1 % formic acid in water, 1 mL/min; carbon compensation 15 % methanol; 40 µL sample injected.



A11 HPLC/ICPMS chromatograms before silica column, experiment II; chromatographic conditions mentioned in Appendix A10.

A12 Experiment II, chloroform/methanol 2+1 (v/v).

Peak	Retention time [min] (n=3)	SD	ng As/g sample (n=3)	SD
Total	0-45		3780	100
Void volume	1.75	0.01	1323	246
A	18.67	0.04	74	6
B	19.23	0.02	332	12
C	23.10	0.02	552	34
D	25.33	0.04	95	4
E	26.52	0.02	48	10
F	27.16	0.01	642	127
G	27.80	0.02	121	25
H	28.56	0.02	91	15
I	29.62	0.01	78	10
J	31.07	0.02	53	11

A13 Experiment II, chloroform/methanol 2+1 (v/v), crude.

Peak	Retention time [min] (n=1)	ng As/g sample (n=1)
Total	0-45	5504
Void volume	1.73	1875
A+B	18.93	462
C	23.16	675
D	25.41	133
E	26.35	91
F	26.93	1054
G	27.74	233
H	28.55	167
I	29.60	148
J	31.06	106

A14 Experiment II, MTBE/MeOH 10+3 (v/v).

Peak	Retention time [min] (n=3)	SD	ng As/g sample (n=3)	SD
Total	0-45		1921	206
Void volume	1.75	0.01	660	103
A	18.65	0.02	15	1
B	19.22	0.01	62	6
C	23.11	0.01	483	20
D	25.31	0.04	66	4
E	26.52	0.02	21	2
F	27.18	0.02	265	34
G	27.80	0.02	57	8
H	28.56	0.02	40	4
I	29.62	0.04	36	4
J	31.06	0.04	27	4

A15 Experiment II, MTBE/MeOH 10+3 (v/v), crude.

Peak	Retention time [min] (n=1)	ng As/g sample (n=1)
Total	0-45	2383
Void volume	1.73	741
A+B	18.95	39
C	23.14	548
D	25.33	83
E	26.38	34
F	26.95	360
G	27.71	88
H	28.58	63
I	29.67	53
J	31.03	36

A16 Quantification of arsenosugar phospholipids for the chloroform/methanol extract after the silica column for experiment II.

	Unknown A	Unknown B	AsHC 332	AsPL 958
		ng As per g dried alga		
Sample 1*	70	320	523	495
Sample 2	80	340	590	710
Sample 3	70	330	540	720

* stored for 10 days in elution solvent

A17 Experiment III, MTBE/MeOH 10+6 (v/v).

Peak	Retention time [min] (n=3)	SD	ng As/g sample (n=3)	SD
Total	0-45		1888	87
Void volume	1.66	1.66	38	6
A+B	19.44	0.02	81	9
C	23.78	0.02	508	30
D	25.94	0.04	62	3
E	26.59	0.04	43	2
F	27.20	0.02	553	18
G	27.81	0.02	131	4
H	28.58	0.04	89	5
I	29.56	0.04	81	4
J	31.02	0.08	39	2

A18 Experiment III, chloroform/methanol 2+1 (v/v).

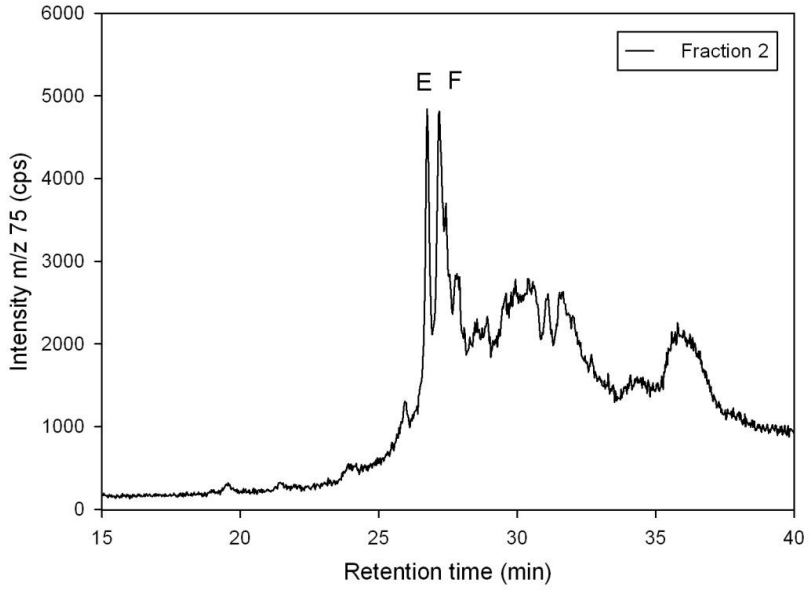
Peak	Retention time [min] (n=3)	SD	ng As/g sample (n=3)	SD
Total	0-45		2308	53
Void volume	1.66	0.03	33	15
A+B	19.46	0.01	83	6
C	23.81	0.03	555	23
D	25.95	0.03	79	4
E	26.58	0.04	51	2
F	27.22	0.03	765	46
G	27.82	0.03	177	7
H	28.59	0.04	122	4
I	29.62	0.03	106	5
J	31.08	0.05	49	4

A19 Experiment A, chloroform/methanol 2+1 (v/v); purified extract, fraction 3.

Peak	Retention time [min] (n=3)	SD	ng As/g sample (n=3)	SD
Total	0-45		4804	41
Dead Volume	1.54	0.01	241	49
A	18.95	0.03	101	5
B	19.48	0.03	495	43
C	23.69	0.04	770	21
D	25.91	0.03	161	7
E	26.60	0.02	69	7
F	27.19	0.03	1422	76
G	27.80	0.02	324	17
H	28.54	0.03	218	18
I	29.57	0.03	149	8
J	31.12	0.08	62	11

Appendix 20 Experiment A, comparison of the arsenolipid species.

Peak	Extract before water partitioning (n=1)	Extract after water partitioning, before SiO_2-column (n=1)	Extract after SiO_2-column (n=3)
Total	7108	6383	4804 ± 41
Dead Volume	217	26	241 ± 49
A	31	12	101 ± 5
B	138	32	495 ± 43
C	663	653	770 ± 21
D	205	163	161 ± 7
E	207	122	69 ± 7
F	1771	1773	1422 ± 76
G	563	499	324 ± 17
H	441	371	218 ± 18
I	324	371	149 ± 8
J	7108	6383	62 ± 11



A21 HPLC/ICPMS chromatograms of experiment A, chloroform/methanol 2+1 (v/v); fraction 2; stationary phase: ZORBAX XDB-C8 4.6 mm x 150 mm; 5 µm; mobile phase: 10 mmol acetic acid in water with NH_3 at pH 6.0 and methanol, 1 mL/min, gradient elution; gradient profile: 0-25 min, 50-95 % methanol; 25-40 min, 95 % methanol; post column split, 10 % to ICPMS; additional flow: 5 % methanol, 0.1 % formic acid in water, 1 mL/min; carbon compensation 15 % methanol; 20 µL sample injected.

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