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in Chemistry

Distribution of hydrophilic and lipophilic arsenic species within the macroalgae

Rebecca Jane Sim

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School of Engineering and Natural Sciences

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Rebecca Jane Sim

Dissertation submitted in partial fulfillment of a
Philosophiae Doctor degree in Chemistry

Ph.D. Committee
Ásta Heiðrún Pétursdóttir
Guðmundur Haraldsson
Jörg Feldmann
Karl Gunnarsson

Opponents
Barbro Kollander
Kristmann Gíslason

Faculty of Physical Sciences
School of Engineering and Natural Sciences
University of Iceland
Reykjavik, May 2024

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Faculty of Physical Sciences
School of Engineering and Natural Sciences
University of Iceland
Dunhagi 3
107, Reykjavik
Iceland

Telephone: 525 4000

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Author ORCID: 0000-0001-7398-8062
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Abstract

In recent years seaweed has gained popularity as a health food due to its high content of minerals and vitamins. However, seaweeds may also accumulate high levels of potentially toxic elements – in particular arsenic, which may become incorporated into larger biological molecules such as sugars and lipids. It is unclear how these organic arsenic compounds are formed/stored and if they may serve a biological purpose (i.e., detoxification or energy storage). However, toxicological studies into arsenic-containing lipids have demonstrated cytotoxicity comparable to that of arsenite, a known carcinogen, and arsenic-containing sugars are suspected to display toxicity with chronic exposure. This thesis aims to investigate variations in the distribution of arsenic compounds throughout several classes and species of seaweed. Samples of brown, red and green macroalgae were collected from two locations in Iceland across two different months and analysed for several potentially toxic elements as well as hydrophilic arsenic speciation using HPLC-ICP-MS. Brown macroalgae were additionally sectioned into anatomical parts to determine if the distribution of arsenic species differs throughout the thallus. Select samples were chosen for state-of-the-art lipophilic arsenic speciation using HPLC-ICP-MS/ESI-MS/MS and HPLC-qToF-MS. Limited information is available on arsenic speciation in seaweed thus it is hoped that this extensive profiling of several different species will help elucidate how these unusual compounds are formed and stored. The data from this project will also contribute to the necessary information needed for the risk assessment of arsenic species in seaweed for human consumption and may have an impact on future food safety legislations.

Útdráttur

Þörungar geta tekið upp mikið magn af frumefninu arsen úr sjónum á efnaforminu ólífrænt arsen sem er þekktur krabbameinsvaldur. Í þörungunum greinist arsen einnig á formi fjölbreyttra lífrænna efnasambanda arsens t.d. arsenósykrur og arsenólípíð, en lífrænar arsentegundir hafa verið taldar hættulausar. Nýlegar rannsóknir á arsenólípíðum hafa þó sýnt að þau geta verið jafn frumudrepandi og ólífræna arsenið og mögulegt er að arsenósykur hafi langvarandi neikvæð áhrif við reglubundna neyslu. Margt leikur á huldu um uppruna arsenólípíða en upphafspunktur framleiðslu þeirra er talinn eiga sér stað í þörungum. Þörungar njóta stöðugt meiri vinsælda á Vesturlöndum. Brýn þörf er á frekari upplýsingum um þessi efnasambönd til að meta til hlítar áhættuna sem fylgir neyslu þeirra og tryggja að settar séu viðeigandi reglur um hámarksmagn þeirra í matvælum. Sýnum af rauð-, græn- og brúnþörungum var safnað nálægt Grindavík og Kjalarnesi. Sýnin voru ítarlega rannsökuð m.t.t. þungmálma og framkvæmd var tegundagreining arsens til að afla upplýsinga um efnaform arsensins. Tegundargreining arsenólípíða er flókin og var framkvæmd í völdum sýnum með massagreinunum HPLC-ICP-M/ESI-MS/MS og HPLC-qToF-MS. Aukinheldur var brúnum stórþörungum skipt í líffræðilega hluta til að ákvarða hvort dreifing arsentegunda sé jöfn um þangið. Takmarkaðar upplýsingar eru til á heimsvísu um arsenólípíð í þangi, svo þessi umfangsmikla prófílgreining þeirra í mismunandi tegundum þörunga mun styðja við að skýra hvernig þessi dularfullu lífrænu efnasambönd arsens myndast og hvar þau eru geymd. Gögnin geta einnig nýst við áhættumat á arsentegundum í þangi til manneldis og geta því haft áhrif á framtíðarlöggjöf um matvælaöryggi.

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List of Manuscripts

The manuscripts on which the thesis will be based are detailed below.

Sim, R., O'Brien, L., Weyer, M., Gunnarsson K., Sveinsdóttir H. I. & Pétursdóttir, Á. H., Potentially toxic elements in Icelandic seaweeds. *Submitted to Marine Pollution Bulletin* (2024).

Sim, R., Weyer, M. & Pétursdóttir, Á. H., Inorganic arsenic in seaweed: a fast HPLC-ICPMS method without co-elution of arsenosugars. *Accepted by Analytical and Bioanalytical Chemistry* (2024).

Sim, R., Feldmann, J. & Pétursdóttir, Á. H., The impact of different sample preparation methods on the arsenic speciation in Laminaria digitata. *To be submitted to Journal of Chromatography Open* (2024).

Sim, R., Feldmann, J., Stengel, D. B. & Pétursdóttir, Á. H. Temporal and intra-thallus variation in arsenic species in the brown macroalga Laminaria digitata. *Environmental Chemistry.* **20**, 55–65 (2023).

Sim, R. & Pétursdóttir, AH., A comprehensive analysis of water-soluble arsenicals in Icelandic macroalgae. *Submitted to Environmental Chemistry* (2024).

Sim, R., Raab, A., Feldmann, J., & Pétursdóttir, AH., A comprehensive analysis of arsenic-containing lipids in several species of brown, red and green seaweeds collected in Iceland. *To be submitted to Science of the Total Environment* (2024).

The author and co-authors contribution to each manuscript is stated below.

Potentially toxic elements in Icelandic seaweeds
Sim, R. –sample collection and preparation, conceptualisation, acquisition of data, analysis and interpretation of data, visualisation of data and drafting of manuscript.
O'Brien, L. – sample collection and preparation, and acquisition of data.
Weyer, M. – acquisition of data.
Gunnarsson, K. – identification of seaweed species, revision of manuscript.
Sveinsdóttir H. I. – revision of manuscript.
Pétursdóttir, Á. H. – sample collection, conceptualisation, interpretation and visualisation of data, and revision of manuscript.

Inorganic arsenic in seaweed: a fast HPLC-ICPMS method without co-elution of arsenosugars
Sim, R. - sample collection and preparation, conceptualisation, method development, acquisition of data, analysis and interpretation of data, visualisation of data and drafting of manuscript.
Weyer, M. – method development and acquisition of data.

Pétursdóttir, Á. H. – conceptualisation, interpretation and visualisation of data, and revision of manuscript.

The impact of different sample preparation methods on the arsenic speciation in Laminaria digitata
Sim, R. - sample collection and preparation, conceptualisation, method development, acquisition of data, analysis and interpretation of data, visualisation of data and drafting of manuscript.
Feldmann, J. - conceptualisation, interpretation and visualisation of data, and revision of manuscript.
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Temporal and intra-thallus variation in arsenic species in the brown macroalga Laminaria digitata
Sim, R. - sample collection and preparation, conceptualisation, method development, acquisition of data, analysis and interpretation of data, visualisation of data and drafting of manuscript.
Feldmann, J. - conceptualisation, interpretation and visualisation of data, and revision of manuscript.
Stengel, D. B. - interpretation and visualisation of data, and revision of manuscript.
Pétursdóttir, Á. H. - conceptualisation, interpretation and visualisation of data, and revision of manuscript.

A comprehensive analysis of water-soluble arsenicals in Icelandic macroalgae
Sim, R. - sample collection and preparation, conceptualisation, method development, acquisition of data, analysis and interpretation of data, visualisation of data and drafting of manuscript.
Pétursdóttir, Á. H. - conceptualisation, sample collection, interpretation and visualisation of data, and revision of manuscript.

Analysis of arsenic-containing lipids in several species of brown, red and green seaweeds collected in Iceland
Sim, R. - sample collection and preparation, conceptualisation, acquisition of data, analysis and interpretation of data, visualisation of data and drafting of manuscript.
Raab, A. – method development, acquisition of data, interpretation and visualisation of data, and revision of manuscript.
Feldmann, J. - interpretation and visualisation of data, and revision of manuscript.
Pétursdóttir, Á. H. - conceptualisation, sample collection, interpretation and visualisation of data, and revision of manuscript.

Abbreviations

Abbreviation	Definition
AAS	atomic absorption spectrometry
AB	arsenobetaine
AC	arsenocholine
AES	atomic emission spectroscopy
AFS	atomic fluorescence spectrometry
As(III)	arsenite
As(V)	arsenate
AsCer	arsenoceramide
AsDAG	arsenodiacylglyceride
AsFA	arsenofatty acid
AsHC	arsenohydrocarbon
AsPC	arsenophosphatidylcholine
AsPG	arsenophosphatidylglycerol
AsPI	arsenophosphatidylinositol
AsPL	arsenophospholipid
AsSugar	arsenosugar
AsSug-gly	2-(2,3-dihydroxypropoxy)-5-(dimethylarsorylmethyl)tetrahydrofuran-3,4-diol (*arsenosugar derivative with glycerol r-group*)
AsSug-PO_4	2,3-dihydroxypropyl [3-[5-(dimethylarsorylmethyl)-3,4-dihydroxy-tetrahydrofuran-2-yl]oxy-2-hydroxy-propyl] hydrogen phosphate (*arsenosugar derivative with phosphate r-group*)
AsSug-SO_3	3-[5-(dimethylarsorylmethyl)-3,4-dihydroxy-tetrahydrofuran-2-yl]oxy-2-hydroxy-propane-1-sulfonic acid (*arsenosugar derivative with sulphonate r-group*)
AsSug-SO_4	3-[5-deoxy-5-(dimethylarsoryl)-a-d-ribofuranosyl]oxy-2-hydroxypropyl hydrogen sulphate (*arsenosugar derivative with sulphate r-group*)
AsSugPeL	arsenosugar phosphoacyl ether
AsSugPL	diacyl-arsenosugar phospholipid
AsSugPhytol	arsenosugarphytol
AsTAG	arsenotriacylglyceride
bw	body weight
CE	capillary electrophoresis
CRM	certified reference material

CT	cryogenic trapping
DC	direct current
DCM	dichloromethane
DDMMA	5'-deoxy-5'-dimethylarsinoyl-adenosine
DL	detection limit
DMA	dimethylarsinate
DMAA	dimethylarsinoyl acetate
DMAE	dimethylarsinoyl ethanol
DNA	deoxyribonucleic acid
DoE	design of experiment
EDTA	ethylenediaminetetraacetic acid
EFSA	European Food Safety Authority
EM	electron multiplier
ESI	electrospray ionisation
EU	European Union
EXAFS	extended X-ray absorption fine structure
FI	flow injection
GC	gas chromatography
GFAAS	graphite furnace atomic absorption spectroscopy
HG	hydride generation
HPLC	high performance liquid chromatography
HR-CS	high resolution continuum source
IARC	International Agency for Research on Cancer
iAs	inorganic arsenic (sum of arsenite and arsenate)
ICP	inductively coupled plasma
LOD	limit of detection
LOQ	limit of quantification
mAsSugPL	monoacyl-arsenosugar phospholipid
ML	maximum level
MMA	monomethylarsonate
MIP	microwave-induced plasma
MS	mass spectrometry
MS/MS	tandem mass spectrometry
NEE	non-exhaust emissions
OES	optical emission spectroscopy
PCA	principal component analysis
PA	phosphatidic acid
qqq	triple quadrupole
qToF	quadrupole time of flight
PTE	potentially toxic element

REE	rare earth element
REML	restricted maximum likelihood
RF	radio frequency
RNA	ribonucleic acid
ROS	reactive oxygen species
RSME	root mean squared error
SAM	S-adenosyl methionine
TMA	trimethylarsine
TMAsFOH	trimethyl fatty acid alcohol
WHO	World Health Organisation
XANES	X-ray near edge structure
XAS	X-ray atomic spectroscopy

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Chapter 1 – Thesis Introduction, Aims and Structure

1.1 Introduction

Seaweed is becoming increasingly popular as a health food or supplement in Western countries, and has been regarded by many as a 'superfood' due to its high content of minerals, vitamins, and antioxidants (1,2). Whilst seaweeds may be rich in essential elements such as iron and calcium, they may also accumulate high levels of arsenic – a toxic element widely associated with its use as a poison throughout history. Whilst arsenic is ubiquitous throughout the environment, it displays particularly unique behaviour in marine ecosystems where it may take on several chemical forms ranging from small, inorganic molecules to complex sugars and phospholipids. Seaweeds are thought to be the starting point for many of these organic arsenic compounds as algae serve as a primary food source for many higher marine organisms. However, the mechanisms by which these compounds are formed are still largely unknown and there is debate over whether they may be a product of detoxification or serve a biological function i.e., energy storage or conservation of phosphorous under limiting conditions (3,4). Investigating variations in arsenic compounds between seaweeds species may be useful to further our understanding of how and why these compounds are formed, as previous studies have demonstrated large variations in arsenic metabolism (4,5). Thallus sections within large brown macroalgae often differ in biological function, i.e., the holdfast provides support, and the meristem is responsible for new frond growth, therefore the distribution of arsenic species throughout the thallus was also investigated in order to uncover any possible links between the production of these compounds and biological function.

This thesis will contribute significantly to current understanding by being the first to report a comprehensive study of the arsenic speciation in several species of brown, red and green seaweed collected in Iceland. Arsenic-containing sugars and arsenosugarphospholipids were both found to be most abundant in the reproductive tissues of the brown seaweeds, which may be evidence that the former are the starting product of the latter as both share a dimethylarsenoribose moiety. The lipids are perhaps produced by the binding of AsSugars to phosphatidic in the membrane which would provide an explanation as to why these lipids also contain a phosphate moiety and two acyl groups. Whether these species are produced accidently as a result of biological infidelity or are produced with a biological purpose is unclear. It does not appear that arsenosugars serve as energy storage as is displayed with other sugars, however, their inclusion in the cell wall matrix may have yet to be discovered benefits (i.e., increased resistance to environmental changes).

Additionally, this thesis may serve to address the lack of occurrence data currently available for arsenic speciation in seaweed – in particular the lipid-soluble species. Previously, the organic arsenic compounds were generally considered to be less toxic than the inorganic, but this has recently been disproven; Several arsenic-containing hydrocarbons have been

demonstrated to exert cytotoxicity comparable to arsenite in vitro(6), and exert developmental toxicity in the in vivo models *Drosophila melanogaster* and *Caenorhabditis elegans* (7,8). Thus, access to occurrence data will be crucial in the future for monitoring and regulatory purposes to ensure consumer safety.

1.2 Structure of the Thesis

The thesis will be based on a collection of articles that have been or are to be submitted to peer-reviewed journals. The thesis will begin with an introductory chapter describing the relevance and significance of the work, followed by a detailed literature review explaining the scientific background and current state of knowledge. The second chapter will be a summary of all results and findings, and the 6 manuscripts on which the thesis is based. The manuscripts have been organised as such that the methodology used increases in complexity, beginning with the measurement of total arsenic (and other metals) and ending with the sophisticated instrumental set-up required for the analysis of arsenolipids species. The final chapter will discuss the implications of the results on the current state of knowledge in the field, and any future implications the results may have on legislation on maximum levels of arsenic species in food for human and livestock consumption.

1.3 Aims and Objectives of the Thesis

The aim of the thesis was to address several research questions regarding the arsenic speciation in macroalgae:

- In what proportions are the different arsenic species stored within the thalli?
- Does this relate to how complex arsenic compounds such as arsenosugars and arsenolipids are formed?
- Do other variables such as season and location have an impact on the arsenic speciation in macroalgae?
- Will Rhodophyta and Chlorophyta behave differently to Phaeophyta in terms of arsenic speciation?
- Are there different mechanisms for the formation of these compounds that are dependent on seaweed type?

To answer these research questions, a large sample set of brown, red and green seaweed specimens were collected from two locations in Iceland across two sampling months. The large brown thalli were divided into sections based on anatomy and function (i.e., sporophyll for reproductive tissues). This sample-set was then analysed for total element concentrations, water-soluble arsenic speciation and a select few for lipid-soluble speciation.

Chapter 2 – Scientific Background

2.1 Seaweed

The term seaweed is commonly used to describe the thousands of species of macroalgae growing in the world's seas and oceans. These organisms have outlasted most forms of prehistoric life - with the oldest species of multi-cellular algae discovered thought to be approximately 1 billion years old (9). Humans have long understood the potential value of seaweed; For example in 701 AD the Japanese were allowed to pay their taxes to the emperor in kelp (10), and *Palmaria palmata* (dulse) was widely used as a trading commodity in Iceland in the 700s (11). Seaweed seems to be a resource with endless potential applications, and nowadays is used for everything from combatting climate change (by feeding cattle the methane reducing *Asparagopsis* species) to thickening agents for toothpaste and ice-cream (12–14). Seaweed is taxonomically categorized into 3 classes depending on pigmentation: brown (Phaeophyceae), red (Rhodophyta), and green (Chlorophyta), Figure 1. Brown seaweeds are the most abundant class by biomass and have large thalli with distinct features such as holdfasts, stipes, and fronds. These seaweeds are typically dark brown to olive green in colour, depending on the ratio of fucoxanthin to chlorophyll (15). However, the red pigmentation in Rhodophyta is due to the presence of phycobilins in the chloroplasts, which mask the green colour of chlorophyll (16). The morphology of red macroalgae is diverse, ranging from the complex branched thalli found in *Vertebrata lanosa*, to the solid, calcareous Coralline species. Green seaweed species are typically smaller in size than the other classes, and often have no distinguishable features such as a holdfast or midrib. From comparison of chloroplast genomes, it is thought that Chlorophyta are closely related to terrestrial plants, sharing a common ancestor approximately 470 million years ago (17).

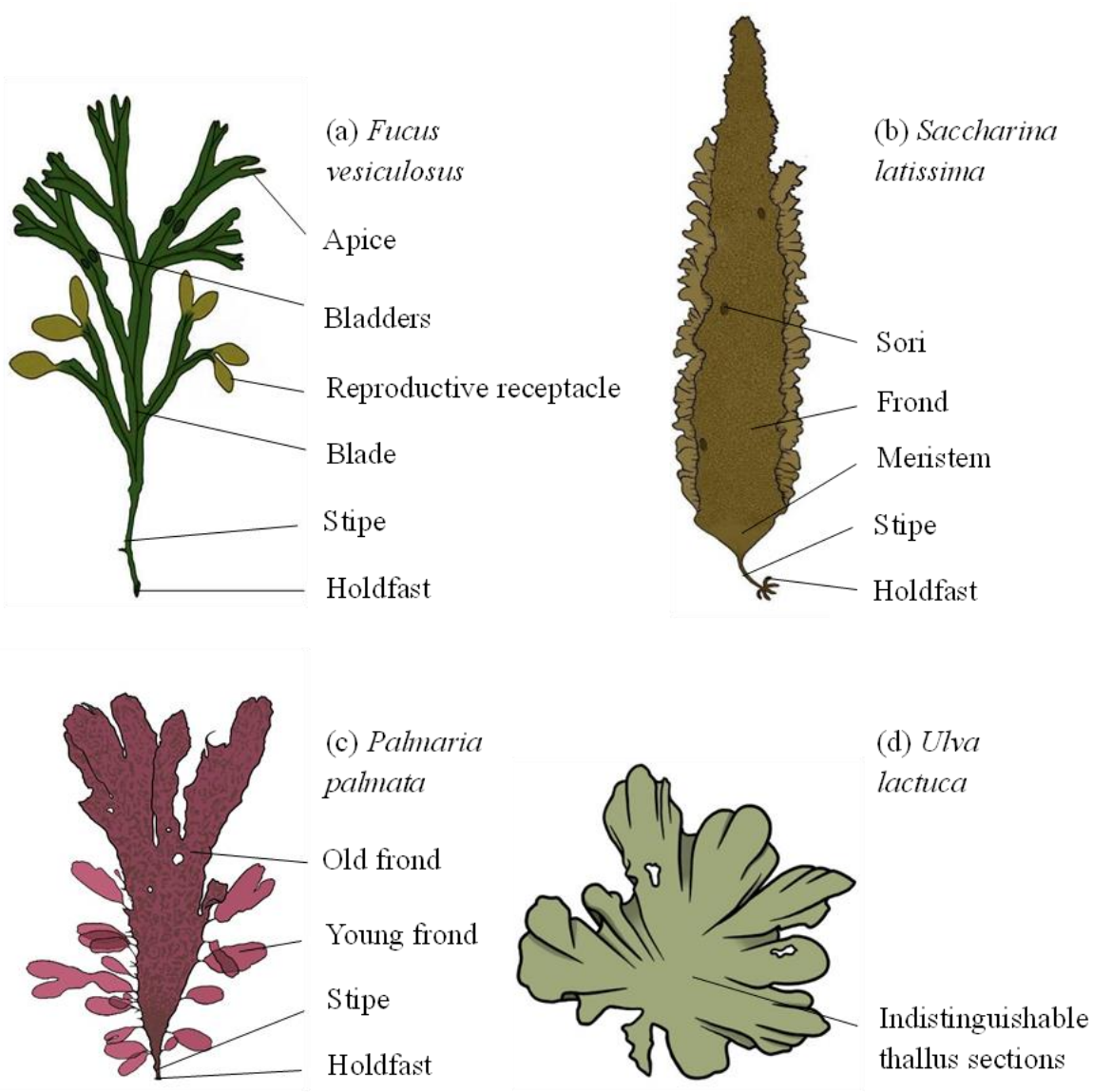

Figure 1 – The anatomy of several common seaweeds found in Iceland, although morphology can vary greatly between species within the same class. (a) *Fucus vesiculosus* (Phaeophyta), (b) *Saccharina latissima* (Phaeophyta), (c) *Palmaria palmata* (Rhodophyta), (d) *Ulva lactuca* (Chlorophyta).

There are approximately 269 benthic marine algae species native to Iceland, many of which may have significant economic value (18). The global seaweed market was worth over 15 billion USD alone in 2021, and this is expected to increase to 25 billion USD by 2028 (19), and seaweed cultivation is set to be one of the fastest growing sectors in the marine industry in the coming years. Iceland has been described as an ideal location for the aquacultures which produce the majority of globally harvested seaweed, and the country is thought to have one of the largest under-utilised seaweed resources in the world. Traditionally, species such as *Ascophyllum nodosum* has been used as fertilizer by the coastal communities due to its natural abundance (20), but now this seaweed has found more outlandish uses such as in fabrics for clothes that claim to be able to nourish dried skin with vitamin E and antioxidants (21). Other species such as *Laminaria hyperborea* are harvested for their alginates, and the red seaweed *Chondrus crispus* for carrageenan, both of which are used as gelling agents or emulsifiers in food products such as nut milks, processed meats, and yoghurts. Recently there has been increased interest in seaweed for pharmaceutical and cosmetic purposes as consumers favour products derived from natural sources, and the bioactive compounds extracted from algae have been shown to have various anti-inflammatory, anti-bacterial and anti-viral properties (22). For example, the sulphated polysaccharides extracted from *Saccharina latissima have* been shown to exhibit high anti-tumour behaviour (23), and those extracted from *Sargassum fusiforme* were shown to inhibit the activity of the enzyme responsible for collagen breakdown, thereby reducing skin sagging and wrinkle development (24). Seaweed is also growing increasingly popular as a health food in Western countries due to its high content of minerals and iodine, the latter of which European populations are often deficient in. Thus, as it becomes more widely consumed, analysis from a food safety perspective is required to assess the risk from toxic elements and the inorganic and organic compounds into which the elements can become incorporated.

2.2 Potentially Toxic Elements in Seaweed – with a Focus on Arsenic

Seaweeds have been widely shown to accumulate high levels of potentially toxic elements (PTEs) (25–28). These may be essential trace elements such as zinc, cobalt and selenium which are required for cellular function (e.g., serve as enzyme co-factors) or non-essential elements such as arsenic, cadmium, and lead which have no known biological function and can exert toxicity at low levels. The ability of macroalgae species to accumulate such a wide array of elements is thought to be due to the polysaccharide composition of the cell walls – all of which have a range of electronegative functional groups (i.e., carboxyl) that can complex a variety of cations (29). In Phaeophycea, alginates and cellulose are typically the main components of the extra-cellular wall matrix, along with sulphated polysaccharides such as fucoidans and fucans. In Rhodophyta the cell wall is typically comprised of cellulose, xylans and sulphated galactans such as carrageenan. Chlorophyta cell walls show perhaps the most variation of the three classes, but are generally composed of cellulose and hydroxyproline, as well as xylans and mannans. Cell wall polysaccharides are often over produced by algal cells as a means of avoiding the accumulation of PTE inside cells, however, low levels are still observed in the intracellular matrix (30). The PTE are thought to enter cells by means of molecular or ionic mimicry, whereby the carrier proteins and ion channels are unable to distinguish between chemical species with similar radii or structure. Such is thought to be the case for arsenic, where arsenate and phosphate share similar chemical structures and almost identical pKa values (31,32).

Arsenic (As) is the 33rd element in the periodic table, has a mass of 75 atomic mass units and no stable isotopes. This element has long had a deadly history and is most associated with use as a poison for rats or undesirable husbands in the 19th century. During the Victorian Era As ($CuHAsO_3$) was also used to create a popular vibrant dye – 'Scheele's green'- which was widely used to colour everything from wallpaper to children's toys before being pinpointed as the cause of many mysterious, painful deaths (33). However, this elements reputation was redeemed in 20th century, as the first drug that proved effective in the treatment of syphilis was As based (34) and the element is now widely used in drugs to treat ailments such as leukaemia and joint disease (35). As may be released into the environment from natural processes such as volcanic eruptions and weather erosion of mineral bearing rocks, or from anthropogenic activities such as smelting. As pollution can have detrimental effects on the environments, and contaminated ground waters are a huge problem for the 2.5 billion people that rely on these as a source of drinking water (36). Marine ecosystems are also disproportionately affected by As contamination as all waste streams eventually lead to the world's oceans, and as such many marine organisms contain high levels of As. Seaweeds in particular have been shown to contain high levels of As - where internal concentrations may reach upwards of 100 mg kg^{-1} (4,37). The As metabolism of seaweeds is largely of interest as they are unique in their ability to tolerate elevated levels of As, and furthermore can synthesize a range of unusual organic As metabolites not produced by terrestrial plants.

2.3 Arsenic Species Found in Seaweed

The toxicity of As, or any element for that matter, is highly dependent on chemical form and oxidation state. As such, the speciation analysis of elements has become more popular and advanced in recent decades, and is now an important tool in health and safety, and food analysis (38–40). Seaweed is widely known to accumulate considerable concentrations of As, often magnitudes higher than the surrounding environment. This As may occur as multiple different species in the marine environment, ranging from inorganic acids to larger biomolecules such as phospholipids – where its incorporation is likely due to structural similarities between phosphate and arsenate (41). When such a large range of species are present, measuring the total As content alone is not enough to provide a full assessment of the toxicological risk. For example, in fish, arsenobetaine (AB) which often makes up the majority of the As content, is considered non-toxic and is excreted unchanged in urine after consumption by humans. Conversely, the inorganic species which only account for a minor fraction of As in fish, are carcinogenic (42). Each individual As species has also been shown to have a different mode of toxicity (43), and where combinations of these toxic compounds are present there could be the potential for a "cocktail effect". Thus, it is evidently necessary to consider each of the As species individually in different food matrices, including algae, to provide an accurate picture of the risks to consumers.

2.3.1 Inorganic Arsenic

The term 'inorganic arsenic' (iAs) is commonly used to describe the sum of arsenite (As(III)) and arsenate (As(V)) which are the naturally occurring forms found throughout the environment. ThioAs(III) and thioAs(V) have also been reported to occur in sulfidic environments (i.e., geothermal waters and peatlands) and polluted groundwaters (44–46). ThioAs(V) may also occur in marine sediments as a product of bacterial degradation of

organic matter (47). Arsenite is suspected to enter seaweed cells through aquaglyceroporin channels in the cell membrane in a similar fashion to those found in rice plants, where these channels are responsible for the passive efflux of neutral species (i.e., glycerol) and water (48). As(V) is thought to accumulate in seaweed due structural similarities with phosphate - where both anions have been shown to be taken up in same ratio without distinction (49), Figure 2.

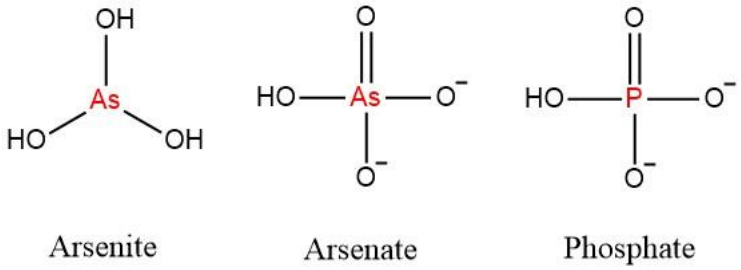

Figure 2 – The structure of arsenite, arsenate and phosphate at pH 8.08 to 8.33 (i.e., pH of sea water) (50). At this pH, arsenite is a neutral species, however arsenate and phosphate are both doubly deprotonated.

Inorganic arsenic is classed as a level 1 human carcinogen by the World health organization (WHO), and long-term exposure has been linked to bladder, lung, and skin cancers as well as diabetes and cardiovascular disease (51–56). Trivalent As is a weak mutagenic agent thought to exert toxicity by increasing the levels of reactive oxygen species which may result in DNA lesions (57). As(III) may also inhibit DNA repair by binding to cysteine residues in critical proteins (58), and in doing so act as a cocarcinogen for other mutagens (59). As(V) is thought to exert toxicity by replacing phosphate in biochemical reactions, i.e., uncoupling adenosine triphosphate (ATP) synthesis by binding to adenosine diphosphate (ADP) in the presence of succinate leading to the formation of an unstable and unusable compound (60). Although the iAs typically accounts for a minor fraction of the total As in most seaweed species, *Sargassum furisome* (hijiki) and *Laminaria digitata* are unique in their ability to accumulate high amounts of inorganic As, where it can comprise over 50% of the total As (37,61,62). Due to this, consumption of hijiki has been discouraged worldwide for many years (63). The European food safety authority (EFSA) has estimated that on average an adult in the European population is exposed to 0.03-0.15 µg kg^{-1} body weight of iAs daily from ingestion of drinking water, grain-based products, and marine-based foods (64). Seaweed was not found to contribute significantly to this exposure, but this was largely due to the low levels consumed rather than the iAs content of the seaweed, where several unspecified species were found to contain close to 100 mg kg^{-1} of iAs (64). However, this may change in the future as seaweed consumption has increased in recent years fuelled by demand for meat and dairy alternatives – a study carried out in the UK found a 2.3-fold increase in the availability of seaweed products from 2015 to 2023 (65).

2.3.2 Arsenosugars and Other Water-soluble Arsenicals

Generally, the most abundant As species in seaweeds are arsenosugars (AsSugars), with only a small amount of inorganic or methylated species present, Figure 3. Arsenobetaine (AB) and arsenochloline (AC) are common in fish and bivalves, but not typically in algae - although AB may be present in extremely low concentrations from epiphytic species (66,67). Other marine organisms such as bivalves, crustaceans and sea cucumbers may also contain

AsSugars (68–70), however these are thought to be the result of accumulation after seaweed consumption by these organisms.

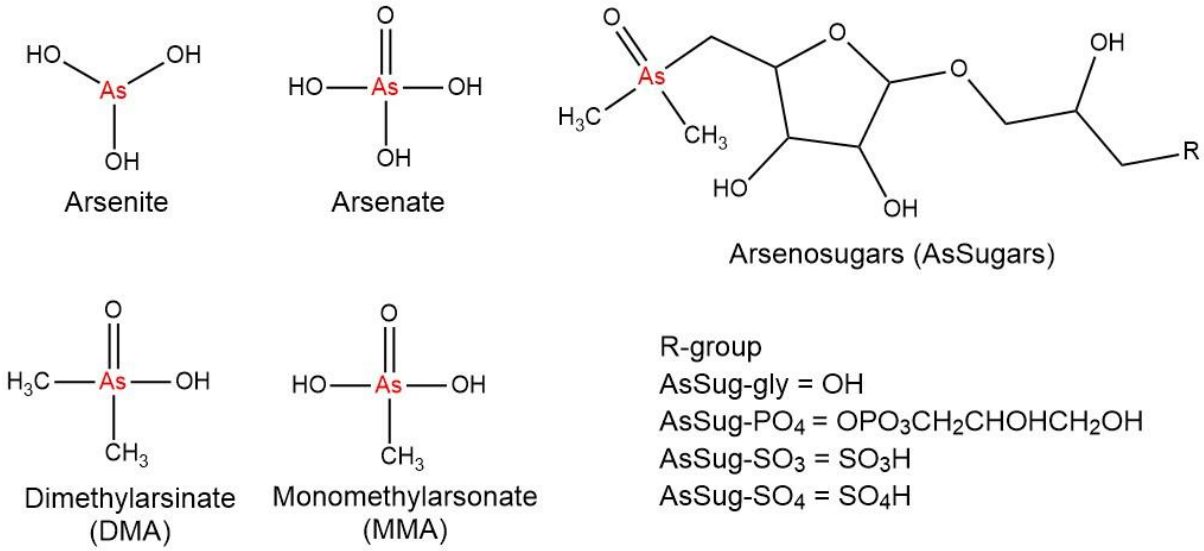

Figure 3 – The water-soluble arsenic species commonly reported in seaweed.

Four dimethylarsenoribose derivatives are commonly reported in seaweed, Figure 3, all differing only by R-group on the side chain of the ribose. Sulphated riboses (AsSug-SO_3 and AsSug-SO_4) tend to dominate in brown algae, whilst the glyercol (AsSug-gly) and phosphate (AsSug-PO_4) derivatives are most abundant in red and green algae. Regardless, all seaweeds appear to contain some concentration AsSug-gly and AsSugPO_4 and it has been suggested the former is the degradation product of the latter binding to phosphatidic acid in the cell membrane (68). The concentrations of AsSugars in seaweed have been shown to vary significantly between species, section of thallus and season (49,61) – with the highest levels found in the most biologically active tissues of the thalli (4,61). Whilst the highest phosphorous demand and consequently highest As concentrations are also found in these parts of the thallus, the implications this may have on the potential origins of AsSugars is still unclear.

The pathway for the formation of AsSugars has been partially described in literature, and begins with the methylation of As(III) (71). An S-adenosyl methionine donor and methyl transferase enzyme carry out the methylation of As(III) to produce monomethylarsonous acid (MMA(III)) and subsequently dimethylarsinous acid (DMA(III)) after further methylation (71). As(V) may also serve as the substrate after reduction. This differs from the widely accepted Challenger pathway for biological methylation of As, where a series of methylation and reductive elimination steps were proposed to explain the production of methylated species from iAs (72), Figure 4. However it should be noted that the Challenger pathway is based on the conversion of As(III) and As(V) to trimethylarsine (TMA) in fungi, and that AsSugars are rarely detected in fungi but abundant in cyanobacteria (73,74).

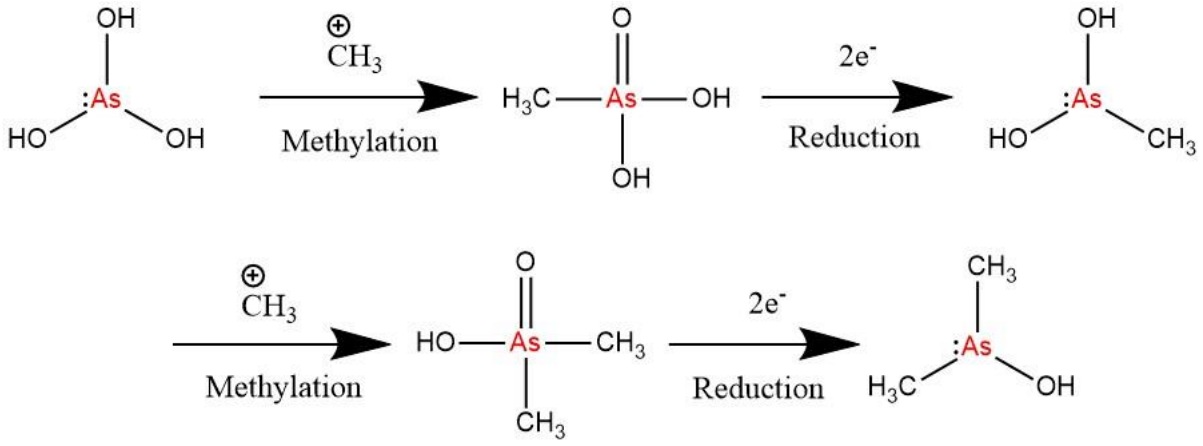

Figure 4 – The Challenger pathway for the methylation of As(III) to DMA(III) (72).

DMA(III) is then thought to undergo adenosylation resulting in a 5'-deoxy-5'-dimethylarsinoyl-adenosine intermediate (DDMMA) from which the adenosine moiety will be cleaved to produce the dimethylribose structure (71). In the bacterial species *Synechocystis*, the gene encoding the enzyme responsible for catalysing the adenosylation reaction has been mapped and a similar situation is thought to occur in seaweed. Further gylcosidation reactions are thought to be responsible for producing the side chain of the ribose, where differing R-groups are thought to be due to species specific enzymes able to carry out the glycosidation reaction (75).

With regards to the toxicity of these compounds, in vitro experiments with two AsSugar derivatives have shown no acute toxicity (76), however there is concern over toxicity from chronic exposure of AsSugars (77) as they can be metabolised to cytotoxic As species (i.e., thio-DMA) after ingestion (78). Ingested AsSugars may also be converted to their thioxo analogues which have demonstrated higher bioavailability in vitro (79). ThioAsSugars have been reported to occur naturally in some marine organisms but are generally unstable and susceptible to oxidation.

2.3.3 Arsenic-containing Lipids or Arsenolipids

A small percentage of the As in seaweed is present in the form of various lipid-soluble species, which are collectively known as arsenolipids (AsLipids). There are 13 main structural groups of AsLipids, Table 1, some of which were first reported as recently as 2019 (80). The nomenclature for As-containing lipids is currently under debate as traditionally compounds have been labelled using the class of compound followed by the nominal mass, i.e., AsHC360 refers to an arsenohydrocarbon with a mass of 360 amu. However, this naming system offers no information regarding the degree of saturation and a similar convention to normal lipids is being proposed (81) (e.g., 22:6 is docosahexaenoic acid – a 22 carbon alkyl chain with 6 unsaturated double bonds) and so AsHC360 would be followed by C16:0.

Table 1 – The 13 main structural groups of arsenolipids that have been reported in marine organisms in literature. This number may be increased to 14 if mono-acyl arsenosugarphospholipids are considered separate to the di-acyl forms.

Arsenic-containing lipid group	**Marine organisms**	**Reference**
Arsenohydrocarbons	Fish and oils: blue whiting, capelin, cod, herring, tuna, capelin Crustaceans: krill, lobster Macroalgae: *Alaria esculenta, Laminaria digitata, Saccharina latissima*, *Sargassum fusiforme* (Phaeophyta) *Palmaria palmata* (*Rhodophyta)* Plankton: *Picocystis sp.* Environmental: Sediment	(3–5,37,82–87)
Arsenofatty acids	Fish and oils: capelin, cod, herring, tuna Crustaceans: krill, lobster Macroalgae: *Laminaria digitata, Saccharina latissima* (Phaeophyta), *Palmaria palmata* (Rhodophyta)	(5,37,82,85,87,88)
Arsenodiacyl-glycerides	Macroalgae: *Coccomyxa spp.* (Chlorophyta)	(80)
Arsenotriacyl-glycerides	Fish oil: blue whiting (suspected due to presence of AsFA ether degradation products) Macroalgae: *Coccomxya spp.* (Chlorophyta)	(80,83)
Trimethylarsenofatty acid alcohols	Fish and oils: capelin, herring Macroalgae: *Palmaria palmata*	(5,89)
Arsenosugar phospholipids	Crustaceans: Brine shrimp, lobster Macroalgae: *Alaria esculenta*, *Laminaria digitata, Saccharina latissima* (Phaeophyta), *Palmaria palmata* (Rhodophyta) Fresh-water algae: C*hlamydomonas reinhardtii* Unicellular algae: *Dunaliella tertiolecta* Plankton: *Picocystis sp.* Higher marine organisms: Ringed seal Environmental: Seawater and sediment	(3,4,85,88,90–92)
Arsenosugar phytol	Fresh-water algae: C*hlamydomonas reinhardtii* Unicellular algae: *Dunaliella tertiolecta* Plankton: *Picocystis sp.* Environmental: Sediment	(3,88,93)
Arsenosugar-phosphoacyl ether	Environmental: Seawater	(90)

Arsenoceramides	Macroalgae: *Coccomxya spp.* (Chlorophyta)	(80)
Arsenophosphatidylcholines	Fish: Herring, herring caviar Crustacean: krill (lyso-AsPC also detected), lobster Higher marine organisms: Ringed seal, star spotted shark	(5,92,94)
Arsenophosphatidylethanolamines	Macroalgae: *Coccomxya spp.* (Chlorophyta) Fish: herring, salmon caviar Crustacean: lobster	(5,80,85,95)
Arsenophosphatidylglycerols	Macroalgae: *Coccomxya spp.* (Chlorophyta)	(80)
Arsenophosphatidy-linositols	Macroalgae: *Coccomxya spp. (Chlorophyta)*	(80)

Di-acyl arsenosugarphospholipids (AsSugPLs) are the most abundant species of AsLipids reported in brown macroalgae where concentrations may reach several mg kg^{-1} of seaweed dry weight (4,96). AsSugPL958 (C16:0/C16:0) has been reported as the most abundant in brown algae however the unsaturated AsSugPL982 (C16:0/C18:2) often dominates in Rhodophyta (4,5,96). These differences are suggested to arise from the variations in normal lipid composition. The mono-acyl arsenosugarphospholipids (m-AsSugPLs) are thought to be artefacts from the degradation of the di-acyl forms (97). Regardless, AsSugars are thought to be the starting point for AsSugPL species as they share a dimethyl arsenoribose moiety – where in one study the authors suggest they may be formed from AsSug-gly binding to phosphatidic acid in the cell membrane (68).

Arsenosugarphytols (AsSugPhytols) are perhaps the most unusual AsLipid structure and contain a dimethyl ribose group which is bound to a phytyl side chain. The normal phytol is found in chlorophyll and is required for tocopherol synthesis (98), so it is unclear when the ribose moiety becomes bound. If these compounds are produced from the degradation of chlorophyll all algae species should have the ability to synthesise it – however these compounds have so far only been reported in unicellular algae, phytoplankton, freshwater algae, and sediments (3,88,91,99). Arsenosugar phosphoacyl ethers have only thus far been detected in seawater and are thought to arise from bacterial origins (90).

The arsenohydrocarbons (AsHCs) and arsenofatty acids (AsFAs) are of particular concern due to their high bioavailability (100,101). In vitro testing with synthesised AsHCs has demonstrated toxicity comparable to that of arsenite as well as neurological and developmental toxicity in the in vivo model *Drosophila melanogaster* (7). These compounds also have the potential to cross blood-brain barriers whilst facilitating the transport of other toxins by increasing membrane permeability. Fatty acids have also been demonstrated to exert cytotoxicity, but higher levels of toxicity were caused by metabolism to thio-DMA (102). Trimethylfatty acid alcohols (TMAsOHs) were first reported in capelin oil and are the only group of lipids to contain a trimethyl As atom (the other groups are dimethylated). Seaweeds have also been demonstrated to contain this class of compounds, where TMAsOH374 was found to be the major AsLipid species in *P. palmata* (4,5).

Arsenodiacylglycerides (AsDAGs), arsenotriacylglcerides (AsTAGs), arsenoceramides (AsCers), arsenophosphatidylinositols (AsPIs) and arsenophosphatidylglycerols (AsPGs)

have only been reported thus far in *Coccomyxa spp.* (Chlorophyta) (80), however, retention times upwards of four hours were required to elute these compounds. The existence of AsTAGs was previously suggested by Taleshi et al., after the discovery of carboxylic acid methyl-esters produced during the clean-up procedure of blue whiting oil (83). Arsenophosphatidylcholines (AsPCs) were first identified in herring caviar and have been reported to occur throughout a wide range of marine species including fish, crustacean and algae (albeit in lower concentrations) (5,95). The toxicity of phosphatidyl-containing AsLipids has not been well studied but is assumed to arise from cleavage of the As-containing fatty acid moiety (103). Arsenophosphatidylethanolamines (AsPE) are similar in structure to AsPCs where an ethanolamine group is present in place of the choline head. AsPEs have been identified in several marine organisms, Table 1.

The formation of these compounds is yet to be understood, with some potential theories suggesting biological infidelity during normal lipid synthesis or dedicated synthetic pathways. However, more evidence is beginning to surface suggesting the latter, and that these compounds may impart useful properties to cell membranes not provided by normal lipids. A study using NanoSIMs to image the spatial distribution of As in *L. digitata* found that nearly all of the As was stored in the cell wall (37) – however this specimen contained low levels of lipophilic As (1.5% of total As content) so it is possible these compounds may be stored elsewhere, i.e., as functional lipids in the cell membrane.

Although algae are the likely starting point of AsLipids (and AsSugars) the significant focus has been on the AsLipid speciation of seafoods, oils and fish meal. The available literature on AsLipids focuses on brown macroalgae and there is a severe lack of information on red and green seaweeds. Furthermore, there is only a single study reporting spatial variations in AsLipids throughout macroalgae thallus and nothing on impacts of seasonal variations on lipids. In order to understand more about these compounds there is a dire need for more information on the occurrence of these species throughout different algal species and marine environments.

2.4 Analysis Methods

2.4.1 Sample Preparation

Sample preparation is an important step in any analysis, but even more so during speciation analysis as it is integral the chemical form of the analytes is preserved. The storage of samples before analysis is often overlooked although it may significantly impact final results. For example, repeated freeze-thawing of samples has been shown to cause materials to lose water and may alter concentrations of total As and inorganic species As(III) and As(V) (66). Drying is used to prevent this and any chemical changes that could occur due to microbial growth or decomposition. Freeze-drying is widely accepted as the best method of sample preservation where oxo-organoAsals have been shown to remain stable throughout the process (104,105). Freeze-drying also minimises the effects of oxidation so the potential for loss of As(III) is reduced (106). Oven-drying may be appropriate in some cases (i.e., feed) however it is not often used when preparing biological samples as concentrations can be altered through loss of volatile species and the speciation can be destroyed with the use of high temperatures (66). Homogenisation is also routinely performed after sample drying, however, the particle size to which the material has been homogenised is not frequently

reported. Lower extraction efficiencies of water-soluble species have been reported for fresh material (107), but it is unclear whether this is due to changes in chemical speciation during drying (i.e., degradation of lipid-soluble species to water-soluble after drying) or if cells membranes and walls are more intact than would be expected if sample material was lyophilised.

2.4.2 Extraction and Clean-up

It is not known how the inorganic As species, AsSugars or methylated species are stored within the seaweed cells, but all can be readily extracted using aqueous solutions. The most commonly used "gentle" extractants include water or a water-methanol mixture (90:10) to increase the solubility of less polar species (108–110). A partial speciation approach can also be taken, whereby hydrogen peroxide is added to oxidise As(III) to As(V) so both may be quantified as the sum of iAs. The addition of hydrogen peroxide may additionally oxidise thiolated As species and their oxo counterparts (77). Extractions with dilute acids such as nitric or hydrochloric acid are considered more "aggressive" and have been shown to extract higher levels of iAs by releasing them from protein bonds (111), as well as causing degradation of the side chain of AsSug-SO_4 and AsSugPO_4 to the base AsSugars (i.e., hydroxyl in place of side chain) (112). This however may be useful in the quantification of iAs in *A. nodosum* and *Fucus sp*. as they may contain high concentrations of AsSug-SO_4 that elute at a similar time to iAs using anion exchange chromatography with basic mobile phases (49,110). Extractions are typically performed under moderately high temperatures (~90°C), and with the aid of agitation, high pressure, sonication, or microwaves (113). Extraction time can be anywhere from 2 minutes to several hours, although somewhere in the middle is considered optimal (113). Clean-up procedures are not commonplace when measuring water-soluble speciation, however one study does report unsuccessful experimentation with activated carbon as a clean-up procedure in seafood samples after digestion with pepsin (114).

Unsurprisingly, different solvents are required to extract lipid-soluble arsenicals from biological samples. The most common extraction solvent reported is a mixture of dichloromethane (DCM) and methanol (2:1), where DCM is used to increase the solubility of the non-polar lipids (4,96,115). Methyl tert-butyl ether and pyridine have also been reported (3,80). Some studies additionally include an extraction with hexane to extract non-polar lipids, however this fraction rarely contains significant amounts of As (5,83). Extraction with non-polar organic solvents is not typically performed on seaweeds as these have very low concentrations of lipids in general in comparison to fish tissues and oils. Clean-up and preconcentration are performed on lipid extracts as the arsenic-containing lipids only make up a trace amount of the total lipids present in the samples. The most common method of sample clean-up is solid phase extraction with a silica or bentonite column, where lipids with an O=As moiety are retained while interfering matrix components are washed away. Although there are concerns over whether this procedure may degrade arsenotriglycerides or arsenophosphatidylcholines to free fatty acids (100). Other clean-up procedures may include the use of hexane to disrupt the phospholipid bilayer of the cell membrane (116).

The unextractable or "residual" As has also been recovered from sample residues after extraction for water-soluble and lipid-soluble As species. This fraction has been recovered in the form of As(V) by a previous study using a more aggressive extraction solvent – with authors suggesting the original form may have been As(III) bound to thiols in proteins within the cell (117). Being that there are several fractions of As within seaweed as previously

described, a mass balance approach is normally followed, where the sum of lipid-soluble, water-soluble, and residual As are compared to the total As concentration of the sample material (4,5,88).

2.4.3 Inductively Coupled Plasma Mass Spectrometry (ICP-MS)

Inductively coupled plasma mass spectrometry (ICP-MS) is one of the most common methods used for measuring total concentrations of metals and metalloids, and as an element-specific detector in speciation measurements (81,118). The plasma created by the inductive heating of argon gas can reach temperatures hotter than the surface of the sun (up to 10,000 K) (119), allowing elements with high ionisation potentials to be analysed in a compound independent fashion – with the only limitations being fluorine, neon, and helium. A basic ICP-MS setup will include a sample introduction system, a plasma torch, quadrupole mass analyser and a detector, Figure 5.

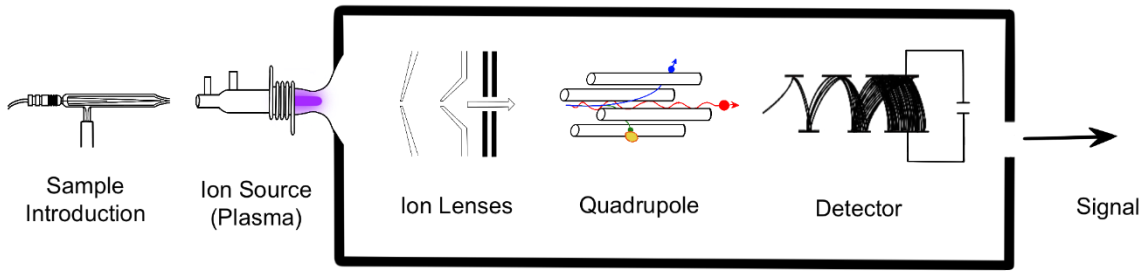

Figure 5 – The components of a basic ICP-MS setup, including a sample introduction system, a plasma torch, quadrupole mass analyser and a detector.

An inert quartz nebuliser is first used to convert liquid samples to an aerosol before introduction to the plasma. A stream of argon gas breaks up the liquid sample entering the nebuliser to produce a fine aerosol rich in small droplets. All droplets then pass through the spray chamber, where larger droplets exit to the waste under gravity and those smaller than 10 µm (approximately 1% of sample) are carried into the plasma (120).

The plasma is formed at the end of the torch, which consists of 3 concentric quartz cylinders fitted with a copper induction coil at the end, Figure 6. Argon is most frequently used however helium also performs well as a plasma gas but is not used due to costs (121). The sample aerosol is introduced into the centre-most cylinder by a carrier flow of argon gas (~1 L min^{-1}), the plasma is formed from an auxiliary argon flow between the two inner cylinders (~15 L min^{-1}), and an additional flow of argon (~1 L min^{-1}) between the outer cylinders cools the torch (122). To create the plasma a high-voltage discharge is applied to the flow of gas causing a small number of argon atoms to ionise and produce free electrons. These electrons are accelerated by the electromagnetic field produced by the induction coil and collide with more argon which are then stripped of electrons. This process proceeds in a chain reaction generating huge amounts of heat. The plasma discharge is sustained by power supplied to the induction coil by a radio frequency (RF) generator. As the sample aerosol proceeds through the plasma, the water molecules are vaporised from the droplets leaving behind small solid particles which enter the gaseous phase before atomisation and ionisation from the collision of argon electrons.

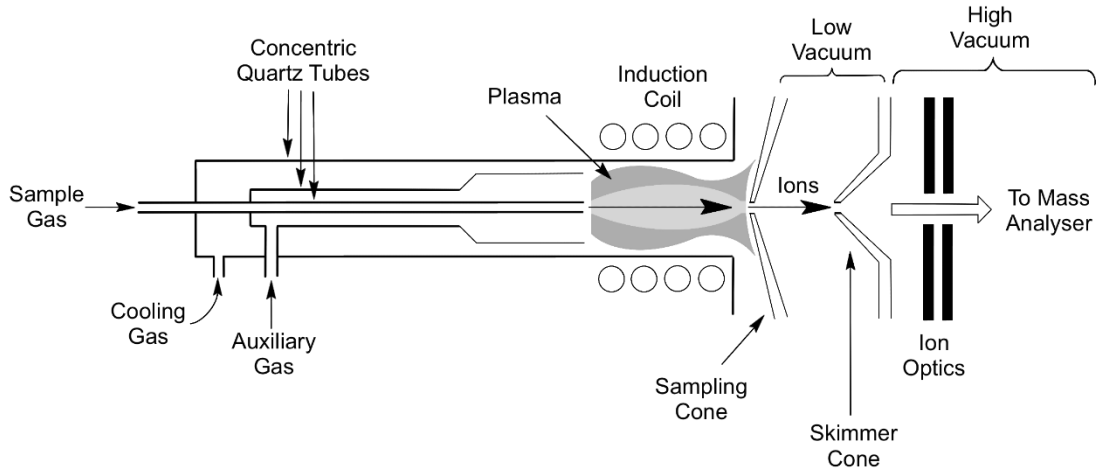

Figure 6 – The plasma torch and interface.

The ions, as well as other neutral atoms and photons emerge from the plasma and are extracted through a 1 mm diameter orifice in the sampling cone (123), Figure 6. The region between the sampling and skimmer cones is maintained at a rough vacuum (100-300 Pa), however the region behind the skimmer cone is maintained at a high vacuum ($1x10^{-3}$-$1x10^{-5}$ Pa) and the difference in pressure causes supersonic expansion of the ion beam (122,124). A portion of the ions are extracted through an even smaller orifice in the skimmer cone (0.45 mm diameter) and are directed by electrostatic lenses to the quadrupole mass analyser (122). The quadrupole filters out undesired interfering ions and matrix, allowing only ions with the selected mass-to-charge ratio (m/z) to be transmitted. The quadrupole consists of four parallel conductive rods (i.e., molybdenum alloy) spaced about a central axis, Figure 7. A radiofrequency with a DC offset voltage is applied between pairs of rods as such that opposing pairs have potentials of the same sign (125). This generates a dynamic electrostatic field through which only m/z with stable trajectories will pass through the quadrupoles whilst others are scattered. The electrostatic field can be easily manipulated to allow scanning across a mass range or to jump between selected m/z.

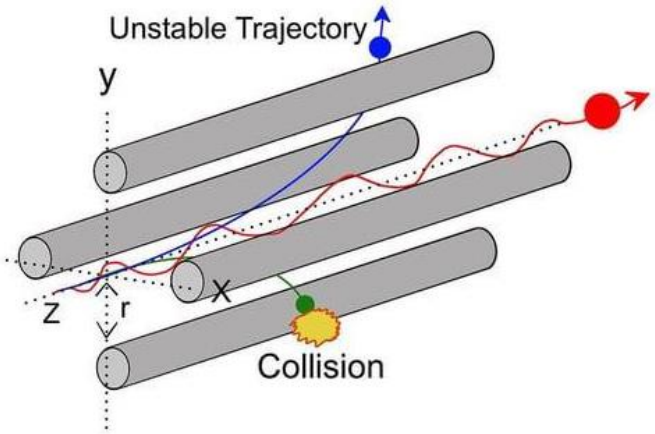

Figure 7 – The configuration of a quadrupole mass analyser. Ions with unstable trajectories are scattered outside the system (blue) or collide with the quadrupole rods (green). Ions with the correct mass-to-charge ratio (red) pass through the quadrupoles in a spiralling motion about the central axis, r.

ICP-MS are prone to isobaric interferences from polyatomic species and doubly charged metal ions. The measurement of As is plagued by interferences on ^{75}As from $^{40}Ar^{35}Cl$, which may falsely inflate As concentrations in matrices with high chlorine content. To overcome the issue of isobaric interferences, additional quadrupoles or collision cells may be included in the design. A collision cell is the simplest method of removing interferences and is installed before the quadrupole mass analyser. The collision cell is a hexapole or octopole rather than a quadrupole as the former have larger regions of stability which allow the transmission of almost all ions through the cell without scattering (126). The principle of kinetic energy discrimination is used to separate analytes from the interferences, whereby the cell is pressurized with a light gas (i.e., helium) which collides with the entering ions (127). The polyatomic interferences are larger than the analyte ions and will thus undergo more frequent collisions and emerge from the cell with lower energy. Alternatively, ICP-MS may also be fitted with three consecutive quadrupoles (ICP-MS/MS) which allows analysis to be run in mass-shift mode. The first of the three quadrupoles is used normally, however the second serves as a reaction cell where the analytes are combined with a reactive gas (i.e., oxygen, ammonia, or nitrous oxide) (128,129). The third quadrupole then selects for the m/z of the analyte plus the reaction gas, e.g., $^{75}As^{16}O^{+}$ = m/z 91. ICP-MS/MS is widely preferred over the use of a collision cell, however both methods will experience lower sensitivity due to the fewer number of ions that are transmitted to the detector.

The final destination for ions which have successfully navigated through the quadrupole(s) is the detector. The most frequently used detector for ICP-MS applications is a discrete dynode electron multiplier (EM) which converts incident ions to electrical current (130). The incoming positively charged ions strike the surface of the first dynode in the EM which is maintained at a high negative voltage. This interaction causes the emission of several electrons from the surface of the first dynode, which in turn strike further dynodes releasing more electrons and triggering a cascade which gives rise to a measurable signal. EMs are able to operate in pulse mode where the signal is measured in counts per second at the first dynodes and analogue mode where the signal is measured as voltage from the final dynodes. This vastly increases the linear dynamic range of the detector and means ICP-MS can be used for concentrations ranging from parts per trillion to parts per million (130).

An internal standard is monitored throughout analysis to ensure response stays relatively consistent between standards and samples and no drift in the signal is observed. The internal standard should ideally have a similar mass and similar first ionisation potential to the analyte of interest (131) – for As measurements germanium is frequently chosen (132,133).

2.4.4 Arsenic Speciation with HPLC-ICPMS

Speciation analysis of As has become an increasingly popular research topic in recent years due to the vast number of intriguing chemical forms As can take in the marine and terrestrial environment. Additionally, it has become a crucial tool in food safety analysis as As toxicity is highly dependent on chemical species. In order to perform speciation measurements, the analytes of interest must first be separated before reaching the detector. This is accomplished by coupling a chromatographic method such as high-performance liquid chromatography (HPLC) to the ICP-MS.

For water-soluble As speciation in seaweed, anion exchange chromatography is commonly employed as cationic species such as AB or AC are generally present in very low concentrations (49,66). The mobile phase used is most often an aqueous solution of a basic ammonium salt (e.g., bicarbonate, carbonate) adjusted to the desired pH with ammonia solution (108–110). Volatile salts are chosen to reduce the deposition of carbon on the sampling and skimmer cones during analysis which can reduce sensitivity. Analytes may alternatively be eluted under acidic conditions, e.g., an ammonium dihydrogen phosphate mobile phase which is pH adjusted with trifluoroacetic acid or citrate (134,135). Regardless, an isocratic or gradient elution can be used depending on the complexity of the matrix, the latter of which is arguably more suitable for seaweed matrices which may contain a large number of components requiring separation (136). A volatile, low mass organic modifier (i.e., methanol or acetonitrile) may also be added to the mobile phase to increase signal response through the carbon enhancement effect (137). This is done in low concentrations (1-8%) as high levels of organic solvents can result in signal depression by reducing the temperature of the plasma (110).

In comparison, lipid-soluble As speciation is less frequently reported in the literature as the instrumental set-up of the ICP-MS requires modification to run in the organic mode required to work with high volumes of organic solvents (138). These modifications can include switching to a narrow bore torch (1.5 mm rather than standard 2.5 mm) (139), lowering spray chamber temperatures below 0°C (87), post-column dilution (e.g., 1:10) (88) or the use of oxygen as an optional gas to volatise carbon and reduce deposition on cones which in turn requires the use of expensive platinum tipped cones as long term use of oxygen as an optional gas can be corrosive (140). For the analysis of AsLipids, the majority of studies utilise reverse-phase HPLC with a C18 column and a gradient elution with water (0.1% v/v formic acid) and methanol (0.1% v/v formic acid) (4,88,96). One interesting study reports the use of ≤30% 1,2-hexanediol as a substitute for 100% organic mobile phase which allowed authors to use the ICP-MS in “normal mode” (i.e., as would be used for aqueous mobile phases) (141). The 1,2-hexanediol mobile phase was also found to have the same eluting power as 100% methanol and authors could use increased flow rates as high as 1.5 mL min^{-1} without post-column dilution(141). However, the use of HPLC-ICP-MS alone is not sufficient due to the lack of commercially available standards for AsLipid speciation, and some form of high-resolution mass spectrometry (i.e., qToF, Orbitrap) must be performed alongside to provide identification of the individual compounds.

2.5 Different Approaches to Arsenic Speciation

Whilst HPLC-ICP-MS remains the most popular technique used for quantitative As speciation due to its robustness across a range of sample matrices, several other methods have been reported in the literature, Table 2. Hydride generation (HG) can be used when performing water-soluble speciation to eliminate interferences from large organoarsenicals, where post-column and prior to detection with ICP-MS, a strong reducing agent such as $NaBH_4$ is used to transform inorganic As species or methylated species into volatile hydride compounds. The inclusion of a HG step allows for increased sensitivity and lower detection limits (DL), where for example instrumental DLs for high performance liquid chromatography – atomic fluorescence spectroscopy (HPLC-AFS) methods in literature range from 0.39-0.64 µg L^{-1} but were lower for HPLC-HG-AFS at 0.03-0.14 µg L^{-1} (142–144). However, analyte specific standards are needed for quantification as the efficiency of hydride formation differs for each compound, and so HG is typically limited to the quantification of As(III), As(V) and methylated species DMA and MMA (145). HG has also been suggested as an alternative to chromatographic separation where only the quantification of iAs is required, however it appears the method is more suited for screening purposes. For instance, one study reports almost 2-fold higher concentrations of iAs in a range of *Saccharina latissima* samples when using HG-ICP-MS (0.24-0.45 mg kg^{-1}) compared to HPLC-HG-ICP-MS (0.12-0.27 mg kg^{-1}) (146). A separate study additionally reported similar issues with fish matrices DOLT-4 and DOLT-5, where iAs concentrations were 0.047 and 0.076 mg kg^{-1} respectively when measured with HG-ICP-MS but 0.025 and 0.029 mg kg^{-1} when measured with HPLC-HG-AFS (142).

Alternative separation methods to HPLC have also been reported in literature for quantitative As speciation, most commonly gas chromatography (GC) or capillary electrophoresis (CE). Three studies were able to achieve similar DLs using CE-ICP-MS (0.03-1.8 µg L^{-1}) to that of HPLC-ICP-MS (<0.01-1.0 µg L^{-1}) for rice, marine reference materials, ground water and poultry (147–149). The method was found to be applicable for the quantification of several arsenicals including AB, AC, As(III), As(V), DMA and MMA, and additionally several phenylarsonic acids used as veterinary drugs for poultry (147–149). GC separations are typically only used for volatile analytes such as AsH_3 and AsHCs (116,150,151), however one study reports the analysis of As(III), As(V), DMA and MMA using GC-MS after chemical derivatization using ethylthioglycolate to form volatile ring structures (152).

Alternative, lower cost detectors to ICP-MS have also been reported in the literature including AFS, atomic absorption spectroscopy (AAS), microwave-induced plasma mass spectrometry (MIP-MS), inductively coupled atomic emission spectroscopy (ICP-AES) (143,144,153–155), Table 2. The use of graphite furnace atomic absorption spectroscopy (GFAAS) has also been reported for As speciation but is typically only used for relatively uncomplex water matrices (156–158). However, all of these alternative detectors have the major drawback of exhibiting compound specific responses meaning standards for each individual analyte are needed - this is not observed for As speciation with ICP-MS. Nonetheless, a novel study published in 2023 reported a GC-MS method for compound independent quantification of arsenohydrocarbons with a commercially available triphenylarsine standard (151). Low signal intensities were previously observed due to the incomplete thermal conversion of the AsHCs to their trivalent state in the injection port of the GC, however reduction prior to analysis produced results comparable to those obtained using HPLC-ICP-MS (151). This method could present an accessible, low-cost alternative for the

analysis of the most toxic class of AsLipids – with the potential for wide implementation across laboratories.

Table 2 – Examples of arsenic speciation techniques reported in literature for a range of biological and environmental matrices.

Analysis method	**Matrix**	**Analyte**	**Instrumental detection limit (µg L^{-1})**	**Reference**
HPLC-ICP-MS	Rice, seaweed, seafood, and marine oil	iAs	1.0*	(159)
	Rice, tuna fish and wheat	iAs, DMA, MMA	0.03-0.05*	(160)
	Urine and serum	AB, As(III), As(V), DMA, MMA	0.3-5	(161)
	Urine	AsFA362, AsFA418	<0.01	(141)
HPLC-ICP-MS/ESI-MS	*Holcus lanatus* and *Pteris cretica* (plant)	As(III)-(PC2)2, GS-As(III)-PC2, As(III)-PC3, and As(III)-PC2	-	(162)
	Saccharina latissima and *Alaria. esculenta* (seaweed)	AsHCs, AsFAs, AsSugPLs	0.25*	(4)
HPLC-HG-ICP-MS	TORT-2	iAs	0.01*	(163)
	TORT-1 and CRM 627 oyster, cockle, mussel and fresh water algae	AB, As(III), As(V), DMA, MMA	0.1-0.3	(164)
HPLC-HG-AFS	TORT-3, DORM-4, DOLT-5	iAs, DMA, MMA	0.03-0.1*	(142)
	TORT-1 and fresh bivalve tissues	AB, As(III), As(V), DMA, MMA	0.1-0.14	(143)
HPLC-HG-AAS	TORT-1 and fresh bivalve tissues	AB, As(III), As(V), DMA, MMA	1.1-3.9	(143)
	Ground water	As(III), As(V)	1.5-2.2	(165)
	Urine	AB. AC, As(III), As(V), DMA, MMA As(V), TMAO	0.1-0.9	(153)
HPLC-HG-GFAAS	Water	As(III), As(V)	0.2	(166)
HPLC-ICP-AES	Soil	As(III), As(V), DMA, MMA	0.1-1.0	(167)
	Standards mixture	As(III), As(V), DMA, MMA	7-18	(155)
HPLC-AFS	Kelp, shrimp,	As(III), DMA, MMA,	0.39-0.64	(144)

	squid	As(V)		
HPLC-N_2-MIP-MS	Urine	AB, AC, As(V), MA, DMA, TMAO,	0.68-22	(168)
HPLC-USN-MIP-MS	Urine	AB, AC, As(V), DMA, MMA, TMAO	0.21-3.6	(154)
HG-ICP-MS	Seaweed	iAs	0.06	(146)
HG-ICP-MS	Fish and seaweed	iAs	0.1*	(142)
HG-CT-ICP-MS	River and seawater	iAs, DMA, MMA	<0.01	(169)
HG-ICP-AES	Rice	iAs	0.7*	(170)
FI-HG-AAS	Seaweed	iAs	0.22*	(171)
FI-HG-AAS	Seaweed	iAs	0.04*	(172)
HG-AAS	Rice	As(III), iAs	0.07-0.14*	(173)
HG-GFAAS	Gasoline	AsH_3	<0.01	(158)
HR-CS-GFAAS	Water	iAs	0.6	(156)
GFAAS	Water and snow	As(III)	<0.01	(157)
GC-ICP-MS	Fish oil	AsHCs	0.05	(116)
GC-ICP-MS	Natural gas	AsH_3	<0.01	(150)
GC-MS	Seaweed, fish oil	AsHCs	-	(151)
GC-MS	Seafood	As(III), As(V), DMA, MMA	6-14	(152)
CE-ICP-MS	Rice	As(III), As(V), DMA, MMA	0.15–0.27	(148)
CE-ICP-MS	DOLT-3 (dogfish liver)	AB, As(III), As(V). DMA, MMA,	0.6–1.8	(147)
	TORT-3 (lobster hepatopancreas), DORM-4 (fish protein), ground water, plant, poultry	AB, AC, As(III), As(V), DMA, MMA, Phenylarsonic acids	0.03–0.1*	(149)
HPLC-ESI-MS/MS	Ancient marine sediment	AsLipids and thio-AsLipids	-	(174)
Micro-XANES	Animal fur, bird feathers	As(III)-S complexes, endogenous As(V)	-	(175)
XANES/EXAFS	*Pteris vittata* (plant)	As(III)-S complexes		(176)
	Contaminted soil and solid waste	As(III) and As(V) adsorption to metal oxides	-	(177)

*Instrumental detection limit estimated from method detection limit, sample weight and volume of extractant used.

More sophisticated high resolution mass spectrometry techniques such as qToF-MS and Orbitrap are often used for the identification of As species – most often AsLipids due to the lack of available standards (4,87). If both identification and quantification are required, the post-column flow can be coupled to both ICP-MS and some form of high resolution or tandem mass spectrometry, Table 2. This approach has been successfully used to detect new species of AsLipids in several marine organisms (96,115,178). An alternative approach to

the identification of novel AsLipids was described by Liu et al. that used data-processing software to link product ions with their precursors using product ions as the grouping models (87). This approach allowed authors to detect 23 AsLipids – 10 of which were novel - in 4 types of seafood. Whilst the characteristic product ions selected here (103 m/z and 105 m/z) are typical fragments observed from arsenohydrocarbons and arsenofatty acids, this technology has the potential to be expanded to larger arsenicals such as glycerophospholipids, which are known to fragment into larger product ions still containing the dimethyl arsenoribose moiety (i.e., 237 m/z, 391 m/z and 409 m/z).

The major limitation of these hyphenated techniques is that they must rely upon extraction procedures to remove analytes from samples prior to analysis which may mean valuable information regarding the oxidation state and coordination of As within compounds is lost (179). Additionally, As(V)-O complexes have been demonstrated to be more efficiently extracted than those containing As(III)-S, so it is likely these compounds are not extracted and therefore remain undetected (179). This can be overcome by the use of X-ray atomic spectroscopies (XAS), such as X-ray absorption near edge structure (XANES) and extended X-ray absorption fine structure (EXAFS) where sample materials are analysed directly, and analytes can be observed in-situ. These methods may also be used quantitatively through the use of linear combination fitting models (180,181). The application of XAS to As speciation has previously been used to identify As(III)-S complexes in biological tissues (i.e., fur, feathers, plants), to determine endogenous As(V) on animal fur, and to observe the adsorption of As(III) and As(V) to metal oxides in contaminated soils and waste (175–177).

Overall, it appears that a combination approach is best taken to As speciation, where ICP-MS techniques are used for robust quantification, high resolution MS (i.e., orbitrap) is used for identification and finally XAS is used to elucidate oxidation state and coordination.

2.6 Current Legislation, EFSA Opinion and Difficulties

As of 2024 under EU commission regulation 2023/915, maximum levels are in place for the levels of total As in salt (0.5 mg kg^{-1}) and iAs in infant formula and baby food (0.01-0.02 mg kg^{-1}), rice-based products (0.03-0.3 mg kg^{-1}), and fruit juices (0.02 mg kg^{-1}) (182). The legislation does not include maximum levels for total As or iAs in any seafood or seaweed product for human consumption. This differs significantly to other food safety regulations across the world where potential exposure risks to As in seafood have been addressed. For example, in Australia and New Zealand, there are limits of 1 mg $kg^{-1}$of iAs for seaweed and molluscs for human consumption and 2 mg kg^{-1} for fish and crustaceans(183). Total As is also limited in cereals (1 mg kg^{-1}) and salt (0.5 mg kg^{-1}) (183). China has perhaps the most extensive legislation which covers iAs in several matrices: rice (0.2-0.25 mg kg^{-1}), seafood (0.1-0.5 mg kg^{-1}), mushrooms (0.5-0.8 mg kg^{-1}), fish oils (0.1 mg kg^{-1}) and cereal foods for infants (0.2-0.3 mg kg^{-1}). Additionally total As limits are defined for meat products (0.5 mg kg^{-1}), milk (0.1-0.5 mg kg^{-1}) and fresh vegetables (0.5 mg kg^{-1}). It could be argued that these strict regulations are necessary in China due to the frequent consumption of foods naturally high in As (i.e., rice and hijiki seaweed), something which is not typical of the European diet. Currently, France is the only European country to have implemented nationwide limits for iAs out with EU regulation 2023/915. A limit of 3 mg kg^{-1} for iAs in seaweed condiments has

been established for 21 species of seaweed allowed for human consumption (including *L. digitata*) (184).

An updated risk assessment on inorganic As in food was published by the European Food safety Authority (EFSA) in January 2024, and was developed based on epidemiological studies reported in scientific literature. A significant increase in the likelihood of developing skin cancer was observed when daily intakes exceeded 0.16-0.31 µg kg^{-1} body weight (bw) per day, where for reference the daily tolerable intake is currently set at 0.3 µg kg^{-1} bw per day. The assessment also found sufficient evidence linking low to moderate exposure with bladder and lung cancers, stillbirth, congenital heart disease, chronic kidney disease and ischemic heart disease. Additionally, there was emerging evidence of negative impacts on foetal development and reduced birth weight from inorganic As exposure. This was derived from epidemiological studies in Bangladesh, although the relevance of this to Europe is unclear as malnutrition is less common. It is difficult to say whether the results of this risk assessment will prompt further EU legislation as many of the epidemiological studies used in the assessment were primarily based in areas of the world where ground waters are highly contaminated with As (e.g., Bangladesh, Chile). Whilst these studies have aided in our understanding of the impacts of iAs exposure through ingestion, whether this will prompt more expansive legislation in the future is unclear.

With regards to organoarsenicals, significantly less is known about the potential health impacts after exposure. The trivalent forms of the methylated species MMA(III) and DMA(V) demonstrate similar toxicity levels to that of As(III), whereby they may induce direct damage to DNA (185), and DMA(V) has been shown to promote bladder carcinogenesis in rats (186). However, there is debate over the relevance of this animal model to humans, as DMA may bind to rat haemoglobin and thus be retained in the body for a longer period of time (187). AsSugars have demonstrated no acute toxicity in vitro but are suspected to exert some form of chronic toxicity (76,77). Similar behaviour may be expected of arsenogycerophospholipids which also contain a dimethylribose moiety. AsSugars are largely metabolised to small methylated species (e.g., DMA) after consumption before excretion through urine, where large variations were observed between participants with regards to the metabolites produced and total amount of As recovered (188,189). AsLipid metabolism also appears to be specific to individuals, but instead results in a range of water-soluble and lipid-soluble arsenicals (190). These major differences between individuals with regards to As metabolism are likely to present significant difficulties when assessing risk to consumers for organic As species as well as inorganic – as the toxicity may be underestimated for certain groups.

EFSA are in the process of preparing a risk assessment for the combined exposure to small and complex organoarsenicals which will be finalised by the beginning of 2025. This report will be based on available occurrence data for organic As or literature and will assess the risk from methylated species (i.e., MMA and DMA), and in a separate opinion the risk from AsSugars and AsLipids species. Thioarsenicals are also expected to be covered by this assessment as there is concern over their higher bioavailability compared to the oxo-analogues. It is unlikely that the report will prompt the implementation of maximum levels for the organoarsenic species due to the lack of knowledge on health effects from exposure. Additionally, any legislation implemented for AsSugars and AsLipids would be difficult to adhere to due to the lack of commercially available standards, reference materials and accessible methods for lipid measurements.

Chapter 3 – Papers I-VI

3.1 Summary of Results

Paper I: Potentially toxic elements in Icelandic Seaweeds

Seaweed is becoming an increasingly popular food source due to its high mineral and antioxidant content, and the European demand for seaweed is expected to rise during the coming years. However, seaweeds may accumulate high levels of potentially toxic elements (PTEs) such as As, Cd and Pb, which may pose a threat to the safety of consumers. This study aims to investigate trends in PTEs in several species of brown, red and green seaweeds collected in Iceland, and how these elements may vary between thallus section, location, and season.

Brown seaweeds generally contained the highest levels of As, Cd and U, Figure 8, all of which were highest in the reproductive tissues during February. High levels of As may be of concern for *Laminaria digitata*, as a large portion may be in the form of carcinogenic inorganic species (61). As concentration was also negatively correlated with the concentration of several essential elements (Mn, Fe, Co and Cu) suggesting that As accumulation may have an impact on the uptake of other elements. Concentrations of Cd were also found to be elevated in commercially relevant species when compared with those grown in France and Norway – in particular *Alaria esculenta* and *Palmaria palmata* – and may attributed to the abundance of basalt rock found in Iceland. Green seaweeds were found to contain the highest concentrations of Cr and Fe, but seasonal comparisons could not be drawn due to the lack of specimens available during the winter months.

Seaweed grown in Iceland was found to be relatively high in essential trace elements such as Cu, Zn and Fe, and all samples contained low levels of toxic Hg – where all were below the ML of 0.1 mg kg^{-1} allowed in food supplements. With regards to other toxic elements, over half of the samples contained levels of As higher than the ML of 40 mg kg^{-1} for seaweed-derived animal feed, and 25 samples exceeded the 3 mg kg^{-1} ML for Cd in food supplements - levels of Cd in *A. esculenta* sporophyll were as high as 18 mg kg^{-1}. Seaweeds grown or cultivated in Iceland have the potential to provide a source of essential minerals and trace elements, however the levels of Cd should be monitored, and certain species avoided for human consumption (i.e., *A. esculenta*).

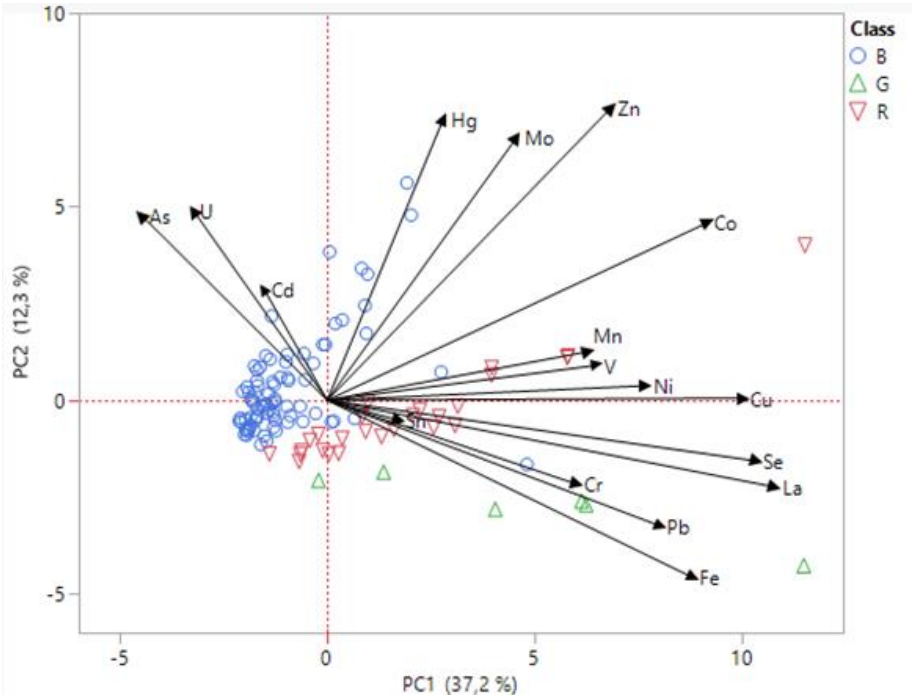

Figure 8 – Principal component analysis of the concentration of 17 elements in samples of Phaeophyta (B), Rhodophyta (R) and Chlorophyta (G) measured by ICP-MS.

Paper II: Inorganic arsenic in seaweed: a fast HPLC-ICPMS method without co-elution of arsenosugars

Seaweed is known to accumulate high levels of As – which may be in the form of carcinogenic iAs. If iAs levels are to be regulated for this complex matrix, it is crucial that there are straightforward, fast methods available to ensure analysis is widely accessible. Here we propose a fast method for the routine measurement of iAs in seaweed without co-elution of AsSugars that may complicate quantification. The addition of nitric acid and hydrogen peroxide to the extraction solution allows for a 'partial speciation' approach where iAs is quantified as the sum of both inorganic species oxidised to As(V) and interferences from high levels of sulphur-containing AsSugars (AsSug-SO_3 and AsSug-SO_4) are removed by degradation to a dimethylarsenoribose with no side chain that elutes with AsSug-gly (112).

The developed method was optimised using a custom fractional factorial design of experiment (DOE) and tested on a range of reference materials including TORT-3 (0.36 ± 0.03 mg kg^{-1}), DORM-5 (0.02 ± 0.003 mg kg^{-1}), DOLT-5 (0.07 ± 0.007 mg kg^{-1}) which all showed good agreement with previously reported values (109,142). Additionally, for the reference material Hijiki 7405-b which has a certified value for iAs (191), the recovery was found to be 99 ± 9%. The method was found to be suitable for high throughput analysis of iAs in a range of food and feed matrices, Figure 9, including *Asparagopsis taxiformis* seaweed, grass silage and insect proteins, with an LOD of 0.006 mg kg^{-1} and LOQ of 0.018 mg kg^{-1}.

The method requires minimal sample preparation and provides acceptable separation of iAs from other analytes in under 7 minutes, Figure 9. However, it may be limited with regards to the quantification of DMA in seaweed, as the acidic extraction may cause overestimation of this analyte by causing degradation of lipid species that are typically more abundant in seaweed than other marine matrices such as AsSugPLs. However, the concentrations of DMA quantified using this method may provide a better estimation with regards to exposure after ingestion and subsequent digestion of seaweed.

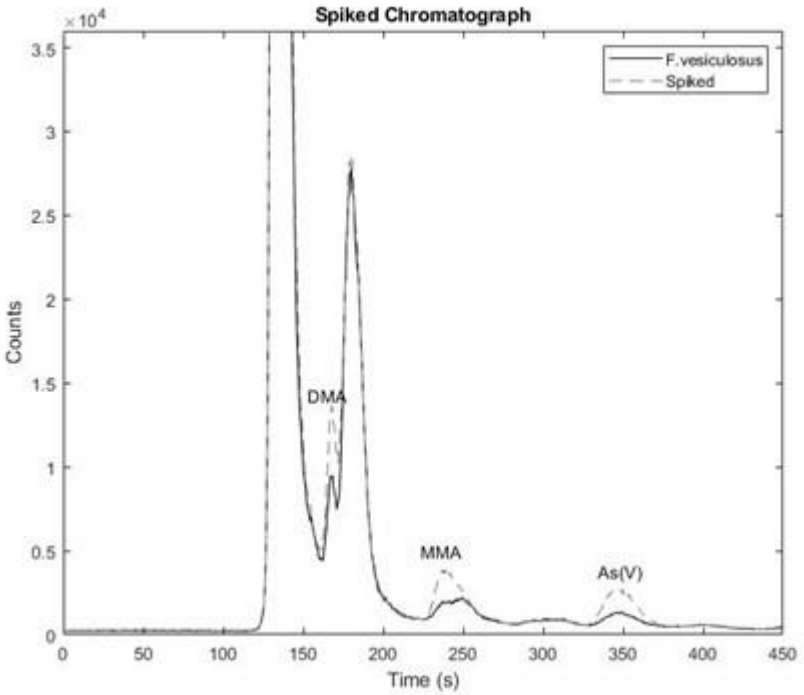

Figure 9 – The separation of iAs (as As(V)) from other analytes using the developed HPLC-ICP-MS method. Extraction solution: 1% (v/v) nitric acid, 3% (v/v) hydrogen peroxide. Chromatography: 60 mM ammonium carbonate, 3% (v/v) methanol, isocratic elution. Column: PRP-X100 anion exchange, 250 x 4.1 mm, 10 µm.

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Paper III: The impact of different sample preparation methods on the arsenic speciation in *Laminaria digitata*

Brown seaweeds are known to accumulate high concentrations of As which may be metabolised to complex structures such as arsenic-containing sugars and lipids. Preservation of these large As compounds throughout sample preparation is crucial to ensure accurate results. The aim of this study was to investigate the impact of different drying methods on the water-soluble As speciation in *L. digitata*, as well as the fractionation of As before and after drying.

The distribution of As throughout the lipid-soluble, water-soluble, and residual fractions was changed significantly after drying, Table 3, where the polar lipid-soluble As accounted for 19.3% of the total As before drying and 1.1-3.8% after drying (t-test, $p<0.03$). The residual As was also found to increase after drying depending on the method used, where oven drying at 110°C resulted in the highest concentration of non-extractable As (9.12 ± 1.81 mg kg^{-1}) compared to air-dried (7.0 mg kg^{-1}) and freeze-dried (6.7 mg kg^{-1}) samples. As has been demonstrated to be stored in the cell wall, with smaller amounts in the intracellular matrix in *L. digitata* (37). Perhaps the evaporation of water from the cytosol during drying does not allow cells to be opened sufficiently during extraction leading to an increase in residual As, particularly if residual As is bound as As(III) (or DMA(III)/MMA(III)) to sulphur-containing groups within the cell (3,117).

The water-soluble speciation was found to remain similar between drying methods, although lower levels of AsSug-gly were extracted from fresh material (10.5 mg kg^{-1}) than dried (13.4-15.1 mg kg^{-1}). Freeze-drying removed higher levels of AsSug-PO_4 (32-42%) and AsSug-SO_3 (11-14%) than fresh, air-drying and oven-drying – suggesting that these analytes may be stored in the cell wall matrix which is damaged during freeze-drying due to the formation of crystals (192). AsSug-SO_4 was not detected. Oven-drying was found to increase DMA concentrations, likely due to thermal degradation of large As compounds (AsSugars or even AsLipids) to unknown, smaller arsenicals and DMA. As(V) concentrations were similar between fresh and dried samples (22.1-25.4 mg kg^{-1}) - which means accurate results can be obtained for this carcinogenic species regardless of how the sample was prepared.

Table 3 – The arsenic fractionation in *Laminaria digitata* after sample preparation by freeze-drying, air-drying, oven-drying or no drying. Non-polar lipid soluble: hexane extraction, polar-lipid soluble: DCM/methanol extraction, Water-soluble: water extraction, and Residual: non-extractable (digested). CRM Hijiki (7405-b) is also shown.

Sample	**Non-polar lipid-sol As (mg kg^{-1})**	**Polar lipid-sol As (mg kg^{-1})**	**Water-sol As (mg kg^{-1})**	**Non-extractable As (mg kg^{-1})**	**Total As (mg kg^{-1})**	**Recovery (%)**
Fresh *L. digitata*	<LOQ (<0.1%)	12.3 ± 1.11 (19.3%)	51.1 ± 0.42 (80.3%)	2.61 ± 0.03 (4.1%)	63.6 ± 3.16	104 ± 2
Freeze-dried *L. digitata*	<LOQ (<0.1%)	2.39 ± 0.32 (3.8%)	51.9 ± 1.32 (81.6%)	6.71 ± 0.60 (10.6%)	63.6 ± 3.16	96 ± 1
Air-dried *L. digitata*	<LOQ (<0.1%)	1.41 ± 0.11 (2.3%)	57.1 ± 0.93 (93.5%)	7.04 ± 1.45 (11.5%)	61.1 ± 0.53	107 ± 1
Oven-dried *L. digtata*	<LOQ (<0.1%)	0.72 ± 0.15 (1.1%)	53.2 ± 0.68 (79.5%)	9.12 ± 1.81 (13.6%)	66.9 ± 4.61	94 ± 4
Hijiki 7405-b (*Sargassum fusiforme*)	<LOQ (<0.1%)	5.62 ± 0.17 (%)	21.5 ± 0.33 (%)	19.7 ± 0.91 (%)	50 ±	95 ±

Paper IV: Temporal and intra-thallus variation in arsenic species in the brown macroalga *Laminaria digitata*

AsSugars account for the majority of total As in common seaweed species, yet it is unclear whether these are formed through some detoxification pathway for iAs or are precursors/degradation products of arsenic-containing phospholipids in the cell wall. The aim of this study was to compare temporal and intra-thallus variations in water-soluble As and total non-polar and polar AsLipids in *L. digitata* to offer potential insight into the metabolism of As.

In general, lower levels of total As were detected in the samples collected in May (39.2-74.5 mg kg^{-1}) compared to those collected in February (72.6-151 mg kg^{-1}). The concentration of As(V) was found to consistently increase along the thallus from the holdfast/stipe (0.78-1.82 mg kg^{-1}) to the decaying fronds (44.4-61.0 mg kg^{-1}) in both months, and AsSug-SO_3 was the dominant AsSugar in the majority of samples, Figure 10. AsSug-SO_4 was not detected in any samples. The extraction efficiency was lower in fresh samples (64-77%) than in freeze-dried (95-116%) from the same month.

Water-soluble, polar AsLipids, and residual As concentrations were generally highest in February, and the non-polar AsLipids accounted for <0.42% of total As in all samples. The levels of polar AsLipids were significantly higher during February than in May, which is in line with the higher levels of total lipid content found in edible seaweeds during colder months (193). The residual As was higher in February where a maximum of 26.1 mg kg^{-1} was found in the old frond compared with maximum of 10.1 mg kg^{-1} in the young frond in May. The water-soluble fraction followed a similar trend to the As(V) concentration, where levels increased from holdfast/stipe to decaying frond and were highest in February.

This study is the first to report not only temporal, but intra-thallus variations in the water-soluble As speciation and distribution of As in *L. digitata*. This dataset adds significantly to our understanding of As metabolism of *L. digitata*, and how this may fluctuate temporally and spatially within algal thalli.

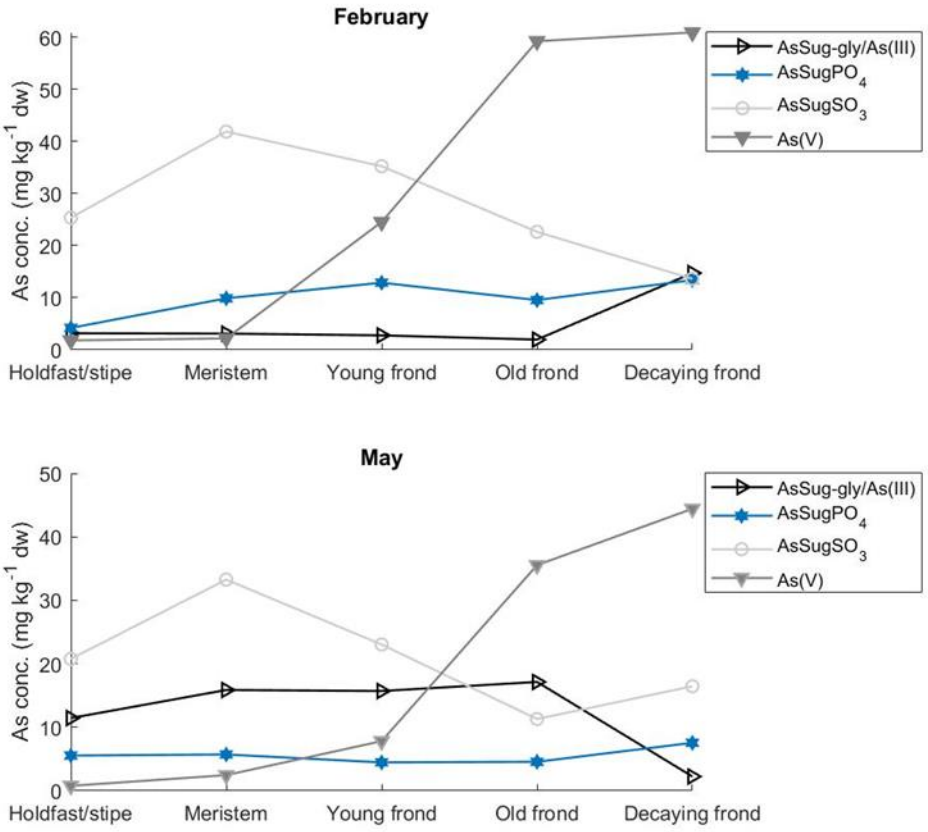


Figure 10 – The average concentrations of water-soluble As species in February and May 2022 in different sections of the *Laminaria digitata* thalli. Samples were analysed in duplicate (n=2) and all concentrations are expressed per kg of dry sample weight. Analysed by HPLC-ICP-MS following extraction with water.

Paper V: A comprehensive analysis of water-soluble arsenicals in Icelandic macroalgae

Seaweeds are abundant in arsenic-containing sugars, where they may comprise over half of the total As (49). The mechanism of AsSugars production in macroalgae is still largely unknown but has been shown to vary between algae species, thallus section and season. This study reports the As speciation of brown, red and green macroalgae collected in Iceland across two sampling months (May and February).

AsSugars were found to account for the majority of the extracted As in all seaweed samples (>50%), and only small amounts of DMA (<LOQ-0.41 mg kg^{-1}) and MMA (<LOQ-0.07 mg kg^{-1}) were present. All samples contained low levels of toxic, iAs – where only in *Ulva intestinalis* (Chlorophyta) did concentrations exceed 1 mg kg^{-1}. The sulphate and sulfonate ribose derivatives were the dominant arsenicals in brown algae, whereas the phosphate ribose was generally the dominant As compound in red and green algae, Figure 11. The majority of seaweed species appear to have the ability to synthesise all four AsSugar derivatives, however it is unclear why some are produced to higher abundances than others. The glycerol and phosphate riboses were detected in every sample analysed, which might suggest that AsSug-gly is the starting point for AsSug-PO_4, which could be formed by AsSug-gly binding to phosphate-containing lipids in the cell membrane.

The production of AsSugars may be an efficient way of excluding As from the intracellular matrix, or they may serve as useful components in the cell wall similar to polysaccharides in the cell wall matrix, as extremely high levels of AsSugars (<100 mg kg^{-1}) can be tolerated by the algae with no ill effects.

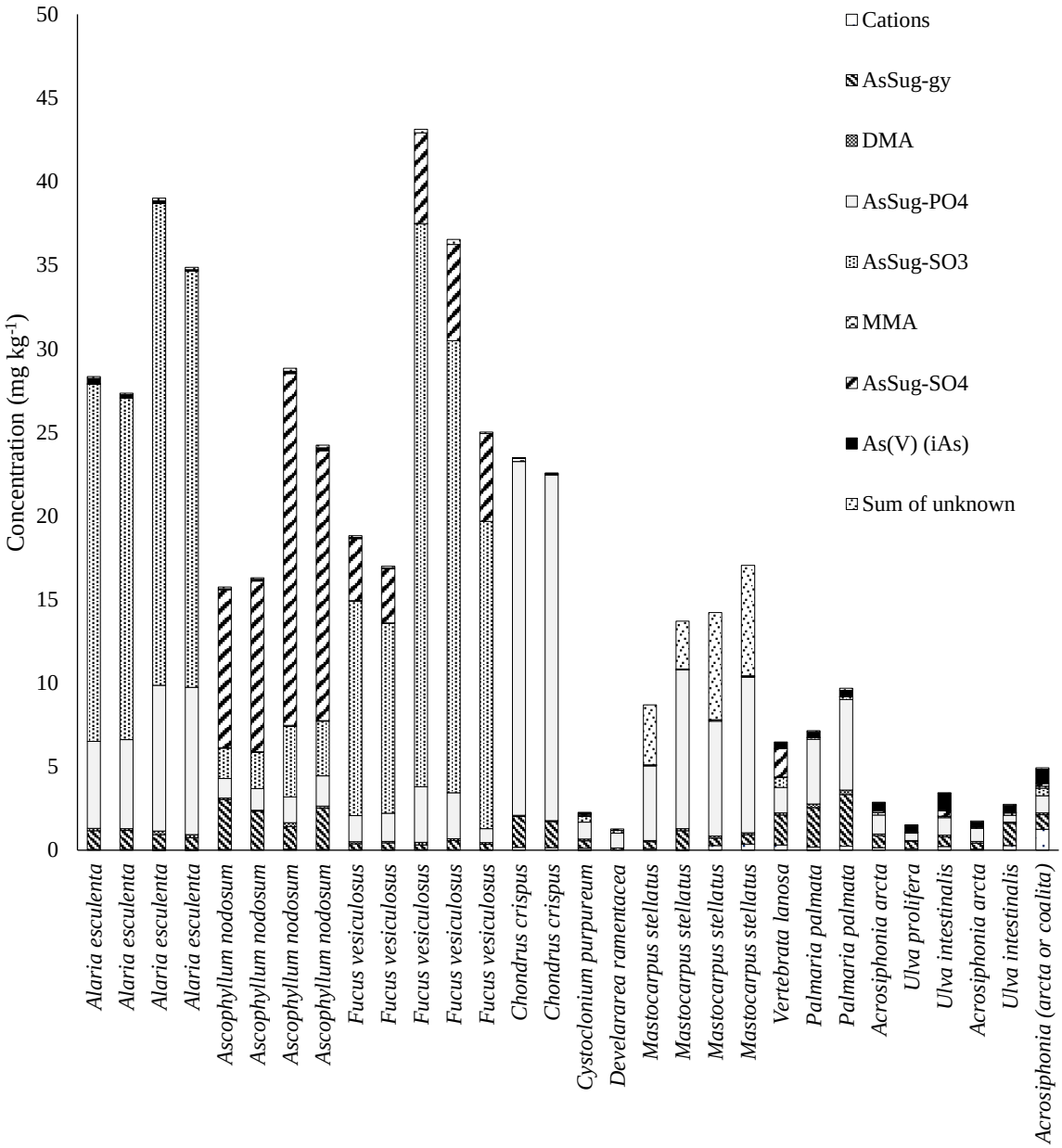


Figure 11 – The water-soluble composition of several species of seaweed collected in Iceland. Phaeophyta: *Ascophyllum nodosum*, *Alaria esculenta* and *Fucus vesiculosus*. Rhodophyta: *Cystoclonium purpureum*, *Devaleraea ramentacea*, *Vertebrata lanosa*, *Chondrus crispus*, *Mastocarpus stellatus*, *Palmaria palmata* and *Porphyra dioica*. Chlorophyta: *Ulva prolifera*, *Ulva intestinalis* and *Acrosiphonia arcta*.

Paper VI: Analysis of arsenic-containing lipids in Icelandic seaweeds

Seaweeds are known to sequester high levels of As - a potentially toxic metalloid - which may be metabolised to an array of complex lipid structures including hydrocarbons and phospholipids. The mechanisms of formation of these lipids are largely unknown but are thought to vary between brown, red and green seaweeds – where the latter two have been infrequently investigated. This study aims to investigate the distribution of AsLipids throughout marine macroalgae to help aid in understanding their formation.

In total, 35 different AsLipid species were identified across all samples, including 2 novel compounds (AsHC380 and AsFA398) identified in *A. nodosum*. AsSugPLs were found to dominate in brown algae where AsSugPL958 (C16:0/C16:0) was most abundant in all except *L. hyperborea* where AsSugPL984 (C16:0/C18:1) dominated, Figure 12. AsSugPLs were most concentrated in the reproductive tissues of the brown algae, where concentrations of AsSugPL958 ranged from 0.38-2.3 mg kg^{-1}. AsHC360 was also detected in relatively high concentrations in several samples where levels were highest in *F. vesiculosus* (0.16-0.64 mg kg^{-1}) and *Devaleraea ramentacea* (0.23 mg kg^{-1}).

Two AsSugarPhytol compounds were detected in the green seaweeds (AsSugPhytol546 and AsSugPhytol562), where the AsSugPhytol546 compound was generally the most abundant species found in *Ulva prolifera*, *Ulva intestinalis* and *Acrosiphonia arcta* but showed a dependence on sampling location for *U. intestinalis*. This arsenosugars phytol was also detected at trace levels in several samples of red seaweed including *Chondrus crispus* and *Porphyra dioica*, but it is unclear if this is produced by the algae or some epiphytic species that may reside on the surface of the seaweeds. These findings may have significant implications on the current understanding of As metabolism in Chlorophyta, as this is the first instance of AsSugarPhytols being reported in macroalgae.

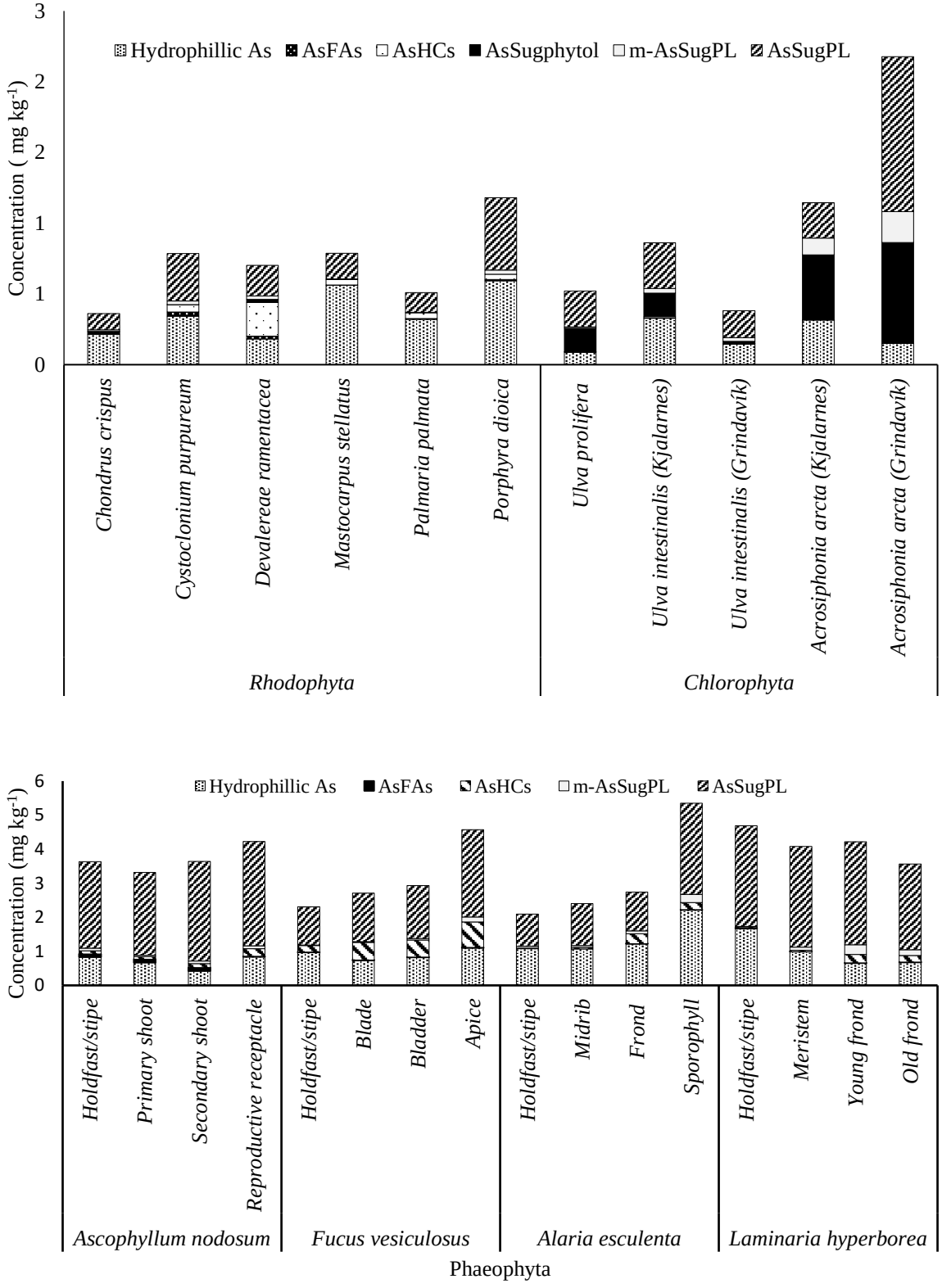


Figure 12 – The distribution of arsenic species based on retention time region, extracted using DCM/methanol in several samples of Rhodophyta, Chlorophyta and Phaeophyta collected in Iceland and analysed with HPLC-ICP-MS/ESI-MS and HPLC-qToF-MS. AsFAs: arsenofatty acids, AsHCs: arsenohydrocarbons, mAsSugPL: mono-acylarsenosugarphospholipid, AsSugPL: di-acylarsenosugarphospholipid.

3.2 Paper I – Potentially Toxic Elements in Icelandic Seaweeds

Rebecca Sim [A B], Liberty O'Brien [A C], Marta Weyer [A C], Karl Gunnarsson [D], Hildur I. Sveinsdóttir [A E] and Ásta H. Pétursdóttir [A*]

Author Affiliations:
[A] Matís, 12 Vínlandsleið, 113, Reykjavík, Iceland
[B] Faculty of Physical Sciences, University of Iceland, Dunhagi 7, 103, Reykjavík, Iceland
[C] The School of Natural and Computing Sciences, University of Aberdeen, Meston Walk, Aberdeen, Scotland
[D] Marine and Freshwater Institute, Fornubúðir 5, 220, Hafnarfjörður, Iceland
[E] Faculty of Food Science and Nutrition, Aragata 14, 102 University of Iceland, Reykjavík, Iceland

Abstract

Seaweed is becoming an increasingly popular food source due to its high mineral and antioxidant content, and the European demand for seaweed is expected to rise during the coming years. However, seaweeds may accumulate high levels of potentially toxic elements (PTEs) such as As, Cd and Pb, which may pose a threat to the safety of consumers. This study aims to investigate trends in PTEs in several species of brown, red and green seaweeds collected in Iceland, and how these elements may vary between thallus section, location, and season. Concentrations of As (3.8-265 mg kg^{-1}), Cd (0.06-18 mg kg^{-1}) and U (0.03-1.9 mg kg^{-1}) were found to be highest in the reproductive tissues of brown seaweeds during February, whilst certain green *spp.* contained the highest levels of Pb (0.02-1.8 mg kg^{-1}) and Fe (25-13607 mg kg^{-1}). Samples were found to contain relatively high levels of essential trace elements such as Fe and Zn but contained elevated levels of Cd – with 19 samples exceeding the maximum limit (ML) of 3 mg kg^{-1} in food supplements. As levels were also high where over half of samples exceeded the 40 mg kg^{-1} ML for As in seaweed-derived animal feed. Certain seaweed species (*Alaria esculenta*, *Fucus vesiculosus* and *Vertebrata lanosa*) grown in Iceland may be prone to high levels of Cd and thus, may not be suitable for human consumption in large quantities.

Introduction

Potentially toxic elements (PTEs) are naturally occurring throughout the environment, but anthropogenic activities such as agriculture and manufacturing have resulted in widespread contamination of marine ecosystems. Seaweeds are well known for their ability to accumulate these PTEs, and concentrations within cells can reach many orders of magnitude higher than that of the surrounding environment (194). Elements such as Mn, Fe and Mo are essential trace nutrients, and have important cellular roles, e.g., as enzyme co-factors. Selenium, for example, is an essential building block of the amino acids selenomethionine and selenocysteine. Nonetheless, all of these elements can be toxic in high concentrations, whereby they induce oxidative stress and disruption of normal cellular function (195). Conversely, non-essential elements such As, Cd, Hg and Pb can cause cellular toxicity at low levels and are especially harmful due to their persistence in the environment. Once inside cells these elements can cause damage to DNA, RNA, lipids, and proteins by increasing the production of reactive oxygen species (ROS) (196). ROS are produced as a product of normal

metabolism, and a delicate balance between these and protective antioxidants is usually maintained within cells (197). Disruption of this balance from exposure to PTEs is associated with various illnesses such as cancer, cardiovascular disease, and neurodegenerative diseases such as Alzheimer's disease (198). Seaweeds can also sequester a variety of rare-earth elements (REE) such as La – although these are less frequently reported (199,200). Nevertheless, these may become more relevant in the future as the manufacture of consumer electronics which require the use of REE increases. The ability of algae species to sequester this wide array of cations is due to the polysaccharide composition of the cell walls and brown seaweeds are particularly efficient at sequestering metals (29). As such, species like *Padina durviaelli* and *Sargassum muticum* have been investigated as pollution bio monitors and absorbent materials to clean up waste-water streams (201–203). The variations in metal(loid) accumulation capabilities between species is likely due to the differences in polysaccharide compositions of the cell wall, with each exhibiting different degrees of branching, cross linking, and differing functional groups. As well as variations between species, the abundance and composition of these polysaccharides has been shown to vary between season, location, and section of the thallus, and as such the PTE concentrations also have a dependence on these variables (204). These polysaccharides have a range of electronegative functional groups (i.e., sulphate, carboxyl, amide) that can complex a variety of metal and metalloid cations. Cell wall polysaccharides are often over produced by algal cells as a means of avoiding the accumulation of PTEs inside cells, however, low levels are still observed in the intracellular matrix (30). The PTEs are thought to be transported into cells by means of molecular or ionic mimicry, whereby the carrier proteins and ion channels are unable to distinguish between chemical species with similar radii or structure. This is thought to be the case for arsenate, which is analogous in structure to phosphate – a mineral essential for all life on earth (49).

Despite the potentially high concentrations of toxic elements it may contain, the popularity of seaweed for human consumption has shown considerable increase in recent years and is viewed as a sustainable food source for the growing population (205). Seaweed also contains an abundance of bioactive compounds (i.e., antioxidants) and essential trace- and macro-minerals that are often lacking in consumers diets (205,206). The demand for seaweed in Europe is expected to reach 8 million tonnes by 2030, and Iceland in particular is thought to have one of the largest underutilized seaweed resources in the world (207,208). Thus, if seaweed production in Iceland is to grow, it is crucial levels of PTEs are investigated to ensure consumer safety. This study aimed to investigate the effects of species, location, and anatomical section on concentrations of PTEs in species of Icelandic seaweed using principal component analysis (PCA). This dataset was used to determine if location and season have an impact on the levels of essential and non-essential elements in seaweeds grown in Iceland, and whether these elements may be in high enough concentrations to be of toxicological concern.

Experimental

Chemicals and Reagents

Ultrapure water (18 Ω m) was obtained from a milliQ water dispenser (Millipore) and used throughout unless otherwise stated. Nitric acid (HNO_3) (≥69%, TraceSELECT) was purchased from Honeywell (Germany) and hydrogen peroxide (≥30%, for trace element analysis) was supplied by Carl Roth (Germany). Standards for total metal analysis (As, Cd, Co, Cr, Cu, Fe, Hg, Mn, Ni, Pb, Se, Sn and Zn) were supplied by LabKings (Netherlands) and (Ba, La, Mo, Sb, Ti, V, U and Zr) Peak Performance (CPI international, USA). An

internal standard was prepared from a 1000 mg L^{-1} stock solution of indium also obtained from Peak Performance.

Samples and Reference Materials

A range of brown (Phaeophyta), red (Rhodophyta), and green (Chlorophyta) seaweed species were collected from two locations in Iceland during May 2021 and February 2022, Table 4. The Reykjanes peninsula is thought to have one of the largest abundances of seaweed resources in Iceland; therefore, this location was chosen for sampling along with another location North of Reykjavík. Fewer samples were collected in February due to the lack of availability of green and red species during the winter months. The samples were transported back to the lab in plastic bags and were stored at -18°C until preparation. Samples were rinsed sparingly with tap water to remove sand, and any epiphytic species were removed manually using a stainless-steel blade rinsed with ultrapure water and a citric acid/ethylenediaminetetraacetic acid (EDTA) solution.

Table 4 – The species of seaweed collected in May 2021 and February 2022.

	Grindavík (63°50 55.5 N 22°18 23.5 W)	**Kjalarnes (64°13 34.2 N 21°48 50.7 W)**
May 2021	*Fucus vesiculosus, Laminaria digitata, Ascophyllum nodosum, Alaria esculenta*	*Fucus vesiculosus, Laminaria digitata, Ascophyllum nodosum, Saccharina latissima*
	Palmaria palmata, Mastocarpus stellatus, Porphyra dioica	*Palmaria palmata, Devaleraea ramentacea, Cystoclonium purpureum, Chondrus crispus*
	Acrosiphonia arcta, Ulva intestinalis	*Acrosiphonia arcta, Ulva prolifera, Ulva intestnalis*
February 2022	*Fucus vesiculosus, Laminaria digitata, Ascophyllum nodosum, Saccharina latissima, Alaria esculenta*	*Fucus vesiculosus, Laminaria hyperborea, Ascophyllum nodosum*
	Palmaria palmata, Mastocarpus stellatus, Vertebrata lanosa, Chondrus crispus	*Palmaria palmata, Chondrus crispus*
	*Acrosiphonia sp. (arcta or coalita)**	-

*DNA results inconclusive on exact species.

Phaeophyta and Rhodophyta species with larger thalli were divided into anatomical sections such as meristem and young frond, Appendix F1. The holdfast and stipe sections were pooled due to the small amount of material. The reproductive sori in the *Laminaria* and *Saccharina* species were only present in the February samples. Chlorophyta specimens were not sectioned as they are mostly annual, and the old and new parts are indistinguishable from one another. DNA testing was additionally used to confirm the exact Chlorophyta species, Appendix T1, however the exact *Acrosiphonia spp*. of one sample could not be determined and will be referred to as *Acrosiphonia sp.*. DNA testing was not required for other samples as all have easily identifiable morphologies (i.e., *F. vesiculosus* bladders on either side of midrib). After sectioning, parts from multiple thalli were pooled and freeze-dried until constant mass. Samples were then ground to a fine powder using an IKA tube mill (Germany) and stored in airtight containers at room temperature, away from sunlight.

Certified reference material 7405-b (Hijiki) was purchased from the National Meteorology institute of Japan and TORT-3 (lobster hepatopancreas) was supplied by the National research Council of Canada. Both reference materials were analysed alongside each batch of samples.

Sample preparation

Approximately 200 mg of sample material was weighed into quartz digestion vials in triplicate before adding 1 mL of nitric acid and 1 mL of hydrogen peroxide. The digestion procedure was performed using an Ultrawave microwave digestion system (Milestone, Italy). A loading pressure of 40 bars was added to the reaction chamber of the Ultrawave system, before the temperature was increased to 240°C over a period of 20 minutes. The reaction chamber was held at 240°C for 10 minutes, which was then followed by a 15-minute cool-down period. The digests were then quantitatively transferred to 50 mL falcon tubes and made up to 50 mL with water. The solutions were then analysed directly, or stored at -18°C, and subsequently defrosted before analysis.

Analyte quantification

The determination of total element concentration was carried out using an Agilent 7900 ICP-MS with octopole collision cell and Agilent SPS 4 autosampler, Table 5. The analysis was performed in helium gas mode to eliminate isobaric interferences and a 1000 µg L^{-1} indium internal standard in 1% HNO_3 was introduced post-autosampler to monitor fluctuations in the signal response. An external calibration in the range 0 – 200 µg L^{-1} was used to measure concentrations of elements in the digested samples. The ICP-MS was tuned daily to minimise interferences from doubly charged species and oxides.

Reference materials hijiki 7405-b and TORT-3 were run in triplicate alongside each batch of samples. The limit of detection (LOD) and limit of quantification (LOQ) were respectively calculated as 3.3 and 10 multiples of the standard deviation of the element concentrations in 10 digestion blanks, Appendix T2.

Table 5 – The ICP-MS operating conditions.

ICP-MS operating conditions	
RF power	1550 W
RF matching	1.20 V
Plasma gas	15 L min^{-1}
Carrier gas	1.07 L min^{-1}
Make-up gas	0.80 Lmin^{-1}
Spray chamber temperature	2°C
Octopole collision cell	Pressurized, He gas (5 mL min^{-1})
Integration time	1000 ms
Peak pattern	3
Replicates per analysis	3
Isotopes monitored	Ti^{47}, Cr^{52}, Mn^{55}, Fe^{56}, Co^{59}, Ni^{60}, Cu^{63}, Zn^{66}, As^{75}, Zr^{90}, Mo^{95}, Cd^{111}, In^{115} (internal std), Sb^{121}, Ba^{137}, Hg^{201}, Pb^{208}

Data analysis

Scaling of the data, followed by mean centring was performed in Microsoft Excel. JMP Pro 16 was used to perform principal component analysis (PCA) of the data using a restricted maximum likelihood (REML) estimation method to account for missing values, i.e., element concentrations that were below the LOQ of the method.

Results and Discussion

Quality control

The reference materials Hijiki 7405-b and TORT-3 showed generally good agreement with the certified values for the elements measured, Table 6.

Table 6 – The total element concentrations in Hijiki and TORT-3.

	Hijiki 7405-b (mg/kg) (n=9)	**Certified value (mg/kg)**	**TORT-3 (mg/kg) (n = 9)**	**Certified value (mg/kg)**
Vanadium (V)	1.29 ± 0.13	-	9.76 ± 0.87	9.1 ± 0.4
Chromium (Cr)	5.14 ± 0.43	5.5	2.03 ± 0.18	1.95 ± 0.24
Manganese (Mn)	22.04 ± 1.70	22.6 ± 0.5	16.45 ± 1.48	15.6 ± 1.0
Iron (Fe)	235 ± 18.29	210	176.05 ±19.64	179 ± 8
Cobalt (Co)	1.75 ± 0.21	1.9	1.19 ±0.14	1.06*
Nickel (Ni)	2.70 ± 0.24	3	5.47 ± 0.50	5.3 ± 0.24
Copper (Cu)	3.82 ± 0.48	4.48 ± 0.12	455 ± 54.2	497 ± 22
Zinc (Zn)	14.29 ± 1.64	13.6 ± 0.5	149 ± 13.3	136 ± 6
Arsenic (As)	51.01 ± 3.92	49.5 ± 1.0	71.5 ± 6.02	59.5 ± 3.8
Selenium (Se)	<LOQ	-	12.28 ± 1.26	10.9 ± 1.0
Molybdenum (Mo)	0.28 ± 0.03	-	3.68 ± 0.34	3.44 ± 0.12
Cadmium (Cd)	1.22 ± 0.11	1.25 ± 0.04	45.18 ± 4.49	42.3 ± 1.8
Tin (Sn)	0.04 ± 0.01	-	0.02 ± 0.00	0.029*
Mercury (Hg)	0.04 ± 0.02	-	0.34 ± 0.07	0.292 ± 0.022
Lead (Pb)	0.24 ± 0.04	0.2	0.25 ± 0.03	0.225 ± 0.018
Lanthanum (La)	0.16 ± 0.02	-	3.37 ± 0.41	-
Uranium (U)	1.03 ± 0.08	-	0.12 ± 0.01	-

*Information value.

Overview of results

There were large variations in Fe concentrations between all samples, Table 7, where *Chlorophyta spp.* generally had highest levels followed by *Chondrus crispus*, and the holdfast/stipe sections of the Phaeophyta. These samples contained concentrations of Fe in the order of thousands of mg kg^{-1} and so 1-2 g of this seaweed as dried material would provide the recommended daily intake for adult men (209). Other essential trace elements such as Zn, Co and Mn were most abundant in *Palmaria palmata*, and the inclusion of this seaweed in the diets of those who do not consume meat (i.e., vegan) may be beneficial to increase intake of these elements. The highest levels of V were also found in *P. palmata* samples collected in winter (134-334 mg kg^{-1}). V is not considered an essential element for humans, and the maximum tolerable intake of V is 1.8 mg a day for adults. Therefore, it is likely the consumption of *P. palmata* in excess of approximately 10 g would exceed this. *Vertebrata lanosa* contained the highest concentration of Se, although levels were relatively similar across classes and species, ranging from <LOD-1.3 mg kg^{-1}.

In relation to food safety regulations set by the European Commission, all samples contained well below the maximum level (ML) of 0.1 mg kg^{-1} set for Hg in food supplements (182), Table 7. Over half of the samples analysed exceeded the ML for As in feed derived from seaweed (210), with the brown seaweed species typically containing the highest concentrations. Whilst the majority of this As may be in the form of arsenic-containing riboses for most seaweed species, in *Laminaria digitata*, carcinogenic inorganic As (iAs) may account for over 50% of the total As (37,49,61). The levels of Cd were high in some species of seaweeds, namely *Alaria esculenta*, *F. vesiculosus* and *V. lanosa*, where 25 samples exceeded the ML of 3 mg kg^{-1} for seaweed-derived food supplements (182). In particular, the *A. esculenta* sporophyll of samples collected at Grindavík in February contained the highest concentrations of Cd (18 mg kg^{-1}) and exceeded this ML 6-fold. Concentrations of Pb were generally low, where only a single *P. palmata* sample and *Acrosiphonia sp*. – both of which were collected in February - exceeded 1 mg kg^{-1}. Additionally, all samples contained between 0.01-1.1 mg kg^{-1} of Sn which would mean the consumption of these seaweeds would not contribute significantly to the provisional tolerable weekly intake of 14 mg kg^{-1} of body weight (211). With regards to more uncommonly reported elements, La concentrations in all samples were not high enough to be of toxicological concern, and concentrations of U with levels over 1 mg kg^{-1} were only found in Fucales – suggesting this species may contain polysaccharides with an affinity for U.

Table 7 – The range and median value of total element concentrations measured in Phaeophyta, Rhodophyta and Chlorophyta. Minimum-maximum values (**Median value**).

Element	Phaeophyta (mg kg^{-1})	Rhodophyta (mg kg^{-1})	Chlorophyta (mg kg^{-1})
Vanadium (V)	0.32-21 (**1.7**)	1.4-334 (**19**)	9.4-28 (**26**)
Chromium (Cr)	0.14-48 (**0.89**)	0.43-21 (**3.6**)	2.7-26 (**10**)
Manganese (Mn)	1.1-312 (**19**)	8.1-980 (**34**)	36-183 (**153**)
Iron (Fe)	25-5521 (**193**)	169-3310 (**651**)	2247-13607(**7474**)
Cobalt (Co)	0.05-6.2 (**0.83**)	0.19-7.8 (**0.85**)	0.9-6.2 (**4.1**)
Nickel (Ni)	0.21-26 (**2.4**)	3.6-77 (**9.5**)	3.3-26 (**12**)
Copper (Cu)	0.74-32 (**4.8**)	3.1-46 (**18**)	5.0-110 (**22**)
Zinc (Zn)	9.5-129 (**31**)	13-303 (**48**)	8.5-39 (**28**)
Arsenic (As)	26-265 (**61**)	5.0-49 (**11**)	3.8-9.5 (**6.3**)
Selenium (Se)	<LOQ-0.52 (**<LOQ**)	<LOQ-1.3 (**0.37**)	0.26-1.3 (**0.80**)
Molybdenum (Mo)	0.28-12 (**0.74**)	0.45-6.1 (**1.4**)	0.48-1.8 (**0.88**)
Cadmium (Cd)	0.06-18 (**1.6**)	0.28-7.0 (**0.78**)	0.09-0.74 (**0.36**)
Tin (Sn)	<LOQ-1.5 (**0.02**)	0.01-0.2(**0.04**)	0.02-0.12 (**0.09**)
Lanthanum (La)	0.01-1.3 (**0.06**)	0.04-1.8 (**0.41**)	0.33-1.6 (**1.1**)
Mercury (Hg)	<LOQ-0.13 (**<LOQ**)	<LOQ-0.04 (**<LOQ**)	<LOQ-0.03 **(<LOQ**)
Lead (Pb)	<LOQ-0.61 (**0.02**)	0.02-0.45 (**0.14**)	0.08-1.8 (**0.31**)
Uranium (U)	0.07-1.9 (**0.48**)	0.03-0.19 (**0.07**)	0.06-0.17 (**0.14**)

Large variations in the total concentrations of elements were observed between classes of seaweed, and as such grouping could be seen after application of the PCA, Figure 13. Brown seaweeds generally contained the highest concentrations of As, Cd and U, and lowest concentrations of Pb and Mn. High levels of As are typical of Laminariales, with one Icelandic study reporting concentrations in the range of 93-116 mg kg^{-1} for *Saccharina latissima* and *A. esculenta* fronds (4). Phaeophyceae biomass is extremely fast-growing and requires high levels of minerals to facilitate this growth (212), thus it is not surprising that they contain high levels of As and Cd if both are taken up by P and Ca transporters respectively (49,213). The uptake of U is largely unknown, but is expected to occur through large anionic transporters such as bicarbonate or phosphate (214), which may explain the acute angle between the As-U loadings, Figure 13. Hg, Mo, and Zn had little to no effect on the grouping but were responsible for intra-class variations within the brown algae samples. The red seaweed species exhibited large intra-class variations in many elements but most notably, Cu and Ni, where concentrations ranged from 5.65-62.1 mg kg^{-1} and 4.14-117 mg kg^{-1} respectively, where *C. crispus* typically contained the highest levels of Ni and brown algae holdfast/stipes the most Cr. The green seaweeds contained high levels of Cr and Fe, a trend which has been previously reported in *Enteromorpha spp.* (215) but low levels of toxic elements As, Cd, Hg, and Pb - potentially due more specific uptake pathways in Chlorophyta.

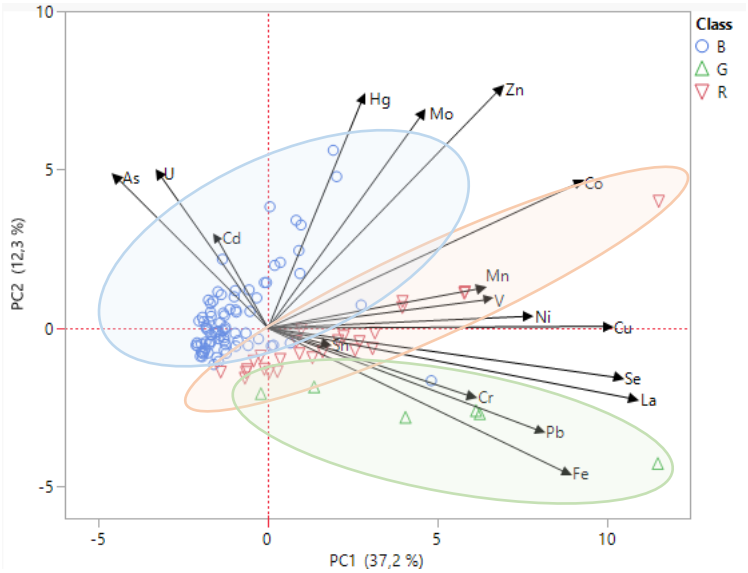


Figure 13 – The variable loadings and scores of all samples as a biplot with the first two PC as axes. B (blue), G (green) and R (red) denote samples from the Phaeophyta, Rhodophyta and Chlorophyta classes of seaweed respectively.

When considering correlations between elements, Figure 14, As appears to correlate negatively with a number of elements (V, Cr, Mn, Fe, Co and Cu), suggesting that As accumulation may impair the uptake of essential metals. This has been demonstrated in terrestrial plants whereby As accumulation was found to reduce Zn, Cu and Fe uptake, as well as chlorophyll concentration and plant growth (216,217). The same appears to be true for Cd and U to a lesser extent, where there are negative correlations between these two elements and V, Fe, Cu and Se. Cd has been shown to inhibit the biosynthesis of proteins in algae, therefore the demand for the metal ions required to produce metalloproteins would be expected to decrease under exposure to Cd (218). There also appears to be a strong positive correlation between La-Fe, and La-Se, potentially indicating there may be competitive uptake between these ions.

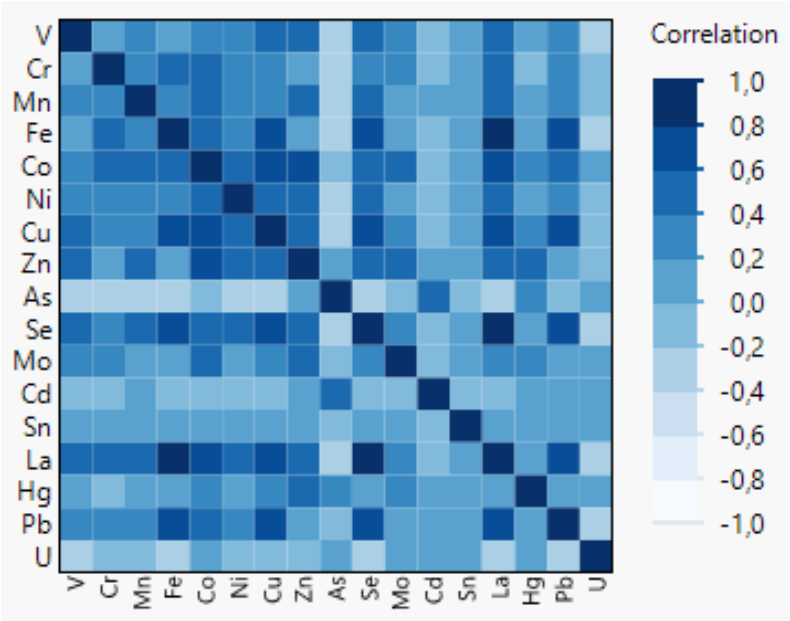


Figure 14 – Correlation colour map displaying correlations between elements estimated by Row-wise method. Darkest blue indicates a strong positive correlation between two elements, and lightest blue indicates a strong negative correlation.

Seaweed Class

Samples were additionally grouped by class (e.g., Phaeophyta or Rhodophyta) prior to the analysis, Figure 15, to investigate trends between seaweed species. The analysis was not performed for the samples of green seaweed as the number of specimens was too low to achieve a positive correlation matrix (n=7). Briefly, specimens of *Ulva intestinalis* contained both the highest and lowest As concentrations (3.8 and 9.5 mg kg^{-1}) of all Chlorophyta, highest levels were found in samples collected at Kjalarnes. *Acrosiphona* samples contained the highest concentrations of Cd (0.45-0.74 mg kg^{-1}) and Mn (150-161 mg kg^{-1}).

F. vesiculosus samples generally contained higher levels of U (0.24-2.3 mg kg^{-1}) and Mn (28-295 mg kg^{-1}) than the other brown species, as well as high levels of Cd (1.1-12 mg kg^{-1}) – where the highest Cd concentrations were detected in February samples from Grindavík. All species from the Laminariales order, *A. esculenta*, *L. digitata*, *Laminaria hyperborea* and *S. latissima* approximately group in the left-most quadrants, Figure 15 (left), characterised by low concentrations of essential elements (i.e., Cu, Fe, Mo, and Zn) and large intra-species variations in As and Cd. *Ascophyllum nodosum* samples displayed the most intra-species variation, particularly with regards to Cr and Zn where levels ranged from (0.38-34 mg kg^{-1})

and (20-129 mg kg^{-1}) respectively and were most concentrated in the holdfast/stipe tissues. As previously mentioned, *P. palmata* samples contained a wide range of concentrations of V (56-334 mg kg^{-1}), but also exhibited a large degree of variation between samples for the majority of elements, Figure 15 (right). *P. palmata* samples tended to group in the lower left quadrant with other Palmariales *sp. Devaleraea ramentacea* and *Mastocarpus stellatus* (Gigartinales). *C. crispus* had the highest levels of As and Ni, whilst *Porphyra dioica* was generally lowest in essential elements such as Fe, Zn, Se, and Mo.

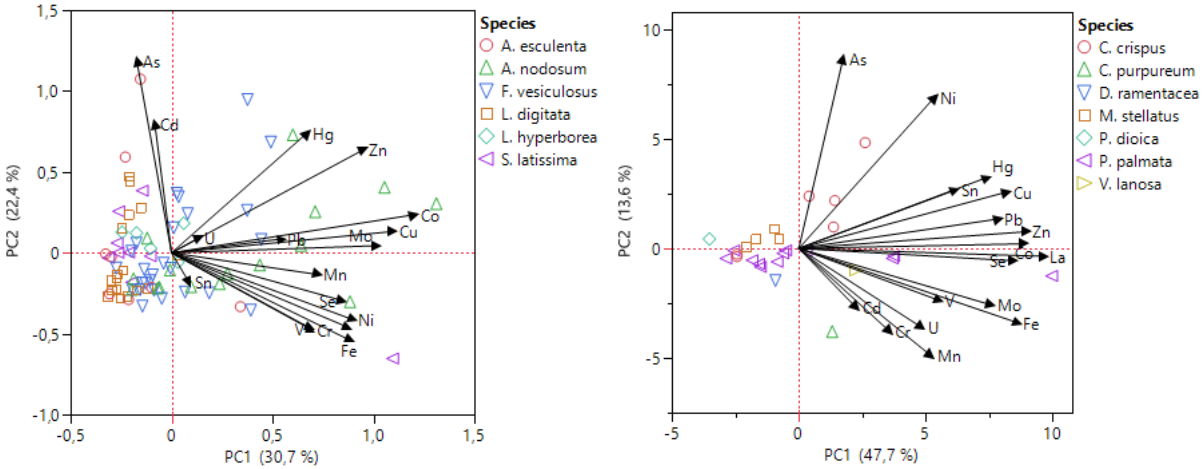


Figure 15 – (left) The variable loadings and scores of Phaeophyta samples as a biplot with the first two PC as axes. (right) The variable loadings and scores of Rhodophyta samples as a biplot with the first two PC as axes

Season

When samples were grouped by season before PCA, the concentrations of As, U and Cd, as well as Zn, Mo, Co, and Cu were generally higher during February, Appendix F2. Many brown seaweed species grow during this time of year when nitrate availability is not a limiting factor for growth (219), and so higher levels of essential elements and those taken up by ionic mimicry would be expected at this time of year. The concentrations of Cr and Fe appear to be higher in May, but this trend is likely driven by the green seaweed *spp*. collected during that sampling month.

Thallus section

Brown samples were also grouped by anatomical function, e.g., support for stipe and photosynthesis for frond, Appendix T3 and Appendix F3. Although there was no distinct grouping between the different biological functions, those with high biological activity (photosynthesis and reproduction) appear to accumulate higher levels of As, Cd and U, whereas sections associated with support accumulated higher levels of Cr and Fe – perhaps due to the age of this thallus section.

Location

Location was not found to be a significant contributor to the difference in PTE concentrations between samples, suggesting both sampling locations were relatively uncontaminated sites. However, samples from the Grindavík location typically had higher levels of As and Cd, a trend which was even more apparent during February. This can likely be attributed to the

differences in geology, as basalt rock is abundant in the south of Iceland where Grindavík is located (220). Basalt rock is known to contain As and Cd, but additionally high levels of Hg, however, the seaweed species may have a low affinity for Hg cations in the presence of other metals (220,221). Additionally, the recent volcanic activity in the Reykjanes peninsula may have contributed to the high levels of Cd and As observed in Phaeophytas as these events are known to release potentially toxic elements into the surrounding environment. Higher levels of Cd in seaweeds collected in Iceland relative to those in Norway and France have also previously been reported (222). Brown algae collected at Kjalarnes had particularly high concentrations of Cr, Ni, Fe and Zn localised in the holdfast/stipe section of the thallus. These high levels may be explained by the proximity of the sampling location to a major road as these elements are often associated with non-exhaust emissions (NEE) and would likely accumulate in the oldest section of the thallus. Therefore, brown seaweed samples from both locations were additionally analysed for elements associated with NEE (Ba, Sb, Ti and Zr). Briefly, levels of Sb were low in all samples (<LOQ-0.18 mg kg^{-1}) and *A. nodosum* generally contained the highest concentrations. Ti and Zr followed similar trends, where both were most concentrated in the older parts of the thallus (holdfast/stipe and decaying frond) and highest during May at Kjalarnes. Trends in Ba concentrations were not as distinct; In the *Laminaria spp*. levels of Ba were highest in the older frond sections and holdfast/stipe with similar concentrations found across both locations, but in *F. vesiculosus* Ba concentrations were higher at Kjalarnes than at Grindavík. In *A nodosum*, there were increases in Ba concentrations across all sections between May and February, but relatively similar levels between locations. These results may have interesting implications for the use of seaweeds as bio-monitors for NEE and other PTE pollution, i.e., specific seaweed species may have to be selected for certain elements, and thallus sections must be considered individually.

Conclusion

In general, brown seaweeds contained the highest levels of As, Cd and U, and these elements were found to be in higher abundance in reproductive tissues of samples during the winter. As concentration also appeared to be negatively correlated with that of several essential elements (Mn, Fe, Co and Cu) suggesting As accumulation may have negative effects on nutrient uptake. Green seaweeds were found to contain the highest concentrations of Cr and Fe, but seasonal comparisons could not be drawn due to the lack of specimens available during the winter months. Seaweed grown in Iceland was found to be relatively high in essential trace elements such as Cu, Zn and Fe, and all samples contained low levels of toxic Hg – where all were below the ML of 0.1 mg kg^{-1} allowed in food supplements. With regards to other toxic elements, over half of the samples contained levels of As higher than the ML of 40 mg kg^{-1} for seaweed-derived animal feed, and 25 samples exceeded the 3 mg kg^{-1} ML for Cd in food supplements, where 1 sample was found to exceed this limit 6-fold. Concentrations of Cd were also found to be elevated in commercially relevant *spp*. when compared with those grown in France and Norway – in particular *A. esculenta* and *P. palmata* – and may attributed to the abundance of basalt rock found in Iceland. *P. palmata* also contained high concentrations of V - particularly during February – which may be of toxicological concern if consumed in large quantities.

3.3 Paper II – Inorganic Arsenic in Seaweed: a Fast HPLC-ICPMS Method without Co-elution of Arsenosugars

Rebecca Sim [A B *], Marta Weyer [C] and Ásta H. Pétursdóttir [A]

Author Affiliations:
[A]Public Health and Food Safety, Matís, Vínlandsleið 12, 113, Reykjavík, Iceland
[B]Faculty of Physical Sciences, Dunhagi 3, 107, University of Iceland, Reykjavík, Iceland
[C]Department of Chemistry, University of Aberdeen, Meston Walk, Aberdeen, Scotland

Abstract

Seaweed is becoming increasingly popular in the Western diet as consumers opt for more sustainable food sources. However, seaweed is known to accumulate high levels of arsenic – which may be in the form of carcinogenic inorganic arsenic (iAs). Here we propose a fast method for the routine measurement of iAs in seaweed using HPLC-ICP-MS without co-elution of arsenosugars that may complicate quantification. The developed method was optimised using design of experiment (DOE) and tested on a range of reference materials including TORT-3 (0.36 ± 0.03 mg kg^{-1}), DORM-5 (0.02 ± 0.003 mg kg^{-1}), DOLT-5 (0.07 ± 0.007 mg kg^{-1}). The use of nitric acid in the extraction solution allowed for the successful removal of interferences from arsenosugars by causing degradation to an unretained arsenosugar species, and a recovery of 99 ± 9% was obtained for iAs in Hijiki 7405-b when compared with the certified value. The method was found to be suitable for high throughput analysis of iAs in a range of food and feed matrices including *Asparagopsis taxiformis* seaweed, grass silage and insect proteins, and offers a cost effective, fast, and robust option for routine analysis that requires minimal sample preparation. The method may be limited with regards to the quantification of dimethylarsinate (DMA) in seaweed, as the acidic extraction may lead to overestimation of this analyte by causing degradation of lipid species that are typically more abundant in seaweed than other marine matrices (i.e., arsenophospholipids). However, the concentrations of DMA quantified using this method may provide a better estimation with regards to exposure after ingestion and subsequent digestion of seaweed.

Introduction

Arsenic is a naturally occurring element released from the erosion of mineral-bearing rocks and anthropogenic activities such as smelting and mining. The toxicity of arsenic-containing compounds is highly dependent on chemical speciation and oxidation state, with the inorganic species being classified as carcinogenic by the International Agency for Research on Cancer (IARC) (42). The sum of the inorganic species, arsenite and arsenate, is often referred to as inorganic arsenic (iAs) and exposure has been linked to bladder, lung, and skin cancers, as well as diabetes, cardiovascular disease, and pulmonary disease (51,52,54,55,223–226). Ingestion of food or drinking water is the major exposure route to iAs, with rice and cereals being significant contributors to dietary intake (64). Seafood may also contribute to dietary intake depending on cultural and geographic differences in consumption (64,227). The speciation analysis of seafood matrices can be particularly

challenging due to the number of components that require separation from iAs, such as arsenobetaine (AB), arsenosugars (AsSugars) and small, methylated arsenicals like dimethylarsinate (DMA) and monomethylarsonate (MMA). Seaweeds are widely known to contain high levels of arsenic - predominantly in the form of AsSugars where they may comprise over 85% of the water-soluble As (228). High concentrations of these sugars can hinder the quantification of iAs species by coelution during chromatographic analyses (i.e., anion exchange). Sample introduction techniques such as hydride generation (HG) have been developed to overcome this issue but involve the use of strong oxidising agents such as $NaBH_4$ under acidic conditions which may be hazardous - as well as additional apparatus (229). Alternatively, separation of these closely eluting analytes using anion exchange chromatography often requires long runtimes or the use of a mobile phase gradient (108,110). However, previous studies have shown that using a low concentration of a protic acid in the extraction step can cause hydrolysis of the R-group of the ribose ring, producing an AsSugar that elutes in the void volume of the anion exchange column, thus simplifying the chromatography (112). The use of nitric acid in the extraction solution has been successfully applied to seafood and rice matrices, and has also been shown to increase the recovery of iAs from seaweed (49,230).

Additionally, design of experiment (DOE) can be used to optimise chromatographic separation and runtime, whereby a systematic approach is taken to model the effects of a large number of factors in fewer runs. DOE can be used for method development, optimisation or robustness testing, and has previously been applied to optimise the extraction of arsenic species in seafoods, rice and soils (110,231,232), however, is most commonly used with pharmaceutical analyses (233). Optimisation of extraction parameters such as time, volume of solvent and temperature have been previously reported, as well as instrumental variables such as flow rate, column temperature and pH (110,234).

Whilst in 2015 the EU introduced regulations for maximum levels of iAs allowed in rice and derived products, similar legislation is yet to be proposed for seaweed for human consumption. France is currently the only European country to have implemented nationwide maximum levels for iAs, currently set at 3 mg kg^{-1} for seaweed condiments, and in Australia and New Zealand this limit is even lower at 1 mg kg^{-1}. Additionally, an updated risk assessment by the European Food Safety Authority (EFSA) in 2024 determined that current exposure levels to inorganic arsenic raised health concerns for adult consumers – highlighting the need for the investigation of iAs levels in foods for human consumption (235). Seaweed poses a unique challenge due to the large variations in iAs concentration between species. For example, in *Laminaria spp.* the total As content can reach upwards of 100 mg kg^{-1}, and in *Laminaria digitata* specifically iAs can comprise over 50% of this total, however, in *Laminaria longicruris* iAs has been shown to account for less than 1% of the total As (37,49,236). As such, the implementation of maximum total As limits would not be nearly specific enough to assess the full toxicological risk posed by the consumption of seaweed.

There is an increasing demand for sustainable, alternative food sources in an effort to reduce the effects of climate change, and as such seaweed production is now the fastest growing aquaculture sector (237). Besides being a popular health food with a high nutrient content, seaweed is also finding increased uses in the food production, cosmetics, and pharmaceutical industries (238–240). Although the current iAs levels in the majority of seaweed species pose no imminent threat to human health, outliers such *Laminaria digitata* or *Sargassum fusiforme*

(hijiki) mean it is crucial that levels are monitored, and occurrence data is gathered as the consumption of seaweed and derived products becomes more widespread (62). Here, we propose a fast chromatographic method for the routine measurement of iAs in seaweed suitable for high throughput analysis and use across a wide range of food and feed matrices. DOE was used to optimise the acid concentration and chromatographic parameters to achieve an acceptable separation of As(V) from other analytes in under 7 minutes.

Experimental

Chemicals and Standards

All chemicals used were of analytical grade or better and Milli-Q water from a Millipore water dispending system (MilliPore, France) was used throughout unless otherwise stated. Hydrogen peroxide (H_2O_2) (for trace analysis, ≥30%) and nitric acid (HNO_3) (ROTIPURAN Supra, 69%) were supplied by Supelco (Sigma-Aldrich, France) and Carl Roth (Germany) respectively. Ammonium carbonate was obtained from BDH (UK) and methanol (HPLC grade) was purchased from Honeywell (USA). Arsenate standards used for external calibration of the iAs analysis were prepared from a stock solution of disodium hydrogen arsenate heptahydrate (98.5%) supplied by Argus-Chemicals (Italy) dissolved in Milli-Q water. DMA and MMA standards used for spiking experiments were prepared from dimethylarsinic acid disodium salt (100%) supplied by Argus-chemicals (Italy) and disodium methyl arsonate hexahydrate (99.5%) purchased from Chem-Service (USA), respectively. Standards for total As analysis were prepared from 1000 mg L^{-1} As standards supplied by LabKings (Netherlands). Indium and germanium internal standards were prepared from 1000 mg L^{-1} stock solutions purchased from Peak Performance (CPI International, USA).

Sample and reference materials

A range of brown (Phaeophyta), red (Rhodophyta) and green (Chlorophyta) macroalgae were analysed to ensure the method could be applied to all species of seaweed. During May 2021, samples of *Cystoclonium purpureum* (Rhodophyta) and *Palmaria palmata* (dulse, Rhodophyta) were collected from the intertidal zone of a beach near Kjalarnes, Iceland, and *Porphyra dioicaa* (Rhodophyta) was collected near Grindavík, Iceland. In February 2022, *Fucus vesiculosus*, *Laminaria digitata*, *Saccharina latissima* and *Ascophyllum nodosum* (at least 10 individuals per species, all Phaeophyta) were collected near Grindavík, Iceland. The samples were transported to the lab in sterile plastic bags, where they were kept refrigerated at 2°C prior to cleaning. Any epiphytes were manually removed from the seaweed using a stainless-steel knife rinsed with Milli-Q water, and samples were rinsed sparingly with tap water to remove sand. Samples were freeze-dried (Christ, Germany) to constant mass before being milled to a fine powder and stored at room temperature away from sunlight. Brown macroalgae have large thalli with easily distinguishable parts, and so the species of brown seaweed collected were dissected into anatomical parts before drying, e.g., meristem, frond, reproductive tissues. A detailed description of the sectioning can be found in Appendix F1.

A sample of the tropical seaweed *Asparagopsis taxiformis* (Rhodophyta) was harvested in June 2020, in Azores. A grass silage used for livestock feed (oven-dried, Iceland), freeze-

dried mussels (Iceland) and white rice were milled to a fine powder (IKA tube mill, China) before being included in the analysis. Certified reference materials DOLT-5 (dogfish liver), DORM-5 (fish protein), TORT-2 (lobster hepatopancreas), TORT-3 (lobster hepatopancreas), BFLY-1 (blackfly), VORM-1 (worm) and CAME-1 (canola plant) were obtained from the National Research Council of Canada. Hijiki (7405-b, Phaeophyta) which is certified for As(V) was purchased from the National Meteorology Institute of Japan. *Ulva lactuca* (CRM BCR-279, Chlorophyta) was obtained from the Institute of Reference Materials and Measurements in Belgium. A sample of *A. nodosum* collected in Iceland was used as an in-house reference material for the identification of AsSugars.

Sample preparation

Total As. Samples were also analysed for total As. Briefly, 200 mg of material was added to quartz digestion tube with 1 mL HNO_3 and 1 mL H_2O_2 and digested using and Ultrawave microwave digestion system. The digestion mixture was then quantitatively transferred to 50 mL falcon tube and made up to 50 mL with Milli-Q water. A 1 mL of the supernatant from the iAs method was also digested in order to calculate the column recovery.

iAs. Briefly, 100 mg of freeze-dried ground material was added to quartz digestion tubes with 10 mL of 1% (v/v) HNO_3 and 3% (v/v) H_2O_2 solution. H_2O_2 was included in the extraction solution to oxidize As(III) to As(V) so the sum of both inorganic arsenic species could be quantified as As(V), without conversion of organoarsenic species to iAs (230).The extraction was performed using an Ultrawave microwave digestion system (Milestone, Italy), in which the samples were extracted for 40 min at 90°C before a 15 min cool-down period. The extracts were then transferred to 50 mL falcon tubes and centrifuged (Eppendorf, Switzerland) at 4000 rpm for 15 min. A 1 mL aliquot was transferred to a microcentrifuge tube and centrifuged at 15000 rpm (Heraeus, US). The final supernatant was then generally analysed directly using HPLC-ICP-MS but was diluted 1:5 with the extraction solution for CRM hijiki (7405-b) and *L. digitata* frond and sori.

Analyte quantification

Total As. Total arsenic measurements were performed using an Agilent SPS4 autosampler and Agilent 7900 ICP-MS with octopole collision cell in He gas mode. An external calibration in the range 0-200 µg L^{-1} was used for quantification and standards were prepared by serial dilution in 2% HNO_3. Tuning of the ICP-MS was performed daily, and a 1000 µg L^{-1} indium internal standard was introduced continuously during analysis through a T-piece.

iAs. Quantification of iAs was performed using an Agilent Infinity II 1290 HPLC coupled to an Agilent 7900 ICP-MS with the octopole collision cell pressurized with He gas, Table 8. The ICP-MS was manually tuned before each use with a 50 µg L^{-1} As solution. Separation of the As species was achieved using a Hamilton PRP-X100 column (250 x 4.1 mm, 10 µm) and corresponding guard column under an isocratic elution with 60 mM ammonium carbonate buffer (3% MeOH). An internal standard containing 50 µg L^{-1} Ge was continuously added post-column during the analysis. The method LOD and LOQ were calculated as 3.3 and 10 times the standard deviation of ten method blanks spiked with 0.1 µg L^{-1} As(V), multiplied by an average dilution factor. Data acquisition and manual integration of peaks was performed using the Agilent Masshunter software. All calculations were performed in excel.

Table 8 – The instrument operating parameters for the ICP-MS method used for the quantification of total As and the HPLC-ICP-MS method used for the quantification of iAs.

Instrument operating parameters	
ICP-MS settings	Agilent 7900 ICP-MS
RF power	1550 W
RF matching	1.26 V
Plasma gas flow	15.0 L/min
Carrier gas flow	1.07 L/min
Make-up gas flow	0.8 L/min
He gas flow	5.0 L/min
Spray chamber temperature	2 °C
Isotopes monitored	As^{75}, In^{115} (internal standard)
HPLC-ICP-MS settings	Agilent 1290 Infinity II HPLC and Agilent 7900 ICP-MS
Isotopes monitored	As^{75}, Se^{77}, Ge^{72} (internal standard)
Anion exchange column	PRP-X100 (250 x 4.1 mm, 10 µm)
Guard column	PRP-X100 Guard cartridge
Mobile phase	60 mM $(NH_4)_2CO_3$, 3% MeOH, adjusted to pH 9.0 with ammonia solution
Flow rate	1 mL min^{-1}
Injection volume	40 µL

Method development

The final method (Table 8) used for the extraction and quantification of iAs was optimised using a custom fractional factorial DOE set up in JMP Pro 16, Table 9, that was used to determine the main effects. The design consisted of 4 factors investigated at 3 levels and 1 factor at two levels: flow rate (0.5, 0.75 and 1 mL min^{-1}), buffer concentration (40, 50 and 60 mM ammonium carbonate), HNO_3 % (0, 1 and 2% v/v) in extraction solution, pH of mobile phase (9, 9.5 and 10) and column length (150mm or 250 mm PRP-X100, 10µm). The effects of these 5 factors on both the chromatographic run time and the resolution of the iAs peak to AsSug-SO_4 were studied. The resolution was calculated using Equation S1 in the Electronic Supplementary Material. Two centre points were also included in the design giving a total of 18 runs that were performed in a randomised order. As the experiments were conducted over two separate days, runs were split into two blocks and block number was included as a variable.

A sample of *A. nodosum* (primary shoot) was chosen to produce the extracts to be used for the DOE due to the high concentration of AsSug-SO_4. As before, 100 mg of sample material was accurately weighed out into quartz 12 mL tubes before the addition of 10 mL of an extraction solution containing either 0, 1 or 2% (v/v) HNO_3 and all containing 3% (v/v) H_2O_2. The mixtures then underwent extraction as previously described in an Ultrawave microwave digestion system and further centrifugation before analysis. The unfiltered extracts were then injected directly.

Quality Control

Identification of DMA, MMA and As(V) peaks were carried out by spiking with the respective standards, Figure 16, and retention time comparison to CRM 7405-b (hijiki) which has a characteristically high As(V) concentration (24.4 mg kg^{-1}). Identification of AsSugars was carried out by retention time comparison of an in-house reference material: a sample of *A. nodosum* that had previously undergone LC-MS/MS analysis (Quantiva, Thermo), Electronic Appendix F4. Column recoveries were acceptable for all chromatographic runs and calculated to be between 82 and 107%, suggesting all extracted species were sufficiently eluted from the column. Certified reference materials were analysed alongside each batch of samples.

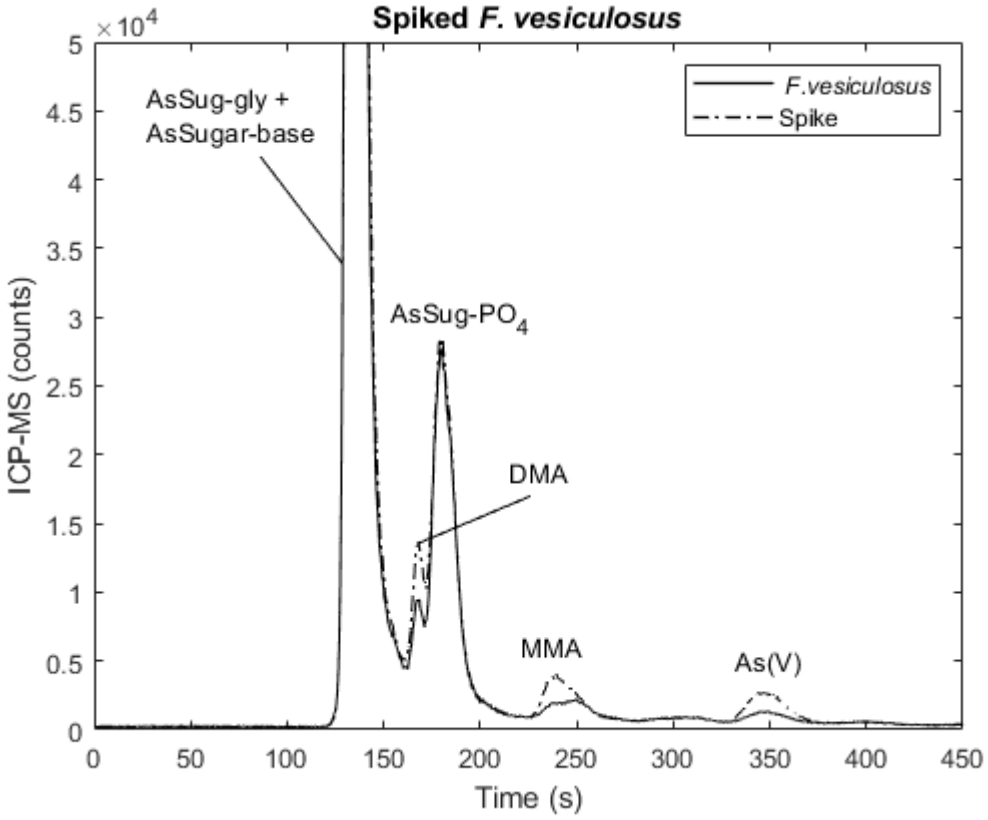


Figure 16 – A sample of spiked *Fucus vesiculosus* extracted using 1% (v/v) HNO_3 and 3% H_2O_2 and analysed with HPLC-ICP-MS. The sample was spiked with 0.5 µg L^{-1} DMA, MMA and As(V).

Results and Discussion

Design of experiment

The resolution of As(V) and the closest eluting peak (which was consistently AsSug-SO_4 in the sample of *A. nodosum* used), as well as chromatographic runtime were optimised using a custom design in JMP Pro 16. In order to estimate main effects, the acid concentration in the extraction solution and mobile phase flow rate, pH and buffer concentration were all studied at 3 levels. Two different column lengths were also evaluated. One sample of *A. nodosum*

(primary shoot) was used to produce all extracts used for testing, as this species is known to contain high concentrations of AsSug-SO_4 which may co-elute with As(V) (49). Extracts were prepared as previously described but with differing HNO_3 concentrations. The runtime and separation were used as response factors, Table 9, where the runtime was defined as the time the final peak returns to baseline and the separation was defined as the resolution between As(V) and the closest eluting peak to the left.

Table 9 – The parameters for each run of the experimental design and responses. n = 1 for all runs with the exception of centre points where n = 3.

Run	Block	Flow rate (mL min^{-1})	Buffer conc. (mM)	HNO_3 conc. (v/v %)	pH	Column length (mm)	Runtime (min)	Separation
1	1	0.75	40	0	9	150	9.10	1.08
2	1	0.5	60	0.5	9	150	7.23	1.15
3	2	1	40	1	9	150	4.84	0
4*	1	0.75	50	0.5	9.5	150	4.30	0.41
5	2	0.5	60	1	9.5	150	4.25	0.42
6	2	0.5	40	0	10	150	8.11	1.18
7	1	1	60	0	10	150	3.5	0.49
8	1	0.5	40	1	10	150	6.47	0.39
9	2	1	50	1	10	150	3.54	0
10	2	0.5	50	0	9	250	21.01	3.57
11	1	1	60	0	9	250	11.17	2.09
12	1	0.5	40	1	9	250	14.78	3.20
13	2	1	60	1	9	250	7.43	2.49
14	2	1	40	0	9.5	250	13.05	1.52
15*	1	0.75	50	0.5	9.5	250	11.42	1.93
16	2	0.5	60	0	10	250	14.60	0.80
17	1	1	40	0.5	10	250	7.24	1.93
18	1	0.75	60	1	10	250	9.57	2.18

*Centre point.

Two separate linear models were generated for each response based on the data in Table 9. It was assumed that all interactions obeyed strong hereditary. The regression model for separation had an $R^2 = 0.95$ and a low root mean squared error (RMSE) = 0.33, both of which suggest the model adequately describes the observations. The significant factors effecting the separation were found to be the column length and pH of the mobile phase, Figure 17, where increasing pH was found to have a negative effect on the separation. The term pH*pH being included in the model suggests pH has a parabolic relationship with separation rather than linear. However, as the pKa values of As(V) are 2.19, 6.98 and 11.53 and this experiment is restrained to a pH range of 9-10, linear behaviour can be assumed (118). This is additionally reflected by the *p* value of the pH*pH term, Figure 17. Block, acid concentration and flow rate were not found to be significant factors.

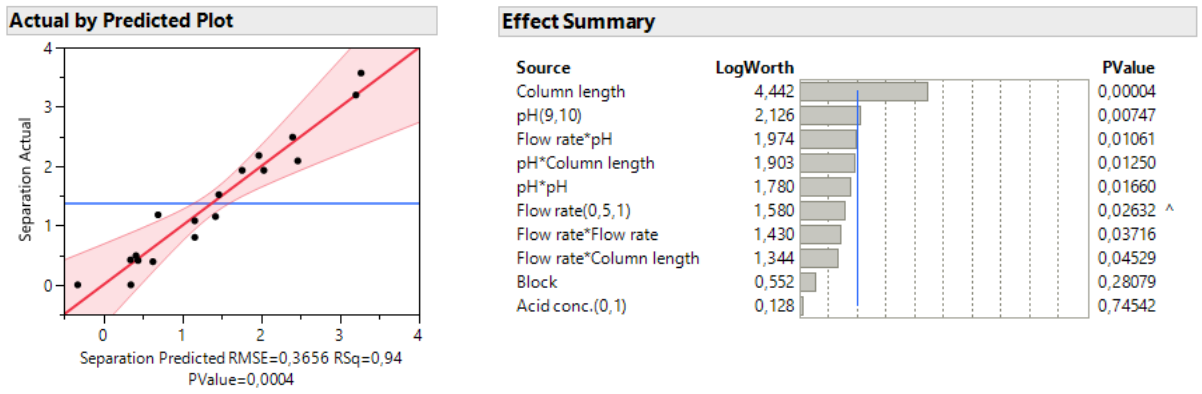


Figure 17 – The plot of actual by predicted for the linear regression model for the separation, and summary of effects. The LogWorth values are transformed p-values for visual clairty (i.e., LogWorth>2 is significant at p=0.01 level).

The regression model for runtime had an R^2 = 0.98 and a RMSE = 0.87. This suggests the linear regression is a good fit, but the higher RMSE value means the model may produce less precise predictions than the model for the separation, Figure 18. Column length, flow rate, pH, buffer concentration and acid concentration were all found to be significant factors affecting the runtime, where column length had the strongest effect. The block number was not found to be a significant factor.

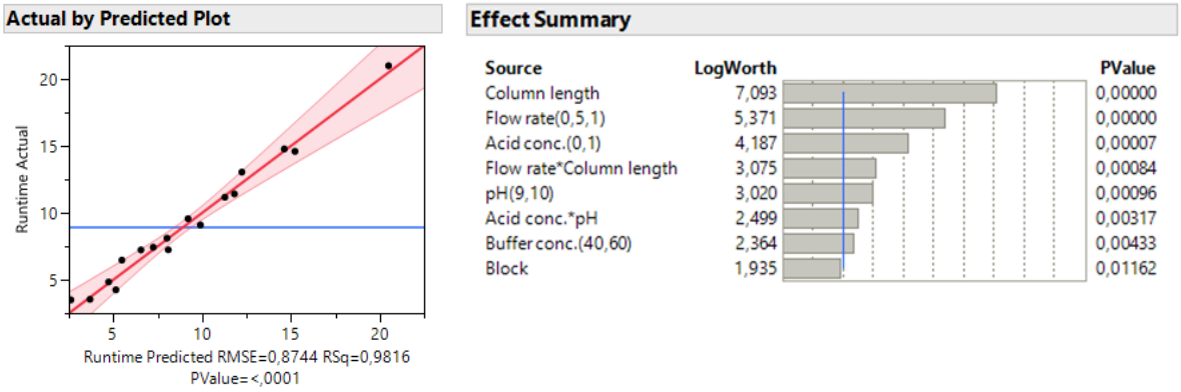


Figure 18 – The plot of actual by predicted for the linear regression model for the runtime, and summary of effects. The LogWorth values are transformed p-values for visual clarity (i.e., LogWorth>2 is significant at p=0.01 level).

The separation of As(V) from other analytes was deemed more important than the runtime, and so the significant factors for this response were optimised to maximise the separation, and these results used as constraints for the optimisation of the runtime. The 250 mm column and pH 9 gave the highest predicted separation, Appendix F5. When these values were used as constraints to minimise the runtime, the model predicts the optimised parameters to be a flow rate of 1 mL min^{-1}, buffer concentration of 60 mM and acid concentration of 1% (v/v). These parameters were then used for the analysis of all samples. The predicted runtime for these parameters was 6.57 minutes, and the actual runtime for the seaweed matrices was approximately 6.90 minutes.

Method validation

As per Eurachem recommendations for method validation, the selectivity, linear range, trueness, and precision of the developed method were tested (241). The selectivity of the method for iAs measurement was determined by analysis of TORT-3 (lobster hepatopancreas) and hijiki (7405-b) which are confirmed to contain the potential interferent AsSug-SO_4 (110). The use of HNO_3 in the extraction was found to sufficiently remove the interference and allow accurate quantification of iAs, where AsSug-SO_4 was no longer detectable and the results obtained for iAs in these reference materials were comparable to previously reported using longer chromatographic methods, Table 10 (110). The trueness was determined by comparison of results obtained for reference material hijiki (7405-b) with the certified value, where the recovery was found to be acceptable (99 ± 9%). Hijiki is currently the only marine reference material with a certified value for iAs, therefore spiking recovery experiments were performed with a sample of *F. vesiculosus* known to contain high concentrations of AsSug-SO_4 relative to the iAs concentration (49). Spiking with 1, 5 and 10 µg L^{-1} yielded acceptable recoveries of 112, 92 and 101% respectively. Additionally, to rule out any matrix effects an internal calibration was also performed for the same sample in triplicate, where samples were spiked at 3 levels (0.5, 5 and 10 µg L^{-1}) using an As(V) standard. No significant difference was found between the results obtained using an internal calibration vs. an external calibration ($p = 0.46$), where concentrations were calculated as 0.054 ± 0.009 mg kg^{-1} and 0.055 ± 0.004 mg kg^{-1}, respectively.

Precision is defined as the repeatability (within batch or run) and the reproducibility (between laboratories). As this study is a single laboratory validation the reproducibility was not tested, however samples showed good repeatability between replicates where RSD between replicates of reference materials ranged from 7-15%. The LOD achieved was comparable to others reported (108,142,236) and is considered acceptable for the determination of iAs in rice products to comply with European legislation, whereby levels must not exceed 0.04 mg kg^{-1} for white rice (182). Additionally, calibration within the working range of the method was shown to be linear, Electronic Supplementary Material Fig S4.

Reference materials

There is a clear lack of consensus in the literature over the value of iAs in marine reference materials solutions - with multiple sources reporting values with order of magnitudes difference for same material. For the reference material TORT-2, iAs values as low as 0.09 mg kg^{-1} and as high as 1.23 mg kg^{-1} have been reported when using HPLC-ICP-MS (113,242,243). This study quantified the iAs concentration in TORT-2 as 0.59 mg kg^{-1} which is well within this range of values, Table 10, and similar to values obtained using a microwave assisted extraction, an acidic solvent and subsequent quantification with LC-ICP-MS (0.50-0.78 mg kg^{-1}) (113). The results for TORT-3 were lower than those reported using an extraction solution containing methanol (0.631 mg kg^{-1}) (110) and also those using HNO_3 in the extractant (0.43 mg kg^{-1}) (142), and were more similar to results from extraction procedures using water as the solvent (0.341 mg kg^{-1}) (136). A similar trend is observed for DOLT-5, where extractions using aqueous methanol report greater iAs values (0.218 mg kg^{-1}) in comparison with this study (0.068 mg kg^{-1}). Previous research has reported DOLT-5 iAs concentrations in the range of 0.029-0.076 when using low concentrations of HNO_3 and/or

H_2O_2 (109,142). Concentrations of iAs in DORM-5 and hijiki were in good agreement with certified or previously reported values.

MMA concentrations were generally lower than reported values for all reference materials, possibly due to partial separation from an unknown compound (UK), Figure 16. DMA values were slightly lower than reported previously, with the exception of hijiki which was consistently higher than the literature values, 0.73 ± 0.10 mg kg^{-1} compared to 0.24 ± 0.01 mg kg^{-1} (109,191). This increase in DMA is unlikely the result of AsSugars degradation as the breakdown is acid induced rather than microbial. Thus, the reaction would be expected to proceed via the AsSugar derivative with no side chain rather than the DMA and other intermediates observed during microbial degradation (112,244). Higher marine organisms may also contain AsSugars and a similar trend was not observed in these matrices. The lower concentrations of DMA reported in the other marine reference materials, e.g., DOLT-5, may potentially indicate that a compound unstable under acidic conditions is co-eluting with DMA, for example dimethylarsinoyl acetate (DMAA) is often present in higher marine organisms but not algae, and has been shown to have a similar retention time to DMA (110,136).

All reference materials showed good extraction efficiencies (72-101%), however, the value for hijiki is higher than previously reported with milder aqueous extractions (42-53%) (191,245). The use of acid in the extraction solution may have potentially caused degradation of arsenic-containing lipids to water-soluble species, as Hijiki *spp.* have been shown to contain significant concentrations of lipid-soluble As, however this requires further investigation (246). Similar extraction efficiencies are observed for marine animal matrices regardless of the use of acid in the extraction, as the main arsenic-containing lipid species are fatty acids and hydrocarbons (247). High extraction efficiencies have been reported for the *U. lactuca* CRM when HNO_3 was used in the extraction solvent (248). The same study reported significantly lower values for DMA (0.04 mg kg^{-1}), however, a shorter extraction time was used, and a value of 0.08 mg kg^{-1} for DMA was reported when a methanol/water extraction solution was used. Additionally, the authors report a value of 1.2 mg kg^{-1} for As(V) (this study: 1.36 mg kg^{-1}) and did not detect AsSug-SO_3 or AsSug-SO_4 (248).

Table 10 – The concentration of DMA, MMA and iAs (mg kg^{-1}) in a range of certified reference materials determined using HPLC-ICP-MS. Concentrations of iAs are compared with those previously reported in literature.

Reference material	DMA conc. (mg kg^{-1})	MMA conc. (mg kg^{-1})	iAs conc. (mg kg^{-1})	Total As extracted (mg kg^{-1})	Total As (mg kg^{-1}) (certified value)	Extraction efficiency (%)	Column recovery (%)	Reference
DORM-5 (n = 5)	0.19 ± 0.02	<LOQ	0.02 ± 0.003 (0.015-0.019)	13 ± 0.31	13.3 ± 0.7	101 ± 2	93 ± 3	(109)
DOLT-5 (n = 5)	1.8± 0.12	0.10 ± 0.01	0.07 ± 0.007 (0.03-0.04)	32 ± 0.65	34.6 ± 2.4	92 ± 2	97 ± 1	(109)
TORT-2 (n = 10)	0.30 ± 0.08	0.08 ± 0.01	0.59 ± 0.04 (0.50)	18 ± 1.4	21.6 ± 1.8	85 ± 7	84 ± 3	(113)
TORT-3 (n = 5)	1.1 ± 0.06	0.12 ± 0.02	0.36 ± 0.03 (0.51-0.62)	45 ± 3.4	59.5 ± 3.8	72 ± 6	105 ± 1	(109)
Hijiki (7405-b) (n = 5)	0.73 ± 0.10	0.05 ± 0.01	24 ± 2.2 (24.4 ± 0.7)	39 ± 1.4	49.5 ± 1.0	78 ± 3	85 ± 4	(191)
U. lactuca (CRM BCR-279)	0.07 ± 0.01	<LOQ	1.4 ± 0.20	2.43 ± 0.46	3.1 ± 0.21 (certified value)	78 ± 14	98 ± 17	-

LOD = 0.006 mg kg^{-1}, LOQ =0.018 mg kg^{-1}. Previously reported values for iAs in brackets.

TORT-2 showed large differences in extraction efficiency dependent on the sample weight used, and so the effects of two sample to solvent ratios on analyte concentration and total As were investigated, Table 11. Significantly higher concentrations of total As were extracted when less sample material was used, as well as significantly higher levels of iAs. The same trend was not observed for other analytes extracted from TORT-2 suggesting that either the iAs binding differs from the other arsenic compounds, or that the As(V) is in a form with a low solubility coefficient and is thus not extracted quantitatively when high sample weights and low solvent volumes are used.

Table 11 –The iAs and total As concentrations in TORT-2 from two different sample to solvent ratios.

Sample to solvent ratio (mg:mL)	n	iAs (mg kg^{-1})*	Tot As extracted (mg kg^{-1})*	Extraction efficiency (%)*
50:10	5	0.63 ± 0.04	19.68 ± 0.62	91 ± 3
100:10	5	0.52 ± 0.03	17.25 ± 0.83	80 ± 4

*Significant difference between ratios ($p<0.05$).

Seaweed samples

The developed method worked well for all species of seaweed tested, with no interferences observed from the high concentrations of AsSug-SO_4 that can be present in some species of brown seaweeds (4). The *L. digitata* reproductive tissues (sori) contained the highest levels of iAs (54 mg kg^{-1}), followed by the *L. digitata* meristem and *A. taxiformis*, Table 12. The iAs concentration of all seaweed samples collected in Iceland were found to be below maximum levels set by France for seaweed-based condiments (3 mg kg^{-1}) with the exception of the *L. digitata* sori, which exceeded this by many multiples. Levels of MMA in the seaweed samples varied between <LOQ and 1.5 mg kg^{-1}, where the Laminariales typically had the highest concentrations. The concentrations of DMA ranged from 0.16 – 1.1 mg kg^{-1}. The extraction efficiency was particularly low for *C. purpureum* (49%) and several brown seaweed samples, Table 12. Low extraction efficiencies have also been reported for species of red seaweed collected off the coast of China, where as little as 15.4% of the total arsenic from *Eucheuma denticulatum* was extracted using water (107). It may be that the As is not in water-soluble form, i.e., arsenic-containing lipids or As(III) strongly bound to sulphur residues in proteins, so will not be extracted using aqueous extraction solvents. Brown seaweed species collected in winter months have previously been reported to contain lower amounts of water-soluble As than those found in summer (61).

Table 12 – The concentration of iAs (mg kg^{-1}) in seaweed samples.

Seaweed (n = 3)	DMA conc. (mg kg^{-1})	MMA conc. (mg kg^{-1})	iAs conc. (mg kg^{-1})	Total As extracted (mg kg^{-1})	Total As (mg kg^{-1})	Extraction efficiency (%)	Column recovery (%)
P. dioica	0.16 ± 0.02	<LOQ	0.05 ± 0.00	24 ± 0.62	22 ± 0.93	111 ± 3	99 ± 3
A. taxiformis	0.73 ± 0.07	0.14 ± 0.01	2.4 ± 0.03	13 ± 1.4	14 ± 0.17	92 ± 10	100 ± 3
P. palmata (Dulse)	0.23 ± 0.03	<LOQ	0.36 ± 0.01	7.9 ± 2.7	8.3 ± 0.21	95 ± 18	92 ± 12
C. purpureum	0.18 ± 0.02	<LOQ	0.45 ± 0.01	2.8 ± 0.40	5.6 ± 0.16	49 ± 7	105 ± 13
F. vesiculosus (apice)	1.4 ± 0.14	0.38 ± 0.04	0.35 ± 0.06	72 ± 10	78 ± 0.74	92 ± 13	107 ± 6
F. vesiculosus (blade)	0.31 ±0.04	0.13 ± 0.01	0.08 ± 0.01	21 ± 3.7	32 ± 4.5	66 ± 12	105 ± 9
A. nodosum (primary shoot)	0.52 ± 0.04	0.03 ± 0.02	0.05 ± 0.01	19 ± 2.6	33 ± 1.1	59 ± 8	98 ± 2
A. nodosum (reproductive receptacle)	0.77 ± 0.06	0.14 ± 0.02	0.10 ± 0.02	48 ± 0.61	113 ± 23	43 ± 1	105 ± 2
L. digitata (meristem)	0.73 ± 0.06	1.3± 0.40	2.6 ± 0.01	97 ± 1.5	130 ± 7.5	74 ± 1	94 ± 3
L. digitata (sori)	0.31 ± 0.04	0.35 ±0.06	54 ± 1.3	85 ± 3.9	210 ± 25	40 ± 2	107 ± 2
S. latissima (stipe)	0.63± 0.03	0.54± 0.04	0.12 ± 0.01	49 ± 1.1	76 ±3.4	65 ±1	100 ± 3
S. latissima (frond)	0.71 ± 0.05	1.5 ± 0.43	0.05 ± 0.01	142 ± 9.3	214 ± 27	66 ± 4	105 ± 6

*Literature value (248), LOD = 0.006 mg kg^{-1}, LOQ =0.018 mg kg^{-1}.

As previously discussed, the concentrations of DMA detected in seaweed matrices increased with the use of acid in the extraction solution. This trend was investigated further by performing two separate extraction and analysis methods to quantify arsenic species in one sample *of A. nodosum*, Table 13. The use of an acidic extraction solution results in higher concentrations of DMA than the use of a water/H_2O_2 solution, but the increase does not appear to arise from the degradation of AsSugars. AsSug-SO_4 appears to degrade to the base sugar resulting in a quantitative increase in the peak associated AsSug-gly, and AsSug-SO_3 now appears to co-elute with AsSug-PO_4 (112). Thus, it is likely that the increase in DMA is the result of an additional compound being extracted and degraded – such as a lipid species unique to seaweed. Although further analysis with mass spectrometry would be needed to confirm exact products created during extraction, previous studies have reported DMA as a product of lipid degradation in seaweed (115). It could also be argued that the acidic extraction provides a closer estimation of the DMA concentration that individuals would be

exposed to after the consumption of seaweed, as similar concentrations of HNO_3 have been previously used to simulate gastric juice (112).

Table 13 – As speciation of *Ascophyllum nodosum* (primary shoot) sample collected from the Grindavík location May 2021 using two separate extraction methods and analyses.

	Method 1 (n = 2)	**Method 2 (n =3)**
Extraction solvent	3% (v/v) H_2O_2	1% HNO_3 (v/), 3% (v/v) H_2O_2
Extraction method	Mechanical shaking RT Overnight	Microwave-assisted 90°C 40 min
HPLC method	Gradient Ammonium carbonate 0.5 mM, 50 mM (3% MeOH, pH 9.2)	Isocratic Ammonium carbonate 60 mM (3% MeOH, pH 9)
Total As extracted (mg kg^{-1})	15.31	18.13
AsSug-gly (mg kg^{-1})	3.00	12.84*
DMA (mg kg^{-1})	0.08	0.41
AsSug-PO_4 (mg kg^{-1})	1.17	4.07
AsSug-SO_3 (mg kg^{-1})	1.81	ND
AsSug-SO_4 (mg kg^{-1})	9.44	0.22
iAs (mg kg^{-1})	0.06	0.05

*Sum AsSug-gly and base sugar (no side chain). The base sugar has been demonstrated to elute at same time as AsSug-gly (112,248). ND – not detected.

Application to other matrices

To test ruggedness, the developed method was applied to other marine and terrestrial matrices. All terrestrial samples and the mussel sample contained reproducibly low levels of iAs, ranging from 0.018-0.15 mg kg^{-1}, Table 14. In the grass-based feed and CAME-1 As(V) was the only analyte detected, and in VORM-1 small cationic peaks were additionally detected in the void volume. Although reference materials VORM-1, BFLY-1 and CAME-1 were only recently introduced, the results are in good agreement with values published from an inter-laboratory study, with the exception of BFLY-1 which is higher than previously reported (109). The concentration of arsenicals detected in the rice sample are typical of this matrix (249–251), and the mussel sample contained relatively high levels of DMA, low levels of MMA along with several AsSugars, Appendix T4. An unidentified late-eluting compound was also detected in the mussel sample, Figure 19, similar to an unknown reported in blue mussels from Norway (110), where the authors used a methanol/water extractant. The extraction efficiencies were high with the exception of CAME-1, where less than half of the total arsenic was extracted in the form of As(V). The recalcitrant arsenic is likely to be in the form of As(III) bound to sulphur-containing proteins rather than lipid soluble species, as CAME-1 is comprised of the high protein residue left over after the extraction of canola oil from *Brassica napus* (109,252).

Table 14 – The iAs concentration (mg kg^{-1}) of other matrices quantified by the developed method.

Sample material (n = 3)	DMA conc. (mg kg^{-1})	MMA conc. (mg kg^{-1})	iAs conc. (mg kg^{-1})	Total As extracted (mg kg^{-1})	Total As (mg kg^{-1})	Extraction efficiency (%)	Column recovery (%)
Grass silage (feed)	ND	ND	0.03 ± 0.00	0.03 ± 0.00	0.04± 0.0	72 ± 7	107 ± 12
Rice	0.10 ±	<LOQ	0.15 ± 0.01	0.25 ± 0.03	0.29 ±0.01	89 ± 10	101 ±10
Mussel	1.2 ± 0.04	0.05 ± 0.00	0.15 ± 0.00	7.08 ± 0.04	13	55 ± 0.3	91 ± 2
Worm (VORM-1)	ND	ND	0.09 ± 0.00 (0.11^{A})	0.10 ± 0.01	0.13^{A} ± 0.00	75 ± 7	100 ± 3
Blackfly (BFLY-1)	<LOQ	<LOQ	0.08 ± 0.01 (0.05^{A})	0.09 ±0.01	0.10^{A}± 0.00	88 ± 12	90 ± 5
Canola plant (CAME-1)	ND	ND	0.02 ± 0.00 (0.01^{A})	0.02 ± 0.00	0.04^{A}	64 ± 4	94 ± 9

[A]Literature value (109), LOD = 0.006 mg kg^{-1}, LOQ =0.018 mg kg^{-1}. ND is not detected.

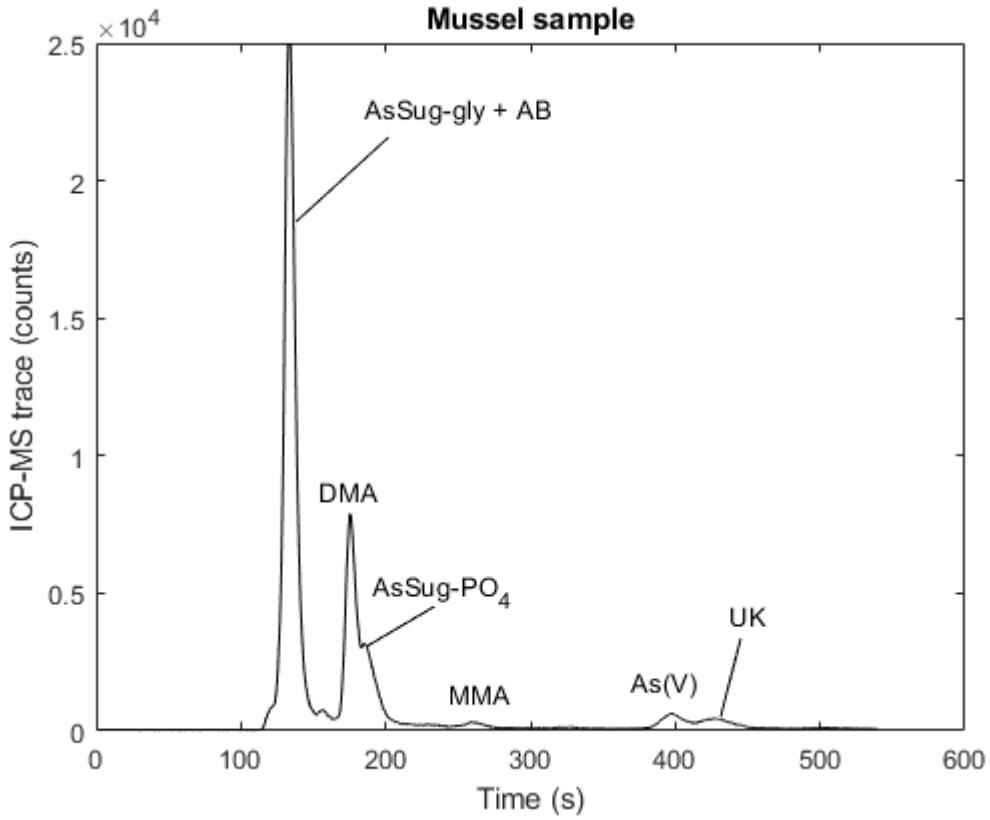


Figure 19 – A chromatograph of mussel sample extracted with 1% (v/v) HNO_3 and 3% H_2O_2 solution and analysed with HPLC-ICP-MS.

Conclusion

Routine measurement of the iAs levels in seaweed is likely to become crucial in the future as European legislation is expanded to include these matrices – highlighting the need for accessible methods for routine analysis. The method developed using DOE is suitable for the high throughput analysis of iAs in a range of matrices (i.e., seaweed, rice, insect and feed), and can provide adequate separation of compounds from As(V) in a similar chromatographic time frame to that of HG-ICP-MS (142). The use of HNO_3 and H_2O_2 allows for a 'partial speciation' approach where iAs is quantified as the sum of both inorganic species oxidised to As(V) and interferences from high levels of AsSugars are removed by degradation to an unretained AsSugar species. The method produced accurate and repeatable results, where the recovery of iAs in hijiki was 99 ± 9% when compared with the certified value and spiking experiments with *F. vesiculosus* yielded acceptable recoveries (92-112%). The applicability of this method to a diverse range of matrices allows for a 'one-fits-all' extraction and analysis that is cost-effective, fast, and robust and requires minimal sample preparation. However, this method is limited in its determination of DMA in seaweed matrices, and care should be taken when using HNO_3 in the extraction solution if the aim is to quantify this analyte, as this may provide an overestimation by degrading unknown As species. However, the concentrations of DMA quantified using this method may provide a better estimation with regards to exposure after ingestion and subsequent digestion of seaweed. Further research should investigate the application of this method to other food matrices which are currently regulated (i.e., fruit juice concentrates and infant foods) (182).

3.4 Paper III –The Impact of Different Sample Preparation Methods on the Arsenic Speciation in *Laminaria digitata*

Rebecca Sim [A B], Jörg Feldmann [C] and Ásta H. Pétursdóttir [A]

Author Affiliations:
[A]Matís, Vínlandsleið 12, 113, Reykjavík, Iceland
[B]Faculty of Physical Sciences, University of Iceland, Dunhagi 7, 103, Reykjavík, Iceland
[C] TESLA-Analytical Chemistry, Universitity of Graz, Universitätsplatz 1, 8010, Graz, Austria

Abstract

Brown seaweeds are known to accumulate high concentrations of arsenic which may be metabolised to complex structures such as sugars and lipids. Preservation of these large arsenic compounds through sample preparation and extraction is crucial to ensure accurate results, and so no species are destroyed, under- or over-estimated. This study aimed to investigate the impact of different drying methods on the water-soluble arsenic speciation in *Laminaria digitata* by comparing the concentrations of arsenicals extracted from fresh samples to those from freeze-dried, air-dried and oven-dried materials. Overall, water-soluble speciation remained similar between drying methods, however lower levels of AsSug-gly (10.5 ± 0.42 mg kg^{-1}) were extracted from fresh material than dried (13.4-15.1 mg kg^{-1}), and the same was not observed for the other arsenosugars detected. Oven-drying also caused thermal degradation of a small quantity of arsenosugars to unknown arsenic compounds and DMA. The arsenic fractionation before and after drying was also investigated, and a signifcant portion of the polar lipid-soluble arsenic was found to be lost to other fractions after drying – where the polar lipid-soluble arsenic accounted for 19.3% of the total arsenic before drying and 1.1-3.8% after drying (*t*-test, $p<0.03$). Additionally all drying methods were found to increase the concentration of non-extractable arsenic relative to fresh samples. The reason for this is unknown. Common sample preservation techniques (i.e., freeze-drying) appear to be suitable when measuring water-soluble speciation, but should be carefully considered when analysing the potentially unstable lipid-soluble fraction.

Introduction

Arsenic is a potentially toxic element that is ubiquitous throughout the environment. Brown seaweeds (Phaeophyta) are known to accumulate high levels of this element, which may be metabolised to a wide variety of arsenic-containing molecules including lipids and sugars. Arsenosugars (AsSugars) comprise the majority of total As in most seaweeds, with the exception of *Laminaria digitata* and *Sargassum fusiforme* (Hijiki) where the inorganic arsenic (iAs) species arsenite and arsenate may account for over half of the total arsenic (37,62,236). Phaeophyta may additionally contain an array of lipid-soluble arsenic compounds, where the arsenosugarphospholipids (AsSugPLs) are the most frequently reported lipids (4,37,246). AsSugars are suggested to be the starting point for AsSugPL production as both share a dimethyl ribose moiety (Figure 20). For example, the binding of the AsSugar-gly derivative to phosphatidic acid in the cell membrane has been suggested as a pathway for their formation (68).

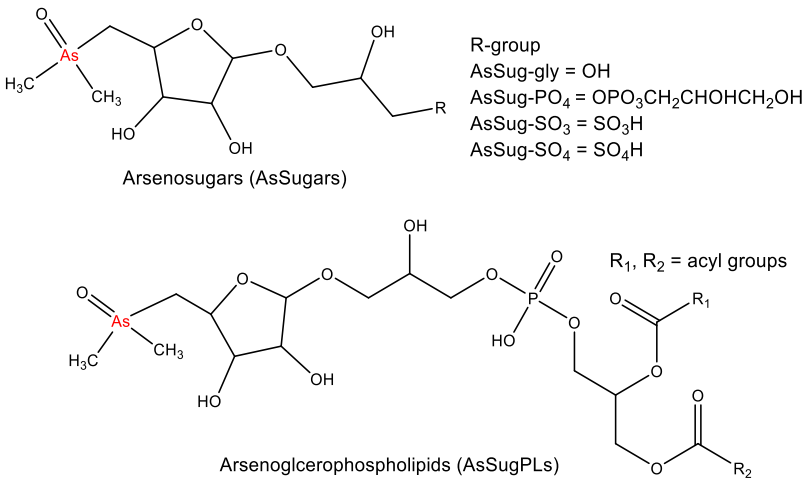


Figure 20 – The structure of common arsenosugars and arsenosugarphospholipids reported in seaweed.

However, the AsSugPL compounds have been reported to be unstable (4), with possible degradation to mono-acyl analogues and potentially water-soluble species over time. Total arsenic concentration and speciation has been reported to remain relatively stable during storage after processing of seaweed and seafood (105,253), although changes that may occur during sample preservation methods are often neglected. Whilst freeze-drying is widely regarded as the most effective method of preserving the speciation of arsenic compounds within sample material, its effect on larger arsenicals in complex matrices is largely unknown. This study aims to investigate the impact of different sample preservation methods (e.g., air-, freeze- and oven-drying) on the arsenic fractionation and water-soluble speciation in *L. digitata*.

Experimental

Chemicals and Reagents

Water (18 Ω cm) from an ultrapure water dispenser system (Millipore, Germany) was used throughout.
Arsenic fractionation solvents heptane and dichloromethane were purchased from Honeywell and methanol (HPLC grade) was obtained from Honeywell (USA). Ammonium carbonate from BDH (UK) was used as the mobile phase buffer for the HPLC-ICP-MS analysis. For the digestion procedure, nitric acid (ROTIPURAN Supra, 69%) from Carl Roth (Germany) and hydrogen peroxide (for trace analysis, ≥30%) supplied by Supelco (Sigma-Aldrich, France) were used.

Speciation analysis was performed using an external calibration prepared from dimethylarsinic acid disodium salt (100%) from Argus-Chemicals (Italy) dissolved in water as needed. An arsenic stock solution for total concentration measurements (1000 mg L^{-1}, 2% HNO_3) was supplied by LabKings (Netherlands) and the stock solutions for the internal standards germanium and indium (1000 mg L^{-1}, 2% HNO_3) were purchased from Peak Performance (CPI International, USA).

Samples and reference materials

Ten *L. digitata* specimens were collected from Grindavík in May 2022, as described in Sim et al. 2023 (61). Briefly, samples were washed with tap water to remove sand, and visible epiphytes removed manually with a stainless-steel knife. Thalli were divided down the middle, and half from each of the 10 specimens pooled together and homogenised using a GM 300 blender (Retsch). The other thalli halves were used for a separate study (61). The homogenised sample material was then dried in one of three ways – freeze-dried, air-dried or oven dried – and a fourth batch underwent no drying and immediate extraction with water (within 2 hours) Air-drying was performed in a dark room at approx. 30°C, oven at 110°C, all performed until constant mass (24- 72 hours depending on method). A subsample of both air-dried and freeze-dried material was dried in an oven at the same temperature as oven-dried samples to constant mass to correct for the differing amounts of water lost from each method.

Certified reference material 7405-b hijiki (National Meteorology Institute of Japan, Japan) was analysed in duplicate alongside all batches for speciation and total arsenic measurements. The arsenic fractionation experiment was also performed on hijiki for comparison to samples.

Total arsenic determination. In triplicate, approximately 200 mg of dried sample material was weighed into 12 mL quartz digestion tubes before the addition of 1 mL of nitric acid and 1 mL of hydrogen peroxide. The quartz tubes were then transferred to the reaction vessel of an Ultrawave microwave digestion system (Milestone, Italy) for digestion. Briefly, a loading pressure of 40 bars was added to the reaction vessel with nitrogen gas before heating to 240°C over 20 minutes. This temperature was maintained for 10 minutes before a 15-minute cool-down period. Digests were then quantitatively transferred to polypropylene tubes and made up to 50 mL with water.

Water-soluble speciation. In duplicate, approximately 200 mg of dried sample material (2000 mg wet) was added to 50 mL centrifuge tube before the addition of 10 mL of water. Mixtures were shaken for 2 hours then centrifuged at 4000 rpm for 10 minutes. An aliquot (1 mL) of the supernatant was transferred to a microcentrifuge for further centrifugation at 15000 rpm for 5 minutes, after which the supernatant was analysed directly. A 1 mL portion of the original supernatant was also diluted to 10 mL with 0.2 mL of nitric acid and water to measure total extracted arsenic.

Arsenic fractionation. Based on the method described in Pétursdóttir et al. (5) In triplicate, 500 mg of sample material was extracted in 5 mL of heptane in a glass vial and left to stand for 2 hours. The supernatant was then removed, and the extraction repeated, before combining the supernatants evaporating to dryness in a fume hood. The same procedure was repeated with a dichloromethane/methanol (2:1) mixture once the sample material had completely dried. For the final extraction, the sample material was transferred to a 50 mL centrifuge tube and 10 mL of water added. The mixture was shaken for 2 hours and centrifuged at 4000 rpm for 15 minutes before removing the supernatant. A 1 mL portion of the supernatant was diluted as previously described for total arsenic measurements. The remaining sample residue was washed with 2 x 15 mL of water to avoid over estimation of the water-soluble fraction, and then dried in an oven at 70°C overnight before being digested as previously described.

The dried heptane and dichloromethane/methanol supernatants were transferred to 12 mL quartz digestion vials by reconstitution in 200 µL of the respective solvent, repeated twice to ensure complete transfer. The solvents were re-evaporated before digestion.

Analyte quantification

Total As. The quantification of total arsenic was carried out using an Agilent 7900 ICP-MS with octopole collision cell and an Agilent SPS 4 autosampler. The ICP-MS was run in He gas mode (5 mL min^{-1}) with Ni cones and an Agilent MicroMist nebuliser. The plasma gas flow was set to 15 L min^{-1}, the carrier gas flow set to 1.07 L min^{-1} and the make-up gas flow to 0.8 L min^{-1} of argon. The spray chamber was maintained at 2 °C and a 1000 ug L^{-1} indium internal standard solution was introduced post-autosampler flow. Standards for external calibration were prepared in the range 0-100 µg L^{-1}, in 2% HNO_3 and the ICP-MS was tuned daily.

Water-soluble speciation. Analysis was performed using an Agilent Infinity II 1290 HPLC coupled to an Agilent 7900 ICP-MS in no gas mode. The instrument was tuned before each use to optimise counts on masses 75 (As^+), 72 (Ge^+) and 77 ($^{40}Ar^{37}Cl^+$) using a solution containing 50 µg L^{-1} of As and Ge. Separation of analytes was performed on a Hamilton PRP-X100 column (250 x 4.1 mm, 10 um) and corresponding guard column under isocratic conditions with a 20 mM ammonium carbonate and 3% (v/v) methanol mobile phase. An injection volume of 40 mL and flow rate of 1 mL min^{-1} were used. Data acquisition and manual integration of peaks was performed using Agilent Masshunter software.

Quality Control

The identification of arsenite, dimethylarsenate (DMA), monomethylarsenate (MMA) and arsenate was performed using spiking experiments. Identification of AsSugars was based on retention time matching to a sample of *Ascophyllum nodosum* that had previously undergone LC-MS/MS measurement (Thermo, Quantiva) and hijiki 7405-b. Column recoveries were acceptable and calculated to be between 98-108% for samples (n =2, n = 3 for fresh) and 84-94% for hijiki (n = 4). The LOD and LOQ for the quantification of total arsenic were calculated from the external calibration curve multiplied by an average dilution factor and were 0.02 and 0.05 mg kg^{-1} respectively.

Statistical analysis

Where possible, statistical tests were used to determine significant differences between results. Analysis was performed in excel using paired, two-tailed t-tests.

Results and Discussion

Arsenic fractionation

Mass balance recoveries for all samples were acceptable and found to be between 94-107%. Total arsenic concentrations were not found to be significantly different between drying methods (p-value 0.1-0.3).

The concentration of As in the non-polar fraction was found to <LOQ in all samples, including the reference material hijiki 7405-b. This is lower than what has been previously reported for a similar reference material hijiki 7405-a, where concentrations of non-polar arsenic extracted by hexane were reported to be between 0.02-0.1 mg kg^{-1} (4). The concentration of polar lipid-soluble arsenic appears to be negatively impacted by sample drying, Table 15, where the concentration was 12.3 ± 1.1 mg kg^{-1} and decreased to 2.39-0.72 mg kg^{-1} depending on the drying method. Freeze-drying appeared to conserve the highest amount of arsenic-containing polar lipids, and the concentration was shown to decrease as the temperature of the drying method increases. The arsenic lost from the polar-lipid soluble fraction after drying appears to be re-distributed into the water-soluble and non-extractable fractions.

The total concentration of water-soluble arsenic was similar between fresh and freeze-dried (51.1-51.9 mg kg^{-1}) but increased after air-drying (57.1 mg kg^{-1}) and oven-drying (53.2 mg kg^{-1}). The significant increase in concentration after air-drying may mean that bacterial action is able to convert lipid-soluble species to water-soluble compounds as the other drying methods do not provide optimal temperatures for bacterial activity. The small increase in water-soluble As observed after oven-drying is likely due to thermal degradation of larger lipid species. For reasons unclear, the unextractable residual arsenic displayed the opposite trend to the polar lipid-soluble fraction, where the amount of recalcitrant arsenic increased with the drying method temperature. Fresh sample material had the lowest level of recalcitrant arsenic (2.6 ± 0.03 mg kg^{-1}), and freeze-drying and air-drying approximately doubled this value to 6.7 mg kg^{-1} and 7.0 mg kg^{-1} respectively whilst oven-drying increased the recalcitrant arsenic over 3-fold to 9.1 mg kg^{-1}. As has been demonstrated to be stored in the cell wall, with smaller amounts in the intracellular matrix in *L. digitata* (37). Perhaps the evaporation of water from the cytosol during drying does not allow cells to be opened sufficiently during extraction leading to increase in residual arsenic, particularly if residual As is bound as As(III) (or DMA/MMA(III) to sulphur-containing groups within the cell

Table 15 – The distribution of arsenic within the lipid-soluble, water-soluble, and residual fractions of *L. digitata* samples dried using different methods: air-, freeze-, oven-dried and fresh (no drying). n = 3, and the % contribution of each compound to the total arsenic concentration is also shown.

Sample	Non-polar lipid-sol As (mg kg^{-1})	Polar lipid-sol As (mg kg^{-1})	Water-sol As (mg kg^{-1})	Non-extractable As (mg kg^{-1})	Total As (mg kg^{-1})	Recovery (%)
Fresh *L. digitata*	<LOQ (<0.1%)	12.3 ± 1.1 (19.3%)	51.1 ± 0.42 (80.3%)	2.61 ± 0.03 (4.1%)	*63.6 ± 3.2	104 ± 2
Freeze-dried *L. digitata*	<LOQ (<0.1%)	2.39 ± 0.32 (3.8%)	51.9 ± 1.3 (81.6%)	6.71 ± 0.60 (10.6%)	63.6 ± 3.2	96 ± 1
Air-dried *L. digitata*	<LOQ (<0.1%)	1.41 ± 0.11 (2.3%)	57.1 ± 0.93 (93.5%)	7.04 ± 1.5 (11.5%)	61.1 ± 0.53	107 ± 1
Oven-dried *L. digtata*	<LOQ (<0.1%)	0.72 ± 0.15 (1.1%)	53.2 ± 0.68 (79.5%)	9.12 ± 1.8 (13.6%)	66.9 ± 4.6	94 ± 4
Hijiki 7405-b (*Sargassum fusiforme*)	<LOQ (<0.1%)	5.62 ± 0.17 (11.3%)	21.5 ± 0.33 (43.3%)	19.7 ± 0.91 (39.6%)	49.7 ± 2.5 (49.5** ± 1.0)	96 ± 2

*The same total arsenic value was used for fresh and freeze-drying as arsenic concentration has not been shown to change significantly after freeze-drying (254). **Certified value for total As concentration.

Water-soluble arsenic speciation

In general, the total extracted arsenic in samples used for water-soluble speciation was higher than the total water-soluble arsenic measured during the fractionation experiment due to the dichloromethane/methanol step performed prior to the water extraction for the fractionation. Less polar arsenic species such as AsSug-gly may be soluble in both methanol and water, so differences can be attributed to this compound being extracted during the first dichloromethane/methanol extraction step (5,66). The opposite was observed for fresh sample materials, where 51.1 mg kg^{-1} of arsenic was extracted during the fractionation experiement, but only 48.3 mg kg^{-1} when water was used as the extraction solvent first, Table 16.

The drying techniques produced similar results in comparison to the fresh samples for the water-soluble arsenic species with the exception of AsSug-gly and DMA, Figure 21. The concentration of AsSug-gly extracted from fresh *L. digitata* (10.5 ± 0.42 mg kg^{-1}) was lower than when the sample material was dried (13.4-15.1 mg kg^{-1}), whereas the same was not observed for the other arsenosugars (AsSug-PO_4 and AsSug-SO_3). The other common arsenosugar derivative, AsSug-SO_4, was below the LOQ in all samples, which confirms results from Ender et al., where AsSug-SO_4 was not detected in *L digitata* samples. Drying

methods are known to destroy the cell structure (255), thus any arsenicals contained inside the cell would likely be less efficiently extracted in fresh sample material as cells are more stable and prevent the extraction of cystolic arsenic. Imaging with NanoSIMS in *L. digitata* has revealed the majority of arsenic to be stored in the cell wall, with a small amount in the intracellular matrix (37). Whilst it is likely the majority of this intra-cellular arsenic is in the form of As(III) bound to sulphur in cysteine residues or methylated species DMA and MMA, the lower levels of AsSug-gly extracted here before drying might indicate some AsSug-gly is present within cells before it can be transported to the cell wall matrix. AsSug-gly and As(III) co-eluted at the beginning of the chromatograph, however, analysis with a separate chromatographic method determined that neglible levels of As(III) were present in the *L. digitata* samples and that levels did not contribute signifcantly to the concentration of AsSug-gly (61).

The concentration of DMA in the oven dried samples was approximately 10-fold that of all other samples as the high temperatures used likely resulted in thermal degradation of the arsenosugars. This is similar to previously reported for a *Porphyra sp*. where an increase in DMA concentration from 0.44 to 0.69 was observed after baking at 200°C (256). The oven-dried samples also contained the highest concentration and number of unknown (UK) compounds, Table 16, however 2 unknown compounds were present in nearly all samples (UK1 2.9 min; UK7 16.3 min). An additional 5 unknown peaks (UK2 3.3 min; UK3 6.6 min; UK4 8.0 min; UK5 11.8 min; UK6 16.3 min) were detected in oven-dried samples, Figure 22, which are likely to be small, methylated arsenicals such as dimethylarsinoylethanol (DMAE) or dimethylarsinoylacetate (DMAA) which are known to be products of AsSugar decay (244). MMA concentrations were also found to be highest in oven dried samples (0.15 mg kg^{-1}), but were generally low in all samples (0.05-0.09 mg kg^{-1}).

Additionally, freeze-drying resulted in higher concentrations of AsSug-PO_4 (5.1 mg kg^{-1}) and AsSug-SO_3 (12.5 mg kg^{-1}) than the other drying methods, where concentrations ranged from 3.56-3.84 mg kg^{-1} and 11.0-11.26 mg kg^{-1} respectively. High concentrations of mannitol are typically found in *L. digitata* (up to 30% of dry mass), and the crystallisation of mannitol during freeze-drying has been demonstrated to generate mechanical stress which disrupts the lipid bilayer of the cell membrane (192). If AsSug-PO_4 is a product of lipid decay as previously suggested (68) it would be logical to assume more would be extracted after freeze-drying. However, a similar trend was observed for AsSug-SO_3 yet no arsenic-containing lipids with an AsSug-SO_3 have been reported, so it is more likely the increase in AsSug-PO_4 and AsSug-SO_3 extracted after freeze-drying is due to damages to the cell wall structure after drying, as the majority of arsenic is stored here (37). As(V) concentrations were similar between drying methods and ranged from 22.1-25.4 mg kg^{-1}, where extractions with fresh sample (22.1 ± 1.17 mg kg^{-1}) material yielded the lowest concentrations and freeze-dried the highest (25.4).

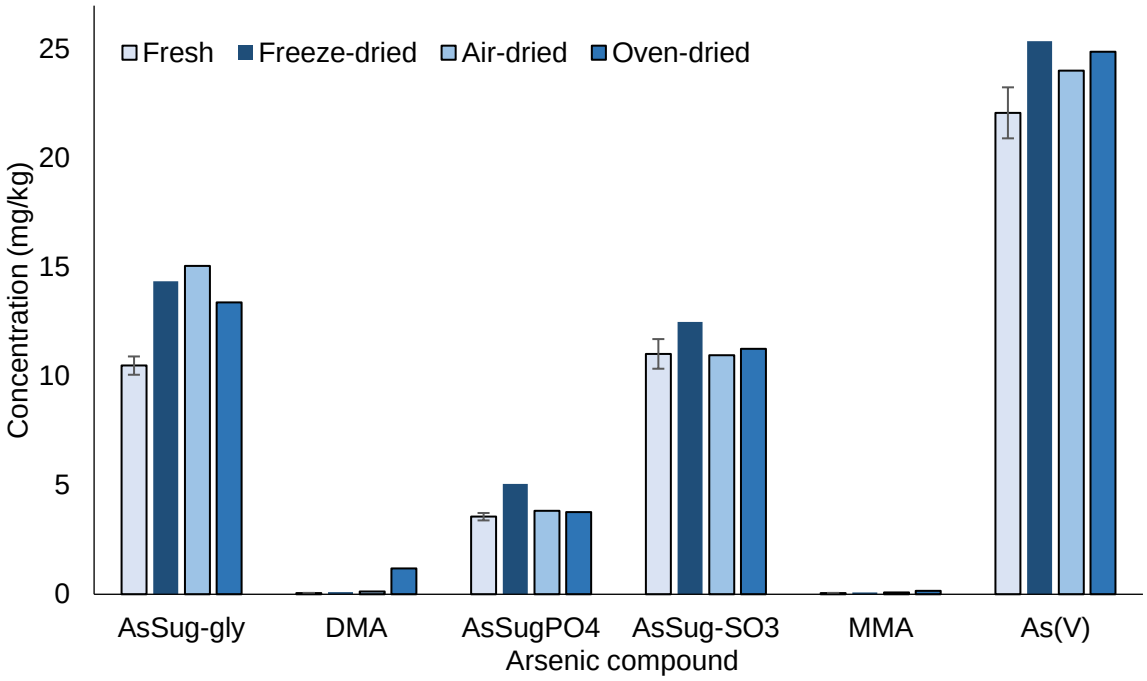


Figure 21 – The water-soluble speciation of of *L. digitata* samples dried using different methods: air-, freeze-, oven-dried and fresh (no drying). n = 2 for all samples except fresh where n = 3 and error bars represent 1 standard deviation.

Table 16 – The concentrations of water-soluble species extracted from *L. digitata* samples dried using different methods: air-, freeze-, oven-dried and fresh (no drying). The % contribution of each compound to the total extracted As is also shown.

	AsSug-gly (mg kg^{-1})	**DMA (mg kg^{-1})**	**AsSug-PO4 (mg kg^{-1})**	**AsSug-SO3 (mg kg^{-1})**	**MMA (mg kg^{-1})**	**As(V) (mg kg^{-1})**	**Sum of unknown compounds (mg kg^{-1})**	**Total As extracted (mg kg^{-1})**	**Extraction efficiency (%)**
Fresh *L. digitata* (n = 3)	10.5 ± 0.42 (22%)	0.05 ± 0.01 (0.1 %)	3.56 ± 0.17 (7.4%)	11.0 ± 0.68 (23%)	0.05 ± 0.01 (0.1%)	22.1 ± 1.17 (46%)	0.80 ± 0.05 (1.7%)	48.3 ± 1.6	77 ± 3
Freeze-dried *L. digitata* (n = 2)	14.4 (25%)	0.10 (0.2%)	5.06 (8.8%)	12.5 (22%)	0.09 (0.2%)	25.4 (44%)	0.64 (1.1%)	57.6	91
Air-dried *L. digitata* (n = 2)	15.1 (25%)	0.14 (0.2%)	3.84 (6.4%)	11.0 (18%)	0.08 (0.1%)	24.0 (40%)	0.52 (0.9%)	60.0	98
Oven-dried *L. digtata* (n = 2)	13.4 (23%)	1.19 (2.0%)	3.77 (6.4%)	11.3 (19%)	0.15 (0.3%)	24.9 (42%)	2.11 (3.6%)	58.8	88
*Hijiki 7405-b (*Sargassum fusiforme)* (n = 4)	0.70 ± 0.07 (**0.45 ± 0.02)	0.26 ± 0.06	0.26 ± 0.07 (**0.20 ± 0.01)	0.18 ± 0.03 (**0.16 ± 0.01)	0.07 ± 0.02	23.2 ± 1.6 (**24.4 ± 0.7)	0.12 ± 0.02	26.1 ± 2.5	53 ± 5

*Measured values for hijiki have been previously reported as analysis was carried out alongside that reported by Sim et al. (2023) (61).
**Certified values for hijiki 7405-b (191).

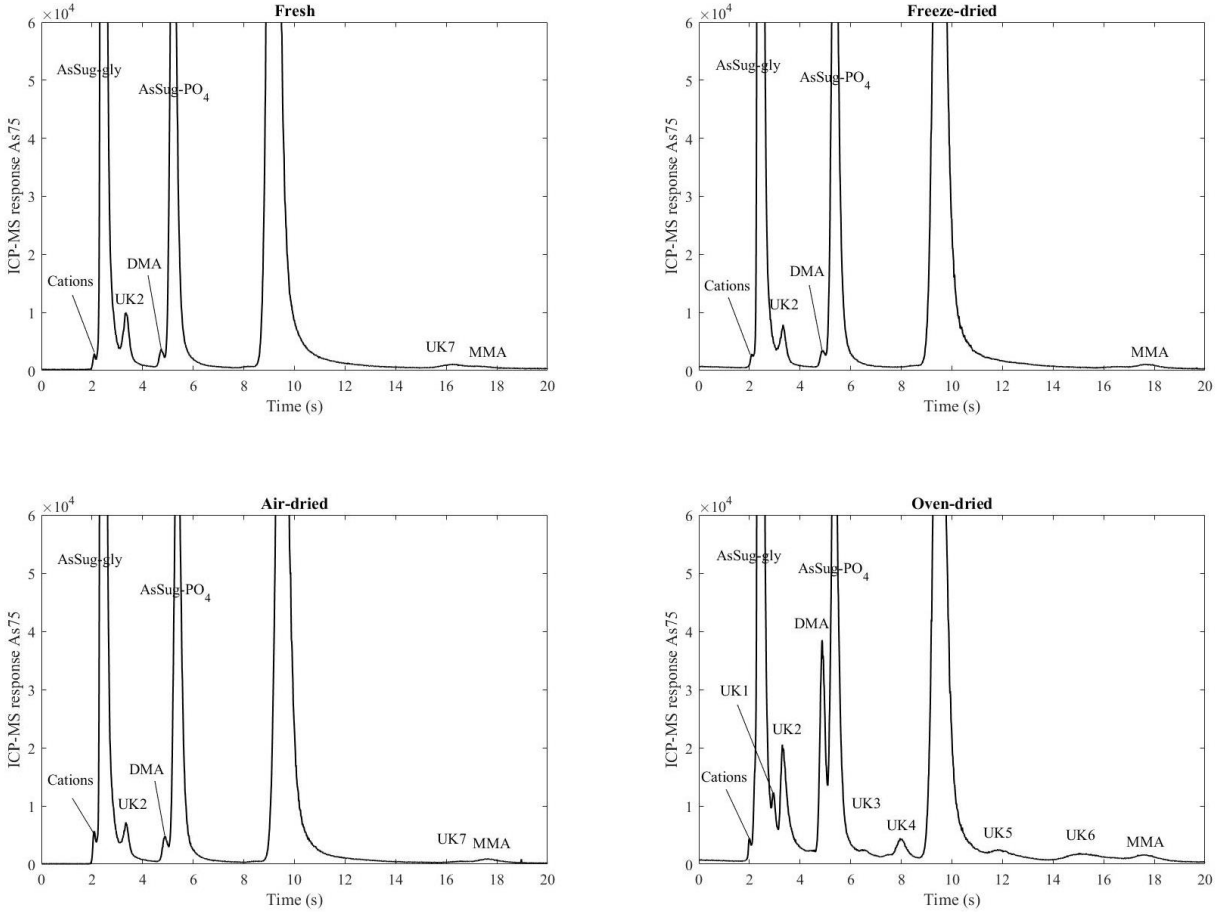


Figure 22 – The chromatographs of fresh, freeze-dried, air-dried and oven-dried *L. digitata* samples from 0-1200 seconds. Method: PRP-X100 anion exchange column (250 x 4.1 mm; 10 µm), isocratic elution with 20 mM ammonium carbonate buffer mobile phase with 3% (v/v) methanol, and ICP-MS detector.

Conclusion

The aim of this study was to investigate the impact of different drying methods on the water-soluble arsenic speciation in *L. digitata*, as well as the fractionation of arsenic before and after drying. The water-soluble speciation was found to remain similar between drying methods, although lower levels of AsSug-gly were extracted from fresh material than dried and the differences in extraction efficiency demonstrated for AsSug-gly appear to be dependent on the level of disruption to the cell wall/membrane structure. Freeze-drying extracted higher levels of AsSug-PO_4 and AsSug-SO_3 than the other methods as it likely caused more damage to the cell wall structure where AsSugars are thought to be stored (37). Oven-drying was found to cause thermal degradation of large arsenic compounds (AsSugars or arsenolipids) to unknown, smaller arsenicals and DMA. Despite this, oven-drying did not appear to impact the concentrations of the 3 AsSugars detected or As(V). The most important result is that As(V) concentration was independent of the sample drying method - which means accurate results can be obtained for this carcinogenic species regardless of how the sample was prepared.

The arsenic fractionation before and after drying was also investigated. A significant portion of the polar lipid-soluble arsenic was found to be lost to either water-soluble or residual fractions after drying – where the polar lipid-soluble fraction accounted for 19.3% of the total arsenic prior to drying and 1.1-3.8% after drying. Drying sample material also appears to increase the concentration of residual arsenic, where an increase from 4.1% of total arsenic to 10.6-13.6% was observed after drying, but the reason for this is still unclear. These results suggest that sample preservation techniques should be carefully considered depending on the speciation analysis to be carried out - in particular for the lipid-soluble arsenic compounds which appear to be sensitive to drying.

3.5 Paper IV – Temporal and Intra-thallus Variation in Arsenic Species in the Brown Macroalga *Laminaria digitata*

Rebecca Sim [A B], Jörg Feldmann [C], Dagmar B. Stengel [D] and Ásta H. Pétursdóttir [A]

Author Affiliations:
[A]Matís, Vinlandsleid 12, 113 Reykjavík, Iceland
[B]Faculty of Physical Sciences, Dunhagi 3, University of Iceland, Reykjavík, Iceland
[C]TESLA-Analytical Chemistry, Institute for Chemie, Universitätsplaz 1, University of Graz, 8010 Graz, Austria
[D]Botany and Plant Science, School of Natural Sciences, University of Galway, University Road, Galway, Ireland

Abstract

Environmental context. Arsenic contamination has a disproportionate effect on marine ecosystems. Organisms such as some marine macroalgae, which accumulate potentially toxic elements from the surrounding environment, have developed an internal conversion process that is not yet fully understood. Are arsenic-containing sugars a product of detoxification, or simply the result of phospholipid degradation?

Rationale. Arsenosugars (AsSugar) account for the majority of total arsenic in common seaweed species, yet it is unclear whether these are formed through some detoxification pathway for inorganic arsenic or are precursors/degradation products of arsenic-containing phospholipids in the cell wall.

Methodology. Temporal and intra-thallus variations in water-soluble arsenic were measured by HPLC-ICP-MS, as well as total non-polar and polar arsenic-containing lipids by ICP-MS in *Laminaria digitata* to offer potential insight into the origins of arsenosugars. Water-soluble speciation with and without freeze-drying was also compared to determine whether freeze-drying changes the water-soluble As speciation.

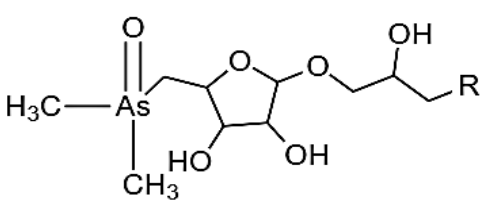
Results. In general, lower levels of total As were detected in the samples collected in May (39.2-74.5 mg kg^{-1}) compared to those collected in February (72.6-151 mg kg^{-1}). The concentration of arsenate was found to consistently increase along the thallus from the holdfast/stipe (0.78-1.82 mg kg^{-1}) to the decaying fronds (44.4-61.0 mg kg^{-1}) in both months, and AsSug-SO_3 was the dominant AsSugar in the majority of samples. The extraction efficiency was lower in fresh samples (64-77%) than in freeze-dried (95-116%) from the same month. Water-soluble, polar AsLipids, and residual As concentrations were generally highest in February, and the non-polar AsLipids accounted for <0.42% of totAs in all samples.

Discussion. Our results suggest that the arsenosugars are not a product of arsenic detoxification, but a by-product of normal biological activity. It is probable that the arsenosugars are bound to the cell membrane within the *Laminaria digitata* cells, and lyophilisation is required to release them quantitatively. Future research should focus on speciation of polar lipid-soluble As extracted from fresh samples to determine if the lower extraction efficiency observed in this study is due to the As being in an unextractable form, i.e. lipids, and thus is not removed from cells during water-based extractions.

Introduction

Arsenic is released into the environment from natural and anthropogenic sources, and contamination has a disproportionate effect on marine ecosystems. Marine macroalgae (seaweeds) are efficient in accumulating elements from the surrounding seawater and can sequester arsenic to reach internal concentrations as high as 150 mg kg^{-1} dry weight (107). Arsenite (As(III)) is thought to accumulate in seaweeds through hexose permeases and aquaglyceroporins in the plasma membrane (in a similar manner to rice plants), but arsenate (As(V)) is thought to enter the cell due to phosphate transporters inability to distinguish between the two anions (49,257,258). Multiple studies have also demonstrated higher arsenate uptake during phosphate limitation (259,260). Through still unclear and likely species-specific mechanisms, the arsenic is incorporated into larger biological molecules such as sugars and lipids. Arsenosugars (AsSugars) are typically the most abundant species found in seaweed, with only small amounts of inorganic, methylated or lipid species present (261). A few brown seaweed species, however, contain higher concentrations of inorganic arsenic (iAs), e.g. *Hizikia fusiforme* (hijiki) and *Laminaria digitata* (Phaeophyceae, Ochrophyta) which can comprise over 50% of total arsenic (37,49,236). Whereas consumption of Hijiki has been discouraged in recent years, *L. digitata* is still commonly eaten, and used as animal feed and fertilizer. There are four main dimethylarsenosugar derivatives found in seaweed, Figure 23, and variations in compositions between species is likely due to differing enzyme systems capable of catalysing the glycosidation reaction responsible for the side chain of the furanose ring (75). It is unclear whether these AsSugars are formed through some detoxification pathway for iAs or are precursors/degradation products of arsenic-containing phospholipids, which contain the same dimethylribose moiety, Fig 1 (43,93,262). It has even been suggested that AsSugars may be produced by bacteria on the surface of the seaweed (263). The majority of evidence points towards detoxification, and a pathway for the biosynthesis of AsSugars was proposed after identifying a gene involved in AsSugar production in the cyanobacterium *Synechocystis* (71). The donation of methyl groups from S-adenosylmethionine (SAM) to arsenite (or arsenate after reduction) is first catalysed by the enzyme SsArsM, before the addition of the deoxyribose moiety by the radical SAM enzyme SsArsS (71,264). It is possible a similar pathway exists in seaweeds, and *Fucus serratus* (Phaeophyceae, Ochrophyta) specifically has been shown to produce DMA and AsSugars as major metabolites after increased exposure to As(V) (263). However, at external As concentrations of 100 µg L^{-1} the authors suggest that conversion of As(V) to As(III) becomes saturated, and As(III) becomes the major metabolite detected at levels high enough to exert toxicity (263).

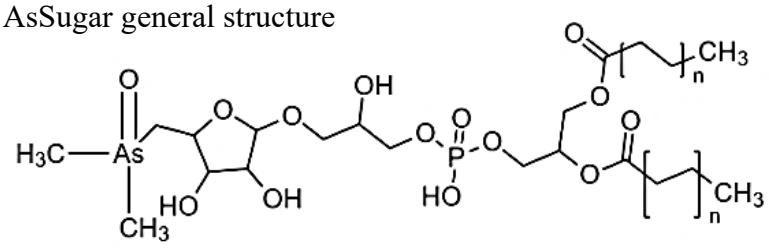
A recent study by Ender et al. used NanoSIMS to investigate the localisation of As within *L. digitata* cells and found that nearly all of the arsenic was present in the cell wall, with only a minor fraction in the internal cellular matrix (37). This would suggest that AsSugars are likely a component of the polysaccharides of the cell wall, and that their production is an efficient way of removing toxic arsenic from inside of the cell.



R-groups

AsSug-Gly = OH
AsSug-SO_3 = SO_3H
AsSug-SO_4 = OSO_3H
AsSug-PO_4 = $OPO_3CH_2CHOHCH_2OH$

A) AsSugar general structure



B) Arsenophospholipid general structure

Figure 23 – A) The structure of the four arsenosugar derivatives typically reported in seaweed. B) The structure of arsenosugar phospholipids reported in seaweed.

The total arsenic concentration has been shown to vary by season and section of thallus for the majority of Phaoephyceae (194,265,266). In *L. digitata* inorganic As specifically has been reported to increase along the thallus from stipe to decaying distal frond, but a similar trend was not observed for *Ascophyllum nodosum* from the same location (236). Spatial variations in arsenic species have also been reported for another member of the Laminariales, *Saccharina latissima*, where the most biologically active tissues contained the highest portions of arsenolipids (4). There is a lack of data available regarding the seasonal variations in AsSugar and arsenolipids (AsLipids) composition, but it is likely these compounds experience seasonal changes as they have been reported to vary with differing nitrate, phosphate, and oxidative stress levels (Pétursdóttir *et al.*, 2016).

The aim of this study was to compare temporal and intra-thallus variations in water-soluble arsenic and total non-polar and polar AsLipids in *L. digitata* to offer potential insight into the metabolism of arsenic. Extractions using fresh, undried sample materials are not often reported in literature, and so the water-soluble speciation before and after drying was compared to determine whether freeze-drying has an impact on the water-soluble speciation (267). The aim of this study was to provide insight into the arsenic metabolism of *L. digitata*, and how the arsenic speciation may fluctuate temporally and spatially within the thallus. Understanding these fluctuations may be useful for aquaculture industry and means harvesting approaches can be adapted to select *L. digitata* biomass that contains the least harmful composition of arsenic speciation.

Experimental

Chemicals and Reagents

Ultrapure water (18.2 M Ω cm) was obtained from a milliQ water dispenser system and was used throughout unless stated otherwise. Ammonium carbonate was used as the mobile phase buffer and was obtained from BDH (UK). Methanol (HPLC grade) supplied by Honeywell (USA) was added to the mobile phase and was used as an extraction solvent. Other extraction solvents dichloromethane and heptane were both obtained from Honeywell. Nitric acid (ROTIPURAN Supra, 69%) supplied by Carl Roth (Germany) and hydrogen peroxide (for trace analysis, ≥30%) from Supelco (Sigma-Aldrich, France) were used for the digestion of samples for total arsenic measurements. Dimethylarsinic acid standards used for external calibration of HPLC-ICP-MS were prepared from a stock solution of dimethylarsinic acid disodium salt (100%) supplied by Argus-Chemicals (Italy) dissolved in utrapure water. The standards for total arsenic measurements used were 1000 mg kg^{-1} As in 2% HNO_3 and were obtained from LabKings (Netherlands). Indium (1000 mg L^{-1}) and germanium (1000 mg L^{-1}) supplied by Peak Performance were used as internal standards for both water-soluble and total arsenic measurements.

Samples and reference materials

Ten individual, attached, *L. digitata* specimens comprising intact holdfast, stipes, and blades, were collected from the low intertidal zone of a beach near Grindavík, Iceland (63.85, -22.31) in February 2022, and again in May 2022. The samples were transported back to the lab in seawater and were washed sparingly with tap water to remove loose sand and salt that could cause chromatographic interferences. Any visible epiphytes were removed manually. All thalli collected were divided into the sections illustrated in Figure 24 and pooled to create samples representative of all ten specimens.

The holdfast and stipe sections were combined due to the small amounts of sample material. The decaying frond was characterised by discolouration and damage, and all other sections were morphologically distinct. Samples collected in February were freeze-dried to constant mass and milled to a fine powder. The samples collected in May were washed and sectioned in the same way as previously described, but the wet material was first homogenised and divided into two sub-samples. The first sub-sample underwent immediate extraction (approximately 2 h after harvesting) without any prior freeze-drying, and the second sub-

sample was freeze-dried until constant mass and homogenised further before extraction. All samples were milled into a fine powder to ensure subsamples were representative of the ten thalli that were combined.

Certified reference materials 7405-b hijiki (National Meteorology Institute of Japan, Japan) was analysed in duplicate during each analytical run for both total arsenic and water-soluble arsenic measurements. CRM 7405-b also underwent the same sequential extraction procedure as samples. A sample of freeze-dried *A. nodosum* from a previous study was collected in Eskifjörður, Iceland and was used as an internal laboratory reference throughout.

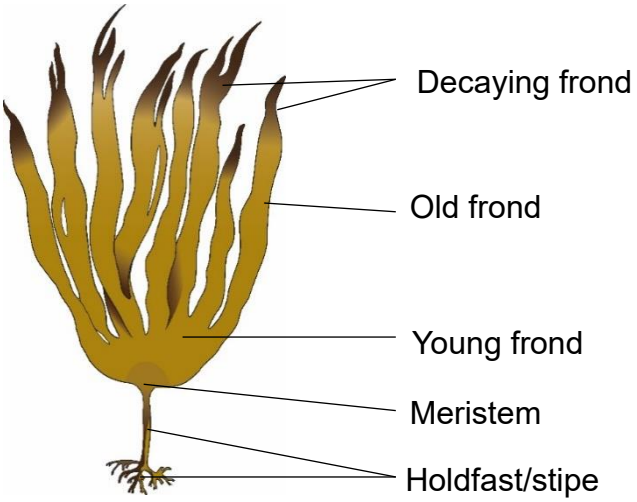


Figure 24 – The sectioning of *Laminaria digitata* samples.

Total arsenic determination

The total As in samples was determined using ICP-MS. In triplicate, 0.2 g of sample material were weighed into quartz digestion tubes before adding 1 mL of nitric acid and 1 mL of hydrogen peroxide. Digestion was performed using an UltraWave Microwave digestion system (Milestone, Italy) and digests were quantitatively transferred to polypropylene falcon tubes and diluted to 50 mL with milliQ water.

Extraction

Arsenosugars. Extractions were performed by adding 0.2 g of dry sample material and 10 mL of water to 50 mL polypropylene tubes in duplicate. For the fresh samples 1 g of material was used, and the extraction was performed approximately 2 h after collection of the thalli. Samples were mechanically shaken for 2 h before centrifuging at 4000 rpm for 10 min. A 1 mL aliquot of the supernatant was transferred to plastic microcentrifuge tubes for further centrifugation at 15000 rpm for 5 min and the samples directly analysed on HPLC-ICP-MS. The total As was also determined in the extracts using the method previously described for digestion.

Sequential extraction. Method based on that described in elsewhere (5). Briefly, in triplicate 0.2 g of dry sample material was extracted twice, each time using 5 mL aliquots of heptane in

borosilicate-glass scintillation vials, with the first extraction left to stand for two hours after swirling to wet the entirety of the sample material. The second extraction was allowed to stand for 30 min, and the supernatants were then combined and left to stand until evaporated to dryness. The sample residue was dried before the extraction procedure was repeated with two 5 mL aliquots of a DCM/MeOH (2:1) mixture. The dried heptane and DCM/MeOH supernatants were then transferred to digestion vials by redissolving in 200 µL of the respective solvent, this was repeated 3 times to ensure complete transfer. The solvents were then re-evaporated before digestion. The final extraction was performed by transferring the dried residual sample material to 50 mL polypropylene tubes and adding 10 mL of water. The mixture was mechanically shaken for 2 h and produced a slurry that was centrifuged at 4000 rpm for 15 min before removing the supernatant. A 1 mL aliquot of the water extract was digested. The residue was washed twice with 15 mL of water to avoid over-estimation of the remaining arsenic, and a portion was digested to analyse for total As content.

Analyte quantification

Total As. Quantification of total arsenic was carried out using an Agilent 7900 ICP-MS with octopole collision cell in He gas mode with an external calibration in the range 0-200 µg L^{-1} prepared by serial dilution. Standards were all prepared in 2% HNO_3. The ICP-MS underwent automatic tuning before each use.

Water-soluble As. Speciation analysis of the water-soluble arsenic species was performed using an Agilent Infinity II 1290 HPLC coupled to an Agilent 7900 ICP-MS in no gas mode. The instrument was manually tuned before each use with a 50 µg L^{-1} As solution, and separation of the species was achieved using a Hamilton PRP-X100 column (250 x 4.1 mm, 10 µm) and corresponding guard column. An isocratic elution with 20 mM ammonium carbonate (3% MeOH) was used, and an internal standard of 50 µgL^{-1} Ge was introduced post-column to normalise counts for fluctuations in plasma. Masses 77 and 82 were monitored as well as 75 to check for $Ar^{40}Cl^{35}$ interferences on mass 75. Data acquisition and manual integration of peaks was performed using Agilent Masshunter software. The instrumental parameters are shown in Appendix T5.

Data treatment

Two-way, paired t-tests were performed on the total As measurements to determine whether differences in mean concentrations between months and thallus section were significantly different, where a $p \leq 0.05$ was considered statistically significant.

Quality Control

Identification of the methylated and inorganic arsenic species were performed using spiking experiments. The identification of AsSugars was based on retention time matching with an internal laboratory reference material and hijiki 7405-b. Hijiki has certified values for two of the four main arsenosugars derivatives, and the lab reference material refers to a sample of *Ascophyllum nodosum* (Phaeophyceae, Ochrophyta) from a previous study that underwent identification using LC-MS/MS (Quantiva, Thermo), Figure 25. This sample was chosen as a reference material as it contained all four arsenosugars derivatives. Column recoveries (the sum of the quantified As species divided by total As in the extract) were acceptable and

calculated to be between 83 and 115% for all chromatographic runs, suggesting all extracted species were eluted from the column. The reference materials also showed good agreement with certified values for both total arsenic and arsenate, Table 17. The LOD and LOQ for the quantification of water-soluble As were found to be 1.4 µg kg^{-1} and 4.3 µg kg^{-1} dry weight correspondingly. These values were calculated as the standard deviation of the concentration of three 0.5 µg L^{-1} calibration standards, multiplied by an average dilution factor. The LOD and LOQ for the quantification of total arsenic was calculated from the external calibration curve multiplied by an average dilution factor and were found to be 0.02 and 0.05 mg kg^{-1} respectively. The detection and quantification limits of both methods were found to be comparable to those reported by groups using similar techniques in the literature (268–270).

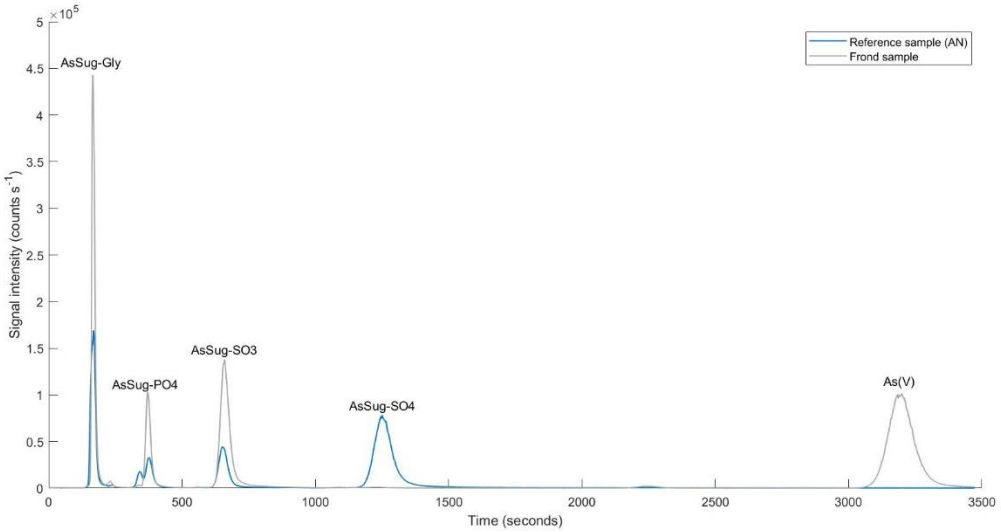


Figure 25 – The sample of *A. nodosum* used as an internal laboratory reference material and a *L. digitata old* frond sample.

Results and Discussion

Reference materials

Certified reference material 7405-b (hijiki) was analysed alongside samples during total and speciation measurements, Table 17.

Table 17 – The concentrations of total As and As(V) found in the reference material analysed alongside samples (191). Errors are 1 SD, where n = 4.

Sample	**Total As (mg kg^{-1})**	**AsSug-gly/ As(III) (mg kg^{-1})**	**AsSug-PO_4 (mg kg^{-1})**	**AsSug-SO_3 (mg kg^{-1})**	**AsSug-SO_4 (mg kg^{-1})**	**As(V) (mg kg^{-1})**
7405-b (hijiki) (n=4)	45.2 ± 2.46 (*49.5*[A] ± 1.00)	0.70 ± 0.07 (0.45[B] ± 0.02)	0.26 ± 0.07 (0.20[B] ± 0.01)	0.18 ± 0.03 (0.16[B] ± 0.01)	1.75 ± 0.06 (1.41[B] ± 0.04)	23.2 ± 1.57 (*24.4*[A] ± 0.7)

[A]*Certified values*, [B]literature values (191).

The values for hijiki show good agreement with the certified values for total As and As(V). The values for arsenosugars show relatively good agreement with the literature values with

the exception of the AsSug-gly, where the value is higher than that which has been previously reported. This is due to the fact that As(III) was not oxidised during the extraction and co-eluted with AsSug-gly during analysis, and as such these analytes were quantified as the sum of both.*Total As*

In general, lower levels of total As were detected in *L. digitata* samples collected in May (39.2-74.5 mg kg^{-1}) than in those collected in February (72.6-151 mg kg^{-1}). Increasing As concentrations from stipe to decaying frond were noted in May but not in February samples, Figure 26. Previous studies have also reported higher concentrations of internal As during winter months for other brown seaweeds, *Alaria esculenta* and *Saccharina latissima* (4). Typically seaweeds are likely to accumulate nutrients during winter when nitrate is most abundant in ocean and not a limiting factor for growth (212). Increased nitrate uptake has also been shown to increase phosphate uptake, which would explain the increase in As during February if both are accumulated by same method without distinction (49,271).

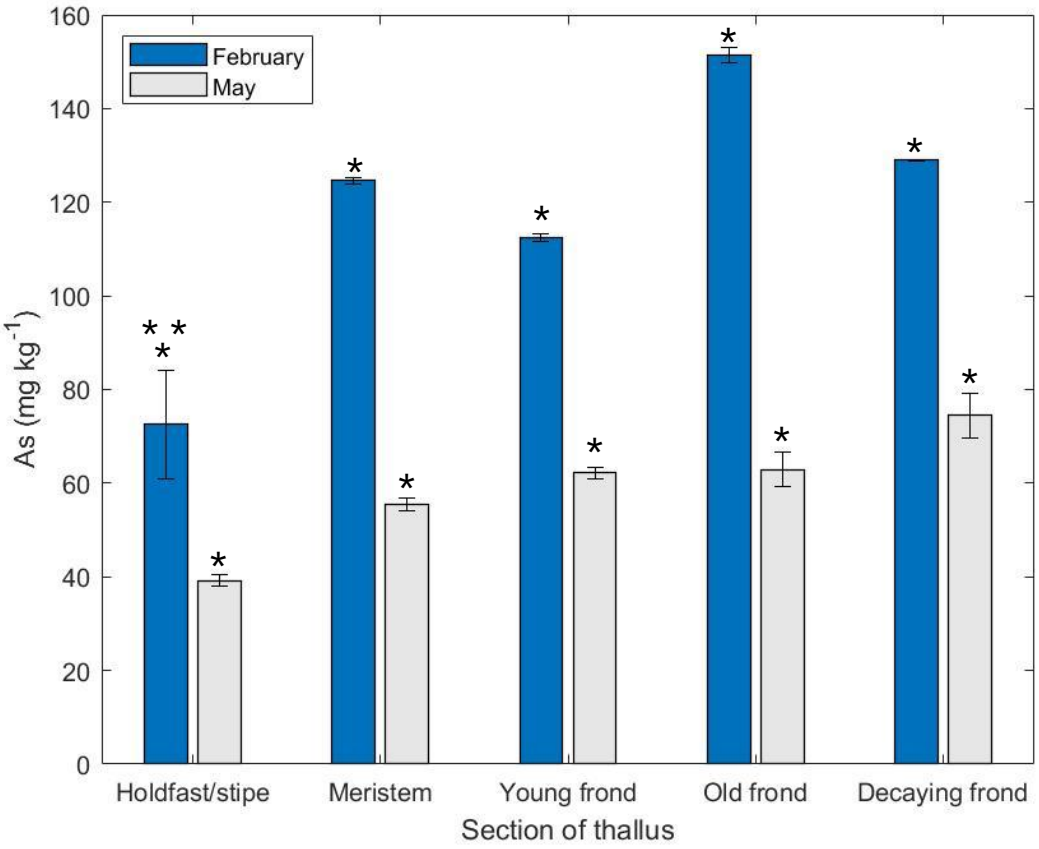


Figure 26 – The total As concentrations in sections of *L. digitata* thalli in February and May 2022. The error bars represent 1 SD, where n=3. Asterisk (*) denotes significant differences between months ($p<0.05$), double asterisks (**) denotes significant differences between adjacent thallus section of same month ($p<0.01$).

Temporal and intra-thallus differences in water-soluble As

The AsSugars contents of brown algae have been shown to vary greatly between seasons and sections of seaweed thallus for some species (Lai et al., 1998). When studying the water-soluble speciation in each section of the thallus individually, Figure 27. AsSug-SO_3 was found to be the dominant AsSugar in all sections for both months, with the exception of the decaying frond in February. The meristem section had the highest concentration of AsSug-SO_3 during both months. The levels of AsSug-PO_4 showed no obvious trend with regards to season or thallus section, and concentrations were between 4.21 mg kg^{-1} and 13.4 mg kg^{-1} for all samples. The AsSug-SO_4 concentration was below LOD in all *Laminaria* samples, and only low levels of DMA and MA were present. DMA and MA were identified using spiking experiments, Appendix F6. The extraction efficiencies were lower in the samples collected in February (47-83%) than those in May (95-116%). As no oxidising agent was used in the extraction solution, AsSug-gly and As(III) co-eluted near the start of the chromatographic run, so were therefore quantified as the sum of both analytes. In samples collected in February, this sum was found to be highest in the decaying frond (14.8 mg kg^{-1}) and low in all other sections (1.97 – 3.19 mg kg^{-1}), whereas the opposite trend was observed for May where the sum was 2.3 mg kg^{-1} in the decaying frond, and 11.5-17.1 mg kg^{-1} in the other thallus sections. The observed differences are likely attributable to AsSug-gly, as the As(III) in all samples was found to ≤0.15 mg kg^{-1} when samples were analysed specifically for this analyte using a different method (110), Appendix T6 and Appendix F7. Another study has also previously reported reported low concentrations of As(III) in other *Laminaria spp*. (273). As(III) is also more cytotoxic than As (V), and is quickly methylated as a detoxification mechanism by SAM enzymes (262). Since winter is when the highest levels of growth occur in the non-decaying parts of the thallus, the AsSug-gly is not likely be a product of detoxification as the concentration would then be expected to increase with increasing total As.

The meristem section of the thallus consistently had the highest concentration of unidentified compounds regardless of season, Appendix T7. Although the unknown compounds made up only a minor fraction of the total As (<2.4 %), based on the retention times these compounds are likely small, methylated DMA analogues such as dimethylarsinoyl acetate (DMAA), dimethylarsinoyl ethanol (DMAE), or dimethylarsinoyl propionate (DMAP), Appendix F8. Wolle et al. previously reported DMAA and DMAP eluting around a similar retention time to DMA using anion exchange chromatography with a similar mobile phase composition (108). DMAE in particular is known to be a product of AsSugar degradation under anaerobic conditions, and this could have potentially been produced by bacteria on surface of *L. digitata* (244). Irrespective of season, there appears to be a trend of increasing As(V) concentration from holdfast/stipe to the decaying frond, similar to results previously for *L. digitata* (236). It has been suggested that arsenate is bound to fucoidans in the cell wall in the form of arsenoesters – not dissimilar to the sulfuric acid esters that form with fucoidan – which would immediately be hydrolysed upon extraction with water (37). This may explain why most of the As(V) is found in the fronds, as the fucoidan content is typically highest in this thallus section with the exception of reproductive tissues (274). Mature parts of the seaweed also typically contain higher levels of fucoidan, and the polysaccharide composition is known to vary seasonally which could account for the vast differences in arsenate between February and May samples (275). However, this offers no explanation as to why *L. digitata* in

particular can accumulate such high levels of As(V) compared to other species within the Laminariales (276).

The As(V) concentrations were between 0.78-59.3 mg kg^{-1}, which alone is high enough to be of relevance to food safety concerns (Pétursdóttir et al. 2015).Whilst there are currently no Europe-wide regulations for inorganic arsenic in seaweed products for human consumption, France have implemented a limit of 3 mg kg^{-1} for algae condiments and there is a 2 mg kg^{-1} limit in the EU for seaweed products to be used as animal feed (*Directive 2002/32/EC of the European Parliament*, 2002; CEVA, 2019).The majority of these samples exceed both of these regulations by many multiples with their As(V) content alone.

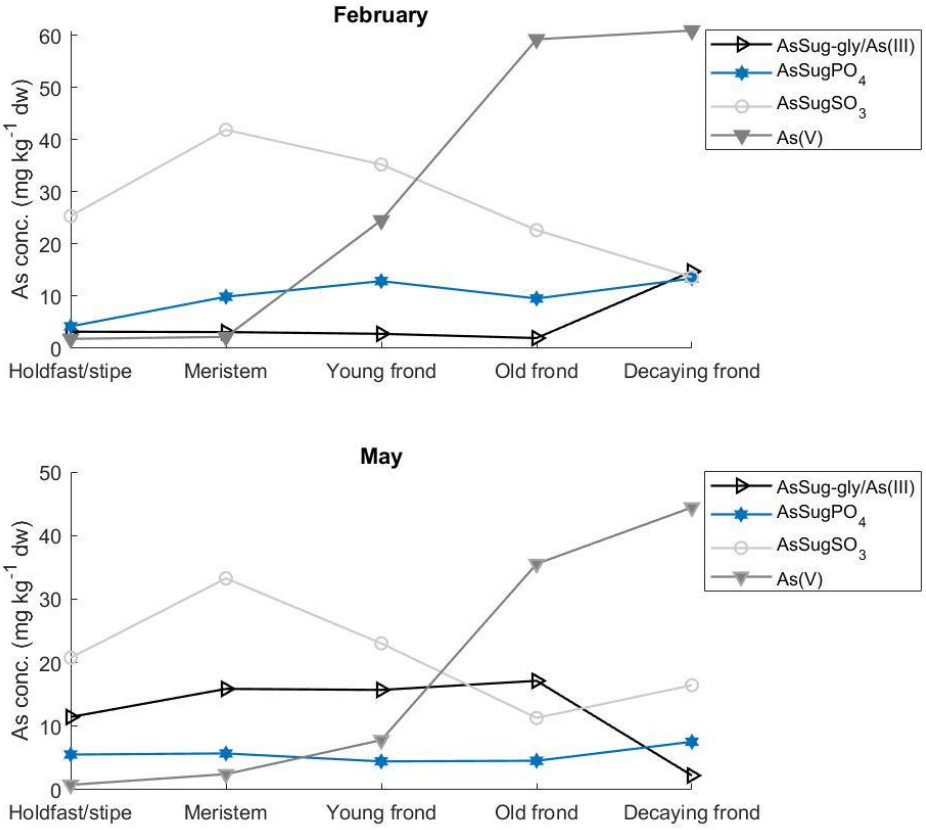


Figure 27 – The average concentrations of water-soluble As species in February and May 2022 in different sections of the *L. digitata* thalli. Samples were analysed in duplicate (n=2) and all concentrations are expressed per kg of dry sample weight.

Comparison of fresh and freeze-dried seaweed from May

As very few studies report extractions using materials from fresh samples, the speciation of water-soluble As in fresh material was analysed in an effort to determine the effects of freeze-drying on the speciation. When comparing the extraction efficiency for sections of the thallus in fresh and freeze-dried samples from this study, Figure 28, it is apparent that it is much lower for fresh samples. On average the extraction efficiency when using fresh sample material was 68% compared to 103% for freeze-dried, suggesting that freeze-drying is potentially required to destroy the cell wall and membrane, and release the AsSugars quantitatively. The lower extraction efficiency observed using fresh samples could also be due to As being in an unextractable form using an aqueous extractant, i.e. as Aslipids, and thus is not removed from cells during water-based extractions. Although more As was extracted from freeze-dried material, the intra-thallus trend for AsSug-SO_3 is similar for both fresh and freeze-dried material, suggesting that the extraction of this analyte is largely dependent on the degree of homogenisation the sample has undergone. It is unlikely the AsSugars are stored inside the cells, as low levels of As in the cytosol have been reported using NanoSIMS (37). Instead, it is likely the AsSugars are bound to the cell membrane. A similar trend is seen for AsSug-gly/As(III) and AsSug-PO_4 with the exception of the decaying frond and meristem, respectively. Again, there is a clear trend of increasing As(V) along the thallus from the holdfast and stipe material to the decaying frond, but only the old and decaying fronds show markedly different extraction efficiencies between the two materials. When considering the holdfast/stipe - a section which is difficult to homogenise due to its tough, fibrous structure – similar levels of As(V) were extracted from both fresh and freeze-dried samples (0.63 and 0.78 mg kg^{-1}). This is not true for the old frond and decaying frond sections, where an approximately 2-fold improvement in extraction efficiency was observed after freeze-drying. The higher levels of As(V) in the older frond sections may mean that not all of the As(V) could be bound by the polysaccharides in the cell wall and may be inside the cell. Thus, the freeze-drying may be required to release all As(V) that is present.

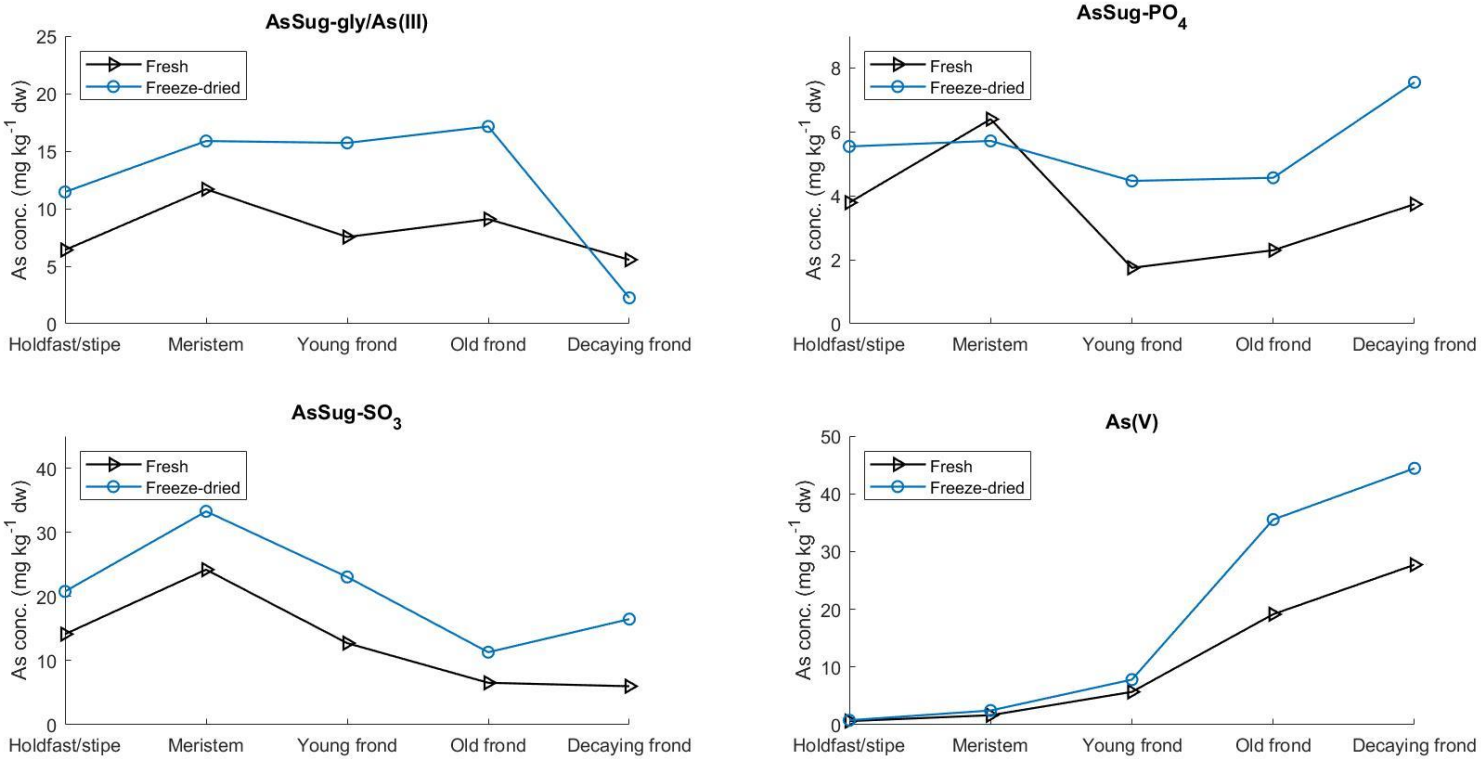


Figure 28 – Comparison of the concentrations of water-soluble As species extracted from sample material collected in May, without and with freeze-drying. All concentrations are expressed per kg of dry weight.

Temporal and intra-thallus variations in As distribution

Analysing the water-soluble As species alone is not enough to understand the full extent of seasonal or spatial changes in arsenic metabolism. Thus, the total arsenic content of fractions extracted with solvents of differing polarity was determined, as well as the quantification of total As in the unextractable As fraction. The total water-soluble arsenic content was found to follow the same trend as the arsenate for both months, Table 18. The concentration of water-soluble As extracted during the sequential extraction scheme was lower than during the speciation analysis, which is most likely due to the order of extractions. During the sequential extraction the DCM/MeOH step will extract a small amount of water-soluble species into the solvent, thus, when performing quantification of the individual water-soluble As species, it is crucial that the water extraction is performed first to give accurate quantification (5). Conversely, if the water extraction is performed first, there is a risk of hydrolysis of lipophilic arsenic species to polar, water-soluble species.

Recoveries were between 80 ± 11% and 106 ± 8% (n=3). The levels of polar AsLipids were significantly higher during February than in May, Figure 29. Similar trends were seen in both months, with the exception of the holdfast/stipe tissue in February, where the meristem and young frond both had higher levels than the surrounding frond tissues. These two parts are the youngest and metabolically most active thallus sections and are associated with new growth (278). Therefore, if polar AsLipids are by-product of biological activity, the highest concentrations would be expected in these areas. Residual As was generally lowest in the holdfast and highest in the frond sections of the *L. digitata*. The residual As was higher in February where a maximum of 26.1 mg kg^{-1} was found in old frond compared with maximum of 10.1 mg kg^{-1} in the young frond in May. The unextractable As species have been suggested to be As(III) bound to thiol groups, e.g., to cysteine groups in lipoproteins within cells. As(III) has been shown to bind strongly to multiple thiol groups in proteins in *Escherichia coli* bacteria - a similar situation could occur in seaweed (252). Stronger extraction methods have been able to recover the recalcitrant arsenic in the form of As(V) (117)·(179). The non-polar AsLipids accounted for <0.42% of totAs, and concentrations were generally below 0.1 mg kg^{-1}, with the exception of the old frond during May where the non-polar As was 0.26 mg kg^{-1}. The speciation of arsenic extracted by non-polar solvents has previously been reported in the literature for fish meal, where arsenic-containing hydrocarbons were found to be the major constituent of a hexane extract, followed by small amounts of arsenic-containing fatty acids (84). These species are also commonly reported, albeit in higher concentrations, in the DCM/MeOH extracts of seafood and algae (4,87,279,280).

Table 18 – The distribution of As in sections of the *L. digitata* thalli in freeze-dried samples from February and May 2022.Errors are 1 SD, where n=3. The percentage fraction contributes to total As concentration is also listed in brackets.

Month	**Thallus section**	**Non-polar AsLipids (mg kg^{-1})**	**Polar AsLipids (mg kg^{-1})**	**Water-soluble As (mg kg^{-1})**	**Residual As (mg kg^{-1})**	**Total As (mg kg^{-1})**	**Recovery (%)**
February	Holdfast/stipe	0.02±0.01 (<1%)	32±1.6*,** (43%)	36±0.9** (49%)	8,3±0.5*,** (11%)	73±2.9	103±2
	Meristem	0.03±0.01 (<1%)	11±0.6* (9%)	74±5.1** (59%)	15±0.6* (12%)	125±7.5	80±11
	Young frond	0.01±0 (<1%)	11±0.7* (10%)	83±1.7* (74%)	20±5.4* (18%)	112±1.0	102±12
	Old frond	0.02±0 (<1%)	9.2±0.1*,** (6%)	92±6.1* (60%)	26±2.1*,** (17%)	151±24	84±10
	Decaying frond	0.06±0.03** (<1%)	6.1±0.53*,** (5%)	93±6.7* (72%)	15±1.6 (12%)	129±13	89±3
May	Holdfast/stipe	0.02±0.01* (<1%)	2.3±0.16** (6%)	33±2.6** (84%)	5.3±1.3 (14%)	39±1.3	104±9
	Meristem	0.07±0.02 (<1%)	6.7±0.04** (12%)	42±3.3** (75%)	8.3±1.8** (15%)	55±1.3	102±3
	Young frond	0.03±1.11 (<1%)	5.1±0.7** (8%)	50.4±5.1 (81%)	10±1.2** (16%)	62±1.3	106±8
	Old frond	0.25±0.04* (<1%)	2.2±1.0** (4%)	54±0.2 (85%)	8.0±0.4** (13%)	63±3.7	102±8
	Decaying frond	0.13±0.06** (<1%)	3.7±1.2** (5%)	65±1.9** (87%)	8.4±0.3** (11%)	75±4.7	103±9
CRM	7405-B (hijiki)	<LOQ (<1%)	5.6±0.2 (11%)	22±0.3 (43%)	20±0.9 (40%)	*49.5*±1.0 [A]	95±1

[A]*Certified value.* Asterisk (*) denotes significant differences between months ($p<0.05$), double asterisks (**) denotes significant differences between adjacent thallus section of same month ($p<0.05$).

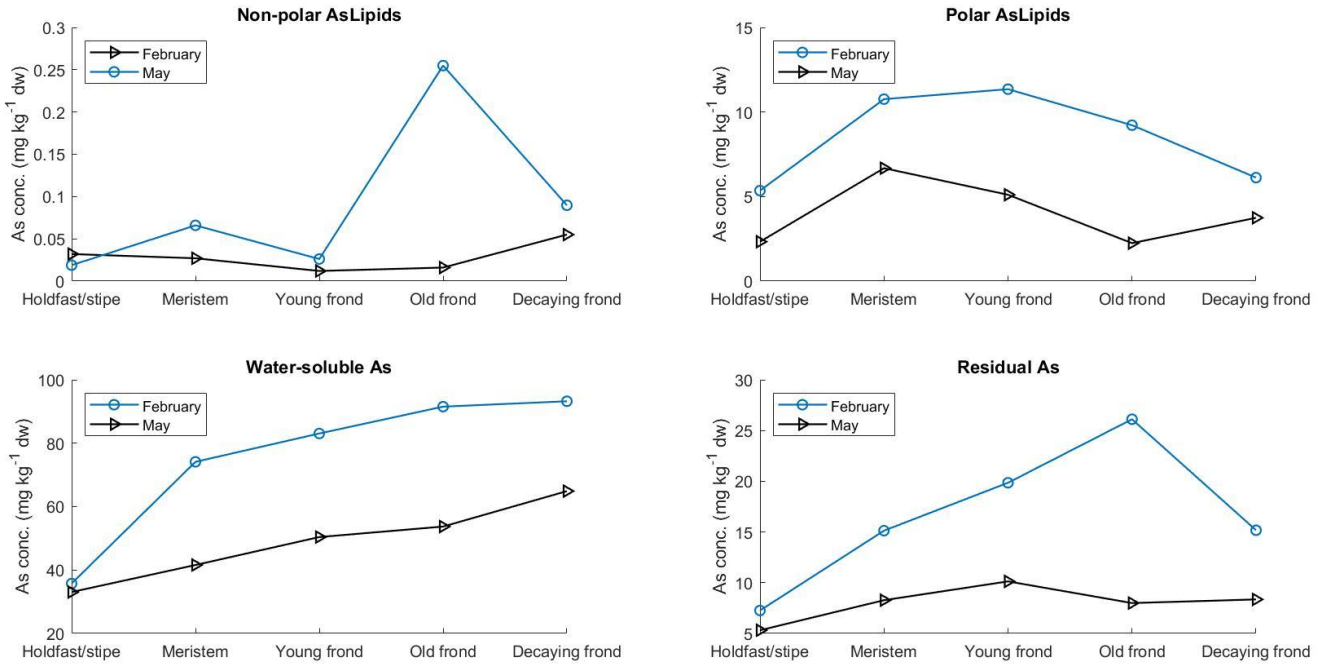


Figure 29 – The arsenic fractionation of fresh material collected in May (n=3). All concentrations are expressed per kg of dry sample weight.

To summarise, lower levels of total As were detected in the samples collected in May (39.2-74.5 mg kg^{-1}) compared to those in February (72.6-151 mg kg^{-1}) where all sections were significantly different between months ($p<0.01$). The concentration of arsenate was found to consistently increase along the thallus from the holdfast/stipe to the decaying fronds in both months, and AsSug-SO_3 was the dominant AsSugar in the majority of samples. The concentration of As(V) was consistently found to be lowest in the holdfast/stipe section (0.78- 1.82 mg kg^{-1}) and increases along the thallus with the highest values always found in the decaying fronds (44.4-61.0 mg kg^{-1}) – a trend which could potentially be explained by the differing polysaccharide content in each section. The extraction efficiency was lower in fresh material than for freeze-dried, 64-77% and 95-116% respectively and depended on thallus section. Similar intra-thallus trends for both fresh and freeze-dried materials were observed when studying the concentrations of the extracted AsSugars individually, whereas this was not seen for As(V). This suggests that AsSugars may require lyophilisation to be quantitatively released from cells and that they may be bound in the cell membranes. Levels of water-soluble, polar lipid-soluble, and residual levels of As were generally highest in February, and the non-polar lipid-soluble As accounted for <0.42% of totAs in all samples. The higher levels of polar AsLipids in February (6.12-31.5 mg kg^{-1}) compared to those in May (2.24-6.68 mg kg^{-1}) are in line with the higher fat contents typically found in seaweed during winter.

This study is the first to report not only temporal, but intra-thallus variations in the water-soluble As speciation and distribution of As in *L. digitata*. This dataset adds significantly to our understanding of arsenic metabolism of *L. digitata*, and how this may fluctuate temporally and spatially within algal thalli. Future research should focus on analysing the speciation of polar lipid-soluble As extracted from fresh samples to determine if the differences in extraction efficiency observed in this study were due to the As being in an unextractable form, i.e. lipids, and thus is not removed from cells during water-based extractions.

3.6 Paper V – A Comprehensive Analysis of Water-soluble Arsenicals in Icelandic Macroalgae

Rebecca Sim [A B*] and Ásta H. Pétursdóttir [A]

Author Affiliations:
[A] Matís, Vínlandsleið 12, Reykjavík 113, Iceland
[B] Faculty of Physical Sciences, University of Iceland, Dunhagi 7, Reykjavík 105, Iceland

*Correspondence to: rebecca@matis.is

Abstract

Seaweeds are known to accumulate high levels of arsenic, which may be subsequently metabolised to small, methylated compounds or become incorporated into large biological molecules such as arsenosugars. The mechanism of arsenosugars production in macroalgae is still largely unknown but has been shown to vary between algae species, thallus section and season. This study reports the arsenic speciation of brown, red and green macroalgae collected in Iceland across two sampling months (May and February). Arsenosugars were found to account for the majority of the extracted arsenic in all seaweed samples (>50%), and only small amounts of DMA (<LOQ-0.41 mg kg^{-1}) and MMA (<LOQ-0.07 mg kg^{-1}) were present. The sulphate and sulfonate ribose derivatives were the dominant arsenicals in brown algae, whereas the phosphate ribose was generally the dominant arsenic compound in red and green algae. The majority of seaweed species appear to have the ability to synthesise all four arsenosugar derivatives, however it is unclear why some are produced to higher abundances than others. The production of AsSugars may be an efficient way of excluding As from intracellular matrix, or they may serve as useful components in the cell wall similar to polysaccharides in the cell wall matrix, as extremely high levels of arsenosugars (<100 mg kg^{-1}) can be tolerated by the algae with no ill effects. The glycerol and phosphate riboses were detected in every sample analysed, which might suggest that AsSug-gly is the starting point for AsSug-PO_4, which could be formed by AsSug-gly binding to phosphate-containing lipids in the cell membrane. Further studies should use a combination of imaging and speciation techniques to fully investigate how AsSugar derivatives may be formed, and through which mechanisms.

Introduction

Arsenic (As) is a naturally occurring metalloid introduced into the marine environment through the erosion of mineral-bearing rocks and anthropogenic activities. Whilst the concentration of As in the open ocean is expected to range from 1 to 3 µg L^{-1} (281,282), macroalgae can accumulate levels many magnitudes higher than the surrounding environment, with As concentrations as high as 130 mg kg^{-1} dry weight reported in *Laminaria digitata* and *Saccharina latissima* (4,236). The accumulation of As in seaweed has been shown to vary between species and season, and may be influenced by temperature, pH, and abundance of other nutrients in seawater (61,272,283). As such, it would be expected that the range of arsenic species found in seaweed, Figure 30, will also display a dependence on these variables.

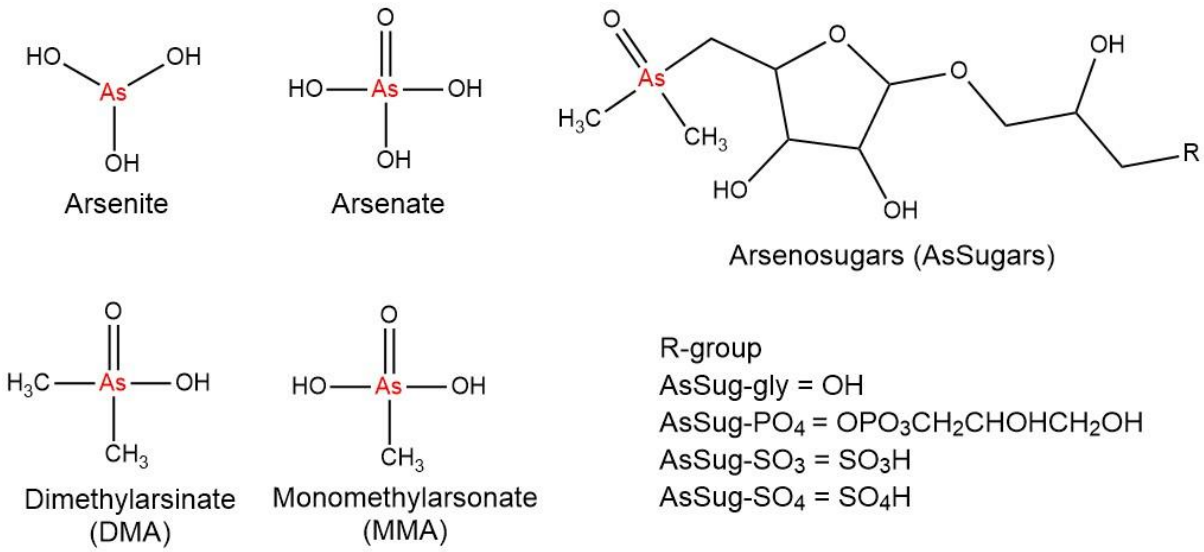


Figure 30 – The most common water-soluble arsenic species reported in seaweed.

The inorganic forms arsenite and arsenate, collectively referred to as inorganic arsenic (iAs), are the naturally occurring forms found in the environment. The iAs content of seaweed is generally low, but in certain species such as *L. digitata* and *Sargassum fusiforme*, iAs may account for more than 50% of the total As (37,276,284). Arsenite has been shown to enter cells through aquaglyceroporin channels in eukaryotes (285) and arsenate uptake in algae is thought to occur through phosphate transporters, as both anions are analogous in structure (286). Competitive uptake between As and P has also been demonstrated in incubation experiments with *Fucus gardneri*, but similar results were not reported in experiments with *Ascophyllum nodosum*, suggesting more than one arsenate uptake mechanism may exist (287,288). iAs exposure in humans has been linked to bladder, skin, and lung cancers (53); Though the mode of toxicity is yet to be fully understood, proposed mechanisms include genotoxicity, inhibition of DNA repair and co-carcinogenesis (60). After assimilation by algae, iAs may be metabolised through direct methylation of arsenite or reduction and subsequent methylation by a S-adenosyl methionine (SAM) methyl donor and methyl transferase enzyme (289). Although it is commonly accepted that organisms use methylation as a detoxification mechanism, there is controversy over whether this holds true for As, as the methylated species monomethylarsonate (MMA(V)) and dimethylarsinate (DMA(V)) both display some degree of toxicity, e.g., both MMA(V) and DMA(V) induced mitotic arrest in cultured hamster cells (290). The trivalent forms, MMA(III) and DMA(III), also display considerable genotoxicity, however, are only intermediates in the methylation process (291). DMA(III) is also thought to be an intermediate in the production of arsenosugars (AsSugars) - the most abundant group of arsenicals found in seaweed – which consist of a 5-dimethylarsinoyl ribose and 2'-side chain with differing R-groups. The exact mechanism of AsSugar production in seaweed is unknown, but in *Synechocystis sp*. a gene encoding the enzyme responsible for catalysing the adenosylation of DMA(III) to 5'-deoxy-5'-dimethylarsinoyl-adenosine (DDMMA) has been sequenced (292). Cleavage of the adenosine moiety and further glycosidation reactions are thought to produce the final ribose structures, and differences in R-groups are likely due to species specific enzymes able to carry out the glycosidation reaction (75). For example, Laminariales typically contain high levels of the sulfonate ribose (AsSug-SO_3) but no sulphate ribose (AsSug-SO_4), whereas Fucales tend to

contain both (49). In vitro experiments with AsSugars have shown no acute toxicity (76), however there is concern over toxicity from chronic exposure of arsenosugars (77).
This study reports the water-soluble arsenic speciation for a range of brown, red and green seaweeds collected from two locations in Iceland during two different seasons. By investigating variations in water-soluble arsenicals between different species, sections of thallus and seasons, this study aims to provide insight into the metabolism of As by seaweeds, and the mechanisms by which AsSugars are formed and stored. Additionally, the results from this study may serve as occurrence data to aid legislative bodies in assessing the risk of chronic exposure to AsSugars from consumption of seafood – something which is severely overlooked with regards to the current regulations regarding arsenic in foodstuffs.

Experimental

Chemicals and standards

All chemicals used were of analytical grade or higher unless otherwise stated. Ultrapure water (18 Ω cm) was obtained from a milliQ water dispenser (Millipore) and was used throughout. Nitric acid (ROTIPURAN Supra, 69%) was purchased from Carol Roth (Germany) and hydrogen peroxide (for trace analysis, ≥30%) was obtained from Supelco (Sigma-Aldrich, France). Ammonium carbonate and methanol (HPLC-grade) were purchased from BDH (UK) and Honeywell (USA) respectively. Dimethylarsenic acid standards used for the quantitation of water-soluble arsenic species were prepared from dimethylarsinic acid disodium salt (100%) supplied by Argus-chemicals (Italy) and diluted as necessary. An arsenic stock (1000 mg kg^{-1}, 2% HNO_3) used for the external calibration of total arsenic concentration and cross calibration of HPLC-ICP-MS standards was purchased from LabKings (Netherlands), and indium and germanium stock solutions (1000 mg kg^{-1}, 2% HNO_3) were obtained from Peak Performance (CPI International, USA). Disodium methyl arsonate hexahydrate (99.5%) purchased from Chem-Service (USA) and disodium hydrogen arsenate heptahydrate supplied by BDH (UK) were both used in spiking experiments to identify MMA(V) and As(V).

Samples and reference materials

Briefly, several species of brown, red and green macroalgae were collected from two locations in Iceland (Kjalarnes and Grindavík) during May 2021 and February 2022, Table 4. All brown and red thalli were harvested with the holdfast and stipe intact, and specimens were transported to the lab in plastic bags where samples were rinsed sparingly with tap water. Any visible epiphytes were removed manually using a stainless-steel knife rinsed with a 2% citric acid in EDTA solution and ultrapure water. Brown macroalgae *spp.* have large thalli with distinct anatomies, and so were sectioned into anatomical parts e.g., frond or sporophyll, Appendix F1. The holdfast and stipes were combined due to the lack of sample material.

Certified reference material 7405-b (hijiki) was obtained from the National Meteorology Institute of Japan and was analysed in duplicate alongside each batch of samples for both speciation and total arsenic measurements.

Sample preparation

Total As. In triplicate, 200 mg of sample material was weighed into 12 mL digestion vials before the addition of 1 mL of nitric acid and 1 mL of hydrogen peroxide. The vials were then transferred to an Ultrawave Microwave Digestion System (Milestone, Italy) for digestion, as described in previous studies (61). Digests were then transferred to polypropylene tubes, the final volume made up to 50 mL with water and analysed directly.

Water-soluble As extraction. In duplicate, between 100 and 200 mg of sample material was weighed into sterile 50 mL falcon tubes before adding 10 mL of 3% (v/v) hydrogen peroxide solution. This partial speciation approach was used to simplify quantification of iAs by conversion of As(III) to As(V), and to convert potential thiolated arsenic species to their oxo-forms. The sample and solvent mixtures then underwent mechanical shaking overnight at room temperature. Mixtures were centrifuged at 4000 rpm for 15 minutes and 1 mL aliquot of the supernatant transferred to microcentrifuge tubes and further centrifuged at 15000 rpm for 5 minutes. The final supernatant was analysed directly. A 0.5 mL aliquot of the final supernatant was diluted to 10 mL with 0.2 mL of nitric acid and water for total arsenic analysis to calculate column recovery.

Instrumental parameters

Total As. The total As concentrations for this sample set have previously reported (293). Briefly, the quantification of total As was carried out using an Agilent 7900 ICP-MS and Agilent SPS 4 autosampler, Appendix T8. The collision cell of the ICP-MS was pressurized with He gas (5 mL min^{-1}), and a Ge internal standard (1 mg L^{-1}, 1% HNO_3) was introduced post-autosampler throughout the duration of the analysis. An external calibration was used to quantify concentrations of total As.

Water-soluble As Speciation. The method used for the analysis of water-soluble arsenicals was based on a previously reported method with minor modifications (110). Speciation analysis was carried out using an Agilent 1260 Infinity II coupled to an Agilent 7900 in no gas mode, Appendix T9. A Hamilton PRP-X100 anion exchange column (5 µm; 250 mm x 4.6 mm) and an ammonium carbonate mobile phase gradient (A:0.5 mM 3% MeOH, B:50 mM 3% MeOH) were used to separate the analytes. The pH of the mobile phase was adjusted to 9.2, rather than the previously reported 9.3, to achieve better separation of MMA and other unidentified co-eluting analyte, Figure 31. Although the first pKa value of As(III) is 9.23, oxidation to As(V) by hydrogen peroxide in the extraction solution ensures that As(III) does not elute in the void volume.

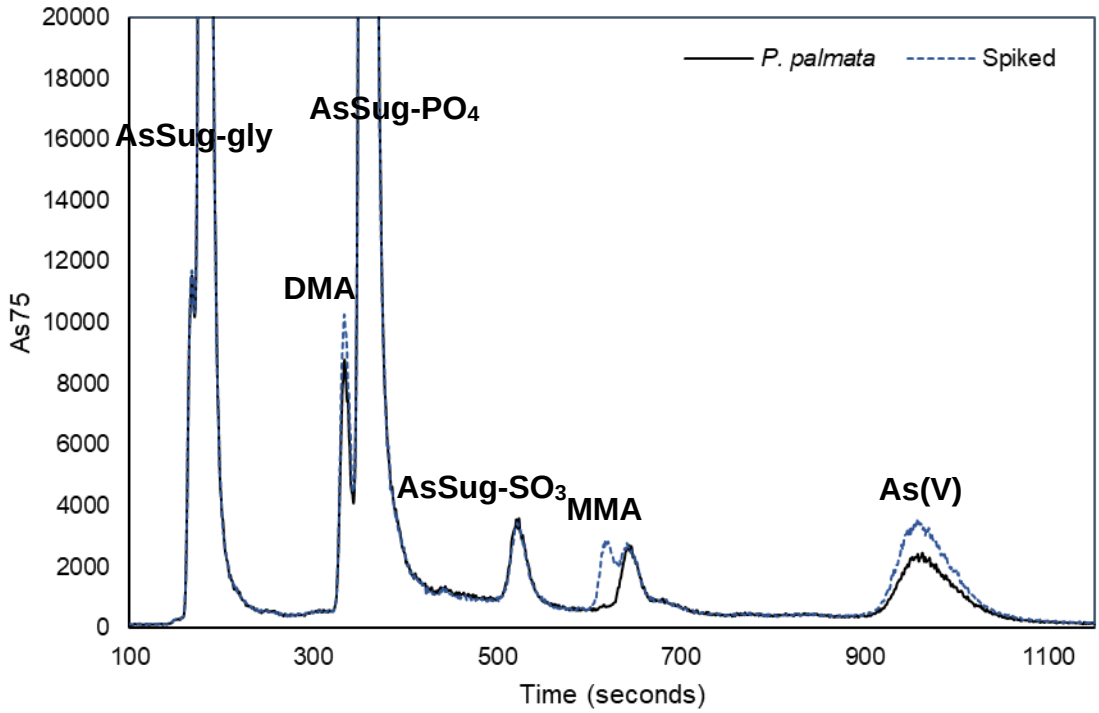


Figure 31 – A sample of *Palmaria palmata* spiked with 2 µg L^{-1} of DMA, MMA and As(V).

Quality control

Identification of DMA, MMA and As(V) (sum of oxidized As(III) and As(V)) was carried out using the respective standards. A sample of *Ascophyllum nodosum* that had previously undergone LC-MS/MS analysis (Quantiva, Thermo) served as an in-house reference material and was used to identify the arsenosugars. Column recovery was acceptable and calculated to be between 80-109% for all samples. The reference material, hijiki, that was used throughout the study showed good agreement with certified values and previously reported values for water-soluble As species and total As. The LOD and LOQ for the method were found to be 0.007 mg kg^{-1} and 0.02 mg kg^{1} respectively and were calculated as 3.3 and 10 times the standard deviation of 3 consecutive injections of 0.1 µg L^{-1} DMA standard, multiplied by an average dilution factor.

Data analysis

Integration of speciation data was performed using MassHunter Quanititaive Analysis.

Results and Discussion

Analysis of reference material 7405-b (hijiki) showed good agreement with certified values for total As, iAs and AsSug-SO_4, Table 19. The concentration of AsSug-gly reported here is higher than the certified value, 0.50 mg kg^{-1} compared with 0.44 mg kg^{-1} but is still within the acceptable range. There are no certified values for DMA, AsSug-SO_3 or AsSug-PO_4, however all results were in agreement with those previously reported (191), Table 19. The results for MMA in this study are lower than those reported by one previous study - 0.05 mg kg^{-1} and 0.08 mg kg^{-1} respectively – where an aqueous methanol extraction was used (110).

Table 19 – The concentrations of total As and As species in the reference material hijiki.

Analyte	7405-b (hijiki) (n = 12)	Certified value
Total As (mg kg^{-1})	51.01* ± 3.92	49.5 ± 1.0
AsSug-gly (mg kg^{-1})	0.50 ± 0.04	0.44 ± 0.02
DMA (mg kg^{-1})	0.22 ± 0.03	0.24**
AsSug-PO_4 (mg kg^{-1})	0.23 ± 0.02	0.20**
AsSug-SO_3 (mg kg^{-1})	0.14 ± 0.03	0.16**
MMA (mg kg-1)	0.05 ± 0.01	-
AsSug-SO_4 (mg kg^{-1})	1.43 ± 0.20	1.41 ± 0.04
iAs (mg kg^{-1})	23.24 ± 1.25	24.4 ± 0.7
Column recovery (%)	90 ± 5	-

*n = 9 for total As measurement, **literature value (191).

Phaeophyta

Alaria esculenta was only identified at one of the two sampling locations - Grindavík. All samples of *A. esculenta* contained <LOQ levels of MMA regardless of thallus section or season, and AsSug-SO_4 was not detected in any *A. esculenta* samples. Previous studies that report arsenic speciation in *A. esculenta* report very low concentrations (0.01 mg kg^{-1}) or did not detect AsSug-SO_4 (4,49). Concentrations of DMA and iAs were also relatively low and ranged from 0.11-0.46 mg kg^{-1} and 0.03-0.34 mg kg^{-1} respectively, Table 20. Levels of iAs were highest in May, and the greatest concentration was always found in the frond section of the thallus. This differs from results previously reported for other Laminariale, *L. digitata*, where levels were found to be highest during winter (61). AsSug-gly, AsSug-SO_3 and AsSug-PO_4 were all detected in varying concentrations, Table 20, with AsSug-SO_3 being the dominant arsenical in each section of the thallus followed by AsSug-PO_4. Similar to the total As, concentrations of AsSugars were generally highest in samples collected during February, with the exception of AsSug-gly, where levels remained similar between the two sampling months (0.48-1.2 mg kg^{-1} in February and 0.70-1.2 mg kg^{-1} in May). The distribution of all arsenicals stayed relatively constant throughout the sampling months in the holdfast/stipe and midrib sections of the thallus, however there were pronounced differences in the frond and sporophyll (reproductive tissues) between May and February. Concentrations of AsSug-SO_3 increased approximately 3-fold from May to February in these sections, and a nearly 2-fold increase was observed for AsSug-PO_4. Extraction efficiencies ranged from 41-79%, where the sporophyll had lower efficiencies (42-73%) than all other tissues (69-83%), which may potentially be explained by the higher proportions of lipid-soluble As found to be present in the sporophyll (4). Extraction efficiencies were generally highest in May, which is line with previous observations for other Phaeophyceae *Fucus spiralis* and *L. digitata* (Lai et al., 1998; Sim et al., 2023).

Concentrations of MMA were consistently low in all samples of *A. nodosum* and ranged from <LOQ-0.07 mg kg^{-1}. iAs concentrations ranged from 0.06-0.35 mg kg^{-1} in all samples and were highest in the holdfast/stipe and reproductive receptacles during both sampling months. DMA levels were generally higher in February (0.13-0.42 mg kg^{-1}) than May (0.05-0.22 mg kg^{-1}) in *A. nodosum* and followed a similar trend as iAs, Table 20. The concentration of AsSug-gly and AsSug-PO_4 were similar across months and thallus sections, ranging from 1.4-3.0 mg kg^{-1} and 1.2-3.1 mg kg^{-1} respectively. Dissimilarly to the *A. esculenta*, AsSug-SO_4

was the dominant arsenic compound in all samples of *A. nodosum* analysed, followed by AsSug-SO_3, where both were higher in February than May. AsSug-SO_3 was also consistently found in the highest concentrations in the reproductive receptacles. No clear trends were observed for the extraction efficiencies, and similar results were found for *A. nodosum* collected at Kjalarnes during the same months, Appendix T10. Extraction efficiencies ranged from 37-71%.

In *Fucus vesiculosus* samples, concentrations of iAs and MMA were low throughout the whole thallus in both months, where levels never exceeded 0.08 mg kg^{-1} and 0.07 mg kg^{-1} respectively. DMA was lowest during May where levels ranged from 0.09-0.16 mg kg^{-1}, and higher in February where concentrations were between 0.12-0.38 mg kg^{-1}. AsSug-SO_3 was the dominant arsenical in all samples of *F. vesiculosus* - similarly to *A. esculenta* - and was most concentrated in the apice section of the thallus and reproductive receptacles. This sugar remained at similar concentrations in the bladder and frond throughout both months but increased up to 2-fold between May and February in all other sections. A similar trend was observed for AsSug-gly in all sections, where concentrations approximately doubled from May to February. Levels of AsSugars were consistently lowest in the bladders, however, the main function of the bladders are to maintain buoyancy, and so little biological activity would be expected to occur here. Extraction efficiencies ranged from 54-89% in May and 40-88%, where the holdfast/stipe samples displayed the highest efficiencies. Similar results were observed for samples of *F. vesiculosus* collected from Kjalarnes, Appendix T1

zTable 20 – The concentrations of water-soluble arsenicals in species of Phaeophyta collected at Grindavík in May 2021 and February 2022.

Species	Sampling month	Section (n = 2)	AsSug-gly (mg kg^{-1})	DMA (mg kg^{-1})	AsSug-PO_4 (mg kg^{-1})	AsSug-SO_3 (mg kg^{-1})	MMA (mg kg^{-1})	AsSug-SO_4 (mg kg^{-1})	iAs (mg kg^{-1})	Total As extracted (mg kg^{-1})	Total As* (mg kg^{-1})	Extraction efficiency (%)
Ascophyllum nodosum	May	Holdfast/stipe	2.5	0.13	1.8	3.3	0.03	16	0.17	23	40 ± 3.0	58
		Primary shoot	2.3	0.05	1.3	2.2	0.03	10	0.11	17	30 ± 1.3	58
		Secondary shoot	3.0	0.08	1.2	1.8	0.03	9.4	0.06	17	35 ± 0.40	50
		Reproductive receptacle	1.4	0.22	1.6	4.2	0.07	21	0.14	30	51 ± 0.66	59
	February	Holdfast/stipe	2.3	0.29	3.1	4.1	0.06	24	0.26	35	73 ± 8.5	48
		Primary shoot	2.7	0.13	1.2	2.6	0.03	9.7	0.08	18	33.0 ± 1.1	71
		Secondary shoot	1.7	0.17	1.6	3.6	<LOQ	15	0.08	23	48 ± 2.3	37
		Reproductive receptacle	1.4	0.42	2.8	10	<LOQ	54	0.35	68	130 ± 8.1	52
Fucus vesiculosus	May	Holdfast/stipe	0.33	0.10	0.83	18	<LOQ	5.3	<LOQ	29	32 ± 1.3	89
		Blade	0.41	0.09	1.7	11	<LOQ	3.2	0.05	21	34 ± 9.6	74
		Bladder	0.37	0.11	1.6	13	<LOQ	3.7	0.08	22	33 ± 4.6	65
		Apice	0.28	0.16	3.3	34	<LOQ	5.4	<LOQ	45	83 ± 14	54
		Reproductive receptacle	0.57	0.10	2.8	27	<LOQ	5.8	<LOQ	40	46 ± 0.51	87
	Feb	Holdfast/stipe	0.72	0.20	1.4	42	<LOQ	10	<LOQ	52	59 ± 13	88
		Blade	0.82	0.15	1.1	12	<LOQ	5.0	<LOQ	21	32 ± 4.5	67
		Bladder	0.55	0.12	0.67	7.8	<LOQ	2.7	<LOQ	13	32 ± 3.6	40
		Apice	1.3	0.38	2.6	53	<LOQ	8.7	<LOQ	62	78 ± 0.74	80
		Reproductive receptacle	1.4	0.27	1.3	47	<LOQ	7.7	<LOQ	56	123 ± 2.9	45
Alaria esculenta	May	Holdfast/stipe	0.70	0.19	8.8	25	<LOQ	-	0.06	37	44 ± 1.6	83
		Midrib	1.2	0.11	5.3	20	<LOQ	-	0.21	27	34 ± 0.99	78
		Frond	1.1	0.15	5.2	21	<LOQ	-	0.34	27	35 ± 4.0	76
		Sporophyll	0.90	0.21	8.7	29	<LOQ	-	0.11	39	54 ± 0.38	73
	Feb	Holdfast/stipe	0.48	0.17	10	24	<LOQ	-	0.08	37	48 ± 2.5	79
		Midrib	0.56	0.16	6.8	34	<LOQ	-	0.03	46	66 ± 1.4	69
		Frond	1.0	0.46	12	73	<LOQ	-	0.10	96	125 ± 5.0	76
		Sporophyll	1.2	0.24	15	88	<LOQ	-	0.07	108	265 ± 9.9	42

Rhodophyta

MMA was low for all red seaweed species, ranging from <LOQ-0.06 mg kg^{-1}. Concentrations of DMA decreased from May to February in both *Mastocarpus stellatus* and *Palmaria palmata* but stayed similar in *Chondrus crispus* throughout sampling months, Table 21. AsSug-SO_4 was found in low concentrations in all samples ranging from <LOQ to 0.05 mg kg^{-1} and was similar to previously reported concentrations in *P. palmata* and *C. crispus* which were 0.01 mg kg^{-1} and <LOD-0.12 mg kg^{-1} respectively (49). AsSug-PO_4 was the most abundant arsenical in the majority of Rhodophyta samples, and concentrations stayed relatively constant between sampling months in *M. stellatus* but increased between May and February in both *C. crispus* and *P. palmata*. However, the *C. crispus* compared here are from two locations so the increase cannot solely be attributed to season. *Cystoclonium purpureum* and *Devaleraea ramentacea* were only found during May at one location, and *Vertebrata lanosa* is an epiphytic species removed from samples of *A. nodosum* collected at Grindavík in February along with *Porphyra dioica*. AsSug-PO_4 was the dominant As compound in *C. purpureum* and *D. ramentacea* – similar to *C. crispus*, *M. stellatus* and *P. palmata*, Table 21. The most abundant arsenic compounds in *V. lanosa* were AsSug-gly closely followed by AsSug-SO_4, and similar distributions have previously been reported for this seaweed species (49). The concentrations of the latter are atypical of red seaweed and may originate from the symbiosis between *V. lanosa* and *A. nodosum*, as AsSug-SO_4 is abundant in the latter (49). *V. lanosa* also contained higher levels of iAs (0.39 mg kg^{-1}) than is typically reported for Rhodophyta species. The iAs levels in *V. lanosa* are similar to a previously reported value of 0.26 mg kg^{-1} for samples collected in New England (49), but are much higher than in the *A. nodosum* shoot from which the *V. lanosa* sample grew (0.08 mg kg^{-1}). DMA, MMA and iAs were all detected in *C. purpureum* and *V. lanosa*, whereas only MMA was detected in *D. ramentacea* and in low concentrations (0.01 mg kg^{-1}). Extraction efficiencies ranged from 26-73% for all species of Rhodophyta, where *P. palmata* samples had the highest efficiency that stayed similar during sampling months (72- 73%). *C. purpureum* and *V. lanosa* displayed reasonable extraction efficiencies of 50 and 57% respectively, whereas for *D. ramentacea* the efficiency was much lower at 24%. No literature is available on the lipid-soluble As species of *C. purpureum and D, ramentacea*, thus it is difficult to say whether the low extraction efficiencies observed here are due to high concentrations of lipid-soluble arsenicals. Rhodophyta typically have high protein content (294,295), therefore the authors suggest the residual As is likely in the form of As(III) bound to sulphur-containing proteins. In contrast to the other Rhodophyta, *P. dioica* had an extraction efficiency of 99% where nearly all of extracted As was in the form of AsSug-PO_4 (20 mg kg^{-1}) with minor contributions from DMA and AsSug-gly.

Table 21 – The concentrations of water-soluble arsenicals, total arsenic and extraction efficiency of several species of Rhodophyta collected from Kjalarnes (K) and Grindavík (G) in May 2021 and February 2022.

Sample (n =2)	AsSug-gly (mg kg^{-1})	DMA (mg kg^{-1})	AsSug-PO_4 (mg kg^{-1})	AsSug-SO_3 (mg kg^{-1})	MMA (mg kg^{-1})	AsSug-SO4 (mg kg^{-1})	iAs (mg kg^{-1})	Total As extracted (mg kg^{-1})	Total As* (mg kg^{-1})	Extraction efficiency (%)
Cystoncloniu m purpureum (May, K)	0.45	0.11	1.0	0.34	0.06	0.08	0.10	2.8	5.6 ± 0.16	50
Develarea ramentacea (May, K)	0.13	<LOQ	0.89	0.16	<LOQ	0.09	<LOQ	2.0	7.6 ± 0.92	26
Vertebrata lanosa (Feb, G)	1.8	0.13	1.5	0.58	0.03	1.7	0.39	7.3	10 ± 1.2	71
Chondrus crispus (May, K)	0.41	0.06	4.1	<LOQ	<LOQ	<LOQ	0.07	4.4	6.5 ± 0.27	67
Chondrus crispus (Feb, G)	0.68	0.07	10	0.02	<LOQ	<LOQ	0.11	12	23 ± 0.51	54
Mastocarpus stellatus (May, G)	0.40	0.07	4.5	<LOQ	<LOQ	0.04	<LOQ	8.0	17 ± 2.7	48
Mastocarpus stellatus (Feb, G)	0.44	0.13	6.9	<LOQ	<LOQ	0.07	<LOQ	15	22 ± 2.0	69
Palmaria palmata (May, G)	1.6	0.19	3.3	0.08	0.02	0.05	0.08	6.5	9.1 ± 0.06	72
Palmaria palmata (Feb, G)	2.6	0.14	6.4	0.40	0.05	<LOQ	0.06	9.4	13 ± 1.5	71
Porphyra dioica (May, G)	0.87	0.15	20	<LOQ	<LOQ	0.02	<LOQ	22	22 ± 0.93	99

*n = 3

Green seaweed species

All samples of green seaweed with the exception of the unidentified *Acrosiphonia* sp. were collected during May due to the low abundance of Chlorophyta during the winter months. AsSug-PO_4 was the dominant arsenical in all of the green *spp.*, Table 22, with the exception of *Ulva intestinalis* from Grindavík where AsSug-gly was most abundant by approximately 3-fold. The *U. intestinalis* from Kjalarnes and *Acrosiphonia sp*. contained relatively high levels of iAs (0.80-1.1 mg kg^{-1}) in comparison to the other green *spp.* (<LOQ-0.43 mg kg^{-1}). Extraction efficiencies were also highly dependent on seaweed species, where in *U. intestinalis* from Grindavík over 80% of the total As was in a water-soluble form and for all other Chlorophyta, the extraction efficiencies were lower at 35-59%. A recent study with C*hlamydomonas reinhardtii* (Chlorophyta) determined that 52-59% of the total As was present as polar, lipophilic arsenic species (88) - similar may be true of the *Acrosiphonia arcta* and *Ulva spp.* investigated here.

Table 22 – The concentrations of water-soluble arsenicals in species of Chlorophyta collected from Kjalarnes (K) and Grindavík (G) in May 2021, and *Acrosiphonia sp.* collected from Grindavík (G) in February 2022.

Sample (n = 2)	**AsSug-gly (mg kg^{-1})**	**DMA (mg kg^{-1})**	**AsSug-PO$_4$ (mg kg^{-1})**	**AsSug-SO$_3$ (mg kg^{-1})**	**MMA (mg kg^{-1})**	**AsSug-SO$_4$ (mg kg^{-1})**	**iAs (mg kg^{-1})**	**Total As extracted (mg kg^{-1})**	**Total As* (mg kg^{-1})**	**Extraction efficiency (%)**
Acrosiphonia arcta (K)	0.71	0.10	1.2	0.15	<LOQ	0.12	0.43	3.3	6.5 ± 0.31	51
Ulva prolifera (K)	0.44	0.03	0.45	0.04	<LOQ	<LOQ	0.40	1.9	5.3 ± 0.62	35
Ulva intestinalis (K)	0.56	0.12	1.1	0.08	<LOQ	0.31	1.1	3.9	9.5 ± 0.38	41
Acrosiphonia arcta (G)	0.36	0.11	0.78	0.07	<LOQ	0.06	0.22	2.2	6.1 ± 0.56	36
Ulva intestinalis (G)	1.3	0.07	0.41	0.15	<LOQ	0.02	0.41	3.0	3.8 ± 0.37	80
Acrosiphonia sp. (G, Feb)	0.86	0.11	1.04	0.44	0.12	0.17	0.80	4.5	7.68 ± 0.21	59

*n = 3

Discussion

The differences in AsSugar compositions between seaweed species is thought to be due to differing enzymes capable of carrying out the glycosidation reaction on the side chain of the ribose (75). The results presented here in combination with those reported in the literature suggest it is likely that all Phaeophyta, Rhodophyta and Chlorophyta have the pathways required to synthesise the four AsSugar derivatives – though some may be produced in low quantities (i.e., 0.01 mg kg^{-1} AsSug-SO_4 reported in *A. esculenta* (49)) (37,49,70). The concentrations of AsSugars appear to be affected by external factors such as season as well as species and thallus section. Phosphate demand is highest during winter months in Phaeophyta due to higher levels of growth and when nitrogen abundance is not a limiting factor (219). If arsenate and phosphate are taken up by the same method without distinction (49), it is logical to assume that higher As concentrations would be expected during the winter.

The AsSug-PO4 and AsSug-gly derivatives were present in all samples analysed across the three classes (Phaeophyta, Rhodophyta and Chlorophyta) which may support the theory that AsSug-PO_4 is a degradation product of arsenic-containing phospholipids that are formed when AsSug-gly binds to phosphatidic acid in the cell wall (32). Additionally, increased external phosphorous concentrations have been reported to increase the production of both AsSug-PO_4 and arsenosugarphospholipids (AsSugPL) and decrease the concentration of AsSug-gly in unicellular algae, *Dunaliella tertiolecta* (91). Whilst AsSug-gly and AsSug-PO_4 have been widely reported to occur in phytoplankton, cyanobacteria, fresh-water unicellular algae, the sulphated and sulphonated AsSugar derivatives are only typically reported in marine macroalgae (3,71,74,296). In macroalgae, AsSug-SO_3 is generally more abundant than the AsSug-SO_4 (with the exception of *A. nodosum*) which is unusual as the C-O-S bond is common in metabolites produced by the macroalgae, i.e., the polysaccharide fucoidan which contains O-SO_3 branches. Sulphated polysaccharides are additionally abundant in seaweeds (297), thus a range of enzymes are expected to be available to carry out the transfer of SO_3 to a hydroxyl group on the side chain producing a sulphate moiety. It is unclear why AsSugSO_3 which contains a C-S-O bond is more abundant - perhaps a thiol group is first present on the side chain of the ribose, which is then irreversibly oxidised to SO_3 as is seen with thiols in cysteine residues in proteins (298).

An imaging study using NanoSIMS has shown that arsenic within *L. digitata* (Phaeophyta) cells is stored primarily in the cell walls which suggests that arsenosugars must also be stored here (37). Storage in the cell wall could be an efficient way of excluding toxic As from the intra-cellular matrix, or alternatively the AsSugars could serve as useful components of cell wall matrix in the same manner as polysaccharides (e.g., alginates). Polysaccharides are an integral part of the cell wall matrix and impart anti-freezing properties in cold temperatures, assist with osmotic regulation and may complex potentially toxic cations reducing their accumulation in the intra-cellular matrix (204). Regardless, seaweeds appear to tolerate high concentrations of these AsSugars (this study: sum 0.9-104 mg kg^{-1}) with no ill effects as all seaweed specimens collected during sampling were healthy.

As(V) specifically is thought to be stored in the cell wall of seaweeds as arsenoesters which rapidly hydrolyse upon extraction with aqueous solvents, similarly to sulphates bound as sulfuric acid esters to fucoidan. However, this was theorised after NanoSIMS imaging of *L. digitata* which contained elevated levels of iAs (62 mg kg^{-1}) (37). *L. digitata* and *Sargassum fusiforme* (hijiki) have consistently been demonstrated to contain high levels of iAs – so much so that consumption has been discouraged for many years (62). However, the theory proposing the existence of arsenoesters may not hold true for all species as the levels of iAs

here rarely exceeded 1 mg kg^{-1} and several species such *as F. vesiculosus* also contain similar levels of fucoidans (274,299). Additionally, in *Alaria spp.* fucoidan levels are reported to peak in January, with the highest % of dry matter found in the sporophyll – however the iAs concentration remained low throughout the thallus and both sampling months. Thus, it is likely As(V) is stored in a different manner in *A. esculenta* or that the seaweed species-specific composition of fucoidan does not allow for complexation of large concentrations of iAs (274,300,301).

The low levels of MMA and DMA observed here are likely to due to the fact these compounds are intermediates in the production of AsSugars (and further, possibly arsenic containing lipids). DMA has previously been demonstrated as an intermediate in the production of AsSugars, where an enzyme catalysing the adenosylation of DMA(III) to DDMAA was identified (292).

Conclusion

This study aimed to provide insight into the mechanisms by which AsSugars are formed and their storage. Where comparisons could be drawn, AsSugars concentrations were generally highest in February and in the most biologically active parts of the thallus. Phosphate demand may be higher in winter months due to growth, and if arsenate and phosphate are taken up by the same method without distinction it is logical that the concentration of arsenic species would be higher during this time (49). All seaweed species appear to have the capacity to produce all four AsSugar derivatives as evident by the variety of results presented here and in the literature (37,49,70). Additionally, the low levels of MMA (<LOQ-0.07 mg kg^{-1}) and DMA (<LOQ-0.41 mg kg^{-1}) detected in all samples relative to the AsSugar levels suggests that the conversion to AsSugars is fast. The production of AsSugars may be an efficient way of excluding As from intracellular matrix, or they may serve as useful components in the cell wall similar to polysaccharides in the cell wall matrix, as extremely high levels of AsSugars can be tolerated by the algae with no ill effects. Further study using a combination of imaging and speciation techniques is necessary to fully investigate how AsSugar derivatives may formed in of the cell, and through which mechanisms.

3.7 Paper VI – Analysis of Arsenic-Containing Lipids in Several Species of Brown, Red and Green Seaweeds Collected in Iceland

Rebecca Sim [AB], Andrea Raab [C], Jörg Feldmann [C] and Ásta H. Pétursdóttir [A]

Author Affiliations:
[A]Matís, Vinlandsleid 12, 113 Reykjavík, Iceland
[B]Faculty of Physical Sciences, Dunhagi 3, University of Iceland, Reykjavík, Iceland
[C]Institut für Chemie, Universität Platz 1, Universität Graz, Graz, Austria

Abstract

Seaweeds are known to sequester high levels of arsenic - a potentially toxic metalloid - which may be metabolised to an array of complex lipid structures including hydrocarbons (AsHCs), fatty acids (AsFAs) and phospholipids (AsSugPL). The mechanisms of formation of these lipids are largely unknown but are thought to vary between brown, red and green seaweeds – where the latter two have been infrequently investigated. This study aims to investigate the distribution of arsenolipids throughout marine macroalgae to aid in understanding their formation. Samples collected in Iceland were analysed for lipophilic arsenic speciation by HPLC-ICP-MS/ESI-MS and HPLC-qToF-MS. In total, 35 different arsenolipid species were identified across all samples, including 2 novel compounds (AsHC380 and AsFA398) identified in *Ascophyllum nodosum*. Arsenosugar phospholipids were found to dominate in brown algae where AsSugPL958 (C16:0/C16:0) was most abundant in all except *Laminaria hyperborea* where AsSugPL984 (C16:0/C18:1) dominated. AsSugPLs were most concentrated in the reproductive tissues of the brown algae, where concentrations of AsSugPL958 ranged from 0.38-2.3 mg kg^{-1}. AsHC360 was also detected in relatively high concentrations in several samples where levels were highest *Fucus vesiculosus* (0.16-0.64 mg kg^{-1}) and *Devaleraea ramentacea* (0.23 mg kg^{-1}). Two arsenosugar phytol compounds were detected in the green seaweeds, where (547 m/z) compound was generally the most abundant species found in *Ulva prolifera*, *Ulva intestinalis* and *Acrosiphonia arcta* but showed a dependence on sampling location for *U. intestinalis*. This arsenosugars phytol was also detected at trace levels in several samples of red seaweed including *Chondrus crispus* and *Porphyra dioica*, but it is unclear if this is produced by the algae or some epiphytic species that may reside on the surface of the seaweeds.

Introduction

Arsenic is a ubiquitous metalloid released into the environment through geological processes and anthropogenic activities. Seaweeds are known to sequester high levels of arsenic, where cellular concentrations may reach several orders of magnitude higher than that of the surrounding waters (49,282). The uptake of arsenic is thought to occur through transport of arsenite through aquaglyceroporins and glucose permeases, and through phosphate transporters due to the structural similarities between arsenate and phosphate. The arsenic may then become incorporated into larger molecules such as arsenic-containing sugars (AsSugars) and arsenic-containing lipids (AsLipids). Approximately 13 structural groups of AsLipids have been identified in marine organisms, however the mono- and di- acyl

arsenoglycerolphospholipids (AsSugPLs), arsenohydrocarbons (AsHCs), and arsenofatty acids (AsFAs) are the most commonly reported species, Figure 32 (81,302). The mechanism of formation of arsenolipids is still largely unknown. It has been suggested that the production of AsLipids may be a method of detoxification for removing arsenic from inside the cell or a means of conserving phosphorous during limiting conditions (140,303). The phosphate-containing arsenosugar derivative is thought to be the starting point for the formation di-acyl AsSugPLs, where both compounds share a common dimethylarsenoribose moiety (140). Additionally, it has been suggested that the binding of the glycerol arsenosugar derivative with phosphatidic acid in the cell membrane results in di-acyl AsSugPLs (68), which may then degrade to the related mono-acyl structures (97), Figure 32. AsSugPLs are generally the most abundant lipid class found in Phaeophyta, where they were found to comprise over 50% of the total lipid-soluble arsenic in *Alaria esculenta* and *Saccharina latissima* (4). Rhodophyta and Chlorophyta have been less frequently investigated than Phaeophyta, but the lipid-soluble arsenic speciation appears to vastly differ from that of brown macroalgae; In *Chlamydomonas reinhardtii* (Chlorophyta) an arsenosugar phytol (AsSugPhytol) compound was the most abundant species reported (88), and in Palmaria palmata (Rhodophyta) this was a trimethyl arsenofatty acid alcohol (TMAsFOH) (5). AsHCs may also account for a significant portion of the lipid-soluble arsenic found in seaweed depending on the algal species. For example, in Hijiki (*Sargassum fusiforme*, Phaeophyta) and Wakame (*Undaria pinnatifida*, Phaeophyta), AsHCs accounted for 27-34% of the total lipid-soluble arsenic (304) but only 8% in *Laminaria digitata* (Phaeophyta) (37). Arsenodiglyercides (AsDAGs), arsenoceramides (AsCer) and arsenophosphatidylinositols (AsPIs) have only thus far been reported in *Coccomyxa spp*. (Chlorophyta) cultivated in a medium with high arsenic concentrations (80). Arsenotriacylglycerides (AsTAGs) were also identified in the same study (80), and free medium chain AsFAs that were detected in samples of fish oil were suspected to be conjugates to triacylglyceride species degrading during sample clean-up, as only long chain AsFAs were detected prior to sample clean-up (178). Arsenosugar phosphoacyl ether lipids isolated from particulate matter in seawater have also recently been described in the literature, but are thought to be produced by bacteria rather than algae due to the ether linkage of one of the acyl changes rather than an ester linkage (90).

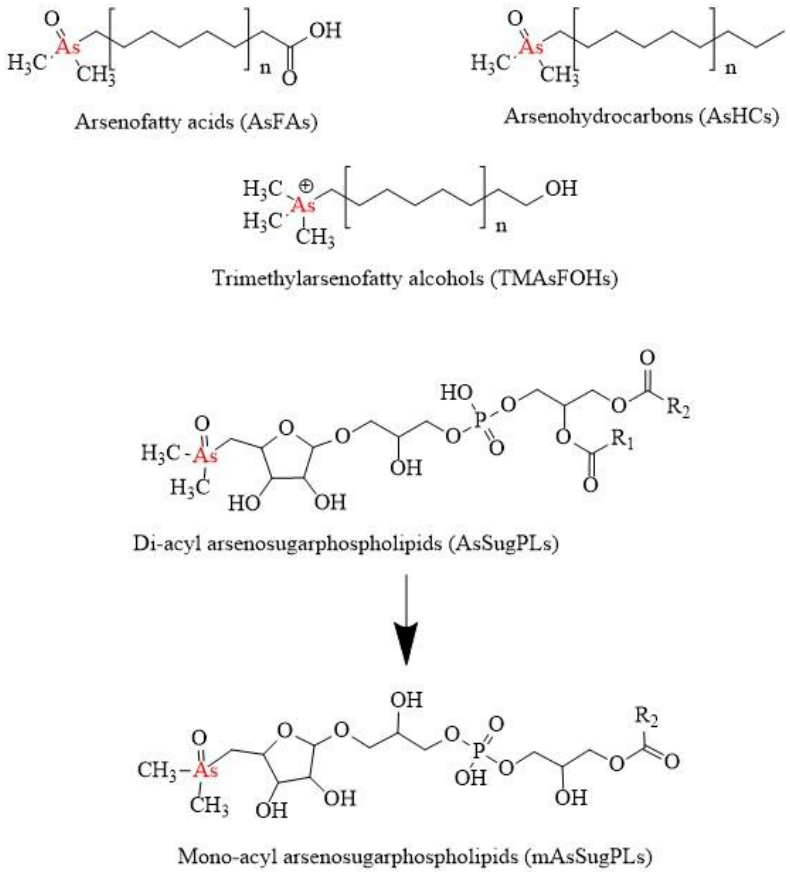


Figure 32 – The most commonly reported arsenolipids in marine organisms: arsenohydrcarbons (AsHCs), arsenofatty acids (AsFAs) and di-acyl arsenosugar phospholipids (AsSugPL) which may degrade to the corresponding mono-acyl species.

With regards to toxicity, AsHCs and AsFA are of the most concern as both have demonstrated in vitro toxicity comparable to that of arsenite (6,102). AsHC332 has also been shown to cross the blood-brain barrier in *Drosophila melanogaster* (305) and disrupt the integrity of these barriers facilitating the transport of other contaminants to the brain (306). Less is known about the toxicity and bioavailability of other arsenolipids groups, but the metabolism of these has been shown to differ vastly between individuals due to differences in gut microbiomes (190,279).

Lipid-soluble arsenic speciation for seaweed matrices is not commonly reported, and the focus has largely been directed towards marine matrices with high fat contents such as fish oils, tissues, and fish meal. Seaweed serves as a primary food source for many marine organisms and is thought to be the starting point for AsLipid structures, and so should accordingly be investigated to learn more about the origins of these compounds. Additionally, seaweed is growing in popularity in Western countries and is seen by many as a sustainable and nutritious food source – thus there is a need to collect data on the AsLipid content of edible seaweeds as they may pose a risk to consumers. The aim of this study was to identify and quantify AsLipids in 13 species of seaweed collected in Iceland to investigate differences in lipid-soluble speciation. Brown seaweed thalli were additionally divided into anatomical sections (e.g., meristem or frond) to investigate the relationship between biological function and distribution to understand how and why these compounds are formed. This study is the

first to report AsLipid speciation for *Ascophyllum nodosum*, *Fucus vesiculosus* and *Laminaria hyperborea* which have not yet been investigated despite widespread use as fertilisers, foods, and livestock feeds.

Experimental

Chemicals and standards

All chemicals were of analytical grade or higher unless otherwise stated. Ultrapure water (18 Ω cm) was obtained from a milliQ water dispenser (Millipore, Italy). Dichloromethane was supplied by Chem-Lab NV (Belgium) and methanol (CHROMASOLV, ≥99.9%) was purchased from Honeywell (Germany). Formic acid (ROTIPURAN, ≥98%) was provided by Carl Roth (Germany) and nitric acid (analytical reagent grade, 65%) was supplied by Fisher Scientific (France). Dimethylarsenate (DMA) standards used for the quantification of arsenic species and total arsenic were prepared serial dilution of disodium dimethylarsenate salts dissolved in ultrapure water.

Samples and reference materials

Thirteen species of brown, red and green seaweed were collected from Kjalarnes and Grindavík in Iceland during May 2021 and February 2022. Where possible, seaweed species were identified based on thallus morphology (i.e., *Fucus vesicul*osus is identifiable due to bladders on either side of midrib) and for those that could not be identified in this manner DNA testing was used (i.e., *Ulva spp*.), Appendix T1. Samples were originally part of a larger dataset where selected samples were chosen for analysis of AsLipids. Upon collection, samples were transported to the lab in plastic bags and stored at -18°C and thawed at 5°C before cleaning. Samples were washed sparingly with tap water to remove debris, and any visible epiphytes were removed manually using a stainless-steel knife rinsed with an ethylenediamine tetra acetic acid (EDTA) and 2% citric acid solution, and ultrapure water. The large brown macroalgae thalli were sectioned into anatomical parts, e.g., meristem or frond, Appendix F1, before freeze-drying to constant mass (Christ, Germany). All samples of green seaweeds were dried whole, as they are mostly annual and new and old parts are indistinguishable from one another. After freeze-drying all sample materials were milled to a fine powder using an IKA tube mill (IKA, China) cleaned with an EDTA and 2% citric acid solution between samples.

The reference material TORT-2 (lobster hepatopancreas) was obtained from the national research council of Canada (NRC) and was analysed in duplicate alongside all batches of samples, as well as for the total arsenic measurements.

AsLipid extraction for speciation measurements

In duplicate, 200-300 mg of sample material was weighed into 50 mL polypropylene tubes before the addition of 5 mL of a DCM/MeOH mixture (2:1). Samples were swirled to ensure all material was in contact with solvent before undergoing mechanical shaking for 2 hours at 30°C. Samples were then centrifuged at 4000 rpm for 15 minutes. Two 1 mL aliquots of the supernatant were transferred to an Eppendorf microcentrifuge tubes and the solvent evaporated using a rotation vacuum concentrator (Christ, Germany). One 1 mL aliquot of the

supernatant was used for speciation measurements and the other for total arsenic measurements to calculate column recovery. The dried extracts were stored at -20°C. Prior to analysis, the dried sample extract was re-constituted in 200 µL of methanol, mechanically shaken for 1 hr and centrifuged at 15000 rpm. One hundred µL of the supernatant was then transferred to a HPLC vial and analysed directly.

Digestion for total As measurements

In triplicate, 50-100 mg of sample was weighed into 15 mL quartz digestion tubes before adding 1 mL of nitric acid and 1 mL of hydrogen peroxide. Samples were vortexed and added to the reaction chamber of an Ultrawave microwave digestion system (Milestone, Italy). A loading pressure of 40 bars was added to the chamber using nitrogen gas before the temperature was increased to 240°C over a 20-minute period. The reaction chamber was maintained at this temperature for 10 minutes, before a 15-minute cool down. The digests were then quantitatively transferred to polypropylene falcon tubes and diluted to 50 mL with ultrapure water.

To calculate column recovery, the dried sample extracts were re-constituted in 200 µL of methanol before shaking and centrifugation as before. One hundred µL of the supernatant was then transferred to a quartz digestion tube and left to stand overnight to allow the solvent to evaporate. 0.3 mL of nitric acid was then added to residues and digested as before using an Ultrawave microwave digestion system. Digests were then quantitatively transferred to polypropylene falcon tubes and made up to 15 mL with ultrapure water.

Instrumental parameters

Speciation analysis. Untargeted identification and quantification of arsenolipids in the algae samples was performed using a combination of HPLC-ICP-MS/ESI-MS and HPLC-qTOF-MS. The HPLC-ICP-MS/ESI-MS set up allowed for the simultaneous identification and quantification of AsLipids, and high-resolution mass spectrometry (qTOF-MS) was used to confirm the identity of the lipids under identical chromatographic conditions (i.e., same column and mobile phases).

The first stage of analysis was performed using an Agilent Infinity II 1260 HPLC coupled to both an Agilent 8800 ICP-MS/MS and an Agilent G6460A ESI-MS/MS (qqq), and as previously reported by Raab et al. (2022) (88) The post-column LC flow was split 1 part to the ICP-MS/MS and 9 parts to ESI-MS/MS. Analytes were separated on an ACE C18 excel 3 column (150 x 4.6 mm) using a gradient elution with 0.1% formic acid in water and 0.2% formic acid in methanol, where the sample injection volume was 50 µL, flow rate was 1 mL min^{-1}, and column compartment temperature was maintained at 40°C. The ICP-MS was used with platinum cones and a 1 mm single piece narrow bore injector torch. An internal standard containing 10 µg L^{-1} germanium and 5% acetone was introduced to the plasma post-column by a separate LC pump at a rate of 0.8 mL min^{-1}. The constant addition to acetone to the plasma was used to reduce any carbon enhancement effects due to the differing carbon content of the mobile phase, and additionally results were normalised using the response of the Ge internal standard, Figure 33. The ICP-MS/MS was run in mass-shift mode where O_2 was used as a reaction gas (0.3 mL min^{-1}), and masses 47 ($^{31}P \rightarrow {}^{31}P^{16}O$), 48 ($^{32}S \rightarrow {}^{32}S^{16}O$), 88 ($^{72}Ge \rightarrow {}^{72}Ge^{16}O$) and 91 ($^{75}As \rightarrow {}^{75}As^{16}O$) were monitored. The ESI-MS/MS was used in positive scan mode with a gas temperature of 350°C, gas flow of 12 L min^{-1}, nebuliser

pressure of 45 psi, sheath gas temperature of 400°C, sheath gas flow of 11 L min^{-1}, capillary voltage of 4.5 kV and a fragmentor voltage of 135 V.

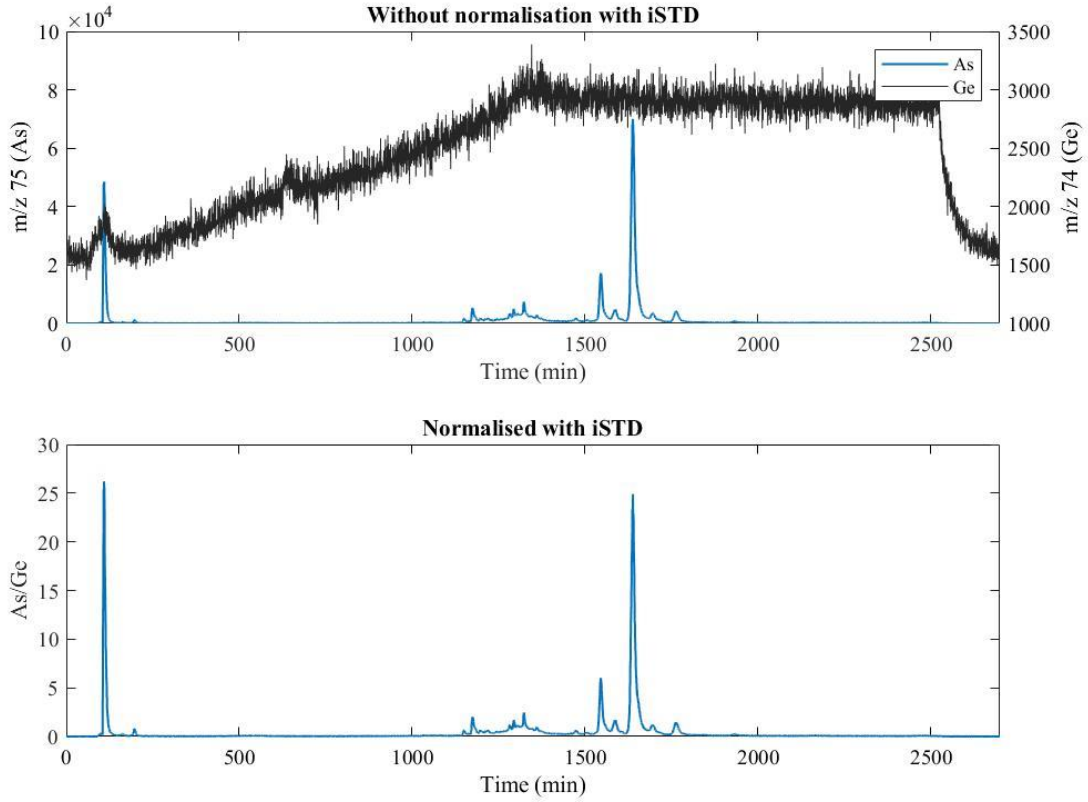


Figure 33 – (top) A chromatograph of *Ascophyllum nodosum* primary shoot analysed by HPLC-ICP-MS (m/z 91 as AsO^+) before normalisation with 10 µg L^{-1} germanium internal standard, (top) and after normalisation with 10 µg L^{-1} germanium internal standard.

The second stage of the analysis was performed using an Agilent infinity II 1290 coupled to an Agilent G6546A qTOF-MS. Identical chromatographic conditions as before were employed, and the qTOF-MS was used in positive scan mode (100-1100 m/z) with a gas temperature of 280°C, gas flow of 8 L min^{-1}, nebuliser pressure of 30 psi, sheath gas temperature of 380°C, sheath gas flow of 12 L min^{-1}, capillary voltage of 4 kV and a fragmentor voltage of 110 V. All instruments were tuned before use.

Total arsenic analysis. Measurements for total arsenic content of samples was carried out using an Agilent 7900 ICP-MS with octopole collision cell and Agilent SPS4 autosampler. The instrument was run in He gas mode (5 mL min^{-1}), with Ni cones and masses 75 (As) and 115 (In – internal standard). Measurement of total arsenic in extracts was carried out under the same conditions using an Agilent 7800 ICP-MS/MS.

Data analysis

Integration of chromatographic data generated from the HPLC-ICP-MS/MS was performed using Origin 2023b Analysis of MS data was performed using MassHunter Qualitative analysis 10.0. Eluted compounds were identified by accurate mass, fragmentation spectra where possible and comparison to literature.

Quality control

There are currently no certified reference materials for AsLipids, however, reference material TORT-2 was run alongside each batch of samples when performing speciation measurements. This material was produced from lobster tissues which have been demonstrated to contain lipid classes not commonly found in seaweeds, i.e., arsenophosphatidylcholines (AsPCs) (25), and crustaceans may contain relatively high levels of free AsFAs which are generally present at trace levels in algae matrices (86,307). Therefore, this reference material was selected to aid with the identification of these compounds in samples through retention time comparison. With regards to quality control for the quantification of arsenolipids, all column recoveries were found to be between 80-118% for all samples. The limit of detection and quantification were calculated as 3.3 and 10 times the standard deviation in the response of ten injections of a 1 µg L^{-1} dimethylarsinate (DMA) standard.

Results and Discussion

Mass balance

A reasonable mass balance (81-118%) was achieved for all samples when comparing the total arsenic to the sum of As extracted using DCM/MeOH and residual As. The highest levels of As were generally extracted from Phaeophyta, with *Laminaria hyperborea* tissues having the highest levels of extractable arsenic. The concentrations of As extracted from *Alaria esculenta* during this study (2.2-5.3 mg kg^{-1}) are notably lower than what has been previously reported for this species (3.7-13 mg kg^{-1}) (4) - likely due to differences in sampling months (May vs. January). Brown seaweeds have also been shown to have higher arsenic and lipid contents during the winter months (61,295). Even higher concentrations of As were extractable from TORT-2 but were largely due to the extraction of less polar water-soluble species. This was confirmed by the large peaks eluting in the void volume of the column, Appendix T11 and F9. With regards to the red and green algae, *Acrosiphonia arcta* samples (1.4-2.7 mg kg^{-1}) and *Porphyra dioica* (1.9 mg kg^{-1}) had higher levels of DCM/MeOH extractable As than the other Rhodophyta and Chlorophyta, which ranged from 0.41-0.93 mg kg^{-1}.

Table 23 – The concentration (mg kg^{-1}) of extracted As, residual As and total As in the Phaeophyta, Rhodophyta and Chlorophyta species analysed. The recovery (%) is also shown. Where thallus section is not described seaweed was analysed as a whole.

Seaweed species (thallus section)	**Sampling month**	**Extracted As (mg kg^{-1}) (n = 2)**	**Residual As (mg kg^{-1}) (n = 2)**	**Total As (mg kg^{-1}) (n = 3)**	**Recovery (%) (n = 2)**
Ascophyllum nodosum (holdfast/stipe)	May	3.6	35	41 ± 1.4	94
Ascophyllum nodosum (primary shoot)	May	3.3	28	43 ± 0.30	85
Ascophyllum nodosum (secondary shoot)	May	4.1	30	33 ± 1.8	104
Ascophyllum nodosum (reproductive receptacle)	May	4.5	42	42 ± 3.4	116
Fucus vesiculosus (holdfast/stipe)	May	2.4	33	32 ± 1.3	111
Fucus vesiculosus (blade)	May	1.5	34	34 ± 0.89	103
Fucus vesiculosus (bladder)	May	2.9	31	33 ± 4.6	102
Fucus vesiculosus (apice)	May	4.0	76	83 ± 14	96
Alaria esculenta (holdfast/stipe)	May	2.2	38	44 ± 1.6	90
Alaria esculenta (midrib)	May	2.5	35	34 ± 0.99	109
Alaria esculenta (frond)	May	2.9	29	35 ± 4.0	90
Alaria esculenta (sporophyll)	May	5.3	57	54 ± 0.38	115
Laminaria hyperborea (holdfast/stipe)	February	6.1	93	114 ± 2.5	87
Laminaria hyperborea (meristem)	February	4.9	86	102 ± 13	89
Laminaria hyperborea (young frond)	February	6.2	93	112 ± 4.5	88
Laminaria hyperborea (old frond)	February	5.0	95	84 ± 4.6	118
*Laminaria hyperborea (sori)**	February	5.6 ± 0.90	73 ± 5.5	96 ± 5.4	81 ± 6
Porphyra dioica	May	1.9	18	22 ± 0.93	94
Chondrus crispus	May	0.43	8.5	8.7 ± 0.70	101
*Cystoclonium purpureum**	May	0.93 ± 0.04	4.4 ± 0.24	5.6 ± 0.16	95 ± 5
Devaleraea ramentacea	May	0.88	5.6	7.6 ± 0.92	85
Palmaria palmata	May	0.41	9.13	12 ± 1.6	81
Mastocarpus stellatus	May	0.86	16	16 ±	107
Acrosiphonia arcta (1)	May	1.4	5.0	6.5 ± 0.31	98
Acrosiphonia arcta (2)	May	2.7	3.8	6.1 ± 0.56	106
Ulva intestinalis (1)	May	1.2	7.2	9.5 ± 0.38	88
Ulva intestinalis (2)	May	0.46	3.7	3.8 ± 0.37	111
*Ulva prolifera**	May	0.65± 0.07	3.9 ± 0.14	5.3 ± 0.62	87 ± 10
TORT-2 (lobster hepatopancreas)**	-	14.10±3.48	5.2 ± 0.9	19.3 ± 2.40 (**21.6**)	100 ± 5

**All measurements performed in triplicate. **All measurements performed in quadruplicate. Certified value in bold for reference material TORT-2.*

Phaeophyta

The quantification and assignment of peaks detected in Phaeophyta samples is shown in Figure 34 and Tables 24-26. AsSugPLs were the major group of lipophilic arsenic compounds extracted from the brown algae accounting for 42-83% of the total extracted As across all samples, where the majority of compounds identified were saturated di-acyl AsSugPLs. Several AsHCs and AsFAs were also identified including a novel unsaturated hydrocarbon with theoretical m/z 381.2139 ($C_{21}H_{38}OAs^+$ $[M+H]^+$) and a saturated fatty acid with theoretical m/z 399.2244 ($C_{21}H_{40}O_2As^+$ $[M+H]^+$). The novel hydrocarbon was detected in all sections of *Ascophyllum nodosum* whereas the novel fatty acid was only present in the holdfast/stipe tissues. The positions of the unsaturated bonds of the hydrocarbon could not be determined from the mass spectra, Appendix F10 and F11. Fatty acids were only detected as

minor traces, Appendix T12, with the AsFA422 the most frequently occurring. AsFA424 was also identified but was only present in some *Fucus vesiculosus* samples.

A. nodosum contained several species of AsSugPLs, Table 26, however those which differ by a CH_2 group tended to co-elute (i.e., AsSugPL930 major and AsSugPL944 minor) which is a common occurrence throughout the literature (11,12,28). Regardless, similar concentrations and compositions of AsSugPLs were observed across each thallus section, a trend unique to A. nodosum and not observed in the other brown macroalgae analysed. AsSugPL958 (C16:0/C16:0) dominated (1.7-2.3 mg kg^{-1}), followed by AsSugPL930 (C16:0/C14:0) (0.38-0.42 mg kg^{-1}) and AsSugPL986 (C16:0/C18:0) (0.10-0.16 mg kg^{-1}). The AsHCs were in vastly lower abundance than the AsSugPLs. The reproductive receptacles contained the highest levels of AsHC, where AsHC402 was the most abundant species, with minor contributions from AsHC360, AsHC358 and AsHC332.
In *F. vesiculosus* the concentration of all AsSugPLs identified were highest in the apice – totalling approximately 2.3 mg kg^{-1} compared to 0.95-1.3 mg kg^{-1} in the rest of the thallus. The blade, bladder and holdfast/stipe contained similar compositions of phospholipids whereas the apice contained approximately 2-fold higher levels of AsSugPL930 and AsSugPL958. *F. vesiculosus* contained relatively high levels of toxic AsHC (0.18-0.75 mg kg^{-1}) which is atypical of macroalgae, where concentrations were highest in the apice followed by the blade. AsHC360 was the most abundant species with minor levels of AsHC332, AsHC374, AsHC402. The identity of AsHC374 was assigned as the hydrocarbon and not the TMAsOH isomer based on fragmentation pattern, where the $AsO(CH_3)_2^+$ fragment was present at m/z 137, Appendix F12. Both *A. nodosum* and *F. vesiculosus* have not before been reported in the literature, however the results show similar speciation AsSugPL speciation with those for other Fucale, such as Sargassum fusiforme (hijiki), but differ slightly in hydrocarbon compositions (11,29).
Alaria esculenta was similar to *F. vesiculosus* and *A. nodosum* in AsSugPL composition, but results differed from those previously reported where concentrations of AsSugPLs were significantly higher. In this study concentrations of AsSugPL930 and AsSugPL944 (C16:0/C15:0) ranged from 0.08-0.27 mg kg^{-1} and 0.46-1.4 mg kg^{-1} respectively whilst Pétursdóttir et al. report concentrations of 0.6-0.9 mg kg^{-1} for AsSugPL930 and 0.2-2.5 mg kg^{-1} for AsSugPL958 (4). Although the previous study used similar thallus sectioning, sampling was performed during the winter months vs. spring during this study – suggesting AsLipids may vary seasonally as has been demonstrated for hydrophilic arsenic species and total As where both are typically highest in the winter months (61).
In *L. hyperborea*, AsSugPL984 (C16:0/C18:1) dominated rather than AsSugPL958 where concentrations for both compounds ranged from 0.56-0.79 mg kg^{-1} and 0.24-0.56 mg kg^{-1} respectively. Other unsaturated species AsSugPL956 (C16:0/C16:1) and AsSugPL982 (C16:0/C18:2) were also in higher abundance in *L. hyperborea* than the other brown macroalgae. AsHC360 was the most abundant hydrocarbon - as has been previously reported for other Laminariale *Saccharina latissima* – with the highest levels occurring in the sori (reproductive tissues)(4,96). AsHC346, AsHC388 and AsHC402 were also detected and quantified. The mono-acyl AsSugPL720 was also identified in all Phaeophyta samples (0.03-0.21 mg kg^{-1}). This is unsurprising as all samples contained high abundances of AsSugPL958 which the mono-acyl compound is thought to be is a degradation product of (97). Other mono-acyl AsSugPLs were also identified as minor traces, Appendix T13.

AsSugPL concentrations were consistently highest in the apice tissues of *F. vesiculosus* and reproductive tissues of the other brown seaweeds – all areas associated with high levels of biological activity (i.e., growth or reproduction). A similar trend has been observed for levels of total arsenic which are thought to increase due to increased phosphorous uptake – where arsenate and phosphate are both taken up indiscriminately by the same transporter (49,61). AsSugPL production in phytoplankton has been shown to increase in high phosphorous abundancies (3), however this could be result of increased arsenic uptake due to increased phosphorous uptake which then requires the detoxification of more arsenic or alternatively, the increased phospholipid production under abundant conditions would lead to higher AsLipids as a result of biologically infidelity.

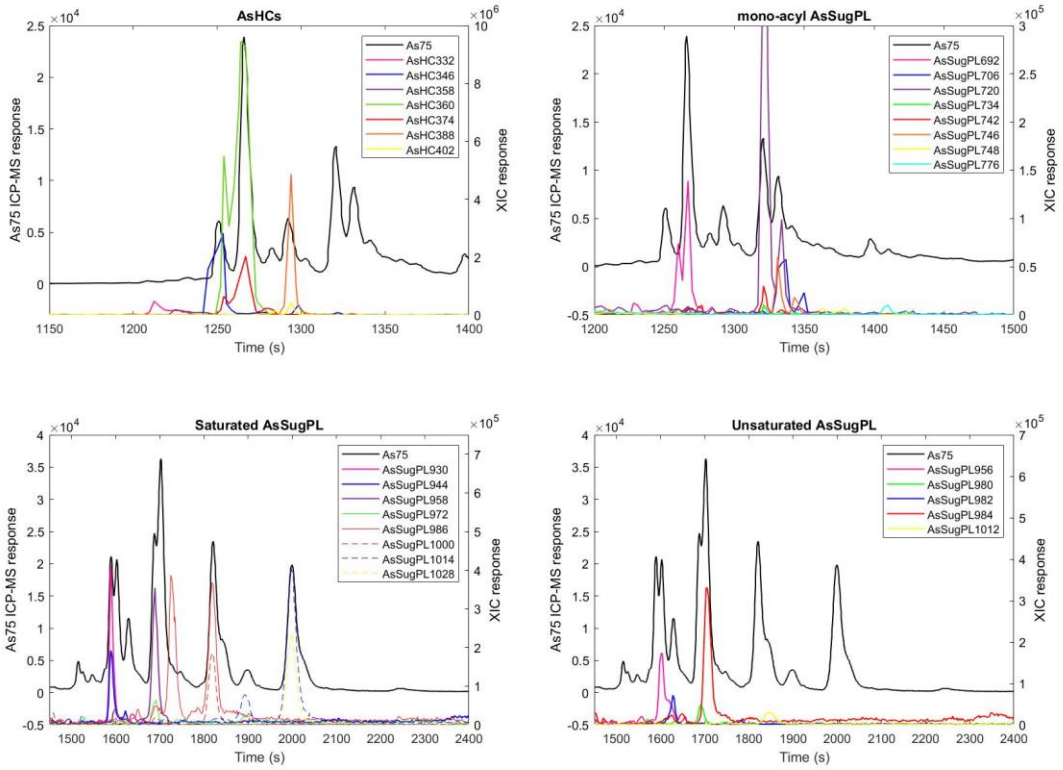


Figure 34 – The peaks eluted from *L. hyperborea* after extraction with DCM/MeOH (2:1). HPLC-ICP-MS trace (m/z 91 as AsO^+) in black, HPLC-qToF-MS extracted ion chromatographs for AsLipid masses ± 3 ppm. EIC were shifted based on retention time of peak during HPLC-ICP/ESMS. Analytes were separated on an ACE C18 excel 3 column (150 x 4.6 mm) using a gradient elution with 0.1% formic acid in water and 0.2% formic acid in methanol where the sample injection volume was 50 µL, flow rate was 1 mL min^{-1}, and column compartment temperature was maintained at 40°C.

Table 24 – The quantification of major peaks in *Ascophyllum nodosum* and *Fucus vesiculosus*. Concentrations are in mg kg^{-1} of sample dry weight. n = 2 for all samples. LOD = 0.002 mg kg^{-1}, LOQ = 0.006 mg kg^{-1}. Peak A is unretained water-soluble arsenicals.

		Ascophyllum nodosum				*Fucus vesiculosus*			
RT (s)	**Peak**	**Holdfast/ stipe**	**Primary shoot**	**Secondary shoot**	**Reproductive receptacle**	**Holdfast/ stipe**	**Blade**	**Bladder**	**Apice**
109	A	0.78	0.63	0.4	0.8	0.94	0.66	0.79	1.0
1215	B	0.018	0.009	0.007	0.021	0.016	0.043	0.016	<LOQ
1220	C	0.016	0.006	<LOQ	0.007	0.009	0.022	0.015	0.032
1250	D	0.018	0.016	0.03	0.052	-	-	-	0.014
1270	E	0.01	0.007	0.007	<LOQ	0.16	0.44	0.43	0.637
1290	F	0.021	0.019	0.029	0.11	0.008	0.012	0.018	0.062
1320	G	0.061	0.029	0.062	0.063	0.008	0.016	0.035	0.118
1330	H	0.013	0.011	0.011	0.018	<LOQ	<LOQ	0.013	0.028
1400	I	0.008	0.016	0.034	0.01	0.007	0.009	0.018	0.022
1550	J	0.007	0.012	0.012	0.006	<LOQ	0.007	0.01	0.014
1590	K	0.39	0.32	0.32	0.32	0.13	0.12	0.13	0.2
1600	L	0.062	0.081	0.053	0.066	0.021	0.017	0.023	0.03
1630	M	0.077	0.081	0.165	0.093	0.033	0.054	0.05	0.075
1690	N	1.8	1.7	2.0	2.3	0.57	0.76	0.81	1.1
1700	O	0.031	0.048	0.092	0.059	0.13	0.16	0.2	0.24
1820	P	0.097	0.12	0.16	0.12	12	0.12	0.15	0.23
1900	Q	<LOQ	0.006	0.07	<LOQ	0.006	<LOQ	0.008	0.023
2000	R	0.022	0.018	0.023	0.027	0.022	0.028	0.027	0.054
2240	S	0.009	0.01	0.01	0.018	0.012	0.011	0.013	0.019

Table 25 – The quantification of major peaks in all *Alaria esculenta* and *Laminaria hyperborea*. Concentrations are in mg kg^{-1} of sample dry weight. n = 2 for all samples with the exception of *L. hyperborea* sori where n = 3. LOD = 0.002 mg kg^{-1}, LOQ = 0.006 mg kg^{-1}. Peak A is unretained water-soluble arsenicals.

		Alaria esculenta				***Laminaria hyperborea***				
RT (min)	**Peak**	**Holdfast/stipe**	**Midrib**	**Frond**	**Sporophyll**	**Holdfast/stipe**	**Meristem**	**Young frond**	**Old frond**	**Sori**
109	A	1.0	1.0	1.1	2.1	1.6	0.95	0.62	0.65	0.25 ± 0.01
1215	B	-	-	-	-	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
1220	C	-	-	-	-	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
1250	D	-	-	-	<LOQ	0.008	<LOQ	0.018	0.019	0.05 ± 0.00
1270	E	0.001	0.002	0.013	0.011	0.025	0.022	0.13	0.11	0.21 ± 0.01
1290	F	0.012	0.048	0.28	0.19	<LOQ	0.008	0.055	0.037	0.05 ± 0.00
1320	G	0.017	0.019	0.015	0.21	0.011	0.054	0.12	0.068	0.11 ± 0.01
1330	H	0.036	0.031	0.073	0.004	0.005	0.041	0.16	0.102	0.08 ± 0.00
1400	I	-	-	-	-	<LOQ	<LOQ	0.018	0.01	0.01 ± 0.00
1550	J	0.006	0.007	<LOQ	0.012	0.028	0.027	0.029	0.017	0.02 ± 0.00
1590	K	0.13	0.16	0.082	0.27	0.27	0.42	0.24	0.17	0.29 ± 0.02
1600	L	0.023	0.006	<LOQ	<LOQ	0.16	0.17	0.21	0.22	0.37 ± 0.02
1630	M	0.015	0.026	0.037	0.095	0.18	0.14	0.15	0.1	0.34 ± 0.01
1690	N	0.46	0.61	0.56	1.4	0.46	0.56	0.36	0.24	0.38 ± 0.02
1700	O	0.091	0.18	0.26	0.39	0.61	0.56	0.67	0.57	0.79 ± 0.05
1820	P	0.13	0.14	0.1	0.075	0.42	0.43	0.6	0.5	0.83 ± 0.07
1900	Q	-	-	-	-	0.079	0.083	0.084	0.085	0.14 ± 0.01
2000	R	0.076	0.082	0.055	0.23	0.43	0.37	0.5	0.52	0.78 ± 0.03
2240	S	<LOQ	<LOQ	<LOQ	0.12	0.1	0.006	<LOQ	0.013	0.01 ± 0

Table 26 – The theoretical masses and error (Δ ppm) of AsLipid species identified in Phaeophyta. *Ascophyllum nodosum* (AN), *Fucus vesiculosus* (FV), *Alaria esculenta* (AE) and *Larminaria hyperborea* (LH). Thallus sections: HFS = holdfast/stipe, PS = primary shoot, SS = secondary shoot, RR = reproductive receptacle, BLD = blade, BLR = bladder, API = apice, MID = midrib, FR = frond, SPO = sporophyll (reproductive tissue), MER = meristem, YF = young frond, OF = old frond, DEF = decaying frond and SOR = sori (reproductive tissue). AsLipids identified as minor traces are listed in T13.

Peak	Analyte	Theoretical mass $[M+H]^+$	AN-HFS	AN-PS	AN-SS	AN-RR	FV-HFS	FV-BLD	FV-BLR	FV-API	AE-HFS	AE-MID	AE-FR	AE-SPO	LH-HFS	LH-MER	LH-YF	LH-OF	LH-SOR
B	AsHC332	333.2139		0.0[A]	2.1[A]	-1.2[A]	1.2[A]	1.5	1.2	1.2[A]					-1.5	1.2	1.5	-2.4	1.5
C	AsHC358	359.2295	0.8[A]	2.2[A]	2.2	2.0[A]									-0.6	1.7		0.3	-1.4[A]
D	AsHC346	347.2295								0.6					1.2	0.6		0.3	1.7
E	AsHC360	361.2452	0.3[A]	0.6[A]	1.1[A]	0.6	0.28	0.0	0.3[A]	0.3[A]	1.7	1.4	0.6	1.4	-0.6	0.3	1.1	-0.3	0.6
	AsHC374	375.2608					0.8	0.5	0.5	1.1				-0.5	-1.9	0.5	-0.8	0.0	1.6
F	AsHC388	389.2765					1.3[A]	0.5	-0.3	1.0	0.3	-0.3	-0.8	0.5	-1.5	1.3	0.0	-1.3	-0.5
	AsHC402	403.2921	-2.2	-0.7	-1.2	2.0	2.2	1.2	-3.2		1.24	0.7	0.0	-0.3	2.0		0.0	-2.0	-0.3
G	AsSugPL720	721.2909	3.7	2.1	2.8	-2.7	-2.6	-1.5	0.0	1.9	0.7	0.8	1.9	1.0	-1.1	2.5[B]	-0.7	-0.7	1.7
H	AsSugPL734		0.3			0.6	2.3	-2.3		1.8		0.5	0.5		0.5	0.0	1.1	1.1	-2.3
J	AsSugPL954	955.4893								1.6	-2.1	1.9	0.8	0.9	-0.1			2.0	
K	AsSugPL930	931.4893	0.0[B]	0.5[B]	0.3[B]	2.5	2.6[B]	0.75	-0.1[B]	0.6	0.3	0.0	-0.5	-1.2	-0.1	1.9	1.0	-0.5	-0.2
	AsSugPL944	945.5049	-0.2[B]	-0.1	1.0[B]	1.5	1.2	0.63	0.0	0.8	0.3	-0.1	-0.9	1.0	-1.1	1.2	-0.5	1.8	0.6
L	AsSugPL956	957.5049	0.6	-2.5	-1.5	-1.5	-0.2	-1.2	0.7	-2.3	-0.9	-0.9	0.9	1.9	1.2	0.8	-2.3	-0.2	-0.4
M	AsSugPL982	983.5206	0.4	-0.9	1.2	1.8[B]	1.3	0.6	-3.9	-2.4	0.4	-0.9	0.3	1.2	-1.4	0.4	0.7	-1.2	0.9
N	AsSugPL958	959.5206	0.3	-0.6[B]	0.0[B]	0.5[B]	1.3	-1.0	0.2	0.6[B]	0.0	0.3	0.3	0.7	-1.0	0.8	0.6	1.8	1.4
	AsSugPL972	973.5362	0.1	0.1[B]	0.0[B]	-0.5[B]	0.21	-0.10	-0.6	-0.3	-0.2	-0.9	-0.7	-0.1	1.3	0.2	-0.3	0.2	-0.2
	AsSugPL980	981.5049		-2.5	2.7		0.3	1.2		-1.0	1.7	2.9	2.6	3.3	0.7			2.8	-2.9
O	AsSugPL984	985.5362	0.2[B]	2.4	-1.5	-1.5	1.0	0.1	2.1	1.7[B]	0.10	1.2	-1.3	1.0	-0.4	-3.3	-1.0	0.6	0.8
P	AsSugPL986	987.5519	-0.5	0.7	0.5	-0.7[B]	2.1	-1.7	-2.3	1.9	-0.2	1.7	0.4	0.5	1.9	-1.0	-1.0	1.2	0.6
	AsSugPL1000	1001.5680	-0.5[B]	0.7	0.5	1.5	-0.50	-0.60	-2.9	2.0	-0.1	0.3	-0.3		1.9	-1.0	-0.6	0.8	0.5
	AsSugPL1012	1013.5675	1.9	1.0	-2.5	1.5	1.0			2.4	2.6	-1.5	-1.8		-1.8	-2.1	0.2	-0.1	-2.3
R	AsSugPL1014	1015.5832	1.6	-0.2	-2.9	1.6	-2.0	2.3	1.6	0.7	-0.10	1.6	-2.0		1.5	-1.0	-1.4	-0.4	1.4
	AsSugPL1028	1029.5990				2.0					2.0					2.4	-2.2	-0.6	0.9

[A]103,105 or 123 m/z fragments detected. [B]237,391 or 409 m/z fragments detected.

Chlorophyta and Rhodophyta

The AsLipid composition in Chlorophyta was significantly less diverse than that of the Phaeophyta, with a fewer number of AsSugPLs identified, Table 27. The concentrations of lipids did not appear to be influenced by the total arsenic concentration, where *U. intestinalis* had the highest arsenic content (9.5 mg kg^{-1}) but contained lower concentrations of all lipids than *A. arcta*. Two arsenosugar phytol species (AsSugPhytol546 and AsSugPhytol562) were detected in all species of Chlorophyta and identified based on fragmentation, Appendix F13 and F14. The dimeric form of AsSugPhytol546 ($[M+H]^+$ = 1093.66 m/z) was also present in several samples. AsSugphytol546 was first reported to occur in unicellular algae *Dunaliella tertiolecta* and has since been reported in ocean sediments, plankton and most recently in green freshwater algae *Chlamydomonas reinhardtii* (3,88,308). This compound was the dominant lipid species in all Chlorophyta with the exception of *Ulva intestinalis* collected at Grindavík, Fig 3, where concentrations were 10-fold lower than *U. intestinalis* collected at Kjalarnes (0.014 mg kg^{-1} and 0.15 mg kg^{-1}). *A. arcta* contained the highest concentrations of AsSugPhytol546 where it comprised 8.6-17% of the total arsenic, concentrations for other Chlorophyta were lower and contributed only 0.4-4% to the total arsenic concentration. AsSugPhytol562 was a minor component, and concentrations appeared to increase with increasing AsSugPhytol546. The concentration of AsSugPhytol546 has previously been demonstrated to show dependence on external factors such as phosphate concentration and ocean depth (in the case of phytoplankton) (3). However, *A. arcta* specimens were collected at both locations and had similar AsSugPhytol546 content (0.43-0.66 mg kg^{-1}), therefore changes in arsenic speciation in response to the environment is likely specific to each seaweed species. A pathway for the biosynthesis of AsSugPhytol was proposed by Glabonjat et al., where phytol diphosphate is suggest as the source of the phytol, which becomes attached to a methoxy-dimethylriboside (91). Phytol diphosphate is an intermediate in production of vitamin E, however, brown macroalgae have been shown to produce higher levels of vitamin E than red and green (309), so it unclear why AsSugPhytol synthesis is limited to Chlorophytes. Perhaps Rhodophyta and Phaeophyta both lack a mechanistic pathway to produce arsenic-containing phytyl riboses, as both contain chlorophyll which may be degraded to be produce a source of phytol, Figure 35 (98).

No AsHCs and AsFAs were detected in any Chlorophyta, which is similar to previously reported for *C. reinhardtii* (Chlorophyta) but differs from results for *Coccomyxa spp*. where several AsFAs were detected (80,88). Only AsSugPLs with C16:n and/or C18:n fatty acid residues were identified in Chlorophyta – where unsaturated AsSugPLs generally dominated. Unsaturated AsSugPL have been found to dominate in *Synechocystis sp.* (cyanobacteria) (310) but not in brown macroalgae – where saturated AsSugPL930 and AsSugPL958 were most abundant in *A. esculenta* and *S. latissima* (4).

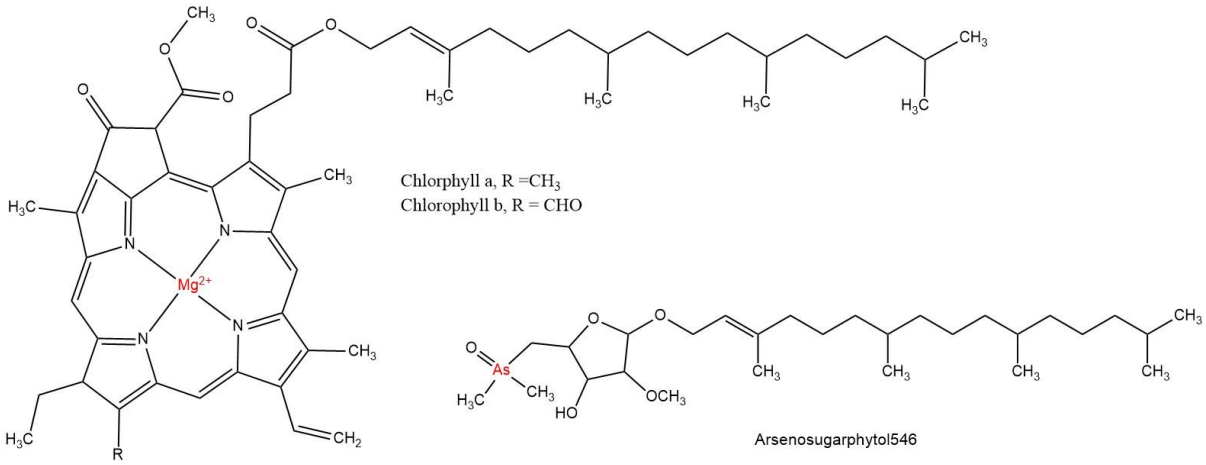


Figure 35 – The structure of arsenosugarphytol546 and chlorophyll a and b. Chlorophyll a is present in Phaeophyta, Rhodophyta and Chlorophyta, whilst chlorophyll b is only present in Chlorophyta (311).

Table 27 – The quantification and assignment of major peaks in Chlorophyta samples. All concentrations are shown in mg kg^{-1} dry sample weight and mass accuracy is shown (Δppm). n = 2 for all samples with the exception of *Ulva prolifera* where n = 3. LOD = 0.002 mg kg^{-1}, LOQ = 0.006 mg kg^{-1}. AsLipids identified as minor traces are listed in Appendix T13.

Retention time (seconds)	**Analyte**	**Theoretical mass $[M+H]^+$**	***Acrosiphonia arcta* (Kjalarnes)**	***Acrosiphonia arcta* (Grindavik)**	***Ulva intestinalis* (Kjalarnes)**	***Ulva intestinalis* (Grindavík)**	***Ulva prolifera** (Kjalarnes)**
1230	AsSugPhytol562	563.3290	0.027 (2.0)	0.053 (1.3)[c]	0.018 (0.9)	<LOQ (2.7)	0.006 ± 0.001 (0.0)
1260	AsSugPhytol546	547.3337	0.43 (0.4)	0.66 (0.4)[c]	0.15 (-1.6)	0.014 (2.2)	0.16 ± 0.018 (0.4)
1300	mAsSugPL720	721.2909	-	0.20	0.016	0.023	<LOQ
1500	AsSugPL954	955.4893	0.030 (2.7)	0.21 (1.4)	0.006 (1.9)	0.057 (1.3)	0.023 ± 0.002 (-0.6)
1540	AsSugPL980	981.5362	-	-	0.031	-	-
1610	AsSugPL956	957.5049	0.033 (0.3)	0.054 (2.2)	0.10	0.019	0.048 ± 0.006 (1.0)[b]
1640	AsSugPL982	983.5206	0.031 (-1.0)	0.25 (-0.1)	0.02 (-0.3)[b]	0.011 (-0.2)[b]	0.048 ± 0.005 (0.6)[b]
1720	AsSugPL958	959.5206	0.029 (0.3)	0.076 (0.0)	0.067 (0.7)	0.054 (2.0)	0.020 ± 0.001 (-1.4)
1760	AsSugPL984	985.5362	0.050 (-1.7)	0.216 (2.8)	<LOQ (-1.4)	-	0.091 ± 0.009 (-0.3)[b]

[a]103,105 or 123 m/z fragments detected. [b]237,391 or 409 m/z fragments detected. [c]111, 251 and 195 m/z fragments detected.

Table 28 – The quantification and assignment of peaks in Rhodophyta samples. All concentrations are shown in mg kg^{-1} dry sample weight and mass accuracy is shown (Δppm). n = 2 for all samples with the exception of *Cystoclonium purpureum* where n = 3. LOD = 0.002 mg kg^{-1}, LOQ = 0.006 mg kg^{-1}. AsLipids identified as minor traces are listed in Appendix T13.

Retention time (min)	**Analyte**	**Theoretical Mass ($[M+H]^+$)**	***Porphyra dioica***	***Chondrus crispus***	***Cystoclonium purpureum****	***Devaleraea ramentacea***	***Palmaria palmata***	***Mastocarpus stellatus***
1040	AsFA376	377.2037	-	0.013 (0.27)	0.012 ± 0.0003 (1.1)	0.017 (1.33)	-	-
1220	AsHC360	361.2452	0.03 (0.6)	<LOQ (0.6)	0.042 ± 0.002 (0.0[a])	0.23 (0.8[a])	0.038 (0.6)[a]	0.039 (-2.5)
	TMAsFOH374	375.608	-	-	(0.3)	(-0.8)	(1.3)	(0.27)
1260	AsSugPhytol546	547.3337	<LOQ (-0.9)	<LOQ (-2.2)[c]	<LOQ (0.2)	<LOQ (-0.7)[c]	-	-
1280	AsHC388	389.2765	<LOQ (0.0)	-	0.007 ± 0.002		<LOQ (0.5)	-
1320	mAsSugPL720	721.2909	-	-	0.019 ± 0.001 (-1.8)[b]	0.017 (-1.9)[b]	-	-
1480	AsSugPL954	955.4893	0.20 (0.3)	0.008 (-0.6)	0.0024 ± 0.005	-	0.026 (1.9)	0.011 (2.1)
1578	AsSugPL956	957.5049	0.020 (0.0)	-	0.049 ± 0.003 (-0.5)[b]	-	0.025 (0.6)	0.07 (0.1)
1590	AsSugPL930	931.4893	-	<LOQ (0.2)	0.01 ± 0.002 (0.1[b])	-	<LOQ (-0.9)	-
1640	AsSugPL982	983.5206	0.12 (-0.4)	-	-	0.028 (1.7)[b]	0.031 (2.1)	-
1720	AsSugPL958	985.5362	0.053 (1.3)	-	0.11 ± 0.012 (1.2)	0.047 (0.1)	0.014 (-1.8)	0.020 (0.7)
1760	AsSugPL984	985.5256	<LOQ (-1.0)[b]	-	0.058 ± 0.005 (-0.4)	0.061 (3.1)	-	-
1780	AsSugPL986	987.5519	-	0.031 (-0.8)[b]	0.042 ± 0.005 (0.2)	0.021 (-1.6)	-	-

[a]Fragments 103, 105 or 123 detected. [b]Fragments 237, 391 or 409 detected. [c]AsSugphytol dimer detected m/z = 1093.66 for $[M+H]^+$.

Rhodophyta samples displayed the most intra-class variations, where AsSugPL composition was different for all species, Table 28. The concentrations of AsHCs were generally low (0.007-0.042 mg kg^{-1}) with the exception of *D. ramentacea* where the concentration of AsHC360 was 0.23 mg kg^{-1}. As before, an arsenic compound with m/z 375.2608 co-eluted with AsHC360, however, no fragmentation data was available for the arsenic compound with m/z 375.2608, so it is difficult to determine which isomer is present. Based on what has previously been reported in *P. palmata* (5), it appears the TMAsFOH is more likely to be present than the hydrocarbon. With regards to AsFAs, only AsFA374 has been previously reported in red seaweed (*P. palmata*, <LOQ-0.017 mg kg^{-1}) but was not detected here, however, AsFA376 was identified in *C. crispus*, *C. purpureum* and *D. ramentacea* (0.013-0.017 mg kg^{-1}) (5).

The lipophilic arsenic speciation of *C. crispus* was particularly un-diverse, where only 3 AsLipids (AsFA376, AsSugPL954 and AsSugPL986) could be quantified among the 6 detected. Similar was true of *M. stellatus* where only 5 AsLipids were identified but not *C. purpureum* where 12 AsLipid species were identified. These 3 species are all in the order Gigartinales and were collected from the same sampling location, which would suggest that the arsenic metabolism of Rhodophyta displays a higher dependency on algal species than in Phaeophyta, where similar AsLipid composition has been demonstrated across several Laminariales (*A. esculenta*, *S. latissima*, and *L. digitata*) (4,37,96).

Minor traces of AsSugPhytol546 were detected in four Rhodophyta species – *Porphyra dioica*, *Chondrus crispus*, *Cystoclonium purpureum* and *Devaleraea ramentacea*. Whilst the signals were not intense enough for fragmentation, the compound and dimer ($[2M+H]^+$) were detected at the same retention time as in Chlorophyta samples. Due to the low concentrations, it is unclear whether the compound is produced by the Rhodophyta or potentially epiphytic species on the surface of the seaweeds, as this is the first report of this compound's occurrence in this class of seaweed. AsSugPhytol546 has been reported in microalgae (*D. tertiolecta*) which may exist as epiphytes on macroalgae thalli to seaweed (91,312). Additionally, Rhodophyta have small thalli and thus a larger surface area to colonise.

Red and green macroalgae are more closely phylogenetically related than brown macroalgae (313), so it is perhaps unsurprising that both display an un-diverse AsLipid composition in comparison to brown. Additionally, it possible the identification method used here is not identifying the full range of arsenic-containing lipids that are present, as a study with *Coccomyxa spp.* (Chlorophyta) has reported several arsenophospholipids (i.e., AsPI, AsCer and AsPE) and arsenoglycerides that were not identified during this study (80).

Conclusion

The aim of this study was to investigate the occurrence of arsenic-containing lipids in several species of seaweed collected in Iceland. The composition of AsLipids was shown to vary between classes of seaweed, with AsSugPLs dominating in Phaeophyta (42-83% of extracted As) and Rhodophyta (23-43% of extracted As), and AsSugPhytols were generally most abundant in Chlorophyta (5-32%). AsSugPL958 (C16:0/C16:0) was found to dominate in *A. nodosum*, *F. vesiculosus* and *A. esculenta* but not in *L. hyperborea* where AsSugPL984 (C16:0/C18:1) was marginally more abundant. AsSugPLs were consistently most concentrated in the thallus sections expected to have the highest biological activity (i.e., reproductive tissues). The dominant AsSugPL varied between species of red and green seaweeds, however AsSugPL958, AsSugPL982 (C16:0/C18:2) were most abundant throughout.

Two AsSugarPhytol species (563 m/z and 547 m/z) were detected in 3 species of Chlorophyta (*U. intestinalis, U. prolifera* and *A, arcta*) but showed variations between the same species collected from differing locations. Additionally, this study is the first to report the occurrence of AsSugPhytol546 in Rhodophyta, a compound which was thought only to be produced by Chlorophyta, unicellular algae and plankton. These findings may have major implications on the current understanding of arsenic cycling in macroalgae, as AsSugPhytols have not previously been reported in green macroalgae (and potentially Rhodophyta). This study expands current knowledge on lipid-soluble arsenic speciation in seaweed but highlights the need further studies in order to understand the mechanisms involved in producing these compounds.

Chapter 4 – Discussions and Conclusions

4.1 Discussion

The aim of this thesis was to investigate the hydrophilic and lipophilic arsenic speciation in several species of Phaeophyta, Rhodophyta and Chlorophyta. Variations in arsenic speciation between species, thallus sections and seasons were analysed to help elucidate how these compounds may be formed, and if they serve a biological purpose (i.e., storage, detoxification, or phosphorous conservation).

Total As was found to be most concentrated in the Phaeophyta (brown seaweed) - where accumulation was highest in the reproductive tissues in February (p-value<0.05 when compared with surrounding tissues) for all samples except *L. hyperborea* and *S. latissima* where total As was highest in the old frond and holdfast/stipe respectively. The reproductive tissues of brown seaweeds are known to grow during the winter and would be expected to have a higher demand for phosphorous in order to synthesise phospholipid membranes for spores and for RNA involved in protein synthesis (314). Thus, if phosphate and As(V) are taken up indiscriminately by the same transporters (49), higher As concentrations would be expected here. Phosphorous uptake is an active, energy-driven process as cells must work against a steep concentration gradient. Similarly, As uptake has also been shown to require energy expenditure, where algal cells kept in low light conditions did not accumulate as high concentrations as those exposed to light under identical conditions, and As uptake in *Picocystis sp.* has been demonstrated to increase under low phosphate conditions (3,288). Thus, the uptake of As is likely directly linked to the phosphorous demand.

The accumulation of As additionally appears to interfere with the uptake of several other elements, where higher levels of As were negatively correlated with levels of Mn, Fe, Co, and Cu. This may mean the metal cations are either competing with As for binding sites in polysaccharides in the cell wall, or that As toxicity inside the cell is interfering with the uptake of these metals. Increased As accumulation in rice has previously been shown to decrease levels of Fe so the latter option may be possible (315). The negative correlation between As and Co is more unusual as Co is not considered an essential nutrient for seaweed (316).

The naturally occurring forms of As in the environment are the inorganic species As(III) and As(V) which are directly taken up by algae. iAs concentrations were generally low in all samples analysed except for *L. digitata*, where it comprised over 50% of the total As. This is similar to previously reported results for this species where concentrations were also found to increase along thallus from holdfast to distal fronds (37,236). Similar intra-thallus trends were observed for *A. nodosum* and *F. vesiculosus* except on a significantly smaller scale (<1 mg kg^{-1}) but not for *S. latissima* where the highest concentration was found in the holdfast tissues. Whilst all brown seaweeds accumulated high levels of As, *S. latissima* accumulated similar levels of As to *L. digitata*, therefore the differences in iAs accumulation may mean the latter does not have as efficient system for methylating As - which is further slowed in distal parts of the frond, or that *L. digitata* has a unique cell wall polysaccharide composition that is able to complex high levels of As(V) (i.e., as arsenate esters which are hydrolysed upon extraction with water (37)).

Methylation is considered a detoxification process for many PTEs, but there is doubt over whether this holds true for As as the methylated compounds MMA and DMA still display some level of toxicity – particularly in the trivalent forms which are produced initially after methylation (60). Low levels of MMA and DMA relative to the AsSugar concentrations were detected in all samples analysed. This may suggest that the conversion from methylated species to ribose derivatives is fast. All *M. stellatus* samples contained high concentrations (approximately 6 mg kg^{-1}) of an unknown compound that eluted at a similar time to MMA. The elution time (~650 s, Figure 31 in Paper V) indicates this may be a low molecular weight arsenical such as dimethyl arsinoyl ethanol (DMAE) which has previously been reported in unicellular algae *D. tertiolecta* and is associated with bacterial degradation of seaweed tissues (93,317,318) - it is not possible for the unknown to be a thiolated species due to the presence of hydrogen peroxide in the extraction solution. Thus this compound must come from bacteria on the surface of the seaweed (i.e., small thallus so large surface area for bacteria to colonize) or from degradation of the AsSugars (317).

Unlike MMA and DMA, the AsSugar composition of seaweeds was shown to vary significantly between species, where in *A. esculenta*, *F. vesiculosus* and *L. digitata* the sulphonate sugar derivative (AsSug-SO_3) was most abundant whereas in *A. nodosum* the sulphate derivative (AsSug-SO_4) was most abundant. The C-O-S bond present in AsSug-SO_4 is common in algae metabolites, i.e., saccharides fucose and fucoidan where the addition is catalysed by sulfotransferases (204), however the direct C to S bond in AsSug-SO_3 is not. Perhaps AsSug-SO_3 begins with a thiol group as the side chain, which is then oxidised to SO_3 in an irreversible process often observed with cysteine residues (298), or perhaps it is synthesised through a different pathway to the other derivatives. Why the As speciation of several brown seaweeds (with the exception of *A. nodosum*) is dominated by AsSug-SO_3 also remains unclear. In Chlorophyta and Rhodophyta the phosphate sugar derivative generally dominated. The AsSug-PO_4 and AsSug-gly derivatives were present in all samples analysed across the three classes (Phaeophyta, Rhodophyta and Chlorophyta). The differences in AsSugar compositions between seaweed species is thought to be due to differing enzymes capable of carrying out the glycosidation reaction of the ribose side chain. However, the results presented in this thesis in combination with those in the literature appear to suggest that every species of seaweed (Phaeophyta, Rhodophyta and Chlorophyta) has the ability to synthesise all four derivatives even if they are only detected at very low levels (37,49,70). The concentrations appear to be affected by external factors such as season as well as species. Perhaps the enzymes responsible for the glycosidation of monosaccharides (e.g., mannose or fucose) are also capable of carrying out the final stage of AsSugars synthesis, as the production of these non-As containing sugars have additionally shown significant variations between species and season (319,320). Additionally, imaging studies have shown that As within *L. digitata* (Phaeophyta) cells is stored primarily in the cell walls – meaning AsSugars must be stored here (37). Their exclusion from the intra-cellular matrix would suggest they do not serve any energy storage purposes as observed with other sugars; however, they may be transported outside of cell as part of a detoxification process to exclude As from the cytosol or to serve as cell wall components similarly to structural polysaccharides (i.e., alginate and fucoidan). The polysaccharides provide resistance from mechanical stress, impart anti-freezing properties in cold temperatures and assist with osmotic regulation (204). Polysaccharides in the cell wall may also complex potentially toxic cations, and thereby reduce accumulation in the intracellular matrix. The impact of AsSugars in the cell wall matrix on other biological processes is unknown, yet seaweeds appear to be able to withstand high levels (>60 mg kg^{-1}).

With regards to the lipid-soluble speciation, Phaeophyta contained the highest levels of lipophilic As – where the majority was in the form of AsSugPLs. Additionally, these were most concentrated in the reproductive tissues similarly to the AsSugars. If AsSugars are the starting point for these lipids, then high concentrations of both would be expected in the same areas of the thallus. Perhaps the AsSugPLs structures arise from the binding of AsSug-gly to phosphatidic acid (PA) in the cell membrane, which would provide rationale for the attachment of a phosphate group and two acyl chains. However, as AsSugPL958 (C160/:C16:0) was generally the most abundant, it would imply that a PA with two palmitic acid residues would be the most abundant PA – something which has not previously been described in the literature. The AsHCs and AsFAs produced by seaweed has been suggested to mirror the natural composition of free fatty acids and hydrocarbons – i.e., C17:0 hydrocarbons are abundant in algae which have the same chain length and saturation level as AsHC360 (321).

There were clear differences between classes of seaweed, where in Chlorophyta an AsSugPhytol species (AsSugPhytol546) dominated in the majority of samples. This compound was also detected in several Rhodophyta in trace amounts, so is perhaps produced by bacteria on the surface of the cell rather than the seaweed itself. This lipid has an identical phytyl chain to that found in chlorophyll, so is likely produced from the free phytol after the degradation of chlorophyll, but how the free phytol would become attached to a ribose is less clear. Non-As containing sugars are typically produced in the chloroplasts, so perhaps the synthesis of AsSugars also takes place within this organelle.

Phosphorous is the second-most limiting nutrient in the ocean, so it may be possible algae have evolved to move past this by utilising As for membrane lipids. AsSugPLs have been demonstrated to increase in *Ectocarpus sp*. under low phosphorous conditions (140) and additionally the incorporation of As into complex molecules such as sugars and lipids does not appear to be a logical final step in a detoxification process. AsSugars are non-toxic to algae, and thus if detoxification were the rationale no further reactions (which produce AsLipids) would be required. Thus, it seems that the only options left to explain the formation of these compounds is biological infidelity or a dedicated biological pathway. However, it remains unclear which is the correct theory.

4.2 Limitations of the current study

The major limitations with regards to As speciation are the measurement techniques, lack of available standards and reference materials. The measurement of inorganic arsenic in seaweed can be severely hindered by the presence of high levels of AsSugars which may co-elute with As(V). Often long run times and gradient elutions are required to separate species which is not practical for routine measurements. As demonstrated within this thesis, acid can be used to degrade certain AsSugar species and the addition of an oxidising agent means As(III) and As(V) are quantified as the sum of both. However, in seaweed matrices the addition of nitric acid had the unexpected effect of increasing DMA concentration, potentially due to the degradation of lipid-soluble species only present in seaweed. Thus, this method is limited with regards to the quantification of methylated species.

For AsLipids, both HPLC-ICP-MS and some form of high-resolution mass spectrometry (preferably coupled together) are required due to the lack of available standards. This set-up is

not widely available and expensive - with only a handful of laboratories in the world having access to this equipment. Therefore, only a select number of samples were analysed for lipophilic speciation which perhaps has not offered as much information into seasonal and locational variations in lipids as was hoped for.

The sample preparation may additionally be an unexpected limiting factor – particularly for lipophilic species – as drying has been shown to significantly reduce the amount of lipid-soluble As and increase the residual As. This may mean the analysis of AsLipids is an underestimation of the amount of lipophilic As as the samples were freeze-dried before analysis. The reason for this increase in residual As after drying is unclear – perhaps it leads to degradation to smaller arsenicals which are then bound to thiols and rendered unextractable, or the As atoms are reduced to their trivalent form and become linked to polysaccharides through sulphylhydryl groups. Drying has also been shown to cause severe damage to cell membranes and protein aggregation, the latter of which is where proteins misfold and become insoluble (322). Fatty acids or phospholipids bound covalently to these proteins become unextractable (323) – similar may be possible for AsLipids.

4.3 Implications on current knowledge and legislation

These findings may have major implications on what is currently known about As cycling in marine macroalgae – most specifically the As-containing lipids – as this is the first time AsSugPhytols have been reported to occur in macroalgae. Whilst these compounds were predicted to dominate in Chlorophyta due to their similarities with unicellular green algae, it is unusual to discover that these compounds are also potentially produced by Rhodophyta which contain different compositions of photosynthetic pigments.

The results from this study may additionally serve as comprehensive occurrence data for hydrophilic and lipophilic As species in edible seaweeds – something which is currently not available but necessary for legislative bodies. The levels of iAs were below the ML of 1.0 mg kg^{-1} set for seaweed products in Australia and New Zealand (183) in all seaweeds with the exception of *L. digitata*, one *U. intestinalis* sample, *U. lactuca* (CRM BCR-279) and the tropical *A. taxiformis*. Only the *L. digitata* samples exceeded the less strict regulations for iAs in France (3 mg kg^{-1}) (184), and in some cases this value was exceeded by many multiples. This may suggest that specific recommendations or regulation should be put forward for this species of seaweed to avoid risks to consumers.

High levels of AsSugars were detected in the seaweeds analysed, where these concentrations reached several tens of mg kg^{-1} (dry weight) in brown seaweeds. AsSugars are suspected to exert some form of toxicity after chronic exposure (77), regular consumption of seaweed has been shown to lead to the accumulation of As in the tissues of seaweed-eating sheep (324). However, these animals do not live long enough to fully assess the cancer risk to humans upon long-term exposure through ingestion. If epidemiological studies do prove significant risks from ingestion of AsSugars, this data set may serve as occurrence data for edible seaweed.

The lipid-soluble As speciation in seaweed has only previously been reported for *L. digitata*, *S. latissima*, *A. esculenta*, *P. palmata* and *Coccomyxa spp.* (4,5,37,80,96), which is far from

the number of species that are considered edible or used as feed additives. AsSugPLs were generally most abundant in brown seaweed (sum: 0.95-5.0 mg kg^{-1}) and *F. vesiculosus* frond and apice tissues specifically were found to contain relatively high levels of toxic AsHCs (sum 0.5-0.7 mg kg^{-1}). Whilst the toxicity of AsHCs are widely known (6), the levels of AsHCs and AsFA in samples pose little to no threat to consumers, particularly at the current seaweed consumption rates estimated by EFSA (26). No toxicity studies have been carried out on AsSugPLs but their structure perhaps suggests they may exert similar toxicities to AsSugars (where both share a dimethylarsenoribose group) but may have higher bioavailability due to the acyl groups (97).

4.4 Future research into arsenic speciation

Further research should focus on the use of imaging techniques and genetic experiments to understand more about the cycling of As in seaweed. To date, only one study has used imaging (NanoSIMS) to determine the localisation of As within seaweed cells – and furthermore this was performed for *L. digitata* where approximately 50% of As was in the form of inorganic species (37). Imaging studies with Chlorophyta specimens containing high proportions of AsSugarPhytols would also be of major scientific interest to understand more about the formation and storage of these unusual compounds (i.e., are they stored in the chloroplasts along with phytol containing chlorophyll?). In some species of Chlorophyta, a significant portion of the As may additionally be non-extractable (i.e., not extractable with organic or aqueous solvents) and so imaging or XAS may be used to determine whether any of this As is bound to sulphur-containing proteins inside the cell.

Genetic experiments in combination with speciation measurements have proven useful in determining which genes are involved with As metabolism – and thus may determine if algae have a dedicated pathway for the formation of As species. For example, the gene encoding the radical SAM enzyme responsible for the production of an intermediate in the formation of AsSugars was identified in *Synechocystis sp.* (cyanobacteria) (71). Another recent study using gene 'knockout' in *Chlamydomonas sp.* was able to determine that the gene thought to be responsible for As methylation in algae did not play a role (88). Previously the major limiting factor for gene editing in macroalgae was the gene transfer method, where this was only previously possible for *Ulva spp.* and the model alga *Ectocarpus* (325,326). However emerging technologies may mean this technique may soon be applicable to larger macroalgae species (327).

4.5 Conclusions

The aim of this thesis was to investigate differences in As speciation between several species of Phaeophyta, Rhodophyta and Chlorophyta. Hydrophilic As species were shown to vary significantly between seaweeds – where high concentrations of iAs were only detected in *L. digitata* and AsSugar composition was highly dependent on species and season. All macroalgae appear to have the ability to synthesise all four AsSugar derivatives, but their production may be influenced by external factors. Lipophilic species additionally varied between species; However, differences were more pronounced between classes of seaweeds where AsSugPLs were dominant in Phaoephyta and Rhodophyta (albeit in lower

concentrations than Phaeophyta) and AsSugPhytols were most abundant in Chlorophyta. This would imply that the cycling of As is highly dependent on algal species, where different seaweed classes may have evolved different mechanisms and pathways for the metabolism of As.

Analysis of the variations in arsenic speciation between species, thallus sections and seasons was hoped to offer additional insight into how these compounds may be formed. AsSugars and AsSugPLs were both most concentrated in the reproductive tissues of the brown seaweeds, which may be evidence that AsSugars are the starting product of AsSugPLs as both share a dimethylarsenoribose moiety. The lipids are perhaps produced by the binding of AsSugars to phosphatidic in the membrane which would provide an explanation as to why these lipids also contain a phosphate moiety and two acyl groups. Whether these species are produced accidently as a result of biological infidelity or are produced with a biological purpose is unclear. It does not appear that AsSugars serve as energy storage as is displayed with other sugars, however, their inclusion in the cell wall matrix may have yet to be discovered benefits (i.e., increased resistance to environmental changes).

This study has contributed significantly to understanding the occurrence of these compounds in marine macroalgae. Further research should focus on the use of imaging techniques to investigate the localisation of arsenic within cells, and the use of genetic techniques (i.e., gene ‘knockout’) in combination with speciation measurements to understand if algae have developed dedicated pathways for arsenic metabolism.

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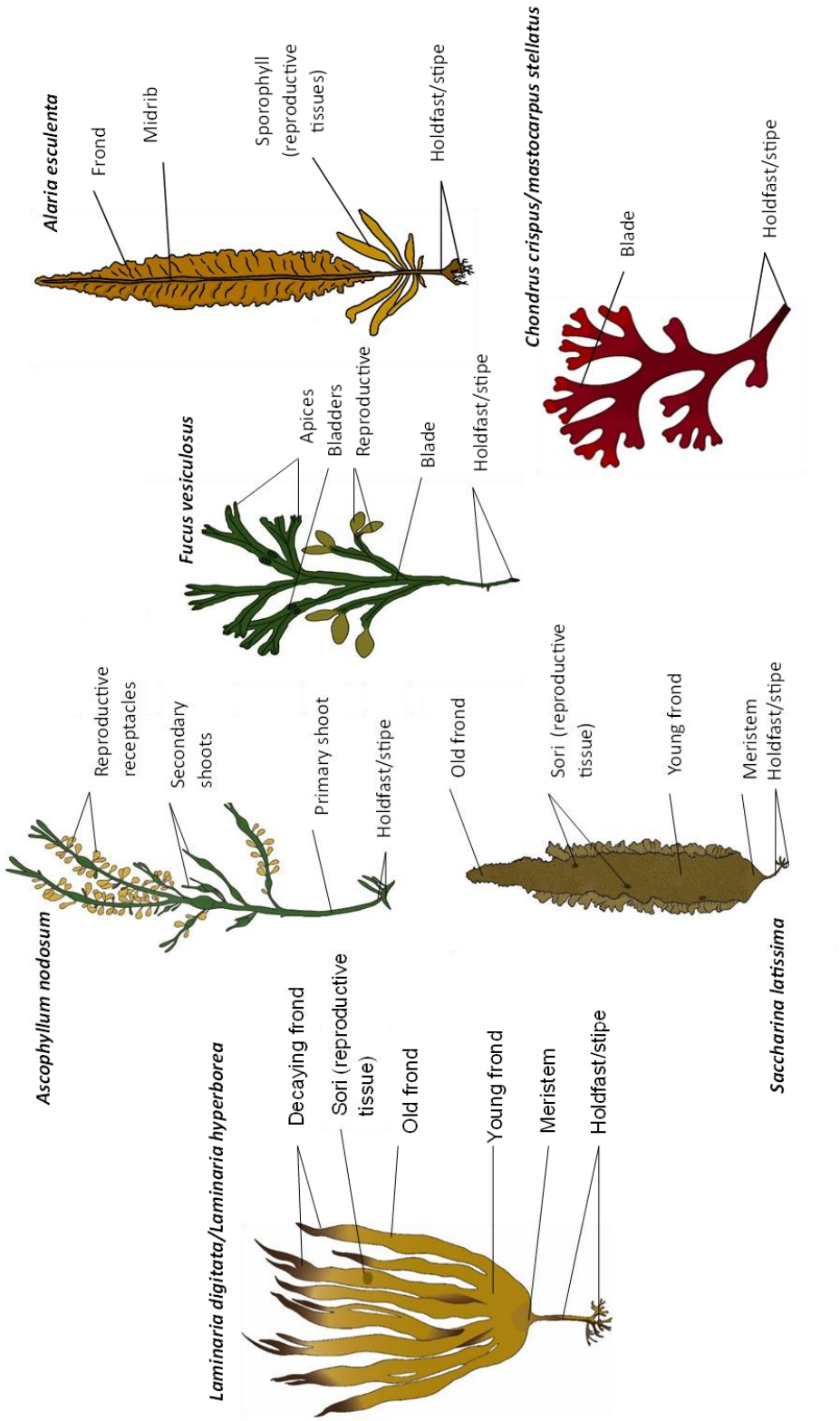
Appendices



Appendix F1 – The sectioning of large macroalgae thalli.

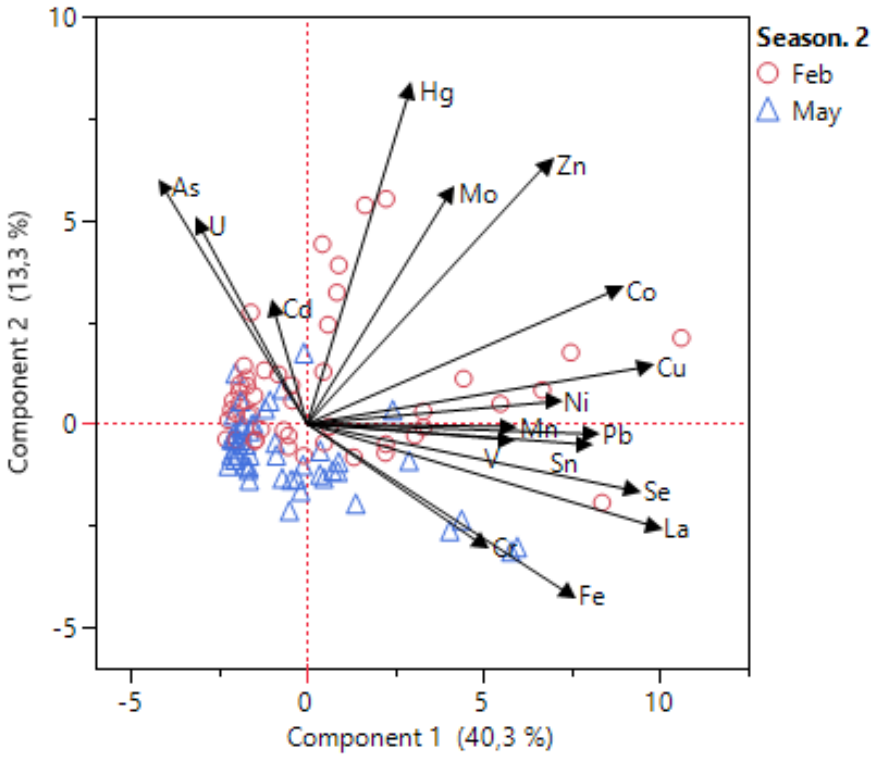
Appendix T1 – The results of DNA sequencing of Chlorophyta samples.

Sample number	BLAST results	Class	Sampling date/location
GR29 (presumed Acrosiphonia sp.)	*Acrosiphonia arcta*	Chlorophyta	May 2021/Kjalarnes
GR30 (presumed Ulva sp.)	*Ulva prolifera*	Chlorophyta	May 2021/Kjalarnes
GR31 (presumed Ulva sp.)	*Ulva intestinalis*	Chlorophyta	May 2021/Kjalarnes
GR32 (presumed Acrosiphonia sp.)	*Acrosiphonia arcta*	Chlorophyta	May 2021/Grindavík
GR33 (presumed *Ulvaria* sp.)	*Porphyra dioica*	Rhodophyta	May 2021/Grindavík
GR34 (presumed *Ulva sp.*)	*Ulva intestinalis*	Chlorophyta	May 2021/Grindavík

DNA extraction, PCR and sequencing. DNA was extracted from approximately 0.2 g of the tissue of selected kelp species using the DNeasy Plant Mini Kit from QIAGEN (ref: 69104) following the manufacturer protocol. Concentration of extracted DNA was assessed using NanoDrop 2000 (Thermo Scientific) spectrophotometer. Internal spacer region (ITS) was amplified and sequenced using the following primer pair: ITS5 (5'-GAAAGTAAAAGTCGTAACAAGG-3') and ITS4 (5'- TCCTCCGCTTATTGATATGC-3') (White et al. 1990). The PCR reaction was performed in a mixture consisting of: 2 µL DNA, 2 µL of 10X Standard Buffer, 2 µL of 10 mM DTP, 0.12 µL of forward and reverse primers at 100 µM concentration each, 13.6 µL of ddH2O and 0.15 µL Taq (Taq DNA Polymerase with Standard Taq Buffer, NEB cat. M0273L). PCR amplifications were done in a MiniAmp™ Thermal Cycler (Applied Biosystems) using the following PCR conditions: An initial denaturation step at 95°C for 5 min, followed by 40 cycles of denaturation at 95°C for 20 s, annealing at 52°C for 40 s and an extension at 72°C for 1 min, and then a final extension at 72°C for 7 min. PCR success was assessed by gel electrophoresis on 1% Agarose gel. PCR products were purified, subjected to a standard BigDye terminator sequencing chemistry (v3.1, ThermoFisher) and sequenced on an ABI 3730 DNA Analyzer (Applied Biosystems). Chromatograms were visually inspected, and quality trimmed. Quality trimmed sequences were subjected to a BLAST search using NCBI's nt/nr nucleotide database and used to verify and assist in species identification of the selected kelp species.

Appendix T2 – The limit of detection (LOD) and limit of quantification (LOQ) of the ICP-MS method for the determination of total element concentrations (paper I).

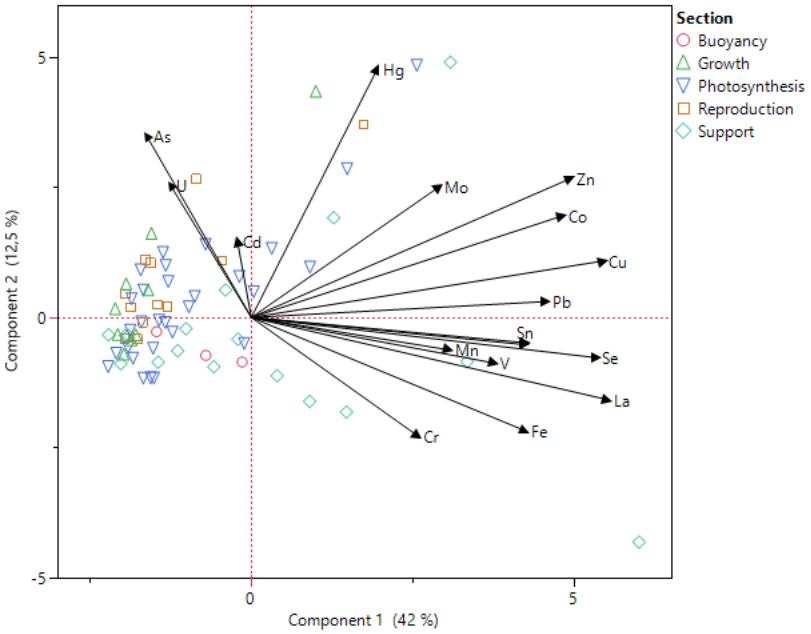
	Limit of Detection (mg kg^{-1})	**Limit of Quantification (mg kg^{-1})**
Ti	0.7	2.1
V	0.02	0.07
Cr	0.02	0.05
Mn	0.02	0.05
Fe	0.4	1.2
Co	0.003	0.01
Ni	0.02	0.05
Cu	0.01	0.04
Zn	1.2	3.8
As	0.01	0.04
Se	0.06	0.2
Zr	0.02	0.06
Mo	0.004	0.01
Cd	0.003	0.01
Sn	0.001	0.002
Sb	0.01	0.03
Ba	0.04	0.11
La	0.003	0.01
Hg	0.007	0.02
Pb	0.003	0.01
U	0.001	0.002



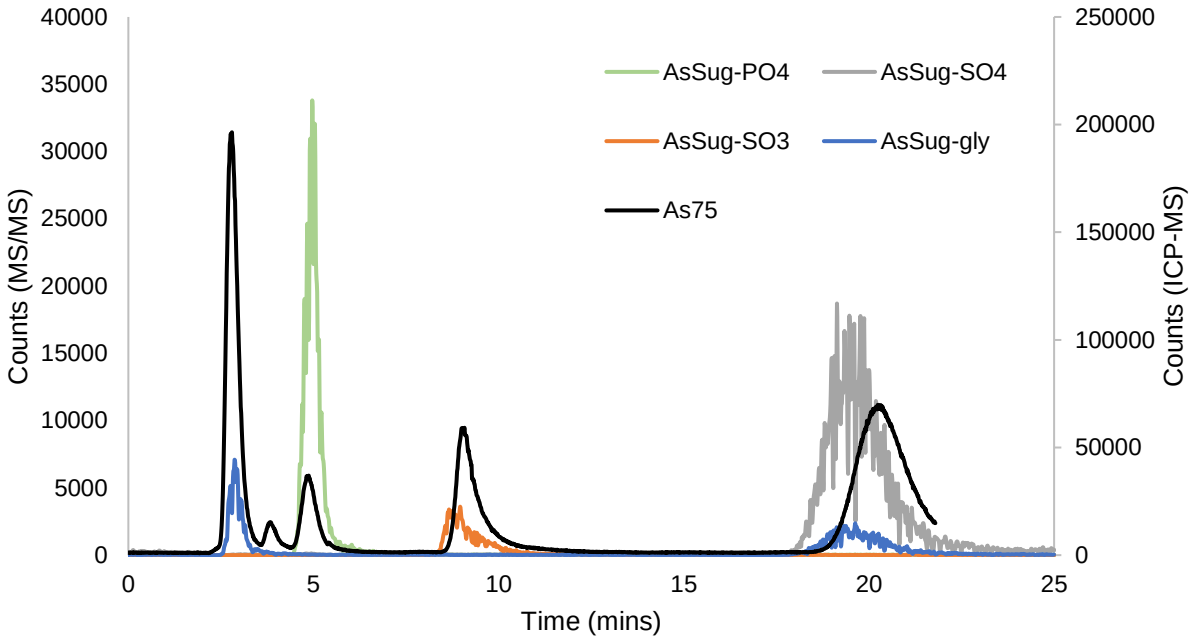
Appendix F2 – PCA of concentration of 17 elements in the sample set, where samples were grouped by sampling month (e.g., February or May) (paper I).

Appendix T3 – The grouping of sections of brown seaweeds by biological function before PCA (paper I).

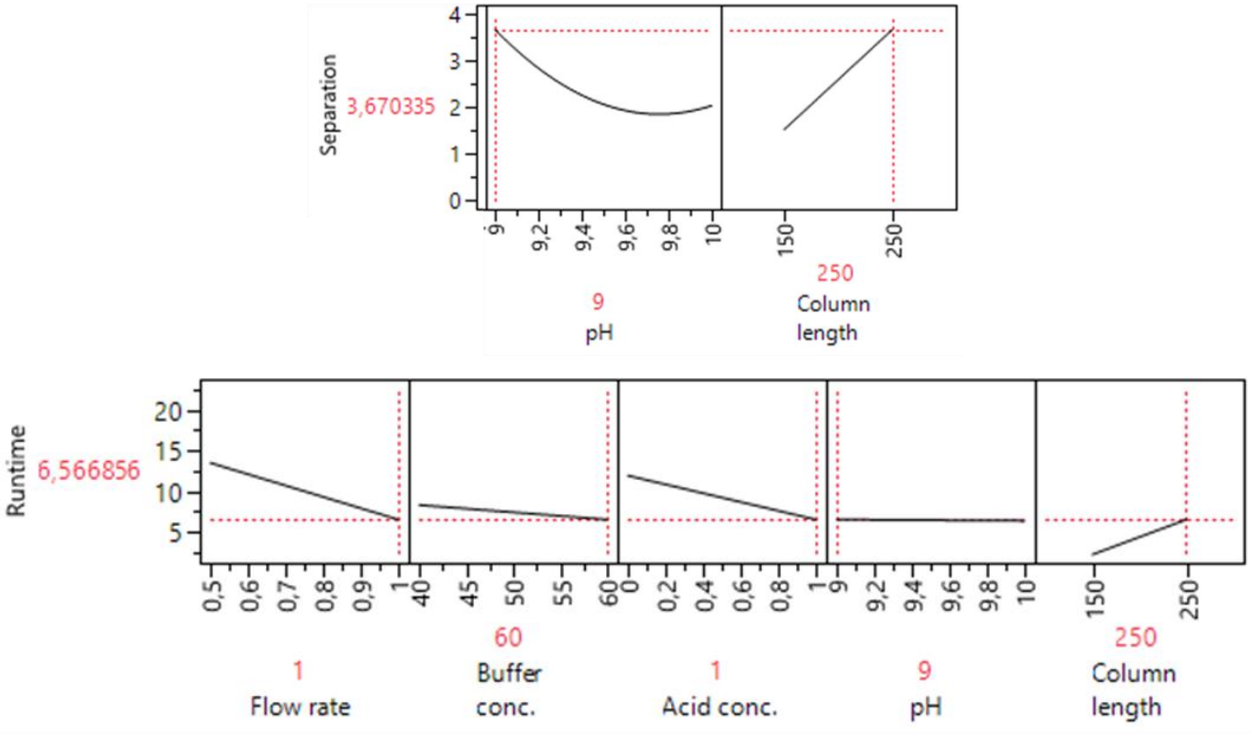
Biological function	Section
Buoyancy	Bladders (*Fucus vesiculosus* only)
Growth	Meristems and apices
Photosynthesis	Fronds and blades
Reproduction	Reproductive receptacles, sori and sporophyll
Support	Holdfasts/stipes



Appendix F3– PCA of the concentrations of 17 elements in the sample set, where samples were grouped by biological function (paper I).



Appendix F4 – The identification of arsenosugars in a sample of *Ascophyllum nodosum* used as an in-house reference material by LC-MS/MS (Quantiva, Thermo). Note: results are from a previous project and were used as reference for AsSugar identification (paper II).



Appendix F5 – The prediction plots for the significant factors affecting the separation and runtime for the iAs method optimised using DOE (paper II).

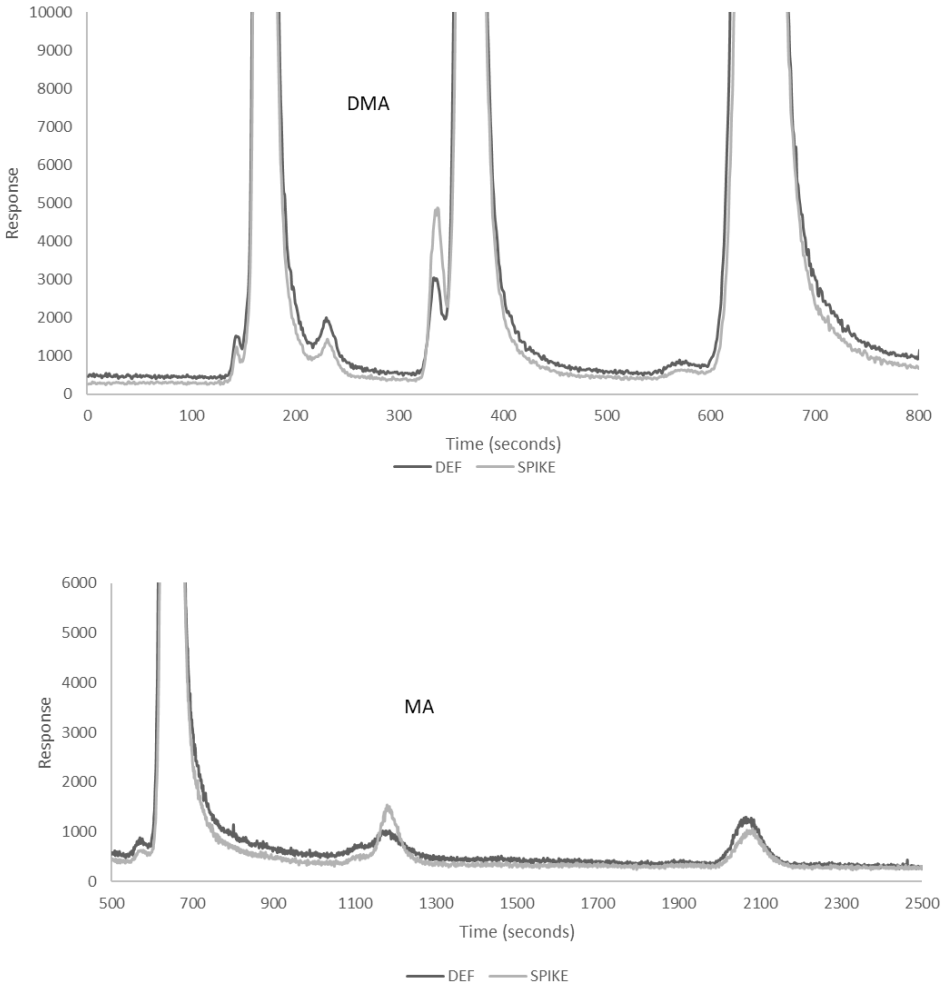
Appendix T4 – Additional results from the seaweed and mussel samples analysed using the iAs method developed with DOE (paper II).

Sample	AsSug-gly (mg kg^{-1})	DMA (mg kg^{-1})	AsSug-PO4 (mg kg^{-1})	MMA (mg kg^{-1})	iAs (mg kg^{-1})	Sum of unknown (mg kg^{-1})	Total As extracted (mg kg^{-1})	Column recovery (%)
Sea lettuce (CRM BCR-279	0.822 ± 0.161	0.071 ± 0.006	ND	<LOQ	1.359 ± 0.198	-	2.425 ± 0.455	98 ± 17
P. dioica	21.885 ± 0.513	0.163 ± 0.016	1.295 ± 0.150	<LOQ	0.050 ± 0.003	0.172 ± 0.014	23.938 ± 0.621	99 ± 3
A. taxiformis	7.542 ± 0.581	0.731 ± 0.072 3	2.00 ± 0.355	0.141 ± 0.008	2.382 ± 0.027	0.173 ± 0.012	13.003 ± 1.419	100 ± 3
P. palmata (Dulse)	5.162 ± 0.420	0.234 ± 0.033	1.298 ± 0.191	<LOQ	0.361 ± 0.013	0.030 ± 0.003	7.905 ± 2.725	92 ± 12
C. purpureum	1.426 ±0.064	0.184 ± 0.019	0.748 ± 0.029	<LOQ	0.450 ± 0.013	0.043 ± 0.002	2.778 ± 0.399	105 ± 13
F. vesiculosus (apice)	63.167 ± 6.129	1.376 ± 0.140	11.112 ± 0.929	0.376 ± 0.035	0.352 ± 0.056	0.450 ± 0.114	72.085± 10.258	107 ± 6
F. vesiculosus (blade)	17.068 ± 1.674	0.307 ±0.04 1	3.878 ± 0.568	0.133 ± 0.014	0.082 ± 0.013	0.137 ± 0.002	20.844 ± 3.685	105 ± 9
F. vesiculosus (reproductive tip)	48.913 ± 1.102	0.453 ± 0.044	7.375 ± 0.533	1.166 ± 0.393	0.197 ± 0.020	0.514 ± 0.068	50.484 ± 7.776	120 ± 17
A. nodosum (primary shoot)	12.786 ± 1.030	0.517 ± 0.038	4.895 ± 0.994	0.030 ± 0.015	0.059 ± 0.013	0.274 ± 0.049	19.357 ± 2.558	98 ± 2
A. nodosum (reproductive receptacle)	40.393 ± 0.479	0.766 ± 0.058	7.690 ± 0.111	0.139 ± 0.023	0.101 ± 0.017	1.448 ± 0.168	48.264 ± 0.613	105 ± 2
A. nodosum (secondary shoot)	17.829 ± 1.998	0.407 ± 0.026	4.760 ± 0.611	0.036 ± 0.014	0.060 ± 0.003	0.407 ± 0.090	22.053 ± 1.403	101 ± 4
L. digitata (meristem)	81.880 ± 1.890	0.730 ± 0.064	3.314 ± 0.353	1.259 ± 0.402	2.599 ± 0.012	1.039 ± 0.063	96.528 ± 1.453	94 ± 3
L. digitata (sori)	25.177 ± 0.545	0.311 ± 0.035	10.829 ± 0.415	0.352 ±0.05 8	54.20 3 ± 1.343	0.260 ± 0.021	84.889 ± 3.890	107 ± 2
L. digitata (old frond)	42.876 ± 0.484	0.635 ±	5.015 ±	0.600 ±	77.91 7 ±	0.329 ± 0.005	134.439 ± 7.157	95 ± 7

L. digitata (decaying distal frond)	27.500 ± 0.984	0.015 0.510 ± 0.067	0.677 5.651 ± 1.347	0.090 0.409 ± 0.034	3.275 80.04 4 ± 1.574	0.252 ± 0.025	108.031 ± 6.423	106 ± 4
S. latissima (stipe)	41.621 ± 0.717	0.633 ± 0.025	6.333± 0.223	0.542 ± 0.036	0.116 ± 0.006	0.336 ± 0.019	49.450 ± 1.082	100 ± 3
S. latissima (old frond)	137.00 6 ± 2.248	0.713 ± 0.045	8.044 ± 0270	1.501 ± 0.433	0.046 3 ± 0.006	0.863 ± 0.038	141.736 ± 9.298	105 ± 6
S. latissima (young frond)	118.14 7 ± 1.152	0.448 ± 0.009	7.037 ± 0.523	0.861 ± 0.032	0.035 ± 0.002	0.849 ± 0.089	112.030 ± 11.7523	115 ± 11
S. latissima (meristem)	59.417 ± 0.123	0.253 ± 0.012	2.932 ± 0.026	1.176 ± 0.095	0.027 ± 0.003	0.236 ± 0.037	63.430 ± 3.437	101 ± 5
S. latissima (sori)	139.00 6 ± 2.428	0.467 ± 0.046	6.053 ± 0.277	2.071 ± 0.196	0.053 ± 0.002	1.430 ± 0.160	138.037 ± 13.769	109 ± 13
Mussels	*4.443 ± 0.041	1.18 ± 0.04	0.481 ± 0.020	0.05 ± 0.002	0.150 ± 0.002	0.142 ± 0.020	7.083 ± 0.039	91 ± 2

Appendix T5 – The instrumental operating parameters for the ICP-MS and HPLC-ICP-MS for water-soluble arsenic speciation (paper IV).

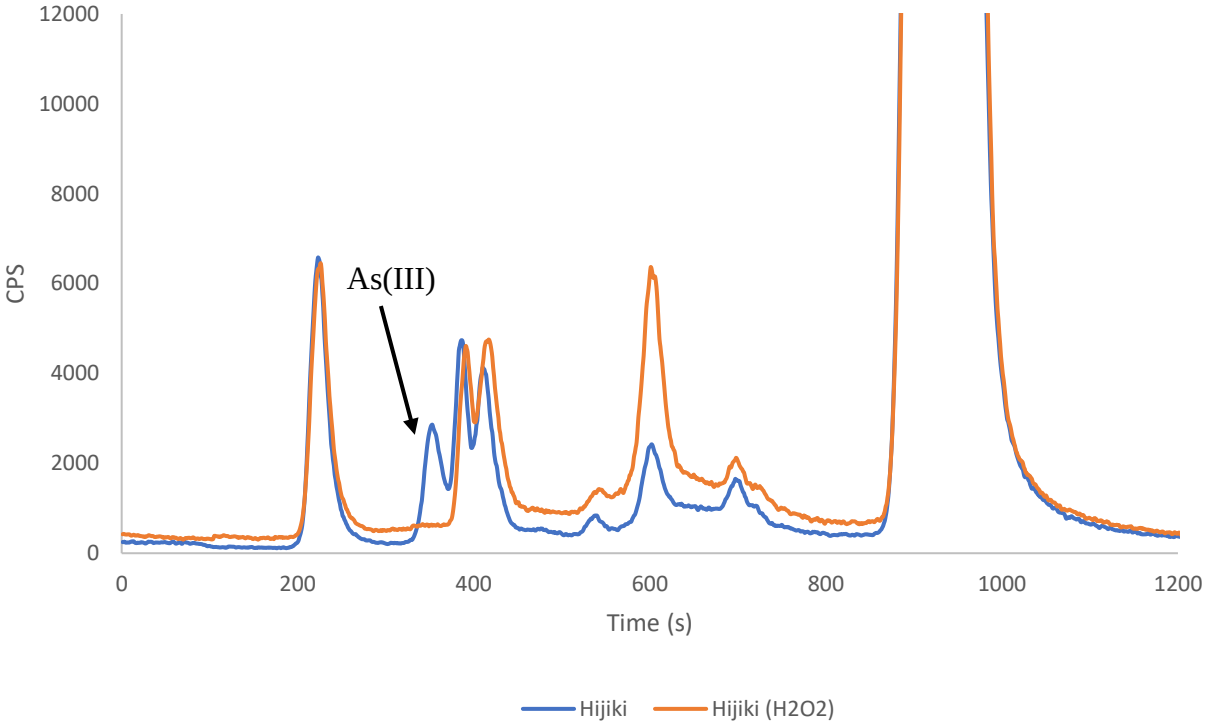
Instrument operating parameters	
ICP-MS settings	Agilent 7900 ICP-MS
RF power	1550 W
RF matching	1.25 V
Plasma gas flow	15.0 L min^{-1}
Carrier gas flow	1.07 L min^{-1}
Make-up gas flow	0.8 L min^{-1}
He gas flow	5.0 L min^{-1}
Spray chamber temperature	2 °C
Isotopes monitored	As^{75}, In^{115} (internal standard)
HPLC-ICP-MS settings	Agilent 1290 Infinity II HPLC and Agilent 7900 ICP-MS
Isotopes monitored	As^{75}, Se^{77}, Se^{82} Ge^{72} (internal standard
Anion exchange column	PRP-X100 (250 x 4.6 mm, 10 µm)
Guard column	PRP-X100 Guard cartridge
Mobile phase	20 mM $(NH_4)_2CO_3$, 3% MeOH
Flow rate	1 mL min^{-1}
Injection volume	40 µL



Appendix F6 – Spiked samples used to identify DMA and MMA (paper IV) (top) Decaying frond sample, normal and spiked with 50 µg L^{-1} DMA solution. (bottom) Decaying frond sample, normal and spiked with 20 µg L-1 MA solution.

Appendix T6– The As(III) concentrations present all samples of *Laminaria digitata*, analysed using a different HPLC-ICP-MS method (paper IV).

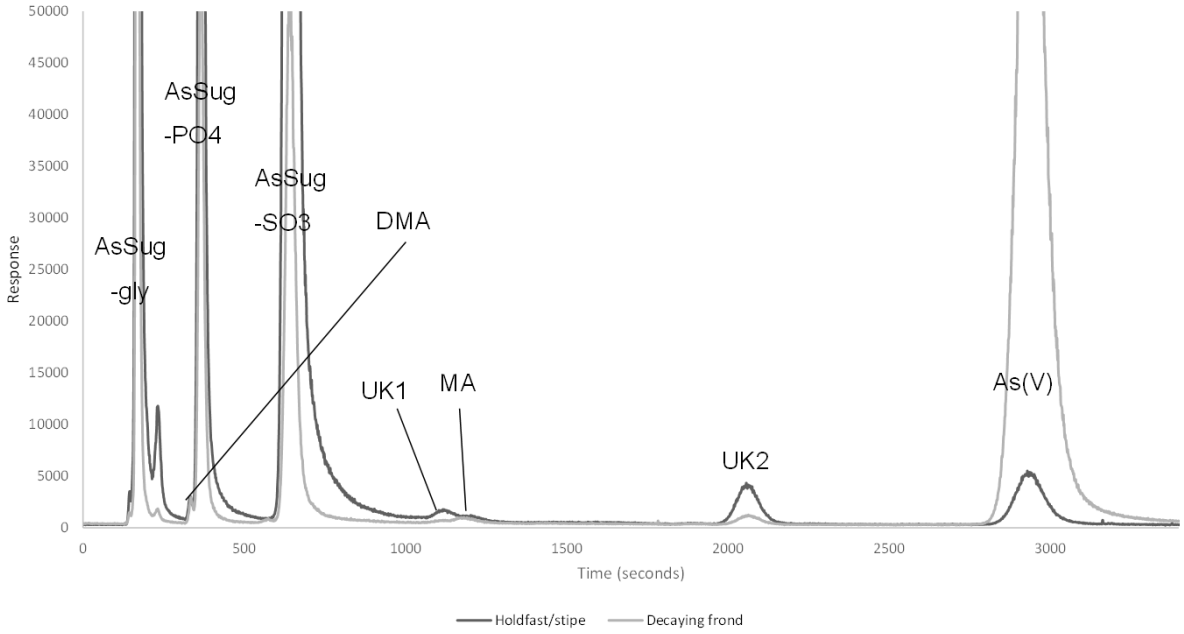
Thallus section (n =2)	As (III) conc. in February (mg kg^{-1})	As (III) conc. in May (mg kg^{-1})
Holdfast/stipe	0.12	0.10
Meristem	0.12	0.13
Young frond	0.15	0.13
Old frond	0.06	0.05
Decaying frond	0.07	0.03



Appendix F7 – Identification of As(III) by spiking with hydrogen peroxide using a different HPLC-ICP-MS method (paper IV).

Appendix T7 – The average concentration of AsSugars and other water-soluble As species in each thallus section of *Laminaria digitata* during both months (paper IV).

Month (drying method)	Thallus section	AsSug-gly/As(III) (mg kg^{-1})	DMA (mg kg^{-1})	AsSug-PO_4 (mg kg^{-1})	AsSug-SO_3 (mg kg^{-1})	MA (mg kg^{-1})	As (V) (mg kg^{-1})	Unknown (sum) (mg kg^{-1})
February (freeze-dried)	Holdfast/ stipe	3.19	0.19	4.21	25.4	0.05	1.82	0.47
	Meristem	3.12	0.22	9.89	41.9	0.09	2.19	1.50
	New frond	2.78	0.28	12.9	35.3	0.05	24.6	1.02
	Old frond	1.97	0.32	9.55	22.7	0.03	59.3	0.85
	Decaying frond	14.8	0.19	13.4	13.6	0.05	61.0	0.71
May (freeze-dried)	Holdfast/ stipe	11.5	0.05	5.55	20.8	0.06	0.78	0.65
	Meristem	15.9	0.07	5.72	33.3	0.07	2.46	1.90
	New frond	15.7	0.06	4.47	23.0	0.06	7.82	0.77
	Old frond	17.1	0.12	4.57	11.3	0.07	35.5	0.75
	Decaying frond	2.2	0.26	7.55	16.5	0.04	44.4	0.43
May (fresh)	Holdfast/ stipe	6.41	0.03	3.79	14.1	0.03	0.63	0.86
	Meristem	11.7	0.03	6.40	24.2	0.03	1.65	1.43
	New frond	7.54	0.06	1.75	12.7	<LOQ	5.69	0.62
	Old frond	9.09	0.08	2.30	6.54	0.02	19.1	0.59
	Decaying frond	5.56	0.09	3.74	6.00	0.13	27.7	0.37



Appendix F8 – Chromatographs of the holdfast/stipe and decaying thallus sections from extractions with fresh *Laminaria digitata* sample material (paper IV).

Appendix T8 – Instrument parameters for analysis with the ICP-MS for total arsenic in Paper V.

ICP-MS operating conditions	**Agilent 7900 ICP-MS (He gas mode)**
RF power	1550 W
RF matching	1.20 V
Plasma gas	15 L min^{-1}
Carrier gas	1.07 L min^{-1}
Make-up gas	0.80 Lmin^{-1}
Spray chamber temperature	2°C
Octopole collision cell	Pressurized, He gas (5 mL min^{-1})
Isotopes monitored	As^{75}, In^{115} (internal std)

Appendix T9 – Instrument parameters for analysis with the HPLC-ICP-MS set-up for water-soluble arsenic speciation (Paper V).

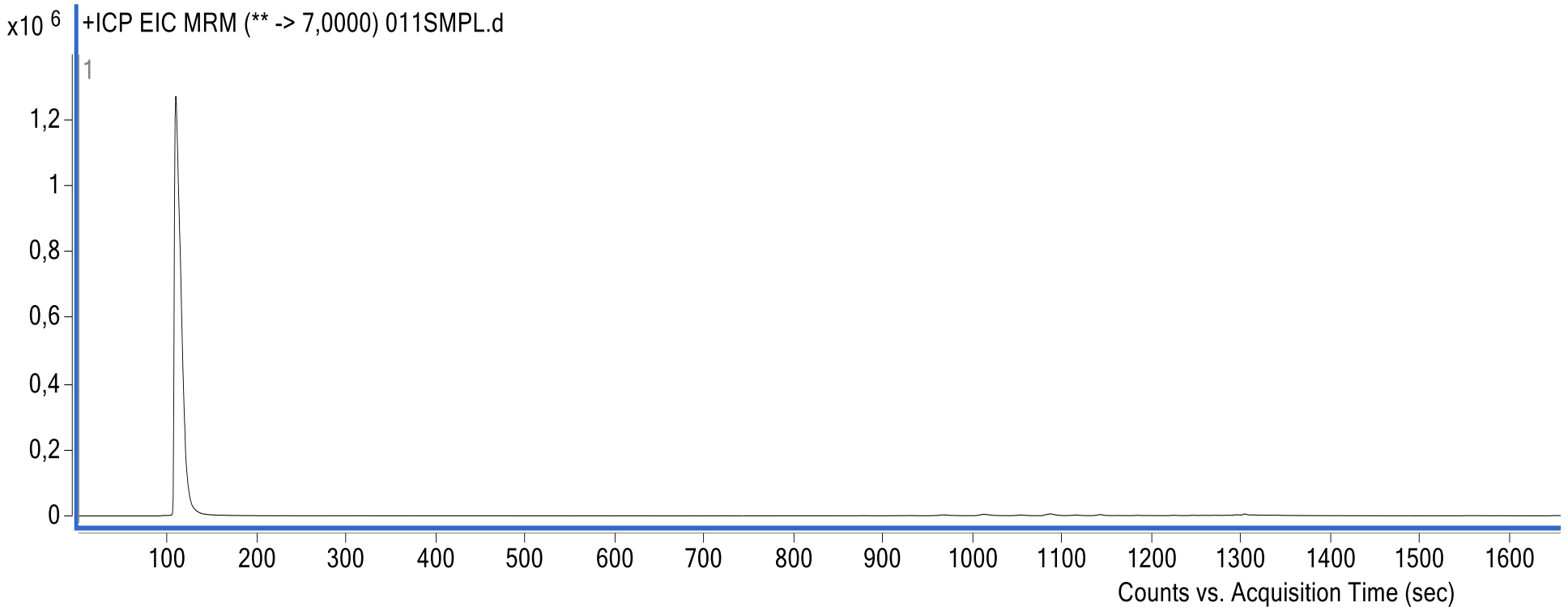
HPLC-ICP-MS settings	**Agilent Infinity II 1290 HPLC and Agilent 7900 ICP-MS (No gas mode)**
RF power	1530 W
RF matching	1.25 V
Nebulizer gas flow	1.07 L min^{-1}
Plasma gas flow	15.0 L min^{-1}
Spray chamber temperature	2°C
Isotopes monitored	^{75}As, ^{77}Se, & ^{72}Ge (internal standard)
Integration time	1 s
Guard column	Hamilton PRP-X100 Guard cartridge
Analytical column	Hamilton PRP-X100 (250 × 4.6 mm;10 µm)
Mobile phase	A: Ammonium carbonate (0.05 mM, pH 9.2) 3% MeOH B: Ammonium carbonate (50 mM, pH 9.2) 3% MeOH
Gradient	0-6 min: A 80% B 20% 6-17 min 100% B 17-23 min A 80% B 20%
Flow rate	1 mL min^{-1}
Injection volume	40 µL

Appendix T10 – The water-soluble arsenic speciation of all samples measured. Extraction: water and hydrogen peroxide (3% v/v). Analysis. HPLC-ICP-MS with anion exchange column and ammonium carbonate buffer (gradient elution). All concentrations in mg kg^{-1} (paper V).

Species	Location	Season	Section	Total As	Total As error	Total As extract	Cations	AsSug-gy	DMA	AsSug-PO4	AsSug-SO3	MMA	AsSug-SO4	As(V) (iAs)	Unknown sum	Column recovery	Extraction efficiency
Ascophyllum nodosum	K	May 2021	Holdfast/stipe	40.75	1.36	27.84	0.00	2.33	0.49	2.26	2.97	0.01	16.82	0.08	0.11	88	68
Ascophyllum nodosum	K	May 2021	Primary shoot	42.77	0.30	16.30	0.00	1.99	0.12	1.22	1.50	0.00	8.58	0.07	0.16	82	38
Ascophyllum nodosum	K	May 2021	Secondary shoot	32.63	1.77	17.67	0.00	1.54	0.10	1.69	1.14	0.01	11.25	0.02	0.15	87	54
Ascophyllum nodosum	K	May 2021	Reproductive receptacle	41.63	3.41	29.15	0.00	1.57	0.21	2.26	3.71	0.01	19.91	0.00	0.14	93	70
Ascophyllum nodosum	K	Feb 2022	Holdfast/stipe	60.76	3.03	19.08	0.00	2.45	0.09	0.98	1.74	0.00	5.44	0.03	0.02	97	31
Ascophyllum nodosum	K	Feb 2022	Primary shoot	34.51	4.59	18.72	0.00	6.34	0.15	1.55	2.04	0.00	6.35	0.04	0.09	89	54
Ascophyllum nodosum	K	Feb 2022	Secondary shoot	87.69	4.31	23.11	0.00	3.30	0.20	1.82	4.34	0.01	13.39	0.08	0.15	99	26
Ascophyllum nodosum	K	Feb 2022	Reproductive receptacle	195.16	11.11	43.17	0.00	1.88	0.31	2.27	7.85	0.01	31.68	0.09	0.28	102	22
Fucus vesiculosus	K	May 2021	Holdfast/stipe	48.62	0.66	44.55	0.02	0.50	0.30	1.24	34.11	0.00	2.28	0.00	0.11	87	92
Fucus vesiculosus	K	May 2021	Blade	46.58	7.30	26.78	0.03	0.39	0.17	1.69	18.02	0.01	2.46	0.00	0.07	85	58
Fucus vesiculosus	K	May 2021	Bladder	31.44	1.57	25.24	0.00	0.41	0.14	1.66	17.62	0.01	4.15	0.00	0.06	95	80
Fucus vesiculosus	K	May 2021	Apice	71.15	4.72	48.65	0.00	0.32	0.14	1.97	39.63	0.03	3.75	0.00	0.10	93	68
Fucus vesiculosus	K	May 2021	Reproductive receptacle	96.03	25.01	40.37	0.00	0.60	0.07	3.23	32.57	0.00	5.65	0.00	0.20	105	42
Fucus vesiculosus	K	Feb 2022	Holdfast/stipe	43.36	2.51	38.16	0.00	0.85	0.23	1.33	30.87	0.00	5.53	0.06	0.11	102	88
Fucus vesiculosus	K	Feb 2022	Blade	73.95	5.72	28.32	0.00	0.84	0.24	1.47	19.65	0.03	3.79	0.04	0.10	92	38
Fucus vesiculosus	K	Feb 2022	Bladder	36.92	2.09	27.16	0.00	0.83	0.20	1.44	21.07	0.02	3.73	0.03	0.12	101	74
Fucus vesiculosus	K	Feb 2022	Apice	92.72	8.00	53.10	0.00	1.24	0.40	2.12	52.84	0.00	4.96	0.06	0.17	116	57
Fucus vesiculosus	K	Feb 2022	Reproductive receptacle	115.10	13.12	67.32	0.00	1.08	0.38	2.46	55.92	0.02	6.73	0.03	0.32	99	58

Appendix T11 – Tort-2 results n = 4 AsPC940 was also detected as well as AsFA390 which comprises one of the fatty acid residues in the arsenophosphatidyl choline (paper VI)

Analyte	Theoretical mass $[M+H]^+$	Concentration (mg kg^{-1})	Error (delta ppm)
AsFA307	307.1254	0.052 ± 0.008	1.3
AsFA374	375.1880	0.044 ± 0.000	0.5
AsFAFA376/AsFA388	377.2037/389.2037	0.079 ± 0.002	0.5/1.8
AsFA390	391.2193	0.019 ± 0.004	1.3
AsHC360	361.2452	0.015 ± 0.0025	1.9
AsPC940	940.5413	-	-2.2



Appendix F9 – The ICP-MS trace (mass shift mode: $^{75}As^{16}O^{+}$) for reference material TORT-2 (lobster hepatopancreas). The majority of the extracted As was in the form of less polar water-soluble species that eluted in the void volume of the column. Inset: the lipid-soluble species (paper VI).

Appendix T12a – All compounds identified in Phaeophyta by accurate mass (HPLC-qTOF-MS) and where possible fragmentation data (paper VI)

Analyte	Theoretical mass $[M+H]^+$	*A. noodsum holdfast/stipe*	*A. nodosum primary shoot*	*A. nodosum secondary shoot*	*A. nodosum reproductive receptacle*	*F. vesiculosus holdfast/stipe*	*F. vesiculosus blade*	*F. vesiculosus bladder*	*F. vesiculosus apice*	*A. esculenta holdfast/stipe*	*A. esculenta midrib*	*A. esculenta frond*	*A. esculenta sporophyll*	*L. hyperborea holdfast/stipe*	*L. hyperborea meristem*	*L. hyperborea young frond*	*L. hyperborea old frond*	*L. hyperborea sori*
AsHC332	333.2139		0.0 A	2.1[A]	-1.2[A]	1.2[A]	1.5	1.2	1.2[A]					-1.5	1.2	1.5	-2.4	1.5
AsHC346	347.2295								0.6					1.2	0.6		0.3	1.7
AsHC358	359.2295	0.8[A]	2.2[A]	2.2	2.0[A]									-0.6	1.7		0.3	-1.4
AsHC360	361.2452	0.3[A]	0.6[A]	1.1[A]	0.6	0.3	0.0	0.3[A]	0.3[A]	1.7	-0.3[A]	0.6	1.4[A]	-0.6	0.3	1.1	-0.3	0.6
AsHC374/ TMAsFOH374	375.2608					0.8	0.5	0.5	1.1						0.5		0.0	1.6
AsFA376	377.2037											2.7[A]						
AsHC380	381.2139	1.6[A]	0.0	-0.8	0.5													
AsHC388	389.2765					1.3[A]	0.5	-0.3	1.0	0.3		-0.8[A]	0.5	-1.5	1.3	0.0	-1.3	-0.5
AsFA398	399.2244	2.0[A]																
AsHC402	403.2921	-2.2	-0.7	-1.2	2.0	2.2	1.2			1.2		0.0	-0.3	2.0	4.0	0.0	-2.0	
AsFA422	423.1880		1.4			0.47	1.0	0.5	0.7					1.4			1.9	
AsFA424	425.2030					-1.2	0.5		0.0									
AsSugPhytol546	547.3337					0.0												
AsSugPhytol562	563.3290																	
mAsSugPL692	693.2596				-2.7[B]							-1.9		0.0	-1.7		1.4	
mAsSugPL706	707.2753				0.6	0.9	1.6	-0.6	2.7	-2.8	-2.8		-1.3			0.3	-0.9	0.9
mAsSugPL720	721.2909	-3.7	2.1	2.8[B]	-2.1[B]	-2.6	-1.5	0.0[B]	1.9	0.7	0.8[B]	1.9	1.0	-1.1	2.5[B]	-0.7	-0.7	1.7[B]

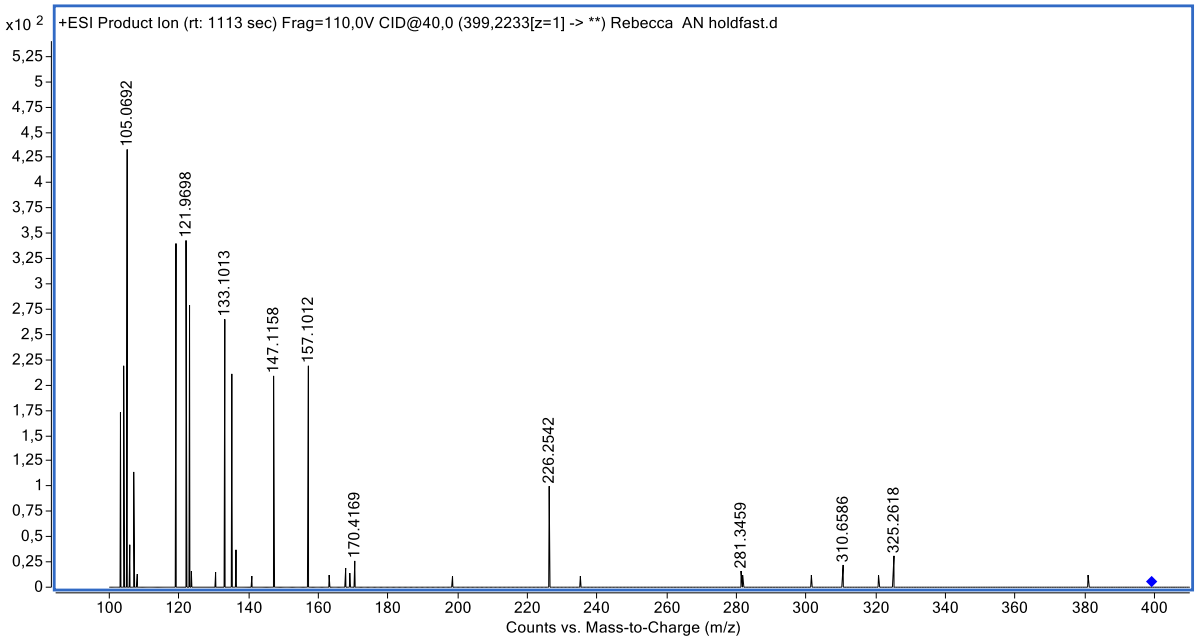
mAsSugPL734	735.3066	0.3			-0.3	2.3	-2.3		1.8		0.5	0.5		0.5	0.0	1.1	1.1	-2.3
mAsSugPL742	743.2753				1.2				-0.1				1.75		-1.4	1.8		
mAsSugPL746	747.3066				0.3								0.4					
mAsSugPL776	777.3535				0.4					-1.7	-1.7	1.2	1.9		-2.7	2.2		-1.3[b]
AsSugPL930	931.4892	0.0[B]	0.5[B]	0.3[B]	2.5	2.6[B]	0.8	-0.1[B]	0.6	0.3[B]	0.0	-0.5	-1.2	-0.1	1.9	1.0	-0.5	-0.2
AsSugPL944	945.5049	-0.2	-0.11	1.0[B]	1.5	1.2	0.6	0.0	0.8	0.3	-0.1	-0.9	1.0	-1.1	0.8[B]	-0.5	1.8	0.6
AsSugPL954	955.4893					3.2			1.6	-2.1	1.9	0.8	0.9	-0.1			2.0	
AsSugPL956	957.5049	0.6	-2.5	-1.5	-1.5	-0.2	-1.2	-0.7	-2.3	-0.9[B]	-0.9[B]	0.9	1.9	1.2	0.8	-2.3	-0.2	-0.4
AsSugPL958	959.5206	0.3	-0.6[B]	0.0	0.5	1.3	-1.0	0.2	0.6[B]	0.0[B]	0.3[B]	0.3[B]	0.7	-1.0	0.8[B]	0.6	1.8	1.4
AsSugPL972	973.5362	0.1	0.1	0.0	-0.5[B]	0.2	-0.1	-0.6	-0.3	-0.2	-0.9[B]	-0.7	0-0.1	1.3	0.2	-0.3	0.2	-0.2
AsSugPL980	981.5049	3.5	-2.5[B]	2.7	3.2	0.3	1.2		-1.0	1.7	2.9	2.6	3.3	0.7	3.3		2.8	-2.9
AsSugPL982	983.5206	0.2	-0.9	1.2	1.8[B]	1.3	0.6	-3.9	-2.4	0.4	-0.9	0.3	1.2	-1.4	0.4	0.7	-1.2	0.9
AsSugPL984	985.5362	0.2[B]	2.4	-1.5	-1.5[B]	1.0	0.1	2.1	1.7[B]	0.1	1.2	-1.3[B]	1.0	-0.4	-3.3	-1.0	0.6	0.8[B]
AsSugPL986	987.5524	-0.5	0.7	0.5	-0.7[B]	2.1	-1.7	2.3	1.9	-0.2[B]	1.7	0.4[B]	0.5	1.9	-1.0	-1.0	1.2	0.6
AsSugPL1000	1001.5680	-0.5	0.7	0.5	1.5	-0.5	-0.6	-2.9	2.0	-0.1	0.3	-0.3		1.9	-1.0	-0.6	0.8	0.5
AsSugPL1012	1013.5675	1.9	1.0	-2.5	1.5[B]	1.0		2.4	2.4	2.6	-1.5	-1.8		-1.8	-2.1	0.2	-0.1	-2.3[B]
AsSugPL1014	1015.5832	1.6	-0.2	-2.9	1.6	-2.0	2.3	1.6	0.7	-0.1	1.6	-2.0[B]	1.5[B]	1.5	-1.0	-1.4	-0.4	1.4
AsSugPL1028	1029.5990									2.0					2.4	-2.2	-0.6	0.9

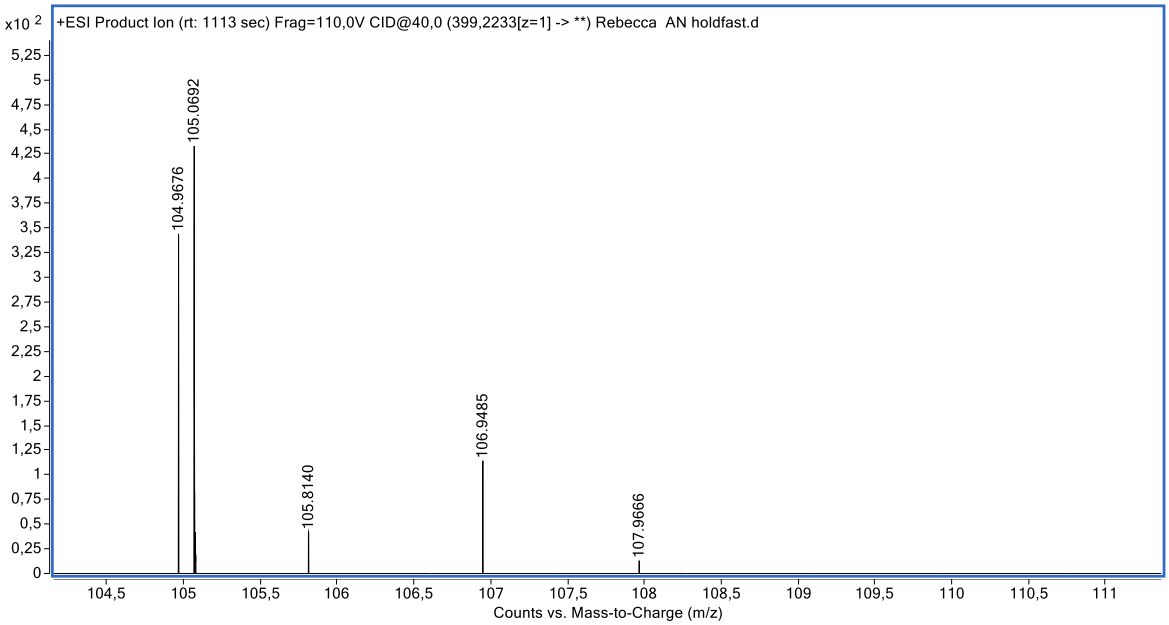
A103,105 or 123 m/z fragments detected. B237,391 or 409 m/z fragments detected.

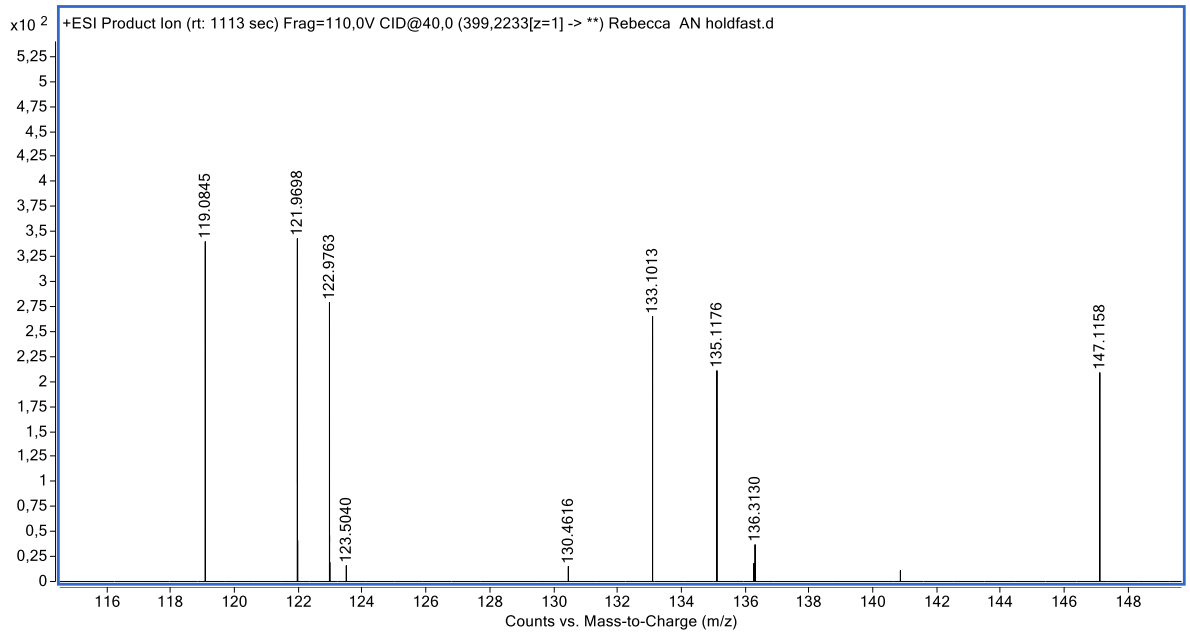
Appendix T12b – All compounds identified in Rhodophyta and Chlorophyta by accurate mass (HPLC-qTOF-MS) and where possible fragmentation data (paper (VI).

Analyte	Theoretical mass $[M+H]^+$	*U. prolifera*	*U. intestinalis (Kjalarnes)*	*U. intestinalis (Grindavík)*	*C. purpureum*	*C. crispus*	*D. ramentacea*	*P. dioica*	*P. palmata*	*M. stellatus*
AsHC332	333.2139				0.6[A]					
AsHC346	347.2295					1.4				
AsHC358	359.2295						2.2		0.8	-3.1
AsHC360	361.2452				0.0[A]	0.6	0.8[A]	0.6[A]	0.6[A]	-2.5
TMAsFOH374	375.2608				0.3		-0.8		1.3	0.3
AsFA376	377.2037				1.1	0.3[A]	1.3[A]			
AsHC388	389.2765				0.8			0.0	0.5	
AsHC402	403.2921						-2.2			
AsSugPhytol546	547.3337	0.4	-1.6[C]	2.2	0.2	-2.2	-0.7	-0.9		
AsSugPhytol562	563.3290	0.0	0.9	2.7						
mAsSugPL720	721.2909	-1.5	0.9		-1.8[B]		-1.9[B]			
AsSugPL930	931.4892				0.1[B]				-0.9	
AsSugPL954	955.4893	-0.6	-1.9	1.3		-2.6		0.3	1.9	2.1
AsSugPL956	957.5049	1.0	1.7		-0.5[B]			0.0	0.6	0.1
AsSugPL958	959.5206	-1.4	0.7	2.0	1.2[B]		0.1	1.3	-1.8	0.7
AsSugPL972	973.5362									
AsSugPL980	981.5049				1.8[B]		-0.2	2.6		
AsSugPL982	983.5206	0.6	-0.3[B]	-0.2	2.8[B]	1.2	1.7[B]	-0.4	2.1	
AsSugPL984	985.5362		-1.4		-0.4[B]		-1.6[B]	-1.0[B]		
AsSugPL986	987.5524	-0.91	-0.2		0.2[B]	-0.8[B]			0.3	

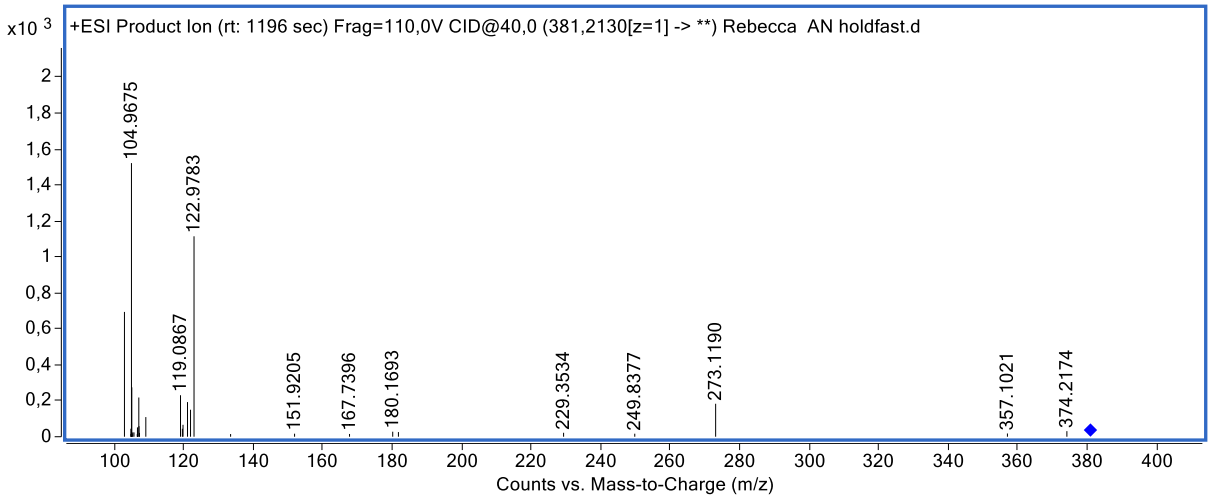
[A]103,105 or 123 m/z fragments detected. [B]237,391 or 409 m/z fragments detected.


x10 2
+ESI Product Ion (rt: 1113 sec) Frag=110,0V CID@40,0 (399,2233[z=1] -> **) Rebecca AN holdfast.d
105.0692
121.9698
133.1013
147.1158
157.1012
170.4169
226.2542
281.3459
310.6586
325.2618
Counts vs. Mass-to-Charge (m/z)

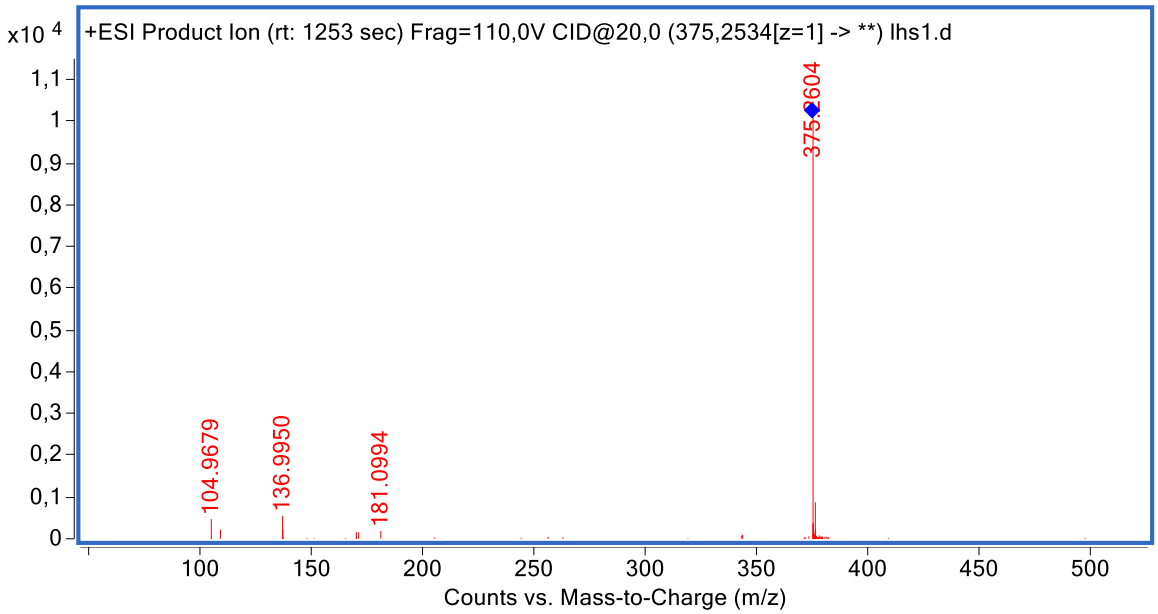

x10 2
+ESI Product Ion (rt: 1113 sec) Frag=110,0V CID@40,0 (399,2233[z=1] -> **) Rebecca AN holdfast.d
104.9676
105.0692
105.8140
106.9485
107.9666
Counts vs. Mass-to-Charge (m/z)



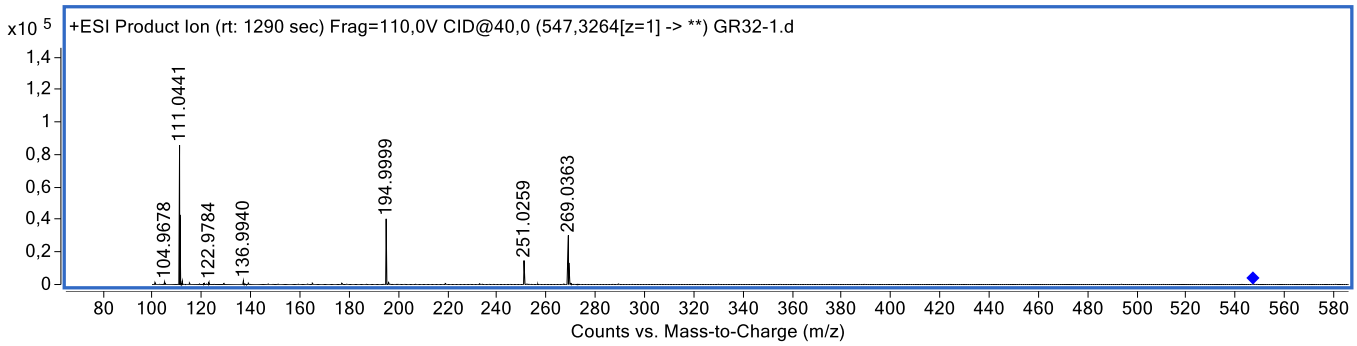
Appendix F10 (top) The mass spectrum of novel AsFA398 detected in *Ascophyllum nodosum.* Fig b. (middle) Fragment 104.9679 m/z is $(CH_3)_2As^+$ is present but generated in lower levels than 105.0692 m/z fragment which is common for fatty acids. Fig c. (bottom) Fragment 122.9763 is $C_2H_8AsO^+$ (paper VI).



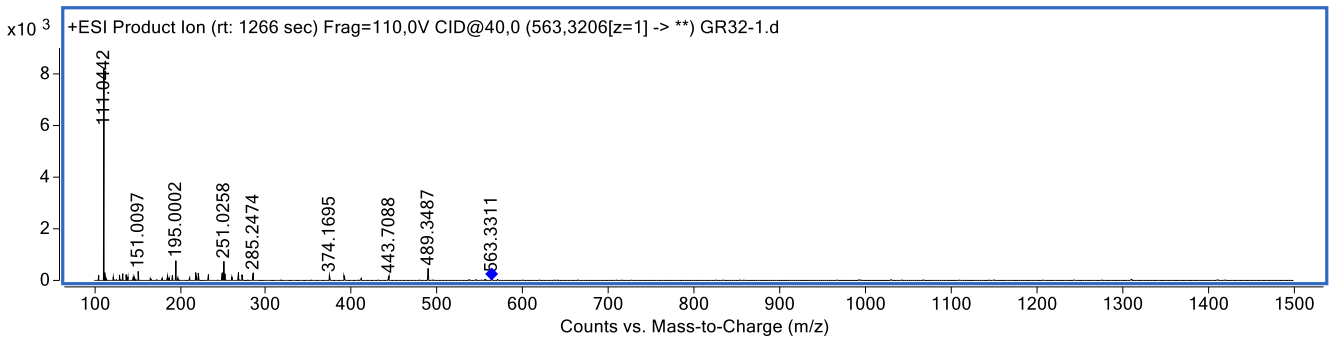
Appendix F11 – The mass spectrum of novel unsaturated AsHC380 (C19:3) detected in *Ascophyllum nodosum*. Fragment at 102.9519 is CH_4As^+, 104.9679 is $(CH_3)_2As^+$ group and 122.9783 is $C_2H_8AsO^+$ group (paper VI).



Appendix F12 – The fragmentation spectra of AsHC374. The fragment at 136.9950 m/z is a $AsO(CH_3)_2^+$ group which would not be present if compound was the TMAsFOH isomer (paper VI).



Appendix F13 The fragmentation of AsSugarphytol546 paper (VI). Fragment at 104.9679 m/z is $(CH_3)_2As^+$ group and 122.978 m/z is $C_2H_8AsO^+$ group. Fragment at 111.044 m/z is $C_6H_7O_2^+$ from ribose and fragment at 195.000 m/z is $C_5H_{12}O_3As^+$.



Appendix F14 The fragmentation of AsSugarPhytol562 (paper VI). Fragment at 104.9679 m/z is $(CH_3)_2As^+$ group and 122.978 m/z is $C_2H_8AsO^+$ group. Fragment at 111.044 m/z is $C_6H_7O_2^+$ from ribose and fragment at 195.000 m/z is $C_5H_{12}O_3As^+$.