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

*Correspondence to: asta.h.petursdottir@matis.is

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.

Keywords: arsenosugars, arsenic speciation, trace element speciation, seaweed, arsenic, trace elements, macroalgae, inorganic arsenic

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 (Huang *et al.*, 2022). 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 (Meharg, 2004; Taylor and Jackson, 2016; Mitra, Chatterjee and Gupta, 2017). Multiple studies have also demonstrated higher arsenate uptake during phosphate limitation (Rahman *et al.*, 2008; Mamun *et al.*, 2019). 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 (Borak and Hosgood, 2007). 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 (Taylor and Jackson, 2016; Ronan *et al.*, 2017; Ender *et al.*, 2019). 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, Fig 1, 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 (Francesconi *et al.*, 1991). 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 (Duncan, Maher and Foster, 2014; Wang *et al.*, 2015; Chen and Rosen, 2020). It has even been suggested that AsSugars may be produced by bacteria on the surface of the seaweed (Geiszinger *et al.*, 2001). 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* (Xue *et al.*, 2019). 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 (Xue *et al.*, 2017, 2019). 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) (Geiszinger *et al.*, 2001). 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 (Geiszinger *et al.*, 2001). 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 (Ender *et al.*, 2019). 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.

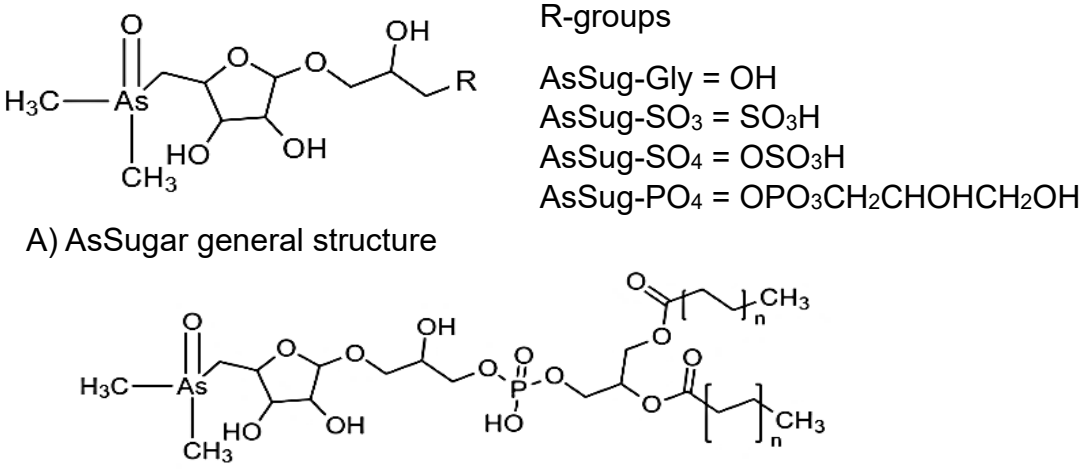

Fig 1. A) The general structure of AsSugars in seaweeds, B) the general structure of arsenic-containing phospholipid.

The total arsenic concentration has been shown to vary by season and section of thallus for the majority of Phaoephyceae (Burger *et al.*, 2007; Ometto *et al.*, 2018; Kreissig *et al.*, 2021). 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 (Ronan *et al.*, 2017). 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 (Pétursdóttir *et al.*, 2019). 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 (Rubio *et al.*, 2010). 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 Fig 2, and pooled to create samples representative of all ten specimens.

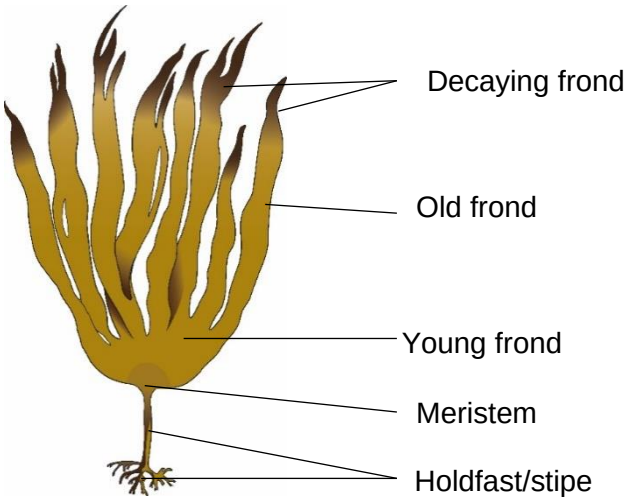

Fig 2. The sectioning of *L. digitata* samples.

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.

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 (Pétursdóttir *et al.*, 2018). 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 2 x 5 mL 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 SI Table 1.

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), Fig. 3. 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 1. 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 (Moreda-Piñeiro *et al.*, 2016; Matsumoto *et al.*, 2018; Kim *et al.*, 2020).

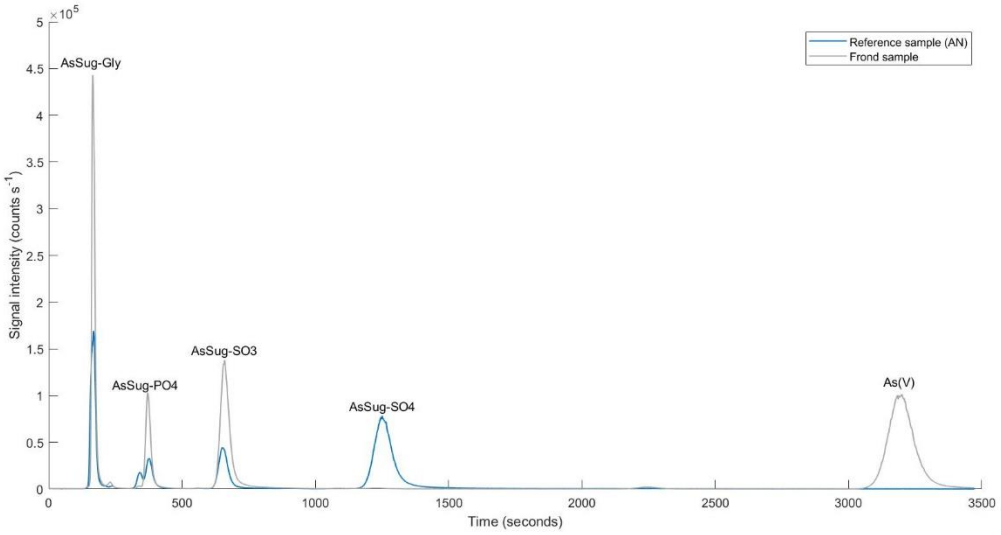

Fig 3. The sample of *A. nodosum* used as an internal laboratory reference material and a *L. digitata old* frond sample.

Results and discussion

Reference materials

Certified reference material 7405-b (hijiki) was analysed alongside samples during total and speciation measurements, Table 1.

Table 1 – The concentrations of total As and As(V) found in the reference material analysed alongside samples (Narukawa *et al.*, 2020). 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 (Narukawa *et al.*, 2020).

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, Fig 4. Previous studies have also reported higher concentrations of internal As during winter months for other brown seaweeds, *Alaria esculenta* and *Saccharina latissima* (Pétursdóttir *et al.*, 2019). Typically seaweeds are likely to accumulate nutrients during winter when nitrate is most abundant in ocean and not a limiting factor for growth (Roleda and Hurd, 2019). 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 (Perini and Bracken, 2014; Taylor and Jackson, 2016).

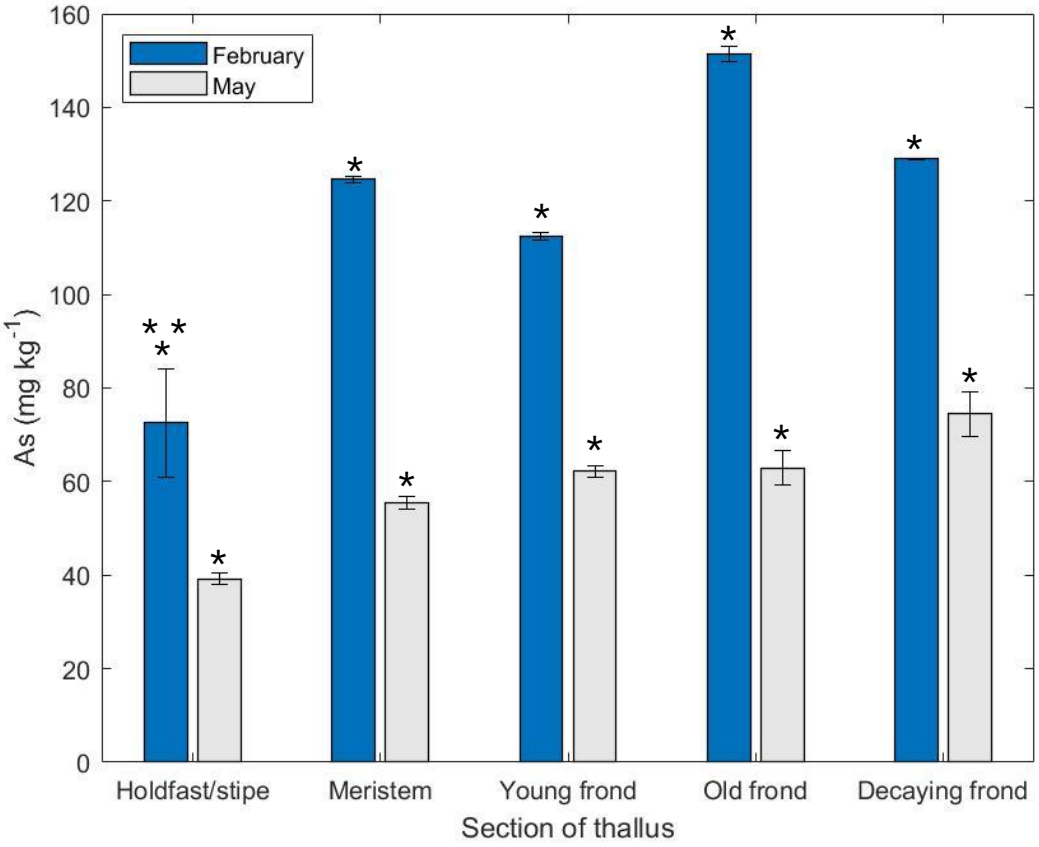

Fig 4. The total As concentrations in sections of *L. digitata* thalli in February and May 2022. The error bars represent 1 SD, where n=3. Asterisk (*) denotes significant differences between months ($p<0.05$), double asterisks (**) denotes significant differences between adjacent thallus section of 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, Fig 5, 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, SI Fig 1. 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 (Tibon *et al.*, 2021), SI Table 4 and Fig 4. Another study has also previously reported reported low concentrations of As(III) in other *Laminaria spp*. (Llorente-Mirandes *et al.*, 2011). As(III) is also more cytotoxic than As (V), and is quickly methylated as a detoxification mechanism by SAM enzymes (Chen and Rosen, 2020). 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, SI Table 3. 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), SI Fig 2. 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 (Wolle and Conklin, 2018). 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* (Pengprecha *et al.*, 2005). 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* (Ronan *et al.*, 2017). 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 (Ender *et al.*, 2019). 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 (Bruhn *et al.*, 2017). 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 (Allahgholi *et al.*, 2020). 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 (Almela *et al.*, 2006).

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.

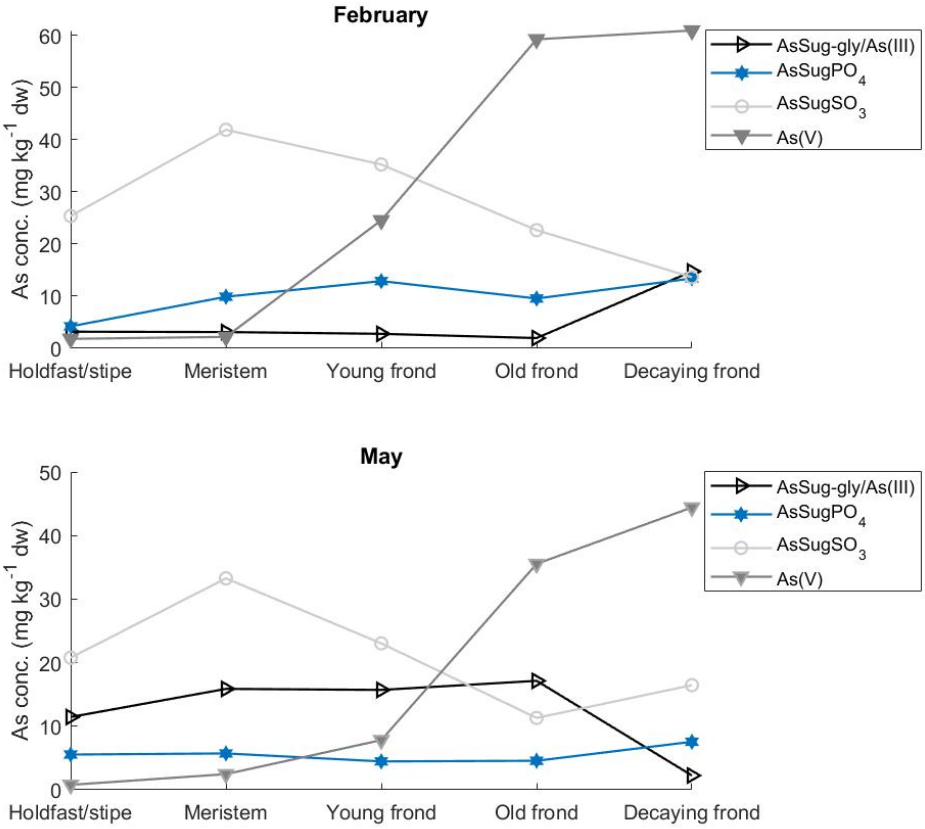

Fig 5. The average concentrations of water-soluble As species in February and May 2022 in different sections of the *L. digitata* thalli. Samples were analysed in duplicate (n=2) and all concentrations are expressed per kg of dry sample weight.

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, Fig 6, 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 (Ender *et al.*, 2019). 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.

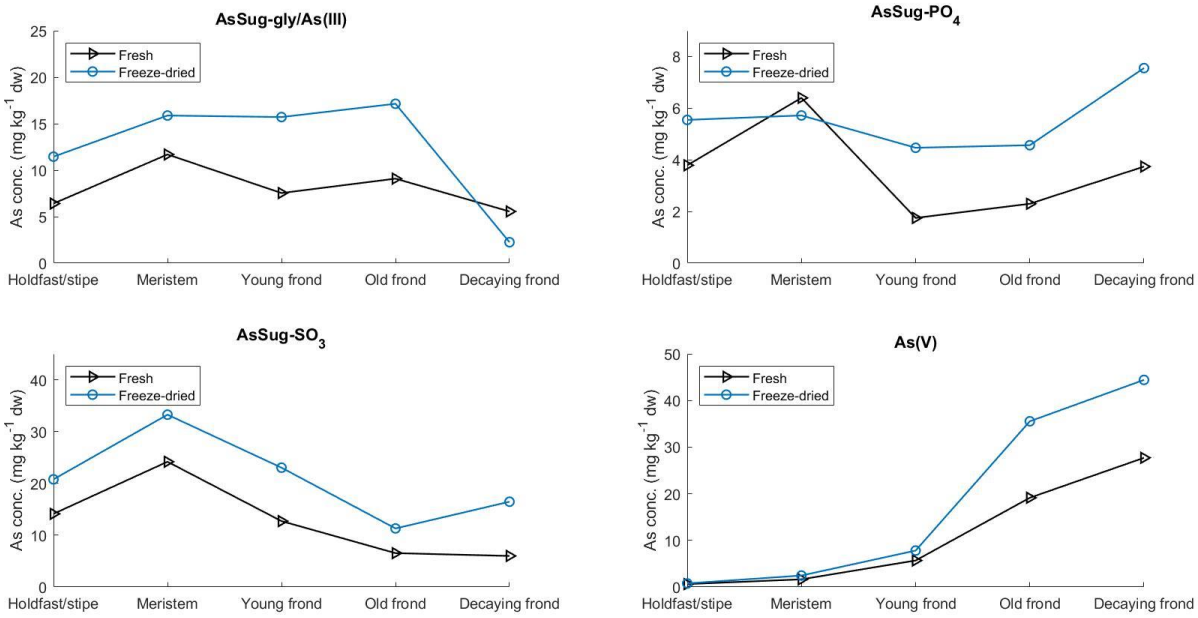

Fig 6. Comparison of the concentrations of water-soluble As species extracted from sample material collected in May, without and with freeze-drying. All concentrations are expressed per kg of dry weight.

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 3. 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 (Pétursdóttir *et al.*, 2018). 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, Fig 7 which is in line with the higher levels of total lipid content found in edible seaweeds during colder months (Madden *et al.*, 2012; Schmid, Guihéneuf and Stengel, 2017). 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 (Stengel et al., 2005). 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 (Shen *et al.*, 2013). Stronger extraction methods have been able to recover the recalcitrant arsenic in the form of As(V) (Navratilova *et al.*, 2011),(Nearing, Koch and Reimer, 2014). 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 (Amayo *et al.*, 2011). These species are also commonly reported, albeit in higher concentrations, in the DCM/MeOH extracts of seafood and algae (Arroyo-Abad *et al.*, 2016; Pétursdóttir *et al.*, 2019; Al Amin *et al.*, 2020; Liu *et al.*, 2021).

Table 3. 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 ($p<0.05$).

Fig 7. Comparison of the concentrations of water-soluble As species extracted from the same sample divided intro fresh and freeze-dried sample 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.

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Data availability statement

The data that supports this study will be shared upon reasonable request to the corresponding author.

Conflicts of interest

One of the co-authors (Jörg Feldmann) is a Guest Editor for Environmental Chemistry.

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