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Arsenic speciation in sea cucumbers: water-extractable species

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Highlights

- First study of As speciation in sea cucumber body parts
- Water-extractable As shown significant variation between sea cucumber species
- Arsenobetaine is dominant species in *Apostichopus californicus* and *Apostichopus japonicas*
- Main fraction of recovered As in *Cucumaria frondosa* is inorganic As
- Steaming of sea cucumbers removed only small fraction of total As

Keywords

sea cucumber, arsenic speciation, HPLC-ICP-MS, arsenic pollution, health supplement

Abstract

With the constant quest for new sources of superfoods to supplement the largely nutrient deficient diet of the modern society, sea cucumbers are gaining increasing popularity. Three species of sea cucumbers, *Cucumaria frondosa*, *Apostichopus californicus* and *Apostichopus japonicas* were collected from three geographical regions, Atlantic and Pacific coast of Canada and Yellow sea/East China sea in China, respectively. These organisms were sectioned into parts (body wall, tentacles, internal organ, skin and muscle) and analysed for total arsenic (As) by inductively coupled plasma mass spectrometry (ICP-MS) and As species by high-performance liquid chromatography (HPLC) coupled to ICP-MS. Sequential extraction was performed to address As species distribution between lipids (polar and non-polar) and water-extractable fractions. Two extraction methods for water-extractable As were compared in terms of the number and the amount of extracted species. The results revealed that total As concentration and As species distribution varies significantly between sea cucumbers species. Total As in studied body parts ranged between 2.8 ± 0.52 and 7.9 ± 1.2 mg kg^{-1}, with an exception of the muscle tissue of *A. californicus*, where it reached to 36 ± 3.5 mg kg^{-1}. Arsenobetaine (AsB) was the most abundant As species in *A. californicus* and *A. japonicas*, however, inorganic As represented over 70% of total recovered As in the body parts of *C. frondosa*. Arsenosugars-328 and 482 were found in all studied body parts whereas arsenosugar-408 was only found in the skin of *A. californicus*. This is the first time that such a variation in As species distribution between sea cucumber species has been shown.

Summary capsule

Sea cucumbers accumulate appreciable quantities of As, with significant variation in the abundance of water-extractable As compounds between three studied sea cucumbers species.

Introduction

Sea cucumbers have been used in traditional medicine of East Asia for many centuries to treat hypertension, rheumatism, asthma, sinus congestion and anemia [1]. However, it is very recently that they became globally known as health supplements. Nutritionally, they have low fat but high protein content and are rich in essential amino acids, minerals (Ca, Mg, Fe and Zn) and vitamins (A, B1, B3 and B3) [2, 3] . Their dry body wall is the most commonly consumed body part which is rich in collagen and polyunsaturated fatty acids such as eicosapentanoic acid and docosahexanoic acid [4]. Chemical and

bioactive extracts made from sea cucumbers have been used in surgeries to heal cuts and applied on burns and wounds [5] and they were found to contain anti-cancer compounds [6]. Sea cucumbers are also a valuable source of saponins, an important class of natural products with a large spectrum of pharmacological effects [7]. A recent study showed that saponin-enriched sea cucumber extracts act as anti-obesity agent through inhibition of pancreatic lipase [8]. Additionally, sea cucumber used as additives in dietary supplements have shown to lowers serum lipids and causes weight loss in rodents [9, 10].

Sea cucumbers (Figure 1) are soft bodied marine invertebrates belonging to the class of *Holothuroidea*. They have an elongated tubular body with a single branched gonad and leathery skin. There are over 1700 species of sea cucumbers with the greatest diversity found in the Asia Pacific region [2]. In the wilderness, they live and feed in the bottom of the seabed thus contribute to mixing of upper sediments, nutrient recycling and stimulation of algal growth which makes them an important part of the coastal ecosystem [11]. Sea cucumbers are commercially fished in more than 40 countries and exported mainly to Asian markets [12, 13]. Due to high international demand for sea cucumbers some species have become very rare or locally extinct [14, 15]. Their decline in the natural habitat is offset by aquaculture sources which have seen a dramatic increase in recent years and is led by China [16]. The total Chinese production of sea cucumbers in 2017 was 200 000 metric tonnes (comparable with annual shrimp exports from Thailand) with the majority of aquaculture operations being located in Bohai and the Yellow sea [17]. Sea cucumbers are generally non-selective deposit feeders and certain species live within the sediment which serves as the main sink for marine pollutants; as such, sea cucumbers can be under high toxic stress. Densely populated and industrialised coastal regions of Northern Bohai and the Yellow sea are heavily polluted with domestic and industrial effluents containing arsenic (As) and other metals [18]. Consequently, juvenile sea cucumbers were found to bioaccumulate As at much faster rate compared to other heavy metals such as mercury or lead [19], reaching levels above 15 mg kg^{-1} in adult organisms [20, 21]. Arsenic toxicity varies among its species with iAs (sum of $As^{(III)}$ as arsenite and $As^{(V)}$ as arsenate) being a Class 1 carcinogen and arsenobetaine (AsB) exhibiting low toxicity [22]. However, there are over 100 As species naturally occurring in the environment, and toxicity that can fall anywhere between highly toxic to benign [23]. Based on the toxicity of AsB, organic As species were thought to be non-toxic until several As hydrocarbons were found to have similar cytotoxicity as iAs [24, 25]. Subsequently, As hydrocarbons were found to be blood-brain barrier permeability enhancers [26] and As lipids were discovered in human breast milk [27]. Abundance of As species between aquatic biota also displays high variation. Fat soluble As lipids were found in fish oils and seaweed [28-33], AsB is the largest fraction of As species in shellfish and finfish, while arsenosugars (AsSug) and iAs are dominant in seaweeds and molluscs [23, 34, 35]. Other

As species such as arsenocholine (AsC), trimelylarsine oxide (TMAO), dimethylarsonic acid (DMA) and monomethylarsinic acid (MMA) were reported in various seafood at trace levels [23].

There are very few publications discussing As levels in sea cucumbers with very limited information about As species. Therefore, we found it compelling to address this knowledge gap especially considering the "superfood" status of sea cucumbers. In present study, we collected and analysed three sea cucumber species including *Cucumaria frondosa*, *Apostichopus californicus* and *Apostichopus japonicas* from the Atlantic and Pacific coasts of Canada as well as from the Yellow sea and East China sea in China, respectively for total As and As species. We compared different As extraction protocols and assessed the impact of cooking on As species prior to consumption.

Material and methods

Chemicals and reagents

Optima grade hexane, methanol, dichloromethane and ACS grade trifluoroacetic acid and hydrogen peroxide were purchased from Fisher Scientific. Analytical grade nitric acid (69 – 70%) by J. T. Baker was obtained from VWR and further purified by sub-boiling distillation in-house. Ammonium carbonate (99.999% trace metals basis), ammonium bicarbonate (≥99.5%, BioUltra), malonic acid (99%, Reagent Plus), arsenic pentoxide (99.9%), cacodylic acid (99.0%), disodium methyl arsenate (99%) were purchased from Sigma Aldrich. ABET-1 [36] was used for preparation of arsenobetaine calibration standards and together with certified reference materials (CRMs) DORM-4 [37] and SQID-1 [38] CRMs were obtained from National Research Council Canada. Arsenosugars 328, 408 and 482 were kindly provided by FDA's Division of Bioanalytical Chemistry. Deionized water (> 18 MΩ cm Milli-Q Element, Millipore) was used in all experiments. All glassware were acid cleaned using 10% HNO_3.

Sample collection and preparation

C. frondosa samples were obtained from two different provinces of Atlantic Canada, Nova Scotia and Newfoundland. Samples from Nova Scotia were obtained by commercial fishers from Northwest Atlantic Fisheries Organization (NAFO) fishing area 4Vn, offshore of Louisburg, Nova Scotia, in September to October 2019. After being fast-frozen upon landing, the samples were stored at -25°C. Before processing, sea cucumbers were thawed with running sea water, and dissected to obtain 3 parts: body wall, tentacles

and internal organs. Raw samples for each body parts were then kept frozen and shipped to the National Research Council of Canada laboratory in Halifax and freeze-dried. Processed sample sets were prepared by steaming the body parts for 24 minutes, and then dried in an oven at 38°C. Samples of *C. frondosa* offshore of the southern coast of Newfoundland and Labrador were obtained from NAFO fishing area 3Ps in February 2019. The sampling was carried out in triplicate and all raw samples were freeze-dried. Samples of 5 individual *A. californicus* were collected at Snake Island (4912.845'N, 12353.552'W) near Nanaimo, British Columbia on October 15, 2018. It should be noted that this is along a British Columbia ferries route, and near container ship anchorage i.e. it is not an area typically commercially fished for sea cucumbers. Upon landing, the samples were dissected to obtain 2 body parts: skin and muscle, and stored frozen. Frozen raw samples were then shipped on dry ice to the National Research Council of Canada laboratory in Halifax and freeze-dried. Samples of *A. japonicas* were collected from 3 different aquaculture sites (a) Haiwangjiu Island, Zhuanghe, Dalian, Liaoning Province during October 2017; (b) Yangma Island, Yantai, Shandong Province during October 2017; and (c) town of Xinan, Xiapu, Ningde, Fujian Province during March 2018. Upon landing, they were dissected to obtain body wall and freeze-dried.

All dry samples were milled using a coffee grinder and sieved (35 mesh, 425 µm) to obtain a homogenous powder for analysis..

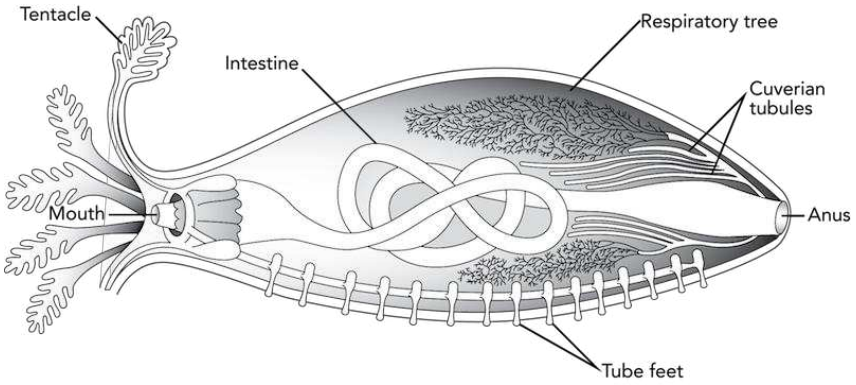


Figure 1. Diagram of cross-section of sea cucumber. Figure from [39]

Sample preparation

Total As

Approximately 0.1 g of sea cucumber body parts (triplicate) were accurately weighed into Teflon digestion vessels and predigested overnight with 7 mL of HNO_3 and 2 mL of H_2O_2. Samples were then digested in microwave digestion system (Ethos EZ, Milestone) using a temperature programme of 0 – 15 min ramp to 200 °C and hold at 200 °C for 15 min. After digestion the samples were transferred into 50 mL vials, evaporated to dryness and reconstituted in 2% HNO_3. Samples were stored at room temperature until further analysis. One CRM from NRC Canada, SQID-1 and 1 method blank were included in each digestion cycle.

Sequential extraction of As species

Sequential extraction was adapted from a previously published method [31]. One to 4 g (depending on sample availability) of sea cucumber was accurately weighed into 40 mL glass vial and extracted 2 x with 35 mL of hexane on a rotary shaker for 3.5 hours. The samples were centrifuged after each extraction and the combined supernatants were evaporated to 1 mL (Hexane fraction). The residue was dried and reweighed between each subsequent extraction step. Dry residue was then extracted 2 x with 35 mL of DCM/MeOH (2:1) on the rotary shaker for 12 h for the first and 30 min for the second extraction step. Samples were centrifuged, both supernatants were combined, evaporated to dryness and reconstituted in 1 mL of MeOH (MeOH fraction). Dry residue following the DCM/MeOH extraction was divided into two subsamples and transferred into 50 mL vials. One aliquot was extracted with 40 mL of MilliQ water, the other with 40 mL of 2 mM TFA with 1% H_2O_2, both in a water bath at 90 °C for 1 h. The samples were brought to room temperature, centrifuged and the supernatant was collected (water/TFA fraction). The residue was washed with 2 x 20 mL of MilliQ water on the rotary shaker for 10 minutes and left to dry overnight at 60 °C. Dry residues were predigested overnight in 1 mL of HNO_3 acid and 1.5 mL of H_2O_2 and subsequently digested in water bath at 90 °C for 1 h. Digests were evaporated to dryness and reconstituted in 2% HNO_3 (residue fraction).

In a second set of experiment the extraction sequence going from non-polar analytes to more polar analytes was reversed. The extraction cascade was performed by first obtaining water/TFA fraction, then MeOH, hexane and residue fraction.

One CRM, DORM-4 and 1 blank sample was carried with each batch of sequential extraction.

Total As in sequential extraction fractions One hundred µL of hexane and MeOH fraction was accurately weighted into Teflon digestion vessels, evaporated to dryness and digested with 4 mL of HNO_3 and 3 mL of H_2O_2 following the procedure for total As. One mL of water/TFA extract was digested with 1 mL of HNO_3

and 1.5 mL of H_2O_2 in a water bath at 90 °C for 1 h. All digests were evaporated to dryness, reconstituted in 2% HNO_3 and stored at room temperature until analysis. One CRM, SQID-1 and 1 method blank were included in each digestion cycle.

Instrumental setup and analysis

Total As All samples were analysed using ICP-MS/MS (Agilent 8900, Agilent Technologies, Mississauga, ON, Canada) in O_2 mode which was optimised daily for sensitivity and stability. Monitored masses were *m/z* 91 (*m/z* 75 As + *m/z* 16 O) for As, and 71 for Ga which was used as an internal standard (IS). Quantitation was performed using external calibration, quality control samples of low and intermediate As concentration were measured every 7 samples and the blank was analysed every 5 samples.

As speciation by anion exchange chromatography (AEC) Agilent 1200 HPLC coupled with ICP-MS/MS (Agilent 8800, Agilent Technologies, Mississauga, ON, Canada) was equipped with a Hamilton PRP-X100 column (10 µm, 4.1 x 250 mm) and identical guard column. Isocratic elution was carried out using 25 mM carbonate buffer at 1 mL min^{-1}.

As speciation by cation exchange chromatography (CEC) Agilent 1200 HPLC coupled with ICP-MS/MS (Agilent 8800, Agilent Technologies, Mississauga, ON, Canada) was equipped with a Metrosept C6 column (5 µm, 4.0 x 250 mm) and identical guard column. Gradient elution was carried out as previously described [40]; mobile phase A: MilliQ water; B: 50 mM pyridine (pH 2) with following gradient: 0 – 22 min (0% B, 0.7 mL min^{-1}), 22.5 – 34 min (10% B, 1 mL min^{-1}), 34.5 – 44 min (0% B, 1.2 mL min^{-1}).

Monitored masses were *m/z* 91 (*m/z* 75 As + *m/z* 16 O) for As, and 71 for Ga which was used as IS. Quantitation was performed by using external calibration standards of DMA, MMA, AsB and iAs in a concentration range of 5 – 100 µg kg^{-1}. Due to limited availability, AsSug-328, 408 and 482 were added into selected samples to confirm the retention time. The quantitation of AsSugs was accomplished using the calibration curve of neighbouring As compounds. Mixed calibration standard was injected every 5 samples and the peak area of individual As species was used for drift correction. Following above method, every 5 samples were quantified using new calibration curve. Blank sample was injected every 6 samples to monitor possible carry over.

Results and discussion

Selection of chromatographic method

Several chromatographic methods were tested for baseline separation of water-extractable As species in sea cucumbers extracts. Water and TFA extracts either defatted or containing naturally present lipids were tested on PRP-X100 and Metrosep C6 columns. Isocratic elution with 2 mM malonic acid previously used for As speciation in rice and tuna [41] showed significant iAs peak broadening and peak splitting when applied on non-defatted extracts. Increasing the molarity of malonic acid didn't improve the peak shape although it shortened the retention time as demonstrated by the authors. Recently, Wolle and Conklin [42] reported on a chromatographic method using a bicarbonate-carbonate gradient which allowed for separation of a large number of As species. Indeed, this method provided very good separation of several As species in the sea cucumbers extracts but a peak with retention time of $As^{(V)}$ was observed in each chromatogram including the blank. A similar observation was noted by others when using a nitric acid gradient with a benezene-1-2-disulfonate modifier [42] but also with a bicarbonate-carbonate gradient [43]. It is assumed that impurities in mobile phase are a source of $As^{(V)}$ which is pre-concentrated on the column during the equilibration and first gradient step and subsequently eluted with increasing molarity of the mobile phase. Changing of bicarbonate-carbonate salts from different suppliers as well as various ratios of mobile phases during the gradient elution were not able to eliminate this peak from the chromatogram. Considering the implications of $As^{(V)}$ coming from mobile phase impurities on the methods' LOD and quantitation of low $As^{(V)}$ levels in sea cucumbers, isocratic elution with 25 mM carbonate buffer was selected for separation of As anions (Figure 2a).

To find a suitable method for separation of extracted cationic species, several methods were tested [44, 45] and the Metrosep C6 column with identical pre-column and gradient elution using 50 mM pyridine (pH 2.7) method as reported by Sloth et al. provided a suitable separation [46] (Figure 2b). It should be noted that while using both anion and cation exchange chromatography a slight shift in retention time of later eluting species was observed between analytical standards and samples. Due to the high dilution factor of As species during the extraction step the extracts were not further diluted in water, but directly injected on the column. The difference between sample and calibration standard matrix is the most probable cause of the observed retention time shift. To verify the retention time in the sea cucumber extracts, samples were spiked with individual standard solutions.

Selection of extraction method

In development of the extraction method for water-soluble As species, the extraction yield, number of extracted species and quality of chromatographic separation were compared. Extraction was performed in neutral pH, in MilliQ water which is frequently used for As extraction from marine samples [30, 47, 48] and in low pH using 2 mM trifluoracetic acid (TFA) with 1% H_2O_2 pH 1.7 [41]. The addition of H_2O_2 was chosen for oxidation of $As^{(III)}$ to $As^{(V)}$ thus detecting iAs species as $As^{(V)}$. Both extractions were carried out in a water bath at 90 °C [41]. In terms of extraction efficiency, no significant difference was observed although TFA with 1% H_2O_2 extracted slightly larger portions of As compounds in all tested samples apart from muscle tissue. Lower extraction efficiency of water was reflected in higher As concentration found in the digested residue, however the sum of water extractable As species with the As residue was in good agreement between tested methods. Higher extraction efficiency using acidic conditions was also reported by Wolle and Conklin who conducted extensive comparison of numerous extraction methods on various marine tissues [42]. Their results indicated, that the extraction efficiency is likely affected by the samples matrix. Furthermore, they observed conversion of AsSug-392, 408 and 482 to AsSug-328 under acidic and alkali conditions, although another study showed that these AsSug degrade to AsSug-254 [49]. Comparison between extraction methods implemented in this study found that 2 mM TFA with 1% H_2O_2 was more efficient in extraction of AsSug-482 from all studied tissues as it can be seen in Figure 2a. The concentration of AsSug-482 was found to be higher in majority of TFA extracts in comparison with water extracts which is at odds with Wolle and Conklin's observations [42].

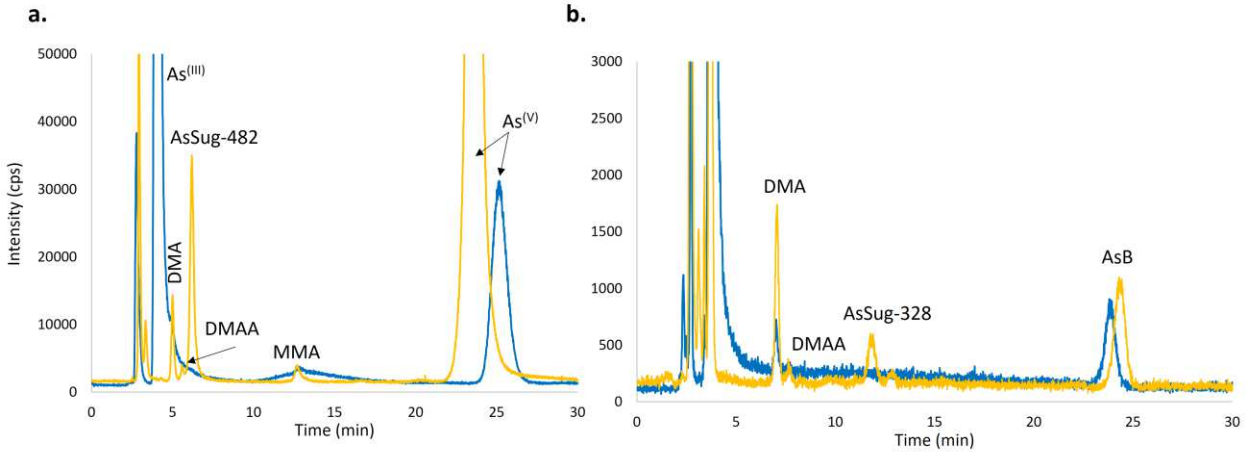


Figure 2. Anion (**a.**) and cation (**b.**) exchange HPLC-ICP-MS chromatograms of water (blue) and TFA (yellow) extracts of *C. frondosa* tentacle.

Traces of AsSug-408 were found in skin tissues of all TFA extracts but interestingly only some skin water extracts contained this As species. Additionally, AsSug-328 was found in all studied samples but in concentrations below the method LOQ. Comparing AsSug-328 peak areas between the TFA and water extracts showed no significant differences. It should be noted that acidic extraction methods tested by Wolle and Conklin [42] used acids at higher concentrations, thus a lower pH and 3 times higher percentage of H_2O_2 than conditions in the present study. The milder acidic conditions used in this study might be responsible for increased extraction efficiency compared to water extraction and seemingly reduced rate of AsSug conversion if this in fact occurs in system presently studied. As the studied samples contained a large portion of $As^{(III)}$ it was necessary to use H_2O_2 under given chromatographic conditions. The shoulder of the $As^{(III)}$ peak in water extracts overlapped the peak of DMA either completely or partially, depending on $As^{(III)}$ concentration which interfered with the quantitation of $As^{(III)}$, DMA and in some tissues AsSug-482. In terms of number of extracted peaks, we found traces of MMA and DMAA in addition to other unknown species in TFA but not in water extracts. Therefore, based on the evaluated conditions, TFA extraction was found more suitable for the purpose of our study.

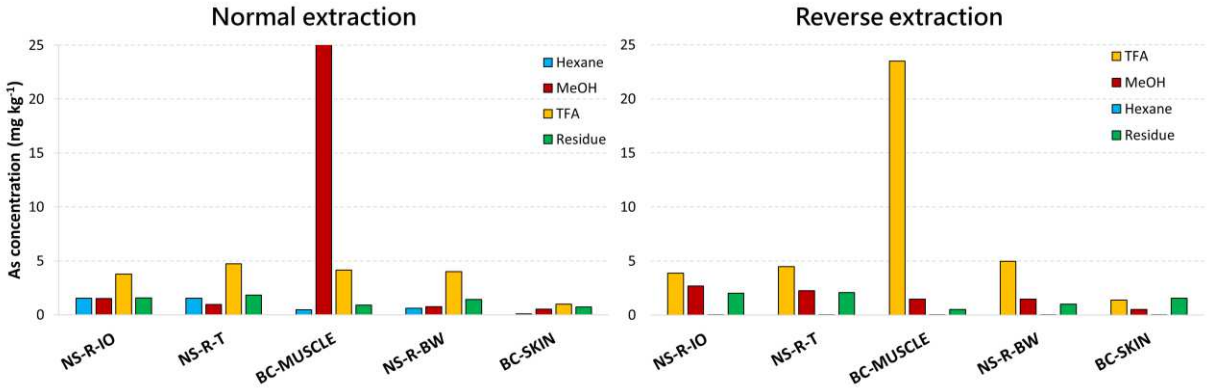


Figure 3. Total As in extracted fractions following normal (non-polar → polar) and reversed (polar → non-polar) extraction steps. Colours of the bars represent individual extraction steps and their order represents the sequence of extraction steps. NS – Nova Scotia; BC – British Columbia; R – raw; IO – internal organs; T – tentacle; BW – body wall

To address the importance of lipid extraction prior to the extraction of water-soluble species, normal and reversed extraction protocols on selected set of samples was performed (Figure 3). Each extracted fraction was digested and analysed for total As and TFA extracts were speciated using AEC and CEC. Upon visual comparison of the extracts colour differences were observed in extracted lipid fractions. Following normal extraction, polar and non-polar lipids were of dark orange to brown colour. Using the reverse extraction method, polar lipids were a very light orange and non-polar lipids were colourless suggesting a potential chemical alteration and/or degradation of the lipids. In terms of As distribution, no significant difference in total As concentration in TFA extracts and the residues was found, with the exception of BC-muscle samples. On the contrary the amount of As extracted in hexane fraction significantly decreased when the TFA and MeOH were applied prior to hexane i.e. during reversed extraction protocol. A similar observation was previously reported by Pétursdóttir et al. who defined lipids found in hexane fraction of the reversed extraction as stable non-polar lipids [50]. The fraction of non-polar lipids that was extracted into MeOH after the reversal of the extraction steps was defined as non-stable non-polar lipids which got hydrolysed upon contact with TFA or water. The notion of potential degradation of As lipids exposed to TFA during reverse extraction was also supported by an increase in the concentration of DMA and iAs in the TFA fraction. Conversion of As lipids under acidic and alkali condition to DMA, AsC, iAs and other unknown species was previously reported [42, 51, 52]. Increase in AsB was also observed with reversed extraction steps which is caused by partial extraction of AsB into the MeOH fraction during the normal extraction protocol. Thus when the steps were reversed, the majority of AsB was extracted into TFA fraction. This explained the significant shift of As between MeOH and TFA fractions in BC-muscle sample (Figure 3) in which AsB constituted of over 90% of total As.

Arsenic distribution in the body parts of sea cucumbers

The total As concentration in the individual body parts of sea cucumbers varied between sea cucumber species (Table 1). SQID-1 CRM (n = 21) used as a quality control, was digested with each extracted fraction as well as sea cucumber tissues for total As analysis. Individual measurements of CRM were in good agreement with the certified value 13.3 ±1.1 mg kg^{-1} (cert. 14.1 ± 2.2 mg kg^{-1}). *C. frondosa*, which was harvested off the Atlantic coast of Canada had total As concentration in the range of 5.2 ± 0.81 mg kg^{-1} in the body wall to 8.7 ± 0.91 mg kg^{-1} in tentacles. Sea cucumber harvested off the Pacific coast of Canada, *A. californicus* showed much larger range of total As, from 2.8 ± 0.52 mg kg^{-1} in the skin tissue to 36 ± 3.5 mg kg^{-1} in the muscle tissue. Only body wall of *A. japonicas* harvested in China was available in which As

ranged between 5.0 ± 0.12 mg kg^{-1} from aquaculture in Liaoning Province, to 7.7 ± 0.090 mg kg^{-1} from aquaculture in Fujian Province. The total As concentration in *A. japonicas* was lower than reported in the literature, 12.4 mg kg^{-1} in the muscle tissue [21] or 15.5 mg kg^{-1} in the whole body of sea cucumber [20]. It is possible that lower concentration reported in presented study are due to different geographical location, seasonal variation or the actual growth stage of sea cucumbers and body parts which were analysed. To the best of our knowledge there are no available data for the comparison of total As in *C. frondosa* and *A. californicus*. The significant difference in As accumulation between these two species could be either due to As rich diet as a result of high concentrations in local environment or differences in As metabolism between sea cucumber species. Specific environmental exposures in the aquatic compartments are reflected in increased concentration of As in the sediments which can be caused by industrial activities or geological conditions. The Pacific coast has limited industrial activities, including forestry and shipping that are minor relative to the scale of industrial activities in the Yellow and East China Seas. The Atlantic coast of Canada is dominated by fisheries and aquaculture. However, some coastal location such as Seal Harbor in Nova Scotia were historically exposed to high As concentrations from mining effluents resulting in sediment As levels as high as 770 mg kg^{-1} [53]. Thus, while increased As concentration can be expected in marine biota harvested from coastal regions, it would not account for As found in *C. frondosa* which are collected further from the coast. From a geological perspective, Nova Scotia is located on meguma metasedimentary terrane which is associated with gold-quartz and arsenopyrite veins [54] resulting in significantly higher As concentration in the local well water [55]. Therefore, As concentration in the sediments could be higher at this coastal regions, hence biota can be exposed to higher As concentrations. Similarly, As rich groundwater was reported on Bowen Island, British Columbia as a consequence of several geological and geochemical processes [54, 56]. Therefore it appears that both coastal regions have geological predisposition to elevated concentration of As such that the observed differences in As accumulation in studied sea cucumbers is more probably rooted in the biological response to As.

Biochemical variations and different responses to environmental stresses between sea cucumber species have been previously reported. Studies looking at accumulation of metals and metalloids in three species of sea cucumbers observed higher accumulation rate in gut when compared with body wall [57]. Another study, looking at saponin distribution in the body parts of large number of sea cucumbers, found that the organ specific distribution is species dependent [7]. Research by Guo et al. showed that not all sea cucumber species are suitable for development of anti-obesity drugs because only 3 out of 10 studied species exhibited pancreatic lipase inhibitory activity [8]. Different types of sulfate polysaccharides with

potential bioactivity, were found amongst sea cucumber species. *Stichopus hermanni* contained significant amount of sulfated glycosaminoglycan, whereas fucoidan, and fucosylated chondroitin sulfate were found in *Thelenota ananas* [4]. Thus it appears that various sea cucumber species may have very different potential to metabolise specific groups of bioactive compounds and hence their response to environmental stressors may be also species specific. Considering the large number of variables in present study, it is not possible to conclude with confidence what are the driving factors behind observed differences in As accumulation between *C. frondosa* and *A. californicus*.

Table 1. Total As in each extracted fraction and total As in different body parts of sea cucumbers in mg kg^{-1}.

		Hexane	MeOH	TFA	Residue	Sum		Total As	
Body part/ location	**n**	**Av (SD)**	**Av (SD)**	**Av (SD)**	**Av (SD)**	**Av (SD)**	**n**	**Av (SD)**	**% bias**
Apostichopus japonicas									
Body wall Liaoning Province	1	0.13	2.1	1.4	1.08	4.7	3	5.0 (0.12)	-7%
Body wall Shandong Province	1	0.10	7.1	2.4	0.99	11	3	7.1 (0.035)	50%
Body wall Fujian Province	1	0.23	7.7	1.80	3.1	13	3	7.7 (0.090)	66%
Cucumaria frondosa									
Internal organs NS	3	1.7 (0.16)	1.6 (0.21)	3.9 (0.68)	1.6 (0.12)	8.8 (0.85)	9	7.9 (1.2)	11%
Internal organs NL	3	1.8 (0.28)	1.4 (0.21)	3.4 (0.74)	1.9 (0.15)	8.4 (1.2)	9	6.2 (0.57)	36%
Internal organs NS - processed	3	2.6 (0.11)	0.61 (0.08)	3.5 (0.32)	1.9 (0.14)	8.4 (0.22)	9	6.2 (0.26)	37%
Tentacles NS	3	1.6 (0.09)	0.90 (0.27)	5.0 (1.6)	2.2 (0.48)	9.6 (1.0)	9	8.7 (0.91)	11%
Tentacles NL	3	1.2 (0.43)	1.6 (1.00)	4.6 (1.3)	1.5 (0.10)	8.9 (2.3)	9	6.3 (1.1)	40%
Tentacles NS - processed	3	1.9 (0.16)	0.30 (0.04)	3.4 (0.24)	1.3 (0.30)	6.8 (0.36)	9	5.6 (0.19)	21%
Body wall NS	3	0.53 (0.13)	0.47 (0.25)	3.4 (0.52)	1.4 (0.21)	5.8 (0.95)	9	6.0 (1.1)	-3%
Body wall NL	3	0.58 (0.09)	0.38 (0.08)	4.0 (1.0)	1.3 (0.16)	6.2 (1.1)	9	5.2 (0.81)	19%
Body wall NS - processed	3	0.67 (0.19)	0.41 (0.12)	2.7 (1.6)	1.5 (0.44)	5.3 (2.2)	9	4.3 (1.6)	22%
Apostichopus californicus									
Muscle BC	5	0.44 (0.04)	32 (4.1)	5.2 (0.90)	0.83 (0.16)	39 (4.7)	15	36 (3.5)	8%
Skin BC	5	0.11 (0.04)	0.60 (0.17)	1.0 (0.19)	0.86 (0.072)	2.6 (0.36)	15	2.8 (0.52)	-7%

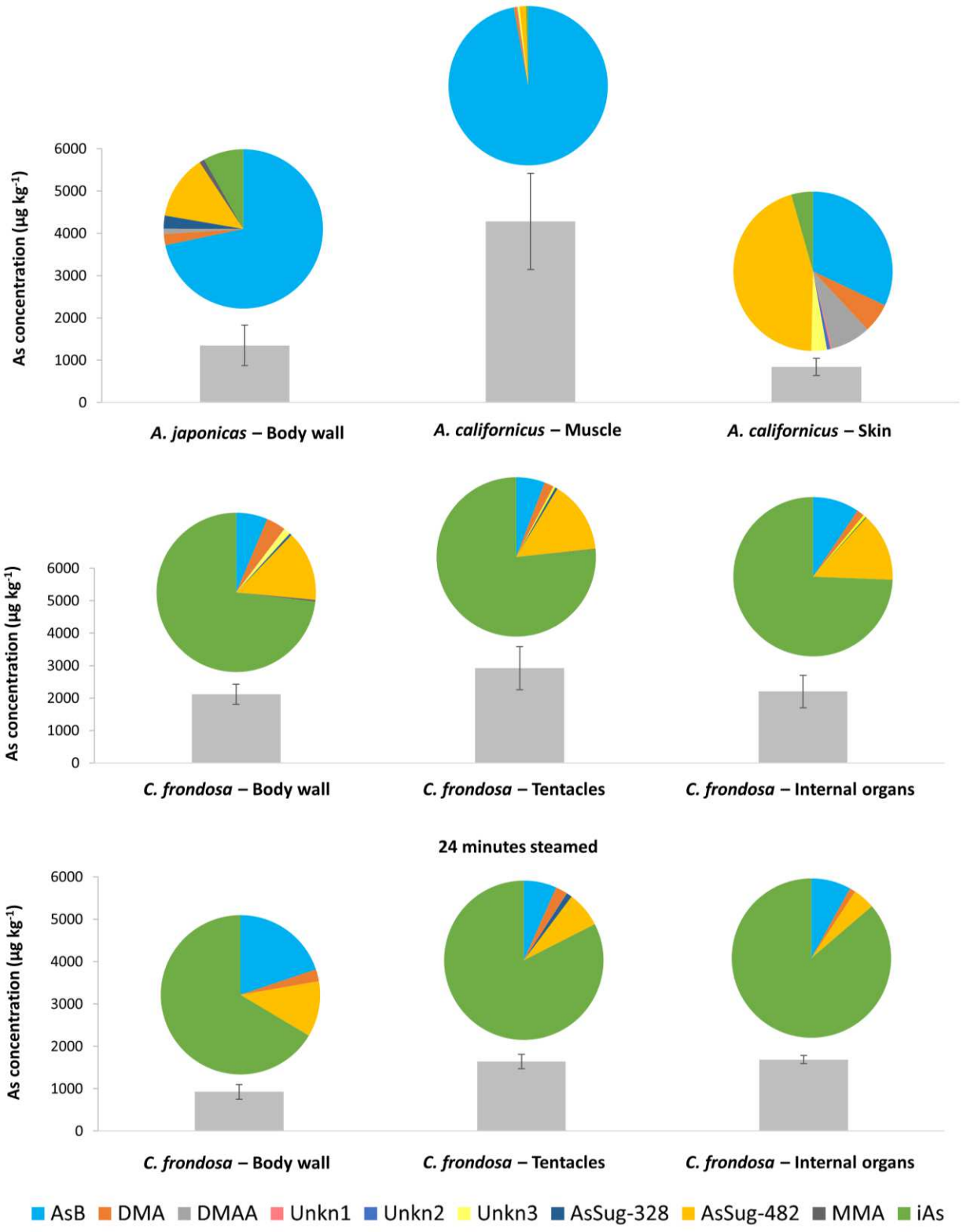
NS – Nova Scotia (Atlantic coast); NL – Newfoundland and Labrador (Atlantic coast); BC – British Columbia (Pacific coast)

Total As was measured in each fraction collected from sequential extraction to account for total As mass balance of the method. The sum of As from the four extraction steps was in good agreement with total

As in the sea cucumber body parts where the recovery bias ranged generally from –7% to +22%. The large bias in body wall Shandong and Fujian province samples is most probably due to overestimation of MeOH fraction, which is over 3 folds higher than in body wall from Liaoning Province. Similarly, DORM-4 CRM (n=4) used for sequential extraction to monitor extraction showed on average 30% bias with certified total As concentration 9.07 ± 0.88 mg kg^{-1} (cert. 6.87 ± 0.44 mg kg^{-1}). Arsenic appears to be uniformly distribution within polar and non-polar lipid extracts of the *C. frondosa* but in the *A. californicus* polar lipids shown higher abundance. Interestingly, 90% of As in muscle tissue of *A. californicus* was extracted in MeOH fraction.

Water-extractable As species in the body parts of sea cucumbers

Speciation of the water-extractable As compounds showed very large variation between the organs and between sea cucumbers species. Arsenobetaine, previously reported in *A. japonicas* [21], was also found to be the most abundant species in our study, followed by AsSug-482 and iAs (Figure 4). Only low quantities of AsSug-328, DMA, DMAA and MMA were found in body wall of *A. japonicas*. Over 95% of total recovered water-extractable As from muscle of *A. californicus*, was found to be AsB and only traces of AsSug-482, DMA, DMAA and iAs were found. The skin of *A. californicus* contained the largest variety of As species and the major fraction was AsSug-482, followed by AsB, DMAA, DMA, iAs and 3 unknown compounds. Other species were found in low concentration and AsSug-408 was present only in trace levels. On the contrary, in *C. frondosa* iAs was the most abundant species in all body parts representing on average 75% of total recovered water-extractable As. AsSug-482, AsB and DMA were other three main As species found in substantial quantities.


As concentration (µg kg-1)
6000
5000
4000
3000
2000
1000
0
A. japonicas – Body wall
A. californicus – Muscle
A. californicus – Skin
C. frondosa – Body wall
C. frondosa – Tentacles
C. frondosa – Internal organs
24 minutes steamed
C. frondosa – Body wall
C. frondosa – Tentacles
C. frondosa – Internal organs
AsB
DMA
DMAA
Unkn1
Unkn2
Unkn3
AsSug-328
AsSug-482
MMA
iAs

380

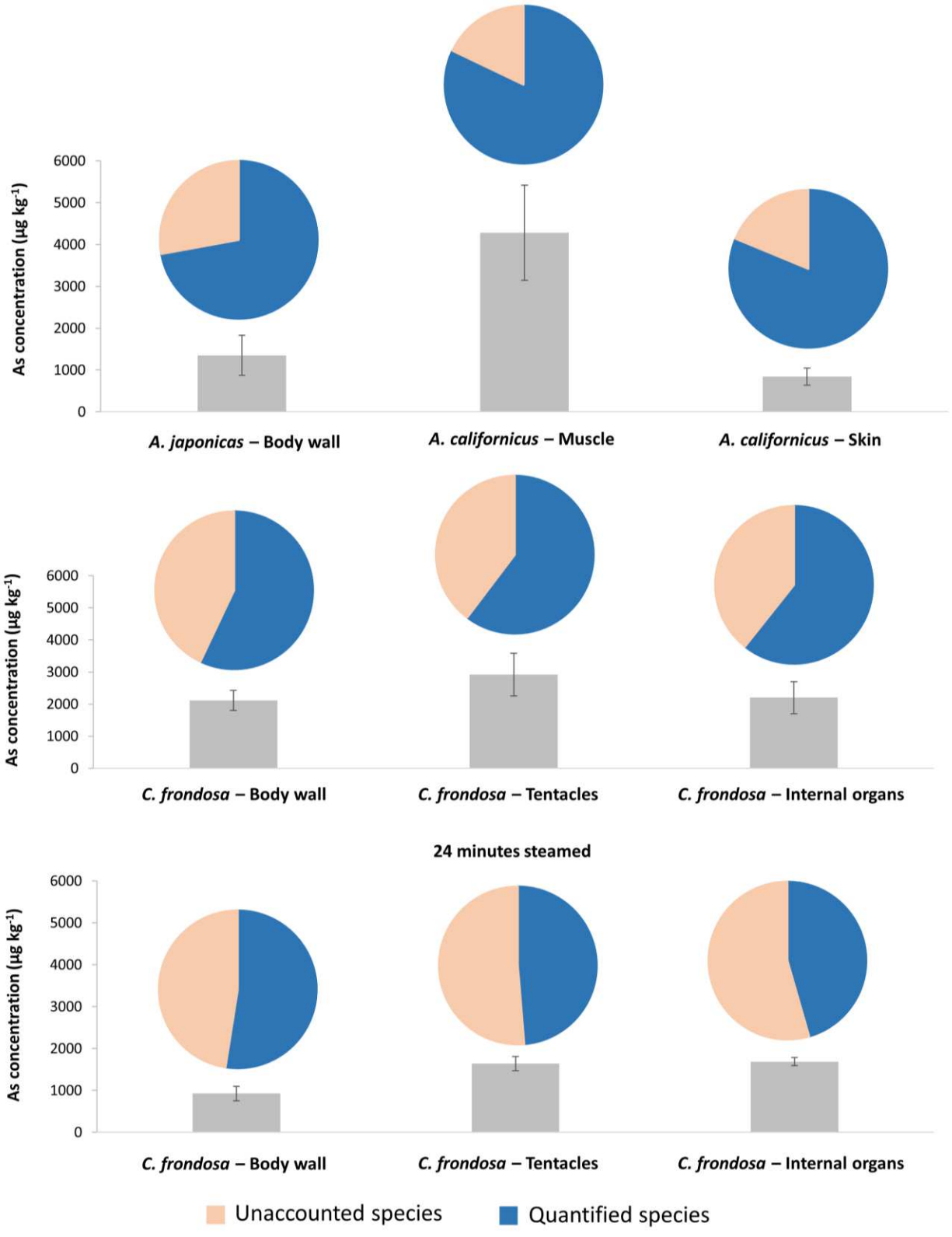
Figure 4. Total As concentration and percentage fraction of individual As species in TFA extracts in body wall of *A. japonicas*, muscle and skin of *A. californicus* and internal organs, tentacles and body wall of *C. frondosa*. Processed body parts belong to *C. frondosa*. Error bars represent SD of replicate samples; n=3 or 5.

Arsenic present in sea cucumbers is of dietary origin. Sea cucumbers feed on algae, phytoplankton and other decaying organic matter on the sea floor. Algae are reported to contain major portion of As as AsSug [30, 47, 58, 59], although several studies reported small quantities of AsB [60, 61]. It was found that AsB is not readily taken up and retained by microalgae and thus it is most likely associated with topical epifauna and bacteria on the damaged blades of algae [62]. Therefore, some dietary intake of AsB can be assumed and indeed it is supported by AsB found in food pellets of sea urchins [58], belonging to the same phylum as sea cucumbers. However, sole dietary intake could likely not explain the large quantities found in the studied sea cucumbers. Several pathways for AsB bio-synthesis from AsSug have been proposed which could take place in the digestive tract of aquatic herbivores facilitated by bacteria present on the consumed algae [60]. Large quantities of trimethylated, oxo and thio-dimethylated AsSug found in sea urchins support this hypothesis [58, 63], however, only trace amounts of some unknown As species were found in the extracts of sea cucumbers which could not be identified due to the lack of standards. It is possible that these As species were not quantitatively eluted from the column as the sum of As species in body parts of *A. japonicas* and *A. californicus*, which are rich in AsB amounted to between 72 and 82 % of total As (Figure 5) in the water-extractable fraction.

Considering AsSug being the predominant As species in algae [30, 47, 58, 59], it is reasonable to assume that their presence as observed in sea cucumbers is of a dietary origin. However, AsSug are also a main structural blocks of As containing lipids (AsLipids). Thus, AsSug can be either an intermediates in bio-synthesis of AsLipids or an artefacts of AsLipids degradation induced by the extraction method. Lipids are essential components of skin and thus it should be expected to find larger variety of AsLipids in this body part. Accordingly, in skin of *A. californicus*, AsSug-482 was found to be the dominant As species and AsSug-408 was only found in this body part. A high concentration of AsSug-482 was generally found in all sea cucumber body parts as seen in Figure 4, and this has also been reported in the body parts of sea urchins [58]. The authors argue that synthesis of AsSug-482 is likely to take place *in vivo* through bonding of AsSug-328 with phosphatidic acid in the cell membrane. Newly formed As containing phospholipids can be then hydrolysed to AsSug-482. Lipid hydrolysis can be induced by trace amounts of water present in the extraction solvents during the lipid extraction step.

412 Interestingly, a very different As species profile was found in *C. frondosa* where iAs dominated the water-
413 extractable As in all body parts. While some algae are known to contain large quantities of iAs, e.g. in hijiki
414 (*Sargassum fusiforme*) reaching over 100 mg kg^{-1} [47, 64], Foster et al. argued that as $As^{(V)}$ interferes with
415 phosphorylation, its uptake and distribution would not be beneficial and a mechanism for its uptake
416 exclusion would exist [58]. Another possible source of iAs is a rapid break down of dimethylarsinylethanol
417 (DMAE), which is a degradation product of AsSug in macroaglae under anaerobic conditions [65].
418 However, the large quantities of iAs found in the present study are most probably an accumulative result
419 of various sources. While sea urchins were found to contain only small fraction of iAs [58, 60, 63], several
420 researchers reported elevated concentrations of iAs in bivalves and gastropods [66-68]. Additionally, it
421 should be noted that the sum of As species in *C. frondosa* body parts, accounted for between 57 and 61%
422 in raw and 46 and 54 % in processed body parts (Figure 5) with respect to total As in these fractions.
423 Considering that more As species are accounted for when cations are the major fraction of As species
424 suggests that significant portion of anions is not being eluted from the chromatographic column.
425 Consequently, without the knowledge of the identity of these As species it is challenging to pinpoint the
426 bio-synthetic pathways that may contribute to the observed As species variation.

427


As concentration (µg kg-1)
6000
5000
4000
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0
A. japonicas – Body wall
A. californicus – Muscle
A. californicus – Skin
C. frondosa – Body wall
C. frondosa – Tentacles
C. frondosa – Internal organs
24 minutes steamed
C. frondosa – Body wall
C. frondosa – Tentacles
C. frondosa – Internal organs
Unaccounted species
Quantified species

428

Figure 5. Total As concentration and percentage fraction of quantified As species in TFA extracts in body wall of *A. japonicas*, muscle and skin of *A. californicus* and internal organs, tentacles and body wall of *C. frondosa*. Processed body parts belong to *C. frondosa*. Error bars represent SD of replicate samples; n=3 or 5.

Impact of thermal processing on As species

Raw body parts of *C. frondosa* were steamed for 24 min (Method section) in order to determine the impact of common thermal processing used in the industry. On average, the processed samples were found to have 28% less total As than unprocessed samples (Table 1). In terms of individual As fraction, steaming reduced total As in all sequential fractions apart from non-polar lipids. Speciation of water-extractable As showed a decrease in AsSug-482, AsB and DMA in all body parts. On the contrary, iAs concentration increased as well as AsSug-328 in tentacles. The change in concentration of the individual As species is similar to the trend observed when reversed extraction was applied. It appears that thermal treatment degrades AsSug and possibly polar As lipids which is reflected in higher fraction of iAs. Thus it could be concluded that cooking of sea cucumbers, in excess water as a proxy to water extraction is the preferred preparation method to reduce As levels rather than steaming which removes only small portion of As.

Conclusion

In present study, comparison between normal and reversed sequential extraction of As species with increasing / decreasing polarity, respectively from the body parts of sea cucumbers revealed a presence of non-stable non-polar As containing lipids. Steaming of sea cucumber body parts, resulted in As species alteration and a small decrease in total As indicating that such processing method would have negligible impact on As. Comparison between extraction of water-extractable As compounds using water and TFA showed that the later reagent can extract larger variety of As species. Speciation of water-extractable As from the body parts of sea cucumbers showed large As species diversity between *A. californicus, A. japonicas* and *C. frondosa*. While AsB and AsSug-482 were the major species in *A. californicus* and *A. japonicas*, iAs was predominantly accumulated in the body parts of *C. frondosa*. Sequential extraction of non-polar and polar lipids showed the presence of As in these fractions indicating that some As in sea cucumbers is present as lipophilic compounds. The identity of these compounds remains a subject of future research. Overall the results showed that a generalised approach to As speciation in individual classes of aquatic fauna doesn't apply to sea cucumbers and it is very probable that similar differences

will be observed in other biota. In order to shed more light on the observed variation in As species distribution between studied sea cucumbers it would be beneficial to speciate As in algae species that are the presumptive food source for sea cucumbers from the respective geographical regions. Furthermore given the difference in total As and As species between sea cucumber species, additional data is required to further understand the associated metabolic processes.

Word count: 6466 excluding authors' names and affiliations

Conflict of interests

No conflict of interest is declared.

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