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Arsenobetaine and thio-arsenic species in marine macroalgae and herbivorous animals: Accumulated through trophic transfer or produced *in situ*?

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ABSTRACT

Arsenobetaine (AB) and thio-arsenoribosides were measured in common macroalgae species (8 phaeophyta, 4 rhodophyta and 2 chlorphyta), along the Australian south east coast line. As well, arsenic species profiles were measured for two common marine herbivores, the sea urchin *Centrostephanus rodgersii* and the fish *Odax cyanomelas* that graze on these macroalgae to understand if trophic transfer of these species would account for their presence in marine herbivores. AB was found in seven of the fourteen macroalgae species investigated but does not contributed significantly to any of the macroalgae arsenic content (0.01-1.2 μg/g). AB was found in only two of the brown macroalgae and all the red and green macroalgae (with the exception of *Corallina officinalis*). Thio-arsenic species were found sporadically, but not in high concentrations in any of the macroalgae investigated. AB present in macroalgae is likely to be associated with epiphytic organisms while thio-arsenoribosides are likely to be produced by decaying parts of damaged macroalgae. A laboratory feeding experiment in which the herbivorous gastropod, *Austrocochlea constricta*, was fed macroalgae containing thio-arsenoribosides for a 24 hr period every three days showed that these are readily accumulated over a short period. Thio-arsenoribosides in herbivores are therefore probably obtained through trophic transfer. Some AB is also obtained through trophic transfer; however, the presence of trimethylated arsonioribosides, a hypothesized precursor of AB formation in herbivores, suggests that some AB is produced within herbivores from the transformation of arsenoribosides accumulated from their diet.

Introduction

Marine macroalgae are a major source of arsenic for herbivorous marine animals in the marine environment. The arsenic species present in marine macroalgae are, therefore, of great importance in the cycling of arsenic within shallow water marine environments.

Marine macroalgae contain mostly arsenoribosides, with minor amounts of inorganic arsenic, dimethyl arsenic and thio-arsenic species (Tukai et al., 2002b). Arsenobetaine (AB), however, has been reported as a minor component of macroalgae (Nischwitz and Pergantis, 2005). Several pathways have been proposed for the formation of AB from arsenoribosides (Fig. 1) although the formation of AB via

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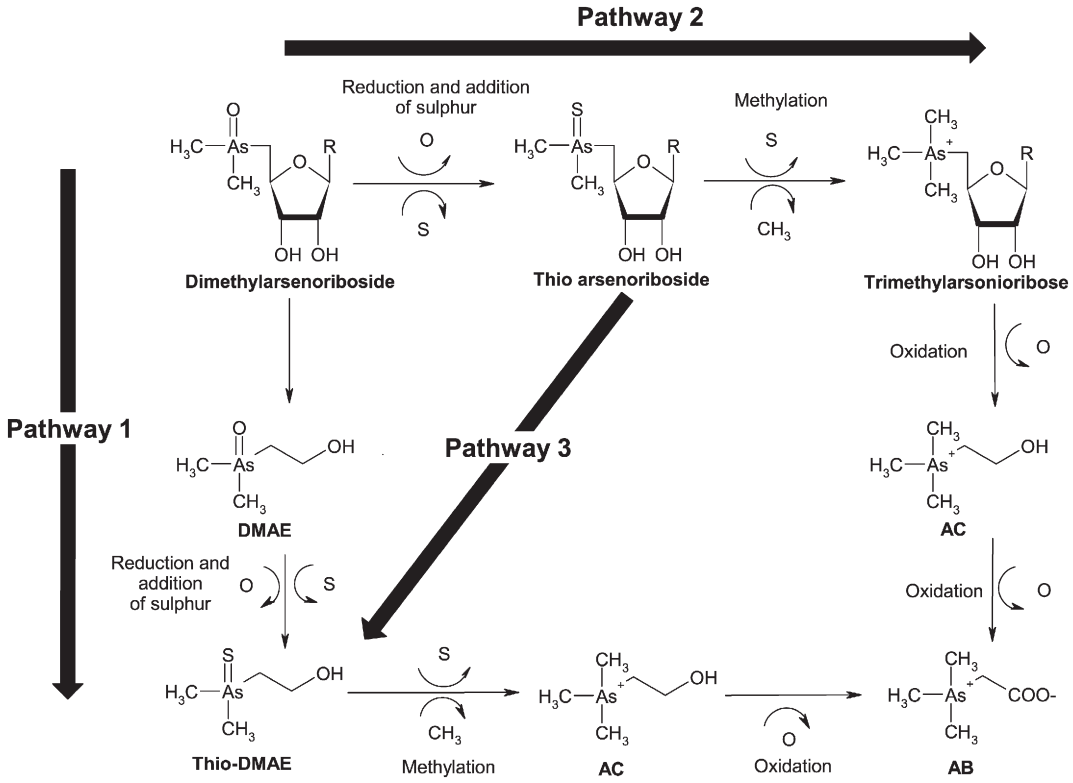

Fig. 1 – Proposed pathways for the metabolism of arsenoribosides and the formation of arsenobetaine in marine animals.

trimethylated arsonioribosides directly from algae is not likely as only one study has reported the presence of trimethylated arsonioribosides in macroalgae (Shibata and Morita, 1988). It would be more likely that these are formed during digestion in organisms. In terms of the trophic transfer and metabolism of arsenic, the reporting of AB must be assessed, as the direct accumulation of arsenobetaine from dietary consumption would explain the occurrence of AB in marine herbivores (Foster et al., 2006, 2008; Kirby et al., 2005). Epiphytes and bacteria are commonly associated with macroalgae (Armstrong et al., 2001; Paerl and Pinckney, 1996) and if not thoroughly removed could be responsible for the presence of some if not all AB in marine herbivores. There is some uncertainty how samples with reported AB concentrations have been cleaned and prepared for chemical analysis. Similarly, thio-arsenoribosides are commonly found in degrading algae (Foster and Maher, 2010) and may have been reported in live algae as it is possible degraded and not healthy live macroalgae specimens were analyzed.

This study reports the occurrence of AB and thio-arsenic species in previously analyzed red, green and brown macroalgae (Tukai et al., 2002a, 2002b) and two herbivores, a sea urchin *Centrostephanus rodgersii* and a fish *Odax cyanomelas* that extensively graze on these macroalgae to understand if trophic transfer of these species would account for their presence in marine herbivores. A feeding experiment was also conducted in which the herbivorous gastropod, *Austrocochlea constricta*, was fed decaying macroalgae containing thio-arsenoribosides to determine if they readily accumulate these arsenic species.

1. Materials and methods

1.1. Sampling

Macroalgae, fish (*O. cyanomelas*), black sea urchin (*C. rodgersii*) and gastropods (*A. constricta*) were collected from Mosquito Bay, Wimbie, and Lilli Pilli on the south coast of New South Wales Australia. Macroalgae were collected by hand from the intertidal zone (Tukai et al., 2002b) and care was taken to select only living undamaged specimens. Sea urchins were collected by hand from the adjacent subtidal zones and fish from the subtidal zone using a spear gun. All samples were placed in clean plastic bags and transported to the laboratory on ice in a cooler.

1.2. Thio-arsenic feeding experiment

Approximately 20 g of fresh *Hormosira banksii* was placed into 500 mL plastic storage containers with 100 mL of unfiltered seawater. The algae were allowed to decay at room temperature (22–25°C) over an initial period of 11 days before being fed to the gastropods. *A. constricta* were kept in an aerated aquarium and placed on the decaying algae at day 12, day 15 and day 21. The gastropods were allowed to feed for 24 hr before being removed, deshelled and frozen. The algae and seawater were collected from each of the containers at the time of collection of the gastropods (data has been previously reported for the water and algal degradation products (Foster and Maher, 2010)).

1.3. Sample preparation

Following collection, marcrolgae were rinsed with deionized water, and scrubbed with nylon brushes to remove salts and epiphytes; the samples were again rinsed with deionized water and placed into 20 mL plastic capped vials. *C. rodgersii* was depurated in seawater collected from sampling sites for 12 hr. *C. rodgersii* samples were dissected into gonad, visceral tissue and gut contents (food pellets) and *O. cyanomelas* samples were dissected into gill, muscle, gonad, gut and gut contents. All samples were frozen at −20°C. All samples were lyophilized then ground to a fine powder with a Retsch ZM100 mill (0.2 mm stainless steel mesh, Retsch), and stored in clean polyethylene vials in a desiccator at room temperature (22–25°C) until further processed for arsenic species.

1.4. Sample analysis

1.4.1. Reagents and standards

Nitric acid (HNO_3) (Aristar, BDH) was used for the determination of total arsenic concentrations. Ammonium dihydrogen phosphate (Suprapur, Merck) pyridine (Extra Pure, Merck) and methanol (HiPerSolv, BDH) were used in the preparation of HPLC mobile phases. Formic acid (Extra Pure, Fluka), phosphoric acid (AR, BDH) and ammonia solution (>99.9%, Aldrich) were used for the adjustment of mobile phase pH. Methanol, acetone (HiPerSolv, BDH) and deionized water (18.2 MΩ cm, Millipore) were used for the extraction of arsenic species.

Arsenobetaine (BCR-626, Institute for Reference Materials and Measurements) was diluted with deionized water to desired concentration. Sulfonate arsenoriboside, phosphate arsenoriboside and sulfate arsenoriboside (SO_3^- arsenoriboside, PO_4 arsenoriboside, OSO_3^- arsenoriboside, respectively) were obtained from a previously characterized fucus extract (Madsen et al., 2000). Glycerol arsenoriboside (OH arsenoribose) was isolated from *Ascophyllum nodosum*. All arsenoribosides were isolated on a preparative PRP-X100 (150 mm × 21 mm, 12–20 μm) using ammonium carbonate buffer, pH 10, 7 mL/min flow rate and an injection volume of 2 mL, the flow was split post column 0.5 mL/min to the ICP-MS and 6.5 mL/min was collected as peak fractions. Thio-OH arsenoriboside, thio-PO_4 arsenoriboside, thio-SO_3– arsenoriboside, and thio-OSO_3– arsenoriboside were synthesized by bubbling H_2S through an extract of *Fucus* previously characterized (Madsen et al., 2000) for approximately 30 sec with a 100% conversion rate. Hydrogen sulfide was generated by adding concentrated HCl to iron sulfide in an enclosed tube and directing the gas to the sample through Teflon tubing.

1.4.2. Total arsenic analysis

Tissues were digested using a microwave digestion procedure described previously by Baldwin et al. (1994). Approximately 0.07 g of freeze–dried tissue was weighed into 7 mL Teflon polytetrafluroacetate digestion vessels (A. I. Scientific, Australia) and 1 mL of concentrated HNO_3 (Aristar, BDH, Australia) added. Digestion was carried out using an MDS-81D microwave oven (CEM, USA) with a time program consisting of three steps: 2 min at 600 W, 2 min at 0 W and 45 min at 450 W. After digestion, vessels were allowed to cool at room temperature (25°C) for approximately 60 min and then diluted with deionized water to 10 mL in polyethylene vials. Total arsenic (*m/z* 75), concentrations were determined with a Perkin-Elmer Elan-6000 inductively coupled plasma mass spectrometer (ICP-MS). Internal standards (^{45}Sc, ^{103}Rh) were added on-line to compensate for any acid effects and instrument drift.(Maher et al., 2003) The potential interference to arsenic (*m/z* 75) from $^{40}Ar^{35}Cl^+$ was determined by monitoring chloride at *m/z* 35, $^{35}Cl^{16}O^+$ at *m/z* 51, $^{35}Cl^{17}O^+$ at *m/z* 52 and $^{40}Ar^{37}Cl^+$ at *m/z* 77. Selenium was monitored at *m/z* 82 as a cross check on $^{40}Ar^{37}Cl^+$. Calibration standards (0, 1, 10, 100, 1000 μg/L) for the determination of total arsenic were prepared daily by appropriate dilution of the multi element calibration standard (Accu Trace, Calibration Standard 2, 10 mg/L).

Certified reference materials analyzed for arsenic was Sargasso (NIES No. 9). Measured arsenic concentrations (mean ± SD; *n* = 3) were: 144 ± 5 μg/g As, compared to the certified value of 155 ± 9 μg/g As.

1.5. Arsenic speciation

1.5.1. Acetone extraction

Approximately 0.2 g of homogenized freeze–dried whole-tissue was added to 50 mL polypropylene vials and 10 mL of acetone added. The samples were then agitated on a mixing wheel for 1 hr at 120 r/min and the supernatant removed after centrifuging at 4500 *g* for 10 min. The extraction procedure was repeated twice, with the supernatant removed after each centrifugation. After the final acetone extraction, the residue pellet was dried to a constant mass in a fume cabinet at room temperature (25°C).

The entire combined acetone supernatant was evaporated in a fume cabinet at room temperature (25°C) to dryness. The residue was resuspended in 0.5 mL concentrated HNO_3 and digestion was undertaken in a hot water bath (90°C) for 1 hr. Digested acetone extracts were allowed to cool at room temperature (25°C) and then diluted with deionized water to 5 mL in 10 mL polyethylene vials. Total arsenic concentration was determined by ICP-MS.

1.5.2. Methanol–water extraction

Water-soluble arsenic species were extracted from biological material by a microwave extraction procedure developed by Kirby and Maher (2002). Approximately, 0.1 g of the dried acetone extracted pellet was weighed into 50 mL polypropylene vials and 10 mL of 50% (V/V) methanol-deionized water added. Mixtures were loaded into the carousel of an MDS-200 microwave oven (CEM, USA) and heated to 75°C for 10 min. The supernatant was removed after centrifuging at 4500 ×*g* for 10 min. The procedure was repeated twice and the supernatants combined. Thirty milliliters of the methanol–water supernatant was evaporated to dryness using a RVC 2-18 rotational vacuum concentrator (50°C) (Christ, Australia) and stored in a freezer (−18°C) until speciation analysis.

1.5.3. Water-soluble arsenic speciation

Prior to chromatography, previously stored methanol/water extracted residues were resuspended in 4 mL deionized water. Total arsenic was determined by digesting 1 mL of the resuspended extract with 1 mL of HNO_3 and 0.5 mL of H_2O_2 in a water bath at 90°C. The digests were diluted to 10 mL

Table 1 – AB, Gly-riboside and thio-arsenoribosides in brown, red and green macroalgae species. Percentage of sum of arsenic species in brackets.

Species	Phyla	Total As [a]	Extracted As	OH-riboside from Tukai et al. (2002b) vs the sum of AB and OH-riboside [b]	AB [c]	OH-riboside	Thio-SO_3^--riboside	Thio-PO_4^--riboside	Thio-OSO_3-riboside	Thio-Gly-riboside
		(μg/g)	(μg/g) ± SD [d]	%	(μg/g) ± SD [d]	(μg/g) ± SD	(μg/g) ± SD	(μg/g) ± SD	(μg/g) ± SD	(μg/g) ± SD
Lobophora sp.	*Phaeophyta*	19.7	19.0 ± 2.9	6 *vs.* 6	0.69 ± 0.10 (3.5)	0.43 ± 0.06 (2.2)	0.06 ± 0.01 (0.3)	n.d.	n.d.	n.d.
Sargassum sp.	*Phaeophyta*	120.4	119 ± 17	1 *vs.* 4	n.d. [d]	4.9 ± 0.7 (4.1)	n.d.	n.d.	n.d.	n.d.
Hormosira banksii	*Phaeophyta*	17.4	17.7 ± 2.6	39 *vs.* 35	n.d.	6.2 ± 0.9 (35)	n.d.	n.d.	n.d.	n.d.
Ascophyllum nodosum	*Phaeophyta*	51	53 ± 2	NA [e]	n.d.	8.5 ± 1.3 (17)	0.17 ± 0.03 (0.3)	0.13 ± 0.02 (0.3)	n.d.	n.d.
Ecklonia radiata	*Phaeophyta*	42.5	42 ± 7	2 *vs.* 4	n.d.	1.9 ± 0.3 (4.5)	n.d.	n.d.	n.d.	n.d.
Macrocystis pyrifera	*Phaeophyta*	131	127 ± 22	NA	n.d.	23 ± 4 (18)	n.d.	n.d.	n.d.	n.d.
Padina fraseri	*Phaeophyta*	5.6	6.3 ± 2.7	7 *vs.* 8	0.50 ± 0.08 (4.2)	0.39 ± 0.06 (3.3)	n.d.	n.d.	n.d.	n.d.
Durvillaea potatorum	*Phaeophyta*	131	127 ± 18	NA [e]	n.d.	14 ± 2 (11)	n.d.	n.d.	n.d.	n.d.
Amphiroa anceps	*Rhodophyta*	2.8	2.7 ± 0.4	16 *vs.* 24	0.23 ± 0.03 (8.3)	0.44 ± 0.07 (16)	n.d.	n.d.	0.027 ± 0.004 (1.0)	n.d.
Martensia fragilis	*Rhodophyta*	1.1	1.1 ± 0.2	18 *vs.* 25	0.15 ± 0.02 (14)	0.12 ± 0.02 (11)	n.d.	n.d.	n.d.	n.d.
Laurencia sp.	*Rhodophyta*	15.5	14.9 ± 2.0	13 *vs.* 22	0.56 ± 0.08 (3.6)	2.8 ± 0.4 (18)	0.16 ± 0.02 (1.0)	n.d.	n.d.	n.d.
Corallina officinalis	*Rhodophyta*	2.9	3.0 ± 0.4	7 *vs.* 8	n.d.	0.23 ± 0.03 (8)	n.d.	n.d.	0.027 ± 0.004 (0.9)	n.d.
Codium lucasii	*Chlorophyta*	8.2	8.5 ± 1.4	24 *vs.* 22	0.71 ± 0.11 (8.6)	1.1 ± 0.2 (13)	n.d.	n.d.	n.d.	n.d.
Cladophora subsimplex	*Chlorophyta*	13.5	13.8 ± 2.2	21 *vs.* 33	1.2 ± 0.2 (8.7)	3.2 ± 0.5 (24)	n.d.	n.d.	n.d.	n.d.

[a] Total arsenic has been previously measured.
[b] % of Gly-riboside reported in Tukai et al. (2002b) versus the sum of AB and Gly-riboside found in the current study.
[c] AB = arsenobetaine; OH-riboside = glycerol arsenoriboside; thio-SO_3^--riboside = thio-sulfonate arsenoriboside; thio-PO_4^--riboside = thio-phosphate arsenoriboside; thio-OSO_3-riboside = thio-sulfate arsenoriboside; thio-Gly-riboside = thio-glycerol arsenoriboside.
[d] SD is based on the analysis of triplicate samples.
[e] NA: not analyzed in the study by Tukai et al. (2002b); n.d. (not detected) = <0.01 μg/g As based on 40 μL injection.

with deionized water and analyzed for arsenic by ICP-MS as previously described. All extracts were filtered through a 0.20 μm RC syringe filter (Millipore). Aliquots of 40 μL were injected onto a high pressure liquid chromatography (HPLC) system consisting of a Perkin Elmer Series 200 mobile phase delivery and auto sampler system (Perkin Elmer). The eluant from HPLC columns was directed by PEEK (polyether-ether-ketone) (*i.d.* 0.02 mm) (Supelco) capillary tubing into a Rhyton crossflow nebulizer of a Perkin Elmer Elan-6000 ICP-MS, which was used to monitor the signal intensity of arsenic at *m/z* 75. Potential polyatomic interferences were checked by monitoring for other ions as described for total arsenic analysis.

The column conditions used for the separation of arsenic species were:(1) For the separation of AB and OH arsenoribose a SCX cation column (250 mm × 4.6 mm, 10 μm) with 20 mmol/L pyridine formic acid buffer (pH 3.5) and a flow rate of 1.5 mL/min at 40°C with an injection volume of 40 μL. (2) For the separation of the thio-arsenoribosides an Atlantis dC18 (150 mm × 4.6 mm, 5 μm) with a 20 mmol/L ammonium phosphate, phosphoric acid buffer (pH 3) and a flow rate of 1 mL/min at ambient room temperature with an injection volume of 40 μL. (3) For the separation of As(V), MA, DMA, PO4 arsenoriboside, SO_3– arsenoriboside and OSO_3– arsenoriboside a PRP-X100 anion column (250 mm x 4.1 mm, 10 μm), with a 20 mmol/L ammonium phosphate, ammonium hydroxide (pH 5.6) and a flow rate of 1 mL/min at ambient room temperature with an injection volume of 40 μL (Kahn et al., 2005; Kirby et al., 2004).

External calibration curves for quantification of arsenic species were prepared by diluting AB for the thio and cationic species to 0, 0.5, 1, 10, and 100 μg/L daily. The peak area response of oxo-OH arsenoribose (0.86 ± 0.05) was assumed to be similar to that using a cation buffer of the same molarity at pH 2.6 and this peak area response was used in the quantification of oxo-OH arsenoribose. Thio-arsenic species were quantified relative to the calibration of AB. The purity of arsenic species was periodically determined by HPLC-ICP-MS.

The chromatography package Total Chrom (Perkin Elmer) was used to quantify arsenic species by peak areas. Arsenic species were identified by spiking with known standards, oxidation with H_2O_2 and comparison of retention times.

The accuracy of arsenic speciation procedure was determined by the analysis of the certified reference material, DORM-2. The concentrations (mean ± SD, $n = 3$) of AB (16.3 ± 0.5 μg/g) and TETRA (0.241 ± 0.005 μg/g) measured in DORM-2 tissues were similar to certified values (AB, 16.4 ± 1.1 μg/g; TETRA, 0.248 ± 0.054 μg/g). The reproducibility of the peak times is presented in Tables 1, 3–5.

2. Results and discussion

2.1. AB and thio-arsenoribosides in macroalgae

Arsenobetaine was found in seven of the fourteen macroalgae species investigated (Table 1). As a proportion of the total extracted arsenic, AB did not contributed significantly to any of the macroalgae arsenic content (0.01–1.2 μg/g). AB was only found in two of the brown macroalgae. AB was found in all the red and green macroalgae with the exception of *Corallina officinalis*.

Comparison of the percentage sum of AB and OH-arsenoriboside from this study with the percentage sum of OH-arsenoriboside by Tukai et al. (Tukai et al., 2002b) revealed that AB contributed to the reported concentrations of OH-arsenoriboside in the study by Tukai and co-workers (Table 1).

Arsenobetaine is likely to be associated with epifauna and bacteria on the surface of the algae. Cleaning algae of all associated epifauna and bacteria could not be performed without total destruction of the integrity of the algae. Red and green macroalgae tend to have finer intricate fronds with larger surface areas that are difficult if not impossible to remove epiphytes from. Brown macroalgae tend to be simpler to clean as they have large blades that can be scraped clean of epifauna. Brown algae can contain fungi incorporated into the macroalgae structure (Deckert and Garbary, 2005; Flewelling et al., 2013; Kohlmeyer and Demoulin, 1981; Oliveira et al., 2012) that cannot be removed. Fungi have been reported to produce AB in natural environments (Kuehnelt and Goessler, 2003; Kuehnelt et al., 1997). The percentage of fungi that are associated with macroalgae can, however, vary greatly (1%–38% fungi) and not all fungi can biomethylate arsenic (Bentley and Chasteen, 2002). Recent studies have shown that it is likely that biomethylation is by bacteria associated with fungi and not the fungi themselves (Bentley and Chasteen, 2002; Dembitsky and Rezanka, 2003; Nearing et al., 2016).

The question remains is AB formed by macroalgae or associated with epiphytes. Duncan et al. (2010) found that AB was not readily taken up and retained by the microalgae *Dunaliella tertiolecta* which are similar phyla to macroalgae. The relatively small concentrations of AB found indicated that AB is not a major metabolite, if at all, given the large quantities of arsenoribosides present in macroalgae. Phaeophyta contain endophytic fungi that grow within the tissues of the algae (Flewelling et al., 2013; Oliveira et al., 2012). *Ecklonia radiata*, had most of its arsenic present as arsenoribosides and no AB (Table 2) indicating that any fungi

Table 2 – Methanol–water extractable cationic and anionic arsenic species from *E. radiata*.

		Total As	Sum of species	Column recovery (%)	OH-riboside	DMA	PO_4 ribose	As(V)	SO_3^- ribose	OSO_3^- ribose
Ecklonia radiata ($n = 12$)	As (μg/g)	62 ± 20	60 ± 8		1.6 ± 0.6	0.28 ± 0.13	7.7 ± 3.6	5.1 ± 0.8	45 ± 3	0.44 ± 0.23
	As (%)			97 ± 12	4.2 ± 2.2	0.64 ± 0.33	16 ± 6	13 ± 1	77 ± 10	0.40 ± 0.10

OH-riboside = glycerol arsenoriboside; DMA = dimethylarsinate; PO_4 ribose = phosphate arsenoriboside; As(V) = arsenate; SO_3^--ribose = sulfonate arsenoriboside; OSO_3^--ribose = sulfate arsenoriboside.

Table 3 – Methanol–water extractable cationic arsenic species from *C. rodgersii* and *O. cyanomelas* tissues.

Sample	Tissue	Total As (As μg/g)	Extracted As (As μg/g)	Column recovery %	AB [a] (As μg/g)	U 1 [b] (As μg/g)	OH-riboside (As μg/g)	TriMeOH (As μg/g)	TMAP (As μg/g)	DMAE (As μg/g)	U 2 (As μg/g)	AC (As μg/g)	TETRA (As μg/g)
C. rodgersii	Visceral	53 ± 1 [d]	51 ± 4	109 ± 4	7.9 ± 0.4 (14)	n.d.	0.93 ± 0.04 (1.7)	n.d.	n.d.	1.1 ± 0.1 (2.0)	n.d.	n.d.	n.d.
	Gonad	13 ± 2	12.2 ± 0.8	85 ± 6	3.9 ± 0.2 (38)	0.11 ± 0.01 (1)	0.51 ± 0.01 (4.9)	n.d.	0.19 ± 0.01 (1.8)	n.d.	n.d.	0.03 ± 0.01 (0.3)	0.06 ± 0.01 (0.5)
	Food pellets	10 ± 1	2.5 ± 0.2	104 ± 7	0.58 ± 0.03 (23)	n.d.	0.08 ± 0.01 (3.1)	n.d.	n.d.	0.31 ± 0.01 (12)	n.d.	n.d.	n.d.
O. cyanomelas	Muscle	5.8 ± 1	4.3 ± 0.3	94 ± 7	0.15 ± 0.01 (4)	n.d.	0.14 ± 0.01 (3.5)	n.d.	0.07 ± 0.01 (1.8)	n.d.	n.d.	n.d.	0.08 ± 0.01 (1.9)
	Liver	12 ± 1	6.4 ± 0.5	97 ± 7	0.50 ± 0.02 (8)	n.d.	0.26 ± 0.01 (4.1)	n.d.	n.d.	n.d.	0.08 ± 0.01 (1.2)	0.04 ± 0.01 (0.6)	n.d.
	Digestive	22 ± 1	15.8 ± 1.1	89 ± 6	0.21 ± 0.01 (1.5)	n.d.	1.6 ± 0.1 (11)	n.d.	n.d.	n.d.	0.50 ± 0.02 (3.6)	0.04 ± 0.01 (0.3)	0.05 ± 0.01 (0.3)
	Gill	11 ± 1	7.5 ± 0.4	89 ± 6	0.96 ± 0.05 (15)	n.d.	0.66 ± 0.03 (10)	n.d.	n.d.	n.d.	1.5 ± 0.1 (24)	n.d.	n.d.
	Gut contents	29 ± 7	25.8 ± 1.7	87 ± 6	n.d. [c]	n.d.	2.6 ± 0.1 (12)	n.d.	n.d.	n.d.	0.81 ± 0.04 (3.6)	n.d.	n.d.
Peak time (min) mean ± SD					4.09 ± 0.06	5.8 ± 0.2	6.8 ± 0.1	7.9 ± 0.1	8.8 ± 0.1	8.9 ± 0.3	10.1 ± 0.1	11.0 ± 0.2	13.1 ± 0.2

[a] Arsenic species abbreviations: AB = arsenobetaine; OH-riboside = glycerol arsenoriboside; TriMeOH = glycerol trimethylarsonioriboside; TMAP = trimethylarsoniopropionate; DMAE = 2-dimethylarsinoyl ethanol; AC = arsenocholine; TETRA = tetramethylarsonium ion.

[b] Not identified.

[c] n.d. = <0.01 μg/g As bases on 20 μL injection.

[d] Arsenic species concentration ± calculated uncertainty (reproducibility of the measurement was calculated based on repeated measurements of samples (n = 3).

present was not producing AB. AB, albeit at low levels, will be transferred to animals consuming macroalgae in associate epiphytes so would account for low concentrations on AB in animals.

Thio-arsenic species were found sporadically, but not in high concentrations in any of the macroalgae analyzed (Table 1). This is consistent with findings of other studies where fresh undamaged macroalgae contain little or no thio-arsenoribosides (Meier et al., 2005). We have undertaken laboratory degradation studies of the macroalgae, *H. banksii* and *E. radiata*, and shown that thio-arsenoribosides are formed as part of the decomposition of arsenosugars to DMA and subsequently arsenate (Duncan et al., 2014; Foster and Maher, 2010).

Marcoalgae are damaged from wave action and many gastropods, for example *A. constricta*, graze on damaged algae, possibly as bacteria colonizing damaged parts of algae are a food source (O'Brien et al., 2011), resulting in small amounts of thio-arsenoribosides in gastropods (Foster et al., 2006). Thus similar to AB, thio-arsenoribosides are likely to be accumulated from the diet rather than synthesized by herbivores.

2.2. AB and thio-arsenoribosides in herbivores

Arsenobetaine was found at relatively low concentrations in the herbivores *C. rodgersii* and fish *O. cyanomelas* that exclusively feed on macroalgae (Table 3). This is in contrast to most other marine animals that contain nearly all their arsenic as AB (Maher et al., 2009). There are exceptions, for example, Shibata and Morita (1992), reported the absence of arsenobetaine in the marine bivalve *Corbicula japonica* and Edmonds and co-workers found little AB in the fish Silver Drummer (*Kyphosus sydneyanus*) (Edmonds et al., 1997). Generally we have found that animals containing low quantities of AB contain oxo-dimethyl arsenoribosides and/or trimethyl arsenoribosides (Tables 3 and 4) and thio-arsenoriboside species (Table 5). Given that macroalgae naturally have epiphytes and bacteria that would contain some AB, this would contribute to the AB seen in the macroalgae (Table 2) and that AB is efficiently accumulated to animals (Amlund et al., 2006; Francesconi et al., 1989) some AB would be obtained by trophic transfer.

Given that large quantities of oxo and thio-dimethylated arsenoribosides and trimethylated arsenoribosides are present in some herbivores (Foster et al., 2008; Kirby et al., 2005), it is probable that AB might also be synthesized within the guts of some herbivorous animals via pathways outlined in Fig. 1. It has been shown that trimethylated arsonioribosides are quantitatively converted to AC (Francesconi et al., 1992), which in turn can be quantitatively converted to AB (Devesa et al., 2005; Francesconi et al., 1989). Ritchie et al. (2004) have also shown that the bacteria *Pseudomonas* sp. was able to convert dimethylarsinoylacetate to AB, while Nearing et al. (2016) have shown that fungi can accumulate AB from inorganic arsenic amended to their growth media. What is not clear is if AB is being biosynthesized by the fungi or the associated microflora within the growth media (Nearing et al., 2016). Thus it is conceivable that gut microorganisms could synthesis AB by as yet uncharacterized pathways.

2.3. Thio-arsenic feeding experiment

A. constricta fed on decaying *H. banksii* with high concentrations of thio-arsenoribosides accumulated low but significant

Table 4 – Methanol–water extractable anionic arsenic species from *C. rodgersii* and *O. cyanomelas* tissues.

Sample		DMA [a]	U 3 [b]	DMAA/MA	PO_4 ribose	As(V)	SO_3– ribose	U 4	OSO_3– ribose
		(As μg/g)	(As μg/g)	(As μg/g)	(As μg/g)	(As μg/g)	(As μg/g)	(As μg/g)	(As μg/g)
C. rodgersii	Visceral	2.4 ± 0.3 [c] (4.3)	n.d. [d]	n.d.	42 ± 5 (75)	0.28 ± 0.03 (0.5)	0.48 ± 0.05 (0.9)	0.39 ± 0.04 (0.7)	n.d.
	Gonad	0.72 ± 0.08 (6.9)	0.57 ± 0.06 (5.5)	n.d.	4.2 ± 0.5 (41)	n.d.	0.04 ± 0.01 (0.4)	n.d.	n.d.
	Food pellets	0.49 ± 0.05 (19)	n.d.	n.d.	0.80 ± 0.09 (31)	0.07 ± 0.01 (2.6)	0.18 ± 0.02 (7.1)	0.03 ± 0.01 (1.2)	n.d.
O. cyanomelas	Muscle	0.29 ± 0.03 (7.2)	n.d.	n.d.	2.6 ± 0.3 (64)	n.d.	0.56 ± 0.06 (14)	n.d.	n.d.
	Liver	1.7 ± 0.2 (27)	n.d.	0.10 ± 0.01 (1.6)	2.3 ± 0.2 (36)	n.d.	0.98 ± 0.11 (16)	n.d.	0.26 ± 0.3 (4.1)
	Digestive	3.5 ± 0.4 (25)	n.d.	1.3 ± 0.1 (9.1)	2.1 ± 0.2 (15)	n.d.	4.2 ± 0.5 (30)	n.d.	0.25 ± 0.3 (1.8)
	Gill	0.45 ± 0.05 (7.1)	n.d.	n.d.	1.6 ± 0.2 (25)	0.25 ± 0.03 (4.0)	0.82 ± 0.09 (13)	n.d.	0.08 ± 0.01 (1.2)
	Gut contents	4.9 ± 0.5 (22)	n.d.	n.d.	3.3 ± 0.4 (15)	n.d.	9.8 ± 1.1 (43)	n.d.	1.2 ± 0.1 (5.2)
Peak time (min) mean ± SD		2.8 ± 0.1	3.6 ± 0.2	4.6 ± 0.1	5.0 ± 0.1	8.5 ± 0.3	8.87 ± 0.40	15.8 ± 0.1	19.4 ± 0.9

[a] Arsenic species abbreviations: DMA = dimethylarsinate; DMAA = 2-dimethylarsinoyl acetic acid; MA = methylarsonate; PO_4 ribose = phosphate arsenoriboside; As(V) = arsenate; SO_3^--ribose = sulfonate arsenoriboside; OSO_3^--ribose = sulfate arsenoriboside.
[b] n.d. = <0.01 μg/g As bases on 40 μL injection.
[c] Arsenic species concentration ± calculated uncertainty (reproducibility of the measurement was calculated based on repeated measurements of samples (n = 3). Total As, extracted As and column recoveries are reported in Table 3.
[d] Not identified.

Table 5 – Methanol/water extractable thio arsenic species from *C. rodgersii* and *O. cyanomelas* tissues.

Sample		U 5 [a]	Thio-SO_3 ribose [b]	Thio-PO_4 ribose	Thio-OSO_3 ribose	Thio-OH-ribose	U 6
		(As μg/g)	(As μg/g)	(As μg/g)	(As μg/g)	(As μg/g)	(As μg/g)
C. rodgersii	Visceral	0.04 ± 0.01 [c] (0.1)	n.d. [d]	0.11 ± 0.01 (0.2)	n.d.	0.02 ± 0.01 (0.04)	0.04 ± 0.01 (0.1)
	Gonad	n.d. [a]	n.d.	n.d.	n.d.	n.d.	n.d.
	Food pellets	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
O. cyanomelas	Muscle	0.02 ± 0.01 (0.5)	n.d.	0.12 ± 0.01 (3.0)	n.d.	n.d.	n.d.
	Liver	0.02 ± 0.01 (0.3)	n.d.	0.05 ± 0.01 (0.8)	n.d.	0.04 ± 0.01 (0.7)	n.d.
	Digestive	0.02 ± 0.01 (0.2)	0.06 ± 0.01 (0.4)	0.07 ± 0.01 (0.5)	n.d.	0.09 ± 0.01 (0.6)	n.d.
	Gill	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
	Gut contents	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Peak time (min) mean ± SD		6.4 ± 0.2	7.6 ± 0.1	8.3 ± 0.4	10.0 ± 0.7	13.5 ± 0.4	16.1 ± 1.0

[a] Not identified.
[b] Arsenic species abbreviations are as presented in Table 1.
[c] Arsenic species concentration ± calculated uncertainty (reproducibility of the measurement was calculated based on repeated measurements of samples (n = 3). Total As, extracted As and column recoveries are reported in Table 3.
[d] n.d. = <0.01 μg/g As bases on 20 μL injection.

quantities in their tissues after feeding on the algae for up to 10 days (Fig. 2). Total thio-arsenic species increased from approximately 1 μg/g in the control organisms to 4 μg/g in the treated organisms after 10 days of exposure to decaying algae as their sole dietary source. This, however, only accounted for 1%–5% of the total As in the organisms. The only species of thio-arsenic to increase in *A. constricta* was thio-phosphate arsenoriboside with thio-glycerol arsenoriboside remaining fairly consistent throughout the experiment (Fig. 2). Therefore organisms feeding on decaying macroalgae should accumulate thio-arsenoribosides.

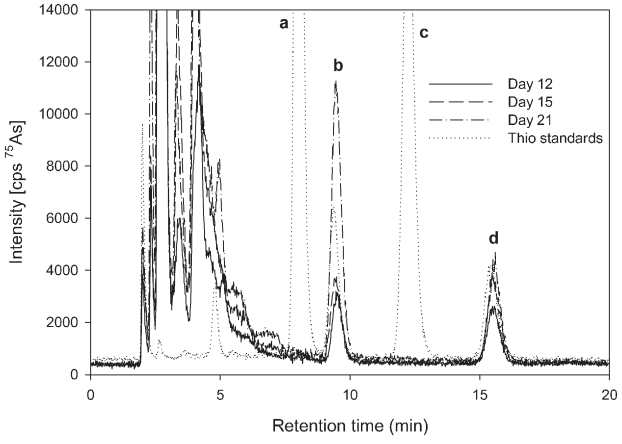

Fig. 2 – Accumulation of thio-arsenoribosides in *Austrocochlea constricta* fed decaying *Hormosira banksii* in laboratory mesocosm. Peak labels correspond to: a: thio sulfate arsenoriboside; b: thio phosphate arsenoriboside; c: thio sulfonate arsenoriboside; d: thio glycerol arsenoriboside. Chomatographic conditions were: Atlantis dC18 column (150 mm × 4.6 mm, 5 μm) with a 20 mmol/L ammonium phosphate, phosphoric acid buffer (pH 3) and a flow rate of 1 mL/min at ambient room temperature with an injection volume of 40 μL.

3. Conclusions

Arsenobetaine present in macroalgae is likely to be associated with epiphytic organisms while thio-arsenoribosides are produced by decayed parts of damaged macroalgae. There is little evidence that an indigenous symbiotic fungi associated with the brown algae *E. radiata* produce AB.

Thio-arsenoribosides in herbivores are likely to be obtained through trophic transfer from macroalgae while some AB is also obtained through trophic transfer, however, the present of trimethylated arsonioribosides in herbivores, a possible precursor of AB formation, suggests that some AB may be produced within herbivores from transformation of arsenoribosides accumulated from their diet. Bacteria present in herbivore digestive systems may also synthesis AB by as yet uncharacterized pathways.

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