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Enhanced bioaccumulation and biotransformation of As in coral reef organisms surrounding a marine shallow-water hydrothermal vent system

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ABSTRACT

The marine shallow-water hydrothermal system in Tutum Bay, Ambitle Island, Papua New Guinea discharges as much as 1.5 kg of arsenic (As) per day into a coral reef ecosystem. Despite the amount of As released, coral reef organisms do not seem to be affected. We investigated the uptake and bioaccumulation of geothermally-derived inorganic As by the soft coral *Clavularia* sp., the calcareous algae *Halimeda* sp., and the sea squirt *Polycarpa* sp., by measuring the total As concentration (TAs) in tissues from each organism and comparing it to the same type of organism collected from a nearby control site. All organisms collected from the hydrothermal area displayed distinctly higher (2 to 20 times) TAs compared to the control site. Concentrations were typically higher in samples collected closer to the focused hydrothermal venting, which is the first direct evidence for enhanced bioaccumulation of As in organisms living within an area of hydrothermal influence. To assess As biotransformation to organoarsenicals, anionic and cationic As species were determined by IC-ICP-MS in methanol/water tissue extracts. The concentrations of several of the organoarsenic species were much higher at the hydrothermal vent site compared to the control site, and several organoarsenic species were present only in the hydrothermal samples, including some unidentifiable species. While intriguing, these speciation results cannot be interpreted robustly due to poor extraction efficiencies. Future researchers should attempt to improve the extraction efficiency to closer to 100%, which would allow a more accurate description of As biosynthesis pathways for the marine organisms living in these environments.

1. Introduction

Arsenic is a toxic element at elevated concentrations for most organisms, although many have evolved detoxification mechanisms which allow them to cope with the toxic effects. For example, marine organisms can take in As by two major pathways; 1) cell diffusion from the water column, and 2) trophic transfer. In the case of cell diffusion, the organism can either take in As(V), mistaking it for phosphate using specific transporters found in the cell wall, or as As(III), via aquaglyceroporins, pore proteins that facilitate the efficient and selective flux of small solutes across biological membranes (Oremland and Stolz, 2003; Hub and de Groot, 2008). The inorganic forms taken in by coastal organisms are then transformed to less toxic forms – organoarsenicals. These organoarsenicals are either excreted as part of the detoxification mechanism or bioaccumulated within the organisms tissue (Francesconi and Edmonds, 1998).

The two major organoarsenic species occurring in natural waters, and which can occasionally be detected in seawater and sediment pore-waters as a result of planktonic and/or microbial interactions, are dimethylarsinate (DMA) or methylarsonate (MA). In addition to DMA and MA, other organoarsenic species can be found in tissues of marine organisms, and over 50 have been identified (Maher et al., 2009). These include trimethylarsine oxide (TMAO), tetramethylarsonium ion (TETRA), arsenobetaine (AB), arsenocholine, (AC), and the 4 major As-carbohydrate compounds, referred to collectively as arsenosugars or arsenoribosides (AR). These are glycerol sugar (GLY-sug), phosphate sugar (PO_4-sug), sulfonate sugar (SO_3-sug), and sulfate sugar (SO_4-sug) (For structures refer to Francesconi and Kuehnelt (2004)).

Marine algae contain mainly arsenosugars, while almost all other organisms analyzed to date contain AB and DMA as the two major species. Thus, these As species are considered the end products of the organism's metabolism. However, biosynthesis and biotrans formation patterns, particularly for AB, are unclear (Khokiattiwong et al., 2009; Maher et al., 2009).

Investigating As bioaccumulation and biotransformation of marine organisms living in marine hydrothermal vent systems, where geothermal As is often enriched relative to background concentrations, provides an opportunity to improve our fundamental understanding of these phenomena. For example, marine shallow-water hydrothermal

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fluids can have a considerable impact on the chemical composition of the often biologically important coastal surface waters, pore fluids and sediments (Pichler et al., 1999a; Price et al., 2007). Diffuse venting may also influence benthic species diversity and abundance at very large distances away from focused venting (Karlen et al., 2010). Thus, researchers attempting to understand biotransformation pathways will be better served by collecting and analyzing organisms living in these environments, and the knowledge gained from these investigations can then be applied to coastal ecosystems impacted by anthropogenic pollution.

The present work focuses on the bioaccumulation and biotransformation of As in coral reef organisms living in the marine shallow-water hydrothermal system in Tutum Bay, Ambitle Island, Papua New Guinea, where as much as 1.5 kg of As were estimated to be discharged daily (Pichler and Dix, 1996). Despite the high amount of As released, corals, clams, and fish did not show a visible response (Pichler et al., 1999b). However, substantial amounts of As were found to be bioavailable from (1) surface sediments, (2) ambient seawaters, and (3) from direct contact with diffusively discharging hydrothermal fluids (Price and Pichler, 2005). Sediments along a transect contained easily extractable As as much as 54 mg kg^{-1}, with a mean of 19. Surface seawaters of Tutum Bay contained as much as four times the average seawater concentration, but As in seawater just above the sediment/water interface is near normal, suggesting that exposure is primarily from diffuse venting and easily-exchangable As from sediments. However, plankton in the surface may accumulate higher concentrations of As, and therefore those organisms on the reef which are filter feeders (e.g., sea squirts) may obtain some of their As from falling plankton. Due to the very elevated bioavailable concentrations of As throughout the hydrothermal environment, enhanced bioaccumulation and biotransformation were suspected in surrounding reef organisms, even though they showed no visible response. Thus, several coral reef organisms living in this environment were collected and analyzed for total As and As speciation to determine if in fact they show evidence of hydrothermal venting of As, and to what extent.

1.1. Previous research

The hydrothermal system in Tutum Bay was previously described in detail (Pichler and Dix, 1996; Pichler et al., 1999a; Price and Pichler, 2005). Several hot springs are located on the west side of Ambitle Island, and extend into Tutum Bay, where submarine hydrothermal venting occurs about 30–40 m offshore in water depths of 5–10 m (Fig. 1). There are four main vent sites discharging through discrete orifices, as well as with abundant diffuse venting of hydrothermal fluids trough sediment (Figs. 1 and 2). Vent fluid temperatures reach as high as 98 °C, and have an approximate pH of 6.1. This location is unique in that it is one of the most As enriched marine hydrothermal systems discovered to date. Arsenic concentrations in hydrothermal fluids reached as high as 950 μg l^{-1}, occurring mainly as As(III), although sediment pore waters can also contain appreciable amounts of As(V) (Price et al., 2007). This geothermally-derived As oxidizes after discharge from the vents to As(V) and quickly and efficiently adsorbed onto the abundant hydrous ferric oxide (HFO) occurring throughout the area (Pichler et al., 1999b). Furthermore, the As concentrations in Tutum Bay sediments averaged 527 mg kg^{-1} of which an average of 19.2 mg kg^{-1} were considered bioavailable (Price and Pichler, 2005).

While several studies have investigated As species occurrence and distribution in marine organisms, their focus was primarily on edible organisms living in unimpacted environments due to obvious health implications (Francesconi, 2010). As a consequence, there are only few reports on As speciation in coral reef organisms. One of the most compelling studies to date compared stony corals from an impacted site to a pristine reef near Phuket, Thailand (Khokiattiwong et al., 2009). The hard coral *Goniastrea aspera* was collected in Phuket Bay, near a tin smelting work, and contained a total As concentration of 29 mg kg^{-1} dry weight. The hard corals *Goniastrea aspera* and *Porites lutea*, were collected from a pristine coral reef, and contained lower total As concentrations of 18 and 13 mg kg^{-1} dry wt, respectively, suggesting increased As retention in the impacted site (although no water or sediment data were reported). Miao et al. (2001) reported an As concentration of 17 (12–19, $n=4$) mg kg^{-1} dry wt., in the coral *Porites evermanni*, collected from French Frigate Shoals, Hawaii. This site was contaminated with metals including As from previous military activity (a control site coral sample contained 12 mg kg^{-1} dry wt. As). Another interesting study compared the distribution of trace metals between the tissue, zooxanthellae, gametes, and skeleton of corals from an uncontaminated site and showed that the zooxanthellae samples were higher in Al, Fe, Mn, Ni, Cu, Zn, As, Cd and Pb than the tissue samples, and the tissue samples were generally higher in trace metals than the skeleton samples (Reichelt-Brushett and McOrist, 2003). The tissue for the hard coral *Acropora tenuis*

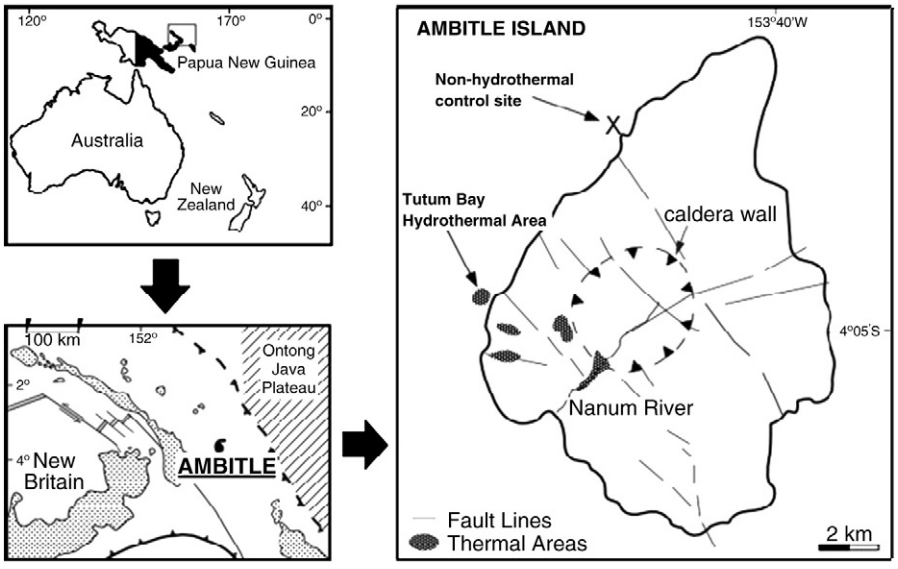

Fig.1. Location of Ambitle Island and the shallow-water hydrothermal vents studied. The Tutum Bay hyrothermal area and the reference site are indicated (modified from Pichler and Dix, 1996).

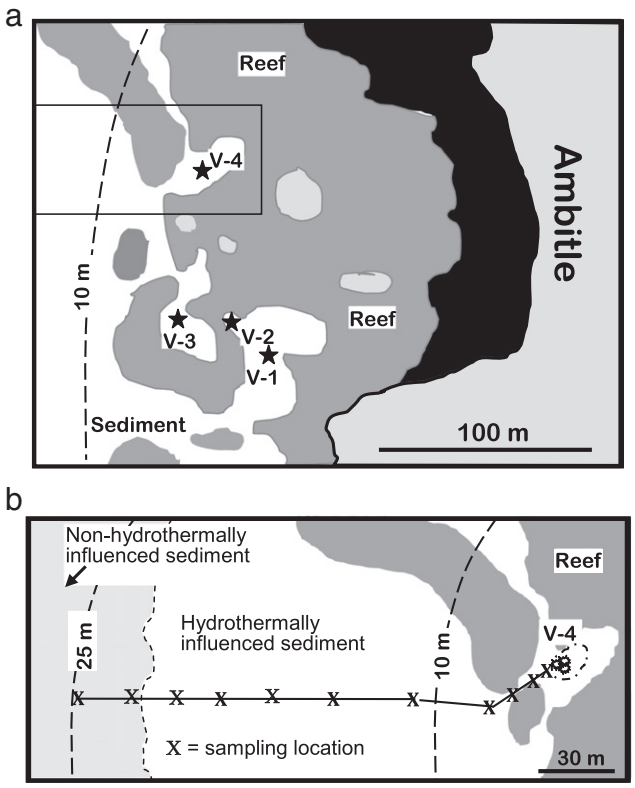

Fig.2. (a) Location of the main venting areas of the hydrothermal system at Ambitle Island. The box in the upper left is enlarged in (b) (modified from Pichler et al., 1999a). (b) Location of transect along which tissue samples were collected for this study.

contained between 1.12 and 2.69 mg kg^{-1} As, dry weight, while zooxanthellae and skeleton were from 2.01 to 3.46 and below detection, respectively. No investigations to date have evaluated bioaccumulation of geothermally-derived As in organisms from marine hydrothermal vents.

2. Methods

2.1. Sample collection and pre-treatment

To investigate the bioaccumulation and biotransformation of hydrothermally-derived inorganic As in Tutum Bay coral reef we chose 3 organisms: the soft coral *Clavularia* sp., the calcareous algae *Halimeda* sp., and the sea squirt *Polycarpa* sp. These organisms were chosen primarily because they were not only present at our control site, but also throughout the hydrothermal environment, making them ideal candidates for this type of investigation.

Clavularia is a genus of soft coral, commonly called star polyp, which is widespread in the Indo-Pacific and Atlantic (Fabriciuis and Alderslade, 2001). This organism is found throughout Tutum Bay in abundance, not only at the control site, but also very near focused hydrothermal venting, particularly where abundant gas bubbles are being discharged through the rocks and sediments. *Clavularia* are zooxanthellate, and thus obtain most of their fixed carbon through photosynthesis byproducts of symbiotic algae and direct uptake from the water column, although filter feeding is possible (Borneman, 2001). For example, these soft corals are common in salt-water aquaria, where feeding is not required, and nitrogen, phosphorus, calcium, strontium, and iron, along with adequate light, are the only key elements required for *Clavularia* to grow.

Halimeda is a green calcareous macroalgae, and thus obtains energy and fixes carbon through photosynthesis. It is very common in tropical marine environments, particularly in lagoonal areas between coral reefs and the shore, and the remains of *Halimeda* and other calcareous algae often make up the primary sediment component in these areas. *Halimeda* are found throughout the coastal environment of Ambitle Island, particularly where there is abundant coral growth. Due to low pH conditions and lack of a suitable hard substratum in the immediate vicinity of focused hydrothermal venting, *Halimeda* were not very common in these areas. Samples were therefore collected from rubble and reefs near diffuse and/or focused venting when necessary.

Polycarpa (a.k.a., tunicate or sea squirt) is a filter feeder with incurrent and excurrent siphons, whose primary food source is plankton (Borneman, 2001). The incurrent siphon is used to intake food and water and the excurrent siphon expels waste and water. The *Polycarpa* in Tutum Bay were primarily located on elevated reefs, above the influence of diffuse venting and hydrothermal sediments. They are members of the phylum Chordata.

The organisms were collected by SCUBA diving along a transect of declining hydrothermal influence out to a distance of 300 m (Figs. 1 and 2). This is the same transect described in Price and Pichler (2005). Samples were also collected from a coral reef located approximately 1.6 km north of Tutum Bay (Fig. 1), which was considered a non-hydrothermal control site.

Once samples were on board the research vessel, the organisms were dissected and washed thoroughly with milli-Q water, placed in 1.5 mL centrifuge tubes and kept frozen during transport to the University of South Florida. Once at the University of South Florida, each sample was freeze-dried, homogenized using a pestle and mortar, and then prepared for As abundance and As speciation measurements.

2.2. Laboratory methods

2.2.1. Total As concentration

Digestion of tissues was carried out by weighing freeze-dried tissue (50–100 mg) into 50 mL digestion vials, adding 1 mL HNO_3 (16 mol L^{-1}) and 0.5 mL H_2O_2 (10 mol L^{-1}), and letting the reaction proceed for 1 h at room temperature, followed by 1 h at 80 °C in a water bath. This two-step digestion procedure was employed to reduce foaming. After cooling, the digest was diluted to 50 mL with milli-Q water, filtered to <0.2 μm, and then diluted further with HNO_3 (0.3 mol L^{-1}) to a degree that corresponded to 25 mg solid tissue sample (e.g., 1:1 for 50 mg sample weight before digestion). All reagents were trace metal grade or better and were obtained from Fisher Scientific. Analytical standards were purchased from High Purity Standards.

Total As concentration (TAs) in the tissue digests was determined by a Perkin Elmer inductively-coupled plasma-dynamic reaction cell-mass spectrometry (ICP-DRC-MS) using O_2 to eliminate the $^{40}Ar^{35}Cl^+$ interference on $^{75}As^+$ by measuring As as AsO^+ at $m/z = 91$ (Bandura et al., 2001). It was important to normalize the amount of tissue sample represented by the final diluted digest aliquot because the apparent measured As concentration (both for samples and analytical spikes) was proportional to the digested sample weight, since it is known that organic carbon enhances the signal for As in ICP-MS (Larsen and Sturup, 1994). This suggests that while the digestion procedure was effective at dissolving the tissue material, it did not break down all organic carbon in solution. ^{193}Ir was used as internal standard.

2.2.2. Extraction and As speciation

Extraction of inorganic and organic As species was carried out using an 80:20 (v/v) methanol/water solution on the DI-rinsed, homogenized, freeze-dried sample splits (Cullen and Reimer, 1989; Ackley et al., 1999; Yeh and Jiang, 2005). Twenty to 200 mg of sample was weighed into 50 mL centrifuge tubes, and 10 mL of the extractant

solution was added. The samples were placed on a spinning shaker for 14 to 16 h (Goessler et al., 1997; Kuehnelt et al., 2001). The resulting mixtures were filtered through a 0.45 μm filter. After the extraction procedure was completed, total leachable As (TLAs) for each extract solution was measured by ICP-DRC-MS following the procedure for TAs outlined above, including matrix matching of the calibration standards to compensate for the methanol's effect on detection sensitivity.

Anionic As species were determined in the filtered MeOH/water extracts (100 μL sample loop) by anion-exchange chromatography-ICP-MS (AEC-ICP-MS), using a Dionex GP50 pump and gradient elution with NaOH solution at pH 11.4–13.0 (0–3 min: 2.5 mmol L^{-1}; 3 → 5 min: 2.5 → 20 mmol L^{-1}; 5 − 10 min: 20 mmol L^{-1}; 10 → 20 min: 20 → 100 mmol L^{-1}; 20–24 min: 2.5 mmol L^{-1}) with 2.4% MeOH at 1.2 mL/min on an AG-16/AS-16 (4 mm) column set (Dionex, Sunnyvale, CA).

Cationic As species were determined in a second extraction performed on freeze-dried tissue sample splits following the same protocol outlined above. Filtered MeOH/water extracts were analyzed (20 μL sample injection) by cation-exchange chromatography-ICP-(HR)MS (CEC-ICP-(HR)MS) (Accela 600 Pump and Element 2 High resolution ICP-MS, Thermo), using a 20 mM pyridine solution at pH = 2.6 at 1.5 mL min^{-1} on a Zorbax 300-SCX (250 × 4.1 mm 5 μm) protected with its corresponding guard column. Total leachable As was determined in the extracts.

Arsenic species were quantified by external calibration with standards from As(III), As(V), DMA, MA, AB and AC. TMAP and TETRA were identified by comparison with retention time in the certified reference material DORM-2 and quantified with the calibration curve of AB purchased from High Purity Standards and Fisher Scientific

An extract of the brown alga *Fucus serratus*, known to contain arsenosugars with glycerol (GLY-sug), phosphate (PO_4-sug), sulfonate (SO_3-sug), and sulfate (SO_4-sug) moieties (Madsen et al., 2000), was provided by Dr. Kevin Francesconi of Karl-Franzens University, Graz, Austria, and was used for the identification of arsenosugars by comparison with the retention time. For the quantification we used the calibration curve of the nearest standard in the chromatogram. The elution order of these four arsenoriboses has never been reported for the eluant/column combination used for our anionic speciation analysis, so we assigned the identities of SO_3-sug, PO_4-sug and SO_4-sug based on the known relative concentrations in the extract. GLY-sug was determined by cation exchange chromatography with a similar method than the reported by Madsen et al. (2000).

2.3. Method quality control

2.3.1. Total arsenic analysis

Sample preparation (method) and analytical (instrument) quality control (QC) included the analysis of duplicate samples, spiked samples and certified reference standards. Total As analysis was assessed by using certified external standards NIST 1566b, NRCC DORM-2, NRCC TORT-1, and IAEA 392. These are oyster, fish, lobster, and algae tissue, respectively. A certified reference material (CRM-TMDW, a reference drinking water containing trace metals) was obtained from High-Purity Standards (Charleston, SC, USA), and used to evaluate instrument accuracy.

Instrumental reproducibility for TAs analysis averaged 2.0% relative standard deviation (% RSD), with a range of 1.0–3.8%. Method reproducibility for 10 duplicate samples, split in the field, averaged 8.4% RSD, and ranged from 0.1 to 26.8%, indicating some sample heterogeneity. However, method reproducibility, or the duplicate analysis for TAs for the control site *Clavularia*, measured 2.07 and 2.125, with a standard deviation of 0.08, and percent relative standard deviation of 3.7, with similar reproducibility for *Halimeda* and *Polycarpa*. The instrument detection limit for TAs was estimated to be approximately 0.017 μg l^{-1}, calculated as three times the standard deviation of deionized water blanks (background signal). Thus, the method detection limit for tissue samples, following dilution to normalize for C content, was 0.036 mg kg^{-1}. TAs concentrations in all samples were well above this detection limit. The recovery for blank and matrix spikes ranged from 102.9 to 104.2%, with an analytical RSD of 1.4%. TAs in certified reference materials showed recovery of 95.2% for DORM-2 (17.1 ± 0.15 mg kg^{-1}; $n = 6$), 91.6% for TORT-2 (19.8 ± 0.3 mg kg^{-1}; $n = 4$), 87.1% for NIST 1566b (6.7 ± 0.1 mg kg^{-1}; $n = 4$), and 107.4% for IAEA 392 (0.19 ± 0.02 mg kg^{-1}; $n = 4$). Thus, for our TAs analysis, the uncertainty intervals of the measured and certified concentrations overlapped for three out of four certified reference materials; only for NIST 1566b (oyster tissue), the measured concentration was slightly below the certified range (87% recovery). This suggests that accuracy of the measured As concentrations in tissues was acceptable.

Spiking experiments were carried out using As(III), As(V), DMA, MA, AC and AB purchased from High Purity Standards and Fisher Scientific. Spikes were also performed with the AR extract to assess/discount co-elution. Spikes, blanks and selected samples were prepared in duplicate to assess analytical reproducibility and sample homogeneity. These duplicates included two samples from Ambitle Island, and the standard DORM-2. Method (preparation) and instrument blanks were prepared and analyzed for As abundance and As speciation analyses, and all sample results were then blank corrected. Accuracy was checked with the analysis of DORM-2, certified for AB (16.4 ± 1.1 mg kg^{-1}) and TETRA (0.248 ± 0.054 mg kg^{-1}). Good agreement between the certified and the obtained value (AB: 15.8 ± 0.4 mg kg^{-1}; TETRA: 0.234 ± 0.024 mg kg^{-1}) was reported.

We estimated our instrument detection limit (eIDL) as three times the standard deviation of the baseline noise in the elution window of each As species, and obtained 0.02 μg l^{-1} for As(V), and 0.01 μg l^{-1} for all other species. Thus, on a dry weight basis, the detection limit for As species detected in *Clavularia* was 0.01 mg kg^{-1} for As(V), and 0.005 mg kg^{-1} for all other species. The detection limit for extracted As species in *Halimeda* ranged from 0.005 to 0.01 mg kg^{-1} in most samples, and from 0.041 to 0.082 mg kg^{-1} in one sample where there was only a small amount of tissue available. Extracted As species in *Polycarpa* had detection limits ranging from 0.005 to 0.032 mg kg^{-1}. Data points below these calculated detection limits are not reported, even though in some cases a peak was clearly visible.

3. Results and discussion

The bioaccumulation of As is very likely enhanced in areas where it is enriched, such as marine hydrothermal vents. Marine shallow-water hydrothermal vents are often shown to contain higher concentrations of As relative to their deep sea counterparts (e.g., Douville et al., 1999; Price et al., 2013-this issue). An organism living in these environments could take in As as As(III) or As(V) from surrounding waters, and As(V) from sediments, and metabolize it to organoarsenic species which will then be accumulated in their tissues (Price and Pichler, 2005).

The present study analyzes organisms living in a shallow water hydrothermal system in an effort to better understand the potential toxicity, biogeochemical cycle, bioaccumulation and biotransformation patterns in a coastal marine organisms affected by As-rich hydrothermal discharge.

3.1. Total As concentration (TAs) in PNG samples

Total As concentrations in *Clavularia* tissue samples from the hydrothermally-influenced sites ranged from 2.9 to 20.9 mg kg^{-1} dry weight, compared to 2.1 in the control site sample (Table 1), and concentrations were generally higher in samples collected closer

Table 1
Total arsenic concentration (TAs) vs. distance for samples investigated in this study.

Distance	*Clavularia*	*Halimeda*	*Polycarpa*
Meters	mg kg^{-1}	mg kg^{-1}	mg kg^{-1}
0	20.4	8.5	5.2
30	2.9	19.0	6.8
60	13.7	20.2	5.5
120	20.9	13.3	4.2
140	7.0	14.7	6.9
180	*n.a.	11.6	n.a.
240	4.8	n.a.	5.3
300	4.2	n.a.	4.4
EF range	1.4–9.8	10.6–25.4	2.0–3.2
Control	2.1	0.8	2.2

* n.a. = not analyzed.

to focused hydrothermal venting. This indicates that enrichment of up to 10 times above background concentrations may occur in *Clavularia* tissue samples as a result of bioaccumulation of hydrothermally-derived As. Variability in total As concentrations is possibly a combination of several factors, including specimen age (increased time to accumulate more As), and sampling of some organisms with varying degree of direct contact with the hydrothermal plumes. The average TAs for samples from the hydrothermal transect is 10.6, thus giving an enrichment factor of ~5.0, on average, with a range of enrichment factors from 1.4 to 9.8. Differences between TAs concentrations in *Clavularia* in the hydrothermal system and at the control site are well above analytical uncertainty, demonstrating that *Clavularia* soft corals take up and store higher concentrations of As from the hydrothermal vent emissions compared to unexposed *Clavularia*.

Unfortunately, *Clavularia* has not been analyzed for TAs or As speciation by other researchers, making data comparison with the literature impossible. The studies mentioned previously had only focused on hard corals.

Total As concentrations in the *Halimeda* collected along the hydrothermal transect ranged from 8.5 to 20.2 mg kg^{-1} dry weight (Table 1). All samples collected from the hydrothermal area contained elevated concentrations of TAs compared to the one collected at the control site (0.8 mg kg^{-1}). Enrichment, and thus bioaccumulation, of As over the control site samples ranged from 10.6 to as high as 25.4 times. The average TAs concentration measured in *Halimeda* collected along the hydrothermal transect was 14.5, giving an average enrichment factor of 18.4, over the concentration of TAs in *Halimeda* collected from the control site. Thus, it is clear that significant bioaccumulation is occurring in *Halimeda* from the hydrothermal environment. *Halimeda* tissue samples were collected from sediments along the transect, but approaching hydrothermal venting, it was necessary to collect the samples from slightly above the sediment/water interface. This is due to the fact that *Halimeda* were not growing in direct contact with hot, acidic hydrothermal fluids. This could account for the lower TAs concentration for the 0 m sample. As mentioned for *Clavularia*, the age of each *Halimeda* specimen may also play an important role and for the TAs accumulated by the organism, but this relationship has not yet been investigated.

No studies of As total concentrations in calcareous algae were previously reported. However, several investigations report TAs for edible types of brown or green *Fucas* (seaweed) species (i.e., non-calcareous algae), and concentrations in these macroalgae have a very large range (e.g., 0.1 to 382 mg kg^{-1} dry weight (e.g., see references (Maher, 1983), (Maher and Butler, 1988), and (Neff, 1997)). Khokiattiwong et al. (2009) reported 26 mg kg^{-1} dry weight for a brown alga (*Padina* sp.), which was collected from the site located near a tin smelter. Investigations of calcareous algae should be given more attention since these organisms contribute significant amounts of carbonate mud to surrounding sediments (Drew, 1983), and could be a source of As in carbonate aquifers.

Arsenic abundance in the tunicate *Polycarpa* collected along the roped transect ranged from 4.2 to 6.9 mg kg^{-1} dry weight (Table 1). There is no clear increasing trend in TAs for samples collected closer to focused hydrothermal venting although higher concentration compared with the control were observed in most of the samples. The average concentration for samples collected along the hydrothermal transect is 5.8 compared to 2.2 mg kg^{-1} in the control site sample. This calculates to an average enrichment factor of 2.7, with a range from 2.0 to 3.2. Thus, As may be enriched slightly in tunicates surrounding hydrothermal venting, but seemingly not as much as in the other 2 organisms. *Polycarpa* only grow on the reef and on coral mounds above the sediment/water interface; thus the opportunity for enhanced bioaccumulation as a result of direct contact with hydrothermal fluids discharging through sediments is less.

Sea squirts are the only organisms in this study for which published As abundance data actually exists. For example, Shiomi et al. (1983) published an average As concentration of 25 mg kg^{-1} for *Halocynthia roretzi*, and Shinagawa et al. (1983) reported total As abundance of 5.0 mg kg^{-1} for the ascidian *Halocynthia roretzi*. These are of course a different genus and species of tunicate with drastically different As exposures, and thus comparison is not ideal. These investigations were for normal, unimpacted environmens, and no reports were found describing As uptake in tunicates living in environmentally impacted environments.

3.2. Arsenic speciation analysis

Table 2 presents concentrations of As species extracted from each organism in this study, while Table 3 is an evaluation of the anionic and cationic As speciation data, and presents extraction efficiency (%) and column recovery (%), arranged by distance from focused hydrothermal venting. Column recovery calculation is a good practice in speciation studies for mass balance purposes and they are calculated as the ratio between the sum of species eluted from the chromatographic column and the total arsenic in the injected extract. Extraction efficiency is calculated as the ratio between the extracted and the total arsenic.

For *Clavularia*, column recovery ranged from 72.4 to 111.2% (Table 3), indicating that most of the extractable As was present in the form of simple small molecular weight compounds. Very good analytical spike recoveries for As(III), As(V), MA and DMA (102.9 to 104.2%, with a % RSD of 1.4) also suggest that there were no major matrix compounds extracted that bind As species. Arsenic extraction efficiency for *Clavularia* ranged from 13.3 to 76.9% for samples from the hydrothermal vent site, and was only 12.3% for the control site (Table 3). Since matrix spikes of As(III), As(V), MA and DMA were recovered satisfactorily through the extraction procedure, this indicates that *Clavularia* contains much of its As as non-extractable forms, particularly at the control site and from the highest concentration samples from the hydrothermal site. Although total extraction of all species is the objective of speciation analysis, this is frequently not accomplished (Francesconi, 2003; Francesconi and Kuehnelt, 2004), because it is difficult to find an extraction fluid in which species of very different polarities (e.g., water- vs. lipid-soluble species) are equally soluble. The wide range of extraction efficiencies observed cannot be explained at this time, but may be a function of specimen age, or the organism's location relative to focused and diffuse hydrothermal venting. Additionally, very unpolar arsenicals are poorly extracted by methanol (Francesconi, 2003), so such As species (including lipid-bound As) may represent the “non-extractable As” in the studied tissues, and the relative abundance of these unpolar arsenicals may vary as a function of uptake in the samples near hydrothermal activity (although this logic does not hold for the control site sample).

The column recovery for *Halimeda* was comparable to that for *Clavularia*, ranging from 82.1 to 114.2% for hydrothermal samples, and

Table 2
Concentations of extractable arsenic species expressed as mg kg, vs. distance for samples investigated in this study.

		Anions								Cations						
	Distance	4.1	4.7	5.5	6.5	7.7	8.5	9.2	14.5	16.1	3.2	4.1	5.1	5.5	6.3	7.2
Clavularia	Meters	DMA	PO4-sug	SO3-sug	SO4-sug	UNK 1	UNK 2	MA	UNK 3	As(V)	AB	GLY-sug	UNK 4	TMAP	AC	TETRA
	0	0.30	0.06	0.01	0.76	0.17	0.03	0.02	<d.l.	0.91	0.65	0.50	<d.l.	0.06	<d.l.	<d.l.
	30	0.16	0.10	0.01	0.30	0.09	0.01	0.02	<d.l.	0.21	0.88	0.12	<d.l.	0.05	<d.l.	0.04
	60	0.24	0.15	0.01	0.52	0.09	0.03	0.01	<d.l.	0.32	0.48	0.26	<d.l.	0.06	0.12	<d.l.
	120	0.21	0.10	0.01	0.17	0.06	0.02	0.02	<d.l.	1.00	1.14	0.19	<d.l.	0.05	0.05	0.06
	140	0.30	0.08	<d.l.	0.20	0.10	0.01	0.02	<d.l.	0.09	1.00	0.20	0.04	0.06	<d.l.	<d.l.
	240	0.26	0.06	<d.l.	0.47	0.08	0.02	0.02	<d.l.	<d.l.	0.68	0.14	<d.l.	0.03	0.02	0.04
	300	0.19	0.06	<d.l.	0.22	0.06	0.01	0.01	<d.l.	0.11	1.53	0.17	<d.l.	0.10	0.09	<d.l.
	Control	0.02	<d.l.	<d.l.	<d.l.	<d.l.	<d.l.	0.01	<d.l.	0.07	0.09	<d.l.	<d.l.	<d.l.	<d.l.	<d.l.
Halimeda	0	0.08	0.02	<d.l.	<d.l.	0.02	<d.l.	<d.l.	3.05	0.02	<d.l.	<d.l.	0.75	<d.l.	0.18	<d.l.
	30*	0.21	<d.l.	<d.l.	<d.l.	0.07	<d.l.	0.06	<d.l.	0.12	n.a.*	n.a.	n.a.	n.a.	n.a.	n.a.
	60	0.19	0.03	<d.l.	<d.l.	0.08	<d.l.	0.04	<d.l.	0.09	0.39	0.34	11.85	<d.l.	<d.l.	0.42
	120	0.16	0.02	<d.l.	<d.l.	0.08	<d.l.	0.03	<d.l.	0.09	0.11	0.11	8.64	<d.l.	<d.l.	0.11
	140*	0.21	0.10	<d.l.	<d.l.	0.04	<d.l.	0.14	<d.l.	0.05	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
	180*	0.16	0.14	<d.l.	<d.l.	0.07	<d.l.	0.06	<d.l.	<d.l.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
	300	0.08	0.02	<d.l.	<d.l.	0.02	<d.l.	0.01	0.05	<d.l.	0.17	<d.l.	1.40	<d.l.	<d.l.	0.30
	Control	0.01	<d.l.	<d.l.	<d.l.	<d.l.	<d.l.	<d.l.	<d.l.	0.02	0.03	<d.l.	<d.l.	<d.l.	<d.l.	<d.l.
Polycarpa	0*	0.41	<d.l.	<d.l.	0.07	0.06	<d.l.	<d.l.	<d.l.	<d.l.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
	30*	0.31	<d.l.	<d.l.	0.07	0.05	<d.l.	<d.l.	<d.l.	<d.l.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
	60*	0.51	0.15	<d.l.	0.08	0.07	<d.l.	<d.l.	<d.l.	<d.l.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
	120	0.27	0.09	<d.l.	0.07	0.03	<d.l.	<d.l.	<d.l.	<d.l.	2.36	0.76	<d.l.	0.25	0.14	0.14
	140*	0.28	0.24	<d.l.	0.05	<d.l.	0.02	0.04	<d.l.	<d.l.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
	240	0.24	<d.l.	<d.l.	0.09	0.04	<d.l.	0.04	0.04	<d.l.	2.89	0.60	<d.l.	0.48	0.34	0.31
	300	0.24	0.16	<d.l.	0.11	0.04	<d.l.	<d.l.	<d.l.	<d.l.	2.37	0.37	<d.l.	0.20	0.10	0.12
	Control	0.07	<d.l.	<d.l.	0.06	0.01	<d.l.	<d.l.	<d.l.	<d.l.	0.61	0.10	<d.l.	0.03	0.04	0.04

*n.a. = not analyzed for cations; <d.l. = below detection limit.

was 36.8% for the control site sample, again confirming the absence of major extractable non-chromatographable As species (Table 3). Extraction efficiency was generally higher for *Halimeda* than for *Clavularia*, and was again better in hydrothermal transect samples (39.1–75.2%) compared to the control site (21.5%). This suggests that *Halimeda* also converts As into non-extractable species, and that this capability decreases at high As exposure. Column recovery for *Polycarpa* ranged from 72.6 to 96.7%. Extraction efficiency was higher than for the other two organisms and ranged from 61.1 to 116.5%. The higher extraction efficiencies obtained could be due to the fact that *Polycarpa* contains mainly AB, which is easily extracted by MeOH/water mixtures.

Table 3
Extraction efficiency (%) and column recovery (%) for cation and anion speciation in the studied samples arranged by distance from focused hydrothermal venting.

	Distance	Extraction efficiency	Column recovery
	Meters	%	%
Clavularia	0	24.4	69.9
	30	76.9	88.9
	60	18.9	88.3
	120	13.3	111.2
	140	36.1	82.9
	240	41.6	90.7
	300	61.3	99.8
	Control	12.3	72.4
Halimeda	0	59.4	82.1
	30	71.9	n.a.
	60	65.7	101.3
	120	73.7	95.4
	140	39.1	n.a.
	180	75.2	n.a.
	300	n.a.	114.2
	Control	21.5	36.8
Polycarpa	0	86.7	n.a.
	30	86	n.a.
	60	102.2	n.a.
	120	112.9	86.3
	140	84.2	n.a.
	240	116.5	82
	300	86.7	96.7
	Control	61.1	72.6

*n.a. = not available.

The major As species in *Clavularia* tissue, which together – on average – constituted approximately 90% of the sum of species, were DMA (average = 10%), SO_4-sug (15.6%), an unknown anionic As species eluting at 7.7 min with the anions (UNK 1) (3.8%), As(V) (15.2%), AB (38%) and GLY-sug (8.8%). Average concentrations for each of these species were 0.24, 0.38, 0.09, 0.44, 0.91, and 0.23 mg kg^{-1}, respectively (Table 2). Thus, AB is the most abundant organoarsenic species present in *Clavularia*, followed by SO_4-sug and As(V). Most importantly, transect samples for the most abundant species, as well as most of the other minor species, were consistently much higher compared to the control site samples (Table 2). For example, their maximum enrichment factors between the hydrothermal transect samples and the control site are 15.0 (DMA), 14.3 (As(V)), and 12.7 (AB), while GLY-sug, SO_4-sug and UNK species 1 were not present at all in the control site samples. Some other minor As species only occurred in transect samples, but not in the control (Table 2). Although the concentrations of the extracted species in the *Clavularia* from the control site sample were very low, the similarities in relative abundances (i.e., %) for most of the species compared to the transect samples suggests that the organism may be detoxifying As by a similar biosynthesis pathway in both the high As hydrothermal environment and the control. Furthermore, SO_4-sug, UNK 2, and other minor species only found in transect samples, may be intermediates in the 'normal' biosynthesis pathway for *Clavularia*, but are below detection limit in the control sample for our particular method. Alternatively, these species could specifically be formed in response to stress by high As exposure, and thus are not present in the control.

No As speciation data have, to our knowledge, been published for *Clavularia*, although it, like most other marine organisms, contains AB as the dominant species present in most marine organisms (Francesconi and Edmonds, 1998). The only As speciation data published for any coral to date to our knowledge is from Khokiattiwong

et al. (2009). The major As species in the two hard corals from the pristine reef was by far AB, constituting 79% of the sum of species. AB was also the dominant species in the impacted site described in the paper (28%), but this sample had much less extraction efficiency (40% vs. 93%). Overall, the coral from the impacted site also contained additional species not present in the sample from the pristine site, and the As species which were present in both samples, other than AB, were generally elevated in the impacted site. Extraction efficiency in our coral samples showed also a wide range (12.3–76.9%, Table 3), which suggests that even within the same species extraction efficiencies can be problematic for interpreting speciation patterns. For example, the organisms living within the hydrothermally impacted site may store different As species with different extraction efficiencies, relative to control site samples. Improving the extraction efficiency to closer to 100% would allow a more accurate description of As biosynthesis pathways in these organisms (Francesconi and Kuehnelt, 2004).

AB is considered to be the final metabolic product for most marine organisms. Thus, much higher concentrations of this species in organisms from environments with elevated As concentrations should occur. Arsenoribosides are technically DMA conjugates, and may be formed in marine organisms as byproducts of the methylation process, possibly as a precursor for AB (Maher and Butler, 1988). However, it is very likely that the arsenoribosides present in *Clavularia* are being produced by their symbiotic algae (zooxanthellae), while the remaining organoarsenicals are being produced within the cell tissue of *Clavularia* itself. The differences in AR for coral samples in the study by Khokiattiwong et al. (2009) are also explained by the presence of symbiotic zooxanthellae. It would be very intriguing to use the separation methods outlined in Reichelt-Brushett and McOrist (2003) and measure the As species in each of these different parts of the coral, since alga (zooxanthellae) often contain different species (typically arsenosugars) compared to other organisms (typically AB).

Halimeda samples collected from the hydrothermal transect contained several major extractable As species. The most abundant of these were an unknown species (UNK 3) found at 14.5 min retention time in the anion exchange separation present as the major species in the sample collected closest to the hydrothermal vent) and a second unknown cation (UNK 4) found at 5.1 min in the cation exchange column. This species was found in most of the samples collected in the hydrothermal transect and it elutes very close to As(III) in the anion exchange separation. Thus, this compound can behave both as a cation and an anion, depending on the elution conditions (pH ~11–12 for the anion, and pH ~5 for the cation), which allows us to speculate as to which As species it may be. For example, other authors have used similar cation exchange chromatographic conditions and in similar retention times found TMAO and/or a trimethylated sugar (Foster et al., 2006), but we did not have available any of these standards. The nature of UNK 4 was investigated by molecular mass spectrometry (LC-MS), but due to the low concentrations found in the extract and the low amount of extract available, the purification and accurate identification of this species could not be performed.

Other major species include GLY-sug, and TETRA (Table 2). In samples where both anion and cation analyses were performed, the major species present are DMA, the unknown species (UNK 4), AsV, AB, GLY-sug, and TETRA. On average, these species constituted approximately 98% of the sum of species. The UNK 4 species was relatively the most abundant species in the extract (~75% of the sum of species), and also occurred in high concentrations, around a maximum of 10 mg kg^{-1}, and each of these species were much elevated compared to the *Halimeda* control site sample (Table 2). It must be highlighted here that due to low sample amount cation analysis was only performed in some selected samples, so the presence of this unknown species was not confirmed in all the samples.

As(V) and As(III) are readily (bio)available in seawater for uptake by algae. Previous research has shown that As(V) does not accumulate in algae, but is rapidly detoxified by a process of methylation and alkylation, and the organism then accumulates the end products of this detoxification, namely arsenoriboses (Francesconi and Edmonds, 1998). Most macroalgae investigated to date contain predominantly the 4 major arsenosugars discussed in the introduction, although up to 15 have been detected, with minor amounts of AB and DMA (Francesconi and Edmonds, 1998; Madsen et al., 2000). There is, to our knowledge, no previous information on the uptake and metabolism of As by calcareous algae. Our *Halimeda* samples contain predominantly only the arsenoriboside GLY-sug, but the identity of the other major species remains unknown.

The most abundant extractable As species in *Polycarpa* was by far was AB (57–64%; 0.6 to 2.9 mgkg^{-1}), but also contained substantial amounts of DMA, GLY-sug, TMAP, AC, and TETRA. Again, as a typical marine organism, higher concentrations of AB are not unexpected. *Polycarpa* is the only organism in our study that is distinctly a filter feeder. Maher and Butler (1988) suggested that the primary As species encountered in higher trophic level organisms are those which are contained within their diet, so the lack of inorganic As species in *Polycarpa* indicates no direct As uptake from the water. AB, arsenoriboses and DMA are the typical main metabolites of As in marine organisms, and since the TAs concentrations in plankton collected over the vent site are as high as 240 mg kg^{-1} (Price et al., unpub. data), it is quite possible that *Polycarpa* takes up significant amounts of these As species from the plankton after they have been methylated (i.e., AB is synthesized not only internally, but also externally, and arsenoribosides may be synthesized externally by planktonic algae).

The only As speciation data available for comparison are for another species of sea squirt, *Halocynthia roretzi*, with the suggested presence of one acidic and two basic As compounds without further structural identification, but interestingly indicate the absence of arsenobetaine (Shiomi et al., 1983).

4. Summary and conclusions

Here we have shown that the major extractable As species in *Clavularia* and *Polycarpa* is AB. In *Clavularia*, As speciation can be explained by a combination of increased presence of intermediates as part of this organism's normal metabolism, along with contribution of arsenoribosides from its symbiotic zooxanthellae, or most compellingly a novel metabolic pathway due to increased As exposure on evolutionary time scales. Arsenic speciation patterns in *Polycarpa* suggests uptake of As via trophic transfer, containing neither As(III) nor As(V), but abundant organoarsenicals. *Halimeda* from the hydrothermal system contained very elevated concentrations of an unknown As species (max 11.7 mg kg^{-1}, ~80% of TAs, vs. non-detect in the control site sample). It is possible that the biosynthesis pathway for this organism is drastically altered and thus stores excess As in its tissues as this as yet uncharacterized species.

Although total extraction of all species is the objective of speciation analysis, this is frequently not accomplished (Francesconi, 2003; Francesconi and Kuehnelt, 2004), because it is difficult to find an extraction fluid in which species of very different polarities (e.g., water- vs. lipid-soluble species) are equally soluble. Improving the extraction efficiency to closer to 100% would allow a more accurate description of As biosynthesis pathways in marine organisms.

In conclusion, this investigation suggests that marine shallow-water hydrothermal vent systems, which are often enriched in As and other toxins, can be used as long-term natural laboratories to monitor geothermal As inputs into coastal ecosystems. As bioaccumulation is enhanced and biotransformation patterns may be significantly altered due to elevated As concentrations in these impacted sites. There is clear evidence of As bioaccumulation along a gradient of increasing As concentrations in a hydrothermal system. This is the first time TAs and As species were measured in the coral reef

organisms *Clavularia*, *Halimeda*, and *Polycarpa*, a soft coral, calcareous algae, and sea squirt, respectively.

As pointed out by Maher and Butler (1988) it is critical for us to understand the natural, coastal biogeochemical cycle of As and other contaminants, as it will allow us to better detect, predict, and evaluate changes arising from human activity. More investigations of this type are required in order to increase our understanding of biotransformation of toxins in these diverse ecosystems.

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