



## Arsenic occurrence and speciation in *Cyclope neritea*, a gastropod inhabiting the arsenic-rich marine shallow-water hydrothermal system off Milos Island, Greece

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### ABSTRACT

Around Milos Island marine shallow-water hydrothermal activity can be found and in Paleochori Bay, where high arsenic concentrations were reported for the hydrothermal fluids. There the gastropod *Cyclope neritea* was present at high densities despite the extreme conditions associated with hydrothermal venting, feeding on white microbial mats. Surprisingly, *C. neritea* collected from the hydrothermal vent area accumulated comparatively less arsenic in its tissues than specimens of the same species collected from an area not affected by hydrothermal activity. Inorganic arsenic was found at high percentages with respect to the sum of arsenic species both in the gut and muscle of *C. neritea* and the amount of arsenobetaine (AB) was less than in specimens collected at an area away from hydrothermal activity. Trimethylarsoniopropionate (TMAP) was found at relatively high percentages in *C. neritea* and in the surrounding environment (sediment and plankton) in Paleochori Bay. Tetramethylarsonium ion (TETRA) was found at high percentages in the gut and muscle of *C. neritea*. It was most likely biosynthesized in the gastropod gut, and accumulated in the muscle, because it was not found in any other compartment studied. Based on these observations we conclude that *C. neritea* is arsenic tolerant. The data suggests that arsenic species distribution is strongly influenced by arsenic species exposure and/or different detoxifying mechanisms, although the exact metabolic pathways could not be completely resolved.

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### 1. Introduction

Because hydrothermal vents in shallow water often contain high concentrations of arsenic (e.g. Pichler et al., 1999) they were used to investigate arsenic bioaccumulation and speciation in coastal marine organisms and ecosystems (Price and Pichler, 2005; Pichler et al., 2006; Price et al., 2013–this issue). Nevertheless, information about arsenic speciation remains limited. Only one other study exists about arsenic speciation in shrimps and mussels from a deep-sea hydrothermal vent system of the mid-Atlantic Ridge (Larsen et al., 1997) and one study for a shallow-water system (Price et al., 2013). Larsen et al. (1997) reported the presence of arsenosugars, which were dominantly found in photosynthetic organisms like algae. Thus, that finding suggested additional pathways for the biosynthesis of arsenosugars. The biosynthesis of arsenobetaine (AB), although reported for the first time more than 30 years ago (Edmonds and Francesconi, 1977), is not completely understood. It was postulated that arsenosugars are precursors of AB, and the finding of dimethylarsinylethanol, a possible intermediate, gives confidence to this hypothesis (Edmonds and Francesconi, 2003). However, it was also hypothesized that other biosynthetic pathways exist for AB,

because AB was found in marine organisms inhabiting environments where algae are not the dominant source (Khokiattiwong et al., 2009). Another pathway, which involves the arsenylation of 2-oxo acids was proposed and would explain also the presence of other compounds, like trimethylarsoniopropionate (TMAP) (Edmonds, 2000).

In Palaeochori Bay on the south side of the island of Milos (Greece) exists a marine shallow-water hydrothermal system in 1 to 20 m depth with abundant gas discharge surrounded by large areas of diffusively venting fluids (Dando et al., 1995a). High levels of arsenic were reported for the hydrothermal fluids in Paleochori (2955  $\mu\text{g L}^{-1}$ ) and nearby Spathi Bay (5850  $\mu\text{g L}^{-1}$ ) (Fig. 1). Arsenic speciation revealed dominantly arsenite (49%) and dithioarsenate (29%), followed by arsenate, monothioarsenate, and trithioarsenate (9, 9, and 2%, respectively). Despite the elevated concentrations of arsenic, elevated temperatures (as high as 80 °C), high salinities (~50‰), low pH (~5.2) and elevated concentrations of dissolved hydrogen sulfide (3.1  $\text{mmol L}^{-1}$ ) (Price et al., 2010; Price et al., 2013–this issue), a small (10 to 15 mm diameter) burrowing nassariid gastropod with a flattened shell, *Cyclope neritea*, can be found at very high densities in the hydrothermal area (Dando et al., 1995b; Southward et al., 1997). The gastropods were feeding on microbial mats though to be caused mainly by the sulfide oxidizing bacterium *Achromatium volutans* (Dando et al., 1995b). It is unclear however how *C. neritea* is able to tolerate these extreme environmental conditions and in particular the high arsenic levels. Thus, the objective of this work was to investigate

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arsenic occurrence and metabolism in this short food chain, and to obtain insight about the tolerance and/or detoxifying strategies of this gastropod.

## 2. Materials and methods

### 2.1. Reagents, standards and certified reference materials

All solutions were prepared with double deionized water obtained from a Millipore water purification system (MilliQ Advantage A10, 18 M $\Omega$  cm<sup>-1</sup>). Nitric acid and hydrochloric acid were obtained with a sub-boiling distillation system from Milestone. Methanol (HPLC grade) and Acetone were purchased from Sigma Aldrich. H<sub>2</sub>O<sub>2</sub> (30%, suprapur), formic acid (p.a.), (NH<sub>4</sub>)H<sub>2</sub>PO<sub>4</sub> (suprapur) and pyridine (p.a.) were purchased from Merck. H<sub>3</sub>PO<sub>4</sub> (85%, p.a.) was purchased from Riedel-deHaen.

Stock standard solutions were prepared as follows: arsenite (As(III)) from As<sub>2</sub>O<sub>3</sub> (Sigma Aldrich) dissolved in 4 g L<sup>-1</sup> NaOH (Merck). Arsenate (As(V)) from Na<sub>2</sub>HAsO<sub>4</sub> · 7H<sub>2</sub>O (Sigma-Aldrich) dissolved in water, methylarsonate (MA) prepared from (CH<sub>3</sub>)<sub>2</sub>AsO(ONa)<sub>2</sub> · 6H<sub>2</sub>O (Chemservice), dissolved in water. Dimethylarsinate (DMA) prepared from (CH<sub>3</sub>)<sub>2</sub>AsO(ONa)<sub>2</sub> · 6H<sub>2</sub>O (Merck) dissolved in water. AB and AC were purchased at Sigma Aldrich and Argus Chemicals respectively and dissolved in water. For total arsenic species measurement a CertiPur® (100 mg L<sup>-1</sup>) multi-element standard solution XVI containing arsenic (Merck) was used. All the stock solutions were kept at 4 °C and diluted solutions were prepared.

The following certified reference materials were used as quality control: DORM-2 (Dogfish Muscle Certified Reference Material for Trace Metals), supplied for NRCC (National Research Council Canada), had a certified total arsenic content of 18.0 ± 1.1 mg kg<sup>-1</sup>. This material was also certified for AB (16.4 ± 1.1 mg As kg<sup>-1</sup>) and tetramethylarsonium ion (TETRA) (0.248 ± 0.054 mg As kg<sup>-1</sup>); BCR-627 (Tuna Fish tissue) certified reference material, supplied by IRMM (Institute for Reference Materials and Measurements) had a certified total arsenic content of 4.8 ± 0.3 mg kg<sup>-1</sup>. It was also certified for AB (3.9 ± 0.2 mg As kg<sup>-1</sup>) and DMA (0.15 ± 0.02 mg As kg<sup>-1</sup>); GBW7310 (Stream sediment) certified reference material, supplied by NRC CRM (National Research Center for Certified Reference Materials), had a certified total arsenic content of 25 ± 3 mg kg<sup>-1</sup>.

An aliquot of freeze dried extract of *Fucus serratus* was kindly donated by Prof. K.A. Francesconi (Karl-Franzens University, Graz, Austria) (Madsen et al., 2000). This extract contained the four arsenosugars, specifically phosphate (PO<sub>4</sub>-sug), sulfate (SO<sub>4</sub>-sug), sulfonate (SO<sub>3</sub>-sug) and glycerol (Gly-sug) and was used to assign the arsenosugar peaks in the chromatograms.

### 2.2. Instruments

A digestion system (Milestone Ethos) with a microwave power of 1000 W and temperature control was used for digestion of gastropods and sediments. An Element 2 high resolution-inductive coupled plasma-mass spectrometer (HR-ICP-MS) (Thermo, Germany) with a microflow nebulizer was used to measure total arsenic content. To carry out arsenic speciation, the Element 2 was coupled to an Accela 600 pump (Thermo, Germany), equipped with an autosampler (HPLC-HR-ICP-MS). The chromatographic columns Hamilton PRP-X100 (250 × 4.1 mm, 10 μm) and Zorbax 300-SCX (250 × 4.1 mm, 5 μm) were protected with guard columns with the corresponding stationary phases. The chromatographic conditions are listed in Table 1 and were similar to Ruiz-Chancho et al. (2011). The outlet of the HPLC column was connected via PEEK capillary tubing to a “conikal” nebulizer of the Element 2. The ion intensity at *m/z* 75 (<sup>75</sup>As) was monitored at low resolution and the possible interference of argon chloride (<sup>40</sup>Ar<sup>35</sup>Cl) was checked in high resolution mode, where that interference is resolved from the <sup>75</sup>As peak.

**Table 1**  
Chromatographic conditions used in the present study.

	Anion exchange	Cation exchange
Column	Hamilton PRP-X100 (250 × 4.1 mm, 10 μm)	Zorbax 300-SCX (250 mm × 4.6 mm, 5 μm)
Mobile phase	20 mM NH <sub>4</sub> H <sub>2</sub> PO <sub>4</sub> pH = 5.8 (adjusted with aqueous ammonia)	20 mM pyridine pH = 2.6 (adjusted with formic acid)
Flow rate (mL min <sup>-1</sup> )	1.5	1.5
Injection volume (μL)	20	20
Arsenic species analyzed	Arsenite, arsenate, MA, DMA, PO <sub>4</sub> -sug, SO <sub>3</sub> -sug, SO <sub>4</sub> -sug	AB, AC, TETRA, TMAP, Gly-sug

### 2.3. Sampling and sample preparation

#### 2.3.1. Sampling

Sampling campaigns were performed in Paleochori Bay in September 2009 and August 2010 and in February 2010 in Ria do Alvor, in the south of Portugal. Approximately 1000 specimens from *C. neritea* were collected in Paleochori Bay by SCUBA diving in September 2009 (Fig. 1). This gastropod was found in Paleochori Bay at around 8 m depth in or adjacent to areas influenced by hydrothermal hypersaline discharges, where it constituted a large part of the biomass. It was crawling on the sediment surface where relatively thick layers of microbial mats occurred, apparently feeding on the mats. Although *C. neritea* usually emerges from the sediment only at night (Southward et al., 1997), in Paleochori Bay it was encountered in the daytime, an exception to its normal habit.

To obtain background information and food chain relationships, a surface sediment sample from the same area where the gastropods live, as well as sediment from an area not directly affected by hydrothermal discharge were collected.

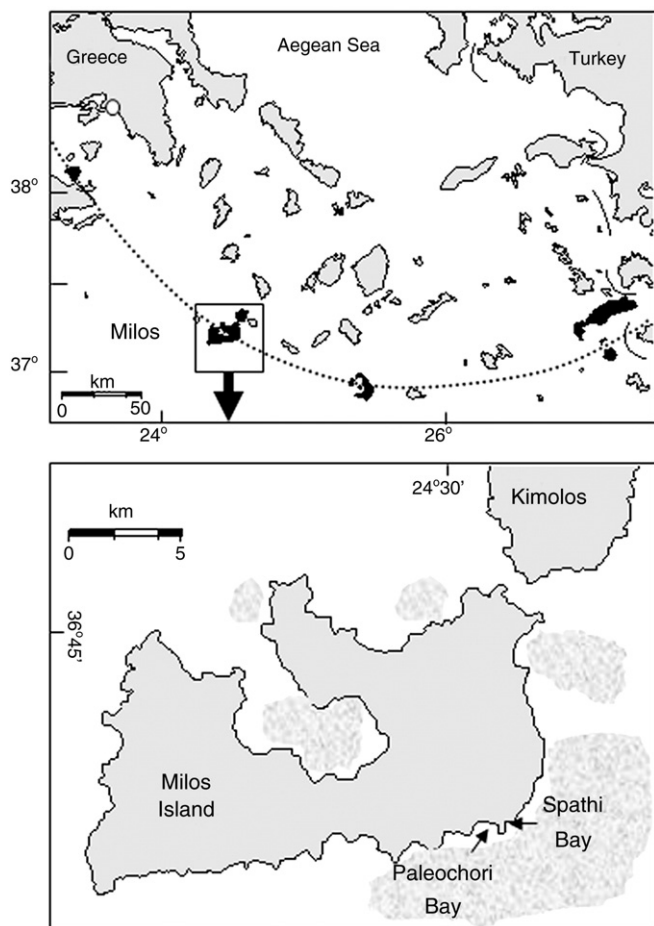
In August 2010, microbial mats and plankton were collected. Microbial mats were collected from the surface of the sediment with the help of a polypropylene 60 mL syringe. Plankton was collected into a plankton net from surface waters throughout Paleochori Bay.

To compare, *C. neritea* from an area not affected by hydrothermal activity, about 200 specimens were collected in Ria do Alvor, a small bay in the south of Portugal (37°07'29.70"N, 08°37'51.52"W) (Calado and Dinis, 2008). There the animals remained buried in the sand during the day and thus, chopped pieces of squid were impaled in small wooden sticks and placed in the substrate to bait the gastropods. Sediment samples were also collected in the same area where the gastropods were collected.

Gastropods and sediments were stored frozen and transported to the University of Bremen, Germany. Microbial mats and plankton were stored in 60 mL syringes, frozen and transported to the same destination.

#### 2.3.2. Sample preparation

Once the gastropods were at room temperature, they were thoroughly washed from sediment with double deionized water. The shells were cracked with a G-clamp, dissected under a binocular microscope, and the gut was separated from the muscle. It was observed that although the gastropods were from the same genus and species that the ones collected in Paleochori Bay were smaller (≈5 mm vs. 10 mm diameter) and also had a more fragile shell, a likely consequence of the surrounding environmental conditions. To have enough sample for arsenic total and speciation analyses, approximately 500 specimens from Milos and 200 from Portugal were processed and a mixed sample was prepared. The muscle and gut samples were freeze-dried, homogenized with mortar and pestle and stored at room temperature until analysis. Sediments were freeze-dried and stored at room temperature until analysis. Microbial mats and plankton, once at room temperature, were filtered through an ash-free filter and rinsed



**Fig. 1.** Map of Greece and the Aegean Sea (upper). The curved dashed line indicates the Hellenic Volcanic Arc. The black islands are islands where hydrothermal activity occurs. The bottom figure shows a map of Milos Island. The dotted areas indicate the presence of submarine hydrothermal activity.

with double deionized water. The clean samples were freeze-dried and stored at room temperature until analysis.

## 2.4. Procedures

### 2.4.1. Total arsenic analysis

For the measurement, Element 2 was used and the measurement was performed at high resolution mode using  $^{115}\text{In}$  as internal standard. Triplicate analyses were performed for each sample together with procedure blanks and certified reference materials.

**2.4.1.1. Digestion of gastropods.** 0.2 g aliquots of the samples (gut and muscle) and the certified reference materials (CRMs) (CRM627 and DORM-2) were weighed in the digestion vessels and 5 mL of concentrated nitric acid and 2 mL of hydrogen peroxide were added. Mixtures were digested according to the following program: 2 min from room temperature to 80 °C, maintained for 1 min at 80 °C, 2 min from 80 °C to 110 °C, 3 min from 110 °C to 140 °C, 4 min from 140 °C to 180 °C, 6 min from 180 °C to 190 °C, maintained for 12 min at 190 °C. After cooling to room temperature, the clear digested samples were transferred and diluted in water up to 50 mL. The analysis of CRMs (CRM627 and DORM-2) showed good agreement between the certified and the obtained values.

**2.4.1.2. Aqua regia digestion of the sediments.** 0.2 g aliquots of the sample and the CRM (GBW07310) were weighed in the digestion vessels and 4.5 mL concentrated hydrochloric acid and 1.5 mL concentrated

nitric acid were added. Mixtures were digested according to the following program: 2 min from room temperature to 80 °C, 1 min maintained at 80 °C, 2 min from 80 °C to 110 °C, 3 min from 110 °C to 140 °C, 4 min from 140 °C to 160 °C, 6 min from 160 °C to 180 °C, maintained for 12 min at 180 °C. As the same way, after cooling to room temperature, the resulting digestates were filtered through ash-free filters and diluted to 50 mL.

### 2.4.2. Arsenic speciation analysis

**2.4.2.1. Arsenic species extraction from gastropods.** For the extraction of arsenic species from gastropods, a two-step sequential extraction with acetone and MeOH/water was used similar to that used by Foster et al. (2006). The freeze-dried pulverized and homogenized samples (0.2 g for all samples and CRMs) were weighed in triplicate in 30 mL PTFE (poly(tetrafluoroethylene)) tubes. Ten milliliters of acetone were added to each tube. The tubes were placed in an end-over-end shaker operating at 20 rpm for 1 h at room temperature. The resulting mixtures were centrifuged at 4500 rpm for 10 min. The supernatants were transferred to a 50 mL polypropylene tube. The extraction procedure was repeated twice. After the second acetone extraction, the residue pellet was dried in a fume cabinet at room temperature for 24 h. The entire combined acetone supernatants were evaporated at room temperature to dryness. The residue was resuspended in 4 mL  $\text{HNO}_3$  and transferred to a microwave vessel. 3 mL of water were added and the mixture was digested according to the following program: 10 min from room temperature to 80 °C, 5 min from 80 °C to 110 °C, 15 min maintained at 110 °C. After cooling to room temperature, the clear digested extracts were diluted up to 15 mL.

0.1 g of the acetone-extracted pellet was weighted in a PTFE tube and 10 mL of MeOH/water (1:1, v/v) were added. Tubes were placed in an end-over-end shaker operating at 20 rpm for 16 h. The resulting mixtures were centrifuged at 4500 rpm for 10 min. The supernatants were filtered through a 0.22  $\mu\text{m}$  filter. The extracts were analyzed the same day by HPLC-HR-ICP-MS.

**2.4.2.2. Arsenic species extraction from microbial mats and plankton.** Due to reduced amount of sample (plankton: 140 mg; microbial mats; 70 mg), only the second step of the sequential extraction process was performed. Then, 50 mg of sample were weighed in PTFE tubes and extracted with 5 mL MeOH/water (1:1, v/v) the same way as for the gastropods.

**2.4.2.3. Arsenic species extraction from sediments.** For the extraction of arsenic species from sediments two processes were used. Sediments were extracted by using the sequential extraction process described above. Moreover, an extraction with 0.3 mol  $\text{L}^{-1}$   $\text{H}_3\text{PO}_4$  was carried out (Montperrus et al., 2002). For this, 0.2 g of sediment were weighed in a PTFE tube and 10 mL of the extractant were added. Tubes were placed in an end-over-end shaker and operating at 20 rpm for 1 h. The mixtures were centrifuged at 4500 rpm (10 min) and the supernatants were filtered through a 0.22  $\mu\text{m}$  filter. The extracts were analyzed within 24 h after the extraction to avoid arsenic species transformation.

**2.4.2.4. Analysis of arsenic species.** Total arsenic was determined in all the speciation extracts with HR-ICP-MS for mass balance calculations. MeOH/water (1:1, v/v) and  $\text{H}_3\text{PO}_4$  0.3 mol  $\text{L}^{-1}$  extracts were analyzed with HPLC-HR-ICP-MS by using two chromatographic separations (Table 1). Arsenic species in the extracts were identified by comparison of retention times with standards. External calibration curves were used to quantify arsenite, arsenate, MA, DMA, AB and AC with the corresponding standards. Identification of TMAP and TETRA was performed by comparison of retention time with DORM-2, which was analyzed in every batch together with the samples. Arsenosugars were identified by comparison of retention times with the arsenosugars present in the *F. serratus* extract (Madsen et al., 2000). TMAP, TETRA and the arsenosugars were quantified with the calibration curve of the

closest arsenic species in the chromatogram. This is justified because previous research has shown that in ICP-MS the response is independent of the species (Francesconi and Sperling, 2005). Moreover, the presence of thio-arsenic compounds was investigated in all the samples by the addition of hydrogen peroxide.

In the analysis of CRM627 and DORM-2 good agreement between the certified and the obtained value was obtained. CRM 627 is certified for AB and DMA, although in the present work also TMAP and TETRA were identified. DORM-2 is certified for AB and TETRA. Moreover, it was used for the identification of TETRA because a standard of this species was not available. Furthermore, although it is not certified for TMAP, it was used for the identification of this species, as there are in the literature several reports, which apply very similar chromatographic conditions and identify and quantify TMAP. An unknown species at a similar retention time as TMAP, and similar concentration ( $0.16 \pm 0.01 \text{ mg kg}^{-1}$ ), was reported by Goessler et al. (1998). TMAP was later quantified unequivocally in DORM-2 by Francesconi et al. (2000), and a concentration of  $0.17 \pm 0.05 \text{ mg kg}^{-1}$  was reported. Kirby and Maher (2002) also reported a concentration of  $0.17 \pm 0.01 \text{ mg kg}^{-1}$ . The results of the present study are in agreement with the reported values of the literature for this species.

### 3. Results and discussion

#### 3.1. Total arsenic content

The concentration of arsenic was determined in gastropods and sediments to obtain information about its accumulation and pathways in a hydrothermal system. When comparing total arsenic concentrations in the aqua regia extracts from Milos versus those from the Ria do Alvor sediments, a much higher concentration of arsenic was found in the Milos sediments (Table 2). In Milos, two sediment samples were compared; one from the area where the gastropods are feeding and another one in an area not directly affected by hydrothermal activity. The concentration of arsenic in these sediments was not significantly different ( $t$  test,  $\alpha = 0.05$ ). This suggests that arsenic can be accumulated in the sediments around the hydrothermal areas in the same range as in some of the areas with hydrothermal fluid

discharge and consequently, living organisms colonizing the area may be exposed to high arsenic concentrations through the sediment even far from the hydrothermal source. A more detailed study needs to be performed to obtain information about the extent of the influence of the high arsenic concentrations expelled by the hydrothermal system in Paleochori Bay.

In contrast to the sediments, higher amounts of arsenic were found in the gut and muscle of *C. neritea* collected in Ria do Alvor (Table 2), although the gastropods from Paleochori Bay were exposed to concentrations up to  $650 \mu\text{g L}^{-1}$  As (Price et al., 2010; Price et al., 2012) through the fluids. The gastropods collected as “control” in Portugal had similar amounts of arsenic in gut and muscle, while those from Milos had more arsenic in the gut. An explanation could be the accidental ingestion of sediment when feeding on the microbial mats (Southward et al., 1997). Thus, the relative differences observed in gut and muscle could be attributed to different feeding habits and/or different arsenic tolerance/metabolism.

There are no references in the literature about arsenic concentrations in *C. neritea* although data exists for other gastropods. Goessler et al. (1997) reported a concentration of  $74.4 \text{ mg As kg}^{-1}$  in a herbivorous gastropod and a concentration up to  $233 \text{ mg kg}^{-1}$  in a carnivorous gastropod. Francesconi et al. (1998) reported concentrations between 112 and  $339 \text{ mg kg}^{-1}$  in three species of gastropods. Another gastropod species, *Hemisfusius ternatus* can accumulate arsenic to high concentrations, exceeding  $100 \text{ mg kg}^{-1}$  (Phillips and Depledge, 1986). Gastropods from a tropical marine ecosystem contained values up to  $360 \text{ mg kg}^{-1}$  (Khokiattiwong et al., 2009). The highest concentration (up to  $1360 \text{ mg kg}^{-1}$ ) was reported in whelks collected in Fogo Island (Newfoundland) (Lai et al., 2002). Surprisingly, when compared to other gastropods species, *C. neritea* accumulated relatively little arsenic. The uptake and metabolism of arsenic, however, can be directly related to the species of arsenic, which is dominant in a given environment. Thus, it is necessary then to speciate arsenic in both the sediments and gastropods from the hydrothermal and non-hydrothermal environments.

Total arsenic could not be determined in the microbial mats and plankton due to the limited amount of sample material. Since both may play an important role in the cycling of arsenic, those were only used for speciation analysis. An order of the concentration of

**Table 2**

Total arsenic concentrations ( $n = 3$ ), acetone extraction percentages, MeOH/water extraction efficiencies and column recoveries for samples and CRMs.

		Total As	Acetone Extraction efficiency	MeOH/water Extraction efficiency	Column recovery
		$\text{mg kg}^{-1}$	%	%	%
Milos	Gastropods gut	$24.2 \pm 0.4$	4.53	37.5	114.3
	Gastropods muscle	$15.2 \pm 0.6$	1.8	62.3	105.8
	Sediment	$32.6 \pm 1.4$	1.6	2.1	129.7
	Sediment $\text{H}_3\text{PO}_4^a$	–	–	3.7*	93.9
	Sediment control	$33.5 \pm 1.3$	0.78	0.79	83.6
	Microbial mats 1	n.a.	n.a.	n.a.	63.2
	Microbial mats 2	n.a.	n.a.	n.a.	67.3
	Microbial mats 3	n.a.	n.a.	n.a.	87.7
	Plankton	n.a.	n.a.	n.a.	80.0
	Ria do Alvor	Gastropods gut	$41.3 \pm 2.5$	0.92	67.7
Gastropods muscle		$38.8 \pm 0.3$	1.3	82.6	100.5
Sediment		$6.5 \pm 0.5$	0.2	0.25	115.2
Sediment $\text{H}_3\text{PO}_4^a$		–	–	35.2*	91.3
CRMs	CRM627	$4.20 \pm 0.03$	3.4	92.1	100.6
	CRM627 certified	$4.8 \pm 0.3$	–	–	–
	DORM-2	$17.0 \pm 0.7$	2.1	95.3	98.6
	DORM-2 certified	$18.0 \pm 1.1$	–	–	–
	GBW7310	$23.2 \pm 0.8$	–	–	–
	GBW7310 certified	$25 \pm 3$	–	–	–

<sup>a</sup> Extracted with  $\text{H}_3\text{PO}_4$   $0.3 \text{ mol L}^{-1}$ ; n.a.: not available.

total arsenic can be obtained by adding the individual species of a given sample.

### 3.2. Arsenic species extraction

Arsenic species were extracted in two steps to obtain information about the lipidic and the polar fraction in the different compartments of the Milos hydrothermal system. Only a small fraction was extracted with acetone (Table 2), which agrees with data from the literature (Kirby and Maher, 2002; Foster et al., 2005, 2008). The highest extraction efficiencies were for the Milos gastropods gut, reaching almost 5% of the total arsenic. Moreover, higher percentages compared to the Ria do Alvor sediment were extracted in all sediments from Milos.

Much higher extraction efficiencies were obtained with the MeOH/water extraction for gastropods from both sites. However, when directly compared, efficiencies were higher for samples from the control area (Ria do Alvor). In general, extraction efficiency was higher in muscle than in gut. The lowest extraction efficiency was obtained in the gut of the gastropods from Milos, most probably due to the presence of sediments which were accidentally ingested.

The extraction efficiency for the sediments was very low (Table 2), which indicated that arsenic was strongly bound to the matrix.

### 3.3. Column recoveries and quality assessment

In speciation analysis, a good quality control procedure and a good indicator for the presence of unknown species are mass balance calculations, like column recovery, which is the ratio between the quantified species and the total injected arsenic. In general, column recovery was close to 100% (Table 2), which indicated that all the major species, which were extracted, were identified. Relatively low column recovery was obtained for the extraction of the microbial mats, which could be an indication for the presence of one or more unknown species. The unknowns could be one or more thio-arsenic species. It is known that thio-arsenicals present lower polarity compared with other organoarsenicals (Raml et al., 2006), and it could be that they were extracted in the first step with acetone or transformed to their oxo-analogues during the extraction. This hypothesis was discarded because Foster et al. (2006) who used a very similar extraction process could identify thio-arsenic species in the MeOH/water fraction after an acetone extraction step. The presence of

thio-arsenic compounds, however, was expected because Price et al. (2010) reported the presence of thio-arsenates in the hydrothermal fluids. An explanation for the absence of thio species could be that they are not absorbed or metabolized by *C. neritea*. In fact, it was reported that *C. neritea* has a high tolerance to sulfide, because it avoids uptake by absorption (Southward et al., 1997). Another explanation is that arsenosugars were not present in the extracts and so far the most common thio-arsenic compounds found in gastropods were thio-arsenosugars (Foster et al., 2006). Actually it is not clear which is the role of the thio-arsenic species in the arsenic metabolism in the marine environment and more studies need to be carried out (Schmeisser et al., 2004).

Instrumental detection limits (DLs), calculated as three times the standard deviation of the base line noise and divided by the slope of the calibration curve, were in the low  $\text{ng L}^{-1}$  level, ranging from 8 to  $155 \text{ ng L}^{-1}$  arsenic, depending on the species. Instrumental quantification limits (QLs) were calculated in the same way but considering ten times the standard deviation and ranged from 28 to  $520 \text{ ng L}^{-1}$  arsenic.

### 3.4. Arsenic species occurrence and metabolism

Arsenic species determination provides information about transformation and accumulation of arsenic in the different compartments of this short food chain in an extreme environment. To our knowledge there were no other studies dealing with arsenic speciation in *C. neritea*. The obtained results are shown in Tables 3 and 4. Additionally, the species distribution in gastropods, microbial mats, plankton and sediments collected both in Paleochori Bay and Ria do Alvor are shown in Fig. 2.

The major arsenic species in the gastropods from Ria do Alvor was AB (Fig. 2(B)). Other authors have identified AB as the major arsenic species in other gastropods (Goessler et al., 1997; Lai et al., 2002; Foster et al., 2005). Additionally, gastropods from Ria do Alvor presented small amounts of other organoarsenic species, like DMA, AC, TMAP, TETRA or Gly-sug. These arsenic species were also reported at low percentages in other marine organisms (Edmonds and Francesconi, 2003). In contrast, the dominant species in the Milos gastropods was inorganic arsenic, which is typically found at low percentages in most marine organisms (Schoof et al., 1999). In Milos, inorganic arsenic was found not only at high percentages in *C. neritea*, but also in microbial mats and plankton. In *C. neritea* gut and muscle, arsenite was the major inorganic species, while microbial mats had higher percentages of arsenate and in

**Table 3**  
Arsenic species concentration ( $n = 3$ , except for microbial mats ( $n = 1$ ) and plankton ( $n = 2$ )) with respect to the sum of species, obtained after anion exchange chromatography for samples and selected CRMs.

		Anions								
		As(III)	DMA	MA	As(V)	PO <sub>4</sub> -sug	UNK1 (Rt 5.1 <sup>b</sup> )	UNK2 (Rt 5.5)	UNK3 (Rt 10.8)	UNK4 (Rt 18.4)
		$\text{mg kg}^{-1}$	$\text{mg kg}^{-1}$	$\text{mg kg}^{-1}$	$\text{mg kg}^{-1}$	$\text{mg kg}^{-1}$	$\text{mg kg}^{-1}$	$\text{mg kg}^{-1}$	$\text{mg kg}^{-1}$	$\text{mg kg}^{-1}$
Milos	Gastropods gut	$3.2 \pm 0.8$	$0.17 \pm 0.01$	$0.13 \pm 0.03$	$2.2 \pm 0.2$	<DL	<DL	<DL	$0.48 \pm 0.06$	<DL
	Gastropods tissue	$2.9 \pm 0.5$	<DL	<DL	$0.17 \pm 0.03$	<DL	<DL	<DL	$0.10 \pm 0.01$	$0.53 \pm 0.12$
	Sediment	$0.64 \pm 0.07$	<DL	<DL	$0.100 \pm 0.007$	<DL	<DL	<DL	<DL	<DL
	Sediment H <sub>3</sub> PO <sub>4</sub> <sup>a</sup>	$0.56 \pm 0.05$	<DL	<DL	$0.36 \pm 0.06$	<DL	<DL	<DL	<DL	<DL
	Sediment control	$0.11 \pm 0.02$	<DL	$0.006 \pm 0.001$	$0.10 \pm 0.01$	<DL	<DL	<DL	<DL	<DL
	Microbial mat 1	0.41	0.008	0.084	1.74	<DL	<DL	0.252	<DL	<DL
	Microbial mat 2	0.14	0.006	0.035	0.64	<DL	<DL	0.150	<DL	<DL
	Microbial mat 3	1.34	0.010	0.102	6.58	<DL	<DL	0.331	<DL	<DL
	Plankton	<DL	$0.075 \pm 0.006$	<DL	$3.07 \pm 0.07$	$0.038 \pm 0.005$	<DL	<DL	<DL	<DL
	R. Alvor	Gastropods gut	<DL	$0.12 \pm 0.02$	<DL	<DL	<DL	$0.44 \pm 0.09$	<DL	<DL
	Gastropods tissue	<DL	<DL	<DL	<DL	<DL	$0.36 \pm 0.09$	<DL	<DL	$0.49 \pm 0.08$
	Sediment	<DL	<DL	<DL	<DL	<DL	<DL	<DL	<DL	<DL
	Sediment H <sub>3</sub> PO <sub>4</sub> <sup>a</sup>	$0.048 \pm 0.008$	<DL	<DL	$2.0 \pm 0.2$	<DL	<DL	<DL	<DL	<DL
CRMs	CRM627	<DL	$0.17 \pm 0.03$	<DL	<DL	<DL	<DL	<DL	<DL	<DL
	CRM627 certified	n.a.	$0.15 \pm 0.02$	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
	DORM-2	<DL	$0.36 \pm 0.06$	<DL	<DL	<DL	<DL	<DL	<DL	<DL
	DORM-2 certified	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.

<sup>a</sup> Extracted with H<sub>3</sub>PO<sub>4</sub> 0.3 mol L<sup>-1</sup>; <DL: below detection limit.

<sup>b</sup> Retention time; n.a.: not available.

**Table 4**

Arsenic species concentration (n = 3, except for microbial mats (n = 1) and plankton (n = 2)) with respect to the sum of species obtained after cation exchange chromatography for samples and selected CRMs.

		Cations						
		AB	AC	TMAP	TETRA	Gly-sug	UNK5 (Rt 5.3 <sup>b</sup> )	UNK6 (Rt 5.6)
		mg kg <sup>-1</sup>	mg kg <sup>-1</sup>	mg kg <sup>-1</sup>	mg kg <sup>-1</sup>	mg kg <sup>-1</sup>	mg kg <sup>-1</sup>	mg kg <sup>-1</sup>
		%	%	%	%	%	%	%
Milos	Gastropods gut	1.78 ± 0.09	<DL	0.51 ± 0.03	1.69 ± 0.04	0.27 ± 0.02	<DL	<DL
	Gastropods tissue	2.8 ± 0.2	<DL	1.2 ± 0.1	2.4 ± 0.2	<DL	<DL	<DL
	Sediment	0.076 ± 0.004	<DL	0.060 ± 0.005	<DL	<DL	<DL	<DL
	Sediment H <sub>3</sub> PO <sub>4</sub> <sup>a</sup>	0.073 ± 0.001	<DL	0.127 ± 0.001	<DL	<DL	<DL	<DL
	Sediment control	<DL	<DL	<DL	<DL	<DL	<DL	<DL
	Microbial mats 1	0.054	<DL	<DL	<DL	<DL	<DL	<DL
	Microbial mats 2	0.018	<DL	<DL	<DL	<DL	<DL	<DL
	Microbial mats 3	0.047	<DL	<DL	<DL	<DL	<DL	<DL
	Plankton	0.59 ± 0.03	<DL	0.24 ± 0.01	<DL	0.114 ± 0.007	<DL	<DL
	R. Alvor	Gastropods gut	24.9 ± 2.5	0.42 ± 0.05	0.15 ± 0.03	0.25 ± 0.01	0.61 ± 0.07	<DL
	Gastropods tissue	30.5 ± 1.4	0.18 ± 0.03	0.33 ± 0.03	0.16 ± 0.02	<DL	0.11 ± 0.01	0.12 ± 0.02
	Sediment	0.018 ± 0.001	<DL	<DL	<DL	<DL	<DL	<DL
	Sediment H <sub>3</sub> PO <sub>4</sub> <sup>*</sup>	0.023 ± 0.003	<DL	<DL	<DL	<DL	<DL	<DL
CRMs	CRM627	3.6 ± 0.1	<DL	0.057 ± 0.004	0.079 ± 0.004	<DL	<DL	<DL
	CRM627 certified	3.9 ± 0.2	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
	DORM-2	15.2 ± 1.2	<DL	0.17 ± 0.02	0.25 ± 0.02	<DL	<DL	<DL
	DORM-2 certified	16.4 ± 1.1	n.a.	n.a.	0.248 ± 0.054	n.a.	n.a.	n.a.

<sup>a</sup> Extracted with H<sub>3</sub>PO<sub>4</sub> 0.3 mol L<sup>-1</sup>; <DL: below detection limit.

<sup>b</sup> Retention time; n.a.: not available.

plankton, only arsenate was detected. An explanation is that microbial mats and gastropods were exposed to higher levels of arsenite because of their proximity to hydrothermal venting. The species TMAP and TETRA were found at relatively high percentages (4.9 to 11.9% for TMAP and 16.2 to 23.8 for TETRA) in gastropods, microbial mats and plankton from Milos. Only two arsenosugars (Gly-sug and PO<sub>4</sub>-sug) were determined at low concentrations in gastropod gut and plankton (Tables 3 and 4).

Inorganic arsenic was the dominant species extracted from the sediments from Milos and Ria do Alvor (Fig. 2, Table 3). The only organoarsenic species found in sediments from Ria do Alvor was AB, although only at trace concentrations. This species was also identified in those sediments from Milos where the gastropods were feeding. But it was not found in the sediment from the area not affected by hydrothermal activity in Paleochori Bay. There the only organoarsenic species detected was MA. TMAP was found at a similar concentration as AB in those sediments where the gastropods were feeding.

Only limited information is available on arsenic compounds in marine sediments. Geiszinger et al. (2002a) found arsenite and arsenate in the sediments together with a major unknown species (70% of the extracted arsenic). Rottanachonkiat et al. (2004) have analyzed arsenic species in river and estuarine sediments and they found mainly inorganic arsenic with traces of a methylated species (MA) in the river sediment. A study dealing with degradation products of arsenosugars in sediments only found the inorganic and the simple methylated (MA and DMA) species after 106 days (Pengprecha et al., 2005). Takeuchi et al. (2005) reported relatively high percentages of organoarsenicals in surface marine sediments, including AB, simple methylated species (MA and DMA) and TMAO and TETRA at lower concentrations. Ellwood and Maher (2003) found together with inorganic arsenic in some cases also dominant amounts of arsenosugars.

In this study several unknown arsenic species were quantified, but mostly at low proportions. Only in the Milos microbial mats an unknown arsenic species was found at higher proportions (UNK2: 10–15%). Two unknown cationic species were found in gastropods from Ria do Alvor. When comparing retention time, one of them appears just after Gly-sug, and with the used chromatographic conditions it could be a trimethylated sugar, which was found at trace levels previously in gastropods (Francesconi et al., 1988).

When the distribution of arsenic species from Milos short food chain is compared to Ria do Alvor gastropods and sediments, it can be observed that inorganic arsenic plays an important role in the system. Gastropods were exposed to high inorganic arsenic concentrations through the hydrothermal fluids (Price et al., 2010), and through their feeding habit, ingesting mainly microbial mats and sediments. Although the role of plankton in this short food chain is expected to be minor, it could be a source of inorganic arsenic. The species AB, although the most important species in marine animals, was found at relatively low concentrations in Milos, particularly when compared to *C. neritea* from Ria do Alvor (Table 4). Accumulation of AB in marine organisms is through the food chain and thus, the low amounts of AB available in Paleochori Bay would explain its low values in *C. neritea*.

An arsenic species found at similar percentages as AB in gastropod gut and muscle from Paleochori Bay was TETRA. This organoarsenic species is a common constituent of marine animals and is particularly prevalent in molluscs (Cullen and Reimer, 1989). Geiszinger et al., 2002b found TETRA at relatively high percentages in marine polychaetes and concluded the possibility that after exposure to arsenate, microbes in the intestine of the polychaete transformed arsenate to TETRA. In our study we only found TETRA in the gut of the gastropod and in the muscle, which suggests that it was biosynthesized by the organism or produced in the gut and then accumulated in the muscle. TETRA was not found in any other compartment (e.g. sediments, plankton or microbial mats), which suggests the possible transformation of another arsenic species, for example inorganic arsenic, to form TETRA in the gut of *C. neritea* as a detoxification mechanism. Tetramine, the nitrogen analogue of TETRA has been reported as a common component of coelenterates and it is also found in the salivary gland of marine gastropods from the genus *Neptunea* (Francesconi et al., 1988). The similarities between arsenic and nitrogen are known, and the absorption of AB, similar to its nitrogen analogue, the betaine. This would indicate that TETRA could be a product of a similar biosynthetic pathway as Tetramine. It was also proposed that TETRA could be accumulated from seawater, because it was experimentally shown that mollusks can directly accumulate TETRA (Gailer et al., 1995). Some authors (Goessler et al., 1997) have proposed that TETRA could have as a precursor a trimethyl(ribosyl)arsonium compound, which experienced an exchange of the ribosil group for a methyl group. It was also proposed that TETRA

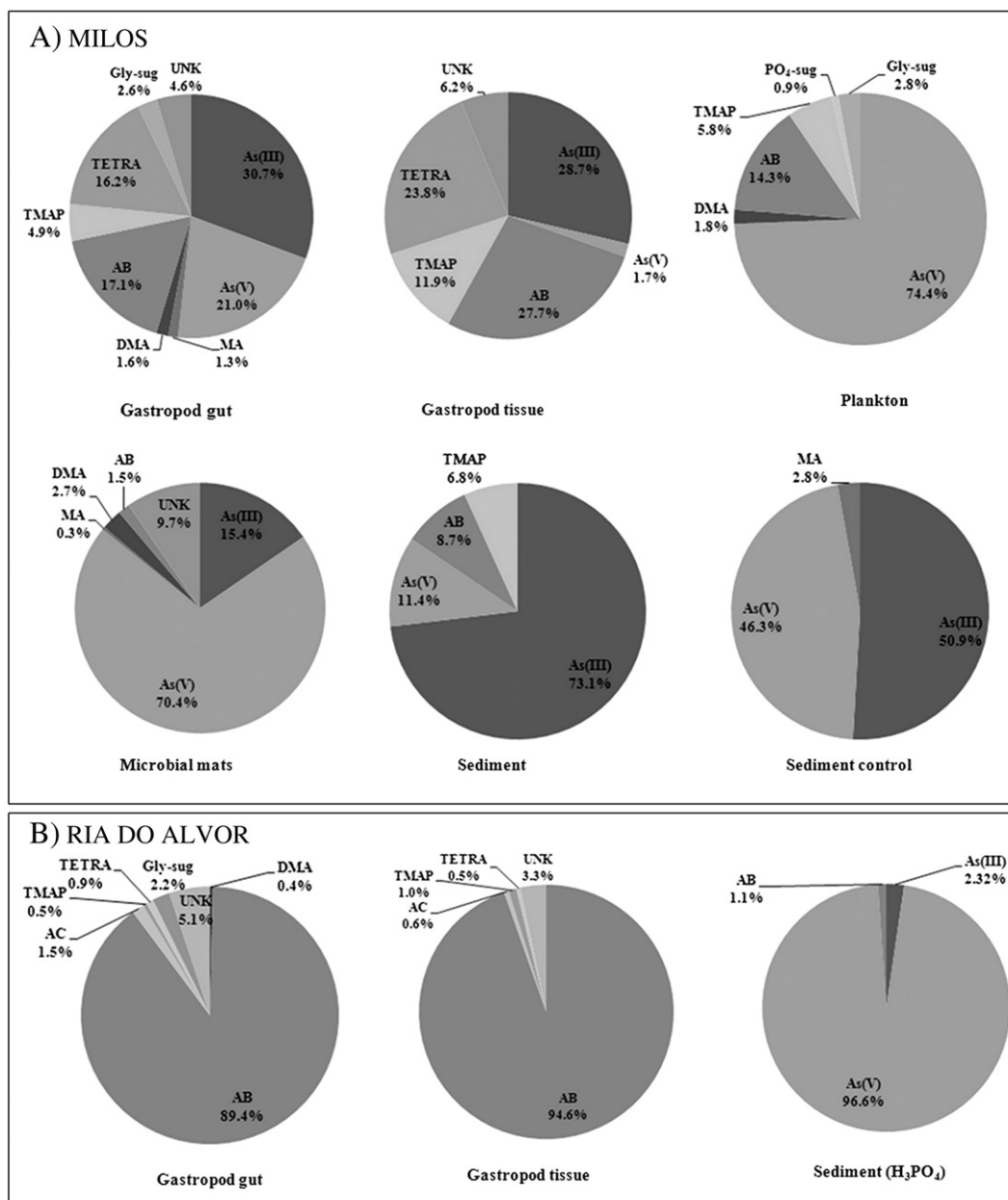


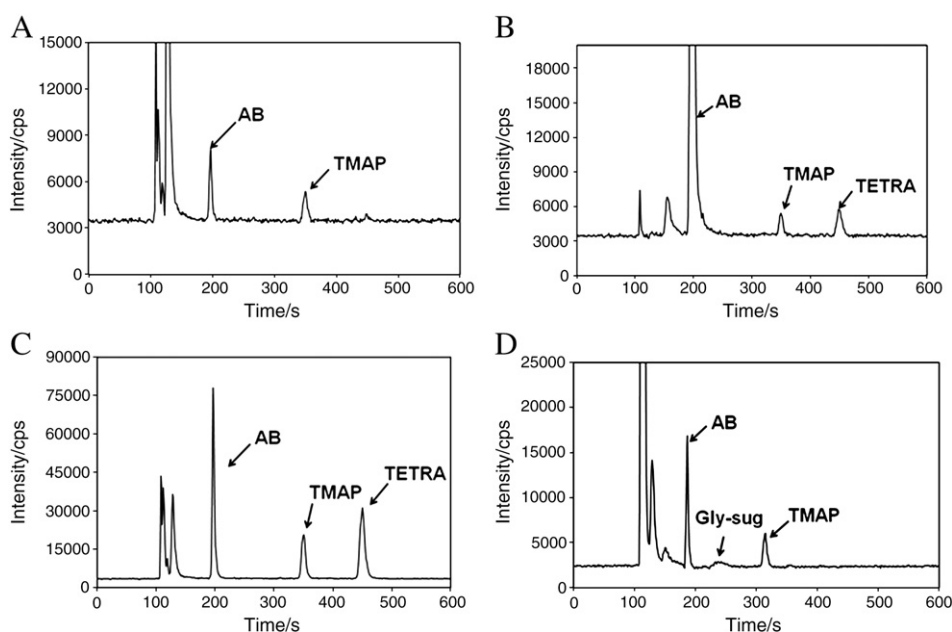
Fig. 2. Arsenic species percentages with respect to the sum of species for the different compartments of Milos Paleochori Bay (A) and Ria do Alvor in Portugal (B).

could be an end product of degradation of AB, but this fact remains unproven (Kaise et al., 1998).

The distribution of TMAP in the compartments studied in Milos hydrothermal system can give us additional information about the arsenic pathways. TMAP was reported for the first time in fish by Francesconi et al. (2000) and constituted 8% of the total arsenic extracted from the fish *Abudefduf vaigiensis*. Compared to AB, TMAP was found always at low percentages but it seemed that it is a common constituent of marine organisms (Ninh et al., 2008). To our knowledge there are no reports of TMAP in marine sediments or plankton. In our study, TMAP was found at low concentrations in sediment and plankton, but in similar concentrations to AB. In *N. neritea* percentages were higher, between 4.9 and 12% in gastropods, because they were feeding on sediments, which contained TMAP. However TMAP was not found in the microbial mats. Thus, the presence of TMAP in the sediments could be attributed to deposition of plankton or could be biosynthesized by the gastropod, excreted and accumulated in the sediment. Although there were similar percentages of TMAP and AB in the sediments with respect

to the sum of quantified arsenic species, AB was accumulated at higher percentages in the gastropod than TMAP. This could be due to the fact that TMAP, which is a betaine but with two methylene units in the carboxialkyl group instead of one (AB), is absorbed in lower proportions than AB (Francesconi et al., 1999). As an example, Fig. 3 shows the cation exchange chromatograms from sediment, *C. neritea* muscle and plankton, where the presence of TMAP can be observed.

The presence of TMAP and AB together in marine organisms offers the possibility of a different biosynthetic pathway of AB. It was proposed that AB might be derived from arsenosugars, commonly found in algae (Edmonds and Francesconi, 2003). Arsenosugars, though, seem less likely to be implicated in the biosynthesis of TMAP, as it is not possible through the AB proposed pathway to obtain a betaine with two methylene groups, which brings into question the proposal about the sugars as precursors of AB. The different compartments studied in the present work showed no or very low amounts of arsenosugars, which could suggest an alternative synthesis of AB by marine organisms. Another possible synthetic pathway was proposed by Edmonds (2000), who suggested



**Fig. 3.** Chromatograms of the cation exchange chromatography of the MeOH/water extracts of (A) Milos sediment collected in the hydrothermal area, (B) CRM DORM-2, (C) *C. neritea* muscle, (D) plankton.

the arsenilation of 2-oxoacids, where DMA is combined with oxaloacetate or glyoxylate to obtain TMAP and/or AB as final products. However, the synthesis of AB in marine organisms has not been completely explained. The hydrothermal system in Milos could be an interesting site to study arsenic metabolism pathways in greater detail, as the amounts of arsenosugars available to *C. neritea* are relatively low and TMAP is present in relatively high proportions in several compartments. Why arsenosugars are not present could be due to the fact that algae, the main source of arsenosugars in the marine environment are not present in the hydrothermally affected area. It could be also related with the environmental conditions, as maybe arsenosugars are not long lived in an environment with low pHs and high temperatures.

#### 4. Conclusions

The occurrence and distribution of different arsenic species in organism of the same species showed that arsenic absorption and metabolism are a complex process. Our study showed that environmental factors can have a strong influence. The exposure to elevated inorganic arsenic concentrations through the hydrothermal fluids and sediments, and also through the food (bacterial mats), caused an unusual speciation speciation in *C. neritea* from Paleochori Bay. The control gastropod from Ria do Alvor, on the other hand, contained almost exclusively AB, likely derived through the food chain. The finding of relatively high percentages of TMAP is an important result, especially with regard to arsenic metabolism implications, as it opens the door to study alternative pathways for AB. Any future study should include experiments under controlled conditions, so that food source can be targeted. This will provide greater insights into the arsenic metabolism in organisms inhabiting hydrothermal vent areas.

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