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## Effects of sample processing on arsenic speciation in marine macroalgae

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The stability of arsenic species in environmental samples during sampling, storage and processing is crucial for speciation studies. Losses, interconversion and degradation of arsenocompounds can occur during these steps as a result of interaction with the container material, microbial activity and temperature or light. In order to study the possible effects of storage and processing on arsenic compounds, subsamples of alga *Cystoseira mediterranea* Sauvageau were stored under different conditions (non-frozen; frozen at -18°C for 24 h and 45 days; and frozen at -80°C for 24 h and 45 days) and subjected to one of the following processing methods: chopping into fine pieces with a knife; grinding by hand in a glass mortar under N<sub>2</sub>(l); drying under an air current at room temperature (25°C); drying in an oven at 40°C; and lyophilization. The total contents of arsenic and arsenic compounds were then determined in these subsamples. Non-frozen subsamples registered 150 mg As kg<sup>-1</sup> while frozen-defrosted samples registered are 92 mg As kg<sup>-1</sup>. Nevertheless, in spite of this loss in content, the proportion of arsenic compounds was maintained in both cases. Our results indicate that freezing is an unsuitable storage method for macroalgae because losses of arsenic may occur. We recommend a drying method for this purpose.

### Introduction

The stability of chemical species of trace elements during sample handling and storage is a prerequisite to obtain reliable information in speciation studies. Given that biological samples are especially vulnerable to storage and handling conditions, these merit special care to preserve losses or changes in some elemental species.

A large number of studies have addressed arsenic speciation in marine organisms, including algae, over recent have generated high number of publications in the last decades. Many are dedicated to examining new compounds and their behavior, transformations and metabolic pathways because algae are crucial organisms in arsenic biogeochemistry and furthermore many algae are selected for human consumption.<sup>1-5</sup> Studies and reviews have reported on the influence of the storage, drying and powdering conditions on total arsenic and arsenic speciation in general or for particular compounds.<sup>6-9</sup> On the basis of these studies, it can be deduced that no common treatment is reliable and that handling and storage protocols should be established in function of the matrix composition, environmental conditions where the sample is collected (e.g. microbial activity, aquatic or dry media), chemical species to be determined, and analytical technique for their measurement.

Here we sought to ascertain the influence of sample storage conditions and further processing on the results of arsenic speciation in the brown alga *Cystoseira mediterranea*. For this purpose, we tested the effect of freezing algal samples at two

temperatures (-18°C and -80°C) for a range of periods, and of non-freezing storage methods. We also tested five sample processing methods (chopping, grinding under liquid nitrogen, air drying, drying at 40°C, and lyophilization) before the extraction of arsenic species. The same extraction method (water extraction) was applied in all cases in order to assure that the differences in the results are only attributable to the pre-treatment. We selected *C. mediterranea* Sauvageau (heterokonphyta, fucales) because of its abundance, availability, environmental interest and arsenic content; specifically, we worked with the alga branches because they are deciduous structures that can completely regenerate from the stipe. Communities of *C. mediterranea* grow along the northern Atlantic coast of Morocco, the Mediterranean coast of Spain, France, Tunisia, and Sicily and the southern Tyrrhenian coast of Italy.<sup>10</sup> This alga is characterized by perennial structures, such as holdfast and stipe, and annual deciduous branches. Several studies<sup>11, 12</sup> have proposed that this macroalga could be used as a bioindicator of the quality of coastal water as part of the European Water Framework Directive.<sup>13</sup> Some have reported values for total arsenic and arsenic species content in stipe medulla of *C. mediterranea*:<sup>14</sup> Total As 39.0 ± 1.2 mg As kg<sup>-1</sup>; As(III) 0.49 ± 0.19 mg As kg<sup>-1</sup>; DMA 0.26 ± 0.03 mg As kg<sup>-1</sup>; As(V) 1.17 ± 0.06 mg As kg<sup>-1</sup>; PO<sub>4</sub>-sug 0.39 ± 0.04 mg As kg<sup>-1</sup>; SO<sub>3</sub>-sug 19.6 ± 0.2 mg As kg<sup>-1</sup>; gly-sug 2.21 ± 0.21 mg As kg<sup>-1</sup>; and unknown anion 1.17 ± 0.10 mg As kg<sup>-1</sup>.

### Materials and Methods

## Reagents and Standards

All chemicals were of analytical grade. Millipore Milli-Q Plus Water (18.2 M $\Omega$  cm) was used for all solution preparations. Ammonium dihydrogen phosphate (Panreac, p.a.) and pyridine (Scharlau, p.a.) were used for anionic and cationic mobile phase preparation, respectively. pH was adjusted using ammonia solution (25%, Panreac, p.a.) and formic acid (98%, Panreac, p.a.). Sample digestion was achieved with nitric acid (69%, Panreac, Hiperpur) and hydrogen peroxide (31%, Merck, Selectipur). Potassium iodide (Panreac, p.a.), ascorbic acid (Panreac, p.a.), hydrochloric acid (37%, Panreac, p.a.) and sodium borohydride (Aldrich, purum, tablets) were used for arsenic determination by HG-AFS (hydride generation atomic fluorescence spectrometry). A solution of  $^9\text{Be}$ ,  $^{103}\text{Rh}$ ,  $^{205}\text{Tl}$  20  $\mu\text{g l}^{-1}$  (NIST High-Purity Standards) was used as internal standard for ICP-MS (inductively coupled plasma mass spectrometry) measurements.

## Arsenic standards

The following were used as arsenic standards in speciation: arsenite from  $\text{As}_2\text{O}_3$  (NIST, USA, Oxidimetric Primary Standard 83d, 99.99%); arsenate from  $\text{Na}_2\text{HAsO}_4 \cdot 7\text{H}_2\text{O}$  (Carlo Erba); methylarsonic acid (MA) as  $(\text{CH}_3)\text{AsO}(\text{ONa})_2 \cdot 6\text{H}_2\text{O}$  (Carlo Erba); dimethylarsinic acid (DMA) as  $(\text{CH}_3)_2\text{AsNaO}_2 \cdot 3\text{H}_2\text{O}$  (Fluka); arsenocholine (AC) as  $(\text{CH}_3)_3\text{As}^+(\text{CH}_2)\text{CH}_2\text{OHBr}^-$  supplied by the "Service Central d'Analyse" (CNRS Vernaison, France); arsenobetaine (AB) as  $(\text{CH}_3)_3\text{As}^+\text{CH}_2\text{COO}^-$  provided by BCR (CRM 626, standard solution) and trimethylarsenic oxide (TMAO) from  $(\text{CH}_3)_3\text{AsO}$  (Argus Chemicals srl). Standardized stock solutions of the arsenic compounds containing about 1000 mg  $\text{l}^{-1}$  were prepared in water, except arsenite, which was dissolved in 4 g  $\text{l}^{-1}$  NaOH solution (Merck, Suprapure). All solutions were stored in the dark at 4°C. Arsenate standard solution with a certified concentration of  $1000 \pm 2$  mg As  $\text{l}^{-1}$  from NIST High-Purity Standards was used for external calibration for the determination of the content with ICP-MS as well as for the standardization of MA, DMA, AC, AB and TMAO stock solutions. Arsenate standard was used for total arsenic determination in seawater by HG-AFS. An aliquot of freeze-dried extract of *Fucus serratus* dissolved in water was used as internal laboratory reference material for the identification of the major arsenosugars: phosphate ( $\text{PO}_4\text{-sug}$ ), sulfate ( $\text{SO}_4\text{-sug}$ ), sulfonate ( $\text{SO}_3\text{-sug}$ ), and glycerol ( $\text{Gly-sug}$ ).<sup>15</sup> The certified reference material BCR CRM 279 Sea Lettuce (*Ulva lactuca*), from the Institute for Reference Materials and Measurements (IRMM) of the European Commission, with a certified value  $3.09 \pm 0.20$  mg As  $\text{kg}^{-1}$ , was used for quality control purposes in total arsenic determinations.

## Instruments and apparatus

A closed microwave digestion system, Milestone Ethos Touch Control, was used for sample digestion and CMR CRM BCR 279. HG-AFS Millennium Excallibur (PS Analytical, UK) was used for total arsenic measurement in seawater. The chromatographic system, consisting of an Agilent 1200 LC quaternary pump equipped with an autosampler, was connected to an anion-exchange and cation-exchange column and guard-column and coupled to an Agilent 7500ce ICP-MS (Agilent,

Germany) equipped with Ari Mist HP nebulizer (Burgener, Canada). The chromatographic program, instrumental operating conditions of LC-ICP-MS and the arsenicals that can be separated with each chromatographic system are given in Table 1. A LyoQuest -80 freeze-dryer (Telstar) was used during sample preparation. A P. Selecta oven was used in sample drying and humidity determination. A CVF 220/86 ultrafreezer was used to keep samples at  $-80^\circ\text{C}$ , and for  $-18^\circ\text{C}$  a Liebherr Economy GT3602 was used.

**Table 1** Chromatographic conditions used for arsenic speciation.

	Anion exchange	Cation exchange
Column	PRP-X100 (250 mm x 4.1 mm, 10 $\mu\text{m}$ ) (Hamilton, Reno, USA)	Zorbax SCX300 (250 mm x 4.6 mm, 5 $\mu\text{m}$ ) (Agilent, Waldbronn, Germany)
Pre column	PRP-X100 (20 mm x 2.0 mm, 10 $\mu\text{m}$ )	Zorbax SCX300 (12.5 mm x 4.6 mm, 5 $\mu\text{m}$ )
Mobile phase	$\text{NH}_4\text{H}_2\text{PO}_4$ 20mM	Pyridine 20 mM
pH	5.8	2.6
Injection volume	20 $\mu\text{l}$	20 $\mu\text{l}$
Flow rate	1.5 ml $\text{min}^{-1}$	1.5 ml $\text{min}^{-1}$
Column temperature	Room temperature	Room temperature
As species	As (III), DMA, MA, As (V), $\text{PO}_4\text{-sug}$ , $\text{SO}_3\text{-sug}$ and $\text{SO}_4\text{-sug}$	AB, TMAO, AC and Gly-sug

## Sampling and sample preparation

*Cystoseira mediterranea* (2 kg, pooled sample) was collected on 19<sup>th</sup> July 2011 in Lloret de Mar on the coast of Catalonia (Spain) in the Western Mediterranean Sea. This alga was collected in a rock platform close to the beach. Branches were cut with a sharp knife to prevent damage to the perennial regenerative structures.<sup>16</sup> Seawater (1 l) was also collected in PET bottles to obtain additional information on arsenic content of the growth medium of algae. The whole amount of sample was immediately transported to the laboratory and divided into five subsamples of 50 g each (Fig. 1). Subsample A was processed within 10 h of sampling (Day 0), subsamples B and C were stored at  $-18^\circ\text{C}$  and D and E at  $-80^\circ\text{C}$  in sealed plastic bags. Subsamples B and D were defrosted the day after freezing (Day 1) and C and E 45 days later (Day 45). After these periods of storage, the common cleaning consisted of removing epiphytes and other substances with tweezers and a short wash in deionized water (<10 s). Each subsample was then divided into five fractions and the following procedures were applied, as summarized in Fig. 1:

- **Chopping** into fine pieces with a stainless steel knife
- Grinding by hand in a glass mortar under  $\text{N}_2(\text{l})$
- Drying under an **air current** at room temperature ( $25^\circ\text{C}$ )
- Drying in an **oven at  $40^\circ\text{C}$**
- **Lyophilization**

Dried subsamples were ground by hand in an agate mortar and stored at room temperature in amber glass bottles until analysis. Non-dried samples were shortly stored at  $4^\circ\text{C}$  in the dark until acidic digestion and extraction.

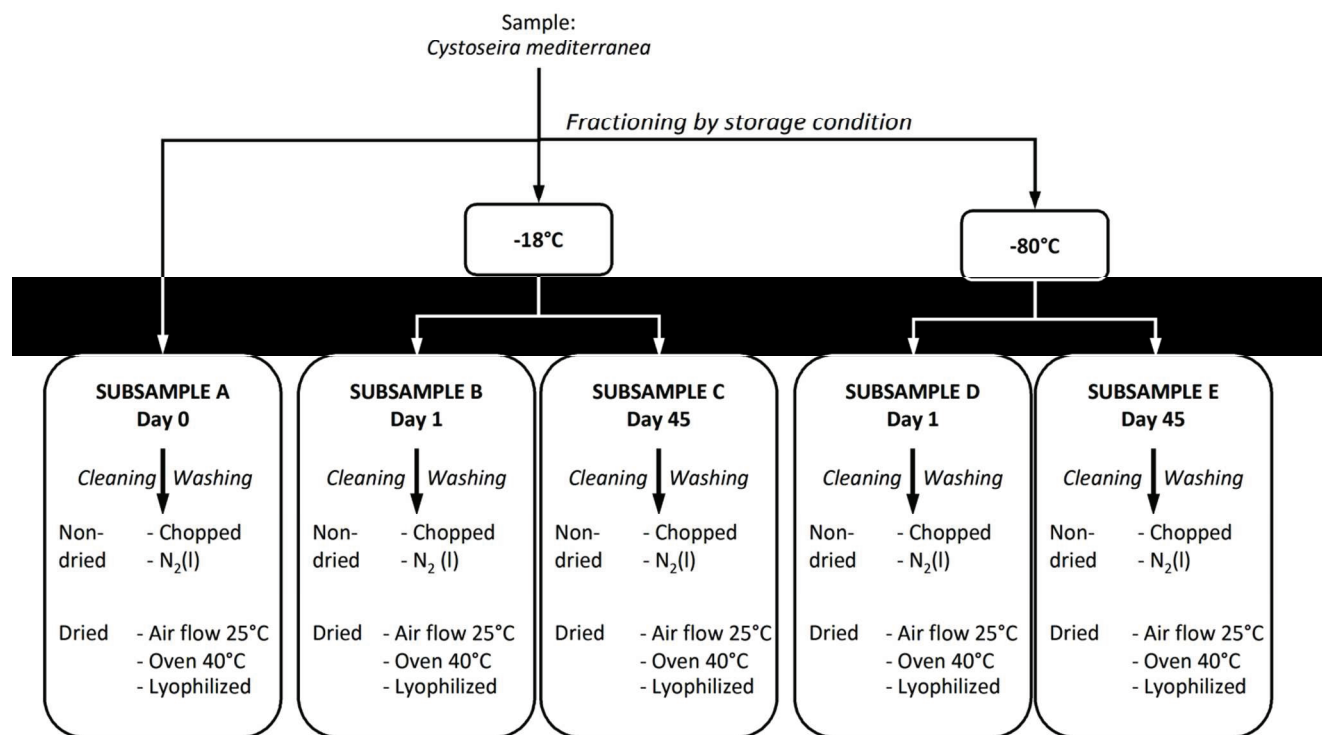


Fig. 1 Scheme of the storage and processing applied to subsamples.

### Total arsenic determination

Acidic digestion was performed in a middle-pressure closed microwave system. Samples (0.8 g of non-dried alga or 0.2 g of dried alga) were digested using the procedure reported for marine algae.<sup>14, 17</sup> Non-dried samples were digested within 2 days after sample processing and dried sample in a week lapses. Digests were diluted 1:50 with 1% HNO<sub>3</sub> before total arsenic measurement, which was performed by ICP-MS (external calibration, <sup>103</sup>Rh as internal standard, He as collision gas). All samples and BCR CRM 279 Sea Lettuce (*Ulva lactuca*) were analyzed in triplicate with their corresponding digestion blanks.

Arsenic from seawater was measured in triplicate by HG-AFS. Pre-reduction to increase the arsine-generation yield was performed as follows: 35 ml of seawater or arsenate standard (for calibration) was acidified with 7.5 ml of 37% HCl. We then added 5 ml of a reducing solution (10% KI, 2% ascorbic acid, 2 M HCl) and diluted this up to 50 ml. The resulting solution was diluted up to 50 ml and kept at 37°C for 2 h to ensure complete reduction of arsenate to arsenite before measurement. Quantification was performed by the standard addition calibration method.

### Arsenic speciation

Aqueous extracts of alga were obtained in an end-over-end shaker (35 rpm, 16 h) from 1 g of non-dried sample or 0.1 g of dried samples and 10 g of water. Extracts were centrifuged (3000 rpm, 15 min) and passed through PET syringe filters (Chromafil PET®, Macherey–Nagel, 0.45 μm) and stored at -80°C until analyses were performed. Arsenicals were extracted from non-dried samples the day they were collected (fresh-pretreated) or defrosted.

Extracts were defrosted immediately before measurements and homogenized under a vortex. Arsenic speciation was carried out in extracts by LC-ICP-MS using the method reported for marine algae.<sup>14, 17, 18</sup> Extracts were diluted with water (1:10 for dried samples and 1:25 for non-dried samples). LC-ICP-MS was used for measurement using anionic exchange chromatography for diluted extracts and while non-diluted extracts were analyzed by cationic exchange chromatography. Multispecies standards of As(III), DMA, MA and As(V) for anionic exchange chromatography and AB, AC and TMAO for cationic exchange chromatography covering the range 1 - 100 μg As l<sup>-1</sup> were prepared fresh daily for speciation analysis. Chromatographic peaks were identified on the basis of their retention times by comparison with standards. Arsenic species were quantified by external calibration curves. As(III) was quantified by subtracting to the front peak of anionic exchange chromatography the species quantified in cationic exchange chromatography. In addition, total arsenic in the extracts was measured in the conditions used for algal acidic digests.

For those species for which a quantification standard was not available, quantification was performed with the calibration curve of the closest standard in retention time as Francesconi and Sperling<sup>19</sup> suggested though this assumption has been questioned.<sup>20, 21</sup> Thus SO<sub>3</sub>-sug was quantified with the calibration curve of the As (V) standard and Gy-sug with the calibration curve of the AC standard; even though AB is the closest standard, AC does not show overlapping problems.

LODs and LOQs were estimated. The former is the lowest concentration of an analyte that can be reliably differentiated from background noise (signal-to-noise ratio greater than 3). The LOQ is the lowest concentration that can be measured (signal-to-

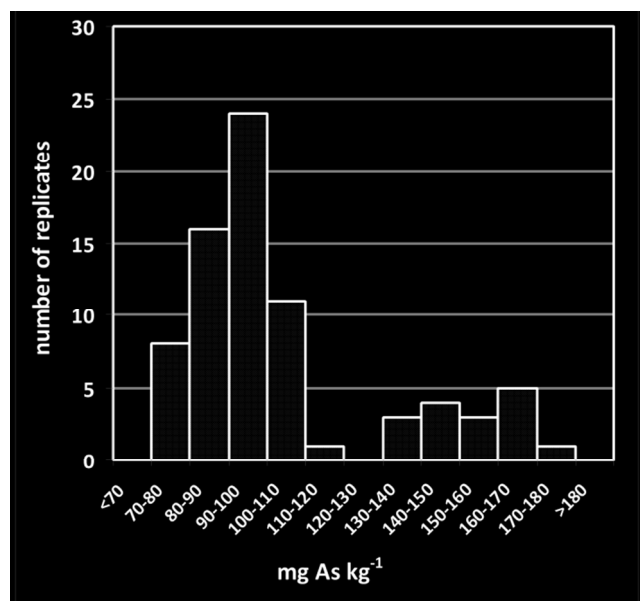
noise ratio greater than 10). For calculating LOD and LOQ, the standard deviation of the base line and the peak base of each analyte multiplied by 3 or 10 (LOD and LOQ respectively) were interpolated in the height calibration curve. The arsenosugar LOD

and LOQ were estimated through a correction factor, which is the relation within the concentration of arsenosugar in *F. serratus* and the peak height.

**Table 2** Total arsenic, arsenic species, extraction efficiency, column recovery and limits of detection and quantification (mean  $\pm$  standard deviation, n=3, mg As kg<sup>-1</sup>, results expressed on dry weight basis)

Subsample	Storage	Processing <sup>a</sup>	Total As	As(V)	SO <sub>3</sub> -sug	gly-sug	Extraction efficiency	Column recovery
DAY 0		C	140 $\pm$ 10	47 $\pm$ 3	11 $\pm$ 1	2.16 $\pm$ 0.08	57% $\pm$ 8%	78% $\pm$ 15%
		N	150 $\pm$ 20	105 $\pm$ 2	15.3 $\pm$ 0.6	3.1 $\pm$ 0.2	97% $\pm$ 14%	87% $\pm$ 15%
		A	163 $\pm$ 2	110 $\pm$ 10	14.1 $\pm$ 0.8	3.37 $\pm$ 0.07	94% $\pm$ 6%	83% $\pm$ 2%
		O	160 $\pm$ 10	112 $\pm$ 4	13.5 $\pm$ 0.4	3.8 $\pm$ 0.2	97% $\pm$ 6%	82% $\pm$ 2%
		L	140 $\pm$ 3	101 $\pm$ 4	13.2 $\pm$ 0.3	3.5 $\pm$ 0.2	104% $\pm$ 4%	81% $\pm$ 2%
DAY 1	-18°C	C	82 $\pm$ 5	53 $\pm$ 3	10.4 $\pm$ 0.6	2.3 $\pm$ 0.2	91% $\pm$ 10%	90% $\pm$ 9%
		N	83 $\pm$ 5	52.3 $\pm$ 0.5	9.18 $\pm$ 0.05	2.36 $\pm$ 0.02	97% $\pm$ 12%	80% $\pm$ 3%
		A	101 $\pm$ 5	67 $\pm$ 2	10.5 $\pm$ 1	2.6 $\pm$ 0.1	99% $\pm$ 1%	80% $\pm$ 4%
		O	103 $\pm$ 3	65 $\pm$ 6	10.5 $\pm$ 0.7	2.4 $\pm$ 0.1	94% $\pm$ 8%	80% $\pm$ 2%
		L	97 $\pm$ 5	64.75 $\pm$ 0.01	10.3 $\pm$ 0.4	3.05 $\pm$ 0.08	102% $\pm$ 4%	79% $\pm$ 1%
DAY 1	-80°C	C	100 $\pm$ 10	61 $\pm$ 0.3	10.3 $\pm$ 0.8	2.47 $\pm$ 0.09	90% $\pm$ 11%	82% $\pm$ 3%
		N	85 $\pm$ 1	65 $\pm$ 4	11 $\pm$ 0.3	2.5 $\pm$ 0.1	104% $\pm$ 12%	89% $\pm$ 3%
		A	100 $\pm$ 2	61 $\pm$ 2	11.2 $\pm$ 0.3	2.45 $\pm$ 0.01	92% $\pm$ 4%	82% $\pm$ 3%
		O	105.2 $\pm$ 0.4	67.1 $\pm$ 0.7	10.5 $\pm$ 0.5	2.9 $\pm$ 0.1	92% $\pm$ 5%	83% $\pm$ 4%
		L	91 $\pm$ 1	55 $\pm$ 2	9.9 $\pm$ 0.1	3 $\pm$ 0.2	96% $\pm$ 1%	77% $\pm$ 2%
DAY 1	-18°C	C	89 $\pm$ 6	66 $\pm$ 3	9.7 $\pm$ 0.6	2.9 $\pm$ 0.2	99% $\pm$ 4%	91% $\pm$ 1%
		N	79 $\pm$ 3	54.6 $\pm$ 0.5	8.9 $\pm$ 0.1	3 $\pm$ 0.2	96% $\pm$ 3%	88% $\pm$ 3%
		A	95.8 $\pm$ 0.5	58 $\pm$ 1	9.3 $\pm$ 0.3	2.6 $\pm$ 0.2	90% $\pm$ 1%	81% $\pm$ 1%
		O	93.6 $\pm$ 0.5	59 $\pm$ 1	10 $\pm$ 0.1	2.9 $\pm$ 0.1	93% $\pm$ 4%	82% $\pm$ 4%
		L	84 $\pm$ 8	57 $\pm$ 1	9.3 $\pm$ 0.6	2.47 $\pm$ 0.08	95% $\pm$ 10%	86% $\pm$ 2%
DAY 45	-80°C	C	94 $\pm$ 10	77 $\pm$ 3	11.6 $\pm$ 0.6	3.1 $\pm$ 0.3	106% $\pm$ 11%	93% $\pm$ 1%
		N	76 $\pm$ 4	50 $\pm$ 1	10 $\pm$ 0.6	2.7 $\pm$ 0.1	91% $\pm$ 3%	91% $\pm$ 1%
		A	94 $\pm$ 5	63.8 $\pm$ 0.4	10.5 $\pm$ 0.5	2.68 $\pm$ 0.03	99% $\pm$ 6%	83% $\pm$ 2%
		O	101 $\pm$ 7	65 $\pm$ 1	11.3 $\pm$ 0.3	2.8 $\pm$ 0.1	93% $\pm$ 6%	84% $\pm$ 3%
		L	90 $\pm$ 2	62 $\pm$ 1	10.4 $\pm$ 0.5	2.73 $\pm$ 0.04	99% $\pm$ 1%	85% $\pm$ 3%
Limit of detection			0.003	0.08	0.41	0.016		
Limit of quantification			0.01	0.22	1.36	0.05		

<sup>a</sup> C: non-dried, chopped; N: non-dried, ground with liquid N<sub>2</sub>; A: dried under air flow 25°C; O: dried in an oven at 40°C, L: lyophilized



**Fig. 2** Histogram representing total arsenic content in *C. mediterranea* subjected to various storages and processings

## 15 Results

### Seawater

Arsenic concentration in the seawater sample was  $1.4 \pm 0.2 \mu\text{g As l}^{-1}$ . This content is within the range of arsenic concentrations commonly reported for the Mediterranean Sea, which typically shows concentrations of  $1.1\text{-}1.9 \mu\text{g l}^{-1}$ .<sup>22-24</sup>

### *Cystoseira mediterranea*

All results are given on dry weight basis. Moisture was estimated as the weight loss at  $105 \pm 5^\circ\text{C}$  until constant weight.

Mean values for total arsenic ranged from  $76 \text{ mg As kg}^{-1}$  to  $163 \text{ mg As kg}^{-1}$  (see Table 2). An histogram was built with the data of all the replicates (Fig. 2) and two populations were identified: samples that were not frozen presented  $150 \text{ mg As kg}^{-1}$  and samples stored at  $-18^\circ\text{C}$  or  $-80^\circ\text{C}$  had a lower arsenic concentration ( $92 \text{ mg As kg}^{-1}$ ). A two factors ANOVA analysis of all data revealed that both, freezing and processing of samples, were significant ( $\alpha=0.05$ ). Subsequent Post-hoc analyses (DHS Turkey;  $\alpha=0.05$ ) found two defined subgroups for processing. Oven and air dried samples presented a mean of 110 and  $113 \text{ mg As kg}^{-1}$ , respectively; whereas non-dried and lyophilized sample presented the following means:  $94 \text{ (N)}$  and  $100 \text{ (C and L)}$   $\text{mg As kg}^{-1}$ .

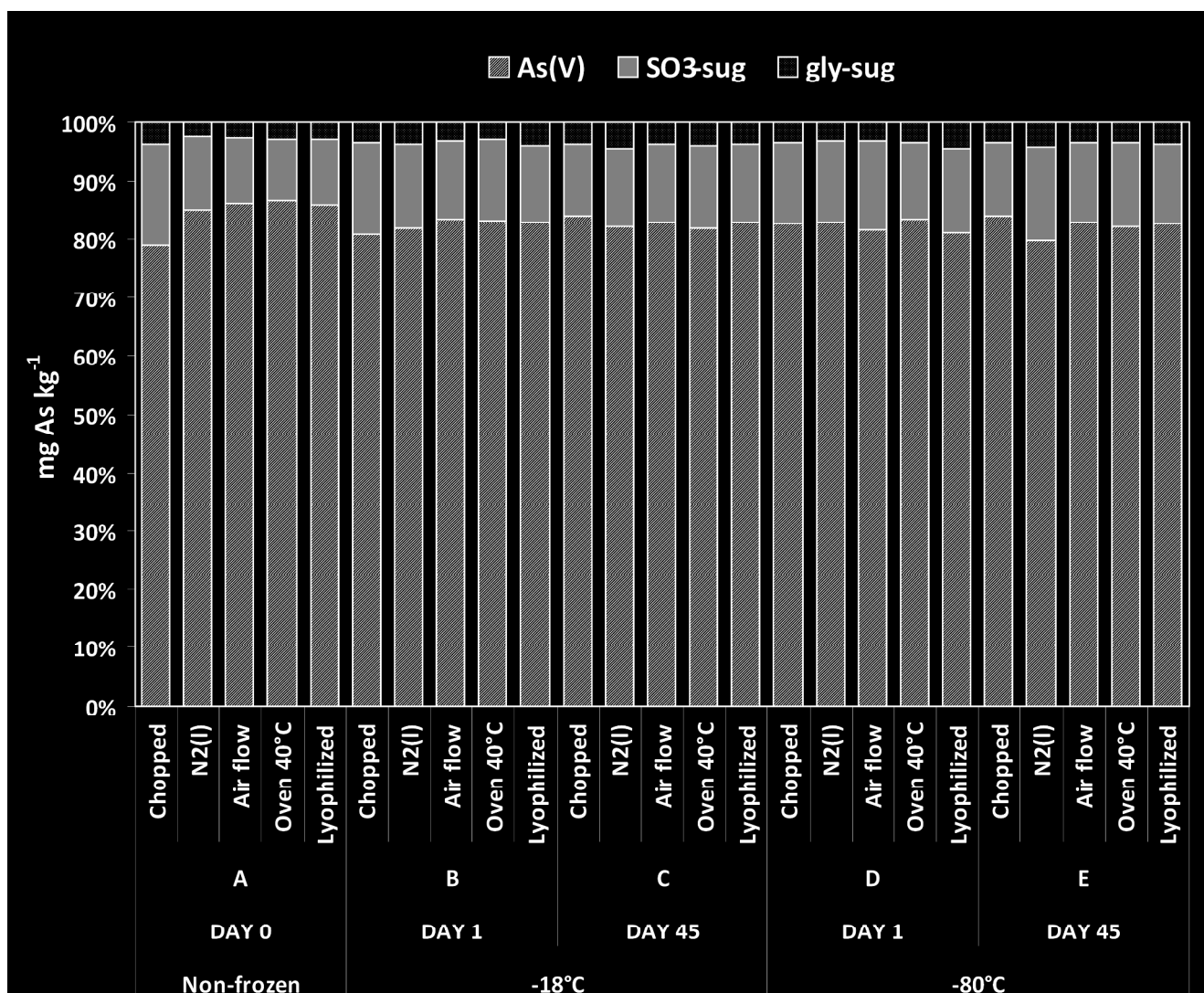


Fig. 3 Percentages of species in *C. mediterranea* subjected to various storages and processings

For arsenic speciation, extraction efficiency (calculated as total arsenic in the extract to total arsenic in digests) ranged from 89.5% to 105.9% except for non-frozen chopped samples, which registered an extraction efficiency of 57.0% (Table 2). All subsamples presented As(V), SO<sub>3</sub>-sug and Gly-sug in the same proportion with respect to the sum of species (Fig. 3). As an example, chromatograms of subsample A (ground under N<sub>2</sub>(l)) are shown in Fig. 4. It can be seen that an Unknown peak is eluting after AB, so that it cannot be attributable to this compound.

#### Quality assessment in the determination of arsenic and arsenic species

##### Column recovery

For quality assessment in speciation studies, column recovery must be evaluated in order to guarantee the correct chromatographic separation. With this aim, we calculated the ratio of the sum of the species eluted from the chromatographic columns to the total arsenic in the extract injected into the column. This parameter allowed us to properly evaluate the quantification of arsenic species. Column recoveries ranged between 77.3% and 92.9% (Table 2).

##### Certified reference material (CRM)

To check accuracy, total arsenic concentration was determined in CRM BCR 279 Sea lettuce (*Ulva lactuca*). The result obtained ( $2.9 \pm 0.3$  mg As kg<sup>-1</sup>) was consistent with the certified value ( $3.09 \pm 0.20$  mg As kg<sup>-1</sup>), thereby demonstrating the accuracy of the analytical method. Results for arsenic speciation for the CRM are shown in Table 3; in addition values found in the literature are given for comparison purposes.<sup>25, 26</sup> Although we used the simplest extraction method (water and end-over-end extraction), we obtained the same extraction efficiencies (57%) and only one reported result showed higher extraction but it was performed with MeOH:H<sub>2</sub>O<sub>2</sub> (1:1) at 75°C and assisted microwave.

##### Analysis of *F. serratus* extract

We used an extract from the brown seaweed *F. serratus*<sup>15</sup> to identify arsenosugars present in our algae samples. For quality control purposes, we quantified As species in *F. serratus* extracts. Our results (DMA:  $0.01 \pm 0.01$  µg; Gly-sug:  $0.07 \pm 0.01$  µg; PO<sub>4</sub>-sug:  $0.07 \pm 0.01$  µg; SO<sub>3</sub>-sug:  $0.56 \pm 0.04$  µg; SO<sub>4</sub>-sug:  $0.37 \pm 0.02$  µg; values for *F. serratus* extract are given as absolute amount for extract µg.) confirming those reported by Madsen *et al.*<sup>15</sup> and other values in the literature on the same extract.<sup>14, 27-29</sup>

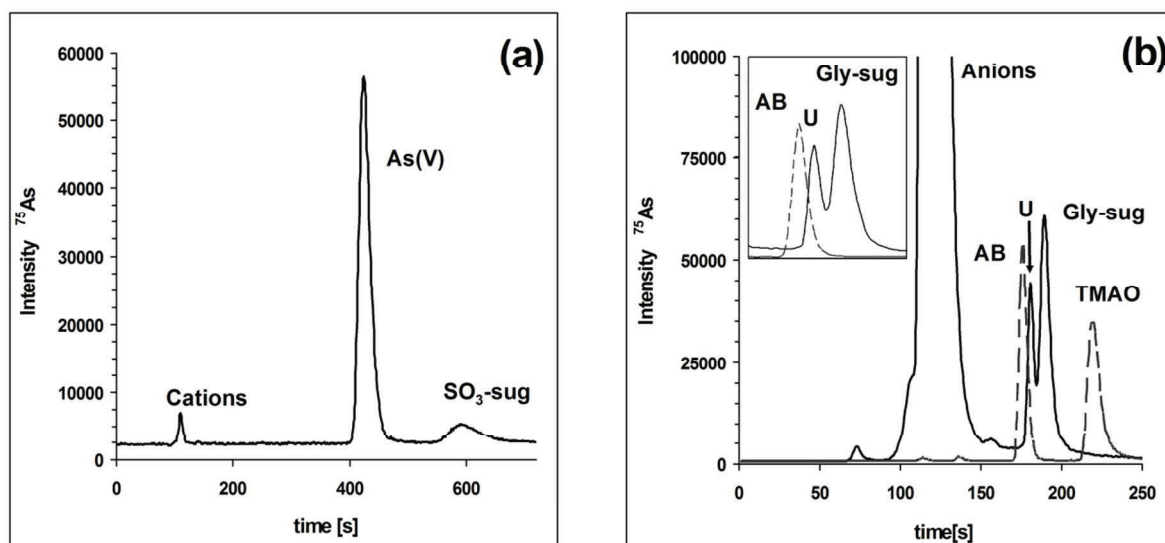


Fig. 4 Anionic (a) and cationic (b) exchange chromatograms of subsample A, which was non-dried and ground under  $N_2(l)$ . In figure 4b, the solid line corresponds to the sample and the dashed line to the AB and TMAO standard ( $10 \mu g As l^{-1}$ )

## Discussion

Total arsenic in *C. mediterranea* branches was  $150 \pm 10$  ( $n=15$ )  $mg As kg^{-1}$ . This value is the mean of the 15 replicates analyzed for subsample A not considering the processing method. This subsample was processed few hours after sampling and presented the highest arsenic concentration.

Extraction efficiencies ranged from 57% to 106% and are higher than usual values reported for alga,<sup>8</sup> thereby indicating that *C. mediterranea* presents most arsenic in water-soluble forms; thus we conclude that water is a suitable extracting agent for this macroalgae. However, fresh samples cut with knife give low extraction efficiencies. This observation can be attributable to the presence of unbroken cell structures that have the capacity to retain arsenic species.

Level of arsenic in all frozen samples was  $\sim 60\%$  lower than the non-frozen samples. Regarding processing methods, it should be taking into account that moisture is a crucial factor and little differences can result in wide variation when correcting for dry weight. For that reason, further studies are required to assess the effect of the processing methods. Furthermore, arsenic compounds did not show changes in their relative contents when we compared frozen and non-frozen samples. This behavior suggests that losses of total arsenic are related directly to the freezing/defrosting process. We postulate that algal cell structures might have broken during this process and arsenic compounds were removed when broken cells were rinsed away after defrosting. This is in agreement with the findings reported Le *et al.*<sup>30</sup> in a deep study of the effect of freezing as storage method. The authors also observed losses of total As in the range of 3-40% when defrosting samples. The same study reported that losses of As affected all species without discrimination. In the present study (see Fig. 3), no significant differences between the species in the extract of defrosted samples and those in the extract

of non-frozen samples were detected.

Each processing method has strengths and weaknesses; therefore, the choice of the method should depend on the kind of sample involved, the amount collected equipment availability, and period of storage before analyses, among other factors. Non-drying methods proved to be demanding and conditioned to the location of the laboratory to the sampling point. In addition, fresh samples cannot be preserved for long periods without damage to the algae and to the arsenic species and freezing showed to produce losses of large amounts of arsenic. Given all of the above points, drying methodologies are more suitable for arsenic speciation studies. Air-drying is a simple method; however, air-dried samples are difficult to pulverize. It is a suitable procedure when samples cannot be transported to the laboratory, and cleaning and drying should be performed *in situ* but avoided when further equipment is available. Lyophilization and oven-drying facilitate grinding but reports of losses of volatile compounds and species interconversion respectively are found in the literature;<sup>6</sup> however, we did not detect these processes.

## Conclusions and recommendations

On the basis of our results, we conclude that freezing is not a suitable storage method for macroalgae since this procedure leads to loss of arsenic. These losses are not detected in drying procedures.

We found that drying to be the most convenient processing method, we recommend lyophilization. Although this process requires a suitable system, it facilitates further pulverization of the sample, which is necessary to assure representativeness. Air-drying is recommended when sampling is performed in a remote location due to its simplicity. We do not recommend working with non-dried samples. Freezing proved to be a process to be avoided, so we do not recommend it.

**Table 3** Speciation results for CRM BRC 279 Sea lettuce (*Ulva lactuca*). Values for CRM reported on the literature are shown with comparison purposes.

Reference	Extracting agent	Extracting method	As(V) (mg As kg <sup>-1</sup> )	As(III) (mg As kg <sup>-1</sup> )	DMA (mg As kg <sup>-1</sup> )	MA (mg As kg <sup>-1</sup> )	TETRA (mg As kg <sup>-1</sup> )	AB (mg As kg <sup>-1</sup> )	gly-sug (mg As kg <sup>-1</sup> )	PO <sub>4</sub> -sug (mg As kg <sup>-1</sup> )	SO <sub>3</sub> -sug (mg As kg <sup>-1</sup> )	Unknown (mg As kg <sup>-1</sup> )	Extraction efficiency	Column recovery
Present study <sup>a</sup>	H <sub>2</sub> O	End-over end agitation	0.53 ± 0.04	0.06 ± 0.03	0.06 ± 0.03	0.04 ± 0.01		0.14 ± 0.02	0.096 ± 0.004	0.08 ± 0.01		0.07 ± 0.02	57%	81%
Foster <i>et al.</i> <sup>23</sup>	MeOH : H <sub>2</sub> O (1 : 1, v/v)	MW-assisted 70°C 15 min	0.52 ± 0.03		0.08 ± 0.01	0.07 ± 0.01	0.04 ± 0.01		0.05 ± 0.01	0.21 ± 0.03		0.01 ± 0.01	77%	
Foster <i>et al.</i> <sup>25</sup>	2% HNO <sub>3</sub>	MW assisted 95°C 6 min	1.2 ± 0.06		0.04 ± 0.01	0.03 ± 0.01			0.24 ± 0.03			0.16 ± 0.02	57%	92%
Caumette <i>et al.</i> <sup>26</sup>	H <sub>2</sub> O	60°C 4 h 15 min sonification	0.7					Traces	0.2	0.1	0.2		53%	87%

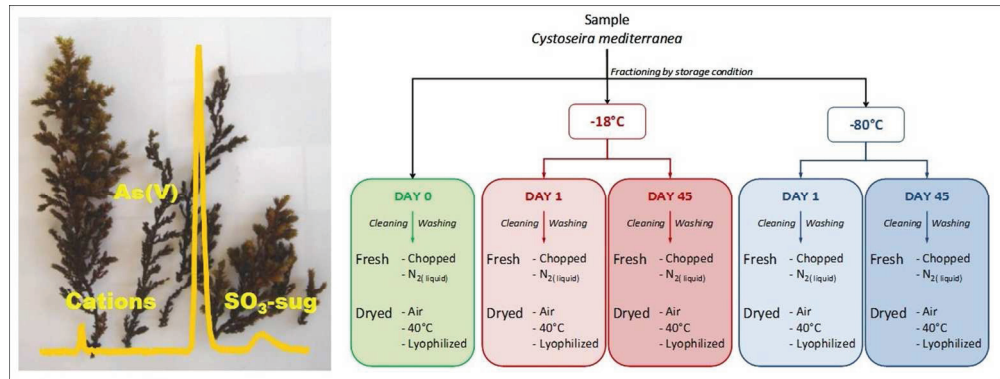
<sup>a</sup> (mean ± standard deviation, n=3, results expressed on dry weight basis).

## Acknowledgements

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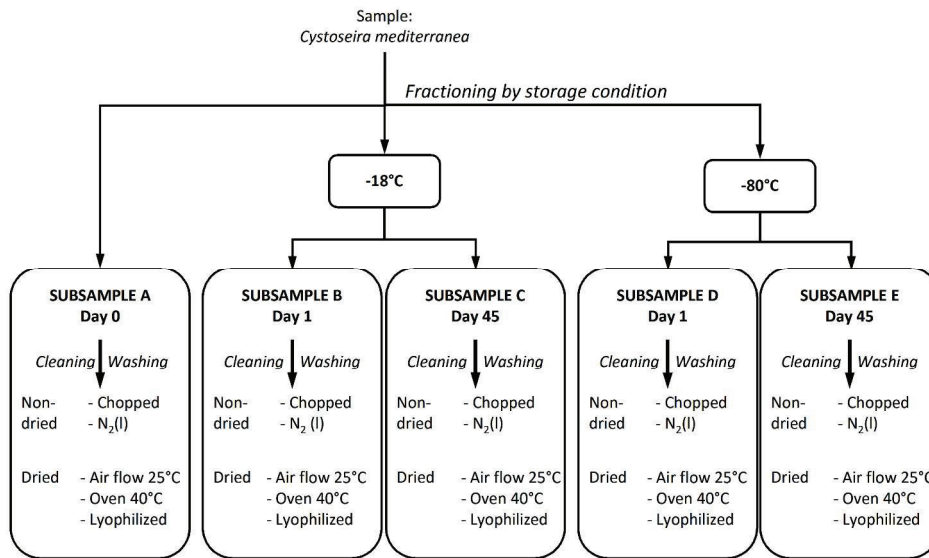
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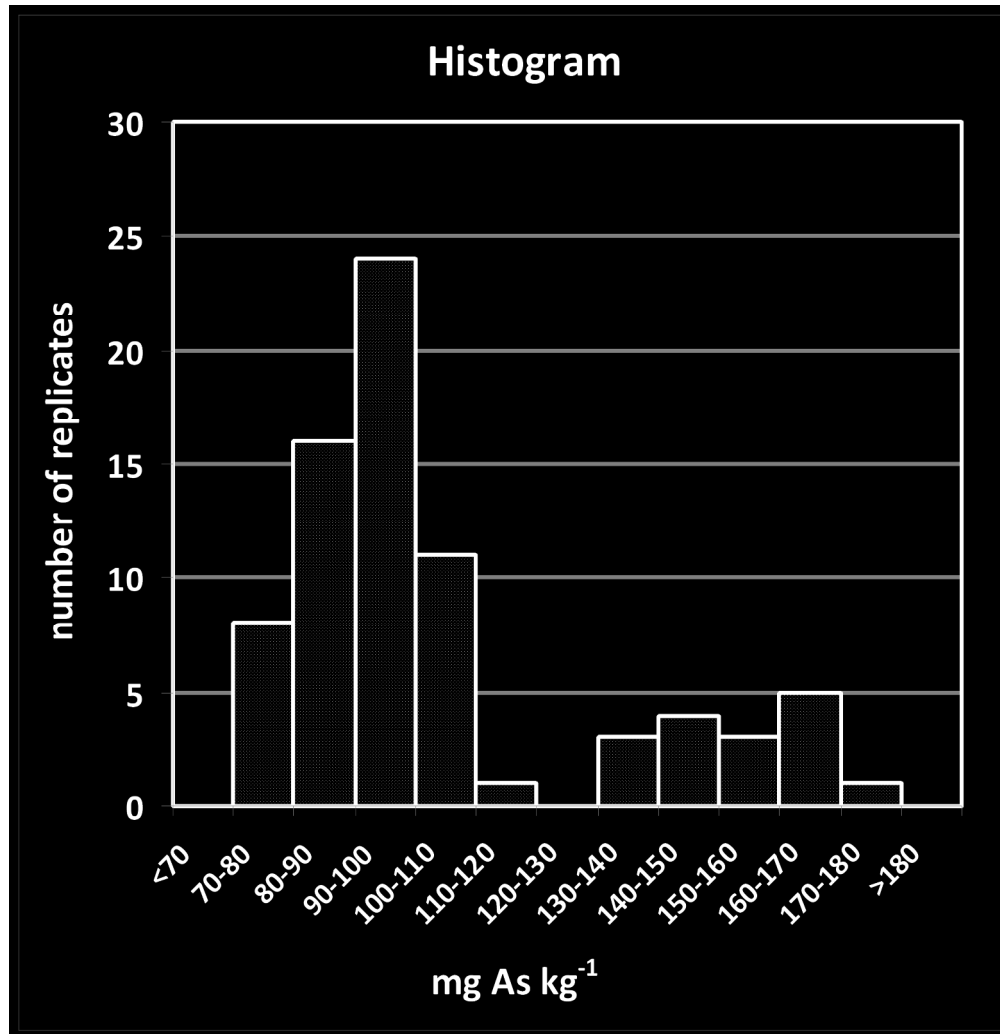


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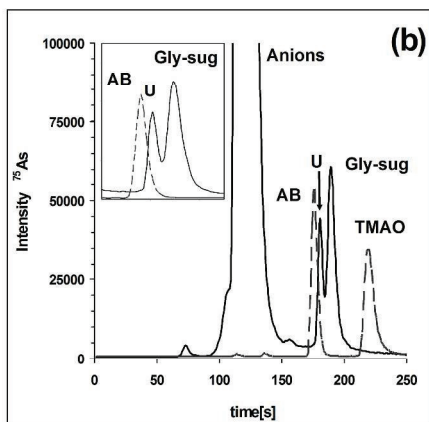
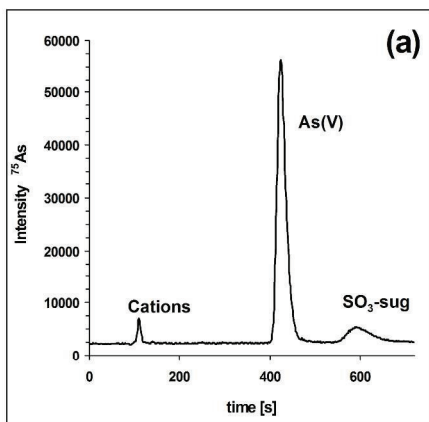




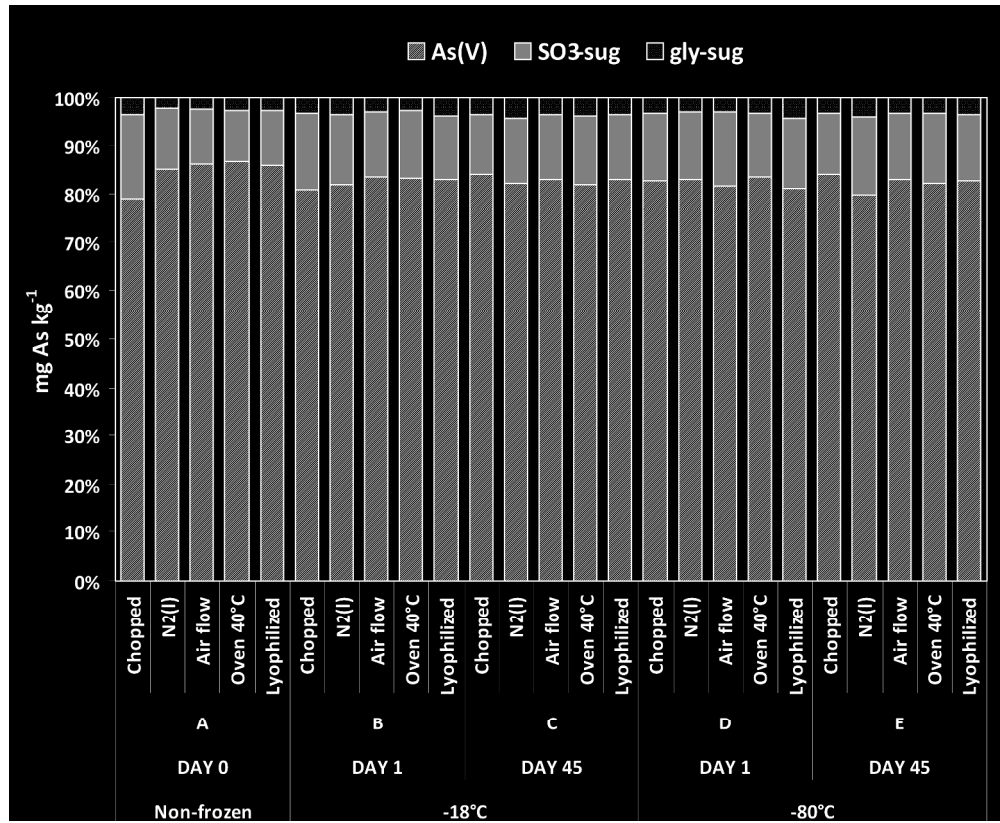
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**Referee 1**

1: There was a mistake in the cell alignment of Table 1. Standard deviation was added for extraction efficiency and column recoveries.

2: We modified the paragraph exposing the statistical analyses (line 24-37) in order to explain the performed tests.

Page 6, Fig 4B: We modified Fig 4, so that more information is added. As can be seen, an unknown peak cannot be attributed to AB since it runs after the standard. So the corresponding sentence on the discussion of Fig 4 has been added.

Test: Effects inter-subjects					
Dependent variable: Total As					
Origin	Square Sum (Type III)	Degrees of freedom	Quadratic Mean	F	Sig
Corrected model	45 055.857 <sup>a</sup>	9	5006.206	96.589	.000
Intersection	703 995.812	1	703 995.812	13 584.003	.000
Freezing	40 212.278	1	40 212.278	775.919	.000
Processing	3383.926	4	845.982	16.919	.000
Freezing*Processing	974.907	4	243.727	16.324	.002
Error	3368.648	65	51.825	4.703	
Total	85 561.861	75			
Corrected Total	48 424.505	74			

<sup>a</sup>R<sup>2</sup>=0.930 (Corrected R<sup>2</sup>= 0.921)

Statistical analyses made with SPSS®

**Referee 2**

Page 1, line 38: We agree with the suggestion of the Referee and we decided to delete the sentences: "In some cases contradictory conclusions have been drawn".

Page 1, line 54: We are working only with the water soluble species. We also used the same extraction for all samples, so the differences in the results would only be attributable to one of the factors we studied. We agree with the Referee that an interlaboratory comparison would have provided more robust findings, but in the aim of the present study does not consider such strategy.

Page 4, Table 2: There was a mistake in the cell alignment of Table 1. Standard deviation was added for extraction efficiency and column recoveries.

Page 4, Line 35. According to the comments of both Referees a new paragraph has been written to explain the performed statistical tests.

Page 5, line 40: The change for the gerund "confirming" was done as the Referee suggested.

Page 6, Fig 4B: We modified Fig 4, so that more information is added. As can be seen, an unknown peak cannot be attributed to AB since it runs after the standard. So the corresponding sentence on the discussion of Fig 4 has been added.

Page 6, Line 8: We removed the passage since we agree with the Referee on the lack of relevance here.

Page 6, Line 20: “~” was added

Page 6, Line 25: In the case of not washing the samples analyzed, epiphytes and other substances will be not removed. Our samples presented a high amount of 2-3 mm long crustaceans living on the surface of algae, it is known that these animals usually contain high amounts of arsenobetaine; therefore, the results obtained might not be comparable with those of washed algae.

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ARTICLE TYPE

## Effects of sample processing on arsenic speciation in marine macroalgae

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The stability of arsenic species in environmental samples during sampling, storage and processing is crucial for speciation studies. Losses, interconversion and degradation of arsenocompounds can occur during these steps as a result of interaction with the container material, microbial activity and temperature or light. In order to study the possible effects of storage and processing on arsenic compounds, subsamples of alga *Cystoseira mediterranea* Sauvageau were stored under different conditions (non-frozen; frozen at -18°C for 24 h and 45 days; and frozen at -80°C for 24 h and 45 days) and subjected to one of the following processing methods: chopping into fine pieces with a knife; grinding by hand in a glass mortar under N<sub>2</sub>(l); drying under an air current at room temperature (25°C); drying in an oven at 40°C; and lyophilization. The total contents of arsenic and arsenic compounds were then determined in these subsamples. Non-frozen subsamples registered 150 mg As kg<sup>-1</sup> while frozen-defrosted samples registered are 92 mg As kg<sup>-1</sup>. Nevertheless, in spite of this loss in content, the proportion of arsenic compounds was maintained in both cases. Our results indicate that freezing is an unsuitable storage method for macroalgae because losses of arsenic may occur. We recommend a drying method for this purpose.

### Introduction

The stability of chemical species of trace elements during sample handling and storage is a prerequisite to obtain reliable information in speciation studies. Given that biological samples are especially vulnerable to storage and handling conditions, these merit special care to preserve losses or changes in some elemental species.

A large number of studies have addressed arsenic speciation in marine organisms, including algae, over recent have generated high number of publications in the last decades. Many are dedicated to examining new compounds and their behavior, transformations and metabolic pathways because algae are crucial organisms in arsenic biogeochemistry and furthermore many algae are selected for human consumption.<sup>1-5</sup> Studies and reviews have reported on the influence of the storage, drying and powdering conditions on total arsenic and arsenic speciation in general or for particular compounds.<sup>6-9</sup> On the basis of these studies, it can be deduced that no common treatment is reliable and that handling and storage protocols should be established in function of the matrix composition, environmental conditions where the sample is collected (e.g. microbial activity, aquatic or dry media), chemical species to be determined, and analytical technique for their measurement.

Here we sought to ascertain the influence of sample storage conditions and further processing on the results of arsenic speciation in the brown alga *Cystoseira mediterranea*. For this purpose, we tested the effect of freezing algal samples at two

temperatures (-18°C and -80°C) for a range of periods, and of non-freezing storage methods. We also tested five sample processing methods (chopping, grinding under liquid nitrogen, air drying, drying at 40°C, and lyophilization) before the extraction of arsenic species. The same extraction method (water extraction) was applied in all cases in order to assure that the differences in the results are only attributable to the pre-treatment. We selected *C. mediterranea* Sauvageau (heterokonphyta, fucales) because of its abundance, availability, environmental interest and arsenic content; specifically, we worked with the alga branches because they are deciduous structures that can completely regenerate from the stipe. Communities of *C. mediterranea* grow along the northern Atlantic coast of Morocco, the Mediterranean coast of Spain, France, Tunisia, and Sicily and the southern Tyrrhenian coast of Italy.<sup>10</sup> This alga is characterized by perennial structures, such as holdfast and stipe, and annual deciduous branches. Several studies<sup>11, 12</sup> have proposed that this macroalga could be used as a bioindicator of the quality of coastal water as part of the European Water Framework Directive.<sup>13</sup> Some have reported values for total arsenic and arsenic species content in stipe medulla of *C. mediterranea*:<sup>14</sup> Total As 39.0 ± 1.2 mg As kg<sup>-1</sup>; As(III) 0.49 ± 0.19 mg As kg<sup>-1</sup>; DMA 0.26 ± 0.03 mg As kg<sup>-1</sup>; As(V) 1.17 ± 0.06 mg As kg<sup>-1</sup>; PO<sub>4</sub>-sug 0.39 ± 0.04 mg As kg<sup>-1</sup>; SO<sub>3</sub>-sug 19.6 ± 0.2 mg As kg<sup>-1</sup>; gly-sug 2.21 ± 0.21 mg As kg<sup>-1</sup>; and unknown anion 1.17 ± 0.10 mg As kg<sup>-1</sup>.

### Materials and Methods

## Reagents and Standards

All chemicals were of analytical grade. Millipore Milli-Q Plus Water (18.2 M $\Omega$  cm) was used for all solution preparations. Ammonium dihydrogen phosphate (Panreac, p.a.) and pyridine (Scharlau, p.a.) were used for anionic and cationic mobile phase preparation, respectively. pH was adjusted using ammonia solution (25%, Panreac, p.a.) and formic acid (98%, Panreac, p.a.). Sample digestion was achieved with nitric acid (69%, Panreac, Hiperpur) and hydrogen peroxide (31%, Merck, Selectipur). Potassium iodide (Panreac, p.a.), ascorbic acid (Panreac, p.a.), hydrochloric acid (37%, Panreac, p.a.) and sodium borohydride (Aldrich, purum, tablets) were used for arsenic determination by HG-AFS (hydride generation atomic fluorescence spectrometry). A solution of  $^9\text{Be}$ ,  $^{103}\text{Rh}$ ,  $^{205}\text{Tl}$  20  $\mu\text{g l}^{-1}$  (NIST High-Purity Standards) was used as internal standard for ICP-MS (inductively coupled plasma mass spectrometry) measurements.

## Arsenic standards

The following were used as arsenic standards in speciation: arsenite from  $\text{As}_2\text{O}_3$  (NIST, USA, Oxidimetric Primary Standard 83d, 99.99%); arsenate from  $\text{Na}_2\text{HAsO}_4 \cdot 7\text{H}_2\text{O}$  (Carlo Erba); methylarsonic acid (MA) as  $(\text{CH}_3)\text{AsO}(\text{ONa})_2 \cdot 6\text{H}_2\text{O}$  (Carlo Erba); dimethylarsinic acid (DMA) as  $(\text{CH}_3)_2\text{AsNaO}_2 \cdot 3\text{H}_2\text{O}$  (Fluka); arsenocholine (AC) as  $(\text{CH}_3)_3\text{As}^+(\text{CH}_2)\text{CH}_2\text{OHBr}^-$  supplied by the "Service Central d'Analyse" (CNRS Vernaison, France); arsenobetaine (AB) as  $(\text{CH}_3)_3\text{As}^+\text{CH}_2\text{COO}^-$  provided by BCR (CRM 626, standard solution) and trimethylarsenic oxide (TMAO) from  $(\text{CH}_3)_3\text{AsO}$  (Argus Chemicals srl). Standardized stock solutions of the arsenic compounds containing about 1000 mg  $\text{l}^{-1}$  were prepared in water, except arsenite, which was dissolved in 4 g  $\text{l}^{-1}$  NaOH solution (Merck, Suprapure). All solutions were stored in the dark at 4°C. Arsenate standard solution with a certified concentration of  $1000 \pm 2$  mg  $\text{As l}^{-1}$  from NIST High-Purity Standards was used for external calibration for the determination of the content with ICP-MS as well as for the standardization of MA, DMA, AC, AB and TMAO stock solutions. Arsenate standard was used for total arsenic determination in seawater by HG-AFS. An aliquot of freeze-dried extract of *Fucus serratus* dissolved in water was used as internal laboratory reference material for the identification of the major arsenosugars: phosphate ( $\text{PO}_4\text{-sug}$ ), sulfate ( $\text{SO}_4\text{-sug}$ ), sulfonate ( $\text{SO}_3\text{-sug}$ ), and glycerol (Gly-sug).<sup>15</sup> The certified reference material BCR CRM 279 Sea Lettuce (*Ulva lactuca*), from the Institute for Reference Materials and Measurements (IRMM) of the European Commission, with a certified value  $3.09 \pm 0.20$  mg  $\text{As kg}^{-1}$ , was used for quality control purposes in total arsenic determinations.

## Instruments and apparatus

A closed microwave digestion system, Milestone Ethos Touch Control, was used for sample digestion and CMR CRM BCR 279. HG-AFS Millennium Excalibur (PS Analytical, UK) was used for total arsenic measurement in seawater. The chromatographic system, consisting of an Agilent 1200 LC quaternary pump equipped with an autosampler, was connected to an anion-exchange and cation-exchange column and guard-column and coupled to an Agilent 7500ce ICP-MS (Agilent,

Germany) equipped with Ari Mist HP nebulizer (Burgener, Canada). The chromatographic program, instrumental operating conditions of LC-ICP-MS and the arsenicals that can be separated with each chromatographic system are given in Table 1. A LyoQuest -80 freeze-dryer (Telstar) was used during sample preparation. A P. Selecta oven was used in sample drying and humidity determination. A CVF 220/86 ultrafreezer was used to keep samples at  $-80^\circ\text{C}$ , and for  $-18^\circ\text{C}$  a Liebherr Economy GT3602 was used.

**Table 1** Chromatographic conditions used for arsenic speciation.

	Anion exchange	Cation exchange
Column	PRP-X100 (250 mm x 4.1 mm, 10 $\mu\text{m}$ ) (Hamilton, Reno, USA)	Zorbax SCX300 (250 mm x 4.6 mm, 5 $\mu\text{m}$ ) (Agilent, Waldbronn, Germany)
Pre column	PRP-X100 (20 mm x 2.0 mm, 10 $\mu\text{m}$ )	Zorbax SCX300 (12.5 mm x 4.6 mm, 5 $\mu\text{m}$ )
Mobile phase	$\text{NH}_4\text{H}_2\text{PO}_4$ 20mM	Pyridine 20 mM
pH	5.8	2.6
Injection volume	20 $\mu\text{l}$	20 $\mu\text{l}$
Flow rate	1.5 ml $\text{min}^{-1}$	1.5 ml $\text{min}^{-1}$
Column temperature	Room temperature	Room temperature
As species	As (III), DMA, MA, As (V), $\text{PO}_4\text{-sug}$ , $\text{SO}_3\text{-sug}$ and $\text{SO}_4\text{-sug}$	AB, TMAO, AC and Gly-sug

## Sampling and sample preparation

*Cystoseira mediterranea* (2 kg, pooled sample) was collected on 19<sup>th</sup> July 2011 in Lloret de Mar on the coast of Catalonia (Spain) in the Western Mediterranean Sea. This alga was collected in a rock platform close to the beach. Branches were cut with a sharp knife to prevent damage to the perennial regenerative structures.<sup>16</sup> Seawater (1 l) was also collected in PET bottles to obtain additional information on arsenic content of the growth medium of algae. The whole amount of sample was immediately transported to the laboratory and divided into five subsamples of 50 g each (Fig. 1). Subsample A was processed within 10 h of sampling (Day 0), subsamples B and C were stored at  $-18^\circ\text{C}$  and D and E at  $-80^\circ\text{C}$  in sealed plastic bags. Subsamples B and D were defrosted the day after freezing (Day 1) and C and E 45 days later (Day 45). After these periods of storage, the common cleaning consisted of removing epiphytes and other substances with tweezers and a short wash in deionized water (<10 s). Each subsample was then divided into five fractions and the following procedures were applied, as summarized in Fig. 1:

- **Chopping** into fine pieces with a stainless steel knife
- Grinding by hand in a glass mortar under  $\text{N}_2(\text{l})$
- Drying under an **air current** at room temperature ( $25^\circ\text{C}$ )
- Drying in an **oven at  $40^\circ\text{C}$**
- **Lyophilization**

Dried subsamples were ground by hand in an agate mortar and stored at room temperature in amber glass bottles until analysis. Non-dried samples were shortly stored at  $4^\circ\text{C}$  in the dark until acidic digestion and extraction.



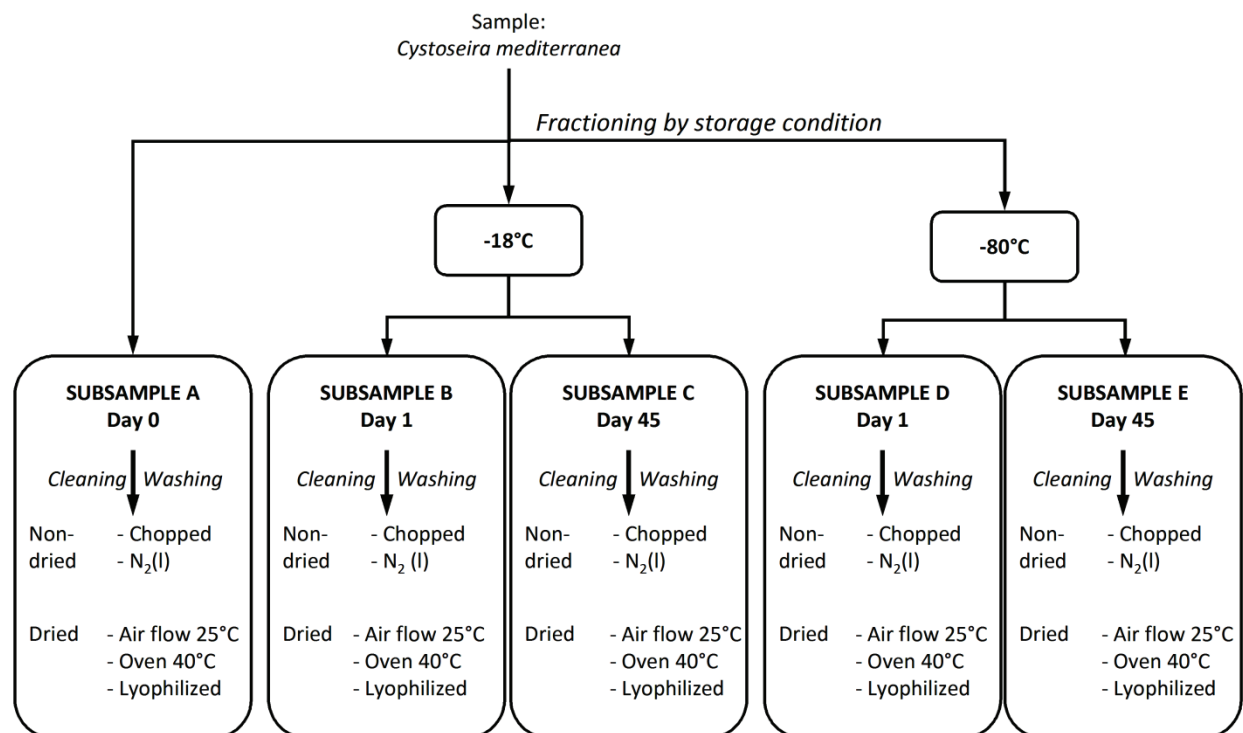


Fig. 1 Scheme of the storage and processing applied to subsamples.

### Total arsenic determination

Acidic digestion was performed in a middle-pressure closed microwave system. Samples (0.8 g of non-dried alga or 0.2 g of dried alga) were digested using the procedure reported for marine algae.<sup>14, 17</sup> Non-dried samples were digested within 2 days after sample processing and dried sample in a week lapses. Digests were diluted 1:50 with 1% HNO<sub>3</sub> before total arsenic measurement, which was performed by ICP-MS (external calibration, <sup>103</sup>Rh as internal standard, He as collision gas). All samples and BCR CRM 279 Sea Lettuce (*Ulva lactuca*) were analyzed in triplicate with their corresponding digestion blanks.

Arsenic from seawater was measured in triplicate by HG-AFS. Pre-reduction to increase the arsine-generation yield was performed as follows: 35 ml of seawater or arsenate standard (for calibration) was acidified with 7.5 ml of 37% HCl. We then added 5 ml of a reducing solution (10% KI, 2% ascorbic acid, 2 M HCl) and diluted this up to 50 ml. The resulting solution was diluted up to 50 ml and kept at 37°C for 2 h to ensure complete reduction of arsenate to arsenite before measurement. Quantification was performed by the standard addition calibration method.

### Arsenic speciation

Aqueous extracts of alga were obtained in an end-over-end shaker (35 rpm, 16 h) from 1 g of non-dried sample or 0.1 g of dried samples and 10 g of water. Extracts were centrifuged (3000 rpm, 15 min) and passed through PET syringe filters (Chromafil PET®, Macherey–Nagel, 0.45 μm) and stored at -80°C until analyses were performed. Arsenicals were extracted from non-dried samples the day they were collected (fresh-pretreated) or defrosted.

Extracts were defrosted immediately before measurements and homogenized under a vortex. Arsenic speciation was carried out in extracts by LC-ICP-MS using the method reported for marine algae.<sup>14, 17, 18</sup> Extracts were diluted with water (1:10 for dried samples and 1:25 for non-dried samples). LC-ICP-MS was used for measurement using anionic exchange chromatography for diluted extracts and while non-diluted extracts were analyzed by cationic exchange chromatography. Multispecies standards of As(III), DMA, MA and As(V) for anionic exchange chromatography and AB, AC and TMAO for cationic exchange chromatography covering the range 1 - 100 μg As l<sup>-1</sup> were prepared fresh daily for speciation analysis. Chromatographic peaks were identified on the basis of their retention times by comparison with standards. Arsenic species were quantified by external calibration curves. As(III) was quantified by subtracting to the front peak of anionic exchange chromatography the species quantified in cationic exchange chromatography. In addition, total arsenic in the extracts was measured in the conditions used for algal acidic digests.

For those species for which a quantification standard was not available, quantification was performed with the calibration curve of the closest standard in retention time as Francesconi and Sperling<sup>19</sup> suggested though this assumption has been questioned.<sup>20, 21</sup> Thus SO<sub>3</sub>-sug was quantified with the calibration curve of the As (V) standard and Gy-sug with the calibration curve of the AC standard; even though AB is the closest standard, AC does not show overlapping problems.

LODs and LOQs were estimated. The former is the lowest concentration of an analyte that can be reliably differentiated from background noise (signal-to-noise ratio greater than 3). The LOQ is the lowest concentration that can be measured (signal-to-

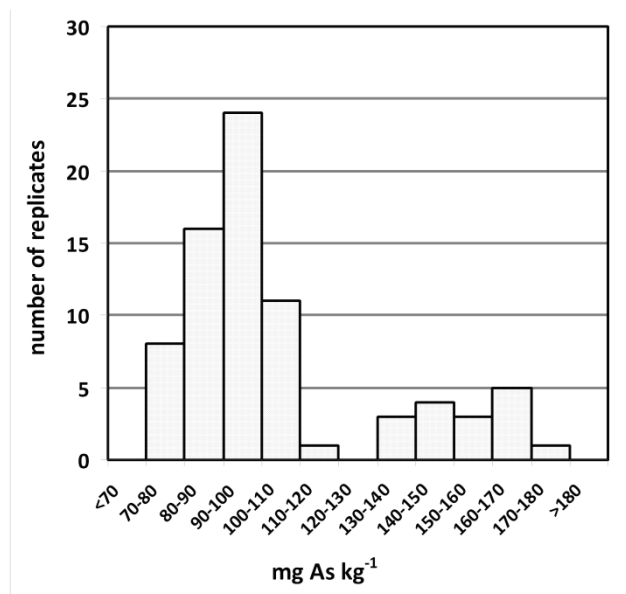
noise ratio greater than 10). For calculating LOD and LOQ, the standard deviation of the base line and the peak base of each analyte multiplied by 3 or 10 (LOD and LOQ respectively) were interpolated in the height calibration curve. The arsenosugar LOD

and LOQ were estimated through a correction factor, which is the relation within the concentration of arsenosugar in *F. serratus* and the peak height.

**Table 2** Total arsenic, arsenic species, extraction efficiency, column recovery and limits of detection and quantification (mean  $\pm$  standard deviation,  $n=3$ , 10 mg As kg<sup>-1</sup>, results expressed on dry weight basis)

Subsample	Storage	Processing <sup>a</sup>	Total As	As(V)	SO <sub>3</sub> -sug	gly-sug	Extraction efficiency	Column recovery		
DAY 0		C	140 $\pm$ 10	47 $\pm$ 3	11 $\pm$ 1	2.16 $\pm$ 0.08	57% $\pm$ 8%	78% $\pm$ 15%		
			N	150 $\pm$ 20	105 $\pm$ 2	15.3 $\pm$ 0.6	3.1 $\pm$ 0.2	97% $\pm$ 14%	87% $\pm$ 15%	
		A	A	163 $\pm$ 2	110 $\pm$ 10	14.1 $\pm$ 0.8	3.37 $\pm$ 0.07	94% $\pm$ 6%	83% $\pm$ 2%	
			O	160 $\pm$ 10	112 $\pm$ 4	13.5 $\pm$ 0.4	3.8 $\pm$ 0.2	97% $\pm$ 6%	82% $\pm$ 2%	
			L	140 $\pm$ 3	101 $\pm$ 4	13.2 $\pm$ 0.3	3.5 $\pm$ 0.2	104% $\pm$ 4%	81% $\pm$ 2%	
			C	82 $\pm$ 5	53 $\pm$ 3	10.4 $\pm$ 0.6	2.3 $\pm$ 0.2	91% $\pm$ 10%	90% $\pm$ 9%	
		B	N	83 $\pm$ 5	52.3 $\pm$ 0.5	9.18 $\pm$ 0.05	2.36 $\pm$ 0.02	97% $\pm$ 12%	80% $\pm$ 3%	
			A	101 $\pm$ 5	67 $\pm$ 2	10.5 $\pm$ 1	2.6 $\pm$ 0.1	99% $\pm$ 1%	80% $\pm$ 4%	
			O	103 $\pm$ 3	65 $\pm$ 6	10.5 $\pm$ 0.7	2.4 $\pm$ 0.1	94% $\pm$ 8%	80% $\pm$ 2%	
			L	97 $\pm$ 5	64.75 $\pm$ 0.01	10.3 $\pm$ 0.4	3.05 $\pm$ 0.08	102% $\pm$ 4%	79% $\pm$ 1%	
		DAY 1		C	100 $\pm$ 10	61 $\pm$ 0.3	10.3 $\pm$ 0.8	2.47 $\pm$ 0.09	90% $\pm$ 11%	82% $\pm$ 3%
					N	85 $\pm$ 1	65 $\pm$ 4	11 $\pm$ 0.3	2.5 $\pm$ 0.1	104% $\pm$ 12%
C	A			100 $\pm$ 2	61 $\pm$ 2	11.2 $\pm$ 0.3	2.45 $\pm$ 0.01	92% $\pm$ 4%	82% $\pm$ 3%	
	O			105.2 $\pm$ 0.4	67.1 $\pm$ 0.7	10.5 $\pm$ 0.5	2.9 $\pm$ 0.1	92% $\pm$ 5%	83% $\pm$ 4%	
	L			91 $\pm$ 1	55 $\pm$ 2	9.9 $\pm$ 0.1	3 $\pm$ 0.2	96% $\pm$ 1%	77% $\pm$ 2%	
	C			89 $\pm$ 6	66 $\pm$ 3	9.7 $\pm$ 0.6	2.9 $\pm$ 0.2	99% $\pm$ 4%	91% $\pm$ 1%	
D	N			79 $\pm$ 3	54.6 $\pm$ 0.5	8.9 $\pm$ 0.1	3 $\pm$ 0.2	96% $\pm$ 3%	88% $\pm$ 3%	
	A			95.8 $\pm$ 0.5	58 $\pm$ 1	9.3 $\pm$ 0.3	2.6 $\pm$ 0.2	90% $\pm$ 1%	81% $\pm$ 1%	
	O			93.6 $\pm$ 0.5	59 $\pm$ 1	10 $\pm$ 0.1	2.9 $\pm$ 0.1	93% $\pm$ 4%	82% $\pm$ 4%	
	L			84 $\pm$ 8	57 $\pm$ 1	9.3 $\pm$ 0.6	2.47 $\pm$ 0.08	95% $\pm$ 10%	86% $\pm$ 2%	
DAY 45				C	94 $\pm$ 10	77 $\pm$ 3	11.6 $\pm$ 0.6	3.1 $\pm$ 0.3	106% $\pm$ 11%	93% $\pm$ 1%
					N	76 $\pm$ 4	50 $\pm$ 1	10 $\pm$ 0.6	2.7 $\pm$ 0.1	91% $\pm$ 3%
		E	A	94 $\pm$ 5	63.8 $\pm$ 0.4	10.5 $\pm$ 0.5	2.68 $\pm$ 0.03	99% $\pm$ 6%	83% $\pm$ 2%	
			O	101 $\pm$ 7	65 $\pm$ 1	11.3 $\pm$ 0.3	2.8 $\pm$ 0.1	93% $\pm$ 6%	84% $\pm$ 3%	
			L	90 $\pm$ 2	62 $\pm$ 1	10.4 $\pm$ 0.5	2.73 $\pm$ 0.04	99% $\pm$ 1%	85% $\pm$ 3%	
			C	94 $\pm$ 10	77 $\pm$ 3	11.6 $\pm$ 0.6	3.1 $\pm$ 0.3	106% $\pm$ 11%	93% $\pm$ 1%	
		Limit of detection			0.003	0.08	0.41	0.016		
		Limit of quantification			0.01	0.22	1.36	0.05		

<sup>a</sup> C: non-dried, chopped; N: non-dried, ground with liquid N<sub>2</sub>; A: dried under air flow 25°C; O: dried in an oven at 40°C, L: lyophilized



**Fig. 2** Histogram representing total arsenic content in *C. mediterranea* subjected to various storages and processings

## 15 Results

### Seawater

Arsenic concentration in the seawater sample was  $1.4 \pm 0.2 \mu\text{g As l}^{-1}$ . This content is within the range of arsenic concentrations commonly reported for the Mediterranean Sea, which typically shows concentrations of  $1.1\text{--}1.9 \mu\text{g l}^{-1}$ .<sup>22-24</sup>

### *Cystoseira mediterranea*

All results are given on dry weight basis. Moisture was estimated as the weight loss at  $105 \pm 5^\circ\text{C}$  until constant weight.

Mean values for total arsenic ranged from  $76 \text{ mg As kg}^{-1}$  to  $163 \text{ mg As kg}^{-1}$  (see Table 2). An histogram was built with the data of all the replicates (Fig. 2) and two populations were identified: samples that were not frozen presented  $150 \text{ mg As kg}^{-1}$  and samples stored at  $-18^\circ\text{C}$  or  $-80^\circ\text{C}$  had a lower arsenic concentration ( $92 \text{ mg As kg}^{-1}$ ). A two factors ANOVA analysis of all data revealed that both, freezing and processing of samples, were significant ( $\alpha=0.05$ ). Subsequent Post-hoc analyses (DHS Turkey;  $\alpha=0.05$ ) found two defined subgroups for processing. Oven and air dried samples presented a mean of 110 and 113 mg As kg<sup>-1</sup>, respectively; whereas non-dried and lyophilized sample presented the following means: 94 (N) and 100 (C and L) mg As kg<sup>-1</sup>.

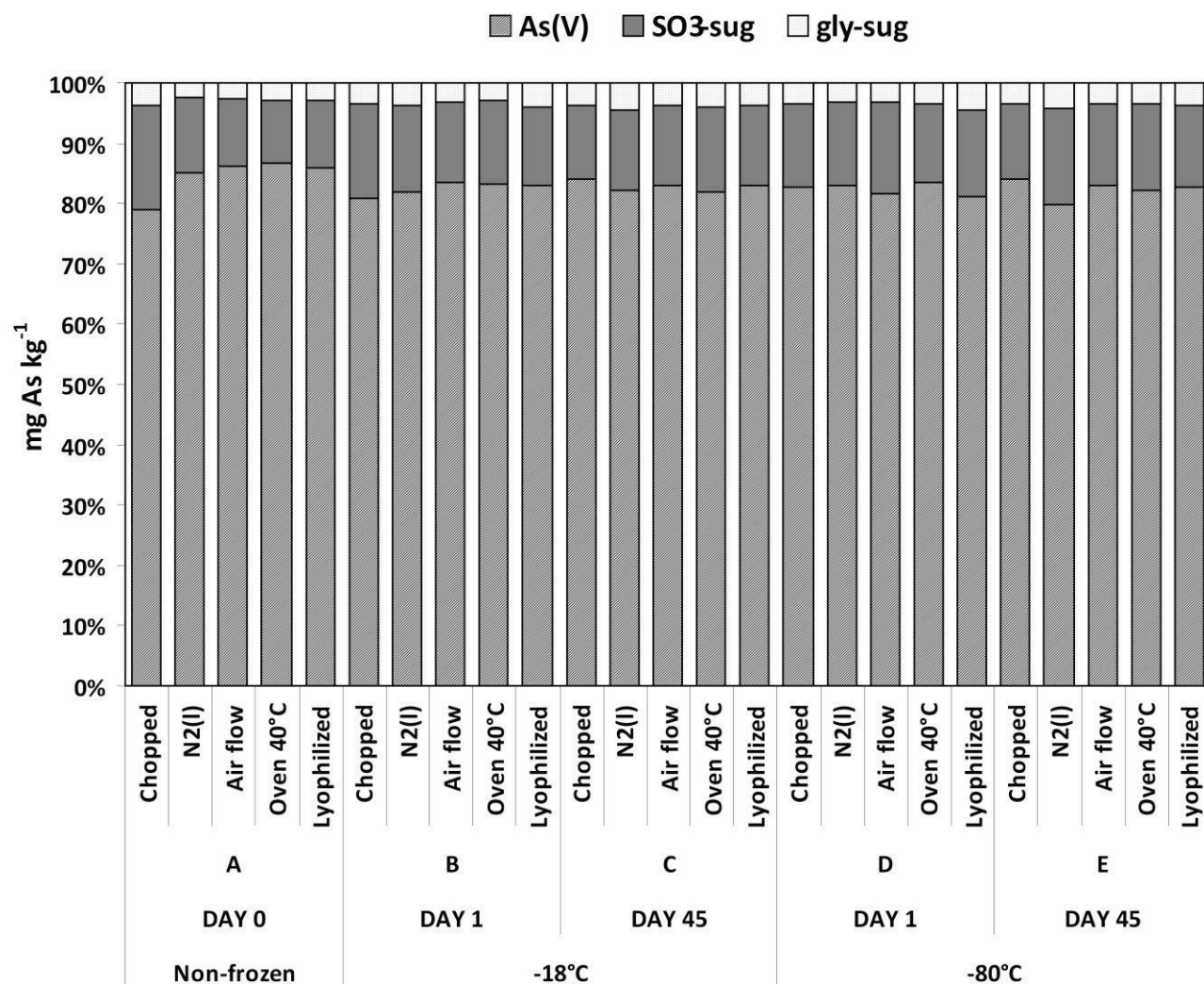


Fig. 3 Percentages of species in *C. mediterranea* subjected to various storages and processings

For arsenic speciation, extraction efficiency (calculated as total arsenic in the extract to total arsenic in digests) ranged from 89.5% to 105.9% except for non-frozen chopped samples, which registered an extraction efficiency of 57.0% (Table 2). All subsamples presented As(V), SO<sub>3</sub>-sug and Gly-sug in the same proportion with respect to the sum of species (Fig. 3). As an example, chromatograms of subsample A (ground under N<sub>2</sub>(l)) are shown in Fig. 4. It can be seen that an Unknown peak is eluting after AB, so that it cannot be attributable to this compound.

#### Quality assessment in the determination of arsenic and arsenic species

##### Column recovery

For quality assessment in speciation studies, column recovery must be evaluated in order to guarantee the correct chromatographic separation. With this aim, we calculated the ratio of the sum of the species eluted from the chromatographic columns to the total arsenic in the extract injected into the column. This parameter allowed us to properly evaluate the quantification of arsenic species. Column recoveries ranged between 77.3% and 92.9% (Table 2).

##### Certified reference material (CRM)

To check accuracy, total arsenic concentration was determined in CRM BCR 279 Sea lettuce (*Ulva lactuca*). The result obtained ( $2.9 \pm 0.3$  mg As kg<sup>-1</sup>) was consistent with the certified value ( $3.09 \pm 0.20$  mg As kg<sup>-1</sup>), thereby demonstrating the accuracy of the analytical method. Results for arsenic speciation for the CRM are shown in Table 3; in addition values found in the literature are given for comparison purposes.<sup>25, 26</sup> Although we used the simplest extraction method (water and end-over-end extraction), we obtained the same extraction efficiencies (57%) and only one reported result showed higher extraction but it was performed with MeOH:H<sub>2</sub>O<sub>2</sub> (1:1) at 75°C and assisted microwave.

##### Analysis of *F. serratus* extract

We used an extract from the brown seaweed *F. serratus* to identify arsenosugars present in our algae samples. For quality control purposes, we quantified As species in *F. serratus* extracts. Our results (DMA:  $0.01 \pm 0.01$  µg; Gly-sug:  $0.07 \pm 0.01$  µg; PO<sub>4</sub>-sug:  $0.07 \pm 0.01$  µg; SO<sub>3</sub>-sug:  $0.56 \pm 0.04$  µg; SO<sub>4</sub>-sug:  $0.37 \pm 0.02$  µg; values for *F. serratus* extract are given as absolute amount for extract µg.) confirming those reported by Madsen *et al.*<sup>15</sup> and other values in the literature on the same extract.<sup>14, 27-29</sup>

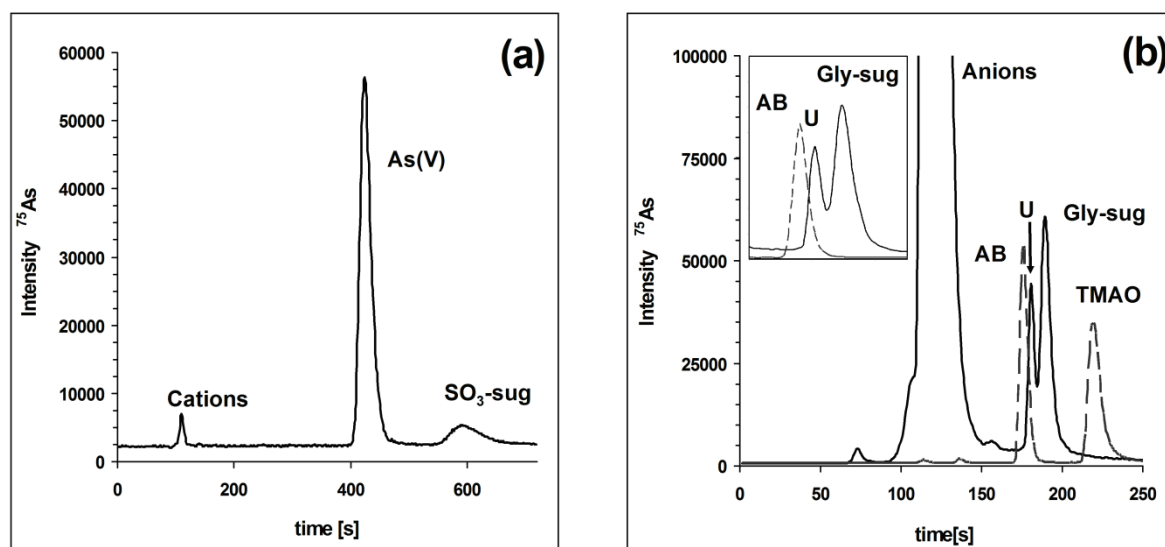


Fig. 4 Anionic (a) and cationic (b) exchange chromatograms of subsample A, which was non-dried and ground under  $N_2(l)$ . In figure 4b, the solid line corresponds to the sample and the dashed line to the AB and TMAO standard ( $10 \mu g As l^{-1}$ )

## Discussion

Total arsenic in *C. mediterranea* branches was  $150 \pm 10$  ( $n=15$ )  $mg As kg^{-1}$ . This value is the mean of the 15 replicates analyzed for subsample A not considering the processing method. This subsample was processed few hours after sampling and presented the highest arsenic concentration.

Extraction efficiencies ranged from 57% to 106% and are higher than usual values reported for alga,<sup>8</sup> thereby indicating that *C. mediterranea* presents most arsenic in water-soluble forms; thus we conclude that water is a suitable extracting agent for this macroalgae. However, fresh samples cut with knife give low extraction efficiencies. This observation can be attributable to the presence of unbroken cell structures that have the capacity to retain arsenic species.

Level of arsenic in all frozen samples was  $\sim 60\%$  lower than the non-frozen samples. Regarding processing methods, it should be taking into account that moisture is a crucial factor and little differences can result in wide variation when correcting for dry weight. For that reason, further studies are required to assess the effect of the processing methods. Furthermore, arsenic compounds did not show changes in their relative contents when we compared frozen and non-frozen samples. This behavior suggests that losses of total arsenic are related directly to the freezing/defrosting process. We postulate that algal cell structures might have broken during this process and arsenic compounds were removed when broken cells were rinsed away after defrosting. This is in agreement with the findings reported Le *et al.*<sup>30</sup> in a deep study of the effect of freezing as storage method. The authors also observed losses of total As in the range of 3-40% when defrosting samples. The same study reported that losses of As affected all species without discrimination. In the present study (see Fig. 3), no significant differences between the species in the extract of defrosted samples and those in the extract

of non-frozen samples were detected.

Each processing method has strengths and weaknesses; therefore, the choice of the method should depend on the kind of sample involved, the amount collected equipment availability, and period of storage before analyses, among other factors. Non-drying methods proved to be demanding and conditioned to the location of the laboratory to the sampling point. In addition, fresh samples cannot be preserved for long periods without damage to the algae and to the arsenic species and freezing showed to produce losses of large amounts of arsenic. Given all of the above points, drying methodologies are more suitable for arsenic speciation studies. Air-drying is a simple method; however, air-dried samples are difficult to pulverize. It is a suitable procedure when samples cannot be transported to the laboratory, and cleaning and drying should be performed *in situ* but avoided when further equipment is available. Lyophilization and oven-drying facilitate grinding but reports of losses of volatile compounds and species interconversion respectively are found in the literature;<sup>6</sup> however, we did not detect these processes.

## Conclusions and recommendations

On the basis of our results, we conclude that freezing is not a suitable storage method for macroalgae since this procedure leads to loss of arsenic. These losses are not detected in drying procedures.

We found that drying to be the most convenient processing method, we recommend lyophilization. Although this process requires a suitable system, it facilitates further pulverization of the sample, which is necessary to assure representativeness. Air-drying is recommended when sampling is performed in a remote location due to its simplicity. We do not recommend working with non-dried samples. Freezing proved to be a process to be avoided, so we do not recommend it.

**Table 3** Speciation results for CRM BRC 279 Sea lettuce (*Ulva lactuca*). Values for CRM reported on the literature are shown with comparison purposes.

Reference	Extracting agent	Extracting method	As(V) (mg As kg <sup>-1</sup> )	As(III) (mg As kg <sup>-1</sup> )	DMA (mg As kg <sup>-1</sup> )	MA (mg As kg <sup>-1</sup> )	TETRA (mg As kg <sup>-1</sup> )	AB (mg As kg <sup>-1</sup> )	gly-sug (mg As kg <sup>-1</sup> )	PO <sub>4</sub> -sug (mg As kg <sup>-1</sup> )	SO <sub>3</sub> -sug (mg As kg <sup>-1</sup> )	Unknown (mg As kg <sup>-1</sup> )	Extraction efficiency	Column recovery
Present study <sup>a</sup>	H <sub>2</sub> O	End-over end agitation	0.53 ± 0.04	0.06 ± 0.03	0.06 ± 0.03	0.04 ± 0.01		0.14 ± 0.02	0.096 ± 0.004	0.08 ± 0.01		0.07 ± 0.02	57%	81%
Foster <i>et al.</i> <sup>23</sup>	MeOH : H <sub>2</sub> O (1 : 1, v/v)	MW-assisted 70°C 15 min	0.52 ± 0.03		0.08 ± 0.01	0.07 ± 0.01	0.04 ± 0.01		0.05 ± 0.01	0.21 ± 0.03		0.01 ± 0.01	77%	
Foster <i>et al.</i> <sup>25</sup>	2% HNO <sub>3</sub>	MW assisted 95°C 6 min	1.2 ± 0.06		0.04 ± 0.01	0.03 ± 0.01			0.24 ± 0.03			0.16 ± 0.02	57%	92%
Caumette <i>et al.</i> <sup>26</sup>	H <sub>2</sub> O	60°C 4 h 15 min sonification	0.7					Traces	0.2	0.2	0.1	0.2	53%	87%

<sup>a</sup> (mean ± standard deviation, n=3, results expressed on dry weight basis).

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