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Arsenic speciation in fish from Greek coastal areas☆

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ABSTRACT

Arsenic speciation analysis was conducted on fish samples (sardine and anchovy) collected from six areas along the Greek coastline, *i.e.* Artemisium Straits, Thermaikos Gulf, Amvrakikos Gulf, Strymonian Gulf, Thracian Sea, and Elefsina Gulf. Total arsenic levels ranging from 11.8 to 62.6 mg As/kg dry weight were determined. Arsenobetaine, a non-toxic form of arsenic, was found to be the main arsenic species, present at 8.6 to 58.8 mg As/kg dry weight, accounting for 67–95% of the total arsenic. Also detected in all fish samples was dimethylarsinic acid, although at considerably lower concentrations, ranging from 0.072–0.956 mg As/kg dry weight. Monomethylarsonic acid was detected at low levels in all anchovy samples, and only in sardines from one area. Finally, inorganic arsenic in the form of arsenate was detected only in fish at one area, indicating the possible effect of an environmental parameter on its presence at detectable amounts. Statistical analysis revealed the environmental variables, such as salinity, total organic carbon and nitrogen, ammonium, phosphate, total phosphorus, dissolved oxygen and pressure index, are potentially correlated to As species concentrations. Furthermore, based on factor analysis, the biological parameters, such as fish weight, lipids, protein and ash content, that are correlated to As species concentrations of fish were also identified. The interrelationship of arsenobetaine and dimethylarsinic acid concentrations within each fish species was evaluated.

Introduction

Greeks rely heavily on seafood diets, especially fish and crustaceans, as a result their main exposure to arsenic (As) is *via* the consumption of seafood that contains elevated levels of As in various chemical forms, *i.e.* arsenic species (Cullen and Reimer, 1989; Edmonds and Francesconi, 1987). It is therefore critical from a food safety point of view for As speciation analysis to be conducted in order to determine As species and their quantities that are being ingested by humans and thus enable more accurate risk assessments (Fowler et al., 2015; Francesconi, 2010; Moreda-Piñeiro et al., 2012). Because many of the marine organisms in question originate from Greek coastal areas with considerable industrial activity, it is of interest to establish As species concentration levels and thus be able to identify if and when marine organisms are being exposed to elevated As concentrations in their environment.

Detection of As contamination in seafood and other marine organisms is critical both because of health risk and environmental issues. This is because different As species exhibit

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different physicochemical properties and toxicities (Cullen and Reimer, 1989; Hughes, 2002; Moe et al., 2016), *i.e.* inorganic As species, arsenite iAs(III) and arsenate iAs(V), are more toxic than the organo-As(V) forms, such as arsenobetaine (AsB), a compound that has been established to be non-toxic and the main As component in fish (Neff, 1997; Sharma and Sohn, 2009). However, it should be stressed that detecting As contamination in fish present in the natural environment is particularly difficult, mainly because fish bioaccumulate As naturally. Therefore, even when marginally elevated As concentrations are found in marine biota, it is extremely difficult to associate these levels directly to anthropogenic contamination or even naturally occurring elevated As levels. This is because the source of AsB, the main As species in marine fish, is not completely understood (Popowich et al., 2016; Zhang et al., 2016b), and thus it is believed to originate from their diet (Azizur Rahman et al., 2012; Cullen and Reimer, 1989; Larsen and Francesconi, 2003; Zhang et al., 2012) or as some studies have suggested to form as part of their metabolism (Foster and Maher, 2016; Zhang et al., 2016b). So the dietary status of fish may be affecting the extent of As bioaccumulation and thus As species levels (Maher, 1985; Maher et al., 1999; Zhang et al., 2016c), especially AsB levels (Amlund et al., 2006a; Larsen and Francesconi, 2003; Zhang et al., 2016b). In addition, environmental factors can affect dietary supplies/food availability (Bonanno et al., 2014; Martino and Houde, 2010; Rumolo et al., 2016) and thus indirectly influence As species levels.

So far only a limited number of As speciation studies in fish collected from Greek coastal areas have been conducted, especially for fish species that are of high consumption such as the sardines and anchovies analyzed here. To the best of our knowledge only the study by Schaeffer et al. (2005) has so far reported As speciation for fish originating from Greek coastal areas. Also, limited reports for As speciation in these fish species, from other areas around the world, have been made so far (Kucuksezgin et al., 2014; Moreda-Piñeiro et al., 2012; Muñoz et al., 2000; Özcan et al., 2016; Rattanachongkiat et al., 2004; Schoof and Yager, 2007).

The objective of the present study was to determine As species levels in two fish species, *i.e.* sardine and anchovy, originating from various areas along the extended Greek coastline. These results are compared to those found in a 2005 study involving the same fish species and similar Greek coastal regions (Schaeffer et al., 2005). It is also of interest to investigate the correlation of environmental and fish biological parameters with As species in fish. This is expected to provide improved insight into the factors affecting the bioaccumulation and metabolism of As species in fish by distinguishing them in terms of fish diet and fish metabolism. Detailed statistical analysis was performed in order to help reveal existing correlations between As species and biological and environmental parameters.

1. Materials and methods

1.1. Sample collection and preparation

Sardine (*Sardina pilchardus*) and European anchovy (*Engraulis encrasicolus*) samples (n = 180) were collected from six different coastal areas in Greece, Artemisium Straits (ART), Thermaikos Gulf (THE), Amvrakikos Gulf (AMV), Strymonian Gulf (STR), Thracian Sea (THR) and Elefsina Gulf (ELE), shown in Fig. 1. All fish samples belonged to a gonad stage level 1. These fish species are consumed in high amounts (approximately 40% of the population is a high consumer of these fish) (Schaeffer et al., 2005). Sampling from all areas took place from September to October 2013.

Immediately after collection, total length and total weight of fish were measured, all edible parts (skin, flesh and bone) were dissected, placed in zip-lock bags and stored at −20°C. All samples were subsequently freeze-dried and stored under dry conditions. For each fish species and area, three composites were prepared by mixing and homogenizing five individuals per composite. Chemical determinations were carried out in triplicate for each composite, thus providing nine replicate analyses for each fish species and area.

1.2. Reagents and standards

Aqueous solutions containing individual arsenic compounds were prepared from sodium arsenite (iAs(III)), $NaAsO_2$ (BDH Chemicals Ltd., Poole, England), from sodium arsenate dibasic heptahydrate $Na_2HAsO_4{\cdot}7H_2O$ (iAs(V)), from dimethylarsinic acid (DMA) or cacodylic acid (Fluka) and from monosodium acid methane arsonate sesquihydrate (Chem Service, West Chester, PA) referred to in the remainder of this manuscript as monomethylarsonic acid (MMA). Arsenobetaine (AsB) or 2-(trimethylarsonio)acetate stock solution was obtained from Sigma-Aldrich. These As species stock solutions were diluted with deionized water (Milli-Q 18.2 MΩ cm) to the desired concentrations before use. For the analytical procedures the following chemicals were used: di-ammonium hydrogen phosphate from Sigma-Aldrich, and hydrogen peroxide and nitric acid from Merck. Total As standard of 10,000 μg/mL from CPI International was used for making standards for total As determination. Indium standard of 1000 μg/mL from CPI International was used to prepare the internal standard for total As.

1.3. Instrumentation

A Flexar high performance liquid chromatography (HPLC) pump (Perkin Elmer) was used for mobile phase delivery at 1 mL/min for the HPLC separations. The pump outlet was connected to a Rheodyne injection valve fitted with a 20 μL loop, followed by an anion exchange HPLC column. The column effluent was introduced into the inductively coupled plasma mass spectrometer (ICP–MS) *via* a pneumatic nebulizer (Meinhard, Elemental Scientific Glassblowing, Golden, CO). The ICP–MS used was a NexION 300xx ICP–MS (PerkinElmer, Shelton, CT, U.S.) which had been optimized for maximum. In sensitivity, CeO^+ levels below 3%, and Ce^{++} levels below 2.5%. Additional m/z ratios were also monitored in order to detect any $ArCl^+$ interferences (m/z 77), as well as Se (m/z 78 or 82) in case corrections were needed.

1.4. Total arsenic determination

Concentration of total arsenic was determined in each composite separately using a modification of the US EPA

Fig. 1 – Six sampling areas (★) along the Greek mainland coastline, at which both sardine and anchovy samples were collected (Artemisium Straits: ART; Thermaikos Gulf: THE; Amvrakikos Gulf: AMV; Strymonian Gulf: STR; Thracian Sea: THR; and Elefsina Gulf: ELE).

Method 3052 for microwave-assisted acid digestion of organically based matrices. After predigestion with concentrated acids, acid digestion was performed in a closed, high pressure, microwave system (Multiwave 3000, Anton Paar, Austria). For the measurement of As concentration in the sample digests, an ICP–MS (NexION300, PerkinElmer, Shelton, CT, U.S.) was used, according to US EPA Method 6020A. An optimal sample dilution factor of 600 was chosen. An internal standard containing indium (10 μg/L) was added to each sample. The applied protocols are described in detail elsewhere (Kalantzi et al., 2013, 2016). Dataset quality assurance was performed by analyzing one blank and one certified reference material (CRM) with every 6 samples digested. The following CRMs were used: DORM-4 (fish protein), LUTS-1 (non-defatted lobster hepatopancreas) certified by the National Research Council of Canada and BCR-668 (mussel tissue) certified by the Joint Research Centre of European Commission. Average recoveries (± standard deviation) for total As from DORM-4, LUTS-1 and BCR-668 were 91.1 ± 3.7% (n = 7); 111.8 ± 4.1% (n = 6); and 103.2 ± 2.3% (n = 5), respectively. The limits of detection (LOD) of the procedure for total As determination were calculated by multiplying the standard deviation of the blanks (n = 19) by three and for As was determined to be 0.05 mg/kg dry weight (dw).

1.5. Arsenic speciation analysis: extraction and separation

Freeze-dried fish powder (0.03 g) was placed in 1.5 mL polyethylene tubes and extracted with 1.2 mL of $(NH_4)_2HPO_4$ 10 mM, pH 7.9. The mixture was then shaken at ambient temperature for 1 min using a vortex mixer, followed by sonication for 3 min in an ultrasonic bath (Ultrasonic Cleaner Elmasonic S025EL) at 40°C. This 2-step procedure was repeated 10 times. At the end of the 40 min procedure an additional 10 min sonication cycle, under the same conditions, was applied. In total each sample was extracted for 50 min. The resulting mixtures were then centrifuged at 3700 rpm and the supernatants were filtered through 0.45 μm nylon filters (Pall IC Acrodisc 13 mm syringe filter) before HPLC analysis.

Anion exchange chromatography (PRP-X100, 250 mm × 4.1 mm i.d., Hamilton, USA) was used for As species separations. A mobile phase consisting of 10 mM $(NH_4)_2HPO_4$ at pH 7.9 was delivered isocratically at a flowrate of 1 mL/min using a PerkinElmer Flexar HPLC system. Samples were injected onto the column *via* a 20 μL injection loop.

1.6. HPLC–ICP–MS As speciation method validation

In the present study, an HPLC–ICP–MS method suitable for determining five As species, *i.e.* AsB, DMA, MMA, iAs(V), and

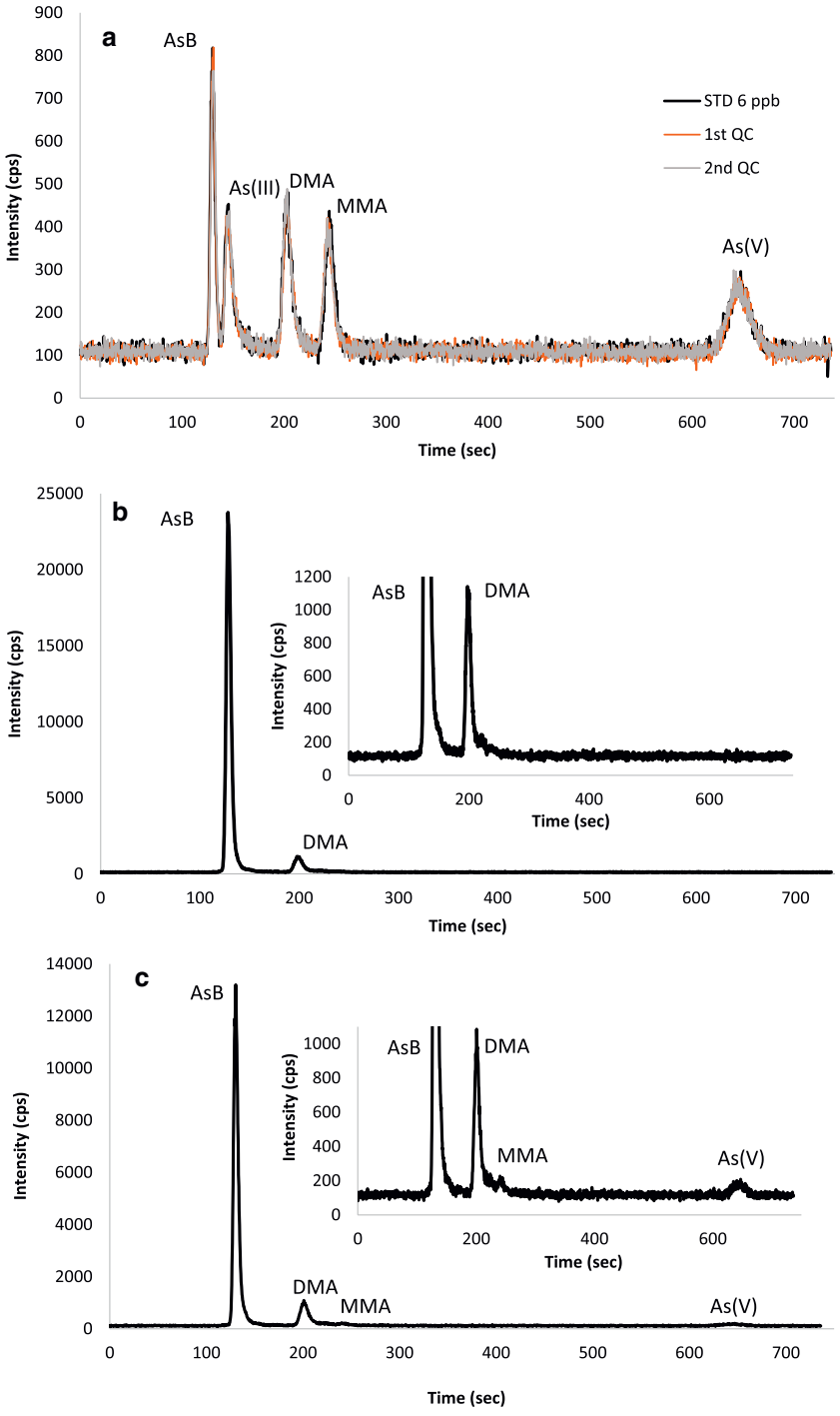

Fig. 2 – HPLC–ICP–MS chromatograms of As species in quality control samples (a); representative fish extract (b) and DORM-4 certified reference material (c). AsB: arsenobetaine; DMA: dimethylarsinic acid; MMA: monomethylarsonic acid; iAs(V): arsenate; iAs(III): arsenite.

iAs(III) was used (Fig. 2a). This method provided solution LODs in the low to sub μg As/ L range for all five species, *i.e.* 0.4 for AsB, 0.2 for iAs(III), 0.3 for DMA, 0.1 for MMA, and 1.0 for iAs(V), for 20 μL sample injections. LODs were calculated from the calibration graph sections close to the origin (from 0 to 12 ng As/ L for each As species) using the slope and the $s_{y/x}$ value. The term $s_{y/x}$ estimates random errors in the measured signal intensity (y-direction) and can be used instead of s_B (standard deviation of blank measurements) (Miller and Miller, 2000). LOD concentrations were calculated as (3 · slope) / $s_{y/x}$. These values gave the following As species LODs in the dry weight fish material: 20 μg As/ kg for AsB,

10 μg As/ kg for iAs(III), 10 μg As/ kg for DMA, 5 μg As/ kg for MMA, 30 μg As/ kg for iAs(V). A representative HPLC–ICP–MS chromatogram of a fish extract is presented in Fig. 2b.

For method validation several extracts from the reference material DORM-4 (fish protein) certified by the National Research Council of Canada (NRCC) were analyzed with each batch of sample fish extracts. The applied method showed DORM-4 extracts to contain AsB, DMA, MMA and iAs(V) at average values (±SD) of 4.020 ± 0.009, 0.607 ± 0.008, 0.054 ± 0.020 and 0.086 ± 0.035 mg/kg dw, respectively (Fig. 2c). The average recovery (±SD) for AsB in DORM-4 was 101.8 ± 8.8% (n = 3), based on the certified values provided by NRCC for AsB (National Research Council Canada, 2015).

1.7. Environmental and biological variables

All the areas reported in the present publication were characterized in terms of ecological indices for nutrients and chemical contaminant stress factors, data used here were taken from Simboura et al. (2016), hereinafter referred to as environmental variables, based on monitoring activities in the framework of the implementation of Water Framework Directive 2000/60/EC (WFD) in Eastern Mediterranean (Greece) during 2012–2014 (Simboura et al., 2016). For each sampling area of the present study, a wide range of stations in the surrounding area was selected in order to estimate the variance of each environmental variable in each sampling area. Environmental variables with non-significant differences amongst areas were excluded (data not shown). The mean values of the selected environmental variables for each area, including salinity, total organic carbon (TOC) and total organic nitrogen (TON), transparency (Secchi depth), dissolved oxygen (DO), phosphate (PO_4), ammonium (NH_4), total nitrogen (TN) and total phosphorus (TP), particulate organic carbon (POC) and particulate organic phosphorus (POP), chlorophyll-a (Chl-a) and pressure index (PI), are summarized in the Supplementary Data (Appendix A Table SD-1). The pressure index (PI) of the water bodies, presented in Appendix A Table SD-1, estimates the magnitude of the anthropogenic pressures imposed and was calculated as the average intensity of all pressure types and ranges from: 0 (no or minor pressures); 0.1–0.44 (slight pressure); 0.56–1 (moderate pressure); 1.11–1.44 (high pressure); 1.56–2 (heavy pressure) (Borja et al., 2011; Simboura et al., 2015, 2016). The classification of the pressures was based on the Water Information System for the Europe (WISE-SoE) for the coastal and marine waters (Simboura et al., 2015, 2016).

Several biological parameters of the examined fish were estimated according to the following procedures. Total moisture and ash content were determined by weighing mass differences before and after heating (AOAC, 1990). Lipid content of the freeze-dried samples was estimated gravimetrically (Folch et al., 1957). Protein content was estimated using the Dumas combustion procedure (Nitrogen Analyzer: Leco Model FP-528) (AOAC, 1990).

1.8. Data analysis

For statistical analysis purposes, the concentrations of total arsenic and arsenic species found to be below their LOD were set equal to 0.5 × LOD. This was done if more than 50% of samples had a concentration of residue that exceeded the LOD; otherwise these analytes were excluded from the analysis (USEPA, 1991). Differences in concentrations of total arsenic and arsenic species between anchovies and sardines for each sampling area and between sampling areas for each fish species were tested by non-parametric Kruskal–Wallis test. Differences of the values of the environmental and biological variables between the examined sampling areas were also checked using the Kruskal–Wallis test. Factor analysis was applied in order to detect any significant correlations of the concentrations of total arsenic and arsenic species in fish with (i) the selected environmental variables for each species and all areas and (ii) the fish biological parameters for each area for both species. All data were normalized to avoid misclassifications arising from the different orders of magnitude of both numerical values and variance of the parameters analyzed. Statistical analysis was performed using STATISTICA v.8.0 (StatSoft INC) software.

2. Results and discussion

2.1. Total As in sardines and anchovies samples from Greek coastal areas

Arsenic concentrations ranging from 11.8 to 62.6 mg/kg dw were determined in digested fish samples (Table 1). More specifically, of the two fish species examined, anchovies were found to contain the highest As concentrations at four of the sampling areas (ELE, THR, AMV and THE), whereas higher but not significant As concentration differences were observed at the remaining two areas (STR and ART) (Appendix A Table SD-2; Kruskal–Wallis $p < 0.05$). The highest total As concentration in anchovies was 62.6 mg As/ kg dw for fish originating from ELE, compared to the highest sardine value of 45.2 mg As/ kg dw, also originating from ELE (Table 1; Appendix A Table SD-3; Kruskal–Wallis $p < 0.05$).

These findings were compared to those reported in a study by Schaeffer et al. (2005) in which total As was determined in the same fish species, originating from some common regions in Greece (Appendix A Table SD-4). In that study anchovies were also found to contain the highest As concentrations ranging from 13 to 26 mg As/ kg dw, whereas sardines contained 9.48 to 13.5 mg As/ kg dw. From these values we observe that average As concentrations are elevated in the present study. More specifically, both sardines and anchovies from ELE showed elevated concentrations of total As by 4 to 6 times compared to the concentrations reported in the 2005 study. On the other hand, for fish from THE, the concentrations of total As in anchovies showed a decrease by a factor of 2 in the present study compared to the respective concentrations measured in the Schaeffer et al. (2005) study. However, it is difficult to accurately explain these differences because the exact areas within the reported sampling regions are unknown, as is the fish age and the time of year samples were obtained for the earlier 2005 study.

In a study by Li et al. (2003) on As in sardines taken from Chinese supermarkets, originating from the East Sea, it was reported that they contained significantly lower total As

Table 1 – Biological variables and concentrations of arsenic species (mean ± SD) in edible parts of sardine and anchovy (*n* = 15) from the six different sampling areas.

	Area	Total wet weight	Total length	Protein content	Lipids content	Moisture	Ash content	Total As	AsB	DMA	MMA	iAs(V)	iAs(III)	AsB to total As	EE
		g	mm	% ww	% ww	% ww	% ww	mg As kg^{-1}dw						%	%
Sardine	ART	12.1 ± 3.1	121.5 ± 10.0	22.5 ± 0.5	7.0 ± 1.5	68.3 ± 1.6	2.7 ± 0.1	18.9 ± 1.2	15.0 ± 1.2	0.462 ± 0.167	nd	0.028 ± 0.016	nd	79 ± 2	82 ± 1
	THE	10.3 ± 3.3	111.7 ± 10.4	26.1 ± 1.1	6.6 ± 1.7	64.8 ± 1.6	3.4 ± 0.1	11.9 ± 0.7	8.6 ± 0.5	0.523 ± 0.157	nd	nd	nd	72 ± 2	77 ± 1
	AMV	12.1 ± 2.1	115.7 ± 7.3	21.6 ± 0.2	8.8 ± 0.5	60.7 ± 0.7	2.2 ± 0.1	18.1 ± 1.4	12.2 ± 0.5	0.956 ± 0.158	0.073 ± 0.010	nd	nd	67 ± 3	73 ± 3
	STR	11.7 ± 3.6	117.1 ± 10.2	25.0 ± 0.6	5.9 ± 1.1	67.3 ± 0.8	2.9 ± 0.1	12.9 ± 0.2	10.6 ± 0.7	0.311 ± 0.063	nd	nd	nd	82 ± 4	85 ± 5
	THR	13.9 ± 2.5	120.1 ± 5.8	22.4 ± 1.5	8.8 ± 1.8	65.3 ± 1.2	2.9 ± 0.2	11.8 ± 0.3	8.0 ± 0.1	0.623 ± 0.195	nd	nd	nd	68 ± 1	73 ± 2
	ELE	9.5 ± 2.1	108.2 ± 7.7	27.6 ± 1.7	5.4 ± 1.1	64.4 ± 2.2	2.8 ± 0.5	45.2 ± 3.4	41.4 ± 9.2	0.295 ± 0.016	nd	nd	nd	91 ± 19	92 ± 19
Anchovy	ART	5.5 ± 1.0	102.4 ± 4.9	25.4 ± 0.7	1.8 ± 0.1	70.3 ± 0.7	2.9 ± 0.1	19.9 ± 0.6	18.1 ± 1.6	0.066 ± 0.022	0.020 ± 0.011	0.046 ± 0.015	nd	91 ± 6	92 ± 6
	THE	6.8 ± 0.8	107.5 ± 3.4	29.4 ± 0.6	1.9 ± 0.2	65.8 ± 0.6	3.2 ± 0.2	13.1 ± 0.5	12.4 ± 1.0	0.072 ± 0.032	0.025 ± 0.002	nd	nd	95 ± 7	96 ± 7
	AMV	3.9 ± 0.6	87.1 ± 4.6	30.4 ± 0.6	3.1 ± 0.1	61.4 ± 0.4	3.6 ± 0.2	23.8 ± 3.9	23.3 ± 4.2	0.147 ± 0.058	0.037 ± 0.000	nd	nd	97 ± 2	98 ± 2
	STR	7.3 ± 2.1	107.0 ± 9.8	25.1 ± 0.1	2.1 ± 0.1	70.1 ± 0.3	2.7 ± 0.1	13.4 ± 0.9	10.8 ± 2.4	0.112 ± 0.017	0.025 ± 0.005	nd	nd	80 ± 15	81 ± 15
	THR	8.2 ± 1.3	113.3 ± 5.2	27.7 ± 1.8	1.7 ± 0.1	67.6 ± 2	2.9 ± 0.2	13.3 ± 0.7	11.8 ± 1.0	0.098 ± 0.053	0.007 ± 0.003	nd	nd	89 ± 3	90 ± 2
	ELE	4.6 ± 2.0	91.3 ± 10.9	33.2 ± 3.5	1.9 ± 0.1	59.3 ± 3.3	3.9 ± 0.4	62.6 ± 12.2	58.8 ± 9.0	0.084 ± 0.010	0.023 ± 0.001	nd	nd	94 ± 4	94 ± 4

nd: not detected; dw: dry weight; ww: wet weight; AsB: arsenobetaine; DMA: dimethylarsinic acid; MMA: monomethylarsonic acid; As(V): arsenate; As(III): arsenite; EE: extraction efficiency (=sum of identified As / Total As × 100); ART: Artemisia; THE: Thermaikos Gulf; AMV: Amvrakikos Gulf; STR: Strymonian Gulf; THR: Thracian Sea; ELE: Elefsina Gulf.

concentrations at 0.64 mg As/ kg (expressed in wet weight, as opposed to dry weight concentrations presented here). Suner et al. (2001) reported total As in sardine samples purchased for local retail outlets (Valencia, Mediterranean Sea) to contain 3.9 mg As/ kg dw, which are also values significantly lower than those determined in the present study. However, direct comparisons are also difficult because of lack of any additional information about environmental and biological parameters. A recent study on As speciation in fish obtained from markets in 2010 originating from the Black Sea coast of northern Turkey reported anchovy to contain a total of 12.6 μg As/ g dw (Özcan et al., 2016). Also, comparable total As concentrations, to the present study, of 5.999 μg As/ g wet weight were reported in Japanese anchovy (Choi et al., 2015).

2.2. As speciation in sardines and anchovies samples from Greek coastal areas

The results of the As speciation analysis in the fish extracts are summarized in Table 1. Arsenobetaine (AsB) was determined in all analyzed samples at concentrations ranging from 8.0 to 58.8 mg As/ kg dw, which corresponds to 67–97% of the total As in these fish. DMA was also determined in all fish samples in the range of 0.066 to 0.956 mg As/ kg dw, corresponding to only 0.14–5.3% of the total As. MMA was detected in all anchovy samples, but only in sardines from AMV. Arsenate, iAs(V), in the range of 0.028–0.046 mg As/ kg (dw) was detected in sardines and anchovies only from ART, but was not detected in fish from any of the other areas. It should be noted that the iAs(V) concentrations are close to the method LOD, but clearly observed in the recorded chromatograms (Fig. 2c). Whereas arsenite, iAs(III), was not detected in any of the fish samples analyzed in the present study (LOD for As(III) in fish material was determined to be 0.01 mg As/ kg dw). Finally, arsenocholine (AC) could not be detected using the method we applied, however, its presence in fish has only been reported in a few sporadic cases at extremely low levels (Choi et al., 2015; Ciardullo et al., 2010; Schaeffer et al., 2005).

Overall, the determined As species accounted for 73 to 92% of the total As in sardines and from 81 to 98% of the total As in anchovies (Table 2). This combined As species extraction/column recovery percentage is to be expected for a water-based extraction procedure, as was used in the present study, for fish having a considerable lipid content. In fact, the extraction/column recovery efficiency showed a strong negative correlation with fish lipid content for all areas, except ELE (Table 2). Overall, higher As extraction/column efficiencies were observed for the anchovy (lower lipids), compared to sardine (higher lipids), except for fish sampled from the STR and ELE (Appendix A Table SD-2). Higher lipid content is also strongly associated with elevated arsenolipids (Amayo et al., 2014), which would not be expected to be extracted using the approach adopted in the present study.

All of the As speciation data reported here have been obtained from fish originating from the same season of the year, *i.e.* Sep.–Oct. 2013. This is important to note because even though there could potentially be a seasonal cycle effect on the concentrations of inorganic and organic arsenic in tissues of marine organisms, nothing about it has so far been reported in the literature. In most studies on As speciation in

Table 2 – Factor analysis results of the arsenic species and the fish biological variables from each sampling areas. Strong (>0.75), moderate (0.50–0.75) and weak (0.30–0.50) factor loadings are marked in bold, bold-italics and italics, respectively.

		ART		THE		AMV		STR		THR		ELE	
		Factor1	Factor2	Factor1	Factor2	Factor1	Factor2	Factor1	Factor2	Factor1	Factor2	Factor1	Factor2
As species	AsB	0.51	**0.84**	**0.84**	−0.48	**0.82**	0.50	0.46	−0.72	**0.95**	0.26	0.15	**−0.98**
	DMA	**−0.87**	−0.40	**−0.99**	0.11	−0.80	**−0.58**	**0.92**	0.34	**−0.83**	**−0.52**	**−0.73**	*0.64*
	MMA					**−0.75**	**−0.65**						
	iAsV	0.23	**0.92**										
	Total As	0.12	**0.97**	0.49	**−0.84**	0.37	0.37	−0.15	**−0.70**	**0.98**	0.00	0.07	**−0.92**
Extraction efficiency	EE	*0.61*	**0.70**	**0.89**	−0.34	**0.83**	**0.56**	**0.65**	−0.49	**0.87**	0.36	0.12	−0.35
Fish biological variables	Total weight	**−0.97**	−0.17	−0.25	**0.95**	**−0.97**	−0.21	0.38	**0.85**	**−0.57**	**−0.82**	**−0.93**	0.36
	Total length	**−0.97**	−0.20	0.15	**0.96**	**−0.98**	−0.18	0.23	**0.92**	−0.19	**−0.96**	**−0.97**	0.22
	Moisture	**0.76**	0.26	0.49	−0.19	0.21	**0.97**	**−0.92**	−0.20	**0.91**	−0.06	**−0.98**	0.01
	Lipids	**−0.91**	−0.36	**−0.98**	0.11	**−0.86**	**−0.50**	**0.90**	0.33	**−0.77**	**−0.63**	**−0.70**	*0.71*
	Proteins	**0.76**	*0.61*	**0.94**	−0.02	**0.91**	0.40	−0.09	−0.48	0.49	**0.87**	**0.97**	−0.19
	Ash	**0.82**	0.27	−0.21	**0.51**	**0.88**	0.43	**0.98**	0.06	−0.27	**0.94**	**0.93**	−0.30
Factor analysis	Eigenvalue	8.23	1.64	5.93	2.20	9.71	0.82	4.85	2.80	7.06	2.40	6.95	1.83
	%Total variance	74.81	14.92	59.32	21.97	88.26	7.45	48.45	27.99	70.59	24.02	69.48	18.30
	%Cumulative variance	74.81	89.73	59.32	81.29	88.26	95.71	48.45	76.44	70.59	94.61	69.48	87.77

ART: Artemisium Straits; THE: Thermaikos Gulf; AMV: Amvrakikos Gulf; STR: Strymonian Gulf; THR: Thracian Sea; ELE: Elefsina Gulf; DMA: dimethylarsinic acid; MMA: monomethylarsonic acid; EE: extraction efficiency.

fish, information on sampling season are rarely provided, as is the case with all the sardine and anchovy studies we have been able to find. However, it has been reported that fish do show significant seasonal variations in their total As tissue concentrations (Frantzen et al., 2015).

2.2.1. Factor analysis

Factor analysis, a multivariate statistical method, yields the general relationship between measured variables by showing multivariate patterns that may be helpful to classify the original data (Liu et al., 2003). In the present study, factor analysis was performed twice on the normalized data sets in order to identify the variables influencing the arsenic composition of fish: (i) for each area for the two fish species to recognize the biological factors (12 variables) and (ii) for each fish species for the six areas to identify the environmental variables (18 variables). Factor analysis generated significant factors (those with eigenvalues of 1 or greater), and revealed important correlations between the examined variables.

For factor analysis of each area for both fish species including the biological parameters (Table 2), two significant factors were recognized for each area which explained 87.6 ± 7.5% of the variance in the dataset for all areas. In general, factors had positive loadings on AsB, total As, extraction efficiency, moisture, protein and ash content and negative loadings on DMA, total weight, total length and lipid content. However, these loadings were strong, moderate, weak or non-significant depending on the area. iAs(V) was detected only in ART and MMA was only detected in both fish species from AMV. Thus, in ART, Factor 2 had positive loadings on iAs(V) and protein content. In AMV, factors had negative loadings on MMA, total weight, total length and lipids and positive on moisture, protein and ash content.

For factor analysis of each fish species from the six areas including the environmental variables (Table 3), three significant factors were recognized for each fish species which explained the 84.9 ± 2.3% of the variance in the dataset. For both fish species, Factor 1, explaining 43–46% of total variance, had negative loadings on salinity and water transparency and positive on TN, TP, POC, POP and Chl-a. This factor can be interpreted as representing inter-correlations of the environmental variables. Factor 2, explaining 30–32% of total variance, had positive loadings on AsB, total As, salinity, TOC, TON, NH_4 and PI. It is suggested that this factor represents the influences of the environmental variables on the AsB and total As concentrations. Factor 3, explaining 8–11% of total variance, had positive loadings on DMA, MMA, PO_4, TP and POP and negative on salinity and DO, which can be interpreted as representing environmental influences on DMA and MMA concentrations in fish. Further discussion for these results is given in the following sections of this manuscript in which the detected individual As species are discussed.

2.2.2. Arsenobetaine in sardine and anchovy

AsB concentrations were found to be higher in anchovy compared to sardine for all sampling areas (Table 1, Fig. 3a). AsB concentrations ranged between 10.8 and 58.8 mg As/ kg dw for anchovy and between 8.6 and 41.4 mg As/ kg dw for sardine. The radar graph in Fig. 3a shows similar trends for AsB concentrations across the areas for the two fish species.

Table 3 – Factor analysis results of the arsenic species and the environmental variables of each fish species (anchovy, sardine) from the different sampling areas. Strong (>0.75), moderate (0.50–0.75) and weak (0.30–0.50) factor loadings are marked in bold, bold-italics and italics, respectively.

		Anchovy			Sardine		
		Factor1	Factor2	Factor3	Factor1	Factor2	Factor3
As species	AsB	−0.07	**0.95**	−0.09	−0.19	**0.92**	−0.16
	DMA	0.25	−0.07	**0.68**	**0.54**	−0.23	**0.66**
	MMA	−0.14	0.30	**0.88**			
	Total As	−0.10	**0.94**	−0.11	−0.14	**0.93**	−0.09
Extraction efficiency	EE	0.35	0.41	0.18	−0.48	**0.55**	−0.26
Environmental variables	Salinity	**−0.70**	0.41	**−0.54**	**−0.58**	0.45	**−0.64**
	TOC	−0.25	**0.87**	0.25	−0.19	**0.91**	0.17
	TON	−0.17	**0.91**	0.27	−0.12	**0.93**	0.22
	Transparency	−0.45	0.21	−0.47	**−0.53**	0.15	−0.30
	DO	−0.28	−0.28	**−0.83**	−0.13	−0.22	**−0.95**
	PO_4	0.47	0.23	**0.83**	0.36	0.18	**0.91**
	NH_4	0.37	**0.82**	0.33	0.30	**0.77**	0.48
	TN	**0.92**	0.08	0.27	**0.86**	0.04	0.40
	TP	**0.75**	0.14	**0.64**	**0.65**	0.08	**0.75**
	POC	**0.96**	0.00	0.27	**0.91**	−0.04	0.39
	POP	**0.79**	0.18	0.48	**0.79**	0.15	**0.52**
	Chl-a	**0.97**	−0.11	−0.19	**0.97**	−0.15	−0.07
	Pressure index	0.25	**0.80**	0.02	0.33	**0.77**	0.04
Factor analysis	Eigenvalue	7.71	5.31	1.98	7.89	5.45	1.37
	%Total variance	42.82	29.50	10.99	46.41	32.05	8.06
	%Cumulative variance	42.82	72.32	83.31	46.41	78.46	86.52

TOC: total organic carbon; TON: total organic nitrogen; DO: dissolved oxygen; TN: total nitrogen; TP: total phosphorus; POC: particulate organic carbon; POP: particulate organic phosphorus; Chl-a: chlorophyll-a; DMA: dimethylarsinic acid; MMA: monomethylarsonic acid; EE: extraction efficiency.

However, differences in concentrations between the two fish for the same area are also evident (Appendix A Table SD-2). Also, significant AsB concentration differences were observed for fish of the same species sampled from different areas (Appendix A Table SD-3). Essentially these two fish species tend to behave in a similar way towards AsB when at the same area. This may be because both fish have similar but not the same diets (Costalago et al., 2014), whereas, differences in dietary availability between areas may be having a significant effect on AsB concentration levels in these fish. Caumette et al. (2012) have reported that carnivorous zooplankton contain AsB as a major arsenic compound, whereas herbivorous zooplankton contain mainly arsenosugars and AsB as a minor compound, phytoplankton and microalgae contain inorganic arsenic and arsenosugars, but no detectable AsB. Therefore differences in available dietary sources may have a significant effect on the type of As uptaken and bioaccumulated by the fish. This diet based AsB differentiation may be further supported by the fact that even though both sardines and anchovies feed on zooplankton which has a high AsB content, only, sardines feed on phytoplankton (Costalago et al., 2014; Karachle and Stergiou, 2014), which has no detectable AsB, but high levels of arsenosugars. As a result it

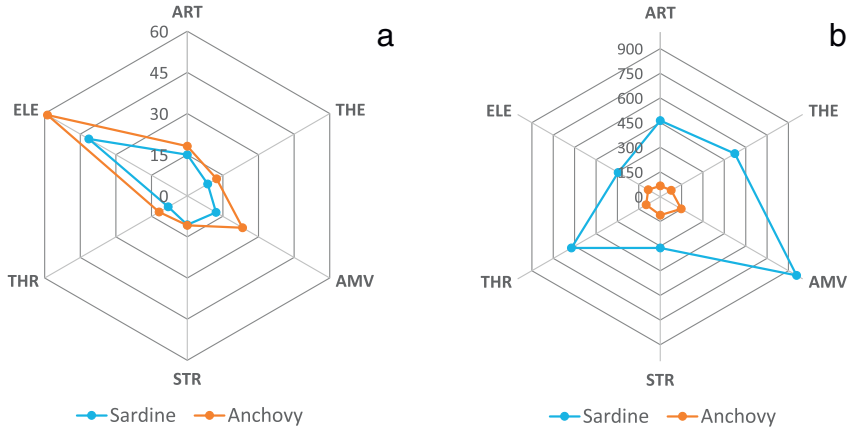

Fig. 3 – Radar graphs showing AsB concentrations mg As/ kg (a) and DMA concentrations μg As/ kg (b) for each fish species sampled at six Greek coastal areas. Area abbreviations are explained in the sampling areas paragraph of the Materials and methods.

can be hypothesized that the higher content of AsB in anchovy, compared to sardine, is affected by the phytoplankton diet of sardines which does not contribute to their AsB content. Furthermore, species-specific differences in the kinetic behavior (accumulation and elimination) of AsB in fish has been observed for other fish species (Amlund et al., 2006a, 2006b), as well as a correlation between fish family and arsenic speciation patterns (Šlejkovec et al., 2004).

However, these findings cannot exclude metabolic reasons for the higher AsB present in anchovies. In a recent feeding experiment, the accumulation of AsB in two marine fish (seabream and grunt) was attributed primarily to biotrasformation than to trophic transfer (Zhang et al., 2016b). It has been reported that, inside the fish organism, biotransformation and detoxification mechanisms, such as the reduction of inorganic arsenic, received through the diet or the water, followed by methylation to less toxic organic forms (Zhang et al., 2016a), as well as the synthesis of metal-binding proteins such as metallothionein-like proteins (Zhang et al., 2012), may occur.

Factor analysis for As species, with respect to fish biological parameters and environmental variables for the different sampling areas was performed in order to further investigate the possible causes for AsB concentration variations (Tables 2 and 3). AsB content was found to have a strong positive correlation with total organic carbon (TOC), total organic nitrogen (TON) and NH_4 and a weak positive correlation with seawater salinity. A strong positive correlation between AsB and the environmental pressure index (PI) was also observed.

Several studies on As speciation in fish have shown positive correlations between AsB content and water salinity. Most recently Hong et al. (2014) showed the correlation between salinity and AsB in biota from estuaries and seawater. This involved biota taken from water having salinities from <0.1 to 33 ppm. In the present study salinity values ranged from 34 to 39 ppm for all sampling areas. However, even in this significantly narrower salinity range AsB bioaccumulation seems to be influenced in a weak positive manner (Table 3). Also, when compared to other studies, here we report both significantly higher AsB concentrations and higher salinity values. In the literature, it has been suggested that AsB may function as a cellular osmolyte, in a similar fashion to glycine betaine which is thought to maintain ionic and osmotic homeostasis during seawater adaptation (Popowich et al., 2016). However, it has also been reported that changes in salinity did not affect muscular retention of AsB in Atlantic salmon (Amlund and Berntssen, 2004). Therefore the hypothesis that AsB is an osmoregulator (Larsen and Francesconi, 2003; Nearing et al., 2016; Šlejkovec et al., 2014), requires further investigation in order to be verified, even though positive correlations between AsB and salinity have been reported in numerous studies (Clowes and Francesconi, 2004).

AsB was also positively associated with variables related to higher food availability like TOC (Bonanno et al., 2014) and TON. Thus, the results of the present study imply that there is an increase of AsB concentration in fish with increasing concentrations of TOC and TON in the water, *e.g.* increase of food availability. This may indicate that sardine and anchovy receive AsB mainly through their diet. However, these conclusions should be cautiously interpreted as the non-organismal origin of TOC and TON, *e.g.* anthropogenic inputs, cannot be ignored.

AsB was also found to have a positive correlation with fish protein content, except for the fish originating from STR and ELE (Table 2). Protein content was higher in anchovies, compared to sardines, at all areas, which is what has been observed for AsB concentrations as well (Fig. 3a; Appendix A Table SD-2). It was also observed that areas at which fish of the same species had lower protein content also exhibited lower AsB values (Appendix A Table SD-2).

An observed negative correlation between AsB and fish lipid content, except for fish sampled from ELE (Table 2), is also in-line with the observation that sardines, having higher lipid content, contain less AsB compared to the anchovies (Appendix A Table SD-2). In a study by Choi et al. (2015), a negative correlation was also found between AsB and lipid content of fish even though it was not statistically significant.

AsB was also found to have a negative correlation with total weight of fish (Table 2). Our results are contradictory to literature data where they imply an accumulation of AsB with the increasing fish weight (age) (Šlejkovec et al., 2014). In the present study, a biodilution effect of AsB is more pronounced.

AsB was also found to have a positive correlation with total As (Table 3) which is consistent with several other studies on fish (Šlejkovec et al., 2014; Zhang et al., 2016b, 2016c).

2.2.3. DMA and monomethylarsonic acid in sardine and anchovy

DMA, the second in concentration As species, found in all analyzed fish samples, showed a distinct distribution difference compared to AsB (Fig. 3b). First of all it was present at significantly lower concentrations ng As/ g dw levels). Also, DMA concentration differences between fish species from the same area, are pronounced. DMA concentrations followed a different trend compared to AsB, being higher in sardine than in anchovy and ranging from 0.066 to 0.956 mg As/ kg dw (Table 1; Appendix A Table SD-2; Kruskal–Wallis $p < 0.05$). DMA values from 0.270 to 0.410 mg As/ kg dw were found for most anchovy and sardine samples in the Schaeffer et al. (2005) study, however, several anchovy samples contained non-detectable amounts.

One of the main differences between DMA and AsB is that the former exhibits a positive correlation with fish lipid content (Table 2). Sardines, having the higher lipid content amongst the two fish species, were observed to have a higher DMA content compared to the anchovies (Appendix A Table SD-2). Some DMA may also be originating from the lipid arsenic or arsenolipids, and thus higher levels of DMA with more lipid content (Molin et al., 2015). Furthermore, there were significant positive correlations between fish weight and DMA. However, DMA was found to have a negative correlation with protein and moisture content, which is the opposite behavior compared to AsB. Similar results have also been reported for fish collected from coastal cities in Korea, where a positive correlation of DMA with lipid content and a negative with moisture content was found even though it was non-significant (Choi et al., 2015). Sardines also feed on phytoplankton which is known to contain elevated levels of arsenosugars, which can degrade to DMA (Caumette et al., 2012).

DMA was also found to have a strong positive correlation with PO_4 and TP, a weak positive correlation with POP and a strong negative correlation with salinity and DO (Table 3).

However, these correlations have received limited attention, if any, in the literature so far.

For MMA the situation is not clear as it is detected at low concentrations in all anchovy samples, but only detected in one of the sardine samples. More specifically, MMA concentrations in sardines were <LOD, except for one sample found to contain 73 μg As/ kg dw, which is the highest MMA value for all samples. MMA in anchovy varied from 7 to 37 μg As/ kg dw and was found in all anchovy samples (Table 1). Factor analysis revealed that MMA has a positive correlation with factors such as fish weight and length, lipids content, PO_4 and TP, and a negative correlation with protein, ash, moisture content, salinity and DO (Tables 2 and 3). However, to the best of our knowledge, there are no literature data to further support these correlations.

MMA and DMA were found to have a positive correlation (Table 3). This can be supported by current theories on the formation of methylated As species. One suggests that inorganic arsenic is methylated *via* oxidative methylation, first forming MMA and then DMA (Thomas, 2007; Thomas et al., 2001). An alternative methylation scheme in which MMA and DMA are produced *via* a common As(III)-triglutathione complex has also been proposed (Hayakawa et al., 2005; Thomas, 2007). In both cases the sequential formation of MMA and DMA may explain their positive correlation.

2.2.4. Inorganic as in sardine and anchovy

Inorganic As(V) was found in both fish species but only from the ART area, at similarly low levels. Whereas fish from no other area contained detectable levels of iAs(V). This indicates that perhaps an environmental parameter at this area may be responsible for the presence of detectable iAs(V). Literature reports have also found iAs(V) to occur sporadically in different fish species from various areas (Choi et al., 2015; Hong et al., 2014; Jankong et al., 2007; Maher et al., 1999; Miyashita et al., 2009; Moreda-Piñeiro et al., 2012). Even though iAs(V) is one of the most toxic forms of As it is difficult to associate it at the levels detected here with the occurrence of As contamination or elevated health risks associated with fish consumption. The study by Schaeffer et al. (2005) on these fish species from Greek coastal areas also did not report any detectable inorganic As(V) in any of the fish samples they analyzed.

Arsenate was found to have a positive correlation with protein content of fish even if this was observed only from one area (ART).

Inorganic As(III) was not detected in any of the fish samples, which is in agreement with what has been reported in the literature. For example, a recent study using a sensitive hydride generation atomic absorption spectrometry technique for As speciation in white salmon, flathead gray, and white ship, having a LOD of 5 μg/kg did not report the detection of any inorganic As (Oliveira et al., 2016).

These findings support the fact that the iAs present in these fish does not constitute reason for concern for consumer health. In agreement with this are several other studies as well, in which case iAs was below the LOD of 0.003 mg/kg for most samples and ranged from 0.0031–0.0077 mg/kg wet mass when detected (Larsen et al., 2005). However, the detected iAs(V) in the fish from Artemisium Straits (ART) is a finding that requires additional follow-up analyses for better understanding of its presence.

3. Conclusions

In this study we have demonstrated the arsenic speciation of highly consumed sardine and anchovy fish taken from various areas along the Greek coastline. High As concentrations were determined in these fish samples. More specifically, most of the As was in the form of AsB which is considered non-toxic. Anchovy samples were found to contain higher concentrations of total arsenic, AsB and MMA and lower concentrations of DMA, compared to sardine samples. Statistical analysis revealed that As speciation is affected by both fish biological and environmental parameters. It was found that total As and AsB concentrations in fish samples are mainly influenced by the existing dietary conditions, and to a lesser extent by salinity, the total organic carbon and nitrogen, the ammonium and the pressure index of the sampling area. MMA and DMA are primarily influenced by the salinity, the dissolved oxygen, the phosphate and the total phosphorus as well as the lipids, protein and moisture content of the fish. Finally it was found that the arsenate is correlated to protein content of the fish, even though only one sampling area gave detectable levels of iAs(V). Compared to a ten year prior study, it seems that AsB and total As in fish were higher in ELE and lower in THE, whereas there were no significant differences amongst the other investigated areas. DMA concentrations in fish did not follow any particular trend.

The presence of iAs(V) in both fish at one of the areas prompts for further evaluation of this particular area. Also, for a more complete assessment of the As burden in these two fish species it is necessary to conduct arsenolipid analysis (Khan and Francesconi, 2016). This is particularly important as recent toxicity studies cannot exclude risks to human health related to the presence of arsenic-containing fatty acids (arsenolipids) in marine food (Meyer et al., 2014, 2015). The high lipid content of these fish points to the relevance of this type of arsenic speciation analysis.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.jes.2017.03.033.

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