Universidade de Aveiro Departamento de Química

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Vanessa Alexandra Teixeira Reis

Contribuição para a avaliação da acumulação de arsénio e desenvolvimento de um método de especiação no processo de produção de algas

Contribution for assessment of arsenic accumulation and development of a speciation method in an algae production process

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Dissertação apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Biotecnologia, realizada sob a orientação científica do Doutor Armando da Costa Duarte, Professor Catedrático do Departamento de Química da Universidade de Aveiro e da Doutora Maria Helena Trindade de Abreu, co-fundadora da empresa ALGAplus, Lda

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agradecimentos

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palavras-chave Arsénio, arsénio inorgânico, bioacumulação, especiação, macroalgas

resumo

O crescente aumento no interesse de consumo de algas marinhas devido aos respetivos benefícios nutricionais, tem realçado a preocupação com a saúde humana devido à eventual presença de contaminantes e substâncias indesejáveis, nomeadamente o arsénio (As).

O presente trabalho reporta o estudo da bioacumulação de arsénio por macroalgas (*Fucus vesiculosus*, *Ulva rigida*, *Porphyra dioica* e *Gracilaria sp.)* cultivadas de forma sustentável num sistema de aquacultura multi-trófica integrada (IMTA) operado pela empresa, ALGAplus, Lda.

O interesse pela determinação de As inorgânico (iAs), arsenito e arsenato, nos alimentos é regido pelo conhecimento dos seus efeitos tóxicos em seres humanos, mesmo em baixas concentrações. Tornou-se assim importante a contribuição deste estudo para o desenvolvimento de um método de especiação de baixo custo (HPLC-HG-AFS), capaz de detetar e quantificar a presença de iAs nas macroalgas cultivadas pela ALGAplus, Lda.

Os resultados evidenciaram que a maior acumulação de As ocorre no inverno presumivelmente devido à baixa taxa de crescimento das espécies, causando um aumento na concentração de As. Os resultados mostraram também que o aumento da acumulação de As seguiu a seguinte ordem: algas castanhas> algas vermelhas> algas verdes, sugerindo que as diferenças na concentração de As podem resultar das diferentes ordens taxonómicas.

A metodologia analítica HPLC-HG-AFS demonstrou ser eficiente e adequada para o processo de especiação revelando ser uma possível alternativa aos métodos de elevado custo. As baixas concentrações de arsenito e arsenato obtidas nos estudos de especiação, evidenciaram, tomando como base a legislação francesa, que a ingestão das macroalgas cultivadas na ALGAplus, Lda não é tóxica para humanos.

keywords Arsenic, bioaccumulation, inorganic arsenic, macroalgae, speciation

abstract

The increasing interest in edible macroalgae due to its nutritional benefits, has promoted the research about harmful contaminants and other unwanted substances, such as arsenic (As).

The present work reports the study of the bioaccumulation of As by macroalgae (*Fucus vesiculosus, Ulva rigida, Porphyra dioica* and *Gracilaria sp*.) produced on a sustainable integrated multi-trophic aquaculture (IMTA) system, operated by ALGAplus, Lda.

The interest in the determination of inorganic As (iAs), arsenite and arsenate, in food is governed by the knowledge of its toxic effects on humans, even at low concentrations. Thus, the development of a relatively inexpensive speciation method (HPLC-HG-AFS), reported in this study, contributed to an improvement in detection and quantification of the presence of iAs in macroalgae cultivated by ALGAplus, Lda.

The results highlighted that the highest accumulation of As occurs in winter, presumably due to the low growth rate of the species, causing an increase in As concentration. The increase in As accumulation in macroalgae showed the following order: brown> red> green; suggesting that the differences in the concentration of As is the result of the different taxonomic orders.

HPLC-HG-AFS has proved to be efficient and fit for purpose in this study of speciation, thus becoming a possible alternative to high costly methods. The low concentrations of iAs obtained during speciation, revealed, based on French legislation, that the ingestion of macroalgae produced in ALGAplus, Lda is not toxic to humans.

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List of Abbreviations

AB	Arsenobetaine
AC	Arsenocholine
As	Arsenic
As-sug	Arsenosugars
CONTAM	Panel on Contaminants in the Food Chain
DMA	Dimethylarsinic acid
EFSA	European Food Safety Authority
EPA	Environmental Protection Agency
ESMS	Electrospray Mass Spectrometry
HG-AAS	Hydride Generation Coupled to Atomic Fluorescence Spectrometry
HG-AFS	Hydride Generation Coupled to Atomic Fluorescence Spectrometry
HPLC	High Performance Liquid Chromatography
IARC	International Agency for Research on Cancer
iAs	Inorganic arsenic
ICP-AES	Inductively Coupled Plasma Atomic Emission Spectroscopy
ICP-MS	Inductively Coupled Plasma Mass Spectrometry
IMTA	Integrated Multi-Trophic Aquaculture
IUPAC	International Union of Pure and Applied Chemistry
IRMM	Institute for Reference Materials and Measurements
JRC	Join Research Centre
LD	Limit of Detection
LQ	Limit of Quantification
MMA	Monomethylarsonic acid
oAs	Organic arsenic
OH-sug	Glycerol-arsenosugar
PO_4-sug	Phosphate-arsenosugar
SO_3-sug	Sulfonate-arsenosugar
SO_4-sug	Sulfate-arsenosugar
SPE	Solid Phase Extraction
WHO	World Health Organization

1. Introduction

1.1. General aspects

Nowadays, more than 25 million tonnes of macroalgae are annually collected worldwide, being China and Japan the biggest producers, followed by United States of America. In Europe, Norway leads the production with approximately 140 tonnes of seaweed[1]. Algae were firstly used as biofertilizers but currently they are used in many countries also for several other purposes, such as, directly as food, in pharmaceutical and cosmetic industries, and in the extraction of antiviral and antibacterial compounds[1, 2]. The health benefits of seaweeds and/or isolates of macroalgae origin is, nowadays, generally agreed in nutrition sciences. Macroalgae are naturally rich in essential nutrients and in various health-promoting compounds, representing promising candidates for the development of functional foods, in order to take advantages of their benefit[3, 4].

On the other hand, despite the associated nutritional properties, algae may also contain harmful contaminants and other unwanted substances, such as arsenic (As). Therefore, it is important to set a proper limit of As concentration in various environmental compartments because this metalloid can become toxic at low concentrations. However, due the differences in toxicity among the different arsenicals, there is a need for speciation, that is, for assessing the distribution of the different species of As with a view to properly assess the risk due to the amount of arsenic ingested from macroalgae consumption. Furthermore, the speciation studies should always be closely followed by the identification of biotic and abiotic factors influencing As accumulation in an algal production process.

In above mentioned context, this dissertation has two main goals: firstly, to evaluate the accumulation of As in algae during the production process, as well as the factors that promote this accumulation; and secondly, to contribute for the development of a low cost and efficient speciation method, in order to evaluate the distribution of arsenic species present in edible macroalgae.

1.2. Physical and chemical properties of arsenic compounds

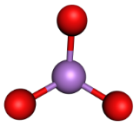
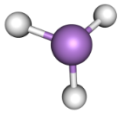
Arsenic is a metalloid, element characterized by metallic and non-metallic properties but often incorrectly mentioned to as a metal, with symbol As, atomic number 33 and atomic weight 74.922 g mol^{-1} within Group 15 of the Periodic Table. Elemental As comprises the following

allotropic forms: grey, yellow, and black. Yellow As is a waxy solid that convert into grey As after exposure to light at room temperature and it is the most stable form[5].

Arsenic occurs in four oxidation states, –3 as in arsine gas or arsenic hydride (AsH_3), 0 as in crystalline arsenic (As), +3 as in arsenite (AsO_3^{3-}), and +5 as in arsenate (AsO_4^{3-}), for a large variety of organic and inorganic As compounds[6, 7]. However, in the environment, inorganic arsenic (iAs) is usually present under the form of either trivalent arsenic (arsenite, As(III)) or pentavalent arsenic (arsenate, As(V))[6, 7] while the dominant organic As forms found include monomethylarsonic acid (MMA; $CH_3AsO(OH)_2$), dimethylarsinic acid (DMA; $(CH_3)_2AsO(OH)$), arsenobetaine (AB, $C_5H_{11}AsO_2$), arsenocholine (AC, $C_5H_{14}AsO^+$), and arsenoribosides (arsenosugars, As-sug)[6, 7].

The CAS number, chemical name, formula of arsenic and arsenic compounds most relevant for this work are shown in Table 1.

Table 1: CAS number, chemical name, formula and structure of relevant arsenic compounds in this work. (Structure obtained from: http://molview.org/)

CAS number	Chemical name	Formula	Structure
7440-38-2	Arsenic	As	
15584-04-0	Arsenate	AsO_4^{3-}	
15502-74-6	Arsenite	AsO_3^{3-}	
7784-42-1	Arsine	AsH_3	
124-58-3	Monomethylarsonic acid	$CH_3AsO(OH)_2$	
75-60-5	Dimethylarsinic acid	$(CH_3)_2AsO(OH)$	

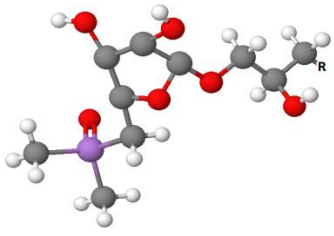
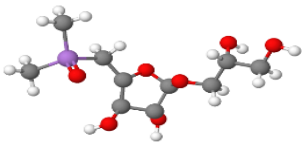
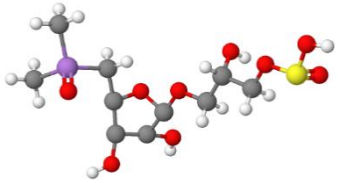
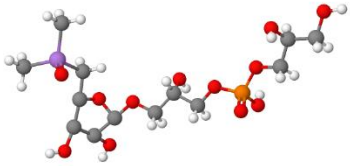
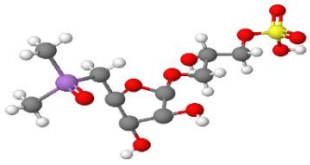
Arsine gas (AsH_3) can be found only in very highly reducing environments, while arsenite and arsenate, are the most common oxidation states in slightly reducing and oxygenated conditions, respectively[8].

Inorganic As can be biotransformed into organic As (oAs), such as methylated arsenicals, with an associated toxicity distinctly lower than that of the inorganic species and into arsenosugars, which are chemical species without reported toxicity. The mechanisms of As biotransformation including oxidation, reduction, and methylation will be discussed in Section 2.

The macroalgae, that are the object of this study, contain high concentrations of As in organic forms, which depend generally on the species of algae, the geographical location and seasonal variations[9]. While the arsenobetaine is the major arsenic species found in fish, mollusc, and crustacean, in marine plants like algae, the most frequently arsenic species are arsenosugars. Besides these species, also DMA, MMA, AC, and iAs can be found, but in significantly lower amounts when compared to AB and As-sug[10, 11, 12].

Most of the As in algae is bound to carbohydrate molecules and these As compounds are collectively referred to as As-sug; marine algae are considered to be the principal producers of As-sug and over 20 different chemical species have been reported in different classes of algal organisms[11, 13]. There are various types of arsenosugars dominant in algae, which are differentiated by end groups containing glycerol (OH), phosphate (PO_4) sulfonate (SO_3), and sulphate (SO_4) as shown in Table 2.

Table 2: Main types of arsenosugars present in algae. (Structure obtained from: http://molview.org/)

Arsenoribosides 	**R=** OH 	**R=** SO_3H 
	R= $OPO_3CH_2CHOHCH_2OH$ 	**R=** OSO_3H 

Glycerol-arsenoriboside and phosphate-arsenoriboside are common to all macroalgae species, while sulfonate-arsenoriboside and sulfate-arsenoriboside are limited to brown and a few red macroalgae species[14].

1.2.1. Acid-base equilibrium: pH as a master variable

Two of the most important factors controlling arsenic speciation are pH and redox potential[15]. As shown in Figure 1, the distribution of the several forms of arsenite (H_3AsO_3, $H_2AsO_3^-$, $HAsO_3^{2-}$, AsO_3^{3-}) depends on the value of pH and the same happens for the several forms of arsenate (H_3AsO_4, $H_2AsO_4^-$, $HAsO_4^{2-}$, AsO_4^{3-}), as shown in Figure 2. Figures 1 and 2 are the diagrams translating the acid-base equilibrium relationships, respectively,

for arsenite:

$$H_3AsO_3 \rightleftharpoons H_2AsO_3^- + H^+ \qquad pK_{a1} = 9.22 \qquad \textbf{(1)}$$

$$H_2AsO_3^- \rightleftharpoons HAsO_3^{2-} + H^+ \qquad pK_{a2} = 12.1 \qquad \textbf{(2)}$$

$$HAsO_3^{2-} \rightleftharpoons AsO_3^{3-} + H^+ \qquad pK_{a3} = 13.4 \qquad \textbf{(3)}$$

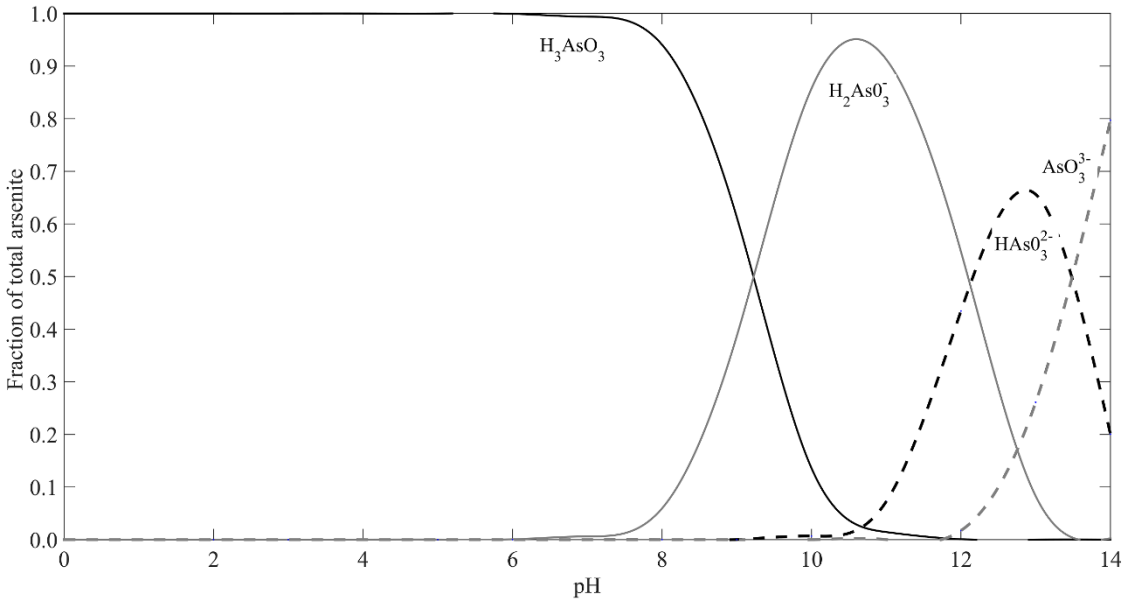


Figure 1: Distribution of arsenite species as a function of pH at 25 °C

and for arsenate:

$$H_3AsO_4 \rightleftharpoons H_2AsO_4^- + H^+ \qquad pK_{a1} = 2.20 \qquad \textbf{(4)}$$

$$H_2AsO_4^- \rightleftharpoons HAsO_4^{2-} + H^+ \qquad pK_{a2} = 6.97 \qquad \textbf{(5)}$$

$$HAsO_4^{2-} \rightleftharpoons AsO_4^{3-} + H^+ \qquad pK_{a3} = 11.5 \qquad \textbf{(6)}$$

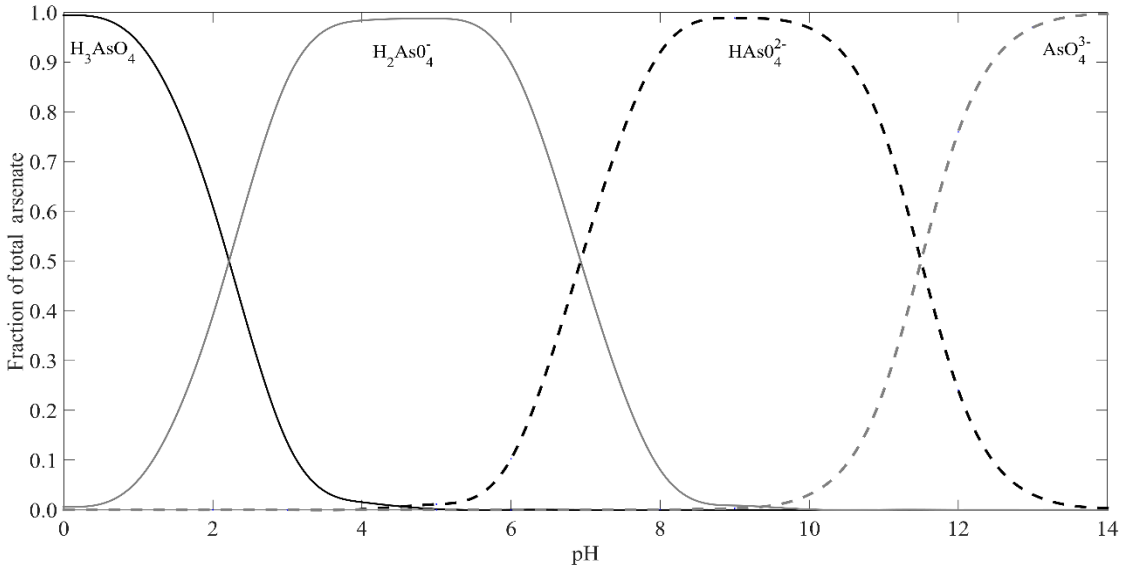


Figure 2: Distribution of arsenate species as a function of pH at 25 °C

Figures 1 and 2 shows that the degree of protonation of both arsenite and arsenate is an important modulation factor for the mobility of those chemical species. For example, the pH of groundwater is often between 6.5 and 8.5[15], and within this range, H_3AsO_3 and ($H_2AsO_4^-$ + $HAsO_4^{2-}$) are the dominant species.

In the present work, the study area is inserted in an estuary where the pH values vary between 7.5 and 9.9, and within this range, the dominant species are about the same as those found in groundwater: H_3AsO_3 for the arsenite while $HAsO_4^{2-}$ becomes predominant for the arsenate.

1.2.2. pH-Eh equilibrium

The value of redox potential (E_h) indicates the strength of oxidation or reduction reaction set in the environment under study. Its negative value confirms that solution demonstrates reducing properties, while positive value indicates about oxidizing reactions in the solution. Figure 3 shows the distributions of the inorganic species as a function of both pH and E_h.

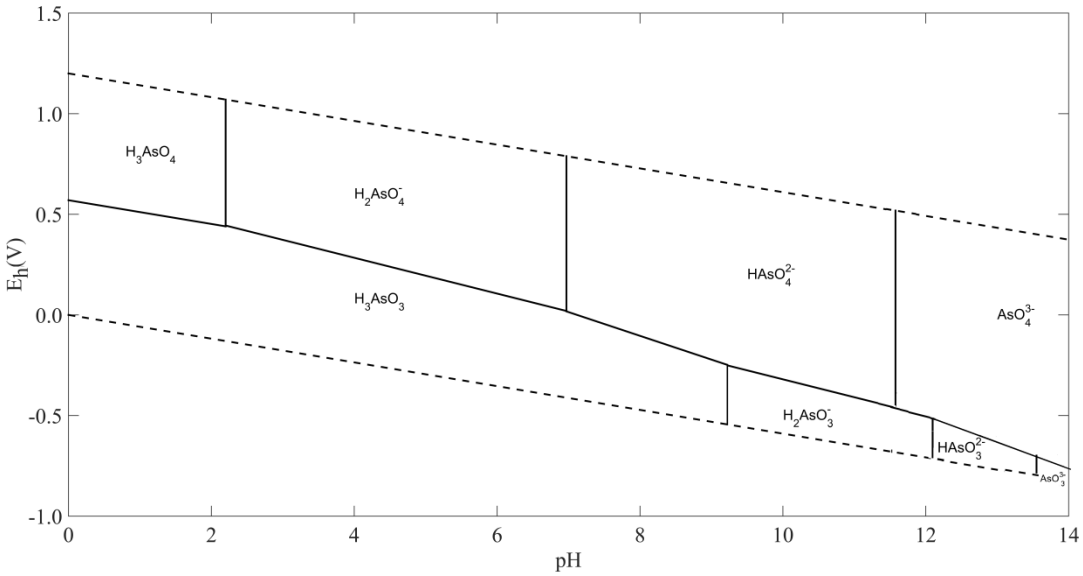


Figure 3: Eh-pH diagram of aqueous arsenic species at 25 °C and 1 bar total pressure

Under oxidizing conditions, H_2AsO_4 is dominant at low pH (less than about pH 6.9), while at higher pH, $HAsO_4^{2-}$ becomes dominant. Chemical compounds containing iAs might undergo a series of transformations and turn into one another under the influence of different processes. Most of these conversions are caused by change of pH[15].

The E_h of groundwater is often between -0.100 and 0.200 V[15], and within this range $HAsO_4^{2-}$ and H_3AsO_3 are the predominant species. However, in the present work, the study area is inserted in an estuary where the Eh values vary between 0.119 to 0.446 V, and within this range, the dominant species are about the same as those found in groundwater: H_3AsO_3 for the arsenite and $HAsO_4^{2-}$ becomes predominant for the arsenate.

1.2.3. Effect of ionic strength

In the Figures 1, 2, and 3, the ionic strength (I) has been considered zero whereas for estuaries it is necessary to apply corrections because the I derived from the salt concentration is much higher than that of the groundwater. Truesdell-Jones[16] proposed a model for application in NaCl-containing solutions and concluded that the ionic strength of seawater is approximately 0.7 M and the coefficient of activity (γ_i) is approximately 0.3, thus causing activity (a_i) to decrease comparatively to concentration (C_i). Thus, the distribution of the species is not greatly affected, as opposed to their activities which decrease substantially due to an increase in ionic strength

($I = \sim 0.7$ M $\Rightarrow \gamma_i = \sim 0.3 \Rightarrow a_i = \sim 0.3\ C_i$, contrary to the ideal situation, where $I = \sim 0$ M $\Rightarrow \gamma_i = 1 \Rightarrow a_i = C_i$). Therefore, it is possible to deduce that the relative distribution of the species is not much affected, in contrast to their activities, which decrease due to the increase in ionic strength. However, the influence of ionic strength on estuarine systems should be studied in order to understand in detail and more accurately its influence on the activity and actual distribution of arsenic species.

1.3. Sources of arsenic in environment

Arsenic is the 20th most common element in the earth´s crust. Normal occurrence concentrations are 0.2-15 mg kg^{-1} in the lithosphere, less than 15 mg kg^{-1} in soils, 0.02-2.8 ng/m^3 in the atmosphere, and in the order of µg/L in the aquatic environment where the concentrations are typically around 1.5 µg/L in open seawater and 4 µg/L in estuarine water[7, 17]. In the marine environment, As occurs mainly in the inorganic forms (arsenite and arsenate), with a large predominance of arsenate[18].

Arsenic has been made available mostly by natural sources, such as rock weathering and volcanic emissions, and it is widely distributed in soil, sediments, water, atmosphere, minerals, and biological tissues[19]. The rocks are the principal source of As, occurring primarily in sulphide form in complex minerals containing also Ag, Pb, Cu, Ni, Sb, Co, and Fe. Although present in more than 200 mineral species, the most common arsenic minerals are arsenopyrite (FeAsS) and sulphides, such as realgar (As_4S_4) and orpiment (As_2S_3)[19]. The volcanic activity is the second major source of As due to release of highly volatile compounds of As into the atmosphere.

Nevertheless, there is also a significant contribution from anthropogenic processes to the dispersion of As compounds into the environment, As it has been used in medicine (i.e. drugs), agriculture (i.e. arsenical pesticides, insecticides, wood preservatives, and soil sterility), livestock (i.e. feed additive), industry (i.e. pharmaceutical substances and electrophotography), electronics (i.e. solar cells and optoelectronic devices), and metallurgy (i.e. plates and alloys)[5].

1.4. Toxicity of arsenic compounds

In general, the toxicity of As compounds depends on several factors such as oxidation number, physical state, particle size, rate of absorption into cells, and rate of elimination[6, 20]. Usually the lower the oxidation number the higher the toxicity, and the higher the methylation

the lower the toxicity[21, 22], thus producing the following order of decreasing toxicity: arsine > arsenite > arsenate > monomethylarsonic acid > dimethylarsinic acid.

The arsenic compounds are also toxic to humans and the effects depend primarily on the chemical specie, route of entry, age, sex, dose, and duration of exposure. Humans are directly exposed to various forms of As, mainly through food and water. Although the As bioavailability depends also on the type of matrix in which it is ingested, the daily intake is considered to be about 20-300 µg for this type of exposure. Finally, the inhalation of As from ambient air is generally low, assuming a breathing rate of 20 m^3d^{-1}, the estimated daily intake is about 20-200 ng in rural areas and 400-600 ng in cities without significant industrial emission of arsenic[8].

According to World Health Organization (WHO), the immediate symptoms of acute As poisoning include vomiting, abdominal pain, and diarrhea. These are followed by numbness and tingling of the extremities, muscle cramping and death, in extreme cases[23]. For long term exposure to high levels of inorganic As (iAs), the first changes occur in skin pigmentation and then skin lesions with patches on the hand palms and feet; it may also occur other pathologies such as peripheral neuropathy, gastrointestinal problems, conjunctivitis, diabetes, renal system failure, enlarged liver, bone marrow depression, destruction of erythrocytes, high blood pressure, and cardiovascular disease[23].

Furthermore, there is also some evidence showing that As can lead to the development of cancers in skin, bladder, and lungs[23], the International Agency for Research on Cancer (IARC) has classified iAs compounds as carcinogenic to humans (Group 1), while DMA and MMA are considered possibly carcinogenic (Group 2B)[24].

1.5. Limits for arsenic compounds in drinking water and foodstuffs

High concentration of total As in drinking water is a potential health danger with serious toxicological concerns for human health. The Environmental Protection Agency (EPA) and WHO state that the threshold level of total As in drinking water is 10 µg L^{-1}, while the limit values for different As species has not been established[25], which makes a compelling need for regulation based on the individual As compounds.

Moreover, there are no general accepted limits for algae based products. However, since the analysis of iAs is reliable for rice and rice based products, maximum levels were delimited and the European Union (EU) introduced regulations of 0.2 mg kg^{-1}, expressed as As, for iAs in white rice and 0.1 mg kg^{-1} for iAs in rice-based foods aimed at infants and children[26]. In 2009, the

scientific Panel on Contaminants in the Food Chain (CONTAM) of the European Food Safety Authority (EFSA) identified a range of benchmark dose lower confidence limit values between 0.3 and 8 μg kg^{-1} body weight per day for inorganic arsenic species, but this range still is under revision[26].

France was the first and only European country to lay down specific regulations on the consumption of seaweed, stipulating maximum values of 3 mg kg^{-1} for iAs[27]. Therefore, in several European countries, such as Portugal, there is no specific legislation regarding contaminants in algal products.

2. Biotic and abiotic factors influencing arsenic accumulation in algae

2.1. Introduction

Bioaccumulation of As in aquatic organisms can derive from water, suspended particles, sediments, and through the food chain. The occurrence, distribution, and As speciation are of utmost importance for assessing the effects of bioaccumulation and trophic transfer through the food chain[28].

Bioaccumulation is a combined result of two different processes: a) bioconcentration, where absorption of pollutants from water into organisms occurs across the whole trophic chain; and, b) bioamplification, where there is an increase in pollutant concentration as the trophic level increases across the trophic chain, thus higher trophic levels show progressively higher degree of contamination until reaching the top of the chain[29].

2.2. Factors influencing arsenic bioaccumulation and detoxification

The accumulation rate depends not only on the availability of arsenic but also on biological, chemical, and environmental factors. Biotic and abiotic parameters such as, species, stage of life cycle, biological activities, biomass density, water temperature, water salinity, pH, light exposure, light intensity, dissolved oxygen, seasonal variation, and concentration of nutrients, namely nitrogen (N) and phosphorus (P) influence the bioavailability and bioaccumulation of As in algae[28, 30, 31]. Some studies demonstrate that As can be accumulated from water, food, and sediment, while seaweed accumulates arsenic directly from water, marine animals incorporate arsenic from feeding algae and can biotransform this iAs into oAs compounds[28, 32, 33, 34].

Maugh[35] has attempted to explain the processes influencing As bioaccumulation in macroalgae and they have shown that the principal mechanism responsible for bioaccumulation is the competition between AsO_4^{3-} and PO_4^{3-} for absorption into the cells of algae. Arsenate is a chemical analogue of phosphate, in size, geometry and ability to enter biochemical reactions thus facilitating its entry into the cells by transport mechanisms, unable to discriminate between PO_4^{3-} and AsO_4^{3-}. Hence, macroalgae absorb AsO_4^{3-}probably due to their inability to distinguish it from the essential anion PO_4^{3-} [36, 37].

Sanders and Windom[36], found that the major inputs of As to the marine and estuarine environment were freshwater inflow and atmospheric deposition, resulting that macroalgae are exposed to arsenic as AsO_4^{3-} at comparable concentrations of PO_4^{3-}, which is an essential and

often growth-limiting nutrient in surface waters, and therefore accumulating dissolved AsO_4^{3-} via a cellular PO_4^{3-} transport system.

Sanders[38] determined that PO_4^{3-} concentration affected the AsO_4^{3-} uptake at the different arsenate concentrations tested. Increased phosphate significantly decreased the arsenate uptake in the culture and reduced the total arsenic concentration, while the increased uptake of AsO_4^{3-} as PO_4^{3-} uptake increases at low PO_4^{3-} concentrations is attributed to the increasing PO_4^{3-} metabolism and indiscriminate AsO_4^{3-} uptake. One of the parameters previously mentioned that can influence the bioavailability and bioaccumulation of As is the species of algae. According to Sanders[38] there is a general trend: brown seaweeds (phaeophyta) contain higher concentrations of arsenic than red (rhodophyta) and green (chlorophyta) macroalgae. This trend may be due to the fact that brown algae have a higher phosphorus concentration in their cell and consequently accumulate more arsenic.

However, Klumpp[30] considered that macroalgae exhibit a different mechanism for AsO_4^{3-} and PO_4^{3-} absorption, since in some marine algae the AsO_4^{3-} does not compete with PO_4^{3-}. He studied the mechanisms and influence of various parameters in As uptake by *Fucus spiralis* and concluded that parameters like pH and salinity had no effect, however, an increase in temperature enhanced the uptake. The same author concluded also that the effect of PO_4^{3-} at low levels is of little importance in determining As levels in organisms in the natural environment.

In relation to the effect of light as a parameter of As bioaccumulation, Bottino et al.[39], showed that As uptake in marine alga T*etruselmis chui* is light-dependent, and more light causes higher AsO_4^{3-} uptake, suggesting that AsO_4^{3-} adsorption is an endergonic process that may compete with cell growth for available photosynthetic energy. However, contradictory results have been reported by Klumpp[30] because for the macroalga *Fucus spiralis*, As uptake in the dark or in the presence of photosynthetic inhibitors is greater than in the presence of light. With this information it becomes possible conclude that the duration of exposure influences As concentration, depending on the macroalgae specie.

According to Challenger et al.[40], after AsO_4^{3-} uptake, algae reduce AsO_4^{3-} to AsO_3^{3-}, and then subsequent methylation leads to the formation of methylated forms, such as MMA and DMA. Based on mechanism of As transformation by algae proposed by Challenger et al.[40], Hellweger et al.[41] proposed the mechanism depicted in Figure 4. In Figure 4 (**a**), under P-limiting conditions, the following steps are assumed: AsO_4^{3-} uptake by algae, reduction of AsO_4^{3-} to AsO_3^{3-}, sequential methylation of AsO_3^{3-} to MMA and DMA, and finally excretion. Nevertheless, under non-P-limiting conditions, as shown in Figure 4 (**b**), As is taken up as AsO_4^{3-} via the PO_4^{3-}

active transport system and AsO_4^{3-} is incorporated into the metabolic cycle of algae due to the similarity between the PO_4^{3-} and AsO_4^{3-}, within the cell, the reduction of AsO_4^{3-} to AsO_3^{3-} is fast, but the methylation is slower, causing AsO_3^{3-} to build up in the cell. The consequence is the occurrence of a peak in the intracellular AsO_3^{3-} concentration and, because AsO_3^{3-} is excreted, inducing a peak in extracellular AsO_3^{3-} concentration.

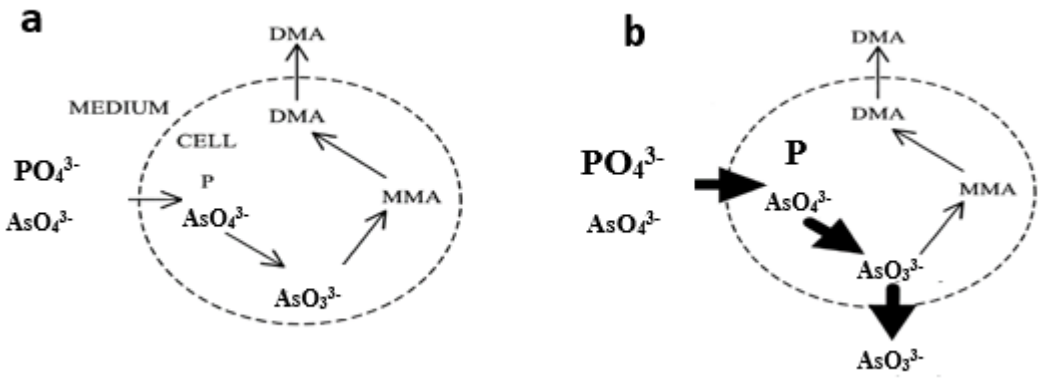


Figure 4: Mechanism of As transformation by algae. **a)** P-limiting conditions and **b)** non–P-limiting conditions[41].

Challenger et al.[40], refers that in seaweeds AsO_4^{3-} accumulation is four times higher than AsO_3^{3-} accumulation, and this fact suggests more toxic AsO_3^{3-} is excreted after the reduction mediated by membrane associated transporter protein. Furthermore, methylated arsenic, MMA and DMA, are excreted rapidly, indicating that As methylation is a powerful detoxification mechanism present in algae.

Although the presence of As-sug in algae was not explained by Challenger et al.[40], the presence of high concentration of this organic arsenical and the mechanism of As-sug synthesis need to be enlightened. Thus, Edmonds and Francesconi[42], proposed a possible pathway for the biotransformation of As(V) to As-sug by marine algae based on the sequential reduction of arsenate followed by oxidative methylation. These authors suggested that As-sug are produced by transferring the methyl and adenosyl groups of the methylating agent S-adenosylmethionine (SAM) to suitable agents, such as DMA. However, the mechanism for As-sug biosynthesis in seaweed involves no clear but complex mechanisms and it has not been completely established[43].

3. Cultive of macroalgae in ALGAplus

3.1. Integrate Multi-trophic Aquaculture system (IMTA)

Integrated Multi-trophic Aquaculture (IMTA) systems are recognized as a suitable tool to sustainably increase animal aquaculture. The concept is based on the recycling of residues derived from the production of upper trophic levels by lower trophic organisms, with a production of an added value[44]. This approach has been used to convert monoculture into an ecological and more sustainable aquaculture[45, 46, 47], with positive environmental and socio-economic benefits for the aquaculture industry.

The cultivation of seaweeds in IMTA promotes higher productivity and less variability than natural seaweed beds due to the higher availability of a constant supply of nutrients. As depicted in Figure 5, seaweeds assimilate the fish-excreted ammonia (NH_4^+), PO_4^{3-}, and carbon dioxide (CO_2), converting them into potentially valuable biomass. With this integrated approach, effluents can be recycled back into the fish ponds or can be discharged without endangering the environment[48, 49]. The IMTA can then minimize the load of nutrients normally discharged by fish aquaculture processes into coastal waters, since seaweeds can remove up to 90% of the nutrient load from an intensive fish farm[50].

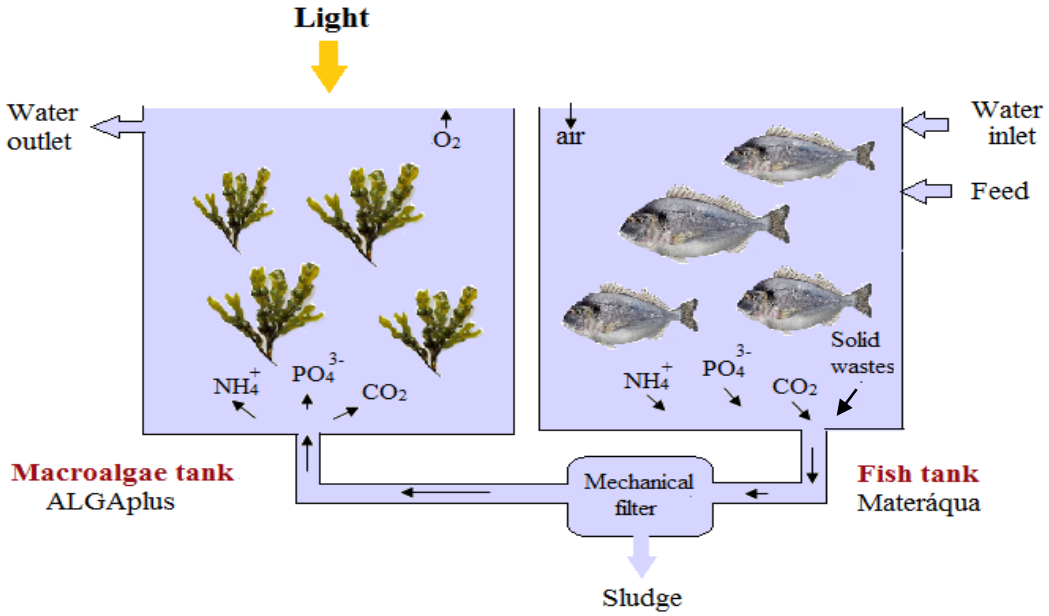


Figure 5: Concept of Integrated Multi-Trophic Aquaculture. (adapted from Pereira and Abreu [51])

ALGAplus (http://www.algaplus.pt/) is a company devoted to the production of seaweed and seaweed based products in controlled environment and with organic certification sited at Ílhavo (Aveiro, Portugal). This company is based at Ria de Aveiro (40° 38' N, 8° 44' W), one of the

most important and extensive coastal lagoon in the northern west Atlantic coast, of Portugal. This estuarine system is considered as a highly productive ecosystem due to its richness in nutrients and organic matter[52]. The lagoon has a maximum width of 8.5 km and a length of 45 km. The facilities of ALGAplus are sited in Ílhavo channel, which is an elongated shallow arm, of 15 km length.

ALGAplus is a pioneer Portuguese company in implementing a land-based seaweed cultivation system, under the IMTA sustainable concept, that is within a fish farm producing sea bass and sea bream. Infra-structures for algae production include an indoor nursery, a land-based tank cultivation system with several capacities, as well as earthen-ponds with variable sizes with water renewal related to the tidal cycles. Green, red, and brown seaweed species are grown with manipulation of production factors, such as stocking density, water renewal, harvest time, and aeration. ALGAplus has a strong focus in R&D associated with the production of macroalgae adapted to the food market needs also developing the crop species of interest. Processing currently includes washing, low-temperature drying and milling, and commercializes sustainable and high-quality seaweeds and seaweed-based products.

3.2. Macroalgae cultivated in ALGAplus

Algae are unicellular or multicellular autotrophs comprising a large diversity of organisms in relation to morphology, complexity, and size. There are two main types of algae: a) the microalgae, which constitute the phytoplankton found in euphotic zone and at the base of food chain; and, b) the macroalgae, which are also primary producers at the base of the food chain but can attain very large sizes, since some stalks of algae can achieve 65 m of extension when they are not subject to predation[1].

Under the scope of this work, four species of macroalgae were selected from 3 distinct phyla, *Phorphyra dioica* and *Gracilaria sp.* (Rhodophyta), *Ulva rigida* (Chlorophyta) and *Fucus vesiculosus* (Phaeophyta). They were cultivated in IMTA system and supplied by ALGAplus, Lda.

The excess of nutrients released in aquaculture production are used for the cultivation of the macroalgae *U. rigida*, *Gracilaria sp*. and *P. dioica*, although *P. dioica* is cultivated in laboratorial environment at ALGAplus, being placed in tanks to grow like *U. rigida* and *Gracilaria sp*.. *F. vesiculosus* is captured from populations that grow naturally in Ílhavo channel and placed in the tanks for a prior wash and also marketed in the same way, like the other species.

The following sub-headings displays the visual features of the macroalgae under study (*Fucus vesiculosus, Ulva rigida, Porphyra dioica,* and *Gracilaria sp.*) as well as the respective morphological characterization.

3.2.1. *Fucus vesiculosus*

Fucus vesiculosus (Figure 6) are very well known brown seaweeds which live completely submerged as an intertidal marine alga commonly found on the coasts of the North Sea, Baltic Sea, and the Atlantic and Pacific Oceans[53]. They are useful as sources of bioactive compounds, and besides becoming a common food in Japan it is used as an additive and flavoring agent in various food products in Europe. Recently, fucoidan extracted from *F. vesiculosus* gained interest because of its biological activities and potential medical applications[54].

Figure 6: Brown macroalgae, *Fucus vesiculosus*

Morphologically, *F. vesiculosus* varies in color from olive green to olive brown and it is typically about 40 cm in length. It is characterized by the small nearly spherical gas–filled vesicles which look like bubblewrap and it occurs in pairs[55].

3.2.2. *Porphyra dioica*

Porphyra (Figure 7), known as *nori,* is considered the most valuable seaweed in the world, with a continuous increase in production. In Asian continent these red algae have a high economic value, being widely used in human alimentation and they one of most eaten seaweeds[56]. *Porphyra* are rich in proteins, vitamins and minerals such as iron, zinc, sodium, potassium, and calcium[57].

Figure 7: Red macroalgae, *Porphyra dioica*

Porphyra dioica is also of interest in this study since this specie is common in the North of Portugal[58], inhabits the intertidal zone of rocky beaches throughout the year, and is able to grow

within a wide range of temperatures, photoperiod, and light intensity[59]. Morphologically are membranous, monostromatic, olive-green to brown-purple or blackish fronds, depending on the species and can reach 5 to 35 cm in length[59].

3.2.3. *Ulva rigida*

Ulva rigida (Figure 8) comprise the genus Ulva, order Ulvaves and phylum Chlorophyta. This green algae are found in a variety of habitats and on several different substrates[60]. They have a good vitamin and mineral profile and are especially rich in glutamic and ascorbic acids, alanine, and iron[61, 62]. *Ulva* has been widely used as a source of food in the farming of invertebrates, as fertilizer, biofilter, as well as complement ingredient in traditional food[63, 64].

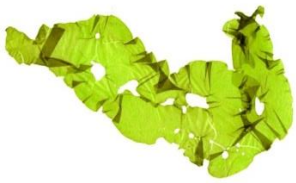


Figure 8: Green macroalgae, *Ulva rigida*

Morphologically, it is a green colored seaweed, with a laminar thallus with a rigid surface with waved margins and it is slippery to the touch. *Ulva rigida* can reach up to 10 cm in height, but size and blade shape are both highly variable[65].

3.2.4. *Gracilaria sp.*

Gracilaria sp. (Figure 9) are one of the seaweed most exploited worldwide[66]. They are a non-indigenous Asian red algae naturalized in Ria de Aveiro, where it became the dominant *Gracilaria* genus[67], since they are well adapted to estuaries and highly resistant to various stressful factors such as darkness, sedimentation, desiccation, and variable nutrients conditions[45, 68]. *Gracilaria* species are also efficient biofilters due to their good capacity to remove ammonia and nitrate from the water[45, 69].

Figure 9: Red macroalgae, *Gracilaria sp.*

Morphologically, *Gracilaria* is a red macroalga that is cartilaginous, cylindrical and up to 50 cm long. Besides, it is coarsely branched[70, 71].

4. Inductively Coupled Plasma Mass Spectrometry for total arsenic determination

Inductively Coupled Plasma Mass Spectrometry (ICP-MS) was used in this work to determine the total concentration of arsenic in samples of algae grown in ALGAplus.

ICP-MS is an instrumental technique that in the last two decades has contributed significantly to studies involving the analysis of environmental, biological and geological samples, and allowed to identify and quantify the different chemical elements that compose a substance. This method can attain a very fast and high throughput multi-elemental analysis (10 - 40 elements per minute per sample) with a detection limit in the sub parts per trillion (ppt) range, and it enables quantitation at the high parts per million (ppm) level. ICP-MS also works over eight orders of magnitude detection level as a result of its higher sensitivity[72].

ICP-MS technique consists of sample introduction into the plasma through the aid of a peristaltic pump so that the sample is aspirated into the nebulizer and converted into an aerosol using a gas flow (argon). The larger aerosol droplets are separated from finer droplets, which is optimal for efficient ionization in the ICP. Then, the finer aerosol droplets can then be swept into the ICP torch via argon gas flow. These newly formed ions then travel out of the torch and come to the ICP-MS interface where ions enter in the vacuum system and proceed to the mass spectrometer. Then the ions travel in the lens system where they are directed or focused to enter the quadrupole analyzer where they are separated based on their mass/charge ratio[72].

5. Analytical speciation of arsenic accumulated in algae: introduction and challenges

Chemical speciation can be considered as the determination of the concentration of the various chemical forms of an element in a matrix, and these species, together, constitute the total concentration of the element in the sample. The mobility, bioavailability, toxicity, and even biological metabolism of As depend on its chemical forms and respective structure. Therefore, the development of analytical techniques not only for the determination of total As concentration but for the selective determination of As compounds in different matrices such as, algae, becomes of extreme importance to acquire an adequate knowledge of the effects of As compounds on the environment, namely in marine and estuarine environmental compartments helping too in the introduction of regulations for iAs in seafood.

The importance of As speciation in algae led to important advances in the development of new analytical methodologies, that when used are subjected to a validation to ensure that analytical method generates reliable and interpretable information about the sample. The validation studies involve the evaluation of the performance characteristics of the method under defined experimental conditions in order to guarantee its applicability, quality control and quality assurance in the different steps of speciation. In addition, to ensure the reliability of the analytical results, it is necessary to take into account possible sources of error that may occur during the various steps prior to the quantification of the different As components, namely: sample collection and preparation, extraction, separation, and detection.

Table A. 1 in the Annex section depicts a summary of the work carried out by several researchers and it includes information about methods for sample preparation, extraction, preservation, separation, detection, and quantification of arsenic species in macroalgae.

5.1. Sample preparation

The setup of procedures for sampling is extremely important for the interpretation of the results in a survey. In order to sample representatively it should be included information on what the samples represent and how they were selected, as well as the weight and number of samples. Sampling procedures often involve a risk of contaminating the sample itself because the transportation and storage of samples involve risks of overheating or freezing, contamination, and chemical changes of matrix. Therefore, a clear description of these procedures becomes

necessary, which will make it possible to conclude where and how the eventual contamination occurred.

Sample preparation is the preliminary step of the analytical methodology and its adequate development is crucial for achieving results fit for purpose. The first step in this operation is the removal of epiphytic communities living in symbiosis with algae and other substances that might interfere in further stages of the analytical methodology: sample washing is often performed either with deionized and ultra-pure water or saline solutions similar to seawater. Whenever it is not possible to remove the epiphytic only by simple washing, the process is carefully performed manually or with the help of tweezers[73]. After the cleaning operation, algal material is generally dried and crushed until obtaining a fine powder. Freeze-drying and thermal treatments are the two options used for removing water from samples. For freeze-drying, the temperature conditions are from -30°C to -60°C, and the time of operation ranges between 24 and 48 hours and for thermal treatments, the samples are kept at temperature between room temperature and 60 °C for 18h to 48h[73, 74].

According to Michalke[75] and Rajakovic et al.[76] problems such as stability, contamination or losses of samples, are frequent and they need to be well thought of and solved in advance. Salgado et al.[77] investigated the stability of total arsenic, arsenite, and arsenate in *Sargassum fulvellum* and *Hizikia fusiformis*, as well as in their aqueous extracts, and the results suggested that samples remained stable for at least a period of 12 months without showing any degradation of the analytical signals. This information becomes essential when it comes to establish the most suitable preservation conditions to ensure the accuracy of the analytical results.

In the extraction process for the purpose of As speciation, the water and mixtures of MeOH:water are the extractants most widely-used. However, the extraction conditions vary significantly, for the following aspects: the range of extractants ratio volume to algal mass; sample exposure to thermal heating; conditions for microwave extraction; ultrasound aided extraction; and mechanical shaking. Not only those conditions influence the extraction efficiency and also the integrity of the native arsenic species during extraction, but also the extract may need further treatment prior to separation, like filtration, because particulates could damage chromatographic columns[78].

5.2. Separation of arsenic compounds followed by detection and quantification

Unlike detection and quantification of total elemental As concentration, speciation is only possible when detection methods are associated with a previous separation step. However, according to Welz[79] , for samples containing only arsenite and arsenate the chromatographic separation is not necessary and the determination of these components can supposedly be performed only by hydride generation coupled to atomic adoption spectrometry (HG-AAS). This technique is assumed to be efficient and consists on the determination of total inorganic arsenic (As(III) + As (V)) after pre-reduction of arsenate to arsenite generally with thiourea, ascorbic acid, or L-cysteine, while the determination of As(III) is conducted generally using hydride generation in controlled conditions, namely, HCl and $NaBH_4$ concentrations in order to inhibit the reduction of arsenate to arsine. Finally, the As(V) concentration is determined by the difference of both measurements. However, although this technique is accepted by various researchers[79, 80, 81], several drawbacks have been reported[82, 83], since this technique is extremely laborious, the reaction conditions must be well controlled and needs high amount of sample.

Hence, the methods available for the As speciation of macroalgae are generally a combination of separation techniques with systems of detection. The separation method most employed is high performance liquid chromatography (HPLC), due to the ease of coupling it with several detection systems, such as: HG-AAS, hydride generation coupled to atomic fluorescence spectrometry (HG-AFS); inductively coupled plasma atomic emission spectrometry (ICP-AES); or inductively coupled plasma mass spectrometry (ICP-MS)[84, 85]. Furthermore, the ICP-MS coupled with HPLC is the most popular technique used, because it is more efficient and provides very low quantification limits (1 µg L^{-1}) and high selectivity for several species of arsenic, besides avoiding several issues associated with matrix effects. However, this technique may have problems associated with interference from Cl^- which depending on the respective concentration can produce $^{40}Ar^{35}Cl^+$ in the plasma and this interference is proportional to the concentrations of Cl^- in the sample. Another disadvantage associated with this technique is the high costs of instrumentation and operation, leading many companies to attempting to find alternative and suitable methods for As speciation.

The combination of HPLC either with HG-AAS or HG-AFS is considered simple, fast and relatively inexpensive for As speciation, thus providing a tool that allows the separation and identification of known As species from their retention times as well as their detection and quantification for quantitative purposes[86, 87].

The principal difference between HPLC-HG-AAS and HPLC-HG-AFS is the sensitivity of the method. Although detection limits, in the order of μg L^{-1}, are achieved with the couplings HPLC-HG-AAS these levels are sometimes not low enough to detect the As species present in biological samples. However, the sensitivity can be further increased with the HPLC-HG-AFS coupling, with limits of detection below μg L^{-1} similar to ICP-MS detection[79].

5.3. Specific parameters for analytical quality control and quality assurance

The quality control always plays a dominant role in any study of chemical speciation if the results are intended for example in the assessment of environmental quality or food safety. The key to successful speciation is the preservation of species information during the analytical procedure from sampling to the final result[75]. According to IUPAC[88], the total analytical procedure must not modify the integrity of species and the result must reflect the original species information. Also, the choice of the method must be fit for the analytical purpose, thus becoming necessary to make the correct description and a statement on the degree of method validation. With respect to the method used it is important to clearly assess the predominant interferences present[89].

The analytical performance of an analytical method must be evaluated through validation protocols and two of the most important parameters are the LD (limit of detection) and the LQ (limit of quantification). Through those limits, it is possible to define the lowest concentration of an analyte that can be reliably detected and quantified[76, 89]. Finally, a validation study is indispensable for the proper characterization of the analytical method used and it is also fundamental for achieving not only reproducibility but also trueness in results.

5.4. Reference materials, certified reference materials, and the matrix effect

The certified reference materials (CRM) are materials sufficiently homogeneous and stable with respect to one or more specified properties, which have been established to be fit for its intended use in a measurement process characterized by a metrologically valid procedure for one or more specified properties, accompanied by a certificate that provides the value of the specified property, its associated uncertainty, and a statement of metrological traceability[90].

One of the solutions to solving frequent methodological and analytical problems is the use of CRMs, which should be regularly used to ensure an appropriate level of quality assurance. However, the main problem associated with the determination of As species and their toxicological evaluation in seaweeds is the lack of reference materials for analysis of speciation, which have certified values of different chemical forms of the element analyzed[78]. Unfortunately, CRM certified species are only available for total As and arsenate, and for arsenate, the price of such materials is very high.

Table 3 lists the reference materials available for validation of As determinations and their certified values. The material NMIJ CRM 7405-a presents certified values not only for total As but also for arsenate, which allows some degree of checking for speciation analysis, at least for As(V).

Table 3: Reference materials available for validation of As determinations in algae

Material Reference	Certified values
ERM-CD200 ***Fucus vesiculosus***	As: 55 ± 4 mg kg^{-1}
NMIJ CRM 7405-a ***Sargassum fusiforme***	As: 35.8 ± 0.9 mg kg^{-1} As(V): 10.1 ± 0.5 mg kg^{-1}

The Institute for Reference Materials and Measurements (IRMM) of the Joint Research Centre (JRC), appointed seventy-four laboratories from 31 countries including the National Reference Laboratories which focused on the determination of total and iAs in wheat, vegetable food, and algae (*Fucus vesiculosus*)[91]. Most of the participants performed satisfactorily for total and inorganic As in vegetable food and wheat (85 and 60%, respectively), but only 20 % of the laboratories reported satisfactory results in the algae test material.

The matrix algae were considered much more challenging than the others, especially because they contain a large variety of organic As species that make the determination of iAs much more complex. Just only 20% of the laboratories involved in the study manage to fully operate under optimal analytical conditions and although an agreement on a value for iAs in algae could be reached, the low number of laboratories obtaining a satisfactory score suggests that this matrix poses special problems for this type of analysis.

Raab et al.[92] performed a study where seven algae samples were distributed by 13 laboratories, and 5 of them attempted a full characterization of the water-soluble fraction regarding to the As species. From the 5 labs, only 3 produced results acceptable in what concerns

the identification and quantification of As species, although different chromatographic methods were used. The extraction efficiency was largely dependent on the algae species and varied from 3% to 96%. The results demonstrated that the three samples of *Hijiki* seaweed have a nearly quantitative extraction of As, while As in the *Sea lettuce* is only 30% extractable and even less in the freshwater algae *Black moss* (below 5%).

Khan et al.[93] determined the levels of total As by ICP-MS and iAs speciation by LC-ICP-MS in various edible species of seaweed. For the analysis of CRM (NMIJ CRM 7405-a) the spiking recovery for arsenic species was 102% which further confirmed that there was no significant loss or gain for each analyte during the digestion/extraction procedures. The mean values of recovery for the CRM was found to be within the interval of confidence ($p < 0.05$) calculated for the certified values, confirming the applicability of the analytical method[94]. Based on these results for the analytical quality parameters, the methods were found to be very efficient for application to the determination of for As species in edible seaweed samples.

However, through the study of the literature, it is possible to perceive the difficulties in obtaining credible and consistent values for the iAs. It is important to reinforce the need to develop CRMs to assist in the validation of analytical methods, especially in complex matrices such as edible algae.

6. Analytical speciation of arsenic in algae: focus on fit for purpose methods

6.1. Sample clean-up with emphasis in solid phase extraction (SPE)

The various techniques of clean-up of samples aim at selectively separating the analyte of interest from all the other components of the sample matrix which may interfere in the analysis.

Although there is already some information about the series of important steps involved in the analytical process before the chromatographic separation of As species, in order to achieving results fit for purpose, the literature often neglects the relevance of clean-up of sample extracts prior to their injection into the HPLC system. This is a crucial step in the whole analytical process because residual matrix components that are introduced to the column along with the analytes can be problematical and interfere heavily both the separation and detection steps. The extract clean-up procedure is particularly important and difficult for the biological samples, like macroalgae. The organic matter due to the presence of significant amounts of lipids, polysaccharides, proteins, pigments, and salt in the sample matrix can result in some deleterious effects in column chromatographic, such as blockage inside the column and loss of resolution between the chromatographic peaks, thus shortening drastically the overall column life.

In order to achieve a cleaner extract, free of pigments and to preserve the chromatographic system, Choi et al.[95], incorporated a single clean-up step in sample extracts was by passing the final extract (50% (v/v) MeOH in 1% HNO_3) through Oasis® MAX (150 mg). After cleanup, Choi et al.[95] verified that most of the seaweed pigments were removed and the eluate was clearer, concluding that this method provided a quick and suitable clean-up for the crude seaweed extract with good recoveries (104-120%).

Yuan et al. [96], in a study on As speciation in rice, reported that the large amount of organic matter in extracts can origin interferences and loss of resolution between the chromatographic peaks during the chromatographic separation. Thereby, the researchers investigated three clean-up methods: C_{18} cartridges, activated carbon, and hexane. They concluded that when C_{18} cartridges were used, the hydrophobic matter and large organic molecules could be removed and the recoveries for As(III) and As(V) were nearly 100%, while the recoveries when hexane was used were 80.4 ± 6.2 to 95.9 ± 2.9%, and finally the activated carbon no recovery detected. Narukawa and Chiba[97] reported similar undesirable effects when they observed the tendency for peak width to increase and peak height to decrease with increasing number of replicates. The measurement errors occurred because the viscosity of the rice flour extract was high and the problem was

solved with internal standardization. There was no need for cleanup, other than centrifugation and filtration.

Moreda-Piñero et al.[98], in a study about pre-treatment for the As speciation in seafood, considered the use of a clean-up procedure based on C_{18} for preventing polar substances and salts to reach the analytical column during the analysis of extracts, and verified that C_{18} produced the highest extraction efficiencies, with recoveries around 100%.

López-Gonzálvez et al.[99] highlighted the importance of a clean-up procedure in the analysis of urine due to the high amount of salt and organic material content with deleterious consequences for the analytical system, when the urine sample is directly injected into a HPLC system. These investigators referred the importance of the use of C_{18} to partially clean-up the urine prior to injection into the HPLC column in order to avoid lack of species separation, peak splitting, and peak broadening owing to column overload which causes a dramatically reduction in column lifetime. Low et al.[100], in a similar study, documented the presence of high chloride concentration in urine and seawater samples. They verified notorious consequences in the separation of As species by ion chromatography, leading to the splitting of a single peak into two or more discrete peaks, as a result of the large differences in the ionic strengths of the mobile phase and injected volume of sample. The authors concluded that the peak splitting can be minimized by inclusion, in the mobile phase, of a column surface modifier, di-n-butylamine phosphate. This modifier enhances the selectivity of As speciation by anchoring positively-charged amine molecules on the column surface, then allowing the modified column surface to accommodate a larger concentration of anions.

Therefore, according to the studies of the literature reported above, solid phase extraction method (SPE) is widely used in biological, clinical, and environmental sample cleaning. The steps involved in a SPE procedure are dependent on the type of matrix, and consists initially of conditioning the cartridges in order to wet and settle the stationary phase, and activate the packing materials, to increase the effective surface area and to remove interferences. After conditioning, the sample matrix is retained in the sorbent and the analyte elutes through the cartridge.

6.2. Development of fit for purpose methodologies for arsenic speciation

6.2.1.1. Anion exchange chromatography

Ion exchange chromatography is a process wherein a solution of an electrolyte is brought into contact with an ion exchange resin and active ions on the resin are replaced by ionic species of a similar charge from the analyte solution[14]. Competition between ions of the stationary phase and those of the analyte allow chromatographic separation to occur based on the difference in migration rates among the analyte components. To achieve a successful separation, the effects of pH and ionic strength of the eluent can be manipulated until optimum conditions are obtained.

The anion character of the species of iAs under particular pH and redox conditions determined that greater flexibility and suitable separation would be obtained using anion-exchange chromatography. Thereby, a Phenomenex Phenosphere SAX 80 A (150 x 2.0 mm) was used in this work, wherein the anion exchanger used in the stationary phase is a silica gel resin having a strong basic quaternary ammonium functional group. The quaternary ammonium functional groups are positively charged and when the sample is injected into the mobile phase the present anions (arsenite and arsenate) interact with the cations in the stationary phase. The differences of adsorption, ion exchange and size lead to different degrees of interaction, resulting in the separation of the ionic compounds. The higher the degree of interaction of the analyte with the stationary phase, the longer it takes to elute, thus exhibiting a longer retention time. A good example is the arsenic speciation, where the arsenite elutes rapidly due to little interaction with the stationary phase, while arsenate elutes slower due to the high interaction with the stationary phase.

6.2.2. Fundamentals of separation and detection by HPLC-HG-AAS

According to the previous study of the methods used for the speciation of arsenic (Section 5.2), it was decided to couple HPLC with HG-AAS, because this method is a simple methodology requiring relatively inexpensive and versatile instrumentation, with a good detection power for inorganic arsenic.

An HPLC pump (Jasco PU-2089 Plus quaternary gradient pump) was coupled with a continuous flow hydride generation system installed in an atomic absorption spectrometer (GBC HG3000 Avanta). For coupling the chromatographic and the detection systems, it was necessary

to connect the column effluent to the bottom of the mixing manifold, while the HCl and $NaBH_4$ were fed on the left side of the mixing block as shown in Figure 10.

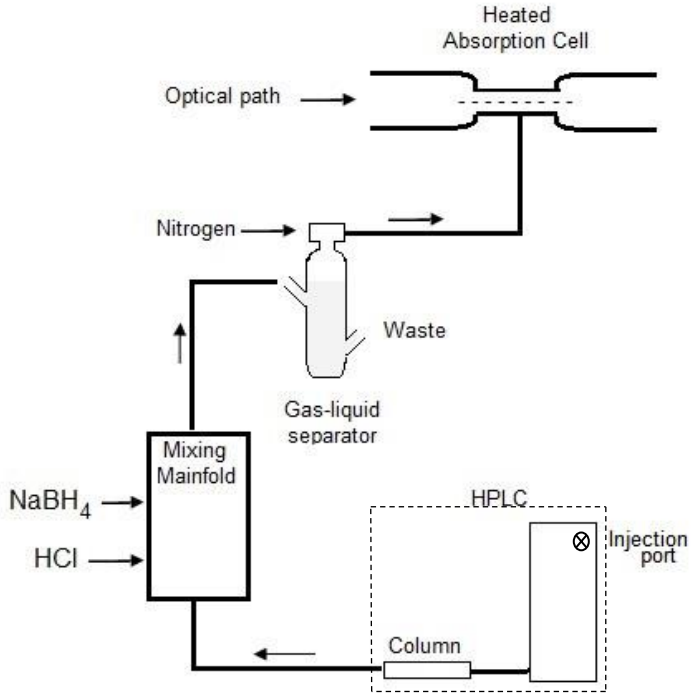


Figure 10*:* Schematic diagram of HPLC-HG-AAS

The principle of this method consists in injecting the sample into the HPLC injection port and separation the different components of inorganic arsenic in a chromatographic column. Then, after separation, inorganic arsenic compounds will react with $NaBH_4$ in acid medium (HCl) to produce volatile arsenic hydride, AsH_3. The generated hydride is separated from the liquid phase in the gas-liquid separator and subsequently transferred by an inert carrier gas (nitrogen), to the adsorption quartz cell atomizer, heated up by a blanket (900 °C) (EHG3000) which provides more accurate temperature control than the flame. For detection, an arsenic hollow cathode lamp (Orpington, Kent, UK) was used, with a wavelength of 193.7 nm and bandpass of 2.0 nm.

6.2.2.1. Mechanisms and operational conditions

The chromatographic conditions used in the determination of iAs compounds were attained with the use of a Phenomenex Phenosphere SAX 80 A (150 x 2.0 mm) anion exchange column. The mobile phase was 8 mM di-Sodium hydrogenphosphate (Na_2HPO_4, Scharlau) and 8 mM Sodium di-Hydrogenphosphate-monohydrate ($NaH_2PO_4H_2O$, Panreac) adjusted to pH 6, with ortho-phosphoric acid (85%).

However, some problems arose due to the choice of this column, because the optimal flow rate of this column is 0.2 mL/min, and to perform the As speciation, the appropriate flow rate should be 1.0 mL/min. In order to prevent the occurrence of sample dilution until reaction with HCl and $NABH_{4,}$ and to obtain adequate peak areas corresponding to As species. Therefore, as expected, the first results during the injection of arsenite and arsenate standard solutions only began to appear after increasing the flow rate to the maximum allowed of 0.8 mL/min.

The setup of the HPLC-HG-AAS system was improved after some initial utilization, since after the arsenite standards injection, there were no satisfactory results, the dependence of the peak area on the concentration of HCl (37%) was investigated. HCl was used as the sample carrier in the hydride generation system and experiments were carried out to evaluate the influence of HCl concentration on the signal. The study was developed using different concentrations of HCl (1 M, 4 M, and 6 M) and a fixed concentration of $NaBH_4$ at 0.4% (w/v). In conclusion, concentrations of 4 M yielded the highest peak areas for As (III) whereas, for HCl concentrations of 1 M, no peaks were revealed, and for 6 M HCl no significant improvements were noticed relatively to 4 M HCl. These results are in accordance with literature[82, 101], where it is reported that is possible to obtain optimal signals of arsenate and arsenite with HCl concentrations between 4-6 M. However, the increase of concentration is more effective in the arsenite than arsenate, because when close to the pH of neutrality is more favorable to the hydride generation of trivalent species. Thus, it is important to be careful in increasing HCl concentration because increasing the strength of the HCl promotes the reduction of arsenate to arsenite.

Despite all the improvements applied in the system, it could not be demonstrated that even at high concentrations of arsenite and arsenate standards (above 100 µg L^{-1}), the AAS performance would be acceptable. On the other hand, for lower concentrations, the performance of the AAS became significantly worse resulting in peaks near the baseline with background noise, revealing that the detection system is not as sensitive as it would be expected.

6.2.3. Fundamentals of separation and detection by HPLC-HG-AFS

6.2.3.1. Mechanisms and principles of operation

Consequently, from to the previous study of the methods used for the As speciation (Section 5.2), it was possible to immediately choose the HG-AFS technique coupled to HPLC as a replacement for HPLC-HG-AAS. The first reason for choosing this method is due to be relatively inexpensive and easy to maintain in a laboratory and AFS represents a suitable detection

alternative to the other atomic and MS techniques. AFS has been considered as superior to AAS and similar to ICP-MS[79]. Linear calibration range and detection limits, in the μg L^{-1} level or even lower, can be easily achieved with HPLC-HG-AFS[102], with further advantages with respect to simplicity, lower acquisition, and running costs for As speciation.

The Jasco, PU-980 intelligent HPLC pump, was coupled with a PSA 10.055 Millennium Excalibur (Orpington, Kent, UK) as shown in Figure 11. Equipped with a continuous flow hydride generation system and a boosted discharge hollow cathode arsenic lamp (Orpington, Kent, UK), as the radiation source of atomic fluorescence detector.

Figure 11: HPLC-HG-AFS system for arsenic speciation

The principle of this method is based on the injection of the sample into the HPLC loop valve and then, the sample is pumped along the chromatographic column, occurring the separation of the different components of As. The exit of the column is connected to a reactor block where the reagents HCl and $NaBH_4$, after passing through the peristaltic pumps, are also connected to the same reactor block. The sample is then mixed with a stream of HCl, in order to acidify the solutions before mixing with $NaBH_4$, followed by the reduction of the As species to form volatile covalent hydrides. This stream follows into the gas-liquid separator, where the volatile hydrides are removed from the liquid and flushed by a stream of argon (Ar), through the Perma Pure® membrane dryer into the detector. Subsequently the hydrides are entrained by the gas flow to the atomizer, which consists of a flame of hydrogen. Then the atoms formed are excited by the radiation from the source of excitation (hollow cathode lamp) and fluorescence

signal reach the photomultiplier, where signal intensity is directly proportional to the As concentration in the sample.

6.2.3.2. Operational conditions

The analytical column used to separate iAs was an anion exchange Hamilton PRP-X100 (250 x 4.6 mm). In this column, the anion exchange resin used in the stationary phase is the strong base anion with the trimethylammonium functional group. This resin is made from polystyrene (PS), which is a plastic material soluble in several solvents, and as a crosslinking agent, divinylbenzene (DVB). In addition, this column had the capacity to reach flow rates higher than the previously used column (SAX 80 A (150 x 2.0 mm)), due to the superior diameter and therefore to allow higher flows. The mobile phase used in this method did not differ from that used in the previous method (HPLC-HG-AAS).

Hydride generation was performed starting from the use of the following reagents: 1.5% (w/v) NaBH4 (Scharlau) stabilized with 1.0% (w/v) NaOH (JMGS, Lda.) and 2M HCl (37%). An argon flow was used to carry the As hydrides to the gas-liquid separator, and a hydrogen stream was used to obtain a good diffusion flame. In order to dry the As hydride, a Permapure system (PS Analytical, Orpington, Kent, UK) was used. The primary current of the discharge hollow cathode lamp was set at 27.5 mA and the boosted current at 34.9 mA. The instrumental operating parameters are listed in Table 4.

Table 4: Instrumental operating conditions for As speciation by HPLC-HG-AFS

Parameter	Operating conditions
HPLC	Jasco, PU-980 intelligent HPLC pump
Column Mobile phase Injection volume	Hamilton PRP-X100, 250 x 4.6 mm, 5 µm 8 mM Na_2HPO_4 and $NaH_2PO_4H_2O$, pH 6, 1.0 mL/min 200 µL
HG	PSA 10.055 Millennium Excalibur
$NaBH_4$ HCl	1.5% (w/v) $NaBH_4$ stabilized with 1% (w/v) NaOH, 1.5 mL/min 2.0 M HCl (37%), 1.5 mL/min
AFS	PSA 10.055 Millennium Excalibur
Wavelength Primary current Boost current	193.7 nm 27.5 mA 34.9 mA

7. Materials and methods

7.1. Sampling and sample preparation

Seasonal samplings were performed between August 2016 and May 2017. The sampling sequence started in August (representing the seasonal conditions of summer) with dehydrated samples of *U. rigida*. In autumn, on November 10th, fresh algae of *F. vesiculosus, P. dioica, U. rigida,* and *Gracilaria sp.* were collected, and part of these macroalgae were washed with salt water treated with UV, while another part was washed with ultrapure water. On December 13th, the dehydrated samples corresponding to these macroalgae were collected. In winter, on January 15th, dehydrated samples of *F. vesiculosus, P. dioica*, and *Gracilaria sp.* were collected, and on March 1st, only dehydrated *U. rigida* was collected. The same sampling procedure was followed in spring, that is, on April 13th, dehydrated samples of *F. vesiculosus, P. dioica,* and *Gracilaria sp.* were collected, and on May 30th only dehydrated *U. rigida* were collected.

All samples collected were transported to the laboratory in plastic bags in a refrigerated box. At the laboratory, half of fresh algae samples were washed with 18.2 MΩ cm, Milli-Q water to remove interfering epiphytes, epifauna, salts and sediment attached. All samples were weighted, frozen and then freeze-dried to prevent further biological activity from modifying the nature of the sample. Once freeze-dried, the samples were homogenized with a blender and stored in a cool and dry place, until the analysis.

Throughout November 2016 and July 2017, physicochemical properties, such as, water temperature (°C), salinity (Practical Salinity Units - PSU), dissolved oxygen (% of saturation), Eh (mV), and pH were measured *in situ* by using previously calibrated Hanna HI 9828 multi-parameter analyzer.

Elemental analysis was performed using a CHNS analyzer (LECO Truspec 630-200-200), that measures carbon, hydrogen and sulfur by means of infrared detection while nitrogen is measured in a thermal conductivity detector system. Determinations were undertaken directly in winter samples of around 2 mg of algae.

7.2. Acid digestion for total arsenic determination

The procedure to perform acid digestion for total arsenic determination it was based in EPA method 3050B. For the total As determination, the digestion of samples was performed using a block digestion system (DigiPrep). Approximately 0.5 g of macroalgae sample was weighted into

a digestion vessel with 5 mL of HNO_3 (65 %) and 5 mL of 18.2 Milli-Q water, which were then mixed and covered with a vapor recovery device.

The samples were heated up to 95°C with reflux for 15 minutes without boiling and, when cooled, 5 mL of HNO_3 was added, being heated again at 95°C with reflux for 30 minutes. Then the samples were allowed to evaporate to 5 mL at 50 °C. Once the samples were evaporated and cooled, 2 mL of Milli-Q water and 3 mL of H_2O_2 (30%) was added and were heated to 50 °C with reflux for 5 hours. After the samples cooled down, 1 mL of H_2O_2 was added until effervescence was minimal in the digestion vessel. The vessel was once again covered with a vapor recovery device and heated until the volume has been being reduced to approximately 5 mL at 50°C. The samples were then left to evaporate to dryness over the next 1-2 days. Finally, the samples were diluted to a final volume of 40 mL with acidified water (HNO_3, 2%) and stored at 4 °C until analysis. Acid digestion procedure was prepared with blanks and the CRM: ERM®-CD200: Bladderwrack (*Fucus vesiculosus*) seaweed.

7.3. Total arsenic determination

Total As determination was performed in GEOBIOTEC laboratory of Department of Geosciences at the University of Aveiro by an ICP-MS, Agilent Technologies 7700 Series (Figure 12).

Figure 12: Inductively Coupled Plasma-Mass Spectrometry

Before the real measurements in the ICP-MS it was necessary to perform a pre-reading in order to suit the dilution to be made due to very high concentrations in the samples. The purpose of the dilutions is to adjust the concentration of As to fit the calibration curve, since only the results that are within the calibration curve are guaranteed in terms of analytical quality. Calibration curves for As was obtained using standards (1.01, 5.18, 19.82, 48.85, 102.11, 206.62

µg L^{-1}) prepared by dilution of certified standard solutions of arsenic in nitric acid (2%). Analytical quality control was performed by using CRM: ERM®-CD200: Bladderwrack (*Fucus vesiculosus*) seaweed.

7.4. Standards and reagents for arsenic speciation

All material used in experiments was previously washed in Derquim 5%, rinsed in Milli-Q water (18.2 MΩ cm^{-1}), soaked in 25% HNO_3 for at least 24h, and subsequently rinsed with Milli-Q water.

All standard solutions were prepared using Milli-Q water. Arsenic trioxide, from M&B, and arsenic pentoxide solution with 1000 ± 0.002 mg L^{-1}, from Merck, were used to prepare the standard solutions. A stock solution of arsenite was prepared by dissolving 0.132 g arsenic trioxide (As_2O_3) in water containing 2 mL HCl, and diluted to 100 mL in a volumetric flask. After preparation of the stock solution, an intermediate solution was prepared with a final concentration of 1 mg L^{-1}. A stock solution of arsenate was prepared from arsenic pentoxide solution, by the addition of 100 µL in a 100 mL volumetric flask in order to obtain a concentration of 1 mg L^{-1}.

From stock solutions of arsenite and arsenate, six standard solutions containing As concentrations between 1 to 30 µg L^{-1} were prepared based on the experimental design of Kang et al.[103]. The uniform design employs a notation $U_n(n^s)$, where U stands for Uniform Design (UD), n for the number of runs and, s for the number of factors. Such experimental design was utilized to reduce the number of standard solutions while keeping the experimental design at an optimal level with a minimal of runs. This work consisted only in the preparation of six samples, in which the six concentration levels of the two analytes of interest (arsenite and arsenate) are enough by a $U_6(6^2)$ uniform design. The preparation of 6 calibrators based on the UD is shown in Table 5. Different volumes of stock solutions of arsenite and arsenate were mixed in the volumetric flasks diluting to 50 mL to obtain concentrations of different proportions in the two species of arsenic.

Table 5: Concentration and volume of iAs for calibration curve based on the uniform design by Kang et al.[103]

Uniform Design		Concentration (µg L^{-1})		Volume (µL)	
Arsenite	Arsenate	Arsenite	Arsenate	Arsenite	Arsenate
2	2	5	5	250	250
3	6	10	30	500	1500
4	1	20	1	1000	50
5	5	25	25	1250	1250
6	3	30	10	1500	500
1	4	1	20	50	1000

7.5. Extraction for arsenic speciation

Extractions were carried out by weighing 2 g of sample into 50 mL centrifuge tubes, adding 50 mL of Milli-Q water, sonicating for 30 minutes, and centrifuging for 20 minutes at 5000 rpm. The extraction procedure was repeated three times with a fresh solvent each time, and the supernatant (50 mL total) was combined in a clean centrifuge tube. The first and second extractions were carried out by adding 20 mL of Milli-Q water and only on the third extraction, 10 mL was added to the centrifuge tubes in order to obtain 50 mL of extract.

The extract was initially filtered using a 0.45 µm filter (Durapore® membrane filters, Merck) in order to remove the suspended particulate matter, followed by a filtration using a 0.22 µm filter (Durapore® membrane filters, Merck) and transferred the filtrated extracts into 250 mL round bottom flasks. The extract solutions for each sample were evaporated to dryness and 2 mL of Milli-Q water was added to the round bottom flasks of the *Gracilaria sp., F. vesiculosus* and *P. dioica* and 3 mL of Milli-Q water to *U. rigida*. This amount of water was added to be removing the dried extract from the round bottom flasks walls.

In the final extract, the SPE method was applied, in order to clean-up the organic content, due to high viscosity of the extracts and the presence of significant amounts of lipids, polysaccharides, proteins, and pigments. To the SPE cartridges was added about 1 g of sorbent (DSC-18, Supelco) fixed in the tube between two filters (Replacement Teflon® Frits, Sigma-Aldrich®). The sorbent was conditioned with 10 mL of methanol and 10 mL of Milli-Q water, pumped through each cartridge at a slower flow to increase the contact time, using the SPE vacuum pump.

The sample extract, containing the analyte of interest, was placed on the top of the cartridge and aspirated with a vacuum pump. As a result, the analyte is collected as the sample passes through the tube, while the interfering impurities remain on the sorbent. Finally, the samples were kept in a fridge, until speciation analysis.

7.6. Phosphate determination

For phosphate determination, water samples were taken from the algae tanks on February 16th and April 11th in order to establish the relationship between the arsenate concentration in the algae and the phosphate concentration in the tanks.

The procedure to perform phosphate determination it was based in EPA method 365.2. Each water sample was filtered using a vacuum filtration device including a vacuum pump, and the pH of the samples were then adjusted to 7 ± 0.2 with a pH electrode.

After this process, the reagents for phosphate determination were prepared. Firstly, the combined reagent was prepared by mixing 50 mL sulfuric acid solution (H_2SO_4), 5 mL of antimony potassium tartrate solution ($K(SbO)C_4H_4O_6.1/2H_2O$), 10 mL of ammonium molybdate solution ($(NH_4)_6Mo_7O_{24}.4H_2O$), and 30 mL of ascorbic acid (1 M). A stock phosphorus solution was prepared weighing 0.1099 g of potassium dihydrogen phosphate (KH_2PO_4) and dissolved in 500 mL of distilled water. Then, in a 50 mL volumetric flask was added 2.5 mg L^{-1} of stock phosphorus solution. From this standard solution were prepared standards with a concentration range from 0.010 to 0.150 mg L^{-1}, finally 8 mL of combined reagent was added to each sample, and after 15 minutes the samples were transferred to optical glass cuvettes and absorbance were read at wavelengths 880 nm using the blank as the reference.

8. Results and discussion

8.1. Characterization of samples by elemental analysis (CHNS)

Elemental analysis is very important in the determination of chemical composition of a compound and of its elemental proportions which allows the calculation of an empirical formula. In this work elemental analysis was used for the determination of the percentages of carbon (C), hydrogen (H), nitrogen (N) and sulfur (S) in different algae species, while the percentage of oxygen was determined by difference (Table 6).

Table 6: Elemental analysis for *Ulva* rigida, *Porphyra dioica, Fucus vesiculosus*, and *Gracilaria sp.* (n=2)

Sample	%C Mean MAX-min	%H Mean MAX-min	%N Mean MAX-min	%S Mean MAX-min	%O Mean MAX-min	% $\frac{C}{N}$	Empirical form
U. rigida	28.5 28.5-28.3	5.19 5.28-5.11	3.31 3.26-3.36	4.79 4.80-4.78	58.3 58.1-58-4	8.61	$C_{17}O_{26}H_{36}NS$
P. dioica	38.3 38.4-38.1	5.79 5.82-5.76	4.68 4.73-4.64	2.23 2.20-2.26	49.0 48.8-49.3	8.18	$C_{46}O_{44}H_{84}N_3S$
F. vesiculosus	32.5 32.3-32.7	4.53 4.54-4.52	1.87 1.93-1.81	1.96 1.95-1.97	59.2 59.3-59.1	17.4	$C_{44}O_{61}H_{75}NS$
Gracilaria sp.	32.9 32.8-32.9	4.66 4.68-4.64	3.77 3.92-3.62	2.41 2.53-2.29	56.3 56.1-56.5	8.73	$C_{37}O_{47}H_{62}N_2S$

The results depicted in Table 6, show that in all species the elements with the highest percentage are C and O while the other elements, N, H and S have low percentages. Among the four macroalgae studied the highest percentage of nitrogen, carbon and hydrogen was obtained for the *P. dioica* (4.68, 38.3 and 5.79%, respectively) while the highest percentage of oxygen and sulfur were obtained for the *F. vesiculosus* (59.2%) and *U. rigida* (4.79%), respectively. Relatively to the smallest percentages, *F. vesiculosus* obtained the smallest percentage of N, H and S (1.87, 4.53 and 1.96%, respectively), while the smallest percentage of C and O were obtained for the *U. rigida* (28.5%) and *P. dioica* (49.0%), respectively.

The higher values of N in red and green algal tissues, as compared to those found in brown algae, suggest that the *F. vesiculosus* was less efficient than other species in the assimilation of nitrogen. Due to the considerable decrease in N concentration, the C:N ratio values were twice as high as in the other species. Low nitrogen concentrations in the growth media

decreases the protein content, while increasing carbohydrate content in the biomass. Although the concentrations of nutrients in this growth media were not measured, it is expected that red and green macroalgae contain a greater amount of protein while brown macroalgae contain a high content of carbohydrates.

8.2. Influence of the washing in the total As concentration in macroalgae

As mentioned in Section 7.1, the first sampling was carried out in two phases: a) fresh samples collection, which consisted in washing a part of these samples with salt water treated with UV, while another part was washed in the University of Aveiro with ultrapure water; and b) dehydrated sample collection. Sampling of algae under different conditions was performed to understand whether there was an influence on the accumulation of As in algae. In order to decide whether this washing step influenced the concentration of As in algae, a statistical treatment was performed using ANOVA (Two Factor Without Replication) and it was concluded that there is no significant effect ($p > 0.05$) between washing with salt water and ultrapure water on the As concentration. Therefore, it was decided to use dehydrated samples provided by the company, because in addition to post-harvesting, the samples are analyzed with the same characteristics of the samples that reach the final consumer.

8.3. Physical and chemical parameters determined *in situ*

The evolution of water temperature, salinity, pH, redox potential, and dissolved oxygen in *P. dioica, F. vesiculosus, Gracilaria sp.,* and *U. rigida* culture tanks were recorded between November 2016 and July 2017 and these data are shown in Figure 13, and in more detail in the Table A.2 in Annex section. For *U. rigida* the parameters were measured in 12 tanks where the macroalgae are cultivated, and due to the enormous amount of values obtained, only the median, maximum and minimum values of the measurements are displayed in Figure 13.

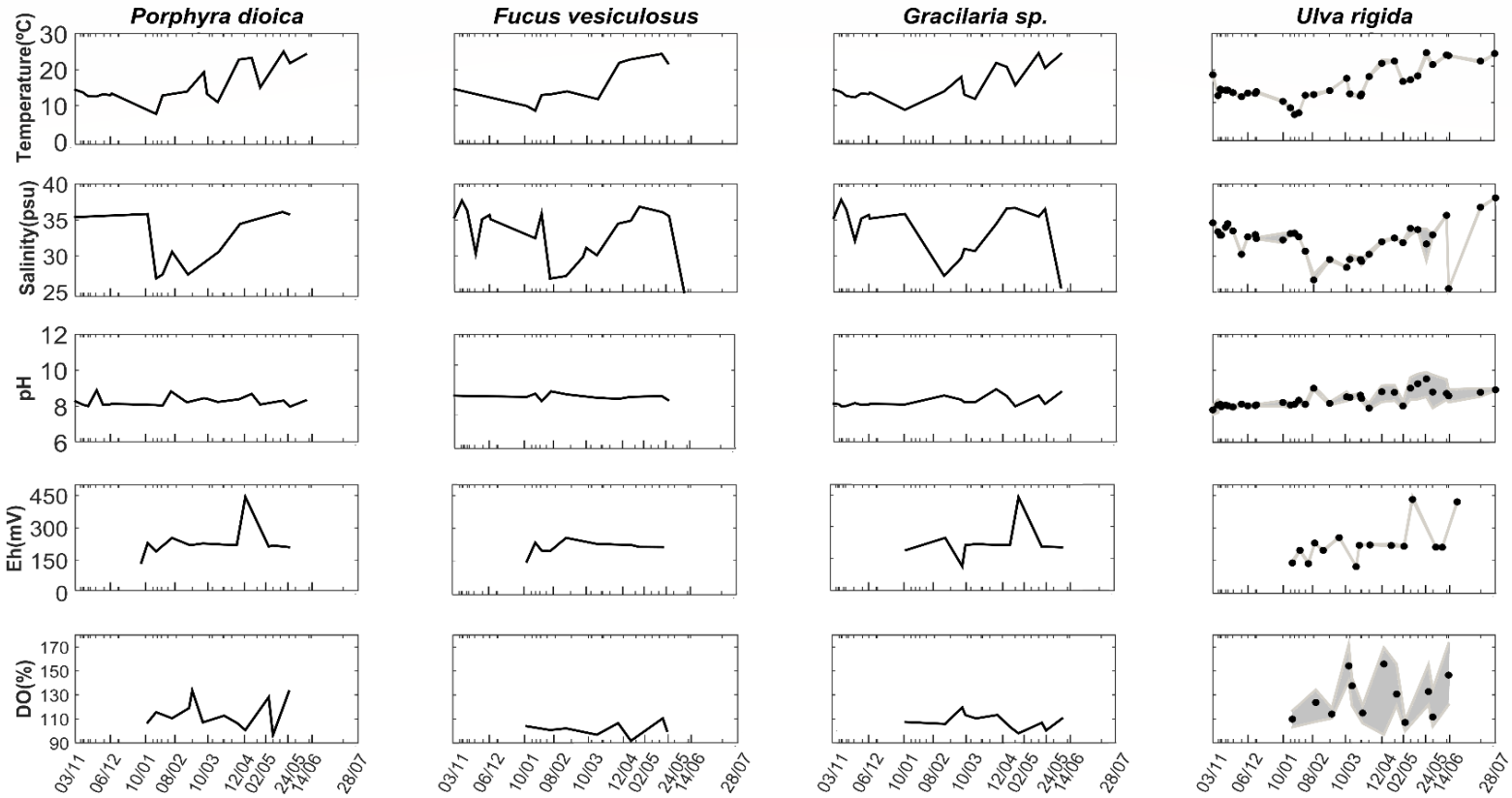


Figure 13: Seasonal variation of temperature (°C), salinity (PSU), pH, Eh (mV), and dissolved oxygen (%) in the tanks of *P. dioica, F. vesiculosus, Gracilaria sp.,* and *U. rigida*. For *U. rigida*, black points are medians and the shadow zone is the distance between the maximum and minimum values

The temperature evolution follows the same trend in all tanks over time. There is a decrease in the temperature range from autumn to winter and an increase in temperature values from winter to summer season which reflects the seasonal evolution of air temperature. The minimum value for water temperature (7.3 °C) was recorded in January and the maximum value (25.0 °C) in May, reflecting the seasonal trend. The salinity values are similar in all tanks and the smallest values were observed during winter season (24.4 psu), due to intense rainfall that occurred in this period, which lead to the consequent increase of fluvial flow increasing the contribution of fresh water in the Ílhavo channel that feeds the algae culture tanks of ALGAplus. Meantime, higher salinity values were recorded during summer (37.8 psu) period with exception of the June 14th in which the value of salinity decreased drastically (25.6 psu) due to an intense precipitation that preceded this period of sampling.

The pH of the algaculture media plays an important role in regulating the uptake of essential nutrients, such as nitrate and phosphate. The common pH range in algaculture systems is between 7 and 9, with the optimum range between 8.2 and 8.7[104]. In this work the pH values obtained are quite constant over time and ranged from 7.7 to 9.0 in tanks of all species except for *Ulva rigida* which reached values higher than 9 during May. The increase of pH in *Ulva rigida* tanks during the mentioned period was probably due to the low aeration, namely in tanks 31 to 37, consequently leading to limitations on the CO_2 consumption.

With respect to the redox potential, there is no considerable variation over time in the *P. dioica, Gracilaria sp.,* and *F. vesiculosus* tanks, where the range of values for this parameter varied between 137.7 to 253.6 mV, except on May 2nd that Eh reached values of 443.1 and 441.8 for *P. dioica* and *Gracilaria sp.*, respectively. Relatively to the values of Eh obtained for *U. rigida* tanks, the greatest oscillations were observed over time, with the highest value occurring on May 2nd reaching a maximum of 446.1 mV and the lowest on March 10th, with a minimum of 119.1 mV.

The diagram in Figure 14, shows that the values of pH (7.5-9.9) and Eh (0.119-0.446 V), indicate that the predominant As specie in algae culture tanks is $HAsO_4^{2-}$. This result suggest that As is incorporated into the cells of algae in the form of arsenate, as reported in the literature[35, 36].

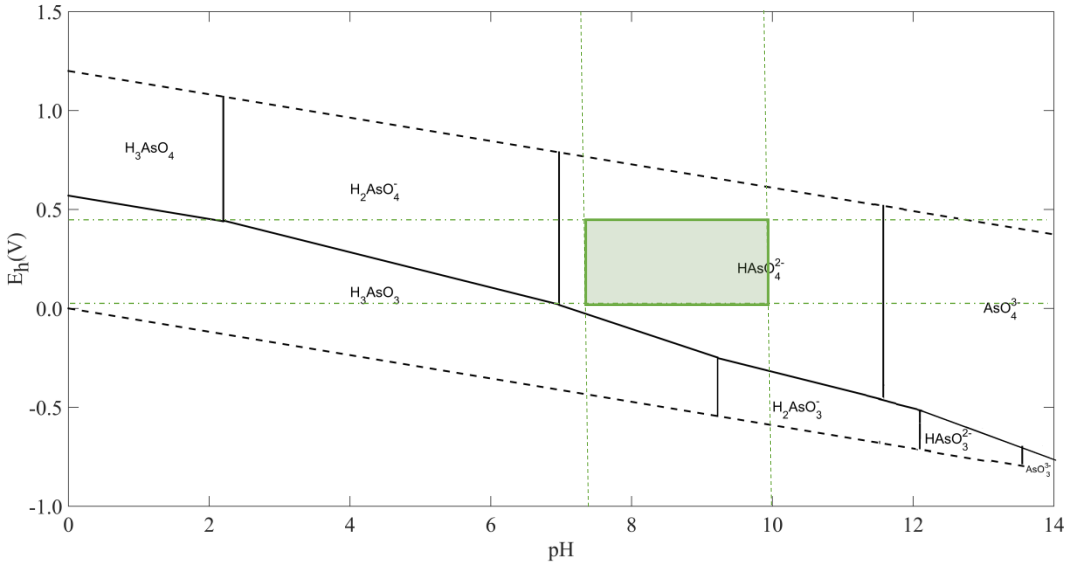


Figure 14: Operating intervals of pH and Eh during physicochemical characterization of algae tanks

The values of percentage of dissolved oxygen were constant over time with a range between 91.7 to 133.6% in the *Porphyra dioica, Gracilaria,* and *Fucus vesiculosus* tanks, while some oscillations were observed in *Ulva rigida* tanks with the highest value (173.1%) obtained in the last three measurements (summer). These values demonstrate the good oxygenation of water due to the high production of O_2 during the process of photosynthesis, mediated by macroalgae.

8.4. Concentration of total arsenic in macroalgae

Seasonal sample collection was performed to determine the total As concentration in macroalgae of different species. Three replicates from each tank of *P. dioica, F. vesiculosus,* and *Gracilaria* sp. were analyzed during autumn, winter, and spring in a total of 27 samples.

For *Ulva rigida* only sample per tank was analyzed, in a total of 12 tanks. In the latter case the sample per tank procedure was adopted because the physical and chemical conditions were kept constant in four groups of tree tanks. The analyses for this species were performed in summer, autumn, winter, and spring, resulting in a total of 48 samples.

Total arsenic concentrations measured in *P. dioica, F. vesiculosus,* and *Gracilaria sp.* are displayed in Figure 15 and for *U. rigida* in Figure 16. The detection limit obtained by blank measurements was 0.02 mg kg^{-1}. The obtained arsenic of CRM content was 51.4 ± 0.8 mg kg^{-1}

which agrees well with CRM of 55 ± 4 mg kg^{-1} with percentages of recovery within the range 89-96% (n=6).

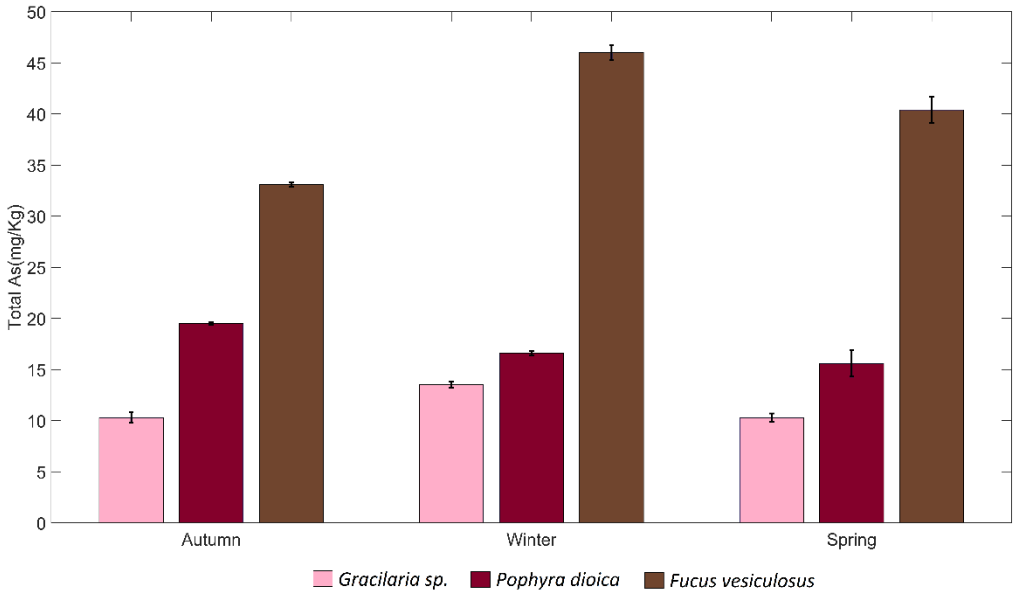


Figure 15: Concentration of total As in autumn, winter, and spring for *P. dioica, F. vesiculosus,* and *Gracilaria* sp.

The As content of the different species varied between 1.19 and 48.0 mg kg^{-1}, where the lowest of concentration of As was found in *Ulva rigida* during the summer and the highest values of concentration of As was observed in *Fucus vesiculosus* during the winter.

Figure 15 showed a decrease of As concentration in *P. dioica* from autumn to spring, where the concentration in autumn was 19.5 ± 0.3 mg kg^{-1} followed by winter and spring with 16.7 ± 0.2 mg kg^{-1} and 15.3 ± 0.7 mg kg^{-1}, respectively.

Relatively to *F. vesiculosus* and *Gracilaria sp.* the pattern found was similar, total As concentration increased from autumn to winter and decreased in the spring. For *F. vesiculosus*, As concentration in winter was 46.5 ± 1.29 mg kg^{-1} followed by spring and autumn with 40.2 ± 1.28 mg kg^{-1} and 33.1 ± 0.4 mg kg^{-1}, respectively. The concentration of *Gracilaria sp.* was lower than those of *F. vesiculosus* and *P.dioica* throughout the seasons: in winter, *Gracilaria sp.* attained 13.6 ± 0.1 mg kg^{-1} followed by autumn and spring with 10.6 ± 0.5 mg kg^{-1} and 10.3 ± 0.2 mg kg^{-1}, respectively.

Figure 16 includes a set of 4 graphs showing that the As concentration in the 4 seasons: summer, autumn, winter, and spring. Comparatively to the others macroalgae species, *U. rigida* presented de lowest As concentration throughout the seasons, where the total As varied between 1.19 to 3.69 mg kg^{-1}. Throughout all the seasons, total As is not much variable during summer,

spring and autumn; however in winter, the concentrations of As attain high values consistently in all the aquaculture tanks.

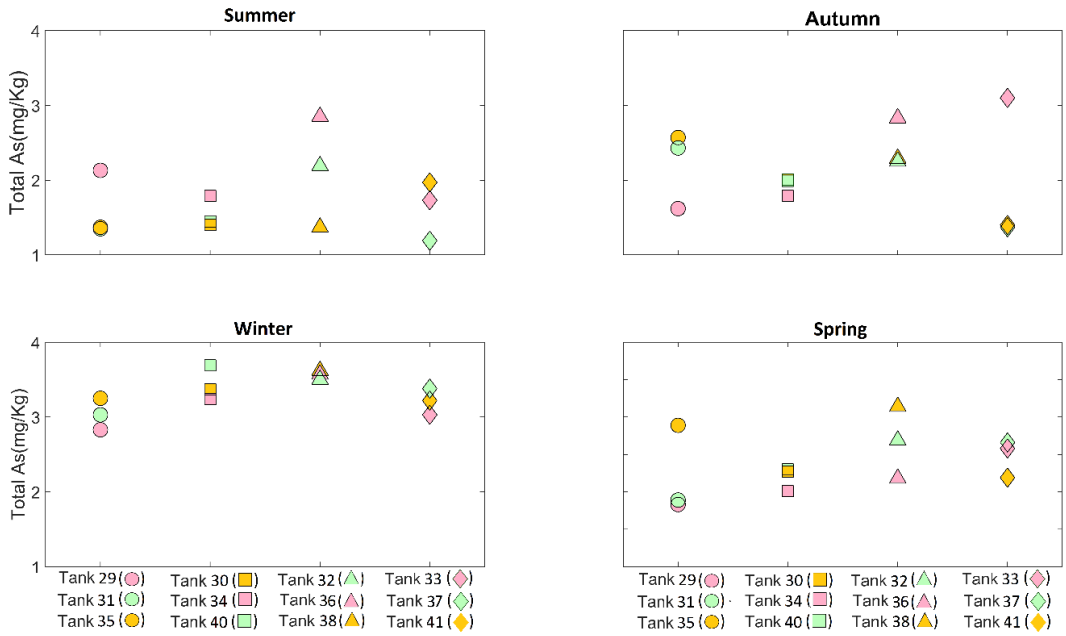


Figure 16: Seasonal concentration of total arsenic in *Ulva rigida*

Figure 16 also shows the As concentration obtained from each tank individually, in order to observe the relationship between the tank and the As incorporation. It is possible to verify that the second lot of tanks, 30, 34 and 40, shows the lowest As concentrations in summer, autumn and spring, but in winter it reaches the highest concentrations of As.

Despite red and brown algae have a low number of analyzed samples (3 x 9) comparatively to green algae, it was possible to see a clear correlation between total As content during the different seasons and algae species. Brown algae accumulated higher levels of As than green or red algae and in winter this concentration is higher than in other seasons, with exception for *P. dioica,* which has accumulated more in autumn. This exception may be due to the fact that this species is cultivated in the laboratory, therefore the time of contact with the natural environment is shorter. Those results were according to the data available in the literature[9] showing that algae naturally accumulate more As in winter than in warmer seasons, probably due to the rate of growth of the species, which is lower in winter, thus causing an increase in As concentration. According to Klumpp[30] algae accumulate more As in periods of low light in the presence of photosynthetic inhibitors, which is in agreement with the fact of algae accumulate more As in winter. However, more studies should be made in order to establish the relationship between the total As content, season and the increase in biomass in warmer seasons.

Besides the seasonal influence, taxonomy may also play a significant role in the distribution of the As compounds among algae[105]. In this study, brown algae contain higher concentrations of As than those observed in red and green algae, as also reported in studies by Francesconi and Edmonds[37], and Sanders[38]. Apparently brown algae are unable to regulate the trace elements due to presence of a large number of compounds with anion groups in their cell walls[38], and this fact is influenced by factors such as temperature, pH, light, life cycle of algae, and relative abundance of phosphate in surrounding water[106]. Sanders[38] suggests that a high accumulation of As by brown algae varies directly with uptake of phosphate, because they contain higher concentrations of phosphate than red and green macroalgae. In this work the phosphate concentration was measured in the water of the macroalgae tanks but no correlation was found and the phosphate concentration was very low (0.01-0.05 mg L^{-1}). Although in the literature it is relatively easy to find results of As concentration in the genus of the algae studied, the vast majority do not admit seasonal variations. However, despite this omission, all concentrations of total As found in the literature are within the range of values obtained in this work.

The results obtained for *U. rigida* are consistent with the results presented by Díaz et al.[107], Pell et al.[85], Llorente-Mirandes et al.[108], Šlejkovec et al.[109], and Al-Masri et al.[110], where the concentration ranged from 1.4 to 5.5 mg kg^{-1}.

The same happens for the concentrations of As in *F. vesiculosus*, where García-Salgado et al.[77] obtained concentration of 36 ± 2 mg kg^{-1} similar to those obtained by Almela et al.[105] of 40.4 mg kg^{-1}. Only Taylor et al.[111] and Maehre et al.[112] made the determination of As in *Fucus sp.* taking into account the seasonality, and value obtained in winter was 32 mg kg^{-1} and in the spring 41 mg kg^{-1}. However, although the concentration of total As in current study was very similar to obtained by Maehre et al.[112] in spring the result in winter is quite superior (45 ± 1.3 mg kg^{-1}).

For the genus *Gracilaria*, the concentration obtained by Pell et al.[85] ranged from 7.1 ± 0.4 and 12.2 ± 0.5 mg kg^{-1}, and for the genus *Porphyra*, Díaz et al.[107], Al-Masri et al.[110], and Taylor et al.[111], obtained 23.8 ± 15.0, 27.3, and 20.7 mg kg^{-1}, respectively. Therefore, also the values obtained for the genus *Porphyra* and *Gracilaria* were concordant with those obtained in the present work.

Table A.3 in the Annex section, presents the total and inorganic As concentrations for different geographic locations in algae covered in this study, obtained by various authors.

8.5. Speciation of inorganic arsenic compounds

8.5.1. Clean-up of samples prior speciation analysis

For the determination of As compounds by HPLC-HG-AFS generally the injection of samples extracts into the chromatographic column occur after its filtration through the syringe filters (0.22µm). However, after performing the extraction procedure for the speciation it was observed that the extracts obtained had a high concentration of pigments and organic matter. There was a need for sample clean-up procedures in order to ensure that the chromatographic separation and results were not compromised. Nevertheless, as previously mentioned, there is no reference in the literature to cleaning procedures for algal extracts and cleaning procedures for other types of biological samples were there considered for the propose of this study.

Thereby, it was concluded that the application of the SPE method could have remarkable effects on the cleaning of algae extracts as it did in other biological matrices[96, 98, 99]. Figure 17 shows the visual results of the different cleaning steps of the extracts obtained from the species: *Gracilaria sp., Fucus vesiculosus,* and *Porphyra dioica* in autumn, winter, and spring. The presence of high amounts of organic matter during the cleaning process is evident due to the high percentage of pigments, proteins, polysaccharides and sugars that characterize macroalgae species[113].

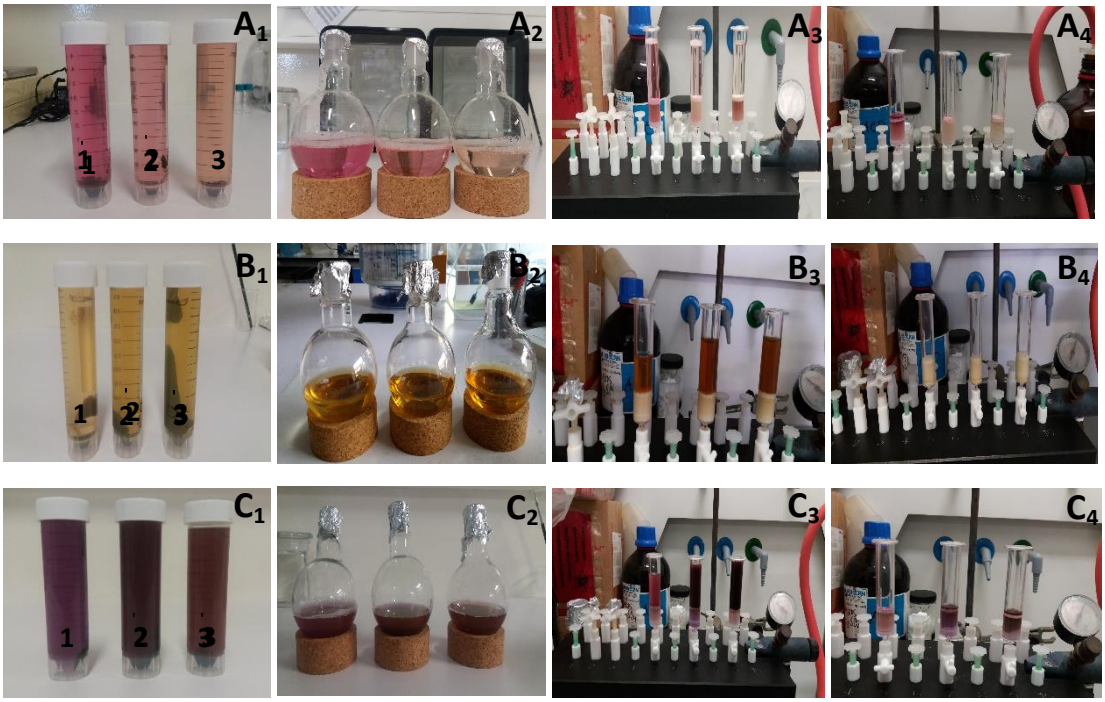


Figure 17: Stages of the cleaning of the extracts of the different species and observation of the different pigmentation during different seasons. **A_{1-4}:** *Gracilaria sp.*; **B_{1-4}:** *F. vesiculosus*; **C_{1-4}:** *P. dioica*. **1:** Autumn; **2:** Winter and **3:** Spring

As previously referred (section 7.5), the sorbent (stationary phase) used in this work in order to retain the interfering matrix was DSC-18, constituted by a polymerically bonded octadecyl with high carbon loading for greater binding capacity based in a reversed-phase retention mechanism. The mobile phase used is polar, water and methanol, allowing the retention of nonpolar interferences in the sorbent.

Figure 17 (**A_1, A_2, B_1, B_2, C_1, C_2**) shows the extract of macroalgae species after the extraction process, where it is clear the presence of organic matter such as, pigments, while Figure 17 (**A_3, A_4, B_3, B_4, C_3, C_4**) shows a large amount of organic matter retained in the sorbent of macroalgae extracts after applying the SPE clean-up process.

8.5.1.1. Effects of sample cleaning by the SPE method

Two replicates of *Gracilaria sp.* extracts in autumn were performed for assessing improvement by SPE cleaning in the speciation results. The first extract was injected only with the filtration through the syringe filter (0.22 µL) while in the second extract was cleaned by SPE method and filtrated by syringe filter (0.22 µL) before the injection into the loop. The chromatogram obtained without SPE cleaning, depicted in Figure 18 (**1**), shows a peak splitting, suggesting the presence of a high amount of organic material and high viscosity in the injected extract, which consequently led to an increase in the width of the peak and a decrease in height of the peak. In order to confirm this hypothesis, about peak splitting, the chromatogram where the SPE method was applied for the cleaning of the extracts is presented in Figure 18 (**2**). From the significant improvement in the appearance of the peak it can be concluded that the proposed clean-up procedure is highly efficient.

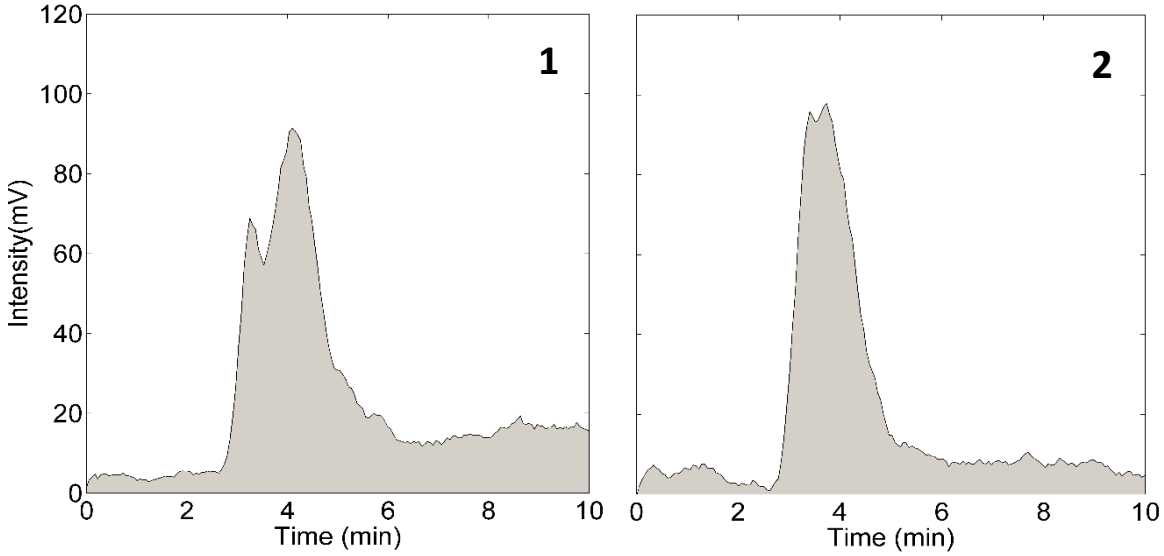


Figure 18: Representation of the arsenite elution chromatogram before and after the SPE method cleaning of the *Gracilaria sp.* extract in the autumn. **1:** No cleaning; **2:** Cleaning

In more detail, Figure 18 (**1**) shows that, as the arsenite is eluted, its peak experiences a higher degree of splitting and there is an evident difficulty in the removal of the adsorbed layer by the mobile phase until eventually the column is able to reach its original state, while in the Figure 18 (**2**), after sample cleaning by the SPE method, it is evident that the split peak is less pronounced and apparently the sample layers adsorbed on the stationary phase are removed much faster.

In general terms, the changes in the chromatographic peak of the two chromatograms presented, are essentially governed by the large differences between the concentration of chloride and organic matter present in the extract of the sample injected into the chromatographic column. When these samples are injected into the loop, they cause an increase in pressure in the chromatographic column due to the high viscosity. Moreover, when a sample with a high amount of organic material interacts with the stationary phase, it changes its surface characteristics and consequently the chromatographic behavior is affected, causing unbalances on its surface and consequently the adsorbed layer is slowly withdrawn by the mobile phase because the viscosity makes it more difficult to transport the sample due to the resistance and surface tension.

On the other hand, when the stationary phase is clean and the sample is injected, there is an interaction between the sample analytes and the exchanges sites of the stationary phase that

compete with each other and the analyte, with less interaction / binding power with the exchange sites, elutes faster.

8.5.2. Separation and detection of arsenic compounds

Arsenic speciation study has been performed for *F. vesiculosus, P. dioica* and *Gracilaria sp.* in autumn, winter, and spring and for *U. rigida* for summer and autumn. Arsenic species in the chromatograms were identified by comparing the retention times with of the standards available for this work. The standard solution used contain a mixture of two species: As(III) and As(V) with known concentrations and the retention time of As(III) and As(V) were identified to be around 3.40 and 7.20 min, respectively. In practical terms and for quantification purposes, the evaluation of peak area associated with As (III) was based on a range of retention times between 2.6 and 5.2 minutes, while for As(V) was based on a range between 6.2 and 8.5 minutes (see i.e. Figure 19).

8.5.2.1. Arsenic compounds detected in *Gracilaria sp.*

The chromatograms of Figure 19 and is possible to conclude from the retention time of the match standard that the As compound detected is arsenite. *Gracilaria sp*. contains arsenite in every season, where the highest concentration is in autumn, being able to be indicator that this season have abiotic or biotic factors that provide the higher accumulation of arsenite. No more As species were detected in this algae regardless of the season, unlike studies of Pell et al.[85] where they detected arsenite, arsenate and, DMA in winter samples while in other seasons they did not detect any these chemical species.

It can be also observed in Figure 19 (**B2**) that SPE cleaning was highly efficient in winter extract, probably due the low concentration of organic matter in this season.

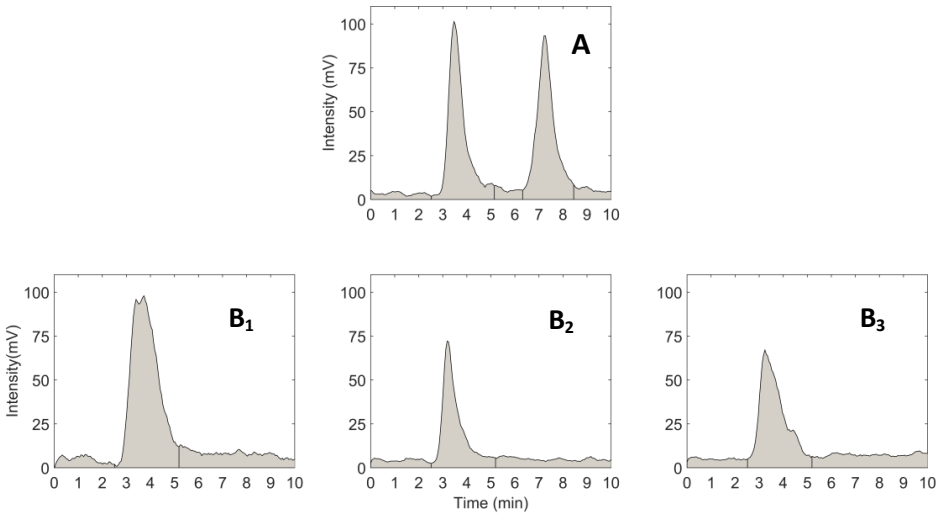


Figure 19: HPLC-HG-AFS chromatograms of arsenite and arsenate standard solution (10 µg L^{-1}) **(A);** and *Gracilaria sp.* extract in autumn **(B_1)**, winter **(B_2),** and spring **(B_3)**

8.5.2.2. Arsenic compounds detected in *Porphyra dioica*

Figure 20 shows the chromatograms of *P. dioica* extract where is a peak of As(III), and a probably a peak of DMA are marked on a range of retention time between 5.2 and 6.2 minutes.

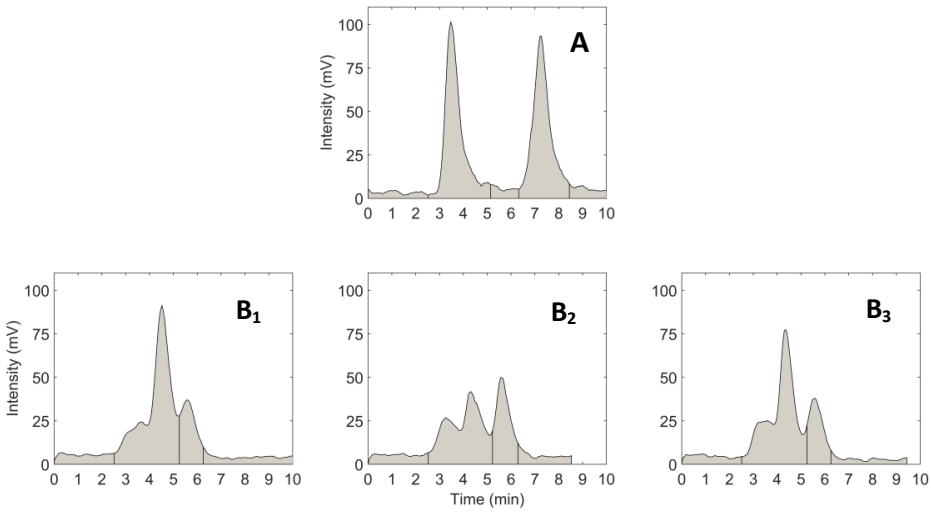


Figure 20: HPLC-HG-AFS chromatograms of arsenite and arsenate standard solution (10 µg L^{-1}) **(A);** and *P. dioica* extract in autumn **(B_1)**, winter **(B_2),** and spring **(B_3)**

The chromatograms show that the elution pattern is the same in all seasons, the first peak to elute is a peak splitting of arsenite where the retention time is consistent with the retention time of the arsenite standard. However, an unknown anion was well separated and found in every

season, and despite its clear identification is difficult to perform due to the lack of appropriate standards, such can be assessed by comparison with similar studies[13].

Based on the study of Šlejkovec et al.[13], same the retention time of this compound matches the retention time of the unknown peak when using the Hamilton PRPx-100 anion exchange column in the same chromatographic conditions.

The distribution of arsenic compounds in Figure 20, in winter (**B_2**) *P. dioica* extract contain low proportions of the most toxic compound, As(III), while in autumn and spring (**B_1** and **B_3**) there is an increase of As(III). For the suspected DMA peak the concentration is similar throughout all the seasons. Arsenate, which has relatively strong toxicity, was not detected in any *P. dioica* sample. The results obtained for this macroalga do not agree with those obtained in similar studies[105, 111, 114] for genus *Porphyra*, the majority of which can detect arsenate and does not detect arsenite. This fact according to Llorente-Mirandes[115] is due to the extraction method used, where it can occur a high interconversion between As(III) and As(V). However, through the study of different extraction methods, the same author concluded that the extraction method used in this work (water extraction) is the most effective in extracting the As species from samples and no significant transformation were observed, thus concluded also that this extraction method preserves the original state of oxidation of As(III) and As(V).

Thus, the presence of arsenite may be related to the cultivation conditions to which this species is subject. *P. dioica* is cultivated in the laboratory of ALGAplus and the stress conditions which the alga is subjected during the cultivation process may induce the higher accumulation of arsenite.

8.5.2.3. Arsenic compounds detected in *Fucus vesiculosus*

The chromatograms obtained for *Fucus vesiculosus* extracts (Figure 21) shown the pattern similar throughout the seasons. In every season it is possible to conclude that the first peak corresponds to the arsenite by the retention time of arsenite standard. The winter chromatogram shows a peak splitting for arsenite compound and the spring chromatogram shows a poor peak where the decrease of height and increase of width of the peak is obvious. In this peak there is an evident difficulty in reaching the baseline, probably attributable to the effect of the matrix. These results suggest that the SPE method is not enough for *F. vesiculosus* extract clean-up, possibly due to the higher concentration of carbohydrates in this specie.

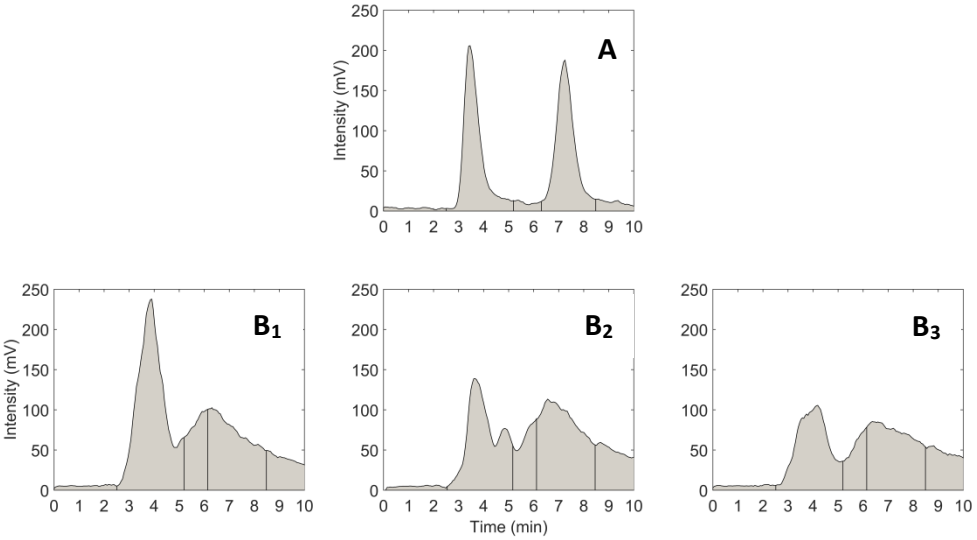


Figure 21*:* HPLC-HG-AFS chromatograms of arsenite and arsenate standard solution (20 µg L^{-1}) **(A);** and *F. vesiculosus* extract in autumn **(B_1)**, winter **(B_2),** and spring **(B_3)**

In relation to the second peak there is a great possibility of being arsenate by comparing the retention time with the correspondent standard. However, a similar study by Fitzpatrick[116] for the speciation of As in *Fucus sp*. resulted in a series of poorly defined peaks and after the application of the electrospray mass spectrometry (ESMS) as detector, concluded that these peak were As-sug and these compounds do not appear solely at their characteristic retention times but elsewhere in the chromatogram. Also, Pell et al.[85], in a similar study concluded that *F. vesiculosus* extract reveals the presence of arsenite and As-sug compounds which are common in brown algae. Therefore, it would not be surprising if at the same time of the elution of arsenate there was a co-elution of As-sug. The probability that co-eluting species with the same molecular ions may be present in a sample cannot be overlooked, and therefore it would be necessary to carry out further studies to prove the presence of these compounds.

8.5.2.4. Arsenic compounds detected in *Ulva rigida*

Finally, two chromatograms are presented for *U. rigida* (Figure 22), one corresponding to the summer sample (**B_1**) and another to the autumn sample (**B_2**), where it is possible to verify that in the two seasons presented the peaks of the chromatograms have poor resolution, such would be due to the matrix effect and low concentrations of As species. While in the summer **(B_1)** chromatogram arsenate is detected, arsenite is detected in autumn (**B_2**). There are studies[107, 108]

about *U. rigida* where they found both arsenite and arsenate in extremely low concentrations but no effect of the season as shown.

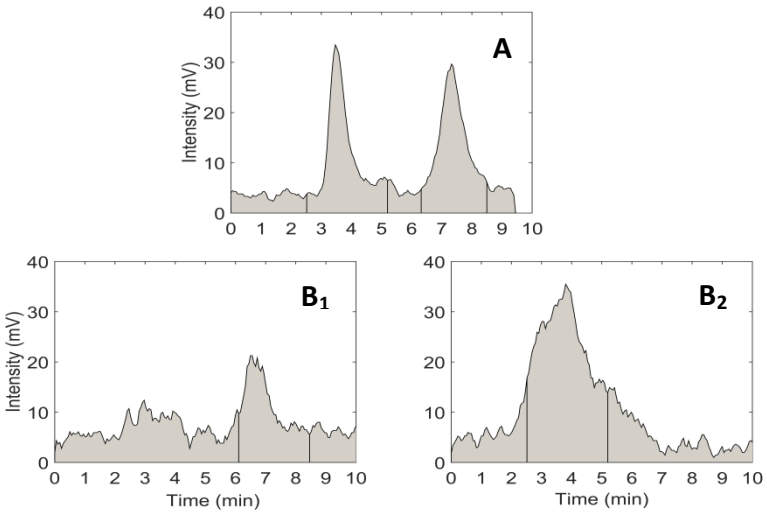


Figure 22: HPLC-HG-AFS chromatograms of arsenite and arsenate standard solution (2 µg L^{-1}) **(A);** and *U. rigida* extract in summer **(B_1)** and autumn **(B_2)**

8.5.3. Quantification of arsenic compounds

Each species of As were quantified by using the calibration curves as shown in Figure A.1 in the Annex section and the speciation results are shown in Table 7.

The concentration of iAs in the edible seaweed analyzed was very low, ranging from 3.8 ± 0.5 to 28.5 ± 0.2 µg kg^{-1} for arsenite and 13.4 ± 1.6 to 17.9 ± 0.4 µg kg^{-1} for arsenate. The main compound quantified was arsenite and it was present in all samples of the different algal species while arsenate was present in all samples of *F. vesiculosus* only. However, despite the range of 6.2 to 8.5 minutes have been considered the arsenate retention time, it is possible that the quantification of the arsenate concentration was affected by the elution of organic species, namely, As-sug.

Table 7: Results of arsenic speciation in macroalgae from ALGAplus (mean ± standard deviation, µg As kg^{-1}, n=2)

Macroalgae	Seasons	Arsenite	DMA	Arsenate
Gracilaria sp.	Autumn	12.1 ± 0.5	-	-
	Winter	3.8 ± 0.2	-	-
	Spring	5.8 ± 0.3	-	-
F. vesiculosus	Autumn	28.5 ± 0.2	-	13.4 ± 1.6
	Winter	19.3 ± 3.0	-	17.9 ± 0.4
	Spring	13.8 ± 1.3	-	13.9 ± 0.7
P. dioica	Autumn	7.64 ± 0.2	*	-
	Winter	4.3 ± 0.2	*	-
	Spring	8.9 ± 2.2	*	-
U. rigida	Summer	-	-	<LD
	Autumn	4.7 ± 0.2	-	-

**possibly detected*

The brown algae, *F. vesiculosus*, shows the highest content of As(III) and As(V), although in autumn, arsenite concentration was more pronounced, while in winter there was a higher concentration of arsenate.

For green algae, *U. rigida*, the iAs content in summer and autumn were very low, which is expected because this species has very low concentration of total arsenic in all seasons.

For red algae, *P. dioica* and *Gracilaria sp*., in winter the value of iAs content was the lowest (4.3 ± 0.2 and 3.8 ± 0.2 µg kg^{-1}, respectively) and about the same for both species. In autumn the Gracilaria sp. presents slightly higher values of arsenite (12.1 ± 0.5 µg kg^{-1}) while in spring *P. dioica* presents slightly higher values of arsenite (8.9 ± 2.2 µg kg^{-1}).

Although all the concentrations obtained were in agreement with the different taxonomies, it was expected that *F. vesiculosus* and *Gracilaria sp.* would acquire more iAs in winter, and *P.dioca* in autumn, due to the highest concentration of total As in these seasons. However, contrarily to the expectations the highest concentrations of As(III) for *F. vesiculosus* and *Gracilaria sp.* were obtained in autumn, and for *P. dioca* in spring. It is possible to conclude that

the highest accumulation of total arsenic does not imply the greatest accumulation of As(III). Nevertheless, this pattern holds true for arsenate.

Comparing the results above mentioned with those obtained by other authors (Table A.3 in Annex section), it is possible to conclude that the macroalgae produced in ALGAplus have very low concentrations of iAs and the As in these macroalgae is present mainly in the organic form.

Finally, through the comparison of these results with the values established by French legislation, none of the samples exceeded the limit (3000 μg kg^{-1}). Therefore, *F. vesiculosus, P. dioica, U. rigida,* and *Gracilaria sp.* are authorized for human consumption in France.

9. Conclusions and further research

This dissertation has two main goals. Firstly, to evaluate the accumulation of As in algae during the production process in IMTA system and the factors that promote this accumulation. Secondly, to contribute for the development of a relative inexpensive speciation method, able to provide relevant information on the concentration of iAs (arsenate and arsenite) present in edible algae cultivated by ALGAplus, Lda.

On the basis of the obtained results for total As concentrations, it has been concluded that brown macroalgae species accumulate higher concentrations than red and green macroalgae. This fact may be due to the taxonomic differences, namely physiological and biochemical factors, allowing an easier accumulation of this metalloid. It was also found a particular trend in algae: they naturally accumulate more As in winter than in warmer seasons, which may be related to the decrease of species growth during the winter, thus facilitating the concentration of As. However, during this work, it has become clear that there are several issues to be solved regarding the accumulation of As by algae, and further studies associated with seasonal and taxonomic effects have to be performed.

Furthermore, to increase the knowledge about the toxicity in each species and establish a correct risk evaluation associated with their chemical form it becomes essential to develop and implement a method of speciation of arsenic. An HPLC separation system was coupled to an HG-AFS detection system, which did not imply the use of sophisticated and expensive instrumentation and can be easily implemented unlike the most commonly used method: HPLC-ICP-MS. HPLC-HG-AFS proved to be adequate for this purpose, revealing suitable efficiency in the speciation of As, despite the complexity of matrix algae. Nevertheless, the complexity of matrix presented a challenge for the chromatographic separation of As species due to the significant amounts of lipids, polysaccharides, proteins, pigments and salt, which are common in macroalgae. These high contents of organic matter result in some deleterious effects for the chromatographic system, such as blockage inside the column and loss of resolution besides peak splitting, compromising the results obtained and shortening drastically the overall column life. Although in this study the SPE was used to clean the extracts, with positive effects in the final results, further studies are necessary to establish procedures for macroalgae extract cleaning in order to avoid erroneous results and problems in the column, during the separation process.

Despite all problems associated with the complexity of the matrix algae during the speciation process, the quantification of arsenite and arsenate was attained with success for assessing whether edible seaweeds produced by ALGAplus contain high potential toxicity. The

concentration of iAs found in all macroalgae are much lower than the maximum concentration legislated by France (3 mg kg^{-1}) for algae-based products.

The industry related to the distribution and production of algae as a source of food is very concerned about the lack of maximum values for As concentration in seafood, which reveals the importance of this work in scientific terms but also from the socio-economic point of view. This is the first study, considering seasonality, in the determination of total arsenic, arsenite and arsenate in macroalgae produced by ALGAplus, Lda. Overall, the findings of the present MSc work become an useful baseline for further understanding of arsenic behavior in an IMTA system.

Finally, the main obstacle associated with the determination of As species and their toxicological evaluation in foodstuffs is the unavailability of certified reference materials for analysis of speciation, compromising the validation of results. Such validation studies are fundamental not only for the proper characterization of the method performance but also for providing a suitable framework for setting up limits of As for environmental and public health protection.

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11. Annex

Table A.1: Sample pre-treatment, digestion and extraction, separation, detection, and arsenic species determined in macroalga. In bold, species of interest for ALGAplus, Lda.

Algae	Pre-treatment	Digestion and extraction method	HPLC		Detection	Arsenic species	Reference
			Stationary phase	Mobile phase			
Cystoseira barbata *Cystoseira spinosa* *Padina pavonica* ***Gracilaria sp.*** *Gracilaria gracilis* *Hypnea musciformis* *Codium fragile* *Ulva intestinalis* ***Ulva rigida*** *Ulva fasciata*	Freeze (-20°C) Wash Dry (40°C)	**Digestion** 0.1 g of sample Mineralization with 8 mL of HNO_3, and 2 mL of H_2O_2 Oven to 10', 90 °C; 5', 90 °C, 10', 90 °C; 120 °C; 10', 120 °C; 10', 190°C Dilution with 20 mL of H_2O Stored at 4°C **Extraction** H_2O Centrifugation at 2800 rpm, 10' Store at 4°C	**Anion exchange** PRP-X100 (250 x 1.4 mm), 10 µm (Hamilton, USA) **Cation exchange** Zorbax 300-SCX (150 x 4.1 mm), 5 mm (Agilent, Germany)	**Anion exchange** 20 mM $NH_4H_2PO_4$, pH 5.8 **Cation exchange** 20 mM pyridine, pH 2.6	**LCP-MS** Agilent 7500ce (Agilent Technologies, Germany)	**Anion exchange** As (III), DMA, MA, As (V), PO_4-sug, SO_3-sug, SO_4-sug **Cation exchange** AB, AC, TMAO Gly-sug	85

Table A.1: Sample pre-treatment, digestion and extraction, separation, detection, and arsenic species determined in macroalga. In bold, species of interest for ALGAplus, Lda. (cont.)

Algae	Pre-treatment	Digestion and extraction method	HPLC		Detection	Arsenic species	Reference
			Stationary phase	Mobile phase			
Colpomenia marina *Ascophyllum nodosum* *Fucus spiralis* *Agarum clathratum* ***Fucus vesiculosus*** *Alaria esculenta* *Laminaria digitata* *Laminaria longicruris* *Saccharina latissima* *Porphyra umbilicalus* *Heterosiphonia sp.* *Polyiphonia lanosa* *Chondrus crispus* *Phyllophora sp.* *Gracliaria sp.* *Palmaria palmata* *Chaetomorpha sp.* *Gayralia oxysperma* *Ulva lactuca*	Air dry Homogenize	**Digestion** 0.25 g of sample Add 5 mL of HNO_3, microwave (mw) at 180 °C, 10' Dilution to 50 mL of H_2O Dilution with 1% HNO_3 (3x) **Extraction** MeOH/H_2O, sonication (1h) Oven at 50°C, add 10 mL of H_2O Filtration Add 10 mL of HNO_3 (1%), sonication (30') Mw (55°C, 10'; 75°C, 10'; 95°C, 30'**)**	**Anion exchange** PRP-X100 (4.6 x 250 mm), 10 µm (Hamilton, Reno, NV) **Cation exchange** Supercosil SCX, (4.6 x 250 mm), 5 µm (Sigma Aldrich, St. Louis, MO)	**Anion exchange** 20 mM $(NH_4)_2CO_3$, pH 9, 40 °C, 1.5mL min^{-1} **Cation exchange** 20 mM pyridine, pH 2.5, 40°C	**LCP-MS** (7700x, Agilent, Santa Clara, CA)	iAs, tAs, DMA, MMA, AB, PO_4-sug, SO_3-sug, SO_4-sug, Gly-sug	111

Table A.1: Sample pre-treatment, digestion and extraction, separation, detection, and arsenic species determined in macroalga. In bold, species of interest for ALGAplus, Lda. (cont.)

Algae	Pre-treatment	Digestion and extraction method	HPLC		Detection	Arsenic species	Reference
			Stationary phase	Mobile phase			
Ceramium sp. *Gelidium sp.* *Polisyphonia sp.* ***Ulva rigida*** *Enteromorpha sp* *Cystoseira barbata* *Fucus virsoides* *Padina pavonica*	Store at 4°C Wash Dry Crush and homogenize Freeze (-18°C)	**Extraction** 0.25-0.75 g of sample Add 6 mL of HNO_3 (65%) and 2 mL of H_2O_2 (30%) Mw (1', 250W; 2', 0W; 5', 250W; 2', 400W; 10' 600W)			**LCP-MS** (7700x, Agilent, Santa Clara, CA)	tAs	13
		Extraction 2-3 g of sample Add 30 mL of H_2O Centrifugation 3000 rpm, 10' Add 5-10 mL of H_2O to the dry residue Filtration with a 0.45 µm membrane filter	**Anion exchange** PRP-X100, (250 x 4.1 mm), 100 µL (Hamilton) **Cation exchange** SCX, (250 x 4.6 mm), 5 µL (Alltech Adsorbosphere)	**Anion exchange** KH_2PO_4 solution, 15 mM, pH 6.0 (NH_4OH), 1 mL min^{-1} KH_2PO_4 solution, 20 mM, pH 65.8 (NH_4OH), 1 mL min^{-1} **Cation exchange** Pyridine, 2.5 mM, pH 2.65 (HCl), 1 mL min^{-1}	**UV-HG-AFS** **UV** 2% of $K_2S_2O_8$ NaOH (2%) for As-sug, 1.35 mL min^{-1}; NaOH (4%) for cations, 1.35 mL min^{-1} **HG** HCl (3M), 3.0 mL min^{-1}; $NaBH_4$ (1.5%) in NaOH (0.1%), 3 mL min^{-1} **AFS** Detector Excalibur (PS Analytical, Kent, UK)	**Anion exchange** As(V), As(III), DMAA, MMAA PO_4-sug, SO_3-sug, SO_4-sug, Gly-sug **Cation exchange** TMAO, AsB, AsC, TETRA	

Table A.1: Sample pre-treatment, digestion and extraction, separation, detection, and arsenic species determined in macroalga. In bold, species of interest for ALGAplus, Lda. (cont.)

Algae	Pre-treatment	Digestion and extraction method	HPLC		Detection	Arsenic species	Reference
			Stationary phase	Mobile phase			
Eisenia arbórea ***Fucus vesiculosus*** *Himanthalia sp.* *Hizikia fusiformis* *Laminaria sp.* *Laminaria digitata* *Undaria sp.* *Porphyra sp.*		**Digestion** Mw with HNO_3 and H_2O_2 **Extraction** 0.2 g of sample. Add 8 mL H_2O Heat at 90°C for 5' (x3) Centrifugation at 14.000 × g for 10' and mix Dilution up to 25 mL with H_2O Centrifugation			**UV–HG–AFS** **HG** HCl (8M) and NaBH4 (1.4%), 1.4 mL min^{-1} **AFS** Detector Millennium Excalibur (PS Analytical)	Gly-sug, As(III), DMA PO4-sug, MMA, SO3-sug As(V), SO4-sug As(III), As(V), AsB, TETRA, TMAO	114

Table A.1: Sample pre-treatment, digestion and extraction, separation, detection, and arsenic species determined in macroalga. In bold, species of interest for ALGAplus, Lda. (cont.)

Algae	Pre-treatment	Digestion and extraction method	HPLC		Detection	Arsenic species	Reference
			Stationary phase	Mobile phase			
Porphyra tenera Laminaria japonica Undaria sp. Hizikia fusiforme Sargassum sp.	Wash Air dry Dry (105°C). Powder Refrigerator (-20°C)	**Digestion** 0.25–0.5 g of sample. Add 7 mL HNO_3 (70%) and 2 mL H_2O_2 Mw to 1000 W, 80°C, 5'; 1000 W, 50°C, 5'; 1000 W, 190°C, 20'; and 0 W, 30' for cooling Dilution to 25 mL with H_2O **Extraction**, 1 g of sample Add 8 mL MeOH in HNO_3 (1%) Sonication bath at 30°C for 30' Centrifugation at 5980 x g, 10' Filtration Dilution with 50% MeOH in HNO_3 (1%).	PRP X-100 (4.1 x 250 mm), 10 µm (Hamilton)	**i)** 2 mM, NH_4HCO_3, MeOH (1%), pH 8 **ii)** 20 mM (NH_4) (NO_3), 20 mM, $H_{12}N_3O_4P$, MeOH (1%), pH 9	**ICP-MS** Elan 6100 DRC II (Perkin-Elmer SCIEX, Norwalk, CT, USA)	As(III), As(V), DMA, MMA, AsB, AsC	93

Table A.1: Sample pre-treatment, digestion and extraction, separation, detection, and arsenic species determined in macroalga. In bold, species of interest for ALGAplus, Lda. (cont.)

Algae	Pre-treatment	Digestion and extraction method	HPLC		Detection	Arsenic species	Reference
			Stationary phase	Mobile phase			
Sargassum sp. H*izikia fusiformis*		**Digestion** 2.5g of sample Add 10 mL HNO_3 (70%) Mw over 30', 225psi, 210 °C Dilution H_2O to 25 mL **Extraction** 2g of sample Add 8 mL of H_2O Mw at 90°C, 5' Centrifugation for 10' at 14000g (x3). Mix Dilution to 25 mL of H_2O	PRP-X100 (25 × 2.3 mm), 12–20µm (Hamilton) PRP-X100 (250 × 4.1 mm), 10µm (Hamilton)	Phosphate buffer 17 mM, pH 5.5	**HG-ICP-AES** **HG** 4M HCl, NaBH4 (0.5%) with NaOH (0.5%)	As(III), As(V), MMA and DMA	[117, 118]

Table A.1: Sample pre-treatment, digestion and extraction, separation, detection, and arsenic species determined in macroalga. In bold, species of interest for ALGAplus, Lda. (cont.)

Algae	Pre-treatment	Digestion and extraction method	HPLC		Detection	Arsenic species	Reference
			Stationary phase	Mobile phase			
Dictyota sp., *Hypnea spinella*, *Laurencia sp.*		**Extraction** 5 g of sample Add 5 mL of MeOH MW (10' at 150 W) Centrifugation at 5000 rpm, 5'	**Anion exchange** PRP-X100, (250 × 4.1 mm) (Hamilton) **Cation exchange** SCX 5U, (250× 4.6 mm) (Alltech Adsorbosphere)	**Anion exchange** KH_2PO_4 solution, 15 mM, pH 6 (NH_4OH) **Cation exchange** Pyridine, 2.5 mM, pH 2.7 ($C_6H_8O_7$)	**UV-HG-AFS** On-line **UV** <u>Anion exchange</u> $K_2S_2O_8$ (2%) in NaOH (2%), <u>Cation exchange</u> $K_2S_2O_8$ (4%) in NaOH (4%) **HG** 4.4 M HCl, 3.0 mL min^{-1}, $NaBH_4$ (1.5%) in NaOH (0.1%), 3 mL min^{-1}.	As, MMA, DMA, TMAO, TETRA, AsB, AsC	119, 109

Table A.1: Sample pre-treatment, digestion and extraction, separation, detection, and arsenic species determined in macroalga. In bold, species of interest for ALGAplus, Lda. (cont.)

Algae	Pre-treatment	Digestion and extraction method	HPLC		Detection	Arsenic species	Reference
			Stationary phase	Mobile phase			
Hijikia fusiforme		**Extraction** 5 g of sample Mix with 10 mL, C_2H_6O (20%) Centrifugation at 3000 rpm, 15' Separation of the supernatant Add 10 mL, C_2H_6O (20%). Centrifugation Filtration. Dilution with H_2O	**Anion exchange** Gelpack GL-IC-A15 (150 × 4.6 mm), 50 µL **Cation exchange** Shodex RSpak NN-614 (150 × 4.6 mm)	**Anion exchange** 3 mM NaH_2PO_4, pH 6 with NaOH 50 µL **Cation exchange** 5 mM HNO_3; 6 mM NH_4NO_3;	**ICP-MS** (Agilent, USA)	As(III), As(V), MMA, DMA	120

Table A.1: Sample pre-treatment, digestion and extraction, separation, detection, and arsenic species determined in macroalga. In bold, species of interest for ALGAplus, Lda. (cont.)

Algae	Pre-treatment	Digestion and extraction method	HPLC		Detection	Arsenic species	Reference
			Stationary phase	Mobile phase			
Iridaea cordata *Ascoseira mirabilis* *Adenocystis utricularis* *Desmarestia menziesii* *Gigartina skottbergii*	Wash Freeze-dry Homogenize	**Extraction** 1 g of sample MeOH: H2O (1:1) Sonication, 3h, 30 °C Centrifugation at 3600 rpm, 3' **Digestion** 0.25 g of sample Add 5 mL of HNO_3, 2 mL of H_2O, and 1 mL of HF Mw at 1200 W, 10', and 1 mL of HF	**Anion exchange** Dionex IonPac AS7 (4.0 x 250 mm) IonPac AG7 (4.0 x 50 mm) **Cation exchange** PRP-X200, (4.1 x 150 mm) Hamilton	**Anion exchange** 25 mM NH_4HCO_3, pH 10 with NH_4OH **Cation exchange** 4 mM, C_5H_5N, pH 2.4 with CH_2O_2	**ICP-MS** (VG Elemental, Franklin, MA, USA) **ESI-ITMS** Agilent 1100 Series LC/MSD Ion Trap Mass Spectrometer (Agilent Technologies, Tokyo, Japan)	As(III), As(V), MMA, DMA, Gly-sug, PO4-sug, SO3-sug, SO4-sug	121

Table A.1: Sample pre-treatment, digestion and extraction, separation, detection, and arsenic species determined in macroalga. In bold, species of interest for ALGAplus, Lda. (cont.)

Algae	Pre-treatment	Digestion and extraction method	HPLC		Detection	Arsenic species	Reference
			Stationary phase	Mobile phase			
Fucus sp.		**Digestion** Heat to 120 °C, 1–2 h. Heat to 140 °C, 6 h. Add 2 mL H_2O_2 and heat for 1–3 h. Dilution **Extraction** Shaker at 150 rpm 37 °C for 1 h	**Anion exchange** PRP-X100 (4.6 ×150 mm) 10 µm Hamilton **Cation exchange** Chrompack, (3 × 100 mm) or (3 × 150 mm), 5 µm	**Anion exchange** 20 mM NH_4HCO_3, pH 7, 0.8 mL min^{-1} **Cation exchange** 20 mM C_5H_6N, pH 2.7, 1.0 mL min^{-1}	**ICP-MS** Thermo Instruments X-Series **HG-AAS** Thermo Instruments SOLAAR	As(III), As(V)	122

Table A.1: Sample pre-treatment, digestion and extraction, separation, detection, and arsenic species determined in macroalga. In bold, species of interest for ALGAplus, Lda. (cont.)

Algae	Pre-treatment	Digestion and extraction method	HPLC		Detection	Arsenic species	Reference
			Stationary phase	Mobile phase			
Callophyllis variegata *Chondracanthus chamissoi* *Gracilaria chilensis* *Gymnogongrus disciplinalis* *Iridaea spp.* *Iridaea laminarioides* *Mastocarpus papillatus* *Mazzaella laminaroides* *Porphyra columbina* *Durvillaea antarctica* *Macrocystis piryfera* ***Ulva rigida***	.	**Digestion** 0.25 g of sample Add 2.5 mL of $MgNO_3$ (20%) and MgO (2%) 5 mL of HNO_3 (50%) Evaporation on a sand bath Over, 12h at 425 ± 25 °C Dissolve white ash in 5 mL of 6 M HCl, and 5 mL of pre-reducing solution (5% KI and 5% $C_6H_8O_6$) Filtration after 30' Add 4.1 mL of H_2O and 18.4 mL of HCl			**FI-HG-AAS** $NaBH_4$ (0.2%) in NaOH (0.05%), 5 mL min^{-1} HCl (10%), 10 mL min^{-1}	tAs. iAs	107

Table A.1: Sample pre-treatment, digestion and extraction, separation, detection, and arsenic species determined in macroalga. In bold, species of interest for ALGAplus, Lda. (cont.)

Algae	Pre-treatment	Digestion and extraction method	HPLC		Detection	Arsenic species	Reference
			Stationary phase	Mobile phase			
Lessonia nigrescens *Durvillaea antarctica*	Wash Dry (40°C) Homogenize	**Digestion** 0.2 g of samples Add 8 mL of HNO_3 and 2 mL of H_2O_2 Room temperature at 90°C, 10', maintained for 10' from 90°C-120°C, 10' from 120°C - 190°C and 10' at 190°C Filtration Dilution	**Anion exchange** PRP-X100, (250 × 4.1 mm), 10 µm (Hamilton, USA) **Cation exchange** Zorbax 300-SCX (150 × 4.1 mm), 5 µm (Agilent, Germany)	**Anion exchange** 20 mM $NH_4H_2PO_4$, pH 6 **Cation exchange** 20 mM pyridine, pH 2.6	**ICP- MS** (Agilent 7500ce) with He as the gas in the collision cell	**Anion exchange** As(III), As(V), DMA, MA, PO4-sug, SO3-sug, SO4-sug **Cation exchange** AB, AC	123

Table A.1: Sample pre-treatment, digestion and extraction, separation, detection, and arsenic species determined in macroalga. In bold, species of interest for ALGAplus, Lda. (cont.)

Algae	Pre-treatment	Digestion and extraction method	HPLC		Detection	Arsenic species	Reference
			Stationary phase	Mobile phase			
Sargassum fulvellum *Chlorella vulgaris,* *Hizikia fusiformis* *Laminaria digitata*	Freeze-dry Homogenize	**Digestion** 0.25 g of sample Add 10 mL of HNO_3 (70%) Mw for 30', 210-225 °C Dilution to 25 mL of H_2O **Extraction** 0.2 g of sample Add 25 mL with H_2O Centrifugation Sonication for 30'' Centrifugation for 10' at 14000 x g (x 3 times) Mix and evaporation Dissolve residue in 4 mL of H_2O	PRP-X100 (Hamilton)	Phosphate buffer 17mM, pH 5.5, 100 µL	**ICP-AES** (Varían Australia Pty Ltd., Mulgrave, Vic, Australia)	As(III), As(V), MMA, DMA	124

Table A.2: Physical and chemical parameters determined *in situ* in the tanks of the algae species under study, *Ulva rigida, Porphyra dioica, Gracilaria sp.* and *Fucus vesiculosus.*

Date	Tank		Water salinity (‰)	Water temperature (°C)	pH	Eh (mV)	0_2 (% sat)
03/11/2016	*U. rigida*	29	37.8	18.4	8.0	-	-
		30	37.8	18.3	8.0	-	-
		31	37.8	18.6	7.8	-	-
		32	37.7	18.6	7.8	-	-
		33	37.8	18.6	7.7	-	-
		34	37.8	18.6	8.0	-	-
		35	37.8	18.6	7.8	-	-
		36	37.8	18.7	7.5	-	-
		37	37.7	18.7	7.9	-	-
		38	37.8	18.8	7.7	-	-
		40	37.7	18.4	8.0	-	-
		41	37.8	18.5	8.1	-	-
	Gracilaria sp.		-	-	-	-	-
	F. vesiculosus		-	-	-	-	-
	P. dioica		-	-	-	-	-
08/11/2016	*U. rigida*	29	36.1	13.0	7.6	-	-
		30	36.3	12.5	7.9	-	-
		31	36.4	12.3	8.4	-	-
		32	36.2	12.7	8.0	-	-
		33	36.1	12.9	8.0	-	-
		34	36.3	12.5	8.3	-	-
		35	36.2	12.7	8.2	-	-
		36	36.0	13.0	8.1	-	-
		37	36.0	12.9	8.1	-	-
		38	36.0	13.1	8.1	-	-
		40	36.2	12.7	8.2	-	-
		41	36.1	13.0	8.1	-	-
	Gracilaria sp.		-	-	-	-	-
	F. vesiculosus		-	-	-	-	-
	P. dioica		-	-	-	-	-
09/11/2016	*U. rigida*	29	35.3	14.7	8.1	-	-
		30	35.4	14.5	8.1	-	-
		31	35.6	14.4	8.2	-	-
		32	35.4	14.6	8.0	-	-
		33	35.4	14.6	8.1	-	-
		34	35.6	14.4	8.2	-	-
		35	35.5	14.5	8.2	-	-
		36	35.5	14.6	8.1	-	-
		37	35.5	14.6	8.1	-	-
		38	35.5	14.6	8.1	-	-
		40	35.6	14.5	8.1	-	-
		41	35.5	14.5	8.1	-	-
	Gracilaria sp.		-	-	-	-	-
	F. vesiculosus		-	-	-	-	-
	P. dioica		-	-	-	-	-

Table A.2: Physical and chemical parameters determined *in situ* in the tanks of the algae species under study, *Ulva rigida, Porphyra dioica, Gracilaria* and *Fucus vesiculosus* (cont.)

Date	Tank		Water salinity (‰)	Water temperature (°C)	pH	Eh (mV)	0_2 (% sat)
10/11/2016	*U. rigida*	29	35.5	14.4	8.0	-	-
		30	35.5	14.1	8.0	-	-
		31	35.5	14.1	8.0	-	-
		32	35.5	14.2	8.0	-	-
		33	35.5	14.3	8.0	-	-
		34	35.5	14.3	8.0	-	-
		35	35.5	14.2	8.0	-	-
		36	35.5	14.2	8.1	-	-
		37	35.5	14.4	7.9	-	-
		38	35.5	14.4	8.0	-	
		40	35.4	14.4	8.0	-	-
		41	35.5	14.3	8.0	-	-
	Gracilaria sp.		35.3	14.5	8.2	-	-
	F. vesiculosus		35.4	14.6	8.0	-	-
	P. dioica		35.4	14.4	8.3	-	-
15/11/2017	*U. rigida*	29	36.8	14.2	8.0	-	-
		30	36.8	14.3	8.0	-	-
		31	36.8	14.2	8.2	-	-
		32	37.0	14.4	8.0	-	-
		33	37.0	14.3	8.0	-	-
		34	36.9	14.1	8.1	-	-
		35	36.9	14.1	8.2	-	-
		36	37.1	14.4	8.1	-	-
		37	37.1	14.3	8.1	-	-
		38	37.1	14.3	8.1	-	-
		40	37.0	14.2	8.1	-	-
		41	37.1	14.3	8.0	-	-
	Gracilaria sp.		37.1	14.0	8.1	-	-
	F. vesiculosus		-	-	-	-	-
	P. dioica		-	-	-	-	-
17/11/2017	*U. rigida*	29	37.7	14.1	8.0	-	-
		30	37.7	14.3	8.0	-	-
		31	37.7	14.3	8.0	-	-
		32	37.6	14.3	8.0	-	-
		33	37.7	14.3	8.1	-	-
		34	37.6	14.3	8.1	-	-
		35	37.7	14.3	8.1	-	-
		36	37.7	14.4	8.0	-	-
		37	37.6	14.3	8.1	-	-
		38	37.5	14.3	8.0	-	-
		40	37.6	14.3	8.1	-	-
		41	37.5	14.3	8.0	-	-
	Gracilaria sp.		37.8	13.8	8.0	-	-
	F. vesiculosus		-	-	-	-	-
	P. dioica		37.7	13.7	8.1	-	-

Table A.2: Physical and chemical parameters determined *in situ* in the tanks of the algae species under study, *Ulva rigida, Porphyra dioica, Gracilaria* and *Fucus vesiculosus* (cont.)

Date	Tank		Water salinity (‰)	Water temperature (°C)	pH	Eh (mV)	0_2 (% sat)
22/11/2016	*U. rigida*	29	36.1	13.9	7.8	-	-
		30	36.3	13.8	7.9	-	-
		31	36.2	13.7	7.9	-	-
		32	36.3	13.6	8.0	-	-
		33	36.2	13.8	7.9	-	-
		34	36.3	13.6	7.9	-	-
		35	36.3	13.6	8.0	-	-
		36	36.4	13.5	8.0	-	-
		37	36.3	13.6	8.0	-	-
		38	36.4	13.5	8.0	-	-
		40	36.4	13.4	8.0	-	-
		41	36.4	13.4	8.0	-	-
	Gracilaria sp.		36.4	12.7	8.0	-	-
	F. vesiculosus		-	-	-	-	-
	P. dioica		36.3	12.6	8.0	-	-
30/11/2016	*U. rigida*	29	31.9	12.5	8.1	-	-
		30	32.0	12.6	8.0	-	-
		31	31.8	12.2	8.2	-	-
		32	32.0	12.5	8.1	-	-
		33	32.0	12.5	8.1	-	-
		34	31.9	12.4	8.2	-	-
		35	32.0	12.4	8.2	-	-
		36	32.0	12.6	8.1	-	-
		37	32.2	12.8	8.1	-	-
		38	32.1	12.7	8.1	-	-
		40	31.9	12.3	8.2	-	-
		41	32.1	12.6	8.1	-	-
	Gracilaria sp.		32.1	12.3	8.2	-	-
	F. vesiculosus		-	-	-	-	-
	P. dioica		30.3	12.6	8.9	-	-
06/12/2016	*U. rigida*	29	35.3	13.5	7.9	-	-
		30	35.3	13.6	7.9	-	-
		31	35.2	13.5	8.1	-	-
		32	35.3	13.6	7.9	-	-
		33	35.2	13.5	8.0	-	-
		34	35.2	13.5	8.1	-	-
		35	35.2	13.5	8.1	-	-
		36	35.2	13.5	8.0	-	-
		37	35.3	13.6	7.1	-	-
		38	35.3	13.6	8.0	-	-
		40	35.1	13.4	8.1	-	-
		41	35.2	13.5	8.0	-	-
	Gracilaria sp.		35.2	13.4	8.1	-	-
	F. vesiculosus		-	-	-	-	-
	P. dioica		35.1	13.2	8.1	-	-

Table A.2: Physical and chemical parameters determined *in situ* in the tanks of the algae species under study, *Ulva rigida, Porphyra dioica, Gracilaria* and *Fucus vesiculosus* (cont.)

Date	Tank		Water salinity (‰)	Water temperature (°C)	pH	Eh (mV)	0_2 (% sat)
13/12/2016	*U. rigida*	29	35.6	13.4	8.0	-	-
		30	35.6	13.5	8.0	-	-
		31	35.6	13.5	8.0	-	-
		32	35.6	13.4	8.0	-	-
		33	35.5	13.4	8.1	-	-
		34	35.6	13.4	8.1	-	-
		35	35.6	13.4	8.0	-	-
		36	35.6	13.4	8.1	-	-
		37	35.6	13.4	8.1	-	-
		38	35.6	13.5	8.1	-	-
		40	35.7	13.4	8.0	-	-
		41	35.6	13.3	8.1	-	-
	Gracilaria sp.		35.7	13.2	8.1	-	-
	F. vesiculosus		-	-	-	-	-
	P. dioica		35.7	12.9	8.1	-	-
14/12/2016	*U. rigida*	29	34.8	14.0	8.0	-	-
		30	35.0	14.0	8.0	-	-
		31	35.0	14.0	8.0	-	-
		32	34.8	13.9	8.1	-	-
		33	34.8	13.9	8.1	-	-
		34	34.9	13.9	8.1	-	-
		35	34.9	13.8	8.1	-	-
		36	34.9	13.9	8.1	-	-
		37	34.9	13.8	8.1	-	-
		38	35.0	13.9	8.1	-	-
		40	35.1	13.9	8.1	-	-
		41	35.0	13.6	8.1	-	-
	Gracilaria sp.		35.2	13.7	8.1	-	-
	F. vesiculosus		-	-	-	-	-
	P. dioica		35.1	13.4	8.1	-	-
10/01/2017	*U. rigida*	29	34.1	11.4	8.2	136.8	-
		30	34.2	11.3	8.2	137.8	-
		31	34.3	11.2	8.3	134.6	-
		32	35.7	11.4	8.0	149.1	-
		33	35.7	11.3	8.2	137.9	-
		34	34.4	11.0	8.2	139.9	-
		35	34.5	11.0	8.3	134.8	-
		36	35.9	11.2	8.2	141.0	-
		37	35.9	11.2	8.3	137.0	-
		38	35.9	11.3	8.2	140.0	-
		40	34.6	11.0	8.0	148.4	-
		41	34.7	11.1	8.3	135.1	-
	Gracilaria sp.		-	-	-	-	-
	F. vesiculosus		-	-	-	-	-
	P. dioica		-	-	-	-	-

Table A.2: Physical and chemical parameters determined *in situ* in the tanks of the algae species under study, *Ulva rigida, Porphyra dioica, Gracilaria* and *Fucus vesiculosus* (cont.)

Date	Tank		Water salinity (‰)	Water temperature (°C)	pH	Eh (mV)	0_2 (% sat)
17/01/2017	*U. rigida*	29	35.8	9.2	8.2	192.2	-
		30	35.8	9.1	8.2	192.8	-
		31	35.8	9.2	8.2	195.1	-
		32	35.6	9.7	7.9	195.8	-
		33	35.8	9.7	8.1	196.0	-
		34	35.7	9.5	8.1	196.1	-
		35	35.7	9.3	8.2	195.9	-
		36	36.0	9.4	8.2	195.9	-
		37	35.9	9.6	8.1	197.1	-
		38	35.9	9.6	8.1	196.6	-
		40	35.8	9.4	8.1	196.1	-
		41	35.9	9.4	8.1	195.9	-
	Gracilaria sp.		35.8	8.8	8.1	196.6	-
	F. vesiculosus		35.8	9.9	7.9	143.6	-
	P. dioica		-	-	-	-	-
21/01/2017	*U. rigida*	29	35.7	7.6	8.2	-	-
		30	35.7	7.6	8.2	-	-
		31	35.8	7.4	8.2	-	-
		32	35.8	7.4	8.1	-	-
		33	35.9	7.4	8.1	-	-
		34	35.9	7.5	8.1	-	-
		35	35.9	7.3	8.1	-	-
		36	36.0	7.3	8.0	-	-
		37	36.0	7.5	8.0	-	-
		38	36.0	7.5	8.1	-	-
		40	36.0	7.6	8.2	-	-
		41	36.0	7.6	8.1	-	-
	Gracilaria sp.		-	-	-	-	-
	F. vesiculosus		-	-	-	-	-
	P. dioica		-	-	-	-	-
25/01/2017	*U. rigida*	29	35.1	8.0	8.4	132.2	-
		30	35.2	7.9	8.3	137.4	-
		31	35.2	7.9	8.4	131.7	-
		32	35.2	8.1	8.2	142.3	-
		33	35.2	7.9	8.3	133.6	-
		34	35.2	8.0	8.3	134.3	-
		35	35.2	8.0	8.4	133.2	-
		36	35.2	8.0	8.3	134.2	-
		37	35.2	8.1	8.3	133.9	-
		38	35.2	8.1	8.3	134.1	-
		40	35.2	8.0	8.3	136.3	-
		41	35.2	7.9	8.3	134.6	-
	Gracilaria sp.		-	-	-	-	-
	F. vesiculosus		-	-	-	-	-
	P. dioica		-	-	-	-	-

Table A.2: Physical and chemical parameters determined *in situ* in the tanks of the algae species under study, *Ulva rigida, Porphyra dioica, Gracilaria* and *Fucus vesiculosus* (cont.)

Date	Tank		Water salinity (‰)	Water temperature (°C)	pH	Eh (mV)	0_2 (% sat)
31/01/2017	*U. rigida*	29	35.5	12.9	8.1	229.0	-
		30	32.5	12.8	8.2	230.6	-
		31	32.5	12.8	8.2	230.8	-
		32	32.6	12.9	7.9	230.9	-
		33	32.5	12.9	8.1	230.1	-
		34	32.5	12.8	8.2	229.5	-
		35	32.6	12.9	8.1	228.6	-
		36	32.6	12.9	8.1	228.5	-
		37	32.6	12.9	8.1	228.6	-
		38	32.6	12.9	8.1	229.3	-
		40	32.5	12.9	8.1	228.0	-
		41	32.5	12.9	8.1	229.7	-
	Gracilaria sp.		-	-	-	-	-
	F. vesiculosus		27.4	13.0	7.7	194.7	-
	P. dioica		35.9	12.8	8.0	229.1	-
8/02/2017	*U. rigida*	29	28.3	12.6	9.1	191.9	131.7
		30	27.6	13.0	9.2	195.9	107.7
		31	27.3	13.1	9.0	197.4	134.4
		32	27.8	13.0	9.0	197.4	129.3
		33	27.3	13.1	9.0	197.0	132.5
		34	27.3	13.1	9.1	196.2	121.3
		35	27.1	13.3	9.0	196.3	123.0
		36	27.1	13.2	9.5	196.1	117.3
		37	28.0	13.3	8.9	196.0	123.9
		38	27.2	13.1	9.0	195.4	122.1
		40	26.9	13.4	8.9	193.6	124.2
		41	27.1	13.3	8.9	193.4	123.5
	Gracilaria sp.		-	-	-	-	-
	F. vesiculosus		30.6	13.2	8.3	191.9	100.7
	P. dioica		26.9	13.2	8.8	191.0	115.6
23/02/2017	*U. rigida*	29	27.2	14.1	8.1	259.3	119.0
		30	27.2	14.1	8.2	256.7	110.4
		31	27.2	14.1	8.2	255.9	113.1
		32	27.3	14.2	8.2	255.5	113.3
		33	27.3	14.2	8.1	255.1	114.7
		34	27.1	14.2	8.2	254.5	117.2
		35	27.2	14.2	8.2	254.8	113.5
		36	27.3	14.2	8.2	254.8	115.3
		37	27.4	14.3	8.1	254.8	115.9
		38	27.4	14.3	8.1	254.8	112.4
		40	27.2	14.2	8.2	253.8	112.7
		41	27.4	14.3	8.1	253.6	115.0
	Gracilaria sp.		27.2	14.0	8.6	253.6	105.8
	F. vesiculosus		27.4	14.0	8.1	253.6	102.1
	P. dioica		27.2	13.9	8.2	253.2	110.5

Table A.2: Physical and chemical parameters determined *in situ* in the tanks of the algae species under study, *Ulva rigida, Porphyra dioica, Gracilaria* and *Fucus vesiculosus* (cont.)

Date	Tank		Water salinity (‰)	Water temperature (°C)	pH	Eh (mV)	0_2 (% sat)
10/03/2017	*U. rigida*	29	29.6	17.5	8.6	119.1	159.6
		30	29.6	17.5	8.6	121.3	154.4
		31	29.6	17.6	8.6	122.0	151.3
		32	29.5	17.7	8.5	120.7	172.2
		33	29.5	17.7	8.5	120.3	164.9
		34	29.5	17.6	8.6	119.8	163.3
		35	29.5	17.6	8.7	119.7	159.2
		36	29.6	17.6	8.7	119.6	152.9
		37	29.7	17.6	8.7	119.7	154.0
		38	29.6	17.6	8.7	119.9	148.4
		40	29.6	17.5	8.6	120.6	141.5
		41	29.6	17.5	8.6	120.8	145.3
	Gracilaria sp.		29.7	18.0	8.4	121.5	119.6
	F. vesiculosus		-	-	-	-	-
	P. dioica		29.9	19.3	8.5	221.2	119.0
13/03/2017	*U. rigida*	29	31.0	12.9	8.3	222.3	129.9
		30	31.9	12.9	8.3	219.9	121.0
		31	31.0	13.2	8.4	218.5	137.0
		32	31.0	13.1	8.4	218.6	135.6
		33	31.0	13.3	8.4	218.7	135.5
		34	31.0	13.5	8.4	218.9	140.0
		35	31.1	13.5	8.5	219.1	140.7
		36	31.1	13.3	8.6	219.0	141.5
		37	31.1	13.3	8.5	219.0	138.1
		38	31.0	13.3	8.6	219.2	144.3
		40	31.3	12.6	8.4	219.6	136.2
		41	31.1	13.6	8.3	219.0	142.9
	Gracilaria sp.		31.0	13.1	8.2	220.0	113.2
	F. vesiculosus		-	-	-	-	-
	P. dioica		31.1	13.2	8.4	220.8	133.6
23/03/2017	*U. rigida*	29	31.0	12.6	7.8	223.8	116.9
		30	31.0	12.7	7.9	221.4	113.6
		31	30.9	12.8	7.9	220.7	114.1
		32	31.0	12.7	8.1	220.0	121.2
		33	31.0	12.7	8.1	219.9	120.3
		34	31.1	12.6	8.6	219.9	119.0
		35	31.1	12.7	7.9	220.1	112.8
		36	31.3	12.5	8.2	220.4	115.7
		37	31.1	12.7	7.9	221.0	108.2
		38	31.3	12.6	7.9	221.5	106.4
		40	30.8	12.8	8.1	222.5	119.1
		41	30.9	12.9	7.9	223.1	112.6
	Gracilaria sp.		30.7	11.9	8.2	224.7	110.4
	F. vesiculosus		30.6	11.8	7.9	225.4	96.8
	P. dioica		30.1	11.0	8.2	227.5	107.1

Table A.2: Physical and chemical parameters determined *in situ* in the tanks of the algae species under study, *Ulva rigida, Porphyra dioica, Gracilaria* and *Fucus vesiculosus* (cont.)

Date	Tank		Water salinity (‰)	Water temperature (°C)	pH	Eh (mV)	0_2 (% sat)
24/03/2017	*U. rigida*	29	30.0	13.6	8.3	-	-
		30	30.3	13.5	8.4	-	-
		31	30.4	13.4	8.5	-	-
		32	30.5	13.3	8.7	-	-
		33	30.6	13.2	8.7	-	-
		34	30.6	13.1	8.8	-	-
		35	30.7	13.2	8.5	-	-
		36	31.0	12.9	8.7	-	-
		37	30.8	13.1	8.4	-	-
		38	30.9	13.1	8.4	-	-
		40	30.7	13.1	8.8	-	-
		41	30.7	13.2	8.6	-	-
	Gracilaria sp.		-	-	-	-	-
	F. vesiculosus		-	-	-	-	-
	P. dioica		-	-	-	-	-
31/03/2017	*U. rigida*	29	32.3	18.0	-	-	-
		30	32.5	18.0	-	-	-
		31	32.1	18.1	-	-	-
		32	32.0	18.1	-	-	-
		33	32.3	18.1	-	-	-
		34	31.9	18.2	-	-	-
		35	32.1	18.0	-	-	-
		36	32.0	18.1	-	-	-
		37	31.8	18.1	-	-	-
		38	31.9	18.0	-	-	-
		40	32.0	18.0	-	-	-
		41	32.0	18.1	-	-	-
	Gracilaria sp.		-	-	-	-	-
	F. vesiculosus		-	-	-	-	-
	P. dioica		-	-	-	-	-
12/04/2017	*U. rigida*	29	34.4	21.8	8.4	220.0	96.80
		30	34.6	21.4	8.8	219.1	107.1
		31	34.3	21.8	8.8	218.8	153.6
		32	34.3	21.8	8.8	218.7	162.6
		33	34.3	21.8	8.7	218.2	158.0
		34	34.3	21.9	9.1	218.1	151.4
		35	34.5	21.9	8.4	217.3	169.6
		36	34.4	21.7	8.9	217.4	160.7
		37	34.3	21.8	8.9	217.6	159.0
		38	34.1	21.7	9.2	217.3	158.5
		40	34.1	21.6	9.1	215.8	149.5
		41	34.1	22.0	8.2	227.0	129.5
	Gracilaria sp.		34.5	21.9	9.0	220.8	113.3
	F. vesiculosus		34.5	21.9	7.8	222.2	106.5
	P. dioica		34.5	22.8	8.4	222.2	112.7

Table A.2: Physical and chemical parameters determined *in situ* in the tanks of the algae species under study, *Ulva rigida, Porphyra dioica, Gracilaria* and *Fucus vesiculosus* (cont.)

Date	Tank		Water salinity (‰)	Water temperature (°C)	pH	Eh (mV)	0_2 (% sat)
24/04/2017	*U. rigida*	29	35.0	22.4	8.9	217.2	136.6
		30	35.0	22.3	9.2	211.8	122.3
		31	35.0	22.4	8.9	211.3	133.2
		32	35.0	22.3	8.8	211.6	129.0
		33	35.0	22.5	8.7	212.3	154.2
		34	35.1	22.3	8.7	212.9	155.7
		35	35.1	22.4	9.2	214.0	127.7
		36	35.0	22.4	8.8	215.0	123.4
		37	35.0	22.4	9.1	216.3	125.0
		38	35.0	22.5	8.3	217.2	121.1
		40	34.9	22.6	8.7	218.4	133.9
		41	35.0	22.5	8.7	219.1	132.5
	Gracilaria sp.		36.6	20.8	8.6	221.0	103.3
	F. vesiculosus		34.9	22.8	7.9	220.9	91.70
	P. dioica		34.9	23.3	8.7	221.0	106.7
02/05/2017	*U. rigida*	29	36.5	16.2	8.0	437.2	99.80
		30	36.6	16.3	8.1	430.9	102.8
		31	36.6	16.4	8.2	430.1	103.4
		32	36.5	16.7	7.9	430.3	106.9
		33	36.6	16.7	7.9	430.5	106.7
		34	36.6	16.6	8.3	431.0	107.9
		35	36.5	16.7	8.1	431.3	107.7
		36	36.4	16.7	8.0	432.4	108.8
		37	36.5	16.7	7.9	433.9	107.1
		38	36.6	16.8	7.9	434.5	107.0
		40	36.6	16.7	8.1	434.2	107.4
		41	36.5	16.9	7.9	446.1	107.8
	Gracilaria sp.		36.7	15.7	8.0	441.8	98.00
	F. vesiculosus		-	-	-	-	-
	P. dioica		36.9	15.1	8.1	443.1	100.7
09/05/2017	*U. rigida*	29	34.0	17.1	8.4	-	-
		30	34.0	17.3	8.4	-	-
		31	34.1	17.1	9.0	-	-
		32	34.3	17.1	9.5	-	-
		33	34.2	17.2	9.6	-	-
		34	34.2	17.2	9.6	-	-
		35	34.0	17.2	9.0	-	-
		36	34.5	17.4	9.0	-	-
		37	34.2	17.3	9.2	-	-
		38	34.3	17.2	9.1	-	-
		40	34.0	17.2	8.5	-	-
		41	34.0	17.3	8.5	-	-
	Gracilaria sp.		-	-	-	-	-
	F. vesiculosus		-	-	-	-	-
	P. dioica		-	-	-	-	-

Table A.1: Physical and chemical parameters determined *in situ* in the tanks of the algae species under study, *Ulva rigida, Porphyra dioica, Gracilaria* and *Fucus vesiculosus* (cont.)

Date	Tank		Water salinity (‰)	Water temperature (°C)	pH	Eh (mV)	0_2 (% sat)
16/05/2017	*U. rigida*	29	35.5	18.8	8.4	-	-
		30	35.5	18.8	8.4	-	-
		31	36.9	18.3	9.8	-	-
		32	36.8	18.1	9.7	-	-
		33	36.9	18.0	9.7	-	-
		34	36.8	18.1	9.6	-	-
		35	36.7	18.2	9.2	-	-
		36	36.8	18.2	9.1	-	-
		37	36.9	18.1	9.6	-	-
		38	36.9	18.3	9.3	-	-
		40	35.6	18.9	8.5	-	-
		41	35.5	19.0	8.5	-	-
	Gracilaria sp.		-	-	-	-	-
	F. vesiculosus		-	-	-	-	-
	P. dioica		-	-	-	-	-
24/05/2017	*U. rigida*	29	32.6	23.8	8.5	208.6	155.5
		30	32.5	23.8	8.5	209.0	142.8
		31	33.2	23.8	8.5	209.8	151.7
		32	34.6	24.7	9.9	208.2	132.1
		33	31.6	24.8	9.4	210.4	128.3
		34	30.7	24.8	9.9	210.5	133.5
		35	32.8	24.8	9.9	210.6	125.6
		36	34.9	24.8	9.7	210.5	131.9
		37	36.7	24.8	9.7	210.3	130.0
		38	36.4	24.9	9.9	210.1	121.9
		40	36.0	23.9	8.6	211.2	145.2
		41	36.1	23.8	8.6	211.3	145.2
	Gracilaria sp.		35.5	24.6	8.6	211.9	106.8
	F. vesiculosus		36.1	24.4	8.0	211.3	110.5
	P. dioica		36.1	25.0	8.3	213.3	128.1
30/05/2017	*U. rigida*	29	35.6	21.0	8.0	207.9	134.9
		30	35.5	21.4	7.9	208.3	134.6
		31	35.7	21.2	8.2	208.8	124.4
		32	35.6	21.3	9.0	209.1	108.6
		33	35.4	21.3	9.6	209.3	112.2
		34	35.4	21.4	9.5	209.8	111.0
		35	35.3	21.4	9.7	210.0	109.0
		36	35.5	21.4	9.8	210.1	110.9
		37	35.7	21.5	8.6	211.0	103.6
		38	35.7	21.4	9.4	211.2	109.1
		40	35.8	21.4	8.2	211.8	119.9
		41	35.6	21.4	8.1	211.9	121.0
	Gracilaria sp.		36.5	20.5	8.1	212.2	100.4
	F. vesiculosus		35.8	21.8	7.7	210.8	100.1
	P. dioica		35.5	21.8	8.0	218.1	96.50

Table A.2: Physical and chemical parameters determined *in situ* in the tanks of the algae species under study, *Ulva rigida, Porphyra dioica, Gracilaria* and *Fucus vesiculosus* (cont.)

Date	Tank		Water salinity (‰)	Water temperature (°C)	pH	Eh (mV)	0_2 (% sat)
12/06/2017	*U. rigida*	29	36.1	23.9	8.9	-	-
		30	36.1	23.9	9.2	-	-
		31	36.2	24.0	9.5	-	-
		32	36.2	24.1	8.4	-	-
		33	36.2	24.1	8.6	-	-
		34	36.2	24.1	8.4	-	-
		35	36.2	24.0	8.5	-	-
		36	36.2	24.1	8.9	-	-
		37	36.2	24.1	8.6	-	-
		38	36.3	24.1	8.6	-	-
		40	36.2	24.1	8.8	-	-
		41	36.2	24.1	8.8	-	-
	Gracilaria sp.		-	-	-	-	-
	F. vesiculosus		-	-	-	-	-
	P. dioica		-	-	-	-	-
14/06/2017	*U. rigida*	29	25.6	23.9	8.5	416.5	173.1
		30	25.6	24.0	8.8	417.3	142.2
		31	25.7	24.0	8.9	418.6	142.7
		32	25.6	23.9	8.4	417.3	134.4
		33	25.6	23.9	8.7	419.7	161.7
		34	25.6	23.9	8.2	420.3	121.8
		35	25.6	23.8	8.3	420.6	125.1
		36	25.7	24.0	8.9	420.6	162.5
		37	25.6	24.0	8.5	421.3	157.3
		38	25.6	23.9	8.6	421.3	148.8
		40	25.6	23.9	8.6	423.0	144.1
		41	25.6	23.8	8.6	423.2	151.2
	Gracilaria sp.		25.6	24.4	8.8	210.1	110.3
	F. vesiculosus		-	-	-	-	-
	P. dioica		24.4	24.3	8.3	210.4	132.9
14/07/2017	*U. rigida*	29	36.5	22.4	8.8	-	-
		30	36.6	22.4	9.0	-	-
		31	36.5	22.5	8.5	-	-
		32	36.7	22.4	8.7	-	-
		33	36.6	22.4	8.7	-	-
		34	36.6	22.4	8.8	-	-
		35	36.7	22.3	8.8	-	-
		36	36.7	22.4	8.7	-	-
		37	36.7	22.4	8.8	-	-
		38	36.7	22.3	8.8	-	-
		40	36.7	22.3	8.7	-	-
		41	36.8	22.0	8.8	-	-
	Gracilaria sp.		-	-	-	-	-
	F. vesiculosus		-	-	-	-	-
	P. dioica		-	-	-	-	-

Table A.2: Physical and chemical parameters determined *in situ* in the tanks of the algae species under study, *Ulva rigida, Porphyra dioica, Gracilaria* and *Fucus vesiculosus* (cont.)

Date	Tank		Water salinity (‰)	Water temperature (°C)	pH	Eh (mV)	0_2 (% sat)
28/07/2017	*U. rigida*	29	37.7	24.0	8.9	-	-
		30	37.7	24.0	9.0	-	-
		31	37.8	24.1	9.0	-	-
		32	37.7	24.0	9.0	-	-
		33	37.8	24.0	9.0	-	-
		34	37.7	24.2	8.9	-	-
		35	37.7	24.1	8.9	-	-
		36	37.8	24.1	8.9	-	-
		37	37.8	24.0	8.9	-	-
		38	37.8	24.0	9.0	-	-
		40	37.7	24.0	8.9	-	-
		41	37.8	24.0	8.9	-	-
	Gracilaria sp.		-	-	-	-	-
	F. vesiculosus		-	-	-	-	-
	P. dioica		-	-	-	-	-

Table A.3: Concentrations of arsenic species (mg kg^{-1}) in macroalgae. In bold, species of interest for ALGAplus

Species	Geographical locations	Inorganic As	Total As	References
		(mg kg^{-1})		
U. rigida	Thermaikos Gulf		1.39–2.7	85
	Lloret del Mar	As (V): 0.11 ± 0.01	5.3	108
	Piran		1.35 ± 0.07	13
	Tartous		5.03 ± 0.04	110
	Venice Lagoon		12 ± 2	125
	Chilean coastal zones	0.40 ± 0.29	3.1 ± 0.8	107
U. lactuca	Sydney		2.9	126
	New Hampshire	As (V): 35.5	5.34	111
	Maine	0.02	4.13	111
	Norway	0.12	7.9	112
P. umbilicalis	Great Bay Estuary		20.73	111
	Spain	0.12	34.5	105
	Spain	0.239	25 ± 3	114
	Japan		14 ± 2	114
P. tenera	Japan	0.280	24.1	105
	Japan	0.167	23.2	
Porphyra sp.	Japan	0.189	32.7	105
	Spain	0.383	24.3	
	Korea	0.176	20.8	
	South Korea	0.131	18.4	
	South Korea	0.116	23.5	
	China	0.402	41.7	
	China	0.223	58.3	
F. vesiculosus	France	As(v): 11 ± 1	36 ± 2	114
	New Hampshire	0.06 ± 0.04	28.89 ± 2.16	111
	Maine		32.76 ± 3.73	111
	Norway		41	112
	Valencia	0.34 ± 0.04	50.0 ± 0.3	127
F. virsoides	Venice Lagoon		73 ± 13	125
	Piran		12.1 ± 0.6	13
	Piran		8.42 ± 0.42	13
F. spiralis	New Hampshire	0.04 ± 0.05	16.27 ± 1.41	111
Gracilaria sp.	Viamyl		7.1 ± 0.4	85
G. gracilis	Venice Lagoon		32 ± 1	125
	Viamyl		9.0 ± 0.2	85
	Kalochori		12.2 ± 0.5	85

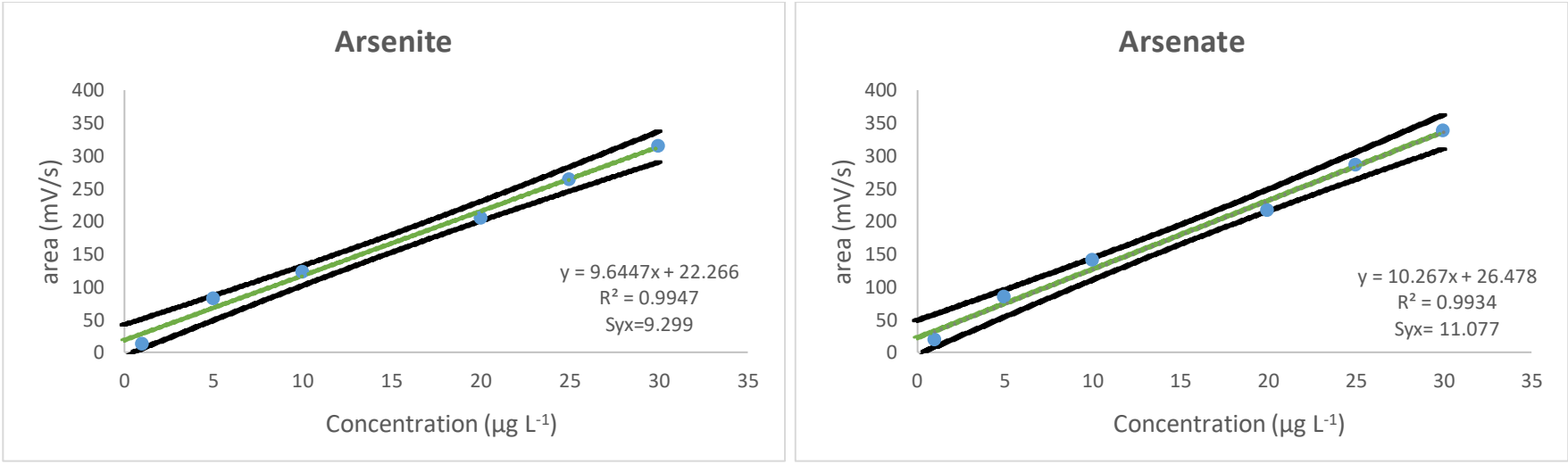

Figure A.1: Calibration curves for arsenite and arsenate and respective confidence intervals (95%)

Limit of detection (LD) was estimated for arsenite and arsenate. The former is the lowest signal of the analyte that can be reliably differentiated from background noise, and it is calculated based on: LD=ya+3xSy/x, where ya is the intercept and Sy/x is the standard deviation of the residuals.

Thus, the limit of detection in concentration units for arsenite is 2.9 µg L-1 and for arsenate is 3.2 µg L^{-1}.