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Research Article

Arsenic K-edge X-ray absorption spectroscopy of arsenic in seafood

Graham N. George[1], Roger C. Prince[2], Satya P. Singh[1] and Ingrid J. Pickering[1]

[1] Department of Geological Sciences, University of Saskatchewan, Saskatoon, SK, Canada
[2] ExxonMobil Biomedical Sciences Inc., Annandale, NJ, USA

It is well known that seafood contains high levels of arsenic. For marine animals arsenic is predominantly present as arsenobetaine and related compounds which are not metabolized and are thought to present no toxic hazard to humans. With edible seaweeds, arsenic is present in different forms, of which arsenosugars are the best known. These species may be metabolized by mammals, and the high arsenic contents of these materials represent a significant environmental source for human exposure to arsenicals in some populations. In this work, we explore the utility of As K-edge X-ray absorption near-edge spectroscopy as an *in situ* probe of the chemical forms of arsenic in seaweeds. We find that three different chemical types of arsenic are present, consistent with arsenate, an arsenosugar or tetra-alkyl-arsonium species, and a trivalent arsenic species.

Keywords: Arsenic / Arsenobetaine / Arsenosugars / Seaweed / Toxicology

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1 Introduction

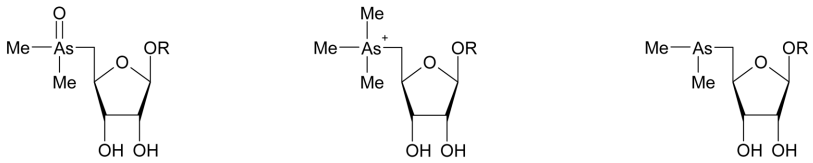
All varieties of seafood typically contain substantial levels of arsenic, which vary from around 1 to 100 mg/kg dry weight [1]. The predominant arsenic form in marine animals, such as fish, crustaceans, and mollusks, is arsenobetaine ($(CH_3)_3As^+CH_2CO_2^-$), with lower quantities of arsenocholine ($(CH_3)_3As^+CH_2CH_2OH$) [1]. Even whale meat contains these compounds, and the higher acid ($(CH_3)_3As^+CH_2CH_2CO_2^-$) can also be present [2]. These formally trivalent arsenic compounds are readily absorbed into the human body, but are not metabolized, and in due course are excreted unchanged into the urine [3]. It is thus well established that the arsenic of marine animals consumed by humans as seafood presents no toxic hazard.

In contrast, in marine algae (seaweed), the arsenic occurs predominantly in pentavalent arsenic ribose derivatives, collectively known as arsenosugars [1], most of which are dimethylarsinylribosides (Fig. 1, 1.) and are formally tri-alkyl arsine oxides. Formally trivalent arsonium arsenosugars (dimethylarsonioribosides) have also been reported (Fig. 1, 2.). While marine algae do not form a very major part of the diet for any human population, and although arsenosugars are clearly not acutely toxic, they are, at least to some extent, metabolized by mammals [4, 5] and their intestinal flora [6]. Thus there is concern about the toxic risk that human consumption of seaweed might present [7]. Nori is the Japanese name for various edible seaweed species of the red alga *Porphyra* the most commonly used of which are *Porphyra yezoensis* and *P. tenera*. Red algae also form part of the diet for communities in China and Korea. Western consumption of Nori has increased markedly in recent years because of the increasing popularity of Japanese foods such as sushi. Green marine algae are also consumed by humans, especially in Japan, and in particular *Enteromorpha* (commonly known as sea grass), which has recently been demonstrated to be a subspecies of *Ulva* [8]. Finally, edible varieties of kelp are known (*e.g.*, *Laminaria saccharina*), and again are consumed in Japanese, Chinese, and Korean food. The arsenic content of these materials is of some concern because, although only small amounts are typically consumed, their high arsenic levels result in a significant environmental source for human exposure to arsenicals.

X-ray absorption spectroscopy (XAS) provides an *in situ* probe of the general chemical forms of a particular element [9]. Its strength is that it can be performed on intact samples, requiring essentially no pretreatment such as digestion or solvent extraction, and has in several cases revealed details that were missed by conventional analysis [10, 11]. It has also, for example, proved essential in understanding the

Correspondence: Professor Graham N. George, Department of Geological Sciences, University of Saskatchewan, 114 Science Place, Saskatoon, Canada SK S7N 5E2
E-mail: g.george@usask.ca
Fax: +1-306-966-8593

Abbreviations: EXAFS, extended X-ray absorption fine structure; **XAS,** X-ray absorption spectroscopy



1. dimethylarsinylribosides **2**. dimethylarsonioribosides **3**. dimethylarsonylribosides

Figure 1. Structures of dimethylarsinylribosides (1) and dimethylarsonioribosides (2) arsenosugars found in seaweeds [1], and of reduced arsenosugars, dimethylarsonylribosides (3). A large number of arsenosugars are known, differing in the nature of the substituent group OR, which is typically a polar group such as glycerol, glycerol-3-sulfate, propane-1,2-diol-3-sulfonate, or carboxyamino-acetic acid.

molecular nature of the toxicological antagonism As and Se [12]. In this report, we explore the utility of As K-edge as a probe of the chemical forms of arsenic in seaweeds. We find that three different arsenic chemical types of arsenic are present in the seaweeds investigated, the spectra of which are consistent with arsenate, arsenosugar, or tetra-alkyl-arsonium species, and a trivalent arsenic species.

2 Materials and methods

2.1 Caution

Many arsenic compounds are toxic and or carcinogenic, and precautions against human exposure should be taken when using these materials.

2.2 Sample preparation

Trimethylarsine ($(CH_3)_3As$) was obtained from Strem Chemicals, and crystalline trimethylarsine oxide ($(CH_3)_3AsO$), $(CH_3)_2AsOH$, and $(CH_3)AsO(OH)$, were provided by Dr. Juergen Gailer, University of Calgary. All other reagents were obtained from Sigma–Aldrich and were of the highest quality available. Low molecular weight arsenic compounds were prepared as 1 mM solutions, dissolved in 50 mM HEPES/NaOH buffer containing 30% v/v glycerol at pH 7.5. Samples of the seaweeds purple laver (*P. perforate*), sea lettuce (*Ulva lactuca*), and fronds of Bull Kelp (*Nereocystis luetkeana*) were collected from the California coastline at Santa Cruz county public beaches. Nori was the commercial dried material, otherwise intended for human consumption, and was purchased from a local fish market. Samples were loaded into acrylic XAS sample cuvettes and frozen in liquid nitrogen prior to data acquisition.

2.3 X-ray absorption spectroscopy

XAS measurements were carried out at the Stanford Synchrotron Radiation Laboratory (SSRL) using the 2T permanent magnet wiggler beamline 9-3, with the storage ring SPEAR containing 80–100 mA at 3 GeV and employing a Si(220) double-crystal monochromator. Beamline 9-3 is equipped with a rhodium-coated vertical collimating mirror upstream of the monochromator, and a downstream bent-cylindrical focusing mirror (also rhodium-coated). Harmonic rejection was accomplished by setting the angle of the mirrors to correspond to a cutoff energy of 15 keV. Incident and transmitted X-ray intensities were monitored using nitrogen-filled ionization chambers. X-ray absorption spectra were measured as the As Kα fluorescence excitation spectra using a 30-element germanium array detector [13] with high-speed analog electronics (Canberra). To avoid problems with nonlinearity of the detector, Germanium filters of six absorption-length thickness were used to preferentially absorb scatter, with silver Soller-slits (EXAFS, Pinoche Nevada) optimally positioned between the sample and the detector. To minimize radiation damage (*e.g.*, photoreduction and photooxidation) samples were maintained at a temperature of 10 K in an Oxford instruments flow cryostat. The energy scale was calibrated with reference to As K-edge spectrum of an elemental arsenic foil measured simultaneously with the data, the lowest energy inflection of which was assumed to be 11867.0 eV.

2.4 Data reduction and analysis

Data reduction and analysis were performed using the EXAFSPAK suite of computer programs (http://ssrl.slac.stanford.edu/exafspak.html) using standard methods [9]. In many cases near-edge spectra of complex mixtures can be used to obtain a quantitative analysis of chemical type by least-squares fitting of a linear combination of suitable model spectra to the near-edge spectrum of the mixture. In this procedure, all spectra are typically normalized to the height of the As K-edge jump so that the fractions of each component obtained in the fit correspond to mole fractions of each component in the mixture. We employed the EXAFSPAK program BACKSUB to generate arsenic K-edge normalized spectra by fitting the experimental spectra to a theoretical atomic curve using a linear scaling factor and a quartic polynomial to remove background using the EXAFSPAK program BACKSUB. In cases where overlapping features are present in the spectra examination of higher derivatives is useful to ensure that fitting reproduces subtle inflec-

tions in the data [14]. Higher derivative spectra were calculated from the normalized absorption spectra using a least-squares cubic polynomial fit over a 1.4 eV energy range.

3 Results and discussion

X-ray absorption spectra arise from excitation of a core electron (*e.g.*, a 1s electron for a K-edge). They can be arbitrarily divided into two overlapping regions – the near-edge spectrum which is the structured region within approximately 50 eV of the absorption edge and the extended X-ray absorption fine structure (EXAFS) which is an oscillatory modulation of the absorption on the high-energy side of the absorption edge and which can be interpreted in terms of a local radial structure [9]. Near-edge spectra are comprised of transitions from the core level (1s for a K-edge) to unoccupied molecular orbitals of the system. Intense transitions are dipole-allowed $\Delta l = \pm 1$, and thus for a K-edge are to levels with a lot of *p*-orbital character. Near-edge spectra are therefore sensitive to electronic structure, and give a fingerprint of the chemical species of the metal or metalloid concerned. The advantage of the near-edge region of the spectrum is that it can be quickly collected with good S/N, whereas EXAFS is more difficult to collect with adequate S/N, and is not always practical on dilute samples. A unique benefit of XAS is that it requires no pretreatment or extraction, and thus provides a tool which can probe chemical species *in situ*. In this paper, we employ arsenic K near-edge spectra to examine the chemical types of arsenic in seaweeds.

Figure 2 shows the X-ray absorption near-edge spectra of ten selected arsenic compounds related to those expected to be found in seafoods. Some of these spectra have been reported previously [11, 15], and in these cases the spectra shown in Fig. 2 are broadly similar to the earlier ones, except for a slightly improved energy resolution, yielding somewhat sharper spectra. The spectra are expected to be sensitive to the chemical environment of the arsenic, but not to the specific compound in which the arsenic is present [9]. Thus, species with different aliphatic substituents (*e.g.*, methyl or a more extended aliphatic group) will give spectra that are effectively identical, and the technique can only identify broad chemical types and not specific molecular species. For example, arsenobetaine and arsenocholine give essentially identical spectra (not illustrated), while aromatic arsonium species (*e.g.*, tetraphenylarsonium chloride) have quite distinct spectra from these (not illustrated). Figure 2 shows that the spectra of all ten species are significantly distinct from each other, and afford unique spectral signatures suitable for fingerprint-type spectroscopic analysis of unknowns. As expected, As^{V} species generally gave spectral features falling at higher energies than those containing As^{III}. A significant exception to this are the formally As^{III} arsonium species (*e.g.*, arsenobetaine), and the formally As^{V} trimethylarsine oxide (Fig. 3). These two species also

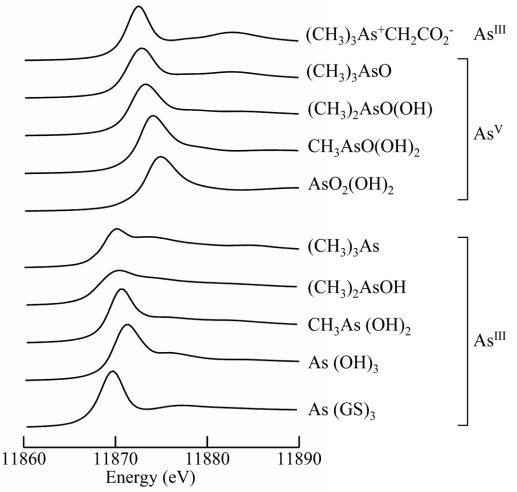


Figure 2. As K-edge X-ray absorption near-edge spectra of a series of biologically relevant arsenic compounds. All species are in solution at pH 7.5; for the oxy acids the formulae for the expected predominant protonation state is given.

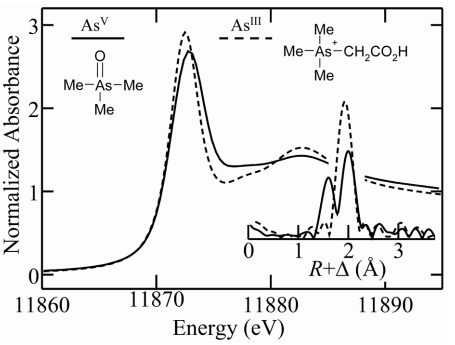


Figure 3. Comparison of the As K-edge X-ray absorption near-edge spectra of trimethylarsine oxide, R_3AsO (solid line) and arsenobetaine, R_4As^+ (broken line). The inset shows the respective As K-edge EXAFS Fourier transforms (phase-corrected for As–C backscattering, *k*-range 1–14 $Å^{-1}$).

happen to correspond to the expected predominant arsenic forms in marine animals and seaweeds, respectively, and we will therefore discuss this similarity further. The positive charge on the arsenic atom of the $[AsR_4]^+$ group effectively shifts the Fermi level and the energies of the bond-state transitions to within the expected range normally expected for formally As^{V} species, which coincidentally falls close to the As^{V} trimethylarsine oxide, making distinguishing these species by As K near-edge spectroscopy difficult. Figure 3 overlays the spectra of arsenobetaine and trimethylarsine oxide, showing the similarity in the near-edge spectra. The inset shows the EXAFS Fourier transforms which give a single peak for $[AsR_4]^+$ corresponding to four equivalent As–C interactions and two peaks for R_3AsO, corresponding to As=O and three As–C interactions. Ideally, EXAFS spectra of unknowns should be used in combination with the near-edge to identify species. EXAFS spectra, however,

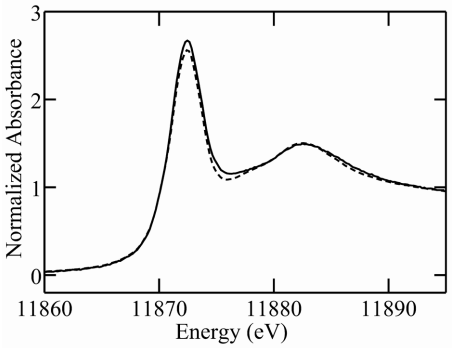


Figure 4. Comparison of As K-edge X-ray absorption near-edge spectra of dogfish skeletal muscle (solid line) and arsenobetaine (broken line).

require data of significantly better S/N and this is not in all cases practical. We note that with data of very good S/N, and the spectra of pure species, the identity can still be suggested solely by comparison of subtle features of the near-edge spectra. Figure 4 compares the spectra of a sample of dogfish skeletal muscle and a solution of arsenobetaine. The near-identity of the spectra confirms the already well-established conclusion [1] that the predominant arsenic form in dogfish is arsenobetaine (or more accurately, we confirm the presence of an aliphatic arsonium species).

Figure 5A compares the arsenic K near-edge spectra of four seaweed samples. The spectra are indicative of the presence of a mixture of species, particularly for *Ulva* and *Nereocystis* (Kelp). Analysis of spectra of species containing several components can be assisted by examining higher derivatives, and Fig. 5B shows the second derivative spectra for the seaweed samples. The presence of multiple features in the spectra, in particular for the *Ulva* and *Nereocystis*, clearly indicates the presence of at least three different arsenic species.

Analysis of the near-edge spectra of complex mixtures can be done using least-squares fitting of a linear combination of suitable model spectra to the near-edge spectrum of the mixture [9]. When $[AsR_4]^+$ and R_3AsO were both included in the refinements, very large estimated SDs were obtained for these components, and examination of the correlation matrix indicated close to 100% correlation, indicating that both cannot be simultaneously included in a fit. Thus, sets of three candidate model spectra, including either $[AsR_4]^+$ or R_3AsO, were fitted to both the absorbance and second derivative spectra, and those which gave the best fits were used for the final fits; in all cases R_3AsO gave a slightly better fit. For the second derivative fits allowing slight energy shifts (*ca*. 0.1 eV) for the R_3As and R_3AsO components gave subtle improvements in the fit, although without significantly changing the estimated percentages of the components concerned. The lines in Fig. 5 show the results of least-squares fitting, and Fig. 6 shows the breakdown of the fit into individual components for the *Ulva* spectrum. Table 1 shows the results of such analysis on the seaweed samples of Figs. 5 and 6.

We note that the presence of three components is clear from the fitting, but that the exact identity of these components is not unambiguously identified in all cases. The highest energy component corresponds to arsenate, with ~20% in *Ulva* and *Porphyra* but with none in *Nereocystis*. Arsenates are difficult to confuse with other species because their near-edge spectra fall at higher energies than other plausible components, and the presence of approximately 20% in green and red algae is an unexpected finding, although consistent with high inorganic arsenic contents that have recently been reported in edible seaweeds

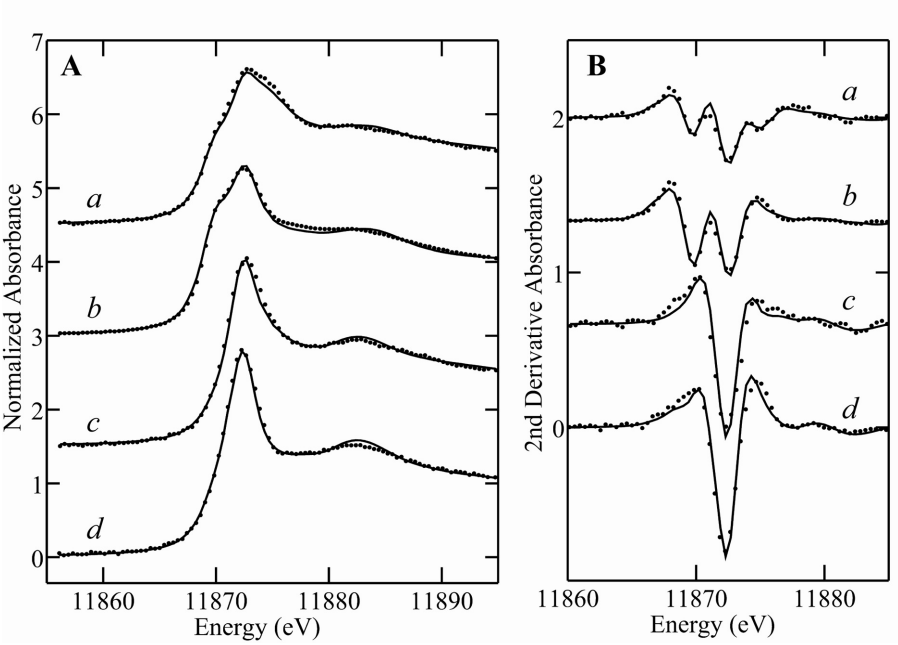


Figure 5. As K-edge X-ray absorption near-edge spectra of seaweeds. (A) The normalized absorption spectra and (B) the second derivative spectra for (a) *Ulva*, (b) *Nereocystis*, (c) *Porphyra*, and (d) Nori. The points show the experimental seaweed spectra, while the lines show the best fits from a linear combination of three models (Table 1).

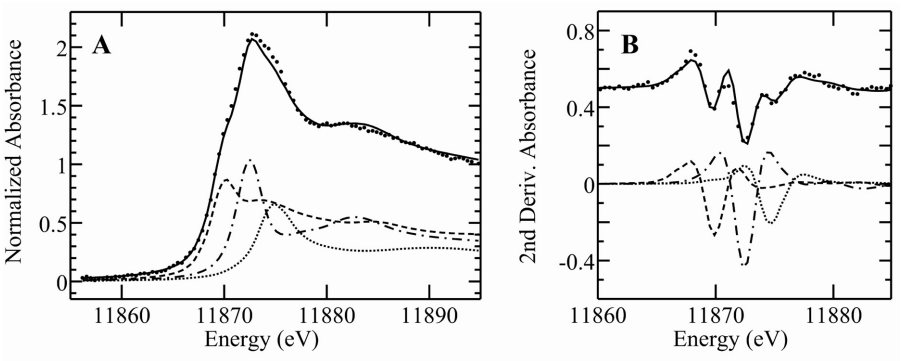


Figure 6. Deconvolution of the least-squares fit of the near-edge spectrum of *Ulva* showing both normalized absorbance (A) and second derivative spectra (B). The points show experimental seaweed data and the solid lines the best fits, with components shown as dashed lines (R_3As), dot-dashed lines (R_3AsO), and dotted lines (arsenate). The fit and experimental points have been vertically offset in (B) for clarity.

Table 1. Least-Squares fitting of As K X-ray absorption near-edge spectra of seaweeds[a)]

	R_3As	R_3AsO	Arsenate	Fit-error $\times 10^{2}$[b)]
Ulva	41(3)	36(2)	23(2)	0.099
Nereocystis	63(2)	37(2)	0	0.087
Porphyra	11(3)	69(3)	20(2)	0.148
Nori	19(3)	74(3)	7(2)	0.100

a) Values given are percentages of total arsenic estimated by fitting the normalized absorption spectra. The values in parentheses are $3\times$ the estimated SDs (e.s.d.) obtained from the diagonal elements of the covariance matrix. Both normalized absorbance and second derivative data were fitted, and gave quantitatively very similar estimates for the levels of the components. In all cases percentages of the components estimated from second derivative spectra overlapped with those from normalized absorption within $3\times$ the e.s.d. (99% confidence limit).

b) The fit error is defined as $\sum(\mu_{expt}-\mu_{calc})^2/(N-1)$ where μ_{expt} and μ_{calc} are the normalized experimental and calculated X-ray absorptions, respectively and N is the number of data points.

[16, 17] and in seaweeds from contaminated waters [18]. As we have discussed, the presence of pentavalent R_3AsO is expected from earlier work, but the presence of a reduced As^{III} species, which fitted to trialkylarsine (R_3As), is more unexpected, although the presence of As^{III} has been deduced using conventional analysis methods [17]. We note that the presence of thio-arsenosugars (R_3AsS) has recently been reported in marine brown algae with up to 10% of this species being detected [19]. While no spectra of any R_3AsS species is presently available to us, we can anticipate that the presence of a thio group will shift the near-edge position of R_3AsS to lower energies. We can estimate this shift from other As K near-edge spectra from compounds in which an oxygen ligand to arsenic is replaced by sulfur as being between –1.0 and –0.6 eV. The low-energy component detected in the seaweed is displaced from R_3AsO by about –2.25 eV, and thus is unlikely to be due to thio-arsenosugars. Furthermore, this reduced arsenic comprises about 40 or 60% of the total arsenic (for *Ulva* and *Nereocystis*, respectively) which is much higher than reported levels for thio-arsenosugars, and in the brown algae sample this was more than 60% of the total. The possibility of a significant fraction of trivalent arsenic in seaweeds must therefore be considered. It is possible that this trivalent arsenic is not detected by other techniques due to a resistance to extraction as has previously been reported [10]. Thiols are known to chemically reduce pentavalent arsenosugars to trivalent arsenosugars (Fig. 1, 3.), and such trivalent arsenosugars have recently been reported to be more toxic than their pentavalent counterparts [7]. The spectra of the *Porphyra* and Nori indicate lower quantities of the reduced component, suggesting that any toxic hazard from consuming trivalent arsenicals in seaweed will be less in red algae than in the other varieties generally consumed.

Any discussion of the hazards of consuming seaweed would be incomplete without mentioning the remarkable series of studies examining sheep eating a diet consisting almost entirely of seaweed. These sheep have been maintained for generations upon a diet of seaweed (chiefly *Laminaria*) on the island of North Ronaldsay (the most northern of the Orkney Islands) [20, 21]. Typically animals consume 45–90 mg of arsenic *per* day with no apparent health effects, although long-term illness could not be ruled out as the sheep were typically slaughtered for human consumption. It is worth noting that these sheep also have an abnormally high sensitivity to copper [22, 23], and can die from copper poisoning if fed upon a normal terrestrial diet [22]. Whether or not the extraordinary tolerance to arsenic is unique to this species of sheep, or whether it is linked to the defect in copper metabolism or just a coincidence, are at present unknown.

4 Concluding remarks

We conclude that As K near-edge XAS is a potentially useful tool for investigating arsenic chemical forms in marine algae that could provide information on the possible health impacts of these arsenic-rich foods. Ambiguities in the K-edge XAS method mean that some components cannot be uniquely identified, and in particular $[R_4As]^+$ and R_3AsO cannot be unambiguously distinguished within a mixture. Future work will include the measurement of an enlarged library of model compound spectra (*e.g.*, including the spe-

cies such as R_3AsS, discussed above), and investigation of the utility of other X-ray absorption edges (*e.g.*, the arsenic L-edges) as an aid to distinguishing such species. The use of microfocus X-ray beams combined with X-ray excitation at carefully selected energies, has previously been extended to provide chemically specific maps of arsenic localizations in higher plants, including intracellular localization [24], and such techniques would be ideally suited to study the localization of different arsenic forms in seaweeds. In particular, many seaweeds grow in structures (*e.g.*, *Enteromorpha* tubes) that are only one cell thick, and provide an ideal system for these techniques.

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