Bioaccumulation and Biotransformation of Arsenic by Marine Algae in Hawaii

Problem and Research Objectives

For several decades arsenical compounds were used in the Hawaiian Islands as pesticides in the sugar industry. Although their use was basically discontinued in the 1940s much arsenic remains in the soil of former sugar cane fields and has been continually transported mostly bound to soil particles with water into the coastal waters of the state.

Seaweeds, or *limu* in Hawaiian, are an important part of Hawaiian cuisine and several species are highly prized by local cooks. It is common practice in Hawaii to gather these algae along the shorelines where they wash up.

Arsenic is famously toxic in several of its forms and therefore there are justifiable concerns about the safety of consuming algae from waters subject to arsenic contamination. This concern is reinforced by the fact that some seaweeds are known to concentrate arsenic (Diaz et al., 2011, Granchinho et al., 2001).

Recent research conducted on behalf of The Nature Conservancy in the State has revealed that seaweed collected at certain shoreline areas in the islands contains relatively high concentrations of arsenic.

Algae can transform arsenic between a number of states, metabolizing the arsenate and arsenite to less toxic methylated forms (Granchinho et al., 2001). This has implications for the risk posed by consumption of algae and its use as a soil amendment.

The objective of this study is to measure the arsenic content in algae collected from nearshore waters around the island of Oahu and to characterize the speciation of any arsenic to see what form it is occuring in.

Methodology

We collected preliminary samples of an alga believed to be *Gracilaria salicornia* (Figure 1) from the shore at Waikiki beach in Honolulu (21.265253°N, -157.822206°W, Figure 2). *Gracilaria salicornia* is one of the most successful invasive algae on reef flats in Hawaii. It is related to, and competitive with, the popular edible alga *Gracilaria coronipfolia.* The collected samples were freeze dried the day after sampling and the freeze dried material sent to our collaborating laboratory at the Illinois Sustainable Technology Center (ISTC) at the University of Illinois at Urbana-Champaign for analysis. Preliminary analyses were not able to speciate fully the total arsenic found in the sample. Subsequent work by John W Scott, ISTC Senior Analytical Chemist, and his team resulted in a fuller accounting of the different species that made up the total arsenic found in the sample.

Figure 1: *Gracilaria salicornia*

Figure 2: Sample collection site

Sample Preparation

The sample was homogenized with the aid of a gyromill and was milled to a fine powder (Figure 2).

Figure 3: Milled *G. salicornia* sample

Total Arsenic Digestion: To prepare the sample for total arsenic analysis by inductively coupled plasma mass spectrometry (ICP-MS), a microwave digestion procedure was employed. A quarter gram of homogenized sample was digested in a CEM microwave digestion system for 40 minutes with the aid of 10 ml nitric acid and 1 ml hydrogen peroxide. After cooling the sample was transferred to a centrifuge tube and diluted to a final volume of 50 ml. In addition, a reagent blank, an arsenic standard matrix spike, and a certified dogfish muscle tissue sample were processed in parallel to the sample to verify sample preparations. A matrix effect was observed for these samples; therefore a second microwave procedure was performed (See Total Arsenic Analysis Section). The second microwave digestion employed was identical to the first with the exception that one tenth of a gram homogenized sample was processed. In addition, the trifluoroacetic acid (TFA) extracted solids (0.078 g) for one algae sample was digested in this batch as well.

Extraction Methods: To prepare the algae sample for arsenic speciation analysis by liquid chromatography inductively coupled plasma mass spectrometry (LC-ICP-MS), solid-liquid extractions were performed. A quarter gram of homogenized sample was treated with 5.0 ml TFA at 65^{o}C for two hours. Afterwards, the sample was shaken on a laboratory mixer for fifteen minutes. The TFA was collected and the procedure was repeated two more times while the TFA phases were pooled. The pooled fraction was then centrifuged for twenty minutes at 2000 RPM and the final TFA fraction was decanted into a drying tube. The TFA was then removed under a gentle stream of nitrogen and the residue was re-constituted in 10 ml 0.2% hydrochloric acid. A reagent blank, an inorganic arsenic matrix spike, and a certified dogfish muscle sample were processed in parallel to verify sample extraction.

Total arsenic analysis of the TFA extracts indicated that the extraction of arsenic was incomplete; therefore a second extraction of one of the TFA extracted solids was performed. The second extraction was identical to that of the first with the exception that a methanol-water (3:1) extraction fluid was utilized and the extraction was performed at 55^{o}C. A reagent blank, an inorganic arsenic matrix spike, and a certified dogfish muscle sample were processed in parallel to verify sample extraction.

Total Arsenic Analysis: Total arsenic analysis was performed with a VG Elemental PQ Excel ICP-MS. Yttrium was utilized as an internal standard and the instrument was calibrated daily with reference materials procured from SPEX Certiprep. Verification of instrument calibration was achieved with preparations and analysis of two independent reference materials from the same vendor, but with different lot numbers. These check standards were analyzed post calibration and post sample analysis. Each ICP-MS measurement was conducted in triplicate and a sample duplicate and an analytical sample spike was performed during each assay.

Arsenic Speciation Analysis: Arsenic speciation was achieved with a liquid chromatography system interfaced to the ICP-MS instrument operated in a transient acquisition mode. Separation of the arsenic compounds was achieved with a Phenomenex Luna C18 100A column (250 x 4.40 x 5μ) with a isocratic mobile phase of 2.5 mM oxalic acid, 10mM 1-heptanesulfonic acid (ion-pairing agent) and 0.1% methanol adjusted to a pH of 4 with ammonium hydroxide. The mobile phase flow rate was set at 1.0 ml/min and an injection volume of 30 μl was used. Yttrium prepared at a concentration of 150 ng/ml in mobile phase was employed as an internal standard. Injection of 10 μl of the internal standard was performed post-column and is necessary since mobile phase and sample salts dampen the signal intensity over the course of the assay. Calibration of the instrument was conducted with reference materials obtained from SPEX Certiprep, Sigma, and Chem Service. Verification of instrument calibration was achieved with preparations of reference materials by another chemist other than the one who prepared the calibration standards. Check standards were analyzed post calibration and post sample analysis.

Principal Findings and Significance

Total Arsenic Results: Table 1 presents the final results for total arsenic in digested samples and extraction samples. A significant matrix effect was observed during measurements of total arsenic in digestion batch one. This was indicated by low recoveries of the digestion matrix spike, digestion certified reference material (SRM), and the analytical spike. Therefore, a method of standard addition was performed on one sample, the matrix spike, and the SRM. Digestion quality controls were much improved under these conditions and confidence in the final arsenic result was obtained. When a smaller digestion mass was utilized in digestion batch 2, the matrix effect was not observed and method of standard addition was not necessary.

Table 1: Total Arsenic Results for Digested and Extracted Algae Samples

	Arsenic, mg/g	Digestion / Extraction Duplicates %RSD/%RPD	Matrix Spike / Reagent Blank Spike, %Recovery	Dogfish Muscle (DORM-2) SRM, %Recovery
Digestion Batch 1	13*	5.3%	60%* / NA	90%
Digestion Batch 2	10.2	17%	83% / 87%	88%
Extraction 1 (TFA)	5.9	8.0%	NA / 83%	92%
Digestion Batch 2 (TFA Solids)	4.7	NA	NA	NA
Extraction 2 (MeOH-Water)	2.6	NA	NA / 59%	87%

* - Determined by Method of Standard Addition
NA - Parameter Not Available

Total arsenic analysis of the TFA extract indicated that 51% of the total arsenic is extracted under these conditions. This was further verified by digestion and analysis of the extraction raffinate to account for 41% of the missing arsenic. Analysis of the second extraction (Methanol-Water 3:1), accounted for 22% of the unextracted arsenic from the first extraction. By summing the percent arsenic extracted by method one and method two, a total arsenic extraction of 73% was achieved.

Arsenic Speciation Results: Table 2 presents the final results for arsenic speciation of the algae extracts. The TFA extraction blank showed significant arsenate signal with regards to arsenate signals observed for samples. Most likely this is due to a argon-chloride interference (Ar40Cl35 at As75). This is also the most likely culprit for the high SRM recoveries for this species as well. Please refer to the discussion section of this progress report for a more details. The methanol-water extraction reagent blank spike recovered low for arsenite, however this was also seen in a low recovery for total arsenic in this sample as well. The most likely cause for this low recovery is that the sample was inappropriately spiked with arsenite.

Known arsenic species measured in this experiment were very low and do not account for the majority of arsenic species present in the algae sample.

Table 2: Arsenic Speciation Results for Algae Extracts (Concentration Units mg/g Unless Otherwise Noted)

	Arsenate (As^{+5}) as Arsenic	**Arsenite (As^{+3}) as Arsenic**	**Monomethylarsinic acid (MMA) as Arsenic**	**Dimethylarsinic acid (DMA) as Arsenic**	**Arsenobetaine as Arsenic**
TFA Extraction Blank	0.21	< 0.1	< 0.1	< 0.1	< 0.1
TFA Algae Extract 1	0.46	0.56	< 0.1	0.11	< 0.1
TFA Algae Extract 2	0.32	0.49	< 0.1	< 0.1	< 0.1
TFA Reagent Blank Spike	105% Recovery	84% Recovery	NA	NA	NA
TFA DROM-2 SRM Extract	323% Recovery	< 0.1	< 0.1	79% Recovery	2% Recovery
MeOH-Water Extraction Blank	< 0.1	< 0.1	< 0.1	< 0.1	< 0.1
MeOH-Water Algae Extract 1	< 0.1	< 0.1	< 0.1	< 0.1	< 0.1
MeOH-Water Algae Extract 2	< 0.1	< 0.1	< 0.1	< 0.1	< 0.1
MeOH-Water Reagent Blank Spike	117% Recovery	12% Recovery	NA	NA	NA
MeOH-Water DROM-2 SRM Extract	198% Recovery	< 0.1	< 0.1	85% Recovery	97% Recovery

Discussion: Data obtained in this experiment are inconclusive if TFA is the best solvent for extraction of arsenic species in algae. This solvent extracted only 51% of the total arsenic contained in the algae sample. In addition, recoveries of arsenobetaine in the SRM were extremely low and reagent blanks for arsenate in this solvent were significant with regards to sample. Smith et al. (2008) reported success with TFA extraction of rice plants, however there is no mention of extraction blanks, no mention of extracted SRMs, and the research was only concerned with arsenate, arsenite, monomethylarsinic acid (MMA), and dimethylarsinic acid (DMA) (1). The use of a chloride interference correction may remedy the issue (see below discussion on interference corrections), however this solution has yet been demonstrated. Kohlmeyer et al. (2002) have reported that marine algae lack arsenobetaine and contain mostly arsenosugars (2). Given that, the effect of TFA may be moot point, however there is still concern that TFA will affect arsenosugars and one wonders, what is happening to the arsenobtaine? Increasing the current arsenic speciation data acquisition time and re-analyzing the TFA extracts to look for later eluting arsenic compounds may be an experiment worth conducting. In addition, setting up the current LC-ICPMS system to run arsenosugars would also be a direction worth heading. However, obtaining arsenosugar reference materials may prove futile (see below discussion on reference materials).

Methanol-water (3:1) extracted arsenic species that were un-extractable with TFA. The reagent blank indicated that the reagents used to prepare the extraction fluids produce less of interference. This result was further demonstrated by a lower recovery of arsenate in the SRM extracted by methanol-water, than that obtained for TFA (198% versus 323%). Arsenobetaine recoveries using the methanol-water extraction procedure were much superior to the TFA extracted counterpart and the DMA extraction recovery was greater as well. Increasing the current arsenic speciation data acquisition and re-analyzing the methanol-water extracts to look for later eluting arsenic compounds may be an experiment worth conducting. Also, setting up the current LC-ICPMS system to run arsenosugars would be a direction worth pursuing. An initial extraction of the algae material with methanol-water (3:1) is planned and results from this experiment are eagerly anticipated.

Observation of the argon chloride interference warrants concern. Many times this can be corrected by subtraction of the reagent blank, however since chloride is anticipated to be variable in samples this practice is unacceptable. Another approach to this issue is to utilize an interference correction equation. Arsenic is monoisotopic, with an atomic mass of 75 Daltons. Chloride has two isotopes, 35 Daltons and 37 Daltons, with relative abundances 75.53 and 24.47 respectively. If one monitors the signal at mass 77 Daltons (Ar40Cl37), then an interference equation can be employed to correct for the interference. However, one must still beware because selenium also has an isotope at 77 Daltons with a relative abundance of 7.58. Therefore, monitoring selenium at mass 82 would allow one to correct for selenium interference at the Ar40Cl37 mass. Still yet we are not out of the woods, krypton has an isotope at mass 82 as well, with a relative abundance of 11.56. Krypton is typically found alongside argon and because it is heavier than argon, its presence becomes more prevalent as the liquid argon tank for the ICP-MS depletes. Therefore, another correction can be made if we measure krypton at mass 83. Putting all the interference equations together results in an expression as follows:

Mass 75 signal - (3.1 x mass 77 signal - (0.82 x mass 82 signal - (1.0 x mass 83 signal)))

The signal coefficients are generated from the ratio of the relative abundances of the elements. Use of interference equations are a commonplace in routine ICP-MS analysis, however to date we know of no speciation assays that utilize them. Analysis of extracted blanks and extracted SRM's under these conditions would provide a measure of success or failure to this approach. Regardless, it is fun to think about.

Without the appropriate arsenic reference materials, identification of other arsenic compounds by the current speciation method is not possible. One option is to contact Professor K.V. Francesconi and see if he would be willing to share the four arsenosugars that are in his possession (Madsen et al., 2000). Another option would be to locate synthesis methods for several of the most probable arsenic sugars present as reported by the Kohlmeyer et al. (2002) and prepare them in-house. A third option could be to contact a chemical manufacturer and request to have the most probable arsenic sugar compounds custom made, however chances are that this option would be costly. A fourth option, would be to set-up an arsenic speciation method identical to the McSheehy and Szpunar (2000) methods and identify unknown arsenic compounds relative to the known arsenic compounds.

Addendum – Further experiments to improve speciation

The data from the above experiment indicated that the algae sample contained 10 μg/g total arsenic. This value agreed well with data obtained from independent analyses conducted for The Nature Conservancy for samples collected in Hawaii. A preliminary arsenic extraction experiment performed with trifluoroacetic acid (TFA) as the solvent and duckweed, a marine type plant in Illinois, as the sample showed some promise. However, extraction by this method on the Hawaiian algae sample was only able to recover 51% of the total arsenic. A second extraction with methanol-water performed on the raffinate from the first extraction was able to remove about 55% of the remaining total arsenic. Arsenic speciation of the extracts from these experiments by liquid chromatography inductively coupled plasma mass spectrometry (LC-ICPMS) indicated that the forms of arsenic present were not amendable to the current instrumental methods employed at ISTC. Furthermore, the extraction method was shown to cause changes in the forms of arsenic present.

Objective: To extract arsenic compounds from algae samples by a solid-liquid extraction method. To determine the arsenic species present in the algae extract and their representative concentrations by LC-ICPMS.

Sample Preparation and Total Arsenic Digestion: The sample preparation used in this experiment was similar to that detailed above. The processed sample was stored at –20^{o}C when not in use. Total arsenic result used for calculating recoveries was based on the earlier experiment.

Extraction Methods: To prepare the algae sample for arsenic speciation analysis by LC-ICPMS, solid-liquid extractions were performed. A quarter gram of homogenized sample was treated with 5.0 ml methanol-water (3:1) at 55^{o}C for 1 hour. The extraction solvent was collected and the procedure was repeated two more times and the extraction fluids were pooled. Eight milliliters from the pooled extract were removed and the methanol was evaporated under a gentle stream of nitrogen at 50^{o}C. Following methanol removal, the sample was diluted to 8.0 ml with 0.2% hydrochloric acid. The final sample was then filtered through a 0.2 μ syringe filter to remove any solids. A reagent blank, an inorganic arsenic matrix spike, a certified dogfish muscle sample, and a commercially available kelp sample purchased locally were processed in parallel to verify sample extraction.

Total Arsenic Analysis: Total arsenic analysis was performed with a VG Elemental PQ Excel ICP-MS. Yttrium was utilized as an internal standard and the instrument was calibrated daily with reference materials procured from SPEX Certiprep. Verification of instrument calibration was achieved with preparations and analysis of two independent reference materials from the same vendor, but with different lot numbers. These check standards were analyzed post calibration and post sample analysis. Each ICP-MS measurement was conducted in triplicate and a sample duplicate and an analytical sample spike was performed during each assay.

Arsenic Speciation Analysis: Arsenic speciation was achieved with a liquid chromatography system interfaced to the ICP-MS instrument operated in a transient acquisition mode. The LC operating parameters were obtained from a reference method designed for marine biota (1). Separation of the arsenic compounds was achieved with a Thermo AS7 column (4mm x 250mm) with a nitric acid gradient mobile phase containing 0.05 mM mM benzene-1,2-disulfonic acid dipotassium salt (ion-pairing agent) and 0.5% methanol. The mobile phase flow rate was set at 1.0 ml/min and an injection volume of 30 μl was used. Yttrium prepared at a concentration of 100 ng/ml in mobile phase A was employed as an internal standard. Injection of 10 μl of the internal standard was performed post-column and is necessary since mobile phase and sample salts dampen the signal intensity over the course of the assay. Calibration of the instrument was conducted with reference materials obtained from SPEX Certiprep, Sigma, and Chem Service. Check standards were analyzed post calibration and post sample analysis.

Total Arsenic in Algae Extract Results: Total arsenic analysis of the methanol-water (3:1) extracts was achieved by ICP-MS. The average total arsenic result obtained for duplicate extract of the Hawaiian algae is 11 µg/g. The average total arsenic result obtained for duplicate extract of the kelp is 65 µg/g.

Arsenic Speciation Results: Figure 1 presents the LC-ICPMS chromatogram of the algae sample spiked with five arsenic species at a five-fold dilution. Recoveries for the five arsenic species spiked in an algae extract recovered from 84% to 106%. In addition, two unknown arsenic species were observed at retention times 6.7 minutes and 7.1 minutes. These unknown peaks were not observed in reagent blanks or calibration standards.

Figure 1
LC-ICPMS Chromatgram of Algae Extract Spiked with 5 Arsenic Species
(5-Fold Dilution)

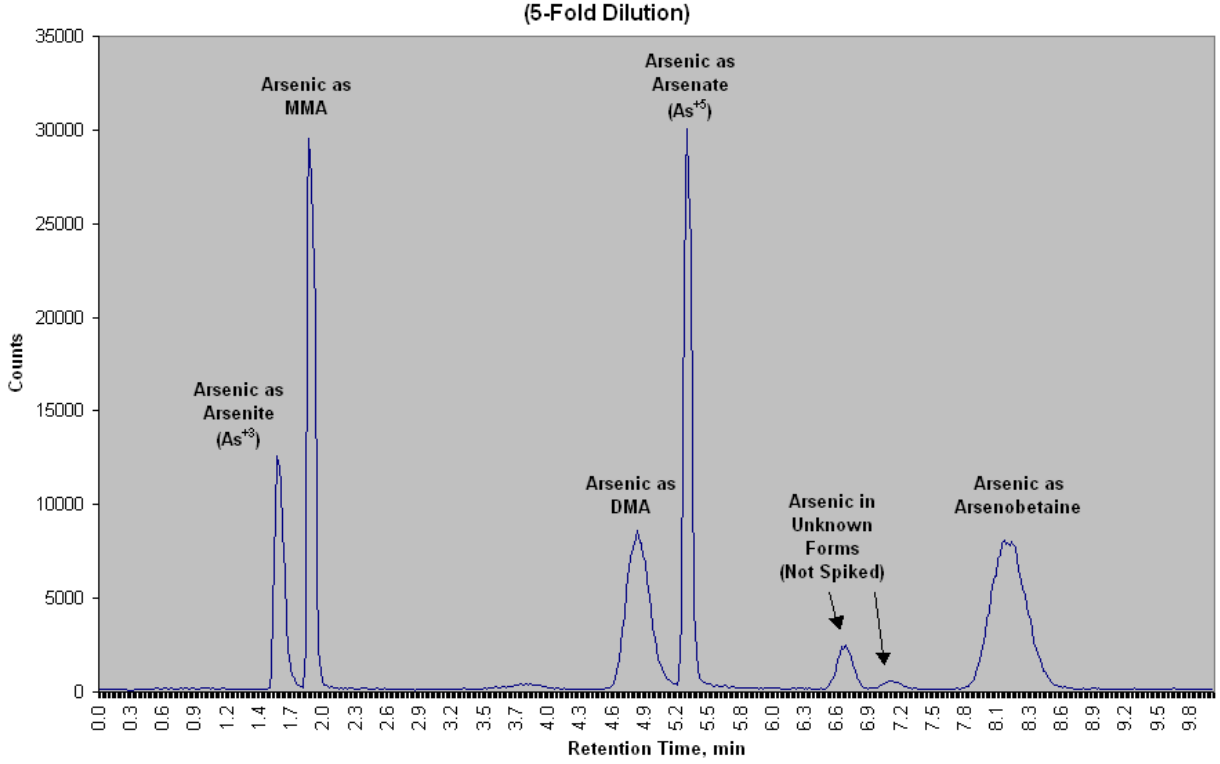

Figure 2
LC-ICPMS Chromatogram of Algae Extract
(5-Fold Dilution)

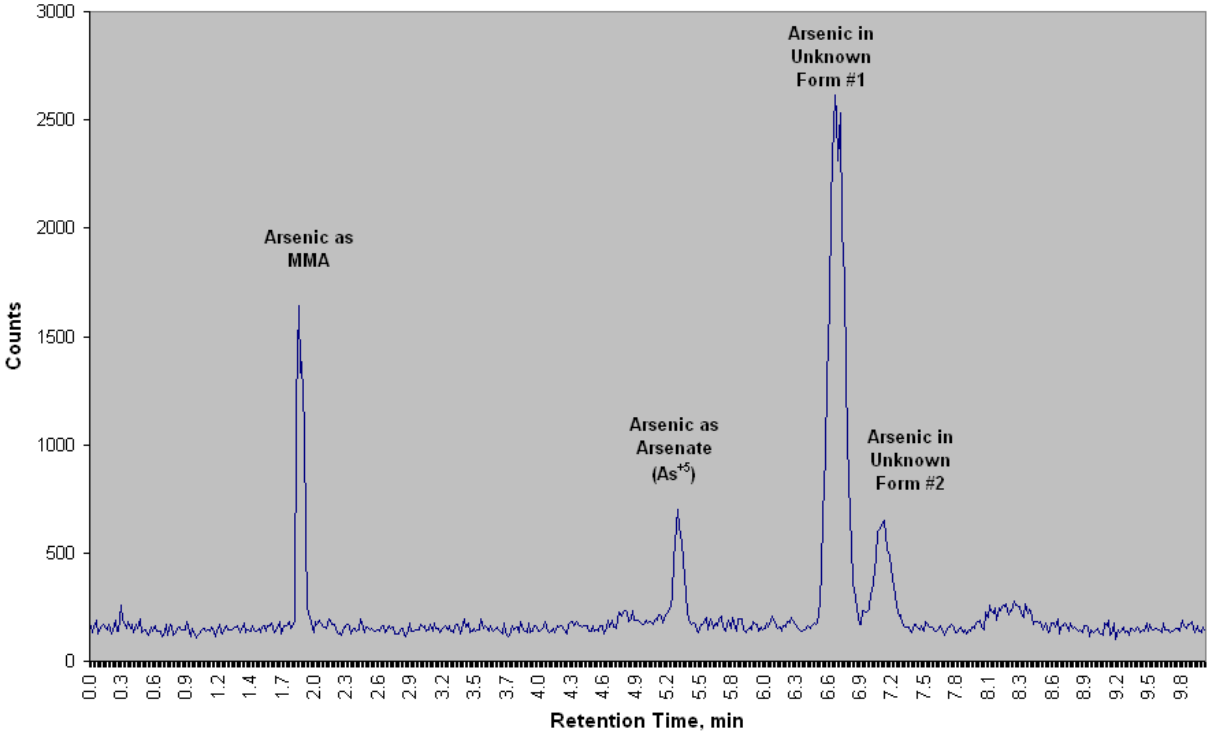

Figure 2 presents the LC-ICPMS chromatogram of an alga extract with no species spike.

The only known arsenic species observed in the extract were arsenic as MMA and arsenic as arsenate. In addition, two unknown forms of arsenic were observed in the extracts. Unknown #2 was found at the greatest concentration. Table 1 presents the final results for arsenic speciation of the algae extracts. Final results are reported as arsenic in concentration unit µg/g.

Table 1: Final Arsenic Species Results for Algae Extracts

	Total Arsenic*, µg/g	Arsenite, µg/g	MMA, µg/g	Unknown Species #1, µg/g	DMA, µg/g	Arsenate, µg/g	Unknown Species #2, µg/g	Unknown Species #3, µg/g	Arsenobetaine, µg/g	Species Sum, µg/g
Gracilaria salicornia (from Hawaii)	11	< 0.3	1.2	< 0.3	< 0.3	0.33	5.5	0.75	< 0.3	7.7
Kombu - Family Laminariaceae (purchased locally)	65	< 0.3	27	20	9.9	6.4	6.5	< 0.3	< 0.3	69

* - Total arsenic in extract measured by ICP-MS

Discussion: Total arsenic analysis of the methanol-water (3:1) algae extract indicates that all of the arsenic present in the algae sample was extracted. The sum of the arsenic species accounted for 70% of the arsenic present in the extract. Two known forms of arsenic were present in the algae extract. Two unknown forms of arsenic were detected in the extract. One of the unknown forms, #2, is the most abundant form of arsenic in this sample. The remaining 30% of the arsenic present in the extract is not detectable by this LC-ICPMS method.

Analysis of total arsenic in the edible kelp sample produced a total arsenic result of 65 µg/g. This result is almost six times greater than the Hawaiian algae sample. The sum of the arsenic species indicates that all the arsenic present in the edible kelp was accounted for by the LC-ICPMS method. Two unknown forms of arsenic were detected in the edible kelp sample. One of the forms, Unknown #2, was identical to one observed in the Hawaiian algae sample. The concentration of this form in the edible kelp was similar to the concentration of this unknown form in the Hawaiian algae sample.

Identification of the unknown arsenic species present is impossible by these methods. In order to isolate and identify these unknown forms, separate methods would have to be developed. Edmonds, et al. were able to isolate and identify unknown forms of arsenic in the edible seaweed *Hizikia fusiforme*, however the method used were extremely labor intensive (2).

Publications Cited

Diaz, Oscar, Yasna Tapia, Ociel Muñoz, Rosa Montoro, Dinoraz Velez, Concepción Almela (2011) "Total and inorganic arsenic concentrations in different species of economically important algae harvested from coastal zones of Chile" *Food Chem Toxicol.* 2011 Nov 25. [Epub ahead of print]

Edmonds, E., Morita, M., Shibata, Y. "Isolation and Identification of Arsenic containing Ribofuranosides and Inorganic Arsenic from Japanese Edible Seaweed *Hizikia fusiforme"*. *J. Chem. Soc. Perkin Transactions 1*. 1987: 577-580

Granchinho, S. C. R., Polishchuk, E., Cullen, W. R. and Reimer, K. J. (2001), "Biomethylation and bioaccumulation of arsenic(V) by marine alga *Fucus gardneri"*. *Applied Organometallic Chemistry*, 15: 553–560.

Kohlmeyer, U., Kuballa, J., Jantzen, E. "Simultaneous separation of 17 inorganic and organic arsenic compounds in marine biota by means of high performance liquid chromatography/inductively coupled plasma mass spectrometery". *Rapid Commun. Mass Spectrom.* 2002; 16: 965-974

Madsen, A.D., Goessler, W., Pedersen, S.N., Francesconi, K.A. Characterization of an algal extract by HPLC-ICP-MS and LC-electrospray MS for use in arsenosugar speciation studies" *J. Anal. Atom. Spectrom.* 2000; 15: 657

McSheehy, M., Szpunar, J. "Speciation of arsenic in edible algae by bi-dimensional size-exclusion anion exchange HPLC with dual ICP-MS and electrospray MS/MS detection" *J. Anal. Atom. Spectrom.* 2000; 15: 79

Smith, E., Juhasz, A.L., Naidu, R. "Arsenic uptake and speciation in rice plants grown under greenhouse conditions with arsenic contaminated irrigation water". *Sci. Tot. Environ*. 2008: 277-283