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Trace element speciation in largemouth bass (*Micropterus salmoides*) from a fly ash settling basin by liquid chromatography-ICP-MS

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Abstract Analytical techniques used to examine the chemical speciation of multiple trace elements are important for the investigation of biological systems. Size exclusion chromatography (SEC) coupled to ICP-MS was used to investigate the speciation of Se, As, Cu, Cd and Zn in tissue extracts from a largemouth bass (*Micropterus salmoides*) collected from a coal fly ash basin and results were compared to a largemouth bass collected at a reference site. Using a Biosil SEC column, with an effective separation range of 100–7 KDa, Cu, Zn and Cd were shown to be bound to metallothionein (MT) in the liver, gill and, to a lesser extent, gonad tissue extract. In liver, muscle and gill of the ash basin bass, Se was predominantly present as low molecular weight species. Only in the gonad extract was the major fraction of Se associated with high molecular weight species. For the liver and gill extracts, further SEC-ICP-MS on a column with an effective separation range of 7000–500 Da was performed, but Se species still eluted near the total volume of the column suggesting a low molecular weight organic or inorganic species. Ion chromatography (IC)-ICP-MS using an AS7 column and HNO_3 gradient elution indicated that the Se and As species in the liver and gill extracts had similar retention times but these retention times did not correspond to retention times for As(III), As(V), dimethylarsenate, arsenobetaine, Se(IV), Se(VI), seleno-methionine, or selenocystine.

Keywords SEC-ICP-MS · Metallothionein · Selenium · Fish tissue

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Introduction

A primary disposal method for coal fly ash is deposition and gravitational settling in aquatic settling basins. Fly ash particles are enriched in inorganic contaminants including As, Se, Cd, Cu, Cr and Zn [1, 2], hence fish that inhabit ash basins and downstream sites are exposed to mixtures of trace elements in the water column and sediments. A number of studies have documented increased body burdens of trace elements in fish, amphibians and reptiles inhabiting fly ash settling basins [3, 4, 5, 6]. Additionally, morphological abnormalities and mortalities have been documented in fauna inhabiting fly ash settling basins [7, 8] and have been ascribed to exposure to excess concentrations of Se, which is consistently elevated in organisms inhabiting the ash basins.

Selenium is present in the water column as selenate (H_2SeO_4) or selenite (H_2SeO_3). The particular species distribution depends upon the prevalent pH and Eh conditions. Selenate is readily soluble, while Se(IV) is more strongly sorbed by mineral phases in the sediment [9]. Selenium may also exist in a variety of organic compounds in the Se(-II) oxidation state [10]. Many of these compounds are analogs of S species and seleno analogs of the two S-amino acids methionine and cysteine are known to occur in nature. In fact, Se is an essential element for plants and animals and a number of proteins, such as glutathione peroxidase and formate dehydrogenase, utilize Se-cysteine in the active site [11]. By contrast, no proteins have been identified that utilize Se-methionine (Se-met). Indeed one proposed mechanism for Se toxicity is that of indiscriminate substitution of Se-methionine for methionine during protein synthesis, which affects subsequent protein structure and function.

Trace element-protein interactions have been studied using liquid chromatography coupled to an element specific detector such as inductively coupled plasma mass spectrometry (ICP-MS) [12, 13, 14, 15, 16]. Size exclusion chromatography (SEC) interfaced with ICP-MS has been used extensively to investigate the interactions of the cys-

teine rich protein metallothionein (MT) with chalcophillic elements Cd, Zn, Cu, Ag and Hg [14, 17]. Size exclusion chromatography has also been used to study specific seleno-proteins such as the plasma protein, seleno-protein P and its possible role in complexing Hg at a 1:1 molar ratio [18, 19] and to study more general Se-protein interactions in urine, liver, kidney and blood of rats fed a Se-enhanced diet [20] and in extracts of muscle tissue from edible fish species [21, 22].

A number of studies in mammals have shown that Se can exert an antagonistic effect on Cd and Hg toxicity and this has been ascribed to either restoration of effective glutathione peroxidase levels and thus prevention of lipid peroxidation [23] or to direct sequestration by a selenoprotein, which has been shown to be the case for Hg [18, 24]. A recent study has shown that accumulation of Cd in mussel hepatopancreas was decreased when Cd and Se were co-administered [25]. In organisms exposed to high concentrations of Se and trace metals the possibility also exists for disruption of sulfhydryl rich proteins via either Se substitution for S-amino acids or Se binding to sulfhydryl groups which may subsequently affect metal transport and binding. Previous studies have suggested that Se can effectively substitute into MT-like proteins, in the laboratory via an automated peptide synthesizer [26], in bacteria cultured in Se(IV) media [27] and in rats co-exposed to Cd and Se [28, 29].

Metabolism of inorganic As to organic species is a mechanism to reduce the toxicity of this element. In marine animals it appears biochemical pathways exist to convert potentially toxic concentrations of inorganic As to relatively non toxic arsenobetaine (AsB, $(CH_3)_3As^+CH_2COO^-$), the latter being identified by ion chromatography (IC)-ICP-MS as the major As species in a number of studies [30, 31]. Ion chromatography-ICP-MS has also been used to investigate the speciation of low molecular weight Se compounds, including Se(IV), Se(VI), Se-cystine (Se-cys) and Se-met [32]. Recently a number of multi-element speciation studies employing ICP-MS have been reported. IC-ICP-MS has been used for As, Se, Sb and Te speciation in a spiked fish CRM extract [33]; HG-ICP-MS has been used for determination of Ge, As, Se, Sn, Sb and Hg species in human urine after fish consumption [34]; and Se and As species in canned tuna fish have been determined by ion-pair chromatography coupled to ICP-MS [35]. The latter study identified AsB and Se-cys as the major As and Se species, respectively.

This study utilizes liquid chromatographic-ICP-MS techniques to examine trace element speciation in organs from largemouth bass (*Micropterus salmoides*) collected from a habitat receiving effluent from the D-area coal fly ash basins at the US DOE Savannah River Site in Aiken, SC. Previous studies conducted at this site have documented physiological and morphological abnormalities in fish, amphibians and reptiles inhabiting the ash basin area. Studies of tadpoles (*Rana catesbeiana*) collected from the ash basins have shown that there is a higher incidence of deformities in mouth structures [4, 36] and in axial malformations as compared to tadpoles collected from a reference site [8]. Fish exposed to sediments from the ash basin exhibited reduced growth rates, severe fin erosion and reduced swimming performance [37]. Concentrations of Se, As, Cr, Cd and Sr are increased in animals taken from the D-area ash basin system as compared to those collected from reference sites [5, 6]. Increased concentrations of As and Se are to be expected in fish exposed to fly ash. These elements occur at elevated concentrations in fly ash in comparison with average concentrations in soils and sediments [2] and both elements are relatively easily solubilized from fly ash [38]. Selenium muscle concentrations of 3 $\mu g\ g^{-1}$ have been reported for largemouth bass exposed to fly ash at a coal-fired power plant at a US Department of Energy facility in Tennessee [39, 40]. Increased Se concentrations have been reported for a number of fish species inhabiting fly ash disposal systems [7, 41]. Elevated Se, Cu, Cd and Zn concentrations in mullet (*Mugil cephalus*) exposed to fly ash discharges in Lake Macquarie, Australia have recently been reported [42].

We hypothesized that Se toxicity may be manifest by the incorporation of Se in proteins, which may affect structure and function; particularly the ability of cysteine rich proteins to sequester trace metals. The objectives of this study were to identify the major protein or non-protein fractions of Se, Cu, Cd, Zn and As in the various tissue extracts of an ash basin bass (AB bass) and compare these to a bass collected from a reference site (FP bass). The use of SEC coupled to ICP-MS allows data on multiple trace elements to be collected simultaneously and thus should help elucidate any interactions between trace elements within the various protein fractions.

Materials and methods

Instrumentation

Total trace element analysis, SEC-ICP-MS and IC-ICP-MS were conducted on an Elan 6100 dynamic reaction cell (DRC) ICP-MS (Perkin Elmer, Shelton, CT). For trace element analysis, the ICP-MS was operated in standard mode (i.e. no reaction gas) and optimization parameters autolens voltage and nebulizer gas flow were optimized daily for maximum signal intensity with a CeO/Ce<0.02. A cyclonic spray chamber and concentric nebulizer were used throughout total trace element and speciation analysis. For both SEC and IC a Dionex GP40 gradient pump (Sunnyvale CA) was used to supply the eluents and was connected to a Thermo Separations AS3500 programmable autosampler. The chromatography columns were connected directly to the concentric nebulizer of the ICP-MS with PEEK tubing. For SEC-ICP-MS the tissue extracts were analyzed twice, once with the ICP-MS operating in standard mode and then with the ICP-MS in DRC mode with CH_4 as the reaction gas. The latter configuration was used to improve sensitivity for Se by reducing Ar dimer formation thus allowing m/z 78 and 80 to be measured [43]. DRC-ICP-MS optimization parameters of cell gas flow and rpq were optimized for lowest detection limits, which is performed by a software algorithm that finds the optimum gas flow and rpq that provides the best combination of high sensitivity and low background noise. Two SEC columns were used; a BioSil 125 SEC column (BioRad, Hercules, CA; optimum separation range 7–100 KDa) and a Superdex HR 10/30 column (Amersham Pharmacia Biotech, Piscataway, NJ; optimum separation range 0.5–7 KDa). The eluent used for both SEC separations was 100 mM NH_4OAc/10 mM Tris-HCl at pH 7.2 (pH adjusted with conc. HCl), the flow rate was 1 mL min^{-1} and the sample injection

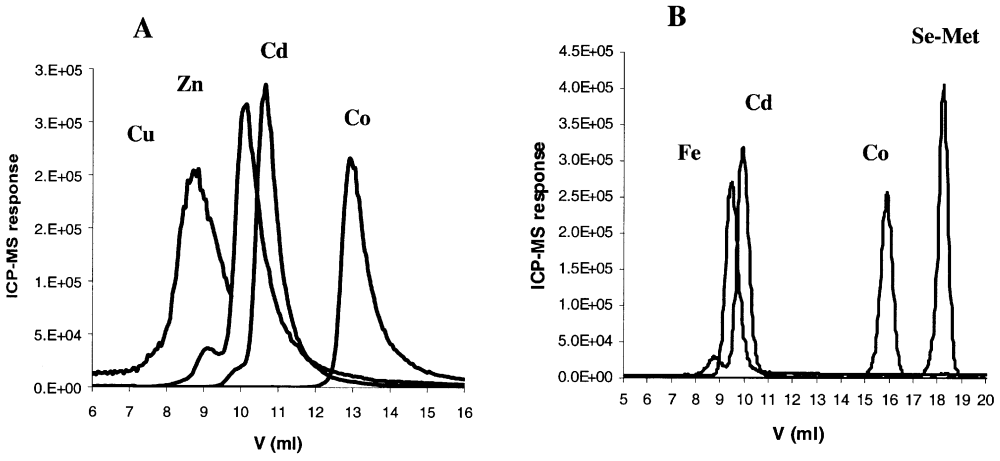


Fig. 1 SEC-ICP-MS chromatograms of metallo-protein standards. **A**. Biosil column showing separation of bovine serum albumin (66 KDa, Cu signal), carbonic anhydrase (29 KDa, Zn signal), metallothionein (10 KDa, Cd signal) and cobalamin (1.3 KDa, Co signal). **B**. Superdex column showing separation of cytochrome c (12.5 KDa, Fe signal), metallothionein (10 KDa, Cd signal), cobalamin (1.3 KDa, Co signal) and selenomethionine (196 Mw)

volume was 100 µL. Example chromatograms for the size exclusion columns generated by monitoring the metal of a pure metalloenzyme standard are shown in Fig. 1A and B.

Inorganic and LMW organic compounds of As and Se cannot be resolved by size exclusion chromatography; however, as many species of these elements occur as anions, they can be resolved by anion exchange chromatography. The separation of arsenite (As III), arsenate (As V), dimethyl arsenic acid (DMA) and AsB has previously been demonstrated on a As7 column (Dionex, Sunnyvale, CA) using a HNO_3 step gradient separation [44]. Using the gradient conditions of Mattusch and Wennrich [44], we attempted to separate Se(IV), Se(VI), Se-cystine (Se-cys) and Se-methionine (Se-met) simultaneously with the As species. However, under these conditions Se-cys did not elute from the column. By including a further gradient step to 300 mM HNO_3 after elution of AsB, Se-cys did elute at retention time of 15.9 min. Fig. 2 shows the separation of standard solutions of the four As and four Se species each at concentration of 10 µg L^{-1} (as either As or Se) and Table 1 provides the conditions of this separation. Monomethyl arsenic acid (MMA) was incompletely resolved from As(III) under these separation conditions. Approximate detection limits (as either Se or As) based on 3σ of baseline noise using a peak height calibration were 20 ng L^{-1} for Se(IV) and Se(VI), 50 ng L^{-1} for Se-M and SeC, 30 ng L^{-1} for As(III) and DMA and 60 ng L^{-1} for As (V) and AsB. IC-ICP-MS was conducted in DRC mode only with CH_4 as reaction gas, a cell gas flow of 0.4 mL min^{-1} and rpq value of 0.4. DRC mode was not necessary for As speciation. However, reasonable detection limits were achieved for As species using the DRC, hence both Se and As were analyzed simultaneously using DRC-ICP-MS as the detector. During sample analysis a number of non-Se interfering peaks were observed at m/z 80 (possibly BrH) and so m/z 78 data is presented for Se in all the chromatograms. Anion exchange chromatography was performed on ethanol:chloroform tissue extracts (see sample preparation section) and an injection volume of 100 µL was used throughout.

Reagents

Protein standards used for calibration of the SEC columns were bovine serum albumin (66 KDa), carbonic anhydrase (29 KDa), cytochrome C (12KDa), metallothionein (~10 KDa) and cobalamin (1.3 KDa) These compounds were obtained from Sigma Chemicals (St Louis, MO) and were prepared by dissolving in deionized H_2O (18 MΩ-cm) and were subsequently stored at –70 °C. Eluent for the SEC-ICP-MS was 100 mM NH_4OAc/10 mM Tris-HCl that was prepared from reagent grade salts (Fisher Scientific, Fairlawn, NJ). The pH of the eluent was adjusted to 7.2 using trace metal grade HCl (Fisher Scientific, Fairlawn, NJ). For IC-ICP-MS As and Se standard solutions sodium arsenite (GFS Chemicals, Columbus, OH), sodium cacodylate, $(CH_3)_2As(O)ONa$, (Sigma, St. Louis, MO), sodium arsenate (Fisher Scientific, Fairlawn, NJ), arsenobetaine (Sigma, St. Louis, MO), sodium selenate (Fisher Scientific, Fairlawn, NJ), sodium selenite (Fisher Scientific, Fairlawn, NJ), selenomethonine (Sigma, St. Louis, MO) and selenocystine (Sigma, St. Louis, MO), were used to prepare stock solutions of each As and Se species in deionized water. Monomethyl arsenic acid (Crescent Chemicals Haupage, NY) was obtained as 100 mg L^{-1} as MMA in methanol. Individual stock solutions of each As or Se species were prepared in deionized water. In general, stock solutions were prepared at concentrations of at least 100 mg L^{-1} As or Se except Se-cystine, which was prepared at a concentration of 60 mg L^{-1}. Individual calibration standards at concentrations of 10–50 µg L^{-1} were prepared daily by serial dilution and analyzed to check that no species transformations had occurred and that retention times had not shifted.

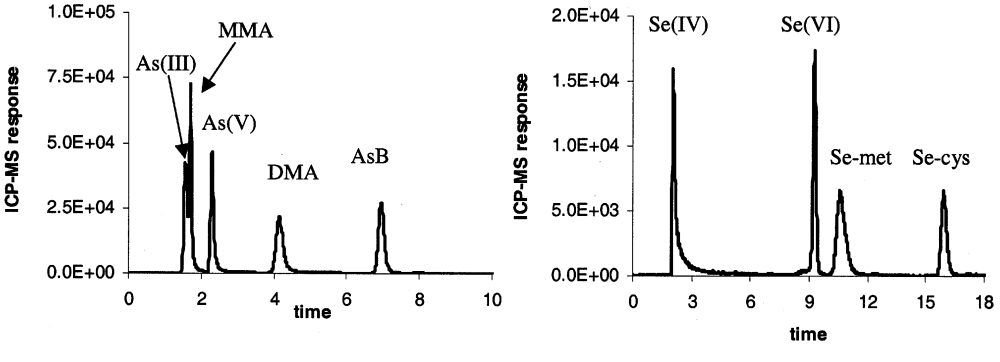


Fig. 2 IC-ICP-MS chromatogram for separation of arsenite (AsIII), arsenate (AsV), dimethyl arsenate (DMA) and arsenobetaine (AsB) concurrent with selenite (SeIV), selenate (SeVI), selenomethionine (Se-met) and selenocystine (Se-cys)

Table 1 Gradient conditions for separation of As and Se species shown in Fig. 2 on the AS7 column

Time (min)	A (DI H_2O) (%)	B (300 mMHNO_3) (%)	Flow rate (mL min^{-1})
Initial	95	5	1
0.00	95	5	1
2.49	95	5	1
2.50	83.2	16.8	1
10.99	83.2	16.8	1
11.00	0	100	2
16.00	0	100	2

Sample preparation and tissue extraction

Female largemouth bass were collected using hook and line from the D-area ash basin system (AB bass; 286 g wet weight, 392 mm total length) and from Fire Pond (FP bass; 876 g wet weight, 420 mm total length), which is a reference site unaffected by fly ash effluent. The fish were returned to the lab alive and killed within 3 h of collection. They were then dissected and samples of liver, gonad, gill and muscle were flash frozen in liquid N_2 and subsequently stored at –70 °C.

Prior to tissue homogenization, the samples were thawed and approximately 300 mg tissue (wet wt.) was homogenized in 3 mL of 100 mM NH_4Oac, 10 mM Tris-HCl pH 7.2 (adjusted with trace metal grade HCl) to which 15 µL of protease inhibitor cocktail (Sigma, St Louis, MO) had been added. The homogenate was then centrifuged at 16 000 rpm for 20 min. A sample preparation methodology used for purifying the metallothionien proteins from a tissue extract was also used [45]. High molecular weight proteins were precipitated from an aliquot of the homogenate by slowly adding an equal volume of 96% ethanol:chloroform (1.08:0.08 v/v) pre chilled to –20 °C. The precipitate was then removed by centrifugation at 16,000 rpm for 20 min.

Total trace element analysis

Samples of gill, gonad, liver and muscle tissue (≈200 mg wet wt.) were acid digested using conc. HNO_3 and H_2O_2 in a microwave digestion procedure and subsequently brought to a volume of 25 mL with deionized water (18 MΩ-cm). Each tissue was analyzed in triplicate and blanks and certified reference materials (DOLT-2; NRC-CNRC, Ontario, Canada) were digested and analyzed as quality control samples. Recoveries were acceptable for Cu, Se and As (96%, 88% and 85%, respectively). Recoveries for Cd and Zn were somewhat lower (82 and 76% respectively), which may be due to matrix suppression in this SRM because an external calibration was used. The triplicate tissue samples were individual dissected sub-samples of the particular organ/tissue (i.e. not replicates from homogenized tissue), consequently variability was fairly high, with an average RSD of 20% for the five elements listed above. However, this is not unexpected given the variability in total trace element concentration within a particular organ.

Results and discussion

Total trace element analysis

Mean trace element concentrations of various tissue and organ samples for the fish from the AB site and the reference (FP) site are shown in Table 2. Increased concentrations for As and Se were observed in the AB bass for all examined tissues. Increased concentrations of Co were found in gill, gonad and liver of AB bass. Greater concentrations of V, Cu and Cd were observed in the gill tissue of AB bass. Iron concentrations in the liver of the FP bass were higher than the AB bass, while Mn was higher in AB bass. The Se muscle concentration reported in this study is comparable to other studies of largemouth bass exposed to fly ash [39, 40]. Similarly, high liver concentrations of Se and Cu and lower muscle concentration have been reported for fish exposed to fly ash [42].

Size exclusion chromatography

Size exclusion chromatograms of liver, gonad, gill and muscle tissue extracts are shown in Figs. 3, 4, 5 and 6, respectively. The retention volume (Vr) for MT on the biosil column is 10.65 (Fig. 1A, Cd signal) and metal-specific MT peaks are clearly evident at this retention time in the liver (Fig. 3) and gill (Fig. 5) and, to a lesser extent, the gonad tissue extract (Fig. 4), as indicated by peaks for Cu, Zn and Cd. On a total element basis (Table 2) there were no significant differences in Zn and Cu liver concentrations between the two fish and the SEC chromatograms for these two elements were also very similar. Metallothionein predominantly binds Cu in the liver and gill. While MT is also a major metallo-protein binding Zn, a significant percentage of Zn is bound by HMW species in the liver and LMW species in the gill. Although total Cd in

Table 2 Mean total trace element concentration in tissues of ash basin and reference fish, n=3

	V	Cr	Mn	Fe	Co	Ni	Cu	Zn	As	Se	Sr	Cd	Pb
	mg kg^{-1} wet wt												
AB bass													
Gill	0.11	0.32	1.24	50.3	0.17	0.88	1.1	15.7	0.19	3.97	4.46	2.46	0.21
Gonad	0.13	1.60	3.41	33.8	0.25	1.18	2.35	43.8	0.13	7.04	1.19	0.01	0.05
Liver	0.26	0.27	4.63	196	0.45	0.24	8.8	30.1	0.54	7.45	0.38	0.59	0.02
Muscle	0.05	0.21	0.15	5.53	0.03	0.26	0.29	3.96	0.08	3.01	0.13	b.d.l	0.01
FP bass													
Gill	0.05	0.33	1.69	67.7	0.07	0.20	0.42	10.4	0.02	0.37	0.28	0.02	0.07
Gonad	0.12	0.38	3.60	43.6	0.08	0.09	2.21	47.7	0.05	1.05	0.306	0.01	0.03
Liver	0.20	0.26	1.56	948	0.21	1.27	10.9	27.4	0.06	0.97	1.41	0.85	0.03
Muscle	0.05	0.16	0.11	5.04	0.02	0.12	0.18	3.59	0.01	0.18	0.093	b.d.l	0.08

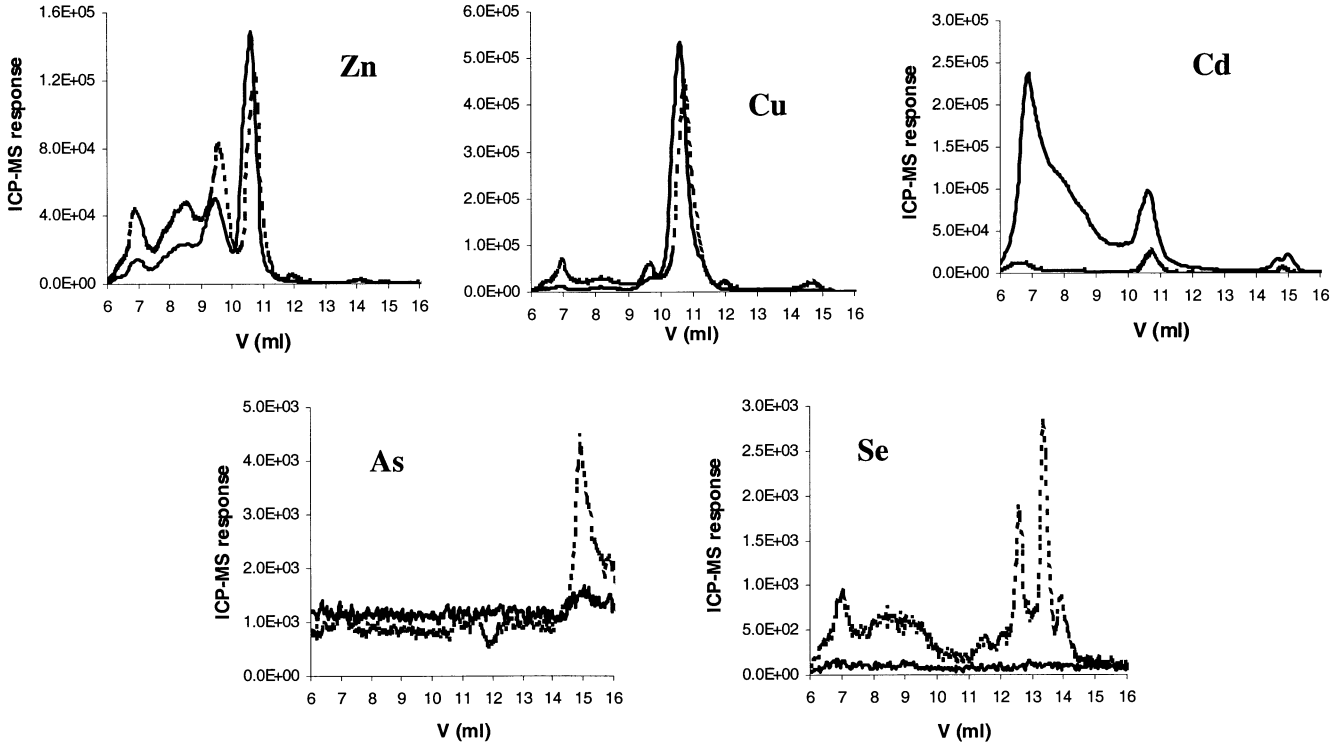
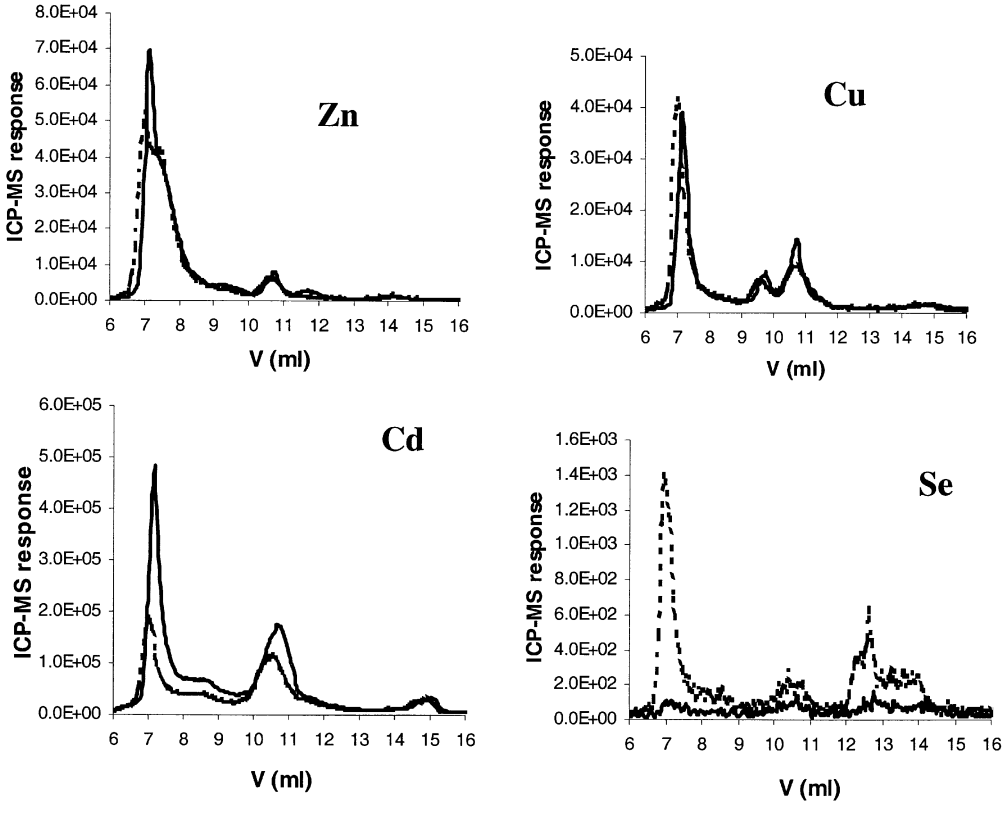


Fig. 3 SEC ICP-MS chromatograms for liver extract on biosil SEC column. *solid line* FP bass (reference), *dashed line* AB bass (ash basin)

Fig. 4 SEC ICP-MS chromatograms for gonad extract on biosil SEC column. *solid line* FP bass (reference), *dashed line* AB bass



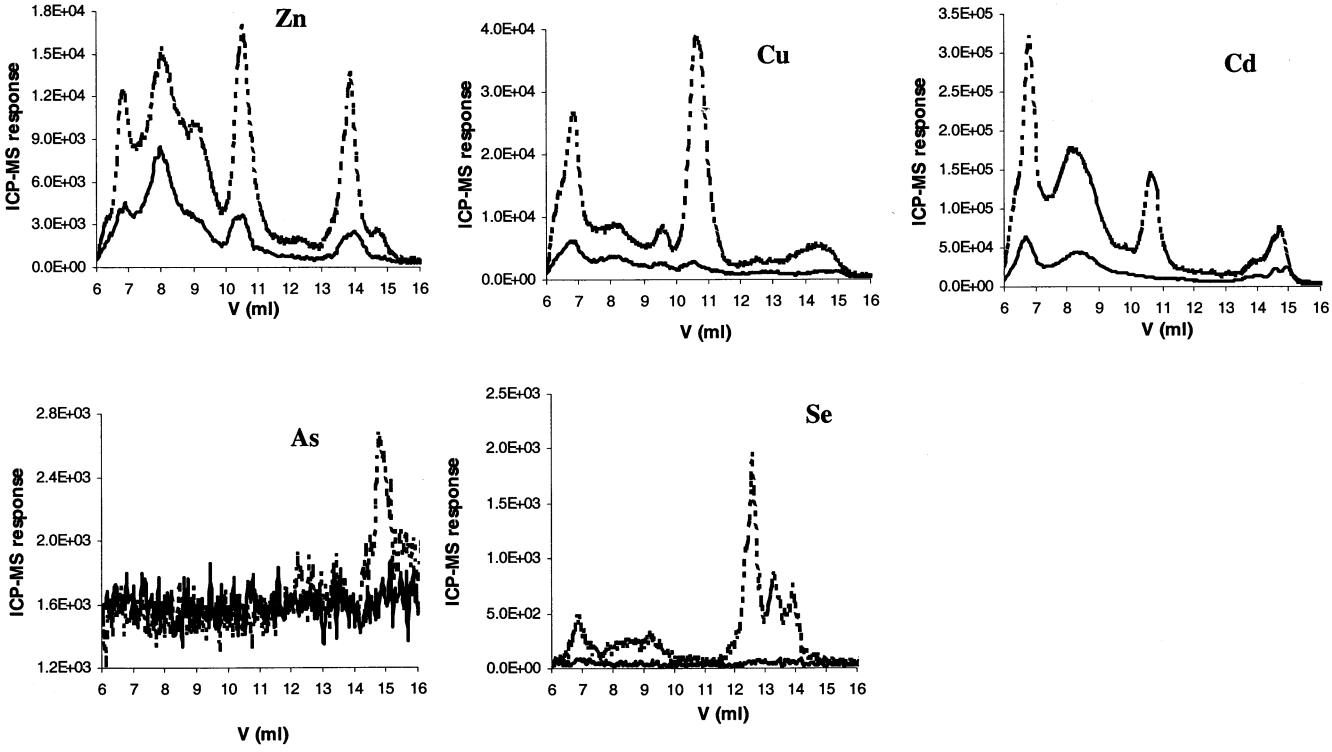
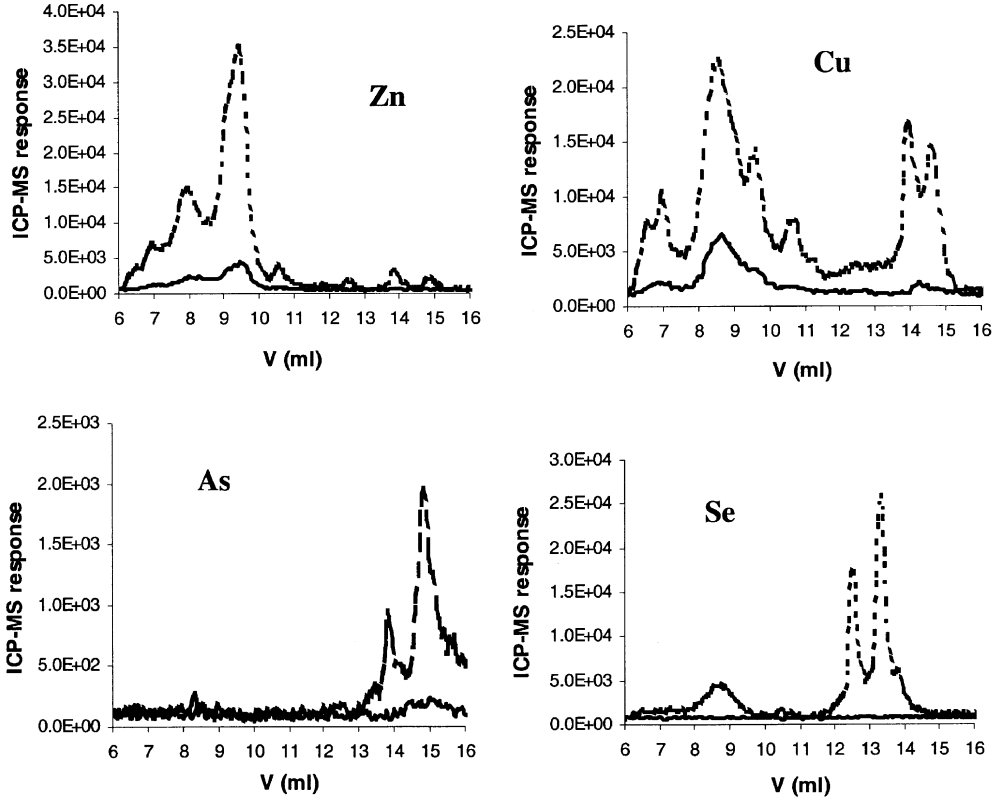


Fig. 5 SEC ICP-MS chromatograms for gill extract on biosil SEC column. *solid line* FP bass (reference), *dashed line* AB bass (ash basin)

Fig. 6 SEC ICP-MS chromatograms for muscle extract on biosil SEC column. *solid line* FP bass (reference), *dashed line* AB bass



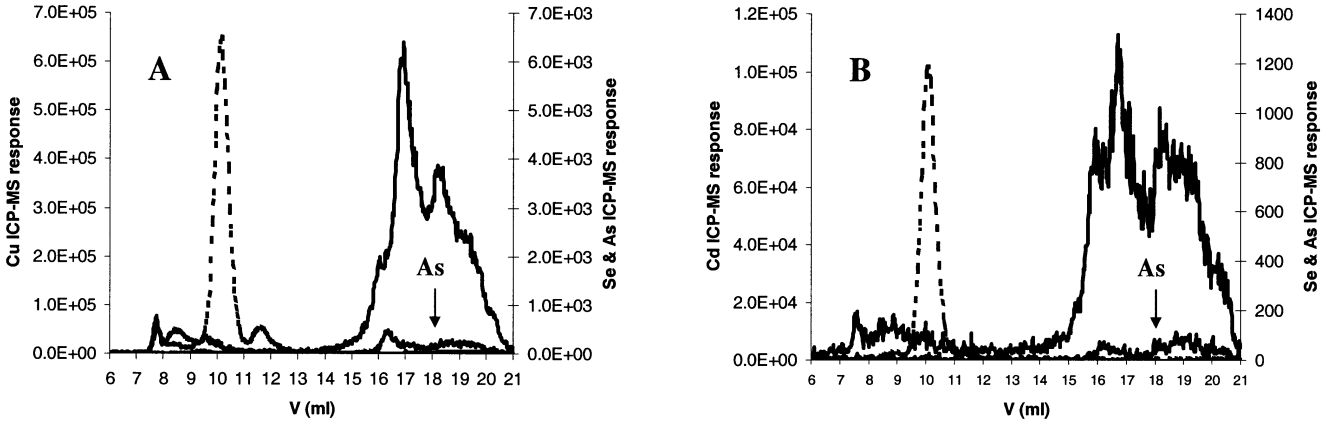


Fig. 7 SEC ICP-MS chromatograms for A. liver extract on superdex SEC column. *dashed line* Cu signal, *solid line* Se (and As) signal. B. gill extract *dashed line* Cd signal, *solid line* Se (and As) signal

the liver of FP bass was not significantly different than the AB bass, much greater concentrations of Cd in the FP bass extract were evident in the SEC-ICP-MS chromatogram. It is possible that Cd in AB bass liver was present as an inorganic species that was retained by the stationary phase of SEC column. However, it is also possible that exposure to both Se and Cd in AB bass prevented the accumulation of Cd in liver proteins. A similar finding has been reported for mussel hepatopancreas, where exposure to Cd and Se reduced protein-bound Cd compared with exposure to an equivalent concentration of Cd alone [25]. Metallothionein bound a small proportion of Cd in the gonad, gill and liver. The majority of Cd was bound in HMW fractions with a small but detectable amount eluting near the total volume of the SEC column, indicative of LMW species. The significantly higher total Cd concentration in the AB bass gill was reflected in the SEC-ICP-MS analysis of the tissue homogenate (Fig. 5), where the major fraction of Cd was bound in HMW compounds. Higher concentrations of Zn, Cu and Cd in the gill presumably reflect exposure to higher water column concentrations of these elements for AB bass.

Selenium was detected by SEC-ICP-MS in all tissue extracts and consistently occurred as 2 or 3 LMW peaks eluting near the total volume of the column. In gill, liver and muscle these LMW Se species were the predominant fraction. However, for the gonad the major Se species (70% of total extracted Se) occurred as a HMW species with a Vr of 7, corresponding to a Mw of 400 KDa. Although Cu, Cd and Zn also co-eluted at this peak, this is near the exclusion volume of the column and thus may be the result of a number of HMW compounds eluting at this Vr. Arsenic was detected in the liver, gill and muscle homogenates of the AB bass and, in each case, eluted at a Vr that would indicate that it was present as a LMW species.

A previous study has identified both HMW and LMW seleno-compounds in edible fish muscle tissue [22]. In that study, cod, salmon, rainbow trout and eel had most Se (76–88%) in compounds >10 KDa, whereas the flat fish species turbot, flounder and dab had 63–72% of Se in compounds with Mw≤10 KDa. The results of our study for Se in muscle tissue are more consistent with the flat fish distribution of Se with >85% occurring with Mw≤10 KDa.

However, the results of this study show little evidence for Se-metal interactions in fish protein fraction. Although strong MT peaks are evident in the SEC of liver, gill and gonad extracts of AB bass, Se does not co-elute at this Vr. Selenium was present predominantly as LMW compounds that were smaller than the resolving power of the BioSil column. Only in the gonad tissue extract was Se predominantly present as a HMW compound and in this case Se co-eluted with Cd, Cu and Zn.

To further elucidate the speciation of As and Se, tissues samples of the liver and gill were homogenized and larger proteins precipitated using ethanol:chloroform [45]. Aliquots of these extracts were then injected onto a Superdex peptide HR 10/30 column that has an effective resolution range of 0.5–7 KDa, with an effective exclusion limit of ca. 20 KDa. An example chromatogram of standard metallo-proteins and selenomethionine is shown in Fig. 1B. The resulting SEC-ICP-MS chromatograms for the ash basin fish liver and gill are shown in Fig. 7. Metallothionein is clearly evident in both the liver and gill extracts as evidenced by the Cu and Cd peaks, respectively, in Fig. 7. However, Se and As elute very near the total volume of the column, indicating that they are present as LMW species. In the liver extract there is some evidence of small Se peak eluting at Vr 9.6 equivalent to an Mw 13 KDa, but clearly the major proportion of Se in both extracts are of lower Mw than the resolving power of the column.

Anion exchange chromatography

Based on the evidence from SEC analysis it was apparent that As and Se in the liver and gill extract were predominantly LMW species, possibly inorganic species. Both Se and As exhibit varied speciation within this molecular weight range in biological samples. In addition to the inorganic Se species the amino acids Se-cysteine and Se-met have been detected in biological samples along with a number of other organo-Se compounds. The conversion of potentially toxic inorganic As compounds, arsenite (As

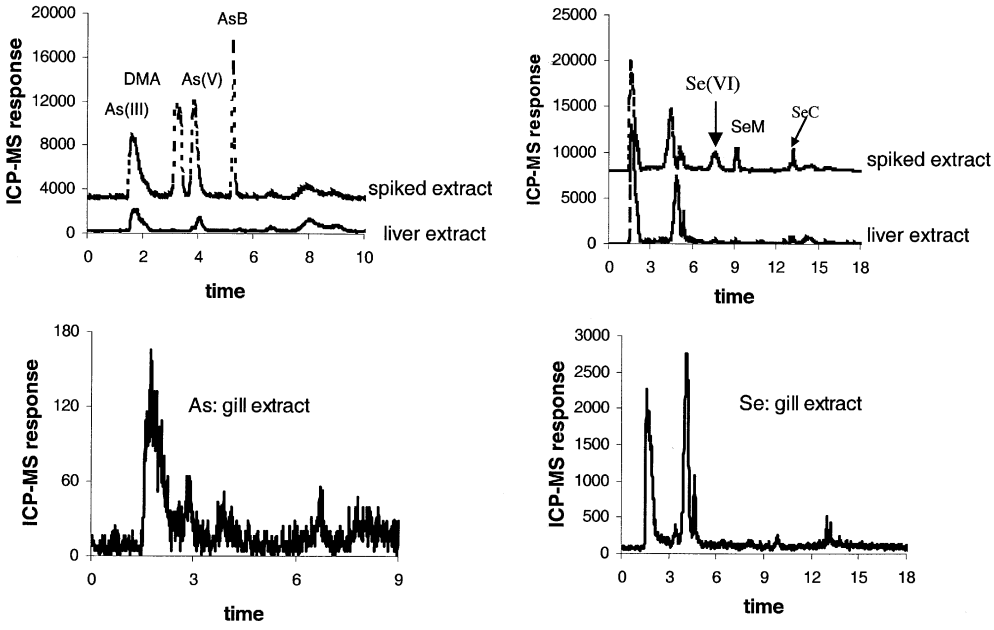


Fig. 8 As and Se IC-ICP-MS chromatograms for AB bass liver and gill extracts. Spike in liver extract was 10 μg L^{-1} As(III), As(V), DMA, AsB, Se(IV), Se(VI), Se-met, Se-cys

III) and arsenate (As V), to the non-toxic species, AsB, is well documented for shellfish and salt water fish species [30, 31]. In an attempt to identify the As and Se species present in the ethanol:chloroform solutions of the AB bass liver and gill, IC-ICP-MS was employed. Using the gradient separation specified in Table 1 it was possible to separate As(III), As(V), DMA and AsB simultaneous with separation and detection of Se(IV), Se(VI), Se-met and Se-cys (the diselenide dimer of Se-cysteine). An example chromatogram of the separation is shown in Fig. 2. The IC-ICP-MS chromatograms of the liver and gill are shown in Fig. 8. Multiple As and Se peaks are evident in both chromatograms; however, these peaks do not correspond to retention times for the standard compounds available to us. Because the sample matrix might have affected retention times of the As and Se species, a standard solution mix of As and Se species was run in the buffer/ethanol:chloroform solution and the liver extract was also spiked with the standard As and Se species mix and reanalyzed. The latter spiked sample chromatograms are shown in Fig. 8. Some retention time changes were evident in the buffer/ethanol:chloroform, specifically, DMA and Se(IV) retention times were increased compared with the aqueous standard (data not shown) while Se-cys retention time decreased. Arsenite is not retained by the AS7 column and elutes in the void volume at 1.6 min. Although a peak is observed at this retention time in both the liver and gill extracts it should be noted that any non-retained As species will elute at the void volume of the column. For example, cationic species such as tetramethylarsonium and arsenosugars have been identified in biological samples [46, 47, 48] and these compounds may elute in the void volume under the low pH conditions employed in our work. There was a small peak in the liver extract that was consistent with the retention time for As(V), but the other broad eluting peaks in the liver sample with retention times between 6–10 min. were not identified. Arsenobetaine has been identified as being the predominant As species in shellfish [31] and salt-water fish species [30], however, AsB was not present in either the liver or gill extracts of the AB bass.

Ion chromatography of extracts also failed to identify the main Se species in the liver and gill extracts. Numerous Se species were detected and, although relative intensity of the peaks differed between liver and gill, the retention times of the major peaks were identical between the two tissue extracts, suggesting that, although unidentified, the main seleno-species in the liver and gill extracts were identical. A major portion of the Se in both extracts eluted at the void volume of the column suggesting that this may be a cationic or neutral species. The second major peak in both the liver and gill extract had an elution time of 4.9 min. and did not match any of the Se standards. However, this peak was shifted in the spiked extract and appeared to co-elute with the spiked Se (IV), which did not occur in the spike chromatogram as a discrete peak. Small peaks were evident in the liver extract with identical retention times to Se-met, Se(VI) and Se-cys.

Conclusions

Selenium and As were elevated in liver, gill, gonad and muscle of a fish exposed to coal fly ash compared with a fish collected from a reference site. Size exclusion chromatography-ICP-MS revealed that As and Se were present as LMW species in extracts of these tissues. Metallothionein and the co-associated metals Cu, Cd and Zn, were identified in the liver, gonad and gill extracts but Se was not associated with this protein. Only in the gonad did Se appear to be associated with a HMW protein that co-eluted with Zn, Cu and Cd. The LMW species of As

and Se were not categorically identified by IC-ICP-MS employing standard inorganic and LMW organic As and Se compounds. Further studies examining different vertebrate species, and utilizing LC-FT-MS to characterize the As and Se species, are on-going.

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