TRACE METAL AND METALLOID ACCUMULATION, DISTRIBUTION, AND, SPECIATION IN LAKE MACQUARIE, N.S.W, AUSTRALIA.

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CERTIFICATE OF AUTHORSHIP

Except where clearly acknowledged in footnotes, quotations and bibliography, I certify that I am the sole author of the thesis submitted today entitled -

Trace Metal and Metalloid Accumulation, Distribution, and Speciation in Lake Macquarie, N.S.W, Australia.

I further certify that to the best of my knowledge the thesis contains no material previously published or written by another person except where due reference is made in the text of the thesis. The material in the thesis has not been the basis of an award of any other degree or diploma except where due reference is made in the text of the thesis.

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In conclusion, I would like to acknowledge that the research undertaken in this thesis would not have been possible without the financial support of the Ecochemistry Laboratory at the University of Canberra.

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PUBLICATIONS RELATING TO THIS THESIS

Kirby J, Maher W, Spooner D.

Arsenic Occurrence and Speciation in Near Shore Macro - Algae -Feeding Marine Animals.

Accepted for publication in Environmental Science and Technology on the 1st May 2005.

Kirby J, Maher W, Ellwood M, Krikowa F.

Arsenic Species Determination in Biological Tissues by HPLC-ICP-MS and HPLC-HG-ICP-MS.

Australian Journal of Chemistry, v57 (2004) pp 957-966.

Maher W, Krikowa F, **Kirby J**, Townsend A, Snitch P.

Measurement of Trace Elements in Environmental Samples using Solution ICP-MS: Current and Future Applications.

Australian Journal of Chemistry, v56 (2003) pp 103-116.

Kirby J, Maher W.

Measurement of Water-Soluble Arsenic Species in Freeze-Dried Marine Animal Tissues by Microwave-Assisted Extraction and HPLC-ICP-MS.

Journal of Analytical Atomic Spectrometry, v17 (2002) pp 838-843.

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Arsenic Concentrations and Speciation in a Temperate Mangrove Ecosystem, N.S.W, Australia.

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Tissue Accumulation and Distribution of Arsenic Compounds in Three Marine Fish Species: Relationship to Trophic Position.

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Archives of Environmental Contamination and Toxicology, v 40 (2001) pp 246-256.

Maher W, Goessler W, **Kirby J**, Raber G.

Arsenic Concentrations and Speciation in the Tissues and Blood of Sea Mullet (*Mugil cephalus*) from Lake Macquarie NSW, Australia.

Marine Chemistry v 68 (1999) pp 169-182.

THESIS ORGANISATION

This thesis is organised into nine chapters that include seven international and national publications (six accepted and one submitted for publication). The initial overview chapter outlines the justification and direction for this thesis. With the exception of chapter 8 (accepted for publication on the 1st May 2005); all chapters are exact duplicates of published articles in international and national refereed journals (chapters 2 to 7). The initial chapters (2 and 3) presents research findings using a marine fish species, mullet *(Mugil cephalus*), to measure trace metal bioavailability in Lake Macquarie, NSW Australia. While subsequent chapters (4 to 8) are presenting research under taken to improve the understanding of arsenic cycling in marine and estuarine environments. The final chapter (chapter 9) is a synopsis of the major findings presented in this thesis. Due to the publication nature of this thesis, an unavoidable degree of replication exists within chapters (publications).

CHAPTER 1

Study Overview

This thesis presents the findings from research undertaken to extend the understanding of trace metal and metalloid accumulation, distribution, and cycling in marine environments. The research in this thesis had two main objectives:

1) To use a benthic feeding marine fish species, mullet (*Mugil cephalus*) to determine the bioavailability of trace metals and metalloids in Lake Macquarie, N.S.W, Australia; and
2) To extend the understanding of the cycling of arsenic compounds in the marine environment.

Lake Macquarie is a large estuarine barrier lake situated near the city of Newcastle, NSW, Australia (Figure 1, page 24). Industrial development to the northern reaches of the lake have been extensive with a lead - zinc smelter, fertiliser plant, steel foundry, collieries (coal mining and storage area) and sewage treatment works (Batley 1987). The lead - zinc smelter that commenced operation in 1897, has resulted in the contamination of the main northern fluvial input (Cockle Creek) with lead, cadmium, zinc, and copper (SPCC 1983). In the southern reaches the lake has two coal - fired power stations, with electric power generated from burning coal at Eraring and Vales Point, and previously at Wangi Wangi. Overflow from ash - dams and stack emissions associated with coal - fired power station practices have been shown to be contributing trace metal and metalloid contamination, especially selenium to the lake (Davis and Linkson 1991). The sediments near these power stations contain fly - ash that has been shown to be elevated in selenium, copper, and zinc (Batley 1987; Swaine 1982, 1985; Davies and Linkson 1991; Crawford *et al.* 1976).

The initial research projects in this thesis were undertaken to gain an understanding of the bioavailability of selenium, cadmium, zinc, and copper in Lake Macquarie, NSW. Mullet (*Mugil cephalus*) a benthic feeding detritivore fish was selected as an indicator species to determine the bioavailability of selenium, cadmium, zinc, and copper because they are known to be a resident species in Lake Macquarie for approximately

three years, they are abundant, easy to collect, and have the potential to be exposed to contamination through both water and dietary pathways.

At the end of 1995, improved fly - ash handling procedures were implemented by the Vales Point power station (Peters *et al.* 1999; Harston 1996). Before this time, fly - ash produced from power generation activities was mixed with lake water and pumped to a nearby ash dam, which drained directly into Wyee Bay via Mannering Bay (Figure 1, page 24). More than 80 % of the water - soluble selenium originally present in the fly - ash was found in the effluent from the ash dam corresponding to a flux of 1.53 ± 0.24 kg day^{-1} (Davies and Linkson 1991). In 1995, these practices were changed to recycle water from the ash dam back to the power station, where it is mixed with cooling water before being discharged into the bay. These practices were expected to raise selenium concentrations in the ash dam, but reduce the amount of suspended and dissolved trace metals and metalloids reaching the lake (Peters *et al.* 1999). We again sampled mullet (*Mugil cephalus*) from the southern basins of Lake Macquarie in 1997 to determine if these improved practices by the Vales Point power station had significantly reduced the bioavailable fraction of selenium, cadmium, copper, and zinc in Lake Macquarie.

The initial projects in this thesis found selenium and to a lesser extent copper to be a major contaminant in the southern basins of Lake Macquarie (Kirby *et al.* 2001a, b). Further studies into selenium speciation did not occur in this thesis due to extensive research already occurring in this area at Lake Macquarie (Peters *et al.* 1999; Harston 1996; Harasti 1997; Maher *et al.* 1992; Maher *et al.* 1997; Deaker and Maher 1995). It was decided to examine arsenic contamination in Lake Macquarie because like selenium this metalloid has been found to be a major contaminant to some terrestrial and aquatic environments from power station activities (Wilhelm *et al.* 2005; Staub *et al.* 2004; Mardon and Hower 2004; Keegan *et al.* 2002; Nerin *et al.* 1994). There are both natural (mostly associated with sulfide minerals e.g. orpiment As_2S_3, realgar AsS, mispickel FeAsS, loellingite $FeAs_2$, cobaltite CoAsS, tennantite $Cu_{12}As_4S_{13}$, and enargite Cu_3AsS_4) and anthropogenic (smelting, coal combustion, and manufacturing e.g. fungicides) sources of arsenic in the environment that have the potential to affect both human and environmental health. Arsenic is a known carcinogen and mutagen

that poses serious human and animal health risks (Mandal and Suzuki 2002). The burning of coal by power stations has been found to be a major source of trace metal and metalloid contamination to the environment, especially for selenium and mercury (Roe *et al.* 2004; Lohner *et al.* 2001; Kirby *et al.* 2001a, b; Pinkney *et al.* 1997). In general, coal contains low arsenic concentrations (< 5 mg kg^{-1}); however, the large amount consumed by power stations makes it a major source of anthropogenic arsenic in the environment (Ng *et al.* 2003). The combustion of coal has been suggested to account for 2 - 5 % of the total arsenic emissions from anthropogenic sources globally (Pacyna and Nriagu 1988).

The measurement of total trace metal and metalloid concentrations has increasingly been recognised as insufficient in providing essential information to understanding bioavailability, mobility, transport, and toxicity in aquatic environments (Batley *et al.* 2004; Caruso and Montes - Bayon 2003; Michalke 2003; Caruso *et al.* 2003; Gong *et al.* 2002). It is now recognised that a detailed knowledge of the diversity of elemental compounds (species), primarily their oxidation states and organometallic forms is essential to understand chemical and biochemical process in the environment (Batley *et al.* 2004; Caruso and Montes - Bayon 2003; Michalke 2003; Caruso *et al.* 2003; Gong *et al.* 2002).

Since the discovery of arsenobetaine in the Western Rock Lobster (*Panulirus cygnus*) by Edmonds *et al.* (1977) there has been continued research and debate into determining the pathways of arsenic in aquatic environments. Arsenic in marine animal tissues has been identified as inorganic (i.e. arsenous and arsenic acids), simple methylated (i.e. dimethylarsinic acid and methylarsonic acid) and complex organic compounds (i.e. arsenobetaine, arsenocholine, tetramethylarsonium ion, trimethylarsoniopropionate, trimethylarsine oxide, and arsenoriboses) (Francesconi and Edmonds 1993; Edmonds *et al.* 1997; Goessler *et al.* 1997; Maher *et al.* 1999; Larsen *et al.* 1993; Kirby and Maher 2002; Kirby *et al.* 2002). The presence of individual arsenic compounds in marine animals will depend on the species, their trophic position and tissue analysed (Maher *et al.* 1999; Kirby and Maher 2002; Kirby *et al.* 2002). An understanding of arsenic compounds in the marine environment is important in assessing their human toxicity (i.e. ingestion) and their potential affect on animal growth, reproduction and survival.

The determination of arsenic compounds in environmental samples requires the use of highly selective and sensitive techniques, such as hydride generation - atomic florescence spectrometry, capillary zone electrophoresis - inductively coupled plasma - mass spectrometry, gas chromatography - mass spectrometry and high performance liquid chromatography - inductively coupled plasma - mass spectrometry (Goessler and Kuehnelt 2002; Karthikeyan and Hirata 2003; Burguera and Burguera 1997; Sarzanini and Mentasti 1997). In the last decade, high performance liquid chromatography - inductively coupled plasma - mass spectrometry has become the procedure of choice for the determination of arsenic compounds in environmental samples (Maher *et al.* 2003; Gong *et al.* 2002; Szpunar *et al.* 2000; Sarzanini and Mentasti 1997). This hyphenated technique has gained wide spread acceptance because of its high selectivity (especially when analysing complex matrixes) and low detection limits (µg l^{-1} to ng l^{-1}). The successful quantitation of all arsenic compounds present in biological extracts has not been achieved using one chromatography run but requires the use of complimentary separation techniques such as reverse phase, anion and cation exchange chromatography and column eluant conditions incorporating different buffer compositions and pH (Geiszinger *et al.* 2002; Kirby *et al.* 2004; Raber *et al.* 2000).

The following are specific study objectives for this thesis:

Biomonitoring Trace Metal Bioavailability in Lake Macquarie, N.S.W.

Study 1

In this study, selenium, cadmium, copper, and zinc concentrations were measured in surficial sediments from the southern basins of Lake Macquarie to determine if contamination was occurring at these locations. The bioavailability of selenium, cadmium, copper, and zinc concentrations in the southern basins of Lake Macquarie were determined using the benthic feeding fish, mullet (*Mugil cephalus)*. Trace metal concentrations in mullet (*Mugil cephalus*) tissues at Lake Macquarie were compared with the same fish species collected at a relatively unpolluted environment on the

south coast of N.S.W and with published literature**.** This data was used to assess threats to human and animal health; and to establish a baseline against which catchment pollution management initiatives can be assessed.

Specific Objectives

- To measure selenium, cadmium, copper, and zinc concentrations in surficial sediments from the southern basins of Lake Macquarie to determine if contamination had occurred at these locations;
- To determine the bioavailability of selenium, cadmium, copper, and zinc in Lake Macquarie using the benthic feeding fish, mullet (*Mugil cephalus)*; and
- To gain an understanding of factors that may affect the accumulation and tissue distribution of selenium, cadmium, copper, and zinc concentrations in the benthic feeding fish, mullet (*Mugil cephalus).*

Study 2

In the previous study, selenium concentrations in mullet (*Mugil cephalus*) muscle tissues were found to be higher than that recommended for safe human consumption (Kirby *et al.* 2001a). Selenium and copper concentrations in mullet (*Mugil cephalus*) tissues were also found at levels likely to have an affect on fish health (Kirby *et al.* 2001a). These elevated selenium and copper concentrations in mullet (*Mugil cephalus*) tissues were attributed to power generation activities and the ingestion of contaminated fly - ash.

In 1995, improved fly - ash handling procedures were implemented at the Vales Point power station in the southern reaches of Lake Macquarie (Peters *et al.* 1999; Harston 1996). Mullet (*Mugil cephalus*) were again sampled from the southern basins of Lake Macquarie to determine if these improved practices by the Vales Point power station had significantly resulted in lower selenium, copper, cadmium, and zinc concentrations in mullet (*Mugil cephalus*) tissues. In addition, we also report findings on the influence of age and gender on bioaccumulation; and verify the relative independence of selenium, cadmium, copper, and zinc with mass.

Specific Objectives

- To determine if improved power station practices at Vales Point power station in 1995 have significantly reduced selenium, cadmium, copper, and zinc concentrations in mullet (*Mugil cephalus)* tissues from the southern basins of Lake Macquarie; and
- To determine the influence of age and gender on the bioaccumulation of selenium, cadmium, copper, and zinc concentrations in mullet (*Mugil cephalus)* tissues.

Arsenic Speciation

Study 3

In general, the majority of arsenic in biological tissues is extracted in the water - soluble fraction. The use of solvents such as water, methanol - water and methanol - chloroform with mechanical agitation, sonication, microwave, and accelerated solvent techniques are traditionally used to extract water - soluble arsenic compounds in biological tissues (Goessler *et al.* 1997; Maher *et al.* 1999; Larsen *et al.* 1993; Geiszinger *et al*. 2002; Londerborough *et al.* 1999; McKiernan *et al.* 1999). These techniques generally show high arsenic extraction efficiencies from muscle tissues (e.g. NRC - CNRC dogfish muscle - 2); however, lower recoveries for other tissues (e.g. liver, hepatopancreas and digestive) and whole animals (e.g. oyster and mussels) (Maher *et al.* 1999; Larsen *et al.* 1993; Kirby and Maher 2002; Kirby *et al.* 2002; Kuehnelt *et al*. 2001; Goessler *et al.* 1998; Angeles Sunar *et al.* 2000; Falk and Emons 2000). This study was undertaken to develop a microwave - assisted technique to improve arsenic extraction efficiency from biological tissues in the water - soluble fraction.

Specific Objectives

- To develop a microwave - assisted technique to improve arsenic extraction efficiency from biological tissues; and

- To examine the robustness of the developed microwave - assisted extraction and high performance liquid chromatography - inductively coupled plasma - mass spectrometry technique for identification and quantification of arsenic compounds in biological tissues by analysing the certified reference materials, dogfish muscle (NRC - CNRC, Dorm - 2, Canada) and lobster hepatopancreas (NRC - CNRC, Tort - 2, Canada).

Study 4

The continued research into determining arsenic cycling in aquatic and terrestrial environments has lead to the discovery of new (e.g. dimethylarsinylacetic acid) and as yet unidentified compounds (Edmonds and Francesconi 2003; Francesconi and Edmonds 1994; Kirby *et al.* 2002). These new and unidentified arsenic compounds have the potential to interfere with high performance liquid chromatography - inductively coupled plasma - mass spectrometry analysis through co - elution as unresolved peaks with known arsenic compounds. This study was undertaken to improve our understanding of the separation of arsenic compounds during high performance liquid chromatography (ion exchange chromatography) - inductively coupled plasma - mass spectrometry with changing column conditions (e.g. pH and buffer strength). These column conditions can then be manipulated in a controlled manner to prevent co - elution with known arsenic compounds and confirm peak identification.

The presence of chloride ions in samples have the potential to interfere with arsenic detection during high performance liquid chromatography - inductively coupled plasma - mass spectrometry (Tukai *et al.* 2002; Larsen *et al.* 1993; Sheppard *et al.* 1992; Raber *et al.* 2000). The elution of chloride ions potentially interfere through the formation of the polyatomic ion, $^{40}Ar^{35}Cl^{+}$, with the same m/z as arsenic (m/z 75). The elution of chloride ions during anion exchange chromatography - inductively coupled plasma - mass spectrometry has the potential to interfere with co - eluting arsenic peaks, such as arsenic acid, sulfonate arsenoribose, and sulfate arsenoribose. High performance liquid chromatography coupled via hydride generation to inductive coupled plasma - mass spectrometry can be used to determine inorganic and simple methylated arsenic compounds in samples containing high chloride ion concentrations

(e.g. urine, macroalgae, mangrove leaves, and seagrass) (Nakazato *et al.* 2000; Alauddin *et al.* 2003; Wei *et al.* 2001). This technique also allows the identification of arsenous acid that cannot be identified using conventional anion exchange chromatography due to co - elution with cationic arsenic compounds in the void volume (Maher *et al.* 2003).

Specific Objectives

- To gain an understanding of the retention behaviour of arsenic compounds during high performance liquid chromatography - inductively coupled plasma - mass spectrometry with changing column conditions (pH, buffer concentration and temperature);
- To develop a high performance liquid chromatography - hydride generation - inductively coupled plasma - mass spectrometry technique to determine inorganic and simple methylated arsenic compounds in biological extracts; and
- To determine arsenic compounds in Tasmanian Kelp (*Durvillea potatorum*), a range of commercially available macroalgae supplements, and sushi seaweeds to determine potential human health risks and for use as in - house quality control materials.

Study 5

The assigning of marine animals into specific trophic positions based on their diet can be a useful tool for modelling pathways of accumulation, distribution, and cycling in aquatic environments (Bernhard and Andrea 1984). Marine animals in higher trophic positions (e.g. pelagic carnivores) have been found to contain the majority of their arsenic in tissues as arsenobetaine (Francesconi and Edmonds 1994), while organisms at lower trophic positions (eg. benthic detritivore and herbivore species) exposed through their diet to sediment, algae, and seagrass also contain appreciable quantities of other inorganic and organic compounds (e.g. arsenic acid, arsenous acid, trimethylarsine oxide, dimethylarsinic acid and arsenoribosides) (Maher *et al.* 1999; Goessler *et al.* 1997; Edmonds and Francesconi 1987; Morita and Shibata 1987). The diet of marine animals and especially their association with sediments can be

important in understanding accumulation, distribution, and cycling of arsenic compound in aquatic environments (Maher *et al.* 1999).

In this study, the tissues of three marine fish species from Lake Macquarie, N.S.W were examined to gain an understanding of the accumulation, distribution, and cycling of arsenic compounds at different trophic feeding positions. Arsenic compounds were measured in mullet (*Mugil cephalus*) a benthic detritivore, luderick (*Girella tricuspidate*) a pelagic herbivore, and tailor (*Pomatomus saltatrix*) a pelagic carnivore using the previously developed microwave - assisted extraction technique and high performance liquid chromatography - inductively coupled plasma - mass spectrometry.

Specific Objective

- To use the developed microwave - assisted extraction technique and high performance liquid chromatography - inductively coupled plasma - mass spectrometry to gain an understanding of the accumulation, distribution, and cycling of arsenic compounds in relation to trophic feeding positions by examining the tissues of three marine fish species (*Mugil cephalus*, *Girella tricuspidata*, and *Pomatomus saltatrix*) from Lake Macquarie.

Study 6

This study was undertaken to extend our understanding of the relationship between arsenic compound accumulation, distribution, and cycling in marine environments in relation to trophic position. The number of animal species in this study was broadened (including gastropod, mollusc, crab, and fish species) to encompass a diverse range of trophic positions, diets, feeding behaviours, and strategies.

Specific Objective

- To examine the marine animal tissues from a temperate mangrove ecosystem using the previously developed microwave - assisted extraction technique and

high performance liquid chromatography - inductively coupled plasma - mass spectrometry to extend our understanding of the accumulation, distribution, and cycling of arsenic compounds in relation to trophic feeding positions.

Study 7

The main pathway (s) for arsenobetaine formation in marine animals is still believed to occur through the conversion of dimethylated arsenoriboses that are common macroalgal constituents (Edmonds and Francesconi 2003; Francesconi and Edmonds 1994). This study was undertaken to determine arsenic compound pathways in the marine animals; fish (*Odax cyanomelas*), abalone (*Haliotis rubra*), and sea urchins (*Heliocidaris erythrogramma* and *Centrostephanus rodgersii*) that are directly exposed through their diets of macroalgae (*Phyllospora comosa* and *Halopteris platycena*) to dimethylated arsenoriboses.

Specific Objective

- To use the previously developed microwave - assisted extraction technique and high performance liquid chromatography - inductively coupled plasma - mass spectrometry to gain an understanding of arsenic compound pathways in the marine animals; fish (*Odax cyanomelas*), abalone (*Haliotis rubra*), and sea urchins (*Heliocidaris erythrogramma* and *Centrostephanus rodgersii*) directly exposed through their diets to dimethylated arsenoriboses in macroalgae (*Phyllospora comosa* and *Halopteris platycena*).

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Chapter 2

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This chapter is available as:

Kirby, J., Maher, W. and Krikowa, F. (2001) Selenium, Cadmium, Copper, and Zinc Concentrations in Sediments and Mullet (*Mugil cephalus*) from the Southern Basin of Lake Macquarie, NSW, Australia. *Archives of Environmental Contamination and Toxicology*, 40(2), 246-256.

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Abstract

Selenium, cadmium, copper, and zinc concentrations were measured in sediments and the tissues of mullet (*Mugil cephalus*) collected from the southern basin of Lake Macquarie, NSW, Australia. Trace metals in surficial sediments are enriched in trace metals relative to background concentrations (selenium, 3–19; cadmium, 14–42; copper, 1.5–3.6; zinc, 0.77–2.2 times background).

Selenium, cadmium, and copper in Lake Macquarie mullet tissues are elevated compared to those in mullet collected from the Clyde River estuary, a relatively pristine location. Selenium and copper concentrations are also elevated compared to those reported in mullet tissues from other nonpolluted coastal environments. Zinc concentrations in Lake Macquarie mullet muscle tissues are significantly higher than those in muscle tissues of mullet from the Clyde River estuary, but mullet from both locations have similar zinc concentrations in other tissues. These results show that contamination of sediment with trace metals has resulted in elevated trace metals in the benthic feeding fish *M. cephalus*.

Little of the variation of trace metal concentrations between fish was explained by variation in mass. Selenium concentrations in mullet are of concern in muscle tissues as they are above recommended acceptable limits for safe human consumption, while concentrations in tissues are at levels that may effect fish growth, reproduction, and survival. Copper concentrations in mullet tissues are also at levels that may reduce fish growth.

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Chapter 3

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This chapter is available as:

Kirby, J., Maher, W. and Harasti, D. (2001) Changes in Selenium, Copper, Cadmium, and Zinc Concentrations in Mullet (*Mugil cephalus*) from the Southern Basin of Lake Macquarie, Australia, in Response to Alteration of Coal-Fired Power Station Fly Ash Handling Procedures. *Archives of Environmental Contamination and Toxicology*, 41(2), 171-181.

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DOI	0.1007/s002440010235

Abstract

Selenium, copper, cadmium, and zinc concentrations were measured in mullet (*Mugil cephalus*) from the southern basin of Lake Macquarie, Australia, in 1997 to determine if improved ash-handling practices at an adjacent coal fired power station, implemented in 1995, had significantly lowered trace metal concentrations in mullet tissues. Mean muscle tissue concentrations of selenium (5.9 ± 0.7 µg/g dry mass), copper (3.6 ± 0.1 µg/g dry mass), and zinc (14 ± 1 µg/g dry mass) are lower than previously reported for mullet analyzed in 1993 (10 ± 2, 21 ± 3, 27 ± 3 µg/g dry mass, respectively). Cadmium concentrations in liver tissues increased from 2.3 ± 0.3 to 6 ± 2 µg/g dry mass. Significant intra-tissue correlations between metal concentrations were found for all tissues except muscle. Strong correlations of selenium, copper, and zinc concentrations were found in liver tissues, indicating a common primary source may exist for these metals, such as fly ash. All trace metals were found to have significant inter-tissue correlations, with strong correlations occurring for selenium between all tissues and for cadmium between all tissues except muscle. Regulation of copper, cadmium, and zinc appears to be occurring in muscle tissue. Selenium concentrations in mullet are still above levels considered to be of concern to human consumers. Trace metal concentrations are below that known to effect the health of fish. Mullet are directly exposed to trace metal concentrations as a result of feeding and the ingestion of contaminated sediment and detritus. Lower metal concentrations found in mullet tissues are attributed to the burial of highly contaminated sediment with material containing lower trace metal concentrations. Little of the variations in trace metal concentrations between mullet was explained by mass, gender, or age.

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Chapter 4

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This chapter is available as:

Kirby, J., and Maher, W. (2002) Measurement of water-soluble arsenic species in freeze-dried marine animal tissues by microwave-assisted extraction and HPLC-ICP-MS. *Journal of Analytical Atomic Spectrometry*, 17, 838-843.

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Abstract

A microwave-assisted procedure is outlined for the extraction of water-soluble arsenic in freeze-dried marine animal tissues. The optimum microwave-assisted conditions were three extractions with 50% (v/v) methanol–water at 70 to 75 °C for 5 min. Quantitative extraction of arsenic in the water-soluble fraction of dogfish muscle (Dorm-2: 103 ± 2%) is consistent with that reported in the literature for this tissue. Lower extraction efficiencies for arsenic were found for liver (e.g. Dolt-1: 75 ± 5%; Pomatomus saltatrix: 80%), digestive (e.g. Tort-2: 92 ± 5%; Mugil cephalus stomach: 58%) and whole (e.g. Mussel CRM 278R: 66.1 ± 0.5%; Bembicium auratum: 76.4%) tissues. Arsenic extraction efficiencies in the water-soluble fraction were slightly higher for Dogfish Liver (Dolt-1) and Oyster (SRM 1566a) compared to that reported in the literature for these tissues. These results indicate that when samples are prepared in a similar manner, the efficiency to extract arsenic in the methanol–water soluble fraction will depend on the marine animal species and tissue analysed. The robustness of the microwave-assisted extraction procedure to identify and quantify arsenic species in freeze-dried marine animal tissues was determined using high performance liquid chromatography-inductively coupled plasma-mass spectrometry and the certified reference materials Dogfish Muscle (Dorm-2) and Lobster Hepatopancreas (Tort-2). Arsenic species determined in Dorm-2 tissue were AsB (16.80 ± 0.14 μg g−1), TMAP (0.17 ± 0.01 μg g−1), AsC (0.023 ± 0.002 μg g−1), TETRA (0.24 ± 0.02 μg g−1) and DMA (0.280 ± 0.004 μg g−1). Arsenic species determined in Tort-2 tissue were AsB (13.10 ± 0.08 μg g−1), TMAP (1.20 ± 0.03 μg g−1), AsC (trace), TETRA (0.055 ± 0.005 μg g−1), DMA (1.03 ± 0.10 μg g−1), MA (0.20 ± 0.01 μg g−1), As+5 (0.41 ± 0.03 μg g−1) and phosphate arsenoribose (0.13 ± 0.03 μg g−1). Two unknown anionic and one cationic arsenic species were also identified in Tort-2 tissue.

Chapter 5

Kirby, J., Maher, W., Ellwood, M. & Krikowa, F. (2004) Arsenic Species Determination in Biological Tissues by HPLC–ICP–MS and HPLC–HG–ICP–MS. *Australian Journal of Chemistry* 57(10) 957–966.

Abstract

The use of high-pressure liquid chromatography coupled directly or by a hydride generation system to an inductively coupled plasma mass spectrometer for the unambiguous measurement of 13 arsenic species in marine biological extracts is described. The use of two chromatography systems; a Supelcosil LC-SCX cation-exchange column eluted with a 20 mM pyridine mobile phase adjusted to pH 2.2 and 2.6 with formic acid, with a flow rate of 1.5 mL min-1 at 40°C, and a Hamilton PRP-X100 anion-exchange column eluted with 20 mM NH4H2PO4 buffer at pH 5.6, with a flow rate of 1.5 mL min-1 at 40°C, was required to separate and quantify cation and anion arsenic species. Under these conditions, arsenous acid could not be separated from other arsenic species and required the use of an additional hydride generation step. Arsenic species concentrations in a locally available Tasmanian kelp (Durvillea potatorum), a certified reference material (DORM-2), and a range of commercially available macroalgae supplements and sushi seaweeds have been measured and are provided for use as in-house quality control samples to assess the effectiveness of sample preparation, extraction, and measurement techniques.

Chapter 6

Kirby, J., and Maher, W. (2002) Tissue accumulation and distribution of arsenic compounds in three marine fish species: relationship to trophic position. *Applied Organometallic Chemistry* 16(2) 108-115.

Abstract

In this study the accumulation and distribution of arsenic compounds in marine fish species in relation to their trophic position was investigated. Arsenic compounds were measured in eight tissues of mullet Mugil cephalus (detritivore), luderick Girella tricuspidata (herbivore) and tailor Pomatomus saltatrix (carnivore) by high performance liquid chromatography–inductively coupled plasma-mass spectrometry. The majority of arsenic in tailor tissues, the pelagic carnivore, was present as arsenobetaine (86–94%). Mullet and luderick also contained high amounts of arsenobetaine in all tissues (62–98% and 59–100% respectively) except the intestines (20% and 24% respectively). Appreciable amounts of dimethylarsinic acid (1–39%), arsenate (2–38%), arsenite (1–9%) and trimethylarsine oxide (2–8%) were identified in mullet and luderick tissues. Small amounts of arsenocholine (1–3%), methylarsonic acid (1–3%) and tetramethylarsonium ion (1–2%) were found in some tissues of all three species. A phosphate arsenoriboside was identified in mullet intestine (4%) and from all tissues of luderick (1–6%) except muscle. Pelagic carnivore fish species are exposed mainly to arsenobetaine through their diet and accumulate the majority of arsenic in tissues as this compound. Detritivore and herbivore fish species also accumulate arsenobetaine from their diet, with quantities of other inorganic and organic arsenic compounds. These compounds may result from ingestion of food and sediment, degradation products (e.g. arsenobetaine to trimethylarsine oxide; arsenoribosides to dimethylarsinic acid), conversion (e.g. arsenate to dimethylarsinic acid and trimethylarsine oxide by bacterial action in digestive tissues) and/or in situ enzymatic activity in liver tissue.

Chapter 7

Kirby, J., Maher, W., Chariton, A. & Krikowa, F. (2002) Arsenic concentrations and speciation in a temperate mangrove ecosystem, NSW, Australia. *Applied Organometallic Chemistry* 16(4) 192-201.

Abstract

Total arsenic concentrations and species were measured in the sediments, vegetation and tissues of marine animals from a temperate mangrove ecosystem. Mean arsenic concentrations ranged from 0.3 to 55 μg g−1 dry mass. Epiphytic algae/fungi associated with mangrove fine roots had relatively higher arsenic concentrations (12 ± 3 μg g−1) than mangrove leaves, bark or main roots (0.3–1.2 μg g−1) and algae/fungi attached to main roots (1.5 ± 0.8 μg g−1). The concentrations of arsenic in detritivores (8.5–55 μg g−1) were significantly higher than in the major primary producers (0.3–1.5 μg g−1), two herbivores (8 ± 1 and 14 ± 2 μg g−1) and omnivores (2–16.6 μg g−1). Most marine animal tissues contained large percentages of arsenobetaine (28–81%). Glycerol arsenoribose was found in all tissues examined (1–23%) except oyster tissues. Relatively large concentrations of this arsenoriboside were found in the digestive tissues of two crab species (13–23%). Small amounts of trimethylarsoniopropionate (1–8%), tetramethylarsonium ion (1–7%), sulfate arsenoribose (2–13%) and trace amounts of arsenocholine (<1%), trimethylarsine oxide (<1%), dimethylarsinic acid (<2%), phosphate arsenoribose (<2%), arsenate (<1%), and sulfonate arsenoribose (<3%) were found in some tissues. Methylarsonic acid was not found in any tissues. Two unknown cationic arsenic compounds (1–2%) and three anionic arsenic compounds (1–17%) were present in some marine animal tissues. The arsenic concentrations and species found in animals could not be attributed to their position in the food web or feeding mode, but are likely to be related to their dietary intake of arsenic and their ability to assimilate, metabolize and retain arsenic species.

Chapter 8

Kirby, J., Maher, W., & Spooner, D. (2005) Arsenic Occurrence and Species in Near-Shore Macroalgae-Feeding Marine Animals. *Environmental Science and Technology,* 39(16) 5999–6005.

Abstract

This study was undertaken to improve the understanding of arsenic species and their pathways of formation in marine animals: fish (Odax cyanomelas), abalone (Haliotis rubra), and sea urchins (Heliocidaris erythrogramma and Centrostephanus rodgersii) that are directly exposed through their diets to dimethyl arsenoriboses in macroalgae (Phyllospora comosa and Halopteris platycena). The identification of dimethyl arsenoriboses (phosphate, sulfonate, and glycerol) in both dominant macroalgae species, and especially digestive tissues of marine animals that consume them, suggests these arsenic species, are to some degree accumulated directly from their diets without degradation or conversion. An unknown arsenic species in H. rubra intestinal tissue was identified using tandem mass spectrometry as 2′,3′-dihydroxypropyl 5-deoxy-5-trimethyl arsonioriboside (trimethyl glycerol arsenoribose). The concentration of trimethyl glycerol arsenoribose in H. rubra intestinal tissue was estimated to account for 28% (5.0 μg g-1 dry mass) of the methanol−water-soluble arsenic fraction. The presence of a trimethyl glycerol arsenoribose in marine animal tissues may be due to microbial-mediated processes that promote the reduction and methylation of dimethyl arsenoriboses released during the breakdown of macroalgae in their diets. Arsenobetaine formation may then occur in the lumen of the digestive tract (i.e., mediated by microorganisms) or in the liver catalyzed by enzymes. The identification of a large amount of trimethyl glycerol arsenoribose in H. rubra intestinal tissue suggests this species is a main constituent in the pathway for arsenic in this marine animal.

CHAPTER 9

Synopsis

Biomonitoring Trace Metal Bioavailability in Lake Macquarie, N.S.W.

The surficial sediments in the southern basins of Lake Macquarie, N.S.W were found to be enriched in selenium, cadmium, copper, and zinc relative to regional determined background concentrations (selenium, 3 - 19; cadmium, 14 - 42; copper, 1.5 - 3.6; zinc, 0.77 - 2.2 times background). This enrichment of selenium, cadmium, copper, and zinc in the southern basins of Lake Macquarie may have resulted from atmospheric deposition due to power generation activities or wide spread dispersion of dissolved and sediment transported trace metals from sources such as fly - ash, urban run off, or sewage.

The muscle tissues of mullet (*M. cephalus*) were found to be a good indicator of the bioavailability of trace metals present in Lake Macquarie. Mullet (*M. cephalus*) collected in 1993 were found to contain selenium, cadmium, copper, and zinc in tissues at significantly higher concentrations than those collected from a relative pristine estuary (Clyde River, N.S.W) and other unpolluted marine environments in Australia. The mean selenium concentrations in mullet muscle tissues (9 ± 2 µg g^{-1} dry mass) collected in 1993 were at levels higher than the recommended acceptable limit for safe human consumption (5 µg g^{-1} dry mass, assuming a wet - dry mass ratio of 5:1) (ANZFA 1992). Mean cadmium, copper, and zinc concentrations in muscle tissues were below the recommended acceptable limits for safe human consumption (copper 50 µg g^{-1} dry mass; zinc 750 µg g^{-1} dry mass; no maximum value has been set for cadmium) (ANZFA 1992). The mean selenium and copper concentrations in mullet (*M. cephalus*) tissues (i.e. liver, kidney, and gonadal) were also found at levels that may affect fish growth, reproduction, and survival (Coughlan and Velte 1989; Finley 1985; Hilton *et al.* 1980, 1982; Hilton and Hodson 1983; Murai *et al.* 1981; Lanno *et al.* 1985).

Tissue distribution of selenium, cadmium, copper, and zinc in mullet (*M. cephalus*) at Lake Macquarie were similar to those reported for other marine fish species (Chernoff and Dooley 1979; Eisler 1981; Hanna 1989; Hornung *et al.* 1993; Vas *et al.* 1993). Mullet (*M. cephalus*) liver tissues were found to accumulate the highest concentrations of all measured trace metals, while gonadal tissues accumulated elevated levels of selenium and zinc. The liver tissue has been reported to be a site of trace metal detoxification and elimination within fish (Miller *et al*. 1992; Sorensen 1991). Elevated trace metals present in gonad tissues are thought to accumulate to aid in its development, to be passed via gametes for utilisation by the developing embryo's and / or to be lost during spawning (Fletcher and King 1978; Zeitoun *et al.* 1976).

Mullet (*M. cephalus*) are detritivores that can be found living and feeding in the benthic reaches of estuarine and marine waters. In this study, the major proportion of the material found in mullet (*M. cephalus*) stomachs consisted of sediments and seagrass (*Zostera capricornia*). The stomachs of mullet (*M. cephalus*) have previously been found to contain epiphytic and benthic micro - algae, seagrass detritus, and sediment (Thompson 1954). Fish are normally unable to breakdown and digest seagrass material (Larkum *et al.* 1989). Thus, the higher concentrations of trace metals accumulated in mullet (*M. cephalus*) tissues may be due to the ingestion of contaminated sediments and associated benthic in - fauna (Dallinger *et al*. 1987), rather than from water, which contains lower trace metal concentrations (Batley 1987).

In 1997, selenium concentrations in mullet (*M. cephalus*) muscle, stomach, and gonadal tissues collected in the southern reaches of Lake Macquarie were significantly less than those analysed in the same fish species in 1993. Selenium concentrations in mullet (*M. cephalus*) muscle tissues decreased from 9 ± 2 µg g^{-1} dry mass in 1993 to 5.9 ± 0.7 µg g^{-1} dry mass in 1997. The average selenium concentrations in mullet (*M. cephalus*) muscle tissues collected in 1997 are still above that recommended for safe human consumption (i.e. selenium 5 µg g^{-1} dry mass, assuming a wet - dry mass ratio of 5:1) (ANZFA 1992). The mean selenium concentrations in mullet (*M. cephalus*) muscle tissues appear to be declining, with the same tissue analysed in 1998 by Barwick (1999) from the same locations at Lake

Macquarie containing even lower selenium concentrations (i.e. 1993, 9 ± 2 µg g^{-1}; 1997, 5.9 ± 0.7 µg g^{-1}; 1998, 4 ± 1 µg g^{-1} dry mass). The mean selenium concentrations in mullet (*M. cephalus*) muscle tissues by 1998 in the southern basins of Lake Macquarie were below that considered to be of concern to human consumers (Barwick 1999). By 1997, selenium concentrations in mullet (*M. cephalus*) tissues (i.e. liver, kidney, and gonadal) were below that shown to cause reduced growth and/or survival in fish species (Coughlan and Velte 1989; Finley 1985; Hilton *et al.* 1980, 1982; Hilton and Hodson 1983).

In 1997, copper concentrations in mullet (*M. cephalus*) muscle, stomach, heart, and gonadal tissues collected in the southern reaches of Lake Macquarie were significantly less than those analysed in the same fish species in 1993. Copper concentrations in muscle tissues decreased from 21 ± 3 µg g^{-1} dry mass in 1993 to 3.6 ± 0.1 µg g^{-1} dry mass in 1997. Similar to selenium, mean copper concentrations in mullet (*M. cephalus*) muscle tissues appear to be declining, with the same tissue analysed in 1998 by Barwick (1999) from the same locations in the southern basins of Lake Macquarie containing even lower copper concentrations (i.e. 1993, 21 ± 3 µg g^{-1}; 3.60 ± 0.02 µg g^{-1}; 1998, 2.8 ± 0.4 µg g^{-1} dry mass). By 1997, copper concentrations in mullet (*M. cephalus*) tissues (i.e. liver, kidney, and gonadal) were below that shown to cause reduced growth and/or survival in fish species (Murai *et al.* 1981; Lanno *et al.* 1985).

The lower selenium and copper concentrations found in mullet (*M. cephalus*) tissues in 1997 may be attributed to improved practices implemented by the Vales Point power station in the southern reaches of Lake Macquarie at the end of 1995 (Peters *et al.* 1999; Harston 1996). These improved practices were expected to reduce the concentrations of suspended and dissolved trace metals, especially selenium, entering the southern basins of Lake Macquarie via ash dam overflows (Peters *et al.* 1999). The lower trace metal concentrations found in mullet (*M. cephalus*) tissues in 1997 may be attributed to the burial of highly contaminated sediments (including fly - ash) with material containing lower trace metal concentrations. Thus, lower trace metal concentrations in mullet (*M. cephalus*) tissues may be due to the ingestion of less contaminated sediments and associated benthic in - fauna. In these biomonitoring

studies little of the variation of trace metal concentrations in mullet (*M. cephalus*) tissues could be explained by mass, gender, or age.

As previously stated, the examination of selenium compounds did not occur at the time of this thesis due to extensive research already occurring in this area at Lake Macquarie (Peters *et al.* 1999; Harston 1996; Harasti 1997; Maher *et al.* 1992; Maher *et al*. 1997; Deaker and Maher 1995). Enzymatic hydrolysis using individual or combinations of different enzymes, such as protease, pepsin, and lipase have been used for the extraction of selenium compounds in biological tissues (Capelo *et al.* 2004; Moreno *et al.* 2001; Moreno *et al.* 2002; Quijano *et al*. 2000; Gomez-Ariza *et al.* 2002; Hinojosa Reyes *et al.* 2004; Goenaga Infante *et al.* 2004). The use of enzymatic hydrolysis techniques is time consuming, tissue dependent, often non - quantitative for selenium extraction, and has the potential to change selenium compounds present in the original samples (Capelo *et al.* 2004). In the future, selenium extraction procedures will need to be developed that provide high selenium extraction efficiencies from all environmental matrices (i.e. sediments and biological tissues) without compound conversion or degradation. The application of selective and sensitive techniques, such as liquid chromatography - mass spectrometry, high performance liquid chromatography - inductively coupled plasma - mass spectrometry, high performance liquid chromatography - hydride generation - inductively coupled plasma - mass spectrometry, and hydride generation - cold trap - inductively coupled plasma - mass spectrometry will also be required to determine known and as yet unidentified selenium compounds, at trace concentrations in complex matrices, such as waters, sediments, atmosphere, and biological tissues. An understanding of selenium compounds (especially selenate, selenite, selenocysteine, selenomethionine, trimethylselenonium ion, dimethyselenide, and dimethydiselenide) present in waters, sediments, and biological tissues in Lake Macquarie is essential in determining their fate and behaviour, and is important in assessing their human (e.g. inhalation and ingestion) toxicity and their potential affect on animal growth, reproduction, and survival.

Arsenic Speciation

The optimum conditions for the removal of water - soluble arsenic in biological tissues were found to be three microwave - assisted extractions at 70 ^{o}C for 5 min with 50 % (v/v) methanol - water. This microwave - assisted technique was found to provide quantitative extraction of arsenic from muscle (e.g. NRC - CNRC Dorm - 2; 103 ± 2 %), with improved recoveries for other tissues (e.g. NRC - CNRC Dolt - 1; 75 ± 5 %) and whole animals (e.g. NIST Oyster SRM 1566a; 78 ± 2 %) compared to those previously reported in the literature (Table 1, page 90 and Table 2, page 93). The improved arsenic extraction efficiency is believed to occur due to efficient sample preparation (i.e. small sample particle size and acetone extraction) and increased mixing of sample and solvent through internal convection currents. Improved arsenic extraction efficiency from < 0.63 µm sample particles is believed to occur due the higher surface area and increased solvent exposure. The acetone extraction of all biological tissues prior to microwave - assisted extraction was found to be an essential step in the extraction of water - soluble arsenic compounds. The removal of fats, lipids, and non - polar soluble cellular constituents increased water - soluble arsenic compound extraction from biological tissues and improved separation, identification, and quantification during high performance liquid chromatography - inductively coupled plasma - mass spectrometry.

High performance liquid chromatography coupled directly to inductively coupled plasma mass spectrometry (HPLC-ICP-MS) is a highly selective and sensitive technique for the determination of arsenic compounds in terrestrial and aquatic environments (Maher *et al.* 2003; Gong *et al.* 2002; Szpunar *et al.* 2000; Sarzanini 1997). This technique can be used to determine all thirteen common arsenic compounds identified in the aquatic environment (i.e. arsenobetaine, arsenocholine, trimethylarsine oxide, trimethylarsoniopropionate, tetramethylarsonium cation, arsenous acid, arsenic acid, methylarsonic acid, dimethylarsinic acid, glycerol - arsenoribose, phosphate - arsenoribose, sulfonate - arsenoribose and sulfate - arsenoribose). The successful quantitation of all these arsenic compounds in biological extracts has not been achieved using one chromatography run but requires the use of complimentary separation techniques such as anion and cation exchange

chromatography; and column eluant conditions incorporating different buffer compositions and pH (Geiszinger *et al.* 2002; Kirby and Maher 2002; Lai *et al.* 2002; Raber *et al.* 2000). It was determined in this thesis that both anion and cation exchange chromatography are required to determine all thirteen common arsenic compounds present in biological tissues (Figure 3, page 112; Fugure 6, page 116). The use of two mobile phase conditions during cation exchange chromatography was also required to determine glycerol arsenoribose and TMAO (Figure 3, page 112). The optimum column conditions determined in this thesis for the determination of all thirteen common arsenic compounds present in biologcal tissues were:

1. Hamilton PRP - X100 anion exchange column (PEEK 250 mm x 4.6 mm, 10 µm) (Phenomenex, USA): A 20 mM ammonium phosphate mobile phase at pH 5.6, flow rate 1.5 ml min^{-1} and a temperature of 40 ^{o}C was used for the identification and quantification of DMA, MA, As^{5+}, phosphate arsenoribose, sulfonate arsenoribose, and sulfate arsenoribose (time = 35 min);
2. Supelcosil LC - SCX cation exchange column (250 mm x 4.6 mm, 5 µm) (Supelco, Australia): A 20 mM pyridine mobile phase adjusted to pH 2.6 with formic acid at a flow rate of 1.5 ml min^{-1} and a column temperature of 40 ^{o}C was used for the identification of AsB, glycerol arsenoribose, AsC, and TETRA (time = 10 min); and
3. Supelcosil LC - SCX cation exchange column (250 mm x 4.6 mm, 5 µm) (Supelco, Australia): A 20 mM pyridine mobile phase adjusted to pH 2.2 with formic acid at a flow rate of 1.5 ml min^{-1} and a column temperature of 40 ^{o}C was used for the identification and quantification of TMAP, TMAO, AsC, and TETRA (time = 10 min).

It was found when analysing for arsenic compounds using cation exchange chromatography at pH 2.6 that it was necessary to determine the effects of high ionisation ions such as sodium. The presence of high concentrations of sodium in biological extractions has the potential to suppress co - eluting arsenic compounds (i.e. arsenobetaine) during cation exchange chromatography at pH 2.6. The monitoring of sodium ions (m/z 23) during cation exchange chromatography-ICP-MS is essential to monitor this potential interference; with biological extracts high in sodium ions requiring dilution (e.g. 1:10 v/v) or removal to prevent peak suppression

during the quantification of cationic arsenic species at pH 2.6 (e.g. Figure 4, page 96).

The presence of chloride ions in biological extracts have the potential to interfere with determination of anionic arsenic compounds during anion exchange chromatography-ICP-MS. This potential interference from chloride ions is due to the formation of argon chloride ($^{40}Ar^{35}Cl^{+}$) with the same m/z as arsenic (^{75}As). The presence of high chloride ion concentrations in biological extracts has the potential to interfere with the determination of co - eluting arsenic compounds such as As^{5+}, sulfonate arsenoribose, and sulfate arsenoribose. The potential interference to arsenic (m/z 75) from chloride ($^{40}Ar^{35}Cl^{+}$) was determined during anion exchange chromatography by monitoring $^{35}Cl^{16}O^{+}$ at m/z 51, $^{35}Cl^{17}O^{+}$ at m/z 52, and $^{40}Ar^{37}Cl^{+}$ at m/z 77. Biological extracts such as urine, macroalgae, mangrove leaves, and seagrass containing high chloride ions will require dilution or removal by techniques such as solid phase extraction using silver nitrate before anion exchange chromatography. Alternatively, ICP-MS instruments containing collision (Agilent) or reaction cells (Perkin Elmer) can be used to minimise formation of $^{40}Ar^{35}Cl^{+}$ polyatomic interferences.

The developed technique of high performance liquid chromatography-hydride generation-inductive coupled mass spectrometry was found to be highly selective and sensitive for the determination of inorganic (i.e. As^{3+} and As^{5+}) and simple methylated (i.e. DMA and MA) arsenic compounds in biological extracts containing high chloride ion concentrations (e.g. macroalgae) (Table 1, page 123; Table 2, page 125). This technique also allows the identification As^{3+} that cannot be determined using conventional anion exchange chromatography-ICP-MS due to co-elution with cationic arsenic compounds in the void volume (Table 1, page 123).

It is important in all arsenic speciation studies that researchers start to incorporate quality control programs such as certified reference material, in-house quality control programs or interlaboratory studies to assess the effectiveness of sample preparation, extraction procedures, and measurement techniques. We have incorporated the certified reference material, dogfish muscle - 2 (NRC - CNRC, Canada; certified for AsB and TETRA) and developed an in - house Tasmanian Kelp (*Durvillea potatorum*) reference material (Table 1, page 123; Figure 10, page 124) into routine

speciation protocols in our laboratory to provide essential information on extraction efficiency, compound stability, and the reliability of results from unknown samples.

High performance liquid chromatography-inductively coupled plasma-mass spectrometry was used in this thesis as a highly selective and sensitive technique to gain an understanding of the cycling of arsenic compounds in the marine environment (chapters 4 to 8). Arsenic was identified in biological tissues as inorganic (i.e. As^{3+} and As^{5+}), simple methylated (i.e. DMA and MA) and complex organic compounds (e.g. AsB, TMAP, AsC, TMAO, TETRA and arsenoriboses) (chapters 4 - 8). The presence of these individual arsenic compounds in biological tissues was found to be dependent on the species, their trophic position, and the tissue analysed.

The tissues of mullet (*M. cephalus*) and luderick (*G. tricuspidate*) collected at Lake Macquarie, N.S.W were found to contain high amounts of TMAO (Tables 2 and 3, pages 140 and 141) (Chapter 6). The presence of TMAO, especially in digestive tissues, may be due to microbial methylation of ingested inorganic arsenic and/or degradation of arsenobetaine. The detritivore mullet (*M. cephalus*) and herbivore luderick (*G. tricuspidate*) may be exposed to inorganic arsenic as a consequence of their feeding habits. Mullet (*M. cephalus*) feed by sucking up detritus from the sediment benthic layer, while luderick (*G. tricuspidate*) consume seagrass that can contain a fine sediment layer adhering to its surface. Some macroalgae contain large quantities of As^{5+} and it is likely that micro-algae consumed by detritivore and herbivore species contain inorganic arsenic as well. Trimethylarsine oxide may also be produced by the degradation of arsenobetaine by microorganisms (Kaise *et al.* 1987; Hanaoka *et al.* 1991). The absence of trimethylarsine oxide from the tissues of tailor (*P. saltatrix*) also collected at Lake Macquarie, N.S.W a pelagic carnivore, which consumes the majority of its arsenic as AsB, suggests that degradation of AsB to TMAO is not a major pathway in marine fish.

Some marine animal tissues in this thesis, especially digestive tissues were found to contain dimethylated arsenoriboses (i.e. phosphate, sulfonate, sulfate, and glycerol) (chapters 6 - 8). The presence of dimethylated arsenoriboses in marine animal tissues has been suggested to occur due to the presence of symbiotic algae and through direct accumulation from their diets (Edmonds and Francesconi 2003; Francesconi and

Edmonds 1994; Kirby and Maher 2002; Kirby *et al.* 2002). The identification in this thesis of dimethylated arsenoriboses (i.e. phosphate, sulfonate, and glycerol) in both macroalgae (i.e. *P. comosa* and *H. platycena*) and especially digestive tissues of marine animals that consume them (i.e. *O. cyanomelas, H. rubra, H. erythrogramma* and *C. rodgersii*) (chapter 8) suggests these arsenic compounds to some degree are accumulated directly from their diet without degradation or conversion. These dimethylated arsenoriboses accumulated in digestive tissues then have the potential to be redistributed to other tissues (e.g. liver, muscle, and kidney), degraded (e.g. dimethylarsinoylethanol, dimethylarsinylacetic acid, AsC, DMA and As^{5+}) and / or eliminated.

The pathway (s) to the formation of AsB in marine animals has still to be established. Arsenobetaine formation based on a pathway using dimethylated arsenoriboses requires the incorporation of a third methyl group (plus decomposition and conversion) that at present has only been shown to occur with a bacterial species (*Pseudomonas* sp) with added dimethylarsinylacetic acid under *in vitro* laboratory conditions (Ritchie *et al.* 2004). The presence of large amounts of dimethylated arsenoriboses in the marine environment (i.e. macro and micro-algae) suggests they are still the likely precursor to AsB in marine animals.

In this thesis, a large unknown arsenic compound identified in *Haliotis rubra* intestine tissues by HPLC-ICP-MS was further characterised using tandem mass spectrometry as 2', 3' - dihydroxypropyl 5 - deoxy - 5 - trimethylatedarsonioriboside (trimethylated glycerol arsenoribose) (Figure 4, page 197). The exact concentration of the identified trimethylated glycerol arsenoribose in *Haliotis rubra* intestine tissues was not determined due to the lack of a pure analytical standard; however, with the assumption of a similar HPLC-ICP-MS response to AsB this compound would account for 27.5 % (5.0 µg g^{-1} dry mass) of the arsenic extracted in the methanol water - soluble fraction. Also, based on the cation exchange chromatography (pH 2.6) retention time for identification of trimethylated glycerol arsenoribose, this arsenic compound would account for low amounts in *H. rubra* muscle (0.4 µg g^{-1}; 0.9 %), and *O. cyanomelas* intestine (0.19 µg g^{-1}; 1.1 %) and muscle (0.023 µg g^{-1}; 0.5 %) tissues. The retention time for trimethylated glycerol arsenoribose using cation exchange chromatography (pH 2.6) was also similar to an unknown arsenic peak found in two

gastropod species, *Pyrazus ebeninius* and *Bembicium auratum* from an Australian mangrove ecosystem (Kirby *et al.* 2002). The role trimethylated arsenoriboses play in the cycling of arsenic compounds in the marine environment may have been underestimated. Identification of a large amount of trimethylated glycerol arsenoribose in *H. rubra* intestine tissues suggests this compound is a main constituent in the pathway for arsenic in this marine animal. It is easier to envision the synthesis of AsB from trimethylated arsenoriboses than dimethylated arsenoriboses in marine animals, as these arsenic compounds do not require further methylation (unlike dimethylated arsenoriboses), only degradation (i.e. AsC) and conversion (oxidation).

In the last decade, HPLC-ICP-MS has become the main instrumental technique used by arsenic speciation research scientists in an endeavour to gain an understanding of the fate, behaviour, and cycling of arsenic compounds in aquatic and terrestrial environments (Maher *et al.* 2003; Gong *et al.* 2002; Szpunar *et al.* 2000; Sarzanini and Mentasti 1997). This increased application of HPLC-ICP-MS has highlighted some limitations to this technique in the analysis of real environmental samples. These limitations include the co-elution of known and unidentified arsenic compounds, co-elution of arsenic compounds with interfering ions, the unavailability of pure standards for all arsenic compounds, and the possible misidentification of unknown arsenic compounds using only retention times. These limitations to HPLC-ICP-MS can be avoided to some degree when analysing complex arsenic compound matrices by using efficient sample preparation techniques (e.g. solid phase extraction, dilution, and acetone extraction), different chromatography techniques (i.e. anion and cation exchange chromatography), and optimised column separations conditions (i.e. cation exchange chromatography at pH 2.6. and 2.2). High performance liquid chromatography - inductively coupled plasma - mass spectrometry will continue to play a major role in determining arsenic compounds in environmental samples, due to its low sample costs, easy of operation, high sample throughput, high sensitivity and selectivity, and robustness to different mobile phases and matrices.

In recent years, the application of molecular analysis techniques such as liquid chromatography-mass spectrometry and liquid chromatography-tandem mass spectrometry to arsenic speciation studies has increasing become popular by

providing high arsenic compound selectivity (i.e. molecular ion) and additional structural information (i.e. fragmentation ions) (McSheehy *et al.* 2003; McSheehy and Mester 2003; Szpunar and Lobinski 2002). These molecular analysis techniques are susceptible to matrix interferences and have higher detection limits compared to ICP-MS techniques (McSheehy *et al.* 2003). Arsenic speciation analysis in the future will need to incorporate complementary atomic and molecular analysis techniques to provide with confidence the identification, quantification, and structural characterisation of known and as yet unidentified arsenic compounds to determine arsenic pathways in aquatic and terrestrial environments.

The important role microorganisms can have in the cycling of arsenic compounds in aquatic environments was again highlighted in this thesis (e.g. inorganic arsenic methylation to TMAO by fish species at Lake Macquarie and the proposed methylation of consumed dimethylated arsenoriboses to trimethylated glycerol arsenoribose in *H. rubra* intestine tissues) (Chapter 6 to 8). The role microorganisms play in the cycling of arsenic compounds in environments may have been underestimated, especially in the formation of AsB. A greater understanding of the role microorganisms play in arsenic compound pathways needs to be established. This may be achieved by using radioactive arsenic (^{74}As) or tagged arsenic compounds to follow the fate, behaviour and cycling in aquatic and terrestrial environments.

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