Are Lipid Soluble Metal Complexes Important in Aquatic Environments?

Daniel Alan Kilgore
B. Env.Sc., B.Env.Sc (Hons)

A thesis submitted in fulfilment of the requirements for the degree

of Doctor of Philosophy

Faculty of Science,

Department of Environment and Geography,

Macquarie University, Sydney Australia

March 2014

Declaration of Authorship and Originality

I certify that this thesis is the result of my own research and that it has not, nor any part of it, been submitted for a higher degree at any other university or institution.

To the best of my knowledge and belief, this thesis is original and contains no material previously published or written by another person, except where due reference has been given in the text.

Daniel Alan Kilgore

March 2014

Acknowledgements

This PhD project would not have been possible without the assistance and support of many people.

This project was undertaken as a collaboration between Macquarie University and CSIRO, Land and Water. I gratefully acknowledge the financial support of the CSIRO and its support in the supply of materials, equipment and technical expertise during the course of this project.

Special thanks and appreciation go to:

My supervisors Dr Simon Apte (CSIRO), Dr Grant Hose and Dr Mark Taylor (Macquarie University). Simon, Grant and Mark thank you for your support, encouragement and advice over the past 4-5 years. This project would not have been possible without the input of each and every one of you. Simon thank you for moulding me into an independent researcher, for pushing me when I needed it, encouraging me when I thought it was impossible, stretching me and teaching me to think outside of the box. Your knowledge and passion for science has inspired me and grown me in ways that I never thought would be possible. Grant, thank you for taking me on as a PhD student and for taking on this project. Thank you for keeping me organised and on track and for always keeping the end in sight. Thank you for always being available and for being so generous with your time. Mark, thank you for sticking with

me through my honours and now my PhD research, your enthusiasm and wealth of knowledge has been invaluable to me throughout this project.

The staff and research students of CSIRO Land and Water, Lucas Heights, where the major part of this project was undertaken. Thank you for your friendship throughout the last 8 years and particularly for your support during the past 4 years. A special thank you to Chad Jarolimek for many years of assistance with the ICP MS and Hannah Osborn for help with the Cooks River fieldwork. Particular thanks goes to Francesca, Hannah, Chris, Darren, Josh, Steve and Cat whose friendship throughout this project has made the long hours feel much shorter. Finally, a huge thank you to Tom, you have shared an office with me for the past 3 years which could not have been easy for you. Thank you for putting up with the tantrums, the mess, the distraction and the noise that came with sharing an office with me. Thank you also for the support and guidance you freely gave, for the lunchtime chats about method development, fieldwork and the difference between lipid soluble and lipophilic. Most importantly thank you for the laughs that have kept me sane when I thought nothing would.

Finally, my family and friends. Mum, Dad, Nana (who is sadly no longer with us), Papa, Sarah, Wayne, Hannah and Dan for you unending love and support. Thank you for trying to understand what I was doing every day, taking the time to listen to my problems, telling me it was possible when I felt it was impossible and pushing me to something far greater than I could have achieved on my own. I would not be where I am today without each of you and this PhD is as much yours as it is mine.

Abstract

Trace metals are normal constituents of natural waters and may be present in a wide range of physico-chemical forms or species. The bioavailability and toxicity of trace metals is dependent on their speciation. In laboratory experiments it has been demonstrated that the toxicity of some trace metals to aquatic organisms is related to the activity of the free metal ion rather than the total metal concentration, however, in a small number of studies it has been shown that toxicity and bioavailability of lipid soluble metal complexes may exceed that of the equivalent free metal ion species. Some toxicity data has shown that these lipid soluble metal complexes can be up to 25 times more toxic than free metal ions. LMSC are chemical complexes containing a metal and a biotic ligand. The nature of the LMSC means that it readily traverses membranes. LSMC are of great importance yet there is currently no method that has been developed that has the ability to accurately quantify the small concentrations likely to occur in the environment.

The aim of this study is to firstly develop a method for the determination of ultra-trace (ng/L) concentrations of lipid soluble cadmium, copper, nickel, lead and zinc complexes in water. Waters were extracted with 1-octanol, a solvent with a similar dielectric constant to that of cell membranes. A preconcentration factor of 50 was achieved by extraction of 250 mL of water into 10 mL of octanol followed by back extraction into a final volume of 5 mL of acidic matrix. Metal concentrations were subsequently determined by Inductively Coupled Plasma Mass Spectrometry (ICPMS). Detection limits (3σ) for the method were 0.002, 0.003,

0.003, 0.001, and 0.011 µg/L for Cd, Cu, Ni, Pb and Zn respectively. The detection limits of the analytical method were lower than those previously reported in the literature.

The developed method was used to conduct a survey of aquatic environments from the Sydney, NSW region. Three locations were surveyed, Centennial Park, Homebush Bay and the Cooks River. Detectable concentrations of lipid soluble Cu, Pb and Zn were found in all three locations, whereas lipid soluble Cd and Ni were only detected in the Cooks River. Lipid soluble Cd concentrations ranged from 0.002 to 0.004 µg/L, lipid soluble Cu concentrations ranged from 0.003 to 0.533 µg/L, lipid soluble Ni concentrations ranged from 0.006 to 0.025 µg/L, lipid soluble Pb concentrations ranged from 0.002 to 0.087 µg/L and lipid soluble Zn concentrations ranged from 0.020 to 0.499 µg/L. The results were comparable to previously reported lipid soluble metal complex concentrations in environmental samples.

The developed analytical method was also used to determine the concentration of lipid soluble metal complexes in mine waste water. Xanthates such as potassium amyl xanthate (PAX) are commonly used in mining processes and are expected to be common in mine waste waters. Potassium amyl xanthate is known to form neutral metal complexes. Detectable concentrations of lipid soluble Cu, Ni, Pb and Zn complexes were measured in the waste water. Lipid soluble Cu concentrations ranged from 70.5 to 84.1 µg/L, Ni concentrations ranged from 0.256 to 0.264 µg/L, Zn concentrations ranged from 0.044 to 0.050 µg/L and a single Pb concentration of 0.006 µg/L was recorded. Of these lipid soluble metal complexes, only Pb and Ni concentrations represented a considerable proportion (a

proportion greater than the limit of detection of the method) of the total dissolved Pb and Ni concentration.

The octanol/water partition coefficients of a number of neutral inorganic complexes were measured to assess the environmental significance of these complexes. In total, 14 complexes were tested. $CdCl_2$, $CuCO_3$, $Cu(OH)_2$, $NiCl_2$, $NiCO_3$, $Ni(OH)_2$, $PbCl_2$, $PbCO_3$, $Pb(OH)_2$, $PbSO_4$, $ZnCO_3$ and $Pb(OH)_2$ all returned low octanol/water partition coefficients (<0.2) indicating that if these complexes are present in natural waters they are unlikely to be lipid soluble. Octanol/water partition coefficients of 3.28 and 0.20 were calculated for the $HgCl_2$ and $B(OH)_3$ neutral complexes. The partition coefficients were comparable to quoted literature values. This result indicates that if present in natural waters the $HgCl_2$ and $B(OH)_3$ complexes may have the ability to passively diffuse across cell membranes.

While a robust method for measuring LSMC concentrations has been developed, the entrainment of colloids within the octanol extracts could be of significant concern. Based on analysis of Al and Fe within the back extracts (used as an indication of the entrainment of colloids within the back extracts) it was determined that, in a number of samples, the entrainment of colloids could be greater than the extraction of LSMC. This was of particular concern when assessing the concentration of lipid soluble Cd, Cu, Ni, Pb and Zn. The use of octanol filled dialysis cells could potentially address these concerns in addition to allowing the concentration of lipid soluble metal complexes to be determined in sediment and biota. This thesis also highlights the frequency of LSMC in the environment. Future work should also be performed to determine the concentration of lipid soluble metal complexes in a

wider range of aquatic environments, both saline and freshwater. Analysis of a wider range of waste waters from industries that used synthetic organic ligands should also be included in this investigation. The stability of both organic and inorganic lipid soluble complexes in laboratory prepared waters and natural waters should be assessed to gain a better understanding about the environmental significance of these complexes. Finally toxicity testing should be performed using a range of lipid soluble metal complexes with the measurement of actual concentration rather than relying on nominal concentrations of lipid soluble metal complexes in test solutions. This may allow for more accurate toxicity data to be generated.

Table of Contents

List of Tables

List of Figures

Chapter 1: General Introduction and Literature Review

1.1 Introduction

This chapter provides a review of the literature relating to the occurrence, toxicity and bioavailability of lipid-soluble metal complexes (LSMC) in aquatic environments. The literature review is structured as follows. First, literature on trace metal speciation in aquatic environments is examined and the significance of LSMC established. Second, literature relevant to LSMC and their formation, the toxicity and bioavailability of free metal ions and the toxicity and bioavailability of LSMC is reviewed. Finally, the literature concerned with methods used to determine the concentration of LSMC in aquatic environments is reviewed, particularly focussing on solvent extraction techniques used for the analysis of LSMC.

1.2 Trace metals in aquatic environments

Trace metals (egg. copper, zinc) are normal constituents of natural waters (Bryan, 1971; Batley et al., 2004). They are important in many metabolic processes and are required by most organisms to survive (Bryan, 1971; Florence, 1982; Ahsanullah and Florence, 1984). Typically, their concentrations in natural waters are in the µg/L range however they can be present in the ng/L range in both freshwaters and seawaters. Contamination from anthropogenic sources, such as mining and industrial processing, can increase the concentrations of trace metals in the environment. Trace metals can be toxic to aquatic organisms and hence their accurate measurement is a necessary precursor to risk assessment and management (Loon and Madgwick, 1995; Mitrovic, 1995). Common trace metals detected as a result of anthropogenic contamination include; copper (Cu), nickel (Ni),

lead (Pb), cadmium (Cd), zinc (Zn), mercury (Hg), and the non-metals arsenic (As) and selenium (Se) are also routinely detected.

Trace metals may exist in natural waters in a variety of physico-chemical forms or species (Bryan, 1971; Apte et al., 1989; Mitrovic, 1995). These forms can include the metal in its elemental state, in various oxidation states, as a free ion, an ion pair, a hydrated ion, metal absorbed to suspended sediment, a metal ligand complex or as a crystalline or amorphous solid (Simkiss and Taylor, 2001).

The bioavailability and toxicity of trace metals depend on their behaviour in ecosystems which is linked to their speciation in that particular water (Haan, 1984; Danielsson et al., 1995; Parent et al., 1996; Carvalho et al., 1999). The speciation of trace metals has a significant effect on their toxicity and bioavailability (Ahsanullah and Florence, 1984; Zorkin et al., 1986; Vasconcelos et al., 1997; Hudson, 1998; Zhang et al., 1998; Allen, 2000; Simkiss and Taylor, 2001), and their biogeochemical cycling and bioaccumulation (Haan, 1984; Blust et al., 1986; Carvalho et al., 1999; Blom et al., 2002; Turner and Mawji, 2005).

A growing amount of data exists to suggest that the most toxic metal species is the free (hydrated) metal ion (Anderson and Morel, 1978; Borgmann and Charlton, 1984) and, therefore, the concentration of free metal ions rather than the total metal concentration should be measured to predict toxicity (Blust et al., 1986; Mann et al., 2002; Batley et al., 2004). Indeed, this paradigm pervades ecotoxicology and environmental science, and led to the development of the free ion activity model and its more recent incarnation the biotic

ligand model (Morel, 1983; Campbell, 1995; Campbell et al., 2002; Paquin et al., 2002). These models predict the biological response of aquatic organisms to a particular water sample based on the concentration of free-metal ions and the fact that complexation of metals with ligands present in the water decreases their bioavailability (Morel, 1983; Boullemant et al., 2004). However, some research has suggested that the complexation of metals by natural and synthetic ligands to form neutral LSMC may represent a more toxic and bioavailable metal species than free metal ions (Florence et al., 1983; Ahsanullah and Florence, 1984; Florence and Stauber, 1986).

1.3 Lipid-soluble Metal Complexes

Trace metals present within aquatic environments can form complexes with naturally occurring complexing-agents, or ligands released from industrial activity (Bryan, 1971; Fernando, 1995). If these complexing agents possess long carbon chains or rings which are hydrophobic in nature and the resulting complex has a neutral charge then a LSMC can form (Phinney and Bruland, 1997; Wong et al., 1997; Zhang et al., 1998). By definition, a LSMC is a complex that is neutrally charged and can traverse (diffuse across) plasma membranes and enter cells by passive diffusion (Batley et al., 2004). This ability to passively diffuse across cell membranes without the need for ion exchange is one factor that makes LSMC potentially more toxic than free metal ions.

1.3.1 Examples of complexing agents that are capable of forming LSMC

A number of synthetic organic compounds have the ability to form LSMC in the presence of certain metals. These complexing agents have a wide range of industrial applications and

include xanthogenates, which are a class of compounds that are used in large quantities by the mining industry as mineral flotation agents and in the purification of mineral ores (Florence et al., 1983; Ahsanullah and Florence, 1984; Lund, 1990; Block and Glynn, 1992; Mitrovic, 1995; Batley et al., 2004; Turner and Mawji, 2005). Xanthogentaes could be present in natural systems if mine tailings or processing water is released. Xanthogenates are also used extensively in the cellulose and rubber industries (Gottofrey et al., 1988; Xu et al., 1988; Dopson et al., 2006). Due to their use in a number of industries, xanthogenates are likely to be the organic ligand of most environmental concern with respect to the formation of LSMC.

8-hydroxyquinoline (also known as Oxine) can also form LSMC. Oxine is a fungicide used in textiles such as tents. This compound does not appear to have wider environmental applications and, therefore, are unlikely to be present in high concentrations in aquatic environments (Phinney and Bruland, 1997).

Diethyldithiocarbamate (DDC) is a heteropolar, sulphur-containing organic compound that forms strong hydrophobic complexes with some metals (Zhang et al., 1998; Fraser et al., 2000; Batley et al., 2004). DDC complexes are widespread in the environment because it is used widely in agricultural fungicides and as a vulcanisation accelerator in the rubber industry (Lund, 1990; Block and Glynn, 1992; Phinney and Bruland, 1997; Zhang et al., 1998; Fraser et al., 2000).

Phenanthrolines (Ahsanullah and Florence, 1984; Turner and Mawji, 2005), lithium aluminosilicate (LAS), caffeine, myristic acid, palmitic acid and nonylphenol (Carlson and Morrison, 1995; Mitrovic, 1995) are additional examples of complexing agents capable of forming LSMC. These compounds are used sparingly in industry and therefore their concentrations in aquatic environments is predicted to be relatively low (Ahsanullah and Florence, 1984; Carlson and Morrison, 1995; Mitrovic, 1995; Turner and Mawji, 2005).

There is also some limited evidence to suggest that in natural waters containing little to no anthropogenic contamination, the toxicity of metals exceeds that predicted by the concentration of free metal ions (Borgmann and Charlton, 1984). Borgmann and Charlton found that in a number of waters they tested the toxicity of the metals of interest exceeded the toxicity predicted when the biotic ligand model was applied. A possible explanation is that in these environments, naturally occurring complexing agents may combine with metals and form complexes that are toxic to aquatic organisms (Borgmann and Charlton, 1984). The formation of LSMC with naturally occurring organic matter may indicate that even in waters which receive little to no anthropogenic contamination LSMC may be present and therefore may be having an impact on the aquatic environment. It is important to remember that this result has only been identified in one study and further investigation of the toxicity of metals in natural waters that receive little to no anthropogenic contamination needs to be performed.

Figure 1.1 below shows the structure of xanthate and diethyldithiocarbamic acid complexed to Cd forming a LSMC. In the two complexes the metal ion (e.g. Cd) is bound between 4

sulphur atoms. Similarly, Figure 1.2 shows the structure of 8-hydroxyquinoline forming a LSMC with Cu. Whilst the Cu ion has a 2^+ charge, the lipid soluble complex has a neutral charge. In the Cu – oxine, complex the Cu ion is bound between two nitrogen and two oxygen atoms (Fig 1.2).

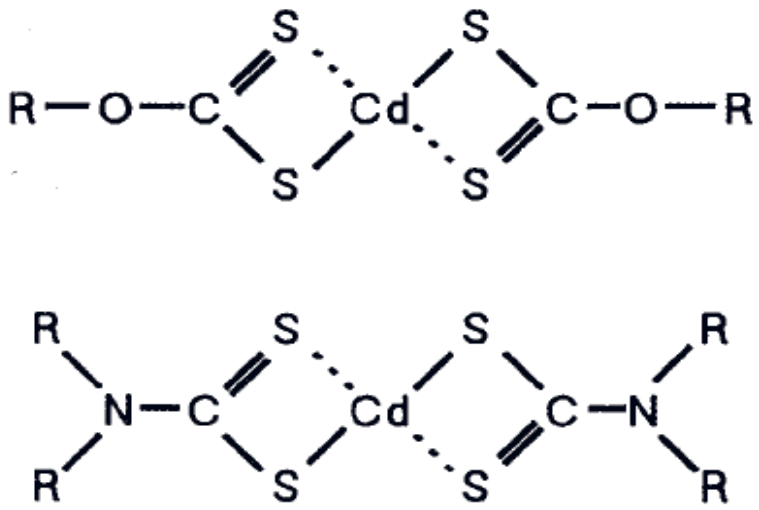

Figure 1.1 Structural figure of xanthate (upper) and diethyldithiocarbamic acid (lower) complexes with Cd. R represents an alkyl chain. Source: (Block, 1991)

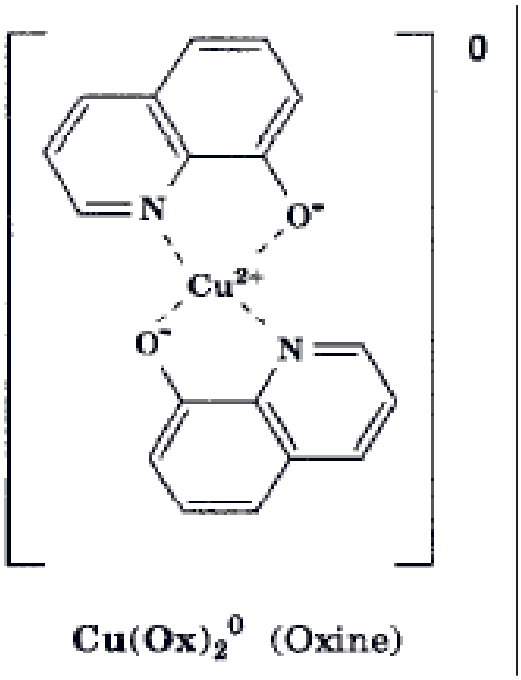

Figure 1.2 Structural diagram of 2 oxine molecules binding to Cu forming a LSMC
Source: (Phinney and Bruland, 1994)

Many metals can act in the same way as the Cd and Cu ions in the figures above, however some metals have a stronger affinity for forming LSMC than others, and they will form more stable complexes (Phinney and Bruland, 1994; Parthasarathy et al., 2010). In waters where a number of metals are present it is often the metal that forms the most stable LSMC that will tend to form first followed by the other metals in order of decreasing stability (Lund, 1990; Mitrovic, 1995). In addition, if a LSMC is already present in a water sample and additional metal contamination occurs, metal replacement may take place particularly if the newly introduced metal forms a more stable LSMC (Lund, 1990; Mitrovic, 1995).

Another class of compounds that exhibit lipid solubility are organometallic and organometalloid compounds (Wong et al., 1997). These include Tetra-ethyl lead used in petrol, Tributyl tin used in antifouling paint and pesticides. These compounds have been found in rain, snow, lakes, and rivers, and can accumulate in plankton and invertebrates and are poisonous to fish (Wong et al., 1997). Limited analysis of organometallic and organometalloid compounds in aquatic environments has been performed but it is likely that the concentrations of these compounds could potentially be enhanced in aquatic environments due to their large range of uses. Additionally, the products that use these compounds have a large amount of contact with aquatic environments (Wong et al., 1997).

1.4 Free metal ions and their toxicological importance

As previously indicated, the bioavailability of metals is dependent on their speciation. This was first established in aquatic systems where it was determined experimentally that the

total metal concentration was not a good indication of toxicity (Sunda and Guillard, 1976). The toxicity of metals to aquatic organisms was demonstrated to be related to the activity of the free metal ion rather than the total metal concentration (Florence and Stauber, 1986; Stauber and Florence, 1989; Phinney and Bruland, 1994; Vasconcelos et al., 1997; Batley et al., 2004).

There is considerable evidence demonstrating that the free metal ion is the more bioavailable and toxic metal species (Zorkin et al., 1986; Zhang and Florence, 1987; Lund, 1990; Block and Glynn, 1992; Florence et al., 1992; Mitrovic, 1995; Parent et al., 1996; Slaveykova et al., 2003; Turner and Mawji, 2004). Therefore, it has become generally accepted to consider the free (hydrated) metal ion as the most toxic and bioavailable chemical species to aquatic organisms (Blust et al., 1986; Turner and Mawji, 2005). In risk assessment studies, the toxicity and bioavailability of aquatic metal concentrations is therefore generally determined by measurement or indirect measurement of the free metal ion and not the total metal concentration.

1.5 Toxicity of Lipid-soluble Metal Complexes

There are a number of exceptions to the general rule that free metal ions are the most toxic chemical species (Poldoski, 1979; Haan, 1984; Blust et al., 1986; Zhang and Florence, 1987; Allen, 2000). In particular, lipid soluble complexes significantly increase Cd, Cu, Ni, Pb and Zn toxicity and bioavailability compared to the free metal ion for a number of aquatic organisms (Poldoski, 1979; Florence and Stauber, 1986; Zorkin et al., 1986; Stauber and Florence, 1989; Carvalho et al., 1999; Turner and Mawji, 2004). Studies have shown that the relative toxicity of a dissolved metal species to an aquatic organism is more specifically

related to the ability of the metal species to transverse a biological membrane (Poldoski, 1979; Florence, 1982).

Florence et al (1983) demonstrated that as little as 2 µg Cu /L in the presence of an appropriate complexing agent led to a depression of marine algal growth greater than that caused by 40 µg Cu /L present as the inorganic metal ions alone (Florence et al., 1983). This indicated a much higher toxicity of LSMC compared to the free metal ion. Consequently, LSMC are likely to exert deleterious environmental and biological effects that are disproportional to their concentrations relative to other metal species (Florence et al., 1992; Mann et al., 2002; Batley et al., 2004; Turner and Mawji, 2005).

Phinney and Bruland (1997) also demonstrated that the addition of synthetic organic complexing agents or ligands such as DDC and oxine to water facilitated the transport of ambient Cu and Ni into saltwater phytoplankton cells (Phinney and Bruland, 1997). Steady-state cellular Cu concentrations were over 10 times and 6 times greater for DDC and oxine treatments (respectively) than in water controls (no added ligands) and more than 6 times and 2 times greater for Ni (respectively) (Phinney and Bruland, 1997). Table 1.1 shows the toxicity of some common synthetic LSMC to fresh water single celled algae and demonstrates clearly that these compounds are up to 25 times more toxic than free metal ions.

Table 1.1 Summary of the toxicity of synthetic LSMC to algae and their ability to be extracted by octanol

Ligand	[Ligand] mol/L	Total Cu mol/L	Toxicity index*	Percentage solvent extractable
Oxine	2.0×10^{-8}	3.20×10^{-8}	13.5	Not Determined
Oxine	5.0×10^{-8}	3.10×10^{-8}	20	92
PAN	5.0×10^{-8}	3.10×10^{-8}	>25	84
TAN	5.0×10^{-8}	3.10×10^{-8}	>25	92
2,9-dmp	5.0×10^{-8}	3.10×10^{-8}	>25	91

PAN = 1-(2-pyridylazo)-2-napthol. TAN = 1-(2-thiazolylazo)-2-naphthol. 2,9-dmp = 2,9-dimethyl-1,10-phenanthroline.
* Relative to inorganic Cu^{2+} = 1.00.
Source: Florence et al, (1992)

Table 1.2 provides a summary of toxicity data derived from the extant literature (Phinney and Bruland, 1997), with the LSMC, the organism it was tested against and the results of the experiment. The results of different toxicity tests are presented as µmol of metal per cell, as a toxicity index (toxicity of the LSMC compared to the free metal ion) or as an EC_{50} (the Effective Concentration that causes a 50% reduction in the light intensity of the bacteria). The toxicity data in table 1.2 indicates that synthetic LSMC are more toxic and bioavailable than free metal ions. It is important to note that whilst some toxicity data exists, demonstrating the increased toxicity of LSMC to aquatic organisms compared to inorganic metal ions, very few toxicity studies have been performed in this area and therefore this data is very limited. The data has also only focused on the toxicity of lipid soluble Cu and Ni complexes and has failed to assess the toxicity of a range of other lipid soluble metal complexes.

Table 1.2 Summary of the toxicity data derived from various literature sources covering a range of synthetic LSMC

Complex	Organism	Results	Source
Cu – DDC	*Thalassiosira weissflogii* (Coastal Diatom)	600 µmol/cell, 10 x greater than Cu^{2+} controls	Phinney & Bruland 1997
Cu – Ox	*Thalassiosira weissflogi*i (Coastal Diatom)	500 µmol/cell, 6 x greater than Cu^{2+} controls	Phinney & Bruland 1997
Ni – DDC	*Thalassiosira weissflogii* (Coastal Diatom)	600 µmol/cell, 6 x greater than Cu^{2+} controls	Phinney & Bruland 1997
Ni – Ox	*Thalassiosira weissflogii* (Coastal Diatom)	200 µmol/cell, 2 x greater than Cu^{2+} controls	Phinney & Bruland 1997
Cu – Ox	*Nitzschia closterium* (Marine Diatom)	Toxicity index = 20. (20 x more toxic than free Cu ions)	Florence et al 1992
Cu – PAN	*Nitzschia closterium* (Marine Diatom)	Toxicity index = >25. (>25 x more toxic than free Cu ions)	Florence et al 1992
Cu – TAN	*Nitzschia closterium* (Marine Diatom)	Toxicity index = >25. (>25 x more toxic than free Cu ions)	Florence et al 1992
Cu – Oxine	*Photobacterium phosphoreum* (light emitting bacteria)	EC_{50} (30 min) = >50 µmol L^{-1}	Carlson-Ekvall & Morrison 1995
Cu – APDC	*Photobacterium phosphoreum* (light emitting bacteria)	EC_{50} (30 min) = <5 µmol L^{-1}	Carlson-Ekvall & Morrison 1995

DDC = Diethyldithiocarbamic acid. Ox = Oxine/8-hydroxyquinoline. PAN = 1-(2-pyridylazo)-2-napthol. TAN = 1-(2-thiazolylazo)-2-naphthol. APDC = Ammonium pyrrolidinedithiocarbamate.

1.6 Mechanism of toxicity and bioavailability

Lipid membranes are virtually impermeable to free metal ions and small hydrophilic solutes (Blust et al., 1986). In contrast, lipid membranes are relatively permeable to LSMC, which forms the basis for the increased toxicity and bioavailability of LSMC (Blust et al., 1986). This increased permeability is thought to be due to the fact that LSMC are neutrally charged and therefore have less resistance when passing through the plasma membrane of cells.

The uptake of LSMC by cells is thought to occur via a process of passive diffusion of the complexes across the plasma membrane and into the cytosol (Ahsanullah and Florence, 1984; Blust et al., 1986; Phinney and Bruland, 1994; Phinney and Bruland, 1997; Phinney and Bruland, 1997; Croot et al., 1999; Turner and Mawji, 2005). This process is much faster than the corresponding free metal ion transport mechanism in which free metal ions must enter cells via active transport through ion channels (Blust et al., 1986; Carlson and Morrison, 1995; Phinney and Bruland, 1997; Croot et al., 1999). This is due to the fact that the lipid membrane that surrounds cells favours the passage of only lipid-soluble materials (Raman et al., 2003). It has not as yet been accurately determined how much faster this process is, however in toxicity studies it has been determined that toxicity due to LSMC does occur more rapidly than toxicity due to free metal ions (Blust et al., 1986; Carlson and Morrison, 1995; Phinney and Bruland, 1997; Croot et al., 1999). Figure 1.3 below shows the phospholipid bilayer of a cell wall. It clearly shows the ion channels that the free metal ions must use to enter the cell. The lipid solubility of LSMC, due to their neutral charge, allows for direct diffusion of these complexes across a cells bilayer, they do not require ion channels to pass through a cell's membrane (Ahsanullah and Florence, 1984; Blust et al., 1986; Phinney and Bruland, 1994).

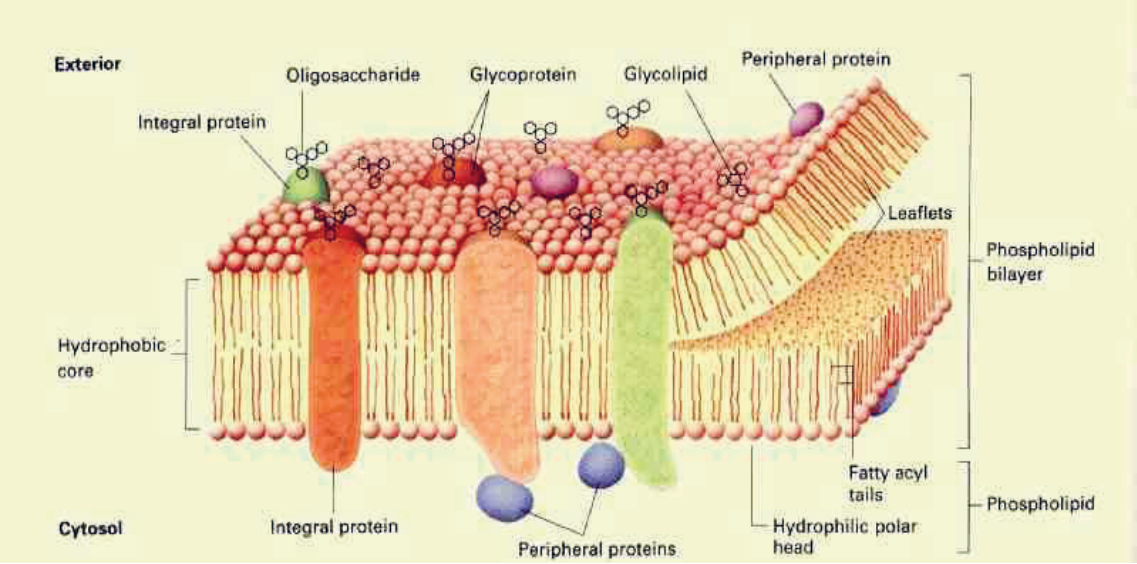

Figure 1.3 The phospholipid bilayer constitutes the basic structure of biological membranes. Integral proteins (ion channels) allow the transport of free metal ions into the cell. LSMC have the ability, due to their hydrophilic nature, and neutral charge to pass straight through cell walls.

Source: (http://www.uic.edu/classes/phys/phys450/MARKO/memb2.jpg)

Once inside the cell the metal is thought to dissociate from the ligands and complex to intracellular metal binding sites such as sulphur-rich phytochelatins as shown in Figure 1.4 (Blust et al., 1986; Phinney and Bruland, 1994; Phinney and Bruland, 1997; Phinney and Bruland, 1997; Croot et al., 1999; Turner and Mawji, 2005). This mechanism is demonstrated by the bioaccumulation of metals in the lipid bodies of algal cells indicating that this dissociation occurs within cells (Wong et al., 1997). It has also been proposed that the ligand is then ejected from the cell and can bind to another metal ion to form another LSMC. This newly formed LSMA can then diffuse back across the cell membrane, thus creating a cyclic transport mechanism. (Florence and Stauber, 1986). This ejection of the ligand from the cell has been observed for 2,9-dimethyl-1,10-phenanthroline in tumour cells and *Nitzschia* cells (Croot et al., 1999). Uptake via the cyclic transport mechanism can continue in this manner

until all the available intracellular binding sites are occupied (Blust et al., 1986; Phinney and Bruland, 1994; Phinney and Bruland, 1997; Croot et al., 1999; Turner and Mawji, 2005), thus maximising the potential metal saturation in a cell, enhancing its toxic effects.

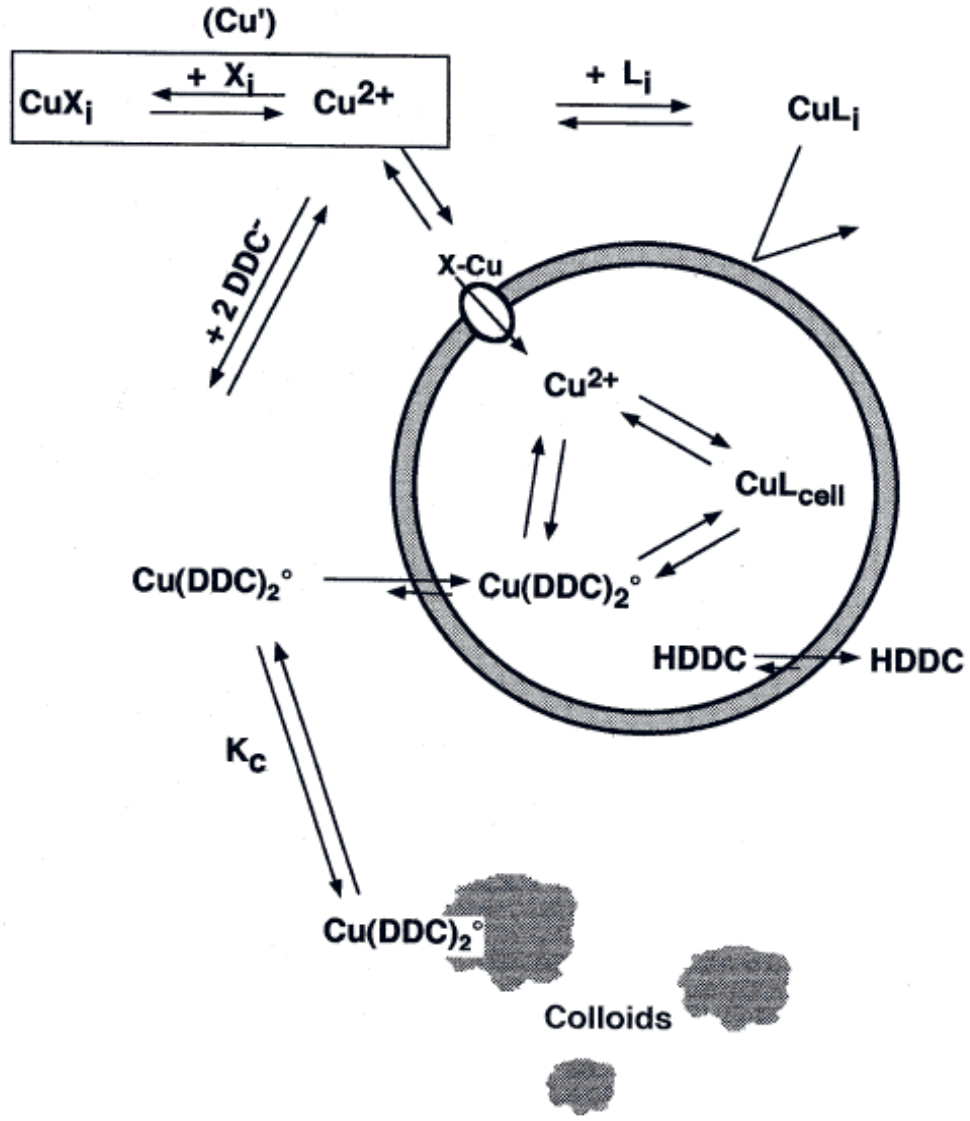

Figure 1.4 Model of the fate of Copper in the presence of DDC
X_i = biotic ligand
Source: (Phinney and Bruland, 1997)

A second proposed mechanism of toxicity involves more stable LSMC that do not dissociate intracellularly. These stable LSMC catalyse the formation of highly damaging hydroxyl radicals from molecular oxygen in a Fenton-type reaction (Florence et al., 1983; Florence and Stauber, 1986; Stauber and Florence, 1987; Barbehenn et al., 2005; Turner and Mawji, 2005). It is proposed in this mechanism that both the LSMC and the hydroxyl radical cause toxicity (Blust et al., 1986; Phinney and Bruland, 1994; Phinney and Bruland, 1997; Croot et al., 1999; Turner and Mawji, 2005).

Florence et al. (1992) suggested that the ligands themselves can exert a toxic effect at certain concentrations. Algal cell death occurred in the presence of 3.1×10^{-8} mol/L of 1-(2-pyridylazo)-2-napthol (PAN) with no added copper. This may provide an explanation for the extreme toxicity of LSMC as they can contain two toxic portions, the metal and the ligand (Florence and Stauber, 1986; Stauber and Florence, 1987; Stauber and Florence, 1989; Florence et al., 1992). Figure 1.4 below shows a model of the fate of Cu in the presence of the ligand DDC, which is capable of forming a LSMC. The fate of the free copper ion, Cu^{2+}, copper bound in a LSMC, $Cu(DDC)_2^0$, and copper bound in an organic complex, CuL_i, can all be seen. The lipid-soluble copper complex is shown entering the cell, dissociating within the cell, the Cu^{2+} ion binding to intracellular binding sites, L_{cell}, and the ligand, HDDC, being ejected. Other metals can act in a similar way to cause toxicity.

1.7 Concentrations of LSMC in aquatic environments

Whilst some data exist about the toxicity and bioavailability of synthetic LSMC on a range of aquatic organisms, they are often still neglected in aquatic contamination and biotoxicity

studies as there are no sensitive, reliable and robust methods for the measurement of LSMC in waters (Florence et al., 1983; Florence et al., 1992; Fraser et al., 2000; Turner and Mawji, 2005). A reliable and sensitive analytical method capable of detecting very small quantities (i.e. sub – μg/L) of LSMC in aquatic environments is needed (Batley et al., 2004). Since LSMC are normally present in low concentrations but can be up to 25 times more toxic than free metal ions (Florence et al., 1992), the detection limits of any technique used to measure LSMC should ideally be at least 25 times more sensitive than those used for other metal species.

The occurrence of natural LSMC in any aquatic environment has not been reported in the literature. Borgmann and Charlton (1984) determined that waters from Hamilton Harbour and Lake Ontario demonstrated greater toxicity than could be predicted from the concentration of free metal ions alone. They concluded that natural complexing agents were capable of forming metal complexes that are toxic to aquatic organisms. Unfortunately, it is difficult to determine if these complexes were lipid soluble, however, their higher toxicity (compare to that of the free metal ion) suggests the ability to transverse the plasma membrane. Research into the structure or concentration of these metal complexes was not performed during the study and therefore it is difficult to accurately determine whether the complexes displayed some lipid solubility.

Research has been conducted however to investigate the effect of naturally occurring humic and fulvic organic matter on cell membrane permeability and the uptake of LSMC (Cambell et al 1995). Investigation into this area is important as it is thought that the presence of

humic and fulvic acids and the complexation of metals with these natural organics, will reduce their toxicity and bioavailability by binding the metals in non-bioavailable complexes. (Parent et al., 1996; Vigneault et al., 2000; Paquin et al., 2002). Parent et al (1996) found that fulvic acid increased the uptake rate of sorbitol (a surfactant) by a unicellular green alga. This indicated that the fulvic acid was increasing the permeability of the cell membrane to organic ligands which exhibit some lipid solubility. Vigneault et al, (2000) investigated the effect of both humic and fulvic acids on the permeability of actual and model phytoplankton membranes. Humic acid had a much more pronounced affect than fulvic acid however some affect was seen with both acids. Results indicated that humic and fulvic acid induced an increase in membrane permeability by up to 30% which could affect the passive diffusion of both LSMC and other neutral inorganic species. This result was observed in both the actual and the model cell membranes and suggests that the interactions between these organic molecules and cell membranes are sensitive to electrostatic interactions (Vigneault et al., 2000). The results were complicated by the fact that natural organic matter generally reduces toxicity and bioavailability which may mask any potential increase in bioaccumulation resulting from the effect of humic and fulvic substances on membrane permeability. This research does however indicate that the presence of natural organic matter may have an effect of the bioavailability and toxicity of LSMC present in waters and this affect should be closely monitored. The investigation unfortunately did not identify if naturally occurring LSMC may be present in waters and if these complexes are likely to be of environmental concern.

Currently, all published literature relating to the structure, toxicity and bioavailability of LSMC relates to the binding of metals to synthetic anthropogenic ligands such as DDC and oxine (Carlson-Ekvall and Morrison, 1995; Phinney and Bruland, 1997). Furthermore, there is a paucity of knowledge regarding the existence of synthetic LSMC in aquatic environments. There is, however, indirect evidence to suggest that these types of complexes may be present in certain bodies of water, particularly those near industrial activities that use complexing agents (Phinney and Bruland, 1994; Phinney and Bruland, 1997). LSMC are likely to occur at very low concentrations (Turner and Mawji, 2004) which makes analysis difficult. Consequently, the presence of LSMC has only been reported in a small number of aquatic environments (Mitrovic, 1995; Turner and Mawji, 2005; Kilgore, 2007). Turner and Mawji (2005) found samples from the River Clyde in southwest Scotland that contained dissolved metals that exhibited some octanol-solubility. It was concluded from this study that metal complexes that are sufficiently lipid soluble, i.e. LSMC, can exist in contaminated rivers like the River Clyde. The LSMC that were analysed by Turner and Mawji (2005) included; Al, Cd, Cu, Mn, Ni, Pb and Zn. The concentrations of these LSMC in the River Clyde are shown in Table 1.3 below.

Table 1.3 Measured Concentrations of LSMC in the River Clyde

Metal	LSMC concentration (μg/L)
Al	0.16 ± 0.009
Cd	0.027 ± 0.040
Cu	0.15 ± 0.063
Mn	0.44 ± 0.032
Ni	0.21 ± 0.023
Pb	0.56 ± 0.11
Zn	0.24 ± 0.021

Source: (Turner and Mawji, 2005)

Lipid soluble metal complexes have been detected in sewage sludge (Carlson and Morrison, 1992; Carlson and Morrison, 1995). The toxicity of Cu in raw and digested sewage sludge was found to be much higher than could be explained by the concentration of free Cu ions alone (Carlson and Morrison, 1992; Carlson and Morrison, 1995). This increased toxicity was attributed to the formation of lipid-soluble copper complexes possibly from products like shampoos, soaps and fats (Carlson and Morrison, 1995). In addition to these sources of complexing agents, LSMC can form in sewage sludge from products such as sodium nitrilotriacetate (NTA), lithium aluminosilicate (LAS) and tri-n-butylphosphate from household cleaners, nonylphenol ethoxylates from industrial cleaners, plasticisers like phthalates, and also coagulators (Carlson and Morrison, 1995). It is likely that caffeine, which is common in municipal sewage also complexed metals. Unfortunately, the concentration of these LSMC was not measured during the experiment and the results of the investigation are complicated by the fact that sewage sludge is likely to contain a range of contaminants (not just metals) which could attribute the higher than expected toxicity. To

accurately determine if LSMC are the reason for the increased toxicity, the concentration of these complexes needs to be accurately determined and not just inferred.

The research of Borgmann and Charlton (1984) identified the presence of toxic copper complexes with natural ligands in waters collected from the Burlington Canal (originating from Hamilton Harbour) and waters collected from Lake Ontario. Canal water collected in both summer and winter and lake water collected during winter all demonstrated greater toxicity to the fresh water cladoceran *Daphnia magna* than that predicted by the free metal ion concentration (Borgmann and Charlton, 1984). The increased toxicity of the waters compared to the concentration of the free copper ion was thought to be due to the presence of natural metal ligand complexes (Borgmann and Charlton, 1984). This study was the first to suggest that natural metal complexes may be potentially toxic and able to transverse plasma membranes. Unfortunately the concentration and structure of these complexes was not determined in this research and, as with the two Carlson and Morrison studies, the increased toxicity was only assumed to be due to the formation of LSMC. No direct analysis of the concentration of LSMC was performed and there no direct link was made between the increased toxicity and the concentration of LSMC.

Boullemant et al. (2004) investigated the effect of changes in pH and the addition of humic acid to the formation and uptake of Cd LSMC by the green algae *Pseudokirchneriella subcapitata*. They determined that uptake of the $Cd(DDC)_2^0$ was lower at pH 6 and 5.5 than at pH 7, however, uptake at these pH values was still greater than the uptake of free Cu ions (Boullemant et al., 2004). In the presence of added humic acid at pH 5.5, uptake of metal

was enhanced, however, uptake was reduced when the pH was increased to 7. These results indicate that in natural waters with varying pH and varying concentrations of natural organic matter, the formation and uptake of LSMC is controlled by a number of factors, some of which increase and some of which decrease the formation and uptake of these complexes (Boullemant et al., 2004). As outlined earlier, the presence of natural organic matter can increase the permeability of membranes to LSMC but at the same time reduce the amount of LSMC by competitive binding effects (Vigneault et al., 2000).

1.8 Analytical methods for detecting LSMC in aquatic environments

At present there is no generally accepted method for determining the nature and extent of the complexation of trace metals in natural waters (Haan, 1984; Fernando, 1995; Blom et al., 2002). Indeed, there are very few direct experimental methods that are available to assess accurately the speciation of metals, and this is particularly true for the measurement of LSMC. Some methods that are capable of determining the speciation of metals in waters include anodic stripping voltammetry (ASV), high performance liquid chromatography (HPLC), ion exchange Chelex-100 resin, ultrafiltration and dialysis (Florence, 1982). Of these techniques, few include a step for the sole determination of metal complexes that are directly lipid-soluble. In particular, LSMC may not be ASV-labile, and may not be dissociated by a chelating resin, therefore other techniques are needed for the detection of LSMC (Florence, 1982; Carlson and Morrison, 1995; Turner and Mawji, 2005).

The release of metals into the environment through anthropogenic activities is widespread and in many parts of the world is escalating (Mann et al., 2002). Additionally, the number of

ligands and complexing agents capable of forming LSMC released into the environment is likely to increase by 1-2% each year due to increases in agriculture, mining and industrial processing (Fernando, 1995). These circumstances are a consequence of the development of new products which require the complexing agents capable of forming LSMC in their manufacture. Consequently, it is desirable to have a method that can determine accurately the concentration of LSMC in aquatic environments (Fernando, 1995). Even taking into account the possible contamination of aquatic environments by trace metals and complexing agents, LSMC concentrations in aquatic environments are expected to be low (ng/L range) and samples could easily be contaminated during collection and analysis (Allen, 2000). To overcome this problem, specialised techniques and equipment must be used when analysing for LSMC in aquatic environments (Allen 2000). Clean techniques are essential and, if not used, can have a significant effect on the accuracy of results. For instance, Allen (2000) found that, in seawater samples, dissolved Cd concentrations up to 30-fold lower, dissolved Cu concentration up to three-fold lower, dissolved Pb concentrations up to 100-fold lower and dissolved Zn concentrations up to 20-fold lower were measured when clean techniques were used as opposed to when they were not (Allen, 2000).

1.8.1 Water analysis methods

Several techniques have been trialled to measure the concentrations of LSMC in waters (Zhang and Florence, 1987). The separation of LSMC from the water is a critical step in the analytical process. Zhang and Florence (1987) trialled an aluminium hydroxide-coated column of sulfonic cation-exchange resin to quantitatively adsorb LSMC. The adsorbed

LSMC were then leached selectively from the column with methanol. This approach was also used for copper complexes; 8-quinolinolate and copper ethylxanthogenate (2 LSMC). While this approach was effective at absorbing LSMC, it was limited in that free metal ions were also adsorbed onto the column meaning that the concentration of LSMC measured was positively biased. The adsorption of the free metal ions was overcome by selectively leaching the free metal ions from the column with Diethylene triamine pentaacetic acid (DTPA) (Zhang and Florence, 1987) but the detection limits of the technique (in the ug/L range) were not low enough to measure the expected concentrations of LSMC in aquatic environments. Unfortunately the method was not tested on environmental samples and its ability to detect LSMC in natural systems remains unproven.

Apte et al. (1989) and Roy and Cambell (1997) independently developed methods that used dialysis cells as a size exclusion barrier to determine metal species in waters. In these methods, a small dialysis cell was filled with water and placed in a water sample or water body under investigation. A positive flux of species able to diffuse through the dialysis membrane occurs until the concentrations on the inside and the outside of the cell reach equilibrium (Apte et al., 1989). By varying the pore size of the dialysis membrane larger or smaller metal species can be selectively determined. The dialysable fraction (i.e., that fraction of metal that diffused into the cell) is comprised of inorganic complexes, the free metal ion and relatively small organic complexes (Apte et al., 1989). A limitation of this technique is its non-selectivity for only lipid-soluble metal species. The concentration of metals in the cell is comprised of a number of species, some of which are not LSMC. A

second limitation of this technique relates to the detection limits afforded, which were not adequate to measure environmentally relevant concentrations of LSMC.

Florence (1982) trialled the Bio-Rad SM2 resin column to remove LSMC from water samples. Bio-Rad SM2 resin is a neutral, non-polar, macroporous, styrene-divinylbenzene co-polymer with a high affinity for molecules containing both hydrophilic and hydrophobic moieties (Florence, 1982). This method involved passing 25-mL of sample through a column of Bio-Beads SM2 resin at a rate of 1.5 mL/min. The total metal concentration of the water was determined by ASV before and after it was passed through the Bio-Rad SM2 resin column. The LSMC concentration was determined indirectly by subtracting the difference between these two measurements (Florence, 1982). Although this method offers some selectivity for LSMC, both hydrophilic and hydrophobic complexes, as well as free metal ions, are simultaneously adsorbed onto the column (Florence, 1982). Selectivity was increased by the addition of a citrate buffer of pH 5.7 to prevent the co-adsorption of free metal ions (Florence, 1982), however, hydrophobic and hydrophilic complexes were still both adsorbed to the column. LSMC are hydrophobic in nature. By using this method the concentration of LSMC would be overestimated by the addition of complexes that are hydrophilic. In order to measure LSMC exclusively, the adsorption of hydrophilic complexes needs to be eliminated.

Turner and Mawji (2004) developed another column resin technique using a C18 column. In this method, 3 x 10-mL aliquots of water sample were passed through a methanol-conditioned reverse phase C18 column. Akin to Florence (1982), the concentration of LSMC

was determined indirectly by calculating the difference between mean metal concentration before and after passing through the column (Turner and Mawji, 2004). Using this method Turner and Mawji (2004) found that retention of metal species onto the column was greater than the extent of metal extraction by octanol, resulting in an overestimation of the LSMC concentrations. In addition, Turner and Mawji (2004) showed that some charged species (inorganic metal complexes) were adsorbed to uncapped silanol groups in the column resulting in further overestimation of the LSMC concentration.

Permeation liquid membranes (PLM) have also been trialled as a method for detection of LSMC in waters. PLM is based on liquid-liquid extraction principles where extraction, back extraction and preconcentration of the target species is achieved in a single step (Parthasarathy et al., 2004; Parthasarathy et al., 2010). Parthasarathy et al. (2010) developed a method capable of detecting the Cu-oxine LSMC in both laboratory prepared samples and tap water samples spiked with the Cu LSMC. The method used a hollow fibre permeation liquid membrane (HFPLM) in which the membrane was impregnated with a 1:1 (v/v) mixture of toluene/phenylhexane. The HFPLM was placed into a water sample and the LSMC then diffused across the PLM and entered a stripping solution (5×10^{-4} mol/L transcyclohexanediaminotetraacetic acid (CDTA)). This stripping solution was removed from inside the HFPLM and analysed for metals by Graphite Furnace Atomic Adsorption Spectroscopy (Parthasarathy et al., 2010). This method was selective for only LSMC and, by having a small stripping solution volume compared to the sample volume, extraction and preconcentration of LSMC was achieved in one step. The detection limits of the developed

method were in the low ng/L range making it acceptable for the determination of LSMC in natural waters (Parthasarathy et al., 2010).

The method of Parthasarathy et al. (2010) provide very positive steps towards the development of a robust method for LSMC quantification. The method had acceptable detection limits, was selective for LSMC and had good recovery of the Cu-oxine complexes from the spiked samples. However, the method has not been tested on natural water samples which are likely to contain a much more complex range of LSMC than just the Cu-oxine complex used in the experiments. In addition, natural waters are likely to have a much more complex sample matrix than tap water. Parthasarathy et al (2010) state that the tap water was chosen "to avoid ambiguity on the possible effect of humic substances on lipophilic complex determination". Without further testing of the method against natural waters it is difficult to determine the effectiveness of the method at measuring LSMC concentrations in waters with a more complex sample matrix.

The method of Parthasarathy et al (2010) was only tested against the Cu-oxine complex, and therefore requires validation against a larger range of LSMC. It is also possible that combination of the toluene/phenylhexane PLM in conjunction with the stripping solution may assist in the diffusion of LSMC across the membrane. If this is the case then determinations made using this method would provide results that are potentially higher than the concentration of LSMC that would actually enter the cells of aquatic organisms. Furthermore, the method appears to be quite complex. Whilst extraction, back extraction

and preconcentration are all achieved in the one step, setting up and carrying out the HFPLM technique can be a complex process. It is important to note that it is possible to make an estimation of the LSMC concentration in water samples using the PLM technique described by Parthasarathy et al (2010). The LSMC concentration can be back calculated from the total metals concentrations measured during this technique, however determining concentrations of LSMC by back calculation has the potential to introduce errors and affect the limit of detection of the technique.

Solvent extraction of waters for the determination of LSMC is the most common method of analysis. This is due to the fact that this method involves the extraction of LSMC into a solvent whose dielectric properties are similar to those of a cells lipid bilayer (Florence et al., 1983; Block, 1991; Friedel et al., 1994; Danielsson et al., 1995; Mitrovic, 1995; Zhang et al., 1998; Turner and Mawji, 2004; Turner and Mawji, 2005). A number of different solvents have been trialled including olive oil (Blust et al., 1986; Turner and Mawji, 2004), chloroform (Florence, 1982), hexanol (Turner and Mawji, 2004), hexane-butane (Florence, 1982; Florence et al., 1983; Turner and Mawji, 2004) and *n*-octanol (Mitrovic, 1995; Turner and Mawji, 2004; Turner and Mawji, 2005). Of these solvents *n*-octanol is most commonly used as it has the most similar dielectric constant to that of a cell's lipid bilayer (Florence et al., 1983; Block, 1991; Friedel et al., 1994; Danielsson et al., 1995; Mitrovic, 1995; Zhang et al., 1998; Steel and Walter, 2003; Turner and Mawji, 2004; Turner and Mawji, 2005). In addition, the extraction of ionic metal species into octanol is not significant and the chemical composition and speciation of a water sample is not changed by the addition of octanol (Turner and Williamson 2005).

Turner and Mawji (2004; 2005) used a shake flask method of solvent extraction with octanol to extract LSMC. This involved the addition of 5 mL of octanol to 5 mL of water sample. The octanol and water were then mixed on a horizontal shaker for 16 hours. The water was then removed from the octanol for metal analysis. The octanol-extractable metal concentrations were determined from the difference between the metal concentration before and after the solvent extraction procedure (Turner and Mawji, 2004). Whilst this technique offered selectivity for only LSMC, detection limits were not adequate to determine ng/L changes in the metal concentration, thus small concentrations of LSMC (like those likely to be present in environmental samples) could not be measured.

Mitrovic (1995) also used a shake flask method of solvent extraction with octanol. Under cleanroom conditions octanol (5 mL) was added to 250 mL of water sample in a separating funnel. The sample was shaken by hand for 5 minutes after which the octanol was removed and the process was repeated. The two 5-mL aliquots of octanol were combined as one solvent extract (Mitrovic, 1995). To analyse the LSMC now present in the octanol they must be transferred to a matrix that is suitable for analysis by typical analytical instrumentation. This was achieved by back extracting the LSMC into an acidic medium before analysis by Graphite Furnace Atomic Absorption Spectrometry (GFAAS). An advantage of Mitrovic's (1995) method over the one developed by Turner and Mawji (2004) is that pre-concentration of LSMC occurs. A total volume of 10 mL of octanol is used to extract LSMC from 250 mL of water (Mitrovic, 1995). This results in a pre-concentration factor of 25, meaning that the concentration of LSMC in the octanol is 25 times the concentration in the original water sample (Mitrovic, 1995). Pre-concentration is furthered by back extracting the

metals into a final back extract volume of 5 mL giving a total pre-concentration factor of 50. Pre-concentration allows easier analysis by GFAAS and increased sensitivity. The technique of preconcentration is essential in ultra-trace analyses and is particularly important when analysing for LSMC due to their low concentrations in aquatic environments. A preconcentration factor of 50 decreases the limits of detection (LOD) for the technique by 50 times allowing for much smaller concentrations of LSMC to be analysed.

Limitations do however exist with solvent extractions as with the other methods mentioned. Turner and Mawji (2005) found that hydrophobic ligands are, to some extent, metal specific, or that the lipid solubility of the resulting complexes are metal dependent (Turner and Mawji, 2005). This suggests that some metal complexes will partition into the solvent to a greater degree than others. This may underestimate the concentration of LSMCs in water samples. In addition, the back extraction of metals from octanol can result in an underestimation of the LSMC concentrations if not all of the LSMC can be removed from the octanol into an acidic matrix. Even with these limitations, solvent extraction has shown the most promise as a method for the determination of LSMC in natural waters.

The method developed by Mitrovic (1995) was further developed by Kilgore (2007). A more robust and reliable back extraction technique was developed, contamination during sample analysis was greatly reduced, and further method performance tests were performed (Kilgore, 2007). The initial method was improved by this research, however, a number of drawbacks with the method were still evident. The major drawbacks revolved around the quality control and quality assurance of the data and the reproducibility of results. Even

with these drawbacks, the method was used to determine the concentration of LSMC in a number of freshwater and marine aquatic environments in the Sydney region (Kilgore, 2007).

1.9 Project Aims

LSMC may be environmentally significant and in laboratory studies their toxicity and bioavailability to aquatic organisms has been briefly documented within the literature (Lund, 1990; Florence et al., 1992; Carlson and Morrison, 1995; Phinney and Bruland, 1997; Vasconcelos et al., 1997). Additionally the toxicity and bioavailability of LSMC has been shown to exceed that of free metal ions (Bryan, 1971; Zhang and Florence, 1987; Block and Glynn, 1992; Mitrovic, 1995; Turner and Mawji, 2004). Whilst evidence of LSMC in different aquatic environments is limited, it has been noted that there is the potential for their formation in a number of environments contaminated by both trace metals and also organic complexing agents (Carlson and Morrison, 1995; Fernando, 1995; Fraser et al., 2000; Turner and Mawji, 2005). The increased toxicity and bioavailability of LSMC compared to free metal ions (Bryan, 1971; Zhang and Florence, 1987; Block and Glynn, 1992; Mitrovic, 1995; Turner and Mawji, 2004) necessitates that LSMC concentrations in aquatic environments be further investigated. With this in mind the major aims of the project are:

(i) To develop a sensitive, accurate, precise and robust method for the determination of LSMC in waters based on that developed by Mitrovic (1995) and Kilgore (2007).

(ii) To conduct an environmental survey of a number of different aquatic environments to establish the environmental significance of LSMC.

(iii) To investigate the importance of neutral inorganic metal complexes through the determination of the octanol/water partition coefficients of a range of neutral inorganic complexes**.**

(iv) To identify the toxicity of the PAX-metal LSMC to aquatic organisms. During this toxicity testing the method developed in (i) should be used to calculate the actual concentration of LSMC in the test waters rather than just relying on the nominal calculated values.

Chapter 2: General Methods

2.1 Introduction

This chapter outlines a number of specialised ultra-trace analytical procedures that were used throughout the project. The procedures were followed as outlined in this chapter unless otherwise stated.

2.2 Cleaning of Equipment

A number of cleaning processes were used to ensure that all the equipment was free from contamination.

Polycarbonate vials (20, 30 and 120 mL vials, ICP – MS vials) were acid soaked for at least 24 hours in 10% nitric acid (v/v). Vials were rinsed thoroughly with Milli-Q water (ultra-clean deionised water 18 MΩ Millipore, Australia) and dried in a laminar flow cabinet before use.

Nalgene 1-L high density polyethylene bottles (both narrow and wide mouth) were washed for 2 hours in detergent (Decon 90) and then rinsed with copious amounts of Milli-Q water before being soaked in 10% nitric acid (v/v) for 24 hours. After 24 hours, the bottles were removed and thoroughly rinsed inside and out with Milli-Q water before being dried in a laminar flow cabinet. Once dry, the bottles were doubled bagged in zip lock bags before being filled with 1% nitric acid (v/v) for 48 hours. After 48 hours the bottles were emptied, the inside was washed 3 times with Milli-Q water and again placed inside the two zip lock bags ready for use.

Sartorius polycarbonate filtration units were cleaned by dismantling the unit and placing the plastic sections in 10% nitric acid (v/v) for 24 hours and the rubber o-rings in 1% nitric acid (v/v) for 24 hours. After 24 hours, the filtration unit was rinsed with Milli-Q water and dried in the laminar flow cabinet. Once dry the filtration unit was reassembled and placed in a zip lock bag ready for use.

Fluorinated ethylene polyethylene (FEP) separating funnels used for solvent extractions were washed by first placing them in 2% detergent (v/v) for at least 2 hours to remove any traces of residual solvent (e.g. octanol) after which they were removed and rinsed thoroughly with Milli – Q water. They were then filled with 50% nitric acid (v/v) and submerged in 10% nitric acid (v/v). The funnels were washed for at least 12 hours, after which, they were rinsed inside and out 3 times with Milli-Q water to remove any traces of acid. The funnels were then used immediately for solvent extractions.

Teflon Oak – Ridge centrifuge tubes were washed in a similar way. Tubes were placed in 2% detergent (v/v) for 2 hours, rinsed with Milli – Q water before being acid washed in 50% nitric acid (v/v) for no less than 12 hours after which they were rinsed inside and out 3 times with Milli-Q water before use. The centrifuge tubes were placed in racks with their lids securely fastened and left in the laminar flow cabinet until required.

Pipette tips (200 µL, 1 mL and 5 mL tips) were acid washed in 10% nitric acid (v/v) and were rinsed thoroughly with Milli-Q water directly before use. A 1% nitric acid solution (v/v) was used to rinse pipette tips between uses.

Glass vials (with Teflon lined lids) used for nitrogen blow down experiments were washed in 10% analytical grade nitric acid (v/v) for 24 hours, rinsed three time with Milli – Q water followed by soaking in 10% trace – pure nitric acid (v/v) for 24 hours before being rinsed 3 times with Milli – Q water. The lids were soaked in 1% trace pure nitric acid for 2 hours before being rinsed 3 times with Milli – Q water.

All the glassware used for vacuum distillation (round bottom flasks, Y shaped connectors and the condenser) was washed in 2% detergent (v/v) for at least 2 hours, rinsed thoroughly with Milli-Q water followed by soaking in 10% trace-pure nitric acid (v/v) for 24 hours. The glassware was then removed from the acid and rinsed 3 times with Milli-Q water before being placed in the laminar flow cabinet to dry.

2.3 Specialised Ultra Trace Apparatus

As it was anticipated that environmental concentrations of LSMC would be in the ng/L range, extreme care was taken to ensure that samples are not contaminated with metals during analysis. To reduce the potential for contamination, an ultra-trace metal Class 300 cleanroom was used. Within the room was a Class 100 laminar flow ultra-clean air cabinet which provided an area where all experimental analysis could be performed free from atmospheric contamination as well as drying of all acid washed equipment before use.

2.3.1 Inductively Coupled Plasma Mass Spectrometry

Inductively coupled plasma mass spectrometry (ICP MS, Agilent, 7500ce) was used for all metal analysis throughout the project. The ICP MS is a multi-elemental analysis instrument

that allows for the rapid detection of a large range of elements. Back extracts (10% HNO_3 (v/v)) and waters (0.2% HNO_3 (v/v)) were analysed in separate batches, each with their own matrix-matched standards. The typical instrument calibration range was 0-500 µg/L. Samples were analysed against internal standards (to correct for minor matrix interferences) and were drift and blank corrected where necessary. The focus of the investigation was on Cd, Cu, Ni, Pb and Zn however Al and Fe and Sr and Li were also analysed to identify any colloidal contamination and ionic metal contamination respectively. A suite of other elements was also analysed as part of the ICP MS analysis programme.

2.4 Solvent Extraction

Solvent extraction was the preferred method of analysis as it incorporates preconcentration of the sample during analysis. Preconcentration was achieved during solvent extraction by using a smaller volume of final aqueous back-extract than the original water sample volume. The initial method that was trialled was developed by Kilgore (2007) based on initial work performed by Mitrovic (1995). Mitrovic (1995) developed a method which afforded some useful results and dealt with much of the early method development involved in solvent extractions (choice of solvent, extraction volume, extraction time, back extraction procedure etc.). There were, however, a number of issues associated with the developed method including issues of quality control, quality assurance, contamination and replication within the method (Mitrovic, 1995). Kilgore (2007) furthered this method and attempted to improve the quality control and assurance issues.

2.4.1 Preparation of the APDC/Sodium bicarbonate solution

The APDC/Sodium bicarbonate solution was added to acidified samples when APDC-metal LSMC needed to be formed. The sodium bicarbonate raised the pH to between 7 and 8 and the APDC was present to complex metals spiked into the solution and form the APDC-metal complexes of interest.

Sodium bicarbonate (12.5 g, BDH 99%) and 0.75 g of ammonium pyrrolidinedithiocarbamate (APDC, Sigma, 99%) were added to a 250 mL volumetric flask. The flask was made up to volume with Milli-Q water. The solution was then transferred to an acid washed 500 mL FEP separating funnel. The solution was purified by solvent extraction. Ten millilitres of 1,1,1-trichloroethane (Sigma, 99%) was added to the flask, the flask was shaken by hand for five minutes after which the phases were allowed to separate for five minutes. The 1,1,1-trichloroethane was removed to waste before extraction with a second 10 mL volume of 1,1,1-trichloroethane. The solvent extraction of the APDC/sodium bicarbonate solution ensures that the solution is low in trace metals and does not contaminate samples it is added to.

2.4.2 Preparation of oxine solution

The synthetic oxine ligand is only sparingly soluble in water and therefore this solution must be made up in methanol. Four grams of oxine (BDH 99% pure) was added to a 100 mL volumetric flask. The flask was made up to volume with methanol (Sigma Aldrich 99%). Due to the use of methanol this solution was unable to be purified in a similar manner to the

APDC solution. Possible metal contamination from this solution should be carefully measured and corrected for if necessary.

2.4.3 Procedure for extraction of LSMC

A 250 mL aliquot of water was accurately weighed into a 500 mL FEP separating funnel. If the pH of the sample was above 7.5, small volumes of nitric acid (Merck, Trace Pure) were used to adjust the pH to between 7 and 7.5. Extraction of the samples was then performed by the addition of 5 mL of 1-octanol (Sigma, 99%); the samples were shaken by hand for 5 minutes after which the two phases were allowed to separate for 5 minutes. The bottom layer (the aqueous phase) was transferred to a second separating funnel whilst the 1-octanol layer (top layer) was removed to an acid washed centrifuge tube (Oak Ridge, FEP). A second extraction was performed on the aqueous phase with a second 5 mL aliquot of 1-octanol. After the phases were allowed to separate the aqueous layer was removed to waste whilst the second extract was combined with the first. Due to interferences caused by the chemical structure of 1-octanol, analysis of the extracts directly by ICP MS was not possible. To overcome this interference, LSMC were back-extracted into an acidic medium which could be successfully analysed by ICP MS.

2.4.4 Microwave Assisted LSMC Back Extraction

Water droplets present in the 1-octanol extracts were first removed using an acid washed 1 mL pipette followed by addition of 0.5 mL of concentrated trace-pure nitric acid. Extracts were then heated to 90°C for 5 minutes using a domestic microwave (1100 Watt, Panasonic Inverter). To ensure that the extracts were at 90°C for 5 minutes an initial heating period

was used. The back extracts were spaced equally around the edge of the microwave plate and heated for 3 minutes on 30% power followed directly by 5 minutes on 20% power. The back extracts were then shaken by hand for 5 minutes. The heating and shaking steps were then repeated. Finally, 4.5 mL of Milli-Q water was added to each of the back extracts before they were shaken by hand for 10 seconds to mix. The phases were allowed to separate for at least 5 minutes before the aqueous back extracts were analysed by ICP MS. The initial volume of 250 mL of water was extracted into a final back extract volume of 5 mL. This resulted in a preconcentration factor of 50 which is adequate to achieve LODs in the low ng/l range.

2.5 Total Dissolved Metals Determination Techniques

In addition to the determination of LSMC, total metals analysis was performed on collected environmental samples. Total dissolved metal concentrations were determined by direct analysis of samples by ICP MS. Milli-Q water based samples were acidified to 0.2% HNO_3, natural freshwaters were filtered to 0.45 µm before being acidified to 0.2% HNO_3 and saline waters were filtered to 0.45 µm, diluted 1:5 (to reduce the matrix interferences of the saline matrix) and were acidified to 0.2% HNO_3. All of these samples could then be analysed directly by ICP MS with matrix-matched standards. Typical detection limits of ICP MS analysis of waters is in the 1 – 10 ng/L range which is sufficiently low enough for the purposes of this investigation.

2.6 Method Blanks

Method blanks for solvent extraction experiments were achieved by extraction of a stripped Milli-Q water sample. A water sample was stripped of metals by initial extraction with 2 volumes of 1-octanol (LSMC method blanks) before it was extracted as per the solvent extraction methods outline in 2.4.3. Method blanks were performed in all solvent extraction experiments and are used to determine the LOD of the method used and to clean the separating funnels before analysis of samples.

2.7 Limits Of Detection

The Limit Of Detection (LOD) is the statistically calculated, lowest possible concentration that the method can detect. It was determined using the formula:

LOD = (3 x Standard Deviation of the Method Blanks)/50.....Equation 2.1

The LOD was calculated for each element in each experiment and is normally calculated based on data from no less the three method blanks. The LOD also takes into account the preconcentration factor of 50 (by dividing the result by 50) that occurs during the extraction and back extraction processes.

Chapter 3: Development of an Ultra-trace Method for the Detection of LSMC in Waters

3.1 Introduction

The long standing paradigm in ecotoxicology and environmental science is that the free ion form of a metal is the most toxic and bioavailable species (Anderson and Morel, 1978). However, there is a growing body of evidence to suggest that metals associated with LSMC may be equally or more bioavailable and toxic (Florence et al., 1983; Ahsanullah and Florence, 1984; Florence and Stauber, 1986) than the free ion form. Consequently, there is a great need to determine the concentration of LSMC in natural waters (Phinney and Bruland, 1994; Fraser et al., 2000; Blom et al., 2002; Turner and Mawji, 2005; Parthasarathy et al., 2010).

Given that currently there are no reliable, robust or sensitive methods for the determination of LSMC, it is not surprising that within the literature there exists only a small amount of data on the concentration of LSMC in many aquatic environments. The concentration of LSMC was determined in contaminated river water from Scotland (Turner and Mawji, 2005) and Mitrovic (1995) determined the concentration of LSMC in a range of aquatic environments in the Sydney area. Mitrovic (1995) measured the concentration of a range of LSMC in waters from seven locations within the Sydney metropolitan area and the concentration of a range of LSMC in sediments from these locations. These locations included both fresh and saline environments and focused on the detection of Cd, Cu, Ni and Pb LSMC. Kilgore (2007) furthered this research and determined the concentration of LSMC in both fresh and estuarine waters from a range of contaminated aquatic environments in the Sydney area. Apart from these studies, no other published research has determined the

concentration of LSMC in aquatic environments and therefore the pool of data on typical concentrations of LSMC in the environment is very limited.

In addition, to the work performed by Turner and Mawji (2005) and Mitrovic (1995), the presence of LSMC has been determined in aquatic environments which include; River water in Canada (Borgmann and Charlton, 1984), Lake Ontario Water (Borgmann and Charlton, 1984), Sewage sludge (Carlson and Morrison, 1992; Carlson-Ekvall and Morrison, 1995), Landfill leachate (Fraser et al., 2000) and San Francisco Bay water (Phinney and Bruland, 1997). These studies identified only the presence of LSMC in these waters but did not attempt to determine the concentration, perhaps due to the lack of a sensitive, reliable and robust method for the determination of these complexes in waters. This issue needs to be addressed if the concentration of LSMC in aquatic environments is to be accurately and routinely determined and the pool of data on typical concentrations of LSMC is to be increased.

The method developed by Mitrovic (1995) and further developed by Kilgore (2007) demonstrated the lowest detection limits and greatest selectivity of all the methods developed and trialled to date. The method used a solvent extraction approach where 250 mL of water sample was extracted twice with two 5 mL aliquots of octanol. The octanol extracts were then combined and back extracted into 5 mL of a 10% nitric acid matrix. This back extraction was performed by addition of 0.5 mL of concentrated nitric acid, heating the sample to 90°C to assist in back extracting the metals and then adding 4.5 mL of Milli-Q water. Analysis of the back extracts was performed by GFAAS or ICP-MS. The method was

capable of detecting ng/L concentrations of LSMC in both fresh and saline waters and selectively extracts only LSMC and not inorganic metal ions (Kilgore, 2007). During research performed by Kilgore (2007) two major limitations within the method were identified. First, the method had only been tested against two laboratory prepared LSMC (the APDC – metal and oxine – metal complexes) with variable quality within the method performance results. Secondly there were concerns that the back extraction technique was not quantitative. The initial focus of this research was to overcome the back extract issues and then perform extensive method validation to ensure that a sensitive, reliable and robust method for the determination of LSMC in waters had been developed.

Initially, Kilgore's (2007) method was tested against the APDC and oxine – metal complexes and then further tested against 3 other laboratory prepared synthetic LSMC; the phthalic acid, pyrocatechal and 2' 2 – bipyridyl – metal complexes. This method testing was performed to validate the robustness of the developed extraction and back extraction techniques and to identify any areas in these two processes where improvements could be achieved. Once a final method had been decided on, the method validation and performance data could then be assessed to ensure that the final method was of the highest quality possible.

Mitrovic (1995) and Kilgore (2007) carried out much of the early method development involved with the solvent extraction technique. Mitrovic (1995) carried out much of the method development around choice of solvent, extraction time and back extraction process. Kilgore (2007) continued with method development focussing on reducing contamination of

the blanks, increasing the effectiveness of the back extraction process and performing further method validation checks. From these two studies a number of important conclusions were drawn. First, octanol is the most appropriate solvent for extraction of LSMC. This is due to the fact that octanol has a similar dielectric constant to that of cell membranes (Florence, 1983; Friedel et al., 1994; Zhang et al., 1998; Turner and Mawji, 2004). This is important as complexes that extract into octanol are likely to partition into or through cell membranes. In addition, octanol shows selectivity to only LSMC, does not affect the speciation of waters it is added to and is only sparingly soluble in water (Turner and Williamson, 2005). Second, extraction into 2 (5 mL) aliquots of octanol ensures >99% extraction of LSMC (Mitrovic, 1995). Third, an extraction time of 5 minutes is suitable for extraction of LSMC into octanol (Mitrovic, 1995).

Further work identified that a major source of contamination within the method was from the solvent itself and therefore the solvent requires cleaning before use (Kilgore, 2007). This cleaning was achieved by 3 successive washing of 200 mL of the solvent with 20 mL of 10% HCl followed by 10% NaOH. The HCl solution removed the metal contamination from the solvent whilst the NaOH returned the pH of the solution back to neutral. In addition, the back extraction procedure was altered slightly, where samples were previously heated by a water bath a new microwave assisted back extraction process was developed (Kilgore, 2007). This further reduced the potential for contamination of the back extracts. This initial work resulted in the development of a solid method for the determination of LSMC in both fresh and saline waters however it was identified that further investigation was still needed into the back extraction technique and further method validation was required before the developed method could be considered reliable, robust and sensitive.

This study has two aims. The first is to develop an effective back extraction process for LSMC. The second is to perform a range of method validation experiments to demonstrate convincingly that the method is sensitive, reliable and robust. These validation experiments will focus on determining the method detection limits, calculating method blank contamination levels, determining the precision of the method and also demonstrating the ability of the method to recover spikes of laboratory prepared LSMC from Milli-Q water samples.

3.2 Development of an improved back extraction technique

Kilgore (2007) developed a back extraction technique which utilised heating by a conventional household microwave oven. The back extraction procedure is outlined in 2.4.4. The work carried out previously by Kilgore (2007) had identified that potentially microwave assisted back extraction may not be capable at transferring all of the LSMC from the octanol into the acidic matrix for ICPMS analysis. During this research an assessment was made of the efficiency of the microwave assisted back extraction technique and an alternative back extraction technique was developed.

3.2.1 Microwave assisted back extraction technique

Mass balance calculations were performed by Kilgore (2007) on the extraction and back extraction of the APDC – metal complex and the oxine – metal complex. This experiment was repeated during this study to verify that the results obtained by Kilgore (2007) were accurate. Table 3.1 below summarises the spike recovery results of the metal – oxine complex achieved during this study, by Kilgore (2007) and by Mitrovic (1995).

Table 3.1 Recoveries of the oxine – metal complex (100 µg/L) from spiked Milli-Q water samples

	% Recoveries				
Sample	**Cd**	**Cu**	**Ni**	**Pb**	**Zn**
This study Mean (n=6)	93 ± 1	19 ± 5	65 ± 4	86 ± 4	65 ± 3
Kilgore 2007 Mean (n=3)	80 ± 6	32 ± 8	95 ± 3	90 ± 5	87 ± 7
Mitrovic 1995 Mean (n= N/A)	5 ± 6	100 ± 2	14 ± 9	4 ± 6	N/D

N/D = the percent recovery for this particular element was not determined.

The method used by Kilgore (2007) was more effective at extracting Ni, Pb and Zn and to a certain degree Cd. However, the method did not seem to extract Cu as effectively as that used by Mitrovic (1995). Of concern is the difference in results between Kilgore (2007) and those obtained during this study. The same method of extraction and back extraction was used yet the results indicate very different recoveries particularly for Cu, Ni and Zn. Mass balance analysis was performed on the above samples where the initial mass of metals, the mass of metals extracted and the mass of metals present in the back extract were all compared. This comparison analyses each of the steps in the method and allows for the loss of metals during each step to be identified and quantified. This mass balance analysis indicated that all of the metals were extracted into the octanol however; the back extraction process was not effectively releasing all of the metals out of the solvent and into the acid matrix for ICPMS analysis. These results indicated that a more thorough back extract method was required.

3.2.2 Alternative back extraction techniques

It was hypothesised that the poor back extraction recoveries were due to the relatively large volume of octanol compared to acid used during the back extraction process (10 mL of

octanol compared to just 0.5 mL of concentrated nitric acid). Octanol is easily oxidised by acid (van Woezik and Westerterp, 2000) and in this oxidation process, the oxidising potential of the 0.5 mL of nitric acid may have been used up oxidising the octanol leaving the metal complex within the octanol phase. An easy solution to this problem would be to increase the amount of acid added during back extraction, however, this would lower the preconcentration factor and reduce the sensitivity of the method. A high preconcentration factor is essential to achieve the required limits of detection to measure the concentration of LSMC in natural waters. Since it was the solvent that was causing the poor back extraction efficiency, the simplest solution to this problem was to remove the solvent from back extraction entirely. It was determined that once the extraction process had been completed the octanol should be removed by evaporation leaving the metal salts behind and available for back extraction into an acid medium. As octanol has a boiling point of 194.5°C at 1 atmosphere (Sciencelab.com, 2011) simple heating of the back extracts as a means of evaporating the solvent would be ineffective. A more sophisticated method was needed to evaporate the 10 mL octanol extracts to dryness. Particular attention was paid to two such methods, evaporation by nitrogen blow down and vacuum distillation.

3.2.2.1 Nitrogen Blow Down

Evaporation by nitrogen blow-down is a process where a narrow stream of nitrogen gas is blown across the surface of a solvent that is to be evaporated. This nitrogen stream removes the solvent vapours from above the sample thus reducing the partial pressure. This reduction in partial pressure forces more solvent to evaporate to equilibrate the pressure differential. In this manner, a solvent can be evaporated at a temperature much lower than

its actual boiling point (Mohrig et al., 2006). Nitrogen blow down is commonly used in organic chemistry to evaporate solvents and concentrate samples prior to analysis (Patton et al., 1989; Chen et al., 1996; Richter et al., 1996; Wu et al., 1999; Cortazar et al., 2005).

Initial research determined that nitrogen blow down could reduce the temperature at which octanol evaporated to 135°C, much lower than its boiling point of 194.5°C. Whilst this temperature is still quite high, heating of back extracts to this temperature could be achieved using a dry block heater and glass evaporation vials. Using this technique it was determined that octanol extracts could be completely evaporated in approximately 2 to 3 hours. Unfortunately, major contamination occurred when using both a simple nitrogen blow down set-up and an off the shelf multi-sample evaporation unit. In addition, the levels of contamination varied greatly between samples evaporated during different experiments and displayed a very random contamination pattern between samples within the same evaporation experiments. For these reasons it was hypothesised that the contamination may be due to particulates from the atmosphere which would explain its random and varied nature but could also be due to contamination entering the samples through the nitrogen stream. This contamination would not likely be due to the nitrogen gas itself but could be due to the dirty lines that the gas passes through before being applied to the samples.

A number of attempts were made to overcome this contamination but unfortunately all of these failed. Initially work investigated using a glass shield above the samples to protect them from any particulate material falling into the vials during evaporation. This decreased the contamination slightly but increased the evaporation time to approximately 6 hours as

the screen trapped octanol and nitrogen vapours above the samples reducing the effectiveness of the blow-down procedure. As the work was being performed in a fume hood it was believed that the particulate contamination was entering the samples as air was being sucked into the hood from the laboratory and not due to particulates falling into the samples from above. A Perspex screen with 3 sides was then built to surround the samples during evaporation and protect them from particulate contamination. This method reduced the contamination further but did not completely eliminate it. The evaporation time using the Perspex shield was approximately 2-3 hours indicating that the screen was having no effect on the effectiveness of the nitrogen blow-down evaporation. As the contamination was not eliminated completely it was decided that the Perspex screen and the glass shield above the samples should be trialled. This reduced the contamination to the lowest levels measured but again did not eliminate it completely and did affect the evaporation time significantly with samples taking up to 7 hours to evaporate.

The final technique that was trialled involved the construction of a Perspex box specifically for the purposes of nitrogen blow-down. The box was completely sealed and contained the dry block heater and the nitrogen blow-down unit). It had a door so that samples could be added, evaporated and removed from the box. A fan small computer fan was used to blow filtered air (1 µm glass fibre filter) into the box to remove any octanol and nitrogen vapours so that evaporation could be performed in the shortest time possible. The nitrogen gas entering the box was passed through a 1 µm glass fibre filter and plastic gas lines used were all acid washed prior to use. Initial testing of the Perspex box indicated that the evaporation time was not affected and that contamination was greatly reduced. Unfortunately, some

contamination of the samples occurred and during subsequent experiments could not be eliminated. Whilst the contamination was only in the µg/L range, as this is the expected range of LSMC concentrations in the environment, this level of contamination is not acceptable.

3.2.2.2 Vacuum distillation back extraction

Vacuum distillation is commonly used to evaporate organic solvents, particularly those with a high boiling point (Lea and Swoboda, 1962; Bryce et al., 1971; Dankert et al., 1981; Takeoka et al., 1988; Hiatt et al., 1994). Vacuum distillation works on the same principle as regular distillation, where a sample is heated, vapours are carried up out of the sample flask and into a condenser in which cold water or ice is used to cool the glass condenser and cause the sample vapours to condense (Mohrig et al., 2006). The liquid formed within the condenser is then allowed to run into a receiving flask. The only difference between regular and vacuum distillation is that this whole unit is placed under reduced pressure, which has the effect of reducing the boiling point of the sample (Mohrig et al., 2006). This reduction in boiling point can be quite significant and allow a solvent with a relatively high boiling point to be evaporated at relatively low temperatures. As the pressure within the distillation unit is reduced, the boiling point of the solvent of interest is also reduced.

Before vacuum distillation could be trialled as an alternative solvent evaporation technique, problems with contamination had to be overcome. The major concern was that atmospheric particulate contamination of the samples would occur as was seen during the nitrogen blow-down experiments. To overcome this problem, a glass blower was commissioned to

build a vacuum distillation unit specifically for the purpose of evaporating octanol back extracts. The unit was made out of high quality borosilicate glass and, due to the small sample volumes to be distilled (10 mL back extracts), was built at a smaller scale than conventional distillation units. All of the parts of the unit that come into contact with solvent (i.e. the sample flask, receiving flask, connector and the condenser) were easily detachable so that they could be acid washed. In addition, a number of sample flasks and connectors (used to connect the sample and receiving flasks to the condenser) were made. These could then be acid washed and one sample flask and one connector were used per sample to reduce any chance of cross contamination between samples. Vacuum distillation could not be performed in a cleanroom due to concerns about the potential build-up of octanol vapours within the cleanroom and as the vacuum pump and recirculating water bath, both of which are metal, were not allowed within the ultra-trace cleanroom environment. The amount of time the unit was exposed to unfiltered air was reduced by always ensuring both sample and receiving flasks were covered and by covering any exposed areas of the vacuum distillation unit with zip-lock bags to reduce the chance of particulate contamination entering the unit. These measures combined to reduce the possible risk of contamination. Vacuum distillation has the added advantage that, unlike nitrogen blow-down, it is a closed system (i.e. once the unit is set-up and the sample and receiving flasks are connected no air exchange occurs) which greatly reduces the possibility of contamination.

The vacuum distillation unit was connected to an Edwards (Shanghai, China) two stage vacuum pump and a 0 – 1000 mbar vacuum gauge. Initially, testing of the vacuum

distillation unit indicated that 10 mL of octanol could be evaporated to dryness in approximately 30 minutes at 70°C. The sample could easily be heated to this temperature through submerging the sample flask in a heated water bath.

3.2.3 Vacuum distillation method performance

3.2.3.1 Blanks and limits of detection

Once the conditions for distillation were determined, the back extraction process could be tested. Solvent was evaporated to dryness and the round bottom flasks then returned to the cleanroom. Concentrated HNO_3 (0.5 mL) was added to each flask and the flasks were swirled for 30 seconds before being allowed to sit for 5 minutes. Milli-Q water (4.5 mL) was then added to each of the flasks before they were again swirled for 30 seconds before being allowed to sit for a further 5 minutes. The 5 mL back extracts were then analysed for the metals of interest using ICP MS.

Contamination originating from the round bottom flasks was tested by addition of acid and milli-Q water directly to acid washed flasks. Vacuum distillation contamination was determined by distilling to dryness 10 mL volumes of octanol and then adding acid and milli-Q water to these flasks. The data was compared to method performance data collected previously during this research using the microwave assisted back extraction process.

Table 3.2 Blank data for the vacuum distillation back extraction procedure

	Cd (µg/L)	Cu (µg/L)	Ni (µg/L)	Pb (µg/L)	Zn (µg/L)
Vacuum distillation blank (n=3)	<0.008	<0.014	<0.023	0.076	0.444
Round bottom flask blank (n=3)	<0.002	<0.023	<0.041	0.100	<0.180
Microwave blank (n=10)	<0.001	<0.012	<0.103	<0.004	<0.120

The results of analysis of the round bottom flask and vacuum distillation blanks (Table 3.2) indicated that that Cd, Cu and Ni results were all lower than the limits of detection of the ICPMS. These low blank results for Cd, Cu and Ni indicated that contamination due to the round bottom flasks and due to the vacuum distillation process for these metals was not of concern and should not affect the methods ability to determine LSMC in collected water samples. The Pb data was of slight concern because the results for the round bottom flasks and for the vacuum distillation process were higher than when the microwave-assisted back extraction process was used and were above the detection limits of the ICPMS. Whilst these results were higher, they are still low enough that they should not affect the methods ability to detect of environmentally relevant concentrations of LSMC in natural waters. Concentrations of Pb lipid soluble complexes are likely to be present in much higher concentrations than those measured during this investigation particularly given the 50 times preconcentration factor of the method. Close attention should be paid however to the Pb concentrations in the vacuum distillation blanks to identify if Pb concentrations continue to be of concern. This high vacuum distillation Zn result (0.444 µg/L) was of concern as it was well above the limit of detection of the ICPMS (0.232 µg/L) and as such is likely to affect the methods ability to detect LSMC in natural waters as their concentration is likely to be within this concentration range.

Limits of detection were calculated from the vacuum distillation blank data and were compared to the limits of detection calculated by Kilgore (2007) using the microwave assisted back extraction process. These results are presented in table 3.3.

Table 3.3 Limits of detection, vacuum distillation back extraction vs. microwave assisted back extraction

	Cd (µg/L)	Cu (µg/L)	Ni (µg/L)	Pb (µg/L)	Zn (µg/L)
LOD, Vacuum distillation (n=6)	0.002	0.003	0.003	0.001	0.011
LOD, Microwave (n=10)	0.002	0.003	0.005	0.001	0.013

The detection limits achieved using the vacuum distillation method are comparable to those achieved using the microwave assisted back extraction procedure of Kilgore (2007). This is an important result as it indicates that process contamination during the vacuum distillation was not significantly different from that which occurred during the microwave assisted back extraction technique.

3.2.3.2 Analysis of APDC – metal complex spiked water samples

The objective of this experiment was to determine if the vacuum distillation process was capable of recovering spiked concentrations of APDC-metal complexes from Milli-Q water samples. Similar experiments were performed using the microwave assisted back extraction procedure. The results from this current study can be compared to those measured previously using the microwave assisted procedure to determine the effectiveness of the vacuum distillation procedure.

An APDC solution was prepared following the method outlined in 2.4.1. Water samples (250 mL) were spiked with 12 mL of the purified APDC solution before a metal spike was added to each sample prior to extraction so the initial concentration was 2 µg/L, resulting in a back extract concentration of 100 µg/L. Extractions were performed following the octanol extraction method (2.4.3). Three method blanks and six spiked samples were extracted. The octanol extracts were retained in round bottom flasks and back extracted using the vacuum distillation method outlined earlier. A spiked control sample was also prepared in order to accurately determine the spike recoveries. The method blank and limit of detection data are summarised in table 3.4 below.

Table 3.4 Method blank and limits of detection data for APDC – metal spiked water samples

	Cd (µg/L)	Cu (µg/L)	Ni (µg/L)	Pb (µg/L)	Zn (µg/L)
Method Blank 1	0.006	0.031	<0.006	0.163	0.995
Method Blank 2	<0.002	0.033	0.010	0.161	1.01
Method Blank 3	<0.002	0.028	<0.006	0.151	1.00
Mean	0.003	0.031	0.007	0.158	1.002
SD	0.002	0.003	0.002	0.006	0.008
Method LOD	0.007	0.008	0.007	0.019	0.023
Microwave method LOD	0.002	0.003	0.005	0.001	0.013

A small amount of contamination for each of the five metals was measured in the method blanks; however, method blank concentrations were overall acceptable. The method blanks were used to calculate a method limit of detection. This limit of detection was very low for all the metals of interest and, except for Ni, were exactly the same as those measured using the microwave assisted back extraction technique. These low limits of detection are due to

very low contamination of the method blanks and also due to the relatively low variability between the three method blanks.

Table 3.5 Spike recoveries for APDC – metal spiked water samples (spiked concentration 100 µg/L)

	Cd (µg/L)	Cu (µg/L)	Ni (µg/L)	Pb (µg/L)	Zn (µg/L)
Mean (n=6)	100	105	107	98	108
SD	1	2	2	1	5

Spike recoveries for all the metals of interest (Table 3.5) were acceptable except for the Cu spike recovery of sample 1 (190 µg/L). The Cu sample 1 result was considered an outlier as all of the other spike recoveries for this sample were acceptable and it was therefore excluded from further analysis. The standard deviations of the six replicates were also very low, ranging between 1 – 5%. This low variability indicates that the method is providing reproducible results and confidence can therefore be placed in the data. Spike additions were performed on all samples prior to ICP MS analysis and no matrix interferences were present indicating that the ICP MS results were reliable.

3.2.3.3 Oxine – metal lipid soluble complex spiked water samples

The previously developed microwave back extraction technique was tested against a number of laboratory prepared synthetic LSMC, one of which was the oxine – metal complex (Kilgore, 2007). Testing of the oxine – metal complex produced mixed results. Percent recoveries varied between studies and in many cases were not acceptable. The oxine recoveries measured by Kilgore (2007) and those measured during this research

indicated the back extraction method was not effective. These poor recoveries were the catalysts for investigating and developing a new, more rigorous and more effective back extraction method.

Normally ligand solutions were prepared in Milli–Q water; however, oxine is only sparingly soluble in water. In contrast, oxine is very soluble (40 g/L) in methanol so methanol was used to make the stock oxine solution. The moles of oxine used must exceed the total number of moles of metal. An initial water concentration of 100 µg/L of Cd, Cu, Ni, Pb and Zn was used. The total number of moles of metal in solution (i.e. summed for all metals) was 0.0007725 Moles in a 250 mL sample. This compares to 0.0013 Moles of oxine in a 250 mL sample.

Using a 4 g of oxine per 100 mL of methanol solution (2.4.2), the volume of oxine solution needed to ensure that oxine is in excess (in the 100 µg/L samples) was calculated as being 5 mL, which contains 0.0013 Moles of oxine.

Initially, three method blanks were extracted following the method outlined in section 2.4.3. Five oxine replicates were extracted, with 5 ml of the oxine solution being added to each sample along with the metal spike to give a final metal concentration of 2 µg/L. Once all of the extractions were performed, back extractions were carried out using vacuum distillation back extraction. Water samples were collected pre- and post-extraction to allow a full mass balance to be performed. The collected water samples were acidified to 0.2% (v/v) HNO_3

immediately after collection and analysed with matrix matched standards by ICP – MS. The back extracts were also analyses by ICP – MS with matrix matched 10% HNO_3 standards.

All samples were analysed by ICP MS and the method blank and limit of detection data is summarise in Table 3.6 below. A summary of the mass balance results is contained in Table 3.7 and a summary of the spike recovery results is presented in Table 3.8.

Table 3.6 Method blank and limits of detection data for analysis of oxine – metal spiked Milli-Q water samples using vacuum distillation back extraction procedure

	Cd (µg/L)	Cu (µg/L)	Ni (µg/L)	Pb (µg/L)	Zn (µg/L)
Method Blank 1	<0.006	<0.003	<0.005	<0.010	0.031
Method Blank 2	<0.006	<0.003	<0.005	0.118	0.362
Method Blank 3	<0.006	<0.003	<0.005	<0.010	0.080
Mean (n=3)	<0.006	<0.003	<0.005	<0.010	0.158
LOD	0.006	0.003	0.005	0.010	0.002

Whilst there was a small amount of zinc contamination, the method blank results are all low enough to allow for the detection of environmental concentrations of LSMC. The limits of detection calculated from this method blank data are also low indicating that the method is reproducible and variability between the results is low. Determination of the LOD for Pb and Zn was performed using only method blank 1 and 3 and the results for method blank 2 were considered to be an outlier.

Table 3.7 Mass balance data from the analysis of oxine – metal spiked Milli-Q water samples using vacuum distillation back extraction procedure

	Cd (µg)	Cu (µg)	Ni (µg)	Pb (µg)	Zn (µg)
Before extraction mean[a] (n=5)	103.9	104.2	105.7	105.5	106.2
After extraction mean[b] (n=5)	1.3	0.8	2.3	0.5	0.4
Amount extracted[c] (difference)	102.6	104.4	103.4	105.5	105.8
Amount in back extract[d]	103.8	104.1	103.4	104.9	105.8
Amount unaccounted for[e]	-1.2	0.3	0.0	0.6	0.0
Amount unaccounted for (% of original spike)	0	0	0	1	0

[a]Mass of metal present in water sample prior to extraction
[b]Mass of metal present in water sample post extraction
[c]Mass of metal that was extracted (a minus b)
[d]Mass of metal that was measured in the back extract
[e]Mass of metal that was extracted but was not present in the back extract (c minus d)

The difference between the before and after extraction water samples clearly indicate that a quantitative extraction (>90%) occurred during the experiment.

Table 3.8 Spike recoveries from the analysis of oxine – metal spiked Milli-Q water samples using vacuum distillation back extraction procedure

	% Recoveries				
	Cd	Cu	Ni	Pb	Zn
Mean (n=5)	95	97	94	100	103
SD	0.3	0.2	0.3	0.3	0.2

The recovery of metals (as metal-oxine complexes) (%) were acceptable (between 85-105%) for every sample across all five metals of interest. In a number of other experiments, both during this research and during previous studies (Mitrovic, 1995; Kilgore, 2007) testing the

oxine – metal complex this result could not be achieved, with unacceptable recoveries and variable results obtained. This current experiment demonstrates that the vacuum distillation process for back extraction is much more thorough and reliable than the microwave assisted method. In addition, the standard deviations of the five replicates were low which indicates that the extraction and back extraction results are reproducible.

There are a number of reasons why the distillation process may achieve better spike recoveries than the microwave assisted back extraction process. During distillation the sample is distilled to dryness resulting in only metal salts remaining in the round bottom flask. This means that when the concentrated nitric acid is added to this flask the acid has direct contact with the metals. This would result in a much more thorough back extraction. In addition as mentioned previously octanol is oxidised by the addition of nitric acid. By removing the octanol before the addition of the acid, more of the acid can solubilise the metals rather than being used up in the oxidation process. Final by removing the octanol prior to back extraction this also reduces the likelihood of and residual octanol present in the final aqueous back extracts from interfering with the ICPMS analysis resulting in much more accurate and representative data.

3.2.3.4 PAX – metal lipid soluble complex spiked water samples

Xanthates are commonly used by the mining industry as floatation reagents and in this process are thought to form LSMC in the presence of several metals (Rao, 1971; Read and Manser, 1976; Gottofrey et al., 1988; Dopson et al., 2006). Potassium amyl xanthate (PAX) is one of the most commonly used xanthates in the processing of mineral ores (Gottofrey et al., 1988). It is therefore likely that aquatic environments surrounding mining operations

that use PAX in their processing steps may receive PAX and PAX – metal complexes by way of chemical spillage, seepage from tailings dams or the release of processing or tailings water (Rao, 1971; Webb et al., 1976; Xu et al., 1988). Given that PAX is such an important and common agent in mineral processing it was important to determine if the method was capable of measuring PAX – metal complexes. This experiment was also used to shed some light on the formation of PAX – metal complexes and the extent of their extraction into octanol. Silver (Ag) was added to the suite of metals used in this experiment because Ag could be present in high concentrations in mine waste water where concentrations of xanthates are also likely to be high. In addition it was expected that Ag could form strong lipid soluble complexes with PAX.

Solution composition: A solution containing 100 µg/L of Cd, Cu, Ni, Pb, Zn and Ag was prepared in Milli-Q water. The concentration of PAX needed to be in excess to ensure that all of the metal was present as the PAX-metal LSMC. Accordingly, the concentration of PAX used was 12500 µg/L. This was done by initially making an intermediate PAX solution of 1.25 g of PAX per 1 L of Milli-Q water. Ten millilitres of this solution was added to the metal solution.

Buffering the Test Solution: A mass of 2.6 g of HEPES buffer in 1 L of water was used to ensure sample pH remained between 7 and 8 during the experiment.

The test solution was prepared by dissolving 2.6 g of HEPES buffer in 990 mL of Milli-Q water. Ten millilitres of the PAX intermediate solution was then added followed by 200 µL of

concentrated HNO_3. The metal spike was then added to give a metal concentration of 100 µg/L of Cd, Cu, Ni, Pb, Zn and Ag.

The adsorption of the PAX – metal complex to the walls of the separating funnel during the extraction process was measured. Adsorption losses were calculated by weighing 250 mL of the test solution into an acid washed separating funnel. The sample was shaken for 5 minutes without any solvent; it was then allowed to sit for 5 minutes before the solution was transferred to a second acid washed separating funnel. The sample was then shaken for a further 5 minutes before being allowed to sit for 5 minutes. This test was repeated on triplicate samples. This process is the standard solvent extraction process just without the addition of any solvent. A 20 mL subsample of the water was taken before and after this process, the difference between these two subsamples is the amount of metal that had adsorbed to the separating funnel walls. The before and after adsorption samples were acidified immediately to 0.2% (v/v) HNO_3. The pH was checked on one of the adsorption samples post shaking to ensure the pH was stable throughout. The pH of adsorption sample was 7.81.

Four metal and PAX spiked samples were then analysed using the standard octanol extraction method (2.4.3). Before and after extraction water samples were collected and acidified immediately to 0.2% HNO_3 so that a mass balance could be performed. The pH was checked on one of the PAX samples post extraction to ensure the pH remained stable throughout the extraction process. This sample had a pH of 7.83 indicating that the pH had remained constant throughout extraction.

All before-extraction samples were collected immediately before shaking was performed. The time from when the metal spike was added to the test solution to the time when the subsample was taken was noted. This was done in case any metal complexes were adsorbing to the bottle walls over the period of the experiment. The initial pH of 1 L test solution was 7.92 and the post experimental pH (after 127 min) was 7.90.

Once all samples had been extracted, back extracts were evaporated using vacuum distillation. Three solvent blanks were distilled first followed by the four PAX samples. The 5 mL back extracts were then removed from the flasks by pipette and transferred to acid washed 5 mL ICP – MS tubes for analysis. Samples were diluted 50 fold prior to analysis to ensure the metal concentrations were within the calibration range of the ICP MS. The before extraction, after extraction and the diluted back extract samples were analysed as a single batch on the ICP – MS with matrix matched standards and spike additions.

The test solution was slightly yellow in colour once the PAX and metals were added together. Post extraction the solution appeared clear and the back extracts were a very vivid yellow colour. This suggests that whatever was causing the colour in the samples had extracted into the octanol.

The results from the solvent blank samples indicate that all samples were back extracted with low levels of contamination (Table 3.9). Results for all six metals that were tested were either below or close to the detection limits of the ICP – MS. Spike additions performed on all of the samples indicated that no matrix interferences were present.

Table 3.9 Solvent blank data, water samples spike with the PAX-metal complex

	Cd (µg/L)	Cu (µg/L)	Ni (µg/L)	Pb (µg/L)	Zn (µg/L)	Ag (µg/L)
Mean (n=3)	0.015	<0.002	<0.001	0.014	<0.013	0.016

The before-extraction samples demonstrated that the initial solution contained close to 100 µg/L of metal (Table 3.10). After the adsorption control extraction was performed, the concentration of Cd, Pb and Zn did not differ significantly. Cd dropped in concentration by 4% whilst Pb and Zn concentrations did not change. However, Cu experienced quite a significant drop in concentration of 23% and Ni experienced a less significant drop of only 9%. Ag experienced the most significant drop in concentration of 49%. The adsorption results indicate that for Cd, Ni, Pb and Zn the loss of metals due to adsorption is less than 10% and therefore should be of little concern. The reduction in concentration of 23% for Cu is of concern and should be taking into consideration when analysing the Cu spike recovery results from the PAX samples. Also the drop in Ag concentration is concerning however it is unclear whether this was due to actual adsorption or an error in making up the initial solution as the initial concentration of Ag was only 8.27 µg/L. The Ag data appears unreliable and it is therefore difficult to draw any conclusions from the data, further analysis of this data was difficult, and as the cause of the significant reduction in the Ag concentration could not be identified, it was eliminated from further analysis.

Table 3.10 PAX – metal lipid soluble spiked water samples adsorption data

	Cd (µg/L)	Cu (µg/L)	Ni (µg/L)	Pb (µg/L)	Zn (µg/L)
Before extraction mean (n=3)	97.64	92.5	102.7	94.0	93.4
SD	0.755	2.28	3.72	1.98	1.13
After extraction mean (n=3)	94.0	71.5	93.7	93.9	93.3
SD	1.52	13.8	3.23	0.644	4.27
Difference (before – after)	3.66	20.9	8.97	0.060	0.136
Difference % of original spike	4	23	9	0	0

Spike recovery results for the adsorption samples were acceptable (85 to 105%) indicating that no matrix interferences occurred during analysis by ICP MS (Table 3.11). The before extraction results were close to the nominal (spiked) concentrations. Concentrations were approximately 100 µg/L, some concentrations had dropped slightly (<10%) but this can be explained by adsorption. The standard deviation between the four replicates was acceptable. The after extraction aqueous phase had significantly lower concentrations of Cd, Cu, Ni, Pb and Zn indicating that either extraction or adsorption had occurred. Less than half the Zn extracted into octanol (concentration dropped from 88 µg/L to only 55 µg/L). This indicates that Zn does not form PAX complexes as effectively as the other metals or that the PAX – Zn complexes that are formed do not extract well into the octanol. The back extracts for Cd, Ni, Pb and Zn all recovered the extracted metals. The expected concentrations for each of these back extracts matched closely the measured concentrations. Percent recoveries for Cd, Ni, Pb and Zn were 97, 95, 103 and 92 respectively. For Cu however, only 43% of the extracted metal was recovered. This indicates that Cu was lost during the experiment either due to incomplete back extraction or adsorption. We know from the adsorption samples that 23% of the copper in solution adsorbs to the container walls during

the experiment. Taking this into account 68%, of the initial Cu can be accounted for. The remaining 32% is unaccounted for.

Table 3.11 PAX – metal lipid soluble spiked water samples

	Cd (µg/L)	Cu (µg/L)	Ni (µg/L)	Pb (µg/L)	Zn (µg/L)
Before extraction mean (n=4)	99.5	90.8	85.6	95.6	88.0
SD	1.79	1.80	8.57	2.16	1.55
After extraction mean (n=4)	0.361	0.242	4.21	0.276	55.4
SD	0.136	0.104	0.174	0.116	1.34
Amount extracted mean (n=4)	99.2	90.6	81.4	95.3	32.6
SD	1.89	1.80	8.50	2.25	1.25
Back extraction mean (n=4)	95.9	39.4	77.7	98.1	30.1
SD	0.692	4.72	7.53	0.540	0.046
Mean % recoveries	97	43	95	103	92

The results indicate that PAX – metal complexes of Cd, Cu, Ni, Pb and Zn do form in solution at a pH of 7.90, which suggests that in environments receiving mine process waters containing PAX or similar xanthates, LSMC may form. The PAX-metal complexes of most metals efficiently extract into octanol; complete mass balances for Cd, Ni, Pb and Zn were achieved whilst some Cu is unaccounted for. The poor Cu recoveries should be investigated further so that this process can be completely understood and explained. The results also indicate that with the exception of Cu, the method can quantitatively determine the concentration of PAX – metal complexes in Milli-Q water samples.

3.3 Summary of the developed method

The concentration of laboratory prepared LSMC spiked water samples were successfully determined using a solvent extraction approach followed by evaporation of the solvent by vacuum distillation before back extracting the metals of interest into an acid matrix for analysis by ICPMS. The developed method was validated using 3 separate synthetic organic ligands; APDC, Oxine and PAX with 5 different metals; Cd, Cu, Ni, Pb and Zn.

Extraction of the spiked LSMC was achieved using 2 extractions with 5 mL of octanol. The octanol aliquots were added to 250 mL of the samples before they were shaken for 5 minutes. The phases were allowed to separate for 5 minutes before the octanol was removed to an acid washed round bottom flask. This process was repeated with a second 5 mL aliquot of octanol and the 2 octanol extracts were combined in the same round bottom flask.

The two 5 mL octanol extracts were combined in an acid washed round bottom flask after the extraction process was completed. These round bottom flasks were carefully placed inside acid washed 120 mL polycarbonate vials. This was done to reduce the risk of contamination of the extracts when they were removed from the cleanroom for distillation. Distillation was performed in a fume cupboard outside of the cleanroom. An acid washed connector was attached to the condenser of the vacuum distillation unit and the receiving flask and the sample flask were than attached to the connector. An insulation pad, made of glass fibre wool, was wrapped around the neck of the sample flask to ensure the octanol remained as a vapour until it reached the condenser. Due to the high boiling point of

octanol, the insulating pad was necessary to avoid the octanol condensing before it reached the condenser and falling back into the sample flask. The sample flask was then carefully placed in a water bath at 70°C and the sample was allowed to warm for 30 minutes. The vacuum pump was then switched on and the vacuum was slowly applied to the distillation unit via the use of a tap at the top of the condenser. This was done to ensure that the pressure in the unit was lowered slowly to avoid any bubbling in the sample. Once the entire vacuum had been applied (pressure dropped to <10 mbar) the sample was allowed to distil to dryness, this took approximately 30 minutes.

Once complete distillation had been achieved, the vacuum pump was switched off, the pressure was released via the tap at the top of the condenser and the sample flask was removed from the water bath. The insulating pad was removed, the sample flask was disconnected and immediately placed back into the acid washed 120 mL polycarbonate vial. The receiving flask was disconnected and the octanol was removed to waste and the connector was disconnected from the condenser. A new acid washed connector was then attached, before the receiving flask and a new sample flask were attached. The distillation process could then be repeated on the next sample.

Once all samples had been distilled, the round bottom flasks (inside their acid washed 120 mL polycarbonate vials) were returned to the cleanroom. The flasks were removed from their polycarbonate vials and 0.5 mL of concentrated HNO_3 was added. The flasks were then swirled individually for 30 seconds before being allowed to rest for 5 minutes. A 4.5 mL volume of Milli-Q water was then added to each flask, the flasks were swirled again for 30

seconds each before being allowed to rest for 5 minutes. After 5 minutes, the back extracts were carefully removed from the round bottom flasks by pipette and placed in acid washed 5 mL vials for analysis by ICP MS.

The vacuum distillation back extraction technique was a crucial component to this research project. The final back extraction technique (outlined above) was used throughout the rest of the research project. Vacuum distillation back extraction was the most rigorous back extraction technique employed for the detection of LSMC in waters. By eliminating the octanol from the back extraction process prior to the addition of the nitric acid the method ensures that the nitric acid has the maximum effect on the metals ensuring that the highest proportion of metal possible is released into the acid matrix for analysis. This technique also reduces any possible matrix effects during ICP-MS analysis as a result of residual octanol in the back extracts. A significant amount of work was performed to develop and test this back extract technique and ensure that it is effectively extracting the tested LSMC.

3.4 Conclusion

The majority of the work performed during this investigation focused on the development of a reliable, sensitive and robust method for the determination of LSMC in waters. This revolved around the development of an effective back extract technique that was capable of back extracting a range of LSMC from octanol extracts. This was achieved through the use of vacuum distillation to remove the solvent before the metal was solubilised in a 10% HNO_3 (v/v) and analysed by ICP MS. The vacuum distillation back extraction technique was

combined with the previously developed extraction technique to produce a method capable of detecting a range of LSMC in waters.

The final method was used to perform a number of validation experiments. These experiments focused on the recovery of APDC – metal, oxine-metal and PAX-metal complexes spiked into Milli-Q water samples. Acceptable spike recoveries were achieved for all complexes across all five metals except for the PAX-Cu complex. The efficient recovery of most metal complexes indicates that both the extraction and, more importantly, the newly developed vacuum distillation back extraction technique are together capable of detecting a range of LSMC in waters. This is a very important result as it was identified that the back extract technique used in previous studies may not have been effectively back extracting all of the LSMC. The development of the vacuum distillation back extraction technique should ensure a much higher quality of data is produced during the analysis of natural waters for LSMC.

Chapter 4: Determination of the Octanol/Water Partition Coefficients of a Range of Neutral Inorganic Metal Complexes

4.1 Introduction

Despite the possibility that NIMC could be ecologically and toxicologically important, there is currently limited understanding of the significance of neutral inorganic metal complexes (NIMC) with respect to their bioavailability to aquatic organisms. A number of NIMC are known to form in natural waters and, to date, particular attention has been paid to the $HgCl_2$ complex and the non-metal $B(OH)_3$ complex (Vigneault et al., 2000; Turner and Williamson, 2005). This past research, however, has failed to adequately investigate whether NIMC have the ability to passively diffuse into cell membranes and therefore whether they pose a risk to aquatic biota.

Kilgore (2007) showed that, despite the absence of an organic ligand, small concentrations of metals extracted into octanol and hypothesised that this extraction may be due to extraction of a small portion of free metal ions. Subsequent speciation calculations revealed that, across the five metals of interest (Cd, Cu, Ni, Pb and Zn), 12 complexes were formed in the laboratory water sample that were neutral and inorganic (Kilgore, 2007) and therefore could potentially extract into octanol just as neutral organic LSMC do. This could explain the detection of metals in the octanol, and indeed supports earlier work of Turner and Williamson (2005) who also found that the extraction of free metal ions into octanol was limited. It was suggested by Kilgore (2007) that these metal complexes that formed in the test solutions could represent a significant class of metal species that, as yet, has not been adequately investigated. If these metal species display significant extraction into octanol, they could potentially pass across cell membranes in a similar manner to organic LSMC (Vigneault et al., 2000).

NIMC in natural waters could represent a significant pool of metals that, under certain circumstances, could exhibit some form of lipid solubility and potentially toxicity. In addition these NIMC may represent an alternate pathway of metal uptake not previous investigated. The environmental significance of these complexes is directly related to two factors. The first is their capacity to form in natural waters and whether this is dependent on a narrow or broad range of physico-chemical conditions. The second is whether the complexes are able to passively diffuse across cell membranes, meaning that they are readily bioavailable and potentially toxic. The ability of a complex to passively diffuse across a cell membrane is indicated by the complexes ability to extract into octanol. It has been shown that LSMC, which have a neutral charge and are of a small enough size, can easily diffuse across a cell membrane via passive diffusion (Blust et al., 1986; Carlson and Morrison, 1995; Phinney and Bruland, 1997; Croot et al., 1999). As NIMC also have no charge they may be able to pass across the cell membrane with similar ease. This could make them as toxic as LSMC (Benoit et al., 1999; Bhamre et al., 2011), consequently, the ability of these complexes to diffuse across cell membranes needs to be determined.

The two factors described above were both investigated as part of the current study. First, the Visual MINTEQ version 3.0 speciation modelling program (Gustafsson, 2006) was used to optimise the solution composition that would be required to favour the formation of a number of NIMC. Visual MINTEQ is a modelling programme in which the concentration of different solution components can be entered and from this the model predicts the metal speciation. The solution composition which would result in the highest percentage of metal present as the NIMC can then be determined (Gustafsson, 2006). Analysis of the solution

composition that results in the formation of the specified NIMC may provide some insight into whether these complexes are easily formed and whether they are likely to form in natural waters.

Second, to determine the environmental significance of the NIMC their octanol/water partition coefficients were determined. The octanol/water partition coefficient or K_{OW} of a complex is a measure of the amount of that complex that partitions into octanol (Turner and Williamson, 2005) and is defined by the formula:

$$K_{OW} = \frac{C_{Octanol}}{C_{Water}}$$

Where,

$C_{Octanol}$ = Concentration of substance e.g. [NIMC] in octanol

C_{Water} = Concentration of substance e.g. [NIMC] in water

The octanol/water partition coefficient has been used extensively to indicate the fate of a compound (Turner and Williamson, 2005; Ingram et al., 2011). It is commonly used in the pharmaceutical industry to determine how well a new drug will penetrate target cells (Ingram et al., 2011) and in ecotoxicology to determine whether the compound is likely to adsorb to sediment and organic matter (Franklin et al., 2001; Apte et al., 2005; Sofyan et al., 2007). In relation to NIMC, the octanol/water partition coefficient can be used to determine the affinity of a complex for octanol and this can be used as a surrogate to assess how easily these complexes might transverse a cell membrane. A high affinity for octanol might indicate that a complex has a high affinity for cell membranes and could easily diffuse through a cell membrane.

The aim of this chapter is to investigate the formation and partitioning into octanol of a number of NIMC. Their formation in aqueous media was assessed by the use of Visual MINTEQ (Gustafsson, 2006) and based on these assessments the solution compositions which maximise the concentration of the NIMC were formulated. The complexes were then formed using this solution composition data and extracted into octanol. The octanol/water partition coefficients were determined for each of the complexes.

4.2 Method

4.2.1 Complexes tested

Kilgore (2007) identified 12 NIMC that might be significant in natural waters. These complexes were chosen based on metals that are commonly measured in aquatic contamination studies and on the basis of the most likely anions that would be present in these samples. This led to 12 complexes being identified as potentially important, which were $CdCl_2$, $CuCO_3$, $Cu(OH)_2$, $NiCl_2$, $NiCO_3$, $Ni(OH)_2$, $PbCl_2$, $PbCO_3$, $Pb(OH)_2$, $PbSO_4$, $ZnCO_3$ and $Pb(OH)_2$. The same suite of NIMC was investigated in this study. Two additional neutral complexes were selected for investigation as outlined below.

4.2.2 Mercury chloride complex

The $HgCl_2$ complex is an important mercury species in the environment in terms of mobilisation and mercury cycling (Hahne and Kroontje, 1972; Mason et al., 1996; Bhamre et al., 2011). Mercury is present in a range of different species in aquatic environments and of these, organic methyl mercury is the most toxic (Snarski and Olson, 1982; Gabriel and Williamson, 2004; Wu and Wang, 2011). The $HgCl_2$ complex is extremely stable, is neutral

and is known to be present in natural waters (Hassett, 1994; Mason et al., 1996; Vigneault et al., 2000; Bhamre et al., 2011).

4.2.3 Boron hydroxide complex

The boron hydroxide, $B(OH)_3$ complex was selected for study because it is known to be present in high concentrations in both seawaters and freshwaters. Boron, a non-metal, is found in seawater at a typical concentration of 4450 µg L^{-1} (Turekian, 1968) and is present as a range of different species. However, this speciation is dominated by the neutral $B(OH)_3$ complex (Klochko et al., 2006). Its formation in seawater is controlled by the following reaction:

$$B(OH)_3 + H_2O \longleftrightarrow H^+ + B(OH)_4^-$$

The pK_a of this reaction is 8.6 (Klochko et al., 2006) which indicates that at a pH less than 8.6 the equilibrium will favour the formation of the neutral $B(OH)_3$ complex. In seawaters with a pH of 8.2, (the typical pH of seawaters (Klochko et al., 2006)) approximately 70% of the boron in solution will be in the form of this neutral complex (Klochko et al., 2006). If this complex extracts into octanol it could potentially indicate a pathway of boron entering a cell and potentially affect marine biota. Turner and Williamson (2005) found partitioning of boron into octanol during the analysis of river water samples and treated sewage effluent. They associated this partitioning to the fact that the dominant form of boron in freshwaters is the neutral $B(OH)_3$ complex. It should be noted that concentrations of B in freshwaters are much lower than in seawater however B concentrations in freshwaters are often

elevated as a result of anthropogenic inputs like sewage discharges (Turner and Williamson, 2005).

The extraction of boron into octanol was observed during fieldwork conducted by Kilgore (2007). During this investigation it was determined that, on average, 0.5% of the total boron in seawater samples extracted into octanol. Whilst this seems a small percentage, due to the high natural concentration of boron in seawater this could represent a significant pool of boron and should be further investigated. To determine the octanol/water partition coefficient of the $B(OH)_3$ complex it was decided that the extraction of boron from seawater would be appropriate. Seawater from Cronulla Beach, NSW, was collected and the octanol/water partition coefficient of boron was determined from this sample. Cronulla was chosen as the sampling location due to its close proximity to the laboratory and the fact that it has been well characterised as a seawater collection site.

4.2.4 Determination of metal speciation in metal aqueous solutions

For metal complexes with Cl^-, the concentration of Cl^- determined by Visual MINTEQ was achieved by the addition of NaCl and the pH was varied by addition of NaOH or HCl. In this way a range of Cl^- concentrations could be achieved at a range of pH values. The carbonate complex concentrations determined with Visual MINTEQ were achieved by the addition of $NaHCO_3$ and the pH of the solution was controlled by the addition of NaOH or HCl. This allowed for a range of CO_3^{2-} concentrations at a range of pH values to be achieved.

Variation in the sulphate concentrations in solution were achieved through the addition of $NaSO_4$ whilst the pH of the solution as adjusted using HCl or NaOH. This allowed for a range of SO_4^{2-} concentrations to be achieved at a range of pH values. Complexes with the OH^- ion were achieved by varying the pH only by the addition of NaOH. A series of stepwise manual iterations were employed to optimise the solution composition for each of the metal complexes of interest.

4.2.5 Preparation of test solutions

All solutions were prepared in Milli-Q water with an initial metal concentration of 100 µg/L so that mass balance calculations could be easily performed well above the limits of detection of the ICP MS. The solution composition was kept as simple as possible so as to reduce any competitive binding effects and, therefore, only the components that were directly required to form the complexes or adjust the pH were added to water samples. A summary of the specific solution compositions and the resulting speciation calculations is given in Table 4.1 below.

Table 4.1 Solution composition data based on Visual MINTEQ determinations and speciation calculations for the NIMC test solutions

Complex	pH	Anion conc. (mM)	Metal conc. (mM)	Complex (%)	Ionic strength
$CdCl_2$	8	Cl^- = 611	0.0009	54.6	0.306
$CuCO_3$	7.5	CO_3^{2-} = 0.008	0.002	2.99	0.00
$Cu(OH)_2$	9	-	0.002	61.0	0.00
$NiCO_3$	9	CO_3^{2-} = 8.14	0.002	83.1	0.005
$Ni(OH)_2$	9	-	0.002	8.16	0.00
$NiCl_2$	2	Cl^- = 532	0.002	0.051	0.273
$PbCl_2$	4.5	Cl^- = 532	0.0005	27.8	0.266
$Pb(OH)_2$	9	-	0.0005	23.3	0.000
$PbCO_3$	8	CO_3^{-2} = 0.027	0.0005	10.2	0.000
$PbSO_4$	2.5	SO_4^{-2} = 43.5	0.0005	63.2	0.080
$ZnCO_3$	8	CO_3^{-2} = 0.360	0.002	6.66	0.0002
$Zn(OH)_2$	9	-	0.002	86.1	0.000
$HgCl_2$	3	Cl^- = 547	0.0005	3.79	0.306

From table 4.1 it is clear that the percent of NIMC formed in each of the solutions is varied. The percentages ranged from 0.051% for the $NiCl_2$ complex up to 86.1% for the $Zn(OH)_2$ complex. This indicates that some complexes are more easily formed and are more stable than others. Those complexes with a higher percentage of formation may be found in higher concentrations in natural waters.

All solutions were prepared in 1 L acid washed nalgene bottles. The pH of each solution was measured before and after extraction with octanol to ensure that an accurate solution pH (listed in Table 4.1) was maintained throughout the experiment. Cronulla sea water was

used as the $B(OH)_3$ test solution, rather than a specific solution being prepared. Consequently, the solution composition data and speciation calculations for $B(OH)_3$ are not presented in Table 4.1.

4.2.6 Extraction into octanol

To determine the K_{OW} of the complexes, the waters were extracted into octanol. A 10 mL volume of the water sample was placed in an acid washed Oak-Ridge centrifuge tube with 10 mL of purified octanol. The samples were then shaken by hand for 10 minutes to ensure adequate interaction between water samples and octanol. The samples were then left to stand for 10 minutes to allow the two phases to separate. The octanol was then removed to an acid washed round bottom flask for back extraction and the aqueous layer was removed to a 20 mL acid washed polycarbonate vial where it was immediately acidified to 0.2% (v/v) HNO_3 for metal analysis by ICP MS. Octanol samples were back extracted following the vacuum distillation back extraction procedure outlined in section 3.3. The 10% (v/v) HNO_3 back extracts were analysed by ICP MS with matrix matched standards and spike additions to correct for any matrix interferences.

4.2.7 Mass balance calculations

Subsamples of 10 mL were taken from the 1 L test solutions prior to extraction into octanol. These subsamples were used to determine the total dissolved metal concentration before extraction. Immediately after collection, subsamples were acidified to 0.2% (v/v) HNO_3 and the concentration of metals was analysed by ICP MS with matrix matched standards and

spike additions. The post extraction dissolved metal concentrations were measured in the 10 mL water samples that had undergone extraction into octanol (section 4.2.6). After extraction, these samples were acidified to 0.2% (v/v) HNO_3 and analysed by ICP MS. The difference between the before and after extraction samples should represent the mass of metals that have extracted into octanol. For a full mass balance to be performed, the concentration in the 10 mL octanol extracts was determined by vacuum distillation and ICP MS analysis. This procedure then allowed for the concentration of metal extracted into octanol to be accurately determined and to be compared to the amount of metal missing from solution due to the extraction process.

Adsorption of metals to the Teflon centrifuge tubes during extraction could mean the concentration of metal that extracted into octanol would be overestimated and a larger K_{OW} would be calculated. To correct for the adsorption of particular species to the centrifuge tube walls, adsorption controls were performed. This was done by placing a 10 mL subsample of the test water into an acid washed Oak-Ridge centrifuge tube and treating it as any regular samples, however octanol was not added to them. In this way the concentration of dissolved metal could be determined in the water directly prior to and after it had been shaken for 10 minutes and then left to stand for 10 minutes. Without the octanol present, any metal lost from solution would be due to adsorption to the centrifuge tube walls. The adsorbed concentration could then be used to correct the data from the octanol extracted samples and therefore the calculated K_{OW} would not contain any adsorption bias.

The extraction of free metal ions into the octanol may also bias the K_{OW} calculations. The extraction of free metals ions during this study should not be significant; however, it was still assessed to ensure it was not biasing the result. This was performed by preparing metal nitrate solutions. Nitrate was chosen specifically because none of the metals of interest from solution complexes with the nitrate ion. The chemistry of the experimental solutions was carefully controlled to ensure the maximum concentration of free metal ions. The Visual Minteq data presented previously was used to determine the solution composition that would result in the maximum formation of free metal ions. The metal results from these ionic control samples could be used to determine the amount of free metal ion that extracted into the octanol and the results from the NIMC solutions could be adjusted accordingly to correct for any bias. The metal nitrate solutions were prepared to have the same pH as the neutral metal complex test solutions with pH adjustments made using dilute solutions of HCl and NaOH.

4.2.8 Determination of the K_{OW} of the test solutions

Once the before extraction, after extraction and octanol metal concentrations had been determined the results were adjusted for any adsorption or free metal ion extraction bias. A mass balance was then performed on the data to ensure all of the metal present in the original sample could be accounted for. If extraction was observed into the octanol phase, the octanol/water partition coefficient of the complex was calculated using the formula described in section 4.1.

4.3 Results

4.3.1 Extraction of NIMC test solutions

The adsorption control samples and the free metal ion control samples indicated that these two biases were not exerting a significant effect on the data. For example the adsorption of metals onto container walls and the extraction of free metal ions into octanol was small enough that it would not significantly affect the NIMC extraction results. Nevertheless all data was corrected for these biases. The corrected results indicated that of the 14 complexes tested only 2 extracted into octanol greater than 0.1% (Table 4.2). The extraction experiments were repeated to ensure the results were reliable and repeatable.

Table 4.2 Percent extraction of the NIMC into octanol for the 14 complexes tested

Cation	Neutral complex	% of NIMC extraction in octanol
Cd	$CdCl_2$	<0.1%
Cu	$CuCO_3$	<0.1%
	$Cu(OH)_2$	<0.1%
Ni	$NiCO_3$	<0.1%
	$Ni(OH)_2$	<0.1%
	$NiCl_2$	<0.1%
Pb	$PbCl_2$	<0.1%
	$Pb(OH)_2$	<0.1%
	$PbCO_3$	<0.1%
	$PbSO_4$	<0.1%
Zn	$ZnCO_3$	<0.1%
	$Zn(OH)_2$	<0.1%
Hg	$HgCl_2$	12.3
B	$B(OH)_3$	13.9

Results were all corrected for absorption and for free metal ion extraction.

A significant amount of Hg extracted from the water sample into the octanol (Table 4.2). Based on the speciation calculations (Table 4.1), this extraction was attributed to the $HgCl_2$ complex.

Table 4.3 Mass balance data for the extraction of the $HgCl_2$ complex

	Hg (µg)
Before extraction (mean n=5)	10.7 ± 0.20
Measured after extraction (mean n=5)	9.29 ± 0.10
Calculated amount extracted	1.40 ± 0.29
Measured amount in back extract (mean n=5)	1.32 ± 0.15
Amount unaccounted for	0.085

The small amount of Hg that is unaccounted for represents <1% of the total mercury. According to the speciation calculations, 3.79% of the mercury in the test solution will be present as the $HgCl_2$ complex. The mass of mercury at equilibrium in solution was 9.29 µg and the mass of mercury present as the $HgCl_2$ complex was equal to 0.40 µg.

As the volume of the water sample and the octanol was the same, the K_{OW} formula can be expressed in terms of mass;

$$K_{OW} = \frac{\text{Mass of Hg in Octanol}}{\text{Mass of Hg as } HgCl_2 \text{ in Water}}$$

$$K_{OW} \text{ of } HgCl_2 = \frac{1.40}{0.40}$$

$$K_{OW} \text{ of } HgCl_2 = 3.28$$

The mass of boron before and after extraction and the mass of boron in the octanol were determined and are summarised in Table 4.4. An octanol/water partition coefficient was calculated based on the data.

Table 4.4 Mass balance data for the extraction of the $B(OH)_3$ complex

	B (µg)
Before extraction mean (n=5)	440 ± 13.9
After extraction mean (n=5)	376 ± 8.75
Amount extracted	64 ± 6.79
Amount in back extract mean (n=5)	61 ± 5.80
Amount unaccounted for	3

According to the literature, 70% of the boron in the seawater will be present as the $B(OH)_3$ complex (Klochko et al., 2006). The mass of boron at equilibrium in solution was measured to be 376 µg and the mass of boron present as the $B(OH)_3$ complex will be equal to 263 µg. The octanol/water partition coefficient of the $B(OH)_3$ complex was then determined.

$$K_{OW} = \frac{\text{Mass of B in Octanol}}{\text{Mass of B as } B(OH)_3 \text{ in Water}}$$

$$\therefore K_{OW} \text{ of } B(OH)_3 = \frac{61.0}{263}$$

$$\therefore K_{OW} \text{ of } B(OH)_3 = 0.23$$

4.4 Discussion

Investigating the extraction of neutral inorganic metal complexes is important to understanding the nature of the LSMC determined during analysis of natural samples. If NIMC demonstrate significant partitioning into octanol, the concentration of LSMC determined during the fieldwork surveys (chapter 5) could comprise not only organic neutral complexes but also neutral inorganic complexes. It is this combination of species extracting into octanol during analysis of natural waters that has been overlooked

previously but could have significant implications for the assessment of bioavailability and toxicity of metals in the environment.

4.4.1 Cd, Cu, Ni, Pb and Zn neutral inorganic metal complexes

None of the twelve NIMC tested significantly extracted into octanol. This may be due to a number of factors. First, the concentrations of the 12 complexes may be too low to extract into octanol. This would be especially true for the $CuCO_3$, $Ni(OH)_2$, $NiCl_2$ and $ZnCO_3$ complexes which make up less than 10% of the total metal in solution. In this experiment, the test solutions were manipulated in an effort to maximise the concentration of the NIMC. In environmental samples were the matrix is much more complex and there is likely to be much more competition between metal species, particularly if dissolved organic matter is present, the percentage concentrations of the NIMC are likely to be even lower.

Many of the 12 complexes tested were present in the test solutions in very low percentages. Seven of the 12 complexes accounted for <30% of the total dissolved metal concentration. These low percentage compositions would result in very low concentrations of the metals of interest being found as part of these complexes. The total mass of metal in the 1 L test solutions was only 100 µg, which corresponds to only 1 µg in the 10 mL test aliquots. If 30% or less of this metal is present as the NIMC, the concentration of metal in solution that would be found as a NIMC is likely to be 300 ng or less. A simple solution to this problem would be to increase the total metal concentration and thus increase concentration of the NIMC, however, this was not an appropriate solution due to solubility concerns of some of the metals including Pb, Zn and Ni. The very low concentration of some of the NIMC may

explain the lack of extraction that was observed during this study. In particular the $CuCO_3$ (2.99%) and $NiCl_2$ (0.051%) NIMC had very low initial concentrations in solution and other NIMC like $Ni(OH)_2$ (8.16%), $PbCO_3$ (10.2%) and $ZnCO_3$ (6.66%) also had relatively low initial concentrations. If the initial concentration of NIMC is low then the amount that extracted into octanol may be below the detection limits of the ICP MS.

The octanol/water partition results for the Cd, Cu, Ni, Pb and Zn complexes indicate that, although a complex has a neutral charge, it may not extract into octanol. This conclusion is likely to be true for both organic and inorganic complexes. Having a neutral charge does not directly result in a high affinity for octanol and other factors, rather, polarity of the complex, is more likely to affect extraction (Vigneault et al., 2000; Turner and Williamson, 2005). Polarity of metal complexes is important as the complex may have a neutral charge but areas of the complex may be slightly positive or negative due to electron grouping. This polarity may affect a neutral complexes ability to diffuse through a cell's membrane. This is a significant result as it demonstrates that not all neutral complexes will extract into octanol and extraction is based on the different octanol/water partition coefficients of complexes.

4.4.2 Mercuric chloride complex

The K_{OW} measured in this study of 3.28 is comparable to the K_{OW} quoted in the literature of 3.33 (Mason et al., 1996). Table 4.5 summarises the octanol/water partition coefficients of neutral mercury complexes quoted in the literature in comparison to that determined for the $HgCl_2$ complex during this experiment.

Table 4.5 Octanol/water partition coefficients of neutral mercury complexes

Source	Complex	K_{OW}
This study	$HgCl_2$	3.28
(Mason et al., 1996)	$HgCl_2$	3.33
(Mason et al., 1996)	Hg^0	4.15
(Benoit et al., 1999)	HgS	0.92
(Mason et al., 1995)	$Hg(OH)_2$	0.05
(Mason et al., 1995)	HgOHCl	1.20

The comparison of the K_{OW} of the $HgCl_2$ complex to the K_{OW} of the other neutral mercury species suggests that if present in the environment, the $HgCl_2$ complex could be environmentally important. The K_{OW} of 3.28 suggests that this complex has the ability to passively diffuse across cell membranes (Vigneault et al., 2000), making it potentially more toxic and bioavailable than a number of other neutral mercury species. The $HgCl_2$ complex may be lipid soluble in natural waters which have a solution composition that will allow for its formation.

4.4.3 Boron hydroxide complex

The calculated boron hydroxide K_{OW} was 0.23. Given the high natural concentration of boron in seawater and the fact that as much as 70% of this boron may be present as the $B(OH)_3$ neutral complex, this relative small partition coefficient could represent a significant boron pool that is potentially bioavailable. The K_{OW} determined during this study was compared to literature values in Table 4.6 below. The literature only contains boron complex partition coefficients from freshwater samples. To date no data on the octanol/water partition coefficients of boron in seawaters is available.

Table 4.6 Octanol/water partition coefficients of boron complexes

Source	Water sample	K_{OW}	pH
This study	Cronulla seawater	0.23	8.20
(Turner and Williamson, 2005)	River Aire (Freshwater)	0.13	7.14
(Turner and Williamson, 2005)	River Beaulieu (Freshwater)	0.15	6.99
(Turner and Williamson, 2005)	River Caldera (Freshwater)	0.11	7.02
(Turner and Williamson, 2005)	Treated sewage effluent (Freshwater)	0.20	6.42

Turner and Williamson (2005) determined the K_{OW} of boron in samples by the difference method. The concentration of boron in the water before and after extraction with octanol was measured and the amount of boron that extracted was equal to the difference between these measurements. This carries inherent errors as this method measures a relatively small difference between two relatively large concentrations or masses. In addition, the composition of the waters tested by Turner and Williamson (2005) were very different to the seawater tested during this study. Turner and Williamson (2005) only tested freshwater samples from rivers that received significant anthropogenic inputs. In addition, changes in pH are likely to affect the amount of $B(OH)_3$ present in a water sample. As the pH is lowered, the concentration of the neutral $B(OH)_3$ complex will be reduced due to the increased concentration of H^+ ions. The pH values measured by Turner and Williamson (2005) were approximately 1 pH unit lower than the seawater used in this investigation and this could have resulted in a significant difference in $B(OH)_3$ concentration and could account for the lower K_{OW} values in the Turner and Williamson (2005) study compared to this study.

The measured K_{OW} of 0.23 suggests that the neutral $B(OH)_3$ complex exhibits a moderate affinity for octanol and therefore may have the ability to passively diffuse across cell membranes. The previous study has also demonstrated the extraction of boron from freshwater samples into octanol (Turner and Williamson, 2005) and returned similar

partition coefficients. The partition coefficients calculated during the previous study were lower than that calculated during the present study (between 10 and 50% lower). These differences could be explained by differences in the water composition i.e. differences in ionic strength, cations and anions present and the pH based on the fact that sea water was used in this experiment and only freshwaters were tested in the previous study. Taking these factors into account and considering the octanol/water partition coefficient calculated during the present study, $B(OH)_3$ could represent a boron complex that historically has been overlooked in terms of the bioavailability of boron.

4.5 Conclusion

The purpose of this investigation was to better understand the composition of the octanol extractable metals fraction from waters. Previous work carried out by Kilgore (2007) identified that neutral inorganic metal complexes may extract into octanol. However, it was shown in this study that the 12 Cd, Cu, Ni, Pb and Zn complexes had very low octanol/water partition coefficients. Nevertheless, the $HgCl_2$ and $B(OH)_3$ complexes did extract into octanol. Octanol/water partition coefficients of 3.28 for the $HgCl_2$ complex and 0.23 for the $B(OH)_3$ complex are consistent with partition coefficients found in the literature and demonstrate that both complexes have an affinity for octanol. This affinity for octanol suggests that LSMC concentrations measured in natural waters may comprise not only organic neutral metal complexes but also some inorganic metal complexes, particularly those complexes with mercury and in the case of boron the extraction of some neutral non-metal species.

Chapter 5: Determination of LSMC Concentrations in Natural Waters

5.1 Introduction

As outlined earlier, a significant body of research has been performed to investigate the relationship between metal speciation and toxicity and bioavailability (Lund, 1990; Campbell, 1995; Paquin et al., 2002; Batley et al., 2004). It is well known that particular metal species are highly toxic to aquatic organisms (Anderson and Morel, 1978; Florence et al., 1983; Ahsanullah and Florence, 1984; Borgmann and Charlton, 1984). LSMC are one metal species that has shown significant toxicity to aquatic organisms (Florence et al., 1992; Phinney and Bruland, 1997) and indeed may be the most toxic metal species (Florence et al., 1992). There is, therefore, a need to acquire reliable environmental data on the concentration of LSMC in a range of aquatic environments. At present only a small amount of data exists on the concentrations of LSMC in natural waters. This lack of data is most likely due to the lack of a sensitive, reliable and robust method for the determination of LSMC.

The aim of this Chapter is twofold. First, to demonstrate that the analytical method developed to measure LSMC (Chapter 3) can effectively determine the concentration of LSMC in natural waters. Second, this Chapter aims to analyse a range of natural waters to identify the relative proportion of LSMC compared to the total metal concentrations in natural waters. This new data should allow for more confident predictions to be made about concentrations of LSMC in particular environments and may also help to identify the types of environments where the concentration of LSMC is likely to be increased and should therefore be further investigated.

5.2 Method

5.2.1 Methodological considerations

Key to reliable and accurate quantification of LSMC in natural waters is understanding the stability of LSMC after collection and the adsorption of LSMC to collection vessels. Little is known about the stability of LSMC in both laboratory prepared studies and in natural waters. Some work has been performed on the stability of the xanthate ligands used in the mining industry and this showed that xanthates in mineral processing waters had a half-life of between 1 and 4 days (Xu et al., 1988). At present, there is a paucity of data on the stability of other ligands and LSMC. Accordingly, it was decided that the samples should be collected, filtered and extracted within 12-24 hours, wherever possible.

Previous research has shown that particular LSMC can absorb to container walls, thus reducing their concentrations in solution (Turner and Williamson, 2005), and resulting in a underestimation of the concentration in the collected natural water samples. This was particularly evident with the PAX – Cu complex during the method development experiments (Chapter 3.2.3.4). Whilst this adsorption was identified in samples made up in Milli-Q water, natural waters are likely to contain numerous organic and inorganic components that may assist in the reducing or eliminating adsorption. It was decided that samples should be collected, filtered and extracted as quickly as possible to minimise any adsorption. It was desirable that samples could be collected, filtered and extracted within 2 hours, however, if samples were to be kept longer they should be stored at 4°C in the dark and should not be stored for more than 24 hours before extraction.

Due to LSMC stability and adsorption issues, the sampling sites for this study needed to be easily accessible. In addition, the sites should not require the use of a boat for waters to be collected and hand or pole sampling should be easily performed. This would ensure that the time between sampling and laboratory filtration and extraction was minimised.

5.2.1.1 Sample site selection

As it was believed that the formation of synthetic LSMC relies on both the presence of organic and trace metal contamination, sampling sites were chosen with this in mind. Sites with historic contamination of organics and/or metals were chosen to test the method. Relatively pristine sites that contain little or no contamination are not likely to contain measurable concentrations of LSMC; naturally occurring LSMC are likely to be present only in concentrations at or near the limits of detection of the method. It was important that the sample locations should represent typical aquatic environments from not only the Sydney region but should be representative of aquatic environments within Australia and throughout the world. These sites should include both fresh and saline waters and should receive a range of anthropogenic contamination from industry and urban development.

5.2.2 Location of sample sites

Samples were collected from three locations across Sydney (Figure 5.1). The locations chosen were Centennial Park, Homebush Bay and Cooks River. These locations cover both fresh and saline aquatic environments. A brief description of each location is given below.

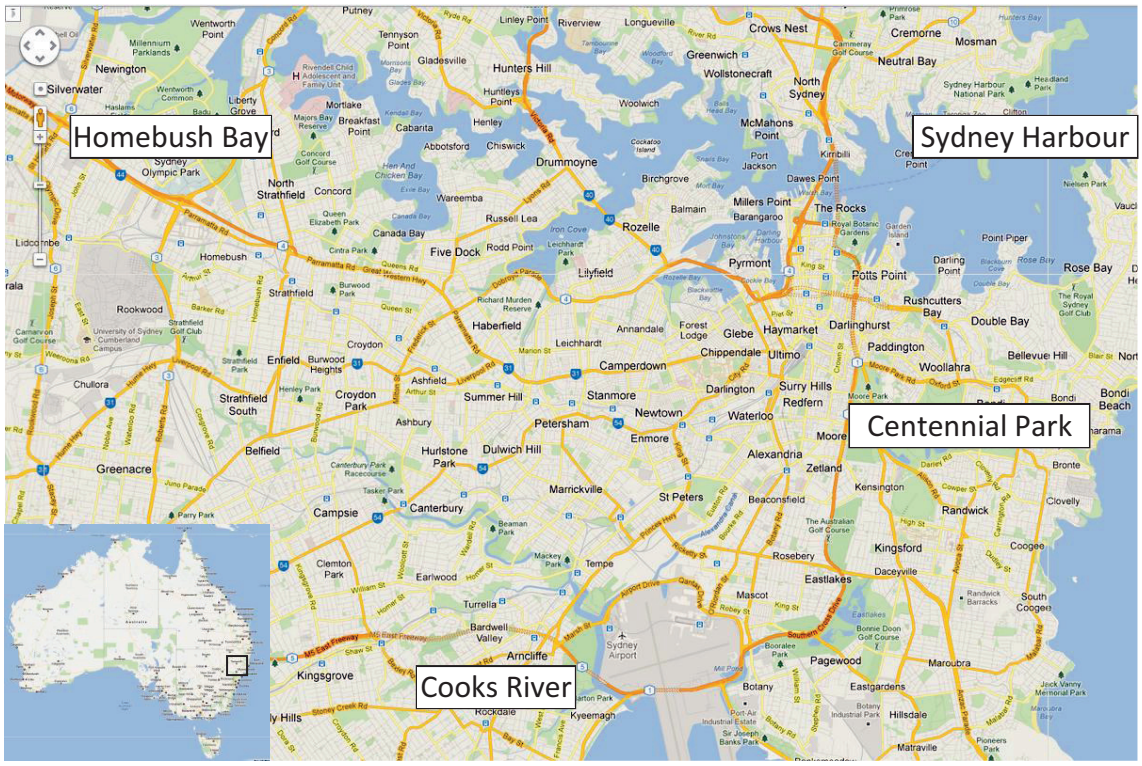

Figure 5.1 Map of Sydney region with sampling locations labelled Source: (Google, 2012)

5.2.2.1 Centennial Park

Centennial Park is a large public urban park that covers approximately 220 hectares in the Eastern suburbs of Sydney, 4 km south-east of the centre of the city. The park was originally set aside as a public area in 1820, however it was not opened as Centennial Park until 1888. The park is now completely surrounded by urban development and is used extensively as a recreational area. The ponds in Centennial Park receive water from the surrounding urban environment. This includes run-off from roads, run-off from playing fields and general storm water from the area. These waters are likely to contain elevated concentrations of organics and trace metals. Centennial Park is representative of a highly urbanised fresh water catchment area.

Five sites were sampled from within the park (Figure 5.2). The ponds situated within the park are all connected through a series of channels and drains and therefore, it was expected that the concentrations of LSMC at each of the sites would be similar.

Figure 5.2 Centennial Park location with 5 sampling sites marked Source: (Google, 2012)

5.2.2.2 Homebush Bay

Homebush Bay is a suburb situated 16 km west of Sydney and contains one of a number of bays along the Parramatta River. At the time of European settlement in 1788, Homebush Bay was a series of tidal wetlands and thick bushland. Since 1879, the NSW Government has gradually purchased or reclaimed the land for various uses which have included; an armament depot, a state hospital, a state abattoir and a correctional facility. For many years the area was also used as state brickworks to supply the building industry. Land filling and

wetland reclamation has resulted in over half of the original wetland area being destroyed. In the 1960's and 70's, large amounts of dredged material from Parramatta River were deposited on the mangrove areas of the Homebush Bay area and this area also became the dumping site for much of Sydney's household rubbish. The Sydney 2000 Olympics and Paralympics saw significant restoration works performed on the Homebush Bay area as Sydney Olympic Park (SOP) was developed as the focal point of the games. The SOP site now consists of a town centre that includes nine world-class sporting and entertainment venues, two hotels, commercial buildings for a growing business community and 430 hectares of parklands.

Homebush Bay is historically contaminated with a range of organic contaminants which include organochlorine pesticides, polychlorinated biphenyls, polycyclic aromatic hydrocarbons and dioxins (Birch and Taylor, 2000; McCready et al., 2000; Birch et al., 2007). High concentrations of heavy metals have also been measured in sediments in the Homebush Bay area (Suh et al., 2004). This makes Homebush Bay an excellent site for the determination of LSMC in waters as historically it has shown both organic and metal contamination. Whilst Homebush Bay itself is an estuarine environment, due to access difficulties, it was impractical to sample from the bay. Therefore, five sites were sampled along Haslam's creek (Figure 5.3) which feeds from the highly urbanised old athlete's village into Homebush Bay. The Bay is likely to contain the highest levels of contamination. Haslam's creek is representative of an urban freshwater environment that is not only receiving contaminants from the urban environment but has also been contaminated from historical industrial use of the area.

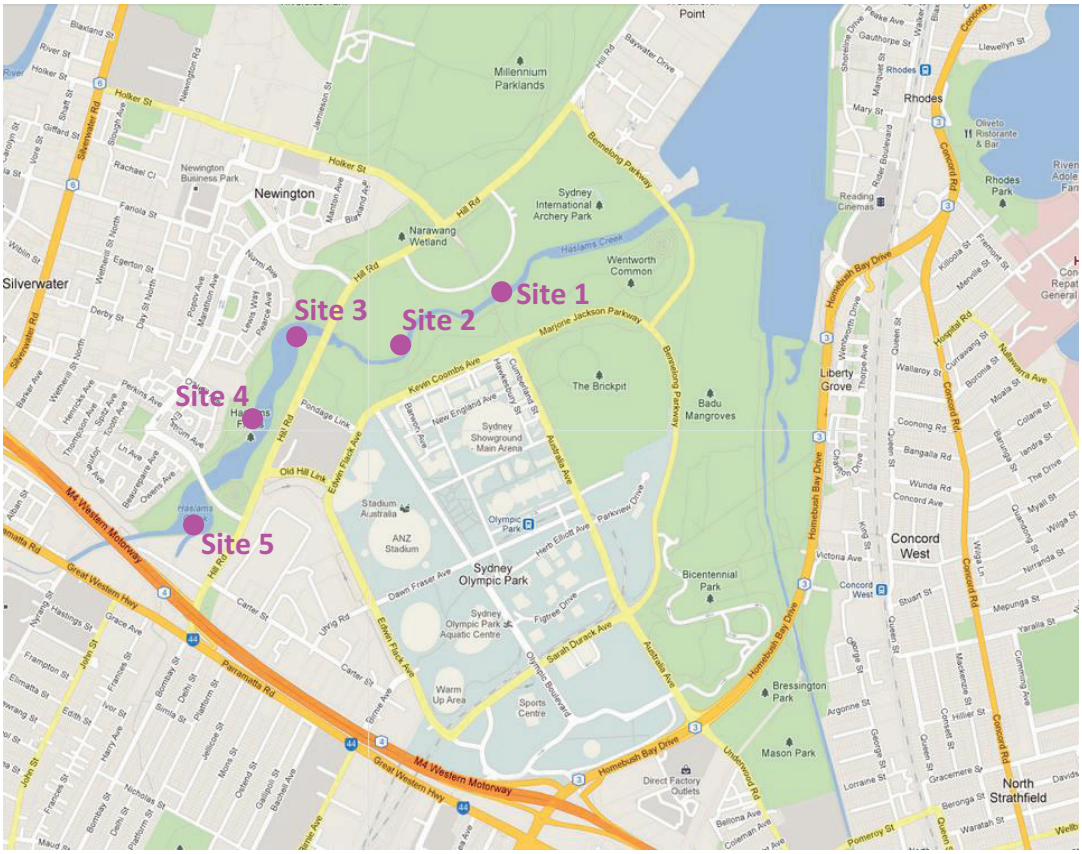

Figure 5.3 Homebush Bay location with 5 sampling sites marked Source: (Google, 2012)

5.2.2.3 Cooks River

The Cooks River is a 23 km long urban waterway in the south-west of Sydney. It serves as part of a stormwater system that services a 100 Km^2 catchment area. Many of the original streams have been replaced with concrete lined channels. The River begins at Yagoona, 20 km south-west of Sydney and empties into Botany Bay only 3 km south of Sydney. The river is highly impacted due to the fact that over 400,000 people live within its catchment area and 100,000 commercial and industrial premises can also be found within the catchment. The river receives a number of pollutant inputs which include; pollution from motor vehicles, litter, sewage, illegal dumping, industrial, commercial and domestic activities. Water quality testing has indicated that the river is contaminated with concentrations of

pharmaceutical and industrial chemicals which are similar to those found in untreated sewage (Cubby, November 2011). This contamination is most likely due to a very old sewage system which is releasing contaminants such as soaps, insecticides, caffeine and anticonvulsants into the waterway (Cubby, November 2011). Twelve sites were selected along the length of the river and in Botany Bay and include both fresh and saline waters (Figure 5.4).

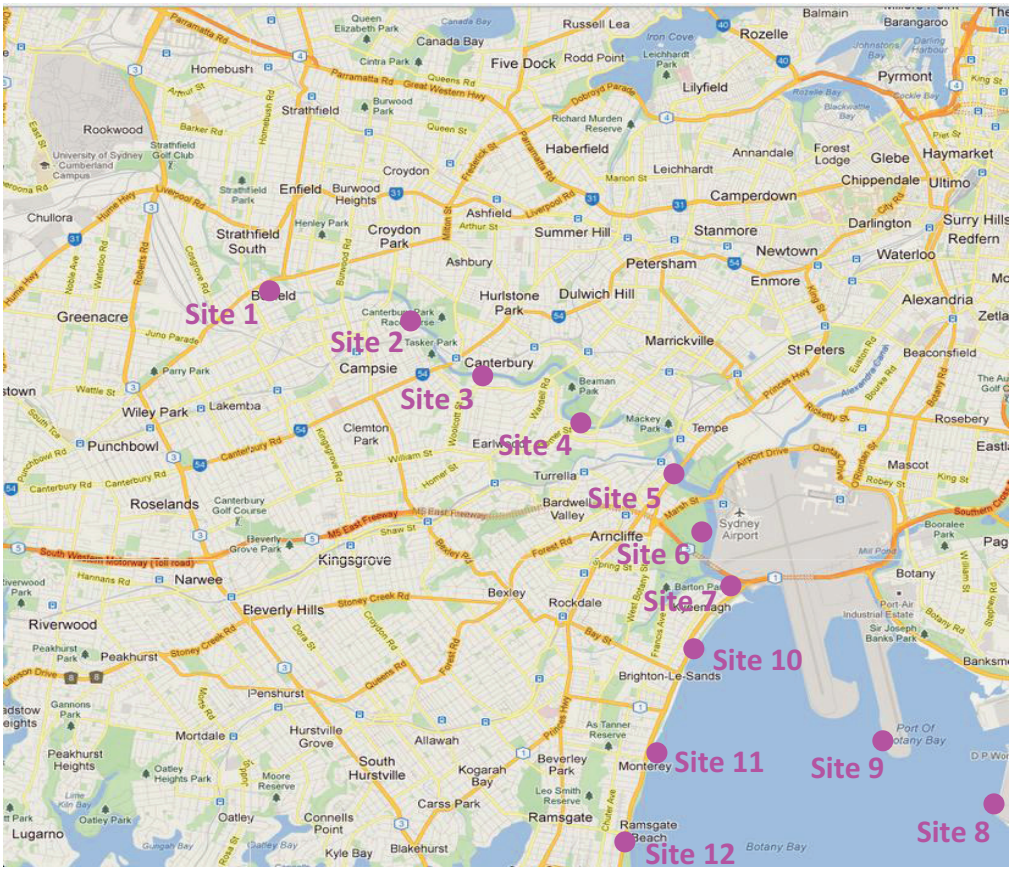

Figure 5.4 Cooks River location with 12 sampling sites marked Source: (Google, 2012)

All three sampling locations were situated within 1 hours drive of the CSIRO Centre for Environmental Contaminants Research laboratory at Lucas Heights where the chemical analysis was performed. As the sampling sites are close to the laboratory it allowed for collection, filtration and analysis to occur within the shortest time possible.

5.2.3 Sample collection and transportation

Samples were collected over 4 days from 9th to the 12th of January 2012. One litre water samples were collected from each site in acid washed Nalgene bottles. Samples were collected by gloved hand where possible or by acid washed pole sampler where access to the waterway was more difficult. The pole sampler is an 8 foot plastic pole with a cradle on the end in which an acid washed Nalgene bottle can be placed. The pole can then be used to submerge the cradle and the Nalgene bottle and collect samples where direct access to the waterway is limited. Samples were immediately placed in a cold, portable cooler after collection and were transported to the CSIRO laboratory. A second 200-mL sample was collected at each site and was used to measure temperature, salinity and dissolved oxygen in the field immediately after collection and was returned to the laboratory for pH analysis.

5.2.4 Sample filtration

On return to the laboratory, samples were immediately filtered to 0.45 µm in acid washed Sartorius filtration units using positive pressure via nitrogen gas. Filter papers were acid washed prior to use by filtering 100 mL of 10% (v/v) HNO_3 followed by 3 x 100 mL aliquots of Milli-Q water. The whole 1-L water sample was then filtered and placed in a new, acid-washed Nalgene bottle. Once filtered, the samples were stored at 4°C until extraction was performed. A 100-mL sample aliquot was placed into an amber glass vial and sent to the National Measurement Institute (NMI), Pymble, NSW, Australia for dissolved organic carbon (DOC) analysis. The vials for DOC analysis already contained sulphuric acid for preservation of the samples.

5.2.5 Measurement of physico-chemical parameters

The pH of the collected water samples was measured using an Orion Thermo pH meter and probe. The temperature and salinity of each of the samples was measured in the field directly after collection using a YSI 30 temperature and salinity meter. The concentration of dissolved oxygen was determined in each of the collected waters directly after collection using a WTW Oxi330 DO meter with a WTW Cell Ox325 probe.

5.2.6 LSMC determination

The collected and filtered water samples were allowed to return to room temperature before they were extracted using the method outlined in section 2.4.3. The average time between collection and extraction was 3 hours, with most samples being collected and analysed within 2 hours. Octanol extracts were combined in acid washed round bottom flasks and stored in the laminar flow cabinet before back extraction by vacuum distillation following the method outlined in section 3.3. Back extracts were analysed directly by ICP MS with matrix matched standards and spike additions to correct for any matrix interferences

5.2.7 Total dissolved metal concentrations determination

Total dissolved metals analysis was performed on all collected samples. A 10-mL subsample of the filtered waters was transferred into an acid washed 20-mL vial and immediately acidified to 0.2% (v/v) HNO_3. Freshwater samples were analysed directly by ICP MS whilst saline waters were diluted 1:5 with Milli-Q water which was acidified to 0.2% (v/v) HNO_3. Dilution was performed to reduce the salt content of the samples which causes matrix interferences and ‘salting up’ of the ICPMS. Undiluted freshwaters and diluted saline waters

were analysed with matrix matched standards and spike additions to identify and correct for any matrix interferences.

5.2.8 Determination of aluminium and iron

Octanol is a very viscous solvent and because of this, colloids may become entrained in the octanol and bias the back extract results. The colloids would remain in the round bottom flasks post vacuum distillation and may leach metals into the back extracts during the addition of concentrated HNO_3 and Milli-Q water. The metals leached from colloids represent a metal concentration that would not be lipid-soluble and thus would positively bias the LSMC concentrations measured in the back extracts. Al and Fe present in natural waters with a pH close to neutral will mainly be found as colloids or attached to colloids. By measuring the concentration of Al and Fe in the back extracts, the severity of colloid entrainment within the octanol could be assessed (Benoit et al., 1994; Dupré et al., 1999) and a determination made about the validity of the LSMC concentrations measured in these back extracts. There may not be a direct relationship between Al and Fe and the other metals of interest in relation to their presence on colloids. This restricts the back extract data from being corrected for the presence of Al and Fe, however, the presence of high concentrations of Al and Fe in the back extracts may indicate that entrainment of colloids is high and the remaining metal results should be viewed within this context.

5.2.9 Statistical analysis of the collected data

Relationships between the concentrations of LSMC and physico chemical parameters and dissolved metal concentrations were examined using Pearson's product moment

correlations. Analysis was done using the Number Crunch Statistical Software (NCSS) package. The significance level (α) was 0.05.

5.3 Results

5.3.1 Physico chemical data

The pH, temperature, salinity and dissolved oxygen values were within the expected range for natural waters and demonstrate that both fresh and saline waters were collected (Table 5.1). The results also indicate that the waters were adequately oxygenated.

Table 5.1 Physical chemistry data from collected fieldwork samples

	pH	Temperature (°C)	Salinity	DO (% Saturation)
Centennial Park 1	7.3	22.4	0.4	78
Centennial Park 2	6.6	23.1	0.2	75
Centennial Park 3	6.5	22.8	0.1	77
Centennial Park 4	7.2	22.5	0.1	79
Centennial Park 5	6.9	23.2	0.2	82
Homebush Bay 1	7.0	22.1	0.4	82
Homebush Bay 2	7.0	21.9	0.1	85
Homebush Bay 3	7.2	23.2	0.1	91
Homebush Bay 4	6.7	22.8	0.3	88
Homebush Bay 5	7.2	22.6	0.1	84
Cooks River 1	8.2	23.8	0.8	93
Cooks River 2	7.8	22.4	1.6	86
Cooks River 3	7.6	23.9	5.0	86
Cooks River 4	7.6	23.5	8.5	74
Cooks River 5	7.9	23.9	15.6	68
Cooks River 6	7.6	24.5	19.2	68
Cooks River 7	7.9	23.2	31.5	92
Cooks River 8	8.0	23.0	30.7	84
Cooks River 9	8.0	24.7	30.9	81
Cooks River 10	7.9	23.9	29.9	87
Cooks River 11	7.8	24.9	30.5	83
Cooks River 12	7.9	24.1	30.9	91

In total, the total dissolved metals samples and LSMC back extracts were analysed for 21 elements. Of these elements only 7 (Cd, Cu, Ni, Pb, Zn, Al and Fe) demonstrated concentrations of LSMC greater than the instrument limits of detection. The remaining elements had concentrations lower than the instrument limits of detection and therefore further analysis of the concentration of these elements in waters was not performed. The instrument limits of detection of all 21 elements are summarised below (Table 5.2).

Table 5.2 Limits of detection of the 21 elements analysed during this research

Element	LOD (µg/L)
Li	0.011
Be	0.001
Ti	0.008
V	0.002
Cr	0.022
Mn	0.004
Co	0.002
As	0.018
Se	0.002
Sr	0.011
Mo	0.003
Ag	0.001
Sb	0.001
Tl	0.001
Cd	0.002
Cu	0.009
Ni	0.009
Pb	0.012
Zn	0.021
Al	0.008
Fe	0.026

LOD = limit of detection

All data were drift corrected if drift during the ICPMS analysis exceeded 10% and were blank corrected if the mean blank exceeded the instrument limits of detection. Only data for metals which returned concentrations greater than the limits of detection have been presented.

Three method blanks were analysed concurrently with samples from each of the three locations and these method blanks were combined to determine the method limits of detection for the analysis of natural waters. The method blank data and limits of detection are only presented for the elements that showed extraction into octanol greater than the limits of detection.

5.3.2 Method performance data

The method blanks concentrations were all low enough to allow for the accurate determination of environmental concentrations of LSMC in natural waters (Table 5.3). Zinc and Aluminium results were slightly elevated compared to the other five metals. This elevation was most likely due to residual contamination within the solvent or due to atmospheric contamination introduced to the samples during the vacuum distillation back extraction process. This apparent contamination was attempted to be eliminated by cleaning the solvent prior to use and ensuring the samples were exposed to the atmosphere for the least amount of time possible however it appears that a minor amount of contamination still occurred. Even though this contamination was present within the nine method blanks the limits of detection of Zn and Al were low enough to allow for the detection of environmental concentrations of lipid soluble Zn and Al.

Table 5.3 Mean method blank results and limits of detection

	Cd (µg/L)	Cu (µg/L)	Ni (µg/L)	Pb (µg/L)	Zn (µg/L)	Al (µg/L)	Fe (µg/L)
Mean Method blank (n=9)	0.045	0.164	0.371	0.105	0.909	9.365	0.461
LOD	0.002	0.008	0.001	0.004	0.028	0.017	0.019

5.3.3 Centennial Park

Measurable concentrations of Cu, Zn, Pb, Al and Fe as LSMC and in solution were detected at all Centennial Park sites (Table 5.4). Concentrations of Cd, Ni, Li, Be, Ti, V, Cr, Mn, Co, As, Se, Sr, Mo, Ag, Sb, Tl were all below detection limits. The percentage of Al and Fe present as a LSMC was relatively low (<4%) indicating that colloid entrainment within the solvent was not significant and the results obtained were most likely due to the extraction of LSMC. Therefore, the lipid soluble concentrations of Cu, Zn and Pb measured are also most likely due to the extraction of LSMC and not colloid entrainment contamination of back extracts. Variability within the duplicate measurements made on the sample collected from Site 1 was calculated (0.10 µg/L) and considered to not be of concern (Table 5.5). The percent relative standard deviation (SD) measured between the two replicate samples, in most cases, was less than 15%, which is not of significant concern considering the very low concentrations of both total metals and LSMC measured in the samples. The percentage of total metal present as a lipid soluble complex was calculated based on results from Table 5.4 and has been displayed as a bar graph (Figure 5.5).

Table 5.4 Total dissolved metals, LSMC metals data and DOC data, Centennial Park

	Total Cu (µg/L)	LSMC Cu (µg/L)	Total Pb (µg/L)	LSMC Pb (µg/L)	Total Zn (µg/L)	LSMC Zn (µg/L)	Total Al (µg/L)	LSMC Al (µg/L)	Total Fe (µg/L)	LSMC Fe (µg/L)	DOC (mg/L)
Site 1 (mean n=2)	2.21	0.140	2.64	0.073	8.10	0.163	90.4	0.901	199	3.99	2.90
Site 2	2.08	0.099	0.184	0.019	8.73	0.175	175	1.75	851	8.51	3.30
Site 3	4.40	0.533	0.488	0.059	5.37	0.215	94.0	2.82	514	15.8	2.90
Site 4	3.80	0.479	0.607	0.085	49.9	0.499	16.8	0.504	139	5.55	3.00
Site 5	7.67	0.076	0.404	0.026	16.1	0.063	34.03	0.340	211	2.12	3.10
LOD	0.001	0.008	0.006	0.004	0.001	0.028	0.010	0.017	0.009	0.019	0.100

Table 5.5 Variation between duplicate analysis of Centennial Park Site 1

	Total Cu (µg/L)	LSMC Cu (µg/L)	Total Pb (µg/L)	LSMC Pb (µg/L)	Total Zn (µg/L)	LSMC Zn (µg/L)	Total Al (µg/L)	LSMC Al (µg/L)	Total Fe (µg/L)	LSMC Fe (µg/L)	DOC (mg/L)
Site 1	2.18	0.120	2.89	0.058	8.11	0.162	91.0	0.910	178	3.58	3.10
Site 1 Dup	2.23	0.159	2.39	0.087	8.08	0.163	89.8	0.891	219	4.39	2.70
Mean	2.21	0.140	2.64	0.073	8.10	0.163	90.4	0.901	199	3.99	2.90
SD	0.035	0.028	0.354	0.021	0.021	0.001	0.849	0.013	29.0	0.573	0.283
SD as % of Mean	1.6	20	13	29	0.3	0.6	0.9	1.4	15	14	9.8

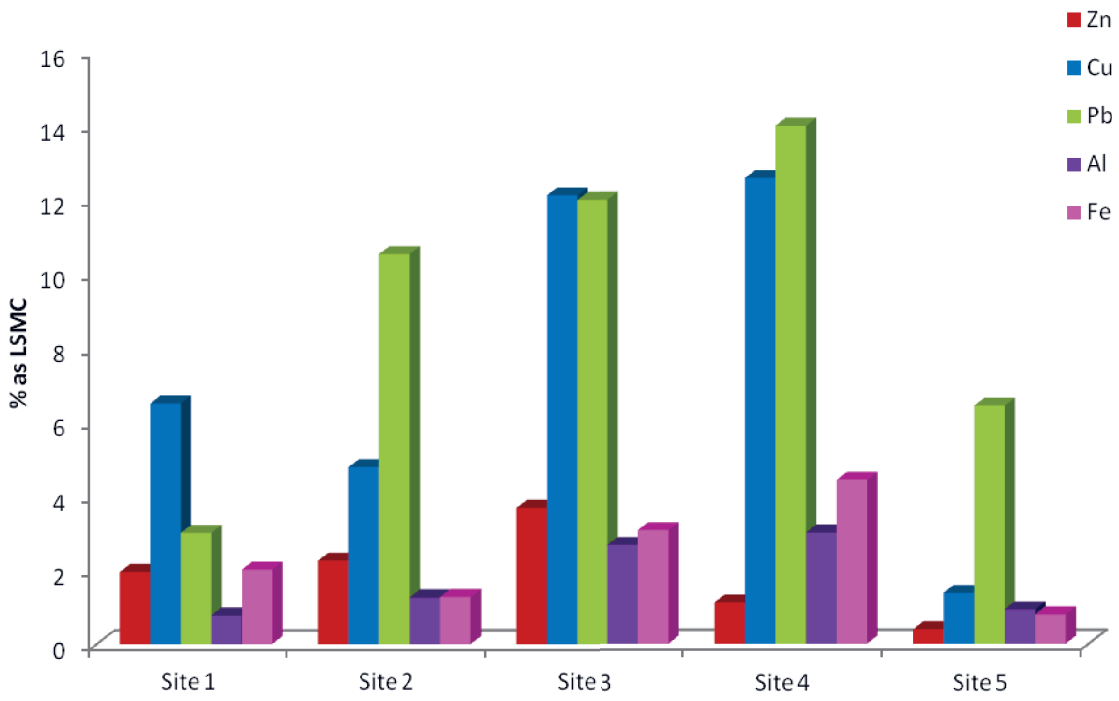

Figure 5.5 Percent of total dissolved metals present as a LSMC, Centennial Park

Visual analysis of the data revealed no significant results; the Cu, Pb and Zn totals metal data and LSMC data did not return any results which were considered to be outliers and the results were relatively similar. There was also little variability within the data which is clearly demonstrated in the above figure. The greatest variation in the percent of metal present as a LSMC was only approximately 12% (Cu and Pb), which can be considered minor due the low concentrations that are being detected and the inherit variability within the LSMC method. The smallest variation was observed within the Zn, Al and Fe data with the highest and lowest percentages differing by approximately 3%. This low variation was expected at Centennial Park because the five ponds sampled are interconnected by a series of overflow drains and during periods of rain, large amounts water flows between them. From Figure 5.5 (and Table 5.4) there appears to be no clear relationship between any of the metals measured and there also appears to be no clear trend in relation to the sites sampled although sites 2, 3 and 4 do appear to have a slightly higher percentages of Cu and Pb present as LSMC compared to sites 1 and 5. This increase is not due to a large increase in the measured concentration of LSMC at these three sites, but rather is due to a decrease in the concentration of total metal measured. In general, the concentration of LSMC was stable and the total metal concentration fluctuated giving rise to some small variation among sites.

There were no significant correlations ($p<0.05$) between the concentrations of LSMC and total metals for any of the metals tested (Table 5.6). The lack of correlation suggests that simply raising the total dissolved metal concentration does not increase the concentration of LSMC. A positive correlation between dissolved and lipid soluble metal concentrations

could indicate that the organic ligands capable of forming LSMC were not saturated with metal ions or that extraction of metals into octanol is not only due to the extraction of organic LSMC but due to neutral inorganic metal complexes and possibly some extraction of other metal species. This is an important result as it suggests that the presence of organic or inorganic ligands may not be the limiting factor in the formation of the LSMC in the Centennial Park samples. There was little significant difference in DOC concentrations between any of the sites (<14%) whilst the concentration of LSMC did fluctuate slightly across the five sites. This suggests that the relationship between dissolved and lipid soluble metal concentrations is not controlled by the total concentration of organic matter in the sample and is more likely related to the type of organic matter present.

Table 5.6 Correlation results for total dissolved metal and LSMC concentrations, Centennial Park

Cu	Pb	Zn	Al	Fe
r = 0.011	r = 0.201	r = 0.879	r = 0.779	r = 0.684
p = 0.986	p = 0.746	p = 0.053	p = 0.120	p = 0.203

There was a significant correlation between the concentrations of lipid soluble Al and lipid soluble Fe (r= 0.931, p=0.022, Table 5.7). This suggests that the extraction of these two metals is related which could indicate an association of these two elements with a common factor, for example, colloidal material within the water samples and/or some complexes with humic material that might extract into octanol. No further significant correlations were found between any of the other LSMC indicating that there extraction into octanol is all independent of each other.

Table 5.7 Correlations between LSMC concentrations of different metals in water samples from Centennial Park.

		LSMC				
		Cu	**Pb**	**Zn**	**Al**	**Fe**
LSMC	**Cu**	-				
	Pb	r = 0.843 p = 0.073	-			
	Zn	r = 0.629 p = 0.256	r = 0.799 p = 0.105	-		
	Al	r = 0.243 p = 0.693	r = -0.137 p = 0.826	r = -0.181 p = 0.771	-	
	Fe	r = 0.568 p = 0.318	r = 0.164 p =0.792	r = 0.099 p = 0.875	r = 0.931 p = 0.022	-

There was no significant correlation between DOC concentrations and lipid soluble Cu, Ni and Pb concentrations (Table 5.8), which suggests that the concentration of LSMC was independent of the total concentration of dissolved organics at the sites. This indicates that, whilst LSMC may contain some organic portions, there formation is most likely related to the types of dissolved organics present and not the total concentration of organics. That is only specific types of organic molecules have the ability to form LSMC with metals in solution.

Table 5.8 Correlation results of DOC measurement and LSMC concentrations, Centennial Park

	DOC	
Sample	**r value**	**p value**
Centennial Park Cu	0.499	p = 0.392
Centennial Park Ni	-0.234	p = 0.705
Centennial Park Pb	0.384	p = 0.523

5.3.4 Homebush Bay

Cu, Pb and Zn concentrations were above the method limits of detection in all sites sampled in Homebush Bay (Table 5.9). Concentrations of Cd, Ni, Li, Be, Ti, V, Cr, Mn, Co, As, Se, Sr, Mo, Ag, Sb, Tl were all below detection limits. The percentage of total metal present as a LSMC is relatively similar across the three metals (Fig 5.6). Of concern is the fact these percentages are similar to those measured for Al and Fe indicating that the extraction of Cu, Zn and Pb may be due to the entrainment of colloids in the octanol extracts and not extraction of LSMC. As the percentage extraction of Al and Fe and Cu, Pb and Zn are relative similar and Al and Fe are used as an indication of the extent of extraction of colloids this similarity makes it difficult to confidently state that the extraction of Cu, Pb and Zn is due to LSMC.

As with the Centennial Park samples, the DOC values were similar across the 5 sites (<3%) which suggests that any differences between the LSMC concentrations across the sites was not due to the total concentration of organics in solution. This result may indicate that if organics within the sample are having an effect on the concentration of LSMC, it is the type of organics present and not just the total concentration of organics that is important. Variability within the duplicate measurements made on the sample collected from Site 1 was low and considered to not be of concern. Similarly standard deviations were all less than 15% of the mean concentrations measured for each of the 5 elements (Table 5.10). This is acceptable variation given the inherent errors of solvent extraction and vacuum distillation back extraction and also the small concentrations of both total metals and LSMC measured in the water samples.

Table 5.9 Total metals data and LSMC metals data, Homebush Bay

	Total Cu (µg/L)	LSMC Cu (µg/L)	Total Pb (µg/L)	LSMC Pb (µg/L)	Total Zn (µg/L)	LSMC Zn (µg/L)	Total Al (µg/L)	LSMC Al (µg/L)	Total Fe (µg/L)	LSMC Fe (µg/L)	DOC (mg/L)
Site 1 (Mean n=2)	2.18	0.093	0.041	0.029	6.15	0.257	13.2	0.322	209	9.34	13.9
Site 2	1.11	0.051	0.037	0.024	7.07	0.212	9.45	0.094	576	11.6	14.00
Site 3	3.72	0.131	0.057	0.029	7.78	0.312	35.9	0.718	223	6.75	13.90
Site 4	1.55	0.075	0.055	0.031	16.4	0.328	30.8	1.54	411	4.25	13.90
Site 5	3.20	0.092	0.044	0.019	15.0	0.144	47.1	0.490	115	3.25	14.10
LOD	0.001	0.008	0.006	0.004	0.001	0.028	0.010	0.017	0.009	0.019	0.100

Table 5.10 Variation between duplicate analysis of Homebush Bay Site 1

	Total Cu (µg/L)	LSMC Cu (µg/L)	Total Pb (µg/L)	LSMC Pb (µg/L)	Total Zn (µg/L)	LSMC Zn (µg/L)	Total Al (µg/L)	LSMC Al (µg/L)	Total Fe (µg/L)	LSMC Fe (µg/L)	DOC (mg/L)
Site 1	2.14	0.100	0.039	0.026	6.29	0.273	13.9	0.297	221	8.87	14.10
Site 1 Dup	2.21	0.086	0.042	0.031	6.00	0.241	12.5	0.346	196	9.80	13.70
Mean	2.18	0.093	0.041	0.029	6.15	0.257	13.2	0.322	209	9.34	13.9
SD	0.049	0.010	0.002	0.004	0.205	0.023	0.990	0.035	17.7	0.658	0.283
SD as % of Mean	2.2	11	4.9	14	3.3	8.9	7.5	11	8.5	7.0	2.0

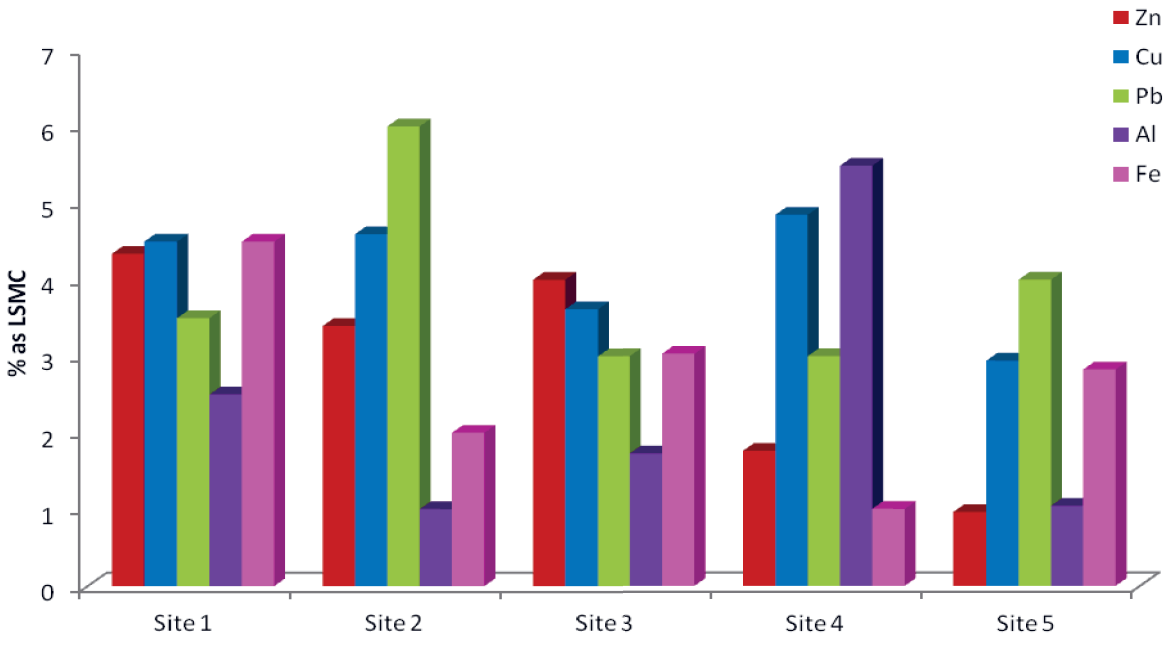

Figure 5.6 Percent of total dissolved metals present as LSMC, Homebush Bay

The bar graph above (Figure 5.6) clearly demonstrates that the percentage of metal present as a LSMC is similar across the 5 sites analysed. There appears to be no trends within the data across the 5 sites and also between the metals analysed. The highest percentage was measured at site 2, where 6% of the Pb was present as a LSMC. This site also returned one of the lowest percentages with only 1% of the Al being present as a LSMC. The spread of the data is quite small with only a 5% spread across the 5 metals and 5 sites analysed. Taking into account the fact that the concentrations are all in the low µg/L range this is a very small range for the data set. The results appear to be varying randomly and independently of each other.

A significant correlation was observed between the total dissolved and lipid soluble Cu and Fe concentrations (Table 5.11). No other statistically significant correlations were found between the other total dissolved and lipid soluble metal concentrations. The r values, of 0.925 and 0.981 for Cu and Fe, respectively, indicate that the relations were relatively strong. The Cu relationships was not surprising as increasing the total concentration of Cu in solution would increase the amount of Cu present that is available for binding with organic and inorganic ligands. The Fe relationship might indicate that as the amount of colloid bound Fe increases (as seen by an increase in the total Fe due to the fact that at natural pH values most of the Fe is associated with colloids); the concentration of Fe in the back extracts also increases due to colloid entrainment within the octanol extracts. This suggests that the issue of colloid entrainment could be of concern and could be affecting the measured LSMC concentration of other metals if they are also attached to these colloids.

Table 5.11 Correlation results for total dissolved metal and LSMC concentrations, Homebush Bay

Cu	Pb	Zn	Al	Fe
r = 0.925 p = 0.024	r = 0.033 p =0.958	r = -0.300 p = 0.624	r = -0.044 p = 0.944	r = 0.981 p = 0.003

A correlation was also identified between the lipid soluble Pb and Zn concentrations with an r value of 0.926 and a p value of 0.024 (Table 5.12). This indicates that as the concentration of lipid soluble Pb increased the concentration of lipid soluble Zn also increased. No other significant ($p<0.05$) correlations were identified between any of the other LSMC concentrations of the other metals analysed.

Table 5.12 Correlation results for LSMC concentration data, Homebush Bay

		LSMC				
		Cu	**Pb**	**Zn**	**Al**	**Fe**
LSMC	**Cu**	-				
	Pb	r = 0.223 p = 0.718	-			
	Zn	r = 0.295 p = 0.630	r = 0.926 p = 0.024	-		
	Al	r = -0.470 p = 0.425	r = 0.451 p = 0.446	r = 0.402 p = 0.503	-	
	Fe	r = -0.817 p = 0.091	r = 0.067 p = 0.915	r =0.174 p = 0.780	r = 0.596 p = 0.289	-

Relationships between the DOC concentrations and lipid soluble Cu, Pb and Zn concentrations measured at the 5 sites were investigated (Table 5.13). No significant correlation ($p<0.05$) was found within the data suggesting that the concentration of LSMC

was independent of the total concentration of dissolved organics at the sites measured as was the case with the data collected from Centennial Park.

Table 5.13 Correlation results of DOC measurement and LSMC concentrations, Homebush Bay

	DOC	
Sample	**r value**	**p value**
Homebush Bay Cu	0.712	p = 0.178
Homebush Bay Pb	0.382	p = 0.526
Homebush Bay Zn	0.203	p = 0.743

5.3.5 Cooks River

Cd, Cu, Ni, Pb, Zn, Al and Fe total dissolved and LSMC concentrations in water samples were above the method limits of detection in a number of the Cooks River samples (Table 5.14). The remaining metals (Li, Be, Ti, V, Cr, Mn, Co, As, Se, Sr, Mo, Ag, Sb, Tl) were all below detection limits

Table 5.14 Total dissolved metals data and LSMC metals data, Cooks River

	Total Cd (µg/L)	LSMC Cd (µg/L)	Total Cu (µg/L)	LSMC Cu (µg/L)	Total Ni (µg/L)	LSMC Ni (µg/L)	Total Pb (µg/L)	LSMC Pb (µg/L)	Total Zn (µg/L)	LSMC Zn (µg/L)	Total Al (µg/L)	LSMC Al (µg/L)	Total Fe (µg/L)	LSMC Fe (µg/L)	DOC (mg/L)
Site 1	0.073	<0.002	4.01	0.061	2.10	0.025	0.140	0.020	17.1	0.225	338	0.595	194	0.742	6.90
Site 2	0.065	<0.002	5.02	0.047	1.27	0.021	0.051	0.008	31.8	0.160	398	0.467	405	1.65	3.00
Site 3	0.028	<0.002	1.24	0.007	1.51	0.009	0.039	0.006	9.84	0.036	394	0.364	103	0.234	3.90
Site 4	0.035	<0.002	0.676	0.006	2.05	0.011	0.052	0.010	5.25	0.116	499	1.17	85.5	0.161	2.50
Site 5	0.060	<0.002	0.569	<0.001	0.750	0.007	0.044	0.005	5.39	0.052	538	0.603	89.0	0.112	1.80
Site 6	0.034	0.003	1.94	0.106	0.837	0.013	0.048	0.007	6.61	0.223	464	0.528	86.4	0.095	1.80
Site 7	0.067	<0.002	1.13	0.006	1.03	0.008	0.039	0.006	3.64	0.112	434	0.269	86.4	0.026	2.50
Site 8	0.043	<0.002	0.682	0.003	0.496	0.008	0.037	0.006	2.28	0.020	311	0.310	94.6	0.017	1.00
Site 9	0.056	<0.002	0.323	0.010	0.534	0.009	0.011	<0.002	2.33	0.090	347	0.393	76.0	0.038	0.90
Site 10	0.079	<0.002	0.516	0.005	0.959	0.007	0.022	0.003	1.87	0.041	322	0.312	72.4	<0.011	1.30
Site 11	0.046	<0.002	0.445	0.027	0.624	0.007	0.016	<0.002	1.51	0.070	381	0.618	73.4	0.017	1.10
Site 12	0.032	0.004	0.404	0.024	0.484	0.006	0.049	0.006	2.24	0.024	398	1.06	81.7	0.012	1.00
LOD	0.008	0.002	0.007	0.001	0.005	0.001	0.002	0.002	0.006	0.008	0.045	0.016	0.047	0.011	0.100

Table 5.15 Percent of total dissolved metals present as LSMC, Cooks River

	Percent						
	Cd	**Cu**	**Ni**	**Pb**	**Zn**	**Al**	**Fe**
Site 1	0.5	1.5	1.2	14.4	1.3	0.2	0.4
Site 2	<0.1	0.9	1.7	15.5	0.5	<0.1	0.4
Site 3	<0.1	0.5	0.6	15.1	0.4	<0.1	0.2
Site 4	<0.1	0.9	0.5	20.0	2.2	0.2	0.2
Site 5	<0.1	0.2	0.9	11.3	1.0	<0.1	<0.1
Site 6	7.7	5.5	1.6	14.6	3.4	<0.1	<0.1
Site 7	<0.1	0.5	0.8	15.6	3.1	<0.1	<0.1
Site 8	<0.1	0.4	1.6	16.3	0.9	<0.1	<0.1
Site 9	0.9	3.0	1.7	8.2	3.9	<0.1	<0.1
Site 10	<0.1	1.0	0.7	15.5	2.2	<0.1	<0.1
Site 11	0.4	6.1	1.2	9.7	4.7	0.2	<0.1
Site 12	11.2	6.0	1.3	12.1	1.1	0.3	<0.1

The concentration of Al and Fe in the LSMC analysis was low (<1.65 µg/L) (Table 5.14). This translated to the percentage of Al and Fe present as LSMC (Table 5.15) also being low (<0.3% for Al and <0.4% for Fe). These low percentages indicate that the entrainment of colloids within the octanol extracts is not a significant concern and therefore confidence can be placed in the Cd, Cu, Ni, Pb and Zn LSMC concentrations being due to the extraction of LSMC. Five of the 12 sites demonstrated percent of Cd as LSMC greater than 0.1% with the highest percent occurring at site 12 (11.2%). All 12 sites demonstrated percentage LSMC of Cu, Ni, Pb and Zn greater than 0.1%. The lowest percent LSMC were measured at site 5 for Cu (0.2%), site 4 for Ni (0.5%), site 9 for Pb (8.2%) and site 3 for Zn (0.4%). The greatest percent LSMC were measured at Site 11 for Cu (6.1%), sites 2 and 9 for Ni (1.7%), site 4 for Pb (20.0%) and site 11 for Zn (4.7%). There were no obvious trends within the measured concentrations of LSMC at the 12 sites (Table 5.14) or the percent of metal present as LSMC

at any of the 12 sites (5.15). There also appears to be no clear relationship between any of the 5 metals measured at each of the 12 sites, with most of the metals having higher and lower percentage of metals present as LSMC at different sites.

It was expected that as the samples were taken along the length of the Cooks River, patterns within the data relating to position along the river might have become apparent. For example sites closer to Botany Bay and in Botany Bay may have lower concentrations of LSMC than upstream sites because of dilution and the reduced impact of anthropogenic contamination. However, such a pattern was not observed, with site 11 having the highest percentage of Cu and Zn present as LSMC. The variability of percent of metals present as LSMC is best seen in Figure 5.7. It is evident from the bar graph that no clear distinct patterns exist within the data.

The high percentages of Cd measured at site 6 and 12 compared to the other 10 sites are a little misleading. The total dissolved concentration of Cd at all 12 sites was relatively low (all <0.079 μg/L) therefore, a very small measured concentration of LSMC (0.003 μg/L at site 6 and 0.004 μg/L at site 12) appears as a large percentage of metal present as LSMC. The limit of detection for Cd is 0.002 μg/L and therefore the concentration of LSMC at these two sites is only slightly higher than the LOD. The percentage of Cu present as LSMC was relatively similar across all 12 sites. There was a slight increase (approximately 4%) from site 6 onwards however this trend did not appear consistent or significant. Ni percentages were similar across all 12 sites and whilst there was a small increase (approximately 3%) in the Zn

percentages from site 6 onwards, this increase was similar to that observed for Cu. This trend does not appear consistent or significant.

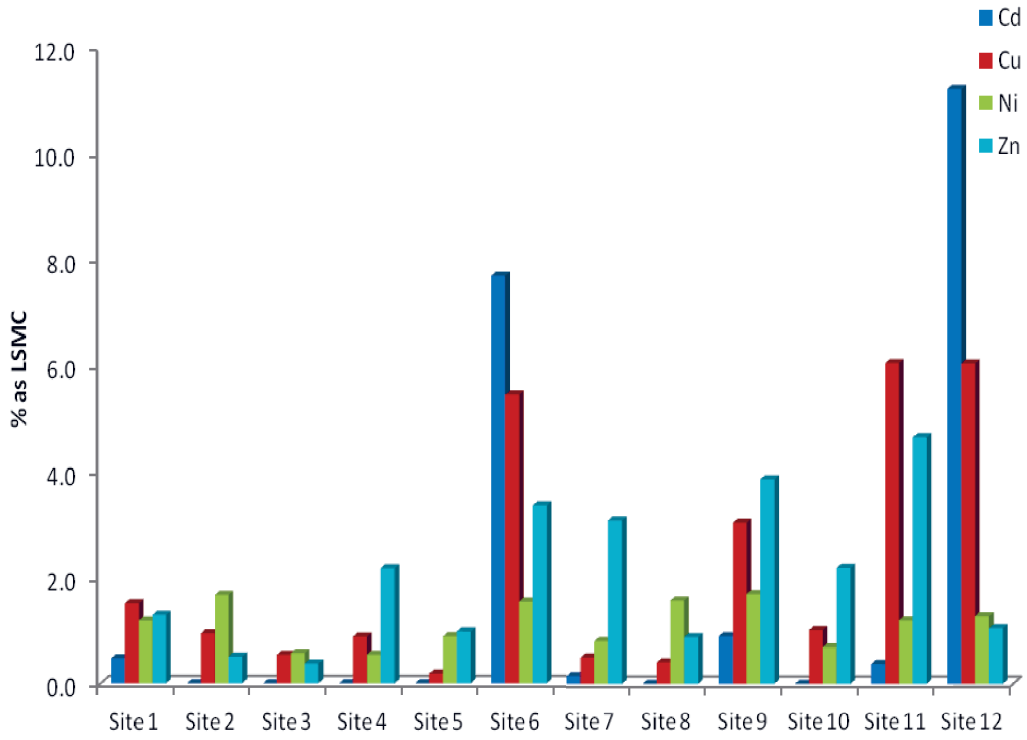

Figure 5.7 Percent of total dissolved metals present as LSMC, Cooks River

The percent of Pb present as LSMC was displayed in its own bar graph (Figure 5.8) due to the fact that the percentages were much higher than those for the other 4 metals. No clear trend was observable within the Pb data. Across the 12 sites the percentage of Pb present as LSMC was relatively similar, and none of the sites displayed substantially different percentages. The lowest percentage of lipid soluble Pb measured was in site 9 with 8% of the total Pb present as LSMC and the highest was in site 4 with 20% of the Pb present as LSMC.

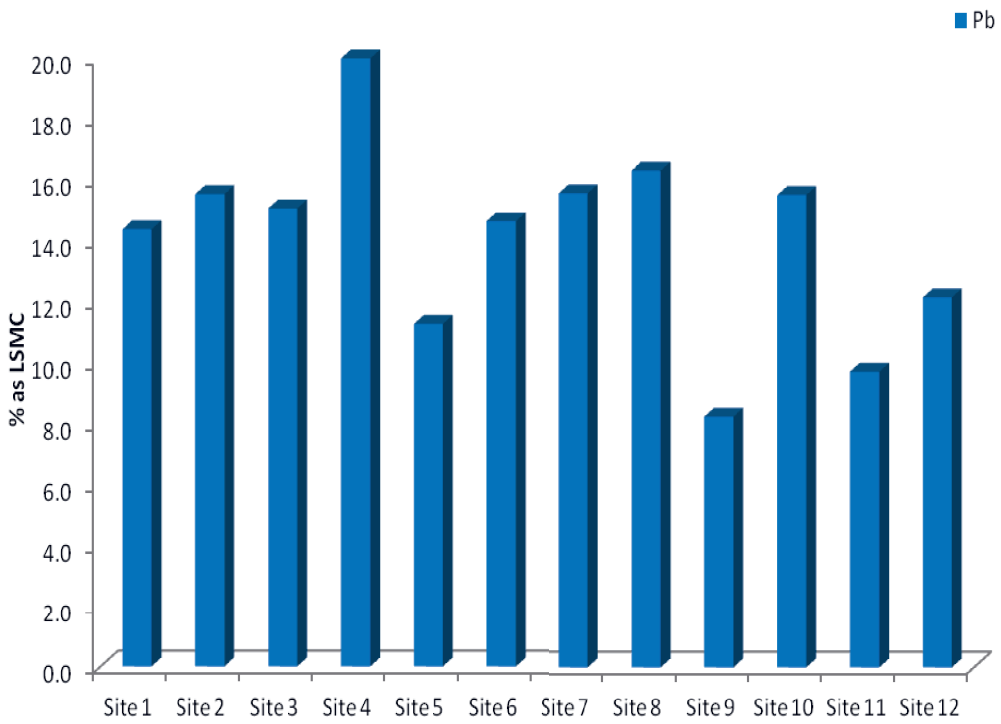

Figure 5.8 Percent of total dissolved Pb present as LSMC, Cooks River

For Cd, Cu, and Zn there was no statistically significant relationship ($p<0.05$) between the concentration of total dissolved metals and LSMC (Table 5.16). This indicates that the concentration of these metals that extracted into octanol was independent of the total dissolved concentration of the metal. A significant relationship ($p<0.05$) was found between the concentration of LSMC Ni and Pb and the total dissolved Ni and Pb concentrations, with r values of 0.645 and 0.979, respectively, and p values of 0.023 and 0.001. From the r and p values it is clear that the Pb relationship is much stronger and more significant than the Ni relationship.

Table 5.16 Correlation results for total dissolved metal and LSMC concentrations, Cooks River

Cd	Cu	Ni	Pb	Zn
r = -0.479 p = 0.115	r = 0.575 p = 0.051	r = 0.645 p = 0.023	r = 0.979 p = 0.001	r = 0.538 p = 0.071

Cu and Zn, Ni and Pb, Ni and Zn and Pb and Zn lipid soluble complexes concentrations were all significantly correlated ($p<0.05$) (Table 5.17). This indicates, that within the Cooks River samples, the amount of these metals that extract into octanol is related.

Analysis of the Al and Fe results was not performed on the Cooks River sample due to the fact that the concentration of both of these metals in the back extracts was not significant. The fact that the percent of Al and Fe present as LSMC in all samples was <0.4% is a significant result in itself. It indicates that for the 12 Cooks River samples analysed, the entrainment of colloids into the octanol extracts was likely to be negligible. This is important as it suggests that the measured concentrations of Cd, Cu, Ni, Pb and Zn in the octanol extracts are free from the potential bias of colloid entrainment. It can therefore be confidently stated that the measured concentrations of these metals were due to extraction of LSMC.

Table 5.17 Correlation results for LSMC concentration data, Cooks River

		LSMC				
		Cd	**Cu**	**Ni**	**Pb**	**Zn**
LSMC	**Cd**	-				
	Cu	r = 0.464 p = 0.129	-			
	Ni	r = -0.401 p = 0.196	r = 0.136 p = 0.674	-		
	Pb	r = -0.100 p = 0.758	r = 0.412 p = 0.184	r = 0.805 p = 0.002	-	
	Zn	r = 0.069 p = 0.831	r = 0.817 p = 0.001	r = 0.807 p =0.002	r = 0.635 p = 0.027	-

Relationships between the measured concentrations of DOC and lipid soluble Cd, Cu, Ni, Pb and Zn were also investigated (Table 5.18). Cd and Cu showed no significant ($p<0.05$) correlation with total dissolved organics, indicating that the lipid soluble concentration of both of these metals is not related to the total concentration of organics as was seen in Centennial Park and Homebush Bay. The concentration of lipid soluble Ni and Pb showed a significant positive relationship with DOC ($p=0.002$ and $p=0.001$, respectively). As the concentration of DOC increased the concentration of both lipid soluble Ni and Pb increased. This relationship demonstrates the possible organic nature of LSMC of Ni and Pb within the Cooks River and Botany Bay. A weaker relationship was identified between DOC and the concentration of lipid soluble Zn ($p=0.048$) indicating that the concentration of Zn LSMC may also be loosely related to the total concentration of dissolved organic matter.

Table 5.18 Correlation results of DOC measurement and LSMC concentrations, Cooks River

	DOC	
Sample	**r value**	**p value**
Cooks River Cd	-0.315	p = 0.319
Cooks River Cu	0.307	p = 0.331
Cooks River Ni	0.795	p = 0.002
Cooks River Pb	0.879	p = 0.001
Cooks River Zn	0.580	p = 0.048

5.4 Discussion

5.4.1 Relationships within the data

Table 5.19 below summarises the relationships within the data from the three fieldwork locations.

Table 5.19 Summary of correlation data from the 3 fieldwork locations

Sampling Location	Correlation	Metals involved	r Value	p Value
Centennial Park	LSMC & LSMC	Al & Fe	0.931	0.022
Homebush Bay	Total & LSMC	Cu	0.925	0.024
Homebush Bay	Total & LSMC	Fe	0.981	0.003
Homebush Bay	LSMC & LSMC	Pb & Zn	0.926	0.024
Cooks River	Total & LSMC	Ni	0.645	0.023
Cooks River	Total & LSMC	Pb	0.979	0.001
Cooks River	LSMC & LSMC	Cu & Zn	0.817	0.001
Cooks River	LSMC & LSMC	Ni & Pb	0.805	0.002
Cooks River	LSMC & LSMC	Ni & Zn	0.807	0.002
Cooks River	LSMC & LSMC	Pb & Zn	0.635	0.027
Cooks River	LSMC & DOC	Ni	0.795	0.002
Cooks River	LSMC & DOC	Pb	0.879	0.001
Cooks River	LSMC & DOC	Zn	0.580	0.048

It was hypothesised prior to the fieldwork that some simple relationships may become apparent within the data, particularly between the LSMC concentrations and the measured DOC values. Due to the body of research indicating that LSMC are organic in nature, with the complex forming when a metal ion is bound between two organic molecules to form a neutral complex, it was hypothesised that a relationship would exist between LSMC and DOC. This relationship was only observed with 3 of the metals and only in the Cooks River samples. The lack of correlation within the data between LSMC and DOC does not necessarily mean that the complexes measure during this study are not organic in nature, it merely illustrates that perhaps the formation of LSMC is not dependent on the total concentration of dissolved organics. The organic molecules needed to form LSMC must have a structure that allows for the binding of a metal ion in the centre of the complex. For this reason, the concentration of total dissolved organics could increase or decrease and if these organics do not have the right structure to form LSMC, the concentration of LSMC may not be affected. Consequently, measurement of the DOC may not be the best predictor of any potential changes in the LSMC concentration.

In addition to considering the structure of the DOC measured, the concentrations measured in the samples must also be considered. In Centennial Park, the concentration of DOC was approximately 3 mg/L for all 5 sites tested and varied very little. At Homebush Bay the concentration was higher, approximately 14 mg/L but again it did not vary considerably between the 5 sites. In the Cooks River samples there was much more variation within the DOC values across the 12 sites. Dissolved organic carbon ranged from 0.90 to 6.90 mg/L. With very little to no variation in the DOC values it is difficult for a correlation to exist with

concentrations of LSMC that are changing from site to site. The small variation in DOC at Centennial Park and Homebush Bay would not allow for a correlation with a measured concentration of LSMC that was increasing and decreasing between the 5 sites.

Within the Cooks River data, a correlation was only identified between Ni, Pb and Zn and DOC and no correlation was found between DOC and Cd and Cu. A correlation was unlikely to exist between the lipid soluble Cd and any other parameter due to the fact that Cd returned a LSMC concentration higher than the LOD of the method at only 2 sites and these two sites were only slightly higher than the LOD. It was interesting that Cu did not return a correlation with DOC especially as Ni, Pb and Zn did. All but one of the sites returned Cu LSMC concentrations higher than the LOD. The lack of correlation could be due to preferential binding of the Ni, Pb and Zn to the organic matter present within the waters analysed. However, Cu is usually considered to be one of the metals which quickly and easily forms LSMC, when the required organic ligands are present.

It was expected that there also might exist strong correlations between the total dissolved metal and LSMC concentrations. It was believed that as the total dissolved concentration of metal in solution increased the concentration of LSMC may also increase. This is however dependent on the water containing organic and inorganic ligands that are available to bind any increased metal concentrations in LSMC. Centennial Park displayed no correlations between dissolved metals and LSMC, A correlation between total dissolved Cu and lipid soluble Cu was identified in the Homebush Bay samples and a similar relationship was identified in the Cooks River samples with total Ni and lipid soluble Ni and total Pb and lipid

soluble Pb. These were the only 3 examples where a correlation was identified between total and LSMC concentrations. This could indicate that in the water samples tested the concentration of ligands present within the water sample, that were capable of forming LSMC, were saturated with metals at some of the lower total metal concentrations and any increase in the total metal concentration could therefore not lead to an increase in the LSMC as there were no available ligands to bind these metals. This conclusion could be tested by taking an aliquot of water from a number of the sites and spiking the sample directly with free metal ions directly prior to extraction. By then comparing the LSMC results from the spiked and non-spiked samples an indication of the waters ability to form further LSMC, i.e. whether the water contains any ligands available to form LSMC, can be made. This technique has been used previously by Mitrovic (1995) and Kilgore (2007) with limited success. The concern with spiking samples with a laboratory prepared ionic metal spike is that by addition of excess metals you are changing the speciation of metals within the sample and therefore during extraction you may be measuring a concentration of LSMC that has no environmental relevance.

The correlations between total dissolved metals and LSMC in the Homebush Bay (Cu) and Cooks River (Ni and Pb) indicate that for these 3 elements, there was a direct relationship between the total concentration and lipid soluble fraction of metals. This could be due to the formation of further organic LSMC as discussed above, but could also be due to the formation of NIMC as discussed in chapter 4. As the concentration of total dissolved metal increases, the likelihood of forming both organic LSMC and NIMC is also increased. In the

case of the samples collected and analysed during this study these NIMC are likely to be similar to those investigated during chapter 4.

Of concern was the relationship identified within the Homebush Bay samples between total and LSMC Fe concentrations. As mention previously, one of the biggest concerns within the method centres on the entrainment of colloidal material within the back extracts and the subsequent leaching of metals from these colloids biasing the LSMC concentrations. For this reason, the concentration of Al and Fe were assessed in all the samples as these two elements are manly associated with colloidal material especially within environmental samples at neutral or near neutral pH's. In addition, these complexes are unlikely to be LSMC and therefore, any Al or Fe present in back extracts is most likely due to colloid entrainment.

A correlation between total and LSMC Fe in the Homebush Bay samples could indicate that colloids are becoming entrained within the solvent and the measured concentrations of LSMC might be biased. This is of concern, especially as the percent of Cu, Pb and Zn present as LSMC was similar to the percent of Al and Fe present as LSMC. As the percentages are similar and a correlation was identified within the total and LSMC Fe data the Cu, Pb and Zn data might be affected by colloid contamination. This result does not automatically mean the Cu, Pb and Zn data should be disregarded, as it has not been proven that a relation exists with colloidal Al and Fe and colloid Cu, Pb and Zn however, the metals data should be viewed in the context of potential bias from colloidal contamination.

A number of relationships were identified between concentrations of different LSMC. Within the Centennial Park samples a relationship was found between Al and Fe LSMC concentrations. This again could indicate that these two metals are associated with colloidal material within the samples and their extraction into octanol is due to entrainment of colloids. In the Homebush Bay samples a relationship was found between Pb and Zn LSMC concentrations indicating that the extraction of these two metals is linked to a common factor. Within the Cooks River samples correlations were identified between Cu and Zn, Ni and Pb, Ni and Zn, and Pb and Zn LSMC concentrations. As Ni, Pb and Zn all demonstrated a correlation to DOC it is likely that the extraction of the metals is linked by this common relationship. The link between the extraction of Cu and Zn is still unclear but could be due to a number of different physico-chemical properties.

5.4.2 Data interpretation

Caution should be used when interpreting the statistical data. Firstly for the Centennial Park and Homebush Bay locations only 5 sites were sampled. With a data set of five, one value that is significantly higher or lower than the rest can skew the data affecting the correlation result disproportionally. A larger data set, like that for the Cooks River samples (12 data points) would be needed to generate more precise and reliable correlation results.

In addition, having a small spread of data limits the generalisations that can be made to areas that are outside the range of the data. This is particularly true for the DOC values which have a very small range and therefore correlations based on this data are not likely to be very robust. Perhaps a way to test whether there is a correlation between DOC and LSMC

would be to artificially enhance the DOC concentration in a natural water sample. By testing the LSMC concentration with varying DOC concentrations, a better understanding of the relationship between DOC and LSMC concentration could be achieved.

In some cases the concentrations of LSMC and even dissolved metals are at or very near the detection limits of the analytical method, particularly for Cd and Ni. These low concentrations result indicate that there may be a loss of precision within the data. The statistical data generated during this research should be view in the context of the low concentrations of LSMC and how these low concentrations reduce the confidence measured data. In general it is acceptable that a result must be at least 10 times greater than the LOD before it is considered reliable. There is a large degree of uncertainty that remains within the data due to the proximity of the measured concentrations with the LOD.

Whilst there were a number of factors to consider when analysing the data, a number of significant correlations were identified. Correlations between total dissolved metals and LSMC concentrations could indicate that as a metal is added to solution, more lipid soluble complexes form and therefore the concentration in the octanol extracts would increase. Relationships between different LSMC concentrations indicate that the metals perhaps do not extract independently and suggests that to a certain point, competition between LSMC to extract into octanol is not significant. Finally the identification of relationships between DOC and LSMC concentrations indicates that as the dissolved organic matter is added to the sample either more LSMC are formed or the membrane becomes more permeable to LSMC.

5.4.3 Variability of LSMC concentrations and percent LSMC results between sites

The percentage of metal present as LSMC for Site 6 and Site 12 at the Cooks River locations are particularly interesting. These two sites show a significantly higher percentage of LSMC then the remaining 10 sites. This was most prominent with respect to the Cd results and to a lesser degree the Cu results. There is no clear reason for the increase in the LSMC concentration in these two sites. The physico-chemistry data does not suggest a reason why sites 6 and 12 would have a higher concentration of LSMC. Site 6 is located towards the end of the Cooks River where it enters Botany Bay; Site 12 is at the southern-most end of Botany Bay. There seems to be a similarity between the two sites that may result in the higher results here and not at the other sites. Site 6 and Site 7 are relatively close spatially yet their concentration of Cd and Cu LSMC are different.

The same relationship can be seen for Sites 11 and 12. All 12 sites from the Cooks River are likely to receive anthropogenic contamination, particularly stormwater along the river and in Botany Bay; however the sites are likely to be affected by these contamination sources to different degrees depending on their proximity to the contamination source. It is likely that localised, point-source contamination of metal and/or ligand may have caused the higher results for Site 6 and Site 12. The total metal concentration at both of the sites was similar to the other 10 sites suggesting that contamination of site 6 and 12 by Cd and Cu is unlikely. It is more likely that the two sites have been contaminated by increased concentration of ligands. It is difficult to confirm this conclusion, however, as the concentration of ligands was not determined during this investigation. The DOC results from sites 6 and 12 do not differ greatly from the other 10 sites which indicates that any changes in the concentration

of ligands present within the 2 water samples is not reflected in the total concentration of organics.

An explanation could be that the total concentration of organics did not vary at these 2 sites but the type of organics present may have varied, with an increase in the concentration of organics capable of forming LSMC present in these two samples. In addition, if the LSMC formed at sites 6 and 12 were significantly more lipophilic (i.e. the LSMC that formed at these two sites had higher octanol/water partition coefficients) than those present at the other 10 sites, they might extract to a greater degree into octanol. The change in the octanol/water partition coefficients of the LSMC could be brought about by the presence of different ligands at these two sites which would lead to the formation of LSMC which have a slightly different structure. Differences in structure that might lead to a higher octanol/water partition coefficient might include the size of the complex, whether the organic molecules that formed the LSMC contain benzene rings (benzene rings due to their coordination might inhibit diffusion through a cell membrane, the polarity of the complex (slight positive and negative charges located around different sections of the complex might inhibit diffusion), the strength of the complex and the stability of the complex. These factors would explain how the total metal concentration and DOC concentration could be similar yet the concentration of LSMC is so different. Again this conclusion is difficult to confirm as the structure and concentration of the ligands at the particular sites was not determined.

At the Centennial Park and Homebush Bay locations, measureable concentrations of Cu, Pb and Zn LSMC were present at all five sites. Al and Fe LSMC concentrations were also detected at all five sites at both locations indicating that some entrainment of colloids could

have occurred during analysis. Centennial Park LSMC percentages ranged from 0 to 14% for Cu, Pb and Zn and 1 to 4% for Al and Fe. At Homebush Bay LSMC percentages ranged from 1 to 6% for Cu, Pb and Zn and 1 to 5 % for Al and Fe. Detectable concentrations of Cd, Cu, Ni, Pb and Zn concentrations were measured in the 12 Cooks River sites indicating that a number of different LSMC are present in the river and in Botany Bay. LSMC percentages ranged from 0 to 11% for Cd, Cu, Ni and Zn and 8 to 20% for Pb. The results from this investigation indicate that a number of LSMC are present in a range of fresh and saline aquatic environments in the Sydney region. The percent of metal present as LSMC ranged from 0 to 20% suggesting that in some cases the LSMC can represent a significant proportion of the metal pool, a proportion that given the data indicating their extreme toxicity could be of concern. These results support the notion that the concentration of LSMC is a significant species for some metals in aquatic environments and should therefore be monitored.

5.4.4 Comparison to previous research

Whilst a small number of studies have attempted to determine the concentration of LSMC in aquatic environments, the concentration of such complexes has only been reported in 3 separate studies. Turner and Mawji (2005) determined the concentration of LSMC from a sample taken from the River Clyde in southwest Scotland. Al, Cd, Cu, Mn, Ni, Pb and Zn lipid soluble complexes were all identified using a solvent extraction approach utilising octanol as the solvent. The River Clyde has received both domestic and trade waste waters from a densely populated and highly industrialised catchment for over a century and is significantly contaminated with both organics and trace metals (Turner and Mawji, 2005). The River

Clyde site is very similar to the Cook River catchment analysed during this study as they are both highly urbanised and receive a number of industrial contaminants and have done over a number of years. The major difference between the two locations is that the Cooks River mainly receives domestic waste waters along its length and a few waste waters from recreational areas like sporting fields and golf courses. The location does not receive trade waste waters until it empties into Botany Bay where it receives contamination from Sydney International Airport, a number of industrial activities that occur along the port at Botany and waste associated with the use of Botany Bay as an important shipping port.

Table 5.20 Concentrations of LSMC in the River Clyde (Turner and Mawji, 2005)

Metal	LSMC concentration (µg/L)
Al	0.16 ± 0.009
Cd	0.027 ± 0.040
Cu	0.15 ± 0.063
Mn	0.44 ± 0.032
Ni	0.21 ± 0.023
Pb	0.56 ± 0.11
Zn	0.24 ± 0.021

Mitrovic (1995) used a shake flask octanol extraction method in which the LSMC were extracted into octanol and then their concentration was determined by back extract of the metals out of the octanol. The concentration of LSMC in 17 different aquatic environments in the Sydney region was performed. Seven of these 17 sites returned detectable concentrations of Cu, Pb, Cd or Ni LSMC (Table 5.21).

Table 5.21 Concentration of lipid soluble Cd, Cu, Ni and Pb in waters collected from the Sydney region (Mitrovic, 1995)

Sample	Cd (µg/L)	Cu (µg/L)	Ni (µg/L)	Pb (µg/L)
Parramatta River		0.102		
Lower Cooks River		0.095		
Botany Bay		0.100		
Woronora Creek				0.035
Garie Beach	0.002			
Powell's Creek			0.126	
Maroubra Beach			0.079	

In addition to determining the concentration of LSMC in waters in the Sydney region, Mitrovic (1995) also measured the concentration of LSMC in a range of sediments collected from the Sydney region. LSMC were extracted from sediments using octanol filled dialysis cells, where a dialysis membrane was filled with octanol and clips were placed on both ends. These cells were placed into collected sediment and left until equilibrium had been reached between the sediment and the octanol inside the cell. Of the 10 sites that were sampled, seven were found to contain lipid soluble Cu, Pb, Cd or Ni complexes. This work is particularly interesting as sediments might represent an environment that could contain much higher concentrations of LSMC than the overlying waters. The results obtained during Mitrovic's (1995) sediment study have been summarised (Table 5.22) to give an indication of the likely concentration of LSMC in sediment porewaters around the Sydney region and in particular in locations similar to those analysed during this current work.

Table 5.22 Concentration of lipid soluble Cd, Cu, Ni and Pb in sediment porewaters collected from the Sydney region

Sample	Cd (µg/L)	Cu (µg/L)	Ni (µg/L)	Pb (µg/L)
Parramatta River		2.50		1.20
Woronora River		4.90	3.50	2.70
Homebush Bay	0.022	42.0		0.900
Port Jackson		2.30		1.30
Towra Point		1.10		
Alexandria Canal		2.20		
Homebush Bay		4.20		
Cook's River				1.80

One final study where the concentration of LSMC was determined in waters was performed by Kilgore (2007). This study employed a modified version of Mitrovic's (1995) method which achieved lower detection limits and lower variability between replicates. Waters were collected from a range of aquatic environments in the Sydney region and the concentration of LSMC was determined using octanol extraction. Concentrations of lipid soluble Cd, Cu, Ni, Pb and Zn were analysed in the collected waters however, only Cu and Zn lipid soluble complexes were detected (Table 5.23).

Table 5.23 Concentration of Lipid soluble Cu and Zn in waters collected from the Sydney region (Kilgore, 2007)

Sample	Cu (µg/L)	Zn (µg/L)
Cronulla	0.021	0.011
Tambourine Bay	0.030	0.036
Five Dock Bay	0.027	0.017
Hen and Chicken Bay	0.032	0.022
Golden Jubilee Oval	0.050	0.110

The sites analysed by Kilgore (2007) represent sites that are the most comparable to those analysed during the current study. The Cronulla site is a marine site that is considered to be relatively pristine and does not receive a significant amount of industrial or domestic waste waters. The area does, however, receive urban storm water runoff and therefore many contain both metal and organic contamination from this source. Cronulla would be most similar to the sites in the south of Botany Bay that are furthest away from the airport and the port. The Tambourine, Five Dock and Hen and Chicken Bay sites are all estuarine environments that receive a significant amount of urban runoff and domestic waste water. These sites are also extensively used for recreational activities and are likely to contain contamination from both aquatic and terrestrial recreational activities. These sites would be relatively similar to the lower reaches of the Cooks River which also receives contamination from similar sources. The Golden Jubilee Oval site represents a freshwater aquatic environment within a highly urbanised catchment that has been historically contaminated as the site had been used as a local rubbish tip before being converted to a recreational area. The aquatic environment is therefore likely to contain historical contamination from the previous tip operations in addition to runoff from the playing fields which may contain organic contamination from fertilisation but also residual contamination from the rubbish tip. This is a similar situation to the Centennial Park and Homebush Bay sites analysed during the current study. Both of these sites have previously been used as rubbish dumps before being converted to recreational areas. Homebush Bay in particular was used as a tip as recently as the 1970's. These two areas are now important recreational areas that are situated within highly urbanised catchments and are likely to suffer from contamination due

to historic uses of the land in addition to receiving urban storm water and domestic waste waters.

In addition to the similar locations analysed by Kilgore (2007), the method used was also similar to the method used during the current study. Consequently, greater confidence can be placed in the data and greater confidence can be placed in any comparisons made between the data sets as differences in methodology are reduced.

The concentrations of LSMC measured in all three locations surveyed in this study were typically in the low ng/L range and were within the range of concentrations measured in previous studies (Table 5.24). Cadmium concentrations greater than the limits of detection were only detected at one of the locations, Cooks River, during the present study and returned mean concentrations approximately 10 fold lower than the concentration of lipid soluble Cd recovered during analysis of the River Clyde (Table 5.24). This could be due to lower contamination of the Cooks River than the River Clyde, or due to the method of LSMC determination used by Turner and Mawji (2005). They determined the concentration of LSMC via the difference in the total metal concentration pre and post extraction into octanol. This method is the least sensitive of the published techniques for the determination of trace metals and trace metal species in water.

Table 5.24 Comparison of measured LSMC concentrations in waters

	Cd (µg/L)	Cu (µg/L)	Ni (µg/L)	Pb (µg/L)	Zn (µg/L)
Turner & Mawji (2005), River Clyde	0.03	0.15	0.21	0.56	0.24
Mitrovic (1995), Parramatta River	-	0.10	-	-	-
Mitrovic (1995), Cooks River	-	0.10	-	-	-
Mitrovic (1995), Botany Bay	-	0.10	-	-	-
Kilgore 2007, Jubilee Oval	-	0.05	-	-	0.11
Kilgore 2007, Tambourine Bay	-	0.03	-	-	0.04
Present Study, Centennial Park (mean n=5)	-	0.24	-	0.06	0.21
Present Study, Homebush Bay (mean n=6)	-	0.09	-	0.001	0.25
Present Study, Cooks River (mean n=12)	<0.002	0.03	0.01	0.007	0.1

The concentration of Cu LSMC at the Homebush Bay location and Cooks River locations were similar to concentrations detected in previous studies (Table 5.24). The sites analysed previously that are most similar to Homebush Bay were Jubilee Oval, Parramatta River and the River Clyde. The concentrations of Cu LSMC measured in Homebush Bay fell within the range of concentrations detected at these three sites. The concentration of Cu LSMC measured during this study in the Cooks River (0.03 µg/L) was at the lower end of the concentration range measured during previous studies but was comparable to concentrations detected in Tambourine Bay. Tambourine Bay is considered a moderately impacted location whilst the Cooks River is considered a highly impacted location receiving waste from a number of sources along its entire length.

The concentration of Cu LSMC in the Cooks River measured during this study is also significantly lower than that detected in the Cooks River by Mitrovic (1995). This is of particularly interest as it shows a direct difference in the concentration of LSMC measured in a waterway after a period of 16 years. However it is difficult to make assumptions from this data as these differences could be due to spatial or temporal variations in the concentration of LSMC. It is also important to view this difference in context of an improved method, improved contamination control, improved sample collection and handling techniques and a much deeper understanding of the need to collect and analyse sample as promptly as possible. All of these factors could give rise to the apparent differences identified within the data. It should also be noted that within the past 16 years it is likely that the condition of the river has improved due to greater awareness of the importance of water quality, tighter control on discharges into the river, routine monitoring and the river may have had a chance to recover somewhat from historical contamination results within this time period.

It is also likely that during these 16 years the catchment has also become much more developed, the airport and Botany port may also have increased traffic which is likely to raise the potential for contamination of the Cooks River. The number of houses situated within the Cooks River catchment is likely to have increased significantly during the past 16 years placing extra strain on the river and also added to the potential contamination load from domestic wastewaters. It has also been noted that the sewage systems in a number of neighbourhoods within the catchment area have not been upgrade for 40 to 50 years and have been found to be leaking directly into the river (Cubby, November 2011) therefore as

urbanisation of the catchment has increase it is likely that more waste water has been passing through the leaking sewage systems leading to a higher incidence of contamination of the river. Traffic through the airport and into and out of the port is also likely to have increased as the population of Sydney has increased over the past 16 years. Pollution from aircraft, ships passing into and out of the port and other industrial activities within the port area are likely to have increased contamination of the Cooks River and Botany Bay even with stricter environmental controls. Taking all of these factors into consideration one would expect to see an increase in the contamination of the Cooks River. Whilst an increase in the total contamination of the waterway does not directly relate to an increase in the Cu LSMC concentration, a significant drop in the Cu LSMC concentration was surprising.

It should also be noted that the value quoted in the above table for the concentration of Cu LSMC in the Cooks River is a mean of 12 values measured along the length of the river these values ranged from 0.003 μg/L to 0.106 μg/L. The most contaminated sites are much more comparable to the value measured by Mitrovic (1995). It is likely that during the study performed by Mitrovic (1995) a sample was collected and analysed which represent the higher end of the contamination range found in the Cooks River. As this study collected and analysed samples along the length of the river and into Botany Bay it is likely that the mean calculated based on this data is more comprehensive. It is very difficult to identify the exact cause or causes of the different results of Cu LSMC within the Cooks River however the method used during the present study is the most reliable, robust and well tested method available for the detection of LSMC in waters.

The concentration of Cu LSMC measured in the Centennial Park location is approximately twice the highest concentration measured during any of the previous studies, even the highly contaminated River Clyde. It was thought that the Jubilee Oval location would be most similar to Centennial Park in terms of historic contamination, current usage, and current sources of contamination. Jubilee Oval had one of the lowest Cu LSMC concentrations (0.05 µg/L) whilst Centennial Park had the highest (0.24 µg/L). Centennial Park was most similar to the River Clyde yet it still had a Cu LSMC concentration 0.09 µg/L lower. Centennial Park is completely surrounded by highly urbanised suburbs and is located within the centre of Sydney. A number of large, busy roads run around the perimeter of the park. As such Centennial Park receives a large amount of storm water runoff and is also likely to receive large amount of domestic waste waters from the surrounding urban environment. The park was once a rubbish dump for the city of Sydney and therefore may contain historical contamination from previous used of the land. All of these factors might explain the much higher concentration of Cu LSMC measured at this location.

Lipid soluble complexes of Ni were only detected in the Cooks River samples during this study and have only been reported in one previous study, that of the River Clyde. No Ni LSMC was detected in the Centennial Park samples or the Homebush Bay samples and was not detected in any of the other environments analysed during previous studies. This suggests that Ni does not readily form lipid soluble complexes in waters or that Ni LSMC is difficult to detect in natural waters. The mean concentration measured during this current study was much lower, 0.20 µg/L, than the concentration detected previously by Turner &

Mawji (2005) in the River Clyde. This is a similar trend as was observed for the Cd results and suggests that the River Clyde may be a more highly contaminated environment than the Cooks River. It could also indicate that the method used during this current study is much more sensitive.

Concentrations of lipid soluble lead were determined at all 3 locations analysed during this current study, analysis of previously reported data reveals that Pb LSMC have only been reported in one location, the River Clyde. As with Cd and Ni, the concentration of Pb LSMC is significantly lower (approximately 0.5 µg/L) in the locations analysed during the current study compared to the concentration measured in the River Clyde by Turner & Mawji (2005). The site that had the largest mean concentration of Pb LSMC was Centennial Park, 0.06 µg/L, whereas the River Clyde contained 0.56 µg/L of Pb LSMC. This could be due to the range of issues mentioned previously including increased contamination in the River Clyde and methodological differences. Again the variability within the data collected during this study was low and therefore is not likely to have had a affected on any comparisons made between the data and previously reported results.

Zinc LSMC have been detected in a number of previous studies and were detected in all 3 locations analysed during this research. The concentration of Zn LSMC measured at the Centennial Park and Homebush Bay locations were most similar to those measured in the River Clyde. Based on the Cd, Ni and Pb results, which have all suggested that the River Clyde is a highly contaminated waterway this is a significant result. Based on the limited amount of previous research performed, the locations of Centennial Park and Homebush

Bay are some of the most contaminated locations in terms of Zn LSMC. It is important when making these determinations that they are made in the context of the methodological differences between the two studies and there likely impact on the concentrations measured by Turner & Mawji (2005). Taking these methodological differences into consideration, Centennial Park and Homebush still returned much higher lipid soluble Zn concentrations then any of the other locations analysed during this research or previously. The concentration of Zn LSMC detected in the Cooks River was similar to that detected by Kilgore (2007) in the Jubilee Oval samples and is approximately 3 times higher than the concentration measured in Tamborine Bay. This indicates that overall the concentrations of Zn LSMC measured during the current study are amongst some of the highest ever measured. This is a significant result given the fact that the previous studies have been much less sensitive and particularly for the River Clyde Cu, Ni and Pb results have consistently return results much higher than those measured during this study.

5.4.5 Difficulties in comparing data from the current study to historical data

Whilst some comparisons have been made between the data collected during the current study and that generated from previous research it is difficult to compare the data for a number of reasons. Firstly it was hypothesised during this study that colloids may become entrained within the solvent during octanol extraction of natural waters. It has been hypothesised that these colloids may leach metals into the back extracts biasing the results. A number of techniques were trialled to reduce and eliminate the issue of colloids contamination; however, none of these were successful.

It was determined that for this study the measurement of Al and Fe within back extracts to indicate the possible extent of colloids entrainment was the most suitable technique. Because it has not been established that there is a direct relationship between the concentration of Al and Fe, the extent of entrainment and the effect on the concentration of the other metals analysed, it is difficult to predict the effect of colloidal contamination. All that can be stated is the degree of extraction of Al and Fe and therefore the likely extent of colloidal contamination of the back extracts. For this reason there is still a level of uncertainty within the data that is not accounted for within the method. This is the first study however, that has identified the issues of colloid entrainment and attempted to assess, eliminate or reduce its impacts on the LSMC concentrations measured. None of the previous studies have identified colloids as being a potential contaminant of the octanol. This could be one explanation as to why the River Clyde samples had some of the highest LSMC concentration recorded.

Comparisons between the current and previous data sets may also have been made difficult due to the time that passed between the different studies. For instance, Mitrovic (1995) measured the concentration of LSMC in the Cooks River in 1995, this study analysed the same environment 16 years later. A large amount of changes will have occurred within the waterway and the surrounding area within this period which may have an effect on the concentration of LSMC. The data generated from the MItrovic (1995) study and other previous studies does provide a ball park figure for the concentration of LSMC in highly urbanised, contaminated aquatic environments.

A number of slightly different methods were used in the studies compared in Table 5.24 which also affects the strength of the comparisons between the data. These different methods all have different sensitivities and contain their own errors. The difference method employed by Turner & Mawji (2005) is the least sensitive method and introduces the possibility of large errors in the data due to the fact the ultra-trace concentration of LSMC is measured by the difference between two relatively large concentrations of total metals. In addition to this, the method employed by Turner & Mawji does not contain a preconcentration step which also affects the sensitivity of the method. The methods used by Mitrovic (1995), Kilgore (2007) and in the current study all determined the concentration of LSMC via extraction into octanol and back extraction into an acidic medium but had different back extraction procedures, contamination control and thus sensitivities. Different sensitivities and levels of contamination can result in data that is less accurate or, more importantly, less representative of the actual concentration of LSMC in the water body under investigation. Different levels of confidence in the data does make comparisons between the data more difficult, however, the data provided by Turner & Mawji (2005), Mitrovic (1995) and Kilgore (2007) has provided the first reliable evidence of the concentration of LSMC in a range of marine and freshwater locations with a range of contamination levels.

The studies listed in Table 5.24 are the only studies which have determined the concentration of LSMC in collected waters. A number of other studies exist where waters prepared in the laboratory have been analysed or natural waters have had concentrations of

LSMC artificially spiked into them. The limited amount of environmental data means that the understanding of the likely concentrations of LSMC in natural waters is quite low that has been performed has been carried out in the Sydney region and in other areas that are exactly the same as those analysed during this study or that are very similar.

5.4.6 Future work

Further work should focus on identifying the concentration of LSMC in a broader range of aquatic environments including those that receive mine wastewaters, treated sewage and high amount of runoff from agricultural practises. These waters would be particularly susceptible to having increased concentrations of LSMC due to the industrial uses of the organic ligands capable of forming LSMC. In addition, pristine environments should also be investigated to determine if naturally occurring organic matter has the ability to form LSMC and at what concentrations these are likely to be found. It may also be important to sample from environments that are likely to contain NIMC in an effort to identify if these complexes are present in the environment and to what degree they extract in natural waters. This further fieldwork would create a much large pool of data on the occurrence and range of LSMC concentrations present in different aquatic environments. This would greatly increase understanding on the types of environments where the concentration of LSMC is likely to be elevated and may help in identifying areas where the concentration of LSMC needs to be determined.

Future work should also focus on further eliminating colloids from the octanol extracts in an attempt to produce data that is unaffected by colloidal contamination. This work could

focus on the use of octanol filled dialysis cells to provide a physical barrier between the water samples and the octanol which would allow the diffusion of LSMC but prevent colloids entrainment. If this technique is unsuccessful centrifugation of the samples prior to the octanol being removed to the round bottom flasks could be investigated. Centrifugation would ensure greater separation between the water and octanol layers after extraction and could reduce or eliminate colloidal contamination; however, its effectiveness at reducing colloidal contamination has never been assessed.

Further field studies at the Cooks River location should be performed to further investigate some of the higher results, particularly those at site 6 and 12. Analysis should be conducted at the same locations over a period of a few consecutive days to identify any daily variations within the data, on the same day over a period of a few weeks and months so that weekly and monthly variation could also be identified and analysed. This data could then be analysed with rainfall, temperature and season data to identify any environmental factors that might be leading to changes in the LSMC concentration. The same physico-chemical measurements taking during this study should also be taken so that any changes within the LSMC concentration can be compared to changes in the waters physico-chemical composition. This would allow for a more thorough evaluation of the causes of changes in LSMC concentrations and might help identify the reasons behind some of the above average results measured at some of the Cooks River sites. It might also help identify any particular point sources of contamination that might be affecting the concentration of LSMC in the river.

Further analysis of the Centennial Park and Homebush Bay sites could also be undertaken. This analysis should perhaps be performed after periods of rain to identify if the increased amount of stormwater and runoff entering these environments has an effect on the LSMC concentrations. The environments could also be analysed on consecutive days after rain to trace any changes in LSMC concentrations in an attempt to identify the residence time and predict the stability of LSMC within these environments. The environments could also be analysed after an extended dry period to determine if the concentration of LSMC becomes concentrated during dry spells. All of this data should be compared to the physico-chemical parameters measured and also weather and climate variables to ensure a complete analysis of the causes of any changes in the LSMC concentration is possible.

To further increase the confidence of results obtained using the current method some further investigation of the stability of LSMC should be performed. By determining the stability of a number of laboratory prepared LSMC, a better understanding of the importance of the amount of time between collection and analysis could be gained. During the present study, the time between collection and analysis was minimised as much as possible to ensure that any changes in the LSMC concentration through adsorption to container walls but also from unstable complexes breaking apart was kept to a minimum. If a better understanding of the stability and fate of some complexes was known, samples could potentially be stored for longer allowing more remote locations. This work would initially involve testing the stability of complexes in Milli-Q water samples in the laboratory. The concentration of LSMC could be assessed in waters over a period of days and or weeks to determine if LSMC concentrations changed. This change could then be attributed to

either adsorption to the container walls (by leaching metals off the container walls and performing a mass balance) or due to unstable LSMC degrading. This could be determined by comparing the concentration of LSMC to the total metal concentration.

If the LSMC concentration decreased and the total metal concentration increased this change would be due to the degradation of unstable LSMC. These same experiments could then be applied to natural waters which have a much more complex sample composition which is likely to affect both adsorption and degradation of LSMC. Finally a water sample that contained a high concentration of LSMC could be collected and the total metal concentration and the LSMC concentration could be determined in the sample over a period of days and weeks. By analysing the changes in LSMC in natural waters that were not artificially spiked into the water sample, an environmentally relevant determination can be made about how the LSMC behave once sampled. At present, there is a distinct lack of knowledge on the stability of LSMC in laboratory prepared waters and natural waters. Making more accurate determinations in this area would greatly benefit the fieldwork and if found to be stable could open up a much larger range of sites for analysis of LSMC.

Finally, in future work, the concentration of Cu and Zn LSMC should be determined in all appropriate samples. From the result of this study, Cu and Zn LSMC were determined to be the most widespread and particularly for Zn, have some of the highest concentrations measured. Therefore, future fieldwork and stability studies should ensure Cu and Zn are included as metals of interest. In addition, the majority of laboratory studies have focused purely on Cu and therefore it should be ensured that future studies also focus on Zn. As yet

it is unclear whether the concentrations of these two LSMC are likely to be of concern in terms of toxicity however; as these two elements have returned some of the most consistently high concentrations of LSMC work should continue to focus on these two metals.

One final area that future work could investigate might be to indentify and measure the key lipid-soluble ligands in water samples. This was well outside the scope of this current research but could provide valuable information about the ability of the waters to form LSMC, how the concentration of lipid-soluble ligands affects the concentration of LSMC and also the ability of the waters to form higher concentrations of LSMC if further metal contamination occurs. This would be a very difficult process as very little is known about naturally occurring lipid-soluble ligands, for instance their structure and typical concentrations. It would also be difficult as little is known about how many anthropogenic organic contaminants have the ability to form LSMC, the structure of these, typical concentrations in contaminated waters and also their stability in natural contaminated waters. This research could potentially form a whole other thesis but would definitely provide valuable information with respect to the formation and concentration of LSMC in aquatic environments.

5.5 Conclusion

The analysis of waters from three locations within the Sydney region revealed the presence of Cd, Cu, Ni, Pb and Zn LSMC in concentrations that were within the range of concentrations measured during previous studies (Mitrovic, 1995; Kilgore, 2007). This

research has shown that aquatic environments in heavily urbanised catchment may contain measurable concentration of Cd, Cu, Ni, Pb and Zn LSMC. In addition, the study has demonstrated that the developed method is reliable and robust at determining the concentration of LSMC in complex natural matrices and that the method is sensitive enough to determine a range of concentrations of LSMC.

Statistical analysis of the data demonstrated that the results obtained were most likely due to the extraction of LSMC and not due to entrainment of colloids into the viscous octanol solvent used. At present it is not possible to determine whether the extracted metals were part of an organic complex, inorganic complex or as some other metal species. It was therefore assumed that any metals that extracted into octanol had the ability to passively diffuse through the cell membrane. Statistical analyses also indicated that the concentration of LSMC was independent of the total dissolved concentration of metal again indicating that the extraction of metals from the collected waters was due to the presence of organic or inorganic complexes that have an affinity for octanol.

Chapter 6: Determination of LSMC Concentrations in Mine Tailing Waste Water

6.1 Introduction

Xanthates are the most widely used synthetic ligand that is capable of forming LSMC (Rao, 1971; Dopson et al., 2006). Xanthates are salts of the dithiocarbon acid – O – ester with a C – O alkyl or aryl chain and are widely used in the metallurgic industry and to a lesser degree the cellulose, agricultural and rubber industries (Gottofrey et al., 1988). They are heteropolar, sulphur-containing organic compounds that form strong hydrophobic complexes with heavy metals (Block, 1991; Block and Glynn, 1992). Xanthates are used to extract minerals from crushed ore (Xu et al., 1988) and, as such, are a key additive in many mineral processing operations. Over 11,000 metric tonnes of xanthates are used in flotation processes each year (Xu et al., 1988). Approximately half of the xanthates added to flotation circuits are discarded with tailings water whilst the other half are consumed during the flotation process as they are removed with the substance of interest from the ore body (Read and Manser, 1976). Concentrations ranging from 4 to 400 µg/L of xanthates can be found in waters receiving runoff from industries that use xanthates (Block, 1991; Block and Glynn, 1992).

Xanthates have the ability to form Lipid Soluble Metal Complexes (LSMC) with metal ions present either in wastewaters or the natural environment (Gottofrey et al., 1988). These LSMC have the ability to cause increased toxicity (compared to free metal ions) to aquatic organisms due to their ability to passively diffuse through cell membranes (Florence et al., 1992).

6.1.1 Study aims

The aim of this chapter is to assess the concentration of LSMC in waste water from a mine where PAX is used in mineral flotation process. Due to the relatively large quantity of PAX used and therefore its increased concentration in the mine waste water, it is assumed that the measured LSMC will most likely be PAX – metal complexes. The concentration of LSMC measured in this waste water would represent a worst case scenario in terms of LSMC contamination, as this waste water would be diluted if released into the surrounding environment. Analysis of mine waste water may give an indication of the typical concentration of some LSMC in these types of waters.

A further aim is to attempt to determine if the concentration of LSMC in mine waste water is sufficiently high to cause an environmental effect in the aquatic environment surrounding the mining operation. By using toxicity data quoted in the literature and the concentration of LSMC measured in the mine waste water, some determinations about the possible toxicity of the collected water may be made.

6.1.2 Toxicity of Potassium Amyl Xanthate

Potassium Amyl Xanthate (PAX) is a sulphur containing 5 carbon chain, in which one end of the molecule contains two exposed sulphur atoms which are essential to binding of metals (Figure 6.1). It has a molecular weight of 409.56 g/mol and is a pale yellow to grey powder or pellet which has a solubility in water of 350 g/L. PAX is one of the mostly widely used xanthates because it is one of the most powerful complexing agents and is relatively non –

selective (Orica, 2010). It is most widely used in the mining industry particularly for the extraction of gold and copper from mineral ores (Gottofrey et al., 1988; Okibe and Johnson, 2002).

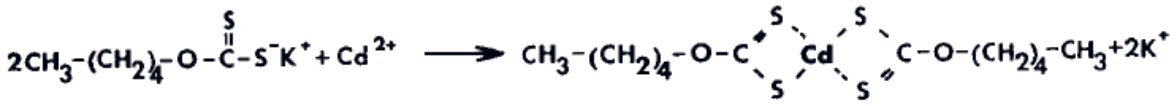

Figure 6.1 Diagram of Potassium Amyl Xanthate (PAX) molecule and an example of a PAX LSMC with Cd

(Source: Gottofrey et al., 1988)

The toxicity of PAX to a range of organisms has been investigated with varying results. Some data has suggested that PAX is relatively non – toxic compared to other xanthates (Dopson et al., 2006) but this contradicts other studies that show PAX to be one of the most toxic xanthates (Webb et al., 1976; Tuovinen, 1978; Block, 1991; Loon and Madgwick, 1995; Okibe and Johnson, 2002). Very few studies, however, have been performed to determine the toxicity of the PAX-metal LSMC.

It has been demonstrated that the uptake of lead in the liver of brown trout (*Salmo trutta*) increased in the presence of PAX (Bertills et al., 1986). This is supported by work that demonstrated increased levels of cadmium in the brain and muscles of brown trout (*Salmo trutta*) exposed to a cadmium and PAX solution compared to control fish (Gottofrey et al., 1988). Similarly, the uptake of cadmium across the gills of both the Eurasian dace (*Phoxinus phoxinus*) and rainbow trout (*Oncorhynchus mykiss*) was greater in solutions containing PAX than in those without (Block and Glynn, 1992). Due to some contradicting results, it is

difficult to definitively state whether or not PAX is toxic to aquatic biota and, indeed, whether the toxicity of PAX is even taxa specific and some variable results have been returned using the same organisms. However, it is clear that the uptake of metals in the presence of PAX is increased compared to exposure to the metal ions alone.

Webb et al (1976) tested the toxicity of 8 xanthates to the Rainbow trout (*Salmo Gairdneri*). In a 96 hour static and 28 day flow-through bioassay Webb et al (1976) found that PAX was one of the most toxic xanthates. The LC_{50} range for the 96 hour static test was 32 – 56 mg L^{-1} for PAX and these were among the lowest LC_{50} values of all the xanthates tested (Table 6.1).

Table 6.1 Summary of acute toxicity of various xanthates to rainbow trout tested for 96 hrs

Xanthate (Source of chemical)	LC_{50} or toxicity range (mg/L)
Sodium sec-butyl (Cyanamid)	100-166
Sodium sec-butyl (Dow Chemical)	~320
Potassium ethyl (Cyanamid)	52
Potassium ethyl (Dow Chemical)	10-100
Sodium isobutyl (Cyanamid)	70-100
Sodium isobutyl (Dow Chemical)	10-100
Sodium ethyl (Cyanamid)	29-37
Sodium ethyl (Dow Chemical)	10-50
Sodium isopropyl (Cyanamid)	217
Sodium isopropyl (Dow Chemical)	100-180
Potassium amyl (Cyanamid)	32-56
Potassium amyl (Dow Chemical)	~40
Potassium isopropyl (Dow Chemical)	32-320
Potassium hexyl (Dow Chemical)	10-100

(Source: Web et al., 1976)

In the long term (28 day) flow – through test where the test subjects were exposed to much lower concentrations (than the 96 hour static tests), the concentration of PAX that caused 100% mortality was 50 times lower than that in the static tests (Table 6.2). For all of the four xanthates tested during the flow-through experiments, the concentration of xanthate required to cause 100% mortality was 50 to 200 times lower than that determined for the static experiments. (Webb et al., 1976). This is most likely due to the half – life of xanthates and that, in flow – through experiments, the organisms were exposed to a water supply with a constant PAX concentration which does not decrease when accumulation or degradation occurs.

Table 6.2 Comparison of concentration of xanthates and duration of exposure required to produce 100% mortality of rainbow trout in static and flow-through bioassays

Chemical tested	Type of bioassay			
	Static		Flow-through	
	Duration (days)	Concentration (mg/L)	Duration (days)	Concentration (mg/L)
Potassium ethyl xanthate	2	100	2	0.5
Sodium isopropyl xanthate	4	180	3	0.3
Sodium ethyl xanthate	4	56	8	1.0
Potassium amyl xanthate	<4	56	28	1.0

(Source: Webb et al., 1976)

Loon and Madgwick (1995) assessed the effect of four xanthates on the leaching of chalcopyrite by *Thiobacillus ferrooxidans*, an acidophilic, autotrophic bacterium that oxidises

ferrous iron and oxidises inorganic sulphur compounds (Loon and Madgwick, 1995). In the concentration range 1 – 10 mM of PAX, a significant drop in the formation of soluble copper and iron was observed indicating growth inhibition. Copper production was suppressed by 77% in the presence of PAX at a concentration of 10 mM. This was the largest suppression of the four xanthates. Maximum growth inhibition of 80 – 90% was observed at 10 mM PAX concentration (Loon and Madgwick, 1995) and, in addition to this, PAX increased the lag phases for growth by approximately 11 times and the maximum cell numbers were much lower (Loon and Madgwick, 1995). PAX was considered the most toxic of all xanthates tested (Loon and Madgwick, 1995). It is important to note that this study was performed at a constant pH of 2 (Loon and Madgwick, 1995) which is not likely to be comparable to aquatic environments surrounding mining operations. This work is still important as during the processing of mineral ore bodies it is likely that waters may have a similar pH however, when the tailings waste water is released into a storage pond or into the environment a significant pH increase is likely to occur. This pH increase will affect the metal speciation and any speciation changes would need to be considered before determining the importance and impact of xanthate-metal complexes.

Touvinen (1978) also investigated the effect of xanthates on the bacterium *Thiobacillus ferrooxidans*. Their results (Table 6.3) indicated that a wide varied of inhibition was displayed by the different xanthates suggesting that their toxicity was dependant on their particular chemical composition and degradation in an acidic medium. PAX caused significant toxicity at both concentrations that were tested. An inhibition of 25% of Fe^{2+} oxidation was measured at a PAX concentration of 0.025% whilst 0.05% PAX caused 83%

inhibition (Tuovinen, 1978). These inhibition results were among the highest of all the eight xanthates tested during this study (Table 6.3).

Table 6.3 Toxicity of xanthates to ferrous-iron oxidation by *Thiobacillus ferrooxidans*

Compound	Concentration (%)	Inhibition of Fe^{2+} oxidation (%)
N-dodecycl mercaptan (DOM)	1.0	57
	0.5	45
	0.1	47
Potassium amyl xanthate (KAX)	0.05	83
	0.025	25
Potassium ethyl xanthate (KEX)	0.05	93
	0.025	86
	0.01	85
Sodium butyl xanthate (NABX)	0.05	1
	0.025	0
Sodium isopropyl xathate (NAIPX)	0.05	62
	0.025	4
	0.01	0
Sodium amyl xanthate (NAX)	0.05	88
	0.025	45
	0.01	1
	0.05	96

(Source: Touvinen, 1978)

A further study carried out by Okibe and Johnson (2002) tested six xanthates against five species of mineral – oxidising, moderately thermophilic and acidophilic bacteria. The study found that the toxicity of the xanthates to the different species of bacteria was quite variable however; PAX demonstrated severe toxicity to 2 of the 5 bacteria species and was slightly less toxic to the remaining 3 bacteria species (Table 6.4). The study hypothesised that the toxicity of PAX could be due to the fact that the flotation chemical binds to the

surface of the sulphide minerals preventing the bacteria from attaching (Okibe and Johnson, 2002). This competitive binding process could account for the reducing in the bacteria's ability to produce energy by oxidisation of the sulphide minerals (Okibe and Johnson, 2002).

Table 6.4 Minimum inhibitory concentrations (in µg/mL) of xanthates to moderately thermophilic, mineral-oxidising acidophilic microorganisms[a]

Xanthate	Microorganism				
	Leptospirillum MT6	*Ferroplasma acidiphilum* MT17	*Acidithiobacillus caldus* KU	*Sulfobacillus acidophilus* NC	*Acidimicrobium Ferrooxidans* ICP
SEX	75 (50)	200 (100)	200 (100)	1000 (500)	200 (100)
SNPX	75 (50)	200 (100)	200 (100)	300 (200)	200 (100)
SIBX	50 (25)	200 (100)	200 (100)	1000 (500)	200 (100)
PNBX	1 (0.5)	25 (10)	500 (100)	200 (100)	200 (100)
PAX	0.5 (0)	25 (10)	200 (100)	200 (100)	500 (200)
X222	1 (0.5)	10 (7.5)	200 (100)	100 (75)	200 (100)

SEX = Sodium ethyl xanthate, SNPX = Sodium n-propyl xanthate, SIBX = Sodium isobutyl xanthate, PNBX = Potassium n-butyl xanthate, PAX = Potassium amyl xanthate, X222 = Mixture of xanthates

[a]Numbers in Parentheses are the greatest concentrations (µg/mL) of xanthate that showed no inhibitory effect on microbial iron oxidation/growth

The capital numbers and letters indicate the stock and source of the microorganisms

(Source: (Okibe and Johnson, 2002))

6.1.3 Toxicity of Potassium Amyl Xanthate – Metal Complexes

Very few studies have been performed to assess the toxicity of metal PAX LSMC. The formation of metal PAX complexes could be significant in terms of the toxicity and bioavailability of metals in aquatic environments given that the industries that discharge PAX are likely to also be discharging water with increased metal concentrations. Bertills et al (1986) investigated the effect of PAX on the uptake of lead in the liver of brown trout (*Salmo truttra*). This investigation revealed that PAX increased the liver lead concentration in brown trout significantly compared to fish exposed to lead alone (Bertills et al., 1986).

This increased uptake was considerable due to the formation of lead – PAX LSMC which increased the uptake of lead across the gills due to passive diffusion of the neutral LSMC (Bertills et al., 1986).

A second study performed to investigate the effect of PAX on the uptake of metal was again carried out on the brown trout. Gottofrey et al (1988) investigated the effect of PAX on the uptake and distribution of cadmium in brown trout compared to trout exposed to cadmium alone. In this study, trout were exposed to water containing 1 µg/L of Cd alone and in the presence of 9 µg/L (44.5 nmol/L, 5:1 molar ratio) and 45 µg/L (222.5 nmol/L, 25:1 molar ratio) of PAX. The results indicate that increased levels of Cd were found in some tissues, most markedly the brain and the muscles, when PAX was present (Gottofrey et al., 1988). The level of Cd in the tissues also increased as the concentration of PAX increased and Cd concentrations of 2 – 3 times higher than the fish exposed to Cd alone were recorded in fish exposed to the highest PAX concentration (Table 6.5).

Table 6.5 Summary of the effects of potassium amyl xanthate on the concentrations of Cd^{2+} in different tissues of the brown trout

Tissues	Tissue concentration of Cd^{2+} (µg/kg wet tissue)[a]		
	Cd^{2+} alone	Cd^{2+} plus PAX 1:5 molar ratio	Cd^{2+} plus PAX 1:25 molar ratio
Gill	822.2 ± 57.1 (13.4)	607.7 ± 45.4 (9.3)	789.3 ± 84.6 (10.3)
Kidney	445.0 ± 64.0 (7.2)	302.7 ± 47.0 (4.6)	375.1 ± 59.7 (4.9)
Liver	377.2 ± 35.9 (6.1)	320.8 ± 57.5 (4.9)	313.3 ± 33.9 (4.1)
Rest/viscera[b]	66.6 ± 8.0 (1.1)	97.0 ± 18.8 (1.5)	96.9 ± 11.2 (11.3)
Brain	4.1 ± 0.9 (0.1)	9.0 ± 1.4[f] (0.2)	12.8 ± 1.8[f] (0.2)
Eye	8.1 ± 0.7 (0.1)	11.9 ± 0.6[f] (0.2)	11.7 ± 1.3[e] (0.2)
Muscle	2.1 ± 0.4 (0.03)	6.6 ± 1.4[e] (0.1)	16.9 ± 5.4[e] (0.2)
Rest/head[c]	38.5 ± 3.9 (0.6)	81.3 ± 7.0[g] (1.2)	99.2 ± 9.0[g] (1.3)
Skin/fin/bone[d]	7.2 ± 1.0 (0.1)	17.9 ± 2.5[f] (0.3)	18.7 ± 3.1[f] (0.2)
Mean conc. in whole fish	61.5 ± 3.7 (1.0)	65.7 ± 6.5 (1.0)	76.9 ± 5.0[e] (1.0)

[a]Mean ± S.E. from 5 fishes
[b]All viscera except the liver and the kidney
[c]All head except the brain and eyes
[d]Skin, fins and bones measured collectively
[e]Significantly different from the fishes exposed to Cd^{2+} alone, $p<0.05$
[f]Significantly different from the fishes exposed to Cd^{2+} alone, $p<0.01$
[g]Significantly different from the fishes exposed to Cd^{2+} alone, $p<0.001$
(Source: Gottofrey et al., 1988)

When the results were expressed as a percentage of the total body burden, values for the brain, the eyes and the rest of the body were higher than in the group exposed to Cd^{2+} only (Table 6.6). Calculations of the bioaccumulation of Cd from the test water shows an average whole–fish concentration factor of 60 -70 for the fish exposed to Cd^{2+} alone and to the low concentration of PAX. For the higher concentration of PAX the whole–fish bioaccumulation factor increased to 80 (Gottofrey et al., 1988). The increased accumulation of Cd was again thought to be due to the formation of lipid soluble Cd – PAX complexes. This hypothesis was confirmed by determination of the chloroform/water partition coefficients of cadmium alone and the cadmium PAX complex. The partition coefficient of Cd alone was 0.002 ±

0.001 with the percent of Cd in the chloroform phase being 0.2%. The partition coefficient of the Cd – PAX complex was 129.9 ± 12.7 and the percent of Cd in the chloroform phase was 99.1%. These results indicated that in the presence of PAX, highly lipid-soluble complexes are formed, which increases the transport of Cd over the gill membranes and across the cell membranes within the trout (Gottofrey et al., 1988). This investigation showed that in the presence of PAX, Cd accumulation is increased due to the formation of LSMC.

Table 6.6 Amounts of Cd^{2+} in different tissues as percentages of the total body burden of the metal after exposure of brown trout to Cd^{2+} alone or Cd^{2+} plus potassium amyl xanthate.

Tissues	Amount of Cd^{2+} in the respective tissues as percentages of the total body burden[a]		
	Cd^{2+} alone	Cd^{2+} plus PAX 1:5 molar ratio	Cd^{2+} plus PAX 1:25 molar ratio
Gill	60.30 ± 2.7	48.02 ± 1.95[e]	49.09 ± 1.89[e]
Kidney	9.46 ± 0.74	5.77 ± 0.83[d]	6.37 ± 0.96[d]
Liver	6.38 ± 0.41	4.20 ± 0.65[d]	3.67 ± 0.38[e]
Rest/viscera[b]	8.49 ± 0.90	8.10 ± 0.83	7.90 ± 0.91
Brain	0.06 ± 0.01	0.18 ± 0.03[e]	0.19 ± 0.04[e]
Eye	0.38 ± 0.03	0.61 ± 0.06[e]	0.45 ± 0.05
Rest/body[c]	14.93 ± 1.64	33.12 ± 1.45[f]	32.33 ± 2.98[f]
Whole fish	100.00	100.00	100.00

[a]Mean ± S.E. from 5 fishes
[b]All viscera except the liver and the kidney
[c]All tissues remaining after removal of the ones specified above
[d]Significantly different from the fishes exposed to Cd^{2+} alone, $p<0.05$
[e]Significantly different from the fishes exposed to Cd^{2+} alone, $p<0.01$
[f]Significantly different from the fishes exposed to Cd^{2+} alone, $p<0.001$
(Source: Gottofrey et al., 1988)

The uptake of cadmium in the presence of PAX was also studied in the Eurasian dace (*Phoxinus phoxinus*) and the rainbow trout (*Oncorhynchus mykiss*) (Block and Glynn, 1992). In this study, both species were exposed to cadmium (0.22×10^{-9} M) alone and cadmium (0.22×10^{-9} M) in the presence of different concentrations of PAX (10^{-7}, 10^{-6}, 10^{-5}, 10^{-4} M). It was determined that uptake at the gills was enhanced from 0.06×10^{-12} to 4.85×10^{-12} mol/g body weight for the Eurasian dace and from 0.25×10^{-12} to 3.11×10^{-12} mol/g body weight for the rainbow trout in the presence of PAX (Block and Glynn, 1992). This increased uptake was attributed to the formation of lipid soluble PAX – cadmium complexes that had the ability to diffuse across the gill membranes (Block and Glynn, 1992). Despite greater uptake across the gills, there was no significant increase in the cadmium concentrations measured in the kidneys. The reason for the lower than expected concentrations of cadmium in the kidneys was because the cadmium became more enriched in the lipid – rich adipose and brown tissue rather than the kidney. Whilst this theory was not supported by measurement of the cadmium in the adipose tissue and the brain, it does support the conclusion of Gottofrey et al (1988) who found that cadmium became enriched in the muscles and brain of brown trout exposed to PAX. Block and Glynn (1992) showed a relationship between cadmium uptake and PAX concentration, clearly indicating that PAX has the ability to form lipid soluble complexes with metals, which increases the bioavailability and hence its uptake of the metal by aquatic organisms.

6.1.4 Stability of Xanthates and Xanthate LSMC

Studies into the stability of xanthates in aquatic environments have indicated that these compounds are not stable and will degrade. However details of this degradation were not

provided (Read and Manser, 1976). The half-life of xanthates can range from 1.16 days to 4.08 days, and decrease with increasing length of the alkyl chain (Xu et al., 1988). As PAX has a 5-carbon alkyl chain length it is likely to have a half-life closer to 1.16 days rather than 4.08 days. In addition to the alkyl chain length, xanthates are easily degraded under even slightly acidic conditions (Tuovinen, 1978). The fact that xanthates are easily degraded and have relatively short half – lives suggests that their potential toxicity within the environment could be low.

Although much is known about the degradation of xanthates, very little is known about the stability and degradation of metal – xanthate complexes. In fact, there are no published studies that have attempted to identify the half-lives of any of the metal – xanthate complexes. This is a significant gap in the scientific knowledge with regards to the stability of xanthate LSMC, and this stability could have a large impact on the effect these LSMC have if released into the aquatic environment.

6.2 Method

6.2.1 Location of sampling site

A gold mine situated in the highlands of Papua New Guinea (PNG) was chosen as the sampling site for this investigation. This research was under a commercial confidentially agreement, so further details of the mine location cannot be given. PAX is used in the processing of a gold ore at this particular mine and the tailings material (or waste water) is stored in a pond on site. Water from this tailings pond was analysed in this study.

6.2.2 Sample collection and transportation

One sample was collected in a 1 L acid washed Nalgene bottle on the 12th of October 2011. The sample was collected directly from the tailings pond using a gloved hand and was immediately placed in a cold, portable cooler. The sample was transported to the CSIRO laboratory by courier.

6.2.3 Sample filtration

On arriving at the laboratory (17th of October 2011), the sample was immediately filtered to 0.45 µm in an acid washed Sartorius filtration unit using positive pressure via nitrogen gas. A filter paper was acid washed prior to use by filtering 100 mL of 10% (v/v) HNO_3 followed by 3 x 100 mL of Milli-Q water. A 100 mL subsample was taken for measurement of physico-chemical parameters before the remainder of the sample was then filtered and placed in a new acid washed Nalgene bottle. Once filtered, the sample was stored refrigerated, overnight, until extraction was performed.

6.2.4 Measurement of physico-chemical parameters

The pH of the water sample was performed using an Orion Thermo pH meter and probe. The temperature of the sample was not measured as it had been stored cold after collection. Conductivity was measured using an YSI 30 meter. The concentration of dissolved oxygen was determined in the collected water directly using a WTW Oxi330 DO meter with a WTW Cell Ox325 probe; however, the dissolved oxygen is likely to be different to that of the tailings pond due to the transportation of the sample from PNG to Sydney.

6.2.5 LSMC determination

The filtered water sample was brought up to room temperature before it was extracted following the method outlined in section 2.4.3. The time between collection and extraction was 6 days. This extended period between collection and analysis should be taken into consideration when interpreting the results. Octanol extracts were combined in an acid washed round bottom flasks and stored in the laminar flow cabinet before back extraction by vacuum distillation following the method outlined in section 3.3. Back extracts were analysed directly by ICP MS with matrix matched standards and spike additions to correct for any matrix interferences. Three replicate extractions were performed on the tailings pond water sample.

6.2.6 Total dissolved metal concentration determination

Total dissolved metals analysis was also performed on the water. Three 10-mL subsamples of the filtered water was transferred into acid washed 20-mL vials and immediately acidified to 0.2% (v/v) HNO_3. The acidified samples were analysed directly by ICP MS with matrix matched standards and spike additions to identify and correct for any matrix interferences.

6.2.7 Determination of aluminium and iron

As outlined in section 5.2.9, entrainment of colloids is a concern when LSMC extractions are performed on natural waters. Al and Fe present in natural waters with a pH close to neutral will predominately be found as colloids or attached to colloids. The concentration of Al and Fe was, therefore, measured in both the back extracts and the filtered water sample.

6.3 Results

6.3.1 Physico chemical data

The pH, salinity and dissolved oxygen values were measured in the laboratory on the collected tailings material (Table 6.7). The results are typical of mine tailings water. The pH was slightly lower than neutral and the salinity was moderate. The temperature and dissolved oxygen results would not be representative of these values at the time of collection as these measurements were made 6 days after the collection of the sample. Transport back to the laboratory would have significantly affected these results.

Table 6.7 Physical chemistry data from collected mine waste water sample

	pH	Temperature (°C)	Salinity	DO (% Saturation)
Waste water replicate 1	6.2	20.1	6.5	85
Waste water replicate 2	6.0	20.3	6.3	89
Waste water replicate 3	6.1	20.3	6.8	92

Nineteen elements were analysed in the total dissolved metals samples and LSMC back extracts. Only metals for which the measured concentration was greater than the limits of detection are presented (Table 6.8 and 6.9).

Table 6.8 Mean method blank results and limits of detection, mine waste water analysis

	Cd (µg/L)	Cu (µg/L)	Ni (µg/L)	Pb (µg/L)	Zn (µg/L)	Al (µg/L)	Fe (µg/L)
Mean (n=3)	<0.001	0.004	0.003	0.002	0.009	0.580	0.114
LOD	<0.001	0.002	0.002	0.001	0.005	0.013	0.014

6.3.2 Method performance data

The measured method blanks concentrations and the calculated limits of detection were sufficiently low to allow for low μg/L concentrations of LSMC to be detected in the samples. The method blank data and limits of detection are only presented for the elements that showed extraction into octanol which was greater than the limits of detection (Table 6.8).

6.3.3 Total dissolved metal and LSMC concentrations in mine waste water

Cu, Ni, Pb, Zn, Al and Fe LSMC above the limits of detection were measured in the mine waste water (Table 6.9). Concentrations of Li, Be, Cr, Mn, Co, As, Se, Sr, Mo, Ag, Cd, Sb and Tl were all below detection limits (Table 6.10). The mine waste water contained particularly high concentrations of total dissolved Cu and Ni. Cu concentrations were in the mg/L range whilst Ni concentrations were in the high μg/L range.

The total dissolved concentrations of Pb and Zn were within the range of concentrations measured during previous fieldwork and therefore do not appear to be elevated however analysis of water used in the ore processing prior to its used would need to be performed to adequately determine whether the concentrations of Pb and Zn are elevated. The total dissolved concentration of Al and Fe were also within the range of concentrations measured during previous fieldwork, this was surprising however as it was hypothesised that these metals may have been present in much higher concentrations as the sample was a mine waste water from the crushing and processing of an ore body. It was thought that this might result in elevated concentrations of Al and Fe.

Elevated concentrations of lipid soluble Cu and Ni were detected within the samples, much higher than any concentrations detected in previous analysis of natural waters (Chapter 5, Table 5.24). The concentration of lipid soluble Pb was low however was within the range of concentrations measured previously, whilst the concentration of lipid soluble Zn was also low and was towards the lower end of concentrations measured during previous studies. The high concentrations of Cu and Ni were to be expected from mine waste water.

Table 6.9 Total dissolved metal and LSMC concentrations in mine waste water

	Total Cu (µg/L)	LSMC Cu (µg/L)	Total Ni (µg/L)	LSMC Ni (µg/L)	Total Pb (µg/L)	LSMC Pb (µg/L)	Total Zn (µg/L)	LSMC Zn (µg/L)	Total Al (µg/L)	LSMC Al (µg/L)	Total Fe (µg/L)	LSMC Fe (µg/L)
Waste water replicate 1	62500	73.6	409	0.264	0.009	0.006	2.93	0.050	84.9	0.176	11.9	0.028
Waste water replicate 2	60700	84.1	393	0.263	0.007	0.006	2.75	0.045	71.5	0.154	13.6	0.025
Waste water replicate 3	63400	70.5	433	0.256	0.008	0.006	2.55	0.044	66.6	0.160	10.2	0.023
Mean	62200	76.1	412	0.261	0.008	0.006	2.74	0.046	74.3	0.163	11.9	0.025
LOD	0.001	0.002	0.003	0.002	0.004	0.001	0.001	0.005	0.016	0.013	0.014	0.014

Table 6.10 Limits of detection of the 13 elements that did not return significant (concentrations greater than the limits of detection) concentrations in the mine waste water

	Li (µg/L)	Be (µg/L)	Cr (µg/L)	Mn (µg/L)	Co (µg/L)	As (µg/L)	Se (µg/L)	Sr (µg/L)	Mo (µg/L)	Ag (µg/L)	Cd (µg/L)	Sb (µg/L)	Tl (µg/L)
LOD	0.003	0.001	0.001	0.002	0.001	0.002	0.001	0.001	0.001	0.002	0.001	0.001	0.001

Lead LSMC percentages ranged from 75 to 87% and the Zn LSMC percentages were 2% for all three replicates (Table 6.11). Whilst detectable concentrations of lipid soluble Cu, Ni, Al and Fe were measured, due to the high dissolved metal concentrations of these elements the LSMC percentages were all less than 1%. It is important to view the Pb percentages in the context of the much lower dissolved metal concentration of Pb compared to other metals and the fact these concentrations (total Pb 0.007 – 0.009 µg/L, LSMC Pb 0.006 µg/L) are close to the limits of detection (total Pb LOD 0.004 µg/L, LSMC Pb LOD 0.001 µg/L).

The extraction of both Al and Fe into octanol was 0.2% (Table 6.11) of the total dissolved concentration however the actual concentrations of Al and Fe are perhaps a more relevant indication of the entrainment of colloids within the solvent extracts. The mean extraction of Al was 0.163 µg/L and the mean extraction of Fe was 0.025 µg/L (Table 6.9).

Table 6.11 Percent of total dissolved metals present as LSMC, mine waste water

	Cu	Ni	Pb	Zn	Al	Fe
Waste water 1	0.1	0.1	75	1.7	0.2	0.2
Waste water 2	0.1	0.1	87	1.6	0.2	0.2
Waste water 3	0.1	0.1	82	1.7	0.2	0.2

6.4 Discussion

6.4.1 Extraction of aluminium and iron

The concentrations of Al and Fe within the back extracts were within the range measured for the extraction of the other 4 metals of interest (between 0.006 µg/L and 76.1 µg/L). The concentrations of Al and Fe present in the back extracts indicate that the extraction of Zn

and Pb may be due to colloid contamination and not the extraction of LSMC as the mean concentration of Zn and Pb in the back extracts was less than that of Al (0.046 µg/L for Zn and 0.006 µg/L for Pb). However, using the less appropriate percent extraction of Al and Fe, the colloid contamination of these two elements did not appear to be of as much concern as it was much less than that of the other metals of interest. The LSMC results for Cu, Ni, Pb and Zn should not, therefore, be attributed to colloidal contamination only. Analysis of the LSMC measured in the back extracts should be analysed in the context of not only the percent extraction of Al and Fe but also the measured concentrations of these elements. The concentrations of lipid soluble Cu, Ni, Pb and Zn measured could therefore be due to a combination of colloidal entrainment and extraction of neutral complexes both organic and inorganic however, given the expected high concentration of PAX in the tailings waste water, it is more likely the lipid soluble complexes measure are the organic PAX – metal complexes.

6.4.2 Extraction of Cu and Ni

The high concentration of total dissolved Cu and Ni was expected in the samples because these waste waters are known to contain high concentrations of dissolved metals due to the enrichment of these elements within the ore that is processed. It was expected that the concentration of LSMC would be high within the samples due to the use of PAX during the mining process. The Cu and Ni results were therefore, not surprising however, the concentration of Cu LSMC was between 2 and 3 orders of magnitude higher than the concentration of Cu LSMC measured during any previous research (Mitrovic 1995, Turner and Mawji 2005, Kilgore 2007). As no data on the concentration of LSMC in mine waste

water could be found a direct comparison with results from this study could not be performed.

The concentrations of total dissolved Ni and Ni LSMC was high. A similar concentration of Ni LSMC (0.21 µg/L) was detected within the River Clyde during the Turner and Mawji (2005); however, this is the only previous study that has detected such a high concentration of Ni LSMC. During fieldwork conducted during this research (Chapter 5), Ni LSMC were only detected from 1 location in the Cooks River at a concentration of 0.01 µg/L, but a direct comparison to a previously analysed mine waste water is not possible.

The percent extraction of both Cu and Ni was low, being 0.1% for both metals. This was a surprising result and was lower than the percent extraction of both Cu and Ni observed in many of the sites sampled during earlier fieldwork (Chapter 5). The low percent extraction of Cu and Ni may indicate that the concentration of PAX in the mine waste water was not as high as expected and therefore the formation of LSMC was not as extensive. It is important to consider the percent extraction of Cu in light of the work performed during the method development in Chapter 3. Poor spike recoveries of the Cu-PAX complex were reported during this work indicating that perhaps the method may not be capable of accurately determining the concentration of the Cu-PAX complex in natural waters. This issue may explain why poor extraction of Cu was observed from the mine tailings sample.

6.4.3 Extraction of lead

The concentration of total dissolved Pb was in the low µg/L range and as such, the concentration of Pb LSMC was also low. Both the total dissolved and lipid-soluble Pb

concentrations were lower than was expected from mine waste water. The concentration of Pb was expected to be elevated within the ore due to enrichment of Pb and this would translate to higher concentrations of Pb within the mine waste water present as both dissolved and lipid soluble Pb. This was not the case, which suggests that perhaps the ore body did not contain a high concentration of Pb or that Pb was removed from the water during the processing steps. It is possible that the Pb also precipitated out of solution during the processing or may have been associated with colloidal material within the waste water and could have settled out prior to collection. The concentration of total dissolved Pb measured during this study was even lower than the concentrations of total dissolved Pb that were reported in chapter 5 suggesting that perhaps some sort of removal (either during processing or due to precipitation or settling out) of the Pb occurred within the mine waste water. The subsequent lipid soluble concentrations of Pb (0.006 μg/L) measured in the mine waste water were lower than the concentrations measured at the Centennial Park and Homebush Bay locations, however, they were within the range of concentrations detected at in the Cooks River and Botany Bay samples. No direct comparison with other mine waste waters was possible as this was the first study that identified the concentration of LSMC in this type of aquatic environment.

The proportion of lead extracted to octanol was relatively large (75 to 87%); however, this is partly due to the relatively low concentration of total dissolved Pb in the mine waste water. The limit of detection for Pb was 0.001 μg/L and the total dissolved metal concentrations ranged from 0.007 to 0.009 μg/L whilst the LSMC concentrations were all 0.006 μg/L. This

suggests that the Pb percentages are subject to large uncertainties and this data should be interpreted with this in mind.

If we were to assume the LSMC Pb results are in fact accurate, it would indicate that a high proportion of the Pb in solution (approximately 81%) was present as the LSMC, and further, that the Pb may be preferentially bound to the PAX compound. It could suggest that Pb has a higher affinity for the PAX than the other metals, particularly as it had a much lower total dissolved concentration, but this is unlikely as previous research has shown that the affinity of metals such as Hg, Cu and Ni for xanthates is much greater than that of Pb (Block and Glynn, 1992). Alternatively, the PAX-Pb complex could be more stable than the other PAX-metal complexes, and so it may be present in the samples for longer allowing for greater extraction of the PAX-Pb complex. This is again unlikely however as metals that have a higher affinity for PAX are also likely to have a higher stability. The high percent extraction of Pb could also be due to the extraction of neutral Pb complexes within the sample that were not formed with PAX, but rather other ligands. However, this is also unlikely as Pb did not form NIMC when tested (under ideal conditions) in Chapter 4.

One thought is that perhaps there was a particular chemical component of the waste water that assisted or enhanced the formation of Pb NIMC, this is difficult to prove given the analyses that were performed during this research. This is unlikely to be the case as the research performed during chapter 4 used conditions that maximised the formation of Pb NIMC and extraction was still not observed. One final explanation for the high percentage extraction of Pb could be that the method was much more capable at extracting and

measuring Pb LSMC than the other metals of interest. This is again unlikely as all of the method development and method validation experiment, particularly those where PAX LSMC were used indicated that the method measured Cd, Cu, Ni, Pb and Zn complexes to the same degree. This is therefore most likely that the percent extraction of Pb is an artefact and the results present in table 6.11 contain a large amount of uncertainty.

6.4.4 Extraction of Zn

It was expected that as with Cu and Ni, the total dissolved concentration of Zn would be particularly high in the mine waste water. This was not the case, and in fact the total dissolved Zn concentration was no higher than concentrations recorded in urban waterways (Chapter 5). The concentration of Zn LSMC was also lower than expected and was at the lower end of the range of Zn LSMC concentrations determined during previous studies (Mitrovic 1995, Turner and Mawji 2005, Kilgore 2007). The low concentration of lipid soluble Zn (0.046 µg/L) was not surprising, given that in the mine waste water sample the initial concentration of dissolved Zn was low. The percent of Zn present as a LSMC was within the range of percentages measured during chapter 5 which suggests that the results obtained during this research may be acceptable. It was however expected that due to the high concentration of PAX used during the processing of the ore this percentage might be elevated. The fact that it was not elevated suggests that perhaps the concentration of PAX in the waste rock was not as high as expected.

6.4.5 Concentration of PAX

The mine tailings waste water was chosen for analysis due to the use of PAX as part of the mine's processing operations. By analysing waste water from a mine that used PAX it was hoped that a better understanding of the likely concentrations of LSMC in waters surrounding industries that use xanthates could be gained. There is a gap in the scientific knowledge surrounding the concentration of LSMC in mine waste waters and this is the first study that has attempted to determine the concentration of a range of LSMC in waste waters from a mine which xanthates are used in the processing operations.

By directly analysing the waste water a worst case scenario for LSMC contamination surrounding the mine could be generated. The total dissolved metals data indicated that the waste water contained elevated concentrations of Cu and Ni, however concentrations of Pb and Zn were within the range of concentrations measured previously during fieldwork. Analysis of the LSMC concentrations revealed that Cu, Ni, Pb and Zn all extracted into octanol and measurable concentrations of all 4 metals were detected in the back extracts. Comparison of the LSMC and dissolved metals concentrations revealed that only Pb and Zn extracted into octanol to a significant degree (greater than 1%). The percentage of Cu and Ni that extracted into octanol was 0.2% indicating that only a small proportion of the Cu and Ni in the samples were present as a LSMC. This was a surprising result as it was believed that the waste water would contain high concentrations of PAX and therefore high concentrations of LSMC.

The lower than expected extraction of Cu and Ni suggests that concentration of PAX may not be as high as initially thought. As mentioned previously there could be a number of

explanations as to why the concentration of PAX was not as high as predicted in the waste water. These reasons include that the PAX may have been consumed during the mineral processing steps. The time between discharge of the mine waste water and collection of the samples was not known and therefore PAX may have degraded prior to collection of the waste waters. The time between collection and analysis of the samples was approximately 1 week, given that some previous work has demonstrated that xanthates have a half-life of between 1 and 4 days and the half-life of PAX is likely to be closer to 1 day (Xu et al., 1988) degradation of PAX is likely to have occurred between collection and analysis. In addition most of the PAX added during the mineral processing operations may have bound to colloidal or fine particulate tailings material removing it from the dissolved phase and making it unavailable to for bind dissolved metals and form LSMC.

During this study the measurement of the concentration of the organic ligand, PAX, was not performed. Determination of the concentration of PAX within the samples would have perhaps assisted in confirming some of these hypotheses about why the concentration of LSMC was not as high as expected. The concentration of PAX was not measured in the samples as this analysis is quite difficult and would require the use of sensitive liquid chromatography mass spectrometry techniques which were not available in the laboratory. If the concentration of PAX was to be determined and these results were to be used in conjunction with the LSMC concentrations to draw conclusions about the LSMC concentrations measured the concentration of PAX would need to be measured at the time of discharge, at the time of collection and at the time of extraction. This would allow for an accurate picture of the changes in PAX concentrations over time and any degradation

behaviour to be generated. In addition to this, determining the concentration of PAX alone would not be sufficient to account for all of the changes in the concentrations of LSMC, the concentration of PAX associated with LSMC would also need to be determined at each time point listed above. Equilibrium is likely to be established between the PAX present in the dissolved phase and any LSMC present. LSMC that degrade or become unstable could potentially break apart releasing the metal back into solution where it could form another LSMC with any available free PAX. PAX associated with colloidal or fine particulates could also participate in this equilibrium and could dissociate and be released back into the dissolved phase. This complicated set of interactions would be difficult to adequately describe even if the concentration of PAX was known at the different time point mentioned above.

6.4.6 Stability of PAX and PAX – metal complexes

Since there was 6 days between collection and analysis, the concentrations of LSMC measured in the samples may underestimate the actual concentration present in the tailings pond. The half-life of xanthates ranges from 1 to 4 days (Xu et al., 1988) and decreases within increasing alkyl chain length (Xu et al., 1988). PAX is likely to have a half-life of closer to 1 day than 4 and it is therefore reasonable to assume that most of the PAX present in the samples may have degraded. Unfortunately, the stability of PAX – metal complexes has not been determined, making it difficult to draw conclusions on the fate of LSMC present in this sample. The stability of xanthates and xanthate lipid soluble complexes could explain why extraction of Cu and Ni was not as high as would be expected from such a mine waste water sample. In addition, a significant amount of time could have passed from when the waste

water was discharged to when the water was collected for analysis, this would increase the amount of degradation that occurred. To overcome these stability concerns it would be necessary to perform the extractions within the shortest possible time.

It is possible to use the half-life of xanthates quoted in the literature to approximately calculate the possible concentrations of LSMC at the time of collection. This would only provide a very rough estimate of the LSMC concentrations and a number of assumptions would have to be made. One needs to assume that the sample was collected directly upon discharge of the mine waste water, that all of the PAX present in the water sample is present as a LSMC, that the concentrations of LSMC measured during this study represent all of the PAX that was present in the sample 6 days after collection and that PAX-metal complexes have the same half-life as PAX does. Based on the concentrations measured and assuming a half-life of 1.5 days ((this is a best guess at the PAX half-life based on literature quoted values of xanthate half-lives) (Xu et al., 1988)), the estimated LSMC concentration at the time of collection is provided in Table 6.12. The concentrations of Al and Fe were omitted from these calculations as it cannot be accurately determined if the concentrations measured during this investigation were due to lipid soluble complexes or colloid contamination.

Table 6.12 Back calculated mean concentrations of the LSMC present in the mine waste water at the time of collection based on a PAX-metal complex half-life of 1.5 days

	Cu (µg/L)	Ni (µg/L)	Pb (µg/L)	Zn (µg/L)
Mean	1220	4.18	0.096	0.736

Using these concentrations and the total dissolved metal concentrations measured during this study an estimate of the proportion of each metal present as a LSMC can be made (Table 6.13)

Table 6.13 Mean total dissolved metal concentrations, back calculated mean LSMC concentrations and percent of metal present as a LSMC based on a PAX-metal half-life of 1.5 days

Total Cu (µg/L)	LSMC Cu (µg/L)	% as LSMC	Total Ni (µg/L)	LSMC Ni (µg/L)	% as LSMC	Total Pb (µg/L)	LSMC Pb (µg/L)	% as LSMC	Total Zn (µg/L)	LSMC Zn (µg/L)	% as LSMC
62200	1220	2.0	412	4.18	1.0	0.008	0.096	1200	2.74	0.736	27

It is important to note that the concentrations of total dissolved metals used in table 6.13 to calculate the proportion of metal present as LSMC were those measured in the laboratory during this study. It is likely that there may have been some changes in the concentration of dissolved metal during the 6 days between collection and analysis particularly as the samples were not kept cold during the whole time of transport back to the laboratory. Some metals may have been lost from solution to the container walls or may have become associated with colloids or particulate material that was removed during the filtration process. These values therefore carry a level of uncertainty that cannot be determined or accounted for but given that the back calculated concentrations of LSMC were only rough estimates some general comments can be made on this new data set.

The estimated proportion of Cu present as a LSMC in the original sample was 2%. This is still a relatively small proportion of Cu given the high dissolved concentration measured in the waste water. This again indicates that even taking degradation into account, the amount of PAX present in the samples may not be as high as expected. The estimated proportion of Ni present as a LSMC in the original sample was 1%. Given that these 2 metals showed a significantly elevated dissolved concentration in the original sample, it is interesting that they have such relatively low proportions present as LSMC. Higher proportions of Cu and Ni LSMC were found in natural waters analysed during chapter 5. The lower than expected concentrations may be because the concentration of PAX in the samples is small and is limiting the formation of LSMC, or something within the water sample is preventing the formation of Cu and Ni LSMC. Perhaps the speciation of Cu and Ni in the dissolved phase is resulting in formation of Cu and Ni species that are stronger or more stable than the Cu and Ni LSMC and therefore the formation of Cu and Ni LSMC is not favored. Further investigation into this area is needed before accurate conclusions can be made about the data. This work should focus on the determination of the Cu and Ni speciation within the dissolved phase to better characterise these metals and should attempt to determine the concentration of lipid soluble Cu and Ni much more quickly so that degradation during transport can be eliminated.

It should also be noted that adsorption of LSMC to the container walls may also be affecting the above results. In previous method development experiments (Chapter 3) it was determined that some LSMC can adsorb to container walls and cause a reduction in the measured LSMC concentration. For this reason, and for stability concerns with regards to

the LSMC, the time between collection and analysis was keep at a minimum during the Chapter 5 fieldwork. During the 6 days between collection and analysis a significant amount of adsorption may have occurred and whilst this would affect both the total dissolved and LSMC concentrations, it would have a different effect on these two concentrations. The LSMC have shown a high affinity for the nalgene bottle walls (Chapter 3) whereas the total dissolved metal species would have a much lower affinity for the container walls. This is largely controlled by the fact that LSMC have a neutral charge and where as many other metal species don't. In addition, the adsorption effect on the LSMC concentration would be much greater due to their concentrations being much smaller. This adsorption that may have occurred during transport could also explain the very small proportion of Cu and Ni that was present as LSMC.

The proportion of Pb present as a LSMC was 1200%, but this is an artifact of the concentration of dissolved and lipid soluble Pb, which, were similar before the degradation correction was performed. This result should be disregarded and, as mentioned earlier, the Pb data contained a large amount of uncertainty due it the fact that all the concentrations of Pb (both lipid soluble and dissolved) were within one order of magnitude of the LOD for Pb. The proportion of Zn increased from 1.7% to 27% after the degradation correction was performed. This was a significant increase and suggests that the proportion of Zn present in the mine waste water at the time of collection was much higher than that measured. This estimate considers only degradation and not adsorption, so the actual concentration in the sample could be even higher.

6.4.7 Presence of cyanide

Cyanide is used in 90% of gold mine operations for the extraction of gold from ore bodies (Akcil, 2003; Hilson and Monhemius, 2006; Gurbuz et al., 2009) and is used at this particular mine. A range of cyanide destruction steps are employed in an effort to reduce the concentration of cyanide in waste waters (Akcil, 2003; Gurbuz et al., 2009), however, even with such steps, it is likely that the waste waters would contain some cyanide and cyanide complexes (Hilson and Monhemius, 2006; Griffiths et al., 2009; Gurbuz et al., 2009; Acheampong et al., 2010). Within the tailings pond, the presence of cyanide could result in competition between the cyanide and PAX to complex metals (Dash et al., 2009; Kamyshny et al., 2012). Complexation of metals by cyanide would result in the formation of charged, both positive and negative, complexes, which would not extract into octanol. This competitive binding could explain the lower than expected extraction of Cu and Ni into octanol. To determine the extent of cyanide competitive binding, it would be necessary to determine the concentration of cyanide in the samples at the time when the LSMC extractions were carried out. In addition to this the speciation of the cyanide would also need to be determined and the presence of any metal-cyanide complexes measured. As this was not performed during the current investigation, it is difficult to confirm whether competition between the cyanide and PAX ligands is causing the low LSMC concentrations of Cu and Ni.

Further work focusing on the concentration of LSMC should focus on identifying and better understanding any competition between PAX and Cyanide and such work could include

some laboratory experiments aimed at measuring the effect of cyanide on the formation and extraction of LSMC. This would help to accurately determine the concentrations at which cyanide does exert an effect on PAX-metal binding and also help to understand what effect it might have on the formation and extraction of LSMC. Future analysis of mine waste waters which are likely to contain cyanide should also aim to identify the concentration of cyanide and cyanide complexes in the samples. These concentrations could then be compared to the result from the laboratory studies to estimate whether the concentration of cyanide present in the samples is likely to cause any affect to the LSMC concentrations measured.

6.4.8 Comparison with previous work

A literature search highlighted the paucity of data available on the concentration of LSMC in mine waste waters or in aquatic environments surrounding mine operations. A relatively large amount of data exists that indicates the concentration of metals and organic ligands in mine waste waters are often above background concentrations (Read and Manser, 1976; Xu et al., 1988; Block, 1991; Block and Glynn, 1992). However, there has been no published study that has directly determined the concentration of LSMC in mine waste water for comparison.

6.5 Conclusion

Extraction of mine waste water revealed measurable concentrations of Cu, Ni, Pb, Zn, Al and Fe. Of these extractable metals, only Pb and Zn had LSMC concentrations that were greater than 1% of the total dissolved metals concentrations. Pb had the greatest percentage

extraction of between 75 to 87% however; there were some reliability concerns within the Pb data due to the measured concentrations being within one order of magnitude of the limits of detection of the method. Zn present as LSMC represented only 2% of the total dissolved Zn concentration. The extraction of metals was lower than expected for a water sample thought to have very high concentrations of PAX. The lower than expected extraction results could be due to a number of reasons which include; a lower than expected concentration of PAX in solution, degradation of PAX or PAX – metal complexes, and competitive binding between PAX and cyanide present in the samples.

Chapter 7: General Discussion, Conclusions and Future Work

7.1 General discussion and conclusions

7.1.1 Method development

A sensitive method for the determination of LSMC in waters was developed (Chapter 3), which improved on previous methods. The detection limits of between 0.001 and 0.011 µg/L were low enough to allow for the detection of LSMC in a range of aquatic environments. The low detection limits were achieved through the use of strict ultra-trace procedures and through the purification of octanol before use. The analytical method was capable of detecting Cd, Cu, Ni, Pb and Zn lipid soluble complexes with APDC, oxine and PAX. Of the five metals tested with three synthetic ligands, only the recoveries of the Cu-PAX complex were low (43%) and considered unacceptable (85% to 105%). The poor spike recovery of the Cu-PAX complex is likely to be linked to adsorption of the complex to the container walls and could possibly be due to some solubility issues with the Cu-PAX complex in the test solution. Even with the poor recoveries of the Cu-PAX complex, the analytical method still performed better than previous methods for the determination of LSMC in waters. The improved method performance is most likely due to the use of vacuum distillation during the back extraction process which removed all of the solvent before back extraction is performed.

7.1.2 Determination of the octanol/water partition coefficients of a range of neutral inorganic metal complexes

The octanol/water partition coefficients of 14 neutral inorganic complexes were determined during this investigation. The results indicated that 12 of the complexes, $CdCl_2$, $CuCO_3$, $Cu(OH)_2$, $NiCl_2$, $NiCO_3$, $Ni(OH)_2$, $PbCl_2$, $PbCO_3$, $Pb(OH)_2$, $PbSO_4$, $ZnCO_3$ and $Pb(OH)_2$, have low

octanol/water partition coefficients (<0.1) and, therefore, are not of environmental significance. Octanol/water partition coefficients of 3.28 and 0.20, for the $HgCl_2$ and $B(OH)_3$ complexes respectively, were determined. These values agreed with octanol/water partition coefficients quoted in the literature and indicated that both of these complexes partition into octanol to some degree. The determination of octanol/water partition coefficients was important as it confirms that the concentrations of LSMC detected in natural waters may include both organic and inorganic neutral complexes for Hg and possibly B.

7.1.3 Determination of LSMC concentrations in natural waters

The analytical method developed in Chapter 3 was used to determine the concentration of LSMC in three different aquatic environments in the Sydney region. Cd, Cu, Ni, Pb and Zn LSMC were detected in samples collected from Centennial Park, Homebush Bay and the Cooks River. Cu, Pb and Zn LSMC were the most widespread LSMC and were detected in all sites sampled. LSMC of Pb showed the most significant concentration with 20% of the total dissolved metal being present as a LSMC in Site 4 of the Cooks River. It is important to note however, that the total dissolved Pb concentrations and LSMC Pb concentrations were all low and were within one order of magnitude of the LOD. This indicates that there is some uncertainty within the data and conclusion drawn from these data would include this uncertainty. Duplicate measurements made at some of the sites during the investigation revealed very little variability. The measured concentrations of LSMC were within the range quoted in the literature, however, this was the first study that identified the presence of Ni and Pb LSMC in waters collected from the Sydney region. The results indicate that waters, both saline and fresh, from aquatic environments with highly urbanised catchments contain measurable concentrations of Cd, Cu, Ni, Pb and Zn LSMC.

Aluminium and iron total dissolved and LSMC measurements were made on the samples collected from the Sydney region. The concentration of Al and Fe in the back extracts and the proportion of Al and Fe extracted was used as an indication of the level of colloid entrainment within the solvent. Al and Fe extraction was measured in the Centennial Park and Homebush Bay locations and percent extraction ranged from 1 to 6%. This extraction indicated that, in these samples, some entrainment of colloids from the waters may have occurred and this may have affected the LSMC results for the other metals of interest due to the transfer of metals from colloids into back extracts. Very little extraction of Al and Fe was observed in the Centennial Park samples which suggests that colloid entrainment within the solvent was not a concern sites sampled at this location. Unfortunately, Al and Fe analysis does not allow the samples to be corrected for colloid entrainment as the association of metals with colloids is not known. The concentration of Al and Fe can only be used to indicate the degree to which colloids may become entrained within the solvent.

7.2 Future Work

There are a number of areas in which future work could be performed. Whilst significant progress has be made during this research there are still a number of areas which require considered investigation. During this future work it may be useful to use an operational definition when referring to metal complexes which extract into octanol. The use of the term “octanol extractable metals” to refer to all species of metals that are extracted by the developed method may be useful. This term would refer to organic LSMC, NIMC and potentially metals from colloidal material. This term would be similar to the term dissolved metals as it is used to describe a range of metal species.

7.2.1 Entrainment of colloids within octanol extracts

The suspected entrainment of colloids within octanol extracts is the largest drawback of the analytical method. Colloids present in natural water samples may become entrained within the solvent extracts and could transfer metals into the back extracts which are not lipid soluble, positively biasing the LSMC results. This issue required further investigation to ensure that the method is measuring the concentration of only LSMC present in water samples. At present, the detection of Al and Fe in octanol extracts can be used to determine the degree of entrainment but no method exists to correct for colloid entrainment. Aluminium and Fe were used to indicate colloid entrainment as it is believed that at the pH of natural waters (between 6 and 8) Al and Fe have a very low aqueous solubility and will be present in the samples as colloidal material or attached to colloidal material. In future studies it may be more appropriate to measure the concentration of silica in back extracts as many colloids will be substantially made of aluminosilicates. Determination of the total dissolved concentration of silica and comparing this to the concentration of silica extracted into octanol may provide a more accurate indication of the entrainment of colloids within octanol extracts and any potential bias this may produce.

It is also possible that some of the results attributed to colloids could also be due to octanol-soluble humic or fulvic acid complexes containing metal ions. It would therefore be important to consider that filtration of the samples may not affect the extraction of Al and Fe from natural water samples. Direct determination of the humics and fulvics present in natural waters and comparison of these values to the concentrations of extracted Al and Fe may indicate whether these organics could potentially be causing error in the determination of LSMC.

Many colloids could be substantially made of aluminosillicates and therefore the measure of silica in future samples will be more informative about the extent of colloidal entrainment.

The technique determined to be most appropriate for preventing the entrainment of colloids in the solvent extracts is through the use of dialysis membrane as a physical barrier between the solvent and the water sample. By placing the octanol inside dialysis tubing and sealing both ends with dialysis clips an octanol filled dialysis can be created. This cell could then be placed into a water sample and left until equilibrium was established between the water sample and the octanol inside the dialysis cell. The dialysis membrane should allow the diffusion of the LSMC into the octanol but prevent colloids from contacting and becoming entrained within the solvent. A technique similar to this, using water filled dialysis cells was used previously for the analysis of different metal complexes in water samples (Apte et al., 1989). In addition techniques like ultra-filtration, filtering the sample through a 0.1 or 0.2 µm filter, or centrifuging the samples during the extraction process to achieve better separation between the octanol and water phases should also be trialled as methods of reducing colloidal entrainment within octanol extracts.

Initial method development experiments were performed investigating the use of octanol filled dialysis cells, however, the results of these experiments were inconclusive and an appropriate method could not be developed. The major issue encountered during these method development experiments revolved around the extraction of the metal complexes into the octanol from the water samples. Extraction of the three complexes tested in Chapter 3, could not be replicated using octanol filled dialysis cells; no extraction of the

APDC, oxine or PAX-metal complexes was detected during any of these method development experiments. The method performance data in Chapter 3 shows that these three complexes do extract into octanol so the presence of the dialysis membrane must be preventing this extraction. A number of techniques were used in an attempt to overcome this extraction issue. These included using dialysis membranes with a range of pore sizes, the use of sample waters with differing ionic strengths and testing a number of equilibrium times. The experiments were all performed on the 3 LSMC used in chapter 3 and were repeated a number of times to ensure data accuracy. Extraction however could not be achieved using octanol filled dialysis cells.

Ultra-filtration was also tested as a means of reducing and/or eliminating colloidal contamination of octanol extracts during this study. Waters collected from centennial park were filtered through 0.45 µm and 0.1 µm HA Millipore filters before being extracted and back extracted using the method developed in chapter 3. Five replicates of each of the filtered waters was analysed to ensure the results were reliable and the results from the samples were compared to determine if the ultra-filtration had any effect on the concentration of Al and Fe that extracted, the extraction of other metals was also compared. The results revealed that by passing the water through the 0.1 µm filter, the concentration of Al and Fe in the back extracts was reduced by 50% whilst there was no statistically significant ($p=0.05$) difference in the concentration of the other metals of interest in the two filtered samples. This reduction in the concentration of Al and Fe could be attributed to the ultra-filtration step as it was the only difference between the two samples. Whilst this reduction is significant, to filter 1 L of water through the 0.45 µm filter

took approximately 20 minutes, using the 0.1 µm filter this time increased to over 4 hrs. This filtration period would be unacceptable for large scale field work surveys where a number of samples from need to be filtered. In addition, a filtration time of approximately 4 hrs exposes the sample to unnecessary risk of contamination. Consequently, it was decided that the potential benefits of reducing colloid contamination did not outweigh the extended filtration time or the risk of contamination and, therefore, ultra-filtration was not investigated further.

Centrifugation of samples after the 5 minute extraction process could be a further technique to reduce the severity of colloidal contamination. Due to the viscous nature of the solvent centrifugation has also been used previously to achieve better separation between the water and the octanol phases (Turner and Mawji 2005) however it has not been used previously to reduce the risk of potential colloid contamination. After the 5 minute extraction period the samples could be centrifuged for a short period of time before the octanol phase is removed to a round bottom flask. There are a number of issues with using the technique. First, the samples should be transferred into a container that would be suitable for centrifugation as the extraction process is performed in 500 mL separating funnels. This would increase the amount of acid washing needed and could increase the risk of contamination. Alternatively, the samples could be extracted in containers that are suitable for centrifugation, a 250 mL nalgene bottle for instance; however this may affect the extraction process itself as complete extraction might not be achieved when a separating funnel is not used. In addition, separating the octanol layer to the round bottom flask would be difficult in a nalgene bottle and this could introduce error within the method

if not all of the octanol is removed to the round bottom flask. Second, centrifugation would need to be performed outside of the cleanroom, samples would need to be removed from the cleanroom environment and transported to a second lab which could introduce further risk of contamination. In addition, contamination of the samples could occur during the centrifugation process itself as the centrifuge contains a number of metal parts. For all of these reasons the centrifugation was not tested as an alternative method for the reduction of colloidal contamination within octanol extracts.

Further work is needed to investigate the use of octanol filled dialysis cells as a method for excluding colloids from the octanol extracts. This work should initially focus on overcoming any extraction issues with the use of dialysis membrane and then should determine the equilibrium time between water and octanol for a range of synthetic laboratory prepared LSMC. Some initial research has been performed into this area by Mitrovic (1995) who measured the concentration of LSMC in sediments and biota using octanol filled dialysis cells. The method employed by Mitrovic (1995), whilst producing some encouraging results, requires further method validation and investigation before it can be applied consistently.

7.2.2 Sample storage and LSMC stability

During research investigating octanol filled dialysis cells as a method for controlling colloid contamination it became apparent that some of the LSMC adsorbed to the container walls over a period of 4 to 5 days. This indicated that analysis of natural water samples that are collected and stored for a number of days may underestimate the concentration of LSMC. This is a consideration that has not been identified or investigated in previous research. A

number of LSMC were tested in addition to a number of container materials to determine if a particular container was more suitable. APDC, PAX and Oxine LSMC were tested in high density and low density nalgene bottles, Teflon bottles and glass bottles and adsorption was observed in all cases. The results varied from adsorption losses of 50% up to complete adsorption of the added LSMC representing significant adsorption. To overcome adsorption concerns, samples collected during the fieldwork component of this project were filtered and extracted as soon as possible, with most samples being extracted within 3 hrs of collection. The issues of adsorption requires further investigation particularly adsorption of LSMC to container walls in natural waters. The test on adsorption performed during this research all used Milli-Q water samples however ionic strength was varied during the tests to investigate its effects on adsorption. In natural waters, which will contain a much more complex matrix, the adsorption results might be significantly different from the preliminary results obtained during this study.

Due to time restrictions this further investigation using natural waters was not performed. Experiments should focus on collecting waters and analyzing the total dissolved and LSMC concentrations in these samples over a number of days. If the LSMC concentration decreases and the total dissolved concentration decreases of this period of time then this indicates that adsorption has occurred. If the total dissolved concentration remains constant but the LSMC concentration decreases, this change would represent degradation of the lipid soluble complexes. Due to the low concentration of LSMC in natural waters, the samples may need to be spiked with laboratory prepared LSMC to ensure changes in the concentration can be measured. In addition, characterization of the waters should be

performed to determine DOC, pH, major cations and anions, metal concentrations and salinity so that potential changes observed in the LSMC concentration can be compared to changes in these other measurements. In addition adsorption should be measured at a number of different temperatures and in light and dark conditions to determine the most appropriate storage method. A more thorough understanding of the adsorption of LSMC to container walls could allow for samples to be stored for longer periods of time which could allow for samples from locations further away from the laboratory to be analysed.

Analysis of the collected fieldwork samples was also performed as soon as possible after collection due to concerns about the stability of LSMC in natural waters. Presently no data exists on the stability of LSMC, however based on limited data; the stability of the organic ligands that can form LSMC indicates they are only stable for 1 to 4 days. By analyzing samples as soon as possible after collection the risk of any changes in the LSMC concentration due to degradation would be reduced. The stability of LSMC requires further investigation and this investigation should initially focus on laboratory spiked water milli-Q water samples. Further experiments should then be performed using natural waters and could be incorporated into the experiments determining the adsorption of LSMC to container walls. Any decrease in the LSMC concentration where the total dissolved metal concentration remains stable would represent the degradation of LSMC within the samples. As with the adsorption of LSMC to the container walls, a more thorough understanding of the stability of LSMC could allow for samples to be stored for longer periods of time and for locations further away from the laboratory to be sampled. In addition, understanding the stability of LSMC is essential for determining the environmental importance of

concentrations of LSMC measured in natural waters and for understanding results from ecotoxicological experiments.

7.2.3 Determination of octanol/water partition coefficients

Octanol/water partition coefficients were determined for the $HgCl_2$ and $B(OH)_3$ neutral complexes and indicated that these complexes partition into octanol and therefore may be of environmental relevance. Further work should be performed to investigate the octanol/water partition coefficients of these two elements in natural waters. Results from natural waters samples would indicate that the complexes are present in natural waters, would indicate if the octanol/water partition coefficients are different between different water types and would indicate if the environmental significance of these complexes changes in different waters. A range of waters should be analysed from both saline and fresh water environments and the octanol/water partition coefficients should be compared to literature values to identify any significant differences. This investigation would allow for a better understanding of the potential toxicity of Hg and B in the environment and potentially shed some light on how these elements enter cells.

Further work could also be performed to investigate the stability of the $HgCl_2$ and $B(OH)_3$ complexes in both laboratory prepared samples and natural waters. The octanol/water partition coefficients of these two complexes could be determined over time and then the stability can be assessed based on changes in these octanol/water partition coefficients. This work should be performed in laboratory prepared samples as the solution composition can be carefully controlled to ensure the maximum concentration of the neutral inorganic complexes and ensure the least amount of competitive binding. Natural waters should also

be used to assess the stability of neutral inorganic complexes as they have a much more complex sample matrix which may have a significant effect on the stability of the complexes. In addition, determining the stability of neutral inorganic complexes in natural waters will produce more environmentally relevant results and may indicate in which samples neutral complexes are more stable and therefore are of more environmental concern.

The bioavailability and toxicity of B and Hg NIMC should be investigated to determine if the concentration of these complexes in natural waters is like to be of environmental concern. This work should initially focus on experiments using laboratory prepared samples so that the concentration of both of the complexes can be carefully controlled and to ensure the concentrations remains relatively constant throughout the test. Work could then be expanded to include natural water samples which could be spiked with B and Hg NIMC. Finally this work could focus on testing the waters which have naturally occurring concentrations of B and Hg NIMC. The results from the laboratory prepared waters, the natural waters spiked with NIMC and the waters which have natural occurring concentrations of NIMC could be compared to determine if the bioavailability and toxicity of B and Hg NIMC to aquatic organisms changes depending on the solution composition. This work would be a significant step in the understanding of the bioavailability and toxicity of B and Hg species and these results may indicate a significant pool of both B and Hg, which previously has not been investigated adequately, as being bioavailable and toxic.

Finally, further work should be performed to investigate whether any other neutral inorganic complexes exist and extract into octanol. Based on the results from this study (chapter 4), further work should focus on the formation of neutral complexes with the less common elements. These elements might include Cr, Ag, Sb and Tl. This could be significant research as these elements are not normally considered in environmental analysis or LSMC analysis. Determination of octanol/water partition coefficients for neutral inorganic complexes with some of these elements could identify a significant pool of bioavailable metals that previously has not been investigated.

7.2.4 Determination of LSMC concentrations in natural waters

Whilst the concentration of a number of LSMC was determined in waters collected from three different aquatic environments, further work should be performed to determine the concentration of LSMC in natural waters. Only a small pool of data exists on the presence of LSMC in aquatic environments and, therefore, future work should address this knowledge gap. Samples from a range of environments impacted by a range of anthropogenic contaminants, both fresh and saline, should be analysed to ensure a comprehensive data set is generated. In particular waste waters from a number of industrial processes, including the mining and agricultural industries could be targeted. In addition household waste waters could also be sampled as some previous work has indicated the presence of LSMC in sewage sludge (Carlson and Morrison 1992, Carlson and Morrison 1995). During this investigation, a range of metals should be measured in addition of the dissolved organic carbon and a range of physico-chemical parameters. Al and Fe should also be measured to determine the extent

of colloid entrainment within the analysed samples so that confidence can be placed in the LSMC results measured.

During analysis of waters collected from around the Sydney region and analysis of mine waste water it became evident that it was important to understand the concentration of organic ligands in the samples. For example, variability in the LSMC concentrations between samples in the Cooks River (Chapter 5) could not be explained by total dissolved metals concentration and was, therefore, attributed to changes in the ligand concentration. In the analysed mine waste water, the concentrations of LSMC were lower than expected, especially for Cu and Ni, which was attributed to the concentration of organic ligand present in the sample (Chapter 5). Unfortunately, it was difficult to prove these assumptions because the concentration of organic ligands was not determined in any of the collected samples. Future work in this area should include developing a technique of measuring, not only, the total concentration of organic ligands present in the samples but also to measure the concentration of particular organic ligands like xanthates, oxine and DDC. A much better understanding of the concentration of organic ligands in natural waters would allow for a more thorough analysis of the collected waters to be performed. Variability within LSMC concentrations could be analysed in more depth if the concentration of organic ligands was determined at the same time.

Previous work performed by Mitrovic (1995) and Kilgore (2007) attempted to assess the concentration of free organic ligands that are capable of forming LSMC in collected samples by analysing the concentration of LSMC in waters before and after spiking the sample with a multi-element metal spike. A collected sample had the concentration of LSMC determined by octanol extraction on a 250 mL aliquot. A second 250 mL aliquot had a multi-element

metal spike added to it and the sample was left to equilibrate before the concentration of LSMC was determined (Mitrovic, 1995; Kilgore, 2007). By performing this process, the ability of a water to form further LSMC can be assess, indicating the concentration of free organic ligand in the sample. Whilst this method does not measure the concentration of organic ligands directly, it can indicate whether a water sample had uncomplexed ligands present. Results obtained from such spiking procedures may be used to better interpret the LSMC results between samples.

As mentioned previously, some variability within the LSMC concentrations was observed in samples collected from the Cooks River. In particular Site 6 and Site 12 had increased concentrations of lipid soluble Cd and to a lesser degree Cu. These increased concentrations could not be explained by the total dissolved metal concentrations of Cd and Cu which did not differ from the other 10 sites. It was hypothesised that these differences were due to the ligands present in the samples collected from these two sites (Chapter 5). Further work could be performed to investigate these differences more thoroughly. Such work should focus on regular sampling of Site 6 and 12 and other sites that had similar increased concentrations. Sampling for LSMC should be performed over a number of months, perhaps with samples collected once a week on the same day at the same time. Samples should have the concentration of LSMC, total dissolved metals and the physico chemical parameters measured. In addition to this, weather conditions at the time of sampling and the days prior to sampling should be noted as rainfall may affect the amount of discharge the sites are receiving. Based on the collected total metal and LSMC results, the physico-chemical results and weather observations, a better understanding of the variability at that particular site

might be achieved. In addition, routine sampling of sites may provide a deeper understanding of what factors affect the concentration of LSMC in natural waters.

The analytical method developed during this research could be further utilised in future work to determine the concentration of LSMC in both sediment and biota. In its current state, the method outline in this research would not be suitable for the measurement of LSMC in sediment and biota due to the viscosity of the solvent which could result in separation issues between the sediment or biota and the octanol. Centrifugation of the sediment/biota solvent mix may result in adequate separation of the two phases, however, a much more appropriate technique would be through the use of octanol filled dialysis cells.

Some initial work was performed by Mitrovic (1995) to investigate the concentration of LSMC in both sediments and waters using octanol filled dialysis cells. Detection limits of the method were in the µg/L range (Table 7.1), which was higher than the detection limits during analysis of waters, due a lack of preconcentration within the method. Even with these higher detection limits this research found that sediments contained lipid soluble Cd, Cu, Ni and Pb complexes in concentrations high enough to be detected using octanol filled dialysis cells. Analysis of 8 biota samples by Mitrovic (1995) did not detect the concentration of any LSMC. Some further method development and method validation would be required before this method could be applied to a wider range of environments as the method was not tested against any laboratory prepared LSMC to assess its performance. Mitrovic's (1995) initial research into this area indicated that the concentration of LSMC is higher in sediments compared to waters. This suggests that the concentration of LSMC in sediments and biota is an issue that requires further attention as these two mediums are likely to

contain much higher concentrations of LSMC than waters and could represent a significant pool bioavailable LSMC.

Table 7.1 Limits of detection for the analysis of sediment and biota

	Cd (µg/L)	Cu (µg/L)	Ni (µg/L)	Pb (µg/L)
Sediment LOD	0.02*	1.00	2.60	0.80
Biota LOD	0.01*	1.00	5.20	0.80

* Sensitivity for analysis of Cd was greater than that of Cu, Ni and Pb Source: (Mitrovic, 1995)

Analysis of eight biota samples by Mitrovic (1995) did not detect the concentration of any LSMC. Some further method development and method validation would be required before this method could be applied to a wider range of environments as the method was not tested against any laboratory prepared LSMC to assess its performance. Mitrovic's (1995) initial research into this area indicated that the concentration of LSMC is elevated in sediments compared to waters. This suggests that the concentration of LSMC in sediments and biota is an issue that requires further attention as these two mediums are likely to contain much higher concentrations of LSMC than waters and could represent a significant pool of toxic and bioavailable LSMC.

7.2.5 Determination of LSMC concentrations in mine tailing waste waters

The concentration of LSMC was assessed in a sample of mine waste water from a gold mine in Papua New Guinea (PNG) that uses PAX, a xanthate, in the extraction of gold. This work was performed to assess the likely concentrations of LSMC in an environment where the concentration of synthetic organic ligand is likely to be high. Despite the extensive use of PAX in the mining industry (Read and Manser, 1976; Okibe and Johnson, 2002; Dopson et

al., 2006), very little data exists on the concentration of PAX-metal LSMC in waste waters or in waters from environments surround mining operations. Data exists on the toxicity of PAX to bacteria and some fish species, however, until now, this toxicity data could not be related to any environmental concentrations of PAX-metal complexes.

Pb and Zn LSMC were detected in the mine waste water sample, however, the concentration of Cu and Ni LSMC were lower than expected from a sample that is likely to contain a high concentration of the organic ligand PAX. To adequately analyse the data the concentration of PAX needed to also be determined in the sample. This again highlights the need for a method capable of detecting the total concentration of organic ligands (e.g. PAX) in waters samples. In addition, it was determined that the stability of the PAX – metal LSMC should be further investigated as this stability would directly determine the environmental importance and the environmental risk posed by of these complexes.

This work should initially focus on conducting some model laboratory experiments in which milli-Q water samples could be spiked with PAX-metal complexes and the stability of these complexes could be measured over time by detection of the total concentration of PAX-metal in solution. Natural waters spiked with PAX-metal complexes could then be tested to determine if the stability of these complexes changes due to the composition of the sample. Components of natural waters (like salinity, dissolved organic matter, pH, colloids, dissolved anions and cations etc.) may increase or decrease the stability of PAX-metal complexes. A suite of physico-chemical parameters should be measured on the natural water sample over the period of the experiment. By testing a range of natural waters and comparing the stability of the PAX-metal complex and the physico-chemical parameters a relationship

between certain parameters and stability may be observed. This would represent a significant body of research as at present the stability of LSMC including the PAX-metal complex has not been adequately performed and is vital in determining the bioavailability and toxicity of LSMC in natural waters.

7.2.6 Determination of the toxicity of LSMC

One area that has received some attention is the toxicity of LSMC to aquatic organisms. The toxicity of a range of LSMC has been assessed against a limited number of aquatic organisms from bacteria to algae to fish (Ahsanullah and Florence, 1984; Gottofrey et al., 1988; Block and Glynn, 1992; Mitrovic, 1995; Fraser et al., 2000). This work has shown that LSMC can be up to 25 times more toxic than free metal ions (Florence et al., 1992) due to their ability to passively diffuse across cell membranes (Ahsanullah and Florence, 1984; Blust et al., 1986; Phinney and Bruland, 1994; Phinney and Bruland, 1997; Phinney and Bruland, 1997; Croot et al., 1999; Turner and Mawji, 2005).

One shortcoming of the current toxicity data is that much of the results are based on the nominal concentration of LSMC in test waters rather than the actual concentration of LSMC. Given that a reliable, robust and sensitive method for the determination of LSMC in waters is now available, toxicity testing can now be performed where the actual concentration of LSMC in the test solutions is measured rather than just relying on just the nominal values. This toxicity testing is critical to making accurate determinations about the toxicity of LSMC to aquatic organisms. A small amount of toxicity testing was performed during this study

testing the toxicity of the PAX – Cu complex against the fresh water algae species *Pseudokirchmeriella subcapitata* and the freshwater Cladoceran species *Ceriodaphnia dubia*. Unfortunately, the results of these toxicity tests were inconclusive and therefore the data was not presented within this thesis. Further work should be performed to assess the toxicity of PAX – metal complexes and a range of other synthetic LSMC using actual concentrations rather than nominal concentration of LSMC. This data can then be compared to historic toxicity data to determine if the values calculated using nominal concentrations are accurately predicting the toxicity of LSMC.

Further investigation of the mechanisms of toxicity of LSMC on aquatic organisms should also be performed. Determining how the LSMC complexes form, their stability, confirming they passively diffuse through the cell membrane and also investigating what occurs once the complex has entered the cell would be beneficial. Having a deeper understanding of LSMC, in particular, the how they behave would help in identifying particular environments where LSMC might be present and would also allow for more informed conclusions to be made about toxicity data. This understanding would also assist in determining the environmental risk posed by concentrations of LSMC detected in natural waters. Mechanistic studies would need to be performed on individual LSMC and should focus initially on PAX due to its extensive use in the mining industry but could also focus on oxine and DDC due to their use as fungicides.

7.3 Concluding remarks

This study has a number of key findings that are expected to improve the understanding of the presence, concentration, composition and toxicity of LSMC. The development of

sensitive method for the detection of LSMC in waters should enable the presence and concentration of LSMC to be accurately determined in a range of aquatic environments. Determination of the octanol/water partition coefficients of some neutral inorganic complexes has identified that the composition of some LSMC detected in natural waters may include organic and inorganic complexes. At present the data only indicates that B and Hg LSMC may contain organic and inorganic complexes however other elements should be investigated in the future. This investigation has also identified a significant pool of potentially bioavailable metals that is poorly understood. The concentration of Cd, Cu, Ni, Pb and Zn LSMC was determined in a number of samples collected from aquatic environments in highly urbanised catchments indicating that LSMC are likely to be elevated in similar aquatic environments within Australia and throughout the world. Analysis of mine waste water likely to contain high concentrations of the organic xanthate ligand, PAX, returned detectable concentrations of Pb and Zn lipid soluble complexes suggesting that further work should focus on the analysis of similar waste waters.

Through the research performed during this study all but one of the project aims was addressed. A more robust and reliable back extraction technique was developed and tested. This new vacuum distillation approach was used to conduct a number of environmental surveys to detect the concentration of LSMC in 3 separate locations, Centennial Park, Homebush Bay and The Cooks River. In addition a small amount of work was performed analysing the concentration of LSMC in mine waste water. This environmental analysis returned a significant amount of high quality data on the occurrence of LSMC in natural

waters, of which there is a distinct lack. The research also investigated the importance of neutral inorganic metal complexes and their ability to extract into octanol. Finally the one aim that was not adequately address was in regards to the toxicity of LSMC. Work was performed to assess the toxicity of the PAX – Cu complex to a marine diatom and a Clodeceran species. Unfortunately the results were inconclusive and due to time constraints the work could not be repeated.

References

Acheampong, M. A., Meulepas, R. J. W. and Lens, P. N. L. (2010). Removal of heavy metals and cyanide from gold mine wastewater. *Journal of Chemical Technology & Biotechnology* **85**(5): 590-613.

Ahsanullah, M. and Florence, T. M. (1984). Toxicity of copper to the marine amphipod Allorchestes compressa in the presence of water-and lipid-soluble ligands. *Marine Biology* **84**(1): 41-45.

Akcil, A. (2003). Destruction of cyanide in gold mill effluents: biological versus chemical treatments. *Biotechnology Advances* **21**(6): 501-511.

Allen, H. E. (2000). Importance of Clean Techniques and Speciation in Assessing Water Quality for Metals. *Human and Ecological Risk Assessment* **6**(6): 989-1002.

Anderson, D. M. and Morel, F. M. M. (1978). Copper sensitivity of *Gonyaulax tamarensis*. *Limnology and Oceanography* **23**(2): 283-295.

Apte, S. C., Batley, G. E., Bowles, K. C., Brown, P. L., Creighton, N., Hales, L. T., Hyne, R. V., Julli, M., Markich, S. I., Pablo, F., Rogers, N. J., Stauber, J. L. and Wilde, K. (2005). A comparison of copper speciation measurements with the toxic responses of three sensitive freshwater organisms. *Environmental Chemistry* **2**(4): 320-330.

Apte, S. C., Gardner, M. J. and Hunt, D. T. E. (1989). An evaluation of dialysis as a size-based separation method for the study of trace metal speciation in natural waters. *Environmental Technology* **10**(2): 201 - 212.

Barbehenn, R., Dodick, T., Poopat, U. and Spencer, B. (2005). Fenton-Type Reactions and Iron Concentrations in the Midgut Fluids of Tree-Feeding Caterpillars. *Archives of Insect Biochemistry and Physiology* **60**: 32-43.

Batley, G. E., Apte, S. C. and Stauber, J. L. (2004). Speciation and Bioavailability of Trace Metals in Water: Progress Since 1982. *Australian Journal of Chemistry* **57**(10): 903-919.

Benoit, G., Oktay-Marshall, S. D., Cantu Ii, A., Hood, E. M., Coleman, C. H., Corapcioglu, M. O. and Santschi, P. H. (1994). Partitioning of Cu, Pb, Ag, Zn, Fe, Al, and Mn between filter-retained particles, colloids, and solution in six Texas estuaries. *Marine Chemistry* **45**(4): 307-336.

Benoit, J. M., Mason, R. P. and Gilmour, C. C. (1999). Estimation of mercury-sulfide speciation in sediment pore waters using octanol—water partitioning and implications for availability to methylating bacteria. *Environmental Toxicology and Chemistry* **18**(10): 2138-2141.

Bertills, U. I., Bjorklund, H. B. and Hornstrom, E. (1986). Biological effects of xanthates. National Swedish Environment Protection Board

Bhamre, P. R., Thorat, S. P., Desai, A. E. and Deoray, B. M. (2011). *Evaluation of Acute Toxicity of Mercury, Cadmium and Zinc to a Freshwater Mussel Lamellidens consobrinus*.

Birch, G. F., Harrington, C., Symons, R. K. and Hunt, J. W. (2007). The source and distribution of polychlorinated dibenzo-p-dioxin and polychlorinated dibenzofurans in sediments of Port Jackson, Australia. *Marine Pollution Bulletin* **54**(3): 295-308.

Birch, G. F. and Taylor, S. E. (2000). Distribution and possible sources of organochlorine residues in sediments of a large urban estuary, Port Jackson, Sydney. *Australian Journal of Earth Sciences* **47**(4): 749-756.

Block, M. (1991). Distribution of Cadmium in an Octanol/Water System in the presence of Xanthates and Diethyldithiocarbamate. *Environmental Toxicology and Chemistry* **10**(10): 1267-1272.

Block, M. and Glynn, A. W. (1992). Influence of Xanthates on the Uptake of 109Cd by Eurasian Dace (*Phoxinus phoxinus*) and Rainbow Trout (*Oncorhynchus mykiss*). *Environmental Toxicology and Chemistry* **11**(6): 873-879.

Blom, L. B., Morrison, G. M., Kingston, J., Mills, G. A., Greenwoog, R., Pettersson, T. J. R. and Rauch, S. (2002). Performance of an In Situ Passive Sampling System for Metals in Stormwater. *Journal of Environmental Monitoring* **4**: 258 - 262.

Blust, R., Verheyen, E., Doumen, C. and Decleir, W. (1986). Effect of complexation by organic ligands on the bioavailability of copper to the brine shrimp, Artemia sp. *Aquatic Toxicology* **8**(3): 211-221.

Borgmann, U. and Charlton, C. C. (1984). Copper Complexation and Toxicity to Daphnia in Natural Waters. *Journal of Great Lakes Research* **10**(4): 393-398.

Boullemant, A., Vigneault, B., Fortin, C. and Campbell, P. G. C. (2004). Uptake of Neutral Metal Complexes by a Green Alga: Influence of pH and Humic Substances. *Australian Journal of Chemistry* **57**: 931-936.

Bryan, G. W. (1971). The Effects of Heavy Metals (other than Mercury) on Marine and Estuarine Organisms. *Proceedings of the Royal Society of London, Series B* **177**: 389 - 410.

Bryce, T. A., Telling, G. M. and Althorpe, J. (1971). Use of vacuum distillation and gas chromatography-mass spectrometry for determination of low levels of volatile nitrosamines in meat products. *Journal of Agricultural and Food Chemistry* **19**(5): 937-940.

Campbell, P. G. C. (1995). *Metal Speciation and Bioavailability*. Chichester: John Wiley.

Campbell, P. G. C., Errécalde, O., Fortin, C., Hiriart-Baer, V. P. and Vigneault, B. (2002). Metal bioavailability to phytoplankton—applicability of the biotic ligand model. *Comparative Biochemistry and Physiology Part C: Toxicology & Pharmacology* **133**(1-2): 189-206.

Carlson-Ekvall, C. E. A. and Morrison, G. M. (1995). Toxicity of Copper in the Presence of Organic Substances in Sewage Sludge. *Environmental Technology* **16**(3): 243 - 251.

Carlson, C. E. A. and Morrison, G. M. (1992). Fractionation and toxicity of metals in sewage sludge. *Environmental Technology* **13**(8): 751 - 759.

Carlson, C. E. A. and Morrison, G. M. (1995). Toxicity of Copper in the Presence of Organic Substances in Sewage Sludge. *Environmental Technology* **16**: 243-251.

Carvalho, R. A., Benfield, M. C. and Santschi, P. H. (1999). Comparative bioaccumulation studies of colloidally complexed and free-ionic heavy metals in juvenile brown shrimp *Penaeus aztecus* (Crustacea: Decapoda: Penaeidae). *Limnology and Oceanography* **44**(2): 403-414.

Chen, C. S., Rao, P. S. C. and Lee, L. S. (1996). Evaluation of extraction and detection methods for determining polynuclear aromatic hydrocarbons from coal tar contaminated soils. *Chemosphere* **32**(6): 1123-1132.

Cortazar, E., Bartolomé, L., Delgado, A., Etxebarria, N., Fernández, L. A., Usobiaga, A. and Zuloaga, O. (2005). Optimisation of microwave-assisted extraction for the determination of nonylphenols and phthalate esters in sediment samples and comparison with pressurised solvent extraction. *Analytica Chimica Acta* **534**(2): 247-254.

Croot, P. L., Karlson, B., Van Elteren, J. T. and Kroon, J. J. (1999). Uptake of ^{64}Cu-Oxine by Marine Phytoplankton. *Environmental Science and Technology* **33**(3615-3615).

Cubby, B. (November 2011). Study keeps up stink over polluted river. Sydney Morning Herald. Sydney, Fairfax Newspapers. **Accessible at: http://www.smh.com.au/environment/water-issues/study-keeps-up-stink-over-polluted-river-20111023-1meii.html**.

Danielsson, L., Zhang, Y. and Sparen, A. (1995). Mechanized Method for Measuring Metal Partition in *n*-Octanol-Aqueous Systems. Partition of Aluminium Complexes. *Analysts* **120**: 2513-2519.

Dankert, J., Zijlstra, J. B. and Wolthers, B. G. (1981). Volatile fatty acids in human peripheral and portal blood: Quantitative determination by vacuum distillation and gas chromatography. *Clinica Chimica Acta* **110**(2–3): 301-307.

Dash, R. R., Gaur, A. and Balomajumder, C. (2009). Cyanide in industrial wastewaters and its removal: A review on biotreatment. *Journal of Hazardous Materials* **163**(1): 1-11.

Dopson, M., Sundkvist, J.-E. and Börje Lindström, E. (2006). Toxicity of metal extraction and flotation chemicals to Sulfolobus metallicus and chalcopyrite bioleaching. *Hydrometallurgy* **81**(3-4): 205-213.

Dupré, B., Viers, J., Dandurand, J.-L., Polve, M., Bénézeth, P., Vervier, P. and Braun, J.-J. (1999). Major and trace elements associated with colloids in organic-rich river waters: ultrafiltration of natural and spiked solutions. *Chemical Geology* **160**(1–2): 63-80.

Fernando, Q. (1995). Metal Speciation in Environmental and Biological Systems. *Environmental Health Perspectives* **103**(Supplement 1).

Florence, T. M. (1982). Development of Physico-Chemical Speciation Procedures to investigate the toxicity of Copper, Lead, Cadmium and Zinc towards Aquatic Biota. *Analytica Chimica Acta* **141**: 73-94.

Florence, T. M. (1983). Trace element speciation and aquatic toxicology. *TrAC Trends in Analytical Chemistry* **2**(7): 162-166.

Florence, T. M., Lumsden, B. G. and Fardy, J. J. (1983). Evaluation of some Physico-Chemical Techniques for the Determination of the Fraction of Dissolved Copper Toxic to the Marine Diatom *Nitzschia Closterium*. *Analytica Chimica Acta* **151**: 281-295.

Florence, T. M., Powell, H. K. J., Stauber, J. L. and Town, R. M. (1992). Toxicity of Lipid-Soluble Copper(II) Complexes to the Marine Diatom *Nitzschia Closterium*: Amelioration by Humic Substances. *Water Research* **26**(9): 1187-1193.

Florence, T. M. and Stauber, J. L. (1986). Toxicity of copper complexes to the marine diatom Nitzschia closterium. *Aquatic Toxicology* **8**(1): 11-26.

Franklin, N. M., Stauber, J. L. and Lim, R. P. (2001). Development of flow cytometry-based algal bioassays for assessing toxicity of copper in natural waters. *Environmental Toxicology and Chemistry* **20**(1): 160-170.

Fraser, J. K., Butler, C. A., Timperley, M. H. and Evans, C. W. (2000). Formation of Copper Complexes in Landfill Leachate and their Toxicity to Zebrafish Embryos. *Environmental Toxicology and Chemistry* **19**(5): 1397-1402.

Friedel, J., Marr, I. L. and Müller, H. (1994). The influence of surfactants on the behaviour of heavy metals in the system n-octanol-water. *Fresenius' Journal of Analytical Chemistry* **350**(1): 74-76.

Gabriel, M. and Williamson, D. (2004). Principal Biogeochemical Factors Affecting the Speciation And Transport of Mercury through the terrestrial environment. *Environmental Geochemistry and Health* **26**(3): 421-434.

Google. (2012, March 1, 2012). "Google Maps." Retrieved March 11, 2012, 2012, from http://maps.google.com.au/.

Gottofrey, J., Björklund, I. and Tjälve, H. (1988). Effect of sodium isopropylxanthate, potassium amylxanthate and sodium diethyldithiocarbamate on the uptake and distribution of cadmium in the brown trout (Salmo trutta). *Aquatic Toxicology* **12**(2): 171-184.

Griffiths, S. R., Smith, G. B., Donato, D. B. and Gillespie, C. G. (2009). Factors influencing the risk of wildlife cyanide poisoning on a tailings storage facility in the Eastern Goldfields of Western Australia. *Ecotoxicology and Environmental Safety* **72**(5): 1579-1586.

Gurbuz, F., Ciftci, H. and Akcil, A. (2009). Biodegradation of cyanide containing effluents by Scenedesmus obliquus. *Journal of Hazardous Materials* **162**(1): 74-79.

Gustafsson, J. P. (2006). Visual MINTEQ. Version 2.40. Stockholm, Sweden, KTH.

Haan, H. D. (1984). Developments in Biochemistry, Complexation of Trace Metals in Natural Waters. *Hydrobiological Bulletin* **18**(2): 165-174.

Hahne, H. C. H. and Kroontje, W. (1972). Significance of pH and Chloride Concentration on Behavior of Heavy Metal Pollutants: Mercury(II), Cadmium(II), Zinc(II), and Lead(II). *J. Environ. Qual.* **2**(4): 444-450.

Hassett, D. J. (1994). Scientifically valid leaching of coal conversion solid residues to predict environmental impact. *Fuel Processing Technology* **39**(1–3): 445-459.

Hiatt, M. H., Youngman, D. R. and Donnelly, J. R. (1994). Separation and isolation of volatile organic compounds using vacuum distillation with GC/MS determination. *Analytical Chemistry* **66**(6): 905-908.

Hilson, G. and Monhemius, A. J. (2006). Alternatives to cyanide in the gold mining industry: what prospects for the future? *Journal of Cleaner Production* **14**(12–13): 1158-1167.

Hudson, R. J. M. (1998). Which aqueous species control the rates of trace metal uptake by aquatic biota? Observations and predictions of non-equilibrium effects. *The Science of the Total Environment* **219**: 95-115.

Ingram, T., Richter, U., Mehling, T. and Smirnova, I. (2011). Modelling of pH dependent n-octanol/water partition coefficients of ionizable pharmaceuticals. *Fluid Phase Equilibria* **305**(2): 197-203.

Kamyshny, A., Oduro, H. and Farquhar, J. (2012). Quantification of free and metal-complexed cyanide by tetrathionate derivatization. *International Journal of Environmental Analytical Chemistry*: 1-12.

Kilgore, D. A. (2007). Lipid Soluble Metal Complexes In Aquatic Environments. Department of Environment and Geography. Sydney, NSW, Australia, Macquarie University. **Honours, Bachelor of Environmental Science**.

Klochko, K., Kaufman, A. J., Yao, W., Byrne, R. H. and Tossell, J. A. (2006). Experimental measurement of boron isotope fractionation in seawater. *Earth and Planetary Science Letters* **248**(1–2): 276-285.

Lea, C. H. and Swoboda, P. A. T. (1962). Simple vacuum distillation procedure for determination of the volatile carbonyl content of autoxidising edible fats. *Journal of the Science of Food and Agriculture* **13**(3): 148-158.

Loon, H. Y. and Madgwick, J. (1995). The effect of xanthate floatation reagents on bacterial leaching of chalcopyrite by Thiobacillus ferrooxidans. *Biotechnology Letters* **17**(9): 997-1000.

Lund, W. (1990). Speciation analysis — why and how? *Fresenius' Journal of Analytical Chemistry* **337**(5): 557-564.

Mann, E. L., Ahlgren, N., Moffett, J. W. and Chisholm, S. W. (2002). Copper toxicity and cyanobacteria ecology in the Sargasso Sea. *Limnology and Oceanography* **47**(4): 976-988.

Mason, R. P., Reinfelder, J. R. and Morel, F. M. M. (1995). Bioaccumulation of mercury and methylmercury. *Water, Air, & Soil Pollution* **80**(1): 915-921.

Mason, R. P., Reinfelder, J. R. and Morel, F. M. M. (1996). Uptake, Toxicity, and Trophic Transfer of Mercury in a Coastal Diatom. *Environmental Science & Technology* **30**(6): 1835-1845.

McCready, S., Slee, D. J., Birch, G. F. and Taylor, S. E. (2000). The Distribution of Polycyclic Aromatic Hydrocarbons in Surficial Sediments of Sydney Harbour, Australia. *Marine Pollution Bulletin* **40**(11): 999-1006.

Mitrovic, S. M. (1995). Lipid - Soluble metal complexes in the aquatic environment. Department of Applied Biology. Sydney, University of Technology. **Bachelor of Applied Science - Environmental Biology (Honours)**.

Mohrig, J. R., Hammond, C. N. and Schatz, P. F. (2006). *Techniques in Organic Chemistry*. W.H. Freeman.

Morel, F. M. M. (1983). *Principles and Applications of Aquatic Chemistry*. New York, NY: John Wiley.

Okibe, N. and Johnson, D. B. (2002). Toxicity of flotation reagents to moderately thermophilic bioleaching microorganisms. *Biotechnology Letters* **24**(23): 2011-2016.

Orica. (2010). "Oricia Mining Chemicals." Retrieved 21/11/2010, from <http://orica-miningchemicals.com/product__services/xanthates.aspx>.

Paquin, P. R., Gorsuch, J. W., Apte, S., Batley, G. E., Bowles, K. C., Campbell, P. G. C., Delos, C. G., Di Toro, D. M., Dwyer, R. L., Galvez, F., Gensemer, R. W., Goss, G. G., Hogstrand, C., Janssen, C. R., McGeer, J. C., Naddy, R. B., Playle, R. C., Santore, R. C., Schneider, U., Stubblefield, W. A., Wood, C. M. and Wu, K. B. (2002). The biotic ligand model: a historical overview. *Comparative Biochemistry and Physiology C-Toxicology & Pharmacology* **133**(1-2): 3-35.

Parent, L., Twiss, M. R. and Campbell, P. G. C. (1996). Influences of Natural Dissolved Organic Matter on the Interaction of Aluminum with the Microalga Chlorella: A Test of the Free-Ion Model of Trace Metal Toxicity. *Environmental Science & Technology* **30**(5): 1713-1720.

Parthasarathy, N., Pelletier, M. and Buffle, J. (2004). Permeation liquid membrane for trace metal speciation in natural waters: Transport of liposoluble Cu(II) complexes. *Journal of Chromatography A* **1025**(1): 33-40.

Parthasarathy, N., Pelletier, M. and Buffle, J. (2010). Transport of lipophilic metal complexes through permeation liquid membrane, in relation to natural water analysis: Cu(II)-8-hydroxyquinoline complex as a model compound. *Journal of Membrane Science* **355**(1-2): 78-84.

Patton, G. W., Hinckley, D. A., Walla, M. D., Bidleman, T. F. and Hargrave, B. T. (1989). Airborne organochlorines in the Canadian High Arctic. *Tellus B* **41B**(3): 243-255.

Phinney, J. T. and Bruland, K. W. (1994). Uptake of Lipophilic Organic Cu, Cd, and Pb Complexes in the Coastal Diatom Thalassiosira weissflogii. *Environmental Science & Technology* **28**(11): 1781-1790.

Phinney, J. T. and Bruland, K. W. (1997). Effects of Dithiocarbamate and 8-Hydroxyquinoline Additions on Algal Uptake of Ambient Copper and Nickel in South San Francisco Bay Water. *Estuaries* **20**(1): 66-76.

Phinney, J. T. and Bruland, K. W. (1997). Trace Metal Exchange in Solution by the Fungicides Ziram and Maneb (Dithiocarbamates) and subsequent uptake of Lipophilic Organic Zinc, Copper and Lead Complexes into Phytoplankton cells. *Environmental Toxicology and Chemistry* **16**(10): 2046-2053.

Poldoski, J. E. (1979). Cadmium bioaccumulation assays - Their relationship to various ionic equilibrium in Lake-Superior water. *Environmental Science & Technology* **13**(6): 701-706.

Raman, N., Muthuraj, V., Ravichandran, S. and Kulandaisamy, A. (2003). Synthesis, characterisation and electrochemical behaviour of Cu(II), Co(II), Ni(II) and Zn(II) complexes perived from acetylacetone and *p*-anisidine and their antimicrobial activity. *Proceedings of the Indian Academic Society (Chemistry Society)* **115**(3): 161-167.

Rao, S. R. (1971). *Xanthates and related compounds*. M. Dekker.

Read, A. D. and Manser, R. M. (1976). Mineral flotation--a study of the re-use and disposal of aqueous solutions containing organic reagents. *Water Research* **10**(3): 243-251.

Richter, B. E., Jones, B. A., Ezzell, J. L., Porter, N. L., Avdalovic, N. and Pohl, C. (1996). Accelerated Solvent Extraction: A Technique for Sample Preparation. *Analytical Chemistry* **68**(6): 1033-1039.

Sciencelab.com. (2011). "1-Octanol MSDS." Retrieved 07/03/2012, 2012, from http://www.sciencelab.com/msds.php?msdsId=9927679.

Simkiss, K. and Taylor, M. G. (2001). Trace element speciation at cell membranes: aqueous, solid and lipid phase effects. *Journal of Environmental Monitoring* **3**: 15-21.

Slaveykova, V. I., Wilkinson, K. J., Ceresa, A. and Pretsch, E. (2003). Role of Fulvic Acid on Lead Bioaccumulation by *Chlorella kesslerii*. *Environmental Science and Technology* **37**: 1114-1121.

Snarski, V. M. and Olson, G. F. (1982). Chronic toxicity and bioaccumulation of mercuric chloride in the fathead minnow (Pimephales promelas). *Aquatic Toxicology* **2**(3): 143-156.

Sofyan, A., Rosita, G., Price, D. J. and Birge, W. J. (2007). Cadmium uptake by *Ceriodaphnia dubia* from different exposures: relevance to body burden and toxicity. *Environmental Toxicology and Chemistry* **26**(3): 470-470-7.

Stauber, J. L. and Florence, T. M. (1987). Mechanism of toxicity of ionic copper and copper complexes to algae. *Marine Biology* **94**: 511-519.

Stauber, J. L. and Florence, T. M. (1989). The Effect of Culture Medium on Metal Toxicity to the Marine Diatom *Nitzschia Closterium* and the Freshwater Alga *Chlorella Pyrenoidosa*. *Water Research* **23**(7): 907-911.

Steel, W. H. and Walter, R. A. (2003). Measuring dipolar width across liquid-liquid interfaces with 'molecular rulers'. *Nature* **424**: 296-299.

Suh, J. Y., Birch, G. F., Hughes, K. and Matthai, C. (2004). Spatial distribution and source of heavy metals in reclaimed lands of Homebush Bay: the venue of the 2000 Olympic Games, Sydney, New South Wales. *Australian Journal of Earth Sciences* **51**(1): 53-67.

Sunda, W. and Guillard, R. R. L. (1976). The relationship between cupric ion activity and the toxicity of copper to phytoplankton. *Journal of Marine Research* **34**(4): 511-529.

Takeoka, G. R., Flath, R. A., Guentert, M. and Jennings, W. (1988). Nectarine volatiles: vacuum steam distillation versus headspace sampling. *Journal of Agricultural and Food Chemistry* **36**(3): 553-560.

Tuovinen, O. H. (1978). Inhibition of <i>Thiobacillus ferrooxidans</i> by mineral flotation reagents. *Applied Microbiology and Biotechnology* **5**(4): 301-304.

Turekian, K. K. (1968). *Oceans*. Prentice-Hall.

Turner, A. and Mawji, E. (2004). Hydrophobicity and Octanol-Water Partitioning of Trace Metals in Natural Waters. *Environmental Science & Technology* **38**(11): 3081-3091.

Turner, A. and Mawji, E. (2005). Octanol-solubility of dissolved and particulate trace metals in contaminated rivers: implications for metal reactivity and availability. *Environmental Pollution* **135**(2): 235-244.

Turner, A. and Williamson, I. (2005). Octanol-water partitioning of chemical constituents in river water and treated sewage effluent. *Water Research* **39**(18): 4325-4334.

van Woezik, B. A. A. and Westerterp, K. R. (2000). The nitric acid oxidation of 2-octanol. A model reaction for multiple heterogeneous liquid-liquid reactions. *Chemical Engineering and Processing* **39**(6): 521-537.

Vasconcelos, M. T. S. D., Azenha, M. A. O. and Cabral, J. P. S. (1997). Comparison of Availability of Copper(II) Complexes with Organic Ligands to Bacterial Cells and to Chitin. *Environmental Toxicology and Chemistry* **16**(10): 2029-2039.

Vigneault, B., Percot, A., Lafleur, M. and Campbell, P. G. C. (2000). Permeability Changes in Model and Phytoplankton Membranes in the Presence of Aquatic Humic Substances. *Environmental Science & Technology* **34**(18): 3907-3913.

Webb, M., Ruber, H. and Leduc, G. (1976). The toxicity of various mining flotation reagents to rainbow trout (Salmo gairdneri). *Water Research* **10**(4): 303-306.

Wong, S. L., Nakamoto, L. and Wainwright, J. F. (1997). Detection of Toxic Organometallic Complexes in Wastewaters Using Algal Assays. *Archives of Environmental Contamination and Toxicology* **32**: 358-366.

Wu, H., Haig, T., Pratley, J., Lemerle, D. and An, M. (1999). Simultaneous determination of phenolic acids and 2,4-dihydroxy-7-methoxy-1,4-benzoxazin-3-one in wheat (Triticum aestivum L.) by gas chromatography–tandem mass spectrometry. *Journal of Chromatography A* **864**(2): 315-321.

Wu, Y. and Wang, W.-X. (2011). Accumulation, subcellular distribution and toxicity of inorganic mercury and methylmercury in marine phytoplankton. *Environmental Pollution* **159**(10): 3097-3105.

Xu, Y., Lay, J. P. and Korte, F. (1988). Fate and effects of xanthates in laboratory freshwater systems. *Bulletin of Environmental Contamination and Toxicology* **41**(4): 683-689.

Zhang, M. and Florence, T. M. (1987). A Novel Adsorbent for the Determination of the Toxic Fraction of Copper in Natural Waters. *Analytica Chimica Acta* **197**: 137-148.

Zhang, Y.-H., Danielsson, L.-G. and Becze, Z. (1998). Partition of copper and cadmium between n-octanol and aqueous phases using segmented flow extraction. *Water Research* **32**(7): 2073-2080.

Zorkin, N. G., Grill, E. V. and Lewis, A. G. (1986). An Ion-Exchange Procedure for Quantifying Biologically Active Copper in Sea Water *Analytica Chimica Acta* **183**: 163-177.