CHARACTERISATION OF COPPER BINDING LIGANDS FROM MARINE CYANOBACTERIAL CULTURES USING VOLTAMMETRY AND MASS SPECTROMETRY

by

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Abstract

Strong copper binding ligands have been produced by *Synechococcus* DC2 grown exponentially under copper stressed conditions. Using pseudo-polarography, all cultures were found to produce a strong copper binding ligand with a log $K'$ of 24.7, assuming that copper is in the +1 oxidation state. Weaker ligands were also present but their binding constants were variable. These strong copper binding ligands were extracted into water-methanol solvents from a hydrophobic resin (XAD-16). Pseudo-polarography was used to track the fractions where the strong ligands were recovered. MALDI and ESI were chosen as soft ionisation sources to access the whole molecule. Both ionisation techniques revealed only one complex that had an isotopic signature characteristic of copper (at $m/z$ 697/699) and this complex was present in two of five culture experiments.

Further investigation of the 697/699 complex showed that it was present in all eluent fractions containing the electrochemically determined strong copper binding ligand. Tandem mass spectrometry using MALDI revealed several fragments where copper was still bound, with the smallest copper containing ion at $m/z$ 281/283. A loss of 64/66 mass units from two different fragments suggests the loss of CuH which also implies that the complex binds copper in the +1 oxidation state. Aspects of the MS/MS spectra suggest that this could be a peptide bound to copper by thiol containing cysteine groups. However, further work is needed to ascertain the true chemical nature of this compound. This work represents the first investigation to combine electrochemistry with mass spectrometry to reveal the much anticipated chemical nature of strong copper binding ligands, present in the surface ocean.
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Glossary

ACTH: adrenocorticotrophic hormone fragment 1–4
ASV: anodic stripping voltammetry
BLM: biotic ligand model
CDTA: 1,2-diaminocyclohexane-N,N,N',N'-tetraacetic acid
CHCA: α-cyano-4-hydroxycinnamic acid
CLE-CSV: competitive ligand equilibration-cathodic stripping voltammetry
CSV: cathodic stripping voltammetry
DHAP: 2′-6′-dihydroxyacetophenone
DMS: dimethyl sulphide
DOM: dissolved organic matter
DPASV: differential pulsed anodic stripping voltammetry
EDTA: ethylenediaminetetraacetic acid
ESI: electrospray ionisation
FIAM: free ion activity model
FT ICR MS: fourier transform ion cyclotron resonance mass spectrometry
GFAAS: graphite furnace atomic absorption spectrometry
HDPE: high density polyethylene
HMDE: hanging mercury drop electrode
HPLC: high performance liquid chromatography
HR ICP MS: high resolution inductively coupled plasma mass spectrometry
ICPMS: inductively couple plasma mass spectrometry

IDMS: isotope dilution mass spectrometry

IMAC: immobilised metal affinity chromatography

LE LL: ligand exchange liquid liquid partition

MALDI QTOF MSMS: matrix assisted laser desorption ionisation quadrupole time of flight tandem mass spectrometry

MALDI TOF MS: matrix assisted laser desorption ionisation time of flight mass spectrometry

MALDI TOF: matrix assisted laser desorption ionisation time of flight mass spectrometry

MALDI: matrix assisted laser desorption ionisation

MS/MS: mass spectrometry / mass spectrometry (tandem mass spectrometry)

PC: polycarbonate

PP: pseudo polarography

RID: reverse isotope dilution

SE: solvent extraction

UV: ultra violet
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Chapter 1

Introduction and background work

Chemical speciation is the determination of the distribution of a chemical element amongst all its possible chemical forms (species) in a given system. The speciation of any element greatly influences its mobility, uptake efficiency and toxicity. This is especially important in the context of biological organisms, since many elements are nutritionally required and must be acquired from the environment. Since metals present at very low concentration (trace metals) are required for general metabolism of all organisms, these elements play an important role in growth dynamics. An important environmental challenge for primary producers is the acquisition of trace metals. In terrestrial and marine environments, the production of metal-binding ligands has been documented for bacteria, cyanobacteria, and other marine protists [1, 2]. An understanding of the speciation of trace metals in the ocean is essential if we are to understand the interactions of trace level marine chemistry and how it affects larger scale patterns in biological productivity.

The work presented here focuses on copper speciation in the ocean. I have chosen copper for several reasons: (i) copper ligands have been well-documented in the marine environment [3] and are readily induced at high copper concentrations [4], thus making them ecologically relevant and well-suited for laboratory work; (ii) recent studies have shown that copper availability may control phytoplankton productivity in the open ocean when iron availability is low (metal-metal interactions) [5]; (iii) copper toxicity is an important problem in many coastal areas, thus making ligands potentially important for bioremediation [6].

While it has been documented that copper-binding ligands exist, their characterization has not yet been accomplished. A better knowledge of copper-binding ligand structure would offer new insights into the mechanism of copper binding, and will further our understanding of organic-metal interactions in the environment.

This chapter will cover an overview of copper and speciation in the marine environment. Past work on marine copper speciation is separated into two parts:
1.1. Introduction: oceanic trace metals

(i) measurements using voltammetry and (ii) methods of structural determinations. Voltammetric analyses inform us about binding constants and concentrations of specific fractions of copper. Previous research that covers structural determinations is presented in two sections: the separation and concentration of the analyte from seawater and the determinations of the chemical nature of the analyte once it has been separated. An overview of past research highlights the analytical challenges of these steps (separation from seawater and structural characterisation). Experimental design presented in this thesis attempts to avoid some of the assertions made when analysing a chemical property of the analyte rather than the preferable, but almost impossible, direct measurement of the analyte itself.

1.1 Introduction: oceanic trace metals

The concentrations of transition metals in the ocean are very low and the requirement of using trace metal clean techniques has only been recognised in the past few decades. Thus, trace metal analysis in marine samples is a relatively new field. Also such very low metal concentrations are dissolved in a highly concentrated matrix and this disparity in concentration has necessitated significant sample preparation (extraction from the seawater matrix and/or preconcentration).

1.1.1 Definitions for speciation

Since most transition metals are present in the ocean at concentrations of less than 1 μM, they are referred to as 'trace' and their concentrations are sometimes noted as a negative log, similar to the pH scale. For example, a concentration of $10^{-13}$ is denoted as pM 13 where M is the activity of any metal ($\{M\}$). The activity of a metal is related to the concentration of a metal whereby a correction factor is used to account for solution interactions between the metal and other components of the solution. Also, seawater itself has a large ionic strength (a measure of the charge density of the solution), which influences the interactions of charged species and this must be accounted for. Binding constants refer to the strength of binding of a metal with an organic complex and are calculated as follows.
1.1. Introduction: oceanic trace metals

For a reaction:

$$M^{n+} + L^{p-} \rightleftharpoons ML^{(n-p)+}$$  \hspace{1cm} (1.1)

then:

$$K = \frac{\{ML^{(n-p)+}\}}{\{M^{n+}\} \{L^{p-}\}}$$  \hspace{1cm} (1.2)

The equilibrium constant, $K$ shows how strongly a metal (M) binds to the ligand (L) in a complex (ML). In a complex saline solution, a metal can bind with several ligands (e.g. Cl$^-$, HCO$_3^-$) that are not of direct interest; these are considered side reactions. This is considered along with ionic strength which also affects the likelihood of a metal to bind. In seawater, important equilibria involve reactants and products that are at a much lower concentration than the major ionic species. Thus, the change in activity of a trace metal ion will not be significantly affected by variations in salinity. The activity of the metal ion is related to concentration by the activity coefficient, $\gamma_{M^{n+}}$.

$$\{M^{n+}\} = \left[ M^{n+} \right] \gamma_{M^{n+}}$$  \hspace{1cm} (1.3)

The activity co-efficient accounts for ionic strength. However there is also a significant decrease in metal ion activity (in this case copper) due to side reactions with major ions. Typically, for seawater the inorganic side reactions are combined into one co-efficient, called the fraction free co-efficient for example, with copper, the co-efficient is $f_{Cu}$.

$$f_{Cu} = \frac{[Cu^{2+}]_{free}}{[Cu^{2+}]_{free} + [CuCO_3] + [CuHCO_3^+] + [CuSO_4] + ...}$$  \hspace{1cm} (1.4)

Therefore, in general, the concentration of the trace metal ([M]) is used and, for convenience $K^c$ is used where 'c' denotes that it is a conditional constant where:

$$K^c = \frac{[ML^{(n-p)+}]_{tot}}{[M^{n+}]_{tot} [L^{p-}]_{tot}}$$  \hspace{1cm} (1.5)
Stability constant | Value | Reference
--- | --- | ---
log K | 18.80 | Cheng [8]
log $K'$ | 17.94 | Croot et al. [9]
log $K^c$ | 10.06 | Campos and van den Berg [10]

Table 1.1: The different stability constants for a copper-EDTA complex in seawater under room temperature (20°C) and pressure. Units for $K$ are M$^{-1}$.

and $K^c$ is related to the true equilibrium constant, $K$ by:

$$K^c = \left( \frac{\gamma_{M^{n+}}f_{M^{n+}}\gamma_{L^{-}}f_{L^{-}}}{\gamma_{ML(n-p)}+f_{ML(n-p)}} \right) K$$

(1.6)

The conditional stability constant, $K^c$, is often represented on a log scale and referred to as log $K^c$. In some cases side reactions are not considered and only ionic strength is considered. Side reactions are corrected for using side reaction co-efficients and the binding constant is denoted as $K'$. This is the ionic strength corrected thermodynamic stability constant:

$$K' = \frac{f_{ML(n-p)}+}[ML(n-p)+]$$

(1.7)

$$f_{M^{n+}}[M^{n+}]f_{L^{-}}[L^{-}]$$

The constant, $K'$, is related to the equilibrium constant, $K$, by:

$$K' = \left( \frac{\gamma_{M^{n+}}\gamma_{L^{-}}}{\gamma_{ML(n-p)+}} \right) K$$

(1.8)

An example of the differences between these three stability constants can be recognised when comparing the stability constants for copper bound to EDTA in seawater and in ideal conditions, this is shown in Table 1.1.

### 1.1.2 Trace metal distribution

Several factors affect the concentration of trace metals with depth. Some metals are only affected by changes in total salt (salinity) and are conservative (such as molybdenum). The total dissolved surface water concentrations of metals that are
non-conservative can change as a result of (i) biological influences: uptake by organisms and their subsequent release back into the water at depth through the remineralisation of biogenic sinking matter and (ii) physico-chemical influences: through the adsorption to particles or by atmospheric deposition of particles (dust or rain). Metals that are dominated by biological mechanisms, such as zinc, can have profiles that are similar to major nutrients such as silicate (as for zinc) or phosphate or nitrate. A typical nutrient profile would have low concentrations in the euphotic zone as a result of biological uptake. Below the euphotic zone, sinking biogenic particles (containing trace metals) are remineralised by bacteria and metal concentration will increase. Aluminum is an example of a metal influenced by physico-chemical factors. Typically surface water concentrations are high, influenced by aeolian input of aluminum. Concentrations then decrease with depth as a result of precipitation and adsorption [11].

### 1.1.3 Copper

Copper has two stable isotopes, 63 and 65, whose percent abundances are 69.2 and 30.8, respectively. In the environment copper typically exists in the +2 oxidation state. However, there are some cases, even in an oxidising environment, where it may exist in the +1 oxidation state [12]. In terms of hardness, copper, in its most oxidised form (+2) is an intermediate element and tends to bind with oxygen and nitrogen and sometimes sulphur donor atoms. Copper (I) is a soft ion and will bind more strongly with sulphur and chloride than nitrogen and oxygen. Sulphur may be able to stabilise Cu(I) even in an oxidising environment [12] and the presence of chloride may slow down the re-oxidisation of copper to the +2 state [13].

#### Distribution of copper

The concentration of copper is low in surface waters, with reported North Pacific surface waters of 0.5 nmol/kg and an increase in deeper waters to 5.3 nmol/kg [14]. Copper is used as a nutrient by phytoplankton [15], although it is toxic to some organisms at very low concentrations. Thus, in surface waters, copper has a nutrient like profile. In the deeper waters the increase in total dissolved copper concentration is considered to be due to influx from the bottom sediments, as well as from
1.2. Biological role of copper

regeneration in medium depth water [14].

1.2  Biological role of copper

Copper is a nutrient and is involved in many metabolic processes, such as the scavenging of the highly reactive oxygen species $O_2^-$ and $O_2^-$ by CuZn containing superoxide dismutase [16]. Copper is also known to be redox active under physiological conditions and, for example, is present in plastocyanin, a photosynthetic protein in the electron transfer chain in cyanobacteria [17], diatoms [18] and green algae [19]. The range of concentrations where copper becomes toxic depends on the organism. It has been found to compete with manganese uptake in *T. pseudonana* [20]. In culture samples the uptake of silicic acid by *T. pseudonana* is also inhibited by the combined metal availabilities rather than individual availabilities [21]; as copper is thought to compete for the active site of the enzyme that catalyses silica uptake.

1.2.1  Speciation

The speciation of copper in the ocean is of particular interest because it is strongly linked with the primary production. Single celled organisms can suffer from copper toxicity in some areas of the ocean, even when copper is at nanomolar concentrations which are typical for the open ocean [22].

Currently, two models exist that describe the bioavailability of a metal. The free ion activity model (FIAM) [23] and the biotic ligand model (BLM) [24]. Both models address toxicity from the viewpoint of the species of metal that an organism can take up. The FIAM assumes that the only species available for uptake is the free metal, and the activity of the free metals quantifies it. The BLM attempts to address the fact that organisms can also utilise metals that are bound to some ligands or to the cell membrane. In short, it is not the total dissolved copper concentration that affects the level of toxicity but the bioavailable concentration of copper. Typically the negative log of the concentration of the free copper (hydrated copper ion) is used to quantify the bioavailable portion. Copper bound to the natural ligands discussed in this dissertation are not considered to be bioavailable, similarly to copper–EDTA complexes [25]. In contrast, other ligands such as dithiocar-
1.2. Biological role of copper

Bamate and 8-hydroxyquinoline, have been found to be bioavailable for a coastal diatom [26]. Therefore FIA will be assumed for this thesis, in agreement with previous copper investigations showing that the free copper ion activity determines copper toxicity in phytoplankton [25].

The dominant form of inorganic copper is CuCO$_3$ [27]. However, in the ocean at least 99.7 percent copper is organically bound [22, 28]. Coale and Bruland [3] reported that the negative log of the activity of copper in the surface waters of the North Pacific is 13–14, while in deeper waters it can be closer to 10–11. In deeper waters the activity of copper is significantly higher (where there is less organic matter). High cupric activities can be brought to the surface during upwelling events which are potentially toxic to some organisms if they reach a pCu of 11 or less.

The very low activity of free copper is related to biologically productive areas, where it is hypothesised that phytoplankton may produce strong copper binding ligands in response to toxic levels of copper. In addition, dissolved organic matter (DOM) originated from the remineralisation of detritus (terrestrial or marine) may also bind copper. However, it is unlikely that the speciation of copper is dominated by these non-specific copper complexes. While the concentration of DOM may be greater than the hypothesised copper binding ligands, the conditional stability constants of some copper-complexes are incredibly high and cannot be explained by generic DOM. Strong copper binding ligands with large conditional binding constants ranging from a log $K_c$ of 9–13 occur only in the surface waters (to around 200 m depth) and are referred to as L1 [3, 22]. Weaker binding ligands, (L2), can have a log $K_c$ of < 9 and occur in the water column from the surface to below the euphotic zone [3]. These two classes are referred to as L1 and L2; decreasing in strength respectively.

These stronger binding molecules tend to only occur in surface waters and over a wide range of copper concentrations, vary in (ligand) concentrations of between 1 and 100 nM [29, 30], where total dissolved copper concentrations range from 0.6 nM to 51 nM, respectively. Absolute values depend on the sample site, although in general, above 200 m, the concentration of L1 is greater than the total dissolved copper concentration [3]. Conditional binding constants range from a log $K_c$ of 8 to 14 [30, 31]. The results suggest that a number of molecules with different binding constants are responsible for the magnitudes of difference in the $K_c$ values.
1.3 Copper complex binding constants

Several studies have shown the production of strong copper binding ligands by laboratory cultures. Strong copper binding ligands have been detected in culture experiments involving some photosynthetic prokaryotes (cyanobacteria [9, 22]) also some eukaryotes including the marine coccolithophorid *Emiliana huxleyi* [22], dinoflagellates and diatoms [4, 9]. Croot et al. [4] conducted experiments to compare copper binding ligand production in several taxa of eukaryotes and prokaryotes [9] and found that laboratory cultures produced ligands with ionic strength corrected binding constants ranging from log $K'$ 16.8 to 36.7.

Comparing binding constants among studies can be difficult as results are operationally defined. Most determinations are made using electrochemistry but the approach taken can differ: anodic stripping voltammetry (ASV), cathodic stripping voltammetry (CSV) or *pseudo*-polarography can all be employed. ASV analyses for the inorganic fraction of copper and determines $K^c$. This can be difficult as the inorganic fraction of copper is determined directly and is often below the limit of detection [32] when samples are natural and contain strong ligands. Conditional stability constants are frequently determined using competitive ligand equilibration cathodic stripping voltammetry (CLE-CSV) as the signal measured is from the copper that is complexed to the added ligand. This signal is more sensitive as it shifts the copper half wave potential away from a region where there is a high baseline due to mercury. This results in a lower limit of detection and makes speciation measurements easier to accomplish [32]. CLE-CSV is also used to determine $K^c$ whereas *pseudo*-polarography determinations have so far been used to determine ionic strength corrected stability constants.

A representative selection of binding constants determined for strong copper complexes in culture and field samples is shown in Tables 1.2 and 1.3. The variation implies that several ligands are present in the strong ligand class (L1). Tables 1.2 and 1.3 show that almost all of the determinations use electrochemistry. Culture samples contain a higher organic content than field samples and so the use of CLE-CSV is less frequent.

These tables demonstrate that the strong binding copper ligands are well documented in terms of presence and binding strength. However, work investigating
1.3. Copper complex binding constants

the structure, and in particular the site directly surrounding the copper atom itself, is presently lacking. In comparison to the conditional binding constant of copper to EDTA, with a log $K^c$ of 10.06 [10], these ligands are clearly strong (see also Table 1.1).
<table>
<thead>
<tr>
<th>Region</th>
<th>Depth (m)</th>
<th>[Ligand] (nM)</th>
<th>log K</th>
<th>Technique</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Atlantic</td>
<td>300</td>
<td>5–13</td>
<td>12.2–13.1</td>
<td>CLE-CSV</td>
<td>Campos and van den Berg [10]</td>
</tr>
<tr>
<td>N. Pacific</td>
<td>200</td>
<td>2</td>
<td>11.6</td>
<td>ASV</td>
<td>Coale and Bruland [29]</td>
</tr>
<tr>
<td>Sargasso Sea</td>
<td>0–140</td>
<td>2</td>
<td>13.2</td>
<td>LE-LL</td>
<td>Moffett et al. [33]</td>
</tr>
<tr>
<td>Eq. Pacific</td>
<td>0–120</td>
<td>1</td>
<td>8.9–9.2</td>
<td>IMAC</td>
<td>Midorikawa and Tanoue [31]</td>
</tr>
<tr>
<td>Central Pacific</td>
<td>1</td>
<td>4</td>
<td>12.3–15</td>
<td>CLE-SE</td>
<td>Miller and Bruland [30]</td>
</tr>
<tr>
<td>Indian Ocean</td>
<td>50</td>
<td>4.1</td>
<td>12.6</td>
<td>CLE-CSV</td>
<td>Donat and van den Berg [34]</td>
</tr>
<tr>
<td>North Sea</td>
<td>32</td>
<td>16.2</td>
<td>12.4</td>
<td>CLE-CSV</td>
<td>Donat and van den Berg [34]</td>
</tr>
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<td>Tyrrhenian Sea</td>
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<td>13.1</td>
<td>CLE-ASV</td>
<td>Scarano et al. [35]</td>
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Table 1.2: The recent studies showing the conditional copper binding constants of ligands found in the field. CLE is competitive ligand equilibration; CSV is cathodic stripping voltammetry; ASV is anodic stripping voltammetry; LE-LL is ligand exchange liquid-liquid partition; IMAC is immobilised ion metal affinity chromatography, in this case titration with copper using an ion selective electrode was used to determine K; SE is solvent extraction.
<table>
<thead>
<tr>
<th>Plankton class</th>
<th>Species</th>
<th>[Ligand]</th>
<th>log K</th>
<th>Technique</th>
<th>Reference</th>
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<tr>
<td>Cyanobacteria</td>
<td><em>Synechococcus</em> DC2</td>
<td>40</td>
<td>36.7†</td>
<td>ASV-PP</td>
<td>Croot et al. [9]</td>
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<td></td>
<td><em>Synechococcus</em> DC2</td>
<td>15-110</td>
<td>12.3-13.3</td>
<td>CLE-CSV</td>
<td>Moffett and Brand [36]</td>
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<td><em>Synechococcus</em> PCC7002</td>
<td>139</td>
<td>12.2</td>
<td>CLE-CSV</td>
<td>Lawrence [37]</td>
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<tr>
<td>Heterotrophic bacteria</td>
<td><em>Vibrio alginolyticus</em></td>
<td>31</td>
<td>11</td>
<td>ASV</td>
<td>Gordon et al. [38]</td>
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<td>Dinoflagellate</td>
<td><em>Amphidinium carterae</em></td>
<td>75</td>
<td>23.7-39.1†</td>
<td>ASV-PP</td>
<td>Croot et al. [9]</td>
</tr>
<tr>
<td></td>
<td><em>Prorocentrum micans</em></td>
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<td>ASV-PP</td>
<td>Croot et al. [4]</td>
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<td>Coccolithophore</td>
<td><em>Hymemomonas carterae</em></td>
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<td><em>Emiliania huxleyi</em></td>
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<td>Leal et al. [39]</td>
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<td>Diatoms</td>
<td><em>Skeletonema costatum</em></td>
<td>47</td>
<td>22.6†</td>
<td>ASV-PP</td>
<td>Croot et al. [9]</td>
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</tbody>
</table>

Table 1.3: The recent studies showing the conditional copper binding constants of ligands found in culture experiments. †These constants are ionic strength-corrected thermodynamic stability constants rather than conditional constants. CLE is competitive ligand equilibration; CSV is cathodic stripping voltammetry; ASV is anodic stripping voltammetry; PP is pseudo-polarography.
1.4 Structural characterisation

Speciation and structural work have presented significant analytical challenges in the past decades (see below Section 1.4.1). However, with the rapid development of more powerful mass spectrometers, speciation measurements are increasingly possible. Typically, for exact mass measurements, a time of flight, (TOF) instrument is used. Exact mass measurements for fragments of ions can be determined using a quadrupole MS coupled with a TOF mass analyser. Fragment ions typically have lower abundance than the parent ion. In such a case, a fourier transform ion cyclotron resonance mass spectrometer FT ICR MS can be employed.

There are a few considerations to be made before reaching conclusions about chemical speciation based on mass spectrometry as each step in sample preparation risks the perturbation of the thermodynamic equilibrium and thus conclusions made based on the experimental data. In order to minimise changes in chemical conditions, one must consider (i) the sample preparation prior to analysis and (ii) the nature of ionisation as the analyte enters the mass spectrometer itself.

1.4.1 Analyte extraction from the seawater matrix

In the case of seawater, analyte extraction is vital as the salt content is so great that it causes unmanageable interference as well as the likelihood of clogging tubing and destroying the sample introduction equipment. Copper binding complexes are naturally present at very low concentrations and so a concentration step prior to analysis is necessary.

If the analyte is detected using mass spectrometry, ionisation of the analyte must be performed using a soft technique to avoid the fragmentation of the molecular ion. Two soft ionization methods are Matrix Assisted Laser Desorption Ionisation (MALDI) and Electrospray Ionisation (ESI). Sample preparation for either of these ionisation sources requires the removal of the analyte from the seawater matrix, and a necessary concentration step to increase signal-to-noise ratios.

Past attempts at isolating a copper complex from seawater have used several different methods of matrix removal. Donat et al. [40] used C-18 columns to isolate hydrophobic organic matter. However, it was found that the extracted complexes were not particularly strong binding, implying that strong copper binding ligands
1.4. Structural characterisation

may have hydrophilic functionalities.

Midorikawa and Tanoue [31] used an ion immobilised metal affinity column (IMAC) to remove copper binding ligands from seawater, and then conducted experiments on the eluents to determine conditional stability constants, complexing capacity and the chemical nature of the extracted ligand (tests for primary amines and carbohydrates). However, titration of these analytes showed that the conditional constants were lower than those expected for the L1 ligand class as found by electrochemical determinations (see Tables 1.2 and 1.3). There are possible explanations as to the discrepancy in binding constants from this method versus electrochemical methods. It is possible that the IMAC column does not retain ligands that are already bound to copper prior to the column extraction. In the ocean, strong ligands are usually present at only slightly higher concentrations than copper concentrations [29] and so the concentration of free (strong copper binding) ligand would be low. Furthermore, the limit of detection may have been an issue in this study. Although, Ross et al. [41] tested the ability of IMAC to extract strong copper complexes. The samples in their study were field samples, making it difficult to distinguish between copper specific complexes and other generic organic complexes. The ability of IMAC to remove copper binding complexes has also been investigated using model ligands by Paunovic et al. [42]. Their findings suggest that ligands with a carboxylic, an amine-carboxylic and non-aromatic primary, secondary and tertiary amine functionalities were not retained. Interestingly, they showed that, in order to retain copper complexes on the column, it was necessary for the IMAC column to form ternary compounds with binding copper complexes. However, the strongest binding ligands in this study did not match the copper binding constants of naturally occurring copper binding ligands by several orders of magnitude. IMAC clearly requires specific functionality in order to accomplish ligand extraction from seawater.

Alternatively, Dupont et al. [43] focused on previous findings [39, 41, 44], suggesting that strong copper binding ligands are thiols. On this basis, the thiol functionality can be derivatised specifically using a fluorescent reagent [45]. Different thiols are then separated using HPLC, and directly infused into an electrospray ion source for analysis using an ion trap mass spectrometer. In this way thiol containing compounds were identified from culture samples exposed to high copper.
1.4. Structural characterisation

Dupont et al. also detected copper containing complexes that were extracted using size exclusion chromatography and HPLC, whereby the analyte is retained by size exclusion and the interfering seawater matrix is lost. Fractions were analysed using GFAAS for copper determinations and MALDI TOF for copper containing thiol identification and quantification. Copper containing thiols were detected in copper-toxic treated cultures but not in control cultures. However, analysis using CLE-CVS showed that these copper-thiol complexes were either weaker chelators or undetectable using the method. This implies that either the extraction method did not concentrate the analyte enough, or these complexes are not the strong copper binding ligands.

1.4.2 Chemical properties of strong copper binding ligands

The strong ligand class is poorly characterised. It is a challenge to extract an analyte from a matrix without knowing the chemical property with which to isolate, measure or identify it. Consequently, there is relatively little information about the chemical properties of L1.

The IMAC extracted copper binding ligands by Midorikawa and Tanoue [31] were in the form of the metal bound species. In order to determine copper binding strength the analyte was demetallated using electrodialysis and then titrated to determine binding strength. As a result of the dialysis, ligands that were less than 1000 Da were lost (1 Da is 1 atomic mass unit). Chemical tests on the resulting demetallated samples were for amino acid and carbohydrate content and fluorescence. The shallower water column samples were found to be non-fluorescent and comprise of di- or tri-peptides with some carbohydrate functionality. These amino acid and carbohydrate functionalities were found to decline with depth implying that these chemicals are easily recycled and/or labile. The fact that they are removed with depth is indicative of strong binding ligands, although this does not provide hard evidence. The binding constants of these ligands were also determined to be lower than those previously determined by other methods (see Tables 1.2 and 1.3).

The combination of IMAC with mass spectrometry was employed by Vachet and Callaway [44] who used high performance size exclusion chromatography (HPSEC) and by Ross et al. [41] using solid phase extraction with an XAD-16 column. Va-
1.5 Summary and thesis overview

Chet and Callaway [44] differentiated weaker ligands from stronger ligands based on the retention times on the IMAC columns. The ‘stronger’ ligands were found to be of lower molecular weights and contained oxygen, and nitrogen, as well as sulphur functional groups. Furthermore, the number of donor atoms was concluded to imply the binding of copper in the (II) oxidation state. Similarly, Ross et al. [41] also found lower molecular weight compounds that contained nitrogen, oxygen and sulphur atoms that could possibly be dipeptides.

From this past work it can be seen that the nature of analyte extraction clearly and inevitably biases the analyte that is detected. The strong copper binding ligands are more difficult to isolate (than generic weaker ligands). Copper binding ligands that are detected have been found to have O, N and S functionality and stronger ligands are thought to be lower in weight. Some work has indicated that the strong copper binding ligands are binding copper (I) [12, 13, 43], whereas others have taken the copper centre to be in the (II) oxidation state [44].

1.5 Summary and thesis overview

Past work using electrochemistry has shown that strong copper binding ligands are present in the ocean. We also know that cultured samples can produce strong copper binding ligands when they are grown at high free copper concentrations.

We are interested in strong copper binding ligands for several reasons. These ligands are so strong that there is no known artificial analogue matching their copper binding strengths. Our understanding of environmental relationships between trace metals and aquatic microorganisms is currently limited by analytical challenges in characterising organic-metal complexes and determining trace metal bioavailability to primary producers. If we are unable to determine their chemical nature then we will not be able to make sound conclusions about the chemical-biological interactions at the cellular level, nor in bulk seawater. Currently our knowledge is limited to the idea of the existence of suites of ligands, collectively determined using voltammetry. In essence, little is known about the chemical nature of these ligands.

The investigation of the chemical properties of strong copper binding ligands is difficult because the analyte occurs in a seawater matrix with high concentrations
1.5. Summary and thesis overview

<table>
<thead>
<tr>
<th>Date</th>
<th>Expt label</th>
<th>Cu treatment (pCu)</th>
</tr>
</thead>
<tbody>
<tr>
<td>April 2003</td>
<td>002</td>
<td>10.3</td>
</tr>
<tr>
<td>October 2003</td>
<td>003</td>
<td>10.3</td>
</tr>
<tr>
<td>October 2003</td>
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<td>12.3</td>
</tr>
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</tr>
<tr>
<td>September 2004</td>
<td>006</td>
<td>12.3</td>
</tr>
</tbody>
</table>

Table 1.4: Grow up experiments of *Synechococcus* DC2 acclimatised to different free copper activities.

of ions that must be removed if structural determinations are to be made. Isolation of the analyte from seawater is difficult because extraction can be selective (such as IMAC) and we do not know the chemical properties of the analyte in order to separate it from interfering compounds. The low concentrations of the analyte present further challenges. The detection limits of mass spectrometers are continually being improved. However, the detection limit combined with the necessary soft ionisation techniques still makes the analyte difficult to identify when using mass spectrometry. Once copper complexes have been identified, additional analysis (such as MS/MS) has been necessary to further identify the complex [41, 44].

The work described here provides one approach that attempts to avoid operationally defined determinations as much as possible. Given the complex nature of trace organics, and the ability of generic organic matter to bind copper, cultured samples are used to simplify the organic component. Pseudo-polarograms are used to identify strong copper binding ligands in these culture samples and to track them during isolation from the seawater matrix and recovery into methanol and water solvent mixtures. Mass spectrometry is then used to identify copper containing ligands and then to gain information regarding their chemical nature. In total, five culture experiments were conducted, with cultures exposed to two different copper treatments as summarised in Table 1.4. These five samples are then followed throughout electrochemical and mass spectrometric analyses in an attempt to grow, isolate and further identify strong copper binding ligands as well as possible. These culture experiments are described in Chapter 2. In Chapter 3 the use of electrochemistry as a tracking device for strong copper binding ligands is described. Electrochemical techniques are used to detect and qualify any ligands. In Chapter 4 the same
1.5. Summary and thesis overview

electrochemical approach is used to assess absence-presence when isolating the analyte from seawater. Chapter 4 also describes the use of soft ionisation techniques. MALDI and ESI are coupled to different combinations of mass analysers depending on the sensitivity and resolution required. MALDI is used to identify complexes that may contain copper based on expected isotopic ratios and is then used to characterise more fully the chemical nature of the copper complex. ESI is also used to help confirm the in-solution speciation of the complex.
Chapter 2

Cultures

The purpose of the work presented here is to produce seawater samples that contain copper binding ligands exuded by cyanobacteria. The intent is that these samples have a higher concentration of copper binding ligands than found in the open ocean. This chapter describes the nature in which these samples were produced.

Culture experiments showed that the cyanobacteria *Synechococcus* DC2, acclimated to toxic copper concentrations, produced copper binding ligands as a detoxification mechanism during log phase growth. This culture based ligand production was to allow easier detection with mass spectrometry and electrochemistry.

2.1 *Synechococcus* DC2

2.1.1 Choice of species

The genus *Synechococcus* is well known for its ability to produce metal binding ligands. It synthesises siderophores, the high affinity iron binding ligands excreted by many bacteria to aid iron uptake in low iron conditions [46]. *Synechococcus* has also been found to produce cobalt binding ligands that aid in cobalt uptake, in the Costa Rica upwelling zone [47]. Most importantly for this work, *Synechococcus*, both coastal and oceanic, are known producers of copper binding ligands, exuded in response to copper toxicity [9, 37]. Cyanobacteria are relatively simple to culture and monitor, as they are autotrophic and fluoresce, enabling quick measurement and easy daily monitoring.

The chosen species is the red clone DC2; an open ocean species originally isolated from the North Atlantic Sargasso Sea collected from 25 m by L. Brand [4] but obtained from Amy Chan (Suttle Laboratory, University of British Columbia). This cyanobacterium strain has been shown to produce copper binding ligands in response to copper toxicity [9].
2.1. Synechococcus DC2

<table>
<thead>
<tr>
<th>Metal nutrient</th>
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</tr>
</thead>
<tbody>
<tr>
<td>Iron</td>
<td>8410</td>
<td>10</td>
</tr>
<tr>
<td>Manganese</td>
<td>120</td>
<td>23</td>
</tr>
<tr>
<td>Zinc</td>
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<td>8</td>
</tr>
<tr>
<td>Copper</td>
<td>10</td>
<td>1</td>
</tr>
<tr>
<td>Cobalt</td>
<td>50</td>
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</tr>
<tr>
<td>Molybdenum</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>Selenium</td>
<td>10</td>
<td>10</td>
</tr>
</tbody>
</table>

Table 2.1: Metal concentrations added to media with [48] and without [49] the presence of EDTA (†EDTA present at 1.0x10⁻⁴ M.)

In these experiments the cultures were not considered to be axenic (a pure culture). Thus, heterotrophic bacteria were monitored throughout two of five experiments to ensure their cell densities were negligible relative to those of the cyanobacteria.

2.1.2 Culture media

The experimental nature of this work requires that the concentrations of metals, and copper in particular, are stringently controlled. Also the introduction of metal chelators is omitted to avoid interference with natural ligand determinations. Typically the media used for trace metal culture experiments is Aquil [48]. This is a synthetic seawater made with major ions, macronutrients and micronutrients (including copper). Two types of media were used in this study: one where metal concentrations are controlled using EDTA as a buffer and one where metal concentrations are lower and no EDTA is added. Metal concentrations for both kinds of media (presence or absence of EDTA) are in Table 2.1.

The total dissolved copper concentrations of the media were determined using isotope dilution mass spectrometry (IDMS described in Section 2.2.3) and based on these values, free ion concentration (pCu) was calculated using MINEQL+ (see next section). IDMS is a powerful analytical method that allows total copper determinations regardless of complete column recovery and irrespective of copper speciation,
2.2. Experimental methods

provided that the enriched spike and natural copper are equilibrated.

MINEQL+ Ver 3.01b

For the purposes of free metal ion concentration calculations, the free ion activity model (FIAM) has been assumed because there is not enough information to determine whether the organic matter present in the culture is partially or completely bioavailable, or unavailable. Also copper toxicity has been shown to be a function of the free ion concentration \[50\] indicating that the FIAM is appropriate. Therefore, the toxicity of copper is related to the free ion concentration: \( p_{\text{Cu}} \).

\[
p_{\text{Cu}} = -\log [\text{Cu}^{2+}]
\] (2.1)

The \( p_{\text{Cu}} \) of any given medium has been calculated using the speciation software MINEQL+ \[51\]. The program uses the input concentrations of all ions present in the media and calculates the quantity of free copper remaining after ionic strength and side reactions with major ions have been accounted for. Organic ligands are accounted for in the same way as inorganic counter ions. MINEQL+ takes these two underlying premises and iteratively solves for the free ion concentration.

2.2 Experimental methods

2.2.1 Culturing protocol

Aquil media is made using the guidelines published by Price et al. \[48\]. Briefly, the major ions are dissolved in distilled deionised water and then cleaned for trace impurities of transition metal doubly charged ions using Chelex-100® beads (Bio-rad). Macronutrients were also cleaned using Chelex-100 beads. The seawater run through the chelex beads was sterilised using a standard microwave protocol \[52\]. Macro and micro nutrients and vitamins were filter sterilised using a 0.22\( \mu \)m swinex filter, and added to the sterile cool media. The media was left overnight to equilibrate and then used as needed.

*Synechococcus* DC2 are used to produce higher concentrations of ligand in batch cultures than in the open ocean. The advantages of using lab cultures include
2.2. Experimental methods

(i) having a well defined system, and (ii) the ability to enhance phytoplanktonic ligand production.

There are a number of approaches to culturing, only some of which will be addressed here. There are two main methods of culturing: batch cultures and continuous cultures.

Continuous cultures are maintained using a chemostat where culture medium is continually renewed and environmental parameters remain constant over a long period of time. A continuous culture can be grown and sub-sampled for chemical analysis over time, while the culture is in log phase growth. In this way the increase in concentration of ligand production, concentration of copper and health of culture can be monitored in parallel. This technique has helped to show the increase in ligand concentration with increase in copper concentration [37] using *Synechococcus* PC700, a cyanobacterium. This has shown that ligand production of strong copper binding ligands is linked to copper concentration, cyanobacteria and toxicity.

Batch cultures can also be used for a similar means, and have been applied here in two ways. Firstly, semi-continuous batch cultures were maintained in order to acclimate the cyanobacteria to toxic copper conditions. Semi-continuous cultures are kept in log phase growth continually by allowing them to grow and then diluting them down with fresh media before they reach senescence. Secondly, batch cultures are used during the ligand production experiments, where the cells only go through one growth cycle. This experimental protocol has been applied to look at the production of dimethyl sulphide (DMS) [53] as well as the production of copper ligands by *Emiliana huxleyi* [43].

The protocol used for ligand production experiments is a batch method adapted from Maldonado et al. [53]. A schematic of the entire procedure can be viewed in Figure 2.1. This culturing protocol was chosen specifically to grow a large biomass under easily controlled conditions; the large biomass producing a large amount of ligand.

Cyanobacteria are known to be sensitive to metal concentrations. As a result, the experimental protocol for growing *Synechococcus* DC2 must have well controlled media in terms of trace metals. Usually, media contains large concentrations of EDTA (sub-mM) that acts as a buffer for metal concentrations and is more forgiving of trace metal contamination. However, the experimental means of determining
2.2. Experimental methods

ligand binding strength, concentration and ultimately structure require the absence of such artificial ligands, which may interfere with detection of natural ligands. Thus, cultures are first acclimated at a well defined copper concentration using EDTA buffered media. Then, during log phase growth the cells are harvested and resuspended in new EDTA free media. The short time exposure of the cultures to the 2 L EDTA free media ensures that the ligand response of the cyanobacteria to the copper levels remains identical to that of the EDTA–controlled 4 L batch media. The two 2 L media batches were set to different copper concentrations. One with zero copper added (and a background of no more than 4.4 nM copper) called ‘C’ for control and one with copper added to gain a pCu 10 called ‘T’ for treatment. In hindsight the ability of the cultures to adjust to the EDTA-free media with different copper concentrations is minimal given that they are incubated in this media for 20 hours only. Thus, for future work, setting different copper levels during the 20 hour resuspension is not necessary.

Five growth experiments were run, three at a high copper concentration, pCu 10, and two at a lower copper concentration, pCu 12. The copper treatments were chosen to represent a toxic (pCu 10) and a non-stressful (pCu 12) condition according to Brand et al. [50]. Copper concentrations were determined using isotope dilution mass spectrometry, addressed in Section 2.2.3.
Chelexed artificial seawater, with EDTA as a metal buffer.

**Culture medium grown to large biomass.**

Harvest at mid-exponential growth; suction filtration.

**Filter**

Inoculum: re-suspension of particulates

In sterile artificial seawater with major ions only. Rinse three times before recombination.

Pipette inoculum into replicate seawater medium.

Chelexed artificial seawater with trace metals but no EDTA.

Collect filtrate Freeze in aliquots immediately.

Filter simultaneously

**Filter**

Incubate for 20 hours.

**Figure 2.1:** Flow chart showing culture experimental protocol used to remove interfering metal buffer (EDTA)
2.2. Experimental methods

All culture samples were kept in an incubator at 18°C, under continuous fluorescent light at an intensity of 25 μEinstiens. Acclimated culture samples in semi-continuous batch cultures were inoculated during log phase growth into the 4 L media containers shown in Figure 2.1. Grow-up experiments were sub-sampled every day to monitor growth and run until a large biomass of exponentially growing cells accumulated. The grow-ups were then filtered using acid washed, sterilised 0.22 μm polycarbonate filters. Filtration of the 4 L culture required 7–8 filters. The collected cells were rinsed on the filters with sterile artificial seawater (run over chelax to remove trace metals) that contained no nutrients. The cells were then re-suspended into a small volume of this media until all were filtered and ready for the second inoculation. This concentrated sample was then inoculated into two 2 L batches of chelator free media and incubated under the same growth conditions for 20 hours. After this time the two batches were filtered simultaneously and the filtrate was kept frozen until later analysis.

2.2.2 Growth monitoring

It is important to demonstrate that the cyanobacterial cells are actively producing ligands during log phase growth as opposed to indirect ligand release during cell lysis. Daily measurements to monitor growth were taken by sub-sampling in a sterile laminar flow hood. Samples were then acclimated in the dark for at least 15 minutes before fluorescence was measured using a Turner Designs model 10-AU fluorometer.

In addition, sub-samples for cell counts were taken from experiments 5 and 6. These samples were fixed with glutaraldehyde (a final concentration of 1% v/v) and flashfrozen for later analysis. Heterotrophic bacteria populations were also monitored in these samples. Cell counts were determined using epifluorescence microscopy (Amy Chan, Suttle laboratory, University of British Columbia). Samples originally stored at −80°C were thawed to room temperature, contents mixed by vortexing. To count the heterotrophic bacteria, each sample (2 mL) was stained with DAPI (1.5 μg/mL final concentration) and left for 15 to 20 minutes. Stained samples were filtered onto 0.22 μm black PCTE filters after the 20 minute period to allow time for the DAPI to bind DNA. Cyanobacteria and heterotrophic bacteria
2.2. Experimental methods

were counted in the same portions of filter under UV light [54].

2.2.3 Total copper determinations

To determine total copper concentrations in the culture media, the seawater copper blanks (prior to copper addition) were measured. The total copper concentration was then calculated from the blank plus the amount of copper that was added (prior to inoculation). In order to determine the blank copper concentration, seawater was sampled from each of the experiments and acidified to a pH < 2 using nitric acid (Seastar grade, 0.012 M). The isotopic spiking concentration (an enriched copper standard containing 99.7% $^{65}$Cu) was chosen in an iterative manner as it was based on an estimate of the copper blank from previous isotope dilution experiments. The concentration of the isotopically diluted solution of the enriched copper plus the natural copper present resulted in a 65:63 ratio of 12. This optimum ratio is calculated according to Heumann [55], Equation 2.2. Analysis at the optimum ratio reduces the propagation of error when calculating the final concentration using the isotope dilution equation.

$$R_{opt} = \sqrt{\left(\frac{h^2}{h^1}\right)_{sample} \times \left(\frac{h^2}{h^1}\right)_{spike}} \quad (2.2)$$

Where $h^2$ is the abundance of $^{65}$Cu and $h^1$ is the abundance of $^{63}$Cu. To ensure that the enriched isotope is in complete equilibration with the copper in the samples, the acidified, spiked samples were then heated to 60°C for at least 8 hours. After this time all samples were cooled and run on an XAD resin modified to chelate doubly charged ions using 8-hydroxyquinoline. The resin and the procedure for its use was made by Sabrina Crispo (graduate student, Orians Laboratory, University of British Columbia). The procedure is found in Section A.1.

Eluent from the columns have a harsh matrix (2 M HCl, 0.5 M HNO$_3$) thus the matrix was changed to 1% HNO$_3$ by evaporating to dryness on a hot plate at ca. 54°C for 8 hours. The samples were then refluxed for 40 minutes with concentrated nitric acid (500 µL) to help remove as much chloride ion as possible. The concentrated nitric acid was removed by evaporation on a hot plate (54°C) for 3 hours. Samples were then redissolved into 1 mL of 1% HNO$_3$, a matrix more
2.3 Results

suitable for ICPMS. To ensure complete dissolution, these closed vials were heated (54°C) for 2 hours.

Samples were run on a Thermo Finnigan ELEMENT2 High Resolution Inductively Coupled Plasma Mass Spectrometer (HR ICP MS). Samples were analysed under medium resolution to separate copper ions 65 and 63 from the polyatomic interferences MgAr + and NaAr + respectively. These polyatomic interferences are particularly significant when analysing samples of seawater origin.

The copper concentrations were determined using isotope dilution:

\[ N_s = \frac{N_{sp}(h^2_{sp} - Rh^1_{sp})}{Rh^1_s - h^2_s} \]

(2.3)

where \( N_s \) and \( N_{sp} \) are the number of copper atoms in the sample and spike, respectively; \( h^2 \) and \( h^1 \) are the abundances of \( ^{65}\text{Cu} \) and \( ^{63}\text{Cu} \), respectively, and the suffix \( sp \) and \( s \) refer to the spike solution and the sample solution, respectively. \( R \) is the ratio of the signal of \( ^{65}\text{Cu} \) to \( ^{63}\text{Cu} \).

Reverse isotope dilution (RID) is routinely carried out on the same day prior to sample analysis. This is to accurately determine the concentration of the enriched spike, and conveniently accounts for mass bias between the two isotopes of copper. Since mass bias is accounted for by the RID a factor to account for it has been omitted from the Equation 2.3. In order to test the column method combined with IDMS a solution of 1% HNO₃ was spiked with 8.48 nM copper standard and run with the other samples.

2.3 Results

2.3.1 Growth monitoring

Log phase growth (the phase of growth when cells are doubling exponentially) can be established using fluorescence or cell numbers. Samples for cell numbers, were taken for experiments 5 and 6. Figure 2.2 demonstrates a tight relationship between fluorescence and cell counts. The slopes for the individual experiments are not statistically different (two-tailed t-test, \( P = 0.05 \)) and so the same linear relationship was used to estimate cell density in experiments 3 and 4.
2.3. Results

Figure 2.2: Calibration plot for experiments 5 and 6 relating fluorescence to cell counts. Linear regression with equation $y = 1.33 \times 10^{-7}X + 3.14^{-3}$. 

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2.3. Results

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Growth rate (d⁻¹)</th>
<th>Intercept</th>
<th>( R^2 )</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td>0.25</td>
<td>-2.80</td>
<td>0.96</td>
</tr>
<tr>
<td>3</td>
<td>0.21</td>
<td>-4.05</td>
<td>0.97</td>
</tr>
<tr>
<td>4</td>
<td>0.21</td>
<td>-4.07</td>
<td>0.98</td>
</tr>
<tr>
<td>5</td>
<td>0.18</td>
<td>-3.56</td>
<td>0.97</td>
</tr>
<tr>
<td>6</td>
<td>0.19</td>
<td>-4.22</td>
<td>0.99</td>
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</table>

Table 2.2: The regression line data for the log plots of fluorescence Figure 2.4 for culture growth monitoring. The growth rate is the slope of the linear regression.

Figure 2.3 displays the daily change in fluorescence used as a proxy for growth in all our experiments. Figures 2.3 and 2.4 show that the rate of growth for experiments three to six were approximately the same. This implies that the difference in copper activity from pCu 12 to 10 has little effect on the growth rates. This indicates that the range of copper concentrations chosen elicit physiological changes to cope with high levels of copper before growth rate reductions were observed. Interestingly, experiment 2 has a shorter lag time compared with the other experiments. Close examination of this experiment revealed that the cell density of its inoculum was higher than that in other experiments. The fluorescence of the inoculum for experiment 2 was 10.9 compared with a range of 6.7–7.9 for the other experiments. This is also reflected in the intercepts of the linear regressions (Table 2.2) of the data in Figure 2.4. High inoculum cell density may result in fast conditioning of the medium and consequently a shorter lag phase.

Each 4 L culture was divided into two containers of two litres, whose growth was also monitored using fluorescence during the 20 hour incubation period. These data points are also on the growth curves (open data points) of the 4 L fluorescence data and lie close to their respective regression line. This shows that the cells in the 4 L have not been affected by filtration and resuspension, and that the cyanobacteria did not change their rate of growth between the two types of media (EDTA based and EDTA free media).
Figure 2.3: Graph showing culture experiment growth curves using fluorescence as a proxy for growth. Hollow data points are before and after the incubation period.
2.3. Results

Figure 2.4: The natural log of fluorescence for all experiments. Hollow data points are before and after the incubation period. Experiments 3–6 have growth rates of 0.17–0.21 d$^{-1}$ while experiment 2 has a rate of 0.25 d$^{-1}$. Regression line equations are in Table 2.2.
2.3. Results

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Fluorescence</th>
<th>Cells per mL</th>
<th>Percent uncertainty†</th>
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<tr>
<td>2C</td>
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<td>N/A</td>
<td></td>
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<tr>
<td>3C</td>
<td>2.15</td>
<td>1.43E+7</td>
<td>16.0</td>
</tr>
<tr>
<td>3T</td>
<td>2.26</td>
<td>1.51E+7</td>
<td>15.3</td>
</tr>
<tr>
<td>4C</td>
<td>2.76</td>
<td>1.89E+7</td>
<td>12.7</td>
</tr>
<tr>
<td>4T</td>
<td>2.62</td>
<td>1.78E+7</td>
<td>13.3</td>
</tr>
<tr>
<td>5C</td>
<td>2.25</td>
<td>1.69E+7</td>
<td>11.4 §</td>
</tr>
<tr>
<td>5T</td>
<td>2.31</td>
<td>1.74E+7</td>
<td>12.9 §</td>
</tr>
<tr>
<td>6C</td>
<td>2.07</td>
<td>1.53E+7</td>
<td>12.4 §</td>
</tr>
<tr>
<td>6T</td>
<td>2.03</td>
<td>1.34E+7</td>
<td>17.0</td>
</tr>
</tbody>
</table>

Table 2.3: Cyanobacterial cell concentrations at the end of each incubation period. †Percent error is the 95% confidence limit based on 3 degrees of freedom for a two-tailed test. §Based on the 95% confidence limit of the mean.

2.3.2 Heterotrophic bacteria determinations in the cyanobacteria cultures

The growth of the heterotrophic bacteria was also monitored. Heterotrophic bacterial populations were never greater than 7 percent of the (ligand producing) cyanobacterial population, and so were not a dominant population at any time during 20 hour period. Data for the heterotrophic bacteria compared to cyanobacteria are shown in Figure A.1. Cyanobacterial population data for these experiments is shown in Table 2.3. Samples 5C, 5T and 6C were counted directly and so the error on these values is based on the 95% confidence limit of the mean. The other samples were interpolated from the line of best fit in Figure 2.2 and so the error values for these are higher and based on a two-tailed test.

2.3.3 Isotope dilution: total copper concentrations

The isotope dilution method allows for the determination of background copper contamination in our non-enriched media. A known sample of 8.48 nM Cu was run to check the method. The determined concentration was 8.7 ±0.12 nM (95% confidence limit: the ICPMS measured the counts at 63 and 65 m/z 45 times per sample). The expected value does not come within the error of the ICP MS determinations,
2.4 Conclusions

implying that there was additional error in the sample preparation. Given that the purpose of this measurement is to determine the total activity of copper in the media, this error is sufficiently low.

Since a large amount of EDTA is used in the media, and the variation of the copper blank is low (0.6-4.4 nM), the activity of copper does not significantly vary. Copper activities were calculated using MINEQL+. The resultant blank concentrations and consequent total copper concentrations can be found in Table A.1 in Section A.2.

2.4 Conclusions

In this chapter the culturing choice and method to allow well controlled copper levels have been described. A batch method combined with a media exchange method [53] is used, aiming to isolate cyanobacteria produced copper ligands.

Growth monitoring with fluorescence has been used to show that the cultures were in exponential phase throughout the 20 hour incubation time in EDTA free media. Cell count data for experiments 5 and 6 show that there is a linear relationship between fluorescence and cell concentration. The relationship is the same for both experiments and has been used to estimate cell concentrations in experiments 3 and 4. Heterotrophic bacteria were monitored in experiments 5 and 6 to ensure that the cyanobacteria were the dominant population (<7% of the cyanobacterial population, by cell number) during the 20 hour incubation period.

Five experiments were run. It was intended that experiments 2, 3 and 5 had a pCu of 10 and experiments 4 and 6 were at a pCu of 12. Each culture was acclimated to the copper activity of the experiment prior to the start of the experiment. The lag time of experiment 2 revealed that an experimental deviation may have occurred. This culture is considered to be an outlier from the rest of the experiments. Culture experiments 3–6 had the same growth rates which may be due to physiological changes in the cells to cope with high copper concentrations. Therefore, these experiments were considered to be replicates. The filtrate of the EDTA–free media was frozen until analysis.
Chapter 3

Ligand detection using electrochemistry

The methods and data discussed here show that *Synechococcus* DC2 produced at least two copper binding ligands in the grow up experiments. The construction of pseudo polarograms allows the measurement of ionic strength corrected stability constants and the estimation of ligand concentrations. It was found that a stronger ligand was produced at a lower concentration and a weaker ligand at a higher concentration. The strong ligand had a log $K'$ of 49.4 $\pm$ 1.1 and the weaker ligand 19.8 $\pm$ 5.5, if these are assumed to be Cu(II) complexes. If assumed to be a Cu(I) complex, the stronger ligand would have a log $K'$ 24.7 $\pm$ 1.1. Ligands were produced at all levels of copper that cultures were exposed to.

3.1 Electrochemical methods for determining ligand concentration and metal complex binding constants

Electroanalytical methods describe a group of techniques that are extremely useful approaches in solving ocean chemical speciation problems. They enable the study of the ‘free’ metal ion directly and indirectly and are successfully used to identify different fractions of the total element. Typically a mercury electrode is used, combined with stripping methods that enable pre-concentration to lower the detection limit. Anodic and adsorptive cathodic methods have both been used to show the presence of copper binding ligands [56] as well as study the speciation of other metals in the ocean, for example, iron [57], lead [58], zinc and cadmium [59]. This section will briefly describe these two approaches, as well as a more detailed
3.1. Electrochemical speciation methods

description of the method used.

Anodic stripping voltammetry

Anodic Stripping Voltammetry (ASV) measures the reduction/oxidation of the free metal ion. Initially the mercury electrode has a negative potential applied to it, reducing the metal analyte into the drop (or thin mercury film) and concentrating it. Then the potential is ramped in the positive direction whereby the metal oxidises back into solution creating a current that is measured. This technique is able to distinguish total dissolved metal concentration from free metal and also from complexed metal. By altering the deposition potential of the electrode the different species of metal can therefore be detected.

Deposition at small negative potentials (e.g. -0.70 V [3]) will give rise to free metal-only determinations. Titration of environmental samples with copper will first saturate any free ligands; until all ligands have been saturated, increasing copper concentration will result in no increase in copper signal. Once free ligands are saturated, further copper titration will give rise to ASV-labile copper and the signal will increase linearly with copper additions. These data can then be used to determine the concentration and binding constant of the ligands present in the sample [56, 60]. Speciation studies using these techniques have been applied to oceanic copper speciation problems over the past few decades [3, 9, 61].

Competitive ligand equilibration–cathodic stripping voltammetry

In Cathodic Stripping Voltammetry (CSV) a positive potential is applied to the solution to plate the analyte onto the drop. The positive potential attracts molecules that are complexed to the metal or are electroactive themselves. The molecules or complexes collect around the drop so that this is a pre-concentration step. The potential is then ramped in a negative direction until the metal in the complex, (or the redox active atom in a molecule – for example thiol containing molecules [62]) is reduced into the drop. The resulting current is measured and is related to the concentration of the metal complex.

In oceanic speciation experiments, CSV is used to indirectly determine natural ligand binding strengths and concentrations. With the use of an added ligand,
3.1. Electrochemical speciation methods

also referred to as the competitive ligand, this method is called Competitive Ligand Equilibration–Cathodic Stripping Voltammetry (CLE-CSV). The concentration and binding constant of the added ligand are well known. Once added and equilibrated, copper titration will at first give no change in signal as the natural ligand, on the basis that it binds copper more strongly, becomes saturated with copper before the added ligand. As the free natural ligand concentration diminishes the added lig­and will start to show an increase in signal as it binds more of the added copper. This will result in a curve that will eventually straighten once the natural ligand is completely saturated.

This technique assumes that the natural ligand is not electroactive itself, based on a difference in structure of the metal centre compared to the added ligand. The choice of added ligand dictates the range of natural ligands that can be detected with this technique. The detection window is dictated by the overall side reaction co-efficient, $\alpha'$, which is a function of the strength and concentration of the competitive ligand. The side reaction co-efficient, $\alpha'$ is defined as the following [3, 10]:

$$\alpha' = \alpha_{Cu'} + \alpha_{CuCL}$$  \hspace{1cm} (3.1)

where CL is the competitive ligand. Each individual $\alpha$ is defined by the side reactions that the molecule (or element) undergoes. For example in the case of inorganic copper the side reaction coefficient is defined as:

$$\alpha_{Cu'} = 1 + \sum \left( \beta_{CuX_i} [X]^i \right) + \sum \left( \frac{\beta_{Cu(OH)l}}{[H^+]^l} \right)$$  \hspace{1cm} (3.2)

where $\beta$'s refer to the stability constants for major anions and water with copper. $\alpha_{CuCL}$ would have a similar definition but would have the stability constant terms for a 1:1 and 1:2 stoichiometric ratio for copper with the competitive ligand. Variation of the detection window changes the detectable natural ligand concentration and binding strength.
3.1. Electrochemical speciation methods

3.1.1 ASV pseudo-polarograms

Traditionally, the half wave potential \( E_{1/2} \) of a metal in an electrolyte is measured from a direct current polarographic wave. The application of this technique to trace metal-complexation determinations in seawater is limited by the sensitivity of the instrument to detect trace metals. Croot et al. [9] presented a method to construct a pseudo-polarogram using Differential Pulsed Anodic Stripping Voltammetry (DPASV) to increase the limit of detection. A pseudo-polarogram is constructed by taking individual measurements at varied deposition potentials. In ASV the deposition potential can be used to distinguish between different species of copper. If copper is inorganically bound, a less negative potential can be used. However as copper binding strength increases, a more negative potential is required to force the copper to reduce into the mercury drop. Strongly bound copper will therefore have a more negative half wave potential than more weakly bound copper. In this way the binding constant can be determined with the advantage of adding relatively few reagents and without assuming a detection window, although it must be within the realms of the stability of water and mercury.

Choice of method

Anodic stripping voltammetry based pseudo-polarography is the chosen method because culture samples contain larger amounts of dissolved organic matter (DOM) compared to ocean samples. The use of CLE-CSV is a disadvantage in this case as DOM interferes with the electroactive competitive ligand copper complex. Several studies have used CLE-CSV to determine natural ligand binding constants and concentrations, however fewer have used this method when applied to cultured samples. Since cultured samples contain a far greater concentration of dissolved organics, the use of cathodic stripping voltammetry is less attractive as organics can interfere with the diffusion of the analyte to the mercury surface, interfering with the signal output. Measuring the copper itself (as opposed to the complex) by plating with a negative potential reduces this interference. Furthermore the direct measurement of copper, as opposed to the indirect approach of CLE-CSV has the advantage that ligated copper is not missed as a result of detection window limitations. Here the only restriction is if the copper-complex redox reactions occur
3.1. Electrochemical speciation methods

outside the stability boundary of the electrodes and electrolyte.

3.1.2 Theory: log K determination

In polarography, any redox reaction with a metal complex will involve the dissociation of the complex and then reduction of the metal to form a metal-amalgam and can be described in two steps:

\[ \text{CuL}_q^{(2-a)+} \rightleftharpoons \text{Cu}^{2+} + qL^{a-} \]  

\[ \text{Cu}^{2+} + 2e^- + \text{Hg} \rightleftharpoons \text{Cu(Hg)} \]  

where \( n \) is the number of electrons. The current that is produced due to the transfer of electrons, is dependant on the concentration of the complex at (but not adsorbed on) the mercury drop surface (Hg\(^0\)) and the dissociation constant of the complex. The current produced, \( i \), at any point along a direct current (dc) wave is therefore proportional to the concentration of the product, \( \text{Cu(Hg)} \) and reactants, \( \text{CuL}_q^{(2-a)+} \) referred to in Equation 3.3 and Equation 3.4 [63], and is given by

\[ i = k_{\text{CuL}_q}([\text{CuL}_q] - [\text{CuL}_q\text{Hg}^0]) \]  

where \( k_{\text{CuL}_q} \) is a constant of proportionality, related to the square root of the diffusion co-efficient as described by the Cotrell equation [64]. The Cotrell equation (for a spherical microelectrode) describes the current as a function of the flux of the analyte [64]. Provided that the reaction is reversible, where the association/dissociation of the species from each other and with the drop are faster than the rate of diffusion, then the relationship between the dissociation constant and the half wave potential using the Nernst equation is as follows [63]:

\[ E_{1/2} = \varepsilon - \frac{2.303RT}{nF} \log \frac{K_{\text{CuL}_q} \gamma_{\text{CuL}_q} k_{\text{Hg-Cu}}}{\gamma_{\text{Hg-Cu}} k_{\text{CuL}_q}} - q \frac{2.303RT}{nF} \log [L] \gamma_L \]  

where \( n \) is the number of electrons transferred; \( K_{\text{CuL}_q} \) is the stability constant of the \( \text{CuL}_q \) complex; \( k_{\text{Hg-Cu}} \) and \( k_{\text{CuL}_q} \) are constants of proportionality; \( \gamma \)'s refer to the activity coefficients of the species; \( \varepsilon \) is the standard potential of the mercury.

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3.1. Electrochemical speciation methods

amalgam; $R$ is the gas constant; $T$ the temperature; $F$ is Faraday’s constant and $q$ is the number of ligand molecules per copper-ligand complex.

Equation 3.6 shows that the half wave potential is shifted by the strength of the copper complex. If one substitutes ionic strength corrected thermodynamic stability constants for the stability constant (for standard conditions) then the equation can be simplified. The shift in half wave potentials, $\Delta E_{1/2}$ can then be described according to\[9, 63\]:

$$\Delta E_{1/2} = (E_{1/2})_{\text{CuL}_q} - (E_{1/2})_{\text{Cu'}} \approx \frac{0.0591}{n} \log K'_{\text{CuL}_q} - q \frac{0.0591}{n} \log [L]$$

(3.7)

where,

$$0.0591 = \frac{2.303RT}{F} \text{ at } 25^\circ\text{C}$$

(3.8)

In Equation 3.7, $[L]$ is the concentration of the ligand and $\text{Cu'}$ is the inorganic copper.

However, the reduction reaction at the mercury drop cannot be assumed to be reversible. To satisfy this Hoyle and West [65] showed that a calibration line could be constructed relating stability constant to the half wave potential. Shuman and Cromer [66] showed that in the case of large reaction rates, irreversible reaction currents become quasi-reversible. Croot et al. [9] have shown that this approach can be successfully applied to seawater based cultured samples. Thus in order to measure half wave potentials a modified Lingane equation taken from Croot et al. [9] will be used that is valid for irreversible reactions:

$$\log[(I_{\text{max}} - i)/i] = \frac{(E - E'_{1/2})\alpha n}{0.059}$$

(3.9)

where $I_{\text{max}}$ is the maximum (diffusion limited) current and, $\alpha$, is the transfer coefficient of the reduction.$^1$ The transfer coefficient reflects the difference in Gibbs free energy between the reactants and the products of the rate determining step at the electrode surface. This equation will be used to determine the half wave potential. This theory has been applied to seawater containing natural binding ligands by Croot et al. [9].

$^1$This use of $\alpha$ is not to be mistaken with the side reaction co-efficient previously described.
3.2 The determination of natural ligand copper complex binding constants

3.2.1 Experimental methods

Before analysing culture samples, several artificial ligands were analysed and their pseudo-polarograms were constructed. Since these ligands bind copper with varying strengths, a calibration line based on the half wave potentials derived from the pseudo-polarograms and ionic strength corrected thermodynamic stability constants (taken from Croot et al. [9]) could be constructed.

Sample preparation

All water was distilled and deionised using a Millipore system, and has a resistance of at least 18 MΩ. In all cases any plastic ware used was first rinsed three times with water, followed by three rinses with methanol (HPLC grade), followed by three rinses with water. This step was taken to minimise any organic based film on plastic ware and any organic contamination. Plastic ware (PC, Teflon or HDPE only) was then acid washed by immersing in hydrochloric acid (4 M) and heating to 55°C for at least 8 hours. PC and HDPE plastic ware was then rinsed three times with water and left, immersed in nitric acid (0.1 M, environmental grade) for at least a week. Equipment was then rinsed with water three times and left in a laminar flow hood to dry.

Calibrant ligands were analysed at high concentrations (10 – 500 μM) with 100 nM copper. These high concentrations were chosen to reduce analysis time, following Croot et al. [9]. Samples were prepared in artificial seawater, the same as that used to make media but without the addition of trace metals. This ensured that the electrolyte solution was the same between calibrant and sample solutions. A buffer was added to keep the pH at 8.1: ammonium borate buffer (100 μL, 1 M boric acid, reagent grade; 0.35 M ammonia, Seastar grade). These samples were left overnight at room temperature in the dark to equilibrate.

For sample preparation prior to electoraanalysis, cultured samples were chemically altered as little as possible to maintain intact speciation. Samples were prepared the day before voltammetric analysis. Some frozen samples were not stored
3.2. The determination of natural ligand copper complex binding constants

in appropriate sample sizes. As a result, samples were sometimes defrosted and
poured into smaller aliquots and refrozen for later analysis. In all cases samples
analysed were defrosted a maximum of two times before analysis. It was found
that further defrosting and refreezing decreased the amount of copper binding lig­
and. Samples that had been defrosted twice showed no difference between those
that had been defrosted only once. Cultured samples (60 mL) were removed from
the freezer, put in a dark bag and allowed to defrost at room temperature. Once
defrosted, an ammonium borate buffer (pH 8.1) was added followed by copper
(40 nM, in 0.01 M hydrochloric acid, Seastar grade). These reagents were shaken
vigorously before pipetting 9.5 mL aliquots into Teflon voltammetric cells. Subse­
quently, these samples were left in the dark at room temperature overnight to ensure
equilibration. Cultured samples were grown in EDTA free water and little or no cop­
per was added to it. As a result, it was necessary to add copper in order for it to
bind to any ligands present.

Instrumental parameters

Voltammetric experiments were run using a Metrohm 506 voltammeter, with a
Princeton Applied Research 174A Analyser; data were recorded using a chart recorder.
Initially parameters were based on those of Croot et al. [9] but were optimised for
the electrode system and analyser in use. The electrode stand was designed for
50 mL voltammetric cups. To reduce the volume of sample used, a smaller 10 mL
capacity Teflon voltammetric cup, designed for a different instrument was inserted
into a larger cup. The stream of nitrogen gas that continues to flow over the sample
during analysis was not impeded by this adjustment. Instrument parameters are in
Table 3.1.

To reduce analysis time, calibrant ligands were analysed with higher ligand
and copper concentrations (compared to cultured samples) so that deposition times
could be minimised. In total, seven calibrant ligands were analysed.

Analysis of cultured samples took about 10 hours each. It was found that the
sensitivity of the voltammeter gradually increased with time. In order to account
for this six sample aliquots were used, each was analysed at six different deposition
potentials, starting and ending with deposition potential -1.00 V. All peak heights
were then normalised to the peak height of the closest run with a deposition poten-
3.2. The determination of natural ligand copper complex binding constants

<table>
<thead>
<tr>
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<tr>
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</table>

Table 3.1: Analyser, electrode and chart recorder parameters for cultured samples. †Calibrant ligands had a different plate time (given in brackets).
3.2. The determination of natural ligand copper complex binding constants

tial of -1.00 V. This change in sensitivity with time was considered to be due to a change in temperature and gradual warming of the solutions. Samples were equilibrated in an air-conditioned lab and then analysed in a non-air conditioned lab that, during the summer was significantly warmer. The laboratory where sample preparation took place was air conditioned at 19–20°C, whereas the sample analysis took place in a laboratory that frequently reached temperatures as high as 26°C.

3.2.2 Results

Calibrant ligands

A total of seven calibrant ligands were used to create the calibration plot. Ionic strength corrected thermodynamic stability constants of the copper complexes were taken directly from Croot et al. [9]. Typical pseudo-polarograms are shown in Figure 3.1.

Figure 3.1 shows that as the copper complex strength increases it requires a more negative potential to reduce the metal onto the mercury drop. By plotting deposition potential versus log[(I_{max} - i)/i] (Equation 3.9) the half wave potential can be determined.

Figure 3.2 shows the determination of the half wave potential of the copper complexes of CDTA and EDTA. The slopes are similar because the number of electrons transferred are the same. The half wave potential is when I_{max} - i = 0.5 (at 50% of the reduction current) so that the $E_{1/2}$ can be interpolated for when $y = 0$.

By plotting all of the half wave potentials, a calibration plot showing the empirical relationship between the thermodynamic stability constant and the half wave potential can be established.

Figure 3.3 summarises these data and the resulting linear regression line has the following equation:

$$\log K' = 2.662(\pm 2.28) - 34.907(\pm 4.80)E_{1/2}$$

(3.10)

Errors (in brackets) are expressed at the 95% confidence level. This equation is fairly close to that of Croot et al. [9], who report that the use of Triton-X results in a small cathodic shift which may account for some of the difference. Triton-X was
3.2. The determination of natural ligand copper complex binding constants

Figure 3.1: Pseudo-polarograms for copper complexes measured in artificial seawater. (Ligands 100 nM, Cu 100 nM).
3.2. The determination of natural ligand copper complex binding constants

Figure 3.2: Determining the half wave potentials for copper complexes of EDTA and CDTA.
3.2. The determination of natural ligand copper complex binding constants

Figure 3.3: The empirical relationship between thermodynamic stability constant of copper complexes and half wave potential. Dashed lines represent the 95% confidence limits.
3.2: The determination of natural ligand copper complex binding constants

not added for pseudo polarogram construction in these experiments.

The effect of deposition time on the half wave potential

The long deposition times for the culture samples are necessary due to the fairly low copper concentrations in the sample. Calibrant ligands had shorter deposition times. This discrepancy must be corrected as deposition time affects the copper signal and will shift the half wave potential. Unlike direct current polarography, the current measured by pseudo-polarography is a function of both the deposition potential and the deposition time. As the deposition time increases, the amount of copper that is reduced increases relative to the amount that is in its oxidised state at the drop surface. The copper amalgam is also diffusing into the mercury away from the drop surface and so the relative concentration of reduced to oxidised is also changing at the surface of the drop with time. As a result the half wave potential, which in pseudo-polarography is when half of the copper is in its reduced form, will shift in a negative direction. Omanovic and Branica [67] presented a treatment regarding deposition time discrepancies. The dependence of the half wave potential with deposition time is given in Equation 3.11 [67].

\[ E_{1/2,2} - E_{1/2,1} = \frac{0.059}{n} \log \frac{t_{dep,1}}{t_{dep,2}} \]  

(3.11)

Omanovic and Branica stated that while in theory the shift of half wave potential should vary linearly with deposition time, in practise the relationship is ‘curvilinear’, the slope decreasing as the deposition time increases. Therefore as the deposition time increases the change in shift in half wave potential decreases. By measuring the signal with change in deposition times the curvilinear relationship can be observed, the shift in half wave potential can be calculated by constructing a linear fit to data points that do vary linearly with deposition time. The discrepancy between the deposition times for the calibrant ligands and the culture samples can be accounted for.

Experimentally the curvilinear relationship has been determined and the correct deposition times are interpolated (as in Figure 3.4). These corrected deposition times (154 s and 295 s) are then used to determine the shift in half wave potential. The shift in half wave potential from this effect is less than -0.1 V, a smaller shift
3.2. The determination of natural ligand copper complex binding constants

Figure 3.4: The dependence of the ASV peak signal on deposition time of copper in seawater at pH=8.1.

Figure 3.4: The dependence of the ASV peak signal on deposition time of copper in seawater at pH=8.1.
3.2. The determination of natural ligand copper complex binding constants

than the error in log $K'$ determinations. It can be seen from Equation 3.11 that
the small shift is a result of the log of the relative difference in corrected plating
times (154 s and 295 s). The larger the plate times used the smaller the difference
in corrected plate time (because of the curvi-linear nature of the graph).

These long deposition times also result in the copper-mercury amalgam diffus­ing
within the mercury drop from the surface and towards the centre of the drop.
Therefore, when the copper is stripped from the mercury drop, a little less than was
plated comes back out into solution. This plating-stripping discrepancy is not no­
ticeable at higher levels of copper. However, after running several voltammograms
in the same solution the sensitivity was observed to decrease. To avoid this prob­
lem, several cups were made and each cup was used six times only. This was found
to be fewer than the number of runs necessary to see a decrease in sensitivity.

Cultured sample log $K'$ determinations

Cultured samples were analysed in the same manner as described above. Pseudo-
polarograms were run in duplicate and averaged to plot. Due to variation in sensi­
tivity, data was normalised to the maximum current on each polarogram. Typical
pseudo-polarograms are shown in Figure 3.5.

Figure 3.5 clearly shows the presence of two waves in both pseudo-polarograms.
Also the waves show fairly good agreement, although they are normalised. Each
of the pseudo-polarograms shows the presence of a stronger ligand and a weaker
ligand. After constructing the log plots, the half wave potentials and their corre­
sponding log $K'$ values are shown in Table 3.2. The half wave potentials for the
stronger ligand(s) are in good agreement, implying that the same ligand strength,
and thus ligand presence are in all of the experiments. It is therefore possible that
the same strong ligand is present in all of the experiments. Given that the same lig­
and was deemed to be present in all experiments the slopes of the log plots should
also be the same, as is the case for the ‘T’ experiments whose slopes have good
agreement (‘T’ for treatment, although these are considered replicates to the ‘C’
batches (Section 2.2.1)). The half wave potentials for the weaker ligands have much
lower agreement implying that different ligand mixtures must be present between
experiments.

For the strong ligands, there is a much larger variation when comparing the ‘C’
3.2. The determination of natural ligand copper complex binding constants

Figure 3.5: Pseudo polarograms from experiments 3 (pCu 10) and 6 (pCu 12). Data points are averages of duplicate samples and error bars represent the range of data.
3.2. The determination of natural ligand copper complex binding constants

experiments. This variation in half wave potential determination is also reflected in the larger error range in the pseudo-polarograms. It was found that the error ranges of the pseudo-polarograms reduced over the weeks when these experiments were run. In short, certain logistical difficulties were overcome with time. The instrument was not run by software, all settings and timings were accomplished manually and so with time, procedure improved greatly. Also, the voltammetric cell set-up had been adjusted from its original design (as described earlier). As a result, the electrode holder was not plush with the plastic beaker unless pushed down with some force. As a consequence when the voltammograms were run there was more vibration as well as possible leakage of the nitrogen blanket. This discovery was made some weeks into experiment runs and following these two improvements the error range reduced, as well as the scatter on the log plots that were used to determine half wave potentials. Chronologically it was generally the ‘C’ experiments that were initially analysed for each experiment. The ‘T’ experiments were then run with an improved experimental procedure. Noisier experiments were not repeated later in an effort to reduce sample expenditure in this part of research. The error was also large when there was a low number of data points, \( n \). When \( n \leq 3 \) no error range is presented and these half wave potentials can only be regarded as qualitative. Although the error ranges are large for individual interpolations, agreement between samples is good, as can be seen from the standard deviation of the averages. The main purpose of these experiments is to determine the presence of a ligand that binds strongly to copper. Any sample saved in the electrochemical part of the research was later used for structural experiments using mass spectrometry.

The agreement between the weaker ligands is much lower. There is a greater standard deviation of the mean as well as greater error on interpolation to determine the half wave potential. The error on interpolation is partially due to a lower number of data points on the log plots. This lower number of data points is a result of prioritising that the stronger ligand wave is better defined at the expense of the weak ligand wave. The fact that the log \( K' \) values for the weak ligand vary more, could be a reflection of this noise in the data, but since the variation is large it is also likely that there are different weak ligands present in the samples, with similar copper-binding stability constants. The strength of the weaker ligand is in the range of the artificial ligands tested and is closer to CDTA than cyclam which is the strongest
3.2. The determination of natural ligand copper complex binding constants

calibrant ligand used here.

It can be seen from the pseudo-polarographs that the weaker binding ligand concentration is always greater than the stronger binding ligand. It is not clear however whether or not the weaker binding ligands are exuded as a direct response to copper toxicity. Since the weaker binding ligands also have varying binding constants it is possible that the weak ligands are not one specific complex but a group of complexes – implying that these ligands may be present coincidentally and not a direct toxic response. This is discussed in more detail in Section 3.4.

Extrapolation with these half waves from the calibration line results in ionic strength corrected log thermodynamic stability constants of $49.4 \pm 1.1$ for the stronger ligand and $19.8 \pm 5.5$ for the weak ligand. The log $K'$ for the strong ligand is high when compared to other determinations (see Tables 1.2 and 1.3), however this method of determination has assumed a transfer of two electrons (copper in the +2 oxidation state). If copper was bound in the +1 oxidation state the log $K'$ of the strong ligand would be 24.7. Since this is the ionic strength corrected thermodynamic constant it does agree more with other conditional constant determinations. This is discussed further in Section 3.4 and in Chapter 4.
<table>
<thead>
<tr>
<th></th>
<th>Strong ligand</th>
<th></th>
<th></th>
<th></th>
<th></th>
<th>Weak ligand</th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Expt</td>
<td>Slope</td>
<td>$E_{1/2}$ ($V$)</td>
<td>log K'</td>
<td>$n$</td>
<td>Expt</td>
<td>Slope</td>
<td>$E_{1/2}$ ($V$)</td>
<td>log K'</td>
<td>$n$</td>
</tr>
<tr>
<td>2C</td>
<td>8.1 (0.8)</td>
<td>-1.34 (0.08)</td>
<td>49.5 (9.4)</td>
<td>5</td>
<td>2C</td>
<td>3.3 (0.2)</td>
<td>-0.55 (0.09)</td>
<td>21.8 (6.0)</td>
<td>6</td>
</tr>
<tr>
<td>3C</td>
<td>7.3 (1.2)</td>
<td>-1.34 (0.10)</td>
<td>49.4 (10.2)</td>
<td>6</td>
<td>3C</td>
<td>4.3 (1.2)</td>
<td>-0.53 (0.34)</td>
<td>21.0 (14.5)</td>
<td>4</td>
</tr>
<tr>
<td>4C</td>
<td>5.0 (0.6)</td>
<td>-1.35 (0.08)</td>
<td>49.6 (9.2)</td>
<td>6</td>
<td>4C</td>
<td>5.4 (0.5)</td>
<td>-0.57 (0.13)</td>
<td>22.7 (7.4)</td>
<td>6</td>
</tr>
<tr>
<td>5C</td>
<td>11.8 (2.1)</td>
<td>-1.35 (0.22)</td>
<td>49.8 (14.1)</td>
<td>4</td>
<td>5C</td>
<td>4.1 (2.1)</td>
<td>-0.49 (0.62)</td>
<td>19.6 (24.1)</td>
<td>4</td>
</tr>
<tr>
<td>6C</td>
<td>12.6</td>
<td>-1.32</td>
<td>48.6</td>
<td>2</td>
<td>6C</td>
<td>2.3 (4.6)</td>
<td>-0.36 (7.58)</td>
<td>15.1</td>
<td>3</td>
</tr>
<tr>
<td>3T</td>
<td>6.8 (1.1)</td>
<td>-1.32 (0.10)</td>
<td>48.5 (9.9)</td>
<td>6</td>
<td>3T</td>
<td>4.2 (0.4)</td>
<td>-0.55 (0.20)</td>
<td>21.8 (9.5)</td>
<td>5</td>
</tr>
<tr>
<td>4T</td>
<td>6.4 (0.4)</td>
<td>-1.34 (0.04)</td>
<td>49.5 (7.7)</td>
<td>6</td>
<td>4T</td>
<td>4.1 (0.4)</td>
<td>-0.56 (0.09)</td>
<td>22.0 (5.8)</td>
<td>6</td>
</tr>
<tr>
<td>5T</td>
<td>5.3 (1.0)</td>
<td>-1.37 (0.12)</td>
<td>50.3 (10.6)</td>
<td>6</td>
<td>5T</td>
<td>1.7 (1.0)</td>
<td>-0.44 (2.01)</td>
<td>18.1</td>
<td>3</td>
</tr>
<tr>
<td>6T</td>
<td>5.9 (0.6)</td>
<td>-1.34 (0.07)</td>
<td>49.4 (8.9)</td>
<td>7</td>
<td>6T</td>
<td>2.9 (2.8)</td>
<td>-0.39 (1.31)</td>
<td>16.4 (47.8)</td>
<td>4</td>
</tr>
</tbody>
</table>

Table 3.2: Half wave potentials and the slopes of the log plots (errors are given in brackets to the 95% confidence limit); $n$ is the number of data points on the log plot used to determine the half wave potential. †Error for averages is presented as two standard deviations of the mean.
3.3 Determination of the concentration of the copper binding ligands

3.3.1 Experimental methods

This section is based on the assumption that copper will bind to the weaker ligand once the copper concentration is greater than the stronger binding ligand capacity. Only then will the weaker ligand be able to bind residual copper.

Standard additions were run on samples that were prepared in the same way as for the construction of pseudo-polarograms (Section 3.2.1). Samples were defrosted in the dark and buffered to pH 8 using a boric acid–ammonia buffer (1 M and 0.35 M respectively). The voltammetric analyser parameters can be found in Table 3.1, except for the deposition time which was 180 s and the deposition potentials which were -0.40 V to determine free copper, -1.00 V to determine the free copper plus that bound to weak ligands and -1.50 V to determine the total copper bound and ‘free’ (or inorganic). The deposition potential of -0.40 V is based on Figure 3.1, where the polarographic wave for ‘free’ copper is complete by -0.40 V. The binding strength for copper and the weak ligand is similar in strength to CDTA and so should also not be measured at this deposition (a comparison of ‘free’ copper and weak ligand like-CDTA is in Figure 3.1).

It has been determined that all of the culture samples contain a compound that likely contains sulphur as it binds copper in the plus one oxidation state. This is known from MALDI TOF data (an ion present at m/z 1329) and is discussed in Chapter 4. The presence of sulphur-containing compounds that bind copper is not surprising as the presence of sulphur compounds in natural seawater samples has been reported [68], as well as in culture experiments [39]. As sulphur complexes can be electroactive, and given the probable presence of a sulphur complex, Triton-X (0.04% v/v) is added for these determinations. Triton-X is a surfactant that minimises current produced from surface active complexes.
3.3. Determination of the concentration of the copper binding ligands

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Figure 3.6: The standard addition of experiment 6 to determine the concentration of electroactive copper at three deposition potentials (-0.40 V, -1.00 V and -1.50 V).

3.3.2 Results

Each culture experiment had a corresponding standard addition. Standard additions were linear. One example of standard addition data is presented in Figure 3.6. The range of concentrations for the strong ligand was 2–6 nM and 5–22 nM for the weak ligand class. The data for experiments 3, 5 and 6 are in Table B.1.

Knowing the amount of electroactive copper at these deposition potentials allows the determination of copper concentration at each of the steps on the pseudo-polarogram. The difference in concentration of copper between the steps of the pseudo-polarographic wave is the amount of copper that is bound to each ligand class. If one assumes a 1:1 stoichiometric ratio then the concentration of the ligand can be inferred. A summary of ligand concentrations is presented in Table B.1. These numbers can only be considered as estimates as the error ranges are large. Al-
though the exact numbers are not reliable this does show that there is significantly more weak ligand than strong ligand and that there is a fairly low concentration of strong ligand. However even at this low concentration, there is still adequate lig­and to buffer the amount of free copper that cyanobacteria were exposed to in these experiments.

3.4 Discussion and conclusions

3.4.1 Pseudo-polarograms

The pseudo-polarograms show that Synechococcus DC2 produces two copper binding ligands while in log phase growth. The agreement in log \( K' \) between several experiments implies that the ligand is likely to be the same compound in all experiments. Furthermore it supports the hypothesis that these ligands have been produced as a direct response to copper toxicity. This ligand binds copper strongly and with a log \( K' \) of 49. This is one of the strongest copper binding ligands found in the literature [9]. One possible explanation for this discrepancy between the binding strength determined here and other research, is the difference in the linear regression of the calibration line compared to Croot et al. [9]. Their much lower slope would result in a lower log \( K' \) value and there is some error involved in such a large extrapolation. Croot et al. [4] have suggested that their large log \( K' \) value of 36.7 for the same Synechococcus strain may be because the complex is binding copper (I) as opposed to copper (II). In this case, the number of electrons transferred in the reaction would be one and so the log \( K' \) would be halved. The likelihood of binding as copper (I) is increased if the ligand contains sulphur. Chapter 4 discusses the presence of sulphur more extensively. It is likely that for the complexes observed here, copper ligands contain sulphur, thus it is possible that the log \( K' \) is closer to 24–25.

It is not clear whether the weaker binding ligands are exuded as a direct response to copper toxicity. Since the weaker binding ligands have varying binding constants it is likely that the weak ligands are not one specific complex but a group of complexes. Also, the strong ligand concentration is high enough to buffer the copper present and so the presence of the weak ligand is not necessary to reduce
3.4. Discussion and conclusions

copper toxicity. This suggests that these ligands are present coincidentally and not a direct toxic response.

3.4.2 Ligand concentrations

The ligand concentration determinations have large error ranges and are semi-quantitative only. The concentrations imply that there is more weak ligand present than strong ligand in all of the experiments. The use of an internal standard would not have improved the quality of data in this instance as the likely candidates for an internal standard (Cd or Pb) are not similar enough to copper, especially if there is a possibility that copper is binding to sulphur-containing complexes. The magnitude of the strong copper complex concentration is lower than in other investigations (see Tables 1.2 and 1.3). This is most likely due to the difference in culturing method. The copper activities that the cyanobacteria are exposed to are relevant to oceanographic concentrations. However, the cyanobacteria have only been exposed to EDTA free media for 20 hours (unlike other experiments). Production rates for these strong ligands are on the order of $10^{-20}$ moles per cell per hour. Production rates for other strong copper ligand experiments are not available. Production (moles per cell) of siderophores [69] by iron limited Synechococcus 7002 is about one hundred times (depending on the iron treatment) greater than the strong copper binding ligands that are produced here and again, the culturing method is different from that presented here.

The concentrations of the strong copper binding ligands are low and of the order of nanomolar. However these low concentrations do buffer the toxic copper that these cultures have been exposed to. Therefore although these determinations have low precision, the results are reasonable.

3.4.3 Conclusions

This chapter shows that cyanobacteria produce copper binding ligands that are strongly binding when acclimated to toxic copper conditions and still in log phase growth. Synechococcus DC2 also produces higher concentrations (compared to the stronger ligands) of weaker ligands which are not necessarily the same ligand mixture in all experiments and are probably not a direct response to copper toxicity.
3.4. Discussion and conclusions

The strong binding ligand has a log \( K' \) of 49.4±1.1 which is extremely high. It is possible that the log \( K' \) is half of that value (24–25) if copper is bound in the (+1) oxidation state.

The use of pseudo-polarography has allowed the measurement of copper binding ligands produced from culture based samples. The application of this method allows direct determination of copper ligands as opposed to other methods which are indirect and involve the addition of further organic reagents. This addition can be problematic when measuring culture based samples which contain more DOM than field samples. Since it is likely that the copper that is bound to strong ligands found in this work is in the +1 oxidation state, these results do agree when comparing the ionic strength corrected constants (found here) with conditional constants (Table 1.3), provided one considers the fundamental difference between the two parameters as illustrated in Table 1.1.
Chapter 4

Analyte extraction and
determinations of the chemical
nature of a copper binding ligand

This chapter describes the method and development used to isolate and concentrate copper binding ligands from seawater. Construction of pseudo-polarograms enables the tracking of the strong copper binding ligand throughout the column extraction. Column eluents are screened using MALDI TOF MS for copper containing complexes based on their similarity to the isotopic signature of copper. Copper containing complexes are then examined further using different combinations of mass spectrometers that use both matrix assisted laser desorption ionisation (MALDI) and electrospray as an ion source.

4.1 Removal of strong copper binding ligands from seawater using solid phase extraction

From Chapter 3 it has been established that all culture samples contain two ligand types: a weaker ligands class and a stronger ligand class. Anodic stripping voltammetry based pseudo-polarograms have been employed to assess the presence of strong copper binding ligands and it is therefore used to find a resin that will remove the strong ligand class from seawater and track this ligand in column eluents.
4.1. Strong copper binding ligand extraction

4.1.1 Choice of resin

The criteria for choosing a resin are (i) that it retains the strong copper binding ligand from seawater and (ii) that there is good recovery from the column. In the past, resins have been chosen for their compatibility with chemical functionality of the complex. It is clear from Chapter 1 that the choice of extraction is inevitably selective. Therefore the choice of resin here is based solely on the fact that the ligand binds copper strongly. Pseudo-polarography has been used to identify a strong ligand and it will be used to determine its removal from the same matrix as well as recovery from the resin.

Two partially hydrophobic resins that were successful for the removal of siderophores from seawater [70] were tested for copper binding ligand removal from the seawater matrix. Extremely hydrophobic resins have been found to be unsuccessful in removing stronger ligands [40]. It has also been shown that DOM isolates from hydrophobic resins co-elute with copper binding ligands when eluted with hydrophilic solvent combinations [71]. This implies that copper binding ligands have some hydrophilic functionalities. Therefore partially hydrophobic resin types successful for siderophores [70] were tested.

Experimental methods: resin selection

Aliquots (20 mL) of cultured samples were defrosted; buffer (pH 8.1–8.3) and copper standard (40 nM) were added prior to over night equilibration. Samples were left at room temperature and kept in the dark over this period.

The apparatus consisted of a peristaltic pump, Teflon tubing and a custom made Teflon column with a bed volume of 0.5 mL. All experiments were undertaken in a laminar flow hood. Before use all tubing was acid washed by running hydrochloric acid (25%, reagent grade) through it overnight at a flow rate of 0.7 ml/min. The two most successful resins for the extraction of siderophores from seawater [70] were tested: SM-2 Biobeads (Bio-Rad Laboratories) and XAD-16 (Amberlite). Prior to loading, the resins were batch-cleaned; in the case of SM-2 Biobeads, cleaning instructions were taken from manufacturer instructions (details are in Appendix C). XAD-16 resin was cleaned using a method modified from Lepane [72] (also found in Appendix C). Once batch cleaned, the resin was loaded onto the column as a
4.1. Strong copper binding ligand extraction

<table>
<thead>
<tr>
<th>Sample</th>
<th>Resin</th>
<th>Pre-column peak height (nA)</th>
<th>Post-column peak height (nA)</th>
<th>Ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>-1.00V</td>
<td>-1.50V</td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>SM-2</td>
<td>190</td>
<td>195</td>
<td>1.02</td>
</tr>
<tr>
<td>Expt 3C</td>
<td>SM-2</td>
<td>207</td>
<td>320</td>
<td>1.55</td>
</tr>
<tr>
<td>Control</td>
<td>XAD-16</td>
<td>same as above</td>
<td>1.02</td>
<td></td>
</tr>
<tr>
<td>Expt 3C</td>
<td>XAD-16</td>
<td>same as above</td>
<td>1.55</td>
<td></td>
</tr>
</tbody>
</table>

Table 4.1: The relative DPASV copper signal for a cultured sample to test the ability of selected resins to remove a strong copper binding ligand. Ratio expresses the relative difference in signals between the deposition potentials, a value of $R > 1$ indicates the presence of a strong ligand.

methanol slurry. Once on-column, the resin was further washed by first running distilled de-ionised water through the entire system (5 min at 3 mL/min) followed by hydrochloric acid (1 M, reagent grade, 20 min at 3 mL/min). Water was then run through the column and the pH monitored until it returned to pH 5.5–6 (that of water). The flow rate was then set to 0.67 mL/min.

In the case of resin tests, artificial seawater was run through the column prior to a sample. Half of the defrosted sample (10 mL) was then run through the column and collected into a Teflon voltammetric cup. Sample run through the column was compared directly to sample not run through the column. A control sample of artificial seawater that was also buffered and with copper added was run through the test resin.

All pseudo-polarograms showed two ligands: a weaker and a stronger. The stronger ligand (the ligand of interest) had a half wave potential of -1.35 V so that by a deposition potential of -1.50 V, the copper that was bound to the ligand was included in the ASV signal. As a result of the presence of the strong ligand, there is an increase in signal when comparing the depositions at -1.00 V and -1.50 V. Thus, voltammograms (DPASV) were run on the samples post-column and pre-column, with a deposition time of 760 s at these two potentials (all other parameters are summarised in Table 3.1).
4.1. Strong copper binding ligand extraction

Results: resin selection

The change in signal resulting from plating at different deposition potentials will indicate the presence of strongly bound copper. A comparison of the copper signal from the same culture sample, run through different columns and a control is presented in Table 4.1. A ratio of greater than 1 is taken to indicate the presence of a strong ligand in seawater. Absolute values cannot be used as sensitivity may well change depending on the removal of other DOM that may change the efficiency of diffusion of copper towards the mercury drop electrode [67]. In the case of XAD-16 the change in ratio from 1.55 to 1 (actually 0.93) when using the partially hydrophobic XAD-16 resin is taken as an indication that copper ligands are removed from the seawater matrix.

The XAD-16 resin is therefore successful in the extraction of strong binding ligands, in agreement with the findings of Ross et al. [41]. The SM-2 resin, however, does not remove most of the strong copper binding ligands, implying that these ligands are different to siderophores. This is not surprising as the binding constants of copper-siderophore complexes are far lower than those found here [9].

4.1.2 Recovery determinations

Experimental methods

Once a successful resin was chosen, culture samples could be extracted and concentrated. Aliquots of frozen sample (1000 mL) were defrosted and buffered to pH 5 with ammonium acetate buffer. Tests were also run at pH 8, however recovery was improved with pH 5 and copper complex speciation was found to be unaffected at this slightly acidic pH as evidenced by mass spectral data. Copper was added to a final concentration of 60 nM to ensure that all ligands are bound prior to extraction. The samples were equilibrated overnight in the dark at room temperature. Samples were then run through the column at a rate of 0.65–0.70 ml/min which took about 25 hours. The column was then eluted using 2 aliquots of water followed by 2 aliquots of a mixture of methanol and water (1:1 v/v) followed by 2 aliquots of methanol. Each aliquot was two bed volumes (1.04 mL). The eluents were collected individually, 200 μL were then reintroduced to artificial seawater for electrochemi-
4.1. Strong copper binding ligand extraction

cal experiments to see if and in which fraction the strong ligands were recovered.

A control solution of artificial seawater (1000 mL) with additional copper (60 nM) and buffer for pH 5 (ammonium acetate) and for pH 8 (ammonium borate) was also run through the column for mass spectral analysis.

Pseudo-polarographic determinations were then carried out using the parameters in Table 3.1, with the exception that the deposition time was 240 s. Since the concentration of recovered ligand is unknown, copper was added to a final concentration of 250 nM with the intention that all ligands would therefore be bound to copper. Much larger copper additions (2.5 μM) were found to be too large and swamped the relatively smaller change in copper signal at different deposition potentials due to copper complexation. Samples were left to equilibrate in the dark overnight at room temperature. Triton-X (0.04% v/v) was added as large amounts of DOM could potentially be present.

Results

Ligands will elute with the first favourable change in solvent polarity. The first two eluent aliquots were considered to be column rinses to remove salts. Thus the first eluent of interest was eluent number 3, the first methanol:water mixture. The second eluent of interest was eluent number 5, the first pure methanol aliquot to run through the column. These eluents were used to construct post-column pseudo-polarograms. However all fractions were investigated using MALDI TOF MS, Table 4.4 (page 81). Pseudo-polarograms of the two column eluents from experiment 6 are presented in Figure 4.1. From these pseudo-polarograms it can be seen that the strong ligand is recovered from the resin but in both fractions. It elutes when it contacts the solvent that has a lower dielectric constant than pure or salty water. This indicates that the ligand is not particularly hydrophobic but rather, moderately hydrophilic.
4.1. Strong copper binding ligand extraction

Figure 4.1: Pseudo-polarograms of two different column eluents from experiment 6 (pCu 12). Data points are averages of duplicate samples and error bars represent the range of data.
4.2 Mass spectrometric screening for copper ligands

4.2.1 Ionisation sources for mass spectrometric speciation determinations

Detection of the molecular ion using mass spectrometry requires the use of a soft ionisation technique. The two main options for dissolved analyte samples are matrix assisted laser desorption ionisation (MALDI) and electrospray ionisation (ESI). Electrospray ionisation as a source is more direct for speciation measurements as the analyte remains in solution throughout the ionisation process. Speciation studies to determine the ability of electrospray to maintain the chemical forms representative of solution equilibria have been undertaken and electrospray has been shown to be suitable for this application. The ability of ESI to reflect the species of metal complexes in aqueous solutions has been investigated by Ross et al. [73] where it was found that the percentages of copper complexes found in aqueous solutions (calculated by the speciation software MINEQL) matched the results from ESI spectra of water-methanol solutions well. The addition of tannic acids (an environmentally relevant group of organic acids) to seawater has also been investigated to see if electrospray results matched those predicted by MINEQL. The resulting good match showed that ESI can be applied to speciation studies in seawater matrices with good agreement [74].

Speciation studies using MALDI have also been applied to seawater problems [43]. However, to date, the ability of MALDI to reflect solution speciation has not been so extensively studied. MALDI involves the drying of the analyte and some crystallisation occurs around the analyte. The advantage to MALDI is that it uses very low sample volumes (1−2 μL) compared to electrospray (unless using nanospray). It also ionises samples by a different mechanism to electrospray and so the ionisation efficiency for the analyte may be better, providing a second option for analyte ionisation.

Ross et al. [74] found that the speciation of copper was affected by the reduction of copper by the solvent acetonitrile. Thus methanol was considered a better solvent to use than acetonitrile for this application and only methanol and water mixtures were used throughout these experiments.
4.2. Mass spectrometric screening for copper ligands

4.2.2 Screening for ligands using MALDI TOF

Analyses with MALDI used two matrix materials (1) α-Cyano-4-hydroxycinnamic acid (CHCA) and (2) 2'-6'-dihydroxyacetophenone (DHAP), an acidic and a neutral matrix respectively. The neutral matrix was used with the intention that speciation would be less affected. The acidic matrix is a more common matrix; several experiments were duplicated in both and since similar results were obtained they were used interchangeably.

Samples were mixed with CHCA (5 mg/mL CHCA in 75% acetonitrile and 0.1% trifluoroacetic acid (TFA)) in a 1:1 (v/v) ratio of sample to matrix. This mixture was then applied to the MALDI target. In the case of DHAP, two matrix mixtures were made. A solution of 20 mg/mL DHAP in isopropyl alcohol was pipetted (1 μL) onto the target and left to dry. Then (1 μL) of a mixture (1:1 v/v) of the analyte with a solution of 10 mg/mL DHAP in 20 mM ammonium citrate and 10% isopropyl alcohol was added over the previous layer. Samples were then analysed on a Voyager-DE™ Workstation MALDI TOF in reflectron mode. The upper mass calibrant (mw: 1296.7) used was angiotensin and the lower mass calibrant (mw: 487.2) was adrenocorticotropic hormone fragment 1-4 (ACTH-4).

Initially when samples were analysed, no ions were detected that matched the expected isotopic signature of copper (a simulation of the expected copper isotopic signature is presented later in this thesis, in Figure 4.7). Thus, samples were evaporated down, using a speed-vac, to a ten fold concentration factor while ensuring that the samples were not evaporated to dryness. These samples were then applied to the MALDI target as before and analysed.

4.2.3 Screening results

Samples from experiments 2 and 5 were extracted under both of the different pH conditions (pH 5 and 8). The resultant spectra showed that the same peaks were present in both experiments but that the intensity of the peaks of interest (m/z 697 and 1329) was increased using pH 5. Although, MALDI TOF data is not quantitative, a comparison of the spectra for the two pH values for experiment 5 are in Figures 4.2 and 4.3.
Figure 4.2: The full MALDI TOF spectrum of Eluent 3, Experiment 5, extraction at pH 8. Note that the total number of counts corresponding to 100% intensity is greater than Figure 4.3.
Figure 4.3: The full MALDI TOF spectrum of Eluent 3, Experiment 5, extraction at pH 5. Note that the total number of counts corresponding to 100% intensity is lower than Figure 4.2.
4.3. Mass spectral characterisation of peaks of interest

<table>
<thead>
<tr>
<th>Experiment</th>
<th>697+</th>
<th>1329+</th>
</tr>
</thead>
<tbody>
<tr>
<td>2C</td>
<td>✗</td>
<td>✓</td>
</tr>
<tr>
<td>3C</td>
<td>✗</td>
<td>✓</td>
</tr>
<tr>
<td>3T</td>
<td>✗</td>
<td>✓</td>
</tr>
<tr>
<td>4C</td>
<td>✗</td>
<td>✓</td>
</tr>
<tr>
<td>4T</td>
<td>✗</td>
<td>✓</td>
</tr>
<tr>
<td>5C</td>
<td>✓</td>
<td>✓</td>
</tr>
<tr>
<td>5T</td>
<td>✓</td>
<td>✓</td>
</tr>
<tr>
<td>6C</td>
<td>✓</td>
<td>✓</td>
</tr>
<tr>
<td>6T</td>
<td>✓</td>
<td>✓</td>
</tr>
</tbody>
</table>

Table 4.2: The presence of peaks of interest in all of the MALDI TOF experiments.

After thorough searching through the spectral data, only two peaks of interest were found (that were not present in the control). (1) The peak at 1329+ was interesting because it was present in all culture samples. (2) The peak at 697+, was interesting even though it was not in all samples, as it had an isotopic signature similar to that expected for copper. A summary of the presence of these peaks of interest is presented in Table 4.2.

4.3 Mass spectral characterisation of peaks of interest

4.3.1 Investigations into the ion at m/z 1329

The ion at m/z 1329 is of interest because it is distinct from the control runs (seawater only) and because it is present in all culture grow up experiments. Electrochemical data has shown that the strong copper binding ligand is in all samples, and that it is probably the same ligand in each experiment (as log K' values are consistent between experiments).

The spacing of the isotopic peaks are one mass unit apart. This shows that this is a +1 ion. Inspection of this peak indicates that it does not have an isotopic structure similar to that of copper (in Figure 4.4). However, further analyses with large additions (20 μM) of copper after elution from the XAD-16 column show
4.3. Mass spectral characterisation of peaks of interest

that this ion can bind copper as displayed in Figure 4.4. MALDI sourced spectra often contain salt adducts of ions present. From this spectrum it is likely that the peaks at m/z 1351 and at m/z 1355 are due to the sodium and aluminium adducts respectively. This is confirmed by the fact that the isotopic signature of these smaller adduct peaks reflect the isotopic signature of the molecular ion cluster. Both sodium and aluminium are monoisotopic elements and have exchanged with a hydrogen ion (effectively monoisotopic) and no change in isotopic signature is possible. The third adduct is at m/z 1391 a mass difference (from the molecular ion) of 62 Da. The mass difference of 62 implies that one hydrogen has been exchanged for a copper ion. This indicates that copper has been complexed in the (+1) oxidation state; copper is the only element that has an isotope at 63 atomic mass units. Although the adduct cluster at 1391 Da does not reflect the isotopic signature of copper or a copper complex (Figure 4.7) this is most likely due to the large number of carbon atoms which give rise to a high abundance of the peak one mass unit higher than the molecular ion peak (M+1). A complex with one copper atom and 80 carbon atoms would have a similar isotopic signature to this adduct.

It is interesting that the addition of large amounts of copper did not result in observations of other peaks that reflect the isotopic signature of copper. Only this molecular ion was found with such a noticeable copper adduct. The low intensity of the copper adduct is a function of both the ionisation efficiency of the complex and its concentration. This is likely a very weakly binding ligand, since high concentrations of copper were necessary for its observation. Furthermore, sodium and aluminum adducts are also present (at m/z 1351 and 1355) implying that this is a non-specific binding site for M⁺ ions.

Binding copper through the (+1) oxidation state is interesting as it gives us information about the complex. Binding Cu(I) is most often via a sulphur atom and to a lesser extent through nitrogen, a slightly harder atom. It has been shown that sulphur containing ligands can reduce copper(II) to copper(I) and that this is aided by the stabilisation of copper(I) by the chloride ions present in seawater [12]. Thus, it is probable that this complex contains sulphur atoms which can be electroactive. It was found during electrochemical experiments that an electroactive complex was present. The electroactive ligand was also shown to contain copper by running direct current polarograms with copper additions. A pre-adsorption wave (reflecting
4.3. Mass spectral characterisation of peaks of interest

electroactivity) was detected and was associated with the addition of copper. The wave occurred in classic polarograms on scans of a single drop (HMDE) to -1.2 V (Figure 4.5) but only at very high concentrations of copper (sub-mM). That this electrochemical pre-adsorption wave occurred only with very high copper concentrations, coincides with the fact that the mass spectrometric 1391 copper adduct ion also only occurs when large concentrations of copper have been added. This ligand is therefore not the strong copper binding ligand as it seems to bind copper only when high copper concentrations are present. As a result, this complex was not investigated using electrospray ionisation.

Figure 4.4: A MALDI TOF spectrum of the m/z 1329 ion. The insert is a zoom in of the copper adduct at m/z 1391 with copper (20 μM) added.
4.3. Mass spectral characterisation of peaks of interest

Figure 4.5: The direct current polarographic pre-adsorption wave of an electroactive complex that contains copper.
4.3. Mass spectral characterisation of peaks of interest

4.3.2 Investigations of the isotopic signature of the ion at m/z 697

MALDI based determinations of the ion at m/z 697/699

This ion was present in two experiments (5 and 6) in both the ‘C’ and ‘T’ batches. The interest in this peak is based on the isotopic signature which is given in Figure 4.6. The isotopic peaks of the ion at m/z 697 are spaced one mass unit apart, this means that this is a +1 ion. This isotopic signature implies that it could contain copper. The slightly elevated M+2 peak compared to that of the elemental copper isotopic signature implies that another element containing a relatively large abundance of an M+2 isotope is also present. The most likely candidate for an or-
4.3. Mass spectral characterisation of peaks of interest

Figure 4.7: The predicted isotopic ratios for Cu (dark) and a copper dimerised-glutathione complex (light).

ganic species is sulphur. The presence of one or two sulphur atoms would increase the M+2 relative intensity as in Figure 4.7 where two sulphur atoms are present in the copper glutathione-dimer complex. Glutathione is a tripeptide with a cysteine amino acid residue, therefore the dimer could be a reasonable approximation of the ion at m/z 697. It can be seen that the M+2 peak of the copper complexed glutathione dimer (C_{20}H_{32}N_{6}O_{12}S_{2}Cu) has contribution from the two sulphur atoms, and that the contribution from two $^{13}$C atoms is low (without the sulphur and copper atoms, the $^{13}$C contribution to the M+2 peak would be 5.4%.

Since the peak intensities are low and variable, and there are many possible chemical formula combinations that could also give rise to this isotopic signature, it is necessary to clarify whether or not this complex contains copper. Two different
4.3. Mass spectral characterisation of peaks of interest

Figure 4.8: MALDI TOF spectra of the 697 Da ion, spiked and non-spiked using enriched copper standards, post column extraction. A is the non-spiked sample with an expected isotopic ratio of 1:0.45; B is the spiked sample with an expected copper isotopic ratio of 1:4.

Copper enrichment experiments were undertaken to ascertain the presence of copper in this complex. The first experiment was with the addition of an enriched copper standard (99.7% $^{65}$Cu) after the ligand had been eluted from the column. Copper was added as a standard (1% HNO$_3$) to a final ratio of 1:4 (63:65); a natural copper standard (1% HNO$_3$) was added to a second aliquot of the same sample and both samples were then left for 45 minutes. Following this period, buffer was added (0.3 M NH$_3$Ac) until the pH was 6.5−7.0. Samples were prepared in the same way as described for the DHAP matrix (Section 4.2.2) and analysed using a Bruker Biflex IV MALDI TOF using a reflectron. The resulting data are in Figure 4.8.
4.3. Mass spectral characterisation of peaks of interest

The spiked sample does not have the ratio expected (copper, 1:4), if it contained copper. However the signal to noise ratio is low. The enriched copper standard is in nitric acid and could degrade organic complexes, particularly those that contain sulphur atoms, explaining the low signal to noise ratio. There is a still an increase in the M+2 peak indicating that some $^{65}$Cu may have exchanged with natural copper without complete degradation.

The second spiking experiment involved spiking the sample before it was extracted onto the XAD-16 column. This time, the amount of copper spike added was limited by the amount of nitric acid that the standard was dissolved in. Nitric acid concentrations were kept the same as natural copper spikes. This resulted in a final 63:65 copper ratio of 1:1. Results were again analysed using MALDI-TOF MS, however this time, samples were analysed using the CHCA matrix because this matrix was fresh. The results of this experiment are in Figure 4.9.

The enriched copper spectrum does not exactly reflect the expected ratio of 1:1. It is therefore likely that this complex does contain copper as well as at least one sulphur atom. These two experiments combined contribute evidence that this complex does contain copper, but it is not a complete confirmation. Also these experiments have all been undertaken using MALDI as an ionisation source. It can be seen from these experiments that copper bound to this complex may be partially or wholly exchangeable. Since acidic enriched copper only was added, the degree of exchange cannot be determined.

**Electrospray based determinations of the ion at m/z 697/699**

It has been observed that some organic copper-complexes (made from CuSO$_4$) are observed as copper(I) complexes when detected using MALDI, fast atom bombardment (FAB) and plasma desorption (PD), implying that copper reduction takes place during ionisation. However electrospray determinations using methanol as a solvent have shown copper complexes in the +2 oxidation state [73]. ESI is known to be a more direct technique for speciation measurements and it is therefore important to detect the presence of the complex using electrospray. This will also provide further clarification of the isotopic signature.

Mass calibration of this sample was achieved by introducing a separate stream of Glu-fibrinopeptide (the doubly charged molecular ion has a mass to charge ra-
4.3. Mass spectral characterisation of peaks of interest

Figure 4.9: MALDI TOF spectra of the 697 Da ion, spiked and non-spiked using enriched copper standards, before column extraction. A is the non-spiked sample with an expected isotopic ratio of 1:0.45; B is the spiked sample with an expected copper isotopic ratio of 1:1.
4.3. Mass spectral characterisation of peaks of interest

tio of 785.8426) orthogonally to the analyte sample using a Lockspray™ interface
giving mass correction in real time (as opposed to external or internal calibration
techniques). The sample solution was not buffered, but following evaporation using
an equal volume of methanol
was added to aid ionisation and evaporation during electrospray. In order to con­
serve sample, nanospray was employed and the sample was directly infused at a rate
of 1 μl/min. The isotopic signature of the molecular ion cluster using electrospray
is in Figure 4.10.
Figure 4.10: The nESI spectrum of the ion at m/z 697.
4.3. Mass spectral characterisation of peaks of interest

<table>
<thead>
<tr>
<th>Ionisation source</th>
<th>697</th>
<th>698</th>
<th>699</th>
<th>700</th>
</tr>
</thead>
<tbody>
<tr>
<td>CHCA (MALDI)</td>
<td>100</td>
<td>45</td>
<td>80</td>
<td>33</td>
</tr>
<tr>
<td>DHAP (MALDI)</td>
<td>100</td>
<td>30</td>
<td>68</td>
<td>25</td>
</tr>
<tr>
<td>ESI</td>
<td>100</td>
<td>35</td>
<td>45</td>
<td>-</td>
</tr>
</tbody>
</table>

Table 4.3: The isotopic signatures of the 697 ion from different ionisation sources

The isotopic signature is reflective of a copper isotopic signature, but in this case the M+2 ion (m/z 699) is not as elevated, suggesting that the complex may not contain sulphur. This ESI based spectrum also shows that the same molecular ion is present in both ESI and MALDI based ionisation sources. This shows that, in this case, the (chemical) species has not altered when using MALDI. It can be seen from this spectrum that there is a lower signal to noise ratio than spectra that were acquired using MALDI (Figures 4.8 and 4.9). Since ESI has been used to clarify the speciation of this complex and because of the difficulty in peak intensities, MALDI was used for further investigations.

MALDI and ESI determinations

The isotopic signature of the 697 ion can help to predict the number of carbon, sulphur and copper atoms. However, the isotopic signature is variable and changes with the nature of ionisation. The percentages of each of the ions is presented in Table 4.3. These values are taken from the figures presented in this thesis. However, in general, all three ionisation techniques have resulted in variable isotopic signatures and this table should be considered a general summary. The lack of agreement between ESI and MALDI is most likely explained by the lower signal to noise ratio in the ESI spectrum.

From Table 4.3 it can be inferred that there are probably around 30 to 45 carbon atoms (based on the abundance of the $^{13}$C abundance of 1.1%). The variation in abundance of the M+2 peak is large and it could be a result of several possible combinations of sulphur or copper atoms. However the presence of two copper atoms would significantly increase the abundance of the M+2 peak to greater than
4.3. Mass spectral characterisation of peaks of interest

Figure 4.11: The variation in isotopic signatures with sulphur and copper content. All plots contain $C_{35}H_{51}N_{6}O_{5}$ and vary in the number of copper and sulphur atoms. X axis is m/z; Y axis is percent intensity.

the molecular ion peak. It is more likely that only one copper atom is present. A series of possible isotopic signatures based on a difference of 0, 1, 2 or 3 sulphur atoms and 0, 1 or 2 copper atoms is in Figure 4.11. These signatures were calculated using Chemputer1.

If a peptide, a molecule of this weight would contain approximately 4–5 amino acids. This would require a minimum of six nitrogen atoms. Thirty-five carbon atoms were chosen to agree with a possible 30–45 carbon atoms taken from Table 4.3 and the other atoms were assigned so that a complex with one copper atom would sum to around 697 Da. Comparison of Figure 4.11 to the 697 signature (Fig-

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1http://winter.group.shef.ac.uk/chemputer/isotopes.html (28 May 2006)
4.3. Mass spectral characterisation of peaks of interest

<table>
<thead>
<tr>
<th>Eluent</th>
<th>697 (intensity)</th>
<th>1329 (intensity)</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td>✓ (very low)</td>
<td>✓ (very low)</td>
</tr>
<tr>
<td>3</td>
<td>✓ (high)</td>
<td>✓ (high)</td>
</tr>
<tr>
<td>4</td>
<td>✓ (high)</td>
<td>✗</td>
</tr>
<tr>
<td>5</td>
<td>✓ (high)</td>
<td>✗</td>
</tr>
<tr>
<td>6</td>
<td>✗</td>
<td>✗</td>
</tr>
</tbody>
</table>

Table 4.4: The presence of peaks of interest in the eluent fraction of experiment 6C, using MALDI TOF.

ures 4.6, 4.9 and 4.10) leads to the conclusion that the 697 complex must contain one copper atom only. From the variation in number of sulphur atoms (plots E–H) it is possible that any of the plots could match the abundance of the M+2 peak presented in Table 4.3. Thus the complex probably contains 0 to 3 sulphur atoms.

4.3.3 Reconciliation between the 697 ion and electrochemical data

In Section 4.1.2 it has been determined that the strong copper binding ligand is present in eluents 3 and 5. Initially only these eluents were screened using MALDI TOF. However, all eluents, 2, 3, 4, 5 and 6 were then screened using MALDI TOF to determine whether or not the presence of the 697 ion or the 1329 ion in the eluent fractions correspond to the presence of the the strong binding ligand, determined using electrochemistry. MALDI TOF data cannot be considered quantitative, so only presence-absence (and a qualifying description) can be determined. All five eluents were screened and the results are presented in Table 4.4.

Table 4.4 shows that the ion 697 is present in the three eluents, 3,4 and 5. Electrochemical determinations have shown that the strong copper binding ligand is present in eluents 3 and 5 (and therefore also likely present in eluent 4). Therefore the strong ligand presence can be correlated to the presence of the 697 ion in mass spectrometric data. This is the strongest evidence that the 697 ion is likely the strong copper binding ligand grown from culture samples, even though this complex was observed in only 2 of 5 culture experiments (this is discussed in more detail in Section 4.5). This evidence is not conclusive, however it does show that
we cannot rule out the 697 ion as a possible strong copper binding ligand.

4.4 Structural determinations from the fragmentation of the 697 ion

Structural information can be gained from the MS/MS fragmentation patterns of an ion. Initially, only the fragmentation of the 697 ion was possible. Fragmentation leads to a lowering of the signal to noise ratio and in the case of the 699 ion (already less abundant than the 697 ion) the fragmentation spectrum became too noisy. In order to overcome this low signal to noise ratio, samples from the second enriched copper spiking experiment were used. These samples were spiked with $^{65}$Cu prior to column extraction, to a final 63:65 copper ratio of 1:1. Isolation and fragmentation of these two ions were then conducted separately by Doug Olson (NRC-PBI, Saskatoon) using a Waters-Micromass Q-TOF Ultima Global hybrid tandem mass spectrometer using MALDI as the ionisation source. Prior to analyte introduction Glu-fibrinopeptide was used as a mass calibrant, both analyte and Glu-fib were introduced using CHCA as the matrix. A comparison of the MS/MS spectra of the 697 and 699 ions is in Figure 4.12, where peak masses are in integer values. More detailed spectra for smaller mass regions can be found in Figures C.1 to C.6.
Figure 4.12: MALDI QTOF MSMS spectra of the 697 and 699 ions.
4.4. Structural determinations from the fragmentation of the 697 ion

<table>
<thead>
<tr>
<th>Ion pair</th>
<th>Δ Mass</th>
<th>Expected</th>
<th>Abs error (Da)</th>
<th>Rel error (ppm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>697–679</td>
<td>18.0184</td>
<td>18.0106</td>
<td>0.0078</td>
<td>433</td>
</tr>
<tr>
<td>679–661</td>
<td>18.0441</td>
<td>18.0106</td>
<td>0.0335</td>
<td>1860</td>
</tr>
<tr>
<td>576–558</td>
<td>18.0240</td>
<td>18.0106</td>
<td>0.0134</td>
<td>744</td>
</tr>
<tr>
<td>558–540</td>
<td>17.9928</td>
<td>18.0106</td>
<td>-0.0178</td>
<td>-988</td>
</tr>
</tbody>
</table>

Table 4.5: The error between the expected mass for water loss in fragmentation data and the observed exact masses.

From this figure a lot of detail is apparent. The fact that this has been obtained by a time of flight analyser should under more ideal circumstances provide good exact mass data. This is implied by the number of decimal places for the fragments in the equivalent Figure C.1 and C.2–C.6. However, only integer values are provided here as the error in more precise values is too high. Typically the acceptable error for these exact mass determinations is 20 ppm at the most, as is the case with MS/MS experiments on model compounds. The spectra presented here, however do not have that precision. This can be displayed by the exact mass differences between ions 697-679 and 679-661 which both clearly correspond to water loss. A summary of the absolute and relative errors on these mass differences is provided in Table 4.5. The larger error for this analyte (unlike other model compounds) is a result of the extremely challenging analytical problems that are associated with this analyte: extremely low concentrations, unknown complex type (e.g. protein or siderophore) and highly interfering salts as well as the presence of generic DOM.

As a result of these large error ranges the spectra have been interpreted using integer values for peak masses only. The exact mass information is difficult to obtain because of low signal to noise ratios due to a low analyte concentration in the presence of high amounts of dissolved organic matter. These spectra were obtained from a combination of eluents 3 and 4 both of which contain significant DOM as they were the first two aliquots that contain methanol, which will extract generic DOM from the column.

In Figure 4.12 there are some peaks that occur in both the 697 MS/MS spectrum and the 699 MS/MS spectrum, for example the peak at m/z 449. As it occurs at the same mass in each spectrum then this ion cannot contain copper. However, if
similar peaks occur in the two spectra but with a mass shift of two units, for example m/z 487 and 489, then this is the same ion but containing a different isotope of copper (as with the 697 and 699 ions). In this way, the ions that contain copper can be identified. Also small losses can be indicative. For example, the mass difference between 697 and 679 corresponds to a loss of 18 Da and is therefore most likely due to the loss of a water molecule. The peaks at m/z 281 and 283 are of particular interest as they are the lowest mass ions that still contain copper. If we know the oxidation state of copper then the mass of the fragment bound to the copper can be determined. Since this is a low mass, the number of possibilities for its structure is lower.

The loss of 64/66 mass units in two places is also of interest. These loses occur at ions m/z 513 \rightarrow 449 and m/z 429 \rightarrow 365. Since the ions m/z 513 and 429 both contain copper (as the 699 spectrum contains the ion shifted by two mass units) and both ions m/z 449 and 365 do not contain copper, it is likely that this loss of mass is due to the loss of copper plus one. Therefore this is most likely due to the loss of copper(I) hydride. Copper hydride is a possible neutral loss as it has a reasonably strong bond energy (61 kcal/mol), stronger than other transition metals (Co, Ni, Fe and Zn) [75]. The loss of copper hydride indicates that copper is dissociating in the +1 oxidation state. This can also be further substantiated by other neutral losses discussed below. If copper dissociates from the ligand in the +1 oxidation state, it is feasible that it is bound in the +1 oxidation state. This is further speculative evidence that copper may be bound in the +1 oxidation state in the 697/699 complex, as well as the electrochemically determined binding constant where it is more conceivable that copper is in the +1 oxidation state than in the +2 oxidation state (Section 3.4). However, it is possible that copper(II) is present in the complex, but that, following ionisation and fragmentation it could have been reduced during collision induced dissociation, particularly when nitrogen and sulphur donor atoms are present in the ligand [76]. The loss of a hydride also results in the formation of a radical ion (as opposed to a protonated ion). This can change the fragmentation patterns of a peptide and make the spectra quite complicated [77].

It is questionable as to whether or not this complex is a peptide. Metallated peptides and fragmentation via radical ions complicate the product ion spectrum compared to the product ion spectra of protonated peptides [77]. As discussed
4.4. Structural determinations from the fragmentation of the 697 ion

<table>
<thead>
<tr>
<th>Amino acid</th>
<th>Residue mass</th>
<th>Neutral loss location</th>
<th>No. observations</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ala</td>
<td>71</td>
<td>558 → 487, 487 → 416, 290 → 219</td>
<td>3</td>
</tr>
<tr>
<td>Pro</td>
<td>97</td>
<td>513 → 416, 219 → 122</td>
<td>2</td>
</tr>
<tr>
<td>Asp</td>
<td>115</td>
<td>531 → 416, 515 → 400†</td>
<td>1</td>
</tr>
<tr>
<td>Glu</td>
<td>129</td>
<td>558 → 429, 531 → 402</td>
<td>2</td>
</tr>
<tr>
<td>Phe</td>
<td>147</td>
<td>576 → 429</td>
<td>1</td>
</tr>
<tr>
<td>Arg</td>
<td>156</td>
<td>558 → 402</td>
<td>1</td>
</tr>
</tbody>
</table>

Table 4.6: The possible amino acids present based on the observation of neutral losses from MALDI QTOF MSMS spectra. †This neutral loss is clear only in the 699 MS/MS spectrum.

above, it is possible that this complex is a metallated peptide, and that it fragments via radical ions. This explains the complicated spectrum and so it is plausible that this is a copper-peptide complex. Given that the error of the exact masses for the fragments is large, these spectra cannot provide clear data for an ultimate structural solution. These spectra have been interpreted given the information provided and the likelihood that this complex is a peptide is discussed, however the true nature of this complex cannot be proven.

The loss of several water molecules is indicative of the presence of a hydroxyl amino acid: either serine or threonine, however there are no direct mass losses that provide direct evidence that they are present. An example of an amino acid that is more likely present is alanine, based on the observation of a neutral loss that corresponds to the residue (Δ71 units between the following pairs: 558 → 487, 487 → 416 and 290 → 219). As the mass difference of 71 occurs before and after the 487 ion, it is also possible that there are two alanine residues present. Other evidence for the possible presence of amino acids can be found in Table 4.6.

The amino acids in Table 4.6 are potentially present, particularly those with a higher number of occurrences. However these amino acids have dissociated individually and are not likely to be bound to the copper atom. It can be noted that none of the amino acids here contain a sulphur atom even though other evidence (discussed above) points towards the presence of sulphur. This implies that sulphur containing amino acids may be bound to copper, which is in the +1 oxidation state.
4.4. Structural determinations from the fragmentation of the 697 ion

<table>
<thead>
<tr>
<th>Amino acid</th>
<th>Residue mass</th>
<th>Neutral loss location</th>
<th>No. observations</th>
</tr>
</thead>
<tbody>
<tr>
<td>Asn</td>
<td>114</td>
<td>531 → 355</td>
<td>1</td>
</tr>
<tr>
<td>Pro</td>
<td>97</td>
<td>281 → 122</td>
<td>1</td>
</tr>
<tr>
<td>Ile</td>
<td>113</td>
<td>540 → 365</td>
<td>1</td>
</tr>
<tr>
<td>Met</td>
<td>131</td>
<td>558 → 365</td>
<td>1</td>
</tr>
<tr>
<td>Cys-cm</td>
<td>161</td>
<td>513 → 290</td>
<td>1</td>
</tr>
<tr>
<td>Trp†</td>
<td>186</td>
<td>697 → 449</td>
<td>1</td>
</tr>
</tbody>
</table>

Table 4.7: The possible amino acids present and potentially bound to copper(I), based on the observation of neutral losses (one amino acid residue mass +62 Da) from MALDI QTOF MSMS spectra. Cys-cm is carboxy-methyl cysteine. †Tryptophan is isobaric with the two amino acid residue pairs (Glu, Gly) and (Asp, Ala).

(deduced from the observation of the loss of copper hydride).

The difference in mass between the ions observed to contain copper and those that do not contain copper, minus 62 Da (the mass for copper in oxidation state +1, in exchange for one proton) can therefore indicate possible amino acids that are bound to copper. These potentially copper binding amino acid residues are presented in Table 4.7. Each of these possibilities occurs just once which decreases the likelihood that these residues are present, unless combined with other observations.

It is possible that copper is present in the +2 oxidation state, a list of these amino acid–copper(II) neutral losses are in Table 4.8. The neutral loss of a single amino acid bound to copper(II) would necessitate the interaction of the metal with the side chain and for the amino acid to have an overall -2 charge when not bound to copper. From Table 4.8, the amino acid alanine can be eliminated as it does not have side chain interaction with copper(II) [78], thus this classification can be ruled out. Of the rest, proline, tryptophan, and asparagine are also present in Table 4.7, this shows that these amino acids may be present, and can bind copper in either oxidation state. Thus, it is possible that this ligand contains copper bound in the +2 oxidation state, however, based on the mass spectral data it is not more likely than with copper bound in the +1 oxidation state.

Furthermore, there is a likelihood that this complex contains sulphur (based on
4.4. Structural determinations from the fragmentation of the 697 ion

<table>
<thead>
<tr>
<th>Amino Acid</th>
<th>Residue Mass</th>
<th>Neutral Loss Location</th>
<th>No. Observations</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ala</td>
<td>71</td>
<td>487 → 355, 281 → 149</td>
<td>2</td>
</tr>
<tr>
<td>Pro</td>
<td>97</td>
<td>513 → 355</td>
<td>1</td>
</tr>
<tr>
<td>Asn</td>
<td>114</td>
<td>540 → 365</td>
<td>1</td>
</tr>
<tr>
<td>Asp</td>
<td>115</td>
<td>531 → 355</td>
<td>1</td>
</tr>
<tr>
<td>Trp†</td>
<td>186</td>
<td>558 → 311</td>
<td>1</td>
</tr>
</tbody>
</table>

Table 4.8: The possible amino acids present and potentially bound to copper(II), based on the observation of neutral losses (one residue mass +61 Da) from MALDI QTOF MSMS spectra. †Tryptophan is isobaric with the two amino acid residue pairs (Glu, Gly) and (Asp, Ala) and this loss is present in the 697 product ion spectrum only.

the isotopic signature) and, there is circumstantial evidence that this complex is the strong copper binding ligand whose stability constant is more conceivable if copper is in the +1 oxidation state. The loss of 64/66 mass units also suggests that copper is bound in the +1 oxidation state. The possible presence of two cysteine residues also implies that copper is in the reduced form, as cysteine will reduce copper, and stability constants for copper(II) and cysteine are often not available because of this redox activity. In seawater, the reduction of copper by a thiol is further substantiated because halides are present and can stabilise copper(I) [78]. Therefore, it is more likely the that 697/699 complex contains copper in the +1 oxidation state.

Typical fragmentation of peptides can result in fragment peaks that correspond to the immonium ion of the amino acid residues. The immonium ion is a result of a rearrangement of the residue and has the general formula H₂N⁺=CHR [79]. Some immonium ions have smaller masses than the mass spectrometer scanning range. The MS/MS spectra presented here scan from m/z 100 to 700 only. Each of the likely amino acids are discussed individually so that all observations can be taken into account. These possible assignments and others referred to below are illustrated in Figure 4.13 and are not confirmed findings.
Figure 4.13: MALDI QTOF MSMS spectra of the 697 ion with potential amino acid assignments. Detailed, expanded spectra are also provided in Appendix C in Figures C.1 to C.6.
4.4. Structural determinations from the fragmentation of the 697 ion

**Alanine (Ala), 71 Da**

The presence of alanine is possible as the residue mass (71 Da) can be seen as a neutral loss in three places. The neutral loss of 71 units is most obvious from peak 290 as both 290 and 219 have high intensities. The corresponding immonium ion (peak at m/z 44) is below the scanning range of the mass spectrometer. The peak at m/z 219 could be due to the amino acid pair alanine-phenyalanine, however this ion could also be due to Cys-Asp or Met-Ser and there is no direct evidence for the presence of phenylalanine. The loss of alanine also occurs twice (sequentially) from the m/z 558 peak implying that there could be two residues present (558 → 487 → 416).

**Aspartic acid (Asp), 115 Da**

The presence of aspartic acid is possible through the direct loss of the neutral residue (531–416). This residue mass loss is also possible through the neutral loss of a residue pair (alanine-aspartic acid) if bound to copper(I) in exchange for a proton (at 697 → 449). The immonium ion (88 Da) and other associated ions are below the scanning range. Further it is possible that the ion at m/z 219 contains aspartic acid with Cys to make up the ion at 219. Aspartic acid has a carboxylic acid functional group which has a pKa of 3.71 [80] so copper could potentially bind through the hydroxy oxygen. However, the affinity of Cu(I) for this amino acid is moderate (lower than histidine and higher than proline or alanine) [81].

**Asparagine (Asn), 114 Da**

The presence of asparagine is possible through the loss of asparagine bound to copper(I), (531 → 355) however these peaks are of low intensity and the neutral loss of asparagine alone is not observed. It is also possible through the loss of asparagine plus copper(II), with higher intensity peaks, however it is not likely that asparagine would lose two protons in exchange for Cu(II) to form a neutral fragment. The immonium ion for asparagine should be observed at m/z 87, however this is below the scanning range. Therefore there is not strong evidence to assert that asparagine is present.
4.4. Structural determinations from the fragmentation of the 697 ion

Cysteine (Cys), 103 Da

Cysteine can be present as the neutral residue, or if there are two residues present, then it can also be present through the oxidation of the two sulphur thiol groups to form a sulphur bridge. If this were the case, then a mass difference of two (corresponding to the loss of two protons) between the oxidised and reduced forms would be observed (e.g., two cysteines would sum to 206 Da, whereas cystine would sum to 204 Da). The oxidised form (cystine) is not observed here. Cysteine has been assigned as present through the peak at m/z 122, where if it were a C-terminal amino acid and fragmented at the peptide bond (between the carbonyl carbon and the secondary amine nitrogen) the mass would be Cys (103) + OH (17) + 2H (2) for a charged species and making it a \( y_1 \) ion. Cysteine is also a candidate for binding copper, particularly in the +1 oxidation state as it has a high affinity for Cu(I) [81].

Cys-Cys-Cu(I) amounts to a neutral loss of 268 units (with copper exchanged for a proton) which must occur from an ion that contains copper to an ion that does not contain copper. This loss can be observed in two places. Between the copper containing ion at m/z 558 (to 290) and at m/z 487. Thus it is possible that cysteine exists at the C-terminal end of the peptide and that there are two cysteine amino acids adjacent to each other along the chain. The immonium ion is below the scanning range of the spectrometer. However, it is also possible that two cysteine residues are present but not adjacent to each other as is discussed below (in the Proline section).

The presence of cysteine as a \( y_1 \) ion is also discussed in the m/z 167 ion section where an alternate explanation for the presence of the 122 ion is considered.

Glutamic acid (Glu), 129 Da

The peaks that indicate the possible presence of this amino acid occur twice between m/z 531 → 402 and m/z 558 → 429. The immonium ion for glutamic acid should occur at m/z 102 and is not observed in the spectrum. Thus it is also possible that this amino acid is not present.
4.4. Structural determinations from the fragmentation of the 697 ion

**Methionine (Met), 131 Da**

Methionine is another candidate for binding copper as a +1 ion, and it has been found to have a higher affinity for copper(I) than cysteine (in the gaseous phase) [81]. Methionine is potentially present through one observation only, and only if bound to copper(I) in exchange for a proton, this occurs from the copper containing ion 558 to the copper free ion at m/z 365. The immonium ion associated with this residue should be at 104 and is not present in the spectra. Thus it is also possible that methionine is not present. However, a second possibility is the ion at m/z 219 whose mass could sum to three possible amino acid pairs (Met-Ser, Asp-Cys or Phe-Ala). If methionine is part of this ion then serine must also be present.

**Proline (Pro), 97 Da**

Proline has been listed as a possible amino acid because the associated neutral loss of the residue (97 Da) occurs twice in these spectra. It occurs firstly from the copper containing ion at m/z 513 to the copper containing ion at m/z 416 as well as a neutral loss from the copper free ion at m/z 219 to the copper free ion at m/z 122 which is possibly a cysteine amino acid. Thus if proline is present it is possible that it is bound to cysteine. Also, it falls off sequentially after the loss of what could be alanine at the m/z 290 peak. This implies that proline could connect the C-terminal cysteine to alanine. However if this were the case, then the second cysteine amino acid could not be directly associated with the first C-terminal cysteine. This is possible as the two cysteine amino acids are only found together when bound to copper. Thus copper could be holding two cysteine amino acids together when they are not bound to each other directly. However the presence of dicysteine is present with and without copper. The presence of proline in the middle of the chain means that it cannot co-ordinate a metal [82], thus the observation that proline exists only as a non-coordinated amino acid is reasonable.

**Serine (Ser), 87 Da and Threonine (Thr), 101 Da**

The evidence for the presence of these amino acid residues is solely the loss of several water molecules from peaks 697 and 576. The hydroxyl group on the side chain of these residues makes this loss more likely [79]. However there is no direct
4.4. Structural determinations from the fragmentation of the 697 ion
evidence for their presence. The immonium ions are m/z 60 and 74 for serine and
threonine, respectively, and are below the scanning range of the mass spectrometer.
Indirectly it is possible that serine is present as part of an amino acid pair with
methionine as the peak at m/z 219. However it is unlikely as the other possible
explanations for this ion have more direct evidence for their presence.

Tryptophan (Trp), 186 Da
Tryptophan is only possibly present if bound to copper and tryptophan does have a
high affinity for copper(I) [81]. Tryptophan is also nearly isobaric with alanine plus
aspartic acid who are already deemed possibly present through the direct neutral
loss of their corresponding residues. The immonium ion for tryptophan would be
at m/z 136 which is not present. Other ions associated with Trp are also not present
(77, 117, 130 and 132 [79]).

The peak at m/z 167
The peak at m/z 167 is an intense peak that can also be seen as a neutral loss from the
697 ion to the 531 ion. This ion does not correspond to an amino acid residue and
cannot be reconciled with the possibility that this complex may be a peptide directly
by using amino acid masses. However, at lower masses there are peaks present
which may be related to it. The peaks at m/z 149 and 139 correspond to the loss of
water and carbonyl groups respectively. Also the peak at m/z 122 corresponds to the
loss of an ammonia group from the m/z 139 ion. These three ions therefore show
that the m/z 167 ion exhibits the loss of water, ammonia and carbonyl groups which
is typical of a b1 ion of a fragmentated peptide. Thus it is possible that if this molecule
is a complexed peptide then the m/z 167 ion could be a modified amino acid. This
would negate the claim that the m/z 122 ion is a y1 cysteine ion. However, with
either explanation the possibility still exists that this could be a peptide.

Summary
These amino acids are presented as possibilities only as are the ions and neutral
losses presented in Figure 4.13. The presence of 2Cys, Ala, Asp, Pro, water and
Cu(I) have been more strongly alluded to, however the sum of these amino acids
4.4. Structural determinations from the fragmentation of the 697 ion

(569 Da) does not account for the mass of the whole peptide. There is a remainder of 127 mass units. Were this a copper(II) complex then it is possible that the remainder 127 mass units could correspond to a glutamic acid amino residue, in exchange for a second proton so that the non-metallated peptide has a negative charge. However, it is quite clear that this is a copper(I) complex and so the remainder 127 mass units cannot be explained by any of the 20 most common amino acids. The presence of the m/z 167 ion also cannot be explained by the 20 essential amino acids.

It is usual that a protein or peptide should fall apart along the backbone of the peptide, where residues are sequentially lost [79, 83]. The possible presence of the m/z 167 peak as an N-terminal ion or cysteine as a C-terminal ion indicates that this complex could be a linear peptide. However the lack of sequential amino acid residue losses makes this spectrum difficult to interpret and it is questionable as to whether or not it is actually a peptide at all. However, if this were a peptide it would not be a typical peptide as it binds copper and potentially quite strongly. Copper could keep certain residues together that are not necessarily bound together if the peptide is wrapped around the copper centre. This is a possible explanation as to why sequential losses of amino acids are not observed.

In summary, this data alone is not enough to elucidate the structure of the ion at m/z 697. However there is information that can be gained about this ion; a summary is provided in Table 4.9.
<table>
<thead>
<tr>
<th>Observation</th>
<th>Location</th>
<th>Inference</th>
<th>Supporting evidence</th>
</tr>
</thead>
<tbody>
<tr>
<td>Loss of water molecules</td>
<td>$697 \rightarrow 679 \rightarrow$</td>
<td>Presence of Ser or Thr in these fragments [79]</td>
<td>none: no direct losses of Ser or Thr (Table 4.6)</td>
</tr>
<tr>
<td></td>
<td>$661, 576 \rightarrow$</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>$558 \rightarrow 540$</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Loss of 64/66 units</td>
<td>$531 \rightarrow 449, 429 \rightarrow 365$</td>
<td>Loss of CuH; copper probably bound in $+1$ oxidation state</td>
<td>Copper(I) also possibly attached to cysteine</td>
</tr>
<tr>
<td>Isotopic signature</td>
<td>$697$</td>
<td>Suggests one copper atom and 0–3 sulphur atoms</td>
<td></td>
</tr>
<tr>
<td>Presence of C-Terminal $y_1$ ion</td>
<td>$122$</td>
<td>A (possible) linear peptide</td>
<td>Conflict: $b_1$ ion at 167</td>
</tr>
<tr>
<td>Presence of N-Terminal $b_1$ ion</td>
<td>$167$</td>
<td>Linear peptide, modified amino acid present</td>
<td>Conflict: $y_1$ ion at 122</td>
</tr>
<tr>
<td>Copper bound to multiple amino</td>
<td>Cys-Cu, Ala-Asp-Cu and Met-Cu</td>
<td>Copper holding together parts of the peptide that are not directly joined</td>
<td>Peptide does not sequentially fragment (typical of non-complexed peptides)</td>
</tr>
<tr>
<td>acid residues</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table 4.9: A summary of observations and inferred conclusions about the chemical nature of the 697 ion.
4.5 Discussion and conclusions

In this chapter two ions, with m/z 1329+ and 697+, have been investigated. The interest in the 1329+ ion is that it was present in all experiments. However, based on the isotopic signature it probably does not contain copper, and is shown to bind copper at high copper concentrations only. It is also probably responsible for some surface activity in electrochemical experiments. Since the 1329 m/z ion was present in the third eluent only (and not eluents four and five) it is most likely not the strong copper binding ligand described in Chapter 3.

The 697+ ion was present in eluents 3, 4 and 5 for experiments 5 and 6. A strong copper binding ligand was tracked using electrochemical pseudo-polarograms and was also found to be present in eluents three and five (and is therefore probably also present in eluent 4). Therefore it is possible that the 697+ ion is a strong copper binding ligand. Further, it was the only ion in all experiments that was found to have an isotopic signature that reflects the presence of copper.

Further investigations into this ion show that it most likely does contain copper, in the +1 oxidation state. This in turn implies that sulphur is present, although the isotopic signature is too variable to confirm this (isotopic peaks suggested 0–3 sulphur atoms). The evidence that copper is present is further substantiated by the fragmentation data of the 697+ and the 699+ ions and by tracking where the fragments are present but two mass units apart (as with the two copper isotopes).

Fragmentation data of the 697+ ion indicate that this complex could be a peptide, but exact characterisation is not possible with the information available. It is possible that two cysteine amino acids are present along with other amino acids including alanine, aspartic acid and proline.

The 697+ ion is present in two grow-up experiments only (experiments 5 and 6), from cultures exposed to pCu 10 and 12 respectively, and it is surprising that these experiments would be similar to each other and dissimilar to culture experiments exposed to the same copper concentrations. The similarity of these experiments (compared to the others) is that they were the most recent culture grow-ups and were grown at times that overlap. Strong copper binding ligands were found at similar concentrations in all five experiments using electrochemistry. However, for the mass spectral determinations, the length of time between defrosting the sample
4.5. Discussion and conclusions

and running it through the column is an extra twenty-four hours compared to those samples defrosted for electrochemical analysis. There is a further twenty-five hours for the entire sample (1000 mL) to run through the column, which is followed by extraction. In contrast, electroanalysis of samples was completed within less than twenty four hours of the original time of defrosting. Thus from the time of removing from the freezer, electrochemical samples were completely analysed thirty-six hours later, whereas complete extraction was accomplished seventy-two hours after removal from the freezer. It is possible that samples that had been in the freezer for a full year longer than experiments 5 and 6 would degrade faster once warmed up. Degradation does not have to be complete but enough that the concentration was lower than the limit of detection. Another possible explanation is that the culture strains had changed between being exposed to high copper concentrations from one year to the next. However, that two independent cultures (acclimated to different pCu concentrations) would change the same way is unlikely and so the former explanation is more likely.

The possibility that copper is bound as a 1+ ion in the $697^+$ ion (as we see the same molecular ion present in MALDI as well as ESI spectra) would also mean that in electrochemical determinations the number of electrons transferred is only one. This would have an effect on the log $K'$ value determined using the pseudopolarograms according to Equation 3.7, in which case the log $K'$ would be halved and the strong copper binding ligand would have a log $K'$ of 24.7 a value that agrees more with determinations of log $K_c$ from other research methods (Tables 1.2 and 1.3). This further suggests that the $697^+$ ion could be the strong copper binding ligand.
Chapter 5

Discussion and conclusions

5.1 Summary

5.1.1 Cultures
Using batch culture experiments, cyanobacteria were maintained in log phase growth and acclimated to two copper concentrations, pCu 12 and 10. The lower copper concentration (pCu 12) was intended to be less stressful. However, the growth rates of all cultures were indistinguishable. Experiment 2, which has a much shorter lag time than corresponding replicates, is concluded to be anomalous. The inoculum for this experiment had a higher cell density and was therefore able to condition the media faster.

The filtrate of all cultures was harvested when the cultures were in log phase growth. This was to ascertain that strong copper binding ligand production was a direct response to copper toxicity as opposed to indirect production through cell lysis during senescence (death phase).

5.1.2 Electrochemistry
Filtrate samples were analysed using pseudo-polarography. Strong copper binding ligands were found in all samples. Two ligand classes were identified in all of the experiments, a weaker ligand class (discussed below) and a stronger ligand class with log K's ranging 24.2–25.5 if a copper(I) is assumed. The ionic strength corrected binding constants of these strong ligands were very similar, implying that the strong ligand may be the same chemical species in all experiments. If this is the case then the production of copper binding ligands as a direct response to copper toxicity is likely.

Weak ligands were also found in all cultures, although their binding constants
5.1. Summary

showed large variability (log K's ranging 15–23). This implies that the weak ligands were not the same mixture (of ligands) in all experiments and were probably not produced because of copper toxicity. The concentrations of the weaker ligand class were greater than the strong ligands. However, the strong ligand concentrations were still enough to buffer the toxic copper that cells had been acclimated to.

The construction of pseudo-polarograms was used as a helpful tool for tracking the strong copper binding ligand throughout the resin selection and ligand extraction procedure. It showed that SM-2 biobeads (Biorad) did not remove the strong ligand from seawater, whereas, the XAD-16 resin does extract the ligand, although elution with methanol-water mixtures does not recover the ligand into one distinct fraction. Instead, the strong ligand elutes continually into three main fractions, as soon as the dielectric constant of the solvent is lowered from that of water. This work is in agreement with Ross et al. [41] as our successful resin, (XAD-16) was also used for copper ligand extraction in their study.

5.1.3 Mass spectrometric determinations

The first methanol-water eluent and the first methanol only eluent (eluents 3 and 5) of all samples were initially screened using MALDI TOF MS, a useful technique for investigating intact molecules, while using little analyte sample. Two peaks of interest were identified: (i) the 1329+ ion because it occurred in all cyanobacterial experiments, and (ii) the 697+ ion, although it was present in only two experiments, it was the only ion detected that had an isotopic signature that reflected that of copper.

Spiking experiments using enriched 65Cu were undertaken to determine whether or not copper is present in the 697/699 complex. This data showed that copper is present, and that the complex also probably contains one or more sulphur atoms. The isotopic signature in unspiked samples implied one copper atom, and 0-3 sulphur atoms.

The ion at m/z 1329 was present in all culture samples. Once column extracted, this ion was present in the first methanol-water eluent only. This indicated that this ion was not the strong copper binding ligand which was found in several eluents. Whereas, the 697/699 complex presence in eluent fractions reflected the presence
5.2 Discussion

of the strong copper binding ligands detected using electrochemistry.

To decipher the chemical nature of the complex, tandem mass spectrometry, using MALDI, was employed to isolate the 697\(^+\) ion and fragment it. The sample used had been spiked with \(^{65}\text{Cu}\) to increase the signal to noise ratio in the 699 ion MS/MS spectrum. Comparison of these two spectra has shown that copper is present in the complex, likely in the +1 oxidation state. The presence of the ion at m/z 122 may indicate that this is an (unusual) peptide. It is possible that cysteine is present as a C-terminal amino acid ion (to sum 122 Da). Alternatively, the loss of ammonia, water and carbonyl groups may indicate the possible presence of a b ion, explaining these high intensity low mass ions. Therefore, it is possible that this complex is a linear peptide that wraps around the metal centre, such that the fragmentation of this complex is unusual for a (non-complexed) peptide. Thus a tentative interpretation of these spectra as a metallated peptide is reasonable.

5.2 Discussion

5.2.1 The ion at m/z 1329

The isotopic signature of the peak at m/z 1329 indicates that this complex does not contain copper. However, it is possible that the isotopic signature can be misleading. The 1329 ion may be a dimer of the 697 ion by sulphur dimerising across a sulphur-sulphur bridge with a complexed copper ion in its +1 oxidation state. Where, if the ion at m/z 697 was represented by CuLSH\(^+\), then the m/z 1329 ion would be CuLSSL\(^+\). It would still be possible to bind a second copper atom in the +1 oxidation state if two thiol functionalities were present in each monomer, as implied by the mass spectral data in Chapter 4. If this were the case then the same complex would be present in all culture batches (in agreement with electrochemical data). However the two complexes (1329 and 697) could not be connected by any further data other than the likely coincidence of the molecular weights.
5.2. Discussion

5.2.2 Oceanic strong copper binding ligands and the 697/699 complex

The oxidation state of copper in the 697/699 complex

Chapter 4 presents the product ion spectra of the 697/699 complex. As the 697/699 complex does not change mass number in the electrospray ionised spectrum it is likely that the speciation of this complex is not altered by ionisation using MALDI. The observed neutral losses have indicated that the presence of thiols in this complex is likely. Thiols are well known for binding copper(I) more strongly than copper(II), and, are also known to reduce copper on complexation. Furthermore, the likely loss of CuH in two places suggests that copper(I) is present in the ions. Observed neutral losses that imply the presence of copper(II) are less frequent. Therefore, there is stronger evidence that the 697/699 complex binds copper in the +1 oxidation state through thiol group(s).

If we assume a copper(I) complex, electrochemically determined log K' values become more reasonable and in line with other studies that have determined conditional binding constants (Table 1.3). A copper(I) complex would have a log K' of 24.7, as opposed to 49.4 for a copper(II) complex. It is possible that the m/z 697 ion detected using mass spectrometry is also the strong copper binding ligand detected using electrochemistry, since the strong copper binding ligands are present in the same eluent fractions as the m/z 697 ion. This was the only copper containing ion detected and the only ion of interest to coincide with the strong copper binding ligand presence in the eluent fractions. This work therefore suggests that strong copper binding ligands bind copper in the +1 oxidation state.

The oxidation state of copper in strong oceanic binding ligands

Stability constants for thiol complexes with copper(I) are greater than those with copper(II), and the log Kc for thiol–Cu(I) complexes are reported to be 10–11 [12]. Given that these are conditional constants, these values are reasonable when compared to the ionic strength corrected values presented here. Therefore, the presence of (L1) copper(I) complexes in the open ocean is possible and, the strong copper binding ligand here (possibly the 697/699 complex) has a comparable binding
5.2. Discussion

strength to them.

In the surface waters of the open ocean, possible mechanisms that will reduce copper(II) to (I) and stabilise it are:(i) the photo-reduction of copper, like iron [84, 85]; (ii) the reduction of copper by sulphite ions brought to the surface waters in atmospheric water droplets [84, 86] and (iii) the stabilisation of copper(I) by chloride ions [12]. Leal and van den Berg [12] investigated the presence of strong copper(I) binding ligands in the ocean using electrochemistry. They showed that, upon titration with copper(II) ions, copper (I) ions were formed within one hour. In their study, the presence of thiols was invoked to be necessary for the reduction of copper within the complex. In culture experiments presented here, copper(II) was added to samples that were kept in the dark throughout defrosting and column extraction. Therefore, while photo-reduction and atmospheric sources are relevant for environmental samples, they are not relevant in this study. However, before both electrochemical and mass spectrometric determinations, the samples were allowed to equilibrate overnight, with copper(II) addition. Since thiols have been shown to be present (mass spectral data), it is likely that the thiols in the sample had enough time to reduce the added copper completely, overnight. The ligands present in this sample are therefore able to reduce copper independently, in a similar way to sulphites in rainwater. Thus, investigations of strong copper binding ligands in the ocean should also examine copper(I) as a likely species.

Detoxification mechanisms and the identity of strong copper binding ligands

There are two possible mechanisms of copper detoxification. Either strong copper binding ligands are exuded as free ligands that subsequently bind copper (siderophore style) or these ligands are exuded when already to bound to copper as an intracellular detoxification mechanism (metallothionein style). As the samples presented here contain two ligand types a simple copper titration will not determine whether or not the strong ligand is free on exudation.

Enriched copper spiking experiments have shown that ligands are either free on exudation and/or complexed copper is exchangeable, therefore a siderophore style detoxification mechanism cannot be ruled out. The 697/699 complex was found to be partially exchangeable under acidic conditions from pre-column spiking experiments (Figure 4.8). If these ligands are free then this is a siderophore-style
5.2. Discussion

(exudation of the free ligand) detoxification mechanism. If these ligands have exchangeable copper, the ligands must be capable of reversible copper redox reactions, in which case, they could be either metallothionein or siderophore style ligands. Metallothionein-style ligands would reduce copper inside the cell to detoxify it and then expunge from the cell. However, electroanalysis of field samples has shown that the strong copper binding ligand class is present at slightly higher concentrations than the total copper concentration suggesting that these are siderophore style ligands. Therefore while both copper binding mechanisms are possible it is likely that the most prevailing mechanism of copper detoxification in the ocean is by sulphur containing ligands in seawater (siderophore-style).

The identity and consequently the specificity of strong copper binding ligands has been the subject of debate. The likelihood that these complexes are siderophores is not very high given that these complexes probably bind copper through the +1 oxidation state (as the complex, 697/699, presented here does), while siderophores have hard donor atoms (O, N and C). Although nitrogen can bind copper(I) fairly strongly [81] it does not have the same capability as sulphur to reduce copper. The reduction of copper (by 697/699 complexation) in samples presented here, points to the presence of a thiol group, capable of copper reduction. As siderophores are catechols and hydroxamates (and not thiols) it is unlikely that they provide the identity to these ligands. Furthermore, in this study the electrochemically determined ligand was found not to extract onto SM-2 biobeads (BioRad) which was found to be the most successful resin on which to extract siderophores [70].

It is also possible that these ligands are phytochelatins. However, the 697/699 complex is not a polymer of glycine and cysteine only, as expected for a phytochelatin. In addition, the copper binding ligands found in this study are stronger binding than phytochelatins [87]. In support of the work presented here, thiol containing dipeptides produced by a copper stressed coccolithophorid have been shown to bind copper [43], and these are also not phytochelatins. The specificity of these ligands (L1) for copper has been shown by Coale and Bruland [3] as titration of natural copper ligands with lead, iron and zinc did not change the free concentration of copper (determined using ASV). This is also an indication that these ligands are not phytochelatins, which are known to bind several metals, and cadmium in particular. The specificity of the 697/699 complex has not been investigated. It has been found
that a diatom has a higher phytochelatin production in response to cadmium and not copper [88, 89], this example illustrates that phytochelatins are non-specific.

The premise of the production of these ligands is that they detoxify copper [36]. The (toxic) mode of copper uptake can be (i) accidental uptake through an ion channel protein at the cell membrane, (ii) formation of ternary complexes at the cell surface and subsequent incorporation into the cell [24], as with some siderophores [90] or (iii) the dissolution of lipophilic complexes (that contain a toxic element) across the membrane [24]. Copper uptake when bound to artificial organic ligands has been identified when the ligand is lipophilic [26] (and therefore hydrophobic). The hydrophobicity of strong copper binding ligands is probably low as the strongest ligands were not well extracted by C-18 SPE columns [40]. The 697/699 complex presented here was also not particularly hydrophobic as it elutes in methanol-water mixes and not just in methanol only. Further, the resin used for extraction (XAD-16) has been found to be successful for siderophores which also exhibit some hydrophilicity. If toxic uptake is by the solubility of the complex through the lipid membrane, then exudation of copper binding ligands to prevent toxicity would not be very effective. This mode of toxicity is therefore not likely. It is likely that accidental uptake is the mode of toxic copper uptake since diatom growth rates have been shown to decrease as a function of the free ion activity and not natural organic fractions (which would be necessary for the formation of ternary complexes) or the total concentration [25]. The prevention of accidental uptake of free copper may elicit a physiological response to either produce metallothioneins or excrete strong binding ligands.

**Availability of copper when bound to a strong ligand**

Even though copper can be toxic, it is also a metal required for growth. The strength of the copper binding ligands calls into question the copper bioavailability. However, although strongly bound to a ligand, redox enzymes may facilitate the oxidation of copper from L1 and release it. If this is the case then the necessity of copper for iron nutrition in iron limited diatoms [5] could be disrupted by the exudation of copper binding ligands. Therefore, if this copper-complex is not bioavailable, then the exudation of this ligand (siderophore-style) may have consequences in terms of competition with other organisms. If iron limited diatoms require more copper,
5.2. Discussion

It could be possible that these copper binding ligands render them copper limited as well, and therefore less successful. However, copper limitation in diatoms does not occur until a pCu of 15 [5], whereas the free copper concentrations in the open ocean where strong copper ligands exist are closer to pCu 13 [22]. Furthermore, diatoms have also been shown to produce strong copper binding ligands [4]. If these ligands are exuded as free molecules, as field data suggests, it still seems to be an expensive way to combat toxicity. Recent studies have modelled that copper availability was very low during the protozoic period (1–2 billion years ago) when cyanobacteria were initially evolving, and that copper(I) was the prevailing ion [91]. Thus, their requirements for copper were minimal reflecting early (protozoic) trace metal availability [92]. It has been hypothesised that cyanobacteria are particularly sensitive to high copper concentrations because they were evolving at a time when copper availability was low (when the planet was reducing) [50]. Yet, it has been shown that *Synechococcus* DC2 does utilise copper in the superoxide dismutase enzyme [16]. Thus, although copper availability was low, *Synechococcus* DC2 still uses copper (present day). It could therefore be possible that these strong copper(I) binding ligands were originally used for a different purpose: for the uptake of copper(I) in true siderophore style. Although it has also been proposed that metal binding ligands have been used to maintain cellular biochemical characteristics that are similar to protozoic sulphidic trace metal availabilities [91], the exudation of free ligands for detoxification remains an overly complicated mechanism compared to simple intra-cellular metallothionein/ phytochelatin production. Therefore, a further question can be posed: did copper utilisation in enzymes evolve later when copper became more available? Whilst highly speculative, the idea that copper binding ligands could have been used for uptake and only later for the prevention of toxic stress is plausible. The same genes (for copper transporters) have been found to be expressed under high copper as well as under low copper in a prokaryotic organism [93]. Also these same copper transporters have been found in a *Synechococcus* strain [94]. The use of the same transporter mechanisms for cell stress and deficiency has also been suggested for other metal transporters [95] including the expression for the Nramps di-cation transporters under iron replete and limited conditions in yeast [96]. Given that a metallothionein-style molecule would probably suffice as a detoxification mechanism, it is surprising that an expensive
molecule, such as L1 would be manufactured and then exuded. If a strong copper binding ligand is synthesised under toxic and limiting conditions, a secondary uptake mechanism would be necessary at the cell surface to access the bound copper. This is possible with the use of redox enzymes, in particular oxidases at the cell surface as has been shown for iron [5]. This secondary uptake mechanism may only be 'switched on' when cells are exposed to low copper conditions thus distinguishing toxic and limiting physiological responses.

5.3 Further work

The 697/699 ion has not been completely characterised. If the complete identity was known then, provided that the synthesis of this molecule is possible, the binding strength of this ligand could be determined independently to ascertain whether or not this is a candidate for the strong copper binding ligand class (L1). In order to determine its structural character more fully, it would be interesting to dissociate copper from the complex to then fragment the free ligand. If the free ligand is a protonated peptide it should more clearly fragment in a way that is typical of a peptide (sequential losses of amino acids along the backbone of the peptide).

Exact mass determinations would also provide further information about the binding site of copper. The exact mass of a small fragment would provide few possibilities of a chemical formula which could reveal the binding site of copper in this complex. Given the low signal to noise ratio, a more powerful instrument than the QTOF analyser would be necessary. The recent increase in availability of fourier transform ion cyclotron resonance mass spectrometers (FT ICR MS) would make this instrument an appropriate choice where resolution is greater than current analysers.

Electroanalysis of the strong copper binding ligand shows that the concentrations are low enough to reflect some open ocean ligand concentrations. It is unlikely that that open ocean samples contain one ligand only (and not a group of ligands). However, it would be possible to run much larger volumes of seawater through a resin to gain a greater concentration factor as has been achieved with siderophores [70] in order to isolate copper binding ligands in environmental samples. The resin method presented here could be combined with on-line analysis for
5.4 Conclusions

Cyanobacterial cultures were grown in high copper conditions so that they produced strong copper binding ligands during the growth phase. In this study, cultures produced two types of ligand: a strong ligand and a class of weak ligands. The ionic strength corrected binding constants for the weaker ligand class were highly variable, implying that they are not all the same ligand. The log $K'$ for the strong copper (such as HR ICP MS) in order to identify copper containing fractions.

The bioavailability of these strong copper binding ligands is of great interest. The first point for clarification is whether or not these ligands are produced as metallothionein-style molecules or siderophore-style molecules. The difference between these two detoxification mechanisms is that the first is intra-cellular and the second is extra-cellular. Therefore it may be possible to determine which mechanism is present based on radioactive copper uptake experiments. If cells were acclimated under toxic conditions and then exposed to radioactive copper, cellular uptake would occur if the detoxification mechanism was metallothionein style. These cells would then have to be harvested and resuspended into non-radioactive media. If the media then starts to contain radioactive copper then this would confirm that the mechanism is metallothionein style. The longest half life of the radioactive copper isotope is $^{67}$Cu of 2.6 days, followed by $^{64}$Cu with a half life of 12.7 hours. The bioavailability of ligated-copper could then be determined based on uptake experiments when cultures are copper limited. Furthermore, given that iron uptake mechanisms are thought to change depending on iron availability [97] it is possible that copper uptake mechanisms could change when under iron limitation given that copper use is increased when iron is low [5]. Also the question as to why expensive molecules have been developed for toxicity prevention (siderophore style) remains unanswered. Possible theories such as the production of true-siderophore style ligands to enable the utilisation of copper under low copper availability (in an evolutionary context) could be answered if the structure of strong copper binding ligands known. Then the age of the corresponding gene for this ligand and other copper centered biological molecules would help to answer these questions.

5.4 Conclusions

Cyanobacterial cultures were grown in high copper conditions so that they produced strong copper binding ligands during the growth phase. In this study, cultures produced two types of ligand: a strong ligand and a class of weak ligands. The ionic strength corrected binding constants for the weaker ligand class were highly variable, implying that they are not all the same ligand. The log $K'$ for the strong
5.4. Conclusions

copper binding ligands had less variability between grow-up experiments and so it is plausible that the same ligand is present in all grow ups.

The use of pseudo-polarography has enabled the challenging electroanalysis of culture based samples that are high in generic DOM. Here, this technique has been used to track the copper binding ligand throughout a solid phase extraction process, from identifying a suitable resin to determining the fractions that contain copper. Pseudo-polarographic based standard additions have been used to estimate the concentration of copper bound to each of the ligand classes and it has been determined that strong copper binding ligands are present in nanomolar concentrations. As these concentrations are lower than anticipated, this method could potentially be applied to open ocean sampling where there are similar ligand concentrations (although probably not the same ligand).

Searching for copper bound ligands amongst other organic compounds is an analytical challenge. Thus it is extremely useful to ascertain the presence of copper ligands prior to mass spectral analysis. The presence of MS detectable copper ligands was established first using the characteristic isotopic signature using MALDI TOF MS. A peak at 1329 m/z was found in all samples but was found to only weakly bind copper. Copper ligands were found in experiments 5 and 6 only. It is possible that this is due to the length of storage time between samples where experiments 5 and 6 were the most recent culture grow ups, as discussed in Section 4.5. This copper complex was at 697 m/z and was then studied in much closer detail. Isotopic signatures of the complex using ESI and MALDI as sources have shown a high degree of variation in intensity of the peak at two mass units higher than the monoisotopic ion (M+2). Enrichment experiments have shown that copper is present and MALDI experiments implied the presence of sulphur due to a higher than expected M+2 peak with and without the $^{65}$Cu enrichment. Tandem mass spectrometry using MALDI shows several fragments where copper is bound. The fragment ion of particular interest is at 281/283 m/z. This is the smallest ion that is still bound to copper. The direct loss of 64/66 mass units from ions at m/z 513 and 429 suggest the loss of CuH and therefore that copper is bound in the +1 oxidation state. Furthermore there is some fragmentation evidence that points to a thiol containing compound. As this complex is the only mass spectrometrically detected copper containing complex, and it is present in all of the eluent fractions fractions that also
5.4. Conclusions

contain the electrochemically detected strong copper binding ligand, it is possible that the 697/699 m/z ion corresponds to a strong copper binding ligand.

The analytical problems faced during this kind of work are numerous, due to the very low concentrations of the analyte, in a highly interfering matrix where we do not know the exact chemical nature of the analyte. As a result the chemical characterisation of copper binding ligands is greatly lacking in information. The work presented here provides one of the first investigations that combines the well documented electrochemical information with much anticipated mass spectral information about these ligands. To date, this is the first investigation to isolate a copper complex whilst assuming nothing about that chemical nature of these ligands other than the fact that they bind copper strongly. The work presented here brings us one step closer to determining the structure of strong copper binding ligands. Such knowledge then provides us with a tool for answering numerous other questions, only a few of which have been put forward here.
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Bibliography


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Bibliography


Appendix A

Additional notes for Chapter 2

This chapter provides further details and data, summarised in Chapter 2.

A.1 Column method for 8-hydroxyquinoline functionalised XAD resin

All reagents referred to are Seastar grade chemicals. This method was devised, and the resin made, by Sabrina Crispo.

Resin preparation

The resin is loaded into a commercially available column with a bed volume of 0.8 mL. These columns have gravity controlled flow as the sample volumes are small.

1. Wet resin with methanol (HPLC grade).
2. Transfer to water (distilled, deionised).
3. Rinse with ca. 30 mL of a solution of 2 N HCl and 0.5 N HNO₃.
4. Adjust the resin to pH7 with a buffer (0.3M NH₄Ac), until the column is dripping at that pH.

Running and eluting the sample

1. Adjust the seawater to pH 7 using buffer (0.3 M NH₄Ac) and a more concentrated buffer (36% by volume HAc; 64% by volume NH₃).

2. Pour sample onto the resin and catch what drips out.
A.2. Total copper determinations

3. Once all sample (10 mL) has gone through let the final amount drip through.

4. Rinse sample bottle with 1 mL buffer (0.3 M NH₄Ac) and pour onto column.

5. Pour a further 2 mL buffer (0.3 M NH₄Ac) onto column.

6. Pour 2 mL of water that has been adjusted to pH 6.5 using a small volume of the buffer (2 N HCl and 0.5 N HNO₃).

7. Elute with 7 mL of a solution of 2 N HCl and 0.5 N HNO₃.

When finished bring column back up to pH 7 for storage using 1 mL water adjusted to pH 6.5 and then 10 mL buffer 2 N HCl and 0.5 N HNO₃.

A.2 Total copper determinations

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Cu blank (nM)</th>
<th>additional Cu (nM)</th>
<th>total Cu (nM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td>1.52 (0.04)</td>
<td>1116 (0)†</td>
<td>1118</td>
</tr>
<tr>
<td>3</td>
<td>1.72 (0.03)</td>
<td>1116</td>
<td>1118</td>
</tr>
<tr>
<td>4</td>
<td>0.61(0.03)</td>
<td>11.2</td>
<td>12</td>
</tr>
<tr>
<td>5</td>
<td>4.41(0.02)</td>
<td>1116</td>
<td>1120</td>
</tr>
<tr>
<td>6</td>
<td>0.65(0.01)</td>
<td>11.2</td>
<td>12</td>
</tr>
</tbody>
</table>

Table A.1: The total copper concentrations added to each experiment (4 L grow up). Errors to the 95% confidence limit are given in brackets.
A.3 Growth monitoring

Figure A.1: Charts to show the populations of cyanobacteria compared to heterotrophic bacteria. The darker colours refer to the cell counts during the 4 L batch. The lighter colours (stripes for the cyanobacteria) refer to the counts before and after the incubation period in metal buffer free media. Horizontal stripes represent the ‘C’ batch, vertical stripes represent the ‘T’ batch.
Appendix B

Electrochemistry data

Standard addition data for the determination of electroactive copper in each culture experiment

<table>
<thead>
<tr>
<th>Experiment</th>
<th>pCu</th>
<th>Ligand concentration (nM)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Strong</td>
</tr>
<tr>
<td>3C</td>
<td>10</td>
<td>2</td>
</tr>
<tr>
<td>3T</td>
<td>10</td>
<td>6</td>
</tr>
<tr>
<td>5C</td>
<td>10</td>
<td>5</td>
</tr>
<tr>
<td>5T</td>
<td>10</td>
<td>5</td>
</tr>
<tr>
<td>6C</td>
<td>12</td>
<td>3</td>
</tr>
<tr>
<td>6T</td>
<td>12</td>
<td>5</td>
</tr>
</tbody>
</table>

Table B.1: Ligand concentration determinations. Error associated with these measurements is $> 100\%$. 
Appendix C

Mass spectrometry data and additional information

SM-2 Biobeads cleaning procedure

The following method was taken from Bio-beads® SM Hydrophobic and polar interaction adsorbents.¹

1. Slurry resin in methanol (HPLC grade).
2. Degas by stirring and aspirating in a vacuum flask.
3. Load into column as a methanol slurry.

XAD-16 cleaning procedure

The following method is modified from Lepane [72].

1. Shake in dichloromethane and then decant off.
2. Shake in methanol (HPLC grade) and decant off.
3. Repeat with methanol two more times.

Mass spectra

The mass spectra here (Figures C.1, C.2, C.3, C.4, C.5 and C.6) are detailed versions of the mass spectra from Figure 4.12. A discussion of these data is presented in Section 4.4.

¹This method is provided by BioRad on purchasing Bio-beads.
Figure C.1: MALDI QTOF MSMS spectra of the 697 and 699 ions in detail.
Figure C.2: MALDI QTOF MSMS spectra of the 697 and 699 ions in detail. A1 and B1 are the MS/MS spectra for the 697 and 699 ions respectively and for the region 610–720 m/z.
Figure C.3: MALDI QTOF MSMS spectra of the 697 and 699 ions in detail. A2 and B2 are the MS/MS spectra for the 697 and 699 ions respectively and for the region 520–720 m/z.
Figure C.4: MALDI QTOF MSMS spectra of the 697 and 699 ions in detail. A3 and B3 are the MS/MS spectra for the 697 and 699 ions respectively and for the region 520–610 m/z.
Figure C.5: MALDI QTOF MSMS spectra of the 697 and 699 ions in detail. A4 and B4 are the MS/MS spectra for the 697 and 699 ions respectively and for the region 300–520 m/z.
Figure C.6: MALDI QTOF MSMS spectra of the 697 and 699 ions in detail. A4 and B4 are the MS/MS spectra for the 697 and 699 ions respectively and for the region 100–300 m/z.