Voltammetric Studies on the Stabilisation of Dissolved Copper in Hydrothermal Vent Fluids

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Abstract

Globally significant amounts of hydrothermally-derived, bio-active copper (Cu) are stabilised against precipitation, whether as dissolved or nanoparticulate species, and are laterally transported over thousands of kilometres across the deep-ocean (Sander and Koschinsky 2011). This stabilisation is facilitated by the formation of strong Cu-complexes with hydrothermally-derived ligands, which together influences the biogeochemical cycling of Cu in the ocean (Sander, Koschinsky et al. 2007). However, despite the recognised importance of these stabilisation processes, their extent and mechanisms, and the chemical nature of the hydrothermally-derived ligands are poorly understood. A better understanding of these processes will strengthen oceanic biogeochemical models, as broad assumptions will be lessened due to data on the distribution of hydrothermally-derived Cu-stabilising ligands. This project aims to provide data to rectify the paucity of information about this important phenomenon, which is the stabilisation of hydrothermally-derived Cu in the deep sea. As such, hydrothermally-influenced seawater from different vent sites along the geologically active, southern Mid-Atlantic Ridge were analysed for Cu-complexing ligands using the competing ligand equilibrium - adsorptive cathodic stripping voltammetry (CLE-AdCSV) technique.

In this study, the CLE-AdCSV technique was assessed to determine its ability to quantify Cu complexation in UV-irradiated seawater to which Cu-binding sulphur-ligands glutathione, L-cysteine and sodium sulphide had been added. It was determined that the competing ligand, salicylaldoxime, has an unexpected and deleterious impact on the Cu-complexing abilities of the sulphur ligands, which invalidates the assumption in CLE-AdCSV that the added ligand has negligible effects on the species in solution; except the trace metal of interest (Cu). The CLE-AdCSV method failed to detect more than one Cu-binding ligand class in mixed-ligand solutions. Also, it was unable to correctly quantify Cu-complexation in these solutions, which is suspected to be due to a quantitative artefact of the method. It was conclusively shown that the CLE-AdCSV technique has profound limitations that may lead to severe misrepresentation of Cu-complexation in seawater samples. As such, it
is recommended that metal-complexation parameters derived from CLE-AdCSV for seawater samples should be reconsidered, and validated against independent experimental approaches and other speciation analysis methods, for example diffusive gradients in thin films (DGT).

Having assessed the quantitative reliability of the CLE-AdCSV technique, the method was then used in this study to determine Cu-complexing ligands in hydrothermally-influenced seawater, after an acidification pre-treatment. These measurements resulted in characteristically, oddly-shaped Cu-ligand titration plots for the hydrothermal samples, which complicated the quantification of the Cu-complexing ligands by the Gerringa data treatment method. Nonetheless, complexation parameters – ligand concentrations and stability constants, were extracted from the data within the limitations of the data not being ideal.

Total dissolved Cu concentrations in the hydrothermally-derived seawater samples were enriched relative to ambient seawater, with values from 1 to 1563 nM. The concentration of Cu-complexing ligands were measured at 0.014 ±0.001 to 10.6 ±2.16 µM, and with log $K_{Cu'\ell'}$ values ranging from 11.13 ±0.23 to 12.80 ±0.38. The more stable Cu-complexes were associated with samples from low-temperature, diffused vent sites, while samples from the high-temperature vents had much higher concentrations of Cu-stabilising ligands. A significant fraction of the Cu-ligand pool was suspected to be inorganic sulphur species in both the high and low temperature vent samples. It was successfully demonstrated that if elemental sulphur was a major part of the Cu-complexing species, then it could explain the analytical oddities of the CLE-AdCSV technique evident during the analysis of the hydrothermally-influenced seawater samples. Finally, the results reported here are taken to be more representative of the eventual products from the reaction of hydrothermal fluids with seawater, rather than at the exact time and location of sample collection.

This current study reported concentrations of hydrothermally-derived ligands up to 10 µM which is similar to that used by Sander and Koschinsky (2011), who used geochemical modelling to simulate the mixing of Cu and Cu-binding ligands from hot black-smoker vents. Sander and Koschinsky reported that up to 4.2% of
hydrothermally derived copper can be stabilised by ligand concentrations up to 10 μM. Since the amount to Cu that is stabilised is primarily dependent on the ligand concentrations, the author of this present study proposes that the hydrothermally-derived Cu measured in this study would be stabilised to the same extent reported by Sander and Koschinsky (2011). That is, it is expected that up to 4.2% of the hydrothermal end-member Cu reported in this study can be stabilised, and contribute to the oceanic Cu flux.
Acknowledgements

It is with deep appreciation that I acknowledge Associate Professor Sylvia Sander who supervised this thesis with enthusiasm and provided valuable advice. Without her guidance, and technical expertise, this project would not have been possible; hence, I am most grateful for the support she rendered. I also express my sincere appreciation to my thesis co-supervisor, Professor Andrea Koschinsky, for her support and for inviting me to her lab in Germany.

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Thanks to Dario Omanovich for providing great assistance and suggestions that helped with my data analysis using MCCSOFT. Also, thanks to Malea Zygaldo who as an intern partly gathered the data for the UV-digestion of humic acid.

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## Glossary

<table>
<thead>
<tr>
<th>Term</th>
<th>Definition</th>
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<tbody>
<tr>
<td>Adsorption</td>
<td>The adhesion of an extremely thin layer of molecules to the surfaces of solid bodies or liquids with which they are in contact.</td>
</tr>
<tr>
<td>Biological Uptake</td>
<td>The absorption by a living species of a substance, such as nutrient, and its permanent or temporary retention.</td>
</tr>
<tr>
<td>Chlorinity</td>
<td>The total amount in grams of chlorine, iodine, and bromine contained in 1 kg of seawater, assuming that the bromine and iodine have been replaced by chlorine.</td>
</tr>
<tr>
<td>Chlorophyll maxima</td>
<td>Chlorophyll maximum layers are common features of vertical stratified water columns. The term is broadly used to describe subsurface maxima of chlorophyll concentration. These are found throughout oceans, lakes, and estuaries around the world at varying depths, thicknesses, intensities, composition, and time of year.</td>
</tr>
<tr>
<td>CLE-AdCSV</td>
<td>Competing Ligand Equilibrium – Adsorptive Cathodic Stripping Voltammetry</td>
</tr>
<tr>
<td>Cu-CYS</td>
<td>Copper-Cysteine Complexes</td>
</tr>
<tr>
<td>Cu-GSH</td>
<td>Copper-Glutathione Complexes</td>
</tr>
<tr>
<td>Cu-SA</td>
<td>Copper-Salicylaldoxime Complexes</td>
</tr>
<tr>
<td>CYS</td>
<td>Cysteine</td>
</tr>
<tr>
<td>Desorption</td>
<td>The reverse process of adsorption whereby the adsorbed matter is removed from the adsorbent.</td>
</tr>
<tr>
<td>DF</td>
<td>Dilution Factor</td>
</tr>
<tr>
<td>Flocculation</td>
<td>Process by which clumps of solids in water aggregate through biological or chemical action.</td>
</tr>
<tr>
<td>GSH</td>
<td>Glutathione</td>
</tr>
<tr>
<td>Langmuir Isotherm</td>
<td>This equation relates the coverage or adsorption of molecules on a solid surface to gas pressure or concentration of a medium above the solid surface at a fixed temperature.</td>
</tr>
<tr>
<td>MAR</td>
<td>Mid-Atlantic Ridge</td>
</tr>
<tr>
<td>Term</td>
<td>Definition</td>
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<tr>
<td>---------------</td>
<td>-------------------------------------------------------------------------------------------------------------------------------------------</td>
</tr>
<tr>
<td>Precipitation</td>
<td>Formation of a separable solid substance from a solution, either by converting the substance into an insoluble form, or by changing the composition of the solution.</td>
</tr>
<tr>
<td>$S^0$</td>
<td>Elemental Sulphur</td>
</tr>
<tr>
<td>SA</td>
<td>Salicylaldoxime</td>
</tr>
<tr>
<td>Salinity</td>
<td>The total amount of solid material in grams contained in 1 kg of water.</td>
</tr>
<tr>
<td>Steric Hindrance</td>
<td>This arises from the fact that each atom within a molecule occupies a certain amount of space. If atoms are brought too close together, there is an associated cost in energy due to overlapping electron clouds and this may affect the molecule’s preferred shape and reactivity.</td>
</tr>
<tr>
<td>Tectonic</td>
<td>The branch of geology that describes the large-scale motion of the Earth’s crust.</td>
</tr>
<tr>
<td>Ultramafic</td>
<td>Igneous and meta-igneous rocks with a very low silica content (less than 45%), generally $&gt;18%$ MgO, high FeO, low potassium, and are composed of usually greater than 90% mafic minerals (dark coloured, high Mg and Fe content).</td>
</tr>
</tbody>
</table>
1.1 Controls of Trace Metals in the Ocean

1.1.1 Introduction

The bulk of living biomass is chiefly made up of a few major elements – carbon, hydrogen, oxygen, nitrogen, phosphorous, sodium, potassium, chlorine, calcium, magnesium, sulphur (and silica in diatoms) – whose proportions vary within a relatively narrow range in most organisms (Morel, Milligan et al. 2003). A number of trace elements, particularly first row transition metals – manganese (Mn), iron (Fe), copper (Cu), zinc (Zn), cobalt (Co), and nickel (Ni) – are also essential for the growth of organisms. In the ocean, most trace metals are present in picomolar to nanomolar concentrations and they play important roles in ocean biogeochemistry by acting as critical micronutrients for primary producers (Sunda 2012). These metals are required in a vast array of enzymatic reactions carried out in marine organisms, including bacterioplankton, phytoplankton, fungi and macroalgae (Vraspir and Butler 2009).

Since the early 1970s, there have been rapid increases in our understanding of the concentrations, distributions and chemical behaviour of dissolved trace metals in the ocean (Bruland and Lohan 2003). Important initiating factors were major advances in analytical chemistry and instrumentation; along with the development and adoption of non-contaminating or “clean” techniques for collection, storage and analysis of seawater samples (Millero 2013). As a result, seawater concentrations of many trace elements have been shown to be factors of 10 to 1000 lower than those previously accepted (Donat and Bruland 1994). This has allowed scientists to show that the concentrations and distributions of trace metals in seawater are consistent with known biological, physical and geochemical processes within the ocean (Bruland and Lohan 2003). These processes, along with the various sources and sinks, superimposed on the general circulation and mixing of the oceans result in characteristic vertical and horizontal concentration profiles of each dissolved trace metal (Bruland and Lohan 2003).
1.1.2 Sources of Trace Metals to the Ocean

In order to understand the biogeochemistry of trace metals, such as Fe and Cu, in the oceans, it is necessary to evaluate their composition, flux rate and subsequent fate when delivered to the ocean. The major sources of copper and other trace metals to the surface ocean, as shown in Figure 1.1, are atmospheric and riverine inputs of weathered products of the exposed continents (Donat and Bruland 1994), and diffusion from continental-shelf sediments. Domestic and industrial wastewaters, sewerage discharges, and urban runoff also contribute some quantities of trace metals to the aquatic environment; however, these are mostly point sources that lead only to excessive local metal burdens (Feely, Massoth et al. 1983). Trace metal inputs to the deep ocean result from hydrothermal activity at mid-ocean ridges, and other seafloor spreading centres (Von Damm 1995).

Figure 1.1 Schematic showing the main sources of particulate and dissolved trace metals in seawater are dust, rivers and shelf sediments, whereas hydrothermal inputs are significantly less. After introduction into sea water, the metals undergo biogeochemical cycling, which causes fractionation and vertical transport from surface to deep waters. At the same time the metals are transported and distributed by surface and deep-water circulation (Frank 2011).

The distribution of dissolved trace metals in river waters are influenced by several factors - geology of the river catchment area; decomposition of organic materials; chemical constraints within the aqueous system itself, chiefly particulate-dissolved equilibria; and for some rivers, anthropogenic inputs (Chester and Jickells
Of these, the geology of the river catchment imposes the fundamental constraint on the amounts of trace metals available for mobilisation from riverine sediments. The main source of these sediments are ultimately continental rocks which have been broken-up and dissolved by physical and chemical weathering; and exposure to biological activity (Füterer 2006). Before reaching the coast, the river-transported materials must pass through the estuarine environment.

**Figure 1.2** Processes critical in controlling the partitioning of chemical species in estuaries with particular emphasis on trace metals (Modified from Turner and Millward 2002).

Riverine dissolved metals that end up in the ocean are highly constrained by complex estuarine processes at the river-sea interface. Estuaries act as filters of the river-transported chemical signals which often emerge from the mixing zone in considerably altered forms due to modification processes as show in Figure 1.2 (Dagg, Benner et al. 2004). Of these processes flocculation, adsorption, precipitation and biological uptake transfer trace metals from the dissolved to the particulate phase (Troup and Bricker 1975). Desorption from particle surfaces and the breakdown of organics add trace metals to the dissolved phase which may be stabilised by inorganic and organic ligands, then flushed out with the water mass into the immediate costal ocean. Most of the sediments transported to the coastline by rivers are deposited on coastal zones, in large estuaries, and on the continental shelves (Chester and Jickells 2012).
This non-conservative behaviour within estuaries makes it difficult to obtain realistic estimates of the actual river input of trace metals to the open oceans (Bruland and Lohan 2003).

The direct input of trace metals to the open ocean is primarily influenced by long-range transport and deposition of atmospheric dust. Natural sources include continental dust and volcanic activity; whereas anthropogenic sources are mainly fossil fuel burning and waste incineration (Chester and Jickells 2012). The mobilisation of dust particles to the atmosphere is strongly dependent on the nature of the surface cover (desert and arid lands vs forest and grassland) in the source area, which itself is dependent on the prevailing geological, weathering and general climatic regimes. Volcanic activity is a sporadic source of metal-rich dust, but large scale eruptions inject very large amounts of minerals into the atmosphere which are transported over the ocean, imposing short term non-steady state conditions on the upper water column (Frogner, Gíslason et al. 2001). Perhaps the greatest uncertainty in estimating the impact of atmospheric input on the oceans is the estimate of trace metals associated with dust that is soluble upon entering the ocean (Bruland and Lohan 2003).

1.1.3 Trace-Metal Profiles in the Ocean

The global ocean can be considered a dynamic steady-state system in which the amount of an element entering per unit time is equal to, or compensated by the settling out of an equivalent amount (Millero 2013). Insights into the behaviour of trace elements in the ocean are obtained by comparing the respective residence times, which is the average time that a substance remains in seawater before removal by some precipitation or adsorption process to oceanic sediments. The elements with long residence times (> 10^5 yr) are characterised by a lack of reactivity of their aqueous ions in the oceans; as such, they are of relatively higher concentrations. Elements with shorter residence times (< 10^3 yr) have a relatively lower concentration in seawater as their aqueous ions are more reactive and are rapidly removed to the sediments (Bruland and Lohan 2003).
The vertical concentration profiles of trace metals in the ocean is mainly influenced by biological uptake or passive scavenging onto either living (e.g. algae), or non-living particulate material (e.g. sand); and the remineralisation of dissolved elements from sinking particles (Bruland and Lohan 2003). The interplay of these processes results in three principal categories of trace-metal depth profiles in the ocean; namely: conservative-type, nutrient-type and scavenged-type (see Figure 1.3).
1. Conservative-type Distributions: These trace metals interact weakly with suspended particles, have oceanic residence times greater than \(10^5\) yr (Donat and Bruland 1994), and have relatively constant concentrations in the ocean. Trace metals in this category include molybdenum (Figure 1.3A), antimony, tungsten and caesium.

2. Nutrient-type Distributions: These trace metals appear to be involved with the internal cellular activities of phytoplankton. Consequently, their concentrations are depleted in surface waters due to direct uptake by phytoplankton and/or passive adsorption onto biogenic particles (Millero 2013). Their concentration then increases with depth as sinking particles undergo microbial decomposition and/or dissolution. The trace metals cadmium (Figure 1.3B), zinc and nickel are good examples of this type of nutrient behaviour.

3. Scavenged-type Distributions: These trace metals interact strongly with suspended particles both by passive adsorption and biological uptake. Consequently they are rapidly removed from seawater and have very short oceanic residence times of \(< 10^3\) yr (Donat and Bruland 1994). Their concentrations are highest near, and decrease with distance from their sources which include rivers, atmospheric dust, hydrothermal sources and bottom sediments. The elements aluminium, lead and manganese (Figure 1.3C) are good examples with this depth profile.

Some trace metals, such as copper and iron, have hybrid depth profiles (Figure 1.3D) in the ocean and are influenced by both nutrient-type and scavenging-type processes (Millero 2013). Similar to nutrient-type trace metals, dissolved Cu and Fe are often depleted in surface waters in areas of high productivity and regenerated at depth. In less productive waters, or in areas with higher external dust inputs, the concentrations of these elements can exhibit surface maxima more indicative of scavenged elements. Instead of a rapid increase in concentration with depth, shown by nutrient-type elements, dissolved copper concentrations only increase gradually with depth due to a balance of remineralisation and particulate scavenging in deep waters. There are also trace metals that exist in more than one chemical form.
(speciation) with substantially different depth profiles. A fascinating example involves the trace element germanium; inorganic germanic acid (H₄GeO₄) has a nutrient-type distribution profile which is in marked contrast to the conservative-type distribution of the methylated species CH₃Ge(OH)₃⁰ and (CH₃)₂Ge(OH)₂⁰ (Bruland and Lohan 2003).

Figure 1.4 Schematic representation of the global thermohaline circulation system showing the movement of water in the global oceans (http://www.earthzine.org).

It has been established that trace metals in the ocean have clear and distinct depth profiles; in addition, they also have very distinct and characteristic horizontal distributions throughout the global oceans. This is primarily driven by the deep-sea thermohaline circulation (Figure 1.4) which is a part of the global-ocean circulation system, driven by changes in oceanic temperature and salinity (Millero 2013). Most of this deep-water formation takes place in the North Atlantic, where the major surface inflow is the trace-metals deficient Norwegian Sea. The North Atlantic has a large outflow of deep-water with higher concentrations of major nutrients and longer-lived trace metals (Sunda 2012). This nutrient-rich deep water flows to the south, and after mixing with bottom-waters, flows into the South Pacific and South Indian Oceans. There are no deep water formations in the North Pacific, instead, it is a sink for North
Atlantic (and other) deep-waters, and so waters in the North Pacific are relatively much older (Millero 2013).

The concentrations of nutrient-type elements increase along the direction of the main advective flow of deep-waters in the global oceans. Consequently, their concentrations in the deep North Pacific are higher, compared to the North Atlantic waters as shown in Figure 1.3B for cadmium (Sunda 2012). In contrast, the concentrations of scavenged-type elements decrease along the direction of deep-water flow as they are uptaken by biological and passive adsorption processes. This is exemplified in Figure 1.3C for the scavenged-type element, Mn, which has a lower concentration in the deep North Pacific relative to the North Atlantic.

1.1.4 Biological Interactions with Trace Metals

Trace metals are essential in many intracellular enzymatic reactions carried out by marine phytoplankton; including essential roles in photosynthetic C-fixation, respiration and N₂-fixation (Sunda 2012). This is primarily due to the ability of trace metals to be readily reduced and accept electrons, making them essential to many biochemical electron transport reactions. Of the trace metal nutrients, Fe is needed in the greatest amount for phytoplankton growth as it is essential in the primary photochemical reactions of photosynthesis and the respiration of organic carbon (Raven 1990). According to calculations, the numerous iron-containing redox intermediates involved in these processes account for the bulk of the relatively high iron requirement of both phytoplankton and heterotrophic bacteria (Morel, Milligan et al. 2003). For manganese, most of its phytoplanktonic requirement is in the water oxidising complex of photosystem II (which contains four Mn atoms), and thus is essential for oxygenic photosynthesis (Sunda 2012). Other trace metal nutrients (zinc, cobalt and copper) have lesser effects on productivity; but may exert an important influence on the species composition of algal communities because of large differences in metal requirements among species.

In the case of Cu, it occurs along with Fe in cytochrome oxidase which is the terminal enzyme of the respiratory electron transport system that reduces O₂ to H₂O
Cu is also a necessary cofactor of an oxidase that is involved in the Fe-transport reaction of some eukaryotic algae, and possibly some oceanic cyanobacteria that possess a high-affinity Fe(III) reductive pathway (Stearman, Yuan et al. 1996). Cu also occurs in plastocyanin which substitutes for the Fe-containing protein, cytochrome c₆, in photosynthetic electron transport in oceanic diatoms (Peers and Price 2006). With some exceptions, the bioactivity of Cu is determined by the concentration of Cu²⁺ and in many cases, the concentration of the kinetically labile dissolved inorganic species. Though Cu is vital for growth, even moderately elevated amounts of the free ion are potentially toxic to marine phytoplankton, but this sensitivity is species dependent (Sunda 2012).

Some biological species in the ocean are able to ameliorate the toxic effects of Cu²⁺ by producing organic ligands that complex Cu²⁺ and effectively render it non-toxic. Evidence suggests that at least some of these Cu-binding ligands in seawater are produced by phytoplankton, especially cyanobacteria, as in the North Pacific and Atlantic these Cu-chelating species often occur at highest levels near the deep chlorophyll maximum where cyanobacteria are abundant (Coale and Bruland 1988; Moffett 1995). The chelators appear to perform an important function of detoxifying Cu, as in their presence, the average Cu²⁺ in surface North Pacific waters occurred at a non-toxic concentration (~10⁻¹³ M), but in their absence Cu²⁺ would have been ~0.5x10⁻¹⁰ M, a level toxic to many marine phytoplankton (Coale and Bruland 1988). It has also been shown in lab cell-culture experiments with *Synechococcus* (a group of cyanobacteria that are extremely sensitive to copper toxicity) that the production of Cu-complexing ligands was greatly enhanced in response to additions of toxic levels of Cu (Brand, Sunda et al. 1986).

One hypothesis is that Cu-sensitive cyanobacteria may regulate the bioavailability of Cu in the open-ocean by producing organic ligands that modify the chemistry of their environment to allow survival. There is a debate whether the ligands bind copper intracellularly and are then released outside the cell as a detoxifying method for high Cu concentrations; or the ligands are first released, and then bind copper extracellularly (Vraspir and Butler 2009). The structures of these ligands are relatively unknown, and though they are important in reducing the toxicity
of Cu to cyanobacteria, they also lessen the bioavailability of Cu to other organisms that are not able to utilise the complexed form. Conceivably, reduced bioavailability of Cu could be deleterious to the growth of eukaryotic phytoplankton or to other species with high Cu requirements, giving the ligand producers an advantage to thrive.

1.1.5 Speciation and Redox Controls

Studies on metal complexing ligands in the ocean propose the existence of two main and distinct classes based on their conditional stability constants (log K). The first class (L1) is generally a small pool of strong ligands, with log K values of 12-14 and concentrations in the range of 1-40 nM (Town and Filella 2000). The second ligand class (L2) is a larger pool of weaker ligands with log K values of 8-10 and concentrations in the range of 6-150 nM. Little is known about the chemical structure or architecture of the organic ligands involved in binding metals in seawater. However, there have been some recent advances into the structure and function of Fe-binding marine siderophores (Velasquez, Nunn et al. 2011). The L1 and L2 concentrations of Cu-complexing ligands were measured using anodic stripping voltammetry in the North Pacific surface waters (<200 m) at concentrations of 1-2 and 5-10 nM, respectively (Coale and Bruland 1988). Their presence led to greater than 99% of the copper being chelated to these ligand classes. Ligand classes weaker than L2 have not been studied systematically, mainly due to methodological and analytical restraints.

Some trace metals in the ocean occur in different oxidation states; each having different solubility, binding strength with organic ligands, and biological activity (Bruland and Lohan 2003). Consequently, the redox chemistry of these metals has a major influence on their chemical behaviour, biological uptake, and biogeochemical cycling. For copper, thermodynamic equilibrium considerations predict that nearly all of the dissolved copper in seawater should exist as Cu(II), and it is this oxidation state which is mostly stabilised by organic ligands. However, Cu(II) may be reduced to Cu(I) by photochemical processes, reduction at biological cell surfaces or by reaction with chemical reducing agents, such as sulphur containing organic ligands (Leal and van den Berg 1998). This reduction of Cu(II) to Cu(I) is very limited as Cu(I) is unstable in
seawater and is readily re-oxidised to Cu(II), on time scales of minutes, by reacting with dissolved O₂ (Moffett and Zika 1988). This occurs even though the Cu(I) state is stabilised to a considerable extent by chloride complexation, therefore some Cu(I) nevertheless occurs in surface seawater (up to 5-10% of total dissolved copper) despite its thermodynamic instability.

![Conceptual diagram of the mutual interactions between trace metal nutrients and marine phytoplankton. In these interactions the chemistry of trace metal nutrients regulates the productivity of marine phytoplankton communities. These communities in turn regulate the chemistry and cycling of the trace metals by cellular uptake and assimilation, vertical transport of biogenic particles, production of organic chelators, and biological mediation of metal redox cycling (Copied from Sunda, 2012).](image)

**Figure 1.5** Conceptual diagram of the mutual interactions between trace metal nutrients and marine phytoplankton. In these interactions the chemistry of trace metal nutrients regulates the productivity of marine phytoplankton communities. These communities in turn regulate the chemistry and cycling of the trace metals by cellular uptake and assimilation, vertical transport of biogenic particles, production of organic chelators, and biological mediation of metal redox cycling (Copied from Sunda, 2012).

While trace metals clearly influence the productivity and species composition of marine phytoplankton, these organisms in turn have a profound reciprocal effect on the chemistry and cycling of these metals (Figure 1.5) on a variety of time and spatial scales (Boyd and Ellwood 2010). As described earlier, an example of this reciprocal interaction is the effect of algal uptake, particulate settling and regeneration cycles on the vertical distribution and inter-ocean transfer of nutrient metals. Marine biota also releases metal chelating ligands through zooplankton grazing and defecation, and through lysis of cells and microbial degradation processes (Sunda 2012). Also, some phytoplankton species are involved in the reduction and oxidation of redox active metals (Fe, Mn, Co and Cu) by various biological processes (Vraspir and Butler 2009). Thus, trace metal nutrients and marine phytoplanktons comprise an interactive system in which each exerts a controlling influence on the other (Sunda 2012).
1.2 Hydrothermal Venting

1.2.1 Geological Setting of Hydrothermal Vents

Figure 1.6 Global distributions of hydrothermal vent fields (http://www.interridge.org).

The understanding of plate tectonics led scientists to speculate on the existence of hydrothermal vents due to a body of overwhelming circumstantial evidence (Wilson 1963). Among these clues were the discovery of anomalously warm water (> 44°C) sampled below 2,000 m in the Red Sea (Morgan 1971), and the discovery of metal-rich seafloor sediments near mid-ocean ridges in the global oceans (Girdler 1965). Perhaps the most convincing evidence for the existence of hydrothermal vents were the measurements of heat flow in seafloor sediments that showed a curious pattern - higher in some areas and cooler in others. This led to the paradigm proposal that hydrothermal circulation could be causing convective cooling of the oceanic crust (Lawver, Williams et al. 1975).
It was not until 1977 that the first hydrothermal vents were discovered at the bottom of the Pacific Ocean along the Galápagos Rift. The seafloor vents were gushing warm, mineral-rich fluids into the cold, dark depths and flourishing with extraordinary biological life (Corliss, Dymond et al. 1979). Since then, a wealth of active and inactive vent sites have been found throughout the oceans (Figure 1.6) in various tectonic settings, including mid-ocean ridges, back-arc rifts, subduction zones and to a lesser extent, fracture zones, and seamounts (German, Baker et al. 1995).

Geological activity along oceanic-divergent plate boundaries results in the formation of slow- and fast-spreading mid-ocean ridges, which is a network of volcanic submarine mountain ranges that extend laterally for ~70,000 km through all of the ocean basins in a nearly continuous seam (Leveille and Juniper 2003). Slow-spreading ridges (< 5.5 cm/yr), such as the Mid-Atlantic Ridge, have a steep and rugged, mountainous topography with a deep and prominent rift valley (~400 - 2500m deep) along the axis (Herzig and Hannington 2006). At faster spreading ridges, (> 9 cm/yr), such as the East Pacific Rise, the ridge topography is relatively smooth and no rift valleys develop (Leveille and Juniper 2003). There are also intermediate-spreading ridges (5-9 cm/yr), such as the Indian Ocean Ridge, where the topography is more subdued and the rift valley is only 50 - 200m deep. Seafloor hydrothermal activities have also been reported on a range of back-arc spreading centres at convergent plate margins, such as the Lau Basin in the southwest Pacific Ocean (Tivey 2007). At these tectonic boundaries one oceanic plate is subducted beneath another, while an asymmetric submarine spreading centre occurs in the overriding plate, lying behind an arc of surface volcanoes above the site of subduction (Elder 1965).

1.2.2 Hydrothermal Circulation

The continued divergence at sea-floor spreading centres causes the crustal material connecting both plates to stretch thin, and eventually pull apart, creating a deep fissure in the earth’s crust. The opening of the fissure reduces the pressure on the superheated magma below, consequently, columns of the molten material rises, often violently, to fill the gap (Hamblin and Christiansen 2004). On contact with the
cold seawater, the superheated substance is rapidly cooled, and solidifies creating new crustal material. This new material continues to spread and creates a rift between both plates (Leveille and Juniper 2003), partly due to the extensional stress exerted by the rising magmatic currents that diverge under the new crustal material, dragging it apart in the direction of flow (Von Damm 1990).

**Figure 1.7** Schematic representation (not drawn to scale) of the major geochemical reactions occurring during the hydrothermal cycling of seawater through the Earth’s crust at ocean floor spreading zones (redrawn from Millero 2013). The reaction zone, deep in the crust, is where most of these geochemical reactions occur.
Thermal contraction and seismic events cause the surface of the newly formed seafloor to be perforated with cracks and fissures (Seyfried and Ding 1995), and these provide sufficient permeability to allow cold seawater to percolate downwards into the oceanic crust. With increasing depth, the seawater is progressively heated and reacts with the surrounding rocks, leading to a change in the chemical composition of the seawater (Mills 1995). These changes are detailed in Figure 1.7 which shows the major chemical reactions that occur during seawater/hydrothermal fluid circulation.

In the first reaction zone (< 350 °C) Mg\(^{2+}\) is removed from the fluid by combining with OH\(^-\) to form Mg(OH)\(_2\), which precipitates as Mg-rich smectite and chlorite at temperatures less than, and greater than 200°C, respectively (Swallow and Crease 1965). The removal of OH\(^-\) creates an excess of H\(^+\) ions, which is the main acid generating reaction that causes hydrothermal fluids to have pH values as low as 3 (Seyfried and Ding 1995). The loss of Mg\(^{2+}\) is balanced by Ca\(^{2+}\), K\(^+\) and Na\(^+\) which are leached from the rocks into the descending seawater (Swallow and Crease 1965). Oxygen is removed by reacting with Fe\(^{2+}\) from basaltic rocks to form Fe\(^{3+}\)-oxyhydroxide species, hence the fluid becomes anoxic. Sulphate is removed mainly by precipitation of anhydrite (CaSO\(_4\)), and partly by reduction to H\(_2\)S. Although reduction of SO\(_4^{2-}\) contributes some H\(_2\)S to the hydrothermal fluid, most of the reduced sulphur is directly derived from fluid-rock interactions (Seyfried and Ding 1995).

The sum of reactions in the low temperature zone results in a highly corrosive fluid that is acidic, anoxic, alkali-rich and Mg-poor relative to the starting seawater before it enters the crust (Yucel, Gartman et al. 2011). At greater crustal depths, within the high-temperature reaction zone (>350°C), the fluid interacts with the hot oceanic basement and leaches most of its transition metals such as Fe, Mn, Cu and Zn, together with Au, Ag and Si (Swallow and Crease 1965). In addition, volatiles like \(^3\)He, CH\(_4\), CO\(_2\), SO\(_2\), H\(_2\)O and H\(_2\) are added to the chemically altered fluid by magmatic degassing, and inorganic synthesis at high temperatures (Swallow and Crease 1965). Under these conditions, some hydrothermal fluids achieve sufficiently high temperatures and pressures to intersect the two-phase boundary of seawater below the critical point (407°C and 298 bars), and separate into a low salinity, vapour-rich phase and a metal-rich, brine phase (Koschinsky, Garbe-Schönberg et al. 2008). Metals
in the super-heated, brine phase are predominantly transported as chloride complexes, but in some low-temperature fluids, metal-bisulphide complexes may dominate, especially for metals such as As, Hg and Sb (Toner, Fakra et al. 2009).

Figure 1.8 Hydrothermal vent structures. (A) Black smoker chimney venting a dense plume of dark-coloured polymetallic particles. (B) Cross section of a high-temperature, black smoker chimney spire, showing the zonation of precipitated minerals (photos courtesy of MARUM, University of Bremen).

This newly formed hydrothermal fluid achieves maximum buoyancy in the high-temperature reaction zone. It then rises rapidly to the seafloor, displacing large volumes of seawater from the major faults and fractures within the upflow zone (Seyfried and Ding 1995). When there is no subsurface mixing with the colder seawater, the hydrothermal fluid erupts from the seabed at high-temperature (350 - 405°C) focused vents (Mills 1995). This high-temperature fluid erupts from the vent and mixes turbulently with the oxygenated, cold seawater (~2°C), causing dissolved metal species to rapidly oxidise, and consequently precipitate from solution (German, Baker et al. 1995). This gives rise to a dense plume of dark-coloured polymetallic particles (“Black smoke”), as shown in Figure 1.8A, with the major minerals being Fe-sulphides, Fe-oxyhydroxides, chalcopyrite, elemental sulphur and amorphous silica (Mottl and McConachy 1990). These minerals eventually settle around the vent orifices to produce large chimney-like constructs, and other mineralogically complex deposits proximal to the vent fields (Lupton, Delaney et al. 1985). The characteristic feature of black-smoker vents is their central chalcopyrite (CuFeS₂)-lined orifice and...
outer anhydrite (CaSO$_4$)-rich wall, as depicted in Figure 1.8B which shows the cross-section of a high-temperature chimney.

Hydrothermal fluids can also exit the seafloor at low-temperature (< 50°C), diffused vents which can sustain a vibrant biological community, but are not generally associated with extensive mineralisation such as chimney structures (Hannington, Jonasson et al. 2013). This low-temperature venting occurs at older tectonised portions of the ocean crust (flanks of mid-ocean ridges) where the hydrothermal fluids are not hot enough to carry sufficient metals and sulphur to the seafloor. Alternatively, it also happens along ridge axes where high-temperature fluids are conductively cooled or subsurfacially mixed, resulting in the deposition of metal and sulphur within the vent complex or beneath the seafloor. Diffused venting at higher temperatures (100 to 300°C) forms “white-smoker” chimneys, so called because of the white particles of silica, anhydrite, and barite that precipitate from solution as the hydrothermal fluids are quenched by cold seawater (German and Von Damm 2006).

1.2.3 Hydrothermal Plumes

The minerals discharged from hydrothermal vents as black/white smoke along with gases and other dissolved ions, are expelled as buoyant plumes above the vents. On rising, the hydrothermal fluid is rapidly diluted by entrainment of ambient seawater, causing temperature and particle concentrations within the plume to exponentially decrease within a short distance from the vent orifice (Baker, German et al. 1995). The hydrothermal plume rises with a continuous increase in volume, until it attains neutral buoyancy relative to the background seawater. The exact height is a function of both the strength of the hydrothermal source itself, and the density stratification of the water column into which it is emitted (Eldridge 1965). Upon attaining neutral buoyancy, the plume disperses laterally with the ocean currents away from the vent field. This creates a distinct hydrographic and chemical layer that stretches up to thousands of kilometres from the hydrothermal spreading centre (Baker, German et al. 1995) as shown in Figure 1.9. Highlighted in the figure is a large plume of dissolved
iron over the Mid-Atlantic Ridge (MAR) with a width in excess of 1,000 km (Saito, Noble et al. 2013).

**Figure 1.9** A zonal section of dissolved Fe (dFe) (nM) in the South Atlantic showing a large plume of dFe over the Mid-Atlantic Ridge (MAR) at ~2,900m depth, 2 km in height and a width in excess of 1,000 km. The dFe plume is thought to be due to long-range transport from MAR hydrothermal vents (Saito, Noble et al. 2013).

In the early stages of the buoyant plume some of the larger sulphide and sulphate particles quickly settle out, leaving only the very fine-grained oxides (Fe and Mn oxyhydroxide), sulphides and sulphates in the neutrally buoyant plume. The precipitation of Fe-oxyhydroxides strongly dominates in the buoyant plume (Mottl and McConachy 1990), while Mn oxidation occurs primarily in the neutrally buoyant plume where the slower oxidation is mediated by bacterial processes (Cowen, Massoth et al. 1990). There is also significant co-precipitation (along with Fe) of other metals enriched in hydrothermal fluids; notable among these are Cu, Zn and Pb. As such, these metals are highly enriched in the plume particles, by factors up to 20 or more relative to the deep-ocean suspended matter (German and Von Damm 2006). Though these metals are generally enriched in plume particles, their concentrations do not follow a linear-dilution pattern in the buoyant and/or neutrally buoyant plume (Lilley, Feely et al. 2013). This has been attributed to differential sedimentation or oxidative dissolution of the sulphide phases in the plume.
Thus far, the processes active in a hydrothermal plume have been discussed as inorganic geochemical processes; however, microbial processes are well known to mediate key chemical reactions in hydrothermal plumes. One particularly good example of such a process is the microbial oxidation of dissolved manganese in which microbes transform dissolved hydrothermal Mn into the particulate form (Campbell, Palmer et al. 1988; Cowen, Massoth et al. 1990). Distributions of dissolved CH$_4$ and H$_2$ in hydrothermal plumes have also been shown to be controlled by bacterially, mediated oxidation (de Angelis, Lilley et al. 1993). That is, CH$_4$ and H$_2$ is rapidly removed from the neutrally buoyant plume by microbial activity.

1.2.4 Hydrothermal Fluxes to the Ocean

Submarine hydrothermal activity plays a critical role in the budgets of heat, water and chemical constituents that are transported along the earth-ocean-atmosphere system (German and Von Damm 2006). Despite this importance, there remain uncertainties as to the net flux of heat and materials from submarine hydrothermal activities to the global oceans. A major problem in confidently quantifying this heat flux is the conflict between the different methods used to estimate heat flux, which is the key determinant in water and chemical fluxes (Elderfield and Schultz 1996). Another uncertainty is both the relationships and the relative importance of axial high-temperature vents with a deeper magmatic source, and of low-temperature vents with a shallower magmatic source. In addition, there is significant temporal as well as spatial variability in the chemistry of vent fluids and hydrothermal plumes (Von Damm, Oosting et al. 1995).

Elderfield and Schultz (1996) reviewed the various geophysical and geochemical methods of estimating hydrothermal fluxes, and concluded that the best global estimate for axial high-temperature hydrothermal fluid flux is 3 (±1.5) x 10$^{13}$ kgyr$^{-1}$. However, a recent study using the isotopic mass balance of thallium in the ocean crust suggests that a more accurate estimate for high temperature hydrothermal water flux is 0.72 x 10$^{13}$ kgyr$^{-1}$, and that from low temperature vents is (0.2-5.4) x 10$^{17}$ kgyr$^{-1}$ (Nielsen, Rehkämper et al. 2006).
Table 1.1 Global estimates of hydrothermal fluxes from high-temperature fluids (Elderfield and Schultz 1996) and low-temperature fluids at ridge flanks (Kadko, Baker et al. 1994); and removal fluxes of elements by hydrothermal plume particles (Rudnicki and Elderfield 1993). Note: positive numbers represent a flux into the ocean and negative numbers a sink to hydrothermal plume particles.

<table>
<thead>
<tr>
<th>Element</th>
<th>High-temperature fluxes ($10^9$ molyr$^{-1}$)</th>
<th>Low-temperature fluxes ($10^9$ molyr$^{-1}$)</th>
<th>Plume particle fluxes (molyr$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Li</td>
<td>12-39</td>
<td>-(2-11)</td>
<td></td>
</tr>
<tr>
<td>B</td>
<td>1.1 - 4.5</td>
<td>-(1.9-19)</td>
<td></td>
</tr>
<tr>
<td>Mg</td>
<td>-1600</td>
<td>-(700-1100)</td>
<td></td>
</tr>
<tr>
<td>K</td>
<td>230 - 690</td>
<td>-(100-640)</td>
<td></td>
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<tr>
<td>Ca</td>
<td>9-1300</td>
<td>200-550</td>
<td></td>
</tr>
<tr>
<td>SO$_4$</td>
<td>-840</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Rb</td>
<td>0.26-0.95</td>
<td>-(0.19-0.28)</td>
<td></td>
</tr>
<tr>
<td>Cs</td>
<td>(2.9-6) x $10^{-3}$</td>
<td>-(2.3) x $10^{-3}$</td>
<td></td>
</tr>
<tr>
<td>Si</td>
<td>430-660</td>
<td>1300-1800</td>
<td></td>
</tr>
<tr>
<td>Al</td>
<td>0.12-0.6</td>
<td></td>
<td></td>
</tr>
<tr>
<td>V</td>
<td>0</td>
<td>-4.3 x $10^7$</td>
<td></td>
</tr>
<tr>
<td>Cr</td>
<td>0</td>
<td>-4.8 x $10^7$</td>
<td></td>
</tr>
<tr>
<td>Mn</td>
<td>11-34</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fe</td>
<td>23-190</td>
<td></td>
<td></td>
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<tr>
<td>Co</td>
<td>(6.6-68) x $10^{-4}$</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cu</td>
<td>0.3-1.3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Zn</td>
<td>1.2-3.2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pb</td>
<td>(2.7-110) x $10^{-4}$</td>
<td></td>
<td></td>
</tr>
<tr>
<td>P</td>
<td>-4.5 x $10^{-2}$</td>
<td>-3.2</td>
<td></td>
</tr>
<tr>
<td>As</td>
<td></td>
<td>-1.1 x $10^{10}$</td>
<td></td>
</tr>
<tr>
<td>U</td>
<td></td>
<td>-1.8 x $10^8$</td>
<td></td>
</tr>
<tr>
<td>Mo</td>
<td></td>
<td>-4.3 x $10^7$</td>
<td></td>
</tr>
<tr>
<td>Be</td>
<td></td>
<td>-1.9 x $10^6$</td>
<td></td>
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<tr>
<td>Ce</td>
<td></td>
<td>-1.7 x $10^6$</td>
<td></td>
</tr>
<tr>
<td>Nd</td>
<td></td>
<td>-1.0 x $10^6$</td>
<td></td>
</tr>
<tr>
<td>Lu</td>
<td></td>
<td>-6.3 x $10^6$</td>
<td></td>
</tr>
<tr>
<td>CO$_2$</td>
<td>100-1200</td>
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<td></td>
</tr>
<tr>
<td>CH$_4$</td>
<td>6.7-24</td>
<td></td>
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<tr>
<td>H$_2$</td>
<td>3-15</td>
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</tr>
<tr>
<td>H$_2$S</td>
<td>85-960</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

A compilation of annual elemental flux estimates by Kadko et al. (1994) is shown in Table 1.1, along with estimates derived by Elderfield and Schultz (1996) for high-temperature fluids. Scavenging by hydrothermal plume particles removes significant quantities of reactive trace elements, such as rare earth elements, from
ambient seawater such that, overall, hydrothermal activity acts as an oceanic sink for such elements (Table 1.1), not a source. Also, the eventual transfer of some gases, input by hydrothermal activity, from the ocean to the atmosphere extend the influence of hydrothermal activity to beyond the oceans themselves.
1.3 Thesis Outline: Aims and Objectives

Significant amounts of hydrothermally-derived, bio-active trace metals such as copper are stabilised, and transported into the deep-ocean 1000s of kilometres away from the vent sites. This is facilitated by high concentrations of metal complexing ligands which are thought to be produced in the hydrothermal environment. However, the extent of the stabilisation/complexation processes and the chemical nature of these ligands are both poorly understood. This project aims to advance our knowledge of this phenomenon by measuring Cu-complexing ligands in hydrothermally-derived fluids from vent sites along the geologically active southern Mid-Atlantic Ridge. This was achieved using the CLE-AdCSV technique, and presented below is an outline of how this was executed.

Chapter 2: Presents details on the theory governing CLE-AdCSV, and the analytical methods for total dissolved copper and copper-complexing ligands. Also presented are quality control data that tests the reliability of the method and a review of the data analysis method.

Chapter 3: Examines the quantitative reliability of the CLE-AdCSV technique in quantifying copper-complexation in UV-irradiated seawater to which Cu-binding ligands glutathione, L-cysteine and sodium sulphide were added.

Chapter 4: Describes the measurement of copper-complexing ligands in hydrothermally-influenced seawater samples from vent sites along the southern Mid-Atlantic Ridge.

Chapter 5: Provides suggestions to explain some of the non-ideal behaviours of the hydrothermal samples during CLE-AdCSV titrations.

Chapter 6: Presents overall conclusions arising from the study along with recommendations for future research.
2.1 Metal-Ligand Complexation

2.1.1 Background

Metal ions are distributed in the marine and fresh water environment as – free aqua ions; complexed with simple organic or inorganic ligands; complexed with macromolecular or colloidal ligands; adsorbed on, or incorporated in suspended particles; and adsorbed on, or assimilated by living organisms (Buffle 1988). The distribution of a metal ion amongst the different environmental and biological compartments, as shown in Figure 2.1, is primarily controlled by the metal speciation, which is the range of chemical forms that the metal may assume. In a given

![Figure 2.1 Schematic representation of the distribution of a metal ion, M, amongst the different compartments of a natural marine system (adapted from Buffle, 1988).](image-url)
environment, the species distribution of a metal depends on the properties of the metal itself, as well as on the availability and nature of potential ligands. Ionic strength, pH, and redox status also play a role in defining species distribution (Reeder, Schoonen et al. 2006). Each chemical species of a given metal assumes very distinct roles in determining the distribution of the metal ion in the natural aquatic environment. Therefore, it is important to distinguish each, or groups of these species from each other.

The fraction of the dissolved metals complexed by the major inorganic ligands in an aquatic sample can be modelled accurately using their respective stability constants (Xue and Sigg 2002), while the portion complexed by natural organic and trace inorganic ligands are more difficult to estimate. For this reason, direct laboratory analysis is required. It is therefore essential to have analytical tools that can measure the total concentration of the metal, and also selectively measure the concentrations and certain properties of the different chemical species of the metal. The selectivity of these methods often depends on kinetic factors that require the definition of certain criteria for distinguishing between kinetically inert and labile complexes. It should be noted that there are undetectable components of soluble trace metals which cannot be measured by a given technique because their chemical state does not possess the physicochemical properties required by the analytical method (Ružić 1982).

The natural metal-complexing species in an aquatic sample can be divided into two categories: simple ligands (Cl\(^-\), CO\(_3\)^{2-}, amino acids) and complex compound groups (fulvic compounds, proteins). The latter often contain a large number of complexes per molecule, and the total molar concentration of these sites can be comparable to the concentrations of many simple ligands (Buffle 1988). For simple ligands, metal complexes can result from two types of interactions, inner and outer sphere electron transfers as depicted in Figure 2.2. Outer sphere complexes result from purely electrostatic attraction between the ligand and the metal ion, both of which conserve their hydration sphere; as such, they are not directly bonded to each other (Haim 1975). Inner sphere complexes result from the formation of covalent bonds between the metal and the ligand, as the lone pairs of the ligand are attracted to the high, concentrated, positive charge on the metal ion. There are additional effects that can
influence the overall complexation process for both categories of ligands, such as electronic structure, steric and conformational effects of the complexation site.

![Diagram of outer and inner sphere coordination](image)

**Figure 2.2** Diagram of outer and inner sphere coordination between a ligand (L⁻) and a metal ion (M⁺). The outer sphere coordination results purely from electrostatic attraction, while the inner sphere coordination results from the formation of covalent bonds between the ligand and the metal.

There are different modes of binding between the central metal ion and the complexing ligand; however, the analytical methods used to study this subject are less interested in the molecular details of the formed complexes. Instead, the focus is on understanding the general reaction of the various species under the experimental conditions, particularly the factors governing the stabilities and reactivities of the complex compounds. This approach is necessitated by practicality; as for example, individual components of homologous groups cannot be easily isolated and studied separately. Therefore, the whole group has to be treated as a single complexing class whose properties are the sum of the individual components, plus certain specific properties resulting from possible synergistic actions between them, such as aggregation reactions. With this understanding, the correct interpretation of analytical data from these measurements requires consideration of all the physical and chemical processes which can occur in natural aquatic systems.

### 2.1.2 Metal-Ligand Complex Formation

The generalised mononuclear reaction between a metal and a coordinating ligand can be represented by the corresponding law of mass action expression:

\[
M + X \rightleftharpoons K_{MX} MX \quad K_{MX} = \frac{[MX]}{[M][X]} \quad (1)
\]
The law of mass action is a mathematical model that explains and predicts the behaviour of dissolved species in a dynamic equilibrium, and is used for calculating the respective equilibrium concentrations (Ringbom 1963). In the given example, $M$ represents the metal, $X$ the ligand with the charges omitted, and brackets denote concentration of the enclosed species. The traditional stability constant, $K_{MX}$, is the thermodynamic equilibrium constant for the formation of the complex, $MX$, and indicates the quantitative strength of the complexing species. Where multiple ligands are bound to the central metal ion, the equilibrium is determined by as many equilibrium constants as there are complexes (Schwarzenbach and Flaschka 1969), and the overall thermodynamic equilibrium constant is the product of the individual equilibrium constants.

In a fundamental sense, bare metal ions (such as Cu$^{2+}$) do not exist in an aquatic sample; instead, they mainly occur as metal-aqua complexes (such as [Cu(H$_2$O)$_6$]$^{2+}$). These complexes are labile and the weakly bound water molecules are quickly substituted, causing trace metals in the aquatic environment to also be complexed by the major inorganic ligands, such as Cl$^-$, OH$^-$, CO$_3^{2-}$ and HCO$_3^-$. For ease of discussion, the notations $X$ and $CuX$ are used respectively to represent the aggregate of all the major inorganic ligands, and the aggregate of the corresponding copper complexes. Shown in equation (2) are the inorganic copper complexes that predominate in seawater, but the list is not exhaustive as there are also contributions of other inorganic, polynuclear and mixed-ligand complexes of copper (van den Berg 1982). However, the concentrations of some of these species are negligible at the relatively low copper concentrations and pH of seawater.

$$[CuX] = [CuCO_3^0] + [Cu(CO_3)_2^{2-}] + [CuOH^+] + [Cu(OH)_2^0] + [CuCl^+] \quad (2)$$

Copper is also complexed by natural organic species in seawater; however, unlike inorganic species, these are more difficult to define as their nature and properties are often very poorly known, and their compositions are not uniform in aquatic systems (Buffle 1988). Those in the marine environment probably consist of a continuous spectrum of dissolved organic carbon (DOC) exuded by planktons, and many components of higher molecular weight such as fulvic and humic substances,
peptides and protein compounds (Tessier and Turner 1995). Though the organic ligand pool is generally difficult to characterise, there are specific analytical methods that allow some species to be fully defined, revealing their nature, structure and other chemical properties. Among these species are simple organic components with a well-characterised structure and a low molecular weight such as amino acids, hydroxyl acids and monosaccharides.

The ill-defined, organic ligands which cannot be fully isolated and characterised due to analytical limitations, are instead grouped into different fractions based on similar complexation properties, and discussed as homologous compound-groups (Tessier and Turner 1995). They comprise relatively small compounds (eg. fulvic acids and peptides) or larger ones (eg. humic acids, polysaccharides, polypeptides, lipids and proteins). The concept of ligand group/class is therefore operationally defined, and though not precise, is necessary to interpret metal complexation reactions in aquatic chemistry. For discussion, $L$ and $CuL$ are respectively used to refer to a homologous organic-ligand group and the corresponding aggregate of copper complexes.

The formation of $CuL$ is shown in equation (3). For simplicity, the charges associated with the different species are omitted here, and in the proceeding discussion as in many cases they are not known with any certainty. The traditional thermodynamic equilibrium stability constant, $K_{CuL}$, pertains to the reaction in an ideal system where the metal and ligand exist only in the forms shown, and they partake in no competing side reactions (Elder 1975).

$$Cu + L \rightleftharpoons CuL \quad K_{CuL} = \frac{[CuL]}{[Cu][L]} \quad (3)$$

However, competing side reactions can, and in most instances do exert considerable influence on the main complexation reaction such that the stability constant is significantly altered (Elder 1975). It is therefore imperative to quantify the inadvertent side reactions to ensure that the calculated stability constant reflects only the main complexation reaction.

It has already been established that copper undergoes side reactions with the major inorganic ligands in seawater. These can be modelled without difficulty as the
required parameters: alkalinity, pH and major ion composition are well known for seawater (van den Berg 1982). The organic ligands of interest also enter into side reactions, as at ambient seawater pH they are probably partially protonised as their free ions only appear above pH 10 (Schwarzenbach and Flaschka 1969). These ligands also form complexes with other competing cations such as Ca$^{2+}$ and Mg$^{2+}$. It is difficult to determine the extent of side reactions of the organic ligands, as normally the degrees of protonisation and of complexation by competing cations are not known in seawater (van den Berg 1982). In this case it is better to use a conditional stability constant ($K'_{CuL}$), which is a realistic quantification of the stability of the main complex ($CuL$) under the given set of conditions.

It is convenient to not prime the conditional stability constant itself, but its subscripts; thus, $K_{Cu'L'}$ indicates that the side reactions of copper and those of the ligand have been taken into account. This establishes a new equilibrium expression:

$$K_{Cu'L'} = \frac{[CuL]}{[Cu'][L']}$$  \hspace{1cm} (4)

$$[Cu'] = Cu^{2+} + CuX$$  \hspace{1cm} (5)

$$[L'] = [L^{p−}] + [LH_i^{−p}] + [M_jL_j^{−p}]$$  \hspace{1cm} (6)

In expression (4), $[Cu']$ denotes the concentration of not only free copper ions, but also all dissolved copper that has not reacted with the ligand of interest. Correspondingly, $[L']$ represents the concentration of all dissolved species of the complexing ligand not bound to copper. These are formally defined by expressions (5) and (6) where $[LH_i^{−p}]$ = total concentration of the protonated forms of $L$, and $[M_jL_j^{−p}]$ = concentration of $L$ complexed by metal ions other than copper and H$^+$. The conditional stability constant ($K_{Cu'L'}$) defines the relationship between the quantities of interest: the concentration of the product formed ([CuL]), and the total concentration of uncomplexed copper ([Cu']) and ligand ([L']). This definition reveals that $K_{Cu'L'}$ is not a real constant, but depends on the ambient conditions, especially on the concentration of the competing species in solution. As such, the calculation of $K_{Cu'L'}$ requires prior determination of the side reaction products of the ligand, $[LH_i^{−p}]$ and
but these are not well known for natural waters. The lack of values for \( LH_{i}^{p} \) and \( M_{i}L_{j}^{p} \) is mitigated by introducing a factor that quantifies the side reactions in each water sample. This factor, the side reaction coefficient (\( \alpha \)), was originally introduced by Schwarzenbach (Schwarzenbach and Flaschka 1969), and is discussed in the next section.

### 2.1.3 Side Reaction Coefficient

Side reactions produce a myriad of compounds in solution, but very rarely is there a desire to know the true concentration of these species. Instead, the sole interest is in knowing their combined effect on the main coordination reaction and the calculated stability constant. As such, the side reaction coefficient (\( \alpha \)) is introduced to simplify the equilibrium calculations, and it quantitatively represents all the competing side reactions without truly knowing the exact nature of the competing species (Ringbom 1963). In this way the equilibrium expression is kept very simple, containing only three terms, and avoids the complications that would arise if the concentrations of all the species actually present were introduced.

To clarify the nature of side reaction coefficients, consider the case established in equation (3), where copper forms a 1:1 complex with an organic ligand. The organic ligand also partakes in side reactions with other cations, including hydrogen; \( M \) will be used to represent the aggregate of these cations. As shown in expression 7, these side reactions have their own equilibrium expression, for which \( \beta_{ML} \) represents the aggregate stability constant.

\[
L + M \rightleftharpoons ML \quad \beta_{ML} = \frac{[ML]}{[M][L]} \quad (7)
\]

\[
\beta_{ML}[M] = \frac{[ML]}{[L]} = \alpha_L \quad (8)
\]

\[
[L'] = \alpha_L[L] = [ML] \quad (9)
\]

From expression (8), the side reaction coefficient for the ligand, \( \alpha_L \), is introduced as the product of \( \beta_{ML} \) and the free cations, [M]. Expression (9) shows that [ML] equals [L'], as essentially, [L'] is the concentration of the organic ligand species not bound to
copper, which is otherwise bound as $ML$ with the competing cations. The ligand also exists as free ions, that is not bound to either Cu or M; however, these are not considered significant at ambient seawater pH (Schwarzenbach and Flaschka 1969).

$$K_{CuL'} = \frac{K_{CuL}}{\alpha_L} \quad (10)$$

$$K_{Cu'L} = \frac{K_{CuL}}{\alpha_{Cu}} \quad (11)$$

The combination of expression (4) with (9) yields expression (10) which shows the influence of the side reactions of $L$ on the stability constant of the main reaction. Using the same logic as above, in the situation where only the side reactions of copper are considered, the effect on the stability constant is reflected in expression (11).

It is possible to define $\alpha_{Cu}$ and $\alpha_{L}$ individually, but this leads to a complicated expression which is overcome by introducing an overall side reaction coefficient ($\alpha_{CuL}$) which is the sum of both (Elder 1975). The value for ($\alpha_{CuL}$) accounts for practically all occurring side reactions on the main complexation reaction and its influence on the conditional stability constant is shown in expression (12).

$$K_{Cu'L'} = \frac{K_{CuL}}{\alpha_{CuL}} \quad (12)$$

An important feature of $\alpha_{CuL}$ is that it is independent of total copper concentration (Elder 1975), and is dependent only on concentrations of the competing species which are in turn dependent on pH. As such, few generalisations can be made about trace metal complexation in aquatic systems as these species are highly variable in due to the composition of different water bodies. Therefore, the application of side reaction coefficients to equilibrium modelling provides a relatively simple way of dealing with the large numbers of simultaneous complexation reactions under the variable pH and ionic conditions of natural waters.
2.1.4 Competing Ligand Equilibrium – Adsorptive Cathodic Stripping Voltammetry (CLE-AdCSV)

2.1.4.1 Theory

The aim of speciation studies is to estimate the complexation parameters (ligand concentrations and conditional stability constants) required to predict the behaviour of different metal species in the natural aquatic environment. This requires analytical techniques that can selectively detect the metal ion of interest from amongst the background matrix which contains all the stable elements of the Periodic Table at a wide range of concentrations; plus several radiogenic elements; dissolved organic matter of mostly unknown structures; suspended particles comprising many different solid phases; and inorganic colloidal matter (Tessier and Turner 1995). It is also essential that the chosen method can be used confidently at the naturally low trace metal concentrations (typically in the nanomolar range). Also, since the free metal ion is the most bio-active species, whichever measurement technique is used, the calculated free ion concentration should be accurate.

The two most commonly used speciation techniques for natural waters employ voltammetric stripping methods: anodic stripping voltammetry (ASV) and adsorptive cathodic stripping voltammetry (AdCSV). ASV is a speciation technique which detects species that react directly with the mercury electrode such as free hydrated metal species (Cu$^{2+}$, Zn$^{2+}$, Cd$^{2+}$, Pb$^{2+}$), and some inorganic complexes such as Cu(CO$_3$)$_{10}^-$ (Bruland, Rue et al. 2000). In contrast, AdCSV is an indirect method suitable also for metals that do not react directly at the Hg-electrode (e.g., Fe$^{3+}$, Co$^{2+}$), but which form an electrochemically-active complex with a ligand added to the sample (Thomas and Henze 2001). An advantage of these techniques is that they are highly sensitive and selective, with detection limits generally in the range of $10^{-11}$ to $10^{-9}$ mol L$^{-1}$ (Thomas and Henze 2001). Also, these methods usually do not require sample pretreatment, such as preconcentration, lowering the possibility of sample contamination, and hence, does not change the ambient speciation.

The AdCSV technique is preceded by the addition of a well-characterised organic ligand (AL – for added ligand) to the water sample; this forms an adsorbable,
electroactive complex with the metal under investigation, in this case copper. This coupling gives the name competing ligand exchange - adsorptive cathodic stripping voltammetry (CLE-AdCSV), and is the speciation technique used in this study. With time, a competing equilibrium is established between $AL$ and any ambient, naturally occurring, Cu-binding organic ligands. This changes the mass balance of total dissolved copper ($[Cu_T]$) according to equation (13), where $[Cu(AL)_x]$ is the concentration of the competing Cu-ligand complexes. For most competing ligands only 1:1 ($Cu(AL)_1$) and 1:2 ($Cu(AL)_2$) complexes need to be taken into account (Xue and Sigg 2002).

$$[Cu_T] = [Cu^{2+}] + [CuX] + [CuL] + [Cu(AL)_x] \quad (13)$$

The $Cu(AL)_x$ complex is subsequently adsorbed to a hanging mercury drop electrode at a fixed potential for a time period appropriate to the copper concentration. After deposition, the complex is stripped from the Hg-drop, and the resulting analytical signal is the current from the reduction of Cu(II) in the adsorbed complex to Cu(0). The resultant current is proportional to the amount of $Cu(AL)_x$ accumulated on the electrode surface, which under constant accumulation and stripping conditions, is in turn proportional to the concentration of $Cu(AL)_x$ in solution (Thomas and Henze 2001).

Based on equilibria, the concentration of the $Cu(AL)_x$ complex is calculated using its stability constant, conditional for the sample matrix, and the free $AL$ concentration as shown in expression (14). This provides a compact way to use equilibrium calculations to relate the labile copper ion concentration, $[Cu']$, to that of the electroactive species, $[Cu(AL)_x]$, and the side reaction coefficient of the $AL$ ($\alpha_{Cu(AL)_x}$).

$$[Cu(AL)_x] = [Cu'] \left( K_{Cu(AL)_x} [AL]^x \right) = [Cu'] \alpha_{Cu(AL)_x} \quad (14)$$

If the conditional stability constants of the competing ligand complexes with copper, proton and major cations are available, then the coefficient ($\alpha_{Cu(AL)_x}$) can be easily calculated. However, if these data are not available, then $K_{Cu(AL)_x}$ and $\alpha_{Cu(AL)_x}$ must be determined in solutions identical to the composition of the natural water sample.
The CLE-AdCSV technique does not directly measure the bio-active Cu$^{2+}$ ion; instead, this is obtained by mathematical manipulations of the voltammetry results after titrating the sample with increasing amounts of copper at a fixed concentration of added ligand. After each copper addition, the Cu(AL)$_x$ reduction current is determined, and a titration curve of the type depicted in Figure 2.3 is plotted. A marked change in the gradient of the titration curve occurs when the Cu-complexing capacity of the natural ligands are saturated (Apte, Gardner et al. 1988). Beyond this point, the slope of the upper portion of the titration curve is theoretically identical to that obtained when no ligand is present (e.g., a UV-irradiated portion of the water). The overall shape of the titration curve indicates the extent and nature of complexation, but other processes besides complex formation can produce the same form of titration curves, such as metal adsorption on colloidal particles (Ružić 1982). On the basis of the direct titration curve, it is not possible to distinguish between these two processes. For this reason, minimum sample treatment such as filtration may be necessary.

The CuL complexes in the sample are assumed to be non-electrochemically labile (van den Berg 1982); as such, at the higher Cu-additions when the natural ligands are saturated, the peak currents are proportional to the ambient Cu$^{2+}$ and the
labile portion of $CuX$. Both of these, as shown in expression (15), makes up the electroactive $Cu(Al)_x$ species that are directly measured. 

$$([Cu_T] - [CuL]) = ([Cu^{2+}] + [CuX]) = [Cu(Al)_x] \quad (15)$$

$$i_p = S([Cu_T] - [CuL]) \quad (16)$$

This establishes a proportionality equation, (16), between the peak current ($i_p$) and the copper concentration, and introduces a proportionality factor ($S$ – sensitivity) which is specific to the analytical method and the sample being titrated. The sensitivity is conventionally obtained as the slope of the linear portion of the titration curve. Also, extrapolation of the linear portion of the titration curve down to the x-axis gives a crude estimate of the ambient organic ligand concentration, assuming a 1:1 complexation stoichiometry (Apte, Gardner et al. 1988).

To introduce more practical terms in the calculation, expressions (15) and (16) are combined to yield expression (17), in which $[Cu(Al)_x]$ is substituted with its equivalent, $[Cu']\alpha_{Cu(Al)_x}$. Equation (17) is modified to include the side reaction coefficient of copper with the inorganic ligands; this yields expression (18) in which $\alpha_{Cu(Al)_x}$ and $\alpha_{CuX}$ are grouped to give combined side reaction coefficient ($\alpha'$). In most cases the factor $(1 + \alpha_{CuX})$ is negligible in comparison to $\alpha_{Cu(Al)_x}$, as such $\alpha'$ is mostly controlled by the concentration of the added ligand. The combined side reaction coefficient is unique for each water sample and establishes the concept of an analytical detection window. After correction with $\alpha'$, the sensitivity can be used to obtain an estimate for $[Cu^{2+}]$ and by extension, the other complexation parameters.

$$i_p = S[Cu'] \alpha_{Cu(Al)_x} \quad (17)$$

$$i_p = S[Cu^{2+}] (1 + \alpha_{CuX} + \alpha_{Cu(Al)_x}) = S[Cu^{2+}] \alpha' \quad (18)$$

The analytical detection window is a unique property of CLE-AdCSV in which the experimental parameters can be tuned to measure different ligand classes, albeit with different stability constants, in the same sample. Basically, the value of $\alpha'$ defines an analytical competition strength which has to be comparable to the magnitudes of the side reaction coefficients of the naturally occurring ligands ($\alpha_{CuL}$) that are detected.
at each analytical window. This leads to an operationally defined method in which the value of $\alpha'$ is tuned to the analytical window of interest by purposely varying the concentration of the added ligand, or by selecting a different added ligand that forms weaker or stronger complexes with copper (Lucia, Campos et al. 1994). It has been shown that where $\log \alpha_{\text{Cu(Al)x}}$ is the centre of the detection window, only values for $\log \alpha_{\text{CuL}}$ within approximately ±1 are thought to be quantifiable accurately (Apte, Gardner et al. 1988).

Natural aquatic samples contain several classes of organic ligands that form Cu-complexes of greatly varying stabilities (Buffle 1988); therefore, speciation measurements at a single detection window gives only part of the data required for accurate speciation calculations. To better describe the heterogeneity of the organic ligand pool, a minimum of two titrations are required (van den Berg and Donat 1992) to define at least two or more ligand classes in the sample. The ligands identified at each detection window are usually termed: L1 – ligands that have a lower concentration but form relatively stronger metal complexes; and L2 - ligands that are at a higher concentration, but form weaker metal complexes (van den Berg 1982).

The analytical data obtained from complexometric titrations need to be further reduced to obtain the required complexation parameters, and for this several mathematical treatments have been developed including various data linearisation and non-linear curve-fitting techniques. The two most commonly used linearisation methods are based on discrete ligand-binding models - one based on Scatchard’s work with small molecules binding by proteins (Scatchard 1949), and the other, van den Berg/Ružić linearisation (Ružić 1982; van den Berg 1982), is based on the Langmuir isotherm. Both linearisation methods were compared with simulated ligand-titration data and it was shown that the Scatchard treatment consistently produced results of poorer precision, for both stability constants and ligand concentrations (Apte, Gardner et al. 1988), as such, this data treatment method is not discussed further. The non-linear curve fitting technique was pioneered by Gerringa (Gerringa, Herman et al. 1995), and the technique is named accordingly.
2.1.4.2 Analysis of CLE-AdCSV Data

The van den Berg/Ružić linearisation equation can take on different forms; in the situation where there is only a single ligand class in solution, within the specific analytical window, that forms a 1:1 complex with copper, the applicable linearisation equation is described as:

\[
\frac{[Cu^{2+}]}{[CuL]} = \frac{[Cu^{2+}]}{[L_T]} + \frac{\alpha'}{[L_T] K_{Cu'L'}}
\]

(19)

For a one ligand system a plot of \([Cu^{2+}]/[CuL]\) vs \([Cu^{2+}]\) gives a straight line, Figure 2.4(A), from which a value for the \([L_T]\) is obtained from the inverse of the slope, whereas the value for \(K_{Cu'L'} = (\alpha' \times \text{slope})/Y\)-axis intercept. If there are two or more ligands classes with different stability constants in the detection window, the resultant plot will show a curvature (van den Berg 1982); this requires an extended form of the van den Berg/Ružić equation to fit a multi-ligand system. However, the quality of the resulting parameters is questionable, and extreme caution is advised (Apte, Gardner et al. 1988); so ideally, a non-linear curve fitting technique is recommended for calculating the complexation parameters for a multi-ligand systems.

Among the disadvantages of the van den Berg/Ružić linearisation is that the axes are not independent of each other, and such is not ideal for error minimisation.
Also, at high values for $K_{\text{CuL}'}$, any measurement variability tends to produce variation in the plot, which sometimes lead to observed negative values for the intercept of the y-axis, and hence, negative values for $K_{\text{CuL}'}$. Also, the observation of curvature in the left hand side of the plot can be inconclusive, as often, it can be attributed to scatter in the data since the linearisation inherently narrows the range of values in this area. Moreover, the scatter obscures the possibility of detecting the presence of more than one ligand class.

In comparing the linear and non-linear fits, it was shown that there is no significant difference between values for [L]$_T$ obtained with the methods (Gerringa, Herman et al. 1995). However, the linear fitting method tends to give a lower value for $K_{\text{CuL}'}$ and often overestimates [Cu$^{2+}$] when an analytical error occurs at low Cu concentrations in the metal titration. The non-linear Gerringa method is preferred as it is more suited to the error structure of the metal titration data, which is of constant absolute magnitude. Moreover, it offers the advantage that the standard error of the estimated parameters can be calculated consistently. The non-linear Gerringa equation that describes a 1:1 complexation is shown in expression (20), and in principle, it can easily be extended to fit for more than one ligand.

$$CuL = \frac{[L_T] [Cu^{2+}] K_{\text{CuL}'} [Cu^{2+}]}{1 + K_{\text{CuL}'} [Cu^{2+}]} \quad (20)$$

A plot of [CuL] vs [Cu$^{2+}$] as shown in Figure 2.4(B) is used to fit the Gerringa non-linear regression; ideally, a mathematical software is required to iteratively solve the equation to determine the complexation parameters.

### 2.1.5 Accuracy and Validity of Voltammetric Techniques

A suitable competing ligand for a given metal should be well characterised with respect to the formed metal-ligand complexes, and these complexes should be able to be accurately and selectively measured by voltammetry. Among the Cu-complexing ligands are catechol, 8-hydroxyquinoline, salicylaldoxime and tropolone (Lucia, Campos et al. 1994); the complexes are shown in Figure 2.5. The stabilities of the Cu-catechol complexes span a large range but catechol is oxidation prone and can only be
equilibrated for a few minutes, which may be too short to achieve thermodynamic equilibrium (M.G. van den Berg 1984). Cu-tropolone complexes are comparatively weak (Donat and van den Berg 1992), whereas the complexes with 8-hydroxyquinoline are very stable making it suited for the detection of very stable natural complexes as weak complexes would be outcompeted fully (Van Den Berg 1986). Salicylaldoxime is the most used AL for Cu as its complexes can be determined in seawater at a better sensitivity than the other ligands, and the stability of its complexes are intermediate to that of 8-hydroxyquinoline and tropolone (Lucia, Campos et al. 1994).

![Figure 2.5](image)

**Figure 2.5** Names and structures of the various Cu-added ligand complexes used in Cu-ligand titrations with CLE-AdCSV.

Throughout the CLE-AdCSV titration, hydrogen ions are liberated during the formation of the copper-ligand complexes and the solution pH decreases, and this is further exacerbated as the Cu-standard solution used in the analysis is usually acidified. Relatively small pH changes can result in large differences in the free copper ion concentration and resultantly in the calculated speciation (Xue and Sigg 2002). Therefore, it is essential to maintain the pH constant, and close to the in situ pH and this is best achieved using an adequate pH buffer. The selected buffer must not complex the specific metal, and at least exhibit no obvious effects on the resulting metal speciation under the working conditions.

When the simple single-ligand model of complexation is used, CLE-AdCSV can provide relatively accurate metal complexation data, but it depends on a number of criteria and assumptions. It is assumed that full thermodynamic equilibrium is
established between the added ligand and the natural ligands within the selected equilibration time. However, such assumption may not always be accurate, due to the dynamic nature of natural water samples. It is also convenient to assume that chemical reactions such as adsorption or oxidation do not significantly decrease the concentration of the added ligand in solution; and that mixed complexes are not formed between the added ligand and natural ligands; and also that competition with other cations does not significantly affect the main equilibrium relationships. These are acceptable and expedient assumptions as long as the parameters obtained are adequate to predict the Cu-complexing behaviour of the natural water sample.

2.2 Materials

2.2.1 Instrumentation

<table>
<thead>
<tr>
<th>Voltammetric Parameters</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Purge time initial/subsequent</td>
<td>120s/30 s</td>
</tr>
<tr>
<td>Accumulation potential</td>
<td>-0.15 V</td>
</tr>
<tr>
<td>Accumulation time</td>
<td>30-60 s</td>
</tr>
<tr>
<td>Voltammetric scan</td>
<td>From -0.15 V to -0.8 V</td>
</tr>
<tr>
<td>Equilibration time</td>
<td>5 s</td>
</tr>
<tr>
<td>Modulation time</td>
<td>0.02 s</td>
</tr>
<tr>
<td>Interval time</td>
<td>0.2 s</td>
</tr>
<tr>
<td>Step potential</td>
<td>0.00405 V</td>
</tr>
<tr>
<td>Modulation amplitude</td>
<td>0.04995 V</td>
</tr>
</tbody>
</table>

The voltammetry setup consisted of a Metrohm 663 VA Stand connected to a µAutolab II Potentiostat, which together were computer-controlled using GPES v4.9 software. The working electrode was a hanging mercury drop electrode (HMDE), the reference electrode was Ag|AgCl|3M KCl, while a glassy carbon rod was used as the auxiliary electrode. The voltammetry was operated in the differential pulse mode at room temperature and each sample was scanned multiple times, but the voltammograms recorded were the first three with peak heights within approximately
5% of each other. Samples were purged with oxygen-free nitrogen gas, and stirred during deposition with a PTFE rotating rod. The voltammetric parameters as shown in Table 2.1, were optimised for the detection of Cu(SA) complexes, and produced stripping voltammograms that were smooth and stable as shown in Figure 2.7. Measurements for pH were obtained using a Metrohm 713 pH Meter, and the values were calibrated against NBS pH buffers 4, 7 and 9. Samples for UV-digestion were placed in acid-cleaned quartz tubes and arranged in an Annular Photoreactor (model APQ 40), then irradiated with a 400 W medium pressure Hg lamp for 4-6 hours.

2.2.2 Reagents

The water used for the preparation of all chemicals was purified by distillation followed by deionization using a Milli-Q® system, with the final water having a conductivity of ≥18 MΩ.cm at 25°C. Analytical grade hydrochloric acid was further purified by sub-boiling distillation using a quartz apparatus. An aqueous stock solution containing 0.025 M salicylaldoxime was prepared in 0.1 M quartz-distilled HCl (q-HCl) and was stored at 4°C for up to 4 weeks. Copper standard solutions were prepared by dissolution of an atomic absorption spectrometry standard (Spectrosol®) in 1 % q-HCl. A pH buffer containing 1M H₃BO₃ (Sigma, ACS grade) and 0.35M NH₃ (analytical grade) was chelexed and UV-irradiated for 6 hours; 100 µL of this buffer in 10 ml of seawater maintained the pH in the region of 8-8.3.

2.2.3 Trace-metal Cleaning

Ultraclean sample handling and analysis techniques are necessary to obtain reliable trace metals data for aquatic environments. As such, the handling and manipulation of samples were done in a vertical-laminar flow class 100 bench, using trace-metal clean practices throughout the entire process to minimise sample contamination. All sample containers were cleaned by soaking in a 2% Citranox® detergent bath for two days, then washed with distilled water. Containers were then transferred to 50% HCl (AR) for 1 week, and then rinsed with deionised water from a Milli-Q® system. This was followed by a further soak for at least 1 week in 10% q-HCl.
Sample bottles were stored filled with 0.1% q-HCl and placed in double bags until use. All other plastic and glass labware not directly used for sample handling were cleaned as above, except they were not soaked in 50% HCl.

2.3 Sample Analyses

2.3.1 UV-Irradiation

Dissolved organic matter (DOM) found naturally in aquatic environments can interfere with the voltammetric determination of total dissolved metals as they may complex the metals, preventing them from reduction/oxidation at the Hg-electrode, or shift the electrochemical reduction or oxidation potential and distort the signal (Golimowski and Golimowska 1996). These organic species may also undergo electrochemical reduction or oxidation, thereby increasing the background current which can make voltammetric analysis difficult. DOM may also adsorb on the Hg-electrode surface thus limiting its active surface area, and this is particularly undesirable for the adsorptive voltammetry method used in this study.

![Figure 2.6 Effectiveness of UV-irradiation in removing dissolved organic compounds from seawater. (A) The decomposition of humic acid (HA) with time as monitored by UV-absorbance. (B) Cu-ligand titration curve of seawater after 4 hours of UV-irradiation.](image)

It is therefore important to remove DOM from the seawater samples before voltammetric determination of total dissolved copper; for this, UV-irradiation was
selected as this method is effective and does not contaminate the sample. In assessing the effectiveness of the UV-irradiation process, humic acid (HA) (467.6 mg/L) with the addition of 30% H₂O₂ to assist the oxidation process, and q-HCl to avoid a precipitation of salts, were added to a series of quartz tubes and exposed to the UV radiation in a batch experiment. The decomposition of the HA was monitored with time by measuring the UV-absorbance at 254 nm. After one hour of irradiation, the active chromophoric component of the HA decreased by about 90% of its initial value and further decreased over the next five hours as shown in Figure 2.6(A).

The dissolved organic carbon (DOC) from the concentration of humic acid used in the experiment was many magnitudes greater than that found in most natural aquatic environment. Therefore, if one hour of UV-irradiation was able to almost fully disintegrate the Cu-complexing abilities of this high DOC concentration, then by extension the same irradiation time is assumed to be sufficient for seawater which has a much lower DOC content. However, to be certain, 4-6 hours irradiation was used for seawater samples throughout the study. The results from a typical Cu-ligand titration of a seawater sample after 4 hours of UV-irradiation is depicted in Figure 2.6(B). It shows that the sample was relatively free of Cu-complexing species, as the characteristic peak height suppression and curvature, typical for when metal-complexing species are in solution were absent in the titration.

2.3.2 Total Dissolved Copper

In the determination of total dissolved copper, [Cu₃], seawater (0.2 µm filtered) was acidified using q-HCl to pH ≤ 2 and then UV-irradiated prior to the analysis. Once cooled to room temperature, an aliquot of 10 ml was pipetted into a PTFE titration vessel, then trace-metal grade NH₄OH (Optima™, Fisher Chemical) was added to neutralise the pH. The volume of NH₄OH was dependent on the extent to which the sample was acidified, but was in the range of 40-90 µL. This was followed by the addition of 100 µL of borate buffer, then 10 µL of 0.025 M salicylaldoxime to give a final concentration of 25µM; the sample was then ready for voltammetric analysis. After the first measurement was completed, two standard additions of copper were used to calibrate the method for total dissolved copper concentration.
Table 2.2 Replicates of total dissolved copper values obtained using the optimised voltammetry method for SAFe D2 reference seawater and a test sample. The bold values are the average for each sample, showing the standard deviation of the many replicates.

<table>
<thead>
<tr>
<th>Total Dissolved Copper (nM)</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>SAFe D2</td>
<td>Test Sample</td>
</tr>
<tr>
<td>2.18</td>
<td>3.98</td>
</tr>
<tr>
<td>2.31</td>
<td>4.48</td>
</tr>
<tr>
<td>2.09</td>
<td>3.73</td>
</tr>
<tr>
<td>2.06</td>
<td>3.80</td>
</tr>
<tr>
<td>2.27</td>
<td>4.25</td>
</tr>
<tr>
<td>2.38</td>
<td>3.79</td>
</tr>
<tr>
<td>2.20</td>
<td>4.37</td>
</tr>
<tr>
<td>2.41</td>
<td>3.79</td>
</tr>
<tr>
<td>2.58</td>
<td>3.95</td>
</tr>
<tr>
<td>2.25</td>
<td>3.75</td>
</tr>
<tr>
<td>2.43</td>
<td>3.94</td>
</tr>
<tr>
<td>2.09</td>
<td>4.09</td>
</tr>
<tr>
<td>2.04</td>
<td>4.51</td>
</tr>
<tr>
<td><strong>2.25 ±0.16</strong></td>
<td><strong>4.03 ±0.28</strong></td>
</tr>
</tbody>
</table>

The method for determining total dissolved copper was optimised using UV-irradiated seawater, subsequent to which the accuracy was assessed by measuring a reference seawater sample, SAFe D2 (Bruland 2013). The reference seawater had a certified value of 2.28 ±0.15 nM for [Cu\textsubscript{T}] after pre-treatment with UV-irradiation. After repeated measurements of SAFe D2 (n=13), [Cu\textsubscript{T}] was determined to be 2.25 ±0.16 nM with a relative standard deviation (RSD) of 7.28 %. The results, shown in Table 2.2 confirm that the optimised voltammetry method is able to accurately determine [Cu\textsubscript{T}] in seawater samples, which shows that contamination from chemicals and procedure were not significant. The precision of the method was assessed by measuring (n= 13) a test seawater sample from the west Pacific, for which [Cu\textsubscript{T}] was determined to be 4.03 ±0.28 nM with a RSD of 7 %. It is reasonable to conclude that the optimised method is able to accurately determine total dissolved copper in seawater with a RSD of approximately 7 %.
2.3.3 Copper-Complexing Ligands

Copper-complexing ligand concentrations and conditional stability constants were determined using the indirect method of CLE-AdCSV (see Section 2.1.4) which detects the electrochemically-active complex formed between the labile fraction of Cu in the sample, and a well characterised organic ligand added to the sample. Salicylaldoxime (SA) was used as the competing ligand because its complexation parameters with Cu in seawater are well characterised, and its sensitivity allows the detection of very low ligand concentrations. In this method, the seawater sample, if frozen, was thawed over 1-2 days at 4 °C and then allowed to reach room temperature just before the procedure was started. From this were pipetted 10 ml aliquots in 10-15 PTFE vials, and to each was added 100 µL of the borate buffer. This was followed by increasing additions of copper standard in the range of 0-400 nM depending on the expected ligand concentration.

![Figure 2.7](image)

**Figure 2.7** Voltammograms of increasing copper concentrations added to a test seawater sample, from the west Pacific, during a Cu-ligand titration using 10 µM SA and deposited at -0.1 V.

The samples were then left to equilibrate for an hour, to allow the added Cu to bind to the natural ligands before an excess of SA was added. Without this initial reaction time, achieving equilibrium would be more difficult, as Cu(SA) would first have to dissociate before the natural ligands can bind to the free Cu. The SA was finally added at a concentration of 1-10 µM depending on the desired analytical window, but was always well in excess of Cu and other metals that may also form complexes.
stable complexes with SA. The samples were then allowed to equilibrate for a minimum of 12 hours, but typically overnight, which has been found to give stable Cu(SA) peaks as shown in Figure 2.7. The voltammetric measurements were made after the competing equilibrium had been established between the added ligand and any ambient, naturally occurring, Cu-binding organic ligands.

2.3.4 Data Manipulation

The Cu-SA peak currents were used in calculations that transformed the data, and generated values for the concentration and conditional stability constant of the Cu-complexing ligands in solution. There are a few transformation methods (see section 2.1.4.2) each with its own limitations, but the most widely used is the Gerringa non-linear fitting method. The manual calculations involved are long, exhaustive and prone to errors; therefore, all calculations and data fitting were done with the software Metal Complexing Capacity Software (MCCSOFT), which is authored by Dario Omanovic from Ruder Boskovic Institute, Zagreb, Croatia. The up-to-date version is available at http://gss.srce.hr/pithos/rest/omanovic@irb.hr/files/Software/. In an attempt to determine if the software is calculating accurate values, four ligand titration data sets were used for manual calculation of the copper complexation parameters, using the van den Berg linearisation method; the values compare relatively well with those calculated by MCCSOFT as shown in Table 2.3.

Table 2.3 Comparison between manual calculation and MCCSOFT in determining ligand concentration, \([L_{Cu}]\), and conditional stability constant, \(\log K_{Cu'L'}\) from the same Cu-ligand titration datasets.

<table>
<thead>
<tr>
<th>Dataset</th>
<th>([L_{Cu}]) nM</th>
<th>(\log K_{Cu'L'})</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Manual</td>
<td>MCCSOFT</td>
</tr>
<tr>
<td>1</td>
<td>23.56</td>
<td>25.41 ±2.2</td>
</tr>
<tr>
<td>2</td>
<td>21.36</td>
<td>22.03 ±1.7</td>
</tr>
<tr>
<td>3</td>
<td>27.49</td>
<td>24.26 ±1.1</td>
</tr>
<tr>
<td>4</td>
<td>23.15</td>
<td>20.33 ±3.5</td>
</tr>
</tbody>
</table>
Chapter 3: Quantitative Assessment of the CLE-AdCSV Technique in Copper-Ligand Titrations

3.1 Introduction

The concept that the chemical speciation of trace metals in natural waters is regulated by forming complexes with natural organic ligands is now a central paradigm of aquatic chemistry (Hunter 2005). With a few exceptions, this is based on results from speciation measurements using electrochemical techniques, most notably CLE-AdCSV. The theory governing CLE-AdCSV is well established (Ružić 1982; van den Berg 1982) and has been used for the past few decades, providing results that redefined the understanding of trace-metal speciation in natural aquatic systems. Though CLE-AdCSV is used almost exclusively to determine trace metals speciation, there are only a few studies that thoroughly investigated, and experimentally verified the quantitative basis underlying this technique (Leal and van den Berg 1998). A major constraint is to find suitable standard reference materials for natural metal-complexing ligands, which is partly due to the complex nature of the ligand pool in natural waters, and the inability to keep them stable during storage.

Within these limitations, an appropriate approach to quantitatively verify the CLE-AdCSV technique is to UV-irradiate a seawater sample, making it relatively free of natural metal-complexing ligands, and then add a metal-specific ligand. The complex with the added ligand should have a known thermodynamic stability constant (log \( K_{\text{CuL}} \)), as determined using other method(s); this is then compared to the log \( K_{\text{CuL}} \) measured with CLE-AdCSV. It is recognised that this is a model system which is different from a natural aquatic sample; but this approach was used to a small extent to assess copper complexation with glutathione (GSH) and cysteine (CYS) (Leal and van den Berg 1998). These thiol species are ideal as they form stable Cu-complexes with known complexation parameters, and they are common in the marine environment.

Thiols are organic compounds that include the sulphydryl group (-SH) and exist in a huge variety of forms such as reduced glutathione and cysteine; plus disulphur dimers which can result in hundreds of possible compounds such as oxidised
glutathione, cystine and phytochelatin. Reduced glutathione (GSH) and cysteine (CYS), as shown in Figure 3.1A and C, are polydentate ligands which offer multiple potential binding sites, i.e., carboxylate oxygens, amino nitrogen, thiol groups and amide groups. The structure of GSH and CYS are such that their binding sites are sterically hindered, and cannot be simultaneously coordinated to the same metal ion. Consequently, their coordination chemistry is characterised by the formation of protonated and polynuclear complexes (Bibhesh 2005).

![Figure 3.1 Some examples of thiol species in the natural aquatic environment. (A) Reduced glutathione, (B) Oxidised glutathione, (C) Reduced cysteine and (D) Oxidised cysteine (cystine).](image)

Glutathione and cysteine are considered to be readily oxidised to dipeptides (oxidised glutathione and cystine) in the presence of enzymes (Yeh, Hou et al. 2006); however, outside the enzymatic environment this reaction can be catalysed by transition metal ions such as copper (Kachur, Koch et al. 1998). It is established that the Cu ion is bound in a 1:2 ratio during the copper-catalysed oxidation of thiols; however, there are uncertainties as to the exact complexation mechanism. As such, there is an active debate as to whether Cu(II) is first reduced to Cu(I) in the presence of thiol and then complexed, or whether the thiol species directly complex Cu(II) and then the latter is reduced to Cu(I) (Cavallini, De Marco et al. 1969; Pecci, Montefoschi et al. 1997; Kachur, Koch et al. 1998).
In the above mentioned study by van den Berg et al. (1998) copper-glutathione (Cu-GSH) and copper-cysteine (Cu-CYS) complexes were successfully characterised using Cu-ligand titrations with salicylaldoxime (SA) as the added ligand. In the absence of conclusive evidence that these thiols bind exclusively to Cu(I), the authors nonetheless suggested that some of the ligands detected in seawater, and previously assumed to be Cu(II) binding ligands, could be complexed to Cu(I). However, there is an ongoing scientific debate (Cavallini, De Marco et al. 1969; Pecci, Montefoschi et al. 1997; Kachur, Koch et al. 1998) as to the exact complexation and redox mechanisms involved in the oxidation of thiols by trace-metal ions. This current study builds on that of van den Berg et al. (1998), but no attempt was made to speculate on the actual redox species of copper involved in the titrations, such requires a detailed investigation beyond the scope of this project.

3.2 Experiments and Observations

To experimentally verify the quantitative reliability of the CLE-AdCSV technique glutathione, L-cysteine and sodium sulphide (Na$_2$S; inorganic Cu-complexing ligand) were added to UV-irradiated seawater and the extent of copper complexation was determined using CLE-AdCSV. Since sulphur species are potentially prone to oxidation once in solution, they were prepared fresh each day from the reagent salts and then spiked in predetermined concentrations. The solutions were neither stabilised nor standardised as the weights were accurate enough within the analytical tolerance for errors. However, given the unstable nature of Na$_2$S, it is possible that the actual concentrations may have been slightly different from that calculated from the total solids dissolved. The copper complexes formed with the sulphur species had specific electrochemical interactions with the Hg-electrode, and caused individual peaks in the voltammograms. As shown in Figure 3.2, Cu-CYS gave a peak at approximately -0.43V, Cu-GSH at -0.53V and that with Na$_2$S (Cu-S) formed a sharp peak in the region of -0.5V. It should be noted that these sulphur related peaks were absent in the voltammetric scan of UV-irradiated seawater during Cu-ligand control titrations (Figure 3.2A).
Sulphur species, whether free or complexed, reacts with mercury from the electrode, giving reduction peaks in the region of -0.4 to -0.6 V (Canterford and Buchanan 1973), similar to those shown in Figure 3.2. It was proposed that in the case of thiols, it is the free thio-group that complexes with mercury giving the observed reduction peak (Laglera and van den Berg 2003). In an earlier paper the same research group had suggested that it is the Cu-thiol complex that forms the reduction peak (Leal and van den Berg 1998). The results from this current study convincingly show that, in general, it is the copper complex of these sulphur compounds that gets reduced at the Hg-electrode and not the free species. However, some observations in
this current study show that the mechanism involved is not a simple, single species electrode reaction. The peaks from the sulphur species can provide useful information about the complexation processes; as such, the voltammetric scans were extended further negative to analyse thiol and sulphide peaks during each measurement.

### 3.3 Results

#### 3.3.1 Single-Ligand Titrations

Glutathione, CYS and Na$_2$S were added separately to UV-irradiated seawater at 50 nM with 2 µM SA, and analysed as per the Cu-ligand titration method in section 2.3.3. As expected, plots of the Cu-SA peak currents (at -0.27 V) gave typical complexometric titration plots (see Figure 3.4A). The Cu-GSH and Cu-CYS peak currents, at more negative potentials, increased with Cu additions but then levelled off as shown in Figure 3.3A for Cu-GSH and Figure 3.4B for Cu-CYS. The peak resulting from the addition of Na$_2$S had a different pattern (Figure 3.3B); it increased to a maximum then decreased until it eventually disappeared at higher copper additions.

![Figure 3.3](image)

Figure 3.3 (A) Changes in the Cu-GSH peak current (at -0.53 V) for 50 and 100 nM GSH with copper additions. (B) Replicates of changes in the Cu-S peak current with addition of 50 nM Na$_2$S. All Cu-ligand titrations were in separate UV-irradiated seawater at pH 8.3, 2 µM SA and deposition at -0.15 V for 30 s.

Copper binds to GSH and CYS in a 1:2 ratio; therefore, 25 nM of Cu-complexing ligands were expected to be measured for titrations with 50 nM thiol additions; Table
3.1 shows the results. Cu-GSH and Cu-CYS complexes were measured at average concentrations of 24 ± 4 and 22 ± 1 nM respectively.

<p>| Table 3.1 CLE-AdCSV determination of Cu-ligand concentrations (L_{Cu}) and conditional stability constants (log K_{Cu'L'}) after separate additions of glutathione, L-cysteine and Na_{2}S to UV-irradiated seawater. Titrations were at 2 μM SA, pH 8.3 and deposition at -0.15 V for 30s. log α_{CuL} (K_{Cu'L'} : [L_{Cu}]) is a measure of the analytical strength and should be within ±1 of the detection window, which is 3.85 ± 0.2 at 2 μM SA. |</p>
<table>
<thead>
<tr>
<th>Ligand</th>
<th>Added (nM)</th>
<th>Measured (nM)</th>
<th>log K_{Cu'L'}</th>
<th>log α_{CuL}</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glutathione</td>
<td>50</td>
<td>25.4 ± 2.29</td>
<td>11.97 ± 0.15</td>
<td>4.37</td>
</tr>
<tr>
<td></td>
<td></td>
<td>22.0 ± 1.73</td>
<td>12.05 ± 0.13</td>
<td>4.39</td>
</tr>
<tr>
<td></td>
<td></td>
<td>25.0 ± 6.6</td>
<td>11.61 ± 0.38</td>
<td>4.01</td>
</tr>
<tr>
<td></td>
<td></td>
<td>24.8 ± 4.59</td>
<td>11.53 ± 0.23</td>
<td>3.92</td>
</tr>
<tr>
<td></td>
<td></td>
<td>21.7 ± 4.17</td>
<td>11.64 ± 0.27</td>
<td>3.97</td>
</tr>
<tr>
<td></td>
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<td>23.78 ± 3.88</td>
<td>11.76 ± 0.23</td>
<td>4.13</td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>43.4 ± 4.21</td>
<td>11.43 ± 0.11</td>
<td>4.07</td>
</tr>
<tr>
<td></td>
<td></td>
<td>55.5 ± 15.5</td>
<td>11.10 ± 0.31</td>
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</tr>
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<td></td>
<td></td>
<td>49.45 ± 9.86</td>
<td>11.27 ± 0.21</td>
<td>3.96</td>
</tr>
<tr>
<td>L-cysteine</td>
<td>50</td>
<td>24.2 ± 1.13</td>
<td>12.86 ± 0.11</td>
<td>5.24</td>
</tr>
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<td></td>
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<td>18.6 ± 0.68</td>
<td>12.95 ± 0.11</td>
<td>5.22</td>
</tr>
<tr>
<td></td>
<td></td>
<td>20.3 ± 3.45</td>
<td>12.61 ± 0.43</td>
<td>4.92</td>
</tr>
<tr>
<td></td>
<td></td>
<td>26.9 ± 1.55</td>
<td>12.54 ± 0.12</td>
<td>4.97</td>
</tr>
<tr>
<td></td>
<td></td>
<td>19.4 ± 1.05</td>
<td>12.10 ± 0.12</td>
<td>4.39</td>
</tr>
<tr>
<td></td>
<td></td>
<td>21.88 ± 1.57</td>
<td>12.61 ± 0.18</td>
<td>4.95</td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>17.4 ± 0.60</td>
<td>12.69 ± 0.08</td>
<td>4.93</td>
</tr>
<tr>
<td></td>
<td></td>
<td>24.1 ± 0.92</td>
<td>12.35 ± 0.08</td>
<td>4.73</td>
</tr>
<tr>
<td></td>
<td></td>
<td>25.0 ± 2.76</td>
<td>12.23 ± 0.20</td>
<td>4.63</td>
</tr>
<tr>
<td></td>
<td></td>
<td>18.2 ± 1.90</td>
<td>12.49 ± 0.20</td>
<td>4.76</td>
</tr>
<tr>
<td></td>
<td></td>
<td>24.4 ± 2.14</td>
<td>12.31 ± 0.17</td>
<td>4.70</td>
</tr>
<tr>
<td></td>
<td></td>
<td>21.82 ± 1.66</td>
<td>12.41 ± 0.15</td>
<td>4.75</td>
</tr>
<tr>
<td>Na_{2}S</td>
<td>50</td>
<td>68.9 ± 18.0</td>
<td>11.48 ± 0.32</td>
<td>4.32</td>
</tr>
<tr>
<td></td>
<td></td>
<td>34.2 ± 15.7</td>
<td>11.58 ± 0.64</td>
<td>4.11</td>
</tr>
<tr>
<td></td>
<td></td>
<td>52.2 ± 5.43</td>
<td>11.53 ± 0.14</td>
<td>4.25</td>
</tr>
<tr>
<td></td>
<td></td>
<td>51.77 ± 13</td>
<td>11.53 ± 0.37</td>
<td>4.23</td>
</tr>
</tbody>
</table>

The solutions were made daily without standardisation; nonetheless, there was good agreement in the measured ligand concentrations. On average, 52 ± 13 nM of
Cu-complexing ligands were measured after the addition of 50 nM Na$_2$S, this suggests an approximate 1:1 complexation ratio between Cu and the resultant sulphur species. These are assumed to be simple Cu-sulphide species, such as CuS (Luther, Rickard et al. 1996). However, Cu also forms sulphide complexes of stoichiometry Cu$_2$S$_3^{2-}$, among other mixed ligand and multinuclear complexes (Helz, Charnock et al. 1993; Thompson and Helz 1994).

Having established that Cu-complexation can be successfully characterised with independent 50 nM additions of model Cu-complexing ligands GSH, CYS and Na$_2$S; the assessment was extended using 100 nM of some species. As expected, an increase in the GSH and CYS concentrations caused a corresponding increase in the Cu-GSH and Cu-CYS peak currents. This is shown in Figure 3.4B which compares the Cu-CYS peak currents after the addition of 50 and 100 nM of CYS. The apparent 1:2 complexation ratio also held true with the addition of 100 nM GSH, as an average of 49.45 ± 9.86 nM of Cu-complexing ligands was measured in these samples. Interestingly, after the addition of 100 nM CYS only an average of 21.82 ± 1.66nM of Cu-complexing ligands was measured. This unexpected result was obtained consistently as shown in Table 3.1. The matter of experimental errors can be ruled out as special care was taken to control all the potential sources of error.

![Graph showing Cu-ligand titration results after the addition of 50 and 100 nM of CYS to separate UV-irradiated seawater.](image)

**Figure 3.4** Cu-ligand titration results after the addition of 50 and 100 nM of CYS to separate UV-irradiated seawater. (A) Cu-SA titration plots (B) Changes in Cu-CYS peak currents. Titrations were at pH 8.3, 2 µM SA and deposition at -0.15 V for 30 s.
The lower-than-expected ligand concentration measured in the presence of 100 nM CYS suggests either a change in the stoichiometry of the Cu-complex with increased [CYS]; or a constraining limit imposed by the chemistry of the sample matrix. These speculations were inferred from the Cu-SA peak currents at -0.27 V (Figure 3.4A) and the classical way of analysing CLE-AdCSV complexometric titrations as detailed in chapter 2. However, analysis of the Cu-CYS peak at -0.43V showed that the peak currents at 100 nM CYS were twice that at 50 nM for the same titration points. This is shown in Figure 3.5 which highlights the deviation around the mean (2.2 ± 0.2) when the Cu-CYS peak currents at 100nM CYS were divided by those at 50 nM for the same titration points. The Cu-CYS peak currents clearly show that there was twice as much Cu-CYS at the Hg-electrode when CYS was increased from 50 to 100 nM; yet this increase in Cu-complexation was not detected by analysing the Cu-SA peak currents.

![Figure 3.5](image)

**Figure 3.5** The deviation around the mean (\(\bar{X}\)) when the Cu-CYS peak currents from 100 nM CYS is divided by those from 50 nM CYS for each titration points. Titrations were at pH 8.3, 2 µM SA and deposition at -0.15 V for 30 s.

A unique property of CLE-AdCSV is the ability to vary the analytical competition strength by using the same competing ligand, but at different concentrations, to detect metal-ligand complexes with different complexing parameters. To investigate this feature the SA concentration was increased to 10 µM, and some titrations repeated with all other parameters remaining unchanged. The Cu-CYS peak currents at 10 µM SA followed the same pattern as at 2 µM SA, except the individual peaks were
lower (Figure 3.6B). This was expected as an increased amount of SA would bind more Cu, reducing that available to CYS; hence, the individual Cu-CYS peak currents would be reduced. In the context of a simple complexation mechanism, this observation suggests that it is the Cu-CYS complex that is responsible for the peak at -0.43 V, and not the free CYS. Otherwise, there would have been an increase in the cysteine peak currents with less free Cu to compete with the mercury from the electrode.

Figure 3.6 Cu-ligand titration results after the addition of 50 nM CYS with 2 and 10 µM SA in separate UV-irradiated seawater. (A) Cu-SA titration plots. (B) Changes in the Cu-CYS peak currents. Titrations were at pH 8.3, and deposition at -0.15 V for 30s.

Cu-complexing ligands were measured at an average concentration of 20.4 ± 2.63 nM (n=2) in solutions containing 50 nM CYS and 10 µM SA, for which Figure 3.6A is a representative titration plot. This shows that the Cu-complexing capacity of CYS did not vary with the detection window, in line with expectations as only one ligand was present in the sample. The absence of a shift shows that the results are unambiguous, and suggests that these results are not an artefact of the measurement. Titrations of 100 nM CYS at 10 µM SA showed clear signs of Cu-complexation, due to the typical curvature in the Cu-SA plot (data not shown), but the scatter in the data was too excessive to make any confident calculation. It should be noted that excessive scatter in Cu-ligand titration data at 10 µM SA was a constant feature throughout the entire study, but this is not widely reported in the literature.
In contrast, glutathione appeared to be fully outcompeted by 10 µM SA due to the absence of a curvature in its complexometric titration plot as shown in Figure 3.7A. However, it was previously reported by Leal and van den Berg (1998) that glutathione competes for copper at this higher detection window. Interestingly, with increased Cu-additions, the progression of the Cu-GSH peaks at 10 µM SA was different from that observed with 2 µM SA. At 10 µM SA the Cu-GSH peak decreased gradually with Cu additions as shown in Figure 3.7B.

![Figure 3.7](image_url)

**Figure 3.7** Cu-ligand titration results after the addition of 50 nM GSH with 2 and 10 µM SA in separate UV-irradiated seawater. (A) Cu-SA titration plots. (B) Changes in the Cu-GSH peak currents. Titrations were at pH 8.3 and deposition at -0.15 V for 30s.

A cardinal assumption in the theory governing CLE-AdCSV is that the competing ligand has a negligible effect on the other species in solution (Xue and Sigg 2002). To investigate this assumption a series of titrations were done without SA to see the changes, if any, in behaviour of the sulphur species in solution. If the above assumption is valid, then it is expected that with no SA in solution the individual Cu-thiol peak currents should be higher, as all the copper would be available to bind with the thiol. Also, no changes in the overall development of the Cu-thiol peak would be expected; as such, the Cu-thiol peak should increase with Cu additions and level off, as before, once the thiols are fully complexed. The latter expectation was observed when 50 nM GSH was titrated without SA as shown in Figure 3.8A. Unexpectedly, with 50 nM CYS the Cu-CYS peak decreased with copper additions to a minimum, then increased until it stabilised as shown in Figure 3.8B. It should be noted that the Cu-CYS
peak was at the same potential (-0.43 V), but was oddly-shaped when no SA was in solution.

![Figure 3.8](image.png)

**Figure 3.8** Cu-ligand titration results after adding 50 nM of each sulphur species with 0 and 2 µM SA in separate UV-irradiated seawater. (A) Changes in the Cu-GSH peak current. (B) Changes in the Cu-CYS peak current. (C) Changes in the Cu-S peak current. Titrations were at pH 8.3 and deposition at -0.15V for 30s.

Titrations were also done with 50 nM Na₂S to which no SA was added, and as shown in Figure 3.8C, the Cu-S peak currents (at -0.5 V) were higher than when 2 µM SA was added. Also, when no SA was added, the Cu-S peak goes to a maximum at approximately 50 nM added copper which corresponds to the [L₃Cu] added to the solution, assuming a 1:1 stoichiometry. In the presence of 2 µM SA, the Cu-S peak goes to a maximum at around 23 nM Cu, and this corresponds to the [L₃Cu] calculated for
In this particular titration. From these observations, it appears that the height of the Cu-S peak gives an estimate of the free \([S^2-]\) in solution. By this reasoning it can be assumed that the presence of SA in solution reduced the amount of free \(S^2-\) in solution; this is developed further in the Discussion.

The Cu-SA titration data were used to calculate values for the conditional stability constants (\(\log K_{CuL}\)) of the Cu-ligand complexes. The calculated \(\log K_{CuL}\) is dependent on the side-reactions of copper and the added ligand, and in this case requires no assumption regarding the inorganic speciation of copper which can occur as Cu(I) or Cu(II). At 2 \(\mu\)M SA the average \(\log K_{CuGSH}\) values as determined when 50 and 100 nM of GSH were added are 11.76 ± 0.23 and 11.27 ± 0.21 respectively. For CYS the corresponding \(\log K_{CuCYS}\) values at 2 \(\mu\)M SA were 12.61 ± 0.18 and 12.41 ± 0.15 when 50 and 100 nM of CYS were respectively added. At the higher analytical detection window using 10 \(\mu\)M SA, the average \(\log K_{CuCYS}\) when 50 nM CYS was added is 13.26 ± 0.22. This shows that the complexes formed with L-cysteine were more stable than those with GSH, but the latter was more stable than the complexes with \(Na_2S\) as at 2 \(\mu\)M SA those had an average \(\log K_{CuS}\) of 11.53 ± 0.37.

### 3.3.2 Mixed-Ligand Titrations

The preceding discourse assessed the reliability of the CLE-AdCSV method in quantifying Cu-complexation after individual additions of L-cysteine, glutathione and sodium sulphide to UV-irradiated seawater samples. These were ideal model-systems; but in reality, natural aquatic samples will not contain only a single Cu-complexing ligand. As such, experiments were undertaken to assess Cu-complexation in mixed-ligand systems. In theory, the data treatment method (see Section 2.1.4.2) can characterise two or more ligand classes in solution once the difference in their \(\log K_{CuL}\) is sufficiently large; such is the case for the Cu-complexing ligands used in this study, as evidenced by the values in Table 3.1. However, it should be noted that the data treatment method does not account for chemical interactions (such as mixed ligand formations) that may occur when multiple ligands are in solution. This project
has identified that the complexation dynamics change when at least two of these sulphur species were in solution.

The thiols species were added in comparable concentrations to ensure none of the individual peaks were disproportionately large, which could otherwise coalesce with, or out-scale the other. Figure 3.9A shows triplicates of a typical voltammetric scan of a mixed-ligand solution with 50 nM each of CYS and GSH. The expected Cu-GSH and Cu-S peaks from a mixture with GSH and Na₂S coalesced into a single peak (Figure 3.9B). This observation reinforces that a peak in the region of -0.5 V is characteristic of general sulphur species, and cannot be used with samples from a natural aquatic as a means to unambiguously identify individual sulphur species.

![Figure 3.9](image)

**Figure 3.9** Triplicates of typical voltammograms for mixed-ligands added to UV-irradiated seawater with 2 µM SA and deposition at -0.15 V for 30 s. (A) 50 nM GSH + 50 nM CYS, (B) 50 nM GSH + 50 nM Na₂S.

In mixed-ligand titrations with GSH and CYS, the Cu-CYS peak development is similar to when only CYS was in solution. That is, it increases with copper additions but does not level-off as distinctly as before (Figure 3.4B). The Cu-GSH peak takes a completely different pattern as shown in Figures 3.10A-C; it goes to a maximum then decreases with further copper additions. The coalesced sulphur peak from the mixture with 50 nM GSH and 50 nM Na₂S also goes to a maximum and then decreases (Figure 3.10D), but the peak does not disappear as it did when only Na₂S was in solution. These observations highlight that the CLE-ACSV method is deficient, as it does not account for possible interaction/competition amongst the multiple ligands in solution.
The proceeding discussion shows that these observations may be directly linked to the quantitative aspects of the mixed-ligand titrations; and may have implications for complexometric titrations of natural aquatic samples containing multiple thiol or sulphur species.

In all of the mixed-ligand titrations, data analysis was unable to resolve for two distinct ligand classes in solution. In titrations with CYS and GSH at 2 µM SA, as shown in Figures 3.10A-C, the Cu-GSH and Cu-CYS peaks were independently affected by Cu additions; yet, only one ligand class was detected. These results conclusively show that the interactions are not necessarily linear when two or more ligands are in solution. This is significant; however, it may be limited to Cu-complexation by organic and inorganic sulphur species. Mixed-ligand solutions of GSH and CYS did not appear to compete with SA for Cu when 10 µM SA was used (data not shown), though CYS did complex Cu at this higher detection window when it was in solution individually.

Table 3.2 CLE-AdCSV determination of Cu-ligand concentrations ($L_{Cu}$) and conditional stability constants ($\log K_{CuL}$) after mixed-ligand additions of glutathione and L-cysteine to UV-irradiated seawater. Titrations were at 2 µM SA, pH 8.3 and deposition at -015 V for 30s.

<table>
<thead>
<tr>
<th>Ligands Added</th>
<th>Measured (nM)</th>
<th>$\log K_{CuL}$</th>
<th>$\log \alpha_{CuL}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>50 nM GSH + 50 nM CYS</td>
<td>19.9 ± 2.55</td>
<td>11.84 ± 0.19</td>
<td>4.13</td>
</tr>
<tr>
<td></td>
<td>25.8 ± 3.82</td>
<td>11.95 ± 0.23</td>
<td>4.36</td>
</tr>
<tr>
<td></td>
<td>21.84 ± 3.29</td>
<td>11.90 ± 0.21</td>
<td>4.25</td>
</tr>
<tr>
<td>50 nM GSH + 100 nM CYS</td>
<td>22.8 ± 1.29</td>
<td>12.20 ± 0.10</td>
<td>4.56</td>
</tr>
<tr>
<td></td>
<td>27.5 ± 0.92</td>
<td>12.29 ± 0.06</td>
<td>4.72</td>
</tr>
<tr>
<td></td>
<td>30.5 ± 1.48</td>
<td>12.22 ± 0.08</td>
<td>4.70</td>
</tr>
<tr>
<td></td>
<td>26.9 ± 1.23</td>
<td>12.23 ± 0.08</td>
<td>4.66</td>
</tr>
<tr>
<td>50 nM CYS + 100 nM GSH</td>
<td>22.8 ± 1.41</td>
<td>12.02 ± 0.11</td>
<td>4.38</td>
</tr>
<tr>
<td></td>
<td>25.5 ± 1.18</td>
<td>11.95 ± 0.08</td>
<td>4.36</td>
</tr>
<tr>
<td></td>
<td>25.7 ± 1.62</td>
<td>11.96 ± 0.10</td>
<td>4.37</td>
</tr>
<tr>
<td></td>
<td>24.67 ± 1.4</td>
<td>11.97 ± 0.10</td>
<td>4.37</td>
</tr>
</tbody>
</table>

The results from the mixed-ligand titrations are shown in Table 3.2. The most significant observation is that the expected amount of ligand was not measured in any of the titrations. The expected amount of ligands to be measured is based on the
already established 1:2 complexation ratio of Cu with both CYS and GSH, and 1:1 with Na₂S. In the case of 50 nM GSH with 50 nM CYS, only an of average 21.84 ± 3.29 nM of the expected 50 nM ligand was measured. For 100 nM GSH with 50 nM CYS, an average of 24.67 ± 1.4 nM was measured instead of 75 nM; the latter was also expected for 50 nM GSH with 100 nM CYS, but only an average of 26.9 ± 1.23 nM was measured. These results clearly show that the method is unable to measure the correct ligand concentration in mixed-ligand samples.

Figure 3.10 Changes in the Cu-GSH (secondary-axis), Cu-CYS and Cu-S peak currents in mixed-ligand titrations at 2 µM SA. (A) 50 nM GSH + 50 nM CYS, (B) 100 nM GSH + 50 nM CYS, (C) 50 nM GSH + 100 nM CYS. (D) Coalesced peak from 50 nM GSH + 50 nM Na₂S. The black dotted line intersects the x-axis at the [Cu] that corresponds to the expected [L₄Cu], and the dashed line corresponds to the measured [L₄Cu].
Interestingly, in all cases the measured ligand concentration was approximately 25 nM which suggests that this value may be an artefact of the method. Explanations for these observations cannot be postulated from the Cu-SA peak current; therefore, analyses of the Cu-GSH, Cu-CYS and Cu-S currents were done to obtain insights into the complexation mechanisms.

Superimposed on each plot in Figure 3.10 are lines that intersect the x-axis at copper concentrations that correspond to the amount of ligand expected to be measured in each titration, and that which was actually calculated from the CLE-AdCSV data. In Figure 3.10A the Cu-GSH peak increases to a maximum at approximately 25 nM of added Cu, and this value corresponds to the amount of ligand measured using the Cu-SA peak analysis. At 50 nM (expected) the Cu-GSH peak then levels off, while the Cu-CYS peak changes slope. Figure 3.10B shows that at 25 nM (actual [L_{Cu}]) there is a change in slope of the Cu-GSH peak and another change in slope occurs at 75 nM (expected [L_{Cu}]) at which Cu-CYS also changes slope. As shown in 3.10C Cu-GSH goes to a maximum at approximately 25 nM Cu (actual [L_{Cu}]) and then levels off at 75 nM (expected [L_{Cu}]) before disappearing; the Cu-CYS peak also changes slope at 75 nM. Figure 3.10D shows the progression of the coalesced peak from 50 nM GSH and 50 nM Na_{2}S, in this particular titration the measured ligand concentration was 17 nM which coincides with the maximum peak current at -0.5 V. These interpretations go beyond the traditional interpretation of metal complexometric titration data (Apte, Gardner et al. 1988), and although not quantitative, they do provide insights into the disparity between the ligand concentration added and measured using CLE-AdCSV.

3.4 Discussion

A cardinal assumption in the CLE-AdCSV method is that the added ligand has no significant impact on the species in solution, except the trace metal of interest. Interactions with other common constituents of seawater are taken into account by the side reaction coefficient (\(\alpha_{CuSA}\)) determined in the calibration of the method (Lucia, Campos et al. 1994). If this assumption is valid, then the addition of salicylaldoxime
should only bind Cu, leaving the sulphur species relatively unaffected. Results from the present study cast doubt on this assumption, at least when using salicylaldoxime in the presence of sulphur species. It was shown that an increase in [SA] reduced the individual Cu-thiol peak currents, as exemplified in Figure 3.6B for CYS. This observation seems logical, as an increased amount of SA would reduce the Cu available to the CYS; hence, the individual Cu-CYS peak currents would be lower. This may explain the individual peak currents, but it does not account for the overall pattern of the Cu-CYS peak current throughout the entire titration when the [SA] was increased.

![Figure 3.11](Figure 3.11) Titration of a sulphur-rich hydrothermal sample from the south Atlantic with salicylaldoxime (SA), showing changes in the Cu-SA and sulphur peak currents. Measurement was at pH 8.3, and deposition at -0.15 V for 30 s.

As shown in Figure 3.6B, the Cu-CYS peak currents stabilised when that CYS was supposedly saturated with Cu, and Figure 3.4B shows that an increase in [CYS] caused the Cu-CYS peak currents to stabilise at higher values. The maximum height of the Cu-CYS peak during a titration is limited by the concentration CYS added to the solution. Therefore, an increase in [SA] to 10 µM should reduce the individual Cu-CYS peak currents, but the maximum peak current that was attained at 2 µM SA should still be attained, albeit at a higher concentration of added copper. This is because SA would bind more copper; hence, a higher amount of copper would have to be added to provide the free concentration required to saturate the CYS in solution. This expectation was not observed; hence, the proposition is that SA reduced the Cu-
complexing ability of the CYS in solution. An increase in [SA] would reduce the CYS available to bind Cu, and this explains why the Cu-CYS peak currents stabilised at lower values when [SA] was increased from 2 to 10 µM.

It has been suggested that an increase in the concentration of salicylaldoxime can saturate the Hg-electrode; thereby, reducing the surface area available to the electroactive sulphur species (Xue and Sigg 2002). This would explain the lower Cu-CYS peak currents when [SA] was increased. However, evidence from the present study suggests that this is not a valid hypothesis. The supposedly SA-saturation of the Hg-electrode was tested using a sulphur-rich hydrothermal sample, which was partitioned in 10ml aliquots and increasing [SA] was added up to 30 µM, followed by the normal voltammetric analysis. The results are presented in Figure 3.11 which shows that the Cu-SA peak current increased with [SA] and then stabilised. This was expected as once all the Cu was complexed by SA, any further increase in [SA] would not cause a further change in the Cu-SA peak current as this is limited by the copper in solution. The sulphur peak current (at -0.5V) was also monitored and it stabilised, then staying relatively constant with increasing [SA].

If increased concentrations of SA were saturating the Hg-electrode then the sulphur peak currents should have decreased with increasing [SA] as there would have been less surface area for the sulphur to react. This was not observed, suggesting that the observed reduction in the Cu-thiol peak currents with increased [SA] is not due to SA saturating the electrode. Further evidence is presented in Chapter 4 which shows that increases in [SA] caused corresponding increases in the sulphur peak for some hydrothermal samples. In summary, the observations discussed in this chapter provide evidence to show that in addition to the metal ion of interest, SA does have an effect on the spectator species in solution, at least for sulphur-rich seawater samples. This further reinforces the proposal that the lowering of the Cu-thiol peak currents was due to a chemical reaction between salicylaldoxime and the thiol species, but this potential reaction was not investigated further.

The Cu-GSH and Cu-CYS peak currents in the presence of 2 µM SA increased with the addition of Cu, and then gradually stabilised potentially when the thiols were
saturated, as shown in Figures 3.3A and 3.4B. This pattern was observed when CYS was added at 100 nM with 10 µM SA as shown in Figure 3.6B. These observations can be explained by a simple complexation mechanism between Cu and the thiol species. When 50 nM of GSH was added with 10 µM SA, there was a consistent decrease in the Cu-GSH peak height (Figure 3.7B), almost opposite to that observed at 2 µM SA. This pattern complicates the already established explanation in this report for the complexation between Cu and thiol species. Hence, it shows that the Cu-complexation dynamics in solution are not as simple as previously assumed. It also reinforces the proposition that SA does have an interfering chemical reaction with the sulphur species in solution.

There is an even more intricate complexation mechanism between Cu and the sulphur species generated after the dissolution of Na₂S. The Cu-S peak currents increased to a maximum with Cu additions and then decreased until it disappeared. It could be argued that the dissolution of Na₂S produced multiple sulphur species, one set of which is active in the ascent of the Cu-S peak current and another in the descent. This, however, does not explain the disappearance of the peak current for species that would have formed during the ascent. Another explanation is that the complex started disintegrating after saturation, which could explain the complete disappearance of the peak signal after the complex was fully disintegrated. An explanation for this potential disintegration of the formed complexes is not fully understood, but the results suggest that it is a likely possibility which warrants further investigation.

The observations reported here about Cu-S complexation draws into question previously published results (Al-Farawati and van den Berg 1999) that measured the conditional stability constants of metal-sulphide complexes in seawater. In their study, sulphide peak currents were measured and found to decrease with metal additions. In the example outlined in that paper, 100 nM of S²⁻ was titrated with Ag⁺ additions of 100, 250, 350 and 2000 nM. Unfortunately, Cu was not used in that part of their experiment, but it can be logically assumed that the behaviour of Ag-S would be similar to Cu-S as they have similar chemistries (Luther, Rickard et al. 1996). As such, it is possible that the initial increase in the Ag-S peak current was missed due to the
large additions of Ag⁺. However, since the metal cation and concentrations were different, this can only be assumed. Nonetheless, the results being reported in the present study for Cu-S gives a different perspective on the metal-sulphide complexation mechanism, which should inform future experiments that attempt to measure metal-sulphide stability constants in natural aquatic samples.

Glutathione additions of 50 and 100 nM, and CYS at 50 nM with 2 µM SA suggest a 1:2 complexation stoichiometry between Cu and the thiols. This seems to confirm some of the basic theory (Leal and van den Berg 1998), assuming that other effects in the solution are minimal. When CYS was increased to 100 nM with 2 µm SA the [L₄Cu] measured was the same as when only 50 nM of CYS was added. However, comparison of the Cu-CYS peak currents (at -0.43 V) showed that it doubled when CYS concentration was increased from 50 to 100 nM. This shows that CYS complexed twice the amount of copper, yet the increase was not detected from the conventional data analysis of the Cu-SA peak currents. This suggests that CYS competes with SA for Cu up to a threshold, after which the competition ends, but Cu-CYS complexation continues. This is unambiguous proof that the CLE-AdCSV method lacks quantitative reliability, and may misrepresent the extent of Cu-complexation in aquatic samples.

In the present study, glutathione did not show any signs of competing with SA for Cu when 10 µM SA was used, though it was reported to have been measured at this higher detection window (Leal and van den Berg 1998). However, the Cu-GSH peak currents in this study did decrease with Cu additions suggesting that GSH was interacting with Cu, though no apparent complexation was detected from the Cu-SA peak currents. The detection windows at 2 and 10 µM SA had log α₄CuSA of 3.85 and 5.04, respectively (Lucia, Campos et al. 1994). These values are a measure of the analytical competition strength and supposedly only complexes with log α₄CuL within ±1 would be detected. As shown in Table 3.1, the Cu-GSH complex had log α₄CuL = 4.13, which falls within both detection windows. However, the α₄CuL is just at the lower end of the detection window at 10 µM SA, which may explain why the Cu-GSH complex was not detected as competing with SA at the higher detection window in this study.
In the mixed-ligand titrations with GSH and Na$_2$S in this present study, the resultant Cu-GSH and Cu-S peaks coalesced into a single peak. This shows that previous studies (Le-Gall and van den Berg 1993; Le-Gall and van den Berg 1998; Laglera and van den Berg 2003) that used this peak to quantify thiols in natural samples should be treated with caution, since the peak may result from a mixture of sulphur species. Though this sulphur peak cannot be attributed to a single species, it could be used as a proxy for the total sulphur species content in a particular aquatic environment, and if monitored could provide information on the changes in the sulphur species content in relation to chemical and physical changes of the water body.

The data treatment method in this present study was unable to solve for two distinct ligand classes using the complexometric titration data from the mixed-ligand experiments. It is possible that if the log $K_{CuL}$ of the complex stabilities are too close, then the method may not resolve for two separate ligands. Instead, they would be detected as a single ligand class but with a concentration equal to the sum of the individual ligands. However, the method was also unable to successfully measure the expected amount of ligand. This suggests that previous ligand titrations on seawater (Coale and Bruland 1988; Lucia, Campos et al. 1994) may have misrepresented the concentration of Cu-binding ligands, especially that for organic and inorganic sulphur species. As a consequence, the reported values may not have accurately reflected the natural system; hence the associated conclusions about the aquatic speciation of Cu would have been flawed. Among these are conclusions on the bioavailability/toxicity of Cu, as determined by the Biotic Ligand Model (Di Toro, Allen et al. 2001), based on Cu$^{2+}$ concentrations derived from CLE-AdCSV measurements of aquatic samples.

### 3.5 Conclusions

The reliability of the CLE-AdCSV technique for quantifying Cu-complexation was assessed using UV-irradiated seawater to which model Cu-binding ligands glutathione, L-cysteine and Na$_2$S were added. Salicylaldoxime was used as the competing ligand. The voltammetric signal of the resulting Cu-GSH, Cu-CYS and Cu-S
complexes were monitored to provide information to augment the traditional complexometric titration data. Among the major finding of this study were the following:

- In general, it is the copper-sulphur complex that is reduced at the Hg-electrode and not the free sulphur species. However, some observations show that the mechanism involved is not a simple, single species electrode reaction.

- The assumption that the competing ligand has no impact on the spectator species in solution (except those already accounted for log $\alpha_{\text{CuSA}}$) is not valid when using salicylaldoxime for sulphur-rich seawater samples. This is due to an unexplained chemical reaction with salicylaldoxime that reduces the Cu-complexing ability of the sulphur species in solution.

- The data analysis method was unable to resolve for two ligands when multiple sulphur species were in solution, though the log $K_{\text{CuL'}}$ values were sufficiently different to allow such.

- The CLE-AdCSV technique was unable to detect the correct amount of ligand added in mixed-ligand titrations. This was always detected at approximately 25 nM, which suggest that this may be an artefact of the method.

- Analysis of the Cu-sulphur peak currents does provide insights into the complexation mechanism; as such, these should be routinely monitored to augment the conventional complexometric titration data.

The present study shows that there are profound limitations to the CLE-AdCSV method, and provides unambiguous proof that the method lacks quantitative reliability and may misrepresent the extent of Cu-complexation in aquatic samples. As such, CLE-AdCSV derived stability constants should be reconsidered, and placed in context with independent experimental approaches and other speciation analysis methods, for example diffusive gradients in thin films (DGT).
Chapter 4: Stabilisation of Dissolved Copper in Hydrothermal Vent Fluids from the Southern Mid-Atlantic Ridge

4.1 Introduction

The widespread occurrence of seafloor hydrothermal activity, mainly along mid-ocean ridges, makes it one of the main potential, but poorly quantified sources of bio-active trace metals to the global oceans (Sander and Koschinsky 2011; Saito, Noble et al. 2013). Hydrothermal vents are focused, point sources that introduce many chemical species into the deep-sea at concentrations many orders of magnitude higher than existing in the background seawater (e.g. H₂, CH₄, ³He, Mn, Fe, Cu), while others are scavenged from seawater by hydrothermal particles (e.g. PO₄³-, V, As, rare earth elements) (Lilley, Feely et al. 2013). The discharging vents are only a minuscule percentage of the seafloor; the actual value is uncertain as many parts of the ocean ridges remain unexplored (German and Von Damm 2006). However, the effects of these vents are enhanced by the rapid entrainment of background seawater, forming buoyant hydrothermal plumes that disperse horizontally away from the vent field with ocean currents once neutral buoyancy is attained.

The mixing of hot, anoxic vent fluids, laden with reduced metals and sulphur, with cold, oxic bottom waters results in rapid oxidation and precipitation of a non-equilibrium assemblage of metal-rich sulphide, sulphate and oxide particulate species (German, Baker et al. 1995). Several types of reactions occur within the plume, including the coprecipitation of some trace species, scavenging of trace elements on Fe and Mn oxide surfaces, and oxidative dissolution of some sulphide phases (Lilley, Feely et al. 2013). The larger particles quickly settle out of the rising plume, leaving only the very fine-grained oxides, sulphides and sulphates to form the bulk of hydrothermal particles in the neutrally buoyant plume. These eventually settle and form polymetallic mineral deposits up to hundreds of kilometres from the vent fields (Lilley, Feely et al. 2013). However, a small unknown fraction of trace-metal species escape these mass precipitation events, and are transported as nanoparticles (<200 nm) in the oceans (Yucel, Gartman et al. 2011).
The contribution of hydrothermal vents to the oceanic inventories of trace metals has been difficult to constrain because of incomplete knowledge of the (bio)geochemical transformations of vent fluid components in plumes (Lilley, Feely et al. 2013). Chief among these uncertainties are the influence of organic (thiol, amines, carboxylic acids and hydroxyl groups) and inorganic (S$^{2-}$, HS$^-$ and Cl$^-$) components on the solid-solution equilibrium of the metals in the hydrothermal fluid. Some of these species are known to complex dissolved trace metals in hydrothermal plumes, and this may delay or prevent metal precipitation and surface adsorption (Sander and Koschinsky 2011); thereby enhancing the flux of dissolved metals away from the vent field as shown in Figure 4.1. This has been reportedly detected by a few researchers as plumes of anomalously high concentrations of dissolved metals in the deep-ocean (Boyle, Bergquist et al. 2005; Wu, Wells et al. 2011; Nishioka, Obata et al. 2013; Saito, Noble et al. 2013).

**Figure 4.1** Schematic representation of the long-range dispersal of a hydrothermal plume. Bottom panel: In a system with purely inorganic metal speciation, sulphide minerals and oxide particles form principal Cu and Fe sinks; only negligible amounts of dissolved metal escapes. Top panel: Strong Cu- and Fe-binding organic ligands of hydrothermal and seawater origin allow for a significant flux of dissolved metals into the ocean (Sander and Koschinsky 2011).

Dissolved iron depth profiles in the tropical/subtropical Pacific showed evidence of deep-water anomalously high iron concentrations (Boyle, Bergquist et al. 2005; Wu, Wells et al. 2011). These depth anomalies are consistent with advective
iron sources via long-range transport of hydrothermal iron from the Loihi seamount (18.92°N 155.27°W) and the hydrothermally active, East Pacific Rise. Similar anomalies in dissolved iron concentration were detected in the Indian Ocean over 3,000 km in the deep layer centred at a depth of approximately 3,000 m (Nishioka, Obata et al. 2013). This anomaly in dissolved iron was also suggested to be due to hydrothermal sources, but from the Indian Ridge segment. A large plume of dissolved iron was also detected over a large geographic scale in the South Atlantic between 1,500 and 3,500 m depth, and this was also attributed to hydrothermal venting, though from the Mid-Atlantic Ridge (MAR) (Saito, Noble et al. 2013).

A study using a global ocean circulation model suggested that the distribution of dissolved iron in the oceans, especially the Southern Ocean, cannot be successfully modelled unless an iron flux from hydrothermal sources is included (Tagliabue, Bopp et al. 2010). The hydrothermal flux used in the model was partly obtained from previously published results (Bennett, Achterberg et al. 2008) in which CLE-AdCSV was used to measure Fe-complexing ligands from non-buoyant plumes overlying the MAR near 5°S. The authors did not state the actual hydrothermal signature of the samples, but assumed a $10^4$-fold dilution of the hydrothermal fluids with background seawater. It is known that inorganic sulphur species interfere with voltammetric measurements but there was no mention of that being controlled in the measurements reported by Bennett et al (2008). Nonetheless, the authors reported Fe-complexing ligands in the range of 2.5 - 4.2 nM (n=3), and these values were extrapolated to make estimates of the hydrothermal contribution to the iron budget of the global oceans.

In another study (Sander, Koschinsky et al. 2007), Cu-complexing ligands were measured in deep-sea (various depths and basins), hydrothermally-influenced samples at concentrations up to 4,000 nM, and conditional stability constants of $\log K_{CuL} = 12.48$ to 13.46. The voltammetric determinations were done after the samples were pre-treated to remove acid volatile sulphur species that would otherwise interfere with the measurements. The authors attributed the apparent copper stabilisation to organic ligands and suggested that thiols may be a part of the Cu-stabilising ligand pool. In a follow-up study (Sander and Koschinsky 2011), geochemical model simulations of hydrothermal fluid-seawater mixing were made using end-member
data from two hydrothermal fields, Turtle pits (5°S) and Rainbow (36°N) on the MAR. The model suggested that 14% of dissolved Cu in the deep oceans could be derived from hydrothermal vents, due to stabilisation by hydrothermally-derived ligands. However, in the absence of hydrothermally-derived ligands the contribution would amount to just 0.2%.

At present, the identities of Cu-stabilising species in hydrothermally-influenced seawater are not well known (Sander and Koschinsky 2011). Considered first is the case where these metal-complexing species are organic compounds, and are a part of the dissolved organic matter (DOM) pool. A first assessment of hydrothermal flux of DOM indicates that it is elevated only in low-temperature (<50°C) diffuse fluids (Lang, Butterfield et al. 2006), owing to the high biological productivity. As such, it is expected that the large quantities of biomass at hydrothermal vent sites could be an important source of organic ligands, such as amino acids, through microbial degradation. Microbes in marine surface waters are known to produce organic metal-binding ligands to reduce metal-micronutrient depletion and metal toxicity (Morel and Price 2003). Therefore, microbes residing at hydrothermal vents may have adopted similar survival strategies (Klevenz, Sander et al. 2012).

Another potential source of metal-complexing organic matter in hydrothermal environment is abiotic production at the high temperature, and low pH conditions. It was concluded from a theoretical (mathematical) work that there is a great possibility for the abiotic production of organosulphur compounds, especially thiols in hydrothermal systems, using CO₂ and CO as the precursors (Schulte and Rogers 2004). The results from Schulte and Rogers indicate that significant amounts of methanethiol could be generated from abiotic reactions, which then acts as a precursor for the formation of other organic compounds. Ultramafic-hosted hydrothermal systems – such as Lost City (30°N, 42°W) and Rainbow on the MAR – are particularly good candidates that provide favourable environments for abiotic formation of organic compounds with low molecular weight (Holm and Charlou 2001).

Inorganic sulphur ligands are also considered important Cu-stabilising species in these environments; among these are sulphide and bisulphide species. These form
relatively stable complexes with several metals, including Cu$^{2+}$ (Luther, Rickard et al. 1996); as such, they may have a major influence on the fate and bioavailability of vent-derived metals in the global oceans. However, the sulphide in some kinetically labile complexes may oxidise over the course of sampling, storage and equilibration during laboratory preparations; hence, they may not be detected during laboratory analysis.

In addition to metal-ligand complexes, nanoparticles may play a role in the speciation and transport of vent-derived metals. These particles behave like dissolved components with respect to aqueous transport and do not settle (Yucel, Gartman et al. 2011); therefore, metals bound to these particles may escape in the neutrally buoyant plume away from the vent site. In one study on hydrothermal plume particles ($\leq 10$ μm diameter, it was determined that there is a strong association between Fe(II) and organic carbon in hydrothermal plume particles, and it was suggested that Fe(II) was stabilised against oxidation and/or precipitation through sorption or complexation by the organic carbon in these particles (Toner, Fakra et al. 2009). Conclusions from another study (Yucel, Gartman et al. 2011) suggest that a substantial fraction of iron entering the ocean from hydrothermal vents is in a stable form as FeS$_2$ nanoparticles ($< 200$ nm), which increases the probability that vent-derived Fe will be long-range transported (laterally) in the deep ocean as shown in Figure 4.1. The same can be expected for hydrothermally-derived copper as it coprecipitates with Fe on the hydrothermal plume particles (Lilley, Feely et al. 2013).

It is now widely accepted that metal-stabilising species allow a small, but globally significant flux of hydrothermally-derived trace metals to enter the open ocean, whether as stabilised dissolved or nanoparticulate species (Kleint, Kuzmanovski et al.; Sander and Koschinsky 2011). These metal-stabilising species influence critical global elemental cycles (such as the carbon cycle), but at present, neither their chemical nature, nor the exact sources are confidently known (Sander and Koschinsky 2011). An accurate understanding of these processes will aid in strengthening oceanic biogeochemical models as broad assumptions will be reduced due to data on the distribution of hydrothermally-derived, metal-stabilising species. This current project addresses some of these fundamental gaps in our knowledge by measuring stable Cu-complexing species in hydrothermally-influenced seawater from different
(geographically and chemically) plume environments. Although Cu and Fe are the metals mostly studied (Kleint, Kuzmanovski et al. ; Sander, Koschinsky et al. 2007; Sander and Koschinsky 2011), it is anticipated that complexation mechanisms also play an important role for other metals including Zn, Co, Ni, Cd and others.

4.2 Study Area and Sampling

4.2.1 Cruise Outline

Selected vent sites along the volcanically and tectonically active southern Mid-Atlantic Ridge (SMAR) were sampled during the MAR-SÜD III cruise (M68/1) from 27 April – 2 June 2006 on R/V Meteor. This cruise was a part of the German SPP 1144 “From Mantle to Ocean: Energy, Material and Life Cycles at Spreading Axes” (http://www.geomar.de/en/research/fb4/fb4-muhs/projects/spp1144). The research objectives of the M68/1 cruise were to:

1. Locate and characterise the vents for the 4°48’S, 8°10’S, 8°18’S and 9°33’S hydrothermal plumes, where the hot vent sources were not yet found.

2. Quantify hydrothermal fluxes (heat, gases, and metals), and investigate the role of ocean mixing on hydrothermal plume dispersal (laterally) and distribution of vent-derived fluids and gases.

3. Assess the origin and lateral dispersal of vent fauna, and investigate the links between the variations in the different communities and the physico-geochemical environment.

4. Determine the factors controlling the composition and temporal variability of hydrothermal fluids and the microbial communities they support.

The work plan included detecting and mapping hydrothermal plumes by CTD and turbidity sensors, and localising the vents using the autonomous underwater vehicle (AUV) ‘ABE’ from the Woods Hole Oceanographic Institution. Bathymetric mapping was carried out with the multibeam system of R/V Meteor, and high-resolution mapping with the AUV. The remotely operated vehicle (ROV), Quest
(MARUM) was deployed in parallel and subsequent to the AUV discoveries in the hydrothermal fields to sample rocks, sulphides, fluids and the biota. Sample recoveries were complemented by onboard chemical analysis of the fluids, microbial experiments, and preparation of biological specimens.

### 4.2.2 Description of Hydrothermal Vent Sites

**Figure 4.2** (A) Location of the study sites. (B) Bathymetric map of the rift valley of the southern Mid-Atlantic Ridge at 4°48'S. (C) The ABE bathymetric map showing the location of the three high-temperature hydrothermal vent fields as well as the Wideawake diffuse vent field, south of which is the other low-temperature vent field, Liliput (not shown).

The northernmost site is the Red Lion hydrothermal field which is centred at 4°47.83’S/12°22.60’W in a water depth of 3,050 m and consists of at least four active black smoker chimneys separated by 10-15 m (Haase, Petersen et al. 2007). Three of the smokers (Tannenbaum, Sugarhead, and Mephisto) are typical 4-6m high pillar-like structures, whereas the fourth, Shrimp Farm, is a 4 m high structure with a 5 m wide plateau of extreme flange growth at the top (see Figure 4.3A). The fauna are dominated by large swarms of vent shrimp, and the chimney sulphides are
predominantly chalcopyrite, pyrite and sphalerite. The end-member chlorinity of the vent fluids at the source was 546 mmol/L which is close to seawater; and the [Si] was 21 mmol/L, comparable to typical fluids from basalt-hosted hydrothermal vents (Von Damm 1995).

Comfortless Cove hydrothermal field is 1 km southeast of Red Lion in a water depth of 2,996 m and comprises of one hot and two diffuse vent sites. The active, high-temperature vent, Sisters Peak, is a 13 m high chimney edifice, and consists of two spires of which the eastern one was inactive. The west spire (see Figure 4.3B) was venting acidic (pH 3.1) low-salinity fluids with a calculated end-member composition.
of 224 mmol/L Cl and 14.5 mmol/L Si. The base of the chimney was characterised by blocks of recrystallised massive sulphides that were colonised by mussels, crabs and shrimps. Two diffuse vent sites, Foggy Corner and Golden valley, are located 60 m to the north and 70 m to the northeast of Sisters Peak, respectively. Both diffused vent sites were densely colonised by mussels as shown in Figure 4.3C for Golden Valley.

Turtle Pits vent field is located 1 km southwest of Comfortless Cove in a water depth of 2990 m on the flank of a volcanic edifice. The vent field consists of three active black smokers (Southern Tower, One Boat and Two Boats). Southern Tower is a 13 m high chimney structure, whereas One Boat and Two Boats are characterised by mounds of sulphide debris with several small chimneys on top, as shown in Figure 4.3D for Two Boats. Exit temperatures at the vents up to 407°C were recorded (Koschinsky, Garbe-Schönberg et al. 2008); which along with observed vigorous boiling, suggests that the fluid was phase-separated close to the critical point of seawater; 407°C, 298 bar. Sulphide deposits are characterised by pyrrhotite, chalcopyrite and isocubanite mineral assemblage. The sample area was populated with shrimps and crabs.

Some 200 m to the east of Turtle Pits is the diffuse vent field, Wideawake, which showed signs of a recent volcanic eruption. The fauna are more diverse than at the high-temperature sites, consisting mainly of vent mussels, clams and limpets. H₂S-oxidising bacteria were observed under the microscope on a lava sample recovered close to the mussel assemblage in the Wideawake field (Haase, Petersen et al. 2007).

The Lilliput diffuse-venting hydrothermal field (not shown on Figure 4.2) was discovered in 2005 as the southernmost known vent field on the MAR (9°33'S/13°12'W) in a water depth of 1,500 m (Perner, Seifert et al. 2007; Haase, Koschinsky et al. 2009). It consists of several low-temperature diffused vents; but no high-temperature vents were found in this field, though there was evidence of the latter, based anomalously high concentrations of gases and metals about 300-400 m above the seafloor. The area is characterised by large mounds of orange-coloured, Fe-oxyhydroxide hydrothermal sediments (Figure 4.4A), and populated by crabs, shrimps and mussel patches with mostly juvenile mussels as shown in Figure 4.4B. Besides the
main Lilliput field, more diffuse, active emanation sites north of Lilliput were discovered and sampled.

![Figure 4.4](image)

**Figure 4.4** Photographs of the main Lilliput diffused-vent site (photographs courtesy of MARUM, University of Bremen). (A) Hydrothermal Fe-oxhydroxide deposits (B) Assemblage of mussels around a vent emanating hydrothermal fluids.

### 4.2.3 Hydrothermal Fluid Sampling

Direct sampling of hydrothermal vent fluids was carried out with the Kiel Pumping System (KIPS), which is a remotely controlled flow-through system (Garbe-Schönberg, Koschinsky et al. 2006). KIPS was mounted on the tool sled of a deep-water remotely operated vehicle (ROV), QUEST 4000 m (Figure 4.5A); the electrical and software control of both systems were fully integrated. The components of KIPS which came in contact with the sample are made entirely of chemically inert materials: perfluoroalkoxy (PFA) and polytetrafluoroethylene (PTFE), and a short sampling nozzle made of titanium (Figure 4.5B). These materials have low adsorption coefficients, preventing systematic errors that could be introduced by contamination or losses due to adsorption. Parallel to the titanium nozzle is a high-temperature sensor delivering real-time, temperature data for the tip of the nozzle. Hot fluid that enters the nozzle is distributed amongst sampling flasks by a motor-driven multiport valve (PTFE/PETP). In addition, Niskin bottles were mounted at the front of the ROV in order to collect fluid samples from discharging vent sites. The water column was
sampled with a rosette that measures conductivity, temperature and depth; this instrument was equipped with 24 bottles, 10 L each.

**Figure 4.5** Hydrothermal-fluid sampling system. (A) QUEST 4000 m ROV with KIPS mounted on the starboard side of the tool sled. (B) Titanium nozzle of the KIPS fluid sampling system (photos courtesy of MARUM, University of Bremen).

Immediately after recovery, all KIPS and Niskin bottles were transferred to a lab equipped with a clean bench (Class 100), where they were sub-sampled. Samples were taken from each flask for the analysis of dissolved Mg as a measure for hydrothermal fluid purity – that is to determine what percentage of the fluid is pure hydrothermal as opposed to seawater entrainment. First on-board analyses showed that hydrothermal fluids at the Turtle Pits and Sisters Peak vents were sampled with high purity: the chlorinity of 285 nM of fluids from the Turtle Pits, *Marker #2 Vent* was close to the value of 269 nM extrapolated from samples taken during the M64/1 cruise in 2005. For subsequent analyses of dissolved ions, the complete volume of one flask was transferred to a \( \text{N}_2 \)-flushed FEP bottle, homogenised, and then sub-sampled for the different analyses. Samples used in this study were filtered and immediately frozen at -20°C. A separate flask was dedicated for the analysis of dissolved gases and for the determination of isotopic composition (determined by other research groups on the cruise).
4.3 Results

4.3.1 Acidification Pre-Treatment Experiments

It was shown in Chapter 3 that the CLE-AdCSV method, in some instances, can successfully determine the concentration of selected copper-complexing sulphur species in seawater. The method however failed to measure the expected ligand concentration when at least two of these sulphur species were in solution; this warrants further investigation. Nonetheless, the method was used to see how it coped with hydrothermally-influenced seawater, as it is the best method currently available. The voltammetric method was further complicated as hydrothermal samples have an inherently high concentration of inorganic sulphur species, which limits voltammetric analysis as:

1. They quickly saturate the mercury electrode and reduce the surface area available to other species in solution.
2. Their voltammetric peak (at approximately -0.5 V) is extremely huge, and it out scales the Cu-SA peak, which is the analytical peak of main interest.
3. They interfere with the normal functioning of the reference electrode, which had to be cleaned after a few measurements.

It was therefore essential to remove a portion of these inorganic sulphur species, without altering the function of the Cu-complexing organic species in solution.

This was achieved using an acidification pre-treatment in which the sample was acidified to pH < 2, which removes most acid volatile sulphides from dissolved metal-(bi)sulphide species, as well as from metastable sulphide minerals (Rickard and Morse 2005). The low pH caused most metal-(bi)sulphide species to dissociate and convert to $H_2S$, which was purged from solution with $N_2$ gas for 30 minutes. The sample was then neutralised with $NH_3$, then passed through a 0.2 µm filter to remove any precipitates from solution. The Cu-ligand titration then proceeded normally as detailed in section 2.3.3.
A series of Cu-ligand titrations were done to assess the effectiveness of this pre-treatment, and whether organic, Cu-complexing species such as thiols were affected by the acidification. In these experiments glutathione, L-cysteine and sodium sulphide were added at 50 nM each to separate UV-irradiated seawater. This was followed by the acidification pre-treatment, and then the sample prepared as per the normal Cu-ligand titration with 2 μM SA. Table 4.2 shows selected results from these experiments. The acidification pre-treatment significantly removed the Cu-binding inorganic Na₂S from solution, though not completely. It is expected that all the HS⁻ and S²⁻ species would have been removed from solution, but it is possible that stable Cu-sulphide species may have been formed; such are reportedly inert, and do not fully dissociate at pH near 2 (Luther, Rickard et al. 1996). Also, the Na₂S reagent was not of the highest purity (technical grade); therefore, it is possible that other stable metal-sulphur species were in the mix.

Table 4.1 CLE-AdCSV determination of Cu-ligand concentrations ([L_{Cu}] ) and conditional stability constants (log K_{Cu'L'}) for the acidification pre-treatment experiments, after separate additions of glutathione, L-cysteine and Na₂S to UV-irradiated seawater. Titrations were at 2 μM SA, pH 8.3 and deposition at -0.15 V for 30s. log α_{CuL} (K_{Cu'L'} \cdot [L_{Cu}] ) is a measure of the analytical competition strength and should be within ±1 of the log α for the detection window, which is 3.85 ± 0.2 at 2 μM SA.

<table>
<thead>
<tr>
<th>Ligand</th>
<th>Added (nM)</th>
<th>Measured (nM)</th>
<th>log K_{Cu'L'}</th>
<th>log α_{CuL}</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glutathione</td>
<td>50</td>
<td>22.90 ± 2.67</td>
<td>11.70 ± 0.18</td>
<td>4.06</td>
</tr>
<tr>
<td></td>
<td></td>
<td>17.30 ± 3.06</td>
<td>11.78 ± 0.36</td>
<td>4.02</td>
</tr>
<tr>
<td></td>
<td></td>
<td><strong>20.10 ± 2.87</strong></td>
<td><strong>11.74 ± 0.27</strong></td>
<td><strong>4.04</strong></td>
</tr>
<tr>
<td>L-cysteine</td>
<td>50</td>
<td>14.90 ± 1.07</td>
<td>12.88 ± 0.19</td>
<td>5.05</td>
</tr>
<tr>
<td></td>
<td></td>
<td>12.40 ± 0.83</td>
<td>13.17 ± 0.23</td>
<td>5.26</td>
</tr>
<tr>
<td></td>
<td></td>
<td><strong>13.65 ± 0.95</strong></td>
<td><strong>13.03 ± 0.21</strong></td>
<td><strong>5.16</strong></td>
</tr>
<tr>
<td>Na₂S</td>
<td>50</td>
<td>12.1 ± 2.74</td>
<td>12.15 ± 0.43</td>
<td>4.23</td>
</tr>
<tr>
<td></td>
<td></td>
<td>8.29 ± 1.84</td>
<td>12.39 ± 0.47</td>
<td>4.31</td>
</tr>
<tr>
<td></td>
<td></td>
<td>9.28 ± 1.45</td>
<td>12.23 ± 0.31</td>
<td>4.2</td>
</tr>
<tr>
<td></td>
<td></td>
<td><strong>9.89 ± 2.01</strong></td>
<td><strong>12.26 ± 0.40</strong></td>
<td><strong>4.25</strong></td>
</tr>
</tbody>
</table>

Glutathione appeared to have been relatively unaffected by the acidification pre-treatment; with the addition of 50 nM, 20.10 ± 2.87 nM (n=2) was measured.
which is close to the expected 25 nM, as established in Chapter 2. For cysteine, only 13.65 ± 0.95 nM (n=2) was measured and this suggests that cysteine might have been affected by the acid pre-treatment. These measurements however need to be repeated in another study to confidently establish the response of these Cu-complexing thiols to the acidification pre-treatment. If these results are to be generalised, then it is expected that the acidification pre-treatment should not have an excessively deleterious effect on the organic Cu-complexing thiol species in the hydrothermally-derived seawater samples. However, unfortunately, some undesirable effects are to be expected from the acidification pre-treatment, but such is a compromise for working with these hydrothermal samples. Therefore, the Cu-complexing species measured in this study are probably an underestimation of the in-situ concentration.
Table 4.2 Sample information and results from Cu-ligand titrations using CLE-AdCSV after acidification pre-treatment. $[\text{Cu}_T]$ is the dissolved Cu concentration (0.2 µm filter). $[\text{L}_{\text{Cu}}]$ is the total concentration of natural Cu-complexing ligand within the sample and $\log K_{\text{Cu'L'}}$ is the stability constant of the complex; both were calculated using the Gerringa non-linear transformation technique with the software, MCCSOFT. Titrations were done with 2 µM SA, and deposited at -0.15 V.

<table>
<thead>
<tr>
<th>Hydrothermal field</th>
<th>Sample ID</th>
<th>% Hydrothermal endmember fluid</th>
<th>$T_{\text{in situ}}$ (°C)</th>
<th>pH</th>
<th>$[S^2-]$ (µM)</th>
<th>$[\text{Cu}_T]$ (nM)</th>
<th>$[\text{L}_{\text{Cu}}]$ (µM)</th>
<th>$\log K_{\text{Cu'L'}}$</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Wideawake</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
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<td>3 ROV 1/2</td>
<td></td>
<td>8.9</td>
<td>17.5</td>
<td>6.7</td>
<td>59.1</td>
<td>48.47</td>
<td>0.35 ± 0.073</td>
<td>11.62 ± 0.32</td>
</tr>
<tr>
<td>12 ROV 10 F</td>
<td></td>
<td></td>
<td></td>
<td>7.8</td>
<td>115</td>
<td>1.44</td>
<td>0.17 ± 0.046</td>
<td>11.37 ± 0.30</td>
</tr>
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<tr>
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<td>16.9</td>
<td>407</td>
<td>3.1</td>
<td>191</td>
<td>12.60</td>
<td>1.51 ± 0.31</td>
<td>11.43 ± 0.23</td>
</tr>
<tr>
<td>Marker#2 Vent</td>
<td>12 ROV B8 F</td>
<td>24.8</td>
<td>407</td>
<td>3.1</td>
<td>217</td>
<td>59.13</td>
<td>3.29 ± 0.46</td>
<td>11.50 ± 0.17</td>
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<tr>
<td>Tannenbaum</td>
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<tr>
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<td>36.4</td>
<td>350</td>
<td>3.1</td>
<td>957</td>
<td>582</td>
<td>4.56 ± 0.72</td>
<td>11.43 ± 0.20</td>
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<td>87.6</td>
<td>380</td>
<td>3.1</td>
<td>&gt; 370</td>
<td>1563</td>
<td>7.07 ± 1.30</td>
<td>11.51 ± 0.32</td>
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<td>9.6</td>
<td>7.0</td>
<td>12.2</td>
<td>10.70</td>
<td>1.20 ± 0.14</td>
<td>12.00 ± 0.28</td>
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</tr>
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<td>Diffuse vent 180m south</td>
<td>39 ROV 8 N</td>
<td>16.0</td>
<td>16.0</td>
<td>7.8</td>
<td>1.00</td>
<td>0.021 ± 0.003</td>
<td>11.86 ± 0.27</td>
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<td>7.8</td>
<td>4.8</td>
<td>7.2</td>
<td>15</td>
<td>3.43</td>
<td>0.033 ± 0.003</td>
<td>12.27 ± 0.23</td>
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<td>LiliPut</td>
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<td>11.8</td>
<td>6.6</td>
<td>6.5</td>
<td>29</td>
<td>8.13</td>
<td>0.090 ± 0.012</td>
<td>11.67 ± 0.21</td>
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<td>13.2</td>
<td>6.5</td>
<td>6.9</td>
<td>45</td>
<td>2.33</td>
<td>0.037 ± 0.005</td>
<td>12.12 ± 0.32</td>
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<td>10.0</td>
<td>10.0</td>
<td>7.6</td>
<td>6.0</td>
<td>7.52</td>
<td>0.014 ± 0.001</td>
<td>12.80 ± 0.38</td>
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</tbody>
</table>
4.3.2 Sample Analyses

It should be noted that the Cu-ligand titrations of the hydrothermal samples were not done under in situ conditions; instead, the measurements are a snapshot of each sample after filtration at normal conditions i.e. room temperature and an oxidising environment. This contrasts the deep-sea hydrothermal sample environment which is usually under high temperature and pressure in reducing conditions. This inevitably led to sample changes such as degassing, precipitation of metal (bi)sulphide and oxide species, and a possible pH change. The changes were prominently observed in some of the high-temperature samples upon handling in the laboratory.

Figure 4.6 Visual changes observed upon laboratory analysis of hydrothermally-influenced seawater. (A) Formation of green precipitates after dilution with ambient UV-irradiated seawater. (B) Orange precipitates recovered after acidification pre-treatment and subsequent neutralisation to pH 8.3.

Samples from high-temperature vents were usually acidic, and cloudy due to white suspended particles. When these samples were diluted with ambient UV-irradiated seawater the sample immediately changed to an intense green colour as shown in Figure 4.6A. Upon acidification, the sample reverted to the original colour and then back to green, though less intense, after N\textsubscript{2}-purge and neutralisation with NH\textsubscript{3}. Surprisingly, upon filtration there were orange precipitates on the filter (see Figure 4.6B); the final colour change was probably due to air exposure. These observations suggest that the changes were pH-dependent. Also, the intensity of the green colour was related to the concentration of S\textsuperscript{2-} in the samples. It is expected that this precipitation during the sample treatment would have caused changes in the
concentration of some dissolved, trace-metal species as they would either co-precipitate or adsorb on the surface of the particles (German and Von Damm 2006).

4.3.2.1 Total Dissolved Copper

Samples for total dissolved copper concentration, $[\text{Cu}_T]$, were diluted with seawater, UV-irradiated and then analysed according to the method described in Section 2.3.2. The analysis of $[\text{Cu}_T]$ was plagued with the same precipitation problem just outlined, as the samples were analysed at ambient seawater pH. It is therefore expected that the analytically determined $[\text{Cu}_T]$ would have been lower (undetermined) than in the original acidic samples. The $[\text{Cu}_T]$ measured were significantly higher than ambient seawater as shown in Table 4.2 where values varied from 1 to 1563 nM. These values are far greater than those reported for deep Atlantic waters, which has a maximum of around 2 nM (Biller and Bruland 2012) indicating a marked copper enrichment in the seawater due to hydrothermal inputs. From the results shown, on average, the low-temperature vents have a lower $[\text{Cu}_T]$ than the high temperature vents. This is expected as Cu is only enriched if fluid was hot enough (350°C) to dissolve the major Cu mineral, chalcopyrite (CuFeS$_2$) (Seyfried and Ding 1995).

4.3.2.2 Copper-Complexing Ligands

Preliminary tests indicated high Cu-binding ligand concentrations in most of the samples; as a result they were diluted with a UV-irradiated batch of seawater for which $[\text{Cu}_T]$ was predetermined. Unlike seawater, samples from hydrothermal vents can vary dramatically in composition from site to site, due to variations in the sub-seafloor chemical processes that control the chemistry of the vent fluid. Therefore, analytical parameters such as dilution factor and copper concentration range had to be optimised for each sample. It should be noted that Cu-ligand titrations were not possible without the acidification pre-treatment. Once this was established none of the samples were analysed without the pre-treatment. Therefore, the behaviour of
the samples during Cu-ligand titrations was not compared before and after the acidification pre-treatments.

Figure 4.7 Cu-ligand titration curves as determined by CLE-AdCSV. (A) Representative seawater sample. (B) Representative hydrothermal sample (7 ROV 13F) after dilution with UV-irradiated seawater and acidification pre-treatment. (C) The effect of dilution factor (DF) on the shape of the titration curve for 7 ROV 13F. (D) The effect of salicylaldoxime concentration, [SA], on the shape of the titration curve for 12 ROV 7F. Titrations were at pH 8.3, deposition at -0.15 V for 30 s and with 2 µM SA; 10 µM SA was used for one titration as shown in D.

The Cu-ligand complexometric titration of hydrothermal samples gave curves that were different from that of normal seawater (see Figure 4.7A). A representative example of titration curves obtained for the hydrothermal samples is shown in Figure 4.7B. These titration curves, with a ‘hump’ at the lower end and a linear portion at higher copper additions, are characteristic of all the samples, regardless of vent origin.
or sample chemistry. The odd shape is dependent on the proportion of hydrothermal signature in the sample; as shown in Figure 4.7C, which compares the titration curves of the same sample at different dilutions. With dilution, the odd shape was less pronounced, and approached that of normal seawater. Another observation is that the hump was more pronounced with increased SA concentration; this is shown in Figure 4.7D which compares the Cu-ligand titrations for the same sample at 2 and 10 µM SA. The linear portion of both curves eventually had the same sensitivity at higher copper additions.

Figure 4.8 Changes in the Cu-sulphur peak currents during CLE-AdCSV of selected hydrothermal samples. (A) 7 ROV 13F at multiple dilutions with 2 µM SA. (B) 7 ROV 13F with no SA, at different dilutions. (C) 7 ROV 13F (DF=100) at 0 and 2 µM SA. (D) 12 ROV 13F (DF=50) at 0, 2 and 10 µM SA. Samples were diluted with UV-irradiated seawater; measured at pH 8.3, and deposition at -0.15 V.
The voltammetric peak at around -0.5 V, characteristic of sulphur species, was still present in all samples after the acidification pre-treatment procedure. It shows that acidification did not remove all the inorganic sulphur species from solution; or the peak was due to organosulphur species, or a mixture of both. The changing height of this peak was monitored with increasing \([\text{Cu}_{\text{T}}]\) for each sample, and as previously observed for \(\text{Na}_2\text{S}\) (see Chapter 3), the peak increased to a maximum and then decreased until it disappeared. As expected, the maximum height of this sulphur related peak is dependent on the dilution of the sample as shown in Figure 4.8A, which compares the peak currents for 7 ROV 13F at different dilutions with 2 \(\mu\text{M}\) SA. Dilution also had the same effect on the sulphur peak current when there was no SA in solution as shown in Figure 4.8B for 7 ROV 13F at different dilutions.

Thus far, the sulphur-related peak in the hydrothermal samples behaves similarly to the sulphur peak when \(\text{Na}_2\text{S}\) was in solution, and so the behaviour in the absence of SA was also assessed. With no SA in solution the sulphur peak currents from \(\text{Na}_2\text{S}\) were much higher (see Figure 3.8C), and disappeared at a much higher concentration of added copper. For some of the hydrothermal samples in the study, this same behaviour was seen, and is exemplified in Figure 4.8C which compares the sulphur peak currents at 0 and 2 \(\mu\text{M}\) SA for 7 ROV 13F. Interestingly, the opposite was also seen for some samples analysed at multiple SA concentrations; that is, the sulphur peak currents were lower when there was no SA in solution, shown in Figure 4.8D for 12 ROV 13F.

Calculations of complexation parameters for the samples were difficult due to the odd shape in the Cu-SA complexometric titration curve. Most of the sample points in the ‘hump’ had to be discarded as they did not fit the Gerringa data analysis method. This is further explained in Chapter 5. The results from sample titrations at 10 \(\mu\text{M}\) SA are not presented as the ‘hump’ was generally larger (see Figure 4.7D), and complexation parameters could not be confidently calculated. Therefore, all the results reported were collected at 2 \(\mu\text{M}\) SA after dilution, up to a factor of 400 for some samples; otherwise, the analyses would not have been possible. As shown in Table 4.2, the ligand concentrations measured were in the \(\mu\text{M}\) range, and are thus
significantly higher than that measured in ambient seawater. Generally, the ligand concentrations in the high-temperature vents were significantly higher than diffused, low-temperature vents. When the values were plotted separately for low and high-temperature vents, there was no clear trend between $[\text{Cu}_T]$ and $[\text{L}_{\text{Cu}}]$ as shown in Figure 4.9.

![Figure 4.9 Correlation of total dissolved copper concentrations with ligand concentrations in the hydrothermal samples. (A) Low-temperature, diffused vents. (B) High-temperature, focused vents.](image)

It is generally assumed that the Cu-complexing species measured by CLE-AdCSV are organic in nature, and are a part of the pool of DOM. However, for the hydrothermal samples it is suspected that portions of the Cu-complexing species are inorganic sulphur species. This was tested for one sample, 7 ROV 13F. The sample was diluted by a factor of 100 and then UV-irradiated for 6 hours, followed by the acidification pre-treatment and then the conventional titration procedure. After UV-irradiation, the sulphur peak was still present in the sample, though smaller than before. The Cu-SA plot also had the odd shape, but the ‘hump’ was less pronounced than before. Calculations revealed a $[\text{L}_{\text{Cu}}]$ of $2.69 \pm 0.53 \mu\text{M}$, this compares to $5.36 \pm 1.23 \mu\text{M}$ before the UV-irradiation. The log $K_{\text{CuL}}$ before and after UV-irradiation were $11.15 \pm 0.34$ and $11.41 \pm 0.3$, respectively. The results of the UV-irradiation experiment confirmed that the Cu-complexing species in the sample are not entirely organic species; otherwise no Cu-complexation should have been measure after exposure to the UV light.
4.4 Discussion

The speciation of Cu in hydrothermal fluids sampled directly from the vents probably varies from that measured under our experimental conditions in which temperature, pH and pressure were modified; and reduced species were given time to oxidise. Also, the in situ fluids have a different ionic composition from the diluted samples, and this will influence the speciation of Cu different to that measured under the analytical conditions (German and Von Damm 2006). Overall, the experimental conditions were towards thermodynamic equilibrium of ambient seawater; therefore, the experimental results reported here more represent the eventual products of the reaction of hydrothermal fluids with seawater, rather than the speciation at the exact time and location of sample collection.

Copper is shown to be enriched in hydrothermal fluids, and the concentration is mainly controlled by the mineralogy of the bed rocks that the hydrothermal fluid interacts with. Temperature is also a major constraint as the amount of Cu leached into the fluid rapidly decreases at temperatures below 350°C due to the reduced solubility of chalcopyrite (CuFeS₂) (Von Damm 1995). Consequently, more copper is dissolved in the hot vent fluids that erupt from the seafloor without much subsurface mixing with seawater. In contrast, some diffused-vent fluids undergo extensive subsurface mixing with seawater, which leads to dilution of the end-member fluids; and dilution of hot, acidic fluids with seawater results in mineral precipitation of chalcopyrite due to cooling and increase in pH. Therefore, the dissolved copper concentration was much lower in diffused-fluids than in hot-vent fluids as shown in Table 4.1. This contrast is evident for the samples from Comfortless Cove. Both samples from the high-temperature, Sisters Peak vent had [Cu₁] values of 582 and 1563 nM, compared to 11 and 3 nM of the two low-temperature, diffused vents in the same area.

The direct relationship between [Cu₁] and in situ fluid temperature was generally observed throughout the entire sample suite. However, this relationship is less clear when the samples are separated into low and high-temperature vents as shown in Figure 4.10. This can be explained by the reported precipitation (see Figure
that occurred when the samples were brought to the ambient pH of seawater for analysis. It is expected that dissolved copper would co-precipitate with, and/or be scavenged from solution by the precipitated minerals, giving a logical explanation for the non-conservative behaviour of dissolved copper with vent fluid temperature. The observed precipitation is an artefact of the current method, and its extent was directly related to the \([S^{2-}]\) and the percentage of hydrothermal fluid in the sample.

Two distinct ligand classes are often observed in ambient seawater with the stronger binding class at a lower concentration than the weaker ligand (van den Berg 1982). Unfortunately, the oddly-shaped Cu-ligand titration plots for hydrothermal samples made it impossible to fit the data for more than one ligand class. It is however expected that there would be multiple ligand classes in hydrothermal vent fluids due to a complex mix of vent-derived organic, inorganic and colloidal species (Sander and Koschinsky 2011). Further details are provided in Chapter 5 to explain the difficulties in calculating the complexation parameters from the oddly-shaped titration plots. Also, beyond the oddly-shaped plots, it is possible that the CLE-AdCSV technique cannot resolve for multiple classes of Cu-complexing ligands in sulphur-rich waters, which is detailed extensively in Chapter 3.
The conditional stability constants of the Cu-complexes in the hydrothermal fluids did not vary extensively (see Table 4.2), regardless of vent origin and chemistry. The calculated log $K_{CuL'}$ values ranged from $11.13 \pm 0.23$ to $12.80 \pm 0.38$. Interestingly, the more stable complexes were associated with the low-temperature vents, which had higher log $K_{CuL'}$ values ranging from $11.37 \pm 0.30$ to $12.80 \pm 0.38$ with an average of $11.95 \pm 0.28$. The high-temperature vents had log $K_{CuL'}$ values of $11.13 \pm 0.23$ to $11.51 \pm 0.32$ with an average of $11.41 \pm 0.27$. This negative correlation between log $K_{CuL'}$ and in situ fluid temperature is shown in Figure 4.11A. Also, the concentrations of Cu-complexing ligands were higher in the high-temperature vents, and this positively correlates with the concentration of $S^{2-}$ in the samples. These trends suggest that the calculated $[L_{Cu}]$ and log $K_{CuL'}$ may provide insights into the vent biogeochemistries.

![Figure 4.11](image)

**Figure 4.11** Correlation between the hydrothermal sample parameters as determined during the laboratory analyses.

The positive correlation between $[S^{2-}]$ and $[L_{Cu}]$ (see Figure 4.11B) suggests that a significant part of the ligand pool may be inorganic sulphur species; this explains the elevated concentrations in the high-temperature vents. Among these may be reduced sulphur species (e.g. sulphide and elemental sulphur), which persist in oxygenated seawater due to the formation of relatively stable metal-sulphide complexes (Rozan, Benoit et al. 1999). In the case of copper, they tend to form a variety of bisulphide, sulphide, and polysulphide complexes which can be mixed-ligand types, as well as mononuclear and multinuclear complexes (Luther, Rickard et al. 1996). The sulphur
species in solution gave a peak at approximately -0.5 V in the voltammogram; but unfortunately, the present voltammetric technique does not provide enough information to qualitatively identify individual species. However, it is shown in Chapter 5 that elemental sulphur is a likely Cu-complexing species in the hydrothermal samples; and it also explains the oddly-shaped complexometric titration plots.

It is shown that the low-temperature, diffuse vents are associated with a pool of stronger Cu-complexing ligands, though at much lower concentrations. Following from the suggestion that a significant part of the ligand pool may be inorganic sulphur species, it is expected that \([L_{\text{Cu}}]\) would be lower in the diffuse, low-temperature vent fluids. This is due to lower \([S^{2-}]\) in diffuse-flow vent fluids because of subsurface dilution and precipitation of sulphur species. The areas around low-temperature vents provide favourable living conditions; as such, there is a higher amount of biological activities around these vents, which may contribute Cu-complexing organic molecules to the ligand pool (Sander and Koschinsky 2011). This contribution may be due to the release of non-specific organic compounds during biomass degradation, or specific synthesis by microbes to mediate metal stress around these low-temperature vent habitats (Klevenz, Sander et al. 2012). The higher concentrations of organic Cu-complexing species (from the vent biota) in the diffuse vent samples may explain the higher overall \(\log K_{\text{CuL}}\). Though speculative, this observation complements the results of chapter 3, in which the organic thiols had a higher \(\log K_{\text{CuL}}\) than Na\(_2\)S which behaves like the inorganic sulphur species present in high-temperature, vent fluids.

Nanoparticulate pyrite and other nanoparticles which are widespread components of hydrothermal black-smoker emissions (Gartman, Findlay et al. 2014) could behave like dissolved species and mimic Cu-complexing ligands during the complexometric titrations (Ružić 1982). Beyond analytical interference, metal-sulphide nanoparticles or clusters are recognised to be important in the stabilisation and transport of metals such as Fe away from the hydrothermal mixing zones (Yucel, Gartman et al. 2011); therefore, the same may be assumed for copper. These nanoparticles are in greater concentration in high-sulphide vent sites, and probably form a large part of the inert and possibly some of the labile dissolved copper.
fractions. It is also possible that metal-rich nanoparticles may be covered on the surface by organics, which adds a further layer of stabilisation to the metals within the sulphide clusters or nanoparticles (Hsu-Kim, Mullaugh et al. 2008). Therefore, metal-sulphide clusters, nanoparticles, and organic-metal complexes may act in concert, together stabilising the flux of hydrothermal copper to the ocean.

Overall, it is suspected that the Cu-complexing ligands that were measured in the present study are predominantly sulphur related species, with a large fraction being inorganic species. There is no evidence to determine whether the ligands are biological degradation products from the vent communities; or whether they were specifically synthesised by vent-microbes to condition their environment as suggested in the study by Klevenz et al. (2011), which showed that Cu-complexing ligands are produced by cultured, vent-microbes in response to a range of copper additions. Also, it was not determined whether the ligands are abiotic molecules formed under hydrothermal conditions as proposed by Schulte (Schulte and Rogers 2004). It is shown in chapter 5 that elemental sulphur could be a part of the ligand pool in hydrothermal environments. However, regardless of what the ligands are, they are very stable species as they endured the acidification pre-treatment process, and still retained the ability to form relatively strong complexes with copper.

The Cu-stabilising ligands will be diluted in the deep ocean once they move away from the vent field, and the extent to which Cu is stabilised is expected to be the same as that modeled by Sander and Koschinky (2011). In the reported work, the authors simulated the processes in the hydrothermal fluid-seawater mixing zone by geochemical modeling of Cu and Cu-binding ligands from hot black-smoker vents. The model used Cu-binding ligand concentrations up to 10 µM, which is similar to that reported here for Southern Tower vent in the Turtle Pits hydrothermal field. Therefore, it is expected that up to 4.2% of the hydrothermal end-member Cu reported in this study can be stabilised, and contribute to the oceanic copper flux. The same may be true for other hydrothermally-sourced metals (such as Zn, Co, Ni and Cd) that form stable complexes in seawater.
4.5 Conclusions

It is now widely accepted that metal-stabilising species allow a small, but globally significant flux of hydrothermally-derived trace metals to enter the deep ocean, whether as stabilised dissolved or nanoparticulate species. This project examined this process by measuring stable Cu-complexing species in hydrothermally-influenced seawater from vent sites along the southern Mid-Atlantic Ridge. The CLE-AdCSV analytical technique did not produce Cu-ligand titrations plots as that of normal seawater, but complexation parameters were determined within these analytical limitations. Overall, the experimental conditions were towards thermodynamic equilibrium typical of ambient seawater; as such, the results are more representative of the eventual products of the reaction of hydrothermal fluids with seawater, rather than the speciation at the exact time and location of sample collection.

Total dissolved copper concentrations in the hydrothermal fluids was enriched to that in ambient seawater, and ranged from 1 to 1563 nM. The concentrations of the Cu-complexing species were measured at concentrations of 0.014 ±0.001 to 10.6 ±2.16 µM with conditional stability constants from 11.13 ±0.23 to 12.80 ±0.38. The more stable Cu-complexes, with higher log $K_{CuL}$ values, were associated with the low temperature vents and this is speculated to be due to a higher degree of biological activities around these vents. The high-temperature vents had lower log $K_{CuL}$ values and this is speculated in this study to be due to the higher concentration of inorganic sulphur species, which is suggested to be the significant part of the Cu-complexing ligand pool in both high and low temperature vents.

These Cu-complexing species are at concentrations that suggest they may influence the copper biogeochemical cycle in the ocean. However, there is no evidence to confidently determine the chemical nature and exact sources of these species.
5.1 Introduction

Sulphur is thermodynamically stable in the form of sulphate (SO$_4^{2-}$) in ambient, oxygenated oceans (Canfield 2004); as such, reduced sulphur species were not regarded as significant in these systems due to their instability in the presence of molecular oxygen (Millero 1991). Nevertheless, the occurrence of metastable reduced sulphur species have been widely reported in a variety of fully oxygenated marine and freshwater systems (Cutter and Krahforst 1988; Bura-Nakić, Helz et al. 2009; Superville, Pižeta et al. 2013). In the context of this report, reduced sulphur species (RSS) comprise a group of compounds that contain sulphur in nominally -2 and 0 oxidation states. Some of the most important RSS in natural waters include sulphides (HS$^-$/S$^{2-}$), organic thiols (e.g. cysteine, glutathione, and phytochelatins), inorganic and organic polysulphides, and dissolved elemental sulphur.

The most widely measured RSS in the ocean are sulphides, the concentration of which decreases with depth, and from coastal to the open ocean. As such, it is suggested that there is biological mediation of the reduction of SO$_4^{2-}$ to sulphides and other RSS (Radford-Knery and Cutter 1994; Rogers and Schulte 2012), possibly by marine sulphate-reducing bacteria or phytoplankton (Walsh, Cutter et al. 1995). Sulphate is also reduced in the marine environment through thermochemical catalysis at high temperatures, such as at hydrothermal vents (Seyfried and Ding 1995). At such high temperatures sulphate reacts as an oxidant for Fe$^{2+}$ and other reduced minerals, thereby being reduced to H$_2$S without the participation of microorganisms. There is also an abundance of reduced organosulphur compounds in the oceans including thiols (Dupont, Moffett et al. 2006); however, it is difficult to make an exhaustive list since sulphur is present in many organic molecules, from amino acids to humic substances.

The occurrence of sulphides and their organic analogues, thiols, in fully oxygenated waters is attributed to kinetic stabilisation resulting from complexation
with trace metals such as iron, copper and zinc (Rozan, Benoit et al. 1999). In the case of copper, it tends to form a variety of stable bisulphide, sulphide, and polysulphide complexes as well as mononuclear and multinuclear complexes (Luther, Rickard et al. 1996). There are strong associations between dissolved Cu and RSS in the ocean, which imply that the biogeochemistries of these two bio-active elements are likely to be linked. However, little is known about the linkages, as such, numerous investigations have been done to further explicate the association between reduced sulphur compounds and metals in natural waters (Zhang and Millero 1994; Al-Farawati and van den Berg 1999; Wang and Tessier 2009; Superville, Pižeta et al. 2013).

Voltammetric techniques have been used to characterise sulphur species in seawater and have provided information on concentration, identity, origin, distribution and behaviour of RSS in seawater. In principle, their determination is based on a two-electron reversible electrochemical oxidation of metallic mercury from the drop (Canterford and Buchanan 1973). The mercury is oxidised from Hg(0) to Hg(II) in the potential range of -0.5V to +0.1V with the immediate formation of insoluble Hg(II) sulphide (HgS), which forms a monolayer on the electrode surface. The amount of HgS formed on the electrode surface is proportional to the deposition time and concentration of reactive sulphur species in solution. Summaries of the reactions for sulphide (Eq. 1) and elemental sulphur (Eq.2) are as follows:

\[ HS^- + Hg^0 \rightleftharpoons HgS(s) + H^+ + 2\bar{e} \]  
\[ S^0 + Hg^0 \rightleftharpoons HgS(s) \]  
\[ H^+ + HgS(s) + 2\bar{e} \rightarrow Hg^0 + HS^- \]  

After being accumulated, the oxidised mercury (HgS) is then stripped cathodically to elemental mercury (Hg^0) and sulphide (Eq. 3), leading to a voltammetric peak in the region of -0.5 to -0.6 vs. Ag|AgCl. This procedure is akin to indirect CSV, as the analyte itself (here the sulphur ion) does not undergo an electron transfer reaction.

The potential (-0.5 to -0.6 V) of the sulphur peak in CSV scans of natural waters depends on the measurement procedure and the sample composition. According to
the literature, this voltammetric peak is usually attributed to a single or a mixture of several RSS (Ciglenečki and Ćosović 1996; Rozan, Benoit et al. 1999; Laglera and Tovar-Sánchez 2012), including free sulphide, metal complexes of sulphide and several thiol compounds. Therefore, the determination of individual RSS by CSV is severely limited by coalescence - the merger in a single peak of the signals due to multiple compounds. Although the peak is most probably due to a mixture of RSS, it can still be used as a marker (or indicator) of sulphur species content in a particular aquatic environment, evidencing their temporal dynamics in relation to chemical and physical changes of the water body (Superville, Pižeta et al. 2013).

Nonetheless, voltammetric techniques were reportedly used to characterise individual RSS in natural waters; however, none was shown to be selective enough to unambiguously identify individual species in such a complex matrix (Rozan, Benoit et al. 1999; Laglera and Tovar-Sánchez 2012; Superville, Pižeta et al. 2013). A sample pre-treatment was used for some studies, in which acid was added to convert dissolved free sulphides to $\text{H}_2\text{S}$ which was then purged with $\text{N}_2$ gas. A stable sulphur-related, voltammetric peak subsequent to this pre-treatment has been attributed to individual metal sulphides in river waters (Rozan, Benoit et al. 1999); elemental sulphur in pore waters (Wang, Tessier et al. 1998); and thiols in hydrothermally-influenced seawater (Sander, Koschinsky et al. 2007). In reality, this peak could be due to several sulphur compounds, and as shown in Chapter 3, glutathione, cysteine and $\text{Na}_2\text{S}$ are examples that endured the acidification pre-treatment. It should be emphasised that direct measurement, without addition of reagents, is the only method that leaves the equilibrium state of the solution undisturbed. In this way a representation of those species which exist under natural conditions can be characterised (e.g. colloidal, particulate and soluble sulphur species).

As discussed in Chapter 4, Cu-stabilising ligands were measured in hydrothermally-influenced seawater using complexometric titrations with the CLE-AdCSV technique. The results suggest that the ligands may predominantly be sulphur species, which are suspected to have caused the resultant Cu-ligand titration plots of the hydrothermal samples to be different from that of normal seawater. Among the
copious observations from the Cu-ligand titrations of the hydrothermally-influenced seawater samples were:

1. The Cu-SA titration plots had a distorting ‘hump’ at lower copper additions but behaved as expected at higher additions.

2. An increase in the concentration of salicylaldoxime caused the distortion in the Cu-SA (salicylaldoxime) titration plots to be more pronounced.

3. The sulphur peak at -0.5 V increased with copper additions to a maximum then decreased until it disappeared, before completion of the titration.

4. When there was no salicylaldoxime in solution the sulphur peak disappeared only after much higher Cu additions.

5. With dilution, the sulphur peak currents were lowered, while the Cu-SA peak currents increased.

6. An increase in salicylaldoxime concentration caused a corresponding increase in the sulphur peak currents for some samples, but lowered it for others.

7. A sulphur peak was still in the voltammogram after UV-digestion, albeit with a much lower current, and the resultant Cu-SA titration plot was less distorted.

These observations have not been previously reported for voltammetric analysis of sulphur-rich natural waters; as such, reported in this chapter is an attempt to understand and replicate these unique behaviours. This was achieved by performing Cu-ligand titrations with CLE-AdCSV on elemental sulphur enriched seawater samples.

5.2 Experiments and Results

Cu-ligand titrations with the RSS glutathione, cysteine and Na₂S (see Chapter 3) did not give the same effects as that observed with the hydrothermal samples. To this effect, elemental sulphur was tested as it is an existing sulphur species in hydrothermal fluids (Raiswell 1992). Elemental sulphur (S⁰) is only sparingly soluble in
water but it can be greatly increased in sulphidic waters by reacting with dissolved sulphide to form polysulphide species (Giggenbach 1972). $S^0$-enriched solutions were freshly prepared by adding sulphur powder to UV-irradiated seawater which was agitated continuously at room temperature for 30 minutes. The fine, visible suspension was separated from the solution by passing through a 0.2 µM filter. The batch of $S^0$-enriched seawater was then separated in two - one to test a particular experimental condition/treatment, and the other was a control that did not undergo any chemical treatment. Results for each experimental condition/treatment were compared to the control, but not across different experiments as the extent of $S^0$-enrichment could have been different for each batch.

The experiments were conducted in a manner such that elemental sulphur made similar contributions to the voltammetric peak current as that recorded in a hydrothermal sample. It must be emphasised that all comparisons to the hydrothermal samples are purely qualitative as the CLE-AdCSV method, as already shown in Chapter 3, is inherently limited in its ability to quantitatively assess Cu-stabilising species in sulphur-rich waters. The overall behaviour of $S^0$-enriched seawater was similar to that reported for the hydrothermal samples under the experimental conditions. That is, Cu-ligand titrations with CLE-AdCSV gave the same oddly-shaped titration plots (Figure 5.1A); and the prominence of the odd shape was dependent on the sulphur enrichment.

The effect of acid pre-treatment and UV-irradiation on dissolved elemental sulphur was tested and the results are presented in Figure 5.1. The acid pre-treatment reduced the Cu-complexing species in solution as evident in Figure 5.1A, which shows the Cu-SA titration plots for a batch of sample – before and after the acid pre-treatment. As expected, the sulphur peak currents, at the same copper additions, were lower for the acid pre-treated sample (Figure 5.1B) due to the loss of some sulphur species upon acidification and N$_2$-purging. Figure 5.1D shows that UV-irradiation removed a significant amount of sulphur species from solution; consequently, the corresponding Cu-SA titration plot (Figure 5.1C) was significantly less distorted than before UV-irradiation. Overall, the UV-irradiation, rather than the
acid pre-treatment, had a much greater effect on reducing the Cu-complexing abilities of $S^0$-enriched solutions. The UV-degradation of $S^0$ was previously reported in laboratory experiments (Superville, Pižeta et al. 2013), and confirms that this sample pre-treatment cannot be used in natural samples to unambiguously partition organic and mineral sulphur species.

![Figure 5.1 Results for Cu-ligand titrations on seawater enriched with elemental sulphur. (A) Cu-SA titration plots and (B) Changes in the sulphur peak currents for a sample with and without acid pre-treatment. (C) Cu-SA titration plots and (D) Changes in the sulphur peak currents for a sample before and after UV-irradiation. The UV-irradiation was for 6 hours, and the sample was not acid pre-treated. All measurements were at pH 8.3, with 2 µM SA, and deposited at -0.15 V for 30s.](image)

Also tested was the effect of salicylaldoxime concentration on the Cu-complexing abilities of $S^0$-enriched seawater; these samples were acid pre-treated to
keep the test conditions similar to the hydrothermal samples. As shown in Figure 5.2A, an increase in salicylaldoxime from 2 to 10 µM, caused the odd ‘hump’ in the Cu-SA titration plot to be more pronounced. Also, as observed with the hydrothermal samples, both plots had relatively similar peak currents at higher copper additions. The increased concentration of salicylaldoxime lowered the sulphur peak currents at the same copper additions (see Figure 5.2B). When salicylaldoxime was not added to the titration, the sulphur peak currents were significantly higher; and as observed with the hydrothermal samples, disappeared only after much higher copper additions.

**Figure 5.2** Results for Cu-ligand titrations on UV-irradiated seawater enriched with elemental sulphur (A) Cu-SA titration plots at 2 and 10 µM SA. (B) Changes in the sulphur peak current at 0, 2 and 10 µM SA. All measurements were at pH 8.3, and deposited at -0.15 V for 30s.

Further analysis of the titration data for the hydrothermal samples shows that the odd shape in the Cu-SA plot is qualitatively related to changes in the sulphur species. It was observed that the Cu-SA plot became linear just after the sulphur peak disappeared as shown in Figure 5.3A for a representative hydrothermal sample (7 ROV 13F); additional plots are presented in Appendix I. Similar observations were made with the titration data from S^0^-enriched seawater samples as exemplified in Figure 5.3B.
Figure 5.3 Representative examples of Cu-ligand titrations (A) Hydrothermal sample (7 ROV 13F) (B) UV-irradiated seawater, enriched with elemental sulphur. The dotted line shows where the sulphur peak disappears, after which the Cu-SA plot is linear. All measurements were at pH 8.3, with 2 µM SA and deposited at -0.15 V for 30s.

As mentioned in Chapter 4, the calculation of Cu-ligand complexation parameters for the hydrothermally-influenced seawater samples was difficult as the data did not fit the data model underlying the evaluation method. This is due to the unusual distribution of data points in the lower end of the Cu-SA titration plots; its effect on the data analysis is examined here using the van den Berg/Ružič (VDB) linearisation technique. The theory (see section 2.1.4.2) shows that the VDB linearisation technique should give a straight line, from which the complexation parameters are calculated. The VDB plot using data from a Cu-ligand titration for a normal seawater sample is shown in Figure 5.4A; also in the figure is the VDB plot of the data obtained for a typical hydrothermal sample. The VDB method does not fully linearise the data for the hydrothermal sample; instead, only the last few data points are linear. These linear points are highlighted in Figure 5.4B, and significantly, they correspond exactly to those in the linear section of the Cu-SA titration plot for the same sample. These observations are characteristic of all the hydrothermally-influenced seawater samples and further plots are presented in Appendix I.
Figure 5.4 Representative examples of van den Berg/Ružić (VDB) linearisation of Cu-ligand titration data. (A) VDB linearisation for normal seawater and that for a hydrothermal sample; the solid line shows the linear sections. (B) VDB linearisation of the same hydrothermal sample data, presented with the corresponding Cu-SA titration plot; highlighted are the points which fit the VDB linearisation model.

5.3 Discussion

The premise of this chapter was to determine the cause of the oddly-shaped Cu-ligand titration plots for hydrothermal samples, since none of the model thiols (i.e., GSH and CYS) and $S^{2-}$ tested in Chapter 3 caused an 'odd-shape' as observed in the hydrothermal samples. However, elemental sulphur ($S^0$) enriched seawater behaves the same as hydrothermal-influenced seawater during Cu-ligand complexometric titrations with CLE-AdCSV analysis. As expected, the sulphur species in both samples gave voltammetric peaks at identical potentials (-0.5 V), since both are assumed to form HgS at the surface of the mercury electrode. Beyond the peak potential; the results overall show that $S^0$ behaves like the sulphur species that were active in the hydrothermal samples. It was seen that the $S^0$-enriched samples gave the same ‘hump’ in the complexometric titration plot; as expected, the prominence of the ‘hump’ was directly related to $S^0$-enrichment. Also, the voltammetric sulphur peak at -0.5V, like the hydrothermal samples, went to a maximum then decreased until it disappeared with further copper additions. It can therefore be concluded that the presence of $S^0$ is the likely reason for the oddly-shaped titration plots observed for hydrothermally-influenced samples.
The acidification pre-treatment degraded some of the Cu-complexing species in the S\(^0\)-enriched solution; however, the reduction was not excessive as this pre-treatment is designed to remove volatile sulphur species such as HS\(^-\). Elemental sulphur is relatively inert under these conditions, and would have resisted the acidification process. This suggests that elemental sulphur could potentially be a component of the Cu-complexing ligand pool that was measured in the hydrothermal samples after the acid pre-treatment. It should be noted that if elemental sulphur was the active species measured in the hydrothermal samples, then it is possible that polysulphides could have also added to the S\(^0\) species measured. This is possible as polysulphides, which are present in hydrothermal fluids (Gartman, Yücel et al. 2011), will decompose at pH<5.5 into additional S\(^0\) and H\(_2\)S (Chadwell, Rickard et al. 2001); the latter is purged from solution. Therefore, the final S\(^0\) signal may be a combination of any original S\(^0\) and that produced for the degradation of polysulphides.

The UV-irradiation treatment was shown to be effective at removing a great portion of Cu-complexing species in the S\(^0\)-enriched solution. This is interesting as it has been suggested repeatedly that UV-irradiation is a pre-treatment specific to the oxidation of organic species in natural waters (Golimowski and Golimowska 1996; Bertilsson and Tranvik 2000; Sander, Kim et al. 2005). The method is effective in removing organic species from solution, but it is non-specific and is shown here to also degrade the Cu-complexing abilities of elemental sulphur. Therefore, results obtained after this pre-treatment should be discussed cautiously, and not be limited to only the removal of organic species, as is common.

The ‘hump’ in the complexometric titration plots of S\(^0\)-enriched solutions was more pronounced with an increase in salicylaldoxime concentration; this was the same for the hydrothermal samples. An increase in salicylaldoxime concentration also reduced the sulphur peak currents. As with the hydrothermal samples, the sulphur peak in the S\(^0\)-enriched solutions only disappear after much higher additions of copper when salicylaldoxime was absent from the solution.

It is proposed here that the voltammetric peak at -0.23 V, attributed to Cu-SA, was due to mixed species incorporating sulphur. At the lower Cu additions it is
suggested that mixed Cu-SA-S species were responsible for the voltammetric peak instead of a pure Cu-SA species. At higher copper additions, when the sulphur peak has decreased, the Cu-SA-S peak shifted to another Cu-SA species with a different sensitivity. A mixed Cu-SA-S complex seems very likely when the coordination chemistry of all the species are considered, and the resultant complex would be expected to have similar reduction potential as the pure Cu-SA complex. The sulphur in the mixed Cu-SA-S species would be expected to react with the Hg-electrode in a similar manner and such would interfere with the peak current at -0.23 V; thereby, explaining the odd shape in the complexometric titration plots at lower copper additions.

Another explanation for the oddly-shaped titration plots is interference from metal sulphides that form directly on the Hg-electrode when the solution composition and the electrochemical conditions (i.e. deposition potential and accumulation time) are suitable. These deposits and their corresponding reduction peaks do not necessarily reflect the bulk situation and that may result in errors in the electroanalysis of natural samples.

### 5.4 Conclusions

The focus of this chapter was to determine the cause of the oddly-shaped Cu-ligand titration plots for the hydrothermally-influenced seawater samples measure in Chapter 4. It was conclusively shown that elemental sulphur enriched seawater successfully replicated the voltammetric behaviours of the hydrothermal samples. Therefore, it was suggest that elemental sulphur in hydrothermal fluids could explain the analytical limitations of the CLE-AdCSV technique in analysing these samples. Also, these observations have not been previously reported for voltammetric analysis of sulphur-rich natural waters.
Chapter 6: Conclusions and Future Research

6.1 Summary and Conclusions

Globally significant amounts of hydrothermally-derived, bio-important trace metals are stabilised against precipitation and are transported over thousands of kilometres into the deep-ocean. This is facilitated by the formation of strong copper-complexes with hydrothermally-derived ligands. However, the extent of this stabilisation process and the chemical nature of the ligands are poorly understood. This project aimed to provide data to supplement the paucity of information about this important phenomenon. As such, hydrothermally-influenced seawater samples from the geologically active, southern Mid-Atlantic Ridge were analysed for Cu-complexing ligands using the CLE-AdCSV technique as detailed in the preceding chapters.

Chapter 1: Detailed in this chapter were the biogeochemistries of copper and other trace metals in the ocean. This included the major sources, distribution profiles, and biological interactions; along with the speciation and redox controls. Also examined was the hydrothermal flux of trace metals to the deep-ocean. Included was the geological setting of hydrothermal vents, seawater/fluid circulation and the dynamics of hydrothermal plumes.

Chapter 2: This chapter examined the analysis of Cu-speciation by voltammetry, specifically the CLE-AdCSV technique. Information on CLE-AdCSV included the underlying theory, accuracy and validity, and methods to analyse the resulting data. Also detailed were instrumentation, quality control, and methods to determine total dissolved copper and Cu-complexing ligands.

Chapter 3: This chapter assessed the reliability of CLE-AdCSV to quantify Cu-complexation in UV-irradiated seawater to which Cu-binding ligands glutathione, L-cysteine and Na₂S were added. It was determined that there are profound limitations to the CLE-AdCSV technique, which may lead to severe underestimates of Cu-complexation in aquatic samples. As
such, it was recommended that CLE-AdCSV derived stability constants should be reconsidered, and validated against independent experimental approaches. Among the specific findings of this chapter was that:

- Salicylaldoxime had an unexpected, deleterious impact on the Cu-complexing abilities of the sulphur ligands. This invalidates the CLE-AdCSV assumption that the added ligand has negligible effects on the species in solution; except the trace metal of interest (Cu).

- Analysis of Cu-ligand titration data did not resolve for more than one ligand class in multi-thiol solutions, though the differences in the log $K_{\text{CuL}}$ values were sufficiently large to allow such.

- The CLE-AdCSV technique was unable to detect the added concentration of ligands in mixed-ligand solutions. This was always detected as ~25 nM, suggesting a possible artefact of the method.

- The Cu-sulphur peak currents provide insights into the complexation mechanisms, and so it is recommended that they are monitored to supplement the traditional complexometric titration data of Cu.

**Chapter 4:** This chapter reported the CLE-AdCSV determination of Cu-complexing ligands in hydrothermally-influenced seawater from sites along the southern Mid-Atlantic Ridge. Included are descriptions of the sea voyage, vent sites and fluid sampling details. The resulting Cu-ligand titration plots were characteristically, oddly-shaped; nonetheless, complexation parameters were extracted from the data within these limits. The results reported are more representative of the eventual products from the reaction of hydrothermal fluids with seawater, rather than an in situ representation. Among the significant results was that:

- Total dissolved copper in the hydrothermally-influenced seawater samples was enriched relative to ambient seawater, and the values ranged were from 1 to 1563 nM.
• The concentration of Cu-complexing ligands were measured at 0.014 ±0.001 to 10.6 ±2.16 µM, and with log $K_{CuL'}$ values ranging from 11.13 ±0.23 to 12.80 ±0.38.

• The more stable Cu-complexes were associated with the low-temperature vent, which is suspected to be due to the higher densities of biological activities around these vents.

• The high-temperature vents had lower log $K_{CuL'}$ values that is thought to result from the higher amounts of inorganic sulphur species, which are suspected to be the significant part of the Cu-ligand pool in both the high and low temperature vent samples.

Chapter 5: Investigated in this chapter was a possible cause for the oddly-shaped Cu-ligand titration plots for the hydrothermally-influenced seawater samples. Elemental sulphur (S⁰) was enriched in seawater, and these solutions successfully replicated the voltammetric behaviours of the hydrothermal samples. As such, it was concluded that elemental sulphur in hydrothermal fluids could explain the analytical oddities of the CLE-AdCSV technique in analysing hydrothermally-influenced samples.

The overall results from this study show that hydrothermal venting releases high concentrations of ligands that form stable Cu-complexes with relatively large log $K_{CuL'}$ values, which suggest that they may have a quantifiable impact on the long-range transport of copper in the global oceans. However, the exact impact can only be determined after refining the different ocean biogeochemical models, as broad assumptions will be reduced due to data on the flux of hydrothermally-derived Cu-stabilising ligands. Although Cu was the metal studied, it is anticipated that hydrothermally-derived ligands also play important roles in the biogeochemical cycles of other metals including Fe, Zn, Co, Ni, Cd and others. This is primarily because these ligands are oftentimes nonspecific and will stabilise other trace metals that they come in contact with.
6.2 Future Research

This thesis has provided new information on the reliability of the CLE-AdCSV technique to quantitatively assess Cu-complexation in sulphur-rich aquatic systems. It also provided extensive information on Cu-complexation in hydrothermally-influenced seawater samples from the southern Mid-Atlantic Ridge. This current research also presented evidence to suggest that elemental sulphur may be among these Cu-stabilising species. However, the results presented suggest that further research needs to be conducted in order to continue improving our understanding of copper biogeochemistry. In view of this, the following new research paths are recommended:

- Extend the quantitative assessments of the CLE-AdCSV technique to other Cu-complexing ligands; this should determine whether the limitations reported in Chapter 3 are due to the nature of the sulphur ligands. Also, these experiments should be extended to other metals such as Fe and Zn.

- Determine the structure of the Cu-stabilising ligands to clarify their inorganic and/or organic nature, using HPLC coupled with mass spectrometry. Advances were made in this project to develop an HPLC/fluorescence technique to quantify thiols in hydrothermal fluids. The information was not presented but, it showed promising results and should be further developed.

- Determine the size fractionation of trace metals, such as Cu, in hydrothermal fluids and plumes. This is to elucidate the role of particulate, nanoparticulate, and dissolved fractions for the export of Cu from hydrothermal vents. This can be achieved with carefully designed ultrafiltration experiments.

- Do more specific lab culture experiments with hydrothermal microbes under increasing metal (e.g. Cu) stress with varied parameters (temperature, pressure, $[S^{2-}]$, oxic and anoxic conditions, etc.), building on the work of Klevenz et al. (2012).

- Develop a speciation model to better represent the actual conditions present during the CLE-AdCSV complexometric titration of Cu in hydrothermal samples.
References


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Kleint, C., S. Kuzmanovski, et al. "Organic Cu-complexation at the shallow marine hydrothermal vent fields off the coast of Milos (Greece), Dominica (Lesser Antilles) and the Bay of Plenty (New Zealand)." Marine Chemistry(0).


Shown in Appendix I are sample information and Cu-ligand titration data for some of the hydrothermally-influenced seawater samples. Only a single data set is shown for each sample, but many samples were analysed in duplicates and at different dilutions, from which the average values are represented in Table 4.1.
This sample was collected at the low-temperature Wideawake hydrothermal vent field. Shimmering water was observed coming out of a crack covered with Bathymodiolus (mussels) and was sampled with a temperature of 17.5 °C.

DF = 10
[SA] = 2µM
Dep. Time = 60s

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This sample was collected at the low-temperature Wideawake hydrothermal vent field with a Niskin bottle at a temperature of 2.8 °C.

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[SA] = 2µM
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This sample was collected at the high-temperature Southern Tower vent in the Turtle Pits hydrothermal vent field.

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[SA] = 2µM  
Dep. Time = 30s

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12 ROV B7

This sample was collected at the high-temperature Turtle Pits hydrothermal vent field at a site labeled Marker #2 with a temperature of 407°C.

DF = 50
[SA] = 2μM
Dep. Time = 30s

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<tr>
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</table>

![Graph showing peak current vs. copper concentration]
This sample was collected at the high-temperature Turtle Pits hydrothermal vent field at a site labeled Marker #2 with a temperature of 407°C.

DF = 20
[SA] = 2µM
Dep. Time = 30s

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<th>Cu-S Current -nA</th>
</tr>
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<tr>
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<tr>
<td>202.71</td>
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</tbody>
</table>
This sample was collected at the high-temperature Tannenbaum vent in the Red Lion hydrothermal vent field with a temperature of 346°C.

DF = 100
[SA] = 2µM
Dep. Time = 30s

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<th>Cu-S Current (-nA)</th>
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<tr>
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<tr>
<td>405.45</td>
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</tbody>
</table>
This sample was collected at the high-temperature Shrimp Farm vent in the Red Lion hydrothermal vent field with a temperature of 185°C.

DF = 2.7
[SA] = 2µM
Dep. Time = 30s

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<tr>
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</table>
20 ROV 5F

This sample was collected at the high-temperature Sister Peak vent in the Comfortless Cove hydrothermal vent field with a temperature of 350°C. Visible boiling was observed.

DF = 40
[SA] = 2µM
Dep. Time = 10s

<table>
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<th>Cu-S Current -nA</th>
</tr>
</thead>
<tbody>
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<tr>
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<tr>
<td>346.55</td>
<td>54.5</td>
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</table>
20 ROV 6F

This sample was collected at the high-temperature Sister Peak vent in the Comfortless Cove hydrothermal vent field with a temperature of 380°C. Visible boiling was observed.

DF = 95

[SA] = 2µM

Dep. Time = 10s

<table>
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<th>Cu Conc. (nM)</th>
<th>Cu-SA Current (-nA)</th>
<th>Cu-S Current (-nA)</th>
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</table>

![Graph showing peak current vs. copper concentration](image-url)