Origins, Distribution, and Ecological Significance of Marine Microbial Copper Ligands

by

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We acknowledge with respect the Lekwungen peoples on whose traditional territory the university stands and the Songhees, Esquimalt and W̱SÁNEĆ peoples whose historical relationships with the land continue to this day.

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Abstract

Copper (Cu) is required by marine microbes for essential biological processes, including photosynthesis and nitrogen fixation, but can be toxic above a certain threshold. Copper bioavailability in seawater is regulated by complexation with dissolved organic ligands of unknown source and structure. Culturing experiments have demonstrated the production of high-affinity Cu-binding ligands by marine algae in response to metal stress or limitation, suggesting they function either as metal ‘sponges’ to reduce copper toxicity or ‘carriers’ that promote uptake. The goal of my thesis research was to develop methods for the recovery and characterization of Cu ligands from seawater that could then be applied to natural samples to investigate sources and structures of recovered ligands. Using natural seawater spiked with model Cu ligands, I developed an immobilized Cu(II)-ion affinity chromatography (Cu(II)-IMAC) protocol which was shown to be effective in quantifying an operationally defined subset of natural Cu ligands. I then applied Cu(II)-IMAC to seawater collected along transects in the Canadian Arctic and NE Pacific Ocean to assess the abundance of this ligand pool across a diverse set of samples. Ligand distribution profiles and their covariance with other components of seawater (e.g. chlorophyll) were consistent with *in situ* biological production of some Cu-binding ligands. Model ligands spiked into seawater and recovered by Cu(II)-IMAC were also used to develop protocols for structural characterization of Cu ligands by solid-phase extraction (SPE) and tandem mass spectrometry (MS/MS). This research provides new tools for the isolation and characterization of copper ligands in natural samples, and new insights into the biogeochemical cycling and ecological significance of Cu in the ocean.

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Abbreviations and Nomenclature

5LM – five-ligand mixture of 8HQ, GSH, His, SHA, Trp

8HQ – 8-hydroxyquinoline

ASV – anodic stripping voltammetry

Chalkophores – copper ligands which facilitate transport of Cu across biological membranes

Copper-binding compounds – organic or inorganic compounds which interact with Cu

Copper ligands – organic compounds which bind Cu with high affinity

CSV – cathodic stripping voltammetry

Dissolved – seawater components which pass through small filters (typically 0.45um or 0.2um)

DOC, DON, DOP – dissolved organic (carbon / nitrogen / phosphorus)

DOM – dissolved organic matter

ESI – electrospray-ionization

GSH – reduced glutathione

His – L-histidine

HLB – hydrophilic-lipophilic balanced resin

HPLC – high-performance liquid chromatography

IMAC – immobilized metal-ion affinity chromatography

ISE – ion-selective electrode

MCX – mixed-mode cation exchange resin

MS – mass spectrometry

MS/MS – tandem mass spectrometry

PPL – Priority PolLutant ® resin

SHA – salicylhydroxamic acid

Speciation – distribution of physicochemical forms in which an element exists in solution

SAX/SCX – strong anion/cation exchange resin

SPE – solid-phase extraction

Trp – L-tryptophan

WAX/WCX – weak anion/cation exchange resin

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Dedication

This work is dedicated to my mother Shirley for her tireless resolve and inspirational empathy, and to my father Duncan for his infectious curiosity and dissective analyticity.

1.0 Introduction

1.0.1 Chapter abstract

Findings of this dissertation must be framed within the broader context of historical knowledge upon which the hypotheses and experiments rest. Chapter 1 provides an overview of relevant past and contemporary research, from foundational basics of oceanography to a synopsis of the available literature on organic copper-binding compounds in the marine environment.

1.1 Introduction to marine biogeochemistry

Oceans are perhaps our planet’s most environmentally and economically important ecosystems. Global oceans are the ultimate carbon and heat sink, trapping CO_2 and regulating our climate. Sea life sustains fishing industries, natural marine ecosystems have cultural importance, beaches provide tourism revenue, hydrocarbon deposits beneath the seafloor are exploited, and global economies depend on seaborne infrastructure. Our collective appreciation of the ocean fuels both pushback against ecologically dangerous practices and support for implementation of sustainable practices by industries which impact it.

Historically, ocean biogeochemistry research has largely been conducted using a limited set of samples taken from discrete depths at fixed stations along a relatively small number of oceanic transects. Inherently these samples only present a snapshot of the dynamic ecosystems which they seek to characterize, and steady-state assumptions are often made despite strong influences of diurnal, seasonal, and multi-year cycles. One strategy to understand such a complex system is to examine the ratios of and covariance between chemical elements, compounds, and organisms which are partitioned in distinct ways by biogeochemical processes. These relationships help to define and reveal how marine ecosystems are shaped by and react to changing inputs.

1.1.1 Major and minor components of seawater

Virtually every natural element is found in the ocean, each at concentrations defined by a dynamic mix of biogeochemical factors. Some elements and compounds are typically present in seawater at millimolar concentrations (Na^+, Ca^+, K^+, Mg^{2+}, Cl^-, and SO_4^{2}). Such components are introduced to and removed from seawater by shared processes, resulting in a relatively constant ratio through the global Ocean regardless of geographic location or depth (Garrison & Ellis, 2016; Libes, 2009; Millero, 2013). These ‘bulk salts’ are responsible for the high ionic strength of seawater, a factor that presents serious analytical challenges for studying chemical species present at low relative abundance.

Components of seawater which are typically present at micromolar concentrations include biologically relevant nutrients such as nitrate, nitrite, phosphate, ammonium, and silicate. Distributions of these ions in seawater depend on their sources, mobility, and sinks in the marine environment, resulting in interpretable concentration gradients across surface, coastal, benthic and oceanic waters (Millero, 2013).

Seawater also contains gases at nanomolar to millimolar levels including dimethyl-sulfide, methane, oxygen, and carbon dioxide. These gases are introduced into the marine environment through atmospheric exchange, biological activity, or hydrothermal venting (Millero, 2013). While O_2 is not very soluble in seawater, CO_2 is very soluble, so most anthropogenic CO_2 emissions partition into seawater across the air-sea interface and enter the global Ocean with significant implications for global climate (Garrison & Ellis, 2016). Once in the aqueous phase CO_2 reacts with H_2O to form an equilibrium with carbonate (HCO_3^-) and bicarbonate (H_2CO_3), releasing H^+ in the process. This results in a net acidification of the ocean over time as anthropogenic CO_2 emissions increase, concomitant with surface temperature increases caused by greenhouse effects. These ongoing human-induced changes will – and already have begun – to have unforeseen consequences on global ecosystems (IPCC, 2014).

Components of seawater can be grouped based on how they are distributed in the water column. Elements and compounds which are found at similar ratios throughout the ocean are said to have ‘conservative’ distribution. Those with lower concentrations near the surface, consistent with biological uptake and utilization in the photic zone, are said to have ‘nutrient-like’ profiles, while those that decrease in concentration with depth are described as being ‘scavenged’ from the water column onto sinking particles (Libes, 2009).

This dissertation examines components of seawater which are present at much lower concentrations than those described above – namely, dissolved organic compounds and trace elements present at low-nanomolar concentrations.

1.1.2 Trace metals

Trace metals are those dissolved in seawater at concentrations less than 10 umol kg^{-1} (Bruland *et al.*, 2013). Metals are introduced into the marine environment through riverine flow, atmospheric deposition, benthic inputs, and hydrothermal vents. The first two of these inputs have been significantly altered by human activity in the industrial era, resulting in an imbalance from steady-state cycling for those trace metals with anthropogenic sources (Libes, 2009).

Distributions of trace metals in the marine environment are often consistent with distributions of other components of seawater which are influenced by similar processes – i.e., conservative, scavenged, nutrient-like, and even hybrid or mixed versions of these profiles (Bruland *et al.*, 2013). Some metals are highly soluble, not very particle reactive, and follow conservative distribution profiles (e.g. Mo) with little variance in concentration relative to salinity across the globe. Other metals have scavenged profiles (e.g. Al) with high levels near their major sources, decreasing with horizontal or vertical distance as metals are scavenged into particulate forms (Millero, 2013). Biologically utilized metals like iron (Fe) exhibit nutrient-like distribution profiles with surface minima due to microbial uptake. Metals with complex sources and sinks in different oceanic regions have distributions that are a hybrid form of these other profile types. A few elements (e.g. Ge) have complex interactions or have multiple species that result in mixed profiles. Copper (Cu) is often described as nutrient-like but has a unique profile because of its relatively high particle reactivity (Mason, 2013).

Trace metal distributions vary significantly in different regions of the ocean. Coastal ecosystems receive an influx of trace metals through riverine systems, especially from large rivers and those draining steep topography. Estuarine processes lead to adsorption of trace metals onto sinking particulates that dramatically reduces export; for example, 99.8% of riverine Fe and 66% of riverine Cu are precipitated during estuarine mixing, never reaching the open ocean (Mason, 2013). Groundwater discharge may also be a significant contributor to trace metal content in coastal waters. Further away from the coast, trace metals are rapidly removed from surface waters by adsorption to sinking organic matter and can only be replenished through atmospheric deposition, upwelling, or remineralization. Hydrothermal vents in the deep ocean contribute significant amounts of trace metals including Cu to the marine environment (Libes, 2009). However, since this work mostly focuses on surface waters, the nature and impact of hydrothermal vents on Cu biogeochemistry will not be discussed.

Partitioning of trace metals between the dissolved phase and the particulate phase also impacts their biogeochemistry. Particulate metals are found in colloids (e.g. iron oxyhydroxides), in marine ‘snow’, bound to algal cell surfaces, incorporated into cellular structures, and within larger organisms. Metals are scavenged into the organic particulate phase through selective biogeochemical reactivity, resulting in distinct partitioning of the various trace metals in distinct oceanic environments (Mason, 2013). This partitioning greatly impacts the biogeochemical cycling and ultimate fate of trace metals and their isotopes, since those with higher particle reactivity are more quickly taken up by both living and nonliving organic matter (Bruland *et al.*, 2013; Libes, 2009).

Trace metals are difficult to study in the marine environment, particularly since research vessels conducting sampling may themselves contaminate the local water column with metals. Anti-fouling paints made of Cu can present a significant analytical hurdle when sampling from ships for Cu biogeochemistry. Protocols for trace metal sampling involve meticulous preparation (Cutter *et al.*, 2010), as trace-level metal contamination is ubiquitous even in a laboratory setting. Labware – especially glass – can leach small amounts of contaminant metals into samples, particularly at low pH. Analysis of trace metal speciation is thus undertaken using an abundance of care. Plasticware with low metal leaching properties is prepared by soaking for long periods or at high temperatures in strong acid, then stored before sampling in ultrapure water containing specialty-grade clean acid.

The extent to which a dissolved trace metal participates in various biogeochemical cycles depends on the speciation of that metal – that is, the various physicochemical forms that it takes (Florence, 1982; Hirose, 2006; Libes, 2009; Millero, 2013). These may include different oxidation states and any inorganic or organic complexes that it forms. Many trace metals in the marine environment – Fe, Co, Ni, Cu, Zn, and Cd – form stable complexes with organic ligands (Kraemer *et al.*, 2015; Vraspir & Butler, 2009). This dissertation focuses on the impact of these ligands on Cu biogeochemistry.

1.1.3 Dissolved organic matter

Organic compounds dissolved in seawater are highly variable and complex and so have historically been described in terms of the elemental components of this dissolved organic matter (DOM). Distinct processes govern the distribution of dissolved forms of carbon (DOC), nitrogen (DON), and phosphorus (DOP), so measurement of the ratios between these forms offer insight into the biogeochemistry of those elements. For example, because microbial utilization of nitrogen and phosphorus as nutrients depletes the organic forms of these elements, productive waters can be identified by their relative absence. Measurements of other processes that covary with indicators of productivity can thus be linked to biology (Libes, 2009; Millero, 2013).

Analysis of DOM using modern spectrophotometric and spectroscopic techniques has revealed the presence of a staggering diversity of compounds including simple sugars, amino acids, proteins, and humic/fulvic acids. Originating largely from biological exudates, though also produced through grazing and viral lysis, DOM is modified through inorganic processes including redox and photochemical interactions (Hansell & Carlson, 2014; Libes, 2009). Some of these compounds are refractory to microbial degradation and persist for long periods of time, while others are quickly utilized or transformed by biological processes. Some organic compounds in seawater (including amino acids and simple sugars) can be measured directly, but most DOM requires a pre-concentration step due to low abundance and interference from salts. Because pre-concentration steps such as solid-phase extraction (SPE) only capture an operationally defined fraction of total DOM (e.g. SPE-DOM), many questions remain about the true composition of dissolved organic compounds in the ocean.

Non-targeted qualitative analyses of marine SPE-DOM by high-resolution electrospray ionization mass spectrometry (ESI-MS) have identified a huge number of dissolved organic compounds. Assigning elemental formulae to the accurate mass-to-charge (*m/z*) ratios determined by high-resolution MS show SPE-DOM is mostly composed of molecules containing only C, H, and O, with a relatively small proportion containing N and/or S. The relative abundance of these elements can be used to infer the biochemical origin of DOM components (Hansell & Carlson, 2014). Each formula may represent a spectrum of isomeric compounds, exponentially increasing in diversity with molecular weight. However, recent work suggests that the percentage of compounds in marine SPE-DOM with multiple isomeric forms may be much less significant than previously suggested (Kaijun Lu, OSM 2020).

Spectrophotometric analysis of DOM typically measures *chromophoric DOM* (CDOM) or *fluorescent DOM* (FDOM). The spectral character of DOM results from the sum contributions of all compounds with photoactivity at visible or UV wavelengths, including interactions between chromophores and fluorophores. Because CDOM is controlled by processes that impact the total DOM pool, changes in CDOM can be used to infer biogeochemical processes occurring along oceanic transects or over time. Moreover, CDOM derived from terrestrial sources has different optical properties than CDOM from aquatic sources, and can thus be used as a biogeochemical tracer of terrestrial input (Hansell & Carlson, 2014).

Microbial communities produce unique contributions to marine DOM through their collective exometabolome. It is well understood that biological processes drive changes in DOC/DON/DOP, but the specific nature of these changes has only been characterized on a superficial level (Libes, 2009; Millero, 2013). Among other considerations, taxonomic composition and growth factor limitations dictate the spectrum of compounds present in DOM from both intentional excretion and incidental grazing or viral lysis. These products can then influence the composition and distribution of microbial communities. Individual components of DOM – for example, nutrient vitamins, amino acids, and polysaccharides - can have profound impacts on the productivity of marine phytoplankton communities. Organic compounds that complex trace metals (e.g. Fe^{3+}) have also been shown to play a crucial role in supporting phytoplankton growth when certain micronutrient trace metals are limiting (Hirose, 2007; Vraspir & Butler, 2009). Indeed, many components of the marine microbial exometabolome may have evolved in an ‘arms race’ of metal acquisition and detoxification strategies.

Marine DOM may be autochthonous, generated *in situ* through cellular exudation and lysis, or allochthonous, produced elsewhere and potentially subject to degradation or photochemical cross-linking before sampling. Compositional changes in DOM measured over spatial or temporal scales can thus reflect biogeochemical processes occurring across marine environments or in response to anthropogenic inputs. Sampling and obtaining an historical dataset for DOM composition thus facilitates insight into oceanic processes, how to model them, and how human activity impacts those processes.

1.2 Marine microbes

Oceans host a staggering diversity of interrelated biology. Much research still needs to be done to characterize these organisms, their relationships, and their ecological and economic significance. Only the microbial realm – more precisely, photosynthetic microbes – will be examined in this work.

1.2.1 Primary productivity

Phytoplankton are photosynthetic autotrophic micro-organisms that convert sunlight and inorganic compounds into biomass that feeds higher trophic levels, sustaining the marine food web. Moreover, half of global O_2 synthesis and CO_2 consumption result from phytoplankton photosynthesis (Garrison & Ellis, 2016).

Sustained growth of these organisms (also known as *primary productivity*) requires a consistent supply of nutrients and light. Coastal waters in particular have nutrient-rich terrestrial inputs and thus support productive and diverse algal growth, as do oceanic regions with significant upwelling of nutrients. Conversely, primary productivity in the open ocean is limited by a paucity of nutrients including Fe. Although natural assemblages of phytoplankton are diverse, many regions of the ocean with high primary productivity are most abundantly represented by eukaryotic *diatoms* (Barsanti & Gualtieri, 2014).

1.2.2 Phytoplankton metabolism

Phytoplankton require a variety of nutrients for growth, many of which are readily available in most natural waters. Carbon dioxide for photosynthesis and oxygen for respiration are typically present in excess in ocean waters, although light can limit photosynthesis. Natural phytoplankton communities are most often limited by nitrogen and phosphorus (Trujillo & Thurman, 2017), but certain regions of the ocean have an abundance of these nutrients yet an absence of productivity and have been described as 'high nutrient low chlorophyll' (HNLC). Growth limitation in these waters is often due to a lack of bioavailable Fe (Bruland *et al.*, 2013), although light limitation may also play a role.

Nutrient uptake rates and cellular quotas vary across species and according to growth conditions. For example, the dependence of diatom growth on the presence of silicic acid for frustule synthesis leads to their higher relative abundance in silicate-rich oceans (Miller & Wheeler, 2012). Distinct groups of phytoplankton also produce unique types and ratios of photosynthetic pigments. Diatoms predominantly generate chlorophyll *a* and *c*, but also utilize beta-carotene and xanthophylls such as fucoxanthin, and other phytoplankton species use a range of diverse and unusual pigments (Graham & Wilcox, 2000; Miller & Wheeler, 2012). Importantly, standard fluorescence assays will only account for chlorophyll-a and not truly reflect the photosynthetic capacity of phytoplankton within the sample (Barsanti & Gualtieri, 2014).

Metabolic activities of natural phytoplankton assemblages form intersections among biological and geochemical cycles in marine ecosystems. Cellular processes requiring certain nutrients drive the acquisition, transformation, or utilization of other nutrients. HNLC regions of the ocean are elevated in nitrogen because of Fe limitation stalling metabolism through an insufficient amount of Fe to act as a cofactor in nitrate/nitrite reductases.

Conversely, too high a concentration of otherwise nutritional compounds can inhibit the growth, distribution, or composition of phytoplankton communities. For example, anthropogenic input of Cu to natural waters can significantly inhibit primary productivity in estuaries and coastal waters.

Marine phytoplankton are intentionally grown in aquaculture for a wide variety of applications. Algae can be harvested for use as feed, utilized to chemically transform wastewater or soil, or for biosynthesis of consumer products and medicine (Lucas *et al.*, 2019).

1.3 Trace metals in the marine environment

1.3.1 Trace metals in phytoplankton

Trace metals play multiple roles in cellular upkeep and metabolism within marine phytoplankton. Many can participate in redox reactions and/or are able to interact with both amino and nucleic acids, with the potential to modulate genetic, cytosolic, and membrane function. Some trace metals including Fe, Mn, Co, Cu, and Zn are considered essential for phytoplankton; if uptake and accumulation of these elements are below a certain threshold, the organism will suffer from a reduction in some physiologically important function(s) (Stumm & Morgan, 1996). Concentrations of essential metals within phytoplankton are approximately 10^7 times higher than ambient external levels, necessitating competitive mechanisms to enhance trace metal sequestration.

Certain trace metals may also become toxic to marine phytoplankton at high enough concentrations. If uptake and accumulation of these elements are *above* a certain threshold, the organism will similarly suffer a reduction in function(s). Copper, which is both essential and potentially toxic to marine phytoplankton, has been referred to as a ‘goldilocks’ element.

Early studies of the effects of toxic trace metals on marine phytoplankton demonstrated that toxicity is not necessarily related to total metal levels but is instead a function of the bioavailable fraction of metal species present in growth media (typically the free aqueous form, e.g. Cu^{2+}, and weakly associated inorganic complexes, e.g. $CuCO_3$).

Later studies into nutrient uptake among marine phytoplankton confirmed that accumulation of essential trace metals is not simply a function of total metal concentration. In particular, uptake of Fe was shown to depend strongly on the presence of Fe-complexing ligands.

Presented with the challenge of obtaining just the right amounts of trace metals, in an environment where metal levels fluctuate widely and rapidly, marine phytoplankton appear to have evolved mechanisms for maintaining metal homeostasis. Bioavailability – and thus uptake and accumulation – is a function of organic speciation, so by introducing new organic compounds that contribute to the speciation of a trace metal, phytoplankton can modify bioavailability to access appropriate levels of nutrient metals under different conditions.

Adsorption of dissolved trace metals onto biological surfaces, including microbial cells and detritus, can significantly deplete the levels of these metals in surface waters. This, in turn, reduces their availability to phytoplankton and limits algal growth. This reciprocal relationship illustrates the importance of marine phytoplankton and algal exudates as biogeochemical factors that influence the distributions of trace metals and their partitioning between dissolved and particulate phases.

Transport of trace metals across cellular membranes can occur by passive, facilitated, or active mechanisms. Trace metals may be transported in their free ionic forms, hydrated or with inorganic complexes, or in the form of organic complexes.

Biological interactions with trace metals can also lead to chemical transformation of metals by, for example, the formation of organometallic compounds. While these interactions can have serious implications such as the formation of toxic products in the marine environment (e.g. methylation of Hg), the focus of this research is on the complexes formed by the association of Cu^{2+} ions with dissolved organic ligands at equilibrium in seawater.

Approximately one third of all marine productivity is limited by the availability of Fe and, potentially, other trace metals. This deficiency represents a significant opportunity to increase productivity through ocean fertilization experiments, discussed below. Consequently, Fe is perhaps the most important trace metal with respect to global primary production, although Cu is also of major importance since it is required for Fe uptake (Annett *et al.*, 2008).

1.3.2 Dissolved copper

Anthropogenic inputs are the predominant sources of Cu in the marine environment, including industrial release of Cu into coastal waters. Human activity accounts for about 65% of Cu inputs to the ocean (Mason, 2013). Polluted natural water systems have been shown to contain dissolved copper levels far above toxicity thresholds for even the most resilient organisms. Understanding the ecological effects of such anthropogenic inputs is crucial for the development of effective regulations to mitigate the effects of Cu pollution.

Copper levels in the open ocean typically range from ~0.5 nM at the surface to ~5 nM in deep waters, while coastal levels vary widely from <10 nM to >150 nM (Mason, 2013). Cu has relatively high particle reactivity, leading to a more complex vertical distribution than for other trace metals (e.g. Zn, Cd) with nutrient-like profiles. Inorganic speciation of Cu^{2+} in seawater is dominated by $Cu(OH)_x$ hydrides, with smaller proportions of Cu existing as free aqueous ions, carbonates, and chlorides (Flemming & Trevors, 1989; Mason, 2013). More than 99% of dissolved Cu^{2+} in natural seawater samples has been shown to exist as organic species formed by high-affinity ligands (Vraspir & Butler, 2009).

1.3.3 Copper as a nutrient and a toxin

Copper is a required cofactor in respiratory enzymes, making it a crucial element in the photosynthetic cascade utilized by marine phytoplankton. Cu^{2+} is also found in oxidases, nitrogen uptake complexes, iron acquisition surface transport proteins, chaperones, and transcriptional regulators of metal stress genes (Festa & Thiele, 2011; Trevors & Cotter, 1990). In aerobic prokaryotes, Cu is used in the respiratory transport chain as part of cytochrome c oxidase. Uptake of iron complexes by phytoplankton involves multi-Cu containing surface transport complexes, resulting in a higher Cu quota in low-iron environments (Annett *et al.*, 2008; Peers *et al.*, 2005).

Chalkophores (copper-binding compounds that facilitate biological transport of copper) have been characterized in some organisms. Notably, methanotrophs have been shown to produce high-affinity copper-binding ligands to promote Cu uptake (Kenney & Rosenzweig, 2018). Such an acquisition strategy is crucial for these microbes since methane mono-oxygenase requires multiple Cu cofactors (Trevors & Cotter, 1990).

The toxicity of copper to marine phytoplankton at relatively low levels is well documented (Brand *et al.*, 1986). Cyanobacteria in particular are sensitive to Cu levels in a laboratory setting. Culture studies using metal chelators have long demonstrated that Cu toxicity is largely dependent on the concentration of free aqueous or weakly bound inorganic forms of copper (Sunda & Lewis, 1978). Copper toxicity can result from the substitution of Cu for other trace metals in metalloproteins or from the production of harmful hydroxyl radicals by free intracellular Cu^{2+} (Festa & Thiele, 2011). Potential mechanisms for alleviating copper toxicity include export, storage, or transformation into less toxic forms (Mason, 2013). Copper toxicity has been utilized by many industries, including as a marine algaecide. Copper sulfate is used as a fungicide for agricultural applications, famous for its use in vineyards in the grape-saving Bordeaux mixture.

1.3.4 Anthropogenic inputs of trace metals

Because Fe is known to be a limiting nutrient in HNLC regions of the ocean, attempts have long been suggested – and recently been made – to boost natural phytoplankton communities through bulk addition of iron (Buesseler & Boyd, 2003; Huesemann, 2008). These efforts are advertised as a way to bolster local populations of fish for fisheries industries and as potential geo-engineering projects for large-scale carbon sequestration (Strong *et al.*, 2009). However, iron fertilization experiments have typically used inorganic forms of Fe(III) such as ferrous sulphate with no organic chelating agent. Since culturing experiments have shown iron uptake strongly depends on its organic speciation, it is not clear if additions of uncomplexed iron would be successful. Most iron addition experiments have resulted in temporarily increased primary production as evidenced by chlorophyll a blooms and increased nutrient uptake in the surface mixed layer, along with compositional shifts in the taxonomy of phytoplankton assemblages favouring larger groups such as diatoms (Williamson *et al.*, 2012).

Copper is rarely a limiting nutrient for primary productivity in natural marine ecosystems, so additions of copper to natural waters are not an avenue to boost phytoplankton growth. However, understanding the effects of Cu additions to seawater or artificial seawater cultures may still have utility. If copper, or certain organic Cu species, preferentially foster the growth of specific desirable phytoplankton, that link could be exploited for aquaculture purposes. Inhibiting the growth of specific unwanted phytoplankton could be used to mitigate harmful algal blooms while promoting the growth of more desirable organisms. Conversely, knowledge of the downstream effects of certain Cu species can guide policies regulating industrial pollution.

In any case, stressing ecosystems poses potential risks until the effects of those stressors can be appreciably understood. Negative impacts of large-scale geoengineering projects could potentially be disastrous due to unforeseen effects. By developing a fundamental understanding of basic cellular mechanisms for maintaining trace metal homeostasis, ambitious projects and meaningful policies can be implemented to promote ocean sustainability.

1.4 Organic speciation of trace metals

Trace metals in seawater have no shortage of potential organic complexation sites. Natural waters contain metal-binding DOM in the form of small carboxylates, amino acids, and larger photo-linked and/or degradation products of biological macromolecules such as humic and fulvic acids. Nonetheless, organic speciation of many bioactive trace metals appears to be dominated by high-affinity compounds present in low concentrations. Recovery of these ligands from bulk DOM presents a significant analytical challenge. Natural ligands are a diverse mix of potentially related molecules, generally present at low concentrations in a very salty and complex mixture of organic matter.

1.4.1 Biogeochemistry of organic metal ligands

High-affinity organic ligands typically form metal complexes through N, O, or S-containing functional groups (Zhang *et al.*, 2019). Nitrogen functionalities in ligands can be particularly diverse, ranging from 1° and 2° amines to highly substituted groups such as hydroxamates or imidazoles, while oxygen also offers many potential moieties including carboxyls and catechols. Sulfur is often implicated in trace metal binding, but is more commonly associated with Cu(I) rather than Cu(II) (Kraemer *et al.*, 2015). Most low-molecular weight metal-complexing ligands are polyprotic acids and the strength of organic complexation with transition metals generally follows the Irving-Williams series (Mason, 2013; Stumm & Morgan, 1996). Cu^{2+}, which has nine d-orbital electrons and can form chelates containing up to six functional groups, tends to interact more strongly with organic ligands than other metal ions (Manceau & Matynia, 2010; Millero, 2013).

Organic compounds that complex trace metals can strongly influence their geochemical cycling. In aerobic seawater Fe(II) is readily oxidized to Fe(III), which has low solubility and quickly precipitates (Hirose, 2007). Iron-binding ligands increase the solubility of Fe(III), thus retaining it within the water column and preventing loss (Bruland *et al.*, 2013). Similarly, removal of Cu(II) by adsorption onto sinking particulate matter is likely decreased by high-affinity small organic ligands. Some researchers have also examined the reverse scenario whereby complexation of metals by ligands slows the rate of ligand degradation (Thompson *et al.*, 2014).

As described below, organic metal-ion complexes can have a profound impact on phytoplankton communities, and phytoplankton communities have a reciprocal impact on organic speciation of trace metals. Because of these nuanced interactions, attempts to understand the biogeochemical cycling and ecological significance Cu or other dissolved trace metals without considering organic speciation are unlikely to succeed (Viljoen *et al.*, 2019).

1.4.2 Siderophores

Siderophores are high-affinity microbial iron-binding ligands that directly facilitate biological uptake of iron by interacting with cell-surface iron transport proteins (Hirose, 2006; Kraemer *et al.*, 2015). This interaction is typically the rate-limiting step for iron uptake, meaning larger phytoplankton with lower surface area : volume ratios may have more difficulty acquiring trace metals at a sufficient rate (Morel & Price, 2003). Although siderophores may potentially contribute to the organic speciation of Cu and/or other trace metals in seawater, they are only discussed here to compare Cu ligands with analogous compounds in seawater that have been more extensively characterized.

Siderophores also increase the bioavailability of iron by stabilizing the oxidized Fe^{3+} species that would otherwise readily precipitate. In fact, organic complexation can maintain dissolved Fe in seawater at higher levels than the calculated maximum solubility of ferric hydroxides (Liu & Millero, 2002). It is unclear if the mechanism by which siderophores promote cellular uptake involves the release of reduced Fe^{2+}, which is much more bioavailable than Fe^{3+}, through a photochemical charge-transfer reaction (Mason, 2013).

Iron-binding ligands in the marine environment are often described as a single pool (L) despite a range of affinity constants which some researchers group into very strong (L_1) and strong (L_2) ligand classes. L_1 ligands are found predominantly in surface waters at <1nM, while L_2 ligands are found above 1nM at a range of depths (Kraemer *et al.*, 2015).

Siderophore production has been associated with metal stress in cultures and natural communities of phytoplankton. Certain bacteria are known to produce siderophores in response to low iron conditions to promote iron uptake, alongside production of surface iron transport proteins (Vraspir & Butler, 2009).

Molecular structures of siderophores produced by phytoplankton have been elucidated through techniques such as high-resolution mass spectroscopy of culture media, using microbes grown in simpler media than seawater (Walker *et al.*, 2017). Two general types of siderophores have been characterized: amphiphilic fatty acids with hydroxamate or carboxyl functionalities, and α-hydroxy carboxylates (Vraspir & Butler, 2009). The latter of these two has been shown to have photoreactivity, resulting in reduction of Fe(III) to Fe(II).

1.4.3 Phytochelatins and thiols

Phytochelatins (PCs) are a class of polypeptides composed of (g-Glu-Cys)$_n$-Gly polymers, with between 2 and 11 repeating units of Glu-Cys, synthesized from glutathione. PCs interact with divalent cations such as Cu^{2+} through their thiol functionality (Satofuka *et al.*, 2001). These peptides are generally thought of as an intracellular storage system for both essential and toxic metals, and metal stress has been associated with the induction of cytosolic PC synthesis in a wide range of phytoplankton (Ahner *et al.*, 1995, 2002). Cu^{2+} in particular has been shown to induce PC production in diatoms (Ahner & Morel, 1995) and green algae (Gekeler *et al.*, 1988). Phytochelatin levels in natural waters have been shown to covary with free Cu^{2+} concentrations (Ahner *et al.*, 1997). Culture studies have shown increased production and exudation of thiols in response to Cu^{2+} stress (Dupont & Ahner, 2005; Kawakami *et al.*, 2006).

A survey of estuarine waters in 2017 suggested thiols and humic substances were a significant source of Cu(II) ligands (Whitby *et al.*, 2017). Correlative studies have shown a link between Cu binding capacity of samples, and thiol content, although computer models suggest carboxylic acids are more likely to contribute to the organic speciation of Cu (Mason, 2013).

1.5 Copper ligands

1.5.1 Early evidence for organic Cu(II) ligands

Before Cu-binding organic components of seawater were directly measured, organic complexation was theorized after studies attempting to quantify Cu levels in seawater detected the presence of ‘labile’ and ‘bound’ fractions of Cu. The latter fraction required a treatment step to liberate complexed Cu prior to analysis – for example by organic extraction (Slowey *et al.*, 1967), oxidation (Williams, 1969), or UV-irradiation (Foster & Morris, 1971). These studies provided the first evidence that most Cu in the marine environment was not in free aqueous ionic form, but rather, in organic complexes.

Direct measurement of Cu complexation by organic ligands was first accomplished by anodic stripping voltammetry (ASV) (Batley & Florence, 1976). Innovations soon led to the development of other electrochemical methods including cathodic stripping voltammetry (CSV) (van den Berg, 1984a) and ion-selective electrodes (ISE) (Swallow *et al.*, 1978). Other early techniques such as MnO_2 adsorption, C18 chromatography, and ligand exchange (LE) methods were generally discarded by the turn of the millennium in favour of voltammetric approaches that dominate the field of Cu speciation to this day. This history is chronicled in Table 1.

Table 1.1 List of publications reporting direct measurement of copper ligands in seawater.

Reference	**Source**	**Depth or species**	**Method**
(Batley & Florence, 1976)	Southern	Surface	ASV
(Duinker & Kramer, 1977)	N Atlantic	Surface	ASV
(Swallow *et al.*, 1978)	Culture	Cyano, green algae	ISE
(McKnight & Morel, 1979)	Culture	Diatoms, cyano, more	ISE
(McKnight & Morel, 1980)	Culture	Cyanobacteria	ISE
(Srna *et al.*, 1980)	N Pacific	Surface	ASV
(Kremling *et al.*, 1981)	N Pacific	Surface	XAD-2
(Hasle & Abdullah, 1981)	N Atlantic	0 – 140 m	ASV
(Mills & Quinn, 1981)	N Atlantic	Surface	C18
(Piotrowicz *et al.*, 1982)	N Atlantic	Surface	ASV
(Nilsen & Lund, 1982)	N Pacific	Surface	ASV
(Plavšić *et al.*, 1982)	Mediterranean	Surface	ASV
(van den Berg, 1982)	N Atlantic	Surface	MnO_2
(Hirose *et al.*, 1982)	N Pacific	Surface	XAD-2
(Mackey, 1983)	S Pacific	Surface	C18
(van den Berg, 1984a)	N Atlantic	Surface	CSV
(van den Berg, 1984b)	N Atlantic	Surface	MnO_2
(Buckley & van den Berg, 1986)	N Atlantic	0 – 3000 m	ASV
(Kramer, 1986)	N Atlantic	Surface, deep	ASV
(Donat *et al.*, 1986)	N Atl, N Pac	Surface, deep	SPE, ASV
(Seritti *et al.*, 1986)	Culture	Green algae	ASV
(Sunda & Hanson, 1987)	N Atl, S Pac	Surface	C18
(van den Berg *et al.*, 1987)	N Atlantic	Surface	CSV
(Moffett & Zika, 1987)	N Atlantic	0 – 180 m	LE
(Coale & Bruland, 1988)	N Pacific	0 – 1400 m	ASV
(Morelli *et al.*, 1989)	Culture	Diatom	C18
(Zhou & Wangersky, 1989)	N Atlantic	0 – 2000 m	C18
(Moffett *et al.*, 1990)	N Atl, Culture	0 – 1000 m, cyano	LE
(Coale & Bruland, 1990)	N Pacific	0 – 500 m	ASV
(Midorikawa *et al.*, 1990)	N Pacific	0 – 1000 m	LE
(Sunda & Huntsman, 1991)	Estuary	Surface	LE
(Donat & van den Berg, 1992)	N Atl, Indian	Surface	CSV
(Midorikawa *et al.*, 1992)	N Pacific	Surface	LE
(Gordon, 1992)	N Atlantic	0 – 1000 m	IMAC
(Donat *et al.*, 1994)	N Pacific	Surface	CSV
(Moffett, 1995)	N Atlantic	Surface	CSV
(Gerringa *et al.*, 1995)	Culture	Diatom	ASV, CSV, C18
(Moffett & Brand, 1996)	Culture	Cyanobacteria	CSV
(Midorikawa & Tanoue, 1996)	Pacific	0 – 1500 m	IMAC, ISE
(Gordon *et al.*, 1996)	Estuary	Surface	IMAC
(Gerringa *et al.*, 1996)	N Atlantic	Surface	ASV, CSV, C18
(Donat *et al.*, 1997)	N Atlantic	Surface	IMAC, ASV
(Moffett *et al.*, 1997)	N Atlantic	Surface	CSV
(Kozelka & Bruland, 1998)	N Atlantic	Surface	ASV
(Midorikawa & Tanoue, 1998)	N Pacific	Surface	IMAC
(Gledhill *et al.*, 1999)	Culture	Brown algae	CSV
(Croot *et al.*, 1999)	N Atl, Culture	Surface, cyano	ASV
(Midorikawa & Tanoue, 1999)	N Pacific	0 – 1000 m	LE
(Leal *et al.*, 1999)	Culture	Coccolithophore	CSV
(Bruland *et al.*, 2000)	N Atlantic	Surface	ASV, CSV
(Gordon *et al.*, 2000)	Estuary, Culture	Surface, cyano	IMAC

(Skrabal *et al.*, 2000)	Estuary	Surface, Sediment	ASV
(Laglera & van den Berg, 2003)	Estuary	Surface	CSV
(Ross *et al.*, 2003)	N Pacific	Surface	IMAC, MS
(Vachet & Callaway, 2003)	Estuary	Surface	IMAC, MS
(Dryden *et al.*, 2004)	Estuary	Surface	CSV
(Dupont *et al.*, 2004)	Culture	Coccolithophore	CSV, MS
(Shank *et al.*, 2004)	Estuary	Surface	CSV
(Buck & Bruland, 2005)	N Pacific	Surface	CSV
(Dupont & Ahner, 2005)	Culture	Coccolithophore	Thiols
(Shank *et al.*, 2006)	Estuary	Surface	CSV
(Dryden *et al.*, 2007)	Estuary	Surface	CSV
(Moffett & Dupont, 2007)	N Pacific	0 – 3000 m	CSV
(Wiramanaden *et al.*, 2008)	Culture	Cyanobacteria	CSV, MS
(Chapman *et al.*, 2009)	Estuary	Sediment	CSV
(Buck *et al.*, 2010)	Southern	180 m	CSV
(Buck *et al.*, 2012)	N Pacific	0 – 3000 m	CSV
(Bundy *et al.*, 2013)	Southern	Surface	CSV
(Thompson & Ellwood, 2014)	Southern	0 – 3500 m	CSV
(Oldham *et al.*, 2014)	N Atlantic	Surface	CSV
(Heller & Croot, 2015)	Southern	0 – 3000 m	CSV
(Li *et al.*, 2015)	N Pacific	Surface, sediment	CSV
(Whitby & van den Berg, 2015)	N Atl, Estuary	Surface	CSV
(Al-Farawati *et al.*, 2016)	Indian	Sediment	CSV
(Nixon & Ross, 2016) **and current work**	**N Pacific**	**Surface**	**IMAC**
(Whitby *et al.*, 2017)	Estuary	Surface	CSV
(Wong *et al.*, 2018)	Estuary	0 – 40 m	CSV
(Whitby *et al.*, 2018)	N Pacific	0 – 1400 m	CSV
(Echeveste *et al.*, 2018)	Culture	*E. huxleyi*	CSV
(Nixon *et al.*, 2019) **and current work**	**Arctic**	**0 – 3000 m**	**IMAC**
(Wong *et al.*, 2019)	N Pacific	0 – 58 m	CSV
Current work	**N Pacific**	**0 – 800 m**	**IMAC**

1.5.2 Electrochemical studies

Directly measuring the association and dissociation kinetics of metal-ion complexes using electrochemistry is a powerful analytical approach. By measuring the concentration and copper-binding affinity of an organic ligand pool, the natural speciation of dissolved Cu in marine systems can be investigated. Electrochemical speciation is, however, limited to complexes that lie within the operationally-defined window of the technique being used. Voltammetric techniques are also destructive to samples, provide no structural data, and cannot differentiate between ligands of similar binding strength (Buck *et al.*, 2012).

Electrochemical detection of copper-binding ligands involves the measurement of free Cu^{2+} in solution by an electrode while either more Cu^{2+} (ASV) or more ligand (CSV) is titrated in. These data provide information on the average binding affinity of a ligand pool for Cu^{2+}. Binding affinity estimates have been used to define two broad classes of ligands. The stronger class, L_1, is typically defined as having log conditional stability constant ($\log K'_{cond\,(Cu')}$) values in the range

11.5-14, whereas the weaker class, L_2, has values in the range 8.5-11.5 (Hirose, 2006; Vraspir & Butler, 2009). Many electrochemical studies of natural seawater have detected both of these classes (Coale & Bruland, 1988, 1990). Concentration estimates for these ligand classes reflect the number of Cu binding sites available, so multi-dentate ligands are accounted for as if they were multiple separate ligands.

Voltammetry-based concentration estimates for strong Cu ligands in seawater vary significantly, particularly among early studies. These variations reflect the diversity of techniques, progress towards improving sensitivity, the complexity of seawater, and the operationally defined manner by which estimates of concentration are obtained. Several early studies report ligand concentrations in the micromolar range (Buckley & van den Berg, 1986); such findings have not been corroborated in modern publications that have estimated L_1 levels to range from around 0.5 to 10 nM and L_2 levels from approximately 1 to 50 nM (Wong *et al.*, 2018, 2019). Estimates have been consistent in suggesting more than 99% of dissolved Cu is organically complexed, particularly in surface waters.

Based on the extremely strong binding affinity of L_1-type ligands for copper, and the presence of these ligands in excess of total dissolved copper levels in surface waters, it could be expected that virtually all dissolved copper in seawater is bound to the L_1 class. However, in copper-enriched deep or coastal waters, copper levels may exceed L_1 and result in a significant population of L_2-bound Cu that also been suggested to play a role in regulating productivity (Hirose, 2006, 2007). It is not well understood if these different pools of ligands play distinct ecological roles.

1.5.3 Cu(II)-IMAC

Originally developed for the extraction and purification of proteins, immobilized metal-ion affinity chromatography (IMAC) was first applied to concentrate dissolved copper ligands in marine DOM by Gordon (1992). In principle, high-affinity copper-binding ligands will form stable interactions with stationary phase Cu^{2+} ions on the column while other DOM is not retained and is discarded as flow-through. After sample loading, elution of target compounds is achieved by disrupting interactions between metal ions and organic ligands. Elution of copper ligands from IMAC columns can be achieved with competitive ligand equilibration or acid dissociation. The former inherently contaminates the sample, making it less desirable for applications with downstream analysis of fractions. The latter is relatively non-destructive, and pH values used for elution (pH 2.2) have been shown electrochemically to completely dissociate natural copper ligands from copper (Gordon *et al.*, 2000).

On-line UV detection may be used to produce Cu(II)-IMAC chromatograms that visualize ligand elution based on their spectral characteristics. It is tempting to suggest that these chromatograms can discriminate between low-affinity (short retention time) and high-affinity (long retention time) ligands. In fact, retention time depends upon a number of factors including stability of the ternary column-metal-ligand complex (Paunovic *et al.*, 2005) and kinetics of ligand association and dissociation within the IMAC column.

The first application of Cu(II)-IMAC to seawater measured two peaks using a detection wavelength of 280 nm, suggesting two classes of copper ligands had been concentrated and fractionated from bulk DOM in Atlantic seawater (Gordon, 1992). Subsequent publications used ASV to demonstrate that IMAC recovers a significant portion of both L_1 and L_2 copper ligands from estuarine and coastal seawater samples (Donat *et al.*, 1997; Gordon *et al.*, 1996, 2000). IMAC analyses of Pacific samples using a detection wavelength of 254 nm reported a single chromatographic peak containing highly concentrated UV-absorbing organic matter that was shown to contain two ligand classes based upon analysis using an ion-selective electrode (Midorikawa & Tanoue, 1996) and fluorescence quenching (Midorikawa & Tanoue, 1998).

Refining Cu(II)-IMAC protocols for the isolation of copper ligands is a key aspect of this dissertation. Chapter 2 covers the development of a multiple step procedure involving IMAC, SPE, and ESI-MS/MS for the isolation, structural characterization, and quantitation of a model copper ligand spiked into seawater at low levels. IMAC is then applied to natural samples from the Arctic Ocean (Chapter 3) and the NE Pacific Ocean (Chapter 4).

1.5.4 Characterization of marine copper ligands

Molecular weights of potential copper-binding ligands have been estimated by size-filtration chromatography and related methods. Size filtration analyses of IMAC fractions suggest high-affinity Cu ligand pools, of both classes but particularly the weaker class, are dominated by small compounds with molecular weight under ~1 kDa (Gordon *et al.*, 1996; Midorikawa & Tanoue, 1998; Vachet & Callaway, 2003). Some studies have suggested high molecular weight compounds may also contribute to the organic speciation of copper in seawater (Hasle & Abdullah, 1981), though these will not be discussed here. Size-fractionation analysis of estuarine waters suggests copper-binding ligands are predominantly part of the ‘truly dissolved’ (<0.015 um) pool of DOM and not dissolved colloids (Adrienne Hollister, presented at OSM 2020).

Some studies have attempted to characterize Cu ligands chemically. Copper ligands have been shown to be sensitive to UV oxidation (Anderson *et al.*, 1984), and ligands recovered by Cu(II)-IMAC in particular are photolabile (Gordon, 1992). Treatment of seawater samples by XAD in an attempt to remove humic acids resulted in lower Cu(II)-IMAC peak area, suggesting a potential role for humic substances in contributing to marine copper speciation (Gordon *et al.*, 1996).

Mass spectrometry (MS) is a powerful analytical technique capable of identifying and quantifying dissolved organic compounds based on the *m/z* of molecular ions. These ions are produced and introduced to the instrument using techniques such as electrospray ionization (ESI), and may be detected as intact ligands or structural fragments produced during tandem mass spectrometry (MS/MS) experiments.

To date, only a handful of researchers have published mass spectrometric analyses of copper-binding ligands in seawater. Two potential Cu ligands were identified in Cu(II)-IMAC fractions of Pacific coastal seawater samples analysed by ESI-MS (Ross *et al.*, 2003). These masses, 259 and 265 Da, correspond to small peptides with amino and thiol functionalities, respectively. Other potential molecular formulae of copper-binding compounds in DOM have been produced using high-resolution MS analysis (Boiteau *et al.*, 2016). Detection of these potential ligands can be enhanced using pre-concentration steps such as solid-phase extraction (SPE). For example, the sorbents HLB, PPL, and C18 were shown to recover ~10% of the Cu-binding portion of DOM in seawater samples (Waska *et al.*, 2015). It is possible that recovery could be improved using pre-concentration steps such as IMAC for extraction of a less complex and more concentrated sample.

MS has also been applied to the detection of organic compounds complexed with Cu in chromatographic extracts from artificial seawater cultures. Eluents of an XAD-16 resin were monitored for their capacity to complex Cu using pseudo-polarography; one of the eluents containing a strong copper-binding ligand also contained a Cu complex of *m/z* 697 (Wiramanaden *et al.*, 2008). More recently, high-resolution MS has been used to identify two potential Cu ligands (*m/z* 696 and 720) produced in response to copper stress in separate cultures of *Synechococcus*, *Prochlorococcus*, *Crocosphaera*, and *Trichodesmium* (Lydia Babcock-Adams, presented at OSM 2020).

Evaluation of ESI-MS/MS for the analysis of marine Cu ligands in both natural and artificial seawater samples is described in Chapter 2 and Appendix 6.1 of this dissertation.

1.5.5 Distribution of copper ligands

Organic speciation of Cu has been estimated in all major ocean basins by various researchers. Geographical distributions (oceanic transects and depth profiles) offer potential insights into sources of copper ligands in seawater by assessing their covariance with biogeochemical measurements. Covariance of copper ligand content with indicators of primary productivity would indicate a biological source. Covariance with geochemical signatures – isotope ratios, rare earth elements, and so on – would allow more exhaustive investigations of ligand stability and circulation.

Most depth profiles of organic copper speciation have been obtained using ASV (Buckley & van den Berg, 1986; Coale & Bruland, 1988, 1990; Hasle & Abdullah, 1981) or its modern incarnation, CSV (Buck *et al.*, 2012; Heller & Croot, 2015; Moffett & Dupont, 2007; Thompson & Ellwood, 2014; Whitby *et al.*, 2018; Wong *et al.*, 2018, 2019). Other methods that have been applied to seawater to probe vertical profiles of copper ligands are C18 chromatography (Zhou & Wangersky, 1989), LE (Midorikawa *et al.*, 1990; Midorikawa & Tanoue, 1999; Moffett *et al.*, 1990; Moffett & Zika, 1987), ISE (Midorikawa *et al.*, 1990) and, as continued in this work, IMAC (Gordon, 1992; Midorikawa & Tanoue, 1996).

Distinct pools of copper ligands may be distributed according to different factors influencing their production and circulation. A depth profile of the upper 1000 m obtained by Cu(II)-IMAC in the N Atlantic showed the weaker (L_2) class of ligands to be evenly distributed at all depths while the stronger (L_1) class reached a maximum in shallow waters (Gordon, 1992). Similar profiles taken in the NE Pacific had significantly higher levels of L_2 below 50 m relative to surface waters (Whitby *et al.*, 2018). Other studies have found L_2 to be less variable than L_1 (Wong *et al.*, 2018).

Copper ligands have been detected in benthic waters deeper than 1500 m. Studies from the N Atlantic (Buckley & van den Berg, 1986; Zhou & Wangersky, 1989), N Pacific (Buck *et al.*, 2012; Moffett & Dupont, 2007), and Southern Ocean (Heller & Croot, 2015; Thompson & Ellwood, 2014) suggest that, even in the deepest parts of the ocean, the majority of dissolved copper is complexed by organic ligands. Copper ligands in these waters are likely allochthonous DOM, composed of stable compounds transported to depth by ocean currents. It is possible that benthic sources of copper ligands exist, perhaps near hydrothermal vents, but this will not be further explored here.

Distributions of copper ligands have been frequently associated with dissolved copper levels. Many studies have shown a covariance between copper ligand levels and total dissolved Cu concentrations (Whitby *et al.*, 2018; Wong *et al.*, 2018, 2019), leading to suggestions that ligands are released by natural phytoplankton communities as a copper detoxification strategy.

Relatively few studies have examined the horizontal distribution of copper ligands along oceanic transects (Coale & Bruland, 1990; Wong *et al.*, 2018, 2019; Zhou & Wangersky, 1989), or changes in copper ligand content over time, across seasons and years. One study showed copper ligands have little diurnal variation, but high variability and high seasonal dependence, reaching a maximum during a picoplankton bloom in the late summer (Gordon *et al.*, 1996).

This dissertation presents distribution profiles of marine copper ligands as measured by Cu(II)-IMAC in samples collected during a scientific cruise in the Canadian Arctic (Chapter 3) and four cruises in the NE Pacific (Chapter 4).

1.5.6 Culturing studies

Copper ligand distribution studies have implicated phytoplankton as a potential ligand source in natural seawater, and these studies have spurred interest into algal culturing. Strong ligands have been shown to reach a maximum coincident with depths of highest biological productivity (Gordon, 1992; Gordon *et al.*, 1996). Highly productive waters also have the highest copper complexing capacity when measured by C18 (Elbaz-Poulichet *et al.*, 1994; Zhou & Wangersky, 1989) and by ligand exchange methods (Moffett *et al.*, 1990). A robust study of copper ligands in the Southern Ocean suggested a variety of phytoplankton sources (Thompson *et al.*, 2014). However, not all studies have linked copper ligands to primary productivity, suggesting other sources might play a significant role in some waters (Coale & Bruland, 1990).

Covariance of copper ligand pools with indicators of biological activity is not in itself proof that phytoplankton products are impacting organic copper speciation. Careful interrogation of axenic algal cultures offers a promising avenue for the characterization of copper ligand synthesis and function. Biological production can be demonstrated simply if an organism is shown to accumulate a ligand or ligand pool in culture media over a time course. Spiking ligands bound to radiolabeled ^{67}Cu into culture media has been used to probe copper uptake mechanisms (Semeniuk *et al.*, 2015). Unfortunately, these relatively simple analytical methods are compromised by a lack of meaningful structural information about natural ligands.

Rudimentary electrochemical analysis of algal culture media provided the earliest evidence that phytoplankton detoxify copper through the release of copper chelating ligands (McKnight & Morel, 1979; Swallow *et al.*, 1978). Iron limitation was also shown to induce copper ligand production in cultures of marine phytoplankton (McKnight & Morel, 1980).

A wide variety of microbes have since been implicated in copper ligand production, often in response to high copper levels. Cyanobacterial *Synechococcus* species have been shown to produce a ligand with affinity in the L_1 range in culture media (Croot *et al.*, 1999; Gordon *et al.*, 2000; Moffett *et al.*, 1990; Moffett & Brand, 1996). Diatoms such as *Ditylum brightwellii* (Gerringa *et al.*, 1995) and *Skeletonema costatum* (Morelli *et al.*, 1989), and brown algae such as *Fucus vesiculosus* (Gledhill *et al.*, 1999) produce ligands in the L_2 range in culture. A survey of a wide range of strains of *Emiliania huxleyi* found that the most Cu-tolerant strains produced the most copper-binding organic material (Echeveste *et al.*, 2018).

Many of these studies provide compelling evidence that copper speciation in the marine environment is significantly impacted by dissolved organic compounds of biogenic origin. Even when metal stress is shown to induce production of ligands, however, the mechanisms by which they are produced are unclear. Ligands could be direct products of a dedicated anabolic pathway, or lysis products, or inorganically photolinked aggregates, or cleavage/degradation products, and so on. Moreover, natural assemblages of phytoplankton in open oceanic ecosystems clearly have a wide variety of potential sources for copper ligands. However, it is unlikely that organic copper speciation is simply a result of the abundance and diversity of DOM, given that Cu ligands of relatively high-affinity tend not to vary in abundance with total DOM concentration.

1.5.7 Emerging data

Several research groups are actively studying organic copper speciation in seawater, largely driven by the international collaborative GEOTRACES program (Boiteau *et al.*, 2016; Buck *et al.*, 2012; Heller & Croot, 2015; Jacquot & Moffett, 2015; Nixon *et al.*, 2019; Whitby *et al.*, 2018). A recent electrochemical analysis of seawater from the NE Pacific detected a class of particularly high binding affinity compounds that the authors suggest are potential chalkophores (Whitby *et al.*, 2018). High-resolution mass spectrometry was used in another study to identify molecular formulas of potential Cu^{2+}-binding natural organic compounds across a broad library of samples (Boiteau *et al.*, 2016).

An intercomparison study examining methods to measure copper speciation in seawater samples found freezing samples may disrupt ambient speciation and thereby interfere with downstream analysis (Buck *et al.*, 2012). Such disruptions may or may not decrease recovery of ligands by Cu(II)-IMAC, even if derivative breakdown products of those ligands would otherwise be recovered by SPE.

Copper uptake experiments have been conducted using radiolabeled ^{67}Cu and phytoplankton cultures. Diatom culture studies investigating copper acquisition have suggested organically bound copper may actually be bioavailable, that diatoms have both low-affinity and high-affinity Cu transport systems, and that these uptake systems require Fe and are distinct between coastal and oceanic species. (Guo *et al.*, 2010). Uptake of copper by natural assemblages from Line-P in the N Pacific was shown to depend on the presence of synthetic and natural ligands, rather than on free Cu^{2+} as historically believed (Semeniuk *et al.*, 2009, 2015). These studies also showed that copper uptake is particularly significant in small phytoplankton, and that distinct ligand pools can have differential effects on eukaryotes relative to prokaryotes. It was also hypothesized that copper uptake may involve reduction of Cu(II) prior to uptake.

1.6 Dissertation Summary

Identification of the copper-binding ligands present in seawater and marine microbial cultures remains key to addressing critical questions concerning the biological uptake, geochemical cycling, and ecological significance of Cu in the ocean. Meeting this challenge through the development and validation of new methodology for recovering and profiling marine Cu ligands is the central theme of this dissertation.

Chapter 2 presents an evaluation of Cu(II)-IMAC-SPE-MS/MS for the recovery and analysis of marine organic copper ligands.

These validated Cu(II)-IMAC protocols are applied in Chapter 3 to natural seawater samples taken from the Canadian Arctic Ocean to assess the distribution of copper ligands.

Cu(II)-IMAC is used in Chapter 4 to analyze Pacific Ocean samples collected over a multi-year study, presenting distribution profiles for copper ligands and their covariance with other data.

Appendix 6.1 describes further efforts to refine Cu(II)-IMAC-SPE-HPLC-MS/MS methods for structural characterization of natural copper ligands. Appendix 6.2 contains preliminary results of phytoplankton culturing for copper ligand production and analysis.

1.6.1 Research questions and hypotheses

Research into the speciation of copper in the marine environment is currently limited by a lack of structural information about the organic complexes involved. Developing a reproducible method to investigate the chemical functionalities or molecular identities of any of these compounds would significantly accelerate the study of their source, distribution, and ecological roles. Several hurdles stand in the way of identification, namely the low concentration and diversity of copper-binding ligands and the high ionic strength and heterogeneity of seawater. The first question explored in this dissertation – whether a series of protocols that isolate and characterize natural copper ligands in seawater can be developed – seeks to address this knowledge gap and overcome those hurdles. Separation techniques including Cu(II)-IMAC and SPE are known to isolate and concentrate strong copper ligands for downstream analysis, and detection methods including UV spectrophotometry and mass spectrometry are known to provide meaningful information about the chemical structures of copper ligands. Thus, the first hypothesis of this dissertation is:

> *Some of the dissolved organic compounds contributing to Cu(II) speciation in seawater can be characterized and mapped using appropriate chromatographic and spectroscopic techniques.*

There is strong evidence that Cu(II) in natural seawater is mostly found in organic complexes, and that marine phytoplankton in artificial seawater cultures produce strong copper-binding ligands. Meaningful characterization of ligands in natural waters is mostly limited to electrochemical methods that measure concentration and binding affinity without elucidating chemical functionalities or molecular identities. Such data are insufficient to answer the second research question explored in this dissertation - whether phytoplankton influence the speciation of copper in the marine environment. Few studies prior to the beginning of this research project had attempted to answer this question. However, if a protocol can be developed that reproducibly provides data on ligand functionality or structure, the impact of marine phytoplankton communities on organic copper speciation could be demonstrated by assessing the covariance of copper ligands with biological indicators such as chlorophyll content or microbial taxonomy. If these same techniques could demonstrate the production of similar ligands in artificial cultures then the production of ligands in response to copper stress would strongly suggest a role in copper homeostasis. Thus, the second hypothesis of this dissertation is:

> *Some of the dissolved organic compounds contributing to Cu(II) speciation in seawater are phytoplankton products that impact primary productivity by regulating copper cycling.*

1.6.2 Approach and challenges

Successful recovery of unidentified copper ligands from seawater, as required by the first hypothesis, necessitates the development of analytical methods capable of meaningfully characterizing a diverse set of potential compounds. While Chapter 2 describes development of a protocol that allows accurate quantitation of a model copper-binding compound spiked in seawater at a low level, only a single ligand is examined. Appendix 6.1 details the continued development of Cu(II)-IMAC for multiplexed recovery of a variety of model ligands from seawater and quantitative analysis by HPLC-MS/MS.

Data from Chapter 2 regarding the analysis of natural copper ligands suggested Cu(II)-IMAC alone may provide meaningful information about organic copper speciation in samples taken from distinct sampling sites. It became apparent that the measured chromatographic peak area for seawater samples from one location was a reproducible value distinct from samples taken elsewhere. This suggests that spectrophotometry of fractionated seawater could meaningfully characterize the presence of an operationally defined subset of natural organic copper ligands (Fig. 1.1), partially addressing the first hypothesis. Chapter 3 describes the distribution of this subset of copper ligands along an oceanic transect covering the Canadian Arctic Ocean based on Cu(II)-IMAC data, while Chapter 4 describes their distribution along a transect of the Pacific Ocean.

Biological origins and biological influences of copper ligands – two aspects of the second hypothesis – would result in covariance of copper ligand distribution with biological indicators. To probe the potential sources and sinks of copper ligands in the Arctic and Pacific Ocean, relationships between copper ligand distribution and other biogeochemical data are examined in Chapters 3 and 4.

Preliminary data from experiments into culturing diatoms for the production of copper ligands are presented in Appendix 6.2. A well-defined artificial seawater culture media is used to demonstrate the effects of copper toxicity and copper limitation on growth rates. A technique is being refined to remove EDTA from cultures for subsequent recovery and analysis of copper ligands by Cu(II)-IMAC.

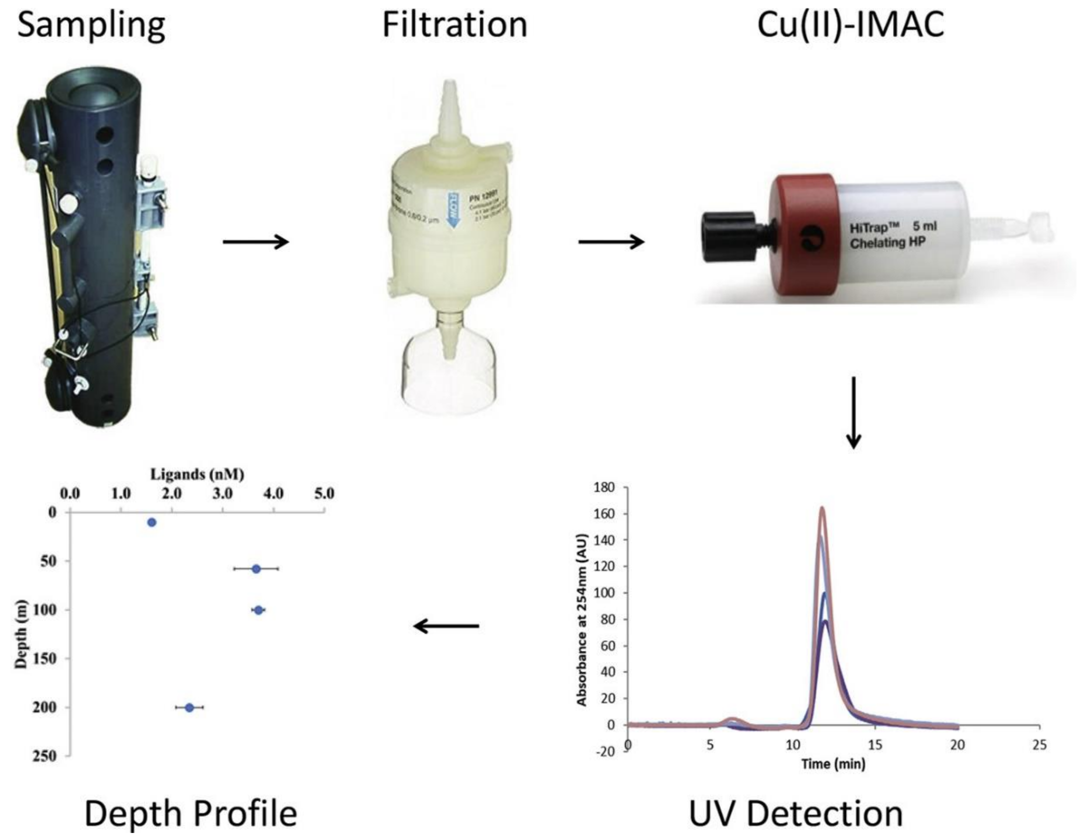


Figure 1.1 Probing the distribution of marine copper ligands with Cu(II)-IMAC. Seawater samples are collected using a trace-metal-clean rosette, gravity-filtered, and immediately frozen. Samples are thawed and fractionated by Cu(II)-IMAC, and peak areas from the resulting chromatograms are used to estimate copper ligand concentration in natural waters.

2.0 Evaluation of immobilized metal-ion affinity chromatography and electrospray ionization tandem mass spectrometry for recovery and identification of copper(II)-binding ligands in seawater using the model ligand 8-hydroxyquinoline

Adapted from: Nixon, R. L., & Ross, A. R. S. (2016). Evaluation of immobilized metal-ion affinity chromatography and electrospray ionization tandem mass spectrometry for recovery and identification of copper(II)-binding ligands in seawater using the model ligand 8-hydroxyquinoline. *Frontiers in Marine Science*. https://doi.org/10.3389/fmars.2016.00246

Contributions: I wrote the manuscript and performed the data analysis and all experiments. I mentored co-op students Jose Campos and Crystal Sommer, who contributed towards the development of MS/MS protocols.

2.0.1 Chapter abstract

Complexation by organic ligands dominates the speciation of iron (Fe), copper (Cu), and other bioactive trace metals in seawater, controlling their bioavailability and distribution in the marine environment. Several classes of high-affinity Fe-binding ligands (siderophores) have been identified in seawater but the chemical structures of marine Cu-complexing ligands remain unknown. Immobilized metal-ion affinity chromatography (IMAC) allows Cu ligands to be isolated from bulk dissolved organic matter (DOM) in seawater and separated into fractions that can be characterized independently using electrochemical and spectroscopic techniques. Attempts have been made to combine IMAC with electrospray ionization mass spectrometry (ESI-MS) to characterize marine Cu ligands, but results have proven inconclusive due to the lack of tandem mass spectrometry (MS/MS) data to confirm ligand recovery. We used 8-hydroxyquinoline (8-HQ), a well-characterized model ligand that forms strong 1:2 metal:ligand complexes with Cu^{2+} at pH 8 ($\log \beta_2 = 18.3$), to evaluate Cu(II)-IMAC and ESI-MS/MS for recovery and identification of copper(II)-complexing ligands in seawater. One-liter samples of 0.45 μm-filtered surface seawater were spiked with 8-HQ at low concentrations (up to 100 nM) and fractionated by IMAC. Fractions eluted with acidified artificial seawater were desalted and re-suspended in methanol via solid-phase extraction (SPE) to obtain extracts suitable for ESI-MS analysis. Recovery of 8-HQ by Cu(II)-IMAC was confirmed unambiguously by MS/MS and found to average 81% based upon accurate quantitation via multiple reaction monitoring (MRM). Cu(II)-IMAC fractionation of unspiked seawater using multiple UV detection wavelengths suggests an optimal fraction size of 2 mL for isolating and analyzing Cu ligands with similar properties.

2.1 Introduction

Organic complexation of dissolved trace metals like iron (Fe) and copper (Cu) exerts a major influence on their mobility, toxicity, and bioavailability in the marine environment (Bruland *et al.*, 2000; Florence, 1982; Kraemer *et al.*, 2015; Sunda, 1988). Several classes of biogenic high-affinity Fe-binding ligands (siderophores) have been identified in seawater and are thought to facilitate iron uptake in marine bacterioplankton (Hutchins *et al.*, 1999; McCormack *et al.*, 2003). Copper-carrying analogs (chalkophores) such as methanobactin (log KCuL ~20; (El Ghazouani *et al.*, 2011)) are known to be produced by certain methanotrophic bacteria (Bandow *et al.*, 2012; Kim *et al.*, 2004). Culturing experiments have shown that marine phytoplankton are also capable of producing strong, extracellular Cu-binding ligands (Croot *et al.*, 2000; Dupont *et al.*, 2004; Dupont & Ahner, 2005; Leal *et al.*, 1999; McKnight & Morel, 1979, 1980; Moffett *et al.*, 1990; Moffett & Brand, 1996; Wiramanaden *et al.*, 2008). Some of these exudates are thought to act like chalkophores (Semeniuk *et al.*, 2015), promoting biological uptake and utilization of Cu for e.g., photosynthesis, radical detoxification, and iron acquisition (Maldonado *et al.*, 2006). Others may be released to moderate concentrations of free, bioavailable Cu^{2+} ions (Dryden *et al.*, 2004; Leão *et al.*, 2007) that would otherwise be toxic to marine phytoplankton. Although numerous studies have shown that Cu in seawater exists primarily in the form of organic complexes (Vraspir & Butler, 2009), no marine copper ligands have been positively identified.

Originally developed for protein isolation, immobilized metal-ion affinity chromatography (IMAC) was first applied to the study of marine Cu-complexing ligands in 1992 (Gordon). The principal advantage of Cu(II)-IMAC is that it enables compounds with an affinity for copper to be isolated from other organic compounds that could interfere with their analysis. IMAC is also capable of resolving compounds into fractions with differing affinities for copper, allowing electrochemical or spectroscopic analysis to be applied specifically to each fraction. Gordon successfully demonstrated concentration and partial purification of Cu-complexing organic compounds from the Sargasso Sea using IMAC with UV detection, resolving these compounds into two chromatographic peaks or fractions based on their relative affinities for copper. Vertical profiles showed that the lower-affinity (earlier-eluting) peak was invariant with depth whereas the higher-affinity (later-eluting) peak reached a maximum at 60 m. Subsequent applications of Cu(II)-IMAC to natural waters (Donat *et al.*, 1997; Gordon *et al.*, 1996, 2000; Midorikawa & Tanoue, 1996, 1998; Paunovic *et al.*, 2005, 2008; Wu & Tanoue, 2001) employed UV absorbance, fluorescence, chemical analysis, and electrochemical methods such as differential pulse anodic stripping voltammetry (DPASV) to characterize the recovered ligands. Two classes of marine Cu-complexing ligands have been distinguished: a stronger (L_1) class (log K′ = 12 to 14) present at relative low concentrations (below 10 nM) and found primarily in surface waters, and a weaker (L_2) class (log K′ = 9 to 12) found at higher concentrations (above 100 nM) and more broadly distributed in the water column. Donat *et al.* (1997) demonstrated removal of L_1 and L_2 from seawater by Cu(II)-IMAC and detection of L_1 in pooled IMAC eluents. More recently, Cu(II)-IMAC has been used in combination with electrospray ionization mass spectrometry (ESI-MS) to isolate and identify intracellular copper-binding proteins from marine algal cells (Smith *et al.*, 2014), some of which (e.g., chaperones) are thought to bind Cu^+ in vivo.

Early attempts to characterize marine Cu-binding ligands using Cu(II)-IMAC and ESI-MS were successful in obtaining molecular weight information from seawater extracts (Ross *et al.*, 2003; Vachet & Callaway, 2003), paving the way for structural analysis using tandem mass spectrometry (MS/MS) (Wiramanaden *et al.*, 2008). However, results obtained using ESI-MS alone were insufficient to positively identify or optimize the recovery of marine Cu ligands. The latter is important given the typically low concentrations at which organic ligands are present in seawater, and the need for intermediate steps such as solid-phase extraction (SPE) to desalt and concentrate samples prior to MS analysis (Waska *et al.*, 2015). We describe a method for recovery and structural characterization of Cu(II)-complexing ligands in seawater using IMAC, SPE, and ESI-MS/MS. We validate our approach by demonstrating recovery and unambiguous identification of a model organic Cu-binding ligand from seawater at low concentrations (below 100 nM). The compound chosen was 8-hydroxyquinoline (8-HQ), a well-characterized model ligand that is amenable to ESI-MS and forms strong 1:2 metal:ligand complexes with Cu^{2+} at pH 8 ($\log \beta_2 = 18.3$; (Ross, 1998)). The ability of 8-HQ to form uncharged, lipophilic complexes makes it a viable model for biological metal-ion carriers as well as for marine Cu-complexing ligands. We also present Cu(II)-IMAC data for oceanic and coastal surface waters using multiple detection wavelengths, which enable fractionation of marine Cu ligands based on UV absorbance and IMAC retention times.

2.2 Materials and methods

2.2.1 Reagents

All reagents and solvents were analytical and high-performance liquid chromatography (HPLC) grade, respectively, unless otherwise noted. Hydrochloric acid (12N) was obtained from Anachemia (Vancouver, BC), acetonitrile and methanol from VWR (Mississauga, ON), and ethylenediamine tetraacetic acid (EDTA) and ammonium acetate from Fisher (Pittsburgh, PA). Copper (II) sulfate (99.995% trace metal free), 8-hydroxyquinoline, glutathione, histidine, and tryptophan were obtained from Sigma-Aldrich (St. Louis, MO). Ultrapure deionized water (16–18 MΩ-cm) was prepared using an EMD Super-Q system from Millipore (Billerica, MA). Artificial seawater (ASW) was prepared by dissolving 32 g of Instant Ocean (Blacksburg, VA) in 1 L of ultrapure water, and acidified (aASW) by adding HCl to a concentration of 10 mM (pH 2.1).

2.2.2 Collection of seawater samples

Natural seawater was collected from inshore coastal and open ocean surface waters of the Neast Pacific. Oceanic surface seawater (OSW) was collected at a depth of 5 m near station P22 (49° 41.92′ N, 140° 39.98′ W) during the June 2015 Line-P cruise aboard the CCGS John P. Tully and transferred directly to a 50-gallon plastic drum using a high-volume Teflon® pump (model PFD1 210, Saint-Gobain ASTI, Nanterre, France). Coastal surface seawater (CSW) was collected in plastic tubs lowered by hand from the dock at the Institute of Ocean Sciences (IOS), Sidney, British Columbia during the spring and summer of 2015. Samples from both sources were stored unfiltered in the dark at room temperature, and were pumped through a 0.45-μm filter (GWV high-capacity groundwater sampling capsule, Pall Corporation, Port Washington, NY) prior to any treatment or analysis.

2.2.3 Immobilized copper(II)-ion affinity chromatography

Cu(II)-IMAC involves passing filtered aqueous samples through a chelating column charged with Cu^{2+} ions, which are immobilized by tethered functional groups such as iminodiacetate (IDA) or nitrilotriacetate (NTA). Compounds with an affinity for divalent copper are selectively retained on the column, then eluted and recovered using an acidic or competing ligand solution. We performed Cu(II)-IMAC fractionations using two systems. The first was a manually operated dual-column system built in-house during previous studies (Ross *et al.*, 2003) utilizing a single wavelength UV detector (LKB Bromma Uvicord S, Pharmacia Biotech, Uppsala, Sweden), henceforth referred to as the LKB system. The second system was an automated single-column commercial unit developed primarily for protein purification (AKTA PrimePlus, GE Healthcare, Mississauga, ON), which we refer to as the AKTA system. This system was supplied with multiple UV filters and software (PrimeView v5.31) for chromatographic analysis. Both systems were fitted with 5-mL Hi-Trap Chelating Sepharose HP columns (part no. 17-0409-03, GE Healthcare).

The same experimental protocol was applied to each system, all reagents and samples being delivered at a flow rate of 0.9 mL/min (LKB) or 1.0 mL/min (AKTA). Seawater samples and the solutions used to condition, charge, elute and regenerate the IMAC columns were prepared in 1-L acid-washed high-density polyethylene (Nalgene®) bottles. After rinsing with 30 mL of deionized water the Hi-Trap columns were charged with 50 μmoles of Cu^{2+} ions by passing 5 mL of a 10 mM copper(II) sulfate solution through each column. The columns were rinsed with 5 mL of ultrapure water and equilibrated with 20–30 mL of acidified artificial seawater (aASW). One-liter samples of natural or artificial seawater, with or without added model ligands, were then passed through each column. When the sample containers were nearly empty, desorption of retained compounds was achieved by eluting the columns with aASW for 20–30 min, during which the UV absorbance of the eluent was monitored at 254 nm (both systems), 214 or 280 nm (AKTA only). Eluent fractions corresponding to major chromatographic peaks were collected in glass scintillation vials (Wheaton, Millville, NJ) and stored at 5°C. The absorbance spectra of selected IMAC fractions were obtained using a UV-Vis spectrophotometer (model 8453, HP Agilent, Mississauga, ON) to confirm the extraction of model ligands. Following elution the IMAC columns were regenerated by passing 50 mM EDTA in ultrapure water through each column for 15 min to remove the Cu^{2+} ions before repeating the procedure, at all times taking care to avoid entraining air into the system.

2.2.4 Solid-phase extraction

We used SPE for off-line desalting of IMAC fractions as this method has the potential to recover unknown organic compounds of varying chemical composition. SPE was performed using a RapidTrace Workstation (Caliper Life Science, Hopkinton, MA) and Oasis 3 cc HLB (hydrophilic-lipophilic) cartridges (Waters Limited, Mississauga, ON). The cartridges contained a polymeric reversed-phase sorbent designed to extract a wide range of acidic, basic, and neutral compounds. HLB cartridges were conditioned with one volume (3 mL) of methanol and rinsed with 3 mL ultrapure water and 3 mL aASW (the IMAC eluent) before loading 2–4 mL of IMAC fraction. Loaded cartridges were rinsed with 3 mL aASW and 3 mL of 5% (v/v) methanol in ultrapure water before eluting with 2 mL methanol, which provided the highest concentration of recovered ligand. The cartridges were then regenerated with 3 mL of methanol and 5 mL ultrapure water, followed by a blank extraction using unspiked aASW to minimize carryover between IMAC fractions (see Section Characterization of Natural Ligands below). Extracts were collected in polypropylene culture tubes (VWR) and stored at 5°C prior to ESI-MS analysis. Selected extracts were also analyzed by ESI-MS to confirm and measure the recovery of model ligands using standards prepared in methanol.

2.2.5 Mass spectrometry

Model ligand standard solutions and SPE extracts were analyzed by flow-injection (FI) ESI-MS using a tandem quadrupole mass spectrometer (model API 5000, AB Sciex) fitted with an electrospray ion source (Turbo Ionspray; AB Sciex) connected to a high-performance liquid chromatography (HPLC) pump and autosampler (model P680 and ASI-100, Dionex). ESI-MS parameters were optimized while infusing a 0.68 μM solution of 8-HQ in the FI carrier solvent at 100 μL/min using a syringe pump (Harvard Instruments). The carrier solvent consisted of 1 mM ammonium acetate in 70% (v/v) aqueous acetonitrile (pH 7.6). Optimal ESI-MS conditions were: DP 120, EP 10, CAD 5, CUR 30, GS1 60, GS2 30, and IS 5500. Fragmentation of the 8-HQ molecular or precursor ion of mass-to-charge ratio (m/z) 146.2 to produce diagnostic fragment or product ions via collision induced dissociation (CID) tandem mass spectrometry (MS/MS) was optimized using the same solution. Two precursor-to-product ion transitions corresponding to loss of water (m/z 146.2 > 128.2) and formation of the tropylium ion (*m/z* 146.2 > 91.2) from 8-HQ were selected for quantification and confirmation, respectively, via multiple reaction monitoring (MRM) using the instrument software (Analyst v1.6.2). MRM provides specific, sensitive, and accurate quantification of target compounds during FI (or on-line HPLC) analysis. Optimal MRM conditions were CE 40, CXP 19 (146.2 > 91.2), and CE 35, CXP 24 (146.2 > 128.2). Standard solutions containing 0.5–50 μM 8-HQ were prepared by serial dilution of a stock solution containing 100 μM 8-HQ in methanol (the SPE eluent). The system was calibrated for FI-ESI-MS/MS analysis of SPE extracts using the HPLC pump and autosampler to deliver 10 μL of each 8-HQ standard to the ESI source at flow rate of 100 μL/min using the FI carrier solvent. Refrigerated SPE extracts were allowed to equilibrate at room temperature, filtered through a 0.22-μm syringe filter (part no. 179718, Canadian Life Science, Peterborough ON), transferred to 2-mL glass vials (Sigma), loaded into the autosampler, and analyzed by MRM under the same conditions as the calibration standards.

2.2.6 Treatment of seawater samples

The properties of natural copper-binding compounds were investigated using data from replicate Cu(II)-IMAC experiments. Retention times and peak areas for coastal and oceanic surface seawater samples were compared using different UV detection wavelengths (214, 254, and 280 nm) before and after different treatments. The photochemical and thermal stabilities of natural ligands were examined by placing oceanic seawater samples in water-cooled 1-L PTFE containers within an artificial sun chamber (~700 W/m2; Suntest CPS, Atlas, Mount Prospect, IL) designed to reproduce the spectrum of natural sunlight, with minimal transmission below 280 nm, or immersing and heating them in a water bath (~60°C; Isotemp 3016D, Fisher Scientific) for up to 24 h prior to IMAC. The effects of ionic strength and prior complexation on the recovery of natural ligands were also examined by decreasing salinity with ultrapure deionized water, increasing it to approximately 38‰ with artificial sea salt (Instant Ocean), or spiking the seawater sample with copper(II) sulfate to a concentration of 100 nM and allowing it to equilibrate for several hours before IMAC fractionation.

2.3 Results

2.3.1 Immobilized copper(II)-ion affinity chromatography

UV spectrophotometric analysis of 8-HQ standards and OSW samples spiked with 8-HQ showed that the model ligand absorbs strongly at 254 nm, and that the absorbance at this wavelength is proportional to ligand concentration. Using this wavelength to monitor the elution of Cu(II)-IMAC extracts from OSW samples spiked with up to 100 nM 8-HQ produced chromatograms with two significant peaks (Fig. 2.1). The earlier-eluting peak remained essentially unchanged whereas the later-eluting peak increased in area with increasing 8-HQ concentration ($r^2 = 0.99$).

Chromatograms obtained during Cu(II)-IMAC of ASW spiked with 8-HQ contained only the later-eluting peak, while UV absorbance spectra of collected fractions corresponding to this peak matched those of standards contained 8-HQ in aASW (the IMAC eluent).

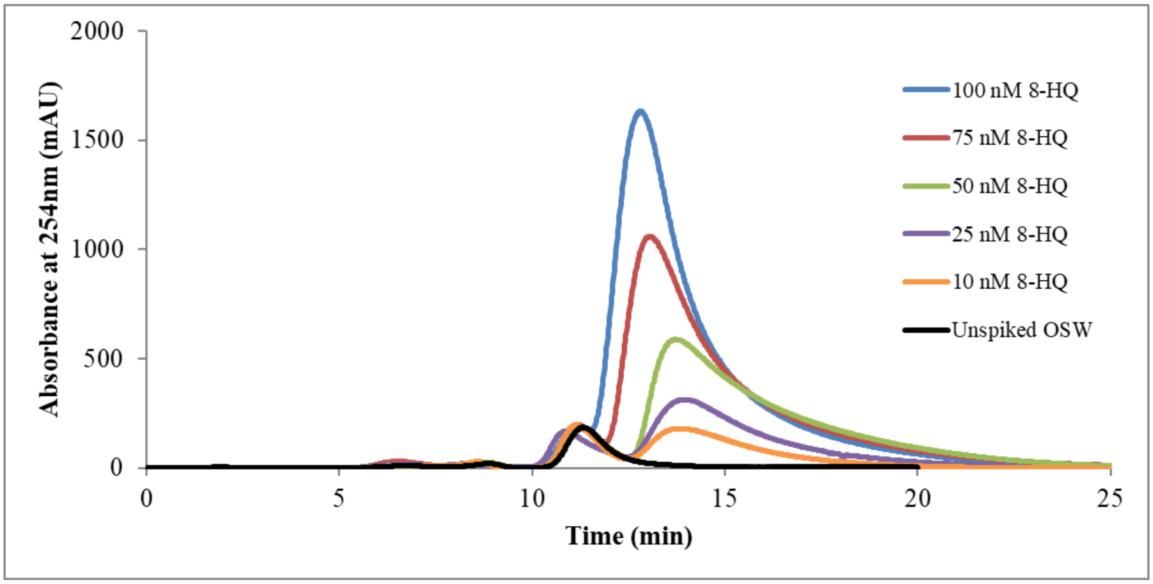


Figure 2.1 Immobilized copper(II)-ion affinity chromatography (Cu(II)-IMAC) of oceanic surface seawater (OSW) spiked with the model ligand 8-hydroxyquinoline (8-HQ). One-liter OSW samples containing up to 100 nmoles of added 8-HQ were fractionated by Cu(II)-IMAC while monitoring UV absorbance of the eluent at 254 nm using the LKB system (see text). All samples gave rise to a peak at 11 min, attributed to natural copper ligands present in OSW, whereas samples spiked with 8-HQ gave rise to a second elution peak at 12–14 min with area proportional to the amount of ligand added to the OSW sample ($r^2 = 0.9927$).

Our decision to elute the IMAC column with acidified seawater (Midorikawa & Tanoue, 1996) was based on the need to avoid introducing large concentrations of a competing ligand such as glycine, which would interfere with subsequent ESI-MS analysis, while ensuring that our results could be used to validate those of earlier experiments performed using the same eluent (Ross *et al.*, 2003). Previous studies suggest that eluting Cu(II)-IMAC columns with acidic solution may displace copper from the column, making it available to form complexes with ligands recovered in the eluent. Because this may affect the quantification of 8-HQ in IMAC fractions using UV absorbance spectrophotometry we relied upon specific, accurate quantification by MRM (see Section Electrospray-Ionization Mass Spectrometry below) to estimate the recovery of model ligands during IMAC.

2.3.2 Solid-phase extraction

Since marine Cu ligands have not yet been identified we chose to evaluate a type of SPE cartridge (Waters Oasis HLB) designed to recover as broad a range of organic compounds as possible. SPE conditions were optimized using FI-ESI-MS/MS with MRM to quantify 8-HQ in methanol extracts collected following SPE of 10 μM 8-HQ standards prepared in 2 mL of aASW (the IMAC eluent). The dependence of SPE recovery on elution volume was investigated by using different amounts of methanol (0.5–3.0 mL) to elute the HLB cartridge, and MRM to quantify the amount of 8-HQ in the resulting extracts. Recoveries ranged from less than 10% using 0.5 mL to 90% using 3 mL of methanol. However, the highest concentration of 8-HQ in the SPE extract was obtained using the recommended elution volume of 2 mL (Waters Corporation, 2014), which gave an average recovery (n = 4) of 74.4 ± 2.3 %. Rinsing the eluted HLB cartridge with methanol did not appear to retrieve any residual 8-HQ. During preliminary experiments, however, recoveries obtained after extracting several samples using the same cartridge were found to be greater than those obtained using a new cartridge. Carryover was henceforth minimized by performing blank extractions with 2 mL of unspiked aASW between samples. Cu(II)-IMAC fractions were desalted and concentrated for FI-ESI-MS/MS analysis using the optimized SPE protocol, applying a value of 74.4% to correct for recovery during SPE (Table 2.1).

Table 2.1 Recovery of the model ligand 8-hydroxyquinoline (8-HQ) from seawater by immobilized copper(II)-ion affinity chromatography (IMAC) and solid-phase extraction (SPE).

8HQ added to SW sample (nmoles)	8HQ measured in SPE extract (nmoles)	8HQ corrected for SPE recovery	% of IMAC fraction used for SPE	8HQ in IMAC fraction (nmoles)	% added 8HQ in IMAC fraction	% total IMAC peak in IMAC fraction	% added 8HQ recovered in IMAC peak
25	8.0	10.8	46	23.5	94.0	95	96.0
25	8.2	10.8	53	20.4	81.8	97	84.3
50	12.5	16.8	46	36.5	73.0	99	73.8
100	56.2	75.5	100	75.5	75.5	90	83.9
				Mean	81.1		85.2

2.3.3 Electrospray-ionization mass spectrometry

ESI-MS analysis of 8-HQ in methanol detected the protonated molecular ion at *m/z* 146.2. This was fragmented inside the collision cell of the mass spectrometer to generate a characteristic product-ion MS/MS spectrum for the model ligand (Fig. 2.2). Two discrete and relatively abundant fragments (*m/z* 128.2 and 91.2) were selected for MRM. The instrument was first calibrated using standards containing 0.5–50 μM 8-HQ in methanol (the SPE eluent). These were analyzed in quadruplicate and the results averaged to generate a linear plot (r^2 = 0.9794) of

MRM peak area vs. 8-HQ concentration. The lower limit of quantification (LLOQ) was determined by performing replicate analyses (n = 4) of the solvent blank, calculating the standard deviation of these measurements, multiplying by 10, and adding this value to the average blank response. The result was substituted into the linear calibration equation to determine a LLOQ of 0.54 μM.

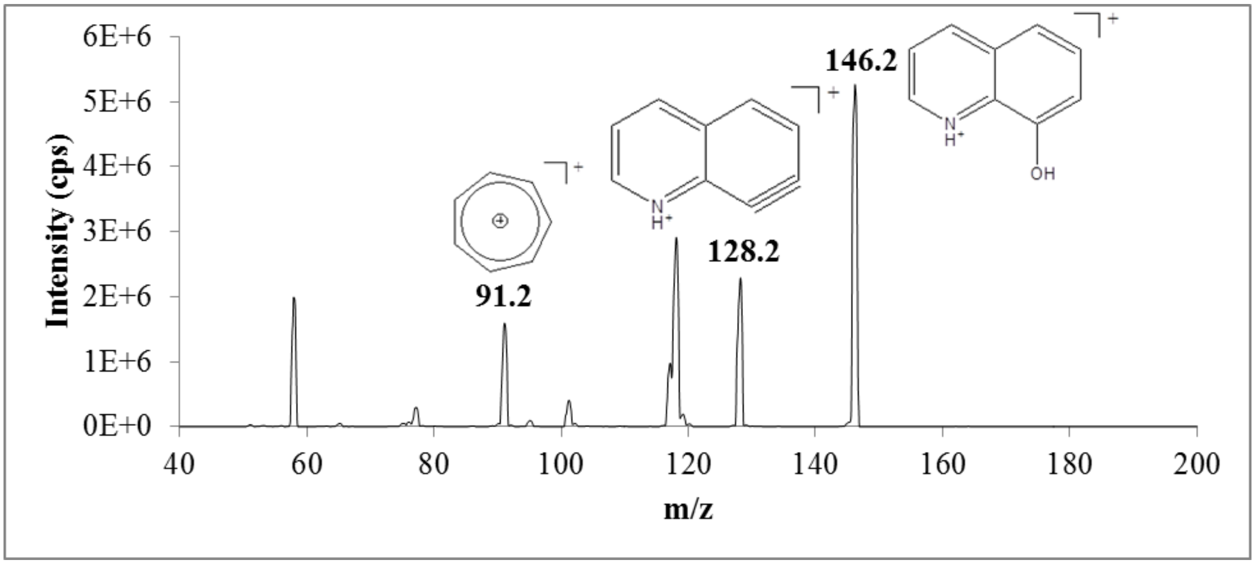


Figure 2.2 Product-ion mass spectrum of the protonated molecular/precursor ion (*m/z* 146.2) of 8-hydroxyquinoline (8-HQ) obtained by electrospray ionization-tandem mass spectrometry (ESI-MS/MS). Precursor-to-product ion transitions corresponding to loss of water (*m/z* 146.2 > 128.2) and formation of the stable tropylium ion (*m/z* 146.2 > 91.2) were selected for multiple reaction monitoring (MRM) to provide accurate quantification and unambiguous identification of 8-HQ in methanol extracts obtained by solid phase extraction (SPE) (see text).

The calibrated FI-ESI-MS/MS system was used to analyze Cu(II)-IMAC fractions obtained from 1-L seawater samples spiked with up to 100 nmoles 8-HQ (Fig. 2.3). Broadening of the IMAC peak at higher concentrations (Fig. 2.1) meant that the volume of the collected fraction varied from 1 to 10 mL, with a maximum of 4 mL being used for SPE. Variable volumes were accounted for by applying the appropriate corrections during calculation of the 8-HQ concentration in each IMAC fraction. For example, if a 1-L seawater sample spiked with 100 nmoles 8-HQ gave rise to a collected peak fraction of 8 mL, and 4 mL of that fraction were subjected to SPE, the measurement of 25 nmoles 8-HQ in the resulting SPE extract by MRM would indicate that 25% of the spiked ligand had been recovered in the SPE extract and 67% in the original IMAC fraction, following correction for 74.4% recovery during SPE (see Solid-Phase Extraction above). IMAC fractions collected from OSW spiked with 25–100 nmoles 8-HQ were subsequently found to contain an average of 81 ± 8% of the initial spike (Table 2.1). By applying a further correction based on the total area of the observed IMAC peak and the area corresponding to the fraction actually collected, it was possible to estimate a total recovery of 85 ± 9% for the initial Cu(II)-IMAC step (Table 2.1).

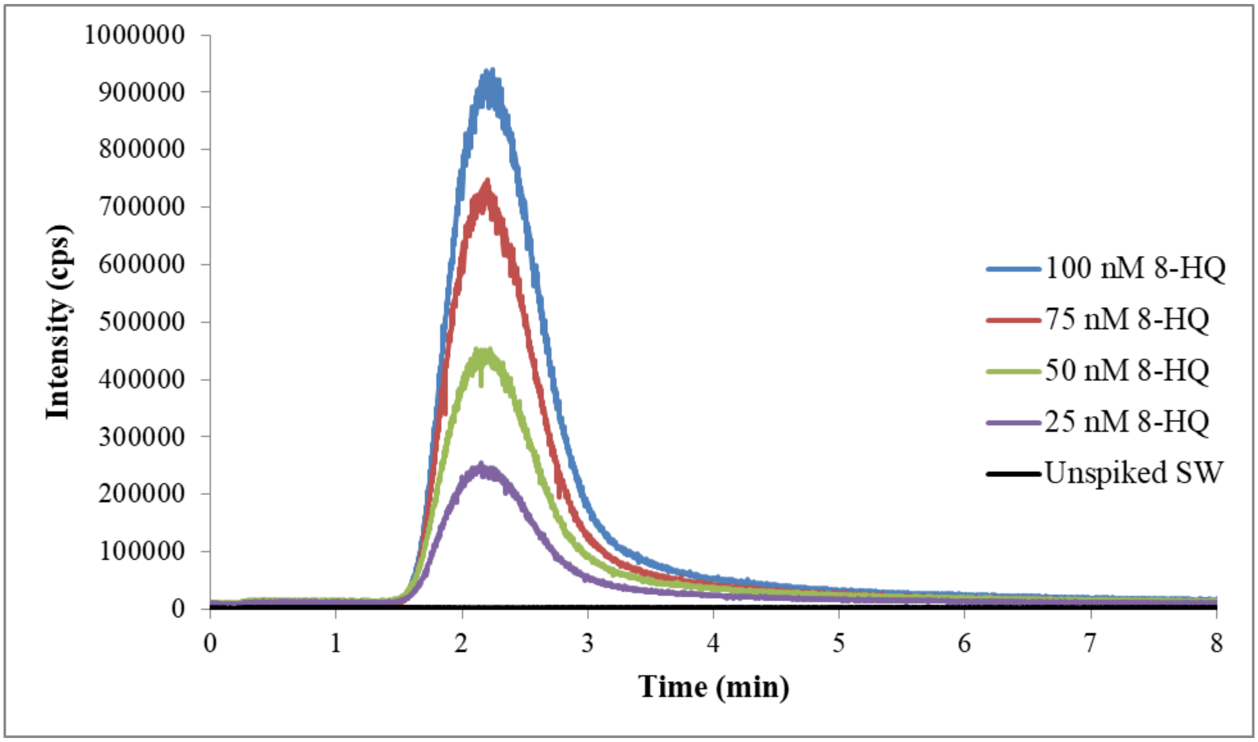


Figure 2.3 Multiple reaction monitoring (MRM) chromatograms for the *m/z* 146.2 > 91.2 transition used to quantify the model ligand 8-hydroxyquinoline (8-HQ) in Cu(II)-IMAC fractions desalted by solid-phase extraction (SPE). The fractions correspond to the model ligand peaks observed in the Cu(II)-IMAC chromatogram obtained by fractionating 1-L surface seawater samples spiked with 0–100 nmoles 8-HQ. Ten microliter of each SPE extract were analyzed by flow-injection electrospray ionization tandem mass spectrometry (FI-ESI-MS/MS) using 1 mM ammonium acetate in 70% acetonitrile at 100 µL/min as the carrier solvent.

2.3.4 Characterization of natural ligands

Cu(II)-IMAC was used to investigate the properties of natural copper ligands from coastal (CSW) and OSW by fractionating unspiked seawater samples and monitoring the IMAC eluent at different wavelengths (214, 254, or 280 nm) using the AKTA system. A representative IMAC chromatogram for CSW (Fig. 2.4A) shows that the natural ligands absorbing most strongly at each wavelength had different retention times, and that peak area was greater for ligands absorbing primarily at 214 nm than for those absorbing at 254 or 280 nm (although differences in molar absorptivity for individual chromophores preclude any inference as to relative ligand concentrations). Similar results were obtained for OSW samples; however, peak area was greater for CSW than for OSW samples (Fig. 2.4B). Retention times for extracted ligands that absorbed most strongly at a particular wavelength were consistent between samples, but those absorbing primarily at 280 nm had longer retention times (Fig. 2.4C). To investigate the thermal and photochemical stabilities of natural ligands, OSW samples were exposed to heat or artificial sunlight prior to Cu(II)-IMAC. Irradiation of the samples for 24 h decreased the area of the predominant IMAC peak by 40% whereas incubation at 60°C for 24 h without irradiation reduced peak area by 15%. In contrast, changing the salinity of seawater samples or spiking them with Cu^{2+} ions to a concentration of 100 nM had no significant effect on peak area.

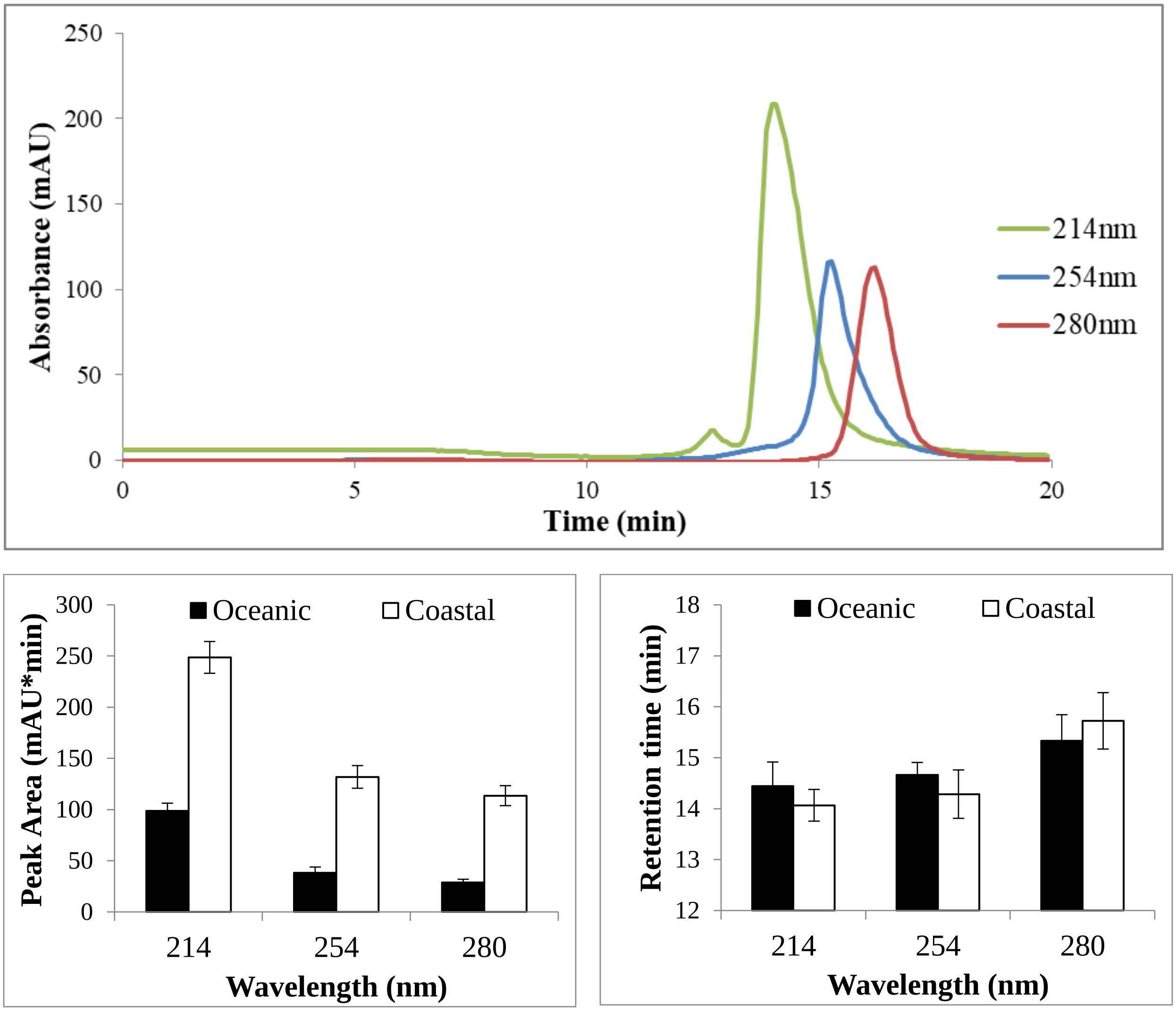


Figure 2.4 Immobilized copper(II)-ion affinity chromatography of coastal (CSW) and oceanic surface seawater (OSW) samples using the AKTA system (see text) to monitor the elution of replicate samples at 214, 254, and 280 nm. Top panel: extracted compounds that absorb most strongly at each wavelength were found to elute at different times. Left panel: peak areas were reproducible (n = 10; error bars represent 1 SD) and were consistently greater for coastal than for oceanic surface seawater. Right panel: retention times were significantly greater for compounds that absorbed most strongly at 280 nm but were no different for CSW than for OSW samples.

2.4 Discussion

Cu(II)-IMAC fractionation of surface seawater samples spiked with 8-HQ gave rise to a UV chromatographic peak with a retention time greater than that observed for natural Cu ligands (Fig. 2.1). The area of this peak was proportional to the mass of 8-HQ originally added to the seawater sample. Analysis of collected IMAC fractions by SPE and FI-ESI-MS/MS confirmed that IMAC fractions corresponding to the model ligand peak contained 8-HQ at a concentration proportional to that in the original sample, with an average recovery of 81%. To our knowledge this is the first time that a model Cu-binding ligand has been recovered, positively identified, and accurately quantified in seawater by MS/MS. Our results validate the previous assignment of measured molecular weights to marine Cu-complexing ligands using an earlier version of the same method (Ross *et al.*, 2003). The product-ion spectrum for 8-HQ (Fig. 2.2) also serves to illustrate the kind of structural information that MS/MS can provide for ligands isolated using this method. The order in which model and natural ligands elute from the Cu(II)-IMAC column is consistent with a higher stability constant for the Cu(II) complex of 8-HQ (see Section Introduction). Indeed, the greater binding strength of 8-HQ forms the basis for its use in determining total Cu concentrations in seawater by cathodic stripping voltammetry (CSV) and other techniques (Coale *et al.*, 1992; Kramer, 1986). Results obtained using 8-HQ suggest that natural Cu ligands of similar binding strength (log K′ ~18) may be recovered from seawater using Cu(II)-IMAC, assuming that they are able to form stable ternary complexes with IDA-Cu^{2+} (Paunovic *et al.*, 2005).

The LLOQ of 0.54 µM achieved using MRM is equivalent to 1.08 nmoles of 8-HQ in a 2-mL SPE extract. Assuming that 100% of the collected IMAC fraction is used for SPE this corresponds to an original concentration of about 1.8 nM in a 1-L seawater sample, based upon 74.4 % recovery during SPE and 81.1 % during IMAC. Although this is on the order of concentrations measured voltametrically for the stronger L1 class of marine Cu ligands, detection and analysis of a specific ligand by ESI-MS/MS will depend on its individual concentration in seawater. Should greater sensitivity be required the concentration of methanol extracts obtained by SPE can be increased by partial evaporation of the solvent prior to analysis. Pooling Cu(II)-IMAC fractions from replicate seawater samples could also be used to increase the quantity of isolated ligands.

Adding 100 nM copper(II) sulfate to spiked and unspiked OSW did not affect the area of the model or natural ligand peaks observed during IMAC, suggesting that prior complexation with Cu in seawater has no significant effect on ligand recovery (Fig. 2.5, see also Paunovic *et al.*, 2005). Similarly, reducing the sample flow to 0.5 mL/min produced only a slight increase in the areas of model and natural ligand peaks. This suggests that the rates of exchange within the IMAC column are sufficient to achieve good recovery of Cu ligands at 1.0 mL/min despite the relatively short contact time with the sample (~5 min), as indicated by results for 8-HQ (Table 2.1). Repeated fractionation of seawater samples has been shown to provide incremental increases in the recovery of natural ligands (Donat *et al.*, 1997). However, we found that running seawater samples through the column a second time produced a peak no more than 0.15 times as large as that obtained during the initial extraction, which nevertheless accounts for the incomplete recovery of 8-HQ observed during IMAC (Table 2.1).

Having evaluated Cu(II)-IMAC for recovery of Cu ligands we proceeded to investigate the fractionation of natural Cu ligands in unspiked CSW and OSW, using the AKTA system to monitor different UV wavelengths. The peak area at a given wavelength and retention time was always greater for coastal that for oceanic samples (Fig. 2.4B). Results obtained previously for Neast Pacific coastal waters, using the more sensitive LKB system showed that the IMAC peak corresponding to stronger Cu ligands reduced in size with increasing depth and distance from the mouth of the Fraser River. This is consistent with the production of strong biogenic Cu ligands in surface waters and with riverine input of organic ligands from terrestrial sources, both of which suggest that ligand concentrations are likely to be higher in CSW than in OSW samples. Chromatograms observed for CSW and OSW were consistent for a given wavelength and location, each chromatogram containing a single predominant peak. If this peak represented a single compound, or class of compounds, we would expect peak area to change according to the UV absorbance characteristics of the ligand(s) but the retention time to remain unaltered. This is exactly what we observed for the model ligand 8-HQ in spiked seawater (data not shown). For unspiked surface seawater, however, the retention time for the most abundant ligand peak changed with the detection wavelength (Fig. 2.4A,C).

Gordon *et al.* (1996) found that the main difference between the two major peaks observed during Cu(II)-IMAC of estuarine samples was that the later-eluting peak absorbed strongly at 280 nm, which they attributed to the presence of proteins or peptides containing aromatic amino acids. They also found that the size of the earlier-eluting peak was reduced by prior treatment with the hydrophobic resin XAD-16. This was attributed to the presence of humic substances, which are known to absorb strongly at 254 nm. The relative retention times of the peaks detected at 254 and 280 nm in our CSW and OSW samples appear to be consistent with these findings. However, we also observed an earlier-eluting peak using a detection wavelength of 214 nm (Fig. 2.4A), which is characteristic of the peptide bonds found in all proteins. Furthermore, of the two retained fractions observed by Paunovic *et al.* (2005) in terrestrial organic matter, the earliest-eluting fraction was attributed to peptides and proteins and the later-eluting fraction to aromatic substances, based on comparisons with model ligands. Recovery and analysis of these fractions using a specific detection method like ESI-MS/MS would help to resolve any uncertainties as to the nature of the ligands present. Our results for OSW and CSW suggest that using multiple detection wavelengths can assist in identifying such fractions, and that 2 mL is an appropriate fraction size for capturing ligands with similar UV absorption and Cu-binding characteristics (Fig. 2.4A,C).

Paunovic *et al.* (2005) suggest that IMAC is an effective tool for fractionating Cu ligands capable of forming ternary complexes with Cu^{2+} and IDA. Although our results for the bi-dentate ligand 8-HQ support this conclusion, we also performed Cu(II)-IMAC on OSW before and after spiking with the hexa-dentate model ligand EDTA, which absorbs at 214 nm in the IMAC eluant. The resulting (214 nm) UV chromatograms were identical, suggested that EDTA was unable to form stable ternary complex with IDA-Cu^{2+}. Previous studies have shown that both stronger (L_1) and weaker (L_2) natural ligands are recovered from seawater by IDA-Cu^{2+} (Donat *et al.*, 1997). However, results for EDTA suggest that recovery of natural multi-dentate ligands including, potentially, chalkophores like methanobactin (Kim *et al.*, 2004) may be limited by steric factors. Nevertheless, model natural ligands tryptophan, histidine, and glutathione were recovered from seawater by Cu(II)-IMAC during preliminary experiments, as indicated by the appearance of additional chromatographic peaks which increased in area with increasing ligand concentration (Chapter 6.1). The recovery and analysis of natural ligands by SPE and FI-ESI-MS/MS is currently being investigated, along with the use of phytoplankton cultures to produce Cu ligands for further method development and analysis.

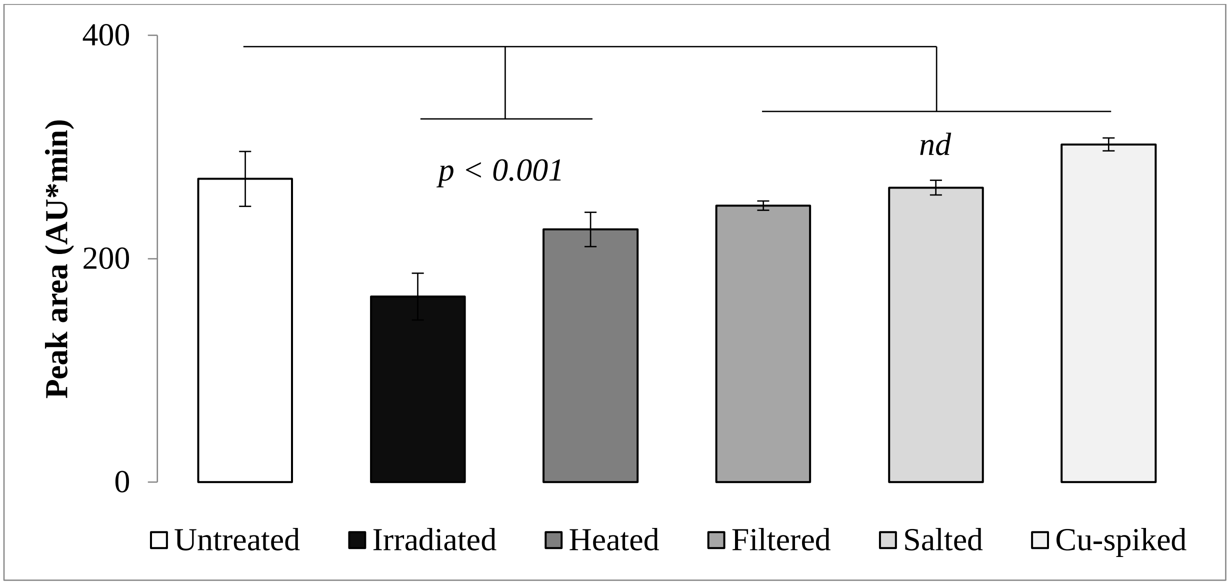


Figure 2.5 Sensitivity of Cu(II)-IMAC recoverable ligands to irradiation and other treatments. Seawater sample replicates were either: exposed to artificial UV light for 72 hours; heated to 60° C for 18 hours; filtered to 0.22 µm (instead of 0.45 µm); amended with salt to increase ionic strength; or spiked with Cu(II) to bind ligands. *nd*, no statistical difference

2.5 Conclusion

A method for isolating, identifying, and quantifying Cu(II)-complexing ligands in seawater has been developed. The method, which employs Cu(II)-IMAC fractionation, SPE recovery, and FI-ESI-MS/MS analysis, was evaluated using the strong model ligands 8-HQ and EDTA. An average recovery of 81% was obtained for the bi-dentate ligand 8-HQ, which was positively identified and quantified in seawater by MRM. The hexa-dentate ligand EDTA was not recovered by Cu(II)-IMAC, suggesting that natural multi-dentate ligands may be under-represented by this method. Cu(II)-IMAC of surface seawater samples using multiple detection wavelengths showed that natural Cu ligands can be resolved into fractions suitable for SPE based on UV absorption and retention time. Identification of these natural ligands by FI-ESI-MS/MS is currently being investigated.

3.0 Distribution of copper-complexing ligands in Canadian Arctic waters as determined by immobilized copper(II)-ion affinity chromatography

Adapted from: Nixon, R. L., Jackson, S. L., Cullen, J. T., & Ross, A. R. S. (2019). Distribution of copper-complexing ligands in Canadian Arctic waters as determined by immobilized copper(II)-ion affinity chromatography. *Marine Chemistry*. https://doi.org/10.1016/j.marchem.2019.103673

Contributions: I wrote the manuscript and participated in sample collection from all CB stations, CAA-8, and CAA-9. I conducted all Cu(II)-IMAC experiments and performed the data analysis.

3.0.1 Chapter abstract

Complexation by dissolved organic ligands affects the bioavailability and distribution of copper and other bioactive trace metals in seawater. However, relatively little is known about the origin and identity of marine copper-complexing ligands, particularly in Arctic waters. We used immobilized copper(II)-ion affinity chromatography (IMAC) to isolate dissolved (< 0.2 μm) copper ligands from seawater samples collected during the 2015 Canadian Arctic GEOTRACES expedition. UV detection at 254 nm was employed to monitor elution of the compounds retained by IMAC. The areas of the resulting peaks were used to generate depth profiles that show, for the first time, how copper ligands are distributed across the Canadian Arctic. Copper ligand concentrations ranged from 0.9 to 4.8 nM, the depth of highest ligand concentration often coinciding with the chlorophyll maximum depth. Correlations between ligand concentration and in situ chlorophyll-a fluorescence suggest that marine phytoplankton or cyanobacteria could be an important source of copper ligands. A correlation was also observed between copper ligand and dissolved copper concentrations in Baffin Bay. Comparison of these results with published data for humic substances and other dissolved organic matter suggests that terrestrial input contributes to the pool of ligands captured by IMAC, particularly in the Canada Basin and Canadian Arctic Archipelago.

3.1 Introduction

Dissolved organic ligands that form stable complexes with trace metals in natural waters play an important role in the biological utilization and geochemical cycling of these metals (Kraemer *et al.*, 2015; Sunda, 1988). In seawater, dissolved copper (dCu) exists almost exclusively as copper(II) complexes formed by organic ligands whose origin, identity, and function remain poorly defined (Vraspir & Butler, 2009). Previous studies have shown that copper(II)-complexing ligands are often concentrated near the chlorophyll maximum (Coale & Bruland, 1988; Donat *et al.*, 1986; Moffett *et al.*, 1990), implying that growing phytoplankton assemblages are a significant source of these ligands. Culturing experiments (Buck *et al.*, 2010; Croot *et al.*, 2000; Dupont *et al.*, 2004; Gledhill *et al.*, 1999; Gordon *et al.*, 2000; Leal *et al.*, 1999; McKnight & Morel, 1979, 1980; Moffett *et al.*, 1990; Moffett & Brand, 1996; Semeniuk *et al.*, 2015; Wiramanaden, 2006; Wiramanaden *et al.*, 2008) support the hypothesis that certain marine algae produce ligands to regulate copper uptake.

Electrochemical methods have been used to estimate the concentrations and binding affinities of dissolved copper(II) ligands in the Atlantic (van den Berg, 1984b; Buckley & van den Berg, 1986; Donat & van den Berg, 1992; Huizenga & Kester, 1983; Kramer, 1986; Moffett *et al.*, 1990; Sunda & Hanson, 1987), Pacific ((Buck & Bruland, 2005; Coale & Bruland, 1990; Donat *et al.*, 1986; Midorikawa & Tanoue, 1996), Indian (Donat & van den Berg, 1992) and Antarctic Ocean (Bundy *et al.*, 2013) and in the sub-Arctic waters of the N Pacific and Bering Sea (Coale & Bruland, 1988; Moffett & Dupont, 2007; Whitby *et al.*, 2018) as well as in coastal and estuarine waters (Ahner *et al.*, 1997; Donat *et al.*, 1994; Dryden *et al.*, 2004; Gordon *et al.*, 1996; Laglera & van den Berg, 2003; Skrabal *et al.*, 2000; Whitby & van den Berg, 2015). We are not aware of any published studies of copper ligands in the high Arctic.

Copper ligands can be isolated from other components of marine dissolved organic matter (DOM) using immobilized copper(II)-ion affinity chromatography (IMAC) (Donat *et al.*, 1997; Gordon, 1992). We have shown that IMAC can be used to determine the relative abundance of copper ligands in coastal and oceanic waters of the Neast Pacific based upon their UV absorbance (Nixon & Ross, 2016)In addition to measuring the distribution of copper ligands this relatively simple and robust method provides fractions suitable for ligand characterization by mass spectrometry (Nixon & Ross, 2016; Ross *et al.*, 2003; Vachet & Callaway, 2003) and other analytical techniques (Cottrell *et al.*, 2014; Midorikawa & Tanoue, 1996).

As part of the Canadian Arctic GEOTRACES program we used IMAC to isolate and quantify dissolved copper(II)-binding ligands in samples collected at different depths from 10 locations in the Canada Basin, Canadian Arctic Archipelago (CAA), and Baffin Bay. Results were used to create depth profiles for this operationally-defined fraction of copper ligands. Two types of profiles were observed; one with mid-depth maxima below 30 m, seen in the Canada Basin and western CAA, and one with near-surface maxima above 30 m, observed in the eastern CAA and Baffin Bay. The depth of highest ligand concentration was generally found to coincide with the chlorophyll maximum depth. Results constitute one of only a few studies of copper speciation in Arctic waters and the first comprehensive survey of copper ligands across the Canadian Arctic.

3.2 Materials and methods

3.2.1 Reagents

Reagents and solvents were analytical and high performance liquid chromatography (HPLC) grade, respectively, unless otherwise noted. Hydrochloric acid (HCl) was obtained from Anachemia (Vancouver, BC), ethylenediamine tetraacetic acid (EDTA) from Fisher (Pittsburgh, PA), and copper (II) sulfate (99.995% pure) and 8-hydroxyquinoline from Sigma-Aldrich (St. Louis, MO). Ultrapure deionized water (18 MΩ-cm) was prepared using an EMD Super-Q system from Millipore (Billerica, MA). Artificial seawater was prepared by dissolving 32 g of Instant Ocean (Blacksburg, VA) in one litre of ultrapure water and acidified by adding HCl to a concentration of 10 mM (pH 2.1).

3.2.2 Collection of seawater samples

Samples were collected from the CCGS Amundsen as part of GEOTRACES sections GN02 (10/07/2015–20/08/2015) and GN03 (4/09/2015–1/10/2015) covering an area from 56°N to 77°N and 53°W to 150°W in the Canadian Arctic. Sampling was carried out at 10 stations along a transect between the Canada Basin and Baffin Bay (Fig. 3.1, Table 3.1). Samples were collected at depths ranging from 10 m to a maximum of 240 m in the CAA and Canada Basin and 500 m in Baffin Bay. A trace-metal clean sampling system consisting of a powder-coated aluminium frame holding twelve 12-L Teflon-coated Go-Flo bottles and tethered by a 4000-m 4-member conducting Vectran cable encased in polyurethane (Cortland Cable Company, Cortland, NY) was used to collect seawater samples. These were gravity-filtered through 0.2-µm Acropak filters (Pall Corporation, Port Washington, NY) on board the ship in a HEPA filtered environment. The filtrate was collected in 1-L high density polyethylene (HDPE) bottles (Nalgene®) and stored at −20 °C for copper ligand measurements. Where possible, two or more replicate samples were collected for ligand analysis at each depth. Filtrate was also collected in 500-mL low density polyethylene (LDPE) bottles (Nalgene®) and acidified to pH 1.7 using SeaStar Baseline HCl (SeaStar Chemicals, Sidney, BC) for storage and dCu analysis. Sampling bottles were pre-cleaned according to GEOTRACES protocols (Cutter *et al.*, 2010).

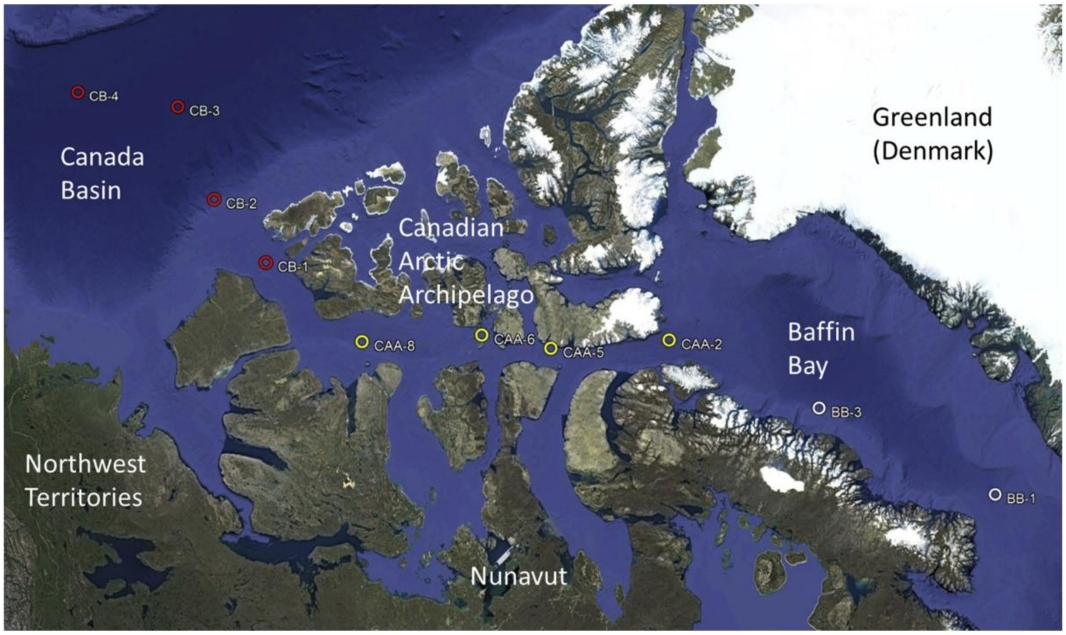


Figure 3.1 Selected locations at which samples were collected for copper ligand and dissolved copper analysis in the Canada Basin (CB), Canadian Arctic Archipelago (CAA), and Baffin Bay (BB) during the 2015 Canadian Arctic GEOTRACES expedition (map: Google Earth).

Table 3.1 Location, sampling date and ice cover for stations in the Canada Basin (CB), Canadian Arctic Archipelago (CAA) and Baffin Bay (BB).

Station	**Latitude (N)**	**Longitude (W)**	**Sampling date (2015)**	**Ice cover (/10)**
CB-4	75° 00.0′	150° 00.0′	14-Sep	0
CB-3	76° 58.8′	140° 03.0′	11-Sep	1
CB-2	75° 48.6′	129° 13.8′	9-Sep	4
CB-1	75° 07.8′	120° 34.2′	7-Sep	1
CAA-8	74° 08.4′	108° 49.8′	23-Sep	0
CAA-6	74° 45.0′	97° 27.6′	15-Aug	0
CAA-5	74° 32.4′	90° 48.6′	13-Aug	0
CAA-2	74° 19.2′	79° 30.0′	10-Aug	0
BB-3	71° 24.6′	68° 36.0′	5-Aug	0
BB-1	66° 51.0′	59° 03.6′	3-Aug	2

3.2.3 Analysis of seawater samples

IMAC was carried out as previously described (Chapter 2: Nixon & Ross, 2016) using a flow rate of 1 mL/min and a manually operated dual-column system incorporating a single wavelength UV detector (LKB Bromma Uvicord S, Pharmacia Biotech, Uppsala, Sweden). Briefly, two 5-mL Hi-Trap Chelating Sepharose HP columns (part no. 17-0409-03, GE Healthcare, Mississauga, ON) were rinsed with 30 mL of deionized water and charged with 50 µmoles of Cu^{2+} ions by passing 5 mL of a 10 mM copper(II) sulfate solution through each column. The columns were rinsed with 5 mL of ultrapure water and equilibrated with 20 to 30 mL of acidified artificial seawater before passing 1 L of filtered seawater sample through each column. Retained compounds were then eluted with acidified artificial seawater for 20 to 30 min, during which the UV absorbance of the eluent was monitored at 254 nm. Absorbance was plotted digitally using custom-built software and peak areas determined using the Riemann method. The columns were regenerated with 15 mL of a 50 mM EDTA solution before rinsing with deionized water.

Chromatograms recorded during IMAC of Arctic seawater samples contained a peak (Fig. 3.2) corresponding to the elution of UV-absorbing DOM with an affinity for immobilized Cu^{2+} ions (Nixon & Ross, 2016). This peak represents an operationally-defined fraction of the dissolved copper(II)-binding ligands present in seawater (see 3.4 Discussion). Peak area was converted to ligand concentration using a linear calibration ($y = 44.16\times + 88.17$, $r^2 = 0.998$) generated by analyzing, in triplicate, seawater samples spiked with 0 to 100 nmol/L of 8-hydroxyquinoline, a model ligand used to develop and validate the IMAC method (Nixon & Ross, 2016).

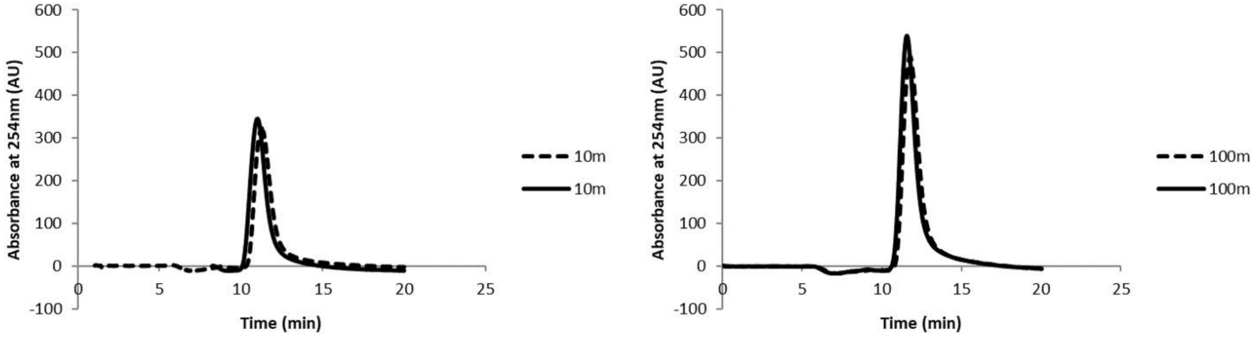


Figure 3.2 Immobilized copper(II)-ion affinity chromatography of duplicate samples collected at station CB-3 in the Canada Basin.

Dissolved copper was measured by triple-quadrupole inductively coupled plasma-tandem mass spectrometry (ICP-MS/MS) following offline pre-concentration by solid-phase extraction.

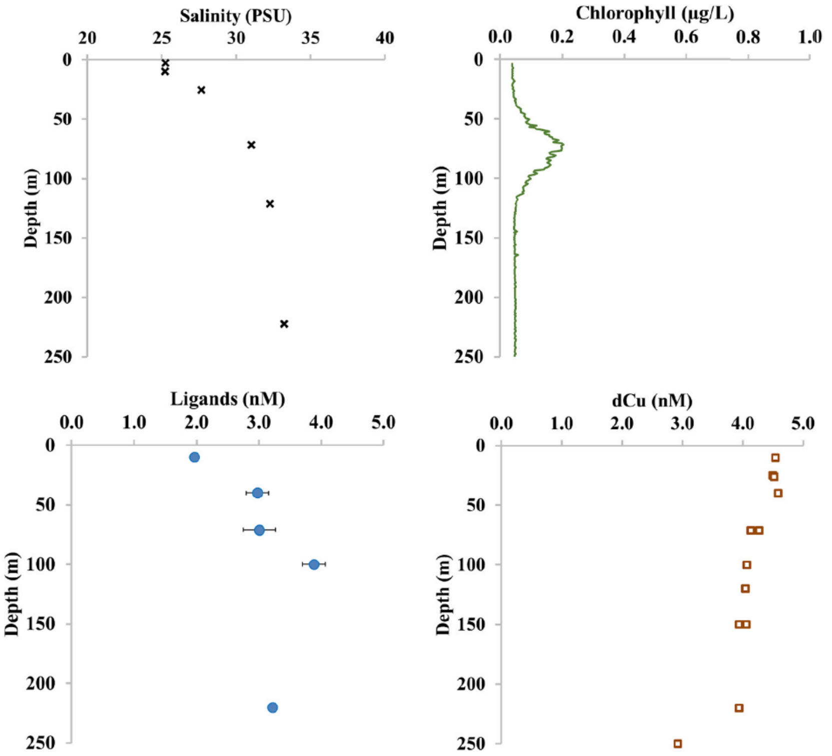
Salinity was measured at each station using a sensor (Sea-Bird, Belleville, WA) attached to a conductivity/temperature/depth (CTD) rosette. In vivo chlorophyll-a fluorescence was measured in the Canada Basin and at station CAA-8 using a fluorometer (Seapoint, Brentwood, NH) attached to the CTD rosette, and with a separate fluorometer (Turner Designs, Sunnyvale, CA) at other stations. Fluorescence measurements were converted to chlorophyll-a concentrations using calibration factors based upon chlorophyll analysis of discrete waters samples from selected stations (Michel Gosselin; personal communication). These and other oceanographic data, including hydrographic measurements (Al Mucci; personal communication), were made available by Amundsen Science Data Collection (2015) and by participating GEOTRACES scientists via a shared database hosted by the University of British Columbia (Kristina Brown; personal communication). Contemporaneous measurements of thiol, humic substances, and fluorescent dissolved organic matter in the Canada Basin and CAA, published by Gao and Guéguen, were also used to interpret the copper ligand profiles determined by IMAC.

3.3 Results

Hydrographic data collected during the 2015 Canadian Arctic GEOTRACES expedition identified three main water masses in the Canada Basin and CAA. Surface waters (SW; top 30 m, salinity <32 PSU) were dominated by runoff and ice melt in the Canada Basin and western CAA and influenced by warmer, more saline Atlantic waters in the eastern CAA. Arctic outflow waters (OW; 30 to 300 m, salinity <33.7 PSU) incorporated Pacific summer and winter waters in the Canada Basin and river input and ice melt in the CAA. Deep waters (DW; below 300 m, salinity >34.5 PSU) were dominated by warm, saline Atlantic water. Salinity profiles help to relate these water masses to the distributions of chlorophyll, copper ligands, and dCu across the Canadian Arctic (Fig. 3.3, Fig. 3.4, Fig. 3.5).

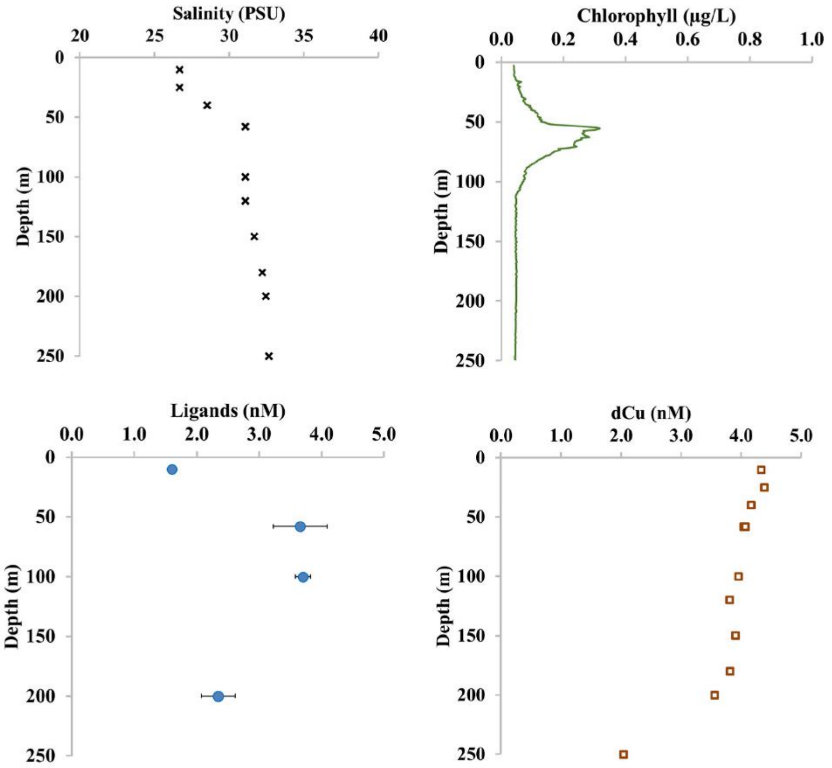
(a)

CB-4


Salinity (PSU)
20
25
30
35
40
Depth (m)
0
50
100
150
200
250
Chlorophyll (µg/L)
0.0
0.2
0.4
0.6
0.8
1.0
Ligands (nM)
0.0
1.0
2.0
3.0
4.0
5.0
dCu (nM)

(b)

CB-3


Salinity (PSU)
20
25
30
35
40
Depth (m)
0
50
100
150
200
250
Chlorophyll (µg/L)
0.0
0.2
0.4
0.6
0.8
1.0
Ligands (nM)
0.0
1.0
2.0
3.0
4.0
5.0
dCu (nM)

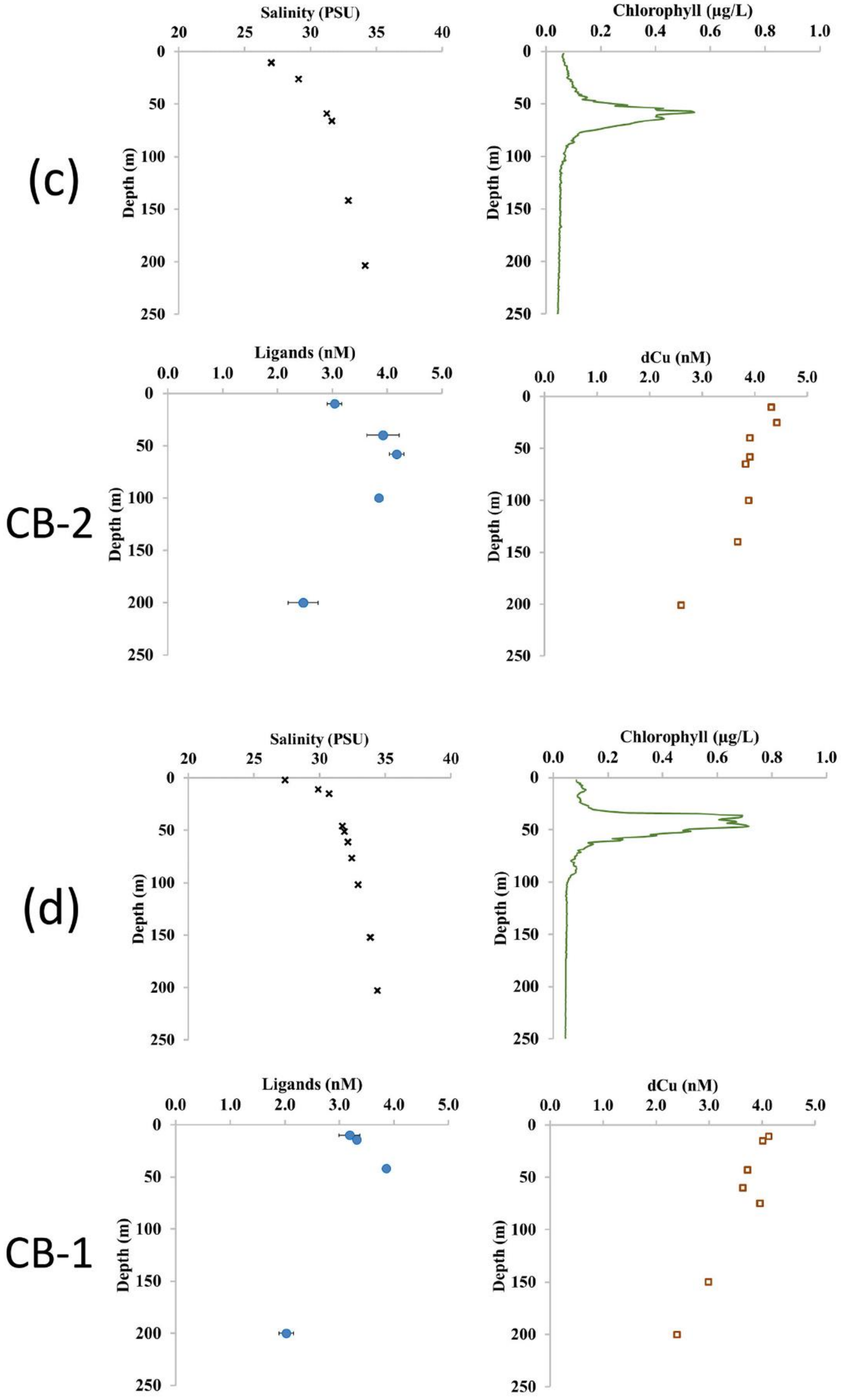


Figure 3.3 Profiles of salinity (PSU), chlorophyll-a (μg/L), copper ligand and dissolved copper concentration (nM) from west (CB-4) to east (CB-1) in the Canada Basin.

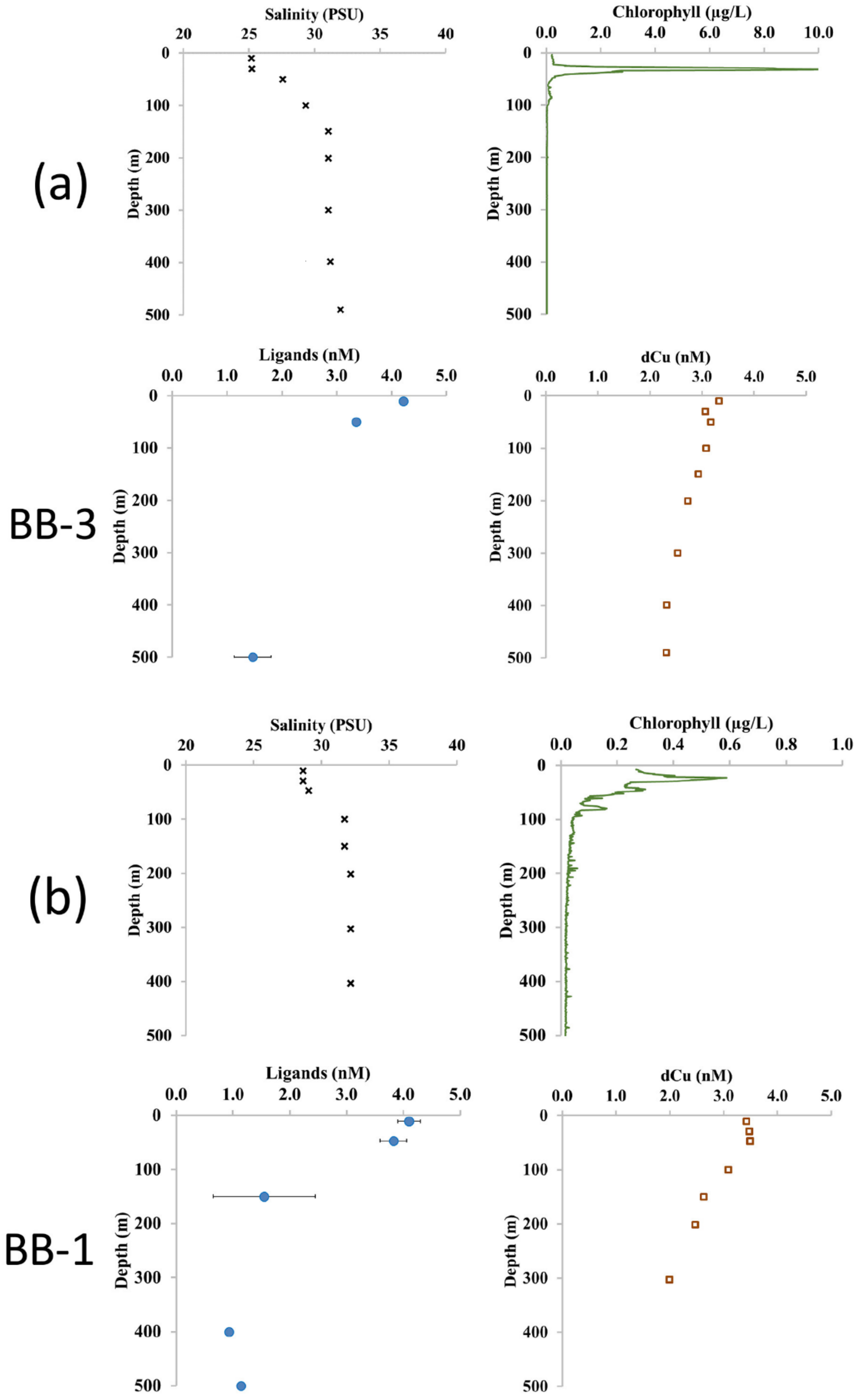
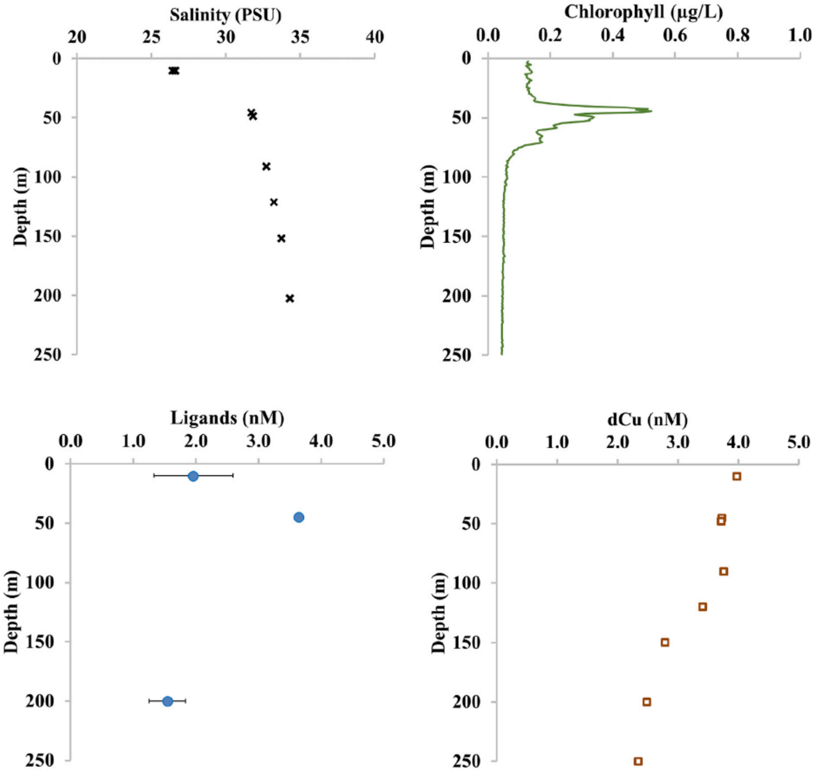


Figure 3.4 Profiles of salinity (PSU), chlorophyll-a (µg/L), copper ligand and dissolved copper concentration (nM) from west (BB-3) to east (BB-1) in Baffin Bay.

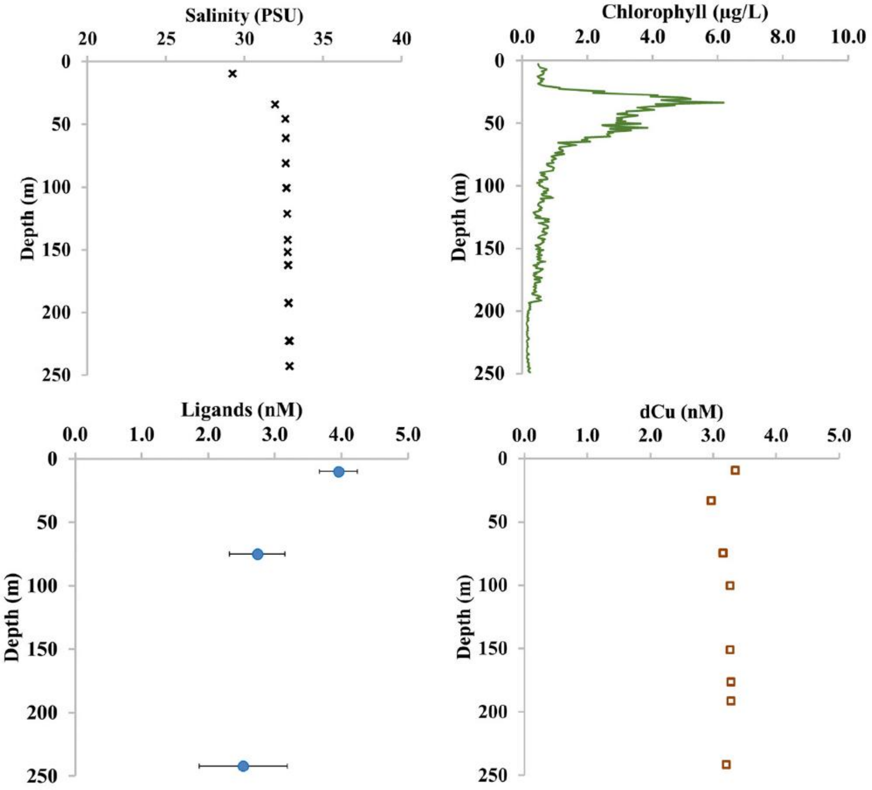
(a)

CAA-8


Salinity (PSU)
20
25
30
35
40
Depth (m)
0
50
100
150
200
250
Chlorophyll (µg/L)
0.0
0.2
0.4
0.6
0.8
1.0
Ligands (nM)
0.0
1.0
2.0
3.0
4.0
5.0
dCu (nM)
0.0
1.0
2.0
3.0
4.0
5.0

(b)

CAA-6


Salinity (PSU)
20
25
30
35
40
Depth (m)
0
50
100
150
200
250
Chlorophyll (µg/L)
0.0
2.0
4.0
6.0
8.0
10.0
Ligands (nM)
0.0
1.0
2.0
3.0
4.0
5.0
dCu (nM)
0.0
1.0
2.0
3.0
4.0
5.0

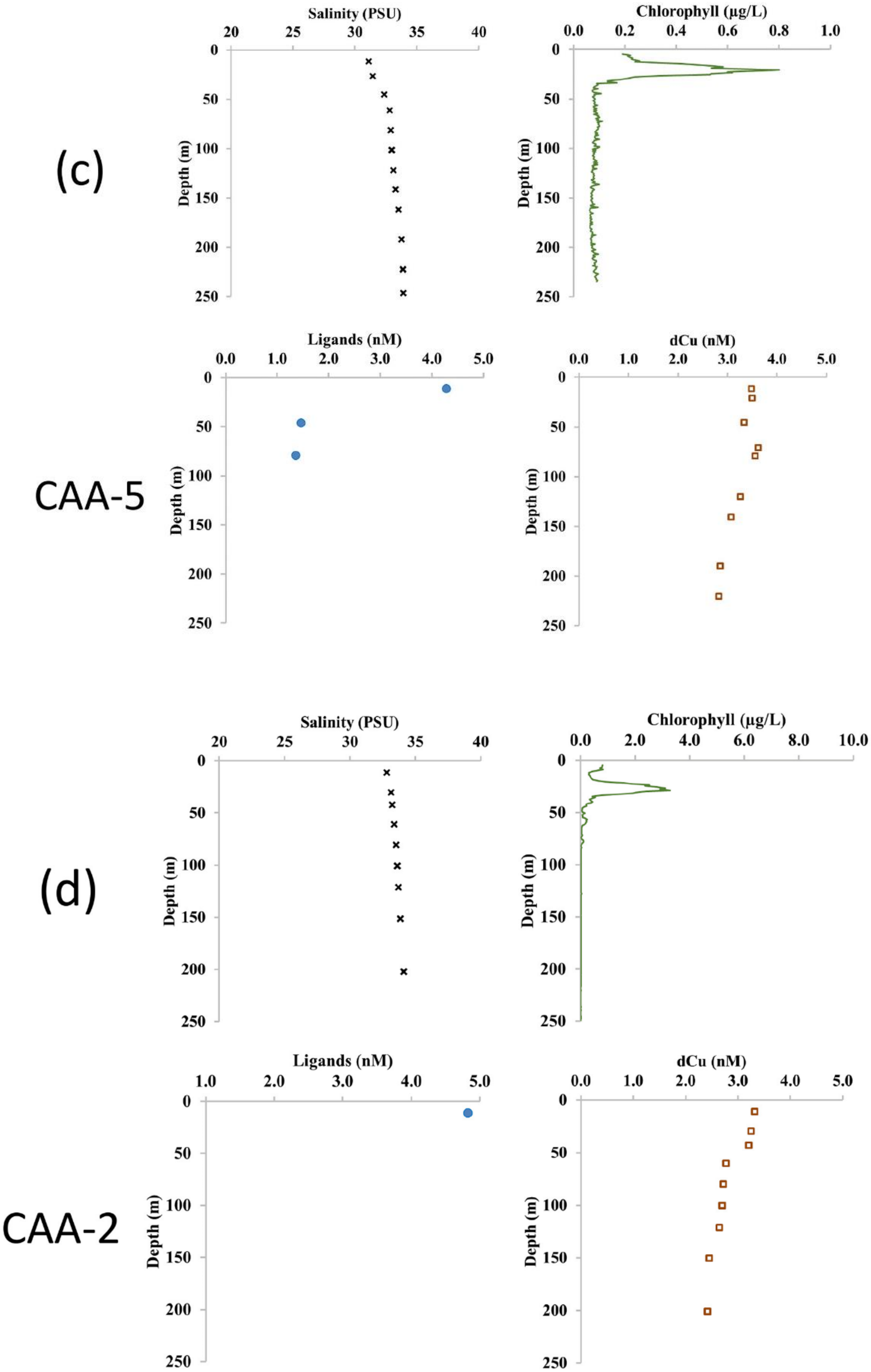


Figure 3.5 Profiles of salinity (PSU), chlorophyll-a (µg/L), copper ligand and dissolved copper concentration (nM) from west (CAA-8) to east (CAA-2) in the Canadian Arctic Archipelago.

3.3.1 Canada Basin

Ligand depth profiles in the Canada Basin were similar in shape, featuring a mid-depth maximum of between 3.7 and 4.2 nM that shoaled eastwards from 100 m at CB-4 (Fig. 3.3a) to 42 m at CB-1 (Fig. 3.3d). Analysis of duplicate samples (Fig. 3.2) showed that ligand concentrations were reproducible with an average relative standard deviation of 10.9%. Error bars indicating plus or minus one standard deviation from the mean are shown wherever replicate samples were analyzed (Fig. 3.3, Fig. 3.4, Fig. 3.5). Stations CB-3 and CB-4 had surface ligand concentrations of 1.6 and 2.0 nM, respectively, compared with 3.2 nM at CB-1 and 3.0 nM at CB-2, where ice cover was greatest (Table 3.1). Chlorophyll maxima in the Canada Basin were close to the depth of highest ligand concentration (Fig. 3.3). The ligand maximum extended below the chlorophyll maximum at stations CB-2, CB-3 and CB-4. The ligand concentration at 220 m was 3.2 nM at CB-4 whereas ligand concentrations at 200 m ranged from 2.0 to 2.5 nM at other CB stations. Concentrations of dCu in the Canada Basin were > 4 nM at the surface and remained at around 4 nM until a point below the chlorophyll maximum at which dCu started decreasing with depth to <3 nM at 250 m.

3.3.2 Baffin Bay

Ligand profiles in Baffin Bay were distinct from those observed in the Canada Basin, the highest concentrations (> 4 nM) being found near the surface (Fig. 3.4). Chlorophyll maxima in Baffin Bay were again close to the depth of highest ligand concentration. Baffin Bay had relatively low ligand concentrations below 200 m (between 0.9 and 1.5 nM). Surface concentrations of dCu were lower in Baffin Bay than in the Canada Basin with maximum values approaching 3.5 nM. Dissolved copper concentrations gradually decreased with depth to between 2.0 and 2.3 nM at 500 m.

3.3.3 Canadian Arctic Archipelago

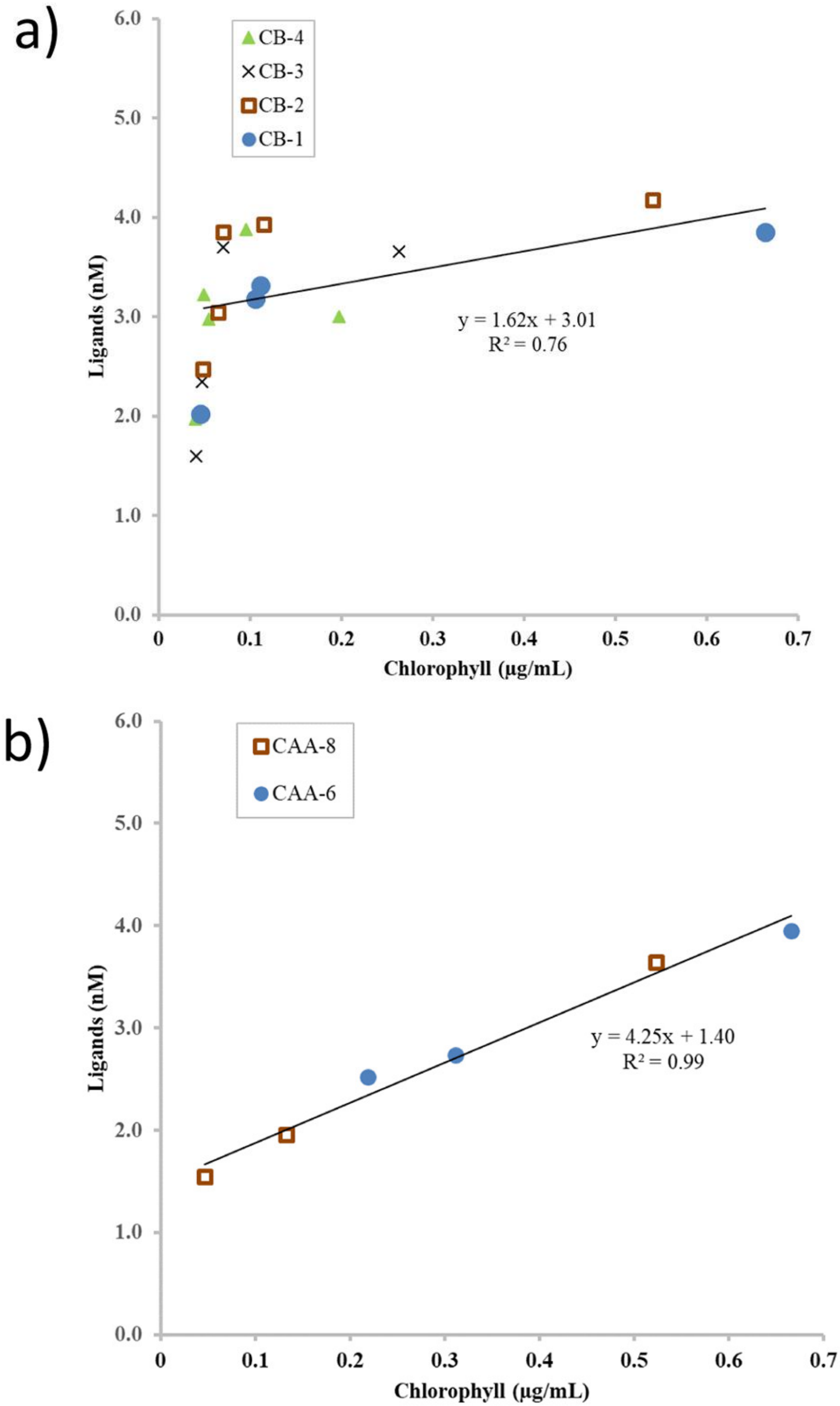
Ligand profiles featuring mid-depth and near-surface maxima were both observed in the CAA. The profile at CAA-8 (Fig. 3.5a) resembled those seen in the neighboring Canada Basin with a maximum ligand concentration of 3.6 nM at 45 m, the chlorophyll maximum depth. The dCu profile at CAA-8 was also similar to those observed in the Canada Basin, decreasing with depth from a surface maximum of 4.0 nM to 2.5 nM at 200 m.

Ligand profiles at stations CAA-5 and CAA-6 (Fig. 3.5b,c) resembled those seen in Baffin Bay with highest ligand concentrations (~ 4 nM) near the surface. Ligand concentrations below 50 m showed little variation but were significantly lower at CAA-5 (< 2 nM) than at CAA-6 (2 to 3 nM), approaching those observed at depth in Baffin Bay. Again, chlorophyll maxima at these stations were close to the depth of highest ligand concentration. Water collected from 11 m at station CAA-2 (Fig. 3.5d) had the highest recorded ligand concentration of 4.8 nM (no other samples were collected at this station). Profiles of dCu at CAA-5 and CAA-6 showed relatively little variation with depth, averaging 3.2 nM between the surface and 250 m. At CAA-2, dCu decreased sharply from 3.2 nM at 43 m to 2.8 nM at 60 m before falling steadily to 2.4 nM at 200 m.

3.4 Discussion

3.4.1 Operational definition of copper ligands

Electrochemical studies have confirmed that marine copper-binding ligands can be isolated from seawater using IMAC; for example, Donat *et al.* (1997) demonstrated removal of both stronger (L_1) and weaker (L_2) classes of copper ligands from seawater and detection of L_1 in pooled IMAC eluents. Nevertheless, it is possible that certain ligands may not be retained or detected during IMAC, including multi-dentate ligands unable to form stable ternary complexes with $IDACu^{2+}$ (Nixon & Ross, 2016; Paunovic *et al.*, 2005) or those that show weak UV absorbance. However, relative retention times for natural and model ligands observed using the same IMAC method (Nixon & Ross, 2016; Ross *et al.*, 2003) suggest that we are monitoring ligands with an affinity for Cu^{2+} comparable to the L_1 class. We have also shown, by spiking samples with excess Cu^{2+}, that most ligands entering the IMAC column in complexed form are retained (Nixon & Ross, 2016), presumably via dissociation of the complex or interaction of the complexed copper with the column resin. Hence, the predominant peak observed in the IMAC chromatograms of all Arctic samples represents an operationally defined fraction of UV-absorbing ligands with similar and relatively strong affinities for Cu^{2+}. The contributions to IMAC peak area made by different types and sources of ligands will depend upon their relative abundance and UV absorbance. However, the consistency of our ligand measurements in terms of their relationship with chlorophyll (Fig. 3.3, Fig. 3.4, Fig. 3.5, Fig. 3.6) and with L_1 ligand concentrations determined by voltammetry in the sub-Arctic Neast Pacific and Bering Sea (Moffett & Dupont, 2007; Whitby *et al.*, 2018) suggest that they provide a reliable indication of the distribution and concentrations of copper(II)-complexing ligands in Arctic waters.


a)
CB-4
CB-3
CB-2
CB-1
Ligands (nM)
6.0
5.0
4.0
3.0
2.0
1.0
0.0
y = 1.62x + 3.01
R² = 0.76
0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
Chlorophyll (µg/mL)
b)
CAA-8
CAA-6
Ligands (nM)
6.0
5.0
4.0
3.0
2.0
1.0
0.0
y = 4.25x + 1.40
R² = 0.99
0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
Chlorophyll (µg/mL)

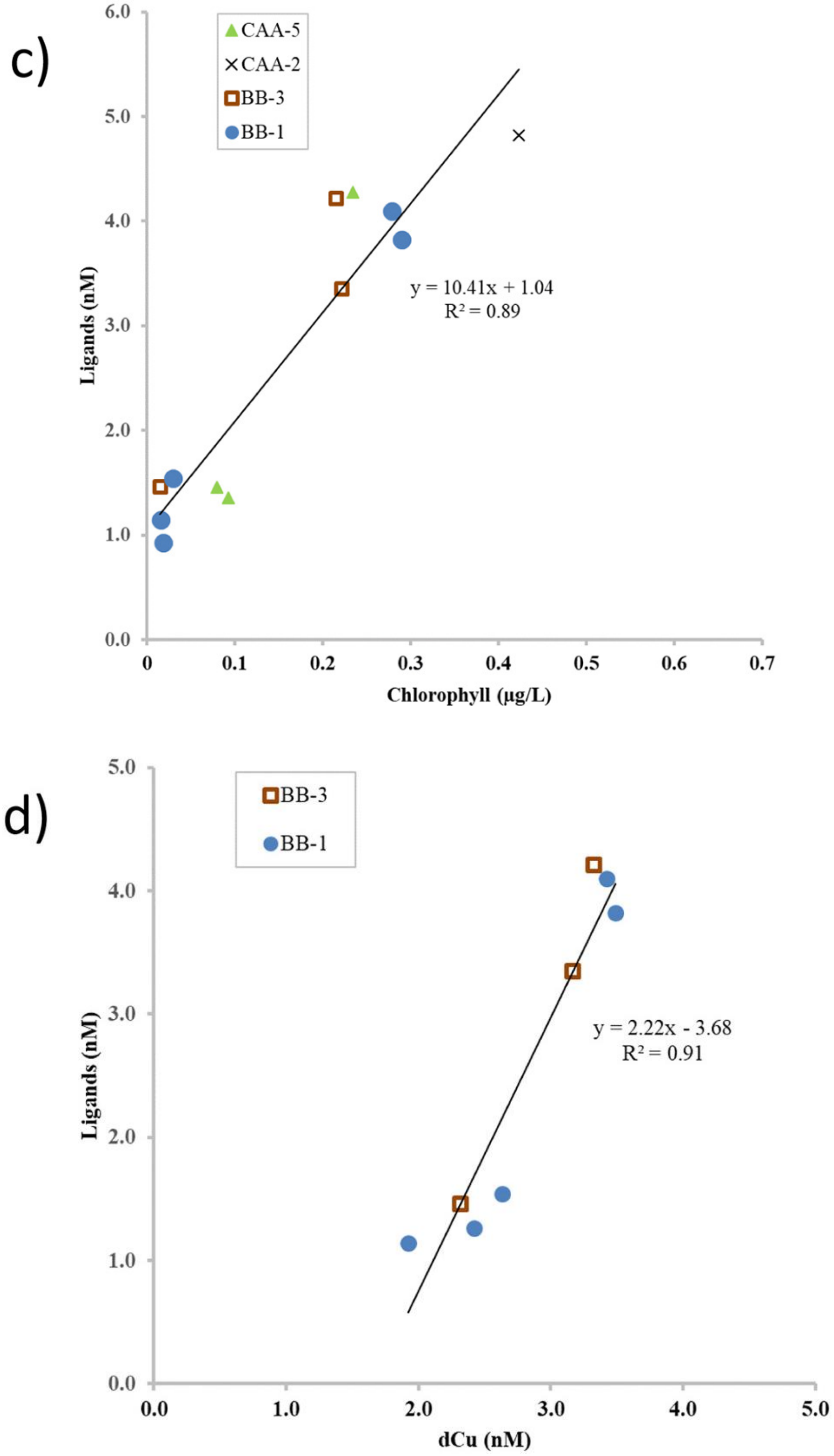


Figure 3.6 Plots of ligand concentration vs. chlorophyll concentration for the Canada Basin (a), Canadian Arctic Archipelago stations CAA-8 and CAA-6 (b), CAA-5, CAA-2 and Baffin Bay (c), and plot of ligand concentration vs. dissolved copper for Baffin Bay (d).

3.4.2 Profiles with maxima below 30 m

Mid-depth ligand maxima were observed in the Canada Basin and at station CAA-8, coinciding with the chlorophyll maximum in the OW layer. The shallowing of these maxima between station CB-4 (Fig. 3.3a) and station CAA-8 (Fig. 3.5a) is consistent with an eastward shoaling of the OW layer, as indicated by the corresponding salinity profiles. Ligand concentrations were relatively low (< 2.0 nM) near the surface at stations CB-4, CB-3 and CAA-8 where lack of ice cover (Table 3.1) may promote photo-oxidation of certain ligands. However, stations CB-1 and CB-2 (Fig. 3.3) had similar ligand concentrations (around 3 nM) near the surface despite low ice cover at CB-1, suggesting that other factors are important in determining ligand abundance.

Complementary studies (Gao & Guéguen, 2018) indicate that thiols, which include biologically-derived ligands like glutathione, are relatively abundant at the chlorophyll maximum in the Canada Basin and eastern CAA. Naturally occurring thiols have been shown to complex copper in estuarine waters (Laglera & van den Berg, 2003) and are known to be produced by some cyanobacteria under conditions of metal stress. We have shown that glutathione can be recovered from seawater by IMAC (Nixon & Ross, 2016) although absorbance at 254 nm is relatively weak. Analysis of fluorescent dissolved organic matter using UV–vis spectrophotometry and parallel factor analysis (Gao & Guéguen, 2018) found that protein-like component C4 (associated with in situ biological production) and the absorption coefficient at 355 nm (a_{355}, a proxy for terrestrial DOM) were also relatively high in the OW layer of the Canada Basin and western CAA. Taken together, these results suggest that (i) marine phytoplankton or cyanobacteria associated with the chlorophyll maximum could be a significant source of the copper ligands captured by IMAC, and (ii) humic substances and other terrestrial DOM contribute to the pool of copper ligands recovered from the OW layer.

3.4.3 Profiles with maxima above 30 m

Ligand profiles with near-surface maxima were observed in Baffin Bay and at CAA-5 and CAA-6 where the chlorophyll maximum was relatively shallow. Ligand concentrations measured at 10 m in Baffin Bay were among the highest recorded (> 4 nM) whereas those below 150 m were among the lowest (1.5 nM or less). The difference in ice cover at stations BB-1 and BB-3 (Table 3.1) appeared to have little effect on ligand concentrations near the surface, implying that other factors are more important in determining ligand abundance. Indeed, relatively high a355 values in surface waters at CAA-2 (Gao & Guéguen, 2018) suggest that contributions from terrestrial DOM to copper ligands in the eastern Canadian Arctic are likely to be greater near the surface. This is consistent with the high ligand concentration (4.8 nM) measured at CAA-2 (Fig. 3.5d).

3.4.4 Sources of copper ligands

The highest ligand concentration at each station was generally observed in the vicinity of the chlorophyll maximum. This is consistent with ligand profiles generated using electrochemical methods (Coale & Bruland, 1988; Moffett *et al.*, 1990) and the hypothesis that marine phytoplankton are potential significant sources of copper-complexing ligands in seawater. To further investigate the relationship between ligand abundance and phytoplankton biomass we plotted ligand concentration against chlorophyll concentration at each station. The results fall into three geographically distinct groups.

The first group consists of the four stations in the Canada Basin (Fig. 3.6a). A positive linear correlation ($y = 1.62\times + 3.01$, $r^2 = 0.76$) between ligand and chlorophyll concentrations was observed for nine of the eighteen samples in this group. These include samples collected from within the chlorophyll maxima, and from near the surface at CB-1 and CB-2. The intercept, which represents the extrapolated ligand concentration in the absence of chlorophyll, may serve as a proxy for humic substances (HS) and other copper-binding DOM not associated with phytoplankton. To test this hypothesis we converted the intercept (3 nM) to IMAC peak area (221 AU*min) using the original calibration curve (see Methods and Materials). We then generated a second IMAC calibration by analyzing seawater spiked with 0 to 200 µg C/L of an equal mixture of three HS standards (3S101H, 2S101F and 3S101F; International Humic Substance Society, St. Paul, MN) based upon the carbon content of each standard (International Humic Substance Society, 2019). We used this calibration ($y = 1.63\times + 16.20$, $r^2 = 0.995$) to convert the intercept peak area to an equivalent average HS concentration of 126 µg C/L. This value lies within the range of HS-like concentrations (55 to 145 µg C/L) measured by voltammetry in the OW layer of the Canada Basin (Gao and Guéguen, 2018), implying that a significant proportion of HS binds copper.

The remaining Canada Basin samples fall into two sub-groups, each within a narrow range of low chlorophyll concentrations (Fig. 3.6a). The sub-group with ligand concentrations >3.7 nM included samples from below the chlorophyll maximum at stations CB-2, CB-3 and CB-4. The highest ligand concentrations at CB-3 and CB-4 were actually measured below the chlorophyll maximum depth (Fig. 3.3a,b). Zooplankton grazing of phytoplankton is known to occur in the Canada Basin and may release metal-binding DOM into the water column. Capture of this material by IMAC below the chlorophyll maximum would result in ligand concentrations higher than those expected on the basis of chlorophyll measurements, as seen for this sub-group. Samples with ligand concentrations <2.5 nM include those collected near the surface at stations CB-3 and CB-4 and from 200 m at stations CB-1, CB-2 and CB-3. Photo-oxidation of copper ligands in open surface waters and bacterial degradation of copper-binding DOM at depth may have contributed to lower than expected ligand concentrations in these samples.

The second group of stations consists of CAA-6 and CAA-8 (Fig. 3.6b) which lie to the east of the Canada Basin. These showed a strong linear correlation between ligand and chlorophyll concentrations ($y = 4.25\times + 1.40$, $r^2 = 0.99$). Applying our HS calibration to the intercept gave an equivalent value of 82 µg C/L, which lies within range of HS-like concentrations (19 to 190 µg

C/L) measured in the Canada Basin and CAA (Gao & Guéguen, 2018). The third group consists of CAA-2, CAA-5 and the two stations in Baffin Bay (Fig. 3.6c). These also showed a strong linear correlation between ligand and chlorophyll concentrations ($y = 10.41\times + 1.04$, $r^2 = 0.89$) with a steeper slope and a smaller intercept than for stations to the west. Applying the HS calibration to this intercept gave an equivalent value of 72 µg C/L. The apparent decrease in copper-binding HS between the Canada Basin and Baffin Bay is consistent with complementary studies (Gao & Guéguen, 2018) that found terrestrial DOM to be highest in the western part of the study region. Although the source of mid-depth humic-rich waters remains unknown, previous studies have implicated terrestrial runoff as a significant contributor to bulk DOM in the Canadian Arctic. On the other hand, an increase in the slope of ligand vs. chlorophyll plots between the Canada Basin and Baffin Bay (Fig. 3.6a-c) may indicate greater production of copper ligands by phytoplankton in the eastern part of the study region.

The distribution of copper ligands across the Canadian Arctic can be seen more clearly by combining ligand profiles from station CB-4 in the Canada Basin to station BB-1 in Baffin Bay (Fig. 3.7). The resulting section shows that the depth of highest ligand concentration shoals steadily from west to east across the Canada Basin and through the CAA to Baffin Bay, tracking both the chlorophyll maximum and apparent inputs of terrestrial DOM to the OW layer in the west and SW layer in the east.

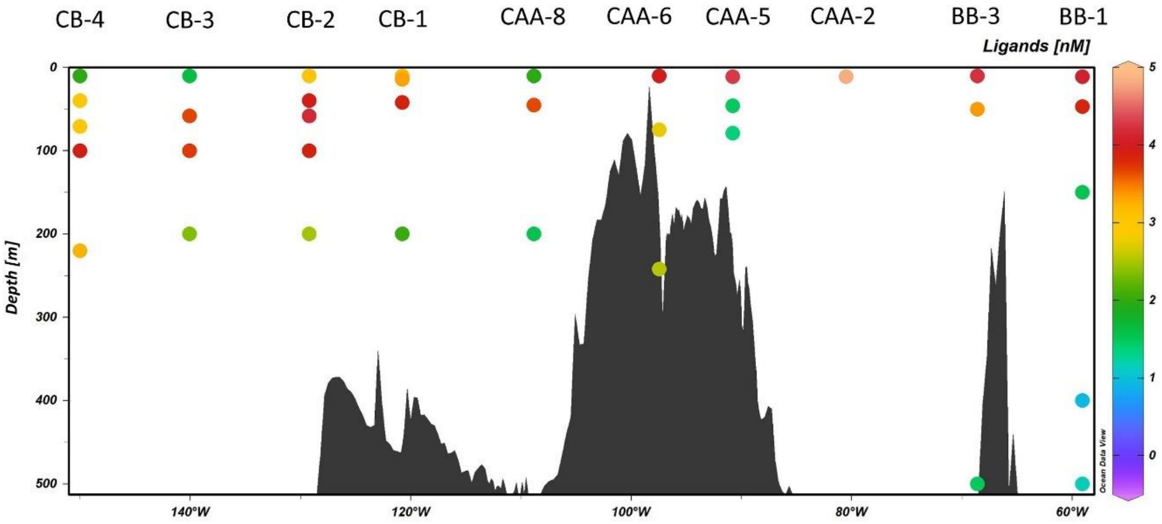


Figure 3.7 Distribution of copper ligands (nM) along a transect between station CB-4 in the Canada Basin and station BB-1 in Baffin Bay (Fig. 3.1) as determined by immobilized copper(II)-ion affinity chromatography (visualization: Ocean Data View).

3.4.5 Comparison with dissolved copper

Given that exposure to copper can influence the production of copper-complexing ligands by phytoplankton (see Introduction) we also examined the relationship between chlorophyll, ligand and dCu concentrations. Dissolved copper concentrations were generally found to be highest at the surface, decreasing steadily to a point below the chlorophyll maximum at which dCu begins to decline more rapidly. The depth at which this occurs in the Canada Basin decreases from about 200 m at CB-4 (Fig. 3.3a) to 75 m at CB-1 (Fig. 3.3d) which is consistent with a shoaling of the OW layer, and the associated chlorophyll and ligand maxima. The highest dCu concentrations measured at these stations (~ 4.5 nM) are unlikely to induce toxicity or limitation among phytoplankton assemblages, based upon in vitro studies (Gordon *et al.*, 2000; Moffett & Brand, 1996). Nevertheless, the fact that ligand maxima occurred at depths where higher chlorophyll and dCu concentrations were found is consistent with the release of copper-binding DOM by phytoplankton, whether in response to the presence of copper or not. This is also true of stations in the CAA and, in particular, Baffin Bay where higher dCu concentrations coincide with ligand maxima.

The possibility that some of these ligands may be produced in response to copper was further investigated by plotting ligand concentration against dCu (Fig. 3.6d). A strong correlation ($y = 2.22\times - 3.68$, $r^2 = 0.91$) between ligand and dCu concentrations was observed in Baffin Bay, where phytoplankton appear to be a significant source of copper ligands (Fig. 3.6c). The plot shows that ligand concentration increases with dCu in a ratio of approximately 2:1, which is consistent with complexation of Cu^{2+} by smaller ligands of the kind known to be recovered by IMAC (Nixon and Ross, 2016). An extrapolated dCu concentration of 1.7 nM in the absence of any copper ligands captured by IMAC is consistent with the recovery of a sub-set of copper-binding DOM by this method (see Operational definition of copper ligands). The omission of certain ligands by IMAC may also have contributed to the apparent lack of a consistent relationship between ligand concentration and dCu in the Canada Basin and CAA (not shown).

3.4.6 Comparison with phytoplankton taxonomy

A wide variety of organisms have been shown to produce copper-complexing ligands in seawater including eukaryotic phytoplankton (Anderson *et al.*, 1984; Croot *et al.*, 2000), diatoms (Croot *et al.*, 2000; Gerringa *et al.*, 1995; Morelli *et al.*, 1989; Zhou & Wangersky, 1985), dinoflagellates (Croot *et al.*, 2000) and coccolithophores (Croot *et al.*, 2000; Echeveste *et al.*, 2018; Leal *et al.*, 1999). Culturing experiments have also shown that high-affinity copper ligands can be produced by copper-sensitive marine phytoplankton (Leão *et al.*, 2007) in response metal stress and to regulate copper uptake (Semeniuk *et al.*, 2015).

Phytoplankton are the most significant contributors to bulk DOM in the Canadian Arctic, although comprehensive taxonomic data are not readily available. Heterotrophic dinoflagellates and other flagellates represent most of the biomass in the water column whereas diatoms are abundant near sea-ice interfaces, which were largely absent at our sampling locations (Table 3.1). Large phytoplankton are highly productive near ice-rich archipelagic stations while small phytoplankton dominate in the low productivity open waters of the Canada Basin and Baffin Bay (Varela *et al.*, 2013). Decreasing C:N ratios further suggest an ongoing increase in the relative abundance of small phytoplankton and heterotrophs in the Canada Basin, due to freshening of surface waters (Crawford *et al.*, 2015).

Available data from the 2015 GEOTRACES cruise in the eastern Canadian Arctic (Michel Gosselin: personal communication) show that most of the chlorophyll detected in Baffin Bay is associated with cells between 0.7 and 5 µm in diameter concentrated at a depth of about 30 m, whereas a significant proportion of the chlorophyll signal at stations in the central and eastern CAA is associated with cells larger than 20 µm residing at greater depths. Similarities between the distribution of this chlorophyll signal and of the copper ligands measured by IMAC in the eastern Arctic (Fig. 3.7) suggests that picoplankton, which consist mainly of eukaryotes in these waters, may be a significant source of copper ligands (Croot *et al.*, 2000; Gordon *et al.*, 2000; Moffett *et al.*, 1990; Moffett & Brand, 1996; Wiramanaden, 2006). Techniques to identify and hence determine the origin of ligands captured by IMAC are being developed (Nixon & Ross, 2016).

3.5 Conclusion

Immobilized copper(II)-ion affinity chromatography (IMAC) was used to profile the distribution of an operationally-defined fraction of dissolved copper ligands across the Canadian Arctic. Significant correlations between ligand and chlorophyll concentrations suggest that marine phytoplankton could be an important source of copper ligands, particularly in the eastern Canadian Arctic. The correlation between ligand and dissolved copper concentrations in Baffin Bay is consistent with biological production and IMAC recovery of small organic ligands. Comparisons with published data for humic substances and other marine dissolve organic matter suggest that both *in situ* biological production and terrestrial input contribute to the pool of copper ligands captured by IMAC.

4.0 Copper ligands in the NE Pacific Ocean

Contributions: I wrote the manuscript and performed data analysis. I collected samples from the 2017-06 cruise. I performed most Cu(II)-IMAC experiments, and mentored co-op student Jasper George who completed IMAC of some samples from the 2018-04 cruise.

4.0.1 Chapter abstract

Copper-complexing components of marine dissolved organic matter are of uncertain structure but have been hypothesized to originate from phytoplankton exudates, terrigenous material, and/or photo-linked humic matter. Determining the sources of Cu ligands is key to understanding their role in copper cycling and marine microbial ecology. Following our previous study in the Arctic Ocean (Chapter 3), we used immobilized copper(II)-ion affinity chromatography to quantify dissolved ligands in samples collected during four cruises from June 2016 to September 2018 along the Line-P transect in the NE Pacific Ocean. Copper ligand levels correlate to some extent with chlorophyll levels, phytoplankton pigment, *in situ* chl-a fluorescence, and other data measured by collaborators during the same cruises. Depth profiles at stations P4 and P26 are presented with estimated copper ligand levels ranging from 1.8 to 9.5 nM. Together, these data suggest the pool of copper-binding ligands in oceanic samples is only partially derived from growing phytoplankton communities.

4.1 Introduction

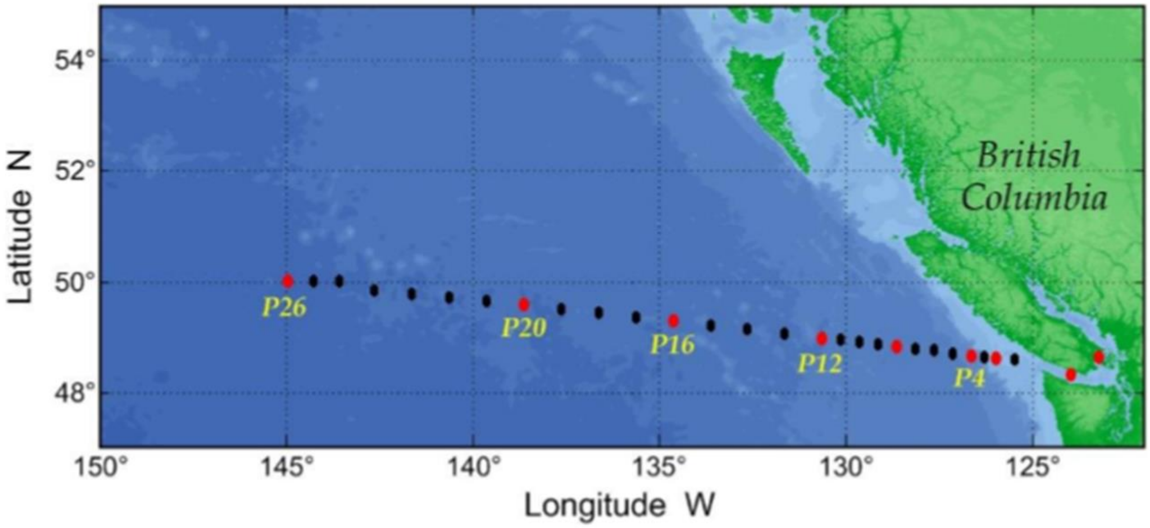
Components of marine dissolved organic matter (DOM) which form strong complexes with copper (Cu) play a role in biological copper uptake and utilization of this element, which can act both as a micronutrient and a toxin to marine phytoplankton. Culture experiments suggest Cu-complexing ligands can be produced by marine organisms in response to Cu stress or limitation (Croot *et al.*, 2000; Gledhill *et al.*, 1999; Gordon *et al.*, 2000; Leal *et al.*, 1999; Mawji *et al.*, 2008; Moffett *et al.*, 1990; Moffett & Brand, 1996; Wiramanaden *et al.*, 2008), while studies of natural seawater samples have noted that Cu(II) speciation may also be supplemented by terrigenous DOM and humic substances (Abualhaija *et al.*, 2015; Muller & Batchelli, 2013; Nixon *et al.*, 2019; Whitby & van den Berg, 2015).

We have studied the distribution of copper-complexing ligands in the Canadian Arctic Ocean as determined by immobilized Cu(II)-ion affinity chromatography (IMAC) (Nixon *et al.*, 2019). This protocol is appealing both as a simple analytical tool and as a preparatory step in the isolation of marine copper ligands for analysis by voltammetry (Donat *et al.*, 1997) or mass spectrometry (Nixon & Ross, 2016; Ross *et al.*, 2003; Vachet & Callaway, 2003).

Here we use Cu(II)-IMAC to investigate the distribution of Cu ligands along a transect in the NE Pacific Ocean. Results include depth profiles at coastal station P4 and oceanic station P26, which lies in the high nutrient-low chlorophyll (HNLC) waters of the Alaska Gyre. Correlation of IMAC data with measurements of chlorophyll, copper, and *in situ* fluorescence offer insight into potential sources of these ligands. Results suggest that copper-complexing components of marine DOM are derived from a variety of sources including phytoplankton assemblages as well as marine and terrestrial humic substances.

4.2 Materials and methods

4.2.1 Collection of seawater samples



Station	P26	P20	P16	P12	P4
Location	*50˚00'N, 145˚00'W*	*49˚34' N 138˚40' W*	*49˚17' N 134˚40' W*	*48˚58' N 130˚40' W*	*48˚39' N 126˚40' W*

Figure 4.1 Sampling locations along Line P in the NE Pacific Ocean. Image courtesy of DFO.

Collection and filtration of seawater samples for Cu(II)-IMAC were carried out during four cruises aboard the CCGS *John P. Tully* as part of the Line P Iron Program, a GEOTRACES process study (GPpr07). Seawater was collected from coastal station P4 (48˚N, 126˚W), oceanic station P26 (Stn Papa, 50˚N, 145˚W), and three intermediate stations along the Line-P transect (Fig. 4.1) at depths ranging from 5 m to 800 m during cruises in June 2016 (2016-06), June and August 2017 (2017-06 and 2017-08), and September 2018 (2018-40) (Table 4.1). To maintain trace-metal cleanliness, sampling bottles (General Oceanics) were acid-cleaned before the cruise, affixed to and lowered with a Kevlar line, and triggered using acid-cleaned Teflon messengers.

Samples were immediately gravity-filtered using 0.2-µm Acropak filters (Pall Corporation, Port Washington, NY) and stored at -20 ˚C in 1-L high density polyethylene (HDPE) bottles (Nalgene) pre-cleaned according to GEOTRACES protocols (Cutter *et al.*, 2010).

4.2.2 Analysis of seawater samples

Copper ligands were isolated from filtered seawater by Cu(II)-IMAC as previously described (Chapter 3: Nixon and Ross, 2019). Approximately 1 L of each sample was pumped at 1 mL/min through a 5-mL Hi-Trap Chelating Sepharose HP column (GE Healthcare, Mississauga, ON) charged with 50 µmoles of Cu^{2+} ions in the form of copper(II) sulfate (Sigma-Aldrich, St. Louis, MO) and eluted for 25 minutes with artificial seawater (Instant Ocean, Blacksburg, VA) acidified to pH 2.1 with hydrochloric acid (SeaStar Chemicals, Sidney, BC). Absorbance at 254 nm was monitored using a UV detector (LKB Bromma Uvicord S, Pharmacia Biotech, Uppsala, Sweden) and the area of the predominant elution peak was measured using custom-built software. After correcting for volume of sample, IMAC peak area was converted to ligand concentration ([L], nM) using a linear calibration ($y = 44.16x + 88.17$) obtained with the model ligand 8-hydroxyquinoline (Chapter 2: Nixon & Ross, 2016).

4.2.3 Oceanographic data

Other data collected during each cruise were obtained from the DFO Water Properties website (https://waterproperties.ca/linep/cruises.php). These include *in situ* measurements of salinity, temperature, chlorophyll-a fluorescence (SeaPoint sensor), and dissolved oxygen obtained during CTD casts carried out immediately before or after the collection of samples for Cu(II)-IMAC. Discrete measurements of chlorophyll, phaeopigment, dissolved oxygen, and dimethylsulfide (DMS) concentration were also carried out using bottle samples collected during CTD casts.

4.3 Results

4.3.1 Cu(II)-IMAC data

Among all samples from all cruises, Cu(II)-IMAC peak area ranged from ~100 to 500 AU*min, corresponding to Cu ligand concentrations between 1.8 and 9.4 nM, with most samples falling between 2.0 and 4.0 nM (Table 4.1). Depth profiles of Cu ligand concentration measured by Cu(II)-IMAC at all stations, and at P4 and P26, are shown in Figure 4.2.

Table 4.1 Cu ligand concentrations measured by Cu(II)-IMAC along Line P during four cruises.

Cruise	**Station**	**Depth (m)**	**[L] (nM)**
June 2016	P4	40	3.54
	P26	10	2.02
		40	2.25
June 2017	P26	5	3.26
		25	3.24
		50	3.70
		75	2.60
		100	2.89
		150	2.00
		200	2.43
		400	2.16
		800	2.96
August 2017	P4	25	6.30
	P12		1.84
	P16		2.24
	P20		2.68
	P26		3.91
September 2018	P4	10	9.42
		25	7.70
		50	4.31
		75	4.11
		100	3.00
	P12	40	4.78
	P16	40	6.19
	P20	40	4.12
	P26	10	3.91
		25	3.84
		40	4.50
		50	3.46
		75	2.74
		100	2.00

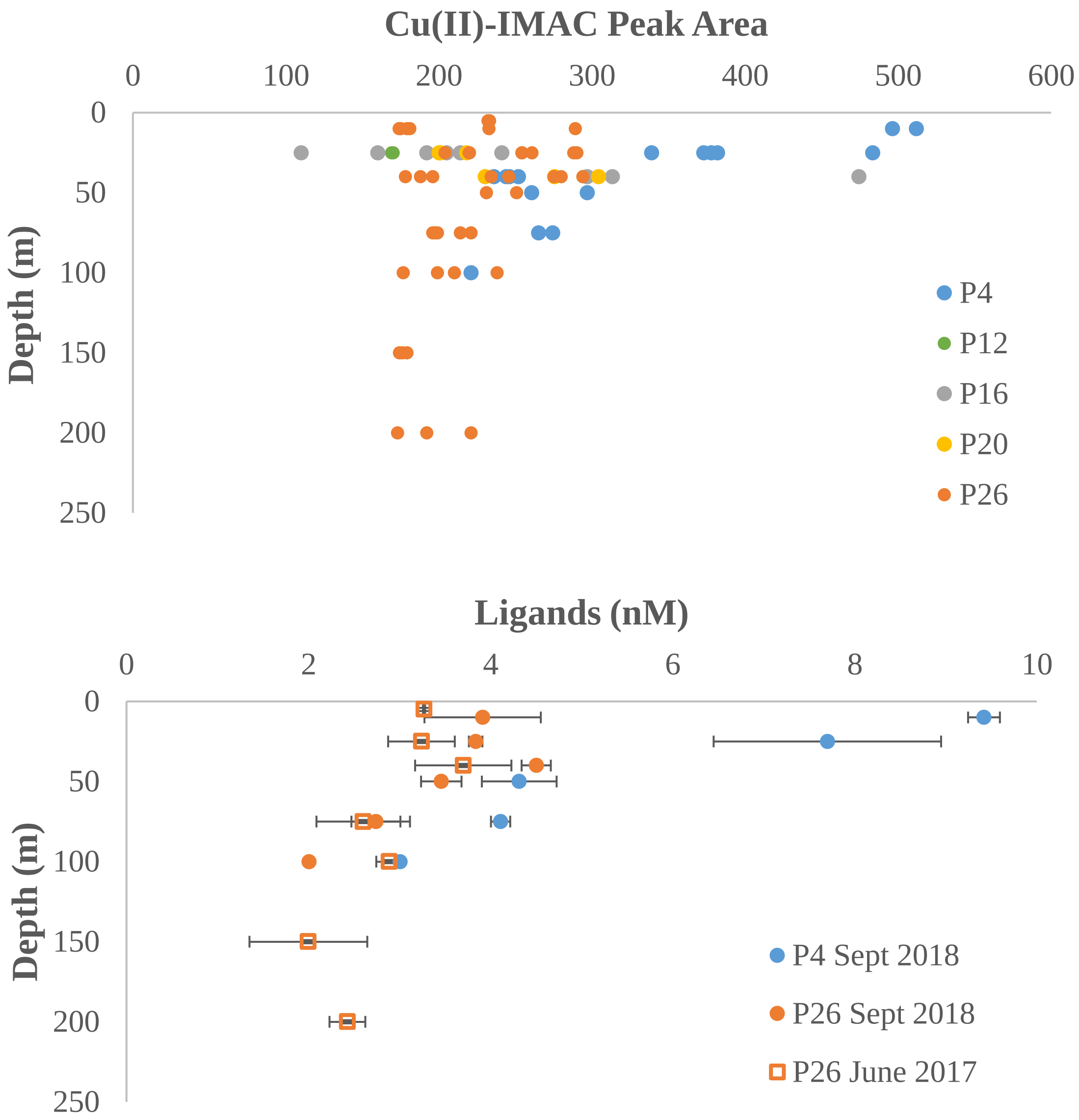


Figure 4.2 Depth profiles of Cu ligand abundance measured by Cu(II)-IMAC in samples from Line P. Upper panel: Cu(II)-IMAC peak area from all stations and all cruises. Lower panel: Cu ligand depth profiles at coastal station P4 and oceanic station P26 during the June 2017 and September 2018 cruises with standard deviations shown by error bars.

Cu ligand concentrations were consistently higher at P4 than all other stations, where a near-surface (10 m) maximum of 9.4 nM measured in September 2018 was the highest [L] reported in this study. Ligand concentrations observed during this cruise decreased with depth, reaching 7.7 nM at 25 m, 4.3 nM at 50 m, and 3 nM at 100 m. Similar values were measured at 25 m (6.3 nM) in August 2017 and at 40 m (3.5 nM) in June 2016. At P26, Cu ligand levels reached a maximum at a depth of 40 m in September 2018 (4.5 nM) and 50m in June 2017 (3.7 nM). Ligand content at P26 ranged from 2.0 to 4.5 nM across all cruises.

Samples were collected at a single depth from five stations during the August 2017 and September 2018 cruises to examine the horizontal distribution of ligands along Line P. Coastal station P4, and to a lesser extent oceanic station P26, showed elevated ligands levels relative to all other stations at 25 m in August 2017. Ligand levels ranged from 4.1 nM (P20) to 6.2 nM (P16) across all stations at 40 m in September 2018.

4.3.2 Comparison with other data

Measurements of chlorophyll a fluorescence, both in bottle samples and *in situ*, from the August 2017 cruise are presented in Figure 4.3. Coastal station P4 exhibited a chlorophyll maximum (2.8 mg/m^3, 1.6 ug/L) at 15 m while a deeper chlorophyll maximum depth of 25 m (3.6 mg/m^3, 1.3 ug/L) was observed at oceanic station P26. Intermediate stations generally had lower levels of chlorophyll and deeper maxima closer to 40 – 50 m. These data are largely comparable to measurements taken at each station during the September 2018 cruise (data not shown).

Linear regressions were used to determine the correlation between Cu ligand abundance in depth profiles of P4 and P26, and biologically relevant parameters including chlorophyll fluorescence (bottle and *in situ*), phaeopigment, dissolved oxygen, and DMS (Fig. 4.4 and 4.5).

Cu ligand concentrations at P4 showed a strong positive correlation with bottle chlorophyll (R^2=0.98), *in situ* fluorescence (R^2=0.96), and phaeopigment (R^2=0.90) (Fig. 4.4). Y-intercepts for these regressions range from 2.6 to 3.6 nM.

Cu ligand concentration at P26 were positively correlated with bottle chlorophyll (R^2=0.42) and *in situ* fluorescence (R^2=0.37) but not with phaeopigment concentration (Fig. 4.4). Y-intercepts for these regressions are 2.3 and 2.5 nM.

No significant correlation was observed between Cu ligand abundance and dissolved oxygen or DMS (Fig. 4.5).

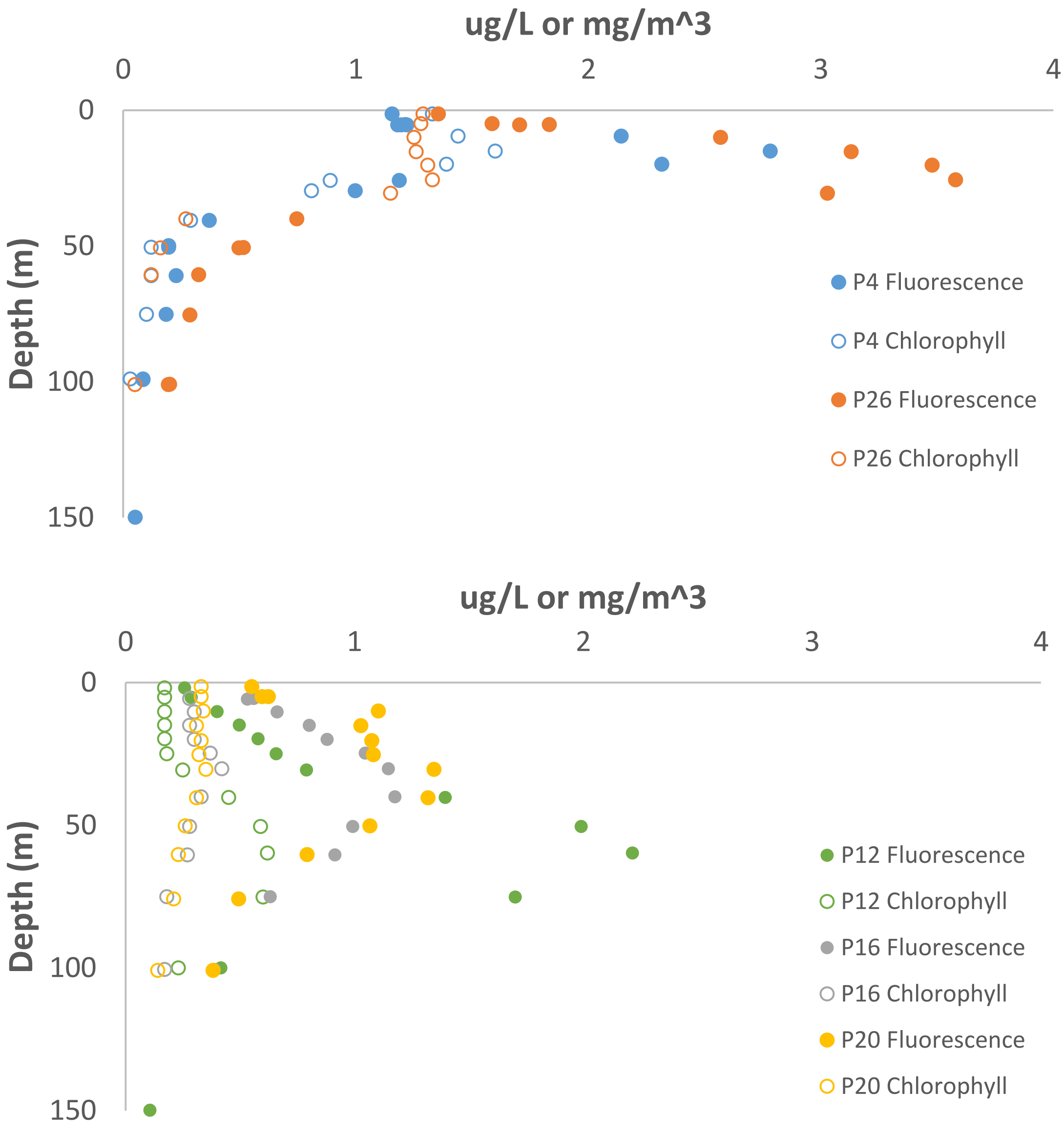


Figure 4.3 Depth profiles of *in situ* fluorescence (mg/m^3) and extracted chlorophyll (ug/L) at five Line P stations measured during the August 2017 cruise.

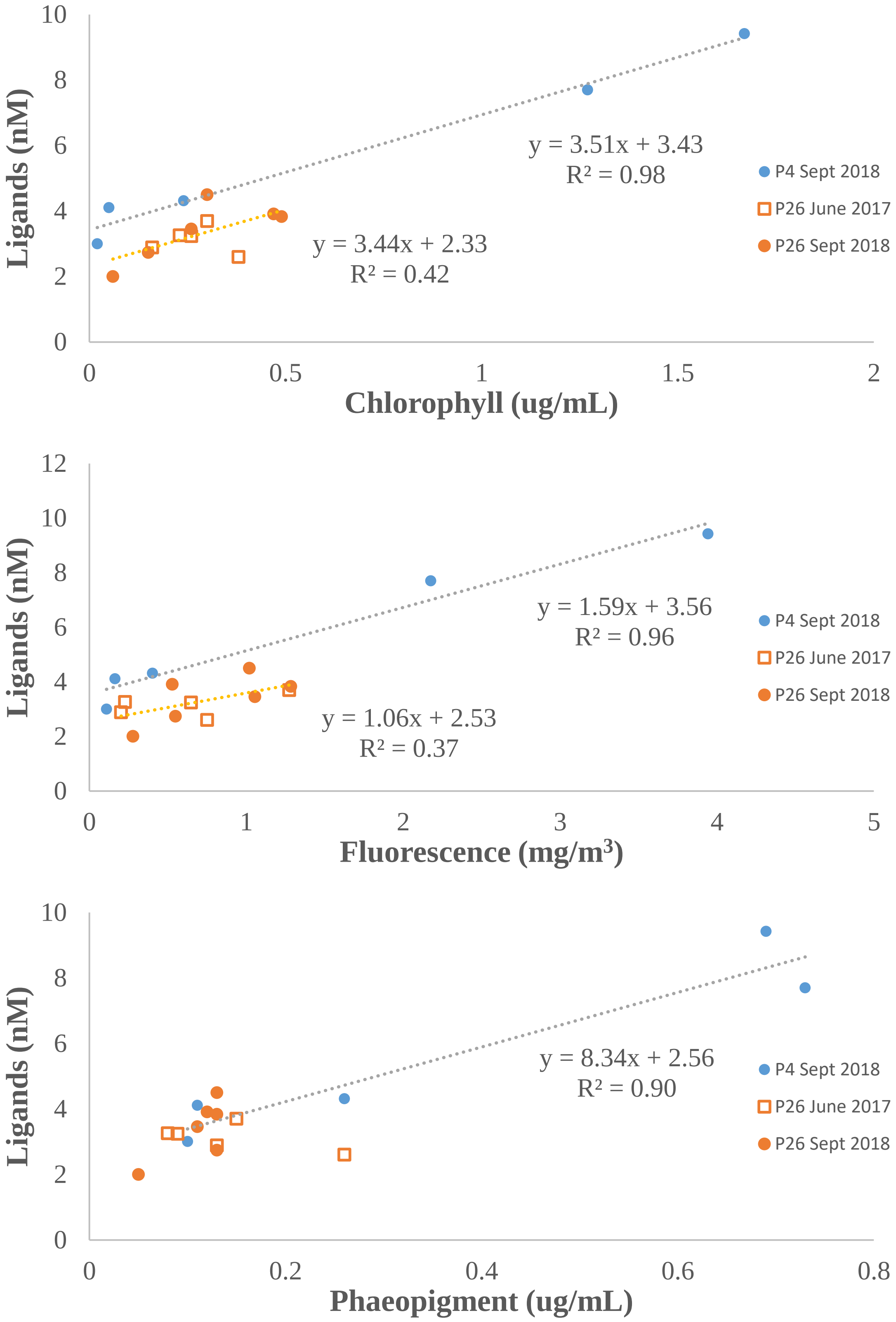


Figure 4.4 Covariance of [L] as determined by Cu(II)-IMAC with chlorophyll, *in situ* fluorescence and phaeopigment at stations P4 and P26 during June 2017 and September 2018.

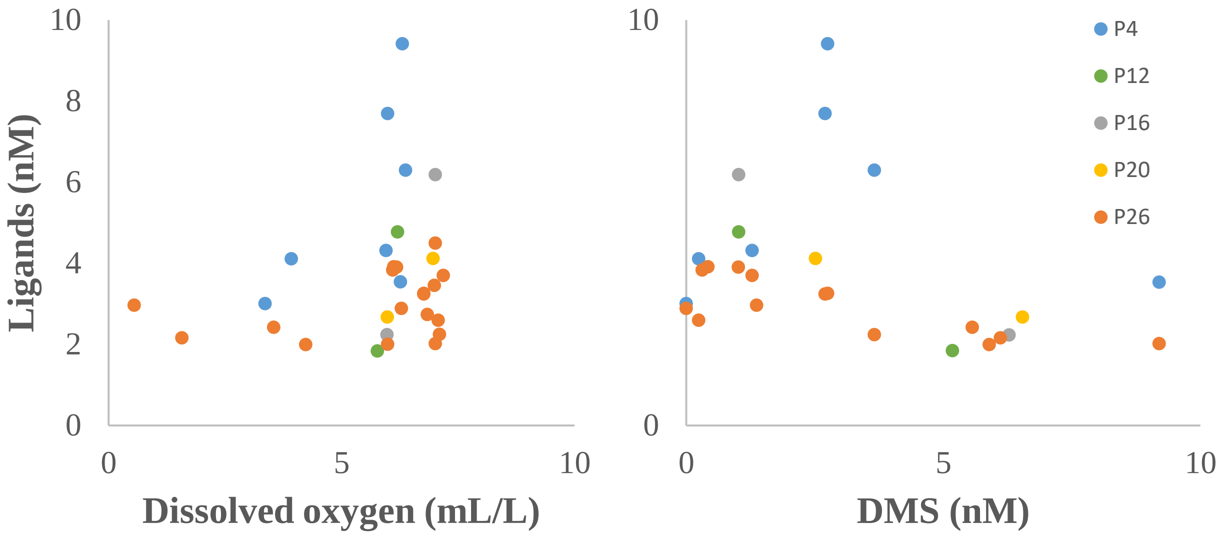


Figure 4.5 Covariance of [L] as determined by Cu(II)-IMAC with dO_2 and DMS across all Line P stations and cruises.

4.4 Discussion

4.4.1 Cu ligand distribution

Copper ligand concentrations measured by Cu(II)-IMAC in this study ranged from 1.8 and 9.5 nM with most samples falling between 2.0 and 4.0 nM, comparable to results from our study of Canadian Arctic Ocean samples (Chapter 3: Nixon *et al.*, 2019) and previous electrochemical studies of Cu ligand concentration in the NE Pacific (Coale & Bruland, 1988, 1990). The highest Cu ligand levels were observed in surface samples, particularly those at coastal station P4. Depth profiles taken at oceanic station P26 during cruises in June 2017 and September 2018 were similar, suggesting low inter-annual variability.

Depth profiles of copper ligands at stations P4 and P26 were previously assessed using voltammetry on samples collected during a 2012 cruise (Whitby *et al.*, 2018) which found L_1 copper ligand levels ranging from 1 to 4 nM, following similar profiles but at lower abundance than our estimates by Cu(II)-IMAC. Whitby *et al.* also measured slightly higher surface concentrations of L_1 ligands at P4 relative to P26, and a similar depth profile to ours at P4, but a quite dissimilar depth profile at P26. Discrepancies may reflect differences in the ligand pools measured by CSV relative to Cu(II)-IMAC.

4.4.2 Cu ligand covariance with other parameters

We investigated potential sources of Cu ligands by plotting ligand concentration against other data collected during each cruise (Fig. 4.4 and 4.5). Chlorophyll and phaeopigment concentration, which represent phytoplankton biomass and grazing by zooplankton respectively (Taguchi *et al.*, 1993), were assessed in discrete bottle samples while fluorescence was measured *in situ*. Covariance was measured based on linear regression analyses.

Coastal station P4 and oceanic station P26 are subject to different inputs, so ligand concentration covariance with chlorophyll, phaeopigment, and *in situ* fluorescence was assessed for P4 and P26 separately (Fig. 4.4). [L] was more strongly associated with chlorophyll, phaeopigment, and fluorescence at P4 than at P26, suggesting phytoplankton are a predominant source of Cu ligands in coastal waters but not in the open ocean. Slopes of covariance with chlorophyll and fluorescence are similar between the two stations, suggesting phytoplankton at both stations contribute similar amounts of Cu ligands relative to their productivity. Y-intercepts of these regressions show P4 has higher extrapolated [L] in the absence of both chlorophyll and fluorescence, consistent with more inputs of ligands from sources other than phytoplankton including terrestrial humic substances.

In general, Cu ligand levels reached a maximum coincident with the chlorophyll maximum depth. This agrees with our findings in the Arctic Ocean (Nixon et al., 2019) and suggests that a portion of the Cu ligand pool recovered by Cu(II)-IMAC is produced *in situ* by growing phytoplankton communities. Stronger correlations between [L] and chlorophyll at coastal station P4 than at oceanic station P26 may also reflect contributions of Cu ligands from riverine inputs which also contain components that boost primary productivity.

As DMS is a phytoplankton byproduct which has been associated with metal stress (Sunda *et al.*, 2002) we had expected to observe a correlation between copper ligand levels and DMS. We observed a negative correlation (Fig. 4.5) that could suggest phytoplankton populations which produce more DMS also produce fewer copper ligands, potentially as a consequence of zooplankton grazing, but this correlation was not statistically significant. Dissolved oxygen, also produced by phytoplankton, had no correlation with Cu ligand levels.

4.5 Conclusion

Understanding potential sources and diversity of copper-complexing components within marine DOM helps guide future studies into sampling and characterizing potential chalkophores. Cu ligand concentration at coastal station P4 had strong correlations with indicators of primary productivity including *in situ* chlorophyll fluorescence and extracted chlorophyll a, suggesting growing phytoplankton communities are a significant source of copper ligands. Weaker correlations at oceanic station P26, and extrapolations from linear regressions, suggest sources other than phytoplankton may contribute Cu ligands to the total DOM pool.

5.0 Conclusion

5.1 Summary of results

The development and application of Cu(II)-IMAC for the characterization of natural copper ligands in seawater have been demonstrated successfully in this dissertation. These data contribute to the available literature on marine Cu ligands and carefully explore both proposed hypotheses.

In Chapter 2, the model ligand 8HQ is used to evaluate and refine Cu(II)-IMAC-SPE-MS/MS protocols for recovery and unambiguous quantitation of low abundance, low molecular weight marine organic compounds capable of forming stable complexes with Cu^{2+}.

Appendix 6.1 extends this work, demonstrating simultaneous recovery and quantitation of five diverse model ligands using a refined set of protocols. Results suggest that combining HPLC separation with SPE using PPL and/or MCX resin prior to high-resolution MS or MS/MS analysis should enhance recovery and promote identification of natural copper ligands in seawater or marine phytoplankton cultures. Chapter 2 and Appendix 6.1 together address the first hypothesis of this dissertation, presenting chromatographic and spectroscopic techniques for recovery and characterization of natural copper-binding ligands in seawater.

Cu(II)-IMAC is used to assess the distribution of copper ligands in the Canadian Arctic Ocean in Chapter 3, and in the NE Pacific Ocean in Chapter 4. Consistent depth profiles are observed in geographically related sampling sites, while profiles differ significantly between unrelated sites. Copper ligand concentrations estimated using Cu(II)-IMAC are comparable to estimates made by CSV.

Covariance plots of copper ligand distribution with biogeochemical data are presented in Chapters 3 and 4 to investigate potential ligand sources. These data suggest that marine phytoplankton are likely a significant source of strong Cu ligands in seawater, but that other sources, including allochthonous DOM and terrestrial inputs, also exist.

Preliminary investigations into culturing phytoplankton in artificial seawater media for copper ligand analysis by Cu(II)-IMAC are presented in Appendix 6.2. These suggest that *Phaeodactylum tricornutum* may be a good candidate for producing Cu ligands in sufficient quantities for identification using the methods described in Chapter 2 and refined in Appendix 6.1.

Cu(II)-IMAC fractions of natural seawater samples have been extracted by SPE and are awaiting analysis. Once an HPLC-MS/MS protocol has been established, this library of extracts will provide valuable material for high-throughput analysis of a wide diversity of natural seawater samples during future studies.

5.2 Relevance

Copper has a variety of unique relationships with aquatic ecosystem health, all of which are moderated by organic complexation. This dissertation examines Cu primarily through a biological lens: in particular, how phytoplankton community distribution, composition, and productivity rely on a tolerable level of bioavailable copper. Biological roles of copper in the marine environment are relevant to aquaculture management, anthropogenic activity, and perhaps the future of climate engineering.

Phytoplankton communities support the marine food web and thus have an obvious impact on fishing and shellfish industries, but these organisms also have widespread utility outside their natural ecosystems. Artificial cultures of phytoplankton, which require inputs of organically complexed Cu, are used as feed for farmed finfish and shellfish, while genetic engineering of pesticide-resistant cyanobacteria has been explored for inland agricultural purposes. Algae have been used in treatment plants to reduce anthropogenic pollution into natural ecosystems. Healthy algal communities are essential for maintaining coral and cetacean-based tourism, while hobbyist pond owners and aquarists intentionally grow algae to stabilize artificial ecosystems. Other industries have also explored algal cultures for the production of cosmetics and biopharmaceuticals including antibiotics. All of these economic and ecological applications could benefit from enhanced understandings of mechanisms that maintain metal homeostasis.

Anthropogenic release of copper into aquatic ecosystems represents a potentially serious threat to biological communities. Most importantly, industrial release of copper into the atmosphere and water systems is the primary source of new copper into the ocean. Potential impacts of Cu pollution on marine phytoplankton continue to result from the frequent use of copper in industrial and household algaecides. Governmental policies regulating this sort of pollution would likely be ineffective if organic complexation were not taken into account. However, intentional negative regulation of algal growth by exploiting the toxicity of free Cu^{2+} could be explored as an avenue to mitigate damage associated with harmful algal blooms. Conversely, if Cu^{2+} is a limiting nutrient in natural or cultivated phytoplankton communities, inputs of appropriately organically complexed Cu(II) may be desirable to stimulate growth. These policies and strategies all benefit from a comprehensive understanding of copper-algae interactions.

Organic complexation of dissolved copper in seawater plays a broader role than can be covered here. Geochemical partitioning of Cu between water masses, into sediments, and between DOM and non-biotic POM are influenced by organic speciation. This may also be true for atmospheric deposition of copper and/or loss by ocean spray. Studies into how organic speciation impacts the geochemical mobility of dissolved Cu would benefit physical oceanographers in development of accurate computer models for the depiction and forecast of trace metal circulation and partitioning in the environment. Organic ligands may also modulate isotopic fractionation of $^{65}Cu/^{63}Cu$ within organic or inorganic pools. High-affinity metal-binding compounds may actually play other unknown roles – siderophores, for example, have been shown to have antimicrobial or drug resistance activity. Research into these possibilities would be simplified if protocols for their recovery and characterization reached a higher level of development.

5.3 Future Directions

Much remains to be understood about the sources, ecological roles, structures, and distribution of copper-binding components of marine DOM. Developing methods to accurately and unambiguously quantify individual Cu ligands in natural seawater or algal cultures is crucial for meaningful characterization of their biogeochemical properties.

Distribution profiles of Cu ligands in natural seawater samples can provide insight into their sources. Correlative analyses, including those presented here in Chapters 3 and 4, can associate ligand content with other relevant biogeochemical properties. Future studies could correlate individual Cu ligands or subsets of them with biological taxa or markers of terrestrial inputs. Sample collection could also be expanded geographically, to new ocean ecosystems such as estuaries and hydrothermal vents, and temporally, to examine diurnal or seasonal variations in Cu ligand abundance. Future studies should also investigate the impact of freezing samples on ligand content. Assessing ligand content in seawater immediately after sampling and filtration relative to samples frozen and thawed could be achieved using shipboard instruments.

Cultures of marine phytoplankton in natural or artificial media will also be essential to evaluate the ecological roles of Cu ligands. Initial studies might focus on determining environmental factors stimulating ligand production such as Cu stress or limitation. Impacts of organic Cu speciation on primary production and cellular uptake mechanisms could be assessed with additions of known ligands and radiolabeled ^{67}Cu to culture media. Microbiological analyses could determine if ligands are cellular exudates or lysis products. Transcriptomic assays could identify potential genetic elements responsible for ligand production.

Cu(II)-IMAC in this study quantified ligand content based on absorbance at 254 nm. Incorporating a multiple wavelength diode-array detector would significantly expand the analytical potential of IMAC. Peak areas at different wavelengths could reflect distinct pools of Cu ligands recovered from samples, facilitating fraction collection and permitting distribution analyses of each of these pools among seawater or culture media samples.

Voltammetry of Cu(II)-IMAC fractions and SPE extracts could provide Cu ligand binding affinity and concentration estimates to further develop these protocols. IMAC and SPE recovery bias and efficiency could be estimated by examining the abundance of ligand classes in fractions and extracts relative to unfractionated seawater.

Determining the molecular structures of marine Cu ligands will most likely be achieved by a combination of chromatography and mass spectrometry. High-resolution MS methods excel at identifying molecular formulae (Boiteau *et al.*, 2016) while MS/MS is necessary for unambiguous structural characterization (Nixon & Ross, 2016). As a caveat, off-line pre-concentration steps such as Cu(II)-IMAC and SPE, and on-line HPLC separations, have the potential to bias results in favour of easily recoverable ligands. Preparing pure standards of identified ligands would be helpful as these could be used as internal standards to assess recovery efficiency and permit accurate quantification.

6.0 Appendices

6.1 Appendix A: Refining Cu(II)-IMAC-SPE-HPLC-MS/MS

Contributions: I trained and directed co-op student Jacob Davis, who conducted most of the experiments and data analysis. I wrote the discussion.

Cu(II)-IMAC-SPE-MS/MS was developed using the model ligand 8-HQ as described in Chapter 2 for the recovery and characterization of natural Cu ligands from seawater. Here, these protocols are refined using multiple model ligands in an attempt to maximize recovery of a potentially diverse set of natural ligands.

6.1.1 Model ligands

Model copper-binding ligands were selected from a study of Cu(II)-IMAC for fractionation of soil solutions (Paunovic *et al.*, 2005). The five compounds shown in Figure 6.1 were selected for the development of a multiplexed Cu(II)-IMAC-SPE-MS/MS protocol; although an assortment of other model ligands were tested, these will not be discussed here.

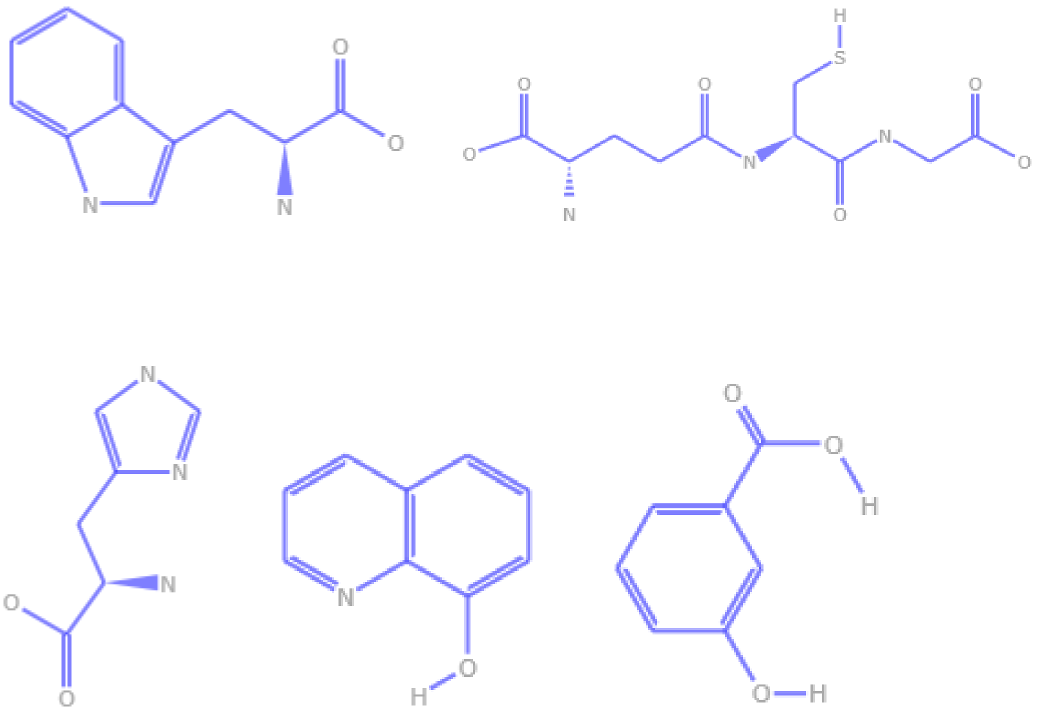


Figure 6.1 Structures of model ligands recovered by Cu(II)-IMAC. Trp (top left), GSH (top right), His (bottom left), 8HQ (centre), and SHA (bottom right) are depicted.

6.1.2 Cu(II)IMAC

Natural seawater samples are likely to contain a wide range of copper ligands which are retained on a Cu(II)-IMAC column. Recovering all of these in a single fraction would be problematic if retention times of these ligands are considerably different.

A five ligand mixture (5LM) containing model ligands with a range of functional groups (8HQ, GSH, SHA, Trp, His) was added to artificial seawater to assess Cu(II)-IMAC for multiplexed recovery of a diverse set of copper ligands. Figure 6.1 shows that chromatograms taken at three wavelengths all share a single major peak with retention time of approximately 14-18 minutes. The presence of a single peak suggests two important things: collecting a single fraction representing the major peak may suitably recover a meaningful proportion of the total ligand pool; and Cu ligands with significantly different copper binding affinities may have similar retention times.

6.1.3 SPE-MS/MS

A variety of SPE resins, each capable of recovering a different subset of organic compounds based on their sorbent groups, are available for solid-phase extraction of aqueous mixtures. Protocols for fractionation and recovery of a wide variety of analytes can be developed by sequentially adjusting the pH or polarity within the resin to form or break interactions between analyte and resin to isolate desired compounds from contaminants.

Sorbents may contain acidic or basic functional groups capable of interacting with cations or anions, respectively. Strong cation exchange (SCX) and weak cation exchange (WCX) sorbents are available, as are their anionic equivalents SAX and WAX. Sorbents may also have complex structures. Hydrophobic-lipophilic balanced (HLB), mixed-mode cation exchange (MCX) and BondElut Priority PolLutant (PPL) resins contain stationary phases with both polar and hydrophobic character. These sorbents are frequently used for concentration of marine DOM for downstream analysis (Sandron *et al.*, 2015).

We sought to determine which SPE sorbent type would be ideal for extracting natural copper ligands from Cu(II)-IMAC fractions by using five model ligands. Up to 50 µM of the 5LM was added to 2mL of acidified artificial seawater (IMAC elution buffer), and extractions with each sorbent were manually optimized to maximize recovery of all ligands. Figure 6.3 shows the results of these experiments, which highlight the importance of sorbent selection. PPL, HLB, and MCX sorbents were shown to recover at least three of five ligands appreciably. Though MCX recovered all five ligands, losses averaged more than 50%. These data suggest that MCX, PPL, or a combination of multiple sorbents should be used to maximize Cu ligand extraction from Cu(II)-IMAC fractions.

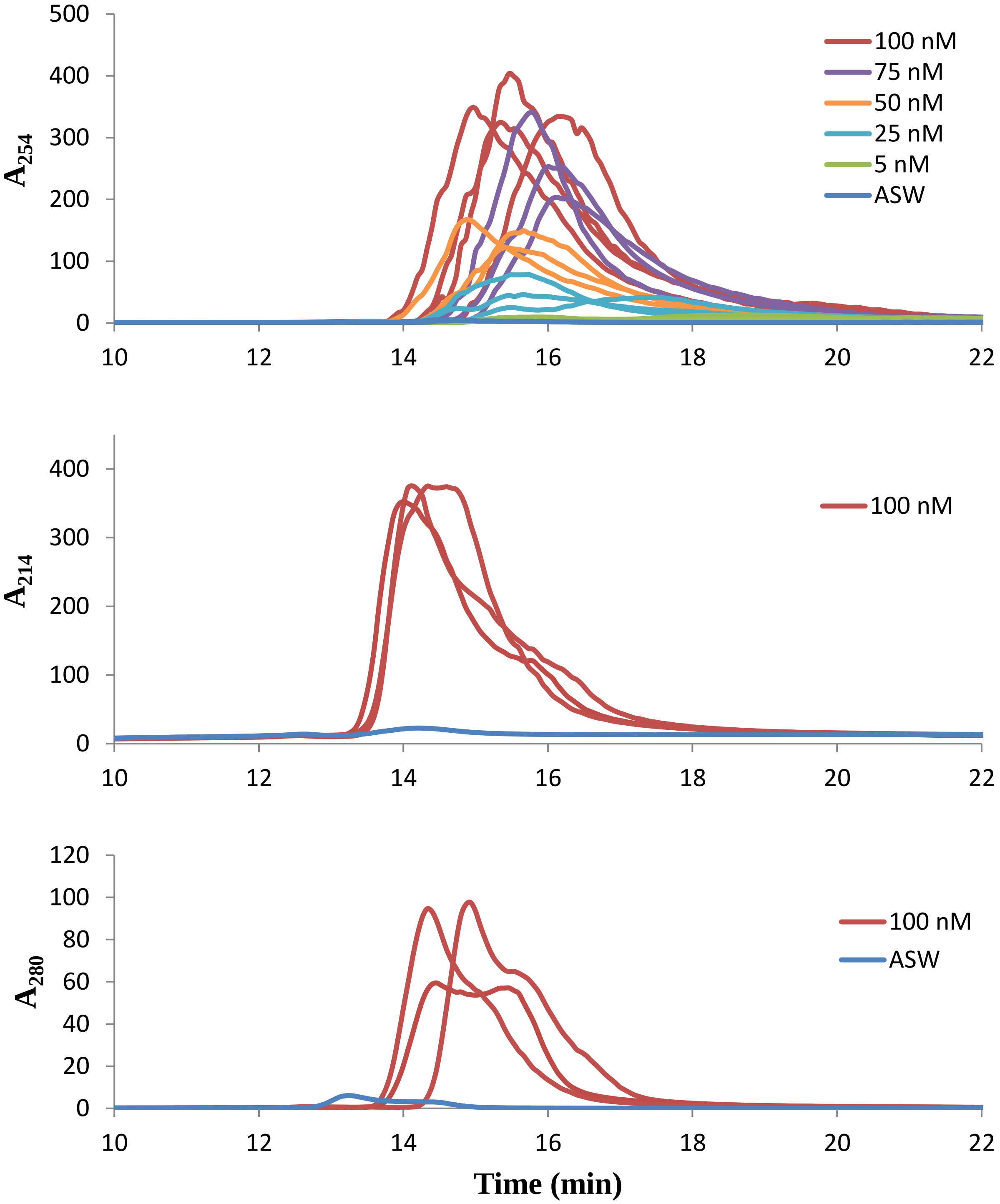


Figure 6.2 Cu(II)-IMAC chromatograms at three wavelengths of a mixture of model ligands in artificial seawater. 1 L of ASW was spiked with up to 100 nmoles each of 8HQ, Trp, His, SHA, and GSH before fractionation and acid elution. (214, 280: 0 or 100 nmol, AKTA system; 254: 0, 25, 50, or 100 nmol, LKB system; see Chapter 2 for a description of each system).

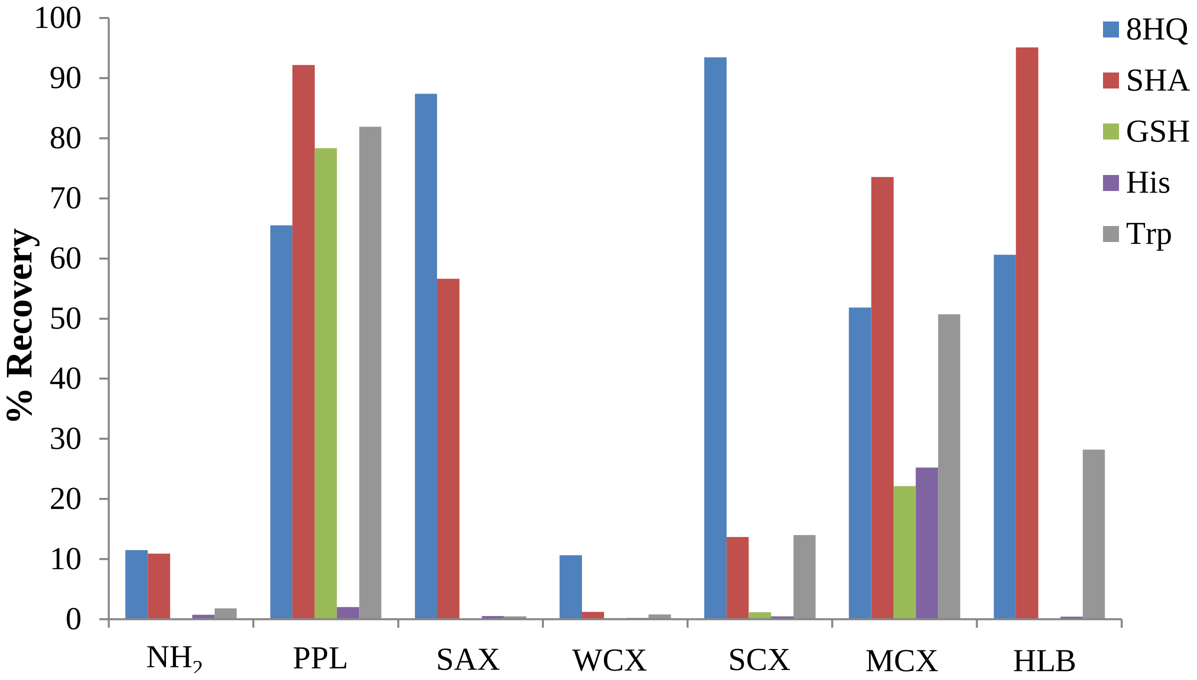


Figure 6.3 Recovery of five model ligands by different SPE sorbents. 8HQ, SHA, GSH, His, and Trp were each spiked into 2 mL acidified artificial seawater and extracted using optimized protocols for each sorbent. Recovery is given as % signal relative to 5LM prepared at equivalent concentrations in the SPE elution buffer.

To assess potential matrix and solvent effects on ionization during MS/MS, combinations of MS carrier solvents and SPE elution buffers were evaluated in terms of their capacity to detect and quantify multiple copper ligands simultaneously. The 5LM was added to each SPE sample matrix and four ligands (8HQ, GSH, His, Trp) were quantified by MS/MS in each mobile phase, as shown in Table 6.1. SHA was not included in these analyses due to poor ionization in positive-ion mode. These data show that while mobile phase choice is relatively unimportant, some SPE elution buffers strongly interfere with ionization. Organic and acidic elution buffers (e.g. 2% formic acid in methanol) were the most efficient at ionizing all four ligands.

Table 6.1. MS/MS detection of model ligands prepared in different SPE elution buffers and monitored using different mobile phases. 100 nmoles each of 8HQ, GSH, Trp, and His were added to 2 mL of SPE sample matrix and quantified using optimized parameters by MS/MS. Recovery is colour-coded: green, >50%; yellow, >10%; red, <10%. NOAC: ammonium acetate; FA: formic acid; MeOH: methanol; ACN: acetonitrile; N.D.: no data.

		MS/MS Mobile Phase							
Solid phase extraction sample matrix	**Ligand**	0.1% FA in 70% ACN	1 mm NOAc in 70% ACN	2 mM NOAc in 70% ACN	50% ACN	1 mM NOAc +0.1% FA in 70% ACN	1 mM NOAc +0.1% FA in 50% ACN	1 mM NOAc +0.1% FA in 20% ACN	1 mM NOAc +0.1% FA
2% FA in 10% MeOH	8HQ	🟩	🟩	🟩	🟩	🟩	🟩	🟩	🟩
	GSH	🟨	🟨	🟥	🟥	🟥	🟥	🟥	🟥
	His	🟩	🟩	🟨	🟨	🟩	🟨	🟨	🟨
	Trp	🟩	🟩	🟨	🟨	🟩	🟨	🟨	🟨
2% FA in MeOH	8HQ	🟩	🟩	🟨	🟩	🟩	🟩	🟩	🟩
	GSH	🟩	🟩	🟨	🟩	🟨	🟩	🟨	🟨
	His	🟩	🟩	🟩	🟩	🟩	🟩	🟩	🟩
	Trp	🟩	🟩	🟩	🟩	🟩	🟩	🟩	🟩
Methanol	8HQ	🟩	🟩	🟨	🟩	🟩	🟩	🟩	🟩
	GSH	🟨	🟨	🟥	🟨	🟥	🟨	🟨	🟥
	His	🟩	🟨	🟩	🟩	🟩	🟩	🟩	🟩
	Trp	🟩	🟨	🟩	🟩	🟩	🟩	🟩	🟩
70% ACN	8HQ	🟩	🟨	🟩	🟩	🟩	🟩	🟩	🟩
	GSH	🟨	🟨	🟨	🟨	🟥	🟨	🟨	🟥
	His	🟩	🟩	🟩	🟩	🟩	🟩	🟩	🟩
	Trp	🟩	🟩	🟩	🟩	🟩	🟩	🟩	🟩
50% ACN 50% MeOH	8HQ	🟩	🟩	🟨	🟩	🟩	🟩	🟩	🟩
	GSH	🟨	🟥	🟥	🟨	🟥	🟨	🟨	🟥
	His	🟩	🟩	🟨	🟩	🟨	🟩	🟩	🟨
	Trp	🟩	🟨	🟨	🟩	🟩	🟩	🟩	🟩
0.5 M NH4OH in 10% MeOH	8HQ	🟨	🟨	🟥	N.D.	N.D.	N.D.	N.D.	N.D.
	GSH	🟥	🟥	🟥					
	His	🟩	🟥	🟥					
	Trp	🟥	🟥	🟥					
5% NH4OH in ACN/ MeOH	8HQ	🟥	🟥	🟥					
	GSH	🟥	🟥	🟥					
	His	🟥	🟥	🟥					
	Trp	🟥	🟥	🟥					

6.1.4 HPLC

Detecting low abundance unknown compounds is a difficult task using MS/MS, even after multiple pre-concentration steps. To enhance signal-to-noise ratio, on-line HPLC was explored to further fractionate Cu(II)-IMAC-SPE extracts. As shown by preliminary results in Figure 6.4, appropriate chromatographic columns and protocols can result in efficient separation of model Cu ligands.

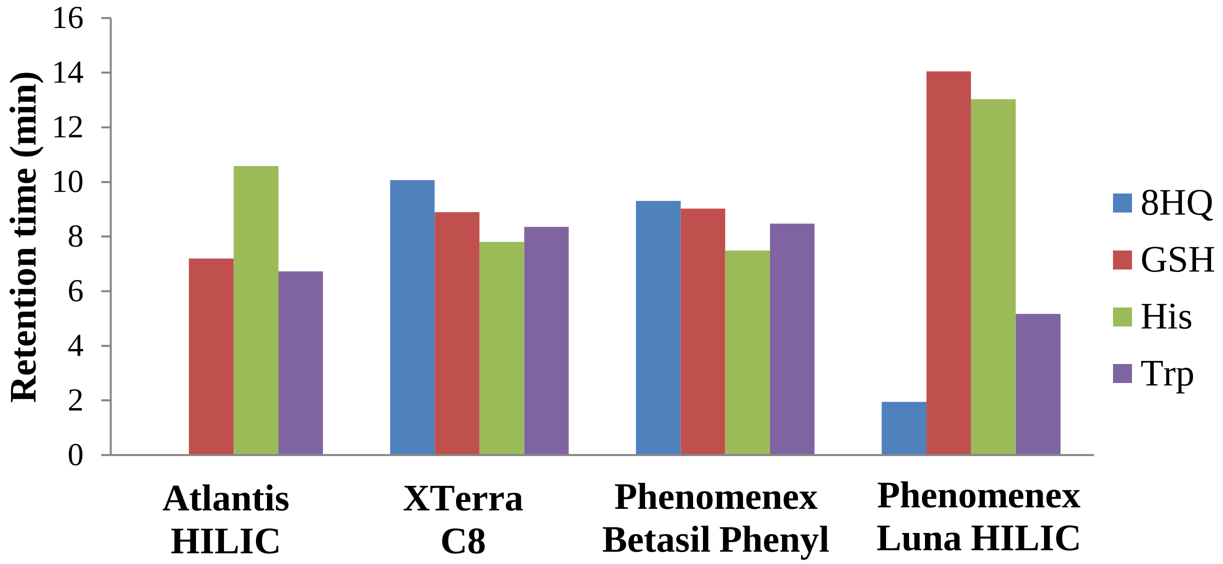


Figure 6.4 Retention times of model ligands fractionated by different HPLC columns, monitored by MS/MS.

Further studies into on-line HPLC separation of Cu(II)-IMAC-SPE samples prior to ESI-MS/MS should refine protocols and column selection for optimal recovery and separation of natural Cu ligands. These improvements could be facilitated through the use of a ligand mixture or mixtures of DOM components such as humic acid standards.

6.2 Appendix B: Culturing diatoms for copper ligand analysis

Contributions: I designed and established the culturing laboratory. I trained and directed co-op student Jasper George, who conducted most of the experiments and data analysis. I wrote the the discussion.

6.2.1 Trace metal clean marine diatom culturing with Aquil media

To further investigate the biological origin of Cu ligands, and potentially obtain enough material for ligand identification, two species of marine phytoplankton were grown and harvested in late log-phase for analysis by Cu(II)-IMAC.

Thalassiosira pseudonana (CCMP 1335) and *Phaeodactylum tricornutum* (CCMP 630) were obtained from Laurie Keddy at the DFO Pacific Biological Station in Nanaimo, BC for culturing experiments. These diatoms were chosen because their genomes are annotated and they are relatively robust and simple to grow in culture. *P. tricornutum* in particular was chosen as a previous study noted production of copper-complexing organic material by this organism, particularly in the stationary phase, though this study did not directly measure copper complexation (Zhou & Wangersky, 1985).

Because copper ligand production is associated with metal stress, diatom cultures were grown in a culture media with well-defined levels of trace metals. Aquil medium was prepared using a modified protocol based on the original recipe (Morel *et al.*, 1979). Artificial seawater was prepared as described in Table 6.2, gravity-filtered (0.45 µm) and cleaned of trace metals using a Chelex-100 column. Culture media was sterilized by boiling in a microwave, after which vitamins and trace metals were added. Cu^{2+} levels in media used to induce metal stress were ‘low Cu’ (no added $CuCl_2$, estimated pCu ~16) to ‘high Cu’ (estimated pCu 11), while cultures being maintained without metal stress were kept at ‘optimal Cu’ (pCu 14) based on previous work (Maldonado, personal communication).

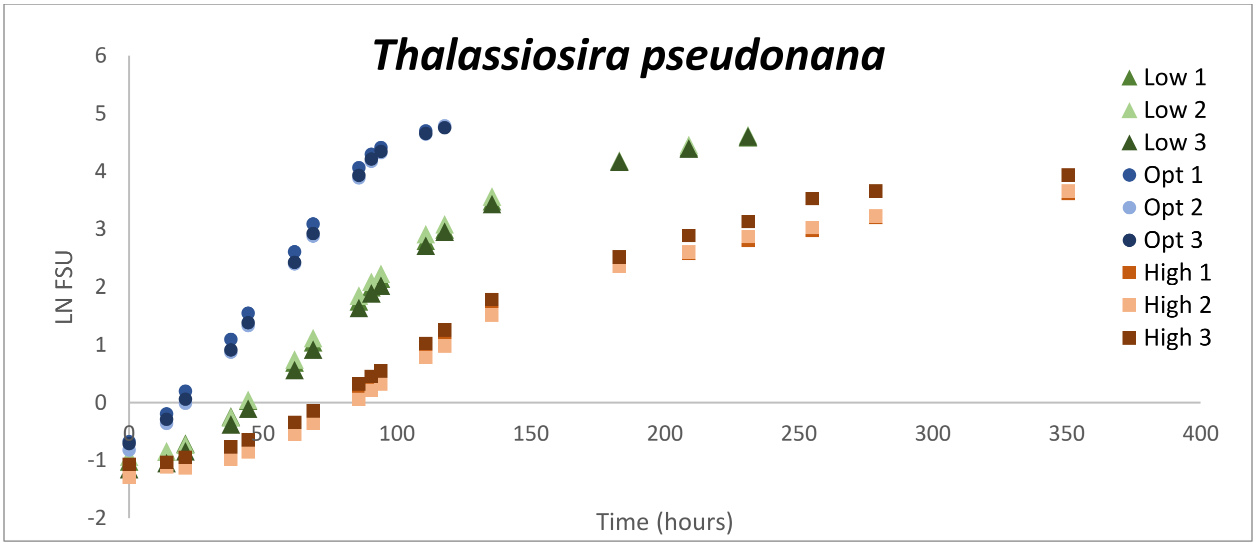
Both species of diatom were maintained in log phase by inoculating a small volume (10-100 uL) of culture into 25 mL of fresh ‘optimal Cu’ media approximately once weekly and incubating cultures at room temperature under 24-hour light with PAR values of ~105 $\mu E\ m^{-2}\ s^{-1}$. Growth was monitored based on chlorophyll fluorescence using a Turner 10-AU fluorometer (in fluorescence standard units, FSU). Growth curves plotting ln FSU against time (Fig. 6.5) were assessed over multiple generations to calculate doubling times and determine when cultures were in log phase.

Table 6.2 Phytoplankton culture media recipe based on Aquil media.

Component	Molarity in media	Mass (g) added per 20L media
NaCl	4.20E-01	**490.90**
Na_2SO_4	2.88E-02	**81.82**
KCl	9.39E-03	**14.00**
$NaHCO_3$	2.38E-03	**4.00**
KBr	8.40E-04	**2.00**
H_3BO_3	4.85E-04	**0.60**
$MgCl*6H_2O$	5.46E-02	**222.00**
$CaCl_2*2H_2O$	1.05E-02	**30.87**
$SrCl_2*6H_2O$	6.38E-05	**0.34**
$NaH_2PO_4*H_2O$	1.00E-05	**0.03**
$Na_2SiO_3*9H_2O$	1.00E-04	**0.57**
NaF	7.14E-05	**0.06**
NaNO3	3.00E-04	**0.51**
$FeCl_3$	1.37E-04	*Added 1 mL/L of a 1000x mixture of 6 metals in 100 µm EDTA*
K_2MoO_4	1.00E-07	
$MnCl_2$	1.10E-07	
CoCl	4.42E-08	
$ZnSO_4$	7.00E-08	
Na_2SeO_3	7.00E-08	
$CuCl_2$	varies	

6.2.2 Growth rates of *T. pseudonana* and *P. tricornutum* at a range of pCu^{2+}

Culture media containing increasing levels of copper were prepared in an attempt to induce metal stress in two marine diatoms and determine their growth characteristics in 25 mL cultures. *T. pseudonana* exhibited a significant reduction in growth rates when subcultured into ‘low Cu’ or ‘high Cu’ relative to ‘optimal Cu’ media, suggesting these preparations of Aquil media are capable of inducing both Cu stress and Cu limitation (Fig. 6.5). *P. tricornutum* did not experience any reduction in growth rate regardless of pCu, suggesting this organism is either less sensitive to Cu^{2+} or has mechanisms allowing it to tolerate levels that would otherwise be toxic. Such mechanisms may include the production and release of copper-complexing ligands to regulate the availability of Cu^{2+} in the growth medium.



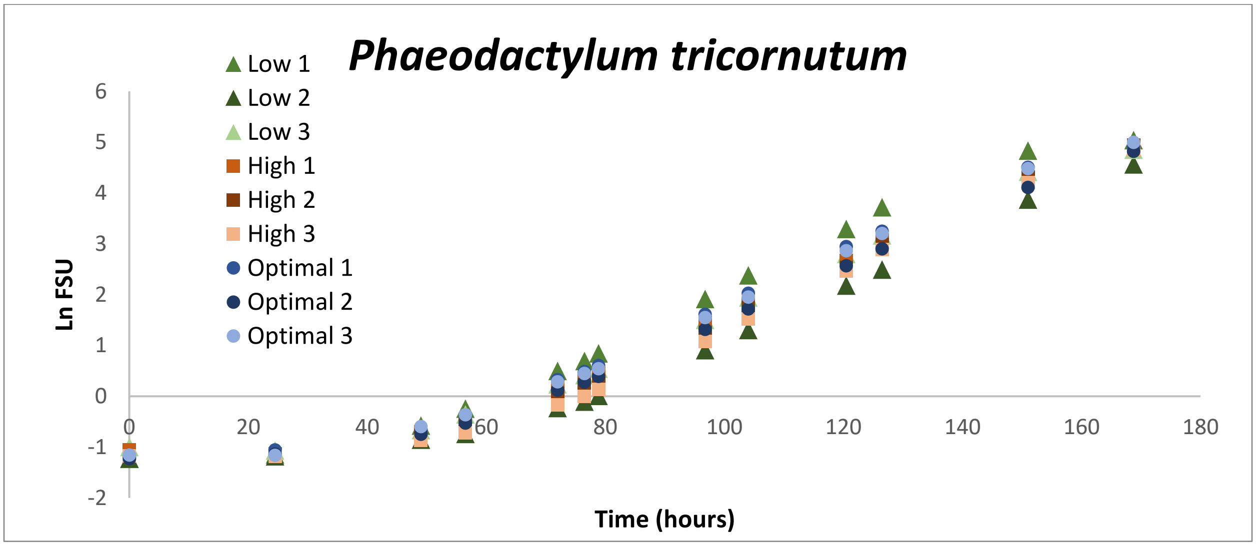


Figure 6.5 Growth curves of *Thalassiosira* and *Phaeodactylum* grown at different copper levels. 25 mL cultures of Aquil at each pCu were inoculated with 10 uL of late log-phase cultures grown in optimal Aquil media. Growth was monitored based on chlorophyll a fluorescence.

6.2.3 Copper ligand recovery from culture media by Cu(II)-IMAC

Algal culture media recipes generally include metal-chelating ligands that prevent metal toxicity but interfere with downstream analyses. Aquil media used in this work typically contains EDTA, a very strong copper chelator that interferes with Cu(II)-IMAC. Some culture studies have utilized low EDTA culture media, often by ‘weaning’ cells through media dilutions. Culture media from *Synechococcus* species containing 2 nM EDTA was shown by ASV to not significantly contribute to copper speciation (Gordon *et al.*, 2000), and this method was shown to result in the production of extremely high (2 µM) copper ligand levels. Other researchers

(Maldonado, personal communication) have attempted to filter cells out of cultures for re-suspension in EDTA-free media.

We have attempted to remove EDTA from cultures by filtration. 25 mL *P. tricornutum* cultures grown to late log-phase were passed by suction filtration through a 0.2 µm polycarbonate (PC) filter. Cells captured on the filter were added to a fresh equal volume of EDTA-free culture media with or without added trace metals. After an incubation period to allow cells in EDTA-free media to release Cu ligands in response to metal stress, media was again filtered through a 0.2 µm PC filter to prepare it for Cu(II)-IMAC.

2 L optimal Aquil media was inoculated with 25 mL of a late log-phase culture of *P. tricornutum* and grown to mid log-phase. All 2 L of culture were then filtered onto a single 0.2 µm PC filter and the filter disc was introduced into 2 L of Aquil media with no added EDTA or trace metals. After 24 hrs of incubation 1 L of this culture was filtered and assessed by Cu(II)-IMAC, for which the peak area was 47 mAU*min. After 72 hrs another 1 L was filtered and measured to have an increased peak area of 79 mAU*min, suggesting the culture was actively producing copper ligands. Further experiments need to be conducted to verify this preliminary result.

Future studies should assess by fluorometry and microscopy the growth characteristics of diatoms grown in 2 L cultures at each Cu level and in EDTA-free media amended with copper. Voltammetry of EDTA-free culture media would be an appealing avenue to confirm Cu(II)-IMAC data on Cu ligand production.

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