

Evidence for the production of copper-complexing ligands by marine phytoplankton in the subarctic northeast Pacific

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ABSTRACT

The availability and toxicity of copper (Cu) to marine phytoplankton involve complexation of Cu by dissolved organic ligands of uncertain origin and structure. As part of a GEOTRACES process study we used immobilized copper(II)-ion affinity chromatography (IMAC) to isolate and obtain information about the distribution and potential sources of Cu-complexing ligands along Line P in the subarctic northeast Pacific. Filtered seawater samples were collected from up to five stations during cruises in June 2016, June 2017, August 2017, and September 2018. Copper ligand concentrations were consistently higher at coastal station P4 than at other stations, particularly in surface waters. Ligand concentrations in the upper 40 m at station P26 increased between 2016 and 2018 following the 2014–2016 warming anomaly in the northeast Pacific, whereas an increase in ligand concentration from June to August 2017 coincided with seasonal increases in dissolved organic carbon (DOC) and net community production. At all stations, the depth of highest ligand concentration and maximum chlorophyll concentration coincided. A positive linear correlation between ligand and chlorophyll concentrations suggests that marine phytoplankton are a significant source of Cu ligands along Line P. The lower than expected ligand concentrations at station P26 in June 2016 are consistent with changes in phytoplankton ecology associated with the 2014–2016 warming anomaly. Comparing results from coastal and oceanic waters with those obtained previously in the Canadian Arctic suggests that terrigenous organic matter and marine humic substances contribute to the pool of Cu ligands captured by IMAC.

1. Introduction

Components of marine dissolved organic matter (DOM) that form stable complexes with copper (Cu) play an important role in the biological uptake and utilization of this element, which can act both as a micronutrient and as a toxin to marine phytoplankton. Culture experiments suggest that copper-complexing ligands can be produced by photosynthetic marine organisms in response to Cu stress or limitation (Crook et al., 2000; Echeveste et al., 2018; Gledhill et al., 1999; Gordon et al., 2000; Leal et al., 1999; Mawji et al., 2008; Moffett and Brand, 1996; Moffett et al., 1990; Wiramanaden et al., 2008). In addition, studies of natural seawater samples indicate that organic speciation of Cu may be supplemented by terrigenous DOM or marine humic substances formed by bacteria and phytoplankton (Abualhaija et al., 2015;

Mellet et al., 2018; Muller and Batchelli, 2013; Nixon et al., 2019; Romera-Castillo et al., 2011; Whitby and van den Berg, 2015).

We have studied the distribution of copper-complexing ligands in Canadian Arctic waters using immobilized Cu(II)-ion affinity chromatography (IMAC) (Nixon et al., 2019), an approach that has also been applied successfully to soil and freshwater samples (Paunovic et al., 2005, 2008; Wu et al., 2001; Wu and Tanoue, 2001). This technique is appealing both as a simple tool for detecting marine Cu ligands in situ (Gordon, 1992; Gordon et al., 1996; Midorikawa and Tanoue, 1996) or in cultures (Gordon et al., 2000; Crook et al., 2003; Smith et al., 2014) and as a preparatory step in their analysis by voltammetry (Donat et al., 1997; Gordon et al., 2000), spectroscopy (Midorikawa and Tanoue, 1998) or mass spectrometry (Nixon and Ross, 2016; Ross et al., 2003; Vachet and Callaway, 2003). Results from our earlier study (Nixon et al.,

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2019) suggested that phytoplankton assemblages and terrestrial DOM contribute to the pool of Cu ligands captured by IMAC in Canadian Arctic waters.

Here we use the same IMAC procedure to investigate the distribution of Cu ligands along Line P, a transect that extends from the coastal waters of British Columbia to Ocean Station Papa (OSP) in the high nutrient, low chlorophyll (HNLC) waters of the Alaska Gyre. Oceanographic data have been collected along Line P since 1959, providing one of the few time series of sufficient quality and duration to be useful in examining long term variability of the ocean. Line P cruises operated by Fisheries and Oceans Canada (DFO) from the Institute of Ocean Sciences (IOS) in Sidney, British Columbia take place each winter, spring and summer, providing a platform for process studies as well as regular monitoring of ocean conditions. We used this platform to study the distribution of Cu ligands along Line P during consecutive spring (2016/17) and summer (2017/18) cruises. Our study includes depth profiles of ligand concentration at coastal station P4 and outer station P26 (OSP) as well as ligand concentrations at specific depths along Line P. Although seasonal studies of Cu speciation have been carried out before (e.g. Croot, 2003; Dryden et al., 2007) we believe this is the first study to investigate spatial and temporal changes in the distribution of marine Cu ligands along an open ocean transect.

2. Materials and methods

2.1. Reagents

Copper (II) sulfate (99.995% pure) was obtained from Sigma-Aldrich (St. Louis, MO), ethylenediamine tetraacetic acid (EDTA) from Fisher (Pittsburgh, PA), and SeaStar Baseline hydrochloric acid (HCl) from SeaStar Chemicals (Sidney, BC). 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 for

use as the IMAC elution buffer by adding HCl to a concentration of 10 mM (pH 2.1).

2.2. Collection of seawater samples

The collection and filtration of seawater samples were carried out in June 2016, June 2017, August 2017 and September 2018 during DFO cruises aboard the CCGS John P. Tully as part of the Line P Iron Program, a GEOTRACES process study (GPpr07). Ligand samples were collected from various depths (Table 1) at stations P4 (48°39' N 126°40' W), P12 (48°58' N 130°40' W), P16 (49°17' N 134°40' W), P20 (49°34' N 138°40' W), and P26 (50°N, 145°W) (Fig. 1). During 2016 and 2017 cruises, 12-L Go-Flo sampling bottles (General Oceanics), which had been cleaned according to GEOTRACES protocols (Cutter et al., 2010), were deployed on a Kevlar line and triggered using acid-cleaned Teflon messengers. In September 2018 samples were collected using a trace-metal clean rosette consisting of a powder-coated aluminium frame holding twelve 12-L Teflon-coated Go-Flo bottles and tethered by a 4000-m 4-member

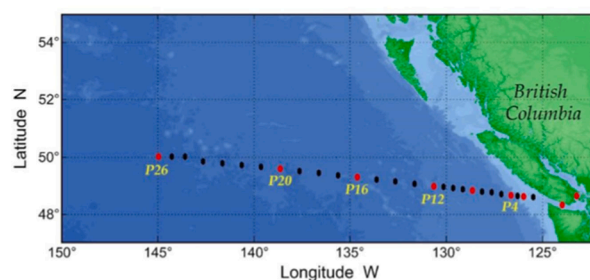


Fig. 1. Stations at which copper ligand and trace metal samples were collected along Line P.

Table 1

Temperature, salinity, average IMAC peak area, ligand, chlorophyll, and dissolved copper concentrations at the five Line P stations.

Cruise	Station	Depth (m)	Temperature (°C)	Salinity (g/kg)	IMAC Peak Area (AU-min)	Ligands (nM)			Chlorophyll-a (µg/L)		dCu (nM)		
						Mean	SD	n	Mean	SD	Mean	SD	
June 2016	P4	40	10.1	32.5	244.5	3.5	0.1	4	0.46	0.02	1.74	0.01	
		P26	10	9.3	32.9	177.3	2.0	0.1	4	1.18	0.04	1.92	0.01
June 2017	P26	40	7.6	32.9	187.3	2.2	0.2	3	1.30	0.05	1.91	0.02	
		5	8.9	32.7	232.5	3.3	0.0	2	0.23	0.01	–	–	
		25	8.8	32.7	237.1	3.4	0.4	2	0.26	0.01	1.35	0.02	
		40	6.2	32.7	251.5	3.7	0.4	3	0.30	0.01	1.60	0.02	
		75	5.3	32.7	203.0	2.6	0.2	3	0.38	0.01	2.09	0.02	
		100	5.5	33.1	204.5	2.6	0.1	2	0.16	0.01	2.61	0.03	
		150	6.2	34.0	176.4	2.0	0.0	3	0.07	0.00	1.93	0.03	
		200	5.6	34.0	182.5	2.1	0.2	2	0.06	0.00	2.07	0.01	
August 2017	P4	400	4.3	34.1	183.7	2.2	0.4	3	0.05	0.00	2.62	0.02	
		800	3.4	34.4	219.1	3.0	0.3	2	0.04	0.00	–	–	
		25	11.8	32.5	366.3	6.3	0.4	2	0.89	0.03	1.94	0.01	
		P12	25	16.2	32.7	169.5	1.8	0.0	2	0.18	0.01	1.00	–
		P16	25	15.1	32.8	203.7	2.6	0.2	3	0.37	0.01	0.90	0.01
		P20	25	14.2	32.7	206.3	2.7	0.2	3	0.32	0.01	–	–
		P26	25	13.1	32.6	289.0	4.5	0.0	2	1.33	0.05	1.44	0.01
		September 2018	P4	10	15.1	32.4	504.1	9.4	0.2	2	1.67	0.06	2.47
25	13.4			32.5	428.2	7.7	1.2	2	1.27	0.05	2.17	0.01	
50	8.9			32.8	278.7	4.3	0.4	2	0.24	0.01	2.76	0.02	
75	8.4			33.5	269.6	4.1	0.1	2	0.05	0.00	1.81	0.00	
100	8.3			33.8	220.9	3.0	0.0	1	0.02	0.00	1.78	0.05	
P12	40			14.7	32.6	299.1	4.8	0.1	2	0.75	0.03	1.17	0.01
P16	40			10.2	32.6	361.6	6.2	1.8	3	0.37	0.01	1.08	0.01
P20	40			7.7	32.7	270.0	4.1	0.7	3	0.33	0.01	1.34	0.01
P26	10			14.2	32.5	260.9	3.9	0.6	2	0.47	0.02	1.51	0.01
	25			14.1	32.5	257.6	3.8	0.1	2	0.49	0.02	1.40	0.01
	40	9.9	32.6	286.9	4.5	0.2	2	0.30	0.01	1.48	0.01		
	50	8.2	32.7	240.9	3.5	0.2	2	0.26	0.01	1.53	0.01		
P26	75	6.3	32.8	209.2	2.7	0.3	2	0.15	0.01	1.47	0.01		
	100	5.5	33.1	176.6	2.0	0.0	1	0.06	0.00	1.61	0.01		

conducting Vectran cable encased in polyurethane (Cortland Cable Company, Cortland, NY) (Measures et al., 2008).

Seawater was gravity-filtered on board using 0.2- μm Acropak filters (Pall Corporation, Port Washington, NY). Samples for ligand analysis were collected in 1-L high density polyethylene (HDPE) bottles (Nalgene) pre-cleaned according to GEOTRACES protocols (Cutter et al., 2010) and immediately stored at $-20\text{ }^{\circ}\text{C}$. Where possible, replicate samples were collected at each depth (Table 1). Frozen ligand samples were returned to IOS where they were thawed overnight at $4\text{ }^{\circ}\text{C}$ and analyzed promptly at room temperature in the months following each cruise. Samples for dissolved Cu analysis were collected in pre-cleaned 500-mL low density polyethylene (LDPE) bottles (Nalgene®) and acidified to pH 1.7 using SeaStar Baseline HCl prior to analysis.

2.3. Analysis of seawater samples

Copper ligands were isolated from filtered seawater samples by Cu (II)-IMAC as previously described (Nixon et al., 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+} and eluted for 25 min with acidified artificial seawater. Absorbance at 254 nm was monitored using a UV detector (Model 2138 Uvicord S, LKB Bromma, Uppsala, Sweden) fitted with an 8- μL flow cell (Model 80-1251-83; LKB) of path length 2.5 mm. The area of the predominant elution peak was measured (AU-min) using custom-built software. After correcting for the volume of sample analyzed, IMAC peak area (y) was converted to ligand concentration (x) using a linear calibration ($y = 44.16x + 88.17$ AU-min, $r^2 = 0.998$) obtained with the model ligand 8-hydroxyquinoline (Nixon and Ross, 2016). Applying the principle of standard additions (Miller and Miller, 1988) and extrapolating the unweighted calibration to $y = 0$ indicated a background ligand concentration of $x = 2$ nM in the artificial seawater used to prepare the standards and elution buffer. The IMAC column was regenerated with 15 mL of 50 mM aqueous EDTA.

Replicate IMAC ligand fractions obtained from station P26 in June 2016 were analyzed by ultra-high performance liquid chromatography-high resolution mass spectrometry (UHPLC-HRMS), following solid-phase extraction (SPE) as described by Nixon and Ross (2016). Extracts were dried under vacuum and reconstituted in 100 μL of 10% aqueous methanol, of which 10 μL were injected onto a BEH C18 column (2.1×100 mm, 1.7- μm ; Waters, Mississauga, ON) using an Ultimate 3000 UHPLC system coupled to an LTQ-Orbitrap Velos Pro mass spectrometer (ThermoFisher, Mississauga, ON) via an electrospray (ESI) ion source. Separations were performed at $45\text{ }^{\circ}\text{C}$ using a flow rate of 0.35 mL/min and a binary solvent system consisting of 0.01% formic acid in water (solvent A) and 0.01% formic acid in 2:1 v/v acetonitrile:isopropanol (solvent B) with a gradient of 2 to 75% B over 15 min followed by 2 min at 100% B and 4 min at 2% B to re-equilibrate the column. Full-scan negative-ion HRMS from mass-to-charge ratio (m/z) 80 to 1250 was carried out in Fourier Transform mode with a resolution of 60,000 FWHM at m/z 400 and a mass error of less than 3 ppm using a reference mass of m/z 112.98563 for real-time calibration. The acquired data were processed using XCMS software (Smith et al., 2006) and a customized R script for peak detection, retention time correction, and peak grouping.

Dissolved copper (dCu) was measured by triple-quadrupole inductively coupled plasma-tandem mass spectrometry (ICP-MS/MS) following offline pre-concentration by solid-phase extraction, as previously described (Jackson et al., 2018).

2.4. Collection of oceanographic data

Hydrographic and 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 and temperature (Model 911plus CTD; Sea-Bird Electronics, Bellevue, WA) and chlorophyll-a fluorescence (Chlorophyll Fluorometer, Seapoint

Sensors Inc., Kingston NH) obtained during CTD casts carried out immediately before or after the collection of seawater samples for ligand and dCu analysis. In situ salinities were converted to absolute salinities using the routines provided by McDougall et al. (2012). Discrete measurements of chlorophyll concentration were carried out using bottle samples collected during the CTD casts. Chlorophyll samples were filtered onto 25-mm GF/F filters and stored in glass scintillation vials at $-80\text{ }^{\circ}\text{C}$ prior to analysis. Samples were extracted in 90% acetone at $-20\text{ }^{\circ}\text{C}$ for 24 h and analyzed using a 10 AU fluorometer (Turner Designs, San Jose, CA) calibrated with a pure chlorophyll-a standard (Sigma). Fluorescence readings were used to calculate chlorophyll concentrations (Holm-Hansen et al., 1965) with an average CV of 3.8%. Copper ligand concentrations were also compared with the abundance of main taxonomic groups of phytoplankton, derived from pigment concentration samples collected during CTD casts and measured by high-performance liquid chromatography (Zapata et al., 2000) and Chemtax v1.95 (Mackey et al., 1996). Following the grouping of Higgins et al. (2011) and the analysis procedures detailed in Peña et al. (2019), eight taxonomic groups were included in the Chemtax analysis: diatoms 2, haptophytes 6–8, chlorophytes, pelagophytes, prasinophytes 3, dinoflagellates 1, cryptophytes, and cyanobacteria.

3. Results

3.1. Hydrographic data

Observations along Line P show strong gradients in physical-biogeochemical properties. Stations near the coast ($<130^{\circ}\text{W}$) are highly dynamic year-round and strongly influenced by coastal currents, seasonal upwelling, and other shelf/slope dynamics (Bif and Hansell, 2019; Peña and Varela, 2007). At P4 salinities measured in the upper 20 m ranged from 31.9 g/kg in June 2016 to 32.4 g/kg in September 2018 and are typical of surface waters on the west coast of Vancouver Island (Vindeirinho, 1998) which are influenced by riverine inputs. Below 100 m salinities at P4 ranged from 33.4 g/kg in June 2016 to 34.0 g/kg in September 2018, reflecting the influence of the California Undercurrent that flows northwards during the summer (Freeland and Denman, 1982). The outer Line P station P26 lies at the southern edge of the Gulf of Alaska and is characterized by strong seasonality in upper layer temperature, nutrients, dissolved oxygen and net community production (Harrison, 2002; Peña and Varela, 2007; Pelland et al., 2018; Bif and Hansell, 2019). During summer a shallow seasonal pycnocline develops mostly due to summer heating and is found at about 30 m. During winter the seasonal pycnocline is mixed away leaving a winter mixed layer that overlies the top of the permanent pycnocline at about 120 m depth, which is associated primarily with salinity stratification (Cummins and Ross, 2020). The salinity and depth of the upper mixed layer (ML) at P26 ranged from 32.5 g/kg and 30 m in August 2017 and September 2018 to 32.9 g/kg and 80 m in June 2016 and 2017. Salinity increased across the seasonal thermocline that forms below the ML between April and November before reaching the top of the halocline (HC), a layer of strong salinity stratification present throughout the subarctic NE Pacific (Pelland et al., 2016). The upper boundary of the HC (salinity 33.9 g/kg) ranged in depth from 125 m in June 2017 to 150 m in August 2017 and September 2018, when similar hydrography was observed at stations P12, P16 and P20. Temperatures in the ML were highest at P12 ($16\text{ }^{\circ}\text{C}$) and coolest at P26, where they ranged from $14\text{ }^{\circ}\text{C}$ in September 2018 to $9\text{ }^{\circ}\text{C}$ in June 2016 and 2017. Temperature profiles help to relate these hydrographic features to the distributions of chlorophyll, copper ligand, and dissolved copper concentrations in Line P surface waters (Figs. 2, 3 and 4).

3.2. Coastal station P4

The ligand depth profile obtained at coastal station P4 in September 2018 (Fig. 2) reached a maximum of 9.4 nM at 10 m, the highest

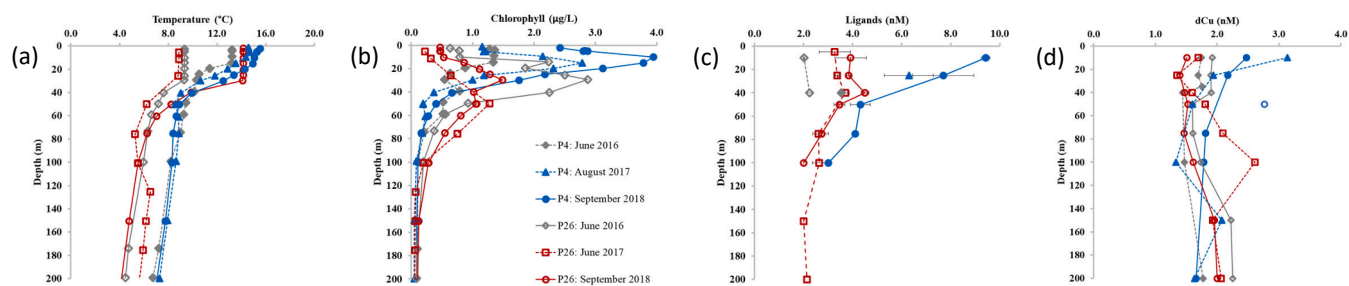


Fig. 2. Temperature (a), chlorophyll (b), copper ligand (c), and dissolved copper concentrations (d) measured at station P4 in June 2016, August 2017 and September 2018 and at station P26 in June 2016, June 2017 and September 2018.

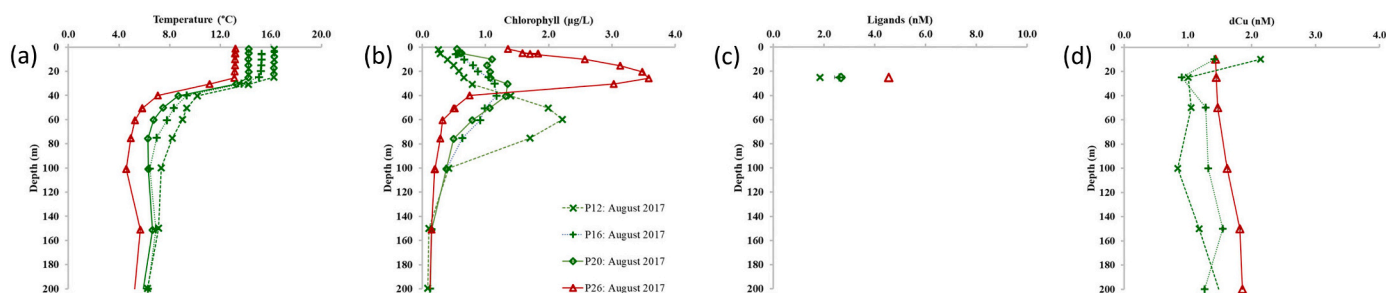


Fig. 3. Temperature (a), chlorophyll (b), copper ligand (c), and dissolved copper concentrations (d) measured at stations P12, P16, P20 and P26 in August 2017.

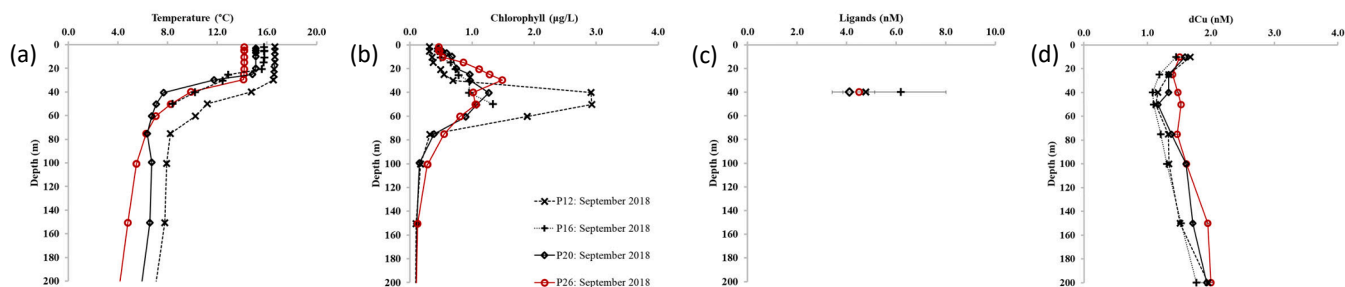


Fig. 4. Temperature (a), chlorophyll (b), copper ligand (c), and dissolved copper concentrations (d) measured at stations P12, P16, P20 and P26 in September 2018.

concentration observed in this study. The next highest values of 7.7 and 6.3 nM were measured at 25 m in September 2018 and August 2017, respectively. Concentrations of 3.5 and 4.3 nM were measured at 40 m in June 2016 and at 50 m in September 2018, respectively. In all cases, the chlorophyll maximum at P4 was observed at a depth of approximately 10 m (Fig. 2b), coinciding with the depth of highest measured ligand concentration in September 2018. Copper ligand concentrations at P4 were consistently higher than those measured at the same time and depth at other stations. Dissolved Cu at P4 was generally highest in surface waters (Fig. 2d) with concentrations at 5 m ranging from 1.7 nM in June 2016 to 2.5 nM in September 2018 and 3.1 nM in August 2017. Except for a value of 2.8 nM measured at 50 m in September 2018 (which appears to be an outlier) dCu at P4 decreased with depth during all cruises to a concentration of between 1.6 and 1.8 nM at 200 m.

3.3. Oceanic station P26

Ligand profiles obtained at outer station P26 in September 2018 and June 2017 were similar with maximum values of 4.5 and 3.7 nM, respectively, observed at 40 m (Fig. 2c). The ligand maxima appear to lie within the ML (Fig. 2a) between the chlorophyll maximum depths of 30

m and 50 m observed at P26 in September 2018 and June 2017, respectively (Fig. 2b). The ligand concentration of 2.2 nM measured near the chlorophyll maximum at 40 m in June 2016 is slightly greater than that measured at 10 m during the same cruise. These concentrations are significantly lower than those measured at the same depths in June 2017 and September 2018. The ligand concentration of 4.5 nM measured at 25 m in August 2017 (Fig. 3c) is greater than the values of 3.8 and 3.4 nM recorded at the same depth in September 2018 and June 2017 (Fig. 2c) when the chlorophyll maximum was below 25 m (Fig. 2b). Dissolved Cu at P26 was lowest in September 2018 (Fig. 2d) and August 2017 (Fig. 3d), increasing steadily with depth from 1.4 nM at 25 m to about 2.0 nM at 200 m. In the upper 40 m dCu was highest, at around 1.9 nM, in June 2016 and between 1.4 and 1.7 nM in June 2017 (Fig. 2c). Below 40 m dCu was somewhat higher in June 2016 than in September 2018 and August 2017, whereas dCu in June 2017 increased to 2.6 nM at 100 m before decreasing to 2.0 nM at 150 m.

3.4. Intermediate stations P12, P16 and P20

Ligand concentrations at the three intermediate stations were measured at 25 m in August 2017 and at 40 m in September 2018 in

order to explore the horizontal distribution of Cu ligands along Line P. The lowest ligand concentration recorded during this study was 1.8 nM, measured at 25 m at station P12 in August 2017 (Fig. 3). The concentration of 2.6 nM measured at stations P16 and P20 is also lower than the values recorded at P4 and P26, where 25 m corresponded to the chlorophyll maximum depth in August 2017 (Fig. 3b). In September 2018 the chlorophyll maximum depth was close to 40 m at all stations except P4 (Fig. 4). Ligand concentrations measured at 40 m in September 2018 ranged from 4.1 to 4.8 nM except at station P16, which averaged 6.2 nM (Fig. 4c). Dissolved Cu measured at intermediate stations P12 and P16 in August 2017 (Fig. 3d) was highest at 10 m, decreasing to 1.0 nM at the chlorophyll maximum depth of 25 m (Fig. 3b) below which dCu was generally lowest at P12. Likewise, dCu at intermediate stations P12, P16 and P20 in September 2018 (Fig. 4d) decreased from around 1.6 nM at 10 m to 1.1 nM at the chlorophyll maximum depth of 50 m (Fig. 4b) below which dCu was generally highest at P20.

4. Discussion

4.1. Operational definition of copper ligands

To help define the fraction of organic ligands captured by IMAC along Line P we turned to a recent study of copper speciation in the subarctic Pacific (Wong et al., 2021) carried out between June and August 2017 along two transects, one of which intersects with Line P at station P26 (referred to as CL-16 in the study). Concentrations and stability constants ($\log K'$) of Cu-binding organic ligands in dissolved ($< 0.2 \mu\text{m}$) and truly soluble ($< 0.03 \mu\text{m}$) fractions were determined by Wong et al. using competitive ligand exchange adsorptive cathodic stripping voltammetry (CLE-AdCSV) with a competing ligand (salicylaldehyde) concentration of $5 \mu\text{mol/L}$. Two classes of Cu-binding ligands defined as strong (L_1) and weak (L_2) based on a cutoff ($\log K' = 13.5$) established in previous studies (Buck and Bruland, 2005; Jacquot et al., 2013) were detected at P26/CL-16 in waters shallower than 800 m. These data afford a direct comparison with dissolved Cu ligand concentrations of 3.4, 2.6, 2.0, 2.1, and 3.0 nM measured by IMAC at 25, 100, 150, 200, and 800 m, respectively, in June 2017 (Table 1). The concentrations of L_1 measured at these depths by CSV in July 2017 were 1.31, 1.44, 1.58, 2.28 and 2.53 nM whereas L_2 concentrations were 7.26, 2.83, 4.07, 7.26 and 27.0 nM, respectively (Wong, K.H., personal communication). In each case the concentration of ligands measured by IMAC was greater than or close to the concentration of L_1 and less than or similar to the concentration of L_2 measured at the same depth.

Using 8-HQ to calibrate the IMAC method may underestimate natural ligand concentrations, given that the molar absorptivity of 8-HQ at 254 nm ($3 \times 10^4 \text{ L cm}^{-1} \text{ mol}^{-1}$) is at least twice that of other small aromatic molecules like ferulic acid ($1.4 \times 10^4 \text{ L cm}^{-1} \text{ mol}^{-1}$), caffeine ($1.6 \times 10^4 \text{ L cm}^{-1} \text{ mol}^{-1}$) and tryptophan ($0.9 \times 10^4 \text{ L cm}^{-1} \text{ mol}^{-1}$) used to model natural organic ligands (European Food Safety Journal, 2011; Wünsch et al., 2015). Hence, the average L_1 :IMAC concentration ratio of 0.7 for ligands measured at the same depth at CL-16/P26 suggests that IMAC captures at least some of the L_2 ligands detected by CSV. This is borne out by an average L_2 :IMAC ligand concentration ratio of 3.5, which is more in keeping with the expected difference in molar absorptivities between 8-HQ and natural Cu ligands.

The concentration of L_2 increases rapidly with depth below 100 m whereas ligand concentrations measured using IMAC appear more in step with L_1 between 100 and 800 m. A corresponding decrease in average $\log K'$ for L_2 (Wong et al., 2021) suggests that IMAC may be targeting a sub-set of stronger L_2 ligands which, like L_1 , remain relatively stable below 100 m. These observations are consistent with initial work by Gordon (1992) who found that IMAC captured two types of Cu ligands, a stronger fraction with a maximum concentration at 60 m and a weaker fraction that showed little variation with depth. In contrast, the increase in L_2 below 100 m observed by Wong et al. (2021) coincides with increasing amounts of bacterial remineralization and Cu-binding

humic substances in deeper waters (Whitby et al., 2018). Donat et al. (1997) observed that stronger natural ligands captured by IMAC were not retained using a hydrophobic (C-18) resin (Donat et al., 1986) suggesting that they are more hydrophilic in nature. To be retained by IMAC such ligands must be capable of forming stable ternary complexes with immobilized Cu^{2+} ions (Paunovic et al., 2005) which may be inhibited, particularly for larger molecules, by steric limitations or competitive binding of other metal ions (e.g. Ca^{2+}). Midorikawa and Tanoue (1996, 1998) also observed that IMAC appears to trap smaller ligand complexes, suggesting that only the low molecular weight fraction of humic material is retained by IMAC. This is consistent with IMAC recovery of selected L_2 ligands at P26, which may include smaller humic substances, bacterial ligands or remineralization products, and with retention of 8-HQ (Nixon and Ross, 2016) which could be considered a model ligand for low molecular weight humic substances. The positive correlation between chlorophyll abundance and IMAC ligand concentration suggests that IMAC-recoverable ligands are more directly associated with phytoplankton than L_1 or L_2 , although the L_2 profile in July 2017 (Wong et al., 2021) features a sub-surface maximum close to the chlorophyll maximum depth seen in August 2017 (Fig. 3b).

4.2. Sources of copper ligands

The depths of highest ligand concentration measured by IMAC along Line P generally coincided with the chlorophyll maximum depth. This is consistent with similar observations made in the Canadian Arctic during the fall of 2015 (Nixon et al., 2019) and with previous studies involving electrochemical measurements of Cu ligand concentration in seawater (Donat et al., 1986; Coale and Bruland, 1988; Moffett et al., 1990; Moffett, 1995; Thompson et al., 2014), implying that marine phytoplankton are a significant source of Cu ligands. This hypothesis is supported by dissolved ligand concentrations of 3.4 and 4.5 nM measured at 25 m at station P26 in June and August 2017 (Table 1), which reflect an increase in dissolved organic carbon (DOC) from about 60 to 72 μM linked to the seasonality in net community production at OSP (Bif and Hansell, 2019). However, there are instances in which the relative abundance of Cu ligands measured by IMAC deviates from that expected on the basis of chlorophyll measurements, notably at station P26 in June 2016 (Fig. 2). This suggests that factors other than phytoplankton biomass may affect the distribution of Cu ligands along Line P. To investigate this further we plotted ligand concentration measured by IMAC against chlorophyll concentration measured in discrete water samples collected at the same time (Fig. 5).

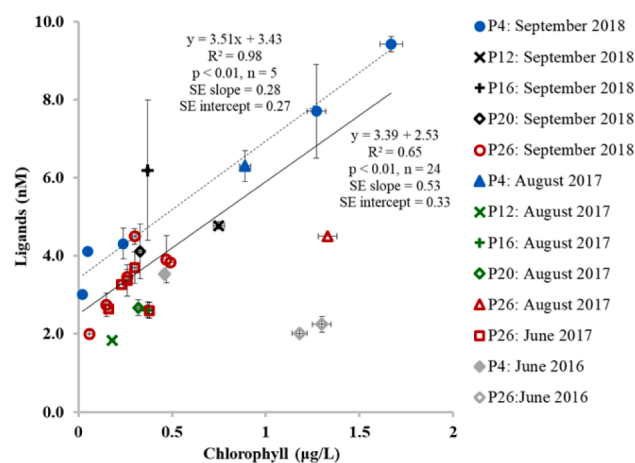


Fig. 5. Linear correlation between copper ligand and chlorophyll concentrations for all cruises and stations except P26 in June 2016 (solid line) and for P4 in September 2018 (dotted line). The standard errors (SE) of the slope and intercept are included for each regression.

A positive linear correlation ($r^2 = 0.65$, $n = 24$) was observed between ligand and chlorophyll concentrations, with the exception of those measured at station P26 in June 2016 where ligand concentrations were lower (Fig. 2c) and chlorophyll concentrations higher (Fig. 2b) than during other cruises. Temperature profiles (Fig. 2a) suggest that conditions in June 2016 were similar to those in June 2017. However, pigment analysis (Peña et al., 2019) revealed that the average abundances of prasinophytes and cyanobacteria in the upper 40 m at station P26 were lower in June 2016 (0.009 and 0.007 $\mu\text{g/L}$ Chl-a) than in June 2017 (0.026 and 0.019 $\mu\text{g/L}$ Chl-a, respectively). A corresponding change in the composition of the Cu ligand pool may explain the lower abundance of ligands captured by IMAC in June 2016, when the 2014–2016 North Pacific marine heatwave was still affecting plankton ecology in this region (Peña et al., 2019). A general increase in Cu ligand concentrations measured in the upper 40 m between 2016 and 2018 (Fig. 2c) may reflect a return to more normal conditions following this event. In any case, results suggest that ligand concentrations at P26 are subject to seasonal and inter-annual variability but persist in the upper water column, with the potential to modify the speciation and bioavailability of Cu and possibly other metals transported there via eddies (Johnson et al., 2005), dust (Schallenberg et al., 2017), or volcanic ash (Hamme et al., 2010).

To gain insight into the possible nature of these ligands, IMAC peak fractions obtained from P26 in June 2016 were analyzed by ultra-high performance liquid chromatography (UHPLC) and high resolution mass spectrometry (HRMS) using negative electrospray ionization (ESI). This identified a deprotonated molecular ion of m/z 210.00425 not found in the elution buffer that had the same peak area (arbitrary units) in duplicate samples ($37,917 \pm 841$; RSD 2.2%) from 10 m and in triplicate samples ($58,343.7 \pm 1653$; RSD 2.8%) from 40 m, the chlorophyll maximum depth (Fig. 2b). The closest match (± 0.8 ppm) to this m/z is the empirical formula $\text{C}_8\text{H}_5\text{NO}_6$ (211.01172 Da) which corresponds to one or more isomers of pyridine-tricarboxylic acid (e.g. tripicolinic acid). This molecule closely resembles the bacterial ligands pyridine-2,4-dicarboxylic acid (Sliemandagger and Nicholson, 2001) and pyridine-2,4-dithiocarboxylic acid (Kenney and Rosenzweig, 2018), which forms Cu complexes and is capable of redox cycling (Cortese et al., 2002). The proposed structure of this small aromatic ligand is also consistent with IMAC recovery of low molecular weight complexes containing carboxyl groups (Midorikawa and Tanoue, 1996; Midorikawa and Tanoue, 1998) and with production of microbial ligands in surface waters at OSP (Semeniuk et al., 2015; Semeniuk et al., 2009; Whitby et al., 2018).

Turning to ligand and chlorophyll profiles in September 2018, the linear regression for outer station P26 ($y = 4.13x + 2.22$, $r^2 = 0.61$, $n = 6$) is similar to that for all data points combined ($y = 3.39x + 2.53$, $r^2 = 0.65$, $n = 24$) excluding June 2016 whereas the regression line for coastal station P4 ($y = 3.51x + 3.43$, $r^2 = 0.98$, $n = 5$) is transposed in the direction of higher ligand concentrations. Consequently the intercept, which represents the extrapolated ligand concentration in the absence of chlorophyll (Nixon et al., 2019), is greater for P4 than for P26. This is consistent with terrestrial input of Cu-binding DOM in coastal waters, whereas the presence of marine Cu-binding humic substances (Sohn and Weese, 1986; Whitby et al., 2018) accounts for the intercept observed at P26. The similarity in the slopes of these regression lines suggests that the relationship between the abundance of phytoplankton and of associated ligands captured by IMAC remains fairly stable along Line P, an observation generally associated with passive release of low molecular weight DOM by phytoplankton (Livanou et al., 2019). On the other hand, incubation studies carried out in the surface waters of the California Current (Mellett et al., 2018) suggest that exopolymeric and terrestrially-derived humic substances may contribute to Cu ligand concentrations as high as 9 nM in coastal waters, comparable with the dissolved ligand concentration measured at P4 in September 2018 (Fig. 2c).

4.3. Comparison with dissolved copper

The possibility that some of these ligands may be produced in response to copper was investigated by plotting ligand concentration against dCu (Fig. 6). A positive linear correlation ($r^2 = 0.69$, $n = 13$) was observed between ligand concentration and dCu measured at coastal station P4, and at outer station P26 in August 2017 and September 2018. The dCu value for 50 m at P4 in September 2018 (blue circle) was confirmed as an outlier by comparing the depth profile for dCu (Fig. 2d) with those for other trace metals, which showed no such anomaly. An extrapolated dCu concentration of approximately 1 nM in the absence of ligands captured by IMAC is consistent with the recovery of a sub-set of Cu-binding DOM using this method (cf. Section 4.1), as seen previously when applying IMAC to samples from Baffin Bay (Nixon et al., 2019). The omission of certain ligands may also have contributed to the apparent lack of a consistent relationship between ligand concentration and dCu at intermediate stations or at P26 in June 2016 and June 2017, when dCu profiles were more variable than in August 2017 or September 2018. Taken together, these results suggest that the abundance of Cu ligands captured by IMAC may be linked to dCu concentrations in coastal waters, and at OSP in late summer, while the correlation between ligand and chlorophyll concentrations (Fig. 5) indicates that some of these ligands may be associated with marine phytoplankton.

4.4. Comparison with phytoplankton taxonomy

To further investigate the role of phytoplankton ecology in ligand production, the concentration of ligands measured by IMAC during each cruise (Table 1) was plotted against the contribution of each taxonomic group to total chlorophyll *a* concentration at the closest available depth (Table 2). A positive linear correlation (Fig. 7) was observed between ligand concentration and the abundance of prasinophytes ($r^2 = 0.73$) pelagophytes ($r^2 = 0.56$), cryptophytes ($r^2 = 0.54$) and cyanobacteria ($r^2 = 0.42$) across all stations and cruises ($n = 24$), implying a balance between exudation and biomass for these groups within the ML (Livanou et al., 2019). The inclusion of outlying data points at higher concentrations may affect the validity of the correlations observed for

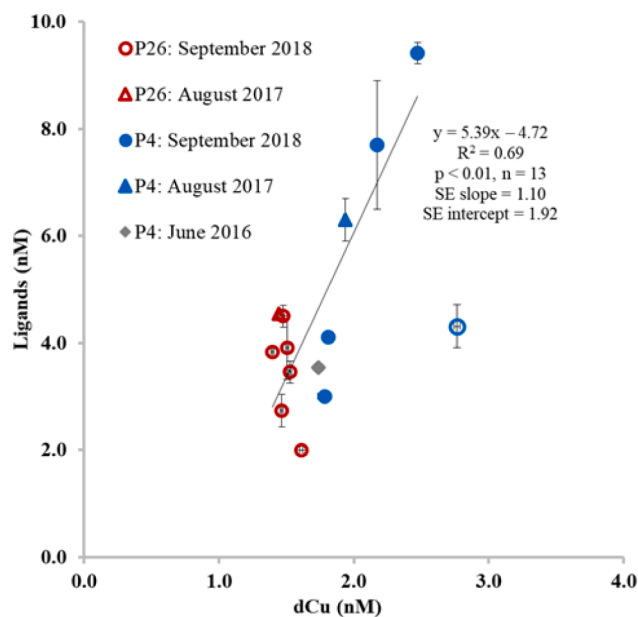
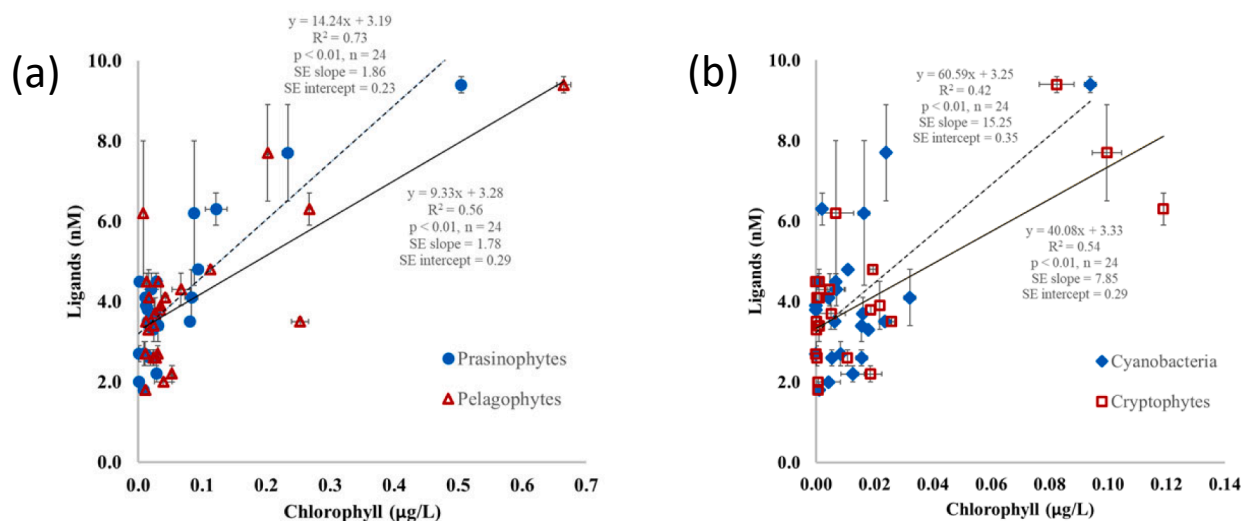


Fig. 6. Linear correlation between copper ligand and dissolved copper concentrations at coastal station P4, and at outer station P26 in August 2017 and September 2018.

Table 2Abundance of phytoplankton taxa that correlate with dissolved copper ligand concentration. Mean and standard deviations (SD) are from duplicate samples ($n = 2$).

Cruise	Station	Depth (m)	Prasinophytes ($\mu\text{g/L Chl-a}$)		Pelagophytes ($\mu\text{g/L Chl-a}$)		Cryptophytes ($\mu\text{g/L Chl-a}$)		Cyanobacteria ($\mu\text{g/L Chl-a}$)		
			Mean	SD	Mean	SD	Mean	SD	Mean	SD	
June 2016	P4	39	0.081	0.006	0.253	0.013	0.026	0.001	0.024	0.002	
		P26	14	0.001	0.001	0.039	0.002	0.001	0.000	0.004	0.004
			40	0.029	0.008	0.053	0.002	0.019	0.004	0.013	0.004
June 2017	P26	5	0.023	0.001	0.016	0.002	0.000	0.000	0.018	0.001	
		30	0.031	0.005	0.024	0.002	0.001	0.001	0.016	0.001	
		40	0.027	0.000	0.026	0.002	0.005	0.005	0.016	0.000	
		60	0.023	0.003	0.028	0.002	0.000	0.000	0.016	0.002	
August 2017	P4	30	0.122	0.017	0.267	0.004	0.119	0.000	0.002	0.001	
		P12	30	0.009	0.001	0.012	0.001	0.001	0.000	0.001	0.001
	P16	30	0.019	0.002	0.024	0.000	0.011	0.000	0.005	0.000	
		P20	30	0.001	0.000	0.031	0.002	0.000	0.000	0.000	0.000
	P26	30	0.002	0.001	0.031	0.005	0.000	0.000	0.000	0.000	
September 2018	P4	15	0.505	0.006	0.665	0.011	0.082	0.006	0.094	0.002	
		30	0.234	0.009	0.202	0.001	0.100	0.005	0.024	0.003	
		50	0.021	0.003	0.067	0.014	0.005	0.005	0.007	0.003	
		60	0.011	0.000	0.042	0.005	0.000	0.000	0.004	0.001	
		P12	40	0.093	0.001	0.112	0.001	0.019	0.001	0.011	0.001
		P16	40	0.087	0.004	0.008	0.000	0.007	0.006	0.016	0.002
	P20	40	0.083	0.010	0.017	0.001	0.001	0.000	0.032	0.002	
		P26	15	0.013	0.000	0.039	0.002	0.019	0.000	0.000	0.000
		30	0.015	0.000	0.034	0.001	0.019	0.001	0.000	0.000	
		40	0.028	0.002	0.013	0.002	0.001	0.000	0.007	0.001	
		50	0.015	0.001	0.012	0.001	0.000	0.000	0.006	0.001	
		60	0.009	0.000	0.011	0.001	0.000	0.000	0.008	0.000	

**Fig. 7.** Linear correlation between copper ligand concentration and the abundance of prasinophytes and pelagophytes (a) and of cryptophytes and cyanobacteria (b).

cryptophytes and cyanobacteria (Fig. 7b), which appear to produce more ligands per cell than prasinophytes or pelagophytes (Fig. 7a). However, correlation coefficients were greater ($r^2 = 0.86, 0.72, 0.72$ and 0.67 , respectively) for September 2018 alone ($n = 12$) and suggest that prasinophytes (green flagellates) are a significant source of the Cu-complexing ligands captured by IMAC. This is consistent with the relatively low abundance of ligands (Table 1) and prasinophytes (Table 2) observed at P26 in June 2016 and August 2017 (Fig. 5). A study of copper sensitivity in marine microalgae (Levi et al., 2007) found the prasinophyte *Micromonas pusilla* to be among the smallest and most sensitive to dissolved Cu whereas *Tetraselmis gracilis* was found to produce the antioxidant superoxide dismutase in response to metal exposure. The need for these sensitive nanoplankton to regulate Cu exposure is consistent with the production of Cu-binding ligands detected by IMAC along Line P, and the measurement of such ligands in the surface waters of Baffin Bay where small phytoplankton species predominated

(Nixon et al., 2019). Pelagophytes, which have been identified as potential source of zinc-binding organic ligands in the Tasman Sea (Sinoir et al., 2016), may also produce ligands capable of binding Cu, which would account for the relationship between pelagophytes and the ligands captured by IMAC along Line P. Likewise, the correlation between ligands and cyanobacteria is consistent with previous studies suggesting that cyanobacteria are a significant source of strong Cu-binding ligands (Mann et al., 2002; Moffett and Brand, 1996; Moffett et al., 1997), some of which may be retained via interaction of their stable Cu complexes with IMAC (Crook et al., 2003). No significant correlation was observed between Cu ligand concentration and the abundance of chlorophytes, haptophytes, dinoflagellates, or diatoms despite the relatively high abundance of the latter at P26 in June 2016 (Peña et al., 2019). Mass spectrometric analysis of Cu ligands isolated from seawater and phytoplankton cultures using IMAC (Nixon and Ross, 2016) may help to shed light on the associations between specific ligands and taxonomic groups.

5. Conclusion

Immobilized copper(II)-ion affinity chromatography was used to quantify dissolved copper ligands at selected stations during consecutive spring (2016/17) and summer (2017/18) Line P cruises. Significant correlations between ligand and chlorophyll concentrations suggest that small marine phytoplankton including prasinophytes and cyanobacteria could be a source of copper ligands along Line P. Comparing results for coastal and oceanic waters suggests that marine humic substances and terrigenous organic matter contribute to the pool of ligands captured by IMAC, which may include smaller ligands of the L₂ class defined by voltammetry. Changes in phytoplankton ecology associated with the 2014–2016 North Pacific warming anomaly are consistent with the lower than expect ligand concentrations observed near the surface at OSP in June 2016. This ligand pool, which shows seasonal and inter-annual variability, has the potential to maintain the solubility of trace metals in the high nutrient, low chlorophyll waters of the subarctic northeast Pacific.

Declaration of Competing Interest

None.

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