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Detection, characterization and dynamics of dissolved organic ligands in oceanic waters

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Abstract—Dissolved organic matter in oceanic waters was investigated in terms of the interaction with copper. Three classes of organic ligands, namely, L_1, L_2 and L_N that were concentrated by repeated rounds of lyophilization and dialysis, were distinguished from each other by differences in their complexing abilities for copper. Ligands L_1 and L_2 appeared to be assigned to the group of weak ligands in the literature. The conditional stability constant of ligand L_N for Cu(II) was extremely high and was comparable to that of EDTA (ethylenediaminetetraacetic acid).

Two types of ligands that were similar to weak ligands L_1 and L_2, were extracted directly from seawater using immobilized metal ion affinity chromatography (IMAC). IMAC gave a new insight in that the weak ligands were a mixture of at least two types of different organo-chemical ligands and that their dynamics might be active in the water column.

INTRODUCTION

During the past 3 decades, increased attention has been paid to the chemistry of metal-organic compounds. The ability of dissolved organic matter to form complexes with metal ions in natural water is of interest because of the associated biological implications, such as the bioavailability and toxicity of metals to living organisms (JACKSON and MORGAN, 1978; ANDERSON and MOREL, 1978), and because of its relevance to efforts at understanding geochemical cycles of metals in the environments (WANGERSKY, 1986). The term metal-organic has been used to cover broadly those compounds with metal-carbon bonds (usually known as organometallic compounds), the metal-oxygen-carbon compounds (the alkoxides or alcoholates), and the coordination compounds of metals and organic molecules. The authors' particular interest in this field is the metal-organic coordination in natural aquatic systems, in which a metal cation is bound by an organic molecule containing one or more sites for either ionic or coordinate bond formation.

Although much excellent research has been carried out on the physico-chemical principles of trace metal adsorption on model substrates, the applicability of the results to the "real world" is still hampered by our lack of knowledge on the surface properties of organic materials (SALOMONS and FÖRSTNER, 1984). Approaches have been followed mainly along the metal speciation, along the interaction between the metal and humic substance that is a polydiverse mixture of structurally uncharacterized

organic materials, and along the interaction between the trace metal and organisms. Despite metal complexation being one of the geochemical function of organic matter in seawater, organic chemical characterizations of ligands in seawater have not been well documented. Indeed, less than 30% of the component molecules of dissolved organic matter (DOM) has been identified as to combined amino acids, carbohydrates and solvent-extractable lipids (WILLIAMS and GORDON, 1970; WILLIAMS and DRUFFEL, 1987, 1988). Such molecular-level analyses typically involve chemical degradation of macromolecules, thus, may not give much information in terms of the complexing ability of organic matter. The current study of metal-organic association needs to be focused not only on the study of metal speciation but also on the characterization of organic ligands in seawater. Various steps are necessary to understand metal-organic association in seawater. The first step is to detect organic ligands and to measure their levels and metal-complexing properties in seawater. The second stage is to trace the dynamics, i.e., production, transformation, decomposition as well as transportation, of organic ligand in the water column. To clarify their chemical properties relevant to functional groups, specificity for a metal, etc. are also key factors for understanding the mechanisms of metal-organic association. Describing a molecular structure in terms of complexation in seawater will be a final goal. In the foregoing section, we intend to summarize our recent works on the interaction between metals and organic ligands, and to discuss characterization and dynamics of organic ligands in the water column. An attempt is also made to itemize what we know and what we don't know about metal-organic association, and to argue problems by which our efforts to understand metal-organic association in the sea, have been hindered.

INTERACTION BETWEEN METALS AND ORGANIC LIGANDS

In marine, especially, in oceanic environments, the concentrations of metals and of organic ligands are extremely low. Moreover, seawater contains high levels of inorganic salts with a complex composition. Therefore, analytical difficulties are inherent in efforts at measuring the ability of DOM to form complexes with metals in natural seawater.

Two general techniques have been used in attempts to determine the complexing ability of natural ligands in seawater. The first approach involves the separation of organic and inorganic forms of metals, for example, by means of liquid-liquid extraction or adsorption on synthetic resin or some other adsorbent. Such methods have been reviewed in detail (MANTOURA, 1981; NEUBECKER and ALLEN, 1983; BUFFLE, 1988). The approaches are convenient for isolation of metal-organic complexes from relatively large volumes of seawater. However, shifts of prevailing equilibria, induced by the separation and concentration steps, may occur and they may be difficult to quantify (MIDORIKAWA and TANOUE, 1994a). Thus, there is no general agreement whether the metal-organic complexes separated from seawater are indigenous or artifactual. Such procedures also do not circumvent the risk of chemical and biological denaturation of the isolated ligands during the process of separation.

The second approach involves the characterization of natural ligands by

determining their concentrations and stability constants for the formation of complexes with metals, usually by electrochemical techniques. Although improved voltammetric techniques have been developed (VAN DEN BERG, 1984; BRULAND *et al.*, 1985), the sensitivity of many electrochemical techniques is not sufficient for the determination of electroactive metal ions that occur at the low levels in nature, especially in the presence of strong chelators or of relatively high concentrations of chelators. Even if such a technique, utilizing the second approach, has adequate sensitivity, the stability constants measured for natural ligands are essentially "conditional" (BUFFLE, 1980). The measurements of the conditional stability constant are useful to investigate metal speciation in seawater. Such measurements, however, provide little information on the characteristics of organic ligands, because numerous side-reactions need to be considered in order to characterize natural ligand based on results of the direct metal-titration method (MIDORIKAWA *et al.*, 1990; MIDORIKAWA and TANOUE, 1993c). We developed a new method for the determination of the complexing ability of natural organic ligands in seawater in which any interference by ambient and contaminating metal ions or side-reactions was excluded (MIDORIKAWA *et al.*, 1990).

Procedures

Our technique involves three separate steps (Fig. 1): 1) concentration and

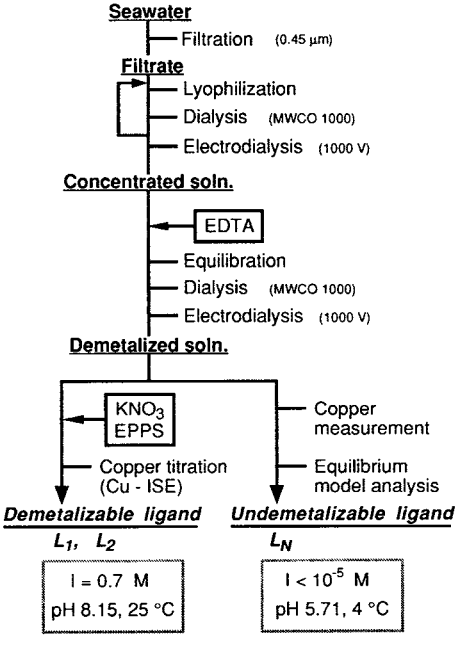

Fig. 1. Schematic representation of the procedures for the concentration, desalting, demetallization and measurements of natural ligands from seawater.

desalting of DOM from seawater; 2) removal of metals bound to DOM; and 3) measurement of complexing ability.

The concentration of DOM in seawater was carried out by lyophilization and desalting in dialysis tubing [nominal molecular mass cutoff, 1000 daltons (Da)]. By the repeated rounds of lyophilization and dialysis, 5 L of seawater was concentrated to a final volume of a few milliliters at the first step. Removal of metals bound to DOM in the concentrated solution was performed by the ligand-exchange reaction using excess EDTA (ethylenediaminetetraacetic acid) (abbreviated demetallization hereafter). After addition of EDTA, the equilibrated solution was dialyzed and then subjected to electrodialysis. The level of exogenously added EDTA decreased to less than 10^{-7} M and did not interfere with the subsequent metal titration. The concentrations of alkaline and alkaline-earth metals in the concentrated and demetallized sample decreased to less than 1/2000 of those in the original seawater after electrodialysis.

The complexing ability of the demetallized solution was determined by metal titration using the ion-selective electrodes (ISEs). For calculations, we applied the discrete-ligands model of DZOMBAK *et al.* (1986), based on a 1:1 stoichiometry in the metal-ligand complexation, and used the linear transformation method of RUZIC (1982) to calculate the total concentrations (C_L) of natural ligands and the conditional stability constants (K'_{ML}) of their complexes with metal ions. For each metal titration, the total concentration of metal ion, C_M, was calculated and the concentration of free metal ion, [M], was obtained from the measured electrode potential (E) of the ISE.

Sample treatments were always performed with a control during the course of the demetallization procedure, and corrections were made for every measurement, using the control data. As the control experiment, the dialysis tubing that included 6 ml of a solution containing approximately 10^{-7} M Cu(II) and 33 mM EDTA without the sample of seawater, was treated in the same manner as the sample at the same time.

Complexing ability for copper

A typical titration curve of surface sample after demetallization is shown in Fig. 2(a). The large deviation of the potential to more negative values in the curve indicates that some ligands in the sample solution have the ability to form complexes with Cu(II). The abilities of natural ligands to form complexes are summarized in Table 1. The two types of ligand for Cu(II) were characterized. The values were determined in the ranges of 8.41–9.60 (conditional stability constant: $\log K'_{ML_1}$) and 1.1–3.9 nM (concentration of ligand: C_{L_1}) for the stronger ligand L_1 and in the ranges of 7.09–7.94 ($\log K'_{ML_2}$) and 4.0–11 nM (C_{L_2}) for the weaker ligand L_2, respectively.

The conditional stability constants of organic copper complexes in seawater are summarized in Table 2. On the basis of the conditional stability constants of organic-Cu(II) complexes, HIROSE (1994) conveniently divided organic ligands into the three classes: the strongest ($\log K'_{CuL} > 13$), the strong ($\log K'_{CuL} \cong 12$) and the weak ($\log K'_{CuL} < 10$) ligands and commented that the concentrations of the organic ligands

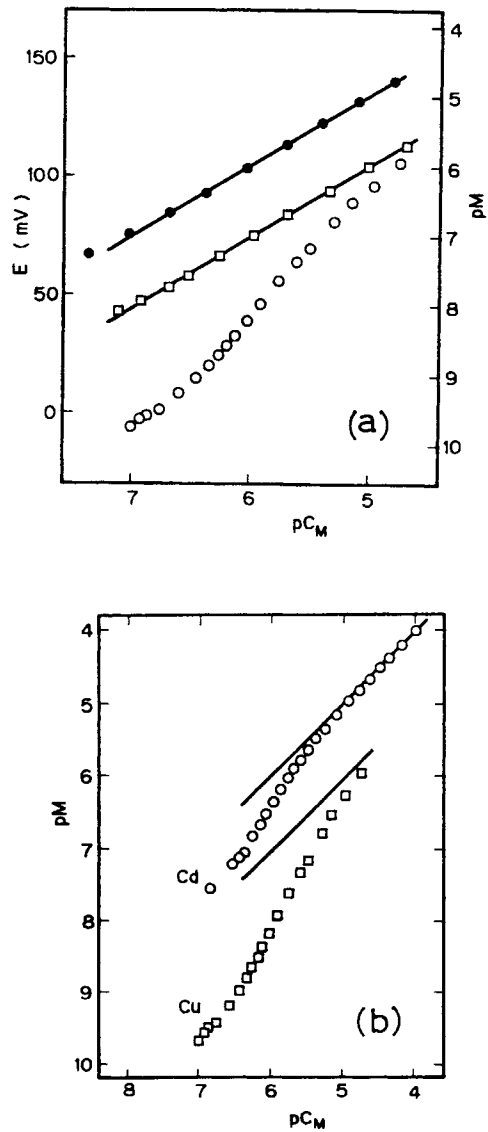

Fig. 2. Copper titration of a sample of surface water (site P, 0 m) in solution (pH8.15, EPPS) (○) at an ionic strength of 0.7 M (KNO_3) and a temperature 25.0°C (a). Results for the nonbuffered solution (pH4) (●) and the buffered solution (pH8.15, EPPS) (□) are also shown. Repeated measurements from a single sample of seawater (site P, 0 m) for two metal ions after repeated demetallization (b). The solid line represents the control values for Cu(II) (□) and Cd(II) (○), respectively.

Table 1. Conditional stability constants (K'_{ML}) and concentrations (C_L) of natural ligands in samples of seawater at pH8.15 (EPPS), at an ionic strength of 0.7 M (KNO_3), and at a temperature of 25.0°C. The concentration of ligand has been converted to that in seawater. The volume of the original sample of seawater used was about 5 l, except in the case of site S-1, 0 m (+: 1.71, #: 1.01)

Site[a]	Depth	Cu(II)								Cd(II)	
		C_{L1} (nM)		log K'_{ML1}		C_{L2} (nM)		log K'_{ML2}		C_L (nM)	log K'_{ML}
	(m)	B	A	B	A	B	A	B	A	A	A
S-1	0+	9.4	8.6	8.34	8.61	62	19	6.38	7.25	-	-
	0#	7.9	7.0	8.48	8.68	45	17	6.57	7.30	-	-
P	0	-	2.2	-	8.89	-	7.3	-	7.09	2.7	6.81
	191	5.1	3.9	8.26	8.41	20	5.5	6.55	7.75	3.8	6.74
J	0	1.9	1.2	9.36	9.60	7.6	6.5	7.50	7.57	1.2	7.21
	523	1.3	1.1	9.20	9.44	5.4	4.0	7.59	7.94	1.4	6.75
	1071	*	3.5	*	9.05	14	11	7.15	7.77	4.5	6.82

[a]Site S-1: at 34°56′ N, 138°41′ E on Apr. 26, 1988. Site P: at 41°32′ N, 147°00′ E on Aug. 13, 1987. Site J: at 44°15′ N, 130°58′ E on Aug. 26, 1987.

Key: *, not detected; -, not determined; B, before demetallization; A, after demetallization.

complexed with Cu(II) are of the following order: the strongest < the strong << the weak ligands. The two types of ligands for Cu(II) characterized in the present study may not be compared simply with those found in the literature (Table 2), because experimental conditions were different for each other. However, the conditional stability constants of both ligands L_1 and L_2 in the present study appear to be assigned to the weak ligand L_W in the literature. Three explanations can be suggested to reason out why the stronger ligands were not detected in the present study. 1) The loss of the strong ligands during the dialysis may have occurred because of the low molecular weight of the strong ligands (RAMAMOORTHY and KUSHNER, 1975; BUFFLE *et al.*, 1977; GIESY *et al.*, 1977), and/or adsorption of the organic materials to the dialysis membrane or glassware used in the procedure (Tanoue, unpublished data). More relevant information is clearly required about the recovery of natural ligands from seawater. 2) The demetallization procedure in the present method is not completely effective because insufficient time is allowed for equilibration or the feedback of equilibrium during the separation of EDTA from material in the dialysis tubing. 3) Low concentrations of members of a group of strong ligands were not detected because of the inadequate sensitivity of the ISEs. If a highly sensitive technique could be employed, the missing ligand that has a stronger affinity than L_1 for copper might be detectable.

Recent understanding of copper speciation based on the experimental studies has revealed that organic ligands whose conditional stability constants are strong enough for complexation with copper, occur in seawater (Table 2), and more than 99% of Cu(II) in the surface water are present as organic complexes. Since the total concentration of dissolved Cu(II) is usually less than that of the organic ligand, L_1,

Table 2. Conditional stability constants of organic Cu(II) complexes in seawater. Organic ligands were grouped into three classes, namely the strongest, L_S, the strong, L_I, and the weak, L_W, ligands according to HIROSE (1994)

Sampling location	Conditional stability constant			Ligand concentration (nM)			References
	log K_S'	log K_I'	log K_W'	L_S	L_I	L_W	
Irish Sea	----	----	8.9-9.7	----	----	1000	MANTOURA *et al.* (1978)
Adriatic Sea	----	----	8.8	----	----	130-150	PLAVSIC *et al.* (1982)
Atlantic	----	----	9.9	----	----	170	VAN DEN BERG (1982)
Western North Pacific	13.8	11.8	----	7	21	----	HIROSE *et al.* (1982)
Southeastern Gulf of Mexico	----	>12	9.8	----	5	15	SUNDA and FERGUSON (1983)
Sargasso Sea	----	----	8.8	----	----	28	WAITE and MOREL (1983)
South Atlantic	----	12.2	10.2	----	11	33	VAN DEN BERG (1984)
Atlantic	----	----	9.9, 9.0	----	----	31,87	VAN DEN BERG *et al.* (1984)
North Sea	----	----	8.9-9.1	----	----	80-103	KRAMER and DUINKER (1984)
North Atlantic	----	----	9.1-9.5	----	----	50-82	KRAMER (1986)
North Atlantic	----	9.8-12	7.4-9.4	----	4-144	2-420	BUCKLEY and VAN DEN BERG (1986)
Biscayne Bay	----	12.0	10.4	----	5.1	110	Moffett and Zika (1987)
Naragansett Bay	----	12.2-12.4	10.1-10.2	----	20-50	100	SUNDA and HANSON (1987)
Coastal Peru	----	12.3	9.2	----	3.8	75	*ibid*
Chistiansen Basin	----	11.7	9.1	----	50	68	HERING *et al.* (1987)
Montauk Point	----	11.7	9.1	----	20	50	*ibid*
Sargasso Sea	----	----	9.8	----	----	7.5	HANSON *et al.* (1988)
Gulf Stream	----	----	9.7	----	----	7.5	*ibid*
North Pacific	13.0±0.6	----	10.0±0.4	1-3	----	5±2	COALE and BRULAND (1988 and 1990)
Southwestern Sargasso Sea	13.2	----	9.7	2.0	----	80	MOFFETT *et al.* (1990)
Severn Estuary	----	11.4-12.8	----	----	13-196	----	APTE *et al.* (1990)
Plymouth Channel	13.1-15.1	11.2	----	6-27	83	----	VAN DEN BERG *et al.* (1990)
North Carolina shelf	13.2	----	10.0	3.3	----	26	SUNDA and HUNTSMAN (1991)
Indian Ocean	----	12.6	----	----	4.1	----	DONAT and VAN DEN BERG (1992)
North Sea	15.6	12.4	----	7.8	16	----	*ibid*
Sargasso Sea	14.0	----	9.8	0.88	----	7.4	DONAT and Bruland (1992)
Sargasso Sea	13.6-15.9	12.4	10.5-10.7	1.5-2.0	3.5-5.4	44-73	VAN DEN BERG and DONAT (1992)
North Sea	14.2-15.6	12.0-12.2	----	1.2-9.1	16-21	----	*ibid*
Central Pacific	13.3-≥15	12.2-12.7	10.2-10.8	1-3	3-4	10-15	MILLER and BRULAND (1994)
San Francisco Bay	≥13.5	----	9.0-9.6	13	----	60-80	DONAT *et al.* (1994)
Mediterranean Sea	13.0-14.8	----	----	3.2-14	----	----	CAMPOS and VAN DEN BERG (1994)
Northeast Atlantic	12.2-13.1	----	----	4.9-13	----	----	*ibid*
Northeast Atlantic	12.0-13.0	----	----	5-12	----	----	ACHTERBERG and VAN DEN BERG (1994)

L_S, or $L_S + L_I$, most of the dissolved Cu(II) is present as complexes with the organic ligands, L_S and/or L_I. The organic ligand, L_W, may not bind to Cu(II) under the conditions of seawater. Thus, organic ligands L_1 and L_2 characterized by copper-titration of the demetallized sample in the present study may not participate in the complexation of copper in seawater.

Complexing ability for cadmium

One of the advantages of the method in the present study is that repeated measurements can be made by using the same sample after removal of the exogenously added metal ions. The same procedure also enables this technique to be applied to the binding by natural ligands of different metal ions. The measurements for Cd(II) were carried out after removal of Cu(II) from titrated sample solutions. Figure 2(b) shows titration curves for the same sample for Cu(II) and for Cd(II). The Nernstian response of the Cd(II) ISE was obtained in the control solution (solid line in Fig. 2(b)). The response with the same slope was also observed in the sample solution below $pC_M = 5$ ($= -\log C_M$). The decrease of the potential in the curve is also evident in the case of Cd(II). However, the degree of decrease is smaller than that in the curve for Cu(II), indicating that the complexing ability for Cd(II) is lower than that for Cu(II) under these conditions. The results for Cd(II) are also given in Table 1. The values were determined in the ranges of 6.74–7.21 for $\log K'_{ML}$ and 1.2–4.5 nM for C_L, respectively. If we apply the discrete-ligands model of DZOMBAK *et al.* (1986) and use the data-fitting procedure of RUZIC (1982), it appears that at least two ligands, which have different stability constants, are necessary to explain the results for Cu(II) as mentioned previously, while only one ligand is necessary to explain the results for Cd(II). These characteristic features are similar to the results obtained by GUY and CHAKRABARTI (1976) with isolated humic acid.

Undemetallizable ligand

During the course of experiments, we found out that copper was reproducibly detected in the demetallized sample collected from both coastal and oceanic areas (MIDORIKAWA *et al.*, 1992; MIDORIKAWA and TANOUE, 1994a). The concentrations of copper in concentrated solutions inside the sample bags before and after demetallization and in the corresponding control bags are summarized in Table 3. Amounts of copper in the sample bag after demetallization (Table 3, column A) were usually low, compared with the values before demetallization (Table 3, column B). However, a significant difference in levels of copper was observed between the results for the control (Table 3, column C) and those for the sample after demetallization (Table 3, column A). This phenomenon suggests that the copper detected after demetallization may bind strongly to some natural ligands in the sample solution even in the presence of excess amounts of EDTA. A methodology based on the combination of the demetallization procedure and the simple equilibrium model was developed to confirm the occurrence of the strong ligand and to estimate its complexing ability. The model used in this section was provided in detail by MIDORIKAWA and TANOUE (1993a, 1993b, 1993c, 1994a).

Table 3. Concentrations of copper in original and concentrated samples of seawater before and after demetallization and in the dialysis bag of the corresponding control experiments

Site[c]	Depth (m)	Original seawater C_{Cu}[a] (nM)	Concentrated seawater: Seawater volume (l)	Bag volume (ml)	C_{Cu}[b] B (nM)	C_{Cu}[b] A (nM)	Control: Bag volume (ml)	C_{Cu}[b] C (nM)
S-1[d]	0	1.20 ± 0.02	0.49	5.9	100	79	6.0	13
	0	1.49 ± 0.03	0.49	6.4	115	71	6.0	11
	0	1.44 ± 0.04	0.25	5.1	69	47	5.0	8.4
	0	1.33 ± 0.10	0.15	7.4	27	25	6.5	10
S-2[d]	0	4.14 ± 0.14	0.10	6.9	60	21	6.8	5.4
	0	4.09 ± 0.12	0.10	6.1	67	36	6.0	19
A	0	0.49 ± 0.02	1.05	8.9	58	62	8.4	26
P	0	2.70 ± 0.03	4.33	6.2	1890*	145	5.6	5.3
	191	0.55 ± 0.03	4.32	6.4	371*	53	5.6	5.3
J	0	0.33 ± 0.03	6.16	7.0	290*	22	6.2	13
	523	0.68 ± 0.03	7.23	5.9	833*	68	5.6	6.5
	1071	0.87 ± 0.03	4.75	5.6	738*	151	5.6	6.5

B: Before demetallization. A: After demetallization. C: Control.

[a]The total concentration of copper is given as that in the original seawater: An amount of copper detected in the dialysed sample before demetallization was converted to the concentration in seawater after subtraction of the blank value for copper measurement. The uncertainty represents a standard deviation (1σ).

[b]The concentration of copper is given as that in the dialysis bag. The precision in the determination of copper is generally ±2 nM (1σ). However, it is ±22 nM (1σ) for samples marked with "*".

[c]Site A, 33°17′ N, 134°14′ E on Feb. 12 1988; S-2, 35°01′ N, 138°30′ E on Apr. 26 1988; locations of other sites, see Table 1.

[d]Multiple measurements made on subsamples of surface seawater from Suruga Bay.

Three chemical forms of copper would occur in the concentrated sample, namely, copper complexed with EDTA, copper complexed with inorganic ligands and copper complexed with natural organic ligands. The concentrations of each copper species in the concentrated sample after demetallization were estimated from the experimental results and from calculations using an equilibrium model (Table 4; MIDORIKAWA and TANOUE, 1994a). The level of Cu(II)-EDTA in the sample was calculated to be almost equal to that of the controls ($C_{Cu,C}$: Table 3, column C). The value $C_{Cu,A}$ (Table 3, column A) for copper in the sample after demetallization was corrected by subtracting the control value $C_{Cu,C}$ (Table 3, column C). The corrected value was called the excess copper, $[Cu]_{ex}$, and is given by:

$$[Cu]_{ex} = C_{Cu,A} - C_{Cu,C}. \quad (1)$$

Table 4. Concentrations of excess copper detected and each copper species evaluated in the sample solution inside the dialysis bag at an ionic strength below 10^{-5} M, at pH5.71 and at 4°C

Site	Depth (m)	Concentration (nM)						Upper limit (nM)
		$[Cu]_{ex}$[a]	[CuY][b]	[Cu']	$[CuL_1]$	$[CuL_2]$	[Y'][c]	$[L_N']$
S-1	0	66	13	4.5×10^{-6}	0.001	0.0001	48	173
	0	60	11	3.7×10^{-6}	0.001	9×10^{-5}	50	159
	0	39	8.4	2.6×10^{-6}	0.0004	4×10^{-5}	53	99
	0	15	10	3.3×10^{-6}	0.0002	2×10^{-5}	51	42
S-2	0	16	5.4	1.6×10^{-6}	0.0003	1×10^{-5}	56	64
	0	17	19	7.5×10^{-6}	0.002	7×10^{-5}	42	72
A	0	36	26	1.2×10^{-5}	0.003	0.0002	35	45
P	0	140	5.3	1.6×10^{-6}	0.002	0.0001	56	473
	191	48	5.3	1.6×10^{-6}	0.001	0.0003	56	305
J	0	9	13	4.5×10^{-6}	0.02	0.001	48	592
	523	61	6.5	2.0×10^{-6}	0.007	0.0009	54	407
	1071	144	6.5	2.0×10^{-6}	0.007	0.001	54	675

[a, b, c]The precision (1σ) is as follows: a) ±3 nM; b) ±2 nM; c) ±17 nM.

The excess copper ($[Cu]_{ex}$) in the demetallized sample is free from the Cu-EDTA complex from Eq. (1). In Table 4, the concentrations of both inorganic ([Cu']) and organic copper complexes $[CuL_1]$ and $[CuL_2]$ are negligible as compared with the level of the excess copper. In conclusion, the excess copper found in the demetallized sample cannot be derived from an EDTA complex, from an inorganic complex, or from natural organic complexes with L_1 and L_2. Thus, the idea begins to emerge that the excess copper may be in the form of a more stable complex with an alternative and uncharacterized natural ligand—an undemetallizable ligand—that is stronger than natural ligands L_1 and L_2.

Conditional stability constant of the undemetallizable ligand

If we assume that the natural ligand bound to copper after demetallization is a single unique species of undemetallizable ligand, designated "L_N", the conditional stability constant, K'_{CuL_N}, can be estimated from the concentration of each species of copper in the sample after demetallization. The equilibrium with respect to the ligand L_N in the solution of the demetallized sample is expressed by the following equation:

$$CuL_N + Y' \overset{K_N}{\rightleftharpoons} L_N' + CuY \qquad (2)$$

Therefore

Table 5. Estimated lower limits for the concentration of the undemetallizable ligand, L_N, and the conditional stability constant for copper in various samples of seawater at an ionic strength below 10^{-5} M, at pH5.71 and at 4°C.

Site	Depth (m)	Lower limit C_{L_N}[a] (nM)	Lower limit log K'_{CuL_N}
S-1[b]	0	0.79 ± 0.04	13.9 ± 0.2
	0	0.77 ± 0.04	14.0 ± 0.2
	0	0.80 ± 0.06	14.2 ± 0.2
	0	0.74 ± 0.15	14.0 ± 0.3
	ave.	0.78 ± 0.04	14.1 ± 0.1
S-2[b]	0	1.08 ± 0.20	14.2 ± 0.3
	0	1.04 ± 0.18	13.5 ± 0.3
	ave.	1.06 ± 0.13	14.0 ± 0.2
A	0	0.31 ± 0.03	13.8 ± 0.3
P	0	0.200 ± 0.004	14.3 ± 0.3
	191	0.071 ± 0.004	14.0 ± 0.3
J	0	<0.01	*
	523	0.050 ± 0.002	13.9 ± 0.3
	1071	0.170 ± 0.004	14.0 ± 0.3

*Not estimated.

[a]Concentration of ligand is that in seawater. The values of $[Cu]_{ex}$ was taken as the lower limit for C_{LN} (MIDORIKAWA and TANOUE, 1994a).

[b]Values estimated from multiple subsamples of seawater from Suruga Bay are averaged.

$$K_N = \frac{K'_{CuY}}{K'_{CuL_N}} = \frac{[L_N'][CuY]}{[CuL_N][Y']} \tag{3}$$

where Y is EDTA, K_N is equilibrium constant, K'_{CuY} and K'_{CuL_N} are conditional stability constants corresponding to CuY and CuL_N complexes, respectively. For K'_{CuL_N}, Eq. (3) can be rewritten as follows:

$$K'_{CuL_N} = \frac{K'_{CuY} \cdot [CuL_N][Y']}{[CuY][L_N']}. \tag{4}$$

In Eq. (4), the concentration of the CuL_N complex $[CuL_N]$ is given as $[Cu]_{ex}$ in Table 4. The concentrations of the copper-EDTA complex [CuY] and of free EDTA [Y'] are obtained from the control experiment (Table 4). The conditional stability constant of copper-EDTA, namely, $\log K'_{CuY}$, is 13.78. For the concentration of free L_N (concentration of ligand not bound to copper), $[L_N']$, only its upper limit was estimated (MIDORIKAWA and TANOUE, 1994a). Thus, K'_{CuL_N} was calculated from Eq. (4) as a minimum estimate. The conditional stability constant for the ligand L_N was evaluated to be higher than a range of $10^{13.8}$–$10^{14.3}$ (Table 5). Concentrations of

this ligand in the coastal waters are higher than those in the oceanic waters, but the conditional stability constants are the same for each other. The conditional stability constant of ligand L_N is comparable to or a little higher than those of the L_S in the literature, but levels of the ligand L_N is one or two orders of magnitude lower than those of the L_S in the literature (Table 2).

Binding sites

DOM is a very poorly characterized organic reservoir in the ocean. Our methodology involves dialysis and, consequently, organic matter with apparent molecular mass greater than 1000 Da is concentrated and demetallized. Let us briefly consider the molecular mass distributions of DOM obtained by the most commonly adopted ultrafiltration technique. In the 1970's, there are discrepancies between the data of OGURA (1974) and WHEELER (1976) who reported that the majority of DOM had a high molecular mass (more than 1000 Da) and the data of ANDREN and HARRISS (1975) and MAURER (1976) who indicated that low-molecular-mass materials, of less than 1000 Da, comprised the bulk of the DOM. Recently, it appears clearer that relatively limited proportions, approximately 20–40%, of DOM had a molecular mass greater than 1000 Da (CARLSON *et al.*, 1985; BENNER *et al.*, 1992). Furthermore, it was observed that less than 50% of organic ligands had a molecular mass greater than 1000 Da as sensed by UV-absorption (see below), and relative abundance of organic ligands alone in each molecular mass fraction (>10 kDa, 10 kDa–1000 Da, and <1000 Da) is approximately proportional to the abundance of DOM in each fraction (Midorikawa and Tanoue, unpublished data). Thus, recovery in the present study may present less than half of the total DOM as well as total ligands in dissolved phase.

Despite the fact that the quantitative information about the recovery of DOM during the dialysis processes could not be provided, carbohydrates and amino acids were detected in the dialyzed samples (Table 6). Concentrations of carbohydrates and amino acids were high at the surface and low in the deep waters at site J. The values for amino acids at the station located in the western North Pacific (site B)

Table 6. The concentrations of carbohydrates and amino acids in dialyzed samples from site J. Concentrations of carbohydrates and amino acids are expressed as μg/l of glucose and glycine equivalents, respectively

Depth (m)	Carbohydrate	Amino acid [a]
0	109	11.7
500	37.6	1.2(?)
1000	19.3	8.2

[a]In the western North Pacific (site B: 31°33′ N, 145°21′ E), concentrations of total amino acids (glycine equivalent) were determined to be as follows: 0 m, 28; 28 m, 17; 69 m, 16; 120 m, 21; 210 m, 14; 500 m, 9.0; and 1000 m, 9.0.

ranged from 14 to 28 μg/l at the depth above 210 m and 9 μg/l at depths of 500 m and 1000 m, respectively (see footnote in Table 6). Concentrations of amino acids in the dialyzed samples from two different oceanic areas also resemble one another. Repeated rounds of metal-titration measurements using exactly the same sample gave the same titration curves (MIDORIKAWA *et al.*, 1990). The procedure is reproducible with respect to the recovery of the organic matter in seawater, although the organic matter in the dialyzed sample may represent less than half of total DOM.

Table 1 gives invaluable information about the binding sites on the natural ligands in seawater. It is not necessary to assume that the binding sites for the two metal ions are the same. However, the stability constants for Cu(II) of synthetic ligands, such as EDTA and NTA (nitrilotriacetic acid), are usually higher than those for Cd(II) (MARTELL and SMITH, 1974). Concentrations of ligand (C_L) for Cd(II) were found to be similar to those of the stronger ligand (C_{L_1}) for Cu(II) in the present study (Table 1). Thus, it is reasonable to interpret the results as indicating that the stronger binding site for Cu(II) corresponds to that for Cd(II). It is likely that the weaker ligand for Cd(II) may be too weak to detect.

Because of the lack of the suitable methodology (see below), little information is available about metal-binding functional groups. The conditional stability constants of ligand L_1 in the present study were compared with those of model ligands as well as humic substances through the linear free energy relationships (LFER) between Cu(II) and Cd(II) (Fig. 3). Many researchers have considered that the ligands that possess complexing ability in natural waters are variants of so-called

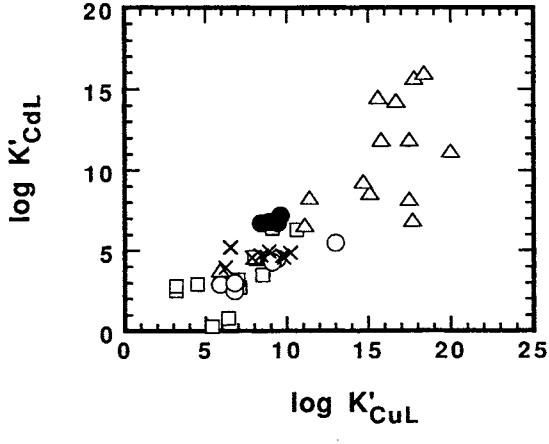

Fig. 3. Comparisons of formation constants for copper and cadmium complexations of (a) weak ligand L_1 (●) at pH8.15 and model ligands (□, bidentate; ○, tridentate; △, more than tetradentate) as well as humic substances (×). The values for model ligands were taken from MARTELL and SMITH (1974) and were corrected that results to values corresponding to conditions in the present study. The values for humic substances are those measured at pH5–7, taken from the following literature: a) MANTOURA *et al.* (1978), SOHN and HUGHES (1981); b) CHEAM and GAMBLE (1974), RAMAMOORTHY and KUSHNER (1975), GUY and CHAKRABARTI (1976), BRESNAHAN *et al.* (1978), and SAAR and WEBER (1979).

humic acids or fulvic acids, and their major complexing sites are generally assumed to involve phenolic, hydroxy or carboxylic groups, such as those on salicylate, phthalate, pyruvate, and glycolate moieties (SCHNITZER and KHAN, 1972). However, ligand L_1 was distinguished from the humic substances (Fig. 3).

ISOLATION AND CHARACTERIZATION OF ORGANIC LIGAND

Studies on metal-organic association in the oceanic waters have been focused on the metal speciation *in situ*. It should be noted that any study on metal speciation has been based on a measure of the complexing ability of the organic ligand and, then, metal species were calculated as to whether a given metal was in such form of complexes with the ligand, or not, under the conditions present in seawater. Therefore, a knowledge of organic ligands in seawater is essential for understanding metal speciation in seawater. Beside the chemical aspects of metal-organic association, distributional characteristics of organic ligands are basic knowledge for understanding the dynamics of organic ligands as well as metal speciation in seawater. However, a little information is available for distribution profiles of organic ligands in oceanic waters. Attempts to isolate and characterize organic ligands in seawater have been focused on so-called marine humic materials that are extracted from seawater with hydrophobic resin such as XAD and C_{18} SEP-PAK (e.g., MANTOURA *et al.*, 1978; PIOTROWICZ *et al.*, 1983; MILLS and QUINN, 1981). These methods as well as the procedure described in the previous section are available for detecting organic ligands in seawater, but such samples obtained have the organic ligands as a minor constituent and the major constituent has no significant complexing ability. Thus, organic characteristics of such samples do not represent those of the organic ligands only. The need for isolation of organic ligands alone from seawater is apparent if we are to understand their molecular characteristics.

Immobilized metal ion affinity chromatography (IMAC) that was originally used for protein separation (PORATH *et al.*, 1975; SULKOWSKI 1985), was applied for the isolation of organic ligands from seawater by GORDON (1992). Transition-metal ions are immobilized on Sepharose derivatized with iminodiacetic acid (IDA) chelating groups and allowed to interact with ligands in the mobile phase. Only organic ligands for a high affinity with the particular immobilized metal ion are chemically adsorbed by forming the complexation with unoccupied coordination sites on the metal. The adsorbed ligands are washed to be free from other organic materials in seawater and are eluted by either lowering the pH of the mobile phase or introducing a displacing ligand in the buffer. Thus, the principle of IMAC is suitable for both isolation and concentration of organic ligands from seawater.

Procedure

Chelating Sepharose® Fast Flow gel (Pharmacia, Uppsala, Sweden) coupled with copper was applied to isolate and concentrate organic ligands in oceanic seawaters. An entire procedure of IMAC involves four steps: 1) IMAC column conditioning; 2) immobilized copper affinity adsorption of organic ligands from seawater (abbreviated IMA-adsorption). It is customary to include sodium chloride,

0.1–1.0 M in buffer (pH7–8) used in IMAC to suppress ionic interaction between sample and the gel. Thus, filtered seawater was directly applicable to a column of IMAC; 3) IMA-desorption. Seawater, whose pH was lowered to pH2 using HCl, was used as the mobile phase and eluted organic ligands from the IMAC column were monitored with UV-absorbance (wavelength, 254 nm); and 4) regeneration of the column according to the manufacture's instruction (PHARMACIA, 1988).

Ligand fractions were subjected to chemical analyses. A solution of 3.5% NaCl containing EDTA (50 mM) was also used as the mobile phase. In this case, naturally occurring organic ligands were IMA-desorbed quantitatively, however, the eluent comprised not only organic ligands from seawater but also exogenously added EDTA, Cu-EDTA and copper. The organic ligand fraction was demetallized to remove exogenously added EDTA and copper, and was subjected to measurements of complexing ability for copper as mentioned in the previous section. More detailed descriptions of the methodology will be reported elsewhere (MIDORIKAWA and TANOUE, 1994b).

Isolation of organic ligands

Figure 4(a) shows a typical elution pattern of organic ligands in oceanic water on IMAC, when acidified seawater was used as the mobile phase. A large peak with elution volume of approximately 19 ml, was always detected in all the seawater samples examined. Since no peak was observed upon elution when seawater samples were loaded onto Chelating Sepharose® Fast Flow gel without copper, organic matter responsible for UV-absorption is not an artifact but so are organic ligands that are concentrated by mechanisms of IMA-adsorption and -desorption on IMAC. UV absorption of the ligand fraction was significantly higher than those of the original seawater as well as the column blank within a spectral region 200–400 nm (Fig. 4(b)). DOM is the most significant contributor to the total UV absorbance in seawater in the Ultra-violet region (ARMSTRONG and BOALCH, 1961). At wavelengths below 250 nm, the only effective inorganic species in seawater were nitrate and bromide and the absorbance of both inorganic species were particularly apparent at wavelengths below 235 nm (OGURA and HANYA, 1966). Organic ligands that were eluted with the solution of 3.5% NaCl containing EDTA and were demetallized, also exhibited similar UV spectra (data not shown). In this case, inorganic species and organic compounds with molecular mass of less than 1000 Da were eliminated during the process of demetallization. Differences in UV spectra between eluate and column blank are again observed which could be attributed to the presence of natural organic ligands in the eluate. Thus, organic ligands in a few liters of seawater were successfully isolated and concentrated to a few milliliters of their fractions.

For further verification, the ligand fraction that was eluted with a solution of 3.5% NaCl containing EDTA, was subjected to copper-titration after demetallization. Two ligands having different stability constants, were again characterized from the titration curve (data not shown). Two ligands, L_1 and L_2, in the ligand fraction of IMAC appear to resemble the L_1 and L_2 determined in concentrated DOM in the previous section. Stronger ligands L_S and L_I in the literature and L_N in the previous

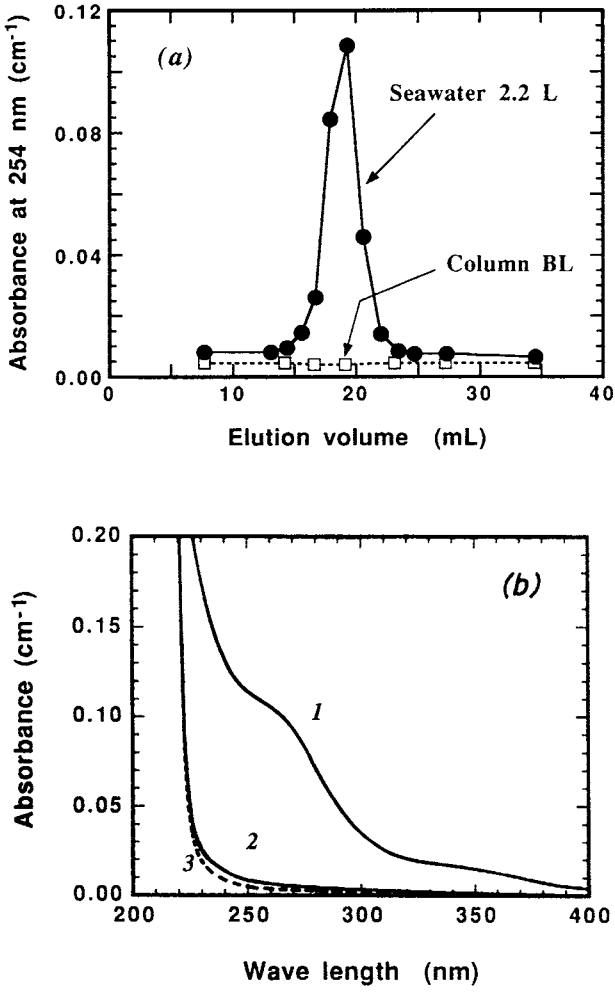

Fig. 4. Elution pattern of organic ligand fraction detected at 254 nm (a) and UV spectra of the organic ligand fraction (b) from IMAC column. Sample was 2.2 L of surface water collected from site M-3 (0°00′ S, 155°57′ E on Jan. 3, 1992). Seawater at pH2 (HCl) was used as the mobile phase. Curves 1, 2 and 3 in (b) represent a peak fraction (volume; 1.4 mL) of organic ligand, corresponding fraction (1.4 mL) of column blank and seawater at pH2 (mobile phase), respectively.

section were not detected in the ligand fraction of the IMAC. As mentioned previously, stronger ligands L_S and L_I in the literature, and ligand L_N have complexed with copper and other metals in seawater, while L_W in the literature and weaker ligands L_1 and L_2 detected in concentrated DOM were not bound to copper nor other metals but might be bound to Mg(II) and Ca(II) in seawater (HIROSE, 1994). Thus,

it is reasonable that IMAC isolates and concentrates the weaker ligands from seawater. Apparently further methodological improvements of IMAC are required if we are to isolate and concentrate stronger ligands in seawater.

Organic chemical characterization of isolated ligand

Up to date, there is no information available for organic chemical characteristics of specified organic ligands because of a lack of methodology for the isolation of the ligand from seawater. Organic ligands corresponding to L_W in the literature (Table 2) were detected from surface through deep waters (COALE and BRULAND, 1988, 1990; MIDORIKAWA *et al.*, 1990). The apparent lack of vertical structure in profiles of their concentrations and the conditional stability constants appeared to indicate that the weak ligand L_W was refractory and their dynamics was conservative and the water column residence time must be long (COALE and BRULAND, 1988). Successful isolation of organic ligands using IMAC enables chemical analyses of organic ligands in seawater. Organic chemical compositions of ligands extracted by IMAC which were assigned to the weak ligands L_W in the literature, gave a new insight to the dynamics of weak ligands, as mentioned below.

Figure 5 shows vertical profiles of chemical properties of organic ligands that were eluted with acidified seawater. Levels of amino acids and carbohydrates are high in the surface water and decrease rapidly with depth, while UV-absorbance shows no significant vertical trend. Ratios of fluorescence to UV-absorbance was low in the surface water and increased with depth. Since UV-absorbance was almost constant, the vertical increase in the ratio was responsible for the increase in fluorescent intensity of the organic ligand in the mid-depth and deep waters. The observations indicate that the organic ligand fraction extracted from seawater is a mixture of at least two types of ligands, even if the two types of ligands were indistinguishable from each other with respect to their concentrations and their conditional stability constants. One type represents molecules having a relatively low fluorescent emission but are rich in amino acids and carbohydrates. Vertical profiles showed levels of this type which were high in the surface water and decreased rapidly with depth. This type of ligands was likely derived from living organisms, especially phytoplankton, in the surface water. Steep gradients in amino acids and carbohydrates below the euphotic layer indicated this type of ligands were easily remineralized by microbial activity, thus, its dynamics may be active.

Another type was predominant in deep water and this type of ligands appeared to have reverse chemical characteristics, namely, poor contents of amino acids and carbohydrates but relatively high fluorescent emission. The vertical characteristics of fluorescent emission of organic ligand isolated by IMAC are quite similar to those of bulk seawater fluorescence in the oceanic water column (HAYASE *et al.*, 1988; CHEN and BADA, 1992), namely low fluorescence in surface waters and significantly higher in deep waters. HAYASE *et al.* (1987) reported that the absorption spectra (250–400 nm) continuously increased with decreasing wavelength, with no apparent absorption band or shoulder. They also found that fluorescence/absorbance ratios were nearly constant in Tokyo Bay waters, and these characteristics were similar to

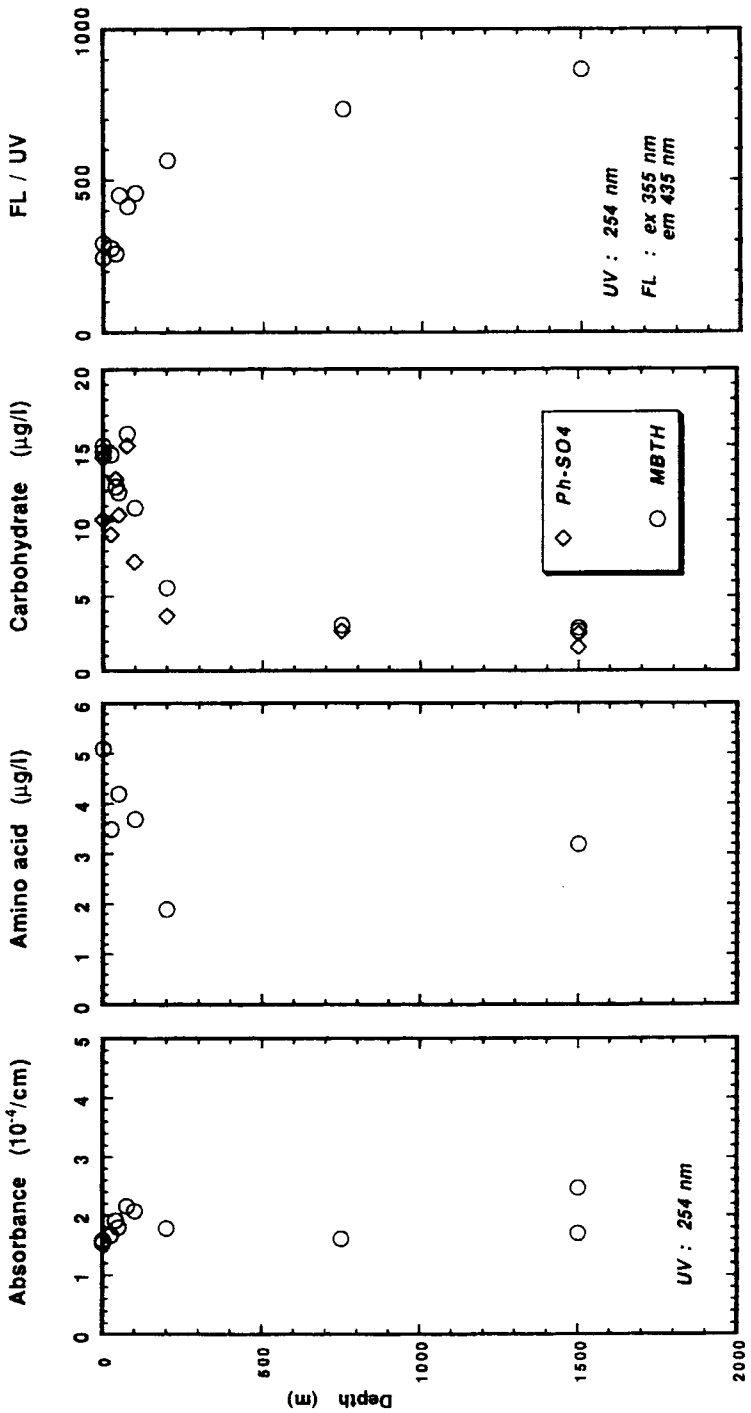

Fig. 5. Vertical profiles of organic chemical characteristics of organic ligand fraction extracted by IMAC in the Equatorial Pacific. Water samples at sites N-12 (00°01′ N, 170°00′ E on Feb. 12 1991) and M-2 (00°00′ S, 150°01′ E on Dec. 30 1991) were combined. The fluorometric method (UDENFRIEND *et al.*, 1972) was used for determination of total amino acids. Total carbohydrates were determined by both the phenol sulfuric acid method (HANDA, 1966) and the MBTH method (BURNEY and SIEBURTH, 1977; PAKULSKI and BENNER, 1992).

those of sedimentary fulvic acid from Tokyo Bay. The absorption spectrum of the organic ligand in the present study has a broad shoulder with an absorption maximum near 254 nm (Fig. 4(b)) and the fluorescence/absorbance ratios significantly increased with depth. The observations demonstrate that the organic ligand isolated by the IMAC is different from the humic type fluorescent compounds in coastal waters. This is consistent with the result from the LFER analysis that ligand L_1 in concentrated DOM was clearly distinguished from the humic substances (Fig. 3). CHEN and BADA (1992) discussed the dynamics of the fluorescent compounds in the sea and commented that one possible source and explanation for the fluorescence of mid-depth and deep waters are the release of fluorescent molecules from sinking particles in correlation with nutrient remineralization (HAYASE *et al.*, 1987, 1988) and *in situ* formation by microbial activity. It is well documented that the major sink of the fluorescent compounds was photochemical bleaching (e.g., KRAMER, 1979; HAYASE *et al.*, 1988; KIEBER *et al.*, 1990; CHEN and BADA, 1992). The similar vertical trends between the fluorescence of organic ligands in the present study and bulk fluorescence measurements in the literature, indicate that the dynamics of the latter type of organic ligands isolated by the IMAC is closely related to those of bulk fluorescent organic materials. The source of this type of ligands is probably the release from particulate organic matter in surface water, but they readily degraded because of photochemical bleaching *in situ*. The residence time of this type of ligands may be shorter than that of the former type of organic ligands in surface water. However, once they are transported from the surface to mid-depth and deep waters, through sinking particles, their residence time is longer compared with those of former type of ligands because of their bio-refractory nature (CHEN and BADA, 1992).

Levels of organic ligands corresponding to strong ligand L_S as determined by metal titration method in the literature (Table 2) were high in the surface and decreased with depth rapidly below the mixed layer. These characteristic profiles suggested that the strong ligand L_S was likely derived from the phytoplankton in the euphotic layer (COALE and BRULAND, 1988, 1990). It is very likely that the chemical structure of strong ligand L_S is that of a highly ordered chelating compound, and therefore such a ligand must be derived directly from organisms. However, such profiles do not always indicate the direct evidence of the source of the strong ligand, because such strong ligands were possibly complexed with metals *in situ* and they could not be detected, even if they occurred in the intermediate and deep waters (BRULAND, 1989; MIDORIKAWA and TANOUE, 1994a). The facts that the strong ligand was detected below the mixed layer when methods other than metal-titration method were employed (MIDORIKAWA and TANOUE, 1994a; CAMPOS and VAN DEN BERG, 1994; ACHTERBERG and VAN DEN BERG, 1994), also supported the observation that vertical profiles of strong ligand L_S determined by metal titration were method-oriented results. To date, IMAC could not extract the strong ligand and the source and fate of strong ligands remain to be in question which will be addressed in future studies. Organic chemical characterization of the strong ligand will provide key information for understanding the dynamics, i.e., production, decomposition, transformation and transportation, of the strong ligand in seawater.

SUMMARY AND CONCLUSIONS

The three types of ligands for Cu(II), namely, L_1, L_2 and L_N, were characterized in the DOM that was concentrated by repeated rounds of lyophilization and dialysis. Ligands L_1 and L_2 appeared to be assigned to the group of weak ligands in the literature. The conditional stability constant of ligand L_N for Cu(II) was extremely high and was comparable to that of EDTA.

Two types of ligands that were similar to weak ligands L_1 and L_2 in the concentrated DOM sample, were extracted directly from seawater using immobilized metal ion affinity chromatography (IMAC). The weak ligands have been thought to be conservative in the water column because of the lack of vertical structure in their concentrations and conditional stability constants. However, IMAC gave a new insight that the weak ligands were a mixture of at least two types of organochemically different ligands and their dynamics might be more active, even if the two types of ligands were indistinguishable to each other in terms of their conditional stability constants.

It is quite clear that we have only begun to understand the role and relevant chemical characteristics of organic ligand in the sea. Studies of metal-organic association have been focused on the metal speciation *in situ*. The metal speciation has been based on a measurement of the complexing ability of organic ligand. However, the characterization of organic ligands has been left out of investigation, up to date. Therefore, the need to clarify the dynamics of organic ligand is apparent in future studies if we are to understand the biogeochemical cycle of metals in the water column.

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