Table 1. Comparison of toxicity for representative PACs in the atmospheric environment

	Promoting activity[b]		Carcinogenicity		Relative mutagenic potency[g]	Relative AhR potency[h]	CYP1A1 mRNA induction[k]
Compound[a]	Present study	Previous study	EPA[e]	IARC[f]			
PAHs							
Phe		−[c]	D	3	0	ni[i]	
FA			D	3	0	u[j]	
Py		+[c]	D	3	0	n[i]	
B[*a*]A		+[c]	B2	2B	0.082	0.26	29
Chr	+	+[c]	B2	2B	0.017	0.35	29
CPP				2A	6.9		
B[*b*]FA	++		B2	2B	0.25	4.7	48
B[*k*]FA	++		B2	2B	0.11	84	56
B[*a*]P	−	±[c]	B2	1	1	1	40
B[*e*]P	+	+[c]		3	0.0017		
DB[*a*,*h*]A		−[d]	B2	2A	0.29	9.0	52
IdP			B2	2B	0.31	2.5	51
B[*ghi*]Pe		+[c]	D	3	0.19	u	3
DB[*a*,*l*]P	−			2A	24		
Oxy-PAHs							
NphQ	+						
PhQ	−				0	u	
BAQ	+				0	0.028	17
NCQ	+				0	0.13	21
B[*a*]FO	+					0.37	3
B[*b*]FO	+					0.072	15
BPO	++				0.32	u	0.4
Nitro-PAHs							
1-NPy	+	+[c]		2A	0.025		
1,6-DNPy	+			2B	0.28		
1,8-DNPy	+			2B	0.046		
2-NFA					0.050		
3-NFA	+			3	0.0026		
8-NFA	+						
6-NChr	++			2A			
3-NBAO	++			2B			

[a] Phe, phenanthrene; FA, fluoranthene; Py, pyrene; B[*a*]A, benz[*a*]anthracene; CPP, cyclopenta[*cd*]pyrene; DB[*a*,*h*]A, dibenz[*a*,*h*]anthracene; IdP, indeno[1,2,3-*cd*] pyrene; B[*ghi*]Pe, benzo[*ghi*]perylene; 2-NFA, 2-nitrofluoranthene.

[b] Using Bhas 42 cells. ++, powerful positive; +, positive; −, negative; ±, equivocal.

[c] Previously reported by Asada *et al.* (29).

[d] Previously reported by Sasaki *et al.* (30).

[e] Environmental Protection Agency in USA; B2, human probable carcinogen; D, unclassifiable (5).

[f] International Agency for Research on Cancer: 1, human carcinogen; 2A, human probable carcinogen; 2B, probable carcinogen; 3, unclassifiable (4, 21).

[g] Using human B-lymphoblastoid cell constitutively expressing CYP (h1A1v2) from Durant *et al.* (7).

[h] Measured transcriptional activation via promotor region of CYP1A1 using mouse hepatoma (H1L1) cell by Misaki *et al.* (40).

[i] No activity.

[j] Less than 25% of maximum activity with TCDD.

[k] Fold-induction of CYP1A1 mRNA with 5 μM PAC to DMSO control using human hepatoma (HepG2) cells by Misaki *et al.* (41).

Many ROS-producing metals, metalloids and catechol exhibit tumour-promoting activities (30) and regulate cell proliferation (55). ROS are reported to regulate protein kinase C (PKC) and protein tyrosine phosphatase (PTP) followed by mitogen-activated protein kinase (MAPK) activation and E-cadherin regulation, leading to EMT (56). Several nitro-PAHs (38,42) and quinone compounds (15–19,38,42,57) have been reported to induce ROS production. Several quinones have been reported to inhibit PKC, a regulator of cell proliferation and tumour promotion (58). Several oxy-PAHs express ARE-mediated enzymes such as AKR1C1 (15,41). Therefore, the high tumour-promoting activity of BPO could be induced *via* AREs, considering the RT-PCR results for HO-1 mRNA in Bhas 42 cells (Figures 2E and 3B). It has been reported that several PAHs were metabolically activated to form ROS-generating metabolites, and before that ARE-mediated gene activation (AKR1C1 mRNA) was induced by many PAHs in HepG2 cells (41). Hence, the tumour-promoting activities of B[*k*]FA and B[*b*]FA may be partly mediated by the ARE pathway (Figures 2A and 3B).

B[*a*]P, DB[*a*,*h*]A and DB[*a*,*l*]P are known to have powerful carcinogenic potency, probably resulting from their DNA adduct formation abilities followed by their mutation inducibility (Table 1) (4,5,7). Repeated doses of B[*a*]P and 3-methylcholanthrene have been reported to promote tumour development (59). Strong initiating activities for these compounds were found by Asada *et al.* although no tumour-promoting activities were confirmed using Bhas 42 cells (29,30). It is thought that the tumour development and initiating activities of Bhas 42 cells result from the fixation and accumulation of genetic damage after cell divisions (29). Whereas in the present study, DB[*a*,*l*]P and PhQ showed no tumour-promoting activity and B[*a*]P exhibited no significant activity though both CYP1A1 and HO-1 mRNA were significantly induced (Figures 2A, C and 3). These results may have been caused by growth arrest and apoptosis following the accumulation of DNA adducts and oxidative stress (Figure 2B and D). In rat liver WB-F344 (60) and cancerous human lung A549 cells (61), an increase in cell proliferation at low concentrations was reported for DB[*a*,*l*]P, PhQ and NphQ which exhibited cytotoxicity (significant decrease of cell proliferation) at high concentrations. In comparison with these cells, Bhas 42 cells are more sensitive to cytotoxicity, and an increase in cell proliferation could not be detected at low concentration levels for these acutely toxic compounds (Figure 2B and D). In Figure 3, the induction of CYP1A1 and HO-1 mRNA in treated cells at 24 h was predicted to be reflected by AhR-mediated and oxidative signalling of the compounds themselves or their metabolites, respectively, even if they were cytototoxic (39,40). Epidermal hyperplasia associated with the induction of cytokines and hypersensitivity was required for tumour promotion in mouse skin exposed to DB[*a*,*l*]P at low concentrations (62); however, this promoting effect could not be evaluated with Bhas 42 cells in the present study. Tumour-promoting activities were detected for many compounds in the present study, as reflected by the release from contact inhibition *via* the dysregulation of signal transduction (32–34,52–56,58), although an accumulation of genetic damage may be partially responsible. It was thought that the contribution of genetic damage was limited to tumour-promoting activities in Bhas 42 cells for several oxy-PAHs (BAQ, B[*a*]FO and B[*b*]FO (Figure 2C and E and Table 1), which are not or only slightly mutagenic (7,13).

The tumour-promoting activities of these compounds may be mediated by other receptors and signal transduction pathways (63–66). For example, it has been demonstrated that non-dioxin-like polychlorinated biphenyls (PCBs) may activate tumour promotion in liver epithelial cells *via* the constitutive androstane receptor (CAR) with the degradation of E-cadherin (adherence junction) and inhibition of Wnt/β-catenin/Tcf signalling (65,66). Interactions of other nuclear receptors with Wnt/β-catenin/Tcf signalling and their effects on adhesion, morphogenesis and oncogenesis have been reviewed (63), and tumour promoters in liver cells acting through the peroxisome proliferator activated receptor α have also been reported (65).

In summary, we found that B[*k*]FA, B[*b*]FA, BPO, 3-NBAO and 6-NChr had powerful tumour-promoting activities and most of the other oxy-PAHs and nitro-PAHs also exhibited these activities when using Bhas 42 cells. It was predicted that many of these compounds induced CYP1A1 or HO-1 mRNA in Bhas 42 cells. Accordingly, AhR activity or antioxidant signalling may be relevant to the tumour-promoting activities of these compounds on release from contact inhibition. Several studies have identified genes related to the promotion of cell growth and tumour induction, as demonstrated by transfection and knockdown of these genes in cellular and experimental animal models (67–70). We next plan to elucidate in detail the genes and mechanisms involved in the tumour-promoting activities of several PACs. Lübcke-von Varel *et al.* (71) showed the inhibition of GJIC in the polar fractions of sediment polluted with petroleum extracts. It is predicted that several polar PAH derivatives, such as oxy-PAHs, significantly contribute to tumour-promoting activities in the atmospheric environment surrounding industrial areas because of combustion. Future studies are warranted to identify the primary compounds that contribute to tumour-promoting activities of particulate matter in the atmospheric environment.

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RESEARCH PAPER

Novel speciation analysis of copper in river water: observation of soluble anionic copper–ligand complexes

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Abstract In natural water, copper is believed to be complexed with organic materials such as humic and fulvic acids, as well as anthropogenic substances such as ethylenediaminetetraacetic acid (EDTA). The nature of the complexes is known to be a main factor in determining copper toxicity to aquatic biota, as well as its geochemical transport. In this study, organically chelated copper species in river water were revealed to be classified into hydrophobic and anionic components using column cartridge separation techniques based on C18 and anion exchange. The amounts of anionic ligated copper species in the tested water samples ranged from 0 to 46 nM, and the average contribution of the anionic ligated copper to the total dissolved copper concentration was around 30 %. Several water samples contained discharged sewage effluents with high contents of EDTA, which may be the main chelating agent responsible for copper retention on the anionic ion exchange cartridge column. However, most of the river water samples were not contaminated with EDTA, and therefore, fulvic substances are expected to be responsible for retention. As this is the first observation of anionic copper species in the natural aquatic environment, further geochemical and biological studies of these substances should be undertaken.

Keywords Copper speciation · River water · Sewage plant treatment water discharge · Anionic organic chelated copper

Introduction

The biological effects of copper have been well described in published studies, and its environmental toxicity has been revealed as quite variable (Flemming and Trevors 1989). Although mammals are relatively resistant to copper exposure, several aquatic biota are very sensitive to free copper ions at the ppb level (Nor 1987). These biological effects are profoundly affected by the chemical speciation of the copper in the water (Borgmann and Ralph 1983; Campbell et al. 2014; Connan and Stengel 2011; Flemming and Trevors 1989; Huebert et al. 1993; Nor 1987; Winner 1985). Several studies have shown that copper complexed with ligands such as humic and fulvic substances or anthropogenic compounds such as ethylenediaminetetraacetic acid (EDTA) exhibited low or no toxicity to aquatic biota, leading to the hypothesis that the free copper ion is the main determinant of toxicity (Huebert et al. 1993; McKnight 1981; Winner 1985). For example, algae growth in a Japanese lake was influenced by the presence of organic chelators (e.g., EDTA) and was well correlated with copper complexation ability (Croot et al. 2003; Nanjo et al. 2000).

Copper speciation analysis has been recently performed for several estuaries (Buck et al. 2007; Coale and Bruland 1988; Waeles 2004; Waeles et al. 2009). In surface water from an estuarine area in the Northeast Pacific, the extent of copper ion chelation by organic compounds was estimated to be as high as 99 % (Coale and Bruland 1988). Waeles (2004) showed that ~40 % of the total copper was bound by non-polar hydrophobic organic materials in the

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Loire estuary and North Biscay continental shelf of France. In copper-contaminated streams, organic substances such as fulvic acids and EDTA bind to copper ions, and ~90 % of the total copper in these sources is in the organically complexed form (Breault et al. 1996). Because streams and rivers can be highly contaminated with drainage effluents from sewage plants in highly developed areas, man-made chelating substances can also influence copper ion speciation, and affect algal or microorganismal growth in water as well the geochemical mobility of copper ions.

The determination of copper speciation has been performed in a variety of ways. Classical electrochemical techniques including stripping voltammetry and polarography have been used to detect inorganic copper ions or labile organically chelated copper (Croot et al. 1999; Masuda and Boyd 1993; Muller et al. 2001; Scarano et al. 1992; Sodré and Grassi 2007). A direct comparison between the concentration of inorganic copper ions and the total copper ion content, which was measured by spectroscopic methods, enabled the determination of organic copper in water (Jones and Bolam 2007). Ligand competition is another candidate for estimating the concentration of inorganic copper ions (Scarano et al. 1990, 1992). More recently, organic copper was successfully extracted using C18 cartridge columns; the adsorbed materials, i.e., the hydrophobic organic copper (HOC), could be distinguished from the inorganic or hydrophilic organic copper ions that passed through the column (Abbasse et al. 2002; Mills and Quinn 1981; Mills et al. 1982).

In this study, to obtain precise knowledge about the copper speciation in river water flowing through highly populated areas, the above-mentioned C18-filtered fraction containing inorganic and hydrophilic organic copper ions was further subjected to an anion exchange resin followed by a chelating resin consisting of iminodiacetate groups. These processes allowed the division of the copper species into anionic organic copper (AOC) and inorganic copper ion or readily labile cationic organic copper (IOC) species, respectively. The copper concentrations in the obtained fractions were determined spectrophotometrically using 4-(3,5-dibromo-2-pyridylazo)-*N*-ethyl-*N*-(3-sulfopropyl)-aniline (3,5-DiBr-PAESA) (Abe et al. 1989). Using this novel system, the copper speciation in river water was determined, and the obtained data was compared with the EDTA quantification results from the same water samples.

Material and methods

Materials

The anion exchange column cartridge, an Oasis MAX 3 cc (60 mg) extraction cartridge, and ODS column cartridge, a Sep-Pak® Vac RC (500 mg) C18 cartridge, were obtained from Waters (Milford, MA, USA). For inorganic copper ion extraction, Empore™ High Performance Extraction Disk Cartridges (Chelator 6 ML/10 MM) were obtained from the 3 M Company. 3,5-DiBr-PAESA was synthesized according to the literature with a slight modification, in which an ODS column purification was employed instead of precipitation mediated by CO_2 gas bubbling (Ohshita et al. 1981, 1985). For the working solution to determine copper ion, 3,5-DiBr-PAESA (1 mg) was dissolved in methanol (1 mL). Other reagents were analytical grade and used without further purification unless otherwise noted.

River water sampling

The sampling was performed from October 2014 to January 2015 at 13 sites in eight rivers, including the Kaname River (sampling point 1), Suzu River (sampling points 2 and 3), Onzo River (sampling point 4), Koayu River (sampling points 5 and 8), Ogino River (sampling point 6 and 7), Nakatsu River (sampling point 9), Sagami River (sampling point 10), and Sakai River (sampling points 11–13), as illustrated in Fig. 1. The locations of sewage plants are also shown in the same figure. Sampling sites 1, 3, 11, 12, and 13 were located downstream from the sewage plants, and sampling site 2 was located upstream.

Speciation of copper in river water

River water (1 L) was filtered through a 0.45 μm polyethersulfone membrane filter (Millipore Express Plus, 0.45 μm) immediately after sampling. The filtrate (500 mL) was passed through a Sep-Pak Vac RC (500 mg) C18 cartridge that had been pretreated by the sequential addition of methanol and water. The material retained on the C18 column was eluted with 1:1 methanol/water (3 mL) solution, evaporated to dryness, and redissolved in 3 M HNO_3 (1 mL). This retained material was designated as highly HOC. The C18-filtered water (500 mL) was next passed through an OASIS MAX anion exchange column that had been prewashed by sequential additions of water (5 mL), methanol (5 mL), and water (5 mL). This filtrate was then passed through the chelating resin of an Empore Disk column cartridge that had been pretreated according to the manufacturer's instructions. To recover the adsorbates from the column cartridges (OASIS MAX and Empore Chelating Disk), each cartridge was washed with distilled water (5 mL) and the retained materials were eluted twice with 3 M HNO_3 (0.5 mL each for a total volume of 1 mL). The adsorbed material on the OASIS MAX had an anionic nature due to the chelation of organic substances, and corresponded to AOC. The copper retained on the Empore disk was a combination of inorganic and

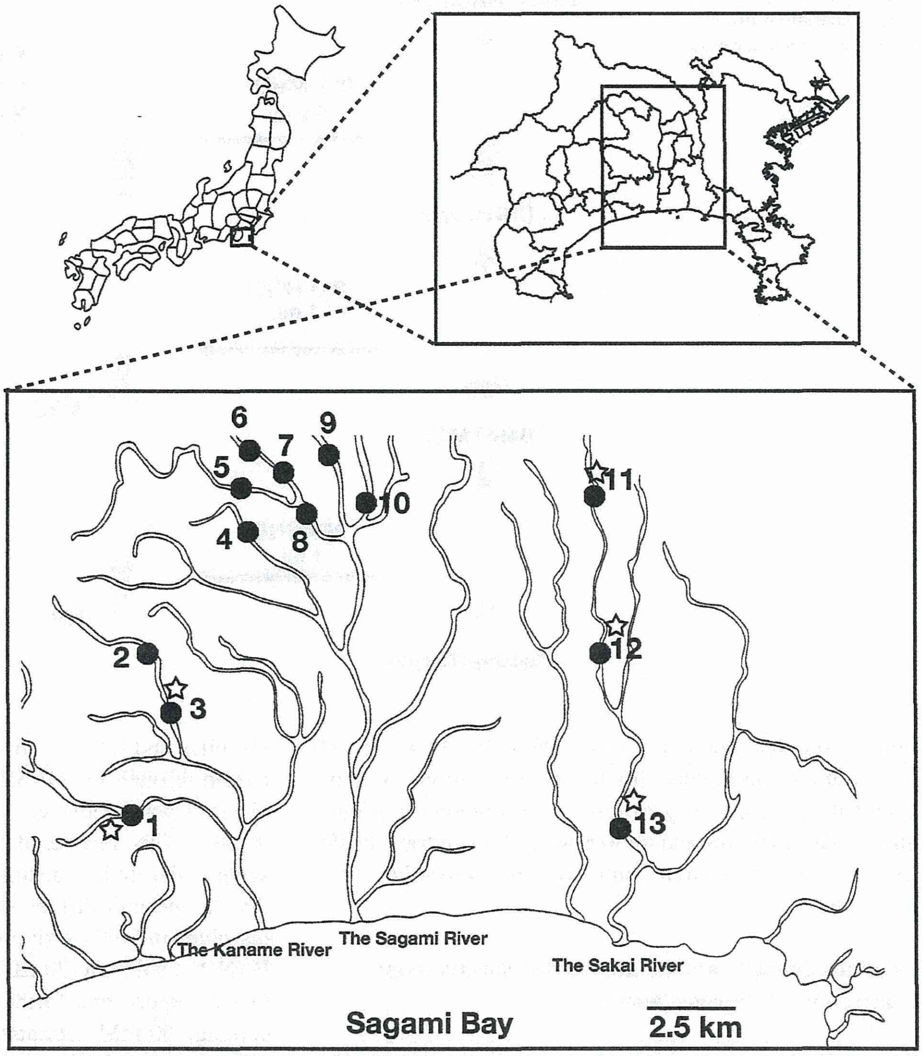

Fig. 1 Sampling points of river water. Sewage plants are also shown as *star marks*

readily labile organic copper, abbreviated together as IOC. The speciation procedures are illustrated in Fig. 2.

Determination of Cu^{2+} using 3,5-DiBr-PAESA

The copper-sensitive colorimetric detection reagent 3,5-DiBr-PAESA was originally developed for the determination of copper in serum. Because of the easy, simple handling and copper specificity of the reagent, a determination kit has recently been provided by several manufacturers to detect copper ions at sub-ppm levels. Although cobalt and nickel ions can interfere with the spectroscopic determination of copper, the concentrations of these ions are markedly lower than that of copper in the natural environment. For these reasons, 3,5-DiBr-PAESA can be considered as specific for copper ions. This reagent, however, has a moderate chelate formation constant, and the measurement of copper can be strongly disturbed by chelating substances such as EDTA when using the originally described method as provided in the manufacturer's protocol. This weakness was overcome in this study by an exchange of the buffer solution, which made it possible to distinguish strongly and weakly chelated copper species in the water samples. The detailed procedure is described as follows.

To a plastic tube (1.5 mL), the following were sequentially added: the sample (100 μL), 0.5 M sodium citrate buffer (pH 4, 54 μL), and 0.7 M L-ascorbic acid (30 μL). After 60 min, 3,5-diBr-PAESA solution (15 μL) and 10 % SDS solution (45 μL) were sequentially added and the solution was heated at 37 °C for 5 min. Each solution (200 μL) was transferred to the well of a 96-well plate and the absorbance at 570 nm was measured using a plate reader (Tecan Infinite 200). When the

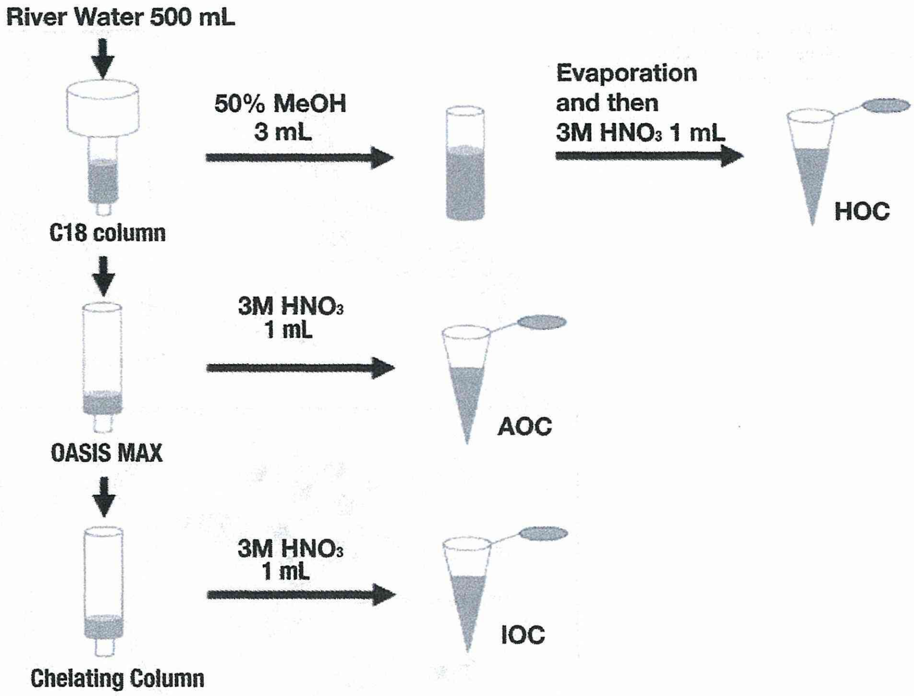

Fig. 2 Schematic diagram of copper speciation using cartridge column separation

sample solution contained 3 M HNO_3, 6 M NaOH (50 μL) solution was added. In the determinations of inorganic/labile organic copper ions, 3 M ammonium acetate buffer was used instead of 0.5 M sodium citrate buffer (pH 4). The detection limit of this procedure was 0.15 μM.

Graphite furnace atomic absorption spectroscopy analysis of Cu in river water

To evaluate the accuracy of the Cu concentrations obtained by the 3,5-DiBr-PAESA system, the river samples were analyzed by atomic absorption spectroscopy (AA) with graphite furnace equipment. The total copper concentration of the filtered river samples was also determined by AA analysis using an AA6300 (Shimadzu, Kyoyo). A Cu hollow-cathode lamp was used as the radiation source at 324.8 nm. The temperature program was set for drying at 150 °C (Ramp 20 s), ashing at 800 °C (Ramp 13 s,), atomizing at 2300 °C (Ramp 0 s, hold 2 s), and cleaning at 2500 °C (Ramp 0 s, hold 2 s). The detection limit was 2.5 nM.

Analysis of EDTA in river water

To determine the concentration of chelating substances in river water, EDTA was analysed by HPLC according to the published method (Geschke and Zehringer 1997; Nowack et al. 1996). An OASIS MAX 3 cc (60 mg) extraction cartridge was pretreated with water (5 mL), methanol (10 mL), and water (5 mL). A water sample (200 mL) was passed through the OASIS MAX column and the retained material was recovered with 50 % formic acid/methanol (4 mL). This recovered solution was evaporated in the sample vial under vacuum and further dried in a DTU-2B dry thermo unit (Taitec Corp.) at 90 °C. The residue was dissolved in 5 mM sodium formate (1 mL) and then, 1 mM $Fe(NO_3)_3$ solution (20 μL) was added. The sample vial was tightly capped and further heated at 90 °C for 1 h. After cooling, 50 mM tetrabutylammonium bromide (TBABr) solution (40 μL) was added, and 20 μL of this solution was injected into the HPLC system. The HPLC system consisted of an LC-10ADvp HPLC pump (Shimadzu, Kyoto) and an SPD 10 Avp UV detector (258 nm) equipped with a COSMOSIL 5C18-AR II column (4.6 × 250 mm, Nacalai Tesque, Kyoto, Japan). The eluent consisted of 8 % acetonitrile containing TBABr (0.001 M), sodium formate (0.005 M), and formic acid (0.015 M). The detection limit of this analysis procedure was 0.01 μM EDTA.

Results

Optimization of Cu quantification procedure using 3,5-DiBr-PAESA

Because 3,5-DiBr-PAESA is only commercially available as a part of a kit to detect copper in biological samples, we synthesized it according to the published method, for use in

Cu^{2+} quantification. The physicochemical data of the prepared 3,5-DiBr-PAESA was fully coincident with reported values (Supplementary Fig. 1). Although the literature procedure reproducibly allowed the detection of copper ion at sub-ppm levels, the addition of EDTA to the copper standard solution clearly disturbed the formation of the 3,5-DiBr-PAESA–Cu^+ complex due to the formation of the highly stable EDTA–Cu^{2+} complex, resulting in a decrease in the UV absorbance from the expected value (Fig. 3a). The original quantification procedure, therefore, should be revised to detect chelated copper species.

In this study, three experimental parameters, including reaction temperature, kinds of buffer solution, and reducing reagents, were re-examined. Because Cu^+ does not form a particularly stable complex with EDTA, an exchange of reducing reagents was the first choice to overcome the lower-than-expected results obtained by the original procedure in the presence of EDTA. Hydroxylamine hydrochloride is generally used to reduce metal ions, such as Fe^{3+} to Fe^{2+}. Compared to the ascorbic acid used in the original procedure, hydroxylamine gave slightly better results at room temperature; however, it offered no benefits for 3,5-DiBr-PAESA complex formation in the presence of EDTA. Elevated reaction temperature did not improve the quantification results. At higher reaction temperatures, the reproducibility of the quantification value was quite low. However, the simple exchange of the buffer solution from an acetate buffer to a citrate buffer gave excellent results: the absorbance values for Cu^+–3,5-DiBr-PAESA in the presence and absence of EDTA were quite similar (Fig. 3a). In this case, the large excess of citric acid enhances the preferential formation of the citric acid–copper complex rather than the EDTA–Cu complex. A calibration curve using citrate buffer showed a linear response from 0 to 5 ppm (Fig. 3b). This finding indicates that a simple exchange of buffer solution would be applicable to Cu speciation analysis. The results obtained in acetate buffer probably reflect the concentration of IOC, whereas those obtained in citrate buffer showed the sum of concentrations of both IOC and strongly chelated copper.

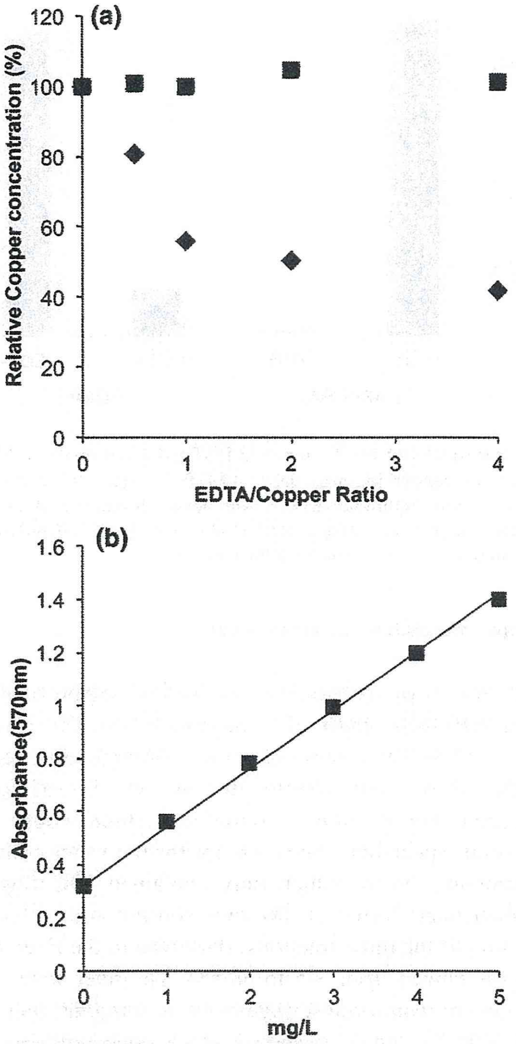

Fig. 3 **a** Effects of EDTA on the analysis of Cu ion by 3,5-DiBr-PAESA at 37 °C using acetate (*diamonds*) or citrate (*squares*) buffer solutions; and **b** Cu^+–DiBr-PAESA calibration curve in citrate buffer system

Development of speciation method for copper via column separation

Because the Cu concentrations in the river water were predicted to be at sub-ppb levels, it was necessary to condense the Cu species in the samples. To achieve this, several column cartridges were tested. Also, the efficiency of the copper speciation using column cartridges was confirmed using an EDTA–Cu complex that was prepared by mixing a solution of 2 ppm Cu^{2+} with an equimolar amount of EDTA. The obtained EDTA–Cu complex solution (500 mL) was passed through the OASIS MAX column and the retained material was eluted with 3 M HNO_3 (1 mL total). The copper concentration in the eluate was determined by the described improved colorimetric method using 3,5-DiBr-PAESA. Without EDTA, no Cu was found in the eluate, whereas with EDTA, a nearly quantitative recovery of Cu was confirmed (Fig. 4). Moreover, although the Cu–EDTA complex was not retained on the iminodiacetate chelating cartridge column, free copper ion could be recovered by the same chelating disk. Furthermore, the Cu–EDTA complex was not retained on the C18 column. Because EDTA has four carboxylate groups, the total net charge of Cu–EDTA is estimated to be −2. Therefore, an anionically charged Cu complex (AOC) such as Cu–EDTA can be speciated by the anion exchange column.

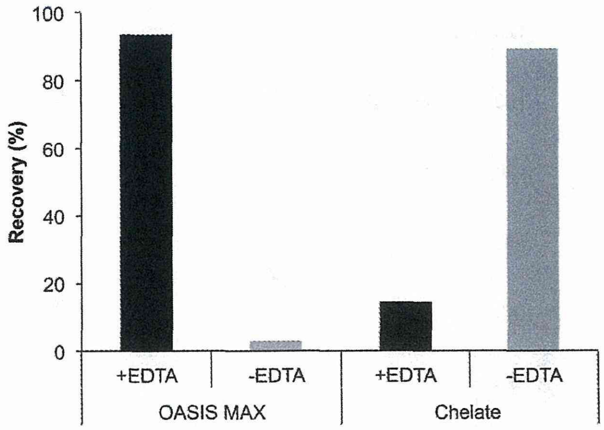

Fig. 4 Recovery of free Cu ions and EDTA–Cu complexes. −EDTA: 2 mg/L of copper ion was used. +EDTA: 2 mg/L of copper ion premixed with equimolar EDTA was passed through each column. Retained copper was eluted 1 M HNO_3 and the recovered copper concentration was determined with PAESA

Copper speciation of river water

River water was collected in the central region of Kanagawa Prefecture, Japan. The Sagami, Sakai, and Kaname Rivers cross from north to south through this region. Several rivers were selected for the analysis of copper speciation. For 12 out of 13 samples, we found copper ions in various speciation states, except for the water collected at sampling site 6, which only contained IOC (Fig. 5a; Supplementary Table 1). The total concentration of copper (the sum of the three fractions) dissolved in the river water samples ranged from ~5 to 68 nM. The river water sampled downstream from sewage plants at sampling sites of 3, 11, 12 and 13, tended to show high Cu concentrations. The HOC concentration was ~1–5 nM at most of the tested sampling sites, but in the Sakai River, where three sewage plants are located, HOC was present at over 5 nM. AOC was also found in relatively high amounts (above 10 nM) at a sampling site 8. IOC was found at a concentration of around 10 nM. The relative ratios of IOC/AOC/HOC are shown in Fig. 5b. The AOC/IOC values were relatively comparable at each sampling point except 6, whereas the HOC percentages were relatively high in rivers affected by sewage effluents at sampling sites 11, 12 and 13.

Because the results for nearly all the samples approached the detection limits of our AA system, the raw samples after filtration through a 0.45 μm filter were analyzed by AA. The total copper concentrations obtained by AA were well coincident with the data obtained from the improved 3,5-DiBr-PAESA system in this study (Supplementary Fig. 2). Moreover, randomly selected extracted samples were also examined by AA, and showed very close values to the 3,5-DiBr-PAESA system.

Concentration of EDTA in river water

The concentrations of EDTA were determined by HPLC after preconcentration, following the method developed by Geschke et al. with slight modification (Geschke and Zehringer 1997). Here, an OASIS MAX anion exchange cartridge column was used for the preconcentration of EDTA instead of the commercially available SAX-based extraction column (J. T. Baker) used in the original method. The EDTA recovery with the OASIS MAX cartridge column was around 90 %. Because this method requires evaporation of the eluent, 50 % HCOOH/MeOH was selected as solvent. The obtained HPLC chromatograms showed the presence of EDTA peak; however, the EDTA concentrations in almost all samples were near the detection limit of our system, except for three sample locations. These three sampling points were downstream of the drainage water discharges, and contained 3.7–16 nM EDTA. At other sampling points, only trace amounts of EDTA (below 1 nM) were detected (Fig. 6; Supplementary Table 1).

Discussion

In this study, we developed a new spectroscopic analysis method to distinguish between chelated and free Cu ions with a simple exchange of buffer systems. The exchange of the acetate buffer system by the citrate buffer system probably promotes copper–citrate complex formation in the presence of large amounts of citrate, resulting in smooth reaction with 3,5-DiBr-PAESA. Moreover, we found that the dissolved copper could be classified into three speciation states, including hydrophobic C18-retained copper (HOC); hydrophilic, anionic organic-chelated copper (AOC); and inorganic or labile cationic organic-chelated copper (IOC). This is the first report of the observation of anionic chelated copper species. The determined copper concentrations were comparable to those of San Francisco Bay surface water that had been affected by drainage (~30 nM), and Petaluma River water (at a median value of ~50 nM) (Buck et al. 2007). As mentioned in the results section, the AA results were well coincident with the data obtained by the DiBr-PAESA method. We conclude, therefore, that the concentrations of the three copper species (i.e., IOC, AOC, and HOC) obtained in this study were quite reliable.

To date, copper speciation methods have only classified free copper ions or organically chelated copper ions. Almost of all the copper in oceanic surface waters was reported to be complexed with organic materials (Coale and Bruland 1988). Dissolved organic carbon concentrations were reported to be well correlated with those of total