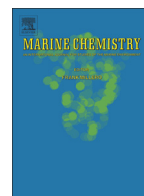




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## Evidence for copper-binding humic substances in seawater

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## ABSTRACT

Copper-binding humic substances (Cu–HS) in seawater were determined using a novel method based on cathodic stripping voltammetry (CSV). Cu–HS adsorb on the mercury (Hg) surface and are quantified on the basis of the reduction current for complexed Cu. Humic acid reference material (Suwannee River humic acid, HA) was used to calibrate the voltammetric response for the HS. The method was used to determine the concentration of Cu–HS in estuarine and coastal seawater, giving very good agreement with HS determined by UV spectrophotometry. Optimised conditions include a Cu concentration of 20–50 nM, a deposition potential of 0.05 V, differential pulse mode and  $\text{pH}_{\text{NBS}}$  8.2. The presence of Cu–HS indicates that this may be an important species of Cu in estuarine waters. The complex stability of Cu–HA was determined by two methods giving a value of 12.08 for  $\log K'_{\text{Cu-HA}}$  and a binding capacity of 18 nmol Cu for 1 mg of the HA. This complex stability is less than that ( $\log K'_{\text{CuL}} = 14$ ) of the strongest Cu complexes thought to occur in ocean waters suggesting that the Cu–HS could represent the weaker class of Cu-binding ligands. Iron was found to compete with copper for complexation with the HA.

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## 1. Introduction

Trace metals, such as iron (Fe) and copper (Cu), are important micronutrients that can impact the growth of microorganisms. Cu is an essential part of various enzymatic pathways, such as in carbon and nitrogen cycles and in Fe transport (Jacquot et al. 2014; Maldonado et al. 2006; Peers et al. 2005) and reportedly alleviates stress from Fe limitation to coastal diatoms (Biswas et al. 2013). Conversely, ionic  $\text{Cu}^{2+}$  is also toxic to marine microorganisms (Morel et al. 1978; Sunda and Guillard 1976), affecting some cyanobacteria at  $[\text{Cu}^{2+}] > 10^{-12}$  (Brand et al. 1986).

The chemical speciation of copper in seawater is dominated by organic complexation (Bruland et al. 2000; Buck and Bruland 2005; Buck et al. 2012; Jacquot et al. 2014; Skrabal et al. 1997). The identity of the ligands is still poorly known but Cu-binding thiols have been identified emanating from reducing marine sediments and occurring in estuarine and ocean waters (Chapman et al. 2009; Laglera and van den Berg 2006; Le Gall and van den Berg 1998; Ross et al. 2003). Thiols are known to be produced by microorganisms (Dupont et al. 2004) sometimes in response to Cu stress (Leal et al. 1999; Moffett and Brand 1996). Humic substances (HS) are known to bind Cu in both freshwater and seawater (Kogut and Voelker 2001; Shank et al. 2004; Xue and Sigg 1999) and may represent a second component of the pool of copper-binding ligands. Fulvic (FA) and humic (HA) acids make up the soluble components of HS, which in turn is often a major component of dissolved organic matter in freshwaters (15–100%

(Chanudet et al. 2006). FA and HA in natural waters can be quantified on the basis of their absorbance (Abbt-Braun and Frimmel 2002; Stevenson 1982), chemiluminescence (Tian et al. 2005), elemental analysis (Abbt-Braun and Frimmel 2002; Huffman and Stuber 1985) and voltammetric response for complexes with Fe (Laglera et al. 2007) and molybdenum (Mo) (Quentel and Elleouet 2001). The voltammetric method has been used to demonstrate that HS-like substances bind Fe in the marine system (Laglera and van den Berg 2009).

Here it is demonstrated that Cu–HS species in seawater also adsorb on the electrode and are detected by voltammetry. This response is used to quantify the concentration of Cu-binding HS in seawater and leads to a method to further identify Cu-complexing ligands in natural waters. The method was tested on samples from estuarine waters demonstrating that Cu-binding HS is present. The complex stability ( $\log K'_{\text{CuHA}}$  values of  $\sim 12$ ) is comparable to that for copper complexing ligands in other river and estuarine waters (Dryden et al. 2007; Gordon et al. 2000).

## 2. Materials and methods

## 2.1. Equipment

The voltammetric system used was a  $\mu$ Autolab-III potentiostat (Ecochemie, Netherlands) connected to a hanging mercury drop electrode (HMDE, Metrohm model 663VA). The reference electrode was Ag/AgCl with a salt bridge filled with 3 M KCl, with a glassy carbon rod counter electrode. Solutions were stirred during the deposition step by a rotating PTFE rod and the instrument was controlled by GPES software. Oxygen was removed from measured solutions by

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purging with water-saturated nitrogen. Mercury use was minimised by changing the software to discard 2 mercury-drops and using the 3rd instead of discarding the usual 4 drops between scans. Voltammetric cells (glass and PTFE) used for total copper determination were cleaned using 0.1 M HCl and rinsed with deionised water followed by UV-digested sample before determination. Cells used for titrations were normally not rinsed between titrations to minimise de-conditioning of the vials; they were briefly rinsed ~once a week. pH measurements were calibrated against pH 7 and pH 4 standards on the NBS pH scale. A 125-W UV system (Van Den Berg 2014) with 4 30-mL PTFE-capped quartz sample tubes was used to irradiate (45 min) acidified sea water samples prior to determination of total dissolved copper. Absorbance was measured at a wavelength of 355 nm using a Jenway 7315 spectrophotometer in polystyrene cells of a 1 cm path length. Background correction was against UV seawater. The absorbance of each station was calibrated against increasing concentrations of the HA standard to quantify the HS in samples, similar to that used for chromophoric dissolved organic matter (CDOM) (Chanudet et al. 2006; Hong et al. 2005; Laglera et al. 2007).

## 2.2. Reagents

Water used for rinsing and dilution of reagents (MQ) was purified by reverse osmosis (Millipore) and deionisation (Milli-Q). An aqueous stock solution containing 0.01 M salicylaldehyde (SA) was prepared in 0.1 M HCl. Copper standard solutions were prepared by dilution of an atomic absorption spectrometry standard solution (BDH Spectrosol grade) in 0.01 M HCl. A pH buffer containing 1 M boric acid (Aristar grade) and 0.35 M ammonia was UV-irradiated for 45-min to remove organic contaminants. Contaminating metals were removed from the buffer by overnight equilibration with 100  $\mu\text{M}$   $\text{MnO}_2$  (van den Berg 1982) and then filtered (0.02  $\mu\text{m}$  inorganic membrane, Whatman); 100  $\mu\text{L}$  of this buffer in 10 mL seawater gave a pH of 8.2. Reference humic and fulvic acid used for calibrations were Suwannee River HA (International Humic Substances Society (IHSS) Standard II 2S101H) and FA (IHSS standard 1R101F), which were dissolved in MQ water to a concentration of 0.1  $\text{g L}^{-1}$  and stored in the dark at 4 °C when not in use.

All sample containers (fluorinated LDPE, Nalgene) were cleaned in 3 steps: first by soaking for 1 week in 1% detergent in warm MQ-water, followed by soaking for 1 week in 1 M HCl (Analar grade), and finally by soaking at least 1 week in 0.1 M HCl (Analar grade). Containers were then rinsed in MQ-water and stored partially filled with 0.01 M HCl (high-purity grade).

## 2.3. Sample collection and storage

Seawater from the Atlantic (mixed-depth, salinity 35, collected May 2003, 0.2  $\mu\text{m}$  filtered) was used for preliminary experiments to optimise the conditions for determining Cu-binding HS.

A transect of samples from the Mersey Estuary and Liverpool Bay (Irish Sea) was collected using a peristaltic pump. The water inlet tubing was held away from the vessel, the RV Marisa, (May 2013 and April 2014) and used to fill 5-L polyethylene containers. The suspended matter was allowed to settle overnight in the laboratory and the supernatant water was filtered through a 0.2  $\mu\text{m}$  filter (Sartobran cartridge, Whatman) using a vacuum pump, and stored in the dark at 4 °C.

## 2.4. Procedure to determine copper and copper-binding humic substances (HS) using CSV

Acidified seawater was UV-irradiated (giving UV-SW) for 45 min prior to the copper determination, whereas untreated filtered water was used for determining copper-binding humics (Cu-HS). An aliquot of 10 mL seawater was pipetted into the voltammetric cell and 100  $\mu\text{L}$  borate buffer was added (final concentration 0.01 M and  $\text{pH}_{\text{NBS}}$  8.2).

Total dissolved copper was determined by CSV in the presence of 20  $\mu\text{M}$  SA in the UV-SW (Campos and van den Berg 1994) after addition of ammonia and borate buffer to adjust to  $\text{pH}_{\text{NBS}}$  8.2. The deposition potential was  $-0.15$  V (30 s), using a 1-s potential-jump to  $-1.3$  V to desorb any residual organic matter, followed by 9 s equilibration at  $-0.15$  V prior to the voltammetric scan (differential-pulse (DP) mode, modulation time 40 ms, modulation amplitude 50 mV, step potential 5 mV, interval time 0.1 s).

The concentration of Cu-HS was determined after addition of sufficient copper (usually 50 nM Cu) to saturate the HS followed by detection of the Cu-HS complexes by CSV. Calibration was by internal standard additions of SR-HA (IHSS Standard II 2S101H). The deposition potential was  $+0.05$  V, and the deposition time was between 10 and 60 s depending on the concentration of HS. The quiescence time was 9 s and scans were initiated from 0 V and terminated at  $-0.75$  V. The scanning parameters were also differential-pulse (DP) mode, a modulation time of 40 ms, a modulation amplitude of 50 mV, a step potential of 5 mV, and an interval time of 0.1 s.

## 2.5. Procedure to determine $K'_{\text{CuHA}}$ and the concentration of copper complexing sites on the HA

The concentration of copper complexing ligands in a solution of HA in UV-SW was determined by CSV with ligand competition against SA (Campos and van den Berg 1994). Approximately 150 mL UV-SW was transferred to a 250-mL low-density polyethylene bottle (LDPE) (Nalgene), HA was added to 1 and 2  $\text{mg L}^{-1}$ , and 0.01 M borate buffer and 1, 2 or 10  $\mu\text{M}$  SA (actual concentration depending on the detection window) was added. 10 mL aliquots of the solution were pipetted into 12 25-mL PFA (perfluoroalkoxy alkane) (Savillex, Cole-Parmer) vials with lids. Copper was added to each vial in steps of progressively increasing concentration from 0 nM to 150 nM. These were then left to equilibrate overnight (or for a minimum of 8 h). The labile copper concentration (i.e. that which reacted with the added SA) in each cell was then determined by CSV using 30 s deposition time. The deposition potential was  $-0.1$  V, followed by a 9-s quiescence period and the scan initiated at the same potential (no potential jumps were used). The scan was in DP-mode as for total Cu. Data were interpreted using the van den Berg/Ruzic linearization procedure (Campos and van den Berg 1994) and comparative calculations using software from Dario Omanovic (MCC.exe programme, from <http://gss.srce.hr/pithos/rest/omanovic@irb.hr/files/Software/>), which fits the data simultaneously to several fitting methods, linear and non-linear.

## 3. Results and discussion

### 3.1. Preliminary experiments

Preliminary experiments involving measurement of iron-binding HS in seawater by catalytic CSV in the presence of bromate (Laglera et al. 2007) showed the presence of a peak at a potential of  $-0.25$  V, more negative than that of inorganic copper (at  $-0.17$  V), which increased with additions of HA. Separate experiments showed that the peak is obtained without bromate and that additions of both copper and humic acid (SR-HA) increased the height of the peak (Fig. 1), indicating that the peak is due to a complex of Cu with HA. The peak was present in coastal and estuarine samples but was removed upon UV digestion, with no peak in the range of 0 to  $-0.6$  V in UV-SW with 20 nM Cu until HA was added. HA addition to the UV-SW without added copper caused a peak by the Cu-complex with the background Cu concentration in the UV-SW (1.7 nM) which increased significantly with nanomolar Cu additions.

The peaks for Cu-HA and Fe-HA were found to increase simultaneously as HA was added to seawater, with the Cu-HA peak clearly visible without the requirement for bromate addition as required for Fe-HA.

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