Removal of Trace Levels of Cu(II) from Seawater by Co-precipitation with Humic Acids

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To maintain performance related to fuel consumption and maneuverability, the bottom of ships are painted with antifouling paint that contains Cu₂O as a biocidal pigment. However, in enclosed coastal areas around dockyards, some of the Cu(II) contained in the paint is eluted into the surrounding water. The present study examined the removal of Cu from seawater by co-precipitation with humic acids (HAs). After precipitating the HA in seawater, the amount of Cu(II) in the supernatant was colorimetrically measured by a colorimetry using bathocuproine. The removal efficiency (RE%) for micromolar Cu(II) increased with increasing initial concentrations of HAs. An RE of 90% was obtained using an HA derived from hardwood bark compost. Aromatic components in the HA that contained highly substituted acidic functional groups appeared to enhance the removal of Cu(II). The findings reported herein indicate that HAs represent a useful material for removing trace levels of Cu from seawater.

Keywords Humic acid, removal of copper, bathocuproine, seawater, precipitation, aggregation, antifouling paint

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Introduction

Fuel consumption and maneuverability are critical issues in the shipping industry. These factors are affected by the frictional resistance of water against the bottom hull, to which algae and shellfish can become attached. Therefore, to avoid the accumulation and growth of such fouling organisms, antifouling paint containing a biocidal pigment is frequently applied to the bottom of many ships. Cuprous copper oxide (Cu₂O), which functions as a biocidal Cu(II) source, has been used as a common component of antifouling paints since the 1980s. As used in biocidal pigment, cupric copper has a negative effect on organisms by arresting their growth, photosynthesis, mitochondrial respiration and reproduction. For example, the growth of a brown macroalgal sporophyte was inhibited with increasing concentrations of more than about 0.15 μM of Cu. It has been reported that even lower levels of Cu (0.4 μM) caused a 40% decline in germination frequency for a brown macroalga. The EC₅₀ value for Cu for a mussel was reported to be approximately 0.1 - 0.15 μM. However, the contamination by Cu derived from antifouling paint constitutes an environmental problem in enclosed coastal areas. In particular, Cu contamination in areas around a dockyard would be expected to be higher than that in the open sea because the amount of antifouling paint particles released from these ships in such areas would be increased. The leaching of Cu from an antifouling paint is accelerated with increasing surface area of the antifouling paint. Moreover, the contaminants would be easily retained because dockyards are usually located in enclosed coastal areas. Fine antifouling particles were reported to have accumulated in sediment around a dockyard (1280 mg-Cu kg⁻¹), and the leaching of Cu from the particles reached levels of more than 0.25 μM h⁻¹ under seawater-like conditions (4°C, 30% of salinity). It has also been reported that the Cu concentration in seawater around a shipyard situated in an enclosed coastal area significantly increased (ca. 0.01 - 0.37 μM) compared to that value for outside the shipyard (ca. 0.010 ± 0.005 μM). Seawater contains a variety of cations, for example, the concentration of Na⁺, Mg²⁺, Ca²⁺ and K⁺ are about 450, 48, 10 and 9 mM, respectively. Compared with the levels of these cations, even in highly polluted seawater, the concentrations of Cu were low (<1 μM). For this reason, the removal of contaminated dissolved Cu from seawater is a thorny subject in coastal environments.

It is known that humic substances have the ability to bind metals, which can influence metal speciation, solubility, mobility, and bioavailability in various aquatic environments. In particular, it is well known that heavy metals, such as Cu and Pb, form complexes with humic substances. The solubility of humic substances could be influenced by the ionic strength and the presence of multivalent cations. Under conditions of higher ionic strength, such as in seawater, the electric charge on humic substances is partially neutralized, which results in a decreased solubility. In addition, multivalent cations can stimulate the aggregation of humic substances. As a result, humic substances would be precipitated in seawater that contains high levels of Ca²⁺ and Mg²⁺. The binding affinity of humic substances to Cu²⁺ (log K = 6 - 13) is usually significantly stronger than those for Ca²⁺ (log K = 2 - 6) and Mg²⁺ (log K < 2). Therefore, even if the concentration of Cu²⁺ is low compared to Ca²⁺ and Mg²⁺, Cu²⁺ could be expected to preferentially bind to humic substances. After the binding of Cu²⁺, excess metal binding sites may also be occupied by Ca²⁺ and Mg²⁺, resulting in...
in the precipitation of humic substances and the contaminating 
Cu²⁺ associated with them.

In the present study, the removal of trace levels of Cu²⁺ from 
seawater by co-precipitation was examined. Humic acid (HA),
which is a major fraction of humic substances, was used as the 
Cu-removing agent. The ability of such an agent to remove Cu 
would be dependent on its structural features. Therefore, HAs 
derived from three different origins were used in this study. The 
influence of structural features, which were elucidated by UV- 
vis spectrometry and TMAH-py-GC/MS, on removal efficiency 
was examined.

**Experimental**

**Materials**

Hardwood- and softwood-bark composts and natural peat 
powder in Aberdeen shine were obtained as sources of HAs 
from the Sanyo Chip Industry Co., Ltd. (Yamaguchi, Japan), 
Green-Sogyo Co., Ltd. (Iwate, Japan) and Rivers-Import LLC. 
(Shizuoka, Japan), respectively. Before use in the tests, these 
soil samples were freeze dried and large tips were removed by 
filtering through a 2-mm mesh sieve. A stock solution of 
(Shizuoka, Japan), respectively. Before use in the tests, these 
sources of HAs, respectively. Before use in the tests, these 
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**Preparation of humic acids**

Humic acids (HAs) were extracted from peat and compost 
samples and purified according to the protocol of the 
International Humic Substances Society. Total humic 
substances were extracted from peat or compost samples with 
0.05 M aqueous NaOH. An aliquot of 1 M l(+)-ascorbic acid (Wako, 
Japan), which was used as a reducing agent, was prepared 
freshly for each day. An 80% final volume of artificial seawater 
was prepared by dissolving the following salts in 800 g of pure 
water: NaCl 28.0 g; MgSO₄ 7H₂O 7.00 g; MgCl₂ 6H₂O 4.00 g; 
CaCl₂ 2H₂O 1.48 g; KCl 0.70 g. The pH of artificial seawater 
was buffered at 8.00 ± 0.01 using 0.05 M tris-HCl.

**Co-precipitation and quantification of Cu**

A 9.6-mL aliquot of artificial seawater and Cu²⁺ (0 - 30 μM) 
was pipetted into a 15-mL centrifugation tube. HAs were added 
(0 - 500 mg L⁻¹) and the final volume was adjusted to 12 mL 
with ultrapure water. After shaking, the mixture was allowed to 
stand for three days at room temperature. Precipitates and 
aggregates were separated from the supernatant by centrifugation 
(10000 rpm, 10 min and 5°C) and filtration (0.20 μm).

The total Cu in the solution was measured by a method using 
BCP as a colorimetric agent independent from the HA 
concentration. To reduce Cu(II) to Cu(I), a 200-μL aliquot of 
1 M ascorbic acid was added to the filtrates. Cuprous copper 
was then extracted three times with 1 mL aliquots of BCP 
(300 μM) in chloroform. The resulting chloroform phase, 
which is caused by the surface active action of HAs, was 
isolated by centrifugation (10000 rpm, 3 min and 5°C). To 
concentrate and adjust the volume, the solvent was removed 
with a stream of N₂ gas at 80°C, and the dried extract was then 
dissolved in 1 mL of chloroform that was added using a 
volumetric pipet. The concentration of the Cu(I)-BCP complex 
was determined based on the absorbance at 476 nm using a UV-vis spectrophotometer (Jasco V-630, Japan) with a 1-cm quartz window micro-cuvette. The amount of Cu(II) that was co-precipitated with the HAs was determined from the difference between the concentration of Cu 
in the initial solution and that in the filtrate.

**Analysis of UV-vis spectroscopic properties**

UV-vis spectroscopic analyses were carried out on a UV-vis 
spectrophotometer (Jasco V-630, Japan) using a 1-cm quartz 
window micro-cuvette. The filtrate solutions, which were 
diluted to the value of initial HA concentration to 50 mg L⁻¹ 
with artificial seawater, were used for these measurements. The 
absorption and peak shape of the UV-vis spectra before and after the precipitation (Fig. S1, Supporting Information). According to the Beer-Lambert law, therefore, the concentrations of HA in the filtrates were simply estimated 
from the absorbance at 280 or 600 nm (ε₂₈₀ or ε₆₀₀) calculated using the following equation:

$$\varepsilon_{280\text{ or }600} = \frac{(\text{Absorbivity at }280\text{ or }600\text{ nm (cm}^{-1}\text{g}^{-1}\text{-HA})}{\text{HA in filtrate (g L}^{-1})}$$

There was a linear relationship between the absorbivity (cm⁻¹) 
at 220 nm and the concentration of HAs adjusted to 0 - 50 
mg L⁻¹ in 0.05 M tris-HCl buffer at pH 8.00 (r² = 0.999). 
No significant changes were found from the shape of the UV-vis 
spectra before and after the precipitation (Fig. S1, Supporting Information). According to the Beer-Lambert law, therefore, the concentrations of HA in the filtrates were simply estimated 
from the absorbivity (cm⁻¹) at 220 nm based on that value of 
50 mg L⁻¹ HAs in 0.05 M tris-HCl buffer (pH 8.00 ± 0.02).

**TMAH-py-GC/MS analysis for HAs**

Detailed structural features of HAs were investigated by 
TMAH-py-GC/MS, according to the procedure described in 
previous reports. In a typical run, a 1.0 ± 0.1 mg portion of 
powdered HAs was placed in a 50-μL deactivated stainless cup. 
Then, 25 μL of TMAH (40 mg mL⁻¹ in MeOH) and 10 μL of 
onadecanoic acid (0.05 mg mL⁻¹ in acetone) as an internal 
standard were then added to the cup. After removing the solvent 
in a desiccator under reduced pressure, the cup was placed in a 
PY-2020D type Double-Shot Pyrolyzer (Frontier Laboratories, 
Japan) connected to a GC-17A/QP5050 type GC/MS system 
(Shimadzu, Japan). The sample was pyrolyzed at 550°C for 
4.0 min. Pyrolysate compounds were separated on a 100% 
dimethylpolysiloxane capillary column (Quadrex, 0.25 mm i.d. 
× 25 m, 0.25 μm film thickness, TCI, Japan) using high purity helium (99.995%) as the carrier gas. The temperature program 
for the GC oven was as follows: 50°C for 1 min, 50 – 300°C at 
a heating rate of 5°C min⁻¹ and 300°C for 4 min. The 
quantitative analysis was calculated from the relative peak 
areas (%) to that of the internal standard. The structures of 
the detected pyrolysate compounds were determined by comparing 
the obtained mass spectra with NIST mass spectral data.
Results and Discussion

Removal efficiency of Cu(II) with HAs

The influence of incubation period on the removal efficiencies of Cu(II) (RE%) was investigated using HHA (Fig. 1). The RE% value for 3 μM Cu(II) increased during the initial 24 h, and then reached a plateau after a three-day incubation period (Fig. 1). This period was also sufficient for the precipitation of HA (Fig. S2). From the results, subsequent experiments were carried out using a 3-day incubation period.

Figure 2 shows the RE% for 3 μM Cu(II) for different concentrations of HAs. As shown in Fig. 2, the RE% values increased with increasing initial HA concentration for all of the HAs, and they reached a plateau for the initial concentrations of HAs higher than 300 mg L⁻¹. After adding HA to artificial seawater, the pH values for the sample solution of 50 – 500 mg L⁻¹ HA slightly increased to 8.03 – 8.25 from 8.00. Since the Cu(II) binding ability of HA could not be affected by this change of pH, the achieved RE% values varied depending on types of HA added. For HHA, the RE% was about 50% at a low initial concentration of HHA (50 mg L⁻¹), and reached about 90% for 300 – 500 mg L⁻¹ of initial HHA. The RE% values for SHA and PHA were smaller than those for HHA in each corresponding initial HA concentration. Although, in the case of a high initial HA concentration (300 – 500 mg L⁻¹), the RE% values for SHA and PHA were limited to about 50% and 40%, respectively. The amounts of Cu (nmol) in the precipitated HA (mg) at each initial HA concentration are shown in Fig. 3. The concentrations of the co-precipitated Cu (nmol mg⁻¹ of HAs) decreased exponentially with an increasing amount of the precipitated HHA and SHA (Fig. 3), suggesting that HHA and SHA have a similar affinity for the binding of Cu(II). It is noteworthy that the binding sites in HA appear to be more suitable for the removal of Cu by co-precipitation. The precipitability for the PHA (ca. 20%) was smaller than those of SHA (ca. 40%) and HHA (ca. 60%). In addition, the co-precipitation capacity of Cu (nmol mg⁻¹) was significantly lower than the corresponding values for SHA and HHA, suggesting that HA derived from peat soil is not suitable for use as a Cu-removal agent. This result indicates that HHA contains more suitable binding sites for Cu removal, and the removal efficiency appeared to be closely related to the precipitability of the HAs.

Structural features of HAs elucidated by TMAH-py-GC/MS analysis

Pyrolysate compounds were classified into the following groups: n-alkane, fatty acids, aromatic compounds, N- or P-containing compounds, sterols and terpenes, and others. The aromatic groups in the pyrolysates, which are mainly derived from lignin, were phenolic acids, di- and trimethoxybenzoic acids. Lipids in the pyrolysate, such as fatty acids, sterols and terpenes, are a well-known structural unit of humic substances and are of microbial origin or plant waxes. Pyrrole- and indole-containing compounds, which are derived from amino acids, peptides and proteins, are classified as N-containing pyrolysate compounds. Phosphoric acid methyl ester, derived from the cleavage of phospholipids, are classified as P-containing compounds. The ratios of these groups in each of the HA samples are shown in Fig. 4. PHA have high aliphatic structural features. The content of n-alkanes and fatty acids, which were not anticipated to bind Cu, were the major components of PHA. The structural features of PHA make it unfavorable for metal binding, consistent with the results for low RE% (Figs. 2 and 3). Aromatic and N- and P-containing compounds were the major components of pyrolysates in HHA (Fig. 4). The total relative areas of N- and P-containing
compounds for HHA, SHA and PHA were 349, 192 and 75.9, respectively. The percent areas of N-containing compounds in this group for HHA, SHA and PHA were 87.6, 73.2 and 25.3%, respectively. N-Containing compounds can serve as binding sites of Cu(II) with high affinity.\(^1\) There was no significant difference in the total areas of P-containing compounds for HAs. The total relative areas of aromatic groups for HHA, SHA and PHA were 1012, 778 and 722, respectively. The distribution of substitution number and chemical structure of the unique aromatic pyrolysate compounds in HAs are shown in Fig. 5, where the substitution number was determined by counting the number of substituents as follows: \(-\text{OH}, -\text{O-Me}, -\text{COOMe},\) and \(-\text{CO}-\). Di- and tri-substituted aromatic compounds were dominant in all of the HAs (Fig. 5). The amount of tetra-substituted compounds in the HHA was apparently larger than those in SHA and PHA. Also, 4-hydroxy-3,5-dimethoxy-benzoic acid hydrazide, a second major component in HHA (ca. 27% of the tetra-substituted compounds), was detected only in HHA. Mono-substituted aromatic compounds were dominant in the SHA sample. The composition of aromatic groups in pyrolysates in HAs reflected their origin. Sinapyl, coniferyl and coumaryl alcohols are units in lignin molecules and are found in hardwoods, softwoods and grasses, respectively.\(^{18}\) Therefore, the differences shown in Fig. 5 can be attributed to the soil sources. It can be concluded that tri- and tetra-substituted aromatic compounds are advantageous for complex formation and subsequent aggregation. In particular, hardwood lignin derivatives, such as the tetra-substituted compounds in Fig. 5, show a high degree of precipitability and RE% for HHA.

Structural features of HAs, as elucidated by UV-vis analysis

The structural features of the HAs were examined based on UV-visible absorption data (Table 1). Absorption in the UV region (200 - 400 nm) is attributed to \(n\)- and \(\pi\)-electron transitions of C=O bonds and C=C bonds.\(^{29}\) Based on this, the absorptivity at 280 nm (\(\varepsilon_{280}\)) is commonly used as an index of the degree of aromaticity of humic substances.\(^{19,24-26}\) The higher value of the original \(\varepsilon_{280}\) for HHA indicates that it contains a higher degree of aromatic structures than SHA and PHA, consistent with the features of pyrolysates (Fig. 5). After precipitation, the \(\varepsilon_{280}\) values in the supernatants for all HAs were decreased compared to the values for the original solution, suggesting that aromatic rich compounds had precipitated. The aggregation and precipitation of HA in seawater appear to be caused by complex formation between Ca\(^{2+}\) and Mg\(^{2+}\) and with carboxyl groups. The absorptivity at 600 (\(\varepsilon_{600}\)), which is a well-known index of the degree of humification, appears to be related to the development of conjugation in both aromatic and aliphatic structures, as well as the condensation of and inter- or intra-molecular donor-acceptor complexes between chromophores.\(^{18,24}\) The \(\varepsilon_{600}\) values for HAs derived from bark compost were larger than that for PHA. In general, aliphatic structures are relatively more abundant in peat humic acid because the decomposition and conjugation of organic residues would be more limited in the environment of a peat bog than in forest soil.\(^{18,30}\) The \(\varepsilon_{600}\) values in the supernatant of HHA and SHA were decreased compared to the values for unaggregated solutions. The decrease in \(\varepsilon_{600}\) values can be attributed to a decrease in the molecular size of and the content of oxygen-containing functional groups. Therefore, the precipitation of aggregated
fraction would also result in a decrease in $\varepsilon_{600}$. The decrease of $\varepsilon_{600}$ may be the result of the precipitation of the highly conjugated fraction, such as aromatic compounds, consistent with the $\varepsilon_{280}$ values. In contrast with HHA and SHA, the $\varepsilon_{600}$ values for supernatants of PHA were increased from the original values. Since PHA does not easily precipitate in seawater, the increase in $\varepsilon_{600}$ values can be attributed to an increase in particle size caused by aggregation, but not at a level to result in the formation of a precipitate. From the changes in absorbivities, it can be concluded that the aromatic compounds that contain large numbers of acidic functional groups largely contribute to the removal of Cu by co-precipitation.

**Influence of Cu(II) concentration on co-precipitation**

The extent of removal of Cu was largely dependent on precipitability of the added HA. To better understand this, the influence of the concentration of Cu(II) on precipitation was investigated using HHA (Fig. S3, Supporting Information). For initial concentrations of Cu(II) in the range of 0.1 to 30 μM, Cu(II) did not contribute to the precipitation of HHA. The threshold concentrations of Cu(II) for causing the aggregation and precipitation of HA were reported to be 50 and 140 μM, respectively. Then the level of Cu(II) used in this study failed to result in either aggregation or precipitation. Therefore, the precipitation in this study can be attributed to the relatively high concentrations of Ca$^{2+}$ and Mg$^{2+}$ in seawater. Figure 6 shows the relationship between the values for RE% and initial Cu(II) concentration. If Cu(II) competed for the binding sites in HA with Ca$^{2+}$ and Mg$^{2+}$, the complexation of Cu(II) with HA would be predicted to be superior with increasing initial Cu(II) concentration. However, as shown in Fig. 6, the RE% value slightly decreased with increasing initial Cu(II) concentration. Because carboxylic acid and phenolic hydroxyl groups in humic substances are the major part of metal binding sites, the metal binding capacities for HA are related to the content of acidic functional groups. It is also known that humic substances have two binding sites for Cu(II) with different stability constants. The steric configuration of the sites could be largely related to the strong binding, for example, adjacent phthalic and salicylic acids are able to form highly stable Cu(II) complexes by quadridentate chelation. In particular, Cu$^{2+}$ binding has a high binding affinity for phenolic sites, while Ca$^{2+}$ binding occurs predominantly at carboxylic sites. Although less abundant in comparison with the acidic functional groups, N-containing groups strongly favor Cu(II). It was inferred that the content of N-containing groups in HHA was significantly larger than those in SHA and PHA (Fig. 4), suggesting that HHA has high affinity to bind with Cu(II). The stability constant for strong Cu binding sites in humic substances (ca. log $K = 10 - 13$) is significantly larger than the corresponding values for Ca$^{2+}$ and Mg$^{2+}$ (log $K < 6$). Thus, the presence of specific binding sites could explain the high performance of Cu$^{2+}$ removability. When the concentration of Cu(II) is increased, the specific strong binding sites would become occupied, and excess Cu(II) would then bind weak sites in competition with Ca$^{2+}$ and Mg$^{2+}$. This is a plausible reason why the RE% performance decreased with increasing concentrations of Cu$^{2+}$.

**Conclusions**

Trace levels of Cu(II) in seawater could be removed by co-precipitation with HAs. The efficiency of removal was largely dependent on the amount and origin of the HAs. HHA, which originated from a hardwood bark compost, removed up to 90% of 3 μM Cu(II). Aromatic compounds highly substituted with acidic functional groups, which are derived from hardwoods, and N-containing compounds found to be highly effective for removing Cu(II) by co-precipitation, since they contain a relatively large number of strong binding sites. The findings presented herein indicate that humic acids, in particular those obtained from hardwood forests, are useful as an agent to remove traces of Cu(II) from seawater.

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**Supporting Information**

Changes of UV-vis spectra for HHA, SHA and PHA depend on the precipitation, the relationship between decreasing rate of the HHA concentration, and influence of the initially adjusted concentration of Cu(II) on precipitation rate for 500 mg L$^{-1}$ HHA. This material is available free of charge on the web at http://www.jsac.or.jp/analsci/.

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