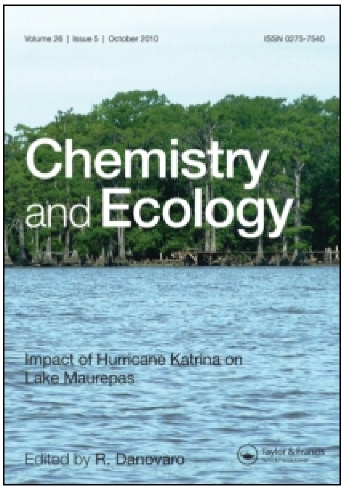

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ENVIRONMENTAL SIGNIFICANCE OF HUMIC ACID IN THE SEQUESTRATION OF METALS

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Change in the fluorescence of humic acid was studied using various metals, such as iron, cadmium, chromium, mercury, manganese, nickel and lead, to test the significance of humic acid in the sequestration of metals. On keeping the concentration of humic acid fixed (10 μg) and varying the concentration of metals (1–40 μg), the following order of magnitude of quenching was noticed: Cu > Fe > Cr > Mn > Ni > Hg > Pb > Cd. However, humic acid concentration (1–40 μg) and metal concentration (10 μg) showed a reverse trend. To test the humic acid–metal interaction in an aquatic system and on aquatic organisms such as fish, the LC_{50} values at 96 h for metals such as iron, copper, mercury, nickel and chromium were determined against common guppy fish (*Poecilia reticulata*). A 20 μg dose of humic acid showed a reduction in toxicity of metals towards fish as compared to a control exposed to metals only. The significance of results in relation to ionic interactions in ecotoxicology are discussed.

Keywords: Humic acid (HA); sequestration; fluorescence; complexation; interaction

INTRODUCTION

The wide use of heavy metals in industry and agriculture and their subsequent intrusion in various amounts may pose a great stress to aquatic organisms in particular, and to the whole ecosystem in general (Ure and Davidson, 1995). The seriousness and longevity of these

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heavy metals in the aquatic environment are compounded by the fact that they are non-degradable, with significant oxidizing capacity and substantial affinity for electronegative nucleophilic sites in the organism. Evidence for strong organic complexation has been demonstrated under controlled experimental conditions with homologous compounds (mainly fulvic and humic acids) extracted from natural environments such as lakes and rivers (Filella *et al.*, 1995). Humic substances are found in natural surface waters, interstitial waters of sediment, soils in both soluble and insoluble forms and play a crucial role in the speciation, transport and deposition of a variety of compounds ranging from metal ions to lipophilic compounds (MacCarthy, 1989). The functional groups present in humic compounds, responsible for their reactivity, complexation ability and solubility are carboxyls, alcohols, ketonic, quinoid compounds and phenols. Metal humate interaction has been shown to have an important role in toxicity, persistence and bioavailability of heavy metals in aquatic environments (Winner, 1985; Fish *et al.*, 1986; Stackhouse and Benson, 1988). The study of metal binding by naturally occurring complexants is of primary importance in understanding the behaviour and fate in aquatic systems. Further, it is noticed that in the presence of complexants, the toxicity of heavy metals and their uptake by aquatic animals can be reduced. Therefore, the role of humic acid in the sequestration of different metals was studied by fluorescence and spectrophotometric methods and an attempt was made to evaluate the influence of humic acid on heavy metal toxicity to fish (*Poecilia reticulata*).

MATERIALS AND METHODS

Humic acid (sodium salt) from Aldrich Chemical Company (USA) was used in all the studies. Other chemicals used in the experiments were from British Drug House (England) and E. Merck (Germany).

To test the effect of different metals on the fluorescence of humic acid, concentrations of metals (1–40 μg) such as $FeCl_3$, $CdCl_2$, $CrCl_3$, $HgCl_2$, $MnCl_2$, $NiCl_2$ and $(CH_3COO)_2$ Pb were prepared. Two sets of experiments were conducted. In the first set, concentration of humic acid (10 μg) was kept constant and that of metal varied, while in the second set, the concentration of metal was 10 μg but the concentration of humic acid (1–40 μg) was changed. The 5.0 ml assay system contained various concentration of metal and humic acid as needed. Distilled water was used as blank. Fluorescence in both cases was measured at 340 nm excitation and 489.6 nm emission on a RF-5000, Ratio Fluorophotometer (Shimadzu Corporation, Japan). Change in fluorescence in the assay system was compared with humic acid fluorescence alone.

To determine the effect of pH on humic acid metal interaction, various concentrations of metals (1–10 ppm) were incubated with humic acid (20 μg) at pH values from 4 to 10. The volume of the medium was 50 ml. The concentration of metal was 1 ppm in the case of cadium, lead, copper and chromium and 10 ppm in the case of iron, nickel, mercury and manganese. After incubation, the readings of humic acid were taken at 500 nm with a Spectronic 1001 Spectrophotometer (Bausch and Lomb, Milton Roy Company, USA).

To determine the effect of humic acid on the LC_{50} of metals towards fish, toxicity testing with the common guppy (*Poecilia reticulata*) was conducted. The fish were purchased from a local aquarium shop and held for three weeks in the laboratory in aged tap water (tap water left in the open for at least three days). The aged tap water used in the study had the following characteristics: pH 7.2–7.4; dissolved oxygen 6.7–7.2 $mg\,l^{-1}$; alkalinity 95–100 $mg\,l^{-1}$; hardness 116–122 $mg\,l^{-1}$ as $CaCO_3$ at 25 ± 1°C as measured by the method of APHA (1995). During laboratory holding, fish were provided with Shalimar Synthetic diet. They were not fed during the experiments because feeding could have increased the rate of metabolism and excretory substances which could influence the toxicity of the test solutions. After acclimatization, ten fish were transferred to 500 ml glass beakers with 300 ml solutions of each metal. The metals used for LC_{50} determination were iron, copper, mercury, nickel and chromium. Range-finding experiments were performed and LC_{50} values at 96 h were determined. Mortality was recorded daily to 96 h and the LC_{50} value and rate of survival were determined by graphic interpolation (Doudoroff *et al.*, 1951). After LC_{50} determination, the same number of fish were exposed to a lethal concentration of various metals in combination with 10 to 20 μg humic acid in a separate set of experiments under similar conditions. Controls were also run simultaneously.

RESULTS

The metal concentrations varying from 1–40 μg affected the fluorescence of fixed concentration of humic acid. Of the eight metals listed (Fe, Cd, Cr, Cu, Hg, Mn, Ni and Pb), copper was the most effective quencher and cadmium the least at all five concentrations. The order of magnitude of quenching was : Cu > Fe > Cr > Mn > Ni > Hg > Pb > Cd (Table I). Further, keeping the concentration of metal fixed (10 μg) and varying the concentration of humic acid from 1 to 40 μg, the fluorescence of humic acid increased and the order of magnitude in this case was maximum in the case of cadmium and minimum in case of copper (Table II).

Upon raising the pH from 4 to 10 a decrease in the optical density of humic acid was noticed at 500 nm in the system treated with the metal as compared to the control in each case excluding iron, where there is increase, indicating that binding is pH dependent (Table III).

The LC_{50} values for different metals and the effect of humic acid are shown in Table IV. Ten μg humic acid did not show any effect on the metal toxicity to fish whereas 20 μg of humic acid showed a reduction in the toxicity of the metal to fish as compared to fish exposed to the metal only.

DISCUSSION

Humic compounds are excellent complexing agents for trace metals (Schnitzer and Khan, 1972; Buffle *et al.*, 1977; Stevenson, 1985). Ionic strength, pH and electron affinity are the three major factors affecting speciation in solution. However, reaction kinetics, the relative concentrations and complexing affinities of cations and anions may be equally important (Stumm and Morgan, 1981). Complexation of metal ions usually reduce their effective concentration in water and in many cases also their biological availability and toxicity to aquatic animals (Reuter and Perdue, 1977). The bioavailability of metal ion decreases when water hardness increases, due to inorganic complexation (Wang, 1987). Deighton and Goodman (1995) reported that not only complexation but kinetic heterogeneity may also be an important factor in determining the bioavailability of metal–humic acid in aquatic system. Humic acid forms charge transfer complexes with Cu (II) (Boyd

TABLE I Quenching of fluorescence of humic acid (10 μg) after addition of various concentration of metals (listed in Table)

Conc. of Metals (μg)	*Fluorescence of humic acid after addition of metals*							
	Fe	*Cd*	*Cr*	*Cu*	*Hg*	*Mn*	*Ni*	*Pb*
1	46±1.90	185±7.60	110±5.70	56±2.25	172±7.6	143±6.5	156±6.2	198±9.2
5	40±1.75	182±7.40	80±3.80	21±1.07	161±6.5	132±6.0	150±5.9	181±8.8
10	32±1.60	179±8.80	71±3.30	18±0.92	155±7.2	116±5.4	126±6.1	150±7.0
20	31±1.50	173±8.60	37±1.70	12±0.60	140±7.0	106±5.2	112±5.4	173±8.2
40	23±1.10	165±8.10	22±1.00	9±0.43	130±6.2	94±3.8	92±4.3	141±6.9

Control value for 10 μg of humic acid = 215.0
Values are represented as arithmetic mean of three replicates ±S. E.

TABLE II Effect of fluorescence of humic acid (1–40 μg) after addition of fixed concentrations of metals (10 μg)

Conc. of Humic Acid (μg)	*Fluorescence of humic acid after addition of metals*							
	Fe	*Cd*	*Cr*	*Cu*	*Hg*	*Mn*	*Ni*	*Pb*
1	7.9±0.29	39.05±1.20	8.7±0.40	6.0±0.32	37.6±1.0	17.9±0.09	26.89±0.08	31.34±1.07
5	20.0±1.05	143.5±6.4	30.0±1.0	14.28±0.09	108.1±4.9	70.99±2.35	93.37±4.0	100.1±4.7
10	34.27±1.45	217.8±10.9	45.4±1.85	28.9±1.20	170.4±5.7	131.13±6.8	154.28±7.8	162.17±6.2
20	92.47±3.8	310.24±17.5	69.4±2.40	43.25±1.65	274.3±12.1	209.63±10.2	247.8±11.25	218.8±10.8
40	185.1±8.9	433.95±22	92.5±3.5	56.44±2.1	358.0±17.5	294.2±12.2	334.3±17.2	362.4±18.5

Control value for 10 μg of humic acid = 215.0
Values are represented as arithmetic mean of three replicates ±S. E.

TABLE III Effect of pH on the humic acid metal interaction

pH	*Control*	*O. D. of humic acid at 500 nm, 20 μg HA + 1 ppm metal*				*O. D. of humic acid at 500 nm, 20 μg HA + 10 ppm metal*			
		Cd	*Pb*	*Cr*	*Cu*	*Ni*	*Hg*	*Mn*	*Fe*
4	0.053	0.044	0.042	0.051	0.049	0.049	0.046	0.051	0.074
5	0.055	0.048	0.045	0.054	0.054	0.047	0.045	0.048	0.075
6	0.058	0.048	0.047	0.054	0.052	0.049	0.047	0.049	0.065
7	0.063	0.054	0.050	0.056	0.053	0.058	0.047	0.051	0.072
8	0.063	0.054	0.054	0.056	0.056	0.048	0.046	0.054	0.071
9	0.065	0.054	0.051	0.051	0.058	0.048	0.045	0.054	0.070
10	0.069	0.050	0.054	0.059	0.065	0.049	0.047	0.061	0.068

Values are represented as arithmetic mean of three replicates.

TABLE IV Effect of humic acid on the LC_{50} of Guppy Fish (*Poecilia recticulata*)

LC_{50} Values 96 h	*LC_{50} + Conc. of HA (μg)*	*No. of fish tested*	*No. of fish died in 96 h*
Fe (10 mg l^{-1})	–	10	5
	10	10	5
	20	10	–
Cr (1 mg l^{-1})	–	10	5
	10	10	4
	20	10	–
Hg (0.1 mg l^{-1})	–	10	–
	10	10	5
	20	10	2
Ni (10 mg l^{-1})	–	10	5
	10	10	5
	20	10	–
Cu (4 mg l^{-1})	–	10	5
	10	10	3
	20	10	1

Values are represented as arithmetic mean of three replicates.

et al., 1981). Senesi *et al.* (1986) have found that soil humic acid has two different classes of binding sites for Cu (II) and Fe (III). A change in the fluorescence of humic acid after addition of metal as shown in Table I and II suggested the possibility of complexation between humic acid and the metals. The quenching or increase in fluorescence after addition of various concentration of metals or humic acid could be due to non-availability/availability of binding sites in humic acid. The complexation capacity of humic substances for cadmium is lower than that of other heavy metals (Lund *et al.*, 1990). Such a type of complexation is believed to occur between the compound containing ketonic, carboxylic and quinoid groups and any transition metal having $3d^5$ to $5d^{10}$ configurations. Since humic acid is a negatively charged polyelectrolyte and polyfunctional ligand, it can complex with positively charged metal ions (Cu, Fe, Cr, Mn, Ni, Cd, Hg and Pb) but the selectivity of metal speciation may be governed by oxidation state, polarizability, size and geometry of the cation. Further, it is suggested that copper, iron, chromium, manganese and nickel preferably bound to humic acid by virtue of their small ionic radius/charge density ratio whereas cadmium, mercury and lead are preferentially complexed by humic acid by virtue of their large ionic radius/charge density ratio. The overall retention energy of these complexants depends on the total metal complexant concentration ratio. The chemical reactivity of humic acid can be predicted from the total acidity value (sum of carboxylic and phenolic group content). The dissociation of protons from the carboxyl groups, which starts at pH 3.0, creates a negative charge. This negative charge increases its value, and becomes larger at pH 9.0 due to dissociation of the phenolic-OH group. The presence of such pH dependent, or variable, changes enable the humic molecule to perform many chemical reactions, e.g. adsorption of cations and water, or complex formation and chelation of metals (Tan, 1994). Decrease in the optical density after the addition of metal with the increase in pH could be due to the stronger binding between humic acid and metals as a result of development of a more negative charge on humic acid due to release of a proton. Winner (1984) found that the addition of humic acid to water decreases the acute and chronic toxicity of Cu(II) to daphnids, but increased the acute and chronic toxicity of Cd(II) to these organisms. He further suggested that these observations may be related to the concentration of free Cu(II) and Cd(II) present in solution, with the uncomplexed Cu(II) concentration reduced in the presence of dissolved humic acid and Cd(II) concentrations are not. Sparks (1995) reported that chromium (III) act as a Lewis hard acid and it forms a stable complex with the carboxyl groups of the humic acid, further limiting its availability for animal uptake. Decrease in the toxicity of metals to fish (*Poecilia reticulata*) after addition of humic acid in our experiment can be explained on the similar basis. Our findings suggest that complex forms of heavy metals with humic acid are generally less available to aquatic animals and considerably less toxic than free metal ions. Other factors responsible for the sequestration of metals by the humic acid may be surface area, dissociation kinetics, cation exchange capacity, surface charge density of humic acid and stability constants of humic acid and metals.

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