Bull. Environ. Contam. Toxicol. (2002) 69:655–661

DOI: 10.1007/s00128-002-0111-9

Bulletin of Environmental Contamination and Toxicology

Combined Effects of Lead and Humic Acid on Growth and Lead Uptake of Duckweed, *Lemna minor*

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Received: 23 May 2001/Accepted: 10 July 2002

Humic substances, the end product of chemical and biological degradation of animal and plant residues, are the most widely distributed natural organic products on the surface of the earth. They occur in all aquatic and terrestrial environments. The complexation of humic substances and metal ions is extremely important in that this affects the behavior and mobility of metals in the environment. The ability of humic acids to form complexes with metal ions can be attributed to their high content of oxygen-containing functional groups with different affinities. The major complexing sites are carboxyl and phenolic groups. If two or more organic functional groups (*e.g.* carboxylate) coordinate the metal ion forming an internal ring structure, chelation, which is a form of complexation, occurs.

The capacity of aquatic plants such as duckweeds (*Lemna* sp.) to remove potentially toxic heavy metals from water is well documented. Duckweeds were shown to take up heavy metals and survive in a highly stressed aquatic environments (Rodgers et al. 1978). Concentration factors for duckweed subjected to various other metals (Ag, B, Cd, Cu, Ni and Zn) range as high as 50,000 depending on metal concentration, synergistic and antagonistic effects, and competition (Lisiecki and McNabb 1975; Hutchinson and Czyrska 1975). Allard (1991) suggested that humic substances, under certain conditions, could initiate abiotic reduction of heavy metals. Humic acid has been shown to influence (either enhance or reduce) heavy metal toxicity as it does with cadmium and chromium in several species of fish, clams and *Daphnia* (Winner 1984; Stackhouse and Benson 1988; Borgmann et al. 1991; Hansten et al. 1996; Hollis et al. 1996). Since little or no information is available regarding the influence of humic acid on heavy metal toxicity in plants, the present study was conducted in order to investigate the combined effects of lead and humic acid on total chlorophyll content, growth rate, multiplication rate and lead uptake of common duckweed, *Lemna minor*.

MATERIALS AND METHODS

All experiments were performed with *L. minor* collected from natural ponds and kept in a reservoir in the greenhouse of the Faculty of Science, Mahidol

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University. Tests were conducted in the laboratory under controlled temperature (25-27°C) and light (40 watt cool-white fluorescence). Before inoculation, the plant fronds were rinsed with distilled water. Modified Steenberg medium (diluted to one quarter strength and maintained at pH 6.0 ± 0.5) was used as growth medium (Kwan and Smith 1988).

The maximum incubation time for all experiments was 12 days. Fifteen prewashed plants with 30 fronds were transferred to each jar which contained 100 mL of modified Steenberg medium inoculated with various concentrations of lead nitrate (50, 100, 200 mg $PbNO_3$/L) combined with each concentration of humic acid (10, 20, 40, 80, 160 mg/L). There were 3 sets of experiments with a total of 18 combinations of lead and humic acid as follows :

Set 1	Set 2	Set 3
50/0	100/0	200/0
50/10	100/10	200/10
50/20	100/20	200/20
50/40	100/40	200/40
50/80	100/80	200/80
50/160	100/160	200/160

L. minor treated with lead only (50/0, 100/0, 200/0 mg/L) served as controls for each set of experiment. All experiments were carried out in triplicate.

On the 3rd, 6th,9th and 12th days, the control (lead, humic acid free) and treated plants (lead and humic acid) were analyzed for total chlorophyll content (Arnon 1949), growth rate (Porath et al. 1979), and multiplication rate (Landolt and Kandeler 1987). The procedures for decomposing plant material followed those of Anderson (1991) and Katz and Jennis (1983). Total lead content in the entire plant was determined using an atomic absorption spectrophotometer (APHA 1992). Differences in total chlorophyll content, growth rate and multiplication rate were determined among different treatments by One-Way Analysis of Variance (ANOVA) with Least Significant Difference (LSD) multiple comparisons.

RESULTS AND DISCUSSION

Lead and humic acid in combination had significant effects on total chlorophyll contents of *L. minor* (Table 1). At 50 mg/L of lead only, total chlorophyll content increased to 18.9 mg/g by day 12, but those plants treated with lead and humic acid had significantly higher total chlorophyll contents ($P < 0.05$) (29.7 –37.4 mg/g). However, at a lead concentration of 100 mg/L combined with various concentrations of humic acid, the total chlorophyll content nonsignificantly increased until day 6, then decreased ($P > 0.05$) (Table 1). Influence of humic acid on lead toxicity was also observed in the 200 mg/L treatment. At the highest humic acid concentrations (80, 160 mg/L), the total chlorophyll content significantly increased throughout (P <

0.01) (Table 1). The addition of high concentrations of humic acid brought about an increase in total chlorophyll content even at high lead concentrations.

Lead and humic acid in combination had significant effects ($P < 0.05$) on growth rate and multiplication rate of *L. minor* (Tables 2, 3). Low lead concentration even without humic acid did not significantly inhibit growth rate and multiplication rate ($P > 0.05$). Lead concentrations of 100 and 200 mg/L significantly inhibited growth of *L. minor* even in the presence of humic acid ($P < 0.01$) (Table 2). Lead at 50 mg/L combined with humic acid did not significantly inhibit multiplication of *L. minor* ($P > 0.05$), while a lead concentration of 100 mg/L, even with humic acid, inhibited multiplication ($P < 0.05$) (Table 3). In the 200 mg/L treatment, multiplication significantly increased in the presence of humic acid at every concentration ($P < 0.05$) (Table 3).

Lead and humic acid in combination had significant effects ($P < 0.01$) on lead uptake by *L. minor* (Table 4). In 50 and 100 mg/L treatments, lead uptake was found to be maximal on day 6, did not significantly differ on day 9 ($P > 0.05$) and, then decreased significantly on day 12 ($P < 0.01$), except for the 50/160 and 100/160 treatments in which there was an increase in lead uptake (Table 4). Hence, humic acid did not significantly decrease lead uptake at 50 and 100 mg/L of lead ($P > 0.05$). In the 200 mg/L treatment, the lowest lead contents were observed in the presence of 160 mg/L of humic acid. Therefore, a high concentration of humic acid could significantly decrease the lead uptake of *L. minor* ($P < 0.01$).

It was shown in the present study that the administration of a high concentration of humic acid (160 mg/L) could decrease the lead uptake by *L. minor* and bring about increases in total chlorophyll content, growth rate and multiplication rate compared with *L. minor* treated with lead nitrate alone. It has been suggested and clearly demonstrated experimentally that natural organic matter, particularly humic substances, under certain conditions could initiate the abiotic reduction of heavy metals such as mercury, cadmium and lead (Allard 1991). The reducing effect of natural organic compounds could be less important in soils and sediments, where microbial processes are likely to dominate. However, in aquatic systems, where concentrations of dissolved humic substances generally range from 1-10 mg/L, abiotic redox processes might be of greater importance (Allard 1991).

Mierle and Ingroum (1991) studied the role of humic substances in the mobilization of mercury from watersheds. They found that the mercury concentration in water was closely related to water color, probably reflecting the concentration of humic and fluvic matter in the streams. The association of mercury with this parameter is most likely due to the complexation of mercury by humic material. The observations reported in this study extend the phenomenon to small headwater streams and clearly indicate that the

Table 1. Influence of different combinations of humic acid and lead (50, 100, 200 mg/L) on total chlorophyll content of *L. minor*.

$PbNO_3$/ humic (mg/L)	Total chlorophyll content (mg/g)				
	Day 0	Day 3	Day 6	Day 9	Day 12
50/0	2.20 ± .23	2.45 ± .28	4.82 ± .23	9.08 ± .20	18.90 ± 1.91
50/10	2.20 ± .23	2.20 ± .25	2.95 ± .34	22.55 ± 1.27	29.76 ± 2.11
50/20	2.20 ± .23	2.27 ± .20	2.96 ± .26	23.67 ± 3.27	32.74 ± .74
50/40	2.20 ± .23	1.91 ± .48	2.61 ± .17	23.20 ± 1.54	32.08 ± 1.17
50/80	2.20 ± .23	4.51 ± .40	4.59 ± .60	18.50 ± 1.72	37.40 ± 1.00
50/160	2.20 ± .23	9.62 ± 1.02	8.10 ± 1.36	18.88 ± 8.69	33.04 ± 1.30
100/0	2.20 ± .23	6.17 ± 1.79	9.72 ± .98	5.98 ± .45	4.07 ± 1.08
100/10	2.20 ± .23	9.50 ± 1.82	9.73 ± .56	7.42 ± .80	12.73 ± .74
100/20	2.20 ± .23	15.87 ± .58	9.90 ± 1.51	8.77 ± 1.45	10.28 ± 1.29
100/40	2.20 ± .23	13.96 ± 6.63	10.46 ± .62	9.49 ± 1.64	11.08 ± 2.74
100/80	2.20 ± .23	13.55 ± .54	12.47 ± 1.63	9.28 ± 4.46	9.46 ± 1.14
100/160	2.20 ± .23	10.09 ± .40	17.81 ± 2.54	14.74 ± 2.78	10.33 ± .43
200/0	2.20 ± .23	3.28 ± .11	3.88 ± .22	1.85 ± .22	1.14 ± .35
200/10	2.20 ± .23	3.63 ± .08	3.98 ± .31	2.25 ± .40	2.51 ± .31
200/20	2.20 ± .23	5.90 ± .50	4.15 ± .42	2.36 ± .65	1.58 ± .13
200/40	2.20 ± .23	3.93 ± .93	3.80 ± .13	3.97 ± .84	1.61 ± .20
200/80	2.20 ± .23	3.25 ± .08	4.61 ± .70	4.70 ± 1.53	12.13 ± 5.38
200/160	2.20 ± .23	8.07 ± .16	13.03 ± 1.38	13.11 ± 1.7	13.59 ± .55

mean ± S.D. ; n = 45

Table 2. Influence of different combinations of humic acid and lead (50, 100, 200 mg/L) on growth rate of *L. minor*.

$PbNO_3$/ humic (mg/L)	Growth rate (g/g/d)				
	Day 0	Day 3	Day 6	Day 9	Day 12
50/0	0	0.03 ± .01	0.06 ± .01	0.12 ± .02	0.12 ± .02
50/10	0	0.11 ± .00	0.17 ± .01	0.17 ± .01	0.18 ± .00
50/20	0	0.15 ± .01	0.18 ± .01	0.19 ± .01	0.21 ± .01
50/40	0	0.15 ± .02	0.15 ± .02	0.2 ± 0.02	0.2 ± .00
50/80	0	0.14 ± .01	0.11 ± .04	0.14 ± .01	0.13 ± .00
50/160	0	0.13 ± .01	0.23 ± .01	0.22 ± .01	0.20 ± .01
100/0	0	0.05 ± .01	0.04 ± .03	0.10 ± .01	0.11 ± .01
100/10	0	0.11 ± .02	0.12 ± .01	0.10 ± .01	0.10 ± .01
100/20	0	0.13 ± .02	0.13 ± .01	0.13 ± .01	0.08 ± .01
100/40	0	0.13 ± .01	0.12 ± .01	0.09 ± .01	0.08 ± .01
100/80	0	0.15 ± .04	0.06 ± .02	0.04 ± .01	0.09 ± .01
100/160	0	0.16 ± .01	0.15 ± .01	0.13 ± .01	0.08 ± .04
200/0	0	0.04 ± .02	-0.01 ± .03	-0.01 ± .01	-0.02 ± .01
200/10	0	0.03 ± .01	0.04 ± .01	0.01 ± .01	0.02 ± .01
200/20	0	0.04 ± .03	0.06 ± .01	0.01 ± .01	0.01 ± .03
200/40	0	-0.07 ± .07	0.02 ± .02	0.01 ± .02	0.02 ± .01
200/80	0	-0.06 ± .04	0.02 ± .01	0.02 ± .02	0.02 ± .01
200/160	0	-0.06 ± .01	0.14 ± .02	0.13 ± .03	0.14 ± .01

mean ± S.D. ; n = 45

Table 3. Influence of different combinaitons of humic acid and lead (50, 100, 200 mg/L) on multiplication rate of *L. minor*.

$PbNO_3$/ humic (mg/L)	Multiplication rate (values are means ± S.D.)				
	Day 0	Day 3	Day 6	Day 9	Day 12
50/0	0	197.7 ± 11.41	206.78 ± 6.76	284.44 ± 2.17	326.11± 2.96
50/10	0	189.67 ± 2.36	269.33 ± 3.93	361.22 ± 9.17	405.22 ± .01
50/20	0	272.56 ± 6.73	275.0 ± 9.62	304.04 ± 5.43	443.0 ± 7.84
50/40	0	194.11± 1.49	244.67 ± 8.05	354.11 ± 3.97	417.22 ± 6.28
50/80	0	205.73 ± 3.42	230.22 ± 8.54	301.89 ± 4.49	337.22 ± 9.08
50/160	0	199.78 ± .89	280.33 ± 3.84	349.44 ± 5.97	396.18 ±9.98
100/0	0	208.44 ± 1.56	104.22 ± 11.57	80.48 ± .81	72.89 ± 2.54
100/10	0	199.56± 6.76	115.17 ± 3.71	84.81 ± 2.91	71.08 ± .73
100/20	0	196.67 ± 1.67	114.67 ± 2.40	90.96 ± 3.25	69.81 ± 1.14
100/40	0	195.78 ± 1.44	111.17 ± 4.87	81.26 ± 3.87	68.11 ± 2.48
100/80	0	193.89 ± 2.0	98.67 ± 2.0	63.93 ± 1.53	64.08 ± .12
100/160	0	191.11 ± 1.18	109.56 ± .72	90.56 ± 3.46	75.31 ± 3.19
200/0	0	41.08 ± 1.2	85.17 ± 1.32	64.52 ± 4.17	42.72 ± 1.90
200/10	0	37.22 ± 2.57	101.14 ± 10.41	75.58 ± .72	60.03 ± 13.87
200/20	0	41.97 ± .77	96.78 ± 2.06	78.41 ± .41	54.69 ± 3.78
200/40	0	36.61 ± 1.27	96.17 ± 1.69	78.26 ± 1.64	56.14 ± 5.75
200/80	0	40.44 ± 1.13	90.0 ± 3.62	74.54 ± 3.06	51.69 ± 1.36
200/160	0	58.22 ± 12.24	115.44 ± 8.88	91.81 ± 4.84	71.44 ± 1.97

mean ± S.D. ; n = 45

Table 4. Influence of humic acid on lead uptake by *L. minor* exposed to lead nitrate concentrations of 50, 100, 200 mg/L.

$PbNO_3$/ humic (mg/L)	Lead content (mg/g) (valus are means ± S.D.)				
	Day 0	Day 3	Day 6	Day 9	Day 12
50/0	0.00 ± .01	1.32 ± .23	2.34 ± .47	1.25 ± .36	0.63 ± .20
50/10	0.00 ± .01	1.17 ± .15	1.43 ± .27	1.25 ± .36	0.45 ± .05
50/20	0.00 ± .01	0.44 ± .22	1.57 ± .35	1.58 ± .70	0.22 ± .04
50/40	0.00 ± .01	0.68 ± .07	1.8 ± .18	1.28 ± .15	0.44 ± .21
50/80	0.00 ± .01	0.71 ± .14	1.19 ± .04	0.73 ± .10	0.31 ± .13
50/160	0.00 ± .01	0.81 ± .08	0.44 ± .05	0.58 ± .08	0.97 ± .23
100/0	0.00 ± .01	2.19 ± .18	7.19 ± 1.46	4.67 ± .98	1.82 ± .57
100/10	0.00 ± .01	1.8 ± .29	4.57 ± .57	3.50 ± .97	2.28 ± .07
100/20	0.00 ± .01	1.76 ± .26	3.14 ± .14	2.78 ± 2.90	2.07 ± .20
100/40	0.00 ± .01	1.47 ± .26	2.57 ± .12	4.08 ± 1.51	3.44 ± .59
100/80	0.00 ± .01	1.85 ± .47	4.75 ± 1.10	4.92 ± .18	3.02 ± .62
100/160	0.00 ± .01	1.38 ± .30	1.48 ± .43	1.64 ± .66	1.91 ± .13
200/0	0.00 ± .01	10.86 ± 2.10	22.10 ± 3.94	13.22± 7.53	13.49 ± 5.61
200/10	0.00 ± .01	14.07 ± .07	21.54 ± .96	21.11 ± 2.10	25.29 ± 6.65
200/20	0.00 ± .01	11.33 ± 1.73	26.94 ± 5.99	14.73 ± .29	43.52 ± .20
200/40	0.00 ± .01	10.76 ± 1.80	16.96 ± .88	19.6 ± 1.46	36.01 ± 5.75
200/80	0.00 ± .01	11.94 ± 1.33	15.75 ± 1.98	10.88 ± 3.62	23.18 ± 3.16
200/160	0.00 ± .01	0.02 ± .14	3.29 ± .33	3.27 ± .59	7.95 ± 4.43

mean ± S.D. ; n = 45

geochemistry of mercury in streams and lakes is dominated by its interactions with humic material (Mierle and Ingroum 1991).

The complex reaction of humic acid and lead ions may be caused by the dissociation of protons from the carboxyl group of humic acid which starts at pH 6. This dissociation increases with increasing pH (Allard 1991). In this study, the addition of humic acid to the lead nitrate solution resulted in an increase of pH. The solution became a weak base when its pH was not more than, or equal to, 9. Presumably, there was a dissociation of a proton from the carboxyl group in the humic acid which then formed complexes with lead ions resulting in precipitation. This formation of complexes with lead decreased the free lead ions available for the plants *L. minor*. This mechanism may explain the decreased lead uptake caused by humic acid and thereby the decreased toxicity of lead in *L. minor*.

The results of the present study suggest that *L. minor* may be used as a bioindicator due to its ability to accumulate metals in measurable amounts, its availability throughout the year and relative ease of collection, and its availability in terms of quantity and distribution, making unbiased sampling possible at a modest cost. Moreover, it can be used in tertiary treatment plants to efficiently remove heavy metals such as lead. The degree of uptake is affected by the amount of organic matter due to the formation of complexes of humic acid with heavy metals that cause the metals which are bound with organic matter, to lose their ionic characteristics. Humic acid may detoxify lead in plants temporarily. The metal ions might become toxic again depending on environmental factors such as pH and temperature. This type of monitoring is best referred to as ecological monitoring and consists of the surveillance of plants and animals of the whole system in order to detect changes in environmental quality. However, humic acid content should be considered as an important factor related to heavy metal pollution assessment.

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