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Effect of Medium Phosphate Levels on the Sensitivity of *Selenastrum capricornutum* to Chemicals

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Algae are the most important primary producers in aquatic ecosystems and potent indicators for environmental water quality. Several standard protocols have been developed and employed as part of a battery of tests for ecotoxicological evaluation of chemicals and waste waters (Nyholm and Källqvist 1989). As for the experimental factors, medium composition is known to have a significant effect on the toxicity of some chemicals (Adams and Dobbs 1984; Millington et al. 1988). Although there were some reports of decreased algal toxicity in responses to increases in macronutrient concentrations, few studies were made on the effect of limiting nutrient on algal sensitivity (Janssen and Heijerick 2003). Algae in the field are grown under low concentrations of nutrients, and phosphate is possibly the main limiting nutrient in freshwater systems. Hall et al. (1989) have indicated that green algae are more sensitive to copper under P limited condition than under the non-limited condition. It has been suggested that algal sensitivity differences in response to the amounts of nutrient available might have ecological implications (Guasch et al. 2004). Therefore, it will be important to understand the changes in relative toxicity made under the influence of varying nutrient level.

We examined the effect of reduced phosphorus nutrient levels on the toxicity of some chemicals by the U.S. EPA standard growth inhibition test with a modification of its medium phosphate concentration using the freshwater green alga *Selenastrum capricornutum*. Two metals (Zn and Cu) and two herbicides (simazine and butachlor) were tested to compare the results obtained from the present experiments with those of previous related works (Hall et al. 1989; Kuwabara 1985; Tubea et al. 1981). Simazine and butachlor are widely used herbicides, and known to be toxic to *S. capricornutum* (Fairchild et al. 1997; Hatakeyama et al. 1994). Compounds also tested were 4-methylcatechol, 4-hydroxybenzoic acid, protocatechualdehyde (3,4-dihydroxybenzaldehyde), protocatechuic (3,4-dihydroxybenzoic) acid, and gallic (3,4,5-trihydroxybenzoic) acid. These are naturally occurring plant polyphenols and possible intermediates formed during the aerobic microbial degradation of aromatic contaminants (Smith 1990), but not fully evaluated for their toxicity to freshwater green algae. Previous toxicity studies of phenols with aquatic organisms have been limited, for the most part, to synthetic monophenols substituted with chlorine atoms.

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MATERIALS AND METHODS

Selenastrum capricornutum NIES-35 was used as described previously (Kamaya et al. 2003).

$CuCl_2 \cdot 2H_2O$, $ZnSO_4 \cdot 7H_2O$, simazine (6-chloro-*N*,*N*-diethyl-1,3,5-triazine-2,4-diamine), butachlor (*N*-butoxymethyl-2-chloro-2',6'-diethylacetanilide), 4-hydroxybenzoic acid, protocatechualdehyde (3,4-dihydroxybenzaldehyde), protocatechuic (3,4-dihydroxybenzoic) acid, and gallic (3,4,5-trihydroxybenzoic) acid monohydrate were obtained from Wako Pure Chemical Industries, Osaka, Japan. 4-Methylcatechol was obtained from Tokyo Kasei Kogyo, Tokyo, Japan. Spectroscopy-grade dimethyl sulfoxide (DMSO) was obtained from Nacalai Tesque, Kyoto, Japan. All compounds had a stated purity of 97% or better and were used as purchased.

The algal growth inhibition test was performed according to the U.S. EPA (1989) method. In P-limited experiments, K_2HPO_4 in the standard U.S. EPA medium was reduced from 6 μ*M* (standard; 100%) to 3 (50%), 1.5 (25%), 1.2 (20%), or 0.6 μ*M* (10%), while other nutrients remained unchanged. Cultures were prepared in 100-mL Erlenmeyer flasks containing 20 mL of the filter-sterilized (0.22 μm) test medium (initial pH: 7.45 ± 0.05). Each agent was tested at six concentrations in triplicate. 4-Methylcatechol and 3,4-dihydroxybenzaldehyde were dissolved in DMSO and added to each culture (final concentration of DMSO: less than 0.3%). Benzoic acid was directly dissolved in test medium, and the medium pH was adjusted to 7.45 before filter-sterilization; this highest test solution was diluted with test medium to prepare a series of test solutions. Both controls and test cultures were inoculated with exponentially growing algae from a culture (standard medium) at an initial concentration of 1 x 10^4 cells/mL. These cultures were incubated at a temperature of 24 ± 1°C, and shaken at 100 rpm under a continuous illumination of 4000 (± 10%) lux. After 72 hr cell density was determined by microscopic direct counting with a hemocytometer. The median inhibition concentration (IC_{50}) values for test chemicals were calculated by the linear interpolation method (US EPA 1989) based on their nominal concentration.

RESULTS AND DISCUSSION

The effect of test medium composition on metal toxicity in algae has been studied extensively (Janssen and Heijerick 2003), and the interaction of metal toxicities with phosphorus limitation has been reported for copper and zinc (Hall et al. 1989; Kuwabara 1985). We examined first the P effects on the toxic responses of *S. capricornutum* to these metals under the present test condition. As shown in Table 1 copper was more toxic in the media containing lower concentrations of P than in the original medium (6 μ*M* P). This P effect was consistent with the results for copper in *Chlorella vulgaris* and *Chlamydomonas geitleri* (Hall et al. 1989). In contrast, the toxic responses to zinc appeared to be similar at all tested P concentrations (Table 1). Kuwabara (1985) reported that P concentrations (2, 4, and 6 μ*M*) did not give significant changes in the toxic responses to Zn in terms of

both the lag phase and the growth rate for *S. capricornutum*. Vasseur et al. (1988) showed no significant differences in the toxicity data (EC_{50} values) for zinc obtained from the four different algal test media in *S. capricornutum*. Both Zn and Cu are essential micronutrients, but algal responses to toxic concentrations of these trace metals were different. As discussed by Kuwabara (1985) different toxic mechanisms may exist within an algal species for these metals.

Table 1. Effect of medium phosphate levels on the metal toxicity expressed as 72-h IC_{50} [μM (μg / L)].

Metal	6.0 μM P	3.0 μM P	1.2 μM P	0.6 μM P
Cu	4.41 (280)	2.31 (147)	2.36 (150)	1.01 (64.2)
Zn	0.69 (44.8)	0.73 (47.8)	0.95 (62.0)	0.73 (47.7)

On the other hand, there are some reports of the effect of medium composition on the toxicity of pesticides (Adams and Dobbs 1984; Shabana et al. 2001). However, little is known about the direct effects of nutrient limitation on the toxic responses to organic chemicals. As shown in Table 2 the toxicities (in terms of IC_{50} values) of simazine and butachlor were not significantly affected by medium phosphate levels. Similar observations were reported by Tubea et al. (1981) studying the combined effects of nutrient and herbicide levels on the growth of the green alga *C. pyrenoidosa*. They showed that the effect of prometryn, a triazine herbicide structurally related to simazine, was similar under both saturated (7.43 μM) and limited (0.01 μM) P levels.

Table 2. Effect of medium phosphate levels on the herbicide toxicity expressed as 72-h IC_{50} [μM (μg / L)].

Herbicide	6.0 μM P	3.0 μM P	1.5 μM P
simazine	0.231 (48.6)	0.365 (73.6)	0.284 (57.3)
butachlor	0.00231 (0.72)	0.00209 (0.65)	0.00301 (0.94)

There are several reports on the toxicity of simple phenols, especially chlorinated ones, to microalgae (Kuivasniemi et al. 1985). However, the toxicity of phenolic acids and related compounds found in soils and aquatic environments have not been fully evaluated for the toxicity toward algae (Dedonder and Van Sumere 1971; Kramer and Trümper 1986). We examined the effects of phosphorus nutrient level on the toxicity of selected phenolic compounds which are naturally occurring plant derived polyphenols and possible intermediates produced by the aerobic microbial degradation of aromatic contaminants (Smith, 1990). All benzoic acids were tested under neutralized condition (intial pH 7.45) to avoid the influence of medium pH on the toxicity. 4-Hydroxybenzoic acid was not toxic at concentration levels tested here (up to 1 mM), whereas other polyphenols were all toxic at micromolar levels (Figure 1, Table 3). The toxicity of these polyphenols determined at full-strength of P (6 μM) in descending order of toxicity was 4-methylcatechol > protocatechualdehyde > gallic acid > protocatechuic acid.

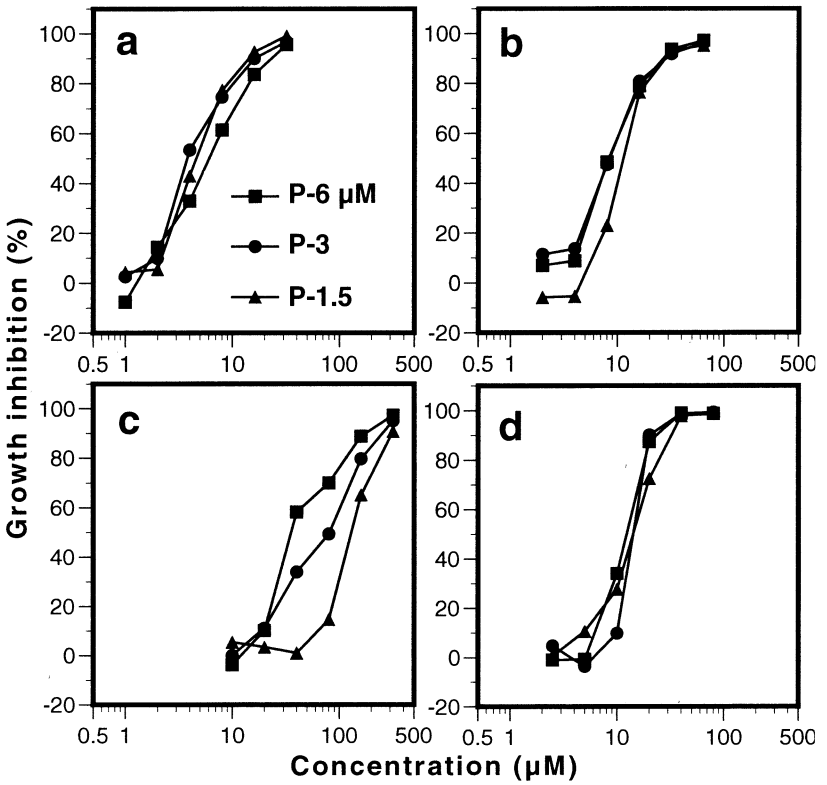

Figure 1. Effect of medium phosphate levels on the growth of *S. capricornutum* at different concentrations of 4-methylcatechol (a), protocatechualdehyde (b), protocatechuic acid (c), and gallic acid (d).

Table 3. Effect of medium phosphate levels on the toxicity of phenols expressed as 72-h IC_{50} [μM (μg / L)].

Phenol	6.0 μM P	3.0 μM P	1.5 μM P
4-hydroxybenzoic acid	> 1000	n.d.[a]	n.d.
4-methylcatechol	6.1 (760)	3.8 (470)	4.8 (600)
protocatechualdehyde	8.4 (1160)	8.6 (1190)	11.8 (1630)
protocatechuic acid	36.2 (5580)	81.8 (12600)	136.0 (21000)
gallic acid	12.9 (2190)	15.0 (2550)	15.6 (2650)

[a] Not determined.

Among the catecholic compounds, the most toxic was 4-methylcatechol with a methyl substituent, and the least was protocatechuic acid with a carboxyl group, suggesting that oxidative transformation of the methyl carbon to formyl and carboxyl groups by microorganisms might be a detoxification process for algae. Furthermore, the toxicities of 4-hydroxybenzoic acid, protocatechuic acid and gallic acid indicated the importance of the number of hydroxyl groups on the benzene ring for their toxicities. Similar toxic responses of *S. capricornutum* to 4-

methylcatecohol, protocatechualdehyde and gallic acid were observed in cultures with three different P levels, although a slight decrease in the toxicity was observed for protocatechualdehyde at 1.5 μ*M* P (Table 3). Thus the levels of P did not significantly alter the toxicity of these phenols; the ratios between the highest and lowest IC_{50} values were less than 2. However, different dose-responses were observed for protocatechuic acid depending on the medium P concentration as shown in Figure 1c. Interestingly, the acid appeared to be less toxic under reduced P concentrations and the IC_{50} values (Table 3) seemed to be negatively correlated with the medium P concentration ($n = 3$, $r^2 = 0.94$). This was an opposite response to that found for copper treatment, although the mechanism was not clear.

The present study indicates that phosphorus nutrient limitation in the test medium influenced the sensitivity of algae to a toxicant in a different manner depending on the type of compound (toxicity mechanism) and the extent of limitation (stress intensity). Therefore, the results obtained from laboratory toxicity tests performed under non-limiting conditions cannot always be extrapolated to field conditions. It has been shown that green algae under phosphorus limitation have higher amounts of lipids and carbohydrates, and increased activity of alkaline phosphatase than non-limited cells (Healey and Hendzel 1979; Kilham et al. 1997). However, implications of these stress responses for changes in the uptake and effects of toxicants are unknown at present. Studies will be needed to understand the physiological and biochemical responses of *S. capricornutum* to potential environmental stressors in relation to the toxicity assessment of environmental contaminants.

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