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Toxicity of Phenanthrene in Freshwater Sediments to the Rooted Submersed Macrophyte, *Vallisneria spiralis*

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Abstract A study was conducted to determine the response of the rooted submersed macrophyte, *Vallisneria spiralis* to phenanthrene in freshwater sediments with initial phenanthrene concentrations from 0 to 80 mg kg^{-1} dry sediment. The sensitivity of various morphological endpoints was evaluated after 90 days of exposure. The most sensitive toxicity test endpoints were those that reflected root growth. Toxicological sensitivity of the endpoints changed with the effect level selected. The toxicity threshold from a plot of the EC_{10} values was 1–2 orders of magnitude lower than those calculated for the threshold from plots of the EC_{25} or EC_{50} values. In addition, stimulatory responses (hormesis) on root growth were observed at subtoxic concentrations of phenanthrene, and a hormetic model should thus be incorporated for ecological risk assessment.

Keywords Endpoint sensitivity · Hormesis · Phenanthrene · Sediment · Submersed macrophyte · Vallisneria spiralis

In the freshwater sediment ecological risk assessment procedure for organic contaminants, such as polycyclic aromatic hydrocarbons (PAHs), most standard test methods designed to determine sediment toxicity recommend animal species (Volpi Ghirardini et al. 2005; Weston et al. 2010) and a relatively small number of species of aquatic plants (Lewis et al. 2001; Turgut and Fomin 2002). The most commonly used aquatic plants in toxicity tests are algae and *Lemnaceae* (Lewis 1995). However, these species are not representative of all plants, and they may be less sensitive than rooted macrophytes to certain organic contaminants (Belgers et al. 2007). Even though the use of rooted submersed macrophytes has been limited in sediment toxicity studies compared to the use of algae and free-floating plants (Biernacki et al. 1997; Cedergreen et al. 2004), the macrophytes do possess some attributes that are lacking in free-floating plants. They are exposed to contaminants in both the sediment and overlying water, and allow for several morphological endpoints to be tested, such as root length and number, stolon length and leaf length (Biernacki et al. 1997; Arts et al. 2008). *Vallisneria spiralis* is a perennial rooted submersed macrophyte with a high adaptive capability and a wide geographical range (Wang and Yu 2007). It has been found to be useful for the biomonitoring of toxic metals (Vajpayee et al. 2001) and organochlorine contaminants (Biernacki et al. 1997). We evaluated its use for phytoremediation of contaminated sediments, and found that it was capable of significantly decreasing the concentration of phenanthrene in sediments (Yan et al. 2011). However, limited information is available on the toxicity of phenanthrene to *V. spiralis*. A growth stimulatory response at toxicant concentrations lower than those that cause growth inhibition (i.e., hormesis) has been reported in some toxicological studies with plants (McCann et al. 2000; Belgers et al. 2007). For example, shoot length of *Myriophyllum spicatum* showed significant stimulation at PAHs concentrations below 13 mg L^{-1} (McCann et al. 2000).

In this study, the sensitivities of various endpoints for *V. spiralis* were evaluated following its exposure to phenanthrene in sediment. Further, the phenomenon of hormesis

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was investigated in terms of its possible role in risk assessment.

Materials and Methods

The rooted submersed macrophyte *Vallisneria spiralis* and bulk sample of surface sediment were collected about 1 week prior to the start of the experiments from East Taihu Lake (31°10′N, 120°24′E) in China. The macrophyte was placed in vessels in a greenhouse for acclimation. Sediment samples were sieved through a 0.5 cm sieve, and then stored at 4°C until use. The background concentration of phenanthrene in the sediment samples was 0.0086 mg kg^{-1}. The phenanthrene-spiked sediments with five different initial phenanthrene concentrations (8, 24, 40, 56, and 80 mg kg^{-1} dry sediment) were artificially prepared using methanol as a carrier, as described previously (Yan et al. 2011). A control group without spiked phenanthrene was also set up. Each treatment had three replicates.

Sediment (110 g wet weight per vessel) was placed at the bottom of plastic vessels (diameter 16 cm, and height 25 cm) in a greenhouse. Three seedlings of *V. spiralis* of similar size (3–4 leaves, <1 cm root, 4.90 ± 0.35 g fresh weight) were transplanted into each test vessel. Vessels were filled with 5.0 L of tap water at a depth of 22.5 cm. Nutrients were added from a stock solution to obtain nutrient concentrations in the overlying water as described previously (Cleuvers and Ratte 2002). These amounts of nutrients and trace elements were added to each vessel once a week for plant growth. In addition, filtered water was also added once a week to compensate for evaporation loss. All test vessels were illuminated by 4 daylight lamps (36 W lamps) producing 2655 Lux illumination at vessel surface in a light regime of 12 h light and 12 h darkness. To ensure that all test vessels received the same amount of light, they were randomly moved around at regular time intervals. The experiment lasted for 90 days. At the end of the experiments, plants in test vessels were harvested, rinsed with distilled water, and separated into leaf, stolon and root components. Twelve endpoints of plant growth and morphology covering leaf, stolon and root development were measured (Table 1).

Data for the various endpoints were evaluated using non-linear regression techniques with Origin software (OriginPro 7.5, OriginLab Corporation, USA). The testing models were Logistic and Hormetic according to a previous report (Hanson and Solomon 2004). To evaluate the significance of hormesis, the effective concentrations (EC_x) of a number of endpoints from *V. spiralis* were calculated using models excluding and including hormesis. Effect measures were calculated at three levels, EC_{10}, EC_{25} and EC_{50} for each endpoint when possible. The threshold of toxicity for phenanthrene was calculated according to documented methods (Hanson and Solomon 2002). Briefly, effect measures at each of the three calculated levels (EC_{10}, EC_{25} and EC_{50}) were plotted as a cumulative frequency distribution using a probability scale on the *y*-axis as a function of the log concentration. Plotting positions were expressed as percentages and calculated from the Weibull formula:

$$100 \times i/(n + 1) \tag{1}$$

where *i* is the rank of the datum and n is the total number of data points in the data set. Data were plotted and linear regressions on the transformed data were calculated using the Origin software. A low centile (1%) from a distribution of EC_{10}s was deemed as the threshold of toxicity.

Phenanthrene in sediment samples was extracted with dichloromethane and analyzed by a high-performance liquid chromatograph (HPLC) (Agilent 1200, USA) according to a procedure described elsewhere (Yan et al. 2011). Chromatography was performed at 30°C with phenanthrene detected at 254 nm. The reproducibility and recovery of the extraction method for the spiked samples were satisfactory, with 89.7 ± 3.6% recovery for sediment.

All data were subject to strict quality control procedures. Statistical analyses were done using the OriginPro software with one-way ANOVA and Fisher LSD for comparison of treatment means with $p < 0.05$. A $p < 0.05$ was considered significant.

Results and Discussion

Relatively small differences were observed in the measured water quality conditions with water temperature at 22 ± 2°C, pH at 7.83 ± 0.92, and dissolved oxygen at 7.42 ± 0.56 mg L^{-1} during the experiment.

The EC_{10}, EC_{25} and EC_{50} values, the equation used to model the relationship, and the corrected r^2 values are shown in Table 1. As shown in Table 1, sensitivities of toxicological endpoints changed with the effect level chosen for comparison. At the EC_{10}, root total length was the most sensitive endpoint, while root dry weight was the most sensitive endpoint at the EC_{50}. Since no single morphological endpoint was consistently the most sensitive in this study, sediment toxicity tests using rooted macrophytes should include various endpoints. In fact, morphology-related variations in endpoint sensitivities to toxicants are common in aquatic macrophytes (Biernacki et al. 1997). In this study, a comparison of the twelve morphological endpoints showed that the submersed macrophyte was generally more tolerant in terms of growth/biomass and shoot endpoints, but less tolerant in terms of root endpoints

Table 1 The laboratory-derived EC_x values (mg kg^{-1}) calculations for endpoints, including 95% confidence intervals (parentheses) and regression variable values

Endpoint	EC_{10}	EC_{25}	EC_{50}	Model	Variables	Corrected r^2
Dry weight of root in sediment (g)	0.049 (0, 0.127)	2.32 (0, 3.62)	4.69 (0, 15.77)	Logistic	a = 0.40; b = 0.94; x = 4.69	0.71
Wet weight of root in sediment (g)	0.24 (0, 0.54)	2.8 (0.86,4.74)	7.47 (3.39, 11.55)	Logistic	a = 1.99; b = 1.27; x = 7.47	0.99
Average length of root in sediment (cm)	0.03 (0, 0.08)	1.85 (0.51,3.19)	6.09 (0, 21.23)	Logistic	a = 8.66; b = 0.51; x = 6.09	0.99
Total length of root in sediment (cm)	0.017 (0, 0.046)	0.36 (0, 0.73)	8.75 (0, 22.12)	Logistic	a = 2784; b = 0.38; x = 8.75	0.99
Average length of root outside sediment (cm)	11.42 (0, 22.42)	16.23 (0,34.21)	26.67 (-23.03,76.38)	Hormetic	t = 0.18; h = $-2.98e^{44}$; b = 0.95; x = 26.67	0.99
	No fit	No fit	No fit	Logistic	–	–
Dry weight of total root (g)	34.50 (21.96, 47.04)	47.54 (33.43, 61.65)	61.89 (47.22, 76.56)	Hormetic	t = 0.39; h = 0.03; b = 2.44; x = 61.89	0.92
	78.4 (x−x)	80.3 (x−x)	82.49 (x−x)	Logistic	a = 0.51; b = 42.2; x = 82.49	0.27
Number of total root	9.08 (7.49, 10.67)	14.00 (11.23,16.77)	21.21 (16.90, 25.53)	Hormetic	t = 182.28; h = 0.09; b = 1.31; x = 21.21	0.99
	No fit	No fit	No fit	Logistic	–	–
Dry weight of stolon (g)	37.09 (28.80,45.38)	41.19 (33.02,49.36)	46.52 (37.52, 55.45)	Hormetic	t = 0.27; h = 0.22; b = 3.17; x = 46.52	0.92
	79.58 (x−x)	79.68 (x−x)	79.94 (x−x)	Logistic	a = 1.03; b = 82.9; x = 79.94	0.17
Total length of stolon (cm)	36.05 (16.15,55.95)	37.53 (22.1,52.97)	39.88 (28.68, 51.10)	Hormetic	t = 10.87; h = 0.60; b = 2.26; x = 39.88	0.92
	No fit	No fit	No fit	Logistic	–	–
Dry weight of the total biomass (g)	57.15 (42.87,71.43)	70.2 (62.2,78.37)	>80	Logistic	a = 2.49; b = 4.30; x > 80	0.81
Relative growth rate (%)	79.12 (x−x)	>80	>80	Logistic	a = 0.02; b = 40.61; x > 80	0.79
	>80	>80	>80	Hormetic	t = 0.02; h = 0.004; b = 32.6; x > 80	0.89
Leaf length (cm)	79.10 (x−x)	79.64 (x−x)	>80	Logistic	a = 40.90; b = 29.36; x > 80	0.48

> means EC_{50} higher than the highest test concentration. a, b, t, h are constants of regression models. *x−x* indicates that it is not possible to calculate a confidence interval. – indicates that no data are available. *No fit* means that no convergence is obtained in the model

(Table 1). Growth or biomass and shoot endpoints were several orders of magnitude less sensitive than the root endpoints. This implied that even at high phenanthrene concentrations where root endpoints were affected, the plant was still viable and would be able to grow again once concentrations of the toxicant decreased.

With a reduction of phenanthrene concentration, the sensitivity of morphological endpoints changed. This variation was also observed in other studies (McCann et al. 2000; Hanson and Solomon 2004). Organic contaminants like phenanthrene are first absorbed by the roots of *V. spiralis*, and then translocated to other parts (Schnoor et al. 1995). Thus, growth limitations induced by phenanthrene might have their first effects on roots. Root endpoints, reflecting root growth in sediments, were most sensitive.

Five endpoints (number and dry weight of total root, average length of root outside sediment, and total length and dry weight of stolon of *V. spiralis*) were found to exhibit a concentration–response pattern that clearly showed the phenomenon of hormesis. In these cases, curve-fitting with a hormesis model to calculate EC_x values was more appropriate than fitting achieved using a logistic model that did not allow for hormesis. The concentration–response relationship generated from data in Table 1 allowed for plotting cumulative frequency distributions of effect measures for *V. spiralis* according to the Weibull equation. The distributions were found to fit a log-normal curve quite well, with r^2 values generally above 0.85 (Fig. 1; Table 2). Root in sediment endpoints were located in the lower end of the curves, whereas growth/biomass

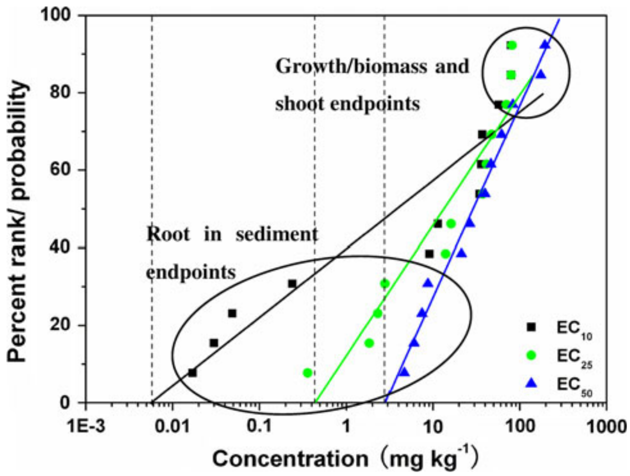

Fig. 1 Measurement distributions about effect of phenanthrene in sediments using the Weibull equation for *V. spiralis* using 90 EC_{10}, EC_{25} and EC_{50} values. The *dashed line* the threshold of toxicity for that distribution

and shoot endpoints were situated in the upper part. In addition, the endpoint distribution curves like EC_{10} showed that concentrations for growth/biomass/shoot endpoints and root in sediment endpoints differed by a factor of 100–1,000.

The distributions were used to estimate a threshold of toxicity, which was defined as a low centile (1%) of the distribution. The threshold was interpreted as the concentration below which no adverse impact should be observed for any endpoint, based on the specific effect level selected. A toxicity threshold (0.0065 mg kg^{-1}) was found to be a more conservative measure of toxicity for risk estimation than the lowest calculated EC_{10} (Table 1). The ratios between the lowest EC_{10} and the corresponding toxicity threshold were equal to 2.63. The toxicity threshold from EC_{10} distribution plotted was 1–2 orders of magnitude lower than those calculated for EC_{25} and EC_{50}. According to these distributions, the toxicological risk that a contaminant might cause in the field to an aquatic macrophyte could be accurately and effectively estimated with laboratory data.

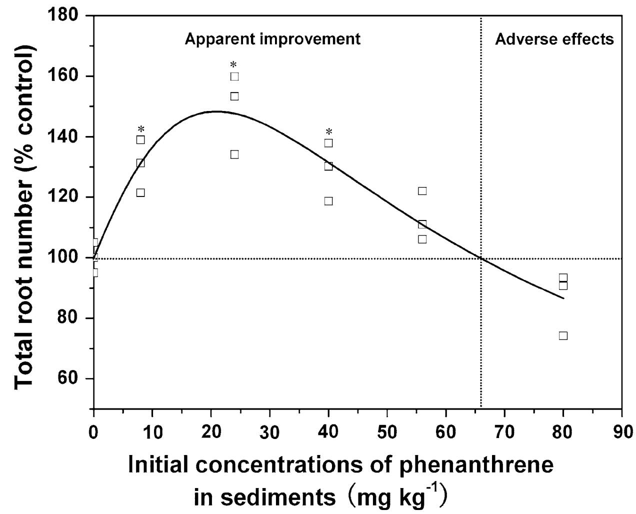

Fig. 2 A representative example of hormetic model used to describe the effect of phenanthrene-contaminated sediments on the total root number of plant. The *asterisks* statistically significant data

Stimulatory responses of phenanthrene on root growth were observed during the experiment (Table 1; Fig. 2). A representative example of responses consistent with the hormetic phenomenon is provided in Fig. 2. A stimulatory response in total root number occurred at the phenanthrene concentrations of 8, 24 and 40 mg kg^{-1}. However, the total root number in the highest treatment of 80 mg kg^{-1} was less than that in the control. This inverted U-shaped curve as shown in Fig. 2 is the most common form of the hormetic concentration–response curve depicting low-concentration stimulatory and high-concentration inhibitory responses (Calabrese and Blain 2009). Interestingly, the number of roots growing above the sediment in the treatments with spiked phenanthrene was greater than the number in the control, which indicated that *V. spiralis* may change its root growth morphology in order to decrease the toxicity on plant growth when exposed to phenanthrene in sediment. In fact, many plants may develop adaptive response mechanisms to toxicants in sediment (Calabrese and Blain 2009). When plants are exposed to toxic

Table 2 Regression coefficients and intercepts for phenanthrene toxicity distributions for *V. spiralis* as calculated using the Weibull equation

Distributions	$y = ax + b$[a]			Regression intercepts (mg kg^{-1})[b]	n[c]
	a	b	r^2	Toxicity threshold	
EC_{10}	39.68	17.67	0.85	0.0065	12
EC_{25}	12.08	33.67	0.90	0.47	12
EC_{50}	−22.30	49.46	0.98	2.96	12

[a] These values are transformed into units of log and probit for the purposes of regression, and backtransforms were used to calculate the intercepts. The distribution units were in mg kg^{-1}

[b] The toxicity threshold is the 1 centile of the toxicity distribution

[c] Number of data points used in the ranking

compounds, they can respond to unfavorable growing conditions by producing more seeds or stolons, giving the next generation a greater opportunity to germinate under more favorable conditions. Therefore, hormetic responses such as low levels of site contamination have been reported to be associated with enhanced growth and enhanced uptake of contaminants from the sediment by plants (McCann et al. 2000; Nwaichi et al. 2010).

Hormesis has usually been ignored in ecological risk assessment (Calabrese and Baldwin 2003). Since hormesis is an unpredictable phenomenon, test models with and without hormesis should be considered in all cases. In this study, EC_x values were calculations based on both the logistic and hormetic models. When endpoints showed significant hormesis, the EC_x values predicted with the hormesis model provided improved estimates of an EC_x value than the logistic model. In addition, several other studies have shown that the hormesis model is superior to other concentration–response models in its ability to predict stimulatory responses and calculate EC_x values, regardless of whether the effects are stimulatory or inhibitory (Calabrese and Baldwin 2003; Calabrese and Blain 2009). Our study has confirmed that the hormetic model should be considered for ecological risk assessment.

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