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NOTE

Quantification of Toxic Effects of the Herbicide Metolachlor on Marine Microalgae *Ditylum brightwellii* (Bacillariophyceae), *Prorocentrum minimum* (Dinophyceae), and *Tetraselmis suecica* (Chlorophyceae)

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Toxic effects of the herbicide metolachlor (MC) were evaluated for three marine microalgae, *Tetraselmis suecica* (chlorophyte), *Ditylum brightwellii* (diatom), and *Prorocentrum minimum* (dinoflagellate). MC showed a significant reduction in cell counts and chlorophyll *a* levels. Median effective concentration (EC_{50}) was calculated based on chlorophyll *a* levels after a 72-h MC exposure. EC_{50} values for *T. suecica, D. brightwellii*, and *P. minimum* were 21.3, 0.423, and 0.07 mg/L, respectively. These values showed that the dinoflagellate was most sensitive when exposed to the herbicide, at a concentration comparable to freshwater algae, suggesting its potential as an appropriate model organism for ecotoxicity assessments in marine environments.

Keywords: marine microalgae, ecotoxicity assessment, EC_{50}, herbicide, metolachlor

In aquatic environments, microalgae constitute the base of the aquatic food chain. Additionally, they are known for their diversity and sensitivity to environmental changes, thus making them potential candidates for environmental risk assessment studies evaluating toxic contaminants (Stauber and Davies, 2000). Toxicity tests mainly utilize endpoints that measure growth rate, cell density, or chlorophyll content of test species (OECD, 2006). However, the algal sensitivity not only varies among toxicants but also among taxonomic groups (Boyle, 1984). Even the sensitivities to the same toxicant are very different between freshwater algae and marine algae (Sverdrup *et al.*, 2001). Thus, toxicity data employing freshwater algae cannot be used as a surrogate for testing in marine environments and vise versa.

Metolachlor (MC) affects the functioning of membrane structural components through the impairment of lipid and protein synthesis (Fuerst, 1987), as well as respiration and photosynthesis in plants (Sloan and Camper, 1986). It has been used as a common herbicide for annual weed control in corn, potato fields, and golf courses (US EPA, 2000). This chemical may cause toxicity to aquatic organisms through distributed-source pollution (Lin *et al.*, 1999). The concentration of MC in the aquatic environment was found to range from 5–80 μg/L, and the variation was dependent on season (Cook and Moore, 2008). In addition, the marine environment may be contaminated with various herbicides as a result of spray-drift, run-offs and accidental spills or from freshwater sources (Brock *et al.*, 2006). The toxicity of MC is well documented for freshwater algae (Fairchild *et al.*, 1997; Ma and Liang, 2001; Roubeix *et al.*, 2011) with a selective study in a marine microalgae, *Nannochloropsis oculata* (Kyriakopoulou *et al.*, 2009). With almost no studies carried out using marine diatoms and dinoflagellates, studies utilizing these marine microalgae as test species are desirable.

In the present study we evaluated the toxic effects of the herbicide MC on three marine microalgae. These included the chlorophyte *Tetraselmis suecica*, the diatom *Ditylum brightwellii*, and the dinoflagellate *Prorocentrum minimum*, because they have been used previously as test organisms for toxicity studies in marine environments (Canterford and Canterford, 1980; Gerringa *et al.*, 1995; Pérez-Rama *et al.*, 2001; Millán de Kuhn *et al.*, 2006). For our toxicity tests, we obtained microalgal cultures of *T. suecica* (P-009), *D. brightwellii* (B-326), and *P. minimum* (D-127) from the Korea Marine Microalgae Culture Center (Busan, Korea). They were cultured in f/2 medium, and maintained at 20°C and a 12:12 h light:dark cycle with a photon flux density of ca. 65 μmol photons/m^2/s. Exponential growth phase cultures (50 ml) were treated in duplicate with different nominal concentrations (~100 mg/L) of MC (Cat. No. 36163, Sigma, USA), and sub-samples were withdrawn at 0, 12, 24, 48, and 72 h. The concentrations used for toxicity assays were 0.01, 0.05, 0.1, 0.5, 1, 10, 20, and 50 mg/L for *D. brightwellii* and *P. minimum*, with additional 75 and 100 mg/L for *T. suecica* based on previous studies (St-Laurent *et al.*, 1992; Juneau *et al.*, 2001; Kyriakopoulou *et al.*, 2009). Cell counts and chlorophyll *a* levels were chosen as endpoints to evaluate the toxicity of MC. The 72-h EC_{50} (median effective

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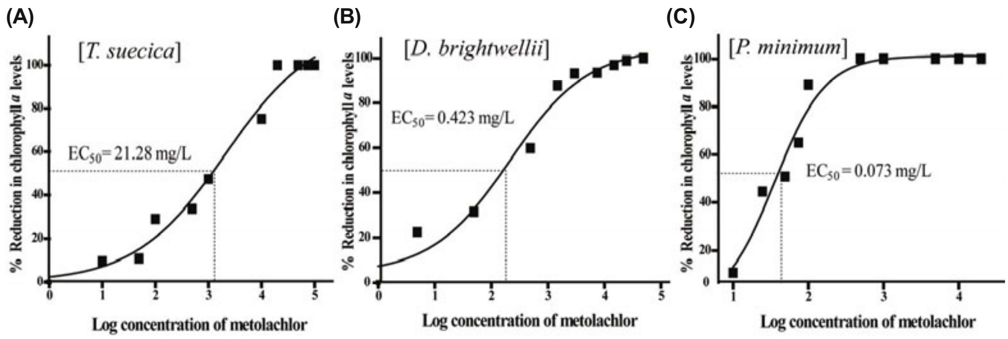

Fig. 1. **Dose response curves of a chlorophyte, *T. suecica* (A), a diatom, *D. brightwellii* (B) and a dinoflagellate, *P. minimum* (C) based on chlorophyll *a* levels.**

concentration) and the percentile inhibition were calculated as recommended in the Organization for the Economic Cooperation and Development (OECD) test guidelines (OECD, 2006). The values of 72-h EC_{50}, including EC_5, EC_{10}, and EC_{20}, were estimated using a sigmoidal dose-response curve. They were plotted using Origin 8.5 (MicroCal Inc., USA) based on the sigmoidal 4-parameter equation (Teisseyre and Mozrzymas, 2006): Log $EC_{50} = a + (b - a)/[1 + 10 \times (x - c)^d]$, where a is the response value at zero or minimum asymptote, b is the response value for infinite concentration or maximum asymptote, c is the mid- range point, d is the steepness of the curve or the Hill slope, and x is the dilution coefficient. Dunnett's analysis was carried out to estimate no observable effective concentration (NOEC) and lowest observable effective concentration (LOEC) by using Graphpad InStat (GraphPad Inc., USA). All the data presented are mean values of duplicates; differences between non-treated and treated cultures were tested by one-way analysis of variance (ANOVA) with post hoc Student's Newmann Keul's test in Graphpad InStat (GraphPad Inc., USA).

The toxic effects of MC on the three microalgae were evaluated by measuring cell counts and chlorophyll *a* levels. Tested microalgae showed marked sensitivities to the herbicide, with patterns depicting dose-dependent reductions in cell counts and chlorophyll *a* levels. Pearson's correlation coefficient (r) was compared between chlorophyll *a* and cell counts. *T. suecica* (0.92, P=0.1247) and *P. minimum* (0.99, P=0.117) showed a positive correlation between the two endpoints. *D. brightwellii*, however, showed a negative correlation (-0.827, P=0.0007), because *D. brightwellii* cells aggregated, thus making it impossible to count individual cells. Hence, we calculated the 72-h EC_{50} values for the three microalgal test species only using chlorophyll *a* levels (Fig. 1). The 72-h EC_{50} values for *T. suecica*, *D. brightwellii*, and *P. minimum* were 21.3±0.2 mg/L, 0.423±0.090 mg/L, and 0.073±0.015 mg/L, respectively (Table 1). As for the threshold effect parameter, we calculated NOEC, LOEC, EC_5, EC_{10}, and EC_{20} values, which represented the initial concentration of the test chemical that triggers an effect on the test microalgae (Table 1).

Based on literature surveys, we summarized the MC toxicity based on EC_{50}, half maximal inhibitory concentration (IC_{50}), and NOEC values (Table 2). With regard to MC toxicity, most available data were for freshwater algae, excluding the marine eustigmatophyte *Nannochloropsis oculata* (Kyriakopoulou *et al*., 2009). Our study provided additional toxicity data for MC to marine microalgae represented by three common taxonomic groups, such as chlorophyte (green algae), diatom, and dinoflagellate. Overall, they responded differentially to MC (see Fig. 1), suggesting that the herbicide causes a heterogeneous response to different microalgal groups (Shi *et al*., 2011).

Upon comparisons of our EC_{50} data with available literature (Table 2), the chlorophyte *T. suecica* was most tolerant to MC. In addition, this species was ~50 to ~200 times more tolerant to MC exposure than *D. brightwellii* and *P. minimum*, respectively. This was in accordance with earlier studies using metals (Millán de Kuhn *et al*., 2006; Debelius *et al*., 2009). *D. brightwellii* has commonly been used as a test species in toxicity evaluation and bioaccumulation studies (Gerringa *et al*., 1995). Although this species was observed to be sensitive to metal exposures (Canterford and Canterford, 1980; Gerringa *et al*., 1995), it displayed moderate toxicity to MC compared to other test species. Interestingly, EC_{50} values recorded for *D. brightwellii* (0.423 mg/L) in the present study were comparable to the OECD recommended freshwater diatom, *Navicula pelliculosa* (0.38 mg/L) (Dobbins *et al*., 2010). MC was most toxic to the dinoflagellate, *P. minimum* (72-h EC_{50}=0.073 mg/L). The sub-lethal toxicity response of the dinoflagellate was comparable to the freshwater chlorophytes, *Ankitrodesmus falcatus*, *Chlorella pyrenoidosa*, and *Pseudokirchneriella subcapitata* (formerly known as *Selenastrum capricornutum*); however, it was more sensitive than the chlorophytes, *Scenedesmus* spp. and *Pediastrum biwae* (Table 2).

Additionally, the toxicity of MC to marine microalgae was compared to other herbicides and insecticides. For example, the EC_{50} values of the herbicide triazine to marine diatoms

Table 1. **The effective concentration after 72 h exposure of metolachlor to three microalgae**

Species	NOEC (mg/L)	LOEC (mg/L)	EC_5 (mg/L)	EC_{10} (mg/L)	EC_{20} (mg/L)	EC_{50} (mg/L)	95% confidence limits
T. suecica	0.088±0.001	0.154±0.030	0.690±0.008	3.427±0.660	10.39±0.001	21.3±0.020	19.85–22.19
D. brightwellii	0.0025	0.001	0	0.008±0.0002	0.085±0.001	0.423±0.090	0.254–0.625
P. minimum	0	0	0.001±0.0004	0.012±0.001	0.035±0.005	0.073±0.015	0.068–0.084

Table 2. Sensitivity of the herbicide metolachlor to common freshwater and marine microalgae

Taxonomic position	Species	EC_{50}/IC_{50}/NOEC (mg/L)	References
Freshwater species			
Chlorophyceae	*Ankitrodesmus falcatus*	0.096	Juneau *et al.* (2001)
Chlorophyceae	*Chlorella pyrenoidosa*	0.068–0.152	Liu and Xiong (2009)
Chlorophyceae	*Psedokirchneriella subcapitata*	0.037–0.084	Fairchild *et al.* (1997), Juneau *et al.* (2001)
Chlorophyceae	*Pediastrum biwae*	0.330	Juneau *et al.* (2001)
Chlorophyceae	*Scenedesmus acutus*	0.1	St-Laurent *et al.* (1992)
	Scenedesmus vacuolatus	0.156–0.598[a]	Kotrikla *et al.* (1999)
Bacillariophyceae	*Navicula pelliclosa*	0.38	Dobbins *et al.* (2010)
Nostocaceae	*Anabaena cylindrical*	4.0[b]	St-Laurent *et al.* (1992)
Chroococcales	*Microcystis aeruginosa*	0.073	Juneau *et al.* (2001)
Nannoplankton	-	0.117	Juneau *et al.* (2001)
River periphytic diatoms	-	0.005–0.03	Roubeix *et al.* (2011)
Marine Species			
Eustigmatophyceae	*Nannochloropsis oculata*	10.5	Kyriakopoulou *et al.* (2009)

[a] IC_{50} value
[b] NOEC

Cyclotella gamma and *Chaetoceros* sp. were 0.494 mg/L and 0.043 mg/L, respectively (Tang *et al*., 1997; Debelius *et al*., 2008). Endosulfan (insecticide) was very toxic to the dinoflagellate, *P. minimum*, for which the EC_{50} value was 0.025 mg/L (unpublished data). EC_{50} of the insecticide, cypermethrin to the marine dinoflagellate, *Scrippsiella trochoidea* was reported as 0.205 mg/L (Wang *et al*., 2012). Moreover, the marine algae, *D. brightwellii* and *P. minimum* were more sensitive to MC than freshwater and marine invertebrates; for example, 24-h LC_{50} values of MC to the freshwater crustacean, *Daphnia magna* and marine crustacean, *Artemia franciscana* were 9.5 mg/L and 168 mg/L, respectively (Kyriakopoulou *et al*., 2009). These results suggest that marine diatoms and dinoflagellates may be very sensitive to herbicide exposures (Védrine *et al*., 2003), and can be used as model test organisms in aquatic toxicity assessment studies.

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