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Fungicides and herbicide removal in *Scenedesmus* cell suspensions

Rachel Dosnon-Olette, Patricia Trotel-Aziz, Michel Couderchet, Philippe Eullaffroy *

Laboratoire Plantes Pesticides et Développement Durable (PPDD), URVVC-SE EA 2069, Université de Reims Champagne-Ardenne, BP 1039, 51687 Reims Cedex 2, France

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ABSTRACT

Remediation capacities of two freshwater microalgae, *Scenedesmus obliquus* and *Scenedesmus quadricauda*, were assessed for the removal of two fungicides (dimethomorph and pyrimethanil) and one herbicide (isoproturon) from their medium. To ensure these studies were performed with healthy algae, pesticide effects where first apprehended on chlorophyll *a* fluorescence emission and growth rate. After a 4 d-exposure to 600 μg L^{-1} of dimethomorph or pyrimethanil, or to 10 μg L^{-1} of isoproturon, algal growth rate and some of their photosynthetic processes were weakly affected (<30% variation). The pesticide removal percentage of *Scenedesmus* cells reached a maximum of 10%, 24% and 58% for pyrimethanil, dimethomorph and isoproturon, respectively. In parallel, the maximum removal rate was 36 and 40 μg × 10^{-9} cells for dimethomorph, 17 and 26 μg × 10^{-9} cells for pyrimethanil, 2 and 2 μg × 10^{-9} cells for isoproturon, in the presence of *Sc. obliquus* and *Sc. quadricauda*, respectively. Results showed that *Sc. quadricauda* was more effective in the removal of dimethomorph and pyrimethanil compared to *Sc. obliquus*.

1. Introduction

In intensively cultivated areas, agriculture and viticulture are significant sources of surface water contamination by pesticides via spray drift and/or run-off (Groenendijk et al., 1994; Kloeppel et al., 1997; Shultz, 2001). Once in the aquatic environment, these chemicals have the potential to induce adverse effects on ecosystem health (Moore et al., 2007). To minimize the impact of this pollution, it is important to develop innovative technologies to clean up the contaminated water. For years, the use of plants to remediate polluted soils and water (so-called phytoremediation) has gained attention and popularity as a cost-effective, environmentally friendly and efficient *in situ* technology for a variety of pollutants (Cunningham et al., 1995; He et al., 2005; Pilon-Smits, 2005; Rai, 2009), and among them, many pesticides (Schröder and Collins, 2002; Dhir et al., 2009).

Phytoremediation with microalgae have been utilized for areas polluted with nutrients or heavy metals (Zhou et al., 1998; Awasthi and Rai, 2005; Perales-Vela et al., 2006), however increasing environmental pollution by pesticides may lead to consider microalgae as good candidates to remove these contaminants from water at low cost (Tang et al., 1998; Friesen-Pankratz et al., 2003; Weiner et al., 2004; González-Barreiro et al., 2006).

In the present study, owing to their common use in agriculture and viticulture, dimethomorph and pyrimethanil (used for control of mildew and grey mould on grapes), and isoproturon (used for controlling broad leaf weeds and grasses in cereal crops), were selected as model pesticides. Considering a phytoremediation approach to remove the selected pesticides, we chose two species of microalgae known to bio-accumulate nutrients and heavy metals: *Scenedesmus obliquus* and *Scenedesmus quadricauda* (Awasthi and Rai, 2005; Pellón et al., 2008; Ruiz-Marin and Mendoza-Espinosa, 2008). Few data exist, however, regarding their capacity to remove pesticides (Guanzon et al., 1996; Cai et al., 2007). Algal sensitivity to selected pesticides was also investigated using growth rate and also chlorophyll *a* fluorescence measurement as a non-invasive, highly sensitive, fast and easy to apply tool to probe the degree of algal injury by pesticides (Dewez et al., 2005; Juneau et al., 2007).

2. Materials and methods

2.1. Algal material

Sc. obliquus and *Sc. quadricauda* (SAG 276-3a and 276-4b; Göttingen, Germany) were maintained in batch cultures containing 400 mL of mineral growth medium (pH 6.5; Couderchet and Böger, 1993) in 1 L Erlenmeyer flasks.

Flasks were maintained in a growth chamber at 23 ± 2 °C under continuous light (65 μmol photosynthetic active radiation m^{-2} s^{-1}) provided by cool white fluorescent lamps (Sylvania Gro Lux F36 W, Germany). Microalgal suspensions were placed on an orbital shaker (110 rpm). All experiments were performed with exponentially growing cell cultures and were inoculated in 250 mL Erlenmeyer flasks containing 100 mL culture medium (Couderchet and Böger,

* Corresponding author. Tel.: +33 3 26 91 85 25.
E-mail addresses: philippe.eullaffroy@univ-reims.fr, philippe.eullaffroy@ec.gc.ca (P. Eullaffroy).

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1993) with an initial concentration of 1.4×10^6 cells mL^{-1} (1.2 μg mL^{-1} of chlorophyll).

2.2. Experimental design and pesticide contamination procedure

We used formulated pesticides: dimethomorph ((E,Z)4-[3-(4-chlorophenyl)-3-(3,4-dimethoxyphenyl)acryloyl]morpholine) as Forum (BASF Agro, Belgique, 150 g L^{-1} of active ingredient, a.i.), pyrimethanil (N-(4,6-dimethylpyrimidin-2-yl)aniline) as Scala (BASF Agro, France, 400 g L^{-1} of a.i.) and isoproturon (3-(4-Isopropylphenyl)-1,1-dimethylurea) as Matin (John et Stephen B., France, 500 g L^{-1} of a.i.). The actual amount of the a.i. in the source products Forum, Scala and Matin was analysed. We found that this amount was 10% higher (165 g L^{-1}), 16% lower (333 g L^{-1}) and 10% higher (550 g L^{-1}) than the nominal concentrations, respectively. The amount of formulated product required to achieve our nominal test concentrations was calculated on the basis of those analyses. Pesticide concentrations given in this study refer to the actual, and not to the nominal concentrations of the a.i. All stock solutions are aqueous solutions and were prepared immediately before initiating experiments.

All experiments were repeated three times and each sample was run in triplicate. Three different controls were carried out in parallel: # 1, algae in a pesticide free medium; # 2, dead-frozen algae in a medium containing pesticides, and # 3, medium containing pesticides but free of algae. In control # 2, the quantity of dead-frozen algae was adjusted to simulate the increase of biomass observed in the experiment using viable algae. One batch was analysed immediately after spiking each exposure container (time point 0 h) and four other batches every 24 h (time point 24–96 h). No pesticide was detected in control # 1.

2.3. Toxicity assessments

Pesticide effects on algal suspension were determined by measuring growth rate and algal chlorophyll fluorescence emission. Growth was determined using spectrophotometric determination of optical density. Optical density was measured at 680 nm (OD680). Kasai et al. (1993) reported that cell numbers and OD680 are highly correlated. A strong correlation between optical densities and cell densities of the algae tested was confirmed in this experiment, with r^2 values >0.97 for all algal species tested. Optical density was then used as a surrogate for growth (cell density) for each freshwater alga. A pulse amplitude modulated spectrofluorometer (PAM-Walz, Effeltrich, Germany) was used for chlorophyll *a* fluorescence measurement. After a 15-min adaptation to the dark at 23 ± 2 °C, the minimal and maximal fluorescence yield (F_o and F_m) were determined by a modulated low light and a saturating flash, respectively. The algal solution was then continuously illuminated and the minimal and maximal light-adapted fluorescence level (F'_o, F'_m) determined. Each measurement was made with a given volume of algal suspension filtered on a glass-fiber filter (Millipore AP20 013) in order to obtain 7×10^6 cells on the filter. The maximum photosynthetic capacity (also called maximum quantum yield of photosystem II primary photochemistry) was estimated by the ratio $F_v/F_m = (F_m - F_o)/F_m$ for dark adapted leaves (Genty et al., 1990). The photochemical ($q_P = (F'_m - F_s)/(F'_m - F'_o)$) and non-photochemical ($q_N = 1 - ((F'_m - F'_o)/(F_m - F_o))$) quenching were determined as in Schreiber et al. (1986).

Growth rate and chlorophyll fluorescence were recorded every 24 h, during the 4-d exposure of the algal suspension up to 800 μg L^{-1} of dimethomorph and pyrimethanil; and to 0, 5, 10 and 20 μg $L^{-1}$of isoproturon.

2.4. Pesticide determination

Pesticide concentration in medium was determined in supernatants after centrifugation of 7 mL aliquots at 3000*g* for 15 min at 4 °C. Acetonitrile (ACN) was added, 10% (v/v), to the supernatant for dimethomorph and pyrimethanil determinations. The samples were stored at −20 °C before HPLC analysis. Isoproturon determination required the addition of 3 mL dichloromethane to 5 mL of supernatant. After vigorous agitation, dichloromethane extracts containing isoproturon were collected. Then another 3 mL of dichloromethane was added to the extract and collected after agitation. The eluent was gently evaporated with nitrogen and residues were dissolved again in 0.5 mL of ACN, and then stored at −20 °C before HPLC analysis.

A 80 mL aliquot of each culture was required to determine the pesticide quantity inside the microalgal biomass. Following a centrifugation at 3000*g* for 15 min at 4 °C, the pellet was resuspended in 40 mL of fresh medium, shaken vigorously and centrifuged (at the end of the process no pesticide was found in the supernatant). This step was repeated twice. The final pellet was resuspended in 5 mL of pure methanol, which was kept in darkness for 48 h. Methanol suspension was centrifuged again to obtain a clean supernatant, *i.e.* free of cell debris. The supernatant was evaporated under nitrogen stream and 0.5 mL of ACN was added before pesticide analysis (González-Barreiro et al., 2006).

The removal rate of the algae was expressed as the quantity of pesticide removed from the medium (μg × 10^{-9} cells). The quantity found in algae was reported as a percentage of the quantity that had disappeared from the medium.

A 20 μL-aliquot of sample was injected into a Varian ProStar 410 HPLC system and analysed using a C18 reversed phase column (100 mm × 3 mm, 5 μm particle size, Kromasil 100, Varian, Les Ulis, France) and eluted isocratically with ACN (60%) and water (40%) acidified with H_3PO_4 (0.1%). Peaks were detected with a diode array detector (Varian, Prostar). Identification of pesticide was confirmed by UV spectrum and concentration was determined by comparison with a standard curve obtained with dimethomorph, pyrimethanil and isoproturon certified standards (Sigma, Saint-Quentin-Fallavier, France). The UV detection was made at 246, 261 and 242 nm for dimethomorph, pyrimethanil and isoproturon, respectively.

2.5. Statistical analysis

In this study, all statistical analyses were performed with SigmaStat 3.5 (Systat Software Inc, San Jose, CA, USA). Significant differences between controls and contaminated samples were determined by One Way ANOVA tests and *P* values <0.05 were considered significant.

3. Results

3.1. Toxicity

3.1.1. Maximum quantum yield of photosynthesis (F_v/F_m)

The effect of dimethomorph, pyrimethanil and isoproturon on plant photosynthesis is reflected in the F_v/F_m ratio inhibition percentage in Fig. 1a, b and c, respectively. After 96 h of experiment, *Sc. obliquus* and *Sc. quadricauda* were slightly or not affected by any of the studied pesticides with an F_v/F_m inhibition always lower than 6%. *Sc. obliquus* and *Sc. quadricauda* were not significantly different in sensitivity.

3.1.2. Photochemical quenching (q_P)

After 96 h, q_P was significantly affected by dimethomorph, pyrimethanil and isoproturon with a maximum inhibition of 9%, 13%