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Impact of the herbicide metolachlor on river periphytic diatoms: experimental comparison of descriptors at different biological organization levels

Vincent Roubeix*, Nicolas Mazzella, Brigitte Méchin, Michel Coste and François Delmas

Cemagref, UR REBX, F-33612 Cestas Cedex, France

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Abstract – A microcosm experiment was carried out in order to test the effect of the herbicide metolachlor on river periphytic diatoms and to find potential diatom bioindicators of contamination. Effects were investigated at different biological organization levels (biofilm, diatom community, population and individual levels). The colonization of glass substrates by natural biofilm in artificial streams did not vary quantitatively between control and contaminated conditions (5 and 30 $\mu g.L^{-1}$). However, non-parametric multivariate analysis of variance revealed a significant difference between contaminated and control diatom communities with regard to species composition. The difference was due to the greater development of probably tolerant species in the presence of the herbicide (*e.g.*, *Planothidium frequentissimum, Planothidium lanceolatum*, *Amphora montana, Surirella brebissonii* and *Nitzschia gracilis*). An increase in the occurrence of abnormal forms was observed in relation to metolachlor concentration. In particular, up to 8% of the frustules of the species *Surirella angusta* exhibited prominent deformities. Monospecific acute toxicity tests were then performed on two species to estimate toxicity parameters based on growth inhibition. These tests also confirmed the teratogenic effect of the herbicide on *S. angusta*. This study shows that low concentrations of metolachlor in natural streams may significantly alter diatom community structure and that abnormal diatom forms should be taken into account in water contamination assessment.

Key words: Community / diatoms / herbicide / metolachlor / toxicity

Introduction

Metolachlor (metolachlor) is a herbicide that belongs to the chloroacetanilide class that has replaced triazines to become one of the most frequently occurring groups of herbicides in the French surface waters survey networks (Dubois *et al.*, 2010). It is commonly used before and after crop emergence to prevent weed growth (Kegley *et al.*, 2010). The concentration of metolachlor in rivers is highly variable, peaking during the field application period and after heavy rain. Time-weighted concentrations of over 1 $\mu g.L^{-1}$ were recorded in some rivers and peak concentrations reached several tens of $\mu g.L^{-1}$ (Battaglin *et al.*, 2000; Clark and Goolsby, 2000; Dubois *et al.*, 2010; Roubeix *et al.*, in press). Since agricultural pesticides contaminate rivers via runoff and spray drift, it has become essential to evaluate their impact on non-target aquatic organisms.

Herbicides contaminating rivers may be particularly toxic to aquatic plants and algae. In shallow rivers, periphytic diatoms are often the dominant group of primary producers; they are at the basis of the trophic food web and any alteration of their communities may have repercussions at higher trophic levels. Diatoms are key factors for water quality evaluation in the European Water Framework Directive (European Commission, 2000). A good ecological status is attributed to rivers where the species composition of periphytic diatom communities is only slightly altered by human activities (Hering *et al.*, 2010). The toxicity of metolachlor has been assessed via monospecific toxicological tests on microalgae (Fairchild *et al.*, 1997; Ma and Liang, 2001; Junghans *et al.*, 2003; Ma *et al.*, 2003) but rarely with diatoms (Battaglin *et al.*, 2000). Such tests, based on a single species and focusing on one endpoint (biomass production), only give partial information about the potential impact of the herbicide in the environment. Although it is not yet a standard method, testing microalgal communities sampled in the field assesses toxic

*Corresponding author: vincent.roubeix@cemagref.fr

effects on the individual species as well as on the whole assemblage.

Communities increase their tolerance to toxicants through two mechanisms: (i) development of tolerant and competitive species and reduction of the relative abundance of sensitive species (Schmitt-Jansen and Altenburger, 2005; Pesce *et al.*, 2010) and (ii) tolerance acquisition of some species to toxic pressure (Ivorra *et al.*, 2002). As diatoms are useful bioindicators of river eutrophication and saproby (Prygiel *et al.*, 1999), there have been many attempts to find specific indicators of toxic pollution in diatom communities. Some field studies have tried to relate periphytic diatom community variations to herbicide concentrations; however, the variations in the field of many interfering factors (*e.g.*, trophic level, shading and current velocity) make the isolation and clear demonstration of the toxic effect difficult (Morin *et al.*, 2009; Roubeix *et al.*, in press). The experimental exposure of diatom communities to a chosen toxicant in enclosed controlled systems is then a suitable complementary approach. A large set of examples can be found in the literature with various herbicide molecules tested in either mesocosms or microcosms (Kosinski, 1984; Hamala and Kollig, 1985; Noack *et al.*, 2003; Schmitt-Jansen and Altenburger, 2005; Mohr *et al.*, 2008; Ricart *et al.*, 2009; Vera *et al.*, 2010).

In this study, microcosm experiments were designed to evaluate the effect of metolachlor on river periphytic diatoms. The objectives were (i) to assess the potential impact of water contamination on diatoms through different endpoints at different biological organization levels and (ii) to find potential indicators among diatoms of pollution by such a herbicide. In order to do this, an experiment using artificial channels was performed to explore the impact of the herbicide on diatom communities grown from river biofilm. Then, the response to metolachlor exposure of single species composing the communities was assessed via acute toxicological tests using the same descriptors as for the channel experiment to better understand the results obtained with complex assemblages.

Material and methods

Experimental design

Artificial channels

Natural diatom communities were collected in rivers by scraping immerged substrates with a scalpel and were maintained in bottled commercial spring water until return to the lab. The diatoms were sampled at four stations located on three small rivers in SW France (the Gèze, Sousson and Save, 'Coteaux de Gascogne') with different exposures to agricultural pollution to obtain maximal species diversity. The upstream station on the Gèze River (Organ, 43.273°N 0.483°E) and the station on the Save River (Montmaurin, 43.230°N 0.654°E) were only slightly impacted by agricultural pollution, whereas significant pesticide concentrations were found at the downstream station on the Géze River (Peyret, 43.327°N 0.535°E) and at the station on the Sousson River (Aujan, 43.380°N 0.505°E) (Roubeix *et al.*, in press).

The diatom samples were mixed before equal inoculation into six glass flasks ('artificial channels') filled with 40 litres of filtered river water (Whatman GF/B filters, 1 µm pore size) from the station with the highest water quality (Organ). In each channel, clean glass slides (6 × 10 cm) were hung from the surface to mid depth and served as substrate for diatom colonization. An immerged pump (NEW-JET 2300) maintained a surface current between the glass slides, and daylight neon lamps placed over each channel gave a homogeneous illumination of 150 $\mu mol.m^{-2}.s^{-1}$ with a 14:10 light–dark cycle. The surface turbulence created by the pumps ensured efficient oxygenation of the growth medium, which remained O_2 saturated during the experiment. The filtered river water was slightly enriched with nutrients following the composition of WC culture medium (Guillard and Lorenzen, 1972), providing oligotrophic growth condition comparable to the study zone (Roubeix *et al.*, in press).

Racemic metolachlor (98%, Dr. Ehrenstorfer, Germany) was dissolved in ultrapure water at 100 $mg.L^{-1}$ and an increasing volume of the solution was poured into the channels; this gave two controls (no metolachlor), two channels at 5 $\mu g.L^{-1}$ and two others at 30 $\mu g.L^{-1}$. In the following, the six channels are called 0a, 0b, 5a, 5b, 30a and 30b, the number corresponding to metolachlor concentration and the letters 'a' or 'b' to the duplicate channels for each concentration.

Phosphate concentration was checked every 2 days and after each diatom sampling. Nutrient consumption by algae was compensated by the addition of concentrated WC medium in the proportion of the loss of PO_4. At the same frequency, 10 mL water samples were taken at the surface of channels 5 and 30 for control of metolachlor concentrations along the time course of the experiment. These samples were filtered with Spartan RC 30 syringe filters (0.45 µm, regenerated cellulose, Whatman) and stored in glass bottles at 4 °C until HPLC-ESI-MS/MS analysis. Two additional samples were taken from the control channels at the end of the experiment to check if there had not been any contamination. After 1 week of growth, a few millilitres of concentrated metolachlor solution was added to channels 5 and 30 to maintain concentration levels. Water temperature was measured daily at different hours of the day and averaged out at 21 °C ($\pm$ 2.4 S.D., $n = 27$). The loss of water volume by evaporation or sampling was compensated daily by addition of distilled water.

Biofilm samples were taken after 2 weeks of colonization from two glass slides. Glass slides were scraped with a cutter blade on both sides and the biofilm was resuspended in 200 mL of commercial spring water. From this suspension, 5 mL was kept in a 5% formaldehyde solution for diatom community examination and the rest was filtered on 2 Whatman GF/F filters for dry mass and chlorophyll-a measurements. After 1 week and following

diatom sampling, 0.5 litre water samples were taken from each channel for chemical analysis.

Monospecific tests

The species *Surirella angusta* and *Achnanthidium minutissimum* were isolated by V. Roubeix from the Gèze (from station Organ, see above) and the Ruiné Rivers (from a little polluted site: 45.4907°N, 0.0172°E), respectively, in summer 2009. These two species were chosen because they are common in the studied area and because they showed contrasting responses to exposure to metolachlor in the channel experiments (see results). Moreover, focusing on the species *S. angusta* allowed a more specific study of the relationship between metolachlor concentration and frustule deformation. The two diatoms were maintained in the same water as used for the artificial channels, enriched with nutrients from WC culture medium (with silicate). This growth medium was used to dissolve the racemic metolachlor and to dilute the concentrated solution (100 $mg.L^{-1}$) to yield five test concentrations ranging from 30 to 38 880 $\mu g.L^{-1}$ on a logarithmic scale with a factor 3. These concentrations were chosen to encompass the highest concentration tested in the channel experiment and concentrations at which the effect of metolachlor on diatom growth is visible. Concentrations were checked at this step by HPLC analysis. For each species, 15 autoclaved 50 mL glass bottles were filled with 30 mL of the obtained metolachlor solutions in triplicate plus five control bottles with metolachlor-free growth medium. Then, 1 mL of preconditioned exponentially growing culture was added to the bottles, which were placed on a rotative disk in a culture chamber at 23 °C with a 130 $\mu mol.m^{-2}.s^{-1}$ illumination and a 14:10 light–dark cycle. Diatom biomass in the bottles was estimated by *in vivo* fluorescence using a bbe Fluoroprobe equipped with a 25 mL cuvette holder. Before pouring the content of the bottles into the cuvette, the bottom of the bottles was gently scraped with a brush to ensure complete resuspension of the benthic algae. Initial biomass was measured in an additional bottle for each species (5 and 3 $\mu g.L^{-1}$ for *S. angusta* and *A. minutissimum*, respectively). Diatom growth during the test was assessed by the final biomass (in μg chl $a.L^{-1}$). Culture samples (5 mL) were conserved in lugol for organic matter digestion and enumeration of abnormal forms.

As it is often hypothesized that Si limitation may be a cause of diatom frustule deformation, a complementary test was performed to demonstrate that the appearance of abnormal forms in the channel experiment could not be attributed to Si depletion. For this, the species *S. angusta* was grown in six further bottles. Three of them contained normal growth medium (controls as above) whereas the three others contained filtered river water enriched with all nutrients except silicate. The cultures were carried out in the same conditions as the toxicological tests but for an extended time of 10 days. At the end, diatom biomass in the cultures without Si addition was 40% less than in the controls (368 ± 88 (S.D.) *vs.* 627 ± 4 μg chl $a.L^{-1}$) and residual $Si(OH)_4$ concentrations were only 0.18 ± 0.05 (S.D.) mg $SiO_2.L^{-1}$, which shows that Si limitation was clearly reached.

Chemical measurements

Basic parameters

Two parameters, ash-free dry mass (AFDM) and chlorophyll-a, were used as biomass indicators. Biofilm dry mass was determined according to the European standard NF EN 872. AFDM was estimated from the difference between dry mass and final mass after sample combustion in a muffle furnace at 500 °C for 1 h. Chlorophyll-a content of the biofilm as well as water pH, conductivity and nutrient concentrations were measured following French and international standards (NF T90-117, NF T90-008, NF EN 27888, NF EN ISO 13395, NF EN ISO 11732, NF T90-023 and NF T 90-007).

Metolachlor determination

For determination of metolachlor concentration, water samples were transferred into 1 mL vials. An HPLC Ultimate 3000 (Dionex, CA, USA) and with a triple quadrupole mass spectrometer API 2000 (Applied Biosystems, CA, USA) were used. The chromatographic separation was done on a Gemini-NX C18 3 µm, 110 Å, 100 × 2 mm equipped with a SecurityGuard from Phenomenex (CA, USA). The two eluents were acetonitrile (A) and ultrapure water with 5 mM ammonium acetate (B), and a linear gradient was applied: an isocratic 10% mixture of A for 1 min, then 30% A till 4 min, 40% A till 8 min, 80% A till 9.5 min, 80% isocratic A till 10.5 min, a decrease of A to 10% till 11 min and then the initial composition was maintained for 4 min. The total running time was thus 15 min and the flow rate was kept constant at 400 $\mu L.min^{-1}$. Multiple reaction monitoring (MRM) mode was used for the metolachlor with a quantitative transition ($284 > 252$) and a second transition for the confirmation ($270 > 176$). Metolachlor-d6 ($290 > 258$) was used as internal standard.

Diatom microscopic examination

Biofilm samples were boiled in H_2O_2 for 30 min to oxidize the organic matter. Then the remaining cleaned diatom frustules were transferred to a glass microscope slide in a high-refractive medium (Naphrax©, Brunel Microscopes Ltd, UK). Diatom species were identified at × 1000 magnification from the taxonomic literature of Central Europe (Krammer and Lange-Bertalot, 1986–1991) and recent nomenclature updates. The relative abundance of each species was calculated from a minimum total count of 400 diatom valves.

The bulk proportion of abnormal forms of diatom frustules was estimated separately from a total count of 1000 valves at × 2000 magnification. Only clear morphological

Table 1. Chemical conditions in the six channels at the start of the experiment (t_0) after 1 and 2 weeks (t_1 and t_2).

t_0	pH	Conductivity ($\mu S.cm^{-1}$)	PO_4 (mg $P.L^{-1}$)	NH_4 (mg $N.L^{-1}$)	NO_2 (mg $N.L^{-1}$)	NO_3 (mg $N.L^{-1}$)	$Si(OH)_4$ (mg $SiO_2.L^{-1}$)
0a	8.0	166	0.049	0.008	0.038	1.10	4.52
0b	8.0	168	0.051	0.006	0.028	1.12	4.88
5a	8.0	170	0.049	0.004	0.036	1.07	4.88
5b	8.0	169	0.049	0.010	0.025	1.05	4.73
30a	8.0	170	0.051	0.005	0.036	1.04	4.27
30b	8.0	170	0.054	0.010	0.026	1.09	4.76
t_1							
0a	7.8	181	0.053	0.004	0.001	1.52	5.13
0b	8.0	177	0.056	0.007	0.004	1.49	5.01
5a	8.0	185	0.046	0.010	0.000	1.59	5.28
5b	8.0	178	0.053	0.002	0.003	1.42	4.98
30a	8.0	178	0.048	0.000	0.003	1.35	5.11
30b	8.0	174	0.049	0.003	0.002	1.24	5.08
t_2							
0a	8.1	189	0.049	0.018	0.010	1.80	0.13
0b	8.2	193	0.054	0.019	0.015	1.83	0.12
5a	8.2	198	0.054	0.019	0.011	1.98	0.17
5b	8.2	194	0.058	0.019	0.010	1.84	0.12
30a	8.2	196	0.059	0.025	0.009	1.82	0.12
30b	8.2	187	0.058	0.023	0.012	1.69	0.67

deformities of the frustule outline were taken into account (Falasco *et al.*, 2009) excluding irregular striation. The proportion of deformed valves of *S. angusta* was specifically estimated from a mean total count of 75 ± 39 (S.D.) valves for the artificial channel samples and among 1000 valves for the monospecific test. In the latter, total cell counts were also performed to establish the linear relationship between chlorophyll-a concentration and cell abundance of *S. angusta* ($\mu g\ chl\ a = 3 \times 10^{-6}$ cell $+ 3$, $R^2 = 0.98$).

Statistical analysis

All statistical analyses were computed using the R software (Ihaka and Gentleman, 1996). Non-parametric multivariate analysis of variance (NP MANOVA) was used to compare diatom communities among treatments (Anderson, 2001). The analysis was performed on Bray–Curtis distances between samples, calculated on log-transformed abundance data of species representing more than 1% of a community in at least one channel (33 species). Homogeneity of multivariate dispersions within treatments was checked using traditional analysis of variance on residuals after principal coordinate analysis (Anderson, 2006). Two factors were tested in the analysis: the concentration of metolachlor (main factor) and the channel (nested factor: two channels per concentration). Permutations were constrained according to the factor tested. For the nested factor test, permutations of the replicates were done across channels within each contamination level and, for the main factor, channels were permuted across contamination levels keeping pairs of replicates associated with each channel. Nine hundred and ninety-nine permutations of raw data were used for each test. A posteriori pairwise comparisons between treatments were performed with the same permutational test (NP MANOVA) but in a one-way design.

The significance of metolachlor effect on biological parameters (chl a, AFDM, species abundances and abnormal forms) was tested using univariate non-parametric Kruskall–Wallis and Wilcoxon tests.

The Hill model (Hill, 1910) was used to fit the data of the monospecific tests by non-linear regression:

$$Chla_f = Chla_0 + \frac{\alpha}{1 + (C_{tox}/EC_{50})^{\beta}}$$

where $Chla_0$ and $Chla_f$ are initial and final diatom biomasses, C_{tox} is the concentration of the herbicide, EC_{50} is the 50% effect concentration and α and β are other model parameters. The NOEC (no observed effect concentration) was determined by comparing each treatment with control using the one-tailed Wilcoxon test.

Results

Chemical conditions

Throughout the experiment pH and conductivity remained approximately constant (Table 1). Phosphate, which is often the limiting nutrient for algal growth in rivers, was well controlled. Its concentration was low (0.05 mg $P.L^{-1}$) and similar to natural concentrations in oligotrophic rivers. Ammonium and nitrite had low concentrations and only slight opposite variations. Nitrate increased from about 1 to almost 2 mg $N.L^{-1}$, whereas silicic acid concentrations fell during the last week in a comparable way in each channel. Overall, in spite of variations of some nutrients over time, for one given time, there was only little difference between treatments.

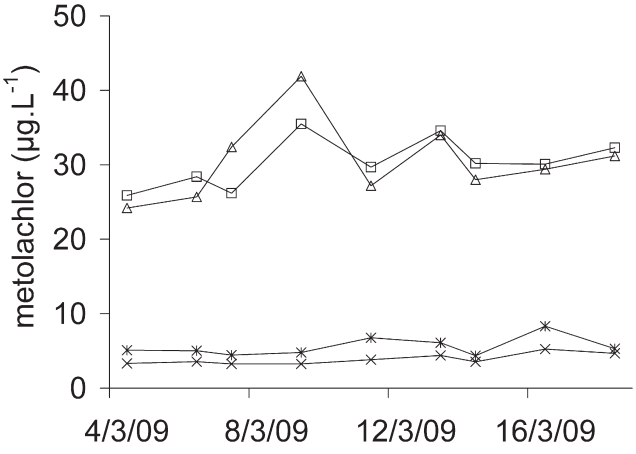


Fig. 1. Concentrations of metolachlor in the four contaminated channels (channels 5a, b and 30a, b) along the duration of the experiment.

Metolachlor concentrations varied only moderately and maintained values close to the nominal concentrations fixed at 5 and 30 µg.L^{-1} (Fig. 1). Time-weighted average concentrations were 5.7 ± 1.1, 3.9 ± 0.9, 30.9 ± 4.8 and 30.6 ± 4.1 µg.L^{-1} ($\pm$ S.D.) for channels 5a, 5b, 30a and 30b, respectively. The herbicide was not detected in the control channels.

Effect on biomass

The growth of biofilm on the glass slides in the artificial channels was assessed through the two biomass indicators: AFDM and chl a. Final biomass did not differ significantly between treatments (Fig. 2) as regards both indicators, and thus no effect of metolachlor was apparent on periphyton biomass. Chl a density and AFDM reached on average 0.04 mg.cm^{-2} and 0.04 mg.dm^{-2}, respectively, at the end of the experiment.

The monospecific toxicological tests showed no apparent effect of metolachlor on diatom growth at concentrations under 100 µg.L^{-1}. EC_{50} values were estimated at 2962 and 1880 µg.L^{-1} metolachlor for *S. angusta* and *A. minutissimum*, respectively (Fig. 3). The NOEC amounted to 180 µg.L^{-1} for both species.

Effect on community composition

The dominant species of diatom communities sampled at the end of the channel experiment are presented in Table 2. The communities were characterized by the predominance of small species (*Nitzschia pusilla*, *Eolimna minima*, *Planothidium frequentissimum*, *Mayamaea permitis* and *Navicula seminulum*) and by the relative importance of the centric *Melosira varians* and some larger species from the genus *Nitzschia* (*Nitzschia palea* and *Nitzschia gracilis*) and *Surirella* (*Surirella angusta* and *Surirella brebissonii*).

The application of non-parametric multivariate ANOVA revealed a significant effect of metolachlor on

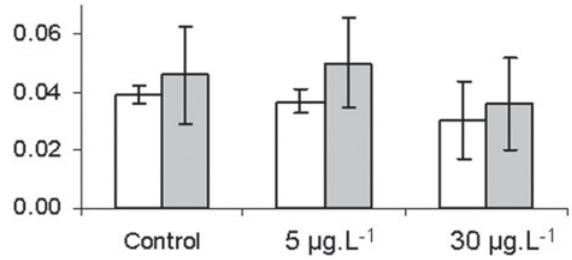


Fig. 2. Biofilm biomass ($\pm$ S.E.) on glass slides after 14 days of experiment, expressed as ash-free dry mass (AFDM, white bars, mg.cm^{-2}) and as chlorophyll-a (grey bars, mg.dm^{-2}).

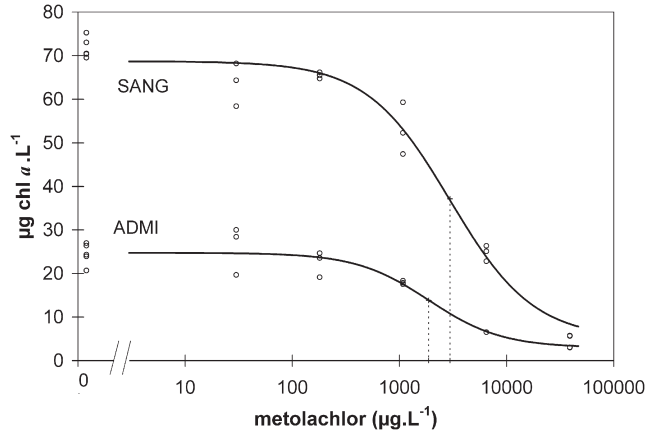


Fig. 3. Results of the acute toxicity tests of metolachlor based on growth inhibition of the diatoms *Surirella angusta* (SANG) and *Achnanthidium minutissimum* (ADMI). The graph presents diatom biomass (in µg chl a.L^{-1}) at the end of the tests. Vertical dashed lines indicate EC_{50} values.

diatom community composition ($p = 0.016$) and the absence of a channel effect ($p = 0.108$) (Table 3). Pairwise comparisons between treatments indicated that most of the difference lay between control channels and the contaminated ones (5 and 30 µg.L^{-1}) that did not significantly differ from each other (Table 2 and Fig. 4).

Individual variations in the relative abundance of some species among treatments can suggest sensitivity or tolerance. The species *Achnanthidium minutissimum*, *Mayamaea permitis*, *Nitzschia palea* and to a lesser extent *N. pusilla* had lower abundance at high contamination levels (Fig. 5) and may be considered sensitive to metolachlor. Other species increased in abundance with metolachlor concentration and therefore appeared rather tolerant, especially *Planothidium frequentissimum*, *P. lanceolatum*, *Surirella brebissonii*, *Amphora montana* and *Nitzschia gracilis*.

Abnormal forms

A higher proportion of abnormal forms of diatom frustules was observed in the contaminated channels (Fig. 6). In whole communities, it remained very low (< 6‰, Fig. 7). Nevertheless the increase with metolachlor concentration was significant. Deformities were particularly

Table 2. Relative abundance (in %) of the dominant species in the 12 diatom communities sampled at the end of the channel experiment. The numbers (1 and 2) refer to the two biofilm samples from each channel.

Metolachlor ($\mu g.L^{-1}$)	Biofilm sample	NIPU	EOMI	MVAR	PLFR	MAPE	NPAL	NSEM	NIGR	SANG	GPUM	PTLA	SBRE	NCPL	ADMI	NHAN
0	0_a1	72.5	6.3	0.5	1.5	6.3	5.5	0.0	0.8	0.3	0.0	1.0	0.0	0.0	1.0	0.0
	0_a2	63.8	6.3	2.0	0.5	13.8	3.5	0.0	0.0	1.3	0.0	0.5	0.3	0.3	1.3	0.0
	0_b1	47.5	4.3	1.0	0.8	15.0	12.0	1.0	1.0	1.0	0.8	0.8	0.5	0.5	0.3	1.8
	0_b2	36.5	7.0	2.8	1.8	8.8	14.8	1.0	0.8	3.3	1.5	1.3	0.5	1.0	3.0	0.5
5_a1		21.8	1.0	8.0	11.8	8.8	4.3	6.3	1.8	1.3	3.0	1.3	0.5	0.5	0.0	
5_a2		44.8	0.0	1.8	6.5	7.0	6.5	3.5	3.0	0.8	1.0	1.0	1.5	3.3	1.5	
5_b1		46.3	0.3	1.0	7.0	6.3	5.8	2.0	2.5	0.8	2.0	1.3	1.0	2.3	2.3	
5_b2		30.3	0.8	1.8	12.3	9.0	1.8	1.5	4.3	4.8	1.5	2.0	1.0	0.0	1.5	
30	30_a1	59.3	4.0	1.0	2.3	5.0	2.8	2.3	2.5	1.8	0.8	2.8	2.8	1.0	0.0	1.3
	30_a2	52.0	8.0	4.5	1.8	7.0	3.0	2.8	1.8	2.8	1.0	1.5	1.3	0.3	0.0	0.3
	30_b1	12.0	25.0	10.5	17.8	3.5	3.0	2.5	1.3	3.8	0.0	4.5	1.8	3.5	0.0	3.3
	30_b2	8.3	20.0	23.3	6.3	1.8	4.8	0.5	1.5	5.8	0.3	4.3	4.5	3.0	0.8	3.0

NIPU, *Nitzschia pusilla*; EOMI, *Eolimna minima*; MVAR, *Melosira varians*; PLFR, *Planothidium frequentissimum*; MAPE, *Mayamaea permitis*; NPAL, *Nitzschia palea*; NSEM, *Navicula seminulum*; NIGR, *Nitzschia gracilis*; SANG, *Surirella angusta*; GPUM, *Gomphonema pumilum*; PTLA, *Planothidium lanceolatum*; SBRE, *Surirella brebissonii*; NCPL, *Nitzschia capitellata*; ADMI, *Achnanthidium minutissimum*; NHAN, *Nitzschia hantzschiana*.

Table 3. Results of non-parametric multivariate ANOVA applied on diatom community samples.

NP MANOVA	df	MS	*F*	*p* value
Metolachlor	2	0.10613	2.145427	0.016*
Microcosm (nested)	3	0.04947	1.839264	0.108
Residuals	6	0.02690		
Pairwise comparisons among treatments				
Control *vs.* 5 $\mu g.L^{-1}$	1	0.098363	3.1254	0.028*
Residuals	6	0.031472		
Control *vs.* 30 $\mu g.L^{-1}$	1	0.131271	3.4224	0.028*
Residuals	6	0.038357		
5 $\mu g.L^{-1}$ *vs.* 30 $\mu g.L^{-1}$	1	0.088753	2.6549	0.058
Residuals	6	0.033429		

*Significant results ($\alpha = 0.05$).

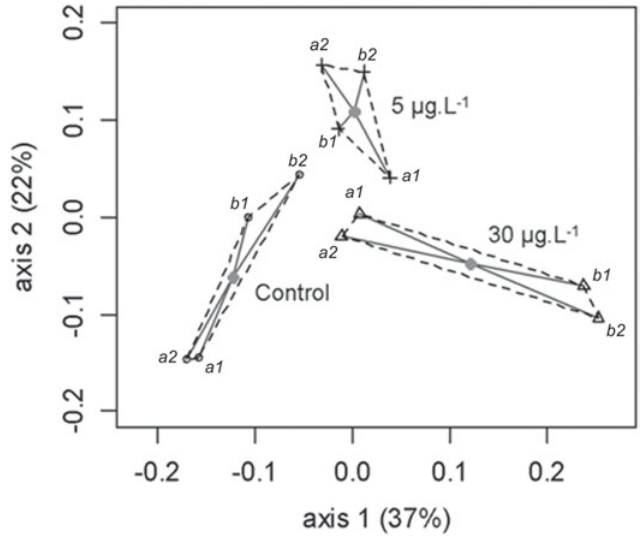


Fig. 4. Principal coordinate plot of diatom communities at the end of the channel experiment derived from Bray–Curtis distances between samples. Sample dispersion around their centroid was not significantly different among treatments (ANOVA, $p = 0.15$). The letters (a and b) and the numbers (1 and 2) refer to the duplicate channels and to the two biofilm samples collected in each channel, respectively.

prominent in the sub-dominant species *Surirella angusta* (Fig. 6). Their frequency in the populations of *S. angusta* increased significantly with contamination to reach 80‰ at the end of the experiment at 30 $\mu g.L^{-1}$ (Fig. 7).

In the monospecific cultures of *S. angusta*, abnormal valves were approximately 2-fold more abundant in the presence of metolachlor but no dose effect was evidenced after 4 days exposure. Si-limited cultures exhibited significantly less abnormal forms than contaminated cultures and did not differ from controls in this aspect (Fig. 8).

Discussion

Structural *vs.* quantitative endpoints

The channel experiment demonstrated an effect of metolachlor from a concentration of 5 $\mu g.L^{-1}$ on the

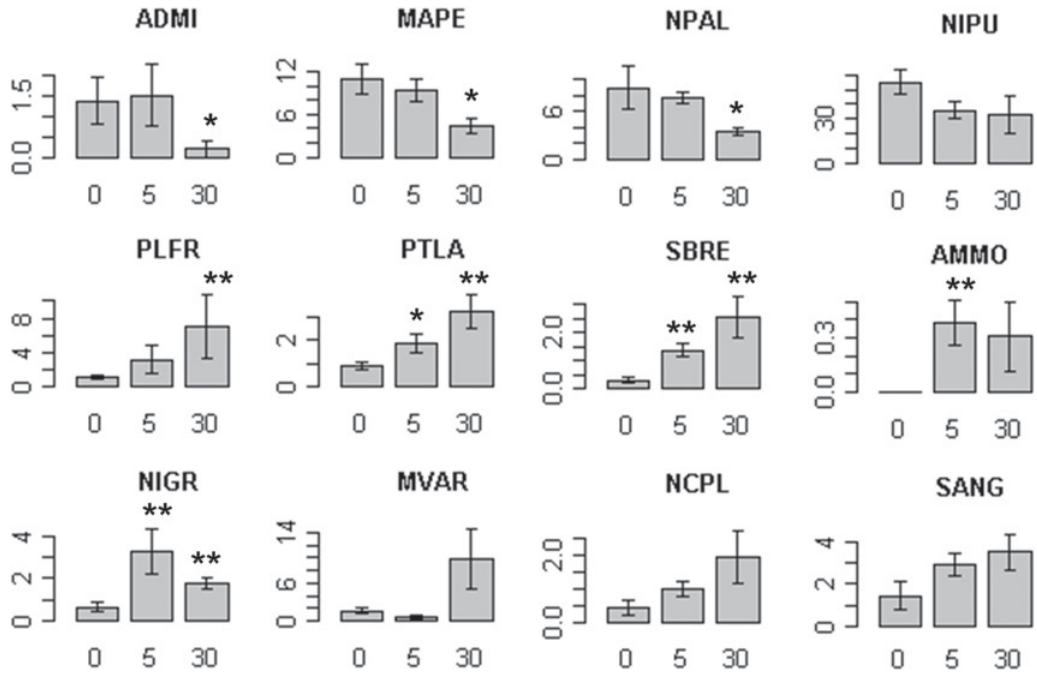


Fig. 5. Bar plots of the relative abundance (in %, ± S.E.) of some diatom species suggesting sensitivity or tolerance to metolachlor. Differences with control conditions were investigated using Wilcoxon's test (*$p < 0.1$, **$p < 0.05$). ADMI, *Achnanthidium minutissimum*, MAPE, *Mayamaea permitis*, NPAL, *Nitzschia palea*, NIPU, *Nitzschia pusillana*, PLFR, *Planothidium frequentissimum*, PTLA, *Planothidium lanceolatum*, SBRE, *Surirella brebissonii*, AMMO, *Amphora montana*, NIGR, *Nitzschia gracilis*, MVAR, *Melosira varians*, NCPL, *Nitzschia capitellata*, SANG, *Surirella angusta*.

species composition of diatom assemblages after 2 weeks of substrate colonization. This effect was revealed by maintaining the contamination level and all other factors that influence diatom communities, namely light, trophic level and current velocity. Starting from realistic oligotrophic conditions, the regular addition of nutrients allowed biofilm growth on the substrates but could not prevent transient Si limitation at the end of the experiment. Indeed as biomass accumulates exponentially, the consumption of nutrient increases accordingly and the frequency of nutrient supply should have been increased likewise. Nevertheless Si limitation occurred similarly in each channel only at the end of the experiment and may not have differentially influenced the effect of the herbicide on diatom community composition.

These results obtained under controlled conditions confirm that the diatom community alterations observed in the field can be the consequence of chronic river contamination by herbicides (Morin *et al.*, 2009; Roubeix *et al.*, in press). Here, the demonstration of the toxic effect of metolachlor on river diatom community composition at environmental contamination levels is clearly evidenced. The relatively low difference between communities formed at 5 and 30 $\mu g.L^{-1}$ of metolachlor suggests that most of the difference from control already lay in the communities at 5 $\mu g.L^{-1}$ and that the effect of the toxicant might be visible at even lower concentrations.

However, descriptors related to periphyton biomass (chl a and AFDM) did not indicate any effect of metolachlor at the tested concentrations. Moreover, EC_{50} values from the monospecific tests and from other similar studies with microalgae based on short-term growth inhibition tests (Junghans *et al.*, 2003; Vallotton *et al.*, 2008; Liu and Xiong, 2009) suggest much higher effect thresholds. Indeed, no significant effect of metolachlor was visible on the growth of *S. angusta* and *A. minutissimum* up to a concentration of 180 $\mu g.L^{-1}$ (NOEC). As tolerant species progressively replace sensitive ones in a community exposed to a toxicant, the effect on the whole community biomass may not be visible unless the tolerance limit of the most robust and competitive species is reached. Therefore community composition change is a more sensitive endpoint to evaluate the toxicity of a pollutant on a natural aquatic environment and deviation from the reference condition, as requested by the European Water Framework Directive.

Species sensitive/tolerant to metolachlor

The changes in composition of diatom communities with metolachlor concentrations are informative about the possible bioindication of herbicide contamination. In such microcosm experiments, the decrease in abundance of a species along a contamination gradient may result from species sensitivity to the tested toxicant or to a change in biological interactions linked to water toxicity. *Achnanthidium minutissimum* is a species indicative of good water quality (Coste *et al.*, 2009), which was shown (as in this study) to maintain its relative abundance at low

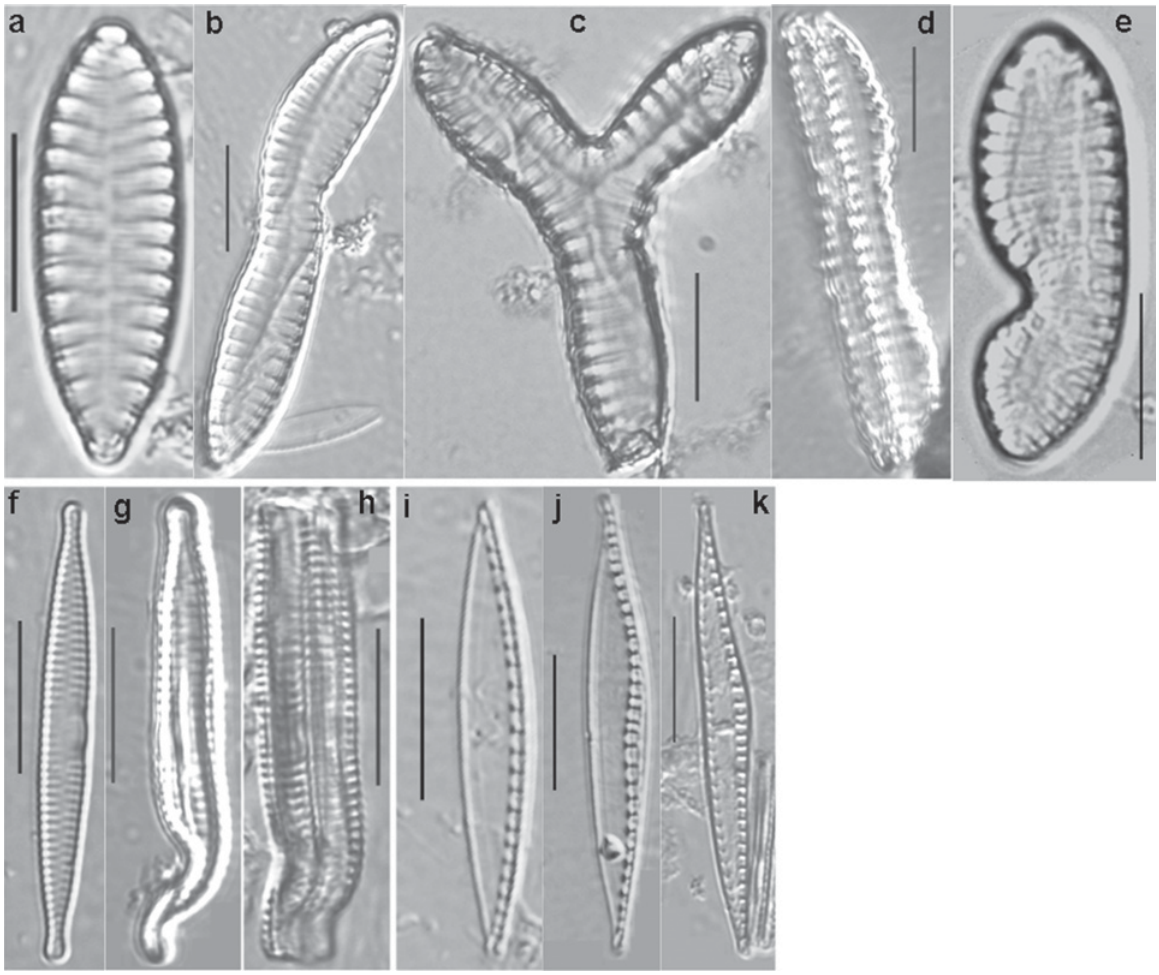


Fig. 6. Abnormal diatom frustules observed in the contaminated experimental units for the species *Surirella angusta* (a to e), *Fragilaria capucina* (f to h) and *Nitzschia sociabilis* (i to k). (a), (f) and (i): normal form of each species; (d) and (h): pair of deformed frustules following vegetative reproduction (OM × 1000, vertical bars indicate 10 µm).

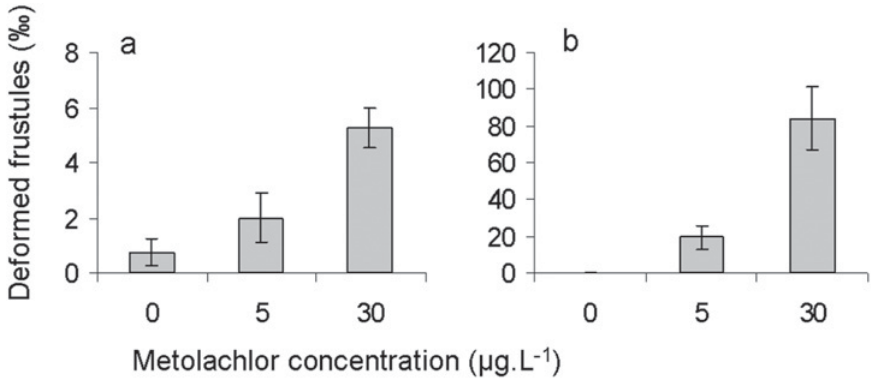


Fig. 7. Relative abundance of deformed frustules (± S.E.) in the whole diatom community (a) and within *Surirella angusta* populations (b). Kruskall–Wallis test: $p < 0.05$ (a) and $p < 0.01$ (b).

herbicide concentration and to be strongly reduced at higher contamination levels (Hamilton *et al.*, 1987; Pérès *et al.*, 1996). Therefore, it seems that *A. minutissimum* is rather sensitive to herbicides. This is supported by the lower EC_{50} obtained for this species than for *S. angusta*, which appeared indifferent to metolachlor in the channel experiment. The species *Mayamaea permitis* and *Nitzschia palea* may bring interesting information for separating toxic *vs.* trophic effects, as they exhibited the same sensitivity pattern as *A. minutissimum* (Fig. 5); however,

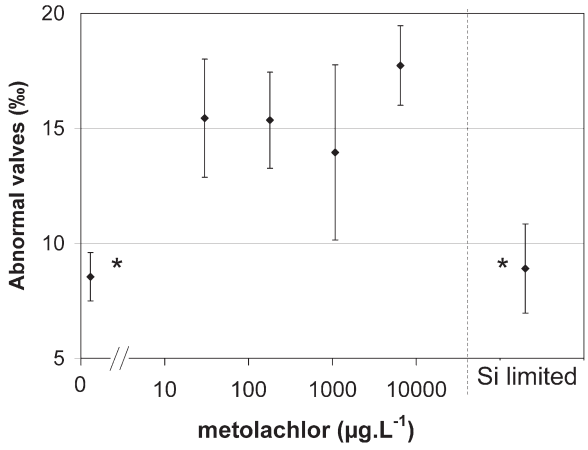


Fig. 8. Variations in the proportion of abnormal valves of the species *Surirella angusta* in relation to metolachlor concentration and Si limitation. Results from the acute toxicity test (*significantly different from contaminated cultures, $p < 0.05$, Wilcoxon test).

they are known to be generally more tolerant to organic and trophic pollution. The species *Nitzschia pusilla* had a lower relative abundance in the two treatments with metolachlor. However, it remained the dominant species in almost all communities sampled. It may be rather indifferent to metolachlor and the drop in relative abundance would result from the greater development of tolerant species in contaminated channels.

Species increasing their proportion in communities formed at higher metolachlor concentrations may be considered tolerant. This is the case of the saprophilous *Surirella brebissonii* as well as *P. frequentissimum* and *P. lanceolatum*, which have already been identified as tolerant to pollution by herbicides including metolachlor in a previous field approach (Roubeix *et al.*, in press). *A. montana*, *N. gracilis* and especially *N. capitellata* are characteristic of low water quality (Coste *et al.*, 2009) and showed tolerance to metolachlor in this study. For *M. varians*, however, general knowledge of its sensitivity to pollution (eutrophication and saproby) is not consistent with the findings of certain herbicide studies. Indeed *M. varians* is generally recorded in clean rivers but appears to accommodate well to high herbicide concentrations (Spawn *et al.*, 1997; Debenest *et al.*, 2009).

Relevance of abnormal forms as an indication of toxicity

This experimental study clearly establishes the link between abnormal forms and the herbicide. The appearance of diatom deformities is often presumed to result from Si limitation (Debenest *et al.*, 2010). However, from this study, this hypothesis can be rejected for several reasons: (1) there was a clear dose effect in the channels and there was most Si left in one of the most contaminated channels (30b), (2) in the monospecific test, deformities were significantly more abundant in the contaminated cultures, and (3) Si limitation did not produce more abnormal forms than control conditions. The duration of the experiments (14 *vs.* 4 days) may be the cause of the difference in proportion and in response to concentration between the channel experiment and the monospecific test. It is likely that the proportion of abnormal forms in a population increases with time at a rate depending on the exposure level. It could also be hypothesized that the progressive degradation of metolachlor may produce metabolites having higher teratogenic effects than metolachlor (Osano *et al.*, 2002; Hladik *et al.*, 2005).

Like other herbicides belonging to the chloroacetanilide class, metolachlor inhibits the biosynthesis of very long chain fatty acids in plants and microalgae (Boger *et al.*, 2000) and consequently blocks cell division and enlargement. Such a mode of action does not *a priori* suggest an effect on diatom frustule structure. However, changes in ultrastructural morphology were reported in a green microalgae following exposure to metolachlor (Liu and Xiong, 2009). As the synthesis of the diatom frustule involves a special intracellular vesicle (SDV) and requires the exocytosis of biogenic silica (Martin-Jezequel *et al.*, 2000), any alteration of the cell membrane system may have repercussions on frustule construction.

The link between abnormal diatom forms and contamination by heavy metals has been well documented (Gold *et al.*, 2003; Cattaneo *et al.*, 2004; Morin *et al.*, 2008; Falasco *et al.*, 2009). However, the literature on the effect of herbicides on diatom morphology is scarce. There are now experimental studies reporting diatom deformities due to herbicides with three different physiological targets: photosynthesis (isoproturon), gene expression (maleic hydrazide) and lipid synthesis (metolachlor, this study) (Schmitt-Jansen and Altenburger, 2005; Debenest *et al.*, 2008). The variety of toxicants giving the same effect on diatoms suggests that toxic stress generally generates abnormal forms.

The frequency of abnormal forms has already been proposed as a bioindicator of heavy metal contamination (Cattaneo *et al.*, 2004). Recent results concerning the effects of herbicides suggest that abnormal forms might also be used to indicate herbicide contamination in the field. However, as the relative abundance of deformities is generally just a few ‰ (Fig. 7) (Morin *et al.*, 2009), this would require a higher counting effort than bioindication on a taxonomic basis (AFNOR, 2004). The example of *S. angusta* shows that it would be easier to focus on widespread sensitive species that could be used as sentinels.

Conclusion

The experiments conducted in this study showed that metolachlor has toxic effects on river periphytic diatoms. The results are interesting for the toxicological assessment of pesticides as well as for the bioindication of contamination. It emerges that effect concentrations may vary according to the species and the endpoint (biomass,

deformities and community composition) considered. Microalgal tests based on biomass can be easily related to ecosystem functions (primary production and trophic pathways). However, diatom community composition seems to be more sensitive to contamination and should be considered as a relevant endpoint. Also the channel experiment provided information about the tolerance of some diatom species to metolachlor. These data will contribute to the knowledge of diatom sensitivity to toxicants, which could lead to the construction of new diatom indices of water quality. Our experimental results support the inclusion of abnormal diatom forms in water quality indices because they may reflect toxic stress.

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