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

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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  $\mu\text{g.L}^{-1}$ ). 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  $\mu\text{g.L}^{-1}$  were recorded in some rivers and peak concentrations reached several tens of  $\mu\text{g.L}^{-1}$  (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

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

64 Communities increase their tolerance to toxicants  
65 through two mechanisms: (i) development of tolerant  
66 and competitive species and reduction of the relative  
67 abundance of sensitive species (Schmitt-Jansen and  
68 Altenburger, 2005; Pesce *et al.*, 2010) and (ii) tolerance  
69 acquisition of some species to toxic pressure (Ivorra *et al.*,  
70 2002). As diatoms are useful bioindicators of river  
71 eutrophication and saprobity (Prygiel *et al.*, 1999), there  
72 have been many attempts to find specific indicators of  
73 toxic pollution in diatom communities. Some field studies  
74 have tried to relate periphytic diatom community varia-  
75 tions to herbicide concentrations; however, the variations  
76 in the field of many interfering factors (*e.g.*, trophic level,  
77 shading and current velocity) make the isolation and clear  
78 demonstration of the toxic effect difficult (Morin *et al.*,  
79 2009; Roubeix *et al.*, *in press*). The experimental exposure  
80 of diatom communities to a chosen toxicant in enclosed  
81 controlled systems is then a suitable complementary ap-  
82 proach. A large set of examples can be found in the  
83 literature with various herbicide molecules tested in either  
84 mesocosms or microcosms (Kosinski, 1984; Hamala and  
85 Kollig, 1985; Noack *et al.*, 2003; Schmitt-Jansen and  
86 Altenburger, 2005; Mohr *et al.*, 2008; Ricart *et al.*, 2009;  
87 Vera *et al.*, 2010).

88 In this study, microcosm experiments were designed to  
89 evaluate the effect of metolachlor on river periphytic  
90 diatoms. The objectives were (i) to assess the potential  
91 impact of water contamination on diatoms through  
92 different endpoints at different biological organization  
93 levels and (ii) to find potential indicators among diatoms  
94 of pollution by such a herbicide. In order to do this, an  
95 experiment using artificial channels was performed to ex-  
96 plore the impact of the herbicide on diatom communities  
97 grown from river biofilm. Then, the response to metola-  
98 chlor exposure of single species composing the commu-  
99 nities was assessed via acute toxicological tests using the  
100 same descriptors as for the channel experiment to better  
101 understand the results obtained with complex assem-  
102 blages.

## 103 Material and methods

### 104 Experimental design

#### 105 Artificial channels

106 Natural diatom communities were collected in rivers by  
107 scraping immersed substrates with a scalpel and were  
108 maintained in bottled commercial spring water until return  
109 to the lab. The diatoms were sampled at four stations  
110 located on three small rivers in SW France (the Gèze,  
111 Sousson and Save, 'Coteaux de Gascogne') with different  
112 exposures to agricultural pollution to obtain maximal  
113 species diversity. The upstream station on the Gèze River  
114 (Organ, 43.273°N 0.483°E) and the station on the Save  
115 River (Montmaurin, 43.230°N 0.654°E) were only slightly

116 impacted by agricultural pollution, whereas significant  
117 pesticide concentrations were found at the downstream  
118 station on the Gèze River (Peyret, 43.327°N 0.535°E) and  
119 at the station on the Sousson River (Aujan, 43.380°N  
120 0.505°E) (Roubeix *et al.*, *in press*).

121 The diatom samples were mixed before equal inocula-  
122 tion into six glass flasks ('artificial channels') filled with  
123 40 litres of filtered river water (Whatman GF/B filters,  
124 1 µm pore size) from the station with the highest  
125 water quality (Organ). In each channel, clean glass slides  
126 (6 × 10 cm) were hung from the surface to mid depth and  
127 served as substrate for diatom colonization. An immersed  
128 pump (NEW-JET 2300) maintained a surface current  
129 between the glass slides, and daylight neon lamps placed  
130 over each channel gave a homogeneous illumination of  
131 150 µmol.m<sup>-2</sup>.s<sup>-1</sup> with a 14:10 light-dark cycle. The  
132 surface turbulence created by the pumps ensured efficient  
133 oxygenation of the growth medium, which remained O<sub>2</sub>  
134 saturated during the experiment. The filtered river water  
135 was slightly enriched with nutrients following the compo-  
136 sition of WC culture medium (Guillard and Lorenzen,  
137 1972), providing oligotrophic growth condition compar-  
138 able to the study zone (Roubeix *et al.*, *in press*).

139 Racemic metolachlor (98%, Dr. Ehrenstorfer,  
140 Germany) was dissolved in ultrapure water at 100 mg.L<sup>-1</sup>  
141 and an increasing volume of the solution was poured into  
142 the channels; this gave two controls (no metolachlor), two  
143 channels at 5 µg.L<sup>-1</sup> and two others at 30 µg.L<sup>-1</sup>. In the  
144 following, the six channels are called 0a, 0b, 5a, 5b, 30a and  
145 30b, the number corresponding to metolachlor concentra-  
146 tion and the letters 'a' or 'b' to the duplicate channels for  
147 each concentration.

148 Phosphate concentration was checked every 2 days and  
149 after each diatom sampling. Nutrient consumption by  
150 algae was compensated by the addition of concentrated  
151 WC medium in the proportion of the loss of PO<sub>4</sub>. At the  
152 same frequency, 10 mL water samples were taken at the  
153 surface of channels 5 and 30 for control of metolachlor con-  
154 centrations along the time course of the experiment. These  
155 samples were filtered with Spartan RC 30 syringe filters  
156 (0.45 µm, regenerated cellulose, Whatman) and stored in  
157 glass bottles at 4 °C until HPLC-ESI-MS/MS analysis.  
158 Two additional samples were taken from the control  
159 channels at the end of the experiment to check if there had  
160 not been any contamination. After 1 week of growth, a few  
161 millilitres of concentrated metolachlor solution was added  
162 to channels 5 and 30 to maintain concentration levels.  
163 Water temperature was measured daily at different hours of  
164 the day and averaged out at 21 °C (± 2.4 s.d., *n* = 27). The  
165 loss of water volume by evaporation or sampling was  
166 compensated daily by addition of distilled water.

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

175	diatom sampling, 0.5 litre water samples were taken from	residual $\text{Si}(\text{OH})_4$ concentrations were only $0.18 \pm 0.05$	232
176	each channel for chemical analysis.	(s.d.) $\text{mg SiO}_2 \cdot \text{L}^{-1}$ , which shows that Si limitation was	233
		clearly reached.	234
177	<b>Monospecific tests</b>		
AQ2178	The species <i>Surirella angusta</i> and <i>Achnanthydium</i>	<b>Chemical measurements</b>	235
179	<i>minutissimum</i> were isolated by V. Roubeix from the Gèze	<i>Basic parameters</i>	236
180	(from station Organ, see above) and the Ruiné Rivers		
181	(from a little polluted site: 45.4907°N, 0.0172°E), respec-	Two parameters, ash-free dry mass (AFDM) and	237
182	tively, in summer 2009. These two species were chosen	chlorophyll-a, were used as biomass indicators. Biofilm	238
183	because they are common in the studied area and because	dry mass was determined according to the European	239
184	they showed contrasting responses to exposure to metola-	standard NF EN 872. AFDM was estimated from the	240
185	chlors in the channel experiments (see results). Moreover,	difference between dry mass and final mass after sample	241
186	focusing on the species <i>S. angusta</i> allowed a more specific	combustion in a muffle furnace at 500 °C for 1 h.	242
187	study of the relationship between metolachlor concentra-	Chlorophyll-a content of the biofilm as well as water pH,	243
188	tion and frustule deformation. The two diatoms were	conductivity and nutrient concentrations were measured	244
189	maintained in the same water as used for the artificial	following French and international standards (NF	245
190	channels, enriched with nutrients from WC culture	T90-117, NF T90-008, NF EN 27888, NF EN ISO	246
191	medium (with silicate). This growth medium was used to	13395, NF EN ISO 11732, NF T90-023 and NF T 90-007).	247
192	dissolve the racemic metolachlor and to dilute the concen-		
193	trated solution ( $100 \text{ mg} \cdot \text{L}^{-1}$ ) to yield five test concentra-	<i>Metolachlor determination</i>	248
194	tions ranging from 30 to 38 880 $\mu\text{g} \cdot \text{L}^{-1}$ on a logarithmic		
195	scale with a factor 3. These concentrations were chosen to	For determination of metolachlor concentration, water	249
196	encompass the highest concentration tested in the channel	samples were transferred into 1 mL vials. An HPLC	250
197	experiment and concentrations at which the effect of	Ultimate 3000 (Dionex, CA, USA) and with a triple quad-	251
198	metolachlor on diatom growth is visible. Concentrations	rupole mass spectrometer API 2000 (Applied Biosystems,	252
199	were checked at this step by HPLC analysis. For each	CA, USA) were used. The chromatographic separation	253
200	species, 15 autoclaved 50 mL glass bottles were filled with	was done on a Gemini-NX C18 3 $\mu\text{m}$ , 110 Å, $100 \times 2 \text{ mm}$	254
201	30 mL of the obtained metolachlor solutions in triplicate	equipped with a SecurityGuard from Phenomenex (CA,	255
202	plus five control bottles with metolachlor-free growth	USA). The two eluents were acetonitrile (A) and ultrapure	256
203	medium. Then, 1 mL of preconditioned exponentially	water with 5 mM ammonium acetate (B), and a linear	257
204	growing culture was added to the bottles, which were	gradient was applied: an isocratic 10% mixture of A for	258
205	placed on a rotative disk in a culture chamber at 23 °C with	1 min, then 30% A till 4 min, 40% A till 8 min, 80% A till	259
206	a $130 \mu\text{mol} \cdot \text{m}^{-2} \cdot \text{s}^{-1}$ illumination and a 14:10 light–dark	9.5 min, 80% isocratic A till 10.5 min, a decrease of A	260
207	cycle. Diatom biomass in the bottles was estimated by	to 10% till 11 min and then the initial composition	261
208	<i>in vivo</i> fluorescence using a bbe Fluoroprobe equipped	was maintained for 4 min. The total running time was	262
209	with a 25 mL cuvette holder. Before pouring the content	thus 15 min and the flow rate was kept constant at	263
210	of the bottles into the cuvette, the bottom of the bottles	$400 \mu\text{L} \cdot \text{min}^{-1}$ . Multiple reaction monitoring (MRM)	264
211	was gently scraped with a brush to ensure complete	mode was used for the metolachlor with a quantitative	265
212	resuspension of the benthic algae. Initial biomass was	transition ( $284 > 252$ ) and a second transition for the	266
213	measured in an additional bottle for each species (5 and	confirmation ( $270 > 176$ ). Metolachlor-d6 ( $290 > 258$ ) was	267
214	$3 \mu\text{g} \cdot \text{L}^{-1}$ for <i>S. angusta</i> and <i>A. minutissimum</i> , respec-	used as internal standard.	268
215	tively). Diatom growth during the test was assessed by the		
216	final biomass (in $\mu\text{g chl a} \cdot \text{L}^{-1}$ ). Culture samples (5 mL)	<b>Diatom microscopic examination</b>	269
217	were conserved in lugol for organic matter digestion and		
218	enumeration of abnormal forms.	Biofilm samples were boiled in $\text{H}_2\text{O}_2$ for 30 min	270
219	As it is often hypothesized that Si limitation may be a	to oxidize the organic matter. Then the remaining cleaned	271
220	cause of diatom frustule deformation, a complementary	diatom frustules were transferred to a glass micro-	272
221	test was performed to demonstrate that the appearance	scope slide in a high-refractive medium (Naphrax®,	273
222	of abnormal forms in the channel experiment could not be	Brunel Microscopes Ltd, UK). Diatom species were	274
223	attributed to Si depletion. For this, the species <i>S. angusta</i>	identified at $\times 1000$ magnification from the taxonomic	275
224	was grown in six further bottles. Three of them contained	literature of Central Europe (Krammer and Lange-	276
225	normal growth medium (controls as above) whereas the	Bertalot, 1986–1991) and recent nomenclature updates.	277
226	three others contained filtered river water enriched with all	The relative abundance of each species was calculated	278
227	nutrients except silicate. The cultures were carried out in	from a minimum total count of 400 diatom valves.	279
228	the same conditions as the toxicological tests but for an	The bulk proportion of abnormal forms of diatom frus-	280
229	extended time of 10 days. At the end, diatom biomass in	tules was estimated separately from a total count of 1000	281
230	the cultures without Si addition was 40% less than in the	valves at $\times 2000$ magnification. Only clear morphological	282
231	controls ( $368 \pm 88$ (s.d.) vs. $627 \pm 4 \mu\text{g chl a} \cdot \text{L}^{-1}$ ) and		



**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 ( $\mu\text{S}\cdot\text{cm}^{-1}$ )	$\text{PO}_4$ ( $\text{mg P}\cdot\text{L}^{-1}$ )	$\text{NH}_4$ ( $\text{mg N}\cdot\text{L}^{-1}$ )	$\text{NO}_2$ ( $\text{mg N}\cdot\text{L}^{-1}$ )	$\text{NO}_3$ ( $\text{mg N}\cdot\text{L}^{-1}$ )	$\text{Si(OH)}_4$ ( $\text{mg SiO}_2\cdot\text{L}^{-1}$ )
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

283 deformities of the frustule outline were taken into account  
 284 (Falasco *et al.*, 2009) excluding irregular striation. The  
 285 proportion of deformed valves of *S. angusta* was specifi-  
 286 cally estimated from a mean total count of  $75 \pm 39$  (s.d.)  
 287 valves for the artificial channel samples and among 1000  
 288 valves for the monospecific test. In the latter, total cell  
 289 counts were also performed to establish the linear relation-  
 290 ship between chlorophyll-a concentration and cell abun-  
 291 dance of *S. angusta* ( $\mu\text{g chl a} = 3 \times 10^{-6} \text{ cell} + 3$ ,  $R^2 = 0.98$ ).

## 292 Statistical analysis

293 All statistical analyses were computed using the R  
 294 software (Ihaka and Gentleman, 1996). Non-parametric  
 295 multivariate analysis of variance (NP MANOVA) was  
 296 used to compare diatom communities among treatments  
 297 (Anderson, 2001). The analysis was performed on Bray-  
 298 Curtis distances between samples, calculated on log-  
 299 transformed abundance data of species representing more  
 300 than 1% of a community in at least one channel (33  
 301 species). Homogeneity of multivariate dispersions within  
 302 treatments was checked using traditional analysis of  
 303 variance on residuals after principal coordinate analysis  
 304 (Anderson, 2006). Two factors were tested in the analysis:  
 305 the concentration of metolachlor (main factor) and the  
 306 channel (nested factor: two channels per concentration).  
 307 Permutations were constrained according to the factor  
 308 tested. For the nested factor test, permutations of the  
 309 replicates were done across channels within each contam-  
 310 ination level and, for the main factor, channels were  
 311 permuted across contamination levels keeping pairs of  
 312 replicates associated with each channel. Nine hundred  
 313 and ninety-nine permutations of raw data were used for  
 314 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 ab-  
 normal forms) was tested using univariate non-parametric  
 Kruskal–Wallis and Wilcoxon tests.

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

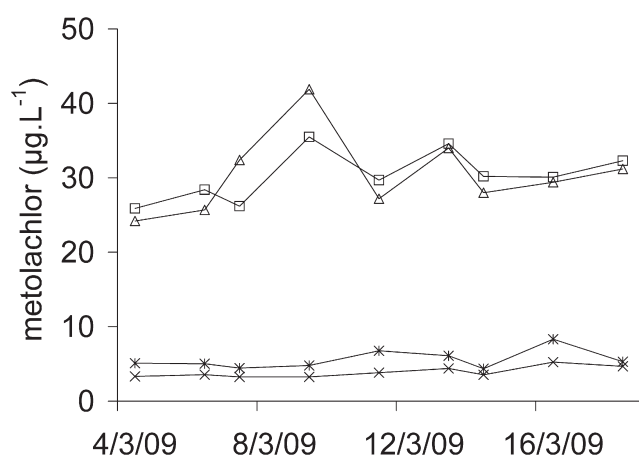
$$\text{Chla}_f = \text{Chla}_0 + \frac{\alpha}{1 + (C_{\text{tox}}/\text{EC}_{50})^\beta}$$

where  $\text{Chla}_0$  and  $\text{Chla}_f$  are initial and final diatom  
 biomasses,  $C_{\text{tox}}$  is the concentration of the herbicide,  
 $\text{EC}_{50}$  is the 50% effect concentration and  $\alpha$  and  $\beta$  are other  
 model parameters. The NOEC (no observed effect con-  
 centration) was determined by comparing each treatment  
 with control using the one-tailed Wilcoxon test.

## 330 Results

### 331 Chemical conditions

332 Throughout the experiment pH and conductivity  
 333 remained approximately constant (Table 1). Phosphate,  
 334 which is often the limiting nutrient for algal growth in  
 335 rivers, was well controlled. Its concentration was low  
 336 ( $0.05 \text{ mg P}\cdot\text{L}^{-1}$ ) and similar to natural concentrations in  
 337 oligotrophic rivers. Ammonium and nitrite had low con-  
 338 centrations and only slight opposite variations. Nitrate  
 339 increased from about 1 to almost  $2 \text{ mg N}\cdot\text{L}^{-1}$ , whereas  
 340 silicic acid concentrations fell during the last week in a  
 341 comparable way in each channel. Overall, in spite of  
 342 variations of some nutrients over time, for one given time,  
 343 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.

344 Metolachlor concentrations varied only moderately  
 345 and maintained values close to the nominal concentrations  
 346 fixed at 5 and 30  $\mu\text{g.L}^{-1}$  (Fig. 1). Time-weighted average  
 347 concentrations were  $5.7 \pm 1.1$ ,  $3.9 \pm 0.9$ ,  $30.9 \pm 4.8$  and  
 348  $30.6 \pm 4.1 \mu\text{g.L}^{-1}$  ( $\pm$ s.d.) for channels 5a, 5b, 30a and  
 349 30b, respectively. The herbicide was not detected in the  
 350 control channels.

351 **Effect on biomass**

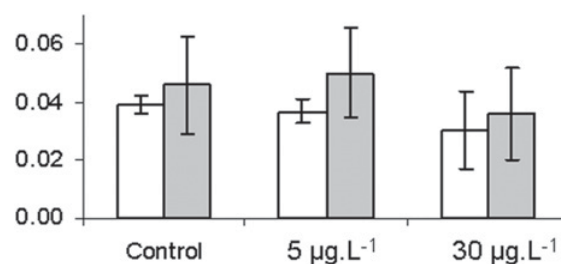
352 The growth of biofilm on the glass slides in the artificial  
 353 channels was assessed through the two biomass indicators:  
 354 AFDM and chl a. Final biomass did not differ significantly  
 355 between treatments (Fig. 2) as regards both indicators, and  
 356 thus no effect of metolachlor was apparent on periphyton  
 357 biomass. Chl a density and AFDM reached on average  
 358  $0.04 \text{ mg.cm}^{-2}$  and  $0.04 \text{ mg.dm}^{-2}$ , respectively, at the end  
 359 of the experiment.

360 The monospecific toxicological tests showed no ap-  
 361 parent effect of metolachlor on diatom growth at con-  
 362 centrations under  $100 \mu\text{g.L}^{-1}$ .  $\text{EC}_{50}$  values were estimated  
 363 at 2962 and 1880  $\mu\text{g.L}^{-1}$  metolachlor for *S. angusta*  
 364 and *A. minutissimum*, respectively (Fig. 3). The NOEC  
 365 amounted to 180  $\mu\text{g.L}^{-1}$  for both species.

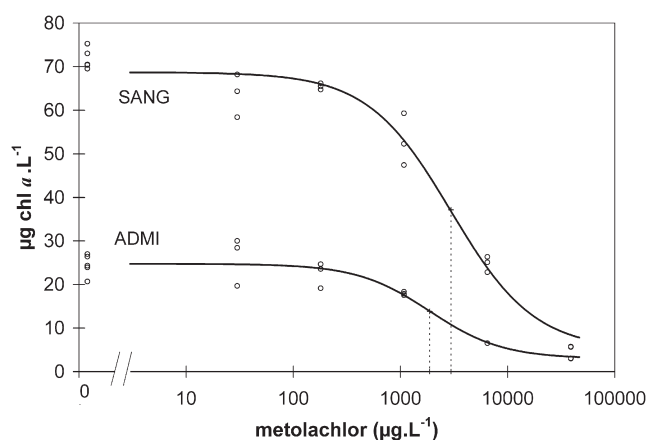
366 **Effect on community composition**

367 The dominant species of diatom communities sampled  
 368 at the end of the channel experiment are presented in  
 369 Table 2. The communities were characterized by the  
 370 predominance of small species (*Nitzschia pusilla*, *Eolimna*  
 371 *minima*, *Planothidium frequentissimum*, *Mayamaea per-*  
 372 *mitis* and *Navicula seminulum*) and by the relative importance  
 373 of the centric *Melosira varians* and some larger species  
 374 from the genus *Nitzschia* (*Nitzschia palea* and *Nitzschia*  
 375 *gracilis*) and *Surirella* (*Surirella angusta* and *Surirella*  
 376 *brebissonii*).

377 The application of non-parametric multivariate  
 378 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,  $\text{mg.cm}^{-2}$ ) and as chlorophyll-a (grey bars,  $\text{mg.dm}^{-2}$ ).



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

379 diatom community composition ( $p = 0.016$ ) and the  
 380 absence of a channel effect ( $p = 0.108$ ) (Table 3). Pairwise  
 381 comparisons between treatments indicated that most of  
 382 the difference lay between control channels and the con-  
 383 taminated ones (5 and  $30 \mu\text{g.L}^{-1}$ ) that did not significantly  
 384 differ from each other (Table 2 and Fig. 4).

385 Individual variations in the relative abundance of  
 386 some species among treatments can suggest sensitivity  
 387 or tolerance. The species *Achnantheidium minutissimum*,  
 388 *Mayamaea permitis*, *Nitzschia palea* and to a lesser extent  
 389 *N. pusilla* had lower abundance at high contamination  
 390 levels (Fig. 5) and may be considered sensitive to  
 391 metolachlor. Other species increased in abundance with  
 392 metolachlor concentration and therefore appeared rather  
 393 tolerant, especially *Planothidium frequentissimum*, *P. lan-*  
 394 *ceolatum*, *Surirella brebissonii*, *Amphora montana* and  
 395 *Nitzschia gracilis*.

396 **Abnormal forms**

397 A higher proportion of abnormal forms of diatom frus-  
 398 tules was observed in the contaminated channels (Fig. 6).  
 399 In whole communities, it remained very low ( $< 6\%$ ,  
 400 Fig. 7). Nevertheless the increase with metolachlor concen-  
 401 tration 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.

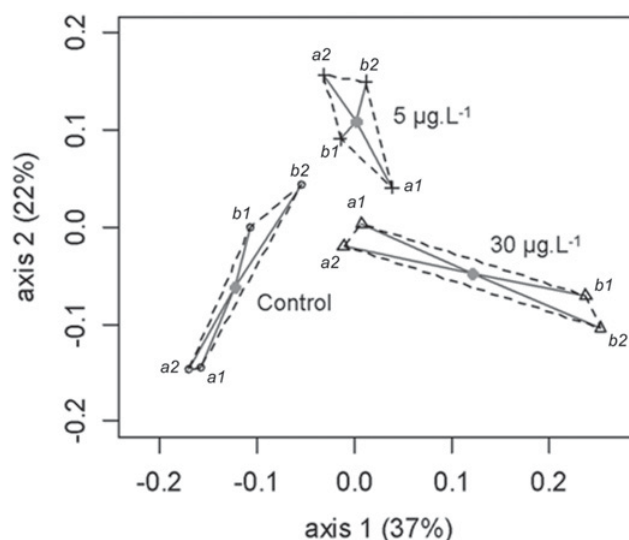
Metolachlor ( $\mu\text{g.L}^{-1}$ )	Biofilm sample	NIPU	EOMI	MVAR	PLFR	MAPE	NPAL	NSEM	NIGR	SANG	GPUM	PTLA	SBRE	NCPL	ADMI	NHAN
0	0_a1	72.5	6.3	0.5	1.5	6.3	5.5	0.0	0.8	0.3	0.0	1.0	0.0	0.0	1.0	0.0
	0_a2	63.8	6.3	2.0	0.5	13.8	3.5	0.0	0.0	1.3	0.0	0.5	0.3	0.3	1.3	0.0
	0_b1	47.5	4.3	1.0	0.8	15.0	12.0	1.0	1.0	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	14.0	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	0.0
5_a2	44.8	3.5	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	0.0
5_b1	46.3	8.3	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	0.0
5_b2	30.3	5.8	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	0.0
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, *Planolithidium frequentissimum*; MAPE, *Mayamaea permitis*; NPAL, *Nitzschia palea*; NSEM, *Navicula seminulum*; NIGR, *Nitzschia gracilis*; SANG, *Surirella angusta*; GPUM, *Gomphonema pumilum*; PTLA, *Planolithidium lanceolatum*; SBRE, *Surirella brevissonii*; NCPL, *Nitzschia capitellata*; ADMI, *Achnanthis 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 $\mu\text{g.L}^{-1}$	1	0.098363	3.1254	0.028*
Residuals	6	0.031472		
Control vs. 30 $\mu\text{g.L}^{-1}$	1	0.131271	3.4224	0.028*
Residuals	6	0.038357		
5 $\mu\text{g.L}^{-1}$ vs. 30 $\mu\text{g.L}^{-1}$	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.

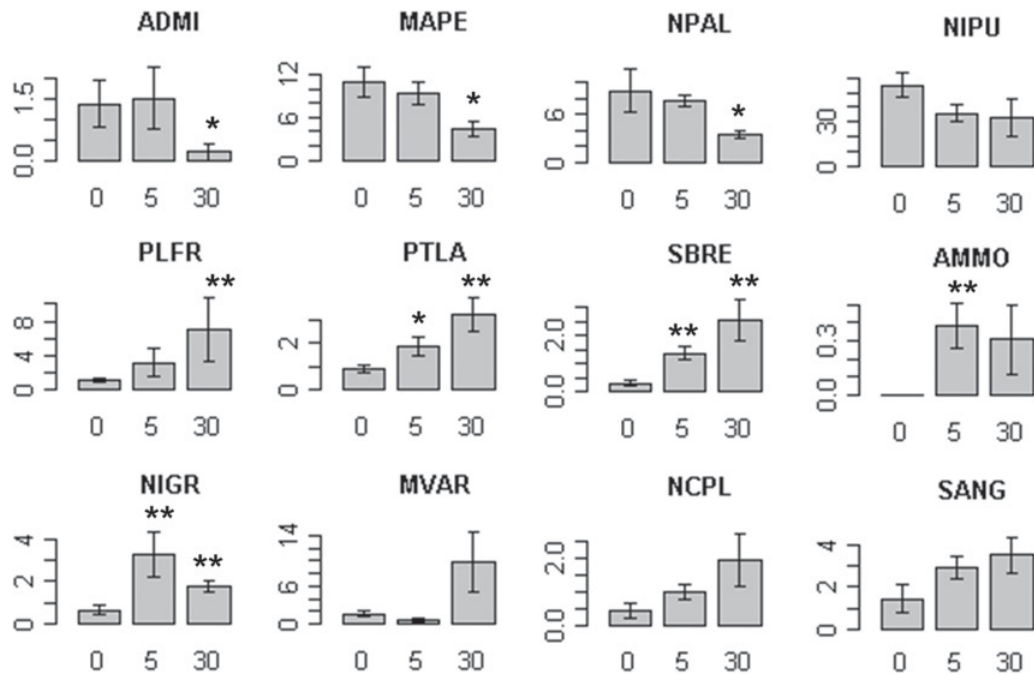
prominent in the sub-dominant species *Surirella angusta* (Fig. 6). Their frequency in the populations of *S. angusta* increased significantly with contamination to reach 80% at the end of the experiment at 30  $\mu\text{g.L}^{-1}$  (Fig. 7).

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  $\mu\text{g.L}^{-1}$  on the



**Fig. 5.** Bar plots of the relative abundance (in %,  $\pm$  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, *Achnantheidium 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*.

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

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

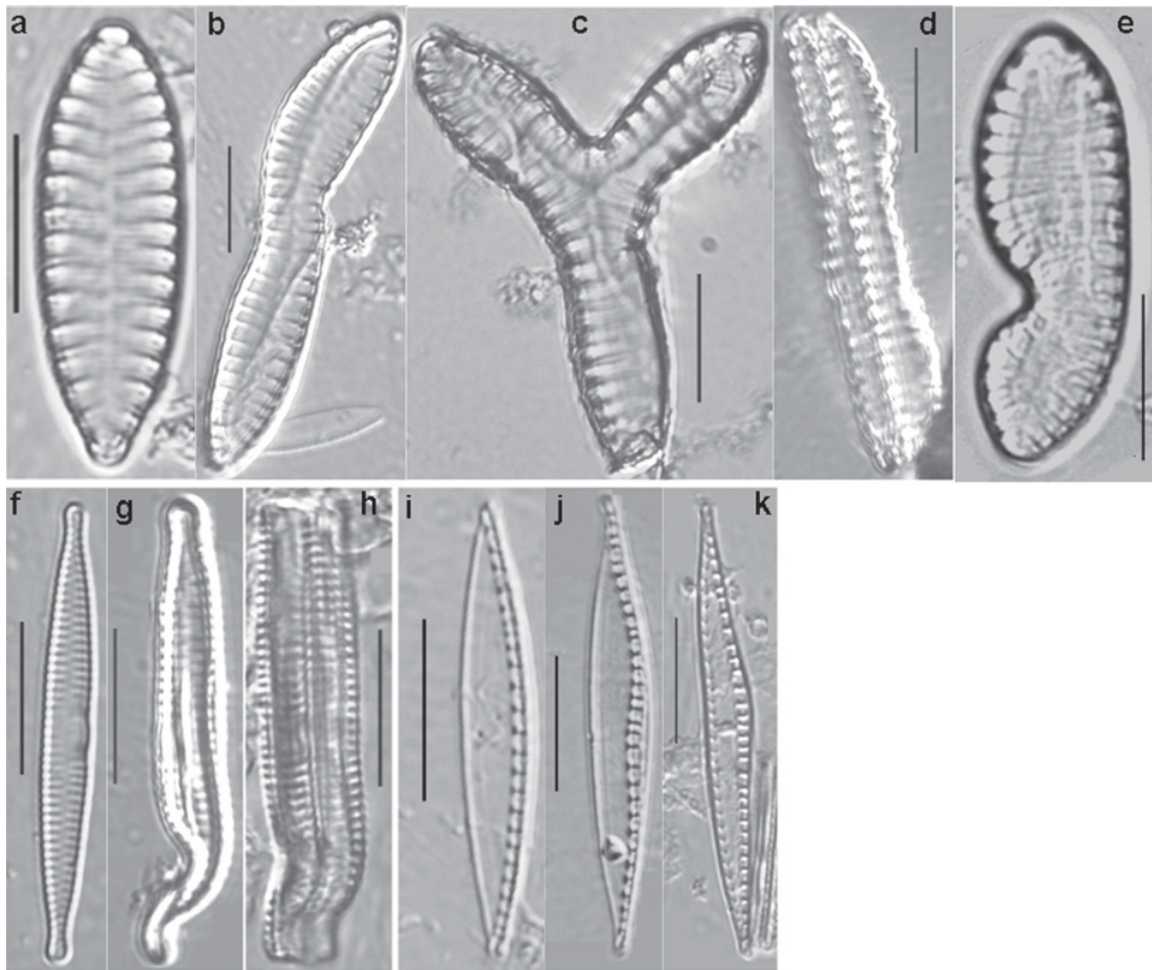
451 However, descriptors related to periphyton biomass  
 452 (chl a and AFDM) did not indicate any effect of metol-  
 453 achlor at the tested concentrations. Moreover, EC<sub>50</sub> values

454 from the monospecific tests and from other similar studies  
 455 with microalgae based on short-term growth inhibition  
 456 tests (Junghans *et al.*, 2003; Vallotton *et al.*, 2008; Liu and  
 457 Xiong, 2009) suggest much higher effect thresholds.  
 458 Indeed, no significant effect of metolachlor was visible on  
 459 the growth of *S. angusta* and *A. minutissimum* up to a con-  
 460 centration of 180  $\mu\text{g.L}^{-1}$  (NOEC). As tolerant species  
 461 progressively replace sensitive ones in a community  
 462 exposed to a toxicant, the effect on the whole community  
 463 biomass may not be visible unless the tolerance limit of the  
 464 most robust and competitive species is reached. Therefore  
 465 community composition change is a more sensitive end-  
 466 point to evaluate the toxicity of a pollutant on a natural  
 467 aquatic environment and deviation from the reference con-  
 468 dition, as requested by the European Water Framework  
 469 Directive.

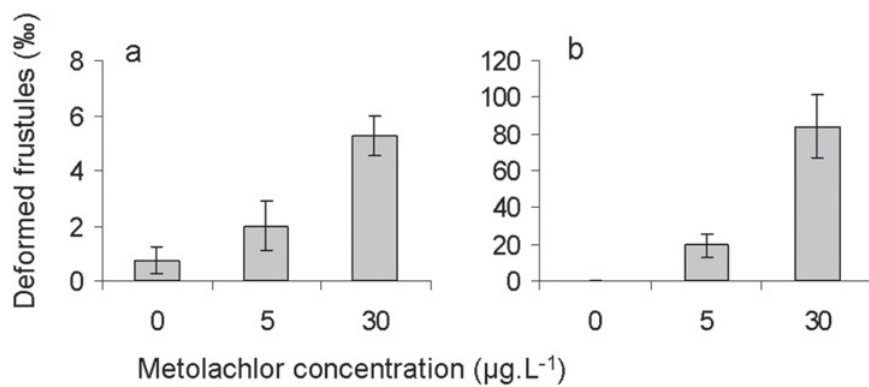
#### Species sensitive/tolerant to metolachlor

470  
 471 The changes in composition of diatom communities  
 472 with metolachlor concentrations are informative about  
 473 the possible bioindication of herbicide contamination.  
 474 In such microcosm experiments, the decrease in abun-  
 475 dance of a species along a contamination gradient may  
 476 result from species sensitivity to the tested toxicant or to a  
 477 change in biological interactions linked to water toxicity.  
 478 *Achnantheidium minutissimum* is a species indicative of  
 479 good water quality (Coste *et al.*, 2009), which was shown  
 480 (as in this study) to maintain its relative abundance at low





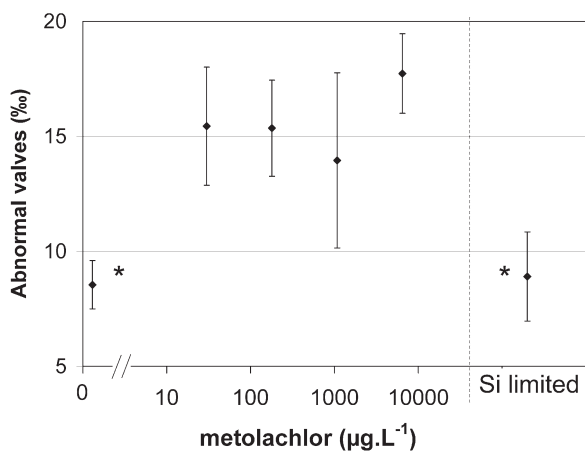
**Fig. 6.** Abnormal diatom frustules observed in the contaminated experimental units for the species *Surirella angusta* (a to e), *Fragilaria capucina* (f to h) and *Nitzschia sociabilis* (i to k). (a), (f) and (i): normal form of each species; (d) and (h): pair of deformed frustules following vegetative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



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

481 herbicide concentration and to be strongly reduced at  
 482 higher contamination levels (Hamilton *et al.*, 1987; Pérès  
 483 *et al.*, 1996). Therefore, it seems that *A. minutissimum* is  
 484 rather sensitive to herbicides. This is supported by the  
 485 lower EC<sub>50</sub> obtained for this species than for *S. angusta*,

486 which appeared indifferent to metolachlor in the channel  
 487 experiment. The species *Mayamaea permitis* and *Nitzschia*  
 488 *palea* may bring interesting information for separating  
 489 toxic *vs.* trophic effects, as they exhibited the same  
 490 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).

491 they are known to be generally more tolerant to organic  
 492 and trophic pollution. The species *Nitzschia pusilla*  
 493 had a lower relative abundance in the two treatments with  
 494 metolachlor. However, it remained the dominant species  
 495 in almost all communities sampled. It may be rather  
 496 indifferent to metolachlor and the drop in relative abun-  
 497 dance would result from the greater development of tol-  
 498 erant species in contaminated channels.

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

515 **Relevance of abnormal forms as an indication of**  
 516 **toxicity**

517 This experimental study clearly establishes the link be-  
 518 tween abnormal forms and the herbicide. The appearance  
 519 of diatom deformities is often presumed to result from  
 520 Si limitation (Debenest *et al.*, 2010). However, from this  
 521 study, this hypothesis can be rejected for several reasons:  
 522 (1) there was a clear dose effect in the channels and there  
 523 was most Si left in one of the most contaminated channels  
 524 (30b), (2) in the monospecific test, deformities were

525 significantly more abundant in the contaminated cultures,  
 526 and (3) Si limitation did not produce more abnormal  
 527 forms than control conditions. The duration of the experi-  
 528 ments (14 vs. 4 days) may be the cause of the difference in  
 529 proportion and in response to concentration between the  
 530 channel experiment and the monospecific test. It is likely  
 531 that the proportion of abnormal forms in a population  
 532 increases with time at a rate depending on the exposure  
 533 level. It could also be hypothesized that the progressive de-  
 534 gradation of metolachlor may produce metabolites having  
 535 higher teratogenic effects than metolachlor (Osano *et al.*,  
 536 2002; Hladik *et al.*, 2005).

537 Like other herbicides belonging to the chloroacetani-  
 538 lide class, metolachlor inhibits the biosynthesis of very  
 539 long chain fatty acids in plants and microalgae (Boger  
 540 *et al.*, 2000) and consequently blocks cell division and en-  
 541 largement. Such a mode of action does not *a priori* suggest  
 542 an effect on diatom frustule structure. However, changes  
 543 in ultrastructural morphology were reported in a green  
 544 microalgae following exposure to metolachlor (Liu and  
 545 Xiong, 2009). As the synthesis of the diatom frustule  
 546 involves a special intracellular vesicle (SDV) and requires  
 547 the exocytosis of biogenic silica (Martin-Jezequel *et al.*,  
 548 2000), any alteration of the cell membrane system may  
 549 have repercussions on frustule construction.

550 The link between abnormal diatom forms and con-  
 551 tamination by heavy metals has been well documented  
 552 (Gold *et al.*, 2003; Cattaneo *et al.*, 2004; Morin *et al.*, 2008;  
 553 Falasco *et al.*, 2009). However, the literature on the effect  
 554 of herbicides on diatom morphology is scarce. There are  
 555 now experimental studies reporting diatom deformities  
 556 due to herbicides with three different physiological targets:  
 557 photosynthesis (isoproturon), gene expression (maleic  
 558 hydrazide) and lipid synthesis (metolachlor, this study)  
 559 (Schmitt-Jansen and Altenburger, 2005; Debenest *et al.*,  
 560 2008). The variety of toxicants giving the same effect on  
 561 diatoms suggests that toxic stress generally generates ab-  
 562 normal forms.

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

574 **Conclusion**

575 The experiments conducted in this study showed that  
 576 metolachlor has toxic effects on river periphytic diatoms.  
 577 The results are interesting for the toxicological assess-  
 578 ment of pesticides as well as for the bioindication of con-  
 579 tamination. It emerges that effect concentrations may vary  
 580 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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